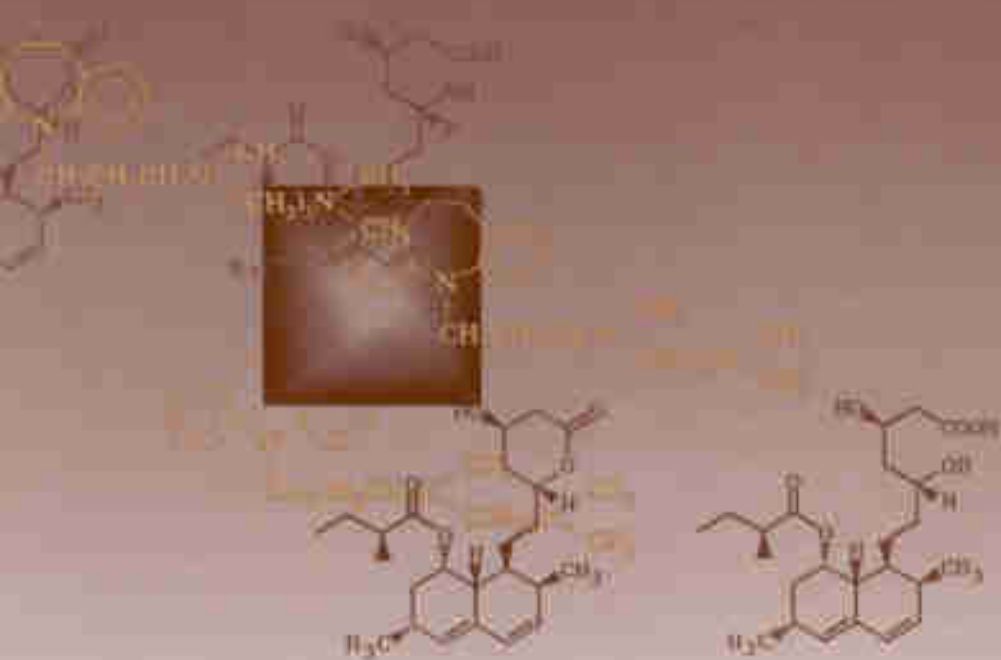# IMPURITIES

EVALUATION OF

PHARMACEUTICALS

SATINDER AHUJA

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Preface

The subject of impurities evaluation of pharmaceutical compounds has been insufficiently addressed in the scientific literature up to this time. Reasons for this shortcoming are many and varied. As a matter of fact, because of the apparent negativity attached to the word, there is no clear definition for impurity in the pharmaceutical world. Terms such as by-products, transformation products, degradation products, interaction products, and related products are frequently used. The impurities related to residual solvents used in the preparation of pharmaceutical compounds or marketed drug products are frequently referred to as organic volatile impurities (OVI). And the impurities relating to the inert ingredients (excipients) used in pharmaceutical formulations or pharmaceutical adjuvants used in the preparation of the marketed drug products are rarely mentioned.

It is necessary to incorporate stringent tests to control the impurities arising from different sources that are variously described, as explained above. This fact is also evident from the requirements of the Federal Food, Drug, and Cosmetic Act and from a large number of pharmacopeias that provide tests for the control of specific impurities. A new drug development process should include an armamentarium of physicochemical tests to fully define

the purity of a pharmaceutical compound prior to performance of extensive pharmacologic and toxicologic studies. This is essential to assure that the observed pharmacologic or toxicologic effects are truly due to the compound of interest and not due to impurities. Furthermore, it is important to ensure that the product formulated for marketing does not generate any impurities during its shelf life that could cause deleterious effects.

This book discusses various sources of impurities, methods utilized to isolate and characterize them (including those methods that do not require pre-separation, such as GC/MS and HPLC/MS), analytical methods used for evaluation and control, and applications to various classes of drug products (marketed products as well as those under development). Chiral impurities are also discussed from the standpoint of their origin, analytical methodology, and regulatory perspective for controlling them.

The book is intended to be a valuable resource for a large number of analysts and administrative personnel involved in assuring the quality of pharmaceutical products. It may be of interest to the academic world as a textbook that aligns teaching with industrial needs. Regulatory authorities are likely to find this book useful, since it attempts to provide state-of-the-art information on the field.

I would like to thank the forerunners in this field for their many contributions. And I thank my wife for her patience during the time this book was being written and published.

Satinder Ahuja

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1
An Overview

There is no precise definition for "impurity," because of the apparent negativity attached to this word in the pharmaceutical sciences (1). Webster's dictionary defines impurity as something that is impure or makes something else impure. A better definition of an impure substance may be as follows: a substance of interest mixed or impregnated with an extraneous or usually inferior substance. Personally, I like a simple definition for an impurity, that is, any material that affects the purity of the material of interest, viz., active ingredient or drug substance. The impurities are not necessarily always inferior. From the standpoint of its usage, the drug substance is compromised in terms of purity even if it contains another material with superior pharmacological or toxicological properties. At first pass this may not be readily clear; however, on further thought it would become apparent that if we are to ensure that an accurate amount of the drug substance is being administered to the patient, then we must assess its purity independently from the extraneous materials. Therefore any extraneous material present in the drug substance has to be considered an impurity even if it is totally inert or has superior pharmacological properties.

1.1 TERMINOLOGY

1.1.1 Commonly Used Terms

A number of terms have been commonly used to describe an impurity or impurities:

> Intermediate
> Penultimate intermediate
> By-products
> Transformation products
> Interaction product
> Related products
> Degradation products

Some of these terms denote potential sources of impurities; e.g., intermediaries; others tend to de-emphasize the negativity, e.g., related products. Let's review them individually:

Intermediates: The compounds produced during synthesis of the desired material are called intermediates, especially if they have been isolated and characterized. The most important criterion is characterization, i.e., they cannot be just potential reaction products theorized to occur (see *By-products* below). The theorized compounds are best designated as potential intermediates.

Penultimate Intermediate: As the name suggests, this is the last compound in the synthesis chain prior to the production of the final desired compound. Sometimes confusion arises when the desired material is a salt of a free base or acid. In the opinion of this author, it is inappropriate to label the free acid or base as the penultimate intermediate if the drug substance is a salt.

By-products: The unplanned compounds produced in the reaction are generally called by-products. It may or may not be possible to theorize all of them. Hence, they present a thorny problem to the analytical chemist.

Transformation Products: This is a relatively nondescript term which relates to theorized and non-theorized products that may be

produced in the reaction. Transformation products are very similar to by-products, except the term tends to connote that more is known about the reaction products.

Interaction Products: This term is slightly more comprehensive and more difficult to evaluate than the two described above, i.e., by-products and transformation products, in that it considers interactions that could occur between various involved chemicals-- intentionally or unintentionally.

Related Products: As mentioned, the term related products tends to suggest that the impurity is similar to the drug substance and thus tends to play down the negativity frequently attached to the term impurity. These products can have similar chemical structure and potentially similar biological activity; however, as we shall discuss later, this by itself does not provide any guarantee to that effect.

Degradation Products: The compounds produced due to decomposition of the material of interest or active ingredient are often referred to as degradation products. We also need to concern ourselves with the products produced from degradation of other compounds that may be present as impurities in the drug substance.

1.1.2 Compendial Terminology

United States Pharmacopeia (USP XXIII, 1995 edition) deals with impurities under several sections:

> Impurities in Official Articles
> Ordinary Impurities
> Organic Volatile Impurities

The pharmacopeia acknowledges that concepts about purity are susceptible to change with time and purity is intimately related to the developments in analytical chemistry. What we consider pure today may be considered impure at some future date if methods are found that can resolve other components contained in a particular compound. Inorganic, organic, biochemical, isomeric, or polymeric components can all be considered impurities. The following terms have been used to describe impurities:

Foreign substances
Toxic impurities
Concomitant components
Signal impurities
Ordinary impurities
Organic volatile impurities (OVI)

Foreign Substances: The materials that are introduced by contamination or adulteration, and not as consequences of synthesis or preparation, are labeled foreign substances, e.g., pesticides in oral analgesics.

Toxic Impurities: These impurities have significant undesirable biological activity, even as minor components, and require individual identification and quantitation by specific tests.

Concomitant Components: Bulk pharmaceutical chemicals may contain concomitant components, e.g., geometric and optical isomers (see section 1.3) and antibiotics that are mixtures.

Signal Impurities: These are distinguished from ordinary impurities discussed below in that they require individual identification and quantitation by specific tests. These impurities include some process-related impurities or degradation products that provide key information about the process.

Ordinary Impurities: The species of impurities in bulk pharmaceutical chemicals that are innocuous by virtue of having no significant undesirable biological activity in the amounts present are called ordinary impurities.

Organic Volatile Impurities: This term relates to residual solvents that may be found in the drug substance.

Inorganic impurities may also be found in compendial articles. These impurities may be as simple as common salt or other compounds that are controlled, such as heavy metals, arsenic, etc.

1.1.3 Chiral Impurities

Compounds having similar chemical structure but different spatial orientation, leading to different optical rotation, are of great im-

portance because of the resulting optical isomers; the undesired optical isomer is considered a chiral impurity; e.g., the d-isomer of a compound can have different pharmacological or toxicological activity from that of the l-isomer (2). As discussed in a later chapter in this book, the number of chiral impurities can be related to the number of asymmetric carbons in a molecule.

The body may metabolize the optical isomers differently, leading to further potential differences in toxicity. For all practical purposes, a metabolite can be considered an impurity produced in the body by transformation of the administered drug. From a methodological standpoint it would be desirable to consider the metabolites as biologically produced impurities in order to enable the bioanalytical chemist to draw upon enormous resources spent by pharmaceutical analysts on developing methods for analysis of structurally related compounds.

Chiral molecules can also occur for a number of other reasons. These are discussed later in this book and must be factored into any evaluation of impurities.

1.1.4 Miscellaneous Impurities

Other sources of impurities are the materials that may be present in the starting material that can be potentially carried into the active ingredient of interest. And the impurities that relate to inert in-gredients (excipients) and solvents used during synthesis must also be considered. Impurities can be produced during various drug product formulation steps. These impurities have the potential of being present in the final drug product. Potential reaction products must also be evaluated.

1.2 REGULATIONS AND REQUIREMENTS

There are moral, economic, and competitive reasons, as well as those of safety and efficacy, to monitor impurities in drug products (3). However, monitoring impurities and controlling them mean different things to different people or to the same people at different times, even those in the pharmaceutical sciences and

industry (see Chapter 2). A unified nomenclature is necessary to ensure that everyone speaks the same language when addressing issues relating to impurities.

There are a number of requirements that impinge on monitoring impurities. For example, a country's pharmacopeia, or the one accepted by it, frequently provides the primary guidance as to how impurities are to be monitored and controlled. In the majority of countries these pharmacopeias are run under the aegis of the government. USP is a notable exception to this case. If a product is considered a pharmacopeial item, then it must meet the compendial requirements.

In the United States, the Federal Food, Drug and Cosmetic Act and its amendments require that a manufacturer demonstrate the safety and efficacy of a new drug prior to introducing it into interstate commerce. The requirements are clearly spelled out in the Notice of Claimed Investigational Exemption for a New Drug (IND) and the New Drug Application (NDA).

INDs require "a statement of the methods, facilities and controls used for the manufacturing, processing, and packing of the new drug to establish and maintain appropriate standards of identity, strength, quality, and purity as needed for safety and give significance to clinical investigation made with the drug."

NDAs require more specific and detailed information, including stability studies to ensure that the identity, quality, and purity of the drug product is preserved until its expiration date.

In 1987 the Food and Drug Administration (FDA) issued a set of guidelines where the question of stereochemistry was approached directly in the guidelines on the manufacture of drug substances (4). The FD&C Act requires a full description of the methods used in the manufacture of the drug, which includes testing to demonstrate its identity, strength, quality, and purity. For chiral compounds this includes identification of all chiral centers. The enantiomer ratio, although 50:50 by definition for a racemate, should be defined for any other admixture of stereoisomers. It is expected that the toxicity of impurities, degradation products, and residues from manufacturing process will be investigated as the

development of the drug is pursued. The same standards should, therefore, be applied to the racemates. For all practical purposes, optical isomers are considered impurities.

The FDA may initiate action, under the Food and Drug Act, to cause removal of a product from the market, or, as is provided in the law, a manufacturer can voluntarily recall from the marketplace batches that do not meet the established specifications.

Regulatory authorities in each country use their own standards for allowing conductance of clinical studies on a new drug product, i.e., a drug substance that has been formulated or the product that is approved for commerce. A detailed discussion is given in Chapter 2 on how regulations vary from country to country and the resultant impact on international commerce. It should be noted here that efforts are being made to unify these approaches, as exemplified by International Conference on Harmonisation (ICH) guidelines; however, much remains to be done.

1.3 CHARACTERIZATION OF IMPURITIES

Once an impurity has been detected, it becomes necessary to estimate its content. Detectability frequently means that a given component provides a signal at least twice that of background noise or the baseline. At times the multiple is set higher for greater assurance. Initial estimations are generally done against the parent compound because in most cases the authentic sample of impurity is not available. It is important that the authentic sample should be used for estimations, when it is available. If the estimations indicate that a given impurity content is greater than 0.1%, then it must be characterized as per the FDA requirements.

Hyphenated methods such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) or a number of other chromatographic-spectroscopic configurations are perfectly suitable for initial characterization of the impurities. Of course, these methods are not always available or applicable--a detailed discussion is included in Chapters 4 and 9 as to why it is not always possible to use these methods. In case it

is necessary to procure authentic material for purposes of structure confirmation, synthesis or isolation methods should be utilized.

It is often necessary to isolate impurities because the instrumental methods mentioned above are not available or need further confirmation. Isolation should be initiated based on simple extraction or partition methods. It may be possible to extract impurities selectively on the basis of acidity, basicity, or neutrality. The extraction process usually involves liquid-liquid extraction, where one phase is an aqueous solution and the other is an organic phase that is nonpolar. By appropriate adjustment of the pH of the aqueous solution, one can extract acidic, basic, or neutral impurities. Further separations can be made by chromatographic methods.

Techniques such as chromatography can provide separation of compounds from each other and from the main compound. Separations are based on properties such as adsorption, partition, ion-exchange, or molecular size. Because of its simplicity, thin layer chromatography (TLC) is often the technique of choice. It generally entails selection of a suitable solvent system (mobile phase) for resolution of impurities on a silica gel plate. If the nature of impurities is not known, systems that are acidic, neutral, and basic are investigated. Universal detectors (UV or spray reagents) are preferred. Details on these methods and more sophisticated preparative separations are discussed in Chapter 3.

1.4 ANALYTICAL METHODOLOGIES

Development of a new drug mandates that meaningful and reliable analytical data be generated at various steps of the new drug development (5). To ensure the safety of a new pharmaceutical compound or drug requires that the new drug meet the established purity standards as a chemical entity or when admixed with animal feeds for toxicity studies or pharmaceutical excipients for human use. Furthermore, it should exhibit excellent stability throughout its shelf life. These requirements demand that the analytical methodology that is used be sensitive enough to measure low levels of

impurities. This has led to analytical methods that are suitable for determination of trace/ultratrace levels, i.e., sub-microgram quantities of various chemical entities (6).

A variety of methods are available for monitoring impurities. The primary criterion is the ability to differentiate between the compounds of interest. This requirement reduces the availability of methods primarily to spectroscopic and separation methods or a combination thereof. At times, titrimetric methods and other simple methods may work and should be utilized. However, to keep the size of this book manageable, these methods have been appropriately discussed.

1.4.1 Spectroscopic Methods

The following spectroscopic methods can be used:

> Ultraviolet (UV)
> Infrared (IR)
> Nuclear magnetic resonance (NMR)
> Mass spectrometry (MS)

Ultraviolet spectrophotometry (UV) at a single wavelength provides minimal selectivity of analysis; however, with the availability of diode array detectors, it is now possible to get sufficient simultaneous information at various wavelengths to ensure greater reliability.

Infrared spectrophotometry (IR) provides specific information on some functional groups that may allow quantitation and selectivity. However, low-level detectability is frequently a problem that may require more involved approaches, which are generally a deterrent to a pharmaceutical analyst.

Nuclear magnetic resonance spectroscopy (NMR) provides fairly detailed structural information on a molecule and is a very useful method for characterization of impurities; however, it has limited use as a quantitative method.

Mass spectrometry (MS) provides excellent structural information and, based on the resolution of the instrument, it may provide an effective tool for differentiating molecules with small differences in the molecular weight. However, it has limited use as a quantitative technique.

IR, NMR, and MS are excellent techniques for characterization of impurities that have been isolated by any of the techniques discussed above. UV has been found to be especially useful for analyzing most samples with high pressure liquid chromatography. This combination is commonly used in pharmaceutical analysis.

1.4.2 Separation Methods

The following separation methods can be used:

> Thin layer chromatography (TLC)
> Gas chromatography (GC)
> High pressure liquid chromatography (HPLC)
> Capillary electrophoresis (CE)
> Supercritical fluid chromatography (SFC)

A brief account of the above-listed methods is given here to provide a quick review of their potential use.

Except for CE all of these methods are chromatographic methods. CE is an electrophoretic method that is frequently lumped with the chromatographic methods because it shares with chromatography many of the common requirements. However, it is not strictly a two-phase separation system--a primary requirement in chromatography. Hyphenated methods such as GC-MS, LC-MS, GC-LC-MS, LC-MS-MS, etc. are all discussed later in this book.

A broad range of compounds can be resolved by TLC by utilizing a variety of different plates and mobile phases. The primary difficulties related to this method are limited resolution, detection, and ease of quantitation. The greatest advantages are the ease of usage and low cost.

Gas chromatography is a very useful technique for quantitation. It can provide the desired resolution, selectivity, and ease of quantitation. However, the primary limitation is that the sample must be volatile or has to be made volatile by derivatization. This technique is very useful for organic volatile impurities (OVI).

High pressure liquid chromatography is frequently casually referred to as high performance liquid chromatography today. Both of these terms can be abbreviated as HPLC, and these terms are used interchangeably by chromatographers. This is a useful technique whose applications have been significantly extended for the pharmaceutical chemist by the use of a variety of detectors such as fluorescence, electrometric, MS, etc.

Capillary electrophoresis is a useful technique when very low quantities of samples are available and high resolution is required. The primary difficulty is relatively lower reproducibility.

Supercritical fluid chromatography offers some of the advantages of GC in terms of detection, and HPLC in terms of separations, in that volatility of the sample is not of paramount importance. This technique is still evolving, and its greatest application has been found in the extraction of samples.

1.5 SYNTHESIS-RELATED IMPURITIES

Impurities in a pharmaceutical compound or a new chemical entity (NCE) originate mainly during the synthetic process from raw materials, solvents, intermediates, and by-products. The raw materials are generally manufactured to much lesser purity requirements than a drug substance. Hence, it is easy to understand why they would contain a number of components that in turn could effect the purity of the drug substance.

Similarly, solvents used in the synthesis are likely to contain a number of impurities that may range from trace levels to significant amounts that can react with various chemicals used in the synthesis to produce other impurities. Intermediates are also not generally held to the purity level of the drug substance--hence the remarks made for the raw materials apply. By-products are

frequently unknown and are very rarely controlled. So they too are a source of concern in terms of monitoring impurities. The "pot reactions," i.e., when the intermediates are not isolated, are convenient, economical, and time saving; however, they raise havoc in terms of the generation of impurities because a number of reactions can occur simultaneously.

The penultimate intermediate is generally controlled in the pharmaceutical synthesis. However, rigorous standards of purity for the drug substance are very rarely applied at this stage. It is important to remember that this step is the last potential source of impurities. Therefore it is very desirable that the methods used for analysis at this stage be rigorous and that the tightest economically and practically feasible specifications be applied.

1.6 FORMULATION-RELATED IMPURITIES

A number of impurities in a drug product can arise out of inert ingredients used to formulate a drug substance. Furthermore, in the process of formulation, a drug substance is subjected to a variety of conditions that can lead to its degradation or other deleterious reactions. For example, if heat is used for drying or for other reasons, it can help accelerate degradation.

Solutions and suspensions are potentially prone to degradation due to hydrolysis or solvolysis (see kinetic studies in Chapter 7). These reactions can also occur in the dosage form in a solid state, such as in the case of capsules and tablets, when water or another solvent has been used for granulation. The water used in the formulation can not only contribute its own impurities, it can also provide a ripe situation for hydrolysis and metal catalysis. Similar reactions are possible in other solvents that may be used.

Oxidation is very possible for easily oxidized materials if no precautions are taken. Similarly, light-sensitive materials can undergo photochemical reactions. Details are provided in Chapter 6 in this book regarding how various excipients can contribute to degradation and the resulting impurities.

1.7 STABILITY STUDIES

It is necessary to conduct stability studies to predict, evaluate, and ensure drug product safety (3). However, stability can mean different things to different people, based on their discipline in the pharmaceutical sciences and industry. A variety of terms are still employed to encompass the what and the how and the why of stability: kinetic study, compatibility study, stability evaluation, stability-indicating assay, expiration dating, outdating, shelf life, storage legend, preformulation study, failure of a batch to meet specifications, microbiological stability, stability of the active ingredient, stability of the formulation, stability in the marketed package, stability in the sample package, stability in the dispensing package, and stability in the hands of the consumer. All of these areas have been referred to as stability. A unified terminology is badly needed to ensure that everyone understands the importance of stability studies.

The objective of Chapter 8, allocated for stability studies in this book, is to review the many facets of stability and to outline what a present-day stability program does and should include. An attempt will be made to interrelate scientific considerations with regulatory requirements. To highlight their importance in monitoring and evaluating impurities, kinetic studies have also been treated in a separate chapter.

1.8 KINETIC STUDIES

Most of the degradation reactions of pharmaceuticals occur at finite rates and are chemical in nature. These reactions are affected by conditions such as solvent, concentration of reactants, temperature, pH of the medium, radiation energy, and the presence of catalysts. The order of the reaction is described by the manner in which the reaction rate depends on the concentration of reactant. The degradation of most pharmaceuticals can be classified as zero order, first order, or pseudo-first order, even though they may

degrade by complicated mechanisms, and the true expression may be of higher order or be complex and noninteger.

An understanding of the limitations of experimentally obtained heat of activation values is critical in stability predictions. For example, the apparent heat of activation of a pH value where two or more mechanisms of degradation are involved is not necessarily constant with temperature. Also, the ion product of water, pKw, is temperature-dependent, and $-\Delta Ha$ is approximately 12 kcal, a frequently overlooked factor that must be considered when calculating hydroxide concentration. Therefore, it is necessary to obtain the heat of activation for all bimolecular rate constants involved in a rate-pH profile to predict degradation rates at all pH values for various temperatures.

It is incumbent upon the chemist to perform some kinetic studies to predict stability of a drug substance. However, it is important to recognize the limitations of such predictions. The importance of kinetic studies and the effect of various additives on the reaction rates are discussed at some length in Chapter 7.

1.9 APPLICATIONS

Numerous applications exist in the areas of monitoring quality, stability, and safety of pharmaceutical compounds, whether produced synthetically, extracted from natural products, or produced by recombinant methods. A large number of applications are included throughout this book to provide ready reference to various methods. In Chapter 9, on applications, the compounds have been classified into the following groups:

> Alkaloids
> Amines
> Amino acids
> Analgesics
> Antibacterials
> Anticonvulsants, antidepressants, and tranquilizers
> Antineoplastic agents

Local anesthetics
Macromolecules
Steroids
Miscellaneous

This classification, which combines chemical grouping with therapeutic usage, has been found by the pharmaceutical chemist to be quite helpful.

The ready availability of this information may be helpful in incorporating stringent tests to monitor the impurities since, as mentioned before, these impurities can originate from various sources. This is evident from the requirements of the Federal Food, Drug and Cosmetic Act and from the various pharmacopeias that provide tests for the control of specific impurities. A new drug development program should include an armamentarium of physicochemical tests to fully define the impurity profile of a pharmaceutical compound prior to performance of extensive pharmacological and toxicological studies. This is essential to ensure that observed toxicological or pharmacological profiles are truly due to compound of interest and not due to impurities.

The level to which any impurity should be controlled is primarily determined by its pharmacological and toxicological effects. Here we should include all impurities: those originating out of synthesis and those originating from other sources such as degradation. For example, penicillins and cephalosporins have been known to undergo facile cleavage of the β-lactam bond in aqueous solution. This is of special interest since some studies on penicillins have shown that their instability may affect possible reactions involved in penicillin allergy (7). The control of low-level impurities is extremely important when a drug is taken in large quantities. Examples are the use of methotrexate (10-20 g) to treat neoplasia or the faddist use of vitamins, especially vitamin C.

Particular attention must also be paid to the detection of DNA in all finished biotechnology products because DNA might be incorporated in the human genome and become a potential oncogene. It may be desirable to show the absence of DNA at the

picogram-per-dose level to ensure the safety of biotechnology products (8).

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2
Regulations and Requirements for Controlling Impurities

Bulk pharmaceuticals or, more appropriately, bulk pharmaceutical chemicals (BPCs), can be obtained from multiple sources and, therefore, it is very important that impurities in them be carefully monitored and controlled (1). This is especially true with generic drugs, which now account for up to 40% of the prescription drugs sold in the United States, up from 24% a decade ago (2). It is important to remember that 70% to 80% of the key ingredients in American-made generics come from foreign suppliers. This is also the case with about 60% of the components in brand-name drugs. Official industrial estimates indicate that a small but growing percentage of finished drugs--possibly 10% to 20%--are also being imported as companies in Canada and Western Europe market more drugs in the United States.

It is entirely possible that the present system of regulations and requirements is inadequate for controlling them. Over the last three years, the Food and Drug Administration (FDA) has doubled the number of its inspections at foreign plants. In the last year alone, the FDA has found serious manufacturing deficiencies, from contaminated water supplies to lax quality controls, in 35% of its overseas inspections, in countries as varied as Switzerland and China, compared with 19% at domestic plants. In one well-known

example at least 15 patients suffered epileptic seizures and 2 died in the late 1980s after taking a generic drug intended to prevent seizures. According to FDA documents, a now-defunct Colorado company that made the drug suspected that defects in the ingredient from Italy made the medication far less potent than it should have been. This clearly points to the great need for monitoring purity and for controlling drug production worldwide.

As mentioned in Chapter 1, the compendia or an accepted compendium in a given country provides guidelines on requirements for monitoring and controlling impurities. However, it is the job of regulatory authorities to ensure that requirements are being met by appropriate inspections and analyses of the materials that are actually being produced. Regulatory agencies also provide direction by requiring that certain purity standards are met when a new drug is filed for clinical investigation or when it is approved for commercial use. These topics are further discussed in some detail here.

2.1 COMPENDIAL REQUIREMENTS

One of the primary objectives of the United States Pharmacopeia (USP) is to ensure the user of official articles, i.e., items included in the USP, that these items meet the requirements of identity, strength, quality, and purity (3,4). However, it is practically impossible to include in each monograph a test for every impurity, contaminant, or adulterant that might be present, including microbial contamination. Impurities may arise from a change in source of material or from a change in processing, or they may be introduced from extraneous sources. USP recognizes that the present monographs (see ordinary impurities for instance) may not adequately portray profiles of multisource BPCs and proposed a comprehensive provision (5).

It should be recognized that the choice of labeling as a route to compliance is not as desirable as the more satisfactory route of correspondence with the committee of revision that leads to the incor-

poration in the monograph of an appropriate purity test, in essence, a second test of ordinary impurities or chromatographic purity. This policy assures a full disclosure of purity. It can be carried out by including all process-necessary monograph tests. Furthermore, it is important to recognize that USP does not distinguish between prescription and over-the-counter articles, and the proposed policy covers both categories. And the scope of USP's proposed coverage of multisource, already marketed drug substances, is limited to synthetic organic medicinals.

2.1.1 Compendial Impurities Tests

The following tests for impurities are included in the USP.

Foreign Substances and Impurities: The tests for the presence of foreign substances and impurities are provided to limit such substances to amounts that are objectionable, under which the article is customarily employed. An impurity, contaminant, or adulterant may arise from a large variety of sources, as discussed earlier. Tests suitable for detecting such occurrences, the presence of which is inconsistent with applicable manufacturing practices or pharmaceutical practice, should be employed in addition to the tests provided in the individual monograph.

Other Impurities: Official substances may be obtained from more than one process, and thus may contain impurities not considered during preparation of monograph assays or tests. Whenever a monograph includes a chromatographic assay or test and that monograph does not detect such an impurity, solvents excepted, the impurity will have its amount and identity stated under the heading "other impurity(ies)" by the labeling of the official substance.

2.1.2 Other Impurities

The presence of an unlabeled impurity in an official substance constitutes a variance from the standard if the content is 0.1% or

greater. The sum of all other impurities combined with the monograph-detected impurities should not exceed 2.0%.

Categories of drug substances excluded from other impurities requirements are fermentation products and semi-synthetics derived therefrom, radiopharmaceuticals, biologicals and biotechnologically derived, peptides, herbals, and crude products of animal or plant origin.

A toxic impurity should not be listed under other impurities. Toxic impurities require specific monograph procedures. In due course of time, the uncertainties about toxicity testing requirements may be clarified in that the International Conference on Harmonisation (ICH) has been working on a schematic for "qualification" of impurities for new molecular entities. When this matter is eventually resolved, the following issues must be addressed (4):

• Are impurities in already marketed substances to be considered as qualified?

• Should the USP subsequently adopt a presumption that ordinary impurities or other impurities have been qualified by considerations appropriate to product histories?

• Would data or drug master files be available, where pertinent, to establish a limit test for a specific impurity?

A total of 464 monographs that contain a chromatographic purity test or chromatographic stability-indicating assay are affected by the proposal to revise the requirements described under other impurities.

2.1.3 Impurities in Official Articles

Purity or impurity measurements on finished preparations present a challenge to those who are setting standards for the pharmacopeia. Where degradation of a preparation over a period of time is an issue, the same analytical methods that are stability-indicating are also purity-indicating. Resolution of the active ingredient(s) from the excipients necessary to the preparation presents the same qualitative problem. Thus, many monographs for pharmacopeial

preparations feature chromatographic assays. Where more significant impurities are known, some monographs set specific limit tests. As discussed in Chapter 1, the USP provides definitions of impurities under various names. The following comments on the requirements may be pertinent here:

Foreign Substances: The presence of objectionable foreign substances that are not revealed by monograph tests and assays constitutes a variance from the official standards. Allowance is made in the USP for detection of foreign substances by the unofficial methods.

Toxic Impurities: These impurities have significant undesirable, biological activity, even as minor components, and require individual identification and quantitation by specific tests. The toxic impurities can arise out of synthesis, preparation, or degradation of compendial articles. It is incumbent on the manufacturer to provide data to support the classification of such impurities.

Concomitant Components: These are not considered impurities in the pharmacopeial sense. Limits on contents, or specified ranges, or defined mixtures are provided in the USP. Examples of these are geometric and optical isomers (or racemates) and antibiotics that are mixtures.

Signal Impurities: They are distinct from ordinary impurities in that they require individual identification and quantitation by specific tests. Based on validation data, individualized tests and specifications are selected that include comparison to reference standard of the impurity, if available. Signal impurities may include some process-related impurities or degradation products that could provide key information about the process. It is incumbent on the manufacturer to provide data for appropriate classification of these impurities.

Ordinary Impurities: These impurities are considered innocuous by virtue of having no significant undesirable biological activity in the amounts present. They can arise out of synthesis, preparation, or degradation of compendial articles. The USP controls these by including tests for them. Tests for related sub-

stances or chromatographic purity may also control the presence of ordinary impurities.

