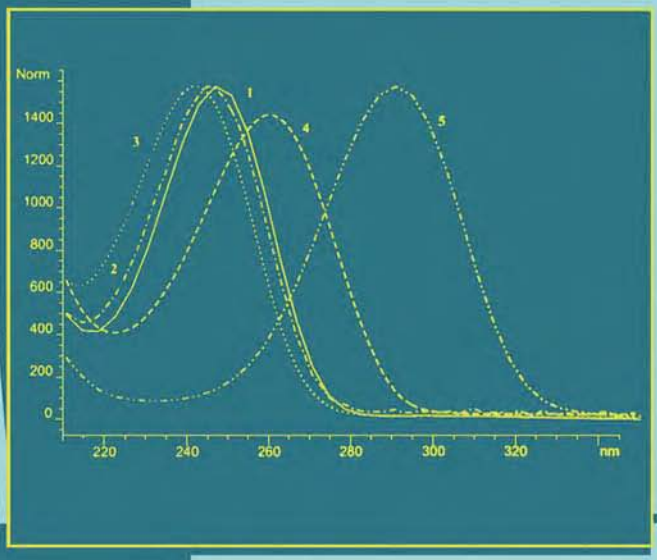
Progress in Pharmaceutical and Biomedical Analysis Volume 4

Identification and Determination of Impurities in Drugs



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Identification and Determination of Impurities in Drugs

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PROGRESS IN PHARMACEUTICAL AND BIOMEDICAL ANALYSIS 4

Identification and Determination of Impurities in Drugs

Edited by

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Preface

Having worked as an analytical chemist in the pharmaceutical industry for 41 years and having written or edited nine books on various topics in this field, I have decided to round this figure up to ten by writing/editing a book summarizing the analytical aspects of impurities in drugs.

Why just impurities? One of the features of the revolutionary changes in the last four decades in pharmaceutical analysis has been the increasing importance of the characterization and quantitative determination of impurities in bulk drug materials and pharmaceutical products by their analytical investigation. The appearance and rapid spreading of a broad and sophisticated array of spectroscopic and chromatographic/electrophoretic techniques, as well as their hyphenated combinations, enables the analyst to determine the structures and concentrations of related organic impurities (degradation products), residual solvents, inorganic impurities down to the ppm level within such a short time. This would have been impossible even to imagine 10–20 years ago. The evaluation of the results thus obtained, amended with further important data on the enantiomeric, polymorphic and microbiological purity, is the best way to characterize the quality of drugs and drug products, thus greatly contributing to the safety of drug therapy.

A consequence of the amazingly large number of analytical techniques necessary for the characterization of the purity of drugs is that no single analyst can be expert in all of these methods, and, therefore, the role of a head of a laboratory dealing with impurity profiling is somewhat similar to the role of the conductor of an orchestra. To remain with this analogy, in the classical period of drug analysis only a few techniques were available to solve the problem using the knowledge of those times. Often a *Bach Brandenburg Concerto*, with but several brilliant, intertwined melodic lines, is performed by a chamber orchestra without a conductor. This is naturally not the case with pieces such as *Mahler's* relatively complex *Symphony VIII* which is also known as the *Symphony of a Thousand* because of the tremendous vocal and orchestral forces required, and where the conductor has a predominant role. It is my personal view based on many decades of experience in impurity profiling that due to the complexity of the problems and the large variety of possibilities to solve them, the analytical challenges in this type of work provides much enjoyment since it affords intellectual pleasure to the analytical chemist.

The main aim of this book is to give an up-to-date survey of the problems in drug impurity profiling and of the many possibilities for their solutions. I

hope that the book will be a useful source of information to chemists and pharmacists dealing with these problems in quality control laboratories of drug manufacturers, drug registration agencies, contract research organizations, in drug research laboratories where impurity-related problems often occur, as well as in university departments where pharmaceutical analysis is part of the curricula.

Due to the great diversity of the problems and methods in drug impurity profiling I invited prominent authorities in their respective specialties to contribute the majority of the chapters. As a citizen of a small country and as an employee of a medium-sized pharmaceutical company, it has been a great honour, and at the same time a satisfying task, for me to work with colleagues from Belgium, Germany, Sweden, The Netherlands, United Kingdom and the USA, among them representatives of some the leading drug manufacturers, and last but not least with the Hungarian contributors, mainly many of my colleagues at the Chemical Works of Gedeon Richter Ltd.

Sándor Görög
Budapest, September 1999

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Chapter 1

VARIOUS ASPECTS OF THE ESTIMATION OF IMPURITIES IN DRUGS

1.1. Introductory Remarks

Sándor Görög

1.1.1. Drug Purity and the Safety of Drug Therapy

Two issues of fundamental importance in drug therapy are *efficacy* and *safety*. Drug analysts play an indirect but very important role in creating the basis for highly efficient drug therapy by giving analytical support to synthetic, biotechnological, pharmacological, pharmaceutical technological, clinical, etc. research to find the most efficacious drug material and its optimal dosage form.

Much more important is the role of drug analysts in the other area, in securing the highest possible *safety* for drug therapy. The safety of drug therapy is determined by two main factors:

- The *pharmacological–toxicological profile of the drug substance*, i.e. the relation of the beneficial and adverse effects of the drug material to the human organism: Since the adverse effects of a pure drug material and the preparations made thereof are their inherent properties, drug analysts cannot do very much to improve the safety of drug therapy from this point of view.
- Adverse effects caused by the *impurities* in the bulk drug material and its dosage forms: The role of analytical chemist in monitoring and controlling these thus greatly contributing to the safety of drug therapy is obvious. This is the reason why analytical activities concerning impurities are among the most important issues in modern pharmaceutical analysis as indicated among other examples by the recent publication of a book [1], a book chapter [2] and excellent reviews [3,4] on this topic.

An *impurity* as defined by the ICH Guidelines [5] is ''Any component of the medicinal product which is not the chemical entity defined as the active substance or an excipient in the product'', while the definition of *impurity profile* in the same is ''A description of the identified and unidentified impurities present in a medicinal product''. Although no definition is given for the term ''impurity profiling'', it is generally considered to be the common name of a group of analytical activities the aim of which is the detection, identification/structure elucidation and quantitative determination of organic and inorganic impurities as well as residual solvents in bulk drugs and pharmaceutical formulations. Further definitions, the classification of impurities, regulatory aspects, treatment of the subject matter by the principal pharmacopoeias and the International Conference on Harmonisation including the toxicological aspects of impurities are discussed in Sections 1.3 and 1.5.

The relation between purity of a certain material and its impurities is obvious. The Physical Chemistry Division of the International Union of Pure and Applied Chemistry (IUPAC) suggested the following definition for the purity of a sample [6]: ''A sample is sufficiently pure when the amount of each of the impurities which may interfere with the specific purpose for which the sample is required is so low that their combined effect is negligible within the desired limits of accuracy''. With two additional remarks this general statement made almost 30 years ago can be applied to the present role of impurity profiling in drug analysis and its contribution to assure the safety of drug therapy.

(a) The final goal of the analytical activities in industrial and regulatory drug control laboratories is to assist the drug manufacturers in providing high *quality* drugs for the therapeutic use. The best way to characterise the quality of a bulk drug sample is to determine its *purity*. There are two possible approaches to reach this goal: the determination of the *active ingredient content* with a highly accurate and precise specific method or the determination of its *impurities*. In the early years of drug analysis when chromatographic techniques were not yet available, the characterisation of the purity of drugs was based on the determination of the active ingredient content by non-specific titrimetric and photometric methods supported by the determination of physical constants and some limit tests for known impurities based mainly on colour reactions. The deficiencies of this approach are well known: in many cases even highly contaminated drug materials could meet the requirements set in the early editions of pharmacopoeias. As a consequence of the enormous development of the analytical technology in the last few decades entirely new possibilities have been created for the determination of the purity of drug materials. In principle, it is now possible to replace all non-specific assay methods with highly specific and precise new (mainly HPLC) methods thus greatly improving the value of the determination of the active ingredient content (assay) of

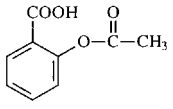
bulk drug materials. In spite of this, the examination of the latest revisions of the principal pharmacopoeias convinces the reader that in the majority of cases the assays for drug substances containing suitable functional groups is still based on the classical non-specific methods, and HPLC methods are prescribed only in a limited number of monographs. There are probably two reasons for this.

One is certainly the enormous difference between the duration and costs of the two approaches. Titrations and spectrophotometric measurements can be carried out within a very short time with minimal costs while a HPLC assay usually requires time demanding system suitability tests, and a high number of parallel runs of the test material and reference standard as well as the use of expensive instrumentation, columns and solvents. On the other hand, of course the value of the results obtained by the two approaches is not comparable with regard to specificity and accuracy.

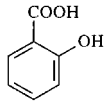
The other reason for the lack of the general breakthrough of HPLC methods in the assay of drug materials is that, as a consequence of the above mentioned development of the analytical technology, the possibilities of evaluating the impurities have been improved to an even greater extent than for the assay methods. As a consequence of this – in accordance with the above cited general IUPAC statement – the quality (purity) of a drug can be much more efficiently characterised by characterising and quantitatively evaluating the impurities than by the most developed assay methods. For example, if the active ingredient content of a bulk drug material is 99.0% and a highly specific, accurate and precise method is used for the assay with a relative standard deviation in the order of 0.5%, the purity is 99.0 ± 0.5%, which means an intolerable uncertainty even in this ideal case for the level of impurities. Conversely, impurities can be much better characterised by their direct determination. As a result of the changes in the analytical technology and the continuously increasing demands regarding the purity of drugs, the way of thinking in pharmaceutical analysis has been changed to a great extent: we are witnessing the decreasing importance of assay methods and the increasing importance of impurity evaluation in contemporary pharmaceutical analysis.

An example of the necessity for the change of the attitudes to drug quality involves the case of one of the most classical drugs, *aspirin* (**1**). Its quality has been characterised in the pharmacopoeias (old and new revisions alike) by titrimetric assay (saponification by sodium hydroxide and back titration of the uncomsumed base). A colour test for free salicylic acid (**2**) as the main impurity/decomposition product has also been included. About 20 years ago when HPLC became widespread in pharmaceutical analysis, it was found that in addition to salicylic acid, bulk drug samples and aspirin tablets from various manufacturers contain three more impurities (**3**–**5**) of which **4** reached in some cases the 1% level [7,8]. It was supposed that these impurities, all capable of

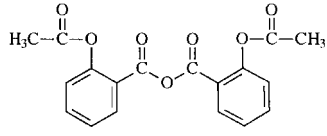
reacting with protein amino functions are responsible for allergic reactions of aspirin [9]. It is readily apparent that all of the above impurities consume sodium hydroxide in the saponification/titration method: thus their presence cannot be detected by this method. As a consequence of this finding a limit test based on a colour reaction with the aminopyrazolone/ferricyanide reagent has been included in the European Pharmacopoeia [10].



aspirin (**1**)



salicylic acid (2)



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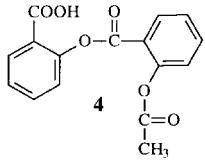

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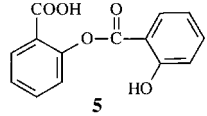

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(b) A difference between the IUPAC definition for purity and the practice of drug analysis is that in the latter field limits are set to control the quantity of not only impurities which ''…may interfere with the special purpose…'' of the drug but also that of harmless compounds. Of course stress is given to the limitation of toxic or potentially toxic impurities.

The great dilemma of the pharmaceutical industry and the regulatory authorities is where to set the limits. The sensitivity of the analytical methods available to drug analysts is no longer a limiting factor: modern instruments enable the detection and quantification of inorganic, organic impurities and residual solvent down to the 1 ppm level or even lower in some cases. One can agree with the statement of Krstulovic and Lee [4] that ''…the role of the ethical pharmaceutical industry is to define an impurity profile that is acceptable for the intended use of a given drug, without compromising its therapeutic safety…''. It is, however, difficult to make general statements about how to define the impurity level which does not compromise therapeutic safety. It is a relatively easy task to set the limits to known toxic (carcinogenic, etc.) impurities. In such cases the limits are often in the low ppm range which means that safety is really assured (often oversecured). It is more difficult to find an acceptable solution for the problem of synthesis-related organic impurities in pharmacopoeias. These depend on the synthetic route and for this reason, in the

majority of cases, general limits have to be defined for unknown impurities. In these cases the usual requirements, e.g. <1–2% of total organic impurities and <0.5% of individual impurities provide generally acceptable safety for the therapeutic use of the drugs.

It has to be emphasised that to meet the requirements of the pharmacopoeias regarding the purity of drugs is a necessary but by no means sufficient condition of the safe use of drugs. The policy of the drug registration agencies and the tendencies for the international harmonisation of this policy with the aim of improving the safety of drug therapy are summarised in Sections 1.3 and 1.5. For the analytical chemist this means that all impurities above 0.1% (in the case of drugs with a high daily dose above 0.05%) have to be identified and determined by sufficiently selective methods.

To deal with impurities below this limit is usually not necessary, unless particularly toxic or carcinogenic impurities are known or suspected to be present. However, it has to be taken into consideration that in the framework of the very sharp competition in the world drug market the purity of drugs is one of the most important issues: the requirements of some customers of bulk drugs are more stringent than those laid down in the pharmacopoeias and ICH Guidelines, and as a consequence of this the importance of analytical information on impurities in the 0.01% range is increasing. This statement is especially valid for analytical investigations where the aim of the study is the identification of ''synthesis markers'', which can prove the illegal use of certain patented reaction routes (cf. Section 1.2.4).

After this short introduction outlining some views about the present situation of purity-related issues in pharmaceutical analysis it may be of interest to look back to the beginnings of the pharmaceutical industry more than 150 years ago. Two quotations from the book published on the occasion of the 200th anniversary of the birth of Emanuel Merck, the founder of the world famous pharmaceutical company will certainly convince the reader that purity issues and the role of analytical chemists in them are as old as the pharmaceutical industry itself [11].

The official report of an industrial exhibition in Mainz in 1842 makes the following statement about Merck's alkaloids: ''…in the unanimous opinion of the referees, in terms of scale and *purity* his products are clearly superior to everything that has yet been supplied by other factories''. The following sentence can be found in a letter written by Emanuel Merck in 1851 to a customer who complained about the quality of a consignment of morphine: ''I herewith guarantee the *purity* of my preparations and undertake to reimburse you for any damage that may arise through one of my preparations being impure; for this I take leave to request that you have the morphium in question investigated by a *competent chemist''*.

1.1.2. Aims and Scope

The aim of this book is to give an overview of the state-of-the-art developments in the field of analytical activities related to impurities, including degradation products, polymorphic modifications and microbiological impurities in bulk drug materials and pharmaceutical formulations. Thanks to the tremendous developments in the analytical methodology in the last few years a large variety of methods are available to enable one to achieve the same goal: detection, identification and determination of impurities. More or less the same results are obtainable by the classical approach, e.g. separation by thin-layer or column chromatography and investigation of the separated and eluted impurities by classical spectroscopic techniques, and by the most modern approaches such as on-line high-performance liquid chromatography, NMR and mass spectrometry (HPLC/NMR/MS) or capillary electrochromatography coupled with tandem mass spectrometry (CEC/MS/MS). In addition to these approaches based on individual impurities, classical, general purity tests based on, e.g. melting point determination and phase solubility testing [3,12] are still in use. The choice between the various possibilities can be motivated by several factors, among others the character and the number of the problems to be solved simultaneously, the capabilities of the different laboratories to purchase new (often very expensive) equipment and to some extent also the personal interest and ambitions of the analysts who are responsible for the purity issues. One of the main aims of this book is to help the readers to chose more wisely among the amazingly large number of possibilities.

This book is methodology-oriented. In Chapter 1 only the most important aspects of the background of impurity-related analytical studies (toxicological, pharmacopoeial aspects, the characterisation of the sources of impurities and the role of impurity profiling in various fields of drug research, production and therapeutic use) are summarised. Chapter 2 deals with related organic impurities, the strategies for impurity profiling, the use of chromatographic (TLC, GC, HPLC, SFC) and related separation methods (CE, CEC), spectroscopic (UV, MS, NMR) and hyphenated techniques such as (GC/MS, HPLC/MS/(MS), CE/MS, CEC/MS, SFC/MS, HPLC/NMR/(MS) as well as differential scanning calorimetry. In accordance with the great importance of this field and the most varied analytical possibilities available this is the longest chapter in the book. The subject of Chapter 3 is the identification and determination of residual solvents from the classical loss on weight determination, and thermoanalytical methods to the different variants of the generally used gas chromatographic and GC/MS measurements and the moderately important NMR approach. The determination of inorganic impurities mainly by atomic spectroscopic techniques and the still widely used classical pharmacopoeial limit tests, supplemented by other approaches is discussed in Chapter 4. The special

problems of degradation products as impurities are dealt with in Chapter 5, where the application of the methods summarised in Chapter 2 is described for problems such as stability tests, elucidation of degradation pathways, etc. A separate chapter (Chapter 6) has been compiled to deal with one of the most up-to-date problems in contemporary pharmaceutical analysis, the estimation of enantiomeric purity of chiral drugs mainly by chromatographic and electrophoretic techniques. In addition, the capabilities of chiroptical and NMR methods are also briefly discussed. Although, as yet, only the first steps have been made by the regulatory agencies to treat polymorphic modifications as impurities, Chapter 7 is devoted to various approaches to solve these problems by infrared, Raman and NMR spectroscopies, X-ray diffraction and thermoanalytical methods. Since in the broader sense of the word the microbiological purity of drugs and drug products also belongs to this circle, the most important information from this field is summarised in Chapter 7. After the mainly methodology-oriented chapters, in the last one, Chapter 9 four groups of drugs (peptides, antibiotics, biotechnological products and steroids) have been selected to demonstrate the use of the methods described in the previous chapters to solve the practical problems arising during the course of impurity profiling of these drugs. A large variety of practical examples taken from other groups of drugs can be found in Chapters 2–7, too, as illustrations of the use of the different analytical techniques are described there.

The relative lengths of the various chapters and sections do not necessarily reflect the importance of the various techniques in contemporary drug analysis. Methods introduced 30–40 years ago which can be considered to be classical methods by now but still form the main component of the complex analytical methodology required in impurity profiling are well covered by monographs. For this reason relatively more space has been devoted to the new, still not widely used but very promising techniques.

It is the aim of this book to cover all important aspects and methods of drug impurity profiling but it is by no means among the goals to present a full bibliography of the very rich literature in this area. The practical examples have been selected in such a way that they should be good illustrations of the application of the various analytical methods reflecting the modern way of thinking necessary for problem solving in this very complicated but beautiful field of pharmaceutical analysis. As a consequence of the large variety of methods to be illustrated and the limitations of this book, the authors were forced to leave out pertinent publications from their examples.

References

1. S. Ahuja, *Impurities Evaluation of Pharmaceuticals*, Marcel Dekker, New York (1998)

2. S. Husain and R.N. Rao, in *Advances in Chromatographic and Electromigration Methods in Biosciences* (Z. Deyl, I. Mikšik, F. Tagliaro and E. Tesařová, Eds.), Elsevier, Amsterdam (1998)
3. D. Giron, *Boll. Chim. Pharm.* **133**, 201–219 (1994)
4. A.M. Krstulovic and C.R. Lee, *J. Chromatogr. B* **689,** 137–153 (1997)
5. ICH Guideline: *Impurities in New Drug Substances*, CPMP/ICH/142/95 (May 1995)
6. L.A.K. Staveley (Ed.), *The Characterisation of the Chemical Purity. Organic Compounds*. Butterworths, London (1971)
7. J.C. Reepmeyer and R.D. Kichhoefer, *J. Pharm. Sci.* **68**, 1167–1169 (1979)
8. R.D. Kirchhoefer, J.C. Reepmeyer and W.E. Juhl, *J. Pharm. Sci.* **69**, 550–553 (1980)
9. H. Bundgaard, *J. Pharm. Pharmacol.* **26**, 18–22 (1974)
10. *European Pharmacopoeia*, p 345. The Council of Europe, Strasbourg (1997)
11. *Modern by Tradition. The history of the chemical-pharmaceutical factory E. Merck, Darmstadt*, p 29. E. Merck, Darmstadt (1995)
12. R.E. Schirmer (Ed.), *Modern Methods of Pharmaceutical Analysis, Vol. III*, pp 157–169. CRC Press, Boca Raton, FL (1982)

1.2. The Nature and Origin of the Impurities in Drug Substances

Sándor Görög

1.2.1. Organic Impurities

Organic impurities, often called related, ordinary or synthesis-related impurities can originate from various sources and from various phases of the synthesis of bulk drugs and the preparation of pharmaceutical dosage forms. A sharp differentiation between synthesis-related or process-related [1] impurities and degradation products is not always possible: degradation products can be formed during the synthesis and the isolation of the endproduct and also upon storage of the bulk drug and especially during formulation and storage of the dosage form (see Section 5.1).

As will be shown in this section, the majority of impurities are characteristic of the synthetic route of the manufacturing process of the drug. Since there are several possible synthetic routes for the preparation of the same drug material and in the case of generic drugs many of them can be in practical use, the set of the structures of possible impurities presented by the European Pharmacopoeia [2] is in many cases far from being complete: different syntheses can give rise to other structures. In the following part of this section several examples will be presented classifying them according to the origin of the impurities [3].

1.2.1.1. Last Intermediate of the Synthesis

Impurities falling into this category are often called ''probable'' or ''expected'' impurities. For example, the last step in the synthesis of *paracetamol* is the acetylation of 4-aminophenol: the latter is a probable impurity (measured photometrically by the European Pharmacopoeia [2]) in the bulk drug material. Various pharmaceutically important 17α-ethinyl-17-hydroxysteroids are prepared by the ethinylation of 17-oxosteroids; for this reason 17-oxosteroids are probable impurities of *ethinylsteroids* (see Figure 1.2.A). Another example also from the steroid field is prednisolone. The final step in its synthesis is the introduction of the Δ^1 double bond usually by microbiological dehydrogenation. For this reason the most likely impurity in *prednisolone* is the 1,2-dihydro derivative (hydrocortisone); for the structures see page 729.

1.2.1.2. Products of Incomplete Reaction During the Synthesis

If the last intermediate has two functional groups and the final step involves the same reaction on both, there is always a possibility that only one of them reacts and a partially reacted impurity appears. Impurities of this kind also fall into the category of probable impurities. For example, in one of the syntheses of *ethynodiol diacetate* (see page 87); the final step is the diacetylation of ethynodiol (17α-ethinylestra-4-ene-3β,17-diol). Since the reactivity of the secondary 3-hydroxy group is much higher than that of the tertiary 17-hydroxyl, a probable (and real) impurity is ethynodiol-3-acetate. Similarly, the final step in the synthesis of *pipecuronium bromide* (2β,16β-bis-(4-dimethylpiperazino)-3α,17β-diacetoxy-5α-androstane dibromide) is diacetylation of the 3α,17β-dihydroxy derivative and the likely impurity is the 17β-monoacetyl derivative.

Incomplete reaction as the source of impurities is not restricted to the final step of the synthesis. For example in one of the syntheses of *enalapril maleate*

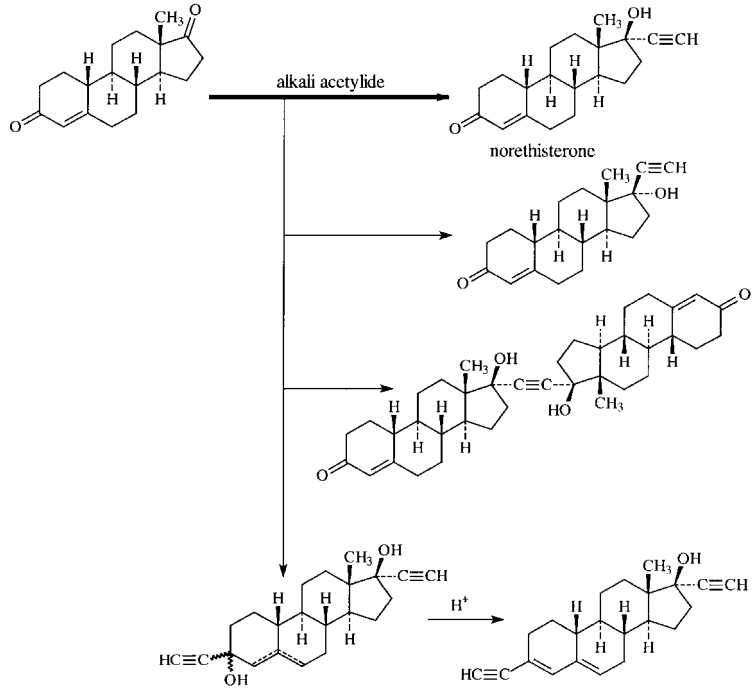
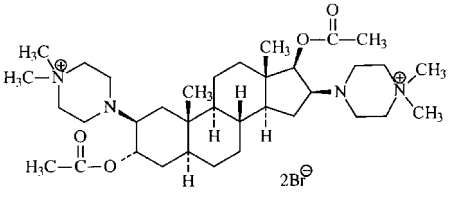


Figure 1.2.A.
Ethinylation of 17-oxosteroids with three side reactions

and *lisonopril* (for the formulae see page 124) the $-CH_2-$Phe unit is introduced into the molecule as –CO–Phe. This is transformed to the $-CH_2-$Phe group by catalytic hydrogenation of the oxo group. Unreduced or partially reduced oxo group (to CH(OH)–Phe) leads to oxo and hydroxy derivatives of enalapril and lisinopril as impurities.



Pipecuronium bromide

1.2.1.3. Products of Overreaction

In many cases the reaction of the final step is not selective enough and the reagent attacks the last intermediate in addition to the desired site. For example in the course of the decanoylation of nandrolone (19-nortestosterone, 17β-hydroxy-estra-4-ene-3-one) to form *nandrolone decanoate* the 4-ene-3-one can also be decanoylated to form an enol ester type impurity estra-3,5-diene-3,17β-diol-bis-decanoate.

Overreaction can take place not only in the final step but also in previous steps of the synthesis. For example, during the course of the above mentioned reduction of the –CO–Phe unit in the synthesis of *enalapril maleate* and *lisinopril* not only can incomplete reaction take place but overreduction, too, leading to the hydrogenation of the phenyl ring; this is the source of cyclohexyl–enalapril and cyclohexyl–lisinopril as impurities; (the latter is listed among the official impurities in the European Pharmacopoeia [2]). Another example for a possible overreduction takes place in the course of the reduction of the 3-oxo group of norethisterone (17α-ethinyl-17-hydroxy-estr-4-ene-3-one) to the above mentioned ethynodiol by complex metal hydrides. This reduction may attack the Δ^4 double bond also leading to the 17α-ethinyl-estrane-3β,17-diol, the diacetate of which is a possible impurity in *ethynodiol diacetate* [4]. In Figure 1.2.A another overreaction involving the 4-ene-3-oxo group in steroids is shown. In the course of the ethinylation of 4-ene-3,17-diones the ethinylation is not entirely regioselective: in addition to the formation of 17α-ethinyl-17-hydroxysteroids, 3,17-diethinyl derivative is also formed as an impurity. The detection, separation and structure elucidation of this impurity is described in detail as a case study in Section 2.1.7.

Other types of overreaction can also take place. As seen in Figure 1.2.B, *tolperisone* is prepared by Mannich condensation (reaction of 4-methylpropiophenone with 1 mole of formaldehyde and piperidine). If two moles of formaldehyde take part in the reaction an impurity containing an additional hydroxymethyl group can appear.

A further example is the chlorination step in the synthesis of *pyridinol carbamate*. The reaction product of the photocatalysed chlorination of 2,6-lutidine is the bis-chloromethyl derivative. This is converted in two steps to the final product. The product of overreaction during the chlorination of 2,6-lutidine is the trichloro derivative. As shown in Figure 1.2.C this is the precursor of a hydroxy derivative in pyridinol carbamate [3].

1.2.1.4. Impurities Originating from Impurities in the Starting Material of the Synthesis

Impurities present in the starting materials of the dug syntheses can also be sources of impurities in the drug material. In these cases the impurity undergoes the same reactions as the main component leading to mainly isomeric impurities. As an example of this the appearance of the isomeric 4-trifluoro-

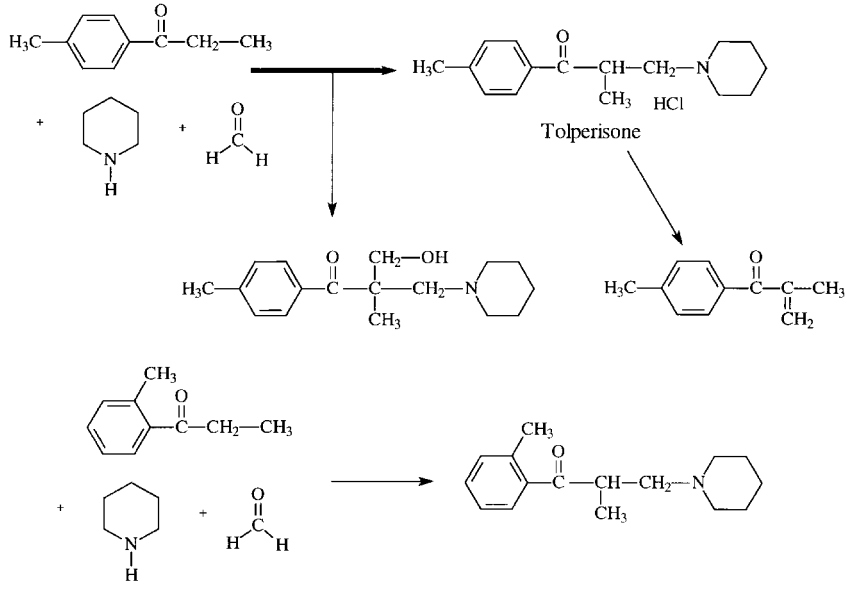


Figure 1.2.B.
Formation of tolperisone and three impurities

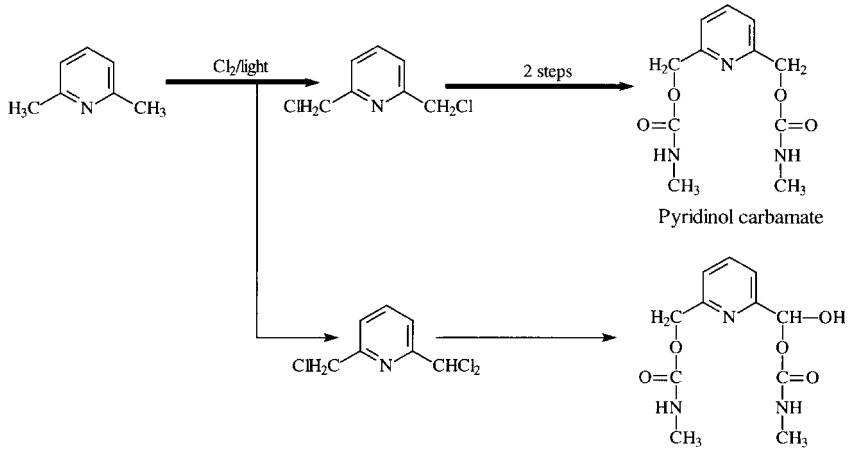


Figure 1.2.C.
Formation of pyridinol carbamate and the origin of its impurity

methyl impurity in 3-trifluoromethyl-α-ethylbenzhydrol (*flumecinol)* is a consequence of the presence of 4-trifluoromethylbromobenzene impurity in 3-trifluoromethylbromobenzene which is the starting material of the synthesis [5,6] is discussed in Section 1.4.1. Another example is related to the synthesis of *tolperisone*, discussed in the previous paragraph. If the starting material of the Mannich reaction 4-methylpropiophenone contains as an impurity 2-methylpropiophenone, the 2-methyl analogue of tolperisone can also be an impurity.

1.2.1.5. Impurities Originating from the Solvent of the Reaction

In some cases the solvent of a reaction or an impurity in the solvent is also transformed during the synthesis leading to an impurity. For example, one of the first steps in the synthesis of the above mentioned *pipecuronium bromide* is the catalytic elimination of methanesulphonic acid from 3β-hydroxy-5α-androstane-17-one methansulphonate to form 5α-androst-2-ene-17-one. In the course of the optimisation of the reaction conditions of this reaction an impurity was identified as 3β-phenyl-5α-androstane-17-one. In this experiment the solvent mixture contained benzene and the catalysts were silica and aluminium chloride. The obvious reason for the formation of the 3-phenyl derivative was a Friedel–Crafts type reaction between the active ester and benzene [7]. In the course of the synthesis of the above mentioned *enalapril maleate* the first step of the synthesis is a Friedel–Crafts reaction between

benzene and maleic anhydride to form 1-phenyl-1-oxobut-2-ene-4-oic acid. If benzene is used in large excess also as the solvent of the reaction, traces of toluene in it lead to a 4′-methyl derivative of the above intermediate and this can be the source of an analogous impurity in the final product [8]. If dichloromethane is selected as the solvent for the reaction this solvent can also participate in the Friedel–Crafts reaction leading to an impurity where two molecules of the intermediate are connected with a methylene bridge at their 4′ positions.

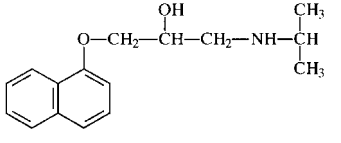
1.2.1.6. Impurities Originating from the Catalyst

The use of homogeneous catalysts may lead to the formation of rarely occurring impurities in which the catalyst molecule is incorporated. An example of this is the tosylation of prednisolone at the 21 position catalysed by pyridine in the course of a synthesis of *mazipredone* (for the formula see page 513). An impurity in the intermediate prednisolone-21-tosylate was found to be the quaternary 21-pyridinium derivative of prednisolone [9].

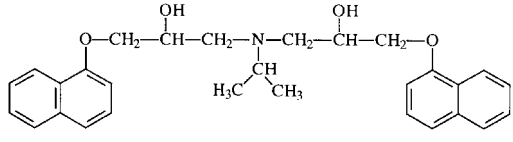
1.2.1.7. Products of Side-Reactions

In the majority of cases side reactions are unavoidable beside the main reactions in organic syntheses even if pure starting materials and reagents are used and the reaction conditions are carefully optimised. Our knowledge of side reactions is increasing as the new analytical technology enables the structure of by-products in the order of 0.01% to be determined. From among the innumerable side-reactions of the syntheses of drug materials just a few are presented here as characteristic examples.

The reaction of the 17-oxo group of steroids with alkali acetylides to form 17α-ethinyl-17-hydroxy steroids is shown in Figure 1.2.A where an overreaction-type side-reaction (formation of 3,17-diethinyl derivatives) is also shown. The reaction scheme in Figure 1.2.A shows two typical side reactions also. These are the formation of the epimeric 17β-ethinyl-17-hydroxy derivative



Propranolol



4-isopropyl-1,7-bis(1-naphthyloxy)-4-azaheptane-2,6-diol

and the acetylene-bridged dimeric derivative [10]. The formation of dimeric derivatives is a frequently occurring side reaction in other drug syntheses also. For example, such an impurity is mentioned among the impurities of propranolol in the European Pharmacopoeia [2].

An interesting side reaction has been found in the course of the acetylation of the sterically hindered 17-hydroxy group in ethynodiol (17α-ethinylestra-4-ene-3β,17-diol), catalysed by 4-dimethylaminopyridine. As seen in Figure 1.2.D, the side reaction leads to the *Z* and *E* isomers of 17α-ethinyl-estr-4-ene-3β,17-diol-3-acetate-17-(3′-acetoxy-2′-butenoate) [3].

Frequently occurring side reactions are the formation of isomers. A typical example for positional isomers as impurities is the formation of isodanazol as an impurity in *danazol*; see Figure 1.2.E. [11]. Diastereomers as impurities occur mainly in peptide derivatives. These and also the diastereomers of their diketopiperazine derivatives (another important group of impurities in peptide drugs) are described as the impurities of *enalapril* and *lysinopril* in the European Pharmacopoeia [2].

Many more examples of impurities originating from side reactions can be found in Chapter 2 as examples of the application of different techniques for their separation, identification and structure elucidation.

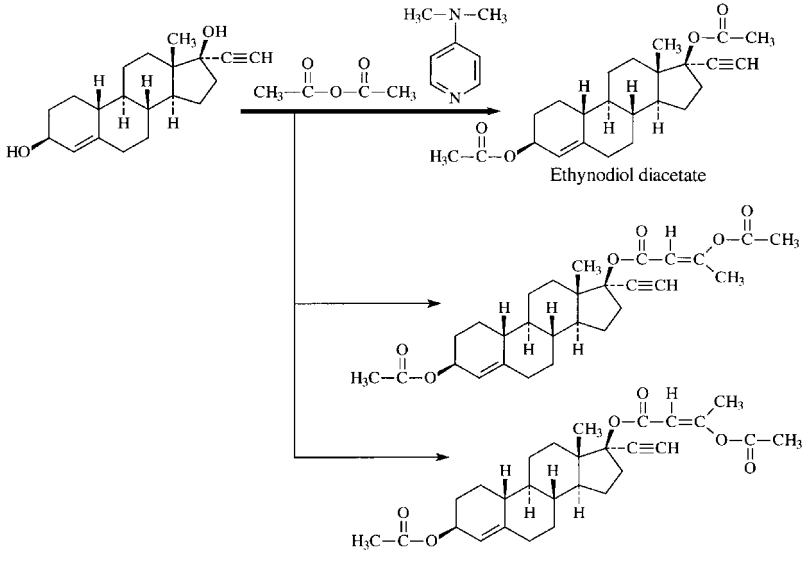


Figure 1.2.D.
Acetylation of the 17-hydroxy group in a 17α-ethinyl-17-hydroxy steroid with two side reactions

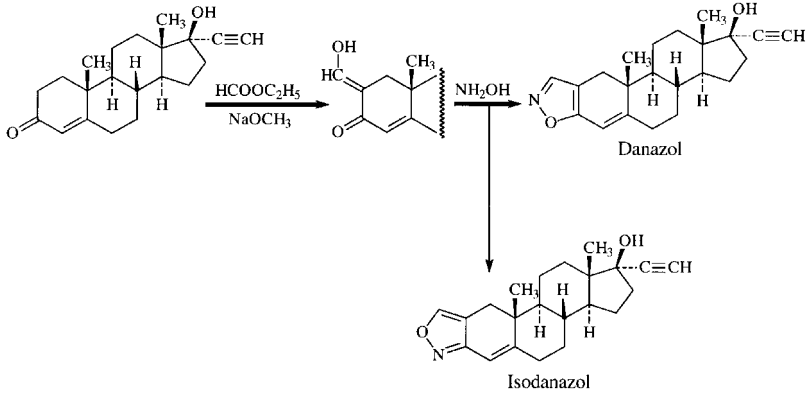


Figure 1.2.E.
Formation of danazol and isodanazol impurity

1.2.1.8. Degradation Products as Impurities

As is described in detail and illustrated by examples in Section 5.1, transformation/degradation of the final product of the drug synthesis can take place in the reaction mixture of the final step or during isolation, drying, etc. For this reason degradation products form a group of impurities in drugs. For example, during the course of the Mannich reaction in Figure 1.2.A leading to *tolperisone,* piperidine and formaldehyde can split off from the drug material to form 1-(4-methylphenyl)-prop-2-ene-1-one. *Papaverine* can be oxidised under the conditions of the final step of the synthesis to papaverinol and papaveraldine. The quantity of these products increases under storage conditions; for this reason they can be considered to be impurities and also degradation products. Sections 5.3–5.5 contain several more examples.

1.2.1.9. Enantiomeric Impurities

In the case of chiral drugs administered as the pure enantiomer the antipode is considered to be an impurity. The regulatory and analytical aspects of this subject are discussed in Chapter 6, where several examples are also presented.

1.2.2. Residual Solvents

Solvents are used in practically all phases of activities in the pharmaceutical industry. Residues of solvents are therefore usually present at least at trace level in bulk drug materials and pharmaceutical formulations. The origin of these solvent residues in *bulk drug materials* are as follows:

- The most likely source of solvent residues is the solvent of the crystallisation of the bulk drug material. There are various factors to be taken into account when selecting this solvent. The crystallisation should reduce the quantity of impurities to the lowest possible level with the highest possible recovery of the drug substance. The crystallisation from the selected solvent should generate the desired crystal morphology. In addition to these it should be volatile enough to be removable by drying at not too high temperature. It should be noted that many of the possible solvents are excluded from being used as solvents of crystallisation due to their high price or their health (Section 1.5.4.2) or environmental hazards. In some cases not pure solvents but their mixtures are used for crystallisation (e.g. different fractions of petroleum ether). In some other cases the solvent may contain other volatile components as impurities (e.g. 2-methylpentane, 3-methylpentane and methylcyclopentane in *n*-hexane). These are also possible trace level impurities in bulk drugs. For this reason the solvent used for the crystallisation should undergo a careful gas chromatographic check prior to its use and prior to the estimation of the residual solvent profile of the bulk drug material.
- In some cases the binding of some solvents to the drug material prior to the crystallisation can be so strong that they can be present at trace level even after the crystallisation. These impurities can be the solvent or volatile products of the last reaction step or volatile reagents used in the last reaction step (e.g. acetic acid or trifluoroacetic acid).
- If the final purification of the bulk drug material is column chromatography, the chromatographic solvent can also be present. If ion-exchange chromatography is used, the above mentioned acetic and trifluoroacetic acids and other volatile reagents can also be present.
- The bulk drug material can bind volatile components by adsorption from the air. Most typically this is water in the case of hygroscopic compounds but sometimes the most sensitive analytical methods can detect trace level volatile impurities which are present in the air which comes into contact with the drug material during the drying, packaging, etc. procedures.

Unless stoichiometric quantities of the solvents are present in all batches of the bulk drug (e.g. 0.5, 1, 2, etc. moles of water of crystallisation) and the solvates can be characterised by X-ray, IR and thermal analysis, any solvent

present, including water is considered to be an impurity and limits are set by the different pharmacopoeias for their quantity. The toxicological, regulatory and methodological aspects of this problem are discussed in Sections 1.3, 1.5.4 and in Chapter 3.

The obvious method for the removal of volatile impurities from bulk drug materials is drying at a suitable temperature preferably under reduced pressure. It must be noted that in many cases the removal of the last traces of solvents can be a very difficult, sometimes impossible task. Drying for too long at too high temperature can result in degradation of the drug molecule. Although the occurrence of oxidative type degradation products can be avoided or at least minimised by using an inert gas atmosphere for the drying, the ease of the reduction of the level of residual solvents below the limits set by the pharmacopoeias and various guidelines is an important factor to be taken into account when the solvent for the crystallisation is selected. Finding the most suitable solvent and the appropriate conditions for drying the bulk drug material is only possible with the active co-operation of analytical chemists to check the residual solvent profile in all steps of this research.

Solid dosage forms may contain volatile components originating from various sources.

- Solvent residues from the bulk drug materials.
- Solvent residues from the excipients. Lactose monohydrate contains stoichiometric quantities of water while others (e.g. starch) may contain adsorbed water up to 10%.
- Water, alcohol, 2-propanol, chloroform, dichloromethane and other solvents are used in wet granulation technologies and in the application of the active ingredients to the powder mixture of excipients by spraying.
- The same solvents are used to dissolve various polymeric materials used for the preparation of filmcoated tablets and various sustained release formulations.

The solvents are removed by drying and their quantity in the solid dosage forms is controlled by the same methods as in the case of bulk drugs.

1.2.3. Inorganic Impurities

There are various possible sources for inorganic impurities in drugs:

- The starting materials, reagents and solvents of the synthetic manufacturing process can be sources for salts of inorganic acids (chlorides, sulphates, phosphates, etc.). The same may contain various heavy metals as well.

- Heavy metals can originate also from the reaction vessels and tubings used in the manufacturing process.
- Filters, filter aids and adsorbents used mainly for decolourising the solutions of bulk drug materials during their crystallisation and chromatographic purification can also release heavy metals and salts of inorganic acids.
- Traces of some inorganic reagents themselves or their transformation products are also possible impurities. For example, in the reaction products of oxidations with oxidising agents such as selenium dioxide, chromium trioxide, permanganate and mercury(II) salts traces of selenium, chromium, manganese or mercury can be detectable. The use of reducing agents, e.g. lithium aluminium hydride or sodium borohydride can lead to the presence of traces of aluminium or boron in the finished products, etc.
- Heterogeneous catalysts such as palladium and nickel may contain as impurities their ionised form or the same can be formed during the reaction; this can be the source of such impurities in the bulk drug material.
- The degradation of the drug material can also be a reason for the presence of inorganic impurities (e.g. phosphate salts from the hydrolysis of phosphate esters, hydrazine from the hydrolytic splitting of hydrazides or hydrazones).

1.2.4. Impurities in Excipients

The estimated number of pharmaceutical excipients, i.e. ingredients used by the pharmaceutical industry to convert drug substances to pharmaceutical formulations is around 1000. The main pharmacopoeias include monographs for about 200 materials used as excipients. The most frequently used excipients are sugars such as lactose and sucrose, carbohydrate-type biopolymers such as starch and microcrystalline cellulose, cellulose derivatives, polymeric materials such as polyethyleneglycol (macrogols), polyvinylpyrrolidone (povidone), various oils of plant origin, stearic acid and its magnesium salt, inorganic materials such as calcium phosphate, various forms of silicic acid, etc.

Only a relatively small proportion of many of these materials is used for the preparation of pharmaceuticals: the majority is consumed by the food and cosmetic industries. As a consequence of this and of the complex nature of most of the excipients their quality and especially their purity is much more difficult to define and control than is the case with drug materials. However, the quantity of these materials in the pharmaceutical formulations is in the majority of cases higher than that of the active ingredient itself and for this reason their purity is an important issue [12–14]. Even minor impurities in not sufficiently

controlled excipients can be hazardous for the human organism and impurities can also influence in an undesired way the stability of the drug product, e.g. traces of metals in the excipient can catalyse the degradation of the active ingredient; peroxide impurities in polyethylene glycols can cause the degradation of oxidisable drugs [15], etc.

In the past few years great efforts have been made to catch up for the backlog of the regulatory aspects of the quality of excipients relative to drug materials [16,17]. This is reflected in the high number of conferences and courses devoted quite recently to this topic [18,19].

A few examples are presented below for impurities in various excipients together with their limits and the methods for their determination in the European Pharmacopoeia [2]. *Chlorides* and *sulphates* are determined by the methods described in Sections 4.2.2 and 4.2.4, but the limits can be higher than in the case of drug materials (e.g. in the case of *magnesium stearate* 0.025% and 0.5%, and *calcium phosphate* 0.15% and 0.5%, respectively). In the latter excipient the limit test for *fluoride* (determined by direct potentiometry) is 50 ppm. A limit test (10 ppm) for *nitrate* based on a colour reaction (diazotisation) is prescribed for *hydroxyethylcellulose*. Up to 1.0% chloride is allowed.

The limit set for *heavy metals* (see Section 4.2.7) is 30 ppm in calcium phosphate, 20 ppm in magnesium stearate, 20 ppm in hydroxyethyl- and hydroxypropylcellulose, 10 ppm in *povidone*, etc. The limits for toxic metals are much lower, e.g. 1 ppm nickel in *hydrogenated arachis oil* (determined by atomic absorption spectroscopy, Section 4.3.3) or 4 ppm arsenic in calcium phosphate (Section 4.2.9).

The determination of *monomers* in polymeric excipients is an important part of their analytical control. For example *1-vinylpyrrolidin-2-one* is determined in povidone by HPLC (limit 10 ppm), ethylene oxide in *macrogol* (1 ppm, head-space gas chromatography). A recent paper describes the determination of monomeric *4,4′-methylenebiscyclohexylamine* in a pharmaceutical polymer after extraction followed by solid phase extraction, transformation to the heptafluorobutyramide and GC-MS analysis [20]. The limit test for *hydrazine* in povidone is based on thin-layer chromatography of its derivative with salicylaldehyde (1 ppm). The determination of aldehydes is described in Section 2.2.1 (limit 500 ppm).

The limit for the *peroxide* content (expressed as hydrogen peroxide), e.g. in hydroxypropylcellulose is 400 ppm (colour test with titanium(III)-chloride).

Of the biopolymers the *protein* content of *potato starch* is determined as ammonia after digestion with sulphuric acid (limit 0.1%). The allowable limits for iron and sulphur dioxide are 10 and 50 ppm, respectively. Not more than 0.05% of *ether-soluble impurities* are allowed in *microcrystalline*

cellulose. Important tests in the analytical investigation of hydroxyethylcellulose are the determination of *ethylene oxide* (1 ppm), *2-chloroethanol* (10 ppm) by head-space gas chromatography and *glyoxal* (20 ppm) by a colour reaction.

The analytical control of fatty oils includes the gas chromatographic determination of *foreign fatty acids* and *sterols.*

References

1. S. Husain and R.N. Rao, *Proc. Control Qual.* **10**, 41–57 (1997)
2. *European Pharmacopoeia, 3rd edn*, Council of Europe, Strasbourg (1997)
3. S. Görög, G. Balogh, A. Csehi, É. Csizér, M. Gazdag, Zs. Halmos, B. Hegedüs, B. Herényi, P. Horváth and A. Laukó, *J. Pharm. Biomed. Anal.* **11**, 1219–1226 (1993)
4. S. Görög, A. Laukó, B. Herényi, G. Czira, É. Csizér and Z. Tuba, *Acta Chim. Hung*. **100**, 377–382 (1979)
5. S. Görög, A. Laukó and B. Herényi, *J. Pharm. Biomed. Anal.* **6**, 697–705 (1988)
6. S. Görög, B. Herényi and M. Rényei, *J. Pharm. Biomed. Anal.* **10**, 831–835 (1992)
7. S. Görög, A. Laukó, B. Herényi, A. Georgakis, É. Csizér, G. Balogh, Gy. Gálik, S. Mahó and Z. Tuba, *Chromatographia* **26**, 316–320 (1988)
8. S. Görög, G. Balogh and M. Gazdag, *J. Pharm. Biomed. Anal.* **9**, 829–833 (1991)
9. S. Görög, in *Steroid Analysis in the Pharmaceutical Industry*. (S. Görög, Ed.), pp 181–211, Ellis Horwood, Chichester (1989)
10. S. Görög and B. Herényi, *J. Chromatogr.* **400,** 177–186 (1987)
11. G. Balogh, É. Csizér, Gy.G. Ferenczy, Zs. Halmos, B. Herényi, P. Horváth, A. Laukó and S. Görög, *Pharm. Res.* **12**, 295–298 (1995)
12. S.C. Smolinske, *Handbook of Food, Drug and Cosmetic Excipients*, CRC Press, Boca Raton, FL (1992)
13. American Pharmaceutical Association, Royal Pharmaceutical Society of Great Britain, *Handbook of Pharmaceutical Excipients, 2nd edn*. The Pharmaceutical Press, London (1994)
14. H.P. Fiedler, *Lexikon der Hilfstoffe für Pharmazie, Kosmetik und angrenzende Gebiete*, Editio Cantor, Aulendorf (1996)
15. J.W. McGinity, J.A. Hill and A.L. La-Via, *J. Pharm. Sci* **64**, 356–357 (1975)
16. Z.T. Chowhan, *Pharm. Technol.* **19**, 43–48 (1995)
17. Z.T. Chowhan, *Pharm. Technol.* **21**, 56–67 (1997)

18. *Excipients in Pharmaceutical Dosage Forms: The Challenge of the 21st Century*, IPEC (International Pharmaceutical Excipients Council), Nice, 14–15 May (1998)
19. *Excipients in Pharmaceutical Formulations. Regulatory and Scientific Requirements,* APV (International Association for Pharmaceutical Technology), Darmstadt, 27–28 February (1997)
20. D.G. Watson, L. Li Xin, J.M. Midgley and D. Carr, *J. Pharm. Biomed. Anal.* **19**, 917–921 (1999)

1.3. Toxicological Aspects

Richard Lee

1.3.1. Introduction

Discussion of the toxicological aspects of impurity profiles for pharmaceutical products requires that several tenets of safety assessment be re-iterated, as a baseline for building the edifice of a reasoned and rational argument that seeks to limit both the number and amount of impurities on safety grounds.

Firstly, the licensing of medicinal products for sale in most countries of the world is based upon three premises, that the product will be efficacious in the disease to be treated, it will be sufficiently safe (taking into account other possible therapy) and will be of as good a quality as can reasonably be achieved, even if this is not an absolute requirement for safety.

Secondly, the route and formulation by which a medicine (and hence any impurity) is delivered can profoundly affect safety. In general, given an effective mechanism of clearance, the slower the rate of input to the blood circulation, the less likely are there to be systemic toxic effects. However, at the site of application of the product, concentration of all components will be at their highest and any local toxic action must always be taken into the safety reckoning for benefit: risk calculation.

Thirdly, the dose of active substance administered will vary considerably between products – at least two orders of magnitude are necessary to cover the range, which extends from micrograms to grams. Excipient doses can also vary – to a lesser degree – with route and formulation of product.

It is obvious, but frequently ignored, that this will influence the amount of impurity administered when, as traditionally, impurities are controlled on a percentage or parts per unit basis. Traditionally, the quality of medicinal products has been expressed as concentration (% or ppm) and their safety (toxicity) in amount (mg or mg/kg).

The toxicological assessor in a regulatory agency cannot necessarily be reassured on safety, simply by the limitation of an impurity to a low percentage level. Some medicinal agents are administered by mouth at doses of >30 g/day, so that even 0.01% of an uncharacterised impurity gives a potential patient exposure of >3 mg daily. A few substances would be lethal at that dose regimen!

Conversely, the analyst may not need to struggle to achieve a low (e.g.

0.1%) limit for detection, quantification, validation or reporting, if the daily dose to humans will only be in the microgram or low milligram range. As a rough guide, the limitation to 1 mg daily oral intake of an uncharacterised or poorly characterised impurity will probably satisfy a safety assessment for regulatory purposes.

With this background in mind, the problems connected with safety and impurity profile are the main topic of this chapter, always remembering that safety cannot be entirely divorced from efficacy and that the quality of a pharmaceutical product must serve both safety and efficacy to be effective.

It is unlikely that the presence of any impurity will have a great influence on efficacy; pharmacological activity in precursors or breakdown products of the active substance may be found for some products, but the contribution to overall effectiveness will remain insignificant if the impurity level is controlled to $<5\%$, since this degree of differentiation is seldom achieved in clinical trials – 90% confidence in a 20% difference between treatment groups being considered acceptable in some cases.

Safety, by contrast, can be affected by relatively low levels of impurity. A toxic impurity may be chemically related to the active substance, or a residue of an agent or solvent used in the synthetic process for the active substance or an excipient, or some contaminant introduced during the purification stage of manufacture – leachates from purifying filtration/separation columns or from packaging materials, for example.

Incidental contamination from external sources has been known, but it would be unreasonable to expect process controls to check for such impurities and thus it is not considered in detail in this chapter.

Enough must be known about the impurity profile (both qualitative and quantitative) to allow a judgement on whether one or all of the Quality Specifications applied to a product are appropriate for their purpose, i.e. the impurity will not present any concern over safety or will be an acceptable risk factor for treating serious diseases where there is no other therapy suitable for a particular patient.

Thus, a toxicologist in a regulatory agency examining the impurity specification for a pharmaceutical product will require reassurance derived from questions similar to those which follow.

- What will the daily intake of any impurity be if the patient complies with the product labelling on the maximum recommended dose regimen?
- Has the toxicity of the impurities in the product been elucidated and evaluated by valid experimental studies (in the scientific literature or specifically for the product)?
- If there are no relevant toxicity data, does the daily exposure combined with the structure of any impurity raise safety alerts from previous experience?

- If there is neither information on safety, nor identification/characterisation of the impurity, is it acceptable from a risk: benefit analysis to permit the level of impurity proposed?
- Is the degradation on storage established and validated as acceptable by argument based on known scientific data or by appropriate preclinical safety studies?

This chapter pursues the above thought process for the various types of impurity possible in a medicinal product, with the objective of making clear what issues of safety should be considered, rationalised and eliminated before an impurity specification is proposed by a pharmaceuticals supplier to a regulatory authority, which has the duty of safeguarding public health.

No attempt has been made to describe the detailed toxicological effects of any of the examples of process impurities commonly encountered in pharmaceutical products. For this the reader is referred to any of the comprehensive text-books on toxicology; the references for this chapter are mainly to guidance that deals with the subject, as an aid to regulators and suppliers.

For this purpose, there are guidelines adopted in the EU, USA and Japan (with tripartite guidance under the auspices of ICH – an international conference to harmonise development requirements for pharmaceutical products in these three areas of the world).

For pharmaceutical manufacturers, developers and regulators concerned with European registration and licensing, guidance can be found in Volume III, Part 1 of ''The Rules Governing Medicinal Products in the European Union'' under the headings 'Quality, Biotechnology and Pharmaco-toxicological Guidelines'; those relevant to limiting impurity levels in drug substances and in the final product (see Ref. [1]) are here taken into account in suggesting what information is necessary to set a logical specification.

More recent ICH guidance (Q6A) considers the problems of setting appropriate impurity specifications and several European guidelines deal with quality issues which bear in mind the need for minimum additional hazard to patients from this source. These are also in the list which concludes this chapter.

It is stressed that all this guidance is subject to change as ICH enters its second phase of review (1998–2000) and work proceeds on ''The Common Technical Document'' (CTD) which recognises some anomalies have emerged from the existing guidelines.

For example, in the field of impurity control and safety issues, there are less stringent proposals in Note for Guidance Q3B (dealing with pharmaceutical products) than in Q3A, which preceded it and restricted its scope to bulk pharmaceutical substances.

The CTD (Sections S3 and P5) will address the use of toxicity testing in

the validation of specifications for active substances and pharmaceutical products, respectively.

1.3.1.1. Biological Pharmaceuticals

This sub-heading is used in each section to consider how the impurity issues may change when the therapeutic agent is not a conventional synthetic or extracted chemical of relatively low molecular weight, but is a complex of high molecular weight, often branch-chained polymers, which act on the body in ways similar to those of native proteins and related substances. They may be derived from these native substances by extraction and purification of body tissues (fluids) or synthesised by recombinant DNA systems in micro-organisms or mammalian cell cultures.

Some of the associated impurities may be specific to these 'biologicals' – DNA fragments, viral residues and other cellular components not cleared by the purification procedures. [1]. Also this class of product has its own array of solvents and detergents that may be residual impurities. It is emphasised in this ICH guidance note, that purification of biological products is an essential part of safety reassurance and that reliance on animal model tests alone is unlikely to meet with regulatory approval

1.3.2. Impurities Closely Related Chemically to the Active Substance

This group of impurities can arise in a variety of ways, depending on the nature of the active substance and the synthetic process involved in its manufacture. The chemical relationship may be homologous, if a series of closely related compounds is produced by the same synthesis, analogous (perhaps the most usual) when a precursor is modified only slightly in the final stage of synthesis or isomeric, where for example, one enantiomer is separated from a racemic mixture.

Any pharmacological activity in such related molecules is often of the same type as that of the active substance, and the presence of these impurities in small amounts adds nothing significant to the overall action of the product. However, enantiomers frequently have different pharmacological profiles.

The addition or subtraction of a single methyl or hydroxyl group, at a strategic position in the molecule, can fundamentally alter the interaction between the agent and the mammalian body. However, many immediate precursors of active pharmaceutical agents have their reactive moieties masked to prevent unwanted side-reactions and so are less likely to contribute pharmacological activity to the final product.

The regulatory authorities consider it desirable to have evidence of how

extraneous pharmacology, or toxicity, could be related to the level of any impurity, similar in chemical structure to the active substance, unless it is a normal metabolite involving small change to the structure such as oxidation, decarboxylation, deamination, etc.

Despite the desire to understand concentration versus effect relationships, it may only be feasible to extrapolate from safety test systems the prediction that the maximum impurity level permitted by the specification is unlikely to contribute any significant adverse effect, when administered to the selected patient population.

It will be necessary to demonstrate that the maximum possible human exposure to an impurity has been justified by toxicology studies that relate exposure to effect in terms of escalating dose, i.e. the impurity has been present proportionately at all dose levels in repeat-dose toxicity studies that exposed the animals to more than the maximum possible human exposure and found at least one dose that was without significant adverse effect.

The optimal conclusion reached by such studies is that the impurity has been present in all material tested preclinically in vitro or in intact animal models, has also been present in appropriate concentration in material used for clinical trials and, according to the final specification, will only be present in material used to generate the marketed product in amounts less than that already shown to be sufficiently safe for the purpose of that product.

Occasionally, a problem arises because the scaled-up manufacturing procedure differs from that used to synthesise the smaller quantities suitable for animal studies and clinical trials and has, as a consequence, a different impurity profile. If this is only a quantitative change, some calculation using toxicokinetic data on exposure levels in the test animals may serve to reassure that safety has not been compromised.

If the change produces novel impurities – so-called ''unqualified'' in the ICH guidance – which have not been tested for adverse action, then the specification (conventionally quoted in terms of maximum percentage) may possibly be based on the guideline dispensation that uncharacterised (or partly characterised) impurities may be acceptable if the daily intake does not exceed 1 mg and there are no 'alerts' from what is known or can be inferred about the structure, or what may be deduced from the new manufacturing route of synthesis and its associated reagent inventory.

Changes in the synthetic route at a late stage of development, which produce new impurities above the dispensation level, can be examined for safety by studies of repeated-dose toxicity and genotoxicity. Details of such studies are given in Section 1.5.

For a developer to return to a preclinical safety testing programme, in order to qualify novel impurities at a late stage in development, is generally held to be economically unsound and a last resort strategy. But if $>0.1\%$ has to

be tolerated for manufacturing reasons, then return to a preclinical toxicity programme of pivotal studies may be the only solution to the problem.

The case where, for example, a racemic active substance is converted to its corresponding eutomer in a new product is a special one and is covered by separate guidance [2] which deals with the distomer as a specific impurity and recommends a number of 'bridging' toxicity studies to be conducted to link the toxicology of the racemate with that of the single enantiomer.

In order to ascertain that $<0.1\%$ is present in an oral dosage form or that it will deliver <1 mg/day to the patient, it will be necessary to demonstrate that the analytical method used to assay the impurity is sufficiently accurate to make the claim. The method optimised for the active substance (which presumably detects the impurity as a percentage in the first instance) may not be good enough to determine the impurity with adequate accuracy. Corroborative evidence will need to fix the molecular weight of the impurity within reasonable limits and provide sufficiently sensitive analysis to translate percentage into maximum possible amount in the dosage form.

If a 'new' impurity is unavoidable and may occur in several products, it can be isolated and identified with regard to structure, when it may be possible to justify the inevitable, but low, percentage levels. If the level is not justifiable, the compound would need to be synthesised and a limited safety testing programme conducted to cover the desired residual level as has been done for solvents [1]. However, there is no guidance as to the content of such a programme that would be acceptable to regulatory authorities, and prior discussion with regulatory assessors is advisable to agree the proposed testing in principle.

1.3.2.1. Biological Pharmaceuticals

The concept, of having synthetic-route impurities that are closely-related in structure to the active substance, frequently does not apply to protein or polymeric macromolecular products, which by nature have a range of related structures making up the totality of active substance. Some of the molecules at the extremes of this range may contribute little to the desired therapy, but are not considered as impurities in the generally accepted sense.

However, in some cases the product should be a monomer and any dimer present would be regarded as an impurity and require qualification, possibly by toxicity testing.

There is also the possibility of finding deaminated, oxidised or sulphide bond mismatched derivatives of the active substance and truncated forms generated by enzyme degradation within the cell culture employed. Removal of these is preferred to qualification by animal toxicology.

Similarly, any attempt to unravel the effects of variable glycosylation or of

having C-terminal lysine variants in monoclonal antibody products by the use of crude animal toxicity-testing systems is to be avoided.

1.3.3. Impurities Less Closely Related in Structure to the Active Substance

The intermediates of earlier stages of synthesis (if they are not removed by further reaction steps and purification processes) and degradation products, which may be dependent upon storage conditions and can determine shelf-life as part of the safety aspects of the product specification fall into this category.

The same dispensations on concentration in the product [1] are available if the impurity is 'unknown' and the same criteria for identification and reporting apply.

Unless the same structures are identified as the products of human biotransformation, or they are, co-incidentally, of known structure and toxicity, there will be a need to ensure that adequate exposure has occurred in toxicity testing, so that the specified residual level combined with maximum human dose does not allow a known safe intake to be exceeded.

The administration of an impurity that is also an identified human metabolite of the active substance has not been considered by the regulatory authorities to require separate safety testing procedures, even if the structure is more remote from that of the administered substance as may occur after hydrolytic cleavage, for example, provided that it has also occurred in the species used for preclinical toxicity testing. In this case, it is considered that the impurity should be well-controlled in manufacturing so that a meaningful specification for level at release can be set in order to retain sufficient dose within the dosage form throughout the shelf-life.

1.3.3.1. Biological Pharmaceuticals

As for closely-related substances, the extremes of the range of molecular variation, within a protein of other macromolecular product, may well cover entities that are, in effect, inactive 'impurities'. To date no information has emerged to implicate such molecules in safety issues and the specification on range of molecular weight is the only practical control of this situation. Any chiral variants within the active substance are also deemed to be part of that substance and not impurities.

1.3.4. Impurities of Known Structure, not Related Chemically to the Active Substance

This group of chemicals usually consists of small molecules, which are residuals of the manufacturing process. They may be heavy metals, solvents, reagents or catalysts, precipitating agents, detergents, etc. depending upon the type of product under consideration.

Some of these are specifically controlled through regulatory guidance. There is, for example, an appendix to the guidance on Residual Solvents [1] that contains a thorough literature search on the toxicity studies conducted on the more commonly used solvents. Another appendix gives the rationale for converting toxicology data into percentage, or parts per million, levels that will likely be acceptable in any product, or can be used to justify impurity levels in a particular product. The information on which safety factors to apply to solvent safety data of different types can be extrapolated to provide a guide for any impurity of known structure with limited toxicity studies.

The toxicology of other reagent impurities can be gleaned from standard text-books or by searching published databases. It is not the objective of this chapter to provide an exhaustive index of detailed toxicity, but rather to illustrate the nature of concerns that form in the minds of regulatory assessors when a perceived hazardous impurity appears in a product specification at a level that appears unjustified.

For conventional medicines the specification has, for a long time, controlled such possible toxins as heavy metals (lead, mercury, platinum being the common examples) based on what is considered to be an acceptable daily intake for humans, i.e. one that will not add significantly to the burden of toxicity attributable to 'normal' living conditions. The same principle is usually applied to other inorganic or small organic molecules, although for some, e.g. benzene (considered to be a human carcinogen) and ethylene oxide (a known mutagen) there have been proposals for zero tolerance, which in practical terms means control at the generally available level of analytical capability currently achievable.

Protection of the environment sometimes impinges upon pharmaceutical development in seeking to restrict the general use, of e.g. chlorinated hydrocarbons, thereby making them uneconomic for inclusion in medicinal products, or indeed to exclude CFCs from formulations such as aerosol inhalers, within a limited time-frame.

It is possible that an impurity in this category will be novel in the sense of having no toxicology to support a specification. In this case, it may be possible to argue that it should be treated as an 'unknown' for the purposes of applying the ICH guidance, especially if it is present at $<0.1\%$.

Alternatively it may have been qualified at the required level of intake by

being present at an appropriate concentration in the batches use to conduct preclinical toxicity testing.

The only reason for a regulatory assessor to challenge either of these qualification criteria would be if the structure contained moieties associated with specific toxic action in other molecules, e.g. primary amines, nitro groups, epoxides or methylating entities might cause the assessor to seek more reassurance. Additionally, the propensity of a small molecule to bind tightly to human proteins could be construed as hazardous, since there may be the potential to form allergenic complexes whose antibodies could cross-react with native proteins. There are computer programs of structure–activity relationships available to be consulted (e.g. TOPKAT and DEREK) that will forecast toxicity (J. Maldacker, Logos GmbH, Germany, 1998, pers. commun.).

1.3.4.1. Biological Pharmaceuticals

In medicinal products from biotechnology, the residuals often give rise to more concern over safety than the active substance. Thus viral removal and validation of the techniques to achieve this have been the subject of specific guidance [1]. Qualifying and specifying levels of viral material in human medicines is achieved by experience plus some animal model testing – short-term administration to detect unanticipated toxicity, pyrogenic effects, etc.

Any residual protein or other macromolecule considered theoretically capable of producing an immune response – and hence the possibility of antibodies that will cross-react with native substances and result in an auto-immunity hazard – will be viewed with suspicion, unless there are appropriate data from sensitive animal models to show the absence of any significant cause for concern.

Solvents and phosphate-based detergents remaining in 'biological' medicines have also given rise to concern about safety when it cannot be demonstrated clearly that these have been removed to insignificant levels, and there was not sufficient toxicology data to support the possible residual amounts that could be administered parenterally to humans according to the proposed methodology.

Other process impurities include inorganic salts, carriers, ligands and substances leachable from columns used in purification.

1.3.4.2. Excipients

The issue of possible impurities in excipients can be dealt with under this heading, since it is unlikely that any such impurities will coincide in structure

with any that derive from the synthetic process. It is the manufacturer of the medicinal product who has the responsibility of ensuring that the product is sufficiently safe for its purpose. In the past, few questions seem to have been asked of excipient manufacturers regarding the impurity profile of their contribution to the whole product. This appears to be changing and, at least in terms of residual solvents, representatives of the excipient manufacturers have been present at ICH discussions on the safety aspects and agreed that mechanisms to provide the necessary knowledge should be put in place.

The nature of the possible impurities that could be introduced with excipients, or indeed with the other components associated with the later stages of manufacture of a pharmaceutical product, is very diverse; experience has shown that the greatest hazard is likely to come from powdered glass after a breakage during the process. This would be difficult to detect or to control by specifications on appearance, odour, etc. especially in opaque products designed for oral or topical administration.

It is not the intention of this chapter to cover the range of possible contaminants that would be unacceptable in a pharmaceutical product. This topic is not covered by regulatory guidelines, but there are several classes of compound that would not be welcome – for example, tetrahydropyridine neurotoxins (including MPTP), mycotoxins (from *Arthrinium* spp. on sugarcane) and cyanogenic glucosides (as in cassava root) might conceivably be introduced. The toxicity of these is described in the Proceedings of the fifth International Congress of Toxicology held in 1989 [3]

1.3.5. Impurities of Unknown Structure

The debate here revolves around what is truly unknown. The mere detection of an extraneous substance in a pharmaceutical product will tell something of its character, e.g. UV absorbance at a certain wavelength, retention time in a particular chromatography separation system and resistance to removal in the purification procedures employed.

Nevertheless, such information is sometimes all that characterises a previously undetected peak in the chromatogram, for example, and may be more or less reassuring on safety depending on circumstances.

However, the current guidance rather generously (from the toxicologist's viewpoint) allows qualification of such impurities at 0.1% for active substances administered at no more than 1 g/day [1].

The basis of this concession is that very few chemicals have been found to be significantly toxic at <1 mg intake daily, but there have been exceptions to this.

Specific problems such as beset tryptophan, in which the so-called peak E impurity appeared to be involved in causing an adverse reaction [4] will not be

covered by the general guidelines designed to control quality and safety on the basis of what is known about the vast majority of pharmaceutical materials; for some details see Section 1.4.2. The salutary lesson to be learned from this experience is that some safety analysis and assessment should be done when manufacturing processes change, e.g. in response to increased demand for product.

Submissions to vary a licence for marketing a pharmaceutical product, which incorporate a change of impurity specification, require a safety assessment – on paper at least – and possibly involving new toxicity studies, although any regulator requesting these would need to have adequate reason, based on suspicions about the nature of any new 'unknown' impurity, derived from its provenance and any defining characteristics.

It will be expected that a good attempt has been made to reduce any such 'unknowns' to a minimum, using conventional purification techniques.

1.3.6. Best use of Resources

Although the number of toxic incidents, shown to be caused by impurities in pharmaceutical products is very small, there are three ICH Notes for Guidance which seek to control the amount and type of impurity acceptable in bulk active substances and finished products or advocate toxicity testing to justify exceeding prescribed limits.

The resource question therefore has two aspects. Should time and money be spent on more sophisticated purification and analytical monitoring procedures which become exponentially more expensive as the impurity level decreases? Alternatively, is there more merit in spending resources on safety testing at all those stages of development which involve any significant change in impurity profile?

The answer to these two questions is neither simple nor universal, but some general advice can be offered. Each case will need to be examined to discover which is the more resource-efficient strategy.

When it is relatively easy to achieve $<0.5\%$ (for example) of impurity in a very potent pharmaceutical agent, whose low dose will carry very little impurity to the patient, this will allow minimal safety testing and a concentration of resource on the analytical method to ensure continued adherence to specification. In this way safety is achieved through consistent quality; this is considered to be particularly important for 'biological' products, in which the differentiation of effect between active substance and uncharacterised impurity may be difficult.

On the other hand, when the active substance has relatively low potency, necessitating a high dose input to achieve efficacy, then quite low percentage of

impurity (<0.05%) results in a considerable dose of impurity to patient, so the more useful resource may be the toxicity testing, rather than attempted removal of impurities at very low concentration, with the concomitant difficulty in determining whether purification has been successful.

1.3.6.1. The Toxicity Testing Programme

Historically, the appearance of a new 'unqualified' impurity in a medicinal substance or product, prompted, at most, a study in mice, the design of which was a single dose of one relatively high dose-level, followed by observation of clinical signs of toxicity, including death, over the succeeding 7 days.

This type of protocol has now been superseded by a study of general toxicity using repeated daily dosing for a minimum of 14 days (and an accepted maximum duration of 90 days) the length of study being governed by the concentration of impurity to be qualified and any knowledge of the nature of the impurity concerned. In general, a 28-day duration is acceptable to regulatory authorities for most circumstances.

If the active substance has been tested in a full preclinical programme previously, the absence of any new toxic effect at one dose level (preferably close to the no observed adverse effect level (NOAEL) established for earlier batches) will suffice to 'link' the new batch to the safety testing of previously-tested material. Otherwise, the study should include three dose levels over the range expected to cover the comparable human exposure in recommended use up to the desirable safety margin. For the purpose of estimating systemic exposure, it will be assumed that bioavailability of the impurity is 100% and is approximately proportional to dose.

The observations made in this study should include weight gain of the animals (one species selected for its relative lack of sensitivity to the effects of the active substance – in order to reveal more readily any action of the impurity), behavioural patterns, blood chemistry, haematology and histopathology of important tissues – according to standard protocols for this type of general toxicity testing.

If any new toxic effects emerge, it may be necessary to re-establish a NOAEL or to adjust the original safety margin, which should remain appropriate for the eventual indications of the product.

Testing the isolated impurity for sub-chronic toxicity would involve the use of three dose levels to give sufficient data for calculation of 'permitted daily exposure' as in the Residual Solvents guidance [1].

The second type of study, considered obligatory by regulatory authorities for reassurance on the safety of novel impurities above the 'qualification' threshold, is the package of genotoxicity assays recommended by ICH as the 'standard battery' [5].

Absence of any positive reaction, at the highest recommended exposure, in the three assays (commonly referred to as the 'bacterial mutation [Ames] test, the mouse lymphoma assay and the rodent micronucleus test') is required to qualify the impurity at the concentration present. It may thus be advantageous, when testing the active substance containing the relevant impurity, to test a batch with the highest impurity level in order to achieve the best level of qualification. Alternatively if the impurity is isolated and tested separately, it may be prudent not to load the test systems beyond what is needed to provide reassurance for the product.

The guidelines also mention the possible need for studies on toxicity to reproductive processes, but no examples are given as to where this would apply. If the product is one whose active substance is commonly prescribed in pregnancy or for women likely to become pregnant, then a study of teratogenic potential should be considered [6].

If it is assumed that the need for new impurity qualification by toxicity testing arises at a relatively late stage of the development of a putative medicinal product, then timing is not an issue – any study should be completed as soon as possible to safeguard any patients or healthy volunteers who are participating in clinical trials. However, the usual timing for toxicity studies (active substances with a relevant impurity profile) is recommended in a further ICH guideline [7] and this may be followed if clinical trials have not commenced.

1.3.7. Concluding Commentary

Past experience of the pharmaceutical industry and regulatory assessors, suggests that too little consideration has been given to the issues linking the initial synthetic chemistry (involving small quantities) with scale-up to anticipated manufacturing levels for sales needs, and the attendant changes of impurity profile and the timing of the pivotal toxicity studies to qualify (validate) the eventual impurity specification of the finished product. Project teams should discuss these elements of the development process and try to anticipate problems and assign the appropriate resources to resolve possible issues as early as is feasible.

The advice to pharmaceutical developers, in the form of ICH and other guidance notes, should now be sufficient to induce an understanding by analytical and synthetic chemists of the concerns of toxicologists and an appreciation by the latter of the difficulties involved in reducing impurities to the desired level for minimum risk and of monitoring these limits by the best available methodology.

This mutual comprehension of all the issues surrounding impurity control

should provide an acceptable rationale for the relevant part of the product specifications proposed in an application to market a new product. Good scientific reasoning, based on the accepted guidance, ought to produce consistent regulatory decisions, content pharmaceutical manufacturers and safe medicinal products.

References

1. P.F. D'Arcy and D.W.G. Harron (Eds.), *Proceedings of The Third International Conference on Harmonisation,* Yokohama 1995, The Queen's University, Belfast, UK
   List of Notes for Guidance
   1.1 Impurities in New Active Substances (ICH Q3A) [Adopted in Europe: May 1995 EudraQ/95/022] (Sometimes referred to as 'Impurities in New Drug Substances')
   1.2 Impurities in New Medicinal Products (ICH Q3B) [Adopted in Europe: December 1996 CPMP/ICH/292/95] (Sometimes referred to as 'Impurities in New Drug Products')
   1.3. Validation of Analytical Procedure: Methodology (ICH Q2B) [Adopted in Europe: December 1996 CPMP/ICH/281/95]
   1.4 Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances (ICH Q6A) [CPMP/QWP/367/96–not adopted at time of writing]
   1.5 Residual Solvents in Pharmaceutical Products (ICH Q3C) [Adopted in Europe: September 1997 CPMP/ICH/283/95]
   1.6 Virus Validation Studies: The Design, Contribution and Interpretation of Studies Validating the Inactivation and Removal of Viruses [Adopted in Europe: February 1996 CPMP/BWP/268/95]
   1.7 Preclinical Biological Safety Testing on Medicinal Products Derived from Biotechnology (ICH S6) [Adopted in Europe: September 1997 CPMP/ICH/302/95]
2. *Clinical Investigation of Chiral Active Substances.* CPMP Note for Guidance Eudra/C/91/038 in 'Rules governing Medicinal Products in the European Union, Volume III, p. 603, January (1996)
3. G.N. Volans, J. Sims, F.M. Sullivan and P. Turner (Eds.), *Basic Science in Toxicology,* Taylor & Francis, London (1990)
4. S. Smith, *Pharm. J.* **261**, 819–821 (1998)
5. Note for Guidance on Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals (ICH S2B) [Adopted in Europe: September 1997 CPMP/ICH/174/95]
6. Note for Guidance on Reproductive Toxicology: Detection of Toxicity to

Reproduction for Medicinal Products (ICH S5A) [Adopted in Europe: September 1993 CPMP/ICH/386/95]
7. Note for Guidance on Non-clinical Safety Studies for the Conduct of Human Clinical Trials (ICH M3) [Adopted in Europe: September 1997 CPMP/ICH/286/95]

The CPMP reference and adoption date in Europe are given for ease of obtaining guidelines from the European Medicines Evaluation Agency (EMEA) based in London.

1.4. The Role of Impurity Profiling in Drug Research, Development and Production

Sándor Görög

1.4.1. Impurity Profiling in Synthetic Drug Research

The use of analytical methods is of utmost importance in all phases of synthetic research and related areas (biotechnology, extraction of materials of plant and animal origin) aiming to introduce new chemical entities into the therapy [1–3]. During the gram-scale preparation of new compounds for pharmacological screening the analytical activity takes place in two main directions: (a) structure elucidation of the reaction products by spectroscopic methods, and (b) estimation of their purity (endproduct and its intermediates). When the synthetic chemist takes a sample from the reaction mixture or the crude reaction product for a rapid chromatographic (mainly TLC) test the primary aim is not yet impurity profiling. On the basis of the chromatogram it is possible to get a picture on the course of the reaction. The questions to be answered are if the reaction is completed (the spot/peak of the starting material disappears) and if so is it unidirectional under the selected conditions (one main spot/peak accompanied with minor ones or commensurable spots/peaks appear). On the basis of the data thus obtained the organic chemist is able to select the suitable reaction to reach the goal and optimise the reaction conditions.

It would be difficult to state at which point of the synthetic research real impurity profiling begins. The requirements for the purity of the samples can be quite different in various research departments. Generally speaking it is not reasonable to prepare the large number of test samples for the first pharmacological screening in highly purified form: it is predictable at this stage that the overwhelming majority of the new compounds will be dropped after the first tests and there is ample time afterwards to estimate the impurity profiles of the few materials which have been selected for further investigations. (This is even more the case with the products prepared by means of combinatorial chemistry where mixtures of large number of components are subjected to high throughput screening. The tasks of analytical chemists in this area are quite different.) It is, however, sometimes the case that organic chemists require the identification/structure elucidation of key impurities already at this stage of the research since the rational strategy for the preparation of the target molecule can only be established in possession of this information.

The drug candidates selected for further pharmacological and toxicological tests should undergo thorough analytical investigations. Generally speaking the analytical information obtained on the selected materials should grow together with the amount of chemical and pharmacological information. After a further decision point the organic chemists have to optimise the synthesis and purification of the materials in order that these can be scaled up to prepare material for the pharmaceutical technologists to develop the drug formulation and for the further, decisive toxicological, preclinical and clinical trials. The most important task in this cooperation between organic chemists and analysts is the estimation of the impurity profile of the potential drug substance as a function of several factors (conditions of the reaction, purification and storage, selection and quality of the starting materials, reagents, solvents, catalysts, etc.). It should be noted that in this stage of the development it is not mandatory to identify the impurities, it should be, however, assured that the same impurities occur in and the same limits apply to the batches used for these trials and for those reported in the registration documents and to be used in the therapy. For details see Section 1.5.3.3. It is, however, the general practice of drug manufacturers that impurity profiling studies even in this earlier phase of the development include the identification of the impurities above the threshold limits (0.05–0.1%) since the organic chemists and pharmaceutical technologists need this information for their work.

In the course of the investigation of the influence of the above listed factors on the impurity profile special attention should be paid to the purity of the starting materials of the synthesis. Organic chemists usually use materials of higher purity during the gram-scale synthesis than it would be reasonable from the point of view of the economy of the production of industrial batches. In some cases the purity of the starting material more or less determines the purity of the endproduct. For example the reaction leading to *flumecinol* (3-trifuoromethyl-α-ethyl-benzhydrol) was the addition of the Grignard reagent prepared from 3-trifluoromethyl-bromobenzene to propiophenone. Any 4-trifluoromethyl-bromobenzene impurity in the reagent causes the appearance of the isomeric 4-trifluoromethyl derivative separable by packed column [4], capillary column [5] gas chromatography and high-performance liquid chromatography [6] from the main component. (See Figs. 2.6.D and E in Section 2.6.6.) Since the price of the reagent depends on its isomer content a compromise had to be reached as regards its quality: it should be acceptable from the point of view of both drug safety and economy. It is a general rule that it is not prudent to use unreasonably pure endproduct for the preclinical studies because the same purity will be required for the clinical and industrial batches.

The estimation of the impurity profile of a drug material includes the identification of the main impurities in the intermediates in their synthesis, too. The registration documentation should contain the description of the

reasons for the presence of the impurity. In the case of a synthesis related impurity this means that it should be proved that it is really a synthesis related impurity and the mechanism of its formation should also be presented. This cannot be done without the identification of the main impurities in the intermediates.

The methodological aspects of the identification and quantitative determination of the impurities in bulk drug materials are outlined in Section 2.1 and discussed in detail in Sections 2.2–2.11. The estimation of enantiomeric impurities is the subject of Chapter 6. The identification of degradation products and determination of degradation pathways also begins already in the course of the analytical studies with the bulk drug material (stability studies under stress conditions). This aspect is outlined in the following section and discussed in detail in Chapter 5. Impurity profiling also includes the identification and quantitative determination of solvent residues and inorganic impurities. These are subjects of Chapters 3 and 4, respectively.

1.4.2. Impurity Profiling in the Production of Bulk Drugs

The analytical activities related to the estimation of impurity profiles do not come to an end after the R&D phase of the introduction of a new drug. It is essential to ensure that no new impurities appear in the course of the scaling up procedure and the quantity of the impurities in the bulk drug material identified during the synthetic research phase remain below the specification limits. For this reason the analytical control of all steps of the scaling up procedure is of key importance. It can happen that a new impurity appears during the scaling up or even more typically the quantity of an impurity which was detected but not identified during the synthetic research period since it was in the low 0.01% range reaches or exceeds the threshold limit where its identification is mandatory. In such a situation the structure elucidation of the impurity is an important task in order that in possession of its structure the technologists can make the necessary steps to avoid its formation or at least reduce its quantity.

More or less the same applies to the cooperation of drug analysts and technologist in the course of the production of the bulk drug material in the routine scale. Even under the strictly controlled conditions of a carefully validated technology the possibility of changes in the impurity profile similar to those described in the preceding paragraph cannot be excluded. As described earlier the impurity profile depends on many factors and even minor changes in one of them can result in considerable changes and the analytical chemist should be prepared to give a rapid answer to any questions of this kind arising during the production of the bulk drug. To be able to do so it is also important to estimate the impurity profile of the (key) intermediates in the synthesis and

build in the necessary steps into the in-process-control protocol in order that the origin of a new impurity in the endproduct of a multistep synthesis can be rapidly identified.

The great importance of carefully controlling the effects of even minor changes in the technology on the impurity profile and as a consequence of this on drug safety can be characterised by a scandal related to the questionable purity of certain industrial batches of L-*tryptophan*. This amino acid which is said to have health benefits is widely used as a dietary supplement. At the end of 1989 an epidemic broke out referred to as eosinophilia-myalgia syndrome (EMS) which affected thousands of the consumers of L-tryptophan killing over 30 of them. Careful investigation revealed that these adverse effects occurred only with some batches of L-tryptophan produced by a single manufacturer prior to the outbreak of the epidemic. This manufacturer produced L-tryptophan by fermentation. It became clear that in the course of the production of these batches a new strain of *Bacillus amyloliquefaciens* had been introduced and at the same time the amount of activated charcoal had been reduced in one of the purification steps. The removal of the suspected batches from the market essentially stopped the epidemic. An extremely wide and intense research was launched to investigate the impurity profile of the suspected batches. With the aid of mainly HPLC, HPLC/MS, HPLC/MS/MS and NMR studies several minor impurities were detected and identified [7–10]. Although this research is still going on [11,12] and the picture is still not completely clear from the epidemiological point of view; some of the impurities, among them 3-(phenylamino)alanine, 1,1′-ethylidenebis(tryptophan), 2-(3-indolylmethyl)-L-tryptophan and 2-(3-indolyl)-L-tryptophan were found to be associated with EMS. Quite recently similar studies were carried out with *melatonine,* too, high doses of which also cause EMS-like symptoms. Some of the impurities found were structural analogues of the above mentioned EMS-related impurities [13].

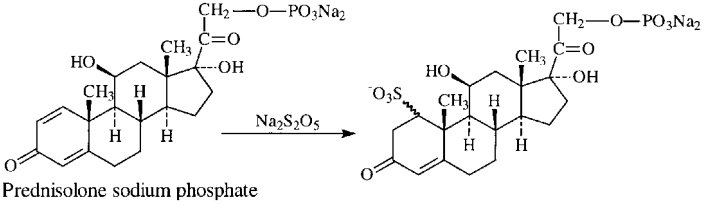
1.4.3. Impurity Profiling in the Research and the Production of Drug Formulations

The identification, structure elucidation and quantitative determination of impurities and degradation products are of prime importance in the course of all phases of research, development and production of drug formulations. The close relationship between impurities and degradation products is discussed in Section 5.1. The pharmaceutical technologist should have a clear picture about the impurity profile of the bulk drug material used for the development of the formulation in order to be able to differentiate between synthesis-related impurities and degradation products. In such a way the stability indicating nature of the analytical method to be used in the course of the development of a drug

formulation can be established. These studies indicate which of the impurities in the bulk drug material are of degradation product type. The increase of these is expected during the stability studies while the synthesis-related impurities are likely to remain constant.

The pharmaceutical technologists should also be aware of the results of the *preliminary stability studies* carried out with the bulk drug material under stress conditions (see Section 5.2). Since these investigations can be considered to be part of the *preformulation studies* [14], in some drug companies these are carried out by the pharmaceutical formulation group while in some others this is part of the responsibilities of the analytical group. These studies reveal the degree of possible sensitivity of the drug molecule to heat, light, humidity, acidic, basic or oxidative conditions (including the investigation of the stability vs. pH profile), traces of metal ions, etc. On the basis of these studies some of the real degradation products of the drug formulation can be predicted while some others may be identified during the course of the stability tests with the formulations.

In possession of suitable analytical methods and the above listed data regarding the possible degradation mechanisms of the drug material the pharmaceutical technologists can begin compatibility studies which are also to be carried out under strict analytical control including the estimation of the impurity profiles. The aim of these studies is to find possible interactions between the active ingredient(s), excipients, antioxidants, etc. As a result of these new impurities can be found. As an example of the immediate adverse effect of excipients the study of Dijkstra and Dekker [15] is mentioned for the stabilisation of the solutions of *prednisolone sodium phosphate* or *dexamethasone sodium phosphate* with their oxidisable dihydroxyacetone-type side chain at position 17. They detected impurities by HPLC when sodium metabisulphite was used as an antioxidant. As seen in Fig. 1.4.A, the impurities were found to be products of an addition reaction between the conjugated double bond system in ring A (Δ^1 double bond) and the antioxidant. In the case of prednisolone



Prednisolone sodium phosphate

Figure 1.4.A.
Reaction between prednisolone sodium phosphate and sodium metabisulphite (from Ref. [15])

sodium phosphate both epimers, the 1α- and 1β-sulphonates were separated and identified; with dexamethasone sodium phosphate only the 1β-derivative was detected.

The *stability studies* of the experimental dosage forms are in the center of the investigations aiming to find the most suitable dosage form with optimal stability and bioavailability. At the beginning of these studies when several variants of the aimed formulation are the subject of the investigation, the main goal is the detection, identification and quantitative determination of degradation products formed under stress conditions. This is the most rapid and most economical way to obtain the data necessary for the selection of the most suitable composition for the formulation. Some of the degradation products are usually identical with those found during the above mentioned stress stability studies of the bulk drug material, but some others can be new and these should be characterised to some extent in order to be able to make a good decision. (It should be mentioned that some of the degradation products found under stress conditions will not occur under the milder conditions of the long-term or accelerated stability studies.) After the selection of the final composition of the dosage form, in the course of scaling up and industrial level production of the formulation the latter forms of the stability studies come to the fore. For details the reader is referred to Section 5.2, the ICH Guidelines [16] and the book of Carstensen [17].

As for the methodological problems of profiling impurities and degradation products in drug formulations it can be stated that in the case of relatively simple solid dosage forms and injectables with not too low active ingredient content there are no particular difficulties as compared with the profiling of bulk drug materials. The situation is, however, much more difficult when solid dosage forms with low active ingredient content or formulations containing complicated matrices (ointments, creams, suppositories, oil-injectables) are investigated as shown for steroid formulations of this kind by Kirschbaum and Cohen [18]. In Sections 5.3–5.5 several practical examples are presented for the identification, structure elucidation and quantitative determination of degradation products among others in pharmaceutical formulations.

1.4.4. Impurity Profiles in Drug Registration: Legal Aspects

As described in the preceding sections of this chapter, the impurity profile of a drug is influenced by several factors, the synthetic route, reaction conditions, source and quality of the starting materials, reagents and solvents used during the synthesis, the purification steps, conditions of crystallisation, drying, distillation and storage of bulk drug materials and drug formulations. For this reason the estimation of impurity profiles of drugs is an excellent means by

which the drug authorities can control the level of the manufacturing process. The number of impurities and their quantities are the best indication of the quality of the drug product. The proportion of identified impurities among the detected (qualified) ones and the standards of the analytical (chromatographic and spectroscopic) dossier describing their detection, identification/structure elucidation and determination enables the authorities to judge the level of analytical activities at the manufacturer. The comparison of the impurity profiles of several batches from the same manufacturer provides a good indication for the constancy of the manufacturing process while the comparison of samples originating from different manufacturers can give a clear picture about the differences between their purity and the levels of the manufacturing procedures.

The comparison between the impurity profiles of drug samples from different manufacturers furnishes at the same time information about the synthesis route used by the different companies. Certain impurities can be considered to be indicators of a certain synthesis pathway (often called ''synthesis markers'') even if they are detected at a much lower level than required by the drug authorities. Due to the very high confidentiality of analytical works done in this area very little if any has been published on these legal aspects of drug impurity profiling, when some companies try to make use of analytical data in providing evidence for the illegal use of their patented synthesis route by another company.

One of the rare published studies where the results of the comparison of the impurity profile of a drug originating from different sources is described is in the recent paper by Lehr et al. [19]. In this study the impurity profile of 22 lots of *trimethoprim* bulk drug substance from five different manufacturers in three countries were compared. Using a gradient RP-HPLC system they detected two major impurities which are not separated and detected by the TLC system of USP 24 [20]. The structures of the two impurities were determined by HPLC/MS and NMR spectroscopy after preparative HPLC separation. The chromatograms of five characteristic examples from among the 22 lots are shown in Fig. 1.4.B. The total quantity of impurity I and II in the various lots reached 2.1% in one of the lots while it was absent in some others. The ''fingerprint-like'' character of the chromatograms in Fig. 1.4.B is evident and certainly betrays the different synthetic/purification procedures used by the various manufacturers.

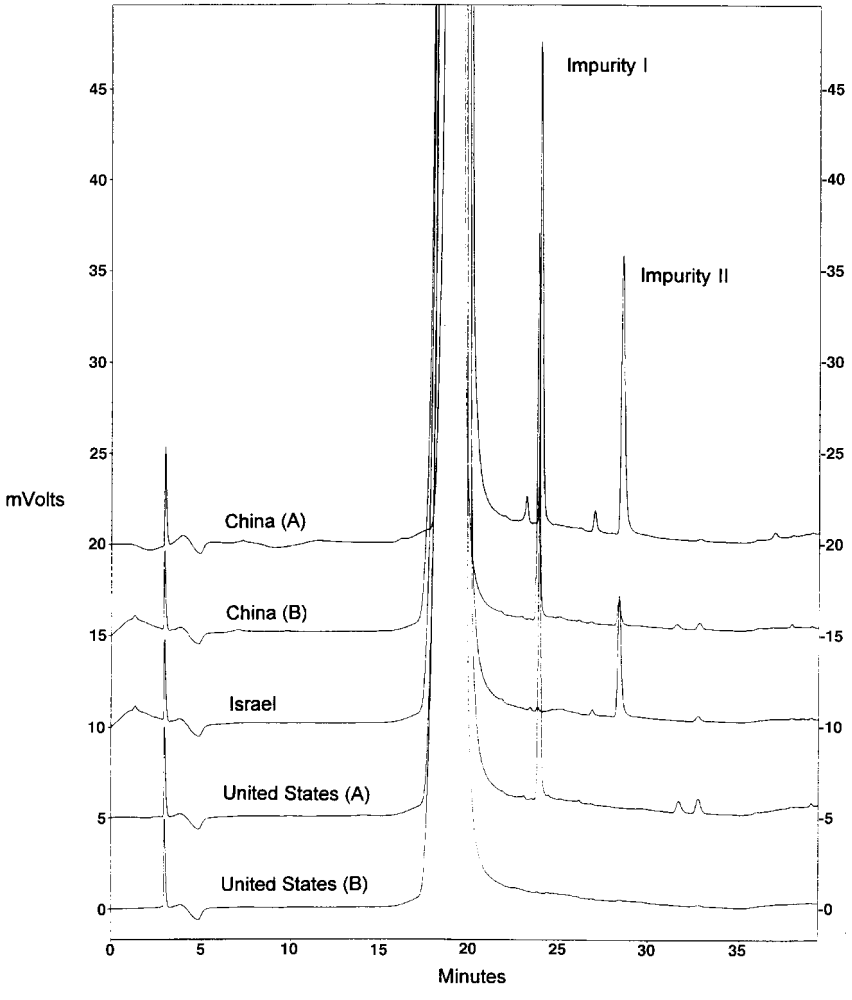
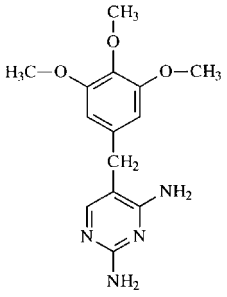
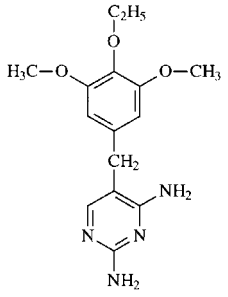


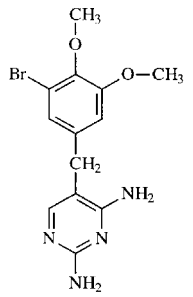
Figure 1.4.B.
HPLC profiles of trimethoprim drug substance from various manufacturers. Column: Beckman Ultrasphere ODS, 5 μm, 4.6 × 250 mm; gradient elution A: 0.25% triethylamine and 1.1% formic acid in water (pH 5.8), B: acetonitrile. 0–10 min 10% B, 20–30 min 25% B, 35–45 min 40% B, 50–60 min 80% B. Flow rate 1.0 ml/min. UV detector 272 nm (from Ref. [19])



Trimethoprim



Impurity I



Impurity II

References

1. H. Möller and H.-H. Donaubauer, *Eur. J. Pharm. Biopharm.* **40**, 53–57 (1994)
2. H. Möller, R. Kirrstetter and M. Worm, *Pharm. Ind.* **56**, 780–784 (1994)
3. J. Ermer and H. Möller, *Reg. Affairs J.* 722–729 (1997)
4. S. Görög, A. Laukó and B. Herényi, *J. Pharm. Biomed. Anal.* **6**, 697–705 (1988)
5. A. Laukó, É. Csizér and S. Görög, in *Proceedings of the 13th International Symposium on Capillary Chromatography*, Vol. II (P. Sandra, Ed.), pp 1548–1556.
6. S. Görög, B. Herényi and M. Rényei, *J. Pharm. Biomed. Anal.* **10**, 831–835 (1992)
7. T. Toyo'oka, T. Yamazaki, T. Tanimoto, K. Sato, M. Sato, M. Toyoda, M. Ishibashi, K. Yoshihira and M. Uchiyama, *Chem. Pharm. Bull.* **39**, 820–822 (1991)
8. A. Müller, E. Busker, K. Günther and B. Hoppe, *Bioforum* **14**, 450–354 (1991)
9. M.W. Trucksess, *J. Chromatogr.* **630**, 147–150 (1993)
10. R.H. Hill, S.P. Caudill, R.M. Philen, S.L. Bailey, W.D. Flaunders, W.J. Driskell, M.L. Kamb, L.L. Needham and E.J. Sampson, *Arch. Environ. Contam., Toxicol.* **25**, 134–142 (1993)
11. T. Simat and H. Steinhart, *J. Pharm. Biomed. Anal.* **16**, 167–173 (1997)
12. B.L. Williamson, L.M. Benson, A.J. Tomlinson, A.N. Mayeno, G.J. Gleich and S. Naylor, *Toxicol. Lett.* **92**, 139–148 (1997)
13. B.L. Williamson, A.J. Tomlinson, P.K. Mishra, G.J. Gleich and S. Naylor, *Chem. Res. Toxicol.* **11**, 234–240 (1998)

14. J. Fairbrother and P. Timmins, in *Steroid Analysis in the Pharmaceutical Industry* (S. Görög, Ed.), pp 300–313. Ellis Horwood, Chichester (1989)
15. J. Dijkstra and D. Dekker, *J. Chromatogr.* **238**, 247–249 (1982)
16. International Conference on Harmonisation (ICH), Stability Testing Guidelines: Stability Testing of New Drug Substances and Products. Step 5. (CPMP/ICH/380/95)
17. J.T. Carstensen, *Drug Stability, 2nd edn*, Marcel Dekker, New York (1995)
18. J. Kirschbaum and B. Cohen, in *Steroid Analysis in the Pharmaceutical Industry* (S. Görög, Ed.), pp 313–340. Ellis Horwood, Chichester (1989)
19. G.J. Lehr, T.L. Barry, G. Petzinger, G.M. Hanna and S.W. Zito, *J. Pharm. Biomed. Anal.* **19**, 373–389 (1999)
20. *The United States Pharmacopoeia 24*, p 1713. USP Convention, Inc., Rockville (2000)

1.5. Regulatory Aspects: ICH and Pharmacopoeial Perspectives

Judy P. Boehlert

1.5.1. Introduction

Identification, qualification, and quantification of impurities are critical tools for assessing the safety and quality of pharmaceutical drug substances and their associated dosage forms. Safety and quality, with respect to impurity profiles, must be demonstrated on initial registration of a pharmaceutical dosage form, monitored on an ongoing basis, and reconfirmed whenever changes that may impact safety or quality are made in the drug substance synthesis or drug product formulation or process. For many years, pharmacopoeias around the world have addressed the topic, primarily through the inclusion of requirements for impurities in monographs for drug substances and/or drug products, but also by including general chapters and/or general notices dealing with different aspects of the topic. Since 1990, considerable attention has been focused on this topic by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) resulting in three impurity guidelines. ICH guidelines address new drug substances and new drug products not previously registered in one of the ICH regions, while pharmacopoeias address requirements for marketed drug substances, excipients, and/or drug products.

1.5.2. International Conference on Harmonisation (ICH) [1]

1.5.2.1. Process

The ICH process was initiated in 1990 by the regulatory agencies and research industry trade groups representing: Europe – European Commission (EC) and European Federation of Pharmaceutical Industry Associations (EFPIA); Japan – Ministry of Health and Welfare (MHW) and the Japanese Pharmaceutical Manufacturers Association (JPMA); and the United States – Food and Drug Administration (FDA) and Pharmaceutical Research and Manufacturers of America (PhRMA). The process was initiated as a means of harmonising the technical requirements for registration applications in these three major pharmaceutical markets. ICH activities are co-ordinated by the

ICH Secretariat under the auspices of The International Federation of Pharmaceutical Manufacturers (IFPMA). ICH conducts its work through Expert Working Groups (EWGs) who report to a Steering Committee. Each of the six parties provides members of the EWGs and a co-ordinator to ensure that documents are appropriately distributed, reviewed, and comments prepared. Observers to ICH, including, The World Health Organisation (WHO), the European Free Trade Area (EFTA) and the Drugs Directorate, Health Canada, also provide representatives to the EWGs as do other invited groups, for example, the generic industry and USP.

ICH guidelines progress through a step-wise process.

- Step 1: A rapporteur, designated from one of the six parties, prepares a draft which is forwarded to the Steering Committee when consensus is reached in the EWG.
- Step 2: The Steering Committee signs the draft and forwards it to the three Regulatory Agencies for formal consultation in each region.
- Step 3: A rapporteur, designated from one of the three regulatory agencies, revises the draft based on comments received.
- Step 4: The three regulatory agencies sign the final draft and it is recommended for adoption in the three regions.
- Step 5: The draft is incorporated into regional guidelines/regulations.

1.5.2.2. ICH Topics

ICH has addressed three principal areas: quality, safety, and efficacy. Under quality seven general areas of concentration have been identified: Stability; analytical validation; impurities; pharmacopoeias; biotechnological quality; specifications; and, CGMPs for active pharmaceutical ingredients. Under impurities, three guidelines have been developed covering new drug substances, new drug products and residual solvents; all have reached Step 5 of the ICH process, however revisions may occur as new information becomes available.

1.5.3. ICH Impurity Guidelines for New Drug Substances and New Dosage Forms [2–7]

1.5.3.1. Introduction

These first two ICH impurity guidelines address the quantification, reporting, identification and qualification of impurities in new drug substances and new drug products. Excluded from consideration in these guidelines are a

number of classes of new drugs: biological/biotechnological, peptide, oligonucleotide, radiopharmaceutical, fermentation, and semi-synthetic products derived therefrom, herbal products, and crude products of animal or plant origin. Although the concepts described in the ICH impurity guidelines are useful for considering and evaluating impurities in all pharmaceutical drug substances and drug products, inclusion of such a wide range of products would have made it impossible to reach a common set of requirements. The third ICH impurity guideline covers residual solvents which may have been used in the drug substance synthesis, excipient production, or drug product formulation, and, remain in the drug product at the end of the manufacturing process. This guideline does not apply during the clinical research process or for existing marketed products, however, the recommendations should be considered in determining appropriate limits for residual solvents based on safety considerations. This guideline applies to all dosage forms and routes of administration and will be covered later in this chapter.

1.5.3.2. Classification of Impurities

Drug substance impurities are classified as organic, inorganic, and residual solvents.

- Organic impurities: Generally related to the drug substance or the synthesis, for example, starting materials, by-products of the synthesis, intermediates, degradation products, and materials used in the synthesis such as reagents, ligands, and catalysts.
- Inorganic impurities: Generally related to the drug substance synthesis and may include reagents, ligands, catalysts, heavy metals, inorganic salts, and processing materials used in the synthesis such as filter aids or charcoal that remain in the final drug substance.
- Residual solvents: Organic or inorganic liquids which are used in the manufacturing process synthesis and remain in the final drug substance. Residual solvents classified as organic volatile chemicals are covered by the ICH guideline discussed later in this chapter.

Drug product impurities are defined as degradation products.

- Degradation products: Degradation products of the active ingredient and reaction products of the active ingredient with an excipient and/or the immediate container/closure system. Impurities present in the new drug substance that are not also degradation products need not be measured in the dosage form. To simplify the discussion in this chapter, the generic term impurities is used at all times.

Polymorphic form and enantiomeric impurities are not covered in the

guidelines although it can be helpful to refer to the general concepts in setting standards for these properties. Extraneous or foreign contaminants are not covered since these are more properly dealt with as GMP concerns and setting general limits is not appropriate.

1.5.3.3. Reporting and Control of Impurities in the Registration Application

The following information should be provided.

1. A description of the laboratory studies conducted to elucidate degradation pathways and determine potential impurities that could arise during the synthesis, purification, and storage of the new drug substance and new drug product.

2. Tabulations should be provided listing total and individual, identified and unidentified organic impurities for all batches of the new drug substance and new drug product used, where applicable, for development, clinical, safety, and stability testing, including batches representative of the proposed commercial process, when available. Impurities should be identified by name, where known, code number or by an analytical descriptor such as HPLC retention time. Batch information should be listed, including: batch identity and size; dosage form strength; dosage form immediate container/closure system; date of manufacture; site of manufacture; manufacturing process; use of batches; storage conditions; and, reference to analytical procedures used.

In the drug substance, the original ICH guideline states that impurities present, but below the validated limit of quantitation, need not be reported. This effectively sets the limit of quantitation, no matter how low, as the reporting threshold for drug substance impurities. Although the original ICH drug substance guideline does not contain a reporting threshold for impurities, values of 50% of the identification or qualification threshold, whichever is lower, have been recommended [8]. This section of the guideline may change to achieve consistency with the drug product guidelines which does have reporting thresholds. In the drug product, impurities at or above the reporting threshold (Table 1.5.A) should be reported. Exclusion of impurities from the drug product specifications, for example, drug substance impurities, should be discussed in the registration application. Unless otherwise justified in the application, reporting levels for the drug product should be below the identification level as described in Table 1.5.B.

3. Inorganic impurities in the drug substance should be controlled using pharmacopoeial or other appropriate procedures and limits. Catalysts that may carry over to the final drug substance should be evaluated during development and tests included where warranted.

Table 1.5.A.
Thresholds for reporting of degradation products in new drug products [5–7]

Maximum daily dose (g)[a]	Threshold (%)[b]
≤1	0.1
>1	0.05

[a] The amount of drug substance administered per day
[b] Threshold is based on percent of the drug substance

4. Residual solvents should be controlled using pharmacopoeial or other appropriate procedures. Appropriate limits for a large number of solvents are covered in the ICH guideline discussed in Section 1.5.4. The solvents included and, even the limits, may change as new information becomes available.

5. A description of the analytical procedures used should be provided along with appropriate validation information, including representative chromatograms showing separation and detectability of impurities. Differences in the analytical procedures used during development and those proposed for the commercial product should be discussed. Although impurities present in the new drug substance need not be measured in the new drug product, it is good analytical practice to provide this information in the analytical procedure for the analyst who may test the product, for example, a notation in the analytical procedure that a peak appearing at a designated relative retention time is an impurity (identified by name when known) and need not be estimated in the dosage form nor included in the sum of total impurities.

Analytical procedures used to detect and quantitate impurities in new drug substances should be validated according to the principles described in the ICH guidelines on the validation of analytical procedures: Guideline on Validation

Table 1.5.B.
Thresholds for identification of degradation products in new drug products [5–7]

Maximum daily dose[a]	Threshold[b]
<1 mg	1.0% or 5 μg TDI[c] whichever is lower
1–10 mg	0.5% or 20 μg TDI whichever is lower
>100 mg to 2 g	0.2% or 2 mg TDI whichever is lower
>2 g	0.1%

[a] The amount of drug substance administered per day
[b] Threshold is based on percent of the drug substance
[c] Total daily intake

of Analytical Procedures: Definitions and Terminology [9–11]; and, Guideline on the Validation of Analytical Procedures: Methodology [12–14]. As part of method validation, it should be demonstrated that impurities unique to the new drug substance do not interfere with or are separated from specified and unspecified degradation products in the new drug product. Analytical procedures should be capable of quantitating impurities, at a minimum, at the reporting threshold, preferably at 50% of this amount. For those impurities known or expected to be unusually potent or to produce toxic or unexpected pharmacological effects, the quantitation/detection limit of the analytical procedures used should be commensurate with the level at which the impurities must be controlled and the general reporting thresholds do not apply.

The guidelines note that although it is preferable to use authentic, characterised impurity reference standards to quantitate impurity levels, it may be acceptable to use the drug substance itself. Where the response factors for the drug substance and impurity are not close, correction factors should be applied wherever possible. The manner in which the impurities are quantitated and any analytical assumptions, such as equivalent detector response, should be described.

6. Specifications should be set for identified and unidentified impurities expected to be present in the new drug substance and new drug product over the period of intended use and under recommended storage conditions. These impurities are known as *specified impurities* and they should be individually listed in the specifications. Stability studies, chemical development studies, and routine batch analyses can be used to establish impurities likely to occur in the commercial new drug substance and new drug product. A general specification limit of not more than 0.1% for any unspecified impurity should also be included. A rationale for why impurities were included or excluded from the specifications for the new drug substance and new drug product should be provided. Limits for impurities should be set no higher than the level which can be justified by safety data, and unless safety data indicate otherwise, no lower than the level achievable by the manufacturing process and the analytical capability.

In summary, specifications for the new drug substance should include: residual solvents; inorganic impurities; organic impurities, including each specified identified impurity, each specified unidentified impurity at or above 0.1%, any unspecified impurity, with a limit of not more than 0.1%, and, total impurities. A summation of assay value and impurity levels generally may be used to obtain mass balance. The mass balance need not add to exactly 100% due to the analytical error associated with each analytical procedure. Specifications for the new drug product should be set for each specified impurity; any unspecified impurity, and, total impurities.

Impurities observed in the new drug substance at an apparent level at or

above 0.1% should be identified, that is, structurally characterised. It may also be prudent to identify those impurities below the 0.1% identification threshold which may be anticipated to exceed that threshold in future batches. The original ICH guidelines stated that, although it is common practice to round impurity values such as 0.05% or 0.09% to 0.1%, for the purposes of these ICH guidelines, such values are not rounded to determine if thresholds have been met or exceeded, and, identification of these impurities, therefore, is not normally required. This section of the guidelines may change to incorporate conventional rules of rounding. Only those impurities observed on both initial drug substance testing as well as those observed during stability studies under recommended storage conditions need be identified. When it is not possible to conclusively identify impurities at the 0.1% level, these unsuccessful attempts should be described.

7. Impurities observed in new drug product stability studies conducted at recommended storage conditions should be identified when the threshold limits in Table 1.5.B are met or exceeded. When identification is not feasible, a summary of the unsuccessful laboratory studies should be included in the registration or marketing application.

Impurities that are expected or known to be unusually potent, producing toxic or pharmacologic effects at a level less than 0.1%, should be identified, qualified, and reported at whatever level the adverse effect is expected or observed.

1.5.3.4. Qualification of Impurities

Qualification is the process of acquiring and evaluating data which establishes the biological safety of an individual impurity or a given impurity profile at the level(s) specified in the new drug substance or new drug product specifications. Impurity limits should be selected based on safety considerations. The level of any impurity present in a new drug substance or new drug product which has been adequately tested in safety and/or clinical studies or is a significant metabolite present in animal and/or human studies is considered qualified. A level of a qualified impurity higher than that present in a new drug substance can also be justified based on an analysis of the actual amount of impurity administered in safety studies.

When data are not available to qualify an impurity at a proposed specification level, studies may be needed to obtain such data when the usual qualification threshold limits are exceeded (Tables 1.5.C and 1.5.D). Higher or lower threshold limits for qualification of impurities may be appropriate for some individual drugs based on scientific rationale and level of concern, including drug class effects and clinical experience. For example, impurities in certain drugs or therapeutic classes may have been previously associated

Table 1.5.C.
Qualification thresholds for impurities in new drug substances [2–4]

Maximum daily dose (g/day)	Qualification threshold
≤2	0.1% or 1 mg/day intake (whichever is lower)
>2	0.05%

with adverse reactions in patients and a lower qualification threshold may be indicated. In unusual circumstances, technical factors (e.g. manufacturing capability, a low drug substance to excipient ratio in the drug product, control technology, or the use of excipients that are also crude products of animal or plant origin) may be considered as part of the justification for selection of alternative thresholds.

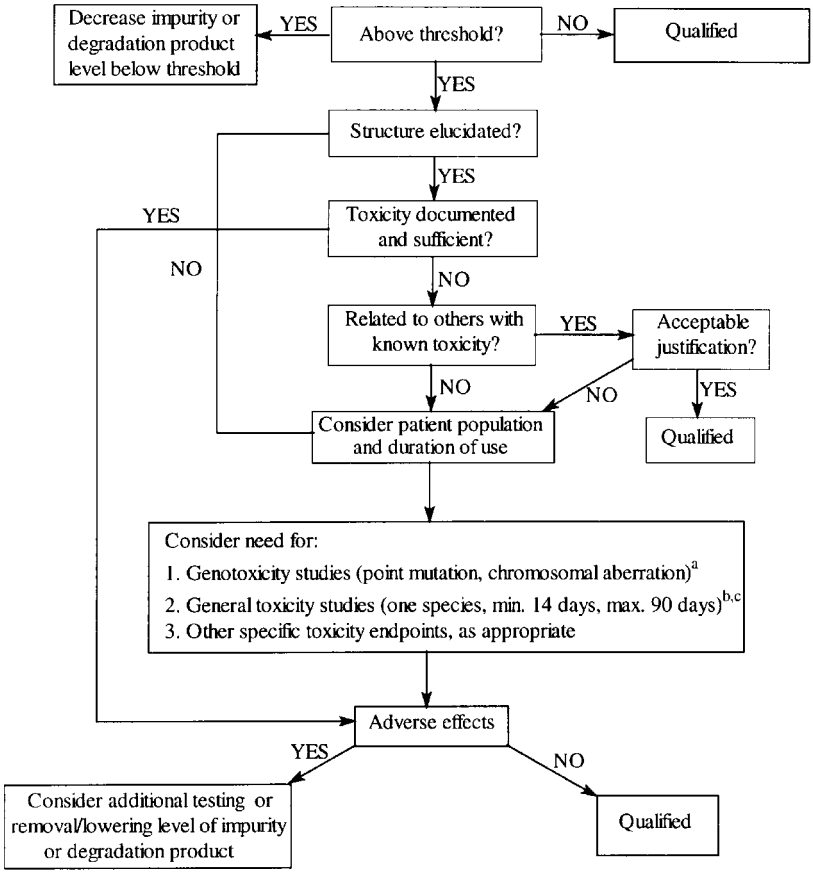
Proposals for alternative thresholds are considered by the registration authorities on a case-by-case basis.

Figure 1.5.A presents the decision tree for safety studies for the qualification of impurities in a new drug substance or a new drug product when thresholds are exceeded. In some cases, decreasing the impurity below the threshold in the drug substance may be preferable to providing safety data. Decreasing the level of impurity must be evaluated on a case-by-case basis, since such efforts can add significantly to manufacturing costs. Reducing impurity levels in drug products can also be considered but, since these are defined as degradation products, may necessitate formulation, container/closure, or storage condition changes. Adequate safety data may be available in the scientific literature to qualify an impurity. If data are not available, additional safety testing should be considered, taking into account a number of factors, including patient population, daily dose, route and duration of drug administration, and drug class

Table 1.5.D.
Thresholds for qualification of degradation products in new drug products [5–7]

Maximum daily dose[a]	Threshold[b]
<10 mg	1.0% or 50 μg TDI[c] whichever is lower
10–100 mg	0.5% or 200 μg TDI whichever is lower
>100 mg to 2 g	0.2% or 2 mg TDI whichever is lower
>2 g	0.1%

[a] The amount of drug substance administered per day
[b] Threshold is based on percent of the drug substance
[c] Total daily intake



a. If considered desirable, a minimum screen for genotoxic potential should be conducted. A study to detect point mutations and one to detect chromosomal aberrations, both in vitro, are seen as acceptable minimum screen.

b. If general toxicity studies are desirable, study(ies) should be designed to allow comparison of unqualified to qualified material. The study duration should be based on available relevant information and performed in the species most likely to maximize the potential to detect the toxicity of an impurity. In general, a minimum duration of 14 days and a maximum duration of 90 days will be acceptable.

c. On a case by case basis, single dose studies may be acceptable, especially for single-dose drugs, and when such studies are conducted using an isolated impurity. If repeat-dose studies are desirable, a maximum duration of 90 days would be acceptable.

Figure 1.5.A.
Decision tree for safety studies [2–7]

effects. Such studies are normally conducted on the new drug substance containing the impurities to be controlled, although studies using isolated impurities are acceptable.

1.5.3.5. New Impurities

During the course of drug development, the qualitative impurity profile may change as a result of, for example, changes in synthetic route, drug product formulation, storage conditions, and, container/closure system. These changes can result in increases in levels of previously observed impurities above the identification and/or qualification threshold limits or new impurities not previously identified or qualified. When these impurities are above the threshold limits, consideration for identification and qualification of the of the level of the impurity is indicated and one of the decision trees for safety studies should be consulted (Fig. 1.5.A). Safety studies should compare the new drug substance containing a representative level of the new impurity with previously qualified materials, although studies using the isolated impurity are also acceptable (these studies may not always have clinical significance).

1.5.4. ICH Residual Solvents Guideline [15–17]

1.5.4.1. Introduction

The ICH guideline recommends levels for residual solvents based on *permitted daily exposure* (PDE) which is defined as a pharmaceutically acceptable intake. The guideline includes the method used to establish PDEs for residual solvents. Higher levels than stated in the guideline may be acceptable in certain cases, for example, short-term (30 days or less) use or topical application. These higher levels should be justified on a case-by-case basis.

Solvents are placed in one of three classes based on safety considerations. The solvents included in the guideline or the class assigned may change as new information becomes available. The guideline recognises that solvents are particularly needed in drug synthesis and may enhance the yield or facilitate production of the desired crystal form, purity, and solubility. However, since there is no therapeutic benefit from residual solvents, they should be removed from pharmaceutical products to the extent possible. Drug products should contain no higher levels of residual solvents than can be supported by safety data. The concepts described in the ICH impurity guidelines should be used to justify the use of solvents not covered in the residual solvents guideline.

Table 1.5.E.
Class 1 solvents [15–17]

Solvent	Concentration limit (ppm)	Concern
Benzene	2	Carcinogen
Carbon tetrachloride	4	Toxic and environmental hazard
1,2-Dichloroethane	5	Toxic
1,1-Dichloroethene	8	Toxic
1,1,1-Trichloroethane	1500	Environmental hazard

1.5.4.2. Classification of Residual Solvents

Residual solvents are placed in one of three classes.

- Class 1: Solvents to be avoided. These known human carcinogens, strongly suspected human carcinogens, and environmental hazards should not be used in the manufacture of drug substance, excipients, or drug products. If their use is unavoidable, the levels should comply with the guideline recommendations (Table 1.5.E).
- Class 2: Solvents to be limited in use. These are non-genotoxic animal carcinogens or possible causative agents of other irreversible toxicity such as neurotoxicity or teratogenicity which should be limited due to their inherent toxicity (Table 1.5.F).
- Class 3: Solvents with low toxic potential to man. No health-based exposure limit is needed since these solvents have PDEs of 50 mg or more per day and can generally be limited by GMP or other quality-based requirements (Table 1.5.G).

The guideline contains a list of solvents (Table 1.5.H) for which no adequate toxicological data was found. Manufacturers wishing to use one of these solvents, should supply justification for their use based on safety considerations. As noted earlier, the guideline may change as new information becomes available.

1.5.4.3. Analytical Procedures

Residual solvents are typically determined using chromatographic techniques such as gas chromatography. Harmonised pharmacopoeial procedures

Table 1.5.F.
Class 2 solvents [15–17]

Solvent	PDE (mg/day)	Concentration limit (ppm)
Acetonitrile	4.1	410
Chlorobenzene	3.6	360
Chloroform	0.6	60
Cyclohexane	38.8	3880
1,2-Dichloroethene	18.7	1870
Dichloromethane	6.0	600
1,2-Dimethoxyethane	1.0	100
N,*N*-Dimethylacetamide	10.9	1090
N,*N*-Dimethylformamide	8.8	880
1,4-Dioxane	3.8	380
2-Ethoxyethanol	1.6	160
Ethylene glycol	6.2	620
Formamide	2.2	220
Hexane	2.9	290
Methanol	30.0	3000
2-Methoxyethanol	0.5	50
Methylbutyl ketone	0.5	50
Methylcyclohexane	11.8	1180
N-Methylpyrrolidone	48.4	4840
Nitromethane	0.5	50
Pyridine	2.0	200
Sulfolane	1.6	160
Tetralin	1.0	100
Toluene	8.9	890
1,1,2-Trichloroethene	0.8	80
Xylene[a]	21.7	2170

[a] Usually 60% *m*-xylene, 14% *p*-xylene, 9% *o*-xylene with 17% ethyl benzene

should be used when feasible. Otherwise, manufacturers can select the most appropriate validated analytical procedure for a particular application. If only Class 3 solvents are present, a non-specific method such as loss on drying may be used.

Analytical procedures used to detect and quantitate residual solvents should be validated according to the principles described in the ICH guidelines on the validation of analytical procedures: Guideline on Validation of Analy-

Table 1.5.G.
Class 3 solvents [15–17]

Acetic acid	Ethyl acetate	Methylisobutyl ketone
Acetone	Ethyl ether	2-Methyl-1-propanol
Anisole	Ethyl formate	Pentane
1-Butanol	Formic acid	1-Pentanol
2-Butanol	Heptane	1-Propanol
Butyl acetate	Isobutyl acetate	2-Propanol
tert-Butylmethyl ether	Isopropyl acetate	Propyl acetate
Cumene	Methyl acetate	Tetrahydrofuran
Dimethylsulfoxide	3-Methyl-1-butanol	
Ethanol	Methylethyl ketone	

tical Procedures: Definitions and Terminology [9–11]; and, Guideline on the Validation of Analytical Procedures: Methodology [12–14].

1.5.4.4. Testing

Testing for residual solvents should be performed when production or purification processes are known to result in the presence of such solvents. It is not possible or rational to test for solvents that may be present due to inadvertent contamination. Such inadvertent contamination should be addressed as a GMP issue. The drug product can be tested or the levels present in the ingredients can be summed to arrive at the drug product total levels. When ingredient solvent levels are summed, no testing of the drug product needs be performed when the calculation results in a level equal to or below that recommended in the guideline. If the calculation results in a level above the recommended level, the drug product should be tested to determine if the solvent levels have been reduced to acceptable levels during the formulation

Table 1.5.H.
Solvents with inadequate toxicological data [15–17]

1,1-Diethoxypropane	Methylisopropyl ketone
1,1-Dimethoxymethane	Methyltetrahydrofuran
2,2-Dimthoxypropane	Petroleum ether
Isooctane	Trichloroacetic acid
Isopropyl ether	Trifluoroacetic acid

process. Testing on the drug product must be performed when solvents are used in the drug product formulation process.

1.5.4.5. Setting Limits

Limits for Class 1 and 2 solvents should be set based on the values in Tables 1.5.E and 1.5.F. For Class 3 solvents a limit of 0.5% is generally acceptable. For Class 2 solvents, two options are provided.

Option 1: Use the concentration limits in ppm shown in Table 1.5.G. This option applies only to doses not greater than 10 g/day and is useful if the daily dose has not been firmly established since the limits are considered acceptable for all substances, excipients, or products. If all excipients and drug substances in a formulation meet the limits given in Option 1, then these components may be used in any proportion. Products that are administered in doses greater than 10 g/day should be considered under Option 2.

Option 2: Calculate the concentration limit using the equation

$$\text{Concentration (ppm)} = \frac{1000 \times \text{PDE}}{\text{Dose}}$$

using the PDE in Table 1.5.F in terms of mg/day with the known maximum dose.

The limits should be realistic in relation to analytical precision, manufacturing capability, and manufacturing standards.

Option 2 may be applied by adding the amounts of a residual solvent present in each of the components of the drug product. The sum of the amounts of solvent per day should be less than that given by the PDE.

1.5.4.6. Reporting Levels of Residual Solvents

The guideline provides examples of how residual solvents could be reported, for example:

- Class 1: Solvents should be identified and individually quantified.
- Class 2: Solvents X, Y,…, are likely to be present. All are below the Option 1 limit.
- Class 3: Solvents are likely to be present. Loss on drying is less than 0.5%.

''Likely to be present'' means the solvents are not consistently removed by a validated process and may remain in the drug substance or drug product. If solvents of Class 2 or Class 3 are present at greater than their Option 1 limits or 0.5%, respectively, they should be identified and quantified.

1.5.5. Pharmacopeial Treatment of Impurities

Pharmacopoeias around the world have long addressed the topic of impurities, chiefly by the incorporation of requirements for impurities into individual monographs but also by inclusion of the topic in general chapters and/or general notices. Pharmacopeial activities will be discussed in the context of the European Pharmacopoeia [18] and The United States Pharmacopoeia [19].

1.5.6. The United States Pharmacopoeia (USP) [19]

1.5.6.1. Introduction

USP contains three General Chapters and a General Notices discussion on impurities: General Chapter ⟨466⟩, *Ordinary Impurities*, official May 15, 1988; General Chapter ⟨1086⟩, *Impurities in Official Articles*; official January 1, 1990; General Chapter ⟨467⟩, *Organic Volatile Impurities*, official November 15, 1990; and, the *Other Impurities* section of the General Notices, official November 15, 1996. USP also incorporates specific requirements for impurities in monographs. As with the ICH guidelines, pharmacopoeias may change as new information becomes available.

1.5.6.2. Ordinary Impurities

General Chapter ⟨466⟩ *Ordinary Impurities* [19a] outlines requirements for an *ordinary impurity* test which may be included in an individual monograph as a test to evaluate the levels of certain impurities. The chapter outlines the analytical method (thin layer chromatography with specified visualisation techniques) to be employed unless the monograph specifies another method. Unless otherwise specified in the monograph, requirements are met if the total of any ordinary impurities observed does not exceed 2.0%. USP distinguishes: *Signal Impurities* which require individual, specific tests; *toxic impurities* which require identification and quantification by specific tests; *concomitant components* which are not considered to be impurities in the pharmacopoeial sense; and, *foreign substances* for which there are no compendial tests. Identification is not required for *ordinary impurities* but is required for *signal impurities*, *toxic impurities*, and *concomitant components*.

1.5.6.3. Impurities in Official Articles

General Chapter ⟨1086⟩ *Impurities in Official Articles* [19b] is an informational chapter and covers concepts related to impurities, including definitions

for *foreign substances, toxic impurities, concomitant components, signal impurities,* and *ordinary impurities*. The chapter contains a section on *industrial concepts* which discusses a rational basis for setting limits on impurities from the initial IND filing to the filing of the NDA. The chapter describes the factors to consider in setting limits for impurities, for example, toxicology of the impurities, route of administration, and daily dose of the drug product. Also described are typical elements that may be included in the IND and NDA filing, for example, limits for individual impurities, total impurities, residual solvents, heavy metals, and enantiomeric purity.

1.5.6.4. Organic Volatile Impurities (OVIs)

Chapter ⟨467⟩ *Organic Volatile Impurities* [20] contains analytical methods and limits for five potential residual solvents in drug substances and excipients and for methylene chloride in coated tablets. Testing need not be conducted when a manufacturer has knowledge that none of the five solvents is a possible contaminant from the manufacturing process, storage, shipment, or handling of the material. However, the manufacturer should assure that the method will work if applied. Table 1.5.I contains USP requirements for OVIs. The limit for methylene chloride in tablets coated with this solvent is 500 μg/day.

1.5.6.5. Other Impurities

The USP *Other Impurities* [21] requirement appears in the *General Notices and Requirements, Tests and Assays – Foreign Substances and Impurities* section of USP. This section of USP is not new, however, the *other impurities* concept first became official in 1996 [22]. This concept addresses the fact that chromatographic analytical procedures for impurities are developed and validated for a material from a particular source and a particular synthetic route. These procedures are not necessarily valid for a material

Table 1.5.I.
USP organic volatile impurity limits [20]

Solvent	Limit (ppm)
Benzene	2
Chloroform	60
1,4-Dioxane	380
Methylene chloride	600
Trichloroethylene	80

produced by another synthetic route since the impurities are often different. These impurities that may be present but not detected by the chromatographic analytical procedure in a monograph are designated as *other impurities.* The manufacturer must declare the amount of these impurities, identified, preferably by name, on their certificate of analysis when the level of individual impurities are at or above 0.1%. The use of the term ''identified'' in this instance is not interpreted to require or imply a requirement for structural characterisation of *other impurities* at the 0.1% level. The sum of all *other impurities* combined with monograph detected impurities does not exceed 2.0% (this is the *ordinary impurities* limit as discussed above) unless other specific requirements are stated in the monograph.

1.5.7. European Pharmacopoeia (Ph. Eur.) [18]

1.5.7.1. Introduction

The Ph. Eur. also contains general chapters, general notices, and monograph requirements for impurities although only drug substances are covered (only general chapters are provided for dosage forms). A major difference between USP and Ph. Eur. handling of impurities is in the inclusion of impurity structures in the EP monograph for impurities that are detected by the designated analytical method. Similar to USP, Ph. Eur. also contains general methods for residual solvents. Both USP and Ph. Eur. provide reference standards for impurities where indicated in the monograph. As noted above, pharmacopoeias may change as new information becomes available.

The *Introduction* to the Ph. Eur. [18a] discusses impurities and states that many monographs have a list of impurities controlled by the tests. Impurities may be known, i.e. observed in batches of the drug substance, or potential, i.e. not observed but expected to occur based on knowledge of the manufacturing process. Identification of impurities by the Ph. Eur. facilitates use of the monograph by a manufacturer, particularly in determining whether or not the monograph impurity tests are valid for their material, i.e. the test will separate their impurities.

1.5.7.2. General Notices

The *General Notices* section of Ph. Eur. makes a number of references to impurities. Section 1.4, discusses *monographs* [18b] and includes a discussion of impurities which states that ''the approximate content of impurity tolerated, or the sum of impurities, may be indicated in parentheses for information only''.

The same section of the *General Notices* also includes a separate discussion on *impurities* and states that the list of impurities controlled by the monograph tests may be divided into *qualified impurities* and *other detectable impurities*. *Qualified impurities* have been accepted by the registration authority as being qualified. *Other detectable impurities* are potential impurities that have not been observed or that occur at levels below 0.1%.

1.5.7.3. General Chapters

General Method 2.4.24, *Residual Solvents* [18c], contains general methods for residual solvents with two alternate GC systems provided for the control of acetonitrile, methylene chloride, chloroform, benzene, trichloroethylene, dioxan, and pyridine.

General Method 2.4.25, *Residual Ethylene Oxide and Dioxan* [23], contains a headspace method for the determination of residual ethylene oxide and dioxan in samples soluble in water or dimethylacetamide. The mean areas of each of the peaks in the sample solution are not greater than half the mean area of the corresponding peak in the reference solution (1 ppm ethylene oxide and 50 ppm dioxan).

The EP published the text of the ICH Guideline on Residual Solvents for review as a Step 2 document [24].

1.5.7.4. Related Substances

Monographs may contain tests for *related substances* with specific limits in parentheses or in some cases, a test for a specific impurity such as dimethylaniline in ampicillin sodium. A test for a specific residual solvent may also be included, for example methylene chloride in ampicillin sodium with a limit of not more than 0.2% m/m.

When appropriate, both USP and EP contain monograph requirements for inorganic impurities, e.g. heavy metals, arsenic, iron and nickel.

References

1. *The ICH Harmonisation Process*, IFPMA, http://www.ifpma.org (1998)
2. *ICH Guideline: Impurities in New Drug Substances*, CPMP/ICH/142/95 (May 1995)
3. *ICH Guideline: Impurities in New Drug Substances,* PAB/PCD Notification No. 877 (September 1995)
4. *ICH Guideline: Impurities in New Drug Substances,* Federal Register, **61**, p 371ff (January 4, 1996)

5. *ICH Guideline: Impurities in New Drug Products*, CPMP/ICH/282/95 (December 1996)
6. *ICH Guideline: Impurities in New Drug Products*, PAB/PCD Notification No. 539 (June 1997)
7. *ICH Guideline: Impurities in New Drug Products*, Federal Register, **62**, No. 96, p 27454ff (May 19, 1997)
8. J.P. Boehlert, *Pharm. Technol.* **21**, 56ff (1997)
9. *ICH Guideline: Validation of Analytical Procedures: Definitions and Terminology*, CPMP/ICH/381/95 (November 1994)
10. *ICH Guideline: Validation of Analytical Procedures: Definitions and Terminology*, PAB/PCD Notification No. 755 (July 1995)
11. *ICH Guideline: Validation of Analytical Procedures: Definitions and Terminology*, Federal Register, **60**, p 11260ff (March 1, 1995)
12. *ICH Guideline: Validation of Analytical Procedures: Methodology*, CPMP/ICH/281/95 (December 1996)
13. *ICH Guideline: Validation of Analytical Procedures: Methodology*, PMSB/ELD Notification No. 338 (October 1997)
14. *ICH Guideline: Validation of Analytical Procedures: Methodology*, Federal Register, **62**, No. 96, p 27463ff (May 19, 1997)
15. *ICH Guideline: Residual Solvents*, CPMP/ICH/283/95 (September 1997)
16. *ICH Guideline: Residual Solvents*, PMSB/ELD Notification No. 307 (March 1998)
17. *ICH Guideline: Residual Solvents*, Federal Register, **62**, No. 247, p 67377ff (December 24, 1997)
18. *European Pharmacopoeia, 3rd edn*, Council of Europe, Strasbourg, (1997). a, p V; b, p 4; c, p 61
19. *The United States Pharmacopoeia 24*, USP Convention, Inc., Rockville (2000). a, p 1876; b, p 2049
20. *The United States Pharmacopoeia 24*, p 1877, USP Convention, Inc., Rockville (2000)
21. *The United States Pharmacopoeia 24*, p 7, USP Convention, Inc., Rockville (2000)
22. *Pharmacopeial Forum,* 14th Interim Revision Announcement, United States Pharmacopeial Convention, Rockville, p 2 (Nov.–Dec. 1996)
23. *European Pharmacopoeia Supplement 1998*, Council of Europe, Strasbourg, p 17 (1998)
24. *Pharmeuropa Supplement* **9**, No. 1 (April 1997)

Chapter 2

IDENTIFICATION, STRUCTURE ELUCIDATION AND DETERMINATION OF RELATED ORGANIC IMPURITIES

2.1. Strategies in Impurity Profiling

Sándor Görög

2.1.1. General Considerations

The origin of related organic impurities in drug materials is the subject of Section 1.2.1 while the toxicological aspects are discussed in Section 1.3. In Section 1.5, where the regulatory-pharmacopoeial aspects of the problem of related organic impurities are discussed in detail, threshold limits are presented above which the identification of the impurities and their quantitation by sufficiently specific methods are mandatory. In the majority of cases this limit is 0.1%, but drug registration authorities, synthetic research chemists (see Section 1.4.1), technologists (see Sections 1.4.2 and 1.4.3) and customers of bulk drug materials are increasingly interested in impurities/degradation products in the range of 0.01–0.1%.

As is described in Sections 1.2.1 and 1.4, the impurity profile is subject to changes caused by changes in the synthetic route, reaction conditions, source and quality of the starting materials, reagents and solvents used during the synthesis, the purification steps, conditions of crystallisation, drying, distillation and storage of bulk drug materials. More or less the same applies to drug formulations, too. As a consequence of these points the impurity profiles of bulk drugs and formulations made thereof have to be checked repeatedly not only during the research and development period but also in all cases when changes take place in the above listed factors. For this reason the estimation of impurity profiles is a frequent task, especially in industrial research and quality control laboratories. Taking into account the time and labour consuming nature

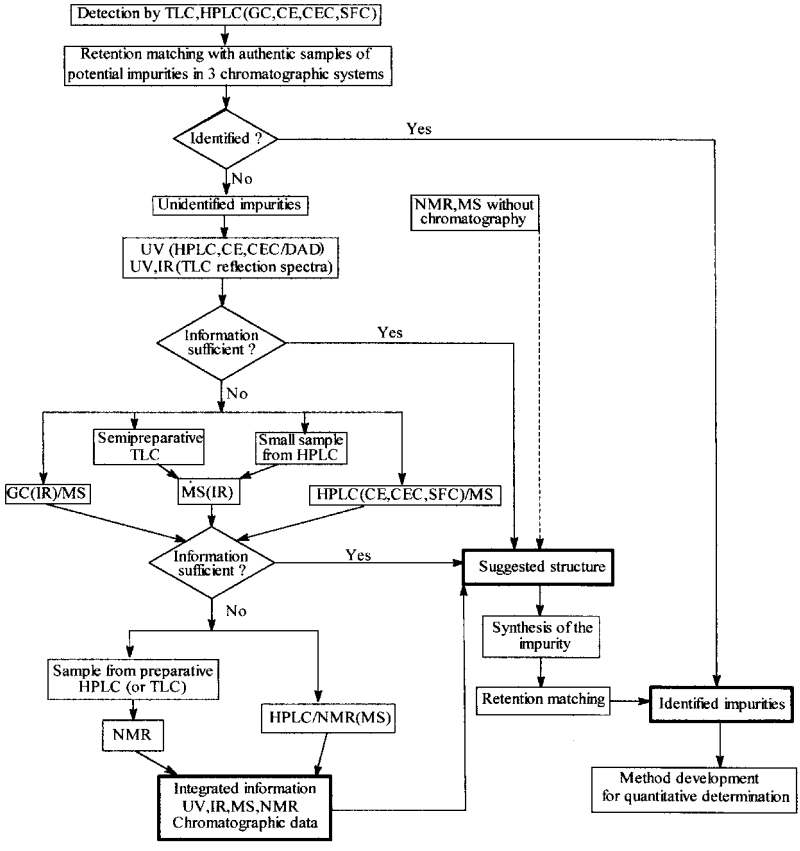


Figure 2.1.A.
A general scheme for the detection, identification, structure elucidation and determination of impurities in drugs

of these studies it is essential to find a strategy which enables the results to be achieved within the shortest possible time with the greatest possible certainty.

The aim of Chapter 2 is the summarisation of general strategies for the identification and quantitative determination of organic impurities meeting these requirements (Section 2.1) and the discussion of the possibilities of individual and hyphenated spectroscopic, chromatographic and some other techniques in Sections 2.2–2.11. In Fig. 2.1.A a scheme is presented containing a proposal for the systematic use of methods available for impurity profiling of drugs. This scheme represents to a great extent the way of thinking in the author's laboratory regarding this matter and the strategy in use there.

It is very difficult to give a general picture about the strategies used in other laboratories for the impurity profiling of drugs. Although of course more or less the same techniques are used in all laboratories, the hierarchy and the manner of the use of the methods in the individual laboratories can be quite different. The reasons for these differences and the difficulties in presenting a general picture about the situation are as follows.

- The overwhelming majority of the research aiming at estimating the impurity profiles of drugs is carried out in the research and quality control laboratories of industrial companies. These companies usually consider the information obtained as a result of this research as the know-how of the company and for this reason only a very small proportion of these results has been published in the literature. For this reason it is rather difficult to give an overview characteristic of the situation in the laboratories of leading drug manufacturers.
- The methodology used in impurity profiling is very rapidly developing. This can be characterised by the continuously increasing size and complexity of the schemes for impurity profiling published in consecutive papers by the author of this section [1–3], preceding the scheme in Fig. 2.1.A. The main impurities in drugs were identified already in the classical period of pharmaceutical research and production. Thirty to forty years ago this was carried out in a very time and labour consuming way, mainly by column chromatographic separation of the impurities followed by the spectroscopic characterisation of the separated materials using the less developed forms of the presently existing spectroscopic techniques. The introduction and rapid spreading of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in the early and late 1960s, respectively, and the appearance of hyphenated techniques, mainly HPLC-MS-(MS) in the early and HPLC-NMR-(MS) in the late 1990s has created an entirely new situation in pharmaceutical research and analysis, and also in the field of impurity profiling. The on-line combination of the great separation power of these chromatographic (and recently also electrophoretic) methods with the spectroscopic techniques making use of the enormous developments in the performances of the latter really enables even minor impurities to be identified and quantified within a very short time with great certainty. The strategy of impurity profiling in different laboratories derived from the rather scattered publications published even in the last decade can reflect different stages of the development in analytical technology.
- The strategy of impurity profiling greatly depends on the character of the laboratory where the work is carried out and on its financial possibilities. In those laboratories where the aim of the work is to give analytical support to research aiming at synthesising new drug compounds and introducing them

to the therapy, the use of the most up-to-date (and very expensive) instrumentation is almost mandatory. At the same time in the analytical laboratories of minor, generic companies it is not by all means necessary to use the most developed instrumentation. It is to be noted that – as described in Section 1.5.3.3 – it is not obligatory to present for the registration authorities the fine structure of the impurity: it is acceptable to describe the results of the partly successful or even unsuccessful trials. This means that the structure of minor impurities is often determined leaving some questions open regarding their fine structure.

- A look at the scheme in Fig. 2.1.A will certainly convince the reader that there are several parallel, in many cases more or less equivalent approaches to reach the same goal, the structure elucidation of the impurity. This situation can be characterised as ''the confusion of abundance'' and as a consequence of this the strategy of impurity profiling in a certain laboratory can depend in addition to the factors discussed above also on subjective factors such as the traditions of the laboratory, moreover on the taste and personal ambitions of the analysts.

What is certainly common in the approaches in different laboratories is that the work begins with the detection of the impurity and its retention matching with possible impurities and if necessary it is concluded with the complex application of chromatographic (electrophoretic), spectroscopic and hyphenated techniques. The summarisation of these studies is the subject of Sections 2.1.2 and 2.1.3.

It is very important to devote a few sentences to the proper selection of samples to be investigated during the course of impurity profiling. Of course the final goal is the identification/structure elucidation and quantitation of impurities being present in the bulk drug substance and/or the drug formulation made thereof. However, especially in the case of minor impurities during the first steps of the research it can be extremely useful to acquire samples in which the relative concentration of these impurities is higher (crude products, mother liquor products in the case of bulk drugs and drug formulation degraded under stress conditions). Using these samples the sensitivity-related difficulties often occurring in this research can be overcome. If the isolation of the impurity is necessary, it can be very advantageous to use these samples as the starting material. However, it is not a general rule that the use of these samples is beneficial because drawbacks can also occur which should also be taken into consideration. For example, it is often the case that there are impurities in these samples which are not present in the finished product (due to the effective purification step in the preparation of bulk drugs or the good stability of the formulation). These sometimes overlap with the real impurities and the difficulties of their separation may exceed the advantages of their use. Even if

problems of this kind are not met, it is important to check by the methods outlined in the subsequent section that the impurity which is looked for is identical with the one in the crude, etc. sample.

2.1.2. Detection of Impurities

As seen in Fig. 2.1.A, the first step of impurity profiling is the detection of the impurities. Although (as will be discussed in Sections 2.1.3, 2.3 and 2.4) high resolution NMR and mass spectroscopy can play an important role in getting a fingerprint-like picture on the purity of a drug sample, the impurities to be characterised in the subsequent steps of impurity profiling are detected by one of the chromatographic (or electrophoretic) techniques. The careful selection of the separation techniques to be used is essential even in this first step. Impurities with extremely low or high polarity/mobility can migrate too slowly or rapidly to be reliably detected and even if this is not the case, an impurity is not necessarily separated from the main component or another impurity and hence there is a danger of not observing and thus neglecting it if only a single separation technique is used. For this reason it is very important to use as many separation techniques with different separation mechanisms as possible to obtain the number of impurities to be dealt with during the course of impurity profiling. The use of planar and high-performance liquid chromatographic (TLC and HPLC) techniques (possibly both in the normal and reversed phase mode) is mandatory but supercritical fluid chromatography (SFC), in the case of charged molecules capillary electrophoresis (CE) and for sufficiently volatile and thermally stable materials gas chromatography (GC) can also afford useful data. Another important aspect of the success of the detection of all impurities is the sensitivity of the detection. It is usually possible to estimate impurities at the 0.01% level if the above mentioned techniques are used under optimised conditions. If not, there are various possibilities to increase this. For example, pre-column derivatisation of the primary amino group with fluorescamine to form highly fluorescent derivatives greatly improves the detectability of spectrophotometrically poorly active impurities after HPLC separation [4].

In this stage of the research it is often necessary to find connections between the various separation techniques. This means that it is necessary to ascertain *which TLC spot, HPLC or GC, etc. peak belongs to the same impurity*. TLC spot elution and injection of the obtained solution into the high-performance liquid chromatograph, gas chromatograph or capillary electrophoresis instrument for retention matching can be done without major technical difficulties. The same applies to HPLC fractions, too. However, in the case of using buffered reversed-phase HPLC systems it is often necessary

to extract the fraction with an apolar solvent prior to injecting it into the gas chromatograph or applying it onto the TLC plate. Although not frequently used, it is worth mentioning that the problem of applying HPLC or GC fractions onto TLC plates has been solved [5,6] and on-line coupling of RP-HPLC with GC-MS has also been described [7]. In addition to the chromatographic data, the identity (or close similarity) of the HPLC(CE, CEC)/diode-array UV spectrum with the TLC reflection spectrum or the identity of the mass spectra obtained in the on-line or off-line mode from TLC, HPLC or CE fractions can also afford useful evidence for the identity of the impurity behind various peaks and spots.

2.1.3. Attempted Identification of Impurities by Chromatographic Retention Matching with Known Potential Impurities

After having obtained a clear picture about the number of impurities to be dealt with and being in possession of chromatographic (electrophoretic) methods suitable for their separation from the main component and from each other the next step in the procedure of impurity profiling is attempting their identification with known potential impurities.

The success of this step depends on the level and intensity of co-operation of analytical chemists with synthetic chemists. With a sufficiently high number of samples of *potential impurities* the analytical chemist has a good chance of identifying the impurities or at least a part of them in a very easy way thus avoiding or at least decreasing the necessity of further time and labour consuming chromatographic and spectroscopic work. Potential impurities are the last intermediate(s) in the synthesis of the drug material, products of uncompleted reaction (e.g. monoacetate of a dihydroxy compound, where the drug material is the diacetate), products of overreaction (e.g. diacetate in an analogous case where the drug material is the monoacetate), by-products (products of side reactions), degradation products which are likely to be formed during the last reaction step, the isolation of the endproduct, etc. (See Section 1.2.1.). Synthetic chemists and technologists usually have several analogous compounds from the earlier period of the research (isomers, etc.) which can also be considered to be potential impurities. It is very important that all these samples be available for the analytical chemists.

With the samples of potential impurities in their hands it is the task of drug analysts to attempt to prove the identity of the real impurities detected earlier with these potential impurities. The easiest and for this reason most generally used method for this is *retention matching*. The necessity of using simultaneously several chromatographic and related separation techniques was already emphasised in the preceding section when the analytical goal was

the separation and detection of the impurities. Of course the responsibility of the analytical chemist at this stage is even greater: it is mandatory not to rely upon a single chromatographic technique because this can be misleading. The identification based on the identity of chromatographic retention times, electrophoretic migration times or R_f values can be considered successful only if identical values are found in at least two but even more preferably in three different systems based on different separation mechanisms. In these experiments it is not sufficient to compare the retention/migration times found in two chromatographic/electrophoretic runs: it is necessary to run separations where the solution of the drug sample is spiked with the potential impurities.

In addition to the successful identification based on retention matching further evidence for the identity of the potential and real impurities is affordable on the basis of comparing easily accessible spectral data. For example, the diode-array UV spectra after HPLC or CE separation, the reflection UV or fluorescence spectra (or at least the colour of the spots at daylight or under short or long wavelength UV lamp) after TLC separation should also be identical. Checking the identity of GC/MS or HPLC/MS pattern can greatly improve the reliability of the identification. In this respect the papers of Nicolas and Scholz merit special mentioning. These workers generated libraries of the HPLC/diode-array UV spectra [8] and HPLC/MS/MS spectra [9] of related compounds, potential and real impurities synthesised in the course of the research for a new drug compound. The UV match factor analysis and fingerprinting using the MS/MS spectra greatly contributed to proving the identity by HPLC retention matching of impurities as low as 0.01% in different lots, prepared by using different synthetic methods.

2.1.4. Complex Application of Chromatographic, Spectroscopic and Hyphenated Techniques for the Structure Elucidation of Impurities

As seen in Fig. 2.1.A the first spectroscopic data which are usually obtained during the course of the complex application of chromatographic and spectroscopic techniques in drug impurity profiling are the *UV spectra* of the impurities. These (and also fluorescence spectra) are easily accessible by means of taking reflection spectra when planar chromatographic techniques are used for the separation of the impurities (for details see Section 2.5.4). Quite recently *reflection FT-IR spectra* taken after planar chromatographic separation has also appeared in the arsenal of analytical methods affording useful information for the structure elucidation of impurities (see Section 2.5.5). When HPLC or one of the capillary electrophoretic techniques are used for the separation of the impurities, rapid scanning (usually diode-array) UV detectors can produce good-quality UV spectra. Gas chromatography is the only

separation technique where no UV spectra can be taken by the commercially available instruments.

The conditions for the usefulness of the above mentioned UV spectra are described in detail in Section 2.7.3. In this section and also in Section 5.3.2 several examples are presented where important conclusions could be drawn from the UV spectra. Sometimes quite small differences between the spectra of the impurity and the main component are of diagnostic value. In some advantageous cases it is even possible to suggest a structure for the impurity at this point if in addition to the UV spectrum the chromatographic behaviour of the impurity and the information on the chemistry of the synthetic procedure are also taken into consideration. Generally speaking, however, the answer to the question formulated in Fig. 2.1.A, namely whether the information obtainable from the UV spectra afford sufficient information to suggest a structure for the impurity is in the majority of cases *no*. It is, however still useful to spend some time and energy on the evaluation of UV spectra since the information thus obtained can be regarded as a useful complement to that obtained from the mass- and NMR spectra.

If the information obtainable from the UV spectrum is not sufficient, the next step in the procedure of the structure elucidation of impurities is usually to take the *mass spectrum* of the impurity. The most effective way to do this is to make use of the on-line GC/MS and HPLC/MS facilities available in the sufficiently developed laboratories dealing with impurity profiling. A great benefit of these techniques is that data can be obtained simultaneously on several impurities down to the 0.01% level. A special advantage of the *GC/MS* technique (Section 2.6.6) is that reliable molecular weight information is obtainable using chemical ionisation and, in addition information on fragmentation necessary for the solution of more delicate structure elucidation problems can also be obtained using the electron impact ionisation technique. A disadvantage is that due to volatility and thermal stability problems the applicability of this technique is limited. Derivatisation reactions widely used in other areas of GC/MS analysis can only be used here if the quantitative nature of the reaction can be ascertained thus avoiding the danger of confusing the side-products of the derivatisation reaction with real impurities.

Great advantages of the *HPLC/MS technique* (Section 2.7.4) are its general applicability and the possibility of coupling it with diode-array UV detectors (*HPLC/UV/MS*). A disadvantage is that using the first generation instruments the soft ionisation techniques usually give only molecular weight information. In modern instruments fragmentation is also obtainable. The most effective devices for impurity profiling are *HPLC/UV/MS/MS* instruments which can simultaneously furnish all information discussed so far. For those laboratories where this technique is available, moreover the number of these instruments enables all simultaneously arising impurity profiling problems to

be dealt with using this technique, the strategy of impurity profiling can be greatly simplified. What is described in the HPLC/UV/MS/(MS) technique more or less applies to techniques where HPLC is substituted by SFC (Section 2.10), capillary electrophoretic techniques, CE (Section 2.8) and CEC (Section 2.9.2). Of these relatively new techniques especially the latter is expected to have a bright future in drug impurity profiling.

As is seen in Fig. 2.1.A, in addition to the hyphenated techniques, there are several other possibilities, too, to make use of the information obtainable from the mass spectra. Of these at first *direct mass spectroscopic* investigation of the sample without any preliminary chromatographic, etc. separation is mentioned. The possibilities of this method which can result in good (preliminary) data is discussed in Section 2.3.2. In laboratories where the modern hyphenated techniques are not available the mass spectra of the impurities are usually taken in the *off-line mode* after separation by planar or high-performance liquid chromatography. As a result of the high sensitivity of mass spectrometry this does not require special instrumentation: impurities scraped off and eluted after TLC separation can be directly investigated. Almost the same applies to HPLC where it is not by all means necessary to obtain the impurity sample by preparative HPLC: the quantity of impurities in fractions obtained from a sufficiently highly loaded analytical column is usually sufficient for the MS investigation (Section 2.3.3) unless the eluent contains inorganic salts and buffers. This approach can be occasionally beneficial even in those laboratories where the hyphenated techniques are at disposal. In such a way information is rapidly obtainable thus avoiding the necessity of developing and optimising suitable systems for, e.g. the HPLC separation of the impurity compatible with the HPLC/MS system.

At this point in the complex procedure of impurity profiling, being in possession of the information obtained from the UV, (IR) and mass spectra the drug analyst should make a very important decision. It should be decided whether the careful evaluation of this integrated information together with the chromatographic data of the impurity in relation to the main component and the full knowledge of the chemistry of the synthesis make it possible to suggest a structure for the impurity or not. If the answer to this question is *yes*, the procedure runs further as shown in Fig. 2.1.A and described in the subsequent sections (synthesis of the suggested structure, etc.). If the answer is *no*, time and labour consuming NMR studies should be carried out prior to this. The experience in the authors laboratory and the scattered data published in the literature show that in a considerable proportion of cases it is possible to stop here (leaving sometimes some questions regarding the fine structure of the impurity open).

The ultimate method for elucidating the structure of the impurity is *NMR spectroscopy*. For the possibilities of NMR spectroscopy without preliminary

chromatographic separation see Section 2.4.3, while Section 2.4.5 summarises the aspects of NMR spectroscopy as a structure elucidation tool in the off-line mode after chromatographic separation. Although the introduction of new techniques have greatly decreased the demand for sample size to obtain good quality NMR spectra, generally speaking it is much higher than that of mass spectroscopy. It is usually not possible to take the NMR spectrum of impurities after separation on ordinary TLC plates or analytical HPLC columns: (semi)preparative scale separation is required to obtain the suitable sample size with minimum level of spectral background caused by ill-defined artefacts. Another possibility is to make use of the latest development in this field, the commercial availability of *on-line HPLC/NMR*, moreover *HPLC/NMR/MS* instruments. So far these techniques are available only in a very limited number of laboratories and only a few papers have been published containing the first results in impurity profiling (Section 2.7.5). At the present time it would be yet difficult to predict to what extent and how rapidly these new techniques will spread in the future and to what extent they will create new situations in drug impurity profiling.

With the NMR spectrum and all other spectra obtained in the previous steps of the work outlined above a structure can be, moreover, should be proposed for the impurity. At this point it should be emphasised that the structure elucidation of impurities is a typical team effort which requires the close co-operation of spectroscopists, chromatographers and synthetic organic chemists. The role of chromatographers is not only to develop systems for the separation and detection of the impurities and for the generation of spectra by the hyphenated techniques, it is also very important to take into consideration during the course of this work not only the spectral data but also the TLC R_f values and HPLC or GC retention times. The comparison of these with those of the main component and other potential impurities furnish useful data regarding the polarity of the impurity and this is also an important source of information to propose the structure for the impurity. It is also essential that a member of the team be an organic chemist who is familiar with all aspects of the synthesis of the drug in question whose opinion should also be taken into account in problematic cases.

2.1.5. Synthesis of the Impurities

It was mentioned in the last sentence of the preceding section that the synthetic organic chemist is an important member of the team during the structure elucidation of the impurity. As shown in Fig. 2.1.A, in the following step of the procedure the organic chemist plays the main role in the synthesis of the impurity with the proposed structure.

At this point the responsibility of the analytical members of the team has to be emphasised. It is not unusual that the synthesis of the impurity with the proposed structure is a more difficult task than that of the main component itself and the multistep synthesis may require several weeks of intense work. For this reason, especially in the case of complicated structures the proposal for the structure to be synthesised should be made after extremely thorough and careful considerations. This is, however, not a general rule. In other instances a structure proposable at an earlier stage of the work can easily be synthesised. It is often the case that the synthesis of a structure proposed on the basis of UV + mass spectrum is easier and less time consuming than its preparation by preparative HPLC for further NMR studies. The question of how to proceed at various stages of the procedure of impurity profiling has to be answered individually from case to case taking into account all these points.

The reasons for the necessity of the gram-scale synthesis of the impurity are as follows:

- After the successful synthesis and full spectroscopic and analytical investigation of the synthesised material this will undergo chromatographic and spectral matching with the impurity found in the drug material. In Section 2.1.3 it is described in detail how the identification of the real impurities on the basis of chromatographic and on-line spectral matching with known potential impurities should be carried out. The same rules apply to this case also. In the case of successful matching final evidence is given to the suggested structure.
- The quality of the spectra obtained from the synthesised material is usually better than that of those taken in the on-line mode or from small isolated samples. After having proved their identity the spectra of better quality can be used for registration and publication purposes.
- The gram-scale quantity of the synthesised impurity can be used as the *impurity standard*. This means that in possession of this it is possible to develop selective analytical methods for the quantitative determination of the impurity (see Section 2.1.6). When such a selective method becomes part of the analytical testing protocol for every batch this impurity standard has to be used routinely. This also means that it may be necessary to submit this sample to drug authorities and customers of bulk drugs may also require the impurity standard.
- The synthesised impurity can be subjected to toxicological testing. In the case of major impurities this is mandatory (see Section 1.3).

Sometimes the synthesis of the impurity can be very problematic, moreover impossible. In these exceptional cases the synthesis can be omitted from the protocol of impurity profiling and the impurity standard can be prepared (often with great difficulties) using preparative HPLC.

2.1.6. Quantitative Determination of the Impurities

The reliability and mainly the accuracy of the quantitative determination of impurities (degradation products) highly depend on whether an impurity standard is available or not. The pharmacopoeias usually do not consider the availability of impurity standards and prescribe non-selective, general quantitative HPLC or semiquantitative TLC and much less frequently GC methods for this purpose. More or less specific methods are prescribed for the determination of only a very limited number of named impurities. In the majority of cases, however, the impurities are not named and even if they are, no selective methods are presented for their determination and hence the quantity of the individual impurities and their sum are expressed as the main component when the above listed methods are used for their determination.

The reliability of these general tests and the value of the results thus obtained depend on several factors and can vary between quite acceptable and absolutely misleading results. The results can be acceptable when HPLC or TLC densitometry are used with a single-wavelength UV detector and the UV spectra of the main component and of the impurity are close to each other and the wavelength of the detection is properly selected. Using the same techniques misleading results are obtained if the spectra are different and in addition to this the wavelength of the detector is not optimal. A 10-fold overestimation can easily occur in the case of the determination of a spectrophotometrically active impurity in an inactive drug and of course similar underestimation of the impurity may take place if the drug is spectrophotometrically active and the impurity is inactive. The same applies to non-pharmacopoeial CE and CEC methods as well. Seriously erroneous results can be obtained during the visual comparison of spots on the thin-layer chromatogram especially if this is done after spraying the plate with a reagent which may react in a different way with the drug and its impurity and may result in different colours. By using GC (and SFC) with flame ionisation detector the differences between the signals of the drug and its structurally related impurity are usually not too large.

All these difficulties and uncertainties can easily be overcome when in possession of the synthesised impurity standard. There are three general possibilities to achieve this.

- As described above, in many cases the differences between the detector signals of the drug and its impurity are not too large: the relative detector signal (response factor) is close to unity, and for this reason it can be neglected. According to the British Pharmacopoeia [10] this principle is applicable when the detector signal is between 0.8 and 1.2.
- If the response factor is larger (e.g. between 0.2 and 5 [10]), it has to be

determined under strictly defined experimental conditions and used as a correction factor in all cases when the quantity of the impurity is primarily obtained from the chromatogram or electropherogram expressed as the main component. The advantage of this simple approach is that after the development and validation of the method no further, often very expensive, impurity standard is required when the method is used routinely.

- In the case of even larger differences between the detector signals and in the general case when the basis of the semiquantitative determination of the impurity is the visual comparison of spots on TLC plates, the method for the determination of the impurity should be developed and validated using the impurity standard and it should be used even afterwards when the method is used routinely.

These general approaches are described in detail and demonstrated on examples in the various sections of Chapter 2 where the application of different analytical techniques are presented to the quantitative determination of impurities in drugs.

2.1.7. A Case Study: Structure Elucidation of Two Impurities in Norgestrel

The following example has been taken from the practice in the author's laboratory [11] with the aim of demonstrating the necessity of the complex use of a variety of techniques (analytical and preparative TLC and HPLC, GC, UV, NMR, MS, HPLC/diode-array UV, GC/MS) in order to obtain reliable results within the shortest possible time. The subject of the study was the structure elucidation of an impurity in norgestrel. This impurity appeared at an R_f value of 0.83 using the TLC system of USP 24 [12] and its quantity was about 0.1% or less expressed as the main component using the non-selective semiquantitative test of USP 24 after visualisation of the spot with phosphomolybdate spray. The R_f value of the main component was 0.56. Retention matching with known potential impurities of norgestrel (3-methoxy-17α-ethinyl-13-ethyl-gona-1,3,5(10)-triene-17-ol and 13-ethyl-gon-5(10)-ene-3,17-dione revealed that these are not separated from the unknown impurity. The TLC reflection spectrum of the spot at $R_f = 0.83$ had a maximum at 268 nm. This clearly indicated that this impurity did not contain either the chromophoric 4-ene-3-oxo group of the main component ($\lambda_{max} = 240$ nm) or the above mentioned phenol-ether type chromophore of one of the above mentioned potential impurities ($\lambda_{max} = 280$ nm). Since the other potential impurity is spectrophotometrically inactive, a new TLC system had to be developed which was more selective than that of USP 24 (Kieselgel 60 F254/chloroform–acetone (95:5

v/v)). In this system the following R_f values were obtained for norgestrel, its apolar potential impurities and the unknown: norgestrel (0.55), 3-methoxy-17α-ethinyl-13-ethyl-gona-1,3,5(10)-triene-17-ol (0.79), 13-ethyl-gon-5(10)-ene-3,17-dione (0.62), 4β,5β-epoxynorgestrel (0.74). The above listed named potential impurities were absent or their quantity was below 0.02%.

The HPLC investigation of the same norgestrel samples (column: LiChrosorb 10 C-18, 250 × 4.6 mm; eluent: acetonitrile–water (85:15 v/v) at 1 ml/min) an apolar impurity appeared at 8.4 min (main component 4.0 min) with a diode-array UV spectrum very similar to the above mentioned reflection UV spectrum of the unknown (maximum at 268 nm with side maximum at 279 nm). On the basis of the UV characteristics and the highly apolar nature of the impurity it was presumed that the 3-oxo group is lacking and the molecule contains a conjugated bonding system in rings A/B. The mass spectrum obtained after TLC spot elution contained a molecule peak at m/z = 320 which corresponds to a 3-deoxo-A/B-diene-3,17-diethinyl structure.

The formation of this type of impurity is easily derivable as a side reaction of the last reaction step: it is the result of the not completely regioselective ethinylation of the 3,17-dione-type intermediate (see Fig. 2.1.B). The product of this side reaction is the mixture of 13-ethyl-3,17α-diethinyl-3,17-dihydroxy-gon-4-(and 5)-enes. In the course of the work-up of the reaction mixture acid-catalysed dehydration takes place very much simplifying the situation leaving uncertainties only in the position of the double bonds: 3,5 or 3,5(10) or 2,4. On the basis of the Woodward rule [13] the 3-ethinyl-3,5-diene structure was the most likely. The λ_{max} calculated for this structure was 264 nm (taking 30 nm as the increment of the linearly conjugated ethinyl group). For the final evidence NMR spectroscopic support was necessary. At this point we found it reasonable to synthesise the impurity by diethinylating the starting material followed by acid-catalysed dehydration and fractionation of the reaction product by preparative HPLC rather than isolating this minor impurity from the drug material.

The main component of the synthesised impurity was found to be identical with the unknown impurity on the basis of TLC R_f values, HPLC retention time, TLC reflection and HPLC/diode-array UV as well as mass spectrometric spectral matching. The most characteristic signals in the NMR spectrum of the synthesised material were singulets of the 3- and 17-ethinyl groups at 2.97 and 2.58 ppm, ^{13}C signals of the same at 85.9 + 77.2 and 88.0 + 74.0 ppm, respectively, multiplets of the vinylic H-4 proton at 6.40 ppm and of the H-6 proton at 5.59 ppm. On the basis of these data the structure of the impurity was considered to be proved: 13-ethyl-3,17α-diethinyl-gona-3,5-diene-17-ol.

It is interesting to note, that the synthesised impurity contained the isomeric 3,5(10)-diene derivative as a minor component. The ^1H-NMR signals characteristic of this structure are that of the 3-ethinyl group at 3.03 ppm and

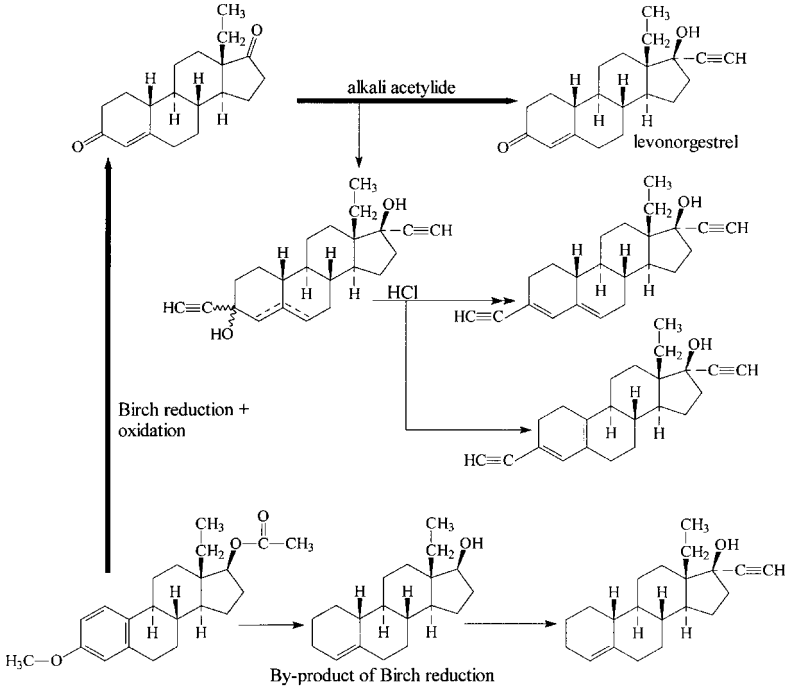


Figure 2.1.B.
The reaction scheme of the last step in the synthesis of norgestrel with the formation of three impurities

the H-4 proton at 6.13 ppm. This isomeric material is poorly resolved from the 3,5-diene (HPLC retention time 8.0 min). The maximum of the diode-array UV spectrum of this *cis*-dienyne type derivative was found to be at 305 nm (calculated on the basis of the Woodward rule at 303 nm). This minor impurity was also found in the investigated norgestrel samples but only at trace level (below 0.02%).

With the selective HPLC method and the impurity standard the quantitative determination of 3,17α-diethinyl-gona-3,5-diene-17-ol became possible and the results could be compared with the above mentioned TLC densitometric results after visualisation with phosphomolybdate. No sufficient mass balance was achieved: the HPLC results were somewhat lower. This was an indication of the presence of another, presumably UV-inactive impurity under the spot of 3,17α-diethinyl-gona-3,5-diene-17-ol. After having developed an even more selective TLC system (mobile phase: 60:40 v/v mixture of *n*-hexane

Table 2.1.A.
Relative retention times (4-ene-3-oxo steroids/3 deoxo analogues). For the HPLC system see text

4-ene-3-oxo steroid/ 4-ene-3-deoxo steroid	Relative retention time
Norethisterone/lynesterol	3.1
3-Ketodesogestrel/desogestrel	3.3
Norgestrel/3-deoxo-norgestrel	3.5

and chloroform) the two impurities could be separated. The R_f values for norgestrel, 3,17α-diethinyl-gona-3,5-diene-17-ol and the new unknown impurity were 0.12, 0.48 and 0.52. The reflection UV spectrum and the HPLC diode-array UV spectrum of the new impurity (retention time at 14.0 min) have proved that this impurity is really spectrophotometrically inactive. The molecular peak in the mass spectrum of the new impurity obtained after TLC spot elution was at m/z = 298 ($M_{norgestrel}$-16). From this, the UV-inactivity and the very low polarity of the new impurity it was concluded that its structure was 3-deoxo-norgestrel (13-ethyl-17α-ethinyl-gon-4-ene-17-ol). Further evidence was furnished by the comparison of the HPLC relative retention times of various pairs of 4-ene-3-oxo and 4-ene-deoxo derivatives; as seen in Table 2.1.A the value of 3.5 was in good agreement with the values found for other pairs. 3-Deoxo-norgestrel was detectable also by gas chromatography. Using an Ultra 1 capillary (12 m × 0.22 mm × 0.33 μm) at a column temperature of 210°C the retention times of norgestrel and 3-deoxo-norgestrel were 31.2 and 11.6 min, respectively. The mass spectrum obtained from the GC/MS scan was in good agreement with that obtained after TLC spot elution. The gas chromatographic method was eminently suitable for the quantitative determination of 3-deoxo-norgestrel while the most selective and sensitive method for the determination of 3,17α-diethinyl-gona-3,5-diene-17-ol was found to be HPLC at 268 nm. (3-Deoxo derivatives are well known impurities of 19-nor-4-ene-3-ones and originate from a side reaction of the Birch reduction step in their synthesis [14–16].)

References

1. S. Görög, *Pharmacon (Seoul)* **21**, 190–197 (1991)
2. S. Görög, J. Brlik, A. Csehi, Zs. Halmos, B. Herényi, P. Horváth, F. Dravecz and D. Bor, *Anal. Methods Instrum.* **2**, 154–157 (1995)

3. S. Görög, M. Babják, G. Balogh, J. Brlik, A. Csehi, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *Talanta* **44**, 1517–1526 (1997)
4. J.C. Berridge, *J. Pharm. Biomed. Anal.* **14**, 7–12 (1996)
5. J.W. Hofstraat, M. Engelsma, R.J. Van de Nesse, C. Goojier, N.H. Velthorst and U.A.T. Brinkman, *Anal. Chim. Acta* **186**, 247–259 (1986)
6. C.T. Banks, *J. Pharm. Biomed. Anal.* **11**, 705–710 (1993)
7. E.C. Goosens, K.H. Stegman, D. de Jong, G.J. de Jong, and U.A.Th. Brinkman, *Analyst* **121**, 61–66 (1996)
8. E.C. Nicolas and T.H. Scholz, *J. Pharm. Biomed. Anal.* **16**, 813–824 (1998)
9. E.C. Nicolas and T.H. Scholz, *J. Pharm. Biomed. Anal.* **16**, 825–836 (1998)
10. *British Pharmacopoeia 1998, Vol. II*, pp A318–321. Her Majesty's Stationery Office, London (1998)
11. P. Horváth, G. Balogh, J. Brlik, A. Csehi, F. Dravecz, Zs. Halmos, A. Laukó, M. Rényei, K. Varga and S. Görög, *J. Pharm. Biomed. Anal.* **15**, 1343–1349 (1997)
12. *The United States Pharmacopoeia 24*, p 1204, USP Convention Inc., Rockville (2000)
13. S. Görög, *Ultraviolet-Visible Spectrophotometry in Pharmaceutical Analysis*, pp 49–50. CRC Press, Boca Raton, FL (1995)
14. H.L. Dryden, in *Organic Reactions in Steroid Chemistry, Vol. I* (J. Fried and J.A. Edwards, Eds.), pp 1–60. Van Nostrand Reinhold, New York (1972)
15. H. Nagoshi and K. Kinugasa, in *Steroid Analysis in the Pharmaceutical Industry* (S. Görög, Ed.), pp 253–265. Ellis Horwood, Chichester (1989)
16. S. Görög, M. Babják, G. Balogh, J. Brlik, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *J. Pharm. Biomed. Anal*. **18**, 511–525 (1998)

2.2. UV-VIS Spectroscopy and Fluorimetry

Sándor Görög

2.2.1. Applications Without Chromatographic Separation [1]

2.2.1.1. Methods Based on Natural UV Absorption

The use of UV-VIS spectroscopy and spectrofluorimetry as tools for the *identification and structure elucidation* of impurities in drugs without chromatographic separation is of very little importance. The applicability of UV-VIS spectroscopy for this purpose is restricted to those instances where the drug material itself is spectrophotometrically completely inactive and the impurity absorbs selectively in the ultraviolet region above 220 nm. Due to the non-selective nature of the light absorption below 220 nm it is impossible to draw valuable conclusions regarding impurities from this spectral region: many of the functional groups occurring even in the spectrophotometrically inactive drug materials are chromophoric groups (isolated double bond, carboxyl group and its derivatives, etc.), which have their π–π^* band in this region. Even the n–σ^* band of auxochromic groups such as amines and halogen derivatives falls into the region below 220 nm.

Although in principle the situation is more favourable in the spectral range above 220 nm, in fact one has to be very cautious with drawing conclusions regarding impurities from the spectra in this range also. For example, if the drug to be tested contains an isolated carbonyl group, its n–π^* band is around 280–290 nm. Although this band is very weak ($\varepsilon < 100$) it can easily be confused with a strong band of an impurity falling into this spectral range. To draw any conclusion regarding impurities from the spectrum of a drug material requires its comparison with that of a sample of high purity. The spectra published in the literature should be treated with great caution. As an example the spectra of *lynestrenol* are shown in Fig. 2.2.A. Curve 1 is taken from a spectrum atlas [2] while curve 2 is recorded in the author's laboratory. Taking into consideration the lack of the shoulder around 240 nm in curve 2 and that the chromophoric groups in the molecule of lynestrenol are restricted to an isolated double and triple bond it can be stated that this shoulder in curve a was due to a conjugated impurity in the sample used for taking the spectrum published in the spectrum atlas. Although it is naturally impossible to say anything about the other parts of the molecule on the basis of the UV spectrum,

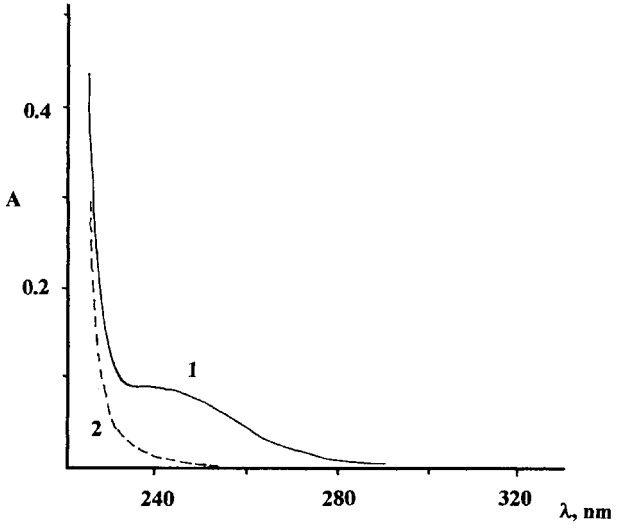
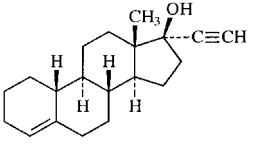
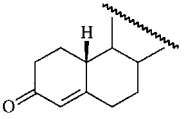


Figure 2.2.A.
Spectra of lynestrenol. (1) Spectrum taken from the literature [2]; (2) Spectrum taken in the author's laboratory, c = 0.05%; solvent: methanol

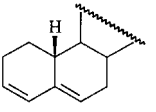
it is probable that it was the 3-oxo derivative of lynestrenol (about 0.25% norethisterone) but the possibility of the presence of some 3,5-diene derivative cannot be excluded either.



Lynestrenol



3-oxo impurity
(norethisterone)



3,5-diene impurity

The use of UV spectrophotometry as a tool for the *quantitative determination* or at least *limitation* of certain impurities in bulk drug materials or other compounds of pharmaceutical interest is of somewhat greater importance: even the latest editions of the pharmacopoeias contain several tests based on absorbance measurements. The following examples have been taken mainly from the European Pharmacopoeia [3–5], British Pharmacopoeia 1998 [6] and the US Pharmacopoeia 24 [7].

As the first example the paragraph ''Conjugated compounds'' in the monograph of *ethynodiol diacetate* is mentioned: the absorbance at 236 nm of the 0.05% solution should not exceed 0.47 [8] or 0.5 [7a]. In this case the term ''conjugated compounds'' can cover the 3,5-diene derivative (λ_{max} = 236 nm) which is the product of the acid-catalysed decomposition of ethynodiol diacetate and also the 4-ene-3-oxo derivative (norethisterone acetate, which is an intermediate of the synthesis; λ_{max} = 240 nm). Fig. 2.2.B shows the spectra of ethynodiol diacetate spiked with increasing quantities of the 3,5-diene. It can be seen that at 236 nm at the given concentration the absorbance of pure ethynodiol diacetate is 0.15, about 0.1% of the impurity can be detected and above 0.2% it is easily measurable, moreover, at this level even the very characteristic fine structure in the spectrum of the impurity is detectable. The absorbance limit of 0.47 corresponds to about 1% of the 3,5-diene impurity.

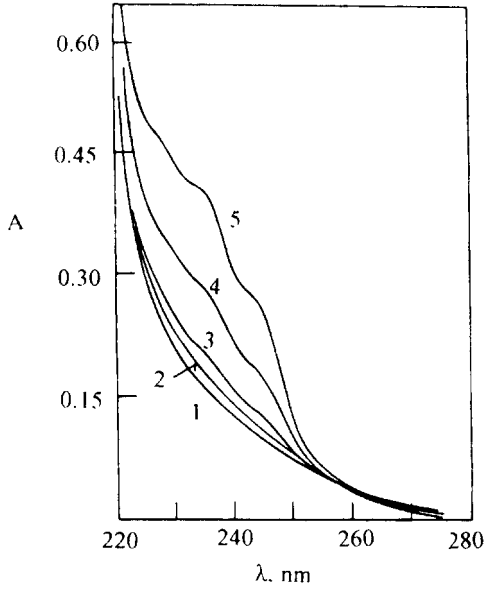
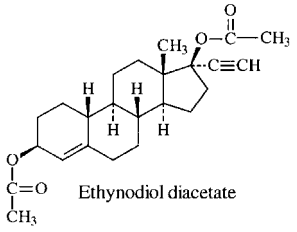
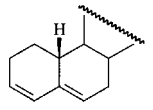


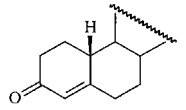
Figure 2.2.B.
Spectra of ethynodiol diacetate (0.05% w/v in ethanol) spiked with the impurity 17α-ethinylestra-3,5-diene-17-ol diacetate; (a) 0%; (b) 0.1%; (c) 0.2%; (d) 0.4%; (e) 0.8%)



Ethynodiol diacetate



3,5-diene impurity

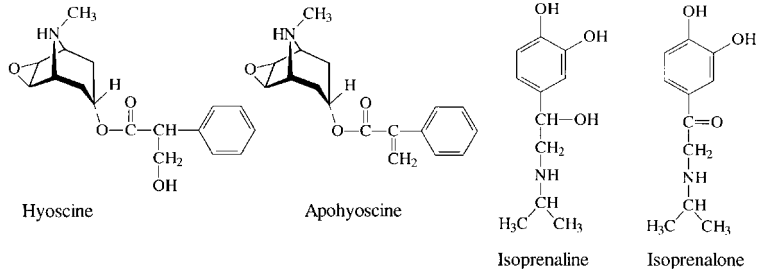


3-oxo impurity

Another example for the measurement of spectrophotometrically active impurities in inactive materials is the testing of *dimeticone* (methyl siloxane polymer) for phenylated compounds [3a] on the basis of the absorbance in the range of 250–270 nm, where the α-band of the phenyl moiety can be found. The selectivity and the sensitivity of the determination of the unsubstituted phenyl group can be improved by using derivative spectrophotometry. For example, the benzene content in 96% *ethanol* [6a] is determined by second-derivative ultraviolet spectrophotometry. The measurement of the amplitude of the peak at 253 nm in the ^{2}D spectrum enables as low a benzene concentration as 2 ppm to be determined. The highly absorbing 5-hydroxymethylfurfural ($\varepsilon_{283\ nm} = 16.300$), degradation product of *sugars* (e.g. *fructose*) is determined at its absorption maximum [3b].

The intense band of the conjugated system of a phenyl ring and a double bond, e.g. in apohyoscine at 245 nm is the basis for the determination of this impurity in *hyoscine hydrobromide* [3c] which contains only an isolated phenyl group poorly absorbing at this wavelength. The selectivity can be improved by using the absorbance ratio method (A_{246}/A_{263}) in the course of the determination of apo-ipratropium in *ipratropium bromide* [3d]. The determination of as little as 0.1% of 1-isopropylamino-3-(2-prop-1-enylphenoxy)propan-2-ol impurity in *alprenolol hydrochloride or benzoate* (the isomeric non-conjugated 2-allyl derivative) is based on the same principle ($\lambda_{max} = 297$ nm) [3e]. The problem of the direct determination of related aromatic ketones (phenones) in benzyl alcohol derivatives is quite similar. If there are no hydroxyl groups on the phenyl ring (phenylpropanonamine impurity in *phenylpropanolamine hydrochloride* [3f,7b] or piperidylpropiophenone impurity in *trihexyphenidyl hydrochloride* [6b]) the wavelength of the measurement is 285 and 247 nm, respectively, while in the case of phenol derivatives (isoprenalone in *isoprenaline sulphate* [3], adrenalone in *epinephrine* [7c], noradrenalone in *noradrenaline hydrochloride* [3g,7d], related phenones in *terbutaline sulphate* [3h,7e], *phenylephrine* [3i], *metaraminol tartrate* [6c], *isoxsuprine hydrochloride* [3j], *isoetharine hydrochloride* [7f], *isoproterenol hydrochloride* [7g], *metaproterenol sulphate* [7h]) it is in the range of 310–330 nm. Because of the not

completely selective absorption of the phenones at these wavelengths the measurement is suitable for the limitation rather than exact determination of the impurities.



Due to its biphenyl moiety the dimeric derivative of *droperidol*, 4,4′-bis[1,2,3,6–tetrahydro–4–(2–oxo–1–benzimidazolinyl)–1–piridyl]butyrophenone absorbs selectively at 330 nm. This is the basis for the limit test (1.5%) of this impurity [7i]. The dimeric derivative of *haloperidol* can be measured in a similar manner at 335 nm [7j].

In some cases the selection of a suitable pH for the solvent enables the selective ionisation of the impurity and on the basis of this its selective determination. For example, the absorption maxima of *estramustine sodium phosphate* where the phenolic hydroxyl at position 3 is in the carbamoyl form exhibits maxima at 267 and 275 nm. This spectrum is not changed upon alkalinisation while that of free estradiol 17β-phosphate impurity undergoes bathochromic shift in 0.1 M sodium hydroxide thus enabling its selective determination at 300 nm [6d]. The determination of phenol in *phenoxyethanol* [6e] and of (3-hydroxyphenyl)trimethylammonium bromide impurity in *neostigmine bromide* [3k] in alkaline medium at 287 and 294 nm, respectively, is based on the same principle.

In other instances the selectivity of the determination of the impurity can be assured by selective extraction prior to the spectrophotometric measurement. As the first example the determination of free chloramphenicol in *chloramphenicol palmitate* is presented. The sample to be tested is dissolved in xylene, the water-soluble impurity is extracted with water. After purification of the aqueous phase by extracting it with toluene the absorbance of the aqueous phase is measured at 278 nm thus enabling the selective determination of low quantities of free chloramphenicol impurity: the limit is set at 450 ppm [3]. Another example is the determination of non-quaternerised 3-dimethylaminophenol as an impurity in *edrophonium chloride* (ethyl(3-hydroxyphenyl)-dimethylammonium chloride). The impurity is extracted with chloroform from

the aqueous solution of the drug buffered to pH 8. Following this, the impurity is re-extracted with 0.1 M aqueous sodium hydroxide and measured as the phenolate at 293 nm [6f]. The principle of the determination of non-quaternerised impurities (expressed as 4-aminoquinaldine) in *dequalinium chloride* is similar. The impurity is extracted from alkaline solution with ether and after re-extraction with 1 M aqueous hydrochloric acid its quantity is calculated from the absorbances of the latter measured at 319 and 326.5 nm [6g]. When dimethylaminophenol impurity is measured in its quaternary ammonium derivative *edrophonium chloride*, the extraction is carried out with chloroform and the absorbance of the extract is directly measured at 252 nm [7k]. The free prednisolone content of *prednisolone sodium phosphate* can be measured at 241 nm after extraction with dichloromethane from an aqueous solution [7l]. A limit test for lumiflavin in *riboflavin and its 5′-phosphate* sodium salt includes the treatment of the test substance with alcohol-free chloroform, filtration and absorbance measurement of the filtrate at 440 nm [7m].

Ultraviolet spectrophotometry is suitable for the solution of even more delicate problems. For the determination of tetraene-type minor component (nystatin, amphotericin A) in the heptaene-type macrocyclic antibiotic drug *amphotericin B* the absorbances are measured at two wavelengths (282 and 304 nm) and calculation is based on the classical Vierordt method [1]. The limit is set to 10% [6h], 5% (parenteral preparations) or 15% (oral and dermatological preparations) [7n]. Another example is the limit test for bacitracin F and related substances in *bacitracin*. This comprises the absorbance measurement at two wavelengths: $A_{290\ nm}/A_{252\ nm}$ should be less than 0.20 [3m].

Quite often the aim of the absorbance measurement above the cut-off wavelength of the drug substance to be tested is not the determination of a well defined impurity but the limitation of impurities absorbing in that spectrum range in general. For example, the paragraph ''Light-absorbing impurities'' in the monograph of *oxytetracycline* ($\lambda_{max} = 353$ nm, A 1%, 1 cm 300) prescribes the measurement of the absorbance at 430 and 490 nm in the 99:1 mixture of methanol and 1 M hydrochloric acid. The specific absorbances at these wavelengths must not exceed 1.25 and 0.2, respectively [3n]. The absorbance of *warfarin sodium* (1.25 g in 10 ml of 1 in 20 solution of sodium hydroxide; 1-cm cell) should be less than 0.1 [3o]. The limit test of non-hydrogenated alkaloids in *ergoloid mesylates* is based on the absorbance ratio $A_{317.5}/A_{280}$ [7o]. Sometimes the requirements are related to wavelength ranges rather than well-defined wavelengths. For example the absorbance of a 1% w/v aqueous solution of *betadex* (β-cyclodextrin) must not exceed 0.10 at any wavelength between 230 and 350 nm and 0.05 between 350 and 750 nm [3p].

2.2.1.2. Methods Based on Colour Reactions

As seen from the examples discussed in this section, the use of ultraviolet spectroscopy in the direct determination of related impurities in drugs is restricted to a limited number of advantageous cases. These possibilities can be somewhat expanded by using *selective chemical reactions* to shift the spectra of the impurities towards the longer wavelengths, usually to the visible spectrum range. In the early period of pharmaceutical analysis these *colour reactions* were the most important means for the detection and as far as possible determination of impurities in drugs [1,9–13]. As a consequence of the introduction and spreading of chromatographic methods their importance has been greatly decreased, but – as it will be demonstrated on the examples taken from the principal pharmacopoeias [3–7] – many of them are still in use. In some cases these colour reactions are followed by absorbance measurements enabling the limitation of the quantity of the impurity on an objective basis. In other cases they are used as limit tests based on the visual comparison of the colours of the test and reference solutions.

The classical colour reaction based on complex formation of salicylic acid with iron(III) reagents is used among others for the determination of free salicylic acid impurity in *carbasalate calcium* (equimolecular compound of calcium-di(acetylsalicylate) and urea). The reagent is iron(III)nitrate and the analytical wavelength is 525 nm. The absorbance limit of 0.115 of the test solution with a carbasalate calcium concentration of 0.2 g/100 ml is equivalent to 0.5% [4a]. In the limit tests for salicylic acid in *aspirin* [3r,7p] and *benolirate* [6i] iron(III)ammonium sulphate or iron(III)chloride is used as the reagent and visual comparison of the colours is carried out. The same method is used for the estimation of 5-chlorosalicylic acid in *niclosamide*. The high sensitivity of the visual method can be characterised by the low limit for the impurity (60 ppm) [3s]. Complexation with iron(III)chloride is used for the determination of meconic acid (3-hydroxy-4-oxo-4*H*-pyran-2,6-dicarboxylic acid) impurity in *morphine* ($\lambda_{max} = 480$ nm) [4b] and streptomycin impurity in *dihydrostreptomycin* ($\lambda_{max} = 550$ nm) [7q]. Other methods based on metal complex formation are, e.g. the determination of peroxides in *copovidone* and *crospovidone* [5a] or in *ether* [7r] with the aid of titanium(IV)chloride reagent ($\lambda_{max} = 405$ and 410 nm, respectively), the determination of non-tertiary amine impurities in *mebeverine hydrochloride* using the copper(II)chloride–pyridine reagent ($\lambda_{max} = 405$ nm) [6j], determination of glycerol in *gold sodium thiomalate* with copper(II)chloride reagent [7s] and the determination of halogenated compounds in *benzyl alcohol* after splitting the carbon–halogen bond by nickel–aluminium alloy followed by reaction with the mercury(II)thiocyanate reagent [3t].

Another group of methods is based on the classical colour reaction of

diazotisation with nitrous acid of primary aromatic amine-type impurities in acylamino type drug materials followed by azo coupling to form azo dyes. A reaction of this type is Fig. 2.2.C taking the determination of free sulfathiazole in *succinylsulfathiazole* as the example. The coupling agent is *N*-(1-naphtyl)-ethylenediamine [3u]. The absorbance of the forming dye is compared at 550 nm with that of a standard solution of sulfathiazole. Sulfathiazole is measured in *phthalylsulfathiazole* in a similar manner [3v]. The same reaction is used also in the limit tests with visual comparison of the intensities of the colours, e.g. reddish-blue colour in the estimation of 4-chloroaniline impurity in *chlorhexidine* [3w] or pinkish-violet in the estimation of 2-chloro-4-nitroaniline impurity in *niclosamide*. The high sensitivity of the method enables to set the limit in the latter case to as low as 100 ppm [3x]. Further applications of the same reaction are the estimation of volatile diazotisable impurities in *4-aminobenzoic acid* [7t], diazotisable impurities in *hydroflumethazide* [7u] as well as free aromatic amines in *diatrizoate* [7v], *iopamidol* [7w], *iothalamic acid* [7x] and *iohexol* [7w]. Another coupling agent is α-naphtol which is used for the determination of free aromatic amines in *amidotrizoic acid* ($\lambda_{max} = 485$ nm) [5b].

An even more sensitive method is the estimation of 4-aminophenol impurity in *benorilate* [6i] and *acetaminophen* [7y] by using the sodium nitroprusside–sodium carbonate reagent (visual comparison of the colour intensities; limit: 20 ppm [6i] or absorbance measurement at 710 nm; limit 50 ppm [7z]). The same reagent is used for the estimation of primary amines and ammonia in *piperazine* [7z] and *N*-aminohexamethyleneimine in *tolazamide* [7a′]. An example for the limit test for non-aromatic primary amines in tertiary amines is their estimation in *benzydamine hydrochloride*

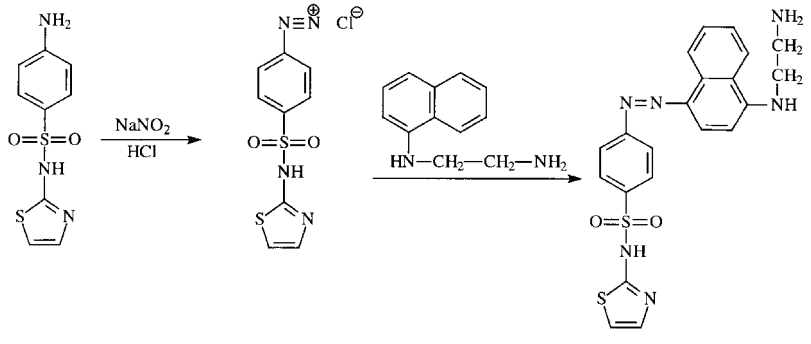


Figure 2.2.C.
Transformation of sulfathiazole to an azo dye

based on the yellow colour of the Schiff's base formed with the 4-dimethylaminobenzaldehyde reagent [6k].

The limit tests of aldehyde-type impurities can be based on their condensation with phenylhydrazine, e.g. in the test for nicotinaldehyde in *nicotinyl alcohol tartrate* (λ_{max} of the forming hydrazone is 370 nm) [6l] or their reducing properties can be utilised. The first example for the latter is the limit test in *betadex* [3p] for reducing sugars, where the latter reduce the copper(II)–tartrate reagent and the forming copper(I) is measured by the molybdenum blue reaction using a sodium arsenate–ammonium molybdate reagent. The absorbance at 740 nm is compared with that of a glucose reference solution (limit: 0.2%). Free fructose in *inulin* is measured with the aid of the blue tetrazolium reaction ($\lambda_{max} = 530$ nm) [7b′]. Another example is the enzymatic oxidation of aldehydes in *povidone* using NAD (nicotinamide-adenine-dinucleotide) as the oxidising agent and aldehyde dehydrogenase enzyme as the catalyst. The test is based on the absorbance at 340 nm of the formed NADH (limit: 500 ppm expressed as acetaldehyde) [3y,7c′]. Formaldehyde is measured in *tyloxapol* with the phenylhydrazine–potassium ferricyanide reagent (limit: 75 ppm, $\lambda_{max} = 520$ nm) [7d′]. For the determination of the same in *oxidised cellulose* chromotropic acid reagent is used (limit 0.5%, $\lambda_{max} = 570$ nm) [7e′].

The classical detection methods for free morphine in *codeine* are still in use: formation of 2-nitromorphine with nitrous acid followed by the estimation of the orange-red colour in ammoniacal solution (limit 0.1%) [3z] and its oxidation with the iron(III)chloride–potassium ferricyanide reagent and estimation of the blue colour [7f′]. The latter method is in use for the estimation of morphine in *noscapine* [7g′]. Another classical reagent (for reducing substances) is tetrazolium blue. It is used for the very sensitive detection of unspecified reducing substances in *ergocalciferol* ($\lambda_{max} = 525$ nm; limit 2 ppm expressed as hydroquinone) [3a′,7h′]. The limit test (300 ppm) for oxalate in *sodium citrate* begins with the reduction of the latter to glyoxalic acid with zinc/hydrochloric acid followed by condensation with phenylhydrazine and terminated with oxidation of the phenylhydrazone with potassium ferricyanide reagent to form a pink reaction product [3b′]. Another reagent for oxalate, iron salycilate is applied for its estimation in *cromolyn sodium* ($\lambda_{max} = 480$ nm) [7i′]. The reagent for the detection of iminodibenzyl in *desipramine hydrochloride* [7j′] is furfural–hydrochloric acid.

The chemistry behind the estimation of two impurities in *fosfestrol sodium* (bis-orthophosphate tetrasodium salt of stilbestrol) is quite interesting [14]. Unesterified free stilbestrol is measured after extraction of this impurity from an aqueous solution with dichloromethane, followed by transformation by short-wave UV irradiation to a tricyclic conjugated tetraene-dione structure measurable at 418 nm (limit 0.15%); see Fig. 2.2.D. The reducing properties of the free phenolic moiety of the semi-esterified stilbestrol sodium monopho-

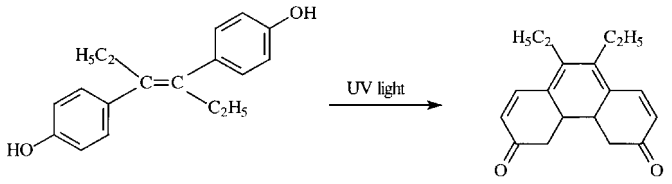


Figure 2.2.D.
UV-light induced transformation of stilbestrol

sphate impurity are the basis for the estimation of this impurity in the aqueous phase of the above extraction using the phosphomolybdotungstic reagent ($\lambda_{max} = 660$ nm; limit 1%) [6m].

There are many more methods for the spectrophotometric estimation of related impurities in drugs in other pharmacopoeias and in the (usually early) literature [1,9–13]. Generally speaking, however, the importance of these methods is expected to further decrease in the future: the TLC or HPLC methods are suitable limit tests even for those named impurities which are now still measured by spectrophotometric-colorimetric methods (in the majority of cases in parallel with the chromatographic purity tests). It is to be noted that in addition to the above discussed related impurities colorimetric methods are widely used as the limit tests for inorganic impurities (arsenic, various metals, sulphite, hydrazine, etc.). These are described in Section 4.1.

2.2.1.3. Fluorimetric Methods

Fluorimetry without preliminary chromatographic separation is only seldom applied for the estimation of impurities in drugs. Two examples are shown; in both instances the measurement is carried out after derivatisation reactions. The limit test (0.5%) for hydroxylamine in *acetohydroxamic acid* is based on derivatisation with pyridoxal 5-phosphate and measurement of the forming aldoxime; excitation at 350 nm, emission at 450 nm [7k′]. The quantity of aminobutanol in *ethambutol hydrochloride* is limited to 1%. Its primary amino group is selectively derivatised with fluorescamine followed by fluorimetric measurement (excitation: 385 nm, emission: 485 nm) [7l′] (see Fig. 2.2.E).

2.2.2. Applications after Chromatographic Separation

UV-VIS spectroscopy and fluorimetry are important tools for the identification, moreover, structure elucidation of impurities after their planar chro-

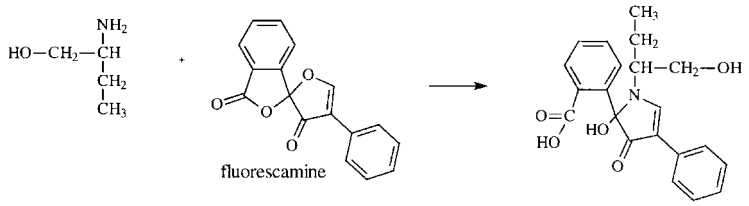


Figure 2.2.E.
Derivatisation of aminobutanol for the fluorimetric measurement

matographic separation from the main component and from each other. Spot elution techniques for these purposes can be regarded to be obsolete. However, in situ UV-VIS spectra (especially in the reflection mode) and fluorescence spectra without or after colour development with suitable spray reagents are widely used in this field. The evidence for the identity of a separated material with a reference standard can be greatly strengthened by adding to the identity of the R_f values the identity of these spectra. In addition to this the spectra can contribute also to the structure elucidation of unknown impurities. These aspects are discussed in Sections 2.1.2–2.1.4.

More or less the same applies to the use of UV-(VIS) spectroscopy and fluorimetry after column chromatographic separation. Before the introduction and spreading of high-performance liquid chromatography (HPLC) this was the most reliable and widely used method for the separation of impurities in drugs for their identification, structure elucidation and quantitative determination. Of the immense literature on this technique two monographs [15,16] and a review are mentioned [17].

At present the technique of classical column chromatography has been almost completely superseded by its modern variant, the HPLC method. Using this technique the identity of the spectra easily obtainable with the aid of diode-array UV detectors greatly improves the value of identification of the impurities on the basis of retention matching with reference standards (see Section 2.7.3). As for the application of the spectra for structure elucidation of unknown impurities, it is usually unnecessary to take the spectra after (semi)-preparative column chromatographic separation (usually preparative HPLC). The reason for this is that in the majority of cases a good-quality diode-array UV spectrum is available already at the beginning of the search for the structure of the impurity. The role of these spectra in this research is discussed in Section 2.1.4 while in Section 2.7.3 several examples are presented to demonstrate the possibilities and limitations of these spectra in the structure elucidation of related impurities in drug materials. The quantitative aspects of HPLC

(where almost exclusively UV or fluorimetric detectors are used for the determination of impurities in drugs) are discussed in Section 2.7.1. What is described for the role of UV spectroscopy and spectrophotometry as well as fluorimetry in high-performance liquid chromatography, almost exactly applies to capillary electrophoresis and capillary electrochromatography, too (see Sections 2.8 and 2.9, respectively).

References

1. S. Görög, *Ultraviolet-Visible Spectrophotometry in Pharmaceutical Analysis*, CRC Press, Boca Raton, FL (1995)
2. H.-W. Dibbern, *UV and IR Spectra of Some Important Drugs, Vol. III*, Edition Cantor, Aulendorf, No. 2221 (1980)
3. *European Pharmacopoeia, 3rd edn*, Council of Europe, Strasbourg (1997). Page numbers: a, 753; b, 881; c, 993; d, 1048; e,368; f, 1055; g, 1242; h, 1608; i, 1322; j, 1064; k, 1225; l, 593; m, 436; n, 1272; o, 1721; p, 465; r,345; s, 1229; t, 457; u, 1547; v, 1329; w, 599; x, 1229; y, 1370; z 673; a′, 802; b′, 1483
4. *European Pharmacopoeia, 3rd edn, Supplement 1998*, Council of Europe, Strasbourg (1998). Page numbers: a, 222; b, 389
5. *European Pharmacopoeia 3rd edn, Supplement 1999*, Council of Europe, Strasbourg (1999). Page numbers: a, 399; b, 251
6. *British Pharmacopoeia 1998*, The Stationery Office, London (1998). Page numbers: a, 548; b, 1330; c, 863; d, 542; e, 1022; f, 522; g, 432; h, 94; i, 147; j, 847; k, 1312; l, 929, m, 619
7. *The United States Pharmacopoeia 24*, USP Convention Inc., Rockville (2000). Page numbers: a, 698; b, 1319; c, 645; d, 1196; e, 1604; f, 919; g, 928, h, 1051; i, 614; j, 804; k, 624; l, 1384; m, 1480; n, 135; o, 655; p, 161; q, 568; r, 692; s, 783; t, 110; u, 835; v, 533; w, 899, x, 908; y, 17; z, 1340; a′, 1674; b′, 888; c′, 1372; d′, 1727; e′, 358; f′, 461; g′, 1207; h′, 651; i′, 475; j′, 505; k′, 43; l′, 689
8. *British Pharmacopoeia 1993*, p 270, The Stationery Office, London (1993)
9. M. Pesez and J. Bartos, *Colorimetric and Fluorimetric Analysis of Organic Compounds and Drugs*, Marcel Dekker, New York (1974)
10. B. Kakac and Z.J. Vejdelek, *Handbuch der Kolorimetrie, I, II (Kolorimetrie in der Pharmazie), III (Kolorimetrie in der Biologie, Biochemie und Medizine)*, Gustav Fischer Verlag, Jena (1962–1966)
11. B. Kakac and Z.J. Vejdelek, *Handbuch der photometrischen Analyse organischer Verbindungen*, Verlag Chemie, Weinheim (1974)
12. Z.J. Vejdelek and B. Kakac, *Farbreaktionen in der spektrophotome-*

trischen Analyse organischer Verbindungen, I, II Ergänzungsband I, II, Gustav Fischer Verlag, Jena (1969–1973, 1980–1982)
13. F.D. Snell and C.T. Snell, *Colorimetric Methods of Analysis, III, IV (Organic Compounds) IIIA, IVA*, Van Nostrand, Princeton (1948–1970)
14. T.D. Doyle, W.R. Benson and N. Filipescu, *J. Am. Chem. Soc.* **98**, 3262–3267 (1976)
15. O. Mikes (Ed.), *Laboratory Handbook of Chromatographic and Allied Methods*, Ellis Horwood, Chichester (1979)
16. Z. Deyl, K. Macek and J. Janák, *Liquid Column Chromatography*, Elsevier, Amsterdam (1975)
17. T.D. Doyle and J. Levine, *J. Assoc. Off. Anal. Chem.* **61**, 172–191 (1978)

2.3. Mass Spectrometry in Impurity Profiling

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2.3.1. Introduction

Mass spectrometry, with its reproducibility, specificity and especially with its high sensitivity, is an indispensable tool in the trace analysis and structural elucidation of pharmaceutical compounds. Over the last decade rapid and extensive methodological developments have made mass spectrometry applicable to a wide range of organic compounds. The introduction of soft ionisation methods rendered the analysis of thermally unstable, non-volatile and high-molecular-weight compounds accessible. The methodologies and types of mass spectrometers currently used in the pharmaceutical sciences have been described in a recent book and review [1,2].

When analysing impurities, sensitivity is of prime importance. Sensitivity depends on the ionisation methods applied. In electron ionisation (EI) full mass spectra can be taken when a nanogram or more of substance is available. Fast atom bombardment (FAB) mass spectra are characteristically simple, often consisting only of protonated molecular ions. Since these ions account for a large percentage or all of the total ion current, the method is both specific and sensitive, allowing analysis in the picogram range. Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) techniques enable detection of compounds in the picogram range. Below this level the selected ion monitoring (SIM) method is required, in which the compound of interest is identified on the basis of the presence of one or more m/z values known to be in the mass spectrum of that compound. This method is also useful in biochemistry and in environmental sciences, i.e. in those cases in which the compound(s) to be analysed are present in complex mixtures. The SIM method can be applied with or without chromatography.

Combined chromatography–mass spectrometry provides a very effective tool for the qualitative characterisation of complex mixtures, such as for trace and impurity analysis, by exploiting the resolving power of chromatography and the strength of mass spectrometry in identifying the separated compounds. Both combined techniques GC/MS and HPLC/MS (discussed in detail in Sections 2.6.6 and 2.7.4) are capable of obtaining complete mass spectra of a few picograms or nanograms of each component. Below this level analysis is possible but special scanning techniques (such as SIM) are necessary. The

latest development in the field of hyphenated chromatographic–mass spectrometric techniques, capillary electrochromatography–mass spectrometry (CEC/MS) is discussed in Section 2.9.2.

Mass spectrometry without chromatographic separation serves also as a useful method to analyse impurities, especially if the different ionisation techniques can be combined with tandem mass spectrometry. For the direct and semiquantitative analysis of biological mixtures the matrix assisted laser desorption ionisation (MALDI) can be applied. Some application examples for the mass spectrometric analysis of impurities before and after chromatographic separation are discussed in the next section.

By characterising and identifying impurities, all of these methods can play a crucial role in the task of optimising the production process such that impurities are eliminated or minimised.

2.3.2. Applications Without Chromatographic Separation

The use of mass spectrometry for the identification of impurities in bulk drugs without chromatographic separation is a viable and effective approach. Unisolated impurities may be detected by applying different mass spectrometric ionisation methods and their structures can be established by the examination of decomposition pathways (tandem MS technique).

The advantage of this approach is that the laborious preparative HPLC isolation becomes unnecessary. In this way solvent contaminants which tend to accumulate in the final product during preparation can also be avoided. The only disadvantage is that isomers of the same molecular mass cannot be differentiated.

Tandem mass spectrometry (MS/MS), a useful method for the analysis of impurities without chromatography, involves separation by mass. A component of the mixture is separated by selecting a single ion (in most cases M^+ or MH^+) at one specific m/z value with the first analyser. The fragments of this ion is monitored by a second analyser. Further details of this methodology, as used for quantitative analysis, are discussed in Refs. [3–5]. Examination of induced fragmentation of normal ions by tandem MS enables the highly specific analysis of complex mixtures down to the ppm level. The deliberate induction of decomposition of normal ions can be carried out by a variety of techniques [6]. Further details of this new efficient area of mass spectrometry, the examination of decomposition pathways by MS/MS is described by Lattimer *et al*. [7]. An example of the excellent sensitivity and specificity that can be attained in trace analysis by using MS/MS methods is the determination of the highly toxic and carcinogenic compound, bis(chloromethyl)ether, which could be detected even at 4 ppt [8].

Drug impurities of synthetic origin (intermediates, by-products, precursors and degradation products) usually have similar structures while contaminants from raw materials and solvents are structurally unrelated to the drug substance. As the ionisation efficiency depends partly on molecular structure, a given ionisation method may produce different responses for the different impurities. To achieve the complete analysis of impurities, the combined use of different ionisation techniques and the application of tandem mass spectrometry proves to be the best approach.