The estimation of these impurities is generally done by relative methods rather than strict comparison to individual reference standards. A general limit of 2.0% is used where documentation does not support adoption of other values.

It is important to remember where a monograph sets limits on concomitant components, signal impurities, and/or toxic impurities; these species are not to be included in the estimation of ordinary impurities unless so stated in the individual monograph.

Organic Volatile Impurities: These impurities constitute residual amounts of the solvents used in the synthesis of drug substances. The test for ethylene oxide is conducted where specified in the individual monograph. The limit is 10 ppm. The USP has the following position on the other organic volatile impurities: Unless otherwise specified in the individual monograph, the amount of each organic volatile impurity present in the bulk pharmaceutical chemical does not exceed the limits shown below in Table 2.1.

Table 2.1   Organic Volatile Impurities*

ORGANIC VOLATILE IMPURITY	LIMITS** (ppm)
Acetonitrile	50
Benzene	100
1,2-Dichloroethane	50
1,4-Dioxane	100
Methylene chloride	500
Pyridine	100
Trichlorethylene	100

*See reference 5.
**The limits of less than 100 ppm are based on relatively greater toxicity concerns.

The USP also includes a special test and limitation of methylene chloride in coated tablets.

2.2 INDUSTRIAL PERSPECTIVE

Pharmaceutical manufacturers recognize that it is their responsibility to ensure that the drug products available to the public are both safe and efficacious. To achieve this objective, they interact with regulatory agencies in developing new drug substances and new drug products, and cooperate with the compendia in writing official monographs for the compendial articles that manufacturers produce.

The goal of high-quality drug products can be achieved when rational approaches are applied to the complex process of drug development. It is important to recognize that the tests used at various stages of drug development and marketing should not be interpreted individually but should be evaluated on the basis of total available information. The safety and efficacy of drug products can be ensured only by employing suitable controls on raw materials, drug substances, and manufacturing, coupled with well-planned and executed toxicological and clinical studies. A rational approach to identify impurities and set limits must rely on the scientific judgments of manufacturers, the compendia, and regulators. This is necessary both for identified and unidentified impurities. An approach for setting limits, based on various factors detailed below, may be preferable over utilization of some arbitrary limits (5):

1. The toxicology of a drug substance containing typical levels of impurities and/or the toxicology of impurities relative to a drug substance
2. The route of administration, e.g., oral, topical, parenteral, or intrathecal
3. The daily dose of a drug substance, i.e., frequency and amount (micrograms or grams) administered
4. The target population (age and disease state), e.g., neonates, children, or senior citizens
5. The pharmacology of an impurity, when appropriate
6. The source of a drug substance, e.g., synthetic, natural product, or biotechnological

7. The duration of therapy, i.e., administration over a long period (treatment of chronic conditions) versus administration intended for a short duration (treatment of acute conditions)
8. The capability of a manufacturer to produce high-quality material at a reasonable cost to consumers.

The basic reason for setting limits is that levels of impurities in a drug substance must be controlled to ensure its safety and quality throughout its development into a drug product and its use as a drug product. It is generally recognized that the concepts for setting impurity limits in bulk drug substances should be the concern of the regulatory and compendial agencies as well as that of the pharmaceutical industry. The concepts can be derived from issues and experiences with drug substances from traditional sources and technologies. The issues arising from biotechnologically produced drug substances, e.g., recombinant DNA and hybridomas, are continuously being defined and therefore are not necessarily covered by these concepts. However, the concepts can serve as a general foundation to address specific issues arising from biotechnology.

Two more terms have been used in the industry for describing impurities and are described here to interrelate with those given above:

Related Substances: These substances are structurally related to the drug substance and may be identified or unidentified degradation products or impurities arising from a manufacturing process or during storage of a material.

Process Contaminants: These are identified or unidentified substances (excluding related substances and water), including reagents, inorganics (heavy metals, chloride, or sulfate), raw materials, and solvents. Substances of this type may be introduced during manufacturing or handling procedures.

2.3 REGULATORY FILING

The Food and Drug Administration has the assigned responsibility of ensuring the safety and efficacy of drugs. This requires that an

investigational drug application (IND) be filed with the FDA prior to the initiation of any clinical studies. A new drug application (NDA) has to be filed and approved before a drug can be commercialized. The following information on chemistry, manufacturing and control (CMC) is generally filed in the IND as per 21 CFR 312.23 (a) for a drug substance and drug product (7):

Drug Substance:

1. Description/Characterization
     Nomenclature and chemistry sheet
     Physical chemistry of new drug substance
2. Manufacturer
     Name and address of the manufacturer
3. Method of Synthesis/Purification
4. Specifications and Analytical Methods
     Raw material control
     Drug substance quality standard/batch analysis with methods
     Reference standard
     Preparation/purification of reference standard
5. Stability

Drug Product:

1. Components and Quantitative Composition
     Excipients
     Solvents, if any
2. Manufacturer
     Name and address
3. Manufacturing and Packaging
     Manufacturing procedure
     Container description/specification
4. Specification and Analytical Methods
     Quality standards/batch analyses
5. Excipient controls
     Quality standards/batch analyses
     Compendial certification
     Additional controls, if any

6. Stability
   Stability report

The production of a drug product is controlled by the current good manufacturing practices (cGMP) and covers the following elements:

- Organization and personnel
- Building and facilities
- Equipment
- Control of components, drug product containers, and closures
- Production and process controls
- Packaging and labeling controls
- Handling and distribution
- Laboratory controls
- Records and reports

It should be recognized that setting of limits on impurities in drug substances is an evolutionary process, beginning before an IND is filed and continuing until well after the approval of an NDA. Therefore, it may be appropriate to address different stages in drug development as separate issues. There are a number of points in the drug development process where the setting of limits may be significantly different:

- Initial IND Filing
- NDA Filing
- After NDA Approval
- ANDA Filing

The filing of an abbreviated new drug application (ANDA) is another activity in which limits are set on impurities. It is important to ensure that analytical methods used to evaluate impurities in drug substances are suitable for the intended purpose at each stage in development.

2.3.1 Initial IND Filing

At this stage the chemical nature of the bulk substance has been defined. The manufacturing process is in the early stage of development, and materials may be produced on a laboratory scale. Usually a few batches have been made and, therefore, minimal historical data are available. The reference materials of a drug substance may be relatively impure. Limits of the purity of a drug substance are set to indicate drug quality. The setting of limits on related substances and process contaminants can be characterized as follows (5):

1. Limits are set on total impurities, and an upper limit may be set on any single impurity. The limit for total impurities should maintain, if possible, a nominal composition material balance.
2. Impurity profiles are documented. These are profiles of the lots of drug substances used in clinical studies and in toxicological studies that establish the safety of drug substances. The lots used in these studies should be typical products of the manufacturing process in use at that time.
3. Limits for residual solvents are based on the known toxicology of the solvents and on the manufacturing capabilities and dosage regimens.
4. General inorganic contaminants are monitored by appropriate tests such as a heavy-metals limit test and/or a test for residue on ignition. Traditional compendial limits are applied unless otherwise indicated. Specific metal contaminants that appear during manufacturing should be monitored by appropriate analytical techniques, and limits should be set based on the toxicological properties of these metals.
5. Appropriate limits are set for impurities that are known to be toxic.
6. Enantiomeric purity is controlled, if appropriate.

Although water is not classified as an impurity, limits for water content may be needed to ensure the stability or ease of processing a drug substance.

2.3.2 NDA Filing

During the IND phases of drug development, the manufacturing process for a drug substance may undergo a number of revisions. Generally, the size of batch would have changed from laboratory scale to approach full-production batch size. A number of batches would normally have been produced, and a historical data base of the results of testing for impurities would be available. When significant changes in a manufacturing process are made, the impurity profile should be reviewed to determine if the toxicological studies are still supportive.

At the NDA stage a reference standard of defined purity is available, analytical methods have been validated, impurity and degradation profiles are known, and enantiomeric purity has been evaluated. The setting of limits on related substances and process contaminants can be characterized as follows (5):

1. Consistency of the impurity profile of a drug substance has been established.
2. IND limits for total and individual impurities (identified and unidentified) are reviewed and adjusted based on manufacturing experience and toxicological data.
3. Impurities present in significant amounts are identified and individual limits are set. However, it is not always possible to identify and/or prepare authentic substances for impurities. The labile nature of some impurities precludes this possibility. Limits may be set on these substances based on comparison of lots produced and used in toxicological and clinical studies.
4. The impurity profiles of the lots designated for marketing should not be significantly different from those of the lot(s) used for toxicological and clinical studies.

5. The composition material balance should be used, if possible, to evaluate the adequacy of the controls.
6. Limits for residual solvents are based on the known toxicology of the solvents and on the manufacturing capabilities and dosing regimens.
7. Limits are set for inorganic contaminants by appropriate tests such as a heavy-metals limit test and/or by a test for residue on ignition. Traditional compendial limits are applied unless otherwise indicated. Based on toxicological properties, limits may be set for specific metal contaminants that appear during manufacturing.

2.3.3 After NDA Approval

After the approval and marketing of a pharmaceutical product, significant changes may be made in the process of manufacturing the bulk drug substance. There may be technological, ecological, economic, or safety reasons for these changes. If any of these changes occur, the pharmacopeial and NDA impurity limits and rationale should be reviewed; the limits should be revised when indicated to ensure similar or improved quality of the drug substance.

2.3.4 ANDA Filing

The drug substance for a pharmaceutical product that is eligible for ANDA status normally is an official article and should be well characterized analytically. Drug substances are typically available from multiple sources, and each source may have a different manufacturing process. Therefore, it is essential that the dosage-form manufacturer evaluate each supplier's drug substance impurity profiles. Limits can then be set, based on the more detailed concepts described for NDA filing, including a review of compendial monographs for appropriateness.

2.4 ICH GUIDELINES

Draft guidelines on impurities in new drug substances have been published in the Federal Register (6) based on the ICH, organized to provide an opportunity for development of tripartite harmonization initiatives with inputs from both regulatory and industry representatives. The primary concern of ICH is harmonization of technical requirements for the registration of pharmaceutical products among three regions: the European Union, Japan, and the United States.

The information included here from the published document is intended to provide guidance for drug marketing registration on the content and qualification of impurities in new drug substances produced by chemical syntheses that have not been previously registered in a region or member state. It is not intended to apply to the regulations of new drug substances used during clinical research stage of development, e.g., investigational new drugs or clinical trial exemptions. Biological/biotechnological, peptide, radiopharmaceutical, fermentation, and semisynthetic products derived therefrom, herbal products, and crude products of animal or plant origin are not covered by these guidelines.

Impurities in new drug substances have been addressed from two perspectives:

Chemistry: This includes classification and identification of impurities, report generation, setting specifications, and a brief discussion of analytical procedures.

Safety: It covers specific guidance for qualifying impurities that were not present in batches of new drug substances used in safety and clinical studies and/or impurity levels substantially higher than in those batches. Threshold limits are given to preclude qualification of impurities present below these limits.

2.4.1 Classification of Impurities

Impurities are generally simply classified into the following three categories:

- Organic impurities (process and drug related)
- Inorganic impurities
- Residual solvents

Organic impurities may arise during the manufacturing process and/or storage of the new drug substance. They may be identified or unidentified, volatile or nonvolatile, and include the following:

- Starting materials
- By-products
- Intermediates
- Degradation products
- Miscellaneous (reagents, ligands, and catalysts)

Inorganic impurities may occur from the manufacturing process. They are normally known and identified and include:

- Heavy metals
- Inorganic salts
- Miscellaneous (reagents, ligands, and catalysts)
- Other materials (filter aids, charcoal, etc.)

Solvents are organic or inorganic liquids used during the manufacturing process. Since these are generally of known toxicity, the selection of suitable controls is easily accomplished. Excluded from this discussion are extraneous contaminants that should not occur in new drug substances and are more appropriately addressed as good manufacturing practice issues; polymorphic form, a solid state property of the new drug substance; and enantiomeric impurities.

2.4.2 Rationale for the Reporting and Control of Impurities

Organic Impurities: The applicant should summarize those actual and potential impurities most likely to arise during synthesis, purification, and storage of the new drug substance. This summary

should be based on sound scientific appraisal of the chemical reactions involved in the synthesis, impurities associated with raw materials that could contribute to the impurity profile of the new drug substance, and possible degradation products. The discussion need only include those impurities that may reasonably be expected based on knowledge of the chemical reactions and conditions involved.

In addition, the applicant should summarize the laboratory studies conducted to detect impurities in the new drug substance. This summary should include test results of batches manufactured during the development process and batches from the proposed commercial process, as well as results of intentional degradation studies used to identify potential impurities arising during storage. Assessment of the proposed commercial process may be deferred until the first batch is produced for marketing. The impurity profile of the drug substance lots intended for marketing should be compared with those used in development, and any observed differences should be discussed.

The studies conducted to characterize the structure of actual impurities present in the new drug substance at or above an apparent level of 0.1% (e.g., calculated by using the response factor of the drug substance) should be described. It should be noted that identification of all recurring impurities at or above the 0.1% level is expected in batches manufactured by the proposed commercial process.

Degradation products observed in stability studies at labeled storage temperatures should be identified. When identification of an impurity is not feasible, a summary of the laboratory studies demonstrating the unsuccessful effort should be included in the application. Where attempts have been made to identify impurities below the 0.1% level, it is useful to report the results of these studies.

Identification of impurities below apparent levels of 0.1% is generally not considered necessary. However, identification should be attempted for those potential impurities that are expected to be unusually potent, producing toxic or pharmacologic effects at a

level lower than 0.1%. In all cases, impurities should be qualified as described later in this chapter. Although it is common practice to round analytical results of between 0.05% and 0.09% to the nearest number, i.e., 0.1%, for the purpose of these guidelines, such values are not rounded to 0.1% and these impurities do not require identification.

Inorganic Impurities: These impurities are normally detected and quantitated using pharmacopeial or other appropriate procedures. Any carryover of catalysts to the new drug substance should be evaluated during development. The need for inclusion or exclusion of inorganic impurities in the new drug substance specifications should be discussed. Limits are based on pharmacopeial standards of known safety data.

Solvents: The control of residues of the solvents used in the manufacturing process for the new drug substance should be discussed. Any solvents that may appear in the drug substance should be quantified using analytical procedures with an appropriate level of sensitivity. Pharmacopeial or other suitable procedures should be used. Limits are based on pharmacopeial standards or known safety data, taking into consideration dose, duration of treatment, and route of administration. Particular attention should be given to quantitation of toxic solvents used in the manufacturing process.

2.4.3 Analytical Procedures

Differences in the analytical procedures used during development and proposed for the commercial product should be discussed in the registration application.

Organic impurity levels can be measured by a variety of techniques, including those that compare an analytical response for an impurity to that of an appropriate reference standard or to the response of the new drug substance itself. Reference standards used in the analytical procedures for control of impurities should be evaluated and characterized according to their intended use. It is considered acceptable to use the drug substance to estimate the levels of impurities. In cases where the response factors are not

close, this practice may still be acceptable, provided a correction factor is applied or the impurities are, in fact, being overestimated. Specifications and analytical procedures used to estimate identified or unidentified impurities are often based on analytical assumptions, e.g., equivalent detector response. The assumptions should be discussed in the registration application.

2.4.4 Reporting Impurity Content of Batches

Analytical results should be provided for all batches of the new drug substance used for clinical, safety, and stability testing, as well as batches representative of the proposed commercial process. The content of individual identified and unidentified and total impurities, observed in these batches of the new drug substance, should be reported with the analytical procedures indicated. A tabulation, e.g., spreadsheet, of the data is recommended. Impurities should be designated by code number or by an appropriate descriptor, e.g., retention time. Levels of impurities which are present but below the validated limit of quantitation need not be reported. When analytical procedures change during development, reported results should be linked with the procedures used, with appropriate validation information provided. Chromatograms should be provided of representative batches, from method validation studies showing separation and detectability of impurities (e.g., on spiked samples) along with any other impurity. Routinely performed, these may serve as representative impurity profiles. The applicant should ensure that complete impurity profiles, such as chromatograms, of individual batches are available. A tabulation should be provided that links the specific new drug substance batch-to-batch safety study to each clinical study in which it was used.

For each batch of the new drug substance, the report should include:

- Batch identity and size
- Date of manufacture

- Site of manufacture
- Manufacturing process
- Impurity content, individual and total
- Use of batches
- Reference to analytical procedure used

2.4.5 Specification Limits for Impurities

The specifications for a new drug substance should include limits for impurities. Stability studies, chemical development studies, and routine batch analyses can be used to predict those impurities that are likely to occur in the commercial product. The selection of impurities that are to be included in the new drug substance specifications should be based on the impurities found in batches manufactured by the proposed commercial process. Those impurities selected for inclusion in the specifications for the new drug substance are referred to as specified impurities in ICH guidelines. Specified impurities may be identified or unidentified and are individually listed in the new drug substance specifications.

A rationale for the inclusion or exclusion of impurities in the specifications should be presented. This rationale should include a discussion of the impurity profiles observed in the safety and clinical development batches, together with a consideration of the impurity profile of material manufactured by the proposed commercial process. Specific identified impurities should be included along with recurring unidentified impurities estimated to be at or above 0.1%. For impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the sensitivity of the analytical methods should be commensurate with the level at which the impurities are to be controlled. For unidentified impurities, the procedure used and assumptions made in establishing the level of the impurity should be clearly stated. Unidentified impurities included in the specifications should be referred to by some appropriate qualitative analytical descriptive label, e.g., "unidentified" A or "unidentified with relative retention

of 0.6," etc. Finally, a general specification limit of not more than 0.1% for any unspecified impurity should be included.

Limits should be set no higher than the level that 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. Where there is no safety concern, impurity specifications are based on data generated on actual batches of the new drug substance allowing sufficient latitude to deal with normal manufacturing and analytical variation, and the stability characteristics of the new drug substance. Although normal manufacturing variations are expected, significant variation in batch-to-batch impurity levels may indicate that the manufacturing process of the new drug substance is not adequately controlled and validated.

In summary, the new drug substance specifications should include, where applicable, limits for:

- 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%
- Total impurities
- Residual solvents
- Inorganic impurities

A summation of assay value and impurity levels can be used to obtain mass balance for the test sample. The mass balance need not add up to exactly 100% because of the analytical error associated with each analytical procedure. The summation of impurity levels plus the assay value may be misleading, such as when the assay procedure is nonspecific, as with potentiometric titrimetry, and the impurity level is relatively high.

2.4.6 Qualification of Impurities

Qualification is the process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a

given impurity profile at the level(s) specified. The applicant should provide a rationale for selecting impurity limits based on safety considerations.

The level of any impurity present in a new drug substance that has been adequately tested in safety and/or clinical studies is considered qualified. Impurities that are also significant metabolites present in animal and/or human studies do not need further qualification. 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 previous safety studies.

If data is not available to qualify the proposed specification level of an impurity, studies to obtain such data may be necessary when the usual qualification limits given below are exceeded.

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

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, qualification may be especially important when there is evidence that such impurities in certain drugs or therapeutic classes have previously been associated with adverse reactions in patients. In these instances a lower qualification threshold limit may be appropriate. Conversely, a higher qualification threshold limit may be appropriate for individual drugs when the level of concern for safety is less than usual, based on similar considerations (patient population, drug class effects, clinical considerations, etc.). Technical factors (manufacturing capability and control methodology) may be considered part of the justification for selection of alternative threshold limits. Proposals for alternative threshold limits are considered on a case-by-case basis.

The decision process for safety studies (see the decision chart) describes considerations for the qualification of impurities when thresholds are exceeded. In some cases, decreasing the level of impurity below the threshold may be simpler than providing safety data.

Alternatively, adequate data may be available in the scientific literature to qualify an impurity. If neither is the case, additional safety testing should be considered. The studies desired to qualify an impurity will depend on a number of factors, including the patient population, daily dose, and route and duration of drug administration. Such studies are normally conducted on the new drug substance containing the impurities to be controlled, although studies using isolated impurities are acceptable.

1. 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 an acceptable minimum screen.
2. 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.

2.4.8 New Impurities

During the course of a drug development program, the qualitative impurity profile of the new drug substance may change, or a new impurity may appear as a result of synthetic route changes, process optimization, scale-up, etc. New impurities may be identified or unidentified. Such changes call for consideration of the need for qualification of the level of the impurity unless it is below the threshold values as noted above. When a new impurity exceeds the threshold, the decision chart should be consulted. Safety studies

DECISION CHART FOR SAFETY STUDIES

| Decrease impurity level below threshold | YES← | Above threshold? | →NO | Qualified |

↓YES

NO — Structure elucidated?

↓YES

YES — Toxicity documented and adequate?

↓NO

| Related to others with known toxicity? | YES → | Justifiable? |

↓NO   ↙NO   YES↓

Patient population, duration of use   Qualified

↓

Consider need for: 1. Genotoxicity studies
2. General toxicity studies
3. Appropriate specific toxicity

↓

Adverse effects

↙YES   ↘NO

Test further or remove impurity   Qualified

should compare the new drug substance containing a representative level of the new impurity with previously qualified material, although studies using the isolated impurity are also acceptable (these studies may not always have clinical relevance).

2.5 GLOBAL CMC DOSSIER

Based on discussions at a workshop entitled Designing the Global
Chemistry, Manufacturing, and Control (CMC) Dossier, a proposal
was developed that includes the following pertinent points relating
to the discussion of this chapter (7):

- Information on chirality is included.
- Purification/drying conditions are provided.
- Alternate methods of manufacture are given.
- Reprocessing is described.
- In-process controls performed at various stages are described.
- Quality control of raw materials includes specifications and test
methods.
- Specifications and test methods are given for pivotal and key/
critical intermediates.
- Tabulated summary of impurities and their qualifications is
provided:
     a. Potential impurities originating from synthesis, e.g., by-
     products, intermediates, reagents, solvents.
     b. Analytical test procedures and their limits of detection.
     c. Impurities and structural deviants found.

Similarly a host of tests should be performed on the drug prod-
uct, including qualification of degradation products. It should also
be mentioned here that there are stringent regulations and require-
ments on impurities arising out of stability studies. These are dis-
cussed in Chapter 8.

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3
Isolation and Characterization

It is frequently necessary to isolate and characterize impurities in order to monitor them accurately, because approximate estimations of impurities are generally made against the material of interest (i.e., drug substance) and can be incorrect. These estimations are based on the assumption that impurities are structurally related to the material of interest and thus have the same detector response. It is important to test this assumption because impurities frequently have different structures with significantly different detector responses (1). Most of the time it is difficult to ensure that the assumption stated above is correct without first isolating and characterizing the impurity in question.

Another approach that might help get around the isolation difficulties entails utilization of hyphenated methods with mass spectrometry. For example, GC-MS or LC-MS can be used to evaluate impurities (see Chapters 4 and 9). Evaluation of this type can provide structural information, thus minimizing the risks arising out of gross assumptions of equality. However, to gain greater assurance of structure, it is important to have other spectroscopic information, such as NMR and possibly IR, on the impurity. These tests can also be run as hyphenated methods along with the chromatographic methods. However, it may prove to be much more cumbersome and time consuming to develop

appropriate experimental parameters to run all these tests, as opposed to isolating and characterizing the impurities.

The discussion above makes a legitimate case for isolation and characterization of impurities most of the time. Furthermore, once an impurity is available, it easier to conduct day-to-day evaluations whenever or wherever they are necessary. It also allows for performance of toxicology studies, if they are deemed desirable.

A number of methods can be used for isolating and characterizing impurities. The application of any given method depends on the nature of the impurity, i.e., its structure, physicochemical properties, and availability (the amount present in the original material from which it must be isolated). The following methods may be useful in this context.

- Extraction
- Column Chromatography
- Preparative Separations

These methods are discussed below.

3.1 EXTRACTION

Various types of extraction procedures can be used to isolate impurities. For the purpose of this discussion, these procedures can be categorized as follows:

- Liquid/Solid Extraction
- Liquid/Liquid Extraction

3.1.1 Liquid/Solid Extraction

In its simplest form, a solvent is selected that would dissolve the impurity of interest but not the solid matrix. For example, if we want to determine salt in sand, we would simply use water to dissolve it and filter the solution, which on evaporation will produce salt in a reasonably pure form. If, on the other hand, other

water-soluble impurities were present in the sand, then it would be necessary to select a different solvent or it would be necessary to further manipulate the solution (e.g., see the discussion in Section 3.1.2). We must acknowledge that it is not that simple when one is working with impurities in pharmaceutical compounds. Here it is almost always desirable to use an organic solvent for extraction because of its unique properties. Furthermore, it is generally easier to volatilize the organic solvent at low temperatures in order to concentrate the impurity. This provides ease of operation. The selection of solvents can be made from Table 3.1, which lists various solvents on the basis of their boiling points and polarity (dielectric constant).

Table 3.1   Boiling Points and Polarity of Selected Solvents

SOLVENT	BOILING POINT (°C)	DIELECTRIC CONSTANT
n-Hexane	190	1.9
Cyclohexane	81	2.0
Carbon tetrachloride	77	2.2
Toluene	110	2.4
Ethyl ether	35	4.3
Chloroform	61	4.8
Methylene chloride	40	8.9
Ethanol	78	24.6
Methanol	65	32.7
Dimethylformamide	153	36.7
Acetonitrile	82	37.5
Water	100	80
Formamide	210	111

The solvents in Table 3.1 have been arranged in order of increasing polarity. The selection process for an appropriate solvent is simplified if we remember "like dissolves like," i.e., if we want to solubilize a polar compound, it is best to select a polar solvent and vice versa for nonpolar compounds. At times, other solvent

effects must also be considered (the interested reader may wish to review a handy textbook of physical chemistry).

Soxhlet Extraction: Soxhlet extraction is a popular method for extracting compounds of interest from solids, e.g., natural products, by repeated extraction with the choice solvent. The main advantage of this method is that it allows utilization of a small volume of solvent to produce a fairly concentrated extract. The material to be extracted is placed in the Soxhlet extractor, which is secured on top of the extraction vessel. The extraction vessel is heated adequately to ensure volatilization of solvent vapors, which are condensed on the top of the material to be extracted. The condensed solvent percolates through the material and drains back into the extraction vessel to repeat the process.

Steam Distillation: Steam distillation is yet another method that can be used for extracting volatile components from natural materials and other matrixes of interest. In this case steam is allowed to percolate through the natural material, leading to extraction of volatile materials, such as oils, which then remain afloat on top of the condensed aqueous layer and can thus be collected in a relatively pure state.

Supercritical Fluid Extraction: Supercritical fluid extraction (SFE) provides idealized means of extracting materials and is a technique of choice today. Since high solute diffusivity, lower viscosity, and excellent solvating properties can be obtained with supercritical fluids, they provide excellent means of isolating impurities and other compounds of interest in a short period of time. The critical pressure, critical temperature, and density of a few compounds used for SFE are given in Table 3.2.

Table 3.2 Solvents for SFE

SOLVENT	PRESSURE (ATM.)	TEMPERATURE (°C)	DENSITY (g/ML)
n-Pentane	33.3	196.6	0.232
CO_2	72.9		0.448
NH_3	111.3	132.3	0.24

Carbon dioxide is most commonly used for SFE because of its availability, ease of use, and disposition.

3.1.2 Liquid/Liquid Extraction

This simply entails extraction of one liquid with another. Generally one of those liquids is aqueous and the other is organic. The primary requirement is that these liquids be immiscible. This procedure is very useful when the liquid into which the material of interest is being extracted is easy to volatilize, thus permitting concentration of the material. Hence the choice of solvents must be made with that consideration in mind.

In this type of extraction process, a solute is distributed between two immiscible solvents. The extraction is controlled by distribution or partition coefficient which defines the ratio of concentration of the solute in two solvents, a and b:

$$K_d = C_a / C_b \qquad\qquad [Eq.\ 3.1]$$

where K_d is the distribution coefficient or partition coefficient. This coefficient is related to the relative solubilities of the solute in two solvents. When one solvent is water and the other is an organic solvent, the inorganic species as well as polar organic compounds are found largely in the aqueous phase, while nonpolar organic compounds are found mainly in the organic phase. The principle of "like dissolves like" holds here as well. The activities should be used in Equation 3.1 to obtain more precise values; however, in a dilute solution, to a first approximation, the distribution coefficient is independent of concentration. The organic phase is generally placed in the numerator; however, it is not uncommon to place the lighter phase, which may or may not be organic, in the numerator. This clearly shows that assignment of phases is arbitrary and should be explicitly stated to avoid any ambiguity in the stated value of the distribution coefficient.

The distribution coefficient relates to a single species and does not include possible products of side reactions. A good example to

consider is the extraction of benzoic acid from the acidic aqueous solution (w) into ether (e), an organic solvent (2). Since the dissociation of benzoic acid is suppressed in the acidified solution, the distribution coefficient is expressed by the following equation:

$$K_d = [HA]_e / [HA]_w \qquad \text{[Eq. 3.2]}$$

This relationship becomes more complicated if the aqueous layer is not acidified and benzoic acid dissociates:

$$HA \leftrightarrow H^+ + A^- \qquad \text{[Eq. 3.3]}$$

$$K_w = [H^+][A^-]/[HA] \qquad \text{[Eq. 3.4]}$$

It is a good idea to remember that the partition equilibrium relates only to the undissociated benzoic acid molecules in two phases and dissociation equilibrium relates only to the species in the aqueous phase. Other complications can arise where a species such as benzoic acid can dimerize partially in an extraction solvent such as benzene.

When extracting benzoic acid, we primarily want to know how much benzoic acid, regardless of its form, is extracted into each phase. Distribution ratio is an expression that takes all forms into account:

$$D = \frac{\text{Total concentration of benzoic acid in organic phase}}{\text{Total concentration of benzoic acid in aqueous phase}} \qquad \text{[Eq.3.5]}$$

$$D = \frac{[HA]_o + 2[HA \cdot HA]_o}{[HA]_w + [A-]_w} \qquad \text{[Eq. 3.6]}$$

We must remember that separation depends ultimately on differences in the K values of individual sample components. The larger differences allow for easier separations. We can make use of secondary chemical equilibrium to facilitate separation in a system based on either phase or distribution equilibria, but the most im-

portant applications of secondary chemical equilibria is in distribution systems.

For the pharmaceutical analysts, the extraction of acids and bases provide some of the best examples on the use of chemical equilibria to facilitate distribution separations. The conversion of an organic acid or base into a water-soluble salt permits its aqueous extraction from a water-immiscible organic phase. Also, control of the pH of the aqueous phase permits the separation of strong acids or bases from weaker acids or bases.

To illustrate the effect of pH, let us look at the simple case of extraction of an organic acid, HA, from aqueous, w, to an organic solvent, o:

$$(HA)_w \leftrightarrow (HA)_o \qquad \text{[Eq.3.7]}$$
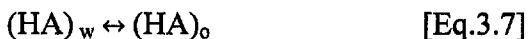
The dissociation of the acid can be represented as follows in the aqueous phase:

$$(HA)_w + H_2O \rightarrow (H_3O^+)_w + (A^-)_w \qquad \text{[Eq. 3.8]}$$
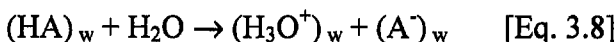
The distribution constant K can be defined as:

$$K = \frac{[HA]_o}{[HA]_w} \qquad \text{[Eq. 3.9]}$$

and the acid dissociation constant from Equation 3.8 as:

$$K_a = \frac{[H_3O^+]_w\,[A^-]_w}{[HA]_w} \qquad \text{[Eq. 3.10]}$$

Since in a dilute solution K is independent of the initial concentration of HA in water, we can calculate D:

$$D = \frac{[HA]_o}{[HA^+]_w + [A^-]_w} \qquad \text{[Eq. 3.11]}$$

Substitution of Equations 3.9 and 3.10 into Eq. 3.11 gives

$$D = \frac{K \quad [H_3O^+]}{[H_3O^+] + K_a} \qquad [Eq. 3.12]$$

Equation 3.12 can be very useful in understanding the disibution of the given acid over the whole pH range in water:

If $[H_3O^+]$ >>Ka, then D = K from Equation 3.12. This is true because the solution is sufficiently acidic so that no ionization occurs in the aqueous phase and HA acts as a simple neutral species, which is distributed between the two phases.