An example for the complex application of the different mass spectrometric methodologies is the identification of impurities in a bulk *spironolactone* sample containing less than 1% of total impurities. For the detection of impurities different ionisation methods were applied in the authors' laboratory [9]. The EI mass spectrum of the sample is shown in Fig. 2.3.A. In the EI experiments the instrument applied allowed the direct probe to be heated independently of the ion source. By this means some degree of fractionation could be achieved by temperature programming. As the compounds evaporated from the probe, beside spironolactone ($M_1 = 416$) another component was detected with a molecular weight of $M_2 = 374$. According to this mass value the compound was assumed to be the deacetyl thiol-derivative of spironolactone. In the tandem MS product ion spectrum of M_2^+ (Fig. 2.3.B) the

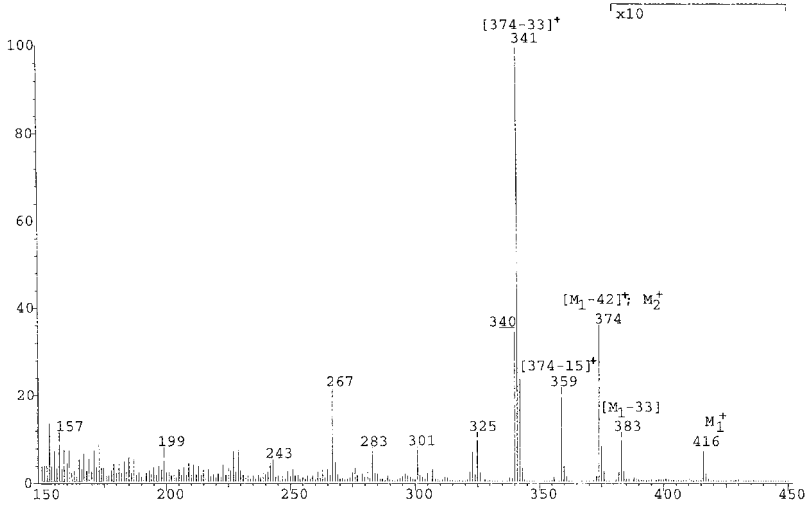


Figure 2.3.A.
EI mass spectrum of a bulk spironolactone sample

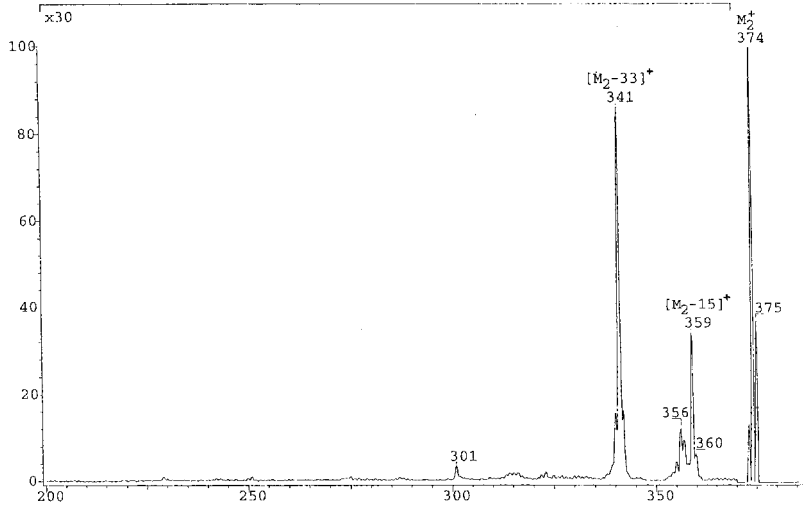


Figure 2.3.B.
EI-daughter ion spectrum of the M_2^+ ion (see Fig. 2.3.A)

loss of an SH-group (33 mass units) was observed in good agreement with the assumed structure. Low energy EI is a useful method to enhance the molecular ions, as the ionisation energy applied is around the appearance potential, therefore the produced molecular ions have little or no excess energy to decompose, i.e. no fragmentation takes place. The low energy (6 eV) EI measurement of spironolactone revealed the presence of a component with a molecular weight of $M_3 = 340$, corresponding to a known impurity (synthesis intermediate and/or degradation product) canrenone. The FAB-MS/MS daughter ion study of M_3H^+ confirmed this assumption as the daughter ion spectrum (Fig. 2.3.C) showed the elimination of water (peak at m/z 323) and peaks at m/z 283 and 267 indicated the partial and total loss of the spiro-ring. The EI fragmentation pathway of the reference canrenone sample proved to be identical.

The well-known impurity 7β-spironolactone [10] could not be detected beside spironolactone by mass spectrometry without chromatographic separation, due to their identical molecular weights.

In the FAB spectrum of the bulk spironolactone sample a further component was detected with a molecular weight of $M_4 = 414$, which was presumed to be Δ^1-spironolactone resulting as an overoxidation product during the preparation procedure.

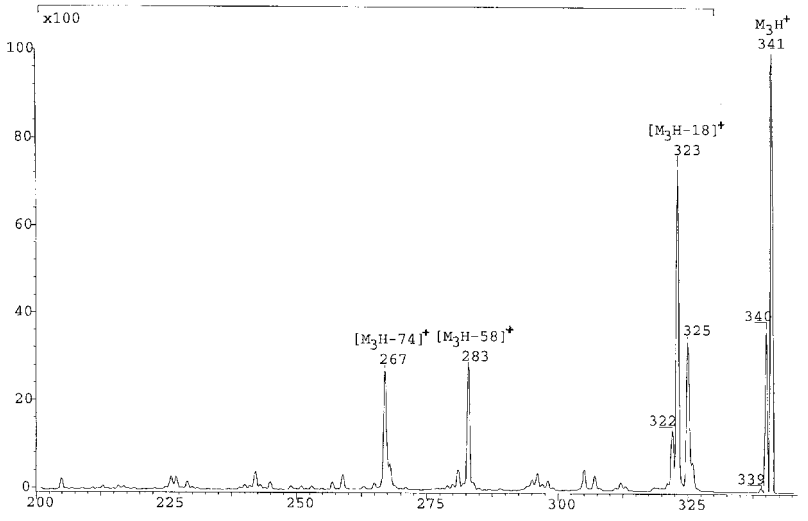
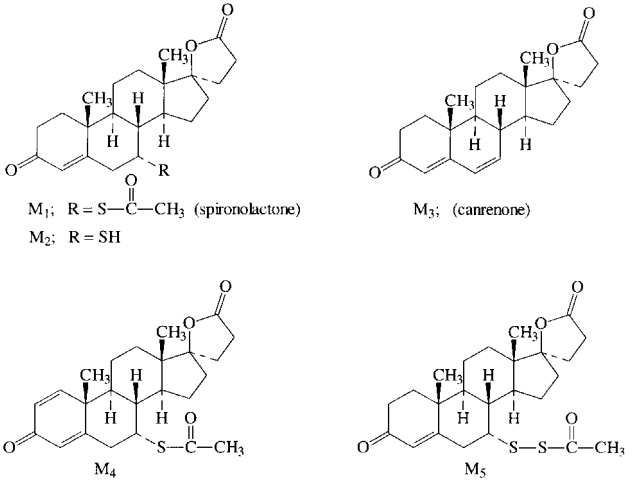


Figure 2.3.C.
FAB-daughter ion spectrum of the M_3H^+ ion (see Fig. 2.3.A)



The ESI-MS technique led to the detection of a further impurity with a molecular weight of $M_5 = 448$, i.e. 32 mass units higher than that of spironolactone (Fig. 2.3.D). This mass difference suggested the presence of an addi-

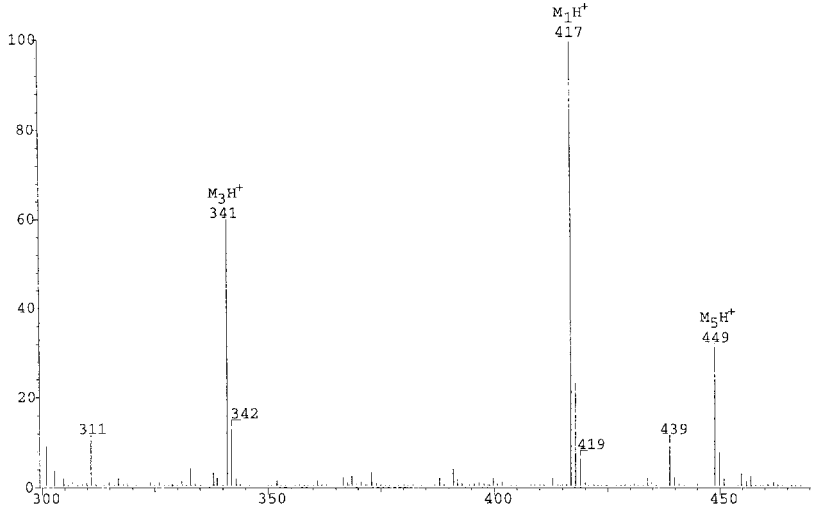


Figure 2.3.D.
ESI-MS spectrum of a bulk spironolactone sample

tional sulphur atom, but the confirmation of this presumption was not possible due to the limited amount of this compound in the bulk drug. Further detailed examination became possible after chromatographic separation (see Section 2.3.3). Electrospray ionisation in the so called ''in-source'' collision induced dissociation (CID) method [11,12] was also used. In this method a growing potential difference between the capillary exit and the tube was applied to achieve collision activation. Application of this technique also confirmed the presence of M_3 in the spironolactone sample.

For the direct analysis of complex biological mixtures the latest ionisation method, matrix assisted laser desorption ionisation (MALDI) combined with a time-of-flight (TOF) mass analyser, has proven to be a powerful and very sensitive tool [13–15]. This technique has the ability to analyse mixtures of peptides and proteins over a broad mass range from the picomole to the high attomole range and has a high tolerance for contaminants, such as salts, buffers and even for some detergents, with a level that would be detrimental to other types of ionisation. Enzymatic digests of proteins or carbohydrate hydrolysis mixtures can be screened by MALDI-TOF with great speed and without a discrimination effect [16]. This ionisation method proved to be a viable technique for the quantitative analysis of low molecular weight antibiotics as well [17]. Due to its advantages MALDI enjoys a wide range of applications throughout biochemistry and biotechnology, in organic chemistry and in pharmaceutical research.

2.3.3. Applications after Chromatographic Separation

Impurity isolation and subsequent off-line mass spectrometry have often been used to confirm the identity of drug impurities and degradates by comparison, if possible, to synthesised reference materials. The general method is to isolate individual components by preparative or – due to the high sensitivity of mass spectrometry – analytical HPLC or TLC and the isolated fractions are subjected to mass spectrometric analysis. In the majority of cases the data obtained from the mass spectra (together with other chemical information) are sufficient to propose a structure for the impurity.

The disadvantage of this approach is that not only the compound of interest but also the contamination from the solvent(s) used for HPLC separation will be accumulated. The compounds originating from the solvent can even suppress the compound to be analysed, especially in FAB ionisation if they are more easily ionised. This undesirable suppression effect can be avoided using continuous flow FAB or ESI and APCI ionisation, respectively.

By the application of mass spectrometry after chromatographic separation the isolated compounds of identical molecular weight can also be analysed, as the MS/MS technique makes it possible to differentiate between stereoisomers and positional isomers. Detection of metastable ions or product ions, sensitive to fine differences in structure, allows the structure identification of different isomers. An example of the application of this principle is the analysis of isomeric carotenoids [18].

The other advantage of this off-line approach, i.e. the mass spectroscopic analysis after chromatographic separation, is that the impurity of interest can be preconcentrated and this makes a detailed examination for structure identification possible. An example of this is the detailed study of one of the impurities (M_5) of the drug *spironolactone*, discussed in Section 2.3.2. After HPLC separation it was possible to perform accurate mass measurement on compound M_5. The measurement indicated an elemental composition of $C_{24}H_{32}S_2O_4$, which was in good agreement with the proposed structure. ESI- and FAB-daughter ion examinations (Fig. 2.3.E) showed that the protonated molecular ion of M_5 eliminated 108 mass units as primary fragment. This means that the whole $C_2H_3S_2O$-group can easily be lost, but the group itself has proved to be stable, not prone to fragmentation. Accordingly we presumed that M_5 was a dithioacetyl-substituted compound. This assumption was confirmed by NMR measurements

Another example, the isolation and identification of two impurities in *cimetidine* present at a level $<0.10\%$, is described by Halmos at al. [19]. The compounds isolated by preparative HPLC were subjected to mass spectrometric and NMR studies. The molecular ion of one of the compounds was identified by FAB-MS, while for the other component the atmospheric pressure

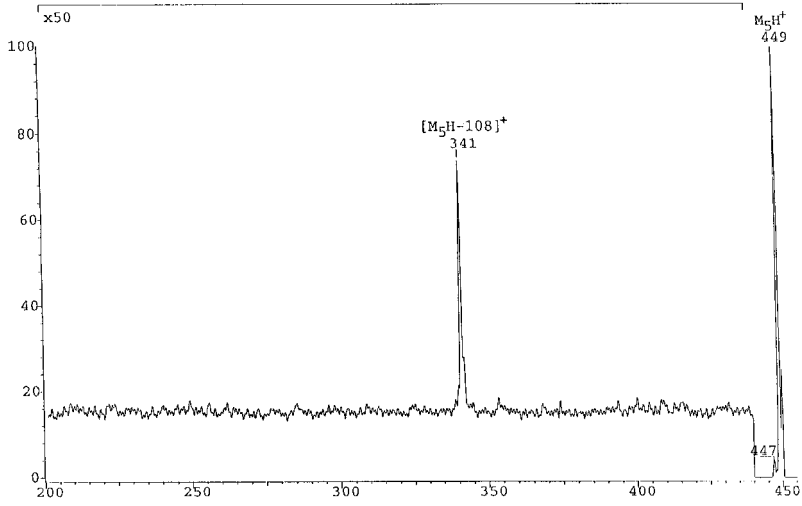


Figure 2.3.E.
ESI-daughter ion spectrum of the M_5H^+ ion

chemical ionisation (APCI) method was used. The fragments gained by EI-MS measurements and also the high field NMR studies, led to the structure elucidation of the two side products, 2,5-bis[(*N′*–cyano–*N″*–methyl)guanidinoethylthiomethyl]-4-methylimidazole and 1,8-bis[(*N′*–cyano–*N″*–methyl) guanidino]3,6-dithiaoctane (impurities **4** and **5** in Section 2.4.7.3).

The following example demonstrates the utility of the off-line approach for isomer recognition. In the HPLC chromatogram of *propanidid* with its impurities two pairs of peaks were found which gave identical protonated molecular ions ($M_1H = 673$ and $M_2H = 689$, respectively) in the FAB mass spectra. M_1 – according to its mass – corresponds to the dimeric version of propanidid. The daughter ion examinations of the two m/z 673 ions (Y_1 and Y_2) showed very similar spectra (Fig. 2.3.F), although the differences observed in the relative intensities of the product ions indicate slight differences in the structures (different linkage of the monomers). M_2 was found to be 16 mass units larger than M_1 and the elemental compositions calculated from the accurate mass measurements proved the presence of an additional oxygen atom. The daughter ion spectra of the two m/z 689 ions were practically identical indicating two oxygen-bridged dimeric structures. This example clearly shows both the power and limitations of mass spectrometry: NMR measurements would be required to identify the exact structures of these isomers.

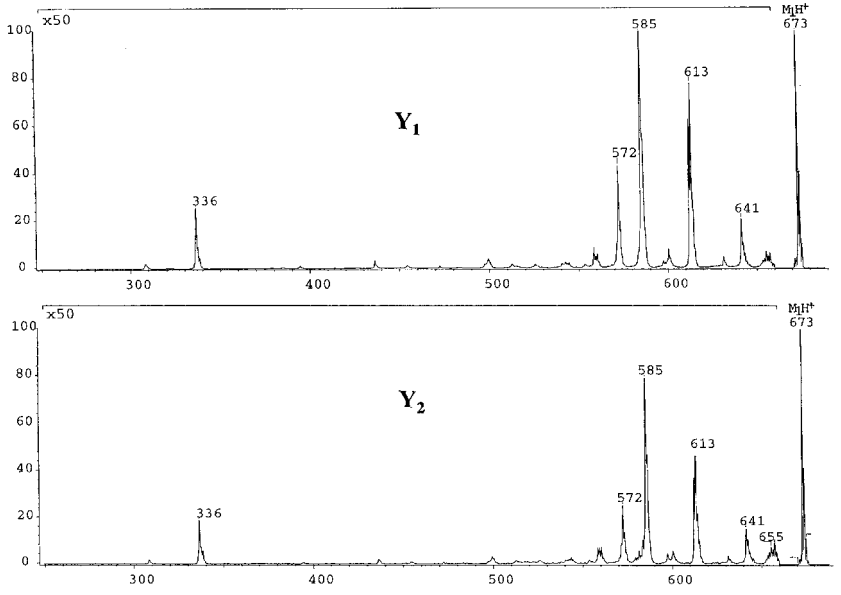
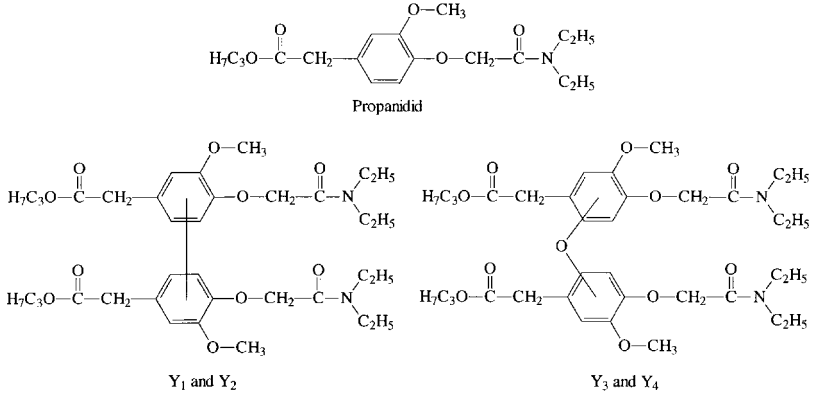


Figure 2.3.F.
FAB-daughter ion spectra of the m/z 673 ions detected as impurities Y_1 and Y_2 of propanidid



Another example shows the structure elucidation of impurities in *allylestrenol* after HPLC separation by EI high resolution and metastable measure-

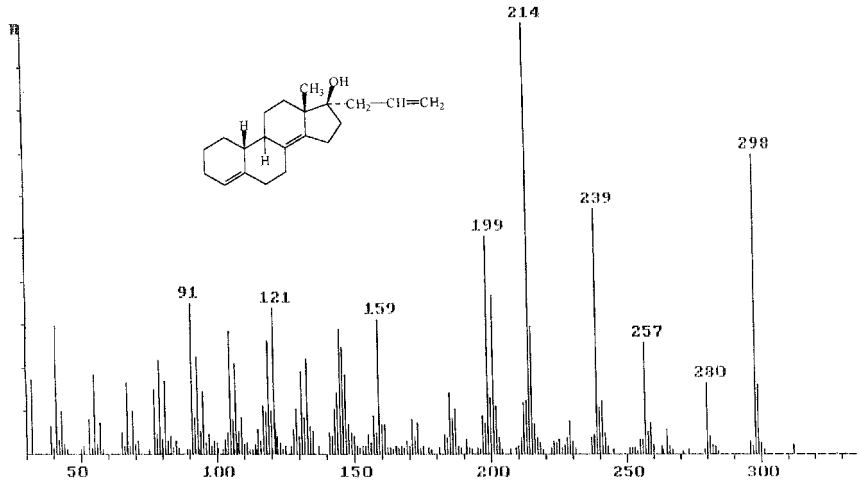
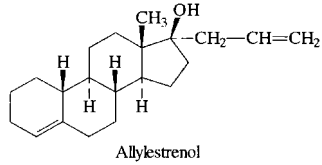


Figure 2.3.G.
EI mass spectrum of impurity **I** in allylestrenol

ments [20,21]. Both unknown components (**I** and **II**) obtained by preparative HPLC have molecular weights of 298 Da. This is two mass units less than the molecular weight of *allylestrenol* indicating an additional double bond in the impurities. This assumption was confirmed by accurate mass measurements. The two impurities showed different fragmentation (see Figs. 2.3.G and 2.3.H), relating to different structures. The common peaks at m/z 280, 257 and 239 correspond to losses of H_2O, CH_2–$CH{=}CH_2$ and H_2O + CH_2–$CH{=}CH_2$, respectively, from the molecular ions. These fragmentation routes were confirmed by metastable measurements.



Allylestrenol

In the spectrum of impurity **I** (Fig. 2.3.G) the base peak at m/z 214 can be explained by the splitting of the C_{13}–C_{17} and C_{15}–C_{16} bonds. This may be due to the presence of the additional double bond at the 8(14) position initialising a β-cleavage. Similar β-cleavages may give rise to the ions at m/z 159 and 121, also characteristic of this isomer.

In the mass spectrum of impurity **II** (Fig. 2.3.H) the main fragment ions

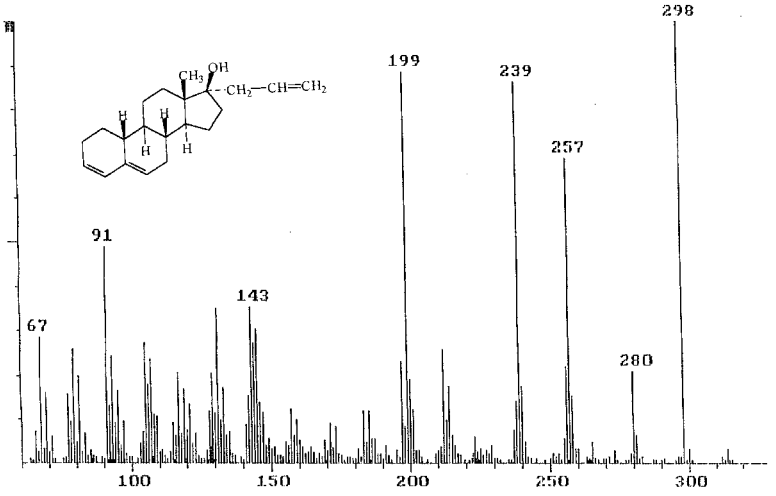


Figure 2.3.H.
EI mass spectrum of impurity **II** in allylestrenol

are shifted by two masses in comparison with the mass spectrum of *allylestrenol* indicating that the additional double bond is not in the C and D rings. NMR and UV studies suggested a 3,5-diene structure. This structure was confirmed by comparing the MS, NMR and UV spectra of the isolated impurity with those of a synthesised standard 3,5-diene sample.

Besides the off-line techniques presented above, up-to-date trends require the on-line application of the different chromatographic and mass spectrometric methods (such as GC/MS, HPLC/MS, CE/MS, CEC/MS, TLC/MS and SFC/MS, described in detail in Sections 2.5–2.10), as the combination of the complementary nature of these individual techniques can be exploited in both the qualitative and the quantitative characterisation of complex mixtures.

References

1. R.A.W. Johnstone and M.E. Rose, *Mass Spectrometry for Chemists and Biochemists*, Cambridge University Press, Cambridge, UK (1996)
2. D.M. Higton and J.M. Oxford, *Appl. Spectroscopy Rev.* **30**, 81–118 (1995)
3. E. Gelpi, *Adv. Mass Spectrom.* 395–415 (1985)
4. S.J. Gaskell and E.M.H. Finlay, *Trends Anal. Chem.* **7**, 202–208 (1988)
5. B.J. Millard, *Quantitative Mass Spectrometry*, Heyden, London (1978)

6. J.H. Scrivens and K. Rollins, *VG Monographs in Mass Spectrometry, No. 4*, VG Organic (now Micromass UK Ltd), Manchester (1992)
7. R.P. Lattimer, H. Muenster and H. Budzikiewicz, *Int. J. Mass Spectrom. Ion Proc*. **90**, 119–129 (1989)
8. T.G. Blease, J.H. Scrivens and W.E. Morden, *Biomed. Environ. Mass Spectrom*. **18**, 775–779 (1989)
9. Unpublished data
10. S. Görög, B. Herényi, P. Horváth and A. Laukó, in *Advances in Steroid Analysis* (S. Görög, Ed.), pp 35–53. Elsevier, Amsterdam (1982)
11. V. Katta, S.K. Chowdhury and B.T. Chait, *Anal. Chem*. **63**, 174–179 (1991)
12. R.D. Voyksner and T. Pack, *Rapid Commun. Mass Spectrom*. **5**, 263–267 (1991)
13. J.S. Andersen, B. Svensson and P. Roepstorff, *P. Nat. Biotechnol*. **14**, 449–457 (1996)
14. M. Mann and G. Talbo, *Curr. Opin. Biotechnol.* **7**, 11–19 (1996)
15. V. Redeker, J.-Y. Toullec, J. Vinh, J. Rossier and D. Soyez, *Anal. Chem*. **70**, 1805–1811 (1998)
16. C.R. Jiménez, K.W. Li, A.B. Smit, J. van Minnen, C. Janse, P. van Veelen, K. Dreisewerd, J. Zeng, J. van der Greef, F. Hillenkamp, M. Karas and W.P.M. Geraerts, in *Mass Spectrometry in Biological Sciences* (A.L. Burlingame and S.A. Carr, Eds.), pp 227–243. Humana Press, Totowa, NJ (1996)
17. Y.-C. Ling, L. Lin and Y.-T. Chen, *Rapid Commun. Mass Spectrom.* **12**, 317–327 (1998)
18. M.E. Rose, in *Carotenoids 6. Proceedings of the Sixth International Symposium of Carotenoids* (G. Britton and T.W. Goodwin, Eds.), pp 167–174. Pergamon Press, Oxford (1982)
19. Zs. Halmos, Cs. Szántay, Jr., J. Brlik, A. Csehi, K. Varga, P. Horváth, M. Kislaki, Gy. Domány, A. Nemes and S. Görög, *J. Pharm. Biomed. Anal*. **15**, 1–5 (1996)
20. J. Brlik, A. Csehi, S. Görög and Gy. Horváth, *13th International Mass Spectrometry Conference Abstracts*, 48, Budapest (1994)
21. S. Görög, M. Babják, J. Brlik, A. Csehi, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *Talanta* **44**, 1517–1526 (1997)

2.4. NMR Spectroscopy

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2.4.1. Introduction

Since the early classical experiments conducted in 1946 by Edward M. Purcell [1] and Felix Bloch [2,3], nuclear magnetic resonance spectroscopy (NMR) has undergone a truly amazing evolutionary process, unparalleled by any other spectroscopic technique. The field of NMR has proved to be such a rich and gratifying source of theoretical research in spin physics, and by now has come to embrace so many powerful and diverse applications, that NMR has become a multidisciplinary subscience in its own right: at least eight Noble prizes in physics and two in chemistry have been awarded to scientists whose work involved, to various extents, magnetic resonance (I.I. Rabi (1944, Physics), E.M. Purcell (1952, Physics), F. Bloch (1952, Physics), A. Kastler (1966, Physics), J.H. Van Vleck (1977, Physics), N. Bloembergen (1981, Physics), K.A. Müller (1987, Physics), N.F. Ramsey (1989, Physics), H.G. Dehmelt (1989, Physics), R.R. Ernst (1991, Chemistry), J. Pople (1998, Chemistry)).

The utilization of NMR ranges from medical diagnostics through resonance imaging to liquid-state applications devoted to establishing molecular structures and geometries, thermodynamic parameters, izomerizational dynamics, intra- and intermolecular interactions, etc. with countless books and specialized scientific journals dedicated to these topics. Given the sheer immensity of the field of NMR, as well as the vast amount of literature already thoroughly covering the topic, it is all the more important to define properly the aim and scope of the present discussion. This endeavor also raises the question: can we, within a short chapter, possibly hope to provide any new insight into the use of NMR regarding drug impurities through the presentation of a few selected concepts and examples? To address these issues, we shall first touch on the two most important uses of NMR (*structure elucidation* and *quantification*), as well as its limitations in terms of *sensitivity* and the required *purity* of the substance, as is relevant to the present book. Within the framework of this short prelude, we shall also attempt to give a brief sketch of the most important concepts, techniques and phenomena that are needed to appreciate the examples to be discussed later.

2.4.2. NMR as a Structure Elucidation Tool

In spite of the diverse applicability of NMR, on a global scale the majority of high-field pulsed Fourier-transform (PFT) NMR spectrometers are dedicated to the liquid-state structural elucidation of chemically synthesized small organic molecules, or those isolated from natural sources, including metabolites. In that regard NMR is mostly employed with a view to finding and/or characterizing new, biologically active chemical entities, to providing the structural information needed for research chemists to develop the pertinent synthetic routes, or to identifying metabolic pathways. With increasing demands being placed on the quality assurance of drug substances, NMR also plays a growing role in identifying small impurities without or after chromatographic separation. For these purposes modern high-resolution NMR offers a stunning range of various experiments (see, e.g. Refs. [4–9]). Since theoretical as well as experimental advances continue to be rapid, this NMR ''toolkit'' is constantly expanding. As a result, NMR spectroscopy proves now in most cases to be the most powerful method in the structure elucidation or conformational analysis of organic molecules, provided that they are available in adequate purity and quantity and their molecular weight does not exceed ca. 50 kDa (this number is constantly increasing together with the unceasing development of NMR).

When considering the use of modern high-resolution NMR spectroscopy, it is essential first to distinguish between the realms of ''small-molecule'' and ''big-molecule'' NMR. A *small* molecule is defined as one with a *fast* rotational tumbling rate ρ_c such that $\rho_c \gg \omega_0$, where ω_0 is the Larmor angular frequency γB_0 (γ denotes the gyromagnetic ratio for the studied nucleus, B_0 is the static external field strength), while the tumbling rate ρ_c represents the reciprocal average time needed for an internuclear vector to rotate through one radian. *Big* molecules (typically MW $>$ ca. 1000), however, tumble slowly so that $\rho_c \ll \omega_0$. Small and big molecules exhibit sharply contrasting relaxational characteristics which manifest themselves in differences in a number of spectral features. For example, as compared to small molecules, in big molecules the linewidths become significantly broader due to dipolar broadening.

Sometimes a molecule can be regarded as small by virtue of its molecular weight (i.e. MW $<$ 1000), but may nevertheless actually behave as a big molecule in NMR if its tumbling rate is slow due to molecular aggregation or high solution viscosity. NMR is in general less suitable for the structure elucidation of big molecules, but is extensively used for the 3D conformational investigation of proteins and DNA or RNA segments of known primary structure.

What makes NMR such a useful technique? First, for typical small organic molecules even the normal one-dimensional (1D) ^{1}H and ^{13}C spectra

are profuse in their information content: chemical shifts, multiplicities, coupling constants, peak areas obtained from the usually well-resolved resonances all provide abundant and easily accessible, geometry-dependent structural information. In many cases a key element in utilizing such data rests on a comparison of the relevant spectral parameters with reference data coming from suitable analogues. In that sense the assignment of resonances, and the process of structure elucidation, are based on a relative approach. Secondly, the advent of a host of multi-dimensional, particularly two-dimensional (2D) and other sophisticated 1D techniques have brought into focus what is the most profoundly important aspect of modern NMR: it can identify through-bond (scalar) and through-space (dipolar) spin–spin *connections*. While scalar couplings give rise to the readily observed multiplet structure of resonances, dipolar couplings can only be observed indirectly, and their most extensively utilized manifestation is the famous nuclear Overhauser effect (NOE) [10]: if i an j are two spatially close spins, selective perturbation by a suitable radio frequency field of the Boltzmann population distribution of i results in a perturbation of j as well, which can be measured as an intensity change in the resonance signal of j. In small molecules NOEs between protons are usually positive with a theoretical maximum of 50%, while in big molecules they are negative with a theoretical maximum of −100%.

While the *size* of both the scalar coupling and NOE can be of profound significance, the detection of their mere *presence* is the source of establishing the topology of the scalar and dipolar coupling networks which relate the various molecular moieties. Such connections provide an absolute basis for structure determination: assuming that a sufficiently large number of connectivities has been experimentally determined, for most small organic molecules the structure can be unambiguously established without the need to make comparisons. This capability of modern NMR has lead to the concept of ''ab initio structure determination''. The development of applied NMR entails a gradual shift from using relative methods toward placing the emphasis on absolute ones, which give far more securely established structures.

Over the last ten years or so the peculiar advantages of PFT NMR have become fully appreciated, leading to the development of many ingenious new ways to measure and exploit these connections. On the technological side, superconducting magnets of increasing field strength (today the strongest commercially available magnets operate at 900 MHz, ^{1}H frequency, and soon the 1 GHz frequencies should be accessible), stability and homogeneity, improved electronics and computer technology, the recent introduction of innovative new devices such as waveform generators, pulsed field gradient modules [11], linear amplifiers, gradient shimming [12–15], supersensitive probes, etc. all contribute to increased sensitivity and resolution, and in some cases dramatically reduce measurement times. On the other hand a

constant development in spin-physics opens new possibilities in ''spin gymnastics'', i.e. the planned manipulation of spin systems with the aim of devising pulse sequences that accomplish a pre-envisioned task. Specialized acquisition and processing techniques such as deconvolution [16], linear prediction (LP) [17] and digital signal processing (DSP) technology which is based heavily on the concept of oversampling [18], have also become useful everyday tools. Such advances have all contributed to the high levels of sophistication now available with the hundreds of different measurement techniques.

With the focus here being placed on small organic molecules, it is noted that NMR structure elucidation mostly involves ^{1}H and ^{13}C spectroscopy. Particularly ^{15}N, ^{19}F, ^{2}H and ^{31}P NMR can in special cases also be powerful tools (for one recent example of applying ^{19}F NMR in drug impurity investigations see Ref. [19]), but this area would merit a discussion in its own right beyond the scope of the present chapter). While the ^{1}H spectrum is richer in dipolar and scalar coupling data, the much wider range of ^{13}C chemical shifts can provide profoundly important information which is not available from ^{1}H NMR data. The fact that a ^{13}C spectrum can be acquired at all often rests on the utilization of the $^{1}H \rightarrow {}^{13}C$ NOE, which can give up to almost 200% intensity enhancement on the detected ^{13}C signal. This enhancement reduces to only a few percent in big molecules, where it therefore often becomes impossible to obtain an informative ^{13}C spectrum. Modern NMR offers basically two strategies for accessing ^{13}C chemical shifts. Following the classical approach, the ^{13}C spectrum may be measured directly, which can be a rather time-consuming process since ^{13}C sensitivity is about 6000 times less than for protons. More recently it has become attractive to obtain the ^{13}C shift information for proton-bearing carbons from a proton-detected PFG-HSQC experiment [20–22]. This approach has the advantage that while giving the ^{13}C shift with better sensitivity than with direct ^{13}C detection, it also provides the direct ^{1}H–^{13}C correlations.

With this brief description given in honor of the formidable powers of NMR, it is easy to form the impression that the resolution of virtually *any* small-molecule structural problem has become an almost straightforward process which ''demotes'' the applied NMR spectroscopist's status from that of creative scientist to highly competent workmanship. However, our everyday experience in determining the structures of large numbers of diverse molecules by the extensive use of state-of-the-art methodologies shows that in practice surprisingly many problems continue to require a dedicated researcher's attitude and commitment on the NMR spectroscopist's part, as well as complementary information coming from other spectroscopic methods. For example, it is rather common that for a small, pure, but unknown molecule *all* important NMR-related pieces of information can be readily obtained. This would mean that, in addition to basic spectral data (typically ^{1}H and ^{13}C chemical shifts, intensities, multiplicities and some coupling constants) it is possible to obtain,

as will be exemplified below, in ''one go'' the direct C–H connectivities (e.g. in a phase-sensitive pulsed-field-gradient-selected heteronuclear single quantum coherence (PFG-HSQC) experiment [20–23]), the H–H scalar connectivities (e.g. in a gradient-selected double-quantum-filtered phase-sensitive correlation spectroscopy (PFG-DQFCOSY) experiment [23–25]), the long-range C–H connections (e.g. in a gradient-selected heteronuclear multiple bond correlation (PFG-HMBC) experiment [25–28]) and the NOE connections (NOE difference [29], double pulsed-field-gradient spin-echo NOE (DPFGSE-NOE) [30] or with sufficiently large molecules a nuclear Overhauser enhancement spectroscopy (NOESY) experiment [31–33]). Even with these data at hand, the structure may still remain undefined, and it is often difficult to devise or envisage an experiment that can add any useful further information with regard to the problem of resolving an unknown structure! Successful structure determination then becomes the creative art of assembling a structure that is consistent in terms of *constitution*, *configuration* and *conformation*, with the above pieces of data. This may turn out to be quite a difficult task calling for chemical intuition and imagination, and often the capacity to readily envisage different structure-candidates in three dimensions.

In reality, added complications often arise from a number of factors, such as impurities, congested spin systems, or the presence of some form of chemical exchange. Actually the latter ensues a far more common problem than is generally realized, particularly in the salt form of drug molecules, and should therefore merit a few comments. For simplicity, we consider a two-site exchange system $A \rightleftharpoons B$ in which species A and B interconvert with the respective forward and backward first-order rate constants k_A and k_B, and a frequency difference $\Delta\omega = |\omega_A - \omega_B|$ exists between the non-exchanging sites. If exchange is fast on the chemical shift timescale, i.e. $k_A, k_B \gg \Delta\omega$, the observed average resonance is sharp and shows no direct indication of the ongoing exchange process. In the case of slow exchange, i.e. $k_A, k_B \ll \Delta\omega$, the resonances due to site A and B are observed as two separate sharp signals. In the intermediate case when k_A and k_B are comparable to $\Delta\omega$, resonances become extensively exchange-broadened. If chemical exchange gives rise to either line broadening or multiple signal sets, both scenarios can seriously hamper structure determination (see below), and often require the use of sophisticated molecular modeling calculations to complement the structure elucidation process.

Such complications limit the amount of data that provide unambiguous information in assembling the structure. In those cases the construction of a structure is often based on a series of premises which may or may not lead to the required consistency. It is quite surprising how many times this process needs complementary input coming from MS and/or IR spectroscopy (sometimes also UV and titrimetric information).

In addition, one important psychological aspect, what we may call the *seduction of consistency*, also comes into play. Once a structure is found (sometimes after much intellectual effort) that is consistent with the available spectral information, it becomes almost irresistibly tempting to view this as *the* structure. Once the spectroscopist becomes thus biased, the possibility of alternatives, particularly that of structural isomers which might exhibit similar consistency with experimental data, is often neither considered nor pursued with adequate vigor. (Computer programs that search for alternative consistent structures are currently under intensive development, to a great extent motivated by the demands of combinatorial chemistry; such programs may provide unbiased help in this regard, especially if consistency is based on a ''clean'' set of experimental data. However, at the time of writing this book, their real utility has yet to be seen, particularly since in difficult cases the data input may carry, as noted above, much ambiguity and hypothetical factors). Of course once such alternatives *are* considered, the wealth of the NMR toolkit usually offers some experiment that can unambiguously select the real structure.

It should also be pointed out that the informational riches of NMR often allows the same structural conclusion to be arrived at by using completely different NMR measurement techniques and interpretational strategies. As a result, there is a natural world-wide tendency for each NMR team to develop its own preferred *modus operandi* for structure elucidation. This diversity of NMR laboratory cultures offers the basis of a useful cross-check if different NMR groups address the same structural problem.

2.4.3. The Sensitivity Problem

As much as NMR is elected over other spectroscopic methods for its rich information content and diversity of methodological repertoire, this comparison has been notoriously unfavorable with regard to *sensitivity*, i.e. the measured signal-to-noise ratio S/N. When looking at the analytical aspects of drug R&D, sensitivity is of course a crucial issue, since the unambiguous determination of small quantities of isolated natural compounds, metabolites or trace impurities can be of key importance regarding a number of fundamental industrial interests, such as marketing authorization applications, quality control, regulatory aspects and patent right protection.

In NMR, a spin–1/2 nucleus (^{1}H, ^{13}C, ^{19}F, ^{15}N) exhibits, in a B_0 field, two energy levels α and β, whose population ratio N_α/N_β is determined at thermal equilibrium by the Boltzmann relation

$$\frac{N_\alpha}{N_\beta} = \exp\left(\frac{-\gamma h B_0}{2\pi k T}\right)$$

The net observable magnetization vector $\mathbf{M_0}$ is aligned along the direction of $\mathbf{B_0}$ and its magnitude is proportional to the population difference: $N_\alpha - N_\beta \sim M_0$. Following a suitable radio frequency pulse, the measured magnetization vector **M** may be equal to or smaller than the thermal-equilibrium value $\mathbf{M_0}$ and returns to equilibrium while precessing about $\mathbf{B_0}$. The receiver coil surrounds a limited volume (the ''active volume'') of the sample and observes, from right angles to $\mathbf{B_0}$, the precessing and decaying $\mathbf{M_{xy}}$ component which gives rise to a damped oscillatory time-domain signal called the free induction decay (FID) whose Fourier transformation yields the frequency-domain signal S. One important instrumental parameter that relates M_{xy} to S is the quality factor Q of the probe, with Q representing the energy stored in the tuned circuit of the receiver relative to that dissipated into the series resistance.

The relative insensitivity of NMR stems from the fact that the population difference $N_\alpha - N_\beta$ is inherently small. At a field strength of, say, 1.4 T (60 MHz), at room temperature the population excess in the lower state amounts to only 0.001%! This difference grows linearly with B_0, i.e. M_0 increases 10-fold when the field strength is increased to 600 MHz. Even at such high fields, typical limits of detection (LOD, defined as S/N = 3) are in the nanomole (10^{-9}) range, while those of infrared spectroscopy and mass spectrometry are around 10^{-12}–10^{-5} and 10^{-18} moles, respectively.

There are several ways in which NMR sensitivity can be maximized, and such attempts also constitute different lines of research aimed at stretching the frontiers of NMR LOD. Here we note the most important general issues, and some more specific aspects related to the sensitivity problem will be highlighted further below in connection with the use of NMR with and without chromatographic separation.

The intensity of the signal S depends on five important factors that can be more or less controlled by the user and/or the manufacturer of the NMR spectrometer.

1. Standard high-resolution NMR probes today are designed to accommodate sample tubes of 5-mm outer diameter and utilize an active volume length of ca. 10 mm. By maximizing the number of spins within the active volume, the net magnetization M can be increased. This of course may be achieved either by dissolving more molecules (solubility and sample amount permitting) or, in the case of mass-limited samples, by decreasing the sample volume together with the active volume, thereby maximizing the concentration and minimizing the solvent/analyte ratio (the detection of weak signals in the presence of strong solvent resonances is unfavorable both ''horizontally'' because the base of an intense absorption-mode Lorentzian conceals a larger

frequency range than that of a weak resonance, as well as ''vertically'' by increasing the dynamic range – see below). To that end, one area of research uses microcoil technology with very small solenoids being wound directly around a capillary that contains the sample after, say, capillary separation. The volume occupied by these coils may be less than 1 μl and LODs can be brought down to the picomole (10^{-12}) level [34–36]. The idea is currently being approached by manufacturers in a less extreme and more user friendly fashion by introducing several lines of commercially available ''microprobes'' [37] and sample tubes that have smaller outer diameters (most notably 3 and 1.7 mm). Research also targets the problem of reducing the sample size along the direction of the $\mathbf{B_0}$ field, which is not a trivial problem since normally the sample should extend well beyond the length of the active volume to ensure that the magnetic susceptibility of the sample is constant along the entire detected region. While some problems arise from the weak but non-negligible detection of sample regions falling outward the edges of the coil [38], if the sample length is limited to be shorter than the active region sample/air and/or sample/container interfaces are also detected, giving susceptibility artifacts in the form of distorted lineshapes [39]. Probably the best current tube design that alleviates this problem is the ''Shigemi tube'' in which the sample/air interface is replaced by a sample/glass interface via a special susceptibility matched glass plug. A different approach is used by Varian's Nano.nmr probe [39,40] in which the sample is held in a special glass container of ca. 40 μl observe volume and is placed within a 5-mm diameter solenoidal coil. The system is oriented at the magic angle and the sample is spun at (2 kHz in order to minimize the effects of susceptibility disconuities associated with the small size of the container or the high viscosity of the sample – a concept borrowed from solid state NMR. The Nano.nmr probe technology also plays a key role in obtaining high-resolution spectra from gel-phase samples that are the products of solid-phase combinatorial chemical synthesis [41]. These techniques now allow the measurement of microgram-to-nanogram sample quantities on a more or less routine basis.

2. With a given number of spins per unit volume, M_0 can of course be increased by increasing the field strength with the added benefit of obtaining a larger spectral dispersion. Besides these obvious advantages of high magnetic fields, there are some less trivial subtleties that must be taken into account. For example, if the system is in a state of *moderately fast* exchange ($k_A,k_B > \Delta\omega$) and therefore shows extensive exchange broadening at, say at 300 MHz, this broadening becomes even worse at 500 MHz due to the increased $\Delta\omega$ value. It can therefore often happen that an exchange-broadened resonance which is well observed at lower fields becomes virtually undetectable at higher fields. If, however, the system shows *moderately slow* exchange ($k_A,k_B < \Delta\omega$), exchange broadening is alleviated by increasing the field strength. Alternatively,

exchange rates can, within the limits allowed by the probe and/or the sample itself, be influenced by controlling the sample temperature. If $k_A, k_B > \Delta\omega$, the sample requires less heating for exchange broadening to be eliminated at a lower field than at a higher field. Conversely, if $k_A, k_B < \Delta\omega$, the $k_A, k_B \ll \Delta\omega$ condition can be reached with less cooling at a higher field than at a lower field. (This is one reason why successful structure determination in the drug industry often requires the availability of at least two NMR instruments operating at different field strengths). Also, by increasing the field strength, small molecules whose tumbling rate ρ_c is not much faster than the Larmor frequency ω_0, may easily start behaving as big molecules if ω_0 becomes larger than ρ_c, or will shift into the ''small molecule'' → ''big molecule'' transition area where $\rho_c \approx \omega_0$. In the latter case no NOEs can be observed but specialized ''spin-lock'' techniques are now available that efficiently overcome this problem [42–45]. When increasing B_0, the ''small molecule'' → ''big molecule'' transition occurs earlier with regard to the $^1H \rightarrow {}^{13}C$ NOE than with regard to the $^1H \rightarrow {}^1H$ NOE [10], and therefore ^1H NMR work may be easier at higher fields while adequate carbon spectra and/or structurally important $^1H \rightarrow {}^{13}C$ NOE data may be more easily obtained at lower fields (one more reason to have access to different field strengths).

3. The natural Lorentzian shape of a resonance is broadened and therefore its peak height is decreased by some inevitable non-uniformity of the B_0 field within the active volume. Therefore the better the homogeneity of the B_0 field can be made through ''shimming'', the closer the resonance becomes to its natural Lorentzian shape which is crucial to achieve the highest possible S/N (as well as resolution). The usually tedious procedure of shimming, which has been traditionally considered as an ''art'' due to its complexity, reliance on much experience and subjective judgments, is now being revolutionized by the introduction of computer-controlled gradient shimming techniques [12–15] which provide a quick and operator-independent form of achieving very-high quality spectra.

4. The Q value of the probe can be made larger by building more sensitive probes. To that end currently much effort is being directed toward developing low-temperature probes in which the transmitter/receiver coil, and in some configurations even the preamplifier, are cooled to low temperatures in order to reduce electronic thermal noise. In the extreme, the coil can be made superconductive in which case it is cooled by liquid helium. The results can be dramatic: while S/N is typically of the order of a few hundred for ^1H detection on conventional probes, it can be increased to a few thousand by low-temperature probe technology. Such probes are only currently becoming available, but are rather promising in terms of cutting back on the lengthy and expensive process of isolating metabolites, drug impurities or natural products in adequate amounts to make structure determination feasible on conventional probes.

5. S/N is routinely increased by accumulation. However, the relative signal intensities are dependent in a subtle way on a number of acquisition parameters whose proper choice is critical to maintain the relationship between molarity and integral ratios [46].

Two more important aspects of the sensitivity problem should be noted. First, the frequent need to investigate the salt rather than the base form of drug substances brings up the problem that in ionic samples the random Brownian motion of the ions provides a capacitive coupling to the tuned resonant circuit of the receiver, which reduces S/N in these so-called ''lossy'' samples. There is significant research directed at devising probes which resist this effect, whereby ''salt tolerance'' has become one of the important parameters that characterize the quality of a probe.

Secondly, by striving to increase M, B_0 or Q, the interaction between $\mathbf{M}_{xy}$ and the coil can increase to the point whereby the transient magnetic field generated, according to Lenz's rule, by $\mathbf{M}_{xy}$ in the coil back-reacts on $\mathbf{M}$. Thus the return of $\mathbf{M}$ to thermal equilibrium will no longer be governed only by normal relaxation processes, but becomes a function also of the momentary coupling between $\mathbf{M}_{xy}$ and the coil, leading to a non-linear NMR response. The phenomenon has become known as ''radiation damping'' [47] and has many undesired effects (e.g. distorts signal widths and heights) [48,49] which interfere with both structure elucidation and quantification. Intriguingly, in cases where the radiation damping effect is weak, its effects on the lineshape are non-conspicuous, but can nevertheless significantly accelerate the thermalization rate [49]. One implication is that in a weakly damped system the T_1 relaxation times determined by a standard inversion-recovery experiment will be correct, but after a normally used 90° detection pulse recovery will *not* proceed according to this T_1 value but significantly faster, thereby introducing errors in the evaluation of a number of experiments where this effect is not taken into account. There is growing concern that radiation damping might become a major obstacle regarding supersensitive probes. Several schemes have been proposed to eliminate radiation damping [50–59], but these either introduce other undesired drawbacks or quench radiation damping on a single resonance only and not throughout the entire spectrum.

2.4.4. NMR as a Quantification Tool

In addition to its non-destructive nature, the appeal of NMR in the quantitative measurement of impurities in the presence of the main substance rests on the fact that the integral intensity of a given resonance is linearly proportional to the number of nuclei contributing to that resonance. Provided that a few precautions are taken in terms of parameter choice [4,46,60–63], most

importantly an adequate time ($>5T_1$) is left between successive accumulations for the spin system to recover to thermal equilibrium, signal intensities will reflect directly the molar ratios of the pertinent molecules independently of molecular weight. In that respect it is the unique feature of NMR that no calibration is needed which means that a suitable signal of the main substance can be directly used for internal intensity referencing. Very small impurity ^1H signals are usually compared against the intensity of a ^{13}C satellite of a nearby main-compound ^1H resonance. (^{13}C satellites arise from the ^{13}C–^1H protons present in 1.1% natural abundance, and appear on both sides of the main ^{12}C–^1H resonance with 0.55% intensity each). Thus in principle additional internal standards or the method of standard addition are not required for quantification by NMR. This is a major advantage since further sources of errors associated with the purity of the reference standards, extra sample treatment, etc. are greatly reduced. (Nevertheless, the method of standard addition can be a useful cross-check regarding the accuracy of the developed NMR method if very small components must be determined in the presence of large signals – see below). In addition, as compared to quantification based on chromatographic separation, different components are *always* resolved by NMR (except for enantiomers which need a chiral environment for separation). For these reasons NMR is increasingly used in the drug industry either for the direct quantitative determination of impurities, or as an indicator of purity based on a comparison of the structural formula with integrals obtained under quantitative conditions [64].

In spite of the apparent simplicity of NMR in quantification, a number of factors can make it difficult to obtain precise NMR integrals. First of all, the accuracy of quantification depends heavily on the noise level of the spectrum, and this noise can often be reduced adequately only by accumulation – a procedure that increases the signal-to-noise ratio (S/N) but also has many catches in quantitation [46]. Another important factor comes from the slow decay of the Lorentzian NMR lineshape: in order to obtain an integral accuracy of 99.90% the integration range must be 636 times the line width [65]. In general, this integral accuracy becomes unattainable for practical reasons. Therefore, the accuracy of the NMR integral ratios relies on the assumption that the errors cancel each other. This assumption can sometimes be heavily corrupted by interference due to ^{13}C satellite bands. Moreover, the bias in the integral ratios due to ^{13}C satellite bands presents a field strength dependent source of error. With modern NMR instruments the ability to apply band selective carbon decoupling during acquisition offers a unique possibility to overcome this problem (see Section 2.4.7.1). Besides eliminating the interfering satellites, carbon decoupling provides an extra 1.1% gain in S/N.

The precision and accuracy of NMR quantification are both influenced by the quality of shimming, the choice of window functions, phase-, baseline- and

drift correction. Therefore standardized sample treatment and data analysis protocols, as well as the expertise of a trained technician prove to be essential to obtain precise results. Even with modern baseline correction routines the manual adjustment of the integral curve is often required when small signals have to be measured. Manual adjustments inherently involve some degree of subjective judgment, which might lead to reproducibility problems if very precise quantification is required or validated, as is often essential in the drug industry. Even with standardized protocols and with highly qualified NMR operators, the reproducibility of a quantitative NMR method was found in a recent study to vary non-negligibly among different instruments and laboratories [66], leading to the suggestion that ''the reproducibility of any NMR assay measurement, which requires a high precision should be validated by an interlaboratory study''.

The measurement of the signal inevitably involves digitization which is handled by the analog-to-digital converter (ADC). The ADC plays a crucial role in the detection and quantification of weak signals: the ADC has a finite resolution represented by its number of bits, and therefore the digitized values of the signal will be in error of the order of the least significant bit of each sampling operation, degrading after FT the LOD and the accuracy of integration of the pertinent resonance peak [67]. For maximum sensitivity it is thus important to amplify the FID such that it completely fills the ADC, and this is a sensible and routine approach in the so-called *low dynamic range* situation when the analytically important signals are of comparable magnitude and much larger resonances are absent. In contrast, in the case of the *high dynamic range* problem, a weak signal must be detected in the presence of a strong signal. Here the FID cannot be amplified indefinitely to ensure an adequately fine digitization of the weak signal by the ADC. Every sampled point represents the sum of the sinusoids constituting the FID and therefore each point contributes to all of the resonances in the frequency domain. Thus if the FID is amplified beyond the point where the most intensive sinusoid component hits the most significant bit of the ADC, the ensuing truncation of the FID will distort not only the largest resonance but the entire frequency spectrum. Thus an adequate digitization of tiny signals may often be a problem. A newly developed technology that greatly alleviates this problem is digital signal processing (DSP) [68] which offers a spectacular S/N improvement especially at low ADC gain.

If the high dynamic range stems from one or more intense solvent signals (for example non-deuterated solvents remaining after chromatographic separation), these resonances can be suppressed by a number of band- or frequency-selective techniques employing highly sophisticated shaped pulses [69,70]. However, the simplest and most straightforward method that is universally used to suppress a single solvent peak (most typically the HDO signal when

working in water or DMSO) is a simple selective saturation by a long continuous irradiation of the pertinent resonance. Although by solvent suppression the dynamic range can be reduced, it should be noted that the broad base of the solvent signal that originates from the transition band region is highly resistant to suppression [38,71], thereby quantification of a weak signal that happens to reside on the slowly decaying skirt of the residual ''hump'' (see Section 2.4.7.1) will remain to be a problem; it may in such cases be wise to experiment with Shigemi tubes. (A further reason for eliminating strong solvent resonances is that they tend to cover a substantial frequency range due to excessive broadening because of radiation damping). If the high dynamic range stems from small signals being in the presence of a complex set of signals rather than solvent signals (e.g. if impurities must be determined in the presence of a main substance) the dynamic range problem is less easily resolved and can be more limiting – see also below.

2.4.5. Applications of NMR in Drug Impurity Profiling after Chromatographic Separation

If the substance in question has been separated in adequate quantity and purity, the full arsenal of NMR pulse sequences can be deployed and structure determination should by all expectations be successful. Some examples of a comprehensive treatment of structure elucidation strategies by modern NMR are found in Refs. [4–10].

In drug-related research, very often the success of small-molecule structure elucidation is not a question of any limitations in NMR methodologies, but becomes hindered by the inadequate condition of the sample which narrows the available range of structurally important NMR parameters: the sample may be mass-limited or may contain chromatographic or other isolation-related ''debris'', or both. (For example some substances such as isooctyl phthalate are dissolved from plastic containers by certain solvents, and tend to accumulate in the sample during chromatographic separation). It may probably be fairly claimed that often the bottleneck in the NMR structure elucidation of very small amounts of isolated compounds is not the *amount* of the sample, but rather its inadequate *purity* that ultimately hinders access to vital pieces of spectral information.

If mass limitation is the *only* problem, the special sensitivity-enhancing techniques noted above can help. One of the first and rather instructive structure elucidation projects based on using a comprehensive array of ''micro'' NMR technologies was conducted by Martin *et al*. [72] and involved a relatively pure isolated dimeric alkaloid available only in about 100 μg quantity. Development of NMR capabilities along these lines are progressing steadily [73–76].

If informative spectral regions are interfered with, or concealed by resonances due to the fact that purification was not possible, infeasible or simply insufficient, the success of structure determination depends to a great extent on the amount of information that remains accessible. Such cases are prone to become a bone of contention between the spectroscopist and the chromatographist, since they may entertain very different views about the purity issue – see Section 2.4.7.2. Even if significant spectral regions are blurred by such rogue resonances, in favorable cases a number of modern NMR methods can conjure out such pieces of structural data that would otherwise seem to be hopelessly inaccessible from the normal spectra. Of key importance here is ''total correlation spectroscopy'' (TOCSY) whose two-dimensional [77–79] and one-dimensional [80] versions allow the identification of entire spin systems. For example, even if *only* one member of the spin system is resolved well enough, selective excitation of this isolated signal allows in a 1D TOCSY experiment the effect of excitation to propagate via the scalar coupling network through the whole system, whereby it can be observed such that the detection of other unrelated spin systems is removed – see Section 2.4.7.1. Many variants [8] then use the TOCSY-located spin members as stepping stones to gain information on their NOE connectivities (TOCSY-NOESY) [80,81], or H–C connections (e.g. HMBC-TOCSY, HSQC-TOCSY) [80,82–86]. In general, however, such complex sequences are more demanding in terms of sample amount.

2.4.6. Applications of NMR Without Chromatographic Separation

Much of what can be said about the problems in analyzing mixtures by NMR and the techniques that may be used to overcome some of those problems, has already been noted above in various contexts. The investigation of mixtures is in fact an almost everyday problem when NMR is used to support various synthetic activities: the intermediates or end-products in need of structural verification or elucidation may be mixtures containing starting materials, reagents, solvents, diastereoisomeric products, etc. Quite often routine TLC purity-checks do not reveal the non-uniformity of the material and the synthetic chemist only becomes aware of the problem from the NMR spectra.

Obviously, in unseparated mixtures the use of NMR can be quite limited except from lucky instances where useful spectral features are still resolved (Sections 2.4.7.1 and 2.4.7.3). Many techniques noted in the previous section can help deciphering such spectra, but these usually demand considerable extra instrument time. One particular methodology that is currently being intensively developed and must be highlighted in addition to those mentioned above is

diffusion ordered spectroscopy (DOSY) [87], which selects in a mixture those signals that share a common diffusion coefficient. The experiment results in a two-dimensional plot in which one axis corresponds to the normal spectrum, and the other resolves resonances according to relative diffusion coefficients. All signals of the same molecule should therefore appear at the same diffusion coefficient, essentially breaking down the spectra of complex mixtures into subspectra of individual components, and by combining DOSY with other well established multipulse experiments these components can be identified. Differences in the diffusion coefficient as small as 1% can be detected [87]. The main shortcoming of the experiment is that it needs individual resonances to be reasonably well resolved (which condition may not be satisfied in mixtures) in order to be able to measure their associated diffusion rates. The topic is currently being intensively researched and appears very promising for use in suitable cases as a simple alternative to LC-NMR.

2.4.7. Examples

Referring back to the aim of the present chapter, below we wish to give three examples which not only illustrate the powers of modern NMR spectroscopy as applied to drug-impurity-related problems, thus being quite typical in the global pharmaceutical R&D environment, but which also require some extra insight, intuition, or simply involve an intriguing chemical aspect. In that spirit, these are aimed to be examples not simply of problem solving by NMR, but ''beyond'' NMR.

2.4.7.1. Impurity Profiling of Enalapril and Lisinopril

Enalapril (**1a**) and lisinopril (**2d**) (Fig. 2.4.A) are widely used in the clinical practice. Enalapril is a pro-drug which, following oral administration, becomes bioactivated by hydrolysis of the ethyl ester to enalaprilat (**2c**). While lisinopril is formulated from the bioactive di-acid form, due to absorption reasons the maleate of enalapril is used for oral administration. The synthesis of enalapril and lisinopril, as patented by G. Richter Ltd., follows analogue routes; the synthesis of lisinopril involves an extra hydrolysis step **1b** → **2d** [88].

It has been shown that the cyclic diketopiperazine (DKP) derivatives of enalapril (**3a**) and lisinopril (**3d, 4d)** may potentially appear as synthetic impurities or degradation products in the drugs [89–91]. With the aim of providing reference compounds for HPLC analysis of the bulk drug impurities, the synthesis of enalapril and lisinopril DKPs (**3a**, **3d** and **4d**) was initiated at Gedeon Richter. The DKP synthesis was accomplished analogously to the synthesis of

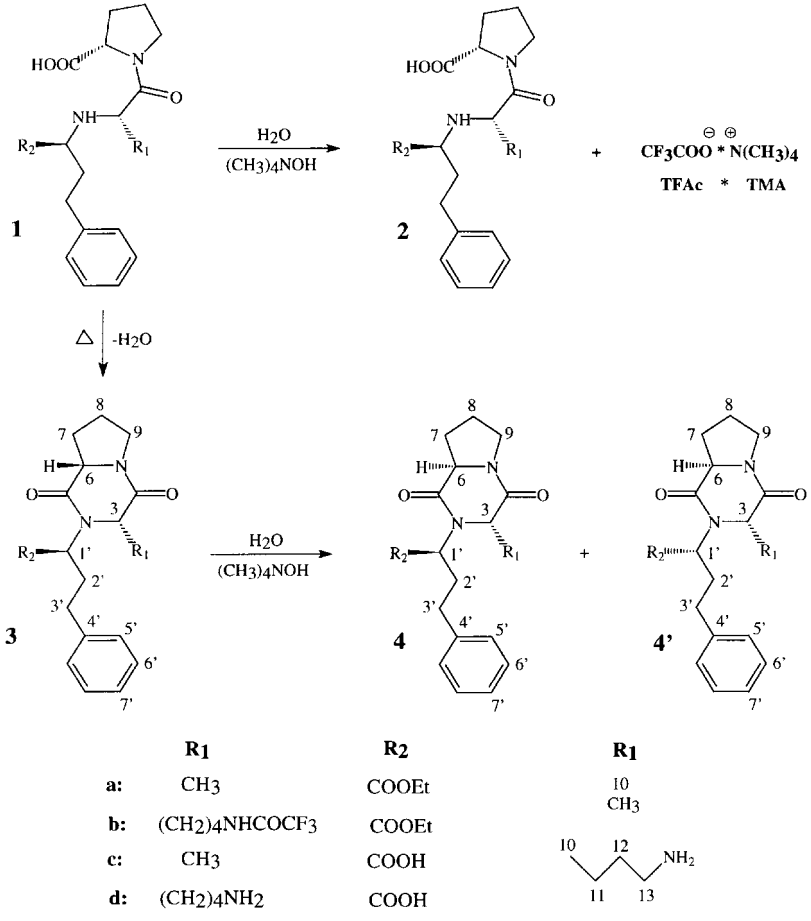


Figure 2.4.A.
Enalapril (**1a**), lisinopril (**2d**) and their transformations

the respective drugs. In solution, enalapril (**1a**) and the last synthetic intermedier of lisinopril (**1b**) readily yielded, upon heating, the respective DKP derivatives **3a** and **3b** (**1** → **3**). However, the base-catalyzed hydrolysis (**3** → **4**) of **3a** and **3b** resulted in a mixture of two stereoisomers (**4c, 4′c** and **4d, 4′d**), which in turn opened a ''can of worms'' in terms of the subsequent NMR spectroscopic efforts. From an impurity profiling point of view, the identification and spectral characterization of the different DKP epimers of enalapril and lisinopril receives special importance due to the continuously increasing

demands concerning the purity of bulk drug substances. We have recently given a detailed account of the spectral and stereochemical characteristics of different enalapril and lisinopril DKP epimers by means of NMR spectroscopy [92]. With regard to that work, here we intend to summarize the key arguments relevant to analyzing diastereomeric mixtures.

The first NMR investigations were conducted, without chromatographic separation, on a mixture of **4d** and **4′d** (major (M):minor (m) = 9:1) as obtained from the synthesis. Figure 2.4.Ba shows the aliphatic region of the ^{1}H NMR spectrum of the mixture of **4d** (major) and **4′d** (minor). MS studies could reveal only one molecular ion ($M^{+} = 387$) that corresponds to the DKP derivative of lisinopril. Therefore the minor component in the mixture was proposed to be a stereoisomer of the main compound. This is a plausible assumption since DKPs are susceptible to configurational changes induced either thermally or upon base-catalysis. In order to verify this scenario selective 1D TOCSY and 1D DPFGSE-NOE measurements were utilized. Figure 2.4.Bb,c,d show three selective 1D TOCSY experiments (measured by exciting the ^{m}H-1′, ^{m}H-6 and ^{m}H-3 + ^{M}H-1′ methin protons, respectively) that map out the minor ^{m}[H–1′–H_2–2′–H_2–3′], ^{m}[H–6–H_2–7–H_2–8–H_2–9] and ^{m}[H–3–H_2–10–H_2–11–H_2–12–H_2–13] (and also the major ^{M}[H–1′–H_2–2′–H_2–3′]) spin systems. By varying the mixing time in the TOCSY experiments, we could assign the minor resonances unambiguously.

Figure 2.4.Cb,c shows two 1D DPFGSE-NOE experiments measured by exciting the minor ^{m}H-6 and major ^{M}H-6 methin protons selectively. The lack of NOEs between H-3 and H-6 and the measured NOEs between H-6 and H_2-10 as well as H_2-11 provides evidence that, with respect to the starting configuration of lisinopril, in both the minor and major species the configuration underwent inversion at either C-3 or C-6. Therefore, the selective 1D TOCSY and 1D DPFGSE-NOE experiments allowed a preliminary stereoanalysis of the mixture. At this point the minor and major species were presumed to be stereoisomers due to epimerization occurring at C-3 and C-6, although epimerization at C-1′ could not be ruled out. In order to determine the absolute configurations, extensive further research was carried out. Deuterium exchange experiments revealed that C-6 rather than C-3 is responsible for the observed inversion and that C-1′ undergoes partial epimerization during hydrolysis, thus giving a mixture of C-1′ epimers **4d** and **4′d**. Since determination of the C-1′ absolute configuration requires a detailed conformational analysis, the minor stereoisomer was isolated by HPLC for the sake of further NMR investigations. Due to ambiguities ensuing from sidechain dynamics, however, the C-1′ absolute configurations in **4d** and **4′d** could be securely resolved only by a comparison with the respective enalapril analogues (**4c** and **4′c**) that possess less degree of conformational freedom. For a detailed analysis the reader is referred to Ref. [92].

Despite the success of NMR efforts that revealed the stereochemistry of the DKP derivatives of enalapril and lisinopril, investigations took a turn in an other direction. This is due to one surplus singlet peak discovered at δ 3.20 ppm in the ^{1}H spectrum of the lisinopril DKP mixture (Figs. 2.4.B and 2.4.C). In the phase-sensitive HSQC spectrum this signal correlates with a carbon signal at δ 54.6 ppm, in phase with the methyl and methin protons, and it does not show any long-range correlations in the HMBC spectra. Since the hydrolysis of **3b** into **4d** and **4′d** was accomplished by a solution of tetramethylammonium

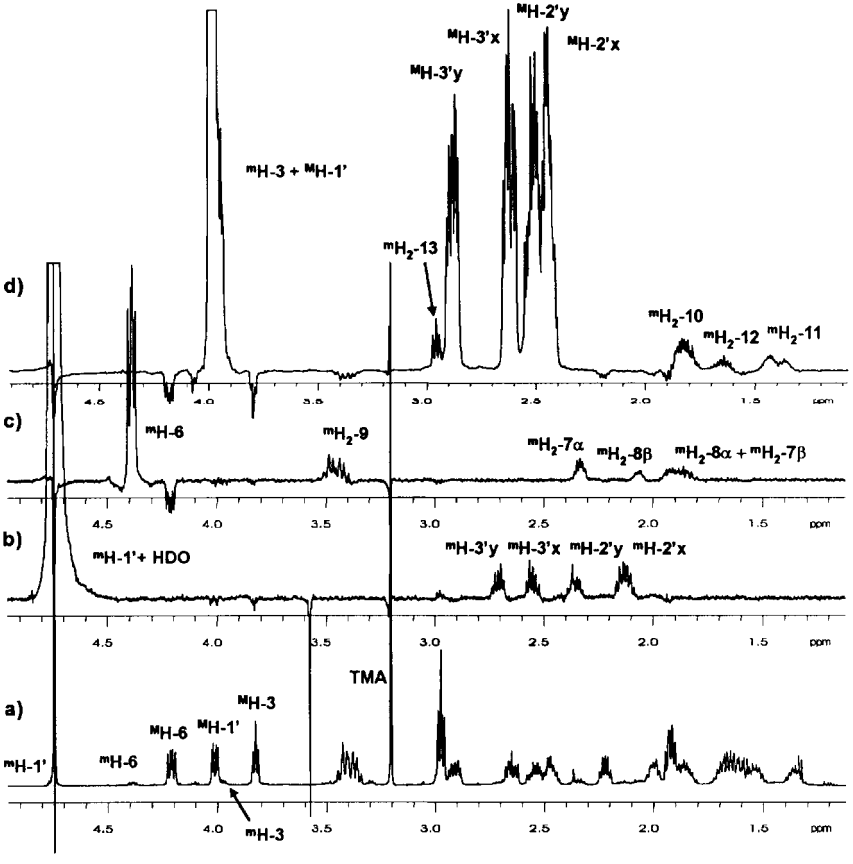


Figure 2.4.B.
(a) Aliphatic region of the ^{1}H NMR spectrum of the mixture of **4d** (major) and **4′d** (minor) and selective 1D TOCSY experiments obtained by exciting the ^{m}H-1′ (b), ^{m}H-6 (c) and ^{m}H-3 + ^{M}H-1′ (d) methin protons, respectively. Selective excitation was achieved by eBurp1 shaped pulses

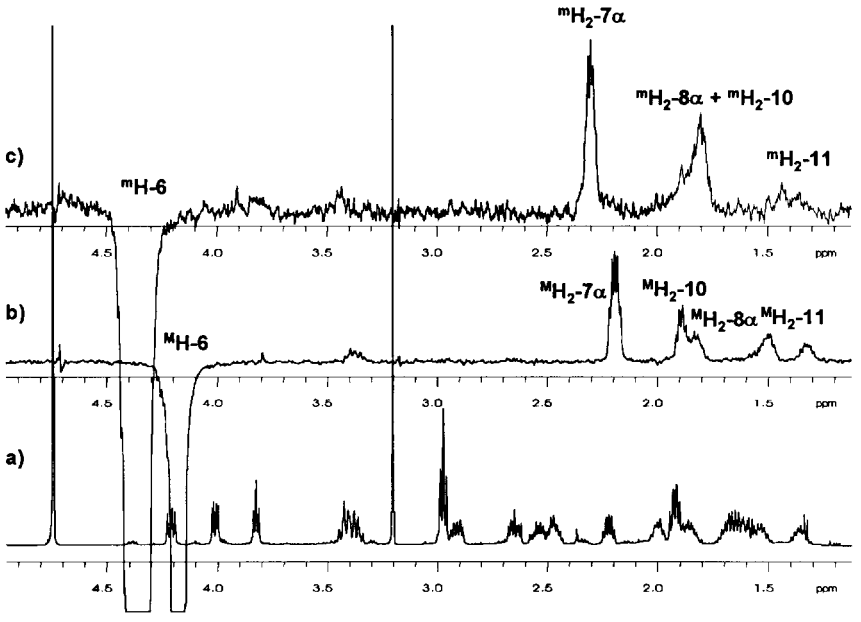


Figure 2.4.C.
(a) Aliphatic region of the ^{1}H NMR spectrum of the mixture of **4d** (major) and **4′d** (minor) and selective DPFGSE-NOE experiments obtained by exciting the ^{M}H-6 (b) and ^{m}H-6 (c) methin protons. Selective excitation was achieved by iBurp2 shaped pulses

hydrox-ide, this singlet peak was attributed to the tetramethylammonium (TMA) ion [$^{+}$N(CH$_3$)$_4$]. The TMA ion generally gives a singlet peak lacking any proton–proton coupling. It is interesting to note, however, that due to the symmetrical nature of this ion a small quadrupole coupling with the nitrogen atom (^{14}N) appears that results in a triplet-like pattern [93]. The coupling (^{2}J[^{14}N,H] = 0.5 Hz) measured on the signal at δ 3.20 ppm verified that this peak is due to the TMA ion.

Due to the fact that tetramethylammonium hydroxide is used for hydrolysis in the final synthetic step that leads to lisinopril (**1b** → **2d**) the TMA ion presents a potential impurity of the drug. A possible counterion of TMA is the trifluoroacetate ion (TFAc) coming from the hydrolysis. Since this impurity eludes any other readily applicable analytical detection technique, the determination of the TMA content of bulk lisinopril batches was based on NMR, providing a unique example of its use, including some pitfalls and limitations, as a quantitative analytical tool.

Figure 2.4.D shows the ^{1}H NMR spectrum of a lisinopril batch spiked with TMA*TFAc salt. The signal due to the TMA ion appears at δ3.16 ppm ($\delta_{DSS} = 0.00$ ppm) as a singlet peak (the quadrupole coupling with the nitrogen (^{14}N) atom is clearly visible in the inset of Fig. 2.4.D).

The quantification of TMA is in this case hampered by two peculiar spectral features.

The NMR spectra of lisinopril exhibit two sets of signals (major:minor = ~5:1) owing to a slow two-site chemical exchange between the proline *cis* and *trans* isomers (Fig. 2.4.D). The most convenient and also the most precise way of determining the TMA content is to relate the integrated TMA intensity to that of a nearby ^{13}C satellite of a suitable lisinopril signal, namely the ^{13}C satellite of the 1^{M} resonance. To this end a knowledge of the fractional population of the major isomer is required.

In the ^{1}H NMR spectrum the TMA signal coincides with the downfield ^{13}C satellite of the $^{M,m}H_2$-Lys-ε protons. This becomes clearly visible for a purified lisinopril sample where the intensity of the TMA signal is comparable to that of the ^{13}C satellite (Fig. 2.4.Fa). Therefore, in order to obtain an accurate

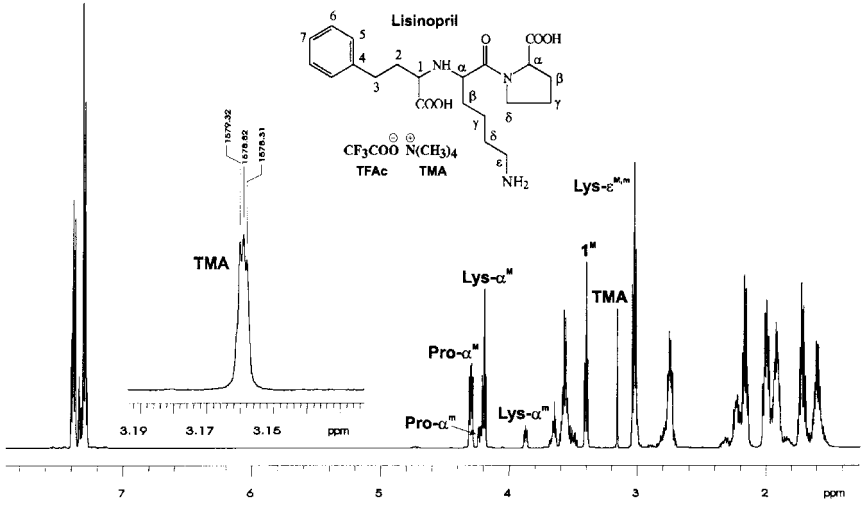


Figure 2.4.D.
^{1}H NMR spectrum recorded in D_2O of a lisinopril sample obtained by intentionally adding some amount of tetramethylammonium (TMA) trifluoroacetate (TFAc) salt to a lisinopril batch. The signal due to the TMA ion appears at 3.16 ppm as a singlet. Upon expansion small splittings on the TMA signal due to quadrupole coupling with the nitrogen atom (^{14}N) become clearly visible (inset)

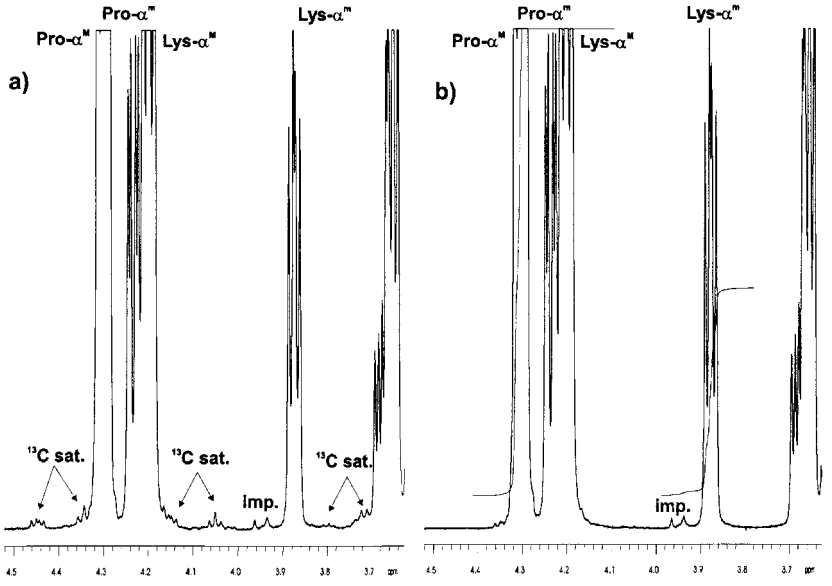


Figure 2.4.E.
Aliphatic region of the ^{1}H NMR spectrum of lisinopril showing the H-Pro-α and H-Lys-α signals (a) without and (b) with band-selective ^{13}C decoupling during acquisition. The WURST shaped decoupling scheme [94] was applied on the aliphatic carbon spectral region. Without ^{13}C decoupling it is hard to define spectral regions sharing the same amount of satellite bands, a prerequisite for an adequate integration of the ^{M}H-Pro-α + ^{m}H-Pro-α + ^{M}H-Lys-α and the ^{m}H-Lys-α signals. However, with ^{13}C decoupling the baseline is purged spectacularly

value for the measured TMA intensity, either the measured integrated intensity corresponding to the sum of the TMA and the pertinent ^{13}C satellite signals needs to be corrected, or the experimental conditions should be altered (see below).

In order to quantify the TMA content of lisinopril, it proves useful to introduce the following notations:

- MI_1 = measured integrated intensity of the ^{M}H-Pro-α + ^{m}H-Pro-α + ^{M}H-Lys-α signals
- MI_2 = measured integrated intensity of the ^{m}H-Lys-α signal
- MI_3 = measured integrated intensity of the upfield ^{13}C satellite of the ^{M}H-1 signal

- MI_4 = measured integrated intensity of the TMA signal (i.e. without the coincident ^{13}C satellite contribution)
- P_M = calculated fractional population of the major isomer
- n_{TMA} = content of TMA in molar%

By making use of these notations we have developed the following protocol.

First, the fractional population of the major isomer is determined from eq. (2.4.1). (Under the applied experimental conditions this value is about 0.8).

$$P_M = \frac{MI_1 - MI_2}{MI_1 + MI_2} \quad (1)$$

Referring back to the above-discussed potential utility of ^{13}C decoupling in quantitative ^{1}H NMR work, Fig. 2.4.E shows the ^{1}H spectral region of the H-Pro-α and H-Lys-α signals (a) without and (b) with ^{13}C decoupling. For **4b** the WURST shaped decoupling scheme was applied on the aliphatic carbon band [94]. Without ^{13}C decoupling it is hard to define spectral regions sharing the same amount of satellite bands, a prerequisite for an adequate integration of the ^{M}H-Pro-α + ^{m}H-Pro-α + ^{M}H-Lys-α and the ^{m}H-Lys-α signals. However, with ^{13}C decoupling the baseline is purged spectacularly. It should also be noted that even with ^{13}C decoupling, minor impurities could still present a problem during integration – see Fig. 2.4.F.

Secondly, to obtain the integrated intensity of the TMA signal which happens to be coincident with the ^{13}C satellite of the $^{M,m}H_2$-Lys-ε signals, two approaches may be used. On the one hand, we can obtain MI_4 from the normal ^{1}H spectrum (Fig. 2.4.Fa) by subtracting from the total measured integral the intensity of the ^{13}C satellite signal which can be calculated if P_M and MI_3 are known. This approach becomes infeasible when very small amounts of TMA need to be determined. Alternatively, the application of band selective ^{13}C decoupling again allows a unique solution to removing the ^{13}C satellite interference. Selective decoupling of the $^{M,m}H_2$-Lys-ε ^{13}C satellite signals without affecting the H-1^{M} ^{13}C satellite permits a direct way to the determination of the TMA integral thus providing a superior method in terms of precision, accuracy and detection limit (Fig. 2.4.Fb). It also has the advantage that decoupling of the ^{13}C satellites due to $^{M,m}H_2$-Lys-ε and TMA can be achieved simultaneously, thus giving a 1.1% gain in the TMA signal intensity. The TMA content n_{TMA} is thus calculated from eq. (2.4.2) as

$$n_{TMA} = \frac{MI_4/12}{(100 \times MI_3/0.55 \times P_M) + MI_4/12} \times 100 \quad (2)$$

The counterion of the TMA ion was established by ^{19}F NMR measure-

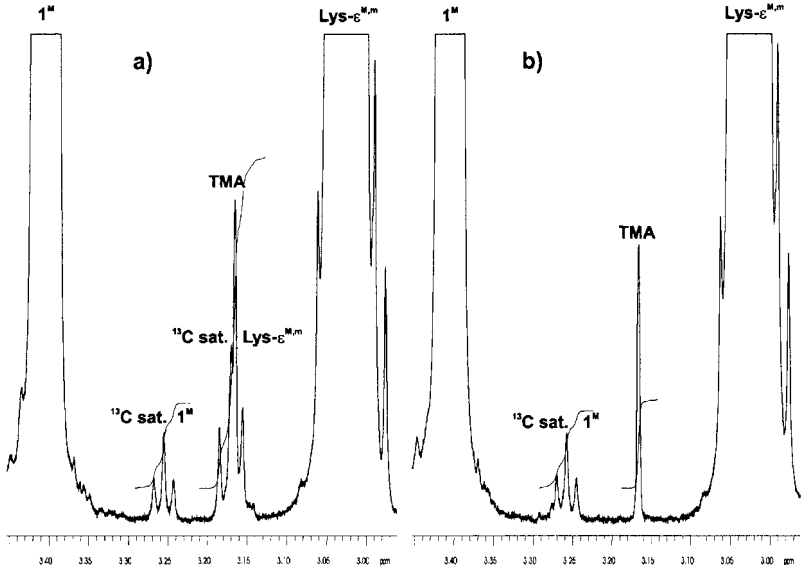


Figure 2.4.F.
Aliphatic region of the ^{1}H NMR spectrum of a purified lisinopril sample showing the H-1^{M} and H-Lys-$\varepsilon^{M,m}$ signals (a) without and (b) with band-selective ^{13}C decoupling during acquisition. The TMA signal happens to be coincident with the ^{13}C satellite of the $^{M,m}H_2$-Lys-ε signals. The WURST shaped decoupling scheme [94] applied on the ^{13}C spectral region containing the H-Lys-$\varepsilon^{M,m}$ and TMA signals allows a unique solution to removing the ^{13}C satellite interference and thus permits a direct way to the determination of the TMA integral. It also provides a 1.1% gain in sensitivity during the detection of the TMA ion

ments. In the ^{19}F NMR spectrum of crude lisinopril batches (not shown), one singlet appeared at -74.4 ppm ($\delta_{CFCl3} = 0.00$ ppm). The intensity of this signal has increased on addition of trifluroacetic acid. By using the method of standard addition the TFAc content was determined and compared to the TMA ion content. The ratio of TMA ion to TFAc was, within experimental error, equal to one in seven different batches. On this basis we concluded that the counterion of TMA is the trifluoroacetate ion.

The precision and accuracy of the method were tested on five different samples obtained from the same lisinopril batch. The five test samples were prepared as follows: in each case 50 mg of the same purified lisinopril batch was dissolved in 1.2 ml of D_2O and 0.7 ml of the resulting solution was filtered into the NMR tube. The TMA content of each sample was determined follow-

ing the protocol described above. The results of the quantitative NMR measurements are summarized in Table 2.4.A. The following conclusions can be drawn: the TMA content of the investigated lisinopril batch is 0.04 ± 0.002%. The relatively small variance of the data suggests that the developed method reliably measures the TMA content at a 0.04% level. Standard addition measurements using TMA-TFAc stock solutions confirmed the accuracy of the results.

As far as the detection limit is concerned, the TMA ion has the advantage that its full proton intensity (12 H) is combined in one intense singlet peak thus allowing the detection of unusually low levels of impurity. On a reference lisinopril sample in which NMR could not detect the TMA ion we have investigated the detection limit by the addition of TMA stock solution. The detection limit was found to be below 0.01%.

In this example the ^{1}H NMR measurements were carried out in D_2O at 30°C on a ^{UNITY}*INOVA* instrument operating at ^{1}H 500 MHz. The residual *H*DO signal at 4.73 ppm was eliminated by presaturation (the small residual ''hump'' comes from transition-band spins which are highly resistant to saturation [38,71]). We employed digital signal processing with an oversampling factor of 68. With our method of combining presaturation and oversampling a ca. 30% gain in S/N could be achieved. The collected number of transients was 32 and the total recycle delay was 37 s (7 s acquisition time and 30 s relaxation delay). The data was processed without apodization and baseline correction. After Fourier transformation automatic phase correction and drift

Table 2.4.A.
NMR signal intensities and TMA content of one lisinopril batch as obtained from five measurements[a]

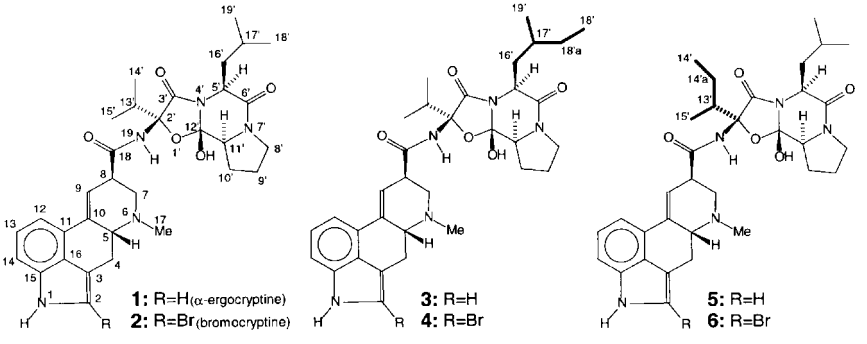
Sample	MI_1	MI_2	MI_3	MI_4	P_M	n_{TMA}
1	1884.45	200.10	75.43	80.12	0.8080	0.0392
2	1848.63	195.62	79.17	83.61	0.8086	0.0391
3	1964.96	210.16	73.96	83.22	0.8068	0.0416
4	1835.85	195.14	76.05	87.63	0.8078	0.0426
5	1837.61	195.24	81.61	88.65	0.8079	0.0402
$\bar{x}$	1874.30	199.30	77.20	84.60	0.8078	0.0406
σ_{n-1}	54.3 (2.9)	6.4 (3.2)	3.1 (4.0)	3.5 (4.1)	0.0007 (0.09)	0.0015 (3.7)

[a] Here $\bar{x}$ denotes the average value, σ_{n-1} stands for the variance of the results. Numbers in parentheses show the variance in percent. The integrated intensities (MI_{1-4}) are given in arbitrary units

correction were applied. In each case the same spectral regions were used for integration and the slope of the integral curve was manually adjusted. In our practice minimal data manipulation proved to be adequate (Fourier transformation without any window functions, automatic phase correction and manual adjustment of the integral slope).

2.4.7.2. Structural Elucidation of two Novel Ergot Alkaloid Impurities in α-Ergocryptine and Bromocryptine

One of the most important members of the ''ergot'' alkaloid family, α-ergocryptine (**1**), is produced naturally by the parasitic fungus known as *ergot* (*Claviceps purpurea*). Bromination of **1** leads to the drug substance bromocryptine (**2**). During the industrial-scale production of bromocryptine, it was noticed a few years ago that the *ergot* strain used at Gedeon Richter started to produce, along with **1** two new alkaloids (**3** and **5**) that are different side-chain homologues of **1**. These compounds began to appear as impurities in the extracted product **1**, while they had previously been absent. Both molecules gave rise to the pertinent brominated derivatives (**4** and **6**) and therefore appeared as new impurities.



The isolation and structural determination of compounds **3–6** required considerable effort. The main difficulty came from the fact that it took several cycles of HPLC and NMR before isolation could be perfected to the point that the compounds became available in adequate purity to make an NMR-based structure determination feasible (for details see Section 2.7.2). The problem is illustrated in Fig. 2.4.G which shows the high-field ^{1}H and ^{13}C NMR spectra of compound **4** (in $CDCl_3$) obtained after the initial isolation effort (Fig. 2.4.Ga,c) and at a later stage (Fig. 2.4.Gb,d) when the separation methodology had been perfected as motivated by the initial NMR results. The quality of the first spectra are obviously rather poor: many signals are broad (possibly because residual impurities coming from the chromatographic system slow down some

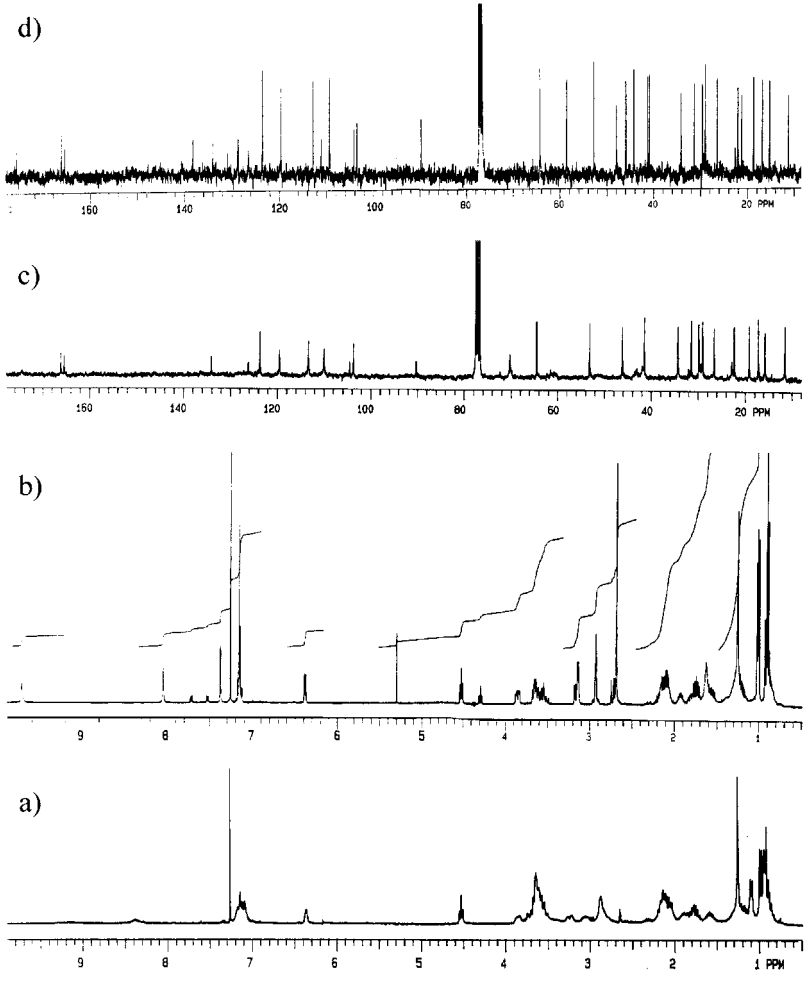


Figure 2.4.G.
High-field ^{1}H and ^{13}C NMR spectra of compound **4** (in $CDCl_3$) obtained after the initial isolation effort (a,c) and at a later stage (b,d) after the separation methodology had been perfected

of the internal motions of the molecule), allowing for much ambiguity in the structure determination. Once the substances became available in adequate purity (Fig. 2.4.Gb,d) and the molecular weights were established by FAB MS, the structures could be determined unambiguously by the concerted use of ^{1}H and ^{13}C NMR data, together with two-dimensional ^{1}H–^{1}H and ^{13}C–^{1}H

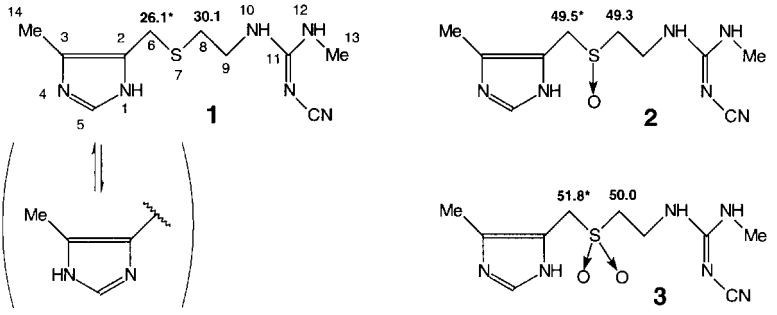


Figure 2.4.H.
Cimetidine (**1**), cimetidine sulfoxide (**2**) and cimetidine sulfone (**3**). Representative ^{13}C chemical shifts are denoted in bold characters. *, signal broadened (30°C, DMSO solvent, 125 MHz for ^{13}C) by the tautomeric exchange process

correlation experiments as well as $^{1}H \rightarrow {}^{13}C$ and $^{1}H \rightarrow {}^{1}H$ NOEs [95] (the quantity in this case was not a limiting factor).

This example is uniquely interesting not only because of the required iterative steps between HPLC and NMR, but also because the production of **3** by the fungus requires homoisoleucine as a building block, which is *not* one of the 20 protein amino acids. This is rather surprising, since homoisoleucine had previously been found only in higher plants [96] and meteorites [97]. It only adds to the mystery that another pharmaceutical research group in the Czech Republic, situated in the geographical proximity of Hungary, has independently and simultaneously isolated and determined by NMR and X-ray analysis the structure of compound **3** [98] which seems to have also been absent from their strains before.

2.4.7.3. Cimetidine Impurities

The NMR structural characterization of cimetidine **1** (Fig. 2.4.H) is complicated [99] by the fact that under routine experimental conditions some of the NMR signals are substantially broadened due to a slow exchange process between the two tautomeric forms of the imidazole ring and/or may also involve slow motions associated with an intramolecular hydrogen bonding of the N(12)H proton to the basic imidazole nitrogen. (The presence of such a bonding was described on the basis of IR [100] and X-ray [101] studies). Extensive exchange broadening affects mainly the imidazole carbon resonances and this suggests that the tautomeric process is more likely to be responsible for the observed exchange broadening.

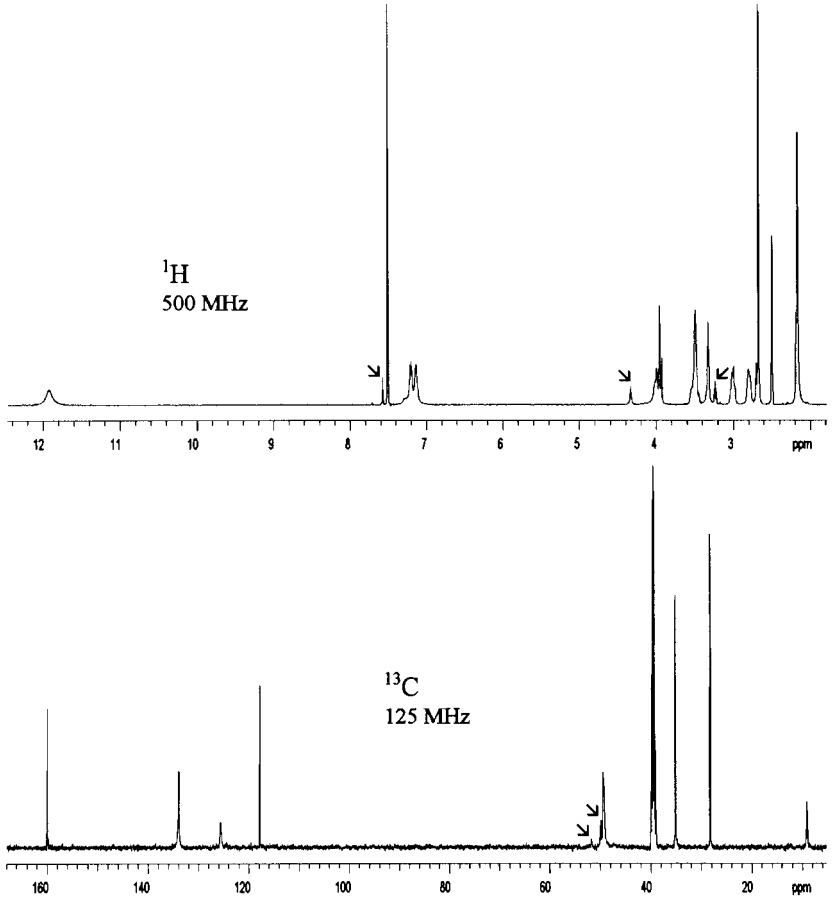


Figure 2.4.I.
High-field ^{1}H and ^{13}C NMR spectra (30°C, DMSO-d_6 solution) of sulfoxide **2**, containing ~10 molar% of the sulfone **3**. The ^{13}C resonances of C(6) (at around 50 ppm) as well as C(2) and C(3) (between 120 and 140 ppm) are exchange-broadened due to the tautomerism of the imidazole ring. Arrows indicate those signals of **3** that are the most separate from **2**; most of the other resonances of **3** are either not visible due to their low peak height or (partially) overlap with those of compound **2**

One well-known impurity of the synthesized drug substance is cimetidine sulfoxide (**2**) which is an oxidative degradation product [102]. During the routine determination of the quantity of **2** in **1**, it was noticed that the HPLC peak associated with **2** was *occasionally* accompanied by another very closely

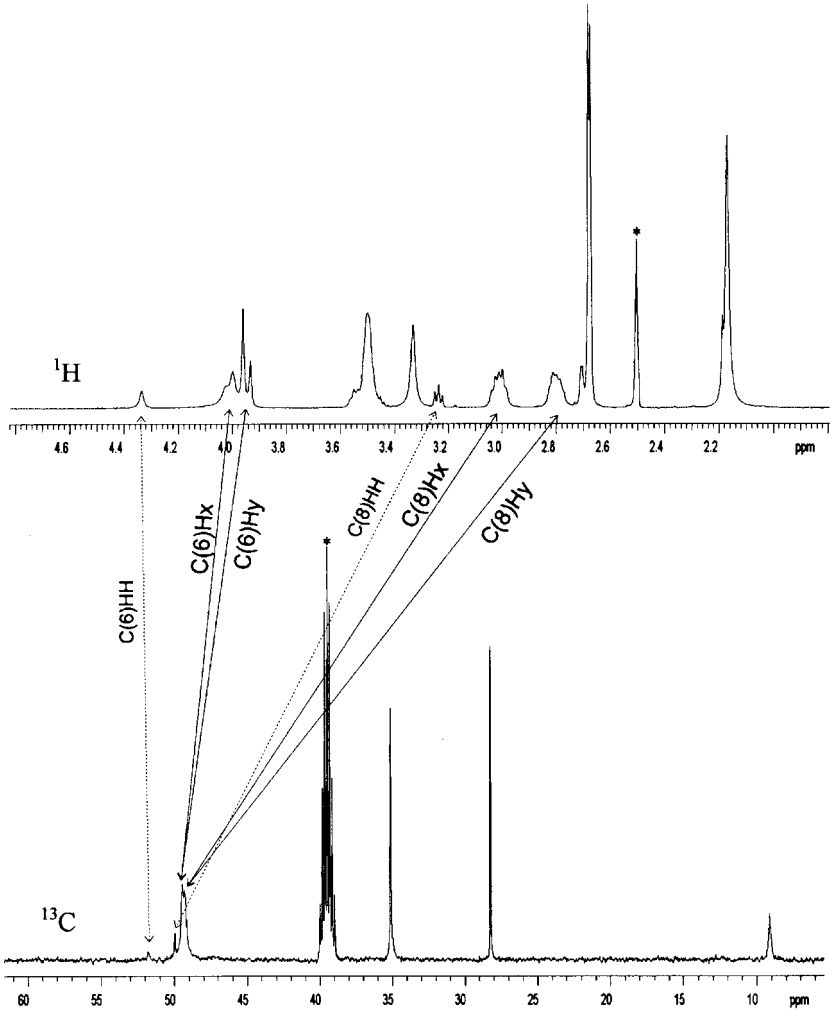


Figure 2.4.J.
Upfield regions of the spectra in Fig. 2.4.I. Solid arrows indicate the one-bond C–H correlations (see also Fig. 2.4.K) of $C(6)H_2$ and $C(8)H_2$ in **2**, while dotted arrows indicate those in **3**. (The multiplets marked by an asterisk are due to DMSO)

spaced peak which was later identified to originate from cimetidine sulfone (**3**). Whether the pertinent peaks could be resolved depended extremely sensitively on experimental conditions and therefore the separation of **3** proved infeasible.

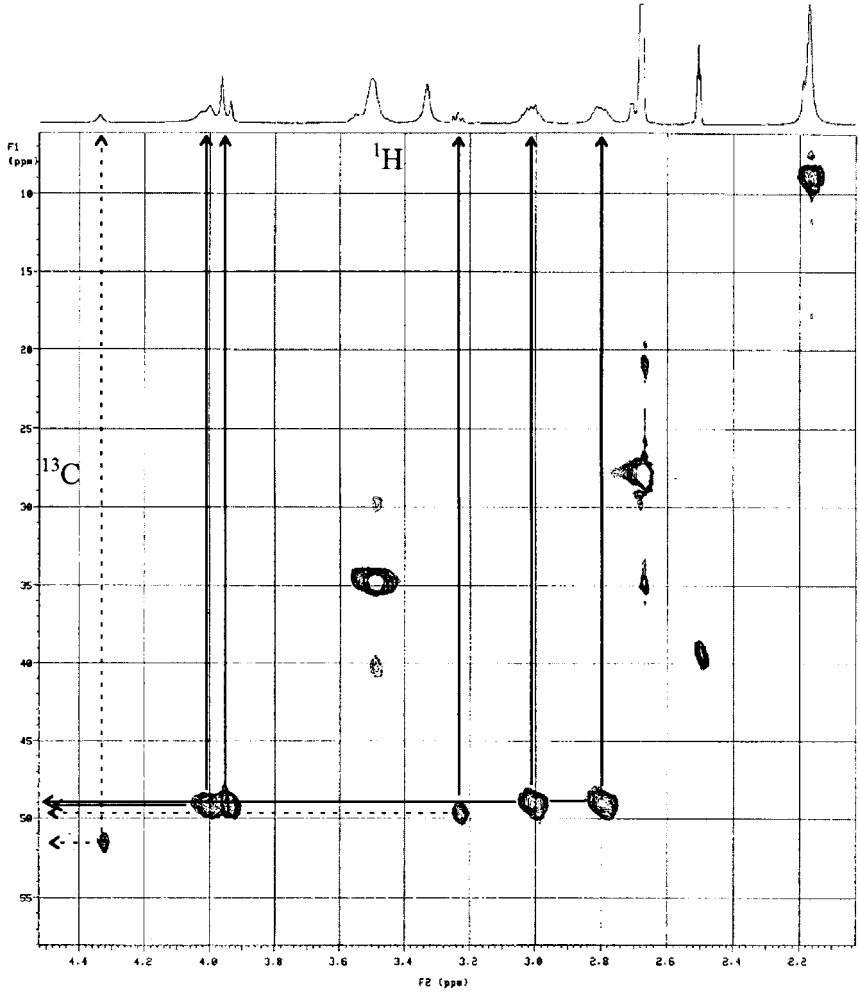


Figure 2.4.K.
Part of a two-dimensional HSQC spectrum with the selected crosspeaks corresponding to the one-bond C–H correlations shown in Fig. 2.4.J

The structure determination of **3** was first attempted from a **2**:**3** mixture of ca. 9:1 molar ratio.

Both compounds are of course also affected by the imidazole ring tautomerism which causes exchange broadening on some resonances (Fig. 2.4.I). Only a limited number of cimetidine-sulfone (**3**) resonances are clearly visible

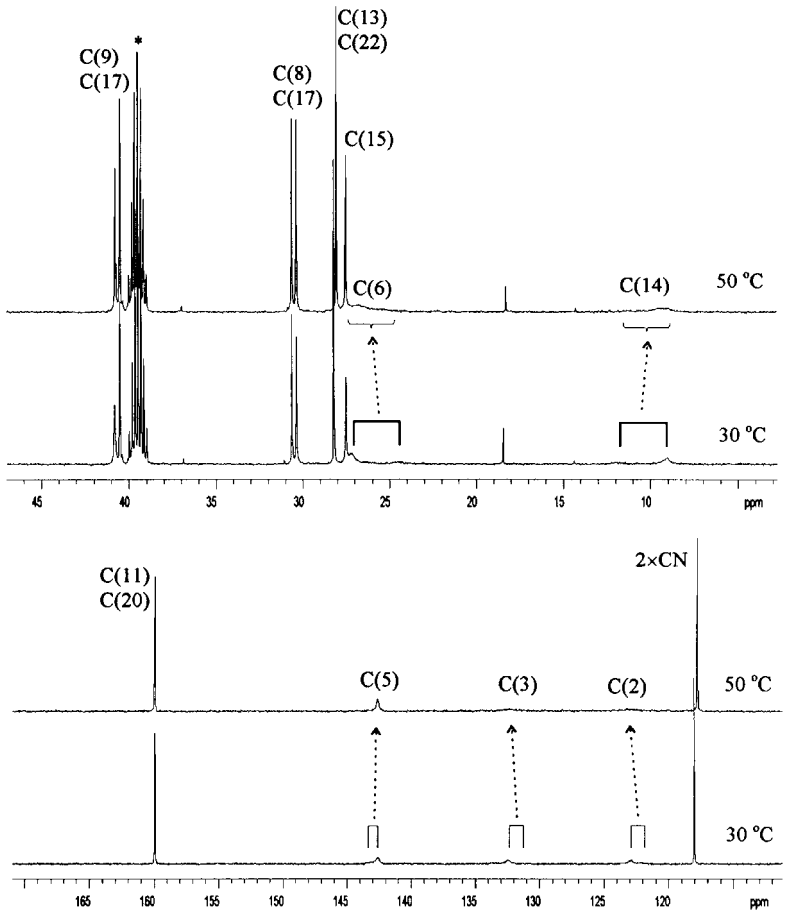
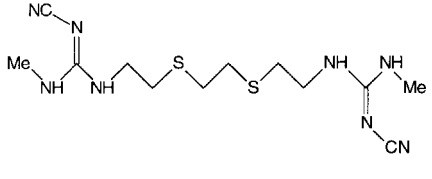


Figure 2.4.L.
Upfield (upper traces) and downfield (lower traces) regions of the ^{13}C NMR spectrum for compound **5** (125 MHz, DMSO-d_6 solvent). The C(2), C(3), C(5), C(6) and C(14) ^{13}C resonances, being the most sensitive to the imidazole ring tautomerism, are split at 30°C into two hardly detectable broad peaks of uneven intensity. Note that with regard to these broad signals the exchange rate is fastest for the resonances due to C(5), which have the least chemical shift difference in the two different tautomeric forms. By increasing the temperature to 50°C, the C(2), C(3), C(6) and C(14) resonances move closer toward coalescence and show even more broadening, while the resonance due to C(5) has already surpassed the coalescence condition and is therefore sharper. (The multiplets marked by an asterisk are due to DMSO-d_6)

in the mixture (Fig. 2.4.I), but one important piece of information that can readily be drawn from the ^{1}H NMR multiplicities is that on each methylene carbon of **3** the geminal protons are enantiotopic, whereas in **2** they are diastereotopic (Fig. 2.4.J). This is in line with the fact that in **3** the SO_2 moiety is symmetric while in **2** the sulfoxide S atom is stereogenic. (It is notable that in **2** the doublet due to H_x-6 is more exchange broadened than the doublet of its diastereotopic partner H_y-6, which is because the effects of exchange on these protons must also be ''diastereotopic'', i.e. non-identical). In the ^{13}C spectrum the peaks due to **3** are hardly detectable, and even the ones suspected of belonging to **3** are quite ambiguous. This ambiguity is resolved by an HSQC experiment (Fig. 2.4.K) which clearly identifies the broader C(6) and narrower C(8) ^{13}C resonance (Fig. 2.4.J). The chemical shifts of these ^{13}C signals are of key importance: relative to **1**, oxidation of the S atom shifts the C(6) and C(8) resonances downfield by ca. 15–20 ppm in *both* the sulfoxide and sulfone compounds, which accords with predictions [103] and again points to structure **3** for the impurity in **2**.

Recently we isolated and identified [99] two further impurities (**4** and **5**) of cimetidine that had not been described up until then. (One year later impurity **5** was described also by Eckers et al. [104] based on HPLC/MS studies.)



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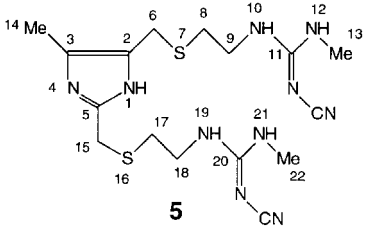

5

Interestingly, in the case of **5** some ^{13}C resonances are actually split at 30°C into a major and minor (~30%) component (Fig. 2.4.L), reflecting an unevenly populated tautomeric exchange process that is even slower than in **1–3**. The difficulties arising from this slow tautomerism are obvious: broad signals are more difficult to detect, consequently if accumulation is carried so far as to ensure good S/N only with regard to the sharp resonances, broad signals may entirely escape detection, possibly leading to faulty structural conclusions. Figure. 2.4.L illustrates how at a given field strength the problem is actually enhanced by raising the temperature to 50°C, which is not high enough for the pertinent resonances to achieve coalescence (except for C(5)), thereby broadening becomes even more extensive (in this case a similar result would be expected if the field strength was decreased but the temperature was kept at around 30°C).

Acknowledgements

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References

1. E.M. Purcell, H.G. Torrey and R.V. Pound, *Phys. Rev.* **69**, 37–38 (1946)
2. F. Bloch, W. Hansen and M.E. Packard, *Phys. Rev.* **69**, 127 (1946)
3. F. Bloch, *Phys. Rev.* **70**, 460–474 (1946)
4. M.L. Martin, J.-J. Delpuech and G.J. Martin, *Practical NMR Spectroscopy*, Heyden, London (1980)
5. S. Braun, H.-O. Kalinowski and S. Berger, *100 and More Basic NMR Experiments, A Practical Course, 2nd edn*, VCH, New York (1996)
6. F.J.M. van de Ven, *Mutidimensional NMR in Liquids, Basic Principles and Experimental Methods*, VCH, New York (1995)
7. H. Friebolin, *Basic One- and Two-Dimensional NMR Spectroscopy, 2nd edn*, VCH, New York (1993)
8. W.R. Croasmun and R. M. K. Carlson (Eds.), *Two-Dimensional Spectroscopy, Applications for Chemists and Biochemists, 2nd edn*, VCH, New York (1994)
9. G.E. Martin and A.S. Zektzer, *Two-Dimensional NMR Methods for Establishing Molecular Connectivity: A Chemist's Guide to Experiment Selection, Performance, and Interpretation*, VCH, New York (1988)
10. D. Neuhaus and M. Williamson, *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, VCH, New York (1989)
11. T.J. Norwood, *Chem. Soc. Rev.* 59–66 (1994)
12. S. Sukumar, M. O'Neil-Johnson, R.E. Hurd and P.C.M. van Zijl, *J. Magn. Reson.* **125A**, 159–162 (1997)
13. H. Barjat, P.B. Chilvers, B.K. Fetler, T.J. Horne and G.A. Morris, *J. Magn. Reson.* **125A**, 197–201 (1997)
14. G.A. Gray and B.K. Fetler, *Magnetic Moments*, Vol. VIII, No. 1, p 16, Varian Associates, Palo Alto (1996)
15. P.B. Chilvers and G.A. Morris, *J. Magn. Reson.* **133**, 210–215 (1998)
16. G.A. Morris and H. Barjat, in *Methods of Structure Elucidation by High-Resolution NMR* (Gy. Batta, K. E. Kövér and Cs. Szántay Jr., Eds.), pp 303–316. Elsevier, New York (1997)

17. J.J. Led and H. Gesmar, *Chem. Rev.* **91**, 1413–1426 (1991)
18. R.A. Beckman and E.R.P. Zuiderweg, *J. Magn. Reson.* **113A**, 223–231 (1995)
19. N. Mistry, I.M. Ismail, M.S. Smith, J.K. Nicholson and J.C. Lindon, *J. Pharm. Biomed. Anal.* **16**, 697–705 (1997)
20. L.E. Kay, P. Keifer and T. Saarinen, *J. Am. Chem. Soc.* **114**, 10663–10665 (1992)
21. A.G. Palmer III, J.Cavanagh, P.E. Wright and M. Rance, *J. Magn. Reson.* **93**, 151–170 (1991)
22. G. Kontaxis, J. Stonehause, E.D. Laue and J. Keeler, *J. Magn. Reson.* **111A**, 70–76 (1994)
23. R.E. Hurd, *J. Magn. Reson.* **87**, 422–428 (1990)
24. I.M. Brereton, S. Crozier, J. Field and D.M. Doddrell, *J. Magn. Reson.* **93**, 54–62 (1991)
25. A.L. Davis, E.D. Laue, J. Keeler, D. Moskau and J. Lohman, *J. Magn. Reson.* **94**, 637–644 (1991)
26. A. Bax and M. F. Summers, *J. Am. Chem. Soc.* **108**, 2093–2094 (1986)
27. W. Wilker, D. Leibfritz, R. Kerssebaum and W. Bermel, *Magn. Reson. Chem.* **31**, 287–292 (1993)
28. J. Ruiz-Cabello, G.W. Vuister, C.T.W. Moonen, P. van Gelderen, J.S. Cohen and P.C.M. van Zijl, *J. Magn. Reson.* **100**, 282–303 (1992)
29. J.K.M. Sanders and J.D. Mersh, *Prog. Nucl. Magn. Reson. Spectrosc.* **15**, 353–400 (1982)
30. K. Stott, J. Keeler, Q.N. Van and A.J. Shaka, *J. Magn. Reson.* **125**, 302–324 (1997)
31. J. Jeneer, B.H. Meier, P. Bachmann and R.R. Ernst, *J. Chem. Phys.* **71**, 4546–4563 (1979)
32. D.J. States, R.A. Haberkorn and D.J. Ruben, *J. Magn. Reson.* **48**, 286–292 (1982)
33. G. Bodenhausen, H. Kogler and R.R. Ernst, *J. Magn. Reson.* **58**, 370–388 (1984)
34. R. Subramanian, M.M. Lam and A.G. Webb, *J. Magn. Reson.* **133**, 227–231 (1998)
35. R. Subramanian and A.G. Webb, *Anal. Chem.* **70**, 2454–2458 (1998)
36. D.L. Olson, T.L. Peck, A.G. Webb, R.L. Magin and J.V. Sweedler, *Science* **270**, 1967–1970 (1995)
37. R.C. Crouch and G.E. Martin, *J. Nat. Prod.* **55**, 1343–1347 (1992)
38. Cs. Szántay Jr., *J. Magn. Reson.* **135**, 334–352(1998)
39. T.M. Barbara, *J. Magn. Reson.* **109**, 265–269 (1994)
40. W.L. Fitch, G. Detre, C.P. Holmes, J.N. Shoolery and P.A. Keifer, *J. Org. Chem.* **59**, 7955–7956 (1994)
41. P.A. Keifer, *Drug Discovery Today* **2**, 468–478 (1997)

42. A.A. Bothner-by, R.L. Stephens, J.-M. Lee, C.D. Warren and R.W. Jeanloz, *J. Am. Chem. Soc.* **106**, 811–813 (1984)
43. A. Bax and D.G. Davis, *J. Magn. Reson.* **63**, 207–213 (1985)
44. T.-L. Hwang and A.J. Shaka, *J. Am. Chem. Soc.* **114**, 3157–3159 (1992)
45. T.-L. Hwang, M. Kadkhodaei, A. Mohebbi and A.J. Shaka, *Magn. Reson. Chem.* **30**, S24–S34 (1992)
46. Cs. Szántay Jr., *Trends Anal. Chem.* **11**, 332–344 (1992)
47. N. Bloembergen and R.V. Pound, *Phys. Rev.* **95**, 8–12 (1954)
48. X. Mao and C. Ye, *Concepts Magn. Reson.* **9**, 173–187 (1997)
49. Cs. Szántay Jr. and Á. Demeter, *Concepts Magn. Reson.* **11**, 121–145 (1999)
50. M. Guéron, P. Plateau, A. Kettani and M. Décorps, *J. Magn. Reson.* **96**, 541–550 (1992)
51. W.E. Maas, F.H. Laukien and D.G. Cory, *J. Magn. Reson.* **113A**, 274–277 (1995)
52. L. Picard, M. Kienlin and M. Décorps, *J. Magn. Reson.* **117A**, 262–266 (1995)
53. P. Broekaert and J. Jeneer, *J. Magn. Reson.* **113A**, 60–64 (1995)
54. D. Abergel, C. Carlotti, A. Louis-Joseph and J.-L. Lallemand, *J. Magn. Reson.* **109B**, 218–222 (1995)
55. C. Anklin, M. Rindlisbacher, G. Otting and F.H. Laukien, *J. Magn. Reson.* **106 B**, 199–201 (1995)
56. V. Sklenar, *J. Magn. Reson.* **114A**, 132–135 (1995)
57. S. Zhang and D. Gorenstein, *J. Magn. Reson.* **118A**, 291–294 (1996)
58. A. Bockmann and E. Guittet, *J. Biomol. NMR* **8**, 87–92 (1996)
59. H. Barjat, D.L. Mattiello and R. Freeman, *J. Magn. Reson.* **136**, 114–117 (1999)
60. D.D. Traficante, *Concepts Magn. Reson.* **4**, 153–160 (1992)
61. D.L. Rabenstein, *J. Chem. Education* **61**, 909–913 (1984)
62. D.J. Cookson and B.E. Smith, *J. Magn. Reson.* **57**, 355–368 (1984)
63. D.L. Rabenstein and T.T. Nakashima, in *Trace Analysis: Spectroscopic Methods for Molecules* (G.D. Christian and J.B. Callis, Eds.), pp 285–393. Wiley, New York (1986)
64. U. Holzgrabe, B.W.K. Diehl and I. Wawer, *J. Pharm. Biomed. Anal.* **17**, 557–616 (1998)
65. C.H. Sotak, C.L. Dumoulin and G.C. Levy, *Topics Carbon-13 NMR Spectr.* **4**, 91–121 (1984)
66. M. Bauer, A. Bertario, G. Boccardi, X. Fontaine, R. Rao and D. Verrier, *J. Pharm. Biomed. Anal.* **17**, 419–425 (1998)
67. J.C. Lindon and A.G. Ferrige, *Prog. Nucl. Magn. Reson. Spectrosc.* **14**, 27–66 (1980)

68. B. John, *Magnetic Moments*, Vol. VII, No. 1, p 16, Varian Associates, Palo Alto (1995)
69. T.-L. Hwang and A.J. Shaka, *J. Magn. Reson.* **112A**, 275–279 (1995)
70. S.H. Smallcombe, S.L. Platt, and P.A. Keifer, *J. Magn. Reson.* **117A**, 295–303 (1995)
71. Cs. Szántay Jr., *Concepts Magn. Reson.* **11**, in press (1999)
72. M.H.M. Sharaf, P.L. Schiff Jr., A.N. Tackie, C.H. Phoebe Jr., L. Howard, C. Meyers, C.E. Hadden, S.K. Wrenn, A.O. Davis, C.W. Andrews, D. Minick, R.L. Johnson, J.P. Shockcor, R.C. Crouch and G.E. Martin, *Magn. Reson. Chem.* **33**, 767–778 (1995)
73. R.C. Crouch, G.E. Martin, S.M. Musser, H.R. Grenade and R.W. Dickey, *Tetrahedron Lett.* **36**, 6827–6830 (1995)
74. R.C. Crouch, A.O. Davis, T.D. Spitzer, G.E. Martin, M.M.H. Sharaf, P.L. Schiff Jr., C.H. Phoebe Jr. and A.N. Tackie, *J. Heterocyc. Chem.* **32**, 1077–1080 (1995)
75. M.H.M. Sharaf, P.L. Schiff Jr., A.N. Tackie, C.H. Phoebe Jr. and G.E. Martin, *J. Heterocyc. Chem.* **33**, 239–243 (1996)
76. G.E. Martin, J.E. Guido, R.H. Robins, M.H.M. Sharaf, P.L. Schiff Jr. and A.N. Tackie, *J. Nat. Prod.* **61**, 555–559 (1998)
77. L. Braunschweiler and R.R. Ernst, *J. Magn. Reson.* **53**, 521–528 (1983)
78. A. Bax and D.G. Davis, *J. Magn. Reson.* **65**, 355–360 (1985)
79. R.E. Hurd, *J. Magn. Reson.* **87**, 422–428 (1990)
80. T.C. Wong, in *Methods of Structure Elucidation by High-Resolution NMR* (Gy. Batta, K.E. Kövér and Cs. Szántay Jr., Eds.), pp 131–147. Elsevier, New York (1997)
81. V. Sklenar and J. Feigon, *J. Am. Chem. Soc.* **112**, 5644–5645 (1990)
82. L. Lerner and A. Bax, *J. Magn. Reson.* **69**, 375–380 (1986)
83. T. Domke, *J. Magn. Reson.* **95**, 174–177 (1991)
84. G.E. Martin, T.D. Spitzer, R.C. Crouch, J.-K. Luo, and R.N. Castle, *J. Heterocyc. Chem.* **29**, 577–582 (1992)
85. B.K. John, D. Plant, S.L. Heald and R.E. Hurd, *J. Magn. Reson.* **94**, 664-669 (1991)
86. W. Wilker, D. Leibfritz, R. Kerssebaum, W. Bermel, *Magn. Reson. Chem.* **31**, 287–292 (1993)
87. G.A. Morris and H. Barjat, in *Methods of Structure Elucidation by High-Resolution NMR* (Gy. Batta, K.E. Kövér and Cs. Szántay Jr., Eds.), pp 209–226. Elsevier, New York (1997)
88. J. Fischer, T. Fodor *et al.*, *Hung. Patents*, Nos. 205.340, 208.026, 214.579 and 214.581
89. O.H. Drummer, S. Kourtis and D. Iakovidis, *Arzneim.-Forsch. Drug Res.* **38**, 647–650 (1988)

90. D.P. Ip, G.S. Brenner and S. Gerald, *Anal. Prof. Drug Subst. Excip.* **16**, 207–243 (1987)
91. D.P. Ip, D. DeMarco and M.A. Brooks, *Anal. Prof. Drug Subst. Excip.* **21**, 233–276 (1992)
92. Á. Demeter, T. Fodor and J. Fischer, *J. Mol. Struc.* **471**, 161–174 (1998)
93. R.J. Abraham, *Analysis of High Resolution NMR Spectra*, pp 16–17, Elsevier, Amsterdam (1971)
94. E. Kupce and R. Freeman, *J. Magn. Reson.* **115A**, 273–276 (1995)
95. Cs. Szántay Jr., M. Bihari, J. Brlik, A. Csehi, A. Kassai and A. Aranyi, *Acta Pharm. Hung.* **64**, 105–108 (1994)
96. S. Hunt, in *Chemistry and Biochemistry of the Amino Acids* (G.C. Barrett, Ed.), Chapman and Hall, London (1985)
97. J.R. Cronin and S. Pizzarello, *Geochim. Cosmochim. Acta* **50**, 2419–2427 (1986)
98. L. Cvak, A. Jegorov, P. Sedmera, V. Havlicek, J. Ondracek, M. Husak, S. Pakhomova, B. Kratochvíl and J. Granzin, *J. Chem. Soc., Perkin Trans.* **2**, 1861–1865 (1994)
99. Zs. Halmos, Cs. Szántay Jr., J. Brlik, A. Csehi, K. Varga, P. Horváth, M. Kislaki, Gy. Domány, A. Nemes and S. Görög, *J. Pharm. Biomed. Anal.* **15**, 1–5 (1996)
100. R.C. Mitchell, *J. Chem. Soc., Perkin Trans.* **2**, 915–918 (1980)
101. E. Haddicke, F. Frickel and A. Franke, *Chem. Ber.* **111**, 3222–3232 (1978)
102. P.M.G. Bavin, A. Post and J. E. Zarembo, in *Analytical Profiles of Drug Substances*, Vol. 13 (K. Florey, Ed.), pp 127–183. Academic Press, Orlando, FL (1984)
103. *The program ACD was used for calculating* ^{13}C *spectra*, Advanced Chemistry Development, 133 Richmond St. West, Suite 605, Toronto, Ontario, M5H 2L3, Canada
104. C. Eckers, N. Haskins and J. Langridge, *Rapid Commun. Mass Spectrosc.* **11**, 1916–1922 (1997)

2.5. Planar Chromatography

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2.5.1. Introduction

Planar chromatography is an essential tool for testing the impurity profile of pharmaceutical drug substances and products [1–3]. When synthesising a new molecule or establishing a new technology this simple method is the first tester in the synthetic chemical research laboratory.

For testing the impurity profile of a new entity, the use of different independent chromatographic techniques is advisable to explore all of the potential known and unknown related substances contained in it as impurities. These methods are usually thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). As HPLC usually works in the reversed phase and TLC in normal phase mode, these methods complement each other and they are suitable for testing all types of impurities, both highly polar and non-polar, of the new entity and an old drug prepared by a new or modified technology.

Due to its advantageous features, planar chromatography has had undiminished popularity in the pharmaceutical industry for many years and is expected to remain so in the future. The main advantages of this method are as follows:

- It is a simple method, it needs no special skills and accessories.
- By this method the simultaneous analysis of many samples is possible, therefore the time and cost of analysis are moderate.
- Samples of different quality and origin can be applied onto the disposable sorbent-layer, therefore different sample pre-cleaning procedures are not required.
- While in the case of HPLC the unknown impurities may be lost in the system remaining on the column or leaving the detector together with the impurities of the solvents, a TLC chromatogram contains all components of the chromatographed mixture.
- Different types of spray-reagents can be used for visualisation of substances having low ultraviolet/visible (UV/VIS) absorbance. The highly active polar surface of the chromatoplate is an ideal medium for in-situ micro-reactions facilitating the differentiation and identification of the impurities by different colours providing useful additional information to the results of HPLC-MS, HPLC-NMR, etc. [4].

- The layer-chromatogram gives a visual experience, therefore it is easy to compare the impurity-profiles of substances of different origin, batches or technologies when chromatographing the substances on the same chromatoplate (Fig. 2.5.A).
- As the planar chromatographic tests are performed by using disposable sorbent layers, there is no possibility for cross-contamination of the different samples (in HPLC the impurity retarded strongly on the column can coelute with the next sample).
- Depending on the task, TLC can be used in different evaluation modes from the qualitative fast identification to the quantitative analysis with sufficient accuracy and precision.

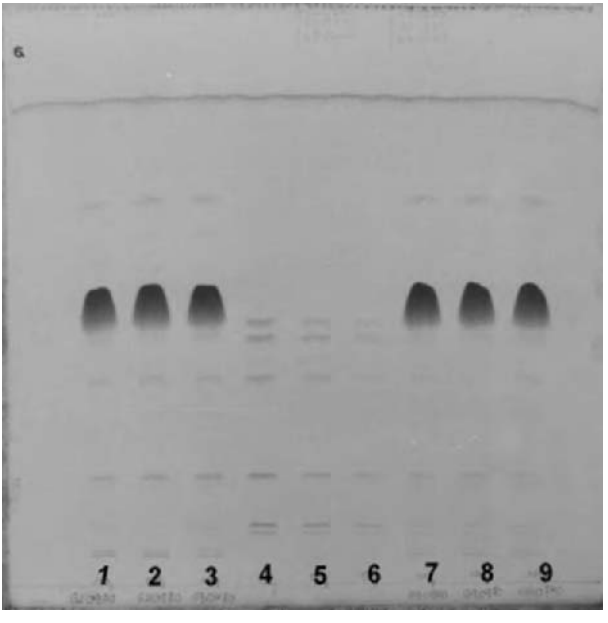


Figure 2.5.A.
Comparison of the impurity-profile of *levonorgestrel* batches by semiquantitative planar chromatography after minor changes in their production technology. Chromatographic conditions in Section 2.5.7. Application: lines 1, 2, 3, batches produced by the original technology; lines 4, 5, 6, calibration with known impurities; lines 7, 8, 9, batches produced by the modified technology

2.5.2. Planar Chromatography in Pharmacopoeias

Due to the above listed advantageous features planar chromatography is a frequently prescribed method in the pharmacopoeias. Although the number of TLC-purity-tests has somewhat decreased in time, it still has great importance even today.

The pharmacopoeial planar chromatographic purity tests are in practically all cases classical, semiquantitative thin-layer chromatographic procedures. The analyte is applied onto a suitable (normal/TLC or fine particle/HPTLC) sorbent layer. The applied quantities of the main components, depending on the impurity-limit and the sensitivity of evaluation, are usually 50–200 μg. The applied volume of the analyte-solution is usually 2–10 μl. Application of a higher quantity of analyte and volume of solutions often results in a distortion (leading or tailing) of the main spot because of overloading. Calibration spots for comparison with the impurity spots are usually the known quantities of a reference substance of the analyte. The analyte and calibration spots are applied in the same volume of solution to avoid the different spot-broadening of different quantities. The development is performed in glass chambers unsaturated or saturated with the vapour of the mobile phase. The development distances are about 15 cm on normal, and 7–8 cm on fine particle sorbent layers. The evaluation of chromatograms depend on the UV/fluorescent-activity of substances investigated. Most types of sorbent-layers contain an indicator-substance having its own fluorescence by irradiation with short wave ($\lambda = 254$ nm) UV-light. UV-active substances absorbing the exciting UV-light can be observed as dark spots on the fluorescent sorbent layer. Substances, having their own fluorescence can be observed as bright spots on a dark background by irradiating with long wave ($\lambda = 366$ nm) UV-light. Coloured substances can be detected in day-light as coloured spots on a white sorbent layer.

Colourless substances having no UV/fluorescence activity are visualised by post-chromatographic derivatisation with different reagents by spraying or dipping.

Pharmacopoeial TLC purity tests are semiquantitative limit tests: the secondary spots of the analyte cannot be more intensive than the comparative spot of the reference substance. These limit-spots are usually 0.2–0.5% of the main component. The task is to decide whether secondary spots are below (''conforms'') or above (''does not conform'') the standards.

Several bulk drug substances are available on the drug market which are prepared by different synthetic procedures by various manufacturers. The pharmacopoeial chromatographic purity tests are usually based on the impurity profile characteristic of the product of the originator or at the most limited number of manufacturers: the impurities in the products of these are separated and detected. The majority of monographs in the European Pharmacopoeia [5]

contains a list of these impurities while in other pharmacopoeias the impurities are named and used as reference spots only in a few cases.

On the other hand, a pharmacopoeial TLC purity test may be unsuitable for separation and detection of impurities in drug samples of different origin. For this reason the verification of the pharmacopoeial chromatographic procedures is very important before their use.

2.5.3. Semiquantitative Planar Chromatography (Advantages and Disadvantages)

In semiquantitative planar chromatography, the impurities of the samples are visually estimated by comparing the area and intensity of spots to those of known quantities of standard substance. Although in such a way the quantity of the impurities can be estimated with a relatively high error, it can be decided with high accuracy whether their quantity is over or under the prescribed limit.

The semiquantitative planar chromatographic purity testing is widely used in those cases when the precision of a semiquantitative analysis is sufficient because it provides a very fast analysis. Using one chromatoplate the estimation of the impurities of 4–5 batches can be performed in a few minutes.

With a certain experience one can perform quite a good estimation if the standard substances of the impurities are available. However this practice would require the use expensive impurity standards, therefore the impurities are usually estimated by comparing their spots with those of small known quantities of the main component. The main sources of error of this general approach are as follows:

- If the main component is UV/fluorimetrically active and the impurity is inactive and vice versa, quite misleading results can be obtained. The degree of the error depends on the degree of the difference between the UV/fluorimetric activities.
- UV-inactive substances can be visualised by using various spray reagents, e.g. sulphuric acid for steroids, but spray reagents are often used for the visualisation of UV-active substances as well. The spray reagents can react with different components to produce different colours. This is an advantage from the point of view of the identification of the impurities but the comparison of different colours for semiquantitative estimation is difficult if not impossible (Fig. 2.5.B). For this reason in such cases the use of spray reagents with lower sensitivity but producing uniform colour (e.g. molybdate reagent) is advisable.
- Substances to be compared may have quite different R_f-values. Due to spot- or band-broadening the substances with higher R_f-values have spots or

Figure 2.5.B.
OPLC-chromatogram of a steroid-compound evaluated in long-wave UV-light after spraying with sulphuric acid. Application: 100 μg of different batches (1, 2), 0.2 μg and 0.5 μg for calibration (3, 4)

bands larger in size and lower in intensity than substances nearer to the origin.

For the above reasons in semiquantitative planar chromatographic purity tests the over- or underestimation of the impurities are frequent when unknown impurities of about 0.1% quantity are calculated on the main component. According to the corresponding guidance of the International Conference on Harmonisation (ICH) [6], a new active pharmaceutical ingredient cannot contain unknown impurities which are equal or more than 0.1%. Due to the error of estimation, impurities are identified and synthesised in some cases unnecessarily because they were strongly overestimated. The contrary of this phenomenon may also happen of course, necessitating the use of other techniques.

Although semiquantitative TLC purity tests are often used in pharmaceutical analysis, methods of this kind are rarely published in the different period-

icals. In one such article a simple and fast purity test for *tetracyclines* is described by Hoogmartens *et al*. [7]. The stationary phase used was silica gel previously sprayed with 10% disodium EDTA solution, its pH being adjusted to 8.0–9.0. The mobile phases were different mixtures of dichloromethane, methanol and water. The evaluation was based on native fluorescence of the substances. The fluorescence was stabilised by dipping the plate in a 30% solution of liquid paraffin in hexane. A sensitive analysis (LOD $< 0.05\%$) can be achieved by this method.

In planar chromatography of drug products (tablet, cream, injection, etc.) the sample-preparation is an important issue. These products are complex mixtures of the active and other ingredients. The other ingredients, e.g. *nipagin* or *nipasol* may have polarity similar to that of the active substance(s). These types of ingredients have to be separated from the active ingredients and their possible degradation products by the TLC procedure. The polarity of other ingredients is very different from that of the main component, but, because their large excess, they can disturb the chromatography. This type of ingredient has to be separated from the investigated components before the chromatographic process.

The most frequently used methods of the pre-chromatographic separation of the inactive ingredients of the products are as follows:

- Dissolution: mainly in solid forms of products (e.g. tablet) the active ingredient(s) are selectively dissolved from the matrix.
- Liquid–liquid extraction: in the case of non-solid forms (injections, creams) the sample is partitioned between two liquid phases. The matrix is concentrated in one phase and the active ingredient(s) in the other one which is to be chromatographed.
- Solid phase extraction: the special packing of the disposable cartridge (2–10 cm^3) selectively retains substances: using this method the active ingredients can be separated from the matrix. Depending on the packing and the eluent the retarded substance(s) can be the matrix or the active ingredients.
- TLC-plate with concentrating zone: the chromatographically inactive and disturbing substances (e.g. inorganic salt) remain in the inactive zone and the components of the cleaned-up sample can be separated in the active zone of the chromatoplate.

After the sample clean-up, the partial evaporation of the sample-solution may be needed to obtain a suitable volume to apply onto the sorbent layer. Due to the variable quality of the solvents available, the application of a blank-sample, similarly prepared as the sample, onto the sorbent layer is also suggested. In this way the possible disturbing impurities of the solvents can be controlled.

In Fig. 2.5.C the chromatogram of an oily injection is shown. The non-

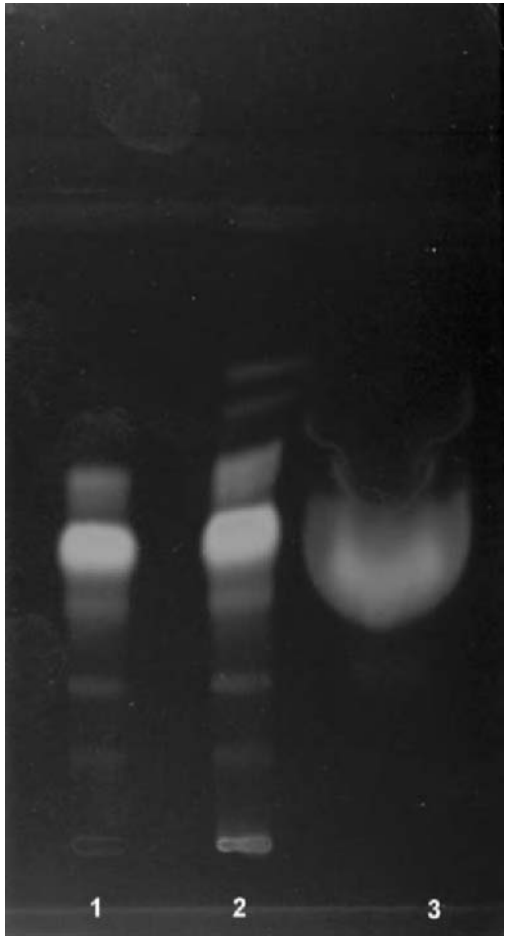


Figure 2.5.C.
TLC-test of *Digoxin* injection. Application: (1) drug substance; (2) injection after solid phase extraction; (3) injection without pre-treatment

sufficient sample preparation resulted in a chromatogram which is not suitable either for a purity test, or for the identification of the active ingredient. After a solid-phase extraction, a chromatogram similar to that of the pure active substance can be obtained.

The planar chromatograms can be stored for some days in a refrigerator but their quality deteriorates (the colours fade) soon. There are different possibilities to preserve the visual information of these chromatograms [8].

- Hand-drawing: fast and cheap, but gives no accurate information especially on the intensities of the different impurity-spots.
- Conventional photography: expensive and time-consuming but in the case of sufficient skill a real and true colour documentation can be provided; its quality does not change for about 10 years.
- Instant photography: an expensive but instant document can be obtained, it can not be duplicated and its quality changes in time.
- Video-documentation: the main advantages of this technique are the fastness and the non-restricted storing-time of the digitised information. In the computer some useful information can be stored together with the chromatogram-picture. It can easily be compiled to complex documentation. Its quality in spatial resolution and colour rendition does not reach the quality of classical photography. The cost of the photo-realistic printing is 2–3-fold higher than in conventional photography.

2.5.4. Quantitative Planar Chromatography, UV/VIS-Densitometry

Although the pharmacopoeial planar chromatographic tests are restricted only to the classical semiquantitative thin-layer chromatographic limit tests, in the practice of the pharmaceutical analysis and quality control, quantitative TLC is also widely used.

As described in the preceding section semiquantitative planar chromatography does not enable the quantitative determination of the impurities and even in the case of limit tests with very low limits the reliability of the semiquantitative evaluation can be problematic. A higher level of information than semiquantitative data is necessary to follow the minor changes in the quantities of the impurities (e.g. in stability studies). Quantitative planar chromatography gives a cost-efficient and versatile alternative to HPLC and GC purity testing.

Although indirect determination, i.e. quantitative measurement of substances eluted from the sorbent layer after chromatographic separation, was frequently used for assay in pharmaceutical analysis (e.g. in the case of testosterone [9]), nowadays the direct (in situ) densitometric determination is the generally accepted method of quantitative planar chromatography.

Quantitative TLC was briefly mentioned as a possibility in the general chromatographic chapter of the 3rd Edition of the European Pharmacopoeia [5] but a detailed description of scanning densitometry as a possible pharmacopoeial method was only recently given in Pharmeuropa by Renger *et al*. [10] and in the last revision of the general chapter on TLC of the European Pharmacopoeia [11].

The mode of the quantitative evaluation of the chromatograms depends on

the characteristics of the tested substances. The most widely used method is the UV/VIS-reflection densitometry.

As in absorption UV/VIS spectrophotometry and in TLC the maximum sensitivity is achievable by selecting the wavelength of the absorption maximum for scanning the densitogram. To be able to do so the remission spectra of the substances to be determined have to be taken and compared. These reflection spectra are usually in close correlation with those determined in solution phase. As an example the solution and TLC/reflection spectra of *cinnarizine* drug substance are shown in Fig. 2.5.D [12]. Therefore the densitometric TLC is also an important tool of structure elucidation of unknown impurities as well as HPLC/MS, GC/MS or NMR methods [13].

If the UV/VIS spectra of the main component and the impurities available are identical, or have a common maximum, the wavelength of densitometric determination is obvious. For example in the case of *famotidine* (Fig. 2.5.E) the spectra of the main component and its impurities contain the (2-diaminomethyleneamino)thiazol moiety with a maximum at 267 nm; (for the structure of famotidine see Fig. 5.1.A in Section 5.1). If the wavelengths of the maximal UV-absorption of the expected impurities and that of the main component are different, a wavelength of a similar UV-absorption is suggested to be used for

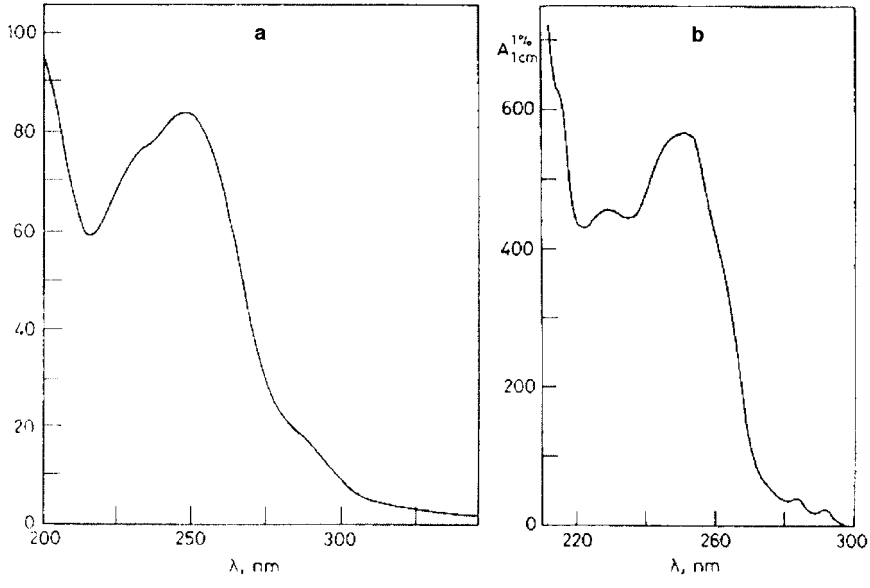


Figure 2.5.D.
Comparison of reflection (1) and absorption (2) UV-spectra of cinnarizine (from Ref. [12])

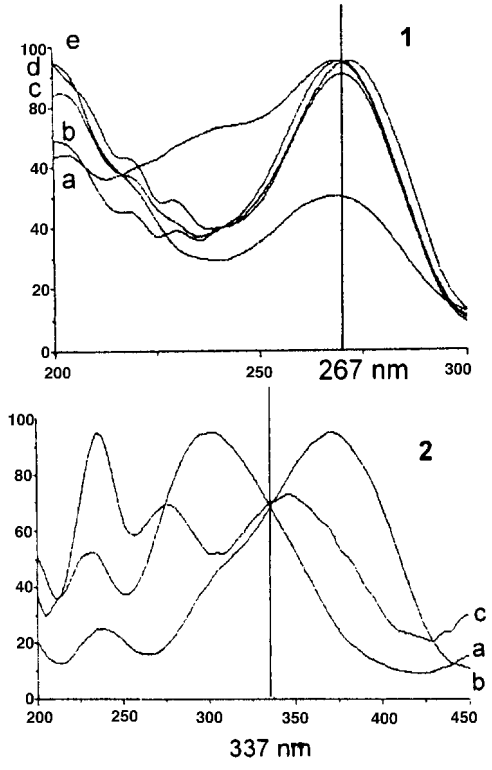


Fig. 2.5.E.
Selecting the wavelength for densitometric determination. (1) Reflection UV-spectra of famotidine (b) and its related substances (a,c–e); (2) reflection UV-spectra of flutamide (a) and its related substances (b,c)

detection such as 337 nm in the case of *flutamide* (α,α,α-trifluoro-2-methyl-4′-nitro-m-propionotoluidide) (Fig. 2.5.E).

The wavelengths of the UV maximum and the intensities of the bands are different in most of cases. Therefore, quantitative determination without positive or negative bias would only be possible in the case of own-substance calibration. As this would be too expensive in routine analysis, the reference standard of the main component is usually used as the calibration substance of the quantitative determination. Based on the slope of the calibration curves of the different impurities and the calibration substance, a correction (response) factor can be calculated in order to decrease the bias originated from the different light-absorption of the different substances.

In quantitative planar chromatography the resolution of the used TLC

system has to be better than that in semiquantitative methods. While two closely-positioned spots can be successfully estimated by visual inspection, this peak-resolution may not be satisfactory to obtain completely separated chromatographic peaks by densitometry. Therefore in quantitative planar chromatography a band-form application is advised in order to obtain better separation of the substances with similar R_f-values. The other advantage of the band-form application is that the positioning of densitometer can be performed by less analytical failure than is the case of spot-application.

The results of the quantitative planar chromatographic analysis are highly influenced by the quality of calibration. The detection is based on measuring the light reflected from the chromatoplate. Since only a small part of the reflected light reaches the detector, the analytical signal is smaller and has a linear relationship with the quantity of sample in a narrower range than in the case of HPLC. The linear calibration graph is very advantageous because of the simplicity of calculation and the necessity of only a few calibration spots. While working in a range proven to be linear, the use of a three-point calibration is satisfactory, a non-linear calibration needs not less than four calibration spots. Although in quantitative planar chromatography the linear range of calibration is narrow, in most cases it is possible to choose the quantity of applied sample in such a way that the quantity of the measured impurities are in the linear range of calibration. If the sample contains impurities of very different concentration, one has usually to work in a non-linear wide range of calibration.

An example for non-linear calibration curves is the testing of impurities in *isometamidium* [14]. The inconveniences caused by this were the reason for the preference of HPLC as the routine method for this purpose.

The non-linear calibration was accepted and used in analysis of a *Co-trimoxazole* tablet. A system, suitable for simultaneous determination of active substances (sulphamethoxazole and trimethoprim) and also the quantity of their degradation products was developed by Agbaba *et al*. [15]. Using a silica gel sorbent layer and chloroform–n-heptane–ethanol (3:3:3 v/v) eluent system, suitable separation was achieved for the quantitative evaluation. As the possible degradation products are expected in different quantities, a wide range of calibration was tested, and a second-degree polynomial regression was used. Excellent repeatability data (0.62–2.2% RSD, $n = 6$) were given for the impurities in the tablet.

The linear range of calibration is wider in the case of band-wise sample-application instead of spots; and by using an unsaturated chamber instead of a saturated one. In quantitative overpressured layer chromatography (OPLC) the linear range of calibration is usually narrower than in quantitative conventional TLC [16].

The analytical error of a quantitative planar chromatographic procedure

is the sum of the errors of different steps of the process. In quantitative purity testing of *danazol* drug substance (for the structure see Fig. 1.2.E in Section 1.2.1) the error of these steps were determined separately [17]. The chromatogram characterising the selectivity of the system and the chromatographic conditions can be seen in Fig. 2.5.F. The errors determined were as follows:

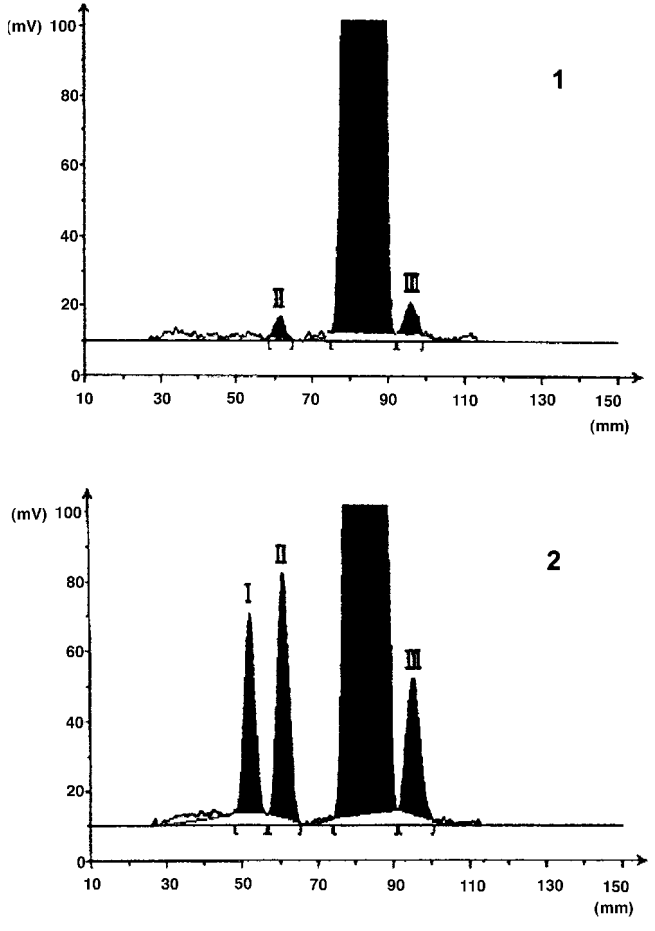
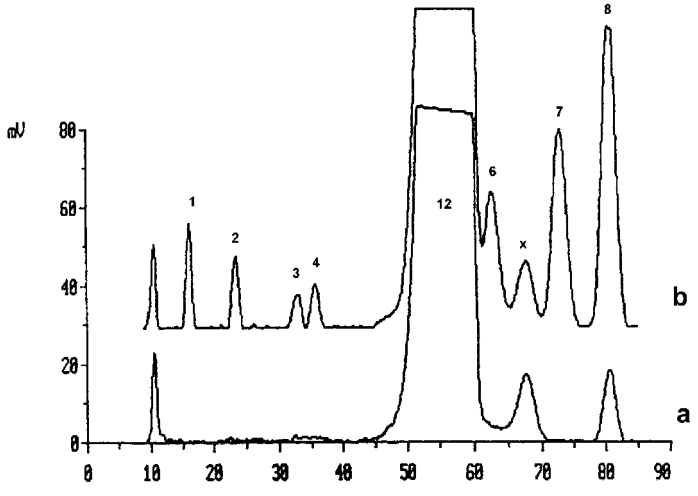


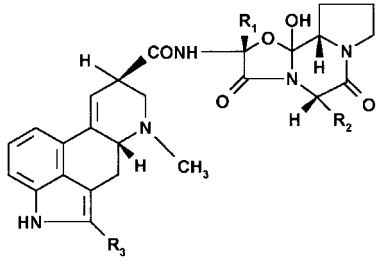
Figure 2.5.F.
Densitograms: (1) danazol sample; (2) danazol spiked with expected impurities I, II, III. Adsorbent: silica gel; mobile phase: chloroform–ethyl acetate 88:12 v/v, containing 0.02% of 2,6-di-tert-butyl-4-methylphenol (from Ref. [17])

- The area of a peak of 0.5 μg danazol was measured (seven times) in the same position of the densitometer. The RSD was 0.22%.
- The area of the same peak was measured (seven times) repositioning the densitometer after each measurement. The RSD was 1.59%.
- The same analytical solution (0.5 μg of danazol) was applied onto the same sorbent layer (seven times) side by side. The RSD of the peak areas was 2.72%.
- The repeatability of the whole procedure was determined by applying different analytical solutions of the same sample (100 μg of danazol) onto the same sorbent layer, and the total amount of the impurities was determined. The RSD characterising the repeatability was 7.28% when the average total amount of the impurities was 0.2%. This value contains the error of different steps of the procedure: sample-preparation, measurement of peak area, positioning of densitometer, the sample-application, calibration and calculation.
- The intermediate precision of the method was determined by performing a purity test of the same sample on five separate chromatoplates on 5 different days by the same analyst. The RSD calculated based on the total amount of the impurities was 8.03%. This value contains errors above the previously mentioned ones that are caused by slight differences in experimental and environmental conditions.

The inhomogeneity of sorbent-layer and vapour phase can cause further analytical error. This type of error can be diminished by the data-pair technique [18]: the application of the samples is performed in duplicates. The units of duplicates are applied onto the two half parts of the sorbent layer. By calculating a mean value, the error caused by the inhomogeneity of the sorbent layer and the vapour phase decreases. Of course the points of calibration are also applied in duplicates, therefore the number of samples roomed on the chromatoplate is strongly limited.

The densitometric determination is also possible in the case of chromatograms visualised by spray-reagents. In this case the quality of quantitative determinations is highly influenced by the uniformity of spraying or dipping into the reagents. The effect of visualisation on the repeatability of a densitometric determination was investigated in the case of *bromocryptine mesylate* [19]. The chromatogram, the expected impurities of bromocryptine mesylate and the chromatographic conditions can be seen in Fig. 2.5.G. The repeatability of the procedure was determined by measuring the sum of the impurities on the same chromatoplate from seven independently weighed analytical solutions. The relative standard deviations were 4.2% by UV detection ($\lambda = 305$ nm) and 11.0% after visualisation by spraying with ammonium molybdate spray-reagent ($\lambda = 685$ nm). The mean values of the sum of the impurities were





		R_1	R_2	R_3
1	Ergosine	CH_3-	$(CH_3)_2$-CH-CH_2	H
2	Bromoergosine	CH_3-	$(CH_3)_2$-CH-CH_2	Br
3	Ergocornine	$(CH_3)_2$-CH-	$(CH_3)_2$-CH	H
4	Ergocrytine	$(CH_3)_2$-CH-	$(CH_3)_2$-CH-CH_2	H
6	Chlorocryptine	$(CH_3)_2$-CH-	$(CH_3)_2$-CH-CH_2	Cl
7	Bromoergocornine	$(CH_3)_2$-CH-	$(CH_3)_2$-CH	Br
8	Bromoergocrytine	$(CH_3)_2$-CH-	C_6H_5- CH_2	Br
12	Bromocryptine	$(CH_3)_2$-CH-	$(CH_3)_2$-CH-CH_2	Br

Figure 2.5.G.
Densitograms of bromocryptin mesylate. (a) Sample; (b) sample spiked with related substances. Sorbent: silica gel; mobile phase: dichloromethane–ethyl acetate–2-propanol–water–ammonia (25:75:2:0.7:0.05 v/v) (from Ref. [19]

0.48 and 0.49%, respectively. In spite of the increased error, the densitometric determination after visualisation by spray-reagent is often used in the case of non-UV-active substances.

In purity-testing of pharmaceutical dosage forms the sample preparation is an additional source of the analytical error. The same quantitative densitometric TLC purity test is used for *Quamatel* tablets and for its active ingredient, *famotidine*. The sample-preparation was a simple dissolution of the powdered tablet. The intermediate precision of the purity tests was characterised by RSD calculated from the sum of the impurities determined by different analysts on different chromatoplates on different days. The intermediate precision was 8.1% in the case of famotidine and 8.5% in the case of Quamatel tablet. If the sample-preparation is more complicated the difference between the precision of the results is higher.

The RSD characterising the precision of a purity test, depends not only on the suitability of the chromatographic and environmental conditions but also on the quantity of the impurities to be determined. In a quantitative densitometric purity test of a new phospholipid the intermediate precision was 5.3–9.6% in the case of a 0.05% level and 4.6–6.4% in the case of a 0.3% level of the different impurities [20]. The chromatography was performed on silica gel $60F_{254}$ (Merck). The mobile phases were chloroform–methanol–p-toluene sulphonic acid (0.25M) in cc. ammonia 70:40:10 v/v and dichloromethane–methanol–cc. ammonia 100:10:2 v/v, respectively. The plates were evaluated by copper(II) sulphate dipping solution and measured densitometrically at 500 nm.

2.5.5. Other Detection Modes

In this section those methods are described which provide higher specificity and, consequently, higher identification power than offered by the conventional UV/VIS densitometry. Only 'quasi on-line' methods are reviewed which are used or can be suitable for impurity testing at least for one lane of the layer chromatogram without removing the sorbent from the chromatographic plate. Planar chromatography coupled with spectroscopic techniques has been reviewed by Somsen and co-workers [21]. Although these methods are frequently used, and have an abundant literature for assay methods, only a few papers deal with their application for impurity determination.

2.5.5.1. Fluorodensitometry

The use of fluorodensitometry in quantitative planar chromatography has been reviewed by Baeyens and Lin Ling [22]. Similarly to the usual arrangement of UV/VIS densitometry, fluorodensitometric measurements are also performed in reflectance mode. The plate is usually illuminated by monochro-

matic UV light and the remitted visible light reaches the detector after passing through a light filter or a second monochromator [23].

Compared to absorption densitometry, fluorodensitometry offers some advantageous features.

- As a consequence of using two wavelengths (excitation and emission), the specificity is enhanced [21,22] and in most cases the background signal is low.
- By appropriate selection of these wavelengths the sensitivity can be increased by a factor of 10–1000 with respect to UV absorption, i.e. the LOD is decreased from, e.g. 10 ng to 500 pg or even lower [22].
- The linear range of the fluorescence intensity is much wider than in the case of UV densitometry: the calibration graph is linear to sample amount over two or three orders of magnitude and passes close or through the origin [22,24–26]

.

Several organic compounds possess strong native fluorescence (e.g. ergot alkaloids). Some others, e.g. (steroids, lipids, carbohydrates, etc.) can be converted into fluorescent derivatives on the chromatographic plate, after separation, by different post-chromatographic treatments [22].

On aluminium-backed amino-modified layers many compound produce fluorescent derivatives upon heating for 7 min at 220°C [27] after separation. Fluorescence can also be induced on a conventional silica chromatoplate by heating it for several hours at 110–150°C in an ammonia atmosphere generated by heating of ammonium hydrogen carbonate [28]. Vapours of strong mineral acids and some Lewis acids can also induce fluorescence [22]. The most generally used fluorogenic reagents are methanolic or ethanolic sulphuric or phosphoric acid. After spraying with or dipping into these reagents the plates are heated for some minutes at about 100–120°C. Sulphuric acid generates different colours of spots, phosphoric acid produces more uniform colours. These colour spots can also be observed in visible light, but the sensitivity can be enhanced when the fluorescence induced by irradiation at 366 nm is measured. Beside these general methods numerous specific reagents are used for fluorescence induction [22,29]. However, it has to be mentioned that the high sensitivity and specificity are advantageous features for assays, but disadvantageous for general purpose impurity profiling.

Several compounds having native fluorescence show an unusually low response when adsorbed onto sorbents compared with the values obtained from solution measurement [22]. This quenching effect is more pronounced for silica gel than for bonded-phase sorbents [30], and can be caused by different phenomena (the adsorption itself, oxidation, catalytic reactions). This problem can be overcome and the fluorescent emission signal on the chromatographic

plate can be enhanced and stabilised by application of either a viscous liquid (paraffin, glycerol, Triton X-100, Fomblin) to the layer before scanning or by antioxidants (e.g. 2,6-di-*tert*-butyl-4-methyl-phenol: BHT) both in the stationary and the mobile phase [30].

The high sensitivity of the fluorodensitometry can only be exploited if the sorbents are thoroughly cleaned, i.e. efficiently pre-developed [31,32] and are free from dust particles [23]. Evaluation of commercially available chromatoplates in respect of sensitivity for fluorodensitometry is also reported [23,31].

As an example for the application of fluorodensitometry in impurity determination, a flavone type anabolic veterinary drug, *ipriflavone* and its impurities were investigated by densitometry in absorbance ($\lambda = 320$ nm) and fluorescence ($\lambda_{exc} = 366$ nm, $\lambda_{em} > 400$ nm) modes [33]. The chromatograms were visualised by treatment with 25% sulphuric acid then heated at 250°C for 1 min. In absorbance mode 14 components were detected whereas in fluorescence mode only four components could be determined but with high sensitivity.

For identification purposes, the fluorescence excitation and emission (FEE) spectra can provide more information compared to UV/VIS absorbance spectrum. The generally used densitometers are not suitable to record full FEE spectra. However, a general purpose spectrofluorometer equipped with a plate scanning accessory can be successfully used to scan the FEE spectra of materials separated by TLC [21]. (In identification of environmental pollutants this set-up was used [34].)

2.5.5.2. In situ TLC-Fourier Transform Infrared (FTIR) Spectroscopy

By using Fourier transform infrared spectroscopy almost all analytes can unambiguously be identified if reference spectra are available [21]. Moreover, the detection and quantification of non-UV-absorbing substances on TLC-plates can also be accomplished without post-chromatographic derivatisation [35]. When reference spectra are not available, valuable information about the molecular structure of the analysed compound may still be obtained by spectral interpretation. On the other hand, difficulties arise because of the strong IR absorbance of the stationary phase (e.g. silica gel). In comparison with the KBr pellet technique, in the case of TLC/FTIR only a rather narrow part of the IR region (3550–1370 cm^{-1}) can be used for the examinations [35].

Since the chromatographic plate is a diffusely reflecting material, a specially designed accessory is needed for the diffuse reflectance infrared Fourier transform (DRIFT) detection on TLC plates. (This technique, also called DRFTIS, is briefly discussed in Section 7.2.2.2.) These methods are reviewed in several papers [21,35,36].

Many years after the pioneering works of others in the mid 1970s, Glauninger

et al. [37] developed a commercially available HPTLC-DRIFT unit (manufactured by Bruker) for *on-line* recording of IR spectra where the entire (10 × 10 cm) chromatoplate is mounted on the computer controlled X–Y stage. Application of this DRIFT unit is described by Kovar *et al*. [38]. In this unit the special mirror arrangement largely eliminates the specular reflectance in the 3600–1350 cm^{-1} region. The diffuse reflectance, which contains the desired spectral information, is directed to a nitrogen cooled MCT (mercury–cadmium–telluride) detector. In order to diminish interferences caused by the IR absorption of atmospheric water and carbon dioxide, the unit is constantly purged with dry, CO_2-free air. The DRIFT unit is attached to an FTIR spectrometer. At the beginning of a measurement, reference interferograms are taken at a blank position of the chromatoplate. During the measurement the stage is moving discontinuously and sample interferograms are continuously recorded and saved. A gas chromatography (GC)-IR software is used for computation of the 'IR-densitograms' and spectra from the interferograms recorded. The blank measurement is used for background subtraction.

TLC-FTIR densitograms can be presented as either the *Gram-Schmidt* or the *spectral window* chromatograms [35]. In the case of computation of the Gram-Schmidt trace, the total integrated IR absorption is measured at all wavenumbers resulting in a universal, substance-non-specific chromatogram. In the case of window chromatograms a user-defined certain wavenumber interval (frequency window) is selected (especially for a substance with known spectrum) and the chromatogram is calculated after Fourier transformation for this range only, resulting in substance-specific and often more sensitive chromatogram. Window chromatograms can be calculated at any desired wavenumber range, so 3D plots (spectrochromatograms) and contour plots can be calculated [38] which enable rapid checking of peak purity [35].

TLC-DRIFT spectra of the spots can be calculated from the interferograms in transmission, quasi-absorption and Kubelka–Munk units. Kubelka–Munk spectra offer higher precision which is advantageous for quantitative work [35].

Due to interactions between the analyte and the stationary phase (both the sorbent and the binder [35]), the TLC-DRIFT spectrum of a compound differs (in positions and shape of absorption bands) from the spectrum obtained by using the KBr pellet or powder technique [21]. For reliable identification of substances, therefore, it is advisable to establish a special spectrum library containing spectra of adsorbed species recorded from chromatographic plates.

Besides the wavenumber limitations, the other major drawback of TLC-DRIFT spectroscopy is its significantly lower sensitivity compared to UV/VIS densitometry. (This is a general problem in almost all coupled techniques of planar chromatography.) The detection limit in a conventional TLC-DRIFT

system [39], ca. 100 ng, was about ten times higher than that of UV densitometry [21]. The identification limit is about 1 μg [36].

In order to improve the poor sensitivity of TLC-DRIFT spectrometry the substance zones (spots) were focused by either a second development perpendicular to the first one [40], or by using the programmed multiple development (PMD) [35], as well as the automated multiple development (AMD) techniques [41]. (The PMD and AMD techniques are briefly described in Section 2.5.6.) By zone-focusing and recording the substance-specific window chromatograms, identification limits could be improved by a factor of 3–6 [40].

The sensitivity of TLC-DRIFT can also be enhanced by improving the IR reflectance of the stationary phase. After the partially successful attempts of others, when zirconium oxide was used as stationary phase [42], Kovar and coworkers in co-operation with a leading manufacturing company of chromatoplates (Merck) recently developed an optimised sorbent for direct HPTLC-FTIR on line coupling [43]. This special (not yet commercially available) HPTLC sorbent layer, comprising 1:1 silica gel 60–magnesium tungstate (a reflection amplifier) having a layer thickness of 100 μm only, improved the detection limit of several pharmaceuticals by a factor of 2–3. Moreover, its evaluable IR range was extended by ca. 100 cm^{-1} into the 'fingerprint' region because the broad interference band at 1337 cm^{-1} in spectra obtained from conventional silica plates is missing (Fig. 2.5.H).

Gram–Schmidt chromatograms in Fig. 2.5.I show that the separation performance of this mixture sorbent is only slightly inferior to that of commercially available chromatoplates but its signal-to-noise ratio is better. To achieve similar retention data as that of commercial silica plates, the eluent strength should be diminished. By using this sorbent the determination and identification of five impurities in *flurazepam* bulk drug substance and capsule (Fig. 2.5.J) were performed [35].

The high identification power of HPTLC-DRIFT is illustrated by the example of the impurity analysis of *chlordiazepoxide* bulk drug substance stored for 8 years as well as a pulverised tablet, which was subjected to a stress test (50°C, 75% relative humidity) for several weeks [40]. Separation of four degradates was carried out on commercial HPTLC silica gel $60F_{254}$ plates by the US Pharmacopoeal TLC method. The impurities were detected by their Gram–Schmidt traces. The substance peaks were identified by calculating quasi-absorbance spectra and comparison with the reference spectra of the library (see Fig. 2.5.K).

The reference standards were only required initially for compilation of the library. Quantitative determination was performed by UV densitometry at 230 nm. Three degradation products (nordazepam, aminochlorobenzophenone and demoxepam) were detected under the stress test, down to 0.3%. The identific-

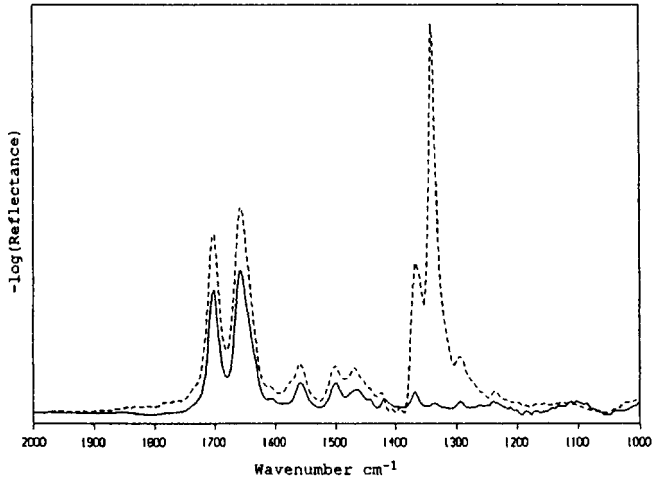


Figure 2.5.H.
HPTLC-DRIFT spectra of caffeine on mixed silica gel 60-magnesium tungstate (—) and on silica gel 60 (- - -) (from Ref. [43])

ation limit could be decreased to 0.1–0.05% by a perpendicular zone-focusing development and recording the window chromatograms.

2.5.5.3. Planar Chromatography coupled with Mass Spectrometry

The methods outlined here are suitable for the on-line recording of mass spectra from chromatoplates providing mass chromatograms from either a strip or from the entire TLC plate. TLC-MS coupling has been reviewed by Busch [44,45] and Somsen *et al*. [21]. Although in principle this technique could be a real alternative to other hyphenated mass spectrometric methods, their use for impurity profiling has not been reported yet.

Since the analytes on a chromatoplate are adsorbed, in the case of direct TLC-MS the soft ionisation methods, e.g. fast atom bombardment (FAB) or liquid secondary ion mass spectrometry (LSIMS) are mainly used. The FAB/LSIMS matrix can be glycerol or 3-nitrobenzylalcohol. To accommodate the chromatoplate and for successive ionisation along the TLC chromatogram, the ion-source housing should be redesigned [46]. Several simple direct TLC-MS probes have been produced by different manufacturers (e.g. Jeol, VG). These probes can hold a ca.10 × 65 mm portion, essentially one track, of a developed plate. This segment of plate, after application of an appropriate FAB/SIMS matrix, is then moved through the FAB beam using a stepping motor [21]. In

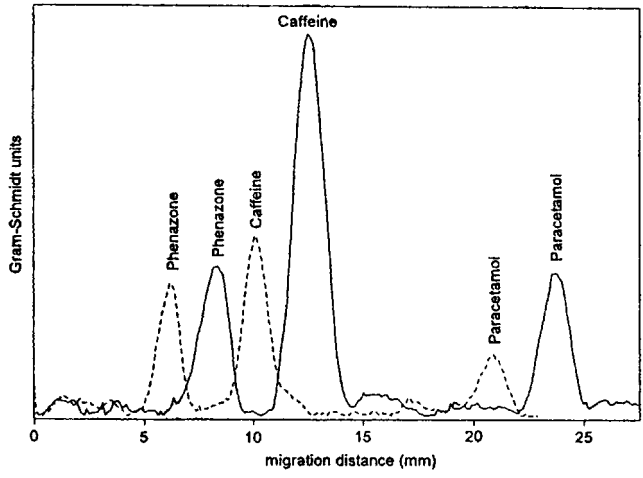


Figure 2.5.I.
Gram–Schmidt chromatograms of test substances on 1:1 mixed silica gel 60-magnesium tungstate (—) and on silica gel 60 (- - -) (from Ref. [43])

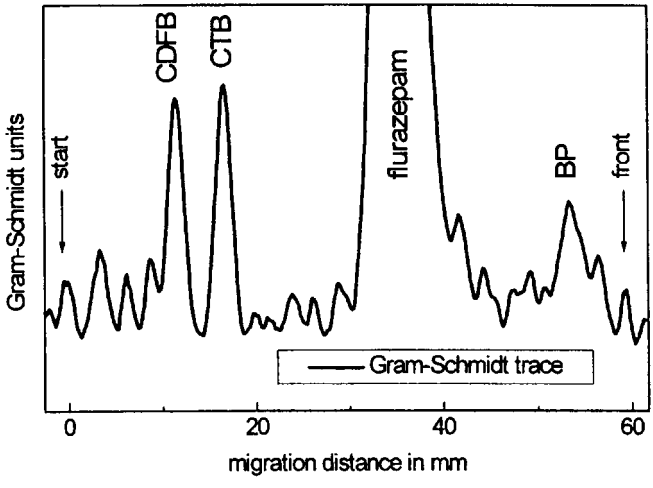


Figure 2.5.J.
Stress test of flurazepam capsule. Gram–Schmidt trace of flurazepam and three degradation products (CDFB, CTB and BP) (from Ref. [35])

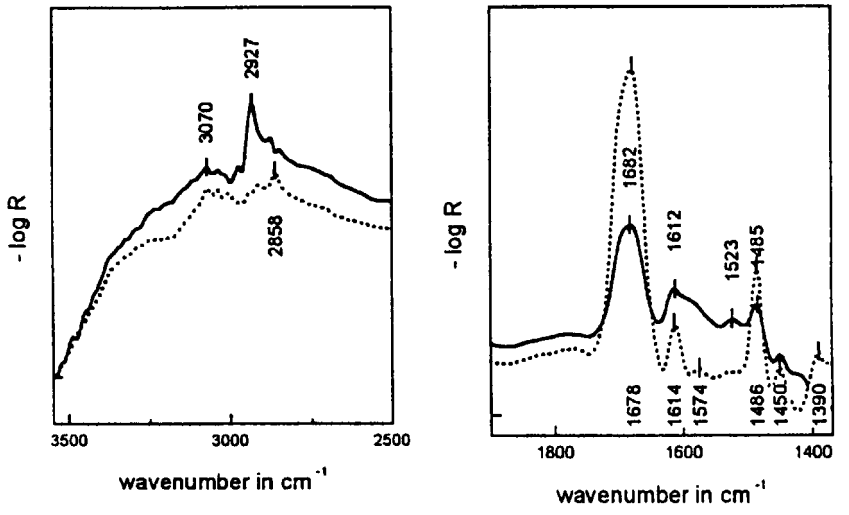


Figure 2.5.K.
Comparison of the DRIFT spectra of the sample (- - -) and of the nordazepam library reference (—). Estimated content: 0.4% (from Ref. [40])

such a way one-dimensional mass spectra and mass chromatograms can be recorded.

A similar scanning device was developed and used by Nakagawa and Iwatani [46] for investigation of several drugs (Fig. 2.5.L). Spectra could be recorded both from glass backed plates and aluminium sheets. No background spectra of sorbents (silica, alumina, cellulose, reversed phase) were found, that might interfere with spectra of analytes.

Two-dimensional mass-imaging was accomplished by Busch [47]. The normally used FAB-LSIMS liquid matrices (e.g. glycerol) provide a limited time for imaging because of spot diffusion. To overcome this limitation, instead of a liquid, a solid matrix [48] (e.g. sorbitol or threitol which is only melted by a primary ion beam) was used by Busch, thus preserving spatial resolution of the chromatogram for a longer time enabling the time-consuming 2D MS-mapping.

2.5.6. Special Techniques: Programmed Multiple Development and Automated Multiple Development

Programmed multiple development (PMD) introduced by Perry *et al.* [49]

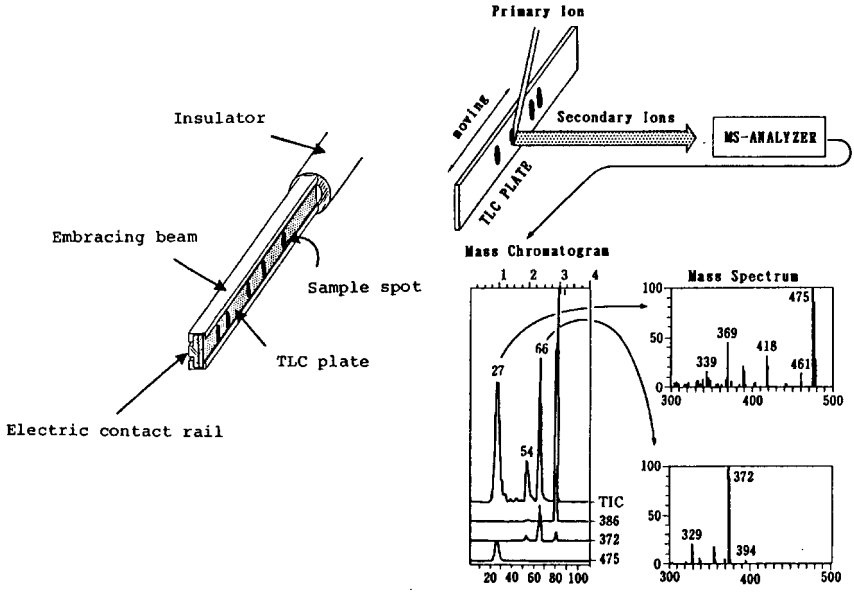


Figure 2.5.L.
A schematic representation of a scanning TLC-MS system (from Ref. [46])

and its improved version, the automated multiple development (AMD) [50–52] are incremental multiple development methods, in which the chromatoplate is repeatedly developed in the same direction. Between the consecutive runs the chromatogram is dried. Eluent flow is only controlled by the capillary forces. The PMD technique uses the *same eluent* in each run and the development distance is increased from step to step.

In the fully automated AMD method, beside the increasing migration distance, a stepwise gradient of *decreasing eluent strength* is also applied. The process begins with the strongest eluent and terminates with the weakest one, enabling a highly efficient zone focusing of the analytes [51]. Since fine particle (HPTLC) plates are used, the maximum development distance can be ca. 9 cm, due to the capillary flow. The whole process can consist of about 10–25 steps, which corresponds to a total development time of 0.5–3 h, and total migration distance of 3–10 cm [52]. Computer controlled AMD equipment is commercially available and manufactured by CAMAG.

The AMD technique in combination with DRIFT was used for impurity determination of an antibiotic injection [41] (cf. Section 2.5.5.2).

In the case of each multiple development method particular care should be taken for the proper selection of the suitable stationary and mobile phases.

Depending on compounds and eluent composition, in certain cases, artefact chromatograms can formed by either extreme adsorption of the analytes [53], or decomposition on the stationary phase.

2.5.7. Overpressured Layer Chromatography

Overpressured layer chromatography (OPLC) is a planar liquid chromatographic method having related characteristics to TLC/HPTLC and HPLC [54–56]. In the case of OPLC, the separation is performed on a sealed TLC/HPTLC chromatoplate, and the eluent is delivered by forced flow with a pump-system, similar to HPLC. In Table 2.5.A the characteristics of OPLC similar to TLC and HPLC as well as special ones are summarised.

Due to the forced flow, the eluent flow-rate is constant and optimal during the whole development. Due to the decreased side-directional diffusion, the spot- or band-broadening are of a smaller extent compared to the conventional TLC/HPTLC even in the case of higher R_f-values, therefore a highly-efficient separation can be achieved. The efficiency of the different planar chromatographic techniques is compared in Fig. 2.5.M [57].

The efficiency of the separation increases with the increasing external pressure [58]. The first commercial OPLC-equipment worked at 10 atm external pressure, the second one at 25 atm. The new, automatic, user-friendly Personal OPLC BS-50 system (Fig. 2.5.N), works at 50 bar (5 Mpa) external pressure. Taking into account the commercial availability of the ready-sealed chromatoplates for OPLC-separations, today OPLC has become a simple, reproducible, really routine-analytical method. Maintaining the advantageous features of TLC, OPLC enables excellent separation in a very short time (a few minutes) with very low eluent-consumption (a few millilitres) and offers the possibility of a cost-efficient, economical analysis.

In classical TLC/HPTLC the developing distance is usually 15 cm on normal particle, and 7–8 cm on fine-particle silica gel plates. Due to the forced flow of the eluent, in OPLC a higher developing distance is possible even on HPTLC sorbent layers without losing the efficiency of the separations. The increased separation distance gives further possibility to improve the resolution. Although the separation distance is restricted by the size of the chromatoplates, there are different possibilities for the longer development by using the OPLC-technique.

- Overrunning or continuous development: the development continues after the eluent reaches the top of the sorbent layer. This method is effective mostly by using the OPLC-BS 50 system, because of forced-flow, the eluent can continuously leave the sorbent layer at the eluent-outlet tube.

Table 2.5.A.
Comparison of OPLC with TLC and HPLC

Similarities to TLC	Similarities to HPLC	Special advantages of OPLC
Separation on TLC/ HPTLC layer	Eluent delivery by forced flow under external pressure	Low eluent consumption (a few millilitres)
Simultaneous separation of several samples	Eluent migration velocity is constant in time	Short development time (a few minutes)
UV-detection is not restricted by the light absorption of the solvents	Closed sorbent surface (no vapour phase)	No preconditioning, no chamber saturation
Eluent change is quick and simple	Automatic operation mode	Off-line + on-line detection modes
High versatility of detection modes and reagents for visualisation	Gradient elution possibility	Viscous eluents can be successfully used also in reversed phase
Samples of complex mixtures can be applied without sample clean-up	Low H-values can be achieved	Combination of off-line and on-line detection and separation modes
	On-line preparative separation facility	

- Multiple development: the development is repeated after drying the sorbent layer between the consecutive developments. The same or different eluents can be used, with the same or increasing development distances. This method results in improved selectivity because of the longer development distances and of the spot-compression [59].
- Two-dimensional separation: although excellent separations can be achieved by this method [60], its use is restricted by the difficulties in the evaluation.
- Long-distance development [61]: it is a special OPLC-technique. The

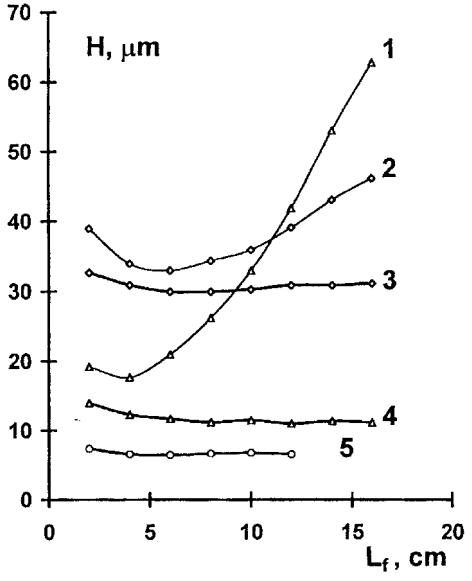


Figure 2.5.M.
Variation of theoretical plate height of PTH-valine along the plate using conventional unsaturated chamber and personal OPLC system. (1) HPTLC silica gel, normal unsaturated chamber; (2) TLC silica gel, normal unsaturated chamber; (3) TLC silica gel, OPLC, 5MPa; (4) HPTLC silica gel, OPLC, 5MPa; (5) Raman silica gel, OPLC, 5MPa. Sample application for silica gel 0.2 μl; for HPTLC silica gel 0.1 μl, and Raman silica gel 0.05μl (from Ref. [57])

sorbent layers are serially connected in a zigzag mode. During the separation process, substances migrate from the upper layer to the other ones through a thin slot of the sorbent layers. Although this technique is used mainly in preparative analysis, it can also be used for analytical separations [62].

An OPLC-purity test was described for *levonorgestrel/norgestrel* drug substances [63]. The selective separation of the 12 impurities was performed on HPTLC silica gel by using two eluent systems. Impurities with higher polarity than the main component were investigated by 4-fold development with increasing developing distances (1000, 2000, 3000, 4000 μl eluent volume) by using a toluene–ethyl acetate–chloroform (50:10:40 v/v) mixture. Impurities with lower polarity than the main component were separated with cyclohexane–ethyl acetate–chloroform (60:20:20 v/v) in a single run; the

Figure 2.5.N.
Personal OPLC BS-50 system for overpressured layer chromatographic analysis (OPLC Ltd., Budapest, Hungary)

eluent volume was 4000 μl and corresponded to about 180 mm developing distance. By this OPLC-method impurities can be selectively estimated, which coelute by the pharmacopoeial TLC-purity tests or having no UV-absorption cannot be detected by HPLC method.

Multiple development using different eluents was used for the fast release purity testing of *Pausogest tablet* having two active substances: *estradiol* and *norethisterone acetate* [64]. The chromatographic conditions were as follows: sorbent layer: HPTLC silica gel (Merck, 5548) sealed for OPLC; development: 1000 and 2000 μl by cyclohexane–ethyl acetate (2:1 v/v) and 3000 μl by toluene–butyl acetate–chloroform (4:2:2 v/v) eluents.

A 10% v/v methanol sulphuric acid was used for visualisation. In Table 2.5.B the R_f-values, detection limits (LOD) as well as categories of the separated substances can be seen.

An HPLC method was changed to a quantitative densitometric OPLC in order to decrease analysis time and cost in the case of *phthaloyl amlodipine*, the last intermediate of a drug substance [16]. The purity test was performed on fine particle silica gel layer by using n-hexane–butyl-acetate–ethyl acetate–chloroform (60:15:15:20 v/v) with overrunning; 6800 μl developing eluent

Table 2.5.B.
Chromatographic data of substances investigated by OPLC purity test of Pausogest tablet

Name	Category[a]	R_f	LOD (μg)	
			UV_{254}	UV_{366}/H_2SO_4
6α-Hydroxy-estradiol	d.p. est.	0.05	0.1	0.02
6β-Hydroxy-estradiol	d.p. est.	0.07	0.1	0.02
6-Hydroxy-norethisterone acetate	d.p. n.a.	0.11	0.05	0.07
6-Oxo-estradiol	d.p. est.	0.18	0.05	0.05
Norethisterone	d.p. n.a.	0.44	0.05	0.02
9,11-Dehydroestradiol	d.p. est.	0.48	0.08	0.05
Estradiol	Main comp.	0.51	0.1	0.07
4-Chloro-estradiol	imp. est.	0.56	0.2	0.05
6-Oxo-norethisterone acetate	d.p. n.a.	0.61	0.08	0.07
Norethisterone acetate	Main comp.	0.68	0.07	0.03
Estrone	d.p. est.	0.81	0.3	0.1

[a] d.p., degradation product; imp., impurity; est., estradiol; n.a., norethisterone acetate

volume was used that corresponded to about a 3200 mm developing distance. The OPLC-method was compared to a similar TLC method by using n-hexane–butyl acetate–ethyl acetate–chloroform (30:15:15:20 v/v) eluent. Both the specificity (Fig. 2.5.O) and precision data (Table 2.5.C) were better in the case of OPLC than in TLC; LOD/LOQ were similar in the two methods, and the calibration lines were linear in a narrower range in OPLC than in the conventional TLC method.

Table 2.5.C.
Precision of purity testing for phtaloyl amlodipine by quantitative TLC and OPLC

Precision	TLC (RSD %)[a]	OPLC (RSD %)[a]
Repeatability (n = 7)	6.1	2.9
Intermediate precision (n = 7)	6.5	3.1

[a] RSD was calculated from the total impurity-data

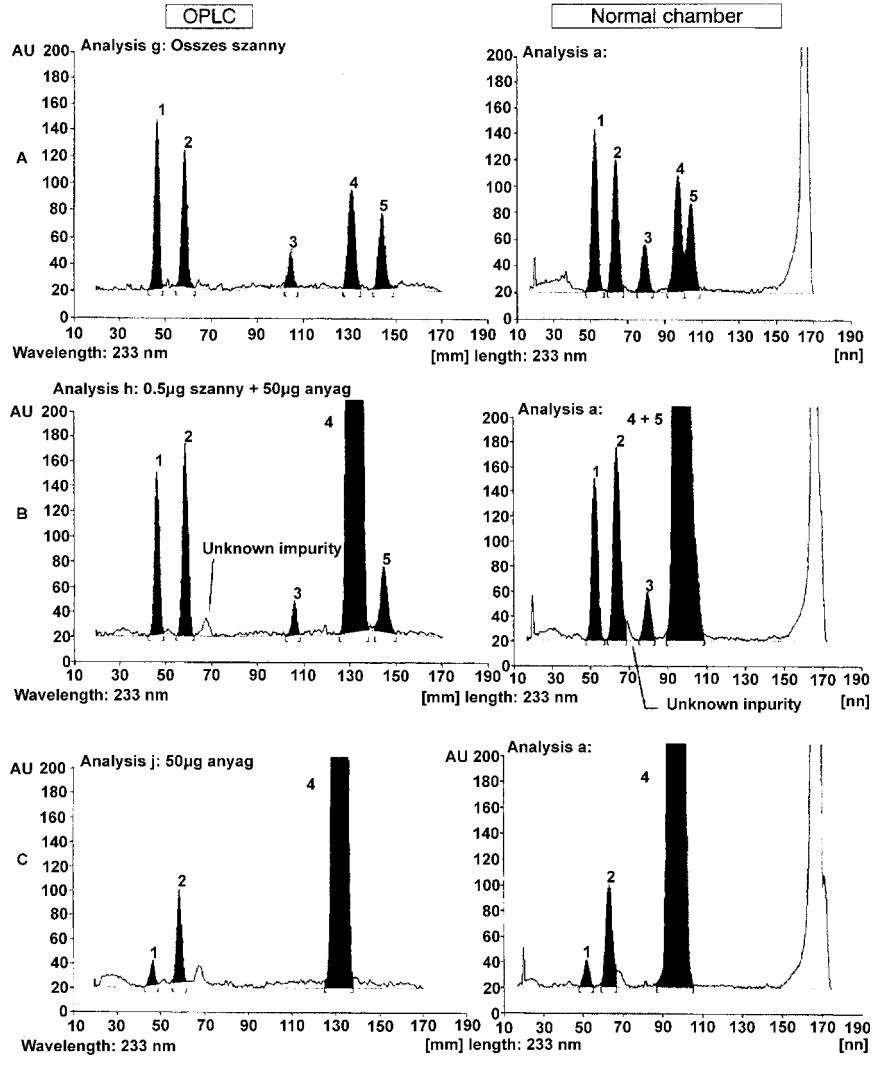


Figure 2.5.O.
Comparison of specificity of an OPLC and TLC purity test for phtaloyl amlodipine (**4**) and related possible impurities (**1,2,3,5**). (A) Model mixture; (B) test substance spiked with the impurities; (C) test substance (from Ref. [16])

In Fig. 2.5.P a TLC and an OPLC chromatogram of 3-keto-desogestrel and its related substances can be seen for determining the detection limits. In TLC an unsaturated chamber, normal particle silica gel sorbent layer and

Figure 2.5.P.
Comparison of band-broadening effect by using OPLC and TLC methods in testing 3-keto-desogestrel and its related substances

cyclohexane–ethyl acetate 7:3 v/v eluent were used at a 15-cm developing distance. In OPLC a fine particle silica gel sorbent and cyclohexane–ethyl acetate–chloroform 7:2:2 v/v eluent were used at 18 cm developing distance. Although the R_f and ΔR_f-values of substances were similar in the two chromatograms, the resolution in the case of OPLC was much better due to the decreased band-broadening.

These examples show that the OPLC-method is superior over classical TLC due to its better selectivity and precision, and can be more favourable even than HPLC in purity testing of substances having no UV/VIS absorbance. Due to its fastness and simplicity, OPLC is particularly suitable for in-process control and cleaning-validation.

2.5.8. Validation of the Planar Chromatographic Purity Tests

In pharmaceutical analysis method validation is based on the recent guidelines of the International Conference on Harmonisation (ICH) [65,66]. However in planning a validation study of an analytical procedure, the analysts have to take into consideration the special characteristics of the used analytical method and also those of the analytes.

The first step is making the plan of validation. In this plan the validation characteristics to be determined and the acceptance limits of those prerequisite for a validated analytical procedure are summarised. Essential to the validation are the available standard substances, impurity-standards and other chemicals in adequate and documented quality as well as the regularly calibrated and controlled instruments. The validation characteristics to be determined in the case of the different analytical tests are summarised in the table of the ICH guideline on validation [65].

Due to its uncertainty, a semiquantitative planar chromatographic purity test can only be considered as a limit test and therefore the validation characteristics to be determined are specificity and detection limit (LOD). The documentation of a semiquantitative planar chromatographic purity test contains the colour photographs of the chromatograms.

In validation of a quantitative densitometric planar chromatographic purity test the specificity, linearity and range of calibration, precision (repeatability and intermediate precision), accuracy, detection and quantitation limits (LOD, LOQ) are determined. The documents of a quantitative planar chromatographic validation are densitograms of the chromatograms.

As the sensitive substances in planar chromatography can often decompose during the chromatographic process, the control of the stability of analyte in the different steps of analysis (sample preparation and application, chromatographic development, evaluation) is highly recommended [17].

In controlling the *specificity* of the system, the analyte and all its expected impurities are chromatographed on the same chromatoplate one by one and also as a common solution. In finished products (tablet, injection, etc.) the effect of the placebo is also tested. The specificity of the system is adequate, if all the expected impurities (product impurities and known degradation products) can be independently determined, in the case of an active substance.

If the analyte is a finished product, the method should be specific only for the degradation products of active ingredient(s), because those are expected to increase during the formulation process [67].

If an analytical procedure is planned to be used in stability studies, its stability-indicating feature has to be proven. This is feasible by testing of stressed samples. The stress-conditions, as discussed in Chapter 5.2, are heat and light in solid and in solution forms; acid, base, light and oxygen (H_2O_2) in solution form. The strength and duration of stresses are chosen to obtain about 2–5% degradation of the analyte, if this is possible. In the case of the stresses, when degradation of the analyte can be detected, the peak purity test of the main component is advisable to control the adequate separation of the degradation product from the main component. The simplest and fastest way to do this in planar chromatography is a two-dimensional separation using the system-eluent in the first direction an eluent of different selectivity in the second direction [17]. By testing stressed samples the specificity of the system for the unknown degradation products can be proven. In Fig. 2.5.Q a stress-test of *terazosine* can be seen.

Although the linear range of calibration is quite narrow in planar chromatography, by the right choosing of sample-application the expected quantity of the impurities to be determined can fall within the linear range. The *linearity*

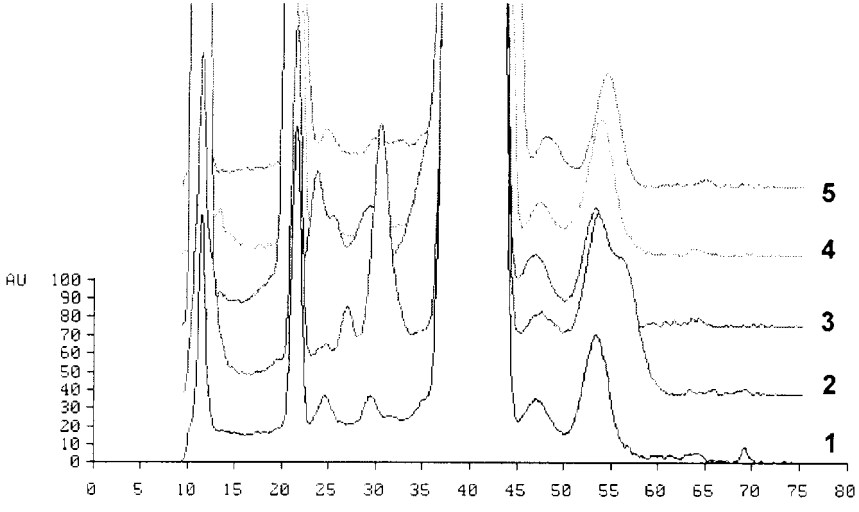


Figure 2.5.Q.
Stress-test of terazosine drug substance in solution. Densitograms of untreated sample (1), stressed by light (2), acid (3), base (4), H_2O_2 (5). Adsorbent: silica gel; mobile phase: ethyl acetate–methanol–ammonia 10:1:1 v/v

of the working *range* of the calibration has to be proven from the LOQ of the impurities to 120% of the individual impurity-limit. The linearity can be simply proven by testing distribution of the residuals.

In testing the *precision* of a quantitative planar chromatographic procedure, the purity test is performed from the same sample from seven different weightings on the same chromatoplate (*repeatability*) or on different chromatoplates in different times by different analysts (*intermediate precision*). The precision of the method is characterised by the RSD-value calculated from the results of different determinations. Typical precision data of a quantitative planar chromatographic method were mentioned in Section 2.5.4.

The *accuracy* of the method is highly influenced by the quality of sample-preparation, therefore this performance-characteristic has a great importance mainly in the case of testing finished products. The accuracy of a purity-test can be characterised by the difference between the added and measured quantities of the impurities available. It is characterised by the recovery rate that is a percentage of the recovered, measured impurity-content. A typical accuracy-limit of a quantitative planar chromatographic purity testing is 80–120%.

The *detection limit* in semiquantitative planar chromatography is determined by testing the intensities of impurity-spots or bands of decreasing quantity applied onto the sorbent layer in equal volumes of solutions of different concentrations. The detection limit, which is the smallest visible quantity should not be higher than the 50% of the individual impurity-limit, but 20% or less is advisable.

The *detection* and *quantitation limits* (LOD, LOQ) in a quantitative planar chromatographic purity test are determined based on the signal-to-noise ratio by the following equations:

$$\mathrm{LOD} = (\bar{x} + 3\mathrm{SD})/s$$

$$\mathrm{LOD} = (\bar{x} + 10\mathrm{SD})/s$$

where $\bar{x}$ is the mean value of the heights of not less than 15 noise peaks, SD is the standard deviation of the x-values, s is the slope of the regression line of three different low quantities applied in triplicate near to the LOQ quantity.

In a quantitative planar chromatographic purity test LOQ should not be higher than 50% of the individual impurity limit and 20% or less is advisable.

Although testing the *robustness* of the method is not compulsory by the ICH-guideline, but should be instructive because useful information may be obtained about the reproducibility and transferability of the method. At the robustness-test the effects of small changes under environmental and experimental conditions on the result are tested. All these conditions are summarised

by Szepesi [68] for thin-layer chromatography. The most critical parameters are usually the quality of the sorbent layers, the exact volume ratio and quality of the eluent-components, the temperature of laboratory, the saturation of the chamber and in the case of OPLC the eluent flow-rate. The effects of different parameters on the TLC and OPLC purity tests were investigated by using experimental design [69,70].

If a new, validated analytical procedure is introduced into the routine analysis, a daily control is advisable to avoid errors caused by unexpected effects (e.g. high temperature in laboratory, poor quality of solvent). The resolution can be controlled by a sample-pair having close R_f-values: the resolution of the chromatogram can be accepted, if this critical sample-pair is well separated.

The sensitivity of the evaluation can be controlled by applying a sample near to LOD ($2 \times$ LOD). The sensitivity of the chromatogram-evaluation can be accepted if this low quantity of the substance can be detected.

The daily control of a quantitative planar chromatographic purity test is performed by determination of a control sample beside the analytical sample on the same chromatoplate. If not less than 20 data points of the control sample are collected, these can be used as a control chart [71]. The result of the sample to be determined can be accepted, if the actual value of the control sample is between the action limits of the mean chart: $\bar{x} \pm 3SD$, where $\bar{x}$ is the mean value of not less than 20 determinations of the control sample, and SD is the standard deviation of these data. By using the mean chart for a control sample information is obtainable on the accuracy of the actual determination. The use of control charts in quantitative planar chromatography has been published [69].

References

1 E. Stahl (Ed.), *Thin-Layer Chromatography, 2nd edn*, Springer, Berlin (1969)
2. L. Treiber (Ed.), *Quantitative Thin-Layer Chromatography and its Industrial Application*, Marcel Dekker, New York (1986)
3. J. Sherma and B. Fried (Eds.), *Handbook of Thin-Layer Chromatography, 2nd edn*, Marcel Dekker, New York (1996)
4. S.D. McCrossen, D. K. Bryant, B. R. Cook and J. J. Richards, *J. Pharm. Biomed Anal*. **17,** 455–471 (1998)
5. *European Pharmacopoeia, 3rd edn*, Council of Europe, Strasbourg (1997)
6. *ICH-guideline, Impurities in New Drug Substances*, CPMP/ICH/142/95
7. W. Naidong, S. Hua, E. Roets and J. Hoogmartens, *J. Planar Chromatogr.* **7,** 297–300 (1997)

8. Z. Végh, A. Wiszkidenszky, A. Narancsik and K. Ferenczi-Fodor, in *Proc. 10th Int. Symp. Instrum. Planar Chromatogr.* (Sz. Nyiredy, B. Szabady, R. E. Kaiser and A. Studer, Eds.), pp 238–246. Visegrád, Hungary, Research Institute for Medicinal Plants, Budakalász (1998)
9. *The United States Pharmacopoeia 24*, p 1610, USP Convention Inc., Rockville (2000)
10. H.R. Altorfer, C.P. Christiansen, W. Dammerz, J. Lang and B. Renger, *Pharmeuropa* **8,** 172–174 (1996)
11. *European Pharmacopoeia 3rd edn*, Supplement 1999, Monograph 2.27 Thin Layer Chromatography, European Pharmacopoeia Commission, Strasbourg (1998)
12. S. Görög, *Ultraviolet-Visible Spectrophotometry in Pharmaceutical Analysis*, p 39, CRC Press, Boca Raton, FLl (1994)
13. S. Görög, M. Babják, G. Balogh, J. Brlik, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *J. Pharm. Biomed. Anal*. **18,** 511–525 (1998)
14. J.N.A. Tettey, G.G. Skellern, J.M. Midgley and M.H. Grant, *J. Pharm. Biomed. Anal*. **17,** 713–718 (1998)
15. D. Agbaba, A. Radovic, S. Vladimirov and D. Zivanov-Stakic, *J. Chromatogr. Sci*. **34**, 460–464 (1996)
16. Z. Szikszay, Z. Végh and K. Ferenczi-Fodor, *J. Planar Chromatogr*. **11,** 428–432 (1998)
17. K. Ferenczi-Fodor, Z. Végh and Zs. Pap-Sziklay, *J. Planar Chromatogr*. **6**, 198–203 (1993)
18. H. Bethke, W. Santi and R.W. Frei, *J. Chromatogr. Sci*. **12**, 392–397 (1974)
19. A. Nagy-Turák, personal communication
20. M. Rischer, H. Schnell, R. Greguletz, E. Wolf-Heuss and J. Engel, *J. Planar Chromatogr*. **10**, 290–297 (1997)
21. G.W. Somsen, W. Morden and I.D. Wilson, *J. Chromatogr. A* **703**, 613–665 (1995)
22. W.R.G. Baeyens and B. Lin Ling, *J. Planar Chromatogr.* **1**, 198–212 (1988)
23. E. Berkhan, *J. Planar Chromatogr.* **1**, 81–85 (1988)
24. V.A. Pollak, in *Handbook of Thin–Layer Chromatography* (J. Sherma and B. Fried, Eds.), p 251. Marcel Decker, New York (1991)
25. H.T. Butler and C.F. Poole, *J. Chromatogr. Sci.* **21**, 385–388 (1983)
26. H.E. Greissler and E. Mutschler, *J. Chromatogr.* **56**, 271–279 (1971)
27. H.E. Hauck, *J. Planar Chromatogr*. **8**, 346–348 (1995)
28. R. Segura and A.M. Gotto, *J. Chromatogr.* **99**, 643–657 (1974)
29. K.G. Krebs, D. Heusser and H. Wimmer, in *Thin–Layer Chromatography, 2nd edn* (E. Stahl, Ed.), pp 854–908. Springer, Berlin (1969)

30. C.F. Poole, S.K. Poole, T.A. Dean and N.M. Chirco, *J. Planar Chromatogr.* **2**, 180–189 (1989)
31. Y. Liang, M.E. Baker, D.A. Gilmore and M.B. Denton, *J. Planar Chromatogr.* **9**, 247–253 (1996)
32. R. J. Maxwell and A.R. Lightfield, *J. Planar Chromatogr.* **12**, 109–113 (1999)
33. D.E. Burton, D.L. Bailey and C.H. Lillie, *J. Planar Chromatogr.* **6**, 223–227 (1993)
34. R.J. van den Nesse, G.J.M. Hoogland, J.J.M. de Moel, C. Gooijer, U.A.Th. Brinkman and N.H. Velthorst, *J. Chromatogr.* **552**, 613–623 (1991)
35. S.A. Stahlmann, *J. Planar Chromatogr.* **12**, 5–12 (1999)
36. P.R. Brown and B.T. Beauchemin Jr., *J. Liq. Chromatogr.* **11**, 1001–1014 (1988)
37. G. Glauninger, K.-A. Kovar and V. Hoffmann, *Fresenius J. Anal. Chem.* **338**, 710–716 (1990)
38. K.-A. Kovar, H.K. Enßlin, O.R. Frey, S. Rienas and S.C. Wolff, *J. Planar Chromatogr.* **4**, 246–250 (1991)
39. O.R. Frey, K.-A. Kovar and V. Hoffmann, *J. Planar Chromatogr*, **6**, 93–99 (1993)
40. S. Stahlmann and K.-A. Kovar, *J. Chromatogr. A* **813**, 145–152 (1998)
41. S. Stahlmann, O.R. Frey and K.-A. Kovar, *Proc. 10th Int. Symp Instrum. Planar Chromatogr.* (Sz. Nyiredy, B. Szabady, R.E. Kaiser and A. Studer, Eds.), pp 158–168. Visegrád, Hungary, Research Institute for Medicinal Plants, Budakalász (1998)
42. N.D. Danielson, J.E. Katon, S.P. Bouffard and Z. Zhu, *Anal. Chem.* **64**, 2183–2186 (1992)
43. G.K. Bauer, A.M. Pfeifer, H.E. Hauck and K.-A. Kovar, *J. Planar Chromatogr.* **11**, 84–89 (1998)
44. K.L. Busch, in *Handbook of Thin–Layer Chromatography* (J. Sherma and B. Fried, Eds.), pp 183–209. Marcel Decker, New York (1991)
45. K.L. Busch, *Trends Anal. Chem.* **11**, 314–324 (1992)
46. Y. Nakagawa and K. Iwatani, *J. Chromatogr.* 5**62**, 99–110 (1991)
47. K.L. Busch, *J. Planar Chromatogr.* **5**, 72–79 (1992)
48. G.C. DiDonato and K.L. Busch, *Anal. Chem.* **58**, 3231–3232 (1986)
49. J.A. Perry, K.W. Haag and L.J. Glunz, *J. Chromatogr. Sci.* **11**, 47–453 (1973)
50. K. Burger, *Fres. Z. Anal. Chem.* **318**, 228–233 (1984)
51. K. Burger, J. Köhler and H. Jork, *J. Planar Chromatogr.* **3**, 228–233 (1990)
52. W. Golkiewicz, in *Handbook of Thin–Layer Chromatography* (J. Sherma and B. Fried, Eds.), pp 135–154. Marcel Decker, New York (1991)

53. K. Ferenczi-Fodor, A. Laukó, A. Wiszkidenszky, Z. Végh and K. Újszászy, *J. Planar Chromatogr.* **12**, 30–37 (1999)
54. E. Tyihák, E. Mincsovics and H. Kalász, *J. Chromatogr.* **174**, 75–81 (1979)
55. E. Mincsovics, E. Tyihák and H. Kalász, *J. Chromatogr.* **191**, 293–300 (1980)
56. E. Mincsovics, K. Ferenczi-Fodor, E. Tyihák, in *Handbook of Thin-Layer Chromatography, 2nd edn* (J. Sherma and B. Fried, Eds.), pp 171–203. Marcel Dekker, New York (1996)
57. E. Mincsovics, M. Garami, L. Kecskés, B. Tapa, Z. Végh, Gy. Kátay and E. Tyihák, *J. Assoc. Off. Anal. Chem.* **82**, 587–598 (1999)
58. E. Tyihák, E. Mincsovics and A.M. Siouffi, *J. Planar Chromatogr.* **5**, 121–125 (1990)
59. E. Mincsovics, M. Garami and E. Tyihák, *9th Danube Symp. Chromatogr.*, Budapest, Hungary (1993), Abstracts, Mo-P-55
60. G. Guiochon, M.F. Gonnord, A. Siouffi and M. Zakaria, *J. Chromatogr.* **250**, 1–20 (1982)
61. L. Botz, Sz. Nyiredy and O. Sticher, *J. Planar Chromatogr.* **3**, 352–354 (1990)
62. E. Tyihák, Gy. Kátay, Zs. Ostorics and E. Mincsovics, *J. Planar Chromatogr.* **11**, 5–11 (1998)
63. K. Ferenczi-Fodor, S. Mahó, S. Pap-Sziklay, I. Török and L. Borka, *Pharmeuropa* **9**, 736–742 (1997)
64. A. Wiszkidenszky, personal communication
65. ICH guideline, Validation of Analytical Procedures: Definition and Terminology, CPMP/ICH/5626/94
66. ICH guideline, Validation of Analytical Procedures: Methodology, CPMP/ICH/281/95
67. ICH guideline, Impurities in New Medicinal Products, CPMP/ICH/282/95
68. G. Szepesi, *J. Planar Chromatogr.* **6**, 259–268 (1993)
69. K. Ferenczi–Fodor, A. Nagy-Turák and Z. Végh, *J. Planar Chromatogr.* **8**, 349–356 (1995)
70. A. Nagy-Turák, Z. Végh and K. Ferenczi-Fodor, *J. Planar Chromatogr.* **8**, 188–193 (1995)
71. D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte and L. Kaufman, *Chemometrics: A Textbook*, p 95. Elsevier, Amsterdam, (1988)

2.6. Gas Chromatography (GC) and Related Techniques

Anna Laukó

2.6.1. Limitations in the Applicability of GC for Impurity Profiling: Derivatisation

The estimation of impurity profiles of drug substances and related materials is always a great challenge for the analyst which can be approached successfully in several ways, from which the analyst can make a deliberate choice. As mentioned in Section 2.1.1, it is very important to use as many separation techniques as possible for the identification of impurities. Gas chromatography is one of the chromatographic methods suitable for the identification and quantitative determination of impurities in drugs and related materials especially if it is coupled with mass spectrometry. The only disadvantage is that due to volatility and thermal stability problems the applicability of this technique in this field is limited.

One reason for the insufficient volatility can be the too high molecular mass which should be less than about 400. Another point is the presence of polar groups, such as carboxyl, thiol, primary and secondary amino, quaternary ammonium, imino or too many hydroxyl groups. These groups, owing to their and tendency to form salts or at least hydrogen bonds, are responsible both for the low volatility of the compounds and for other phenomena that make the application of direct GC either difficult or impossible. In these cases polarity must be decreased by transforming these functional groups to their less polar derivatives by means of suitable derivatising reagents thus increasing their volatility.

Many compounds cannot be analysed by GC because of their thermal instability: they decompose in the injection port of the instrument giving rise to several peaks in the chromatogram due to decomposition products. Caution is necessary since minor peaks originating from the thermal decomposition of the investigated drug material can easily be confused with real impurities. These difficulties can be often overcome by the use of suitable, thermally stable derivatives. Another reason for derivatisation is the increasing demand to use more selective and sensitive methods.

Derivatisation originates from the classical period of analytical chemistry. Almost all reactions that are used for derivatisation are taken from organic syntheses adapted to the micro-scale. In the course of their application in drug impurity profiling attention should be paid to their yield and the possibility of

the occurrence of side-reactions. Minor side reactions of the derivatisation reaction are of no importance when GC is used for the determination of drugs and their metabolites in biological samples and pharmaceutical formulations. However, when this technique is used in impurity profiling side reactions of the possibly simple, single-step derivatisation reaction should be avoided or at least minimised, because the side-products of the derivatisation reaction can also be confused with the impurities.

Several books are available, where the transformation of the above listed polar functional groups to their acyl, silyl, alkyl and other derivatives, suitable for the gas chromatographic analysis is described [1–4]. The most frequently applied derivatisation methods in GC are trimethylsilylation of hydroxyl and carboxyl groups forming silyl ether and ester derivatives, respectively, perfluoroacylation of amines, esterification of carboxyl groups, to enhance the volatility and improve the thermal stability of drugs. For silylation various silylating agents are available for different hydroxy groups, from the reactive primary to the sterically hindered tertiary hydroxy groups. For acylation acetic, trifluoroacetic and heptafluorobutyric anhydrides [10] are widely used. Esterification is usually accomplished using diazomethane or methanol, catalysed by boron trifluoride or various alcohols, mainly methanol, ethanol or isopropanol, catalysed by hydrochloric acid.

In addition to producing stable and volatile compounds for the gas chromatographic analysis, another advantageous feature of chemical derivatisation prior to the GC investigation is that the comparison of the retention characteristics of the derivatised and underivatised compound, supported by the mass spectra of the derivatives obtainable by GC/MS can furnish information about the functional groups in the molecule.