If $[H_3O^+]$<<K_w, then D<<K as a result, and little if any, HA will be extracted. The concentration of HA in the aqueous phase is very low because of the ionization of the acid and hence the amount extracted is very small.

When the pH of the solution is the same as pKa of the acid, i.e., $[H_3O^+]$ = Ka, then D = K/2 and only half of the material would be extracted as compared to the acidic solution discussed above. It is well known that when the pH = pKa, the acid is only half dissociated.

The distribution of acids and bases can, therefore, be markedly influenced by controlling the pH. The larger the difference in their pKa values the easier it is to effectuate their extraction.

3.2 COLUMN CHROMATOGRAPHY

The forerunner of modern chromatography was the separation on an adsorption column that was eluted by letting the solvent flow down by gravity. Column chromatographic methods were commonly used for analytical determinations not very long ago. This technique is now primarily used for the separation of pharmaceutical compounds in preparative chemistry. Depending on the size of the column, the technique can be used for the separation of quantities ranging from micrograms to kilograms.

Detection of the eluent is generally performed by UV spectrophotometry because of the ease of monitoring effluent, either

continuously by using a flow cell or periodically by monitoring the collected fractions from a given sample that alerts the emergence of UV-active components. A large number of publications are available in this area. The reader may find the books by Deyl et al and Mikes useful because they provide abundant examples from the pharmaceutical field (3,4).

Column chromatographic separations are carried out on glass columns of various lengths with diameters upwards of 1 cm. The columns can be packed with almost any stationary phase of choice, ranging from silica gel (or alumina) as used in classic adsorption chromatography through ion exchange resins to chemically modified polydextran gels used primarily for the analysis of biological samples (5). Of course, liquid-liquid partition chromatography columns, widely used for pharmaceutical analysis, can also be used. For this technique, an inert carrier such as Celite or kieselguhr is impregnated with an aqueous buffer or another polar solvent such as dimethyl formamide or dimethyl sulfoxide and elution is carried out with nonpolar solvents. At times, ion pair reagents are also used. The choice of eluents is determined by the detection technique; acetone, benzene, or other UV-absorbing solvents are generally not used. Alternative means of detection can help circumvent this problem. Two examples of column separations are provided.

Testosterone esters are separated from free testosterone, its decomposition product, by the use of a column chromatographic method (6). The tablets containing the testosterone esters are extracted with methanol. The solvent is evaporated to dryness, and the residue is treated with heptane, followed by acetonitrile. The two solutions are mixed with Celite, and the resulting slurry is introduced at the top of the column. Elution is performed with heptane. The first fraction, representing 24% of the eluent volume, is utilized for collecting testosterone esters, and the second fraction, representing the rest of eluent, is used to collect the decomposition product. Monitoring by UV can be performed at 240 nm.

The separation of components of two complex pharmaceutical preparations represents the other example (7). Separation is carried out on a column packed with kieselguhr impregnated with a phosphate buffer. The differences in retention of components with different basicities is utilized effectively to achieve this separation on a column prepared with the buffer at the optimum pH. The optimum pH of the buffer is 6.0 for the combination of antazoline (pKa = 10.1) and naphazoline (pK = 0.9) and is 6.7 for the combination of ephedrine (pK = 9.9) and carbinoxamine (pKa = 8.1). In the former combination, antazoline is eluted quantitatively in a reasonable volume of chloroform, whereas naphazoline is retained by the column. Similarly, carbinoxamine can be eluted with chloroform from the second combination, leaving ephedrine on the column. The retained components can be eluted with 0.1 M hydrochloric acid and can be monitored directly by UV analysis.

Thin-layer Chromatography (TLC): This is a valuable technique for isolation and purification of compounds because of its simplicity. No major equipment is required and method development is relatively easy. All modes of chromatography including adsorption, partition, ion exchange, and gel filtration can be utilized. Ion pair separations, ligand exchange, and chiral separations can also be performed by thin-layer chromatography. The primary limitation is the small number of theoretical plates that are obtained with this method as compared to gas chromatography or high pressure liquid chromatography. However, this limitation can be easily offset by multiple developments. A number of books provide very useful information on this technique (8,9).

In addition to judiciously choosing a sorbent and an eluent for performing TLC, it is necessary to select a suitable method for applying a sample to the plate. Silica gel plates with or without fluorescent indicator are frequently used for most applications. The eluent is generally composed of a mixture of two solvents selected from the elutropic series (see Table 3.1) that may contain an acid or base to provide an appropriate pH. The sample is applied to a minimum surface area in an appropriate volatile solvent. This can be done in the form of circular spots or as strips. The latter

approach allows larger applications of sample on the plate without adversely affecting the separation.

Detection is frequently performed visually or by UV (e.g., 366 nm). The fluorescence-quenching substances absorbing UV light in the short-wavelength region can also be detected if the layer is impregnated with a fluorescent substance. Iodine vapors can help detect most organic substances.

A number of techniques can be utilized to elute the material from the plates. The simplest method is scraping the sorbent containing the material of interest and transfering it to an appropriate extraction vessel, where it is extracted with a suitable solvent. Following filtration or centrifugation, the solvent is removed to collect the desired substance. Vacuum cleaner type of glass devices are also available. If aluminum rather than glass plates are used for TLC, the sample zone can be cut and eluted. Devices are also available such as Eluchrome, which allow direct extraction of zones from the plate without removal from the support.

TLC has been used for monitoring impurities of imipramine and desipramine (10). Silica gel G plates with 254-nm fluorescent indicators are used with the following solvent systems:

- Imipramine: Benzene-ethyl acetate-absolute ethanol-concentrated ammonium hydroxide (50:50:15:3)
- Desipramine: Benzene-ethyl acetate-absolute ethanol concentrated ammonium hydroxide (50:50:20:1)

The distance traveled by solute/distance traveled by solvent and minimum detectability of the impurities are given in Table 3.3.

Table 3.3   TLC of Imipramine and Desipramine (10)

COMPOUND	Rf	MINIMUM DETECTABILITY (MICROGRAMS)
Iminodibenzyl	0.81	0.06
Imipramine	0.67	0.03
Unknown	0.26	0.03
Desipramine	0.36	0.03

TLC allowed characterization of the unknown impurity, and the following structure was determined:
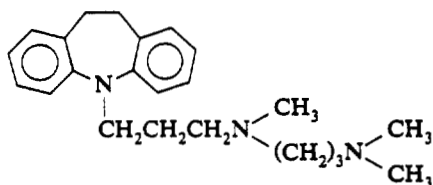
Another interesting study relates to characterization of various new degradation products produced in the matrixes used for pharmaceutical products (11). The new compounds thus produced are resolved by TLC, and their structure is determined by appropriate techniques such as elemental analysis, IR, NMR, or mass spectrometry.

The case history described here deals with pharmaceutical products that contained prednisone, phenylbutazone, and some antacid excipients. On accelerated studies at 40°C, high assay values were obtained for prednisone by the triphenyltetrazolium chloride (TPTZ) colorimetric method. The assay values almost tripled the normal value of 1.25 mg/capsule in some cases after 36 months' storage at 40°C (11). This was a serendipitous discovery in that it clearly indicated that some compound(s) in the formulation was degrading to yield a product that reacts with TPTZ. The reagents, such as TPTZ or BT (blue tetrazolium), provide a stability-indicating method for steroids containing an alpha ketol group. The method is based on quantitative reduction of colorless tetrazolium salts to colored formazans by the alpha ketol group in the presence of a base such as tetramethylammonium hydroxide.

The samples stored under accelerated conditions were analyzed by TLC on silica gel G plates with the cyclohexane-chloroform-acetic acid (40:50:10) system. When the plate was sprayed with potassium dichromate/sulfuric acid reagent, the presence of four impurities (II, III, IV, and VI) was revealed (Scheme I). However, none of these impurities produced color with BT reagent. A number of compounds such as hydrazobenzene, azobenzene, and
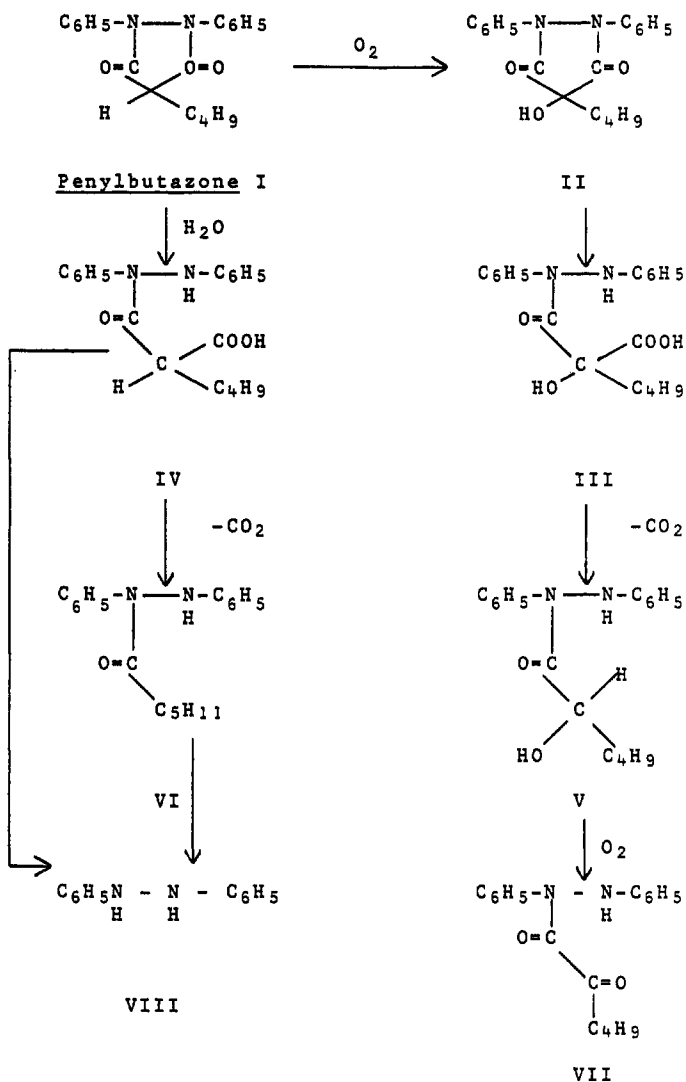
Scheme I. Phenylbutazone degradation.

caproic acid can be theorized as potential degradation products under extreme conditions. Of these compounds, only hydrazobenzene exhibits reactivity with BT.

A blue tetrazolium spray reagent was developed (12), wherein 0.35 % BT solubilized in 95% ethanol is mixed in a 2:1 ratio with 5N aqueous sodium hydroxide to allow direct detection of BT-reacting impurities on a TLC plate. The TLC plate was sprayed with the new BT reagent after development, as described above. A compound with the same Rf value as compound VI was found with BT activity. Since hydrazobenzene has a different Rf value from compound VI, it appears that a new compound(s) has been found, which might be responsible for the high assay values observed for prednisone.

The spot that exhibits high BT activity was isolated by preparative TLC to confirm the observation described above. Care was taken in this case not to spray the components that were to be isolated by the spray reagent. Their location was fixed by spraying a strip on the edge of the plate. The infrared spectrum of the isolated materials did not match with compound VI. A structure was postulated, based on the IR spectrum (see compound V in Scheme I). The structure was confirmed by comparison of various physicochemical data with the authentic compound (13).

The BT activity of compound V is not sufficiently high to explain the cause of this problem. This suggests that the compound responsible for the increased assay value was not being resolved by the TLC system. Thus a new TLC system was developed where acetic acid was replaced with ammonium hydroxide. This resulted in a two-phase mixture—the organic phase saturated with ammonia was used for development. It provides excellent separation of the desired components (see Table 3.4).

The unknown (SA 484-82) was isolated by preparative TLC. It has a melting point of 113°C. The infrared spectrum indicates the presence of two carbonyl groups (1658 cm-1 and 1712 cm-1) and NH function (330 cm-1). The elemental analysis agrees with the empirical formula $C_{18} H_{20} N_2O_2$. Based on NMR and MS, a

structure was assigned to this compound (see compound VII in Scheme I).

Table 3.4   Resolution of Impurities of Interest of Phenylbutazone

COMPOUND	Rf
I, II, III, IV	0.0
V	0.1
Unknown	0.3
VI	0.4

Gas Chromatography: This technique can be very useful for isolation and characterization of volatile components or those components that can be made volatile by derivatization techniques. The latter approach is less useful, considering it is necessary to reverse the process to obtain the original material.

Another point to remember is that the detector used should be nondestructive, i.e., the commonly used flame ionization detector can not be used directly. Splitting the gas stream could allow utilization of this detector. Alternatively, nondestructive detectors such as a thermal conductivity detector can be used. The volatilized component of interest is collected in a suitably cooled trap.

Gas chromatography is more apt to be used in combination with mass spectrometry (GC/MS) for characterization of impurities. Other techniques such as TLC, SFE, or HPLC (discussed below) are better suited than GC for isolation and characterization of nonvolatile components

High Pressure Liquid Chromatography: Specialized instruments are used to carry out HPLC in the preparative mode. However, analytical high pressure liquid chromatographs can also be used for collecting relatively small amounts (mg quantities) of material where repeated injections are made and the fraction or fractions of interest are collected. The specialized instruments have the following characteristics (14):

- Use standardized sample application
- Have small-particle columns
- Generate higher pressure than classical LC
- Monitor eluents continuously
- Collect fractions

For preparative separations, the phase selectivity, or alpha value, of a mixture to be separated is most important. If separation of all components is desired for complex mixtures, the smallest of all alpha values (α) will determine the operation. For example, based on a resolution equation, an α value of 1.2 requires approximately 830 theoretical plates for separation with k' = 4 and a resolution value of 1. This suggests that the sample size of the mixture must be adjusted so that the main component of the pair (with α = 1.2) produces a peak of N = 830. When the sample size increases, the plate numbers decrease rapidly and the peaks become asymmetric and the retention time generally shortens.

Preparative HPLC on Analytical Columns: This type of analytical column (5- to 30-cm long with an internal diameter [i.d.] of 1 to 6 mm) has a much greater capacity than the sample size normally injected on them (14). On a column containing 3 g of C-18 packing material, it should be possible to separate 30 mg of a mixture of compounds. The problem generally stems from the small volume of the flow cell used for the detector. This problem can be circumvented by collecting fractions in the area of overload. For example:

- Fractions 1 to 4 may contain the main compound.
- Fractions 7 to 10 may contain the unknown.
- Middle fractions (5 to 6) may contain the mixture.

An alternate way to achieve this separation would be to make repeated injections at the optimum load capacity and collect the separated fraction with automated fraction collection based on retention time. It is also possible to use long narrow columns or columns with slightly larger dimensions (50 × 0.7 cm).

Preparative Columns: The column dimensions are adapted to the sample size that must be separated. Dimensions can vary from 25×2 cm to 100×10 cm. For separation of pharmaceutical impurities, a 25×2.2-cm column filled with 10 μm of material is recommended (15).

Conventional HPLC instruments can be used. Most commercial HPLC pumps can pump solvent at the rate of 10-15 ml/minute at the optimal velocity for these columns. The same detectors can be used as in conventional HPLC.

The columns can be packed with a variety of materials to allow performance of various modes of chromatography. This means that separation can be converted to the preparatory mode by increasing the injected amount and volume. The use of 10-μm particles is a good compromise between efficiency and speed of analysis. Examples of preparative separations can be found in Chapter 9.

Size Exclusion Chromatography (SEC): Terms such as gel permeation chromatography (GPC) or gel filtration chromatography have been used for this technique. SEC separates molecules based on their size, and it is carried out with rigid materials that can withstand 100 bar pressure. The advantages of SEC are as follows:

- Separations of all soluble compounds can be easily checked because irreversible adsorption or thermal degradation is not a problem.
- Solvents are simple; those selected should suppress adsorption tendencies.
- Separations do not take a long time.
- Information on molecular weights is provided.

For separations based on molecular weights, this technique is highly recommended (see Chapter 9).

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4
Analytical Methodology

It is necessary to be very selective in the utilization of analytical methodology for monitoring impurities in pharmaceuticals, because the content of most individual impurities is likely to be low. A good way to determine which methods can be used is to apply the rule of 0.1%, i.e., the method should be able to reliably determine the impurity of interest at a 0.1% level. This rule is derived from the expectations of the FDA and a number of other regulatory authorities that any impurity present at a 0.1% level or higher should be characterized. It clearly demands that methods must be developed that can detect such impurities at least at 0.05% level to provide assurance for quantitation at the desired level. Ideally the method of choice would be the assay method that resolves all impurities of interest so that it is possible to determine the purity of the main component and simultaneously evaluate all impurities at the same time. Time saving of significant proportion can be made by utilization of this approach. Furthermore, it allows determination of mass balance, thus providing some hints as to whether or not impurities have responses similar to that of the main component.

The approach mentioned above is very useful; however, it is not always workable for a number of reasons:

1. The impurities may have significantly different
    physicochemical properties from those of the main component;

thus the selectivity criteria used for development of the method for the main component may not be useful for monitoring impurities.

2. The impurities may have a different response from the parent compound.

For example, if their response is low by a factor of two, then an impurity that is present at 0.1% level is likely to be only 0.05% based on the detector response and hence may go undetected.

If their response is higher, then detectability may not be a problem. However, the sample will appear to be more impure and the mass balance will be skewed. This will necessitate that quantitation of the impurity in question be made against the authentic standard or an appropriate response factor be determined (see discussion in Chapter 3).

If the impurity has no response or has been unresolved, then one would have the erroneous impression that the sample is more pure than it really is.

The importance of rational method development that covers a variety of possibilities is discussed below.

4.1 STRATEGY FOR METHOD DEVELOPMENT

Method development strategy should start with a close review of the chemical structure of the molecule and its synthesis. It is necessary to determine the penultimate intermediate, because of the potential of its being carried into the component of interest is great. This can be easily done by a quick review of synthesis. A point of caution: For example, if the compound of interest is a salt of the free base with an acid, then the free base is not considered penultimate intermediate. It is necessary to look at the previous step in synthesis to make that judgment. Potential by-products as well as solvent utilized for reaction or crystallization are potential impurities. Additionally, it should be considered whether the molecule has some functionalities that can be easily hydrolyzed in

acid or base. It is also important to know if the molecule is oxidized (see Chapter 7).

The functional groups can also provide valuable clues to the pKa of the molecule and which method(s) may be useful for analyzing it. The pKa in turn can help select the optimum pH for extraction and provide indication of solubility in acidic or basic solutions. The solubility in aqueous or organic solvents may be further indicated by the polarity of the molecule--remembering that like dissolves like. Given below is some of the physicochemical data that should be generated on the pharmaceutical compounds to help develop optimum methods.

4.2 PHYSICOCHEMICAL DATA

For the discussion in this chapter, we will use as a model compound, baclofen, a synthetic antispastic agent that is sold commercially as Lioresal (1,2). It is a white to off-white crystalline powder and has the following structure:

$$Cl - \underset{}{\bigcirc} - \underset{\underset{CH_2NH_2}{|}}{CHCH_2COOH}$$
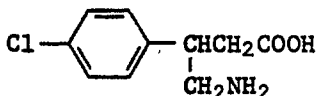
Baclofen

4.2.1 Ionization Constant

It is important to determine the ionization constant of the molecule of interest. The following general discussion may be useful in this context. The dissociation of a molecule, e.g., acetic acid in water, is given by the following expression:

$$HAc + H_2O \leftrightarrow H_3O^+ + Ac^-   \text{[Eq. 4.1]}$$
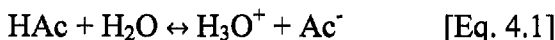
The dissociation constant (K_a) is then given by the following expression:

$$K_a = \frac{[H_3O^+][Ac^-]}{[HAc]} \qquad [Eq.\ 4.2]$$

In dilute solutions of acetic acid, water is in sufficient excess to be regarded as constant at 55.3 moles/liter. The term dissociation is vague in that it includes a vast field of dissociation phenomena. Our primary interest in pharmaceutical analysis is the ionization constants, i.e., those constants which are used to measure the strength of acids and bases. These terms are interchangeable in the pharmaceutical field; however, it is important to recognize the difference. It is very desirable to determine the ionization constant(s) for the compound of interest.

In the pharmaceutical field, we often talk in terms of pKa of a compound, where pKa is -log K_a . It is possible to determine the pKa for bases as well, using the following expression:

$$pKa + pK_b = pK_w \qquad [Eq.\ 4.3]$$

where pK_w = -log of K_w , the auto protolysis constant for water that has a calculated value of 1×10^{-14} at 25°C.

At times, more than one dissociation constant is encountered, depending upon the number and type of functional groups that are present. For example, citric acid, a commonly used ingredient in the pharmaceutical field, has pKa values of 3.15, 4.78, and 6.40.

A number of methods can be used for the determination of pKa values. They range from a simple titration to sophisticated spectroscopic methods such as NMR. The use of simple methods such as potentiometric titration, conductimetry, UV, or solubility are highly recommended. For our model compound, baclofen, the following two values have been reported at 20°C:

$$pK_{a1} = 3.87 + 0.1 \text{ (carboxyl group)}$$
$$pK_{a2} = 9.62 + 0.1 \text{ (amino group)}$$

The pKa values can also be deduced from the published values on related structures.

4.2.2 Solubility

The equilibrium solubility in various solvents commonly used for analysis should be determined. This may be a good time to also determine the solubility values for the purpose of formulation evaluation or dissolution testing. Table 4.1 illustrates baclofen's solubility.

Table 4.1. Solubility of Baclofen

SOLVENT	TEMPERATURE (°C)	mg/ml
Water (pH 7.6)	23	4.3
Methanol	RT	0.045
Ethanol	RT	0.024
Chloroform	RT	0.014
Dimethylformamide	RT	0.008
Acetonitrile	RT	0.004
Phosphate buffer (pH 7.4)	RT	5.0
0.1N HCl	RT	>20
0.1N NaOH	RT	>20

The poor solubility in organic solvents, combined with low solubility in water, adds to the complexity of method development for this compound. High solubility in acidic or basic solvents emphasizes the amphoteric nature of this molecule.

4.2.3 Water Absorption

The information on water absorption is useful in that it reflects the hygroscopicity of the material of interest. The materials that are hygroscopic need to be protected from moisture; otherwise they are susceptible to hydrolytic reactions.

The uptake of water by baclofen at various relative humidities at 40°C, as determined by the Karl Fischer method, is given in Figure 4.1 on the next page. A review of the figure shows that baclofen is not unduly hygroscopic.

4.2.4 Distribution Coefficient

It is desirable to determine the distribution coefficients in various organic phases versus aqueous medium at some defined temperature. Such data is provided for baclofen in Table 4.2 at pH 7.4 (4).
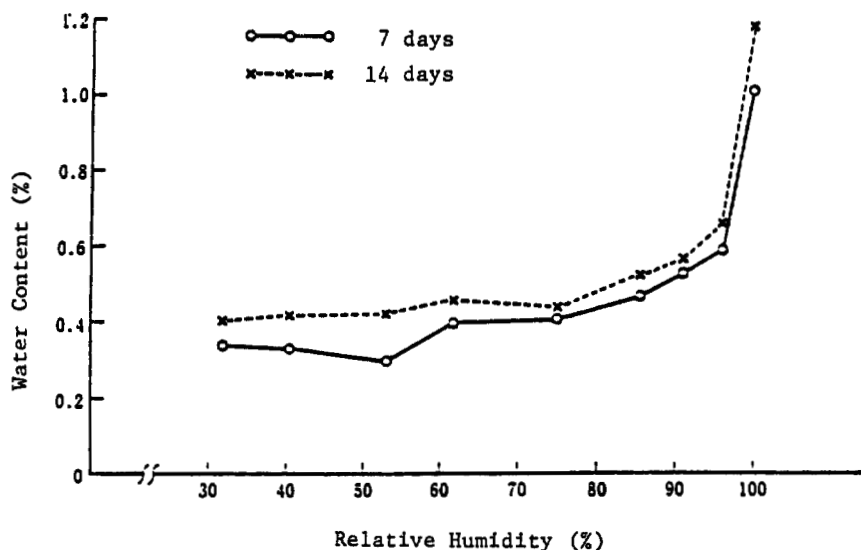
Figure 4.1 Water absorption isotherm of baclofen (3).

Table 4.2 Distribution Coefficients* of Baclofen

ORGANIC PHASE	AQUEOUS PHASE	TEMPERATURE (°C)	$k = C_{org}/C_{aq}$
Butanol	pH 7.4	25	0.67
Octanol	pH 7.4	23	0.11
Methylene chloride	pH 7.4	23	0.003
Ether	pH 7.4	20	0.003
Benzene	pH 7.4	23	0.003
Hexane	pH 7.4	23	0

*The aqueous phase is phosphate buffer.

The data in Table 4.2 suggest that hexane would be the best solvent for extracting any nonpolar impurities that may be present in baclofen. And octanol and butanol may be the solvents of choice to extract baclofen. It is also desirable to generate such data at an acidic and basic pH to provide some indication as to which pH is likely to be more suitable for extraction of the main component from impurities. Based on the pKa value, it is possible to estimate where the main component is not likely to extract into the organic phase: The bases do not extract at pH two units below the pKa value and the acids cannot be extracted at two units above the pKa.

4.2.5 Optical Rotation

It is important to determine whether the molecule of interest is chiral, i.e., does it have one or more asymmetric center? If it is chiral, it is necessary to determine whether the sample at hand is racemic. It should show no optical rotation. On the other hand, if it is racemic, it would be still desirable to show that all enantiomers can be resolved by the selected method and that they are appropriately controlled. Baclofen is sold as a racemate.

4.2.6 Crystal Form

It may be desirable to have information on crystal forms, as various crystal forms can show different solubilities. This type of information can be obtained by running x-ray diffraction or differential scanning calorimetry. No unusual solubility behavior has been attributed to the crystal form of baclofen.

4.3 SPECTROSCOPY

4.3.1 Ultraviolet Spectroscopy

It is desirable to obtain a UV spectrum in a solvent that has a window in the region of λmax, because this wavelength would be

most desirable for the purpose of quantitation if we were to use a UV detector for analysis. It is also desirable at this stage to obtain a UV spectrum in aqueous solution, if possible. If the compound is not soluble in water, a small percentage of methanol or other solvents that are commonly used in HPLC may be used. Furthermore, it is necessary to evaluate the effect of acid or base on the UV spectrum.
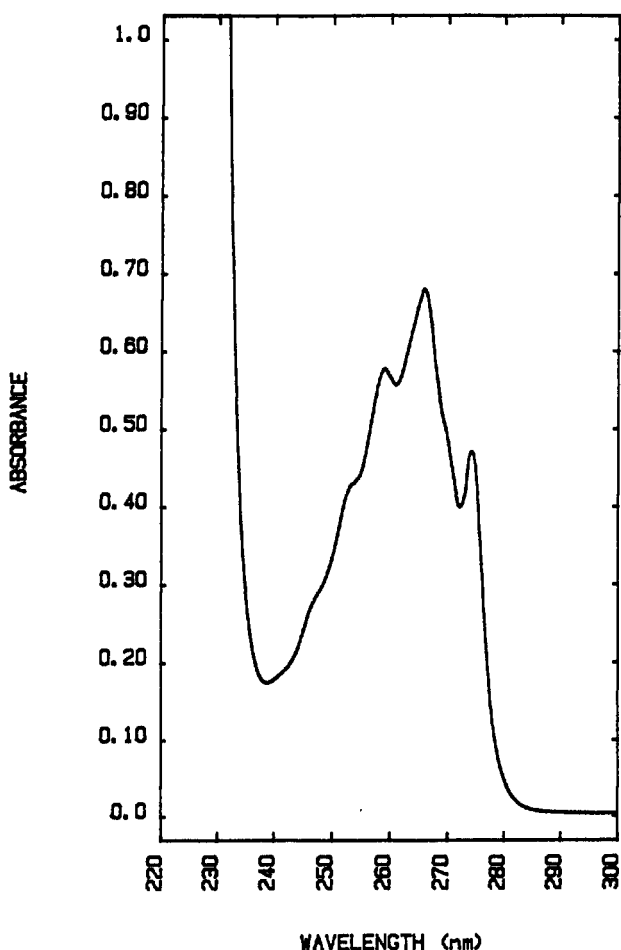
Figure 4.2 UV absorption spectrum of baclofen in 0.1N HCl.

In the case of our model compound, baclofen, the UV absorption spectrum exhibits characteristic aromatic absorption in the region of 258 to 274 nm (5). The bands arise from $\pi \rightarrow \pi'$ transition of the electrons in the phenyl ring. The ultraviolet spectrum in 0.1 N HCl (Figure 4.2) is comparable to that of the aromatic amino acids. It has the following A values (1%, 1 cm) at the indicated λ max:

λ max	A (1%, 1 cm)
274 nm	7.9
266 nm	11.3
258 nm	9.6

The above data clearly suggests that the absorptivity of baclofen is very low, thus placing a premium on method development, especially if it is necessary to utilize UV to monitor it.