In the examples taken from the author's laboratory the transformations prior to gas chromatography with the aim of impurity profiling are mainly based on so called *retro-derivatisation* reactions [5–7]. In contrast to the usual derivatisation reactions where the molecular mass of the analyte increases as a result of the reaction; in this case the molecular mass decreases resulting in smaller, but stable molecules. For example, reaction of some thermally labile compounds with hydrochloric acid which catalyses dehydration, deacylation and other splitting as well as rearrangement reactions may lead to stable derivatives. In the case of *ethynodiol diacetate* (EDDA) or ethynodiol-17-acetate the acid-catalysed splitting of acetic acid and water, respectively, leads exclusively to the stable 3,5-diene derivative (for the structures see Fig. 2.2.B in Section 2.2.1.1), while the decomposition of the untreated sample in the flash heater of the gas chromatograph results in several decomposition products, among them the 3,5- and 2,4-dienes. On the basis of this principle the gas chromatographic determination of the saturated 4,5-dihydro derivative in EDDA and the intermediates in its synthesis could be accom-

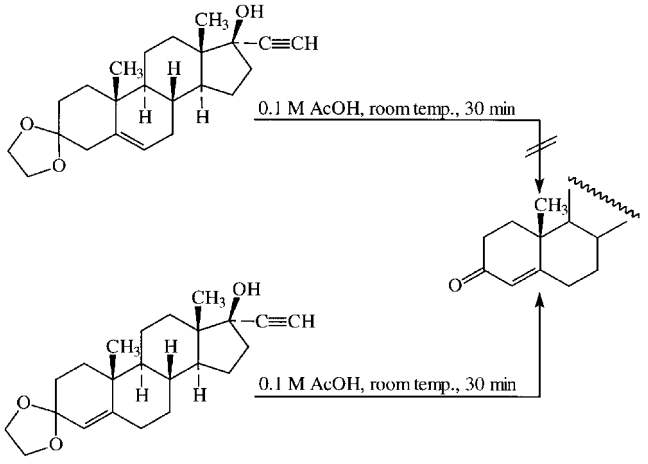


Figure 2.6.A.
Selective transformation of a 4-ene-3-ethylenedioxy steroid to the 4-ene-3-oxo derivative for the GC determination (from Ref. [7])

plished, namely the saturated derivatives are not changed during the treatment with hydrochloric acid [6,8].

Another example is the determination of the isomeric Δ^4 impurity in the steroid intermediate 3-ethylenedioxy-17α-ethinyl-androst-5-ene-17-ol, which is only poorly separated from the main component under ordinary GC conditions. As shown in Fig. 2.6.A, the selective hydrolysis of the ethylenedioxy moiety of the Δ^4 impurity under mild acidic conditions transforms it to the 4-ene-3-oxo derivative which can easily be separated from the unchanged main component [6,7]. This example shows that the aim of derivatisation in GC is not only to increase the volatility and thermal stability but in some cases it increases the selectivity of the measurement, too.

Other examples of this kind include the transformation of the thermally labile steroid 3-methoxy-2,5(10)-dienes by acid catalysed hydrolysis to their stable 4-ene-3-oxo derivatives [9] (see Figs. 9.4.C and 9.4.D in Section 9.4.2.2) and the base-catalysed transformation of the enol ester 17-acetoxy-16-ene group in steroids to their 17-oxo derivative enabling the GC determination of Δ^3 impurity in the Δ^2 derivative [6].

2.6.2. Selection of Columns for the Separation of Impurities by GC

Chromatography is a branch of separation science, and the usual goal is

the separation of complex mixtures into their individual components. During the chromatographic equilibrium, a solute partitions between the mobile and the stationary phases in accordance with its distribution constant. In gas chromatography the mobile phase is the carrier gas, depending on the detector (nitrogen, hydrogen or helium for FID, hydrogen or helium for thermal conductivity detector, argon with some percent of methane for electron capture, and helium for MS). The selectivity of a gas chromatographic column depends on the polarity of the stationary phase which is determined by its chemical structure.

The stationary phase in the case of packed columns are copolymer beads, or a support material – usually acid- and base-washed silanised diatomaceous earth – coated with a polymer (polar, apolar or semipolar), and the glass column for pharmaceutical analysis is filled with this.

The open tubular column was invented by Marcel Golay in 1958, and its introduction has initiated a new era of development of high efficiency separation methods. It is successfully applied also in the pharmaceutical practice of GC. Its importance has increased when the use of fused silica columns spread at the beginning of 1980s. As the outer wall of the quartz capillary is coated with polyimide to prevent moisture attacking the surface, this procedure makes the tube mechanically strong and flexible; it is easy to handle and install it into the oven.

Today fused silica columns are employed in probably over 80% of all GC analyses. In the case of capillary columns the support material is the inner wall of the tube itself, with chemically uniform surface and is coated by a suitable polymer. The thickness, uniformity and chemical nature of the stationary phase have a great effect on the separations obtained. The most common stationary phases are silicone polymers. The polarity of the stationary phases is determined by the type and amount of substituents on the polysiloxane backbone. The most generally used substituents are methyl, phenyl, cyanopropyl and trifluoropropyl groups. Another widely used stationary phase is polyethylene glycol. Its major disadvantage is the high susceptibility to structural damage by oxygen at elevated temperatures.

For increasing the heat stability of polysiloxane phases, the polymer can be chemically bonded to the silanol groups of fused silica, or immobilised by covalent bonding between the polymer chains, i.e. by increasing the molecular weight and viscosity, thus decreasing solute diffusity. These columns can be used even at 350°C.

Strongly polar stationary phases can be produced only as wall coated open tubular columns (WCOT), e.g. CP Sil-88 (cyanopropyl-substituted polysiloxane). In the case of wall coated columns there is only weak interaction between the wall and the stationary phase; these columns are more sensitive to high temperature, to various solvents and their contaminants. Near to the upper

temperature limit bleeding can be considerable. Since avoiding column bleeding is of great importance especially in GC/MS, special stationary phases have been developed, such as DB-5MS, DB-35MS, HP-5MS, where the polar, e.g. phenyl group is in the siloxane chain. These retain the polarity of the ordinary stationary phases but with increased stability and much less bleeding.

The selectivity in gas chromatography is determined by the structure of the stationary phase and solute factors, such as polarisability, solubility, magnitude of dipoles and hydrogen bonding behaviour. In many cases, more than one factor will be significant, thus there will be multiple selectivity influences. Most compound characteristics are difficult to predict and for this reason the separation obtainable for different compounds on a certain column is not easily predictable either. Nevertheless there are some possibilities to make predictions. All stationary phases have polarisability-related interactions. Increased retention time occurs for solutes that are more polarisable. Solubility of the solute in the stationary phase will result in longer retention time. The more soluble a solute in the stationary phase, the greater is its retention. Polyethylene glycols and cyanopropyl substituted polysiloxanes have strong dipole and hydrogen bonding characteristics, trifluoropropyl substituted polysiloxanes have moderate dipole interaction. Generally speaking, however, the separation power of the commercially available modern capillaries is so high that it is in the overwhelming majority of cases possible to find a suitable column for the separation of even structurally closely related (e.g. isomeric) impurities from their main component. For this reason GC is a real alternative to other chromatographic techniques for the impurity profiling of sufficiently volatile and thermally stable drugs.

The length, the internal diameter and the film thickness are also important parameters. In pharmaceutical GC analysis usually 20–30 m. long columns, with 0.1–0.32 mm internal diameter are used. Columns with smaller diameter are more effective, but larger diameter columns can have greater sample capacity, and film thickness. Increasing film thickness will cause a substantial increase in the retention of a solute, and column bleed is also considerable. Columns with film thickness of about 0.1–0.4 μm, are suitable for the analysis of samples with low volatility (e.g. steroids).

As a consequence of the increasing importance of chiral issues in pharmaceutical research, production and analysis (see Chapter 6) several very effective chiral stationary phases have been introduced also in the gas chromatographic practice which can be successfully used for the separation of enantiomers. Some of the most useful GC stationary phases, introduced by the group of Schurig [11] are based on the alkylated α- and β-cyclodextrins. Chirasil-Val introduced by Frank and Bayer [12] and related stationary phases containing chiral amino acid and peptide derivatives bound to a methylpolysiloxane backbone have been successfully used mainly for the separation of

amino acid enantiomers after achiral derivatisation. Reinhardt *et al.* [13] used permethylated β-cyclodextrin dissolved in OV-1701 for separation of the enantiomers of pharmaceutically active benzhydrol-derivatives. These methods are suitable without derivatisation for the enantiomeric purity check of chiral drugs administered as pure enantiomers.

2.6.3. Sample Introduction Systems

''If the column is described as the heart of chromatography, then sample introduction may, with some justification, be referred to as the Achilles heel'' [14]. This statement reflects the reality of gas chromatography: inlets and auxiliary sample introduction devices are often the quality-limiting components of GC. The accuracy and precision of sample introduction have been greatly improved by advances in inlet and sampling technology. Sample introduction has two goals. One is to introduce the sample into the column in such a way that it occupies the shortest section of column. The shorter the sample band is at the beginning of the process, the sharper and narrower the peaks will be on the chromatogram resulting in higher sensitivity and better resolution. The second goal is that no sample degradation or adsorptive losses occur in the injector. There are three injection techniques used for capillary columns in impurity profiling: split, splitless [15] and cool on-column.

Split injection is very simple and the most generally used of the capillary injection techniques. It is used for highly concentrated samples with typical concentrations of 0.1–10 μg/μl per component. The sample is vaporised upon injection and rapidly mixed with the carrier gas; only a controllable, small amount of the injected sample enters the column. The less volatile compounds do not have sufficient time to fully vaporise before they are discarded via the split vent, therefore this injection technique can cause solute discrimination and also decomposition. It is used for general analysis.

Splitless injection is most frequently used for trace analyses or when the per component amounts are not more than approximately 0.1 μg/μl. The injected sample is flash-vaporised in the liner, and sample vapours are carried into the column by the carrier gas where they are recondensed at temperatures below the boiling point of the solvent. The initial temperature of the column has to be below the boiling point of the sample solvent by at least 20°C in order to promote the recondensation effect. When the vaporised solvent leaves the injector and enters the cooler column, the solvent condenses at the front of the column. A solvent film forms, and it will trap and refocus the sample. This is the solvent effect, discovered by K. Grob. After most of the sample has been transferred into the column, vapours remaining in the liner are cleared by opening the split vent which remains open for the duration of the run. Inlet

discrimination is less severe than for split injection. The most important benefit of splitless injection is that the majority of the injected sample is introduced into the column. This results in much higher sensitivity than that achieved using split injection. Splitless injection is also routinely used in drug analysis.

Cool on-column inlet has helped to solve many of the problems associated with vaporising inlets. It gives high accuracy and reproducibility, is sample protecting, has the least solute discrimination among all the inlets, and works by depositing the sample directly into the column. Unlike the other vaporising or ''hot'' sample introduction techniques, the sample is not exposed to high temperatures during injection or transfer to the column. Cool on-column inlets are used for the analysis of samples with a wide boiling-point range or those that are thermally sensitive, and for trace analysis.

Klick [16] investigated the different injection techniques in the gas chromatographic determination of thermolabile trace impurities in *almokalant*.

From auxiliary sampling devices headspace autosamplers are used mainly to determine residual solvents in pharmaceuticals. This technique in which a portion of the gas being in equilibrium with the sample in a thermostated, sealed vial is analysed as described in detail in Section 3.2.3.2.

2.6.4. Detectors [17]

2.6.4.1. General Considerations

The detector is the device, that senses the presence of the components leaving the column, either by measuring the change in some bulk property of the carrier gas, such as thermal conductivity, or by sensing some property unique to the solute itself, such as carbon content. The former is called a bulk property detector, the latter a solute property detector. In general, solute property detectors have much higher sensitivity than bulk detectors. In pharmaceutical GC analysis the most commonly used detector is FID (flame ionisation detector). ECD (electron capture detector) finds less widespread application in pharmaceutical analysis. The mass selective (MSD) detector can be used not only for quantitative, but qualitative (identification, structure elucidation) purposes.

The basic detector parameters to be taken into consideration to permit a prudent choice of detector when investigating and determining impurities in drugs are as follows:

- detector linearity;
- linear dynamic range;
- sensitivity, or lowest detectable amount.

For the main parameters of the various GC detectors see Table 3.2.B in Section 3.2.6.

2.6.4.2. Flame Ionisation Detector (FID)

Since its introduction in 1958 [18], the flame ionisation detector has become one of the most popular measuring devices used in GC. Its use is based on the measurement of variations in the ionisation current in a hydrogen–oxygen flame due to the presence of eluted substances. Chemionisation reaction occurs in the flame, assuming collision of neutral atomic excited states with molecules in their ground state. The magnitude of the ionisation signal is proportional to the number of ionisable carbon atoms, decreased by the electron capture process involving electronegative groups formed as combustion products. FID response is the highest for hydrocarbons, being proportional to the number of carbon atoms, while substances that contain oxygen, sulphur or a halogen yield smaller responses depending on the heteroatom/carbon ratio and on the electron affinity of the combustion products. FID is a universal detector for carbonaceous substances, with the exception of a limited number of small molecular compounds such as carbon disulfide, formic acid, formaldehyde, carbon tetrachloride, etc. This is a very important feature from the point of view of impurity profiling. The main drug component and its structurally closely related impurities are expected to give very similar signals: over- or underestimation of impurities which takes place frequently in HPLC, CE, TLC, etc. due to differences in their UV activities does not occur here. The background current from the flame (ions and electrons formed by the combustion of pure hydrogen and carrier gas) is extremely small, and when an organic compound eluted from the column reaches the detector, the ionisation current increases significantly. Due to the good sensitivity (LOD being in the low nanogram or sometimes in the picogram range), the high dynamic range and the above mentioned uniformity of the detector signals the GC/FID technique is eminently suitable for the detection and quantitative measurement of impurities down to the range of 0.01% impurity level.

2.6.4.3. Electron Capture Detector (ECD)

The electron capture detector is also an ionisation detector. Ionisation of the carrier gas is initiated by the β-radiation of a suitable radioactive source, in most cases ^{63}Ni thereby producing a strong current between two electrodes. If the molecules of the material eluted from the column contain electrophilic groups, e.g. conjugated dienes, nitro group, and halogens, they will capture electrons. The negative ions thus forming recombine with the positive ions of the carrier gas, resulting in a decrease in the intensity of the current. (The

direction of the signal is reversed compared with that of FID.) For this reason ECD is selective to compounds containing the above listed atoms and groups.

ECD is extremely sensitive for these compounds; the sensitivity can be higher by 3–5 orders of magnitude than that obtainable with FID. Compounds which do not contain electron capturing groups but do contain hydroxy, primary or secondary amino groups can be transformed to their perfluoroacyl derivatives greatly improving their detectability. It must be taken into consideration, that not only the compounds to be investigated can be detected, but ECD is also sensitive for other impurities, e.g. trace amounts of oxygen and water must be removed from the carrier gas, because their presence decrease the concentration of free electrons and thus also the probability of the formation of a negative ion of the eluted substance.

As a consequence of the above described features of ECD, it is a useful tool in the ultratrace analysis of certain compounds. Due to its non-general detection capabilities it is of limited importance in impurity profiling but is widely used in the GC analysis of biological samples, halogen containing residual solvents, environmental samples, etc.

2.6.4.4. Mass Selective Detector (MSD)

The mass spectrometer has been a very useful tool for structure elucidation for many years. The idea of coupling it to the gas chromatograph as a detector originated from Holmes and Morell in 1957 [19]. There were two difficulties in coupling GC with a mass spectrometer as a routine analytical device. The first problem was the relatively high flow of the carrier gas, since at that time only packed columns were employed. The other was the data handling system. These problems were solved by the development of open tubular columns and the computer technique. The capability of the on-line GC/MS system has increased exponentially. Nowadays the hyphenated GC/MS system [20] has become one of the most important techniques to investigate impurity profiles and many other areas in routine pharmaceutical analysis. The mass range of a benchtop instrument is up to 600 Da, because molecules with larger molecular mass cannot be chromatographed due to their low volatility. An advantage of the GC/MS method is that both ionisation methods can be easily used. Reliable molecular mass value is obtainable by chemical ionisation (CI) and in addition information on fragmentation necessary to the solution of more complicated structure elucidation problems can also be obtained by electron impact (EI) ionisation technique.

When a molecule is ionised in a vacuum, a characteristic group of ions of different masses occur. When these ions are separated, the plot of their relative abundance versus mass constitutes a mass spectrum. This spectrum can be used for the identification the molecule.

EI is a hard ionisation method, the ionisation chamber is at a very low pressure of about 10^{-8} Torr. Electrons are generated by a heated filament which then pass across the ion source to an anode trap, having a potential of 70 eV. The sample vapour is introduced in the centre of the ion source and the molecules drift, by diffusion, into the path of the electron beam. Molecular ion and ionised fragments are produced by the collision with the electrons, and in most cases the received spectrum helps to elucidate the structure of the molecule. Sometimes only fragments can be observed, and no molecular ion is produced. The level of information obtainable for the structure elucidation can be further increased by using a tandem mass spectrometer coupled to the gas chromatograph. GC/MS/MS benchtop instruments are available on the market, and they are very useful both for qualitative and quantitative analysis.

To complement the structural information obtained from EI, a soft ionisation technique can be employed to get more information about the molecular mass. This soft ionisation technique is the chemical ionisation. Under positive CI conditions ion-molecule interactions occur. A reagent gas (mostly methane or iso-butane) is introduced to the ion source and a relatively high pressure is maintained (typically about 1 Torr). The reagent gas is ionised by the electron beam to produce reactant ions which can interact with the molecules to be investigated. Considerable direct ionisation of the sample molecule by the electron beam does not occur due to the relatively high concentration of reagent gas molecules, but the probability of the interaction of the molecules of the sample with the reactant ions is high because of the high pressure maintained in the source. Ions appearing in the mass spectrum of a compound are due to ion–molecule reactions, which are low in energy compared to EI. The spectra usually contain quasi-molecular ions [MH^+]. Simple fragmentation patterns are often also observed.

After the separation of the ions by the quadrupole or ion trap type mass analyser of the benchtop GC/MS system, the chromatogram can be reconstructed as components eluting from the column by a plot of the total ion current stored and summed by the computer for each scan. The total ion chromatogram shows all the peaks eluted. Mass spectral information can be obtained from the stored spectra, but mass chromatograms can also be plotted in which the ion abundances of a few selected values are extracted from each stored mass spectrum and plotted versus the retention time. The advantage of this technique is that by a judicious choice of the mass values which are plotted, selectivity is achieved for the analysis of the selected compounds thus greatly contributing to their identification.

Problems with manual matching of mass spectra are reduced by computerising the comparison process. Several spectral libraries are available to help the analyst in the structure elucidation work. These libraries can be used successfully in the environmental or clinical analysis, but they are of limited

importance in the impurity profiling of drugs especially if the aim is the estimation of the impurity profile of a new drug. The reason for this is that the libraries usually do not contain the mass spectra of the impurities of a new drug.

The mass selective detector is not only a device to identify compounds, but it is a very selective and sensitive detector which can be employed for quantitative analysis as well, to quantify trace levels of organic compounds. To achieve low detection limits with high selectivity, the GC column must have high efficiency and the characteristic ions of the analyte must be monitored using selected ion monitoring (SIM). It can be used even for the detection of very low ion abundances. In this case, the detector is only tuned to a few selected ions, complete mass spectra are not taken during the run, scanning is valid only for some specific ions and a very good signal to noise ratio can be obtained for the investigated compounds.

As in other branches of gas chromatography, internal standard methods of quantitation are common in GC/MS/SIM analysis, too. The most effective internal standards are isotopically labelled analogues of the substances being analysed. These compounds have the same chemical properties as the corresponding analytes. The isotopically labelled analogue can be used to confirm the identification by its spectrum. However, it must be taken into consideration, that fused silica capillary columns can sometimes separate a compound from its labelled analogue.

2.6.5. Temperature Programming

Temperature programming is a very important tool in gas chromatography. It is used to improve separation power and speed up analysis, especially for complex samples containing many components or having a wide range of boiling points, using splitless or on-column injection mode for trace analysis.

Temperature programming is performed by changing the temperature of the column at a definite rate as the analysis proceeds. Depending on the components occurring in the sample, in some cases temperature can change at different slopes during the run (multi-ramp temperature programming), separating as many components as possible, or using splitless injection, where the sample is reconcentrated by the cold trapping effect. There is one disadvantage of temperature programming: the stationary phase of the column may tend to bleed as temperature is increased. The most modern fused silica capillary columns have chemically bonded, cross-linked or immobilised stationary phases; these are more stable than the stationary phases on the packed columns, but a slight bleeding can be observed even here, especially in the case of thick films. Using FID a baseline drift can be detected, but with

mass selective detectors column bleed will cause many extra ions to appear in the spectra of minor components. These phenomena can be eliminated using the selected ion monitoring technique.

2.6.6. Qualitative Analysis by GC/MS: Identification and Structure Elucidation of Impurities

The role of gas chromatography in impurity profiling is briefly mentioned in Section 2.1 and the importance, possibilities and limitations of GC/MS and its place in the hierarchy of hyphenated techniques for the identification and structure elucidation of the separated impurities are discussed in Section 2.1.4.

Some examples from the author's laboratory are presented here to characterise the role of GC/MS in the identification and structure elucidation of impurities in bulk drugs within the complex use of other chromatographic, spectroscopic and hyphenated techniques.

Propanidid: the impurity profiling of propanidid is an example of using a complex chromatographic system to identify all the related substances in the bulk drug [21].

Figure 2.6.B shows the GC/MS scan of a bulk propanidid sample. It can be seen that three impurities present at about the 0.1% level can be identified; (for the structures see Fig. 2.6.C). Of these two are by-products or originate from the starting material of the synthesis. Impurity **4** merits special mentioning. The difference of 14 between the mass spectra of this impurity and that of the main component indicates that this is an oxidative degradation product. In this case the HPLC/diode-array UV spectrum has also contributed to find the place of oxidation. In addition to the fragmentation pattern the UV spectrum (shift of the maximum from 280 to 307 nm) shows that the active methylene group of propanidid between the phenolic ring and the propyloxycarbonyl group is oxidised leading to a highly conjugated derivative; (for the reaction see Fig. 5.1.B in Section 5.1). The limitations of the GC/MS approach to impurity profiling can be characterised by the fact that the TLC and HPLC analysis of the same sample showed more impurities. These were isolated by semi-preparative HPLC and analysed by direct MS and HPLC/MS. The structures

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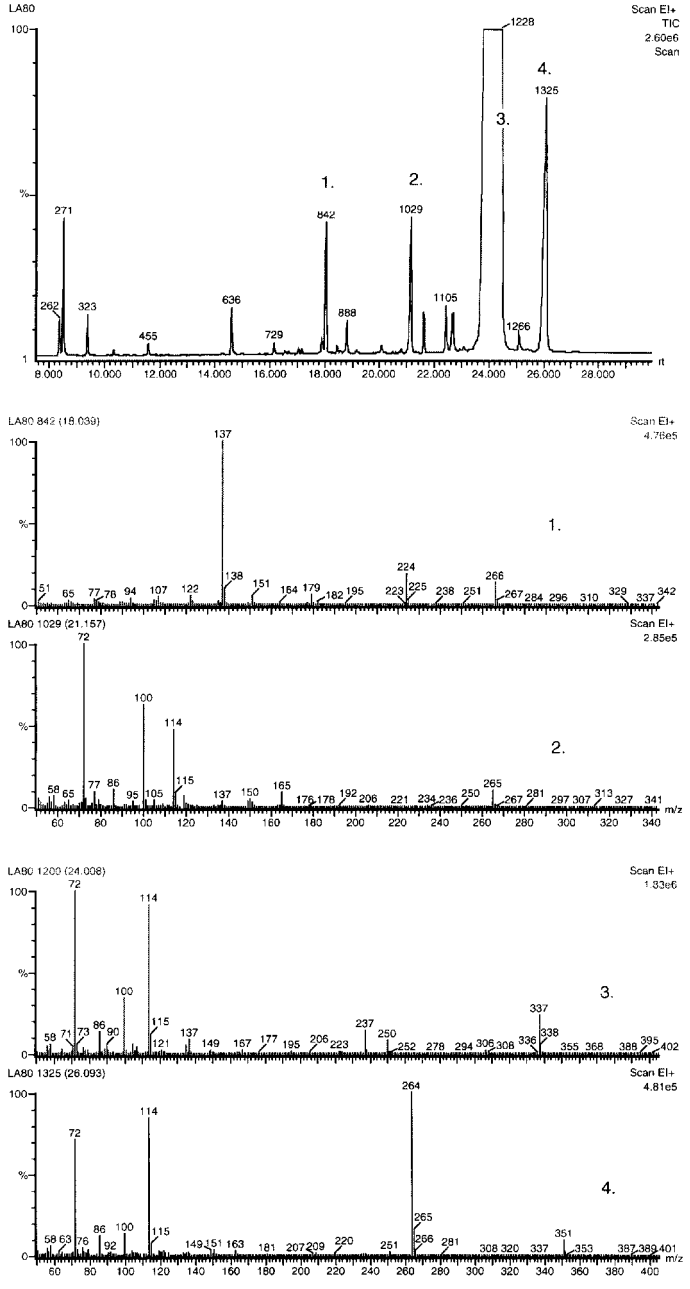
Figure 2.6.B.
Total ion chromatogram of a bulk propanidid sample and the EI spectra of propanidid (**3**) and three main impurities (**1**,**2**,**4**). Column: DB-5MS (J&W) 30 m × 0.25 mm × 0.25 μm. Temperature: injection port: 260°C (splitless), ion source: 200°C, liner: 240°C, oven: 40°C 1 min., 10°C/min up to 280°C. Ionisation: EI, 70 eV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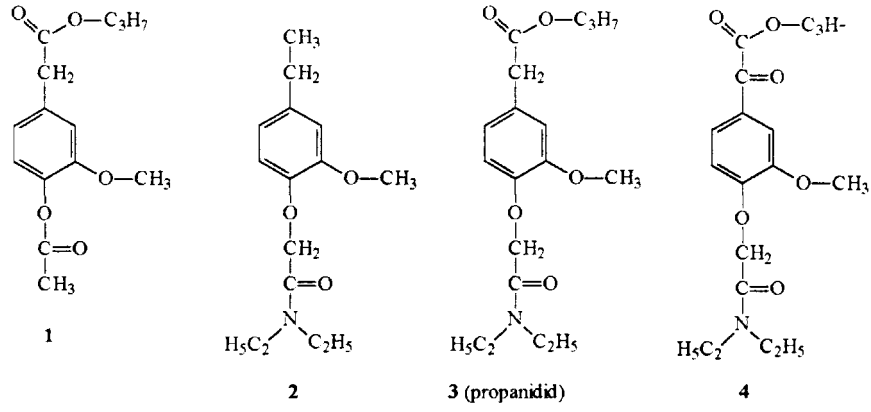


Figure 2.6.C.
Structures of propanidid and three impurities

of the four dimeric impurities with some experimental details are shown in Fig. 2.3.F in Section 2.3.3. Of course the molecular mass of these impurities was too high to appear in the gas chromatogram.

Flumecinol (Zixoryn®, Chemical Works of Gedeon Richter Ltd., Budapest) is prepared from propiophenone and 3-trifluoromethyl-bromobenzene by the Grignard reaction. It is a thermally stable, volatile oily material which is an ideal candidate for impurity profiling by GC/MS [22,23]. The chromatogram of a bulk drug sample is shown in Fig. 2.6.D together with the mass spectra of the main impurities (see Fig. 2.6.E) which were present in the 0.07–0.3% range. These included one of the starting materials (**1**; M = 134)), a typical by-product of the Grignard reaction (**2**; M = 290), a thermal degradation product (**3**; M = 282), a by-product (**4**; M = 234), the isomeric 4-trifluoromethyl derivative of flumecinol (**6**), originating from an isomeric impurity in the starting material (Fig. 2.6.E). As is characteristic of α-ethyl-benzhydrols in general, neither flumecinol (**5**) nor the isomeric impurity (**6**) posses molecule ions but their spectra contain the intense and characteristic peak at m/z = 251; $[M–C_2H_5]^+$.

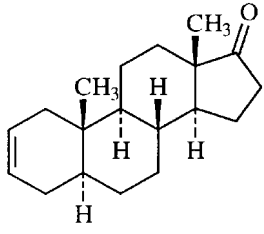
Impurity **4** merits special mentioning. The reason for the formation of this impurity is the presence of 2-hydroxy-tetrahydrofurane impurity in tetrahydrofurane which is used as the solvent of the Grignard reaction. The open form of the impurity (γ-hydroxybutyraldehyde) reacts with the Grignard reagent to form this by-product.

The mass spectrometric identification of the impurities was supported by retention matching with authentic impurity standards.

The applied gas chromatographic system is suitable for the quantitative

determination of the impurities using 3-trifluoromethyl-benzophenone as the internal standard.

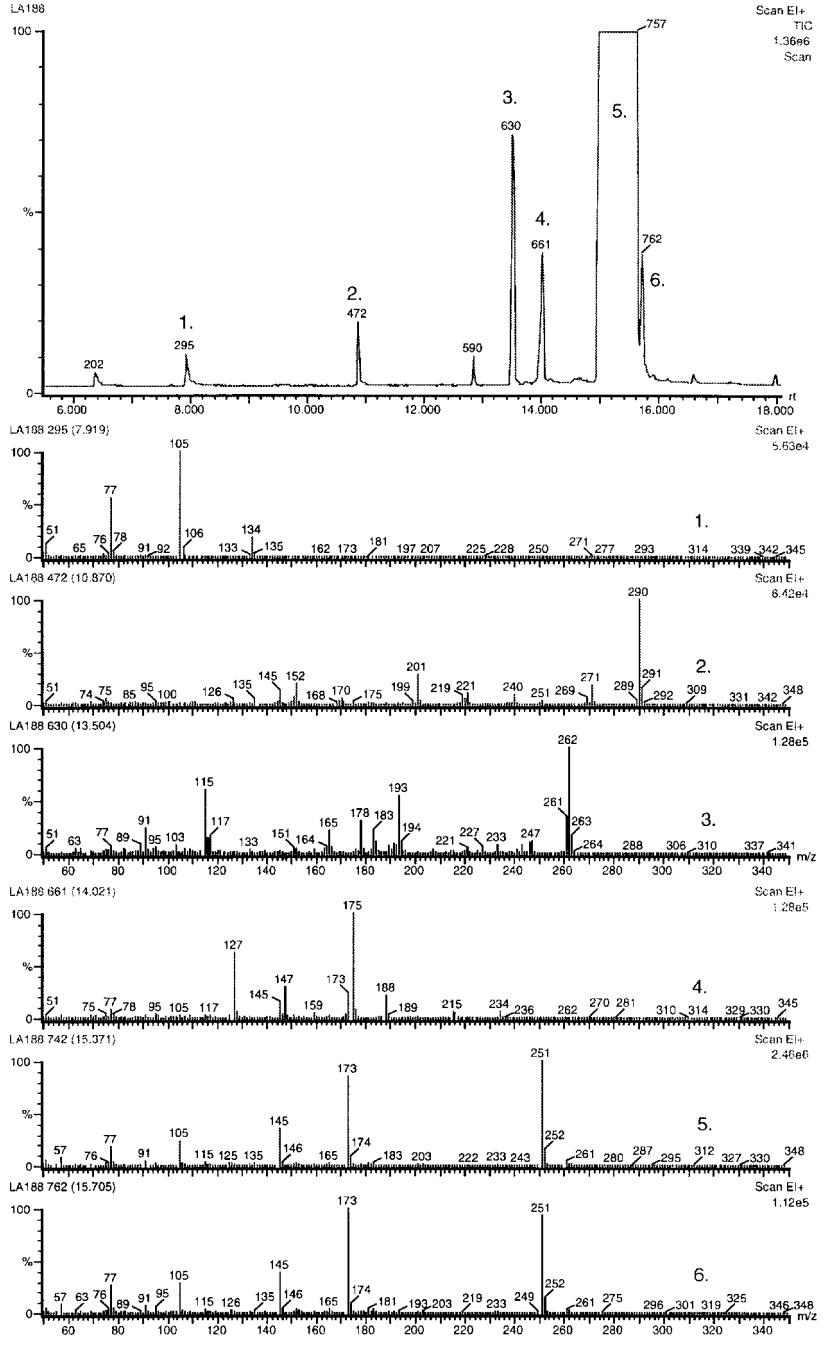
In the course of the synthesis of *pipecuronium bromide* (Arduan^{R}; Chemical Works of Gedeon Richter Ltd., Budapest; for the structure see Section 1.2.1), one of the key steps is the solvolytic splitting of methanesulphonic acid from 3β-methanesulphonyloxy-5α-androstane-17-one to form 5α-androst-2-ene-17-one. The estimation of its impurity profile is an important task as the purity of the final product is greatly determined by the number and quantities of the impurities in this intermediate. It is a simple steroid, having only two functional groups, but its impurity profile is extremely complex.



5α-androst-2-ene-17-one

Capillary GC without derivatisation was selected for the separation of the impurities because of the great resolving power of capillary columns and because of the lack of polar groups on the steroid skeleton [24,25]. As a consequence of their high number and similar structures no single capillary GC system was found to separate all the impurities. Two different capillary columns, the moderately polar DB-5 and the highly polar CP Sil-88 were used for the separation of the impurities. On the phase DB-5 only the main impurity (Δ^3-isomer) can be separated from the main component. The CP Sil-88 column is coated with a highly polar stationary phase (see Section 2.6.2), and it is suitable to separate the saturated (2,3-dihydro) impurity and the other isomers from the main component, with the exception of the Δ^3-isomer. After the identification followed by their synthesis the impurities were controlled by retention matching with authentic standards.

As shown in Fig. 2.6.F, isomeric analogues (1-ene, 3-ene, 4-ene, 5-ene) and the saturated 5α-androstane-17-one were identified. 5α-Androst-2-ene-17-one and all its isomers have the same molecular mass at m/z 272, but their fragmentations are quite different depending on the position of the double bond. For example easy loss of the methyl group at C-10 can be observed, when it is in allylic position (m/z 257, in the case of Δ^4- and Δ^5-isomers). Characteristic loss of mass unit 54 takes place (M-54 = 218) at the Δ^2-isomer. The ion at m/z 108 is significant, corresponding to the cleavage of 9-10 and 6-7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75
78
91
92
106
134
133
135
162
173
181
197
207
225
228
250
271
277
293
314
339
342
345
1.
LA188 472 (10.870)
Scan EI+
6.42e4
290
201
291
2.
51
74
75
85
95
100
126
135
145
152
168
170
175
199
219
221
240
251
269
271
289
292
309
331
342
348
LA188 630 (13.504)
Scan EI+
1.28e5
262
115
193
261
91
117
178
183
263
3.
51
63
77
89
95
103
133
151
164
165
194
221
227
233
247
264
288
306
310
337
341
m/z
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
LA188 661 (14.021)
Scan EI+
1.28e5
175
127
147
173
188
4.
51
75
77
78
95
105
117
145
159
189
215
234
236
262
270
281
310
314
329
330
345
LA188 742 (15.371)
Scan EI+
2.46e6
251
173
145
105
77
57
76
91
115
125
135
146
165
174
183
203
222
233
243
252
261
280
287
295
312
327
330
348
5.
LA188 762 (15.705)
Scan EI+
1.12e5
173
251
145
105
77
57
63
76
89
91
95
115
126
135
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165
174
181
193
203
219
233
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252
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301
319
325
346
348
6.

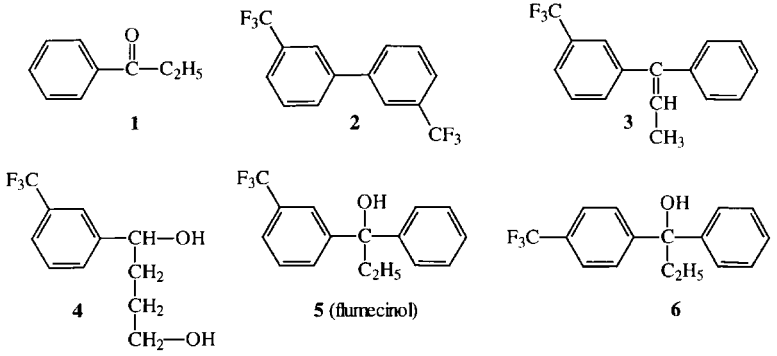

Figure 2.6.E.
Structures of flumecinol (**5**) and impurities **1–4** and **6**

bonds observable in the case of Δ^1- and Δ^4-isomers. This fragment is not present in the mass spectrum of the Δ^5-compound.

The quantitative determination of the by-products was of great importance in the course of the optimisation of the reaction conditions of the splitting of the methanesulphonic acid and is part also of the in-process-control protocol. Due to their similar structures the percentages of impurities can be calculated directly using the area normalisation method. Impurities **1**, **2**, **5** and **6** are calculated directly from the chromatogram obtained using the CP Sil-88 column, while impurity **4** is determined directly using the DB-5 column; (the mass spectrum of **3** in Fig. 2.6.F has been taken from this GC/MS scan).

The most important application of GC and GC/MS is the detection, identification and quantitation of the UV inactive impurities. There are many examples in the field of steroids, e.g. the over-reduction of the 4-ene-3-oxo steroids in the course of the synthesis of ethynodiol diacetate. During the reduction of norethisterone acetate by complex metal hydrides, besides the main product (4-ene-3β-hydroxy derivative) two by-products, the 4,5-dihydro-3β-hydroxy and the 4-ene-3α-hydroxy derivatives are also formed. The saturated impurity has no double bond, it could not be detected by diode-array

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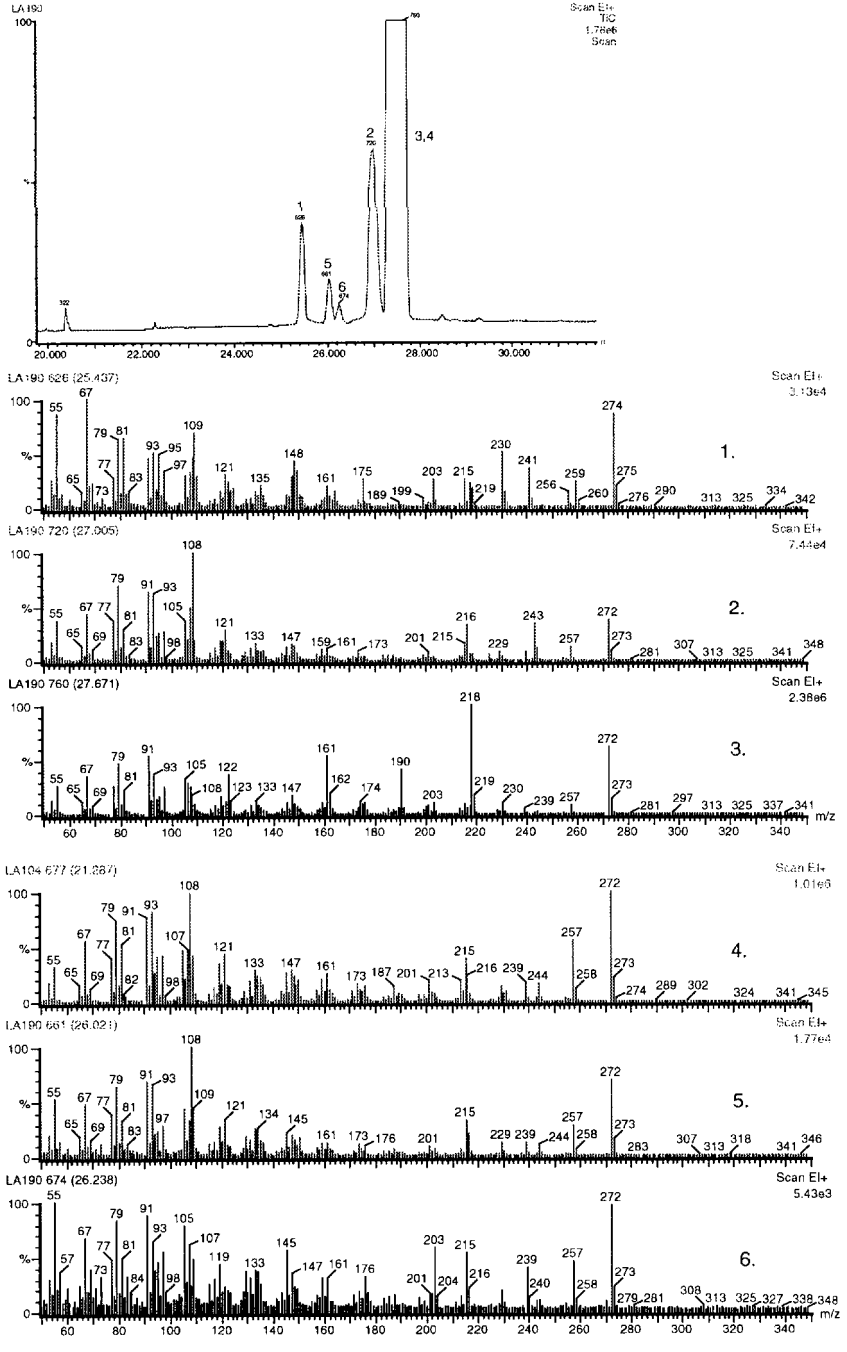
Figure 2.6.D.
Total ion chromatogram of a bulk flumecinol sample and the EI spectra of flumecinol (**5**) and impurities **1–4** and **6**. Column: DB-5MS (J&W) 30 m × 0.25 mm × 0.25 μm. Temperature: injection port: 260°C (splitless), ion source: 200°C, liner: 240°C, oven: 40°C for 1 min 10 °C/min up to 280 °C. Ionisation: EI, 70 eV


LA190
Scan EI+
TIC
1.78e6
Scan
1
2
3,4
5
6
20.000
22.000
24.000
26.000
28.000
30.000
LA190 626 (25.437)
Scan EI+
3.13e4
1.
LA190 720 (27.005)
Scan EI+
7.44e4
2.
LA190 760 (27.671)
Scan EI+
2.38e6
3.
m/z
LA104 677 (21.287)
Scan EI+
1.01e6
4.
LA190 661 (26.021)
Scan EI+
1.77e4
5.
LA190 674 (26.238)
Scan EI+
5.43e3
6.
m/z

HPLC, only indirect evidence could be found for its presence. The qualitative and quantitative determination of the impurities were carried out by GC and GC/MS after hydrochloric acid catalysed transformation of the thermally labile unsaturated derivatives to 3,5-dienes. The 4,5-dihydro-3β-hydroxy derivative is not affected by the acidic treatment. The structures of the impurities were identified by GC/MS [8].

Some more examples of using the GC/MS technique to identify impurities, taken from the literature and from the author's own practice are as follows. GC/MS was successfully used – usually combined with other methods – in the identification of 3-deoxonorgestrel in *norgestrel* [26], epimeric 17α-hydroxy-17β-ethinyl impurity in *norethisterone* [27,28] (for the structures see Fig. 1.2.A in Section 1.2.1), impurities in bulk *cholesterol* [29], an isomeric impurity in *danazol* [30] (for the structures see Fig. 1.2.E in Section 1.2.1).

Isosorbide 5-mononitrate and related impurities, such as isosorbide, isosorbide diacetate and isosorbide 2-acetate-5-nitrate were investigated by Marinkovic *et al.* [31]. A quantitative method was developed to determine the main component and impurities in raw material as well as in dosage form by GC/MS. Due to the hydroxyl groups in the molecules derivatisation was carried out by N,O-bis-trimethylsilyl-trifluoroacetamide.

For the identification of unknown impurities in pharmaceutical products the on-line coupled reversed-phase HPLC-capillary GC/MS is proposed by Ogorka *et al*. [32], by inserting a liquid–liquid extraction step between the RP-HPLC and GC parts of the system. Even poorly resolved HPLC peaks can be selectively transferred to the GC/quadrupole MS system. The combined separation method has a great advantage. In many cases the impurities of the drugs are volatile enough, suitable for GC determination, but not the main component. This technique is a very useful tool for separation of the volatile related substances from the drug.

Goosens *et al*. [33] described a similar system for the identification of impurities in *eltoprazine* and *mebeverine*. After HPLC separation, using 5 mM methanesulphonic acid in acetonitrile-water (84:16) as eluent, methanesulphonic acid was removed on-line from the HPLC eluent via an anion-exchange membrane prior to the introduction into the GC/magnetic sector MS.

Figure 2.6.F.
Total ion chromatogram of a sample of 5α-androst-2-ene-17-one with the mass spectra of the main component and the impurities. Column: CP-Sil 88 (Chrompak) 30 m × 0.25 mm × 0.25 μm. Temperature: injection port: 260°C (splitless), ion source: 200°C, liner: 220°C, oven: 40°C for 1 min, 10°C/min up to 220°C, 20 min isoterm. Ionisation: EI, 70 eV. Key for peaks and EI spectra: (1) 2,3-dihydro; (2) Δ^1; (3) Δ^2 (main component); (4) Δ^3; (5) Δ^4; (6) Δ^5

Related substances of *clonixin* were studied by Esteve and co-workers [34] using capillary GC/MS for the detection and quantitation of impurities (3-chloro-2-methylaniline, 2-chloro nicotinic acid, 2-hydroxy nicotinic acid, N-(3-chloro-2-methylphenyl)-2-chloro nicotinamide and N-(3-chloro-2-methylphenyl)-2-hydroxynicotinamide) after trimethylsilylation using N,O-bis-trimethylsilyl acetamide as the silylating reagent.

Klaffenbach and Kronenfeld investigated the quality of *isopropyl myristate* by GC/MS [35]. Impurities identified by GC/MS were the isopropyl esters of dodecanoic acid and hexadecanoic acid, respectively, and ethyl and methyl ester of tetradecanoic acid. A relatively high level of impurities was found even in the reference standard material specified by German Pharmacopoeia DAB 10 for calibration of response factors for internal standard quantitation.

2-Amino-1,3-propanediol is an important intermediate in the synthesis of *iopamidol*. It is often contaminated by small amounts of related amino alcohols. After derivatisation with trifluoroacetic anhydride followed by GC/MS analysis Musu *et al.* [36] detected 2-aminopropanol, 2-ethanolamine, 1-methoxy-2-aminopropanol, 2-aminomethyl-1,3-propanediol, 1-amino-2,3-propanediol and 2,3-diaminopropanol.

The role of GC/MS in the analysis of cyclic octapeptide drug *atosiban* [38] is described in Section 9.1.2.

Gradient HPLC and GC were applied by Wirth *et al*. [39] as screening methods for the identification and determination of impurities in *fluoxetine hydrochloride* samples and formulated products from different suppliers. In addition of the wide variation of the quality of the samples from different origin, the identification of a new related impurity N-benzyl fluoxetin is worth mentioning.

Further studies include the identification and determination of related impurities in *doxepin* [40] as well as the identification of pyridinedicarboxilic acid-type impurities (as the trimethylsilyl esters) in *niacin* [41].

2.6.7. Quantitative Determination of Impurities

The advantageous features of gas chromatography, discussed in the preceding sections, i.e. extremely high separation power, good sensitivity and wide dynamic range of the flame ionisation detector (FID), generally used in the pharmaceutical applications, and the similarity of the FID signals of structurally related derivatives make gas chromatography eminently suitable for the quantitative determination of impurities in sufficiently volatile and thermally stable drugs and their formulations. Although the frequency of the use of gas chromatography in the purity tests in pharmacopoeias is not comparable with that of TLC and HPLC, it can be stated that GC plays an important

role and is an indispensable tool in checking the purity of many pharmaceutical materials. Of course its main application field is the determination of residual solvents which is dealt with separately in Section 3.2. In addition to this, gas chromatography is often prescribed in the pharmacopoeias for the quantitative determination of small molecules other than solvents and often also for related impurities of large molecules. Due to the high probability of the FID response factors of the impurities related to the main component being close to 1.0, the percentage values for known and even unknown impurities obtained by simple area normalisation are usually much closer to the true values than in the case of HPLC with UV detection or TLC densitometry. It has to be noted, however, that the use of internal standards is more general in the gas chromatographic determination of impurities than in any other branch of chromatography.

In accordance with the general tendencies of and following the development in the technology of gas chromatography the first pharmacopoeial methods were based on packed column separations but in the last decade this has been to a great extent superseded by capillary columns. The detector is usually FID but in some instances the use of mass selective detector is prescribed.

In USP 24 [47a] general gas chromatographic methods are presented for the quantitative determination of *benzyl alcohol*, *chlorobutanol*, *phenol* and *parabens*.

In addition to residual solvents the European Pharmacopoeia contains GC-based limit tests for foreign oils and sterols, N,N-dimethylaniline, ethylene oxide.

The test for foreign oils, among others in *olive oil* [43a], and *soya-bean oil* [43b] is based on the transesterification of the esters (glycerides) of fatty acids (palmitic, stearic, oleic acid and isomers and linoleic acid and isomers) by treatment with anhydrous methanol and potassium hydroxide; the forming methyl esters are chromatographed. For the calibration standard materials (methyl laurate, myristate, palmitate, stearate, arachidate and oleate) are used. The method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydro-epoxy-, cyclopropanic or cyclopropenic group, or those that contain a large proportion of fatty acids with chain length less than eight carbon atoms or to oils with an acid value greater than two. In the method described in Section 2.4.22 in Ph. Eur. [44a] requirements are prescribed for the performance of the gas chromatographic system, e.g. the number of theoretical plates of the capillary column, calculated for the peak corresponding to methyl stearate, should be at least 30 000, the resolution between the peaks of methyl oleate and methyl stearate should be at least 1.8, etc. The requirements regarding the limits of the individual foreign oils, expressed as various fatty acids are specified in the monographs of the various fatty oils. For example in *olive oil* where the main fatty acid components are oleic (56–85%), linoleic (3.5–20%) and palmitic acid (7.5–20%), the limits among others for erucic,

gadoleic, arachidic and saturated fatty acids of chain length less than C_{16} are 0.1, 0.2, 0.5, and 0.1%, respectively.

The determination of *sterols in fatty oils* is described in the Section 2.4.23 of Ph. Eur. [44b]. The sterol fraction of the fatty oil is isolated from the unsaponifiable matter by thin-layer chromatography. Betulin as internal standard is added to the oil before the separation process. The isolated sterols are examined by GC in trimethylsilyl ether form. Fused silica columns (20–30 m × 0.25 mm × 0.25 μm), coated with polymethylphenylsiloxane or polymethylphenylvinylsiloxane are recommended for separation. The peak of betulin must be clearly separated from the peaks of the sterols to be determined. Retention times of sterols relative to β-sitosterol are reported. The composition of the sterol fraction is specified in the individual monographs. The following sterols can be present, e.g. in the sterol fraction of olive oil [43a]: β-sitosterol more than 93%, Δ^7-stigmasterol and cholesterol less than 0.5%, campesterol less than 4% and stigmasterol less than campesterol. In soya-bean oil [43b] not more than 0.3% of brassicasterol is allowed.

Due to its high toxicity very strict limits (20 ppm) are set in Ph. Eur. for the *N,N-dimethylaniline* content in various antibiotics such as *anhydrous ampicillin* [43c], *amoxicillin sodium* [43d,45g] *cloxacillin sodium* [43e,45a], *flucloxacillin sodium* [43f,45b], *tricarcillin sodium* [43g,45c]. Section 2.4.26 of Ph. Eur. [44c] permits the use of both packed columns (2 m × 2 mm i.d. packed with silanised diatomaceous earth coated with 3% of polymethylphenylsiloxane; internal standard: naphthalene) and capillary columns: fused silica capillary (25 m × 0.32 mm × 0.52 μm) coated with crosslinked polymethylphenylsiloxane; internal standard: N,N-diethylaniline.

If manufactured by a process that may leave residues of *2-ethylhexanoic acid* in the product, it is measured by a GC test, using valeric acid as internal standard and a glass column (1.8 m × 4 mm i.d.) packed with a support impregnated with a stationary phase suitable for the separation of free fatty acids thus enabling the impurity to be measured without derivatisation at 145°C. The amount of 2-ethylhexanoic acid in some cases cannot be more than 0.8%, e.g. in *cloxacillin sodium* [43e,45a], *flucloxacillin sodium* [43f,45b], *ampicillin sodium* [43h,45d], but in *tricarcillin sodium* [43g,45c] 0.5% and in amoxicillin sodium [43d,45g] 2.0% is acceptable.

Peptides often contain *acetic acid*: the determination is carried out by GC at 130°C using anhydrous formic acid as the solvent, propionic acid as the internal standard and a glass column (1 m × 4 mm i.d. packed with graphitised carbon for chromatography impregnated with 0.3% of macrogol 20 000 and 0.1% phosphoric acid). The limit for acetic acid in *somatostatin* [43i,45i] and *tetracosactide* [43j,45i] is set to 15.0%. Another method is prescribed for the determination of acetic acid content for *protirelin*, a small tripeptide [43k,45e], where the solvent is water, the internal standard is dioxan, and the acetic acid

content is limited to 2%. Packed column GC is carried out at 150°C using a glass column (2 m × 2 mm i.d.) packed with ethylvinylbenzene-divinylbenzene copolymer.

In pharmacopoeias many special tests can also be found where gas chromatography is used for the determination of the impurities. The most specific and sensitive test in USP 24 and British Pharmacopoeia is the determination of traces of the highly toxic impurity 2,3,7,8-tetrachlorodibenzo-p-dioxin in *hexachlorophene* by GC/MS using a selected ion monitoring method. The limit is as low as 2 ppb! After extraction steps the sample is chromatographed using a capillary column with chemically bonded methyl silicone (CP Sil 5CB, 25 m × 0.2 mm). The inlet system is splitless. The measurement is carried out at an initial temperature of 40°C increasing to 275°C at a constant rate of 40°C/min with the injector temperature 300°C. ^{13}C hexachlorophene is used as the internal standard. The resolution of the mass spectrometer with an electron impact ionisation source cannot be less than 5000. The matching ions are consecutively 320, 322, 324, and 332, 334, 336. The amount of the impurity is calculated from the fragment ratios [45f,47b].

The purity test of *biperiden hydrochloride* [43l,45j] is carried out by GC using a fused silica capillary column (50 m × 0.25 mm i.d.), coated with (vinylphenylmethyl)siloxane maintaining the temperature of the column at 200°C for 5 min, then raising at a rate of 2°C/min to 270°C. The test is not valid unless the resolution between biperiden and its endo-form (main impurity) is at least 2.5, and the principal peak in the chromatogram obtained with 0.1% solution of biperiden has a signal-to-noise ratio of at least 6. For peaks with a retention time relative to biperiden of 0.95–1.05, the area of any other peak cannot be greater than 0.5% of the area of the principal peak and the sum of the area of any such peaks is not greater than 1.0% of the area of the principal peak.

Not identified total impurity limit (1%) is measured also in *bronopol* (2-bromo-2-nitropropane-1,3-diol) [45k]. The examination is carried out in trimethylsilyl ether form at 115°C, using biphenyl as the internal standard and a glass column (1.5 m × 4 mm), packed with acid-washed, silanised diatomaceous support (80/100 mesh) coated with 3% of phenyl methyl silicone fluid.

Three specified related impurities (5-chloro-8-hydroxyquinoline, 5,7-dichloro-8-hydroxyquinoline and 5,7-di-iodo-8-hydroxyquinoline) are measured in *Clioquinol* (5-chloro-7-iodoquinolin-8-ol), [45l] by GC at 190°C after derivatisation with N,O-bis(trimethylsilyl)acetamide in pyridine, using dibutyl phthalate as the internal standard and a column (1.5 m × 4 mm) packed with silanised diatomaceous support (100/120 mesh) coated with methyl silicone gum.

Very low limits of 4-chlorophenol (25–30 ppm) are allowed in *clofibrate* (ethyl 2-(4-chlorophenoxy)-2-methylpropionate) [43n,45m,47c]. For the determination both capillary (USP) and packed column (Ph. Eur., BP) GC are used.

For the determination of the named impurity, 3-mercapto-2-methylpropionic acid and other impurities in *captopril* ({1-[(2S)–3–mercapto–2–methylpropionyl]-L-proline}) [46e] GC is used after trimethylsilylation using capillary column and temperature programming.

The purity test of *desogestrel* (13β-ethyl-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-17β-ol) [45n] is carried out by TLC, but its Δ^3-isomer can be separated and measured only by GC using a CP Sil 5 CB capillary column (10 m × 0.32 mm × 0.25 μm). The limit is set to 0.2%.

Further pharmacopoeial gas chromatographic purity tests include the determination of dechloro-griseofulvin, dehydrogriseofulvin and other related impurities in *griseofulvin* by packed column GC [45o], the determination of 2- and 4-toluenesulphonamide in *saccharin* [43m,45p] by capillary column GC (limit 10 ppm), the determination of traces of aniline (1 ppm), cyclohexylamine (10 ppm) and dicyclohexylamine (1 ppm) in *sodium cyclamate* [43o,45q] by capillary GC and the determination of impurities in *tranylcypromine* [45r] after trifluoroacetylation and using a packed column for the separation.

The problem of the determination of *ethylene oxide* (limit 1 ppm) and *2-chloroethanol* (limit 10 ppm) in excipients by head-space GC is briefly mentioned in Section 1.4.

Of the numerous non-pharmacopoeial methods only two are mentioned here. Volatile, water-soluble amines, such as aziridin and its precursor 2-chloroethylamine at trace level were studied in *flesinoxan* and *befiperide* by De Haan *et al*. [37]. After removal of the bulk of the drug substance by extraction or precipitation, Schotten–Baumann derivatisation (using 4-fluorobenzoyl- or pentafluorobenzoyl chloride) was performed using a water–organic solvent system, to extract the derivatised amines. The organic phase was analysed by capillary GC, applied on-column injection and nitrogen-phosphorus selective detection. Detection limits are 0.2 ppm for aziridine and 0.5 ppm for 2-chloroethylamine.

Matrix effects during standard addition analysis were studied by Sun and Roston [42], through the determination of trace amounts of butyric acid in an experimental drug substance. Butyric acid is a reagent in the synthesis, and because of its noxious smell, its trace amount would effect the drug quality. GC analyses were performed on a fused silica capillary column DB-EEAP of 15 m × 0.53 mm × 1.0 μm without derivatisation, using valeric acid as internal standard. The standard addition method was used to eliminate matrix effects.

References

1. J. Drozd, *Chemical Derivatization in Gas Chromatography* (Journal of Chromatography Library, Vol. 19) Elsevier, Amsterdam (1981)

2. K. Blau and J. Halket J (Eds.), *Handbook of Derivatives for Chromatography,* Wiley, Chichester (1993)
3. A.E. Pierce, *Silylation of Organic Compounds,* Pierce Chemical Co. Rockford, IL (1968)
4. D.R. Knapp, *Handbook of Analytical Derivatization Reactions*, Wiley, New York (1979)
5. S. Görög, M. Rényei and A. Laukó, *J. Pharm. Biomed. Anal.* **1**, 39–46 (1983)
6. S. Görög and A. Laukó, *Magy. Kem. Foly*. **92**, 337–341 (1986)
7. S. Görög, A. Laukó, M. Rényei and B. Hegedüs, *J. Pharm. Biomed. Anal.* **1**, 497–506 (1983)
8. S. Görög, A. Laukó, B. Herényi, G. Czira, É. Csizér and Z. Tuba, *Acta Pharm. Hung.* **100**, 377–382 (1979)
9. S. Görög, M. Babják, G. Balogh, J. Brlik, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *J. Pharm. Biomed. Anal.* **18**, 511–525 (1998)
10. R.J. Siezen and T.H. Mague, *J. Chromatogr.* **130**, 151–160 (1977)
11. V. Schurig and H.P. Novotny, *J. Chromatogr.* **441**, 155–159 (1988)
12. H. Frank, G.H. Nicholson and E. Bayer, *J. Chromatogr. Sci.* **15**, 174–176 (1977)
13. R. Reinhardt, W. Engewald and S. Görög*, J. High Resolut. Chromatogr.* **18**, 259–262 (1995)
14. V. Pretorius and W. Bertsch, J. *High Resolut. Chromatogr.* **6**, 64–70 (1983)
15. K. Grob, *Classical Split and Splitless Injection in Capillary GC*, Huethig, Heidelberg (1986)
16. S. Klick, *J. Pharm. Biomed. Anal.* **13**, 563–566 (1995)
17. J. Sevcik, *Detectors in Gas Chromatography*, Elsevier, Amsterdam (1976)
18. J. Harley, W. Nel, and V. Pretorius, *Nature* **181**, 177–178 (1958)
19. J.C. Holmes and F.A. Morell, *Appl. Spec.* **11**, 86–91 (1957)
20. F.W. Karasek and R.E. Clement, *Basic Gas Chromatography–Mass Spectrometry,* Elsevier, Amsterdam (1988)
21. S. Görög, M. Babják, G. Balogh, J. Brlik, A. Csehi, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *Talanta* **44**, 1517–1526 (1997)
22. S. Görög, A. Laukó and B. Herényi, *J. Pharm. Biomed. Anal.* **6**, 697–705 (1988)
23. S. Görög, G. Balogh, A. Csehi, É. Csizér, M. Gazdag, Zs. Halmos, B. Hegedüs, B. Herényi, P. Horváth and A. Laukó, *J. Pharm. Biomed. Anal.* **11**, 1219–1226 (1993)
24. S. Görög, A. Laukó, B. Herényi, A. Georgakis, É. Csizér, G. Balogh, Gy. Gálik, S. Mahó and Z. Tuba, *Chromatographia* **26**, 316–320 (1988)

25. A. Laukó, P. Horváth, F. Trischler and S. Görög, *Acta Pharm. Hung.* **62**, 82–87 (1992)
26. P. Horváth, G. Balogh, J. Brlik, A. Csehi, F. Dravecz, Zs. Halmos, A. Laukó, M. Rényei, K. Varga and S. Görög, *J. Pharm. Biomed. Anal.* **15**, 1343–1349 (1997)
27. S. Görög, A. Laukó and B. Herényi, *J. Pharm. Biomed. Anal.* **6**, 697–705 (1988)
28. A. Laukó, A. Csehi, G. Balogh, É. Csizér, B. Herényi and S. Görög, *Acta Pharm. Hung.* **61**, 98–105 (1991)
29. A. Laukó, É. Csizér and S. Görög, *Analyst* **118**, 609–611 (1993)
30. G. Balogh, É. Csizér, Gy. G. Ferenczy, Zs. Halmos, B. Herényi, P. Horváth, A. Laukó and S. Görög, *Pharm. Res.* **12**, 295–298 (1995)
31. V.D. Marinkovic, S.S. Milojkovic, J.M. Nedejkovic, J.J. Comor, D. Agbaba and D. Zivanov-Stakic, *J. Pharm. Biomed. Anal.* **16**, 425–429 (1997)
32. J. Ogorka, G. Schwinger, G. Bruat and V. Seidel, *J. Chromatogr.* **626**, 87–96 (1992)
33. E.C. Goosens, K.H. Stegman, D. de Jong, J. Gerhardus and U.A. Brinkman, *Analyst* **121**, 61–66 (1996)
34. M.H. Esteve, R.D. Rofi and G.E. Aldoma*, J. High Resolut. Chromatogr.* **13**, 445–446 (1990)
35. P. Klaffenbach and D. Kronenfeld, *J. Chromatogr.* **767**, 330–334 (1997).
36. C. Musu, L. Fumaigalli, F. Perego, F. Fedeli and F. Uggeri, *J. Chromatogr.* **449**, 432–439 (1988)
37. P.E. de Haan, D. de Jong, J.H.M. Van den Berg and C.G. Kruse, *J. High Resolut. Chromatog.* **12**, 604–607 (1989)
38. D.J. Burinsky, R. Dunphy, A.R. Oyler, J. Shaw and M.L. Cotter, *J. Pharm. Sci*. **81**, 597–600 (1992)
39. D.D. Wirth*,* B.A. Olsen, D.K. Hallenbeck, M.E. Lake, S.M. Gregg and F.M. Perry, *Chromatographia* **46**, 511–523 (1997)
40. T.D. Cyr, R.C. Lawrence and E.G. Lovering*, J. AOAC Int.* **75**, 804–809 (1992)
41. R.D. Kirchhoefer, *J. AOAC Int.* **77**, 117–120 (1994)
42. J.J. Sun and D.A. Roston, *J. Chromatogr. A* **673**, 211–218 (1994)
43. *European Pharmacopoeia, 3rd edn*, Council of Europe, Strasbourg (1997). Page numbers: a, 1257; b,1534; c, 387; d, 383; e, 670; f, 859; g, 1643; h, 390; i, 1516; j, 1620; k, 1405; l, 482; m, 1453; n, 650; o, 1485
44. *European Pharmacopoeia, 3rd edn, Supplement 1999*, Council of Europe, Strasbourg (1999). Page numbers: a, 11; b, 12; c, 20
45. *British Pharmacopoeia 1998*, The Stationery Office, London (1998). Page numbers: a, 372; b, 590; c, 1291; d, 98; e, 1113; f, 670; g, 90; h, 1204; i,

1271; j, 183; k, 197; l, 356; m, 361; n, 438; o, 655; p, 1147; q, 1182; r, 1317
46. *The United States Pharmacopoeia 23*, p 263. USP Convention Inc., Rockville (1995)
47. *The United States Pharmacopoeia 24*, USP Convention Inc., Rockville (2000). Page numbers: a, 1864; b, 810; c, 442

2.7. High Performance Liquid Chromatography (HPLC) and Related Techniques
2.7.1. Separation, Detection and Determination of Impurities by HPLC

Mária Gazdag

2.7.1.1. Introduction

High-performance liquid chromatography (HPLC) was introduced to pharmaceutical analysis not long after its discovery in the late 1960s. By now it has developed into a generally applicable analytical method providing rapid and versatile separation possibilities that meet the increasing requirements for purity testing of bulk pharmaceuticals and pharmaceutical products. It is also suitable for the determination of drugs in biological and environmental samples. It provides a number of highly selective variants to resolve almost every type of separation problem: on the basis of this, HPLC and related techniques can be regarded as the most important analytical method in contemporary pharmaceutical analysis. The great importance of HPLC can be characterised by the very high number of books devoted to this subject. The most important ones published in the last decade [1–7] with emphasis of pharmaceutical and biomedical applications [8–12] are listed.

In the beginning, major attempts were first made to develop highly efficient systems leading to a dramatic improvement in column technology using microparticulate packings and to an optimised design of column hardware resulting in extremely low extra-column peak dispersion through the column. In the later stage of HPLC development, widespread activities have been initiated in the design of phase systems and synthesis of highly selective stationary phases due to the fact that resolution in HPLC is much more dominated by the selectivity of the mobile phase system than by the column efficacy. The fundamentally important steps in creating highly selective phase systems include rigorous control of surface chemistry and adjustment of the final stationary phase properties by appropriate mobile phase selection, leading to specific solute–surface interactions and suppressing undesired interactions. Similarly, significant advances in the instrumentation to further improve separation selectivity (detection, raw data manipulation, etc.) and efficiency (injection techniques, extra-column peak broadening, etc.) have been made. The high level of HPLC instrumentation regarding speed, accuracy, precision and reliability meets the special requirements of purity tests in pharmaceutical analysis.

The main aim of this section is to present a survey on the practical applicability of HPLC in pharmaceutical analysis for purity investigations. Only those theoretical aspects are discussed which can provide help for the analyst in the selection of the most adequate separation system originating from the special requirements of pharmaceutical analysis, such as high selectivity, good efficiency, suitable sensitivity and appropriate accuracy and precision.

2.7.1.2. Column and System Selection for the Separation of Impurities by HPLC

The nature and origin of the impurities in drug substances are discussed and classified in Section 1.2. The subject of this section is mainly focused on the separation, detection and determination of *related organic impurities* mainly by *reverse-phase* HPLC, which is used in the majority of cases for the separation and determination of impurities in drugs.

Column selection is usually the first and very important step in method development, and this decision affects all subsequent aspects of this procedure. The proliferation of different types of columns applied in pharmaceutical analysis may frequently cause a serious problem to the analyst: how to select the best column (nature and particle size of the stationary phase, length and diameter of the column) for a given purpose.

Column selection is generally based on practical experience and knowledge of the analytes. There was a general trend that a longer column provides better separation because of the higher theoretical plate numbers. In contrast to this statement, it has been found that only the first few centimetres of the column determine the dominant effect on the separation [13] and the short columns filled with microparticulate packings of the proper stationary phases can give similar results and resolution to those achieved on longer columns if mobile phase composition is properly adjusted [14]: a continuously increasing trend can be observed to decrease analysis time and to increase the sensitivity by using shorter analytical columns. Another field where great demands have arisen is the performance of the chromatographic hardware and particularly the detection system. Suitably designed column hardware materials (column tubes end fittings, connections, etc.) are also important elements to get minimum peak broadening. The role of temperature is significant because it influences the precision and speed of analysis, the detection limit and in many cases the efficiency and the selectivity of the separation; therefore control of the column temperature is one of the important aspects in the column hardware.

Silica-based stationary phases with octyl and octadecyl groups covalently bonded to the silica surface are the most widely used column packings in

pharmaceutical analysis. These can be characterised as pseudo-liquids, forming a mixture of the bonded moieties and the various mobile phase components on the stationary phase surface [15–20]. Significant differences in retention behaviour may exist not only among identically labelled columns from different manufacturers but also between different column lots from the same manufacturer.

Points to be taken into account when selecting the column include the chemical purity of support materials, particle and pore size, end-capping technology, topography and size of the ligands and the column characterisation presented by the manufacturers.

Polymer-based non-polar stationary phases have some remarkable advantages over silica-based similar supports: no residual surface activity (silanol effects, metal impurities, reactive surface functionalities, etc.), extended pH-range (0–14), extremely high column stability and long life time as well as the possibility of the use of simple eluent composition (low buffer concentration to control the undesired surface activity).

Despite their limited pH-range of usability, silica based reverse-phase columns are still far more widely used than polymer-based columns, owing to their much higher column efficiency.

Mixed-polarity stationary phases can be applied both in reverse- and normal-phase modes. Column packings with intermediate polarities can be produced through the bonding of polar and non-polar groups on the same stationary phase surface. Phases with mixed functional groups can be prepared by various modification methods [21–24].

Other types of stationary phases (used for hydrophobic interaction-, affinity-, ion-exchange- and size-exclusion chromatography) are not discussed in this section because of their limited importance in drug impurity tests. The chiral stationary phases are discussed in Section 6.2.3.

The inertness and stability of chemically bonded reverse-phases enable the use of eluents with very different physicochemical properties. This is the main reason for the very broad field of application of this technique compared with other branches of HPLC [8]. The retention of the solute can be regulated by the concentration of organic components in the aqueous eluent.

To understand the separation mechanism several different effects should be taken into consideration. The interaction between solute molecules and the stationary phase surface responsible for the retention can be characterised on the basis of the solvophobic theory [25]. The forces which are responsible for the binding of the solutes to the long-chain alkyl group of the stationary phase are proportional to the molecular contact area upon binding and to the surface tension in the eluent. The solvophobic theory predicts and the experimental data demonstrate that aqueous eluents are weaker than hydroorganic solvents. Polar substituents enhance the interactions of solutes with polar solvent mole-

cules and decrease the strength of their binding to the non-polar stationary phase surface. The interactions of weakly acidic and basic solutes with aqueous eluents are increased by ionisation, resulting in decreased solvophobic binding and retention. The theory correctly predicts the effects of molecular size and various substituents on the relative retention and provides a good possibility for characterisation of the effect of solvent composition on the eluent strength.

Solvent selection and optimisation are also great problems. The influence of the eluent composition on reverse-phase chromatographic separation of various solutes and the effect of residual silanol groups on the retention of molecules with polar functional groups are the most important factors to be taken into consideration. These can cause unexpected changes in retention behaviour. Several explanations have been published most of them based on the combination of the hydrophobic and silanophilic interactions. Silanophilic interactions are generally considered undesirable in reverse-phase chromatography, and for this reason silanol masking is usually recommended by the addition of various amines to the mobile phase [26–29], end-capping (secondary silanization) [30–32] or special silica pre-treatment procedures [33] as well as the simultaneous blocking of the residual silanols and masking of the functional groups in the solute molecule which can interact with silanols [19] in order to obtain reproducible results and regular retention behaviour.

These methods can reduce silanophilic interaction but cannot completely exclude them. However, it has to be mentioned that in many cases this may be definitely useful to increase the selectivity as demonstrated, e.g. by the separation of four diastereomers of *vincaminic acid ethyl ester* by Szepesi and Gazdag [34].

2.7.1.3. System Optimisation

The theoretical and practical aspects of HPLC method optimisation are studied and described in several textbooks [1–12,35–38]. In this part the practical aspects of method development in pharmaceutical analysis are discussed focusing on selectivity and sensitivity.

For the selection of a suitable chromatographic system for *initial separation* prior to the optimisation, firstly prechromatographic studies are necessary where the physicochemical characteristics of the compounds to be separated (molecular size, chemical properties such as polarity, basicity, lipophilic or hydrophilic character) and the expected interactions with mobile phase components are taken into consideration. The evaluation of the experiences of previous separation studies (TLC, HPLC) is also important. Gradient elution runs are very useful to select the proper isocratic eluent composition

[36,37,39,40]. This type of work is increasingly based on computer assisted expert systems [41–44].

Some important aspects of the *optimisation* of the system are as follows:

- object of optimisation: optimisation of the phase system (combination of stationary and mobile phases, selectivity, capacity) and of the chromatographic system (column, detection, temperature, and other instrumental parameters, etc.);
- factors influencing separations, mainly selectivity: mobile (eluent strength and nature of the organic solvents) and stationary phase composition, pH, ionic strength, flow rate, temperature, secondary chemical equilibria, etc.;
- selection of criteria for selectivity optimisation: the selection of criteria depends on the sample to be separated (known or unknown number of the compounds in known or unknown matrix), on the number of peaks of interest (all peaks have equal importance, or a limited number of peaks are of interest). These aspects are summarised by Schoenmakers [37].

Sometimes the analyst has to work almost ''in the dark'' when a new separation problem has to be solved, and sufficient chromatographic information is not available. It is necessary to ensure that both known and unknown components are separated in the final method. It is necessary to use at least two different separation techniques (normal- and reverse-phase HPLC [45,46], HPLC-TLC [47,48] and HPLC-HPCE [49] separations, etc.) thus minimising the possibility of peak coelution and the failure to recognise the presence of some unknown peaks.

These aspects were taken into consideration by Gazdag *et al.* [45,46] during the course of the optimisation of the separation of steroid mixtures, among them *norgestrel* and its impurities. The experiments were focused on the improvement of the selectivity by controlling band spacing for the maximum resolution. The optimisation criteria were the minimum value of resolution ($R_{S,min}$), the minimum value of normalised resolution ($D_{S,min}$) and the relative concentration-dependent R_S values (R_{sb} and R_{sa})

It was found that in *reverse-phase chromatography* the combination of ''solvent-strength'' and ''solvent-type'' optimisation provides a markedly better separation than either procedure alone, as a global optimum mobile phase composition for band spacing can be determined which requires the optimisation of the solvent strength by varying the percentage of organic component and of the solvent selectivity obtainable by using methanol, acetonitrile, tetrahydrofuran and water.

Using the same optimisation criteria in *normal-phase chromatography* as in the reverse-phase mode the possible combination of ''solvent-strength'' optimisation (variation of the percentage of polar modifier in the mobile phase) and ''solvent-type'' optimisation (measuring the solvent selectivity of

chloroform, acetonitrile, tetrahydrofuran and dioxane) was examined. From the results it can be concluded that the selectivity of the separation can be significantly improved by the proper choice of medium-polarity solvents (chloroform, acetonitrile, tetrahydrofuran and dioxane) in the mobile phase and the elution order is also a function of solvent type. In the separation of the components of steroid mixtures dioxane provides the best properties for improving band spacing.

A larger number of experimental runs is required to attain the global optimum with the solvent strength [40] or iterative lattice [50] method. For samples which do not require solvent type optimisation, the experiments may be finished when the local optimum has been attained by means of solvent strength optimisation.

The above described principles combined with temperature optimisation were applied by Gazdag *et al.* [51] for the purity check of *levonorgestrel* (D-norgestrel) including the separation of the enantiomeric impurity L-norgestrel by reverse-phase chromatography. The effect of the concentration of the organic modifier in methanol–water and acetonitrile–water eluents containing γ-cyclodextrin (γ-CD) as chiral additive at different temperatures was investigated. It was concluded that optimum separations were obtained at 45% methanol or 30% acetonitrile at room temperature. The method is suitable for the simultaneous determination of enantiomeric and other impurities at low concentration (0.1%) with adequate accuracy. For some more details see Section 6.2.2, where the chromatograms in the two systems are also shown in Fig. 6.2.C.

To establish the suitability of a HPLC method in pharmaceutical analysis, further information may be necessary regarding the quality of separations taking into consideration the difficulties created by the analytical problems to be solved. Method validation data provide important information about the system [52]. The quality of separation can be characterised by the *system resolution* (SR) and *system selectivity* (SS). These correlate closely with the applicability of a separation system.

System resolution (SR): The separation of a HPLC system mainly depends on the following parameters.

- The resolution achieved between the peak of the main component and the preceding (R_{sb}) and following (R_{sa}) peaks (specificity). This is of prime importance in the HPLC investigation of impurities in drugs when the difference between the concentrations of the drug and its impurities is 2–4 orders of magnitude.
- The lowest value of resolution ($R_{s,min}$) obtained for any pair of peaks in the chromatogram (selectivity).

The values required for R_{sb} and R_{sa} are functions of the relative concen-

trations of the compounds in the sample (i.e. the peak ratios of the adjacent peaks) and these are dependent also on the analytical problem to be solved. Their recommended values are b for R_{sb} and a for R_{sa}. The most advantageous value for both R_{sb}/b and R_{sa}/a is 1, since a lower value is not sufficient for a perfect separation, and a higher value would increase the analysis time. When all peaks in the chromatogram have equal importance, the minimum resolution measured for the worst-separated peak pair should also be considered.

$$SR = (R_{sb}/b) \times (R_{sa}/a) \times (1 + R_{s,min})$$

System selectivity (SS): This relates to the selectivity of the total system where the number of peaks eluting before and after the main peak is z and v, respectively. SS can be expressed by the following equation:

$$SS = D_a^z/(1 - D_{min}^{z+1})^{1-z} - D_a^v/(1 - D_{min}^{v+1})^{1-v} = Z - V$$

where D_a^z and D_a^v are the average normalised resolution values for the peaks eluting before and after the main component ($\Sigma D^{z+1}/z$ and $\Sigma D^{v+1}/v$, respectively), D_{min}^{z+1} and D_{min}^{v+1} are the lowest values of normalised resolution for peaks z and v.

When Z is higher than V, better separation is obtainable. This is the case when (1) more peaks elute before the peak of the main component, and (2) $D_a^z > D_a^v$ and/or $D_{min}^{z+1} > D_{min}^{v+1}$. When no peak elutes before the main component ($z = 0$), $SS = -V$, while SS has positive value when no peak elutes after the main component ($v = 0$, $SS = Z$). When only one peak elutes before or after the main component, the values of V and Z are equivalent to the corresponding values of the minimum normalised resolution.

The principles of the selection of the most applicable separation system (from among two or more) were investigated on some practical examples in the author's laboratory [53]. The conclusions were as follows:

- The first decision is based on the comparison of the SR values. A higher SR value provides better separation conditions.
- When the systems possess similar SR values (both HPLC methods are capable of solving the analytical task), the SS value are taken into consideration. The recommended separation method can be selected on the basis of the numerical value of SS.
- Finally, the other data elements of method validation are considered in the selection of the most applicable separation system.

After completion of the method development and selection and optimisation of the most applicable chromatographic system for the analytical task it is necessary to discuss the column and instrument optimisation which is also of great importance.

The *efficiency optimisation* includes the selection of the column with a sufficient plate number [39]. The speed of analysis (analysis time) depends on the theoretical plate number (N) and on the particle diameter of the support; by decreasing the particle size the speed of analysis can be increased. To decrease the analysis time the following factors are of importance:

- a good column packing technique: the H value (height equivalent of one theoretical plate) is decreasing;
- low-viscosity solvents in the mobile phase or elevated temperature;
- increased pressure drop over the column.

The *sensitivity optimisation* is an essential aspect of the chromatographic method development. This can be influenced by the injected volume of the sample, the detection parameters, the column diameter and particle size of the stationary phase.

All the parts of the instrument from the injector to the detector (including detector flow cells) contribute to the *extracolumn peak broadening*. It must be reduced especially when small-diameter columns or short columns packed with superfine particles (1.5–3 μm) are used.

Chromatographic optimisation procedures are becoming more multidisciplinary as methods are sought to obtain more and more information on the separations. The chemometric approach, i.e. the use of expert systems is quite general in mobile phase selection [36–38,54]. Further development is expected in this field: chemometrics is or will be involved in all stages of the analytical process and will be as important in the total measurements as the expert systems are presently in method development. Chemometrics is a separate discipline helping in the good utilisation of information technology and in the development of intelligent analysers which automatically select the correct method for the given purpose, carry it out, validate it and interpret the results [55].

2.7.1.4. Detection Problems (Selectivity and Sensitivity)

HPLC is the dominant separation technique in modern pharmaceutical and biomedical analysis because it results in highly efficient separations, and in most cases provides high detection sensitivity. However, detection sensitivity is a function of solute properties. It must contain structural elements that give rise to high UV-absorbance, fluorescence, or electrochemical activity to achieve the required detectability. If no such structural elements are present, the detection problem can be solved by the aid of pre- or postcolumn derivatisation.

The detection is still widely regarded as one of the weakest points in

practical HPLC. Although definite progress has been made in the design of sensitive and specific detectors in recent years, a generally applicable, well-designed, non-specific detector with a satisfactory low limit of detection is still missing.

Two types of HPLC detectors are in use. The response of *selective (specific) detectors* is based on the different and typical physicochemical or chemical properties of the samples investigated (UV, fluorescence, etc.), while *universal (non-specific) detectors* produce a response for all peaks eluted from the column independently of their chemical structure (refractive index, evaporative light scattering, etc.). Selective detectors can be used to minimise the interference of components of the sample which are not subject to the analysis, while universal detectors are useful when a universal response is required and the sample size is large (preparative and size-exclusion chromatography).

The HPLC detectors have some general features:

- the sensitivity (R_D) is defined by the ratio of the detector response to the sample concentration;
- the noise level (N_D) is characterised by the change in its signal during the operation;
- the limit of detection (LOD) is characterised by the smallest change in the measured property which results in a change in the output signal reliably distinguishable from the detector noise;
- the linear dynamic range is defined by the ratio between the maximum value of the measured property for which a linear response is obtained and the LOD.

In the past two decades some types of detectors have been improved considerably. In the field of UV detectors, which are the most generally used detectors in the application of HPLC in pharmaceutical analysis the first step was the development of fast-scanning photometers followed by the introduction of photodiode array detectors (DADs). The linear photodiode array UV detector which is already a standard part of a modern HPLC equipment is a good example for the rapid spreading of a new improvement in instrumentation. DADs provide the UV spectra of the separated components which are very important additional data for the characterisation of the chromatographically separated compounds.

A separate book edited by Huber and George [56] is devoted to diode-array detectors in HPLC characterising the great importance of this excellent detection technique for identification and qualification of impurities in drug substances. These aspects are discussed in Section 2.7.3 with several practical examples from the fields of the structure elucidation of impurities and peak purity testing in method validation mainly based on methods developed in our laboratories at Gedeon Richter Ltd. [52,57–62].

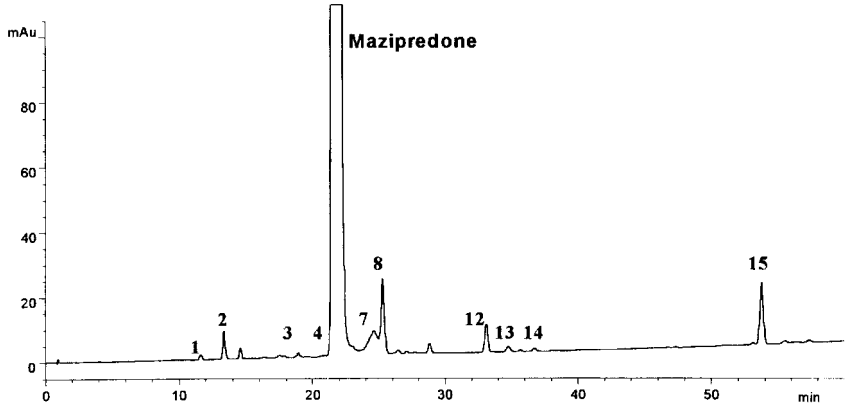


Figure 2.7.1.A.
Chromatogram of a production batch of mazipredone. Conditions: column: Purospher RP-18e, 5 μm, 150 × 4 mm, eluent: A: water–acetonitrile–methanol (85:5:5, v/v/v), B: water–acetonitrile–methanol (20:40:40, v/v/v), both containing 50 mM ammonium acetate, linear gradient from 10% B to 90% in 70 min, flow rate: 1 ml/min, 40°C, detection: 240 nm. For peak numbers see Table 5.5.A (from Ref. [61])

Presently the different hyphenated techniques (HPLC/MS, HPLC/NMR) are the focus of interest in the field of the determination of the impurity profile of drug substances and pharmaceutical formulations (see Sections 2.7.4 and 2.7.5).

In many laboratories in the pharmaceutical industry the bench-top mass spectrometers equipped with HPLC instruments are almost routinely used. These HPLC/MSD systems can be easily and quickly applied combining with diode array UV detectors to identify the unknown impurities and decomposition product in bulk materials and in formulated products. In our laboratory reverse-phase methods (isocratic and gradient systems with different stationary phases, C-8 and C-18 columns) were developed to separate the impurities and degradation products of *mazipredone* [61]. On the basis of HPLC-(APCI)-MS investigations, supplemented by HPLC-DAD data it was possible to describe the structures of all impurities in bulk mazipredone above 0.1% and many of the minor impurities below 0.1% (altogether 13 compounds). Figure 2.7.1.A shows the chromatogram of a production batch of mazipredone. The investigation of the mechanism of the degradation of mazipredone in acidic and alkaline media based on the same methodology is discussed in Section 5.5.3. Its formula appears in Figs. 5.5.E and 5.5.G. Table 5.5.A contains the retention data and the

origin of the impurities/degradation products. These achievements and the elucidation of the mechanism of the decomposition reactions served as the basis for validated purity and stability tests for bulk mazipredone and its liquid formulations.

To identify the impurities of prednisolone we also used the HPLC-(EI)-MSD technique [62]. The details of this study are discussed in Section 9.4.2.5, where HPLC/DAD-UV and HPLC/MS scans are shown in Fig. 9.4.E, the diode-array UV spectra in Fig. 9.4.F and the structures of prednisolone and the impurities in Fig. 9.4.G.

Special detectors and techniques used for enantiomeric investigations are summarised in Section 6.4.

A nice example for the necessity and usefulness of the simulates use of various detectors in the course of drug impurity profiling by HPLC has been presented by McCrossen *et al.* [63]. As shown in Fig. 2.7.1.B all but one of the impurities of the phenol-type drug candidate SKF-99085 are UV-inactive. For

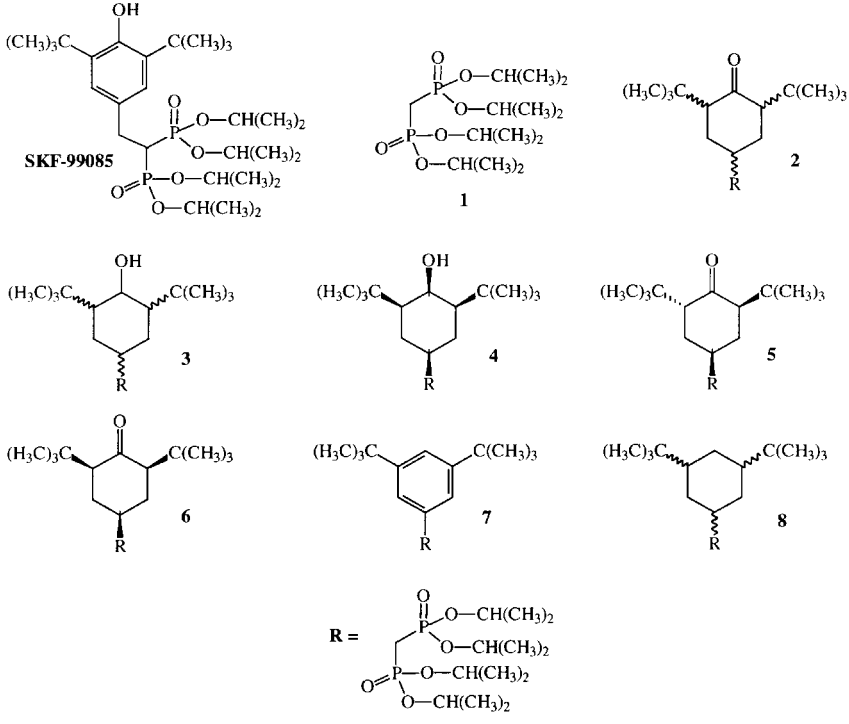


Figure 2.7.1.B.
Structure of SKF-99085 and its related impurities

this reason in the UV trace in Fig. 2.7.1.C where the chromatograms taken by UV, refractive index (RI), MS and evaporative light scattering (ELS) detectors are shown, only one impurity (impurity **7**) is detectable. The MS detector provides the highest sensitivity, and also structural information in the HPLC/MS/MS mode. It is remarkable that the LOD obtainable with the ELS detector is at least ten times lower than when using RI detector. The reason for the absence of the peak of impurity **1** in the ELS trace is the high volatility of this impurity. It is worth mentioning that on-line HPLC/NMR played an important role in the structure elucidation of the impurities.

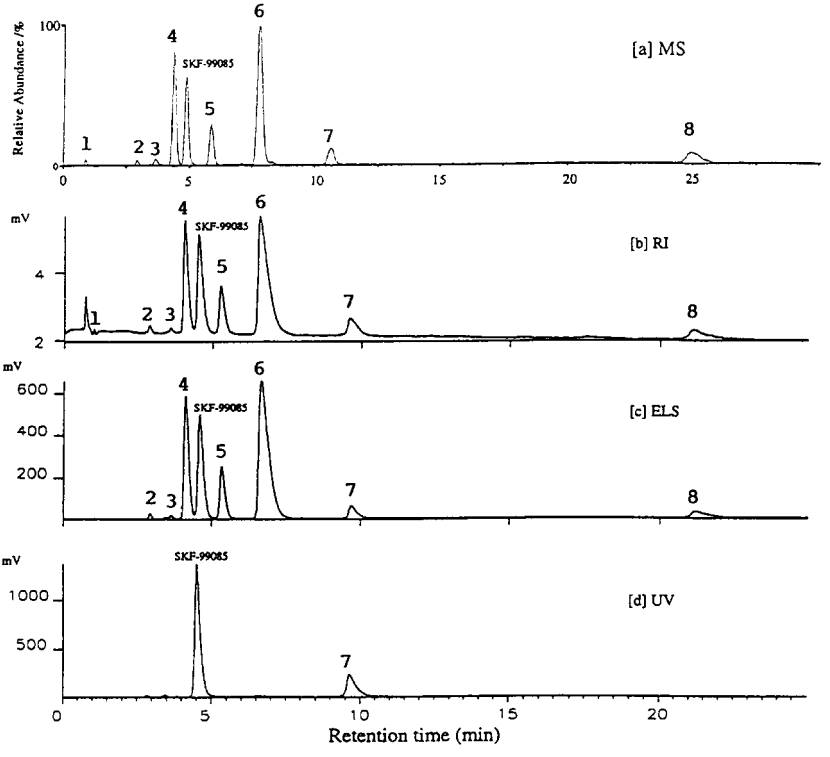


Figure 2.7.1.C.
HPLC investigation of a mother liquor sample of SKFC-99085. Comparison of: (a) MS(TIC); (b) RI; (c) ELS and (d) UV_{220nm} detection. Column: Nova-Pak C_{18} 150 × 3.9 mm, 4 μm; eluent: acetonitrile–water 70:30 v/v, 1.5 ml/min; t: 40°C; 20 μl injection; concentration for MS 10 μg/ml, for RI, ELS and UV 10 mg/ml (from Ref. [63])

2.7.1.5. Quantitative Determination and Method Validation of Impurities by HPLC

The aim of pharmaceutical analysis is to obtain the necessary qualitative and quantitative information about the investigated sample. Since the quantitative analysis includes the total analytical procedure, from sample pre-treatment to the evaluation of the analytical results, each step in this process can be established separately to determine the weakest point which may influence the analytical results. Therefore the quantitative analysis cannot be separated from aspects of the method validation procedure because to select and use an adequate separation method for impurity testing it is necessary to prove the applicability of the intended method.

There are several definitions in the literature to describe the perfect meaning of validation. According to the proposal of the Compendial Assay Validation Committee [64] ''validation of an analytical method is the process by which it is established, by laboratory studies, that the performance of the method meets the requirements for the intended applications''. The method validation is the planning, performance and interpretation of a series of experiments designed to reveal the operational characteristics of an analytical method, which allows its use for a relatively long period of time with acceptable accuracy and reliability formulating criteria for its acceptability or rejection for the intended analytical applications.

Method validation used in the pharmaceutical industry is based on the different guidelines of the International Conference on Harmonisation [65–67]. The overall validation procedure of a HPLC method contains a series of different validation steps (both chromatographic and pre-chromatographic investigations).

The first step in the validation process is the formulation of the *validation protocol* which is to be used during the whole procedure. A systematic evaluation of the planned experiments from application to application ultimately provides the answer as to which experiments are important and which can be omitted. Prior to the preparation of the validation protocol the analyst has to take into consideration the following points:

- sample pre-treatment and sample introduction: selection of the proper solvents for the dissolution of the sample (high purity, miscibility with the eluent, low detector response and good stability for the components in the solution);
- selection of the elution mode: isocratic or gradient elution;
- selection of the proper evaluation methods: peak height or peak area measurement, area normalisation, or external standard or standard addition methods (standards and impurity standards in adequate and documented quality are needed);

- evaluation of the experimental results: precision, accuracy, reproducibility, specificity–selectivity, peak purity testing, sensitivity–detection limit (LOD)–quantitation limit (LOQ), detector linearity, recovery, robustness.

The evaluation of the validation process carried out according to the validation protocol is summarised and documented in the *validation report*.

An important topic in method validation is the quality assurance (QA) of the experimental data. In this procedure QA has multiple functions. If the experimental results indicate failures as compared to the validation protocol, it is the function of QA to reject the analytical results and to give instructions to the analyst to carry out the method development all over again. Other functions of QA are connected to the documentation activity during method validation: assembling the validation package, generating the analytical report, creating standard operating procedures (SOP) for HPLC methods, performing statistical treatment of the experimental data.

The widespread application of HPLC methods and the multitude of commercial sources of columns and packings frequently create problems in assessing comparability during regulatory method validation. This was recognised by the Food and Drug Administration (FDA), which published a ''Guideline for submitting samples and analytical data for method validation'' [68]. In practice each method submitted for validation must include a number of data, defining the necessary characteristics of the system involved. Most HPLC methods submitted for external evaluation require modification as a result of the evaluation, but these are often minor changes only. In some cases, however, the evaluation is very complex, because various factors may affect the success of internal HPLC method validation. The major reasons for delay in successful HPLC method validation can be traced back to the inadequate performance of ruggedness testing or instructions.

The *system suitability* test encompassed in the directions for testing is a measure of the performance of a given system on a given day. The overall test of the system function is performed to recognise whether or not the operation of the system is adequate for the subject procedure. Only if the system passes this test can the analyst start or continue the analytical investigation.

The above discussed aspects of the validation were applied in our laboratory in the course of the separation of *pipecuronium bromide* and its related impurities by normal phase hydrophobic interaction HPLC. Some of these such as sample pre-treatment, recovery of the analyte, its stability during sample preparation and storage, precision of sample preparation, system suitability data, peak purity test, system resolution (SR) and system selectivity (SS) have been described in Ref. [52]. (RP-HPLC and TLC separation systems for the same purpose were also published previously [47,48]).

The formulae of pipecuronium bromide and its related impurities are

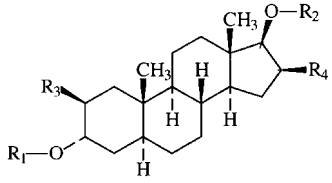
presented in Table 2.7.1.A. The standard chromatogram and the chromatogram used for the system suitability test are shown in Figs. 2.7.1.C and D. The measured system suitability and method validation data are presented in Table 2.7.1.B. The impurities include not quaternerised (**1**) or partially quaternerised (**2**, **3**) derivatives (originating from the synthesis), oxidative degradation product (**4**), partially (**6**, **7**) and totally deacetylated (**8**) derivatives which can either originate from the synthesis or can be products of hydrolytic degradation. Of these the most problematic impurity is the oxidative degradation product (**4**) from the point of view of its identification and separation from the main component [57]; this is why the separation of **4** from the main component is part of the system suitability test (see Fig. 2.7.1.E). It is also to be mentioned that in contrast to the fully saturated main component impurity **4** as an enamine is a strong UV absorber in the short wavelength region ($\lambda_{max} = 235$ nm): this is why its LOD is much lower (see Table 2.7.1.B).

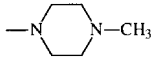
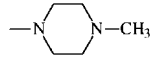
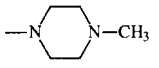
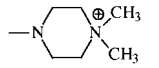
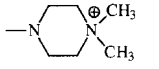
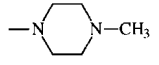
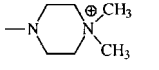
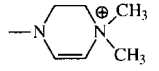
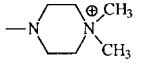
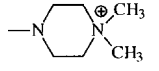
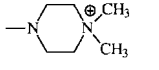
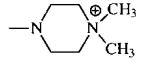
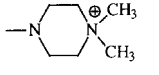
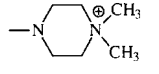
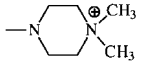
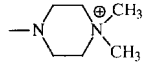
The chromatographic system must be capable of resolving from the peak of interest all other components that give a detector signal. Evidently, the success of the method validation depends mainly on the correct determination of the *peak purity* and the *selectivity* and *specificity* of a given HPLC system. It is clear that acceptable selectivity and specificity can result in pure peak(s). Therefore the appropriate determination of the purity of a chromatographic peak is of primary interest. To improve the assessment of peak purity investigations a combination of the method based on the measurement of the variation in the absorbance ratios in time with the of investigation of samples subjected to appropriate stress conditions so as to produce decomposition products may be applied in order to recognise peak overlapping. A 10–15% decomposition is considered to be adequate. (For the stress conditions used for the peak purity test for pipecuronium bromide see Table 2.7.1.B).

The chromatogram of standard samples and treated samples without and with spiking with known concentration of a standard are recorded at selected wavelengths. The ratio of the absorbances at these wavelengths characterises the peak purity. When the absorbance ratios obtained for non-treated, treated and spiked samples are within the acceptable limit ($\pm$ 5%), the chromatographic peak can be considered to be pure. (For other chemometric methods for peak purity determination see the book of Huber and George [56] and Refs [4–9] in Section 2.7.3) As a typical example chromatograms of pipecuronium bromide are shown in Fig. 2.7.1.F, after one of the treatments summarised in Table 2.7.1.B (treatment at pH 12). The selected wavelengths were 213 and 225 nm. After the investigations it can be stated that our validated method is selective and specific for determination of the main component and it is applicable for stability testing as a stability indicating method.

Other aspects of validation with special respect to impurities (linearity, etc.) are summarised in Section 2.5.8, where the validation of TLC purity tests

Table 2.7.1.A.
Structures of pipecuronium bromide and related impurities



Peak number	R_1	R_2	R_3	R_4	Bromide
1	Ac	Ac			–
2	Ac	Ac			Mono
3	Ac	Ac			Mono
4	Ac	Ac			Di
5 pipecuronium bromide	Ac	Ac			Di
6	H	Ac			Di
7	Ac	H			Di
8	H	H			Di

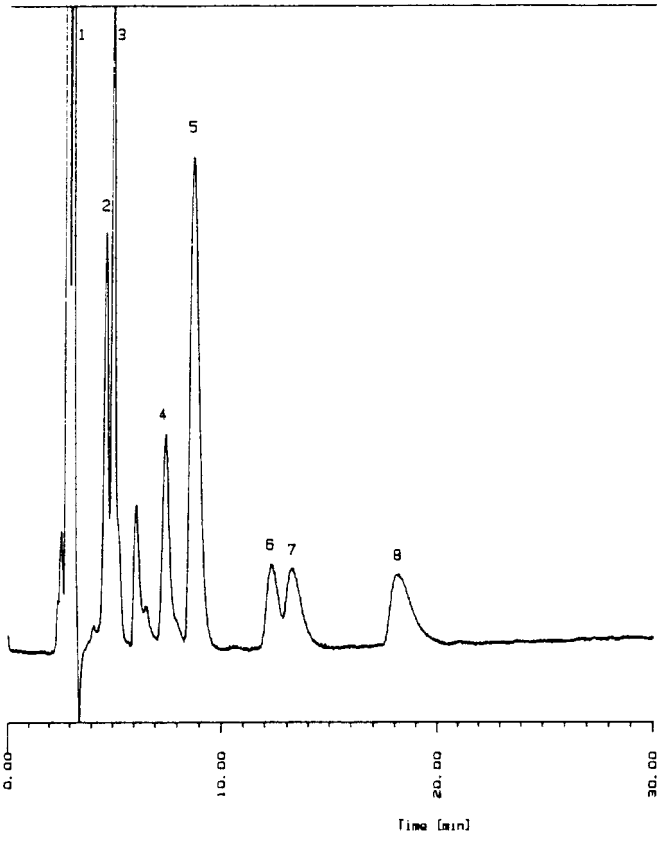


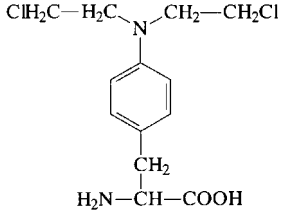
Figure 2.7.1.D.
Separation of pipecuronium bromide and its possible impurities. Conditions: column: LiChrosorb Si 60, 5 μm, 250 × 4.6 mm, eluent: methanol–acetonitrile–concentrated ammonia solution (43:43:14, v/v/v), containing 100 mM each of ammonium chloride and ammonium carbonate, flow rate: 1 ml/min, detection: 213 nm. For peak numbers see Table 2.7.1.A (from Ref. [52])

is summarised. What is described there, can be applied to the validation of HPLC purity tests also.

As described in detail in Sections 2.1.2 and 2.7.1.3 to get a reliable and clear picture about the impurity profile of a drug material it is by no means sufficient to rely upon the data obtained with a single chromatographic system: at least two or even more preferably three chromatographic systems are necessary for this purpose possibly with different separation mechanisms. In addition

to the method described here in detail and the above mentioned RP-HPLC [48] and TLC [47] purity tests another HPLC method based on entirely different separation mechanism (normal phase ion-pair system, using perchlorate as the ion-pairing reagent) was also developed for the purity test of pipecuronium bromide [58]. As seen in Fig. 2.7.1.G the key impurities in Table 2.7.1.A including **4**, the most important impurity, are well separated.

Another example for a robust, sensitive, stability indicating and fully validated method is the reverse-phase gradient HPLC method developed by Brightman *et al*. [69] for the simultaneous determination of the active ingredient content and related impurities in *melphalan* drug substance. The conditions described in the legend of Fig. 2.7.1.H enabled the separation and qualification of all impurities in production batches of melphalan above 0.1%. The method is linear over the column loading range of 0–3 μg of melphalan. The method was applied to melphalan samples stored under stressed conditions. The retention data are presented in Table 2.7.1.C and a typical chromatogram is shown in Fig. 2.7.1.H.



Melphalan (L-enantiomer)

Response factor ruggedness for the determination of impurities in LY297802 tartrate, a muscarinic agonist drug using HPLC separation was investigated by Olsen *et al*. [70]. Figure 2.7.1.I shows the remarkable differences between the UV maxima of the chloro and ethoxy impurities and the main component while the spectrum of the hydroxy impurity is almost identical with the latter. The selected analytical wavelength was 280 nm. At this wavelength the spectrum of the chloro derivative is sloping the opposite direction of the main component and for this reason small changes in wavelength setting can lead to poor reproducibility. The ruggedness of response factors was examined on a single detector and among several different detectors. While the ratio of each impurity to that of the main component was very reproducible (RSD $\leq$ 0.2%) when the wavelength was not changed between injection, the RSD increased by a factor of ten for the chloro and ethoxy impurities when the wavelength was changed and returned to 280 between

Table 2.7.1.B.
Method validation data for the determination of pipecuronium bromide For the reference see Fig. 2.7.1.D

Data	Parameter	Value
System suitability data	Retention time	
	For peak 4	7.3 min
	For peak 5	8.5 min
	Resolution for peaks 4 and 5	Min. 1.5
	RSD for peak 5	Max. 1.0%
	Asymmetry factor for peak 5	Max. 2.0
	Column loadability for peak 5	100 μg
Method validation data	R_{sb} for peaks 4 and 5	1.74
	R_{sa} for peaks 4 and 5	3.81
	$R_{s,min}$ for peaks 2 and 3	1.13
	D_{min} for peaks 2 and 3	0.072
	SS	−0.013
	SR	1.85
	Asymmetry factor for peak 5	1.55
	LOD for peak 5	25 ng

	LOD for peak 4	4 ng
	RSD for peak 5	0.81%
	Day-to-day reproducibility	1.97%
	r^2	0.999
	F	999.9

Peak purity	Stress conditions	Response ratio measured at 213 and 225 nm	
		Treated sample	Spiked sample
	Standard	3.927	
	Reflected light, 14 days	3.999	3.999
	40 °C, 80% RH, 14 days	3.922	3.919
	105 °C, 48 h	3.987	3.975
	UV light (254 nm), 24 h	3.935	3.891
	pH 2, room temperature, 30 min, 2% solution	3.866	3.850
	pH 12, room temperature, 30 min, 2% solution	3.699	3.712
	RSD (n = 13)	3.899 ± 2.51%	

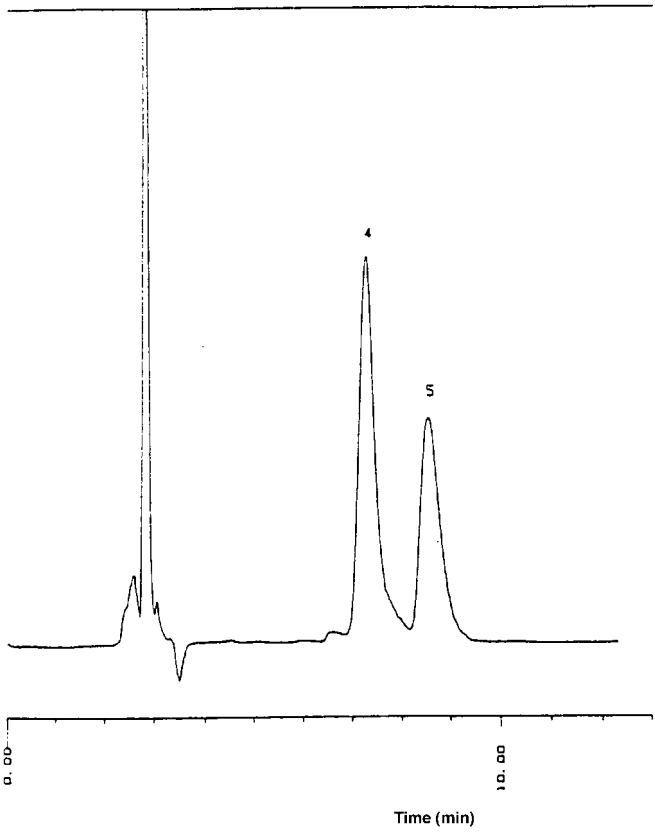


Figure 2.7.1.E.
Chromatogram used for the determination of system suitability. Conditions as in Fig. 2.7.1.D. For peak numbers see Table 2.7.1.A and for the reference Fig. 2.7.1.D

the injections. Even greater variability was found when different detectors were used. Employing a wavelength system suitability sample ensures reproducible impurity response factors for any combination of instrument or analyst. This sample contains the impurities whose responses can be used to set the appropriate wavelength.

Figure 2.7.1.J shows the chromatogram of such a sample scanned at 279, 280 and 281 nm. The wavelength setting of 280 nm is acceptable if the peak area ratio is 1.30 for the chloro to hydroxy and 3.23 for the ethoxy to hydroxy peak area ratios.

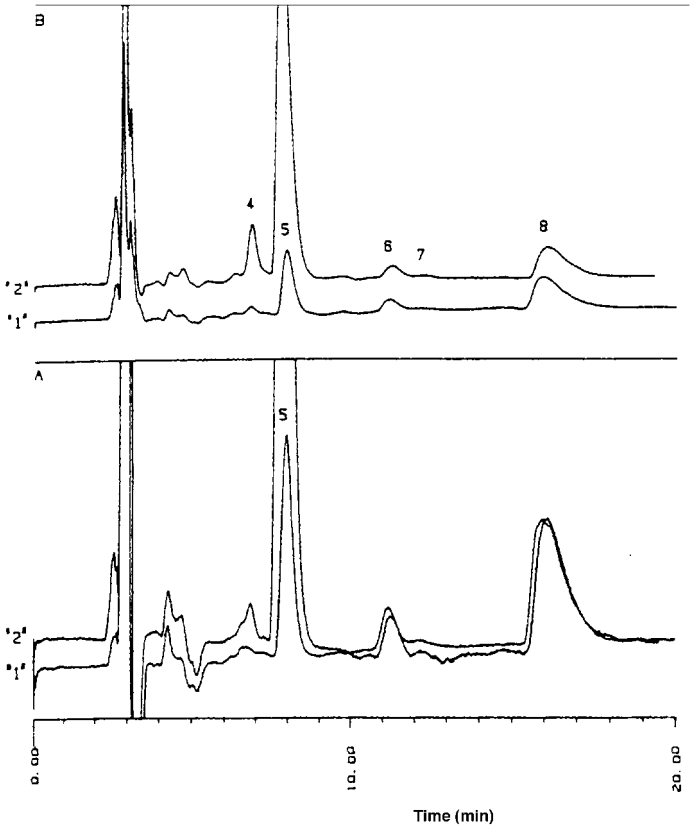


Figure 2.7.1.F.
Assessment of peak purity. (A) Detection at 213 nm; (B) detection at 225 nm. (1) Chromatogram of treated sample (2% aqueous solution at pH 12, room temp., 30 min); (2) chromatogram of treated sample spiked with standard pipecuronium bromide. Conditions as in Fig. 2.7.1.D. For peak numbers see Table 2.7.1.A and for the reference Fig. 2.7.1.D

2.7.1.6. HPLC Purity Tests in Modern Pharmaceutical Analysis: Pharmacopoeial Aspects

As it has already been mentioned in this chapter and in Section 2.1, HPLC and HPLC-related hyphenated methods are the most important methods for the detection, separation, identification and quantitative determination of impurities in drugs. The list of innumerable papers dealing with the results of these

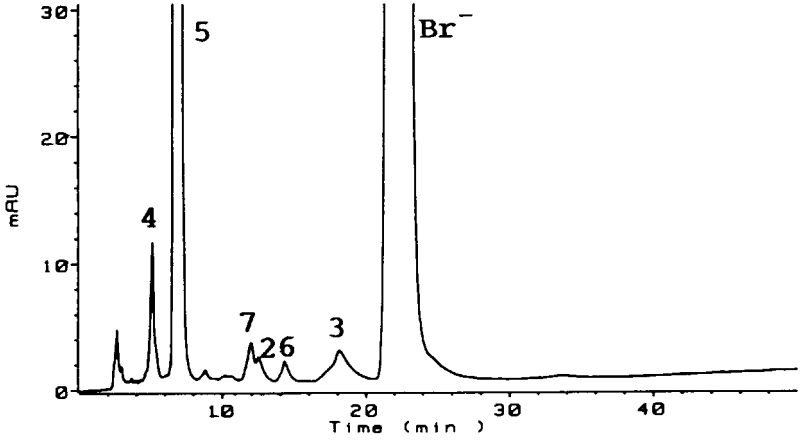


Figure 2.7.1.G.
Model chromatogram of pipecuronium bromide spiked with 0.1% of impurity **4** and 1% of each of impurities **2**,**3**,**6** and **7** (see Table 2.7.1.A). Column: 250 × 4 mm packed with SI 100, 5 μm; eluent: 100 mM sodium perchlorate in acetonitrile–water 96:4 v/v; flow rate: 1 ml/min; detection: 213 nm (from Ref. [58])

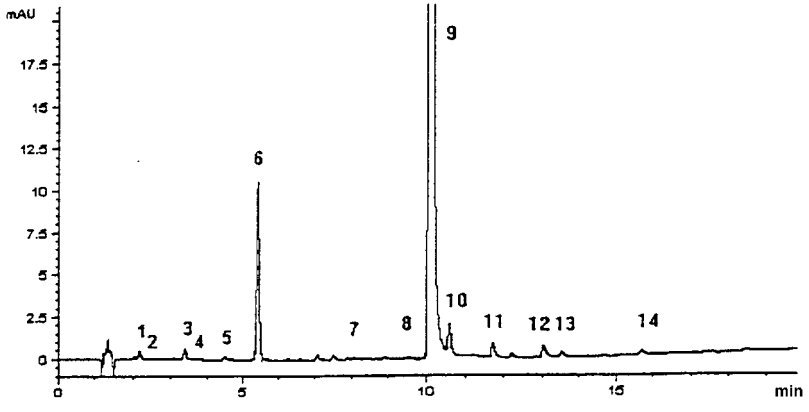


Figure 2.7.1.H.
Typical chromatogram for freshly prepared solution of melphalan. Conditions: column: Hypersil BDS C18, 5 μm, 150 × 4.6 mm, linear gradient from 5 to 60% acetonitrile in water containing 0.05% v/v acetic acid, 0.01% v/v triethylamine, and 0.05% v/v ammonium acetate in 20 min, flow rate: 1.5 ml/min, detection: 260 nm. For peak numbers and reference see Table 2.7.1.C

Table 2.7.1.C.
Chromatographic data of melphalan and related impurities and the origin of the impurities. For the chromatographic conditions see Fig. 2.7.1.H (from Ref. [69])[a]

Peak number	Compound name	Relative retention time	Class
1	Dihydroxymelphalan	0.21	s + d
2	Phthalic acid	0.28	s
3	Morpholino derivative	0.34	s
4	Methoxyhydroxymelphalan	0.35	s
5	Chloroethylamino melphalan	0.44	s
6	Monohydroxymelphalan	0.52	s + d
7	Methoxymelphalan	0.79	d
8	Ethoxymelphalan methyl ester	0.95	s
9	Melphalan	1.00	
10	Chloroethoxy melphalan	1.05	s
11	3-Chloro analogue of melphalan	1.16	s
12	Melphalan dimer	1.27	s + d
13	Melphalan methyl ester	1.31	s
14	Melphalan ethyl ester	1.44	s

[a] s, impurity of synthesis; d, degradation product

studies begun soon after the introduction of HPLC. For example the case study described in the introductory section of this book (detection and determination of new impurities in aspirin – see Refs. [7,8] in Section 1.1) were among the first achievements of HPLC in this field. A few of the hundreds of papers that followed afterwards are mentioned in this section, many more in others, espe-

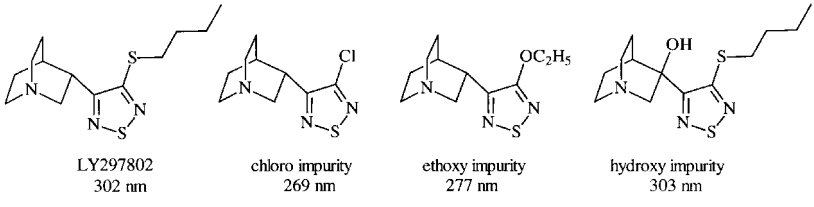


Figure 2.7.1.I.
Structures and UV absorption maxima of LY297802 and its impurities

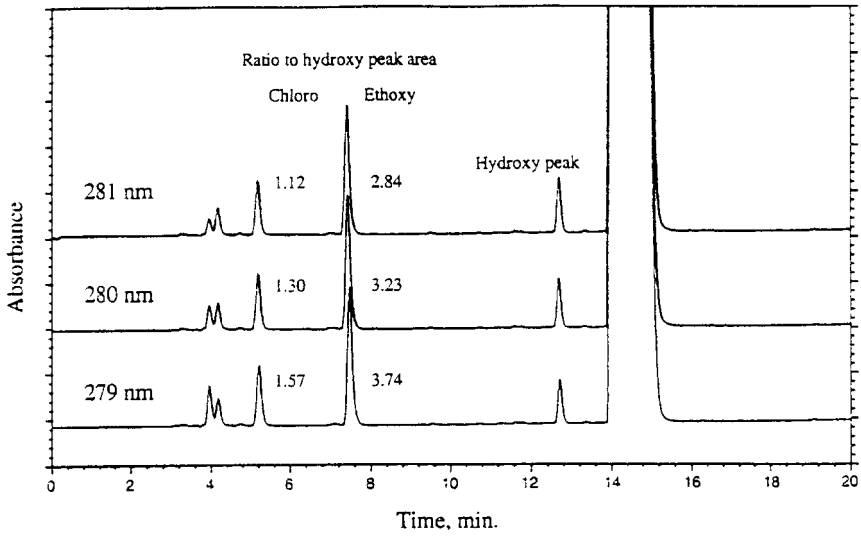


Figure 2.7.1.J.
Chromatogram with absorbance ratios of a wavelength system suitability sample for reproducible determination of impurities in LY297802. Conditions: column: YMC basic, 5 μm, 250 × 4.6 mm, eluent: A: 0.05% trifluoroacetic acid in water, B: 0.05% trifluoroacetic acid in acetonitrile. Gradient program: 25% B to 60% B in 15 min, hold at 60% for 10 min. Flow rate: 1 ml/min, Injection volume: 20 μl. LY297802 tartrate concentration: 4 mg/ml in initial mobile phase (from Ref. [70])

cially in Chapters 5 and 9 as well as in Sections 2.7.3 and 6.3. The sections dealing with the HPLC-based hyphenated techniques (2.7.4 and 2.7.5) also contain several examples for the practical importance of this technique. However, to give a full list of the applications of HPLC in drug impurity profiling would be beyond the scope of this book. Further development is predictable as impurities below 0.1% are becoming the focus of interest and the development of HPLC hardware enables problems of this type to be solved. The combination of this hardware with the continuously developing spectroscopic techniques and chemometric methods shall be certainly a good basis for further developments. To characterise the possibilities of chemometrics in association with HPLC a recent paper [71] is mentioned where a HPLC-based pattern recognition method is described to characterise on the basis of their impurity profile the origin (manufacturer) of various L-tryptophan samples.

The first HPLC method was described in USP XIX Supplement 3 for the assay of *triamcinolone acetonide* [72]. Since that time the role of HPLC tests has been dramatically growing in the monographs of leading and other pharmacopoeias. HPLC is very widely used for the assay of active substances and the formulated products, as well as for the content uniformity and dissolution tests of solid dosage forms. Although TLC methods are more often prescribed for purity testing, the importance and frequency of the use of purity tests by HPLC is increasing. It should be noted, that in many cases (especially in the USP) different HPLC methods are used for assay, content uniformity and dissolution test causing difficulties to the analysts.

With the exception of a limited number of cases when named (toxic, signal) impurities should be measured with the aid of a reference standard of the impurity, unnamed impurities are usually quantitated by comparing their peak area with that of an appropriately diluted solution of the reference standard of the main component. The use of very expensive reference standard materials make these tests rather costly. Where appropriate, these reference standards can be substituted with thoroughly investigated, well-characterised working standards. In some cases impurities (mostly easily formed decomposition products) are made in situ from the main component in the standard solution to control the resolution during the system suitability tests.

In the monographs of European Pharmacopoeia the impurities of active substance are listed and named. The difficulties in using them when samples of different origin are investigated are described in Section 2.5.2 for thin-layer chromatography: the same applies to HPLC purity tests also. To prescribe limits for unnamed individual impurities and their sum in bulk substances and formulated products seems to be generally applicable.

It is general that the pharmacopoeias prescribe the application of a system suitability test. Appropriately described system suitability tests serve to make the HPLC method transfer easier. In practice this means that the column dimensions and the type and particle size of the stationary phase, the eluent system and a recommended ratio of the solvents are specified. The approximate values of the retention times of the main component and a named impurity or a reference substance and the resolution between the peaks corresponding to them are also presented. To reach the latter is obligatory: if the given resolution is not achieved, the ratio of the mobile phase components should be changed until it is reached. In many cases the system suitability tests contain further parameters also. It has to be noted that if all these requirements are fulfilled, no validation is necessary. However, if the same method is planned to be used for different purposes (e.g. assay method for impurity test, etc.) the validation is compulsory.

References

1. L.R. Snyder, J.J. Kirkland and J.L. Gljach (Eds.), *Practical HPLC Method Development, 2nd edn*, Wiley, New York (1997)
2. E. Katz, R. Eksteen, P. Schoenmakers and N. Miller (Eds.), *Handbook of HPLC*, Marcel Dekker, New York (1998)
3. T. Hanai, *HPLC: a Practical Guide*, Royal Society of Chemistry, Cambridge (1999)
4. W.J. Lough and I.W. Wainer (Eds.), *High Performance Liquid Chromatography: Fundamental Principles and Practice*, Balckie, Glasgow (1996)
5. S. Ahuja, *Trace and Ultratrace Analysis by HPLC*, Wiley, New York (1992)
6. U.D. Neue, *HPLC Columns: Theory, Technology and Pr*actice, Wiley-VCH, New York (1997)
7. P.C. Sadek, *The HPLC Solvent Guide*, Wiley, Chichester (1996)
8. G. Szepesi, *How to Use Reverse-Phase HPLC*, VCH, New York (1992)
9. J. Swadesh (Ed.), *HPLC: Practical and Industrial Applications*, CRC Press, Boca Raton, FL (1997)
10. M. Sarsunova, O. Hanc and B. Kakac, *HPLC in Pharmacy and Biochemistry,* Huethig, Heidelberg (1990)
11. G. Szepesi (Ed.), *HPLC in Pharmaceutical Analysis I-II*, CRC Press, Boca Raton, FL (1990)
12. G.W. Fong (Ed.), *HPLC in the Pharmaceutical Industry*, Marcel Dekker, New York (1991)
13. I. Halász and G. Görlitz, *Angew. Chem.* **21**, 50–61 (1982)
14. H.J. Issaq, *J. Liq. Chromatogr.* **7**, 475–482 (1984)
15. K. Karch, I. Sebastian and I. Halász, *J. Chromatogr.* **122**, 3–16 (1976)
16. W.R. Melander, J.F. Erard and Cs. Horváth, *J. Chromatogr.* **282**, 211–228 (1983)
17. C.R. Yonker, T.A. Zwier and M.F. Burke, *J. Chromatogr.* **241**, 257–268, 269–280 (1982)
18. A. Nahum and Cs. Horváth, *J. Chromatogr.* **203**, 53–63 (1981)
19. K.E. Bij, Cs. Horváth, R.W. Melander and A. Nahum, *J. Chromatogr.* **203**, 65–84 (1981)
20. J. Köhler, D.B. Chase, R.D. Farlee, A.J. Vega and J.J. Kirkland, *J. Chromatogr.* **352**, 275–305 (1986)
21. J.B. Crowther and R.A. Hartwick, *Chromatographia* **16**, 349–353 (1982)
22. W.E. Hammers, A.G.M. Theeuwes, W.K. Brederode and C.L. de Ligny, *J. Chromatogr.* **234**, 321–336 (1982)
23. C.H. Loesch and W. Simon, *Chromatographia* **17**, 669–672 (1983)
24. H. Figge, A. Deege, L. Köhler and G. Schomburg, *J. Chromatogr.* **351**, 393–408 (1986)

25. R.W. Melander and Cs. Horváth, in *High Performance Liquid Chromatography*, *Advances and Perspectives, Vol. 2* (Cs. Horváth, Ed.), p 113. Academic Press, New York (1980)
26. C.T. Mant and R.S. Hodges, *J. Liq. Chromatogr.* **12**, 139–172 (1989)
27. P. Spiegel and H.J. Vierstein, *J. Chromatogr.* **294**, 452–455 (1984)
28. P. Cockaerts, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.,* **4**, 367–376 (1986)
29. H.A.H. Billiet, R. Wollers, L. deGalan and H. Huizer, *J. Chromatogr.,* **368**, 351–361 (1986)
30. D.B. Marshall, C.L. Cole and A.D. Norman, *J. Chromatogr. Sci.* **25**, 262–266 (1987)
31. P.C. Sadek and P.W. Carr, *J. Chromatogr. Sci.* **21**, 314–320 (1983)
32. C.H. Lochmüller and D.B. Marshall, *Anal. Chim. Acta* **142**, 63–72 (1982)
33. D.B. Marshall, K.A. Stutler and C.H. Lochmüller, *J. Chromatogr. Sci.* **22**, 217–220 (1984)
34. G. Szepesi and M. Gazdag, *J. Chromatogr.* **204**, 341–348 (1981)
35. J.W. Dolan and L.R. Snyder, *Troubleshooting LC Systems*, Humana Press, Clifton, NJ (1988)
36. J.C. Berridge, *Techniques for the Automated Optimisation of HPLC Separations*, Wiley Interscience, New York (1985)
37. P.J. Schoenmakers, *Optimisation of Chromatographic Selectivity*, Elsevier, Amsterdam (1986)
38. J.L. Glajch and L.R. Snyder, *Computer-assisted Method Development for High-Performance Liquid Chromatography*, Elsevier, Amsterdam (1990)
39. M.A. Quarry, R.L. Grob and L.R. Snyder, *Anal. Chem.* **58**, 907–917 (1986)
40. M.A. Quarry, R.L. Grob, L.R. Snyder, J.W. Dolan and M.P. Rigney, *J. Chromatogr.* **384**, 163–180 (1987)
41. T.P. Bridge, M.H. Williams, G.G.R. Seaton and A.F. Fell, *Chromatographia.* **24**, 691–695 (1987)
42. S.N. Deming and S.L. Morgan, *Experimental Optimisation Methods*, Elsevier, Amsterdam (1987)
43. A.F. Fell, T.P. Bridge and M.H. Williams, *J. Pharm. Biomed. Anal.* **6**, 555–564 (1988)
44. G.G.R. Seaton and A.F. Fell, *Chromatographia* **24**, 208–216 (1987)
45. M. Gazdag, G. Szepesi and E. Szeleczki, *J. Chromatogr.* **454**, 83–94 (1988)
46. M. Gazdag, G. Szepesi and K. Fábián-Varga, *J. Chromatogr.* **454**, 95–107 (1988)
47. M. Gazdag, G. Szepesi, K. Varsányi-Riedl, Z. Végh and Z. Pap-Sziklay, *J. Chromatogr.* **328**, 279–287 (1985)

48. M. Gazdag, Varsányi-Riedl and G. Szepesi, *J. Chromatogr.* **347**, 284–290 (1985)
49. M. Gazdag, K. Mihályfi, P. Kemenes-Bakos and S. Görög, *Acta Pharm. Hung.* **67**, 169–174 (1997)
50. A.C.J.H. Drouen, H.A.H. Billiet, P.J. Schoenmakers and L. de Galan, *Chromatographia* **16**, 48–52 (1982)
51. M. Gazdag, G. Szepesi and K. Mihályfi, *J. Chromatogr.* **450**, 145–155 (1988)
52. G. Szepesi, M. Gazdag and K. Mihályfi, *J. Chromatogr.* **464**, 265–278 (1989)
53. M. Gazdag and G. Szepesi, *J. Chromatogr.* **464**, 279–288 (1989)
54. M. De Smet, A. Peeters, L. Buydens and D.L. Massart, *J. Chromatogr.* **457**, 25–42 (1988)
55. D.L. Massart and L. Buydens, *J. Pharm. Biomed. Anal.* **6**, 535–545 (1988)
56. L. Huber and S.A. George (Eds.), *Diode Array Detection in HPLC*, Marcel Dekker, New York (1993)
57. S. Görög, G. Balogh and M. Gazdag, *J. Pharm. Biomed. Anal.* **9**, 829–833 (1991)
58. M. Gazdag, M. Babják, P. Kemenes-Bakos and S. Görög, *J. Chromatogr.* **550**, 639–644 (1991)
59. S. Görög, M. Babják, G. Balogh, J. Brlik, A. Csehi, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *Talanta* **44**, 1517–1526 (1997)
60. S. Görög, M. Bihari, É. Csizér, F. Dravecz, M. Gazdag and B. Herényi, *J. Pharm. Biomed. Anal.* **14**, 85–92 (1995)
61. M. Gazdag, M. Babják, J. Brlik, S. Mahó, Z. Tuba and S. Görög, *J. Pharm. Biomed. Anal.* **17**, 1029–1036 (1998)
62. S. Görög, M. Babják, G. Balogh, J. Brlik, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *J. Pharm. Biomed. Anal.* **18**, 511–525 (1998)
63. S.D. McCrossen, D.K. Bryant, B.R. Cook and J.J. Richards, *J. Pharm. Biomed. Anal.* **17**, 455–471 (1998)
64. Current Concepts for the Validation of Compendial Assays, *Pharmacopoeial Forum*, USP Convention Inc., Rockville, MD (1986)
65. ICH guideline, Validation of Analytical Procedure: Definition and Terminology, CPMP/ICH/5626/94
66. ICH guideline, Validation of Analytical Procedure: Methodology, CPMP/ICH/281/95
67. ICH guideline, Impurities in New Medicinal Plants, CPMP/ICH/282/95
68. Guideline for submitting samples and analytical data for methods validation, Food and Drug Administration, Center for Drugs and Biologics, Department of Health and Human Services, Rockville, MD (1987)

69. K. Brightman, G. Finlay, I. Jarvis, T. Knowlton and C.T. Manktelow, *J. Pharm. Biomed. Anal.* **20**, 439–447 (1999)
70. B.A. Olsen and M.D. Argentine, *J. Chromatogr.* **762**, 227–233 (1997)
71. I.V. Tetko, T.I. Aksenova, A.A. Patiokha, A.E.P. Villa, W.J. Welsh, W.L. Zielinski and D.J. Livingstone, *Anal. Chem.* **71,** 2431–2439 (1999)
72. *United States Pharmacopoeia XIX,* Suppl. 3, USP Convention, Rockville, MD (1979)

2.7.2. Isolation of Impurities by (semi)Preparative HPLC

Antal Aranyi

2.7.2.1. Introduction

Application of preparative and semi-preparative HPLC becomes necessary when the identification of an impurity cannot be carried out with acceptable certainty by means of the simple use of analytical (chromatographic, spectroscopic, hyphenated) techniques. In this case (semi)preparative HPLC isolation followed by spectroscopic (MS, NMR) investigation is the solution. The quantity of the isolated impurity, needed for the subsequent spectroscopic measurements is generally in the order of 5–50 mg. Theoretical and practical aspects of preparative HPLC are easy to access in excellent monographs [1–4] so they will not be discussed here. This chapter aims to provide a practical guideline, highlighting the special aspects of preparative HPLC for the isolation of impurities.

In order to carry out the preparative HPLC isolation process effectively, it is advisable to perform it in five consecutive steps. These steps are as follows:

- definition of the task, data collection;
- selection of starting material;
- elaboration of isolation strategy, development of preparative HPLC methods;
- performing the isolation;
- analysis of the isolated impurity.

2.7.2.2. Definition of the Task. Data Collection

Precise definition of the task is a prerequisite for successful isolation work. It is useful to do this together with the analysts, who detected the impurity. An analytical chromatographic (preferably HPLC) method has to be chosen which is appropriate for the detection of the impurity during the isolation process. This method must be adopted and the impurity or impurities to be isolated must be marked with an unambiguous identity code.

In order to ease the subsequent work it is highly advisable to carry out very profound data and information collection. This means the collection of the knowledge and data which can be found in the literature and gained in the

course of research, development, analysis and production of the drug. The synthesis route should be known together with the solubility, stability and UV spectroscopic data of the drug and its known impurities. Information on the available analytical liquid chromatographic (HPLC, TLC, OPLC) methods could be of great help in preparative HPLC method development. Most of the data and information are obtainable from the analysts, technologist and synthetic chemists who dealt with the drug in question. It is also necessary to have standard materials of the drug and its known impurities.

2.7.2.3. Selection of the Starting Material

The most obvious and safest possibility is to use the bulk drug as the starting material for the isolation process. It can cause difficulties, however, that these materials contain very small amounts (in some cases below 0.1%) of the impurity to be isolated. In these cases the isolation can be carried out only by processing relatively large amounts of the bulk drug, at considerable expense. If the drug is of very high value and its production volume is very small, it can occur that the quantity, needed for the isolation is not available. It is expedient therefore to consider other possible samples in which the relative concentration of the unknown impurity is higher. Such potential starting materials could be crude products, mother liquors from the final crystallisation step or some fractions from process-scale HPLC purification of the drug. Considerably larger amount of the unknown could be present in products of intentionally misconducted reactions. If the impurity is a degradation product, then bulk drugs, exposed to stress conditions (high temperature, light) could be good starting materials. However, higher relative concentration of the impurity of interest in the potential starting material is not the only point to be considered. In many cases these materials contain numerous other impurities that are not present in the bulk drug. These impurities could overlap with the interesting ones, rendering the isolation process much more difficult. Decisions have to be made by considering both the relative concentration of the impurity of interest in the potential starting materials and the composition of these materials.

Before beginning the isolation, it is essential to check the identity of the impurity of interest in the bulk drug and the chosen starting material. This can be done by *chromatographic retention matching* (see Section 2.1.3) and – if it is applicable – *spectral matching*, using diode-array UV detector for the HPLC measurement (Section 2.7.3).

2.7.2.4. Elaboration of Isolation Strategy: Development of Preparative HPLC Methods

Owing to the low relative concentration of the impurity of interest in the starting material, in most cases it is not possible to perform the isolation in one preparative HPLC separation step, but several consecutive separations must be done, that is an *isolation strategy* has to be elaborated. This strategy consists of one or more *enrichment steps* and a final *purification step*. Different separation steps of an isolation strategy are often carried out with different HPLC methods.

Elaboration of a strategy starts with the *analysis of the task*. On the basis of collected data and information (chemical structure of the drug molecule and its known impurities, stability and solubility data, earlier used chromatographic methods) it is to be taken into account what sort of chromatography (normal-phase chromatography on non-modified or modified stationary phase, reversed-phase chromatography with or without pH-control, reversed-phase ion-pair chromatography, ion-exchange chromatography, chiral chromatography) can be used or is expedient to use for the isolation. The goal is to isolate the impurity in a purity, appropriate for the subsequent MS and NMR investigations: special considerations, arising from this goal must be kept in mind. First, the isolated impurity sample must not contain other compounds, stemming from HPLC separation (mobile phase additives), because these compounds hamper structure elucidation or even make it impossible. For this reason it is preferable to choose HPLC methods which do not use additives in the mobile phase. If this is not possible, the additive used has to be easily removable. The second aspect is that after carrying out the HPLC separation, the enriched or purified impurity has to be *recoverable* from the mobile phase without degradation.

The most important processes generally used to do the recovery of the separated impurity from the mobile phase and – if it is necessary – to remove additives are evaporation, liquid–liquid extraction, crystallisation, precipitation, freeze-drying, column desalting and dialysis. In most cases a certain combination of these processes is needed to solve both tasks. The simplest and most frequently used process is *evaporation*, which is appropriate for both normal- and reversed-phase separations. This process is applicable in itself only if the mobile phase does not contain additives. In order to avoid degradation, evaporation should be done under moderate conditions (high vacuum, room temperature). In some cases non-volatile modifiers can be removed from the totally evaporated fraction by *recrystallisation*, using an appropriately chosen solvent for this purpose. In reversed-phase separations non water-soluble impurities can be recovered by evaporating organic solvents from the eluent and then *filtering* the precipitate. Additives, soluble only in water can

also be disposed of in this way. If the impurity is water-soluble, it can be recovered by *freeze-drying* after evaporating the organic solvents from the mobile phase. This method is applicable for mobile phases, containing volatile additives as well. The most commonly used volatile additives are some organic acids (formic, acetic, trifluoroacetic acid), bases (diethylamine, triethylamine) and some salts (ammonium carbonate, ammonium formate and ammonium acetate). A volatile ion-pairing reagent is heptafluorobutyric acid [5]. In most cases freeze-drying processes have to be repeated several times in order to remove these additives completely. *Liquid–liquid extraction*, followed by evaporation is also a commonly used process which also could be appropriate for getting rid of some additives. *Column desalting* (making a second separation to remove the additive from the recovered material) and *dialysis* – they are quite complicated and time consuming methods – should be used as a last resort.

Having considered applicable HPLC techniques, the next task is to draw up an *isolation strategy* by choosing and developing preparative HPLC methods to be used. As far as possible it is expedient to use existing analytical chromatographic methods as a starting point for preparative HPLC method development, because it results in considerable time and money savings. In fortunate situations scaled up analytical HPLC methods under *overload conditions* can be used for preparative separations but generally further method development work should be done. In most cases it is possible to *redesign* an existing analytical HPLC method so that it meets preparative HPLC requirements. Analytical HPLC methods are optimised to achieve baseline separation of all the bands in the sample. The goal is isolation of an impurity and hence the selectivity factor (α) for the impurity of interest and adjacent peaks (product and/or other impurities) should be as high as possible; the separation of other bands is of no importance. It is also necessary to modify the solvent strength. Generally the k' *value* should be adjusted to between 2 and 6 for the impurity to be isolated. These experiments are carried out on analytical columns. It is advantageous to use the same stationary phase in the analytical column that will be used in the preparative one, but this is not essential in the case of commonly used phases such as silica, C8 or C18 phases. If we want to use special stationary phases (e.g. C8 or C18 phases with low free silanol group content for basic compounds, polymer-based phases, chiral phases, etc.), using the same packing material is inevitable. Another possibility, especially for normal-phase separations is optimising an existing TLC or OPLC method and transforming it to the preparative column [6]. If it is not possible to redesign existing analytical methods, then preparative HPLC methods have to be developed by applying well-known method developing techniques, using TLC and/or analytical HPLC or computer software [6,7].

Since the goal is the isolation of a certain impurity *in a single case* as

quickly as possible, at the lowest possible cost, it is not necessary to optimise other preparative HPLC parameters (*sample load, flow rate*) as thoroughly as it is done in method development of other laboratory-scale or process-scale preparative HPLC separations, intended to be used for a long time. Costs of precise optimisation would exceed the savings from the application of the optimised method. In enrichment steps because of the large amount of starting material to be processed and in order to be able to utilise displacement effect (see later), *highly overloaded chromatography* is applied (typical column load is 0.002–0.02 g/g stationary phase). In purification usually semi-preparative HPLC is used and column load is lower. In some cases it is possible and advantageous to perform *touching band separation* instead of overlapping band separation.

It is practical to carry out final purification from a material that contains at least 50% of the material to be separated. In many cases, if we do not want to loose considerable amounts of this, such material can be obtained in more than one enrichment step. In these enrichment steps it is necessary to process relatively large amounts of material, the volume of fraction, containing the enriched unknown is also large, so it is advisable to choose a method that allows simple recovery. If it is possible, normal-phase HPLC enrichment is preferable to reversed-phase, because recovery can be done by simple evaporation. In order to make enrichment more effective, it is practical to adopt some special preparative HPLC techniques and operating modes. In most cases enrichment is done with *overloaded elution.* If the impurity to be isolated and the main component are eluting close to one another, it is recommended to choose an HPLC method by which impurity is eluting earlier. In this case in overloaded elution the so called *self-displacement effect* can be utilised. The main component pushes the impurity in front of itself and so concentrates it. In the opposite situation, when the impurity to be isolated elutes later, the so-called *tag-along effect* takes place, the main component drags along the impurity, which is detrimental to the separation. A very useful operating mode to improve efficiency of enrichment is simple or multiple *closed-loop recycling*. If the sample contains several impurities to be isolated which have very different elution properties, *gradient elution* has to be chosen. A very effective and interesting possibility to enrich impurities is *displacement chromatography* [1]. Squeezing effect occurs and so the concentration of the impurity is really high in this mode if the impurity is eluting between two major components or a main component and the displacer. In this way an enrichment ratio of several orders can be achieved and this method can be very useful when the relative concentration of the impurity in the starting material is very low.

It is very important that impurities to be isolated must not *degrade* during HPLC separation and recovery from the mobile phase. For this reason *stability*

tests have to be carried out in the mobile phase of the developed method and if degradation of the impurity occurs, another method must be chosen.

2.7.2.5. Performing the Isolation

After having elaborated the strategy and developed the HPLC methods, the next step is performing the isolation. The course of the HPLC separation and recovery process is the same in enrichment and purification in the case of *overlapping band separations*. After having injected the sample, separation is detected by an appropriately chosen detector and – at the critical place of the chromatogram – fractions are collected from the mobile phase leaving the detector. Fraction collection (either manually or computer driven) can be done at regular intervals or on the basis of the detector signal. Fractions are then analysed by analytical HPLC, then suitable ones are *pooled* and recovery is carried out, according to the developed method. If *touching-band separation* is performed in the final purification, there is no need to analyse fractions for pooling the purified impurity. It is advisable to investigate both the pooled fraction and the recovered material, because any degradation of the unknown, which occurred during the recovery process can be detected in this way. Then the recovered material is subjected to the next HPLC separation or, after the final purification to analytical investigation.

The quality of solvents used for the separations is a very important factor, especially when evaporation is applied for recovery. Impurities of the solvent can contaminate the isolated impurity sample, causing difficulties in the structure elucidation. To avoid this it is necessary to use highly pure HPLC grade solvents at least in the final purification.

2.7.2.6. Analysis of the Isolated Impurity

Prior to the spectroscopic investigations it is advisable to check once again the isolated material for identity and homogeneity. Identity checking should be done by the earlier discussed *retention matching* method. The *peak purity test*, performed with a diode-array detector could be a further verification that the sample does not contain other compounds with different UV spectra.

2.7.2.7. A Case Study: Isolation of Two Unknown Impurities in Bromocryptine

In this section the above discussed considerations will be demonstrated on

an example from the author's laboratory, the separation of impurities from *bromocryptine*, the brominated analogue of the peptide-type ergot alkaloid *α-ergocryptine.* (For the structure see Section 2.4.7.2.) Two unknown impurities were detected by HPLC and TLC in the bulk drug material. Their quantity exceeded 0.1%, and hence the identification of these compounds became necessary. Efforts aiming at structure elucidation without isolation gave no satisfactory results. The impurities were isolated by preparative HPLC and then structure elucidation was accomplished by MS and NMR.

The analytical HPLC method, used for the investigation of the bulk drug was as follows: column: NUCLEOSIL C18 7 μm, 250 × 4.6 mm; eluent: acetonitrile–ammonium carbonate (0.01%) 47.5:52.5 v/v, flow rate: 1.0 ml/min, detection: UV, 300 nm. The analytical chromatogram of a typical bulk drug sample is shown in Fig. 2.7.2.A.

Careful analysis of the data available on the material and consultation with synthetic chemists and analysts led to the conclusion that the impurities

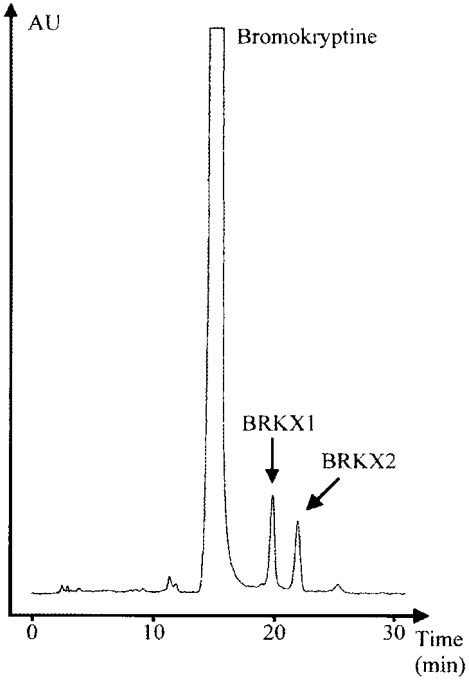


Figure 2.7.2.A.
Analytical HPLC chromatogram of a bulk bromocryptine sample with two impurities (BRKX1 and BRKX2)

were neither degradation products nor by-products of the bromination reaction and they were not enriched in the mother liquor of the last crystallisation step and hence preparative chromatographic isolation had to be made from the bulk drug material itself. A batch with relatively high impurity content (0.4% BRKX1 and 0.2% BRKX2) was chosen as a starting material. It was also ascertained that normal-phase and reversed-phase chromatography with pH-control could be used for preparative HPLC separations.

The elaborated isolation strategy consisted of *two enrichment steps* and a *final purification step*. In the first enrichment normal-phase elution chromatography with *simple recycling* was used. This normal-phase method was developed by modifying and scaling-up an old analytical HPLC method, used for the investigation of α-ergocryptine. The chromatographic parameters of the separation were as follows: WATERS 500A preparative HPLC with two Prep Silica Cartridges (47 × 300 mm) in series, n-hexane–acetonitrile–chloroform–methanol 39.5:35.4:25:2 v/v/v/v mobile phase, 100 ml/min flow rate, RI detection, 5 g sample/injection (0.01 g/g silica). The chromatogram of a run is shown in Fig. 2.7.2.B.

The substance, containing enriched unknowns was recovered by evaporation from the pooled fractions. By processing 50 g of final product, 0.6 g of material with 25% BRKX1 and 16% BRKX2 content was obtained.

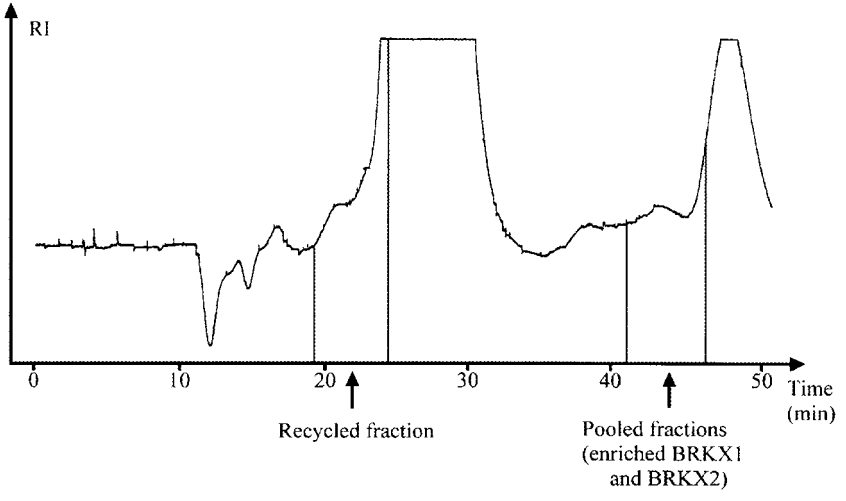


Figure 2.7.2.B.
Preparative chromatogram of normal-phase enrichment of BRKX1 and BRKX2

A highly selective *reversed-phase* method was chosen for further enrichment, which was developed by scaling-up the analytical method described earlier. It was practicable, because enriched unknowns could be recovered from the mobile phase after basification by *extraction* with dichloromethane and evaporation, while the mobile phase additive (ammonium–carbonate) remained in the aqueous phase. The separation was performed on WATERS 500A preparative HPLC with WATERS prep C18 cartridge (47 × 300 mm), 100 ml/min flow rate and RI detection. Column load was 0.6 g/injection (0.0024 g/g stationary phase). Two materials were obtained: 80 mg (80% BRKX2 and 5% BRKX1 content) and 300 mg (50% BRKX1 and 10% BRKX2 content), respectively.

The final purification of these materials was carried out by *reversed-phase semi-preparative HPLC*, using the following instruments: KNAUER preparative HPLC pump and UV/VIS photometer with preparative cell and WATERS U6K injector. Chromatographic parameters were as follows:

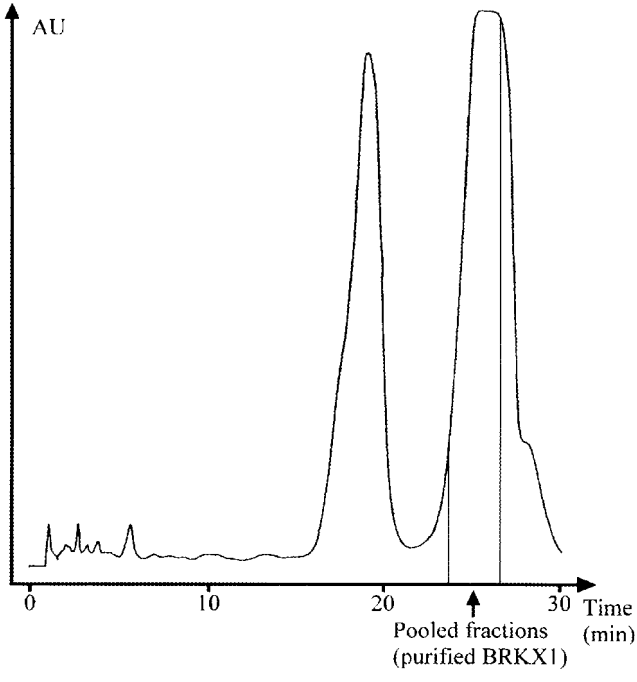


Figure 2.7.2.C.
Chromatogram of semi-preparative purification of BRKX2

KNAUER EUROSPHERE C18 column (18 × 250 mm), acetonitrile–0.01M ammonium-carbonate 47.5:52.5 v/v mobile phase (the same as in the analytical method and in the second enrichment step), 10 ml/min flow rate, UV 254 nm detection. Column load was 40 mg/injection (0.0013 g/g stationary phase) for the first material and 50 mg/injection (0.0016 g/g stationary phase) for the second one. Chromatograms are shown in Figs. 2.7.2.C and D, respectively.

As a result of the isolation process, 50 mg BRKX1 (97.8% purity) and 50 mg BRKX2 (96.9% purity) was obtained. Analytical HPLC chromatograms of the isolated impurity samples can be seen in Fig. 2.7.2.E.

Chromatographic retention matching and peak homogeneity tests proved the identity and homogeneity of the isolated impurities. Structural elucidation was performed successfully by MS and NMR methods [8]. For details and the structure of the impurities see Section 2.4.7.2.

Another case study describing the highly automated isolation of impurities of metoprolol succinate based on mass spectrometric monitoring can be found in Section 2.7.4.13.

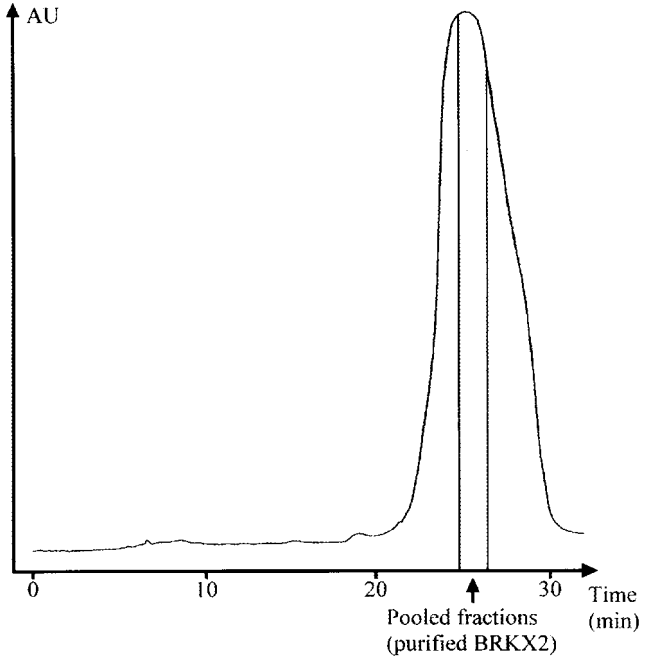


Figure 2.7.2.D.
Chromatogram of semi-preparative purification of BRKX1

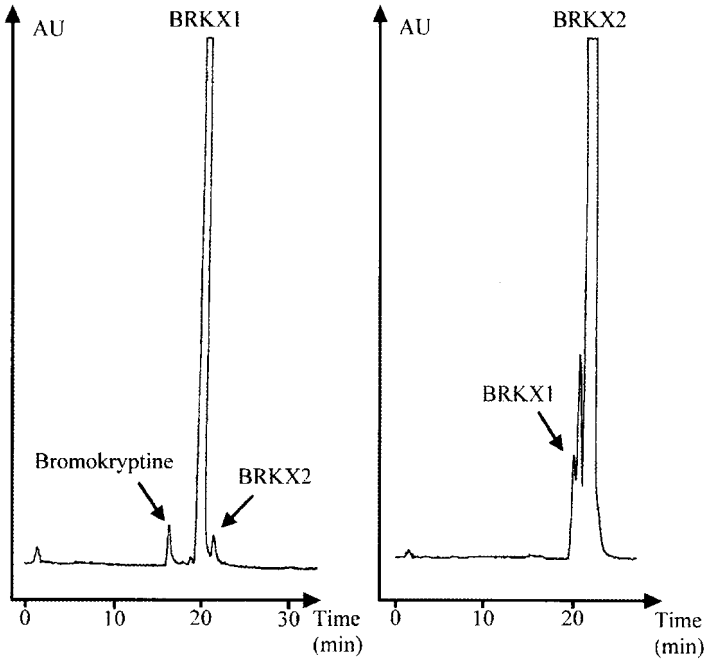


Figure 2.7.2.E.
Analytical HPLC chromatograms of purified BRKX1 and BRKX2

References

1. G. Guiochon, S. Golshan-Shirazi and A. Katti, *Fundamentals of Preparative and Non-linear Chromatography*, Academic Press, New York (1994)
2. K.K. Unger, *Handbuch der HPLC II, Preparative Säulenflüssig Chromatographie*, GIT Verlag Gmbh, Darmstadt (1994)
3. M. Verzele and C. Dewaele, *Preparative High Performance Liquid Chromatography*, TEC, Gent (1986)
4. H. Colin, in *High Performance Liquid Chromatography* (P.R. Brown and R.A. Hartwick, Eds.), pp 415–479. Wiley, New York (1989)
5. D.R.K. Harding, C.A. Bishop, M.F. Tarttelin and W.S. Hancock, *Int. J. Peptide Protein Res.* **18**, 214–220 (1981)
6. P.C. Rahn, M. Woodman, W. Beverung and A. Heckendorf, *Preparative Liquid Chromatography and Its Relationship to Thin Layer Chromatography; Technical Literature*, Waters, Milford, MA (1979)

7. L.R. Snyder, J.J. Kirkland and J.L. Glajch, *Practical HPLC Method Development, 2nd edn,* Wiley, New York (1998)
8. Cs. Szántay Jr., M. Bihari, J. Brlik, A. Csehi, A. Kassai and A. Aranyi, *Acta Pharm. Hung.* **64**, 105–108 (1994)

2.7.3. The Role of the Diode-array UV Spectra in the Identification of Impurities

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2.7.3.1. General Considerations

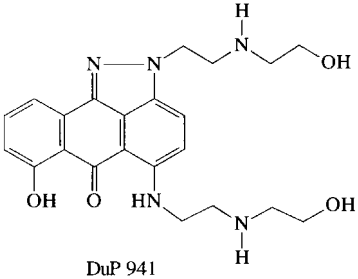
As described in Section 2.2.1 the capability of UV-VIS spectroscopy for the identification and structure elucidation of impurities in drugs without chromatographic separation is very poor. At the same time, however, taking UV spectra in the on-line mode after chromatographic or electrophoretic separation may be very informative and for this reason it can be a useful step during the course of the complex procedure of drug impurity profiling (see Section 2.1.2). The aim of this section is to present examples to characterise the possibilities and limitations of drawing conclusions from the diode-array UV spectra of the separated impurities regarding their structure. As mentioned in Section 2.5 reflection UV spectra taken in the off-line mode after planar chromatographic separation present similar possibilities.

Although – as a consequence of the tremendous developments of NMR and mass spectroscopies – the importance of UV spectroscopy as a structure elucidation tool for organic compounds has dramatically decreased, the introduction of rapid scanning diode-array detectors in the 1980s has created entirely new possibilities in this field. The great importance of diode-array detection in HPLC can be characterised by the fact that this is solely the subject of an excellent book edited by Huber and George [1].

The most important *advantage of the diode-array UV detector* over conventional multiwavelength UV detectors is the speed of scanning the spectra. Using the reversed optics of the diode-array spectrophotometer enables all points in the spectrum to be measured simultaneously on the array of fixed photodiodes. The speed of scanning the spectrum is thus determined by the speed of data acquisition. In modern diode-array UV detectors equipped with powerful computers the time necessary to take the full spectrum from 190 to 600 nm can be reduced to as short as about 10 ms. This speed is more than sufficient in the overwhelming majority of cases in pharmaceutical analysis when the half-band width of peaks separated by HPLC is usually in the order of 1 min and it is only very rarely in the order of 1–10 s in fast HPLC systems and especially in capillary electrophoresis where the peaks are in general narrower.

The *quality of the UV spectrum* of the separated impurities obtained by the diode-array detector is influenced by several factors. One of these is the number

of photodiodes. For example, the number of diodes in the detector of the HP-1090 HPLC instrument is only 205 while in the HP-1100 it is 1024. If the spectrum has fine structure, better quality spectra are obtainable with the latter. In addition to this the quality of the spectra of especially the low level impurities greatly depends on the baseline noise. This can be reduced by using a light source with high intensity, by selecting a suitable reference wavelength (which is as close to the cut-off wavelength of the separated analyte as possible) and a suitable slit width. Generally speaking the sensitivity of the new generation of diode-array detectors is much higher than that of the older ones. Nicolas and Scholz [2] investigated the influence of the above listed factors on the quality of the UV spectra of the impurities of the experimental drug *DuP 941*. It was found that using optimised conditions good quality spectra are obtainable even in the range of 0.001% of highly UV-active impurities. As an illustration the diode-array UV-VIS spectra of two of these impurities taken after HPLC separation using the HP-1090 and HP-1100 chromatographs with the old and new generation of diode-array detectors, respectively, are shown in Fig. 2.7.3.A. As shown, the spectra obtained for the impurities being present at the 0.01–0.02% level with the highly sensitive detector with 1024 diodes are of very good quality while those obtained with the less sensitive detector with 205 diodes are of limited value only in the identification of the impurities.



DuP 941

It has to be noted that rapid-scanning UV detectors other than the above described diode-array detectors are also in use. Generally speaking the quality of the UV spectra taken by these detectors which are based on conventional optics is considerably poorer.

There are three main areas within drug impurity profiling where the advantages of diode-array detectors can contribute to the success of the HPLC (CE) analysis.

(a) *Peak purity determination.* The determination of peak homogeneity is an integral part of the protocol in the validation of any kind of HPLC (and CE) analysis of pharmaceuticals. In the course of impurity profiling studies it is

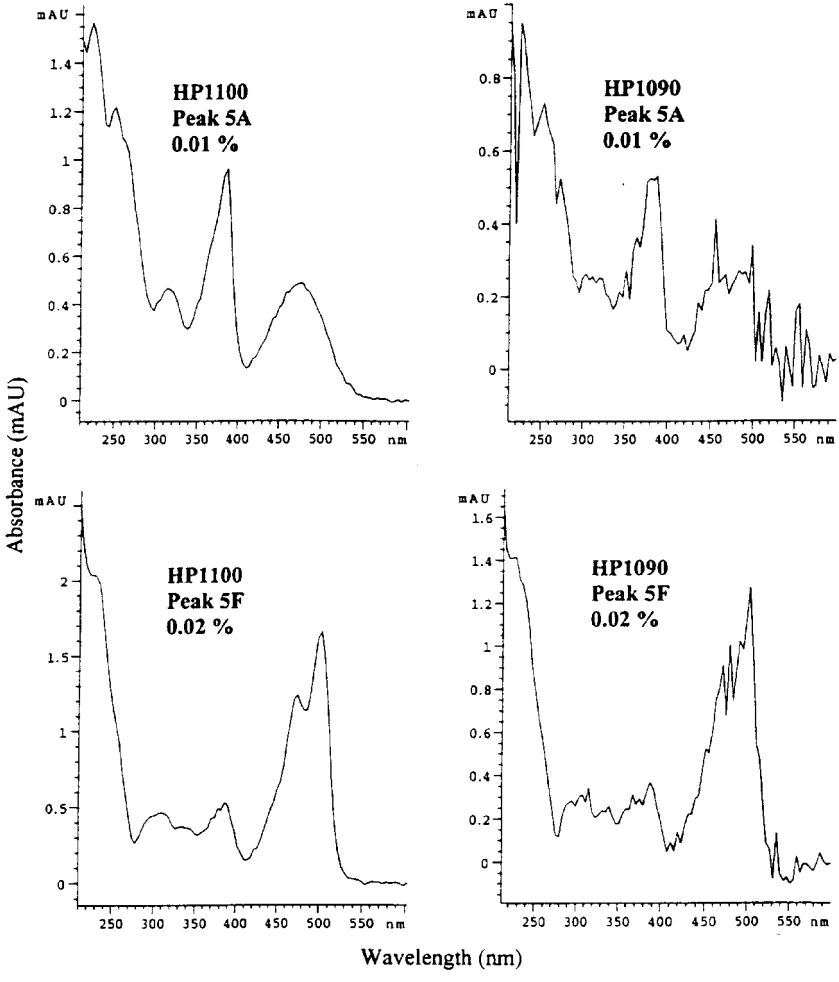


Figure 2.7.3.A.
UV-VIS spectra of impurities 5A and 5F of DuP 941 after HPLC separation using two different diode-array detectors. Column: 150 × 4.6 mm Zorbax SB-C8 3.5 μm. Gradient elution: from acetonitrile–water–trifluoroacetic acid 10:90:0.1 v/v/v to 40:60:0.1 v/v/v in 20 min. Flow rate 1.5 ml/min. Injected amount: 50 μg Dup 941. Retention times: main component: 8 min; impurity 5A: 6.4 min; impurity 5F: 14.2 min (from Ref. [2])

especially important to check the peak of the main component for its homogeneity from the simple and most widely used absorbance ratio method [3,4] to

more sophisticated deconvolution, spectral suppression, spectrum subtraction and other chemometric methods [1a,1b,4–9a]. If any kind of peak inhomogeneity is found (impurity on the leading or tailing edges of the main peak or fused impurity peaks, conveniently demonstrated in the three-dimensional mode) the diode-array spectra themselves furnish further information for the identification of the unresolved impurities. A more detailed discussion of this matter would be beyond the scope of this book.

(b) *Spectral matching.* Matching the diode-array spectra of components separated by HPLC with those taken by computer search from spectral libraries is a widely used method [1a,9a,10] especially in toxicological analysis [11]. This approach is of limited value in drug impurity profiling since it is unlikely that impurities of especially new drugs are included in spectrum libraries. However, matching the diode-array spectra of the separated impurities with standard materials can greatly support the identification of the impurities on the basis of retention matching [12] (see Section 2.1.1).

(c) *Structure elucidation of the separated impurities.* As described in Section 2.1.2 it is reasonable to begin the search for the structure of an unknown impurity separated by HPLC or CE with drawing as many conclusions from its diode-array UV spectrum as possible. Of course there are limitations excluding the possibility of the generalisation of this approach.

- The short-wavelength parts of the (diode-array) UV spectra can be subject of several distorting effects, moreover even false maxima can occur [9b]. In addition to this, short-wavelength UV bands can originate from different chromophoric functional groups and for this reason they are of limited value in the structure elucidation of organic compounds. As a consequence of these factors it is a prerequisite of drawing useful conclusions from the UV spectrum of an impurity that it should have at least one maximum above 210–220 nm.
- Another limitation is that the difference between the structures of the drug material and the impurity should be at or near the chromophoric part of the molecule in order that the difference between their spectra can be of diagnostic value in the structure elucidation of the impurity. For example, the chromophoric group of various steroids is the 4-ene-3-oxo group with an absorption maximum around 240 nm. As it will be shown later, the position of this band is influenced by substituents in the B and C ring of the steroid nucleus but by no means by substituents at C-17. For this reason various esters of 17-hydroxy-4-ene-3-oxo steroids (testosterone, 19-nortestosterone, 17-hydroxyprogesterone, etc.) cannot be differentiated on the basis of their UV spectra.

2.7.3.2. Practical Examples

If the main component and the impurity or impurities do have characteristic absorption above 210 nm and there are considerable differences between their spectra, the information obtainable from these spectra for the structure elucidation varies between modest contribution to the structure obtained with other spectroscopic techniques and a decisive role. Some characteristic examples, mainly from the author's laboratory are as follows.

The aim with the first example is to demonstrate that in advantageous situations, the UV spectrum of the impurity separated by HPLC and recorded by the diode-array detector is so much different from that of the main component and the difference is so characteristic that this can be the basis of the structure elucidation. In the course of one of the steps of the synthesis of *diltiazem* 4-methoxybenzaldehyde is reacted with chloroacetic acid methyl ester. The reaction scheme is depicted in Fig. 2.7.3.B. The crude product of the reaction was investigated by HPLC (Lichrosorb RP-18, 10 μm, 250 × 4 mm; acetonitrile–water 1:1 v/v). In addition to the product of the Darzens reaction (*trans*-(4-methoxyphenyl)-oxirane carboxylic acid methyl ester (**1**), $k' = 3.8$) an impurity (**2**) was detected at $k' = 10.6$. The diode-array spectra can be seen in Fig. 2.7.3.C. From these the following conclusions can be made. The very strong bathochromic shift of the *p*-band in the spectrum of **2** (73 nm relative to the phenolic ether type *p*-band of **1**) under which the α-band of **2** is submerged indicates that two conjugated double bonds are attached to the phenolic ether chromophore. Taking into consideration the simplicity of the molecules of the reactants the only possible molecule with this highly conjugated structure is α-chloro-4-methoxycinnamic acid methyl ester (**2** in Fig. 2.7.3.B). This supposition was later proved by other spectroscopic techniques [13]. Another example for large differences between the spectra of the main component and the impurities easily exploitable for the identification of impu-

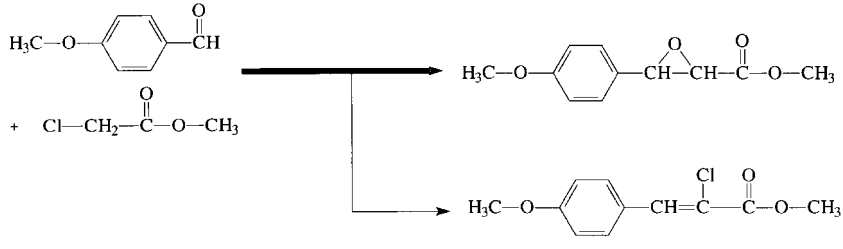


Figure 2.7.3.B.
Scheme of the reaction of 4-methoxbenzaldehyde and chloroacetic acid methylester with a side reaction (from Ref. [13])

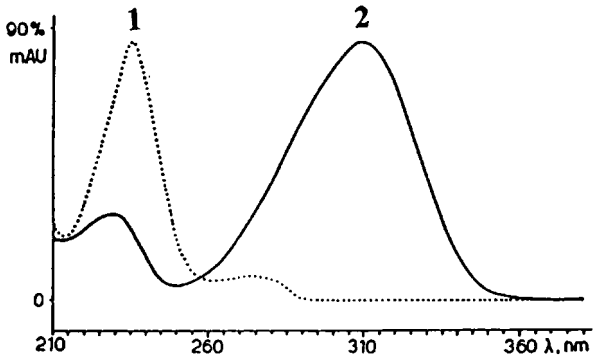


Figure 2.7.3.C.
Diode-array UV spectra of the main product (**1**) and by-product (**2**) of the reaction in Fig. 2.7.3.B. See Fig. 2.7.3.B for the structures and the reference

rities by the HPLC/diode array UV method is the study of the degradation mechanism of *physostigmine salicylate* (one of the degradation products is coloured!) [14].

In especially advantageous cases the main component is spectrophotometrically inactive or only poorly active while the impurity has a characteristic spectrum. For example in the course of the structure elucidation of the main impurity (2′-dehydro-pipecuronium bromide) in the fully saturated, spectrophotometrically only poorly active drug material *pipecuronium bromide*, in addition to NMR and MS studies [15] diode-array UV spectroscopy after HPLC separation in various systems [16,17] played an important role: the enamine-type impurity has an intense absorption band at 235 nm. The identification of a 3,5-diene impurity in *allylestrenol* is another example. The drug material with its isolated double bonds is spectrophotometrically rather poorly active. Of the two impurities containing an additional double bond the one showing a typical conjugated *trans*-diene spectrum with maximum at 234 nm was easily identified as the 3,5-diene derivative [18], while diode-array UV spectrophotometry was of no use in the identification of the isomeric non-conjugated 4,8(14)-diene derivative. For the formulae see Section 2.3.3, where the mass spectra of these impurities are discussed.

Profound differences were found by Görög *et al.* between the phenol-type spectra of *estradiol* [19], *ethinylestradiol* [20], *ethinylestradiol-3-methyl ether (mestranol)* [21] and impurities with analogous structures detected by HPLC. The diode-array spectrum of estradiol (**2**, retention time 6.2 min) and the impurity (**1**, 5.5 min) are shown in Fig. 2.7.3.D. The bathochromic shift of both the α- and *p*-bands by about 30 nm is an indication of the presence of a

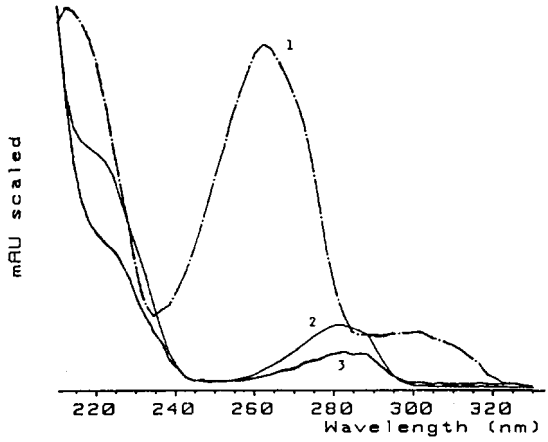
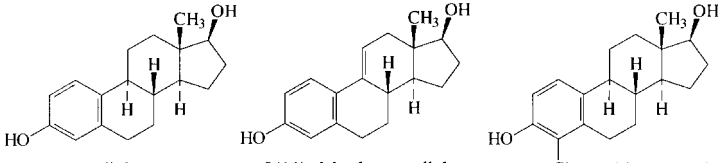


Figure 2.7.3.D.
Diode-array UV spectra of estradiol (**2**), 9(11)-dehydroestradiol (**1**) and 4-chloroestradiol (**3**) after separation by HPLC. Conditions: column: LiChrosorb RP-18, 10 μm, 250 × 4.6 mm; eluent: acetonitrile–water 7:3 v/v; flow rate 1 ml/min (from Ref. [19])

double bond in conjugation with the phenolic ring. The selection of the structure of the impurity from among the possible three regioisomers (Δ^6, $\Delta^{8(9)}$ and $\Delta^{9(11)}$ derivatives) was also possible on the basis of the comparison of the spectrum of the unsaturated impurity and spectral data for unsaturated phenol-steroids in the literature [22]. The possibility of the double bond being in the $\Delta^{8(9)}$ position could be excluded because in this case the conjugation band would appear around 274 nm overlapping the α band. The position of the conjugation band and the α band in the Δ^6 and $\Delta^{9(11)}$ derivatives is similar but the different ratio of their intensities is of diagnostic value: it is 2.9–3.8 for the former and 5.8–6.2 for the latter. Since the ratio for the impurity in ethinyloestradiol was found to be 6.4, its structure being 9(11)-dehydroethinylestradiol was considered to be proven [20]. This was verified by the comparison of the HPLC retention data and spectra of the impurity and the subsequently synthesised impurities of analogous structures in the two other cases also. The mechanism of the formation of the $\Delta^{9(11)}$-derivatives is shown in Fig. 9.4.A.

The diode-array UV spectrum of a second impurity (curve **3** in Fig. 2.7.3.D, retention time 11.2 min) is very similar to that of the main component and at first sight it seemed to be hopeless to draw any conclusion from it for the structure of impurity 3. Mass spectrometric investigations revealed the presence of one chlorine atom at one of the free positions in the phenolic ring (1, 2 or 4). Chlorination of phenols occurs in one of the *o*-positions: for

this reason the presence of the chlorine at position 1 could be excluded. In the literature [23], 285 and 294 nm are described for the wavelengths of the main and satellite peaks of 2-chloroestrone. Since these are at 282 and 288 nm in the spectrum of impurity 3, the chlorine being at position 2 could also be excluded. On the basis of this indirect evidence the impurity was claimed to be 4-chloroestradiol. This was proved by synthesis followed by retention and spectral matching.



Minor structural changes can cause profound differences in the UV characteristics. After successful HPLC separation this can be the basis for the solution of delicate problems by the diode-array detector. For example, during the course of the impurity profiling of crude *19-nortestosterone* (17β-hydroxy-estr-4-ene-3-one) obtained by the Birch reduction of estra-1,3,5(10)-triene-3,17β-diol 3-methyl ether (see Section 9.4.2.1) the GC/MS and HPLC investigations have shown that the main component was accompanied by several stereoisomers and regioisomers [24]. The discrimination between the stereoisomers of 19-nortestosterone and those of its regioisomer 17β-hydroxy-estr-1-ene-3-one was done by HPLC/diode-array UV (250 × 4 mm LiChrosorb SI-60 10 μm column with the 90:10 v/v mixture of n-hexane and 2-propanol as the eluent at a flow rate of 1 ml/min). The basis of the discrimination was that the 4-ene-3-ones at retention times of 11.63 min (19-nortestosterone), 13.35 and 18.08 min have their absorption maxima in the range 236–238 nm, while the maximum absorbance of the 1-ene-3-ones eluting at 8.35 and 10.16 min is at 226 nm.

Minor differences between the spectra of the main component and the impurities can also be the basis of useful conclusions drawn for the structure of the latter. For example, the addition of a methyl group to an isolated phenyl group transforming it from a toluene-like chromophore to a xylene-like chromophore results in a slight bathochromic shift of both the *p* and α bands. Such a shift was found in the case of an impurity in *enalapril* [15] separated by HPLC ($\alpha_{\text{impurity/enalapril}} = 1.78$; octylsilica column, 5 μm, 1:1 v/v mixture of methanol and phosphate buffer, 0.04 M, pH 2). The shift of the α band from 258 to 264 nm indicated the presence of an alkyl substituent presumably at position 4 (*p*-xylene-like spectrum). This supposition was supported by the

mass spectrum and NMR spectrum and finally by synthesis and retention matching.

In the case of the identification of *p*-toluenesulphonic acid as an impurity in *benzenesulphonic acid* (HPLC conditions: column: Vydac C-8, 5 μm, 250 × 4.6 mm; linear gradient, A: 0.02 M triethylammonium phosphate pH 3, B: 0.02 M triethylammonium phosphate–methanol–acetonitrile 20:40:40 v/v/v, 0 min 15%B, 5 min 15% B, 25 min, 68% B, 1 ml/min; t = 60°C) the shift of the *p* band from 213 to 221 nm was of diagnostic value. In this case no further spectroscopic evidence was necessary: the impurity could be identified by means of matching the retention times and UV spectra with a reference standard of the impurity [25].

In the case of complex molecular structures one has to be very careful with drawing conclusions from the location of the bands of the phenyl chromophore. Since the intensity of the α band is low and the *p* band is located in the very short wavelength spectral range, their position is of diagnostic value only in the case of very simple molecules (e.g. the above mentioned benzenesulphonic acid) or in those instances when the part of the molecule attached to the phenyl ring is spectrophotometrically inactive (enalapril). In many cases the bands of other, spectrophotomerically highly active parts of the molecule overlap with these bands thus making it impossible to base the structure elucidation on the shift of the latter. An example for this is the unsuccessful identification of 4′-methyl impurity in *flumecinol* (3-trifluoromethyl-α-ethylbenzhydrol) [26]. In a HPLC system consisting of a 25 × 4 mm LiChrosorb RP-18 column, 10 μm and the 7:3 v/v mixture of methanol and water as the eluent the main peak is eluted at 12 min, while the impurity in question is eluted at 18.5 min. No remarkable difference was found between the position of the α bands in spite of the fact that on the basis of MS and NMR studies the impurity was found to be 3-trifluoromethyl-4′-methyl-α-ethyl-benzhydrol. Spectral convolution studies revealed that the reason for the lack of the shift was that due to the hyperchromic effect of the trifluoromethyl group, the contribution of the trifluoromethylphenyl group to the intensity of their fused band is about 3-fold, compared to the unsubstituted and methylsubstituted phenyl rings.

An example of the special case where not primarily the location of the maximum but rather the shape of the absorption band obtained by the diode-array detector is the basis of the structure elucidation is the identification of 8(14)-dehydronorgestrel in *norgestrel* by Görög and Herényi [20]. The diode-array spectra of the main component and the impurity are shown in Fig. 2.7.3.E. The typical spectrum of the α,β-unsaturated ketone moiety can be recognised also in the spectrum of the impurity. However, the slight hypsochromic shift and especially the strong band broadening is characteristic of the 8(14)dehydro derivatives of 4-ene-3-ones where the two double bonds are not in conjugation

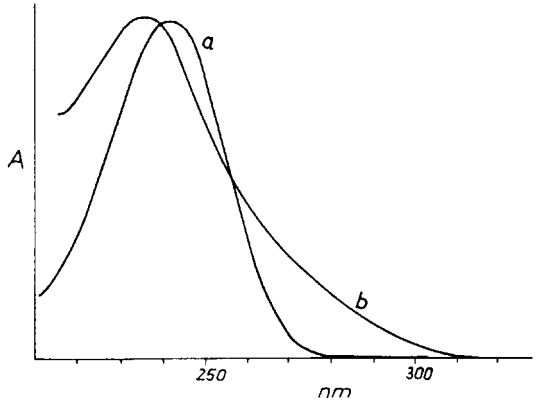


Figure 2.7.3.E.
Diode-array UV spectra of norgestrel (a) and 8(14)-dehydronorgestrel (b) separated by HPLC. Conditions: column: column: LiChrosorb RP-18, 10 μm, 250 × 4.6 mm; eluent: acetonitrile–water 7:3 v/v; flow rate 1 ml/min. Retention times: (a) 9.0 min, (b) 7.4 min (from Ref. [20])

but their ''through space'' interaction results in the distortion of the conjugation band [27]. The formulae of norgestrel and its 8(14)-dehydro derivative and the mechanism of the formation of the latter are shown in Fig. 9.4.A.

Further examples of this type are 4-ene-3-one and 11-deoxy-$\Delta^{9(11)}$ type impurities in cross conjugated 1,4-diene-3-ones, e.g. 1,2-dihydro and 11-deoxy-$\Delta^{9(11)}$ derivative of mazipredone [18,28]. As shown in Fig. 2.7.3.F, the maxima of the three curves are very close to each other but the shapes of the bands are considerably different. The 1,2-dihydro derivative lacking the cross conjugation have narrower bands, while in the case of the 11-deoxy-$\Delta^{9(11)}$ derivative band broadening, moreover an inflection around 260 nm is observable; both are of diagnostic value.

Chromatographic details and chromatograms are presented in Section 5.5.3. Analogous impurities in prednisolone were also characterised by their diode-array UV spectra [29].

As seen in part (a) of Fig. 2.7.3.G the difference between the diode-array UV spectra of triamcinolone acetonide and one of its impurities where the 9α-fluorine was replaced by 9α-bromine was even smaller: slight bathochromic shift accompanied with slight band broadening and an inflection around 270 nm. In order to increase the diagnostic value of these minor differences Cavina *et al.*. [30] found it useful to take the second derivative of the diode-array spectra. Part (b) of Fig. 2.7.3.G shows that the difference between the derivative curves is really much greater.

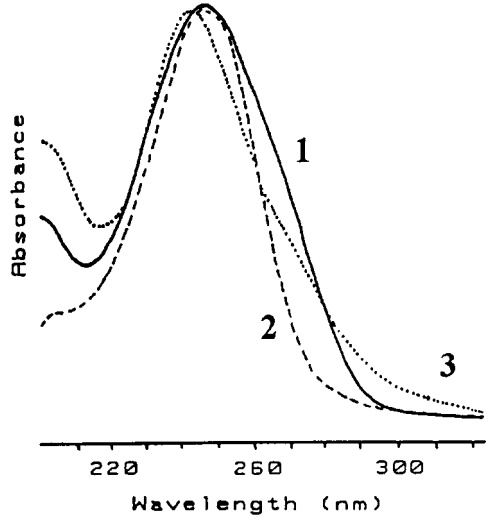
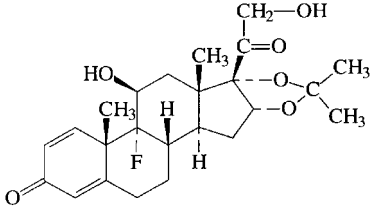
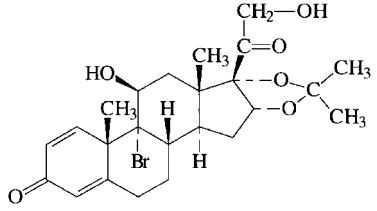


Figure 2.7.3.F.
Diode-array UV spectra of mazipredone (**1**), 1,2-dihydromazipredone (**2**) and $\Delta^{9(11)}$-dehydromazipredone (**3**) after HPLC separation. For the formulae and chromatographic conditions see Section 5.5.3 (from Ref. [18])



Triamcinolone acetonide



9-bromo derivative

The last example relates to the identification of highly polar impurities in norgestrel [24]. As seen in Table 2.7.3.A, their very short retention times related to the parent compound indicate the presence of an additional hydroxyl group in their molecules. It was supposed that these impurities are autoxidation products of norgestrel or originate from the autoxidation products of the intermediates in its synthesis and contain the hydroxyl groups in the vicinity of the 4-ene-3-one group, presumably in the 6 (α and β) and 10β positions. Table 2.7.3.A contains UV absorption maxima of hydroxylated 4-ene-3-oxosteroids

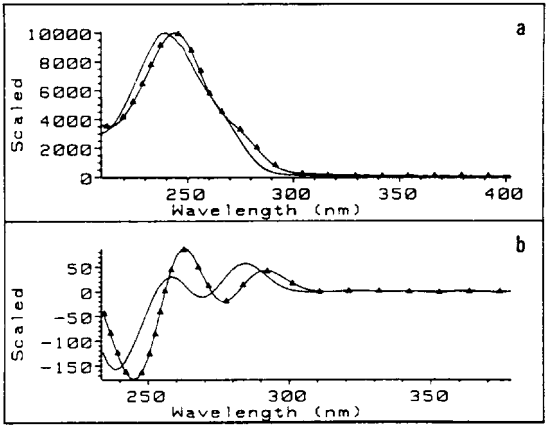


Figure 2.7.3.G.
Diode array UV spectra (a) and second derivative spectra (b) of triamcinolone acetonide (continuous line) and its 9-bromo derivative (triangle line) after HPLC separation. Conditions: column: SpherisorbODS-2, 5 μm, 250 × 4.6 mm; eluent: acetonitrile–water 36:64 v/v; flow rate 1 ml/min. Retention times: triamcinolone acetonide 6.41 min, 9-bromo derivative 8.67 min (from Ref. [30])

Table 2.7.3.A.
Estimation of the position of hydroxyl groups on the basis of the hypsochromic shift of their UV maxima

Hypsochromic shift effected by hydroxyl groups adjacent to the 4-ene-3-one group(nm) [22]		UV maxima of norgestrel and its polar impurities determined by the diode-array UV detector (nm)			
		Norgestrel	Impurities at various retention times (relative to norgestrel, 28.9 min)[a]		
			0.21	0.23	0.34
–	0	246			
6α-OH	1–2		245		
6β-OH	5–6			241	
10β-OH	4–7				239

[a] HPLC column: Hypersil ODS, 5 μm. Linear gradient from 45:55 to 56:44 v/v mixture of methanol and water within 40 min at 1.3 ml/min (from Ref. [24])

taken from the literature [22] and those of the separated impurities taken by the diode-array detector. As seen even the minor differences (hypsochromic shift relative to the non-hydroxylated main component) are of diagnostic value: the supposed structures were supported by the UV spectra. The three compounds were synthesised and successful retention and spectral matching was carried out with the impurities.

Of the numerous further applications of this technique the papers of Ryan dealing with the impurity profiling of various drugs [31], especially barbiturates [32,33] are worth mentioning. Many more examples for the application of diode-array UV spectra with the aim of identifying impurities can be found in the book of Huber and George [1c] and for degradation products in Sections 5.3.2, 5.4.3 and 5.5.3.

References

1. L. Huber and S.A. George (Eds.), *Diode Array Detection in HPLC*, Marcel Dekker, New York, (1993); a, H.-J.P. Sievert and A.C.J.H. Drouden, *Spectral Matching and Peak Purity,* pp 51–126; b, J.K. Strasters, *Chemometrics and Photodiode Array Detection,* pp 127–161; c, R.W. Giuffre, *The Use of Diode Array Detectors in the Pharmaceutical Industry*, pp 163–197
2. E.C. Nicholas and T.H. Schools, *J. Pharm. Biomed. Anal.* **16**, 813–824 (1998)
3. A.C.J.H. Drouen, H.A.H. Billiet and L. De Galan, *Anal. Chem.* **56**, 971–978 (1984)
4. T.D. Wilson, W.F. Trompeter and H.F. Gartelman, *J. Liq. Chromatogr.* **12**, 1231–1251 (1989)
5. A.F. Fell, H.P. Scott, R. Gill and A.C. Moffat, *J. Chromatogr.* **282**, 123–140 (1983)
6. J.G.D. Marr, P. Horváth, B.J. Clark and A.F. Fell, *Anal. Proc.* **23**, 254–256 (1986)
7. H.K. Chan and G.P. Carr, *J. Pharm. Biomed. Anal.* **8**, 271–277 (1990)
8. N.H. Anderson, M.R. Gray and C.J. Hinds, *J. Pharm. Biomed. Anal.* **8**, 853–857 (1990)
9. S. Görög, *Ultraviolet-Visible Spectrophotomery in Pharmaceutical Analysis*, CRC Press, Boca Raton, FL (1995). a, 141–148; b, 24–36
10. T. Alfredson, T. Sheenan, T. Lenert, S. Aamandt and L. Correia, *J. Chromatogr.* **385**, 213–223 (1987)
11. M. Bogusz and M. Wu, *J. Anal. Toxicol.* **15**, 188–197 (1991)
12. E.C. Nicolas and T.H. Scholz, *J. Pharm. Biomed. Anal.* **16**, 813–824 (1998)

13. S. Görög, M. Rényei and B. Herényi, *J. Pharm. Biomed. Anal.* **7,** 1527–1533 (1989)
14. S. Rubnov, D. Levy and H. Schneider, *J. Pharm. Biomed. Anal.* **18**, 939–945 (1999)
15. S. Görög, G. Balogh and M. Gazdag, *J. Pharm. Biomed. Anal.* **9**, 829–833 (1991)
16. G. Szepesi, M. Gazdag and K. Mihályfi, *J. Chromatogr.* **464**, 265–278 (1989)
17. M. Gazdag, M. Babják, P. Kemenes-Bakos and S. Görög, *J. Chromatogr.* **550**, 639–644 (1991)
18. S. Görög, M. Babják, G. Balogh, J. Brlik, A Csehi, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *Talanta* **44**, 1517–1526 (1997)
19. S. Görög, J. Brlik, A. Csehi, Zs. Halmos, B. Herényi, P. Horváth, A. Laukó, F. Dravecz and D. Bor, *Anal. Methods Instrum.* **2**, 154–157 (1995)
20. S. Görög and B. Herényi, *J. Chromatogr.* **400**, 177–186 (1987)
21. S. Görög, A. Laukó and B. Herényi, *J. Pharm. Biomed. Anal.* **6,** 697–705 (1988)
22. J.P. Dusza, M. Heller and S. Bernstein, in *Physical Properties of Steroid Hormones* (L.L. Engel, Ed.), p 95. Pergamon Press, Oxford (1963)
23. E. Schwenk, C.G. Castle and E. Joachim, *J. Org. Chem.* **28**, 136–142 (1966)
24. S. Görög, M. Bihari, É. Csizér, F. Dravecz, M. Gazdag and B. Herényi, *J. Pharm. Biomed. Anal.* **14**, 85–92 (1995)
25. S. Görög, M. Babják, P. Horváth, É. Osztheimer and M. Rényei, *Acta Pharm. Hung.* **69**, 60–68 (1999)
26. S. Görög, B. Herényi and M. Rényei, *J. Pharm. Biomed. Anal.* **10,** 831–835 (1992)
27. E.C. Herrmann and G.-A. Hoyer, *Chem. Ber.* **112**, 3748–3752 (1979)
28. M. Gazdag, M. Babják, J. Brlik, S. Mahó, Z. Tuba and S. Görög, *J. Pharm. Biomed. Anal.* **17**, 1029–1036 (1998)
29. S. Görög, M. Babják, G. Balogh, J. Brlik, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *J. Pharm. Biomed. Anal.* **18**, 511–525 (1998)
30. G. Cavina, R. Alimenti, B. Gallinella and L. Valvo, *J. Pharm. Biomed. Anal.* **10**, 685–692 (1992)
31. T.W. Ryan, *Anal. Lett.* **31**, 651 (1998)
32. T.W. Ryan, *J. Liq. Chromatogr.* **16**, 33–49, 315–329 (1993)
33. T.W. Ryan, *Anal. Lett.* **31**, 2447–2456 (1998)

2.7.4. HPLC/MS for drug impurity identification

Lars Tollsten

2.7.4.1. Introduction

With the introduction of bench-top mass spectrometers for HPLC, controlled by more and more user-friendly software, mass detectors are now routinely used in numerous laboratories in the pharmaceutical industry. The aim of this section is to give a short introduction to present techniques and instrumentation in the field of HPLC/MS, and their use in the identification of drug impurities. Attention will be paid to problems regarding the change of an HPLC/UV method to set up an optimal HPLC/MS method. Examples of MS methods for obtaining structural data for impurities will be given. Although there are many possible instrumental set-ups, the focus will be on electrospray mass-spectrometry (ESI-MS). Details of electrospray fundamentals, the interfacing of electrospray to MS, and practical considerations for ESI-MS analysis are compiled in a book by Cole [1]. Here, some specific examples from recently published studies will be included, mixed with the presentation of data from the author's laboratory. The identification of an impurity in the β-receptor blocker *metoprolol succinate* will be used as one case study, the work with characterising a dimer present in the diuretic *hydrochlorothiazide* and the study of impurities/degradants in a dihydropyridine drug substance are other examples. Several strategies for drug impurity profiling have been presented in the literature [2–4], all stressing the importance of qualitative data from hyphenated chromatography-mass spectrometry like GC/MS and HPLC/MS, in combination with other separation and spectroscopic techniques.

2.7.4.2. History and Development of HPLC/MS Interfaces

Several techniques for interfacing HPLC separations to MS detection have been tried and a first breakthrough came with the introduction of the thermospray interface. Since the atmospheric pressure ionisation (API) source was introduced as an interface for HPLC and MS in the mid and late 1980s there has been a tremendous increase in HPLC/MS applications. The first part of the book by Niessen [5] deals with the history and evolution of HPLC/MS interfacing techniques, followed by a comprehensive presentation of different

interfacing techniques available today. The use of electrospray MS has extended the applicability of mass spectrometry to include a wider range of molecules than could previously be analysed. Most types of molecules evaluated for drug purposes can be analysed using electrospray MS, including medium polar to polar compounds, ranging from low molecular weight amines or acids up to much larger peptides and proteins. Based on the ability of the electrospray ionisation process to produce multiply charged ions, the m/z values of peptides or proteins are seen as distributions of signals within the mass range of the mass analyser, allowing the original molecular weight to be calculated from the envelope of multiply charged ions. The further development of the electrospray interface during the last years, has been aiming in two different directions. One is the effort to solve analytical problems using less and less amounts of analytes, eventually leading to the introduction of nano-electrospray [5,6]. By a passively driven electrospray from a gold-coated disposable spray needle, a flow-rate of only 20–40 nl/min was achieved, and only 1 μl of sample solution was enough for 30 min MS and MS/MS experiments. Another direction of electrospray evolution is to allow higher flow-rates of more complex matrices (like non-volatile buffers) to be sprayed. The use of pneumatically assisted electrospray in combination with the use of heated drying gas allowed for high LC flow-rates. The instrumental design using electrospray ion-sources with off-line spraying geometry, like orthogonal sprays, is less sensitive to high volatile buffer concentrations or non-volatile buffers [5].

2.7.4.3. Mass-Analysers for HPLC/MS

After the introduction of the API ion source for HPLC/MS, most analysers can be easily integrated into HPLC systems to function as HPLC detectors. Basic considerations to be made before purchasing a new MS detector such as the need for skilled operators to handle different analysers, the laboratory resources needed, and most important the type of sample information that can be extracted from an analysis have been reviewed [1,7].

Most commonly used is the quadrupole mass analyser, being present in ''single-quad'' or ''triple-quad'' configurations. A typical m/z interval of a quadrupole is 20–3000, operated at unit resolution (mass accuracy 0.1–0.2 amu), and scanned at about 1 scan/s. With an ESI-interface normally only molecular weight information is obtained from the positive or negative pseudo-molecular ion. By increasing the accelerating voltage through the transfer quadropole/skimmer region, energies of ions are increased, causing gas-phase collisions and fragmentation, allowing some structural information also from a ''single-quad'' analyser. This is known as in-source CID (colli-

sion-induced dissociation). The ''triple-quad'' analyser can be set up in various MS/MS modes for more structural data, caused by gas-phase collisions of higher energy induced by accelerating ions through a gas-containing collision cell.

The ion-trap analyser has some very useful features compared to other analysers. One is the MS^n capability, were a precursor ion is fragmented, one fragment-ion is selected and activated to produce a second generation of fragments, thus resulting in very detailed structural information useful in identification work or mechanistic studies. In theory several generations of ions can be produced, but in practice the intensities of ions are normally enough up to the MS^4 or MS^5 generation. Ion-traps can also be used at increased resolution modes within a limited m/z interval, to obtain information of the charge-state of multiply charged ions, or to obtain higher mass-accuracy of selected ions. The increased resolution mode is software controlled and no other adjustments are needed.

For some time matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) analysers have been successfully used for samples in a matrix absorbing laser energy. Time of flight analysers are now also successfully interfaced with continuous HPLC/ESI sources, as the flow of ions produced can be orthogonally pulsed into the flight-tube of the mass-spectrometer. Advantages of TOFs are many, including very high scan rates, a high mass-range, high sensitivity due to the simultaneous collection of all ions produced, and the capability of producing high-resolution spectra. For structural elucidation, hybrid instruments with a quadrupole and TOF analyser in series, can produce high quality MS/MS spectra including high-resolution data for the determination of molecular formulae.

Magnetic sector instruments are manufactured in a variety of configurations of magnetic sector (B) and electric sector (E) analysers. Their typical advantage in HPLC/MS and MS/MS experiments is the acquisition of high-resolution mass-spectra, for the determination of structural formulae as an aid in impurity identification. An exact mass determination is made using known calibrants (or lock-masses) either in the HPLC mobile phase, or directly in the sample solution during direct infusion experiments.

In Fourier transform mass spectrometers (or ion cyclotron resonance MS) the frequency of rotation of ions trapped in a combined magnetic and electric field is related to their mass. Both single MS and MS/MS experiments can be performed at good accuracy and ultra-high resolution. Interfaced to API or MALDI ion sources FTMS detectors are performing extremely well, but because of their cost they have mainly been used as research instruments.

2.7.4.4. Optimisation of Reversed-Phase HPLC/MS (Buffers, pH, Organic Solvents, Adducts and Ion-Pairing Agents)

2.7.4.4.1. Basic Considerations

The ideal case of method transfer from a traditional analytical HPLC system to an HPLC/MS system, is a direct transfer without the need for any alterations to the separation method. In such cases there would be no doubt about the identity of any minor impurities compared to the HPLC/UV method. This is a desired condition especially for complex samples when impurity references for verification and spiking of the new method are absent. Development of new instrumentation, with special emphasis on API source design, is aiming in the direction of robustness of interfaces to most HPLC mobile phases. However, in reality there are a number of factors to keep in mind when setting up an efficient and robust HPLC/MS method and optimising it for daily operation [1,5,7].

A basic consideration is to judge whether the drug substance of interest is likely to produce any ions at all (see Section 2.7.4.4.3 below). Chemical derivatisation as a method to make possible or enhance the detection of analytes by HPLC/MS is one possibility [8–10], but the detailed chemistry lies outside the scope of this section.

If ions are formed, it should be kept in mind that responses are not straight-forward as in UV-detection, some analytes having detection limits orders of magnitudes lower using MS compared to UV detection, others being hardly detected at all. However, in the evaluation of drug impurities with poor or no UV chromophores, the usage of HPLC/MS may be the best method to study impurity profiles. Several different methods, HPLC/MS, HPLC/NMR, refractive index, evaporative light scattering and indirect detection, were evaluated as alternatives to UV-detection by McCrossen *et al.* [11], in studies of impurities of a drug substance. The identification of the impurities was made by the interpretation of mass-spectra and NMR spectra, and impurities were concluded to show MS response factors close to the drug substance itself, as long as the phosphorous moieties were retained in the impurity structure. Most often, quantitative analyses with HPLC/MS are made with internal standards being chemically very close to the analyte studied. A typical example is to use the deuterated analyte of interest as an internal standard [12], which is not an option when working with unknowns. The best way to quantify an impurity at the 0.1% level, is to dilute the bulk-drug substance 1000 times, and use that peak as an external standard for the HPLC/MS response. This will work for substances showing the same ionisation properties, whereas quantities of other compounds may be either over- or underestimated.

2.7.4.4.2. Mobile Phase Buffer Salts

Buffer salts like phosphates, citrates and borates that are non-volatile should generally be avoided in HPLC/MS methods. These salts can cause space-charging or clogging of sampling cones or capillaries in the API interface, but they also interfere with the electrospray process itself by changing the surface tension of droplets thus affecting the ion-evaporation process [13].

Desalting methods have been used to remove or exchange unwanted buffer salts from HPLC mobile phases, to allow the use of mass detection. Post-column phosphate suppression, using an anion micromembrane suppressor could exchange more than 99% phosphate at 10 mM ammonium phosphate concentration, at a chromatographic flow-rate of 0.1 ml/min, into a particle beam MS [14]. However, the exchange of phosphate to OH^- changed the pH from 7 to 12, which would largely influence the ionisation process on an electrospray MS system. A ''peak tracking'' HPLC/MS system set-up [15] allowed fractions of the chromatogram to be switched into a system using volatile buffers, where the fraction was re-chromatographed on a short column to desalt the sample, and switched into the mass detector for molecular weight determination.

By reducing chromatographic flow rates the total amount of buffer salts entering the ion-source is reduced. This is critical for a stable and robust operation over time. It was demonstrated by Cappiello *et al* [16] that a 1 μl/min flow of 10 mM potassium phosphate into a particle beam interface was possible, using a microscale flow nebuliser, and good quality mass spectra were recorded from the phosphate system.

Modern APCI and ESI interfaces are designed to cope with flow rates of up to 1 ml/min (or above), which are typical for 4.6 mm i.d. analytical columns [5,13]. The APCI interface often works well with flows in the range of 0.5–1.5 ml/min, the ESI interfaces have much lower optima. A conventional electrospray is restricted to flow rates of 1–10 μl/min, whereas commercially available pneumatically assisted electrospray interfaces can be operated at 1 ml/min, but produce optimal signal to noise ratios at 100–300 μl/min [13]. The flow-rate optimisation of a standard HPLC method to an HPLC/MS method could be achieved either by using 1–2 mm i.d. columns, operated at 50–250 μl/min, or by using post-column flow-splitting. The splitter could be a simple T-piece with different length of capillary restrictors for different split-ratios, or a more advanced commercial fixed flow-splitter, directing the optimal flow-rate to the mass-spectrometer and the rest to waste. Optimal flow-rates might be even lower if non-volatile buffers are sprayed, and the robustness of operation over time is considered.

2.7.4.4.3. Mobile-Phase Solution pH

In HPLC/UV analyses the choice of mobile phase pH and organic modifier is made based on achieving the most efficient separation taking into consideration possible interference with the UV signal. On the contrary, when setting up a method for HPLC/ESI-MS, the ability to ionise a particular compound should also be considered. The rule is that basic compounds are analysed in acidic mobile phases to produce protonated positive ions, and acidic compounds are analysed in basic mobile phases to produce negative ions. It should be mentioned that the electrospray ionisation process is not necessarily controlled by the pK_a of a substance, resulting in the protonation of a base being possible both below and above its pK_a value. The term ''wrong-way-round'' electrospray ionisation was used by Mansoori *et al*. [17] to describe the fact that amino acids produced protonated as well as deprotonated ESI-MS signals from mobile phases of a pH range between 3 and 11. However, looking at signal intensities the amino acid analysed followed the general rule of analyte protonation. Preferred volatile mobile phase additives for low pH are normally formic acid, acetic acid or trifluoroacetic acid (TFA). Neutral or basic additives are ammonium acetate, ammonium acetate/ammonia or various volatile amines. Post-column addition could be used to change the solution pH entering the ion-source, allowing the chromatographic separation and MS detection at different pH. The use of TFA, especially in peptide or protein analysis, can result in signal suppression. This is caused by a strong ion-pairing between basic analytes and TFA interfering with the electrospray process. This suppression can be overcome by post-column addition of organic acid/isopropanol [18,19], changing the equilibrium of ion-pairing and reducing surface tension in spray droplet formation. The addition of this ''TFA fix'' consisting of propionic acid/isopropanol in a 75:25 mixture and a 1:2 proportion to the mobile phase, resulted in a 10–50 fold improvement of the electrospray signal. The use of butyric acid showed even higher signals but was ruled out due to its obnoxious smell.

2.7.4.4.4. Organic Solvents

To optimise the HPLC separation the choice of organic solvent in the mobile phase might be essential. Normally acetonitrile or methanol are used for reversed-phase chromatography. However, the type and ratio of organic modifier optimised for the chromatographic separation, is not necessarily optimal for the best response in the electrospray process. An enhancement of up to 20 times signal intensity was seen with methanol based mobile phases compared to acetonitrile based ones, for drug substances under development [20]. If sensitivity is a critical issue, it is advisable to determine the signal intensity

in methanol- or acetonitrile-based solvent mixtures at different pH values. A scheme was followed combining mobile phases with ammonium acetate, formic acid and mixtures, with either methanol or acetonitrile used as an organic modifier. The intensity of protonated, deprotonated and $[M + NH_4]^+$ ions was evaluated. In the evaluation of several organic solvents for negative ion ESI-MS, the most intense signal and less noise was seen in spectra obtained in isopropanol [21]. Less electrical discharge in the ion-source was also obtained using isopropanol with oxygen added as an electron scavenger gas. Another scavenger gas used was SF_6, reducing corona discharge and increasing signal in both positive and negative ionisation mode, especially when mixtures with a high water content were sprayed [22].

2.7.4.4.5. Adduct-ion Formation

Normally, protonated pseudomolecular ions (or deprotonated ions in negative mode) are expected to occur in spectra from ESI-MS experiments. However, adduct formation in the ion source is a quite common phenomenon and several adduct ions or combinations of adducts are to be expected. Some common adducts are $[M + H + CH_3CN]^+$ and $[M + H + CH_3OH]^+$ potentially formed when acetonitrile or methanol are present in the mobile phase, as well as the acetic acid and TFA adducts $[M + H + CH_3COOH]^+$ and $[M + H + CF_3COOH]^+$ where these acids are used to control the pH of the mobile phase. Acetic acid and TFA adducts can also be seen as the negative ions $[M–H + CH_3COOH]^-$ or $[M–H + CF_3COOH]^-$. Some substances have high affinity to sodium, potassium or ammonium ions compared to protons. This results in pseudomolecular ions of $[M + Na]^+$, $[M + K]^+$ or $[M + NH_4]^+$, respectively (see example in Fig. 2.7.4.A). Combinations of the formation of cations and solvent adducts can sometimes cause difficulties in the interpretation of ESI mass-spectra. One way to reduce adduct formation is to modify the mobile phase composition. Another way is to increase the fragmentation voltage (orifice voltage, CID offset) to break-up solvent clusters by adding kinetic energy to the ions. This will not always remove sodium ions from the adducts which are often very stable, and these stable electrospray ions can instead be used to increase the signal response, by adding 10^{-5}–10^{-3} M sodium or other cations to the mobile phase [23]. The enhancement of signal intensity generally seems to be related to the size of the cation used, ranging from lithium up to rubidium and cesium ions. In the case of HPLC/MS methods using non-volatile phosphate buffers, there is a risk that the signal background will contain a series of phosphate clusters that increase the overall baseline noise, interfering with spectra, and thus resulting in a poor detection limit.

Other types of ions seen in electrospray mass-spectra are clusters of dimer complexes formed in the ion-source. These clusters are preferentially formed

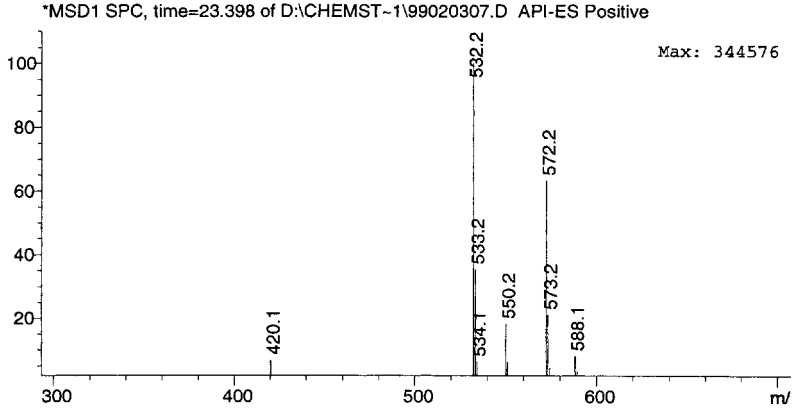


Figure 2.7.4.A.
Positive ion mass spectrum of a drug substance with M = 549.6. The spectrum shows the $[M + H]^+$ ion at 550, $[M + Na]^+$ ion at m/z 572, $[M + K]^+$ ion at m/z 588, as well as the loss of water in the ion-source at m/z 532, indicating the presence of a hydroxyl function in the molecule

when the concentration of the analyte is high in the source, and they can easily be distinguished from covalently bound dimers by their m/z ratio. For example, in the positive mode the dimer complex gives a signal higher by two amu than expected from a protonated dimer. In the same way as peptides and proteins show envelopes of signals due to multiple charging, drug substances with two or more basic functions can be doubly charged or more. The best way to distinguish a double or triple charged ion from a single charged one, is based on the investigation of the distance between the ^{12}C and the ^{13}C isotope peaks, the difference of one for a singly charged species, being reduced to 0.5 and 0.3 for double and triple charged species, respectively.

2.7.4.4.6. Ion-Pairing Agents for HPLC/MS

To increase the retention of polar analytes ion-pairing is often used in HPLC/UV methods. The use of ion-pairing agents like octane sulphonic acid should be avoided in HPLC/MS, as they are non-volatile and likely to result in signal reduction and a high mass spectrometric background. Instead, other ion-pairing reagents can be selected, which are volatile enough to be used in HPLC/MS. For example, various peptides and proteins were successfully analysed using perfluorinated carboxylic acids as the ion-pairing agents: the retention of analytes was significantly increased, with isocratic or gradient elution on a standard C_{18} reversed-phase column [24]. The acids used ranged from perfluor-

opropionic to perfluoroheptanoic acid, in 10 mM concentrations. A trifuoroacetate buffer was used for the identification of several impurities in *tepoxalin* [25]. We used perfluoropentanoic acid in a reversed-phase positive-ion HPLC/MS application, where an acceptable retention was obtained of the polar drug substance, and some of its polar impurities. Some other impurities were sufficiently retained in a standard method and not affected by ion-pairing. No interfering ions were detected in the positive mode. However, when using the mass detector for negative ion analysis the presence of large background ions originating from the ion-pairing agent were detected. Extensive washing of the chromatographic system and of the ion-source was required to remove the elevated background signal.

In the identification of adenine nucleotide containing metabolites of *bisphosphonate drugs* using reversed-phase negative ion HPLC/MS, the use of dimethylhexyl amine as an ion-pairing agent, proved to be a useful tool [26]. The mobile phase used was 10 mM ammonium formate, 10 mM dimethylhexyl amine and 15% acetonitrile at a flow of 0.5 ml/min directly into an ESI source of a Finnigan LCQ ion trap detector. One advantage over other used methods was the higher sensitivity achieved with MS detection, another was the MS/MS capabilities used to distinguish different metabolites. In another study of nucleotides, Witters *et al.* [27] found that tetrabutylammonium bromide could be used as an ion-pairing agent, with a maximum effect on retention of analytes at 50 μM concentration. At a flow rate of 10 μl/min operation was allowed for over 120 h, and the post-column addition of isopropanol did enhance the signal intensity.

2.7.4.5. Straight Phase HPLC/MS

Connecting electrospray or APCI-MS to straight phase or normal phase separations is not yet very common. Applications that can gain from the straight phase HPLC/MS hyphenation are chiral separations, as well as the analysis of samples in matrices that are incompatible with reversed phase chromatography. An alternative to straight phase HPLC/MS is SFC/MS (see Section 2.10.4).

The potential risk of explosion when introducing, e.g. hexane based mobile phases into an API interface with a spray voltage or corona discharge of 4–5 kV must be considered. In two examples of chiral analyses [28,29] it was concluded that a safe operation is achieved by using nitrogen at rather high flow rates as a nebuliser gas, by adding a mixture of isopropanol and water as a post-column modifier, and by keeping HPLC flow rates low. The high flow rates of nitrogen will efficiently remove oxygen from the ion-source, thus reducing explosion risks. The post-column addition of a 1:3 v/v mixture of

aqueous 0.025 M ammonium acetate and isopropanol provides miscibility with hexane based mobile phases, and adds extra water to the ion-source.

We have evaluated an isocratic straight-phase system of heptane–ethanol 90:10 v/v on a YMC PVA-Silica column (4.6 × 150 mm) at 40°C and a flow rate of 1 ml/min, to separate the dihydropyridine drug *clevidipine* and its impurities/degradants. The aim was the development of a direct method for its analysis in a lipid emulsion without complicated sample preparation. The HPLC system was a Hewlett–Packard 1050 instrument with UV-detection at 220 nm. Negative ion ESI mass spectra were recorded by a Finnigan TSQ 7000 API2 in full scan mode, nitrogen sheath gas at 80 psi, spray at 3.5 kV, capillary temperature at 350°C, and a post-column split giving a flow of approximately 100 μl/min to the ESI ion-source. A sheath liquid flow of 100 μl/min of 1 mM aqueous ammonium acetate–acetonitrile 80:20 v/v was added. By using a sheath-flow addition we avoided the need for checking miscibility of phases, but risked a lower efficiency in protonation and a lower signal intensity compared to standard post-column addition. In the analysis of reference drug substance, manual splitting of the drug substance to waste was made to keep the ion-source as clean as possible, and to allow the injection of high amounts of impurities/degradants. The monitoring of the 220 nm on-line UV signal intensity was used to decide when to divert the main peak to waste. The small peaks at 5.4 and 6.0 min are the remaining signals of the main peak. By plotting the extracted ion chromatograms of some known impurities (m/z 354, 382, 452 and 493) these were easily detected down to the 0.1% level (Fig. 2.7.4.B). The structures of clevidipine and its impurities were previously known [30], and are presented in Fig. 2.7.4.C.

2.7.4.6. Micro-Column HPLC/MS

Microcolumn LC has some clear advantages over conventional HPLC. The use of 50–320 μm i.d. columns at flow rates of 0.1 μl to a few μl/min offers high chromatographic efficiency, lower sample consumption and lower mobile phase consumption. As ESI-MS is a concentration sensitive method, the narrower peaks obtained from a sample injected at a capillary-LC compared to conventional HPLC system will result in a better sensitivity. Analyses using down to 2 mm i.d. columns can be made with standard HPLC systems, whereas microbore or capillary LC systems must be specially designed to deal with low flow-rates, to operate robustly and to avoid band broadening caused by the system. Pumps, injectors, detectors and overall extra-column dead-volumes must be suited for low flow rates. Stable isocratic chromatography needs pumps designed to deliver low flow rates. Stable gradients for capillary LC can be produced using a gradient mixing chamber

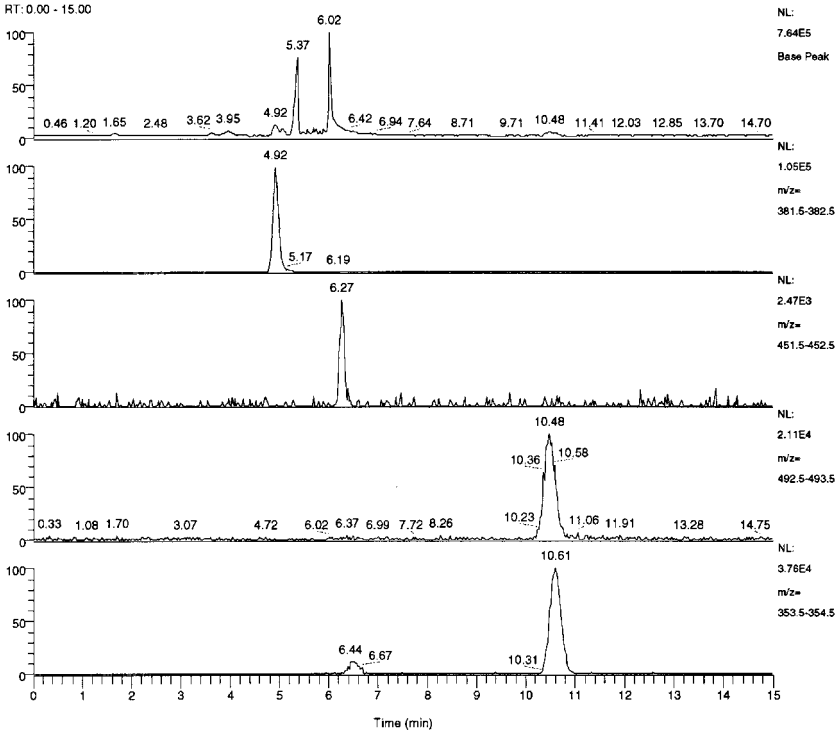
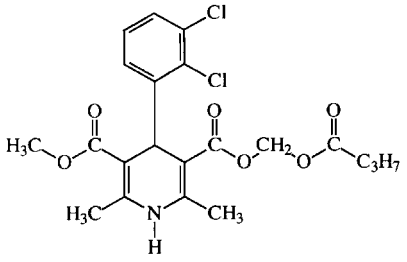


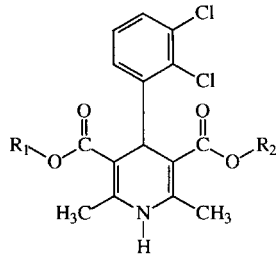
Figure 2.7.4.B.
Straight-phase HPLC/MS of clevidipine and impurities. Top trace is the TIC with the main peak diverted to waste between 5.4 and 6.0 min. The extracted ion-chromatograms of known impurities at m/z 382, 452, 493 and 354 are plotted

of variable volume, in an on-line analysis [31]. It was suggested that the use of a sheath-liquid in a microcolumn LC/MS analysis was efficient to stabilise the electrospray, and a way to increase signal intensity by addition of cations [23] (see Section 2.7.4.4).

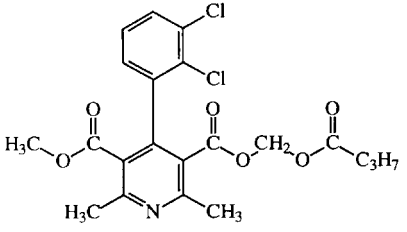
In the analysis of some *β-blockers*, capillary LC/MS was used at a flow rate of 4 μl/min by the post-column addition of 200 μl/min of methanol [32]. The MS sensitivity was high using MS^n and selected reaction monitoring. A microflow splitter was used to create the chromatographic gradient.



Clevidipine M = 455



R_1,R_2 = CH_3, C_2H_5 M = 383
R_1,R_2 = CH_3, H M = 353
R_1,R_2 = CH_3, CH_2-CH_2-CN M = 493



Corresponding pyridine derivative M = 453

Figure 2.7.4.C.
Structure of clevidipine and some impurities detected by straight-phase HPLC/MS

2.7.4.7. Flow-Injection MS

The term flow-injection analysis (FIA) is used here for the sequential injection of the same or different samples, without any separation of analytes on an HPLC column. One application of this technique is in high throughput analysis which is normally not an issue related to impurity identification. Its use in quantitative analysis where linearity and detection limits are crucial should be avoided, since interferences from salts, etc. in the sample solution can be prominent. FIA has been used in the author's laboratory in the control of fractions collected after solid-phase extraction for the concentration of impurities of drug substances (see Section 2.7.4.13) and in the optimisation of electrospray ion-source parameters like spray voltage, nebuliser gas flow and fragmentation voltage. An example with *hydrochlorothiazide* (HCTZ) is presented in Fig. 2.7.4.D. A solution of 0.5 mg/ml HCTZ was injected at 1 min intervals, in a flow of 0.5 ml/min of 50:50 v/v mixture of water and acetonitrile with 1 mM ammonium acetate. The stepwise increase of the frag-

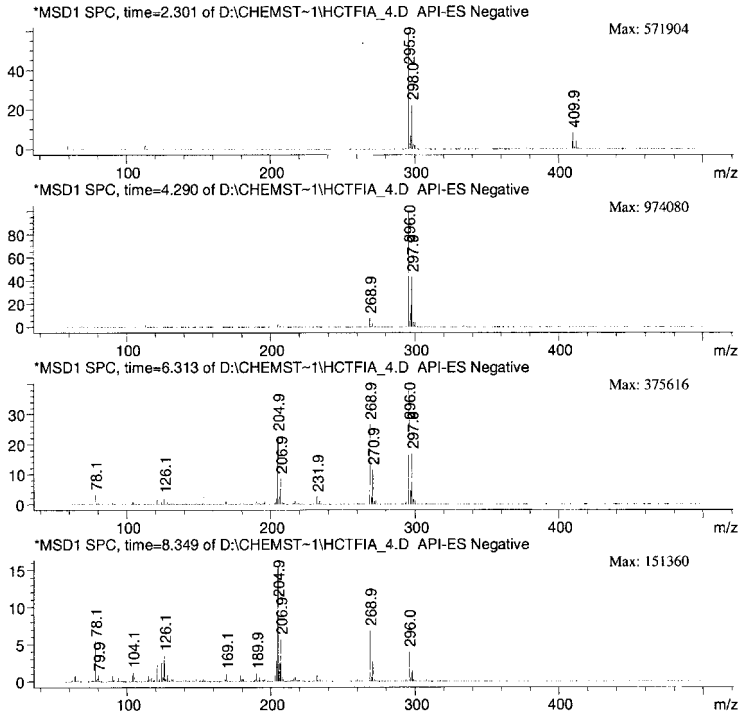


Figure 2.7.4.D.
Flow-injection analysis of 0.5 mg/ml solution of hydrochlorothiazide at different fragmentation voltages for evaluation of MS parameter settings. From top: 70, 110, 150 and 190 V. By a fragmentor setting of 110 V high intensity combined with low fragmentation was achieved. Hewlett–Packard LC/MSD system; negative ion ESI; full scan 50–500; spray voltage 3.5 kV

mentation voltage results in a series of negative-ion mass spectra showing different fragmentation patterns. One spectrum was recorded at a fragmentation voltage of 70 V, showing the $[M–H]^-$ peaks at m/z 296 and 298, and a TFA adduct at m/z 410 and 412. In the following spectra, the fragmentor voltage was increased stepwise by 20 V, first breaking up the TFA adduct, and at higher voltages inducing more fragmentation. The suggested structures of major fragments produced are presented in Fig. 2.7.4.E. The most intense signal was obtained with the 110 V setting. As shown here, flow-injection analysis is a quick and very powerful tool in HPLC/MS method development and optimisation, as well as a direct method for collecting MS fragmentation data.

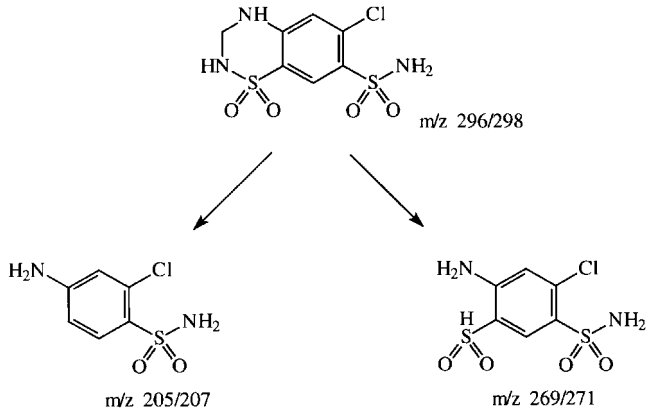


Figure 2.7.4.E.
Hydrochlorothiazide and structures of the most abundant fragment ions produced by FIA and in-source CID. For details see Fig. 2.7.4.D

2.7.4.8. Peak-Purity Determination by HPLC/MS

Modern software for controlling HPLC/MS systems often have built-in functions for peak-purity control. Diode-array UV detectors are generally used for this purpose (see Section 2.7.3); the purity check is based on the comparison of the UV-spectra recorded at the start, apex and end of a chromatographic peak. In a similar way, a comparison of mass-spectra can also be made. An impurity present at the 0.5% level could be detected in the front of the main-peak by comparing spectra, and a plotted extracted single-ion chromatogram showed a peak with a slightly different maximum compared to the main-peak [15].

The inspection of a mass spectrum enables co-eluting impurities to be detected. The detection of an ion with a maximum at or close to the retention time maximum of the main component can indicate a multi-component peak. The additional ion-signal must be evaluated as a potential adduct, fragment ion, or as an impurity co-eluting with the main component. By a comparison with mass spectra of pure drug substances acquired under optimal signal-to-noise conditions, co-eluting impurities down to $<0.1\%$ could be detected [33], and by using different orifice voltages an isobaric impurity could even be distinguished from a drug substance fragment ion.

In an example from a fast chromatographic method for *hydrochlorothiazide* (HCTZ) and its impurities, the main peak was not fully separated from one of its known hydrophilic impurities. Using the peak-purity option of the Chem-

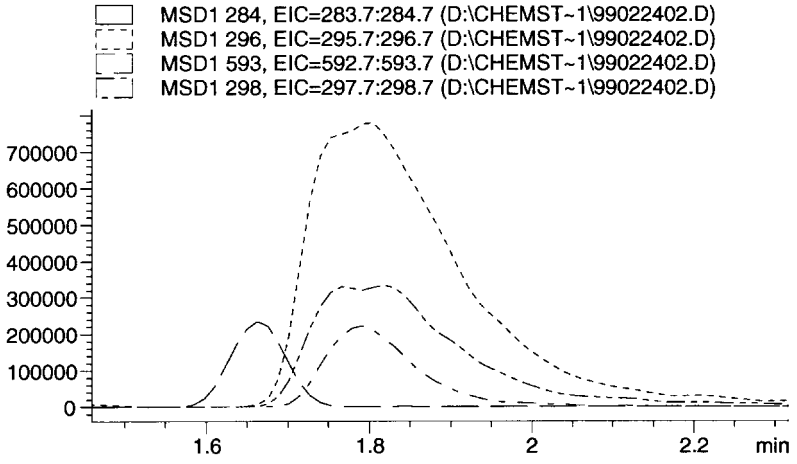


Figure 2.7.4.F.
Plot of peak-purity of hydrochlorothiazide by an automatic purity function searching for all ion-traces having maxima in one peak. The m/z 284 signal is obviously an impurity, whereas other signals are of chlorine isotopes and $[2M + H]^+$ cluster ion. Negative ESI-MS on a Hewlett–Packard LC/MSD system, gradient from 26–60% v/v acetonitrile with 1 mM ammonium acetate at 0.3 ml/min

Station software, a plot was obtained of the different ion-traces found in the peak, resulting in a ''multi-component peak'' warning (see Fig. 2.7.4.F). Four different ion traces were found in the main-peak. These were m/z 296/298 from deprotonated HCTZ chlorine isotopes, m/z 593 from a deprotonated HCTZ dimer complex formed in the electrospray source, and m/z 284 from the deprotonated HCTZ impurity 4-amino-6-chlorobenzene-1,3-disulphonamide. The detected impurity was present at just above the 0.1% level in the batch used (HPLC/UV method).

2.7.4.9. Hydrogen–Deuterium Exchange

In structural elucidation the use of deuterium oxide for the exchange of protons for deuterium on heteroatoms of the molecule has proven to be very useful [34]. The resulting expected mass shifts were confirmed for tested drug substances like *omeprazole* and for shifts up to 17 and 20 amu for *angiotensin I* and *II*. The mass shifts seen using deuterium oxide are indicative of the

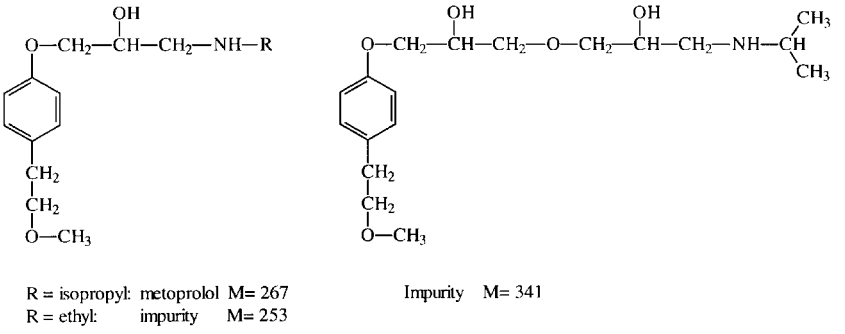


Figure 2.7.4.G.
Structures of metoprolol and its two impurities

number of hydroxyl, amine, amide and thiol groups present, and are of great importance in impurity identification. As an example an unknown by-product of metoprolol, pseudomolecular positive ion with m/z 342, was compared as the protonated and deuterated ion using microcolumn separation HPLC/MS, using 1% v/v acetic acid in acetonitrile or 99.8% pure deuterium oxide–acetonitrile in the mobile phase [35]. The shift of the protonated to deuterated ion was m/z 342 to 346, indicating the presence of three exchangeable protons. The proton carrying the charge of the ion was also shifted as expected. Knowledge of the method of synthesis together with analytical data led to the conclusion that the impurity was one having incorporated an extra carbon chain with one additional hydroxyl moiety (structure in Fig. 2.7.4.G). The direct infusion of a metoprolol succinate mother-liquor solution containing that same impurity as above resulted in the mass-spectra of Fig. 2.7.4.H. The shift obtained for the impurity was an increase of 4 amu, whereas the expected shift for metoprolol itself was an increase of 3 amu. In a similar experiment an unknown metoprolol impurity of M = 347, discussed in sections below, showed a shift indicating two exchangeable protons (data not shown). In a study of drug metabolites, a chromatographic flow rate of 1 ml/min was used with deuterium oxide and deuterated methanol [36], and the effective hydrogen/deuterium exchange ratios using different ionisation methods were compared. Electrospray ionisation was found to most efficiently exchange hydrogen to deuterium.

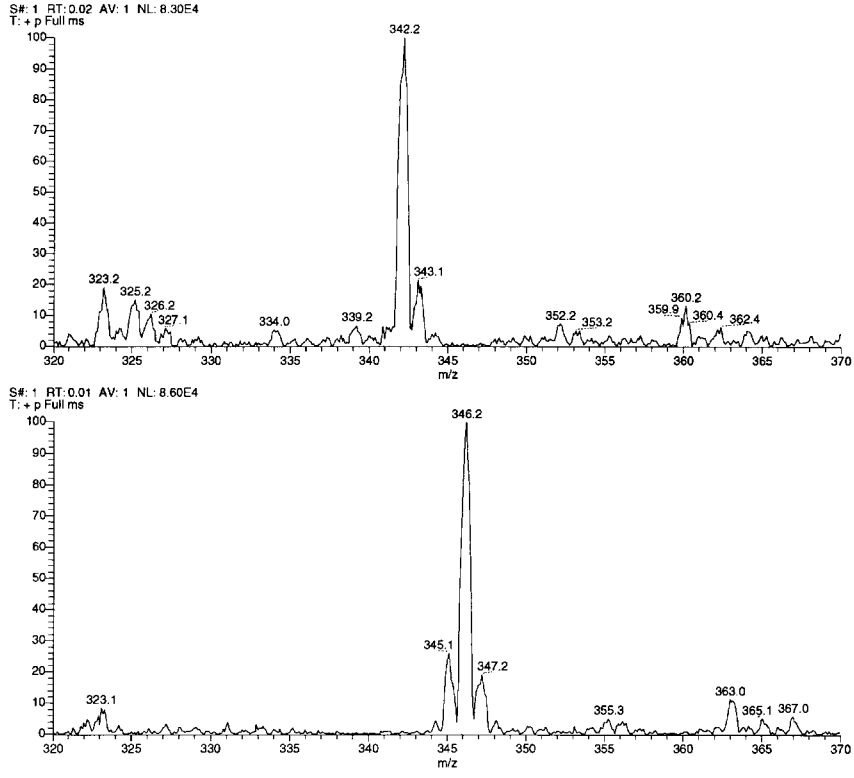


Figure 2.7.4.H.
Deuterium oxide exchange of acidic protons of an impurity in metoprolol. Metoprolol succinate mother liquor was infused at 5 μl/min in either 50:50% v/v water–acetonitrile (top panel) or 50:50% v/v D_2O–acetonitrile (bottom panel) into a Finnigan TSQ 7000 API2 ESI-MS system. The observed shift of 4, from m/z 342 to 346, represents three exchangeable protons

2.7.4.10. HPLC/MS/MS

2.7.4.10.1. Scan Modes for MS/MS

When setting up an MS/MS experiment there are different scan modes at choice for solving different analytical problems. With a triple stage quadrupole instrument several options are available: in a daughter-ion scan the first quadrupole (Q1) is set to transmit only one parent ion, which is fragmented by gas-phase collisions in the second quadrupole (Q2), and the produced fragment

ions are analysed by the third scanning quadrupole (Q3). Collision gases used in the collision cell (Q2) are normally argon or xenon at a pressure of 1 or a few mTorr. A parent-ion scan is the reversed situation setting Q1 to scan mode and Q3 to be locked on one single mass, resulting in the detection of common fragment ions produced from different parents. In a neutral loss scan both Q1 and Q3 are scanning at the same rate and over the same mass range, trying to detect compounds that are related to each other and showing the same neutral losses in their mass spectra. Selected reaction monitoring (SRM) results in highest sensitivity by setting Q1 to transfer one single m/z and Q3 to detect one single fragment of that precursor ion. This is also known as the ''precursor ion mode'' and is the scan-mode of choice for high sensitivity bioanalytical methods. ''Multiple reaction monitoring'' is a term used for an SRM experiment monitoring two or more daughter-ions after fragmentation. The term ''consecutive reaction monitoring'' is used to describe the type of MS^n experiments that can be performed with an ion-trap detector. Ion-traps, FTMS detectors and hybrid quadrupole-TOF detectors can be set up for daughter-ion scans and the SRM type of experiments.

An easy way to obtain MS fragmentation data from a single-stage MS is by using in-source collision induced dissociation (CID). As a complement to normal mass spectra, in-source CID gives useful structural information from the fragmentation induced by an increase in the kinetic energy of ions. The fragmentation is achieved by increasing the fragmentor voltage (CID offset, cone voltage) in the ion-source, causing an increased number of gas-phase collisions to occur. CID mass spectra may be close to identical to daughter-ion spectra for some compounds.

2.7.4.10.2. Data-Dependent MS/MS scan

A first approach to an HPLC/MS/MS experiment is to run a normal HPLC/MS analysis to determine the molecular weights of impurities. A following experiment could be of several kinds. If the impurity is abundant and the selectivity is sufficient for an interference free signal, a direct infusion MS/MS experiment can be made focusing only on the ion of interest. However, a strong suppression of the impurity could be the result of the presence of high amounts of drug substance in the electrospray interface. To avoid the problems of direct infusion methods, a chromatographic separation can be run, using either in-source CID, or a true MS/MS daughter-ion experiment for structural information. The time-programming of the method can set MS parameters for different precursor ions at different chromatographic retention times during the analysis.

A possible way to make a molecular weight confirmation and to collect structural information in one HPLC/MS run is to run a data-dependent scan

experiment. Using the Finnigan TSQ instrument, the software allows the programming of the instrument to make a normal full scan until any ion is detected above a certain threshold. At that point, a switch to the daughter-ion scan mode is made, the first quadrupole being locked at the ion of maximum abundance, the collision offset of Q2 is increased, and a daughter-ion scan is made. The next scan again is a normal full scan, and the cycle is restarted. No collision gas is added into the collision cell in this experiment, as the filling/pumping out of gas takes too much extra time. The disadvantage of leaving collision gas out, is that less energy is available to induce fragmentation. One example of a data-dependent scan experiment of a gradient separation of metoprolol and impurities, is given in Fig. 2.7.4.I. The spikes seen in the mass chromatogram are typical and are caused by the switches between full-scan and daughter-ion scan modes. The daughter ion scan was started at 50 and ranged 20 amu above the selected ion. The experiment illustrates some problems encountered when using automatic methods that are not optimised for individual impurities; peak m/z 254 at 3 min gave a nice daughter-ion spectrum, impurity peak m/z 342 at 3.9 min gave no daughter-ion spectrum, since the most abundant ion was still the tailing signal from m/z 268 of metoprolol, impurity peak m/z 347 at 5.5 min triggered no daughter-ion scan as the

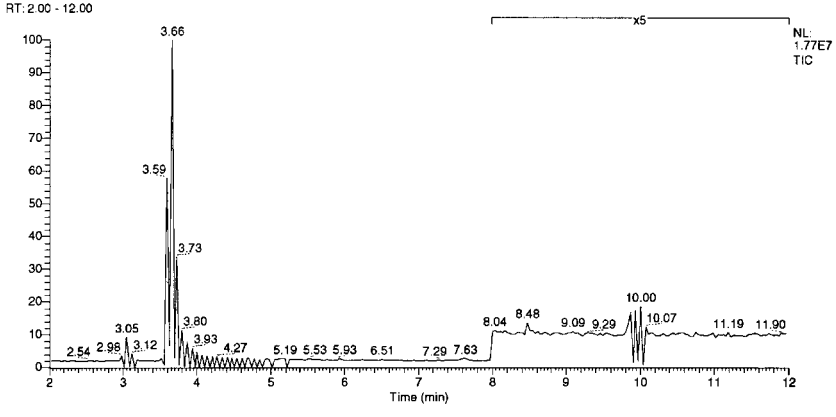


Figure 2.7.4.I.
A typical ion-chromatogram recorded with a data-dependent scan method. Spikes are caused by switching between full scan and daughter-ion scan modes. Three peaks were detected above the threshold limit. HPLC/MS method; gradient of 20–60% v/v acetonitrile from 0 to 20 min with 0.05% v/v TFA; Finnigan TSQ 7000 AP12 ESI-MS; full scan 210–800; spray at 4.5 kV; capillary temperature 350°C. Switch of collision offset from −12 to −30 V between scan modes

signal was weaker than the programmed threshold limit, and the impurity peak m/z 476 at 10 min induced a daughter-ion scan but the collision energy applied was too low to induce fragmentation. The structures of the impurities giving rise to the m/z 254 and 342 signals were previously reported as metoprolol impurities [36] (Fig. 2.7.4.G). The fragment ions of the impurity at m/z 254 (see Fig. 2.7.4.J) confirms that its structure is related to metoprolol, m/z 58, 84 and 102 being shifted 14 amu down compared to metoprolol, and fragments 133 and 159 being common to metoprolol. The impurity has an ethylamino group compared to the isopropylamino group of metoprolol. The use of data-dependent scan experiments with an ion-trap detector is more straightforward,

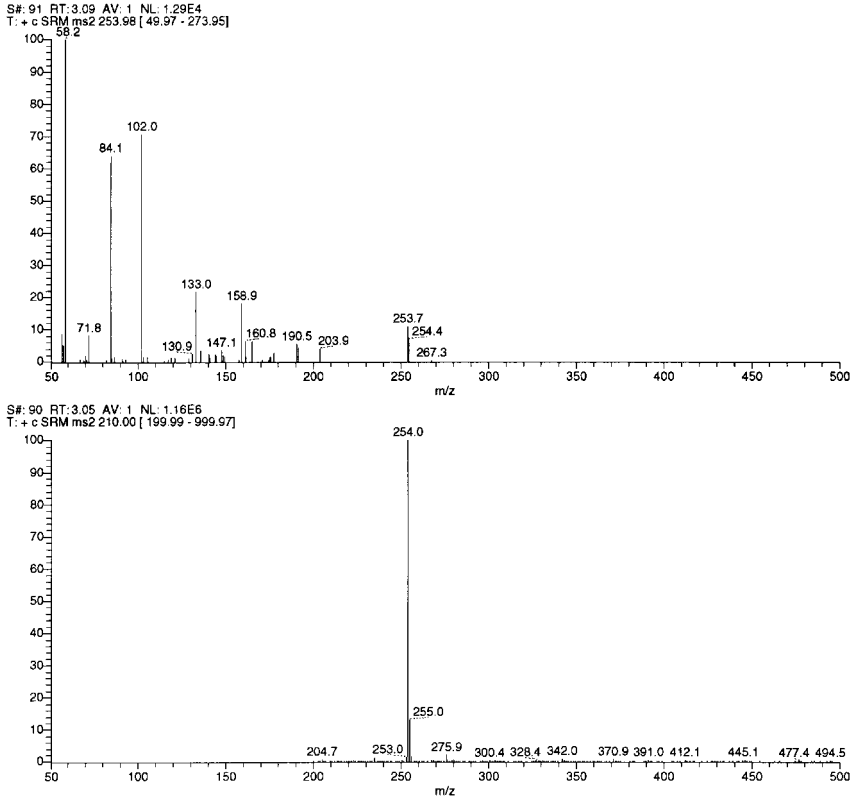


Figure 2.7.4.J.
Automated data-dependent scan method for metoprolol impurities, scan modes alternating between positive ion full-scan MS (bottom) and daughter-ion scan of most intense ion m/z 254 (top). (Peak at 3.05 min of total ion chromatogram in Fig. 2.7.4.I)

as no extra collision gas is needed, and the software can be set to perform several generations of MS^n scans. In the characterisation of metabolites using a Finnigan LCQ ion-trap, it was shown that the structural information from data-dependent MS^n scans was useful in assigning hydroxylation positions of different metabolites [37]. By setting the width of the isolation window to 7 amu, the product ions formed contained chlorine isotope patterns, which is useful information in the structural elucidation.

2.7.4.10.3. Infusion MS/MS and MS^n Experiments

In hydrochlorothiazide (HCTZ), a corrected structure for a dimer-type of impurity was recently suggested [38]. This new structure will be included in the Supplement 2000 of the European Pharmacopoeia (structure in Fig. 2.7.4.K). In the experiment presented here, the HCTZ-dimer was purified by semi-preparative fraction-collection HPLC/MS in the negative ion mode, in a similar way as described for the purification of metoprolol impurities. In a regular daughter-ion experiment the fragment ions produced from m/z 605, are due to the cleavage of the dimer into two different monomers, one HCTZ ion at m/z 296, and another one containing the methylene group of the dimer at m/z 308. Contrary to the CID experiment with HCTZ above, no chlorine isotope ions are seen when looking at daughter ions of one single isotope at the resolution set. By further increasing the capillary temperature, and the collision offset voltage, daughter-ion spectra of the two fragment-ions m/z 296 and m/z 308 could be recorded (Fig. 2.7.4.L). The MS^3 generation of negative ions produced from 296 are m/z 205 and 269, and ions produced from 308 are m/z 216 and 280. The identity of fragments has not yet been fully explained, but the experiment demonstrates the possibility of analysing the third generation ions on a triple-stage quadrupole instrument.

The Finnigan MAT 900S instrument used for the high-resolution MS analysis, is connected to an ion-trap detector (LCQ type) in series allowing

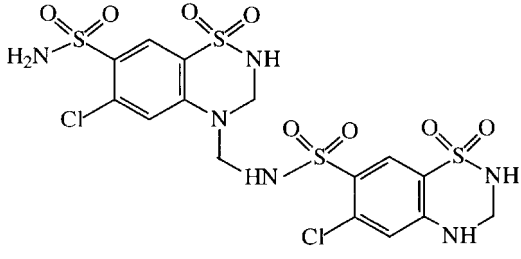


Figure 2.7.4.K.
Structure of the dimer-type impurity in hydrochlorothiazide

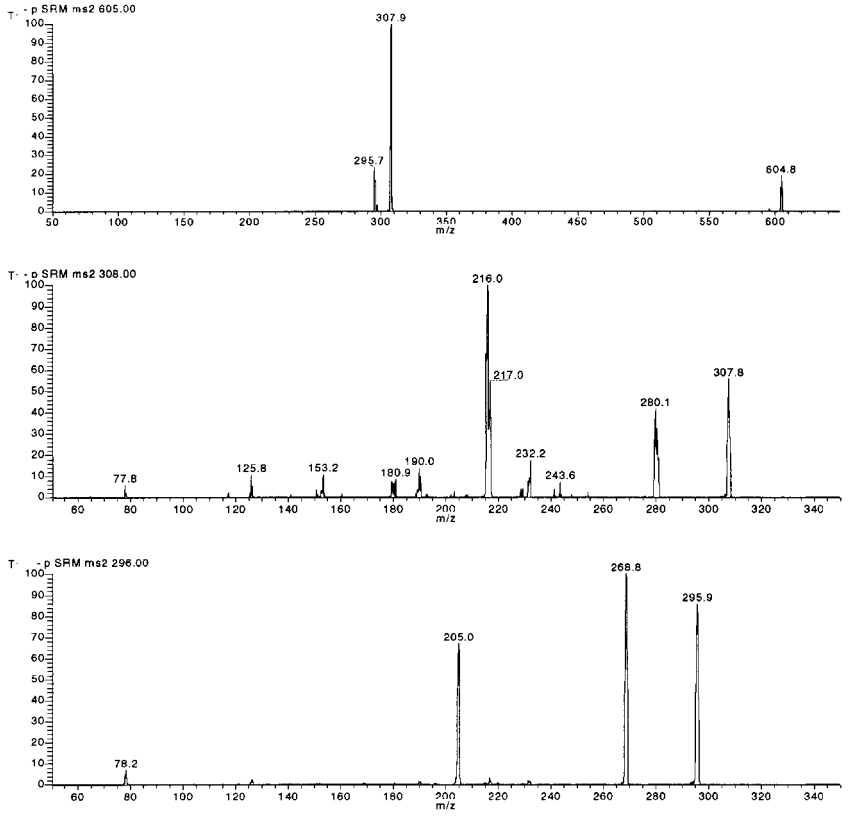


Figure 2.7.4.L.
Negative ion mass spectra recorded by direct infusion of dimer fraction from hydrochlorothiazide (HCTZ). At top is a daughter ion spectrum of m/z 605 of the HCTZ dimer resulting in a simple fragmentation. Mid and lower panels are MS^3 spectra recorded at the m/z 308 and 296 daughter ions, respectively. Experimental set-up: direct infusion into a Finnigan TSQ 7000 AP12; negative ion electrospray; spray voltage 4 kV; heated capillary 250–350°C; collision cell offset 20–25 V; argon collision gas at 1.2 mTorr

MS^n experiments to be performed. The same solution of metoprolol impurity used for high-resolution experiments was also analysed by the ion-trap, this time as the negative m/z 346 ion. The ion of interest was isolated in the ion-trap and fragmented by adding energy causing collisions with helium gas in the trap. Individual fragment ions could stepwise be further isolated and collided to produce MS^n data to the third generation. A scheme of the negative fragment

ions obtained, and the possible structures of these ions are presented in Fig. 2.7.4.M. The positive fragmentation of the m/z 348 ion was less informative, mainly resulting in a loss of 80 amu to form a metoprolol ion.

2.7.4.11. High-Resolution MS and MS/MS

The ultimate high-resolution mass spectrometer is the Fourier transform mass spectrometer, where a resolution of 10^5–10^6 can be achieved, opening possibilities of accurate mass determinations of large molecules and their fragments [39]. When working with drugs and drug impurities the usual molecular weights are well below 1000 amu, and the resolving power of magnetic sector, or even modern time-of-flight instruments, is sufficient.

Using a Micromass Q-Tof instrument, Eckers *et al.* [40], have shown the potential of the identification of drug impurities using accurate mass measure-

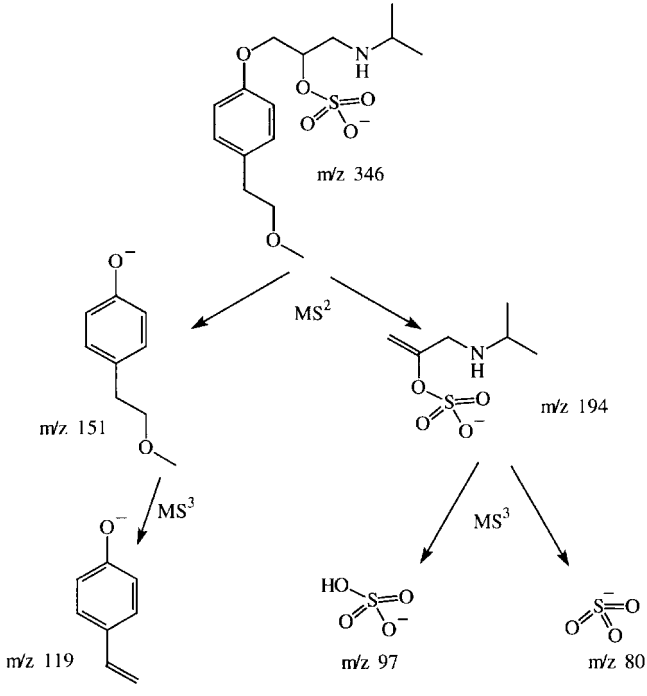


Figure 2.7.4.M.
Negative ion fragmentation of metoprolol impurity M = 347 showing suggested structures of ions formed

ments. The study was made with *cimetidine* drug substance containing several impurities at levels below 0.1%. Using diagnostic ions from known cimetidine impurities, or known typical fragment ions, accurate masses of protonated impurities, and some of their major MS/MS fragments could be determined with a mass error of 2–5 ppm, making the identification of these trace impurities possible.

In impurity profiling studies, the accurate mass determination can be performed directly from the bulk drug substance, but most likely the intensity of low amounts of impurities will be low due to working in high-resolution mode. Our example here is taken from the study of the *metoprolol* impurity positive ion at m/z 348. With the aid of liquid/liquid extraction in dichloromethane–buffer pH 4.5 the wanted impurity could be enriched from 0.05% to approximately 15%, as determined by HPLC with UV detection. The dichloromethane was evaporated and a solution in water–acetonitrile 70:30 v/v was used for the high-resolution experiment. The mass-spectrometer used was a Finnigan MAT 900S, connected in series to a Finnigan ion-trap detector, and equipped with an ESI ion-source. The extract was infused at 1 μl/min using a syringe pump, a solution of polyethylene glycol (PEG) of appropriate molecular weight distribution was pre-mixed in the sample solution, and two PEG peaks were used for accurate mass calibration of the instrument. A series of 10 consecutive measurements were averaged, and the resulting mass was matched against possible theoretical masses using a computer search. The minimum and maximum numbers expected of the different kinds of atoms of the unknown should be given as search criteria to reduce the number of possible suggestions. The resulting hit list contains information on the search criteria used, the result as a best matches list with differences in millimass (mmu) units between expected and measured mass, the R+ value (number of rings and double bonds present), and the suggested elemental composition. In the case of the metoprolol impurity we expected the atoms C, H, O and N to be present, which gave suggestions of elemental compositions that could not be matched to any reasonable by-product. However, when also including sulphur in the search criteria, the suggested elemental composition immediately made sense (see Table 2.7.4.A). An R+ value of 4 matched a structure having one aromatic function (bonds to sulphur not included in R+ value) The theoretical abundance of the ^{13}C and ^{34}S isotopes were calculated for the suggested formula, and checked with the measured ones of the impurity itself in an infusion experiment. The theoretical values of 18.3 and 5.6% matched reasonably well with measured values of approximately 17 and 6%.

Table 2.7.4.A.
Essentials of the results table of high-resolution MS search for elemental composition matching of an impurity of metoprolol (for the structure see Fig. 2.7.4.M). M = 347.1402303; limit: $C_{15}H_{10}$–$C_{30}H_{40}SN_4O_6$ R + D: 0.0 > 20.0

	Δ (mmu)	R+	Formula
1	0.29751	4.0	$C_{15}H_{15}SNO_6$ (correct structure)
2	−0.66293	13.5	$C_{21}H_{19}N_2O_3$
3	2.01721	18.0	$C_{24}H_{17}N_3$
4	2.70989	8.5	$C_{18}H_{23}SN_2O_3$
5	−3.3431	9.0	$C_{18}H_{21}NO_6$

2.7.4.12. Sample Preparation

Before starting the sometimes complex work of impurity identification, it is worth the effort to evaluate the quality of the sample. It is advisable to first check with the organic synthesis laboratory (or other supplier of sample) if there is a mother liquor or crude sample available, where the impurities of interest are present in higher amounts. Another approach is to stress the bulk drug substance to produce a range of degradation products. The optimal stress conditions for different substances vary, but could be as outlined by Rourick *et al*. [41]. They used a scheme for rapid degradation at acid conditions (0.1 M HCl, 2 h), basic conditions (0.01 M NaOH, 1.5 h or 1 M NaOH, 30 min), high temperature (140°C, 6 h), and oxidising conditions (H_2O_2 treatment). An increased UV-irradiation in a ''sun-test'' cabinet could also be used for light-induced degradation. All these stress conditions will likely result in degradation that is of no direct interest in impurity profiling, although some degradation products may be identical to synthesis impurities. Another use of information collected in degradation studies is to create a library of MS/MS data, to be used as fragmentation templates helping the understanding of the mass-spectral fragmentation of the compound classes of interest. Such an approach was described by Lee *et al.* [42] in a study of *buspirone* metabolism, where metabolites were classified into different profile groups based on their MS/MS fragmentation patterns. These profile groups contained information such as relative retention data of compounds, molecular weights, diagnostic MS/MS fragments and common neutral losses.

2.7.4.13. Fraction Collection

2.7.4.13.1. Solid-Phase Extraction

A higher quality sample for impurity identification is obtained from chromatography and fraction collection. A simple way to do this is to use solid-phase extraction (SPE) columns where the bulk drug substance is loaded, and portions of mobile phase are used to elute the drug substance and its impurities. For example, *metoprolol succinate* solution was loaded on Supelclean C_{18} SPE tubes, and eluted with aliquots of 2 ml 0.05% v/v TFA in water with 15% v/v acetonitrile. The SPE tubes were pre-conditioned with acetonitrile followed by the eluting mobile phase. We had access to a set-up handling up to 12 samples in parallel. The quality of the collected fractions was checked by flow-injection ESI-MS. All collected fractions were transferred to vials and 20 μl injected at 1 min intervals into a mobile phase flow of 0.8 ml/min (50:50 v/v water–acetonitrile with 0.025% TFA) in the FIA mode, and positive ions were detected by a LC/MSD system. The comparison of the abundance of the metoprolol m/z 268 signal, and the m/z 348 impurity signal showed that in fractions at 5 and 6 min the impurity concentration was high compared to metoprolol, and these fractions could be used for further MS experiments aiming at structural elucidation (Fig. 2.7.4.N).

2.7.4.13.2. MS Based Fraction-Collection

Recently, integrated fraction-collection HPLC/MS systems have become available, which have advantages compared to UV-signal based fraction-collection. Drexler and Tiller [43] presented an example where the full-scan MS signal of a Finnigan LCQ ion-trap detector triggers both a fraction-collector, and a data-dependent MS^2 and MS^3 scan of *vitamin E-acetate* and *labetalol*. The result is the collection of structural information from both compounds, also allowing additional MS^n experiments by infusion of pure compounds in fractions.

A similar set-up used in our laboratory, allowed the m/z based fraction-collection from a semi-preparative HPLC/MS system. The use of this system, provided sufficient amounts of two impurities of metoprolol succinate (m/z 348 and 360) to perform further MS, moreover also NMR experiments. The software MassLynx/FractionLynx was used to integrate a Micromass Platform LCZ quadrupole detector to a Gilson 233XL autosampler, Gilson 305/306 gradient pump system, a LC Packings Acurate flow-splitter and a Gilson 202 fraction-collector. A solution of approximately 20 mg/ml metoprolol succinate was injected (500 μl) on a semi-preparative Kromasil C_8 column (100 × 20 mm, 5 μm), and a gradient separation at 20 ml/min in 10 mM ammonium acetate from 10 to 80% acetonitrile in 0–15 min was run. The flow-splitter allowed 20 μl/min from the

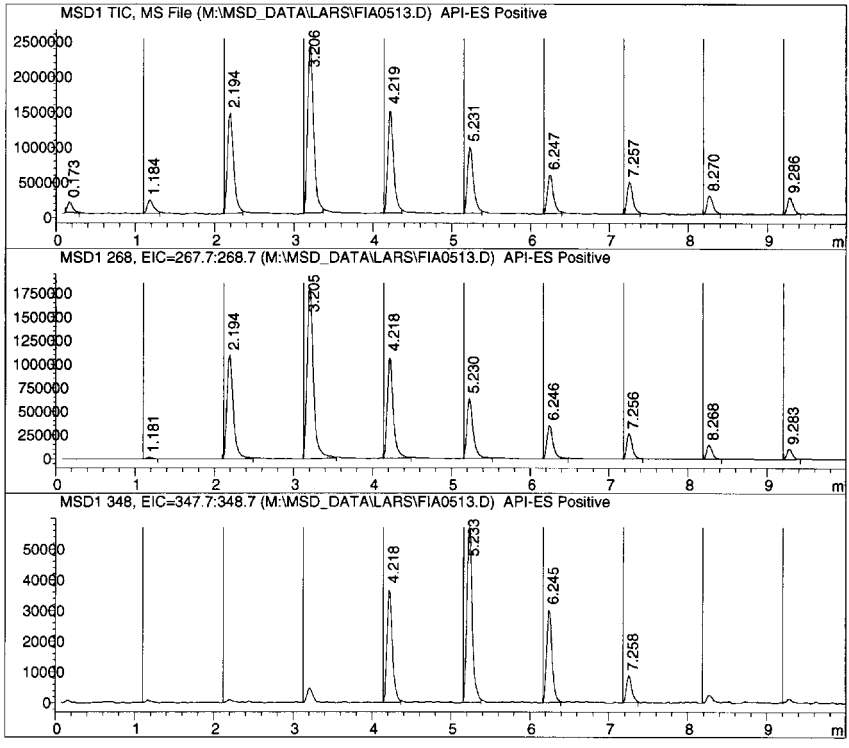


Figure 2.7.4.N.
Flow injection analysis (FIA) of fractions of metoprolol and impurity after separation using solid phase extraction. Traces from the top are the TIC and the extracted ion chromatograms at m/z 268 and 348. Fractions at 5 and 6 min were saved for further experiments. Hewlett–Packard LC/MSD; positive ESI; full scan 200–600; spray voltage 4 kV

column to be mixed with 1 ml/min of methanol make-up solution (Hewlett–Packard quaternary pump off-line), which was again split in a PEEK T-piece for flows to a Gilson 119 UV/Vis detector and the mass detector in parallel. The flow into the Z-spray interface of the MS was approximately 200 μl/min. The remaining flow from the semi-preparative column was directed to waste, except when the target m/z signal was detected. The target signal triggered a valve to switch to fraction-collection mode and fractions were collected into 20 ml test-tubes. Four windows representing the total ion chromatogram, the single-ion traces of m/z 348 and m/z 360 and the analogue square-wave signal for the fraction-collection were monitored (Fig. 2.7.4.O). After a sequence of repeated

injections, the fractions containing the impurities were collected in a round-bottom flask, acetonitrile was removed in a rotary evaporator and the remaining water–ammonium acetate was removed by freeze-drying. The resulting milligram-scale sample could be used for further MS and NMR experiments. As summarised in Section 2.7.4.16, the first impurity thus obtained is the one with a molecular weight of 347 which is shown in Fig. 2.7.4.M and Table 2.7.4.A.

2.7.4.14. Notes on HPLC/APCI-MS

Most set-ups for API mass spectrometry include both an electrospray and APCI interface. The APCI ionisation is a more potent method than electrospray, and the protonation of compounds lacking the typical functional groups needed for electrospray ionisation is possible. The analyte is sprayed through a heated vaporiser and ionised by a 4–5 kV potential applied to a corona discharge needle. The working mass-range of APCI is normally for compounds below 1000 amu, as analytes need to be vaporised in the process. As the vaporiser is heated to 400–500°C some thermolabile substances do not survive APCI ionisation, although the fragmentation induced may be informative. A large part of the approaches for ESI-MS optimisation above also applies to APCI-MS, but the optimisation of pH in the mobile phase is less critical. The use of APCI for qualitative analysis is mostly applied to chemicals in environmental applications [5], but pharmaceutical applications for drug analysis and impurity profiling have also been reported [31,44]. The APCI interface has also been widely used in quantitative bioanalytical methods, as it often has better detection limits to drug substances compared to electrospray [5].

2.7.4.15. Impurities in Drug Formulations

The studies of impurities, degradation products or contaminants in drug-formulations add much complexity to the HPLC/MS analysis. The complexity of a sample matrix containing high amounts of excipients used in the formulation often cause detection problems. Some excipients may not be seen by standard HPLC/UV methods, but are ionised and detected by the MS method of choice. A selective sample work-up aimed at the drug substance and impurities of interest, may solve the problem with excipient background. Impurities may be originating from synthesis, degradation, interaction with excipients or the packing materials used. Any plastic or rubber details being in contact with the formulation should be regarded as potential sources of contaminants [3]. The use of mass-spectral data from a degradation product database is an excellent tool in the evaluation of impurities in formulations

(see Section 2.7.4.12).

The chemical interaction of a drug substance with the enteric coating used in the formulation was found to result in the presence of new impurities, by forming amides with succinate and phthalate moieties of the coating polymers [45]. The structures of impurities were suggested by UV and ESI-MS spectral data from heat and humidity stressed samples. The identities of the impurities were confirmed by synthesis, high-resolution FAB-MS, IR and NMR data.

One example from our laboratory is an extract of a drug substance under dosage formulation development, where an unknown polar degradation product was formed under heat and humidity stress. In spite of sample work-

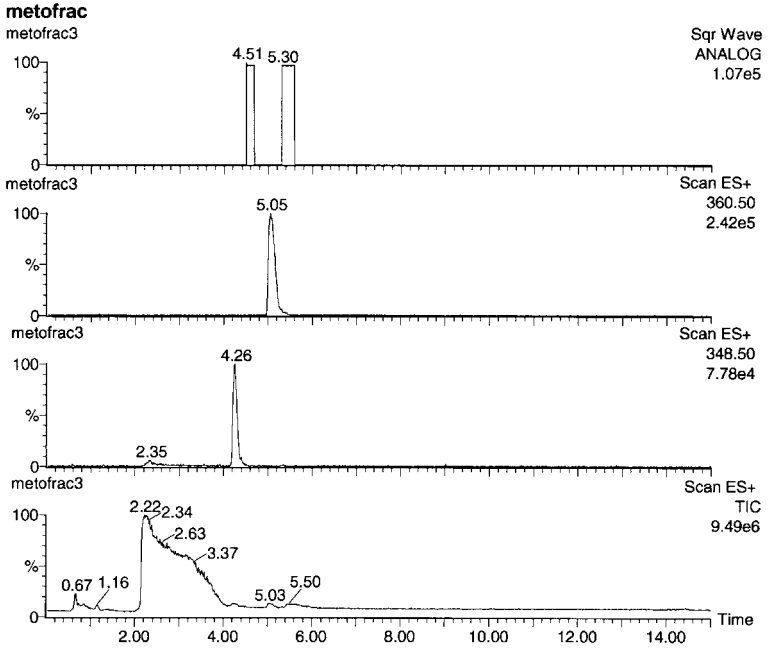


Figure 2.7.4.O.
Fraction collection of metoprolol impurities triggered by selected MS ions. From bottom to top are the TIC, the m/z 348 trace for impurity 1, the m/z 360 trace for impurity 2, and the square-wave signal representing fraction collector valve on/off. Note the time offset between the MS and square-wave signals. Micromass Platform LCZ with Gilson 233XL autosampler and Gilson 202 fraction-collector; positive full-scan electrospray; spray voltage 3.5 kV, cone voltage 30 V

up, the mass-chromatogram was dominated by a range of peaks from remains of polyethylene glycols not seen by UV-detection. Careful inspection of the chromatogram suggested that the ion at m/z 191 was present, and the extracted ion chromatogram shows that it is indeed correlated to the UV peak of the degradant (Fig. 2.7.4.P). A structure of the degradation product could be proposed based on its chromatographic behaviour, UV-spectrum and molecular weight, and verified by synthesis.

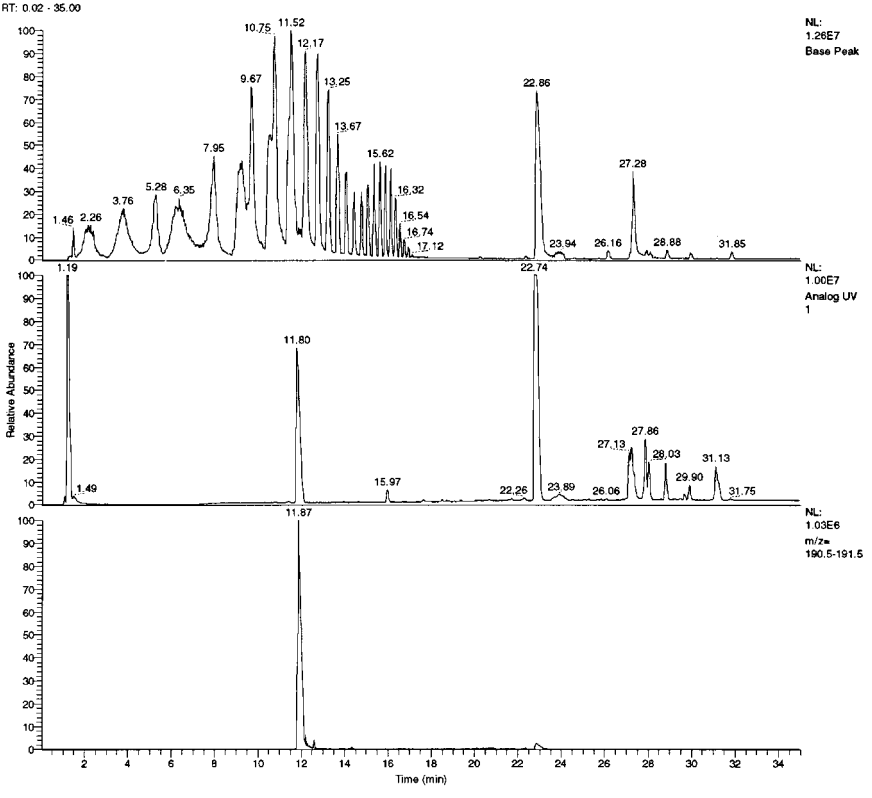


Figure 2.7.4.P.
Drug formulation containing an unknown polar degradation product at m/z 191. Top panel is TIC where Na^+ and NH_4^+ ions of PEG (polyethylene glycol) are dominating. Mid panel shows the UV trace with the impurity at 11.8 min. Bottom panel is the extracted ion chromatogram of m/z 191. HPLC/MS method: gradient from 10 to 60% v/v acetonitrile with 0.05% v/v TFA in 5–35 min; 1 ml/min split to 100 μl/min into Finnigan TSQ 7000 API2-ESI; full scan 200–800; spray voltage 4.5 kV; heated capillary 350°C

2.7.4.16. Conclusions

The aim of this chapter was to give ideas and advice for the solution of qualitative analytical problems related to drug impurity profiling and structural identification. Mass spectrometry of analytes in the liquid phase has been at focus, mainly based on the experiences from actual analytical problems solved with the instrumentation at hand. The overall conclusion is that the selectivity and sensitivity of mass spectrometers makes HPLC/MS one of the first methods of choice in the characterisation of drug impurities in contemporary pharmaceutical analysis.

A good example of this is the structural identification of the *metoprolol* impurity M = 347 (see Figs. 2.7.4.M and N and Table 2.7.4.A). Conclusions leading to the structure of this impurity were drawn from a range of different MS experiments as discussed above. Molecular weight was confirmed by positive-ion HPLC/MS, the MS/MS fragmentation pattern was studied and structures of fragments were assigned, the number of exchangeable protons were measured in a deuterium-exchange experiment, and the molecular formula was confirmed by high-resolution MS. The pure fraction of the impurity collected by semi-preparative fraction collection, was used for ^{1}H NMR experiments. No additional protons could be detected in the NMR spectrum, but in comparison with the spectrum of metoprolol, a shift was observed at protons on and adjacent to the hydroxyl-carbon. Such shift could be expected by the addition of a sulphate group, concluding that NMR data are supporting the proposed structure. Finally, based on the knowledge of the method of synthesis of metoprolol succinate, the impurity was synthesised by the treatment of metoprolol with sulphuric acid in methylbutyl ketone. A new peak of 18% abundance appeared, and proved to have identical chromatographic retention time and molecular-weight as the impurity in the standard analytical gradient HPLC/MS system used in our studies of metoprolol.

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References

1. R.B. Cole (Ed.), *Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation and Applications*, Wiley, New York, 577 pp (1997)

2. S. Görög, M. Babják, G. Balogh, J. Brlik, A. Csehi, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *Talanta* **44**, 1517–1526 (1997)
3. A.M. Krstulovic and C.R. Lee, *J. Chromatogr. B* **689**, 137–153 (1997)
4. E.C. Nicolas and T.H. Scholz, *J. Pharm. Biomed. Anal*. **16**, 825–836 (1998)
5. W.M.A. Niessen, *Liquid Chromatography-Mass Spectrometry, 2nd edn*, Marcel Dekker, New York (1999)
6. M. Wilm and M. Mann, *Anal. Chem*. **68**, 1–8 (1996)
7. R. Willoughby, E. Sheehan and S. Mitrovich, *A Global View of LC-MS, How to Solve Your Most Challenging Analytical Problems*. Global View Publishing, Pittsburgh, PA (1998)
8. J.M.E. Quirke, C.L. Adams and G.J. Van Berkel, *Anal. Chem*. **66**, 1302–1315 (1994)
9. G.J. Van Berkel and K.G. Asano, *Anal. Chem*. **66**, 2096–2102 (1994)
10. G.J. Van Berkel, J.M.E. Quirke, R.A. Tigani, A.S. Dilley and T.R. Covey, *Anal. Chem*. **70**, 1544–1554 (1998)
11. S.D. McCrossen, D.K. Bryant, B.R. Cook and J.J. Richards, *J. Pharm. Biomed. Anal*. **17**, 455–471 (1998)
12. M. Carrascal, K. Schneider, R.E. Calaf, S. van Leeuwen, D. Canosa, E. Gelpi and J. Abian, *J. Pharm. Biomed. Anal*. **17**, 1129–1138 (1998)
13. R.D. Voyksner, in *Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation and Applications* (R.B. Cole, Ed.), pp 323–341. Wiley, New York (1997)
14. A.J.J. Debets, T.J.L. Mekes, A. Ritburg and P.L. Jacobs, *J. High Res. Chromatogr*. **18**, 45–48 (1995)
15. J. Ermer and P.-G. Kibat, *Pharm. Sci. Technol. Today* **1**, 76–82 (1998)
16. A. Cappiello, G. Famiglini, L. Rossi and M. Magnani, *Anal. Chem*. **69**, 5136–5141 (1997)
17. B.A. Mansoori, D.A. Volmer and R.K. Boyd, *Rapid Commun. Mass Spectrom*. **11**, 1120–1130 (1997)
18. A. Apffel, S. Fischer, G. Goldberg, P.C. Goodley and F.E. Kuhlmann, *J. Chromatogr. A* **712**, 177–190 (1995)
19. F.E. Kuhlmann, A. Apffel, S.M. Fischer, G. Goldberg and P.C. Goodley, *J. Am. Soc. Mass Spectrom*. **6**, 1221–1225 (1995)
20. M. Jemal and D.J. Hawthorne, *Rapid Commun. Mass Spectrom.* **13**, 61–66 (1999)
21. R.F. Straub and R.D. Voyksner, *J. Am. Soc. Mass Spectrom*. **4**, 578–587 (1993)
22. F.M. Wampler III, A.T. Blades and P. Kebarle, *J. Am. Soc. Mass Spectrom*. **4**, 289–295 (1993)
23. K.-E. Karlsson, *J. Chromatogr. A* **794**, 359–366 (1998)

24. J.D. Pearson and M.C. McCroskey, *J. Chromatogr. A* **746**, 277–281 (1996)
25. D.J. Burinsky, B.L. Armstrong, A.R. Oyler and R. Dunphy, *J. Pharm. Sci.* **85**, 159–164 (1996)
26. S. Auriola, J. Frith, M.J. Rogers, A. Koivuniemi and J. Mönkkönen, *J. Chromatogr. B* **704**, 187–195 (1997)
27. E. Witters, W. Van Dongen, E.L. Esmans and H.A. Van Onckelen, *J. Chromatogr. B* **694**, 55–63 (1997)
28. T. Alebic-Kolbah and A.P. Zavitsanos, *J. Chromatogr. A*, **759**, 65–77 (1997)
29. A.P. Zavitsanos and T. Alebic-Kolbah, *J. Chromatogr. A* **794**, 45–56 (1998)
30. J. Gottfries, P. Johansson and A. Karlsson, *J. Chromatogr. A* **763**, 115–123 (1997)
31. H. Bauer, *Chromatographia* **28**, 289–292 (1989)
32. K.A. Hutton and D.E. Games, *Rapid Commun. Mass Spectrom.* **11**, 735–744 (1997)
33. D.K. Bryant, M.D. Kingswood and A. Belenguer, *J. Chromatogr. A*, **721**, 41–51 (1996)
34. K.-E. Karlsson, *J. Chromatogr*. **647**, 31–38 (1993)
35. N. Ohashi, S. Furuuchi and M. Yoshikawa, *J. Pharm. Biomed. Anal*. **18**, 325–334 (1998)
36. M. Erickson, K.-E. Karlsson, B. Lamm, S. Larsson, L.A. Svensson and J. Vessman, *J. Pharm. Biomed. Anal*. **13**, 567–574 (1995)
37. P.R. Tiller, A.P. Land, I. Jardine, D.M. Murphy, R. Sozio, A. Ayrton and W.H. Schaefer, *J. Chromatogr. A* **794**, 15–25 (1998)
38. X. Fang, S. Mayr, W. Yin, P.A. Harmon, J.M. Finnegan, R.J. Tyrrell and R.A. Reed, *Pharm. Research*, **14**, Abstract no. 4189 (1997)
39. T. Dienes, S.J. Pastor, S. Schürch, J.R. Scott, J. Yao, S. Cui and C.L. Wilkins, *Mass Spectrom. Rev*. **15**, 163–211 (1996)
40. C. Eckers, N. Haskins and J. Langridge, *Rapid Commun. Mass Specrom*. **11**, 1916–1922 (1997)
41. R.A. Rourick, K.J. Volk, S.E. Klohr, T. Spears, E.H. Kearns and M.S. Lee, *J. Pharm. Biomed. Anal*. **14**, 1743–1752 (1996)
42. M.S. Lee, E.H. Kerns, M.E. Hail, J. Liu and K.J. Volk, *LC-GC Int.* 586–600 (1997)
43. D.M. Drexler and P.R. Tiller, *Rapid Commun. Mass Spectrom*. **12**, 895–900 (1998)
44. M. Gazdag, M. Babják, J. Brlik, S. Mahó, Z. Tuba and S. Görög, *J. Pharm. Biomed. Anal*. **17**, 1029–1036 (1998)
45. P.J. Jansen, P.L. Oren, C.A. Kemp, S.R. Maple and S.W. Baertschi, *J. Pharm. Sci*. **87**, 81–85 (1998)

2.7.5. HPLC/NMR and Related Hyphenated NMR Methods

Ian D. Wilson, Lee Griffiths, John C. Lindon, Jeremy K. Nicholson

2.7.5.1. Introduction

High performance liquid chromatography (HPLC) coupled to a variety of detectors is widely used for the profiling and quantification of impurities in bulk drug and formulated pharmaceuticals. However, before HPLC methods can be used for the routine profiling of pharmaceutical products the identification of any of these impurities has first to be made. Such structural characterisation generally requires a combination of spectroscopic data from both nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Thereafter the identity of the impurity in the product is assumed on the basis of retention and UV properties in the standardised HPLC system. The development of on-line coupling of HPLC with MS has enabled impurities to be at least partially characterised without the need for lengthy and time-consuming isolation procedures. However, in many cases isolation still has had to be performed because further information is needed that can only be obtained via NMR spectroscopy in order to fully elucidate the structure. The introduction of dedicated NMR probes specifically for on-line coupling to HPLC [1–4] promises to greatly reduce the need for the isolation of impurities. Furthermore, in the future the combination of HPLC/NMR/MS in a single system has the potential to greatly increase the efficiency with which impurities might be identified. Currently there are only a few published examples of the use of HPLC/NMR for the detection and identification of impurities and minor components in pharmaceutical mixtures and formulations [5–8]. In part this reflects the relative novelty of the technique, and partly probably results from the commercial pressures on companies not to publish such data. Here therefore we will outline the development of the HPLC/NMR and provide some illustrative examples that will highlight the potential of HPLC/NMR and HPLC/NMR/MS for this type of work.

2.7.5.2. Development of HPLC/NMR

The first attempts at the direct coupling of chromatography, mainly HPLC but also GC [9], with NMR took place in the late 1970s and early 1980s.

However, these pioneering studies were held back by the low sensitivity of the NMR spectrometers, then available, which meant that only high concentration model systems could be studied. Furthermore, dynamic range problems meant that there was a need to use expensive deuterated solvents as chromatographic eluents because the solvent suppression pulse sequences available could not cope with 100% protio solvents. Attempting to get round this problem by limiting the solvents used for chromatography to those, like carbon tetrachloride, which contained no protons placed severe constraints on the separations that could be performed.

The first application of HPLC detection using NMR spectroscopy used a conventional NMR tube and ^{1}H NMR spectroscopy in a stopped-flow mode [10]. Because of the problems associated with the large dead space volumes involved in this methodology the next step was to develop continuous flow probes with Fourier transform instruments to compensate for the poor signal-to-noise ratios seen with on-flow systems. That said, the first continuous flow HPLC/NMR experiments were performed with ionol, anisole and salol as analytes using CCl_4 as eluent and a 90-MHz spectrometer [11]. The HPLC itself was sited 1 m from the magnet. Subsequently a mixture comprising n-butylbenzene, m-xylene, tetralin, naphthalene, dodecane, iso-octane, n-hexane, n-nonane, n-hexadecane and n-pentane was investigated. Separations were obtained with a solvent mixture of Freon 113 ($CF_2Cl{\cdot}CFCl_2$) and $CDCl_3$ (95:5 v/v) on a stationary phase of silica modified to introduce amino- and cyano-functionality.

There has also been a drive to reduce the detection volume in the NMR probe to maintain chromatographic resolution as much as possible. This can only be achieved if the resulting loss of NMR sensitivity can be compensated for by increasing the filling factor, and this has been achieved by mounting the RF coil directly onto the outside of the flow cell. Clearly, this makes it impossible to spin the sample to reduce the effect of magnetic field inhomogeneities, but in practice this does not cause difficulties because the smaller the sample volume the better the magnetic field shimming (below a certain point however, the closeness of the metal detector coil causes magnetic susceptibility effects which distort the magnetic field and cause line broadening). A significant factor in determining the sensitivity or peak heights is the observed lineshape. Thus, if peaks have wide bases a significant part of the signal intensity is found in this part of the peak, resulting in a poor signal-noise ratio (S/N) ratio. This means that good shimming is required for good S/N. However, simply because of the relatively low sensitivity of NMR, the commercially available NMR flow cells still have much larger volumes (ca. 120 μl) than those typical of conventional HPLC detectors. For conventional high field NMR probes, double saddle Helmholtz coils are usually used and hence in HPLC probes the same arrangement with upward flow is employed. Various designs, including a 5-mm NMR

tube, a 1.5-mm capillary tube and an inverted U-shape detector of 2 or 3 mm i.d. (leading to detector volumes in the range 20–120 μl) have been investigated. In general a design employing a glass insert of 2 or 3 mm i.d. and fixed vertically in the NMR magnet is used as the basis for commercially available HPLC/NMR probes. The whole arrangement is enclosed in a glass Dewar with a thermocouple for variable temperature measurements. With current commercial designs, detection volumes of ca. 40–120 μl are needed to obtain continuous flow ^{1}H NMR spectra with a time resolution of 10–20 s depending upon the flow rate. A typical flow rate in the region of 1 ml/min would be used with detector volumes of about 100 μl.

Another major step which allowed useful development of HPLC/NMR involved the development of techniques that eliminated the need for the use of deuterated solvents. Such solvents are used routinely in high resolution NMR spectroscopy but, given the volumes involved, their use for HPLC/NMR has obvious cost implications. Although the use of normal phase, or gel permeation chromatography with NMR and solvents such as deuterochloroform is readily achieved, most separations today are performed in reversed-phase (RP) mode using protonated solvents. The development of HPLC/NMR systems that could deal with reversed-phase solvent systems was therefore critical to making HPLC/NMR feasible. There are a number of NMR techniques used to achieve suppression of the solvent peaks. The simplest involves a secondary RF irradiation at the solvent NMR frequencies before acquiring the NMR data. Because this technique presaturates an extremely narrow range of frequencies, it makes significant demands on the external magnetic field homogeneity. The presaturation can be switched between several discrete frequencies if mixed solvents are used each of which might contain chemically shifted peaks. Other methods involve the use of shaped selective RF pulses which irradiate only the solvent peaks. These can be combined with the application of pulsed magnetic field gradients to improve their effectiveness. One of the most widely used is termed WET [12]. A further method which has been used widely is to adapt the two-dimensional NOE experiment to a one-dimensional mode by using only the first increment. This has the advantage of effectively suppressing the broad wings of the water peak arising from inhomogeneous parts of the magnetic field such as in the vicinity of the leads to the RF detector coil.

On-flow HPLC/NMR is currently only practicable for ^{1}H or ^{19}F NMR detection (unless isotopically enriched compounds are used). It is the usual practice for HPLC/NMR to include an in-line UV detector to enable the quality of the chromatography to be monitored and also to direct the spectroscopist to the peak retention times for stopped-flow HPLC/NMR. With stopped-flow NMR the whole range of high-resolution NMR experiments can be deployed (provided the HPLC/NMR probe can be operated for the desired nucleus) including two-dimensional experiments which provide correlation between

NMR resonances based on mutual spin–spin coupling (e.g. COSY or TOCSY) or heteronuclear correlation studies (e.g. HMQC). In addition, further types of stopped flow experiment can be performed based on either storing peaks in capillary loops as they elute for subsequent NMR (''peak picking'') or stopping the flow at short intervals as a chromatographic peak passes through the probe (''time-slicing'') thereby obtaining sequential spectra through the chromatographic peak. The latter technique is especially useful when there is poor separation of analytes.

In summary, as a result of significant technical advances, HPLC/NMR experiments can be carried out in several ways.

- On-flow, with direct on-line NMR detection of the analytes in the HPLC eluent.
- Stopped-flow, where the chromatography is stopped when peaks of interest enter the flow cell. This includes ''time slicing'' where peaks are examined at, e.g. the upslope, peak maximum and downslope, etc.
- Eluted peaks can be stored in capillary loops for later transfer to the probe for detailed NMR spectroscopic studies (''peak-parking'').

For all of these types of NMR experiment there is no need for compromise between the needs of the chromatographic separation and the spectroscopy such that both isocratic and gradient elution techniques can be employed. There have been improvements in sensitivity due to increases in magnetic field strength (currently 18.8 T is the highest commercially available field strength) and advances in NMR receivers, as well as the improved solvent suppression techniques described above, and modifications to the NMR flow-cell design. All of these factors have combined to yield a limit of detection with solvent suppression of less than 5 μg of compound in the on-flow mode and certainly in the sub-microgram range in stopped-flow mode. The issue of detection limits in HPLC/NMR spectroscopy has been of some interest, and various studies have been performed to determine the performance of current instruments. Although NMR is widely regarded as being of limited sensitivity compared to MS-based techniques, this degree of sensitivity will probably surprise many analysts used to thinking of requirements in the hundreds of micrograms, and several hours of acquisition time. This means that for many applications the currently available probes provide a real HPLC/NMR capability for the analysis of complex mixtures. However, the situation is by no means static and the deployment of higher observation frequencies and technical developments such as digital filtering would be expected to improve the detection limits further of conventional HPLC/NMR systems. In addition, the use of capillary HPLC columns or capillary electrochromatography/electrophoresis (see later) has already demonstrated that spectra can be obtained on ca. 10 ng of analyte using on-flow techniques. New detector technology based on the use of

RF detector coils cooled to liquid helium temperatures promise to provide another 400% increase in sensitivity.

2.7.5.3. Suitable Nuclei for HPLC/NMR

Currently available HPLC/NMR probes can detect a range of nuclei including ^{1}H, ^{2}H, ^{19}F, ^{31}P and ^{13}C. Clearly ^{1}H is the most useful nucleus for general work as it combines sensitive detection with broad applicability as most drugs contain some protons. ^{19}F NMR spectroscopy also has the potential to be useful in impurity profiling and detection, as many modern drugs contain fluorinated substituents. Not only is this nucleus sensitive, but its absence in the excipients used in formulating drugs allows selective detection of the drug after formulation. ^{13}C NMR is probably not suitable for on-flow applications but with modern inverse probes it should be possible to obtain some spectral data in the stopped-flow mode. Examples of the use of ^{2}H and ^{31}P have been shown but the scope for using such techniques for drug impurity work is obviously limited.

2.7.5.4. Experimental Aspects of HPLC/NMR

A schematic showing the instrumental layout of a HPLC/NMR system, with the capability of performing HPLC/NMR/MS, is shown in Fig. 2.7.5.A based on the Bruker HPLC/NMR system (other commercially available systems are available from Varian and Jeol). In our system, a Bruker HPLC system is used comprising a Bruker LC22C pump, an autosampler and a variable wavelength UV detector which is connected in-line to the NMR probe via ca. 2 m of 0.25 mm i.d. PEEK capillary tubing (with more modern ''actively shielded'' magnets the distance between chromatograph and NMR can be reduced). A schematic diagram showing the construction of the Bruker HPLC/NMR probe is shown in Fig. 2.7.5.B.

Typically, HPLC analysis is undertaken in our laboratories using a 50–100 μl sample injection on to a standard 250 × 4.6 mm i.d. reversed-phase column with D_2O/buffer-acetonitrile-based eluents. Flow rates vary between 0.5 and 1.5 ml/min. Generally the acetonitrile is non-deuterated, but on occasion, where there is a requirement to observe signals that would be close to the resonances for this solvent we have substituted either deuterated acetonitrile or deuteromethanol. The eluent from the column is monitored by UV at an appropriate wavelength and ^{1}H NMR spectroscopy has been carried out at between 500 and 800 MHz. The system can be calibrated such that the chromatography software then automatically allows for the delay required between the UV

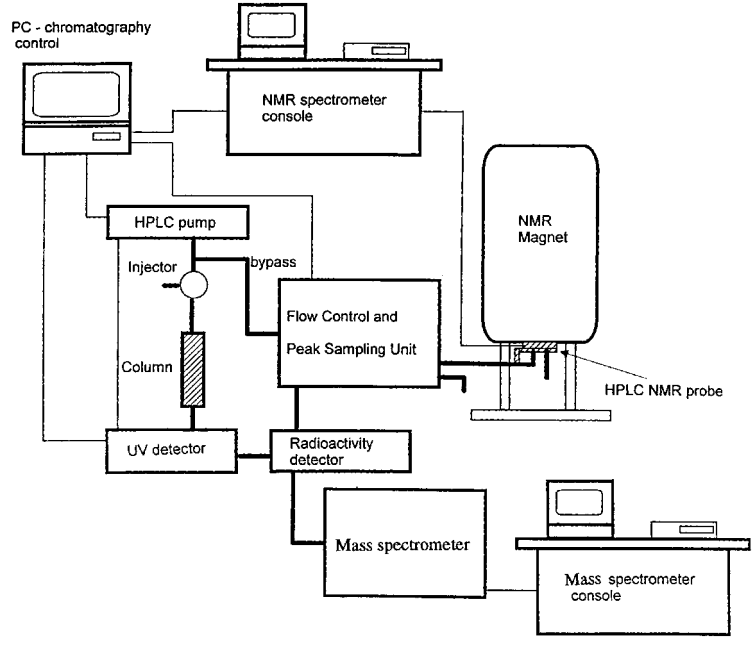


Figure 2.7.5.A.
A schematic representation of a HPLC/NMR/MS installation

detector and NMR flow cell enabling automatic stopped-flow NMR analysis. In general it is advantageous to measure the NMR spectra at the highest available observation frequency as this provides the best S/N. In practice 400 MHz for ^{1}H detection is probably the lowest practicable field strength for routine work. Typically stopped-flow ^{1}H NMR spectra are obtained, with the field locked using the internal D_2O resonance, using solvent presaturation at both the water and acetonitrile resonances. Stopped-flow ^{1}H NMR spectra are then acquired using 128–512 transients with 64 K data points, a spectral width of 10 kHz, and an acquisition time of ca. 3 s with an additional relaxation delay of 3 s. A suitable line broadening can be applied prior to Fourier transformation (FT) to reduce the noise. As well as the conventional one-dimensional NMR spectra, two-dimensional spectra can also be obtained in the stopped flow mode, including phase-sensitive, two-dimensional total correlation (TOCSY) experiments. Typically, we would use 2 s of single frequency saturation power at the water resonance prior to the TOCSY sequence with a spin lock and a mixing time of ca. 80 ms. For optimum resolution, the data are usually apodized using Lorentzian–Gaussian transformation in the acquisition domain and zero-filled by a

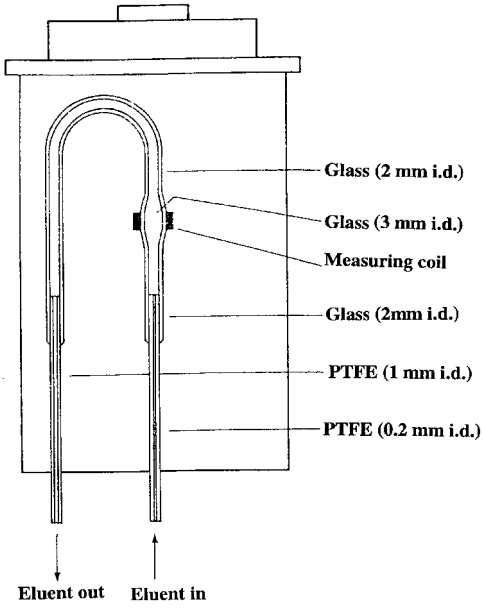


Figure 2.7.5.B.
A schematic representation of a flow through HPLC/NMR probe

factor of 2. In the orthogonal domain the data can be extended using linear prediction and zero-filled but no window function is applied before the second Fourier transformation. During the gradient elution the solvent resonance frequencies change and a blank run has to be carried out first in order to obtain these frequencies. ^{1}H chemical shifts can be referenced to the acetonitrile, at $\delta 2.00$, used as the organic modifier in the HPLC solvent. Although the proportions of D_2O and acetonitrile change during the gradient elution, it has been found that the acetonitrile chemical shift remains relatively constant, changing by only $\delta 0.011$ relative to the standard reference compound TSP (sodium trimethylsilyl-[$^{2}H_4$]-propionate) for up to 30% acetonitrile in D_2O. In addition, in our experience, stopped-flow operation does not cause any problems of band broadening on the chromatographic column due to diffusion if the flow is halted for less than 2 h, even with several stops.

On-flow ^{1}H and ^{19}F HPLC/NMR can also be carried out in a pseudo-two-dimensional mode. Here typical parameters are, 16 scans per experiment, acquisition time 0.25 s, recycle time 1.25 s giving a resolution of 20.0 s per increment, size 4 K data points, and for ^{19}F NMR observation, proton decoupling using the WALTZ-16 method. To gain good resolution in the chemical

shift axis of the NMR chromatogram, the FIDs can be multiplied by an apodization function of the Lorentzian–Gaussian or shifted sine-bell form. ^{19}F chemical shifts can be referenced to external trifluoroacetic acid at −76.8 ppm.

It has proved to be possible to obtain more information from a given set of NMR data by using the post-acquisition data processing method of maximum entropy analysis (the so called ''quantified MaxEnt'' software). This technique may be of particular use in situations where spectra are required for trace components of a mixture where obtaining sufficient signal to noise would otherwise require very long acquisition times. In conventional methods of data analysis the process of extracting information from poorly resolved or low signal-to-noise spectra is approached by trying to remove the result of line-broadening, lineshape distortion or high noise with no regard to how the ''ideal'' data had been perturbed by instrumental artefacts. The maximum entropy method approaches the problem in an iterative manner by starting with trial data sets containing a minimum amount of structure, adding in features as necessary in order to match the observed data. By modifying the distortion model and repeatedly comparing the trial data with that observed, it is possible to reach a point where the difference between the two is within the freedom allowed by the noise in the data. Thus maximum entropy will obtain a fit to the data with the minimum number of spectral features needed to reproduce the measured result and produce a fit which is the most probable solution within the experimental noise. The MaxEnt method can distinguish between NMR resonances and spectral artefacts such as spikes because the line shape and/or phase characteristics of the latter result in low probability in the reconstruction. Like any data processing method, MaxEnt has to be used in the correct manner and the current commercial implementation of the software allows the use of only one line-shape function and a single line-width for any one reconstruction such as Lorentzian or Gaussian or a combination of the two. Nevertheless, for situations where different parts of a spectrum have different line widths or line shapes, these separate parts of the spectrum can be inverse Fourier transformed and modelled with different line shape parameters.

2.7.5.5. Strategies for the use of HPLC/NMR for Impurity Profiling

The major problem in the use of HPLC/NMR for the characterisation of impurities is lack of sensitivity. Where the unknowns are present in large amounts (5–10%), such as might be encountered in ''research'' grade material, sensitivity is perhaps not a major issue but for more realistic problems (0.1–1%) there can be real technical difficulties. There are a number of strategies for overcoming the sensitivity problem. Firstly, the impurity can be isolated in

sufficient quantity for NMR, but in this instance there is no real need for HPLC/NMR. Alternatively, providing that there is a reasonable separation between the peak for the impurity and that for other related compounds the column can be overloaded. In fact analytical HPLC columns have a very high capacity, especially when used in gradient mode, and it has been our experience that 10–15 mg of bulk drug can be loaded onto a C18 bonded analytical HPLC column in order to identify a 0.2% impurity. This is particularly useful in the situation where the minor impurity elutes before the main component.

An alternative approach that we have used [13] is based on column switching technology to deliver the required amount of material into the NMR flow cell and this enables characterisation to be undertaken in a reasonable time (Fig. 2.7.5.C). Essentially, the peak of interest is '' heart-cut'' and concentrated on a short column of an appropriate stationary phase. This can be

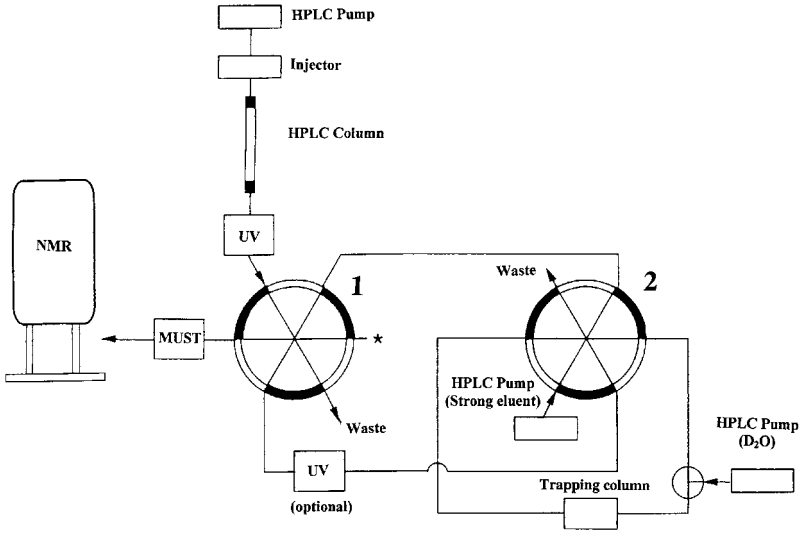


Figure 2.7.5.C.
A schematic representation of the peak trapping system for use in the identification of minor constituents by HPLC/NMR. Key: 1 and 2 are six port Rheodyne valves. Pump 2 provides D_2O as a diluent to reduce the eluotropic strength of the mobile phase and ensure that the component of interest is retained on the ''trapping'' column, from which it can be subsequently eluted with a strong eluent provided by pump 3. The ''MUST'' unit is a Spark Holland Must column switching device with an integral timer used to stop the peak of interest in the flow probe of the NMR spectrometer

repeated a number of times until sufficient material has been accumulated. The impurity is then eluted from the concentrating column with a suitably eluotropic solvent into the NMR probe. This approach is best suited to late-running chromatographic peaks which would otherwise occupy a volume of solution many times the volume of the NMR flow cell.

2.7.5.6. Applications of HPLC/NMR to Impurity Profiling

Whilst many examples of the use of HPLC/NMR in a variety of fields have been described, the body of literature describing the use of HPLC/NMR for drug impurities is not well represented. To date only two examples of the use of HPLC/NMR for this type of work have been described although there have been a number of studies of chemical mixtures of pharmaceutical and biomedical interest. These include the characterisation of peptide libraries [14], natural products [15–17], vitamin derivatives [18], environmental samples [19] and drug metabolites [20,21].

The first study of pharmaceutical impurities used a relatively modest field strength (400 MHz) ^{1}H HPLC/NMR system to identify 3% impurities of a research drug substance [5]. The HPLC/NMR system was a Bruker AMX 400 NMR spectrometer fitted with a 240-μl NMR flow cell. A conventional C-18 bonded column (250 × 4.6 mm) was used with an isocratic solvent system consisting of acetonitrile–0.05 M potassium phosphate buffer (pH 3.5) at 1 ml/min. Initial studies using on-flow NMR were performed on a mixture of the drug substance spiked with two related compounds at the 9 and 4% level respectively (for structures see inset to figures). In this on-flow study, in addition to the ''parent'' drug, the methyl resonances of the 9% spiked compound were detectable and it was possible to abstract rows from the data matrix to provide a weak spectrum for this compound. This led the authors to conclude that an on-flow detection limit of ca. 50 μg of material injected on the column was feasible. With stopped-flow NMR a spectrum from the compound spiked at the 4% level was obtained, and a better quality spectrum for the 9% spike was obtained. Thus, under these conditions ca. 25–50 μg of material on-column was required to obtain good quality ^{1}H NMR data in a reasonable time (ca. 30 min). Having investigated this model system the authors went on to study a real problem where the impurity was deemed to be present at the 3% level. Stopped flow ^{1}H NMR spectra were obtained which enabled a structure to be proposed (see Fig. 2.7.5.D). Diode array detection was also used in this study to provide the corresponding UV spectra for the chromatographic peaks and this confirmed that the impurity was structurally dissimilar to the parent. Interestingly, although the authors attempted to perform HPLC-MS as well this failed to identify the impurity. The authors also stated that, on

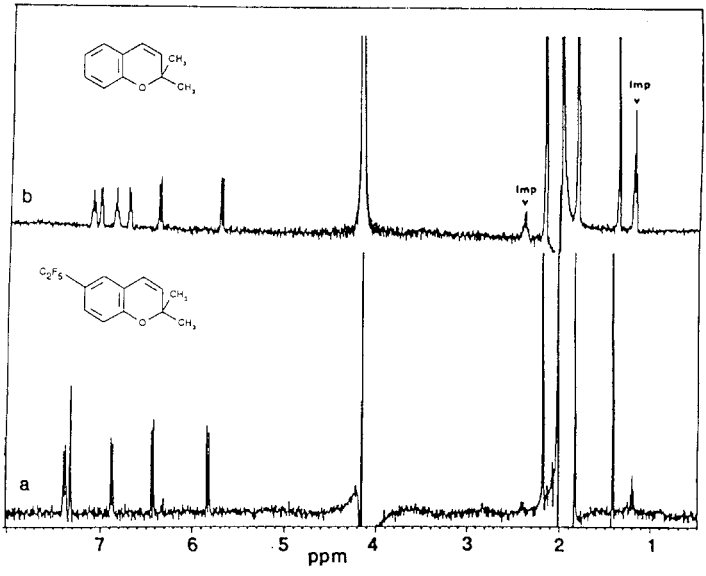


Figure 2.7.5.D.
The on-flow HPLC/NMR spectra of the major component (a) and the stopped-flow spectrum of the related 3% impurity (b). Structures as inset to the figure (from Ref. [5])

the basis of their unpublished work, HPLC NMR ''is particularly powerful for the analysis of compounds which cannot be isolated''.

More recently Mistry *et al.* [6] have described a study in which both HPLC/NMR and HPLC-MS were used for the characterisation of impurities in bulk drug batches of the steroid drug *fluticasone propionate*. This work employed a Bruker AMX-600 NMR spectrometer with a flow probe of 120 μl volume. Reversed-phase gradient chromatography on a C-18 bonded column was performed with D_2O (containing 0.05% trifluoracetic acid)–acetonitrile mixtures at a flow rate of 1 ml/min. The gradients typically began at 45% acetonitrile increasing to 60% in a linear gradient over 25 min and then to 75% by 50 min. Both stopped flow and time-slicing techniques were used and on the basis of characteristic ^{1}H NMR resonances from the stopped-flow HPLC/NMR experiments and from fragmentation patterns in HPLC/MS experiments it was possible to identify a number of late running impurities in the UV chromatogram (Fig. 2.7.5.E) as both symmetric and asymmetric dimeric materials formed by the combination of two fluticasone steroid ring systems. The structures of fluticasone and the part structures of the impurities

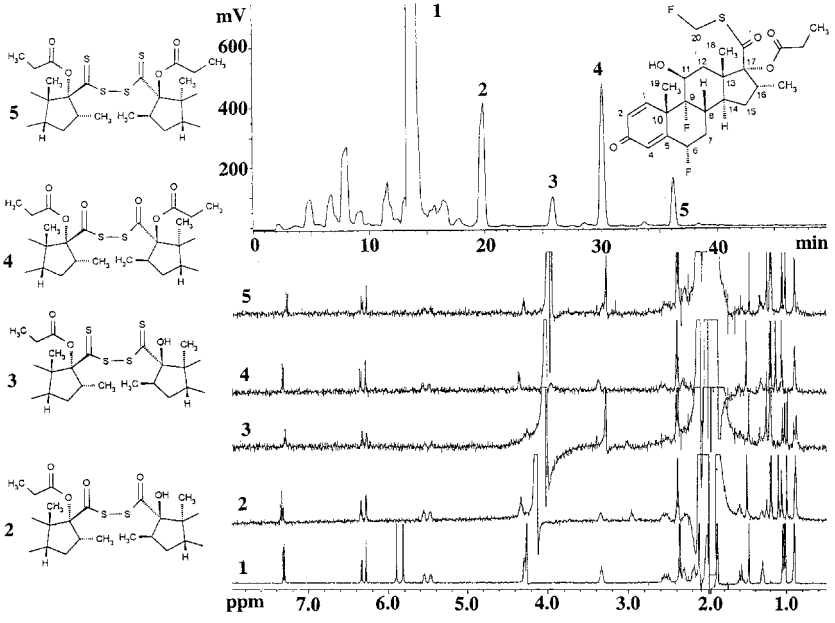


Figure 2.7.5.E.
The HPLC-UV trace (upper) and ^{1}H-NMR spectra (lower) of fluticasone (structure inset to HPLC-UV trace). The part structures of the dimeric impurities are shown to the left of the UV and NMR traces. The numbering of the UV peaks and NMR spectra corresponds to that used for the structures (from Ref. [6])

are shown as insets to Fig. 2.7.5.E together with their HPLC/NMR spectra. The technique of time-slicing was also used over the chromatographic peak of the main component fluticasone in order to investigate the presence of any impurities which might co-elute with the main component.

Another application of HPLC-NMR to impurity profiling examined the use of the technique, in combination with HPLC-MS, HPLC-ELS (evaporative light scattering detection) and HPLC-RI (refractive index detection) to characterise and identify non-UV absorbing impurities of the drug *SKF-99085* [7]. HPLC-NMR was performed using a 500-MHz instrument with deuterated water and acetonitrile forming the mobile phase. In this instance it proved to be difficult to obtain NMR spectra on any but the three most abundant impurities, with the bulk of the identification information deriving from HPLC-MS for which the NMR data provided supporting structural information.

The extension of HPLC/NMR to include chiral separations has recently

been shown with work on *atracurium besylate* [22]. In this study HPLC/NMR was used together with HPLC-CD (circular dichroism) to provide complementary approaches for the identification of structural isomers and enantiomers. Atracurium besylate (2,2′-(3,11-dioxo-4,10-dioxatrideca-methylene)-bis-(2-methyl-1,2,3,4-tetrahydropapaverinium benzenesulfonate), (structure in Fig. 2.7.5.F where the configuration at C1 can be R or S) is prepared from racemic 1,2,3,4-tetrahydropapaverine The drug provides a complex analytical challenge as it exists as a mixture of ten isomers (in various proportions), present as four racemic pairs and two meso compounds. A *cis* compound is defined arbitrarily as one where the benzyl group at C1 and the long chain at N2 are in a *cis* configuration. Stop-flow ^{1}H NMR spectroscopy (at 750 MHz) was used on-line following separation on a chiral HPLC column in order to identify the enantiomeric pairs, to distinguish the meso compounds and to identify key configurational features of the isomers. HPLC/CD experiments were undertaken in parallel enabling the assignment of the enantiomers based on the known CD and absolute stereochemistry of R-laudanosine hydrochloride, (a structural analogue).

In achiral media, different NMR spectra are expected for each of the four types of enantiomer pairs and for the two meso compounds. In general the ratio of *cis* to *trans* residues is about 3.07 which leads to proportions of *cis–cis*, *cis–trans* and *trans–trans* isomers in the ratio of 10.5:6.2:1. Chiral HPLC was performed at 60°C, with diode array UV detection in line to the HPLC/NMR. The HPLC/NMR spectroscopic data were acquired using a Varian INOVA-750 NMR spectrometer equipped with an indirect detection z-gradient ^{1}H flow probe (4 mm i.d. cell of 65 μl volume). ^{1}H NMR spectra were obtained at 750 MHz in the stop-flow mode. Suppression of the solvent signals was achieved using a pulse sequence based on the first increment of a two-dimensional NOESY experiment with dual frequency irradiation for suppression of

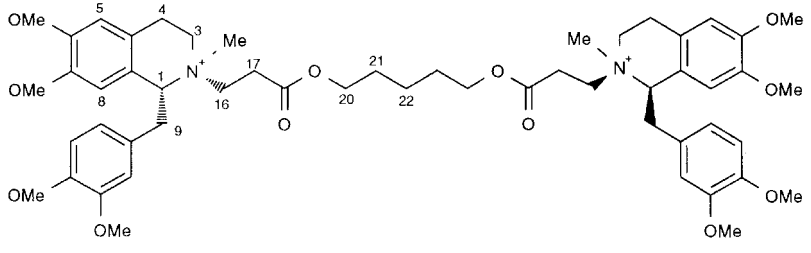


Figure 2.7.5.F.
The structure of atracurium besylate (1). Only the R-*cis*/R-*cis* isomer is shown. In all, the material is formulated as a mixture of *cis* and *trans* isomers (see text for definition) with R and S tetrahydroisoquinoline residues

both the residual water and acetonitrile signals. Separations were obtained isocratically on two 250 × 4.6 mm, 10 μm Chiracel OD-H columns connected to a Chiracel OD guard column of 50 × 4.6 mm i.d. with a mobile phase of 60:40 v/v 0.5 M $NaClO_4$ buffer (pH 2.0):ACN. Whilst it was not possible to get chromatographic baseline resolution of all ten isomers of atracurium, sufficient resolution was achieved to carry out HPLC/NMR.

The HPLC-CD separation (Fig. 2.7.5.G) was performed separately with CD and UV detection obtained using a Jasco J-600 CD spectrometer with a specially constructed Jasco HPLC cell (5 mm path length, 2 mm aperture, 16 μl volume), including quartz double focussing and defocussing optics, connected to the HPLC column via a PEEK capillary. CD spectra of the individual components were likewise acquired using the Jasco J-600 spectrometer by stopping the flow at chromatographic peaks. The UV chromatogram also shows, in addition to peaks for the analyte mixture, some early eluting substances (at retention times between 20 and 35 min) believed to be impurities or degradation products. These impurities require further characterisation.

Expansions of key reporter resonances in the 750 MHz ^{1}H NMR spectra

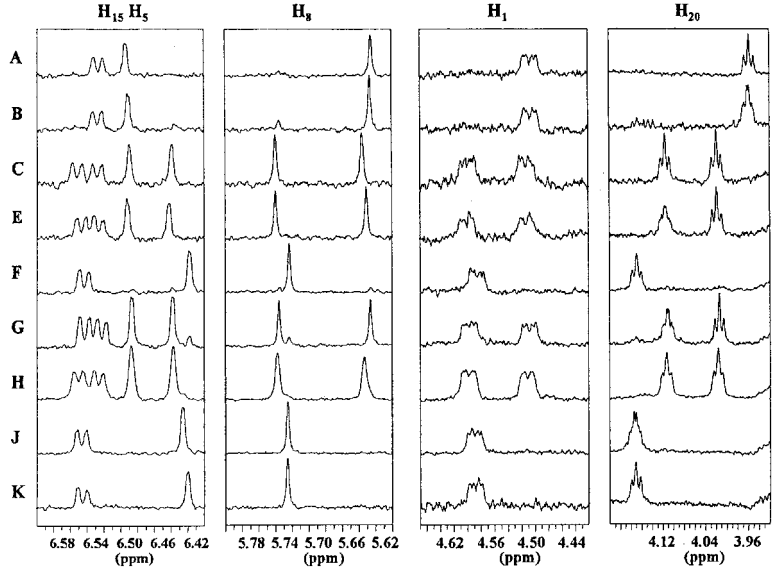


Figure 2.7.5.G.
Expansions of ^{1}H NMR spectra for key peaks in the HPLC/NMR spectra for the different isomers (for chromatogram see Fig. 2.7.5.I). For peak identification (A–K) see Table 2.7.5.A. The protons are numbered according to Fig. 2.7.5.F

obtained in stop-flow mode are given in Fig. 2.7.5.H. It is clear from the chromatogram that whilst some resolution of nine out of the ten peaks has been achieved, most of the peaks show considerable overlap. It might therefore have been expected that NMR spectra produced from the chiral HPLC/NMR experiments would not show clean single isomers, however, in general, they were remarkably pure. This was because although the HPLC peaks were in some cases as much as 8 min wide, the NMR flow probe used in this work only had a 65-μl flow cell. As a result only a fraction from the centre of each HPLC peak was actually subjected to NMR analysis thereby resulting in a spectrum uncontaminated by other components.

On the basis of the number and chemical shifts the ^{1}H NMR peaks arising for H8, peaks A, B and D, were identified as the *trans–trans* isomers, peaks F, J and K as the *cis* isomers and C, E, G and H as the *cis–trans* isomers. The identification of the enantiomeric pair from the three *cis–cis* isomers was possible from the NMR spectra in that the spectra from components F and K were essentially identical and the spectrum from component J showed distinct chemical shift differences. From this it was deduced that peaks F and K were the R/*cis*–R/*cis*, S/*cis*–S/*cis* enantiomeric pair and that peak J was the R/*cis*–S/cis meso compound. Similarly it could be shown that peaks C and H formed a *cis–trans* enantiomeric pair as did peaks E and G (as indicated by resonances for H5 and H20 in particular, as seen in Fig. 2.7.5.H). Although good NMR spectra of only two out of the three *trans–trans* isomers were obtained (peaks A

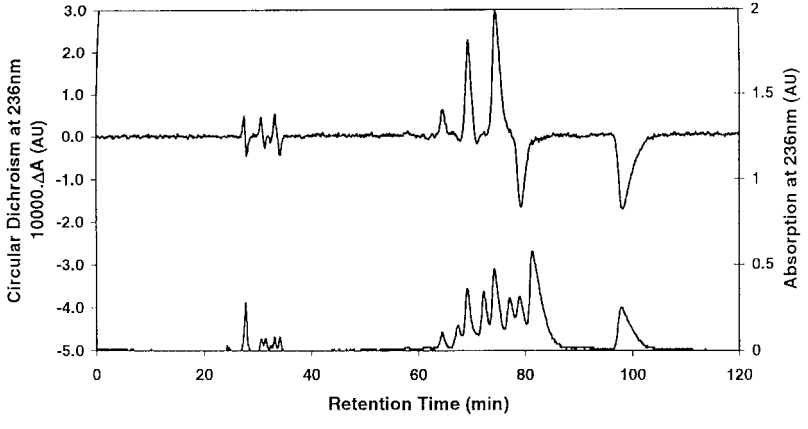


Figure 2.7.5.H.
HPLC separation of the atracurium mixture, with CD and UV detection at 236 nm. The peaks of interest elute between 61 and 95 min (early eluting peaks represent as yet unidentified impurities)

and B), these were clearly different and thus one is from the meso compound and the other is one of the enantiomeric pair isomers.

The results from the HPLC-CD experiment are given in Fig. 2.7.5.G. This shows a HPLC-CD trace on the same time scale as the UV chromatogram. The result was a series of CD peaks, some of which were positive, others negative and some which gave virtually no CD spectrum. By reference to the CD spectrum from R-laudanosine hydrochloride, it was possible to confirm that for the previously identified *cis–cis* isomers, peak F had S/S configuration, peak K was R/R and peak J was the R/S-meso compound. Similar arguments could be applied to the peaks from the *trans–trans* isomers in the chromatogram in that peak A was S/S, peak D was R/R and peak B was the R/S-meso compound. Finally for the *cis–trans* isomers, again in agreement with the NMR results, peak C was S/S, peak E was R/S, peak G was R/S and peak H was R/R. The CD spectra were insensitive to the nature of the configuration of the isomers at the C1–N2 bond. The identity of each isomer is given in Table 2.7.5.A together with HPLC retention times and HPLC UV peak areas.

This study demonstrated the feasibility of directly-coupled chiral HPLC/NMR spectroscopy which has been applied to the separation and identification of the isomers of atracurium besylate. The HPLC/NMR spectroscopy was useful for identifying the nature of the isomeric configuration at the C1–N2 bond and for identifying the enantiomeric pairs of compounds and for distinguishing them from the meso forms. The HPLC-CD experiments were complementary in that, whilst unable to distinguish the C1–N2 isomers (*cis* or *trans*), it was possible to determine the absolute stereochemistry at C1 at each tetrahydroisoquinoline residue as either R/R, S/S or R/S based on the sign of the CD response at a chosen wavelength. A consistent finding was that the S isomers eluted before the R isomers and the *trans* forms eluted before the *cis* forms. By these means a full characterisation of all of the ten isomers of atracurium has been achieved. This approach offers a new method for identification of components of complex pharmaceutical and natural product mixtures and may also be important in studies of racemization of drug metabolites, a process which often occurs in vivo.

The most recent application of HPLC-NMR in this area, in combination with HPLC-MS, was to the characterisation of the degradation products of a protease inhibitor in dosage formulations [8]. A total of six degradation products of *N*-hydroxy-1,3-di-[–4–ethoxybenzenesulphonyl]-5,5-dimethyl-[1,3]cyclohexyldiazine-2-carboxamide, were identified using reversed-phase HPLC with gradient elution, using an acetonitrile-D_2O-formic acid-based solvent system, and spectral acquisition in the stopped-flow mode. Spectroscopy was performed using a 600-MHz NMR spectrometer giving good quality spectra on an estimated 5–50 μg of each component on the column. Whilst the

Table 2.7.5.A.
The chromatographic retention times and identities confirmed by chiral HPLC/NMR and HPLC-CD

Peak	Retention time (min)	HPLC peak area (%)	Isomer by NMR	Sign of CD spectrum	Identity[b]
A	61.95	2.09	*trans–trans*	+	S-*trans*/S-*trans*
B	64.72	2.89	*trans–trans*	0	R-*trans*/S-*trans*
C	66.08	9.61	*cis–trans*	+	S-*cis*/S-*trans*
D[a]	68.39	1.30	*trans–trans*	−	R-*trans*/R-*trans*
E	69.22	8.75	*cis–trans*	0	R-*cis*/S-*trans*
F	70.99	14.81	*cis–cis*	+	S-*cis*/S-*cis*
G	74.09	8.49	*trans–cis*	0	R-*trans*/S-*cis*
H	76.02	9.40	*cis–trans*	−	R-*cis*/R-*trans*
J	78.00	28.29	*cis–cis*	0	R-*cis*/S-*cis*
K	94.90	14.37	*cis–cis*	−	R-*cis*/R-*cis*

[b] Isomer peaks giving a negative CD spectrum have been assigned the R/R configuration by comparison with a closely-related model compound, R-laudanosine hydrochloride

[a] The identity of this peak was assigned by elimination since insufficient chromatographic resolution precluded acquisition of a clean HPLC/NMR spectrum

HPLC-MS data gave good molecular mass information, and fragmentation data enabled structures for the degradation products to be proposed the NMR data was required in order to make more definate structural assignments. As an example the material designated ''degradant 5'' illustrated in Fig. 2.7.5.I could, on the basis of the MS data have been fitted to a number of structures, but the NMR data pointed to the cyclic urea structure shown as an inset to the figure. Similarly, for several of the other degradation products the MS data could have been explained by one or more different structures which were readily resolved with reference to the NMR spectra. This study provides another excellent example of the value of having both HPLC-NMR and HPLC-MS data on the impurities/degradation products to be identified, and clearly points to the value of the doubly hyphenated systems described below.

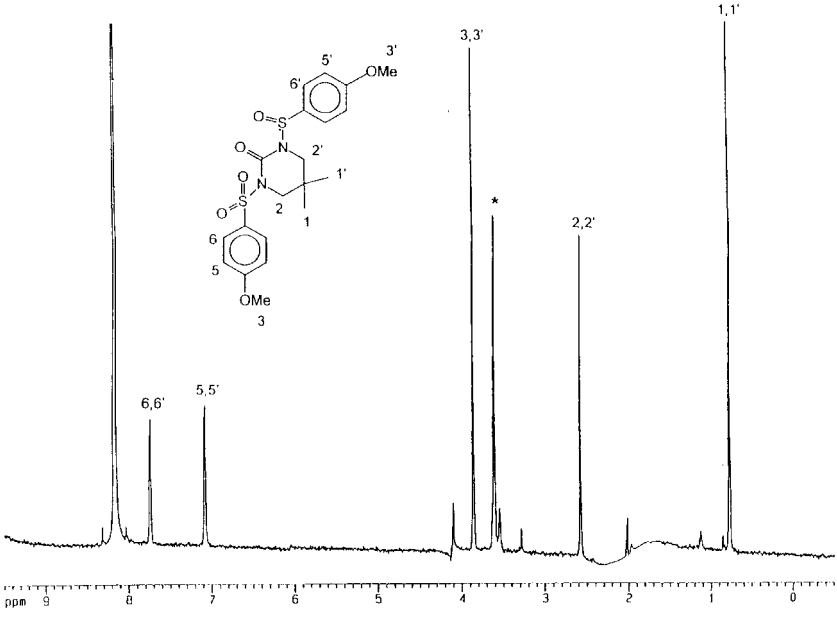


Figure 2.7.5.I.
The HPLC/NMR spectrum of degradant 5 (structure in inset to figure) obtained in stopped-flow mode. The resonance at ca. 3.6 ppm marked with an asterisk is an undefined impurity present in the mobile phase (from Ref. [8])

2.7.5.7. Future Approaches to Impurity Profiling ((CE/CEC)/NMR, SFC/NMR, HPLC/NMR/MS)

2.7.5.7.1. Capillary-HPLC, Capillary Electrophoresis and Capillary Electrochromatography (CE/CEC)/NMR

As mentioned earlier, capillary HPLC/NMR has been described [23,24], and more recently several groups have used capillary electrophoretic or electrochromatographic techniques, to provide high efficiency separations with NMR detection [25,26]. Whilst not yet used for impurity profiling or identification, the techniques can enable spectra to be obtained on a few nanograms or tens of nanograms of material in a few minutes. An example of this is illustrated in Fig. 2.7.5.J where the separation of paracetamol metabolites, present in an extract of human urine, by capillary electrophoresis is shown [26]. The resulting NMR spectra correspond to ca. 10 ng of each of the sulphate and glucuronide conjugates of the drug. Although experimental at the moment such systems clearly show great promise for the future.

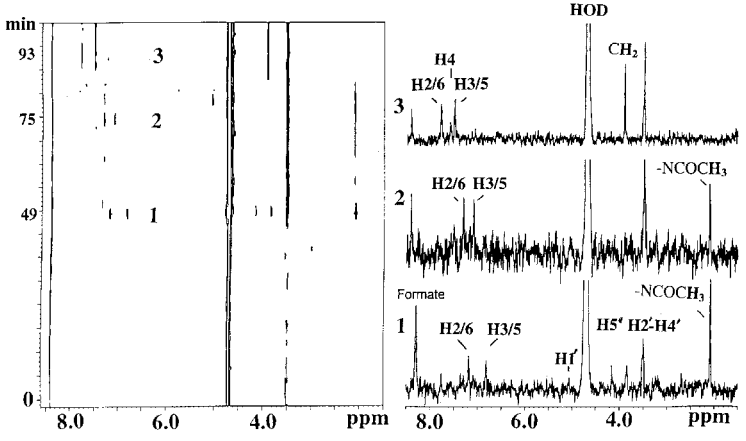


Figure 2.7.5.J.
The on-flow ^{1}H-NMR trace showing the CZE separation of paracetamol metabolites isolated from human urine together with the spectra, obtained from single rows extracted from the continuous flow electropherogram, corresponding to the glucuronide and sulphate conjugates (peaks 1 and 2 respectively) and the endogenous component hippuric acid (peak 3)

2.7.5.7.2. Supercritical Fluid Chromatography (SFC)/NMR

Although not as popular as HPLC/NMR, SFC/NMR has been demonstrated as a practical technique [4,27]. The need to maintain the CO_2 used for the mobile phase in the liquid state means that the probe must be pressurised and this can cause problems in the stopped-flow mode. Furthermore, modifiers such as MeOH often have to be added to increase solubility, re-introducing NMR resonances and removing the main advantage of the technique. SFC is analogous to normal phase HPLC which finds few applications in the pharmaceutical industry. With the possible exception of chiral separations, it is difficult to envisage the development of many applications of SFC/NMR for drug impurity work.

2.7.5.7.3. HPLC/NMR/MS

Linking HPLC with NMR and MS in such a way as to provide both sets of spectroscopic data from a single chromatographic run is an obvious and logical extension of having both types of system available in the laboratory and a number of examples of this have been published [28–30]. Current practical problems of combining both types of spectrometer include compatibility of chromatographic eluents, the deuteration of exchangeable protons, integration of data capture, the type of configuration to use (e.g. spectrometers in-line or in parallel) and the need to make allowance for the fact that the positioning of the mass spectrometer must be carefully considered because of the potential for interference by the stray field of the NMR magnet.

Whilst as yet there are no published studies on the use of this combined methodology for pharmaceutical impurities the power of the combination of HPLC, NMR and MS is provided by some recent studies on urine samples obtained after an oral dose of paracetamol [29]. In the experimental set-up used for these studies conventional RP-HPLC was performed with an acetonitrile–water–trifluoroacetic acid mobile phase and a C18 column. The eluate from the column was split 95:5, with the major portion going to the NMR spectrometer (Bruker, 500 MHz) and the remainder to an ion trap MS (Finnegan). The major metabolites of paracetamol are well known and consist of the sulphate and glucuronide conjugates of the phenolic hydroxyl group on the aromatic ring of drug. The NMR trace (Fig. 2.7.5.J) shows the presence of these metabolites quite clearly, with the anomeric proton for the glucuronide moiety readily visible (7.6 min). In the case of the sulphate conjugate (8.6 min) no new signals are visible in the NMR spectrum to aid identification as the sulphate moiety itself is ''NMR invisible''. However, the mass spectral data obtained for this compo-

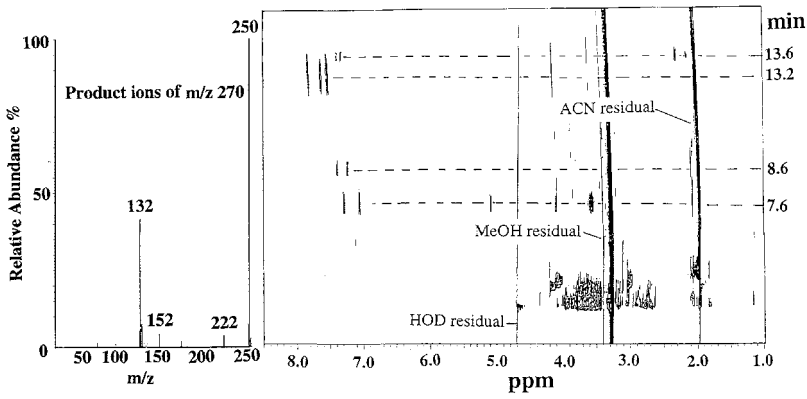


Figure 2.7.5.K.
On flow HPLC/NMR/MS of an extract of urine sample obtained from a human volunteer following the administration of acetaminophen (paracetamol). The pseudo-two-dimensional NMR spectrum show peaks eluting at 7.6 and 8.6 min for the glucuronide and sulphate conjugates, respectively. The prominent aromatic signals eluting at 13.2 min are for the endogenous compound hippuric acid whilst those at 13.6 min are for phenylacetylglutamine, for which the on-flow mass spectrum is shown

nent enabled an unequivocal assignment. However, as well as the paracetamol metabolites some additional aromatic signals were seen eluting at 13.2 (hippuric acid) and 13.6 min. The latter, which were partially overlapped with the hippurate, did not correspond to a usual urinary component. From the NMR spectrum the presence of a monosubstituted aromatic ring (H3, H5 δ7.42, H2,H4H6, δ7.34) with a methylene group appearing as a singlet at δ3.65 was indicated. A further group of signals, at δ4.39, δ2.34, δ2.20 and δ2.00 suggested a glutamine moiety might be present, leading to a tentative assignment of phenylacetylglutamine. This was confirmed by the MS data which gave a molecular ion at m/z 270 (Fig. 2.7.5.K) for the fully deuterated analyte, and appropriate product ions. Thus, the combination of the two techniques however, provided all the required information in a single chromatographic run. The potential for the use of HPLC/NMR/MS for identifying impurities is, we believe, self-evident and it is probable that a similar approach will eventually be applied to CE and CEC.

2.7.5.8. Conclusions

The future of the techniques such as HPLC/NMR (and HPLC-NMR-MS/MS) seems assured. In the case of NMR spectroscopy increasing field strength and improvements in probe design alone will greatly enhance the sensitivity of the technique (e.g. cryogenically cooled NMR signal detection systems are anticipated to yield a 4–5 fold increase in sensitivity). Advances are also apparent in areas such as the use of microbore HPLC/CEC in combination with NMR giving nanogram sensitivity. Even in the absence of further advances.

HPLC/NMR can even now be used as a routine analytical technique. It can be argued that there are disadvantages in the use of NMR coupled to HPLC and it is true that it is generally much less sensitive than detectors such as UV or MS. However, this has to be balanced against the very high information content of the NMR spectra. Improvements in spectrometer dynamic range in NMR experiments mean that the use of deuterated organic solvents is no longer a necessity and H_2O could also be used in place of D_2O. Currently, there are still advantages to using D_2O, and the cost of this is not high when purchased in bulk. As actively shielded magnets become more widely available the length of capillary tubing connecting the HPLC system to the NMR probe will become shorter, reducing problems associated with band broadening.

The increasing automation of HPLC/NMR will increase the value of this approach and HPLC/NMR will find wide applications in areas such as drug metabolism, toxicology and biochemistry and in the pharmaceutical and food industries. Indeed, a time can be envisaged when fully automated HPLC/NMR/MS will allow full structural elucidation of complex mixtures of organic compounds in a single chromatographic run.

References

1. K. Albert and E. Bayer, in *HPLC Detection: Newer Methods* (G. Patonay, Ed.), p 192. VCH, Weinhem, Germany (1992)
2. J.C. Lindon, J.K. Nicholson and I.D. Wilson, *Prog. NMR Spectrosc.* **29**, 1–49 (1996)
3. J.C. Lindon, J.K. Nicholson, U.G. Sidelman and I.D. Wilson, *Drug Metab. Rev.* **29**, 705–746 (1997)
4. K. Albert, *J. Chromatogr. A* **703,** 123–147 (1995)
5. J.K. Roberts and R.J. Smith, *J. Chromatogr. A* **677**, 385–389 (1994)
6. N. Mistry, I.M. Ismail, M.S. Smith, J.K. Nicholson and J.C. Lindon, *J. Pharm. Biomed. Anal.* **16**, 697–705 (1997)

7. S.D. McCrossen, D.K. Bryant, B.R. Cook and J.J. Richards, *J. Pharm. Biomed. Anal*. **17**, 455–471 (1998)
8. S.X. Peng, B. Borah, R.L.M. Dobson, Y.D. Lui and S. Pikul, *J. Pharm. Biomed. Anal*. **20**, 75–89 (1999)
9. T. Tsuda, Y. Ojika, M. Izuda, I. Fujishma and D. Ishi, *J. Chromatogr*. **69**, 194–197 (1972)
10. N. Watanabe and E. Niki, *Proc. Jpn. Acad. Ser. B* **54**, 194–199 (1978)
11. E. Bayer, K. Albert, M. Nieder, E. Grom and T. Keller, *J. Chromatogr*. **186**, 497–507 (1979)
12. S.H. Smallcombe, S.L. Patt and P.A. Keifer, *J. Magn. Reson*. A **117**, 295–303 (1995)
13. L. Griffiths and R. Horton, *Magn. Reson. Chem*. **36**, 104–109 (1998)
14. J.C. Lindon, R.D. Farrant, P.N. Sanderson, P.M. Doyle, S. Gough, M. Spraul and J.K. Nicholson, *Magn. Reson. Chem.* **33**, 857–863, (1995)
15. S. Johnson, E.D. Morgan, I.D. Wilson, M. Spraul and M. Hofmann, *J. Chem. Soc. Perkin Trans*. **1**, 1499–1502 (1994)
16. J.-L. Wolfender, S. Rodriguez and K. Hostettmann, *J. Chromatogr. A* **794**, 299–316 (1998)
17. I.D. Wilson, E.D. Morgan, R. Lafont and B. Wright, *J. Chromatogr. A* **779,** 333–336 (1998)
18. K. Albert, G. Schlotterbeck, L.-H. Tseng and U. Braumann, *J. Chromatogr. A* **750**, 303–307 (1996)
19. M. Godejohann, A. Preiss, C. Mugge and G. Wunsch, *Anal. Chem.* **69,** 3832–3837 (1997)
20. M. Spraul, M. Hoffmann, P. Dvortsak, J.K. Nicholson and I.D. Wilson, *Anal. Chem* **65**, 327–330 (1993)
21. M. Spraul, M. Hofmann, J.C. Lindon, R.D. Farrant, M.J. Seddon, J.K. Nicholson and I.D. Wilson, *NMR Biomed.* **7**, 295–303 (1994)
22. N. Mistry, A.D. Roberts, G.E. Tranter, P. Francis, I. Barylski, I.M. Ismail, J.K. Nicholson and J.C. Lindon, *Anal. Chem.* **71**, 2838–2842 (1999)
23. K. Albert, G. Schlotterbeck, L.-H. Tseng and U. Braumann, *J. Chromatogr. A*. **750**, 303 (1996)
24. G. Schlotterbeck, L.-H. Tseng, H. Handel, U. Braumann and K. Albert, *Anal Chem*. **69**, 1421–1425 (1997)
25. N. Wu, T.L. Peck, A.G. Webb, R.L. Magin and J.V. Sweedler, *Anal. Chem.* **66,** 3849–3857 (1994)
26. J. Schewitz, P. Gfrörer, K. Pusecker, L.-H. Tseng, K. Albert, E. Bayer, I.D. Wilson, N. Bailey, G.B. Scarfe, J.K. Nicholson and J.C. Lindon, *Analyst* **123**, 2835–2837 (1998)
27. K. Albert, U. Braumann, L.-H. Tseng, G. Nicholson, E. Bayer, M. Spraul, M. Hofmann, C. Dowle and M. Chippendale, *Anal Chem.* **66,** 3042–3046 (1994)

28. F.S. Pullen, A.G. Swanson, M.J. Newmann and D.S. Richards, *Rapid Commun. Mass Spectrom.* **9**, 1003–1006 (1995)
29. J.P. Shockor, S.E. Unger, I.D. Wilson, P.J.D. Foxall, J.K. Nicholson and J.C. Lindon, *Anal. Chem.* **68**, 4431–4435 (1996)
30. S. Taylor, B. Wright, E. Clayton and I.D. Wilson, *Rapid Commun. Mass Spectrom.* **12**, 1732–1736 (1998)

2.8. Determination of Drug Related Impurities by Capillary Electrophoresis

Kevin D. Altria

Capillary electrophoresis (CE) is a highly automated form of electrophoresis with on-capillary detection. Free solution CE (FSCE) relies upon an exploitation of differences between the solutes charge and size in an aqueous medium and CE is therefore suitable for analysis of a significant proportion of drugs. Many drugs are either acidic and/or basic, water-soluble and insoluble compounds can therefore be readily analysed using CE. For example an acidic drug could be analysed at a high pH in its anionic form whilst basic drugs may be analysed at low pH as cations. Zwitterionic drugs may be analysed at either end of the pH range. Neutral drugs require use of a form of CE known as micellar electrokinetic capillary chromatography (MECC) in which ionic surfactant is added to the electrolyte. The surfactant molecules aggregate to form micelles thus providing neutral molecules with a basis for partition. Textbooks are available [1,2] giving background details regarding the various separation modes and principles of CE. Readers with internet access are recommended to visit http://www.ceandcec.com which contains extensive information regarding CE.

2.8.1. Introduction to Capillary Electrophoresis (CE)

Figure 2.8.A shows a schematic of a typical CE system set-up. Separations are achieved by filling a capillary with an electrolyte solution. A volume of sample is then injected into the end of the capillary furthest from the detector usually performed by applying a pressure to the sample vial whilst it is inserted into the sample vial. The capillary is then immersed in buffer reservoirs which are placed at either end of the capillary. An electrical field is then applied (between 1 and 30 kV) which causes the compounds in the sample mixture to migrate along the capillary towards the on-capillary detection system. The smaller, higher charged compounds will reach the detector window first.

The fused silica capillary used is usually 25–100 μm wide by 27–50 cm long and typical sample injections range between 1 and 20 nl. Typical detection is UV absorbance although other detection systems are available such as fluorescence or conductivity. The system is PC controlled and the data output is in a plot of detector response with time (as electropherogram). Peak areas are used for calculating purposes.

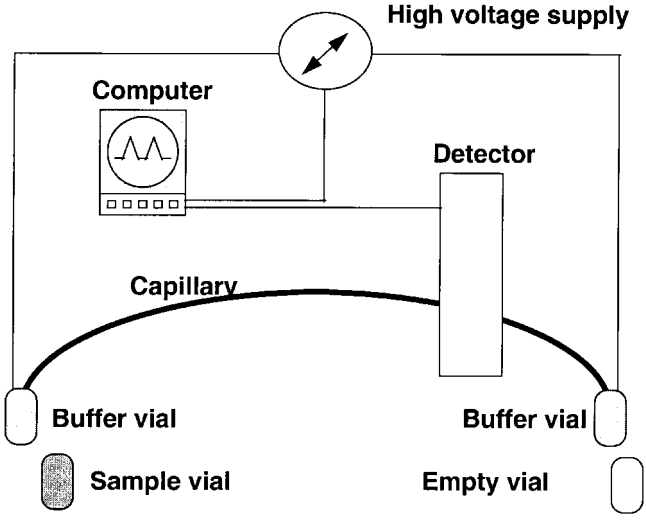


Figure 2.8.A.
Typical CE system

Electrophoresis is the movement of sample ions under the influence of an applied voltage. The ion will move towards the appropriate electrode and pass through the detector. The migration rate or mobility of the solute ion is governed largely by its size and number of ionic charges (Fig. 2.8.B). For instance, a smaller ion will move faster than a larger ion with the same number of charges. Similarly, an ion with two charges will move faster than an ion with only one charge and similar size. The ionic mobility (μE) is therefore related to the charge/mass ratio (Eq. (2.8.1)).

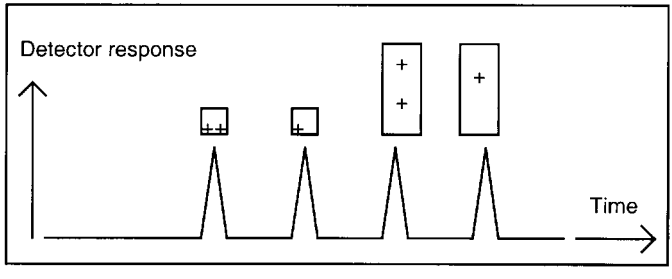


Figure 2.8.B.
Theoretical separation of a range of cations

$$\mu E = \frac{q}{6\Pi\eta r} \tag{2.8.1}$$

where μE is the electrophoretic mobility, q is the number of charges, η is the solution viscosity and r is the radius of the ion.

Therefore, when a mixture of ions having different charges and sizes is separated the smaller more highly charged ions will be detected first (Fig. 2.8.B).

The actual electrophoretic velocity, or speed of the solute ions is related to their mobilities and the magnitude of the applied voltage (Eq. (2.8.2)).

$$V = \mu E \times E \tag{2.8.2}$$

where V is the velocity of the ion and E is the applied voltage (V/cm).

Therefore the higher the voltage the faster the separation.

Application of voltage across a capillary filled with electrolyte causes a flow of solution along the capillary. This flow is called ''electro-osmosis'' and effectively pumps solute ions and the electrolyte in the capillary towards the detector. This flow occurs due to ionisation of the acidic silanol groups on the inside of the capillary when in contact with the buffer solution (Fig. 2.8.C). At high pH these groups are dissociated resulting in a negative charged surface. To maintain electroneutrality cations build up near the surface. When a voltage is applied these cations migrate to the cathode. The water molecules solvating the cations also move causing a net solution flow along the capillary. This effect could be considered as an ''electric pump''.

The extent of the flow is related (Eq. (2.8.3)) to the charge (zeta potential) on the capillary, the buffer viscosity and dielectric constant of the buffer:

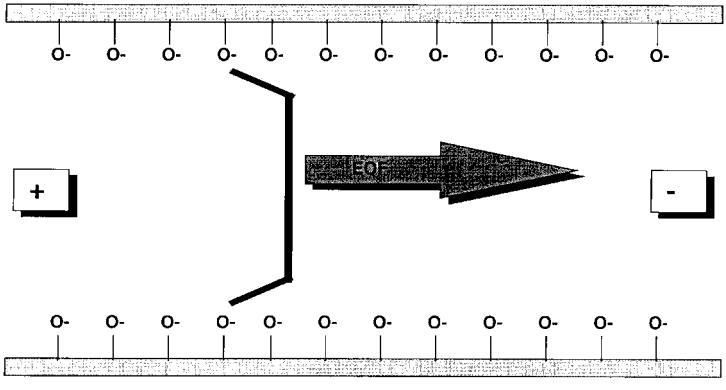


Figure 2.8.C.
Electro-osmotic flow

$$\mu\text{EOF} = (\varepsilon\zeta/\eta) \tag{2.8.3}$$

where μEOF is the ''EOF mobility'', η is the viscosity and ζ is the zeta potential (charge on capillary surface).

The level of EOF is highly dependent upon electrolyte pH as the zeta potential is largely governed by the ionisation of the acidic silanols. Below pH 4 the ionisation is small and the EOF flow rate is therefore not significant; above ~pH 9 the silanols are fully ionised and EOF is strong. The level of EOF decreases with increased electrolyte concentration as the zeta potential is reduced.

2.8.2. Pharmaceutical Analysis by CE

The range of applications of CE is similar to HPLC and includes determination of drug-related impurities, chiral separations, raw material/excipient analysis, drug salt stoichiometry determination, cleaning validation testing, main component/identity confirmation and the analysis of bio-pharmaceuticals. The use of CE in pharmaceutical analysis has recently been covered in a review paper [3] and a book [4]. A recent extensive review by Watzig *et al.* [5] excellently summarises method development approaches in CE and provides over 800 references.

CE offers a completely different selectivity process to that of HPLC and TLC. Therefore it is useful to cross-correlate CE results with those obtained by TLC or HPLC. The simplicity and robustness of CE methods makes them an attractive supplement or replacement to the use of HPLC. CE can offer an inexpensive and rapid test method for a variety of compounds of pharmaceutical interest. Aqueous buffers are generally used in CE and therefore there are considerable environmental benefits to using CE – this aspect has been [6] recognised by the USP as being an important feature of CE.

CE is increasingly being viewed as an alternative and complement to HPLC for the determination of drug related impurities. These determinations are probably the principal role of CE within pharmaceutical analysis and represents a challenge to both selectivity and sensitivity capabilities of the technique. The main component and structurally related impurities often have very similar chemical properties (size and number of ionic charges) which places great requirements on the selectivity necessary. An advantage CE has over its chromatographic counterparts is that high separation efficiencies obtained in CE can often translate a small degree of selectivity into an acceptable resolution. Detection limits of 0.1% area/area are widely accepted as a minimum requirement for a related impurities determination method and this is possible by CE.

This chapter subdivides the reports of drug impurity determinations into low pH, high pH and MECC applications. Examples from each of the three main separation mechanisms employed are discussed. Brief sections are also included on the use of microemulsion CE, non-aqueous CE and applications of indirect UV detection.

2.8.3. Related Impurities Determinations by CE

Currently the vast majority of drug-related impurity determinations are performed by HPLC which can offer the desired sensitivity for trace level determinations and offers a high degree of automation. A wide variety of stationary phases and operating modes makes HPLC applicable to all drug classes. The typical detection limits for drug-related impurities by HPLC are 0.1% or low as 0.01% and this can be routinely met in the majority of circumstances using conventional HPLC UV detectors. It is common practise to employ a secondary support analytical technique to verify HPLC impurity data. In the past this secondary testing has been largely performed by TLC. However, the high degree of automation is not readily available except in highly sophisticated TLC systems. CE also has an additional advantage over TLC in that commercial CE autosamplers and on-line detection allows simultaneous assay of the main component and related impurities by CE.

The ability of CE to give a different selectivity to HPLC and/or TLC provides a further means to characterise the impurity content and profiles in drugs [7]. For example levels of *domperidone* impurities were quantified by TLC, HPLC and CE in drug substance [8]. Table 2.8.A shows the result from HPLC and CE for selected impurities determined in three drug substance batches. Good agreement between the three techniques was obtained for total impurity levels. However, CE resolved two additional components which co-eluted in the HPLC and TLC methods.

One of the attractive features of CE compared to TLC is that peaks are generated which can be integrated and the areas used to calculate the impurity content as area% of the main peak. This makes comparisons with HPLC data much more simple than with TLC determinations. There is however an additional requirement in handling CE data to calculating impurity levels in that it is necessary to divide the observed area of each peak by its migration time [9]. This normalisation is necessary since faster migrating peaks move through the detector at a greater speed than their slower counterparts. Therefore, faster moving peaks have smaller peak widths and correspondingly smaller peak areas as the peak area obtained is related to both the peak height and peak width. The sum of these ''normalised peak areas'' is used to calculate impurities as %area/area. When impurity determinations are to be expressed as

Table 2.8.A.
Determination of impurity content in domperidone drug substance batches by CE and HPLC (from Ref. [8])

Batch number	R45571 content		R48557 content		Unknown impurity	
	CE	HPLC	CE	HPLC	CE	HPLC
1	0.24	0.26	0.15	0.35	0.17	–
2	0.22	0.23	0.15	0.34	0.24	–
3	0.26	0.27	0.15	0.30	0.18	–

%w/w through the use of external standards, providing the precision of migration time is acceptable this normalisation process is not required. This was experimentally demonstrated [9] using a solution of *ranitidine* spiked with a known amount of an impurity. The impurity migrated before the main peak and was spiked at 9.1%, the non-normalised data indicated a result of 6.0% whilst the normalised data confirmed the expected 9.1%.

2.8.4. Separations Using Low pH Electrolytes

Analysis of basic drugs by HPLC can represent problems due to peak tailing due to interactions with stationary phase silanols. This problem does not occur so frequently in CE and impurities of basic drugs are generally well separated at pH 2–4. The selectivity obtained under these conditions is based on differences between the charge/mass ratios for the drug and its related impurities. The majority of degradation impurities and synthetic intermediates tend to be smaller than the drug compound and therefore migrate before the main peak. In addition dimeric impurities may be doubly charged compared to the parent compounds [10,11] at low pH and migrate before the main peak. Conversely in HPLC dimeric impurities are generally strongly retained on column and often require use of gradient elution.

A simple NaH_2PO_4 buffer pH adjusted to pH 2.5 with concentrated H_3PO_4 often gives a useful initial separation for the separation of the related impurities of basic drugs as these are generally water soluble and fully ionised as cations at this low pH. An additional advantage of these simple electrolyte systems is that they have low background UV absorbance and operation [11,12], low UV wavelengths such as 190–210 nm are possible where many drug compounds have significantly enhanced UV absorbance coefficients. For example a low pH phosphate buffer has been used [13] in forensic analysis to analyse 550 basic drugs using a single set of operating conditions.

Many small synthetic intermediates such as *imidazole* are very difficult to analyse by HPLC but present few problems [14] at low pH with detection at 200 nm. It may therefore be possible to detect impurities with very limited chromophores using low wavelength detection in CE that would be undetectable by HPLC which has typical UV cut-off ranges of 205–210 nm depending on the solvent used.

A pH 3 phosphate buffer was used [15] in the identification and quantitation of the main degradation product napthylacetyletylenediamine (NAED) in *naphazoline hydrochloride* bulk drug. The effect of temperature, operating voltage, and electrolyte concentration on the resolution was determined by using a multivariate experimental design. The method was validated and satisfactory specificity, linearity, precision, and accuracy results were obtained.

If mobility differences alone do not provide sufficient selectivity then various complexing agents may be added to the electrolyte to suitably alter the migration speed (mobility) of the drug and its impurities. The most frequently employed approach is the addition of millimolar quantities of cyclodextrin [16,17] into the electrolyte. The migration times obtained then reflect both the solutes electrophoretic mobility and its partitioning with the cyclodextrin.

Other factors that can be optimised in method development include the addition of ion-pair reagents and the ionic strength of the electrolyte. Both of these influence the shape of the main peak and appropriate optimisation may allow resolution of a closely resolved impurity. For instance, various types and concentrations of ion-pair reagent were employed [18] in conjunction with the addition of cyclodextrin in the optimisation of the separation of *remoxipride*. A range of synthetic and degradative impurities of remoxipride were monitored at the 0.1% level. The peak shape and resolution was effectively manipulated by the choice and concentration of these additives. The concentration of both cyclodextrin and ion-pair reagent were simultaneously optimised to adjust the peak shape and selectivity in the separation of remoxipride and eight impurities. The final method conditions used a mixture of tetrapropylammonium (8 mM) and tetrabutylammonium (12 mM) ions and methyl-beta-cyclodextrin (20 mM) which allowed all impurities to be resolved and detected at 0.05% in the presence of the main component.

Resolution can also be adjusted by the addition of organic solvents [19] such as iso-propanol and methanol to the electrolyte as this effects the pK_a values of the basic drugs.

Adjustment of the peak shape to allow resolution of closely related migrating peaks can be achieved by varying the electrolyte constituents. For example to obtain an efficient peak shape for later migrating cationic drugs it is appropriate to use an electrolyte containing triethanolamine as the cationic constituent of the buffer. Conversely if the solute is a small and/or highly charged compound then sodium is as an appropriate cationic constituent. An efficient separation of a basic drug and related impurities was obtained using an electrolyte containing triethanolamine adjusted to pH 2.5 with phosphoric acid. When this analysis was performed using sodium phosphate adjusted to pH 2.5 with phosphoric acid the peak shape obtained [20] was very asymmetric and the impurity peaks were poorly resolved from each other and the main peak. The triethanolamine ion can also become adsorbed onto the capillary surface resulting in a slightly positively charged capillary surface which serves to prevent adsorption of positively charged basic drug ions.

The choice of the anionic component of the buffer can also influence the selectivity obtained. For instance [21] in the method development for the separation of *ranitidine* and its related impurities a wide range of buffer

types were assessed including phosphate, TRIS, acetate, and citrate. Acceptable resolution (Fig. 2.8.D) of ranitidine and seven related impurities was only obtained [21] using a citrate buffer.

In many circumstances there is no need for a full characterisation profile of the test sample and a fast analysis time is more essential. This may be the case for example in the testing of a batch during production where in-process testing is performed. In-process testing involves monitoring the levels of the reactants and products during a synthesis until the batch contains less than a specified level of precursor(s) and/or %purity of the product. In this type of testing speed of analysis is of major importance and the analytical methods used do not generally fully characterise the impurity content of the product. Use of short capillaries coupled with high voltages can allow extremely short analysis times to be attained. *Fluparoxan* impurities were separated within 2 min [12] using a 27-cm capillary whilst use of a 57-cm capillary gave increased resolution but with a longer analysis time of over 10 min.

Water insoluble basic compounds may often be dissolved in a 1:10 dilu-

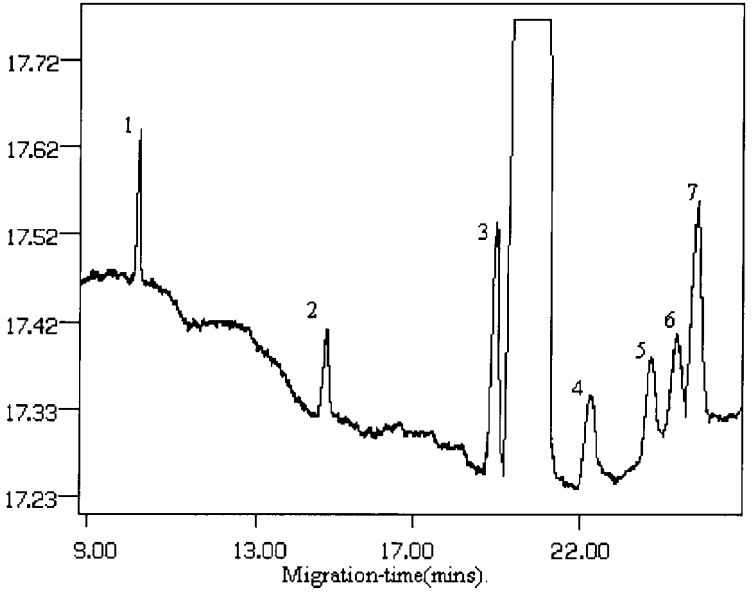


Figure 2.8.D.
Optimised separation of ranitidine and seven related impurities. Conditions: fused silica capillary 27 cm × 50 μm i.d.; buffer equivalent to 190 mM trisodium citrate adjusted to pH 2.6 with citric acid; 6 kV; 230 nm; 25°C (from Ref. [21])

tion of the buffer or pH adjusted water. Amphoteric compounds may [25] be beneficially dissolved in a high pH solution, but analysed using a low pH electrolyte. This approach has been used in the determination of impurities in a water insoluble amphoteric *quinolone antibiotic* [25]. The compound was only soluble at pH extremes of less than 2 and greater than 10. The sample was dissolved in NaOH solution and analysed with a pH 1.5 electrolyte.

Generally samples should be dissolved in pure water or dilute buffer as this maximises resolution and peak efficiencies. Figure 2.8.D shows separation [23] of the impurities of *salbutamol* when the sample is dissolved in either water (Fig. 2.8.Ea) or the buffer (Fig. 2.8.Eb) solution. Higher separation efficiencies and improved detection limits are achieved when the appropriate sample solvents are used. In HPLC the mobile phase is often used to dissolve the sample which is therefore quite different to CE.

The complementary nature of HPLC and CE of the determination of two dimeric salbutamol impurities was highlighted [11]. These relatively large impurities were strongly adsorbed onto the HPLC column and therefore had lengthy retention times. However, in CE the dimeric impurities had a charge $Z = +2$ and therefore migrated before the salbutamol ($Z = +1$). Drug substance batches were tested by CE and HPLC using external standards of the impurities for quantitation. Table 2.8.B shows the good correlation between the two techniques.

Extensive validation has been performed [24] on a CE method for the determination of impurities in *mirtazapine* drug substance present in *Remeron* tablets. An experimental design was used to optimise the separation conditions in terms of the pH, buffer concentration and %methanol content. An experimental design was also used to assess the optimal sample dissolving solvent. It was found that a diluted form of the buffer gave the best performance in terms of repeatability for migration times, consistent current throughout the separation and improved peak area precision. Selectivity was demonstrated by preparing stressed samples by exposure to high levels of temperatures, humidities and light intensity. Detector response linearity (correlation greater than 0.995) for each of the impurities was demonstrated over the range 0.25–2.0% of the expected mirtazapine range. Recovery was demonstrated by spiking placebo tablet formulations (recovery data was well within the range of 90–110%). Use of an internal standard allowed 0.5% RSD values to be obtained for peak area ratios. Limits of detection of 0.02–0.04% were obtained for the individual impurities. A correlation coefficient of 0.9997 was obtained between the CE results and those generated using the established HPLC method.

Impurities have been determined [25] in a water insoluble *quinolone antibiotic* which was only soluble at pH extremes of less than 2 and greater than 10. The sample was dissolved in NaOH solution and analysed with a pH 1.5 electrolyte. A detection limit of 0.1% was demonstrated during validation.

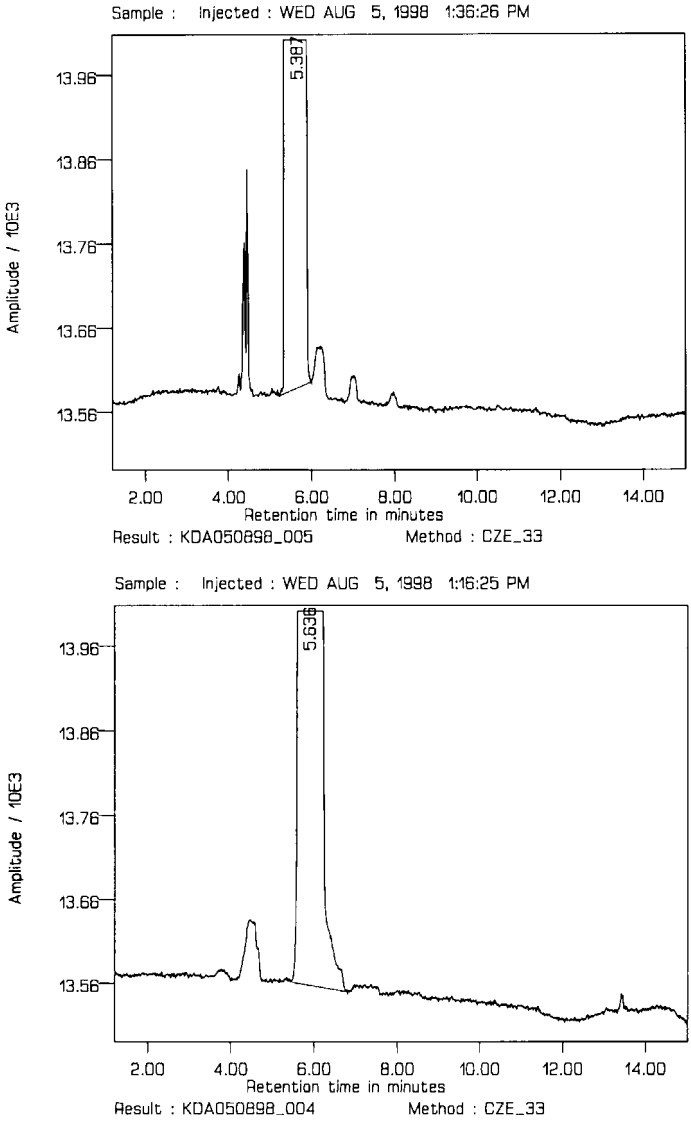


Figure 2.8.E.
Effect of dissolving solvent upon separation quality. (a) Salbutamol sample dissolved in water; (b) salbutamol sample dissolved in run buffer (from Ref. [23])

Table 2.8.B.
Comparison of impurity levels in batches of salbutamol drug substance by CE and HPLC (from Ref. [11])

Batch	Bis ether (% w/w)		Dimer (% w/w)	
	CE	HPLC	CE	HPLC
1	0.14	0.16	0.08	0.08
	0.14	0.16	0.08	0.08
2	0.10	0.11	0.06	0.07
	0.10	0.11	0.07	0.06
3	0.20	0.19	0.13	0.11
	0.20	0.19	0.14	0.10
4	0.12	0.13	0.07	0.06
	0.15	0.14	0.08	0.05
5	0.13	0.14	0.08	0.06
	0.12	0.13	0.07	0.05
6	0.31	0.28	0.18	0.17
	0.31	0.26	0.19	0.15
7	0.07	0.09	0.05	0.04
	0.08	0.10	0.06	0.03
8	0.38	0.38	0.20	0.18
	0.44	0.38	0.22	0.19
9	0.37	0.38	0.19	0.18
	0.37	0.35	0.19	0.19
10	0.75	0.66	0.39	0.33
	0.77	0.67	0.40	0.35

Linearity was measured in two exercises (1–150% and 20–150% of target concentration); correlation coefficients of 0.9990 and 0.9997 were obtained, respectively. A single sample was injected ten times and precision values of 0.4 and 0.6% RSD were obtained for migration time and peak area, respectively.

2.8.5. High pH

At high pH the migration direction of acidic components is against the EOF flow which maximises mobility differences. Operation with standard electrolytes [26,27] such as phosphate (pH 7) or borate (pH 9.5) often lead to useful initial separations for acidic compounds. For example a 100-mM

borate pH 8.5 buffer has been used [13] to separate over 100 acidic drugs. As in low pH separations, selectivity can be altered by addition of cyclodextrins, ion-pair reagents and organic solvents. Addition of organic solvents leads to a decrease in EOF and reduced ionisation of acids which may be beneficial. Alternatively, EOF can be reduced by increasing the viscosity of the electrolyte by the addition [28] of polymeric additives.

Workers from the FDA demonstrated [27] that higher levels of impurities of the aminoglycoside antibiotic *gentamycin* were detected using CE compared with that achieved by the USP registered HPLC method. Gentamycin has a poor chromophore and therefore needs to be derivatised prior to HPLC analysis. However, not all impurities appear to be derivatised to an equal extent. CE allowed the direct analysis of gentamycin and impurities with low UV wavelength detection. High concentrations of borate were used in the buffer as borate complexes with the aminoglycosides which results in the formation of negative charged species which are well separated at high pH. The complexed forms also have significantly increased UV activity. Further work from the same laboratory has been performed [29] on the analysis of various aminoglycoside antibiotics using high concentration borate buffers and detection at 195 nm. For example, 0.4% levels of streptomycin were determined in dihydrostrephtomycin. Potency values were also determined by CE. A cationic surfactant, TTAB, was added to the buffer to reverse the peak migration order to allow trace level determinations of *streptomycin.*

CE can also be applied to the purity testing of intermediate compounds. For example, the impurity content of *diethylenetriaminepentaacetic acid dianhydride* has been determined by CE [30]. These compounds have no appreciable UV activity and are detected by their pre-separation derivatisation with Zn^{2+} ions which was added into the sample solutions. A pH 10 boric acid buffer with detection at 200 nm was used for the determinations and a limit of detection of 0.02% was found for the major degradation products.

A stability indicating method for *enalapril* has been reported [31] which gave detection limits of 0.2% for the monitored impurities. Separation and quantitation of a range of *tetracycline* impurities was achieved [32] by on-capillary derivatisation with EDTA. The anionic complexes were resolved and directly quantified.

Organic solvents can also be used for high pH separations to affect both the selectivity and the EOF rate. For example resolution of *erythromycin* and related substances was achieved [33] using a 50-mM phosphate (pH 7.5) buffer containing 35% v/v ethanol.

A CE method has been used to monitor the solution stability of a *cephalosporin* in solution [26]. Validation included specificity for known degradants and synthetic impurities, linearity over the required range and precision. Analysis was repeated by different analysts on different days. Migration time

precision was less than 1% RSD. Injections were performed from sample solutions every 30 min to monitor the solution stability on-line. The cephalosporin (*Roche compound RO 23-9424*) was found to be twice as stable in a L-arginine–sodium benzoate–saline solution than when prepared in water. The CE data obtained [26] allowed structural elucidation of an unknown degradant formed by hydrolysis.

2.8.6. Micellar Electrokinetic Capillary Chromatography (MECC)

This technique would be adopted when dealing with unchanged solutes or mixtures of charged and neutral species. This approach may also be considered when simple mobility differences prove insufficient in free solution CE. Figure 2.8.F shows the schematic process involved. A high pH buffer is generally used which generates a fast EOF towards the detector. Surfactant, usually SDS, is added to the buffer at a sufficiently high concentration that the surfactant molecules group together to form micelles. These micelles migrate in the opposite direction to the EOF. Compounds can partition with the micelles. Water-insoluble solutes will favour inclusion into the micelle and will therefore be more retained. Separation of neutral compounds in MECC is based purely on chromatographic interactions.

The selectivity can be manipulated in a similar fashion to those parameters employed in reversed phase HPLC and these include addition of cyclodextrin [34], ion-pair reagents [35] and organic solvents [36]. Additional selectivity manipulation can be achieved by varying the type and concentration of surfactant [37].

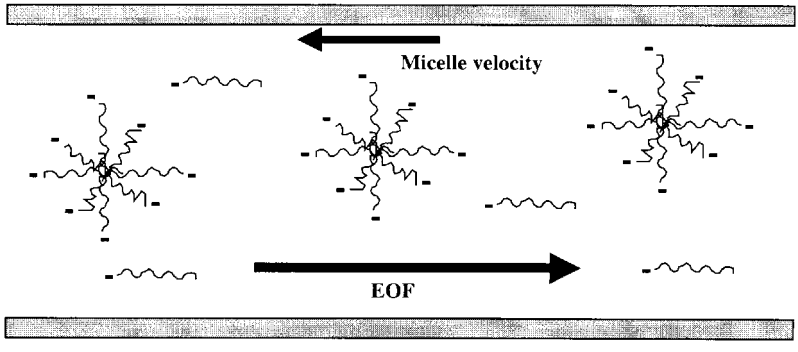


Figure 2.8.F.
Schematic of MECC principles

Water insoluble compounds are generally analysed using MECC. Samples can be prepared in 100% organic solvents but this can produce problems of out-gassing when employing extended injection times. To minimise the potential difficulties it is best to prepare the sample in a solvent containing the minimum percentage of organic solvent required to solubilise the sample. The sample should be soluble in the electrolyte used or on-capillary precipitation can occur.

Ceftazidime related impurities were separated using a 25-mM borate containing 75 mM SDS MECC buffer [38]. The method was validated for both purity determinations and assay of the drug in injection solutions. The use of MECC to separate the charged and neutral impurities in an acidic drug (*SB209247*) has been shown [39]. The impurities were separated using an electrolyte containing 50 mM borate:acetonitrile (65:35% v/v) containing 50 mM SDS. The choice of sample dissolving solvent has a pronounced effect in MECC on the peak efficiency and resolution obtained – particularly if the sample is dissolved in pure solvent as this disrupts the micelles. The optimal resolution being when the sample solvent contained a lower ionic strength than the buffer to permit focusing.

Levels of individual *heroin* impurities separated by MECC were shown to be indicative of the synthetic route and country of origin of the heroin material [40]. MECC has also been used to separate *amoxicillin* and 14 impurities [41]. The amoxicillin method was optimised using experimental design and was validated with an LOD of 0.02%.

An MECC method has been validated to USP guidelines for the simultaneous assay of the antibiotic *cephradine* determination of related impurity content in an aqueous formulation containing sodium carbonate [42]. An RSD of 0.88% was recorded for recovery experiments with average recoveries varying between 99.8 and 100.2. Correlation coefficients of greater than 0.9993 were obtained. Table 2.8.C shows HPLC and MECC assay results for nine sample batches. The assay results showed no significant difference in the 95% confidence limits.

A MECC method (40 mM boric acid pH 10 containing 40 mM SDS and 9% acetonitrile) has been extensively validated [43] for the simultaneous determination of the impurities in both *ibuprofen* and *codeine*. The robustness of the method was assessed using an experimental design. Equivalent assay and purity data was obtained between research and factory based test laboratories. MECC has also been used [44] to monitor the chemical purity of *cefotaxime* drug substance and data were shown to be statistically equivalent to HPLC impurity data. Linearity was demonstrated (correlation coefficient 0.9993) over the range 0.05–1.50 g/l. A limit of detection of 0.02% was obtained for the MECC method. This method was successfully used for the purity determination of *oxazolidinone antibacterials* [45].

Table 2.8.C.
Comparison of HPLC and MECC data for the analysis of cephradine content in an aqueous formulation (from Ref. [42])

Batch no.	HPLC	MECC
057/04	73.5	73.5
064/04	74.2	72.3
065/04	73.2	72.1
066/04	73.6	72.0
067/04	72.5	73.0
055/04	75.3	73.8
058/04	71.8	71.2
059/04	74.0	72.9
060/04	70.4	69.8
Average	73.2	72.3

2.8.7. Microemulsion CE

Microemulsion electrokinetic chromatography (MEEKC) [46,47] is another variant of CE which has received relatively little attention compared to the other modes of CE. The separation principle in MEEKC for neutral compounds is chromatographic and involves solute partitioning with a moving oil droplet. The background to microemulsions and their use in analytical chemistry has recently been reviewed [48]. In MEEKC the microemulsion droplets are generally formed by mixing an immiscible oil such as heptane or octane with water. SDS is added at relatively high concentrations to stabilise the emulsion by coating the outside of the droplet. A co-solvent such butan-l-ol is also added which further stabilises the microemulsion. High pH buffers such as borate and phosphate are generally used in MEEKC as these generate a substantial EOF flow. The droplet is negatively charged due to the SDS coating. The electrophoretic migration of the droplet therefore attempts to oppose the EOF. Hydrophobic solutes favour partitioning into the oil droplet and therefore are more highly retained than water-soluble solutes which have a low partitioning tendency into the oil droplet. Figure. 2.8.G shows the separation of a degraded solution of *phenoxypenicillin* by MEEKC [49]. This sample contained both neutral and charged components.

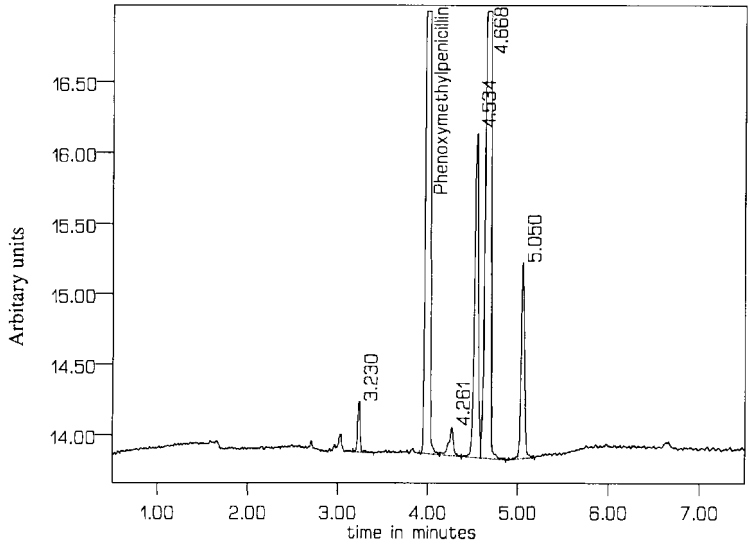


Figure 2.8.G.
Separation of degraded phenoxymethyl penicillin solution. Conditions: 30 cm × 50 μm i.d. capillary (detection window at 22 cm); 0.81% w/w octane, 6.61% w/w butan-1-ol, 3.31% w/w sodium dodecyl sulphate and 89.27% w/w 10 mM sodium tetraborate buffer; 15 kV, 200 nm; 40°C (from Ref. [49])

2.8.8. Non-Aqueous CE

CE can be operated using non-aqueous electrolyte solutions. When organic solvents are used as electrolytes pH readings are no longer accurate and are replaced by pH* which is indicative of the approximate pH of the solution. The main attention in this area has focused on the use of low pH* electrolytes to separate basic drugs [50,51]. Typically ammonium acetate and acetic acid have been added to solvents or solvent mixtures using methanol, acetonitrile, N-methylformanide (NMF) and N,N-dimethylformamide (DMF). These studies have confirmed that improved separation selectivities for basic compounds can be obtained in organic solvents. The improved separation selectivity obtained for these basic drugs allowed purity testing with detection limits of 0.1% or below. The non-aqueous solvents are ideal for use in CE-MS studies [52,53] as the electrolytes are volatile and improved ionisation can occur due to the ease of solvent evaporation from the solution ion.

Separation selectivity can be manipulated in non-aqueous CE if organic solvent mixtures are used. For example Fig. 2.8.H shows separation of a

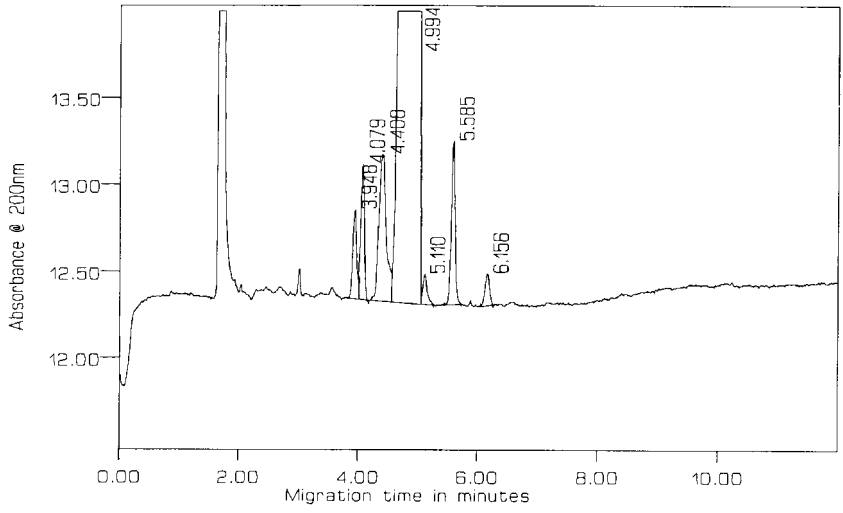


Fig. 2.8.H.
Separation of partially degraded penicillin sample solution. Conditions: 27 cm × 50 μm capillary (20 cm to detector); 1 s injection, using 25:75% v/v acetonitrile–methanol containing 2 mM sodium acetate pH* 9.3 (unadjusted); 25 kV, 200 nm

degraded *penicillin* sample solution using a high pH* electrolyte dissolved in 25:75% v/v acetonitrile–methanol [54]. The separation selectivity was markedly affected when different solvent ratios were used. Another example for the successful application of non-aqueous CE is the separation of *cimetidine* and related impurities [55].

2.8.9. Indirect UV Detection Applications

Indirect UV detection is used to detect ions which have no chromophore. Figure 2.8.I shows the detection principle. An electrolyte containing a species which strongly absorbs UV light is filled into the capillary which also absorbs all the UV light passing through the capillary. When the non-UV active peaks move through the detector the UV signal is decreased and the peak area is related to the solute ion concentration. Indirect UV detection is widely used for the determination of inorganic drug counter-ions (Section 4.3). Similarly indirect UV detection can be used for the determination of impurities that have no

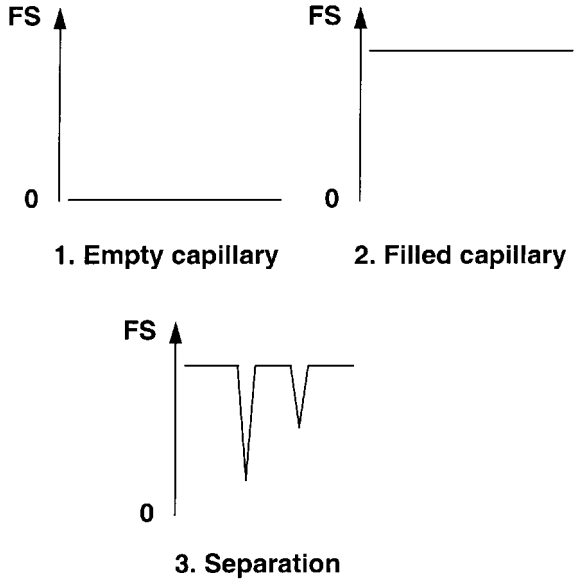


Figure 2.8.I.
Principles of indirect UV detection

chromophores. An electrolyte containing benzyltrimethylammoniun bromide as the UV absorbing ion has been used to determine levels of a non-UV absorbing impurity in *clidinium bromide* drug substance [56]. A limit of detection of 0.008% was obtained for the impurity using indirect UV detection. Results for batch testing obtained by CE and TLC agreed well, for instance a result of 0.44% was obtained by CE which compared with a result of 0.4–0.45% by TLC for the same sample.

A capillary electrophoresis (CE) method has been developed as an alternative method for the determination of the inorganic degradation products sulphate and sulphamate in the antiepileptic drug *topiramate* drug product and drug substance [57]. The anions were separated in a background electrolyte containing potassium chromate and boric acid, followed by indirect UV detection The validation of the method, which was performed according to ICH guidelines (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) comprises specificity, accuracy, linearity, precision, sensitivity and robustness. In addition, the results of an actual tablet sample analysis obtained by this CE method are statistically shown to be in close agreement with those obtained by an ion chromatographic method.

2.8.10. Comparison of CE Performance with HPLC

The data generated on impurity profiling is often compared to that obtained by chromatographic methods (typically HPLC). The principles of separation in CE are entirely different to HPLC and therefore a good agreement between the two techniques strongly supports the integrity of the data. This technique combination is now established in many laboratories and has become a suitable replacement for the conventional use of TLC and HPLC in combination. Apart from routine investigations, this combined use is of particular importance during method validation.

The differences in selectivity between CE and HPLC can result in discrepancies in results with one technique showing an under-estimation in impurity levels. This occurrence signifies that further method optimisation is required. Critical events in the development of a formulation such as synthetic route or process changes or at key drug stability timepoints are times when this may occur. Literature examples include the discovery of additional impurities in *tetracycline* [32] *domperidone* [8] and *fluticasone proprionate* [58].

The principal disadvantages of the use of CE compared to HPLC for determining related impurities are the possible requirements for higher sample concentrations. When operating HPLC and CE at the same wavelength it may be necessary to use 2–5 times more concentrated samples for CE to obtain an equivalent limit of detection. This may represent problems for poorly soluble drugs. This lower sensitivity is principally due to the use of the capillary for on-capillary detection. The section of the capillary used for detection purposes may for example only be 75 μm wide (for a 75-μm capillary) and 200 μm long. This compares very disfavourably with a 1-cm^3 HPLC flow-cell.

Aqueous based electrolytes are often employed in CE which have low UV absorbance coefficients allowing detection wavelengths such as 200 nm to be routinely employed. This can compensate for sensitivity problems. Other approaches include the use of wider bore capillaries, and modified capillaries (z-cells and bubble cell modifications). Many impurities or small intermediates have poor chromophores making their quantitation at traditional HPLC wavelengths difficult or impossible. This may be of particular importance for degradative processes where reactions may lead to the loss of the functionality providing the chromophore.

Alternatively the use of low UV wavelengths may compensate for the inherent poor sensitivity in CE. For example when operating at 200 nm there is a 10-fold increase in signal for *salbutamol* and its impurities compared with that at 276 nm which is the HPLC wavelength [11].

2.8.11. CE/MS

The hyphenation of CE and CEC to MS detectors is well-established [59] and interfaces are commercially available from many MS suppliers. Recent reviews specifically cover the interfacing of CE to specific MS detectors including electrospray [60] and ion-trap [61]. Electrospray is currently the most widely used ionisation technique with positive ion being the most frequent detection mode. The use of non-aqueous CE electrolytes is particularly attractive for CE/MS as sensitivity can be significantly improved. The use of MEEKC or MEKC buffers requires specific operating procedures, or interfaces, to avoid fouling of the MS source with the involatile surfactants.

CE/MS has been used [20] to identify three impurities present at 0.1% of the main component in a drug substance. The mass ions of the separated peaks can be clearly determined at this 0.1% level.

Due to reasons described in detail in Section 2.9.2.1 the related technique CEC/MS is expected to find in the future even wider applications. The details can be found in Section 2.9.2.

References

1. K.D. Altria, *Capillary Electrophoresis Guidebook*, Humana Press, Boca Raton, FL (1996)
2. M. Khaledi (Ed.), *High Performance Capillary Electrophoresis: Theory, Techniques, and Applications* (Chemical Analysis, Vol. 146), Wiley, New York (1998)
3. K.D. Altria, B.J. Clark and M.A. Kelly, *Trends Anal. Chem.* **17**, 214–226 (1998)
4. K.D. Altria, *Quantitative Analysis of Pharmaceuticals by Capillary Electrophoresis* (Chromatographia CE Series, Vol. 2), Vieweg Press, Weisbaden (1998)
5. H. Watzig, M. Degenhardt and A. Kunkel, *Electrophoresis* **19,** 2695–2752 (1998)
6. E.M. Cohen and R.G. Bell*, Pharm. Form.* **20**, 7870–7870 (1994)
7. M.J. Hilhorst, G.W. Somsen and G.J. de Jong, *J. Pharm. Biomed. Anal.* **16**, 1251–1260 (1998)
8. A. Pluym, W. Van Ael and M. De Smet, *Trends Anal. Chem.* **11**, 27–32 (1992)
9. K.D. Altria, *Chromatographia* **35**, 177–182 (1993)
10. K.D. Altria and S.D. Filbey, *J. Liq. Chromatogr.* **16**, 2281–2292 (1993)
11. K.D. Altria, *J. Chromatogr.* **634**, 323–328 (1993)

12. K.D. Altria, *J. Chromatogr.* **636**, 125–132 (1993)
13. J.C. Hudson, M. Golin, M. Malcolm and C.F. Whiting, *Can. Soc. Forens. Sci. J.* **31**, 1–10 (1998)
14. C.P. Ong, CL. Ng, H.K. Lee and S.F.Y. Li, *J. Chromatogr.* **686**, 319–324 (1994)
15. A. Yesilada, B. Tozkoparan, N. Gokhan, L. Oner and M. Ertan, *J. Liq. Chromatogr. Relat. Technol.* **21**, 2575–2588 (1998)
16. B.J. Clark, P. Barker and T. Large, *J. Pharm. Biomed. Anal.* **10**, 723–726 (1992)
17. O. Stalberg, H. Brotell and D. Westerlund, *Chromatographia* **40**, 697–704 (1995)
18. O. Stalberg, D. Westerlund, U.-B. Rodby and S. Schmidt, *Chromatographia* **41**, 287–294 (1995)
19. B.J. Clark, P. Barker and T. Large, *J. Pharm. Biomed. Anal.* **10**, 723–726 (1992)
20. K.D. Altria, *J. Chromatogr.* **735**, 43–56 (1996)
21. M.A. Kelly, K.D. Altria, C.H. Grace and B.J. Clark, *J. Chromatogr. A* **798**, 297–306 (1998)
22. H. Watzig, C. Dette, A. Aigner and L. Wilschowitz, *Pharmazie* **49**, 249–252 (1994)
23. K.D. Altria, *LC-GC Int*. 24–29 (1999)
24. G.S. Wynia, G. Windhorst, P.C. Post and F.A. Mars, *J. Chromatogr. A* **773**, 339–350 (1997)
25. K.D. Altria and Y.L. Chanter, *J. Chromatogr.* **652**, 459–463 (1993)
26. B. Nickerson, B. Cunningham and S. Scypinski, *J. Pharm. Biomed. Anal.* **14**, 73–83 (1995)
27. C.L. Flurer and K.A. Wolnik, *J. Chromatogr.* **663**, 259–263 (1993)
28. F.Y.L. Hsieh, J.C. Cai and J. Henion, *J. Chromatogr*. **679**, 206–211 (1994)
29. C.L. Flurer, *J. Pharm. Biomed. Anal.* **13**, 809–816 (1995)
30. J. Bullock, *J. Chromatogr. B* **669**, 149–155 (1995)
31. X.-Z. Qin, D.P. Ip and E.W. Tsai, *J. Chromatogr*. **626**, 251–258 (1992)
32. C.X. Zhang, Z.P. Sun, D.K. Ling and Y.J. Zhang, *J. Chromatogr.* **627**, 281–286 (1992)
33. A.K. Lalloo and I. Kanfer, *J. Chromatogr. B* **704**, 343–350 (1997)
34. I.S. Lurie, K.C. Chan, T.K. Spratley, J.F. Casale and H.J. Issaq, *J. Chromatogr. B* **669**, 3–13 (1995)
35. C.J. Sciacchitano, B. Mopper and J.J. Specchio, *J. Chromatogr.* **657**, 395–399 (1994)
36. A.E. Bretnall, M.M. Hodgkinson and G.S. Clarke, *J. Pharm. Biomed. Anal.* **15**, 1071–1075 (1997)

37. H. Nishi, T. Fukuyama, M. Matsuo and S. Terabe, *J. Chromatogr.* **513**, 279–295 (1990)
38. R. Porra, A. Farina, V. Cotichini and R. Leece, *J. Pharm. Biomed. Anal.* **18**, 241–248 (1998)
39. M. Giles, *Chromatographia* **44**, 191–202 (1997)
40. V.G. Trennery, R.J. Wells and J. Robertson, *J. Chrom. Sci.* **32**, 1–6 (1994)
41. Y.M. Li, A. Van Schepdael, Y. Zhu, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **812** 227–237 (1998)
42. P. Emaldi, S. Fapanni and A. Baldini, *J. Chromatogr. A* **711**, 339–346 (1995)
43. K.P. Stubberud and O. Astrom, *J. Chromatogr. A* **826**, 95–102 (1998)
44. G.C. Penalvo, E. Julien and H. Fabre, *Chromatographia* **42**, 159–164 (1996)
45. J.P. Scholl and J. De Zwaan, *J. Chromatogr. B* **695**, 147–156 (1997)
46. H. Watarai, K. Ogawa, M. Abe, T. Monta and I. Takahashi, *Anal. Sci.* **7**, 245–258 (1991)
47. S. Terabe, N. Matsubara, Y. Ishihama and Y. Okada, *J. Chromatogr.* **608**, 23–29 (1992)
48. H. Watarai, *J. Chromatogr. A* **780**, 93–102 (1997)
49. K.D. Altria, *Chromatographia* **49**, 457–464 (1999)
50. G.N.W. Leung, H.P.O. Tang, T.S.C. Tso and T.S.M. Wan, *J. Chromatogr. A* **738**, 141–154 (1996)
51. S.H. Hansen, J. Tjornelund and I. Bjornsdottir, *Trends Anal. Chem.* **15**, 175–180 (1996)
52. A.J. Tomlinson, L.M. Benson, J.W. Gorrod and S. Naylor, *J. Chromatogr. B* **657**, 373–381 (1994)
53. L.U. Wenzhe, G.K. Poon, P.L. Carmichael and R.B. Cole, *Anal. Chem.* **68**, 668–674 (1996)
54. K.D. Altria and S.M. Bryant, *Chromatographia* **46**, 122–130 (1997)
55. D.R. Ellis, M.E. Palmer, L.W. Tetler and C. Eckers, *J. Chromatogr. A* **808**, 269–275 (1998)
56. B. Nickerson, *J. Pharm. Biomed. Anal.* **15**, 965–971 (1997)
57. A. Klockow-Beck, A. Nick, S. Geisshuesler and D. Schaufelberger. *J. Chromatogr. B* **720**, 141–151 (1998)
58. N.W. Smith and M.B. Evans, *J. Pharm. Biomed. Anal.* **12**, 579–611 (1994)
59. D. Figeys and R. Aebersold, *Electrophoresis* **19**, 885–892 (1998)
60. J.F. Banks, *Electrophoresis* **18**, 2255–2266 (1997)
61. J.T. Wu, M.G. Qian, M.X. Li, K.F. Zheng, P.Q. Huang and D.M. Lubman, *J. Chromatogr. A* **794**, 377–389 (1998)

2.9.1. Separation, detection and determination of impurities by CEC

Melvin R. Euerby

2.9.1.1. Introduction

Capillary electrochromatography (CEC) is a new and exciting ''hybrid'' separation technique which seeks to exploit the combined advantages of the chromatographic selectivity (mobile and stationary phase) associated with reverse phase (RP) HPLC and the high efficiencies and electromobility of CE. For reviews on CEC theory, instrumentation and operation the reader is directed to Refs. [1–6]. A CEC system briefly consists of a fused silica capillary (50–100 μm i.d. × 8–50 cm length) packed with, typically, 3 μm silica based stationary phase material such as Hypersil or Spherisorb ODS1. This is installed into a conventional or pressurised CE instrument and a voltage is applied across the capillary whose ends are immersed in a suitable mobile phase. The packed capillary is the heart of the system as it provides, in addition to the chromatographic selectivity, the driving force for the separation (i.e. the electrosmotic flow). The technique of CEC is of great interest to the pharmaceutical industry as it promises to generate significantly higher peak capacities than HPLC. In addition, it is possible to exploit the difference in an analyte's partitioning and electromobilities thereby producing an analysis which is truly orthogonal to HPLC. This is highly desirable as regulatory authorities are now requesting an additional mode of separation in order to confirm compound purity. The technique of CEC is still in its infancy with regards to instrumentation and packed capillary technology, however, there have been numerous exciting reports of superior separations using this technique over the more established techniques of HPLC and CE. To date, no group has reported fully validating a CEC method for related substance analysis. However, as most of the CEC research and evaluation has been performed within the pharmaceutical industry where confidentiality is paramount, the current status of the technique is difficult to assess. This chapter will review some of the reported separations achievable with CEC and will discuss the limited data available on the determination of low level impurities/related substances in pharmaceuticals. In addition, it will seek to show that CEC possesses the potential to be a separation technique complimentary to those currently available to the analyst.

2.9.1.2. Analysis of Neutral and Ion Suppressed Pharmaceuticals

To date, most of the reported analyses using CEC have focused on the separation of neutral analytes using traditional stationary phases such as Hypersil ODS and Spherisorb ODS1. These phases possess a relatively large number of acidic silanol groups and, when they are used with mobile phases in excess of pH 7, the silanol groups are fully ionised and hence generate a good EOF.

CEC is an excellent technique for the analysis of neutral and ion suppressed analytes, as can be seen in the numerous reported analyses of structurally diverse pharmaceuticals. Cephalosporin antibiotics [7], prostaglandins [7], diuretics [8,9], steroids [8–11], macrocyclic lactones [10], C- and N-protected peptides [9], nucleosides and purine bases [9], barbiturates [12] and parabens [1] have been successfully analysed by CEC. The CEC analysis of neutral species, weak acids and weak bases yield improved peak shape and higher efficiencies than those obtained with conventional HPLC. In addition, the above analytes exhibit the same linear relationship between ln k and %organic as seen in RP-HPLC.

2.9.1.3. Analysis of Acidic Pharmaceuticals

In order to successfully analyse acidic pharmaceuticals by CEC, the compounds must be chromatographed in their ion suppressed form. This is due to the fact that the high electromobility of the ionised acids is greater than the EOF, as a consequence the ionised acids migrate towards the anode away from the detector. The EOF is dramatically reduced as a result of the need to use a low pH mobile phase in order to maintain the analytes in their ion suppressed mode. However, satisfactory analysis can still be achieved, as highlighted by the analysis of four steroidal diastereoisomers and their acidic precursor (see Fig. 2.9.1.Aa,b). The method is a marked improvement over the current HPLC separation which utilises cyano and amino columns in series, see Fig. 2.9.1.Ac,d. To increase the speed of analysis when low pH mobile phases have to be used, newer type phases have been developed in which a sulphonic acid moiety is bonded onto the same silica particle as the ODS function. These phases generate excellent EOFs which are effectively independent of pH and still retain a good partitioning capacity. We have recently reported the applicability of these phases in the rapid analysis of a range of non-steroidal anti-inflammatory drugs in their ion suppressed mode at pH 2.5 [13].

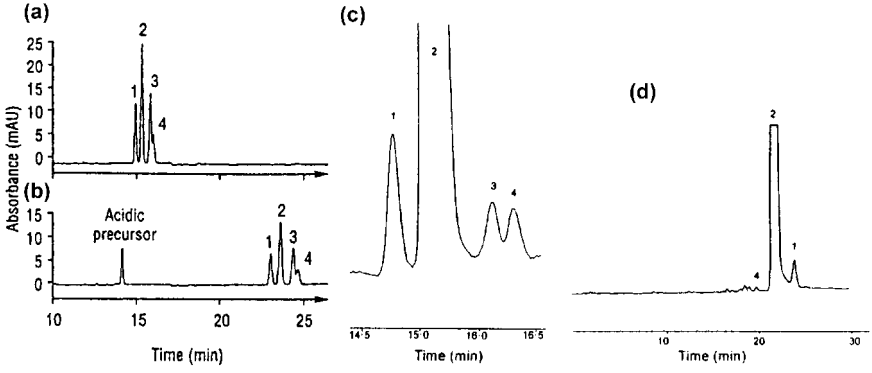


Figure 2.9.1.A.
Isocratic CEC (a–c) and HPLC (d) separation of four diastereoisomeric steroids (peaks **1**-**4**) and their acidic precursor. Analytes: (a,b) the mixture of the four diastereoisomers and their common acidic precursor; (c) the wanted diastereoisomer (**2**) contaminated with **1** (2.4%), **3** (1.1%) and **4** (1.0%); (d) another batch of **2**, contaminated with **1** and **4**. CEC conditions: instrument: HP^{3D} CE system; capillary: 400 mm × 100 μm, 3 μm d_p CEC Hypersil C18; mobile phase; (a,c) acetonitrile–50 mM TRIS (pH 7.8) buffer–water 70:20:10 v/v/v; (b) acetonitrile–50 mM monobasic sodium phosphate (pH 2.3)–water 70:20:10 v/v/v; UV detector 210 nm; temperature 15°C; voltage: 30 kV; electrokinetic injection of the analyte (0.25 mg/ml in acetonitrile–water 1:1 v/v): 5 kV for 15 s; voltage ramp time: 0.5 min; capillary pressurisation: 8 bar. HPLC conditions: columns: Supelcosil LC-CN + Nucleosil NH_2 in series; mobile phase: heptane–ethanol 96:4 v/v (from Ref. [13])

2.9.1.4. Analysis of Pharmaceutical Bases

Bases, not unexpectedly, have been found to exhibit poor peak shape on traditional silica based materials. This is, in part, due to the acidic silanol groups which, although vital for the generation of a good EOF, are detrimental to the analysis of bases [8]. As a consequence of the poor peak shape associated with the analysis of bases, there have been limited examples of such separations published in the literature. In order to circumvent this problem investigations into the use of a strong cation exchange (SCX) phase [8] and bare silica [14] have been made; alas both phases generated non-reproducible results. To date, the most successful approach for the analysis of bases by CEC has been to incorporate competing bases, such as triethylamine, triethanolamine and hexylamine into an acidic mobile phase using traditional RP silicas [15,16].

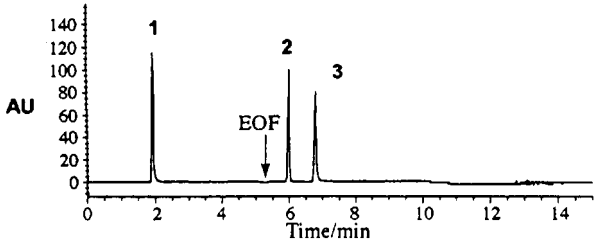


Figure 2.9.1.B.
CEC separation of benzylamine (**1**), caffeine (**2**) and benzoic acid (**3**). Instrument: HP^{3D} CE system; capillary: 250 mm × 100 μm, 3 μm d_p CEC Hypersil C18; mobile phase: acetonitrile–0.05 M triethanolamine phosphate pH 2.5–water 6:2:2 v/v/v; UV detector 214 nm; temperature 15°C; voltage 25 kV; electrokinetic injection of the analyte (100 μg/ml of each component in acetonitrile–water 1:1 v/v), 15 kV for 5 s; voltage ramp time 0.15 min; capillary pressurisation 8 bar (from Ref. [15])

Researchers at Astra Charnwood [15] and the US Drug Enforcement Administration [16] have successfully analysed a wide range of pharmaceuticals and drugs of abuse using this approach. The former group have recently reported that the ''elution order'' of bases using CEC is different to that of HPLC and CE using identical conditions highlighting the truly orthogonal nature of CEC for the analysis of charged bases as electrophoresis is superimposed on the partitioning mechanism [17].

2.9.1.5. Analysis of Mixtures Containing Acids, Bases and Neutrals

In order for CEC to be universally accepted it must be capable of analysing charged and neutral analytes. To date, the only successful solution to this problem is the incorporation of a competing base into the mobile phase (see Section 2.9.1.4). In this case, acids are chromatographed in their ion suppressed mode and hence elute after the EOF with the neutral components, whereas the bases elute before the EOF due to their electromobility [15,16], see Fig. 2.9.1.B.

2.9.1.6. Detection Considerations

The determination of related substances in drug substances and formulations has normally been performed by RP-HPLC where limits of quantification

(LOQ) below 0.05% [18], can be easily achieved in most instances. CEC, in a similar fashion to CE, suffers from the perceived problem of poor concentration sensitivity with UV detection, which is the most widely used mode of detection within the pharmaceutical industry. The problem is due to the inherent short pathlength of the capillary. Although the short pathlength of 50–100μm is a disadvantage in terms of concentration sensitivity, it does permit the use of UV wavelengths in the region of 190–210 nm even with mobile phases containing substantially high proportions of methanol and acetonitrile. The UV extinction coefficients of many compounds are significantly higher at these low wavelengths and hence to a certain extent compensate for the short pathlength.

One recent approach to the sensitivity problem has been to increase the pathlength by the use of a detachable cell with a 1.2-mm pathlength. Preliminary work has shown that improvements up to 10-fold in the signal to noise ratio are obtainable, thereby enabling a lower LOQ and a broader linear dynamic range to be obtained [19].

The amount of analyte loaded is complicated by the fact that, to date, most of the injections have been of the electrokinetic type which depend on the EOF to push the analyte onto the top of the capillary. Hence, any factor which modifies the EOF will affect the amount loaded. For example, more analyte will be loaded at pH 8 on a conventional silica based RP capillary than at pH 2 simply because the EOF is lower at pH 2. This can be circumvented by the use of the mixed mode phases which generate a good EOF at all pHs. The problem is compounded if there are charged analytes present because, depending on the polarity of the applied voltage, the charged species in the sample can either be enriched or not loaded, hence a distorted impurity profile is obtained. Hydrodynamic injections would therefore seem preferable. However, to perform this type of injection requires the use of a modified CE system. The amount loaded onto the capillary is limited by the fact that 3 μm packing material is employed with only a maximum applied pressure of 12 bar being available. This imposes a severe limitation on the level of impurities that can be detected.

Considerable gains in sensitivity can be achieved by simply injecting the sample in a lower eluting strength solvent than the mobile phase [20,21]. It has been reported that up to a 17-fold improvement in peak height could be obtained without a significant decrease in peak efficiency when using an electrokinetic injection technique [20]. In addition to UV detection, in-column fluorescence [22] and laser induced fluorescence [23] have been successfully employed, however, to date these have not been applied to pharmaceuticals. The reader is directed to Section 2.9.2 for MS detection.

Recently there have been encouraging reports of a new signal reprocessing unit which enhances the true signal and reduces the noise. Using this approach workers at LGC have shown an improvement of up to 4-fold in signal

to noise ratios [24]. The fact that CEC produces highly efficient Gaussian shaped peaks suggests that improved detection limits should be possible, especially for small peaks eluting on the tail of major peaks.

2.9.1.7. Selected Examples of the Determination of Impurities by CEC

The literature contains numerous examples of the successful CEC analysis of steroids, a typical example is that reported by workers at Astra Charnwood of the separation of the corticosteroid tipredane from its diastereoisomer and five related substances [9]. As can be seen in Fig. 2.9.1.C, baseline separation of tipredane from its diastereoisomer was achieved with essentially no method development; in contrast, extensive HPLC optimisation failed to resolve these components.

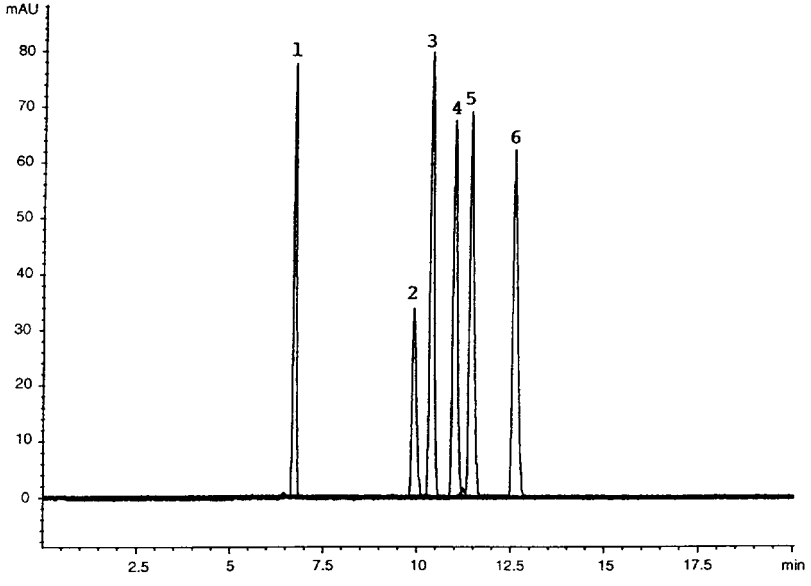
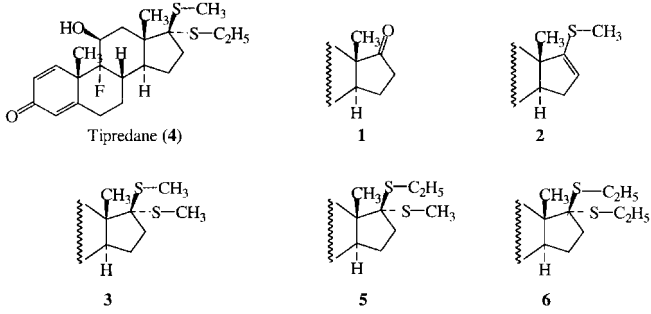
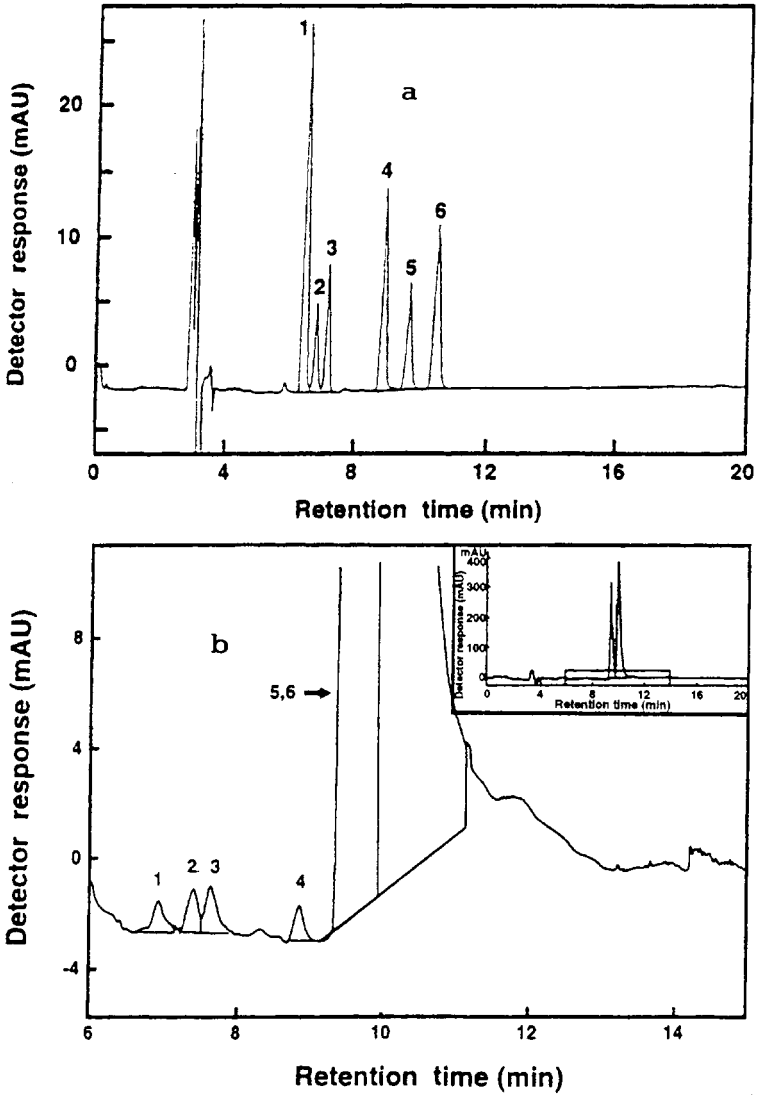


Figure 2.9.1.C.
Separation of tipredane (**4**) from its five related substances (**1**, **2**, **3**, **6** and the C-17 diastereoisomer (**5**)). CEC conditions: unpressurised HP^{3D} CE system using a 250 mm × 50 μm Spherisorb ODS1 3 μm d_p packed capillary; mobile phase: acetonitrile–TRIS (50 mM, pH 7.8) buffer 80:20 v/v; voltage: 15 kV; temperature: 15°C; electrokinetic injection of 5 kV/15 s; UV detector: 240 nm (from Ref. [9], reprinted by permission of John Wiley & Sons, Inc.)



Researchers at Glaxo Wellcome have also reported that CEC is highly efficient in separating the corticosteroid fluticasone from its synthetic related substances [7,10]. Workers at Johnson Pharmaceuticals have recently shown that norgestimate and its related degradation impurities (see Fig. 2.9.1.Da) can be easily separated by CEC [25] using a ternary mobile phase composition (see Fig. 2.9.1.Da). The compounds are extremely difficult to separate even by gradient HPLC due to their differing hydrophobicities. The separation was achieved in less than 15 min, i.e. half the analysis time required by gradient HPLC. In addition, the CEC method which used a 100 μm i.d. capillary and a detection wavelength of 225 nm was reported to be able to quantify 0.1% w/w of individual degradation impurities in norgestimate drug substance (see Fig. 2.9.1.Db).

Figure 2.9.1.D.
CEC separation of norgestimate and its related degradation products. Instrument: HP^{3D} CE system; capillary: 250 mm × 100 μm, 3 μm d_p CEC Hypersil C18; mobile phase: acetonitrile–tetrahydrofuran–25 mM TRIS–HCl (pH 8) 35:20:20:25 v/v/v; voltage 30 kV; temperature 25°C; electrokinetic injection 3 s at 10 kV; UV detector: 225 nm; pressure 8 bar both sides. Peaks: 1, norgestrel; 2, *syn*-norgestrel oxime; 3, *anti*-norgestrel oxime; 4, norgestrel acetate; 5, *syn*-norgestimate; 6, *anti*-norgestimate. (a) Model mixture; (b) norgestimate drug substance spiked with 0.1% of degradation impurities (from Ref. [25] by permission of Preston Publications, a Division of Preston Industries, Inc.)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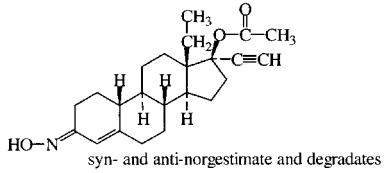
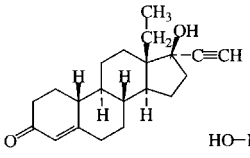
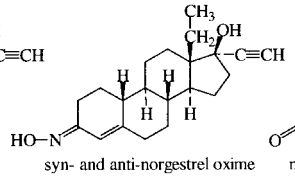


syn- and anti-norgestimate and degradates



norgestrel



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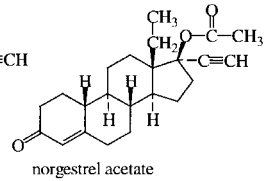


norgestrel acetate

Researchers at Searle have reported the systematic method development of a CEC separation of a mixture of eight polar neutral pharmaceutical compounds (associated impurities and degradants, four of them being closely related positional isomers that are not resolved with all RP supports) [26]. Operational parameters such as stationary phase (C18, C8 and phenyl), mobile phase composition (%organic, type of organic modifier, buffer strength/type and pH), voltage and temperature were systematically optimised. The workers successfully demonstrated the use of the computer optimisation software DryLab® to model the ln k vs. %organic relationship in a predictive manner. An excellent separation of the components was accomplished despite only relatively low k values (0.47–1.33), see Fig. 2.9.1.E; this highlights the power of CEC and its potential to separate multicomponent pharmaceutical mixtures. The paper did not quote the LOQ. However, the workers have subsequently presented results that show that the accuracy and precision of the method was acceptable from a regulatory position [27].

Researchers at DuPont Merck [28,29] have used experimental design to assist in rapid CEC method development and have reported the separation of an antiinflammatory compound DUP 654 from potential impurities in formulations [28]. DUP 654 and its three impurities are phenols or hydroquinones and hence, when analysed at pH values below 8, can be considered to be neutral. The separation was optimised by a stacked mixture design, on a Hypersil ODS capillary, with varying proportions of mobile phase modifiers (such as acetonitrile, methanol and water) and buffer pH. The separation of DU 654 from its three impurities could be achieved in less than 6 min and the results were stated to be comparable to those obtained by HPLC, however, no LOQ was quoted. In another example from DuPont Merck, a modified central composite design was employed to optimise the CEC separation of the anti-

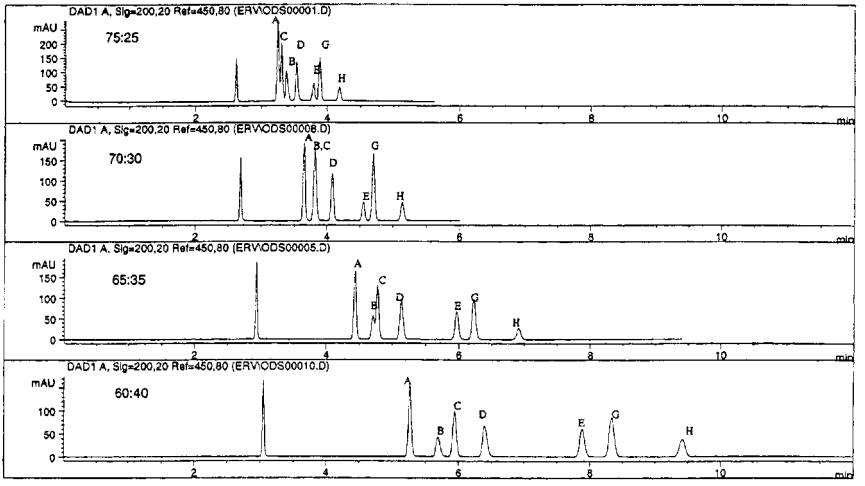


Figure 2.9.1.E.
Effect of organic concentration on the separation of sample compounds. Instrument: HP^{3D} CE system; column: Spherisorb ODS-1 3 μm (250 mm × 75 μm, 345 mm total); mobile phase: acetonitrile–2 mM KH_2PO_4 (pH 3) in ratios as stated; voltage 30 kV; pressure 10 bar; temperature 35°C; UV detector 200 nm; injection 12 s at 2 kV (from Ref. [26])

bacterial DUP 105 (methyl sulphoxide) and its two related compounds (methyl sulphide and methyl sulphone) for analysis speed and quality of separation [29]. The variables examined were applied voltage, buffer concentration and volume of buffer. The end result was the development of a rugged CEC method for the baseline separation of the antibacterial from its impurities in less than 8 min.

Workers at Pfizer have reported the use of CEC for the analysis of the antimycotic agent – fluconazole – which may potentially contain up to five closely related process intermediates [30]. After simple method development, a baseline separation could be achieved using a binary mobile phase composition, although, the analysis time was too long. In order to rectify the excessive analysis time the authors utilised the Snyder selectivity triangle and were able to successfully achieve baseline separation of all components within an acceptable run time of 16 min. This separation is a marked improvement over HPLC and CE which are unable to satisfactorily resolve all these potential compounds in a single analysis. The authors suggested, that given the resolution achieved and the Gaussian peak shape, it should be possible to determine

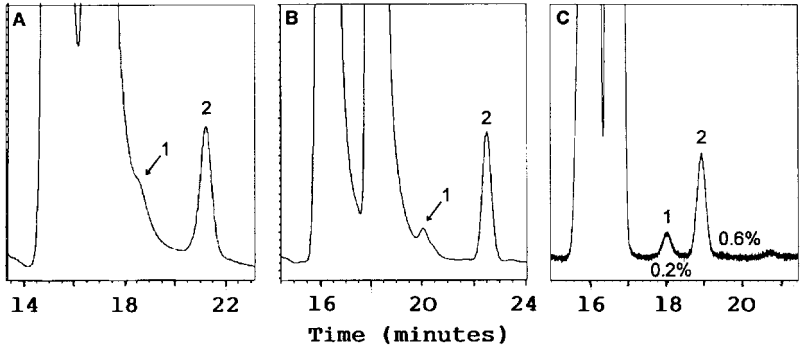


Figure 2.9.1.F.
Comparison of CEC and HPLC for the separation of an artificially degraded sample of budesonide epimers. Mobile phase: acetonitrile–phosphoric acid (20 mM, pH 2.1) buffer 50:50 v/v. (A) HPLC. Column (150 mm) packed with 5 μm Hypersil C18; DP ≈ 80 bar; N = 9600 plates. (B) HPLC. Column (150 mm) packed with 3 μm Hypersil C18; DP ≈ 230 bar; N = 17 000 plates. (C) CEC performed on a HP^{3D} CE system. Capillary: 250 mm × 100 μm packed with 3 μm CEC Hypersil C18; voltage 30 kV; temperature 15°C; pressure 6 bar on both sides; electrokinetic injection 25 s at 30 kV. N = 30 000 plates (from Ref. [21])

impurities down to the 0.5% area/area using a conventional 100 μm i.d. capillary and a detection wavelength of 214 nm.

Researchers at Astra Draco have examined the use of CEC to determine the level of related substances in the pharmaceutically active steroid – budesonide (a 1:1 mixture of epimers) [21]. A reduced factorial design involving temperature, pH and amount of modifier as the variables was employed for the method development. A Hypersil ODS capillary was used throughout the study. By optimising the volume injected electrokinetically onto the capillary, focusing the sample onto the capillary and injecting a highly concentrated sample, the efficiency and the signal to noise ratio can be maximised. With the optimised CEC method, the authors were able to obtain LOQ below 0.1% using a standard 100 μm i.d. capillary and a detection wavelength of 245 nm. The authors also compared the performance of HPLC and CEC and showed that under identical conditions, HPLC generated a LOQ one order of magnitude lower than CEC. However, CEC generated significantly better peak shapes with concomitantly higher efficiencies than LC and a somewhat different selectivity (this can be explained due to differences in the Hypersil ODS used in the LC columns and the CEC Hypersil ODS material). Therefore, by

using CEC it was feasible to separate impurities from the main peaks of an artificially degraded sample that could not be resolved with HPLC, see Fig. 2.9.1.F.

References

1. M.M. Dittmann and G.P. Rozing, *J. Chromatogr. A* **744**, 63–74 (1994)
2. J.H. Knox and I.H. Grant, *Chromatographia* **24**, 135–143 (1987)
3. J.H. Knox and I.H. Grant, *Chromatographia* **32**, 317–328 (1991)
4. M.M. Dittmann, K. Wieand, K. Bek and G.P. Rozing, *LC-GC Int.* **13**, 800–814 (1995)
5. K.D. Altria, N.W. Smith and C.H. Turnbull, *Chromatographia* **46**, 664–674 (1997)
6. M.G. Cikalo, K.D. Bartle, M.M. Robson, P. Myers and M.R. Euerby, *Analyst* **123**, 87R–102R (1998)
7. N.W. Smith and M.B. Evans, *Chromatographia* **38**, 649–657 (1994)
8. N.W. Smith and M.B. Evans, *Chromatographia* **41**, 197–203 (1995)
9. M.R. Euerby, D. Gilligan, C.M. Johnson, S.C.P. Roulin, P. Myers and K.D. Bartle, *J. Microcol. Sep.* **9**, 373–387 (1997)
10. S.J. Lane, R.J. Boughflower, C.J. Paterson and M. Morris, *Rapid Commun. Mass Spectrosc.* **10**, 733–736 (1996)
11. M.R. Euerby, C.M. Johnson, K.D. Bartle, P. Myers and S.C.P. Roulin, *Anal. Commun.* **33**, 403–405 (1996)
12. M.R. Euerby, C.M. Johnson, S.F. Smyth, N.C. Gillott, D.A. Barrett and P.N. Shaw, *J. Microcol. Sep.* **11**, 305–311 (1998)
13. M.R. Euerby, C.M. Johnson and K.D. Bartle, *LC-GC Int.* **11**, 39–44 (1998)
14. P.D. Angus, J.F. Stobaugh, C.W. Demarest, K.M. Payne, K.R. Sedo, L.Y. Kwok and T. Catalano, Presented at the *19th Int. Symp. on Capillary Chromatography and Electrophoresis*, Wintergreen, VA, May (1997)
15. N.C. Gillott, M.R. Euerby, C.M. Johnson, D.A. Barrett and P.N. Shaw, *Anal. Commun.* **35**, 217–220 (1998)
16. I.S. Lurie, T.S. Conver and V.L. Ford, *Anal. Chem.* **70**, 4563–4569 (1998)
17. M.R. Euerby, N.C. Gillott and C.M. Johnson, Presented at the *2nd Int. Symp. on Capillary Electrochromatography*, San Francisco, CA, August (1998)
18. International Conference on Harmonisation (ICH), Validation of Analytical Procedures: Methodology Q2B Guidelines (CPMP/ICH/281/95), November (1996)
19. M.R. Euerby, Presented at the *19th Int. Symp. on Capillary Chromatography and Electrophoresis*, Wintergreen, VA, May (1997)

20. D.A. Stead, R.G. Reid and R.B. Taylor, *J. Chromatogr. A* **798**, 259–267 (1998)
21. Å. Carlsson, P. Petersson and A Walhagen, Presented at the *20th Int. Symp. on Capillary Chromatography*, Riva del Garda, Italy, May (1998)
22. H. Rebscher and U. Pyell, *J. Chromatogr. A* **737**, 171–180 (1996)
23. C. Yan, R. Dadoo, H. Zhao, R.N. Zare and D.J. Rakestraw, *Anal. Chem.* **67**, 2026–2029 (1995)
24. M. Saeed, Personal Communication
25. J. Wang, D.E. Schaufelberger and N.A. Guzman, *J. Chromatogr. Sci*. **36**, 155–160 (1998)
26. P.D.A. Angus, E. Victorino, K.M. Payne, C.W. Demarest, T. Catalano and J.F. Stobaugh, *Electrophoresis* **19**, 2073–2082 (1998)
27. K.M. Payne, C.W. Demarest, K.R. Sedo, L.Y. Kwok and T. Catalano, Presented at the *2nd Int. Symp. on Capillary Electrochromatography*, San Francisco, CA, August (1998)
28. J.H. Miyawa, D.K. Lloyd and M.S. Alasandro*, J. High Resolut. Chromatogr*. **21**, 161–168 (1998)
29. J.H. Miyawa, M.S. Alasandro and C.M. Riley, *J. Chromatogr. A* **769**, 145–153 (1997)
30. M.W. Harrison, A.G. Wright and B.J. Clark, Presented at the *11th Int. Symp. on High Performance Capillary Electrophoresis and Related Microscale Techniques*, Orlando, FL, February (1998)

2.9.2. CEC/MS

Steve J. Lane

2.9.2.1. Introduction

Regulatory authorities today put a significant emphasis on drug purity as well as efficacy requesting full characterisation and identification of any impurities at the level of ≥0.1% of the UV peak area [1]. In addition a truly orthogonal separation technique is required for added confirmation of purity. It is therefore important to obtain good chromatographic resolution of impurities from each other and the main drug peak, as two co-eluting impurities of 0.055% may suddenly need full characterisation as a result of insufficient chromatographic efficiency. This is especially critical if they are isobaric and not distinguishable by mass spectrometry.

In recent times HPLC/diode-array/MS (HPLC/DA/MS) using atmospheric pressure ionisation (API) sources has become the method of choice for the on-line characterisation of low level impurities in drugs and the characterisation of impurity profiles during drug development [2,3]. Typically, nominal molecular weight information is sought from separated components. More recently accurate mass [4] and MS/MS data could be obtained on-line to help improve confidence in the identification of low level impurities. The combination of HPLC/MS and HPLC/NMR increasingly has a role to play in the unambiguous determination of low level impurities [5]. Complementary and improved approaches will invariably include greater sensitivity and improved efficiencies for the chromatographic resolution of structurally similar low level impurities. Micro-separation techniques coupled with electrospray (ESI) mass spectrometry offer many advantages in sensitivity, specificity and chromatographic efficiency. The concentration-dependent response of ESI [6] tabulated in Table 2.9.2.A makes it ideal for coupling with micro-separation techniques, such as capillary LC, capillary electrophoresis (CE) or capillary electrochromatography (CEC) where very low flow rates and high peak concentrations offer maximised sensitivity from hyphenated techniques.

In addition, as the flow rate is proportional to the square of the column diameter, micro-separation techniques coupled with ESI-MS also offer real resource savings in mobile phase consumption and disposal.

The successful utilisation of these advantages presently depends on the

Table 2.9.2.A.
Relative ESI responses against flow rate

Column i.d.	Flow rate (μl/min))	Relative ESI response
4.6 mm	1000	1
2.0 mm	200	5
1.0 mm	50	20
320 μm	5	200
75 μm	0.2	5000

reduction to practice of these micro-separation techniques, operating at low flow rates with existing commercial instrumentation not custom-designed for the job. Further problems lie with their subsequent interfacing with ESI-MS without loss of chromatographic fidelity and finally the robust operation of the combined system in a high throughput manner with ''real world'' samples. The use of microbore HPLC/MS (1.0 mm i.d.) and capillary LC (320 μm i.d.) using 3-μm silica particle size and operating at flow rates of 5–50 μl/min is now becoming commonplace as new purpose built systems are introduced. These developments are driven by the demands of protein sequencing/peptide mapping and split-pool combinatorial synthesis, where highly specific and sensitive analysis is required on small precious samples and almost inevitably the separation system is coupled to ESI MS. Beyond these lower dimensions of column i.d., flow rate and particle size, where sensitivity enhancements are very significant, the technical difficulties associated with running hydraulic pumped systems become prohibitive. Ultra high-pressure LC systems have been reported [7] and demonstrated to provide the excellent efficiencies approaching what is theoretically possible. The physical requirements of a hydraulic system at these extremes of pressure are not trivial and appropriate instrumentation has yet to be commercialised. Electrodrive systems such as capillary electrophoresis (CE) and CEC that are not technologically stretched to the limit offer an attractive alternative to obtain these enhanced performance capabilities from miniaturised systems [8].

Numerous applications of capillary electrophoresis-mass spectrometry (CE/MS) have been published [9] but the technique is still not widely accepted for routine use due to limitations in range of sample volumes that can be analysed without compromising separation efficiency. Poor concentration detection limits resulting from an inability to handle much more than 1–10 nl injections and the non-compatibility of micellar electrokinetic capillary chromatography (MECC) with mass spectrometry for the separation of neutrals suggest that CE/MS may always be a niche technique. Capillary isotachophor-

esis (CITP) and pre-concentration techniques may improve the poor detection limits but not without a time penalty.

Capillary electrochromatogaphy on the other hand offers the high efficiency and electromobility selectivity attributes of CE with the opportunity to utilise a wide range of well understood reverse phase (RP) HPLC stationary phases for partitioning. Relatively few CEC/MS papers have been published to date but this is likely to change rapidly in the near future as the technique is demonstrated in critical application areas including impurity determination in drugs.

This chapter reviews the development of CEC/MS and explores the potential of CEC/MS as a complementary technique to HPLC/MS in the future.

2.9.2.2. Development of CEC/MS

CEC [10–12] is a new high resolution hybrid technique of capillary HPLC and capillary electrophoresis, where the flow through a small i.d. packed column is electrically driven and offers high chromatographic efficiencies with good selectivity and sensitivity when coupled with ESI-MS. The technique has numerous potential advantages in coupling to ESI-MS, since narrower chromatographic peaks produce a higher mass flux and offers a viable alternative to micellar electrokinetic capillary chromatography (MECC) for the separation of neutrals using electrically driven flows. Verheij *et al.* [13] and Hugener *et al.* [14] reported the coupling of pseudo-electrochromatography (PCEC), a combination of both pressure driven and electro-osmotically driven chromatography, with mass spectrometry, where the flow profile approaches that in a pressure driven system [15]. Several authors have subsequently used pressure assisted flow (PCEC) to increase the volumetric flow rate, eliminate the need for make up flow and lessen the problems associated with the column drying out [16,17]. More recently, Wu *et al.* have described the analysis of protein digests using PCEC with an ion trap storage/reflectron time-of-flight mass detector [18,19]. Gordon *et al*. reported true CEC/MS coupling to both continuous flow fast atom bombardment (Cf-FAB) [15] and ESI [20] mass spectrometry. They noted a loss of chromatographic resolution as a result of post detection window dispersion in the length of unpacked capillary necessary for coupling the commercial CE instrument to the MS.

Lane *et al.* demonstrated that if fully packed CEC capillaries are used, then chromatographic fidelity is maintained in the mass spectrometer [21] even when using crude instrumentation consisting of a standard CE tri-axial probe interface with an Isco 3850 commercial CE instrument.

CEC/ESI MS separations of *fluticasone propionate* steroid mixtures (Fig. 2.9.2.A) and diasterioisomeric antibiotics on a 95-cm column were performed,

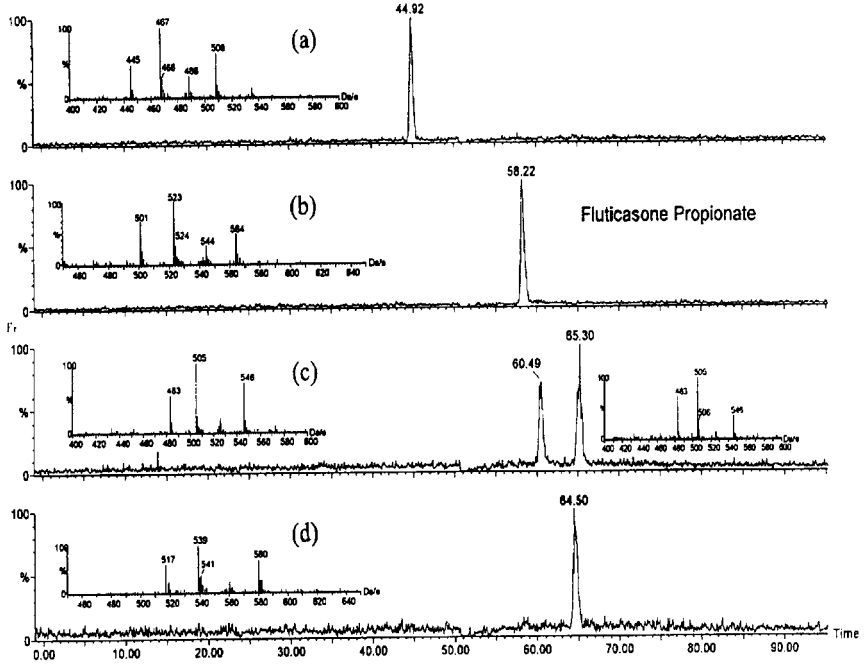


Figure 2.9.2.A.
CEC/MS mass chromatograms with spectra for fluticasone propionate and four related impurities on a 92-cm Hypersil C18 ODSA 3-μm packed capillary. (a) MW 444, (b) MW 500, (c) two isomeric components of MW 482, (d) MW 516, 518 (from Ref. [21] with permission from S.J. Lane, R. Boughtflower, C. Paterson and T. Underwood, *Rapid Commun. Mass. Spectrom.* **9**, 1283–1287 (1995), copyright John Wiley & Sons Limited.)

showing excellent efficiencies with good quality MS data but analysis times were long (70 min). This long analysis time resulted from diluted field strengths across the long columns.