4.3.2 Infrared Spectroscopy

It is desirable to run infrared (IR) spectra, both as a mull in Nujol and as a disc in KBr. This allows for detection of any unusual bands that may be attributable to impurities. Also, KBr spectra, at times, do show variations in crystal forms. The infrared spectrum of baclofen (Figure 4.3) shows the following bands, which are consistent with the structure (Table 4.3):

Table 4.3 Infrared Spectrum Assignments for Baclofen

WAVENUMBER (cm^{-1})	ASSIGNMENT
2650, 2180	$-NH_2$*
1610, 1580	$-NH_2$*, disubstituted benzene
1530	-COOH*
1400	-COOH*
1100	-C-Cl
1020, 830	disubstituted benzene

* Probably occurs as zwitterion.
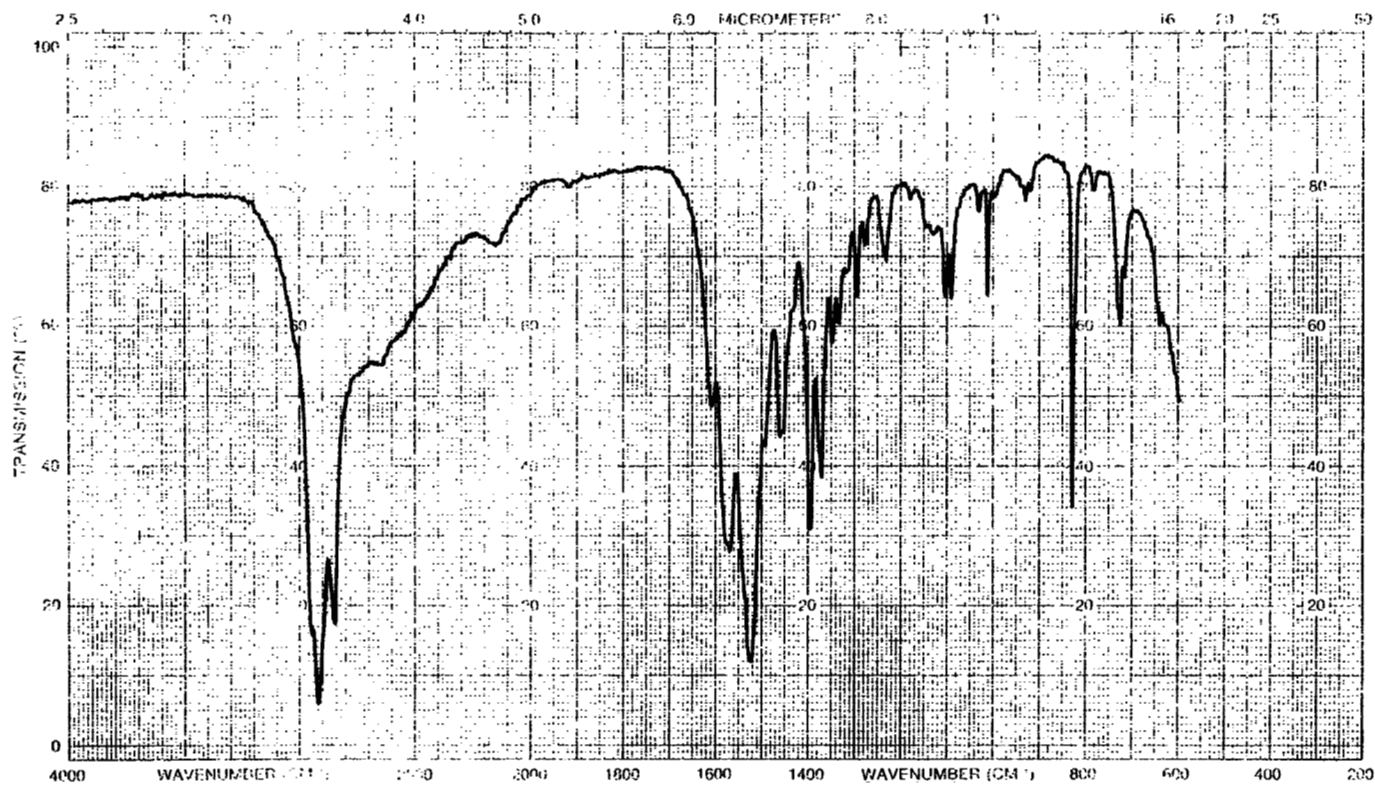
Figure 4.3 Infrared absorption spectrum of baclofen in Nujol.

IR data provides indications as to which wavelength may be usable for quantitation. For example, in the case of baclofen, it would be desirable to monitor NH_2 or COOH groups because that is where the change is likely to occur.

4.3.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectra are generally run for protons. The NMR spectrum (Figure 4.4) of baclofen in deuterated trifluoroacetic acid is consistent with its structure as shown in Table 4.4.

Table 4.4 NMR Assignments for Baclofen

CHEMICAL SHIFT	MULTI-PLICITY	RELATIVE INTEGRAL OF PROTONS	ASSIGNMENT
2.7 to 3.2	Multiplet	2	$-CH-CH_2-COOH$
3.2 to 3.9	Multiplet	3	$-CH-CH_2--NH_2$
6.8 to 7.7	Quartet	--	Disubstituted benzene and exchangeable protons
10.9	Singlet		Exchangeable protons

Sometimes unknown impurities show signals in the NMR spectrum. If this is the case, then a decision must be made as to how the unknown impurity is to be monitored. It is entirely possible that the chromatographic method that has been selected for monitoring impurities would be adequate. If this is not the situation, then, depending upon the content of the impurity in question, either the NMR method can be used or the impurity must be sufficiently characterized to suggest some alternate means of quantitation.
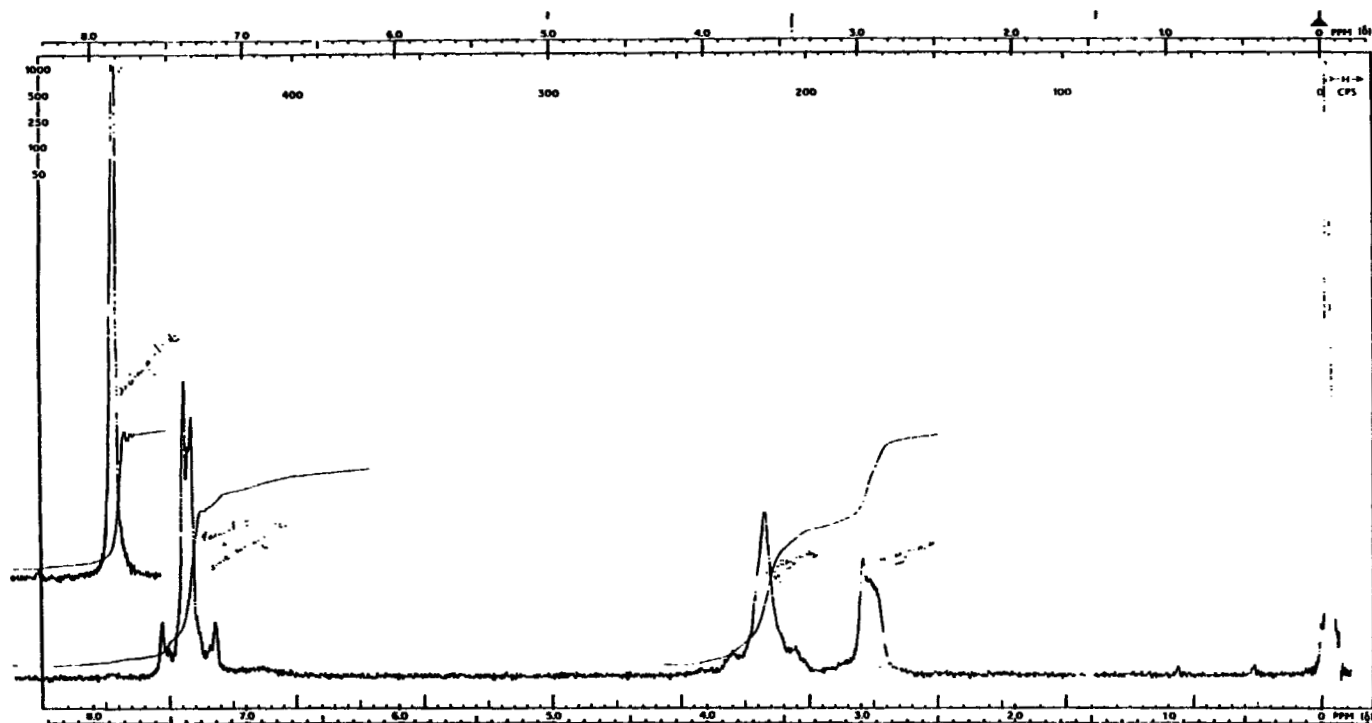
Figure 4.4 NMR spectrum of baclofen in trifluoroacetic acid.
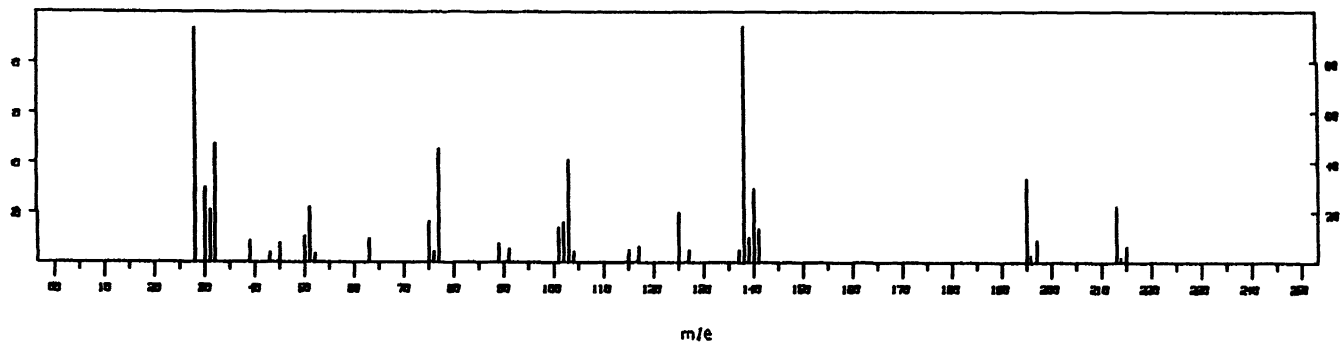
Figure 4.5 Mass spectrum of baclofen.

4.3.4 Mass Spectrometry

Mass spectrometry provides very useful information because it not only allows confirmation of the structure of the parent compound, but it can also indicate the presence of impurities. The mass spectrum of baclofen (Figure 4.5 on previous page) shows a fragmentation pattern, which is compatible with its structure (Table 4.5).

Table 4.5 Mass Spectrometry Assignments for Baclofen

m/e	STRUCTURE
213 195	
138	
103	 138 minus Cl
77	 minus H

Mass fragmentation information is very useful in designing hyphenated methods with MS.

4.4 PRELIMINARY EVALUATION OF IMPURITIES

As mentioned before, the preliminary evaluation of impurities should start from a close review of the synthesis. The penultimate intermediate has the greatest potential to be one of the impurities. In addition, any by-products produced at this stage must be carefully considered. For example, the last step of baclofen synthesis can be carried out in different ways. One process utilizes the reaction between β-(p-chlorophenyl)-glutarimide and bromine in an excess of sodium hydroxide at low temperature. An alternate process is carried out in sodium hydroxide-sodium hypochlorite solution at room temperature:

$$
\underset{\text{Glutarimide}}{Cl-\!\!\!\bigcirc\!\!\!-CH\!\!<\!\!\overset{CH_2-CO}{\underset{CH_2-CO}{}}\!\!>\!\!NH} \xrightarrow[\text{(2) HCl}]{\text{(1) NaOCl}} \underset{\text{Baclofen}}{Cl-\!\!\!\bigcirc\!\!\!-CH\!\!<\!\!\overset{CH_2COOH}{\underset{CH_2NH_2}{}}}
$$
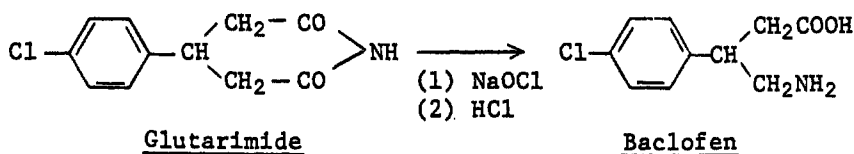
The obvious impurity to be evaluated here is glutarimide. Furthermore, it is conceivable that it could hydrolyze and produce p-chlorophenyl glutaric acid as a potential impurity. It would also be desirable at this stage to review what solvents are being used in the final step, or for crystallization, of the active ingredient because these could be carried as impurities in the material of interest.

4.5 DEGRADATION STUDIES

To monitor impurities, it is essential to decide what type of impurities may be produced by degradation in solution or solid state or when the parent compound is subjected to stress conditions.

4.5.1 Solid-State Stability

Most pharmaceutical compounds are stable in the solid state at ambient temperature. However, as mentioned previously, it is necessary to be vigilant if the material is hygroscopic, because

when that is the case, it is susceptible to hydrolysis as well as oxidation, to name just two potential degradation reactions. In addition, all pharmaceutical compounds should be subjected to high temperature to determine what sort of degradation products may be produced. For example, baclofen is stable under normal storage conditions. Practically no change is observed for the samples stored for 12 months at 35°C. However, at 50°C, formation of the corresponding lactam [4-(4-chlorophenyl)-2-pyrrolidone] is observed as shown below (4,6). Above 160°C, decomposition proceeds at a fast rate.

Baclofen   Lactam
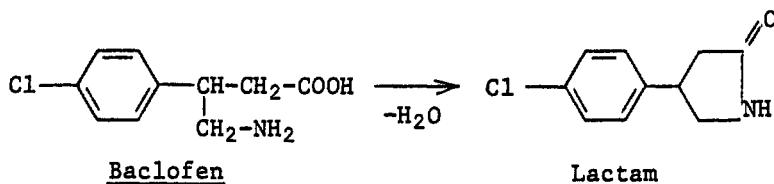
4.5.2 Solution Stability

Degradation studies in solution can be carried out by refluxing the material in acidic and basic solutions for a number of hours. The reflux solutions should then be evaluated by some preliminary chromatographic methods for evaluating any observable degradation products. In this regard, TLC is very useful because it is fast and simple and can handle a variety of samples.

Table 4.6 Solution Stability of Baclofen Active Ingredient

pH	$k \times 10^5$ (days^{-1})	t10%, 25°C
2	8.41	3.4 years
4	5.07	5.7 years
6	3.02	9.6 years
7	2.57	11.2 years
9	14.4	2.0 years
10	14.5	2.0 years
11	11.5	2.5 years
12	4.21	6.9 years

Note: The greatest instability is seen at pH 9 and pH 10.

In the case of baclofen, it is interesting to observe that lactam formation is dependent on the pH of the medium (7). Based on studies performed at 90°, 80°, and 70° C, the pseudo-first order rate constants are given in Table 4.6 on the previous page.

4.6 METHOD DEVELOPMENT AND SELECTIVITY

Based on the above discussion, it should now be possible to make a preliminary decision as to which methods are likely to be suitable for analyzing the parent compound and potential impurities. It may be desirable to conduct a review of the various methods discussed below, to see which of these need to be further evaluated to arrive at the method of choice. With baclofen as an example again, we can see that it is poorly soluble in most organic solvents and it also exhibits low partition coefficients in various organic solvents. This means that simple extraction methods where one might selectively extract impurities are ruled out.

Only very unusual impurities that may be significantly different structurally are extractable. The low absorptivity values for baclofen in the UV region make direct UV determination very difficult because of potential inteferences. Furthermore, glutarimide and lactam exhibit UV spectra in the same region. For a discussion on methods of choice for baclofen, see the various methods described below.

4.6.1 Titrimetry

In general, titrimetry is likely to be insufficiently selective for monitoring impurities; however, it can provide useful information on the purity of the parent compound. This information can then be looked at critically to see if potential impurities were also titratable. If this were the case, then the method needs to be selected in such a manner that the potential impurities are excluded, or the data needs to be interpreted with caution.

Titrimetry is a valuable method for determination of the purity of baclofen because the glutarimide precursor will not titrate when

baclofen is titrated as an acid or a base. The amino group can be titrated in acetic acid with perchloric acid, and the carboxylic acid moiety can be titrated in 80% ethylene glycol monomethyl ether (EGME) with sodium hydroxide after reacting the primary amino group with formaldehyde.

4.6.2 Gas Chromatography

Gas chromatography (GC) is a very useful method for analyzing a variety of compounds. Two primary requirements for its application are that the sample be volatile and thermally stable. This is generally not the case for pharmaceutical compounds, so pharmaceutical analysts tend to use this method primarily for monitoring organic volatile impurities, i.e., residual solvents. However, it is possible to increase the volatility of the pharmaceutical compounds by forming suitable derivatives. This can be demonstrated with baclofen. Since it has both primary amino and carboxylic groups, derivatization of both is necessary prior to analysis by GC (8). The derivatization reaction scheme is as follows:

$$Cl-C_6H_4-\underset{\underset{CH_2NH_2}{|}}{CHCH_2COOH} \xrightarrow[100°C]{n-C_4H_9OH/HCl} Cl-C_6H_4-\underset{\underset{CH_2NH_3Cl^-}{\overset{+}{|}}}{CH-CH_2COOC_4H_9}$$

$$\xrightarrow[RT]{(CF_3CO)_2O} Cl-C_6H_4-\underset{\underset{CH_2NHCOCF_3}{|}}{CHCH_2COOC_4H_9}$$
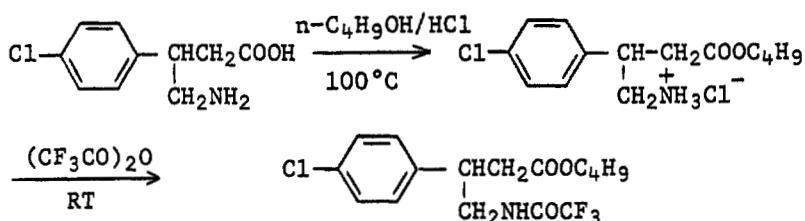
The resulting derivative is chromatographed on a Tabosorb column at 200°C. With this method, lactam is separated from baclofen and thus does not interfere with quantitation of lactam; however, it is not possible to quantitate lactam reliably, as it yields a broad peak.

Ion-pair extraction methods or absorption on charcoal has been used for isolating baclofen from biological fluids (9,10). After elution, baclofen is derivatized and analyzed with an electron capture detector.

4.6.3 High Pressure Liquid Chromatography

A variety of choices are available. The first selection is based on the molecular weight (Figure 4.6). If the molecular weight of the material of interest is over 2000, gel permeation chromatography might be the technique of choice. Select methodology comes into play if the molecule of interest is a protein with high molecular weight. However, a number of proteins can be resolved by ion exchange methods. Discussion here is limited to pharmaceutical compounds that have a molecular weight of less than 2000, which covers a large segment of these compounds.

The method can be chosen from the following list of methods, based on the structure of the compound:

- Adsorption Chromatography
- Normal Phase Chromatography
- Reverse Phase Chromatography
- Ion Exchange Chromatography
- Gel Permeation Chromatography

Some authors do not differentiate between adsorption chromatography and normal phase chromatography. This is incorrect. Admittedly, the mechanisms of separations are not always well defined; however, when a polar stationary phase is being used to confer partition-like properties, it is best to describe this technique as normal phase partition chromatography, as opposed to adsorption chromatography on silica gel, or other adsorbents, where adsorption is the primary mode of separation.

Reverse phase chromatography implies that the stationary phases and polar phases are reversed, as compared to normal phase chromatography, i.e., the stationary phase is nonpolar and the mobile phase is polar.

Ion exchange chromatography deals primarily with ionic compounds or compounds that can be ionized. This technique is further divided into cation or anion exchange chromatography, depending upon whether we want to separate cations or anions. New devel-

opments in this area have led to the technique called ion chromatography, which by virtue of certain modifications allows use of harsh solvents for determination of anions and cations at very low concentrations.

		Solvent	Type	Mode
Sample	MW > 2000	Organic	Various	Size exclusion
		Water	Various	Gel permeation
	MW < 2000	Organic	Polar	Reversed phase / Normal phase
			Nonpolar	Adsorption / Reversed phase
		Water	Ionic	Ion exchange / Ion pair
			Nonionic	Reversed phase / Normal phase / Adsorption

Figure 4.6 Selection of chromatographic
mode based on solubility.
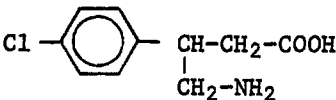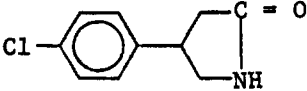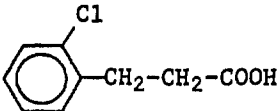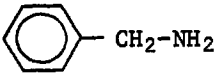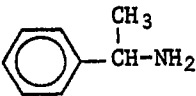
Gel permeation chromatography (GPC) or gel filtration chromatography separates molecules on the basis of molecular weight. It is called size exclusion chromatography (SEC) when nonaqueous solvents are used. This technique is very useful for the separation of macromolecules (11).

Our model compound, baclofen, can be chromatographed as an anion where carboxylate ion is the primary site of exchange or as a cation where the protonated amine is the site of exchange. A successful use of cation exchange separation by HPLC was achieved, which provided excellent separation of baclofen and its

lactam, the primary synthetic and stability-related impurity. The method utilizes a cation exchange column and a mobile phase containing 0.01M tetramethyl ammonium nitrate in 0.02 M nitric acid. Tetramethyl ammonium nitrate helps improve the peak shape. In Table 4.7 a few model compounds demonstrate the selectivity of the method.

Improved separation is possible with an ion-pair reverse phase method (12). Ion-pairing between baclofen and pentane sulfonic acid provides the necessary optimum for the reverse phase separation. The lactam is well resolved because it cannot form the ion pair due to the absence of amino group. More detailed discussion on this method can be found in Chapter 9.

Table 4.7 Selectivity of Ion-Exchange HPLC Method

COMPOUND	STRUCTURE	RETENTION VOLUME (ml)
Baclofen	Cl—⬡—CH–CH$_2$–COOH / CH$_2$–NH$_2$	5.91
4-(4-Chloro-phenyl)-2-pyrrolidone	Cl—⬡— C=O, —NH	1.97
o-Chlorohydro-cinnamic acid	Cl / ⬡—CH$_2$–CH$_2$–COOH	1.97
Benzylamine	⬡— CH$_2$–NH$_2$	9.84
dl-α-Methyl-benzylamine	⬡— CH$_3$ / CH–NH$_2$	18.6

4.6.4 Enantiomeric Separations

Regulatory authorities require that all chiral centers be identified in the molecule and the enantiomeric ratio be defined for any admixture other than 50:50. Furthermore, justification of a racemate or any optically active forms must be made with adequate data. When approval has been obtained for one of the enantiomers, the other enantiomer can be considered an impurity.

In the case of baclofen, it is the racemate that is commercially marketed. The l-form of baclofen is more active than the d-form, based on the results of animal studies (13). However, it is the l-form that is more toxic as well. It is of interest to resolve d- and l-forms to help investigate the chiral purity of baclofen. The separation can be performed on chiral or achiral columns.

Achiral Columns: Spahn et al. (14) have described quantification of baclofen enantiomers in biological materials (urine, plasma, and cerebrospinal fluid) by liquid/solid extraction on Sep-Pak C-18 cartridge. The subsequent derivatization procedure is comprised of two steps; (1) the butyl esters of the enantiomers were formed using butanolic hydrochloric acid (followed by ion-pair extraction of the intermediate products); (2) a chiral derivative was then formed using S-(+)-naproxen chloride as the reagent. The diastereomeric amides were separated by HPLC on a silica gel column, followed by fluorimetric detection. o-phthaldialdehyde and n-acetyl-L-cysteine have been used to prepare a derivative of baclofen. The reaction was carried out at 80°C for 25 minutes. The enantiomers were resolved by reverse phase HPLC on a C-8 column, followed by fluorometric detection (15).

Chiral Columns: Smith and Pirkle (16) resolved baclofen on two different columns: (R) N-pivaloyl-γ-1-(6,7-dimethyl)-naphthyl-11-dodecylamine and (S)-(-)-N-2-naphthyl-valine undecylester. It is necessary to form dual derivatives (methylester + 3,5-dinitrobenzoyl). The mobile phase is composed of 10% isopropyl alcohol and 90% hexane. The values of 1.10 and 1.32 were obtained for the enantiomers on these columns.

Ligand Exchange: Ligand exchange (transition metal complexes) is useful for molecules that are able to form coordination complexes with transition metal ions. Examples of such compounds are amino acids (free and derivatized) and similar compounds such as carboxylic acids.

Various amino acids including phenylalanine which is similar to baclofen can be resolved on a reverse phase C-18 column with a chiral mobile phase of aqueous cupric acetate and N,N-di-n-propyl-L-alanine containing 15% acetonitrile (17,18).

Investigations by Ahuja et al. (19) have revealed that a ligand exchange column may be the simplest and most useful approach for separating enantiomers of baclofen. A Chiralpak WH column was selected for this purpose, since this column was designed to serve as ligand exchanger. It is necessary to optimize the concentration of copper sulfate concentration of the mobile phase, since it significantly influences the separation. Temperature also has a significant effect on resolution. The effect of the addition of methanol relates to the amino acid that is being resolved. The mobile phase is simply 0.25 mM copper sulfate, and the optimum temperature is 50°C. The l-form elutes earlier than the d-isomer and can be quantitated without difficulty.

4.6.5 Hyphenated Methods

Combination of GC and MS or LC and MS can provide powerful methods for separations. Baclofen has been analyzed in cerebrospinal fluid by GC/MS or mass fragmentography (20). The amino acid is derivatized and chromatographed as pentafluoropropyl ester pentafluoropropyl amide. Measurement in the biological fluids is possible down to a concentration of 5 ng/mL.

Other combinations include capillary electrophoresis with MS or supercritical fluid chromatography with MS. These combinations can often be complex and expensive, e.g., LC/MS/MS. A number of these methods are discussed under applications (see Chapter 9).

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5
Synthesis-Related Impurities

A number of impurities that are produced during synthesis can be carried into the new chemical entity (NCE). The most obvious synthesis related impurity is the precursor, or what is more frequently referred to as the penultimate intermediate. Since this is the last compound used in the reaction to produce the NCE, it is easy to see why this compound may still be present: Chemical reactions are generally not 100% complete and do not yield 100% of the final product. As a result, a substantial amount of the precursor has the potential to be in the final product. One or more crystallization steps may be used to minimize this source of contamination; however, it is very difficult to design a crystallization process that would remove all of the materials that can be considered impurities. Hence the need to monitor the penultimate material is paramount, both from the quality and the regulatory standpoints.

Other sources of impurities are intermediates and by-products that may be produced during any of the reaction steps, more particularly in the last step. An impurity from any of the reaction steps also has the potential of further reacting with any of the chemicals that are used in the synthesis. Their presence must be investigated both theoretically and practically. Additionally, the organic solvents used in the synthesis can be carried into the NCE,

and their absence must be demonstrated, or suitable controls must be introduced to monitor them. (The methods for controlling these impurities can be found in the compendia.) In this chapter several examples of drugs that are of commercial interest and the methods that are utilized for monitoring synthesis-related impurities are discussed.

As mentioned before, it is important to recognize at the outset that impurities in pharmaceutical compounds originate mainly during the synthetic process from raw materials, solvents, intermediates and by-products (1). Contaminants of various types, as well as degradation products, comprise some of the other sources of impurities. This suggests that there is a great need to incorporate stringent tests to control impurities in pharmaceutical compounds. The federal Food, Drug, and Cosmetic Act and various pharmacopeias provide requirements for controlling impurities. However, the best approach is to include an armamentarium of physicochemical tests in the new drug development process, fully defining the purity of a pharmaceutical compound prior to performance of pharmacological and toxicological studies (2). This would assure that the observed toxicological and pharmacological effects are truly due to the compounds of interest and not due to impurities.

A variety of analytical methods that can control impurities down to ultratrace levels are available (3,4); however, their application to a given pharmaceutical matrix presents unique problems. Regulatory authorities demand that all impurities should be monitored and those present at 0.1% level or above must be characterized. Realistically, the specifications to which any impurity should be controlled should be primarily determined by its pharmacological and toxicological effects. This should include all impurities originating out of synthesis and those arising out of degradation during synthesis. For example, penicillins and cephalosporins have been known to undergo facile cleavage of β-lactam bond in aqueous solution. This is of great importance because some studies on penicillins have shown that their instability may affect possible reactions involved with penicillin

allergy (5). The control of low level impurities is especially important when a drug is taken in large amounts for therapeutic reasons or as a fad. Examples are the use of methotrexate (10-20 g) to treat neoplasia or the faddist use of vitamins, especially vitamin C.

Specifications for impurities can vary in pharmacopeias. In addition, a pharmaceutical compound can be prepared by a number of different ways thus providing the potential for different impurity profiles or content. This emphasizes the need for methodologies suitable for controlling low levels of impurities and the establishment of rational limits.

5.1 METHOD DEVELOPMENT STRATEGY

It is necessary to study the structure of NCE and the related compounds arising out of synthesis prior to planning a strategy for the method development (see Chapter 4). The functional groups of the NCE, the observed pKa value(s), and other physicochemical properties will frequently indicate which is the best route to take in a given case. As mentioned in Chapter 3, extraction provides the simplest route to monitoring impurities. If it is not possible to extract selectively the NCE, then separation methods such as chromatographic methods should be used since they can allow monitoring of parent compound and impurities in the same run.

5.1.1 Extractable Impurities

The impurities can be extracted frequently on the basis of acidity, basicity, or neutrality. The extraction process usually involves liquid-liquid extraction where one phase is aqueous and the other phase is an organic solvent. For example, the basic impurities will favor the acidic aqueous solution over the organic phase and the opposite case will hold true for the acidic impurities. The situation will be reversed in the basic solution. It can be readily seen by small adjustments over the whole pH range, and it is possible to separate the impurities into a number of groups depending upon

their pKa. This provides a valuable means of extraction of acidic, basic, or neutral impurities by appropriate adjustment of the pH.

5.1.2 Chromatographable Impurities

Chromatography is frequently the technique of choice for separation of impurities from each other and from the main compound. The separations are based on properties such as adsorption, partition, ion-exchange, or molecular size.

Thin-layer chromatography is favored as a qualitative technique because of its simplicity and low operation costs, providing an insight into the nature of impurities. Quantitation is possible; however, good precision (i.e., a relative standard deviation of less than 2%) is generally difficult to obtain with TLC. But exceptions abound, and a number of applications for ultratrace analysis of pharmaceuticals have been reported (6).

Most pharmaceutical analysts prefer to use TLC as a semi-quantitative tool and they generally utilize silica gel plates. If the nature of the impurities is not known, TLC solvent systems (mobile phase) that are acidic, basic, or neutral are investigated. Universal detectors such as UV, iodine vapors, or spray reagents are used. Semi-quantitative estimations are made by comparison with a range of standards run simultaneously on the plate. Quantitation is possible by the scrape-and-elute method, followed by analysis with an appropriate spectrophotometric method or by scanning the plate with a suitable scanner.

Gas-liquid chromatography or, more simply, gas chromatography (GC) is used primarily to resolve volatile compounds. If a compound is volatile, it can be resolved by GC from other related compounds on the basis of its boiling point and/or polarity. Alternatively, derivatization can be used to obtain the desired volatility or polarity.

For a number of reasons, pharmaceutical analysts prefer not to use derivatization methods. Not the least of these reasons is the incompleteness of derivatization reactions, the problem with by-products, and the time required to carry out these reactions. The

last problem can be obviated by carrying out on-column derivatization. It should be noted that GC is used primarily in pharmaceutical analysis today for controlling residual solvents. As mentioned before, suitable methods have been included in the compendia and are not repeated here. However, the combination of GC with a variety of detectors, especially mass spectrometry, should not be overlooked for monitoring impurities (1).

High pressure liquid chromatography (HPLC) is commonly referred to as high performance liquid chromatography albeit incorrectly most of the time. It is very suitable for the analysis of nonvolatile or thermally labile compounds. Although the techniques such as adsorption and ion-exchange chromatography have been used occasionally, the technique of choice is reverse-phase liquid chromatography (RPLC).

In RPLC, the stationary phase and the mobile phase are opposite of normal phase chromatography. Here the stationary phase is nonpolar and the mobile phase is polar. Separations are achieved by varying the polarities of mobile phase and/or pH. This permits separation of a wide variety of compounds. Even the ionic compounds can be resolved by techniques such as ion suppression or ion pairing. A variety of detectors can be used such as UV, fluorescence, electrochemical, or mass spectrometric.

Capillary electrophoresis and supercritical chromatography are new techniques that are finding a number of applications in monitoring impurities. Other strategies to resolve impurities relate mainly to the type of impurities.

A few examples are discussed in the next section to provide readers a perspective of the nature of synthesis-related impurities and the methods of separations that have been used to monitor them. It should be pointed out that minimal information is available in the scientific literature relating to synthesis-related impurities.