The separations were performed using non-volatile buffers at the high pH necessary to generate sufficient electroosmotic flow (EOF) and to achieve reasonable analysis times on the C18 stationary phase used (see Fig. 2.9.2.D). These conditions were not optimal for CEC/MS and were dictated by instrumental restrictions in the coupling of commercial CE instruments with the mass spectrometer using a commercial CE tri-axial probe. A major problem was the lack of CEC-specific phases to promote higher EOF under the lower pH volatile buffer conditions favoured for MS interfacing and the inability to use short columns with high field strengths. A major advantage over CEC/UV

was recognised in that the column format for CEC/MS does not require a detection window or a length of post window open capillary.

Bubble formation and subsequent loss of flow are significantly less, compared with off-line CEC/UV systems. This is probably due, in part, to the absence of the open tube section of the capillary following the packed bed in CEC/UV systems and the consequential differences in EOF at the packed bed/open tube interface [22,23].

On the other hand, an added complication of interfacing CEC with MS is the problem of keeping the column wet at all times whilst having the cathode end of the column ''open'' and not pressurised [8]. Gradient CEC/MS is an elegant solution to this problem [24] but does have its own drawbacks in not being able to handle small volume samples and also the risk of introducing pressure into the column and therefore performing PCEC in reality.

A prototype integral CEC/MS injection/separation interface was designed built and demonstrated by Lane *et al.* [23] that facilitated short CEC columns (minimum 43 cm; see the schematic of the injection/separation interface in Fig. 2.9.2.B). This system obviated the need for a commercial CE instrument and

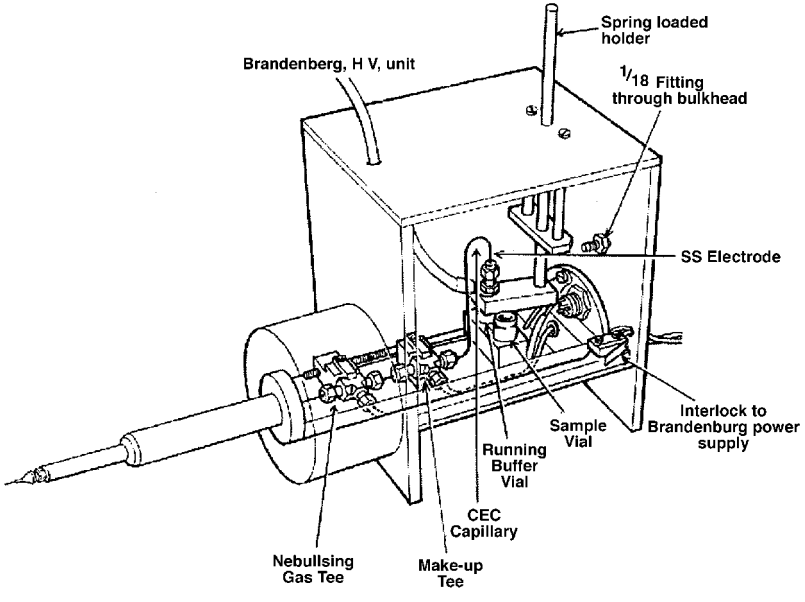


Figure 2.9.2.B.
CEC/MS injection/separation interface (from Ref. [13] with permission from S.J. Lane, R. Boughtflower, C. Paterson and M. Morris, *Rapid Commun. Mass. Spectrom.* **10**, 733–736 (1996), copyright John Wiley & Sons Limited)

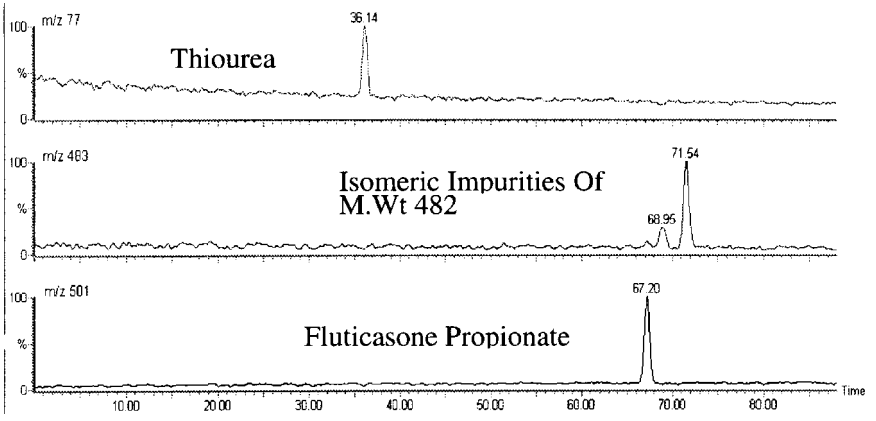


Figure 2.9.2.C.
Mass chromatograms showing the CEC/MS separation of fluticasone propionate (MH^+ 501) and two isomeric impurities (MH^+ 483). Column: 50 cm × 50 μm i.d. × 375 μm o.d.; 3 μm Innovatech ODS2; buffer: 80% CH_3CN/10 mM NH_4OAC, pH 5; make up flow: 20 μl/min of 75% MeOH/0.3% formic acid

the steroid separation was achieved in 11 min, compared to 70 min previously, using the same buffer conditions as before.

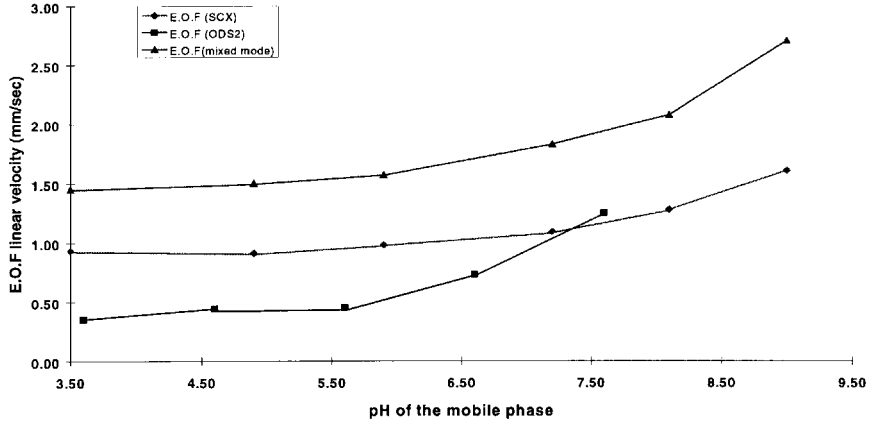


Figure 2.9.2.D.
EOF flow profiles. Columns used: 24-cm Phase Separations SCX packed capillary, 21-cm ODS2 packed capillary and 25-cm Hypersil Mixed Mode packed capillary. Mobile phase: 75%MeCN/50 mM Na_2HPO_4. Marker: thiourea

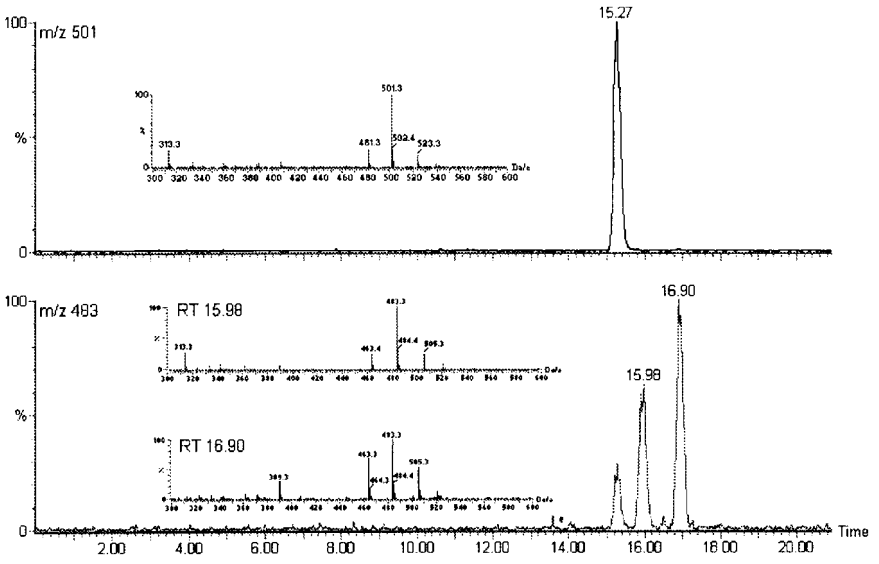


Figure 2.9.2.E.
Mass chromatograms showing the CEC/MS separation of fluticasone propionate (MH^+ 501) and two isomeric impurities (MH^+ 483). Column: 45 cm × 50 μm i.d. × 375 μm o.d. Innovatech experimental 5 μm mixed mode C18/SCX; buffer 75% CH_3CN/25 mM NH_4OAC,pH 5.5; make up flow: 20 μl/min of 75% MeOH/0.3% formic acid. Applied voltage: 28 kV; electrokinetic injection: 28 kV/2 s

Performing the same steroid separation on the interface with an ESI-MS-compatible volatile buffer at lower pH again produced an excessively long analysis time, evidenced by the low EOF and resulting in a long retention time of the unretained thiourea (Figure 2.9.2.C).

To increase the speed of analysis a CEC-specific ''mixed mode'' phase in which a sulphonic acid moiety is co-bonded onto a silica particle with the ODS function was used. This combined phase promotes high EOF whilst maintaining RP selectivity and is pH independent over a wide range, even down to the low pH conducive to optimum CEC/MS mobile phase system (Fig. 2.9.2.D; EOF flow profiles).

Using a C18/SCX (strong cation exchange resin) to promote a high EOF, the separation was achieved in less than 20 min with the above low pH volatile buffer (Fig. 2.9.2.E) with good efficiencies transferred to the MS.

To date we have found that the retention time order for neutrals is typically the same for C18 and C18/SCX.

2.9.2.3. Performance

Figure 2.9.2.F shows the separation of the CEC/MS standard test mixture on an Innovatech Waters Spherisorb S3 C6/SCX column. The resulting positive ion (+ve) ESI spectra for the separated components, shown in Fig. 2.9.2.G, are typified by strong MH^+ molecular ions and the facile loss of HF for fluorinated compounds.

The high chromatographic fidelity is maintained through to the mass spectrometer and efficiencies in excess of 200 000 N/m can be measured on the hydrocortisone peak from the above CEC/MS analysis (Fig. 2.9.2.H).

Negative ion CEC/MS of the mix shows an increased EOF resulting from the higher field strength across the capillary with a negative voltage on the ESI tip (Fig. 2.9.2.I).

The broad low-level response following each peak is thought to be due to excess material adhering to the outside of the capillary and being pulled into the capillary once it is repositioned in the running buffer and the voltage switched on.

Negative ion ESI spectra for these compounds are typified by strong M + $HCOO^-$ anions when formic acid is used in the make-up flow (Fig. 2.9.2.J).

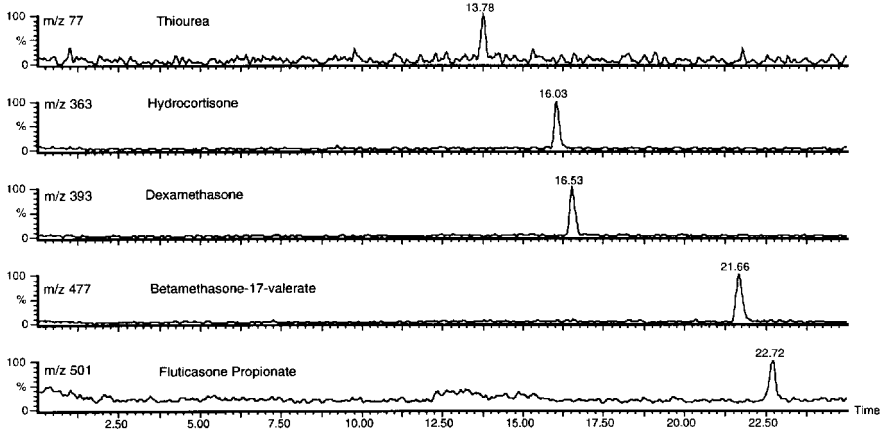


Figure 2.9.2.F.
Mass chromatograms for the CEC/MS separation of a 20-kV/5-s injection of a standard test mix on a 45 cm × 50 μm × 375 μm o.d. Waters Spherisorb S3 C6/SCX column using a buffer of 75% CH_3CN/25 mM NH_4OAc, pH 3.5 with a 20-kV applied voltage

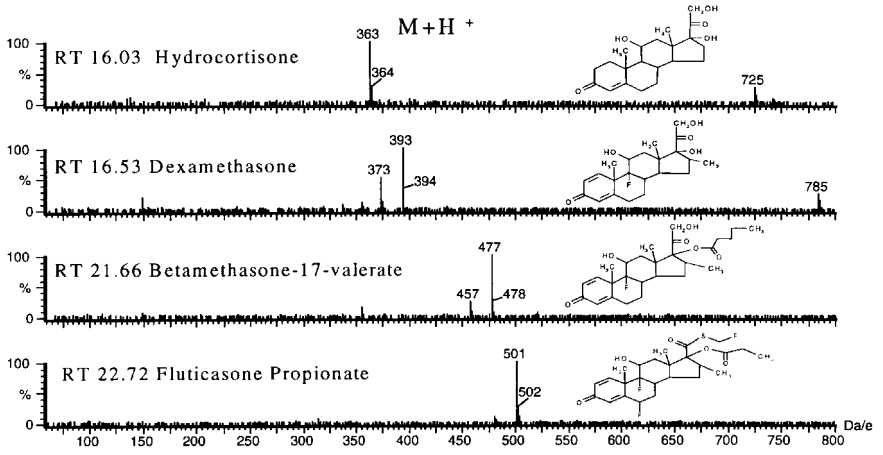


Figure 2.9.2.G.
Positive ion ESI spectra

2.9.2.4. Method Development

Method development for targeted analysis can be approached in a unique way with CEC/MS. Infusing a mixture through a CEC column under EOF (EOF infusion) will provide separation of the components and the opportunity to optimise the MS conditions. Figure 2.9.2.K shows mass chromatograms for

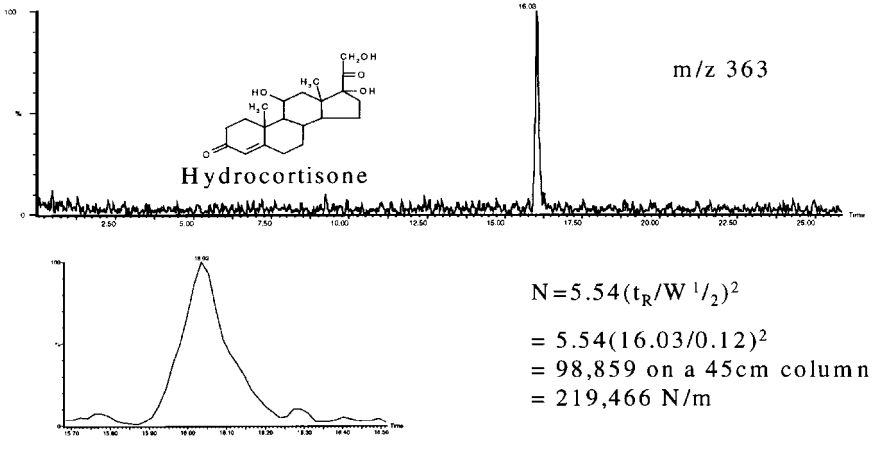


Figure 2.9.2.H.
CEC/MS efficiency from mass chromatogram of m/z 363

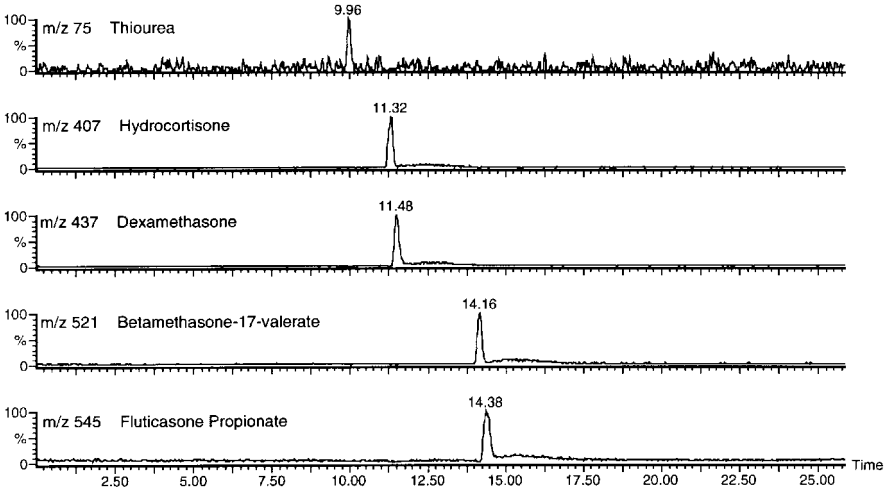


Figure 2.9.2.I.
Mass chromatograms for the CEC/MS separation of a 20-kV/5-s injection of a standard test mix on a 45 cm × 50 μm × 375 μm o.d. Waters Spherisorb S3 C6/SCX column using a buffer of 75% CH_3CN/25 mM NH_4OAc, pH 3.5 with a 20-kV applied voltage

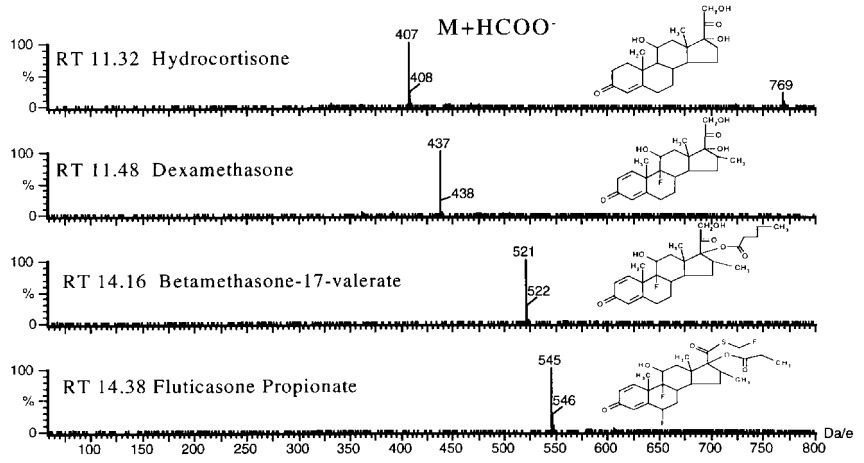


Figure 2.9.2.J.
Negative ion ESI spectra

thiourea and the CEC standard mix on a Micromass Platform LC quadrupole MS system fitted with a CEC/MS injection/separation interface. The capillary has been immersed in the sample solution and a voltage of 20 kV applied

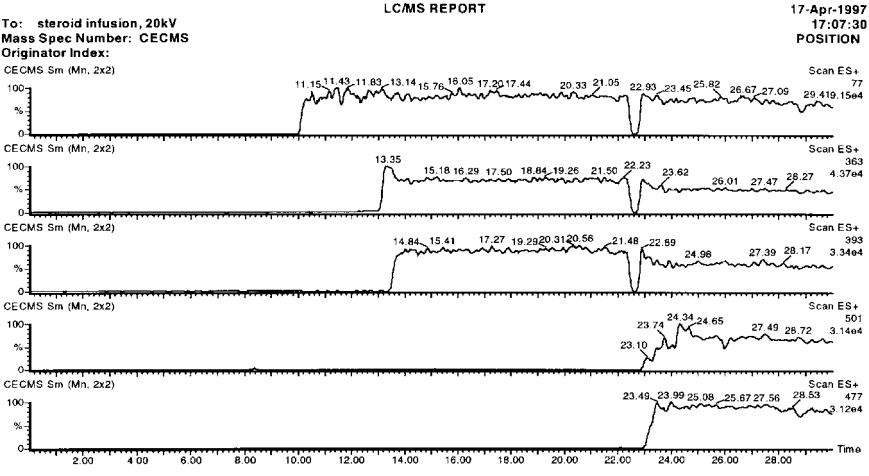


Figure 2.9.2.K.
Mass chromatograms for the 20-kV EOF infusion of the test mixture on a 45 cm × 50 μm × 375 μm o.d. Waters Spherisorb™ S3 C6/SCX column

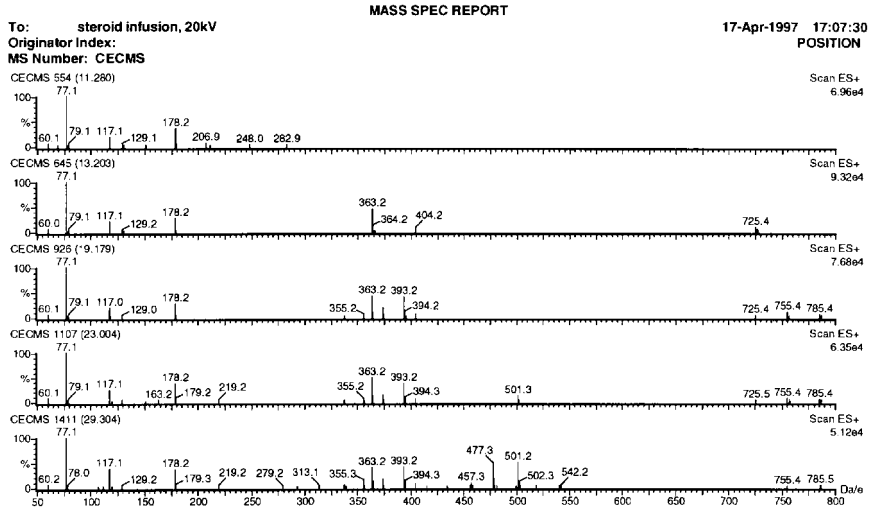


Figure 2.9.2.L.
ESI spectra from EOF infusion

continuously rather than as a discrete injection. The mixture infuses through the packed column at different rates depending on its partitioning and it can be seen that thiourea (m/z 77) is detected first in the source, followed by hydrocortisone, dexamethasone, betamethasone 17-valerate and finally fluticasone propionate (m/z 501).

Early spectra (11 min) show exclusively the MH^+ ion at m/z 77 for thiourea whereas the other extreme at 29 min show all molecular ion species at m/z 77, 363, 393, 477 and 501 allowing optimisation of CEC/MS conditions for maximum response for all ions in the source (Fig. 2.9.2.L).

2.9.2.5. Applications

The increased efficiency of CEC compared to HPLC can be used to great effect in the resolution of low level novel impurities in pharmaceutical compounds as was demonstrated in an early key paper from Smith and Evans [25].

A new low level impurity running between the isomeric s-methyl ester and 6-alpha des fluoro impurities of MW 482 in fluticasone propionate was resolved by CEC/UV on a 3-μm ODS1 column (Fig. 2.9.2.M). The impurity had not been resolved previously by RPLC and therefore never isolated for

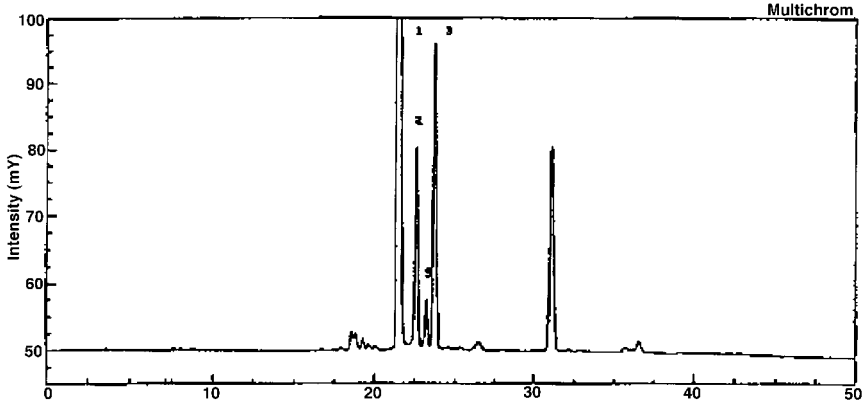


Figure 2.9.2.M.
The separation of fluticasone propionate from three related impurities and an impurity not previously seen. Instrument: modified ABI 270A; capillary: ~60 cm × 50 μm i.d.; packing: 3 μm ODS-1 PC20; detector $\lambda = 238$ nm, 0.05 aufs; rise time = 1.0; applied voltage: 30 kV; temp. 30°C; mobile phase: 80% CH_3CN/20% 5 mM borate, pH 9.0; injection: 0.4 min at 30 kV. (**1**) Fluticasone propionate; (**2**,**3**,**4**) related impurities; (**5**) new impurity (From Ref. [25] with permission from N.W. Smith and M.B. Evans, *Chromatographia*, **32**, 650 (1994))

identification. In this case there is no opportunity for the standard protocol of a preparative RPLC followed by spectroscopic identification, synthesis of the impurity and spiking of the compound in question. On-line LC/MS is problematical as the retention characteristics are unknown as is the MW.

Isolating such a low-level impurity in sufficient amount and purity by CEC is impractical with present sample handling techniques and highlights an inherent weakness of CEC and indeed CE.

The first requirement is to characterise the impurity on-line with CEC/MS. This may lead to identification or at least allow informed HPLC/MS method development

We have looked at the above separation by CEC/MS and developed a protocol that could represent a generic starting point for this type of problem.

In the absence of an in-line UV detector prior to the MS, EOF infusion was used to determine the MW of the impurity. The EOF infusion spectrum is dominated by molecular ions for fluticasone propionate (m/z 501) and the impurities (m/z 483) (Fig. 2.9.2.N). Expansion of the high mass region of the spectrum shows an abundance of $[2M + H]^+$ and $[2M + NH_4]^+$ source

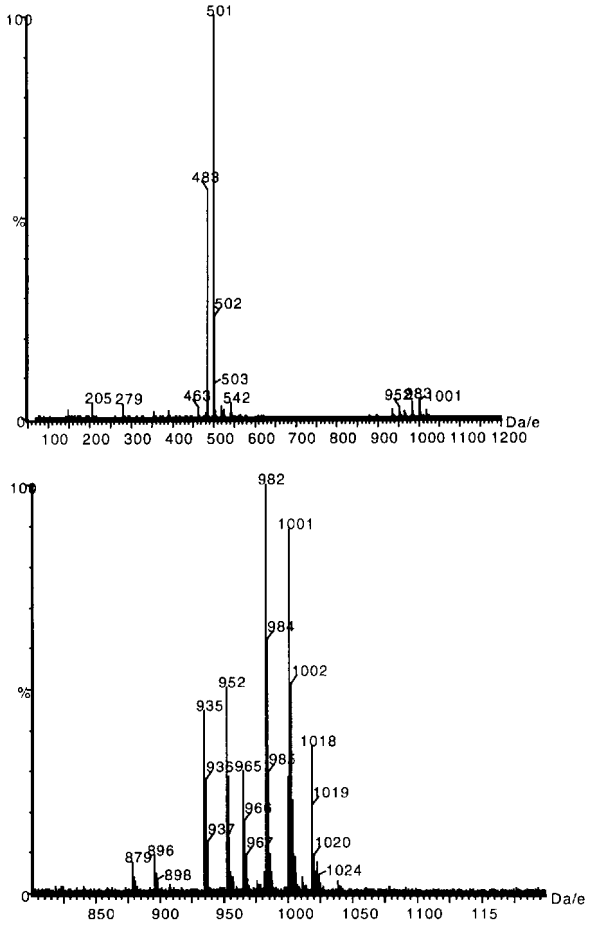


Figure 2.9.2.N.
EOF infusion mass spectrum and expanded high mass portion of the spectrum of fluticasone propionate separated on a 45 cm × 50 μm × 375 μm o.d. Waters Spherisorb S3 C18/SCX column using a buffer of 75% CH_3CN/25 mM NH_4OAc, pH 3.5 with a 22-kV applied voltage

induced dimerisation ions at m/z 965, 982, 1001 and 1018. A further pair of ions at m/z 935 and 952 are characteristic for a known disulphide-dipropionate impurity (see Fig. 2.9.2.Q for structure). A weak pair of ions at m/z 879 and 896 for MH^+ and MNH_4^+, respectively was due to an unknown compound. The column was replenished with fresh buffer and discrete 22 kV injections of the

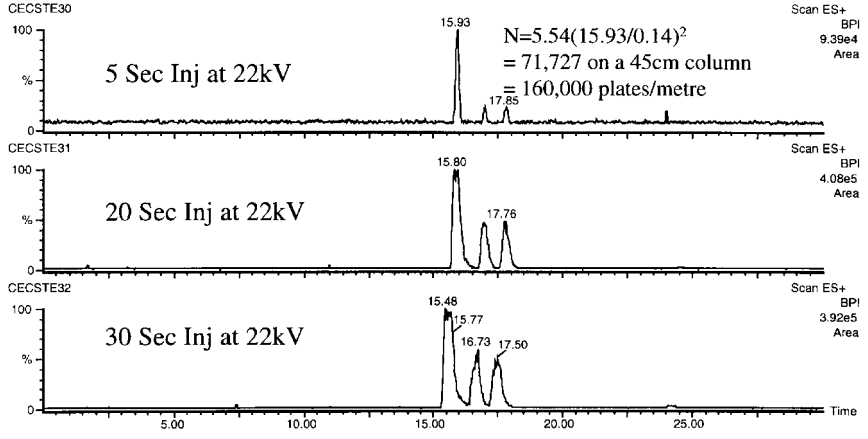


Figure 2.9.2.O.
CEC/MS base peak intensity (BPI) profiles of injections of fluticasone propionate sample on a 45 cm × 50 μm × 375 μm o.d. Waters Spherisorb S3 C18/SCX column using a buffer of 75% CH_3CN/25 mM NH_4OAc, pH 3.5 with a 22-kV applied voltage

sample were made for varying times. Whilst efficiency is lost with increased injection time as the column becomes increasingly overloaded, the resolution is still sufficient to resolve the fluticasone propionate from the isomeric impurities (Fig. 2.9.2.O).

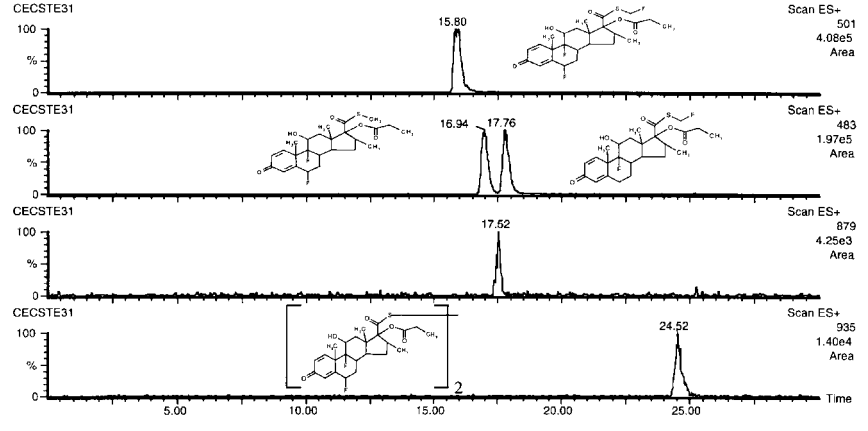


Figure 2.9.2.P.
Mass chromatograms for the 20-s injection

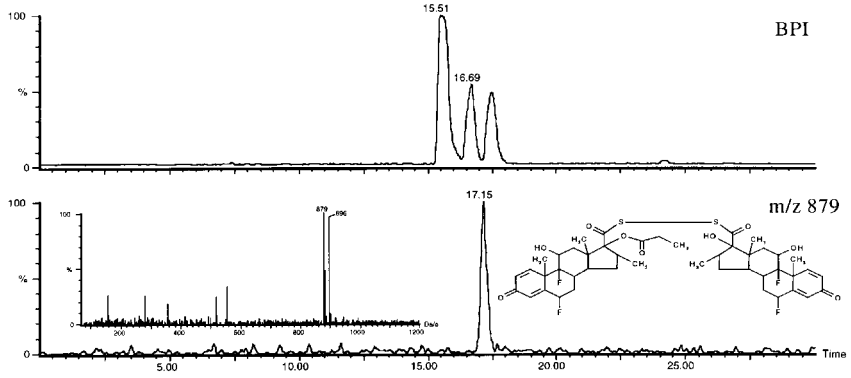


Figure 2.9.2.Q.
Mass chromatogram for disulphide–despropionate at m/z 879

Plotting mass chromatograms for the known (m/z 935) and unknown (m/z 879) compounds show the unknown to elute in between the isomeric pair (Fig. 2.9.2.P) at RT 17.52 min and the structure can be elucidated as the disulphide-despropionate compound (Fig. 2.9.2.Q).

This on-line approach will often be sufficient for full identification especially if coupled with knowledge of the chemistry. The MW knowledge in itself would allow an informed HPLC/MS method development for the resolution of the compounds and subsequent isolation.

Other application areas for CEC/MS include the analysis of DNA adduct mixtures with a limit of detection of 10^{-6} M as reported by Ding *et al*. [26]. Also dansylated secondary amine tags for encoded split-pool combinatorial libraries have been analysed by microelectrospray CEC/MS/MS in the parent ion mode, facilitating the successful decoding of single beads by detection of the surrogate tags at the low femtomole level [27]. The microelectrospray system was designed and constructed to enable short CEC columns to be accommodated in the system.

We have found CEC/MS useful for the analysis of natural product mixtures such as *macrolide antibiotics* [23] and the high resolution analysis of homologues of *carboxymycobactins* in complex matrices. Carboxymycobactins are a group of iron chelating compounds recently discovered and characterised which are present in the culture extracts of the pathogenic mycobacterium *M. avium* [28], *M. tuberculosis* and more recently the non-pathogenic *M. smegmatis* [29]. These compounds are of interest as it is suspected that they play an important role in the transport of iron from the extracellular to the intracellular environment of cells that require iron for growth and survival. The inhibition of this iron transfer, or using the

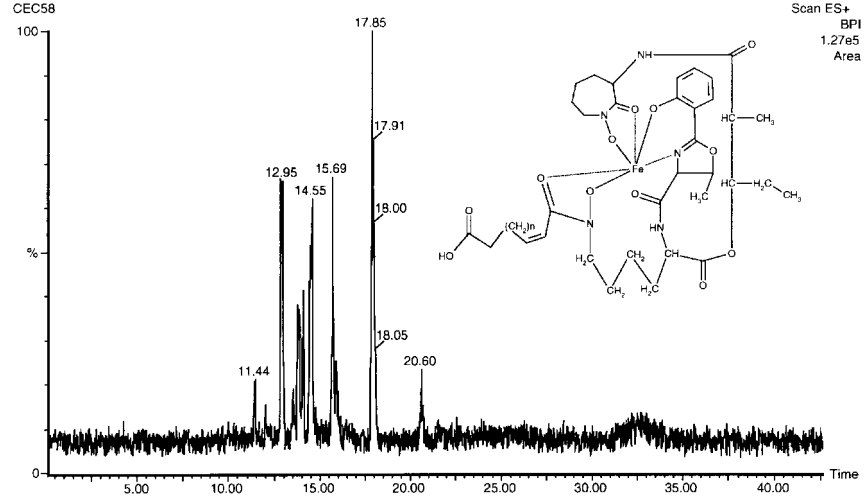


Figure 2.9.2.R.
CEC/MS of carboxymycobactins from a 25-kV/5-s injection of a *M. avium* extract on a 45 cm × 50 μm × 375 μm o.d. Waters Spherisorb S3 C18/SCX column using a buffer of 75% CH_3CN/25 mM NH_4OAc, pH 5.5 with a 25-kV applied voltage

compounds as carriers for antibiotics may be of some therapeutic value in the treatment of tuberculosis. The compounds exist as complex homologous mixtures in several series of isobaric compounds with varying degrees of unsaturation in the fatty acid side-chain. The high chromatographic efficiency of CEC/MS has been useful in separating and characterising these series for each family. Fig. 2.9.2.R shows the base peak intensity (BPI) trace for a methanol extract of *M. avium.*

Fig. 2.9.2.S shows mass chromatograms highlighting the various homologous series between n = 4 and 7, with an example of the ESI-MS spectrum for n = 6, showing the characteristic ^{54}Fe,^{56}Fe isotope ratio.

2.9.2.6. Reduction of Analysis Times and Automation

The manual CEC/MS probe coupled with mixed mode phases has facilitated first generation CEC/MS which has been reduced to practice and shows significant advantages over simply coupling commercial CE instruments to mass spectrometers using commercially available interfaces. However, the

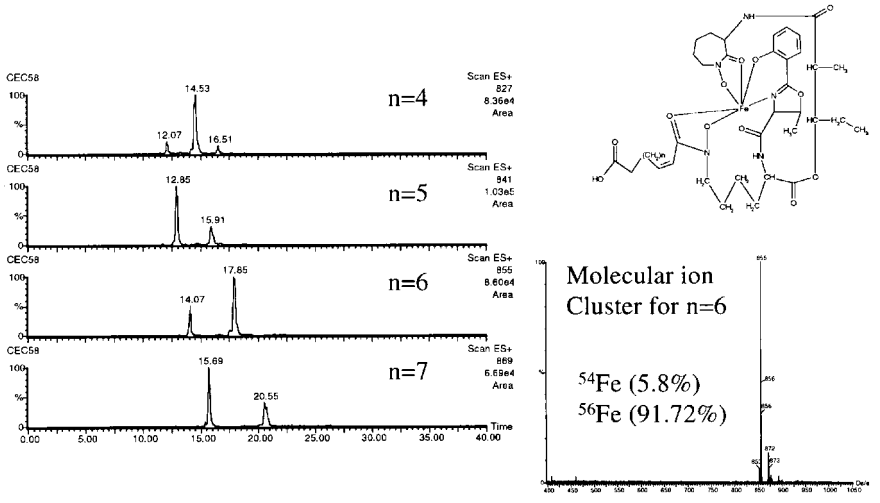


Figure 2.9.2.S.
CEC/MS mass chromatograms for two series between n = 4 and 7

manual source/probe basic design places new limitations on the length of column (45 cm) that can be accommodated and consequently the achievable analysis time using commercially available 30-kV power supplies. The system also lacks robustness as its design involves moving the anode electrode containing the packed capillary to the sample. This puts excessive strain on the fragile capillary, which would be best immobilised within the electrode and the sample moved instead.

Using a Micromass Quattro quadrupole mass spectrometer fitted with a ''z-spray'' source allowed modification to the probe such that it would accommodate 15-cm columns. The analysis time for the original isocratic fluticasone propionate separation was reduced from 70 min in Fig. 2.9.2.A to less than 1.5 min in Fig. 2.9.2.T where the field was 1700 v/cm with a linear velocity of 4.6 mm/min.

A *peptide mixture* was also separated under the same conditions in less than 2 min (Fig. 2.9.2.U).

This speed of analysis, whilst maintaining a degree of chromatographic resolution is of great interest in both combinatorial chemistry and drug metabolism screens and represents the future for CEC/MS.

Rapid analysis using high efficiency electrodriven separation techniques such as CE and CEC yield extremely narrow peak widths in the order of a few seconds. Scanning instruments such as quadrupole mass filters have difficulty obtaining sufficient data points across such narrow peaks. The peak can

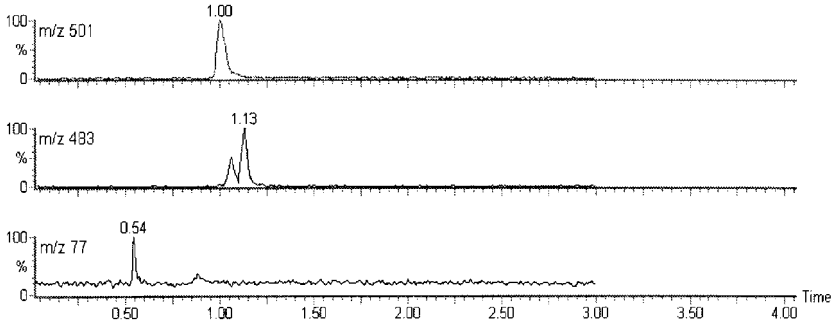


Figure 2.9.2.T.
Fast CEC/MS of fluticasone propionate and isomeric impurities at 1700 V/cm. Column: 15 cm × 50 μm × 375 μm o.d Innovatech 5 μm experimental mixed mode C18/SCX. Buffer: 75% CH_3CN/25 mM NH_4OAc, pH 5.5; make-up flow: 20 μl/min of 75% MeOH/0.3% formic acid; applied voltage: 30 kV. Electrokinetic injection: 20 kV/1 s

become distorted, chromatographic resolution apparently lost and at worst the peak missed completely [30]. Time of flight (TOF) analysers have an inherent ability for fast acquisition rates and consequently a high number of data points can be collected across a narrow peak. They are routinely operational at resolutions of 5000 full width half maxima (FWHM) allowing accurate mass measurement to 5 ppm [4,31]. We have interfaced the manual probe with an early Micromass LCTOF instrument that samples at 10 spectra/s as a proof of principal [31], separating hydrocortisone, dexamethasone and betamethasone 17-valerate. This was not a fast separation with short columns but was designed to evaluate whether the accurate mass of an unknown could be determined using a two-point calibration. The chromatogram was summed and the accurate masses of hydrocortisone and betamethasone17-valerate used as a two point calibration to measure the accurate mass of dexamethasone to 3 ppm, treating it as an unknown [31]. This measurement on-line will be extremely important for degenerate combinatorial libraries and impurity identification and reflects the future for fast separations with MS.

Recently, we have designed and constructed a novel automated electroosmotic flow (EOF) sample introduction and separation interface and successfully coupled it to an electrospray mass spectrometer. The system consists of a small footprint automated CEC injection and separation interface that has an integrated autosampler and connects to the outside world via a proprietary controller for automated CEC/MS, EOF infusion and flow injection analysis (EOFFIA). The system utilises the CEC/MS/MS micro-electrospray. Short

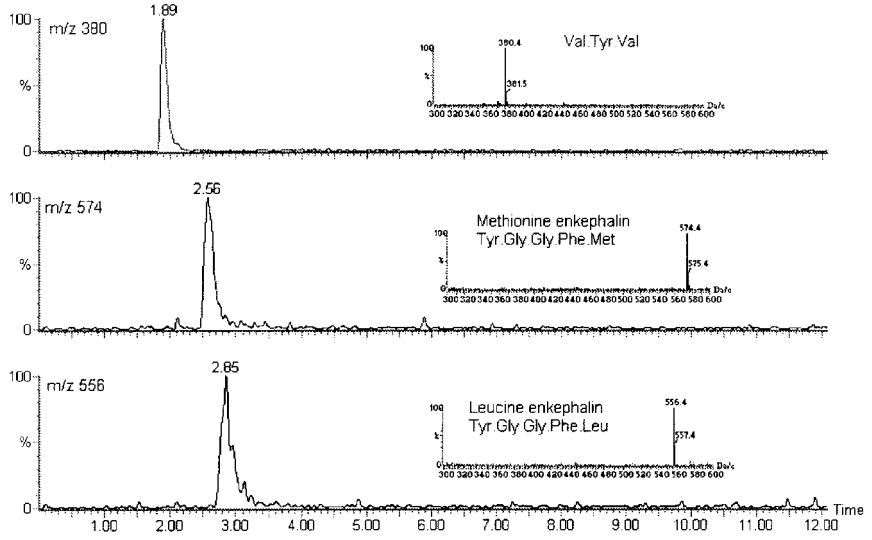


Figure 2.9.2.U.
Fast CEC/MS of a standard peptide mix at 1700 V/cm. Column: 15 cm × 50 μm × 375 μm o.d. Innovatech 5 μ experimental mixed mode C18/SCX. Buffer: 75% CH_3CN/25 mM NH_4OAc, pH 5.5; Make-up flow: 20 μl/min of 75% MeOH/0.3% formic acid. Applied voltage: 30 kV; electrokinetic injection: 20 kV/1 s

CEC columns or open capillaries are immobilised in a hollow stainless steel needle that is located in a pincher bar through which injection and running high voltage is applied. The needle assembly is easily removed for ease of column installation and changeover. At the heart of the automated system is a microprocessor-based controller which allows both manual and automated control via a 12-way key pad.

The controller provides automatic indexing of a 10-position sample carousel, control of kV supply so that injection and running voltages can be set independently for full flexibility and mass spectometer synchronising signals. The system has been made flexible in both mechanical and electronic design to be easily adaptable to a range of ESI mass spectrometers [32]. A schematic of the system is shown in Fig. 2.9.2.V.

This inherent robustness and automation has facilitated easier evaluation of CEC/MS as a technique and we have reported linear calibration lines with good correlation for the β2-agonists salmeterol and salbutamol and tricyclic antidepressants [33] on C18/SCX, C6/SCX and SCX columns using CEC/SIM/MS [34].

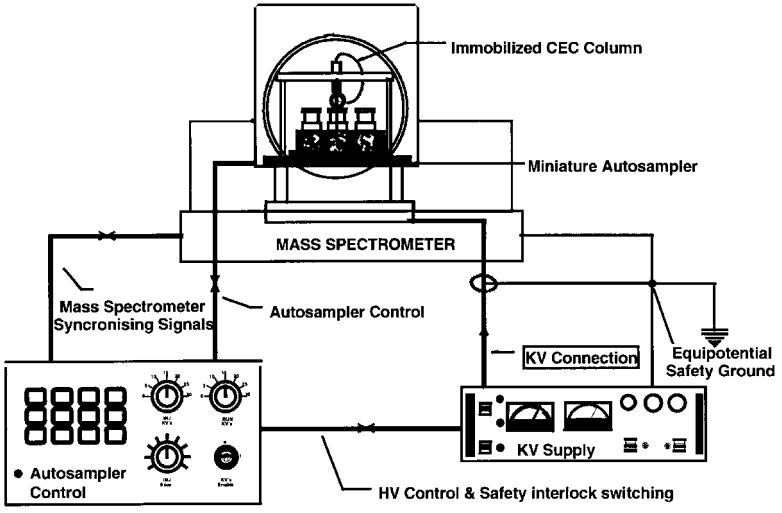


Figure 2.9.2.V.
Schematic of the EOF autosampler (from Ref. [32] with permission from S.J. Lane and M.G. Tucker, *Rapid Commun. Mass Spectrom.* **12**, 947–954 (1998), copyright John Wiley & Sons Limited)

Efficiencies in excess of 350 000 plates/m were reproducibly obtained, where previously the highly basic trycyclic antidepressant compounds had produced extraordinarily high efficiencies that defied chromatographic theory [35] and were thought to result from some type of focussing effect on SCX phases.

Fourteen underivatised tags were also separated by full scan CEC/MS but problems were encountered in setting up a single ion monitoring method for 14 ions with narrow chromatographic peak widths and again CEC/TOF is suggested as a solution [34].

To date there are few CEC/MS papers reporting the quantitation of compounds in biological fluids which so far has necessitated the use of solid phase extraction sample pre-treatment [36] and gradient elution CEC/MS [37].

Recently we have been able to quantitate steroids in dog plasma by CEC/UV and CEC/MS with minimal sample preparation [38]. Efficiencies were maintained after 100 injections of the steroid mix from plasma.

2.9.2.7. Conclusion and Future

All the recent developments in CEC/MS are ultimately applicable to the identification of impurities in drugs and as this combination reaches a stage of robustness and reproducibility, with a reasonable degree of automation, it will undoubtedly become a highly useful orthogonal technique to complement HPLC/MS.

The combination of CEC with TOFMS offers real opportunity for rapid analysis with chromatographic efficiency in a range of application areas. For exquisite sensitivity from a miniaturised system the combination of CEC with nanospray MS [39] holds great promise for the future.

References

1. International Conference on Harmonisation (ICH2), Document on Impurities in New Drug Substances
2. E.C. Nicolas and T.H. Scholz, *J. Pharm. Biomed. Anal.* **16**, 825–836 (1998)
3. J. Ermer, *J. Pharm. Biomed. Anal.* **18**, 707–714 (1998)
4. C. Eckers, N. Haskins and J. Langridge, *Rapid Commun. Mass Spectrom.* **11**, 1916–1922 (1997)
5. N. Mistry, I.M. Ismail, M.S. Smith, J.K. Nicholson and J. Lindon, *J. Pharm. Biomed. Anal.* **16**, 697–705 (1997)
6. G. Hopfgartner, K. Bean, J. Henion and R. Henry, *J. Chromatogr.* **647**, 51–61 (1993)
7. J.E. MacNeir, K.C. Lewis and J.W. Jorgenson, *Anal. Chem.* **69**, 983–989 (1997)
8. M.M. Dittmann, K. Wienand, F. Bek and G.P. Rozing, *LC-GC Int.* **13**, 800–814 (1995)
9. J. Cai and J. Henion, *J. Chromatogr.* A **703**, 667–692 (1995)
10. V. Pretorius, B.J. Hopkins and J.D. Schieke, *J. Chromatogr.* **99**, 23–30 (1974)
11. J.W. Jorgenson and K.D. Lukacs, *J. Chromatogr.* **218**, 209–216 (1981)
12. J.H. Knox and I.H. Grant, *Chromatographia* **24**, 135–143 (1987)
13. E.R. Verheij, U.R. Tjaden, W.M.A. Niessen and J. van der Greef, *J. Chromatogr.* **554**, 339–349 (1991)
14. M. Hugener, A.P. Tinke, W.M.A. Niessen, U.R. Tjaden and J. van der Greef, *J. Chromatogr.* **647**, 375–385 (1993)
15. D.B. Gordon, G.A. Lord and D.S. Jones, *Rapid Commun. Mass Spectrom.* **8**, 544–548 (1994)

16. S.E.G. Deckers, U.R. Tjaden and J. Van der Greef, *J. Chromatogr.* **712** 201–209 (1995)
17. S. Schmeer, B. Behnke and E. Bayer, *Anal. Chem.* **67**, 3656–3658 (1995)
18. J.-T. Wu, P. Huang, M.X. Li and D.M. Lubman, *Anal. Chem.* **69**, 2908–2913 (1997)
19. P. Huang, J.-T. Wu and D.M. Lubman, *Anal. Chem.* **70**, 3003–3008 (1998)
20. G.A. Lord, D.B. Gordon, L.W. Tetler and C.M. Carr, *J. Chromatogr.* **700**, 27–33 (1995)
21. S.J. Lane, R. Boughtflower, C. Paterson and T. Underwood, *Rapid Commun. Mass Spectrom.* **9**, 1283–1287 (1995)
22. J.H. Knox and I.H. Grant, *Chromatographia* **32**, 317–328 (1991)
23. S.J. Lane, R. Boughtflower, C. Paterson and M. Morris, *Rapid Commun. Mass Spectrom.* **10**, 733–736 (1996)
24. M.R. Taylor and P. Teale, *J. Chromatogr.* A **768**, 89–95 (1997)
25. N.W. Smith and M.B. Evans, *Chromatographia* **38**, 649–657 (1994)
26. J. Ding and P. Vouros, *Anal. Chem.* **69**, 379–384 (1997)
27. S.J. Lane and A. Pipe, *Rapid Commun. Mass Spectrom.* **12**, 667–674 (1998)
28. S.J. Lane, P.S. Marshall, R.J. Upton, C. Ratledge and M. Ewing, *Tetrahedron Lett.* **36**, 4129–4132 (1995)
29. S.J. Lane, P.S. Marshall, R.J. Upton and C. Ratledge, *Biometals* **11**, 13–20 (1998)
30. J.F. Banks Jr. and T. Dresch, *Anal. Chem.* **68**, 1480–1485 (1996)
31. Micromass Brochure No. BR20/DAM version 1, May (1997)
32. S.J. Lane and M.G. Tucker, *Rapid Commun. Mass Spectrom.* **12**, 947–954 (1998)
33. N.W. Smith and M.B. Evans, *Chromatographia* **41**, 197–203 (1995)
34. V. Spikmans, S.J. Lane, U.R. Tjaden and J. Van der Greef, *Rapid Commun. Mass Spectrom.* **13**, 141–149 (1999)
35. I.S. Lurie, T.S. Conver and V.L. Ford, *Anal. Chem.* **70**, 4563–4569 (1998)
36. C. Paterson, R.J. Boughtflower, D. Higton and E. Palmer, *Chromatographia* **46**, 599–604 (1997)
37. M.R. Taylor, P. Teale, S.A. Westwood and D. Perrett, *Anal. Chem.* **69**, 2554–2558 (1997)
38. V. Spikmans, S.J. Lane and N.W. Smith, *Chromatographia* **51**, 18–24 (2000)
39. R.N. Warriner, A.S. Craze, D.E. Games and S.J. Lane, *Rapid Commun. Mass Spectrom.* **12**, 1143–1149 (1998)

2.10. Supercritical Fluid Chromatography (SFC)

Olle Gyllenhaal

2.10.1. Introduction to SFC

In SFC the solvating property of the fluid is proportional to the density and consequently the density is the force that regulates the retention in a given system. The density can be regulated by the pressure and/or the temperature. The most commonly used fluid is carbon dioxide and its polarity is rather low. Usually a polar modifier like methanol is added in order to increase the solvating power of the mobile phase. For a comprehensive introduction to the field of analytical SFC the reader is recommended the book by Lee and Markides [1].

2.10.2. SFC using Capillary Columns

The spread and use of SFC took off in the 1980s using capillary columns and flame ionisation detection (FID). Many fascinating separations were presented but in industry few applications were taken into routine use. In the pharmaceutical field some of the limitations were due to limited polarity of neat carbon dioxide, shortcomings of the instrumentation and low overall sample throughput. For the analysis of impurities in the 0.1% region of the parent drug the sample capacity using narrow i.d. columns with thin stationary phase films is not sufficient. Split injections are required if the column efficiency should be maintained. Inert columns show low selectivity and more polar and selective ones have active column walls as was shown for the β-adrenoreceptor blocking drug metoprolol and related compounds [2]. The low sample throughput is partially due to relatively large hold-up times, even if the mobile phase velocity is often well above the optimum and density gradients are used. These gradients demand some time for equilibration before the next injection can be performed. Still, the use of neat carbon dioxide and FID is an attractive combination in the estimation of unknowns that is hard to beat. Recent development of instrumentation has focused on making the technique more user friendly.

2.10.3. SFC using Packed Columns

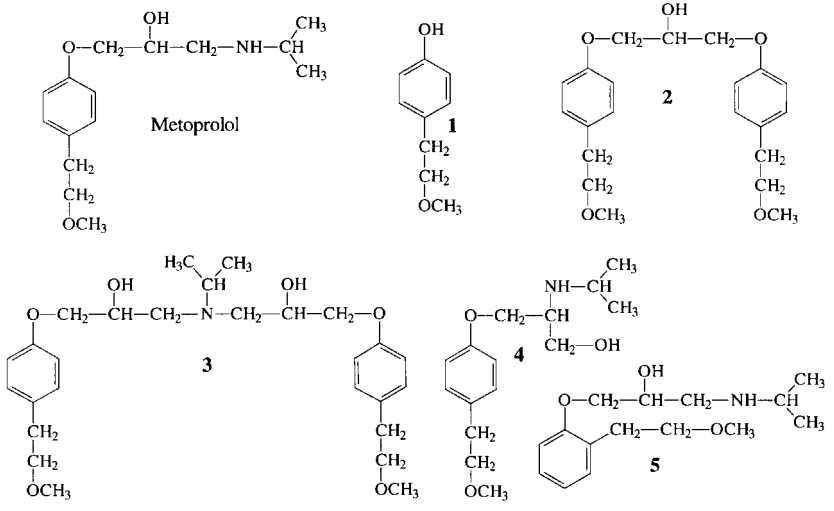
Many of the shortcomings of capillary SFC can be overcome by using instrumentation that is more LC-like. The handling is similar and columns made for LC can be used. There is a wide choice of detectors. The UV detector is the workhorse and with regret the FID can normally not be used since the mobile phase virtually always has a modifier present that generates a high background signal. Other detectors that offer interesting possibilities in SFC include the evaporative light scattering detector [3], the fluorescence detector [4] and the electron capture detector [5]. The former has the advantage of being a general detector that detects compounds that form particles upon evaporation and reflect light. Its main drawback is its inherent non-linear response. The other two detectors are selective and the response of an unknown is therefore difficult to predict. Hence they are not so attractive in the impurity profiling of drugs. This is also true for the nitrogen phosphorous selective detector.

The field of SFC of *drugs* has been thoroughly reviewed [6,7] and is also covered bi-annually in the review of SFC and SFE in *Analytical Chemistry* [8,9]. Most types of drugs have been chromatographed by packed column SFC. Examples that present the simultaneous determination of impurities will be discussed in more detail below.

The most important aspects of method development is the choice of column support and modifier, plus an additive if needed. Fine tuning of difficult separations can often be accomplished by changing the column temperature [10–12] or adding efficiency by connecting a second or third column in series or even ten [13]. The back pressure has a minor importance and the flow-rate can be used to adjust the time for the separation. Modifier gradients are often used in order to analyse samples containing a wide variety of components.

Some characteristic *examples* for the use of packed column SFC in impurity profiling of drugs are as follows.

Omeprazole. Method development was focused on the selection of a suitable column support in order to achieve sufficient selectivity to possible impurities [14]. The separation of the impurities could be performed below the 1% w/w range within 10 min and the elution order was normal-phase LC like. This compared favourably with a LC method [15].



Metoprolol and impurities

Metoprolol was chromatographed using triethylamine in methanol as a modifier [16]. The selectivity was investigated using aminopropyl and diol silica columns and some 12 more or less likely impurities were separated. The final choice was diol and a mobile phase containing acetic acid in excess of the amine. The bulk drug substance was analysed pure and with possible impurities added to the 0.1% w/w level (Fig. 2.10.A). The meta-isomer co-eluted with metoprolol, but by connecting three diol columns in series it was possible to separate this compound as well. Tablets could be analysed with the same chromatographic method after an isolation step. No interference was observed and the procedure could be used as a stability indicating method. The corresponding LC method has some shortcomings with regard to separation of some close analogues [17]. An overview of chromatographic methods for metoprolol and related compounds is in the pipeline [18].

Clevidipine. SFC of this kind of compound is simple [5,19] though for the dosage content determinations of the active substance in an oil in water emulsion careful choice of modifier was required [19]. The SFC method has the advantage over LC methods that no work up procedure is required, and the system can accept aqueous samples. For the analysis of impurities in the bulk drug substance more care was needed in the method development [12]. The final method needed occasional treatment of the silica support with weak citric acid in order to obtain good efficiency of the acid formed upon hydrolysis. Of three investigated silicas from different vendors the selectivity to a symmetric

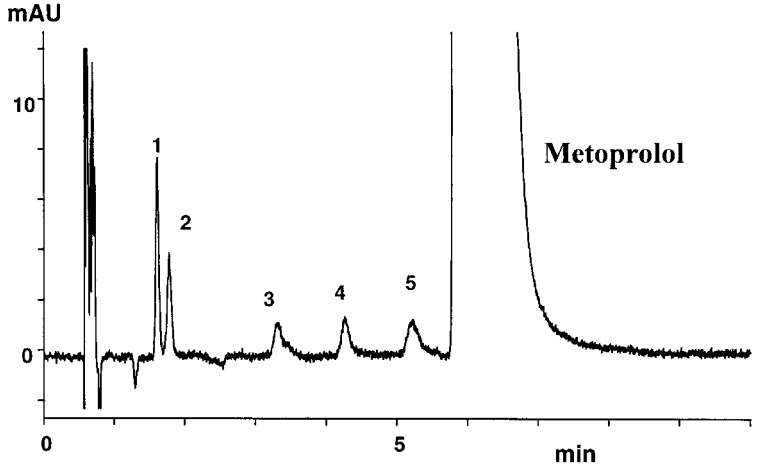


Figure 2.10.A.
Separation of metoprolol and some possible impurities. SFC conditions: 125 × 4 mm i.d. LiChrospher 100 diol at 40°C; 150 bar back pressure; 2.0 ml/min of carbon dioxide with 10% of methanol containing 0.35 M acetic acid and 0.07 M triethylamine; detection at 273 nm. Peaks **1**–**5**: see formulae (from Ref. [16])

ester was superior with Hypersil. The robustness test was performed using statistical experimental design and it was found to be acceptable except for the symmetric ester where no adequate baseline separation was achieved. In Fig. 2.10.B.a,b the comparison with the HPLC method is shown. Compared with HPLC the separation between the pyridine analogue (**1**) and clevidipine is superior, though the symmetric ester (**4**) is on the close side to clevidipine. As seen in the Fig. 2.10.B.b the pyridine analogue (**1**) and the decarboxylated acid (**2**) are not fully separated [20]. The sample throughput is about the same but in HPLC an isocratic system is used whereas in SFC a methanol gradient is performed. The total time in SFC can be significantly reduced if the system is returned to the start after the acid at about 12 min has been allowed to elute.

Isosorbide-5-mononitrate (*5-ISMN*). Method development was fairly simple since there are no functional groups in the molecule that require special attention. The selectivity on three different supports is illustrated by the relative retention data given in Table 2.10.A. The best spread of the analytes was observed with porous graphitic carbon as support. At 40°C 5-ISMN and isomannide mononitrate co-eluted on both Hypersil and diol silica. They were separated after increasing the column temperature (Table 2.10.A).

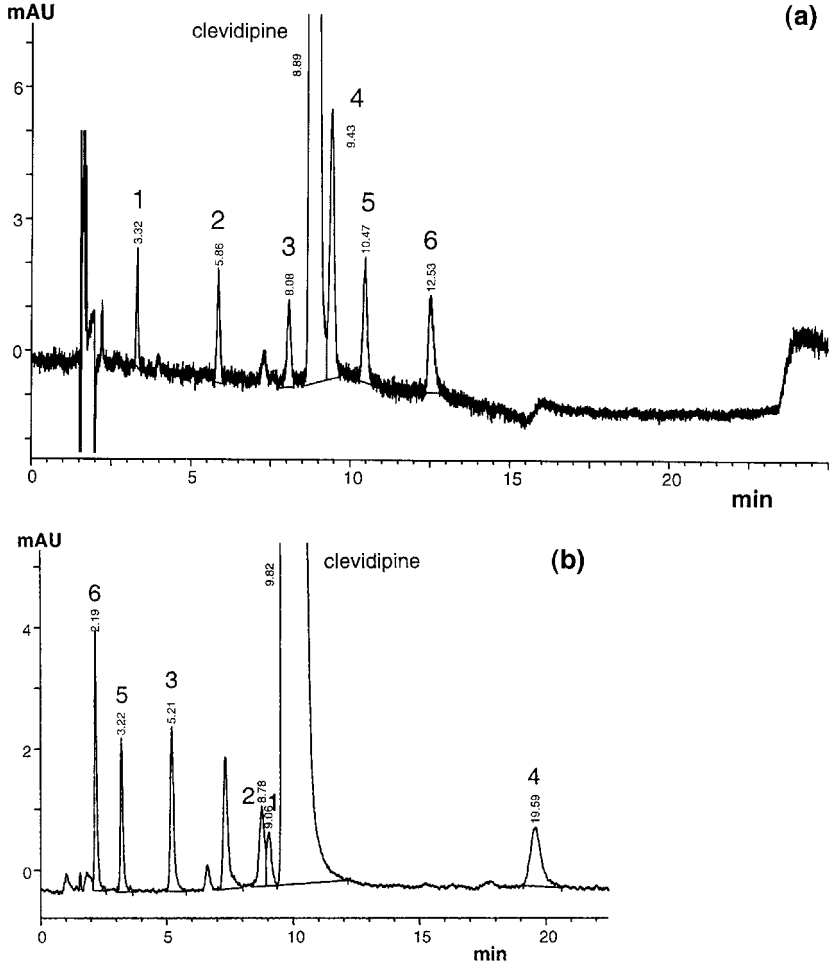


Figure 2.10.B.
SFC (a) and HPLC (b) of clevidipine spiked with its impurities. (**1**) Pyridine derivative; (**2**) decarboxylated acid; (**3**) symmetric methyl ester; (**4**) symmetric ester; (**5**) nitrile; (**6**) acid monomethyl ester. For the formulae see Fig. 2.7.4.C. Detection at 240 nm. SFC conditions: 200 × 4.6 mm i.d. Hypersil column at 50°C and 150 bar back pressure; 1.5 ml/min of carbon dioxide containing 5% of methanol, gradient 0.6%/min to 10% followed by 2%/min to 20%. HPLC conditions: 150 × 3.9 mm i.d. Nova Pak C_{18} with 1.0 ml/min of buffer pH 3.1 as eluent containing 20% v/v of methanol and 40% v/v of acetonitrile [20]

Table 2.10.A.
Relative retention of three possible impurities in 5-ISMN on three different column supports. Conditions: mobile phase carbon dioxide with 6% of methanol and back pressure 150 bar

Column	Isosorbide-2,5-dinitrate	Isosorbide-2-mononitrate	Isomannide mononitrate	Isosorbide-5-mono-nitrate (5-ISMN)	Retention of 5-ISMN (min)	t (°C)	Flow rate (ml/min)
Hypersil (200 × 4.6 mm i.d.)	0.41	0.55	0.93	1.00	7.81	45	1.0
LiChrosorb RP-8 (125 × 4 mm i.d.)	0.41	0.56	0.90	1.00	4.14	50	1.0
Hypercarb (100 × 4.6 m i.d.)	0.83	0.64	0.70	1.00	2.94	40	2.0

Since inorganic nitrate is a possible degradation product, its simultaneous separation and detection is important. This was achieved by including a quaternary ammonium hydrogensulphate salt into the methanol modifier. The shortest retention, but the longest equilibration time, was found with tetramethylammonium hydrogensulphate. The final conditions gave a chromatogram with complete separation within 12 min of the actual possible impurities being added to the 0.1% w/w level of 5-ISMN bulk substance (Fig. 2.10.C).

Salbutamol and six possible impurities and degradates in tablets were separated using a diol column [21]. Back pressure and modifier additives, aliphatic amines, had minor influence on the selectivity whereas the column temperature was adjusted to 70°C in order to optimise the system.

Paclitaxel and 16 possible impurities were separated within 35 min on a diol column using a methanol gradient and UV-detection [22].

Zinc phtalocyanine and two impurities, phthalamide and phthalic acid mononitrile monoamide, below 0.5% were separated using both LC and SFC. In SFC the elution order was reversed and the peaks better separated from the parent compound and furthermore, within a much shorter time as compared with the LC system [23].

Lovastatin and two degradates (dehydrolovastatin and hydroxy acid

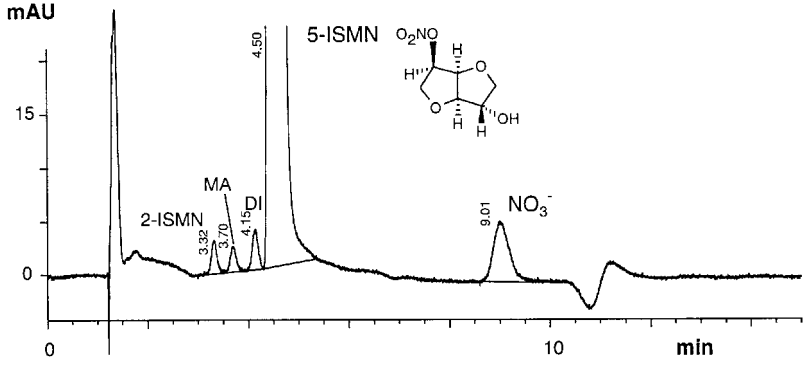


Figure 2.10.C.
SFC of isosorbide-5-mononitrate (5-ISMN) spiked with 0.1% of possible impurities. Conditions: 100 × 4.6 mm i.d. Hypercarb at 40°C; 1.0 ml of CO_2 containing 14% of 5 mM of tetramethylammonium hydrogen sulphate in methanol; back pressure 100 bar; UV-detection at 214 nm. Peak identification: 2-ISMN = isosorbide-2-mononitrate, MA = isomannide mononitrate and DI = isosorbide-2,5-dinitrate

lovastatin) were separated and quantificated within 5 min on a Hypersil silica column using methanol modifier and UV detection [24].

The most important choices in the *development of a method* for the purity evaluation of a drug substance is the selection of a suitable column support and appropriate column temperature. As shown above, the support has a marked effect on the separation of the parent compound versus possible impurities and degradation products. The temperature has also a great influence and can be used for fine tuning of the selectivity. The modifier used in most examples is methanol, which has strong solvating properties, and can be used to regulate the retention. Acetonitrile is rarely used as a modifier in SFC. Minor changes in the selectivity are obtained by altering the back pressure and the additive to the modifier. If resolution is still not sufficient, the efficiency can be increased by coupling of columns in series [13]. The time of analysis can be kept at bay by increasing the flow rate of the system.

2.10.4. SFC/MS using Packed Columns

Through the years mass spectrometers have been hooked up to the capillary and packed column systems used. Few presented papers describe the identification of unknowns at low levels that pose a challenge to the analytical chemist. Good up to date reviews have been published [8,9,25]. It is of great importance to have the possibility to use on-line techniques when new separation techniques are introduced.

A fraction from the column was directly introduced to the ion source [26,27] and an unknown impurity in *clevidipine* present in an early batch at the 0.15% by area level, as determined by UV-detection, was separated and identified. A three-way valve was inserted in the line to the ion source in order to prevent excess of the parent drug clevidipine contaminating the MS instrument [28]. The reconstructed ion chromatogram is shown in Fig. 2.10.D.a with an indication when the valve to the ion source was shut off. The recorded spectrum of the small peak gave the $(M + 1)^+$ ion at 730 which aided in the elucidation of the structure (Fig. 2.10.D.b).

N-methylated *metoprolol* formed by methylation of metoprolol with methyl iodide (see below) was verified by SFC-MS at a low level by a similar approach as in Section 4.2.1 but in this case the noise in the ion source from the amine additive made it necessary to cramp the ordinary 50-μm silica outlet in the ion source to about 20 μm. This system was more sensitive to the temperature of the vaporiser heater.

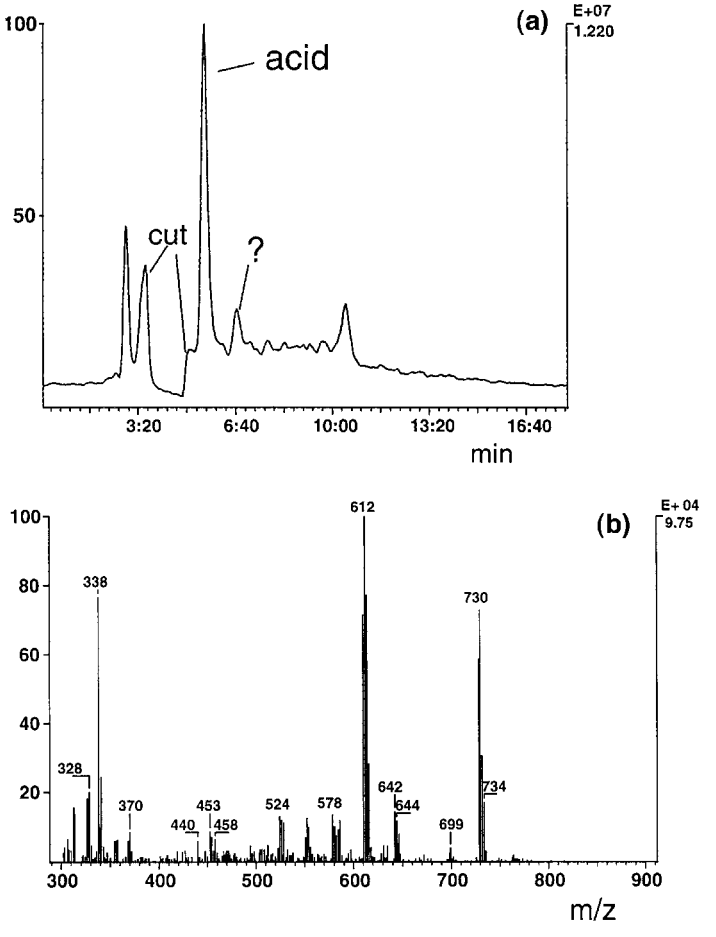


Figure 2.10.D.
Packed column SFC-MS of clevidipine bulk substance using a valve to protect the ion source. (a) Reconstructed ion chromatogram, ions collected from 300–800 (m/z). Conditions: Finnigan TSQ 700 with APCI ion source at 400°C and vaporiser at 200°C, Hypersil column 200 × 4.6 mm i.d. at 50°C, 1.3 ml/min of CO_2 and 0.2 ml/min of methanol (LKB-pumps), Jasco back pressure regulator at 150 bar downstream of the T-coupling, third leg via a three-way valve (Valco) and 50 μm silica into tip of the ion source. The acid is peak **6** in Fig. 2.10.B.a. (b) Spectrum of the unknown from the previous RIC as indicated by ?

2.10.5. Other Detectors used for the Structural Elucidation of Unknowns in SFC

Both NMR and FTIR have been used in combination with SFC. The former requires a specially devised high pressure cell that has a relatively large volume (150 μl). Both continuous detection and stop-flow mode can be used. A drawback with NMR detection for structural elucidation is that the use of deuterated modifiers in carbon dioxide is expensive. An alternative would be to use relatively cheap D_2O [29]. For a more recent review of SFC combined with NMR the paper by Albert is recommended [30]. SFC-FTIR has also been shown to be a powerful tool for obtaining structural information [31]. For on-line detection the fluid must be sufficiently transparent. This problem can be solved by spraying the outlet onto a plate. Later the trace with the deposed substance is scanned by the IR detector [32].

2.10.6. Analysis of Enantiomeric Purity

Many fast and exciting separations of enantiomers have appeared in the literature using normal phase chiral stationary phases [8,9,33,34]. Some authors have presented low level determinations of the enantiomeric impurity in the antipode [35–39] but in most papers the chromatograms show the separation of racemic mixtures.

In our laboratories we have used SFC for the determination of the minor enantiomer of *metoprolol* on Chiralcel OD [37], *clevidipine* and its corresponding acid on Chiralcel OD + Chiralpak AD [38]. A sulfoxide was resolved, and the minor antipode could be determined below 0.1% of the major one, within 5 min on a Whelk O I column [39].

An interesting and different way to separate enantiomers is to use a *chiral selector added to the mobile phase modifier* that can form diastereoisomeric complexes [40,41]. This approach can be cheaper and more flexible than the use of enantioselective columns. As shown below the consumption of the selector can be very low. For example, clevidipine and the corresponding acid formed upon hydrolysis can be chromatographed on porous graphitic carbon with methanol as a modifier and Tween 60 as a deactivating additive. Addition of 0.2 mM of *L-N-benzoyloxyarginine* as the chiral selector to the mobile phase gave a selectivity factor ($\alpha_{R/S}$) higher than 1.2. Fig. 2.10.E shows the chromatogram of the *R*-form of an ethyl-homologue of this acid. The area of the small peak of the *S*-form was below 0.1% of the *S*-form and the analysis time was less than 20 min.

Another example is the chiral chromatography of metoprolol and analogues on a Hypercarb column in the presence of a high concentration of amine

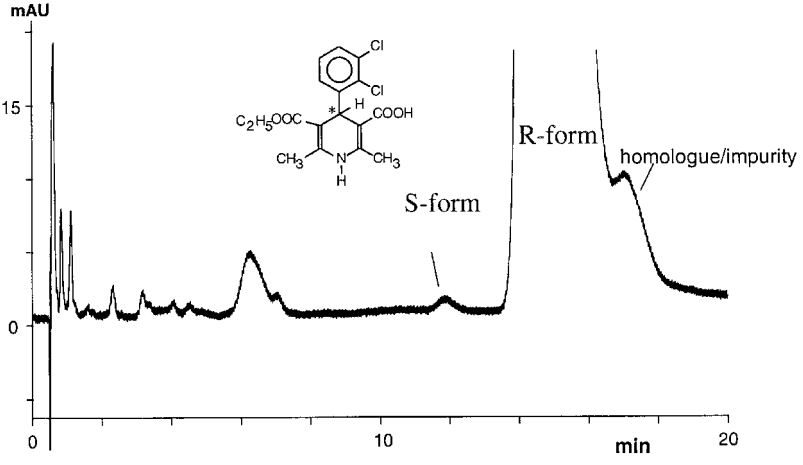


Figure 2.10.E.
Enantioseparation of a dihydropyridine substituted acid using L-N-benzoyloxyarginine as chiral selector. SFC conditions: Hypercarb column 100 × 3 mm i.d. at 70°C; 1.0 ml/min of carbon dioxide with 20% methanol containing 100 μg/ml of Tween 60 and 1 mM of L-N-benzoyloxyarginine; 150 bar back pressure; UV-detection at 240 nm

additive to the polar modifier, e.g. 0.24 M of DMOA in methanol. By adding 0.5 mM of *L-(+)-tartaric acid* as the chiral selector to the mobile phase nearly baseline separation of racemic N-methyl metoprolol ($\alpha \sim 1.12$) was obtained, whereas secondary amines such as metoprolol gave α of only 1.02. This observation was the basis of the separation of the enantiomeric impurity in *S*-metoprolol after N-methylation with methyl iodide, within 10 min (Fig. 2.10.F). The area ratio found and estimation of *R*-metoprolol was then confirmed after analysis on Chiralcel OD.

2.10.7. Advantages and Limitations of SFC in the Purity Analysis of Drugs

A wealth of methods exist for the separation of drugs and related substances by packed column SFC. Basically normal phase-like separations are obtained though many polar solutes can also be analysed [7]. Few papers, however, show the practical utility of the methods for the determination of impurities at real low levels that can be encountered and expected in drug bulk substance as are required by regulations set by the authorities. The reason may in part be due to the fact that such studies are seldom pursued in academia, and many compa-

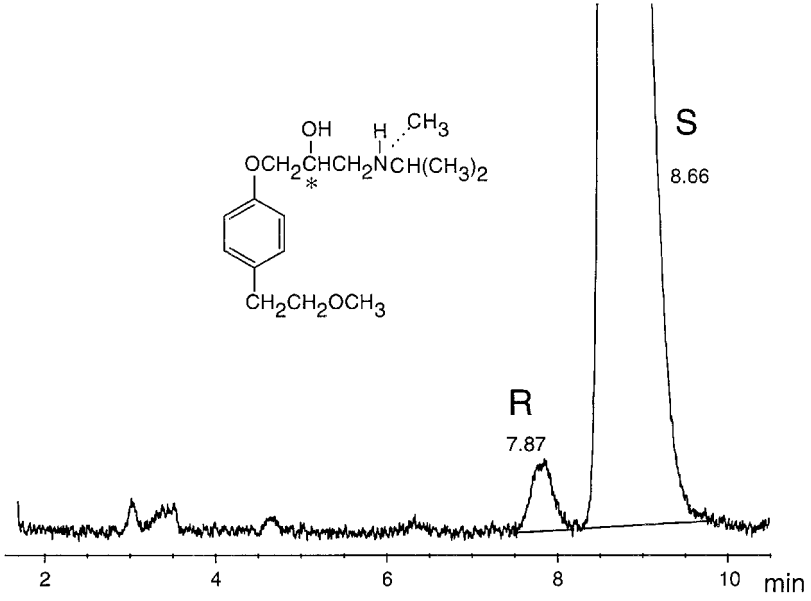


Figure 2.10.F.
Enantiomeric purity of *S*-metoprolol after N-methylation using L-(+)-tartaric acid as selector. Conditions: Hypercarb column 100 × 3 mm i.d. at 30°C; 1.0 ml/min of carbon dioxide with 10% of methanol containing 5 mM of L-(+)-tartaric acid; back pressure 150 bar and UV-detection at 273 nm

nies are reluctant to publish such methods on more interesting candidate drugs. Those examples presented above, however, show the great potential for the use of packed column SFC for practical problems. Some limitations are set by the higher baseline noise encountered for the UV-detector.

A field where SFC is gaining wider acceptance is in the fast determination of enantiomeric purity of drugs as well as in their preparative isolation. Laboratories have set up routine screening procedures for the selection of suitable conditions using packed column SFC.

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References

1. M.L. Lee and K.E. Markides, *Analytical Supercritical Fluid Chromatography and Extraction*, Chromatography Conferences, Utah (1990)
2. O. Gyllenhaal and J. Vessman, *J. Chromatogr.* **516**, 415–426 (1990)
3. J.T.B. Strode, L.T. Taylor, K. Anton, M. Bach and N. Periclès, in *Supercritcal Fluid Chromatography with Packed Columns* (K. Anton and C. Berger, Eds.), pp 97–123. Marcel Dekker, New York (1997)
4. R.M. Smith, O. Chienthavorn, N. Danks and I.D. Wilson, *J. Chromatogr.* **798**, 203–206 (1998)
5. J.T.B. Strode, L.T. Taylor, A.L. Howard, D. Ip and M.A. Brooks, *J. Pharm. Biomed. Anal.* **12**, 1003–1014 (1994)
6. A. Salvador, A. Jaime, G. Becerra and M. De La Guardia, *Fresenius J. Anal. Chem.* **356**, 109–122 (1996)
7. T.A. Berger, *J. Chromatogr.* **785**, 3–33 (1997)
8. T.L. Chester, J.D. Pinkston and D.E. Raynie, *Anal. Chem.* **68**, 487R–514R (1996)
9. T.L. Chester, J.D. Pinkston and D.E. Raynie, *Anal. Chem.* **70**, 301R–319R (1998)
10. T.A. Berger and W.H. Wilson, *J. Pharm. Sci.* **83**, 281–286 (1994)
11. M.T. Combs, M. Ashraf-Khorassani and L.T. Taylor, *J. Chromatogr. Sci.* **35**, 176–180 (1997)
12. O. Gyllenhaal, A. Karlsson and J. Vessman, *J. Chromatogr.* **862**, 95–104 (1999)
13. T.A. Berger and W.H. Wilson, *Anal. Chem.* **65**, 1451–1455 (1993)
14. O. Gyllenhaal and J. Vessman, *J. Chromatogr.* **628**, 275–281 (1993)
15. J. Vessman, *Pharmaceutical Analysis Overview, Encyclopedia of Analytical Sciences*, pp 3798–3808. Academic Press, London (1995)
16. O. Gyllenhaal and J. Vessman, *J. Chromatogr.* **839**, 141–148 (1999)
17. M. Erickson, K.-E. Karlsson, B. Lamm, S. Larsson, L.A. Svensson and J. Vessman, *J. Pharm. Biomed. Anal.* **13**, 567–574 (1995)
18. J. Vessman, O. Gyllenhaal, A. Karlsson, S. Larsson, L. Tollsten and S. Wendsjö, to be published
19. L. Karlsson, O. Gyllenhaal, A. Karlsson and J. Gottfries, *J. Chromatogr.* **749**, 193–199 (1996)
20. L. Tekenbergs-Hjelte, unpublished results
21. J.L. Bernal, M.J. del Nozal, J.M. Rivera, M.L. Serna and L. Toribio, *Chromatographia* **42**, 89–94 (1996)
22. N.K. Jagota, J.B. Nair, R. Frazer, M. Klee and M.Z. Wang, *J. Chromatogr.* **721**, 315–322 (1996)
23. K. Anton, M. Bach, C. Berger, F. Walch, G. Jaccard and Y. Carlier, *J. Chromatogr. Sci.* **32**, 430–438 (1994)

24. J.T.B. Strode, L.T. Taylor, A.L. Howard and D. Ip, *J. Pharm. Biomed. Anal.* **20**, 137–143 (1999)
25. T.R. Baker and J.D. Pinkston, *J. Am. Soc. Mass Spectrom.* **9**(5), 498–509 (1998)
26. D.G. Morgan, K.L. Harbol and N.P. Kitrinos, *J. Chromatogr.* **800**, 39–49 (1998)
27. E. Moyano, M. McCullagh, M.T. Galceran and D.E. Games, *J. Chromatogr.* **777**, 167–176 (1997)
28. O. Gyllenhaal and K.-E. Karlsson, *Abstract from the 8th Int. Symp. on SFC and SFE*, St Louis, MO, July (1998)
29. R.M. Smith and O. Chienthavorn, *Abstract from the 8th Int. Symp. on SFC and SFE*, St Louis, MO, July (1998)
30. K. Albert, *J. Chromatogr.* **785**, 65–83 (1997)
31. J. Yang and P.R. Griffiths, *J. Chromatogr.* **785**, 111–119 (1997)
32. S. Bourne and T.A. Berger, *Pittcon '97*, Atlanta, GA (1997)
33. R.M. Smith, in *Supercritcal Fluid Chromatography with Packed Columns* (K. Anton and C. Berger, Eds.), pp 223–249. Marcel Dekker, New York (1997)
34. K. Williams Phinney and L.C. Sander, *J. Chromatogr.* **785**, 149–158 (1997)
35. P. Biermanns, C. Miller, V. Lyon and W.H. Wilson, *LC-GC Int.* **11**, 744–747 (1993)
36. A. Medvedovici, P. Sandra, L. Toribio and F. David, *J. Chromatogr.* **785**, 159–171 (1997)
37. S. Svensson, A. Karlsson, O. Gyllenhaal and J. Vessman, *Chromatographia* in press (2000)
38. K. Öhlén, O. Gyllenhaal and A. Karlsson, *Abstract from the 7th Int. Symp. on SFC and SFE*, Indianapolis, March/April (1996)
39. A. Nyström and K. Öhlén, unpublished results
40. C. Pettersson and G. Schill, *J. Chromatogr.* **204**, 179–183 (1981)
41. A. Karlsson and C. Pettersson, *Chirality* **4**, 323–332 (1992)

2.11. Purity Check by Differential Scanning Calorimetry

Imre Péter

In the heroic period of pharmaceutical analysis, before the introduction of selective, sensitive and easily performable chromatographic techniques one of the most widely used methods for the purity check of crystalline bulk drug materials was the estimation of their melting point (i.e. melting point depression in comparison with a standard reference material) and the length of the melting range expressed in degrees Celsius. Although no quantitative data are obtainable from these data regarding the purity of the drug material, these are criteria for the acceptance of the purity even in the last editions of pharmacopoeias. In the 1970s and 1980s, due to the development of thermoanalytical techniques, especially *differential scanning calorimetry (DSC)* purity analysis based on the melting process came into the limelight. Although this technique affords quantitative data, in recent years this interest shows decreasing tendency due to the serious limitations in its application. In spite of these limitations, under certain conditions the reasons for its existence in drug analysis are not questionable. This is also supported by the fact that the method is included in the United States Pharmacopoeia [1]. The DSC purity method can be used typically in the case of high purity (>99%) crystalline substances.

This purity analysis is based on the phenomenon that in eutectic systems the melting points of crystalline substances decrease under the influence of the chemical impurity. The thermodynamic relationships are described by the simplified van't Hoff law.

$$T_m = T_0 - \frac{RT_0^2}{\Delta H_0} x_2$$

where T_0 is the melting point of the pure substance, T_m is the sample temperature at equilibrium, x^2 is the mole fraction of impurity in the liquid phase, ΔH_0 is the heat of fusion of pure substance and R is the gas constant.

In the presence of chemical impurity not only the melting point decreases but the shape of the melting curve also changes, it broadens as a function of the amount of the impurity. The amount of the impurities can be calculated from the shape of the curve after certain thermodynamic considerations and with certain arithmetical simplifications of the van't Hoff law [2] (Fig. 2.11.A).

Advantages of the method are the small sample size needed for the measurement and the short measuring time; as well as that it needs no reference substance. However, it has the disadvantage that only the total amount of the impurities can be calculated, and not the individual values.

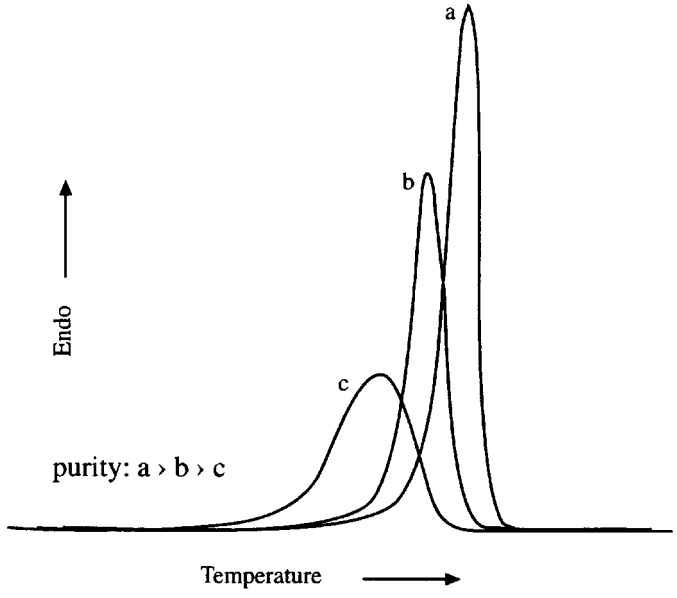


Figure 2.11.A.
The effect of impurity on the shape of the DSC melting curve and melting temperature of cimetidine

Furthermore the law forming the basis of the method is valid only if numerous conditions are fulfilled. These conditions greatly restrict the applicability of the method. Without aiming at completeness, the most important ones are the following [3].

(a) The components should form an eutectic system and the impurities should form an ideal solution in the melt with the main component. The impurity must not form a solid solution. In fact the drugs may contain several impurities and in the majority of cases at least some of them are not identified. In practice, it is impossible to test the eutectic-forming ability of each member of this multicomponent system and to determine the physical–chemical relationship with the main component.
(b) The melting should be accomplished under equilibrium conditions. With a low heating rate (0.2–1°C/min) and small sample mass (1–2 mg), as well as with good thermal contact between the sample and the pan this can be approached to a certain degree.
(c) The heat of fusion of the main component should be constant over the temperature range and independent of the character and amount of the impu-

rities. This can only be an approximation, the deviation from it is most significant in the initial range of the melting.
(d) Evaporation, sublimation, decomposition, recrystallation either of the main component or of the impurities must not occur during the melting.

The aforementioned factors cause a significant uncertainty in the reliability of the results, therefore the results have to be treated with the proper caution. Similarly, the assignment of the baseline also causes difficulty. The initial range of the melting process cannot be evaluated due to the limited sensitivity of the instrument, thus a part of the melting enthalpy is lost. Various mathematical procedures are employed for taking this loss into consideration [3].

The reliability of the DSC purity analysis can be enhanced with the application of the stepwise technique [4]. With its application the measurement can be performed under nearly equilibrium conditions, but the time of the analysis significantly increases. In practice it is not a wide-spread method. Besides the applied measuring conditions (heating rate, sample pan, gas atmosphere) and the calculation method, characteristics of the sample (particle size, sample mass, sample packing) can also influence the results [2,5,6].

Due to the above factors, validation of the method – satisfying the up-to-date requirements – is either impossible or can be performed with great difficulties and is very time-consuming [7]. Therefore, the application of the method has been recently restricted. However, in certain fields of application and under well-defined measuring conditions, the method can still play a fairly significant role.

The impurity profile of samples of various origin can be different. In this case the effect of various impurities on the melting curve can also be different. For this reason in such cases it is difficult to compare the results of the DSC purity analysis. On the contrary, impurity profiles of samples under identical and controlled production are generally identical. In such systems, with cautious handling of the concept of absolute purity, the measured purity data are comparable with one another. Similarly, the method can be suitably applied for controlling the efficiency of the purifying procedures, provided that they always result in totally crystalline substances. In some cases the stability of drugs can also be well monitored during the storage [7]. However, in both latter cases the impurities and degradation products should meet the aforementioned requirements.

In spite of the mentioned difficulties several publications can be found in the chemical literature on the DSC purity analysis of pharmaceutical compounds. A great variety of the compounds can be found among them, independently of their structure. According to comparative tests there is a relatively good correlation with the results obtained with other analytical meth-

ods in the cases of steroids, e.g. *norethisterone, mestranol, ethinyl estradiol* [8], *testosterone*, *testosterone phenylpropionate, testosterone decanoate* [9], *estradiol benzoate* and *phenylpropionate* [10]. While among sulphonamides *sulphathiazole*, *sulphadiazine* or *sulphadimethoxazole* cannot be investigated due to their thermal degradation, the thermal stability of *sulphadimidine* or *sulphamerazine* is suitable thus enabling the DSC method to be applied [11]. In the domain of barbiturates reports can be found on the purity analysis of *barbital* and *allobarbital* [12]. Though polymorphism is generally an undesirable phenomenon, the enantiotropic polymorphic transformation (see Chapter 7.3) of *oxybuprocaine* has no effect on the purity analysis [7].

In the case of certain salt compounds the excess base or acid having a eutectic behaviour can also cause a melting point depression, making possible their determination as impurities [7]. If the additives do not influence the determination, the purity analysis of the active substance can also be performed from the tablets [13].

DSC purity analysis can also be applied for determination of enantiomeric or optical impurity, in cases of both conglomerates and true racemates [14]. However, other chemical impurities are also included in the result.

References

1. *The United States Pharmacopoeia 24*, pp 1999–2000. USP Convention Inc., Rockville (2000)
2. A.A. van Dooren and B.W. Müller, *Int. J. Pharm.* **20**, 217–233 (1984)
3. J.L. Ford and P. Timmins, *Pharmaceutical Thermal Analysis, Techniques and Applications*, pp 108–135. Ellis Horwood Ltd., Chichester (1989)
4. J.P. Elder, in *Purity Determinations by Thermal Methods* (R.L. Blaine and C.K. Schoff, Eds), pp 50–60. ASTM, Philadelphia, PA (1984)
5. A.A. van Dooren and B.W. Müller, *Thermochim. Acta* **66**, 161–186 (1983)
6. J.E. Hunter and R.L. Blaine, in *Purity Determinations by Thermal Methods* (R.L. Blaine and C.K. Schoff, Eds.), pp 29–38. ASTM, Philadelphia, PA (1984)
7. D. Giron and C. Goldbronn, *J. Therm. Anal.* **44**, 217–251 (1995)
8. O.B. Lim, E. Oliver and A. Oliver, in *Purity Determinations by Thermal Methods* (R.L. Blaine and C.K. Schoff, Eds.), pp 39–49. ASTM, Philadelphia, PA (1984)
9. A. Chauvet, R. Pepper and J. Masse, *Thermochim. Acta* **43**, 161–172 (1981)
10. G. De Maury, A. Chauvet and J. Masse, *Thermochim. Acta* **87**, 189–202 (1985)
11. F.I. Khattab, *Thermochim. Acta* **61**, 253–268 (1983)

12. A. Chauvet, G. De Maury, A. Terol and J. Masse, *Thermochim. Acta* **97**, 143–159 (1986)
13. D. Giron and C. Goldbronn, *J. Therm. Anal*. **48**, 473–483 (1997)
14. J. Jacques, A. Collet and S.H. Wilen, *Enantiomers, Racemates and Resolutions*, pp 151–159. Wiley, New York (1981)

Chapter 3

IDENTIFICATION AND DETERMINATION OF RESIDUAL SOLVENTS

3.1. Thermoanalytical Methods

Imre Péter

Various solvents are applied during the preparation and purification of bulk drugs. After the separation from the solvent(s) drug substances – whatever technology is used – always contain more or less of the solvent of the crystallisation and frequently also solvents of the last steps of the synthesis. Generally, the residual solvents are undesirable in the sample, their permitted quantity is usually limited, thus their identification and determination are very important tasks [1]. The general aspects and the origin of residual solvents in bulk drugs and formulations are discussed in Section 1.2.2. In many cases compounds incorporate molecules of solvents stoichiometrically in the crystal lattice, e.g. hydrates or solvates. These are often not identified since the synthetic chemist removes the solvents with drying, before the analyst can examine the crystal structure, or another part may decompose even at room temperature. A part of the solvent bound in this way can be left behind after drying, also. It is a frequent phenomenon that a part of the mother liquor remains entrapped; among the crystals grown together entrapped mother liquor can nearly always be found.

The amount of any volatile substance that is removable under the conditions prescribed by Pharmacopoeias can be determined with the conventional procedure of *loss on drying*, performed under isothermal conditions to a constant mass or for prescribed time. For determination of moisture or other volatile component(s) the isothermal loss on drying method is frequently replaced by *thermogravimetry* (TG) [2]. The concept of this method is the same as that of determination of loss on drying: under the influence of heating the volatile components evaporate and the loss of mass can be measured. In TG the substance is subjected to a controlled temperature program and its mass is determined as a function of temperature or time. Water or other solvents being

in free or bound form, generally evaporate in a certain temperature range which is characteristic of the interaction with the drug substance, similar to the retention time in chromatography. However, thermogravimetry does not supply any information on the quality of the volatile components removed. A different situation is encountered in the case of solvents physically entrapped into crystals, which can leave at the melting point only, at the physical disintegration of the crystal, occasionally at a very high temperature. Entrapped solvent can be determined by thermogravimetry even if decomposition or evaporation processes take place simultaneously with melting.

In the case of overlapping processes the determination of the individual components is accompanied by various errors. However, resolving can be improved with choosing optimal measuring conditions: decreasing the heating rate and the size of the sample, or applying a gas with a great thermal conductivity (e.g. helium) the resolution increases. The shape of the TG curve can also be influenced by the pressure and flow rate of the gas atmosphere, geometry of the sample holder, etc. Characteristics of the sample (sample mass, sample packing, particle size, etc.) can be extremely important having a significant effect on the transport of the volatile components within the sample.

Advantages of the moisture determination with thermogravimetry are the relative quickness, simplicity of the measurement and – owing to the efficiency of the modern thermobalances – the low demand on sample mass. However, due to inhomogeneities the very small sample may not represent exactly the substance to be measured. It is also a disadvantage that due to the low mass of sample the residual solvent of some tenths of a percent corresponds to only a few micrograms that can be measured with a great relative error only as a consequence of the limited sensitivity (0.1–1 μg) and capacity of the applied commercial thermobalances. In each case the performance characteristics of the balance and the expected change in mass should be taken into consideration. The buoyancy effects cause an unavoidable error. If the furnace is heated up, the density of the gas medium inside the furnace decreases. For this reason, the upthrust also decreases which causes an apparent gain in measured mass. Changes in volume of sample during the heating also cause virtual weight changes. Using small sample size and holders, this distortion caused by the buoyancy effects mentioned can be diminished. Similarly, the influence of the purge gas cannot be ignored. In a modern thermobalance the shape of the thermogravimetric curve is reproducible, although dependent on the heating rate, the type of purge gas and its flow rate, the size of the sample holder and the amount of the sample. The buoyancy effects can be compensated by recording a blank curve with the specified measuring conditions and performing a correction with it. Application of a symmetrical thermobalance can also provide a compensation for the above source of error. Figure 3.1.A clearly shows the error caused by the buoyancy and the convection. Using too small an amount of

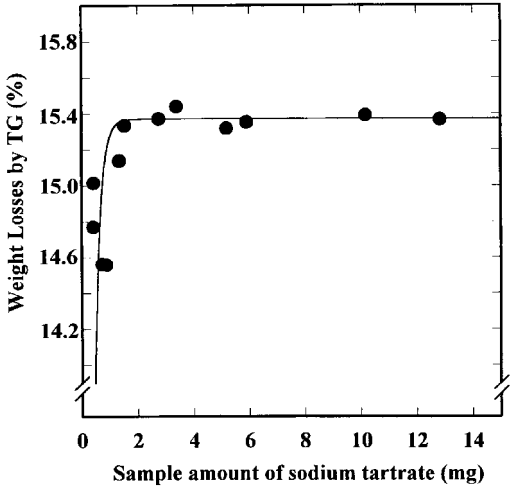


Figure 3.1.A.
Weight losses of sodium tartrate as a function of sample amount measured with thermogravimetry (from Ref. [3])

sample the weight loss – corrected with a blank curve – is also burdened with a serious error, even in the case of high weight change.

Table 3.1.A shows the typical relative standard deviations (RSD) of thermogravimetric methods for moisture content determination, in the case of a monohydrate, a substance containing entrapped solvent not detected with the isothermal loss on drying method and a tablet containing hygroscopic ingredients. These data probably reflect the inhomogeneity of moisture content of the samples, besides the deviation of the method.

When conventional isothermal loss on drying method is applied, the measured decrease in mass may be greater than the real value due to thermal decomposition or sublimation. For example, in the case of *malonic acid* the thermogravimetric moisture measurement (water and other solvent) displays $<0.1\%$, while with the loss on drying method, performed under isothermal conditions at too high a temperature, results of 0.1–0.5% were found [4]. On the other hand, a temperature which is not sufficiently high can also cause an error. Drying of *lysozyme* for 4 h at 60°C gives a lower result than the thermogravimetric determination, due to the low temperature and the short drying period. A similar result is obtained in the case of *deslanoside*, also, if the test is performed according to the Japanese Pharmacopoeia [3].

In some cases the pharmacopoeias also prescribe thermogravimetry for

Table 3.1.A.
Examples of relative standard deviation (RSD) in thermogravimetry using Perkin-Elmer TGS-2 thermobalance (from Ref. [4])

Type	Mean of moisture content (%)	RSD (%)
Monohydrate	3.81 (n = 7)	2.0
Entrapped solvent	0.54 (n = 9)	15
Tablet	4.48 (n = 6)	1.9

the determination of loss on drying. It is applied by the USP 24 [5] for *vincristine sulphate*, *vinblastine sulphate* (heating rate 5°C/min, sample mass 10 mg, nitrogen atmosphere) and *bromocriptine mesylate* (heating rate 10°C/min, sample mass 10 mg, nitrogen atmosphere), as well as by Ph. Eur. [6] for some other biological products: *meningococcal polysaccharide*, *typhoid polysaccharide* and *haemophilus type B conjugate vaccina*, to determine the volatile components and the water content, although the exact measuring conditions are not given for the latter ones.

Resolution of overlapping processes can significantly be improved by decreasing the heating rate thus improving the determination of solvent content bound at different manner, too, although the time of analysis is increased significantly. Another way of amending the resolution is to control the rate of reaction by controlling the rate of mass change. If it exceeds a certain threshold, the heating rate is reduced and kept low until the mass becomes nearly constant. In Fig. 3.1.B the water loss of a hydrate drug candidate can be seen, with the conventional and the Hi-Res™ TGA [7] curves. While three dehydration steps can be observed in the conventional TG curve, then in the Hi-Res™ TGA curve the initial weight loss splits and the resolution of the further two-step dehydration taking place at higher temperature also improves (resolution index 5). However, evaluation of the curves should be performed very carefully, so that the better resolution should not yield artefacts.

Thermogravimetry itself does not supply information on the quality of volatile components or residual solvents. However, application of combined techniques renders their identification possible. For the analysis of the evolving gases (EGA) different, mostly spectroscopic methods, Fourier transform infrared spectroscopy (FTIR) or mass spectroscopy (MS) are used [9]. Although both TG/FTIR and TG/MS methods are applied firstly for the qualitative identification of the volatile components, their application for the quantitative determination is also possible with a suitable apparatus, under reproducible measuring conditions and with adequate calibration. Using coupled techniques

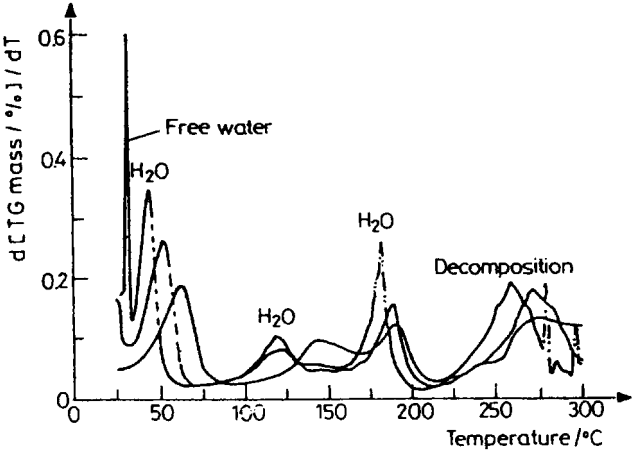


Figure 3.1.B.
DTG curves of Hi-Res™ and conventional thermogravimetry of a hydrated drug candidate. — conventional DTG; - - Hi-Res™ TG resolution index 4; –···– Hi-Res™ TG resolution index 5 (from Ref. [8])

time and effort can be saved. In spite of their advantages the application in the pharmaceutical industry is not wide-spread. In Fig. 3.1.C simultaneously recorded TG curve and the corresponding FT-IR spectra of the methanesulphonic acid salt of an active pharmaceutical ingredient can be seen versus temperature in a three-dimensional figure (wavenumber/absorbance/temperature). The substance was crystallised from butyl acetate. Butyl acetate bound as solvate is released between 60 and 100°C and identified with the aid of a vapour-phase IR spectrum library. However, up to 60°C water can also be detected as the presence of the OH deformation vibration of water molecules at 1600/cm shows.

For the quantitative determination of small amount of residual solvent with adequate precision mercury-cadmium telluride (MCT) detector of suitable sensitivity is needed [11]. Identification of the volatile components is assisted by the richness of details in the spectra, due to the recording being performed in gas phase. In Fig. 3.1.D the simultaneous TG and MS recordings of *haemophilus b polysaccharide* can be seen, made in selected ion monitoring (SIM) mode. Following the water evolution, the loss of weight occurring at 120°C can be explained with release of the residual ethanol, being there as an impurity. A further loss of water takes place together with thermal decomposition, indicated by evolving of carbon dioxide.

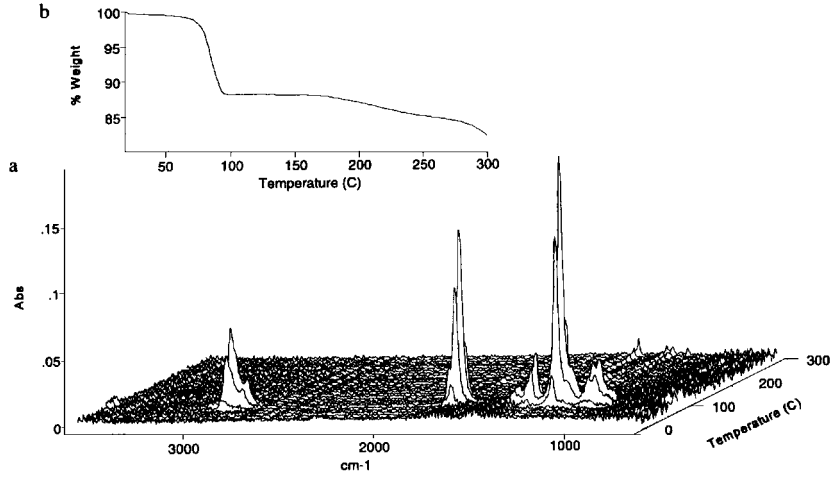


Figure 3.1.C.
Evolved gas profile (EGP) vs. temperature (a) and TG curve (b) for a drug candidate (from Ref. [10])

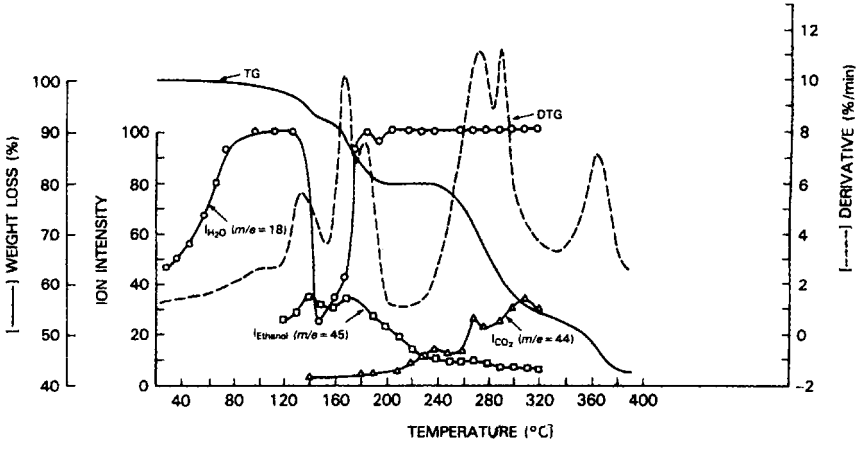


Figure 3.1.D.
TG, DTG and mass spectral ion intensities (I) for water (m/z = 18), CO_2 (m/z = 44) and ethanol (m/z = 45) vs. temperature for *haemophilus b* polysaccharide (from Ref. [12])

Due to its high sensitivity the mass spectrometer is suitable to detect traces of volatile components even in those cases when the thermobalance cannot detect any loss of weight.

In the thermogravimetric curve a group of atoms eliminated in intramolecular reaction due to heating can exhibit a step having a shape virtually identical to weight loss of water of crystallisation or other volatile components. An example for this phenomenon is the elimination of water by splitting off the tertiary hydroxyl group of *4-hydroxy-4-methyl-1-oxa-3,8-diazaspiro[4,5] decan-2-one derivatives* to form 4-methylene-1-oxa-3,8-diazaspiro[4,5]decan-2-ones [13]. Other examples are small peptides, e.g. *aspartame* which undergo solid state aminolysis to form cyclic 2,5-diketopiperazine derivatives; see Section 9.1.1. The weight loss of one mole of water can be observed on the TG curve [14].

The decomposition process is determined first of all by the chemical character of the compounds, thus the elimination of groups of atoms can be different. These processes cannot be elucidated merely by thermal analytical methods, they need chemical tests of the residue to be used.

References

1. ICH Q3C: Impurities, Guideline for Residual Solvents, *Pharmeuropa* **9,** 482–490 (1997)
2. J.G. Dunn and J.H. Sharp, in *Treatise on Analytical Chemistry* (J.D. Winefordner, Ed.), pp 127–266, Wiley, New York (1993)
3. H. Komatsu, K. Yoshii and S. Okada, *Chem. Pharm. Bull*. **42**, 1631–1635 (1994)
4. D. Giron-Forest, Ch. Goldbronn and P. Piechon, *J. Pharm. Biomed. Anal.* **7**, 1421–1433 (1989)
5. *The United States Pharmacopoeia 24*, U.S.P. Convention Inc., Rockville, (2000)
6. *European Pharmacopoeia, 3rd edn*, Council of Europe, Strasbourg (1997)
7. P.S. Gill, S.R. Sauerbrunn and B.S. Crowe, *J. Therm. Anal*. **38**, 255–266 (1992)
8. F. Barnes, M.J. Hardy and T.J. Lever, *J. Therm. Anal*. **40**, 499–509 (1993)
9. S.B. Warrington, in *Thermal Analysis – Techniques & Applications* (E.L. Charsley and S.B. Warrington, Eds.), Royal Society of Chemistry, Cambridge, pp 84–107 (1992)
10. C. Rodriguez and D.E. Bugay, *J. Pharm. Sci.* **86**, 263–266 (1997)
11. D.J. Johnson and A.C. Compton, *Spectroscopy* **3**, 47–50 (1988)
12. J.C. May, R.M. Wheeler and A. del Grosso, in *Compositional Analysis by Thermogravimetry*, *ASTM Special Technical Publication 997* (C.M. Earn-

est, Ed.), pp 48–55. American Society for Testing and Materials, Philadelphia, PA (1988)
13. E. Tóth, B. Kiss, A. Gere, E. Kárpáti and J. Törley, *Eur. J. Med. Chem.* **32**, 27–38 (1997)
14. S.S. Leung and D.J. Grant, *J. Pharm. Sci*. **86**, 64–71 (1997)

3.2. Gas Chromatography and GC/MS

Jürgen Bertram

3.2.1. Introduction

During the final production step solid drug substances are usually isolated by a crystallisation, spray drying or precipitation procedure which require the use of one or more solvents. Therefore residual solvents can be expected to represent a significant part of the impurity profile of solid drugs. Residual solvents can be a health hazard as well as they affect physicochemical properties like particle size, dissolution rate and stability. Physicochemical properties however, can be influenced by residual solvents in a positive way as residual levels of solvents provide better stability and solubility for some products. Thus it seems not generally justified to reduce residual solvents to the lowest possible level. Therefore, GMP rules require to reduce residual solvents to merely reasonable levels. Nevertheless, studies which deal with the optimisation of production processes to eliminate residual solvents [1,2] are useful. Determination of residual solvents e.g. through headspace-gas chromatography with mass spectrometric detection contributes to the characterisation of illicit drugs, supplying information on their source and other relevant forensic parameters [3]. Residual solvents can prove the correctness as well as the authenticity of a production process which means that a number of product characteristics can be indirectly certified and identified through analysis of residual solvents as their fingerprint. For example different lots of EHC, MPHC and carrageenes could be identified by their characteristic volatile components [4,5]. Only a few reviews have been published dealing with aspects like limit and determination of residual solvents in drugs. The most recent one was published by Witschi and Doelker [6]. Especially limits for toxic solvents have given rise to several industrial, pharmacopoeial and governmental activities in the field of foods and drugs. After many years of discussion an international agreement was arranged within the scope of the ''International Conference on Harmonisation'' (ICH) [7] which will hopefully be introduced in all countries which apply either the USP, the European or the Japanese Pharmacopoeia. The document established regulates the limits of toxic solvents on the basis of toxicological risks. The detailed description of this matter can be found in Section 1.7.4. Sometimes, however, the limits required do not satisfy other important aspects like reasonable quality accord-

ing to GMP rules. Because of the strong unpleasant smell of some solvents, especially of aldehydes and ketones, the consumers sensoric demand for good product quality must be taken into account. A general method for the analysis of residual solvents, particularly those covered by the ICH guideline, has been published in the European Pharmacopoeia, Addendum 1999.

Normally solvents are liquids which can be removed from the matrix by evaporation due to their volatile character. Therefore they can be considered as ideal candidates for GC analysis, which generally offers advantages over other analytical methods like HPLC as it can be easily optimised, provides highest separation ability and a linear range up to 10^7 units for detection. The most important advantage of GC is that it enables online isolation and enrichment of volatiles from a nonvolatile matrix through special modes of sample pretreatment.

3.2.2. General Points Valid for the GC Determination of Residual Solvents

Standard and test solutions should be prepared without using pipettes as some of the volatile analyte could be lost. Solvents should be weighed into the flasks, calculating the volumes from the known density. Alternatively Hamilton or other suitable gas-tight syringes should be used for sample dosing.

It is not good analytical practice to prepare aqueous solutions of solvents insoluble in water by shaking the solvent with water. High RSD and low recoveries can result if the test and reference solutions are prepared in such a manner even if excellent equipment is used [8].

For quantitative determination the amount of any added internal standard or of the volatile to be determined should be similar to the amount of the analyte present in the sample which must be determined separately if unknown.

3.2.3. Sample Pretreatment

Sample pretreatment prior to GC analysis spans from the simplest method of dissolving the sample in a suitable solvent to the large scale purge and membrane trap device coupled with the ion source of a mass spectrometer. Table 3.2.A gives an overview of the most important methods for sample pretreatments.

3.2.3.1. Direct Injection

The simplest method of analysing a sample by GC is to inject the sample dissolved in a suitable solvent with a boiling point high enough for the dissolu-

Table 3.2.A.
Advantages and drawbacks of the most important modes of sample pre-treatment

Pretreatment	Advantages	Disadvantages
Direct injection	Very simple and rapid method, requires less validation work	GC-system contamination and column deterioration; unavoidable matrix effects, lower sensitivity; relatively poor limit of quantification (LOQ), usually equal or higher than 0.1%
Static headspace	Very simple technique, if performed manually; much less contamination keeps system clean and extends column life; good precision and sensitivity down to lower ppm; minimised solvent consumption if any, therefore reduced toxic waste production; applicable to solid, insoluble and samples of quite variable composition like biological fluids (MHE); easy calibration by standard addition; reduced artefact formation, variety of techniques like direct desorption and FET which allows adaptation to the sample nature, the partition property of the residual solvent and the required LOD	Special vials and closures; time consuming, therefore automatic devices strongly recommended; much more validation work necessary to optimise equilibration time, temperature, volume of analyte solution, choice of solvent, apparative settings; contaminants can occur at higher temperature during prolonged time through deterioration of the sample and septum related impurities; practically limited to partition ratios between sample and gaseous phase with K-values in the range $<$10–100; danger of leakage especially if applying higher temperatures during long time, memory effects

Table 3.2.A (*continued*)

Pretreatment	Advantages	Disadvantages
Dynamic headspace	Highest sensitivity of all common pretreatment techniques; applicable for volatiles with high partition coefficient; no equilibrium required; sample volume not restricted; thermounstable samples can be analysed at lower temperature	Bake step required; backflush therefore recommended; trap breakthrough if unfavourable type and amount of trap material is used; possible accumulation of a lot of interfering substances from the matrix; (e.g. water is unfavourable for any GC analysis); memory effects if high amounts of volatiles are present in the sample

tion medium to elute later from the GC column than any of the volatiles to be determined. To the 1–5 percentage solution an internal standard for precise quantification should be added which is mandatory in case of split injection. A sample of 1–2 μl of this solution are then directly injected into the injection port of the GC, applying a split technique if capillary columns of smaller diameter are used. The direct injection offers advantages because of the simple sample preparation and the guarantee that all volatile components of the sample are carried to the GC column. For samples of unknown composition, however, this advantage is of minor importance with respect to the drawback that direct injection is unsuitable for screening procedures because unavoidable, variable matrix effects prevent the identification, detection or quantification of many volatiles present in the sample at a concentration lower than 0.1%. A precolumn must be installed to minimise contamination of the analytical column by nonvolatile matters. The injection port can be seriously contaminated by relatively high amounts of the sample which may penetrate slowly through the precolumn or deteriorates in the hot injector producing ghost peaks in the chromatogram. Frequent exchange of the glass liner and the precolumn can be necessary. Additionally to nonvolatile matter high boiling components of the sample migrate through the column requiring high temperature and a long time until they leave the column. Nevertheless, direct injection is frequently performed if known volatiles in nonproblematic samples have to be determined at a concentration around or higher than 0.1%. Some volatiles like toluene with a high FID response or halogen-containing solvents specifically detected by an ECD can be determined at ppm levels and lower. Large-

volume-injectors could improve the sensitivity in special cases allowing the injection of much higher sample amounts and preventing system contamination by backflush. However such injectors are not commonly used in laboratories equipped with GC and examples reported in the literature are mainly for environmental and food analysis [9,10].

Another important aspect to be taken into account, also for headspace techniques, is the right solvent selection for the preparation of the sample and reference solutions. Water free from organic components and therefore without any FID-response seems to be the ideal solvent for the analysis of residual solvents. On the other hand, water has some drawbacks as its high vapour volume can cause an overflow of the injector leading to memory effects and nonreproducible split ratios. The chromatographic separation of early eluting components, like many solvents, can be lost and sensitive column coatings can be destroyed. Therefore organic solvents should be preferred taking care that they are pure and well separated from any sample component to be determined. These requirements are best accomplished by organic solvents eluting later than the volatiles to be determined. For example, test solutions of pharmaceutical bulkware of different pH were prepared with benzyl alcohol and used to study the linearity and recovery of common process solvents [11]. Other suitable solvents are *N*,*N*-dimethylacetamide (b.p. 166°C, normally of high purity, may contain traces of dimethylformamide), dimethylsulphoxide (b.p. 189°C, contains considerable levels of dimethylsulfide and -disulfide as impurities), *N*,*N*-dimethylformamide (b.p. 153°C) and 1,3-dimethyl-2-imidazolidinone (b.p. 108°C at 2.9 Pa). Ethylene glycol (b.p. ~200°C), 2-ethoxyethanol (b.p. 135°C), acetic acid (b.p. 118°C), glycerol (b.p. 182°C at 3.3 Pa) and propylene carbonate (b.p. 240°C) were also mentioned as suitable solvents. It should be kept in mind, however, that some of these solvents may produce artefacts by reaction with the sample which leads to false positive results. A well known example is benzyl alcohol (b.p. 205°C) which can react with methansulfonates to benzene. Side reactions may be prevented in some cases by adjusting the pH of the solution, setting a lower injector temperature or changing the solvent. The residual solvents to be determined can deteriorate as well: ester generally are easily hydrolysed under unfavourable pH-conditions of the test solution and even more stable ethers like ethoxyethanol may deteriorate e.g. in water as well as citric acid solution during prolonged heating.

3.2.3.2. Static Headspace (HS)

Static headspace analysis involves that the sample, placed in a vial closed with a suitable septum, is heated at constant temperature until a thermodynamic equilibrium between the sample and the gas phase is reached. The theory

of static headspace analysis was reviewed by several authors [12,13]. The following fundamental equations are valid:

$$\text{partition coefficient } k = C_S/C_G;\ C_S = k \cdot C_G \tag{3.1}$$

where C_S is the analyte concentration in the sample and C_G is the analyte concentration in the gaseous phase, both after reaching the equilibrium.

$$C_O V_S = C_S V_S + C_G V_G \tag{3.2}$$

where C_O is the original concentration in the sample volume V_S.

$$C_O = k \cdot C_G + C_G \cdot V_G/V_S = C_G(k + V_G/V_S) \rightarrow C_G = C_O/(k + V_G/V_S) \tag{3.3}$$

$$V_G/V_S = \text{phase ratio } \beta \tag{3.4}$$

Equation (3.3) shows that the concentration of the analyte in the gaseous phase is significantly influenced by the phase ratio V_G/V_S only if the k value is small. This means that merely the detection limit of lipophile apolar solvents with low k values may be enhanced by rising the volume of the test solution. If polar solvents with higher k values shall be determined the increasing volume of the test solution has only minor influence on the gas phase concentration of this solvent but prolongs the equilibration time unfavourably. For example k values for most solvents in dimethylacetamide at 105°C generally exceed 200. Therefore only smaller volumes of a sample solution prepared with DMA should be analysed for rapid equilibration. Using water as dissolution medium the k values of some solvents like benzene are very small and a larger sample volume can be chosen to increase the concentration of the volatile in the gas phase (Fig. 3.2.A).

The k value of a specific volatile is influenced by the dissolution medium. From studies of the headspace concentration of representative solvents in relation with several sample dissolution media it was concluded that water and DMI are the best hydrophilic and hydrophobic medium respectively [14]. Organic high boiling dissolution media like DMI, DMA and DMF should, however, be heated only to a maximum of 110°C to prevent serious degradation. Extreme measures were taken to obtain high sensitivity and a short equilibration time by reduction of the sample amount to 1 mg per 25 µl of solution injected into a 3.35 ml vial. Thus 1 ng of the respective solvent which corresponds to a limit of 1 ppm could be detected [15].

The analyte concentration in the gas phase can also be increased by higher equilibration temperature. A sample solution in DMA was equilibrated at different temperatures and the results plotted as temperature versus the ln of

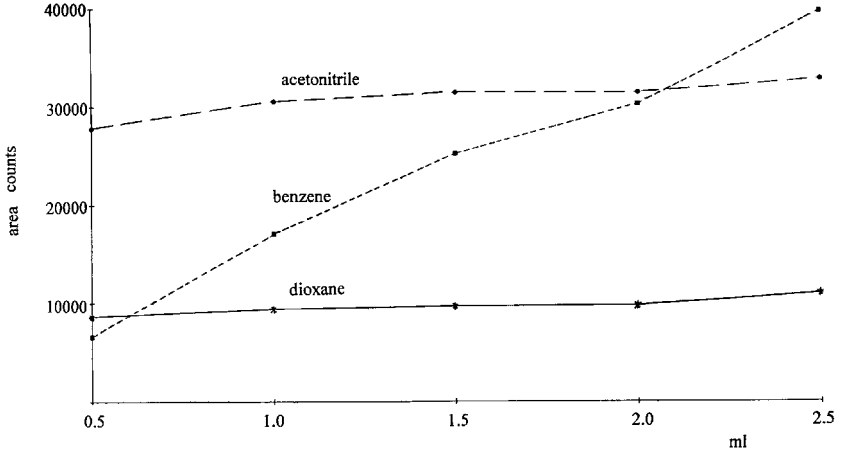


Figure 3.2.A.
Relation between the volume of an aqueous solvent solution and peak area in static headspace analysis: apolar solvent benzene and polar solvents dioxane and acetonitrile

the peak area. A straight line was obtained with a slope of about 3% per °C. It could be estimated that the analyte concentration in the gas phase doubled every 20°C rise in temperature [16]. Static headspace analysis should as far as possible be performed after the thermodynamic equilibrium has been established as generally the accuracy is better then.

On the other hand, good reasons like thermal instability of the sample or loss of analyte by leakage of the vials septum exist to limit time and temperature during the equilibration step. Easily deteriorated solvents are, e.g. esters like ethyl acetate or reactive gaseous compounds like ethylene oxide. Artefacts from unstable samples should be generally taken into account. Some authors reported acceptable linearity and recovery tested for by multiple standard addition as they analysed a sample solution in dimethylformamide equilibrating the solution for 15 min at 60°C (under nonequilibrium conditions) [17]. A fully-automated apparatus should be used, however, in situations where thermally unstable samples demand a compromise regarding the lower temperature and shorter duration of heating.

The main drawback of static HS is its limited sensitivity for samples providing only slow diffusion of the respective volatile into the gaseous phase by which the concentration in the gas phase is diminished. This can be caused by the physical characteristic of a sample (e.g. solid, polymer or highly viscous test solution) or by higher affinity of a solvent to the sample resulting to

a high partition coefficient. Longer injection times and focusing of the analytes at the column head at a temperature as low as possible can improve the limit of detection. Early eluting volatiles may require cryofocussing to obtain good chromatographic resolution as well as acceptable reproducibility and LOD. Thicker column films enhance the capacity for and the retention of early eluting volatiles, however can reduce the sensitivity by a factor up to 10.

On the other hand, if volatiles are present in a sample at high concentrations carry over of the analytes can present a problem. Running blanks between two analyses is a simple and effective way to avoid memory effects. If the headspace is retracted by syringe manually or automatically the gastight syringe should be temperated such as to obtain a sample temperature close to the equilibration temperature at the moment of injection. For loop injection the temperature of the loop should not be set too high, as the concentration of the analyte in the constant volume of the loop would have been diminished because of unnecessary volume expansion. But in any case the temperature of the inlet system – whether this is a syringe, a loop or a pressure controlled valve system – should be set high enough to prevent carry over of any sample component. Equilibration temperature and time can generally be set lower if the sample solution is stirred in a reproducible way. The concentration of the volatiles in the headspace can be enhanced if inorganic salt is added to the sample solution. Unfortunately the extent to which this improves the sensitivity cannot be easily predicted but must be determined by experiment. Influencing factors are the kind and the concentration of the added salt and the nature of the residual solvent to be determined. In this connection it should be mentioned that also addition of non electrolyte to the sample solution influences the partition of volatiles between the liquid and gaseous phase, e.g. addition of water to a sample solution in organic solvent like DMF raises the headspace concentration of lipophilic volatiles. The general rule is that for HS analysis of polar analytes nonpolar solvents should be used and vice versa and that, if this should not be possible due to insolubility of the substance to be examined, test solutions of solvents with low diffusion rates should be analysed by static headspace reducing the volume of the test solution as well as increasing its concentration to the maximal extent. Frequently, however, the main reason for adding salt to the sample solution is to level the inorganic components of complex matrices like biological fluids which show variable composition. Salt addition minimises matrix differences between test and standard solution and thus provides sufficiently precise results by external calibration for such complex samples [18,19]. Apart from this standard addition at single or multiple concentration levels of the added analyte is the usual method for optimal precision of the results.

Several factors which influence the ruggedness of headspace analysis of a solvent mixture were examined. Among instrumental settings like injector and

detector temperature, the liner and column type the ruggedness was significantly determined by the split flow which should be carefully controlled and kept constant [20].

Experimental validation of the lot of parameters to be controlled for headspace analysis can be simplified applying multiple headspace extraction (MHE) through which unfavourable conditions like deterioration or insufficient equilibration can be easily detected.

3.2.3.3. Multiple Headspace Extraction (MHE)

Multiple extraction of the gas phase of a single vial which is under constant conditions in equilibrium with the solid or liquid phase in the vial is called multiple headspace extraction. MHE is really a dynamic extraction procedure nevertheless often mentioned in conjunction with static headspace as performing the procedure needs the same equipment as used for static HS analysis. For the consecutive headspace extraction under strictly identical conditions the software must be capable of controlling the relevant instrumental parameters. For example, after the first extraction the remaining overpressurised gas phase inside the vial must be reset to normal pressure by venting the vial during a short time in a suitable manner. Immediately after this the equilibrium is re-established under exactly the same conditions applied for the first equilibration (pressure, duration and temperature). Then the second extraction is performed.

As some of the analyte has left the vial during the first injection and the venting the following equilibrium concentration of the analyte in the gas phase is lower than prior to the previous injection. By repeating the steps of injection and venting several times and plotting the peak areas versus the number of extraction an exponential function is obtained which plotted as a semilogarithmic graph gives a straight line. If no straight line is obtained the conditions have been shown unsuitable for reaching a stable equilibrium and must be changed accordingly unless good reasons exist to work under nonequilibrium conditions. Thus, MHE is a good validation criteria for robust HS methods despite the fact that also nonequilibrium analysis may give satisfactory results. Additionally MHE under equilibrium conditions is an absolute, matrix independent method for quantification of a volatile. Fundamental aspects of quantification and the theory of MHE were discussed by Kolb and Ettre [21]. According to the equation $A = A_0/1 - e^{-k}$ the peak area A corresponding to the total amount of the analyte in the sample can be calculated from A_0 i.e. the peak area obtained by analysis of the first headspace extract and the slope k of the straight line obtained through a semilogarithmic plot or through the regression analysis of the measured peak areas obtained in the chromatograms of the successive gaseous extracts. If the slope k is not too small a simplified equation

can be applied which requires only two analysis under MHE conditions. The area A corresponding to the total amount of an analyte in the sample can then be calculated from the equation $A = A_1^2/A_1 - A_2$. To calculate the amount of the volatile in the sample the response factor must be determined, e.g. from the peak area obtained after headspace analysis of a known amount of the analyte being completely volatilised in a second vial or, generally, by external calibration or standard addition. Volatile impurities in an insoluble drug were determined by MHE according to the equations mentioned. The results obtained by two and five extraction steps were comparable, the difference in the values being about 16–29% [22].

Generally MHE can be used especially for analysis of solid or insoluble samples and of polymers. Further MHE is the best method for analysis of solutions containing complex samples with varying composition. The usefulness of MHE in static and dynamic Headspace-GC was demonstrated for testing of volatiles in packaging [23,24] and the development of transdermal drug delivery systems [25,26]. The MHE results varied by a RSD of 1–5% for LODs between 0.24 and 0.06 μg. To stabilise the vapour pressure thus increasing the precision of the injectable gas volume it is recommended to add about 10 μl (for 22 ml vial) of a solvent eluting later than that to be determined to the vial containing the solid sample. It would be unfavourable to determine a volatile in such samples simply by standard addition which involves spiking of the sample with the analyte solution. The result would be an inhomogeneous three-phase mixture of solid, liquid and gas which differs with regard to the diffusion rate of volatiles between the three phases and to the respective partition coefficients.

If the graph is either not a straight line or if it is difficult to examine the linearity as the slope k is too small and the variation of the results within the same range the sample should be kept at higher temperature, preferably in an inert atmosphere, or the surface of the solid sample to the gas phase should be extended by cautious grinding of the cooled sample. Polymer films should be as thin as possible to facilitate complete migration of monomers and other volatile components into the headspace. Additionally for some matrix the partition behaviour of a more or less apolar analyte can be improved utilising the displacement effect of a polar high boiling solvent like glycerol of which a small quantity is homogeneously mixed with the sample.

Practically MHE is limited by samples giving rise to very low diffusion rates which reduces the concentration of the volatile in the gas phase to a non detectable level. Examples for this are polymer carbohydrates like alkylhydroxycelluloses and carrageenes which are generally samples of high viscosity [4].

3.2.3.4. Full Evaporation Technique (FET)

FET involves heating of a small sample amount placed inside a closed vial resulting in full evaporation of volatiles into the headspace. This idea can be transferred to small volumes of a sample solution as well as to samples which are volatile to a high extent under the conditions applied (Figs. 3.2.B and 3.2.C).

FET avoids the drawbacks of static headspace analysis if components with high k values shall be determined as neglecting the small volume of any solid residue of the sample only the gaseous phase is present in the vial. Based on the principle and theory as reported [27] the following equations are valid:

$$C_G = C_0 V_S / (kV_S + V_V) \tag{3.5}$$

and

$$C_G = C_0 / (k + \beta) \tag{3.6}$$

where β is the phase volume ratio V_G/V_S and V_G is practically equal to V_V; C_0 is the analyte concentration of the sample, V_S is the sample volume, V_V is the vial volume and k is the partition coefficient between the sample and the gas in the closed vial. The term $V_S \cdot k$ can be neglected in relation to V_G [28] and the calculation performed on the basis of Eq. (3.7).

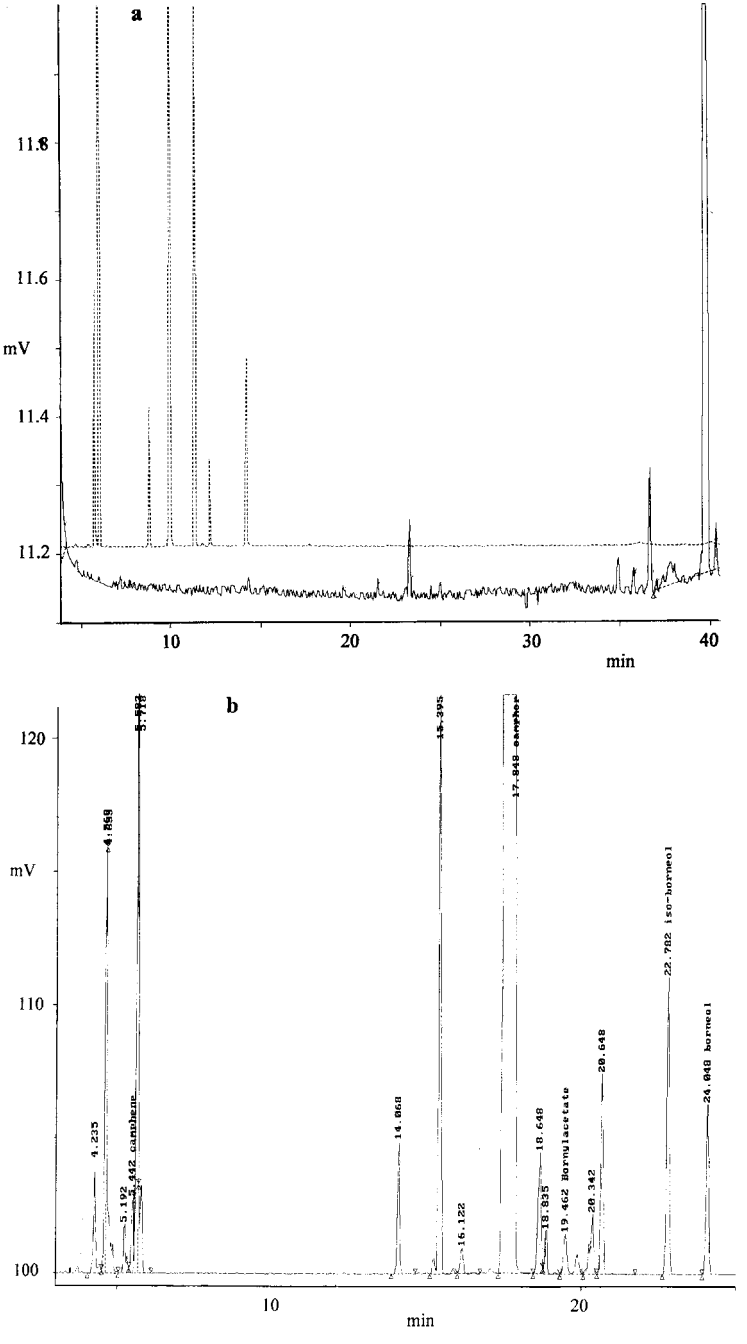
$$C_G = C_0 V_S / V_V \tag{3.7}$$

The main advantage is the short time for complete equilibration due to suppressing any partition process. Limitation of this method is that the absolute amount of the volatile must be high enough to provide a detectable chromatographic peak. Analysing 20–50 mg of a powdered solid 0.4–4 μmol/kg of some solvents could be detected with a mass spectrometer in the SIM mode [28]. Also it must be ensured that a representative sample of the substance to be examined is analysed.

A similar technique with regard to the simple method for the evaporation of volatiles from solid samples in a headspace vial is the direct desorption method. A relatively high sample amount of about 200 mg was heated to a temperature higher than the boiling point of the volatile but well below the melting point of the sample [29]. The LOD was less than 1 ppm.

3.2.3.5. Dynamic Headspace

In a dynamic headspace a continuous gas flow is swept over the surface of a solid sample or through a liquid sample with a rate to provide optimal surface contact between the gas and sample. The volatiles are transported with the gas


a
11.8
11.6
mV
11.4
11.2
10
20
30
40
min
b
120
mV
110
100
4.225
4.868
5.192
5.342 camphene
14.868
15.395
16.122
17.848 camphor
18.648
18.835
19.462 Bornylacetate
20.342
20.648
22.782 iso-borneol
24.848 borneol
10
20
min

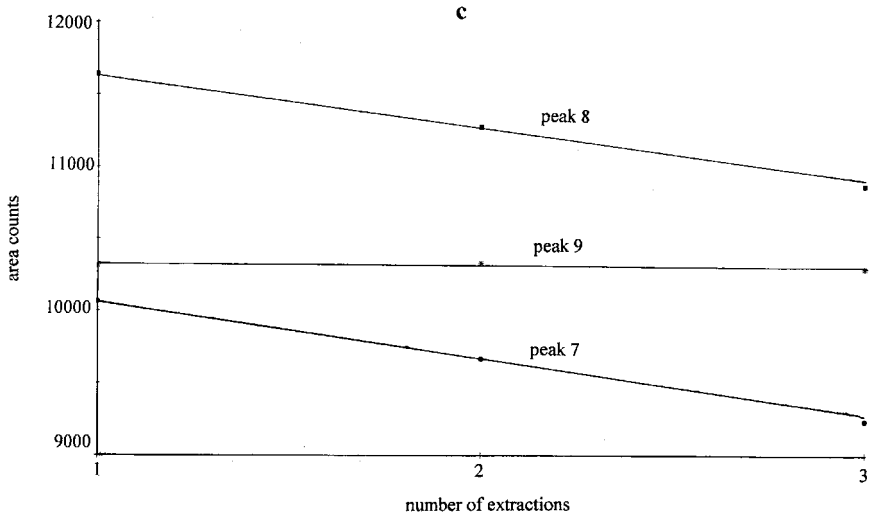


Figure 3.2.B.
FET applied to a sample of camphor for detection of residual solvents and related substances. (a) A 2 μl sample of a 10% methanolic solution equilibrated in a 22 ml vial at 95°C during 20 min; GC analysis using a DB 624 column 30 m × 0.32 mm, 1.8 μm film, under the following conditions: 21 cm/s nitrogen as carrier gas, split 33 ml, inj. 220°C, det. (FID) 300°C; column 40°C, constant 8 min, 10°C/min to 100°C, constant 3 min, 3°C/min to 160°C, constant 10 min; headspace sampler: needle 140°C, transfer line 150°C, injection period 0.02 min. Dotted line shows the chromatogram obtained under identical conditions of a solvent reference solution, containing the solvents (in the elution order) acetonitrile, methylene chloride, chloroform, benzene, trichloroethylene, dioxane and pyridine. (b) Chromatogram of 2 μl of a 10% methanolic solution of camphor equilibrated in a 22 ml vial at 95°C during 20 min; chromatogram obtained with a Carbowax column 30 m × 0.32 mm, 0.25 μm film under the following conditions: 26 cm/s nitrogen as carrier gas, split 37 ml, inj. 200°C, det. (FID) 350°C; column 40°C constant 0.5 min, 5°C/min to 115°C, 2°C/min to 165°C, 10°C/min to 240°C, hold for 3 min; headspace sampler: needle 130°C, transfer line 140°C, injection period 0.05 min. (c) MHE of a crystalline sample of camphor equilibrated at 120°C for 90 min: for some of the related substances (peak 7, RT = 14.07; peak 8, RT = 15.4) suitable equilibration conditions were indicated whereas for peak 9 (RT 18.65) and later occurring peaks (RT 19.46, 20.35, 20.64) the conditions were insufficient. Retention times refer to the chromatogram on the Carbowax column (b)

to a trap where they are cumulated prior to analysis. Dynamic headspace analysis is also designated as purge and trap (P&T) analysis. The purging and trapping can be performed in a closed circuit or in an open system. In a closed system a distinct gas volume is passed several times through the sample and the trap. As the whole system reaches an equilibrium after a while the extent of enrichment of the volatiles in the trap depends on the difference of the k values of the volatile between the gas and the trap compared with the k value

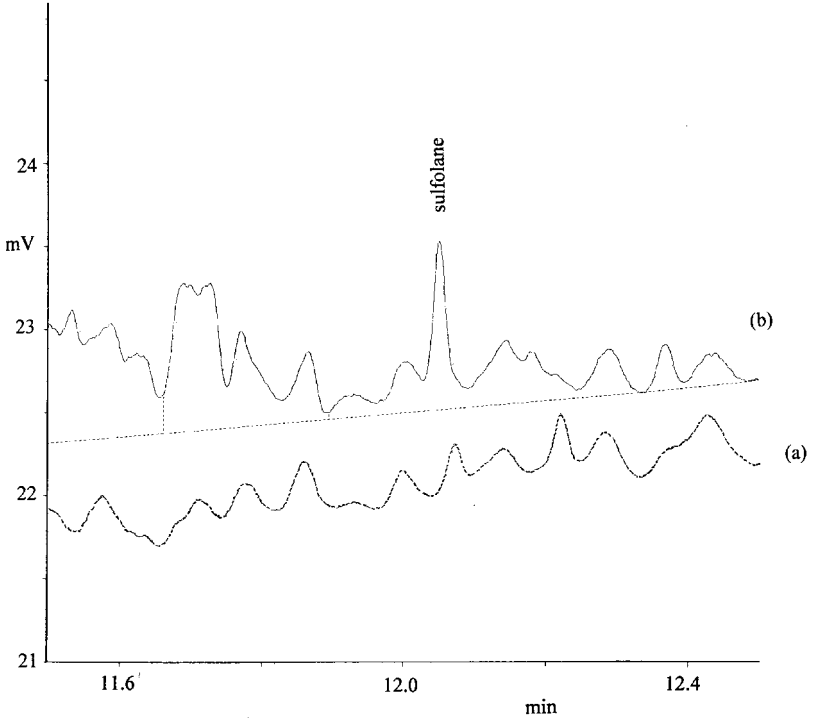


Figure 3.2.C.
FET analysis of sulfolane as an example for a less volatile solvent of class 2 of the Ph. Eur. list. (a) Normal HS with 1 ml of a 20 μg/ml solution of sulfolan, equilibrated at 98°C for 15 min. (b) A 20 μl sample of a 20 μg/ml solution of sulfolane, diluted with 20 μl of water and equilibrated at 120°C for 15 min (FET-method). Carbowax 30 m × 0.32 mm, 0.25 μm film, flow 30 cm/s helium as carrier gas, splitless injection, 1.5 min, then split 15 ml, inj. 260°C, detector 350°C; temp. program: 60°C, 2 min constant, 10°C/min to 100°C, 30°C/min to 240°C, 2°C min to 255 °C; headspace sampler: needle 140°C, transfer line 160°C, injection period 0.3 min

between the gas and the sample. During collection the k value of the trap must be greater than the sample's which apart from the nature of the trap material can be strengthened by heating of the sample and cooling of the trap at the same time. On the other hand, the partition coefficient between the trap and the gas must be as low as possible during the desorption period which is started by switching the outlet of the trap to the GC column followed by rapid heating of the thermostable trap. In an open system the volume of the purge gas is not restricted and the main aspects influencing the enrichment in the trap are the dimension of the trap and the material either filling or coating the inner wall of the trap. The collection can be additionally supported through cooling of the trap. In practice, however, the flow rate as well as the total volume of the stripping gas are limited due to the breakthrough of volatiles less retained by the trap. The breakthrough volume can be experimentally determined at the time when the detector response of the analyte which initially increases with the purging time starts to decrease significantly.

Compared with the static headspace the main advantage of dynamic headspace with concentration of the purged volatiles in a trap is its capability to determine very low concentrations of volatiles due to no restrictions of the sample volume, the solubility of the sample and the volume of the extracting gas. Also heating of thermostable substances is not required. As no equilibrium must be reached under the conditions of P&T this technique is well suitable for volatiles with high partition coefficients ($k > 1000$) between the sample preparation and the purging gas.

The theory of static as well as dynamic headspace (purge and trap) analysis was reviewed by several authors [30–32].

The efficiency of a P&T apparatus is determined by the main components, the purging device with the heatable sample holder and the thermostable trap device. Thus some more parameters than for static HS must be optimised [33,34].

The homogeneous representative sample must be fixed in the sample holder, polymers and solid samples preferably as a thin film or layer to ensure maximum contact with the purging gas and to avoid temperature gradients across the sample material. An optimum between the stripping gas flow and the extraction time should be obtained for the reproducible recovery of the volatile at a constant temperature. Doubling the gas flow has nearly the same effect as doubling of the extraction time.

Purging the sample with a continuous gas flow at elevated temperatures can be so effective that a lot of interference like water and volatiles of a different nature is collected in and desorbed from the trap which causes difficulties during the gas chromatographic analysis. One should keep in mind that 1 g of a sample containing 0.1% of volatile matter will produce 1 mg of volatile compounds which for a single compound is more than adequate for a GC

analysis with capillary columns. Therefore temperature and size of the sample and the volume of the extracting gas should be set such to obtain complete extraction of the components to be determined without serious amounts of other more or less volatile components which would interfere during the gas chromatographic separation. This, however, can often not be achieved in practice. Therefore consideration is necessary with respect to the selectivity of the trapping material. Chromosorb, Porapak, Amberlite XAD resins, activated carbon, molecular sieves and especially the thermostable Tenax are commonly used filling materials for the trap, sometimes also combinations of them in layers [35]. Some of the sorbents provide more selectivity. Tenax, for example, strongly adsorbs aromatics but lets water, low molecular alcohols and hydrocarbons pass through. However too efficient sorbents prevent easy desorption of the retained compounds and require a higher desorption temperature or a following cryogenic cooling trap. Before the gas with the extracted volatiles reaches the analytical trap great excess of water can be eliminated by freezing at -15 to -20°C in a first cold trap filled with glass beads. The last step before the GC analysis is the desorption from the trap. This can be either performed through liquid extraction or preferably through rapid heating of the trap. If a trap wall-coated with a thin film of the trapping liquid polymer is used the desorption is especially fast and the injection band on the column is narrow which is advantageous for good chromatographic separation and less tailing of the peaks. For some liquid samples during gas purge foam prevention is necessary which can be achieved with an inert frit put into the purge gas pathway or by suitable additives like octanol.

For calibration the same procedures are employed as for static headspace analysis, i.e. use of reference standards, standard addition and repetitive gas-phase sampling.

Precision and LOD of some highly volatiles were determined by different modes of sample pretreatment [36,37]. For a specific solvent the following LOD were obtained: determined by dynamic HS 0.04 ppm, by MHE 0.5–1 ppm, by static HS 2 ppm and by direct injection 400–500 ppm The best precision was obtained by static headspace analysis similar to that of direct injection. Comparing liquid extraction, P&T and static HS method it was concluded that static HS was consistently more precise than P&T and provided the best recovery of all methods if the sample was shaken during equilibration.

3.2.3.6. Thermal Desorption

Thermal desorption [38] of volatile components of a sample placed in a rapidly heated special injector (PTV) combined with cold trapping at the head of the column is another powerful dynamic sampling technique for determining volatile substances. LOD in the range 2–90 ng/10 mg sample were reported.

General drawbacks are possible deterioration of the sample by high temperatures which must be much higher than the melting point of the sample to ensure complete and rapid vaporisation of the volatile components. Inertness of the injector surface and of the carrier gas are important requirements to reduce deterioration of the sample matrix and thus avoid artefacts. Additional contamination by other less volatile components with high retention times must be taken into account and should be reduced to a minimum by a precolumn in front of the analytical column or by backflush.

3.2.3.7. Solid Phase Micro Extraction (SPME)

Solid phase micro extraction is a newer technique which involves an equilibrium established among the concentration between the polymer coating on a fused silica fibre and the liquid sample or the headspace above a solid or liquid sample. The fibre is placed in a special holder which protects the fibre during piercing through the septa of the sample vial and the injection port. In a second holder position the fibre is in direct contact with the gaseous or liquid phase containing the analyte which enables easy partition into the thin polymeric coating. After sampling the fibre is retracted to the first position, the protecting needle passed into the injection port of the GC, the needle put into the second position by the holder thus allowing desorption from the fibre into the glass liner space of the injector flushed by the carrier gas. Therefore the most important advantages of this technique are an effective concentration of the analyte in the small volume of the fibre coating and the ideal compatibility with GC injectors which ensures by splitless injection nearly complete desorption and solventless transfer of the collected volatiles to the column. This results in much lower detection limits for volatiles than achieved through static headspace analysis. The concentration process can be optimised choosing between fibre coatings which differ with respect to the selectivity and thickness of the polymeric coating. The possibility of concentrating analytes by immersion of the fibre into the sample solution extends GC analysis to polar substances with high partition coefficients or low volatility. Apart from the selection of the right fibre type mainly the temperature of the sample and the equilibration time must be strictly controlled. The adsorption at the fibre can be accelerated through reproducible stirring or ultrasonification of the sample solution. Applying ultrasonic mixing during the collection period is especially a good choice for quick enrichment of higher boiling volatiles. Especially if the fibre is immersed into the sample solution addition of salt and adjusting a suitable pH-value can improve the concentration of the analyte in the polymer coating.

The main practical problem is the relatively long desorption time ranging from 1 to 5 min which requires specially-designed injector glass liners and effective trapping at the column head especially if early eluting volatile compo-

nents shall be analysed. If due to practical restriction, e.g. by GC/MS it is not possible to provide this by a suitable column type and low starting temperature then cryofocussing is required.

The depth of fibre insertion into the GC injector, the fibre thickness (thick for more volatiles and thin for semivolatiles) and polarity (apolar for hydrocarbons and other nonpolar substances, polar for alcohols and ketones) must be adapted to the special analytical problem. The limited capacity of the fibre coatings and thus quick saturation by the analyte must be taken into account and the concentration of the sample solution diminished accordingly. If the fibre is immersed into the sample solution one should make sure that the vial is well closed and completely filled with the sample solution. Determination of RS by SPME was performed by Perchiazzi and Ferrari [39] who found that SPME and static headspace analysis produced comparable results with respect to linearity and precision. However the recovery differs much more when SPME is applied. Fibres coated with polyacryl were recommended as best choice for RS determination by SPME. Meanwhile a coating of poly(dimethylsiloxane) (PDMS) mixed with highly porous amorphous carbon of optimal porosity is available which shall be better suitable for highly volatiles which are concentrated on the fibre rather by adsorption than by partition. A comparative study with a 100 μm PDMS- fibre lead to the conclusion that solvents were detectable with an LOD range of 0.002–5 ppm and a RSD between 7 and 0.5% depending on the solvent extraction method. SPME in conjunction with static headspace analysis provided nearly the same results as those obtained by immersion into the sample solution after which generally more matrix influences (composition, pH) can be expected [40]. In comparison with purge and trap analysis of substituted benzenes in water headspace SPME gave closely related results [41]. However, SPME was used in combination with cold trapping at the column head before running the temperature program which is not the common routine.

Others [42] experimented with a special gastight SPME syringe and reported superior LOD in the range of 10–200 ppt for RS in pharmaceuticals. They also found out that SPME is more suitable than static headspace analysis for more or less polar substances.

3.2.4. Column Choice for GC Separation of Solvents

No general recommendation for a special column type can be made due to the individual separation requirements caused by matrix-dependent interfering volatile components. However, some types of columns like DB5, Carbowax, RTX 200, DB 1701 and DB 624 column are widely used for the chromatography of solvents. For highly volatile solvents column fillings with strong

retention characteristic, e.g. poraplot Q should be used. Column diameter and thickness of the polymer coating should also be adapted to the low retention of some volatiles. Column inner diameters of 0.32–0.5 mm and film thickness of at least 1 μm seems to be a good compromise between capacity and chromatographic performance.

Taguchi design experiments for improving the separation of a number of solvents are reported [43], however, for a special product it is usually required to separate any solvent rather from possible interfering matrix components than from as many other solvents.

3.2.5. Temperature Program

For screening on residual solvents the starting temperature must be set as low as possible for the column type used. Due to the relatively long time required for complete desorption from the fibre this is essential if SPME is applied. In individual cases one is free in choosing the starting temperature and program due to the separation requirements. Experience of the analyst and commercially available software may help to find out the best solution.

3.2.6. Detection and Identification

The choice of detector is made in accordance with the sample characteristic and the purpose of the analysis whether known or unknown solvents have to be determined, whether a specific solvent must be detected at the lowest level in a complex matrix, whether screening shall be performed or highest selectivity and/or sensitivity are required. Normally a flame ionisation detector (FID) is sufficient. Electron capture (ECD) and nitrogen-phosphorus (NPD) detectors are specific detectors for detection of solvents at low concentration in complex samples. A universal specific and sensitive detector for screening purpose is the MS detector (see Section 3.2.6.2 and Table 3.2.B).

3.2.6.1. Two Column Approach

Principal doubts are opportune with respect to chromatographic identification without specific detection as chromatography itself is by no means an identification method but merely a method which allows some conclusion from the retention behaviour in relation to the characteristic of the analyte. Apart from additional information obtainable by specific detection like ECD or NPD chromatographic verification can be performed by the two column approach in conjunction with authentic reference substances: the chromatography is

Table 3.2.B.
Summary of detector characteristics. (Ref. [6])

Detector type	Characteristics	Approximative detection limit	Linear range
Flame ionisation (FID)	Universal (organic compound)	2×10^{-12} g/s	$>10^7$
Thermoionic emission (TID)	Selective (organic nitrogen and phosphorus)	10^{-13} g/s N	10^5
		5×10^{-14} g/s P	
Flame photometric (PID)	Selective (sulphur and phosphorus)	$<10^{-11}$ g/s S	$\geq 10^3$
		$<10^{-12}$ g/s P	$\geq 10^4$
^{63}Ni electron capture (ECD)	Selective (halogens and other electron capturing groups)	Highly variable, as low as $5 \cdot 10^{-15}$ g	10^4
Photoionisation (PID)	Universal	2×10^{-13} g/s	$>10^7$
Thermal conductivity (TCD)	Universal	4×10^{-10} g/ml (propane)	$>10^5$
Fourier transform infrared (FT-IR)	Universal or selective	200 pg–40 ng	10^4
Mass spectrometer (MS)	Universal or selective	Electron ionisation (EI) 10–100 pg	
		Selected ion monitoring (SIM) $<10^{-13}$ g	10^5

performed on two columns of quite different selectivity (e.g. apolar DB5 or medium polar DB 624 and polar Carbowax) and the retention times are compared with a standard. A well-known example for the two column approach is the European Pharmacopoeia (Ph. Eur.) method.

The Ph. Eur. method is designed partially as a general screening method in accordance with the request of the licensing authorities that distinct solvents (enclosed in list 1 of the general method) are banned for pharmaceutical purposes due to their toxicological and environmental risks. The Ph. Eur. method is in accordance with the ''Guideline for residual solvents'' which was elaborated within the ICH (International Conference on Harmonisation between the USP, Pharm. Jap. and Ph. Eur.) and consists of a toxicological part which describes the methods for establishing exposure limits and the background for the classification of residual solvents by risk assessment. The second analytical part describes a general limit test on residual solvents by static HS-GC which however is not suitable for all solvents of class 2: several solvents like formamide, ethylene glycol, *N*-methylpyrrolidone and sulfolane cannot be limited at the required levels by this method due to their very low volatility and thus concentration in the gaseous phase. If these solvents have to be limited direct injection of the test solution or other suitable methods like FET should be applied if no method is prescribed in the special monograph of the substance to be examined.

Although at the moment this guideline is introduced only by the Ph. Eur. for new active substances, excipients and medicinal products it can be hoped that the numerous different general methods and policies of other pharmacopoeias, especially of the USP, can be substituted in the near future by a common policy and analytical method within the most important market for pharmaceuticals worldwide.

A detailed review of compendial methods for determination of residual solvents was published by Witschi and Doelker [6] and the history of the Ph. Eur. method is reported elsewhere [44].

3.2.6.2. GC/MS

Forensic applications like analysis of illicit drugs and in the context of detection of counterfeited bulkware [45] were the main reason for applying costly GC/MS instruments for the detection of residual solvents. However, in the meanwhile GC/MS combinations are compact instruments much cheaper and widespread in modern laboratories of the pharmaceutical industry. Manufacturers of pharmaceuticals use GC/MS during development and validation of the analytical methods for their products as MS is an ideal detector for the identification of known and the structure elucidation of unknown compounds. Especially if a peak corresponding to one of the solvents of class 1 is detected it

can be necessary to identify this and to exclude any interfering component. Free world trade increases the number of marketed products of unknown origin. Among other unknown impurities also residual solvents must be determined which in addition requires GC/MS for their identification and quantification. Due to the very effective concentration even of less volatile compounds by SPME much more interfering volatiles from the sample as well as from the environment can be detected than with static headspace. Therefore, it can be frequently necessary to identify the solvent to be determined and to combine SPME with the MS detection. GC/MS requires low bleeding columns with an optimal gas flow rate of up to 1 ml. This means that dimension and film thickness of the coating are restricted and cannot be optimal for residual solvent analysis. Columns of 0.25 mm ID and 1 μm film thickness are a good compromise between capacity and favourable flow dynamics. Even then subambient cooling is required for early eluting compounds. Alternatives to the less suitable high bleeding DB624 column are a DB5 or a RTX 200 column the latter one providing improved selectivity for ketones and esters [16] (Fig. 3.2.D and Table 3.2.C).

Mass spectrometric techniques like SIM or MIM (*S*ingle or *m*ultiple *i*on *m*onitoring), mass fragmentography and MS/MS are techniques for specific ion detection and allow the deconvolution of overlapping peaks by recording the specific ion traces.

The sensitivity gain of SIM and MIM depends on the type of the instrument used. Therefore the reported values differ much from 2 up to 100 fold sensitivity in comparison to the total ion current. For an ion trap instrument only little sensitivity gain is obtained by these techniques. However, for this

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Figure 3.2.D.
Chromatograms of class 1 and class 2 solvents of the Ph. Eur. list. (a) TIC (total ion current) of class 2 solvents collected by SPME of the headspace of a concentrated solution in DMSO at room temperature: CH_2Cl_2 (scan number 131); $CHCl_3$ (206); hexane (216); 1,2-dichloroethene (227); ACN (315); ethylcylclohexane (332); trichloroethylene (361); 1,2-dimethoxyethane (416); toluene (615); dioxane (641); pyridine (781); ethylbenzene (892); chlorobenzene (908); xylene (o, p, m, 923, 935, 1040); 2-hexanone (1077). GC conditions: RTX200-column 30 m × 0.25 mm, 1 μm film; 30 cm/s helium as carrier gas; inj. temp. 250°C; temp. prog. 40°C, 5 min constant, 3°C/min to 150°C, 10°C/min to 280°C, 5 min constant. (b) TIC (total ion current) of class 1 solvents collected by SPME of the headspace of a concentrated solution in DMSO at room temperature: 1,1-dichloroethene (scan number 103); carbon tetrachloride (242); 1,1,1-trichloroethane (290); benzene (331); 1,2-dichloroethane (364). GC conditions identical to chromatogram in (a)

type of instrument mass fragmentography improves the signal-to-noise ratio significantly.

MS/MS is a further alternative for specific detection and identification of an unknown volatile in a highly complex sample. MS/MS involves selection of ions of a specific mass-to-charge ratio by the mass analyser. The selected ions are activated through collision with carrier gas molecules in fieldfree chambers

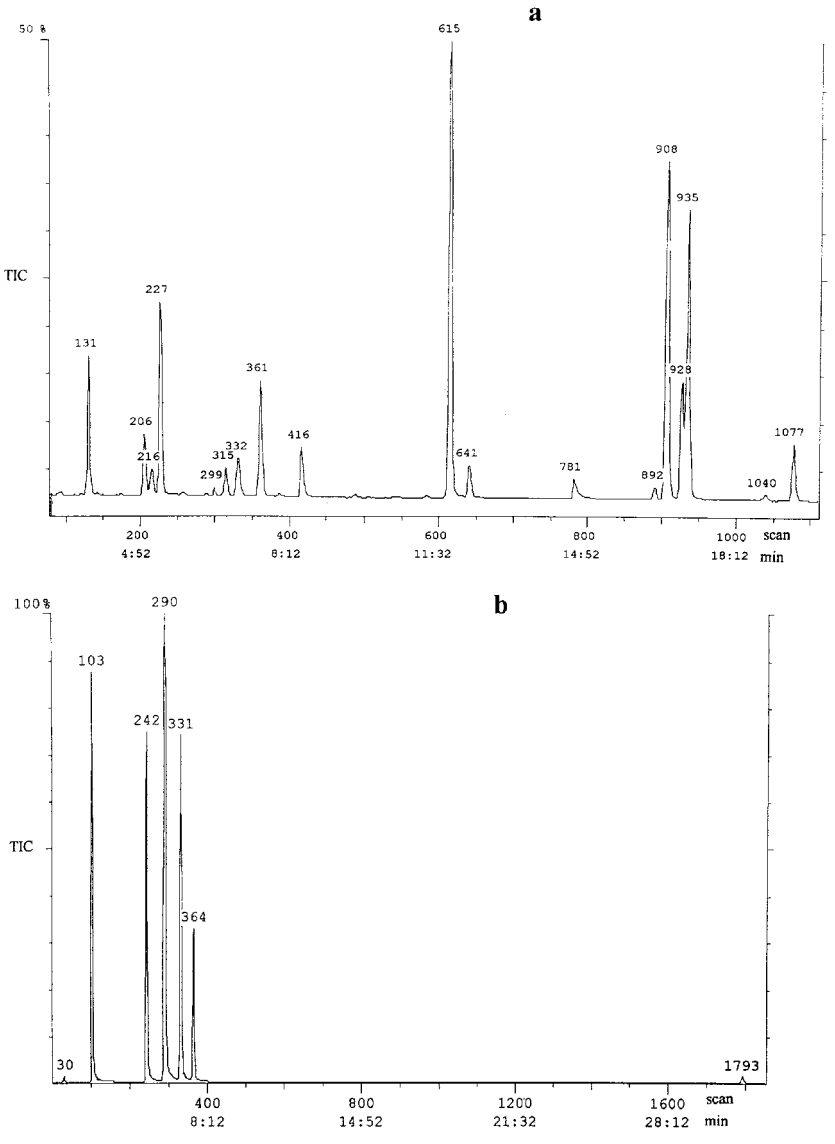


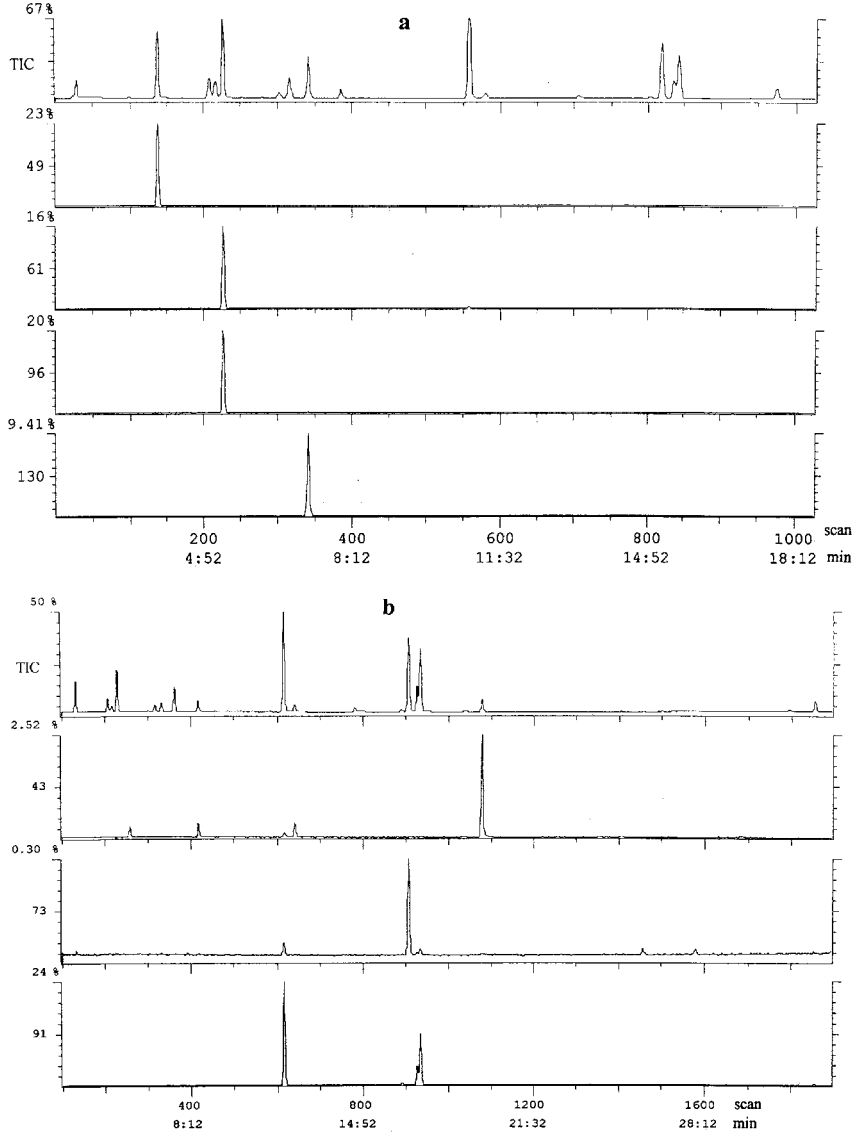
Table 3.2.C.
Retention behaviour for selected compounds on the RTX-200 and the DB-624 capillary column [16]

Analyte	Rtx-200 column			DB-624 column	
	Relative retention index	Retention time (min)	Ratio to benzene	Retention time (min)	Ratio to benzene
Methanol	<500	2.51	0.35	2.32	0.25
Pentane	500	2.87	0.40	–	–
Ethanol	537	3.45	0.48	3.13	0.33
Diethylether	579	3.56	0.50	3.29	0.35
Isopropanol	589	4.23	0.59	3.91	0.42
Methylene chloride	594	4.35	0.61	4.38	0.46
Hexane	600	4.44	0.62	5.46	0.58
2-Methyl-2-propanol	625	4.98	0.70	4.6	0.40
1-Propanol	645	5.39	0.75	6.02	0.64
Chloroform	660	5.70	0.80	7.92	0.84
Carbon tetrachloride	684	6.19	0.87	–	–
Acetone	687	6.25	0.87	3.67	0.39
Heptane	700	6.53	0.91	10.54	1.12
2-Methyl-1-propanol	716	6.86	0.96	9.48	1.01
Benzene	729	7.15	1.00	9.42	1.00

Tetrahydrofuran	736	7.30	1.02	7.74	0.82
Trichloroethylene	745	7.50	1.05	11.64	1.24
1-Butanol	755	7.70	1.08	11.86	1.26
Ethyl acetate	760	7.81	1.09	7.34	0.78
2-Butanone	783	8.33	1.17	7.1	0.75
Octane	800	8.63	1.21	–	–
3-Methyl-1-butanol	833	9.33	1.30	17.34	1.84
Toluene	842	9.53	1.33	17.05	1.81
1,4-Dioxane	848	9.65	1.35	13.04	1.38
1-Pentanol	859	9.94	1.39	–	–
Nonane	900	10.70	1.50	–	–
Methyl isobutyl ketone	936	11.38	1.59	16.51	1.75
Chlorobenzene	942	11.48	1.61	–	–
Decane	1000	12.57	1.76	–	–
Undecane	1100	14.29	2.00	–	–
Cyclohexanone	1148	15.07	2.11	–	–
o-Dichlorobenzene	1167	15.37	2.15	–	–
Dodecane	1200	15.91	2.23	–	–
Tetradecane	1400	18.80	2.63	–	–

between two quadrupols or activated in the ion trap by a radiofrequency. The activated ions decay and produce a pattern of daughter ions which can be recorded in a various manner: either as the whole spectrum or as most intense single fragment ion or some more or less specific ions.

The peaks obtained by recording substance specific ions during the chromatographic analysis are normally not influenced by interfering sample



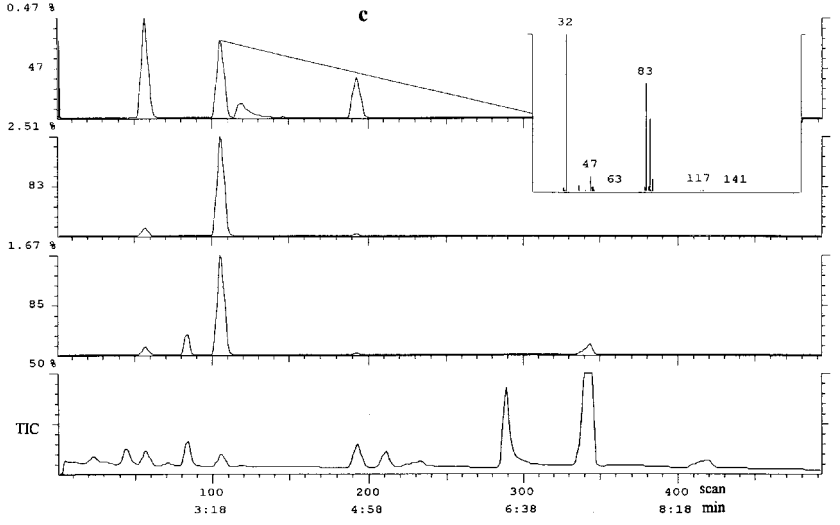


Figure 3.2.E.
Mass fragmentography as a powerful tool for specific and sensitive detection of residual solvents. (a) Specific ions m/z = 49 (methylene chloride), 61 and 96 (1,1-, 1,2-dichloroethene) and 130 (1,1,2-trichloroethene). (b) Less specific ions m/z = 43 (e.g. methylketones, hexane, dioxane), 73 (*N*,*N*-dimethylformamide) and 91 (e.g. toluene, xylenes/ethylbenzene, tetraline). (c) MIM for specific detection, e.g. of chloroform. For TIC (total ion current) chromatogram of class 2 solvents see Fig. 3.2.D.a

components. The same holds true for mass fragmentography which is the subsequent software controlled extraction of specific single or multiple ion traces from total ion current (Fig. 3.2.E and Table 3.2.D).

It should be kept in mind, however, that one condition for an improvement in sensitivity obtained by the techniques mentioned above is the specificity of the chosen masses. Unfortunately mass spectra of residual solvents mainly contain ions in the less specific low mass range which is normally densely occupied by ions of any substance. This makes it difficult to choose a suitable specific and intense mass. For example, the ion m/z = 73 can either be the molecular ion of DMF or a frequently present fragment ion derived from e.g. column wall coating polyalkylsiloxanes or surfaces deactivated with silylating reagents. Similarly m/z = 43 is a rather specific mass. Generally in the low mass range specificity is much lower than is valid for higher masses. Therefore it is also for GC/MS good practice to choose a column of high selectivity and to

Table 3.2.D.
Specific ions of toxic solvents of class 1 and 2 of the Ph. Eur. method (for the limits see Tables 1.5.E and 1.5F)

Solvent	Base peak m/z	Molecular ion m/z	Other m/z
Specific ions of toxic solvents of class 1			
Benzene	78	78	77;51
Carbon tetrachoride	117	152	119;82;47
1,2-Dichloroethane	62	98	100;64;49
1,1-Dichloroethene	61	96	98;63
1,1,1-Trichloroethane	97	132	117;99;61
Specific ions of toxic solvents of class 2			
Acetonitrile	41	41	–
Chlorobenzene	112	112	114:77
Chloroform	83	118	117;85;47
Cyclohexane	56	84	69;41
1,2-Dichloroethene	61	96	98;63
Dichloromethane	49	84	86;51
1,2-Dimethoxyethane	45	90	60:58
N,*N*-Dimethylacetamide	44	87	72;43
N,*N*-Dimethylformamide	73	73	44;42
1,4-Dioxane	88	88	58;43
2-Ethoxyethanol	31	90	72;59;45
Ethylene glycol	31	62	43;33
Formamide	45	45	44;43
Hexane	57	86	43;41
Methanol	31	32	–
2-Methoxyethanol	45	76	–
Methylbutylketone	43	100	85;58
Methylcyclohexane	55	98	83;41
N-Methylpyrrolidone	99	99	98;44;42
Nitromethane	30	61	60;46
Pyridine	79	79	52;51
Sulfolane	41	120	56;55
Tetralin	104	132	117;91
Toluene	91	92	65;39
1,1,2-Trichloroethene	130	130	132;95;60
Xylene (*o*, *p*, *m* + ethylbenzene)	91	106	105;77;39
DMSO	63	78	61;45

take into account the retention behaviour for identification. This can easily be achieved as residual solvents cause no problems with the availability of an authentic reference substance which enables the registration of a library spectrum under the specific conditions of the individual instrument (Fig. 3.2.F).

If MIM and SIM were applied LOD were for some solvents in the range 0.2 mg/l for *n*-octane to 2 mg/l for ethanol. About twice the LOD concentration would be normally sufficient for library searching of any unknown volatile [16]. Masses suitable for SIM or MIM of toxic solvents included into class 1 and 2 of the Ph. Eur. method are summarised in Table 3.2.D.

3.2.6.3. Membrane Inlet Mass Spectrometry

Generally a mass spectrometer can be regarded merely as a special detector. Therefore all methods for sample pretreatment are also applicable to GC/MS. However, during the last time several on-line techniques specifically adapted to mass spectrometers were developed which may be useful for continuos process monitoring during in-process control. *M*embrane *i*nlet or *i*ntroduction *m*ass *s*pectrometry (MIMS) is a method by which the analyte penetrates into the ion source of a mass spectrometer by passive diffusion through a thin membrane of, e.g. dimethylpolysiloxane which is installed as sheet closely to the ion source in a special device [46] or constructed as a hollow-fibre capillary. The sample, either an aqueous solution or a gas, passes by the outer surface of the membrane which separates the high vacuum region of the ion source from the atmosphere. During contact with the membrane more or less apolar small volatile compounds can easily diffuse through the membrane. The analytic characteristics of this method in comparison to others such as purge and trap and static HS have been studied (Table 3.2.E).

Experimental parameters to be optimised are the nature and thickness of the membrane material, the flow rate through the cell, the temperature of the device and the solvent in which the sample is dissolved (usually water). The main drawback is the lack of chromatographic resolution so that a deconvolution program is required.

Purge and Membrane MS [47] is a combination of dynamic headspace analysis with MIMS. The volatiles of the sample purged by a gas are collected in the polydimethylsiloxane membrane as the trap. The membrane is in direct contact with an ion source of a mass spectrometer. By this combination and mass spectrometric single ion monitoring LOD of lower than 0.1–0.3 μg/l were obtained for nonpolar volatiles in water and LOD of 1–20 mg/kg for volatiles in soils. The RSD were 0.5–2.0% for water solutions and 4.8–14.0% for soils, respectively. Special problems can arise with less volatile compounds like PAH which are adsorbed at the surface of the membrane, however due to the lack of accelerating forces penetrate the membrane too slowly towards

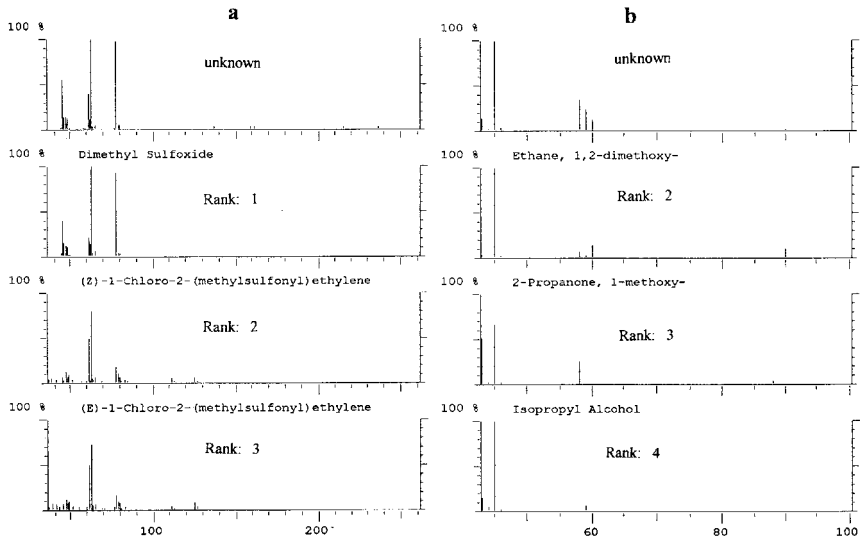


Figure 3.2.F.
Comparison of real solvent mass spectra with respective library (NIST) spectra. (a) DMSO, high correspondence. (b) Dimethoxyethane, lower correspondence

Table 3.2.E.
Characteristic of MIMS in comparison with HS-GC and P&T [46]

Characteristic	MIMS	HS-GC	P&T
Detection limit (μg/l)	<1	<1	1–10
Linear dynamic range	10^4	10^2	10^6
Repeatability (%)	1–11	2–13	1–8
Analysis time (min)	5–10	35–45	35–45
On-line monitoring capability[a]	+++	+++	++[b]
Simplicity of instrumentation[a]	++	+	+++

[a] +++, very good; ++, good; +, fair
[b] Flame ionisation detector used

the high vacuum of the ion source. Techniques like laser desorption are under investigation to deal with this problem [48].

References

1. C. Barthelemy, P. Di Martino and A.-M. Gyot-Hermann, *Pharmazie* **50**, 607–609 (1995)
2. A. Ettabia, C. Barthelemy, M. Ibilou and A.M.G. Hermann, *Pharmazie* **53**, 563–567 (1998)
3. D.R. Morello and R.P. Meyers, *J. Forens. Sci*. **40**, 957–963 (1995)
4. S. Jacobsson and A. Hagman, *Drug Dev. Ind. Pharm*. **16**, 2547–2560 (1990)
5. S. Jacobsson, *J. High Resolut. Chromatogr.* **7**, 185–190 (1984)
6. C. Witschi and E. Doelker, *Eur. J. Pharm. Biopharm.* **43**, 215–242 (1997)
7. A. Artiges, Report on the ICH 4 Conference in Brussels, *Pharmeuropa* **9**, 479–490 (1997)
8. K. Watanabe, H. Seno, A. Ishii and O. Suzuki, *Anal. Chem.* **69**, 5178–5181 (1997)
9. H.J. Stan and M. Linkerhaegner, *J. Chromatogr. A* **750**, 369–390 (1996)
10. J.C. Bosboom, H.-G. Janssen, H.G.J. Mol and C.A. Cramers, *J. Chromatogr. A* **724**, 384–391 (1996)
11. B.S. Kersten, *J. Chromatogr. Sci*. **30**, 115–119 (1992)
12. C.E. Koester and R.E. Clement, *Crit. Rev. Anal. Chem.* **24**, 263 (1993)
13. C.F. Poole and S.A. Schuette, *J. High Resol. Chromatogr.* **6**, 526–549 (1983)
14. L. Hong and H.R. Altorfer, *Pharm. Acta Helv*. **72**, 95–104 (1997)
15. R.B. George and P.D. Wright, *Anal. Chem.* **69**, 2221–2223 (1997)
16. K.J. Mulligan and H. McCauley, *J. Chromatogr. Sci*. **33**, 49–54 (1995)
17. N. Kumar and J.G. Gow, *J. Chromatogr. A* **667**, 235–240, (1994)
18. Z. Penton, *J. High Resolut. Chromatogr*. **15**, 834–836, (1992)
19. K.J. Denis, P.A. Josephs and J. Dokladalova, *Pharm. Forum* **18**, 2964–2972 (1992)
20. G. Wynia, P. Post, J. Broersen and F.A. Maris, *Chromatographia* **39**, 355–362 (1994)
21. B. Kolb and L.S. Ettre, *Chromatographia* **32**, 505–513 (1991)
22. D. Herlitz, G. Herold and H. Nufer, *Pharm. Ind.* **49**, 200–203 (1987)
23. D. Herlitz, G. Herold, W. Neckel and H. Nufer, *Pharm. Ind.* **50,** 251–256 (1988)
24. A. Hagmann and S. Jacobsson, *Anal. Chem*. **61**, 1202–1207 (1989)
25. P. Klaffenbach, C. Coors, D. Kronenfeld, C. Brüse and H.-G. Schulz, *LC-GC Int.* **11**, 166–174 (1998)

26. H. Grote and G. Leugers, *Fres. Z. Anal. Chem*. **327**, 782–785 (1987)
27. M. Markelow and J.P. Guzowski, *Anal. Chim. Acta* **276**, 235–245 (1993)
28. J. Schubert, *Anal. Chem*. **68**, 1317–1320 (1996)
29. A.C. Bicchi and A. Bertolino, *Il Farmaco ed. Pr*. **37**, 88–97 (1982)
30. A.G. Vitenberg, *J. Chromatogr*. **556,** 1–24 (1991)
31. A.G. Vitenberg and B.V. Ioffe, *J. Chromatogr*. **471**, 55–60 (1989)
32. J. Drozd, Z. Vodakova and J. Novak, *J. Chromatogr*. **354**, 47–57 (1986)
33. R. Kostiainen, *Chromatographia* **38**, 709–714 (1994)
34. S.M. Abeel, A.K. Vickers and D. Decker, *J. Chromatogr. Sci*. **32**, 328–338 (1994)
35. T.P. Wampler, *LC-GC Int*. **11,** 653–658 (1998)
36. J. C. Caire, *S.T.P. Pharma Pratiques* **1**, 267–271 (1991)
37. Th.C. Voice and B. Kolb, *J. Chromatogr. Sci*. **32**, 306–311 (1994)
38. M. Sugimoto, H. Suzuki, K. Akinomoto, A. Kuchiki and H. Nakagawa, *Chem. Pharm. Bull*. **43**, 2010–2013 (1995)
39. N. Perchiazzi and R. Ferrari, *Boll. Chim. Farm*. **135,** 434–441 (1996)
40. Application notes from Supelco: New Carboxen TM/PDMS Fibre improves Collection of volatiles; SPME/Capillary GC Analysis of Drugs; Alcohols and Organic Solvents in Biological Fluids; Monitor Organic Volatile Impurities (OVIs) in pharmaceutical products, using SPME/Capillary GC
41. B. Mc. Gillivray, J. Pawliszyn, P. Fowlie and C. Sagora, *J. Chromatogr. Sci*. **32**, 317–322 (1994)
42. C.C. Camarasu, M. Mezei-Szüts and G.B. Varga, *J. Pharm. Biomed. Anal.* **18**, 623–638 (1999)
43. P. Billot and P. Pitard, *J. Chromatogr.* **623**, 305–313 (1992)
44. *Pharmeuropa* **2**, 142–146 (1990); **7**, 111–113 (1995); **5**, 145–148 (1993); **5**, 148–151 (1993)
45. K.J. Mulligan, T.W. Brueggemeyer, D.F. Crockett and J.B. Schepman, *J. Chromatogr. B* **686**, 85–95 (1996)
46. R.A. Ketola, V.T. Virkki, M. Ojala, V. Komppa and T. Kotiaho, *Talanta* **44**, 373–382 (1997)
47. R. Kostiainen, T. Kotiaho, I. Mattila, T. Mansikka, M. Ojala and R. A. Ketola, *Anal. Chem*. **70**, 3028–3032 (1998)
48. M.H. Soni, J.H. Callahan and S.W. McElvany, *Anal. Chem*. **70**, 3103–3113 (1998)

3.3. NMR spectroscopy

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Most of the theoretical and experimental aspects of detecting and quantifying solvent signals by NMR follow directly from Chapter 2.4. However, some issues deserve special note or emphasis.

With regard to the problem of determining residual solvents in a drug substance, we must emphasise the need for extreme care in sample preparation procedures, since minute amounts of solvents may easily (but invisibly) contaminate insufficiently dried glassware. In NMR, residual solvent signals are typically identified on the basis of their characteristic chemical shifts and coupling patterns. These parameters may vary quite significantly depending on the medium in which the NMR measurement is performed, as illustrated in Table 3.3.A. The data in Table 3.3.A were measured in house on a Varian $^{\text{UNITY}}$*INOVA* instrument operating at 500 MHz ^{1}H frequency, with small amounts of ''residual'' solvent being individually mixed into four different NMR solvents. A similar collection of data is also available [1], the main difference being that those data were measured by using solvent mixtures.

Residual solvents are notorious for their long T_1 relaxation times owing to their fast molecular tumbling rates. This may prove to be a major difficulty in directly measuring their (small) ^{13}C signals, since the general insensitivity of ^{13}C detection typically requires a large number of scans, and the efficiency of accumulation depends greatly on how long we must wait after a pulse for spins to relax before the next pulse can be delivered. Non-dipolar relaxation effects may also contribute to this problem, e.g. the spin-rotation mechanism [2] often significantly reduces the intensity of the ^{13}C signal due to methyl groups.

Deuterated NMR solvents always contain small amounts of non-deuterated isotopomers whose ^{1}H signal is typically well detectable, and it will not be obvious whether this signal may partly come from a residual solvent present in the drug substance. It is therefore useful to record the spectrum in different NMR solvents when searching for residual solvent signals. Small ^{1}H solvent peaks are vulnerable to remain undetected due to spectral overlap. If at least one ^{1}H signal of the solvent is clearly visible, this signal can often be used to locate its other scalar-coupled partners, e.g. via a 1D TOCSY experiment [3] (cf. example 2.4.7.1 in Chapter 2.4). Ambiguities remaining as to the identity of the solvent may also be resolved (at least in the case of organic solvents) on the basis of their ^{13}C chemical shifts. Interestingly, rather than directly recording a ^{13}C spectrum, in modern NMR this piece of information often becomes more economically and more reliably available from an HSQC experiment [4–6], as was shown in examples 2.4.7.1 and 2.4.7.3 in Chapter 2.4.

Table 3.3.A.
^{1}H and ^{13}C chemical shifts and ^{1}H multiplicities for several residual solvents as measured in the most commonly used deuterated NMR solvents

Residual solvent/ NMR solvent	Chloroform-D_1		Dimethyl sulphoxide-D_6		Methanol-D_4		Deuterium oxide	
	$^{1}H^{e}$	$^{13}C^{a}$	$^{1}H^{e}$	$^{13}C^{b}$	$^{1}H^{e}$	$^{13}C^{c}$	$^{1}H^{f}$	$^{13}C^{d}$
Methanol	3.48 s	50.6	4.06 q 3.17 d	48.5	3.35 s 4.79 s	49.9	3.34 s	51.6
Ethanol	3.71 q 1.24 t	58.3 18.3	3.44 m 1.06 t 4.31 t	56.0 18.5	3.60 q 1.17 t 4.79 s	58.3 18.4	3.64 q 1.17 t	60.1 19.5
Diethyl ether	3.48 q 1.21 t	65.8 15.2	3.39 q 1.09 t	64.8 15.1	3.49 q 1.17 t	66.9 15.4	3.56 q 1.17 t	68.7 16.8
Ethyl acetate	4.12 q 1.26 t 2.04 s	60.3 14.2 21.0 171.1	4.03 q 1.18 t 1.99 s	59.6 14.0 20.6 170.2	4.10 q 1.24 t 2.01 s	61.5 14.5 20.8 173.0	4.13 q 1.24 t 2.07 s	64.4 16.0 23.2 177.4
1-Propanol	3.60 t 1.59 m 0.94 t	64.6 25.9 10.1	3.34 m 1.42 m 0.84 t 4.31 t	62.4 25.6 10.3	3.50 t 1.54 m 0.92 t 4.79 s	64.7 26.7 10.6	3.54 t 1.53 m 0.88 t	66.3 27.3 12.2

2-Propanol	4.02 sp 1.21 d	64.4 25.3	3.78 m 1.04 d 4.30 d	61.9 25.4	3.92 sp 1.15 d 4.79 s	64.8 25.3	4.00 sp 1.16 d	66.9 26.4
1-Butanol	3.65 t 1.56 m 1.39 m 0.94 t	62.7 34.9 18.9 13.8	3.38 m 1.39 m 1.30 m 0.87 t 4.27 t	60.3 34.6 18.6 13.7	3.54 t 1.51 m 1.38 m 0.93 t 4.79 s	62.7 35.8 20.0 14.2	3.59 t 1.51 m 1.33 m 0.89 t	64.3 36.1 21.1 15.7
Cyclohexane	1.43 s	26.9	1.40 s	26.3	1.45 s	28.0		
n-Hexane	0.88 m 1.29 m 1.27 m	14.1 22.7 31.6	0.86 m 1.26 m 1.25 m	13.8 22.0 30.9	0.90 m 1.30 m 1.29 m	14.4 23.7 32.7		
Acetonitrile	2.00 s	1.8 116.3	2.07 s	1.1 117.9	2.03 s	0.8 118.1	2.06 s	3.5 121.9
Acetone	2.17 s	30.8 206.7	2.08 s	30.6 206.3	2.15 s	30.7 210.0	2.22 s	32.9 218.1
Acetic acid	2.10 s 11.2 vbr	30.7 210.0	1.91 s 11.91 br	21.0 171.9	1.99 s 4.89 br	20.7 175.2	2.08 s	23.1 179.4
Dichloromethane	5.30 s	53.4	5.74 s	54.8	5.48 s	55.3	5.45 s	56.6

Table 3.3.a. (*continued*)

Residual solvent/ NMR solvent	Chloroform-D_1		Dimethyl sulphoxide-D_6		Methanol-D_4		Deuterium oxide	
	$^1H^e$	$^{13}C^a$	$^1H^e$	$^{13}C^b$	$^1H^e$	$^{13}C^c$	$^1H^f$	$^{13}C^d$
Chloroform	7.26 s	77.2	8.30 s	79.1	7.87 s	79.4		
1,4-Dioxan	3.70 s	67.1	3.57 s	66.3	3.66 s	68.1	3.75 s	69.3
Tetra-hydrofuran	3.74 m 1.85 m	67.9 25.6	3.60 m 1.76 m	66.9 25.0	3.72 m 1.87 m	68.9 26.5	3.74 m 1.87 m	70.5 27.7
N,*N*-Dimethyl-formamide	2.88 s(d) 2.96 s(d) 8.02 br	31.3 36.4 162.4	2.73 s(d) 2.89 s(d) 7.95 br	30.7 35.7 162.2	2.86 s(d) 2.99 s(d) 7.98 br	31.7 36.9 164.9	2.85 s(d) 3.01 s(d) 7.93 br	34.1 39.6 167.6
Dimethyl sulphoxide	2.62 s	40.9	2.54 s	40.4	2.65 s	40.5	2.71 s	41.4
Benzene	7.35 s	128.3	7.36 s	128.2	7.32 s	129.3		
Benzyl alcohol	4.67 s 7.35 m 7.35 m 7.29 m	65.3 140.9 127.0 128.5 127.6	4.49 d 7.31 d 7.31 d 7.22 m 5.13 t	62.8 142.4 126.3 127.9 126.5	4.59 s 7.33 m 7.32 m 7.24 m 4.79 s	65.2 142.7 128.0 129.3 128.2	4.63 s 7.41 m 7.44 m 7.39 m	66.6 142.9 130.2 131.4 130.5

Toluene	2.35 s	21.4	2.30 br	20.9	2.31 br	21.5
		137.9		137.3		138.9
	7.17 m	129.0	7.17 m	128.8	7.14 m	129.9
	7.25 m	128.2	7.25 m	128.1	7.21 m	129.2
	7.15 m	125.3	7.14 m	125.2	7.10 m	126.3

[a] $CDCl_3$ centre signal at **δ** 77 (t, $J_{CD} = 31.8$ Hz)
[b] DMSO-D_6 centre signal at **δ** 39.5 (sp, $J_{CD} = 21$ Hz)
[c] CD_3OD centre signal at **δ** 49.0 (sp, $J_{CD} = 21.4$ Hz)
[d] Reference DSS (**δ** 0.0, 17.7, 21.8, 57.1)
[e] Reference TMS (**δ** 0.0)
[f] Reference DSS [**δ** 0.0(s),0.63(m),1.76(m),2.90(m)]

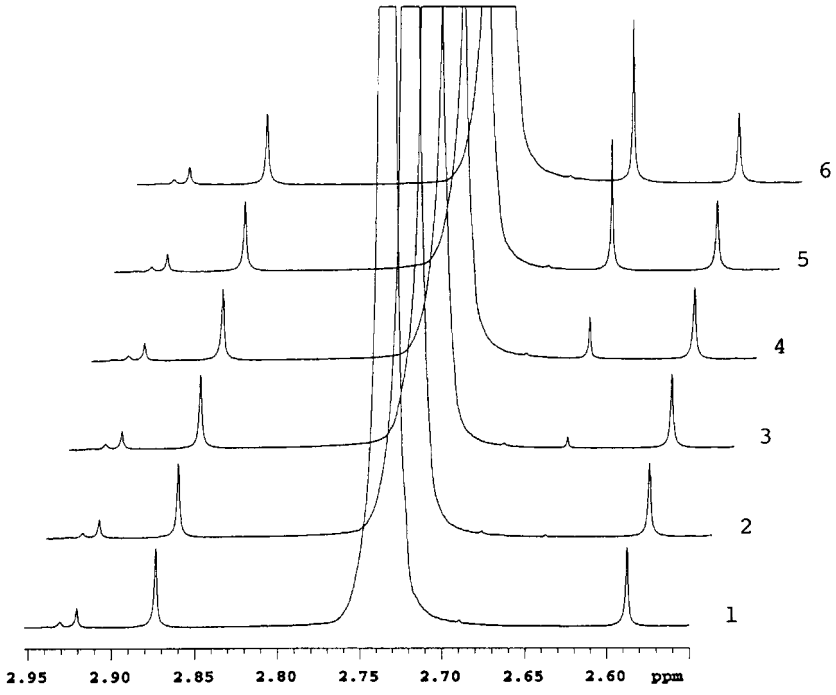

Figure 3.3.A.
NMR spectra of a drug substance spiked with dimethylsulphoxide. (1): 0 ppm; (2): 10.5 ppm; (3): 62.8 ppm; (4): 272 ppm; (5): 795 ppm; (6): 1004 ppm. Solvent: methanol-D_4

Chemical exchange can have a conspicuous effect on the appearance of the ^{1}H signals of solvents with labile protons. E.g. the ^{1}H spectrum of methanol consists in most cases of two singlets due to the OMe and OH protons, respectively. This appearance of the MeOH spectrum may become visually so familiar, that the less commonly observed doublet and quartet multiplicities of the respective resonances in the case of slow exchange may cause some momentary identification problems for the spectroscopist. Both manifestations of the MeOH spectrum can be seen in any NMR solvent, depending on a number of parameters that can influence exchange rates and which are specific to the solution in question. Nevertheless, slow exchange rates are more typically observed in DMSO-D_6 than in, say, $CDCl_3$.

As for the quantification of minor solvents, one of the main difficulties comes, once again, from the slow relaxation of solvent spins, necessitating interpulse delays that may be as long as several minutes. Other than that, all

nuances relating to the quantification of small peaks were already discussed in detail in Chapter 2.4, particularly in example 2.4.7.1.

As an example characterising the possibilities of NMR spectroscopy in the quantitative determination of residual solvents in drugs, the ^{1}H-NMR spectra of a drug material containing an N-CH_3 group spiked with various amounts of dimethylsulphoxide are shown in Fig. 3.3.A. As it is seen, on the basis of the singlet of dimethylsulphoxide at δ 2.65, 10 ppm of dimethylsulphoxide is still detectable and above 50 ppm the integrals of this peak are suitable for quantitation. (Other peaks in the spectrum are that of the N-CH_3 group at δ 2.74 with its ^{13}C satellites at δ 2.59 and δ 2.87, respectively.) This means that in advantageous cases NMR spectroscopy can be considered as a good complement to the most widely applicable GC-(MS) method, especially in the case of less volatile solvents such as dimethylsulphoxide where the GC test is difficult to carry out.

Acknowledgements

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References

1. H.E. Gottlieb, V. Kotlyar and A. Nudelman, *J. Org. Chem.* **62**, 7512–7515 (1997)
2. M.L. Martin, J.-J. Delpuech and G.J. Martin, *Practical NMR Spectroscopy,* Heyden, London (1980)
3. T.C. Wong, in *Methods of Structure Elucidation by High-Resolution NMR* (Gy. Batta, K.E. Kövér and Cs. Szántay Jr., Eds.), Elsevier, New York, pp 131–147 (1997)
4. L.E. Kay, P. Keifer and T. Saarinen, *J. Am. Chem. Soc.* **114**, 10663–10665 (1992)
5. A.G. Palmer III, J. Cavanagh, P.E. Wright and M. Rance, *J. Magn. Reson.* **93**, 151–170 (1991)
6. G. Kontaxis, J. Stonehause, E.D. Laue and J. Keeler, *J. Magn. Reson.* **111A**, 70–76 (1994)

3.4. Miscellaneous

Sándor Görög

The loss of drying or the total amount of volatile impurities determined by thermogravimetric methods (Section 3.1) presents the sum of water and residual solvents in the bulk drug material. If the knowledge of the *water* content is necessary, the pharmacopoeias usually prescribe its determination by modern variants of the classical titrimetric Karl Fischer method [1–4]. The titrant (Karl Fischer reagent [1–3], also called iodosulphurous reagent [4]) is prepared by dissolving iodine and sulphur dioxide in the mixture of dry methanol and pyridine. The reaction between iodine and sulphur dioxide takes place only in the presence of more or less stoichiometric amount of water and on the basis of this the titrimetric determination of the latter in drug substances dissolved in an appropriate solvent (usually methanol) is possible if a suitable endpoint detection method is found. Although the use of the classical visual endpoint detection is not excluded [1–3], more generally amperometric titration is carried out [2–4]. For the determination of very small amounts of water (0.0001–0.1%) the coulometric variant of the method can be used where the iodine is generated by anodic oxidation [3].

It has to be noted that in the course of the gas chromatographic determination of residual solvents water cannot be estimated if the most generally used flame ionisation detector (FID) is used. Since the knowledge of the water content at the trace level is usually not necessary, this problem is in the majority of cases neglected. If a thermal conductivity detector (TCD) is applied, traces of water can be determined together with the organic solvents using the general gas chromatographic techniques, e.g. the headspace method [5] for residual solvents.

In some special instances, e.g. in the case of tablet formulations containing organic acids and sodium hydrogen carbonate, it is just the quantity of water, sometimes even at the trace level, which determines, e.g. the stability of a drug preparation. For example, in a study described by Wasiak and Szczepaniak [6] water was determined in a tablet formulation containing tartaric acid and sodium hydrogen carbonate. It was extracted by dichloromethane making use of the polyethylene glycol content of the tablet and determined down to the 0.1% level by packed column gas chromatography using Porapak Q and TCD.

In addition to the above mentioned detection, another (not very frequently occurring) problem with the generally adopted gas chromatographic determination of residual solvents can be that some of these solvents are not sufficiently volatile among the usual conditions of the GC assays (See Section 3.2).

For example, the quantity of *dimethylsulphoxide* which is the reaction solvent in the final step of the synthesis of *bisnafide* had to be controlled in the bulk drug material. Walker *et al.* [7] found it reasonable to develop a reversed-phase HPLC method with UV detection at 215 nm for this purpose. The limits of detection and quantitation, respectively, were 25.5 and 109.5 ppm dimethylsulphoxide.

References

1. K. Fischer, *Angew. Chem.* **48**, 394–399 (1935)
2. E. Scholz, *Karl Fischer Titration Determination of Water*, Springer, Berlin (1984)
3. *The United States Pharmacopoeia 24,* pp 2003–2005. USP Convention Inc., Rockville, MD (2000)
4. *European Pharmacopoeia*, Council of Europe, Strasbourg, p 66 (1997)
5. B. Kolb and M. Auer, *Fres. J. Anal. Chem.* **336**, 291–302 (1990)
6. W. Wasiak and W. Szczepaniak, *Acta Pol. Pharm. Drug. Res.* **50**, 423–426 (1993)
7. J.T. Walker, D.L. Paolini and J. Segretario, *J. Chromatogr. Sci.* **34**, 513–516 (1996)

Chapter 4

IDENTIFICATION, SEMIQUANTITATIVE AND QUANTITATIVE DETERMINATION OF INORGANIC IMPURITIES

4.1. Classical Methods

Sándor Görög

4.1.1. General remarks

The various types of inorganic impurities in drug substances and the reasons for their occurrence was discussed in Section 1.2.3.

The reason for setting limits for inorganic impurities is on the one hand the toxicity of some of them (mercury, arsenic, hydrazine, etc.). On the other hand, the level of non-toxic inorganic impurities in a drug substance is a good indication for the level of the manufacturing/purification procedure. This was especially important in the classical period of pharmaceutical analysis when the modern chromatographic methods for the detection and quantitation of related organic impurities were not yet available. At that time semiquantitative limit tests for chloride, sulphate and heavy metals were among the important features to characterise the purity of the drug substance (in addition to the value and sharpness of the melting point.) Although the importance of limit tests for non-toxic inorganic impurities as the indicators of the purity of drugs has decreased considerably in our time, even the latest issues of pharmacopoeias [1–3] contain these tests.

Most of the methods used for the detection/determination of inorganic impurities in drug substances even in the modern pharmacopoeias are classical methods based on simple chemical reactions leading to precipitation/opalescence or coloured products [4–6]. The latter can be the basis for limit tests based on visual comparison of the colour with a standard or the absorbance of the coloured solution can be measured by a spectrophotometer thus creating the

basis for a quantitative determination. The selectivity and the sensitivity of the determination of metal impurities can be greatly improved by using atomic spectroscopic methods (Section 4.2). Ion chromatographic, capillary electrophoretic and electroanalytical methods are also in use (Section 4.3).

4.1.2. Residue on ignition

The most general and most rapid method to get a picture about the over-all inorganic impurity content of a bulk substance is the determination of the residue on ignition. One to two g of the material is gently ignited in a weighed (usually platinum) crucible until charred. The residue is then moistened with concentrated sulphuric acid followed by removing the excess of sulphuric acid by gentle heating. The residue (often called *sulphated ash*) is heated at a temperature around 800°C to constant weight, cooled in a desiccator and weighed. The residue on ignition is the difference between the two weightings. The limit specified for the residue on ignition in the majority of the monographs in pharmacopoeias is 0.1%.

4.1.3. Chloride

The semiquantitative limit test for chlorides is based on the formation of silver chloride precipitation upon addition of silver nitrate reagent to the aqueous solution of the sample to be tested acidified with nitric acid. The turbidity/opalescence of the resulting solution is compared visually by viewing against a black background with that of a standard solution containing known quantity of chloride. Bromide and iodide ions also give positive reaction.

The volume of the test solution and the weight of the sample prescribed by the pharmacopoeias are usually in the order of 10–40 ml and a few hundreds of milligrams, respectively. The limit is set to different values in the various paragraphs. The sensitivity of the test is very good: 1 μg chloride in 10 ml volume is detectable [4a]. This enables even the lowest required level (20–30 ppm chloride) to be estimated.

4.1.4. Fluoride

Traces of fluoride ions in drug materials can be estimated by various colorimetric methods [4b,6a]. The European Pharmacopoeia uses a reagent containing the cerium(III) -aminomethylalizarindiacetic acid complex which changes its colour from red to blue in the presence of fluoride ions. Prior to this

test based on visual comparison of the colours, test and standard solutions fluoride ions had to be separated from the sample by steam distillation from strongly acidic solution as hydrogen fluoride [1,2]. This method is very sensitive and enables fluoride to be determined in the ppm range.

4.1.5. Sulphate

The limit test for sulphate is based on the formation of insoluble barium sulphate when barium chloride reagent is added to the solution of the test material acidified with hydrochloric acid. The visual comparison of the turbidities of test and standard solutions is carried out in a similar manner as described for chloride. The sensitivity of the estimation is lower but the method is still sensitive enough to meet the requirements, which is usually in the order of a few hundred ppm.

4.1.6. Phosphate

The quantitative or semiquantitative determination of phosphate ions in drugs (usually phosphate esters) is based on the formation of coloured heteropolyacids [4b,6c]. For example, free phosphate ions in prednisolone sodium phosphate are determined on the basis of the yellow colour of the molybdovanadophosphate formed upon the addition of the molybdovanadate reagent (visual colour comparison) [1a]. The molybdenum blue method (formation of molybdophosphoric acid and its reduction with various reducing agents to a blue complex) is used among others for the determination of phosphate ions in hydrocortisone and in hydrocortisone sodium phosphate (reducing agent methylaminophenol-sulphite; spectrophotometric measurement at 730 nm) [3a], in thioguanine (reducing agent aminonaphtolsulphonic acid, measurement at 620 nm) [3b]. The limit in the latter case is set to 300 ppm. Higher sensitivity is obtainable by the general test where the reagent is sulphomolybdic acid and the reducing agent is tin(II)chloride [1,2].

4.1.7. Sulphite

As low a level as 15 ppm of sulphite (as sulphur dioxide) in glucose is measurable at 583 nm by means of the decolourised fuchsin-formaldehyde reagent [1b].

4.1.8. Heavy Metals

4.1.8.1. General Method

The general limit test for heavy metals is based on the formation of insoluble coloured (usually brown) precipitate when these ions are reacted in moderately acidic solution (pH 3–4) with hydrogen sulphide [3] or thioacetamide which hydrolyses under the conditions of the preparation of the reagent to form hydrogen sulphide [1,2]. If the concentration of the metal ions is very low, a colloidal solution of the forming metal sulphides is obtained: the basis of the comparison of the test and standard solutions is the brown colour of the solutions. As the standard lead(II)nitrate solution is used. In the case of water-insoluble materials the test can be carried out in dioxan or acetone [1,2] or after ignition [1–3] or wet digestion with nitric acid [3]. The reaction is sensitive enough to the test in which the limit is usually set to 10–20 ppm (expressed as lead). For more selective, sensitive and accurate measurements other, usually atomspectroscopic methods are used.

4.1.8.2. Lead

For the selective and sensitive spectrophotometric determination of traces of lead the dithizone method is used [5,6c]. The limit test is based on the selective extraction with chloroform of lead as the dithizone complex in slightly alkaline citrate buffer in the presence of hydroxylamine and visual comparison of the violet colour of the test and standard solutions [3].

4.1.8.3. Iron

Of the large variety of colorimetric methods for the determination of iron [5,6d] the formation of the red complex of iron(III) with thiocyanate ions is generally used for its selective and sensitive determination. The limit test is based on the visual comparison of the colours of test and standard solutions [3]. Any iron(II) should be oxidised to iron(III) prior to the colour reaction. Another colour reaction used in a limit test uses mercaptoacetic acid as the complex forming reagent [1,2].

4.1.8.4. Mercury

Of the several colour reactions of mercury [5,6e] the formation of its dithizonate in strongly acidic solution easily extractable with chloroform is adopted for the determination [3]. The test and reference solutions, respectively, are titrated in a separator with the chloroformic solution of dithizone

discarding the extract after each increment. At the endpoint the colour of the last extract turns to the green colour of dithizone from the orange-yellow colour of the mercury(II)dithizonate complex [6e].

4.1.9. Other Metals

4.1.9.1. Potassium

The limit test includes the comparison of the opalescence produced with sodium tetraphenylborate reagent with that of a standard solution [1,2].

4.1.9.2. Alkaline-Earth Metals

Microtitration with 0.01 M disodium edetate using mordant black as the indicator is suitable for the determination of alkaline-earth metals even at the impurity level [1,2]. A more sensitive determination of *magnesium* (down to the ppm level) is based on its extraction from slightly alkaline solution with the chloroformic solution of 8-hydroxyquinoline containing triethanolamine and *n*-butylamine [1,2,6f]. For the limit test of *calcium* ammonium oxalate solution leading to calcium oxalate precipitate/opalescence is used [1,2]. *Barium* is estimated on the basis of the opalescence produced with sulphuric acid.

4.1.9.3. Aluminium

The very sensitive determination of traces of *aluminium* (about 1 ppm) depends on the measurement of the fluorescence of its complex with 8-hydroxyquinoline after extraction with chloroform (excitation at 392 nm, emission at 518 nm) [1,2].

4.1.10. Arsenic

Due to its high toxicity sensitive methods are required for the determination of as low an impurity level of arsenic as 1 ppm in drug materials. Of the several colorimetric methods [6g] the silver diethyldithiocarbamate method is adopted by the United States Pharmacopoeia [3]. The arsenic content is transformed to arsine by nascent hydrogen generated by zinc/sulphuric acid. This is passed through a solution of silver diethyldithiocarbamate to form a red complex absorbing at 535–540 nm.

Similar sensitivity is attainable by the method adopted by the European

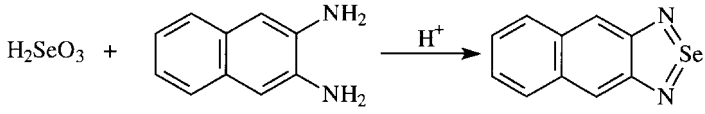

Figure 4.1.A.
Equation for the spectrophotometric determination of selenium

Pharmacopoeia [1]. Here the arsine generated in a similar manner gets in contact with a disk of filter paper impregnated with mercuric bromide where arsine produces a coloured spot (yellow $H(HgBr)_2As$ and black Hg_3As_2) which is the basis for the limit test [6]g. The reagent of a much simpler but much less sensitive test is potassium iodide/hypophosphorous acid [1,2].

4.1.11. Miscellaneous

The reagent for the selective determination of *selenium* after the combustion of the sample is 2,3-diaminonaphtalene; λ_{max} 380 nm [3]. The reaction equation [6h] is shown in Fig. 4.1.A.

A simple but insensitive test for *ammonium ion* is carried out by the estimation with litmus paper [1c] or silver manganese paper [1,2] of the ammonia evolving at 40°C on the effect of heavy magnesium oxide. In a much more sensitive test the reagent is alkaline tetraiodomercurate and the basis of the limit test is the forming yellow colour [1,2].

Traces of *hydrazine*, e.g. in isonicotinoyl hydrazide can be determined at 456 nm after hydrazone formation with 4-dimethylaminobenzaldehyde [7]. Similar reactions with other aldehydes such as benzaldehyde [3c] or salicylaldehyde [1d] are the basis for the determination of hydrazine after the HPLC or TLC separation of the hydrazone derivatives. A gas chromatographic method is presented in Section 5.5.4.

Peroxides (expressed as hydrogen peroxide) are determined spectrophotometrically at 405 nm after a colour reaction with the titanium(III)chloride–sulphuric acid reagent [1e].

References

1. *European Pharmacopoeia, 3rd edn*, Council of Europe, Strasbourg, pp 51–55, (1997). a, p 1382; b, p 903; c, p 409; d, p 1370; e, p 1371
2. *British Pharmacopoeia 1998*, pp A151–A155, The Stationery Office, London (1998)

3. *The United States Pharmacopoeia 24*, USP Convention Inc., Rockville, MD, pp 1857–1863 (2000). a, p 831; b, p 1646; c, p 818
4. D.F. Boltz and J.A. Howell (Eds.), *Colorimetric Determination of Nonmetals*, Wiley, New York (1978). a, p 93; b, p 109; c, p 337
5. K. Burger, *Organic Reagents in Metal Analysis*, Pergamon Press, Oxford (1973)
6. Z. Marczenko, *Separation and Spectrophotometric Determination of Elements*, Ellis Horwood, Chichester (1986). a, p 272; b, p 447; c, p 343; d, p 327; e, 371; f, p 357; g, p 149; h, p 508
7. A.G. Davidson, *Analyst* **107**, 422–427 (1982)

4.2. Atomic Spectroscopy

Alexandra Lásztity

4.2.1. Introduction

Atomspectrometric techniques are selective, sensitive methods for the quantitative determination of metals [1]. They are commonly used for the detection and determination of metals applied as catalysts (e.g. Pd, Ni, Pt, Rh) in the synthesis of pharmaceuticals or metal impurities e.g. Fe, Cr that might arise from stainless steel reaction vessels [2,3]. There are stringent requirements for maximum allowable quantities of impurities in drug substances or products. The sensitivity and selectivity of heavy metal tests of pharmacopoeias (see Section 4.1.8) in many cases do not meet the recent requirements.

4.2.2. Palladium

Various atomspectrometric methods were used for the determination of palladium in drug materials. *Graphite furnace atomic absorption spectrometry (GFAAS)* is a suitable technique for this purpose. It has improved detection capabilities over *flame atomic absorption spectrometry (FAAS).* Wang *et al*. [4] developed a simple, sensitive, accurate and relatively inexpensive Zeeman GFAAS method for the determination of Pd in two drug materials, MK0476 and MK0462 and their synthetic intermediates. A simple sample preparation by dissolution in 70% nitric acid was used because the analysed samples were not soluble in 2% nitric acid. The absorbance was measured at 247.6 nm. The authors claim that high nitric acid level is applicable for GFAAS. The system erosion was negligible, and no matrix effects were observed. The limit of detection (LOD) and limit of quantification (LOQ) were 0.7 and 2 ppm, respectively. Good precision (RSD around 5%) was obtained for these samples at the Pd level close to the LOQ. The analytical recovery of 0.02 μg/ml Pd spike in these substances was between 94 and 109%.

Lévai *et al*. [5] applied a direct GFAAS method with deuterium arc background correction for determination of Pd in enalapril maleate, ethionamide, terazosin hydrochloride and fluoxetine hydrochloride. The samples of enalapril maleate and fluoxetine HCl were dissolved in 0.2 M HNO_3 and 75% methanol/

0.2 M HNO_3. The absorbance was measured at 247.6 nm. The background signal was low after pyrolysis step (1100°C and 30 s hold time): the matrix was decomposed. The analytical recovery of 0.5–2.0 ppm Pd spikes was close to 100%. Slopes of calibration and standard addition graphs in 5% sample solutions of enalapril maleate and ethionamide agreed within experimental errors which means no need for standard addition for determination of Pd. The detection limit was 0.1 ppm. A flow-injection (FI) microcolumn preconcentration method was developed prior to GFAAS determination of Pd using oxime cellulose and iminodiacetic acid ethylcellulose (IDAEC) chelating exchangers. The retained Pd was easily eluted by 0.1 M thiourea/HNO_3 solution. The palladium was retained on chelating celluloses only from terazosin hydrochloride solutions. The percentage recovery of 0.3 ppm Pd spike in 1% terazosin HCl solution at 20-fold enrichment was 95% using calibration curve made by preconcentration of aqueous standards.

The European Pharmacopoeia 3rd Supplement 1999 [6a] also prescribes a direct GFAAS method for the Pd determination in ramipril proposing $Mg(NO_3)_2$ chemical modifier in nitric acid. The limit of Pd content is 20 ppm.

Inductively coupled plasma–mass spectrometry (ICP/MS) was used for the determination of palladium in fosinopril sodium [7]. A simple, rapid and sensitive method was developed without sample preparation. The compound was dissolved in 25:75 (v/v) 2-butoxyethanol/water mixture containing 50 ng/ml indium as internal standard for ICP/MS measurement. The sample concentration was 0.1%. Because of use of organic solvent during ICP/MS measurements the use a platinum sampling cone, a cooled spray chamber and solvent resistant tubes were necessary. A matrix effect was not observed. The precision at 5.5 ng/ml spike was 1.25%. The analytical recovery of Pd spike in the 1.25-40 ng/ml concentration range was between 96 and 107%. The LOQ is 0.1 ppm. ICP/MS and *axial inductively coupled plasma–atomic emission spectrometric (ICP/AES)* methods were proposed for the determination of trace level quantities of Pd in drug substances [8]. The 25:75 v/v mixture of butoxyethanol and water was used also in this case for the dissolution of the compounds in a 0.1% sample concentration. Axial ICP/AES has an improved sensitivity over radial ICP/AES and fewer spectral interferences, but it is more expensive. Internal standard for axial ICP/AES was yttrium and for ICP/MS indium. For both techniques background effects become significant below 5 ng/ml. Relative standard deviations at 5 ppm concentration were 6.36% and 3.41% for axial ICP/AES and ICP/MS, respectively. LOQ was 5 ppm for both techniques.

Of the three techniques GFAAS can provide the required sensitivity with low sample consumption, can handle organic matrices reasonably well, usually without need for sample preparation. If the substance is soluble in water or water/methanol or ethanol mixtures the sample matrix can be decomposed in graphite furnace without loss of the analyte. At higher sample concentrations

(5–10%) it is possible to work without the decomposition of the matrix. Disadvantages of this method are that it is slow and requires special spectral lamp. The analysis of other organic solutions such as butoxyethanol/water mixtures is difficult to perform by autosampler sample introduction. FAAS and ICP/AES in this concentration range can be used after preconcentration of the palladium. Using plasma techniques the concentration of the substance is limited, addition of internal standard is necessary. ICP/MS with high sensitivity is eminently suitable for palladium analysis because no severe isobaric interferences occur but this method is too expensive for single element determination.

4.2.3. Nickel

In a GFAAS method proposed for the European Pharmacopoeia for the determination of nickel in hydrogenated vegetable oils the absorbance of Ni is measured at 232 nm in a pyrolytically-coated tube with a platform on an atomic absorption spectrometer equipped with a Zeeman background compensation system after decomposition of the material in a nitric acid/hydrogen peroxide mixture in a microwave oven. The official sample preparation method for oils (arachis oil hydrogenated [9g], cottonseed oil hydrogenated [6b]) is ashing in a muffle furnace at about 600°C; the limit is 1 ppm nickel.

In the general limit test in Ph. Eur. for ''Ni in polyols'' (e.g. sorbitol, mannitol, maltitol, lactitol) [10a] the Ni content is determined by FAAS after extraction as a complex with pyrrolidine dithiocarbamate (PDTC) into methyl isobutyl ketone (MIBK). Higher sensitivity is attainable with GFAAS.

Atomic absorption spectrometry is used for the determination of Ni in some other drugs. In prazosin hydrochloride the prescribed limit for Ni is 50 and 100 ppm in Ph. Eur. [10b] and USP 24 [11g], respectively, and for Fe 100 ppm in both pharmacopoeias. Both metals are determined by FAAS after ignition of the substance at 800 ± 25°C using sulphuric acid as ashing aid. For the determination of nickel content in calcium stearate GFAAS after digestion in a hydrochloric acid/nitric acid mixture is the official test [10c]. The allowable concentration limit is 5 ppm.

Bermejo-Barrera *et al*. [12] developed a direct method for the determination of nickel in heroin and cocaine illicit drug samples using *electrothermal atomic absorption spectrometry (ETAAS)* with deuterium arc background correction. Cocaine and heroin samples were dissolved in 35% nitric and in water. The temperature program was optimised in presence of chemical modifiers (HNO_3, Pd, Pd + $Mg(NO_3)_2$, $Mg(NO_3)_2$). It was found that $Mg(NO_3)_2$ is the most suitable because at allowable pyrolysis temperature the matrix can be volatilised without loss of Ni. Slopes of calibration and standard addition graphs were similar, this showed the absence of matrix effects. Aqueous stan-

dards could be used for quantification. Precision (RSD%) achieved with spiked cocaine sample (0–30 μg/l) was between 6.96 and 1.48%. The analytical recovery in spiked cocaine samples was close to 100% even at 20% m/v sample concentration. The detection limit was 32 ppb and the limit of quantification 107 ppb.

4.2.4. Lead

The European Pharmacopoeia contains a general limit test for Pb in sugars [10d]. The lowest allowable Pb concentration is 0.5 ppm. The official test for lead determination describes a liquid–liquid extraction of lead(II)pyrrolidine-dithiocarbamate complex into methyl isobutyl ketone and FAAS measurement of Pb absorbance at 283.3 nm in air-acetylene flame by standard addition method. The disadvantage of this method is the high sample size (20 g), and the need for an enrichment technique because the sensitivity of the FAAS method is not sufficient for the determination of lead at this concentration range. In a new limit test for lead in sucrose [9a]. the sample is digested in nitric acid prior to GFAAS determination. The sensitivity of GFAAS is suitable for the determination of lead. A flow-injection IDAEC microcolumn preconcentration method without decomposition of matrix was developed for determination of metal impurities (Cd, Co, Ni, Pb) in sugar samples (sucrose, glucose, lactose) by GFAAS and *total reflection X-ray fluorescence spectrometry (TXRF)* [13]. At tenfold enrichment in 1–5% sample solutions the detection limits were 0.3–29 ppb.

For the determination of lead impurity in metal salts forming coloured sulphides a selective method was necessary; therefore atomic absorption spectrometry is recommended in pharmacopoeias. Direct GFAAS (e.g. for copper gluconate [11a]) or FAAS is used after liquid-liquid extraction into trioctylphosphine oxide (TOPO) from a solution containing ascorbic acid and iodide (ferrous fumarate [11b], ferrous gluconate [11c], manganese gluconate [11d]). In the case of oxprenolol hydrochloride AAS method is prescribed with a limit of 5 ppm [10e]. ETAAS method using palladium as chemical modifier was reported for lead determination in cocaine and heroin samples [14]. The optimum charring and atomisation temperatures were found to be 1000°C and 2400°C, respectively. The limit of detection was 31.4 ppb. Excellent recovery and precision were found. The lead concentration in heroin and cocaine samples were 0.64–2.77 and 0.12–0.57 ppm. To avoid sample preparation, a direct determination of lead in calcium drug samples by ETAAS with molybdenum tube atomiser was developed [15]. The calibration standards contained Pb and matrix elements ($CaHPO_4 \cdot 2H_2O$ or $CaCO_3$). After drying at 100°C, thiourea matrix modifier was added, ashed at 370°C, and atomised

at 2160°C. The absorbance was measured at 217 nm. The samples were homogenised by ultrasonic agitation immediately before injection. The results for an average 3μm particle size sample were found to be in good agreement with those obtained after digestion of samples. The concentration of lead was between 0.28 and 1.53 ppm with RSD% values of 6.6–13.1. Very good recoveries were found indicating that the direct method is suitable for the determination of lead in calcium drug samples. Bozsai *et al.* [16] used Zeeman GFAAS method for determination of lead, arsenic, cadmium and selenium in highly mineralised medicinal waters using the mixture of palladium nitrate and magnesium nitrate as the chemical modifier. The limits of determination of the method were 2.0 μg/l for As, 0.05 μg/l for Cd, 1.0 μg/l for Pb and 1.5 μg/l for Se. High concentrations of sulphate interfere with the determination. This interference can be eliminated by using FI-sulphoxine cellulose microcolumn preconcentration prior to GFAAS measurement. Lead and other heavy metals can be determined in such a way in a highly mineralised medicinal water containing 27 g/l sulphate and 39.5 g/l total solids [18]. The detection limit for lead was 0.063 μg/l after 20-fold preconcentration.

Lead concentration and lead isotope ratios were determined in different calcium supplements by direct ICP/MS using matrix matched lead standards [17]. The samples were digested in nitric acid at 230°C and 12 MPa pressure. Satisfactory recovery was obtained by analysing animal bone (IAEA-H5) reference material. Lead isotope ratios of calcium supplements can be applied for source identification (bone, meal, dolomite, oyster shell, Ca chelates).

4.2.5. Multielement Determinations

An universal temperature programme and calibration were described by Arpadjan and Alexandrova [19] for the modifier-free electrothermal atomic absorption spectrometric determination of trace amounts of As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sb, Sn and V in various drugs including aspirin, dipyrone and paracetamol. The pharmaceuticals behave in graphite furnace as organic chemical modifiers, permitting high pyrolysis temperatures for loss-free thermal pre-treatment of the analyte. The found pyrolysis temperatures at universal temperature programme were: 500°C (Cd), 800°C (Pb), 1000°C (Cu), 1100°C (Sb), 1200°C (Mn, Fe, Sn), 1300°C (Ni, Co), 1400°C (Cr, V). Arsenic was not stabilised by the studied pharmaceuticals. For the determination of arsenic the use of palladium as a chemical modifier was necessary. The standard solutions for universal calibration contained 10% oxalic or tartaric acid. The LOQ values obtained were 0.3 ppb for Cd, 2 ppb for Cr and Mn, 20 ppb for Cu, Fe and Pb, 25 ppb for Co, Ni, Sb and Sn and 100 ppb for V. Because the chemical compositions of pharmaceuticals are very different, it is highly recommended

to test accuracy by spike recovery when the universal temperature programme is applied.

Csikkel-Szolnoki [20] determined Pb, Fe, Cu, As, Cr, Zn and Cd in various pharmaceutical materials at the ppm level by direct and standard addition methods by ICP-AES, GFAAS, and FAAS after dissolution of the sample in water or after digestion in mixtures of nitric acid/hydrogen peroxide or sulphuric acid/hydrogen peroxide.

In the test of Ph. Eur. [9b,c] for extractable Al, Cr, Ti, V and Zr by ICP-AES and Zn by FAAS in polyethylene and polypropylene for containers for parenteral and ophthalmic preparations the material is refluxed in 0.1 M HCl for 1 h in a boro-silicate glass flask. The limits are 1 ppm for Al, Ti and Zn, 0.05 ppm for Cr, 10 ppm for V and 100 ppm for Zr.

ICP/MS is a good tool for the qualitative or semi-quantitative analysis of drugs with sensitivity equivalent to or greater than that of GFAAS and with rapid multielement scanning capability over a wide mass range. A semiquantitative analysis software available with commercial ICP/MS instrumentation can be used for rapid screening of inorganic impurities of samples prior to quantitative determination. The detection limits, precision and accuracy of semiquantitative analysis applied for biological materials was reported [21]. Lewen *et al*. [22] investigated the use of ICP/MS technique as a possible alternative to the USP heavy metal test. The recovery of As, Se, Mo, Ru, Pd, Cd, In, Sn, Sb, Pt, Hg, Pb, Bi in four different drug substances were measured at 10 ppm spike concentration range at 10 μg/l solution concentration (0.1% sample solution in 25:75 v/v mixture of 2-butoxyethanol and water). The recoveries were higher than 90%.

Total reflection X-ray fluorescence (TXRF) spectrometry can be used for multielement determination of elements with atomic numbers $14 < Z < 92$ in pharmaceuticals based on matrix-independent quantification by means of an internal standard. The advantages of this technique are high sensitivity, low detection limits, small sample size requirements, simple quantification in a wide dynamic range [23]. The application of TXRF technique for the screening of heavy metals impurities in drug substances was demonstrated by using direct determination or preconcentration on IDAEC microcolumn prior to the measurement [13]. The element concentrations give fingerprints which make possible the discrimination between different batches of the analysed drugs originating from different production or purification processes [24,25]. Insulin was analysed by direct TXRF with Rb internal standard. Lecithin, procaine and tryptophan samples were digested prior to TXRF measurement. The accuracy of TXRF results for lecithin were verified by ICP-MS. The obtained data were in good agreement for traces of Cr, Fe, Ni, Cu, Zn in the ppm concentration range.

A simple *energy-dispersive X-ray fluorescence analysis* method was

proposed to substitute the heavy metals limit test of Japanese Pharmacopoeia [26].

4.2.6. Other Metals and Elements

In addition to palladium, nickel, and lead tests, pharmacopoeias (Ph. Eur., USP 24) apply atomspectrometric techniques for identification and quantification of Na, K, Ca, Sr, Ba, Mg, Al, Hg, Ag, Cu, Fe, B, Cd, Pt, Sb and Zn. For determination of sodium and potassium *flame photometry*, *atomic emission spectrometry* (*AES*) or FAAS are recommended in pharmacopoeias measuring emission intensity or absorbance at 589.5 nm and 766.5 nm respectively. Sodium and potassium impurities are tested in Ca, Mg Li salts, Na in K-salts and K in Na-salts. Calcium impurities are determined in magnesium salts by FAAS measuring absorbance at 422.7 nm in N_2O-acetylene flame. In calcium salts Mg and Sr traces are tested by FAAS. In the limit test for Ba in protamine hydrochloride by FAAS measuring the absorbance at 553.6 nm in N_2O-acetylene flame the limit is set to 10 ppm [10f]. USP 24 applies the same technique for limit test of 50 ppm Mg in calcium acetate [11e].

Limits by FAAS for copper in Ph. Eur. are as follows: ascorbic acid 5 ppm (for Fe 2 ppm) [10g], mefenamic acid 10 ppm [9d], parnaparin sodium 10 ppm [9e], bleomycin sulphate 200 ppm [10h].

In zinc salts traces of toxic cadmium can be present, therefore Ph. Eur. contains limit tests based on FAAS at 228.3 nm for Cd e.g. in zinc acexamate (2 ppm), zinc stearate (5 ppm), etc.

The limit test for mercury (limit 10 ppm) in penicillamine [10i] is based on FAAS. *Cold vapour atomic absorption spectrometry* (*CVAAS*) is used for the determination of traces of mercury in water for haemodialysis solutions [6c]. The allowable limit is 1 ng/l. The sample is treated with bromine water. After the reduction of mercury(II) with tin(II)chloride, the mercury vapour is transferred with a stream of nitrogen into the spectrometer and the absorbance is measured at 253.7 nm.

GFAAS is applied in the test of Ph. Eur. for traces of aluminium in human albumin solution with a limit of 200 μg/l [10j], and in sodium lactate solution [10k] used for dialysis, haemodialysis and haemofiltration solutions (limit: 0.1 μg/l). The absorbance is measured at 309.3 nm after atomisation in graphite furnace with ammonium nitrate/nitric acid as the chemical modifier. A method was published for quantification of aluminium and strontium in illicit drugs by electrothermal atomic absorption spectrometry using magnesium nitrate or a palladium-magnesium nitrate mixture as chemical modifiers [27]. The obtained limits were 48.0 ppb Al and 16.6 ppb Sr. The analytical recoveries were close to 100% for both elements.

In the limit test for platinum in calcium folinate (limit 10 ppm) *X-ray fluorescence spectrometry* is applied [10l]. In the Supplement of Ph. Eur. [6d] a standard addition AAS method is prescribed. The sample is dissolved in water and the absorbance measured at 265.9 nm.

For dalteparin sodium [9f] a limit test based on ICP/AES of the sample solution acidified by nitric acid is introduced for boron (limit: 1 ppm). The emission intensity was measured at 249.73 nm.

In a direct FAAS test for silver impurity in cisplatin the absorbance is measured at 328.1 nm using fuel-lean air - acetylene flame [10m].

For quantification of zinc the recommended method is FAAS. Pharmacopoeias also apply this method for limit test of zinc in, e.g. glucagon [10n], propyl gallate [10o] and gentian violet [11f]. This method is used also as a limit test for antimony, e.g. in desflurane (limit 3 ppm) [11h]. The absorbance in air–acetylene flame is measured at 217.6 nm.

A rapid *energy-dispersive X-ray fluorescence* (*EDXRF*) method is proposed to substitute the conventional arsenic limit test of pharmacopoeias [28]. This method was successfully applied for the determination of other elements (Si, P, S, Cl, K, Ca, Ti, Mn, Fe, Cu, Zn) in various drug materials [29].

The determination of *tungsten* in bulk drug materials has been recently described by Wang *et al*. [30]. The solution obtained by simply dissolving the sample in 80:20 v/v mixture of nitric acid and water was subjected to ICP-AES (224.875 nm; LOD 1.8 ppm; LOQ 6 ppm) or ICP-MS analysis (W isotope 184; LOD 0.04 ppm; LOQ 0.12 ppm).

The species-selective information in the biomedical field is becoming increasingly important. The atomspectrometric methods determine the total metal content of the sample but provide no information on its chemical identity. There is a need for determination of concentrations of different forms of elements (arsenic, mercury, tin, chromium, etc.) because of the different toxicities of the different species. Separation techniques coupled with atomspectrometric methods are applied for speciation analysis [31]. In the future the importance of such studies is expected to increase even in drug analysis. A speciation analysis of arsenic in urine was published recently based on high performance liquid chromatography, on-line UV photooxidation and continuous hydride generation AAS [32].

References

1. L.H.J. Lajunen, *Spectrochemical Analysis by Atomic Absorption and Emission*, The Royal Society of Chemistry, Cambridge (1992)
2. J.H.M. Miller, *Int. Lab*. 37–47 (1978)
3. L. Ebdon and A.S. Fisher, Pharmaceutical products and drugs, in *Atomic*

Absorption Spectrometry. Theory, Design and Applications (S.J. Haswell, Ed.), p 488, Elsevier, Amsterdam (1991)
4. T. Wang, S. Walden and R. Egan, *J. Pharm. Biomed. Anal*. **15**, 593–599 (1997)
5. Á. Lévai, A. Lásztity, K. Zih-Perényi and Zs. Horváth, *Microchem. J*. **58**, 272–280 (1998)
6. *European Pharmacopoeia, 3rd edn*, Supplement, Council of Europe, Strasbourg (1999). Page numbers: a, p 833; b, p 404; c, p 549; d, p 326
7. N. Lewen, M. Schenkenberger, T. Larkin, S. Conder and H.G. Brittain, *J. Pharm. Biomed. Anal*. **13**, 879–883 (1995)
8. M. Schenkenberger and N. Lewen, Winter Conference on Plasma Spectrochemistry, Scottsdale, AZ, Poster FP24, *ICP Inform. Newslett., Spec. Edn.* **23**, 333 (1998)
9. *European Pharmacopoeia, 3rd edn*, Supplement, Council of Europe, Strasbourg (1998). Page numbers: a, p 473; b, p 46; c, p 49; d, p 378; e, p 417; f, p 259; g, p 183
10. *European Pharmacopoeia, 3rd edn*, Council of Europe, Strasbourg (1997). Page numbers: a, p 55; b, p 1374; c, p 527; d, p 54; e, p 1267; f, p 1402; g, p 412; h, p 486; i, p 1291; j, p 353; k, p 1499; l, p 515; m, p 642; n, p 900; o, p 1396
11. *The United States Pharmacopoeia 24*, USP Convention Inc., Rockville (2000). a, p 470; b, p 711; c, p 713; d, p 1013; e, p 275; f, p 770; g, p 1379; h, p 504
12. P. Bermejo-Barrera, A. Moreda-Piñeiro, J. Moreda-Piñeiro and A. Bermejo-Barrera, *J. Anal. At. Spectom.* **10**, 1011–1017 (1995)
13. Á Kelkó-Lévai, I. Varga, K. Zih-Perényi and A. Lásztity, *Spectrochim. Acta, Part B: Atomic Spectrosc.* **54**, 827–833 (1999)
14. P. Bermejo-Barrera, A. Moreda-Piñeiro, J. Moreda-Piñeiro and A. Bermejo-Barrera, *Anal. Chim. Acta* **310**, 355–367 (1995)
15. S. Ahsan, S. Kaneco, K. Ohta, T. Mizuno, T. Suzuki, M. Miyada and Y. Taniguchi, *Anal. Chim. Acta* **362**, 279–284 (1998)
16. G. Bozsai, G. Schlemmer and Z. Grobenski, *Talanta*, **37**, 545–553 (1990)
17. D. Amarasiriwardena, K. Sharma and R.M. Barnes, *Fresenius J. Anal. Chem.* **362**, 493–497 (1998)
18. K. Zih-Perényi, A. Lásztity, Zs. Horváth and Á. Lévai, *Talanta* **47**, 673–679 (1998)
19. S. Arpadjan and A. Alexandrova, *J. Anal. At. Spectrom.* **10**, 799–802 (1995)
20. A. Csikkel-Szolnoki, *Gyógyszerészet* **40**, 257–261 (1996)
21. D. Amarasiriwardena, S.F. Durrant, A. Lásztity, A. Krushevska, M.D. Argentine and R.M. Barnes, *Microchem. J.* **56**, 352–372 (1997)

22. N. Lewen, S. Mathew and M. Schenkenberger, Winter Conference on Plasma Spectrochemistry, Scottsdale, AZ, Poster FP23. *ICP Inform. Newslett., Spec. Edn.* **23**, 333 (1998)
23. R. Klockenkämper and A. Bohlen, *J. Anal. At. Spectrom*. **7**, 273–279 (1992)
24. M. Wagner, P. Rostam-Khani, A. Wittershagen, C. Rittmeyer, H. Hoffmann and B.O. Kolbesen, *Pharmazie* **51**, 865–868 (1996)
25. M. Wagner, P. Rostam-Khani, A. Wittershagen, C. Rittmeyer, B.O. Kolbesen and H. Hoffmann, *Spectrochim. Acta Part B* **52**, 961–965 (1997)
26. T. Wakisaka, N. Morita, S. Tanaka and T. Nakahara, *Bunseki Kagaku* **45**, 1025–1031 (1996)
27. P. Bermejo-Barrera, A. Moreda-Piñeiro, J. Moreda-Piñeiro and A. Bermejo-Barrera, *Analusis* **24**, 263–266 (1996)
28. T. Wakisaka, N. Morita, S. Tanaka and T. Nakahara, *Bunseki Kagaku* **45**, 1019–1023 (1996)
29. T.P. da Silva de Freitas, E. Ricci Jr. and O.L.A.D. Zucchi, *Rev. Farm. Bioquim. Univ. S. Paolo* **33**, 29–35 (1997)
30. T. Wang, Z. Ge, J. Wu, B. Li and A. Liang, *J. Pharm. Biomed. Anal.* **19**, 937–943 (1999)
31. I.S. Krull (Ed.), *Trace Metal Analysis and Speciation*, Elsevier, Amsterdam (1991)
32. D.L. Tsalev, M. Sperling and B. Welz, *Analyst* **123**, 1703–1710 (1998)

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4.3. Other Methods

Sándor Görög

4.3.1. Electroanalytical Methods

An alternative of the semiquantitative limit test for sulphate impurity based on the formation of barium sulphate, described in Section 4.1.5 is the potentiometric titration with 0.02 M lead(II)acetate using a lead-selective and Ag/AgCl electrodes. No less than 6 g of acetylsalicylic acid is titrated; limit: 0.04% [1a].

Fluoride ions can be determined in drugs by *direct potentiometry* using a fluoride-selective indicator electrode and a silver/silver chloride reference electrode [1b,2,3]. For example, 50 ppm of fluorides is allowed by the European Pharmacopoeia in calcium phosphate used as excipient in solid dosage forms [2a]. After dissolving the sample in 0.1 M hydrochloric acid the measurement is carried out at pH 5.2 in an acetate buffer using a simultaneously run direct five-point calibration. Similar methods have been applied also to calcium ascorbate; limit 10 ppm [2b,3] and desflurane; limit 10 ppm [1b].

The tin(II) content of pharmaceuticals was determined by *amperometric* oxidation after complexation with tropolone [4]. Adsorptive *stripping voltametry* was successfully used in pharmaceutical analysis [5] for the determination of traces of metals in pharmaceutical bulk materials [6] among others for the selective determination of selenium(IV) in pharmaceutical preparations for parenteral administration. The results compare favourably with those obtained by graphite furnace atomic absorption spectrometry (GFAAS) using nickel(II) chemical modifier [7]. Traces of lead in various drugs were also determined by the same technique [8].

4.3.2. Ion Chromatography

Ion chromatography presents various possibilities for the determination of inorganic impurities (both anions and metal ions) in drugs and pharmaceutical preparations [9].

Anions (halogenides, sulphate, nitrate, phosphate) are easily separated from each other and the drug matrix by various ion exchange columns and determined even at the trace level by indirect ultraviolet spectrophotometric detectors (applying usually phthalate ion in the eluent). Another possibility is

the use of conductivity detectors. Ion chromatography with conductivity or (indirect) spectrophotometric detection is suitable for the separation and determination of traces of metals, too. Another possibility is post-column derivatisation with various complex forming reagents followed by spectrophotometric (or fluorescence) detection. For example aluminium(III) was determined in glucose injectables, saline solutions and sterile water by boiling with 0.05 M sulphuric acid for 5 min (to decompose its existing complexes), followed by ion chromatographic separation and quantitation by a fluorescence detector using post-column derivatisation with 8-hydroxyquinoline-5-sulphonic acid [10]. The detection limit in this case was below 0.5 ng/ml. The results agreed reasonably well with those obtained by ICP-AES.

4.3.3. Capillary Ion Electrophoresis

Capillary ion electrophoresis (CIE) is a branch of capillary electrophoresis (see Section 2.8) in which ionisable trace inorganic impurities and organic counter ions are determined in various matrices [11,12]. Among other fields this technique has found wide application also in pharmaceutical analysis [13–18]. Although the main aim of these studies is usually the determination of drug stoichiometry, i.e. the molar ratio of acidic and basic drugs to their cationic and anionic counter ions, attempts have also been made to use this technique for the determination of inorganic ions present as impurities [13,18] An electropherogram from the paper of Nair and Izzo [13] for the detection and quantitation of most important trace impurity level inorganic anions (plus citrate and acetate) is shown in Fig. 4.3.A. Advantages of this technique over the ion chromatographic approach are easy sample preparation, short analysis time and the short time required to regenerate the capillary for the next analysis. In the majority of cases indirect photometric detection is used. The photometrically active component of the carrier electrolyte is usually chromate. The sensitivity of this method is not superior to that of suppression ion chromatography but even in this form it has been found suitable for the determination of small amounts of inorganic anions in bulk pharmaceuticals down to the 0.1% level which is not far from the levels of the requirements of pharmacopoeias. The sensitivity can be improved by replacing chromate with aromatic carboxylates, especially *p*-anisate [12]. If the aim of the study is the determination of trace level anions in drugs, it is usually necessary to use mixtures of organic solvents such as tetrahydrofuran, dimethylacetamide, methanol, 2-propanol, acetonitrile and water. It was found that the migration times, peak shapes and baseline stability are strongly (in some cases favourably) influenced by these solvents. For this reason the solvent composition of the standard solution should be identical with that of the test solution which may contain the above listed solvents up to 10%

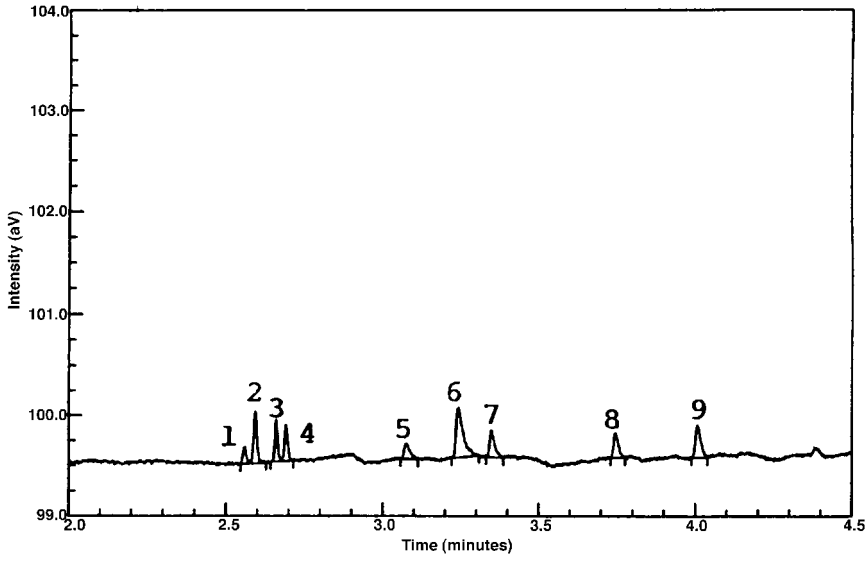


Figure 4.3.A.
Separation of anions at their minimum quantifiable level (1.0 μg/ml). Capillary: 60 m × 75 μm fused silica . Indirect UV detection at 254 nm using as a background electrolyte the aqueous solution of chromate, dilute sulphuric acid and Waters Anion-BT electroosmotic flow modifier. Voltage: 20 kV. Hydrostatic loading time: 20 s. Key: 1, bromide; 2, chloride; 3, sulphate; 4, nitrite; 5, citrate; 6, fluoride; 7, phosphate; 8, carbonate; 9, acetate (from Ref. [13])

v/v [13]. Poorly water-soluble drugs are dissolved in the organic solvent, diluted with sufficient amount of water and centrifuged to remove the precipitated drug material to obtain the test solution [18].

The CIE method was successfully applied by Swartz [17] for the detection of trace level anions (chloride, sulphate, nitrate, citrate, fumarate, phosphate, carbonate and acetate) and for the quantitative determination of cations (calcium, sodium, iron(II) and zinc) in a prenatal vitamin formulation. For anions a negative polarity voltage (20 kV), indirect UV detection at 254 nm and cationic surfactant (5 mM chromate, 0.4 mM CIA-Pak OFM anion BHT, pH 8.0) and for the cations a positive polarity voltage (20 kV), indirect UV detection at 185 nm were applied (5 mM UV-CAT-1, 6.5 mM 2-hydroxyisobutyric acid chelating agent, pH 4.4). The use of the cationic surfactant OFM anion BHT or tetradecyltrimethylammonium bromide [14,18] is to form bilayers on the capillary wall thus causing the reversal of the electroosmotic

flow to support the electromigration of anions. The determination of acetate and trifluoroacetate ions in peptides is also worth mentioning [19].

Wiliams et al. [18] found that the sensitivity of the detection of anions can be increased by one order of magnitude by changing the detection from indirect UV to conductivity detection and used their method for the determination of anions (chloride, nitrate, sulphate) and cations (sodium, potassium, lithium, magnesium and ammonium, using 18-Crown-6 as the chelating agent) in various drug materials.

References

1. *The United States Pharmacopoeia 24*, USP Convention Inc., Rockville, MD (2000). Page numbers: a, p 162; b, p 504
2. *European Pharmacopoeia 1997, 3rd edn*, Council of Europe, Strasbourg (1997). Page numbers: a, p 219; b, Supplement, p 216
3. M. Yuwono and S. Ebel, *Arch. Pharm.* **330**, 348–352 (1997)
4. K. Boutakhrit, G. Quarin, S.A. Ozkan and J.M. Kauffmann, *Electroanalysis* **8,** 789–794 (1996)
5. A. Lozak and Z. Fialek, *Chem. Anal.* **43**, 1–7 (1998)
6. P. Gratteri, S. Pinzauti, G. Papeschi, E. La Porta and S. Furlanetto, *Farmaco* **47,** 695–709 (1992)
7. F. Pellerin, J.F. Letavernier and M. Aubert, *Ann. Pharm. Franc.* **43**, 223–231 (1985)
8. M. Pravda and K. Vytras, *J. Pharm. Biomed. Anal.* **14**, 765–771 (1996)
9. R.P. Haddad and P.E. Jackson, *Ion Chromatography (Principles and Applications),* pp 651–666, Elsevier, Amsterdam (1990)
10. J. Carnevale and E. Jackson, *J. Chromatogr. A.* **671**, 115–120 (1994)
11. P. Jandik and W.R. Jones, *J. Chromatogr.* **546**, 431–443 (1991)
12. P. Jandik, W.R. Jones, A. Weston and P.R. Brown, *LC-GC Int.* **9**, 634–636 (1992)
13. B. Nair and C.G. Izzo, *J. Chromatogr.* **640**, 445–461 (1993)
14. K.D. Altria, D.M. Goodal and M.M. Regan, *Chromatographia* **38**, 637–642 (1994)
15. K.D. Altria, N.G. Clayton, R.C. Horden, J.V. Mackwana and M.J. Portsmouth, *Chromatographia* **40**, 47–50 (1995)
16. K.D. Altria, M.A. Kelly and B.J. Clark, *Trends Anal. Chem.* **17**, 204–226 (1998)
17. M.E. Swartz, *J. Chromatogr.* **641**, 441–444 (1993)
18. R.C. Williams, R. Boucher, J. Brown, J.R. Scull, J. Walker and D. Paolini, *J. Pharm. Biomed. Anal.* **16**, 469–479 (1997)
19. K. Hettiarachchi and S. Ridge, *J. Chromatogr.* **817,** 153–161 (1998)

Chapter 5

DEGRADATION PRODUCTS AS IMPURITIES

Sándor Görög

5.1. The Relation Between Drug Stability Studies and the Estimation of Impurity Profiles

There is a close relationship between drug stability studies [1] and the identification and quantitative determination of related impurities in drugs. Degradation products of drugs are considered to be transformation products of the drug substance forming on the effect of heat, solvents (including high and low pH), oxidising agents, other chemical reagents, humidity, light, etc. On the basis of this definition a considerable overlap can be observed between the categories *related impurities* and *degradation products*: related impurities of degradation product type are often formed in the course of the synthesis and isolation of the bulk drug material. For this reason one of the classes of related impurities in drugs is categorised by the ICH Guidelines as degradation products (see Section 1.5). Some typical examples from the author's experience are as follows.

(a) In many instances the drug substance formed in the last step of the synthetic procedure is subject to various attacks already in the reaction mixture prior to its isolation.

As an example for such a reaction one of the impurities in *famotidine* is mentioned. As is seen in Fig. 5.1.A, in the final step of its synthesis [2] the reaction product famotidine is accompanied by the simultaneously forming cyanamide. The latter reacts in the reaction mixture with the endproduct to form the cyanoguanidine derivative of famotidine, which was separated and detected as an impurity in famotidine by HPLC and TLC and its structure was determined by the complex application of spectroscopic methods [3]. The mechanism of the formation of the impurity was proved by reacting

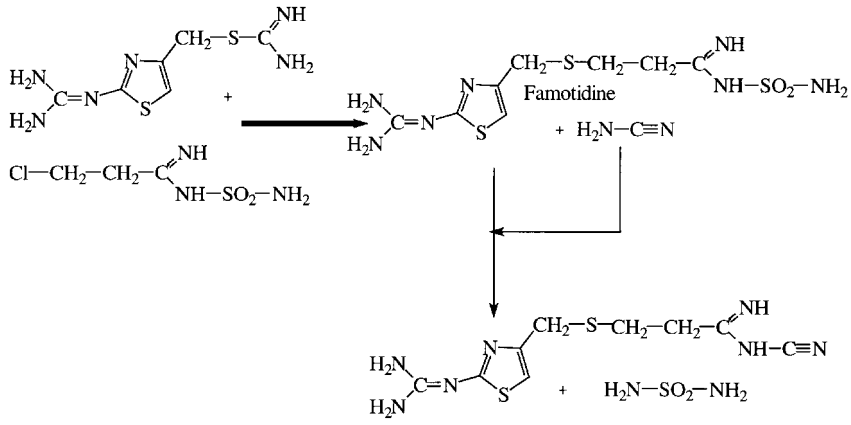


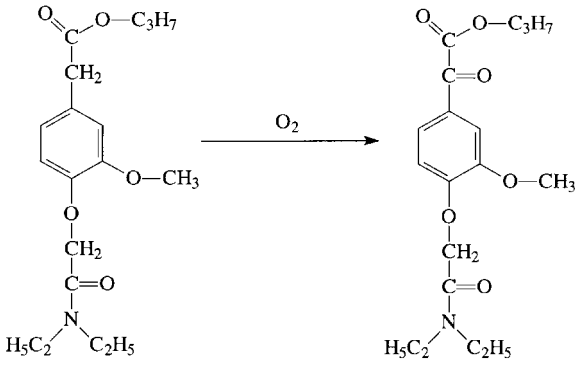
Figure 5.1.A.
Reaction scheme of the last step of the synthesis of famotidine with the formation of an impurity

separately famotidine with cyanamide: the impurity was obtained with good yield.

(b) More typical examples are instances where the reactivity of the endproduct towards, e.g. atmospheric oxygen results in autoxidation products during the final step of the synthesis or in the course of the isolation and drying of the finished product. For example, the oxidation of *propanidid* takes place in the reaction mixture leading to propyl 4-[diethylcarbamoyl(methoxy)]-3-methoxy phenylglyoxylate which has been separated by normal phase HPLC and identified by HPLC/diode-array UV and by mass spectrometry [4] (see Fig. 5.1.B). This is one of the main impurities in propanidid but its quantity is further increased during the storage of the bulk drug material and the formulations and hence it can be considered to be an impurity of degradation product type and also as a real degradation product.

The same applies to 6(α and β)-hydroxy or oxo impurities/degradation products in 4-ene-3-oxo steroids, e.g. *norethisterone* [5], *norgestrel* [5,6], *nestorone* [7], etc. The detailed discussion of these examples can be found in Section 2.7.3. A classical example for impurities/oxidative degradation products is papaverinol and papaveraldin in *papaverine.*

(c) The acid or heat catalysed dehydration of *flumecinol* ((±)-1-(3-trifluoromethylphenyl)-1-phenyl-propan-3-ol) to form the mixture of *Z*- and *E*-1-(3-trifluoromethylphenyl)-1-phenylbuten-1 [8,9] can take place already in the reaction mixture or during the distillation of the bulk drug material but it is also the main decomposition product of flumecinol. The gas chromatographic



Propanidid

Figure 5.1.B.
Oxidative degradation of propanidid

and GC-MS investigation of this decomposition product is described in Section 2.6.

Of course as a consequence of the close similarities moreover overlaps between drug impurity profiles and degradation profiles the analytical methodology used in drug stability studies aiming at detecting degradation products and determining their structure is principally the same as is used for drug impurity profiling (see Chapter 2). In other cases, however, there are considerable differences, too, between the methodologies. If the aim of the study is the determination of the rate of the degradation and estimation of the degradation kinetics, the authors of papers of this kind often find it sufficient to base their study on the determination of the decreasing concentration of the drug material. If the analytical method adopted for this purpose is 'stability indicating', i.e. selective enough to determine the concentration of the undecomposed drug in the presence of the degradation product(s) and other components originating from the matrices, this can be really considered to be an acceptable approach. This is the case, e.g. when simple, single-step, unidirectional reactions such as hydrolysis of esters are investigated. The method for the determination of the undecomposed drug in the early literature was usually spectrophotometry or colorimetry or in the more recent literature derivative spectrophotometry or other background correction methods [10]. The discussion of methods and studies of this kind would be beyond the scope of this book. A few spectrophotometric methods based on the selective determination of the increasing concentration of the degradation product will be shortly mentioned.

More up-to-date approach is, however, the use of chromatographic or electrophoretic methods combined with suitable spectroscopic techniques

which enable the decomposition products to be detected and their structure to be elucidated and the concentrations of both the undecomposed drug and its decomposition product(s) to be simultaneously determined. On the basis of this approach complex degradation mechanisms (degradation pathways) can be studied and the kinetics of the degradation reactions can also be investigated. The detailed discussion of drug degradation kinetics would be of course also beyond the scope of this book. Regarding this matter the excellent book of Carstensen [1] is referred to. The examples to be discussed in the subsequent sections will be from the circle of the identification and structure elucidation of degradation products, estimation of their degradation pathway (see Section 5.3) with some examples on the methodological problems of studies of degradation kinetics (see Section 5.5). Due to the very complex nature of photochemical decomposition of drugs a separate section will be devoted to this topic (see Section 5.4).

References

1. J.T. Carstensen, *Drug Stability, 2nd edn*, Marcel Dekker, New York (1995)
2. *GB Patent* 2 180 237; *US Patent* 4 835 281
3. S. Görög, G. Balogh, A. Csehi, É. Csizér, M. Gazdag, Zs. Halmos, B. Hegedüs, B. Herényi, P. Horváth and A. Laukó, *J. Pharm. Biomed. Anal*. **11**, 1219–1226 (1993)
4. S. Görög, M. Babják, G. Balogh, J. Brlik, A. Csehi, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *Talanta* **44**, 1517–1526 (1997)
5. S. Görög and B. Herényi, *J. Chromatogr*. **400**, 177–186 (1987)
6. S. Görög, M. Bihari, É. Csizér, F. Dravecz, M. Gazdag and B. Herényi, *J. Pharm. Biomed. Anal.* **14**, 85–92 (1995)
7. S. Görög, M. Babják, G. Balogh, J. Brlik, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *J. Pharm. Biomed. Anal*. **18**, 511–525 (1998)
8. S. Görög, M. Rényei and A. Laukó, *J. Pharm. Biomed. Anal*. **1**, 39–46 (1983)
9. S. Görög, B. Herényi and M. Rényei, *J. Pharm. Biomed. Anal*. **10**, 831–835 (1992)
10. S. Görög, *Ultraviolet-Visible Spectrophotometry in Pharmaceutical Analysis*, pp 228–230, CRC Press, Boca Raton, FL (1995)

5.2. Aims, Forms and Conditions of Drug Stability Testing

Sándor Görög

Various kinds of drug stability studies are necessary in the course of the development of new drugs and drug formulations. Even at an early stage of the research for a new drug substance preliminary results for its stability are necessary in order to be able to make a decision whether the intrinsic stability of the material enables further development to be carried out. For this purpose *stress testing* experiments are conducted. The conditions for these are more severe than those for accelerated stability test to be discussed later: i.e. the storage temperature can be 50, 60 or 70°C or even higher at relative humidities up to 75% or greater depending on the individual drug substance or the type of the product. The susceptibility of the material to hydrolysis and oxidation should be checked by treating its solution or suspension at a wide range of pH and also in the presence of an oxidising agent (preferably hydrogen peroxide) also at elevated temperatures. Stress testing should also include testing of the sensitivity of the material to light. The severe conditions are necessary to be able to get a preliminary information on the stability within a relative short period of time (e.g. 2 weeks) rather than at least 6–12 months which is the case with accelerated and long-term stability studies. Another aim of stress testing is to establish the degradation pathway, i.e. to identify the products of degradation under various conditions. Of course this information is of immense importance during the further development of the drug candidate especially in the hands of pharmaceutical technologists before making the development plan for suitable dosage forms. In addition to these points it is important to note that in possession of this information the suitability of the analytical methods to be used for the official long-term and accelerated stability testing, i.e. their stability indicating nature can be checked.

The aim of ''in situ degradation'' studies [1] is similar: generation of ''reference substances'' for system suitability tests in the HPLC purity tests of various drugs. The European Pharmacopoeia [2] contains several descriptions how to transform easily drug substances to their derivatives by ''in situ'' degradation. These include the transformation of *cefuroxime* to descarbamoylceforuxime by heating at 60°C its aqueous solution, *fentanyl* to its despropionyl derivative by refluxing its acidic solution, epimerisation of *minocycline* to 4-epiminocycline at 60°C in aqueous solution, oxidation by iodine of *captopril* to captopril disulphide, etc.

Modelling of stability testing is an interesting new alternative to stress testing. Boccardi [3,4] suggested the use the of the reaction in acetonitrile at

40°C of the drug to be investigated with the radical chain initiator, 2,2′-azobis(2-methylpropanenitrile). By investigating this reaction by HPLC using four substrates (tetrazepam, phenylbutazone, dextromethorphan hydrobromide and trifluoperazine hydrochloride) it was found that there is a good correlation with the reactivity and reaction products under autoxidation conditions (oxidation by atmospheric dioxygen).

Of course stress testing is necessary also during the search for the suitable dosage form: carrying out this test already during the preformulation studies the time necessary for this search can be considerably shortened.

It should be noted that due to the severe conditions degradation products may be observed during the stress testing which will not be observable under accelerated and especially long-term stability testing. In spite of this stress testing is the most important stage of drug stability studies from the point of view of identifying the impurities, establishing the degradation pathways and developing the suitable analytical methods for the further, official stability testing.

The usual conditions for *long-term testing* of bulk drug materials are 25 ± 2°C at a relative humidity of 60 ± 5% for at least 12 months, while the same data for *accelerated testing* are 40 ± 2°C, 75 ± 5% for at least 6 months. The final aim of these tests is to establish the shelf-life of the bulk drug material and the drug product but the discussion of this matter would be beyond the scope of this book. For details see the book of Carstensen [5] and the ICH Guidelines [6].

In addition to the stress testing, long-term and accelerated testing reaction kinetic experiments in the classical sense of the word are also carried out under ''pure'' conditions (absence of excipients, etc.) with the aim of elucidating the mechanism of the degradation reaction and establishing optimum conditions regarding pH, etc. for the drug material. The importance of the elucidation of the mechanism of the degradation reaction, i.e. full knowledge of the structure of the degradation products and the reasons for their formation is emphasised by the potential toxicity of the degradation products. As examples for toxic drug degradation products epianhydrotetracycline in tetracycline, polymeric degradation products in penicillines, 21-aldehyde derivatives in corticosteroids and 3-benzylidenephthalide in phenindione can be mentioned [7].

References

1. U. Rose, *J. Pharm. Biomed. Anal*. **18**, 1–14 (1998)
2. *European Pharmacopoeia, 3rd edn*, Council of Europe, Strasbourg (1997)
3. G. Boccardi, C. Deleuze, M.Gachon, G. Palmisano and J.P. Vergnaud, *J. Pharm. Sci.* **81**, 183–185 (1992)

4. G. Boccardi, *Farmaco* **49**, 431–435 (1994)
5. J.T. Carstensen, *Drug Stability, 2nd edn*, Marcel Dekker, New York (1995)
6. *International Conference on Harmonisation (ICH)*, Stability Testing Guidelines: Stability Testing of New Drug Substances and Products. Step 5. (CPMP/ICH/380/95). Date for coming into operation: 1 January (1998)
7. A.C. Cartwright, *Int. Pharm. J.* **15**, 146–150 (1990)

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5.3. Elucidation of Degradation Pathways

Sándor Görög

5.3.1. Introduction

The subject of this section is the elucidation of degradation pathways of drugs stored under various conditions. The knowledge of the structures of decomposition products of drugs and the mechanism of the reactions by which they arise are important issues in the understanding of the chemistry, stability, and activity of drug substances. The degradation pathways of classical drugs were elucidated already in the early stage of the development of pharmaceutical chemistry. In the course of these studies the analytical methodology available at the time of the studies was used for the detection, isolation, characterisation and structure elucidation of the degradants. Information regarding this matter can be found in standard books [1–4] and in the individual chapters of the series *Analytical Profiles of Drug Substances* [5]. In this section some characteristic, mainly new examples are presented, where the approach to the problem of structure elucidation reflects the up-to-date way of thinking and the use of the presently available instrumental techniques.

5.3.2. The Use of HPLC/Diode-Array UV Spectra

It very rarely occurs in the course of drug degradation studies that the degradation product shows selective absorption in a certain UV spectral range, thus enabling its detection and identification on the basis of its UV spectrum in the presence of a great excess of the parent drug, without preliminary chromatographic separation. A characteristic example of this kind is the acid-catalysed degradation of benzodiazepines to form 2-aminobenzophenones resulting in a strong bathochromic shift in their spectra. For example *nitrazepam* has absorption bands at 257 and 308 nm (ε = 15 700 and 10 300) while the absorption maxima of its acid-catalysed degradation product, 2-aminobenzophenone are at 235 and 358 nm (ε = 17 470 and 18 590). The long wavelength band (causing the yellow colour of the degradant) is eminently suitable for its identification [6].

Much more important but still of limited use is ultraviolet spectroscopy coupled with chromatographic techniques in the estimation of degradation profiles. In some instances, when the difference between the spectra of the

parent compound and the degradation product (obtainable by HPLC/diode-array UV detector or densitometric reflection spectrum scanning after TLC separation) is large and characteristic enough, it is possible to propose the structure of the degradation product. For example, in the course of the stress testing of *mazipredone* by Gazdag *et al*. [7] (treatment with 0.1 M hydrochloric acid at 80°C for 30 min) two degradation products were found at relative retention times of 1.61 and 1.70. For the chromatographic conditions see Fig. 5.3.A, where the diode-array spectra of the parent drug and the two degradation products are shown. The comparison of the diode-array spectra shows that the 1,4-diene-3-oxo moiety (absorption band around 248 nm) is unchanged in the degradation products. On the basis of the long wavelength band of the latter at about 269 and 276 nm, respectively, and from the information obtained earlier regarding the acidic degradation products of prednisolone [8] it was concluded that the degradation products contained the *E* and *Z* form of the chromophoric enol-aldehyde side chain at C-17. (For the chromatogram see Fig. 5.5.D in Section 5.5.3 and for the reaction scheme of the acidic degradation see Fig. 5.5.E.

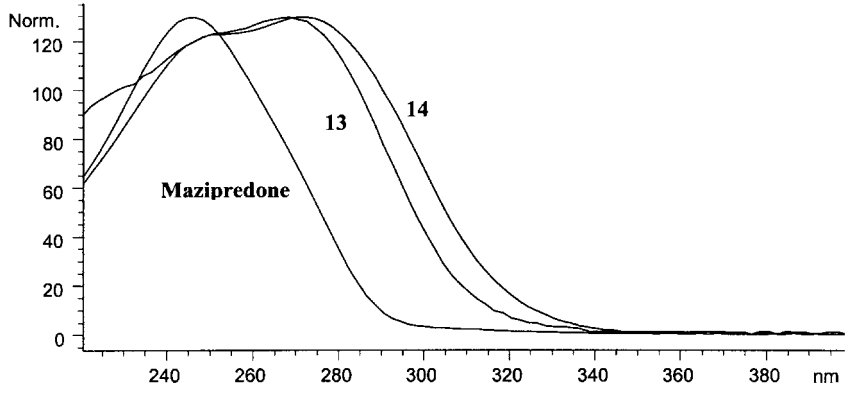


Figure 5.3.A.
Diode-array UV spectra of mazipredone and two acidic degradation products: 11β,20-dihydroxy-3,20-dioxo-pregna-1,4,17(20)*Z*-triene-21-al (**13**) and its 17(20)*E* isomer (**14**) (see reaction equation in Fig. 5.5.E). HPLC conditions: column: Purospher RP-18e, 5 μm, 125 × 4 mm. Eluent: A: water/acetonitrile/methanol 85:5:5 v/v/v, B: water/acetonitrile/methanol 20:40:40 v/v/v both containing 50 mM ammonium acetate. Linear gradient from 10% B at 0 min to 90% B at 70 min. Flow rate 1 ml/min. Temperature 40°C. UV detector: 240 nm (from Ref. [7])

Another example is the identification of the impurities (oxidative degradation products) of *nestorone*, separated form the main component by reversed-phase HPLC (see Fig. 5.3.B) [9]. The diode-array UV spectra of the main component and the separated impurities are shown in Fig. 5.3.C. Impurities where the conjugation chain of the 4-ene-3-oxo moiety is extended by a Δ^6 double bond or a 6-oxo group resulting in bathochromic shifts of 43 and 14 nm, respectively, are easily recognisable on the basis of their characteristic spectra [10,11]. The UV spectra of the more polar impurities with their allylic 6α- and 6β-hydroxy groups show only minor differences as compared with the main component. On the basis of the experiences (relative retention times and hypsochromic shifts) of analogous degradation products of *norgestrel* [12] the identification of these impurities was also possible.

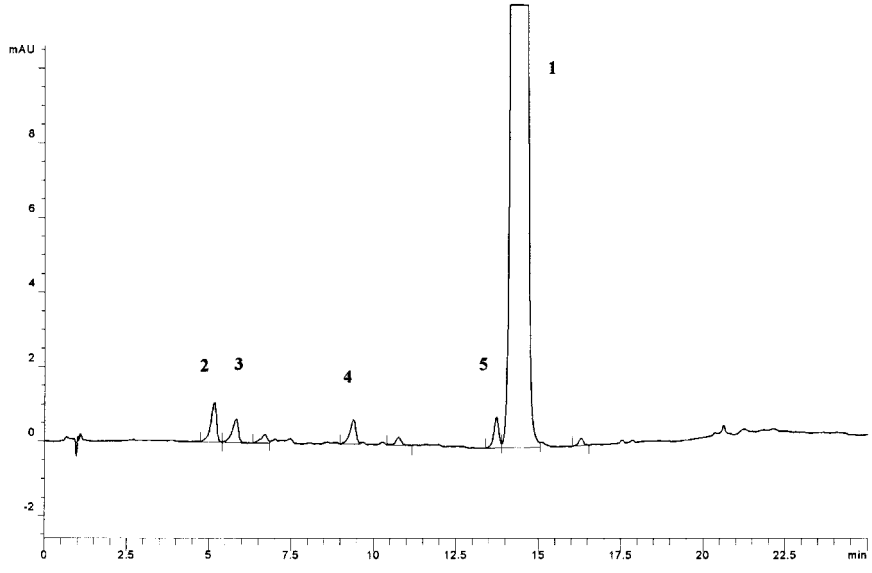


Figure 5.3.B.
HPLC separation of nestorone (**1**) and its impurities, 6α-hydroxy (**2**), 6β-hydroxy (**3**), 6-oxo (**4**), 6-ene (**5**). Column: Nova Pack C-18, 4 μm, 150x3.9 mm. Eluent: A: acetonitrile/water 1:9 v/v, B: acetonitrile. Linear gradient, 0 min 25% B, 14 min 45% B, 20 min 75% B. Flow rate 1 ml/min. UV detector 254 nm (from Ref. [9])

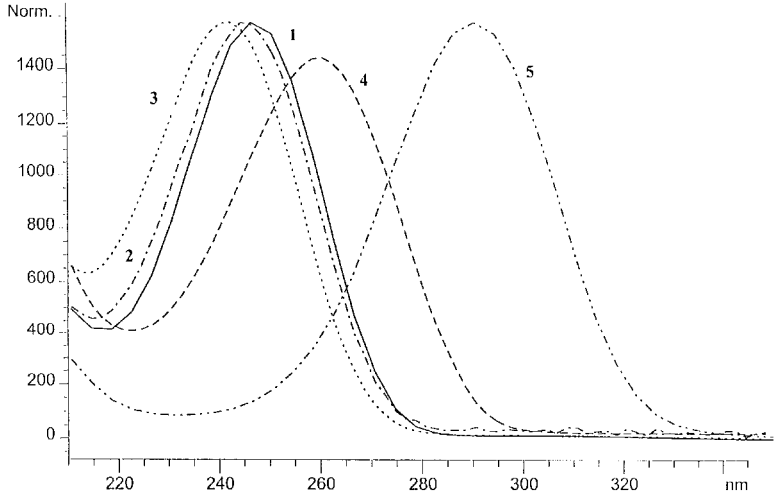
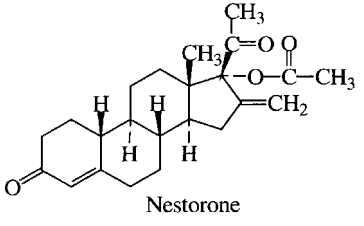


Figure 5.3.C.
Diode-array UV spectra of nestorone and its impurities. For the Key and Reference see Fig. 5.3.B. For the chromatographic conditions see the text



Nestorone

5.3.3. The Use of Mass Spectrometry in Association with Separation Techniques

In the majority of drug degradation studies the elucidation of the degradation pathways, i.e. the determination of the structures of the degradants is based on mass spectrometric investigations. The up-to-date approach is the use of the HPLC/MS technique supplemented by HPLC/MS/MS for substructural studies. As an example the elucidation of the degradation profile of *butorphanol tartrate* is shortly described based on the paper of Volk *et al*. [13]. The

HPLC system enabling the separation of the degradants and their detection by UV detector and structure elucidation by on-line electrospray mass spectrometry including as a second step tandem mass spectrometric studies for substructural analysis consisted of a 2 × 250 mm 5 μm phenyl column with gradient elution (A: 2 mM ammonium acetate pH 4.8; B: acetonitrile; 0 min A:B 65:35, 10–20 min 50:50) at a flow rate of 0.35 ml/min (1:3 split in the HPLC/MS tests) at ambient temperature. The subject for the long-term storage stability study was an aqueous sample of butorphanol tartrate (1 mg/ml) aged for more than three years at 30°C. The stability of the solution was found to be excellent: as is seen in Fig. 5.3.D only minor peaks were observable on the HPLC/diode-array UV scan. Two of the degradants (**2** and **6**) were below the HPLC/UV cut-off criteria but were visible on the HPLC/MS scan. The degradation pathway derived principally from the information furnished by the HPLC/MS (molecular masses) and HPLC/MS/MS studies (fine structures) is depicted in Fig. 5.3.E. It is to be noted that the exact location of the ring

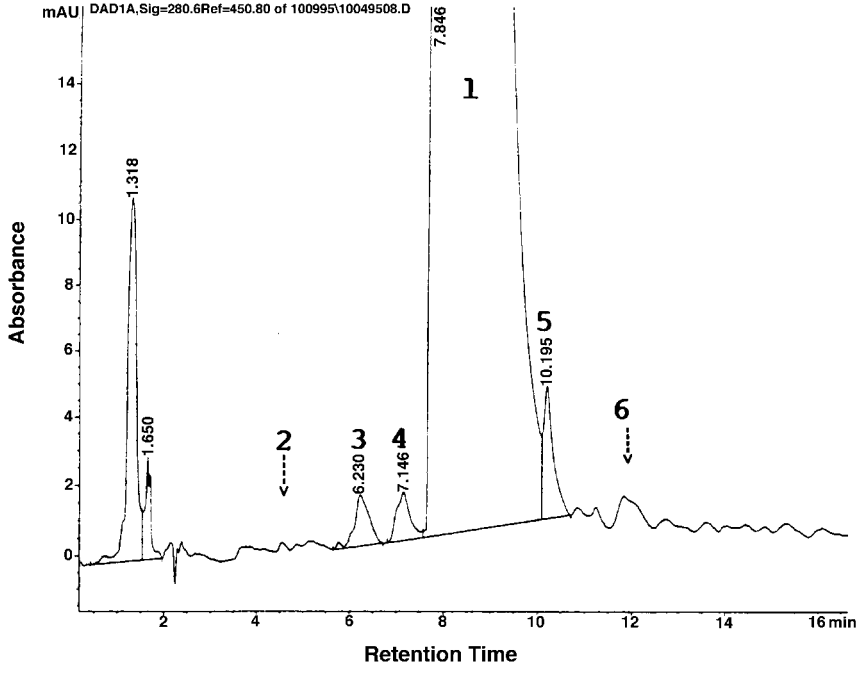


Figure 5.3.D.
HPLC separation of butorphanol tartrate (**1**) and five degradants (**2–6**). For the Key see Fig. 5.3.E (from Ref. [13])

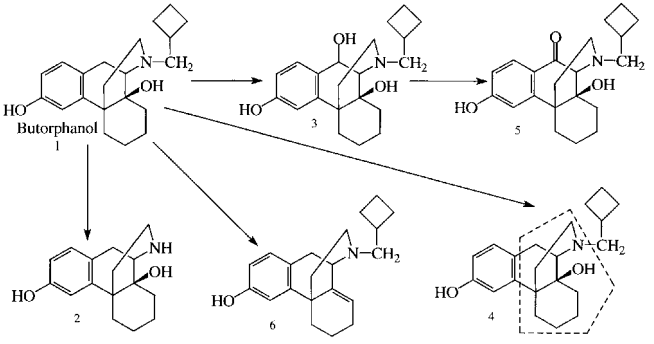


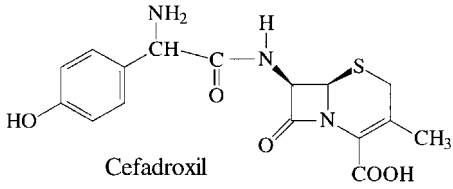
Figure 5.3.E.
Degradation pathway of butorphanol tartrate. Dashed area indicates substructure involved in proposed ring contraction (for the reference see Fig. 5.3.D)

contraction in **4** (molecular weight M-14) was not possible even by HPLC/MS/MS. The diode-array UV spectra were not informative with the exception of Impurity **5**, where the conjugation between the phenolic ring and the oxo group was easily recognisable.

The same group investigated the degradation pathways of *cefadroxil* under stress conditions using the same methodology (HPLC/UV/MS followed by HPLC/MS/MS) with the aim of rapidly obtaining a library of degradants for further studies in the development phase of the drug [14]. The stress conditions included heating of the solid material for 6 hours at 140°C, the aqueous solution for 8 h at 40°C and treating at ambient temperature solutions in 0.3 M hydrochloric acid for 2 h, in 0.01 and 1 M sodium hydroxide for 1.5 and 0.5 h, respectively. It is worth mentioning that using this methodology it is not mandatory to resolve co-eluting components since product ion spectra obtained by the HPLC/MS/MS studies provide evidence even for the structures of unresolved degradation products. In the course of the interpretation of the product mass spectra the main component is used as a substructural ''template'': it is expected that the related impurities and degradants undergo similar fragmentation to the parent compound. The chromatographic data and molecular weights obtained from the HPLC-MS studies and the proposal for the structures of seventeen degradants and impurities obtained from these supplemented by evaluation of the product ion spectra are summarised in Table 5.3.A. As it is seen, for most of the components, among them the minor ones even this rapid screening method enabled structures to be determined leaving, however, a few question marks especially for the fine structure of some of the degradants.

HPLC/particle beam MS technique supplemented by gas chromatography-mass spectrometry (GC-MS) were used by Segarra *et al*. [15] for the

Table 5.3.A.
Profile library of cefadroxil degradant and impurity products[a]



Cefadroxil

	Relative retention time	Molecular weight	Proposed structure
1	0.15	379	Cefadroxil + 16 Da
2	0.20	379	Cefadroxil sulphoxide isomer
3	0.26	397	Cefadroxil sulfoxide isomer
4	0.32		
5	0.40	317	
6	0.62	363	Cefadroxil isomer
7	0.64	363	Δ^2-cefadroxil isomer
8	0.77	233	
9	1.0	381	Cefadroxil with hydrolysed lactam
10	1.0	363	**Cefadroxil**
11	1.98	363	7-epi-Cefadroxil
12	2.12		
13	2.15	363	Piperazinedione cefadroxil isomer
14	2.17	377	Methyl ester of cefadroxil (impurity)
15	2.20	398	
16	2.25	329	
17	2.35	512	Cefadroxil with additional CO-$CHNH_2$-C_6H_4-OH side chain (impurity)
18	2.38	363	Isomer of piperazine dione of cefadroxil (Δ^2 or 7-epi)

[a] From Ref. [14]

identification of the degradation products of *dobupride* (**1** in Fig. 5.3.F) under stress conditions in the solid state and in solution. Although the sensitivity of the former technique is low, an advantage is that the EI (electron impact) mass spectra thus obtained with their rich fragmentation patterns are eminently suitable for the identification of the major degradants (**2**, **4** and **6**). For the identification of the minor degradation products (**3** and **5**) the sensitive GC/MS technique was adopted.

Further studies using HPLC/MS/(MS) technique for the elucidation of degradation pathways are only briefly summarised. Du Preez *et al.* [16] reported on the hydrolytic decomposition of the heterocyclic cage compound *NGP1-01* (8-benzylamino-8,11-oxapentacyclo[5.4.0.0^{2,6}.0^{3,10}.0^{5,9}]undecane) to benzylamine and the corresponding keto-alcohol. Graham *et al.* [17] investigated the degradation of *gentamicin sulphate* in dextrose solution. The HPLC system with electrochemical detection adopted for this study separated the four components of gentamicin and four degradants, among them gentamine C1 and sisomicin the structures of which were determined by HPLC/thermospray MS. During the course of degradation of *mazipredone* under stress conditions (treatment with hydrochloric acid and sodium hydroxide) in addition to the two isomeric C-17 enol-aldehydes already mentioned in Section 5.3.1, Gazdag *et al.* [7] separated and identified several other degradation products including 17-oxo, 20-oxo-21-aldehyde hydrate, 17α-hydroxy-20-carboxylic acid, 17α-hydroxy-20-oxo-21-aldehyde hydrate and 17α,20-dihydroxy-21-carboxylic acid; see Figs. 5.5.D–5.5G in Section 5.5.5. Revelle *et al.* [18] identified 11 degradation products in variously stressed *chlorhexidine digluconate* samples. Zhang *et al.* [19,20] studied the stability of a combination of *cisatracurium besylate* and *propofol* by HPLC/MS. The degradation products of the former included various monomeric derivatives (monoacrylate, acid and alcohol as

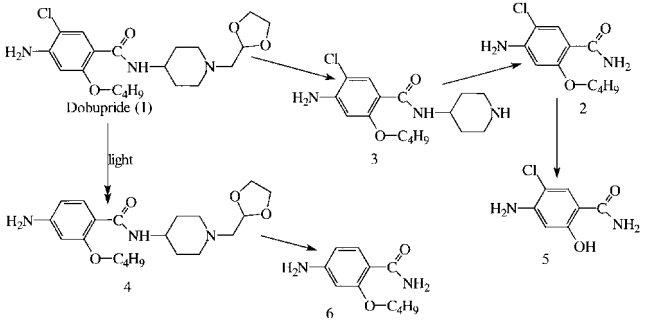


Figure 5.3.F.
Degradation scheme of dobupride (from Ref. [15])

well as laudanosine), while the latter did not degrade. Wu *et al*. [21] identified oxidative degradates of the thrombin inhibitor *3-(2-phenethylamino)-6-methyl-1-(2-amino-6-methyl-5-methylenecarboxamidomethylpyridinyl)pyrazone.* Bartlett *et al*. [22] studied the degradation of *isradipine* effected by 0.1 M hydrochloric acid and sodium hydroxide, hydrogen peroxide and light. The structures of degradates identified by HPLC/MS are shown in Fig. 5.3.G.

The great separation power of capillary electrophoresis (CE) in association with matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) with its ability to provide molecular mass information in the high molecular mass region was successfully utilised by Cruz *et al*. [23] for the elucidation of the very complex degradation mechanism of *nisin*, a peptide antibiotic containing 34 amino acid residues. In this study the fractions obtained by CE were investigated by MALDI-TOF-MS in the off-line mode.

The study of Fischer and Klotz [24] to elucidate the oxidative degradation pathways of *5-aminosalicylic acid* (5-AS) and its main metabolite, *N*-acetyl-5-aminosalicylic acid (Ac5-AS) merits special attention. In this case the aim of the study was not only to obtain data regarding the behaviour of this drug substance under oxidative stress conditions but the results obtained under *in vitro* conditions were hoped to allow a better estimation of the reactivity of 5-AS in terms of its radical scavenging properties under *in vivo* conditions thus contributing to the understanding of its antiinflammatory potency. The hydroxyl radicals were generated by iron(II)/hydrogen peroxide and titanium(III)/hydrogen peroxide systems. The oxidation products were separated by HPLC and the fractions were subjected to GC/MS analysis (both positive- and negative-ion electron-impact modes) after derivatisation of 5-AS and Ac5-AS to their *A*. ethyl ester, N,O-ethyloxycarbonyl, *B.* methyl ester, trifluoroacetyl and *C*. tert.-butyldimethylsilyl derivatives. The primary oxidation products were 2-carboxy-1,4-benzoquinone monoimine and its N-acetyl derivative with a large variety of secondary transformation products.

5.3.4. The Complex Application of Chromatographic and Spectroscopic Techniques

A common feature of the studies outlined in Sections 5.3.2 and 5.3.3 is that the strategy to elucidate the degradation pathways of drugs was based on the on-line application of spectroscopic techniques after chromatographic separation. This approach can be fully successful in relatively simple cases, when the mechanism of the degradation is not too complicated and only a small number of degradants has to be identified. Very useful data are obtainable in such a way even in the case of more complex degradation mechanisms, with the number of degradants in the range of 5–10 or even more if the aim of the

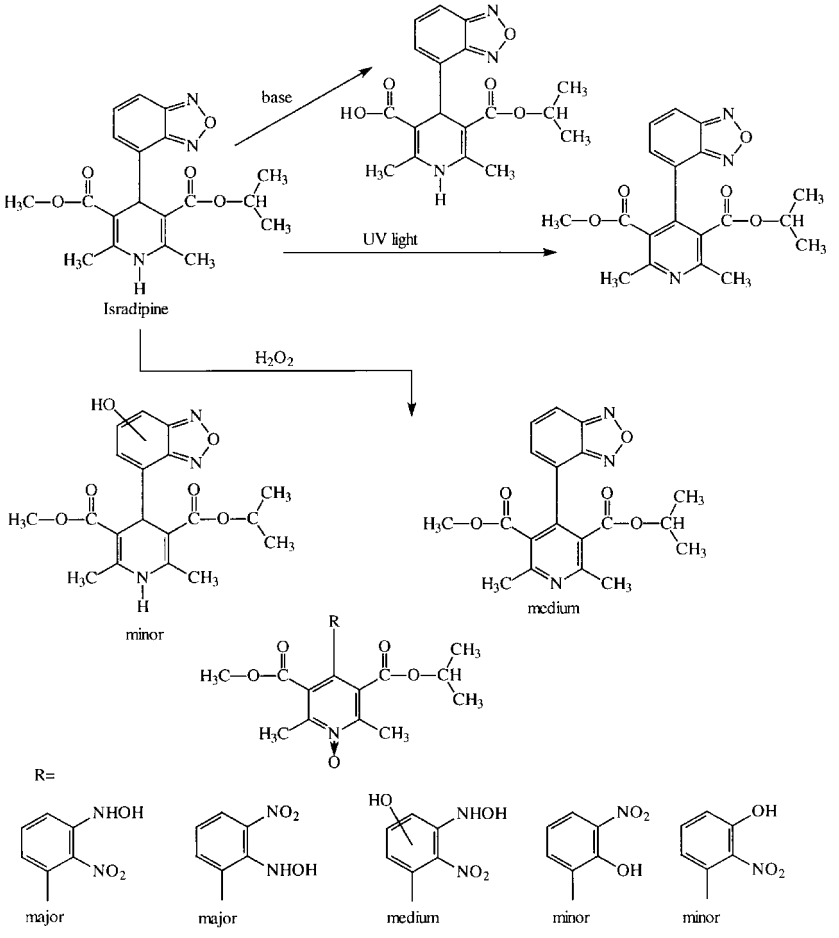


Figure 5.3.G.
Degradation products of isradipin. Degradates in 0.1 M sodium hydroxide; UV light degradation products; degradates in hydrogen peroxide solution (from Ref. [22])

study is to get a rapid information on the degradation pathway sacrificing in some instances some details for the speed of obtaining this information. This level of information is in many cases sufficient for further drug development.

In many other cases, however, the detailed mechanism of the degradation including the fine structures of the degradants (major and minor components

alike) are necessary to get a full picture about the stability of the drug under various conditions. If this is the goal of the study, on-line HPLC/MS/(MS) and GC/MS investigations have to be supplemented by off-line mass spectrometric exact mass measurements enabling the elemental composition of the degradate to be determined and mainly with NMR studies which usually require the isolation of the degradation products by semipreparative HPLC prior to these studies. In connection with this it can be mentioned that the degradation pathway for dobupride (See Fig. 5.3.F) proposed by Segarra *et al*. [15], outlined in the previous section was criticised by Singh [25]. Although this criticism was based mainly on speculations, the author missed the confirmation of the degradation scheme by NMR spectroscopic, CHN and other data.

Another example is the elucidation of the degradation pathway of *cefaclor* described by Baertschi *et al*. [26]. This cephalosporin antibiotic drug is structurally closely related to cefadroxil the degradation pathway of which based on a rapid HPLC/MS/(MS) study [14] was discussed in Section 5.3.3. The authors of this rapid screening system claim that in less than one day their structure elucidation strategy provided detailed information regarding the degradants in the stability samples [13]. As it is seen in Table 5.3.A some of the detected degradants of cefadroxil remained unidentified and some others were characterised but the exact location and stereochemistry of the functional groups in the isomeric degradants were not elucidated. In contrast to these, in the study of Baertschi *et al*. [26] where the subject of the investigation was restricted to the elucidation of the degradation pathways in aqueous solutions (0.1 M hydrochloric acid, buffer pH 5.5 and unbuffered deionised water) the following techniques were used: analytical and preparative HPLC, HPLC/diode-array UV-, IR-, ^{1}H- and ^{13}C-NMR-spectroscopy, EI- and FAB-MS (exact mass measurement for the determination of the elemental composition of the isolated materials), FAB-MS/MS, single-crystal X-ray diffractometry, synthesis of several compounds for spectral and chromatographic studies as potential impurities or model compounds to elucidate the mechanism of the degradation. Although the authors of this study did not characterise the time demand of this study, it can be stated by great certainty that it was two to three orders of magnitude higher than in the case of the above mentioned rapid screening.

The structures for the degradants are collected in Fig. 5.3.H. The complexity of the degradation mechanism can be characterised by the fact that the original paper contains no less that five reaction schemes (among them two full-page and three half-page ones) to describe the mechanism of reactions leading to thiazole-type, pyrazine-type degradation products and the formation of reactive intermediates playing important roles in the degradation pathways.

In contrast to the last examples where several sites in the molecules of the drug substances participate in the degradation reactions under various conditions, in the following example only the oxidative degradation affecting only

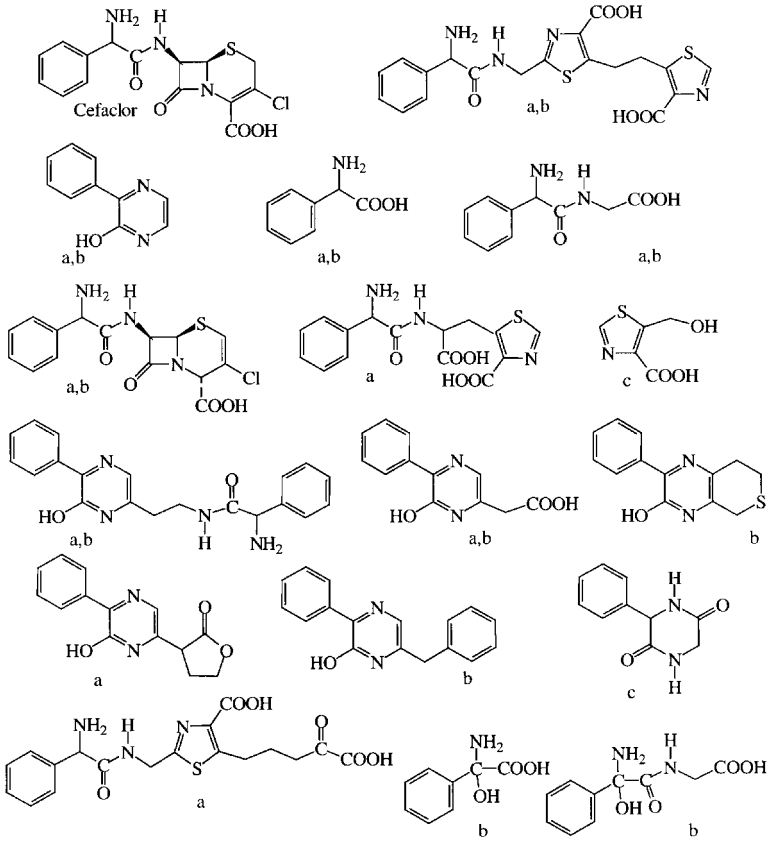


Figure 5.3.H.
Degradation products of cefaclor in 0.1 M hydrochloric acid[a], deionised, unbuffered water[b], buffered to pH 5.5[c] (from Ref. [26])

one single moiety of the molecule of the steroid drug *tipredane,* the C-17 asymmetric dithioketal group was the subject of the study by Euerby *et al.* [27–31]. The acid-catalysed hydrolysis of tipredane is very simple and predictable: it leads by the elimination of the thioketal moiety to the 17-oxo derivative, 9α-fluoro,11β-hydroxy-androst-1,4-diene-17-one. In contrast to this the complex use of chromatographic (HPLC and semi-preparative HPLC), spectroscopic (^{1}H- and ^{13}C-NMR, FT-IR, thermospray- and FAB-MS) techniques and single crystal diffractometry as well as synthesis and separation of the degradates were necessary to elucidate the structure and stereochemistry of

the oxidative degradation products. The primary reaction products of tipredane (**1**) with hydrogen peroxide (or peroxide impurities in polyethylene glycol applied in drug formulations [30]) are sulphoxides at the S-ethyl (**2**) or the S-methyl (**3**) groups both being present as pairs of diastereomers due to the chiral centres introduced by the formation of sulphoxides. Further oxidation leads to the disulphoxide (**4**) with two chiral centres and four diastereomers. Fig. 5.3.I shows the HPLC separation of the eight diastereomers. Tipredane is eluted at 42 min in this system. The mono- and disulphoxides are transformed by thermal elimination of ethyl- or methylsulphenic acid to the corresponding Δ^{16}-methylthio (**5**, 39 min), ethylthio (**6**, 41 min), *R* and *S*-methylsulphoxide (**7**, 15 and 14 min) as well as *R* and *S*-ethylsulphoxide (**8**, 26.3 and 23.7 min) derivatives. Another possibility is the very slow oxidation of the sulphoxides (e.g. **2**) to the corresponding sulphones, (e.g. **9**, 32.1 min). (The time data in parentheses are retention times in the HPLC system in Fig. 5.3.J). The scheme of the degradation is depicted in Fig. 5.3.J.

The elucidation of the stereochemistry of all products and intermediates of the degradation and the well optimised HPLC (using the ICOS system) enabled the exact mechanism of the degradation including the kinetics of the elementary reactions to be established.

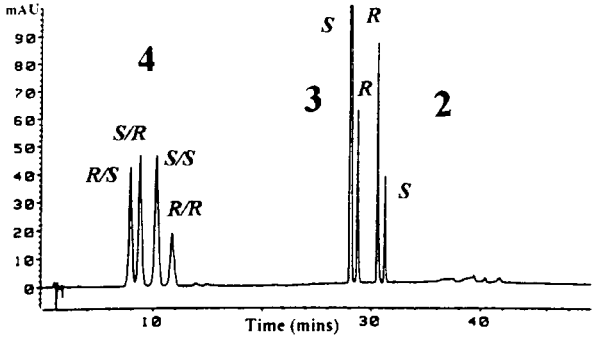


Figure 5.3.I.
HPLC separation of the 8 diastereomers of tipredane sulphoxides. Column: Hypersil Excel ODS, 3 μm, 150 × 4.6 mm. Eluent: 0.025 M ammonium acetate (pH 7.1), in A: tetrahydrofuran/methanol/acetonitrile/water 4.8:16:4:75.2 v/v, B: acetonitrile/water 65:35 v/v. Linear gradient: 0–20 min 0% B, 40 min 100% B, 50 min 100% B. Flow rate: 1 ml/min. Temperature: 40°C. Key: **2**, *R*- and *S* -ethylsulphoxide; **3**. *R*- and *S*-methylsulphoxide; **4**, (peaks 1 and 4: *R*-methylsulphoxide, *S*-ethylsulphoxide, peaks 2 and 3: *S*-methylsuphoxide, *R*-ethylsulphoxide) (from Ref. [31])

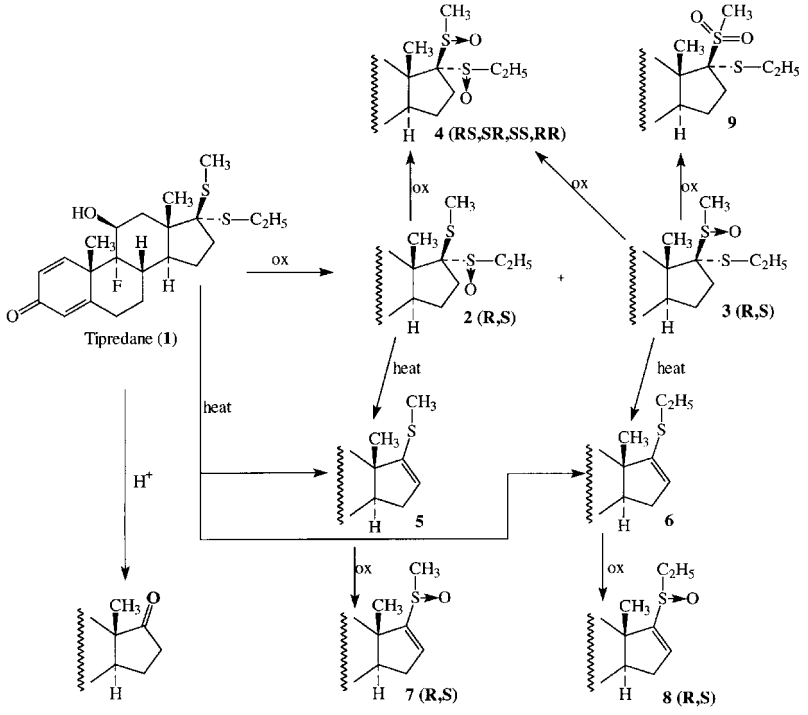


Figure 5.3.J.
Degradation scheme of tipredane

5.3.5. Special Problems of Drug Dosage Forms

In the majority of cases the impurities identified in the course of model degradation studies and those found when dosage forms are investigated are identical or at least there is a close relationship between them. In some cases, however, the identification of degradates in dosage form presents special problems: the interaction between the active ingredient and excipients or package materials have to be taken into consideration.

An example for the interaction with the excipient is the identification of degradation products found during the course of stress stability testing of enteric polymer-coated pellet formulations of *duloxetine hydrochloride* ((*S*)-*N*-methyl-3-(naphthalenyloxy)-2-thiophenepropanamine hydrochloride). Electrospray HPLC-MS investigations revealed that the apolar impurities found during the HPLC test of the coated pellets were *N*-phthaloyl and *N*-succinyl

derivatives of duloxetine, respectively, where the source of the phthaloyl and succinyl residues are the enteric polymers hydroxylpropyl methylcellulose phthalate and hydroxylpropyl methylcellulose acetate succinate used for the coating of the pellets [32].

The example for the impact of the package material on the degradation profile of a drug formulation is an impurity found during the HPLC/APCI-MS/MS investigation of a tablet formulation of *famotidine* (for the formula see page 474). The molecular weight of the degradate (M + 12) suggested that it was a reaction product of famotidine with formaldehyde. The analysis of the product ion mass spectrum of the degradate followed by the synthesis of the proposed structure and detailed spectroscopic investigations indicated that the location of the attack of formaldehyde was at the two primary amino groups of the *N*-(aminosulphonyl)-propanimid-amide moiety leading to a six-membered ring. The origin of formaldehyde was certainly the degradation of the foil used for packaging under stress conditions [33].

References

1. K.A. Connors, G.L. Amidon and V.J. Stella, *Chemical Stability of Drug Substances: a Handbook for Pharmacists, 2nd edn*, Wiley, New York (1986)
2. J.T. Carstensen, *Drug Stability, 2nd edn*, Marcel Dekker, New York (1995)
3. W. Grimm (Ed.), *Stability Testing of Drug Products*, Wissenschaftliche Verlagsgesellschaft, Stuttgart (1987)
4. I. Rácz, *Drug Formulation*, Wiley, Chichester (1989)
5. K. Florey or H.G. Brittain (Eds.), *Analytical Profiles of Drug Substances*, Vols. I–XXV, Academic Press, New York (1972–1998)
6. S. Görög, *Ultraviolet-Visible Spectrophotometry in Pharmaceutical Analysis*, pp 180–183, CRC Press, Boca Raton, FL (1995)
7. M. Gazdag, M. Babják, J. Brlik, S. Mahó, Z. Tuba and S. Görög, *J. Pharm. Biomed. Anal*. **16**, 1029–1036 (1998)
8. M.L. Lewbart, C. Monder, W.J. Boyko, C.J. Singer and F. Iohan, *J. Org. Chem*. **54**, 1332–1338 (1989)
9. S. Görög, M. Babják, G. Balogh, J. Brlik, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *J. Pharm. Biomed. Anal*. **18**, 511–525 (1998)
10. J.P. Dusza, M. Heller and S. Bernstein, in *Physical Properties of Steroid Hormones* (L.L. Engel, Ed.), pp 69–287, Pergamon Press, Oxford, 1963
11. A.I. Scott, *Interpretation of the Ultraviolet Spectra of Natural Products*, pp 364–428, Pergamon Press, Oxford (1964)

12. S. Görög, M. Bihari, É. Csizér, F. Dravecz, M. Gazdag and B. Herényi, *J. Pharm. Biomed. Anal*. **14**, 85–92 (1996)
13. K.J. Volk, S.E. Klohr, R.A. Rourick, E.H. Kerns and M.S. Lee, *J. Pharm. Biomed. Anal*. **14**, 1663–1674 (1996)
14. R.A. Rourick, K.J. Volk, S.E. Klohr, T. Spears, E.H. Kerns and M.S. Lee, *J. Pharm. Biomed. Anal*. **14**, 1743–1752 (1996)
15. V. Segarra, F. Carrera, J.-L. Fabregas and J. Claramunt, *J. Pharm. Biomed. Anal*. **13**, 987–993 (1995)
16. J.L. du Preez, A.P. Lötter and J.K. Guillory, *Pharmazie* **51**, 223–227 (1996)
17. A.E. Graham, E. Speicher and B. Williamson, *J. Pharm. Biomed. Anal*. **15**, 537–543 (1997)
18. L.K. Revelle, W.H. Doub, R.T. Wilson, M.H. Harris and A.M. Rutter, *Pharm. Res*. **10**, 1777–1784 (1993)
19. H. Zhang, P. Wang, M.G. Bartlett and J.T. Stewart, *J. Pharm. Biomed. Anal*. **16**, 1241–1249 (1998)
20. P. Wang, H. Zhang, J.T. Stewart and M.G. Bartlett, *J. Pharm. Biomed. Anal*. **17**, 547–553 (1998)
21. Y. Wu, X. Chen, L. Gier, Ö. Almarsson, D. Ostovic and A.E. Loper, *J. Pharm. Biomed. Anal*. **20**, 471–485 (1999)
22. M.G. Bartlett, J.C. Spell, P.S. Mathis, M.F.A. Elgany, B.E. El Zeany, M.A. Elkawy and J.T. Stewart, *J. Pharm. Biomed. Anal*. **18**, 335–345 (1998)
23. L. Cruz, R.W. Garden, H.J. Kaiser and J.V. Sweedler, *J. Chromatogr. A*. **735**, 375–385 (1996)
24. C. Fischer and U. Klotz, *J. Chromatogr. B*. **661**, 57–68 (1994)
25. S. Singh, *J. Pharm. Biomed. Anal*. **15**, 1801–1803 (1997)
26. S.W. Baertschi, D.E. Dorman, J.L. Occolowitz, M.W. Collins, L.A. Spangle, G.A. Stephenson and L.J. Lorenz, *J. Pharm. Sci*. **86**, 526–539 (1997)
27. M.R. Euerby, J. Hare and S.C. Nichols, *J. Pharm. Biomed. Anal*. **10**, 269–277 (1992)
28. M.R. Euerby, C.M. Johnson, R.J. Lewis and S.C. Nichols, *J. Pharm. Biomed. Anal*. **12**, 761–769 (1994)
29. M.R. Euerby, C.M. Johnson and S.C. Nichols, in *Advances in Steroid Analysis '93* (S. Görög, Ed.), pp 213–220, Akadémiai Kiadó, Budapest (1994)
30. M.R. Euerby, J. Hare and S.C. Nichols, in *Advances in Steroid Analysis '93* (S. Görög, Ed.), pp 605–612, Akadémiai Kiadó, Budapest (1994)
31. M.R. Euerby, J.A. Graham, C.M. Johnson, R.J. Lewis and D.B. Wallace, *J. Pharm. Biomed. Anal*. **15**, 299–313 (1996)

32. P.J. Jansen, P.L. Oren, C.A. Kemp, S.R. Maple and S.W. Baertschi, *J. Pharm. Sci*. **87**, 81–85 (1998)
33. X.-Z. Qin, D.P. Ip, K.H.-C. Chang, P.M. Dradransky, M.A. Brooks and T. Sakuma, *J. Pharm. Biomed. Anal*. **12**, 221–233 (1994)

5.4. Elucidation of Light-Induced Degradation Pathways

Sándor Görög

5.4.1. The Importance of Photodegradation Studies

The investigation of the photostability of drugs and drug formulations is of prominent importance; it is of interest to organic chemists, pharmaceutical technologists, analysts, pharmacologists, toxicologists and clinicians dealing with various aspects of drug research and development. The importance and how current this field is can be characterised by the fact that in the last decade an excellent book [1], three book chapters [2–4], several reviews and a large number of papers have been devoted to this topic.

The main reasons for this high level of interest are as follows:

(a) More than 50% of the monographs for drug materials in the European Pharmacopoeia [5] contain prescriptions for being stored protected from light. This means that the limited photostability of drug materials and formulations is a general problem and for this reason testing the drugs for their stability against light is an integral part of their stability testing under stress conditions [6].

(b) The primary aim of photostability studies is to determine the stability of the drug substance in the solid or dissolved state using either natural sunlight or much more generally light sources (xenon arc, metal halide lamp, cool white fluorescent lamp, near ultraviolet fluorescent lamp) simulating sunlight conditions. The purpose of using these artificial light sources is to increase the intensity of the radiation thus improving the standardisability of the test and shortening its duration. The identification of the photodegradation products and the elucidation of photodegradation pathways is a most interesting task: the direction of the reactions is much less predictable than that of thermal degradation reactions. For this reason in addition to the unquestionable practical importance of the results thus obtained such studies afford most interesting possibilities for chromatographers and spectroscopists to meet with interesting reactions and products and for organic chemists to deal with the interpretation of the reactions and with establishing the reaction mechanisms. This is probably another reason for the popularity of such studies. This applies especially to those studies where the light source is a high-pressure mercury lamp emitting radiation in the much lower wavelength range than outdoor light. Under these conditions the majority of drug substances undergo photodegradation thus affording inexhaustible possibilities for interesting studies.

(c) Phototoxicity and photoallergy are important issues among the side effects in drug therapy. Otherwise non-toxic drugs can be transformed in the human organism to photoproducts upon exposure to light. The importance of this is underlined by the recent deterioration of the ozone layer in the atmosphere and by the spreading of high-intensity artificial light sources with spectral distribution different from that of sunlight. These adverse effects can be well modelled by in vitro photostability studies.

(d) In contrast to the above mentioned phototoxicity and photoallergy, beneficial effects of the interaction of light with chemical agents absorbed by the human body are the basis of phototherapy. The in vitro modelling of this, i.e. the transformation of pharmacologically inactive materials to active drugs is also an important issue.

Despite the efforts to standardise the conditions and requirements of light stability testing [6], the level of standardisation and the uniformity of the practice used in various laboratories are far from those achieved in other fields of drug stability testing [1a]. The main differences are in the energy distribution and intensity of light sources, form and duration of exposure of the test samples to light and the interpretation of the results [7].

5.4.2. Classical Examples

Many of the classical drugs are known to be sensitive to light. Their degradation products have been identified and the often very complex degradation mechanisms clarified already in the early period of the development of pharmaceutical chemistry or in the course of the 1970s and 1980s [1,3,8–11].

Photooxidation is the most frequently occurring degradation pathway. Some examples are as follows. *Catecholamines* such as adrenaline, noradrenaline and isoprenaline are photooxidised to adrenochrome derivatives. The main product of the photooxidation of *morphine* is the dimeric pseudomorphine. *Propranolol* is oxidised at the isopropylamine side chain. *Tetracycline* is oxidised to quinones, *chloramphenicol* to p-nitrobenzaldehyde and other products, *dithranol* to danthron and dianthrones, *nifedipine* and other 1,4-dihydropyridines to aromatic pyridine derivatives. The phototoxic main oxidation product of norethisterone is the $4\beta,5\beta$-epoxide. In addition to isomerisation, epoxidation takes place during the course of the light catalysed degradation of *amphotericin*. Phenothiazines are also sensitive to light. The first step of the photooxidation of e.g. *promethazine, prochlorperazine* and *chlorpromazine* is the formation of the sulphoxides followed by further oxidative degradation steps leading finally to the discolouration of their solutions. The phototoxicity of the degradation products and their intermediates has been subject to extensive studies [4]. The light catalysed addition of water to their 9,10-double bond

to form the 10-hydroxy derivatives (lumiergotamine, etc.) are characteristic degradation reactions of *ergot alkaloids*. Irradiation by ultraviolet light not only plays important role in the preparation of *vitamins D*, but they are transformed to suprasterols and toxisterols upon further irradiation. Further light catalysed degradations among vitamins are the oxidative degradation of *vitamins A, C, E* and *menaquinone,* transformation of *vitamin* B_2 to lumichrome and lumiflavine in acidic and basic solutions, respectively, dimerisation of *vitamin* B_6, transformation of *vitamin* B_{12} to hydroxocobalamine, oxidative deamination of *folic acid* (and *methotrexate*) to pteridine carboxaldehyde and *p*-aminobenzoyl glutamic acid. *Ketoprofen* is decarboxylated followed by oxidation to (3-benzoylphenyl)-ethanol and (3-benzoylphenyl)-ethanone. The products of the photohydrolysis of *frusemide* are saluamine and *N*-furfuryl-5-sulphamoylanthranilic acid.

In contrast to the above listed photooxidations, photoreduction takes place with *nitrazepam* in the presence of transferable hydrogen leading to dimeric products. The main reaction routes in the course of the photodegradation of *hydrochlorthiazide* are dechlorination and hydrolytic ring opening. The short wavelength UV irradiation of *diazepam* leads to benzophenones, 4-phenylquinazolines and 4-phenylquinazolinones. In the case of *chlordiazepoxide* the primary product is a phototoxic oxaziridine derivative which is transformed to quinoxaline and benzoxadiazocine derivatives.

Some of the classical drugs such as *theophylline, apomorphine, thyroxine, imipramine, indomethacin, tetracycline, danthrone* etc. or their solutions undergo discolouration upon exposure to light. The intensity of colour of the degradation product can be very high: the depth of the colour is not necessarily a good indicator of the extent of degradation. Many other important drugs are characterised as being sensitive to light, e.g. *diphenhydramine* (with unstable intermediates responsible for photoallergy), *chloroquine, doxorubicin, metronidazol, nitrazepam, ranitidine, nystatin, trimethoprim, vinblastin, vincristine, warfarin, nitrofurantoin* etc. During the course of the photodecomposition of the latter to 5-hydroxymethylene-2(5H)-furanone nitrite ions are also formed causing methaemoglobinemia [4].

5.4.3. Recent Studies for the Elucidation of Photodegradation Pathways

For the identification of the products formed when *norethisterone* is irradiated by UV-B light chromatographic separation on silica column followed by IR, NMR, MS and CD spectroscopic investigation of the isolated materials was used by Sedee *et al*. [12,13]. In addition to the already mentioned phototoxic $4\beta,5\beta$-epoxide [12] these include further oxidation products e.g. $4\alpha,5\alpha$-epoxide, 5-hydroxy and 5-ethoxy, dihydroxy derivatives, reduction products such as

stereoisomeric 4,5-dihydro derivatives, 3-hydroxy derivative and dimeric products [13]. Even more exotic photodegradation products were identified when the aqueous solution of *methylprednisolone suleptamate* was subjected to irradiation with white fluorescent light [14]. In addition to hydrolysis (methylprednisolone) and oxidation products (17-oxo and 11-oxo derivatives) HPLC/UV/MS revealed the presence of two unknown photodegradates. These were isolated by preparative HPLC (separation on Inertsil Ph followed by desalting on YMC D-ODS). NMR (based especially on NOE investigations and deuterium isotope effect), Cs-FAB and diode-array UV spectroscopic investigation of the isolated degradates led to the structures shown in Fig. 5.4.A.

The photodegradation of fluoroquinolone antibacterial agents has been subject to extensive research up to the present time due to the phototoxicity of the degradation products [15]. Torniainen *et al.* [16,17] used high pressure mercury lamp to induce the photodegradation of *ciprofloxacin,* followed the reaction by TLC and HPLC and subjected the products isolated by flash chromatography on silica column to MS and NMR studies. It was found that the degradation to the 7-amino derivative takes place via the 7-[(2–aminoethyl)-amino] intermediate (see Fig. 5.4.B). The 7-[(2–aminoethyl)amino] derivative

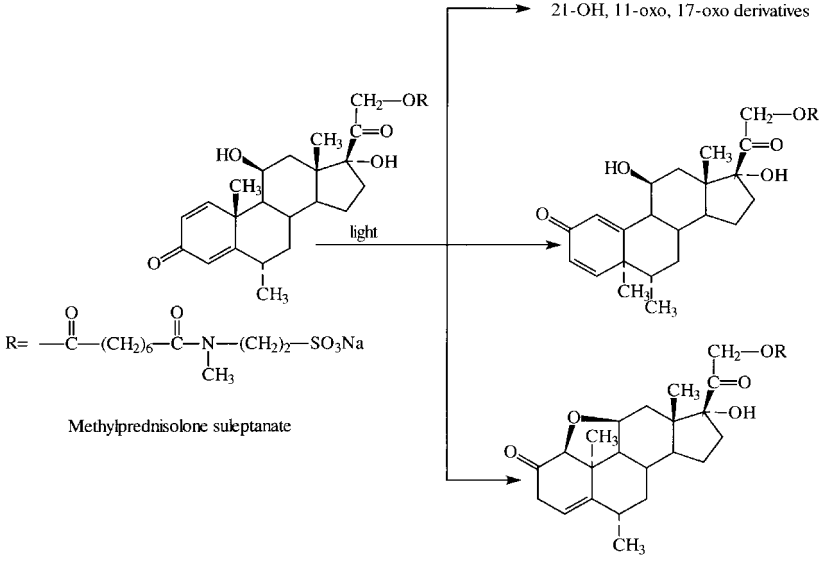


Figure 5.4.A.
Photodegradation scheme of methylprednisolone suleptanate (from Ref. [14])

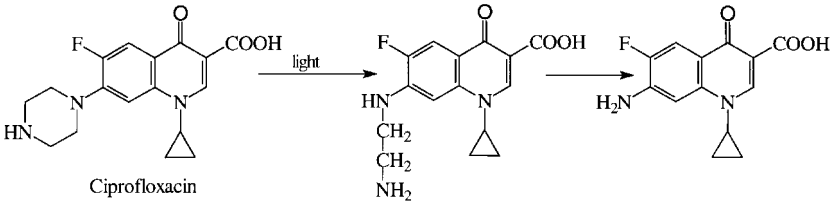


Figure 5.4.B.
Photodegradation scheme of ciprofloxacin (from Ref. [16])

was described and characterised by HPLC also in the case of the 1-ethyl analogue (*norfloxacin*) [18]. The main photodegradation reactions of the structurally related derivative, *sparfloxacin* (5-amino-1-cyclopropyl-1,4-dihydro-7-(*cis*-2,6-dimethyl-1-piperazinyl)-6,8-difluoro-4-oxo-3-quinolinecarboxylic acid) under similar conditions are splitting off of the 8-fluorine and of the 8 fluorine plus the cyclopropyl ring as found by Engler *et al*. [19] on the basis of HPLC/MS/MS studies.

The degradation of *metronidazol* (2-methyl-5-nitro-1*H*-imidazole-1-ethanol) in citrate-phosphate buffer (pH 5), exposed to ambient sunlight was studied by Godfrey and Edwards [20]. Separation by analytical and preparative HPLC followed by the complex application of MS, NMR, IR and UV spectroscopies revealed that the primary, bright yellow (λ_{max} 370 nm) product of the photodegradation is a relatively stable ''excimer ion pair'' formed by the

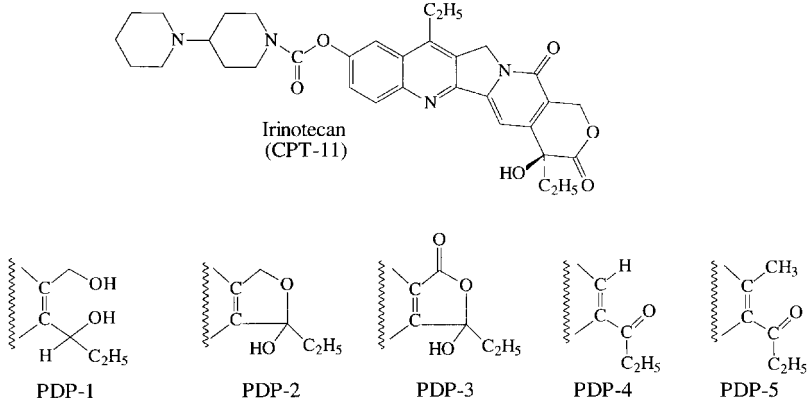


Figure 5.4.C.
Formula of irinotecan (CPT-11) and photodegradation products

interaction of metronidazol with its photoexcited nitro group and citrate ion. This adduct gives a well separable peak in RP-HPLC. Its further transformation by photolytic and thermal degradation to 4-hydroxy-5-desnitro metronidazol and acetone dicarboxylic acid (from the citrate moiety) can be well followed by HPLC.

The elucidation of the photodegradation of the new anticancer drug *irinotecan* (*CPT-11, Campto®, Camptosar®*) merits special mentioning. Two groups dealt simultaneously with this problem, Akimoto *et al.* [21–23] and Dodds *et al.* [24,25]. On the basis of analytical HPLC with UV, fluorescence and MS/MS detection, preparative HPLC followed by IR, NMR and MS studies in was found that the vulnerable part of the molecule is its lactone ring: the structures of the five photodegradation products differ only at this moiety. HPLC/fluorescence/APCI-MS studies enabled the plasma and urine of cancer patients to be screened for these products in order to make comparison

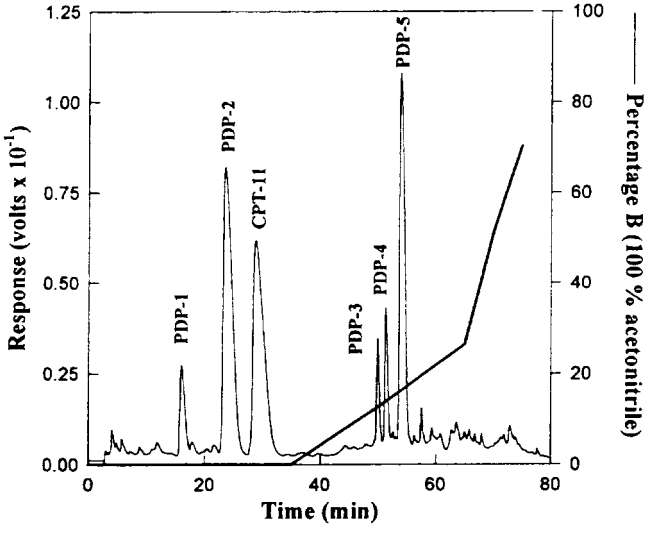


Figure 5.4.D.
Chromatogram of the precipitate formed 5 days after exposure of 100 mg irinotecan (CPT-11) in 50 ml of 0.9% NaCl to white fluorescent light and its five photodegradation products (PDP 1-5). See Fig. 5.4.C for the structures. Column: Nova-Pak phenyl, 4 μm, 150x8 mm. Eluent: A: 0.15 M ammonium acetate (pH 5.2)/acetonitrile/2-propanol 80:17:3 v/v, B: acetonitrile. The solid line represents the gradient profile. Flow rate: 1.4 ml/min. UV detector: 350 nm (from Ref. [24])

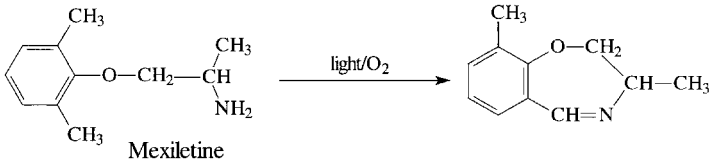


Figure 5.4.E.
Photodegradation of mexiletine

between the photodegradation and metabolism of the drug [25]. The formulae of irinotecan and the five photodegradation products and a typical HPLC scan appear in Figs. 5.4.C and 5.4.D, respectively.

The photodegradation products of *midazolam* (8-chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,5–*a*][1,4]benzodiazepine produced by irradiation by a high-pressure mercury lamp were isolated by flash chromatography by Andersin *et al*. [26] and subjected to complex spectroscopic structure elucidation. The minor degradants were identified by GC/MS. The identified structures included 6-(8-chloro-1-methyl-4,5-dihydro-2,5,10*b*-triaza-benzo[*e*]azulen-6-ylidene)-cyclohexa-2,4-dienone, 6-chloro-2-methyl-4-(2-fluorophenyl)-quinazoline, 6-chloro-2-methyl-4(^{1}H)-quinazolinone, 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one, 2-amino-5-chloro-2′-fluorobenzophenone, 2-acetamido-5-chloro-2′-fluorobenzophenone and 6-(6-chloro-2-methyl-3H-quinazolin-4-ylidene)-cyclohexa-2,4-dienone.

An oxidative cyclisation product of *mexiletine* was identified by Takács [27] formed upon exposure of the solid material or its methanolic solution to sunlight or UV light. The reaction equation is seen in Fig. 5.4.E.

During the course of the stability study by HPLC of *fotemustine* in 5% dextrose solution in PVC infusion bags it was found to undergo a cyclisation reaction when exposed to ambient or solar light (see Fig. 5.4.F) [28].

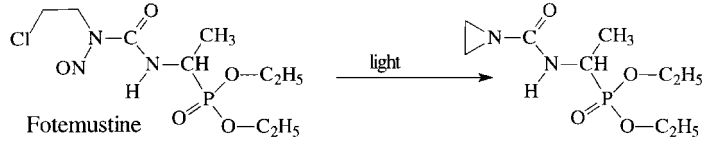


Figure 5.4.F.
Photodegradation of fotemustine

References

1. H. Hjorth Tønnesen (Ed.), *Photostability of Drugs and Drug Formulations*, Taylor & Francis, London (1996). a, pp 287–304; b, pp 83–110
2. J.W. Greenhill and M.A. McLelland, *Photodecomposition of Drugs*, in *Progress in Medicinal Chemistry, Vol. 27* (G.P. Ellis and G.B. West Eds.), pp 51–121, Elsevier, Amsterdam (1990)
3. J.W. Greenhill, *Photodecomposition of Drugs*, in *Encyclopedia of Pharmaceutical Technology*, *Vol. 12* (J. Swarbrick and J.C. Boylan, Eds.), pp 105–135, Marcel Dekker, New York (1995)
4. G.M.J. Beijersbergen van Henegouwen, Medicinal photochemistry: phototoxic and phototherapeutic aspects of drugs, in *Advances in Drug Research, Vol. 29* (B. Testa and U.A. Meyer, Eds.), pp 79–169, Academic Press, San Diego, CA (1997)
5. *European Pharmacopoeia, 3rd edn*, Council of Europe, Strasbourg (1997)
6. *International Conference on Harmonisation (ICH)*, Guideline for the Photostability Testing of New Drug Substances and Products. Step 2. (ICH Topic Q 1 B) (1995)
7. N.H. Anderson, D. Johnston, M.A. McLelland and P. Munden, *J. Pharm. Biomed. Anal*. **9**, 443–449 (1991)
8. K.A. Connors, G.L. Amidon and V.J. Stella, *Chemical Stability of Drug Substances: a Handbook for Pharmacists, 2nd edn*, Wiley, New York (1986)
9. I. Rácz, *Drug Formulation*, Wiley, Chichester (1989)
10. K. Florey or H.G. Brittain (Eds.), *Analytical Profiles of Drug Substances* Vols. I–XXV, Academic Press, New York (1972–1998)
11. W. Lund (Ed.), *The Pharmaceutical Codex, 12th edn*, pp 707–1092, The Pharmaceutical Press, London (1994)
12. A.G.J. Sedee, G.M.J. Beijersbergen van Henegouwen and H.J.A. Blaauwgeers, *Int. J. Pharm.* **15**, 149–158 (1983)
13. A.G.J. Sedee, G.M.J. Beijersbergen van Henegouwen, H. de Vries, W. Guijt and C.A.G. Haasnoot, *Pharm. Weekblad Sci. Ed.* **7**, 194–207 (1985)
14. M. Ogata, Y. Noro, M. Yamada, T. Tahara and T. Nishimura, *J. Pharm. Sci.* **87**, 91–95 (1998)
15. J. Shimada and S. Hori, *Prog. Drug Res.* **38**, 133–143 (1992)
16. K. Torniainen, C.-P. Askolin and J. Mattinen, *J. Pharm. Biomed. Anal.* **16**, 439–444 (1997).
17. K. Torniainen, J. Mattinen C.-P. Askolin and S. Tammilehto, *J. Pharm. Biomed. Anal.* **16**, 887–894 (1997)
18. M. Córdoba-Borrego, M. Córdoba-Diaz and D. Córdoba-Diaz, *J. Pharm. Biomed. Anal.*, **18**, 919–926 (1999)

19. M. Engler, G. Rüsing, F. Sörgel and U. Holzgrabe, *Antimicrob. Agent Chemother.* **42**, 1151–1159 (1998)
20. R. Godfrey and R. Edwards, *J. Pharm. Sci.* **80**, 212–218 (1991)
21. K. Akimoto, A. Kawai, K. Ohya, S. Sawada and R. Aiyama, *Drug Stability* **1**, 118–122 (1996)
22. K. Akimoto, A. Kawai and K. Ohya, *Drug Stability* **1**, 141–146 (1996).
23. K. Akimoto, A. Kawai and K. Ohya, *J. Chromatogr. A* **734**, 401–404 (1996)
24. H.M. Dodds, D.J. Craik and L.P. Rivory, *J. Pharm. Sci.* **86**, 1410–1415 (1997)
25. H.M. Dodds, J. Robert and L.P. Rivory, *J. Pharm. Biomed. Anal.* **17**, 785–792 (1998)
26. R. Andersin, J. Ovaskainen and S. Kaltia, *J. Pharm. Biomed. Anal.* **12**, 165–172 (1994)
27. M. Takács, personal communication
28. T. Dine, F. Khalfi, B. Greisser, M. Luyckx, C. Brunet, L. Ballester, F. Goudaliez, J. Kablan, M. Cazin and J.C. Cazin, *J. Pharm. Biomed. Anal.* **18**, 373–381 (1998)

5.5. Methodological Aspects of Quantitative Drug Stability Studies Based on Degradates

Sándor Görög

5.5.1. General Remarks

The identification and structure elucidation of degradation products of drugs was discussed in Sections 5.3 and 5.4. Another aspect of drug degradation studies is the quantitative measurement of the intact drug material and/or its degradates as a function of time and storage conditions for the bulk drug, its solutions or various dosage forms (see Section 5.1 and 5.2). In this section the methodological aspects of this area are briefly discussed. From among the innumerable studies in the literature only a few (mainly recent ones) have been selected for presentation, where the stability study is based on the measurement of the degradate rather than on the undecomposed drug or the stability indicating analytical method enables the simultaneous estimation of the decreasing concentration of the drug material and increasing concentrations of the degradates as a function of time. The non-analytical aspects of drug stability studies are not covered. Regarding this matter the reader is referred to standard books [1–3], especially to the classical but recently updated, excellent monograph of Carstensen [2]

5.5.2. Spectrophotometric and Spectroscopic Studies

In the classical period of pharmaceutical analysis quantitative drug stability studies were based on *UV spectrophotometric* and mainly colorimetric methods [4]. These have been almost completely replaced by chromatographic and related methods. However, the introduction and rapid spreading of *derivative UV spectrophotometry* about 20 years ago [5] has greatly improved the selectivity of UV spectrophotometry thus creating better possibilities for stability indicating methods to be developed. These are mainly based on the measurement of the undecomposed drug material but in some instances the measurement is based on the selective measurement of the degradant. For example Archontaki [6] studied the hydrolytic degradation of *indomethacin* ([1–(4chlorobenzoyl)–5–methoxy–2–methylindol–3–yl]acetic acid) in alkaline solution. Neither of the hydrolysis products, 5-methoxy-2-methylindol-3-yl acetic acidand 4-chlorobenzoic acid can be determined by UV spectro-

photometry in the presence of the highly conjugated undecomposed drug. There are, however, various wavelength pairs where the fourth derivative spectrum enables the selective determination of 5-methoxy-2-methylindol-3-yl acetic acid ($^4D_{318,311}$, $^4D_{305,310}$, $^4D_{305,300}$) thus creating good possibilities for the stability study. Fourth derivative spectrophotometry enabled the pyridine-type degradation product of *nicardipine* to be determined down to a level of 0.8% in the presence of the undecomposed drug [7]. Another example is the stability study of *phenylbutazone* where using a combination of second and third deriv-atives, four different degradation products can be determined in the presence of one another and the undecomposed drug [8].

In some special cases even *colorimetric methods* can be useful for stability studies. For example, *taurolidine* (4,4′-methylenebis(perhydro-1,2,4-thiadiazine-1,1-dioxide) releases formaldehyde in aqueous solution. For this reason the measurement of formaldehyde by the classical colorimetric chromotropic acid method [5] presents good possibility for checking the stabilisation of taurolidine in aqueous solutions [9].

Fluorimetrically active degradation products of fluorimetrically inactive drugs can be conveniently measured in the presence of the intact drug material. Such a fortunate situation exists in the case of *α-aminopenicillins* (*ampicillin, amoxicillin*) and an *α-aminocephalosporin* (*cephalothin*). Their degradation in methanolic solution catalysed by Cd(II), Co(II) or Zn(II) leads to fluorescent products. For the above listed three antibiotics the wavelength of excitation maximum is 350, 362 and 349 nm, while their emission maxima are at 443, 450 and 447 nm [10].

NMR spectroscopy without chromatographic separation is a very convenient but rather seldom used possibility for drug stability studies. For example, Revelle *et al*. [11] successfully used this technique for the stability determination of *S-adenosyl-*L*-methionine.* The main degradation reaction (deactivation of the *S,S*-drug to the inactive *R,S* epimer) can be followed on the basis of the integration of the SCH_3 singlets (3.00 ppm for the *S,S*- and 2.96 ppm for the *R,S*-epimer), which can be well resolved and integrated by means of 300 MHz NMR. Another example is the stability testing of *furosemide* by NMR spectroscopy [12].

The direct assay and shelf-life monitoring of *aspirin* tablets by *Raman spectroscopy* has been reported by Wang *et al*. [13]. The great advantage of this method is that it does not require the dissolution/extraction of the sample. It is seen in Fig. 5.5.A that the differences between the spectra of aspirin and its degradation product, salicylic acid are large enough (especially in the 1530–1730 cm^{-1} range) to be the basis for their simultaneous determination by least-squares fit and the degradation of the active principle in the tablets exposed to humid air can easily be followed. The method enabled the degradation product to be determined down to the 0.5% level.

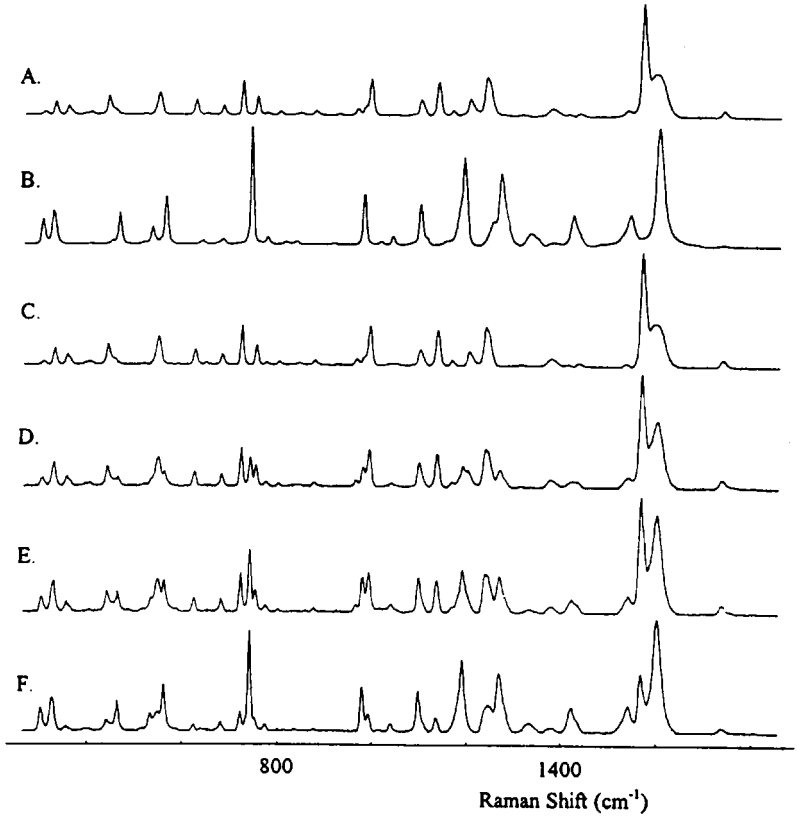


Figure 5.5.A.
Raman spectra of (A) aspirin, (B) salicylic acid, (C) aspirin tablet as received from the supplier. Spectra of tablets after exposure to humid air (D) 2 weeks, (E) 4 weeks, (F) 8 weeks. Resolution about 5 cm^{-1} (from Ref. [13])

5.5.3. HPLC Studies

In the majority of cases drug stability tests outlined in Section 5.2 are performed by high-performance liquid chromatography. The method used for these purposes should be stability indicating, i.e. the peaks of impurities and degradation products should be well separated from each other and from that of the undecomposed drug. In principle the determination of the concentration of the main component is sufficient for the follow-up of the degradation reaction and establish the kinetics of the degradation reaction. However, in studies

striving for higher standards the structures of the degradation products should be previously determined (see Sections 5.3 and 5.4) and the method should enable their quantification with acceptable accuracy and precision. This is mandatory in the case of investigation of complex degradation mechanisms.

As the first example the kinetic investigation of the decomposition of *posatirelin* (L-Kpc-L-Leu-L-Pro-NH_2), an experimental peptide drug is demonstrated [14]. The HPLC system used in this study was as follows. Column: Nucleosil RP-18, 10 μm, 250 × 4 mm; eluent: 0.05 M phosphate buffer (pH 3)/methanol 65:35; flow rate: 1 ml/min; UV detector: 215 nm. The retention time of undecomposed posatirelin under these conditions was 7.7 min. Fig. 5.5.B shows the scheme of its degradation in aqueous hydrochloric acid and sodium hydroxide solutions. As is seen in the scheme and in Fig. 5.5.C where the Arrhenius plots of the reactions are depicted, the main decomposition reaction both in acidic and alkaline media is the hydrolytic opening of the

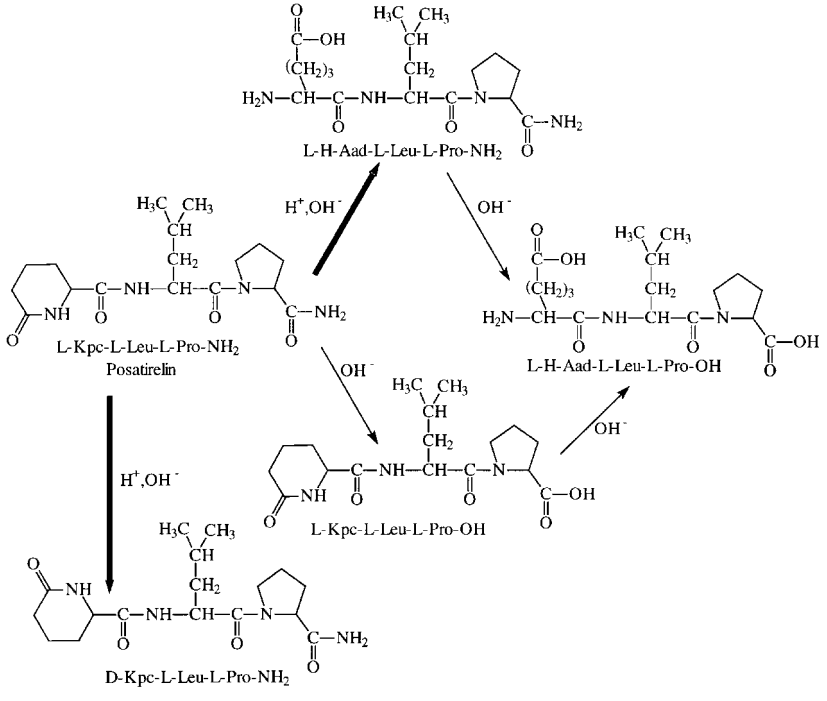


Figure 5.5.B.
Reaction scheme of the degradation of posatirelin in acidic and alkaline media (from Ref. [14])

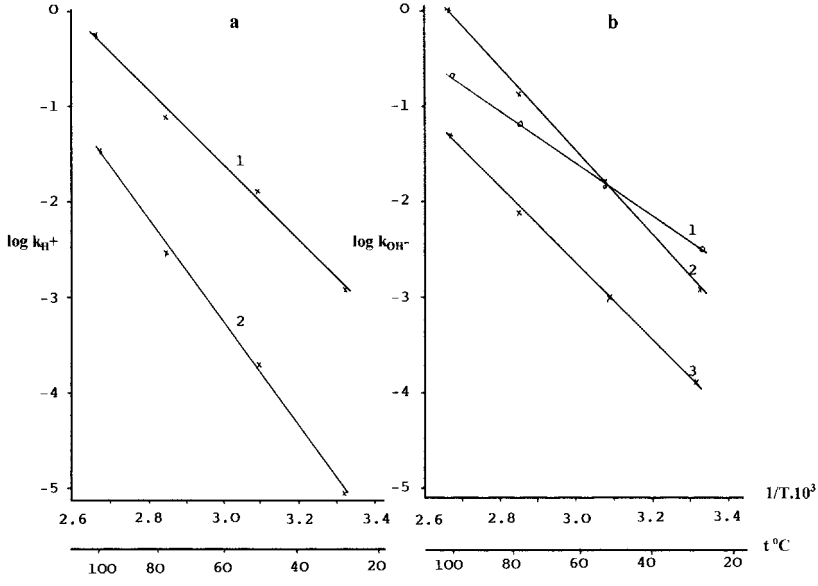


Figure 5.5.C.
Arrhenius plots of the degradation reactions of posatirelin in 0.01 M hydrochloric acid (a) and 0.01 M sodium hydroxide (b) (logarithm of the pseudo-first order rate constants in per hour vs. 1/T). $c_{posatirelin} = 3 \times 10^{-3}$ M. Key. (a): 1: posatirelin ⇒ L-H-Aad-L-Leu-L-Pro-NH_2; 2: posatirelin ⇒ L-Kpc-L-Leu-L-Pro-OH. (b) 1: posatirelin ⇒ L-H-Aad-L-Leu-L-Pro-NH_2;; 2: posatirelin ⇒ D-Kpc-L-Leu-L-Pro-NH_2; 3: posatirelin ⇒ L-Kpc-L-Leu-L-Pro-OH (for the source see Fig. 5.5.B)

lactam ring leading to L-H-Aad-L-Leu-L-Pro-NH_2 (3.6 min), while the splitting of the prolinamide moiety to L-Kpc-L-Leu-L-Pro-OH (12.9 min) and further hydrolysis leading to L-H-Aad-L-Leu-L-Pro-OH (4.6 min) are much slower. Another fast reaction found in alkaline medium was the epimerisation of posatirelin at the L-Kpc unit leading to D-Kpc-L-Leu-L-Pro-NH_2 (10.0 min). The intersection of curves 1 and 2 in Fig. 5.5.C.b due to the higher activation energy of the epimerisation reaction is worth mentioning.

The investigation of complex degradation mechanisms by HPLC usually requires gradient elution systems to be used for the resolution of the large number of degradates. This was the case e.g. in the course of the stability study of *mazipredone* and *Depersolone*® injection made thereof. Gazdag *et al.* [15] developed HPLC systems suitable for the separation and quantitation of as many as 15 impurities and degradation products formed when the drug material

was exposed to acidic, basic media and atmospheric oxygen. The retention data of these are depicted in Table 5.5.A. The chromatograms after acidic and basic treatment and the degradation schemes derived from these are presented in Figs. 5.5.D–5.5.G . (The diode-array UV spectra of mazipredone and two of the acidic degradates are shown as Fig. 5.3.A in Section 5.3.2.)

Another complex degradation mechanism is the oxidative degradation of *tipredane* discussed in detail in Section 5.3.4. On the basis of the gradient

Table 5.5.A.
Retention data of mazipredone, its synthesis related impurities (I), acidic (A) and basic (B) degradation products. For the HPLC system and reference see Fig. 5.5.D

Derivative	Number	Source	RT (min)	RRT
17α-Hydroxy-17-oic acid	1	IB	11.3	0.52
17α,20-Dihydroxy-21-oic acid	2	IB	13.8	0.64
17-Deoxy-20-oxo-21-hydroxy-21-(4-methyl-1-piperazinyl)	3	IB	18.7	0.87
11-Oxo-mazipredone	4	I	21.0	0.97
1,2-dihydro-mazipredone	5	I	21.6	1.00[a]
Mazipredone			21.6	1.00
Prednisolone	6	I	23.0	1.06
17α-Hydroxy-20-keto-21,21-dihydroxy	7	I	24.6	1.14
$\Delta^{9(11)}$-dehydro-mazipredone	8	I	25.5	1.18
11-Deoxy-mazipredone	9	I	26.6	1.23
17-Oxo	10	AB	27.1	1.25
17-Deoxy-20-oxo-21,21-dihydroxy	11	A	32.8	1.52
Prednisolone-21-mesylate	12	I	33.0	1.53
20-Hydroxy-17(20)E-ene-21-al	13	IA	34.7	1.61
20-Hydroxy-17(20)Z-ene-21-al	14	IA	36.7	1.70
Piperazine-bridged mazipredone dimer	15	I	53.7	2.49

[a] 0.97 in the HPLC system in Fig. 5.5.F

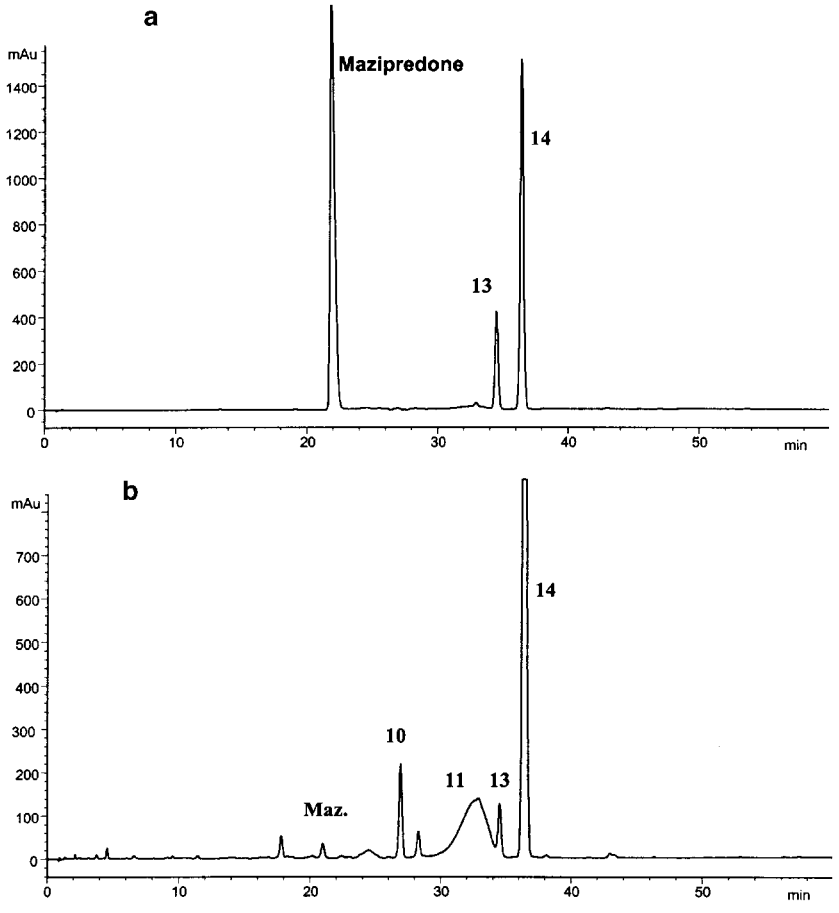


Figure 5.5.D.
Chromatograms of mazipredone after treatment with 0.1 M hydrochloric acid at 80°C. (a) Chromatogram after 30 min reaction time. (b) Chromatogram after 24 h reaction time. For the numbering of the peaks see Fig. 5.5.E. HPLC conditions: column: Purospher RP-18e, 5 μm, 125 × 4 mm; eluent: A: water/acetonitrile/methanol 85:5:5 v/v/v, B: water/acetonitrile/methanol 20:40:40 v/v/v both containing 50 mM ammonium acetate; linear gradient from 10% B at 0 min to 90% B at 70 min; flow rate: 1 ml/min; temperature 40°C; UV detector: 240 nm (from Ref. [15])

HPLC system shown in Fig. 5.3.J all products and intermediates of the degradation scheme in Fig. 5.3.K could be quantitatively measured thus enabling the

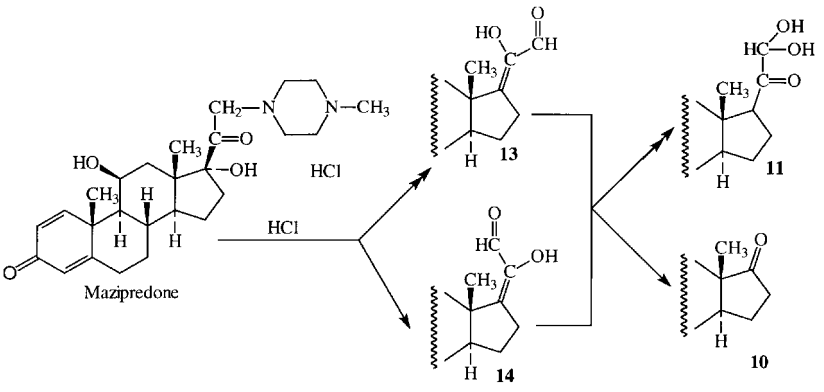


Figure 5.5.E.
5.E Degradation scheme of mazipredone in 0.1 M hydrochloric acid at 80°C (for the reference see Fig. 5.5.D)

rate constants of no less than 14 elementary steps of the degradation reaction to be determined [16].

Further examples include the separation and determination of eight degradates of *phenylbutazone* by Fabre *et al*. [17] using a LiChrosorb RP-18 5 μm column and 52:48 v/v mixture of Tris–citrate buffer (pH 5.25) as the eluent, six degradates of *salbutamol* by Mälkki and Tammilehto [18] using a similar column with either a gradient system (4–9% acetonitrile in 40 mM phosphate buffer containing 5.74 mM triethylamine, pH 3) or an isocratic ion-pair system (40 mM phosphate buffer containing 5 mM tetrabutyl ammonium phosphate, pH 3). Archontaki, Panderi *et al*. [19,20] studied the mechanism and kinetics of the acidic hydrolysis of *prazepam* and *bromazepam* using a C-8 column and acetonitrile, 0.1 M ammonium acetate 68:32 v/v [19] and methanol/acetonitrile/(5 mM potassium dihydrogen phosphate - 0.1 M ammonium acetate, pH 6.2) 26.5:21.5:52 v/v/v [20], respectively.

Some more drug stability studies based on HPLC separation and quantitation of the degradates are the investigation of *norfloxacin* in bulk form and tablets [21], *sunepitron hydrochloride* [22], *isoxazolyl penicillins* [23], photodegradation products of *folic acid* in the presence of riboflavin [24], decarboxylation of *aminosalicylic acid* [25], hydrolysis of *adenosine* in infusion [26], *Nestorone* (16-methylene-17α-acetoxy-19-nor-pregn-4-ene-3,20-dione) in implants and aqueous solutions [27], *chloroquine phosphate* in injections [28].

In all of the studies discussed above UV detector was attached to the chromatograph to monitor the chromatograms and to generate signals suitable

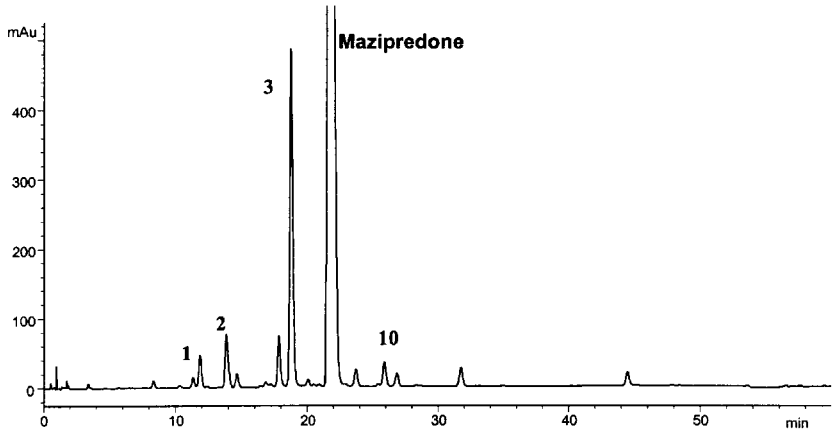


Figure 5.5.F.
Chromatogram of mazipredone after treatment with 0.1 M sodium hydroxide at 80°C for 4 h in the presence of atmospheric oxygen. For the numbering of the peaks see Fig. 5.5.G. HPLC conditions: column: Hypersil BDS C8, 3 μm, 100 × 4.6 mm; eluent: A: ammonium acetate (25 mM), B: methanol; linear gradient to 80% B at 80 min; flow rate: 1 ml/min; temperature: ambient; UV detector: 240 nm (for the reference see Fig. 5.5.D)

for the quantitation of the separated degradation products. Niemi *et al.* [29] used an evaporative light-scattering detector for the study of the hydrolysis of spectrophotometrically inactive *clodronate* (chloromethylenebisphosphonate) esters. The limit of quantitation (S/N = 20) for clodronate was 1.0 μg/injection. Non-linear calibration graphs were obtained. Fluorescence detection was successfully applied by Pena *et al.* [30] for the determination of the major degradation products (epitetracycline, anhydrotetracycline, epianhydrotetracycline) in *tetracycline*. The post-column derivatisation reagent was magnesium acetate in an alkaline borate buffer; the wavelengths of excitation and emission were 385 and 500 nm, respectively.

The study of Shah *et al.* [31] merits special attention. This group built up an automated system to measure the rates of rapid decomposition reactions. The reaction (e.g. the hydrolysis of *2′,3′,5′-triacetyl-6-azauridine*) takes place in a pH-stat, the sampling for the subsequent HPLC analysis is carried out using a microdialysis probe. The system enables the reactions to be followed and the rate constants to be determined even in the case of very fast reactions (half life of about 60 s at pH 9.5 at 67°C).

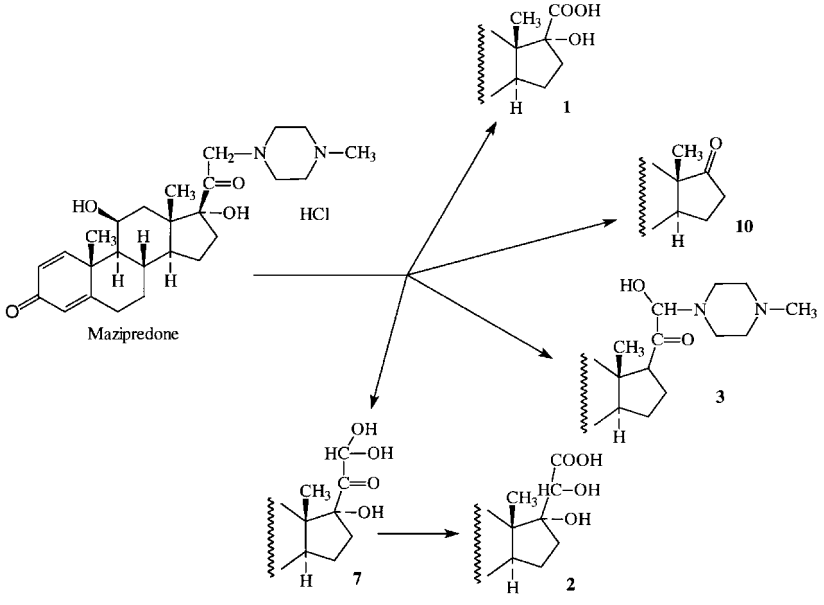


Figure 5.5.G.
Degradation scheme of mazipredone in 0.1 M sodium hydroxide at 80°C in the presence of atmospheric oxygen (for the reference see Fig. 5.5.D)

5.5.4. Gas Chromatographic Studies

Gas chromatography is much less generally used in drug stability studies than high-performance liquid chromatography. For the advantages and disadvantages of the technique see Section 2.6. The following three examples demonstrate that in certain cases it is a very useful method even in this field.

Marinković *et al.* [32] described stability tests of bulk *isosorbide mononitrate* under stress conditions (40°C, 75% rel. humidity). The analyses were carried out by GC/MS after trimethylsilyation by *N,O*-bis-(trimethylsilyl)-trifluoroacetamide. An SPB5 capillary (30 m × 0.32 mm × 0.25 μm) was used starting with a 2 min isothermal period at 100°C followed by temperature programming at 8°C/min to 300°C. The following retention times were found: isosorbide-5-mononitrate (9.1 min), isosorbide (main hydrolysis product; 8.2 min) isosorbide diastereomer (minor hydrolysis product; 7.9 min), 2-chlorobenzoic acid (internal standard; 6.6 min). It has to be noted that the small extent of degradation found in this study would have been difficult to monitor by HPLC with UV detector due to the very poor UV activity of this group of materials.

Liebler *et al*. [33] investigated by GC/MS the oxidative degradation of *vitamin E*. Using stable deuterium-labelled derivatives as the internal standards and trimethylsilylation with *N,O*-bis-(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane this technique has proved to be suitable for the identification and quantitative determination of the main oxidation products, α-tocopherolquinone, 5,6-epoxy-α-tocopherolquinone, 2,3-epoxy-α-tocopherolquinone and α-tocopherolhydroquinone.

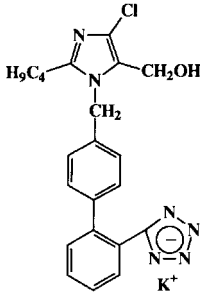
The method of Carlin *et al*. [34] for the determination of traces of the extremely carcinogenic *hydrazine* in *isoniazid* is a gas chromatographic alternative of the spectrophotometric, TLC and HPLC methods mentioned in Section 4.1 for the determination of traces of hydrazine in hydrazides based on its condensation with benzaldehyde and its derivatives. One mole of hydrazine reacts with two moles of benzaldehyde to form benzalazine which can be easily separated and quantitated using a DB5 capillary (30 m × 0.32 mm × 0.25 μm), Ni^{63} electron capture detection and temperature programming from 65°C at 12°C/min until about 10 min, when the peak of benzalazine appears. The internal standard, 3-chlorobenzophenone elutes at 8 min. The method enabled the hydrolytic decomposition of isoniazid in its syrup formulation to be followed down to 1 ppm hydrazine which is the minimum level of quantitation.

5.5.5. Thin-Layer Chromatographic Studies

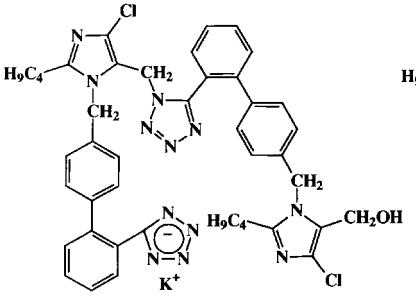
Thin-layer chromatography, discussed in detail in Section 2.5 is an inevitable tool in drug impurity profiling and also in the characterisation of the stability of drugs and the identification of the degradation products. For example in the paper of Evans *et al*. [35] which aimed at presenting a model study for selection, development, definition and validation of selective, stability-indicating procedures for drug substances (through the example of *ranitidine*) and their formulations, thin-layer chromatography was selected for the purity check of the drug substance. 8 impurities, among them 7 degradation products were separated using the following TLC system: 20 × 20 cm chromatoplate silica gel $60F_{254}$, 0.25 mm thick; mobile phase comprising ethyl acetate/2-propanol/ammonia solution 35% (w/w)/water 25:15:4:2 v/v/v/v; tightly sealed glass chromatotank, lined with chromatographic paper and pre-equilibrated for at least 1 h with the mobile phase; loading 200 μg ranitidine (223 μg ranitidine hydrochloride) with 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 1.0% loadings in the standard tracks; running distance 15 cm; visualisation by exposing the previously dried plate to iodine vapour for 15 min in a sealed dry TLC tank; semiquantitative estimation through visual comparison of the intensities of the impurity spots with those in the standard tracks employing a two-point bracketing approach.

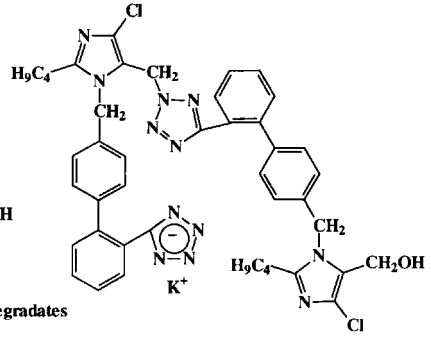
Table 5.5.B shows the structures of the impurities (degradation products) with their relative R_f values, relative response factors and limits of detection. It is worth mentioning that the system suitability tests include the check of the resolution of ranitidine from compound 5 in Table 5.5.B.

In addition to the semiquantitative evaluation shown in the last example more accurate and precise quantitative evaluation is possible by means of using TLC-densitometric evaluation of the impurities, especially if this technique is applied in conjunction with high-performance thin-layer chromatography (HPTLC). As an example the determination of *losartan* and its degradates in a tablet formulation by McCarthy *et al*. [36] is shortly described. The baseline separation of isomers 1 and 2 of a dimeric degradate from each other (R_f 0.31 and 0.54) and from the drug material (0.79) was achieved within as short a developing length as 30 mm using a reversed-phase C-18 silica gel HPTLC plate and 35:25:40 v/v/v mixture of acetonitrile, methanol and 0.1% acetic acid as the mobile phase. The plates were scanned at 254 nm. The method was found to be suitable for the determination of the dimeric degradates down to 0.05%. Quite recently the investigation of the degradation reaction by HPLC/MS(MS) has also been described [37].



Losartan





dimeric degradates

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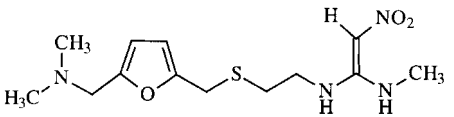
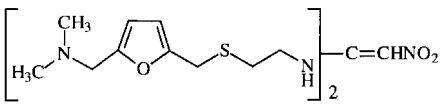
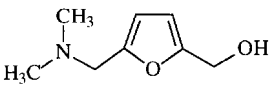
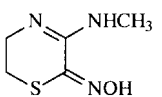
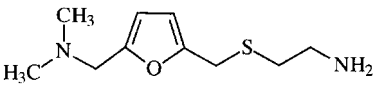
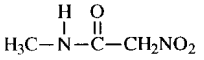
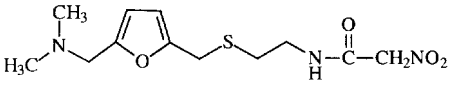
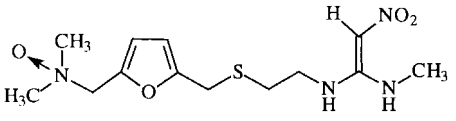
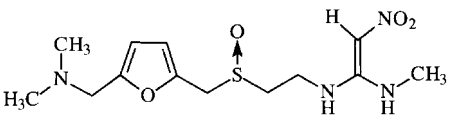
Table 5.5.B.
TLC characteristics of impurities (degradation products) in ranitidine[a]

	Structure	Potential source[b]	Relative R_f	Relative response factor	Limit of detection[c] (%)
1		–	1.00	1.00	0.025
2		S	1.3	1.0	0.1
3		S,D	1.4	0.6	0.1
4		S,D	0.9	1.0	0.1

5		S,D	1.1	1.3	0.1
6		D	0.2	0.4	0.1
7		D	0.4	0.8	0.05
8		D	0.2	0.8	0.05
9		D	0.5	0.8	0.05

[a] (From Ref. [35])
[b] S, from the synthesis; D, degradation product
[c] As percentage of the standard loading of 223 μg of ranitidine hydrochloride

Izer *et al*. [38] investigated the stability of *rifampicin* eye-drops by means of the quantitative determination of its hydrolytic and oxidative degradation products by thin-layer densitometry using a silica gel G plate and the 42:58 v/v mixture of chloroform and methanol and scanning the densitograms at 475 nm for 3-formylrifampicin (R_f 0.66), 25-desacetylrifampicin (0.38), rifampicine itself (0.55) and at 540 nm for rifampicine quinone (0.74).

Stahlmann and Kovar [39] separated the degradates of *chlordiazepoxide* in bulk powder and a tablet formulation by HPTLC, identified the degradates by diffuse reflectance FT-IR spectroscopy (2-amino-5-chlorobenzophenone, demoxepam and nordazepam) and quantitated the degradates by UV densitometry at 230 nm. Another HPTLC densitometric study is the determination of the hydrolysis kinetics of *tinidazole* by Salo and Salomies [40].

5.5.6. Capillary Electrophoretic Studies

As in other fields of pharmaceutical analysis (See Section 2.8) capillary electrophoretic techniques have attracted wide interest in the field of drug stability studies, too, and have become real alternatives to the chromatographic methods. As the first example, the study of Sabah and Scriba [41] on the degradation of *aspartame* (α-L-Asp-L-Phe-OMe) in buffered aqueous solution is presented. The degradation pathway is shown in Fig. 5.5.H. Two CE systems were employed. 1: 47 cm × 50 μm fused silica capillary; 50 mM borate buffer at pH 9.35; voltage 20 kV; internal standard: salicylic acid. 2: 37 cm × 50 μm

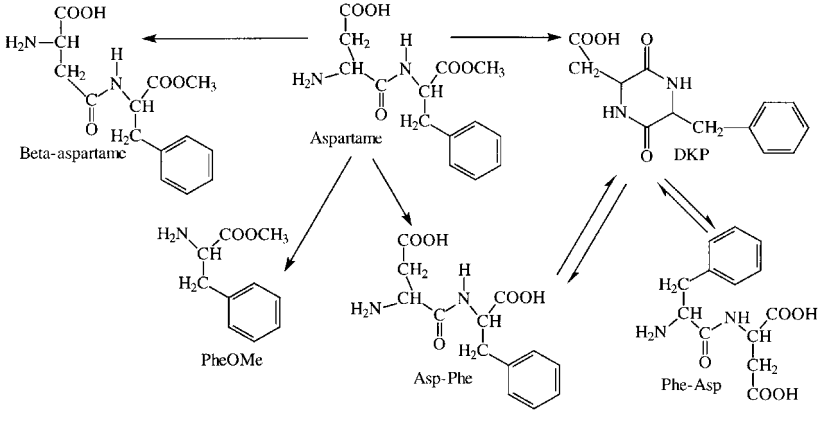


Figure 5.5.H.
Major degradation pathways of aspartame (from Ref. [41])

fused silica capillary; 50 mM phosphate buffer at pH 2.70; voltage 18.5 kV; internal standard: L-Phe-Gly. UV detection (215 nm) was carried out 7 cm from the cathodic end. Sample introduction at the anodic end by hydrodynamic injections at 0.5 p.s.i. Temperature: 20°C.

Fig. 5.5.I is an illustration of the separation of all degradates using system 1 (curve A) and 2 (curve B). As seen in curve A, at pH 9.35 where the carboxyl groups are present in the ionised state, all molecules in the degradation scheme depicted in Fig. 5.5.E (and also phenylalanine, the ultimate degradation product) are well separated. The methyl ester of the latter, which does not contain ionisable group at pH 9.35 migrates with the electroosmotic flow. At pH 2.70 (curve B) where the amino groups are protonated, similarly good separations were achieved. Under these conditions the diketopiperazine (DKP) is not ionised and due to the negligible electroosmotic flow at low pH it does not appear in the electropherogram. At this pH, however, the two diastereomers of β-aspartame (β-L-Asp-L-Phe-OMe and (β-D-Asp-L-Phe-OMe) are resolved. System 2 is suitable also for the separation of the diastereomers of aspartame itself (R_s DL/LL = 1.51), but the diastereomeric D-Asp-L-Phe-OMe is not among the degradation products of aspartame under the conditions described in Fig. 5.5.F. On the basis of the good linearities of the detector responses, good reproducibility and other validation data of the methods it was found to be suitable for the quantitative evaluation of the stability studies of aspartame.

The stability study of *clidinium bromide* (3-hydroxy-1-methylquinuclidinium bromide benzilate) described by Nickerson [42] merits special attention

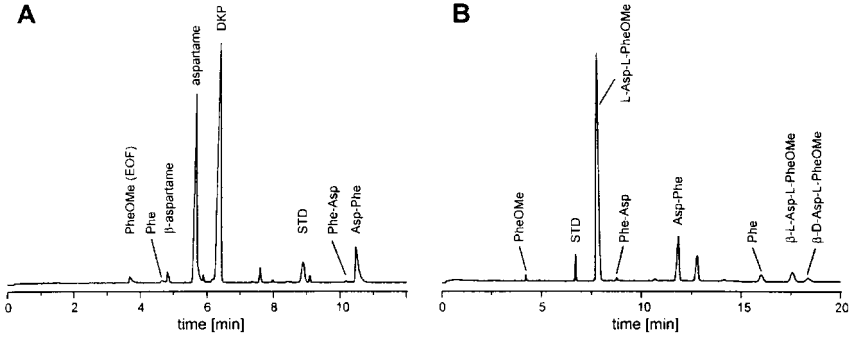


Figure 5.5.I.
Electropherograms of a solution of aspartame following incubation in 50 mM phosphate buffer, pH 7.0) at 70°C for 48 h. (A) Incubations analysed with System 1 (pH 9.35); (B) incubations analysed by System 2 (pH 2.70). For details see text (for the source see Fig. 5.5.H.)

because of the successful use of indirect UV detection. Because of the UV-inactivity of the degradate (3-hydroxy-1-methylquinuclidinium bromide), benzyltrimethylammonium bromide (5 mM) was added to the background electrolyte (10 mM monobasic sodium phosphate). The detection at 205 nm is based on the UV-active benzyltrimethylammonium cation displaced by the UV inactive cation of the degradate. The limit of quantitation (S/N = 10) was found to be 0.04% of the degradant in clidinium bromide.

To extend the possibilities of using capillary electrophoresis to degradation studies of uncharged drugs or drugs with uncharged degradation products its micellar electrokinetic chromatographic version has been successfully used. Studies of this kind (all using sodium dodecyl sulphate as the micelle forming agent) include the degradation study of *pilocarpine* [43], *ifosfamide* [44] and the stability study of a perfusional solution containing *aminophylline*, *methylprednisolone-21-hemisuccinate* and *furosemide* [45].

References

1. K.A. Connors, G.L. Amidon and V.J. Stella, *Chemical Stability of Drug Substances: a Handbook for Pharmacists, 2nd edn*, Wiley, New York (1986)
2. J.T. Carstensen, *Drug Stability, 2nd edn*, Marcel Dekker, New York (1995)
3. I. Rácz, *Drug Formulation*, Wiley, Chichester (1989)
4. S. Görög, *Ultraviolet-Visible Spectrophotometry in Pharmaceutical Analysis*, pp 228–230, CRC Press, Boca Raton, FL (1995)
5. S. Görög, *Ultraviolet-Visible Spectrophotometry in Pharmaceutical Analysis*, pp 114–129, CRC Press, Boca Raton, FL (1995)
6. H.A. Archontaki, *Analyst* **120**, 2627–2634 (1997)
7. G. Ragno and C. Vetuschi, *Pharmazie* **53**, 628–631 (1998)
8. M.G. Quaglia, G. Carlucci, G. Cavicchio and P. Mazzeo, *J. Pharm. Biomed. Anal.* **6**, 421–425 (1988)
9. Y.-S. Sihn, J.K. Guillory and L.E. Kirsch, *J. Pharm. Biomed. Anal.* **16**, 643–650 (1997)
10. P.G. Navarro, A El Bekkouri and E.R. Reinoso, *Analyst* **123**, 2263–2266 (1998)
11. L.K. Revelle, D.A. d'Avignon, J.C. Reepmeyer and R.C. Zerfing, *J. Assoc. Off. Anal. Chem.* **78**, 353–358 (1995)
12. G.M. Hanna and C.A. Lau-Cam, *J. Assoc. Off. Anal. Chem. Int.* **76**, 526–530 (1993)
13. C. Wang, T.J. Vickers and C.K. Mann, *J. Pharm. Biomed. Anal.* **16**, 87–94 (1997)

14. S. Görög, M. Gazdag, B. Herényi, P. Horváth, P. Kemenes-Bakos and K. Mihályfi, in *Reviews in Analytical Chemistry – Euroanalysis VIII* (D. Littlejohn and D. Thorburn Burns, Eds.), pp 349–362, The Royal Society of Chemistry, Cambridge (1994)
15. M. Gazdag, M. Babják, J. Brlik, S. Mahó, Z. Tuba and S. Görög, *J. Pharm. Biomed. Anal*. **16**, 1029–1036 (1998)
16. M.R. Euerby, J.A. Graham, C.M. Johnson, R.J. Lewis and D.B. Wallace, *J. Pharm. Biomed. Anal.* **15**, 299–313 (1996)
17. H. Fabre, N. Hussam-Eddine and B. Mandrou, *J. Pharm. Sci.* **73**, 1706–1709 (1984)
18. L. Mälkki and S. Tammilehto, *J. Pharm. Biomed. Anal.* **11**, 79–84 (1993)
19. H.A. Archontaki, I.E. Panderi, E.E. Gikas and M. Parissi-Poulou, *J. Pharm. Biomed. Anal.* **17**, 739–750 (1998)
20. I. Panderi, H.A. Archontaki, E.E. Gikas and M. Parissi-Poulou, *J. Pharm. Biomed. Anal.* **17**, 327–335 (1998)
21. O.A. Al Deeb, E.M. Abdel-Moety, M.A. Abounassif and S.R. Alzaben, *Boll. Chim. Farm.* **134**, 497–502 (1995)
22. S.T. Colgan, M.L. Dumont and S.G. Ruggeri, *J. Pharm. Biomed. Anal.* **18**, 429–440 (1998)
23. M. Grover, M. Gulati and S. Singh, *J. Chromatogr. B* **708**, 153–159 (1998)
24. M.J. Akhtar, M.A. Khan and I. Ahmad, *J. Pharm. Biomed. Anal.* **16**, 95–99 (1997)
25. P.W. Haney and A.K. Dash, *J. Chromatogr. A* **765**, 233–239 (1997)
26. P. Proot, A. Van Schepdael, A. Raymakers and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **17**, 415–418 (1998)
27. S.M. Ahmed, F. Arcuri, F. Li, A.J. Moo-Young and C. Monder, *Steroids* **60**, 534–539 (1995)
28. E.I.A. Karim, K.E.E. Ibrahim, A.N. Abdelrahman and A.F. Fell, *J. Pharm. Biomed. Anal.* **12**, 667–674 (1994)
29. R. Niemi, H. Taipale, M. Ahlmark, J. Vepsäläinen and T. Järvinen, *J. Chromatogr. B* **701**, 97–102 (1997)
30. A. Pena, A. Carmona, A. Barbosa, C. Lino, I. Silveira and B. Castillo, *J. Pharm. Biomed. Anal.* **18**, 839–845 (1998)
31. K.P. Shah, J. Zhou, R. Lee, R.L. Scowen, R. Elsbernd, J.M. Ault, J.F. Stobaugh, M. Slavik and C.M. Riley, *J. Pharm. Biomed. Anal.* **12**, 993–1001 (1994)
32. V.D. Marinković, B. Gudzić, S.S. Milojković, J.M. Nedeljković and J.J. Comor, *J. Chromatogr. A* **746**, 286–288 (1996)
33. D.C. Liebler, J.A. Burr, L. Philips and A.J.L. Ham, *Anal. Biochem.* **236**, 27–34 (1996)

34. A. Carlin, N. Gregory and J. Simmons, *J. Pharm. Biomed. Anal.* **17**, 885–890 (1998)
35. M.B. Evans, P.A. Haywood, D. Johnson, M. Martin-Smith, G. Munro and J.C. Wahlich, *J. Pharm. Biomed. Anal.* **7**, 1–22 (1989)
36. K.E. McCarthy, Q. Wang, E.W. Tsai, R.E. Gilbert, D. Ip and M.A. Brooks, *J. Pharm. Biomed. Anal.* **17**, 671–677 (1998)
37. Z. Zhao, Q. Wang, E.W. Tsai, X.-Z. Quin and D. Ip, *J. Pharm. Biomed. Anal.* **20**, 129–136 (1999)
38. K. Izer, I. Török, G. Magyar-Pintér, Ė. Varsányi and J. Lipták, *Acta Pharm. Hung.* **66**, 157–163 (1996)
39. S. Stahlmann and K.-A. Kovar, *J. Chromatogr. A* **813**, 145–152 (1998)
40. J.-P. Salo and H. Salomies, *J. Pharm. Biomed. Anal.* **14**, 1261–1270 (1996)
41. S. Sabah and G.K.E. Scriba, *J. Pharm. Biomed. Anal.* **16**, 1089–1096 (1998)
42. B. Nickerson, *J. Pharm. Biomed. Anal.* **15**, 965–971 (1997)
43. K. Persson and O. Aström, *J. Chromatogr. B* **697**, 207–215 (1997)
44. S. Hill, J. Aexiou, P.E. Kavanagh, P.C. Livingstone, R.A. Shalliker and J. Soscic, *Anal. Comm.* **33**, 235–237 (1996)
45. M.G. Quaglia and E. Bossù, *Farmaco* **49**, 403–406 (1994)

Chapter 6

ESTIMATION OF ENANTIOMERIC IMPURITIES

6.1. Introduction

Sándor Görög

Figure 6.1.A shows the distribution of nonchiral and chiral drugs currently used in the therapy and within the latter group the distribution of those which are administered as single enantiomers and as racemates [1,2].

As it is seen more than half of the drug materials are chiral compounds. Whereas the overwhelming majority of natural and semi-synthetic drugs are administered as pure enantiomers, almost the opposite situation exists in the field of synthetic drugs where the majority of chiral compounds are used as the racemates in therapy. However, as a consequence of the continuously increasing knowledge related to enantioselective drug actions and side effects and the wide-ranging concern originating from these, drug registration authorities prefer enantiomerically pure compounds to racemates as new drug entities already at the present time [3–6]. This tendency is expected to increase in the forthcoming years. On the other hand the recent achievements in industrial-scale preparation of enantiomerically pure drugs by stereoselective syntheses or biotechnological methods have created a good basis for the increase of the requirements regarding this matter.

The chirality-related changes in the drug registration policy have brought this field into the focus of interest of synthetic drug researchers, biochemists, pharmacologists. As a consequence of this, chiral analysis is one of the most dynamically developing branches in pharmaceutical analysis. Most of the problems to be solved by drug analysts relate to the simultaneous determination of the enantiomers of chiral drugs and their chiral metabolites in biological samples. Of course these studies are beyond the scope of this book. Impurity-related problems in chiral drug analysis are of two types.

(a) In the case of chiral drugs administered as the single enantiomer the other enantiomer can be considered as an impurity and its relative quantity has

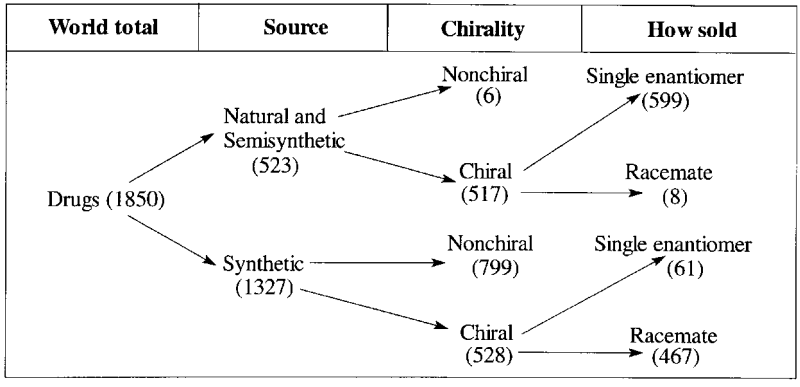


Figure 6.1.A.
Sales distribution of single enantiomer and racemate drugs on the market worldwide (from Ref. [1,2])

to be determined (enantiomeric purity check, determination of (*R*) in (*S*) enantiomer or vice versa). The analytical method for this purpose should enable the enantiomeric impurity to be determined down to the 0.1% level. As is demonstrated in Sections 6.2 and 6.3, this requirement is met by several chromatographic and electrophoretic methods. As demonstrated in Section 6.5, NMR spectroscopy is also suitable for the solution of problems of this kind. The usual requirements of the pharmacopoeias are between 0.1 and 0.5%.

(b) Similar problems may arise in the case of racemates, too. The reasons for this are as follows. In the course of the introduction of a new chiral drug into the therapy the drug registration authorities require a large number of pharmacological, toxicological, pharmacokinetic etc. tests to be carried out with the pure enantiomers in parallel with the same tests carried out with the racemate even if the intention of the manufacturer is the introduction of the racemate. This means that the pure enantiomers have to be prepared (separated) at a rather large scale and the enantiomeric purity check of these is part of the analytical protocol of the above listed tests. The situation can be quite similar with classical drugs administered as the racemates: new drug master files of old chiral drugs or new pharmaceutical preparations made thereof often contain analytical data of the enantiomers.

The paper of Williams *et al.* [7] summarises the analytical, regulatory and R&D aspects of the development of new drugs with one, two and more chiral centres from the discovery phase up to phase III clinical studies leading to new drug application.

Although the majority of analytical papers published in this field deal with the enantiomeric analysis of biological samples, different possibilities for the enantiomeric purity check of chiral drugs have also been well covered and many of the methods described are successfully used routinely. Sections 6.2–6.5 contain the main chromatographic, electrophoretic ORD, CD and NMR spectroscopic approaches but data regarding this matter can also be found in Sections 2.7.5.6 (HPLC/NMR), 2.5 (thin-layer chromatography), 2.10.6 (supercritical fluid chromatography), 2.11 (differential scanning calorimetry) and 7.2 (X-ray diffractometry). Examples from the field of peptide and steroid drugs can be found in Sections 9.1.3 and 9.4.2.1, respectively.

Until quite recently chromatographic methods, mainly HPLC using chiral columns (Section 6.2) seemed to be the most up to date and generally used method for this purpose, but quite recently capillary electrophoresis with chiral buffer additives (Section 6.3) seems to reach, moreover in some cases surpass their importance.

References

1. A. Darbourne, *Scrip Mag.* 16–17 (1993)
2. S. Görög and M. Gazdag, *J. Chromatogr. B* **659**, 51–84 (1994)
3. I.W. Wainer (Ed.), *Drug Stereochemistry. Analytical Methods and Pharmacology*, Marcel Dekker, New York (1993)
4. D.R. Abernethy and N.S. Nabis, Stereoisomeric drugs in herapeutics, in *Drug Stereochemistry. Analytical Methods and Pharmacology* (I.W. Wainer, Ed.), pp 385–397, Marcel Dekker, New York (1993)
5. A.C. Cartwright, *Drug. Inf. J.* **24**, 15–116 (1990)
6. H. Shindo and J. Caldwell, *Chirality* **3**, 91–93 (1991); **7**, 349–352 (1995)
7. R.C. Williams, C.M. Riley, K.W. Sigvardson, J. Fortunak, P. Ma, E.C. Nicolas, S.E. Unger, D.F. Krahn and S.L. Bremner, *J. Pharm. Biomed. Anal.* **17**, 917–924 (1998).

6.2. Chromatographic Methods

Sándor Görög

6.2.1. Separation after Chiral Covalent Derivatisation

Using this general method the enantiomers are transformed to covalently bonded diastereomeric derivatives by reacting them with homochiral derivatising reagents prior to their chromatographic separation (HPLC, GC, SFC, TLC) using achiral stationary and mobile phases. This is the classical but still widely used approach to enantiomeric separation discussed in detail in several books and reviews [1–11]. The main advantages of this approach are the following.

- A large variety of reagents and reactions are available permitting diastereomeric derivatives with good separation and detection possibilities to be formed.
- The proper selection of the homochiral derivatisation agent makes it possible to select the advantageous elution order of the peaks: in possession of both the (*R*) and (*S*) forms of the derivatisation agent it is achievable that the minor peak of the enantiomeric impurity elutes before the main peak thus improving its quantitation.
- It is possible to use inexpensive achiral stationary phases.

Important prerequisites for the successful use of a homochiral derivatising agent are as follows:

- High enantiomeric purity: at least 99.9% if it is intended to be used for enantiomeric purity check of drugs; otherwise the satellite peak originating from the enantiomeric impurity of the reagent can be confused with that originating from the analyte.
- High enantiomeric stability of the reagent and the absence of kinetic resolution (the necessity of equal reaction rates of the two enantiomers with the reagent).
- The absence of racemisation of the reagent, the drug to be derivatised and the diastereomeric reaction product during the derivatisation reaction.
- The good chromophoric and fluorophoric properties of the reagent add very much to its value as a chiral derivatising agent: using a highly UV-active or fluorescent reagent the detection limit of the enantiomeric impurity can be greatly decreased.

Several reviews are available in the literature summarising the main features of the very high number of (usually commercially available) chiral derivatising agents and reactions [1–11]. The reagents include acyl chlorides and anhydrides (e.g. (*S*)-(+)-naproxen chloride), chloroformates (e.g. (−)-menthyl chloroformate and (+)-1-(9-fluorenyl)ethyl chloroformate), isocyanates (e.g. (*R*)-α-methylbenzyl isocyanate), isothiocyanates (e.g. 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate) and others (e.g. 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide, o-phthalaldehyde + *N*-acetyl-L-cysteine) for the derivatisation of *amines* and *amino acids*, acyl chlorides, anhydrides and cyanides (e.g. (*R*)-(+)-α-methoxy-α-trifluoromethyl-phenylacetyl chloride, (*R,R*)-O,O-diacetyltartaric anhydride and (−)-2-methoxy-1,1′-binaphtalene-2′-carbonyl cyanide) for *alcohols and phenols* and amines after the activation of the COOH group (e.g. 1,1-carbonyldiimidazol + (*S*)-(−)-α-methylbenzylamine) for carboxylic acids.

In the overwhelming majority of cases the diastereomers are separated by high-performance liquid chromatography. Especially in the early literature gas chromatographic separations can also be found (e.g. N-heptafluorobutyryl-L-prolyl chloride for the separation of the enantiomers of amphetamines [12]). HPLC [13] and thin-layer chromatography [14] are equally suitable for the separation of the enantiomers of chiral carboxylic acids after their derivatisation with (1*R*,2*R*)-(−)-(4-nitrophenyl)-2-amino-1,3-propanediol or its enantiomer using dicyclohexylcarbodiimide as the activator of the carboxyl group. Although most of the vast quantity of papers published on this topic deal with the general and theoretical problems of the enantiomeric separation and with the analysis of biological samples, in principle the majority of the described methods could be suitable for the determination of the enantiomeric purity, too. As for the sensitivity attainable the method of Demian and Grisphover [15] for the determination of (*R*)-3-aminoquinuclidine in the (*S*)-enantiomer and *vice versa* can be regarded to be characteristic; as little as 0.1% enantiomeric impurity could be determined after derivatisation with (*R,R*)-O,O-dibenzoyltartaric acid anhydride and separation on a C_8 column with as short an analysis time of 3 min.

Although the other possibilities for enantiomeric separations, discussed in the subsequent part of this section seem to be more generally used, new derivatisation reagents are time to time introduced up to the present time. Examples for this are 4-(3-isocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazol and 4-(3-isocyanatopyrrolidin-1-yl)-7-(N,N-dimethylaminosulphonyl)- 2,1,3-benzoxa-diazol [16]. These reagents are available both in the *R* and *S* form and excel with their high enantiomeric stability and the mild conditions for the derivatisation reaction with amines to form diastereomeric thiourea derivatives, which can be well separated on C_{18} columns. The reaction equation of the former reagent with *alprenolol* is depicted in Fig. 6.2.A.

The intense fluorescence of the forming derivatives (excitation/emission wavelengths at 460/550 and 490/530 nm, respectively) enables these reagents to be used for the highly sensitive determination of the enantiomeric impurities in amino acids and β-adrenergic blocking agents.

Another derivatisation reaction which merits special mentioning is the enzyme-catalysed glucuronidation of hydroxy compounds. The high enantiomeric purity of the reagent ((+)-uridine 5′-diphosphoglucuronic acid), the high selectivity and very mild conditions of the reaction catalysed by the 5′-diphosphoglucuronyltransferase enzyme and the excellent separation of the diastereomeric derivatives by RP-HPLC make this method eminently suitable for enantiomeric purity check as exemplified with the study of the drug candidate (−)-2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin (enantiomeric purity 99.89%) and its (+)-enantiomer (99.84%) [17].

As it has been mentioned an advantage of diastereomeric derivatisation is that it enables nonchiral columns to be used. However, if the separation power of a chiral column is combined with the good separability of the diastereomeric derivatives, very delicate problems can be solved as demonstrated by Matuszewski *et al.* [18]. The four stereoisomers of 1[(−)-*SS*-4-ethylamino-5,6-dihydro-6-methyl-7,7-dioxide-4H-thieno(2,3b)thiopyran-2-sulphonamide and its four deethylated stereoisomeric derivatives, among them metabolites, heat and light degradation products were separated on a Pirkle-type chiral column after the derivatisation of the ethylamino or amino groups with (*S*)-(+)-1-(1-naphtyl)ethyl isocyanate to form the diastereomeric urea derivatives.

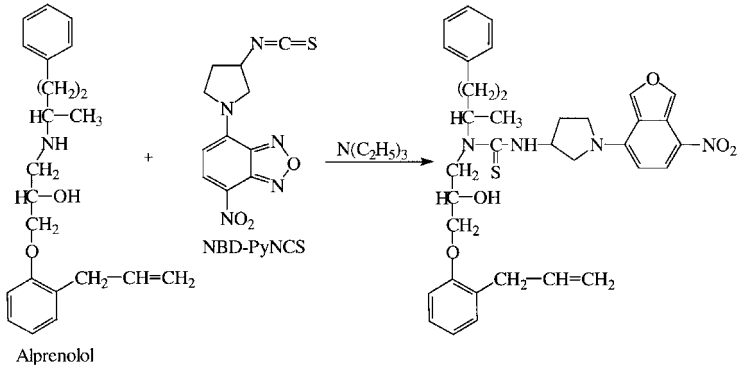
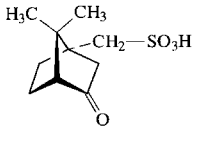


Figure 6.2.A.
Reaction scheme of the chiral derivatisation of alprenolol with 4-(3-isocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazol (NBD-PyNCS)

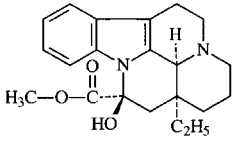
6.2.2. Separation on Achiral Columns using Homochiral Mobile Phase Additives

The dynamic formation of diastereomeric adducts (ion-pairs, ternary complexes, inclusion complexes) with chiral selectors dissolved in the mobile phase followed by their separation on achiral columns is the other widely used method for the separation of the enantiomers of drugs [1–4,6,9–11,19–21]. As in the case of methods discussed in Sections 6.2.1 and 6.2.3, the majority of the published papers in this field also deal with general aspects of this question and the biological applications. On the other hand, however, this general method is one of the most important tools also for the estimation of enantiomeric purity of drugs. Similarly to the situation described in the previous section dealing with covalent chiral derivatisation it is an advantageous feature of also this method that the elution order of the dynamically formed diastereomeric adducts can be controlled by the nature of the chiral selector (*R* or *S*). In addition to this (unlike with the covalent chiral derivatisation) in this case the high enantiomeric purity of the chiral selector is not the prerequisite of the sufficient separation of the enantiomers [22,23]: 1% of (*R*)-*propranolol* in (*S*)-propranolol could be separated and quantificated with benzoxycarbonyl-glycyl-L-alanin as the chiral ion-pair forming agent even if the latter contained as much as 10% of the D-enantiomer [22].

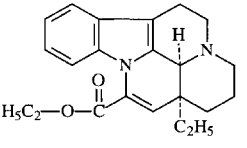
Of the acid-type chiral ion-pairing agent (+)- or (−)-10-camphorsulphonic acid was successfully used among other for the separation of the eight stereoisomers of *vincamine* and for the determination of the enantiomeric purity of the (+)-*cis*-isomer, used in the therapy [24]. Typical chromatograms are depicted in Fig. 6.2.A. The same reagent and chromatographic system were used for the determination of the enantiomeric purity of *vinpocetine* ((+)-cis-apovincaminic acid ethyl ester). The limit of detection for the (−)-cis-enantiomer was 0.1% (Fig. 6.2.B)



10-Camphorsulphonic acid



Vincamine



Vinpocetine

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The enantiomeric purity of *(S)-atropine* was determined by another acid-type chiral ion-pairing agent, (2*S*,3*S*)-dicyclohexyl tartrate. By the application of this reagent the rate of racemisation of (S)-atropine in a tablet formulation was studied over a period of 6 years using Hypercarb column and phosphate

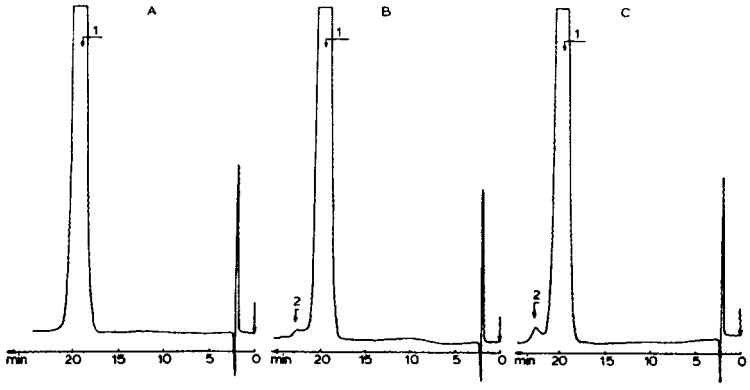

Figure 6.2.B.
Separation of the antipode (−)-cis-vincamine (2) from vincamine (1). A. Pure vincamine; B. Vincamine spiked with 0.5% of (−)-cis-vincamine; C. Vincamine spiked with 1% of (−)-cis-vincamine. Column: Nucleosil 10 CN, 250-4.6 mm. Eluent: hexane/dioxane/1-butanol 70:25:5 v/v/v containing 1 mM diethylamine and 2 mM (+)-10-camphorsulphonic acid at a flow rate of 1.5 ml/min. UV detector: 280 nm (from Ref. [24])

buffer pH 2.8 containing 0.25 mM of the chiral reagent as the eluent. The relative quantity of the (*R*)-enantiomer increased to about 1.5% [25].

The most generally applied chiral mobile phase additives in chiral HPLC are cyclodextrins and their derivatives. The usefulness of these reagents in the determination of the enantiomeric purity of drugs can be illustrated by the determination of the L-norgestrel impurity content in *levonorgestrel* (D-norgestrel, d-norgestrel) (for the formulae see Fig. 9.4.B in Section 9.4.2.1). As is seen in Fig. 6.2.C, taken from the paper of Gazdag *et al*. [26], using γ-cyclodextrin in the eluent the separation of the enantiomers is excellent ($\alpha_{L/D} = 1.14$, $R_{L/D} = 3.03$). The lowest detectable quantity was found to be 0.1%. The RSD for the determination of 1% of L-norgestrel in levonorgestrel was ±3.35% [26]. Similarly good results were obtained with an eluent of water - methanol 38:62 v/v containing 2.62 mM carboxymethyl-γ-cyclodextrin and Nova-Pak C8 column for the separation of L-18-methyl-estra-1,3,5(10)-triene-3,17β-diol 17-acetate impurity in the D-enantiomer ($\alpha_{L/D} = 1.08$, $R_{L/D} = 2.04$, limit of detection: 0.1%) [27].

Functionalised cyclodextrins were successfully used by Gazdag [27] also for the enantiomeric separation of basic drugs as illustrated in Fig. 6.2.C on the example of the enantiomeric purity check of (+)- and (−)-*tolperisone* [27] (Fig. 6.2.D). (For the formula see Fig. 1.2.B in Section 1.2.)

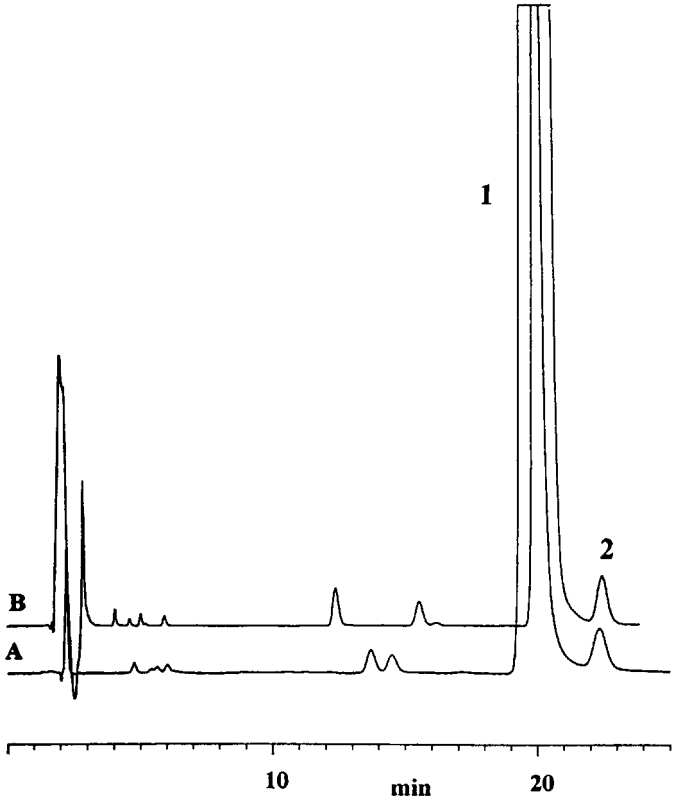

Figure 6.2.C.
6.2.C Separation of 1% L-norgestrel (2) impurity from levonorgestrel (1). Column: Ultrasphere ODS 5 μm, 250 × 4.6 mm. Eluent: methanol/water 45:55 v/v containing 10 mM γ-cyclodextrin (A) and acetonitrile/water 30:70 v/v containing 10 mM γ-cyclodextrin (B). Flow rate: 1 ml/min. UV detector: 244 nm (from Ref. [26])

The use of chiral ligand exchange chromatography using *N*,*N*-dimethyl-L-phenylalanine and copper(II)acetate was used for the separation of the determination of D-DOPA impurity in levodopa [28].

6.2.3. Direct Separation on Chiral Columns

Although due to the high price of the respective HPLC columns the direct

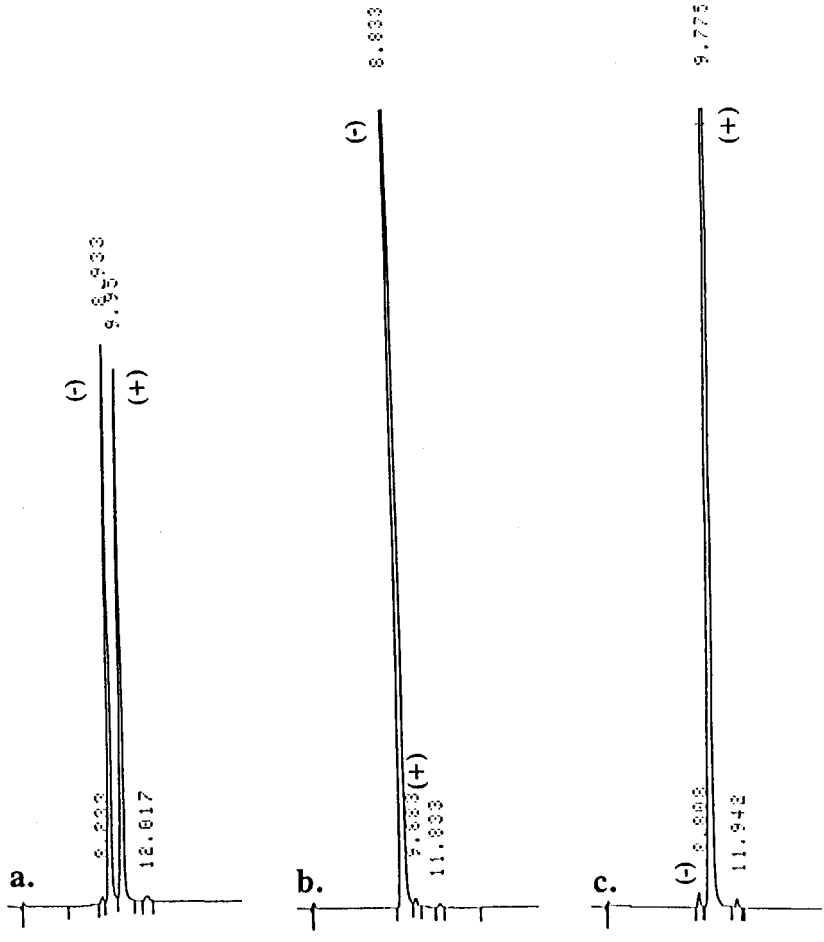

Figure 6.2.D.
Chromatograms of (a) racemic tolperisone; (b) (−)-tolperisone containing 0.52% of the (+)-enantiomer and (c) (+)-tolperisone containing 0.60% of the (−)-enantiomer. Column: YMC-Pack ODS-AQ 3μm 100 × 4.6 mm. Eluent: ''A'' 20 mM triethylammonium phosphate buffer (pH 3.0) and 15 mM carboxymethyl-β-cyclodextrin in water, ''B'' methanol; gradient profile: 0 min 20%B, 20 min 30% B, 22 min 20% B, post run 10 min 15%B. Flow rate: 1 ml/min. UV detector: 260 nm (from Ref. [27])

separation of enantiomers using chiral stationary phases is the most expensive approach to enantioseparation, hundreds of papers have been published on this topic and this seems to be the most generally applied chromatographic technique for this purpose. A large variety of chiral stationary phases (CSPs) are available of which functionalised cellulose, cyclodextrins and glycoproteins bonded to silica seem to be most widely used but several others including Pirkle-type phases, proteins, enzymes, crown ethers, carbohydrates other than cellulose, etc. have also been successfully used. The various leaflets, information booklets, etc. published by the manufacturers of the CSPs contain sufficient information on the conditions suitable for the separation of the enantiomers of the majority of chiral drugs used in the therapy as racemates and pure enantiomers alike. Although a disadvantage of the direct separation is that the elution order of the enantiomers can usually not be influenced, it is not too difficult to find a CSP, where the desired elution order (impurity peak prior to the main peak) is achievable. Even an outlined summarisation of the results of the research aiming at establishing the theoretical basis of the enantioseparation on the different phases and the application of the various CSP-s in biochemistry, pharmacology and pharmacokinetics would be beyond the scope of this book. This experience is, however, usually sufficient to solve the particular problem of determination of enantiomeric purity of drugs administered as the pure enantiomer. For this reason the number of papers dealing with this problem is not comparable with those published from the above mentioned fields.

The most widely used CSPs for the determination of enantiomeric purity of drugs seem to be the group of modified carbohydrates bonded to silica. As a characteristic example the study of Rustum and Estrada [29] for the determination of the enantiomeric purity of *tiagabine* hydrochloride i.e. determination of the unwanted *S*-(+)-enantiomer as an impurity in the *R*-(−)-enantiomer will be discussed. (The pharmacological activity of the latter as an anticonvulsant/antiepileptic drug is higher.)

The first step of the method development and validation was the selection of a suitable stationary phase. No sufficient separation of the enantiomers was obtained using various Pirkle-type and bonded β-cyclodextrin columns. Protein-type columns (bovine serum albumin, α_1-acid glycoprotein) did separate the enantiomers but the elution order was not advantageous (unwanted enantiomer after the main peak) and the ruggedness of the methods was poor. The best results were obtained with the column Chiralcel-OD where the stationary phase is tris-(3,5-dimethylphenylcarbamoyl) cellulose bonded to silica. (With other members of the Chiralcel family such as the OJ and OG columns no sufficient separation was obtained.) The separation efficiency and the peak shape highly depends on the composition of the eluent of the normal-phase separation. From among dichloromethane, chloroform and hexane the latter was selected while from various alcohols 2-propanol + ethanol presented

the best selectivity and resolution if trifluoroacetic acid (selected from among three organic acids) was also added to the eluent. The optimum conditions are presented in the figure caption of Fig. 6.2.E. Under these conditions the following (very advantageous) characteristics and validation data of the method were found: $\alpha = 1.55$; R = 3.4; limit of detection of (*S*) in (*R*): 0.03% (S/N = 3); analysis time: less than 20 min; average recovery of 0.05–1% (*S*) in (*R*): 99.2 ± 1.1% RSD. No change was found in the enantiomeric composition of the sample solution for up to 4 days. The characteristics of the column did not show any significant changes up to 1000 sample injections.

Chiralcel OD column with an eluent of 998:2 v/v mixture of cyclohexane and 2-propanol is used also by the British Pharmacopoeia [30a] for the determination of the chiral purity of *selegiline* (*N*-methyl-*N*-[(1*R*)-1-methyl-2-phenylethyl]prop-2-yn-1-amine hydrochloride). The resolution ($R_{S/R}$) should be at least 1.5. The limit for (*S*)-selegiline is set to 0.5%. The requirements are the same when the unwanted (*R*)-enantiomer is determined in *dexchlorpheniramine maleate* ((*S*)-[3-(4-chlorophenyl)3-(2-pyridyl)propyl]-dimethyla-

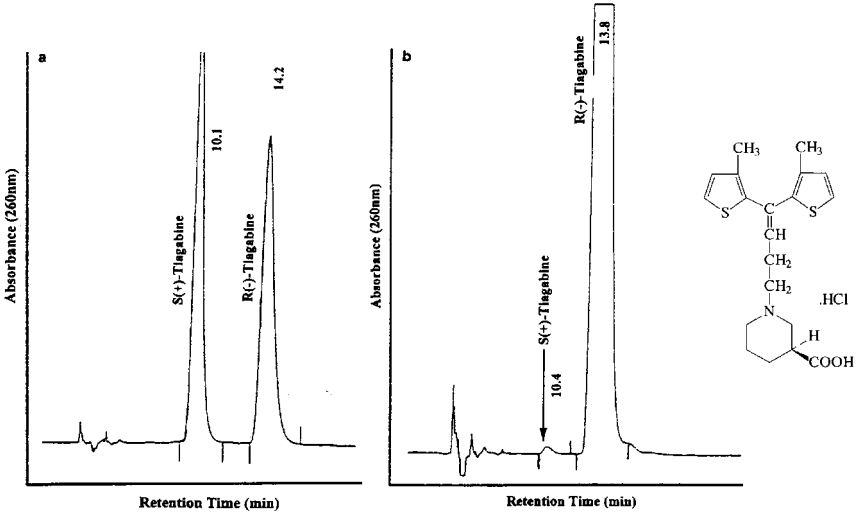

Figure 6.2.E.
Separation of the enantiomers of tiagabine hydrochloride. (a) Racemic mixture; (b) (*R*)-enantiomer containing 0.05% of the unwanted (*S*)-enantiomer. Column: Chiralcel OD 10 μm, 250 × 4.6 mm. Eluent: 800:140:60:5 v/v/v/v mixture of hexane, 2-propanol, ethanol and trifluoroacetic acid at a flow rate of 0.8 ml/min. UV detector: 260 nm (from Ref. [29])

mine maleate) using the related functionalised amylose column [30b]. Quite recently, the complementary behaviour of tris-(3,5-dimethylphenylcarbamoyl) cellulose and amylose phases has been described: racemates which cannot be resolved by one column could be resolved by the other one [31].

The reversed-phase version of the Chiralcel OD column was successfully used by Chilmonczyk *et al.* [32] for the determination of the enantiomeric purity of (*R*)-(+)-*aminoglutethimide*. Ceccato *et al.* [33] used the same stationary phase for the determination of enantiomeric purity of the enantiomers of *pirlindole* (2,3,3a,4,5,6-hexahydro-8-methyl-1H-pyrazino (3,2,1-j,k) carbazole hydrochloride).

The enantiomeric purity test of *paroxetine* hydrochloride ((3*S*,4*R*)-3-(((1,3-benzodioxol-5-yl)oxy(methyl(-4-(4-fluorphenyl)piperidine hydrochloride) to be introduced into the European Pharmacopoeia uses α_1-acid glycoprotein immobilised on silica as the stationary phase [34]. The proposal for the limit of (+)-paroxetine is as low as 0.1%.

Good separations and low limits of detection were found by Doležalová and Tkaczyková [28] in the course of using teicoplanin chiral stationary phase for the enantiomeric purity check of *levodopa* and *methyldopa*.

The paper of Pronce and Tilquin [35] merits special attention: this is the paper where the highest sensitivity has been reported for the determination of enantiomeric purity: 21 ppm of D-aspartic acid was determined in L-aspartic acid and almost the same sensitivity was achieved also for other amino acids. The reasons for this excellent sensitivity were the favourable separation characteristics of the Crownpack Cr(+) column ($\alpha_{D/L}$ values between 1.47 and 3.66) and the high fluorescence of the isoxazole derivative formed with the precolumn *o*-phthalaldehyde/mercaptoethanol derivatisation reaction. (*o*-Phthalaldehyde with a chiral thiol, *N*-acetyl-L-cysteine was already mentioned in Section 6.2.1 as a pair of reagents to form diastereomeric izoxazoles separable on achiral columns.)

An excellent review was recently published by Arai [36] summarising all kinds of liquid phase chiral separations for pharmaceuticals possessing carboxy group.

Chiral stationary phases are available in capillary gas chromatography, too, enabling direct enantioseparations. Especially the use of modified and immobilised cyclodextrins is well documented [37], but several others, e.g. (*S*)-valine-(*R*)-1-(α-naphthylethyl)amide-modified polysiloxane [38] and (*S*)-valine-(*S*)-1-phenylethylamide [39] have also been introduced to produce excellent separations which have not been as widely used for the determination of enantiomeric purity as the chiral HPLC stationary phases. However, it is worth mentioning that the latter has been successfully applied for the separation and quantitation of (*S,R*) and (*R,R*) stereoisomers of *captopril* (1-[3-mercapto-2-(*S*)-methyl-1-oxopropyl]-(S)-proline). The lowest quantifiable levels were 0.08 and 0.09%,

respectively. The sample was transformed to the methyl ester, S-pentafluoropropionyl derivative. The fourth (*R,S*) stereoisomer is separated together with the (*SR*) derivative by achiral GC using a dimethylsiloxane column. The quantity of the latter is subtracted from the sum of the two to obtain the percentage of (*R,S*) (lowest quantifiable level 0.08%) [39].

References

1. W.J. Lough (Ed.), *Chiral Liquid Chromatography*, Blackie, Glasgow (1989)
2. S. Ahuja (Ed.), *Chiral Separations by Liquid Chromatography*, American Chemical Society, Washington, DC (1991)
3. S.G. Allenmark, *Chromatographic Enantioseparation: Methods and Application*, Ellis Horwood, Chichester (1989)
4. I.W. Wainer (Ed.), *Drug Stereochemistry. Analytical Methods and Pharmacology*, Marcel Dekker, New York (1993)
5. S. Görög, Enantiomeric Derivatisation, in *Detection-Oriented Derivatisation Techniques in Liquid Chromatography* (H. Lingeman and W.J.M. Underberg, Eds.), pp 193–216, New York, Marcel Dekker (1990)
6. S. Görög and M. Gazdag, *J. Chromatogr. B* **659**, 51–84 (1994)
7. S. Görög, Enantiomeric derivatisation, in *Encyclopedia of Separation Science* (I.D Wilson, T.R. Alard, C.F. Poole and M. Cook, Eds.), Academic Press, London (2000)
8. M.W. Skidmore, Derivatization for chromatographic resolution of optically active compounds, in *Handbook of Derivatives for Chromatography*, (K. Blau and J. Halket, Eds.), pp 215–252, Wiley, Chichester (1993)
9. R.W. Souter, *Chromatographic Separation of Stereoisomers*, CRC Press, Boca Raton, FL (1985)
10. T. Stevenson T and I.D. Wilson, *Chiral Separations*, Plenum Press, New York (1989)
11. M. Zief and L.J. Crane (Eds.) *Chromatographic Chiral Separations*, Marcel Dekker, New York (1987)
12. H.K. Lim, J.W. Hubbard and K.K. Midha, *J. Chromatogr.* **378**, 109–123 (1986)
13. L. Ladányi, I. Sztruhár, P. Slégel and G. Vereczkey-Donáth, *Chromatographia* **24**, 477–481 (1987)
14. P. Slégel, G. Vereczkey-Donáth, L. Ladányí and M. Tóth-Lauritz, *J. Pharm. Biomed. Anal.* **5**, 665–673 (1987)
15. I. Demian and D.V. Grisphover, *J. Chromatogr.* **466**, 415–420 (1989)
16. T. Toyo'oka and Y.-M. Liu, *Analyst* **120**, 385–390 (1995)
17. T.K. Gerding, B.F.H. Drenth, V.J.M. van de Grampel, N.R. Niemeijer,

R.A. de Zeeuw, P.G. Tepper and A.S. Horn, *J. Chromatogr.* **487**, 125–134 (1989)
18. B.K. Matuszewski, M.L. Constanzer and M. Kiganda, *Pharm. Res.* **11**, 449–454 (1994)
19. A.M. Krstulovic (Ed.), *Chiral Separations by HPLC*, Ellis Horwood, Chichester (1989)
20. G. Subramanian (Ed.), *A Practical Approach to Chiral Separation by Liquid Chromatography*, VCH, Weinheim (1994)
21. K. Jinno (Ed.), *Chromatographic Separations Based on Molecular Recognition*, Wiley-VCH, New York (1997)
22. C. Pettersson, A. Karlsson and C. Gioeli, *J. Chromatogr.* **407**, 217–229 (1987)
23. G. Szepesi and M. Gazdag, *J. Pharm. Biomed. Anal.* **6**, 623–639 (1988)
24. G. Szepesi, M.Gazdag and R. Iváncsics, *J. Chromatogr.* **244**, 33–48 (1982)
25. E. Heldin, N.H. Huynh and C. Pettersson, *J. Chromatogr.* **592**, 339–343 (1992)
26. M. Gazdag, G. Szepesi and K. Mihályfi, *J. Chromatogr.* **450**,145–155 (1988)
27. M. Gazdag, personal communication
28. M. Doležalová and M. Tkaczyková, *J. Pharm. Biomed. Anal.* **19**, 555–567 (1999)
29. A.M. Rustum and V. Estrada, *J. Chromatogr. B* **705**, 111–117 (1998)
30. *British Pharmacopoeia 1998*, p 1153, The Stationery Office, London (1998)
31. U. Selditz, Y. Liao, J.P. Franke, R.A. de Zeeuw and H. Wikström, *J. Chromatogr.* **803**, 169–177 (1998)
32. Z. Chilmonczyk, H. Kascinska and J. Cybulski, *Arch. Pharm.* **328**, 745–749 (1995)
33. A. Ceccato, Ph. Hubert, P. de Tullio, J.F. Liégeois, M. Stachow, J. Géczy and J. Crommen, *J. Pharm. Biomed. Anal.* **17**, 1071–1079 (1998)
34. *Pharmeuropa* **10**, 198 (1998)
35. Th. Pronce and B. Tilquin, *J. Pharm. Biomed. Anal.* **14**, 175–1184 (1996)
36. T. Arai, *J. Chromatogr. B* **717**, 295–311 (1998)
37. W.A. König, *Gas Chromatography with Modified Cyclodextrins*, Hüthig, Heidelberg (1992)
38. I. Abe, T. Nishiyama, T. Nakahara and H. Frank, *J. Chromatogr. A* **694**, 237–243 (1995)
39. D.A. Both and M. Jemal, *J. Chromatogr.* **558**, 257–263 (1991)

6.3. Capillary Electrophoretic (CE) Methods

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6.3.1. Introduction

Over the past few years capillary electrophoresis (CE; see Section 2.8) has been used increasingly for the enantiomeric separation of pharmaceutical compounds [1–9]. CE techniques share with high performance liquid chromatography (HPLC) some interesting properties, such as the absence of limitations with respect to thermal stability and hydrophobic character for both analytes and chiral selectors, giving an enormous freedom of choice among the large number of selectors currently available.

CE has become a very interesting complement to HPLC in the field of enantiomeric purity testing. The benefits of CE compared to HPLC may include higher efficiency, lower operational cost, reduced consumption of chiral selector and faster method development. However, CE cannot compete with HPLC when the preparative separation of even small amounts of pure enantiomers is required, e.g. for the determination of the migration order of the enantiomers or for method validation.

6.3.2. CE Mode and Type of Chiral Selector

The most frequently used modes of CE in chiral analysis are free solution capillary zone electrophoresis (CZE) and capillary electrokinetic chromatography (EKC). The latter mode is capable of separating neutral solutes, due to the presence of an ionic pseudo stationary phase in the separation medium. The most popular EKC approach is micellar electrokinetic chromatography (MEKC), in which charged micelles form the pseudo stationary phase but CD-EKC, employing charged cyclodextrin (CD) derivatives as carriers for neutral compounds, are also increasingly used.

Besides CZE and EKC, capillary gel electrophoresis (CGE), capillary isotachophoresis (CITP) and capillary electrochromatography (CEC) are other CE modes sometimes used for enantiomeric resolution, as can be seen in Table 6.3.A.

In contrast with HPLC, where the use of chiral stationary phases (CSPs) is nowadays the most commonly used approach for the direct enantiomeric

Table 6.3.A.
CE modes applicable to enantiomeric separations

Mode	Chiral selectors
Capillary zone electrophoresis (CZE)	CDs, crown ethers
Capillary gel electrophoresis (CGE)	CDs
Electrokinetic chromatography (EKC)	
Micellar EKC (MEKC)	Chiral surfactants
CD mediated MEKC (CD-MEKC)	CDs
EKC with charged CDs (CD-EKC)	Charged CDs
Affinity EKC (AEKC)	Proteins Charged polysaccharides Macrocyclic antibiotics
Capillary isotachophoresis (CITP)	CDs
Capillary electrochromatography (CEC)	Chiral selectors immobilised on capillary wall or on solid support

separation of chiral compounds, most CE methods involve the addition of the chiral selector to the separation medium, which generally consists of an aqueous electrolyte solution (CZE or EKC) [10–17]. Transient diastereomeric complexes are then formed in the separation capillary between the solute enantiomers and the chiral additive.

An alternative to CZE and MEKC is the use of capillaries filled with a gel (CGE). For example, a CD can be incorporated into a polyacrylamide gel, without covalent bonding [18]. The use of allyl carbamoylated β-CD-acrylamide copolymer gels has also been investigated [19].

The use of a CSP, immobilised either on the capillary wall or on an appropriate support is only applicable in CEC [20].

The indirect separation of enantiomers, which involves their pre-capillary derivatisation with a homochiral derivatising reagent (Marfey's reagent, (+)-FLEC, GITC, etc.; see Section 6.2) and subsequent separation of the resulting covalent diastereomeric derivatives, has also been applied in different CE modes (CZE, MEKC or CGE) [1].

As shown in Table 6.3.A, various types of chiral selectors have been used as buffer additives in CE, such as native cyclodextrins (CDs), a series of CD

derivatives and crown ethers, which tend to form host-guest or inclusion complexes with molecules able to penetrate their cavities, polysaccharides (maltodextrins, amylose), chiral surfactants (e.g. bile acids, digitonin, saponins, amino acid derived or sugar-based synthetic surfactants, used in MEKC) and chiral ligands (e.g. Cu(II)-aspartame).

Proteins (e.g. bovine or human serum albumin, ovomucoid, α_1-acid glycoprotein, avidin, fungal cellulase) charged polysaccharides (heparin, dextran sulfate, chondroitin sulfate) or macrocyclic antibiotics (e.g. vancomycin, teicoplanin, ristocetin A, rifamycin B or SV) represent other classes of chiral selectors which can also be considered as pseudo stationary phases capable of binding drugs stereoselectively by different kinds of intermolecular interactions (affinity-EKC).

Native CDs and CD derivatives are currently by far the most widely used chiral additives in CE, due to their good solubility in aqueous buffers, UV transparency, commercial availability, relatively low cost, stability and absence of toxicity. Most CE applications to enantiomeric purity testing are based on the use of this class of chiral selectors.

CDs are cyclic oligosaccharides consisting of six, seven or eight glucopyranose units and are prefixed α, β or γ, according to the number of these units (6, 7 or 8, respectively). CDs have a toroid shape with a hydrophobic cavity and a polar exterior. Besides its possible inclusion in the inner cavity of the CD molecule, the guest compound can interact with the hydroxyl groups which form the outer rims of this CD. These hydroxyl groups can be derivatised to give selectors with increasing solubility in water and different chiral recognition properties.

In CE, however, chiral recognition in selector-solute interactions, as expressed by the difference in stability for the diastereomeric complexes formed between the solute enantiomers and the chiral selector, does not a priori mean that an enantiomeric separation will be observed, as it is usually the case with chromatographic techniques. In the absence of a (pseudo) stationary phase, there must be a difference in mobility between the free and complexed forms of the solute enantiomers. For instance, no enantioseparation can be observed in CE for neutral analytes if the chiral selector added to the buffer is also neutral.

A series of parameters were found to have an influence on enantiomeric resolution in CD mediated CE: the type and concentration of the CD (native or derivatised) used as chiral selector, the nature and concentration of the buffer ions, the buffer pH, the addition to the buffer of an organic solvent like methanol or of a competing substance like cyclohexanol, the capillary temperature and the voltage applied [10–15,21–27].

An acidic buffer, made of 100 mM phosphoric acid adjusted to pH 3 with triethanolamine and containing different kinds of β-CD derivatives, was found

to be particularly useful for the CE enantioseparation of basic pharmaceutical compounds [14,21,22,28–30] as well as of acidic and neutral drugs [23–27]. At pH 3, triethanolamine is adsorbed on the capillary wall and gives rise to a very low electroosmotic flow (EOF). Furthermore, possible analyte interactions with the capillary wall are minimised and a good mobility match between triethanolamine and most basic drugs is obtained, resulting in high peak efficiency and symmetry.

Among a large number of CDs tested as chiral additives, five β-CD derivatives were found to be especially effective for enantioseparations in CE. Two of these CD derivatives are anionic: sulphobutyl-β-CD (SBCD) and carboxymethyl-β-CD (CMCD), while the three other ones are neutral: dimethyl-β-CD (DMCD), trimethyl-β-CD (TMCD) and hydroxypropyl-β-CD (HPCD).

Charged (anionic or cationic) CD derivatives present some interesting properties such as the fact that they have their own electrophoretic mobility, which allows their use for the enantioresolution of neutral solutes (CD-EKC) [16,23–27,31,32]. On the other hand, ionisable CD derivatives, such as CMCD, provide higher flexibility in the optimisation of CE enantioseparations. They can be used either in uncharged (CZE) or in charged form (EKC) by changing the pH of the running buffer [17,21,22,27]. A reversal of the migration order can often be achieved with this kind of CD derivative by switching from the uncharged to the charged form, which is of particular interest in enantiomeric purity testing.

After selection of the most suitable CD by means of a screening test at a given concentration, the concentration of this CD can be optimised with respect to enantiomeric resolution. The optimal CD concentration corresponds to a maximum mobility difference between the analyte enantiomers [11]. The higher the affinity of the enantiomers for the chiral selector, the lower the optimal selector concentration. The enantioseparation of chiral compounds with very high affinities for a particular CD can often be improved in the usual (mM) CD concentration range by addition of methanol or cyclohexanol to the buffer [14].

At pH values higher than 4, a significant increase of the EOF will be obtained if bare silica capillaries are used. In order to avoid the possible detrimental effect of a high cathodic EOF in this pH range, the latter can be suppressed by dynamic modification of the capillary surface by adding hydrophilic polymers, such as hydroxyalkylcelluloses [15,27,33] or poly(vinyl alcohol) [33], to the running buffer or by permanent coating of the capillary wall [10,33].

According to the acid-base character of the chiral drug, two different strategies were elaborated for the development of chirally selective CE methods, one for basic compounds and the other for acidic and neutral solutes, the

starting buffer being in both cases the pH 3 phosphoric acid-triethanolamine buffer described above [27]. Indeed, enantioselective interactions with CDs were essentially observed with basic compounds in cationic form [14,21,22] while for acidic solutes, higher selectivity and resolution was often obtained with the enantiomers in uncharged form [23–27].

In the case of acidic drugs, essentially present in uncharged form at pH 3, anionic CD derivatives, such as SBCD and CMCD, should be first tested as chiral selectors, in the reversed polarity mode. If enantiomeric resolution is unsatisfactory, dual systems, based on the simultaneous addition to the buffer of a charged CD (SBCD or CMCD) and a neutral CD (TMCD or DMCD), can then be investigated [23–27,34].

Chiral drugs have also been successfully enantioseparated by introduction of CDs in MEKC systems (CD-MEKC), using sodium dodecyl sulfate (SDS) [35] or sodium taurodeoxycholate (STDC) [36] as surfactant.

Other systematic approaches have been proposed for the development of CE methods to determine the chiral purity of drugs [37–39].

6.3.3. Quantitative Aspects and Method Validation

The need to correct peak areas in CE is important for the accurate determination of the level of the minor enantiomer if this level is calculated by comparing peak areas obtained for both enantiomers. Peak areas in CE are related to the analyte residence time in the detection window (on-capillary detection). Since the second enantiomer migrates more slowly in the capillary, it will spend a longer time in the detection window and therefore will have a proportionally greater area than the first enantiomer. This effect can be compensated for by dividing each peak area by its migration time (peak area normalisation) [40,41].

Another phenomenon which should be taken into account for quantitative analysis in CE is the fact that differences in the degree of complexation of the analyte enantiomers with the chiral selector can lead to changes in detector response (absorbance or fluorescence) [42,43]. Therefore response factors should be determined for each enantiomer before their respective levels are calculated by comparison of their peak areas.

If uncoated fused silica capillaries are used, it is advisable to select operational conditions in which the EOF and possible solute-capillary wall interactions are minimised in order to obtain a good reproducibility of the analyte migration times and consequently of the corresponding peak areas. As mentioned above, low pH buffers containing alkylammonium ions such as triethanolamine, should then be preferably used. Another way to cope with this problem is to employ dynamically or permanently coated capillaries,

which is certainly the best solution for CE enantioseparations where the use of buffers with higher pH is required.

The chiral selector can be another cause of variability of migration times, selectivity and resolution in enantiomeric separations. Derivatised CDs are often used as chiral selectors in CE but many of them are mixtures of components with different degrees of substitution and positions of the substituents on the CD molecule [30,37]. It is therefore essential to demonstrate that the enantioseparation can be reproduced using CD derivatives from different origins (different lots from the same supplier or different manufacturers). Clearly it would be preferable to use well characterised single-isomer compounds as chiral selectors [39].

Another source of imprecision in CE is the relatively poor reproducibility of injection compared to HPLC. This is certainly related to the difficulties involved in the precise introduction of nanolitre volumes of samples in the capillary [41]. The best way to solve this problem is to use an internal standard, which should preferably be a non-chiral compound in order to obtain a single peak [43]. However, an internal standard is not necessary if enantiomeric ratios are calculated by comparing the peak areas obtained for the two enantiomers, each enantiomer acting in this case as an internal standard for the other.