Excellent examples of providing detailed information and identification of impurities have been discussed (for example, see Chapter 7 in reference 1). The examples discussed highlight TLC, showing in particular that it is not always necessary to utilize

expensive equipment. Detailed information on characterizing impurities for a number of compounds by different methods is discussed in the next section.

5.2 CASE STUDIES

5.2.1 Metoprolol Tartrate

This compound is a 2:1 salt consisting of a racemic mixture of optical isomers of the base and naturally occurring d-tartaric acid. It is sold under the following trade names: Beloc, Betaloc, Lopressor, Lopresor, Metoros, Seloken, and Selopral.

Synthesis: Metoprolol tartrate is synthesized as follows:

Metoprolol Tartrate
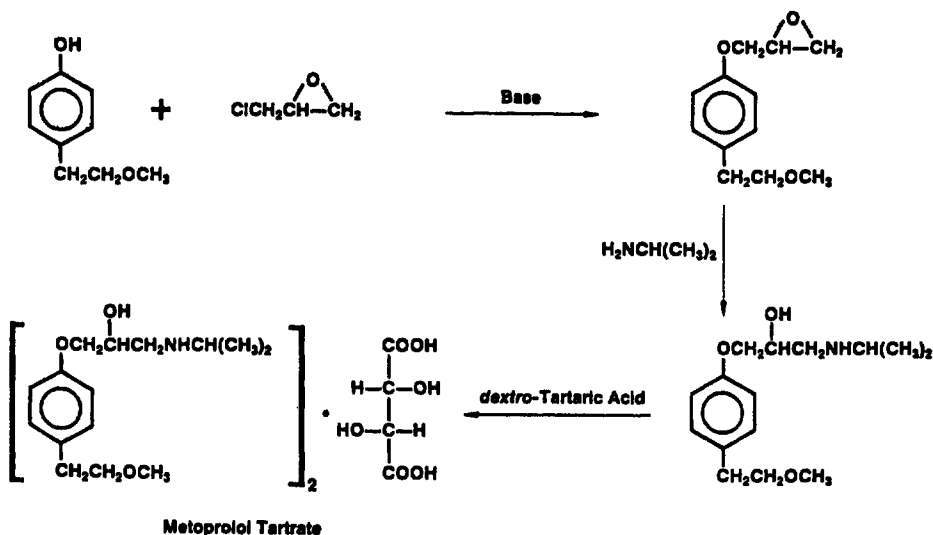
Review of Physicochemical Properties
The pKa of metoprolol is 8.9 in water at 25°C and can be attributed to the secondary amine (8). The pKa's for tartaric acid are 2.93 and 4.23. Metoprolol tartrate is freely soluble in water and methanol and exhibits good solubility even in chloroform. It is a hygroscopic compound that rapidly absorbs water at relative humidities greater

than 70%. The compound exhibits a favorable distribution ratio in chloroform when extracted from 0.1 M sodium hydroxide.

In UV analysis, λ max is observed at 223 nm for the aqueous solution, which shifts to 221 nm in 0.1 N HCl. A solution of metaprolol tartrate in water or methanol exhibits an emission fluorescence at 298 nm when excited at 275 nm.

It contains three asymmetric carbon atoms: one at the 2-propanol position of the base and the other two in the tartaric acid portion of the molecule. The observed optical rotation is due mainly to the d-tartaric acid used in the synthesis.

Analytical Methodology

A variety of methods have been utilized for analyzing metoprolol. Since the analytical profile (8) lists 11 TLC systems, 6 GC systems, 2 GC-MS systems, and 7 HPLC systems (Table 5.1), it is reasonable to expect that these methods have been developed to monitor impurities that are present or potentially can be present.

Table 5.1 HPLC Systems

COLUMN	MOBILE PHASE	DETECTION
μBondapak C-18	Methanol-water (550 ml/470 ml containing 961 mg 1-pentane-sulfonic acid sodium salt (monohydrate), 82 mg anhydrous sodium acetate, and 0.57 ml glacial acetic acid	UV 254 nm
μBondapak C-18	Methanol-water (520 ml/480 ml) containing 1.10 g 1-heptane sulfonic acid sodium salt (monohydrate) and 5.0 ml glacial acetic acid	UV 274 nm
Partisil 10	Methylene chloride-methanol-1M diethylamine in methanol (89/10/1)	UV 277 nm
Silica 5μm	Hexane-isopropanol-methanol-concentrated ammonium hydroxide (850/100/50/1)	Fluorescence λ excitation 224 nm

Table 5.1 HPLC Systems (continued)

COLUMN	MOBILE PHASE	DETECTION
Lichrosorb-Diol	Methylene chloride-1-pentanol (199/1) containing 2.2×10^{-3} M (+)-10-camphorsulfonate	UV 254 nm
Lichrosorb RP 8, 5, or 10 µm particles	100 ml water containing 1.8 ml perchloric acid and 11.2 g sodium perchlorate (monohydrate) with 200 ml acetonitrile diluted to 1000 ml with water	UV 270 nm
Lichrosorb RP 8, 5, or 10 µm particles	100 ml water containing 1.8 ml perchloric acid and 11.2 g sodium perchlorate (monohydrate) with 200 ml acetonitrile diluted to 1000 ml with water	UV 270 nm
µBondapak C-18	Methanol-water-acetic acid (50/49/1) containing 1-heptane-sulfonic acid (one bottle PIC B-7 per liter)	Fluorescence λ excitation 222 nm

Unfortunately, it has not been clarified which impurities are present and at what amounts. No pertinent retention data has been provided. This does not make it easy to assess impurities or degradation products that are likely to be present in the NCE. However, based on the synthesis, one would expect compound preceding formation of metoprolol base to be the potential impurity in metoprolol tartrate.

TLC: The following method has been used in USP XXI (9) for determination of purity of metoprolol tartrate:

TLC plate: silica gel 250 µm
Mobile phase: chloroform-methanol-ammonium hydroxide (80/15/2)
Detection: chlorine gas/ potassium iodide-starch

The impurities are evaluated against a range of standards and the sum of any observed impurities is not greater than 1.0%. Again, even the USP does not indicate which impurities are generally present and which of these is over 0.1%.

Alternate methods include detection systems such as chromate-sulfuric acid, followed by detection at 366 nm or anisaldehyde. Mobile phases such as chloroform-methanol (95/5) or methanol-ethyl acetate (40/20) or butanol-HOAc-water (71/7/22) or methanol-ethyl acetate-diethyl amine (60/35/5) or chloroform-methanol-ammonium hydroxide (80/15/2) have also been used with silica gel plates.

A reverse phase TLC system may be useful for chromatographing metoprolol where the C-18 plate is developed with 0.1 M ammonium acetate in methanol-water (56/44) and detection is by UV light (254 nm) or iodine vapor, followed by UV light.

Gas Chromatography: For determination of metoprolol in pharmaceutical dosage forms, the following chromatographic conditions have been employed:

Column: 210 cm × 5 mm i.d. Glass column packed with 3% JXR on Gas Chrom Q (100-120 mesh).
Column Temperature: The column is operated at 210°C.
Carrier Gas: Nitrogen is used at a flow rate of 45 ml/minute.
Sample: The sample is chromatographed as trimethylsilyl derivative prepared by reacting metoprolol with bis(trimethylsilyl)-trifluoroacetamide.

GC-MS: The GC-MS methods used for monitoring metabolites can also be used for evaluating synthesis-related impurities. In this case a column (100 cm × 2 mm i.d.) containing 3% OV-17 on Gas Chrom Q (100-120 mesh) has been found useful. The column is operated at 200°C and helium at a flow rate of 15 ml/minute is used as the carrier gas. Trifluoroacetyl derivatives of the sample are prepared by reacting metoprolol and related compounds with

N-methyl-bis-(trifluoroacetamide). Mass spectrometry entails electron impact ionization at 60 eV.

High Pressure Liquid Chromatography: USP XXI recommends a C-18 column (30 cm × 3.9 mm i.d., USP column packing L1), for evaluation of metoprolol tartrate and various compounds. The mobile phase is composed of a mixture of methanol-water (550:470) containing 961 mg 1-pentanesulfonic acid sodium salt (monohydrate), 82 mg anhydrous sodium acetate, and 0.57 ml glacial acetic acid. The flow rate is 1 ml/minute, and UV detection is performed at 254 nm.

An alternate system utilizing 15 cm × 4 mm i.d. LiChrosorb RP 8 with either 5- or 10-µm particle size has been used for investigation of purity of metoprolol. The mobile phase contains a mixture of 100 ml of water containing 1.8 ml perchloric acid and 11.2 g sodium perchlorate (monohydrate) with 200 ml acetonitrile diluted to 1000 ml with water. The flow rate is 0.8-0.9 ml/minute and detection is carried out by UV at 270 nm.

5.2.2 Chlorthalidone

Chlorthalidone is a diuretic used in the treatment of edema associated with congestive heart failure. It is known under trade names such as Hygroton, Hydro-Long, Hydroton, and Igroton. Under the trade names of Regroton and Demi-Regroton, it is sold in combination with reserpine.

Synthesis: Chlorthalidone has the following structure:
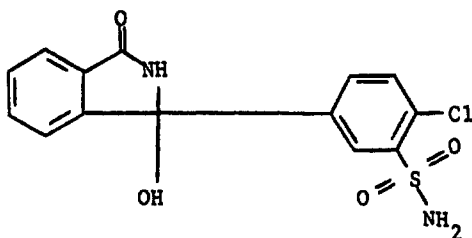
It is synthesized as follows (10): 3-amino-4-chlorobenzo-phenone-2-carboxylic acid is diazotized, and the resulting diazonium chloride is reacted with sulfur dioxide in glacial acetic acid in the presence of cupric chloride to form 4-chloro-2'-carboxy-benzophenone-3-sulfonyl chloride.

The last compound is heated with thionyl chloride to yield 3-chloro-3-(3'-chloro-sulfonyl-4'-chloro-phenyl) phthalide, which is isolated, dissolved in chloroform, and reacted with ammonia in the presence of ethanol. The solvent is removed and the residue is treated with HCl to yield crude chlorthalidone, which is recrystallized from aqueous ethanol.

Review of Physicochemical Properties
Chlorthalidone shows a solubility of 16.7 mg per 100 ml at pH 4.90, which increases with the increasing pH reaching a value of 99.1 mg/100 ml at pH 10.9. Chlorthalidone is freely soluble in methanol and insoluble in chloroform or diethyl ether. The ionization constant for the sulfonamide function has been reported as 9.36 in water at 22°C.

The UV spectrum in dilute HCl-methanol (1/50) shows maxima at 266 nm, 275 nm, and 283 nm.

Analytical Methods
Thin-layer Chromatography: This method allows separation of potential impurities such as chlorthalidone carboxylic acid (CCA). TLC is performed on silica gel GF plate with a mobile phase consisting of ethyl acetate-absolute ethanol-concentrated ammonium hydroxide (50/20/30). The TLC plate is developed to a distance of 15 cm in a saturated chamber and then observed under short wave UV. The Rf values of chlorthalidone and CCA are 0.60 and 0.46 respectively.

Gas Chromatography: GC requires derivatization. For example, chlorthalidone is converted to its tetramethyl derivative by extractive alkylation with tetrahexyl ammonium hydrogen sulfate. Poor availability of this reagent has led to an on-column methylation procedure.

This method employs a 0.2 M solution of trimethylanilinium hydroxide in methanol as the derivatizing agent. The column (1.8 m × 2 mm i.d. with 3% JXR on 100/120 mesh Gas Chrom Q) is operated at 190°C followed by temperature programming to 260°C with helium as the carrier gas at a flow rate of 40 ml/minute. This method allows separation of a close analogue of chlorthalidone, 3-isobutoxy-3-(3'-sulfonamido-4'-chlorophenyl) phthalimidine that is used as an internal standard.

High Pressure Liquid Chromatography: HPLC can be performed on a C-8 column (Zorbax or Partisil) with a mobile phase consisting of 0.01 M dibasic ammonium phosphate and methanol (3:2), adjusted to pH 5.5 with phosphoric acid. The flow rate is maintained at 1 ml/minute, and detection is accomplished with a UV detector at 254 nm. The method resolves CCA, the hydrolysis product of CCA.

5.2.3 Terbutaline Sulfate

Terbutaline sulfate is a synthetic β_2-adrenoceptor that is used as a bronchodilator in the treatment of bronchial asthma. It is commercially sold as a racemic compound under the name of Brethine or Bricanyl.

Synthesis: Terbutaline sulfate can be synthesized as shown on the following page (11):

Review of Physicochemical Properties
Terbutaline sulfate exhibits good solubility in water, 0.1 N HCl, or 0.1 N NaOH (>20 mg/ml). In methanol a solubility value of 2.7 mg/ml has been observed. It shows poor distribution coefficients in various organic phases vs. aqueous phase at room temperature. Terbutaline sulfate is not very hygroscopic. The following pKa values have been reported: 8.8, 10.1, and 11.2. The 10.1 value can be assigned to the amino group. The other two pKa values (8.8 and 11.2) may be attributed to the aromatic hydroxyl groups of this compound.
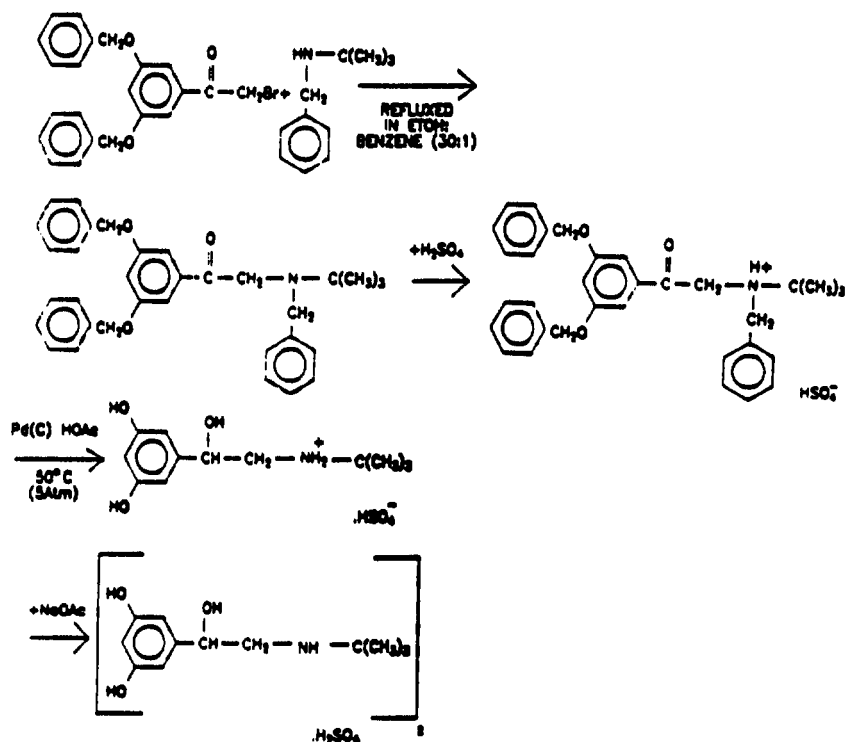
Synthesis of terbutaline sulfate.

The UV spectrum of terbutaline sulfate in 0.1 N HCl exhibits maxima at 276 nm. This absorption arises from the $\pi \to \pi^*$ transition of the phenyl ring.

Analytical Methods: The potential impurity anticipated from the synthesis is 1'-benzyl-t-butylamino-3,5-dibenzyloxyacetophenone. However, in the process of reduction to form terbutaline it can also be debenzylated, so one must also look for that compound as well as any potential impurity. As mentioned before, it is important to consider potential degradation products to assure appropriate monitoring of the impurities.

Thin-layer Chromatography: Terbutaline sulfate has been chromatographed on a silica gel plate with a mobile phase consisting of isopropyl alcohol-cyclohexane-formic acid (13/5/1).

Detection is accomplished with 4-aminoantipyrine and potassium ferricyanide spray reagents.

Gas Chromatography: Terbutaline sulfate cannot be gas chromatographed directly. The molecule has to be converted to its tris-trimethyl silyl ether and then chromatographed on 3% OV-17 coated on a neutralized diatomaceous earth support. The column is operated between 150°C and 190°C.

High Pressure Liquid Chromatography: The potential impurities and potential degradation products of terbutaline sulfate, besides the penultimate intermediate, can be classified into the following four major categories:

- Dihydroxy compounds with t-butylamino side chain
- Cyclized dihydoxyphenyl compounds with basic N in the ring
- Dibenzyloxyphenyl compounds with no amino side chain
- Dihydroxyphenyl compounds with no amino side chain

The physicochemical properties of all of these compounds are very different in terms of polarity, pKas, and partition behavior, and therefore preclude development of one HPLC system that would resolve all of them. Specifically, completely opposite properties in terms of polarity are exhibited by dibenzyloxyphenyl compounds and dihydroxyphenyl compounds. These differences require development of at least two systems which can resolve all of these impurities (11). Both systems (Figures 5.1 and 5.2) utilize an 8 cm × 6.2 mm i.d. C-8 column with 3-μm particle size and detection is carried out by UV at 280 and 235 nm respectively.

System I is composed of water-methanol-tetrahydrofuran (750/140/110) with 0.005 moles 1-octanesulfonic acid per liter. It provides optimum resolution of all the dihydroxyphenyl impurities (Figure 5.1), and as a result, dibenzyloxyphenyl impurities exhibit high retentivities. Optimum resolution of the latter impurities is accomplished with System II (Figure 5.2), which consists of water-tetrahydrofuran-acetonitrile-acetic acid-triethylamine (500/465/35/5/2), where the hydroxy compounds are virtually unretained. System I is therefore useful for assaying and
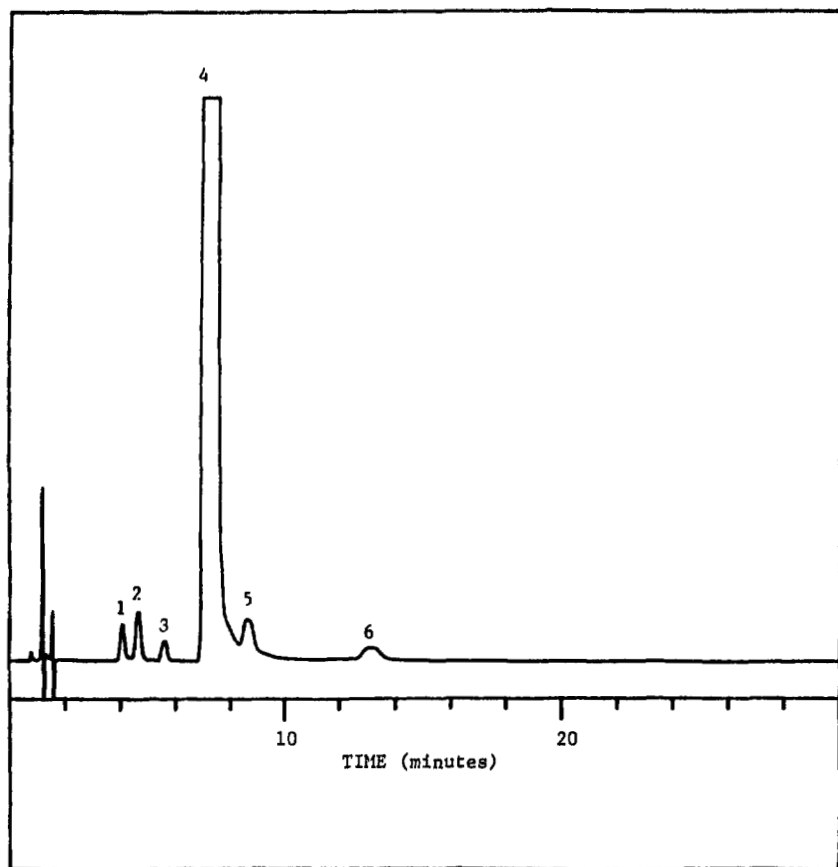
Figure 5.1 Resolution of potential degradation products.

1 = 3,5-dihydroxyacetophenone,
2 = 3,5-dihydroxybenzaldehyde,
3 = 2-t-butyl-4,6,8-trihydroxy-tetrahydroisoquinoline,
4 = terbutaline,
5 = 3,5-dihydroxy-ω-t-butylaminoacetophenone,
6 = 3,5-dihydroxybenzoic acid, ethyl ester
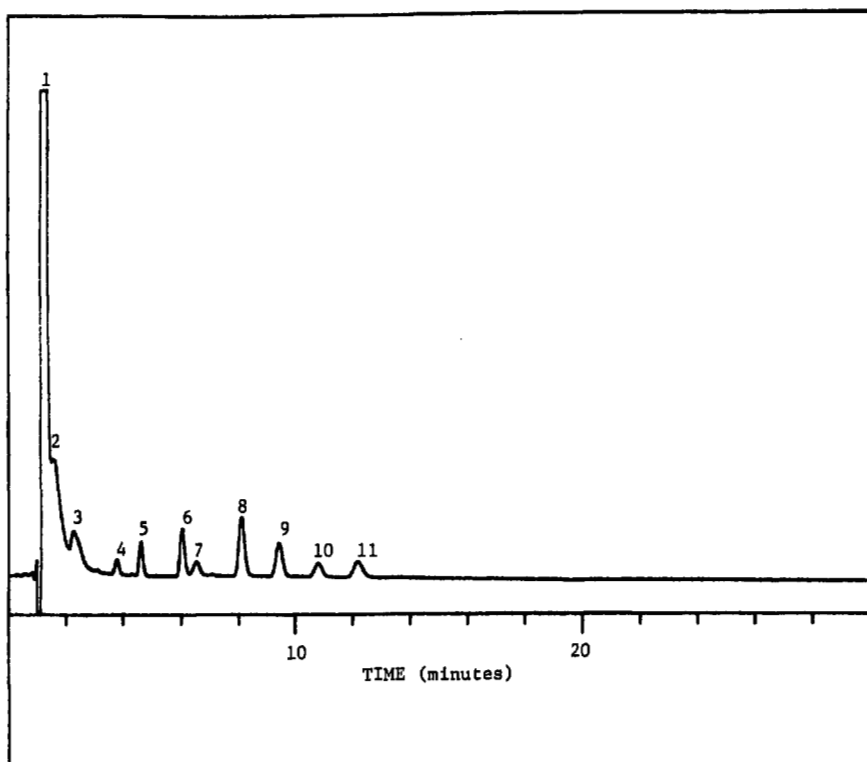
Figure 5.2 Resolution of potential dibenzoyloxyphenyl impurities

1 = terbutaline, 2 = solvent, 3 = solvent,
4 = α-[(t-butylamino)methyl]-3,5-dibenzyloxybenzyl alcohol,
5 = α-methyl-3,5-dibenzyloxybenzyl alcohol,
6 = 3,5-dibenzyloxyacetophenone,
7 = α-[(benzyl-t-butylamino)methyl]-3,5-dibenzyloxybenzyl alcohol,
8 = 3,5-dibenzyloxy-2,6-dibromoacetophenone,
9 = 3,5-dibenzyloxy-1'-bromoacetophenone,
10 = 3,5-dibenzyloxy-2,6,α-tribromoacetophenone,
11 = 1'-benzyl-t-butylamino-3,5-dibenzyloxyacetophenone

monitoring degradation products of terbutaline (4), and System II for monitoring in-process dibenzyloxyphenyl impurities. The minimum detectability for all these compounds is at the nanogram level.

A commercially available chiral column containing α-acid glycoprotein on silica has been used for the resolution of enantiomers of terbutaline (12). The mobile phase contained 0.003 M tetrapropyl-ammonium bromide solution adjusted to pH 7.0. The enantiomers have been resolved in the biological samples on a cyclodextrin column. β-Cyclodextrin has been also used in the resolution of enantiomers of terbutaline sulfate by capillary electrophoresis (13).

A mass fragmentographic method has been used for monitoring terbutaline sulfate in biological fluids (14). The mixed TMS-TFA derivative is chromatographed, and measurement is possible down to the 0.3 ng/ml level.

5.2.4 Lovastatin

Lovastatin is a prodrug with the inactive parent lactone hydrolyzed to the corresponding hydroxyacid form (15).

Lactone   Hydroxyacid
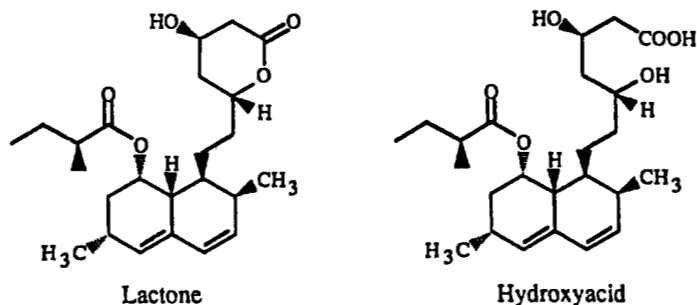
The hydroxyacid is the principal metabolite and a potent inhibitor of 3-hydoxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase. This enzyme catalyzes the conversion of hydroxymethylglutarate to mevalonate, which is an early and rate-limiting step in the biosynthesis of cholesterol.

Commercial Production

Lovastatin is produced commercially in a multistage fermentation process. It is isolated from a strain of *Aspergillus terreus.*

Physicochemical Properties

Lovastatin is insoluble in water and is sparingly soluble in methanol. It exhibits good solubility in acetonitrile and chloroform. No acid-base dissociation constants have yet been observed. In the n-octanol-water system, lovastatin partitions quantitatively into the organic phase. The partition coefficient of the hydroxyacid derivative at pH 7.4 is 14.1.

The UV spectrum of lovastatin shows absorption maxima at 231, 238, and 247 nm. The highest absorbance is observed at 238 nm and is typical of tri-substituted hetroannular diene chromophore.

Lovastatin has eight chiral centers and is optically active. The specific rotation $[\alpha]D$ at 25°C is +330 for a 5.0 mg/ml solution in acetonitrile.

Analytical Methods

Thin-layer Chromatography: Several methods have been used. The separation is achieved with cyclohexane-chloroform-isopropanol (5/2/1) on silica gel 60 plates. Visualization is achieved with UV or by spraying the developed plate with dilute methanolic sulfuric acid solution and application of heat. The latter detection system allows detection of non-UV absorbing impurities.

High Pressure Liquid Chromatography: Separations on a C-8 column with acetonitrile-0.1% phosphoric acid (70/30) followed by detection at 238 nm has been found useful for the drug substance purity. The same system used in a gradient mode with additional detection wavelength at 200 nm has been utilized for measurement of low-level impurities.

5.2.5 Zidovudine

Zidovudine (azidothymidine) is better known as AZT.

Synthesis: Zidovudine or its intermediates can be synthesized as follows (16):

[SI]OCH$_2$ ... OMe

1

[Si] = Ph$_2$^{t}BuSI

ROCH$_2$ ... OMe

N$_3$

2

R= p-Me-C$_6$H$_4$CO

3

4
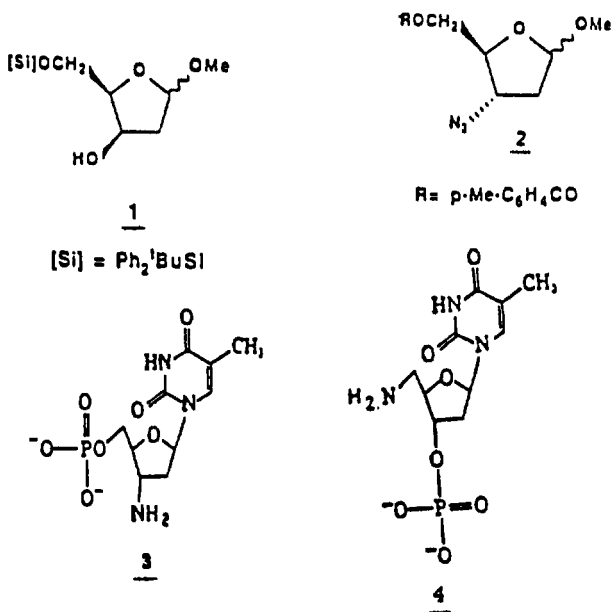
Methyl 5-O-tert-butyldiphenylsilyl-2-deoxy-α,β-D threo pento-furanoside, 1. is prepared from D-xylose as a divergent inter-mediate for the synthesis of azidothymidine. An alternative method of synthesizing azidothymidine has been reported in which a suitable derivative of thymine is coupled with a protected furan-oside of 3-azido-2,3-dideoxy-D-erytropentose, 2. Synthesis of azid-othymidine has been utilized in the synthetic sequence leading to nucleotides, 3-amino-3'-deoxythymidine 5'-phosphate, 3 and 5'-amino-5'deoxythymidine3'-phosphate, 4.

Physicochemical Properties
Zidovudine has solubility in water of 25 mg/ml and exhibits ab-sorption maximum in UV at 266.5 and 234.5 nm. An [α]D of +99 has been observed at 25°C with c = 0.5 in water.

Thin-layer Chromatography: A number of TLC systems have been used:

- Ethyl acetate-ethanol-tetrahydrofuran (1/1/1)
- Chloroform-1% ammonium hydroxide in methanol (3/2)
- 2-Propanol-water-concentrated ammonium hydroxide (7/2/1)

In addition, ethyl acetate and diethyl ether have been used as single solvents. The detection is performed under UV, and compounds containing a trityl group were detected by spraying with aqueous 5% perchloric acid and heating at 80°C for 5 minutes.

High Pressure Liquid Chromatography: This entails chromatography on a C-8 column with 1% acetate buffer pH 5-methanol (82/18) and detection at 265 nm.

5.2.6 Imipramine

Imipramine is a tricyclic antidepressant.
Synthesis: It can be synthesized as follows (17):

Iminodibenzyl   Imipramine Base

Imipramine Hydrochloride
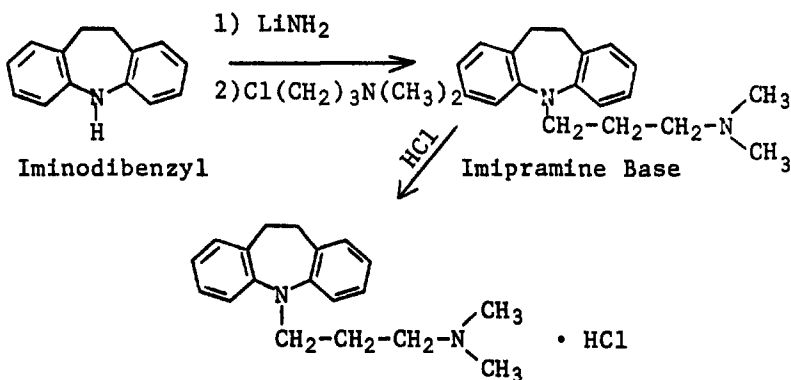
Review of Physicochemical Properties
Imipramine is very soluble in water at pH 4.55 (50 g/100 ml) and ethanol (73 g/100 ml). It exhibits good solubility even in

chloroform. It provides a distribution ratio of >500 in chloroform vs. phosphate buffer (pH 7.4). It has a pKa value of 9.62, based on photometric titration. Imipramine exhibits a UV maximum at 251 nm in 0.1 N HCl.