Chiral CE methods have been validated and were found to give performance levels similar to those obtained with chiral HPLC methods. The validation criteria examined are the same as those considered in the validation of HPLC methods. They include selectivity, linearity of the detector response, limits of detection and quantitation for the undesired enantiomer, accuracy, precision and robustness.

The results obtained for the validation of a CE method developed for the determination of the enantiomeric purity of *S*-naproxen in a pharmaceutical formulation (tablets) are presented in Table 6.3.B [26]. First, the linearity of the calibration curve for *R*-naproxen in standard solutions and in spiked placebos was determined at five concentration levels in the range 0.1–2.0% of the *S*-naproxen nominal concentration. Normalised peak areas (ratios of peak areas to migration times) were plotted versus the analyte concentration. The coefficient of determination (r^2) obtained by linear regression for *R*-naproxen in spiked placebos is given in Table 6.3.B.

The limits of detection (LOD) and of quantitation (LOQ) for *R*-naproxen, corresponding to signal-to-noise ratios of 3 and 10, respectively, were calculated. The LOD was 0.03%, which corresponds to a concentration of 210 ng/ml and an injected amount of 7.3 pg or 32 fmol. The LOQ was 0,10%, corresponding to a concentration of 700 ng/ml and an injected amount of 24.4 pg or 106 fmol.

Method accuracy was assessed by analyzing spiked placebos at three

Table 6.3.B.
Validation of the CE method developed for the determination of *R*-naproxen (enantiomeric impurity) in *S*-naproxen (from Ref. [26])

Linearity	
Calibration range (%)	0.1–2.0
Calibration points	5
Coefficient of determination (r^2)	0.9957
Limits of detection and quantitation	
LOD (%)	0.03
LOQ (%)	0.10
Accuracy ($k = 3$, $n = 6$)	
Mean recovery ± CI[a] (%) at 0.1%	92.7 ± 8.8
1.0%	100.8 ± 4.3
2.0%	99.8 ± 2.3
Repeatability ($k = 3$, $n = 6$)	
RSD (%) at 0.1%	9.0
1.0%	2.8
2.0%	1.9
Intermediate precision ($k = 3$, $n = 18$)	
RSD (%) at 0.1%	9.6
1.0%	3.3
2.0%	2.0

[a] CI, confidence interval

concentration levels (0.1, 1.0 and 2.0%). Mean recoveries for *R*-naproxen with 95% confidence intervals (CI) are given in Table 6.3.B. Since the theoretical value of 100% was included in the confidence interval, the method could be considered accurate over the range studied.

Method precision was determined by measuring repeatability and intermediate precision (between-day precision) for *R*-naproxen in spiked placebos. The study was carried over 3 days at three concentration levels (0.1, 1.0 and 2.0%). As can be seen in Table 6.3.B, acceptable results with respect to precision were obtained, even at the LOQ (RSDs less than 10%), in spite of the fact that no internal standard was used.

Table 6.3.C.
CE applications to the enantiomeric purity determination of drugs[a]

Chiral compound	CE mode	Chiral selector	LOQ (%)	Ref.
Fluparoxan	CZE	β-CD	1	[44]
Naproxen	CGE	HPCD	<1	[45]
Oxamniquine	AEKC	Heparin/CD	0.2	[46]
5,6-Dihydroxy-2-aminotetralin	CZE	Crown ether	0.3	[47]
Propranolol Trimetoquinol	CZE	CMCD	0.1	[21]
Diltiazem	AEKC	Chondroitin sulfate	0.2	[48]
Fenfluramine	CZE	TMCD	0.5	[49]
Isoproterenol	AEKC	Rifamycin	0.1	[50]
Naproxen	CD-EKC	SBCD/TMCD	0.1	[26]
LY231514	CZE	β-CD	0.5	[51]
Ropivacaine	CZE	DMCD	0.1	[28]
Z12231A	CD-MEKC	STDC/HECD	1	[52]
Propranolol	CZE	HPCD	<1	[53]
Tryptophan	CZE	β-CD	0.1	[53]
Terbutaline	CGE	HECD	<1	[54]

[a] HPCD, hydroxypropyl-β-CD; CMCD, carboxymethyl-β-CD; TMCD, trimethyl-β-CD; SBCD, sulphobutyl-β-CD; DMCD, dimethyl-β-CD; STDC, sodium taurodeoxycholate; HECD, hydroxethyl-β-CD

6.3.4. Applications to Enantiomeric Purity Testing

Table 6.3.C includes a number of quantitative CE applications to the determination of the chiral purity of drug compounds in bulk form or in pharmaceutical formulations, with some details about the separation conditions and the quantitation limits [26,28,44–54].

This table clearly illustrates the wide choice of chiral selectors and CE modes which can be used for developing such methods. Even if CZE methods employing neutral CDs as chiral additives are the most commonly used, other kinds of chiral selectors have also been successfully applied, such as crown ethers [47], chiral surfactants (STDC) [52], charged polysaccharides [46,48], macrocyclic antibiotics [50] and charged CD derivatives [26].

In some cases, the CE enantioseparation using CDs has been performed in gels (CGE) [45,54] while in other instances, neutral CDs have been used in combination with other kinds of chiral selectors (heparin, STDC or SBCD) to obtain a further improvement in enantiomeric resolution [26,46,52].

Limits of quantitation for the undesired enantiomer are comprised between 0.1 and 1%, which is adequate for most applications. These limits are often related to the enantiomeric resolution obtained under the CE conditions selected. If the peaks are reasonably symmetrical, a resolution value of 1.5 is often sufficient to determine the minor enantiomer at the 1% level while a resolution higher than two is necessary to reach the 0.1% level. However, the enantiomeric purity determination is usually performed under overloading conditions, so that the peak of the major enantiomer frequently presents a more or less triangular shape.

Under such conditions, the detectability of the CE system will depend on the resolution obtained with the racemic mixture and on the position of the minor peak, as shown in Fig. 6.3.A. In this case, propranolol enantiomers are enantioseparated with a resolution value of 4.4, using carboxymethyl-β-CD (CMCD) at 10 mM concentration in a pH 3 triethanolamine-phosphate buffer [21].

In this CE method, the LOQ (0.10%) for *S*-propranolol, which is the first migrating enantiomer, is lower than that obtained for *R*-propranolol (0.22%) [21]. Since *S*-propranolol is by far the most potent enantiomer, it would be interesting to reverse the migration order of the enantiomers, in order to improve the LOQ of *R*-propranolol.

As mentioned earlier, this can be easily done in CE, especially when the

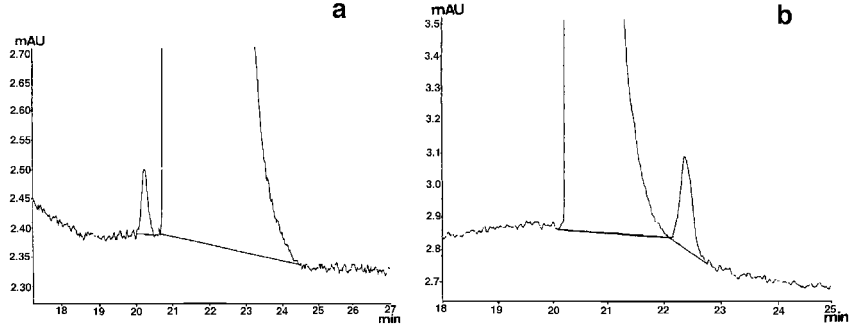

Figure 6.3.A.
Electropherograms of propranolol enantiomers containing low levels of their antipodes. (a) *R*-propranolol spiked with 0.1% of *S*-propranolol. (b) *S*-propranolol spiked with 0.5% of *R*-propranolol. Capillary: uncoated fused silica (48.5 cm × 50 μm i.d; 40 cm to the detector); buffer: 10 mM CMCD in 100 mM phosphoric acid adjusted to pH 3 with triethanolamine; injection: hydrodynamic (5 kPa, 30 s); temperature: 15°C; applied voltage: +25 kV; UV detection: 210 nm. Samples: 25 μg/ml solutions of propranolol enantiomers in diluted buffer (from Ref. [21])

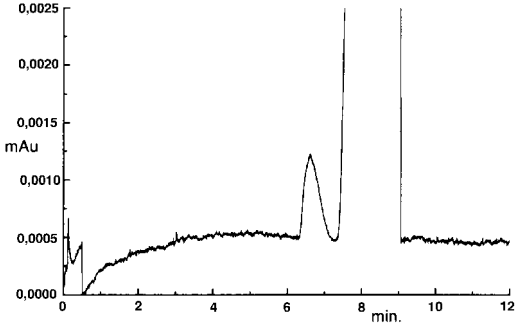

Figure 6.3.B.
Enantiomeric purity testing of *S*-naproxen by CE. Capillary: uncoated fused silica (44 cm × 50 μm i.d., 37 cm to the detector); buffer: 100 mM phosphoric acid/triethanolamine (pH 3) containing SBCD (5 mM) and TMCD (20 mM); injection: hydrodynamic (12 s); temperature: 25°C; applied voltage: −25 kV; UV detection: 210 nm. Sample: 700 μg/ml solution of *S*-naproxen in water/methanol 7:3 v/v (from Ref. [26])

chiral selector is an ionisable CD, like CMCD. With this kind of selector, a change of the buffer pH from 3 to 5 will considerably increase the negative charge of CMCD and consequently, propranolol enatiomers will then start to migrate in the opposite direction, i.e. towards the anode. By switching to the reverse polarity mode, *R*-propranolol will now be the first migrating enantiomer, although it has still the highest affinity for CMCD, because the complexation with CMCD has a accelerating effect on the migration of propranolol enantiomers at pH 5, while it has a decelerating effect at pH 3 [27].

An alternative way to obtain a reversal of the migration order of propranolol enantiomers is to use a polyamine-coated capillary [53]. Under these conditions, the direction of the EOF is reversed and using again the reversed polarity mode, *R*-propranolol will migrate first.

Another example of application is shown in Fig. 6.3.B. In this case, low amounts (down to 0.1%, cf. Table 6.2.B) of the undesired enantiomer (*R*-naproxen) could be determined in *S*-naproxen samples [26]. Naproxen enantiomers are separated in a dual CD system. The polyanionic sulphobutyl-β-CD (SBCD, 5 mM) is used here in combination with a neutral CD derivative, trimethyl-β-CD (TMCD, 20 mM) in a pH 3 triethanolamine-phosphate buffer, giving rise to a resolution value of 5.4 and a favourable migration order for the enantiomers (*R*-naproxen is the first migrating enantiomer). The *S*-naproxen sample shown in Fig. 6.3.B was found to contain 0.93% of the *R* enantiomer.

All these applications clearly confirm that CE can be considered as an

interesting and cost effective alternative to HPLC for the determination of the enantiomeric purity of drugs.

References

1. B. Chankvetadze, *Capillary Electrophoresis in Chiral Analysis*, Wiley, Chichester (1997)
2. K.D. Altria, *Analysis of Pharmaceuticals by Capillary Electrophoresis*, Chapter 4, pp 70–100, Vieweg, Wiesbaden (1998)
3. T.J. Ward, *Anal. Chem.* **66**, 633A–640A (1994)
4. H. Nishi and S. Terabe, *J. Chromatogr. A* **694**, 245–276 (1995)
5. H. Nishi, *J. Chromatogr. A* **735**, 57–76 (1996)
6. S. Fanali, *J. Chromatogr. A* **735**, 77–121 (1996)
7. R. Vespalec and P. Bocek, *Electrophoresis* **18**, 843–852 (1997)
8. K. Verleysen and P. Sandra, *Electrophoresis* **19**, 2798–2833 (1998)
9. B. Chankvetadze, *Trends Anal. Chem.* **18**, 485–498 (1999)
10. S. Fanali, *J. Chromatogr.* **474**, 441–446 (1989)
11. S.A.C. Wren and R.C. Rowe, *J. Chromatogr.* **603**, 235–241 (1992)
12. M.W.F. Nielen, *Anal. Chem.* **65**, 885–893 (1993)
13. Y.Y. Rawjee, D.U. Staerk and G. Vigh, *J. Chromatogr.* **635**, 291–306 (1993)
14. I. Bechet, Ph. Paques, M. Fillet, Ph. Hubert and J. Crommen, *Electrophoresis* **15**, 818–823 (1994)
15. J. Snopek, H. Soini, M. Novotny, E. Smolkova-Keulemansova and I. Jelinek, *J. Chromatogr.* **559**, 215–222 (1991)
16. S. Terabe, *Trends Anal. Chem.* **8**, 129–134 (1989)
17. T. Schmitt and H. Engelhardt, *Chromatographia* **37**, 475–481 (1993)
18. A. Guttman, A. Paulus, S. Cohen, N. Grinberg and B.L. Karger, *J. Chromatogr.* **448**, 41–53 (1988)
19. I.D. Cruzado and G. Vigh, *J. Chromatogr.* **608**, 421–425 (1992)
20. S. Mayer and V. Schurig, *J. Liq. Chromatogr.* **16**, 915–931 (1993)
21. M. Fillet, I. Bechet, P. Chiap, Ph. Hubert and J. Crommen, *J. Chromatogr. A* **717**, 203–209 (1995)
22. M. Fillet, I. Bechet, Ph. Hubert and J. Crommen, *J. Pharm. Biomed. Anal.* **14**, 1107–1114 (1996)
23. M. Fillet, I. Bechet, G. Schomburg, Ph. Hubert and J. Crommen, *J. High Resol. Chromatogr.* **19**, 669–673 (1996)
24. M. Fillet, Ph. Hubert and J. Crommen, *Electrophoresis* **18**, 1013–1018 (1997)
25. M. Fillet, L. Fotsing and J. Crommen, *J. Chromatogr. A* **817**, 113–119 (1998)

26. M. Fillet, L. Fotsing, J. Bonnard and J. Crommen, *J. Pharm. Biomed. Anal.* **18**, 799–805 (1998)
27. M. Fillet, Ph. Hubert and J. Crommen, *Electrophoresis* **19**, 2834–2840 (1998)
28. C.E. Sänger-van de Griend and K. Gröningsson, *J. Pharm. Biomed. Anal.* **14**, 295–304 (1996)
29. C.E. Sänger-van de Griend, K. Gröningsson and D. Westerlund, *Chromatographia* **42**, 263–268 (1996)
30. C.E. Sänger-van de Griend, H. Wahlström, K. Gröningsson and M. Widahl-Näsman, *J. Pharm. Biomed. Anal.* **15**, 1051–1061 (1997)
31. R.J. Tait, D.O. Thompson, V.J. Stella and J.F. Stobaugh, *Anal. Chem.* **66**, 4013–4018 (1994)
32. B. Chankvetadze, G. Endresz and G. Blaschke, *Electrophoresis* **15**, 804–807 (1994)
33. D. Belder and G. Schomburg, *J. Chromatogr. A* **666**, 351–365 (1994)
34. F. Lelièvre, P. Gareil, Y. Bahaddi and H. Galons, *Anal. Chem.* **69**, 393–401 (1997)
35. H. Nishi, T. Fukuyama and S. Terabe, *J. Chromatogr.* **553**, 503–516 (1991)
36. G.N. Okafo and P. Camilleri, *J. Microcol. Sep.* **5**, 149–153 (1993)
37. E.C. Rickard and R.J. Bopp, *J. Chromatogr. A* **680**, 609–621 (1994)
38. S. Boonkerd, M.R. Detaevernier, Y. Vander Heyden, J. Vindevogel and Y. Michotte, *J. Chromatogr. A* **736**, 281–289 (1996)
39. J.B. Vincent and G. Vigh, *J. Chromatogr. A* **817**, 105–111 (1998)
40. K.D. Altria, *Chromatographia* **35**, 177–182 (1993)
41. K.D. Altria, D.M. Goodall and M.M. Rogan, *Electrophoresis* **15**, 824–827 (1994)
42. S. Fanali and P. Bocek, *Electrophoresis* **11**, 757–760 (1990)
43. T.E. Peterson and D. Trowbridge, *J. Chromatogr.* **603**, 298–301 (1992)
44. K.D. Altria, A.R. Walsh and N.W. Smith, *J. Chromatogr.* **645** (193–196 (1993)
45. A. Guttman and N. Cooke, *J. Chromatogr. A* **685**, 155–159 (1994)
46. A.M. Abushoffa and B.J. Clark, *J. Chromatogr. A* **700**, 51–58 (1995)
47. P. Castelnovo and C. Albanesi, *J. Chromatogr. A* **715**, 143–149 (1995)
48. H. Nishi, K. Nakamura, H. Nakai and T. Sato, *Anal. Chem.* **67**, 2334–2341 (1995)
49. R. Porra, M.G. Quaglia and S. Fanali, *Chromatographia* **41**, 383–388 (1995)
50. T.J. Ward, C. Dann and A. Blaylock, *J. Chromatogr. A* **715**, 337–344 (1995)
51. L. Liu, L.M. Osborne and M.A. Nussbaum, *J. Chromatogr. A* **745**, 45–52 (1996)

52. P. Castelnovo and C. Albanesi, *Electrophoresis* **18**, 996–1001 (1997)
53. K.H. Assi, A.M. Abushoffa, K.D. Altria and B.J. Clark, *J. Chromatogr. A* **817**, 83–90 (1998)
54. T. de Boer and K. Ensing, *J. Pharm. Biomed. Anal.* **17**, 1047–1056 (1998)

6.4. Polarimetry, ORD and CD Spectroscopy

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6.4.1. Introduction

The pharmacopoeias generally apply the determination of the specific optical rotation for the characterisation of the optical purity of drugs administered as the pure enantiomer. The measurements are prescribed at D-line of sodium (589.3 nm) or rarely at some wavelenghts in the visible region of the mercury lamp (365, 405, 436, 546, 578 nm) and the value of the specific optical rotation should fall into a range prescribed in the individual monographs. At the shorter wavelengths the values are higher and hence the sensitivity of the measurement can be considerably increased. Generally speaking, however, the sensitivity of the simple polarimetric measurement is low for the estimation of the nonwanted enantiomer as compared with the chromatographic, electrophoretic and NMR methods described in Sections 6.2, 6.3 and 6.5 and due to the possible contribution of other chiral impurities to the measured value the selectivity is also poor. This means that the measurement of the specific optical rotation gives only a rough estimate of the enantiomeric composition.

Of the modern chiroptical techniques the measurement of *optical rotatory dispersion (ORD)* and *circular dichroism (CD)* presents much more sensitive, selective and accurate methods for the determination of the optical purity. This is usually expressed as the enantiomeric excess (*ee*) or enantiomeric purity (EP): $ee(\%) = 100(x^* - x)/(x^* + x)$, where x^* is the mole fraction of the enantiomer in excess. The optical purity (OP) defined as the ratio of the actual specific rotation to the specific rotation of an enantiomerically pure sample is practically the same value. (There is a considerable difference between the terminologies used here and in chiral chromatography and electrophoresis. The *ee* value for the mixture of 99% of *R* and 1% of *S* enantiomer is 98%, i.e. 98% enantiomeric excess + 2% racemate, while in the case of chiral chromatography and electrophoresis the ratio of the areas of the separated peaks of *R* and *S* is 99:1.)

The above mentioned chiroptical methods applied for the estimation of enantiomeric purity of drugs can be divided into two groups:

- From the measurements at the optimal wavelength of the ORD or CD curves of the enantiomers or their derivatives the enantiomeric purity can be calculated (Section 6.4.2).

- ORD and CD spectrometers are also applied as liquid chromatographic (HPLC) detectors (Section 6.4.3).

6.4.2. Determination of Enantiomeric Purity by the Direct Application of ORD and CD Spectroscopy

The optical purity of *α-amino acids* was determined by ORD spectroscopy [1]. L-Acids show a positive whilst the D-isomers a negative Cotton effect in the range of 220-230 nm. The method is 100–700 times more sensitive than polarimetric measurements, at the D-line of sodium.

Erskine *et al*. [2] elaborated a rapid method for the determination of optical purity (OP) using CD and UV spectroscopy. The concentration and OP of *ephedrine* and *mandelic acid* enantiomers and *ephedrine* and *pseudoephedrine* enantiomeric mixtures was determined. In both cases the CD and UV spectra overlap each other in the estimated 256–280 nm region. The data were collected at 0.1 nm intervals and the results were calculated by the partial least-squares method. CD is able to provide information on the amount of excess concentration of one of the enantiomers over that of the other, while from the UV data the total concentration of the two species can be calculated. With the combination of the two methods with a multivariate analysis method such as partial least-squares the concentration of each of a pair of enantiomers was accurately determined in mixtures without the need for chiral separation.

The enantiomeric composition of *phenylglycine* and *mandelic acid* was successfully determined by the application of the anisotropy factor derivable from the ratio of the ellipticity and the absorbance simultaneously measured by CD and UV spectroscopy, respectively [3]. This is an intensive, concentration-independent physico-chemical parameter, which eliminates concentration-related errors caused by the sample preparation, dilution, etc. For the two enantiomers, it has the same numerical value with opposite signs, and is therefore a function of the enantiomeric composition. The procedure based on the determination of the anisotropy factor is rapid, accurate and eminently suitable for routine quality control purposes enabling the determination to be carried out with an accuracy of 0.1%.

A rapid and simple direct spectropolarimeric method was described by Palma *et al*. [4] for the determination of enantiomeric purity of pharmaceutical grade L-*cocaine hydrochloride* and L-*codeine* in tablets by applying a modified Biot–Lowry procedure. Codeine samples were separated from optically active sugars by extraction with chloroform at pH 8–9.

Derivatisation of the analyte has often been used to increase the sensitivity of the determination of enantiomeric purity. For example, the specific rotation

$[\alpha]_D^{25}$ of levodopa is only about $-12°$, which is insufficient to control its optical purity. However, it can be cyclised with formaldehyde, resulting in a more than 10-fold increase of the specific rotation [5].

Complex formation can also be used as an aid to improve the sensitivity of the determination of OP of compounds; for instance, in the case of *ethambutol* complexation with copper(II) was used for this purpose [6]. Optically active *amino acids* when dissolved in an alkaline solution of the potassium salt of the optically active Co(III)-*N*,*N*′-ethylenebis(acetylacetonimine)-glycine complex show multiple Cotton effect curves in the visible region. The OP may then be determined by measurements at 475 nm (CD) and 500 nm (ORD), respectively [7,8].

A method has been elaborated for the determination of enantiomeric purity of *amphetamine* samples [9]. The procedure is based on the derivatisation of amphetamine with 2,4-dinitrofluorobenzene and subsequent measurement of the anisotropy factor by dual (CD/UV) spectroscopy. The advantage of the derivatisation is that due to the bathochromic shift, more selective and sensitive analysis can be performed than it would be possible in the underivatised form. Both ellipticity and absorbance were in linear relation to the concentration in the range of 5×10^{-6}–6×10^{-4} mol/l. By investigating binary mixtures of the amphetamine enantiomers containing 63.3–98.8% of (+)-amphetamine the accuracy of the determination of the enantiomeric purity was found to be 0.2–0.3%.

Purdie *et al*. [10] developed a method for the determination of the ratio of the concentration of enantiomers in binary mixtures of *pseudoephedrine* and *ephedrine* using chiral Cu(II)-tartrate complexes as derivatising agents. The reaction is a simple ligand exchange between tartrate and the chiral drug resulting in the shift of the wavelength of CD detection to the visible range. The change of the CD spectrum is proportional to the concentration of the D-pseudoephedrine and at 527 nm the L-pseudoephedrine complexes have an isobestic point. At this wavelength the D-pseudoephedrine content can be determined. The effects are entirely opposite if L-tartrate is changed to D-tartrate in which case the D-pseudoephedrine complexes will produce the isobestic point and the L-pseudoephedrine content can be determined. The detection limit for the enantiomeric impurity was in the order of 2%.

Complexation of *ketoprofen* to bovine serum albumin (BSA) results in an intensive negative Cotton effect in the n–π^* band of the benzoylphenyl moiety [11] as illustrated in Fig. 6.4.A. It is remarkable, that the CD intensity of the enantiomers bound to BSA is much higher in absolute terms than in the unbound form. The amplification by two orders of magnitude of the CD difference between the antipodes, due to the complexation with the protein can be used to measure the OP of samples with very high accuracy. The values compare well with those obtained with chiral HPLC.

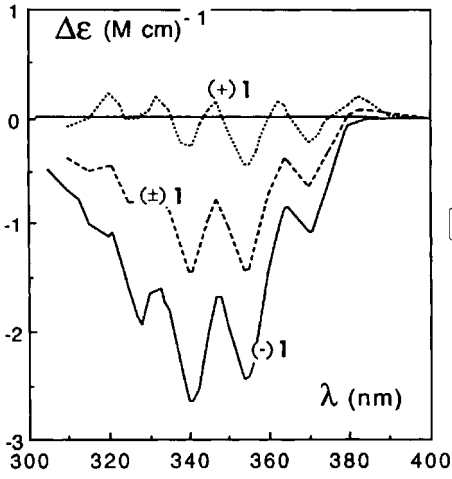

Figure 6.4.A.
CD spectra of (+)-ketoprofen with BSA [(+)**1**], (±) ketoprofen with BSA [(±)–1], and (−)-ketoprofen with BSA [(−)–**1**]. Spectra are obtained after subtraction of BSA contribution. [BSA] = 1.3×10^{-4} M, [ketoprofen] = 0.65×10^{-4} M (from Ref. [11])

6.4.3. The Use of Chiroptical HPLC Detectors for the Analysis of Enantiomeric Purity

Detectors based on optical activity, i.e. optical rotation and circular dichroism have advantageous features in the HPLC analysis of enantiomers [12–14]. One major advantage is their inherent selectivity. Compounds that do not possess optical activity do not interfere even if they co-elute with the analytes. This is particularly useful in the analysis of physiological fluids.

The sensitivity of these detectors is lower than that of the generally used UV detectors, but applying laser light or/and fluorescence detection the sensitivity may be enhanced [15–18]. The most effective way of the application of polarimetric or CD detectors in high-performance liquid chromatography is to use them in series with the conventional UV-VIS and RI HPLC detectors [17,19–27].

Using this experimental set-up the chromatographic separation of the enantiomers is not by all means necessary, because the UV-VIS or RI detector responds to the total amount of the analyte and the response of the chiral detector depends on the actual quantitative ratio of the enantiomers.

L-*Epinephrine* can be specifically analysed in ophthalmic formulation by HPLC using a UV detector in series before a polarimetric detector [19]. This is important as D-epinephrine is inactive in the treatment of glaucoma. The concentrations of both enantiomers in an unknown mixture can be calculated from the detector signals and from the ratio of the two signals measured for an L-epinephrine standard. The enantiomeric purity of *tartaric* and *malic acids* was determined similarly. The sensitivity of detection can be enhanced by complexation with molybdate [26].

Yeung and Reitsma [20] were the first to demonstrate enantiomeric purity determination of some *amino acids* by UV and RI detection coupled with the above mentioned sensitive laser polarimetric detector [20]. A near-infrared (820 nm) semiconductor diode-laser-based OR detector was coupled with a UV detector and the enantiomeric ratio of D- and L-*tryptophan* mixtures was determined [21]. The sensitivity of this technique may be increased by precolumn achiral derivatisation of amino acids with dansyl chloride to increase their specific rotation [22].

The separation of the *SS* and *RR* enantiomers of the racemate drug *Tramadol hydrochloride* as well as its diastereomeric *SR* and *RS* impurities would require a chiral chromatographic method. As another alternative a reversed-phase achiral separation of the diastereomers has been developed [23]. Using argon ion laser-based polarimetric detector in series with a UV detector, the quantitation of the enantiomers without separation has been achieved. The polarimetric detection was accomplished at 488 nm, while the UV detection was at 270 nm. The detection limit was 0.4 μg for the *SS* isomer, and the enantiomeric purity can be determined down to the 0.4% level. Wu *et al*. [24] have used reversed-phase HPLC with dual polarimetric-UV detection to determine the enantiomeric purity of *ephedrine* and *pseudoephedrine*.

Another option for chiroptical HPLC detection is based on circular dichroism (CD) [14,28–31]. CD is an even more selective detection technique than the polarimetric detection, because the CD detectors give a signal only in the region of optically active absorption band.

A relatively simple but very useful information obtainable with the aid of chiroptical detectors is the estimation of the *elution order* of the enantiomers after chiral separation. For example, with the aid of CD and UV detectors connected in series it was found that in the course of the separation of amino acid enantiomers on a Crown Pak CR(−) chiral column L-amino acids were eluted first and detected as positive peaks and all D-amino acids were registered as negative peaks [32].

In addition to the above described simple problems, CD/UV based HPLC detection systems enable also enantiomeric ratios of unresolved chromatographic peaks to be determined. A method of this type was developed by Drake and coworkers [28] for the optical resolution of *pavine* (obtained by

synthesis from papaverine). The determination of the enantiomeric ratio of *ascorbic, lactic* and other *chiral carboxylic acids* based on the same experimental set-up is also described [33].

The CD/UV based HPLC detection systems are capable of recording the enantiomeric composition of the eluates directly during the chromatographic run, on the basis of the dissymmetry ratio or *g*-factor. The absolute value of the dissymmetry ratio is identical at a given wavelength for the two pure enantiomers with different signs and with a maximum value for the optically pure enantiomer. It is independent of the analyte concentration and is linearly related to the enantiomeric purity. The accuracy of the measurement depends on the dissymmetry ratio of the single enantiomer.

As an example the determination of the enantiomeric composition of *3-methyl-desmethyldiazepam* during the chiral chromatographic separation is demonstrated in Fig. 6.4.B [34]. The chromatographic run, the aim of which was the preparative separation of the enantiomers was monitored using simultaneous detection by a CD detector (Jasco, J-600 dichrograph equipped with a 8 μl HPLC cell), and a UV detector operating at 254 nm. The time when baseline resolution is obtained and where the fractions should be collected in preparative HPLC is obvious. This method was used for monitoring the chiral separation of other benzodiazepines as well [35,36].

The HPLC/CD system can be used on line to measure the CD spectrum of enantiomers in eluates from a chiral column by the stopped flow method. Thus, in addition to the estimation of the elution order of the enantiomeric eluates this

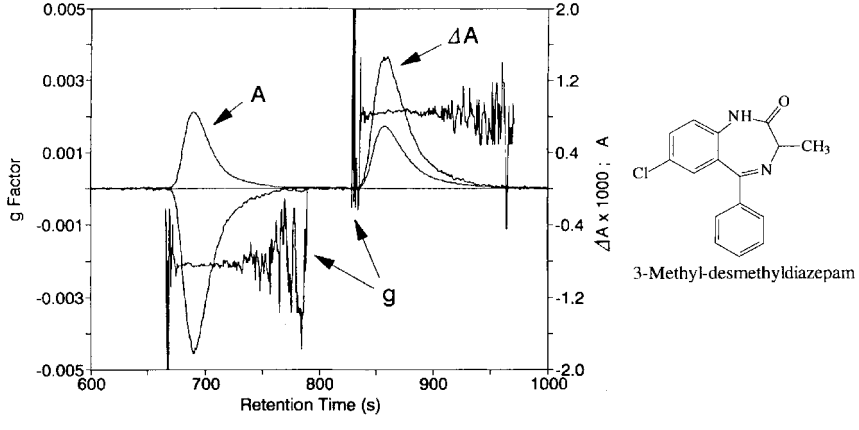

Figure 6.4.B.
Chromatographic resolution of 3-methyl-desmethyl-diazepam on a Pirkle-type column. Mobile phase: hexane/2-propanol 90:10, v/v); flow rate 1 ml/min. UV (A), CD (ΔA),and g (ΔA/A) detection at 254 nm (from Ref. [35])

method can be employed also to the determination of the absolute configuration of enantiomers by applying various empirical and semiempirical rules or by nonempirical methods such as the exciton model or the DeVoe approach [37–41].

6.4.4. Conclusions

The conclusion which can be drawn from the examples presented in the first part of this section is that the direct chiroptical methods as well the HPLC methods based on chiral detection are useful tools for the determination of the enantiomeric purity of drugs. It is characteristic of both techniques that they do not measure directly the enantiomeric impurity but from the measured signal size or from the ratio of chiral and achiral signals the enantiomeric purity can be calculated.

The exact knowledge of the anisotropy factor of the single enantiomers plays an important role in this field; it can be stated that by applying the methods based on this principle the enantiomeric purity can be determined with 0.1–2% accuracy.

The primary aim of coupling HPLC/UV separation/detection with ORD or CD detection (including the possibility of taking the CD spectrum of the separated peaks) is the identification or structure elucidation of the separated materials among them diastereomers and enantiomers from the simple estimation of the elution order of enantiomers to the determination of absolute configurations [42]. (A complex study of this type where HPLC/CD was used along with HPLC/NMR is described in Section 7.5.6.) Another application field is the determination of enantiomeric impurities. On the basis of the experience from the author's laboratory under favourable conditions 0.2–0.5% enantiomeric impurity can be determined by this methods [43].

On the basis of recent tendencies it is predictable that in the near future chiroptical detection will have an increasing role in the determination of enantiomeric impurities in drugs. This is supported by the recent availability of a HPLC/CD/UV detector which enables good-quality CD-chromatograms to be taken after the injection of as low a sample size as 20–100 ng.

References

1. S.S.M. Hassan, *Microchim. Acta* **1**, 9–17 (1981)
2. S.R. Erskine, B.M. Quencer and K.R. Beebe, *Appl. Spectrosc.* **49**, 1682–1691 (1995)
3. P. Horváth, A. Gergely and B. Noszál, *Talanta* **44**, 1479–1485 (1997)

4. R.J. Palma Sr., J.M. Young and M.W. Espenscheid, *Anal. Lett.* **18**, 641–652 (1985)
5. L. Chafetz and T.M. Chem, *J. Pharm. Sci.* **63**, 807–808 (1974)
6. T. Kitagawa, S. Oie and M. Taniyama, *Chem. Pharm. Bull.* **24**, 3019–3024 (1976)
7. Y. Fujii, *Bull. Chem. Soc. Jpn.* **47**, 2856–2861 (1974)
8. Y. Fujii and H. Yoneda, *Chem. Lett.* **1**, 43–46 (1974)
9. H. Hegedüs, A. Gergely, T. Veress and F. Zsila, *Fres. J. Anal. Chem.* **364**, 749–752 (1999)
10. A.R. Engle and N. Purdie, *Anal. Chim. Acta* **298**, 175–182 (1994)
11. M. Zandomeneghi, *Chirality* **7**, 446–451 (1995)
12. A. Gergely, *J. Pharm. Biomed. Anal.* **7**, 523–541 (1989)
13. A. Mannschreck, A.D. Eigelsperger, E. Gmahl and H. Buchner, *Chromatographia* **25**, 182–188 (1988)
14. A. Gergely, The use of circular dichroism as a liquid chromatographic detector, in *Analytical Application of Circular Dichroism* (N. Purdie and H.G. Brittain, Eds.), pp 279–292, Elsevier, Amsterdam (1994)
15. R.E. Synovec and E.S. Yeung, *J. Chromatogr.* **368**, 85–91 (1986)
16. Y.Y. Shao, P.D. Rice and D.R. Bobbitt, *Anal. Chim. Acta* **221**, 239–247 (1989)
17. D.M. Goodall, *Trends Anal. Chem.* **12**, 177–184 (1993)
18. L. Geng and L.B. McGown, *Anal. Chem.* **66**, 3243–3246 (1994)
19. B.S. Scott and D.L. Dunn, *J. Chromatogr.* **319**, 419–426 (1985)
20. B.H. Reitsma and E.S. Yeung, *J. Chromatogr.* **362**, 353–362 (1986)
21. D.K. Lloyd, D.M. Goodall and H. Scrivener, *Anal. Chem.* **61**, 1238–1243 (1989)
22. B.H. Reitsma and E.S. Yeung, *Anal. Chem.* **59**, 1059–1061 (1987)
23. T.J. Edkins, M. Fronheiser, D.R. Bobbit, J.E. Mills and T.M. Rossi, *Enantiomer* **1**, 97–107 (1996)
24. Z. Wu, D.M. Goodall and D.K. Lloyd, *J. Pharm. Biomed. Anal.* **8**, 357–364 (1990)
25. N. Purdie, Chiroptical analytical methods, in *Encyclopedia of Pharmaceutical Technology*, Vol. 18 (J. Swarbrick and J.C. Boylan, Eds.), pp 29–54, Marcel Dekker, New York
26. A. Yamamoto, A. Matsugana, E. Mizukami, K. Hayakawa and M. Míyazaki, *J. Chromatogr. A,* **667**, 85–89 (1994)
27. J.L. Sims, J.A. Carreira and R.A. Bragg, *Chirality* **9**, 122–125 (1997)
28. A.F. Drake, J.M. Gould and S.F. Mason, *J. Chromatogr.* **202**, 239–245 (1980)
29. R.E. Synovec and E.S. Yeung, *J. Chromatogr.* **368**, 85–91 (1986)
30. P. Salvadori, C. Bertucci and C. Rosini, *Chirality* **3**, 376–385 (1991)

31. J. Zukowski, Y. Tang, A. Berthod and D.W. Armstrong, *Anal. Chim. Acta* **258**, 83–92 (1992)
32. K. Takatori, S. Toyama, S. Fujii and M. Kajiwara, *Chem. Pharm. Bull.* **43**, 1797–1799 (1995)
33. O. Zerbinati, R. Aigotti and P.G. Daniele, *J. Chromatogr. A* **671**, 281–285 (1994)
34. P. Salvadori, C. Bertucci, E. Domenici and G. Giannaccini, *J. Pharm. Biomed. Anal.* **7**, 1735–1742 (1989)
35. C. Bertucci, E. Domenici and P. Salvadori, *J. Pharm. Biomed. Anal.* **8**, 843–846 (1990)
36. C. Bertucci, P. Salvadori and L.F. Lopes Guimaraes, *J. Chromatogr. A* **606**, 535–539 (1994)
37. P. Salvadori, C. Rosini and C. Bertucci, *J. Org. Chem.* **49**, 5050–5054, (1984)
38. S.C. Tan, J.A. Baker, N. Stevens, V. Biasl, C. Salter, M. Chalaux, K. Afarinkia and A.J. Hutt, *Chirality* **9**, 75–87 (1997)
39. T.N. Johanssen, B. Ebert, E. Falch and P. Krogsgaard-Larsen, *Chirality* **9**, 274–280 (1997)
40. Gy. Szókán, Sz. Szarvas, Zs. Majer, D. Szabó, I. Kapovits and M. Hollósi, *J. Liq. Chromatogr.* **22**, 993–1007 (1999)
41. H. Takahashi, T. Kashima, S. Kimura, N. Muramoto, H. Nakahata, S. Kubo, Y. Shimoyama, M. Kajiwava and H. Echizen, *J. Chromatogr. B* **701**, 71–80 (1997)
42. F. Brandl, N. Pustet and A. Mannschreck, *Int. Lab.* **29**, 10C–15C (1999)
43. A. Gergely and P. Horváth, unpublished results

6.5. NMR Spectroscopy

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6.5.1. Introduction

Liquid-phase NMR spectroscopy is widely considered as an inherently achiral technique incapable of distinguishing between enantiomers without the aid of an auxiliary chiral probe. Although in most cases the determination of enantiomeric compositions by NMR is based on the use of optically active additives, this may not necessarily be so! The NMR nonequivalence of enantiomers results from the formation of transient diastereomeric solvates which may differ intrinsically in chemical shifts and which may form to different extents if the energy of interaction between homomers differs from that of enantiomers. Even if we assume that no such difference in the interaction energies exists, but we take, for example, an ensemble of molecules where the R form is in excess of the S form, any given S molecule will then experience SR-type interactions in more statistical abundance than SS-types, and an R molecule will be involved in more RR-type interactions than RS-types. The surroundings of ''S'' molecules will thus mostly consist of SR type interactions, while ''R'' molecules will mostly experience RR type interactions. Since these different interactions are of diastereomeric relationship, it follows that enantiomers are inherently nonequivalent in NMR except if they are present in a 1:1 ratio. The resulting spectral nonequivalence in nonracemate enantiomeric mixtures allows in principle the direct measurement of the enantiomeric excess (*ee*) by integration of the pertinent peaks [1–3]. Unfortunately, the intermolecular interactions responsible for this nonequivalence of enantiomers are, in the vast majority of cases, so weak that the ensuing chemical shift differences are in practice smaller than the natural linewidths and are *not* resolved, which is why chiral agents must be employed.

The enantiomeric analytes (R and S) and the chiral agent (CA) form diastereomeric complexes R···CA and S···CA according to the exchange processes R + CA $\rightleftharpoons$ R···CA and S + CA $\rightleftharpoons$ S···CA. The system is then associated with the respective chemical shifts ω_R, $\omega_{R\cdots CA}$, ω_S and $\omega_{S\cdots CA}$. Since these exchange rates are typically fast with respect to the frequency differences $|\omega_R - \omega_{R\cdots CA}|$ and $|\omega_S - \omega_{S\cdots CA}|$, the measured effective chemical shift ω_R^{eff} is the population-weighted time average of ω_R and $\omega_{R\cdots CA}$ while ω_S^{eff} is that of ω_S and $\omega_{S\cdots CA}$ [4]. It is the inherent difference between $\omega_{R\cdots CA}$ and

$\omega_{S\cdots CA}$ which will bring about the desired difference between ω_R^{eff} and ω_S^{eff}, depending partly on the strength of the complexes, on the molar substrate/CA ratio and also on how different the electronic environments of R and S in those complexes are. (Note that the CA molecules themselves quickly exchange sites according to R···CA $\rightleftharpoons$ CA $\rightleftharpoons$ S···CA, therefore each CA molecule will experience the same statistical environment, which is why the enantiomeric ratio is always measured on the signals of the analyte and not on those of the chiral agent). Unfortunately, these exchange processes are the sources not only of the desired enantiomeric distinction, but also broaden, as already pointed out in Section 2.4, the resonances to some extent. This exchange broadening is negligible if the exchange rates are much faster than the frequency differences $|\omega_R - \omega_{R\cdots CA}|$ and $|\omega_S - \omega_{S\cdots CA}|$, but becomes significant if they are comparable. Thus, by introducing a chiral agent we are invoking two, potentially opposing effects. For example, the difference between ω_R^{eff} and ω_S^{eff} is expected to be larger if the R···CA and S···CA complexes are stronger, but this may slow down exchange rates to the extent that the ensuing extensive line broadening renders spectral analysis infeasible. Besides a number of factors (temperature, solvent, concentration, analyte/CA ratio, pH, etc.) which can influence this exchange process, the employed magnetic field strength B_0 is also important: the difference $|\omega_R - \omega_{R\cdots CA} - \omega_{S\cdots CA}|$ increases linearly with B_0, therefore at larger fields $|\omega_R^{eff} - \omega_S^{eff}|$ is also expected to be larger. However, the differences $|\omega_R - \omega_{R\cdots CA}|$ and $|\omega_S - \omega_{S\cdots CA}|$ also increase with B_0 which results in more severe exchange broadening of the average resonance signals (in fact, fast-exchange broadening increases with B_0^2). For very fast exchange rates this broadening is minimal and the use of a large B_0 is favoured, but if exchange rates are only moderately fast, higher fields may actually become counterbeneficial. A ''good'' chiral agent can thus be characterised as one which involves the substrate in the fastest possible exchange, i.e. the complexes are labile, but also incurs the largest possible difference $|\omega_R^{eff} - \omega_S^{eff}|$. Much of the research and practice of chiral recognition by NMR revolves around the art of finding the right compromise between these competing factors.

Clearly, each nucleus within the same enantiomeric form is affected differently by the anisotropic effects of the surrounding functionalities of the chiral medium. In contrast to chromatographic methods where the enantiomeric ratio is measured on two signals only, NMR spectroscopy therefore provides the possibility of a multi-site measure (i.e. with regard to each separate resonance) of the same information. This has the interesting consequence that by varying one physical parameter of the system (pH, solvent composition, temperature etc.) at a time, we have a multiple chance that one or more enantiomeric resonances will differ in chemical shifts to an extent that enables the successful assessment of enantiomeric excess (*ee*).

The techniques currently used in liquid-phase high-resolution NMR spectroscopy offer a wide range of tools utilising the phenomenon of chiral recognition and allowing the determination of enantiomeric purity of chiral drugs. Such methods [5] include the use of chiral solvating agents (CSA) such as modified amino-acids or liquid crystalline solvents or chiral additives like lanthanide shift reagents (LSR), cyclodextrins (CD), cholic-acid derivatives or chiral crown-ethers [6], etc... To improve sensitivity and selectivity, such techniques are often combined with exotic multinuclear (^{2}H, ^{13}C, ^{19}F, ^{31}P) experiments [7–14]. Each of these methods have developed into stand-alone techniques.

There are three main fields of pharmaceutical R&D where NMR plays a key role in the determination of *ee* [15].

(a) Determination of *ee* in drug raw materials as a routine analysis, based on robust test-methods [16] described in pharmacopoeias. Such methods are often optimised for low-field instruments and their advantage lies in the specificity, simplicity, speed, as well as the low cost of the measurement. Among these methods is the application of shift reagents which typically work better at low fields [16] where the exchange broadening [4] they cause on the analyte tends to be less severe.

(b) Monitoring the enantiomeric purity of intermediates of a chiral synthesis in the discovery phase of drug development. Since the structures of such intermediates are often verified by NMR spectroscopy, it might be convenient to check their optical purity ''in situ'' with NMR, instead of developing a chiral chromatographic system. In the case of I.-II. the 0.5–5% accuracy of integrals is usually adequate for the given purpose.

(c) The development of NMR methods of higher accuracy and specificity purely for research purposes is to be mentioned. These special applications can be put into action whenever an independent nonchromatographic procedure is needed. Multinuclear techniques are often successful when the enantiomers cannot be easily separated from other support materials. Besides, the ability to investigate intermolecular interactions via the nuclear Overhauser effect [17,18] enables NMR to support HPLC and CE methods [19–21] in the system development phase and in the investigation of the enantiodiscriminating mechanism that lies behind [22], allowing the determination of absolute configurations [45]. Other NMR parameters, as will be discussed below in the case of cyclodextrins, may also help in revealing this mechanism. It is often the case that within a family of molecules, especially when family members have the same chiral moiety but different achiral sidechains, the discovery of the enantiodiscriminating mechanism for one member of the family allows the confident assignment of absolute configuration to other family members on the basis of the uniform sense of observed spectral nonequivalence [23].

Covalent chiral derivatisation will not be discussed here since it produces covalent diastereomers that are clearly distinguishable in the NMR spectrum and such methods gain more importance in liquid chromatography [24]. However, it is worth briefly noting with regard to case (c) that when several stereogenic centres are present in the molecule, unexpected epimerisation during covalent derivatisation can take place. NMR detects this epimerisation by measuring diastereomers even when the applied LC methods are not specific enough to produce large retention differences.

On the technical side, the applicability of different techniques depends on what spectrometer hardware is available. The era of low-field NMR instruments ($B_0 < 2.3$ T) was almost entirely dominated by the application of chiral lanthanide shift reagents (LSR). The main reason for this was the generally larger shift nonequivalence that was possible to achieve with LSRs compared to CSA-based methods. In today's NMR of very high fields, the user may choose from a large number of more sophisticated techniques and solve the same problem by using many different approaches (cf. below). Research instruments are usually equipped with hardware powerful enough to support most of these techniques. It seems that today many problems related to enantiomeric composition determination are resolved with multinuclear CSA techniques, making good use of the excellent spectral resolution provided by the high magnetic field.

In the following we briefly mention some of the NMR techniques that can be applied in a more or less routine fashion for the estimation of *ee*. Emphasis is placed on the practical aspects that should be considered before deciding on a particular method. It should be noted, however, that none of these methods is universally applicable for every molecular family and many brilliant techniques have been reported in the literature which cannot be included in this section.

6.5.2. Chiral Solvent Additives

The evolution of CSAs has followed a roughly parallel route with the methodological revolution of HPLC and the appearance of high-field NMR instruments. NMR spectroscopy has imported and adapted practically the same reagents that were used either as mobile phase additives or as chiral stationary phases in liquid chromatography. One possible classification of the different chiral solvent methods might be according to the type of molecules used as chiral additives. The choice of chiral additive also determines the choice of solvent. Cyclodextrins, for example, require working in aqueous solutions, whereas lanthanide shift reagents are mostly applicable in apolar media although exceptions are found in the literature [25,26].

6.5.3. Cyclodextrins (CDs) as Chiral Solvating Agents

CDs are widely used in liquid chromatography (LC) for enatiomeric separation either as running chiral mobile-phase additives (CMPAs) or as chiral stationary phases (CSPs). The majority of chiral separations in capillary electrophoresis (CE) is also achieved by the addition of a chiral selector to the running buffer.

The use of cyclodextrins (CDs) as chiral resolving agents in NMR spectroscopy has been well established since the 1980s [27–30]. CDs are cyclic oligosaccharides with an apolar cavity which is large enough to form inclusion complexes with smaller functional units (e.g. an aromatic ring) of the organic solute by means of hydrophobic interactions. Owing to their chirality, the enantioselective interaction with chiral substrates leads to enantiodiscrimination. The toroidal shape of CDs may contain six (α-), seven (β-), or eight (γ-) α-D(+)-glucopyranose units bonded through (1,4) linkages.

The advantages of CDs over LSRs are the following.

- Even at high fields there is no significant line broadening as opposed to LSRs where broadening due to exchange as well as the presence of paramagnetic metal ions may be extensive [7].
- The chemical shift range of the common unmodified CDs is relatively narrow therefore all the aromatic and most of the upfield aliphatic region of the ^{1}H-NMR spectrum remains uneffected by signals arising from the reagent [31].
- The water solubility of CDs allows the measurement of samples of high polarity in D_2O; therefore traces of water remaining in the sample, which is a competing factor in the case of LSRs, does not corrupt the measurement. It must be noted that the improper choice of pH can seriously decrease or even destroy the enantioselectivity of the system, especially when working with anionic cyclodextrins (e.g. SBE-β-CD: sulphobutylether-β-cyclodextrin [20]).

In some cases a large shift nonequivalence can be achieved by this method without significant line broadening. Racemic *dl-norgestrel* and its optically pure component, *d-levonorgestrel*, are presented here as an illustrative example.

Figures 6.5.A and 6.5.B show the ^{1}H-NMR spectrum of *dl-norgestrel* in the presence of γ-cyclodextrin. Singlets of the ethinyl and olefinic hydrogens ($\mathbf{H_b}$ and $\mathbf{H_a}$, respectively) provide an excellent possibility for measuring *ee*. This example also shows how NMR methods can support chromatographic investigations in the development phase since this separation was also achieved successfully by HPLC using cyclodextrins [32–34]. An enhanced degree of enantioselectivity may be found in the case of CDs

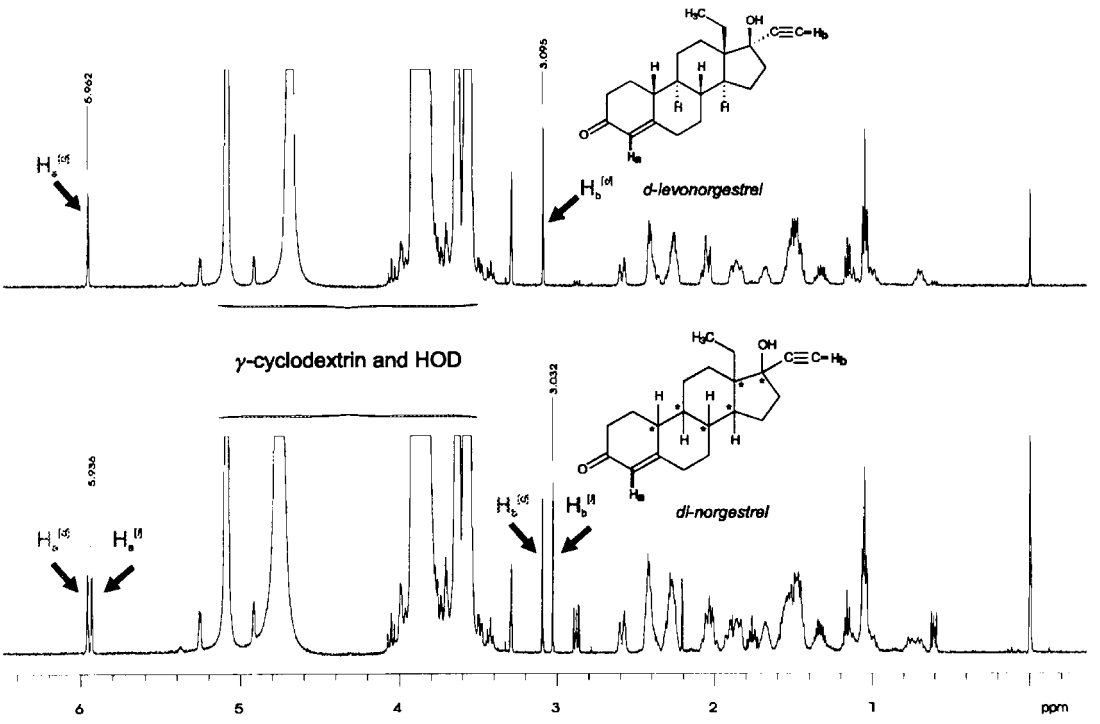

Figure 6.5.A.
Racemic *dl-norgestrel* (lower trace) (≈0.5 mM/l) and *d-levonorgestrel* (upper trace) (≈0.5 mM/l) in the presence of *γ-cyclodextrin* (≈11 mM/l); solvent: D_2O: CD_3OD 50: 1; 500 MHz; 30°C

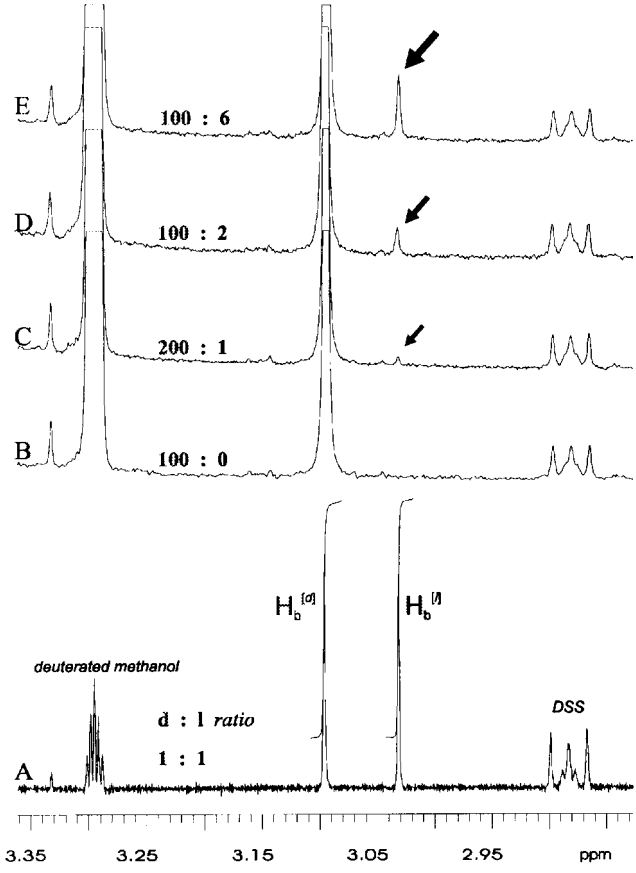

Figure 6.5.B.
Magnified region of the D_2O spectrum of *dl-norgestrel* (≈0.5 mM/l)with five different [*d*]:[*l*] ratios in the presence of *γ-cyclodextrin* (≈11 mM/l).

where a neutral derivative (usually an acyl-group) is randomly bonded to the primary or secondary hydroxyl positions with a defined degree of substitution. Systematically modified CDs were also synthesised to increase the enantioselectivity for a group of compounds, e.g. heptakis(2,3-*O*-diacetyl)-*β*-CD (diac-CD) proved to be successful in the separation of chiral protonated phenethylamines [35–37].

In addition to chiral separation, NMR spectroscopy has proved to be a useful tool for investigating interactions between a chiral analyte and a CD, both inside and outside a CD cavity [20,38–40]. Such investigations are useful in understanding the enantioselective mechanism of CDs in LC and CE

[20,41,42], since they provide information about the nature of the molecular interaction in the inclusion complex, the flexibility of the analyte in the CD cavity, etc. Practically all of the basic NMR parameters and effects may be useful in elucidating the underlying mechanism:

- Most of these investigations are based on the *nuclear Overhauser effect* [17,18] (NOE) and utilise either one- or two-dimensional NOE measurements (NOESY, ROESY [22]).
- Examining the *chemical shift*s for both the analyte and the CD after mixing is useful for investigating the stoichiometry, stability and structure of CD complexes.
- Measurement of the *longitudinal relaxation time* T_1 is also useful for examining the dynamic properties of the analyte in the presence of a CD, since complexation leads to a 10–50% reduction of the T_1 values of the analyte [40].
- The formation of diastereomeric complexes can induce conformational changes in the structure of the probe molecule which may alter the average scalar coupling constant. Similar rules apply to optically active additives other than cyclodextrins.

6.5.4. Application of Lanthanide Chiral Shift Reagents (LSR)

LSRs form an NMR-specific branch of chiral additives. They became successful due to the large shift nonequivalence they produced even at low magnetic fields.

Chiral shift reagents are lanthanide-organic chelates with optically pure chiral organic groups as ligands. The central metal ion has free coordination sites and it can coordinate further ligands, thereby allowing binding of the solute to the shift reagent and formation of a noncovalent diastereotopic complex. Paramagnetic metal ions possess such a property and among them europium and praseodimium was found to produce the smallest line broadening due to the general T_1-decreasing effect of paramagnetic entities [43].

The induction of shift nonequivalence depends on the establishment of a fast reversible molecular association equilibrium between the chiral lanthanide shift reagent and the organic donor substrate functionalities. Therefore, when working with LSRs it is crucial to maximise the interaction (known as the pseudocontact interaction [44,45]) between the LSR and the substrate, and to minimise it with solvent molecules. The approximate order of the binding capabilities of some classes of compounds is as follows: amine > alcohol > ketone > aldehyde > ether > ester > nitrile. DMSO and D_2O, for example, are inappropriate solvents for working with LSRs. Apolar solvents (CCl_4, $CDCl_3$) must be used

and traces of water coming with the sample should be minimised as well.

The method requires a simple but essential optimisation process: the shift reagent/substrate ratio is increased gradually by adding small and known amounts of LSR to the solution of the racemate, typically until the LSR is in molar excess. At each LSR concentration the ^{1}H spectrum is recorded. Signals due to chemically identical functional groups in the diastereomeric complexes become increasingly separated as a function of the LSR concentration. The reasons why such optimisation is needed are many:

- The addition of too much LSR must be avoided because of severe line broadening as discussed above and although some signals are well separated at the peaks, they may still overlap at half-height due to their large linewidth which makes integration highly inaccurate.
- The best separation can rarely be achieved at equimolar concentration, especially when there are several binding sites on the molecule.
- Not only the shift nonequivalence but also the shift displacement should be optimised. It often occurs, that signals that would otherwise be highly suitable for integration and are also well separated become obscured by other badly resolved spectral regions. Once the optimum LSR/substrate ratio is known, this ratio can be applied directly for the measurement of *ee* in nonracemic mixtures of enantiomers [16].

A huge number of chiral shift reagents has been published in the literature [44,45]. *D*-camphorato-europium complexes form a large subclass and among them are the widely used and commercially available compounds of tris[3-(*tert*-butylhydroxymethylene)-*d*-camphorato]europium(III) [46] or tris[3-(trifluoromethylhydroxymethylene)-*d*-camphorato]europium(III) [47].

6.5.5. Chiral NMR Solvents

Examination of the ^{1}H-NMR spectra of polar chiral enantiomeric substances in solutions of optically active alcohols, amines [23] or metal complexes [48] is also a fruitful method for the direct determination of enantiomeric purity [49,50]. Since the active component can be virtually any kind of optically active sample, e.g. quinine, dissolved in high (0.05–0.3 mol/l) concentrations in an achiral solvent, this method clearly gives its user plenty of freedom. The reagents may range from smaller Mosher's acid-like structures [21,51,52] to larger chelates, crown ethers [53] or cholic-acid derivatives. An unwanted drawback of this method could come from the fact that the chiral solvents themselves usually exhibit a complex ^{1}H spectrum potentially overlapping many of the substrate resonances. It is worth noting that such spectral overlaps can be simply and successfully reduced by routine techniques such as

selective decoupling [54] or by modifying the aromatic vs. non-aromatic composition of the solvent.

In some cases good results can be achieved by the combination of *achiral* lanthanide shift reagents and resolving agents [53,55–58].

6.5.6. Multinuclear Approach

So far we have focused on ^{1}H as the primary nucleus utilised in chiral NMR measurements. In principle less sensitive nuclei like (^{2}H, ^{13}C, ^{19}F, ^{31}P) [59,60] also provide the possibility to measure the *ee* if care is taken to set up instrumental conditions such that the linearity of integrals is secured [61]. It should be noted, however, that the ^{1}H nucleus is the most affected by the chemical shift anisotropy of the surrounding medium and therefore most probably it will show the greatest spectral non-equivalence. Nonetheless, by achiral derivatisation the introduction of a ^{19}F- or ^{2}H-containing moiety into the analyte molecule may greatly improve selectivity and sensitivity. Based on this concept, an excellent technique was developed by Courtieu *et al*. in the mid 1990s [9,10]: when a deuterium-labelled analyte is dissolved in a cholesteric-nematic *liquid crystalline solvent*, the observed difference in the quadrupolar splittings for spins > 0.5 (also called the *differential ordering effect*) enables the determination of *ee* or possibly the absolute configuration. Although this method is rather sophisticated and requires deuterium labelling and measurement, it is very efficient and resolves even small enantiodifferentiating groups such as those arising from isotopic substitution [11]. Deuterium labelling can be achieved in good yield e.g. by a perdeutero acyl- or benzoyl group [12,13] in an achiral synthesis. Carbon-13 *chemical shift anisotropy* measured in a lyotropic chiral nematic solvent is also a successful multinuclear NMR tool for the measurement of *ee* [14].

6.5.7. Conclusions

Finally it is worth making a few comments on quantitation and the detection limits of the different methods. Quantitation heavily depends on the precision and accuracy of the NMR integrals, therefore statements of Sections 2.4.3 and 2.4.4 are also valid for the detection limits of enantiomeric separations. On high field instruments equipped with a standard 5 mm probe the smallest detectable optical impurity is usually equal to or less then 0.1% which makes NMR methods an attractive alternative to chromatographic methods.

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References

1. T. Williams, R.G. Pitcher, P. Bommer, J. Gutzwiller and M. Uskokovic, *J. Am. Chem. Soc.* **91**, 1871–1872 (1969)
2. B.S. Jursic and S.I. Goldberg, *J. Org. Chem.* **57**, 7172–7174 (1992)
3. A.B. Uryupin, M.I. Kadyko, P.V. Petrovskii, E.I. Fedin, M. Sanshe, R. Wolf, T. A. Mastryukova and M. I. Kabachnik, *Zh. Obshch. Khim.* **65**, 18–22 (1995)
4. M.L. Martin, J.J. Delpuech and G.J. Martin, *Practical NMR Spectroscopy*, Heyden, London (1980)
5. H.Y. Aboul-Enein, R. Rothchild and A. Sinnema, *Spectrosc. Lett.* **25**, 1367–1385 (1992)
6. J.F. Stoddart, *Top. Stereochem.* **17**, 207–288 (1987)
7. J.W. Jaroszewski and A. Olsson, *J. Pharm. Biomed. Anal.* **12**, 295–299 (1994)
8. C.D. Stevenson, R.M. Fico Jr. and E.C. Brown, *J. Phys. Chem.* **102**, 2841–2844 (1998)
9. I. Canet, J. Courtieu, A. Loewenstein, A. Meddour and J. M. Pechine, *J. Am. Chem. Soc.* **117**, 6520–6526 (1995)
10. I. Canet, A. Meddour, J. Cortieu, J.L. Canet and J. Salaün, *J. Am. Chem. Soc.* **116**, 2155–2156 (1994)
11. A. Meddour, I. Canet, A. Loewenstein, J.M. Pechine and J. Courtieu, *J. Am. Chem. Soc.* **116**, 9652–9656 (1994)
12. J.L. Canet, I. Canet, J. Courtieu, S. Da Silva, J. Gelas and Y. Troin, *J. Org. Chem.* **61**, 9035–9037 (1996)
13. A. Meddour, A. Lowenstein, J.M. Pechine and J. Courtieu, *Tetrahedron: Asymmetry* **8**, 485–494 (1997)
14. A. Meddour, P. Berdague, A. Hedli, J. Courtieu and P. Lesot, *J. Am. Chem. Soc.* **119**, 4502–4508 (1997)
15. U. Holzgrabe, B.W.K. Diehl and I. Wawer, *J. Pharm. Biomed. Anal.* **17**, 557–616 (1998)
16. G.M. Hanna, *J. Pharm. Biomed. Anal.* **15**, 1805–1811 (1997)
17. J.H. Noggle and R.E. Schirmer, *The Nuclear Overhauser Effect*, Academic Press, New York (1971)

18. D. Neuhaus and M. Williamson, *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, VCH, New York (1989)
19. E. Yashima, M. Yamada and Y. Okamoto, *Chem. Lett.* **3**, 579–582 (1994)
20. P. K. Owens, A.F. Fell, M.W. Coleman, M. Kinns and J.C. Berridge, *J. Pharm. Biomed. Anal.* **15**, 1603–1619 (1997)
21. G.M. Hanna and C.A. Lau-Cam, *J. Pharm. Biomed. Anal.* **11**, 665–670 (1993)
22. G. Uccello-Barretta, A. Cuzzola, F. Balzano, R. Menicagli, A. Iuliano and P. Salvadori, *J. Org. Chem.* **62**, 827–835 (1997)
23. W.H. Pirkle and D.J. Hoover. *Top. Stereochem.* 263–283 (1982)
24. S. Görög and M. Gazdag, *J. Chromatogr.* **659**, 51–84 (1994)
25. R. Hazama, K. Umakoshi, C. Kabuto, K. Kabuto and Y. Sasaki, *Chem. Commun.* **1**, 15–16 (1996)
26. R. Hulst, N.K. de Vries and B.L. Feringa, *J. Org. Chem.* **59**, 7453–7458 (1994)
27. B.A. Dawson and D.B. Black, *J. Pharm. Biomed. Anal.* **13**, 39–44 (1995)
28. D. Greatbanks and R. Pickford, *Magn. Reson. Chem.* **25**, 208–215 (1987)
29. A.F. Casy and A.D. Mercer, *Magn. Reson. Chem.* **25**, 765–774 (1988)
30. J. Klein, H. Hartenstein and D. Sicker, *Magn. Reson. Chem.* **32**, 727–731 (1994)
31. A.F. Casy, *Trends Anal. Chem.* **12**, 185–189 (1993)
32. M. Gazdag, G. Szepesi and L. Huszár, *J. Chromatogr.* **351**, 128–135 (1986)
33. M. Gazdag, G. Szepesi and L. Huszár, *J. Chromatogr.* **436**, 31–38 (1988)
34. G. Szepesi and M. Gazdag, *J. Pharm. Biomed. Anal.* **6**, 623–639 (1988)
35. M. Thunhorst and U. Holzgrabe, *Magn. Reson. Chem.* **36**, 211–216 (1998)
36. S.K. Branch, U. Holzgrabe, T.M. Jefferies, H. Mallwitz and M. Matchett, *J. Pharm. Biomed. Anal.* **12**, 1507–1517 (1994)
37. S.K. Branch, U. Holzgrabe, T.M. Jefferies, H. Mallwitz and F.J.R. Oxley, *J. Chromatogr.* **758**, 277–292 (1997)
38. K.B. Lipkowitz, S. Raghothama and J. Yang, *J. Am. Chem Soc.* **114**, 1554–1562 (1992)
39. A.A. Bothner-By, R.L. Stephens, J. Lee, C.D. Warren and R.W. Jeanloz, *J. Am. Chem. Soc.* **106**, 811–813 (1984)
40. N. Azaroual-Bellanger and B. Perly, *Magn. Reson. Chem.* **32**, 8–11 (1994)
41. A.F. Casy, A.D. Cooper, T.M. Jefferies, R.M. Gaskell, D. Dreatbanks and P. Pickford, *J. Pharm. Biomed. Anal.* **9**, 787–792 (1991)
42. Y. Machida, H. Nishi and K. Nakamura, *J. Chromatogr.* **810**, 33–41 (1998)
43. C.C. Hinckley, *J. Am. Chem. Soc*. **91**, 5160–5162 (1969)

44. R.E. Sievers, *Nuclear Magnetic Resonance Shift Reagents*, Academic Press, New York (1973)
45. T.J. Wenzel, *NMR Shift Reagents*, CRC Press, Boca Raton, FL (1987)
46. G.M.Whitesides and D.W. Lewis, *J. Am. Chem. Soc.* **92**, 6979–6980 (1970)
47. H.L. Goering, J.N. Eikenberry and G.S. Koermer, *J. Am. Chem. Soc.* **93**, 5913–5914 (1971)
48. S. Hameed, R. Ahmad and H. Duddeck, *Magn. Res. Chem.* **36**, S47–S53 (1998)
49. W.H. Pirkle and A. Tsipouras, *Tetrahedron Lett.* **26**, 2989–2992 (1985)
50. B.S. Jursic, Z. Zdravkovski and M. Zuanic, *Tetrahedron: Asymmetry* **5**(9), 1711–1716 (1994)
51. J.A. Dale, D.L. Dull and H.S. Mosher, *J. Org. Chem.* **34**, 2543–2549 (1969)
52. C. Garcia-Martinez, Y. Taguchi, A. Oishi and K. Hayamizu, *Magn. Reson. Chem.* **36**, 429–435 (1998)
53. S.E. Weinstein, M.S. Vining and T.J. Wenzel, *Magn. Reson. Chem.* **35**, 273–280 (1997)
54. E. Kupce and R. Freeman, *J. Magn. Res.* **115A**, 273–276 (1995)
55. W.H. Pirkle and D.L Sikkenga, *J. Org. Chem.* **40**, 3430–3434 (1975)
56. W.H. Pirkle, D.L. Sikkenga and M.S. Pavlin, *J. Org. Chem.* **42**, 384–387 (1977)
57. T.J. Wenzel, A.C. Bean and S.L. Dunham, *Magn. Reson. Chem.* **35**, 395–402 (1997)
58. T.J. Wenzel, C.A. Morin and A.A. Brechting, *J. Org. Chem.* **57**, 3594–3599 (1992)
59. R. Hulst, N. Koen de Vries and B.L. Feringa, *Tetrahedron*: *Asymmetry* **5**(4), 699–708 (1994)
60. B.S. Jursic and Z. Zdravkovski, *J. Org. Chem.* **58**, 5245–5250 (1993)
61. Cs. Szántay Jr., *Trends Anal. Chem.* **11**, 332–344 (1992)

Chapter 7

ESTIMATION OF POLYMORPHIC MODIFICATIONS AS IMPURITIES IN DRUGS

7.1. The Phenomenon and Importance of Polymorphism

Béla Hegedűs

7.1.1. Introduction

The term *polymorphism* describes the phenomenon when a substance of a given chemical structure can reproducibly form two or more materials with different submicroscopic spatial arrangements. Such submicroscopic differences may originate from the different conformations of molecules in the elementary cells in the crystalline modifications, or their random positioning in amorphous modifications. At a macroscopic level these differences may either be negligible, or the modifications may exhibit significant deviations in their physico-chemical or biological characteristics. If the habits of the materials are macroscopically different, but these differences do not reach the level of elementary cells, the problem is out of the scope of polymorphism. Similarly, solvates or other adducts of a given substance are not strictly defined polymorphs of that substance, nevertheless the literature often treats them as such – probably on the basis of their identical biological activity and their direct interconvertability [1].

Any specific crystal state represents an energy-minimum of the system as is relevant to the actual temperature and pressure. In other words, at different temperature and pressure values different crystal structures can represent different energy-minima of the system, provided there is a real possibility for the existence of different polymorphic modifications. Polymorphism is *enantiotropic* if the crossing of the limit parameters results in the transformation of one modification into the other and vice versa, and it is *monotropic* if the transition proceeds only in one direction.

Such transitions can be influenced by many different parameters, consequently they cannot be predicted or calculated. Polymorphism may be responsible for a wide range of phenomena affecting drug formulations such as when emulsions diverge, ointments harden into the tube, or in general, formulated products containing drug substances of undefined morphological composition show altered biological efficiency. Formulations consisting of amorphous, consequently ab ovo metastable modifications, are very frequently involved in such problems. In 1965 Kuhnert-Brandstätter [2] examined different groups of drug substances and concluded that some relationship exists between the types of examined drugs and the frequency of appearance of polymorphism. In addition she observed that formulations of metastable products frequently show traces of the more stable modifications, indicating a slow transformation in that direction. For example 67% of the examined steroids were polymorphic with only 17% metastable modifications, while among sulphonamides 40% were polymorphic with 23% altering modifications.

7.1.2. Polymorphism Among the Drug Substances

In 1995 Borka published a review of polymorphic drug substances which appear in the European Pharmacopoeia [3,4]. These papers conclude that neither the number of possible modifications, nor the significance of the differences between the modifications can be predicted. Such differences may be no more than a minor spectroscopic curiosity, or can in other cases be of major pharmaceutical importance. The latest trends clearly show that the issue of morphological purity receives permanently growing attention in the pharmaceutical industry due to the increasing demand for validated manufacturing procedures. Below we highlight, without claiming completeness, the main fields where morphological homogeneity can be a question of vital importance.

7.1.2.1. Bioavailability

The bioavailability of modifications may be dramatically different. As a characteristic example the case of *chloramphenicol palmitate* modifications is often mentioned by citing Aguiar et al. [5], who found great differences in the blood serum level of the drug as a function of the morphological composition. Extreme differences had also been published by Mullins et al. [6] on the blood serum levels of the different modifications of *novobiocine*: 1 h after administering amorphous novobiocine, its concentration in the plasma was found to be 40 μg/ml. However, when novobiocine was administered in crystalline form, no drug was detectable.

In several cases different modifications also show different toxicities, as

found e.g. in the case of *mebendazole*. The known modifications of mebendazole, A, B and C, were administered to mice in a dose of 800 mg/kg. In the case of modification A 0% die off was observed, contrary to this, in the case of modifications B and C the die off was ≈50%, nevertheless the survival was shorter in the case of form B [7].

Such dramatic differences are rare, but instrumental analytical control of the morphological purity of the drug substance is necessary in all cases where any of the important biological characteristics of the different modifications (absorption kinetics, blood serum level as the function of time, kinetics of metabolism etc.) exhibit larger differences than the usual uncertainty of the biological experiments.

7.1.2.2. Pharmaco-Technological Characteristics

Modifications may exhibit markedly different pharmaco-technological characteristics, such as in the case of *chloropropamide*, which was characterised by Tudor et al. [8] as having either long and thin needles in form A, or consisting of roundish small particles in form B. A more detailed presentation of this problem is given in the paper of Hegedűs et al. [9], describing the modifications of *famotidine*. Modification A exhibits a cubic character with rheological properties similar to sand, has a small specific surface and is not capable of electrostatic charging. Contrary to this, modification B has a bulk density half of that of form A, exhibits thin and long needles with a large specific surface, shows an arching tendency, and is prone to electrostatic charging. In the case of any validated tabletting technology, these modifications mutually exclude each other.

7.1.2.3. Manufacturing-Technological Parameters

Modifications may exhibit very different manufacturing-technological parameters as exemplified by *cimetidine*. The most economic synthesis of this drug can be performed in an aqueous medium, but under such circumstances the product crystallises in form B/C by itself [10,11]. Modification D of Cimetidine can be obtained also from aqueous medium under specially modified circumstances [12] However, the crystal structures of these modifications are dramatically different, determining both the filtering and the drying technological characteristics of the forms. While modification B can be hardly filtered and retains a 4–5-fold amount of mother liquor, in the case of form D this amount is approximately 0.4-fold and can be easily filtered and dried. Even the best synthetic procedures may be corrupted if such technological differences are disregarded, therefore permanent control over the morphological characteristics is one of the key points of a stable manufacturing procedure.

7.1.3. Methods for Morphological Examinations

The fact that in the course of the morphological examination of a drug substance the sample has to remain in the solid state, restricts the applicability of the most frequently used instrumental analytical methods [13,14]. Chromatographic measurements, UV and liquid phase NMR spectroscopy require dissolving, mass-spectrometry demands evaporating of the sample, consequently these methods are unusable in this area. The most important instrumental analytical techniques which are currently used for the examination of morphological purity are IR and Raman spectroscopy (Section 7.2), thermoanalytical methods (Section 7.3.) and X-ray diffraction (Section 7.4).

Two further methods are only briefly mentioned here. *Microscopy* is a very useful tool in the field of morphological research. It can be successfully used from the most common crystal habit examinations to the individual analysis of separate crystals on a heatable stage.

A rapidly developing instrumental technique is *CP-MAS (cross-polarisation magic-angle spinning) solid-state NMR* whose detailed description and examples of application in morphological research can be found in the literature [15–18]. This kind of use of CP-MAS NMR in the pharmaceutical industry appears, up until recently, to have been relatively limited, probably because it requires the channelling of NMR resources, usually already fully exploited for other purposes, into a specialised direction. Presumably the benefit/cost ratio of this technique will increase in the future, and it will become a more widely applied powerful tool in morphological investigations. A successful application of CP-MAS ^{13}C-NMR method in the examination of the morphological composition of cimetidine (a good model drug in this field) samples was reported by Middleton et al. [19].

References

1. S. Byrn, R. Pfeiffer, M. Ganey, C. Hoiberg and G. Poochikian, *Pharm. Res.* **12**, 945–954 (1995)
2. M. Kuhnert-Brandstätter, *Pure Appl. Chem.* **10**, 133–145 (1965)
3. L. Borka, *Pharmeuropa* **7**, 586–593 (1995)
4. L. Borka, *Pharmeuropa* **7**, 574–585 (1995)
5. A.J. Aguiar, J. Krc Jr., A.W. Kinkel and J.C. Samyn, *J. Pharm. Sci.* **56**, 847–853 (1967)
6. J.D. Mullins and T.J. Macek, *J. Pharm. Sci.* **49**, 245–252 (1960)
7. K. Láncos, Personal communication
8. A.M. Tudor, S.J. Church, P.J. Hendra, M.C. Davies and C.D. Melia, *Pharm. Res.* **10**, 1772–1776 (1993)

9. B. Hegedűs, P. Bod, K. Harsányi, I. Péter, A. Kálmán and L Párkányi, *J. Pharm. Biomed. Anal.* **7**, 563–569 (1989)
10. *Belgian Patent Specification* No. 893,610 (1981)
11. *British Patent Specification* No. 1,543,238 (1976)
12. *US Patent Specification* No. 4,537,900 (1981)
13. J. Haleblian and W. McCrone, *J. Pharm. Sci.* **58**, 911–929 (1969)
14. H.G. Brittain, *J. Pharm. Sci.* **86**, 405–412 (1997)
15. H.G. Brittain, K.R. Morris, D.E. Bugay, A.J. Thakur and A.T.M. Serajuddin, *J. Pharm. Biomed. Anal.* **11**, 1063–1069 (1993)
16. S.Y. Lin, *J. Pharm. Sci.* **81**, 572–576 (1992)
17. K. Raghavan, A. Dwivedi, G.C. Campbell Jr., E. Johnston, D. Levorse, J. McCauley and M. Hussain, *Pharm. Res.* **10**, 900–904 (1993)
18. P. Gao, *Pharm. Res.* **13**, 1095–1104 (1996)
19. D.A. Middleton, C.S. Le Duff, F. Berst and D.G. Reid, *J. Pharm. Sci.* **86**, 1400–1402 (1997)

7.2. Infrared and Raman Spectroscopy

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7.2.1. Introduction

The last decades gave rapid development in the sensitivity and efficiency of chromatographic procedures as well as in such highly sophisticated structure elucidating techniques as NMR and MS. This development toppled the previous monopoly of vibrational and UV-VIS spectroscopy in organic chemical research. However, vibrational spectroscopy is regaining prestige due to its specific utility in the fast-spreading morphological research of bulk drugs: it is probably the most straightforward method that provides the kind of spectral fingerprint of the molecule that is sensitive to morphological differences stemming from differences in hydrogen bonds or the rotational hindrance of a group. There are of course exceptions where vibrational spectra show no difference between modifications. For example the polymorphism of enalapril maleate [1] comes from the different packing parameters of conformationally identical molecules. In such cases the application of other instrumental techniques like X-ray diffraction or ^{13}C-NMR solid state spectroscopy are recommended.

Over the last few years FT-Raman spectroscopy has been rapidly gaining weight besides the long-established and universally employed FT-IR spectroscopy. Since FT-IR and FT-Raman spectroscopy are both vibrational methods, but utilise different selection rules, they are useful complementary tools as is reflected by their growing simultaneous application in morphological research [2–4].

7.2.2. FT-IR Measurements

7.2.2.1. Standard FT-IR Spectroscopy

The principles and manual methods of the preparation of infrared spectra with proper spectral resolution and intensities using both the KBr and nujol technique are described in the European Pharmacopoeia 1997 [5]. Such spectra are needed for the examination of the morphological purity of bulk drugs. In

this section we only touch upon those aspects of FT-IR where the application of the standard methodology can give misleading or false results and therefore a specialised sample preparation procedure must be employed.

(a) The sample is *hygroscopic.* Bulk drug substances are often salts with a hygroscopic character. These materials are rather unfavourable in respect of controlling their morphological purity by infrared spectroscopy. First, the salt character of the sample generally broadens the infrared absorption bands. Secondly, the broad diffuse absorption band of protonated nitrogens covers the infrared spectrum in the region of 3000–2000 cm^{-1}. In this case the pellet must be prepared under flushing dry nitrogen and measurement must commence as soon as possible. The application of nujol mull can be another means to obtain IR spectra of such materials, but in such cases the strong absorption bands of the suspending nujol will cover other parts of the spectrum in the 3000–2800 cm^{-1} and 1500–1300 cm^{-1} regions.

(b) The morphological composition of the sample *changes under mechanical treatment.* The recrystallisation of metastable modifications into more stable forms provoked by mechanical influences is well known among bulk drug substances. For example, *cefixamine trihydrate* turns into a mixture of crystalline and amorphous forms on grinding [6]. Similarly, *cyclophosphamide monohydrate* looses its crystal water and changes its morphological composition on such mechanical treatments as grinding and tabletting [7].

To take an example from our own experience, modification C of *mebendazole* is a widely used drug in the veterinary practice. In the presence of traces of modification A, modification C tends to recrystallise into the more stable, but inefficient modification A. The latter reaches a well detectable level over a 1–2-min stirring period which is normally needed for the preparation of a homogeneous KBr pellet. If the usual amount of sample is increased 4–5-fold and the homogenising procedure is reduced to a slight agitation with the tip of a spatula, any undesired changing of the sample can be avoided.

The above examples involve interconversions between existing modifications, but it is important to note that mechanical treatment may also cause pseudo-polymorphism in materials which have secondary structures in the solid state, like proteins, hyaluronates, gelatines etc. [8]. As the destruction of the secondary structure is a random phenomenon, the same sample can give different spectra as a function of stirring time and local pressure values. These problems suggest the application of other methods instead of the usual infrared method.

(c) The morphological features of the sample *change without mechanical influences*. Unstable samples may change morphologically with some statistical probability which depends on the light and heat influences reaching the sample [9]. Thus an undesired transition of the sample is more likely to occur as the time between sample preparation and measurement increases. Such a

procedure was detected and followed by the combined use of IR and DSC in the case of six interconverting *mexiletine hydrochloride* modifications [10]. The time-dependent spontaneous recrystallisation of amorphous *indomethacin* was reported by Byrn *et al*. [6]. This uncertainty is one of the reasons behind the fast spread of in situ techniques (see NIR) which do not require any sample preparation.

A rare phenomenon we had the fortune to encounter in our practice involves morphological change induced by the infrared radiation of the FT-IR instrument [11]. A KBr pellet containing a relatively loose crystal-hydrate modification of *cimetidine* was left in the infrared beam of the instrument for some hours, while its spectrum was repeatedly recorded. As a result of this prolonged irradiation the monohydrate modification of cimetidine turned quantitatively into form A.

7.2.2.2. Other Techniques

The rapid and diverse developments in instrumental analytical methods have also affected infrared spectroscopy, particularly in the spread of different reflection and combined methods.

The basic principle of the *ATR (attenuated total reflectance)* technique, first applied by Fahrenfort in 1961 [12] relies on the phenomenon that in the course of total reflection the reflecting beam penetrates to some μm depth into the material touching the outer surface of the reflecting plate. This coaction of the infrared radiation with the outer material results in the attenuation of light. In practice the measurement can be performed with the help of a special accessory, consisting of a quite large ZnSe (or other special material) crystal with a perfectly planparallel flat measuring surface and an inlet and an outlet plate of 45°. As the above depicted coaction attenuates the measuring beam to an extent that depends on the length of the coated measuring crystal, the light leaving the crystal shows a wavelength-intensity profile very similar (but not identical) to the usual infrared spectrum in the analytical region (4000–400 cm^{-1}). The above-noted problem with the recrystallisation of metastable formulations during sample preparation can be easily circumvented in this way, as this examination requires no other sample preparation than the setting of the sample on the measuring crystal. The ATR method is a powerful tool for the examination of bulks, liquids and ointments. The examination of the morphological composition of *ganciclovir* bulk samples with the help of the ATR technique was reported by Salari *et al*. [13]. In a paper of Markovich *et al*. [9] the examination of the solid state amorphous to crystalline transition of an azetidinone derivative drug substance was described. The ATR method proved to be well applicable in monitoring the slow crystallisation of the active agent.

The *DRFTIS (diffuse reflectance Fourier transform infrared spectroscopy)* technique is also applicable in the analytical infrared spectrum region. This method was first described by Fuller and Griffith in 1978 [14]. Its essence also lies in the attenuation of the infrared beam reflected from the sample. The DRFTIS method is also an easily performed technique, as the sample preparation only requires pouring the sample, or its powdered mixture with KBr, into the sample holder and flattening its surface. This flat surface of the sample has to be irradiated by the beam. The reflected radiation, containing the spectral information, is collected by a special optical arrangement and is worked up with the help of a standard software. This method can be a useful tool in the examination of the morphological composition of a drug substance, as demonstrated by Hartauer *et al*. [15] in the case of *sulphamethoxazole*. These authors report less than 4% relative differences between the measured and the theoretical values of the morphological composition of the samples.

Its ease and rapidity makes the *NIR (near infrared reflection)* technique [16] an indispensable tool in the fast identification, and morphological purity control of bulk drugs. This method covers the 10 000–4000/cm region of the spectrum, which contains mainly overtones and combination bands. Nevertheless, owing to the computerised handling of the whole spectrum, NIR is a rather sensitive and reliable method. In the last few years combined measuring heads which are equipped with flexible fibre optics gave a further momentum to this technique. Norris *et al*. [17] report the application of a NIR equipment with an on-line fibre optic probe in the examination of the polymorphic conversion of *trovafloxacin mesylate* modifications in crystal slurry in organic solvents.

The latest *infrared microscopes*, being the most multifunctional and most sensitive tools in morphological research, should also be mentioned [18]. They have a heatable stage and are equipped with a multichannel detector unit which facilitates the examination of the full spectra of separate spots of 20×20 μm as the function of temperature and time both in transmission and in reflection. The spread of these instruments in quality control laboratories is expected.

7.2.3. FT-Raman Measurements

The principles of Raman spectroscopy [19], named after its Nobel awarded discoverer, Sir Chandrasekhara Venkata Raman, were first published in 1928. This technique which measures vibrational spectral transitions of the atoms and atomic groups of the molecules in the 4000–100 cm^{-1} spectral region, is an important complementary method to infrared spectroscopy. The widespread use of Raman spectroscopy had for a long time been hindered by various technical obstacles: at the beginning by the lack of sufficient light

sources, later, when visible light lasers were used, frequently by strong fluorescence. It was the development of a new type of laser (see below), and its application in the well known Michaelson infrared interferometers that gave a real breakthrough in the utility of Raman spectroscopy after 1986.

The Nd:YAG (an yttrium aluminium garnet crystal doped with triply-ionised neodymium) laser produces a near infrared light beam of 9395 cm^{-1} (1064 nm wavelength), and its output is up to 1000 mW power or more. The greatest advantage of this laser comes from its low photon energy, which makes it practically inactive in the field of fluorescence. As the latest instruments are equipped with efficient optical filters cutting off the 10^5–10^8 times more intense Rayleigh component from the reflected light, all the technical obstacles were removed, and an efficient method was established to perform measurements based on the well known Fourier transform method. On a modern FT-Raman equipment (very often combined with an FT-IR unit) Raman spectra can be recorded with arbitrary spectral resolution and a large number of scans.

Details of the technical realisation and the measurement procedures are discussed in excellent handbooks [19,20]. Although the FT-Raman method has not yet been introduced in the field of quality control of bulk drugs by the pharmacopoeias, in the following some important practical aspects of this method will shortly be noted.

Some advantageous features of Raman spectroscopy are as follows.

- The greatest advantage of Raman spectroscopy lies in its high selectivity in respect of the polarity or polarisability of the given groups. This means that highly polarisable groups, including symmetrical vibrations which are inactive in the infrared spectra, are very good Raman scatterers, while the Raman spectrum is nearly blind in respect of highly polarised groups, like a loose O–H or a N^+–H group, which are very intense in the IR spectra, sometimes covering the 3700–2000 cm^{-1} region. This characteristic gives a fairly flat baseline in the Raman spectrum, consequently baseline correction – in contrast to infrared spectra – is normally unnecessary.
- Raman bandwidths are smaller than the corresponding infrared band widths. This results in reduced overlaps and more well-separated symmetrical bands, which can be used in the evaluation of the spectra. As a characteristic example the FT-Raman spectra of five polymorphic modifications and a monohydrate of *cimetidine* are presented in Fig. 7.2.A.
- A further advantage of FT-Raman spectroscopy is its fast and easy use; sample preparation only requires the filling and positioning of the measuring sample holder. After examination the sample can be regained for further use.

Some of the problematic features of Raman spectra are as follows.

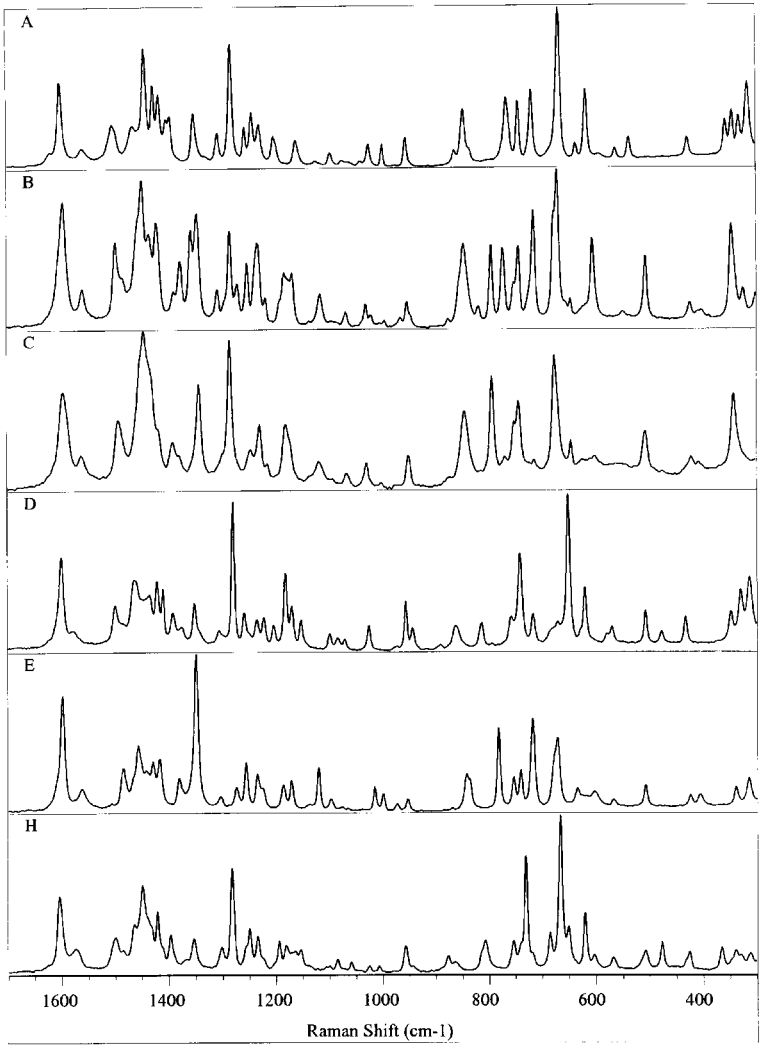

Figure 7.2.A.
1700–300 cm^{-1} region of the FT-Raman spectra of cimetidine modifications A–E [11] and a hydrate H

- Too strong an irradiating laser beam can easily burn the sample. To avoid this, one has to position the sample to a lower beam power (approx. 100 mW). The setting of the measuring power requires special care, and has to be done step-by-step. During this procedure the analyst should watch the

baseline at about 3500 cm^{-1}. A lift of the baseline in this region shows that the irradiating beam is too strong, and overheating of the sample has started and a lower power should be used.

- Between a few very strong bands there are really small peaks in the Raman spectrum. This is only a virtual problem, as the signal to noise ratio, i.e. the baseline reproducibility of the spectrum, can be improved by increasing the number of scans. The above depicted small peaks emerge reproducibly from the baseline and in a properly chosen spectrum window they can be also be analysed.
- The intensity of the scattered light is very small, approximately 10^{-6} fold of the irradiating beam, which can be easily exceeded by the slightest fluorescence that overloads the amplifier of the instrument. A sample contaminated with fluorescent impurities cannot be analysed by Raman spectroscopy; while in the course of a structure elucidating Raman examination one can get rid of the fluorescent contaminant by performing a recrystallisation with clarification, in a morphological examination this is impossible since dissolution of the material destroys the morphological composition.

7.2.4. Quantitative Determination of Morphological Impurities

Concerning the quantitative examinations of morphological impurities in bulk drug substances the following should be noted. In the trade of pharmaceutical bulk materials the requirements regarding the level of morphological impurities are usually not specified. In the majority of cases no well-defined limits and procedures are reported in the literature either. In some cases 10%, in other cases only 1–2% morphological impurity is tolerated. In general the tolerated morphological cross-contamination is about 3–5%. The examples given below are taken mostly from the author's experience.

7.2.4.1. Measurements Based on Well-Resolved Bands

Obviously, the determination of the morphological composition of a mixture from their FT-IR or FT-Raman spectra rests on the availability of those of the pure modifications. In the simplest cases one or more perfectly resolved and properly intense bands can be found in the spectrum of the contaminant modification which do not overlap with any bands of the main component. Whether such diagnostically useful bands can be found depends on some degree of luck. If a diagnostic band exists, a calibration can be carried out with model mixtures using one well resolved band of the main component as an internal standard for the examination.

For example, modification A of *famotidine* may contain modification B as an impurity. As seen in Fig. 7.2.B, modification B has a sharp and intense IR band at 3506 cm^{-1} while the most intense band of modification A can be found at 3452 cm^{-1} [21]. This band of form A can be used as an internal standard for quantitative examinations. In a spectrum where the noise of the baseline was eliminated by accumulating a properly large number of scans (about 50 scans on the given equipment), 0.5% of modification B can be reliably detected in form A [22].

Cimetidine has six well-reproducible [11] modifications amongst which form A is used in the therapy. Fig. 7.2.A shows the region between 1700 and 300 cm^{-1} of the Raman spectra of these modifications. The morphological purity of modification A can be checked near 510 cm^{-1}, where modification A has no scattering, while all other possible forms exhibit one or two bands. With the help of model mixtures, 5% of modification B proved to be well detectable in this way.

7.2.4.2. Measurements Using Spectrum Manipulations

In everyday practice one can hardly find adequately separated and intense spectral bands in the spectra of the modifications. The strong bands usually overlap with smaller ones whereby morphological impurity usually manifests itself only in the form of slight deformations in the IR or Raman bands of the main component. Such spectra can be evaluated either through spectral subtraction or via derivatisaton.

In the case of spectral subtraction the IR spectra of the sample as well as that of the morphologically homogeneous main component, usually presented in transmittance, must be transformed into absorbance, then stored in the proper files of the memory of the computer. Ideally, spectral subtraction results in the perfect elimination of the bands due to the main component, giving a residual low-intensity curve which must be examined whether it contains diagnostic bands due to the contaminating modification. Morphological impurity can be declared only on the positive identification of the most intense 3–4 bands of the contaminating modification in the residual spectrum. It is worth noting that the infrared spectra of solid samples can be slightly influenced both by minor chemical contaminants and by the mechanical circumstances of the preparation of the pellet. This slight deviancy of the spectra sometimes results in sharp and relatively intense peaks in the difference spectra after spectral subtraction. Consideration of these peaks instead of the known main bands of the possible contaminant modification can misguide the spectroscopist.

Scanning the second derivative of the infrared spectrum of the morphologically contaminated sample can also be useful: in the second

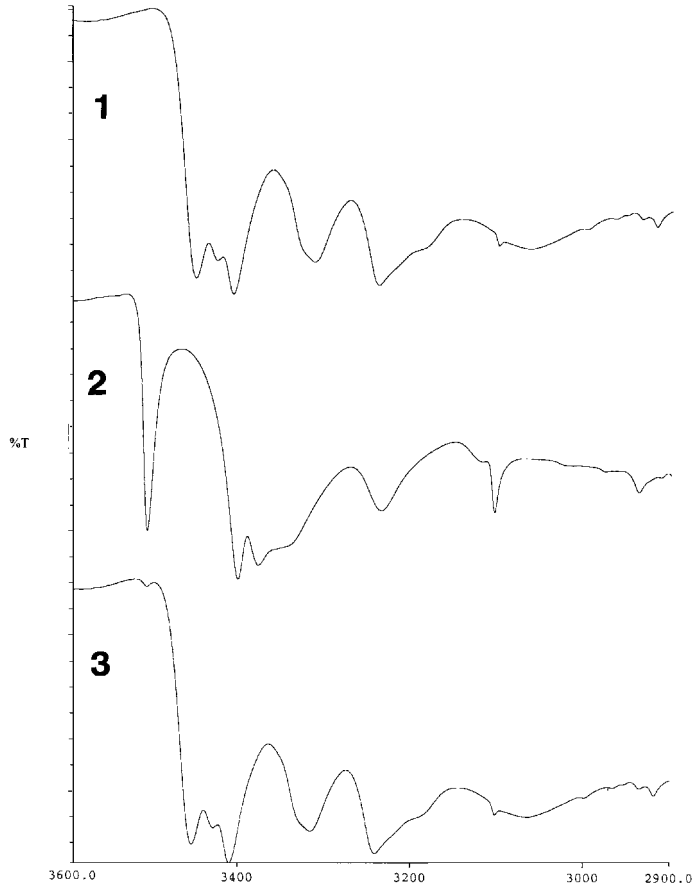

Figure 7.2.B.
Infrared spectra of famotidine. Curve 1: famotidine A; curve 2: famotidine B; curve 3: famotidine A spiked with 1% of form B

derivative positive peaks detect both the bands of the main component and that of the contaminant modification. Identification of the sharpest and most significant bands of the contaminant can verify the presence of the undesired form. An example is given in Fig. 7.2.C.

The quantitation of morphological impurities can be performed by comparing the manipulated spectra with that of identically processed model mixtures.

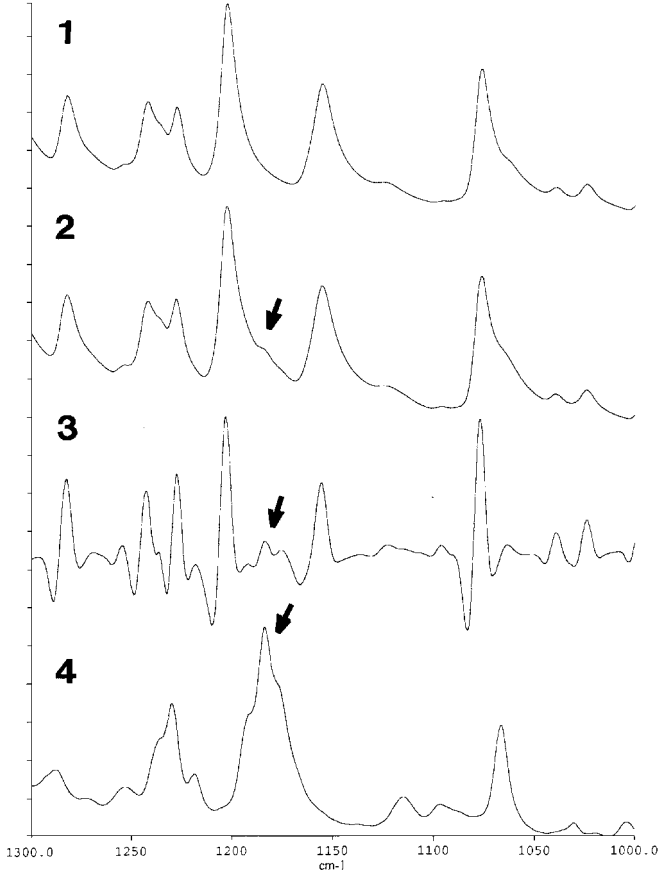

Figure 7.2.C.
Infrared spectra of cimetidine A (**1**), cimetidine A spiked with 5% B modification (**2**), cimetidine B (**4**) and the 2nd derivative spectrum of **2** (**3**). Maxima and shoulders indicating the presence of modification B are marked with arrows

7.2.4.3. Evaluation of Complete Spectrum Curves with Mathematical Methods

There are cases where the modifications exhibit decidedly different spectra, but these spectra do not contain sufficiently strong and well-resolved bands. The examination of morphological purity can then be carried out using curve-fitting procedures. Just as in NIR spectroscopy, the same suitable spectral region of the pure modifications and the mixture is selected and stored in

memory. There are software programs which can compose the mixture spectrum of the sample from the standard spectra of the modifications through least squares curve fitting methods. This procedure gives the relative weights of the composing spectra in the sample of unknown composition. As it is reported by Jalsovszky *et al*. [23] the calculated composition correlates well with the composition of the model mixture: by applying the partial least squares method, even 2% morphological cross-contamination can be determined with less than 10% relative error.

References

1. J. Anwar, S.E. Tarling and P. Barnes, *J. Pharm. Sci.* **78**, 337–342 (1989)
2. C.M. Deely, R.A. Spragg and T.L. Threlfall, *Spectrochim. Acta* **47/A**, 1217–1223 (1991)
3. W.P. Findlay and D.E. Bugay, *J. Pharm. Biomed. Anal.* **16**, 921–930 (1998)
4. A.M. Tudor, M.C. Davies, C.D. Melia, D.C. Lee, R.C. Mitchell, P.J. Hendra and S.J. Church, *Spectrochim. Acta* **47/A**, 1389–1393 (1991)
5. *European Pharmacopoeia 1997*, Council of Europe, Strasbourg (1997)
6. S. Byrn, R. Pfeiffer, M. Ganey, C. Hoiberg and G. Poochikian, *Pharm. Res.* **12**, 945–954 (1995)
7. J. Ketakolainen, A. Poso, V. Viitasaari, J. Gynther, J. Pirttimäki, E. Laine and P. Paronen, *Pharm. Res.* **12**, 299–304 (1995)
8. Unpublished results
9. R.J. Markovich, C.A. Evans, C.B. Coscolluela, S.A. Zibas and J. Rosen, *J. Pharm. Biomed. Anal.* **16**, 661–673 (1997)
10. A. Kiss and J. Répási, *Analyst* **118**, 661–664 (1993)
11. B. Hegedűs and S. Görög, *J. Pharm. Biomed. Anal.* **3**, 303–313 (1985)
12. J. Fahrenfort, *Spectrochim. Acta* **17**, 698–709 (1961)
13. A. Salari and R.E. Young, *Int. J. Pharm.* **163**, 157–166 (1998)
14. M.P. Fuller and P.R. Griffith, *Anal. Chem.* **50**, 1906–1910 (1987)
15. K.J. Hartauer, E.S. Miller and J.K. Guillory, *Int. J. Pharm*. **85**, 163–174 (1992)
16. A.M.C. Davies and P. Williams, *Near Infrared Spectroscopy: The Future Waves*, NIR Publications (1996)
17. T. Norris, P.K. Aldridge and S.S. Sekulic, *Analyst* **122**, 549–552 (1997)
18. P. Ghetti, A. Ghidini and R. Stradi, *Boll. Chim. Farm.* **133**, 689–697 (1994)
19. J.R. Ferraro and K. Nakamoto, *Introductory Raman Spectroscopy*, Academic Press (1994)

20. D.B. Chase and J.F. Rabolt, *Fourier Transform Raman Spectroscopy*, Academic Press (1994)
21. B. Hegedűs, P. Bod, K. Harsányi, I. Péter, A. Kálmán and L. Párkányi, *J. Pharm. Biomed. Anal.* **7**, 563–569 (1989)
22. Under publication
23. G. Jalsovszky, O. Egyed, S. Holly and B. Hegedűs, *Appl. Spectrosc.* **49**, 1142–1145 (1995)

7.3. Thermoanalytical Methods

Imre Péter

7.3.1. General Considerations

Though thermoanalytical methods and first of all *differential scanning calorimetry* (DSC) [1] are routinely applied methods for the identification and characterisation of polymorphic modifications of bulk drugs, the quantitative determination of certain modifications as impurities encounters with numerous difficulties. The main reason for this is that in contrast to IR, Raman and solid phase NMR spectroscopy as well as X-ray powder diffractometry (see Sections 7.1, 7.2 and 7.4), during the calorimetric examinations the sample does not remain unchanged. Under the influence of heat it undergoes phase transition(s) (melting or crystallisation) or even thermal decomposition may take place. Therefore, the modification ratio can also alter during heating. These transitions can be influenced by numerous kinetic factors, hardly predictable during the analyses. Just for this reason the quantitative determination of the modifications in their mixtures by DSC runs into difficulties. This is the explanation why only a few applications are referred in the literature. However just the aforementioned phase transitions render possible the application of DSC under strictly controlled conditions for the quantitative determination of the modifications in their mixtures: the change in enthalpy – accompanying both the solid–solid (recrystallisation) and the solid–liquid (melting) phase transitions – is proportional to the transforming mass.

Thermodynamic relationships among the modifications (A and B) are represented by the free-energy/temperature diagram calculated on the basis of the Gibbs–Helmholtz equation (Fig. 7.3.A). In case of *enantiotropic* systems, in the temperature range of solid phases, at T_0 equilibrium temperature (where the free-energy difference, $\Delta G = G_A - G_B = 0$), the transition is thermodynamically reversible. In the temperature range where $\Delta G < 0$, modification A, whereas at $\Delta G > 0$, modification B is more stable. Under and above the equilibrium temperature the transitions are irreversible. In *monotropic* systems at temperatures lower than the melting points, ΔG is always <0, i.e. modification A is more stable in the thermodynamic sense. In the temperature range of solid phases there is no equilibrium temperature. The transition towards the thermodynamically more stable modification is irreversible.

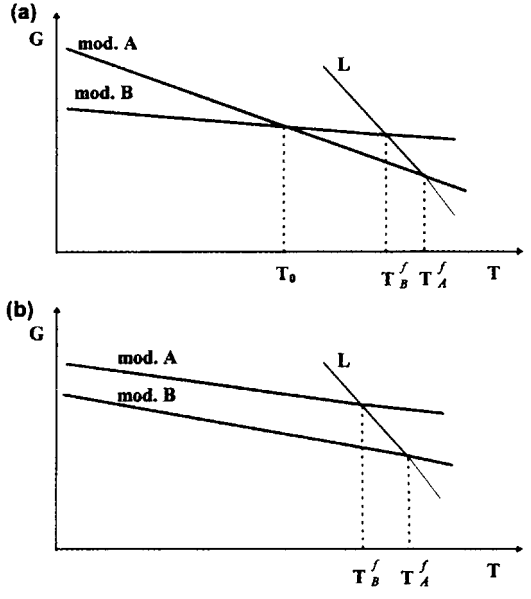

Figure 7.3.A.
Gibbs free energy/temperature diagrams in enantiotropic (a) and monotropic (b) systems in case of dimorphs (modification A and B). T_B^f is the melting temperature of the lower (B) and T_A^f is that of the higher (A) melting form; T_0 is the transition temperature; L is the liquid phase

The thermodynamic stability and the transition temperature can also be studied on the basis of solubility [2] or melting data [3] of the modifications. The Burger's heat of transition (HTR) or heat of fusion rule (HFR) [4] can also offer feasibility for the relatively reliable estimation of thermodynamic stability of the modifications. At the application of thermal methods knowledge of the above relationships is indispensable for the quantitative determination of the modifications. Fig. 7.3.B shows the DSC curves that can be obtained in case of enantiotropy (a) and monotropy (b) [5,6].

In enantiotropic systems (a):

- Lower melting form (B) transforms into higher melting form (A) at or above T_0 in endothermic solid–solid transition and than form A melts at T_A^f;
- Form B melts at T_B^f;
- Form B melts at T_B^f and form A crystallises from the melt and than it melts at T_A^f;

- Below T_0 the thermodynamically unstable form A transforms into form B in exothermic solid–solid transition and then it behaves as above;
- Form A melts at T_A^f.

In monotropic systems (b):

- Higher melting form (A) melts at T_A^f;
- Lower melting form (B) transforms into form A in exothermic solid–solid transition and then form A melts at T_A^f;

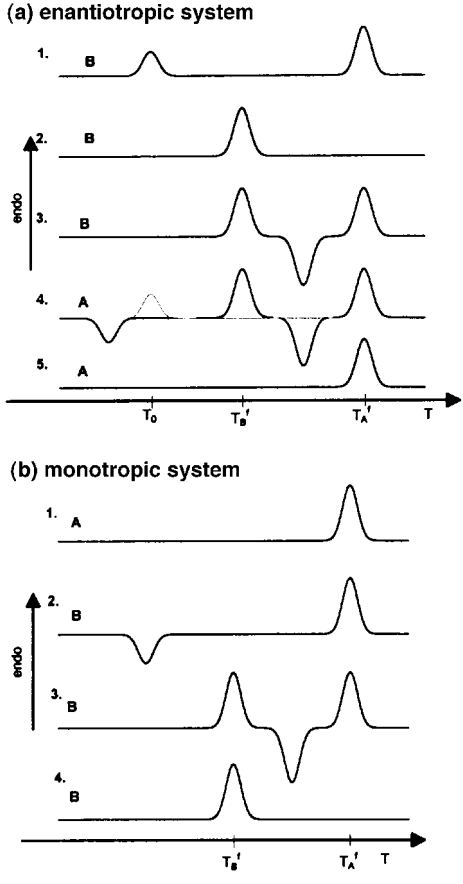

Figure 7.3.B.
DSC curves in enantiotropic (a) and monotropic (b) systems. See text for the explanation of the curves

- Form B melts and form A crystallises from the melt and then melts at T_A^f;
- Form B melts at T_B^f.

DSC records of modifications of chemical compounds show very different patterns. It must not be neglected that the conversion processes are not only under the influence of thermodynamic, but very often under unpredictable kinetic effects, too. This is also influenced on the one hand by the numerous characteristics of the sample (seeds, chemical impurities, solvent residue, defect in the crystal lattice, particle size, etc.), on the other hand by the operating conditions of the DSC measurement (heating rate, sample mass, sample packing, previous handling, etc.). The presence of thermodynamically more stable modification at a given temperature, even in traces, can promote the transition process both in solid or liquid phase.

In case of modification mixture, the main point of the quantitative determination is that the enthalpy change accompanying the phase transitions is proportional to the transforming mass. In addition to the sensitivity of the DSC apparatus [7] the limits of detection and determination depend on the enthalpy of transitions (ΔH), sample mass and heating rate. It often occurs that melting points of the modifications are too near to be distinguished from one another with the help of thermal methods. An example for this is the case of modifications A and D of *cimetidine* [8]. If the processes shown in Fig. 7.3.B are overlapping, the magnitude of enthalpy changes accompanying the individual processes can be evaluated with difficulty or not at all. The resolution can be improved by decreasing the heating rate, the mass of sample or applying a gas with a great thermal conductivity (e.g. helium). On the contrary, using a fast heating rate (25–100°C/min), some phase transitions (e.g. solid–solid transitions or crystallisations from the melt) can as well be bypassed [5]. In case of modification mixture containing more than two polymorphs, the complex stability relations can cause further difficulties in the analysis.

The DSC curves displayed in Fig. 7.3.B offer numerous possibilities for the detection or determination of the individual polymorph components, in case of both enantiotropic and monotropic systems. The suitability of the processes can change from compound to compound, and it is determined by the thermal behaviour of the modifications. In consequence, a generally effective solution cannot be given. Under properly chosen measuring conditions, both the solid–solid transitions and the melting processes can be suitable for the analysis. Due to the facts mentioned before, application of DSC can be recommendable only in well defined polymorphic systems and under well established measuring conditions. Attention should be given that during the processes used for the preparation of modification mixtures the ratio of the modification should not change.

7.3.2. Estimation of Lower Melting Form (B) in Higher Melting Form (A) on the Basis of Solid–Solid Transformation

(a) In case of enantiotropy the endothermic solid-solid transition of lower melting form can be suitable for quantitative determination (Fig. 7.3.Ba, example 1). The heat of transition is proportional to the amount of the lower melting form in the mixture. Due to the aforementioned kinetic reasons the determination is rendered more difficult by the fact that the size of the measured minor endothermic peak can change from lot to lot. For this reason the determination with DSC of lower melting form *I* of *Losartan-K* is not recommendable in the higher melting form *II,* because the measured endothermic heat of transition might as well change between 0 and 4.4 kJ/mol. On the contrary the transition heat is about 7.3 kJ/mol measured with solution calorimetry [9]. The measured transition heat can similarly change in case of modification *II* of *finasteride* [10], too. But in case of well defined systems and under measuring conditions the quantitative analysis is possible. A relevant case is the determination of lower melting form of an *amino-benzoic acid ester* derivative on the basis of its endothermic solid–solid transition, beside the higher melting point modification. The regression coefficient of the calibration curve recorded with the modification mixture of known composition in the range of 10–90% is 0.9954. The enthalpy of transition (ΔH) is 15.6 J/g ($n = 10$, $s = 6\%$) [5]. Of the very few examples found in the literature, the determination of e.g. *sulphathiazole* form *I* modification can be mentioned, which is based on the solid–solid endothermic transition. The applied heating rate is 40°C/min. [11]. As compared to X-ray powder diffraction, the thermal method is more advantageous in case of polymorphic modifications of *stearic acid*, also [12].

(b) In monotropic systems the exothermic solid–solid transition of the lower melting form can be suitable for the quantitative determination (Fig. 7.3.B, example 2). The mentioned factors influencing the transition cannot be neglected in this case either.

7.3.3. Estimation of Lower Melting Form (B) and Higher Melting Form (A) on the Basis of Their Fusions

(a) Very often the crystallisation from the melt of the lower melting form (B) starts already during the heating (Fig. 7.3.Ba,b, example 3). If the endothermic melting process is overlapping with the exothermic crystallisation, the enthalpy change pertaining to the individual processes can be measured hardly or not at all. However, applying a very fast heating rate (25–100°C/min) this transition might as well not occur [5]. In this way,

knowing the melting enthalpy of the pure modification, ratio of the low melting form (B) can be calculated, both in enantiotropic and in monotropic systems.

(b) In the former case (Fig. 7.3.Ba,b, example 3) the measured melting enthalpy of the higher melting form (A) is always greater than it would follow from the composition of the modification mixture. A part of modification A is an artefact originated during the heating. The calibration curve is not linear and depends on several factors (e.g. heating rate, particle size, sample packing, etc.). In the DSC curve, among the endothermic melting curves, an exothermic peak can be frequently observed that is characteristic of the transition. But just because of crystallisation of the artefact, the melting heat of the higher melting form greater than the real one, can render possible the detection of modification A present even in very small quantities. Fig. 7.3.C shows the DSC recordings of *famotidine B* spiked with 0.2, 0.5 and 1% of the higher melting modification *A*. Under the applied measuring conditions the detection limit is about 0.5%. Increasing the heating rate and the sample mass, the detection limit decreases to about 0.2%, but the resolution significantly deteriorates. The transition is monotropic. In the absence of modification *A* the crystallisation is not accomplished from the melt (Fig. 7.3.Bb, example 4) [13].

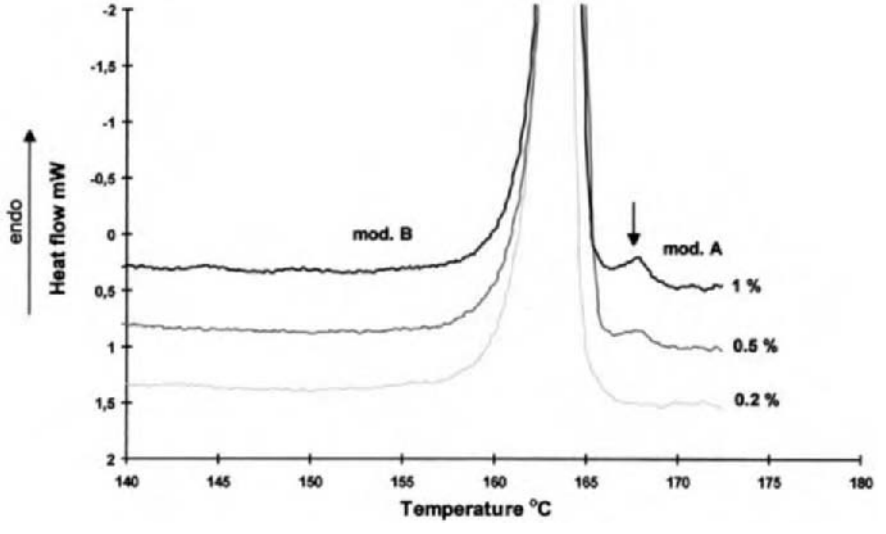

Figure 7.3.C.
DSC scans of famotidine containing 0.2, 0.5 and 1% modification A in modification B (heating rate 5°C/min, sample mass 2 mg)

Applying a mechanical effect (e.g. pressure or grinding in a mortal) the thermodynamically more stable modification can be enriched even if the latter is present at the otherwise not detectable trace level only, thus the detection limit can be decreased [10].

(c) If crystallisation from the melt of the low melting form (B) proceeds slowly or at higher temperature only, or the applied heating rate is relatively high, then its amount can also be estimated on the basis of its melting (Fig. 7.3.Ba,b, example 3). Fig. 7.3.D shows the

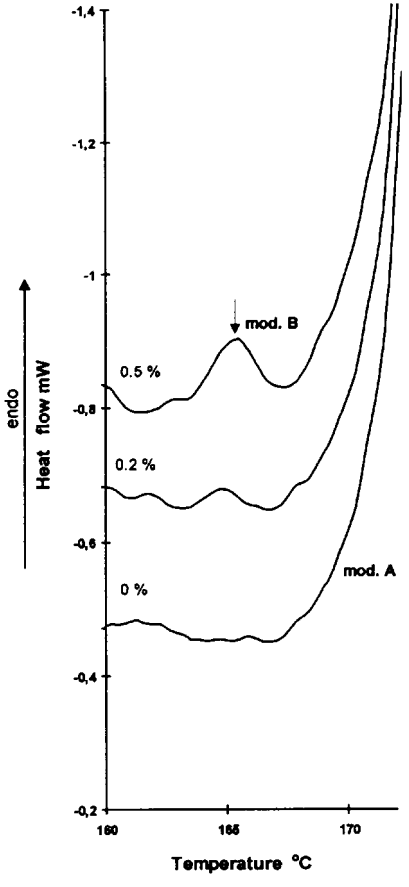

Figure 7.3.D.
DSC scans of famotidine containing 0, 0.2 and 0.5% modification B in modification A (heating rate 20°C/min, sample mass 5 mg)

DSC recordings of modification mixture containing the lower melting point *famotidine B* modification in amounts of 0.2 and 0.5%. Detection limit of modification B is about 0.5%. The recrystallisation process does not disturb the analysis.

(d) If there is a very great difference in the melting enthalpy of the modifications, it can happen that the individual components melt independently from one another without any transition in either the solid or in the melted phase (Fig. 8.3.Ba) examples 2 and 5 and (Fig. 8.3.Bb) examples 1 and 4). This time the melting enthalpy of the modifications is proportional to the amounts of the individual components in the mixture [14].

Hydrates and solvates which are often called pseudo-polymorphic modifications represent a totally different matter. For the determination of bound solvents the most frequently used method is thermogravimetry (see Section 3.1). The loss of mass indicates the presence of pseudo-polymorphs in the sample. Since the dehydration and desolvation are also accompanied by endothermic enthalpy changes, these processes can also be studied with DSC. For example, the dehydration of *α-lactose monohydrate* is characterised by a sharp and intense endothermic peak [15], rendering a possibility for its detection and determination in the *anhydrous* form.

References

1. G. Höhne, W. Hemminger and H.-J. Flammersheim, *Differential Scanning Calorimetry*, Springer, Berlin (1996)
2. D.J.W. Grant and H.G. Brittain, in *Physical Characterisation of Pharmaceutical Solids* (H.G. Brittain, Ed.), pp 321–386, Marcel Dekker, New York (1995)
3. L. Yu, *J. Pharm. Sci.* **84**, 966–974 (1995)
4. A. Burger and R. Ramberger, *Mikrochim. Acta (Wien)* **II**, 259–271, 273–316 (1979)
5. D. Giron, *Thermochim. Acta* **248**, 1–59 (1995)
6. B. Perrenot and G. Widmann, *Thermochim. Acta* **234**, 31–39 (1994)
7. S. Wies, A. Geyer and W. Eysel, *J. Therm. Anal.* **38**, 277–287 (1992)
8. A. Bauer-Brandl, *Int. J. Pharm.* **140**, 195–206 (1996)
9. L.-S. Wu, C. Gerard and M.A. Hussain, *Pharm. Res.* **10**, 1793–1795 (1993)
10. I. Péter, Unpublished data
11. E.G. Shami, P.D. Bernadetto, E.S. Rattie and L.J. Ravin, *J. Pharm. Sci.* **61**, 1318–1320 (1972)
12. N. Garti, S. Sarig and E. Wellner, *Thermochim. Acta* **37**, 131–135 (1980)

13. B. Hegedűs, P. Bod, K. Harsányi, I. Péter, A. Kálmán and L. Párkányi, *J. Pharm. Biomed. Anal.* **7**, 563–569 (1989)
14. D. Giron, *J. Pharm. Biomed. Anal.* **4**, 755–770 (1986)
15. H.G. Brittain, S.J. Bogdanowich, D.E. Bugay, J. DeVincentis, G. Lewen and A.W. Newman, *Pharm. Res.* **8**, 963–973 (1991)

7.4. X-Ray Crystallography

Márta Bálint

7.4.1. The Role of X-Ray Diffraction Methods in Morphological Studies

X-ray diffractometry is a generally applied method in the pharmaceutical industry to investigate the polymorphism of drug substances, as well as to monitor the occasional changes of the applied modification during different steps of the formulation procedure.

Although single-crystal X-ray diffractometers provide the most precise information on crystal structure of drug substances: unit cell data, molecular packing, hydrogen-bonds, conformation and absolute configuration, in the majority of cases the X-ray powder diffraction technique is applied for the identification and quantification of polymorphic phases because it does not need single crystals and it is a considerably less expensive method. The main application field of single-crystal X-ray crystallography is the determination of molecular structures. However, if a single-crystal diffractometer is available, the powder diffraction patterns calculated from the single-crystal data can be used to prove whether the patterns obtained with X-ray powder diffractometry were made of a pure phase or not and whether they were falsified by preferred orientation, i.e. the intensity is smaller or greater than the calculated value.

When a drug substance molecule has two or more polymorphic modifications, then one of them is usually preferred on the basis of their physical, pharmacological and formulation properties. In these cases the other modifications can be considered as impurities of the substance.

Three important fields where the investigation of the morphological purity by X-ray powder diffractometry is necessary are as follows.

(a) The final step of the production of the active substance is usually *crystallisation*. X-ray powder diffraction has crucial importance in cases when thermal and spectroscopic methods cannot make a sufficiently clear distinction among the polymorphs obtained during the crystallisation step.

For example, Doff *et al*. [1] were able to detect the α*-inosine* impurity with a detection limit of 0.4% in the β-polymorph, while the DSC was unsuitable for this task due to the nearness of the melting points. Two modifications of *propyphenazone* cannot be differentiated with IR, at the same time 5% of B modification can be determined with X-ray powder diffraction with a satisfactory precision in the manufactured batches [2].

In advantageous cases the chiral purity test can also be performed with the X-ray powder diffraction method. Estimation of racemic *ibuprofen* in the clinically more advantageous (*S*)-ibuprofen can be performed with a limit of detection at 1% and a limit of quantification at 2% [3], that implies an alternative to that of methods described in chapter 6.

In the author's laboratory the possibility of identification and quantification of *cimetidine* B polymorph in the A form was investigated. As Fig. 7.4.A shows, the B-modification can be detected above 5% making use of the peak at 21.045°2Θ [4].

(b) During stability testing of the active substances a certain modification can undergo phase transition under the effect of heat and humidity. The presence of the unwanted modification in trace amounts can promote the unfavourable transition [2]. In other cases, the metastable modifications can transform to stable ones during the storage. This is exemplified in Fig. 7.4.B by transitions of α- and β-modifications of *phenylbutazone* to the stable δ-polymorph, during storage [5].

Although the anhydrate–hydrate pairs are not true polymorphs, the frequently occurring task of monitoring the transition of anhydrate-to-hydrate merits also mentioning here. For example, the *fluconazole* anhydrate when stored at high relative humidity, is inclined to bond the water molecules into its crystal-lattice. The anhydrate-to-hydrate transition can be monitored with X-ray diffraction, due to their different crystal structures [4].

(c) In various steps of formulation of solid dosage forms phase transition can occur under the effects of technological processes (milling, exposure to pressure and heat etc.). Monitoring of these transitions is often difficult because the active substance is present in very small amounts in the tablet matrix and the other applied crystalline excipients can overlap the diffraction peaks of the active substance. Change of modification of *chloramphenicol* palmitate [6] or the occurrence of the amorphous phase in the crystalline *digoxin* [7] during the milling process are good examples for these types of transitions.

7.4.2. Determination of Polymorphic Impurities with X-Ray Diffraction

There are two main criteria for the successful determination:

(a) The X-ray diffraction pattern of the impurity component should include peak(s) that is (are) missing in the recording of the main component and this (these) should be at a suitable distance (at least 2°Θ) from the neighbouring peaks, i.e. there should be no overlapping. Unfortunately, in some cases there is a not sufficiently pronounced difference among diffraction patters of the modifications. There are numerous mathematical methods to solve the

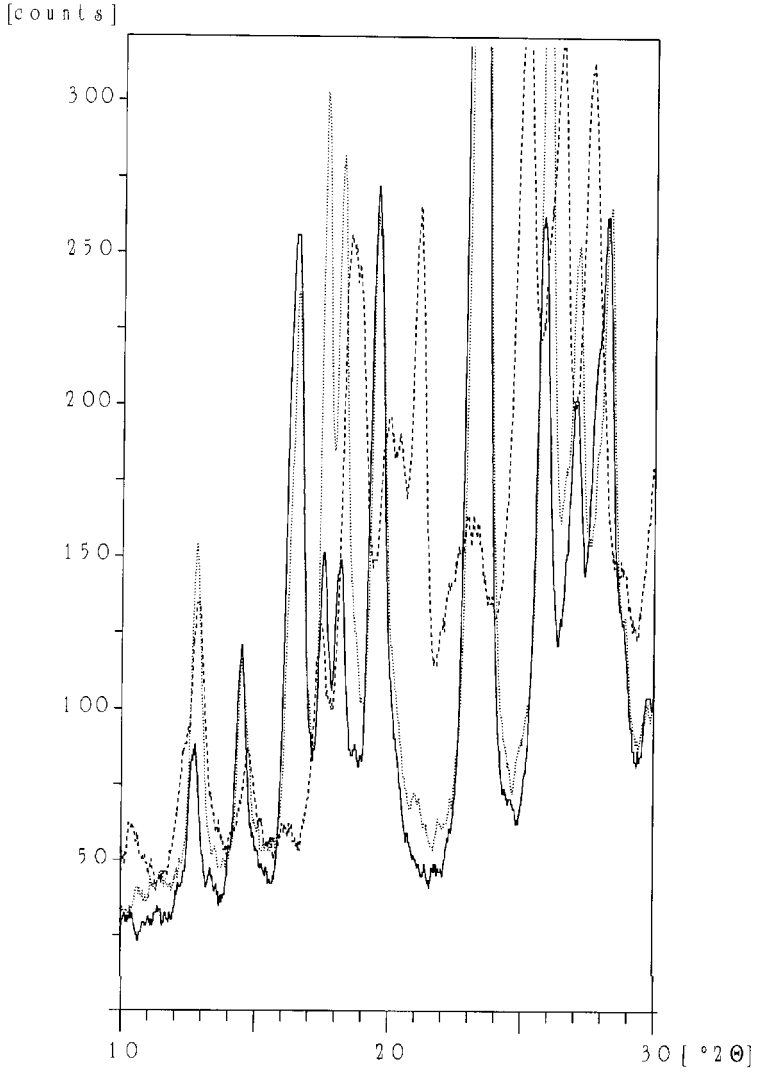

Figure 7.4.A.
Detection of cimetidine B polymorph in A form; —, pure A form; ···, pure B form; - - -, 5% B in A [4]

problem of overlapping peaks, e.g. the profile-fitting method [8]. If this is not applicable, the generalised internal standard method [9] can be used.

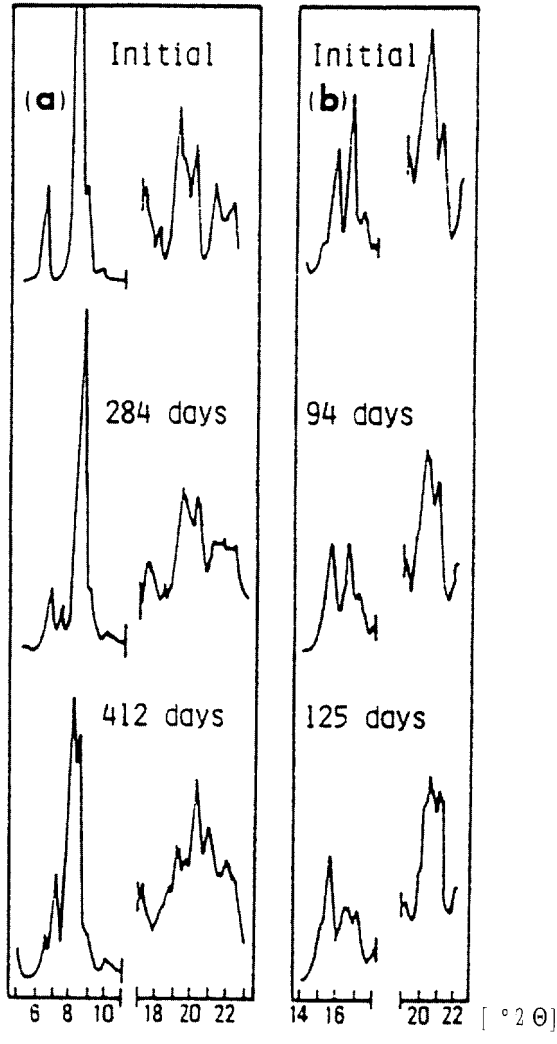

Figure 7.4.B.
Changes in X-ray diffraction intensities of phenylbutazone (form α and β) during polymorphic transformations. (a) α → δ transformation at 40°C and 80% relative humidity, β → δ transformation at 40°C and 30% relative humidity (from Ref. [5])

(b) The other criterion is the precise measurement of intensity of the analytical peak. For the sake of keeping the incident-beam intensity, that influences the absolute intensity of the peaks at a constant value, a voltage stabiliser is needed to be coupled to the diffractometer.

One of the most important factors influencing the peak intensity is the quality of sample preparation. The aim is to prepare a random specimen thus decreasing the preferred orientation to a minimum. In the diffractometers of the Bragg–Brentano geometry the absorption of the sample is the same at all of the degree values, because the radiated volume is constant. However microabsorption should always be taken into consideration. For minimising the preferred orientation, the particle size of the sample should be decreased below 20 μm. On the other hand, with particle size below 1 μm significant line broadening may occur.

To develop a protocol for the quantification of drug polymorphs, the compounds *benzyl* and *benzoic acid* were chosen for a model system by Kidd [10]. The effect of particle size reduction on the preferred orientation was investigated with different sieve fractions of benzyl crystals. The peak area at 12°2Θ decreased from 137271.7 to 13278.2 when the sieve fraction was changed from 355 μm to 38 μm while the relative standard deviation (RSD) of the area measurement decreased from 42.6% to 3.3%. At 22°2Θ the area increased from 11873.8 to 16307.2, while RSD decreased from 65.8% to 1.9%.

The simplest way of particle size reduction is grinding in a mortar with a pestle. This should be done very carefully since soft substances can easily lose their crystallinity. If the polymorph crystals have different hardnesses, the harder particle grinds the softer one, the latter can lose its crystallinity, while the particle size of the harder one does not decrease. Wet-grinding is the recommended method for the suitable sample preparation using a solvent which does not dissolve the components of the sample.

In addition to the particle size the role of the crystal habit is also very important. As the difference between the maximum and minimum dimensions of the particle increases (needles), it becomes more difficult to prepare a random sample. When a modification being present as an impurity and the main component have different habits (e.g. needles and plate-like crystals), the probability of the preferred orientation increases.

Considering the aforementioned general points of view, on the basis of precisely measured intensities the concentration of the polymorphic impurity (C_i) can be calculated from the following formula:

$$C_i = B_i \times I_i \times MAC$$

where B_i is a calibration constant, I_i is the net intensity of the diffraction peak of the impurity and MAC is the mass absorption coefficient of the sample.

If the polymorphic modification to be determined is available in pure form

and there is no overlapping among the peak to be analysed and peaks of the other phases, the spiking method can be applied: a known amount of the impurity modification is added to the sample. Then:

$$C_i = I_o/I_p \times (I_p - I_e)/(I_e - I_o) \times A$$

where I_o is the peak intensity before addition of the polymorphic impurity, I_e is the enriched analyte intensity after addition of the polymorphic impurity, I_p is the intensity of the pure polymorphic modification to be determined and A is the fraction of the added polymorphic modification.

If an internal standard is added to the calibration standard and to the sample, the effect of absorption is eliminated. Then:

$$C_i = (B_i \times I_i)/(B_s \times Is) \times C_s$$

where B_s is the calibration constant of internal standard phase, I_s is the intensity of internal standard phase, C_s is the concentration of the internal standard phase.

Any pure phase may act as an internal standard, but it needs to have one or more fully resolved lines not overlapping any lines in the analyte phases and be of very small particle size or have an absorption similar to the analyte phases. Lithium fluoride is used most widely as internal standard, but in some cases sodium chloride and zinc oxide were also successfully applied.

In case of up-to-date appliance and suitable sample preparation, the detection limit can be smaller than 1%. The precision of quantitative determination performed with X-ray diffraction naturally depends on the amount of the impurity component. In advantageous cases the RSD value can be maintained between 2 and 5%, but in some cases it can exceed 10%.

References

1. D. Doff, F. Bownen and O. Corrigan, *Analyst* **111**, 179–182 (1986)
2. D. Giron, B. Edel and P. Piechon, *Mol. Cryst. Liq. Cryst*. **187** 297–307 (1990)
3. P. Stahuy, A. McKenzie, M. Andres, C. Russel, S. Byrn and P. Johnson, *J. Pharm. Sci*. **86**, 970–971 (1997)
4. M. Bálint, unpublished data
5. Y. Matsuda, E. Tatsumi, E. Chiba and Y. Miwa, *J. Pharm. Sci*. **73**, 1453–1460 (1984)
6. M. DeVilliers, J. van der Watt and P. Lotter, *Drug Dev. Ind. Pharm.* **17**, 1295–1303 (1991)
7. A. Florence, E. Salole and J.Stenlake, *J. Pharm. Pharmac*. **26**, 479–480 (1974)

8. V. Tanninen and J. Yliruusi, *Int. J. Pharm.* **81**, 169–177 (1992)
9. R. Jenkins and R.L. Snyder, *Introduction to X-ray Powder Diffractometry*, p 375, Wiley, New York (1996)
10. Ch. Kidd, P. Varlashkin and Chia-yu Li, *Powder Diffract.* **8**, 180–187 (1993)

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Chapter 8

MICROBIOLOGICAL EXAMINATION OF NON-STERILE DRUGS AND RAW MATERIALS

Hans van Doorne

8.1. Introductory Remarks

The occurrence of micro-organisms in pharmaceutical preparations, harmful to the preparations as well as hazardous to the patient has been demonstrated by the Dutchman Lansberg, as early as 1919 [1]. He concluded that basic knowledge of microbiology is an indispensable tool for the manufacturing and control of pharmaceutical preparations on a scientifically warranted basis. General interest in pharmaceutical microbiology arose after the publication of reports of investigations in Sweden, triggered by the outbreak of infections due to contaminated preparations [2,3].

In the mid-1970s a working group of the Fédération Internationale Pharmaceutique (FIP) published a report suggesting methods for the examination and criteria for different categories of pharmaceutical preparations [4,5]. The proposals were adopted by the European Pharmacopoeia (Ph. Eur.) and have been refined since (cf. Table 8.A).

For those who are not familiar with the Ph. Eur. its structure may be confusing. For 56 raw materials (such as guar, lactose and starch) the microbiological criteria have been included in the respective monographs, and therefore these are mandatory requirements. The microbiological criteria for pharmaceutical preparations have been included in a section of the General Texts (Section 5.1.4); this implies that these are non-mandatory requirements. The methods and media for the microbiological examination of non-sterile drugs are given in the chapter on Biological Tests (Sections 2.6.12 and 2.6.13). This implies that these are mandatory methods. Thus, there is a mix of mandatory and non-mandatory requirements with mandatory methods.

The United States Pharmacopoeia (USP) gives the criteria for the microbiological quality for both raw materials and preparations in almost 190

different monographs. The present methods for the microbiological examination were first described in USP 18 [6] and have remained unchanged ever since.

Table 8.A.
Comparison between original (FIP 1972) and vigorating microbial criteria

Category	Products	Requirements
FIP 1972 [4]		
1a	Injectables	Sterility
1b	Ophthalmic preparations, preparations applied in body cavities normally free from micro-organisms, on burns and severe ulcerations	Absence of revivable micro-organisms in 1 g or 1 ml
2	Preparations for topical application, e.g. on skin lesions, in the nose, throat, ear etc. High risk preparations	Limiting level of revivable micro-organisms: – 10^2 in 1 g or 1 ml – Absence of enterobacteria – Absence of *Ps. aeruginosa* – Absence of *Staph. aureus*
3	Other preparations.	Limiting level of revivable micro-organisms: – 10^3–10^4 aerobic bacteria in 1 g or 1 ml – 10^2 fungi in 1 g or 1 ml Limits for specified micro-organisms: – Absence of *E. coli* In certain cases: – Absence of *Salmonella* in 1 g or 1 ml – Other enterobacteria max. 10^2 per g or per ml – Absence of *Ps. aeruginosa* in 1 g or 1 ml – Absence of *Staph. aureus* in 1 g or 1 ml
European Pharmacopoeia [18]		
1	Preparations required to be sterile by the relevant monograph on the dosage form and other preparations labelled to be sterile	Test for sterility

Table 8.A. (*continued*)

Category	Products	Requirements
2	Preparations for topical use and for use in the respiratory tract, except where required to be sterile	– Total viable aerobic count: not more than a total of 10^2 aerobic bacteria and fungi per gram or per millilitre – Not more than 10^1 enterobacteria and certain other gram-negative bacteria (1 g or 1 ml) – Absence of *Ps. aeruginosa* (1.0 g or 1.0 ml) – Absence of *Staph. aureus* (1.0 g or 1.0 ml)
3	A. Preparations for oral and rectal administration	– Total viable aerobic count: Not more than a total of 10^3 aerobic bacteria and not more than 10^2 fungi (1 g or 1 ml) – Absence of *E. coli*
	B. Preparations for oral administration containing raw materials of natural origin (for which antimicrobial pre-treatment is not feasible and for which the relevant authority accepts a microbial contamination of the raw material exceeding (10^3 viable micro-organisms per gram or per millilitre	– Total viable aerobic count: not more than a total of 10^3 aerobic bacteria and not more than 10^2 fungi in 1 g or 1 ml – Not more than 10^2 enterobacteria and certain other gram negative bacteria in 1 g or 1 ml – Absence of *Salmonella* (10 g or 10 ml) – Absence of *E. coli* (1 g or 1 ml) – Absence of *Staph. aureus* (1 g or 1 ml)
4	Herbal remedies A. Herbal remedies to which boiling water is added before use	– Total viable aerobic count: Not more than a total of 10^7 aerobic bacteria and not more than 10^5 fungi per gram or per millilitre – Not more than 10^2 *E. coli* per gram or per millilitre
	B. Other herbal remedies	– Total viable aerobic count: not more than a total of 10^5 aerobic bacteria and not more than 10^4 fungi per gram or per millilitre – Not more than 10^3 enterobacteria and certain other gram negative bacteria per gram or per millilitre – Absence of *Salmonella* (10 g or 10 ml) – Absence of *E. coli* (1 g or 1 ml)

Recently, the Group of Experts 1CM of Ph. Eur., published a document comparing the methods and media in Ph. Eur., Japanese Pharmacopoeia (JP) and USP, and making proposals for harmonisation [7].

In this chapter the current methods for the examination of pharmaceuticals (raw materials and products) and their validation will be discussed. Wherever possible a comparison with alternatives (e.g. originating from food microbiology) will be made.

8.2. Validation

8.2.1. General Aspects of Validation

Validation of methods is an essential prerequisite for all operations performed under Good Manufacturing Practice (GMP) and Good Laboratory Practice (GLP). The USP 24 [8] describes validation as ''the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the analytical applications''. Six key parameters that should be addressed in a validation protocol are:

- Accuracy
- Precision, including repeatability and reproducibility
- Linearity and range
- Limit of quantitation
- Specificity
- Robustness

The monograph was written for analytical methods and not for microbiological methods. Recently, a draft monograph on ''the validation of microbial recovery from pharmacopoeial articles'' was published [9]. One of the objectives of this draft monograph is to provide guidelines for estimating the number of viable micro-organisms and for the detection of indicators or objectionable micro-organisms. Attention is paid to (i) neutralisation of antimicrobial properties, (ii) recovery of injured micro-organisms, and (iii) estimation of the number of colony forming units. No attention is paid to the problems associated with selective detection and isolation of specific micro-organisms, such as *E. coli* or *Staph. aureus*. For the validation of a bioluminescence method for the microbiological examination of water, a specific interpretation of the above-mentioned key parameters was necessary [10].

Using the USP 24 definitions [8] the following comments can be made with respect to validation of the classical microbiological methods, mentioned in the pharmacopoeias.

(a) *Accuracy* of a method is the closeness of test results obtained by that

method to the true value. The accuracy is difficult to prove in microbiology as it is affected by a number of factors, including:

- Homogeneity of the sample. Micro-organisms may be distributed inhomogeneously over the entire batch (contagious distribution). Such contagious frequency distributions may be due to the presence of bacterial nests or lumps. Sublethal damage to a part of the population may also result in a contagious distribution. Sublethal injury may be invoked to a microbial cell by (mild) heat treatment, drying, or the action of preservatives. Thus, generally micro-organisms are not randomly distributed over the sample, but frequency distributions will rather follow a negative binomial function [11–13]. An important feature of such distributions is their asymmetry. Examination of only one sample may result in unjustified rejection of an entire batch. For products where homogeneity may be a problem the so-called three class sampling plan [14] was introduced in the European Pharmacopoeia.
- Physiological state of the micro-organisms. The chemical composition of the sample, its source and the storage conditions affect the types of organisms present, their number and their physiological state.
- Incubation conditions, including type of medium, temperature and time. The number of micro-organisms that are detected is highly dependent on these factors.
- Statistical errors inherent to the method used. In addition to the distribution of the micro-organisms in the batch as discussed above, experimental errors, such as dilution errors are another source of variation. According to Jarvis [11] the total error of colony count procedures may be derived as follows:

$$\%\ \text{total error} = \pm\sqrt{(\text{A})^2+(\text{B})^2+(\text{C})^2}$$

where A is the % sampling error, B is the distribution error, and C is the % dilution error.

Of these, the distribution error is the most important and dependent on the number of colonies counted. Results of plate count assays follow Poisson distribution, hence the theoretical standard deviation equals the square root of the average value [10,11]. A number of 20–200 colonies per plate will give a standard error as percentage of the mean ranging from 22% to 7%, respectively.

The most probable number techniques (MPN) have even higher standard errors. If an MPN method is performed with three levels of dilution and three tubes per level of dilution (as described in Ph. Eur. and USP) the standard error, expressed as $^{10}\log$ of the average is 0.335 [15]. The 95% confidence limit for a calculated MPN ranges from calculated MPN/4.68 to 4.68 × (calculated MPN).

- Biological nature of micro-organisms. Bacteria, yeasts and moulds are

living organisms. Results of microbiological assays may be an underestimation of the true value, due to microbial antagonism, or they may be an overestimation, e.g. due to growth during a resuscitation step. Moreover, the microbial flora of a product may change qualitatively and/or quantitatively with time due to growth or death of (a part of) the flora.

(b) *Precision* is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogeneous sample. It is a combination of reproducibility (results obtained by different laboratories) and repeatability (results obtained by one analyst). When using selective media, different brands and different sterilisation procedures may cause considerable variation in the results [16,17]. Reproducibility of results of microbiological examinations are known to be low. Faulty results due to lack of precision may lead to unwarranted release or unwarranted rejection of the batch, causing health hazards or economic losses.

(c) *Linearity and range*. These two factors present few theoretical problems, but occasionally experimental difficulties may be encountered. For instance, it is impossible to suspend 10 g of acacia or camomile in 100 ml of medium, due to gelling of the product or to sorption of water by the product. If an analyst would use smaller amounts of sample (e.g. 1 g instead of 10 g per 100 ml of medium) the range of the method would be reduced.

(d) *Limit of quantitation* is defined as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. In principle the limit of detection is one colony forming unit (cfu). As expressed in the definition, the limit of quantitation is affected by the same factors as precision and accuracy, which have been discussed above.

(e) *Specificity* is defined as the ability of a method to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix. For the classical microbiological tests specificity is synonymous with selectivity. Selectivity can be defined as the ability of the method to detect and isolate the specified indicator organism (e.g. *Ps. aeruginosa)* from the sample in the presence of the other natural contaminant (e.g. other pseudomonads).

(f) *Robustness* is a measure of the capacity of the method to remain unaffected by small but deliberate changes in method parameters. An example is the use of a water bath instead of an air incubator. Heating up in a conventional incubator is slower than in a water bath. If the temperature is essential for the selectivity (e.g. 44°C McConkey broth for the detection of *E. coli* [9]) organisms other than the specified indicator may be able to grow before the target temperature is reached, and may thus compromise the selectivity of the medium.

Table 8.B.
Comparison of validation of total viable count

Item	Ph. Eur. [18]	USP[8]
Test organisms	*Staph. aureus* *E. coli* *B. subtilis* *C. albicans* *A. niger*	*Staph. aureus* *E. coli* *Ps. aeruginosa* *Salmonella*
Inoculation	– Make reference suspensions containing about 100 cfu/ml – Use as a control in the presence and the absence of the product	Add 1 ml of not less than 10^{-3} dilution of a 24 h broth culture to test material and follow test procedure
Evaluation membrane filtration and plate count	A count of any of the test organism differing by not more than a factor of five from the calculated inoculum must be obtained	Failure of the organisms to grow in the relevant medium invalidates examination and necessitates a modification of the procedure by: – Increase in diluent volume – Inactivating the agents in diluents – Appropriate combination of modifications
Evaluation MPN method	The calculated value for the inoculum must be within the 95% confidence limits of the results	
Conclusion		If bactericidal activity of the product cannot be inactivated: indication that the article is not likely to be contaminated. Monitoring should be continued to establish antimicrobial spectrum of the article

It may be concluded that there is no answer to the simple question: ''how many bacteria are there in this product?''. Validation of microbiological methods is difficult to achieve. Therefore, harmonisation and rigorous standardisation of the methods in the various pharmacopoeias is essential.

8.2.2. Validation of Total Viable Count

A comparison of the major features of the validation procedures for the total viable count as described in USP 24 [8] and European Pharmacopoeia (Ph. Eur.) [18] is given in Table 8.B.

The Ph. Eur. test is a quantitative test, demonstrating the quantitative recovery and the effectiveness of the neutralisation of the antimicrobial activity. The test organisms are the same as those for the validation of the sterility test, with the exception that *Ps. aeruginosa* has been replaced by *B. subtilis*. Vegetative cells of bacilli are very sensitive to antimicrobial agents. The allowed factor of five between test results and the inoculum has been chosen arbitrary.

The USP test is a qualitative test, that focuses on the effectiveness of the neutralisation of antimicrobial agents. A 10^{-3} dilution of a 24 h broth culture may contain as many as 10^{6}–10^{7} cfu/ml. This is a large inoculum, even larger than allowed in the preservative efficacy test, and the test may therefore be not very sensitive.

8.2.3. Validation of Test for Specified Micro-Organisms

The main characteristics of the validation of the tests for specified micro-organisms in both pharmacopoeias are shown in Table 8.C.

Both procedures may be subject to criticism. The Ph. Eur. validation is a mix between validation and quality control of the selective media. The necessity to repeat a successful validation with each batch of dehydrated medium is questionable, unless the characteristics of the product to be examined (composition, type of antimicrobial treatment) have been changed. Good laboratory practice requires the quality control of the nutrient media, and a ''pharmacopoeia'' of culture media has been published [19–21]. Weenk [22] has statistically evaluated the quality control methods and concluded that solid selective media can be reliably tested with a quantitative method (spread plate, Miles Misra, spiral plater). In order to minimise statistical errors at least two plates with an average count of 100 colonies per plate should be used. For the assessment of liquid media a large number of plates must be used. In order to minimise the laboratory resources, the serial dilution technique in which the broths are tested in triplicate is advocated.

The main objection against the USP method is its large inoculum size. A 10^{-3} dilution of an overnight culture in a non-selective medium contains about

Table 8.C.
Validation of test for specified organisms

Item	Ph. Eur. [18]	USP[8]
Frequency	Each lot of dehydrated medium	Preparatory to conducting the test on a regular basis and as circumstances require subsequently
Test organisms	*Staph. aureus* *E. coli* *Ps. aeruginosa* *S. typhimurium* *(S. abony)*	*Staph. aureus* *E. coli* *Ps. aeruginosa* *Salmonella*
Inoculum	Mixture of 4 test organisms; 100 cfu each	– Separate viable cultures – 1 ml of not less than 10^{-3} dilution of a 24-h culture
Procedure	As in tests in the presence and the absence of the product to be examined	Addition to the first dilution of the test material and following the test procedure
Interpretation	A positive result for the respective micro-organisms must be obtained	Failure of the organisms to grow invalidates that portion of the examination
Suggested modification in case of failure		– Increase in volume of diluent – Incorporation of inactivating agents – Combination of above methods

10^6 c.f.u./ml. Baird [17] suggested as one of the acceptance criteria for selective media that for a test organism, for which the medium has not been designed (e.g. Baird-Parker medium challenged with *E. coli*) that the difference in colony counts on that medium and on a non-selective reference medium should be at least 5 log units. Therefore even unsuitable media may pass the USP validation criteria.

8.3. Total Viable Count

8.3.1. Preparation of the Sample

The main features of the sample preparation of the two pharmacopoeias are given in Table 8.D.

The major differences between the European Pharmacopoeia and the USP are:

- The inclusion of a three class sampling plan in Ph. Eur.
- The status of the MPN method.
- The use of membrane filtration.
- The possibility of using alternative methods.

Table 8.D.
Total viable count: preparation of the sample

Item	Ph. Eur. [18]	USP [8]
Method	– Membrane filtration and plate count method preferred – MPN only where no other method is available – Method must be properly validated – Unless otherwise prescribed: samples of 10 g or ml – Three class sampling plan where homogeneity may be a problem	– For specimens sufficiently soluble or translucent plate count, otherwise MPN method – An automated method may be used provided it has been validated as giving equivalent or better results – Provide separate 10 ml or 10 g specimens for each of the tests
Character of the sample (1)	*Water soluble products*	1. *Solutions or suspensions in water or in water with less than 30% alcohol or,* 2. *Specimens soluble in water, buffer or medium*
	– Dissolve in buffer or another suitable liquid – Neutraliser may be added	– Dissolve in buffer or medium – Perform test for absence of inhibition
	– If necessary prepare further 10-fold serial dilution	– Add to medium within 1 h

Table 8.D. (*continued*)

Item	Ph. Eur. [18]	USP [8]
(2)	*Non-fatty water insoluble products* – Dissolve or resuspend in buffer or other suitable liquid Surface active agent may be added Neutraliser may be added If necessary prepare further 10-fold serial dilution	*Solids that dissolve appreciably but not completely* – Reduce substance to moderately fine powder, suspend in the vehicle specified
(3)	*Fatty products* – Homogenise with not more than half its weight of Tween 80 or other emulsifying agent – If necessary heat to not more than 40°C, in exceptional cases not more than 45°C – If necessary prepare further 10-fold serial dilution	*Water immiscible fluids, ointments, creams, waxes* – Prepare suspension with a minimal quantity of sterile emulsifying agent (such as one of the polysorbates), using a mechanical blender – Warm to 45°C if necessary
(4)		*Fluid specimen in aerosol form* – Chill the container, cut open and allow the propellant to escape

Despite some shortcomings the three class sampling plan has gained wide acceptance for the evaluation of bacterial counts because of its simple application and robust functionality [23]. The definition of the elements of three class sampling plans and the values chosen in Ph. Eur. are summarised in Table 8.E.

This is the most common sampling plan, and it gives very small probabilities of rejection of a lot due to a single result that exceeds M. The application of the suggested three class sampling plan is particularly justified for herbal remedies where homogeneity may be a problem and relatively high levels of contamination are acceptable.

In Ph. Eur. the MPN method is a ''last resort'' to be used for bacterial counts only when no other method is applicable. There are reasons why it has

Table 8.E.
Characteristics of three class sampling plan as used in Ph. Eur. [18]

Element	Definition [23]	Ph. Eur. value
n	Number of samples analysed	5
m	The value of the characteristic (e.g. cfu/g) separating good quality from marginal quality	Limit specified in the relevant monograph (e.g. 10^5 cfu/g for herbal remedies, category 4B)
M	The value of the characteristic separating marginal quality from bad quality	10xm (i.e. 10^6 for herbal remedies)
C_m	The number of samples that can fall between m and M without the lot being considered unacceptable	2
C_M	The maximum allowable number of bad quality items in the sample	0

been retained in this edition of the European Pharmacopoeia:

- It is a recommended method in the USP; deleting the method from Ph. Eur. could be an obstacle for international harmonisation.
- At least one of the instrumental methods relies on the MPN-method [10]. Deletion of the MPN method would prohibit the introduction of such technologies.

In the USP the membrane filtration is not clearly described, it is only mentioned with reference to the sterility test.

For practical reasons the limits of quantitation of pour plates and spread plates are 10 cfu per g or ml and 100 cfu per g or ml, respectively (1 ml or 0.1 ml of a 10^{-1} dilution is added to the agar medium). Thus the limit of quantitation of spread plates is equal to the maximum allowable level of products belonging to category 2 (cf. Table 8.A). The spread plate technique can, therefore, not be used for such products. The limit of quantitation of the MPN method is 3 cfu/g or per ml. As relatively large volumes (e.g. 500 ml) can be filtered through a membrane filter, the filtration method has a much lower limit of quantitation.

The USP allows the use of automated methods, provided that they have been validated as giving equivalent or better results. In the Ph. Eur. a mention is made of automated methods. Many automated analytical instruments for

enumerating (indicator) organisms used in food microbiology have been reviewed by Van der Zee and Huis in't Veld [24]. For the examination of pharmaceutical products and raw materials methods based on impedance [25], bioluminescence [9,26] and solid phase, laser-scanning cytometry [27] seem to be attractive alternatives for the conventional methods. The introduction of the latter technique once more focused the attention to viable but non-culturable cells. Under certain physiological conditions viable cells may confer to a non-culturable state [28]. This so-called VNC-state may have tremendous implications. It has been estimated that the diversity of species cultured from the environment is less than 1% of that actually present in many niches [20]. Moreover, it has been demonstrated that non-culturable cells of some strains of *C jejuni* are capable of infecting mice [29].

8.3.2. Test Procedures

The main features of the test procedures are shown in Table 8.F.

Table 8.F.
Total viable count: test procedures

Item	Ph. Eur. [18]	USP [8]
Membrane filtration	– Use 0.45 μm membrane filters – Wash with 3 × 100 ml (or as validated) of a suitable liquid – Inactivators may be added to wash fluid – Transfer to media	– Only mentioned as last resort, with reference to sterility test
Pour plate method	– Use 2 Petri dishes – Add 1 ml of sample and 15–20 ml of specified media	– Dilute to expect 30–300 colonies – Use 2 Petri dishes – Add 1 ml of sample and 15–20 ml of liquefied agar medium cooled to about 45°C
Surface spread method	– Use 2 Petri dishes with specified media – Spread not less than 0.1 ml of the sample	Not mentioned

Table 8.F. (*continued*)

Item	Ph. Eur. [18]	USP [8]
MPN	– Prepare a three step 10-fold serial dilution – Dilute 3 × 3 aliquots in specified broth medium – If necessary subculture on specified agar	– Prepare a three step 10-fold serial dilution – Dilute 3 × 3 aliquots in specified broth medium
Incubation	– For bacteria and fungi on agar media 5 days at 30–35°C, or 20–25°C respectively – Shorter incubation time allowed if reliable result is obtained – MPN: 5 days at 30–35°C, if subcultured use these results	– Plate counts 48–72 h at 30–35°C – MPN method 24–48 h at 30–35°C
Evaluation	– Select plates with the highest number of colonies (membrane filtration <100 colonies; plate counts <300 colonies, fungi <100 colonies) – Calculate the number of cfu/g or ml – MPN determine by reference to the provided table	– Count number of colonies and express as micro-organisms per g or per ml – MPN: determine by reference to table
Interpretation	– Bacterial/fungal count = average count on specified media – Max. acceptable limit is 5 × limit – Three class sampling plan: no unacceptable and not more than two marginal samples	

Casein soya bean digest agar (CSA) and casein soya bean digest broth (CSB) are used both in the Ph. Eur. and the USP for plate counts and for the MPN method. Sabouraud dextrose agar with antibiotics (SDA-a) is used in Europe for the enumeration of fungi. In the USP yeasts and moulds may be counted on Sabouraud dextrose agar (SDA) or on potato dextrose agar (PDA). The latter medium is made selective by the addition of tartaric acid to a pH of 3.5. Ph. Eur. and USP use different definitions for the total viable count (TVC). In Europe the TVC is defined as the sum of the counts on TSA and SDA-a, whereas in the USP the total viable count is the number of c.f.u.s estimated on CSA. Only occasionally (e.g. vitamin preparations, microcrystalline cellulose and barium sulphate suspension) the USP gives limits with respect to the number of yeasts and moulds.

As discussed before the number of organisms recovered is dependent on the type of medium used. This is especially true for water. It has been demonstrated that nutritionally poor media give higher recoveries than a rich medium such as CSA [30]. The R2A medium developed by Reasoner and Geldreich [31] was shown to yield consistently higher counts than other media when used for the examination of water, or other relatively simple aqueous preparations [32–34]. This medium is presently the medium of choice for the microbiological examination of water in the Standard Methods [35].

The implications of the use of media or technologies that give higher recoveries than the traditional methods are severe. The reported differences between two methods may be large, and therefore batches may be rejected that would have been accepted when analysed with the traditional method. Alternatively, the use of improved methods may lead to complete renovation of e.g. units for the production of purified water, that hitherto had functioned quite satisfactorily.

A second major difference between the two pharmacopoeias is the interpretation of the results. In Europe the relatively low accuracy of microbiological tests was recognised; a TVC exceeding the limit prescribed in the relevant monograph by not more than a factor of five is considered acceptable. No such guidance is given in the USP.

8.4. Tests for Specified Organisms

8.4.1. Introduction

The Ph. Eur. describes tests for *Enterobacteriaceae*, *E. coli*, *Ps. aeruginosa*, *Staph. aureus*, *Salmonella*, and *Clostridium*. The USP has no tests for *Enterobacteriaceae* and *Clostridium*. The tests in Ph. Eur. have been designed to examine the presence/absence of the organisms in 1 g or ml product, whereas

the USP demands that 10 g or 10 ml has to be examined. (In supplement 1999 of the EP the test for *E. coli* is in fact a test on 10 g or 10 ml. This will probably be changed in the supplement 2000.) The organisms are either pathogens, possibly present in pharmaceuticals (*Ps. aeruginosa, Staph. aureus, Salmonella, Clostridium*), indicators for the presence of pathogenic enteric bacteria (*E. coli)*, or index organisms for failing hygienic precautions during production (*Enterobacteriaceae).* As stated in the introductory remarks, the above-mentioned guidelines were set up in the mid-Seventies [5]. In the past two decades other organisms were found to be potentially hazardous contaminants of pharmaceutical preparations, such as *B. cepacia* [36]*, R. pickettii* [37], or higher organisms such as *Acanthamoeba* [38]. Frequently the contamination may have occurred during use, rather than that unopened containers were found to be contaminated. Raw materials may be contaminated with *Enterobacteriaceae* [39]. In particular, contamination of herbal remedies with *Enterobacteriaceae,* exceeding the limits of Ph. Eur. has been reported [40]. Although there is a paucity of reports on the contamination of finished preparations, some information can be found in FDA enforcement reports which can be consulted on the Internet [41]; in Table 8.G the recalls of non-sterile drugs because of microbial contamination in the period '94–'98 are summarised.

From this table it is clear that organisms other than those covered by the pharmacopoeial tests may cause problems. In the process of harmonisation, the relevance of these tests needs to be re-evaluated [42]. In the following sections the test as they appear in USP 24 and Ph. Eur. 3 will be discussed.

8.4.2. Enterobacteriaceae

The family of *Enterobacteriaceae* contains the Gram negative, facultative anaerobic bacteria capable of glucose fermentation. *Enterobacteriaceae* that are normally found in the bowel such as *Escherichia coli* and *Proteus* species may cause disease. Other members, e.g. belonging to genera such as *Enterobacter, Klebsiella,* and *Serratia* are free living. They can be found as contaminants of water and plant material and may cause infections if the normal defence mechanisms of the body are impaired.

The main features of the qualitative test for *Enterobacteriaceae* and certain other bacteria are given in Table 8.H.

Ph. Eur. gives also an MPN method for *Enterobacteriaceae*. As the same media etc. are used for the latter test it will not be discussed here. A test for these types of organisms is only given in the European Pharmacopoeia.

The method follows in principal the classical way for the isolation of *Enterobacteriaceae:* resuscitation in a non-selective medium, enrichment in a medium with brilliant green and bile salts and detection on Violet Red Bile Glucose Agar (VRBG). Lactose Broth is still used as resuscitation although

Table 8.G.
FDA Class I and Class II recalls of non-sterile pharmaceutical preparations due to microbial contamination in the period 1994–1998

Type of preparation	Number of products	Reason
Solid oral preparations	4	Potential microbial contamination (1) Microbial contamination (1) Mould (1) Penicillium (1) Aspergillus (1)
Liquid oral preparations	6	Possible microbial contamination (1) Microbial contamination (2) Bacterial contamination (1) *B. cepacia* (1) *Ps. aeruginosa* (1) *Kl. oxytoca* (1) *C. parapsilosis* (1)
Topical preparations	8	Microbial contamination (1) Mould (2) *Ps. putida* (1) *B. cepacia* (4) *Ps. aeruginosa* (1) *S. marcescens* (1)
Respiratory drugs	4	*R. pickettii* (1) *Ps. putida* (1) *Pseudomonas* (2)

Tryptone Soya Broth was observed to be more productive [43]. For the detection of *Enterobacteriaceae* VRBG is generally used with an overlay technique, providing anaerobic conditions. As the method was designed to allow the

Table 8.H.
Test for enterobacteria and certain other bacteria

Item	Ph. Eur. [18]
Preincubation	2–5 h in Lactose Broth at 35–37°C
Selection	18–24 h in Enterobacteriaceae Enrichment Broth at 35–37°C
Subculture	18–24 h on Violet Red Bile Dextrose Agar at 35–37°C
Evaluation	No growth of Gram negative bacteria

detection of other organisms, particularly *Pseudomonas* species, an anaerobic incubation is less appropriate. However, there are no data showing that relevant *Pseudomonas* species (cf. Table 8.G) can be isolated with this method with acceptable accuracy and limit of detection.

More recently, methods have been developed that detect simultaneously coliform organisms and *E. coli*. In these methods a fluorogenic compound, 4-methylumbelliferyl-α-D-glucuronide (MUG), is combined with a selective agent such as sodium lauryl sulphate, and/or with a compound to detect β-galactosidase activity. Coliforms produce the enzyme β-galactosidase, which hydrolyses ONPG to release the yellow coloured o-nitrophenol. *E. coli* and some strains of *Salmonella, Shigella*, and *Yersinia* produce β-glucuronidase which hydrolyses the MUG to the fluorescent 4-methylumbelliferon. These methods were reported to be at least as effective as the classical methods for the isolation of coliform organisms from water [44,45]. A chromogenic substrate coliform test is recommended for the analysis of drinking water and fresh source water samples [46]. These methods are probably suitable for pharmaceutical preparations as well, particularly because the products likely to be contaminated with coliforms, are generally from animal or plant origin and thus resemble foods.

8.4.3. Escherichia coli

The main features for the isolation and detection of *E. coli* are summarised in Table 8.I.

The methods are different in some aspects. The Ph. Eur. procedure involves a more selective enrichment at 43–45°C, whereas the USP suggests the use of Eosine Methylene Blue Agar, for the confirmation of *E. coli*. Non of the described methods for *E. coli* are suitable for the detection of *E. coli* 0157, because this organism is lactose negative, and therefore will fail to give characteristic colonies on McConkey Agar. So far presence of *E. coli* 0157 in pharmaceutical products has not been reported. The main source for *E. coli* 0157 is cattle, and only a few raw materials are of bovine or porcine origin.

New methods, using media containing MUG or similar compounds have been studied extensively for possible use for the examination of water and foods [45,47–49]. In general these newer media compared favourably with traditional media. ISO standard 118662-2 [50] describes an MPN-technique for the enumeration of presumptive *E. coli* in milk and milk products. Lauryl Sulfate Tryptose broth with MUG is used as the selective enrichment medium. This technique offers several advantages over the present Ph. Eur. method: it allows simultaneous detection of coliform organisms and *E. coli*, it is a rapid method (24–48 h of incubation), and incubation at 44°C is not required. A disadvantage is that organisms such as *Ps. aeruginosa* will not be detected (see

Table 8.I.
Test for *Escherichia coli*

Item	Ph. Eur. [18]	USP [8]
Pre-incubation	18–24 h in Casein Soya Broth at 35–37°C	No preincubation
Selection	18–24 h in McConkey broth at 43–45°C	24–48 h in Lactose Broth at 30–35°C
Subculture	18–72 h on McConkey Agar at 35–37°C	24–48 h on McConkey Agar at 30–35°C
Evaluation	Product passes if no characteristic Gram negative colonies grow on McConkey Agar, or confirmation is negative	Product passes if no characteristic Gram negative colonies grow on McConkey Agar, or confirmation is negative
Confirmation	Suitable biochemical tests such as indole test	– Characteristic colonies on Eosin Methylene Blue Agar – Other suitable biochemical tests

also section on *Ps. aeruginosa*). The use of such media must be considered for next editions of pharmacopoeias.

8.4.4. Salmonella

Several outbreaks of epidemics due to pharmaceuticals contaminated with *Salmonella* have been described. Pancreatic and thyroid preparations, carmine red and potato starch have been found contaminated with this organism [2,51,52].

The main features for the isolation and detection of *Salmonella* are summarised in Table 8.J.

The USP prescribes the use of Lactose Broth for the pre-enrichment step, the reasons for the choice of this medium are obscure, as salmonellae are lactose non-fermentors, but Lactose Broth is also prescribed in at least two AOAC methods for the isolation of Salmonella [53a,b]. In Ph. Eur. only Tetrathionate Bile Brilliant Green Broth, incubated at 41–43°C, is used, whereas in the USP two selective liquid media are used simultaneously. The latter approach is in line with other methods, such as AOAC [53] and ISO/CEN [54] methods for the isolation of *Salmonella* from foods. The ISO method specifies the use of two selective media, viz. Rappaport–Vassiliades medium,

Table 8.J.
Test for *Salmonella.* Comparison of Pharmacopoeial Methods and ISO 6579 Method

Item	Ph. Eur. [18]	USP [8]	ISO [54]
Pre-incubation	18–24 h in Casein Soya Broth at 35–37°C.	24–48 h in Lactose Broth at 30–35°C.	16–20 h in buffered peptone water at 35°C or 37°C.
Selection	1 ml of pre-culture is transferred to 10 ml Tetrathionate Bile Brilliant Green Broth and incubated at 41–43°C	1 ml of pre-culture is transferred to 10 ml Tetrathionate Broth and Fluid Selenite Cystine Medium (or Fluid Rappaport Medium) and incubated for 24–48 h at 30–35°C.	– 1 ml of pre-culture is transferred to 10 ml Rappaport-Vassiliades medium, incubated for 24 h at 42°C and – 10 ml is transferred to 100 ml Selenite Cystine medium, incubated for 24–48 h at 35°C or 37°C.
Subculture	At least on two of the following media:	On three different media:	On two different media (one large, 140 mm, petri dish or two small petri dishes):

	Deoxycholate Citrate Agar (DCA), Xylose, Lysine, Deoxycholate Agar (XLD), Brilliant Green Agar (BGA). Incubate 18–72 h at 35–37°C.	Xylose Lysine Deoxycholate Agar (XLD), Brilliant Green Agar (BGA), Bismuth Sulphite Agar (BSA). Incubate 24 h - 48 h at 30–35°C.	Brilliant Green Agar, Second suitable medium. Incubate 24–48 h at 35°C or 37°C.
Evaluation	Growth of typical colonies of G- negative bacteria indicates possible presence of *Salmonella.* Product passes if no such colonies are present.	Growth of typical colonies of G- negative bacteria indicates possible presence of *Salmonella.* Product passes if no such colonies are present.	Check for the presence of colonies which are considered to be presumably *Salmonella*.
Confirmation	Characteristic growth on Triple Sugar iron Agar (provisional). Appropriate biochemical and serological tests.	Characteristic growth on Triple Sugar iron Agar, 30–35°C, 24–48 h. Footnote: additional confirmation by AOAC-methods.	Appropriate biochemical and serological tests.

incubated at 42°C for 24 h, and Selenite Cysteine medium, incubated at 35°C or 37°C for 24 h.

According to Ph. Eur. subculture must be done on at least two of three-selective agar media. In the USP three media must be used. Deoxycholate Citrate Agar and Brilliant Green Phenol Red Agar are prescribed in both pharmacopoeias. Neither of these media are **is** included in the AOAC methods for salmonella. The other media (Xylose Lysine Deoxycholate Agar (Ph. Eur.), and Bismuth Sulfite Agar (USP)) are prescribed in the relevant AOAC methods [53]. The ISO method prescribes the use of Brilliant Green Phenol Red agar, and a second, not specified, suitable medium.

In the Ph. Eur. the incubation time for the subculture is 18–72 h. This broad time span was chosen to allow incubation over the weekend. A possible alternative is to use the AOAC refrigerated pre-enrichment and selective enrichment method [55]. In the latter method the pre-enrichment or enrichment culture is stored, if necessary, at 4°C during the weekend. It was demonstrated that this did not affect the recovery of Salmonella from products such as whole egg powder, milk powder and animal feeds. The disadvantage of the Ph. Eur. method is that colony characteristics may get lost during the prolonged incubation period.

For a future harmonised version of the pharmacopoeias one of the accepted methods, such as ISO could be adopted.

8.4.5. Pseudomonas aeruginosa

Ps. aeruginosa is an ubiquitous environmental bacterium, and is still considered a major source of hospital acquired infections with a high mortality rate [56]. An outbreak of eye infections caused by the use of an ointment contaminated with this organism was one of the reasons of the Swedish national survey [2]. Its relevance may be restricted to the hospital environment because only a small percentage of clones of *Ps. aeruginosa* possesses the required number of virulence factors to cause infection. The risk to human health is further narrowed by he fact that only specific hosts, such as patients with cystic fibrosis, severe burns, and those subject to foreign device installation are at risk [57].

The main features of the Ph. Eur. and the USP tests are shown in Table 8.K.

Cetrimide Agar, used in both pharmacopoeias, was developed to enhance pigment production by strains of *Ps. aeruginosa*, and it was made selective by the addition of cetrimide [58]. Later, it was demonstrated that the performance could be improved by the addition of nalidixic acid and lowering the concentration of cetrimide from 0.3 g/l to 0.2 g/l [59,60]. Media with this combination of selective agents are prescribed in the USP

Table 8.K.
Test for *Pseudomonas aeruginosa*

Item	Ph. Eur. [18]	USP [8]
Preincubation	18–24 h in Casein Soya Broth at 35–37°C	24–48 h in Casein Soya Broth at 30–35°C
Subculture	18–72 h on Cetrimide Agar at 35–37°C	24–48 h on Cetrimide Agar at 30–35°C
Evaluation	Product passes test if no growth of Gram-negative rods, or confirmation negative	Product passes test if no growth of Gram-negative rods, or confirmation negative
Confirmation	Inoculate in Casein Soya Broth, incubate 18–24 h at 42°C. Product passes if no growth is observed	– Characteristic colonies on media for the detection of fluorescein and pyocyanin – Oxidase test – Other suitable cultural and biochemical tests

and in a draft European standard for the detection and enumeration of *Ps. aeruginosa* in bottled water [61].

Although *Ps. aeruginosa* is still an important pathogenic organism, other micro-organisms, especially those originally included in the genus *Pseudomonas*, but now belonging to separate genera (*Burkholderia* and *Ralstonia*, cf. Table 8.F), were also found to be dangerous contaminants of medical preparations. The media with high concentration of cetrimide or with a combination of cetrimide and nalidixic acid discussed above are less satisfactory for other relevant organisms such as *B. cepacia* (formerly *Ps. cepacia)* [62]. Mead and Adams [63] developed a selective medium (CFC) for the isolation of pseudomonads which contains a lower concentration of cetrimide, but two additional selective agents (fucidin and cephaloridine). A comparison of this medium with the medium prescribed ISO 8360-2 (Modified King's A Broth, MKAB) showed that better isolation of a strain of *Ps. aeruginosa* was obtained with the CFC medium than with the MKAB medium [64]. The medium was also reported to permit quantitative recovery of pigmented and non-pigmented *Pseudomonas* strains from food and environmental samples, whilst at the same time inhibiting most other organisms [65]. The CFC medium is used in ISO standard 13720 for the enumeration of *Pseudomonas* species in meat and meat products [66].

Different confirmation tests are described in the two pharmacopoeias. The European Pharmacopoeia relies exclusively on the growth at 42°C, whereas the

USP specifies the use of a diagnostic medium (Naladixic acid Cetrimide Agar), the oxidase test, and other suitable cultural and biochemical tests.

Ph. Eur. describes tests for *Enterobacteriaceae* and certain other bacteria (including *Pseudomonas* species), *E. coli*, and *Ps. aeruginosa*. It remains to be investigated whether this strategy has to be replaced by a strategy providing tests for coliform organisms, *E. coli*, and *Pseudomonas* species.

8.4.6. Staphylococcus aureus

Staph. aureus is an important pathogen, present on parts of the body, such as skin and mucosa. It causes skin lesions, such as boils, and produces a toxin which, if ingested with food, can give rise to food poisoning. The emergence of methicillin resistant strains (MRSA) has increased its hygienic relevance in the hospital setting. Therefore, its presence in pharmaceutical products is an indicator of failing compliance to GMP, and poses a possible serious health hazard to the consumer.

The main features of the Ph. Eur. and USP tests for *Staph. aureus* are shown in Table 8.L.

There is a difference in the selection step. Ph. Eur. prescribes only the use of Baird–Parker medium, whereas the USP gives three alternatives (Baird–Parker Agar, Vogel–Johnson Agar, or Mannitol Salt Agar). Baird–Parker Agar

Table 8.L.
Test for *Staphylococcus aureus*

Item	Ph. Eur. [18]	USP [8]
Pre-incubation	18–24 h in Casein Soya Broth at 35–37°C	24–48 h in Casein Soya Broth at 30–35°C
Subculture	18–72 h on Baird Parker Agar at 35–37°C	24–48 h on Baird Parker Agar, Vogel Johnson Agar, or Mannitol Salt Agar
Evaluation	Growth of typical colonies of Gram-positive cocci indicates possible presence of *Staph. aureus*. Product passes if no such colonies, or confirmation negative	Growth of typical colonies of Gram-positive cocci indicates possible presence of *Staph. aureus*. Product passes if no such colonies, or confirmation negative
Confirmation	Suitable biochemical tests, such as: – Coagulase test – DNase test.	Coagulase test

(BPA) is the medium of choice for direct plating and enumeration of *Staph. aureus* [67]. Vogel–Johnson Agar (VJA) and Mannitol Salt Agar (MSA) are unreliable for the detection of injured cells [68]. VJA gives reliable counts for untreated cells, but for heated cells, even after a 10-h resuscitation period the counts on this medium were unreliable [69]. However, the difference between the various media may be small, as in both pharmacopoeias a 18–24 h preincubation period is included to allow resuscitation of injured cells and growth to a detectable level.

It may be concluded that the method described in Ph. Eur. is appropriate to detect low numbers (1/g) of *Staph. aureus* in pharmaceutical preparations. Confirmation by means of coagulase and/or Dnase is indispensable as a significant proportion of *Staph. aureus* strains are egg yolk negative [70,71]. The use of bovine fibrinogen [72] as used in a Dutch standard for the examination of milk and milk products [73] has not yet been studied for pharmaceutical products and cannot be recommended.

8.4.7. Clostridium

Only in the European Pharmacopoeia a test for *Clostridium* is prescribed. There are no monographs requiring the absence of these types of organisms. Therefore, the test will not be discussed.

8.5. Concluding Remarks

The microbiological methods described in Ph. Eur. III and USP 24 have remained almost the same for more than twenty years. Instrumental methods and new media developed during that period have not yet gained wide acceptance in the pharmaceutical industry. A number of reasons can be given to explain this reluctance to accept innovations.

- The pharmacopoeial methods are referee tests, and it is difficult, if not impossible, to demonstrate the equivalence of a new method to the vigorating test.
- The introduction of new methods would not only require a vast amount of work for the revalidation of the test for all the relevant products, it might also require retesting products that were released by previous methods but that are not yet beyond the expiration date.
- Due to globalisation of the pharmaceutical market a new test would not be acceptable unless it is a harmonised test.
- Any newly introduced method will raise the question to the relevance of organisms that where hitherto not detected.

As discussed before, the types of organisms for which tests are provided in the pharmacopoeias may not be sufficient to assess the safety of a product, as other pathogenic micro-organisms may be present. Microbiological examination of pharmaceutical preparations is a risk assessment. Guidance is needed on how to perform such a risk assessment and on the types of ''objectionable'' micro-organisms that should be included in such a study. A decision tree for determining what objectionable micro-organism tests are appropriate, based on the character of the product, its intended use, and nature of its components may be useful [42].

References

1. L.M. Lansberg, *Bijdrage tot de Kennis der Bacteriën Flora van Eenige Geneesmiddelen*, Thesis, Leiden (1919)
2. L.O. Kallings, *The 1964 Inquiry by the National Board of Health into Microbiological Contamination of Medical Preparations*, Thule A.B., Stockholm (1966)
3. L.O. Kallings, O. Ringertz, L. Silverstolpe and F. Ernerfeldt, *Acta Pharm. Suec.* **3**, 219–228 (1966)
4. X. Bühlmann, M. Gay and H.U. Gubler, *Pharm. Acta Helv.* **46**, 321–350 (1971)
5. Anonymous, *Pharm. Acta Helv.* **51**, 33–40 (1976)
6. *The United States Pharmacopeia 18*, USP, Rockville (1970)
7. Anonymous, *Pharmeuropa* **10**, 418–447 (1998)
8. *The United States Pharmacopoeia 24* USP Convention Inc., Rockville (2000)
9. Anonymous, *Pharmac. Forum* **24**, 5619–5626 (1998)
10. K. Wills, H. Woods and L. Gerdes, *Pharmac. Forum* **24**, 5645–5664 (1998)
11. B. Jarvis, Statistical aspects of the microbiological analysis of foods. *Progress in Industrial Microbiology*, Vol. 21, Elsevier, Amsterdam (1989)
12. G. Hildebrandt and H. Weiss, *Fleischwirtsch.* **74**, 56–59 (1994)
13. G. Hildebrandt and H. Weiss, *Fleischwirtsch.* **74**, 165–168 (1994)
14. Micro-organisms in foods 2. *Sampling for Microbiological Analysis: Principles and Specific Applications*, pp 19–31, ICMFS, University of Toronto Press (1978)
15. W.G. Cochran, *Biometrics* **6**, 105–116 (1950)
16. H. Van Doorne and F.S.C. Van Voorst Vader, *Pharm. Weekbl. Sci. Ed.* **2**, 186–189 (1980)
17. R.M. Baird, Monitoring microbiological quality: conventional testing

methods, in *Guide to Microbiological Control in Pharmaceuticals* (S. Denyer and R. Baird, Eds.), pp 125–145, Ellis Horwood, New York (1990)
18. *European Pharmacopoeia, 3rd edn*, Supplement, pp 33–41, Council of Europe, Strasbourg (1999)
19. R.M. Baird, L.M. Barnes, J.E.L. Corry, G.W.D. Curtis and B.M. Mackey (Eds.), Quality assurance and quality control of microbiological culture media. *Int. J. Food Microbiol.* **2**, 1–136 (1985)
20. R.M. Baird, J.E.L. Corry and G.D.W. Curtis (Eds.), Pharmacopoeia of culture media for food microbiology. *Int. J. Food Microbiol.* **5**, 187–299 (1987)
21. R.M. Baird, J.E.L. Corry, G.D.W. Curtis, D.A.A. Mossel and N.P. Skovgaard (Eds.), Pharmacopoeia of culture media for food microbiology: additional monographs. *Int. J. Food Microbiol.* **9**, 85–144 (1989)
22. G.H. Weenk, *Int. J. Food Microbiol.* **17**, 159–181 (1992)
23. S. Dahms and G. Hildebrandt, *J. Food Prot.* **61**, 757–761 (1998)
24. H. Van der Zee and J.H.J. Huis in't Veld, *J. Assoc. Off. Anal. Chem.* **80**, 934–940 (1997)
25. P. Silley and S. Forsythe, *J. Appl. Bact.* **80**, 233–243 (1996)
26. P.E. Stanley, B.J. McCarthy and R. Smither (Eds.), *ATP Luminescence. Rapid Methods in Microbiology,* Blackwell Scientific Publications, Oxford (1989)
27. D.L. Jones, M.A. Brailsford and J.-L. Drocourt, *Pharmac. Forum* **25**, 7626–7644 (1999)
28. S.F. Bloomfield, G.S.A.B. Stewart, C.E.R. Dodd, I.R. Booth and E.G.M. Power, *Microbiology* **144**, 1–3 (1998)
29. D.M. Jones, E.M. Sutcliffe and A. Curry, *J. Gen. Microbiol.* 137, 2477–2482 (1991)
30. H. Van Doorne and A. Neuteboom, *Pharm. Weekbl. Sci. Ed.* **6**, 105–110 (1984)
31. D.J. Reasoner and E.E. Geldreich, *Appl. Environm. Microbiol.* **49**, 1–7 (1985)
32. E. Korsholm and H. Sogaard, *Zentralbl. Bakteriol. Hyg. 1. Abt., Orig. B* **185**, 112–120 (1987)
33. G.B. Harding, T. Pass, C. Million, R. Wright, J. DeJarnette and E. Klein, *Artif. Organs* **13**, 155–159 (1987)
34. T. Pass, R. Wright, B. Sharp and G.B. Harding, *Blood Purif.* **14**, 136–145 (1996)
35. Anonymous, Heterotrophic plate count, in *Standard Methods for the Examination of Water and Wastewater, 19th edn*, (A.D. Eaton, L.S. Clesceri and A.E. Greenberg, Eds.), pp 9/31–9/38, American Public Health Association, Washington, DC (1995)

36. A.C. Reboli, R. Koshinsky, K. Arias, K. Marks-Austin, D. Stieritz and T.L. Stull, *Infect. Control Hosp. Epidemiol.* **17**, 741–743 (1996)
37. Anonymous, *Morbid. Mortal. Wkly. Rep.* **47**, 285–286 (1998)
38. C.D. Illingworth, *Surv. Ophthalmol.* **42**, 493–508 (1998)
39. M.C. De la Rosa, M.R. Medina and C. Vivar, *Pharm. Acta Helv.* **70**, 227–232 (1995)
40. L. Kabelitz, *Pharmeuropa* **9**, 570–575 (1997)
41. http//www.fda.gov/opacom/Enforce.html)
42. T.E. Munson, Microbial attributes/microbial limit tests, in *Proceedings of USP Open Conference on Microbiology for the 21st Century, New Orleans*, pp 36–39, USP, Rockville (1998)
43. D.A.A. Mossel and M.A. Ratto, *Appl. Microbiol.* **20**, 273–275 (1970)
44. E.W. Frampton and I. Restaino, *J. Appl. Bacteriol.* **74**, 223–233 (1993)
45. W.O. Pipes, H.A. Minnigh, B. Moyer and M.A. Troy, *Appl. Environm. Microbiol.* **52**, 439–443 (1986)
46. Anonymous, Chromogenic substrate coliform test, in *Standard Methods for the Examination of Water and Wastewater, 19th edn* (A.D. Eaton, L.S. Clesceri and A.E. Greenberg, Eds.), pp 9/64–9/65, American Public Health Association, Washington, DC (1995)
47. D.L. Clark, B.B. Milner, M.H. Stewart, R.L. Wolfe and B.H. Olson, *Appl. Environm. Microbiol.* **57**, 1528–1534 (1991)
48. I.D. Gaudet, L.Z. Florence and R.N. Coleman, *Appl. Environm. Microbiol.* **62**, 4032–4035 (1996)
49. L.J. Moberg, M.K. Wagner and L.A. Kellen, *J. Assoc. Off. Anal. Chem.* **71**, 589–602 (1988)
50. *ISO 11866–2*, Milk and milk products. Enumeration of presumptive Escherichia coli. Part 2: Most probable number technique using 4-methylumbelliferyl-β-D-glucuronide (MUG) (1997)
51. R.P. Mouton, *Ned. Tijdsschr. Geneesk.* **111**, 920–922 (1967)
52. B. Rowe and M.L.M. Hall, *Br. Med. J.* 51–55 (1975)
53. (a) *AOAC Official Method 967.26 Salmonella* in processed foods; (b) *AOAC Official Method 995.20 Salmonella* in raw, highly contaminated foods and poultry feed
54. *ISO 6579*, Microbiology of food and animal feeding stuffs – horizontal method for the detection of *Salmonella* (1993)
55. *AOAC Official Method 994.04 Salmonella* in dry foods. Refrigerated preenrichment and selective enrichment broth culture methods
56. P.A. Rusin, J.B. Rose, C.N. Haas and C.P. Gerba, *Rev. Environ. Contam. Toxicol.* **152**, 57–83 (1997)
57. C. Hardolo and S.C. Edberg, *Crit. Rev. Microbiol.* **23**, 47–75 (1997)
58. E.J.L. Lowbury and A.G. Collins, *J. Clin. Pathol.* **8**, 47–48 (1955)
59. S. Goto and S. Enomoto, *J. Jpn. Microbiol.* **14**, 65–72 (1970)

60. H.A. Lilly and E.J. Lowbury, *J. Med. Microbiol.* **5**, 151–153 (1972)
61. *prEN 12780*, Water quality – detection and enumeration of *Pseudomonas aeruginosa* in bottled water (1997)
62. *The Oxoid Manual*, pp 2/178–2/179, Unipath Ltd, Basingstoke (1990)
63. G.C. Mead and B.W. Adams, *Br. Poultry Sci.* **18**, 661–667 (1977)
64. B.M. Place, H.E. Tillett and N.F. Lightfoot, *J. Appl. Bacteriol.* **80**, 682–686 (1996)
65. C. Jeppesen, *Int. J. Food Microbiol.* **26**, 25–41 (1995)
66. *ISO 13720* Meat and meat products – enumeration of *Pseudomonas* spp. (1995)
67. R.M. Baird and W.H. Lee, *Int. J. Food Microbiol.* **26**, 15–24 (1995)
68. D.L. Collins-Thompson, A. Hurst and B. Aris, *Can. J. Microbiol.* **20**, 1072–1075 (1974)
69. M.E. Stiles and P.C. Clark, *Can. J. Microbiol.* **20**, 1735–1744 (1974)
70. A.T. Willis, J.A. Smith and J.J. O'Connor, *J. Pathol. Bacteriol.* **92**, 345–358 (1966)
71. J.T. Parisi, N.N. Baldwin and M. Sottile, *Appl. Microbiol.* **25**, 558–561 (1973)
72. A.H.W. Hauschild, C.E. Park and R. Hilsheimer, *Can. J. Microbiol.* **25** 1052–1057 (1979)
73. *Dutch Standard EN 6876.* Milk and milk products. Determination of Staphylococcus aureus contents by the pour plate technique

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Chapter 9

SELECTED EXAMPLES (IMPURITY PROFILING OF SOME GROUPS OF DRUGS)

9.1. Peptides

Sándor Görög

9.1.1. Methodological Aspects

There were several reasons to select peptides from among the numerous groups of drug materials amongst those which are discussed in detail in this chapter. In the research, development and production of peptide drugs impurities play a much more important role than in other cases. As a consequence of the specialties of the structure of peptides, the high number of possible isomers, among them stereoisomers, the high number of reactive sites in their molecules, the frequent necessity of the introduction and removal of protecting groups creates a great many possibilities for side reactions and the formation of by-products in the case of both the classical, solid phase and biotechnological peptide syntheses. In addition, the chemical stability of peptides is limited, sometimes definitely poor: they are subject to hydrolytic, oxidative and other attacks. The elucidation of the degradation pathways of peptides and kinetic investigation of the reactions involved are subjects for detailed studies. For this reason the separation, detection, isolation, identification or structure elucidation of impurities and degradation products in this field are very important issues.

Due to the usually poor possibilities for the purification of peptides by crystallisation preparative HPLC belongs to the daily routine here from the beginning of the synthetic research to the industrial-scale production. The analytical control of this is among the frequent tasks of analytical chemists giving support to the peptide research and production. Of course it is an

important rule that in the case of the analytical control by HPLC of the fractions obtained by preparative HPLC an entirely different HPLC system should be used to prevent co-elution of closely related impurities.

The structural characteristics which determine the possibilities and limitations of the use of chromatographic and spectroscopic techniques for the impurity profiling of peptides considerably differ from those of other groups of drugs. The molecular weight of peptides containing more than four to five amino acid units is higher than the average molecular weight of other drugs. Depending on the relative number and the nature of acidic and basic functional groups attached to the peptide chain the properties of these, usually amphoteric compounds can vary from acidic to basic, from hydrophilic to hydrophobic character. The ease of splitting larger peptide molecules hydrolytically or enzymatically to smaller units of even to amino acids is another important feature of peptides often exploited for their analytical characterisation.

In consequence of these features the use of gas chromatography in the estimation of impurities is restricted to smaller peptides and requires (double) derivatisation. Thin-layer chromatography is frequently used especially for smaller peptides. The most important methods for the analysis and among other areas for impurity profiling of peptides are high-performance liquid chromatography [1–6] and related hyphenated techniques. It is also the consequence of the great differences in these structural features that it is difficult to define a general HPLC system suitable for all kinds of peptides. Reversed phase methods are almost exclusively used mainly with porous C_{18} or C_8 supports. For small peptides, up to about 10 amino acid units the recommended pore size is about 100 Å, while for larger peptides 300 Å pore size is suitable. Columns with minimum number of free silanol groups, dedicated to peptide analysis are commercially available. Acidic eluents are generally used which suppress the ionisation of their carboxylic groups to decrease the hydrophilicity of the peptides thus increasing their retention on the apolar stationary phase. However, in the case of peptides with basic functional groups (arginine, lysine, histidine residues) positively charged species are formed under these conditions which are also poorly retained on RP-HPLC columns. The most widely used method to increase the retention and the selectivity and improve the peak shape to make the method suitable for impurity evaluation is the use of ion-pairing reagents in the eluent. In simple cases even the weak ion-pairing properties of the $H_2PO_4^-$ ion being present in the generally used weakly acidic phosphate buffer is sufficient. Trifluoroacetic acid as a volatile ion-pairing agent is used if the aim of the separation is obtaining larger quantities by (semi)preparative HPLC. Stronger ion-pairing agents such as hexane-, heptane- and dodecylsulphonates or perchlorates are also widely used.

Due to the above mentioned structural features peptides are the group

among drug materials where capillary electrophoresis has found wider application than in the case of others.

9.1.2. Synthesis-Related Impurities

The large variety of possible and real synthesis-related impurities and their separation by HPLC and capillary electrophoresis are demonstrated on

Table 9.1.A.
Numbering of the investigated peptides (thymotrinan, thymocartin and their potential and real impurities)

1	Thymotrinan (H-L-Arg-L-Lys-L-Asp-OH)
2	H-L-Arg-L-Lys-D-Asp-OH
3	H-L-Arg-D-Lys-L-Asp-OH
4	H-D-Arg-L-Lys-L-Asp-OH
5	Cyclo-(Asp-Val)[a]
6	Cyclo-(Lys-Asp)[a]
7	H-Asp-Val-OH
8	H-Lys-Asp-OH
9	Ac-Arg-Lys-Asp-OH
10	Cyclo-(Arg-Lys)[a]
11	H-Lys-Asp-Val-OH
12	Iso-BuOCO-Lys-Asp-OH[b]
13	H-Arg-Lys-OH
14	H-L-Lys-(H-L-Arg)-D-Asp-OH
15	Thymocartin (H-L-Arg-L-Lys-L-Asp-L-Val-OH)
16	H-L-Arg-L-Lys-D-Asp-L-Val-OH
17	H-L-Arg-L-Lys-L-Asp-D-Val-OH
18	H-L-Arg-D-Lys-L-Asp-L-Val-OH
19	H-D-Arg-L-Lys-L-Asp-L-Val-OH
20	Ac-Arg-Lys-Asp-Val-OH
21	Isothymocartin (H-Arg-Lys-Asp-(Val-OH)-OH)
22	Iso-BuOCO-Lys-Asp-Val-OH[b]
23	H-Arg-Lys-Asu-Val-OH[c]
24	H-Arg-Lys-Asp-Arg-Lys-Asp-Val-OH
25	H-Arg-Lys-Asp-(Arg-Lys-Asp-Val)-OH

[a] Diketopiperazine derivative
[b] Isobutyloxycarbonyl protecting group at the ε-amino group of the lysine residue
[c] Aminosuccinyl derivative of the aspartyl residue

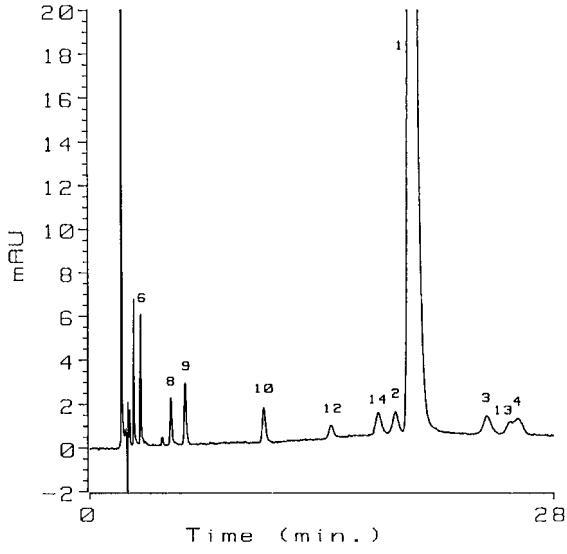


Figure 9.1.A.
HPLC separation of thymotrinan and related peptides (1% each) using hexanesulphonate as the ion-pairing agent. Column: Ultrasphere IP, C-18, 5 μm,250 × 4.6 mm; eluent: 30 mM sodium 1-hexanesulphonate and 20 mM phosphate buffer in water/methanol 70:30 v/v, pH 3.0, set by phosphoric acid; UV detector 210 nm; temperature: 40°C. For the Key see Table 9.1.A (from Ref. [8])

the example of two experimental peptide drugs *thymotrinan* (H-Arg-Lys-Asp-OH) and *thymocartin* (H-Arg-Lys-Asp-Val-OH) [7]. The two drug candidates and the possible and real impurities based on the papers of Gazdag *et al*. [8,9] are summarised in Table 9.1.A.

In Figs. 9.1.A and 9.1.B the RP-HPLC separation of thymotrinan and its potential impurities is shown using acidic eluents containing hexanesulphonate and perchlorate as the ion-pairing agents, respectively. Figures 9.1.C and 9.1.D are illustrations of the separation of thymocartin and its potential impurities with the aid of the same ion-pairing reagents [8].

As an alternative to the HPLC separation the results of the separation of the impurities by capillary electrophoresis is also demonstrated in Fig. 9.1.E [9].

The potential and real impurities summarised in Table 9.1.A represent some of the typical synthesis-related impurities in synthetic peptides. Impu-

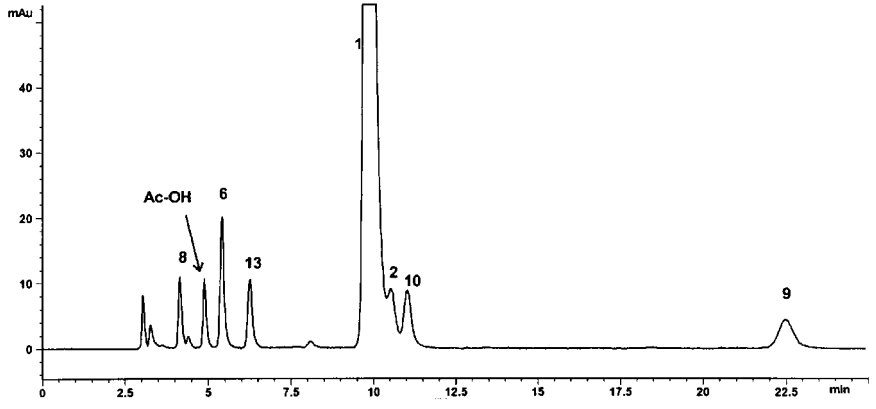


Figure 9.1.B.
HPLC separation of thymotrinan and related peptides (1% each) using perchlorate as the ion-pairing agent. Column: YMC ODS-AQ, 5 μm, 250 × 4.6 mm; eluent: 100 mM sodium perchlorate in water, pH 2.0, set by perchloric acid; UV detector 210 nm; temperature: 25°C. For the key see Table 9.1.A (from Ref. [8])

rities **5**, **6** and **10** are cyclic diketopiperazine derivatives. Due to their entirely different polarities their separation from the main components is not problematic. The situation is similar with impurities containing bulky apolar protecting groups (**12**, **22**). Derivatives in which the α-amino group is acetylated can also be easily separated from the de-acetyl derivatives (**1/9**, **15/20**).The separation of isopeptides is a much more difficult task. Here the peptide chain is transferred from the α-carboxyl group to the β- and γ-carboxyl groups of the aspartic and glutamic acid residues, respectively via a cyclic aminosuccinyl (ASU) derivative (**23**). The separation of iso-thymocartin (**21**), where the valine residue is attached to the β-carboxyl group of the aspartic acid, from thymocartin (**15**) by HPLC requires careful optimisation (Figs. 9.1.C and D). The easy separation of **21** from **15** by capillary electrophoresis (Fig. 9.1.E) is remarkable. This technique separates from each other also the two isomers of the synthesis-related heptapeptide (**24**, **25**) impurity [10].

The most difficult (and most interesting) problems are associated with the separation of diastereomeric derivatives. The source of these can be the not sufficient enantiomeric purity of the starting materials (amino acids or their protected or activated derivatives) and also the inversion of certain amino acid residue(s) (epimerisation) during the synthesis. Under the carefully optimised

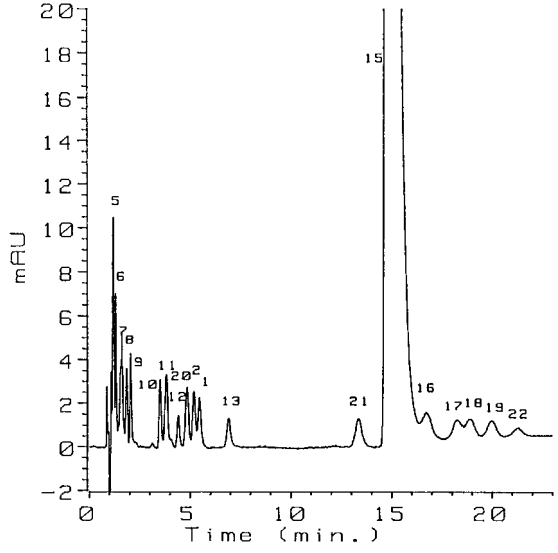


Figure 9.1.C.
HPLC separation of thymocartin and related peptides (1% each) using hexanesulphonate as the ion-pairing agent. Column: Hypersil BDS, C-18, 3 μm, 100 × 4.6 mm; Eluent: 30 mM sodium 1-hexanesulphonate and 20 mM phosphate buffer in water/methanol 68:32 v/v, pH 3.0, set by phosphoric acid; UV detector 210 nm; Temperature: 40°C. For the key see Table 9.1.A (from Ref. [8])

HPLC conditions of Figs. 9.1.A–D, the separation of the diastereomers of both thymotrinan and thymocartin is accessible and the separation from the main component (and partly from each other) is possible by capillary electrophoresis, also. It is worth mentioning, that the diastereomers **3**, **4** of thymotrinan and all diastereomers of thymocartin (**16**–**19**) are only potential impurities and do not occur in real samples. The only real impurity in the early stage of the research was **2** (H-L-Arg-L-Lys-D-Asp-OH) in thymotrinan. The reason for its formation was the epimerisation of the aspartyl residue of the protected thymotrinan (Boc-Arg(HCl)-Lys-Asp(O^{t}Bu)-O^{t}Bu) upon treatment with chloroform, water and dimethyl formamide [11]. It is worth mentioning that the eluent in these early RP-HPLC tests was aqueous phosphate buffer (15 mM) at the unusually high pH of 9. No separation was achieved at pH 8 and below this [12,13]. The diastereomers of the above mentioned protected derivative could not be separated. In this case the determination of the LLD derivative

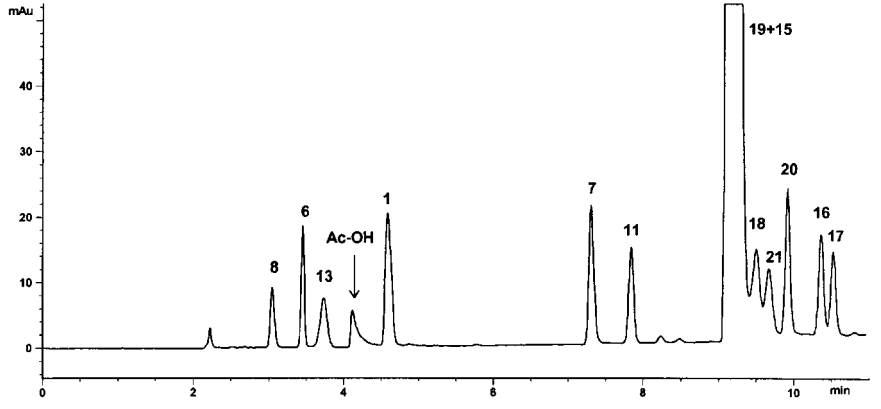


Figure 9.1.D.
HPLC separation of thymocartin and related peptides (1% each) using perchlorate as the ion-pairing agent. Column: Ultrasphere IP C-18, 5 μm, 250 × 4.6 mm; eluent: A = 50 mM sodium perchlorate in water, pH 2.0, set by perchloric acid, B = acetonitrile, linear gradient from 1 to 19% B in 9 min and 5B = 19 for 2 min; UV detector 210 nm; temperature: 25°C. For the key see Table 9.1.A (from Ref. [8])

was traced to the underivatised drug. The protecting groups can be quantitatively removed by treatment with trifluoroacetic acid at room temperature and the neutralised reaction mixture is suitable for direct injection into the chromatograph. It was found that no racemisation takes place during the removal of the protecting groups, hence this indirect method is suitable for the determination of the protected LLD impurity in protected thymotrinan [12].

The estimation of the impurity profile of the thymic humoral factor *THF-γ2* (H-Leu-Glu-Asp-Gly-Pro-Lys-Phe-Leu-OH) by Forino *et al.* [14] is a good example for the necessity of the complex use of chromatographic and spectroscopic methods to determine the structure and origin of impurities in the product of the large-scale synthesis of an experimental peptide drug. These included two different gradient RP-HPLC methods (acidic phosphate buffers with acetonitrile as the organic modifier), thin-layer chromatography, high-voltage paper electrophoresis, isolation of the impurities by preparative HPLC followed by high-resolution NMR (600 MHz) and FAB-MS studies. It has to be emphasised that in this study and all other studies where the impurities are isolated for off-line investigation amino acid analysis also plays important role in the structure elucidation of the impurities. Seven impu-

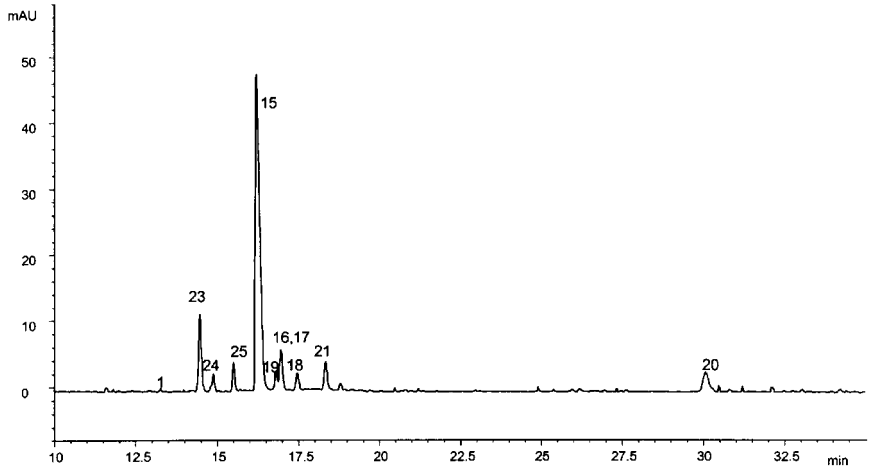
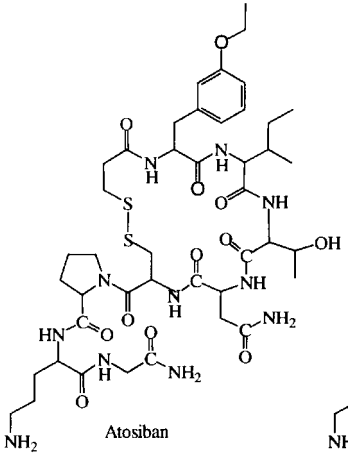


Figure 9.1.E.
Model electropherogram. Separation of thymocartin and related peptides (5% each) by capillary electrophoresis. Fused silica capillary: 70 cm × 50 μm; buffer: 50 mM triethylammonium phosphate containing 10% v/v acetonitrile, pH 3.0, set by phosphoric acid; voltage 20 kV; UV detector (bubble cell) 210 nm; temperature 30°C. For the key see Table 9.1.A (from Ref. [9])

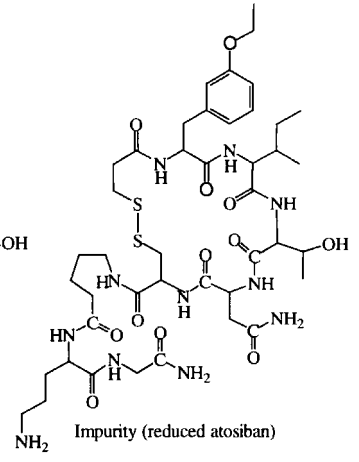
rities were separated and identified. These included two minor fragments (H-Leu-Glu-Asp-OH and H-Gly-Pro-Lys-Phe-Leu-OH) and products of side reactions, similar to those mentioned in connection with the previous examples. One of them was the Asu analogue of the final product: H-Leu-Glu-Asu-Gly-Pro-Lys-Phe-Leu-OH). Two others were isopeptides, an analogue branched at the aspartyl residue: H-Leu-Glu-Asp-OH-(Gly-Pro-Lys-Phe-Leu-OH) and a dodecapeptide branched at the lysine residue: H-Leu-Glu-Asp-Gly-Pro-Lys-(Gly-Asp-Glu-Leu-H)-Phe-Leu-OH. Two further impurities (Z-Leu-Glu(OBzl)-Asp(OBzl)-Gly-OCH_2CHCl_2 and H-Pro-Lys-Phe-Leu(OBzl)-OH) are derivable from the key intermediates.

The structure of a synthesis-related impurity of about 0.1% in *atosiban* (1-(3-mercaptopropionic acid)-2-[3-(p-ethoxyphenyl)-D-alanine]-4-L-threonine-8-L-ornithineoxytocin) was determined by Burinsky *et al.* [15] after preparative HPLC separation by FAB-MS-MS. The molecular mass of the impurity was found to be higher by two mass units than that of the main component. The fragmentation pattern obtained from the MS-MS study revealed that the location of the two additional hydrogens is at the two-

residue Pro-Orn unit. There are two possibilities to explain the increase in the mass of this unit: (1) reduction of the amide carbonyl or (2) opening of the proline ring. Possibility (2) was confirmed by GC-MS. After hydrolysis followed by double derivatisation of the amino acids (preparation of *N*-perfluoropropionyl isopropyl esters) proline was replaced in the chromatogram by 5-aminovaleric acid.