Figure 5.3 Degradation pathway of imipramine hydrochloride (17).
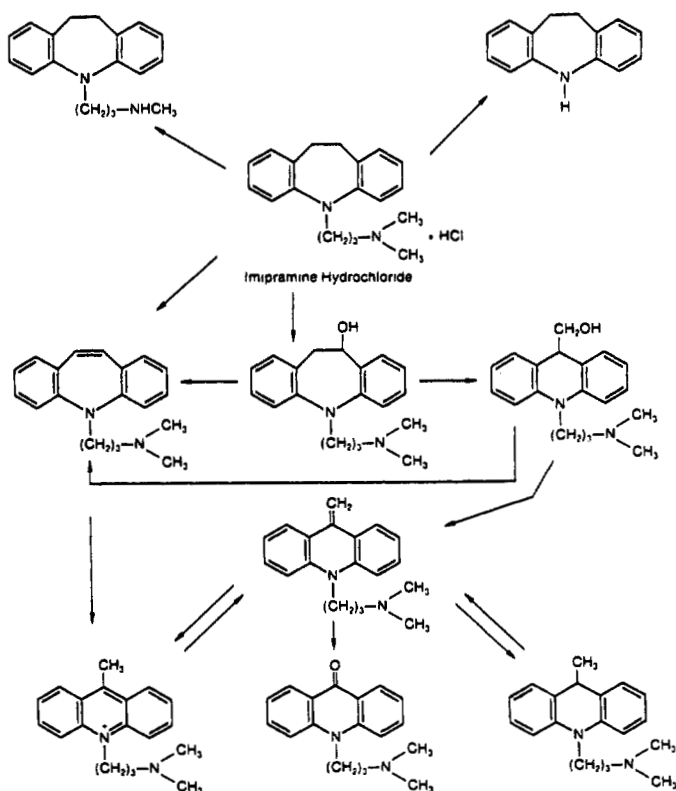
The degradation pathway of imipramine hydrochloride has been delineated (Figure 5.3, on previous page). It is often desirable to study degradation pathways to determine which impurities may be present. Iminodibenzyl has been reported as the major degradation product when samples are heated in aqueous solution of pH. It can also be produced as an unwanted product in derivatization procedures for gas chromatography, so it would be desirable to monitor this impurity.

Analytical Methods
Thin-layer Chromatography: A number of systems have been used, and the two TLC systems that follow have been very useful for monitoring impurities of imipramine:

Acetic acid-ethyl acetate-water-HCl (35/55/5/5)
Benzene-ethyl acetate-ethanol-ammonium hydroxide (50/50/10/5 upper phase).
Detection is performed under UV at 366 nm, followed by spraying with potassium dichromate in sulfuric acid and observing under UV at 366 nm after 2 hours.

Iminodibenzyl is a potential synthesis-related impurity that could also arise from degradation. With the benzene-ethyl acetate-absolute ethanol-concentrated ammonium hydroxide (50/50/15/3) system, Rf values for imipramine and iminodibenzyl are 0.67 and 0.81, respectively (18). In addition, desipramine (Rf = 0.36) and a hitherto unknown impurity with the following structure was resolved at the Rf value of 0.36. The minimum detectability for these compounds is less than 0.1 microgram.
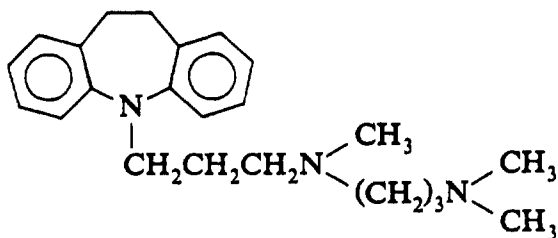
Gas Chromatography: Gas chromatography has been performed with OV-17 or OV-25 columns operated at 230°C to 240°C with a flame ionization detector. The GC-MS method utilizes a 0.9 m × 2 mm column coated with 3% SP 2250 on Supelcoport (80-100 mesh) and is operated at 200°C for 1 minute, followed by temperature programming at 10°C/minute to 250°C.

High Pressure Liquid Chromatography: A number of systems have been used for HPLC of imipramine (Table 5.2). The second system with μBondapak C-18 column is most commonly used.

Table 5.2 HPLC Systems for Imipramine

COLUMN	MOBILE PHASE	DETECTOR
Lichrosorb Si 60	Cyclohexane-ethanol-butanol-ammonium hydroxide (80/20/10/0.4)	UV, 254 nm
μBondapak C-18	Acetonitrile-water (40-60 or 55-45) containing 0.005 M heptanesulfonic acid and 1% acetic acid	UV, 254 nm
Zorbax-SIL	Methylene chloride-methanol-water-diethylamine (850/150/1/0.25)	UV, 251nm

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6
Pharmaceutical Formulation-Related Impurities

The inert ingredients of a pharmaceutical formulation (excipients) are quite complex and can at times be even heterogeneous mixtures (1,2). The excipients can sometimes interact with the main ingredient (i.e., active ingredient) to produce an undesirable product or a product that does not have the same bioavailability. The interaction product for all practical purposes is considered an impurity.

The impurities in the excipients provide a ripe source for many potential reactions, which must be carefully reviewed. These reactions are made more likely by the presence of water, which is invariably present in the active ingredient or excipients as a residual solvent. Varying amounts of water may be present due to the hygroscopicities of these materials. Other solvents may also be present at times if they were used in the synthesis of the active ingredient or excipients.

In semisolid dosage forms and solutions, the effect of solvents may be much more pronounced. Unlike heterogeneous systems such as solids and semisolids, the stability of drugs in homogeneous solutions can be easily predicted with a great degree of accuracy, and the data obtained through basic kinetic studies can often be applied directly to the formulation (see Chapter 7).

However, "extrachemical" or additional reactions can occur with the drug formulated in solution, and they may be overlooked or not considered during the basic studies. All of this points to the need for thorough theoretical evaluations to determine where potential impurities can be produced before a formulation is designed, followed by performance of well-planned preformulation studies.

6.1 PREFORMULATION STUDIES

The selection of a type of dosage form is influenced primarily by the preferred route(s) of drug administration. This places the onus on the development pharmacist to provide a relatively stable formulation under these constraints. Preformulation studies are conducted to ascertain the compatibilities of the drug substance with the excipients, including biological and chemical preservatives that may be necessary for a given formulation. These studies are conducted not only to determine the physical and chemical compatibility of the drug substance with other drug substances that may also be present in a given drug product with several possible excipients, both individually and in combination, but also to forecast the effects of formulation on drug availability.

The methodology, management, and evaluation of a systematic preformulation program for solid dosage forms was described almost thirty years ago (3). It was shown that development of a stable and effective dosage form is determined by the type, quality, and organization of the preformulation studies. Furthermore, it is important to consider interactions between active components and additives, polymorphs, and micelle-forming agents (4).

6.2 WATER VAPOR SORPTION

An interesting example of water vapor sorption is discussed below with regard to aspirin, a commonly used drug. Solid-state hydrolysis of a commercial aspirin sample (Asagran 7016, Monsanto) has been detailed in the scientific literature (5). This sample, described as a single crystal material by the authors,

showed much more water uptake than had been reported previously for other crystalline samples of aspirin under similar conditions (Figure 6.1). There are a number of possible mechanisms by which increased water vapor sorption can occur in a processed crystalline material (6). None of these mechanisms are mutually exclusive, but it should be possible to identify which mechanism contributes most to any observed effect by critical review of data (7).
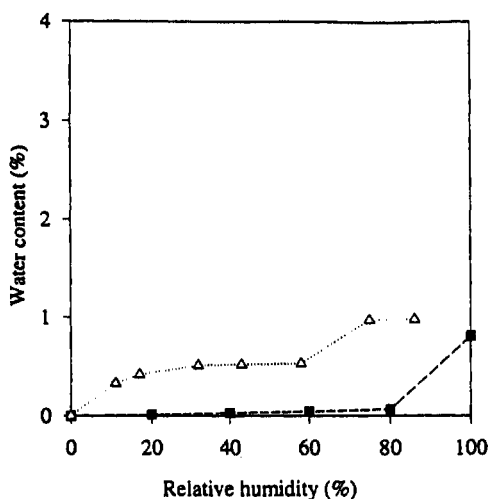
Figure 6.1 Water vapor sorption isotherms reported for aspirin samples: ref 5 (Δ) and ref 5a ($\blacksquare$).

Based on the assumption that the increased level of water sorption by the Asagran sample is due to the formation of an amorphous region during roller compaction, it is possible to calculate the amount of amorphous material required to produce the observed water vapor sorption isotherm. No data are available for water sorption behavior of 100% amorphous aspirin; therefore, it is necessary to assume that aspirin behaves similarly to other amorphous low-molecular weight organic molecules (8). On the

basis of a simple two-component system of lactose that sorbs about 12% water at 50% RH and crystalline lactose which sorbs a negligible amount under these conditions, it has been estimated that the Asagran sample has an amorphous content of 5%. The estimates for much more hygroscopic material are likely to be very high. Based on the knowledge of melting point (135°C), glass transition temperature (-30°C), chemical structure, and aqueous solubility of aspirin, it is expected to behave like lactose and thus the Asagran sample probably had an amorphous content around 10%. This data can be qualitatively corroborated by data from studies of mixtures of crystalline aspirin with amorphous additives, for example, poly(vinyl-pyrrolidone), for which water sorption isotherms are similar to those measured for the roller compacted aspirin (9). It is important to note that such a level of amorphous character normally cannot be detected visually and would be at the limit of detection of X-ray powder diffraction and thermal analytical methods. However, water vapor sorption measurements would be expected to be sensitive to much lower levels of amorphous content than anticipated here.

The formation of amorphous aspirin at some point in the processing history most likely accounts for the increased level of water vapor sorption by the sample. This suggests that solid-state processing can cause the formation of small disordered (amorphous) regions on the surface of aspirin crystals. Such low levels of disorder in crystalline material can markedly increase its tendency to sorb water vapor (8).

6.3 INTERACTION BETWEEN DRUG SUBSTANCES, EXCIPIENTS, AND CONTAINERS

6.3.1 Solutions

The effect of excipients and pharmaceutical aids on solution formulations can be significant. For example, the pH of solutions containing lidocaine hydrochloride changes in the presence of 5% dextrose in saline solution, normal saline, and lactated Ringer

solution (10). Precipitation of the main ingredient can occur as is exemplified by the precipitation of imipramine hydrochloride with sodium bisulfite. A number of other examples of the effects of various excipients and pharmaceutical aids on stability of pharmaceuticals in solution are summarized in Table 6.1.

Table 6.1 Effect of Pharmaceutical Aids on Stability of Active Ingredient

COMPOUND	OTHER INGREDIENTS	REMARKS
Cholecalciferol (vitamin D$_3$)	2% Polyoxyethylene ester Surfactant Polysorbate 80	Polysorbate 80 and pH mainly responsible for observed decomposition
Pyridoxal 5-phosphate	Thiamine, thiamine diphosphate, riboflavin phosphate, adenosylcobalamine, pyridoxal, pyridoxine	Increased decomposition rate at pH 6
Kanamycin	Honey, sugar syrup	Loss of activity at room temperature
Bacillin-3	Honey, sugar syrup	Loss of activity at room temperature
Cephapirin sodium	Mannitol	Less stable with this component
Tetracycline	Calcium Magnesium	Complexation Complexation
Tetracyclines	Urea. thiourea, polysorbate 20, polyethylene glycol 6000	Decreased epimerization
Thimerosal	Bromide, chloride, iodide	Form difficultly soluble halides of cationic mercury compounds

Table 6.1 Effect of Pharmaceutical Aids on Stability of Active Ingredient (continued)

COMPOUND	OTHER INGREDIENTS	REMARKS
Menadione	Sodium metabisulfite	Lower pH due to hydrolysis of sodium metabisulfite followed by oxidation of resulting sodium bisulfite
Apomorphine hydrochloride	Penicillamine at pH 3.6-4.0	Stabilization
Epinephrine (adrenaline)	Boric acid, povidone, erythorbic acid	Stabilization
Epinephrine	Sodium hydrogen sulfite	Stabilization
Tryptophan	Sodium pyrosulfite, oxygen	Discoloration, precipitation

Additional information on instability of solution formulations may be found in Chapters 7 to 9.

6.3.2 Lyophilized Drugs

Many parenteral drug products are lyophilized or dry filled into ampuls since they have limited stability in aqueous solutions. On reconstitution with sterile water or other commonly used diluents or when added to the intravenous fluids, degradation often occurs. Evaluations must be conducted to determine the effects of time and storage conditions in the presence of commonly used vehicles.

6.3.3 Effect of Containers

The effect of containers on the formulation should be investigated also. The "chemical inertness" of commonly used glass containers from the standpoint of composition of various glasses, leaching of

substances from glass by water and other solutions, and the mechanism of such reactions must be considered (11). Stability studies on normal saline solutions in various glass containers has revealed that materials in certain glass types and with certain stoppers cause a significant pH increase on storage due to material release from the stopper on autoclaving (12). Sterilization can also increase the pH of 5% ephedrine hydrochloride solution; this increase is caused by the material leached from ampul glass (13).

6.3.4 Semisolids

Many drugs that would undergo significant degradation if they were formulated as solutions, can be stabilized by formulating the active ingredient into a suspension or emulsion. Gels, ointments, suppositories, creams, and lotions are typical semisolid preparations. Some of these preparations are incorporated into transdermal delivery systems to provide sustained action over a period of time (see Section 6.6).

In addition to chemical inactivation of the active ingredient, semisolid preparations are subject to a wide variety of physicochemical changes such as separation, sedimentation, creaming, or cracking. Since many ingredients are natural products such as fats, oils, waxes, flavoring agents, and perfumes--they are quite susceptible to oxidation (rancidity) and microbiological contamination.

The effect of additives on the kinetics of the interconversion of succinylsulfathiazole crystals has been investigated (14). These investigations show that a physically stable aqueous pharmaceutical suspension may be achieved by including a suitable retardant.

The autoxidation of the oil phase of an oil-in-water emulsion during storage in light is dependent on the type of emulsifier used (15). The observed differences in the extent of autoxidation might have resulted from different solubilities of oxygen in various emulsifier solutions.

6.3.5 Solids

In the absence of excipients and moisture, topochemical and nucleation reactions occur; some of these approximate first-order reaction rates (16). In the presence of moisture, the decomposition kinetics should be dictated by the rates in the saturated solution and should be zero order. It has been shown that first-order decomposition patterns may be possible in the absence of the moisture (17). This effect has been demonstrated with p-aminosalicylic acid as a model system to approximate the situation encountered in dosage forms.

The discoloration of tablets containing a variety of phar-maceutical compounds, such as 8-hydroxyquinoline sulfate, aminopyrine, papaverine, theobromine, and salicylamide has been markedly reduced by using carboxymethylcellulose sodium solution during granulation. Carboxymethylcellulose sodium acts as a scavenger for trace metals, which are a causative factor frequently encountered in the discoloration of pharmaceutical tablets (18).

The moisture sorption and volume expansion of anhydrous α- and β-lactose tablets has been examined under various humidity conditions (19). The moisture adsorption and tablet expansion occurred more readily with α-lactose tablets, leading to the formation of monohydrate. Lactose induces discoloration of several drugs in solid-dosage forms (20).

Effect of Instability of One Compound on Another A capsule dosage form containing phenylbutazone (see Figure 6.2) and alkalizers showed a large increase in prednisone assay values with blue tetrazolium (BT) reagent under accelerated conditions (21). An investigation by TLC revealed the presence of four impurities (II, III, IV, and VI in Figure 6.2); none of these produce color with BT reagent. A BT spray reagent and a new TLC system have been developed by Ahuja and Spitzer (22) to track the degradation product(s) responsible for the problem. Two new transformation products have been characterized (V and VII). Compound VII is found responsible for interference in the prednisone assay.
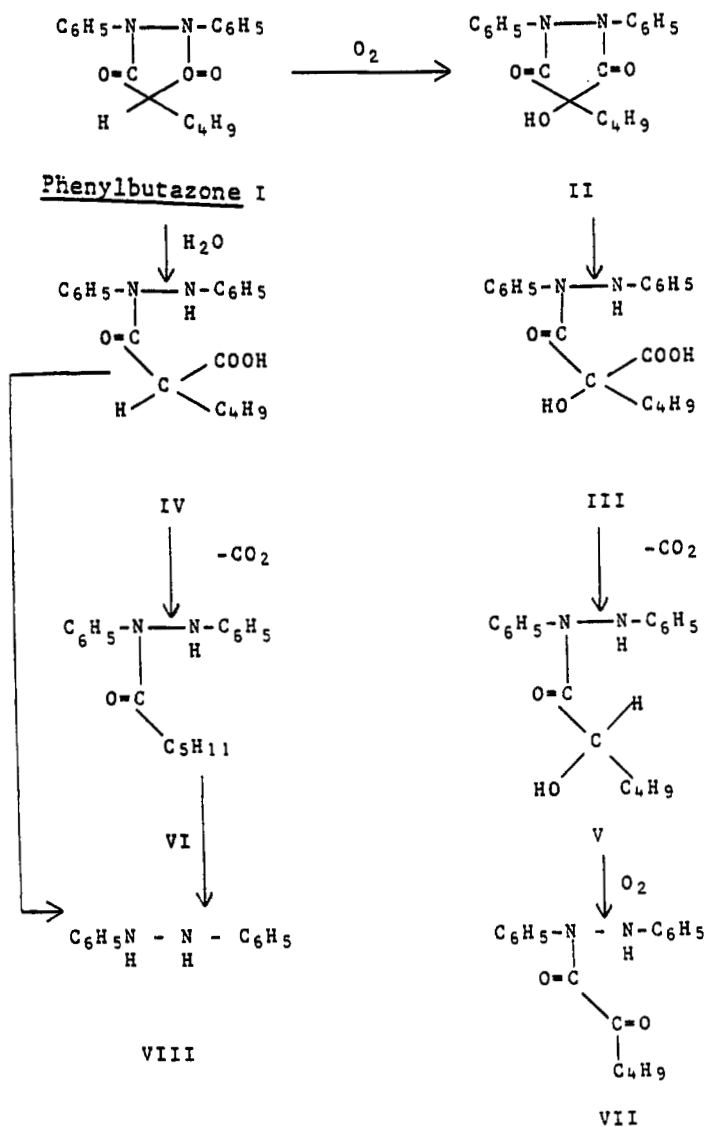
Figure 6.2 Phenylbutazone degradation.

Importance of Selective Methodology Two capsule formulations containing homatropine methylbromide (HMB), antacids, and other ingredients have been investigated for HMB stability under accelerated conditions. TLC, followed by spraying with Dragendorff reagent, revealed the presence of a purple spot. The purple spot is attributable to degradation of HMB to methyltropinium bromide or tropine methylbromide (TMB). This suggests that Dragendorff reaction can be useful for monitoring stability of HMB. The method is stability-indicating because TMB does not yield any precipitate with Dragendorff reagent, and hence would not interfere with the assay. However, it is necessary to stabilize the Dragendorff colorimetric reaction (23) by the addition of methanol to an acetone solution of HMB-iodobismuthate complex in the ratio of 1:1. The stabilization is accompanied by hypsochromic shift from 474 to 382 nm. Stability data on several samples show that a loss as high as 58% degradation of HMB could go undetected by the titrimetric method that has been employed previously (24).

6.4 IMPURITIES IN SOLVENTS

6.4.1 Trace Organic Impurities

Trace organic impurities from solvent(s) can be at times problematic as described in the following case study: O_6-benzylguanine (BG) is an effective substrate for depleting mammalian DNA repair protein belonging to the group alkyltransferase (25). The depletion of alkyltransferase has chemotherapy potential, since it appears that therapeutic effectiveness of certain alkylating agents such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitosourea is greater in cells lacking alkyltransferase. Furthermore, treatment with BG may provide valuable information on the role of the DNA repair protein in carcinogenesis and mutagenesis.

To further evaluate the clinical potential of this poorly water-soluble agent (110 µg/ml), it is necessary to develop a parenteral dosage form at a concentration range of 1-3 mg/ml. An injectable

formulation in PEG 400 (40%) at pH 8 in phosphate buffer developed for further evaluations showed the potential of precipitate formation not attributable to a polymorph or hydrate of BG or the very insoluble degradation product.

The degradation of BG in this solution apparently arises from a reaction between formaldehyde and BG. The formaldehyde is present as an impurity in PEG 400, and it is formed through air oxidation of this solvent. The major product of this reaction is believed to be a methylene-bridged compound containing two BG molecules. This compound is probably produced via an intermediate imine, a Schiff base between one BG molecule and formaldehyde.

6.4.2 Trace Metals

1,3-Benzenediols substituted with aminoalkyl moiety are of significant pharmacological interest. Stability investigations on substituted 5-amino-ethyl-1,3-benzenediol sulfate (AEB), under various exaggerated conditions to find an optimum pH for a stable formulation, revealed that AEB is susceptible to degradation in aqueous solution in the presence of metals (26).

Metal cations such as copper, iron, and calcium have been shown to accelerate the degradation of AEB under an oxygen atmosphere, with concomitant discoloration. (26) The effectiveness of these metal cations in terms of AEB degradation is in the following order: Cu >Fe >Ca. Copper effectively catalyzes AEB degradation down to levels of ten parts per billion in the presence of oxygen.

Details of the kinetic studies may be found in Chapter 7. The results of these studies show that the pH of the solution has a greater effect on AEB degradation (Table 6.2). The rate of degradation is fastest at pH 3 and slowest at pH 5. Based on these studies, it can be concluded that a solution at pH 5 is approximately 6 times more stable than at pH 3.

**Table 6.2 Calculated Apparent First-order
Rate Constants for Kinetic Study**

TEMPERATURE	pH	k_1 (hour^{-1})	k_2 (hour^{-1})
90°C	3.0	*	0.0318
	4.0	0.0029	0.0148
	5.0	0.0019	0.0049
70°C	3.0	*	0.0103
	4.0	0.0003	0.0042
	5.0	0.00008	0.0041
50°C	3.0	--	0.0041
	4.0	0.00026	0.0014
	5.0	0.00003	--

*$k_1 = k_2$

6.5 EFFECT OF BY-PRODUCTS

An enteric-coated tablet that produced a by-product that would blow the cap off the bottle after the cap was loosened has been investigated. This clearly suggested that some gaseous product was being produced that built up on standing. A review of the formulation revealed that the tablets contained cellulose acetate phthalate as a coating, and there was also a subcoating of calcium carbonate. It can be theorized that cellulose acetate phthalate on hydrolysis will produce acetic acid, which can then react with calcium carbonate to produce carbon dioxide. This can be demonstrated in a simple laboratory experiment. The solution to this problem require replacment of the subcoating with a non-reactive compound.

The strength of hydralazine hydrochloride tablets decreases significantly with time if they contain starch as an excipient (27). In a shelf-life study of 10-mg tablets, using HPLC, the levels of usual degradants (i.e., phthalazone, phthalazine, etc.) did not explain the loss as determined by the mass balance. Further studies using HPLC confirmed that the decreased strength of the tablets is due to neither incomplete extraction of hydralazine hydrochloride from the tablet matrix nor to the loss of drug substance during

sample preparation. Binary compatibility tests showed that when hydralazine hydrochloride is heated with each of the excipients separately, the amount of hydralazine hydrochloride decreased significantly in the presence of starch. An investigation to determine the basis of drop in strength showed that starch exposed to the drug exhibited fluorescence at 414 nm. It has been proposed that this fluorescence is due to a triazolophthalazine derivative resulting from hydralazine hydrochloride and starch. Degradation of this moiety generates s-triazolo[3,4-a]phthalazine, which has been detected in aged tablets. This degradation occurs by acid-catalyzed hydrolysis. The quantity of this degradant in the hydralazine hydrochloride tablets explains the loss in observed mass balance due to degradation.

Two common bulking agents, mannitol and lactose, are used for the freeze-dried formulation of methylprednisolone sodium succinate. The effect of these bulking agents has been evaluated at two different ratios of drug to excipients (28). Residual moisture levels were less than 1% in all samples tested, with no significant difference in residual moisture among different formulations. The rate of hydrolysis is significantly higher in mannitol-containing formulations versus lactose-containing formulations, and the rate of hydrolysis increases with an increasing ratio of mannitol to drug.

Thermal analysis and x-ray diffraction data are consistent with a composition-dependent rate of crystallization of mannitol in the formulation and its subsequent effect on distribution of water in the freeze-dried matrix. The increased water content in the microenvironment of the drug decreases the glass transition temperature of the amorphous phase, resulting in an increased rate of reaction. The physical state of lactose remained constant throughout the duration of the study, and the rate of hydrolysis is not significantly different from the control formulation containing no excipient. Thermal analysis and x-ray diffraction data are consistent with formation of a liquid crystal phase in freeze-concentrated solutions of methylprednisolone sodium succinate containing no excipient.

6.6 TRANSDERMAL DRUG DELIVERY

6.6.1 Effect of Vehicle

Topical minoxidil is used for male pattern baldness; however, the mode of penetration of this drug and the site and mechanism of action still remain unclear (29). The commercial formula contains 2% minoxidil in a vehicle composed of 60% ethanol, 20% water, and 20% propylene glycol. The water in the formulation appears to influence the permeation of the solutes far less than the other two solvents. More minoxidil penetrates the skin in hairless mouse skin in Franz diffusion cells experiments as the proportion of ethanol is increased in the mixtures.

Propylene glycol produces both reversible and irreversible changes in protein moieties of skin, enhances solubility of some of the proteins in water, keratin proteins, and changes the cross-link density of keratin within the stratum corneum. The hygroscopic nature of propylene glycol may also draw water into the stratum corneum, increasing the water content of the outermost layers. These experiments indicate that as the concentrations of solvents are altered, they have the potential of influencing other processes that may lead to generation of new impurities and or increase those that are normally produced.

6.6.2 Effect of pH

Various types of polymers have been used as gel-forming agents in hydrophilic matrices for prolonged drug delivery. These polymers include nonionic cellulose ethers such as hydroxypropyl-methylcellulose K4M (HPMC K4M) and acrylic derivatives such as carbopol 974.

Although the influence of formulation on release rate has been investigated (30), the effect of pH on drug release has not been adequately scrutinized. HPMC is not susceptible to pH changes; however, the release rates of drug from HPMC matrices are affected by the pH of dissolution fluid. Furthermore, polymers

possessing carboxyl acid function, e.g., are influenced to a great extent by the pH.

The dissolution of propanol hydrochloride from matrices containing HPMC K4M and carbopol 974 has been investigated using 0.1 M hydrochloric acid or phosphate buffer at pH 4.5 or pH 7.5. In 0.1 M hydrochloric acid, HPMC K4M principally controlled release since carbopol has a low degree of solubility at this pH. As the pH increased, carbopol became increasingly ionized and interacted with propanol hydrochloride to form an insoluble complex, which retarded the release of the drug. DSC and viscometric studies indicate that the two polymers contributed synergistically to the gel network at pH 7.5. It is at this pH that both polymers contribute to matrix integrity and to the control of drug release.

6.6.3 Penetration Enhancers

The usefulness of penetration enhancers in promoting drug permeation across the cornea has been investigated for drugs varying from hydrophilic to lipophilic (31). Four potential penetration enhancers [Azone (aurocapram), hexamethylenelaur-amide, hexamethyleneoctanamide, and decylmethylsulfoxide] are evaluated. Corneal permeation coefficient of drugs that are either hydrophilic (acetazolamide, cimetidine, guanethidine, and sulfacetamide), moderately lipophilic (bunolol and prednisolone), or lipophilic (flubiprofen and its amide analogue) are measured in the presence or absence of various concentrations of Azone. The corneal penetration of hydrophilic compounds is enhanced by at least twentyfold at 0.1% Azone. For prednisolone and bunolol, the maximal enhancement is at 0.025-0.1% Azone and is marginal (two- to fivefold); whereas Azone inhibits rather than enhances the corneal penetration of lipophilic flubiprofen and its amide analogue. All four enhancers behave similarly in enhancing corneal penetration of cimetidine and corneal hydration after incubation.

A computer optimization technique based on response surface methodology has been applied for the optimization of hydrogel

formulation containing indomethacin as a model drug (32). The penetration enhancer comprised a combination of three cyclic monoterpenes: limonene, menthol, and cineole. Pharmacokinetic parameters from an in vivo percutaneous absorption study on rats of model formulations, prepared according to the composite experimental design for five factors, have been determined as the prime response variables. The predicted response values for the optimum formulation can be successfully validated in a repeated in vivo percutaneous absorption study.

The skin permeation of buprenorphine base and HCl salt through cadaver skin have been investigated (33). As expected, buprenorphine free base is more lipophilic than its HCl salt and is practically insoluble in aqueous buffer at pH 8.7. The drug solubility decreases exponentially as the pH of the solution is increased, whereas the permeability coefficient increases as the donor solution pH decreases. The skin flux of buprenorphine HCl is significantly higher than that of the free base from propylene glycol/lauric acid vehicle mixtures. When capric acid, lauric acid, and lauryl alcohol are separately incorporated into an adhesive matrix, the skin flux of buprenorphine·HCl is enhanced by a factor of 2 to 3.5.

In vitro permeation rates of metaproterenol sulfate (MPSD) across hairless mouse skin and TESTSKIN living skin equivalent are very low unless skin permeation enhancers are included in the vehicle (34). A 1:1 molar ratio formulation of MPS and lauric acid has been selected for the in vivo permeation study. The data indicates that lauric acid increases the diffusivity of MPS in the skin by forming a complex and by affecting its partition coefficient between the skin and the delivery system.

6.7 SOFT DRUGS

Soft drugs are biologically active chemical compounds that are used therapeutically as drugs and are characterized by a predictable and controllable in vivo destruction (metabolism) to non-toxic moieties, after achieving their therapeutic role (35). The soft drug

approach combines structure-activity relationship with structure-metabolism relationship to design compounds with higher therapeutic index. They have a metabolically sensitive spot built into their structure to provide a one-step detoxification to metabolites that are inactive and nontoxic. The soft drug approach has been applied to a number of drugs. For example, seven soft drug analogues of propantheline were synthesized, which by design display predictable and controllable decomposition to inactive metabolites. These analogues display facile hydrolysis to an acid, an aldehyde, and an amine. The authors have investigated chemical stability and hydrolytic stability in biological media to assure these compounds are "inactive". It is important to assure that no unknown impurities are being produced that are not being monitored and can be deleterious to humans.

A new type of ultra-short-acting β-blocker which might prove advantageous in treating acute arrhythmia's has been designed, synthesized, and investigated (36). Based on the soft drug "inactive metabolite approach," the inactive phenylacetic acid metabolites of both metoprolol and atenolol were reactivated by esterification with sulfur-containing aliphatic alcohols. Since the sulfur-containing moieties are labile to the ubiquitous esterases, the new compound should be inactivated by one-step enzymatic cleavage back to the inactive phenylacetic acid derivative. The rapid recovery from the β-receptor blockade is believed to be due to fast hydrolysis of soft drugs in the body. This is supported by the in vitro results: hydrolysis studies in phosphate buffer solution indicate that esters are labile to base-catalyzed hydrolysis.

6.8 RECOMBINANT PROTEINS

The successful exploitation of genetically engineered proteins requires preservation of biological activity in all steps of development from purification to storage to administration. Compared to traditional drugs, proteins present many additional problems in preserving their activity because they have higher molecular weight, contain multiple groups, and possess exquisite

three-dimensional structures. The relative fragile nature of most proteins is an obstacle to their processing, storage, and delivery. For example, insulin exhibits instability in both solutions and suspensions.

A critical problem in storage and delivery of proteins is aggregation in the solid state induced by elevated temperature and moisture (37). These conditions are particularly relevant for studies of protein stability during accelerated storage or for proteins loaded in polymeric delivery devices in vivo. When exposed to an environment simulating these conditions, lyophilized insulin shows both covalent and noncovalent aggregation. The covalent process has been elucidated to be intermolecular thiol-catalyzed disulfide interchange following β-elimination of an intact disulfide bridge in the insulin molecule. The process is accelerated by increasing the temperature and water content of the insulin powder or by performing lyophilization and/or dissolution of insulin in alkaline media. The aggregation can be ameliorated by the presence of Cu^{+2}, which presumably catalyzes the oxidation of free thiols. The water sorption isotherm of insulin reveals that the extent of aggregation directly correlates with the water uptake by the lyophilized insulin powder, thus pointing to the critical role of protein conformational mobility in the aggregation process.

The feasibility of spray-drying solutions of recombinant methionyl human growth hormone (hGH) and tissue-type plasminogen activator (t-PA) has been investigated (38). Human growth hormone formulation containing mannitol phosphate buffer and t-PA has been used in an arginine phosphate formulation containing 0.04% (w/v) polysorbate 80. Using filtered air (90-150°C) as the drying medium, hGH could be dried to a residual moisture of $\leq 4\%$. However, 25% of the protein is degraded during the processing. The results of atomization studies suggest that surface denaturation at the air-liquid interface of the droplet in the spray plays a major role in degradation of the protein. The addition of 0.1% (w/v) polysorbate 20 into the hGH formulation reduced the formation of soluble and insoluble aggregates by 90% during atomization. During spray-drying the addition of the same

concentration of polysorbate 20 reduced the formation of soluble and insoluble aggregates by approximately 70 and 85%, respectively. No oxidation, aggregation, or denaturation is observed under severe operating conditions.

Identification of the degradation products of protein is important in optimizing formulations and defining product constituents over the course of their shelf lives. The major aging processes include aggregation/precipitation, oxidation of cysteines and methionines, deamidation of aspargines, peptide bond cleavages at aspartate residues, and aspartate isomerization to isoaspartate (39). Deamidation at aspargine residues, which occurs at neutral to alkaline pHs, involves formation of a succinimide intermediate, followed by loss of ammonia. In addition, peptide chain cleavage at aspartate residue occurs at acidic pH. Isomerization to aspartate residues (with insertion of methylene group into the peptide chain) involves the formation of succinimide intermediate with the loss of a water molecule.

It is important to isolate and characterize degradation products. For example, the degradation products of basic fibroblast growth factor (bFGF) have been isolated by ion exchange HPLC and have been characterized. The predominant product at pH 5 is a succinimide in place of aspartate, as determined by LC/MS, N terminal sequencing, and susceptibility to degradation at pH > 6.5.

Keratinocyte growth factor (KGF) has limited stability in aqueous media because it undergoes denaturation, followed by aggregation at 37°C (40). Anionic polymers and heparin have been shown to increase the denaturation temperatures and extend the half-life of the monomeric, native form of KGF during storage. Salts such as NaCl, sodium phosphate, ammonium sulfate, and sodium citrate have also been found to be highly effective. The fact that the same additives stabilize KGF against both stresses is consistent with the idea that storage stability is determined by denaturation followed by aggregation. Among the osmolytes tested, N,N'-dimethylglycine, trehalose, and sucrose are also effective stabilizers. However, their effectiveness on the thermal

denaturation and the storage stability is not exactly parallel, suggesting that other factors also contribute to the storage stability.

Human epidermal growth factor (EGF) is a single chain polypeptide containing 53 amino acid residues (mw = 6045) and three sulfide bridges (41). It is formulated into ophthalmic and topical preparations for faster and better healing of wounds and burns. In order to use EGF as a therapeutic agent, it is necessary to develop a physically and chemically stable formulation. The most prevalent chemical reaction in EGF is deamidation, a process by which the side chain amide group in glutaminyl or asparaginyl residue is hydrolyzed to form a free carboxylic acid. It is also known that the physical instability of EGF is due to polymerization of monomer into dimer and trimer by disulfide exchange, which may change biological activity or immunological properties. The chemical stability has been improved by utilizing neutral solution (tris buffer) and by the addition of nonionic surfactants (0.01-0.1%) or polymers (0.1-1.0%), at low concentrations. Aggregation can also be prevented by nonionic surfactants (0.01%-0.1%).

Leucine enkephalin (YGGFL) undergoes rapid degradation in sheep nasal mucosa to yield des-tyrosine leucine enkephalin (GGFL) which degrades further (42). The activity of nasal mucosa homogenate against YGGFL and GGFL is greater than that observed with the nasal wash fluid ($t_{1/2}$ 40 and 13 min). The effect of cyclodextrins on the rate of degradation of FGG and YGGFL by leucine aminopeptidase (LAP) and of GGF by carboxypeptidase A (CPA) has been monitored to improve stability. Little effect is observed in the presence of LAP with FGG; however, the half-life of YGGFL is extended from approximately 44 min. to 75 min. in the presence of 25-fold excess of β-cyclodextrin. The stability of GGF is also enhanced: an effect is observable with a 5-fold excess of cyclodextrin and the half-life can be extended by 40-75%.

6.9 ALTERNATE DELIVERY SYSTEMS

Stimuli-sensitive polymers are suitable candidates for the prevention of gastric degradation of oral peptide drug delivery

vehicles, since they will prevent gastric degradation while providing a controlled release of a peptide drug such as calcitonin (a polypeptide with a molecular weight of 3418). The polymeric beads are formed from pH/temperature-sensitive linear terpolymers (poly (N-isopropylacrylamide-co-butylmethacrylic acid) and loaded with the aqueous solution of calcitonin (43). The results show that beads made with high content of acrylic acid (most hydrophilic) provide better loading, stability, and release of human calcitonin.

Biodegradable polymers have been used for the last three decades as drug carriers in implantable devices. Various drugs have been incorporated in hydrolyzable polyesters and polyanhydrides to achieve extended release under physiological conditions (pH 7.4 at 37°C). Some of these drugs contain reactive amines that may compete with the hydrolysis process of the polymer carrier and react with the polymer to form undesired amide derivatives (44). Drug matrix interactions occur in several situations: (a) during drug incorporation in the polymer matrix by injection or compression molding fabrication processes, where high pressure and temperature are applied; (b) during long-term storage; and (c) during the process of biodegradation and drug release. The polymer-drug interaction depends on both the drug and the characteristics of the polymer.

The interaction between primary phenylalkylamines and their hydrochloride salts with several degradable polymers has been investigated under physiological conditions in vitro. Solutions of phenylalkylamines with increasing nucleophilic reactivity have been reacted heterogeneously with representative biodegradable polyanhydride and polyester powders in various pH solutions, and the recovery of the amines from the solutions is determined. Poly(sebacic acid), a reactive polyanhydride, reacted by amide formation with tested amines and their respective HCl salts when exposed to physiological pH 7.4. However, at pH 5.0 no interaction occurred.

The aromatic polyanhydride and the polyester based on lactic acid and caprolactone do react with the amine derivatives at pH

7.4, but at a slow rate. This reaction can be avoided altogether by appropriate selection of salt derivatives of the amines.

6.10 POLYMORPHS, HYDRATES, DESOLVATED SOLVATES, AND AMORPHOUS FORMS

The FDA's drug substance guidelines state that appropriate analytical procedures should be used to detect polymorphic, hydrated, or amorphous forms of drug substance. The guideline also states that it is the applicant's responsibility to control the crystal form of the drug substance and, if bioavailability is affected, to demonstrate the suitability of control methods. The relevance to the pharmaceutical industry lies in the fact that polymorphs and solvates (pseudopolymorphs) of a drug generally display differences in their physical properties such as melting behavior, density, morphology, and solubility. Consequently, properties such as tableting behavior and bioavailability may show considerable variation, depending on which form is chosen for solid drug formulation. Therefore, the effect of formulation, processing, etc. should be carefully considered as any of the material produced that influences bioavailability can be construed as an impurity.

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7
Kinetic Studies

The primary aim of performing kinetic studies for pharmaceutical compounds is to predict the rate of degradation reaction(s) and to understand the mechanism of the reaction(s). Of course other chemical reactions can also occur in a drug product. When the potential of such reactions exists, they should also be evaluated. Furthermore, developing an understanding of these reactions provides us with valuable information as to which degradation products or by-products are likely to constitute significant impurities that need to be monitored (1,2).

It is important to remember that many chemical changes involve two or more reactions that are going on simultaneously in a very complicated manner. Trace amounts of some materials that may originate as impurities from pharmaceutical compounds or excipients can catalyze these reactions. When two substances are mixed together, there are many different products possible according to thermodynamics; however, temperature, concentration, or a specific catalyst can make some reactions more favorable.

Only a few papers on drug kinetics appeared in the literature through the 1940s. Although the theory was well understood and the groundwork in chemical reaction kinetics was laid down, detailed studies on drug kinetics were not undertaken until the

1950s. Two of the main contributors to the improvement of our understanding of kinetic principles as applied to drug development are Higuchi and Garrett (3,4). They applied the principles of chemical kinetics to the evaluation of drug stability. The classical concepts that were brought to bear were the consideration of factors influencing reactions in solution.

The majority of degradation reactions of pharmaceutical compounds in solution occur at finite rates and are chemical in nature. These reactions are affected by conditions such as solvent, concentration of reactants, temperature, pH of the medium, radiation energy, and the presence of catalysts. The manner in which the reaction rate depends on the concentration of reactants describes the order of the reaction. The degradation of most pharmaceuticals can be classified as zero order, first order, or pseudo-first order, even though they may degrade by complicated mechanisms and the true expression may be of higher order or more complex and noninteger.

The quantitative relationship of the specific reaction rate and temperature is given by the Arrhenius expression:

$$k = Ae^{-\Delta Ha/RT} \qquad [Eq. 7.1]$$

where

k = specific rate constant
T = temperature in degrees Kelvin
R = gas constant
A = preexponential factor, a constant associated with the entropy of the reaction and /or collision factors
ΔHa = heat of activation

The equation is usually employed in its logarithmic form:

$$\log k = -(\Delta Ha/2.303RT) + \log A \qquad [Eq. 7.2]$$

The slope of plot of log k against 1/T yields the activation energy. This equation provides the underlying basis that allows

prediction of stability of pharmaceuticals by extrapolation of rate data obtained at higher temperatures.

There are many pitfalls in extrapolation of kinetic data. It is important to understand the limitations of the experimentally obtained heat of activation values prior to attempting stability prediction. For example, the apparent heat of activation at a pH value where two or more mechanisms of degradation are involved is not necessarily constant with temperature. Also, the ion product of water, pKw, is temperature-dependent, and -ΔHa is approximately 12 kcal, a frequently overlooked factor that must be considered when calculating the hydroxide-ion concentration. Hence it is necessary to obtain the heats of activation for all bimolecular rate constants involved in a rate-pH profile to predict degradation rates at all pH values for various temperatures.

7.1 ORDER OF REACTIONS

The rate of reaction can be determined by measuring either rate of decrease of concentrations of the reactants or the rate of increase of concentrations of the products. The rate of decrease in concentration with time is represented as -dc/dt; the increase in concentration, on the other hand, is depicted without the negative sign. Let's briefly review the order of reactions that are more likely to be encountered in pharmaceutical analysis (5,6).

7.1.1 First-order Reactions

This is the simplest case in which the rate of reaction is found experimentally to be directly proportional to the concentration of the reacting substance. Mathematically, it can be expressed as follows:

$$-dc/dt = kc \qquad \text{[Eq. 7.3]}$$

where

$$c = \text{concentration of the material}$$

$$t = \text{time}$$
$$k = \text{reaction-rate constant}$$

On integrating Equation 7.3, we get

$$-\ln c = kt + \text{constant} \qquad \text{[Eq. 7.4]}$$
$$\text{or}$$
$$\log c = \frac{-kt}{2.303} - \frac{\text{constant}}{2.303} \qquad \text{[Eq. 7.5]}$$

A typical plot of first-order degradation is shown in Figure 7.1.
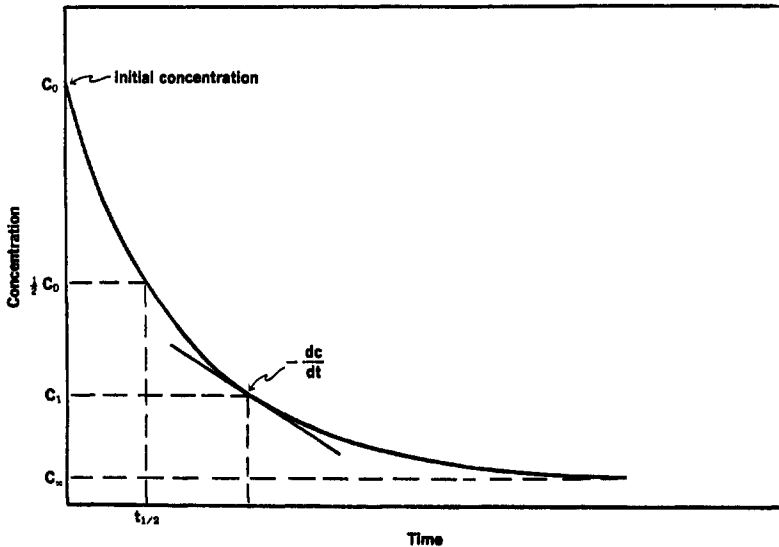
Figure 7.1   Change in concentration of a
decomposing drug with time.

A straight line is produced when the logarithm of concentration is plotted against time. The first-order rate constant k can be determined by multiplying the slope of the line by -2.303.

Integrating Equation 7.3 at concentration c_1 at time t_1, and c_2 at time t_2, we get

$$k = \frac{2.303}{t_2 - t_1} \log \frac{c_1}{c_2} \qquad [Eq. 7.6]$$

The specific reaction rate constant or the velocity constant, k, for a first-order reaction is a number per unit of time and may be expressed in reciprocal seconds or minutes.

The half-life of a reaction, $t_{1/2}$, can provide a quite useful piece of information on reaction, and this half-life can be calculated as follows:

$$k = \frac{2.303}{t_{1/2}} \log \frac{1}{1/2} \qquad [Eq. 7.7]$$

or
$$t_{1/2} = 0.693/k \qquad [Eq. 7.8]$$

For pharmaceutical products, it may be of interest to determine when, for example, 10% degradation ($t_{10\%}$) would occur; $t_{10\%}$ can be calculated by employing the appropriate substitutions in Equation 7.7.

7.1.2 Second-order Reactions

The reaction is considered a second-order reaction when the rate of reaction, as determined experimentally in a laboratory, is proportional to the concentration of each of the two reacting substances. For example, if X and Y react to produce Z as follows:

$$X + Y \leftrightarrow Z \qquad [Eq. 7.9]$$

The reaction is second order if

$$-dc_X/dt = -dc_Y/dt = k\ c_X c_Y \qquad \text{[Eq. 7.10]}$$
$$\text{If } c_X = c_Y, \text{ then} \qquad \text{[Eq. 7.11]}$$
$$-dc_X/dt = kc_X^2 \qquad \text{[Eq. 7.12]}$$

If a and b represent the initial molar concentration of the two reacting substances X and Y, and if x denotes the number of moles per liter that react in time t, the velocity of reaction is expressed by:

$$dx/dt = k(a-x)(b-x) \qquad \text{[Eq. 7.13]}$$

On integration and evaluating the integration constant, it is possible to obtain the following expression for second-order reactions:

$$k = \frac{2.303}{t(a-b)}\ \log\ \frac{b(a-x)}{a(b-x)} \qquad \text{[Eq. 7.14]}$$

When log [b(a-x)/a(b-x)] is plotted against t, a straight line is obtained if the reaction is second order. Its slope is k(a-b)/2.303, and k is then obtained by multiplying the slope of the line by 2.303/
(a-b). The value of k in the second-order reaction depends on the units in which the concentration is expressed. For example, if it is expressed in moles per liter, then k will be in liters/mole/second.

A well-known example of a second-order reaction is hydrolysis of an ester, such as ethyl acetate, by an alkali:

$$C_2H_5OOCCH_3 + OH^- \leftrightarrow C_2H_5OH + CH_3COO^- \qquad \text{[Eq. 7.15]}$$
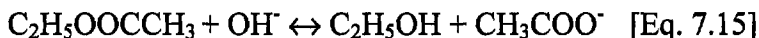
7.1.3 Zero-order Reactions

The reaction in which the rate constant is unaffected by the concentration is described as zero-order reaction. Zero-order reactions are influenced by factors such as absorption of light in

photochemical reactions or the area of catalyst in certain surface reactions. Here

$$-dc/dt = k'$$ [Eq. 7.16]

The concentration of the material remains constant as is exemplified by a saturated solution in contact with a solid. Although c is constant, x (the amount of material reacting) is given by the expression

$$dx/dt = k'$$ [Eq. 7.17]
$$x = k't + constant$$ [Eq. 7.18]

The value of k calculated in this way may include arbitrary constants corresponding to the intensity of light or the concentration of saturated solution or vapor.

7.2 DETERMINATION OF THE ORDER OF REACTIONS

As mentioned before, the reaction rates in pharmaceutical analysis are frequently assumed to occur by the first order or zero order. However, it is important to test these assumptions and determine whether the reaction fits any given order. The order of reaction is determined by the exponents of concentration terms, which determine the rate of reaction. For example, in the following reaction:

$$A + B \leftrightarrow Z$$ [Eq. 7.19]

The rate expression for Equation 7.19 may be

$$dc_Z/dt = kcA^m cB^n$$ [Eq. 7.20]

This reaction is mth order with respect to A, nth order with respect to B, and (m+n)th order for the overall reaction. The rate of consumption of A or B or the rate of production of Z depends on

the concentration c of each of the materials taking part in the reaction, each raised to an exponent which is determined empirically by the experiment. The exponents can be whole numbers or fractions.

7.3 COMPLEX REACTIONS

The discussion above deals mainly with first-, second-, or zero-order reactions. Third-order reactions are similar to second-order reactions except three molecules are involved in the reaction. The fact is very few reactions follow first-, second-, or third- order equations throughout their whole course. More often then not two or more different reactions are taking place simultaneously, so that the mathematical expression of the reaction is the result of several different expressions. The complicating reactions may be consecutive, reversible, or a competing type as shown below.

Consecutive reactions: $A \rightarrow B \rightarrow C$

Reverse reactions: $A + B \rightleftharpoons AB$

Competing reactions: $A \begin{array}{c} \nearrow^{+B} AB \\ \searrow_{+C} AC \end{array}$

Even though the complex reactions may be made of simple steps, it may be exceedingly difficult to describe the overall reaction mathematically. It may be preferable to choose the chemical reactions for laboratory study where all but one of the reactions are negligible. For example, in the consecutive reaction denoted here, if B→C is 100 times faster than the preceding reaction, then the latter will be the rate-determining step. In

reversible reactions, the reverse reaction may be ignored if it does not affect the concentration appreciably. In competing reactions, the product that is formed by the fastest reaction will consume most of the reacting materials and will be the predominant product (see parallel reactions below). In studying chemical kinetics, those reactions are usually chosen in which one reaction predominates and all other reactions, whether consecutive, reverse, or competing, are relatively slow.

7.4 INFLUENCE OF TEMPERATURE

The following rule of thumb is frequently used to explain the effect of temperature on reaction:

The reaction rate doubles for every 10°C rise in temperature.

It should be noted that this is more likely to be true at or near room temperature. The real quantitative relationship, as mentioned above, is given by the Arrhenius expression (Equation 7.1): a straight line is produced when the specific reaction rate is plotted against the reciprocal of absolute temperature, based on the logarithmic form of this equation (Equation 7.2).

For pyrolytic reactions, the heat of activation is likely to be high and the degradation rates obtained at elevated temperatures are of little practical value for extrapolation to room temperature.

7.5 ACTIVATION ENERGY

The theoretical basis of reaction kinetics requires that molecules become activated before they can react. Apparently all molecules are not equally reactive, as is explained by the slowness of some reactions. Since the number of collisions per second is enormous, it might be expected that all reactions would be instantaneous. As a matter of fact, ionic reactions, such as neutralization and precipitation, are immeasurably fast because the energy of activation required for ions to react is usually very small.

In chemical kinetics, a reaction may proceed as follows:

$$X \quad \xrightarrow{\text{slow}} \quad Y \quad \xrightarrow{\text{fast}} \quad Z$$

(reactants)   (activated molecules)   (products)
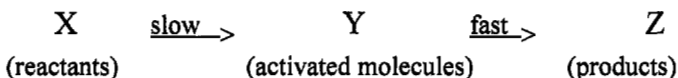
The rate-determining step is the slow reaction requiring an activation energy of ΔHa that can be calculated from the following equation:

$$\Delta Ha = H_Y - H_X \qquad \qquad \text{[Eq. 7.21]}$$

For the reversible process $Z \rightarrow Y$,

$$\Delta Há = H_Y - H_Z \qquad \qquad \text{[Eq. 7.22]}$$

The relationship between heat of activation and heat of reaction is shown in Figure 7.2.
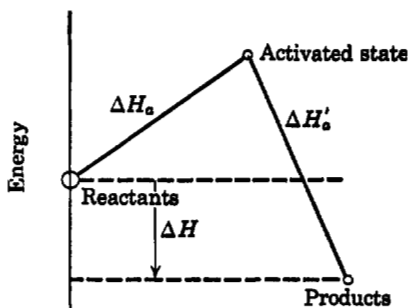
Figure 7.2   Activation energy of forward
and reverse reactions.

The molecules of X require absorption of a definite amount of heat, ΔHa, to reach the activated state Y from which they produce product Z. The reversible reaction demands that molecules Z be supplied with the heat of activation, $\Delta Há$, and, thus in passing

from the activated states of Y to Z, an amount of heat equal to Δ Há is created. The heat of reaction is then equal to the difference between the heats of activation in two directions:

$$\Delta H = \Delta Ha - \Delta Há \qquad [Eq. 7.23]$$

The heat evolved in the step Y to Z is generally greater than the heat absorbed in step X to Y, and the overall reaction going from X to Z results in an evolution of heat. If ΔHa is greater than ΔHá, the reaction X to Z is called endothermic. In the opposite case, the reaction is described as exothermic.

If the reaction is carried out at constant volume rather than constant pressure, then the heat of reaction is ΔE rather than ΔH. In solutions, where there is little change in volume, the two are essentially the same.

The energy required for the activated state is the chief factor in determining the speed of the reaction. In general, for endothermic reactions, the energy required for activation must be at least as great as the endothermic heat of reaction. It is important to remember that heat of activation ΔHa cannot be measured calorimetrically because activated molecules exist only briefly. It is necessary to determine it indirectly by plotting log k against 1/T and multiplying the slope by -2.303R, whereas heat of activation is determined by plotting log K against 1/T, where K is the equilibrium constant.

7.6 PREDICTION OF REACTION RATES

As discussed above, the Arrhenius equation (Eq. 7.1) is very useful for calculating reaction rates of many chemical reactions over a wide range of temperatures. To predict reaction rates, it is generally necessary to split chemical reactions, no matter how complex, into a number of reverse, successive, or competing reactions, in a way that each of these is a unimolecular or bimolecular reaction. Furthermore, it is possible to estimate the frequency factor, A, and activation energy, ΔHa, of each of these reactions and thus a rough

estimation of the rates of fundamental, primary reactions can be made.

The fact that the range of activation energies is limited from 10,000 to 100,000 calories per mole simplifies the prediction of reaction rates. Very few chemical reactions require more than 100,000 calories per mole of activation energy, and those that require less than 10,000 cal are usually too fast to measure. Graphs are available that allow a rough estimate for many simple unimolecular reactions; however, large errors may occur, particularly in first-order reactions in solutions.

7.7 PARALLEL REACTIONS

These reactions are also called competing or side reactions and are quite common in drug products particularly when organic compounds are involved. For example, in some pharmaceutical systems, buffers are used to maintain the solution at a particular pH. Often, in addition to the effect of pH on the reaction rate, there may be catalysis by one or more species of the buffer components. The reaction is then said to be general acid or general base catalysis, depending upon whether the catalytic components are acidic or basic. These reactions also belong to this class of reaction.
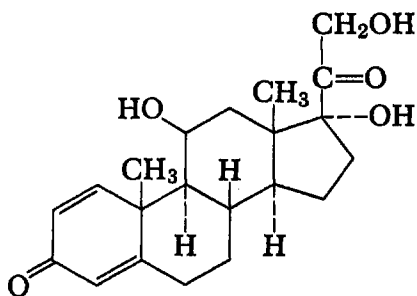
Prednisolone
($\Delta^{1,4}$ pregnadiene-11β,17α,21-triol-3,20-dione)

The base-catalyzed degradation of prednisolone (7) can be used to illustrate these reactions. A study has been carried out at 35°C, and the rate of disappearance of the dihydroxyacetone side chain has been followed by appropriate analytical techniques. The decomposition of prednisolone involves parallel pseudo-first-order reactions, with the appearance of acidic and neutral steroid products.

The mode of reaction may be represented as follows:

$$P \begin{array}{c} \xrightarrow{k_1} A \\ \xrightarrow{k_2} N \end{array}$$
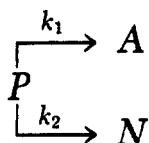
where
P = concentration of prednisolone
A = concentration of acid product
N = concentration of neutral product

The decomposition rate equation is as follows:

$$\frac{-dP}{dt} = k_1 p + k_2 P = kP \qquad \text{[Eq. 7.24]}$$

On integration, we get

$$P = P_0 e^{-kt} \qquad \text{[Eq. 7.25]}$$

The rate of formation of acidic product can be determined as follows:

$$\frac{dA}{dt} = k_1 P = k_1 P_0 e^{-kt} \qquad \text{[Eq. 7.26]}$$

On integration, we get

$$A = \frac{A_0 + k_1 P_0 (1 - e^{-kt})}{k} \qquad \text{[Eq. 7.27]}$$

A_0 can be eliminated from the above equation since no acid product is formed before prednisolone begins to decompose.

Similarly the equation for the neutral product is

$$N = \frac{k_2 P_0 (1-e^{-kt})}{k} \qquad \text{[Eq. 7.28]}$$

The last two equations suggest that for base-catalyzed degradation of prednisolone, a plot of A or N against $(1-e^{-kt})$ should give a straight line and the value of k_1 can be calculated from the slope of the line.

$$k_1 = \text{slope x } k/P_0 \qquad \text{[Eq. 7.29]}$$

The value of k_2, the rate constant for the formation of neutral degradation product is obtained by subtracting k_1 from k.

7.8 STEADY STATE

It is not always possible to exactly integrate a kinetic process. In these cases it is useful to postulate a reasonable reaction sequence and then derive a rate law that applies to a postulated sequence of steps. The observed kinetics of the reaction should match the curve given by the derived rate law if the postulate is reasonable and reflects the actual steps involved in the reaction. An excellent example of this is the Michaelis-Menten equation. (It is assumed that the interaction of a substrate S with an enzyme E to yield product P follows a reaction sequence shown below.)

$$E + S \underset{k_2}{\overset{k_1}{\longleftrightarrow}} (E \cdot S) \overset{k_3}{\longrightarrow} P \qquad \text{[Eq. 7.30]}$$
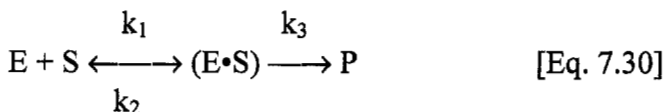
$$\frac{dP}{dt} = k_3 (E \cdot S) \qquad \text{[Eq. 7.31]}$$

Extensive treatment of the preceding yields the Michaelis-Menten equation.

$$\frac{dP}{dt} = \frac{k_2\,SE}{K_m+S}$$ [Eq.7.32]

In an enzyme study, we can usually determine S, P, and E_0, the total concentration of enzyme. K_m is the Michaelis-Menten constant and indicates the tendency of the enzyme substrate complex to decompose to the starting substrate and proceed to the product, relative to the tendency of the complex to be formed.

7.9 EFFECT OF SOLVENTS

The influence of solvents on the rate of degradation is of great importance to all personnel involved with a pharmaceutical product especially the developing pharmacist and the pharmaceutical analyst. The effect of solvents is rather complicated and generalizations are hard to make. However, the following points are of interest:

1. It appears that the reaction of nonelectrolytes is related to the relative internal pressures or solubility parameters of the solvent and solute.
2. Polar solvents (i.e., those with high internal pressures) tend to accelerate reactions that form products having higher internal pressures than the reactants.

A simplified discussion of solvent effects is given below.

7.9.1 Dielectric Constant

When both reactants are ions in a solvent medium or a continuous dielectric, absolute theory gives the following equation:

$$\ln k = \ln k_0 - \frac{N}{RT}\,\frac{Z_A Z_B e^2}{\varepsilon\gamma}$$ [Eq. 7.33]

where ln k is the rate constant at the dielectric constant ε, ln k_0 is the rate constant in the medium of infinite dielectric constant, N is Avogadro's number, Z_A is the charge on ion A, Z_B is the charge on ion B, e is the electronic charge, T is the absolute temperature, R is the gas constant, and γ is proportional to the interatomic distance in the activated complex.

This equation predicts a linear relationship between ln k and $1/\varepsilon$. No effect of the dielectric constants would be noted if one of the molecules were neutral, because Z_A and Z_B would be zero.

The effect of the dielectric constant on the reaction rate between an ion and a neutral molecule is expressed as:

$$\ln k = \ln k_0 + \frac{NZ^2e^2}{2eRT} \left(\frac{1}{\gamma_1 - \gamma_2} \right) \qquad \text{[Eq. 7.34]}$$

where γ is the radius of reactant ions.

Equation 7.34 predicts that the logarithm of the rate constant will vary linearly with the reciprocal of the dielectric constant. However, many drugs are quite complex and do not appear to follow theory, e.g., the solvolysis rate of the aspirin anion increases with an increasing ethanol concentration, but the rates are relatively constant with an increasing dioxane content. Both of these solvents should have produced a decrease in the overall rate. Based on the experimental results, it can be concluded that a possible rate-determining step is the attack of water or ethanol on an unchanged cyclic intermediate (8,9).

Kinetic studies on the hydrogen ion catalyzed degradation of the antibiotic chloramphenicol in water-propylene glycol systems show that a decrease in dielectric constant resulted in an increase in the rate of reaction, a finding that agrees with the requirements for an ion-dipole reaction (10).

The replacement of water by other solvents is often employed in pharmaceutical product development as a means of stabilizing drugs against possible hydrolysis. The results of the above-mentioned study suggest that the use of a solvents mixture with low

dielectric constant may actually increase rather than decrease the rate of decomposition. This suggests that there is a need to perform kinetic studies and to do cautious interpretation of results in stability evaluation of drugs.

7.9.2 Ionic Strength

For reactions involving two ionic species, the rate constant is dependent on the ionic strength, μ. For aqueous solutions at 25°C, Equation 7.35 expresses the rate constant with ionic strength:

$$\log k = \log k_0 + 1.02\, Z_A Z_B \mu^{\frac{1}{2}} \qquad [\text{Eq. 7.35}]$$

A straight line with a slope equal to $1.02\, Z_A Z_B$ is obtained when $\log k$ is plotted versus the square root of μ. Equation 7.35 predicts no effect on a reaction when one reaction is neutral, but the activity coefficient of a neutral molecule is affected by ionic strength, and one can observe a linear relationship between the logarithm of the rate constant and ionic strength:

$$\ln k = \ln k_0 + b\,\mu \qquad [\text{Eq. 7.36}]$$

where b is an empirical constant.

These ionic effects are called the primary salt effect. In addition, a secondary salt effect is observed, which is the effect of ionic strength on the dissociation constant of a buffer species.