Atosiban



Impurity (reduced atosiban)

Solid phase peptide syntheses are also sources of various impurities. For a review of side reactions encountered when Fmoc-amino acids are used in solid phase syntheses see [16]. In the course of the impurity profiling of *Ac-Trp-Asp-Asn-Val-Leu-Asp-His-Leu-Thr-Gly-Arg-OH*, a T cell epitope from the S1 subunit of Pertussis Toxin, Pessi *et al*. [17] utilised sample displacement chromatography for the separation of the impurities which were characterised by FAB-MS. Two main impurities were found both involving the histidine residue: the C-terminal truncated sequence *Ac-Trp-Asp-Asn-Val-Leu-Asp-His-OH* and an analogue where the Asp residue in the vicinity of the histidine underwent cyclisation to the corresponding Asu unit.

Non-analytical papers describing the synthesis of peptides by solid-phase or solution syntheses or their combination followed usually by fractionation and purification by preparative HPLC also contain useful analytical data on the by-products formed during the syntheses. For example, in a recent paper in which Felix *et al*. [18] described the combined synthesis of a 31-residue vasoactive intestinal peptide analogue and characterised the fractions mainly by FAB-MS, amino acid analysis and circular dichroism, the main impurities of the endproduct were found to be diastereomers containing D-Glu^{8} or D-Ala^{19} or D-Asp^{25} residues.

The direct coupling of HPLC with mass spectrometry provides an extremely useful tool for the rapid characterisation of the products and side-products of peptide syntheses. For example, Casazza *et al*. [19] utilised HPLC/electrospray MS for the investigation of the crude product of the solid-phase synthesis of *neuropeptide Y*, a 36 amino acids long peptide. Several impurities were detected by HPLC/UV and identified as incomplete sequences commonly observed in solid-phase peptide syntheses.

Another study based on a similar strategy is the impurity profiling of the synthetic LHRH antagonist *ramorelix* (Ac-[D-3(2-naphthyl)-alanyl-*p*-chlorophenylalanyl-D-Trp-L-Ser-L-Tyr-*0*-à-L-Rhamnosyl-D-Ser-L-Leu-L-Arg-L-Pro-semicarbazide] by Ermer [20]. In Fig. 9.1.F HPLC/UV scans A–D represent four different stages of the development of the drug material from toxicological studies when only the qualification of the impurities is necessary to scaling-up. The main impurities were characterised by HPLC/electrospray MS using an ion trap instrument. The volatile buffer described in the figure legend is eminently suitable for this study.

Although L-*Lysine N-acetylcysteinate* does not contain a peptide bond, it is mentioned here that Toussaint *et al*. [21] successfully used HPLC/atmospheric pressure chemical ionisation MS-MS technique for the separation, characterisation and determination of the impurities (L-cysteine, L-cystine, *N,N'*-diacetylcystine and *N,S*-diacetylcysteine) of this experimental drug.

As mentioned in the introductory remarks of this section capillary electrophoresis (CE) is the method of choice for the separation and characterisation of impurities in peptides. The simultaneous use of HPLC and CE gives orthogonal information which can be very useful in the case of complex impurity profiles. As an example the work of Shane and Hettiarachchi [22] is mentioned describing the impurity profiling of *bradykinin* (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH). For the HPLC study C_{18} columns with water/acetonitrile/trifluoroacetic acid gradient systems were used. It is remarkable that with a support of 90 Å pore size much better resolution of the impurities was attainable than with a 300 Å column. Even better resolution of the impurities was obtained by CE using LPA coated capillaries and phosphate buffer at pH 4.5 as the electrolyte. The CE technique is suitable also for the determination of the acetate counter ion in the peptide [22,23]. For this reason a fused silica capillary was used with phthalate as the UV-absorbing electrolyte and cetyltrimethylammonium bromide as the electroosmotic flow modifier.

Making use of the observations made in the course of previous studies [24], Miller and Rivier [25] confirmed the beneficial effects of using acetonitrile (up to 15% v/v) in the CE buffer in a study aiming at the separation of various GnRH analogues and their impurities. Another important result of this study was the finding that the substitution of the generally used sodium phosphate/phosphoric acid buffers (pH 2.5) by a similar electrolyte but using

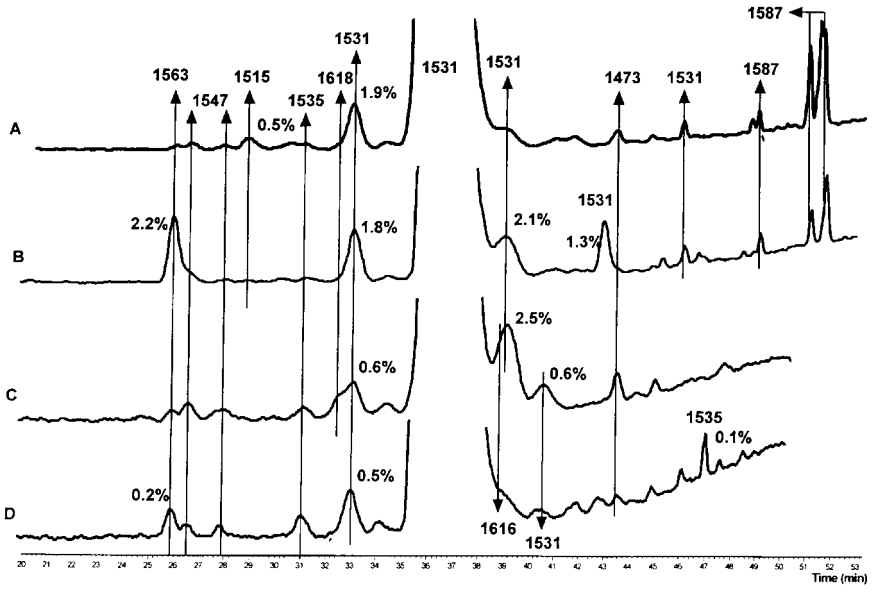


Figure 9.1.F.
HPLC scans of various batches of ramorelix used in toxicological (A) and clinical (B,C) studies and from scale-up (D); m/z ratios for relevant peaks are indicated above or below the arrows; ($M_{ramorelix}$ = 1530.7). The area per cent values of the main impurity peaks (from the UV scan) are also indicated. Column: LiChrospher™ PAH, 5 μm, 250 × 4 mm; eluent: A: acetonitrile-water-trifluoroacetic acid 100:900:2 v/v/v, B: 900:100:1.5 v/v/v. 35 min isocratic period (68% A, 32% B) followed by 20 min gradient elution: 2% B/min; flow rate: 0.5 ml/min; temperature: 30°C; UV detector: 230 nm (from Ref. [20])

triethylammonium phosphate instead of sodium phosphate greatly improves the resolution of the analogous derivatives. For example baseline separation was obtained between *Nal-Glu GnRH* and its position isomeric impurity where the only difference is that it contains a 2-methoxybenzoyl unit instead of the 4-methoxybenzoyl group.

The high selectivity and sensitivity of on-line coupling capillary electrophoresis with mass spectrometry (see Section 2.8.2) can be utilised also for the detection and identification of impurities in peptides. In a model study Hsieh *et al.* [26] investigated the detectability of Lys-bradykinin as an impurity in *Met-Lys-bradykinin.* It was found that while 0.1% of the impurity was not detect-

able by CE/UV, a well evaluable peak was obtained by CE/MS under selected ion monitoring conditions.

Finally the purity tests for two important peptide drugs, *insulin* and *human growth hormone* are shortly discussed. The first HPLC method suitable for the routine-scale separation and quantitation of desamido insulins, proinsulins and other impurities in *insulin* was described by Szepesi and Gazdag [27]. In this method the separation was achieved by a C_{18} column gradient elution with a mixture of organic modifiers and acidic phosphate buffer with high concentration of inorganic salts. In the HPLC method of USP 24 [28] for the determination of desamido insulin and other insulin-related impurities (limit 3%) also acidic phosphate buffer (pH 2) and gradient elution with acetonitrile is used. A similar system is also used by the European Pharmacopoeia [29]. Both pharmacopoeias prescribe a test based on size-exclusion chromatography for the determination of impurities with molecular masses greater than that of insulin (limit 1%). The determination of the proinsulin content in USP 24 is based on radioimmunoassay (limit 10 ppm). The capillary electrophoretic method is also suitable for the separation of desamido insulin and other related impurities from insulin and can be a method of choice for its purity check as described among others by Yomota *et al*. [30,31].

Dupin *et al*. [32] described a capillary electrophoretic method for the purity check of *human growth hormone (hGH).* Figure 9.1.G shows a typical electropherogram. In the same paper a RP-HPLC method is also described for the same purpose.

9.1.3. Enantiomeric and Diastereomeric Impurities

As a consequence of the natural or synthetic origin of polypeptides the determination of enantiomeric purity is not an important issue in the estimation of impurity profiles of high molecular weight peptide drugs with high number of chiral centres therein. There are, however, three lines in peptide analysis, where enantiomeric purity check still plays important role.

(a) In the case of small peptides the probability of the occurrence of enantiomeric impurities increases with the decreasing number of amino acid units. The enantiomers of di- and tripeptides with one or two chiral centres (containing a glycyl residue) were separated by capillary electrophoresis using (+)-18-crown-6-tetracarboxylic acid as the chiral selector [33–35]. Sänger-van de Griend *et al*. [36] used heptakis(2,6-di-*O*-methyl)-β-cyclodextrin for the separation of the enantiomeric DLDD impurity from the μ-opioid receptor selective compound *LEF 553* (*H-Tyr-Arg-Phe-Phe-NH$_2$).* It is interesting to note that in the buffer system (phosphoric acid/triethanolamine, pH 3) of the possible eight enantiomeric pairs of the 16 stereoisomers the separation of the

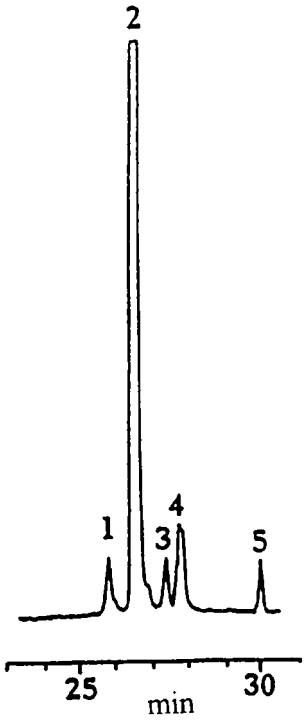


Figure 9.1.G.
Electropherogram of human growth hormone (hGH) spiked with related impurities. CE conditions: fused silica capillary 92 cm × 50 μm I.D.; UV detector (195 nm) at 70 cm; running buffer 0.1 M diammonium hydrogenphosphate, adjusted to pH 6.0 with phosphoric acid.; voltage 20 kV. Key: 1. cleaved hGH; 2. hGH; 3. Gln 18-hGH; 4. deamidated hGH; 5. *N*-succinylated hGH (from Ref. [32])

DLDD/LDLL, DDDD/LLLL, DLLD/LDDL, DDLL/LLDD and DDLD/LLDL pairs was excellent, the separation LLLD/DDDL was fair, DLLL/LDDD was poor and no separation was achieved for the DLDL/LDLD pair (faster migrating/slower migrating). In the study of Bazylak [37] the enantiomeric and diastereomeric impurities of dipeptide-type sweeteners, *aspartame* (α-L-Asp-Phe-OMe), *alitame* (aspartyl-*N*-(tetramethyl-3-thietanyl)alaninamide) and *antiaspartame* (Ac-Phe-Lys-OH) were separated and determined by RP-HPLC using the helical *dl*-[4,4′-(1-methyl-2-propylethane-1,2-diyldiimino)-*bis*(pent-3-en-2-onato)]nickel(II) as the chiral additive in the mobile phase.

(b) As already mentioned earlier one reason for the presence of diastereomeric impurities in synthetic peptides can be the enantiomeric impurities in amino acids, their protected and activated derivatives used as building blocks in the peptide syntheses. For this reason the enantiomeric purity check of these is an important task of analytical chemists working in peptide research teams. The monographs and reviews cited in Section 6.2 contain several possibilities for the determination of the enantiomeric purity of peptides. All the three possibilities described in detail there are widely used. Many reagents are available for the *covalent chiral derivatisation* prior to the HPLC or GC separation on achiral stationary phases (Section 6.2.1). These usually react with the α-amino group of the amino acids. The derivatising agent D- or L-*O*-(4-nitrobenzyl)-tyrosine methyl ester, introduced by Görög *et al*. [38] reacts with the carboxyl group and hence it is eminently suitable for the chiral derivatisation of *N*-protected amino acids and active esters. Dynamic formation of *diastereomeric adducts* is also widely used for the separation of enantiomeric impurities in amino acids by HPLC (Section 6.2.2) and CE (6.3). The use of *chiral HPLC stationary phases* (Section 6.2.3) is also an important method for the chiral purity check of amino acids. The paper of Pronce and Tilquin [39] (described in Section 6.2.3) merits special attention: by the combined use of fluorimetric derivatisation and separation on a chiral stationary phase at little as 21 ppm of D-aspartic acid could be determined in L-aspartic acid.

(c) Racemisation (epimerisation) is one of the most frequently occurring side reactions and degradation reactions in peptide syntheses (see Section 9.1.4) and the estimation of the site of epimerisation is an important task. This problem can be solved by hydrolysing the peptide to amino acid units (usually by 6 M hydrochloric acid at elevated temperatures) and separating the obtained mixture of amino acids by one of the chiral chromatographic methods described in Sections 6.2 and 6.3 which is suitable for the separation of the amino acids and their trace-level enantiomeric impurities. The most generally used method is based on the reaction with the Marfey reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) to form diastereomeric derivatives separable on achiral HPLC stationary phases [40–42]. For example, the LLD impurity in *thymotrinan* (H-Arg-Lys-Asp-OH) discussed earlier in this section [8,11–13] was also detected by hydrolysis, derivatisation by the Marfey reagent and detection of the diastereomeric derivative of D-aspartic acid. A source of error in this method is that racemisation may take place during the hydrolysis. The method of Goodlett *et al*. [43] circumvented this problem by using deuterated hydrochloric acid in deuterated acetic acid as the reagent for the hydrolysis of the peptide. Under these conditions the α-proton is exchanged to deuterium in the amino acids which racemised during the hydrolysis. This means that after the derivatisation by the Marfey reagent the molecular mass of the diastereomers originating from the racemisation during the hydrolysis

will be higher by one mass unit than those which did not racemise under these circumstances. The diastereomers were separated by gradient elution in a RP-HPLC system and the chromatogram was monitored in the ESI-MS mode (no fragmentation) at the molecular masses of the protonated derivatives cancelling out the contribution of those originating from the racemisation during the hydrolysis. The method was successfully used among others for the determination of the enantiomeric purity of the amino acids in a hexapeptide H-Arg-Lys-Lys-Asp-Val-Tyr-OH, isolated as an impurity of *thymopentin* (H-Arg-Lys-Asp-Val-Tyr-OH). Other chiral HPLC derivatisations of the amino acids e.g. with *o*-phthalaldehyde-*N*-acetyl-L-cysteine to form the fluorimetrically active diastereomeric isoxazole derivatives [44], moreover homochiral double derivatisations and separation on chiral GC capillaries [45,46] is also in use.

As already mentioned in the previous section and demonstrated with several examples both there and also in the following section, the separation of diastereomeric peptides is among the most frequently occurring tasks in peptide research and various RP-HPLC and CE methods are available for this purpose. However, as Straub *et al.* [47] have demonstrated, the separation of diastereomers of larger peptides is not always an easy task: in the course of the investigation of the *bradykinin analogue RMP-7 (H-Arg-Pro-Hyp-Gly-Thienylalanine-Ser-Pro-4-Me-Tyr-ψ-(CH_2NH)-Arg-OH)* they could not separate the diastereomers 8D9L and 8L9D with possible D-amino acids in the vicinity of the reduced peptide bond. The problem was solved by the enzymatic splitting of the thienylalanine-serine bond by the enzyme pronase and subsequent separation of the forming diastereomeric tetrapeptides by achiral RP-HPLC.

9.1.4. Degradation Products

Due to the special structural features of peptides i.e. the peptide bonds in the backbone, carboxamide and other bonds in the side chains susceptible to hydrolytic, oxidative, reductive and other attacks stability studies and the identification of degradation products are of pronounced importance in the peptide research and analysis. The analytical aspects of the degradation of peptides and proteins has been reviewed by Reubsaet *et al.* [48]. The following main types of degradation reactions of peptide drugs have to be taken into account:

(a) *Hydrolytic splitting of the peptide chain.* Any peptide bond in the peptide backbone is subject to hydrolytic splitting both in acidic and alkaline medium. N-terminal ends of serine and threonine and both ends of the aspartyl residues are especially sensitive to hydrolytic attacks. In the latter case not only splitting but also isomerisation (transpeptidation) can take place leading to iso-

Asp forms via the cyclic amide intermediate as exemplified in connection with H-Arg-Lys-Asp-Val-OH in Section 9.1.2.

(b) *Deamidation reactions.* Carboxamide groups (C-terminal amides, asparagine, glutamine residues) are subject to hydrolytic attack leading to the free carboxylic acids. In the latter cases this involves the formation of cyclic amide intermediates.

(c) *Degradation of the guanidino group of the arginine residues.* The hydrolytic splitting of the guanidino group in alkaline media transforms the arginine units to ornithine and/or citrulline.

(d) *Cyclisation.* Peptides with N-terminal glutamic acid residue often undergo cyclisation to form pyroglutamyl unit. Cyclisation to diketopiperazine derivatives is also an often occurring degradation reaction of dipeptides or other peptides where two amino acids at the end of the peptide chain are involved in the reaction.

(e) *Racemisation.* This reaction (often called epimerisation in peptide chemistry) may take place at any of the chiral amino acid residues in the peptide chain via a carbanion formed by the loss of the α-methine hydrogen. Aspartyl and glutamyl residues are especially prone to racemisation via cyclic intermediates (see the example of the formation of H-Arg-Lys-Asp in Section 9.1.2). Alkaline media are especially favourable for racemisation but acid catalysed racemisation may also take place.

(f) *Oxidation.* Most typically this involves the formation of intra- or intermolecular disulphide bridges from the sulphydryl groups of cysteine residues and the transformation of the S-CH_3 group in the methionine residue to the sulphoxide. Under more drastic conditions these are further oxidised and the oxidation of other amino acid units also may take place: oxidative ring opening of proline, histidine and tryptophan, dimerisation of tyrosine residues at the o,o'-position and of tryptophane residues at the $2,2'$ position of their aromatic rings, etc.

(g) *Reduction.* This involves the disruption of the disulphide bond effected by various reducing agents, antioxidants, etc.

In the following part a few characteristic examples are shown from among the very high number of papers published on peptide degradation.

The study of Oliyai and Borchardt [49] on the pH-profile of the degradation of the hexapeptide *H-Val-Tyr-Pro-Asp-Gly-Ala-OH,* supported by HPLC and FAB-MS is an example for the pure hydrolytic splitting mechanism (*a*). In strongly acidic solutions (below pH 4) the main reaction was the splitting to H-Val-Tyr-Pro-Asp-OH and H-Gly-Ala-OH with the simultaneous formation of the cyclic H-Val-Tyr-Pro-Asu-Gly-Ala-OH derivative. At pH 4-5 Transpeptidation to form the isopeptide H-Val-Tyr-Pro-Asp-OH-(Gly-Ala)-OH was also observed (via the latter Asu peptide). The isopeptide was found to be the only degradation product above pH 6. Transpeptidation was detected and kinetically

investigated by Vadász *et al*. [50] by HPLC in the course of a study when the hydrolysis of analogues of the *gonadotropin releasing hormone* containing aspartic acid in position 6 was studied.

Pure degradation mechanisms exemplified by the previous studies involving only one of the above listed possible reaction routes are not characteristic of peptide degradations: usually simultaneous reactions with mixed mechanisms take place. As the first example the paper of Reubsaet *et al*. [45] is shortly mentioned. They investigated the degradation of the antitumour hexapeptide *antagonist G (H-Arg-Trp-MePhe-Trp-Leu-Met-NH_2)*. The analytical methods used in this study included HPLC with fluorescence detection after precolumn derivatisation of antagonist G and the arginine-containing degradates with benzoin [51], HPLC/ESI-MS, FAB-MS-MS, capillary electrophoresis and chiral GC (acidic hydrolysis, double derivatisation and chromatography on the chiral stationary phase Chirasil-L-Val). The identified degradates included terminally deaminated substance in acidic solution and the same accompanied by racemisation product at the methionine unit, oxidation product of the same to the sulphoxide and the products of the transformation of the arginine residue to ornithine. The same group investigated as model compounds the degradation of H-Arg-Trp-Phe-OH, H-Arg-Trp-Phe-NH_2, H-Arg-Trp-Phe-OH and H-Arg-Trp-Phe-NH_2, also, in order to get a more detailed insight into the mechanism of the degradation of antagonist G. Deamidation, hydrolysis to dipeptides, formation of diketopiperazines, transformation of the arginine residue to ornithine and racemisation at the Arg and Phe residues were found to be the degradation products [46].

Hoitink *et al*. [44] investigated the degradation products of LH-RH analogues in aqueous solutions under acidic, neutral and basic conditions. The subject of the study was the native hormone *gonadorelin (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2)* and its synthetic analogues *buserelin, goserelin* and *triptorelin*. The 6-Gly residue is replaced by D-Ser(O^tBu) in buserelin and goserelin and by D-Trp in triptorelin, while the terminal Gly-NH_2 residue in the native hormone is replaced by Gly-NHC_2H_5 in buserelin and by NH-NH-CO-NH_2 in goserelin. The methods used were RP-HPLC, chiral amino acid analysis: hydrolysis followed by chiral derivatisation and RP-HPLC, HPLC/MS, FAB-MS-MS of samples separated by RP-HPLC using volatile buffers.

The main degradation pathway of gonadorelin and triptorelin *in acidic solutions* is deamidation while the elimination of the *tert*-butyl moiety of the D-Ser(O^tBu) is the main reaction with buserelin and goserelin. As minor components the products of the hydrolysis or elimination of the pyroglutamyl group were also detected. In *neutral solutions* the main degradation reaction is the hydrolytic splitting of the Trp-Ser bond in all derivatives to form pGlu-His-Trp-OH. This is followed by further splitting/cyclisation/epimerisation to form diketopiperazines cyclo(L-His-L-Trp) and cyclo(D-His-L-Trp). The other part

of the molecules as analogue-specific hexa- and heptapeptides end their epimers at the Ser residue also appear as degradation products. In the case of goserelin the elimination of the semicarbazide moiety is the predominant reaction. The main degradation products of the hydrolysis in alkaline medium are epimerisation at the L-^{4}Ser moiety with some deamidation products of gonadorelin and triptorelin and hydrolysis of the azaglycinamide residue of goserelin.

The aim of the studies taken as examples until now was the investigation of the stability of the peptides under various conditions in order to get acquainted with their properties during the synthesis, isolation and the preformulation studies. The estimation of the stability and degradation profile of real dosage forms of peptide drugs is also an important task. As an example the investigation of the stability of *Pramlintide* injection is shortly discussed. Pramlintide containing 37 amino acids is a synthetic analogue of human amylin, co-secreted with insulin from pancreatic β-cells. Hekman *et al*. [52] investigated heat stressed Pramlintide injection (pH 4; 40°C, 45 days) by strong cation exchange HPLC, RP-HPLC and HPLC/ESI-MS. 13 degradation products were separated. The main degradation product was found to be a deamidation product: [iso–Asp^{21}]-pramlintide. The minor impurities were mainly also deamidation products at various Asn residues but backbone cleavage at His^{18}/Ser^{19} and Ser^{19}/Ser^{20} also took place.

The results of the degradation studies of human and salmon *calcitonin,* a peptide drug with 32 amino acids was reviewed by Cholewinski *et al*. [53]. The critical point of the stability of calcitonin is the disulphide bridge. At high pH values β-elimination takes place leading to dimers and polymers. At low pH the cysteine residue is reduced to two cysteine units. Both calcitonins contain two Asn and 2 Gln residues: their deamidation is also part of the degradation mechanism.

In addition to degradation studies of peptides in the dissolved state the investigation of their stability in the solid state is also an important issue. For example Pikal and Rigsbee [54] investigated the stability of freeze-dried *zinc insulin* by measuring the deamidation by RP-HPLC and the dimerisation by size-exclusion HPLC. The interesting result was that amorphous insulin was far more stable than crystalline insulin. An interesting approach to the stability testing of insulin is the ''fingerprint analysis'' introduced by Oliva *et al*. [55]. In this method the insulin sample undergoes proteolytic splitting by *Staphylococcus aureus* protease followed by HPLC check of the resulting fragments. The fingerprint-like pattern thus obtained is characteristic of the origin of the sample and the degree of its decomposition.

An interesting excipient-induced solid-state degradation product of *Ac-Cys-Asp-(5,5-dimethyl-4-thiazolidinecarbonyl)-(4-aminomethyl-Phe)-Gly-Asp-Cys cyclic disulphide* has been described by Dubost *et al*. [56]. Already the

UV spectrum with its maximum at 257 nm betrayed on a significant change at the 4-aminomethyl-phenylalanine unit of the potential drug molecule. MS, NMR, amino acid analyses and derivatisation of the degradate with 2,4-dinitrophenylhydrazine after preparative HPLC revealed that its 4-aminomethyl group was oxidised to aldehyde to form a benzaldehyde moiety. The oxidising agent was found to be an impurity in mannitol used in the course of lyophilisation of the drug.

For the acid and base catalysed deamidation and epimerisation of *posatirelin* (L-Kpc-L-Leu-L-Pro-NH_2) and the application of the Arrhenius equation to the extrapolation of the degradation data obtained at elevated temperatures to ambient temperature see Figs. 5.5.B and 5.5.C and Ref. [14] in Section 5.5.3.

References

1. M.T.W. Hearn, F.E. Regnier and C.T. Wehr (Eds.), *High-Performance Liquid Chromatography of Proteins and Peptides*, Academic Press, New York (1983)
2. W.S. Hancock (Ed.), *CRC Handbook of HPLC for the Separation of Amino Acids, Peptides and Proteins*, CRC Press, Boca Raton, FL (1984)
3. A. Henschen, K.-P. Hupe, F. Lottspeich and W. Voelter (Eds.), *High Performance Liquid Chromatography in Biochemistry*, pp 139–318, VCH, Weinheim (1985)
4. J. Pick, in *HPLC in Pharmaceutical Analysis*, Vol. II (G. Szepesi, Ed.), pp 1–63, CRC Press, Boca Raton, FL (1991)
5. R.S. Hodges and C.T. Mant, *High-Performance Liquid Chromatography of Peptides and Proteins*, CRC Press, Boca Raton, FL (1991)
6. Gy. Szókán and T. Janáky, Nagyhatékonyságú folyadék-kromatográfia a peptidkémiában *(HPLC in Peptide Chemistry)*, Akadémiai Kiadó, Budapest (1995)
7. L. Kisfaludy, O. Nyéki, I. Schön, J. Dénes, L. Ember, L. Szporny, Gy. Hajós and B. Szende, *Hoppe-Seyler's Z. Physiol. Chem.* **364**, 933–940 (1983)
8. M. Gazdag, K. Mihályfi and S. Görög, *Anal. Chim. Acta* **352**, 231–237 (1997)
9. M. Gazdag, K. Mihályfi, P. Kemenes-Bakos and S. Görög, *Acta Pharm. Hung.* **67**, 169–174 (1997)
10. I. Schőn and O. Nyéki, *J. Chem. Soc., Chem. Commun.* 393–394 (1994)
11. I. Schőn, L. Kisfaludy, O. Nyéki, B. Herényi and S. Görög, in *Peptides 1988* (G. Jung and E. Bayer, Eds.), pp 49–51, Walter de Gruyter, Berlin (1989)

12. S. Görög, B. Herényi, O. Nyéki, I. Schőn and L. Kisfaludy, *J. Chromatogr.* **452**, 317–321 (1988)
13. B. Herényi, S. Görög, O. Nyéki, I. Schőn and L. Kisfaludy, *Chromatographia* **29**, 395–396 (1990)
14. R. Forino, M. Galantino, F. Ciprandi, L. Baumer, E. Arlandini and R. de Castiglione, *Int. J. Peptide Protein Res.* **43**, 513–519 (1994)
15. D.J. Burinsky, R. Dunphy, A.R. Oyler, C.J. Shaw and M.L. Cotter, *J. Pharm. Sci.* **81**, 597–600 (1992)
16. G.B. Fields and R.L. Noble, *Int. J. Peptide Protein Res.* **35**, 161–214 (1990)
17. A. Pessi, V. Mancini, P. Filtri and L. Chiappinelli, *Int. J. Peptide Protein Res.* **39**, 58–62 (1992)
18. A.M. Felix, Z. Zhao, T. Lambros, M. Ahmad, W. Liu, A. Daniewski, J. Michalewsky and E.P. Heimer, *J. Peptide Res.* **52**, 155–164 (1998)
19. A. Casazza, O. Curcuruto, M. Hamdan, A. Bisello and E. Peggion, *J. Chromatogr. A* **715**, 227–240 (1995)
20. J. Ermer, *J. Pharm. Biomed. Anal.* **18**, 707–714 (1998)
21. B. Toussaint, A. Ceccato, Ph. Hubert, J. De Graeve, E. De Pauw and J. Crommen, *J. Chromatogr. A* **819**, 161–170 (1998)
22. S. Ridge and K. Hettiarachchi, *J. Chromatogr.* **817**, 215–222 (1998)
23. K. Hettiarachchi and S. Ridge, *J. Chromatogr.* **817**, 153–161(1998)
24. H.J. Issaq, G.M. Janini and K.C. Chan, in *Advances in Chromatography*, Vol. 35 (P.R. Brown and E. Grushka, Eds.), pp 101–169, Marcel Dekker, New York (1995)
25. C. Miller and J. Rivier, *J. Peptide Res.* **51**, 444–451 (1998)
26. F.Y.L. Hsieh, J. Cai and J. Henion, *J. Chromatogr. A* **679**, 206–211 (1994)
27. G. Szepesi and M. Gazdag, *J. Chromatogr.* **218**, 597–602 (1981)
28. *United States Pharmacopoeia 24*, p 883, USP Convention Inc., Rockville (2000)
29. *European Pharmacopoeia, 3rd edn*, Supplement, pp 590–595, Council of Europe, Strasbourg (1999)
30. C. Yomota, Y. Yoshii, T. Takahata and S. Okada, *J. Chromatogr. A* **721**, 89 (1996)
31. C. Yomota, Y. Matsumoto, S. Okada, Y. Hayashi and R. Matsuda, *J. Chromatogr. B* **703**, 139–145 (1997)
32. P. Dupin, F. Galinou and A. Bayol, *J. Chromatogr. A* **707**, 396–400 (1995)
33. R. Kuhn, D. Riester, B. Fleckenstein and K.-H. Wiesmüller, *J. Chromatogr. A* **716**, 371–379 (1995)
34. D. Riester, K.-H. Wiesmüller, D. Stoll and R. Kuhn, *Anal. Chem.* **68**, 2361–2365 (1996)
35. M.G. Smid and G. Gübitz, *J. Chromatogr. A* **709**, 81–88 (1995)

36. C.E. Sänger-van de Griend, K. Gröningsson and T. Arvidsson, *J. Chromatogr.* **782**, 271–279 (1997)
37. G. Bazylak, *J. Chromatogr.* **668**, 519–527 (1994)
38. S. Görög, B. Herényi and M. Lőw, *J. Chromatogr.* **353**, 417–424 (1986)
39. Th. Pronce and B. Tilquin, *J. Pharm. Biomed. Anal.* **14**, 175–184 (1996).
40. P. Marfey, *Carlsberg Res. Commun.* **49**, 591–596 (1984)
41. G. Szókán, G. Mező and F. Hudecz, *J. Chromatogr.* **444**, 115–122 (1988)
42. G. Szókán, G. Mező, F. Hudecz, Z. Majer, I. Schön, O. Nyéki, T. Szirtes and R. Dölling, *J. Liq. Chromatogr.* **12**, 2855–2875 (1989)
43. D.R. Goodlett, P.A. Abuaf, P.A. Savage, K.A. Kowalski, T.K. Mukherje, J.W. Tolan, N. Corkum, G. Goldstein and J.B. Crowther, *J. Chromatogr.* **707**, 233–244 (1995)
44. M.A. Hoitink, J.H. Beijnen, M.U.S. Boschma, A. Bult, E. Hop, J. Nijholt, C. Versluis, G. Wiese and W.J.M. Underberg, *Anal. Chem.* **69**, 4972–4978 (1997)
45. J.L.E. Reubsaet, J.H. Beijnen, A. Bult, E. Hop, R. Vermaas, Y. Kellekule, J.J. Kettenes-van den Bosch and W.J.M. Underberg, *Anal. Chem.* **67**, 4431–4436 (1995)
46. J.L.E. Reubsaet, J.H. Beijnen, E.H.M. Belshof, M. Bouyakhrichan, A. Bult, E. Hop, Y. Kellekule, R.J. van Maanen, J. Teeuwsen and W.J.M. Underberg, *J. Pharm. Biomed. Anal.* **19**, 277–284 (1999)
47. J.A. Straub, A. Akiyama, P. Parmar and G.M. Musso, *J. Chromatogr.* **679**, 85–91 (1994)
48. J.L.E. Reubsaet, J.H. Beijnen, A. Bult, R.J. van Maanen, J.A.D. Marchal and W.J.M. Underberg, *J. Pharm. Biomed. Anal.* **17**, 955–978 (1998)
49. C. Oliyai and R.T. Borchardt, *Pharm. Res.* **10**, 95–102 (1995)
50. Zs. Vadász, J. Seprődi, J. Érchegyi, I. Teplán and I. Schőn, *J. Chromatogr.* **477**, 377–385 (1989)
51. H. Cui, J.L.E. Reubsaet and A. Bult, *J. Chromatogr.* **736**, 91–96 (1996)
52. C. Hekman, W. DeMond, T. Dixit, S. Mauch, M. Nuechterlein, A. Stepanenko, J.D. Williams and M. Ye, *Pharm. Res.* **15**, 650–659 (1998)
53. M. Cholewinski, B. Lückel and H. Horn, *Pharm. Acta Helv.* **71**, 405–419 (1996)
54. M.J. Pikal and D.R. Ringsbee, *Pharm. Res.* **14**, 1379–1387 (1997)
55. A. Oliva, J. Fariña and M. Llabrés, *Drug. Dev. Ind. Pharm.* **23**, 127–132 (1997)
56. D.C. Dubost, M.J. Kaufman, J.A. Zimmerman, M.J. Bogusky, A.B. Coddington and S.M. Pitzenberger, *Pharm. Res.* **13**, 1811–1814 (1996)

9.2. Biotechnological Products

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9.2.1. Introduction

The European market for biotechnology-derived pharmaceuticals will increase from revenues of $2.34 billion in 1995 to $4.15 billion by 2002 [1]. The world-wide market for approved biopharmaceuticals in 1994 was estimated at about $7.5 billion (about 3% of the entire pharmaceutical industry) [2]. Biopharmaceuticals have reached this significant position in modern medicine because of the high specificity in their activity which could not be matched by low-molecular-weight drugs. This selective biological activity reflects the importance of the three-dimensional structure of the protein/oligonucleotide pharmaceuticals. In order to choose the correct biological system and process steps for expression or production of these biopharmaceuticals it is essential to understand the structural features of the biopolymers that are important to the desired therapeutic activity. A vast number and variety of analytical tools are involved in the structural studies of the products of modern biotechnology such as HPLC in various modes, capillary electrophoresis, mass spectrometry, nuclear magnetic resonance (NMR) spectroscopic techniques, circular dichroism (CD), X-ray diffraction and light scattering studies.

Therapeutic biopolymers include monoclonal antibodies (MAbs), interleukins (IL-s), interferons (IFN-s), growth factors (GF-s), tissue plasminogen activator (tPA), erythropoietin (Epo), tumour necrosis factor (TNF) and blood clotting factors. These proteins may be produced by hybridoma cell lines, by genetically engineered cells or by natural producer lines. The end product of the cell culture or fermentation contains the recombinant product together with impurities which consist of product related degradation products, fragments, or aggregates and contaminants such as host cell proteins, DNA etc. Following the biological expression these proteins are subsequently recovered, purified, and formulated. At each processing step the properties of the active ingredient can be modified and additional contaminants may be introduced while targeted contaminants are removed. The complex array of products along with the complexity of their production technologies has raised unique concerns regarding the safety of these biopharmaceuticals. These concerns result in a redefinition of the requirements for the quality control of therapeutic proteins by the regulatory agencies [3,4].

Table 9.2.A lists the potential impurities and contaminants of a biopharmaceutical production and the most frequently used analytical tools for their detection. Generally speaking all efforts have to be taken to guarantee that any product intended for human use is safe, efficient and will not cause severe side effect or complications.

9.2.2. Therapeutic Proteins

9.2.2.1. General Considerations

The application of recombinant DNA technology to express heterologous proteins in bacteria, yeast mammalian or in insect cells has allowed the production of proteins for therapeutic uses in quantities not attainable in the past. Table

Table 9.2.A.
Impurities and contaminants of concern during biopharmaceutical production and the analytical tools for their detection

	Impurity or contaminant	Analytical technique
1	Proteinaceous contaminants (host cell proteins)	SDS-PAGE, HPLC, HPCE, immunoassays (ELISA, etc.)
2	Endotoxins	Rabbit pyrogen test, Limulus amebocyte lysate (LAL)
3	DNA	DNA dot-blot hybridisation, Polymerase Chain Reaction (PCR)
4	Proteolytic degradation products	Isoelectric focusing (IEF), HPLC, HPCE, SDS-PAGE, N terminal sequencing
5	Presence of mutants and other residues	Tryptic or other enzymatic mapping
6	Deamidated/oxidised forms	IEF, HPCE, HPLC (often combined with mass spectrometry)
7	Microbial contamination	Sterility testing
8	Virus	Viral susceptibility assay
9	Mycoplasma	Code of Federal Regulation (CFR) method
10	General safety	CFR 610.11
11	Not sufficiently glycosylated form	Affinity HPLC, IEF (often combined with mass spectrometry)

9.2.B shows some examples of currently licensed/approved products and their expression systems.

The challenge in protein therapeutics is to acquire a large amount of a purified product under strictly controlled conditions. In order to purify a protein to near homogeneity, the purification protocol should be strictly designed. The purification process must be able to remove all the proteinaceous and nonproteinaceous contaminants from the crude product solution. At the same time further contamination during the purification operation has to be avoided. Lot-to-lot confirmation of product identity is usually conducted using an array of analytical methods including examining chromatographic retention (RP-HPLC), electrophoretic mobility (SDS-PAGE reduced, non-reduced), amino acid composition, peptide maps (reduced, non-reduced), carbohydrate composition and specific biological activity [4]. Regulatory guidelines recommend for the cell culture protein to be 95% pure when examined by an array of analytical techniques. Not one single impurity should exceed 1% of the total protein content. Identification of the unknowns should be attempted. In addition, the product should be pyrogen free and contain less than 10 pg of DNA in each dose received by a patient [4]. Table 9.2.C presents a list of the analytical arsenal recommended by the FDA for the characterisation of MAbs produced in rodent cell lines.

The composition of the starting crude material varies significantly from one product to the other and is affected by the nature of the host organism, type of production and characteristics of the individual cell line used in production. In general bacterial cells are used for producing non-glycosylated proteins. Since bacteria do not secrete the protein of interest, mechanical or chemical cell lysis resulting in additional contaminants (DNA, viruses, lipids) is often necessary. In mammalian cell culture the product is secreted into the surrounding medium. However, mammalian cell cultures require a more complex nutrient medium (serum proteins, peptides, carbohydrates etc.), and therefore the target protein is secreted into a complex matrix. Yeast, fungi and insect cell lines are less frequently used for protein expression. They have their unique cultivation considerations and these always have to be taken into account by downstream processing.

9.2.2.2. Non-proteinaceous Contaminants

During purification process three potential nonproteinaceous contaminants require special consideration: DNA, pyrogens and viruses.

9.2.2.2.1. DNA Impurity

Since normal cells under normal control do not overproduce proteins, biotechnologically derived new products are produced in genetically trans-

Table 9.2.B.
Examples of currently approved/or submitted for approval biopharmaceuticals

Product	Protein	Expressed in	First licensure/ approval
Humulin	Insulin	*E. coli*	1982
Protropin	hGh	*E. coli*	1985
Roferon	IFNα2A	*E. coli*	1986
Recombivax	HbsAg	*S. cerevisiae*	1986
Actimmune	IFNγ	*E. coli*	1990
Neupogen	G-CSF	*E. coli*	1991
Leukine	GM-CSF	*S. cerevisiae*	1991
Leukoprol	M-CSF	*E. coli*	1991
Proleukine	IL2 mutein	*E. coli*	1992
Betaseron	IFNγmutein	*E. coli*	1993
Activase	tPA	CHO	1987
Epogen	Epo	CHO	1989
Saizen	hGh	C127	1989
HB Gamma	HBsAG	CHO	1990
Granocyte	G-CSF	CHO	1991
Recombinate	FVIII	CHO	1992
Kogenate	FVIII	BHK-21	1993
Pulmozyme	DNaseI	CHO	1993
Cerezyme	Glucocerebrosidase	CHO	1994
Gonal F	FSH	CHO	1995
Neumega	IL11	*E. coli*	1995

Proteins and antisense oligonucleotides in clinical trials

Protein	Clinical target
sCD4	AIDS
gB	Herpes vaccine
BMP-2	Bone fractures
IFN-β	Multiple sclerosis
IL-12	Thrombocytopenia
IL-1RA	Rheumatoid arthritis

Table 9.2.B. (*continued*)

Protein	Clinical target
TNF	Cancer
FIX	Hemophilia B
gp160	HIV vaccine
TSH	Thyroid cancer
IL-6	Multiple sclerosis
IL-3	Bone marrow transplantation
Fomivirsen Na (antisense oligonucleotide)	Antiviral
Glucagon	Hypoglycemia

formed cells. These cells are usually tumorigenic and have potential oncogenes [4]. Levels of DNA $<$ 100 pg per dose are considered safe for therapeutic proteins. DNA removal can be obtained by SAX, affinity or HIC chromatography during protein purification. Alternatively, the product may be bound to a cation exchanger in order to wash out DNA and nucleotides. In case of robust proteins, extraction procedures under strongly denaturing conditions may also be suitable [5]. DNA levels in the purified products are determined mostly by the dot-blot hybridisation method. The current level of sensitivity of this method is 1–10 pg DNA/protein [6]. Recently quantitative PCR techniques are competing with the above method providing similar sensitivity and speed on a much lower average analysis cost.

9.2.2.2.2. Pyrogens

The parentheral administration of a medicament may result in the induction of fever, histamine release, altered vascular permeability etc. These reactions are called pyrogenic reactions, and any component, contaminant inducing this reaction is referred as pyrogen [7]. Although potential pyrogenic contaminants can be either of microbial or nonmicrobial origin, one of the most concerns to pharmaceutical production is endotoxin from the family of Enterobacteriacea. They are lipopolysaccharide components of the cell walls of gram-negative cells, and remain biologically active even after autoclaving. Eliminating endotoxins from the protein solution is a very expensive task (separation arsenal usually includes SAX-HPLC, affinity chromatography, detergent treatment etc.). The best strategy for the production of pyrogen free product is to maintain aseptic conditions in the manufacturing process

Table 9.2.C.
Tests for monoclonal antibodies (purified drug substance) from rodent cell lines

Test	Remark
SDS PAGE (reduced and non-reduced, Coomassie blue and silver stain)	Identity, purity, residual amounts of extraneous animal protein <1 ppm, proteolysis, dissociation, cross-linking
Isoelectric focusing	Charge isoforms, deamidation, deglycosylation
Size exclusion HPLC	Aggregation, dissociation, proteolysis, assay
Absorbance (280)	Protein concentration
Amino acid analysis	Amino acid composition
N-terminal sequencing	Identity
Peptide mapping	Identity/purity, deamidation, substitution, oxidation, proteolysis, cross-linking and primary structure
Mass spectrometry	Molecular mass, primary structure, glycosylation
Biophysical methods (NMR, CD, intrinsic fluorescence)	Secondary and tertiary structure, unfolding, denaturation
Immunoassays	Antigen-binding activity, specific contaminants
Binding assay	Potency: antigen-binding activity, neutralisation activity
Western blotting/antisera probes	Specific contaminants (host cell proteins)
HPLC-SAX with amperometric detection	Carbohydrate profiling
Specific tests for potential contaminants	e.g. Fermentation additives, components that may be leached from affinity columns etc.
Tests on adventitious agents	Sterility, endotoxins, DNA

and the subsequent downstream processing (for example pyrogen free chromatographic resins and sterile buffers should be used etc.) [4]. For the detection of pyrogenic substances rabbit pyrogen tests, or Limulus amebocyte lysate (LAL) tests are most frequently used.

9.2.2.2.3. Viral Contaminations

Patients consuming biopharmaceuticals may be immunocompromised due to their disease states and therefore may be more susceptible to potential viral infection from repeated exposure to therapeutic protein products that may contain active viruses or viral particles. The major source of viral impurities in the final product are endogenous viruses that may be present in the host cells. Adequate viral screening of the master cell bank is of greatest importance. Virus removal can be achieved by a series of product treatment such as treatment at low pH, treatment with detergents, filtration, a number of chromatographic techniques (ion exchange, affinity). Usually a combination of these steps may be the best choice. Virus testing for the finished biopharmaceutical product is recommended by all regulatory agencies. Types of viruses that must be tested for vary from cell line to cell line. Human viruses that are often tested for are Epstein–Barr virus, human cytomegalovirus, hepatitis B virus and retroviruses [8].

9.2.2.3. Extraneous Protein Contaminants

All proteinaceous contaminants (for example serum proteins, extracellular proteins etc.) present in the crude solution must be removed from the target protein during purification. In addition, all extraneous protein contaminating the target protein during downstream processes (for example affinity matrix leaking from the affinity column in downstream process), should also be eliminated from the final product. High demand for well-characterised product of biotechnological origin requires powerful analytical methods for successful product characterisation and quality control. An example for removing a leached ligand of plant protein origin from a mammalian protein preparation is demonstrated in Fig. 9.2.A. In this example a cation exchange chromatographic step was used for the impurity removal. In other cases HIC and SEC can also be used for the removal of extraneous proteins from the final product [4]. Capillary electrophoresis can be a very effective analytical tool to analyse cell culture samples containing complex mixture of proteinaceous materials. Figure 9.2.B demonstrates a capillary zone electrophoretic method developed for direct quantification of recombinant Antithrombin III in a CHO cell culture media where other proteinaceous compounds are also present [9].

9.2.2.4. Contaminants of Target Protein Origin

9.2.2.4.1. Quantification of Structurally-Related Components

The stability of protein biopharmaceuticals is critical to the success of any product. A variety of chemical reactions can affect the protein product

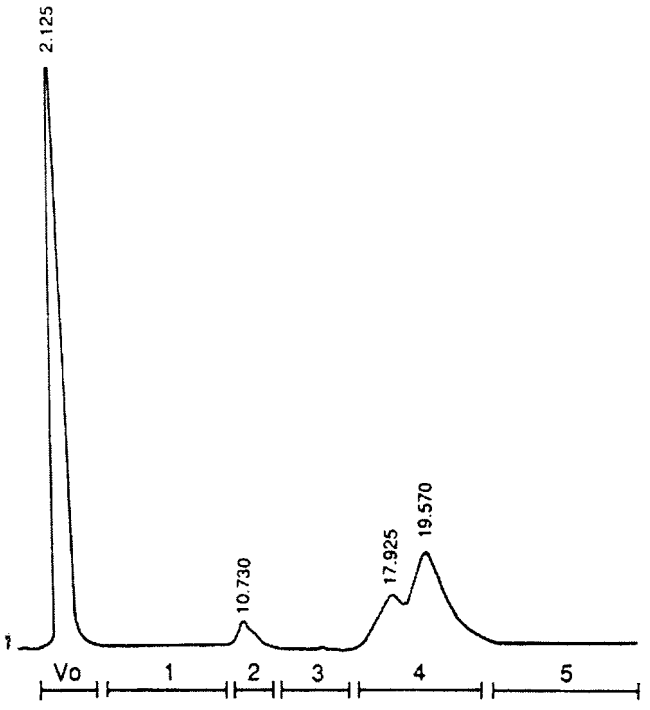


Figure 9.2.A.
Demonstration of removal of ^{125}I labelled extraneous protein from the eluates of a mammalian cell culture product using HPLC. HPLC conditions: Column: Toyo Soda TSK SP-5SW (7.5 × 75 mm); buffer A = 100 mM sodium acetate, pH 5.0; buffer B = 100 mM sodium acetate + 1 M NaCl pH: 7.0; flow rate = 1 ml/min. Gradient: 0% B to 30% B in 10 min. Vo = void volume, 1, 3 and 5 = baseline elutions, 2 = extraneous protein; 4 = mammalian cell culture protein (from Ref. [4])

such as hydrolysis (cleavage of the protein bonds) as well as deamidation of Asn and Gln side chains, oxidation at the Cys or Met residues, beta elimination and racemisation. Typical impurities, structurally related to the protein product are deamidated and/or oxidised forms of the protein resulting from degradation during the process or storage of the product. Analysis of the intact protein is often challenging due to the similar characteristics of the degraded and the intact protein. In determining the stability of proteins, traditional separation methods such as HPLC, gel electrophoresis and isoelectric focusing have been widely used on a routine basis. Peptide mapping is

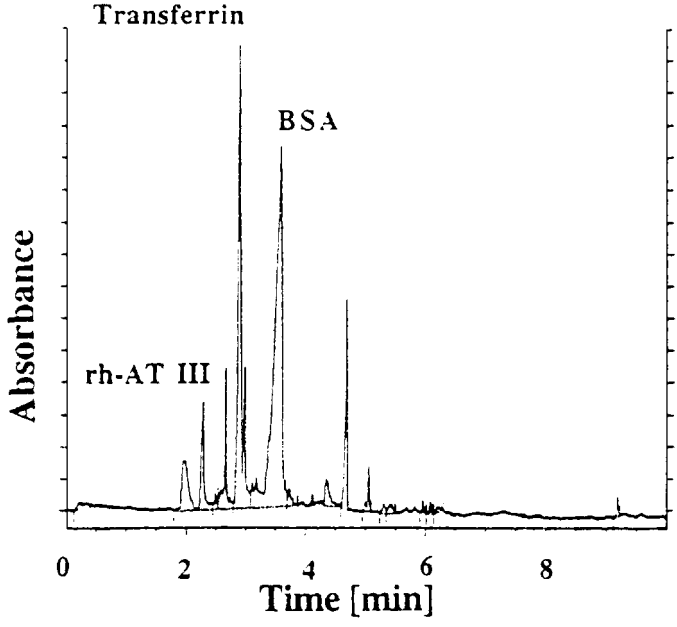


Figure 9.2.B.
CZE -analysis of a CHO cell culture sample containing 16 μg/ml rh-AT. Electrophoretic conditions: 0.05 M borate buffer, pH 9.5 containing 0.1% hydroxypropylmethylcellulose; fused silica capillary of 37/30 cm, i.d. 50 μm; applied voltage 15 kV, t = 22°C. A 5 nl sample was injected by using pressure (50 mbar for 2 s) (from Ref. [9])

frequently used for monitoring protein stability. Peptide mapping can detect only changes greater than 5% in the protein product however, valuable information on the site of the instability of the protein can be obtained [13]. UV spectra of individual peptides, in spite of their overall similarity, can be used as identification tools for peptides obtained from proteolytic digests [11,12]. Automated spectral identification of the tryptic map of rh-GH obtained by capillary electrophoresis has been employed using photodiode array detector combined with automated spectral comparison software [12]. The results were compared to those obtained by using RP-HPLC separation of the same protein [11].

The analysis of a protein or a peptide drug substance in a pharmaceutical dosage form represents several problems. Usually other chemical components of protein origin are added to the formula to enhance chemical stability during storage or manufacturing processes [10]. Two recombinant cytokines, inter-

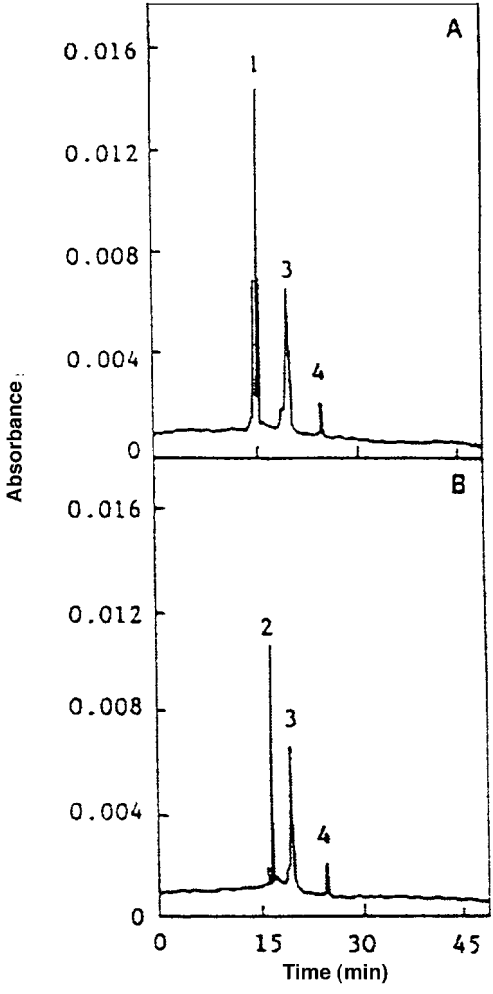


Figure 9.2.C.
Capillary electrophoretic profile of cytokines interferon (A) and interleukin (B) formulation mixtures. (A) Electropherogram of a formulation mixture containing recombinant leukocyte interferon alpha (peak 1), human serum albumin (peak 3), and N-acetyltrytophan (peak 4). (B) Electropherogram of an actual dosage from containing recombinant interleukin- 1α (peak 2), human serum albumin (peak 3), and *N*-acetyltrytophan (peak 4). Buffer: 0.05 M sodium tetraborate buffer (pH 8.3), containing 0.025 M lithium chloride; capillary: 50 cm (effective length) × 75 μm i.d.; applied voltage: 10 kV; detection 200 nm (from Ref. [14])

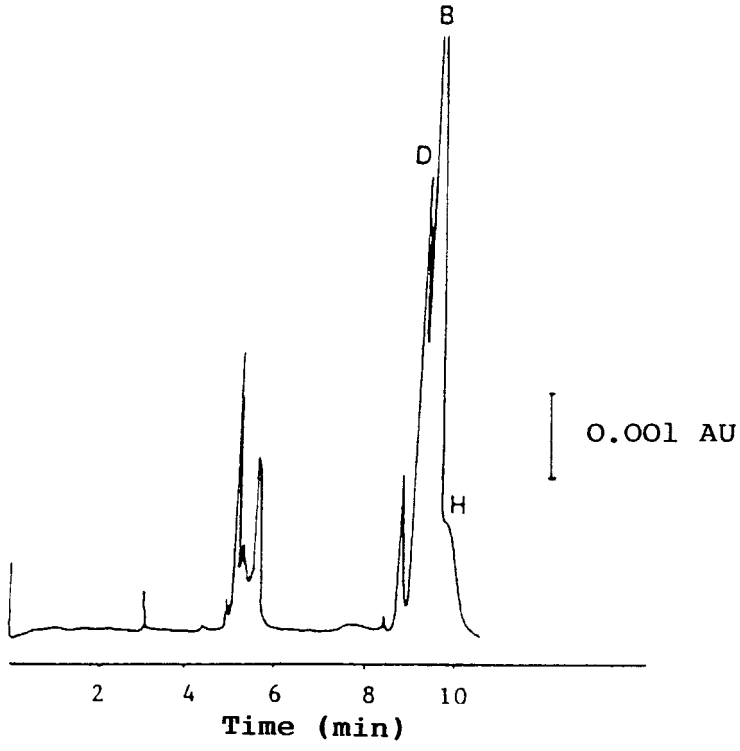


Figure 9.2.D.
CZE analysis of a crude leukinostatin B sample containing also leukinostatin D and H. Buffer: 0.1 M phosphate buffer (pH 2.5); injection, 6 s electrokinetic at 7 kV; applied voltage: 8 kV. Capillary, cartridge with 20 cm × 25 μm coated capillary (from Ref. [15])

leukin-1α and leukocyte interferon alpha were analysed in a typical formulation mixture of a pharmaceutical dosage form. The cytokines were separated and quantified from human serum albumin and *N*-acetyltryptophan [14]. Figure 9.2.C.a,b shows the capillary electrophoretic profiles of the two formulation mixtures. Capillary electrophoresis served as a tool for purity control in the production of Leukinostatin B. Leukinostatins are nonapeptides obtained by submerged cultures of *Paecilomices lilacinus* and *Paecilomices marquandii.* Leukinostatin B could be separated from its D and H variant in the culture broth using capillary electrophoresis within 12 minutes [15] as shown in Fig. 9.2.D.

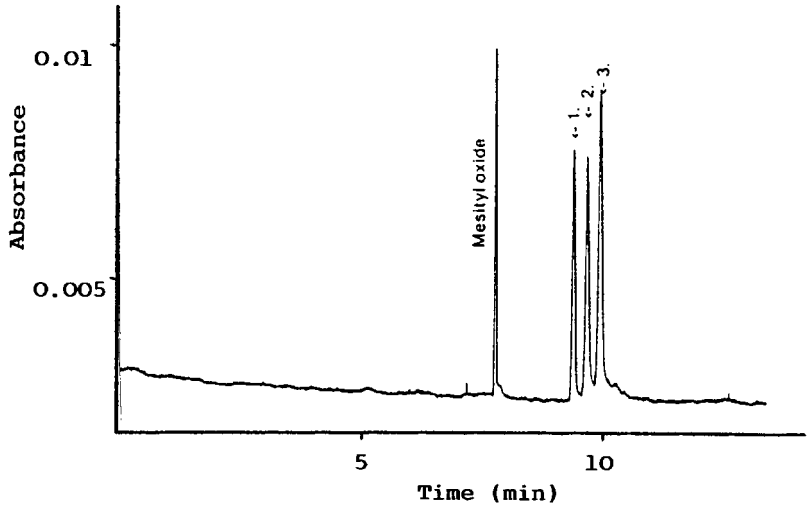
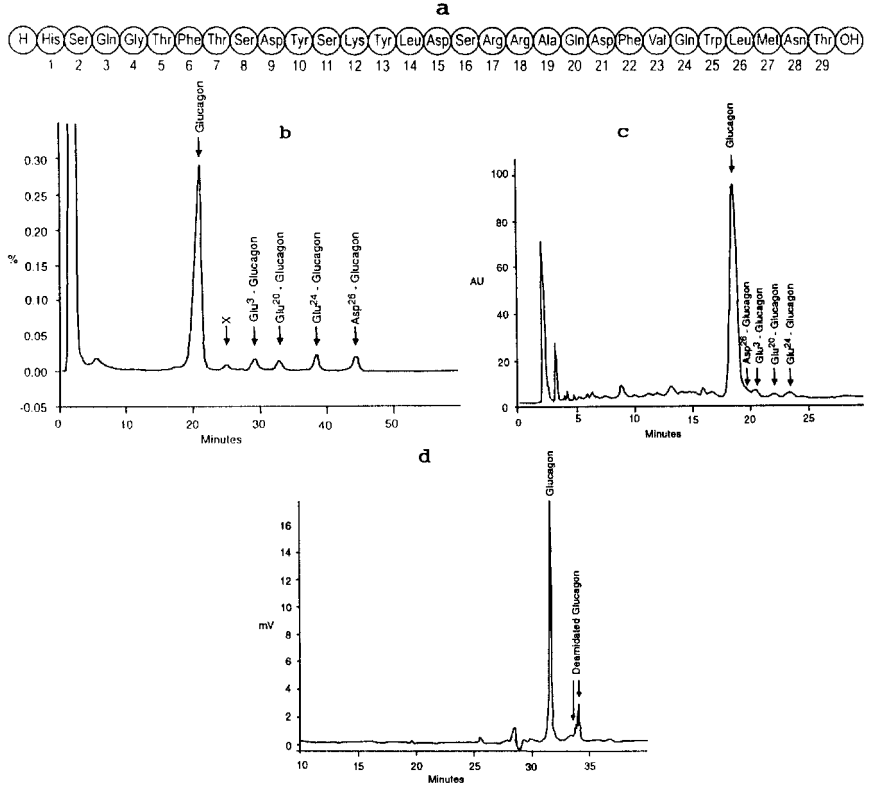


Figure 9.2.E.
Separation of hGH derivatives by CZE. The net charge difference of the derivatives under the experimental conditions of the run with respect to hGH (peak 1) is -1 and -2 for [desamido–149] hGH (peak 2) and [didesamido–149–152] hGH (peak 3), respectively. Conditions: polyimide coated Fusi capillary, 50 μm i.d., 105/81.5 cm long; buffer: 10 mM tricine, 5.8 mM morpholine and 20 mM sodium chloride adjusted to pH 8.0 using 1M NaOH; injection: hydrodynamically for 15 s at 12 cm above the ground electrode buffer reservoir; separation voltage: 30 kV; UV detection at 200 nm (from Ref. [19])

9.2.2.4.2. Deamidation of Proteins

In deamidation reactions, the side chain amide linkage in a Gln or Asn residue is hydrolysed to form a free carboxylic acid via formation of a six-, or five membered ring intermediate, respectively. The resulting product can be a mixture of peptides/proteins in which the polypeptide backbone is attached via an α-carboxyl linkage (Asp) or via a β-carboxyl linkage (iso-Asp). Deamidation frequency of Asn residues, particularly at the Asn-Gly sequence was observed to be higher than that of Gln residues. The rate of deamidation is effected by various conditions; for example the cyclic imide formation is accelerated at neutral or basic pH [10]. Moreover, since the intermediate ring formation involves the participation of the succeeding amino acid in the polypeptide backbone, the size and charge of that neighbouring amino acid plays also an important role in the rate of deamidation.

The rate of deamidation is also influenced by the secondary and tertiary structure of the protein [9,10,14,16,17]. Several examples for deamidation of recombinant proteins have been reported recently. Most authors used CZE of the intact molecule and/or tryptic digestion followed by CZE peptide mapping for monitoring deamidation. Recombinant human growth hormone consists of a single peptide strand with two interchain disulphide bridges. The molecular weight of the molecule is 22 500 Da and the isoelectric point is 5.2. Human growth hormone (rhGH) and its desamido forms have been extensively studied by several authors [18–20]. The protein undergoes deamidation at Asn149 and Asn152. Figure 9.2.E shows the electropherogram of hGH and its deamidated variants. Nielson and Rickard [19] have used two CZE approaches to monitor the deamidation of rhGH: tryptic digestion followed by CZE peptide mapping and CZE of the intact molecule. Significant shift in the migration time of the peptide 15 could be observed upon deamidation. Peak 15 contained two potential deamidation sites and upon multiple deamidation the electrophoretic mobility of that peak



decreased dramatically. The methods developed by Nielsen are reproducible and rugged and can be validated for use within Quality Control groups. An array of hyphenated separation techniques were used by Kornfelt in the study of deamidation of recombinant glucagon, a polypeptide hormone consisting of 29 amino acids in one single chain [17]. The author used anion exchange chromatography, reversed-phase chromatography and capillary electrophoresis to separate and quantitate deamidated forms of glucagon. Anion exchange chromatography provided the best chromatographic separation in this case. Figure 9.2.F.a–d shows the structure, and the separation of glucagon and its desamido forms by HAEX, RP-HPLC and CZE, respectively.

9.2.2.4.3. Oxidation of proteins

The side chains of His, Met, Cys Trp and Tyr residues in proteins are potential oxidation sites. However, Met residues are the most susceptible to oxidation; even atmospheric oxygen can oxidise these residues to Met sulphoxide or even to Met sulphone under drastic conditions. Reactivity of the Met residues toward oxidation is dependant upon their position [21]. The thiol

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Figure 9.2.F.
Primary structure of human glucagon (a), and separation of its desaminoforms by HPLC-AEX (b), HPLC-RP (c) and CZE (d). (a) Amino acid sequence of glucagon. Experimental conditions (b). Column: MONO Q HR 5/5 (Pharmacia Biosystems). Column temperature: ambient. Mobile phase A: 20 mM Tris, 50% v/v ethanol (pH 8.3); mobile phase B: 20 mM Tris, 200 mM sodium acetate, 50% v/v ethanol (pH 8.3). Elution scheme: 0–10 min: isocratic elution with 100% A, 10–30 min: linear gradient from 0% to 27% B, 30–45 min: linear gradient from 27% to 80% B 45–60 min: equilibration with 100% A-buffer; flow rate: 1 ml/min. Detection: 280 nm. Injection load: 200 μg glucagon. Experimental conditions (c). Column: Lichrosorb RP 18 250 × 4.0 mm (Merck). Column temperature: 40°C. Mobile phase A: 200 mM Na_2SO_4, 50 mM H_3PO_4 10% CH_3CN pH (app): 2.5, mobile phase B: 50% CH_3CN. Elution scheme: 0–20 min: isocratic elution with 52/48 A/B followed by linear gradient to 100% B over the next 20 minutes. Finally isocratic elution with 100% B for 10 min; flow rate: 1 ml/min; detection: 215 nm; injection load: 30 μg glucagon. Experimental conditions (d). Capillary: Polyimide coated Fusi 75 μm i.d. 82/75 cm long. Separation Buffer: 100 mM sodium phosphate pH 9.0; injection: at the anode for 10 s app. 10 ng; separation voltage 12 kV 40 min^{-1}; detection: 214 nm (from Ref. [17])

group of the Cys residues can be oxidised to sulphenic acids, disulphides, sulphinic acids or to sulphonic acids depending on the conditions (temperature, pH, buffer, traces of metal ions as catalysts, and the oxygen tension) and the nature of neighbouring groups. The side chains of the above amino acids can also undergo photooxidation by visible light. Recombinant human interleukin-eleven (rhIL-11) is a cytokine involved in stimulating megakaryocytopoieses and platelet production [22]. The protein is relatively small (19 kDa), extremely basic (p*I* ~12), and quite hydrophobic (23% leucine). In developing stable dosage forms of this protein, the stability of the protein has been investigated by various methods [23]. One of the examined areas has been the susceptibility of rhIL-11 to oxidation of the two methionine residues, Met59 and Met123. In addition to chemical modification and a route of generating specific side-chain modifications, the protein has been stressed by incubation at high temperatures for extended periods of time. The samples have been examined by a variety of methods including Asp-N peptide mapping. Figure 9.2.G shows the Asp-N RP-HPLC peptide map of a control and degraded sample. The chromatograms demonstrate that several modifications have occurred that lead to different patterns of the chromatogram. To further analyse these samples, each peptide was purified and examined by capillary electrophoresis. Figure 9.2.H shows the CZE of peptide D12 from the control and degraded samples. The degraded D12 sample shows two peaks, the original D12 peak and a new peak. This new peak corresponds to oxidised D8 peptide, which contains methionine 59.

9.2.2.4.4. Hydrolysis of Proteins

Certain amino acids are more susceptible to cleavage with dilute acids than others. Aspartic acid, (Asp), for example cleaves at least 100 times faster than other peptide bonds, moreover Asp-Pro peptide bonds are hydrolysed under conditions where other Asp-X bonds are stable. Asp-X bonds can also undergo isomerisation via a cyclic imide formation (as mentioned above) to form iso-Asp-X forms [21].

9.2.2.4.5. Carbohydrate Heterogeneity of Glycoproteins

The majority of recombinant proteins manufactured for human or animal therapy are glycoproteins derived from animal (most frequently CHO) cells. They are usually produced as a set of differently glycosylated variants of a common polypeptide backbone [24]. Recombinant tissue Plasminogen Activator (rt-PA) occurs naturally in two major variant forms: Type I (glycosylated on three sites) and Type II (glycosylated at only two sites). The glycoprotein

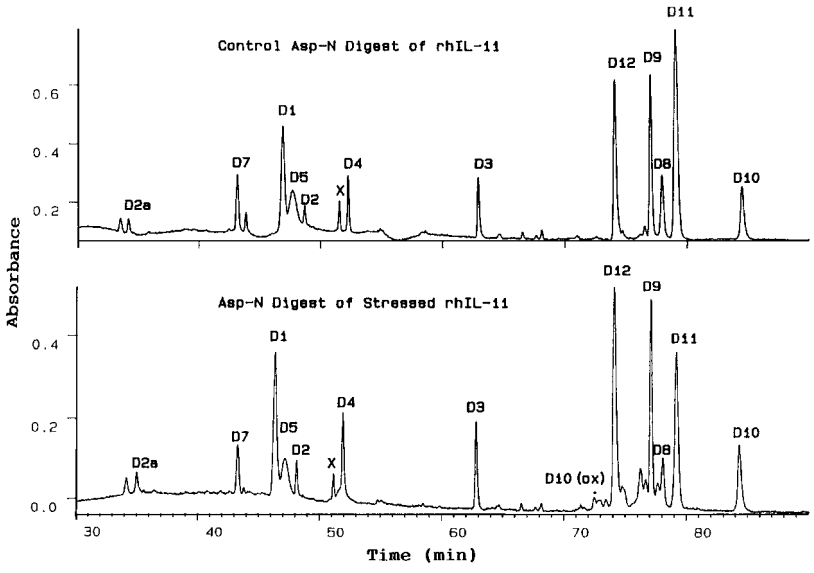

Figure 9.2.G.
RP-HPLC of Asp-N digests of control and degraded rhIL-11 samples. Asp-N (Boehringer Mannheim) digests were performed by incubating the rhIL-11 with Asp-N (50:1 ratio of rhIL-11:Asp-N) in 20 mM Tris (pH 8.3). The digests were quenched with TFA and injected onto a reversed-phase HPLC system: 4.6 × 100 mm C18 column (VYDAC) with a buffer system of 6 mM TFA, 4 mM HFBA (A) and 95% acetonitrile in 6 mM TFA, 4 mM HFBA (B); Waters HPLC pumps, gradient controller; WISP injector; detection: 214 nm

has an isoelectric point between 4.5 and 6.0 [25]. The intact protein gave multiple peaks when separated in borate buffer, due to the carbohydrate heterogeneity on the polypeptide backbone. Honda and co-authors suggested that carbohydrates form complexes with borate ions leading to multiple charges and thus different migration time [26,27]. Carbohydrate heterogeneity can be examined by capillary isoelectric focusing (CIEF) as well, since negatively charged sialic acids attached to the oligosaccharide backbone can result in several isoforms [28–30]. Figure 9.2.I shows the capillary IEF patterns of Type I and Type II rt-PA, respectively.

Recombinant Human Erythropoietin (r-HuEPO) is a glycoprotein hormone promoting and stimulating the production of red blood cells. Its molecular weight is 35 kDa and it is heavily glycosylated (40% of its molecular size is due to the carbohydrate content), containing *N*-, and *O*-linked structures. The presence of the sialic acids is necessary for the biological activity of the

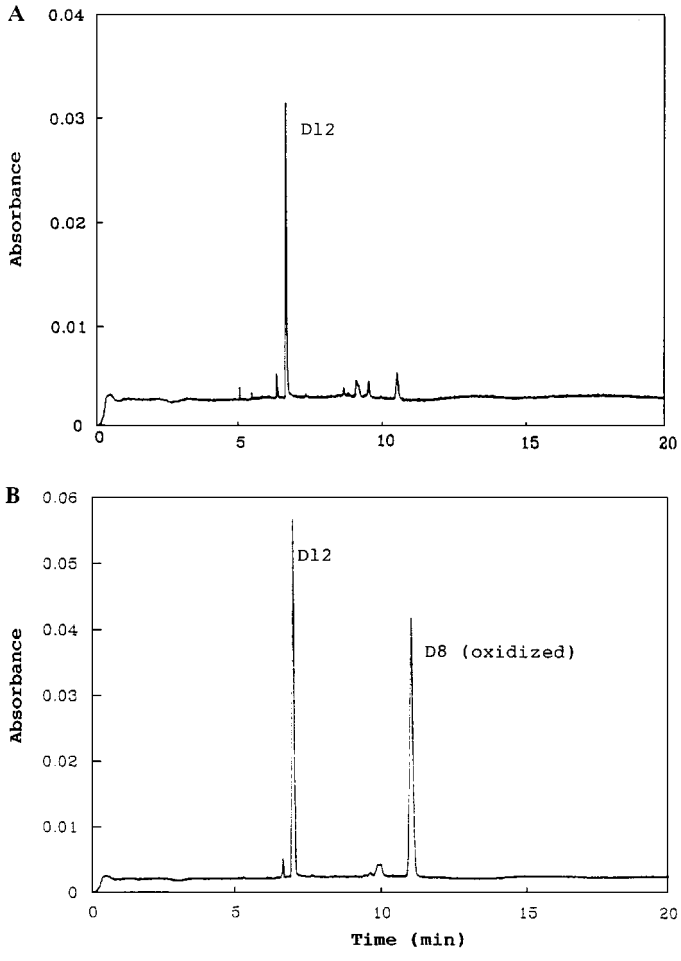

Figure 9.2.H.
CZE of the D12 peptide purified from Asp-N digests of control (A) and degraded (B) rhIL-11 samples. Beckman PACE 2000 CE system was used. Uncoated 50 μm × 50 cm capillary; buffer: 50 mM NaH_2PO_4 (pH 2.5); voltage: 20 kV; detection 200 nm

protein. The intact protein was analysed under different conditions and the results obtained by CE under optimum conditions were comparable to that obtained by traditional isoelectric focusing [28].

At present, glycoproteins are accepted for therapy with some degree of

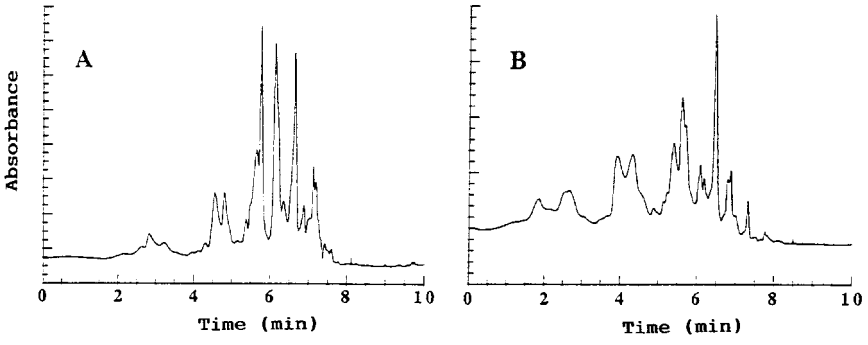

Figure 9.2.I.
CIEF pattern of rt-PA Type I and Type II coated linear capillary (BioRad) 12 cm (effective length), 25 μm i.d.; focusing voltage: 12 kV; mobilisation voltage 8 kV. The ampholyte solution contained 2% ampholyte (pH 6-8), 2% CHAPS (3-((3-cholamidopropyl) dimethylammonio(-1-propane-sulphonate) and 6 M urea; UV detection: 280 nm (from Ref. [28])

glycosylation heterogeneity, however, it is important to define what is the critical glycosylation pattern which is important to the efficacy of the product and therefore, appropriate analytical methods have to be developed to detect heterogeneity.

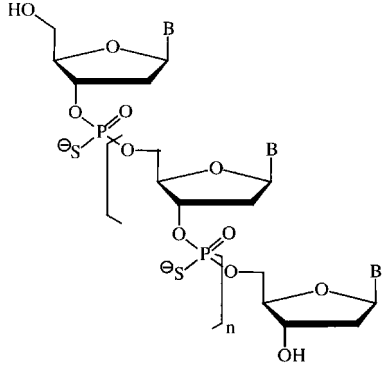

Figure 9.2.J.
Molecular structure of phosphorothioate DNA. Phosphodiester deoxyoligonucleotides (ODNs) possess an O- in place of the S- atom found in SODNs. The length of the oligo = n + 2. *B* represents one of the four nucleotide bases, guanine, cytidine and thymine found in naturally occurring DNA

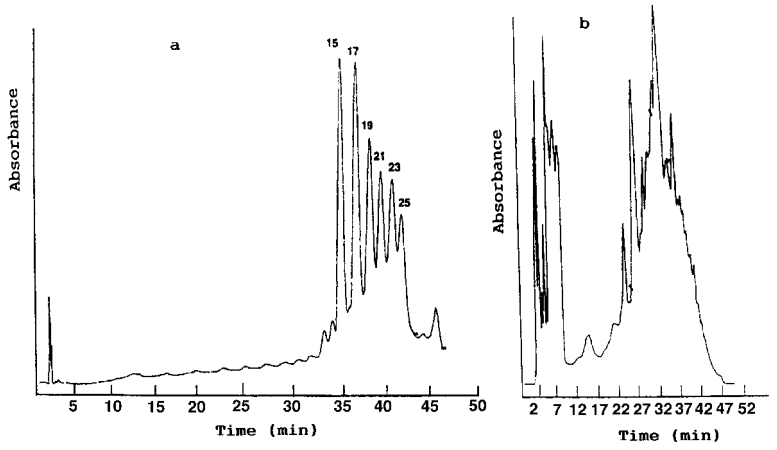

Figure 9.2.K.
Ion exchange HPLC of a mixture containing six SODNs (15–25 meres) differing in length by two nucleotides (a) and of a crude DNA analogue solution (b). Conditions (a): Dionex nucleopac PA-100 column (4 × 250 mm) (Sunnyvale, CA, USA); A: 50 mM Na_3PO_4 (pH 8.1), B: 1.0 M NaSCN, C: CH_3CN; flow rate 1 ml/min, linear gradient of 0.8% min^{-1} B was applied over 50 min, C was kept constant at 30%; UV detection: 280 nm. Conditions (b): Pharmacia mono-Q HR 10/10 column (10 × 100 mm) (Piscataway, NJ, USA); (A) 10 mM NaOH, (B) 1.5 M NaCl, 10 mM NaOH (pH 12.0); flow rate 2 ml/min, linear gradient from 15% to 45% B over 40 min (0.75%/min); UV detection: 290 nm (from Ref. [41])

9.2.3. Oligonucleotides

The past decade has witnessed an exponential development of genetic sequence targeted therapeutic agents. Recent advances in research of gene regulation of cells and molecular mechanism resulted in rational design of drugs that target genetic sequences for both host diseases (such as cancer) and infectious diseases (such as AIDS). Along the pathway of genetic information, from DNA to protein several intervention have been developed to modulate gene expression and regulation, such as duplex or triplex formation using short (8–25 bases) oligonucleotides, in order to block transcription, antisense DNA or antisense RNA binding etc. [31]. All of these approaches have been demonstrated to be effective in vitro, or in some cases even in vivo [32].

Antisense oligonucleotides are short sequences of DNA that form specific hydrogen bonds with complementary DNA or mRNA sequences, allowing

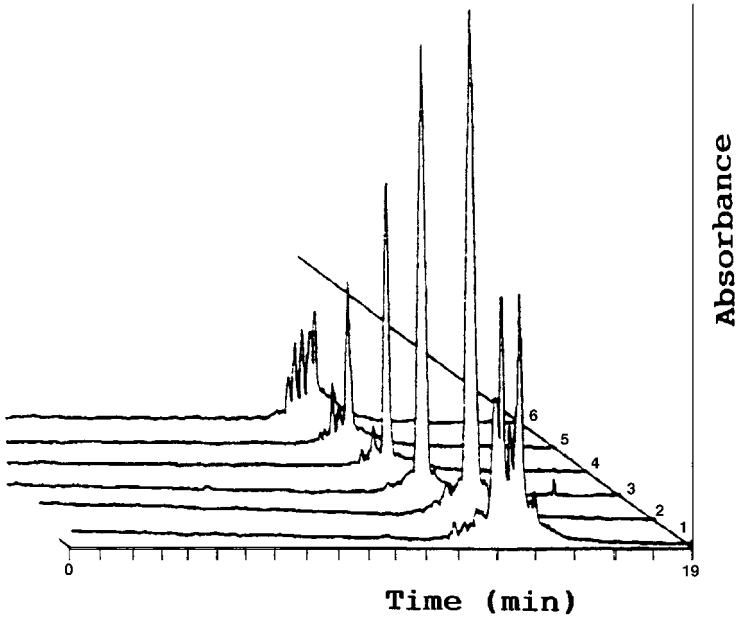

Figure 9.2.L.
Electropherogram of six fractions collected at approximately 31 min from the ion exchange preparative HPLC illustrated in Fig. 9.2.Kb. Conditions: Sample concentration approx. 0.5 AU/ml, injected electrokinetically at −8 kV for 5 s. Constant running voltage: −22 kV in 50 cm × 100 μm fused silica capillaries. Gel matrix: 10% Micro-Gel™ in 35 mM Tris, 5.6 mM H_3BO_3 and 15% ethylene glycol (pH 9.0). For the reference see Fig. 9.2.K

regulation of specific gene of foreign or of cellular origin [33]. The therapeutic utility of antisense compounds were first investigated with unmodified DNA [34]. DNA nucleases however, degrade unmodified DNA-s, therefore chemical modification of antisense is necessary. For example, Phosphorothioate oligonucleotides (Fig. 9.2.J) have higher nuclease resistance and attractive pharmacokinetic and toxicological properties than naked DNA [35]. There are currently more than 10 antisense drugs evaluated in Phase II/III clinical trials for various diseases. One of them Fomivirsen sodium has already passed Phase III trials and New Drug Application (NDA) is submitted to the Food and Drug Administration (FDA) [36].

For clinical trials high amounts of antisense oligonucleotide (from g-scale to kg-scale) are needed. One of the most frequently used technique for anti-

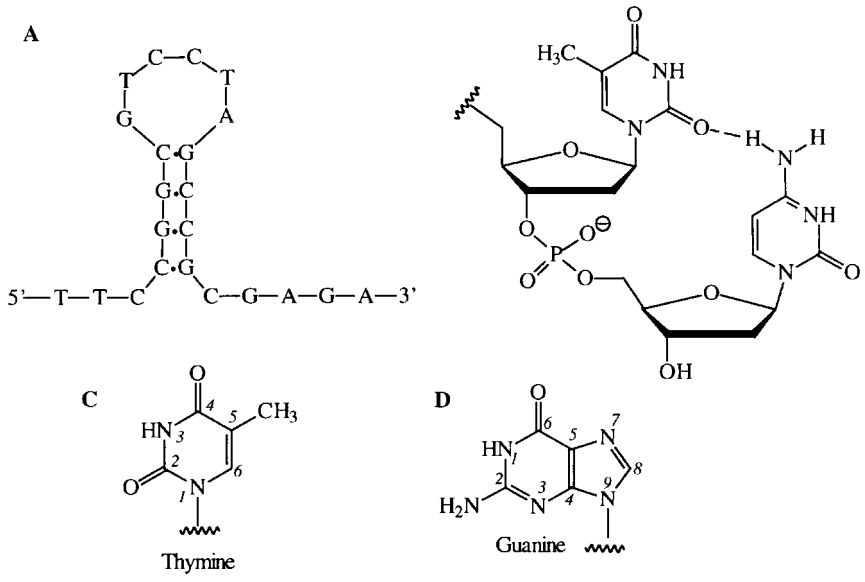

Figure 9.2.M.
Possible secondary structures. (A) Hairpin loop in a single-stranded oligo; (B) hydrogen bonding between 2 adjacent nucleotides (C and T) on the 3′ end; (C,D) the molecular structures of the thymine and guanine moieties. At pH 10.0 the NH position *3* on thymine and position *1* on guanine loses a proton which confers a negative charge on each of these molecules. For the reference see Fig. 9.2.K

sense oligonucleotide production is the automated solid-phase synthesis, where the first protected nucleoside is attached to a solid phase support and the chain elongation is performed in cycles via coupling of the next activated nucleoside block. When the desired length of the oligonucleotide has been reached, cleavage of the oligonucleotide from the solid support and removal of the P, and base protection groups is performed. The production process results in a crude synthetic phosphorothioate oligonucleotide mixture containing the product (the n-mere phosphorothioate), deletion sequences (n-1, n-2, n-…-meres), incompletely sulphurised oligos (where one or more P=O linkage instead of P=S linkages occur), and reaction reagents.

Large scale purification involves HPLC in reversed phase (RP) or in ion exchange (SAX-strong anion exchange) mode. RP-HPLC can differentiate between the correctly sulphurised and the incorrectly sulphurised oligonucleotides, while SAX-HPLC can separate the oligonucleotides differing in charge, thus according to length. SAX HPLC, however does not have the resolving

power to distinguish the length differing by one nucleotide base [37]. Figure 9.2.K.a,b shows the ion exchange separation of a mixture containing six phosphorothioate oligonucleotides and a crude DNA analogue solution, respectively. On the other hand capillary gel electrophoresis (CGE) has been used effectively for size-based separation of both phosphodiester and phosphorothioate oligonucleotides (ODN-s, and SODN-s, respectively) [37–40]. Figure 9.2.L shows analysis of fractions collected from the SAX-HPLC separation of the crude DNA analogue solution. The ability of oligos to form secondary structures or compression artefacts is a function of the oligo's base sequence, and can be defined as hairpin loops or any occurrences of hydrogen bonding between adjacent nucleobases. G-rich oligos are specially apt to form these formations shown in Fig. 9.2.M. Applying denaturing conditions, such as preheating the sample prior injection or using urea in the separation buffer can help reduce compression artefacts [41].

References

1. Forecast To 2002 For Euro Biotech Pharma Market (Monitor), *Marketletter* **February**, 27 (1997)
2. A.S. Lubiniecki, in *Fermentation Process Events Affecting Biopharmaceutical Quality in Drugs and the Pharmaceutical Sciences*, Vol. 85 (J.A. Bontempo, Ed.), pp 11–30, Marcel Dekker, New York (1997)
3. D.A. Baker and S. Harkonen, Regulatory agency concerns in the manufacture and testing monoclonal antibodies for therapeutic use, in *Targeted Diagnosis and Therapy*, Vol. 3 (P. Tyle and B.P. Ram, Eds.), pp 78–98, Marcel Dekker, New York (1990)
4. V.K. Garg, M.A.C. Costello and B.A. Czuba, Purification and production of therapeutic grade protein, in *Purification and Analysis of Recombinant Proteins* (R. Seetharam and S.K. Sharma, Eds.), Marcel Dekker, New York, pp 29–54 (1991)
5. I.S. Johnson, *Science* **219**, 632–637 (1983)
6. A. Riggin, V.T. Luu, J.K. Lobdel and M.K. Wind, *J. Pharm. Biomed. Anal.* **16**, 561–572 (1997)
7. F.C. Pearson, Endotoxin: its structure and function with respect to depyrogenisation, *Depyrogenisation, Parenteral Drug Association Technical Report No. 7*, pp 1–14 (1985)
8. E. Joner and G.D. Christiansen, *Biopharmaceutics* **1**, 36–42 (1988)
9. R. Freitag, Application of HPCE analysis to process monitoring in biotechnology, in *Progress in HPLC-HPCE*, Vol. 5 (H. Parvez, P. Caudy, S. Parvez and P. Roland-Gosselin, Eds.), pp 115–154, VSP, Utrecht (1997)

10. M.C. Manning, K. Patel and R.T., *Pharm. Res.* **6**, 903–917 (1989)
11. H.-J.P. Sievert, S.L. Wu, R. Chloupek and W.S. Hancock, *J. Chromatogr.* **499**, 221–234 (1991)
12. S.L. Wu and M. Herold, Poster 649, presented at the *Sixth International Symposium on Capillary Electrophoresis*, San Diego, CA (1994)
13. P.D. Grossman, J.C. Colburn, H.H. Lauer, R.G. Nielsen, R.M. Riggin, G.S. Sittampalam and E.C. Rickard, *Anal. Chem.* **61**, 1186–1194 (1989)
14. N.A. Guzman, H. Ali, J. Moschera, K. Iqbal and A.W. Malick, *J. Chromatogr.* **559**, 307–315 (1991)
15. M.G. Quaglia, S. Fanali, A. Nardi, C. Rossi and M. Ricci, *J. Chromatogr.* **593**, 259–263 (1992)
16. K. Ganzler, I. Mazsaroff, J.V. Amari and J. Wright, Lecture 85 presented at the *Sixth International Symposium on Capillary Electrophoresis*, San Diego, CA (1994)
17. T. Kornfelt, Characterization of deamidation of glucagon under acidic conditions by capillary electrophoresis, anion exchange HPLC and reverse phase HPLC presented at *HPLC'98*, St. Louis, MO (1998)
18. J. Frenz, S.L. Wu and W.S. Hancock, *J. Chromatogr.* **480**, 379–391 (1989)
19. R.G. Nielsen and E.C. Rickard, in *Analytical Biotechnology – Capillary Electrophoresis and Chromatography* (Cs. Horváth and J.G. Nikelly, Eds.), pp 36–49, American Chemical Society, Washington, DC (1990)
20. R.S. Rush, M.D. McGindley, K.S. Stoney and M.F. Rohde, in *Methods. A Companion to Methods in Enzymology*, Vol. 3, No. 3 (J.E. Wiktorowicz, Ed.), pp 191–204, Academic Press, San Diego, CA (1992)
21. W.S. Hancock, J.E. Battersby, D.A. Lewis, A. Guzzetta and S.L.Wu, Lecture 86 presented at the *Sixth International Symposium on Capillary Electrophoresis*, San Diego, CA (1994)
22. J. Liu, O. Shirota, D. Wiesler and M. Novotny, *Proc. Natl. Acad. Sci. USA* **88**, 2302–2306 (1991)
23. K. Ganzler, N.W. Warne and W.S. Hancock, Analysis of rDNA-derived proteins and their posttranslational modifications, in *Capillary Electrophoresis in Analytical Biotechnology* (Pier Giorgio Righetti, Ed.), pp 183–238, CRC, Boca Raton, FL (1996)
24. R.B. Parekh and T.P. Patel, *Trends Biotechnol.* **10**, 276–280 (1992)
25. M. Taverna, A. Baillet, D. Biou, M. Schluter, R. Werner and D. Ferrier, *Electrophoresis* **13**, 359–366 (1992)
26. S. Honda, S. Iwase, A. Makino and S. Fujirawa, *Anal. Biochem.* **176**, 72–77 (1989)
27. S. Honda, S. Suzuki, A. Nitta, S. Iwase and K. Kakehi, in *Methods. A Companion to Methods in Enzymology*, Vol. 3, No. 3 (J.E. Wiktorowicz, Ed.), pp 233–243 Academic Press, San Diego, CA (1992)
28. K.W. Yim, *J. Chromatogr.* **559**, 401–410 (1991)

29. F. Kilar and S. Hjertén, *J. Chromatogr.* **480**, 351–357 (1989)
30. E. Watson and F. Yao, *Anal. Biochem.* **210**, 389–393 (1993)
31. W.W. Zhang, *J. Mol. Med.* **74**, 191–204 (1996)
32. J.F. Milligan, R.J. Jones, B.C. Froehler and M.D. Matteucci, *Ann. N. Y. Acad. Sci.* **716**, 228–241 (1994)
33. C.A. Stein and Y.-C. Cheng, *Science* **261**, 1004–1012 (1993)
34. P.C. Zamencik and M.L. Stephenson, *Proc. Natl. Acad. Sci. USA* **75**, 280–284 (1978)
35. R.M. Crooke, *Anti-Cancer Drug Design* **6**, 609–646 (1991)
36. R.R. Desmukh, W.E. Leitch and Y.S. Sanghvi, Production scale purification of antisense oligonucleotides, Poster P0629 presented at *HPLC'98*, St Louis, MO, USA
37. A.S. Cohen, M. Vilenchik, J.L. Dudley, M.W. Gembotrys and A.J. Bourque, *J. Chromatogr.* **638**, 293–301 (1993)
38. G.S. Srivatsa, M. Batt, J. Schuette, R.H. Carlson, J. Fitchett, C. Lee and D.L. Cole, *J. Chromatogr.* **680**, 469–477 (1994)
39. L.A. DeDionisio, *Am. Lab.* **July**, 30–34 (1994)
40. A.S. Cohen, D.R. Najarian, A. Paulus, A. Guttman, J.A. Smith and B.L. Karger, *Proc. Natl. Acad. Sci. USA* **85**, 9660–9663 (1988)
41. L.A. DeDionisio and D.H. Lloyd, Capillary gel electrophoresis and antisense therapeutics: the analysis of DNA analogs, in *Progress in HPLC-HPCE*, Vol. 5 (H. Parvez, P. Caudy, S. Parvez and P. Roland-Gosselin, Eds.), pp 293–317, VSP, Utrecht (1997)

9.3. Antibiotics

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9.3.1. Introduction: Official Methods for Analysis of Antibiotics

The antibiotics are an important group of drugs, extensively used in human and veterinary therapy. Some are used on a very large scale to enhance animal growth. By definition antibiotic structures are of natural origin or are produced by synthesis as a structural analogue of a naturally-occurring antibiotic. The chemistry of antibiotics is so varied that a chemical classification is difficult. In this chapter penicillins, cephalosporins, macrolides, tetracyclines, aminoglycosides, polypeptides and polyenes are discussed.

Since most antibiotics are obtained by fermentation or by semisynthesis from natural products, the total amount of impurities present might be rather large because related compounds, also formed during the fermentation process used to produce the antibiotics, often are difficult to remove. Since the biosynthesis route followed during fermentation is not always fully understood, and the side reactions are not always known, the related substances often are not easy to identify. This is what makes identification and determination of impurities in antibiotics (or other substances of natural origin) so different from that of purely synthetic products, where the synthesis route and the reagents are perfectly known and intermediates can be isolated and purified whenever necessary. A number of antibiotics exist as a mixture of components with different activity. For these it is interesting to determine the composition of the mixture.

Official methods to determine the purity of antibiotics, or other drugs, are prescribed by pharmacopoeias. The European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopeia (USP) are the most frequently used [1,2]. If the official monographs prescribe the determination of related substances, the use of separation techniques is almost always used. The number of examples where impurities are determined by chemical reactions, such as colour reactions is rather limited and is decreasing in favour of separation techniques. A typical example of colour reaction is the determination of *streptomycin* in *dihydrostreptomycin* (Ph. Eur.). The same applies to physical techniques such as direct UV measurement of a solution. A typical example is the determination of *bacitracin F* in bacitracin (Ph. Eur.) or the determination of tetraenes in *amphotericin B* (Ph. Eur.). Such techniques will not be reported nor discussed further in this text.

Table 9.3.A gives an overview of the different classes of antibiotics treated by the Ph. Eur. and the USP. Information is given about the total number of monographs on drug substances (bulk active substances) and about the number of monographs where high-performance liquid chromatography (HPLC), gas chromatography (GLC), or thin-layer chromatography (TLC) is used for the identification and determination of related substances, or for the assay. For the more complex antibiotics microbiological assay remains the method of choice. It can be observed that GLC is only rarely used. Most probably it will be replaced entirely by HPLC. TLC is rarely used in the USP and more frequently in the Ph. Eur. TLC never has been popular in the USP, whereas in the Ph. Eur. it has been the method of choice for the determination of impurities. However, TLC tends to be replaced by HPLC in the Ph. Eur.

HPLC is clearly the method of choice in the official monographs. It often allows for simultaneous assay of the active substance(s) and determination of the impurities. Thus, when in the Ph. Eur. HPLC is used for assay, it is generally also used for determination of the impurities in a separate related substances test. The number of impurities which are allowed to occur at a certain level (%) can be limited as well as the total sum of impurities. In the latter case a disregard level is indicated, below which the peaks must not be taken into account. This is generally set at 0.05% or 0.1%. In the USP such related substances tests are often absent. Of course, when a selective HPLC method is used for the assay, the impurities are indirectly limited by the content limit. However, this only is an indication for the total amount of impurities and does not give any information about the individual impurities. Modern analytical practice demands for a separate determination of the impurities. Another difference between the Ph. Eur. and the USP monographs is that the former report a list of potential impurities which can be limited. In older monographs, this information is lacking. The USP rather seldom mentions the name of an impurity limited by a test. In most cases it is not specified which impurities can be limited by the analytical methods prescribed. This means that the user of such a method has no information on the selectivity of the method and the level of validation of its specificity. In general it can be stated that little or no information is available about the validation status of pharmacopoeial methods, unless these are based on published methods or unless such information is published in Pharmeuropa [3] or in Pharmacopeial Forum [4], which is rarely the case.

Capillary electrophoresis (CE) is another very powerful separation technique, but its application in pharmacopoeial monographs is rare. This may be due to the poorer precision of CE but also to the fact that most laboratories are well equipped in HPLC instrumentation and have gained sufficient knowledge of the HPLC technique. There might be no urgent need to install another separation technique and therefore less new methods are developed using

Table 9.3.A.
Antibiotics in the European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopeia (USP)

	Number of monographs		Chromatographic assay method[a]		Chromatographic test method for related substances			
	Ph. Eur.	USP	Ph. Eur.	USP	HPLC		TLC	
					Ph. Eur.	USP	Ph. Eur.	USP
Penicillins	16	23	15	23	15	3	0	0
Cephalosporins	11	24	11	23	6	14	5	1
Macrolides	8	7	3	4	4	5	3	0
Tetracyclines	6	7	6	6	6	5	0	0
Aminoglycosides	9	7	1	4	2	0	6	1
Polypeptides	5	4	0	1	0	0	0	0
Polyenes	2	2	0	0	0	0	0	0
Others	14	14	2 + 3 (GLC)	10 + 1 (GLC)	7 + 2 (GLC)	9	3	1

[a] HPLC, high-performance liquid chromatography; GLC, gas chromatography; TLC, thin-layer chromatography

CE. The application of CE in the analysis of antibiotics has been reviewed [5].

In the following part of the text some more recent developments in the analysis of antibiotics by HPLC and CE will be reported. Most often the impurities are identified by comparison with reference substances. It should be emphasised that the same chemical entity may carry a different name when occurring in different antibiotics. Most of the earlier performed work and more detailed information can be found in excellent books [6–8]. It can be concluded that certainly for the more complex antibiotics, a lot remains to be done.

9.3.2. Penicillins

The penicillins are a large group of bicyclic ring compounds which contain a five-membered thiazolidine ring fused to a β-lactam ring, which is essential for antimicrobial activity. The penicillins are differentiated by a side chain which determines the spectrum of activity and the pharmacokinetic properties. Penicillins are unstable, especially in aqueous solutions, and form a variety of degradation products (e.g. penicilloic and penilloic acids).

Benzylpenicillin or *penicillin G* may contain precursors, side products from the biosynthesis and various degradation products. An evaluation of seven isocratic reversed phase HPLC methods for the analysis of benzylpenicillin and its related substances has been reported. The method which gave the better selectivity was able to separate more than nine components [9]. Based on this method, a gradient elution method was developed which allowed the elution of more strongly retained impurities [10]. Micellar electrokinetic capillary chromatography (MECC) was also explored and found to be as selective as HPLC for the separation of benzylpenicillin from eight related substances [11].

Phenoxymethylpenicillin or *penicillin V* is a natural penicillin too, but it is orally active. Five isocratic reversed phase HPLC methods have been examined for the separation of phenoxymethylpenicillin from its related substances. The best method, which was similar to that used for benzylpenicillin, allowed to separate 9 different components. The most important side product was found to be 4-hydroxyphenoxymethylpenicillin [12,13]. The separation of penicillin V from its related substances was also studied by MECC. Complete separation from eight related substances was obtained [14].

Capillary isotachophoresis (CITP) has been applied for the separation of *penicillin G* and V from their respective precursors [15].

Ampicillin and *amoxicillin* are very closely related semisynthetic aminopenicillins. Beside precursors and side products from the semisynthesis, also degradation products, including oligomers can be present in commercial samples. See Fig. 9.3.A for structures of amoxicillin and its potential impurities.

The selectivity of four isocratic reversed phase HPLC methods for the

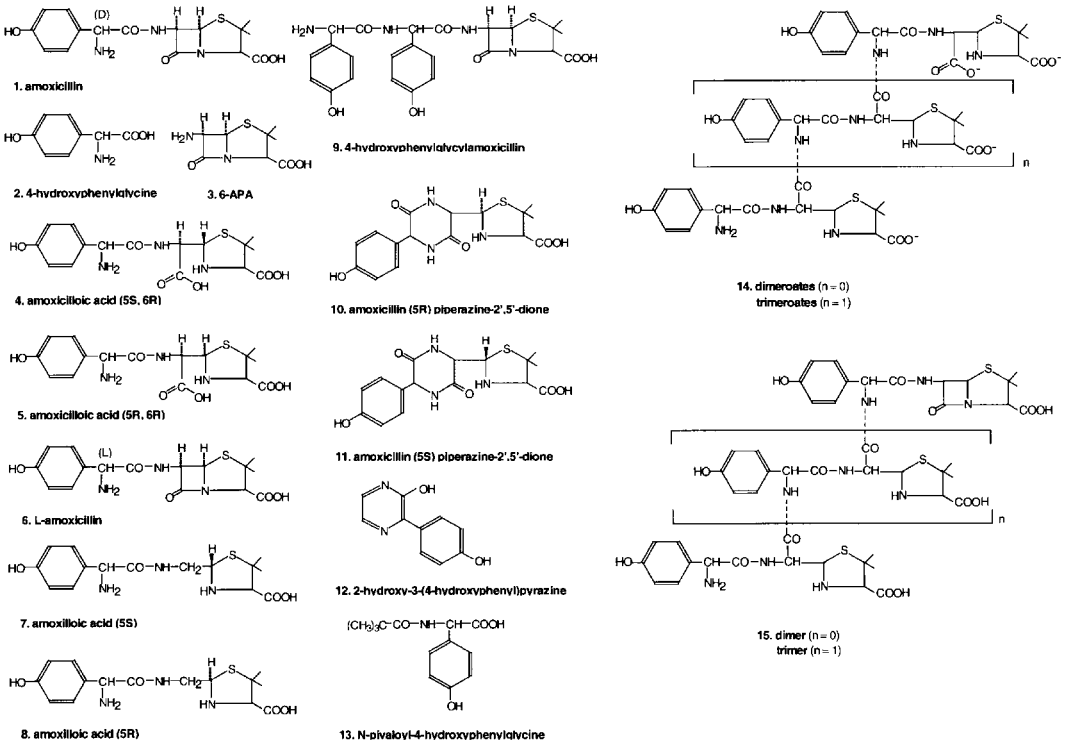


Figure 9.3.A.
Structures of amoxicillin and its potential impurities

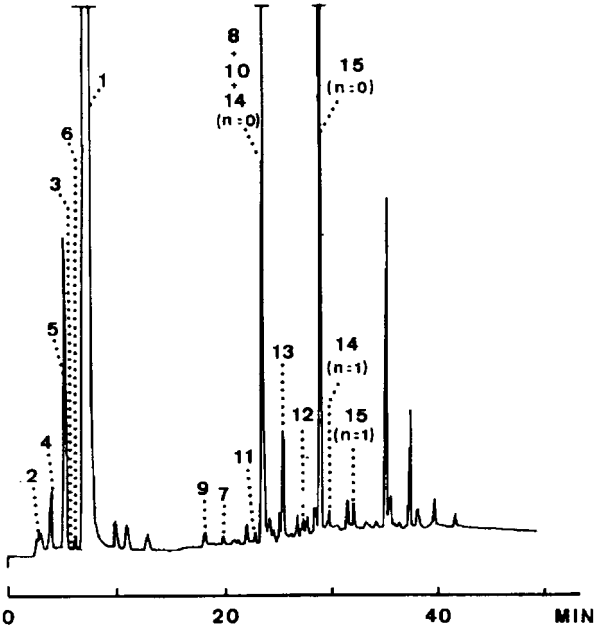


Figure 9.3.B.
Typical chromatogram of amoxicillin sodium with gradient elution. See Fig. 9.3.A for peak identification (from Ref. [18])

separation of *ampicillin* and its related substances was examined. It was found that the USP assay method gave the better selectivity. Based on this method, a gradient HPLC method was developed, which was shown to be suitable for the analysis of related substances in ampicillin [16]. MECC was found suitable for the separation of ampicillin from 15 related substances, four of which were oligomers, reported to be related to the allergenic effect of penicillins [17].

For *amoxicillin* and its related substances, five isocratic reversed phase HPLC methods were examined. Here too, the USP method gave the better results. However, for purity control, gradient elution was necessary. A typical chromatogram of amoxicillin sodium is shown in Fig. 9.3.B [18].

An amoxicillin sodium impurity, unstable in aqueous solutions, was investigated using ionspray MS with flow-injection analysis and HPLC-MS [19]. Five related substances of known structure, of which also a dimer, were separated by MECC. No quantitative data were reported, however [20]. Sixteen known related substances were separated from amoxicillin by MECC. The

quantitative determination of impurities is delicate, because the migration of some of these impurities can change very much upon slight variations of the background electrolyte [21]. An electropherogram of an old commercial sample of amoxicillin sodium salt is shown in Fig. 9.3.C.

MECC was also used to perform a chemical profiling of *ampicillin, amoxicillin* and *penicillin V* samples. Qualitative and quantitative comparisons in terms of the relative percentages of minor components present in the samples, allowed to distinguish samples from different manufacturers [22].

In general, CE methods tend to be faster and show equal or better selectivity than HPLC methods. They are sufficiently precise (RSD $< 1\%$ for the main compound) and sensitive (LOD and LOQ $< 0.1\%$).

Carbenicillin and *ticarcillin* are two closely related semisynthetic carboxypenicillins, both produced as a mixture of two epimers. An ion-pair reversed phase HPLC system to separate carbenicillin from its degradation products has been described [23]. Three other HPLC methods to determine impurities in ticarcillin were compared. Since none of the methods examined gave complete satisfaction, the method giving the better results was modified to obtain a suitable test for the content of impurities using a gradient HPLC procedure [24].

Other semisynthetic penicillins are the isoxazolylpenicillins, *cloxacillin*,

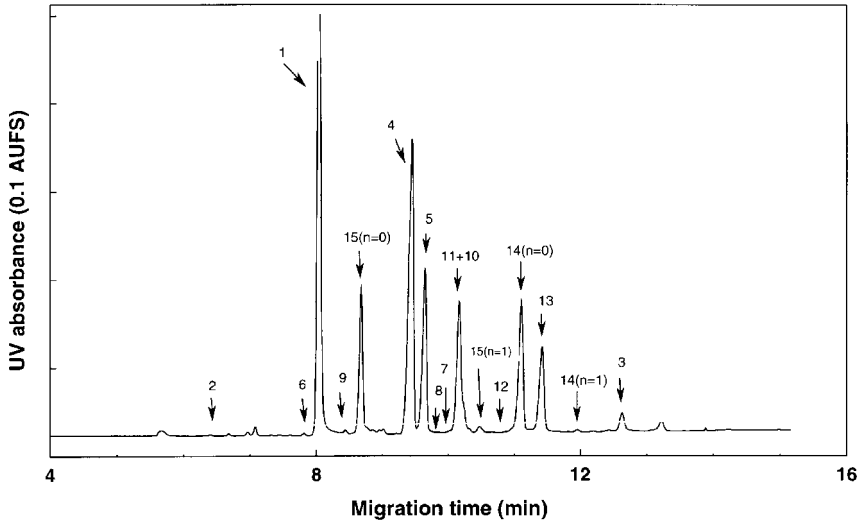


Figure 9.3.C.
Electropherogram of an old commercial sample of amoxicillin sodium salt [21]. See Fig. 9.3.A for peak identification (from Ref. [21])

dicloxacillin and *flucloxacillin*. A comparison of two reversed phase HPLC methods has been reported. Only two methods were compared because other procedures published were very similar. The study demonstrated the suitability of the USP method to determine the content in the drug substances and to control the levels of impurities [25]. Another paper described the identification of degradation products of cloxacillin using HPLC/MS. It was mentioned that care must be taken in the determination of penicillins by HPLC in the absence of specific detectors, because some degradation products have very similar retention times, possibly leading to misidentification [26].

Earlier work on CE of penicillins includes CITP of *sulbactam*. The inclusion complexation of β-cyclodextrin was used to separate a mixture of sulbactam and its synthesis intermediates, as well as a mixture with its alkaline degradation product. The mixtures consisted of equal amounts of the substances [27].

9.3.3. Cephalosporins

Cephalosporins are semisynthetic β-lactam antibiotics, closely related to the penicillins. Beside side products which can arise during semisynthesis, various degradation products can also be formed because cephalosporins are relatively unstable.

The separation of *cefalexin* from six of its related substances using ion-pair HPLC on a C_8 column has been reported [28]. Since C_8 and C_{18} stationary phases did not show a very good reproducibility of the selectivity towards cephalosporins, an isocratic ion-pair HPLC method using poly(styrene-divinylbenzene) (PSDVB) as stationary phase has been developed, allowing the separation of cefalexin from nine potential impurities. Quantitative results for official standards, bulk samples and pharmaceutical dosage forms were included. Peaks were identified by comparison with reference compounds of potential impurities and by photodiode array detection [29]. The same detection technique was also used in another HPLC method to provide more information about the identity of the impurities. This method, using gradient elution, allows the separation of cefalexin from eight potential impurities [30].

An isocratic HPLC method using PSDVB as the stationary phase has been described for the separation of *cefradine* from its impurities. The most important related substance, always present in cefradine, is cefalexin, which also gives origin to degradation products. Applicability of the method for analysis of bulk samples and pharmaceutical dosage forms was demonstrated [31]. An impurity of unknown identity was isolated by HPLC, and its structure was elucidated by NMR and MS to be 4′,5′-dihydrocefradine [32].

Cefadroxil was separated from nine of its decomposition and side

products using an isocratic ion-pair HPLC method on PSDVB. The method was used to compare official standards and to analyse a number of commercial samples [33]. MECC was shown to be suitable for the determination of ten related substances in cefalexin as well as in cefadroxil, and showed a better selectivity and shorter analysis time than HPLC [34,35].

Two isocratic reversed phase HPLC methods for the analysis of *cefalotin* were compared [36]. Since neither of the two methods gave complete satisfaction, the better method was adapted before introduction in the Ph. Eur.

Three isocratic reversed phase HPLC methods were examined for their capacity to separate *cefazolin* from its related substances and decomposition products. The USP method was found to give the best result [37].

The determination of process-related impurities and degradation products of *cefaclor* by HPLC using gradient elution has been reported. The method was applied in stability studies, impurity profiling and analysis of pharmaceutical formulations. The HPLC method was also combined with photodiode array and MS detection to identify some impurities. Other impurities were isolated by preparative chromatography and their structure was determined by MS and NMR [30,38].

Reversed phase HPLC analysis of *cefoxitin* and its related components using gradient elution was reported. Four by-products were determined [39].

A comparison of MECC and HPLC for *cefotaxime* and its related impurities has been published. Structures of cefotaxime and related impurities are depicted in Fig. 9.3.D. Results obtained by both methods were in good agreement. The MECC method, which allowed to determine seven known and three unknown impurities in cefotaxime, was preferred because it is more rapid and economical. Typical electropherograms of a spiked and an unspiked sample are shown in Fig. 9.3.E [40].

Ceftazidime could be resolved by MECC from two major impurities, namely pyridine and the Δ^2-isomer [41].

9.3.4. Macrolides

Reviews on the analysis of macrolides have been published recently [42,43].

Erythromycin A, the main component of *erythromycin* is present together with B, C and E as most important biologically active impurities, and with D and F as minor components. Inactive degradation products and the metabolite *N*-demethylerythromycin A may also be present. Separation of these derivatives from commercial samples by TLC was described. Erythromycin A *N*-oxide and the pseudoerythromycins were also separated [44,45].

Analysis is now carried out mostly by HPLC [46–48]. Degradation

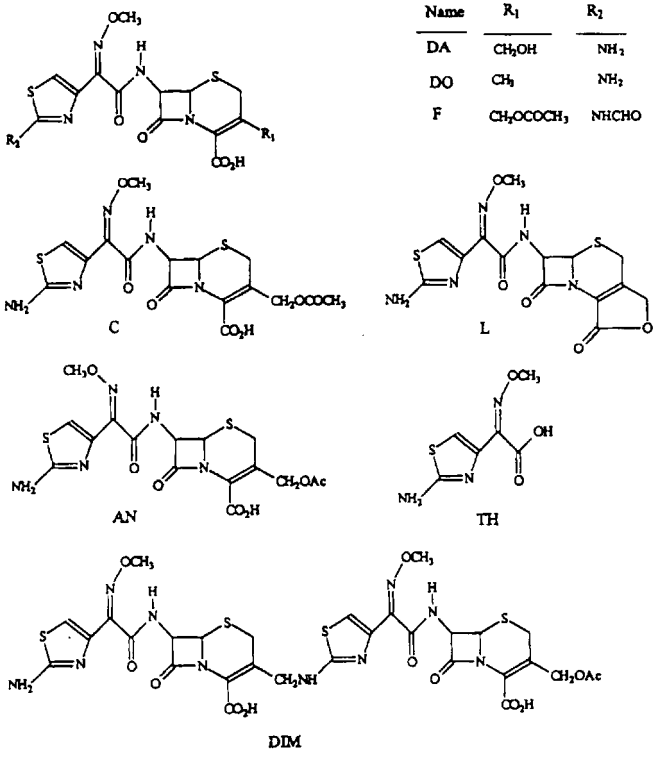


Figure 9.3.D.
Structures of cefotaxime and related impurities [40]

kinetics and stability in neutral and alkaline solutions were investigated [49]. MECC was used to perform a chemical profiling of erythromycin samples. Comparisons in terms of the relative percentages of minor components present in the samples, allowed to distinguish samples from different manufacturers [22]. Erythromycin showed the presence of three impurities when capillary zone electroporesis (CZE) was applied [50]. Five related substances of known structure were also successfully resolved from erythromycin and from each other by CZE. In the presence of large amounts of erythromycin, only three of these impurities could be quantitated [51]. On the contrary, HPLC allows to determine nine known and three unknown impurities [47]. Erythromycin D could not be separated by HPLC or CE.

During analysis of *erythromycin ethylsuccinate*, it was found that *N*-ethyl-

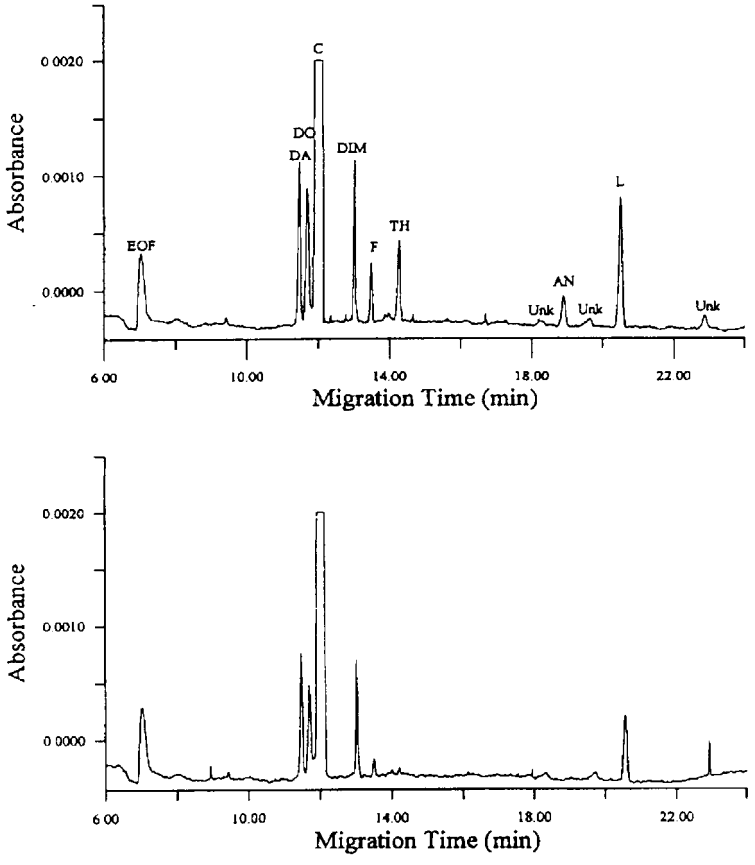
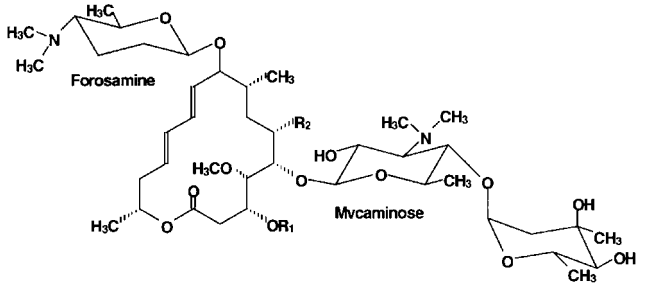


Figure 9.3.E.
Typical electropherograms of a spiked (upper figure) and an unspiked (lower figure) cefotaxime sample. See Fig. 9.3.D for peak identification (from Ref. [40])

succinyl-*N*-demethylerythromycin A was present as a side product from the synthesis [52]. *Erythromycin estolate* contains the corresponding *N*-propionyl-*N*-demethylerythromycin A as a side product [53].

Azithromycin, *clarithromycin*, *roxithromycin* and *dirithromycin* are semi-synthetic erythromycin derivatives. Azithromycin was separated by HPLC from four of its related compounds formed during synthesis and from three acid degradation products [54]. *Clarithromycin* was separated by HPLC from



compound	R_1	R_2	Mycarose
Spiramycin I (SPM I)	H	CH_2CHO	+
Spiramycin II (SPM II)	$COCH_3$	CH_2CHO	+
Spiramycin III (SPM III)	$COCH_2CH_3$	CH_2CHO	+
Spiramycin IV (SPM IV)	H	CH_2CH_2OH	+
Neospiramycin I (NSPM I)	H	CH_2CHO	-
Neospiramycin II (NSPM II)	$COCH_3$	CH_2CHO	-
Neospiramycin III (NSPM III)	$COCH_2CH_3$	CH_2CHO	-

Figure 9.3.F.
Structures of spiramycins and related substances

nine related polar components [55]. It was also separated from seven non-polar components by another method [56] and from nine acid and base degradation products with yet another mobile phase [57]. For *roxithromycin* an official HPLC method allows the separation of nine related substances [1a]. The precursors in the synthesis of *dirithromycin*, erythromycylamine formed by hydrolysis and epidirithromycin formed in an equilibrium process, were separated by HPLC [58].

Oleandomycin showed the presence of two impurities when CZE was applied [50].

Spiramycin, a 16-membered lactone, consists predominantly of three closely-related substances, I with a free hydroxyl group, II and III containing an acetyl and propionyl group, respectively (Fig. 9.3.F). Separation of these major and several minor components has been described [59]. Two novel spiramycins have been isolated from commercial samples and their structure was elucidated [60]. A mixed micellar CE system was applied to separate spiramycin I from six related substances [61]. Though faster, the CE method was less selective than HPLC, as can be seen in Figs. 9.3.G and 9.3.H.

The acid degradation of *josamycin* (leucomycin A3) was studied by HPLC and the molecular weight of each substance formed was determined by thermospray MS. Rearrangement in the aglycone portion giving isoleucine A3 and

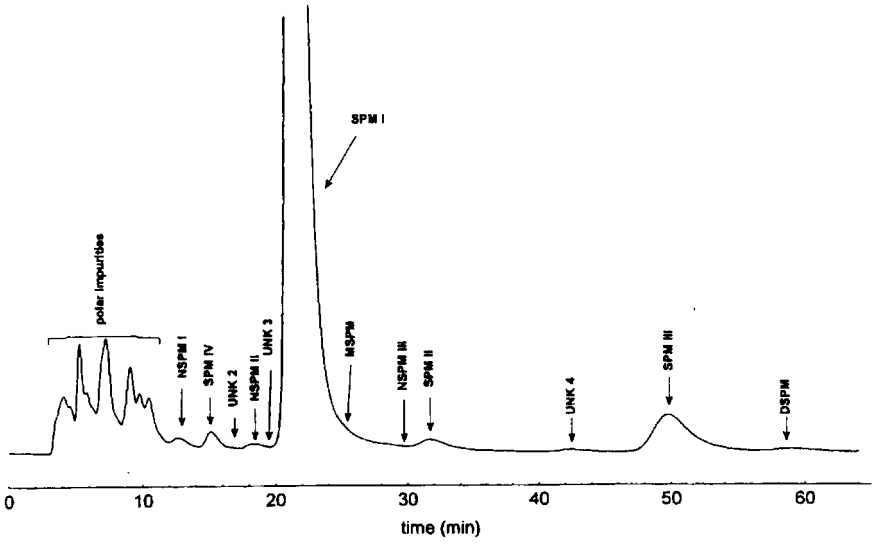


Figure 9.3.G.
Typical chromatogram of commercial spiramycin [59]

cleavage of the glycosidic bond with formation of demycarosyl leucomycin A3 occurred [62]. Ten related leucomycins were separated from josamycin by HPLC using a PSDVB column [63].

Josamycin propionate was separated by HPLC on PSDVB from 7 related substances arising from the biosynthesis of josamycin and from subsequent esterification. Leucomycin A4 propionate and platenomycin A1 propionate are the main biosynthesis impurities while josamycin 2′,9- and 3′′,9-dipropionates are formed by overesterification [64].

Tylosin, with a 16-membered lactone ring, mainly contains the major component A, desmycosin (tylosin B), macrocin (tylosin C) and relomycin (tylosin D). The 4 main components and five minor components were separated using ion-pair chromatography at 55°C [65]. A previously unknown derivative, tylosin A aldol (TAD) was described [66]. The stability of tylosin A in aqueous solution was studied by HPLC on PSDVB and the formation of TAD and a previously unidentified isotylosin A was mentioned [67]. Separation of 2 TAD epimers was realised and for the photochemical reaction product isotylosin A, two isomers were isolated. The structure of the main isomer was elucidated with ^{13}C NMR [68]. Using a mixed micellar CE system, it was possible to separate tylosin from eight known related compounds in a short analysis time [69].

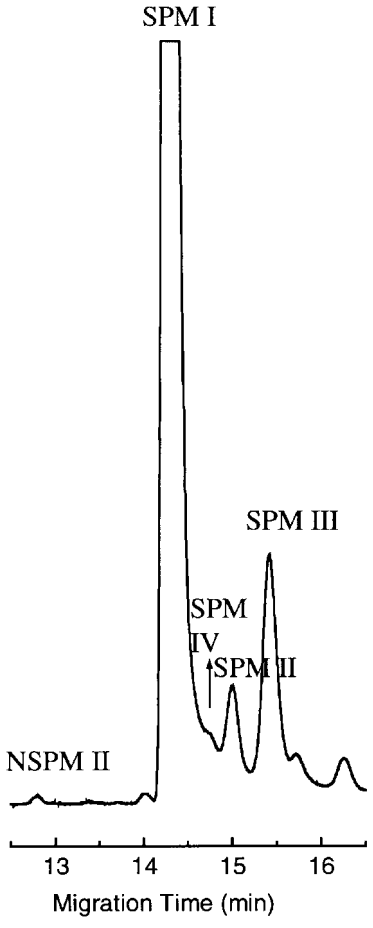


Figure 9.3.H.
Electropherogram of a commercial sample of spiramycin. See Fig. 9.3.F for peak identification (from Ref. [61])

9.3.5. Tetracyclines

Degradation reactions of tetracyclines have been reviewed. These include C-4 epimerisation, dehydration in acidic medium to form anhydrotetracyclines and phthalide formation in basic medium to give isotetracyclines. Oxidation results in enamine formation with subsequent loss of the dimethylamino function to give 4-ketotetracyclines [70]. The primary tetracyclines are all obtained by fermentation and small amounts of related substances are coproduced, e.g.

2-acetyl-2-decarboxamidotetracyclines are present in the corresponding tetracyclines [71]. *Chlortetracycline* contains up to 10 per cent of tetracycline. *Oxytetracycline* contains some tetracycline, while *demethylchlortetracycline* contains mainly demethyltetracycline. *Tetracycline* does not contain another active component.

Analysis and purity control of these natural tetracyclines has been described extensively and a comparison between TLC and HPLC has been carried out [72–74].

Tetracycline was investigated by CZE and three to eight related substances were separated [75–78]. Non-aqueous CE and CZE on C18-coated capillaries were also explored [79,80]. Nice separation of chlortetracycline from six related substances was obtained by CZE [81]. An optimised CZE method with triton-X-100 and methyl-β-cyclodextrin allowed for the separation of demethylchlortetracycline from four related substances, including the acetyldecarboxamido derivative [78]. Eight related substances, including the acetyldecarboxamido derivative, were separated from oxytetracycline using CZE [82].

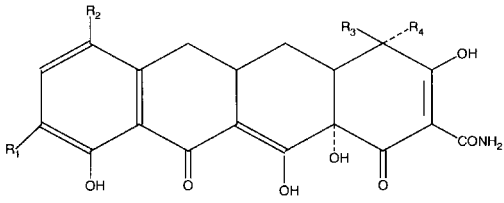
Analysis and purity control for the semi-synthetic derivatives *doxycycline* [83] and *minocycline* [84,85] have also been reported. Five related substances were resolved from doxycycline [86] and also from minocycline [87] by CZE (Figs. 9.3.I–K).

As far as speed and selectivity are concerned, CE methods are generally similar or better than HPLC methods, but they tend to be less precise. For *metacycline*, another semi-synthetic derivative, the related substances doxycycline, 6-epidoxycycline and 2-acetyl-2-decarboxamidotetracycline have been isolated by preparative column chromatography [88]. A comparison between TLC and HPLC for assay and purity control has been carried out [89].

Keto-enol tautomerism of *chlortetracycline* and *doxycycline* was described. The chemical structures of the tautomers were elucidated by on-line and off-line UV spectrophotometry and by ^{13}C and ^{1}H NMR spectrometry [90,91]. The decomposition of tetracycline in methanolic solution under the influence of light and atmospheric oxygen was investigated by HPLC/MS. A molecular structure for eight degradation products was proposed on the basis of their product-ion mass spectra [92].

9.3.6. Aminoglycosides

The aminoglycoside antibiotics, like *neomycin, kanamycin, amikacin, tobramycin, gentamicin, netilmicin, streptomycin* and *dihydrostreptomycin*, all contain amino sugars linked to an aminocyclitol ring. Since most aminoglycosides do not have a UV absorbing chromophore, pre- and postcolumn



	R_1	R_2	R_3	R_4
Minocycline (MC)	H	$N(CH_3)_2$	H	$N(CH_3)_2$
6-Deoxy-6-demethyl-tetracycline (6-DODMTC)	H	H	H	$N(CH_3)_2$
7-Didemethylminocycline (7-DDMMC)	H	NH_2	H	$N(CH_3)_2$
7-Monodemethylmino-cycline (7-MDMMC)	H	$NHCH_3$	H	$N(CH_3)_2$
9-Minocycline (9-MC)	$N(CH_3)_2$	H	H	$N(CH_3)_2$
4-Epiminocycline (EMC)	H	$N(CH_3)_2$	$N(CH_3)_2$	H

Figure 9.3.I.
Chemical structures of minocycline and related substances

derivatisation, refractive index detection and pulsed electrochemical detection (PED) have been used in HPLC analysis.

Neomycin is mainly composed of a mixture of neomycin B and its epimer neomycin C. The Ph. Eur. limits the amount of neomycin C to 3–15%. Neomycin with a content of less than 3% neomycin is called *framycetin* [1]. The USP does not distinguish between neomycin and framycetin [2]. Although beside neomycins B and C, several other components can be present in commercial neomycin samples, most described methods focus on the separation of neomycins A, B and C [93–96]. More recently, an ion-pair reversed phase HPLC method, allowing to separate eight neomycin components, was described. The method was used to analyse a number of commercial samples [97].

Although it was known that *kanamycin*, beside the main compound kanamycin A, contains also several other components, HPLC methods described before 1997 only distinguished between kanamycins A and B [98,99]. In 1997, the separation of eight kanamycin components in commercial samples was described using ion-pair reversed phase HPLC. Two impurities, which were not reported to be present in commercial samples before, were isolated by ion-exchange column chromatography and their structure was determined by MS and NMR [100].

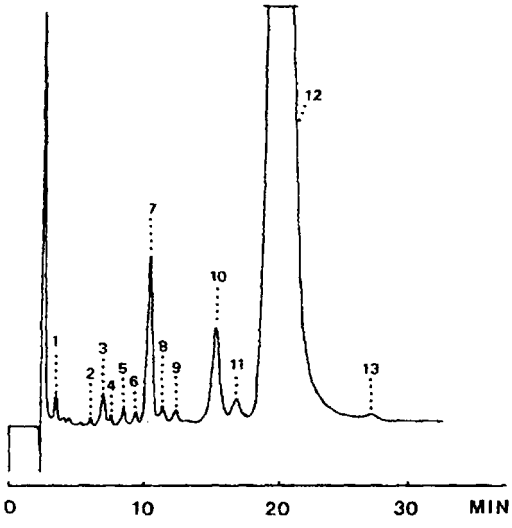


Figure 9.3.J.
Chromatogram of an old sample of minocycline hydrochloride [85]. 1, 7-DDMMC; 2, UNK; 3, 7-MDMMC; 4, UNK; 5, UNK; 6, UNK; 7, EMC; 8, UNK; 9, UNK; 10, 6-DODMTC; 11, UNK; 12, MC; 13, 9-MC; UNK, unknown (from Ref. [85])

Amikacin is a semisynthetic aminoglycoside, obtained by acylation of one amino group in kanamycin A. As kanamycin A has 4 primary amino groups, it is possible to obtain side products that differ only in the position of the acyl group [101]. Beside the positional isomers, which had already been separated by HPLC previously [99], four other substances were also found to be present in commercial samples using ion-pair reversed phase HPLC [102]. Like most semisynthetic antibiotics, amikacin is relatively pure.

Although several HPLC methods have been described to analyse *tobramycin*, nearly all of them focus on the determination of the main peak. Only two papers give information about the presence of minor constituents [103,104]. The separation of nine components was reported. Four of them were identified using reference substances of potential impurities [104].

Gentamicin is a complex mixture of four major components (C_1, C_{1a}, C_2 and C_{2a}) and several minor components, e.g. gentamicin C_{2b}, as shown in Fig. 9.3.L. The separation of component C_{2b} by HPLC has only been reported in a few papers [105–107]. Two give an overview of the composition of commercial gentamicin samples [105,107]. Figure 9.3.M shows a typical chromato-

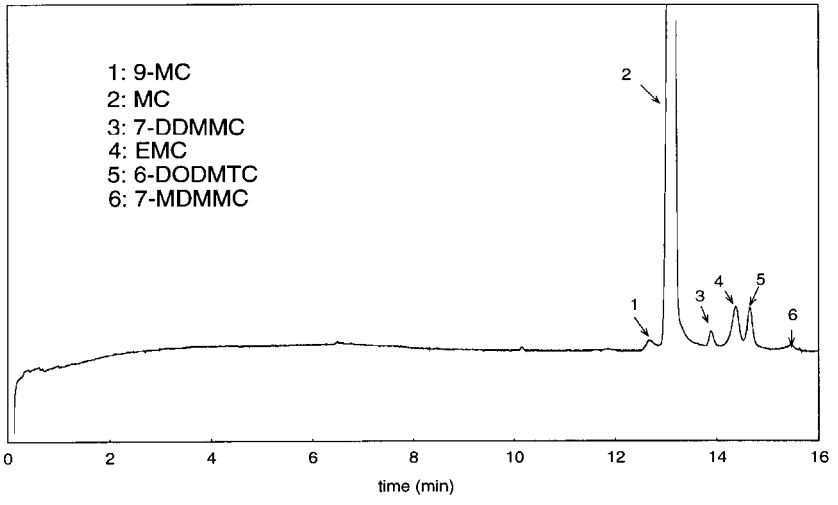


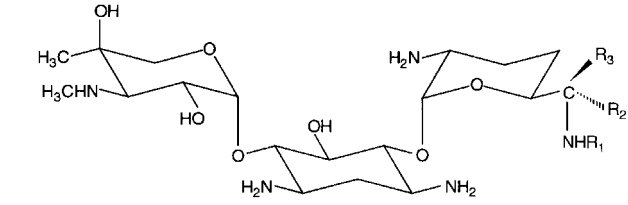
Figure 9.3.K.
Electropherogram of minocycline and related substances. See Fig. 9.3.I for peak identification (from Ref. [87])

gram of a commercial sample obtained by isocratic ion-pair reversed phase HPLC. As can be seen, not all peaks were identified [107].

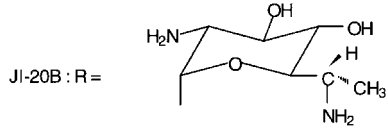
Netilmicin is the N^1-ethyl derivative of sisomicin. Since sisomicin has more than one amino group, other *N*-ethyl derivatives can be formed during synthesis. One HPLC method allows the separation of netilmicin from its potential impurities. Four out of seven impurities separated were identified [108].

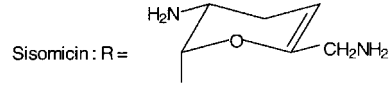
Although *streptomycin* and *dihydrostreptomycin* have been in use for many years, very few HPLC methods were published. An ion-pair reversed phase method combined with UV detection at low wavelength was used to determine streptomycin and dihydrostreptomycin [109]. However, quantitative results were given for only two impurities. Recently, this method was further examined and adapted for dihydrostreptomycin, after which it was used for the analysis of commercial samples. Not only four known impurities could be separated, but also six of unknown identity [110]. The residual quantity of streptomycin in bulk dihydrostreptomycin was also determined by MECC with a cationic surfactant. Other, unidentified compounds, were present in a dihydrostreptomycin standard [111].

So in conclusion, except for streptomycin and dihydrostreptomycin, the



	R_1	R_2	R_3
Gentamicin C_1	CH_3	H	CH_3
Gentamicin C_2	H	H	CH_3
Gentamicin C_{1a}	H	H	H
Gentamicin C_{2a}	H	CH_3	H
Gentamicin C_{2b}	CH_3	H	H





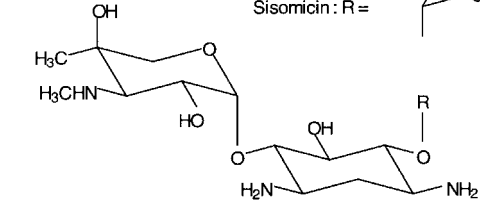


Figure 9.3.L.
Structure of gentamicin components

other aminoglycosides mentioned above can be analysed using ion-pair reversed phase HPLC on PSDVB as stationary phase in combination with PED [97,100,102,104,107,108,110].

The aminoglycosides *butirosin* A and B differ in their structure by the configuration of one sugar hydroxyl group. The impurity butirosin B was quantified in a butirosin A standard by CZE. Other, unidentified species, were detected in a butirosin B standard [111]. The formation of negatively charged complexes between the aminoglycosides and borate was utilised for direct UV detection.

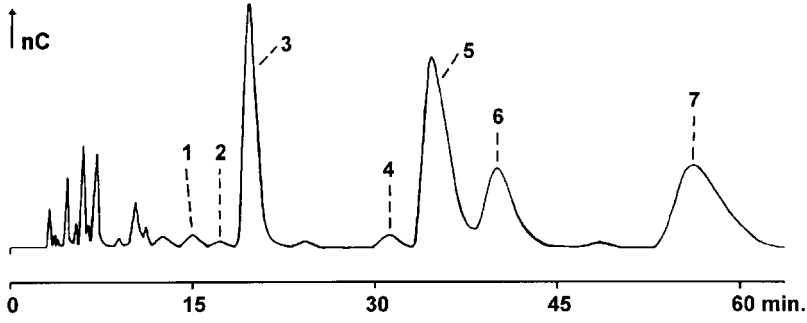


Figure 9.3.M.
Typical chromatogram of a commercial gentamicin sample. 1, JI-20B; 2, sisomicin; 3, gentamicin C_{1a}; 4, gentamicin C_{2b}; 5, gentamicin C_2; 6, gentamicin C_{2a}; 7, gentamicin C_1 (from Ref. [107])

9.3.7. Polypeptides

Bacitracin, used also as a zinc derivative, is a cyclic branched chain polypeptide complex. The major component, up to 80% A, is accompanied by minor ones: B1 to B3, D1 to D3 and E1 to E3, all having antibacterial activity. The corresponding degradation compounds (bacitracins F) are formed by oxidative deamination. The composition was examined by HPLC [112]. Partial separation of individual components was carried out by preparative HPLC [113] and counter-current chromatography [114]. Correlation between some components and antibacterial activity was established [112,115,116]. Structural characterisation of 13 minor components was carried out by HPLC combined with FAB-MS [117].

Polymyxins are a group of cyclic heptapeptides with a fatty acyltripeptide chain. The chemical structures of the main components were proved mainly by hydrolysis and identification of the resulting amino acids, di- and tripeptides and fatty acids. All have five unmasked 2,4-diaminobutyric acid residues making them strongly basic.

Polymyxin B consists of known components B1 to B4. Semi-preparative column chromatography [118] has been reported for isolation of single components from the complex. The presence of polymyxin B0, containing an unknown polar fatty acid was proved after isolation by chromatography on PSDVB [119]. Isolation and characterisation of a polymyxin B1, containing isoleucin in place of leucin, was reported [120]. Several HPLC papers discuss only the presence of the main components, although up to 20 components were

separated [121–125].

Polymyxin E (colistin). The same methodology as for polymyxin B was followed for isolation and characterisation of different components. The presence of three polymyxins E in which leucine was replaced by valine, nor-valine or isoleucine was reported [120]. Counter-current chromatography has been published [126]. Three minor components were isolated and tentatively identified by MS [127].

The separation of polymyxins by MECC was investigated and at least ten compounds were resolved in polymyxin B [128]. Of these, three peaks were identified as polymyxin B2, B3 and B1. Polymyxins A and D showed two main peaks, tentatively assigned to A2, A1 and D2, D1. Polymyxin E contained several other minor species apart from E1, E2 and E3, but these were not identified. Further work is required to identify the components of these complex antibiotics.

Gramicidin is a mixture of linear *N*-formyl pentadecapeptides. Counter-current chromatography [129] and semi-preparative chromatography [130] have been used to fractionate the mixture into the three major groups. Each group consists of two closely related components, containing valine or isoleucine respectively, as was proved by amino acid analysis [129]. Analytical HPLC separation of the gramicidin components has been described [123, 125,131].

9.3.8. Polyenes

A review of the analysis of polyene antifungal antibiotics has been published [132].

Amphotericin B is an amphoteric macrocyclic heptaene macrolide with a typical chromophore. Amphotericin A, a cofermented tetraene, is less active and usually present as a contaminant [133]. Several other, less important components, have been separated by HPLC [134]. A combination of TLC and HPLC has also been used for the investigation of the complex composition of this compound. Beside the two major components, five other heptaenes, one tetraene, a fluorescent pentaene and at least five other components were separated [135].

Nystatin is a polyene macrolide possessing a diene-tetraene chromophore. Isolation of pure components is extremely difficult. Preparative separation on silica gel with aqueous solvents was carried out. Four major components, present in Chinese nystatin, were isolated and their structures were determined by MS. 10-Deoxynystatin A1 was not reported previously [136]. The composition of nystatin was studied by TLC and HPLC with gradient elution showing up to six components. Nystatin consists largely of nystatin A1 while A2, A3

and polyfungin B are minor components. Low amounts of heptaenes are also present [137,138]. The difference in composition of European, American and Chinese samples was shown [139]. Nystatin produced by an improved strain was studied by a combination of HPLC and MS. Nystatin A1, A2, A3 and polyfungin B were shown to be present [140]. A method was described, with a mobile phase that does not affect the antifungal activity of nystatin [141]. It allowed the separation of five peaks. The last eluting peak has a deviating heptaene structure.

References

1. *European Pharmacopoeia, 3rd edn*, and three supplements, pp 1448–1451, Council of Europe, Strasbourg (1997–2000)
2. *United States Pharmacopeia 23*, and nine supplements, USP, Rockville (1995–1998)
3. *Pharmeuropa*, Council of Europe, Strasbourg
4. *Pharmacopeial Forum*, USP, Rockville
5. C.L. Flurer, *Electrophoresis* **18**, 2427–2437 (1997)
6. M.J. Weinstein and G.H. Wagman (Eds.), *Antibiotics, Isolation, Separation and Purification, J. Chromatogr. Library*, Vol. 15, Elsevier, Amsterdam (1978)
7. G.H. Wagman and R. Cooper (Eds.), *Natural Products Isolation, J. Chromatogr. Library*, Vol. 43. Elsevier, Amsterdam (1989)
8. K. Florey or H.G. Brittain (Eds.), *Analytical Profiles of Drug Substances*, Vols. 1–25, Academic Press, San Diego, CA (1972–1998)
9. Zhu Yongxin, A. Verhasselt, E. Roets, A. Perez, E. Porqueras and J. Hoogmartens, *J. Chromatogr. A* **773**, 147–156 (1997)
10. Z. Yongxin, E. Roets, J. Loidl, P. Inama, A. Perez, E. Porqueras, E. Wachberger, C. Lebbe, A.J.W. Smelt, C. Van der Vlies, J.H.McB. Miller, J.M. Spieser and J. Hoogmartens, *J. Chromatogr. B* **707**, 189–193 (1998)
11. Zhu Yongxin, J. Dalle, A. Van Schepdael, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **792**, 83–88 (1997)
12. Zhu Yongxin, E. Merken, M.P. Arevalo, E. Porqueras, E. Roets and J. Hoogmartens, *Eur. J. Pharm. Sci.* **6**, 39–45 (1998)
13. Zhu Yongxin, E. Roets, B. Trippen, Ch.P. Christiansen, M.P. Arevalo, E. Porqueras, B. Maichel, P. Inama, S. Söderholm, J.H.McB. Miller, J.M. Spieser and J. Hoogmartens, *Chromatographia* **47**, 152–156 (1998)
14. Yongxin Zhu, A. Van Schepdael, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **781**, 417–422 (1997)
15. D. Tsikas, A. Hofrichter and G. Brunner, *Chromatographia* **30**, 657–662 (1990)

16. Yongxin Zhu, E. Roets, Zhigang Ni, M.L. Moreno, E. Porqueras and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **14**, 631–639 (1996)
17. Zhu Yongxin, C. Hoogmartens, A. Van Schepdael, E. Roets and J. Hoogmartens, *J. Liq. Chrom. Rel. Technol*. **22**, 1403–1415 (1999)
18. Zhu Yongxin, E. Roets, M.L. Moreno, E. Porqueras and J. Hoogmartens, *J. Liq. Chrom. Rel. Technol.* **19**, 1893–1908 (1996)
19. L. Valvo, S. Alimonti, R. Alimenti, C. De Sena, E. Ciranni Signoretti, R. Draisci and L. Giannetti, *J. Pharm. Biomed. Anal.* **15**, 487–493 (1997)
20. G.N. Okafo and P. Camilleri, *Analyst* **117**, 1421–1424 (1992)
21. Y.M. Li, A. Van Schepdael, Y. Zhu, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **812**, 227–236 (1998)
22. C.L. Flurer and K.A. Wolnik, *J. Chromatogr. A* **674**, 153–163 (1994)
23. P.A. Twomey, *J. Pharm. Sci.* **70**, 824–826 (1981)
24. J.H.McB. Miller and C.J. Nap, *Pharmeuropa* **8**, 185–192 (1996)
25. J.H.McB. Miller and C.J. Nap, *Pharmeuropa* **8**, 193–198 (1996)
26. K.L. Tyczkowska, R.D. Voyksner and A.L. Aronson, *J. Chromatogr.* **594**, 195–201 (1992)
27. I. Jelínek, J. Snopek and E. Smolková-Keulemansová, *J. Chromatogr.* **405**, 379–384 (1987)
28. B. Mason and J. Tranter, *Anal. Proc.* **18**, 310–313 (1981)
29. C. Hendrix, J. Thomas, Li-Ming Yun, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.* **16**, 421–445 (1993)
30. B.A. Olsen, S.W. Baertschi and R.M. Riggin, *J. Chromatogr.* **648**, 165–173 (1993)
31. C. Hendrix, M. Pijcke, Li Ming Yun, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **11**, 145–155 (1993)
32. Zhu Yongxin, C. Hendrix, R. Busson, G. Janssen, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **12**, 1137–1140 (1994)
33. C. Hendrix, C. Wijsen, Li Ming Yun, E. Roets and J. Hoogmartens, *J. Chromatogr.* **628**, 49–58 (1993)
34. Y.-M. Li, Y. Zhu, D. Vanderghinste, A. Van Schepdael, E. Roets and J. Hoogmartens, *Electrophoresis* **20,** 127–131 (1999)
35. Y.-M. Li, D. Vanderghinste, D. Pecanac, A. Van Schepdael, E. Roets and J. Hoogmartens, *Electrophoresis* **19**, 2890–2894 (1998)
36. C. Hendrix, E. Roets and J. Hoogmartens, *Pharmeuropa* **5**, 153–158 (1993)
37. C. Hendrix, R. Anthonissen, E. Roets and J. Hoogmartens, *Pharmeuropa* **6**, 204–207 (1994)
38. L.J. Lorenz, F.N. Bashore and B.A. Olsen, *J. Chromatogr. Sci.* **30**, 211–216 (1992)
39. A. Mangia, A. Scandroglio, S. Silingardi and P. del Buttero, *Il Farmaco – Ed. Pr.* **41**, 107–112 (1986)

40. G. Castaneda Penalvo, E. Julien and H. Fabre, *Chromatographia* **42**, 159–164 (1996)
41. R. Porrà, A. Farina, V. Cotichini and R. Lecce, *J. Pharm. Biomed. Anal.* **18**, 241–248 (1998)
42. H. Vanderhaeghe and J. Hoogmartens, in *Macrolides, Chemistry, Pharmacology and Clinical Uses* (A.J. Bryskier, J.P. Butzler, H.C. Neu and P.M. Tulkens, Eds.), pp 83–100, Arnette Blackwell, Paris (1993)
43. I. Kanfer, M.F. Skinner and R.R. Walker, *J. Chromatogr. A* **812**, 255–286 (1998)
44. Th. Cachet, E. Roets, J. Hoogmartens and H. Vanderhaeghe, *J. Chromatogr.* **403**, 343–349 (1987)
45. I.O. Kibwage, R. Busson, G. Janssen, J. Hoogmartens, H. Vanderhaeghe and J. Bracke, *J. Org. Chem.* **52**, 990–996 (1987)
46. Th. Cachet, I. Quintens, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.* **12**, 2171–2201 (1989)
47. J. Paesen, E. Roets and J. Hoogmartens, *Chromatographia* **32**, 162–166 (1991)
48. M.M. Nasr and T.J. Tschappler, *J. Liq. Chromatogr. Rel. Techol.* **19**, 2329–2348 (1996)
49. J. Paesen, K. Khan, E. Roets and J. Hoogmartens, *Int. J. Pharm.* **113**, 215–222 (1994)
50. C. Flurer, *Electrophoresis* **17**, 359–366 (1996)
51. A.K. Lalloo and I. Kanfer, *J. Chromatogr. B* **704**, 343–350 (1997)
52. Th. Cachet, P. Lannoo, J. Paesen, G. Janssen and J. Hoogmartens, *J. Chromatogr.* **600**, 99–108 (1992)
53. Th. Cachet, M. Delrue, J. Paesen, R. Busson, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **10**, 851–860 (1992)
54. N. Kovacic-Bosnjak, J. Marincel, N. Lopotar and G. Kobrehel, *Chromatographia* **25**, 999–1003 (1988)
55. D. Morgan, P. Cugver, B. Marello, C. Sarocka, D. Stroz and A. Plasz, *J. Chromatogr.* **502**, 351–358 (1990)
56. R.J. Gorski, D.K. Morgan, C. Sarocka and A.C. Plasz, *J. Chromatogr.* **540**, 422–429 (1991)
57. D.K. Morgan, D.M. Brown, T.D. Rotsch and A.C. Plasz, *J. Pharm. Biomed. Anal.* **9**, 261–269 (1991)
58. D.A. Olsen, J.D. Stafford and D.E. Reed, *J. Chromatogr.* **544**, 203–208 (1992)
59. L. Liu, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **764**, 43–53 (1997)
60. L. Liu, E. Roets, R. Busson, A. Vankeerbergen, G. Janssen and J. Hoogmartens, *J. Antibiot.* **49**, 398–401 (1996)

61. R. González-Hernández, Y.-M. Li, A. Van Schepdael, E. Roets and J. Hoogmartens, *Electrophoresis* **20**, 2407–2411 (1999)
62. M. Skinner, R.B. Taylor and I. Kanfer, *Eur. J. Pharm. Sci.* **1**, 61–72 (1993)
63. J. Paesen, A. Solie, E. Roets and J. Hoogmartens, *Fres. J. Anal. Chem.* **352**, 797–800 (1995)
64. E. Roets, X. Lepoudre, V. Van Rompaey, G. Velghe, L. Liu and J. Hoogmartens, *J. Chromatogr. A* **812**, 303–308 (1998)
65. J.H. Kennedy, *J. Chromatogr.* **281**, 288–292 (1983)
66. B.J. Fisch and G.P.R. Carr, *J. Chromatogr.* **253**, 39–50 (1986)
67. J. Paesen, P. Claeys, W. Cypers, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **699**, 93–97 (1995)
68. J. Paesen, W. Cypers, R. Busson, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **699**, 99–106 (1995)
69. K. Tobback, Y.-M. Li, N. Alberto Pizarro, I. De Smedt, T. Smeets, A. Van Schepdael, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **857**, 313–320 (1999)
70. L.A. Mitscher, *Med. Res. Ser.* **9**, 122–164 (1978)
71. G.C. Lancini and P. Sensi, *Experientia* **20**, 83–84 (1964)
72. Weng Naidong, S. Geelen, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **8**, 891–898 (1990)
73. Weng Naidong, C. Hauglustaine, E. Roets and J. Hoogmartens, *J. Plan. Chromatogr.* **4**, 63–68 (1991)
74. Weng Naidong, Sun Hua, E. Roets and J. Hoogmartens, *J. Plan. Chromatogr.* **5**, 152–156 (1992)
75. C.-X. Zhang, Z.-P. Sun, D.-K. Ling and Y.-J. Zhang, *J. Chromatogr.* **627**, 281–286 (1992)
76. S. Croubels, W. Baeyens, C. Dewaele and C. Van Peteghem, *J. Chromatogr. A* **673**, 267–274 (1994)
77. M.F.M. Tavares and V.L. McGuffin, *J. Chromatogr. A* **686**, 129–142 (1994)
78. Y.M. Li, A. Van Schepdael, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **15**, 1063–1069 (1997)
79. J. Tjørnelund and S.H. Hansen, *J. Chromatogr. A* **737**, 291–300 (1996)
80. J.J. Pesek and M.T. Matyska, *J. Chromatogr. A* **736**, 313–320 (1996)
81. Y.M. Li, H. Moons, A. Van Schepdael, E. Roets and J. Hoogmartens, *Chromatographia* **48**, 576–580 (1998)
82. Y.M. Li, A. Van Schepdael, E. Roets and J. Hoogmartens, *J. Liq. Chrom. Rel. Technol.* **20**, 273–282 (1997)
83. K. Dihuidi, M.J. Kucharski, E. Roets and J. Hoogmartens, *J. Chromatogr.* **325**, 431–424 (1985)

84. Weng Naidong, K. Vermeulen, I. Quintens, E. Roets and J. Hoogmartens, *Chromatographia* **33**, 560–566 (1992)
85. Weng Naidong, J. Thuranira, K. Vermeulen, E. Roets and J. Hoogmartens, *J. Liq. Chromatogr.* **15**, 2529–2549 (1992)
86. A. Van Schepdael, R. Kibaya, E. Roets and J. Hoogmartens, *Chromatographia* **41**, 367–369 (1995)
87. Y.M. Li, A. Van Schepdael, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **14**, 1095–1099 (1996)
88. Weng Naidong, K. Verresen, R. Busson, E. Roets and J. Hoogmartens, *J. Chromatogr.* **586**, 61–66 (1991)
89. Weng Naidong, Sun Hua, K. Verresen, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **9**, 717–723 (1991)
90. Weng Naidong, E. Roets, R. Busson and J. Hoogmartens, *J. Pharm. Biomed. Anal.* **8**, 881–889 (1990)
91. Weng Naidong, Sun Hua, E. Roets, R. Busson and J. Hoogmartens, *Int. J. Pharm.* **96**, 13–21 (1993)
92. Y. Liang, M. Bonner Denton and R.D. Bates, *J. Chromatogr.* **827**, 45–55 (1998)
93. K. Tsuji, J.F. Goetz, W. Van Meter and K.A. Gusciora, *J. Chromatogr.* **175**, 141–152 (1979)
94. P. Helboe and S. Kryger, *J. Chromatogr.* **235**, 215–220 (1982)
95. W. Decoster, P. Claes and H. Vanderhaeghe, *J. Pharm. Sci*. **71**, 987–991 (1982)
96. K. Tsuji and K.M. Jenkins, *J. Chromatogr.* **369**, 105–115 (1986)
97. E. Adams, R. Schepers, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **741**, 233–240 (1996)
98. D.L. Mays, R.J. van Apeldoorn and R.G. Lauback, *J. Chromatogr.* **120**, 93–102 (1976)
99. P. Gambardella, R. Punziano, M. Gionti, C. Guadalupi, G. Mancini and A. Mangia, *J. Chromatogr.* **348**, 229–240 (1985)
100. E. Adams, J. Dalle, E. De Bie, I. De Smedt, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **766**, 133–139 (1997)
101. G. Gunawardana, C. Childress, M. Tripp, X. Zhang and P. West, *J. Antibiot*. **50**, 887–889 (1997)
102. E. Adams, G. Van Vaerenbergh, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **819**, 93–97 (1998)
103. H. Russ, D. McCleary, R. Katimy, J.L. Montana, R.B. Miller, R. Krishnamoorthy and C.W. Davis, *J. Liq. Chrom. Rel. Technol*. **21**, 2165–2181 (1998)
104. J. Szúnyog, E. Adams, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal*. submitted for publication
105. G. Seidl and H.P. Nerad, *Chromatographia* **25**, 169–171 (1988)

106. A.E. Graham, E. Speicher and B. Williamson, *J. Pharm. Biomed. Anal*. **15**, 537–543 (1997)
107. E. Adams, W. Roelants, R. De Paepe, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal*. **18**, 689–698 (1998)
108. E. Adams, D. Puelings, M. Rafiee, E. Roets and J. Hoogmartens, *J. Chromatogr. A* **812**, 151–157 (1998)
109. T.J. Whall, *J. Chromatogr*. **219**, 89–100 (1981)
110. E. Adams, E. Roets and J. Hoogmartens, *J. Pharm. Biomed. Anal*. **21** 715–722 (1999)
111. C.L. Flurer, *J. Pharm. Biomed. Anal*. **13**, 809–816 (1995)
112. K. Tsuji and J.H. Robertson, *J. Chromatogr*. **112**, 663–672 (1975)
113. H. Oka, Y. Ikai, N. Kawamura, M. Yamada, K.I. Harada, Y. Yamazaki and M. Suzuki, *J. Chromatogr*. **462**, 315–322 (1989)
114. H. Oka, K.I. Harada and Y. Ito*, J. Chromatogr. A* **812**, 35–52 (1998)
115. V. Pavli and M. Sokolic, *J. Liq. Chromatogr*. **13**, 303–318 (1990)
116. R.G. Bell, *J. Chromatogr*. **590**, 163–168 (1992)
117. Y. Ikai, H. Oka, J. Hayakawa, K.I. Harada and M. Suzuki, *J. Antibiot*. **45**, 1325–1334 (1992)
118. S. Terabe, R. Konaka and J. Shoji, *J. Chromatogr*. **173**, 313–320 (1979)
119. Y. Kimura, H. Kitamura, T. Araki, K. Noguchi, M. Baba and M. Hori, *J. Chromatogr*. **206**, 563–572 (1981)
120. I. Elverdam, P. Larsen and E. Lund, *J. Chromatogr*. **218**, 653–661 (1981)
121. G. Fong and B.T. Kho, *J. Liq. Chromatogr*. **2**, 957–968 (1979)
122. T.J. Whall, *J. Chromatogr*. **208**, 118–123 (1981)
123. B.V. Fisher and R.B. Raya, *Anal. Proc*. **19**, 137–140 (1982)
124. R.B. Taylor, R.M. Richards, A.S. Low and L. Hardie, *Int. J. Pharm*. **102**, 201–206 (1994)
125. E. Adams, R. Schepers, L.W. Gathu, R. Kibaya, E. Roets and J. Hoogmartens*, J. Pharm. Biomed. Anal*. **15**, 505–511 (1997)
126. T.-Y. Zhang, *J. Chromatogr*. **315**, 287–297 (1984)
127. Y. Ikai, H. Oka, J. Hayakawa, N. Kawamura, K.-I. Harada, M. Suzuki, H. Nakarawa and Y. Ito, *J. Liq. Chrom. Rel. Technol*. **21**, 143–155 (1998)
128. H.K. Kristensen and S.H. Hansen, *J. Chromatogr*. **628**, 309–315 (1993)
129. K. Okamoto, H. Yonezawa and N. Izumuja, *J. Chromatogr*. **92**, 147–156 (1974)
130. R.E. Koeppe II and L.B. Weiss, *J. Chromatogr*. **208**, 414–418 (1981)
131. K.S. Axelsen and S.H. Vogelsang, *J. Chromatogr*. **140**, 174–178 (1977)
132. A.H. Thomas, *Analyst* **101**, 321–340 (1976)
133. M. Margosis and A. Aszalos, *J. Pharm. Sci*. **73**, 835–838 (1984)
134. O. Raatikainen, *J. Chromatogr*. **588**, 356–360 (1991)
135. P.M. Kelly, *J. Chromatogr*. **437**, 221–229 (1988)

136. J. Adamovics and S. Unger, *J. Liq. Chromatogr.* **9**, 141–155 (1986)
137. A.H. Thomas, P. Newland and G.J. Quinlan, *J. Chromatogr.* **216**, 367–373 (1981)
138. A.H. Thomas, P. Newland and N.R. Sharma, *Analyst* **107**, 849–854 (1982)
139. J.-W. Ma, Y.-B. Liu, D.-X. Wen, F.-J. Zhu and S.-W. Wang, *Acta Pharm. Sin.* **20**, 294–301 (1985)
140. V.-G. Mitrofanova, Y.D. Shenin, O.A. Mirgorodskaya, A.A. Derzhavets, E.R. Matveeva, G.E. Grinberg, Y.M. Apter and L.S. Golubkova, *Antibiot. Khimiother.* **36**, 9–11 (1991)
141. B. Sauer and R. Matusch, *J. Chromatogr. A* **672**, 247–253 (1994)

9.4. Steroids

Sándor Görög

9.4.1. Methodological Aspects [1–3]

In this section the evaluation of impurities in hormonal steroids and mainly their synthetic analogues is dealt with. Other non-hormonal synthetic derivatives (e.g. quaternary ammonium derivatives) and pharmaceutically less important groups of natural steroids such as sterols and bile acids are only briefly mentioned. Cardiac glycosides and vitamin D having also a steroid skeleton in their molecules but which possess entirely different chemical and pharmaceutical properties are not discussed.

The overwhelming majority of steroid hormones and their synthetic analogues contain moieties with good UV-absorbing properties (4-ene-3-oxo, 1,4-diene-3-oxo groups with λ_{max} around 240 nm or phenolic ring A with absorption maximum around 280 nm etc.). These are very advantageous features from the points of view of their determination by *UV spectrophotometry* in various matrices and the detectability in the course of the TLC or HPLC analysis but are of little importance when the analytical task is the direct determination of impurities/degradation products in bulk drugs and their formulations. Two of the rare exceptions are the limit test for conjugated compounds (4-ene-3-oxo and 3,5-diene type impurities) in ethynodiol diacetate [4,5] discussed in detail in Section 2.2.1 and the difference spectrophotometric determination of free phenolic impurities in estrogenic 3-methoxy derivatives down to about 0.1% (e.g. ethinylestradiol in mestranol) based on the selective shift of the spectrum of the free phenol in strongly alkaline medium [1]. Several spectrophotometric methods based on (colour) reactions are also available [1,2,6] but the importance of their application to the determination of impurities in steroid drugs has been surpassed by modern chromatographic methods.

The native *fluorescence* of estrogens is of some importance in their determination by HPLC. It is interesting to note that the very intense and selective fluorescence of the highly conjugated 3-oxo-4,6,8(14)-trienes (excitation 375 nm, emission 493 nm) enables these steroids as impurities in 4-ene-3-oxo steroids to be determined by direct fluorimetry without separation at the 0.01% level [3a]. The very strong fluorescence of various steroids obtainable by a great variety of reagents containing concentrated solutions of sulphuric, phosphoric acids, antimony trichloride, etc. is an important means for their very sensitive detection and determination by thin-layer chromatography.

Although the *gas chromatography* of steroids is not devoid of difficulties, it is still an important method of their impurity profiling. The problematic points are the volatility and thermal stability. Androstane, estrane and pregnane derivatives containing as functional groups oxo groups and one or at the very most two hydroxy groups can be usually chromatographed without decomposition in their underivatised form. To avoid decomposition is a very important issue since decomposition products can easily be confused with impurities. From this point of view the data in the literature on the use of gas chromatography for the biomedical analysis of steroids can be misleading. Since the mid 1960s gas chromatography was for a long time the most selective and sensitive method widely used for the determination of steroid hormones and the intermediates of their biosynthesis in biological samples and it is still an important method in this field especially in the GC/MS mode. Hundreds of papers have been published on this topic but the majority of these cannot be used for drug impurity profiling. The reason for this is that in these methods steroids are usually chromatographed in their derivatised form (silylation of the hydroxy groups and methoximation of the 20-oxo group of corticosteroids) and it is of no importance if a few tenth of a percent of the steroid to be determined in the biological sample is transformed to a by-product of the derivatisation reaction or decomposed even in the derivatised form. Needless to emphasise that such side reactions of the derivatisation or thermal decomposition are not permissible in drug impurity profiling.

The position of the hydroxy group(s) is an important factor which greatly influences the applicability of gas chromatography to impurity profiling of steroids. Derivatives containing the 5-ene-3-hydroxy group, e.g. *sterols*, secondary hydroxy group at position 17, e.g. *testosterone, 19-nortestosterone*, phenolic hydroxy group at position 3, e.g. *estrone*, or both, e.g. *estradiol*, can be investigated without derivatisation. As an example the estimation of the impurity profile of *cholesterol,* by GC/MS described by Laukó *et al*. [7] is mentioned: major (desmosterol and lathosterol) and minor (5α-cholestan-3β-ol, cholesta-3,5-dien-7-one and cholest-4-ene-3-one) impurities were identified and quantitated. At the same time, however, hydroxy derivatives and even their impurities were identified and quantitated. At the same time, however, hydroxy derivatives and even their acetates where the heat catalysed loss of water or acetic acid leads to conjugated derivatives, e.g. 3,5-diene from *4-ene-3-hydroxy* or 4-ene-3-acetoxy derivatives the decomposition is inevitable. This is why gas chromatography is by no means suitable for the impurity profiling of drugs such as *ethynodiol diacetate* or *spironolactone* where the loss of thioacetic acid from the 7β-thioacetyl-4-ene-3-oxo group leads to the conjugated 4,6-diene-3-oxo derivative.

In some problematic cases derivatisation (mainly trimethylsilylation) of the hydroxy groups can improve the volatility and the thermal stability of

steroids. If this method is used for impurity profiling the very careful optimisation of the derivatisation and chromatographic conditions is necessary to avoid or at least minimise the formation of artefacts. The only way to make sure that the minor peaks appearing in the chromatogram of the test material are real impurities and not derivatisation or chromatographic artefacts, is by parallel investigation of a reference standard material.

Bile acids require double derivatisation (acylation or silylation of the hydroxyls and esterification or silylation of the carboxyl group) but this is of no importance in drug impurity profiling. *Corticosteroids* with their three hydroxy groups and the very unstable dihydroxyacetone-type side chain at position 17 are least suitable for gas chromatographic analysis. Although – as already mentioned – this problem has been solved for biomedical analysis, these methods are by no means suitable for impurity profiling. Exceptions are corticosteroid drugs where the 17α-hydroxy group, the main source of the instability of the side chain is blocked as the cyclic acetonide of the 16,17-dihydroxy moiety (e.g. *triamcinolone acetonide).*

The very important 17α-ethinyl-17β-hydroxy group which can be found in almost all contraceptive steroids, gestogens and estrogens alike merits special mentioning. This group is created by the addition of 1 mol of acetylene to the 17-oxo group (see Fig. 2.1.B in Section 2.1.7). For this reason 17-oxosteroids are the most likely impurities in 17-ethinylsteroids. This reaction is reversible: under certain conditions (e.g. heating with sodium tert.butoxide solution) it can be quantitatively decomposed to the 17-oxo group and acetylene [8]. The decomposition can take place to some extent in the flash heater of the gas chromatograph as well. To minimise this the temperature of the flash heater must not be unnecessarily high and its inertness is also an important issue. Under strictly controlled experimental conditions the majority of 17α-ethinyl-17β hydroxy steroids can be chromatographed but any 17-oxo peak appearing in the chromatogram can be accepted as an impurity peak only if a reference standard not containing the 17-oxo impurity does not produce a similar peak under the same conditions.

Planar chromatographic methods, mainly *thin-layer chromatography* have played important role in the analysis of steroids from the very beginnings of this technique. On the other hand, steroids were ideal subjects to demonstrate the separation, detection and determination of closely related compounds. This is reflected by the high number of books, book chapters and reviews devoted to this subject [1,2,3b,9–11], just to mention a few. For the majority of separation problems simple TLC systems, most typically fluorescent silica layer with solvent systems such as chloroform/acetone, chloroform/methanol, benzene/acetone etc. can be used.

4-ene-3-oxo- and 1,4-diene-3-oxosteroids can conveniently be detected as dark spots on the fluorescent layer by using the line at 254 nm of a mercury

lamp as the light source. Very selective and sensitive detection and quantitation is achievable using various spray reagents (mainly those containing high concentrations of sulphuric or phosphoric acid) which produce various colours with different steroid structures for the visual inspection of the plate or estimating the fluorescence at the other wavelength (366 nm) of the generally used mercury lamp. The excellent separation power and good detection possibilities make thin-layer chromatography an ideal tool for the detection and semiquantitative estimation of related impurities in steroids. This technique is used in the majority of cases as the purity check in the pharmacopoeias. Section 2.5 contains several examples and chromatograms taken from the field of steroid drugs. It is also an important tool in the complex studies aiming to identify the impurities of steroids. A case study of this type is described in Section 2.1.7.

What is described in Section 2.1 for drug impurity profiling in general applies to steroids, too: *high-performance liquid chromatography (HPLC)* (Section 2.7) is the most important and most widely used method for their impurity profiling. Its importance has greatly increased both in general and in the field of steroids in consequence of its successful coupling with mass spectrometry. The great majority of steroid hormone analogue drugs are compounds with medium polarity without acidic and basic functional groups. For this reason their separation both by reversed phase and normal phase systems does not require special techniques: ordinary C_{18} stationary phases with unbuffered water/acetonitrile, water/methanol etc. as the mobile phases or silica columns with hydrocarbon mobile phases containing low percentage of 2-propanol or other alcohols and other simple systems are widely used [1,2,3c,12,13]. In the pharmacopoeias HPLC is usually applied for the assay of bulk steroid drugs and drug formulations but their purity check is also often based on this method.

As described above the majority of steroid hormone analogues are good UV absorbers and hence their detection with a sensitivity sufficient to impurity profiling is usually possible. The selection of a suitable wavelength for scanning the chromatogram is a very important issue. The molar absorptivity of the above mentioned 4-ene-3-oxo steroids at their maxima around 240 nm is about 16 000. If they contain a 3-deoxo 4-ene, 4,5-dihydro-3-oxo or non-conjugated 5-ene-3-oxo or 5(10)-ene-3-oxo derivatives as impurities and the chromatogram is scanned at 240 nm, these impurities are not detectable at all. For this reason it is essential to scan the chromatograms at very low wavelengths (200–205 nm), too, where the absorptivity of even the spectrophotometrically inactive steroids having only isolated double bonds or oxo groups is comparable with that of the conjugated parent compound. The same applies also to (bis)-quaternary ammonium steroids, e.g. pipecuronium bromide (for the formula see Section 1.2.1).

Since under ordinary pH conditions steroid hormone analogues are with a

few exceptions uncharged, neutral molecules, *capillary electrophoresis* (CE) in its original form is unsuitable for their separation. However, micellar electrokinetic chromatography (MEKC) which can be considered to be a mode of CE (see Section 2.8) was successfully used by Terabe *et al*. [14], the inventor of this technique for the separation of steroids and the application of the method for purity check of the corticosteroid *fiuticasone propionate* is also described by Smith and Evans [15]. Another possibility, described by Görög *et al*. [16] is the derivatisation of the oxo group of steroids by positively charged Girard P and T reagents thus enabling their separation by CE. Generally speaking, however, capillary electrophoretic techniques do not play important role in impurity profiling of steroid drugs. Exceptions are of course bile acids. For example, Quaglia *et al*. [17] successfully used CE for the separation of several impurities from *ursodeoxycholic acid* using a pH 8.9 buffer, containing methylated β-cyclodextrins to improve the separation and indirect UV detection.

Although at present only a limited number of examples are available on the use of *capi1lary electrochromatography (CEC)* and *CEC/MS* (see Sections 2.9.1 and 2.9.2) for the separation and identification of impurities in steroid drugs, these techniques seem to have great potential in this field, too.

9.4.2. Selected Examples of Synthesis-Related Impurities

To give a full picture on the numerous, rather complicated reaction routes for the synthesis of various classes of steroid drugs and the impurities originating from these would be beyond the scope of this book. For this reason just a few of the most important reactions are shortly mentioned in order that explanation can be given to the existence of some characteristic impurities.

9.4.2.1. Total Synthesis

Most of the manufacturers use the total synthesis of the steroid skeleton for the preparation of estrogens and 19-norsteroid type contraceptive gestogens [18]. As seen in Fig. 9.4.A the starting materials for this are usually β-naphthol and 2-methyl (or ethyl)-cyclopentane-1,3-dione as precursors of rings A, B and D of the steroid skeleton, respectively. The stepwise hydrogenation of the two double bonds (Δ^8 and Δ^4) in **3** aims the desired stereochemistry at the ring anellations (8β, 9α, 14α). A side reaction of the catalytic hydrogenation of the Δ^{14} double bond in **3** to form **4** is the 1,4-addition of the hydrogen leading to **4a** which is the precursor of impurities 8(14)-dehydro-norethisterone and norgestrel which were separated by HPLC from these gestogenic drugs [19]. (For details and the role of the UV spectra of these impurities taken by the diode-array UV detector see Section 2.7.3 and Fig. 2.7.3.E). This side reaction is the

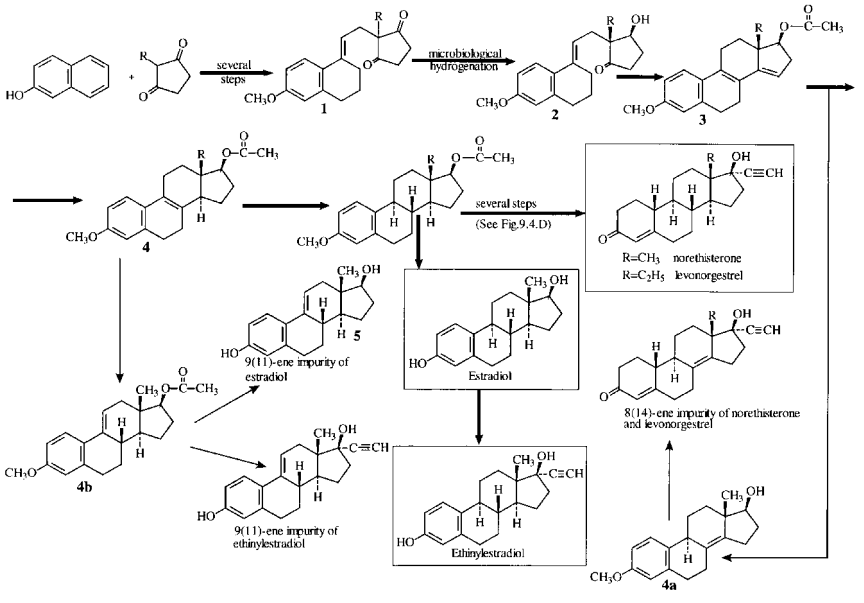


Figure 9.4.A.
An outlined overview of the total synthesis of estrogens and 19-norsteroids

basis also for the formation of 8(14)-dehydro-allylestrenol, discussed in detail in Section 2.3.2.

Another impurity originating from these hydrogenation steps is the 9(11)-dehydro derivative of estradiol and ethinylestradiol. Their precursor is **4b** which is the product of the rearrangement of the Δ^8 double bond to the $\Delta^{9(11)}$ position. These impurities were separated from estradiol [20] and ethinylestradiol [19] by HPLC. The role of the diode-array UV spectra (Fig. 2.7.3.D) in the identification of these impurities is discussed in detail in Section 2.7.3.

The total synthesis can be the source of the enantiomeric impurity L-norgestrel in levonorgestrel (D-norgestrel). If one of the keto groups in the secosteroid **1** (R = ethyl) is reduced to **2** by highly stereospecific microbiological hydrogenation, the D-enantiomer of **2** (R = ethyl) is formed leading to levonorgestrel in the course of the further steps of the synthesis. If, however, the ring closure takes place without preliminary reduction, the racemate (1:1 mixture of the D and L form of **6**) is forming due to the new chiral centre at C-13; see Fig. 9.4.B. In the subsequent steps this is transformed to racemic norgestrel, also used in the therapy. Several methods have been described for the separation of the enantiomers of racemic norgestrel. The first successful

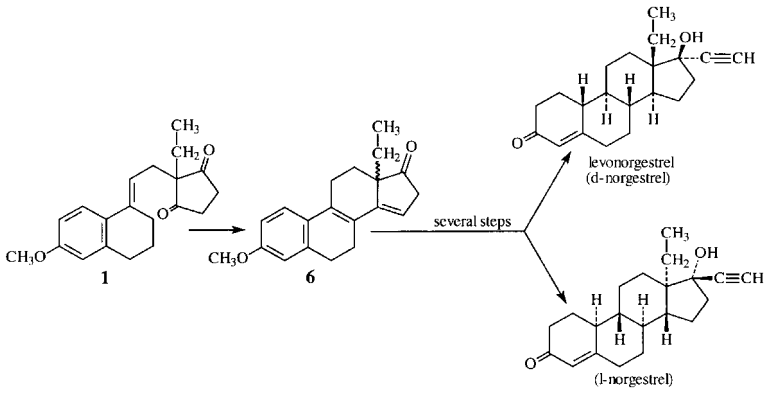


Figure 9.4.B.
Formation of racemic norgestrel (*d,l*-norgestrel). For details see the reaction scheme in Fig. 9.4.A

separations were achieved by HPLC based on the formation of an adduct with γ-cyclodextrin dissolved in the eluent [21,22]. Chemically bonded β-cyclodextrin [23] or its acetylated version [24] and α_1-acid glycoprotein [25] stationary phases, micellar electrokinetic chromatography using sodium dodecyl sulphonate as the micelle forming agent modified by γ-cyclodextrin [26,27] and capillary supercritical fluid chromatography with polysiloxane-anchored permethyl-β-cyclodextrin [28] are also suitable for this purpose. Of these methods the method of Gazdag *et al.* [21] is validated for the determination of traces of L-norgestrel as a possible impurity in levonorgestrel. For details see Section 6.2.2 and Fig. 6.2.B.

The method of Kummer and Werner [29,30] merits special mentioning. They separated no less than 26 pairs of enantiomeric steroids (among them norgestrel enantiomers) using Chiralpak AD (amylose tris(3,5-dimethylphenylcarbamate)) as the chiral stationary phase in the reversed phase mode. Since all the investigated steroids were total synthetically prepared estrogens or 19-norsteroids, in principle they can also contain enantiomeric impurities. The value of their method as a quantitative enantiomeric purity evaluation was checked by validating the determination of enantiomeric estradiol in estradiol. Excellent results were achieved with samples spiked with 0.05-4.8% enantiomeric estradiol.

9.4.2.2. Birch Reduction of the Phenolic Ring A

In Fig. 9.4.A the transformation of **5** to nortestosterone, norethisterone and norgestrel is not described in detail. The transformation of the phenol ether type ring A (3-methoxy-1,3,5(10)-triene) to the 1,4-dihydro derivative (3-methoxy-2,5(10)-diene) system by Birch reduction using metallic sodium dissolved in liquid ammonia as the reducing agent, various higher alcohols as the proton source is the key reaction of the synthesis of 19-nor-4-ene-3-oxosteroids (nandrolone esters, contraceptive gestogens, etc.) [31a,32]. Since the 3-methoxy-2,5-diene group is rather unstable, it is more convenient to investigate the side reactions of the Birch reduction by investigating the 19-nor-4-ene-3-oxosteroid formed in the course of the acid catalysed splitting of the enol ether group followed by the rearrangement of the $\Delta^{5(10)}$ double bond to the Δ^4 position. Although the product of the Birch reduction nor even the 4-ene-3-oxosteroids forming during their treatment with acids are not yet drug substances their impurity profiling is an important task: by identifying the several by-products of this reaction explanation can be given to the appearance of various impurities in the finished products. Crude 19-nortestosterone (nandrolone, 17β-hydroxy-estr-4-ene-3-one) was investigated by Görög *et al*. After fractionation by preparative HPLC, the by-products were identified by GC/MS [33] and HPLC/UV [34].

Figure 9.4.C shows the GC/MS scan of a crude 19-nortestosterone (17β-hydroxy-estr-4-ene-3-one) sample prepared by Birch reduction from estradiol-3-methyl ether (peak 20; M = 286). The reaction equation with several side reactions is shown in Fig. 9.4.D. The conclusions leading to the structures of the by-products could be drawn mainly from the GC/MS data. In addition to the main component (peak 1) several minor components have the molecular mass of 274 (peaks 15–19, 21). These are the 5(10)-ene-3-one isomer (peak 16) and the stereoisomers of the main component and of the 1-ene-3-one isomer [34]. The reason for the formation of the unsubstituted 1,3,5(10)-triene is the addition of two hydrogens at positions 3,10 or 2,3 or 3,4 followed by acid-catalysed elimination of methanol (peak 8; M = 256). Of the other impurities in Figs. 9.4.B and 9.4.C the following products of various types of over-reduction (addition of 4 or 6 hydrogens) have been identified on the basis of the mass spectra from the GC/MS scan and NMR spectra of samples obtained from the crude reduction product by preparative HPLC: (a) stereoisomeric 4,5-dihydro-3-oxo derivatives (peaks 13 and 14; M = 276); (b) 3-methoxy-1(10)-ene or 3-methoxy-5(10)-ene derivative (peak 11; M = 290); (c) 3-deoxo derivatives: 4-ene (peak 5) accompanied by 1-ene and 1(10)-ene and minor isomers (peaks 2, 3, 5, 6) (M = 260). Other minor impurities with molecular masses of 258 (peaks 7 and 12) and 284 (peak 9) contain a further double bond in ring B or C which originates from the total synthetic steps prior to the Birch reduction.

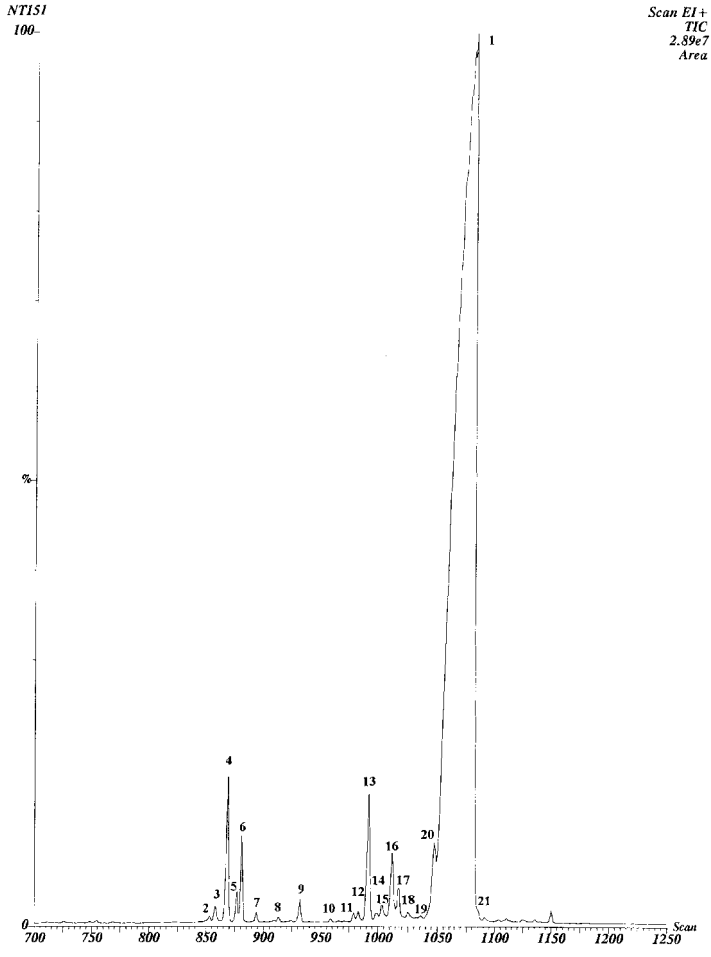


Figure 9.4.C.
GC/MS scan of crude 19-nortestosterone. Column: J&W DB-5 30m × 0.25 mm × 0.25 μm. Temperature: injector 260°C, oven 40°C 1 min, 10°C/min up to 260°C. Injection: 1 μg crude 19-nortestosterone; splitless. Ionisation: EI, 70 eV, TIC. For the Key see the text of Section 9.4.2.2. Scan 1000 is at 18.63 min (from Ref. [33])

Although most of these impurities disappear in the course of the subsequent reactions and purification steps, some of them have been found to be precursors of impurities in various 19-norsteroids. For example the 4,5-dihydro derivative as an impurity in 19-nortestosterone (nandrolone) decanoate was

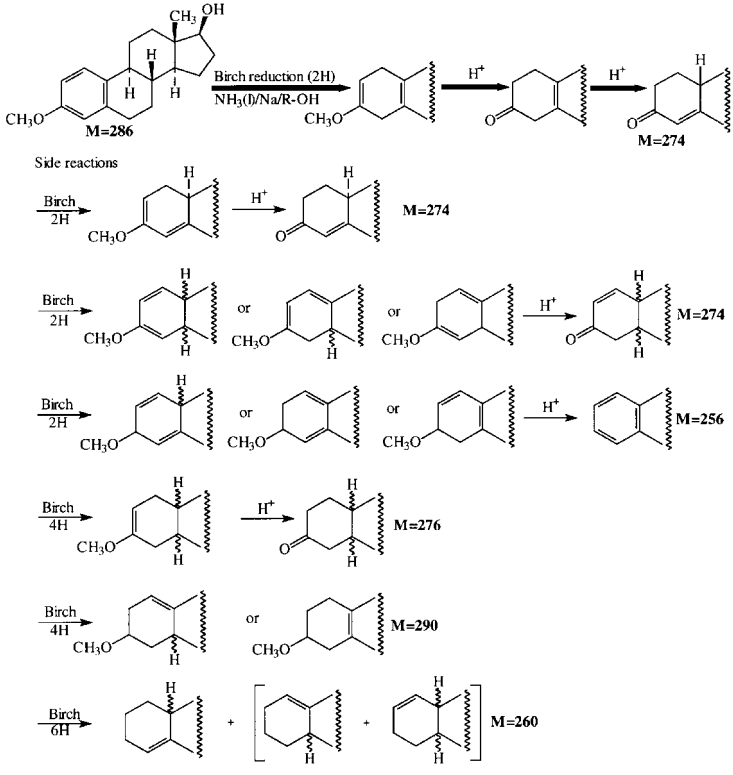


Figure 9.4.D.
Main reaction and side reactions of the Birch reduction of 3-methoxy-estra-1,3,5(10)-triene-17β-ol (estradiol-3-methyl ether) followed by hydrolysis to form 17β-hydroxy-estr-4-ene-3-one (19-nortestosterone, nandrolone)

separated from the main component using 7:3 v/v mixture of hexane and acetone as the mobile phase; (R_f values of the impurity and the main component are 0.66 and 0.53, respectively). The impurity was identified by mass spectrometry after spot elution. 3-Deoxo-norgestrel, discussed in detail in the case study in Section 2.1.7 is also derivable from a side reaction of the Birch reduction.

9.4.2.3. Ethinylation of the 17-Oxo Group

The addition of acetylene to the 17-oxo group to form 17α-ethinyl-17-hydroxysteroids was described already in the heroic period of steroid chemistry

[35,36]. This is one of the most important reactions even in modern steroid chemistry: the introduction of the ethinyl group greatly increases the estrogenic and gestogenic activity of steroids.

The reaction equation of the ethinylation together with three side reactions is shown in Fig. 1.2.A in Section 1.2.1. The most likely impurity in 17-ethinyl-steroids is the unreacted 17-oxosteroid. This is easily separated from the main component by TLC or HPLC. The possibilities and difficulties of the use of gas chromatography for this purpose is discussed in Section 9.4.1. The first side reaction in Fig. 1.2.A is the formation of the epimeric 17β-ethinyl-17α-hydroxy derivative. Impurities of this type were detected only in the case of the ''natural'' 13-methyl series: the steric hindrance caused by the bulky 13-ethyl group prevents the formation of the epimeric impurity in the 13 ethyl series. NMR spectroscopy (after preparative HPLC separation) played a predominant role in the identification of the epimeric impurities [36]. Table 9.4.A shows some characteristic chemical shifts. These data, especially the shift of the angular methyl group as well as DNOE measurements were of diagnostic value in the identification.

The epimeric impurities can be separated and quantitated both by gas chromatography [38] and HPLC [37]. Another side reaction in Fig. 1.2.A is

Table 9.4.A.
Some characteristic NMR chemical shifts of 17α-ethinyl steroids and their 17β-ethinyl impurities (δ, ppm)[a]

		Norethisterone		Ethisterone	
		17α	17β	17α	17β
13–CH_3	^{1}H	0.913	0.933	0.898	0.903
	^{13}C	12.67	16.43	12.68	15.99
≡CH	^{1}H	2.578	2.502	2.576	2.502
	^{13}C	74.09	72.97	74.17	72.89
–C≡	^{13}C	87.32	85.49	87.21	85.54
C-17	^{13}C	79.62	78.38	79.65	79.28
C-16	^{13}C	38.79	39.84	38.30	39.49
C-15	^{13}C	22.87	23.73	23.04	23.91
C-14	^{13}C	49.12	48.84	49.84	48.03
C-13	^{13}C	46.84	47.37	46.65	47.66

[a] Solvent: $CDCl_3$; reference TMS (from Ref. [37])

Table 9.4.B.
GC and HPLC retention data of some 17α- and 17β-ethinyl epimers and their acetylene-bridged dimers[a]

	GC			HPLC		
	Retention time (min)		Rel. retention	Retention time	Rel. retention	
	17α	17β	$r_{\beta/\alpha}$	17α	$r_{\beta/\alpha}$	$r_{dimer/\alpha}$
Norethisterone	18.05	18.67	1.034	6.98	1.31	1.61
Norgestrel	–	–	–	9.36	–	2.50
Ethisterone	18.93	19.57	1.034	7.89	1.38	1.93

[a] See Fig. 2.1A. GC conditions: 20 m × 0.2 mm × 0.33 μm fused silica capillary coated with cross-linked 5% phenylmethyl silicone gum. Temperatures: oven 240–250°C, flash heater, FID 300°C. HPLC conditions: Column LiChrosorb RP-18, 250 × 4 mm; eluent methanol/water 7:3, 1 ml/min (from Ref. [37])

the formation of acetylene-bridged dimers. These can easily be separated from the main component by HPLC. Of course gas chromatography is not suitable for this purpose. It is, however, worth mentioning that the acetylene-bridged dimers decompose in the flash heater of the gas chromatograph to the 17α-ethinylsteroid and the 17-oxo derivative; (another reason to be cautious when evaluating a 17-oxo peak in the gas chromatogram of a 17-ethinylsteroid). The GC and HPLC retention data of some epimers and dimers are summarised in Table 9.4.B.

The formation and identification of two isomeric 3,17-diethinyl derivatives as by-products of the ethinylation reaction in the synthesis of norgestrel [39], also seen in Fig. 2.1.A is described in detail in Section 2.1.7. Two further impurities related to the ethinylation are worth mentioning. As seen in Section 2.5, Ferenczi-Fodor *et al*. [40] identified a 3,4,17-triethinyl derivative in norgestrel which is the ethinylation product of an impurity in the precursor in its synthesis. Levtchenko *et al*. [41] identified 17α-isobutylestradiol as an impurity in ethinylestradiol.

9.4.2.4. Introduction of Fluorine

The introduction of fluorine into the molecules of corticosteroids (particularly at positions 6 and/or 9) greatly increases their pharmacological activity; for this reason the reaction route leading to these is of great importance in synthetic steroid chemistry. The starting material to introduce the 9α-fluorine is the 11β-hydroxy derivative and the usual sequence of the reactions is as follows: 11β-hydroxy $\Rightarrow$ $\Delta^{9(11)}$ $\Rightarrow$ 9α-bromo-11β-hydroxy $\Rightarrow$ 9β,11β-epoxy $\Rightarrow$ 9α-fluoro-11β-hydroxy [31b]. For this reason the above listed intermediates are the most likely impurities in 9-fluorocorticosteroids, such as triamcinolone acetonide, dexamethasone, betamethasone and many others. The separation and identification of the possible and real impurities was thoroughly investigated by Cavina *et al*. using *9α-fluoroprednisolone acetate* [42] as the model compound in the course of the search for impurities in *triamcinolone acetonide* [43]. Both normal-phase (LiChrosorb Si-100 5 μm, chloroform/methanol/water 978.6:20:1.4 v/v/v, detection at 254 nm) and reversed-phase (Spherisorb ODS 5 μm, methanol/water 65:35 v/v or acetonitrile/water 36:64 v/v, detection at 240 nm) HPLC were successfully used. The identification of the impurities was mainly based on the mass spectrometric investigation of the impurities but the minor changes in the UV spectrum of the α,β-unsaturated 3-oxo group caused by the various substituents at the 9 and 11 positions was also of diagnostic value especially if these minor differences are attenuated by using second derivative spectra obtained by the diode-array UV detector. Due to the presence of chloroform in the eluent in the normal phase mode of course the diode-array UV spectra can be taken only in the above described

reversed-phase systems. For details see Section 2.7.3 and Fig. 2.7.3.G. Bernal *et al*. [44] have shown that the use of methyl and ethyl acetates in the eluents of the RP-HPLC mobile phase (e.g. ethyl acetate/methanol/water 2:58:40 v/v/v) is beneficial to the selectivity of the system for the separation of the impurities in 9α-fluoroprednisolone acetate.

The impurity profiling of a 6α,9α-difluoro corticosteroid, *fluticasone propionate* is described in detail in Section 2.7.5 as an example for the use of on-line HPLC/NMR in drug impurity profiling [45].

9.4.2.5. Microbiological Oxidation

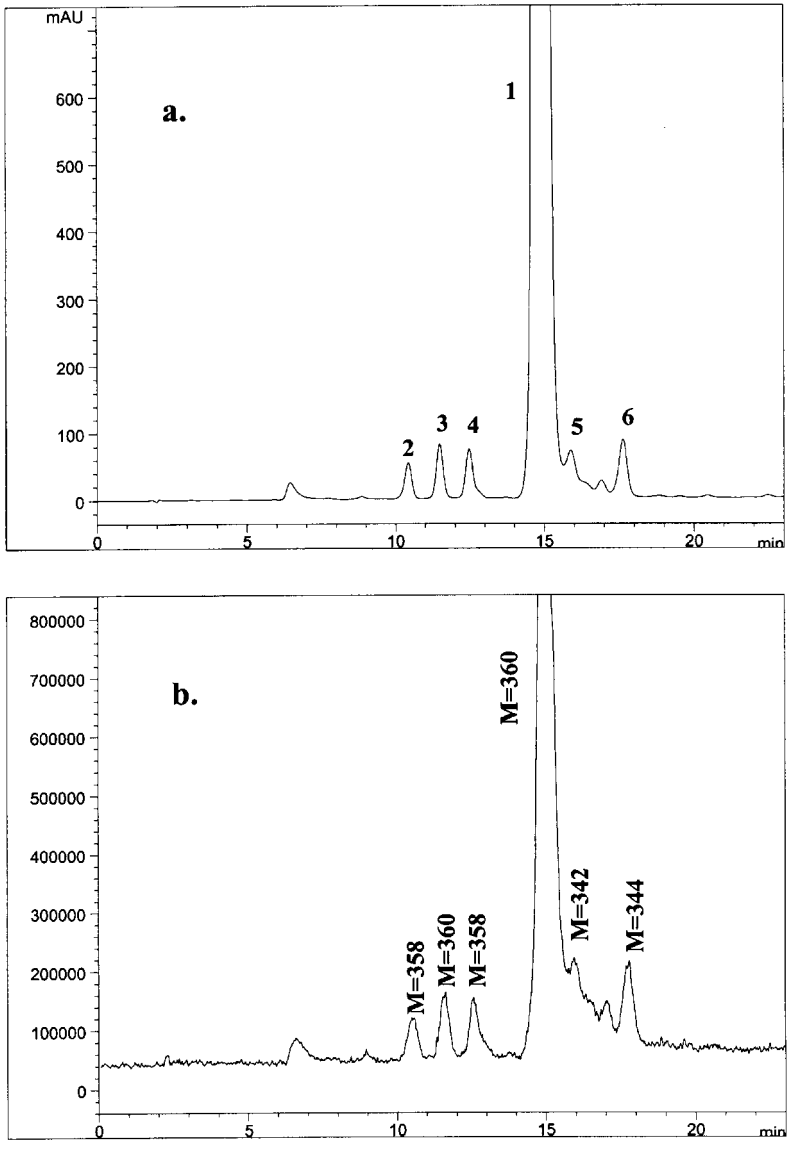
There are two major fields where microbiological oxidation plays predominant role in the industrial-scale preparation of steroid drugs: (a) transformation of plant sterols (mainly β-sitosterol) to 17-oxo derivatives which are intermediates for important steroid syntheses and (b) introduction of the 11β-hydroxy group and the Δ^1 into steroid intermediates to prepare semisynthetic corticosteroid analogues. Of these only the latter affects directly the impurity profiles of the final products.

An early study by Compernolle *et al*. [46] is a good example for the successful application of off-line TLC/MS for impurity profiling. Several impurities of *prednisolone* (11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione) were isolated and characterised. Most of them is related to the above mentioned microbiological steps in its synthesis. Hydrocortisone (the 1,2-dihydro derivative of prednisolone) is the unreacted starting material of the introduction of the Δ^1 double bond. 20-dihydroprednisolone (11β,17α,20β,21-tetrahydroxy-pregna-1,4-diene-3-one) is a well known by-product of the microbiological dehydrogenation. The presence of 11-deoxyprednisolone indicates incomplete 11-hydroxylation in a previous step and the 11-oxo derivative (prednisone) is the product of overoxidation in the same.

In a recent study Görög *et al*. [33] investigated the impurity profile of prednisolone by on-line HPLC/diode-array UV/MS. The UV and mass chromatograms, respectively, can be seen in curve **a** and **b** of Fig. 9.4.E. Figure 9.4.F shows the UV spectra and Fig. 9.4.G the structures derivable from the mass spectra and UV spectra. Although the UV spectra are rather similar, even the observed minor differences were of diagnostic value: shoulder in the spectra of **2** and **5** containing an additional, non-conjugated double bond, minor hypsochromic shift in the 11-oxo derivative (**4**) and a similarly small bathochromic shift in the epimeric 11α-hydroxy derivative (**3**). The smaller bandwidth of the 1,2-dihydro derivative (hydrocortisone, **7**, separated in another HPLC system) is also characteristic.

9.4.3. Degradation Products

The most unstable moiety in steroid hormone drugs is the dihydroxyacetone-type side chain of corticosteroids at position 17. This side chain is sensitive to acidic and basic media as well as it is oxidisable. The very complex



degradation mechanism was thoroughly investigated by several groups already in the early period of steroid chemistry especially on the example of prednisolone. The most important degradation products of the very complex degradation mechanism are the 17-oxo, 17α-hydroxy-20-carboxylic acid, 17α,20-dihydroxy-21-carboxylic acid, 17α,20-dihydroxy-21-aldehyde, 20-hydroxy-17(20)-ene-21-aldehyde [1,2,47]. Similar degradation mechanism was found for *mazipredone* (the 21-deoxy-21-*N*-methylpiparazinyl derivative of prednisolone) by Gazdag *et al*. [48]. This study which was based on HPLC/UV/MS measurements is described in detail in Chapters 5.3 and 5.5.

The 4-ene-3-oxo group is much more stable. It is, however, moderately sensitive to oxidation. The oxidation products (6α and 6β-hydroxy, 10β-hydroxy, 6-oxo, 3-oxo-4,6-diene derivatives are described in Section 2.7.3 and 5.3 on the examples of *norgestrel* and *nestorone* emphasising the usefulness of the diode-array UV spectra in the identification of these degradation product-type impurities [33,34]. The main degradation routes of *norgestimate* (norgestrel oxime acetate) are the acid-catalysed hydrolysis of the ester and oxime groups to form norgestrel and the base-catalysed hydrolysis to norgestrel oxime [49].

Estrogens possessing a phenolic ring A are stable materials. Oxidative degradation products such as the 6α and β-hydroxy, 6-oxo and Δ^6 derivatives of *ethinylestradiol* and *mestranol* were identified by mass spectrometry after TLC separation when these drug materials were exposed to oxygen at elevated temperatures [50].

The vulnerable part of the molecule of *spironolactone* is the 7α-thioacetyl group. Its hydrolysis product (7α-thio derivative) and the splitting product (3-oxo-4-6-dine, canrenone) were separated by HPLC [51]. For the formulae of spironolactone and the degradation products which can be synthesis related impurities as well, see Section 2.3.

Figure 9.4.E.
HPLC separation of prednisolone and its impurities. For the Key see the numbering of the formulae in Fig. 9.4.F. (a) UV scan; (b) MS scan with the molecular masses. Column: Merck LiChrospher 100 RP-18, 5 μm, 125 × 4.0 mm. Gradient elution. A. 0.1 M aqueous ammonium acetate/water/methanol 10:80:10 v/v/v, B: 0.1 M aqueous ammonium acetate/methanol 10:90 v/v. Linear gradient: 0 min 40% B, 40 min 80% B. Flow rate: 0.5 ml/min. UV detector: 240 nm. MS parameters: electrospray positive ionisation mode, drying gas temperature 200°C, flow rate 10 l/min, nebuliser pressure 172 kPa, capillary voltage 3500 V, TIC scan (from Ref. [33])

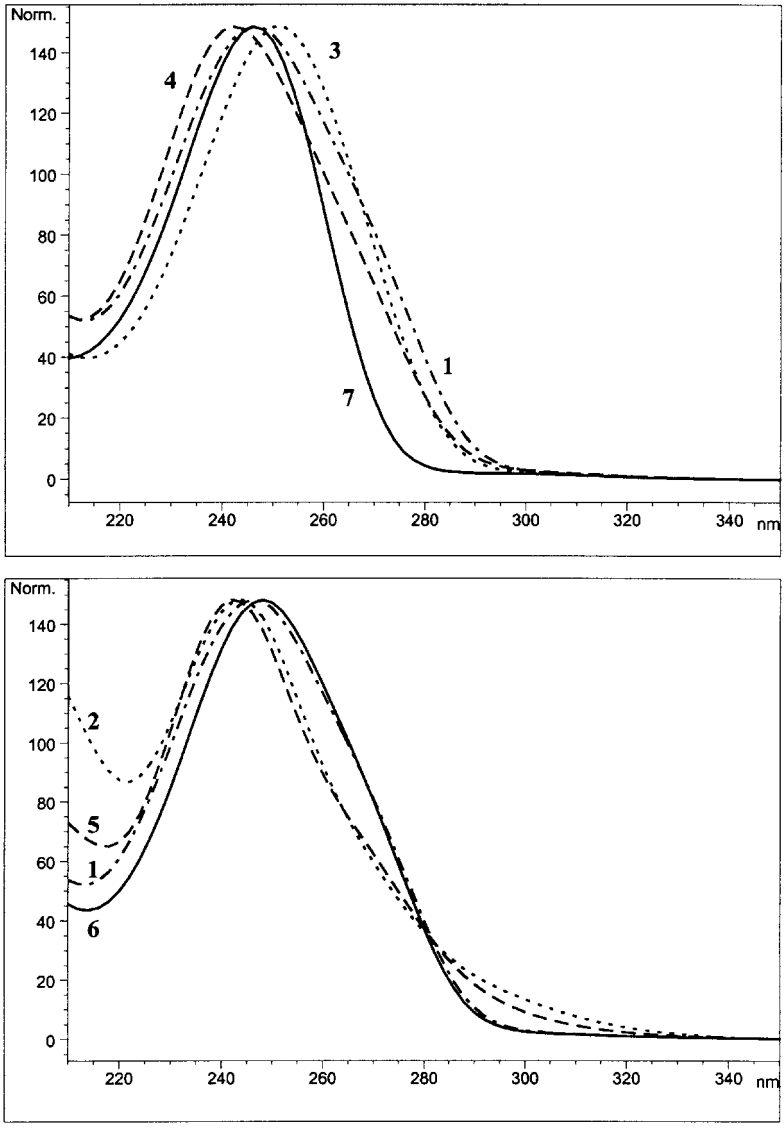


Figure 9.4.F.
Diode-array UV spectra of prednisolone and its impurities. For the key see Fig. 9.4.G. For the reference see Fig. 9.4.E

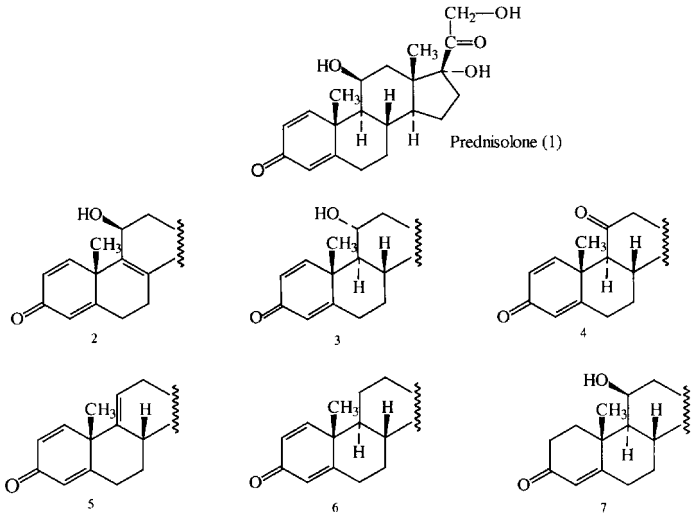


Figure 9.4.G.
Structures of prednisolone and its impurities

References

1. S. Görög and Gy. Szász, *Analysis of Steroid Hormone Drugs*, p 325, Elsevier, Amsterdam (1978)
2. S. Görög, *Quantitative Analysis of Steroids*, Elsevier, Amsterdam (1983)
3. S. Görög (Ed.), *Steroid Analysis in the Pharmaceutical Industry*, Ellis Horwood, Chichester (1989). a. p 17; b. G. Szepesi, pp 103–114; c. G. Szepesi, pp 125–145
4. *The United States Pharmacopoeia 24*, p 698, USP Convention Inc., Rockville (2000)
5. *British Pharmacopoeia 1993*, p 270, Her Majesty's Stationery Office, London (1993)
6. M. Pesez and J. Bartos, *Colorimetric and Fluorimetric Analysis of Steroids*, Academic Press, New York (1976)
7. A. Laukó, É. Csizér and S. Görög, *Analyst* **118**, 609–611 (1993)
8. S. Görög and G. Szepesi, *Analyst* **99**, 218–221 (1974)
9. R. Neher, TLC of steroids and related compounds, in *Thin-Layer Chromatography* (E. Stahl, Ed.), Springer, Berlin (1969)
10. G. Szepesi, Assay of steroids and related materials, in *Quantitative Thin-Layer Chromatography and its Industrial Application* (L. Treiber, Ed.), Marcel Dekker, New York (1986)

11. J.C. Touchstone, *CRC Handbook of Chromatography. Steroids*, CRC Press, Boca Raton, FL (1986)
12. W. Voelter, Steroid hormones, in *High Performance Liquid Chromatography in Biochemistry* (A. Henschen, K.-P. Hupe, F. Lottspeich and W. Voelter, Eds.), pp 481–531, VCH, Weinheim (1985)
13. G. Szepesi, *HPLC in Pharmaceutical Analysis*, vol. II, pp 291–324, CRC Press, Boca Raton, FL (1991)
14. S. Terabe, Y. Ishihama, H. Nishi, T. Fukuyama and K. Otsuka, *J. Chromatogr.* **545**, 359–368 (1991)
15. N.W. Smith and M.B. Evans, *J. Pharm. Biomed. Anal.* **12**, 579–611 (1994)
16. S. Görög, M. Gazdag and P. Kemenes-Bakos, *J. Pharm. Biomed. Anal.* **14**, 1115–1124 (1996)
17. M.G. Quaglia, A. Farina, E. Bossù, C. Dell'Aquila and A. Doldo, *J. Pharm. Biomed. Anal.* **16**, 281–285 (1997)
18. I.V. Torgov, Achievements in the total synthesis of natural steroids, in *Recent Developments in the Chemistry of Natural Carbon Compounds*, Vol. 1, Akadémiai Kiadó, Budapest (1965)
19. S. Görög and B. Herényi, *J. Chromatogr.* **400**, 177–186 (1987)
20. S. Görög, J. Brlik, A. Csehi, Zs. Halmos, B. Herényi, P. Horváth, A. Laukó, F. Dravecz and D. Bor, *Anal. Methods Instrum.* **2**, 154–157 (1995)
21. M. Gazdag, G. Szepesi and K. Mihályfi, *J. Chromatogr.* **450**, 145–155 (1988)
22. H. Lamparczyk, P.K. Zarzycki and J. Nowakowska, *J. Chromatogr. A* **668**, 413–417 (1994)
23. A. Berthod, H.L. Jin, T.E. Beesley, J.D. Duncan and D.W. Armstrong, *J. Pharm. Biomed. Anal.* **8**, 123–130 (1990)
24. C. Kuang, B. Wu and J. Zhou, *Sepu* **7**, 21–23 (1989); *C.A.* **111**, 32908 (1990)
25. Gy. Szász, K. Gyimesi-Forrás and Zs. Budvári-Bárány, *J. Liq. Chrom. Rel. Technol.* **21**, 2535–2547 (1998)
26. Y. Lui, J. Gu, R. Fu and X. Wang, *Sepu* **13**, 387 (1995); *C.A.* **123**, 387–389 (1996)
27. J. Gu, Y. Liu, R. Fu and X. Wang, *Fenxi Huaxue* **24**, 548–550 (1996); *C.A.* **125**, 47937 (1997)
28. M. Jung and V. Schurig, *J. High. Res. Chromatogr.* **16**, 215 (1993).
29. M. Kummer, H.-J. Palme and G. Werner, *J. Chromatogr. A.* **749**, 61–68 (1996)
30. M. Kummer and G. Werner, *J. Chromatogr. A* **825**, 107–114 (1998)
31. J. Fried and J.A. Edwards (Eds.), *Organic Reactions in Steroid Chemistry*, Vols. I–II, Van Nostrand Reinhold, New York (1972). a. pp 1–60; b. pp 423–493
32. A.J. Birch, *Steroids* **57**, 363–377 (1992)

33. S. Görög, M. Babják, G. Balogh, J. Brlik, F. Dravecz, M. Gazdag, P. Horváth, A. Laukó and K. Varga, *J. Pharm. Biomed. Anal.* **18**, 511–525 (1998)
34. S. Görög, M. Bihari, É. Csizér, F. Dravecz, M. Gazdag and B. Herényi, *J. Pharm. Biomed. Anal.* **14**, 85–92 (1995)
35. H.H. Inhoffen, W. Logemann, W. Hohlweg and A. Serini, *Chem. Ber.* **71**, 1024–1030 (1938)
36. L. Ruzicka and K. Hoffmann, *Helv. Chim. Acta* **18**, 986–999 (1938)
37. A. Laukó, A. Csehi, G. Balogh, É. Csizér, B. Herényi and S. Görög, *Acta Pharm. Hung.* **61**, 98–104 (1991)
38. S. Görög, A. Laukó and B. Herényi, *J. Pharm. Biomed. Anal.* **6**, 697–705 (1988)
39. P. Horváth, G. Balogh, J. Brlik, A. Csehi, F. Dravecz, Zs. Halmos, A. Laukó, M. Rényei, K. Varga and S. Görög, *J. Pharm. Biomed. Anal.* **15**, 1343–1349 (1997)
40. K. Ferenczi-Fodor, S. Mahó, S. Pap-Sziklay, I. Török and L. Borka, *Pharmeuropa* **9**, 736–742 (1997)
41. N.K. Levtchenko, D.M. Osikin, I.V. Torgov, A.P. Arzamastzev, T.M. Sokolova and G.I. Tuguntaev, *J. Chromatogr.* **456**, 427–430 (1988)
42. G. Cavina, L. Valvo, B. Gallinella, R. Porrà and A.L. Savella, *J. Pharm. Biomed. Anal.* **10**, 437–446 (1992)
43. G. Cavina, R. Alimenti, B. Gallinella and L. Valvo, *J. Pharm. Biomed. Anal.* **10**, 685–692 (1992)
44. J.L. Bernal, M.J. Del Nozal, G.A. Garcia Buj and J.M. Juárez, *J. Chromatogr.* **607**, 175–181 (1992)
45. N. Mistry, I.M. Ismail, M.S. Smith, J.K. Nicholson and J.C. Lindon, *J. Pharm. Biomed. Anal.* **16**, 697–705 (1997)
46. F. Compernolle, H. Vanderhaeghe and M. Nonclercq, *J. Pharm. Pharmac.* **24**, 429–433 (1972)
47. S. Laik Ali, in *Analytical Profiles of Drug Substances and Excipients* (H.G. Brittain, Ed.), pp 432–435, Academic Press, San Diego, CA (1992)
48. M. Gazdag, M. Babják, J. Brlik, S. Mahó, Z. Tuba and S. Görög, *J. Pharm. Biomed. Anal*. **16**, 1029 (1998)
49. P.A. Lane, D.O. Mayberry and R.W. Young, *J. Pharm. Sci.* **76**, 44–47 (1987)
50. M.L. Cotter, S.D. Levine, R. Mallory and C. Shaw, *Tetrahedron Lett.* **22**, 1939–1942 (1978)
51. A.M. Kaukonen, P. Vuorela, H. Vuorela and J.-P. Mannermaa, *J. Chromatogr. A* **797**, 271–281

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