7.10 CATALYSIS

7.10.1 Acid-Base Catalysis

Many pharmaceutical compounds are subject to general acid, general base, or nucleophilic catalysis in addition to hydrogen-ion or hydroxide-ion catalysis. Several linear free energy relationships quantitate the catalytic rate constant with a property of the species and relate the rate constant for a series of reactions. For acid-base

catalysis, this free energy relationship is the Bronsted catalysis law and can be expressed as:

$$k\ G_A = G_A K_A \alpha \qquad \text{[Eq. 7.37]}$$
$$\text{and}$$
$$k\ G_B = G_B K_B \beta \qquad \text{[Eq. 7.38]}$$

where K_A and K_B are acid and base dissociation constants, respectively, and G_A, G_B, α, and β are constants characteristic of the solvent, temperature, and reaction.

Many drugs have ionizable groups, and the reactions may proceed differently for the ionized and the unionized forms. However, analytically, the total drug concentration is usually measured. For example, for a weak base, the contribution of the ionized and unionized drugs are related through the pKa of the drug and the pH of the medium. The overall reaction rate is the sum of both reactions. Two examples, aspirin and barbiturates, that demonstrate the effect of ionization on the rate constant and the mode of degradation are provided later in this chapter. Some comments on catalysis where a specific catalyst is involved follow.

7.10.2 General Catalysts

A catalyst is defined as a substance that influences the speed of reaction without being chemically altered itself. It is well known that rate of reaction is influenced by the presence of a catalyst. Catalysis is conceptualized to operate this way. The catalyst combines with the reactant known as substrate and forms an intermediate known as a complex, which then decomposes to regenerate the catalyst and yield the products. It appears that the catalyst decreases the energy of activation by changing the mechanism of the process with a corresponding increase in the rate of reaction. Alternatively, a catalyst may act by producing free radicals, which act as intermediates.

Catalytic reactions may be heterogeneous or homogeneous and can occur in a gaseous or liquid state. Heterogeneous catalysis

occurs when the catalyst and reactants form separate phases in the mixture.

Acid-base catalysis is a well known example of homogeneous catalysis in the liquid phase. These reactions are of significant interest in pharmaceutical analysis and are discussed later in this chapter and in Chapter 9.

A number of examples of catalysis are given in Table 7.1 in Section 7.11.

7.11 LIGHT

Exposure to light is known to be deleterious to a number of pharmaceutical compounds. Light can provide the necessary activation energy to induce some degradation reactions. However, radiation of the proper frequency and sufficient energy must be absorbed to activate the molecules. The unit of radiation energy is known as photon and is equivalent to 1 quantum of energy. The reactions affected by light energy are called photochemical reactions. These reactions are not dependent on temperature for activation of the molecules; therefore, the rate of activation in such molecules is temperature-independent. However, after a molecule has absorbed a quantum of radiant energy, it may collide with other molecules, raising their kinetic energy, and this leads to an increase in the temperature of the system. As a result, the initial photo-chemical reaction may be followed by a thermal reaction.

To study photochemical reactions, it is necessary to control the wavelength and intensity of light and the number of photons actually absorbed by the material. Photochemical activation reactions are usually complex and proceed by a series of steps. The rates and mechanisms of various stages can be elucidated through a detailed investigation of all factors involved. Such detailed investigations are generally not necessary for evaluating photochemical kinetic studies on pharmaceuticals.

Sunlight having an intensity of about 8000 footcandles can cause destruction of nearly 34% of vitamin B_{12} in 2 hours (11). Sunlight or normal light at a windowsill is frequently used in the

pharmaceutical industry as a quick screen of light instability of drug products. Light cabinets that provide known footcandles of light are used for planned stability studies.

The photolytic degradation of fumagillin (12) in ethanol has been reported as a first-order reaction that is caused by light of wavelength below 400 nm.

If photolysis is the rate-determining step of the reaction, most often no predictive advantage is obtained by higher-temperature studies, because ΔHa is small and, hence, the effect of temperature is small.

Some examples of the effect of light are illustrated below in Table 7.1.

Table 7.1   Catalysis by Light and Other Agents

COMPOUND	CATALYST
Epinephrine	Sodium metabisulfite, sodium bisulfite, acetone bisulfite
Penicillins	Copper(II)-glycine chelate, copper(II)
Penicillin G potassium	Monohydrogen and dihydrogen citrate ions
Cyclic anhydrides	Perchloric acid
Nalidixate sodium	Light
9-Aminomethylacridan	Light
Phenothiazine	Light
Dihydroergotamine mesylate	Light
Antipyrine (phenazone)	Light
Aminopyrine (aminophenazone)	Light
Dipyrone (noramidopyrine methanesulfonate)	Light
α-[(Dibutylamino)methyl]-6,8-dichloro-2-(3',4'-dichlorophenyl)-4-quinolinementhanol	Light

7.12 HYDROLYSIS

A reaction in which water participates as one of the reactants is called hydrolysis. Well-known examples of such reactions in pharmaceutical compounds are esters and amides. Hydrolytic reactions that are favored in acidic pH are better described as protolysis. However, the term hydrolysis is more often used by pharmaceutical analysts. It must be recognized that molecular hydrolysis reactions proceed much more slowly than the ionic type (protolysis).

Many drugs are derivatives of carboxylic acids or contain functional groups based on this moiety, e.g., esters, amides, lactones, lactams, imides, and carbamates. Pharmaceutical compounds are susceptible to acid and/or base hydrolysis. For example aspirin hydrolysis has been found to be a first-order reaction (13) and is catalyzed by hydrogen and hydroxyl ions. The hydrolysis is much greater at pHs above 10. For a more detailed discussion, see Section 7.14.

Patel and Lemberger (14) have investigated hydroxyl ion catalysis of homatropine, homatropine methylbromide, atropine, and atropine methylbromide. It has been observed that homatropine base decomposes at a rate 5 times that of atropine base. A comparison of the rate constants of quaternary salts with the free bases shows a thirty-five-fold increase in rate over the free bases.

A number of hydrolytic reactions are summarized in Table 7.2

Table 7.2   Hydrolytic Reactions

COMPOUND	REACTION
Salicylamide	Amide hydrolysis
N-Haloaceytlphthalimides	Substituted amide hydrolysis
1-Acylphthalimides	Substituted amide hydrolysis
N-Acylphthalimides	Imide hydrolysis
Meperidine	Ester hydrolysis
Pyridoxine monooctanoate	Ester hydrolysis
Trantelinium bromide	Ester hydrolysis
Salicylanilide N-methylcarbamate	Carbamate hydrolysis
4-Biphenyl-N-methylcarbamate	Carbamate hydrolysis

Table 7.2   Hydrolytic Reactions (continued)

COMPOUND	REACTION
17α-Acetoxy-6α-methyl-4-pregnen-3,20-dione 3-oximino ester	Hydrolysis of oximino ester
Penicillins	Hydrolysis of β-lactam
Cephalosporins	Hydrolysis of β-lactam, intramolecular aminolysis
Clindamycin	Dethiomethylation
5-aminodibenzo[a,d]cycloheptane derivatives	Deamination
Cytarabine (arabinosylcytosine)	Deamination
Cytosine	Deamination
Cytidine	Deamination
5-Azacytidine	Deamination, scission of N–C bond
Chlordiazepoxide	Deamination, scission of C=N linkage
N-Chlorosuccinimide	Dechlorination
N-Chloroquinuclidinimide ion	Dechlorination
N-Chloro-N-methylbenzene-sulfonamide	Dechlorination
N-Chlorinated piperidines	Dechlorination
Iodocytosine	Deiodination, deamination
Δ^9-Tetrahydrocannabinol	Hydration and ether solvolysis
Antimycin A_1	Hydrolytic ring cleavage, loss of CHO group
Dexoxadrol	Hydrolysis of ketal group
Hydrochlorothiazide	Ring opening through hydration of free or cationic imine
Mazindol	Scission of C=N linkage
Methaqualone	Ring cleavage
Coumarinic acid	Lactonization
Canrenone	Scission of C–S bond, lactonization

7.13 OXIDATION

The loss of hydrogen from a molecule is generally called oxidation. More appropriately, it can be defined as the removal of electrons from a molecule. When a reaction involves molecular oxygen, it is commonly called autoxidation because it usually occurs spontaneously under ordinary conditions. Oxidation frequently involves free radicals and accompanying chain reactions. These radicals tend to take electrons from other substances and thus bring about oxidation.

The most common form of oxidative decomposition occurring in pharmaceuticals is autoxidation through a free radical chain process. The free radicals are produced by homolytic bond fission of a covalent bond:

$$A{:}B \rightarrow A\bullet + B\bullet$$

The radicals readily remove electrons from other molecules, and the process is called oxidation. The autoxidation of the free radical chain process can be depicted by the reactions shown in the scheme below.

$$RH \xrightarrow[\text{catalysis}]{\text{heat, light}} R\cdot + H\cdot$$

$$R\cdot + O_2 \rightarrow RO_2'$$

$$RO_2' + RH \rightarrow ROOH + R\cdot$$

$$ROOH \rightarrow RO\cdot + \cdot OH$$

$$RO_2' + X \rightarrow products$$

$$RO_2' + RO_2' \rightarrow products$$
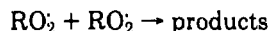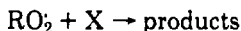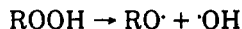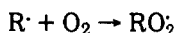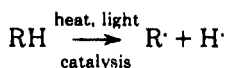
Autoxidation of ascorbic acid has been studied in great detail. Cupric ion has been observed to oxidize ascorbic acid rapidly to dehydroascorbic acid, and KCN CO has been found to break the reaction chain by forming stable complexes of copper. Dekker and

Dickinson (15) suggest that the following equation can be used to calculate the degradation of ascorbic acid by the cupric ion.

$$K = \frac{2.303[H^+]^2}{[Cu^{++}]t} \log \frac{[H_2A]_0}{[H_2A]} \qquad [Eq.\ 7.39]$$

where $[H_2A]_0$ is the initial concentration and $[H_2A]$ is the concentration at time t of ascorbic acid. It is assumed that the initial reaction involves slow oxidation of the ascorbate ion by cupric ion to a semiquinone, which is immediately oxidized by oxygen to dehydroascorbic acid. Nord (16) has shown that autoxidation of ascorbic acid is a function of the concentrations of the monovalent ascorbate anion, the cuprous ion, the cupric ion, and the hydrogen ion in the solution.

Some examples of oxidation are summarized in Table 7.3.

Table 7.3 Oxidation

COMPOUND	SITE OF OXIDATION
Vitamin A esters	Aliphatic chain
Amitriptyline hydrochloride	Dimethylamino side chain
Hydrocortisone	Dihydroxyacetone side chain
Dipyrone	Ethanesulfonate
Dopa	Phenolic groups
Methyldopa	Phenolic groups
Ascorbic acid	Hydroxyl groups
Methylprednisolone	Hydroxyl at C-21
Phenothiazine	5-S in the ring
Chloramphenicol	Combination of hydrolysis and oxidation

7.14 REACTION MECHANISM AND KINETICS

A firm understanding of mechanistic organic chemistry is necessary to any detailed study of drug degradation because most modern pharmaceutical compounds are complex organic mol-

ecules. Degradation studies of many classic drugs have added to our understanding of the mechanism of many organic reactions. Most widely used drugs have been studied and provide a good model for future studies. The complexity and the depth of the required studies can be illustrated by the review of two classic examples, aspirin and barbiturates.

Aspirin Aspirin is an excellent example of a simple compound on which in-depth kinetic studies have been performed and for which reaction mechanisms have been proposed. The first study was reported in 1908; however, it was not until 1950 that the first detailed study was reported by Edwards (23). His work clearly demonstrated specific acid-base catalysis and pH-independent solvolysis of aspirin to salicylic acid. The rate constants for hydrogen-ion and hydroxide-ion catalyses have been found to differ with the charge of the molecule. Edwards explained the relationship between the observed rate constant and pH on the assumption that aspirin hydrolysis occurs according to six simultaneous reactions:

$$CH_3COO\cdot C_6H_4COOH + H_3O^+ \xrightarrow{k_1} HOC_6H_4COOH$$
$$+ CH_3COOH + H^+$$

$$CH_3COO\cdot C_6H_4COOH + H_2O \xrightarrow{k_2} HOC_6H_4COOH + CH_3COOH$$

$$CH_3COO\cdot C_6H_4COOH + OH^- \xrightarrow{k_3} HOC_6H_4COOH + CH_3COO^-$$
$$\text{or } HOC_6H_4COO^- + CH_3COOH$$

$$CH_3COO\cdot C_6H_4COO^- + H_3O^+ \xrightarrow{k_4} HOC_6H_4COOH + CH_3COOH$$

$$CH_3COO\cdot C_6H_4COO^- + H_2O \xrightarrow{k_5} HOC_6H_4COOH + CH_3COO^- \text{ or}$$
$$HOC_6H_4COO^- + CH_3COOH$$

$$CH_3COO\cdot C_6H_4COO^- + OH^- \xrightarrow{k_6} HOC_6H_4COO^- + CH_3COO^-$$
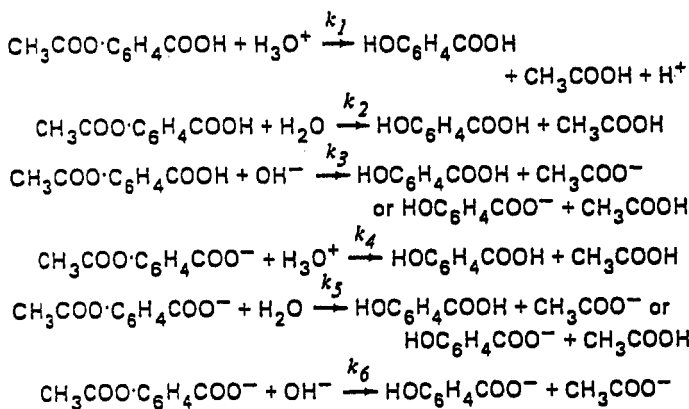
The observed first-order rate constant, k, can then be expressed as a function of the six second-order rate constants and the acid dissociation constant, K, of aspirin:

$$k = \frac{k_1[c_{H+}] + k_2[c_{H_2O}] + k_3[c_{OH^-}]}{1 + K/[c_{H+}]} + \frac{k_4[c_{H+}] + k_5[c_{H_2O}] + k_6[c_{OH^-}]}{1 + [c_{H+}]/K}$$

The pH rate profile for aspirin hydrolysis, particularly in the pH range of 4 to 8, has also been reported by Garrett (8,9). His work points to intramolecular nucleophilic catalysis of aspirin by the ionized carboxyl group catalysis. When the carboxylate ion is intramolecular, it catalyzes a number of ester reactions, although it is not a particularly strong nucleophile. The addition of alcohol increases the solvolysis rate, thus strongly suggesting the involvement of a solvent molecule in the transition state. On the basis of kinetic and isotopic studies, aspirin hydrolysis has been shown to be an intramolecularly nucleophilic catalyzed hydrolysis involving an anhydride intermediate. It is assumed that the transition state of the reaction involves addition of the carboxylate ion to the carbonyl group of the ester, forming a tetrahedral addition intermediate.

Three mechanisms have been proposed on the basis of kinetic studies on the intramolecular catalytic hydrolysis of aspirin by the carboxyl group:

1. It is a unimolecular process in which a carboxylate group acts as a nucleophile.
2. A general acid catalysis takes place in which the undissociated carboxyl group reacts with hydroxide ion.
3. A general base catalysis occurs in which the carboxylate anion reacts with a water molecule.

Barbiturates The barbiturates provide another excellent example of the complex mechanisms by which drugs degrade. Garrett et al (17), in the process of further elucidating the hydrolysis kinetics of several barbiturates, discovered that diethylmalonuric acid (IIa) in basic solution cyclizes to form barbital (Ia). Gardner and Goyan (18) confirmed the reversibility of hydrolysis of the barbituric acid nucleus, and they rationalized the degradation reaction as follows: the unionized barbiturate (III) can be cleaved at the 1,2-position, leading to the production of bisamide (IV), or at the 1,6-(3,4)-position, leading to the ureide (V). However, the ionized barbiturate would cleave only at the latter position leading to the uride (or malonic acid) exclusively.

Ia: $R_1 = R_2 = C_2H_5$
Ib: $R_1 = C_2H_5$, $R_2 = C_6H_5$

IIa and IIb

V

III

IV
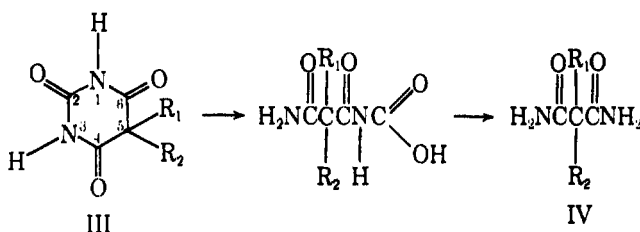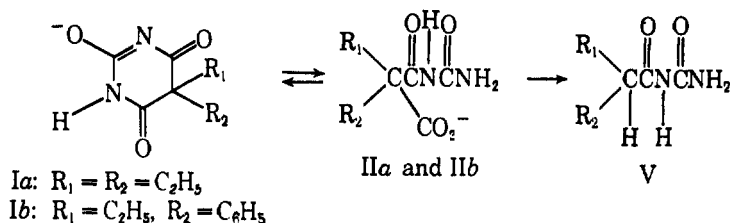
Khan and Khan (19) observed that earlier workers did not kinetically detect the existence of di- and trianionic tetrahedral addition intermediates in the base-catalyzed hydrolysis of barbituric acid, because their alkali concentration range was low. At pH values higher than the pKa_2 of barbituric acid, the equilibrium concentration of undissociated barbituric acid is negligible compared to the concentration of mono- and dianionic barbituric acids. The equations have been developed for the following irreversible first-order reaction path:

Barbituric acid →malonuric acid→ammonia

The rate constants for these reactions show three regions of hydroxide-ion dependence:

1. The reciprocal of the rate constants are linearly related to the reciprocal of the hydroxide concentration at low concentration.
2. The rate constant is independent of the hydroxide-ion concentration at higher concentration of hydroxide ion.
3. The rate constant observes the following relationship at even higher concentration of hydroxide ion:

$$k \, obs = a + b \, [OH^-] + c \, [OH^-]^2 \qquad [Eq. \, 7.41]$$

The empirical parameters a, b, and c are evaluated by the method of least squares. A trianionic tetrahedral intermediate has been proposed to account for the second power of hydroxide in the above equation.

The example below relates to metal catalysis at ultratrace levels.

Substituted Benzenediols 1,3-Benzenediols substituted with an amino-alkyl moiety are of significant pharmacological interest. Stability investigations on substituted 5-amino-ethyl-1,3-benzene-diol sulfate (AEB), under various conditions revealed that AEB is susceptible to degradation in aqueous solution in the presence of metals (20). Metal cations such as copper, iron, and calcium have been shown to accelerate degradation of AEB under an oxygen atmosphere, with concomitant discoloration. The effectiveness of metals in terms of AEB degradation is in the following order: Cu^{+2} >Fe^{+3}>Ca^{+2} (Table 7.4).

Copper effectively catalyzes AEB degradation down to 10 parts per billion (ppb) level in the presence of oxygen.

A significant increase in the rate of AEB degradation occurs when the concentration of cupric ion is increased from 10 ppb to 1000 ppb (Table 7.5).

Table 7.4 Metal Catalysis of AEB in Oxygen Atmosphere

METAL	CONCENTRATION	% AEB REMAINING AFTER STORAGE AT 90°C (O_2)			
		5hr	16hr	22hr	40hr
Cu^{+2}	100 ppb	102	--	74.6	--
	500 ppb	--	--	55.7	--
	1 ppm	88.5	--	42.5	
		(7 hr)		(24 hr)	
Fe^{+3}	100 ppb	--	89.3	--	47.9
	500 ppb	--	76.9	--	33.0
	1 ppm	--	70.7	--	22.4
Ca^{+2}	1 ppm	--	90.7	--	61.2
			(17 hr)		(41 hr)
Fe^{+3}	1 ppm (EDTA: large excess)	--	100	--	83.6
	1 ppm (N_2 atmos.)	--	102	--	99.7
Cu^{+2}	1 ppm	--	--	99.8	--

Table 7.5 Determination of Optimum Concentration of Copper for Kinetic Studies in Oxygen Atmosphere

COPPER CONCENTRATION (ppm)	% AEB REMAINING	
	After 18hr at 90°C	After 41hr at 90°C
10	35.2	8.7
5	45.0	17.7
1	64.7	31.2
0.5	69.9	39.6
0.01	89.9	59.1

The increase of degradation is less pronounced at higher concentrations such as 1 to 10 ppm on a parts per million basis.

Kinetic studies have been performed to determine the effect of pH (in the range of interest) and temperature on the degradation of AEB by monitoring the degradation with a selective high performance liquid chromatographic method. The method entails separation on μ-Bondapak C-18 column (30 cm $\times$ 4.6 mm i.d.) with a mobile phase containing 0.0028 M 1-octane sulfonic acid in methanol-water-acetic acid (36/64/1), followed by detection at 280 nm. The data of kinetic studies is given in Table 7.6.

Table 7.6 Kinetics Studies of AEB Solution (1 mg/mL) in an Oxygen Atmosphere (1 ppm Cu)

TIME (hr)	% AEB REMAINING								
	pH 3.0			pH 4.0			pH 5.0		
	90°C	70°C	50°C	90°C	70°C	50°C	90°C	70°C	50°C
7	88.5	--	--	95.9	--	--	96.9	--	--
24	42.5	85.3	--	93.8	98.6	--	96.5	98.4	--
48	21.7	56.8	101	86.4	98.7	99.3	91.2	101	100
120	2.5	--	--	31.0	--	--	64.0	--	--
123	--	28.2	--	--	--	--	--	--	--
144	--	--	63.8	--	63.8	96.0	--	--	99.1
164	--	12.4	--	--	--	--	--	--	--
167	--	--	--	23.4	--	--	--	--	--
213	--	--	51.1	--	49.7	--	--	--	--
216	--	--	--	--	--	--	40.0	--	--
332	--	--	31.9	--	--	74.2	--	97.1	--
452	--	--	23.1	--	24.0	61.0	--	--	--
787	--	---	--	--	--	47.7	--	66.5	--
1147	--	--	--	--	--	--	--	48.3	--
1892	--	--	--	--	--	--	--	--	94.0

The results of these studies show that pH of the solution has significant effect on AEB degradation. The rate of degradation is fastest at pH 3 and slowest at pH 5. At pH 4 and 5, the degradation reaction appears to proceed in two steps (k_1, k_2) instead of an

apparent single step at pH 3. The postulated second step (k_2) is relatively fast. At pH 3 (90°C), k_1 is indistinguishable from k_2 and its calculated value is 17 times higher than at pH 5 (Table 6.2). It should be noted that since k_1 values are small and are based on only a few data points; these values should be used judiciously. Based on k_2 data at 90°C, it would appear that a solution at pH 5 is approximately 6 times more stable than one at pH 3.

Two degradation products (II and III; Figure 7.3) have been prepared in our laboratory. Their presence cannot be demonstrated in the solution degraded under exaggerated conditions; however, very small peaks for resorcylic acid (VII) and 3,5-dihydroxy benzaldehyde (VI) have been detected by HPLC. This suggests that oxidative degradation is favored (Figure 7.3).
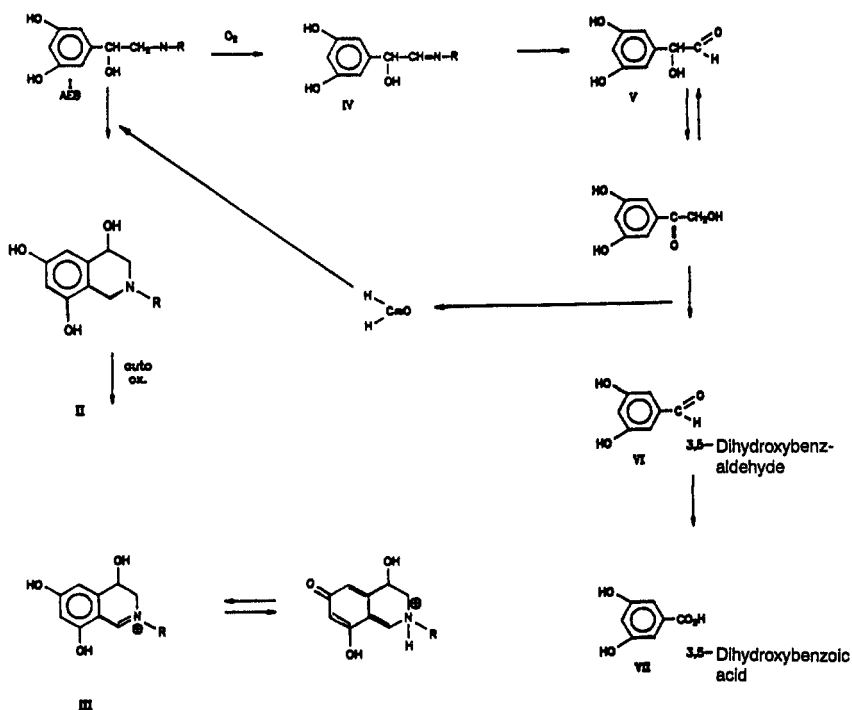
Figure 7.3 Proposed degradation pathway under stressed conditions [Adapted from *Acta Pharm. Suec.,* 9,141 (1972)].

Worst-Case Kinetics A solution of selegiline hydrochloride reference standard that contained no detectable impurities at the time of its preparation, contained a trace of a compound by HPLC at the locus of methamphetamine when analyzed after 1 year (21). Heating selegiline solutions at pH 7 and 105°C produces methamphetamine as the major product at a rate that closely follows the first-order rate equation.
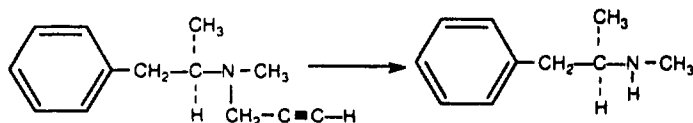
Seligiline and the (-)enantiomer of methamphetamine.

Using only these data and worst-case assumptions, rate constants have been estimated at various temperatures; the activation energy is estimated to be about 25 kcal, and the stability-indicating validity of the assay used has been affirmed. It can be concluded that selegiline undergoes degradation at a negligibly slow rate.

Arrhenius Relationship for Proteins Several papers have described the Arrhenius plot for chemical degradation pathways, such as deamidation, hydrolysis, and racemization of peptides. It has been reported that stability predictions based on the Arrhenius relationship are inappropriate for protein preparations that exhibit complex degradation mechanisms(22). The degradation mechanisms of some protein drugs have been reported to vary as a function of temperature. Extrapolation of stability data obtained with such protein drugs to lower temperatures should be limited to the temperature range over which the same degradation pathway is operative. Furthermore, it has been recommended that the Arrhenius approach should be conducted in the temperature range over which protein drugs are not susceptible to unfolding, usually below 40°C.

The inactivation of the following six protein preparations showed complex kinetics:

- α-Chymotrypsin troche
- α-Chymotrypsin tablet
- Bromelain tablet A
- Bromelain tablet B
- Kallikrein capsule
- β-Galactosidase powder

The reciprocal of the t_{90}, a measure of inactivation rate, exhibited an approximately linear relationship. The results indicate that it may be reasonable in some cases to extrapolate the results obtained at higher temperature to predict the shelf-life of protein preparations.

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8
Stability Studies

The meaning of the word "stability" is self-evident; however, it means different things to different people or to the same people at different times, even those associated with the pharmaceutical sciences and industry. It is generally recognized that there are legal, moral, economic, and competitive reasons, as well as those of safety and efficacy, to monitor, predict, and evaluate drug product stability (1). Although a unified nomenclature has been proposed, the following variations in terminology are still employed to encompass the term stability:

- Failure of a batch to meet specifications
- Stability evaluation
- Stability-indicating assay
- Expiration dating
- Outdating
- Shelf life
- Storage legend
- Preformulation studies
- Compatibility studies
- Kinetic studies
- Stability evaluations

- Stability studies
- Stability of the active ingredient
- Stability of the formulation
- Stability in the marketed package
- Stability in the sample package
- Stability in the dispensing package
- Stability in the hands of the patient

All of these areas have been referred to as stability.

The legal definition of stability is aimed at ensuring that the drug product remains within specifications established to ensure its identity, strength, quality, and purity. Stability is interpreted as the length of time under specific conditions and storage that a product will remain within the predefined limits for all of its important characteristics. It is important to remember that stability is fundamental to all of the product's characteristics, including safety and efficacy. In this chapter we will focus primarily on what is considered stable in the pharmaceutical field and what studies are planned or performed to ensure and evaluate stability of pharmaceutical products. Active ingredient stability, preformulation studies, and kinetic studies have been addressed in earlier chapters.

In the pharmaceutical industry, the disciplines primarily involved with stability are pharmaceutical analysis and product development. It is not possible to monitor stability, determine the reaction rate, or investigate any mechanism without an analytical measurement. Hence the pharmaceutical analyst is involved primarily in stability studies because he or she must develop a method that will quantitatively determine the drug in the presence of, or separate from, the degradation product(s). These methods are frequently referred to as stability-indicating methods. Other terminology, such as selective or specific methods, has also been used. The main point is that the method should be specific enough to distinguish the active ingredient from the degradation product or other by-products produced in storage. To select an appropriate method, an analyst should have a thorough knowledge of the

physicochemical properties of the drug including the understanding of the routes by which a drug can be degraded or transformed.

8.1 STABILITY-INDICATING METHODS

The methodology used for kinetic studies (solid state or solution) can generally be considered suitable for monitoring the stability of pharmaceutical formulations if a similar mode of- degradation is encountered. However, the method may not meet the compendial or regulatory requirements. The effect of drug excipient inter-actions on analytical methodology cannot be ignored (2). Fre-quently, these interactions not only lead to low assay values but also affect drug availability to the patient.

The current trend in stability-indicating methods is based on chromatographic analysis because these methods allow resolution of a variety of compounds. However, stability-indicating methods do comprise a broad span of methods besides electrometric methods, solvent-extraction methods, chromatographic methods and spectrophotometric methods. These methods are discussed briefly below. A number of methods have been detailed throughout this book. The chapter on applications (Chapter 9) includes many examples of these methods as well.

8.1.1 Solvent Extraction Methods

It is possible to extract acidic, basic, or neutral compounds selectively into organic solvents on the basis of the partition behavior of their ionized or unionized species. Double extraction methods have been commonly used in compendia. The extraction is performed into an organic solvent from an acidic aqueous solution, which is then made basic and extracted again with fresh organic solvent. The first extraction removes acid-insoluble impurities that favor the organic solvent and the second extraction allows the extraction of free base relatively free of some or most of impurities. This method has been found useful for the analysis of organic nitrogenous bases.

The approach described above provides some degree of specificity, because it is possible to remove compounds that are neutral or acidic or have more polar substituents that could arise out of degradation. However, it does not eliminate isomers or other closely related basic substances. Therefore, it is essential to demonstrate the validity of this approach. It should be recognized that these methods can provide valuable means to enhance the selectivity of other methods.

8.1.2 Electrometric Methods

Titrimetric methods (aqueous or nonaqueous), commonly used for the precise analysis of the active ingredient, most often do not offer the desired specificity for the analysis of pharmaceutical products. However, if the degradation products do not interfere with the titration, e.g., when the degradation products from an organic amine or amine hydrochloride are not basic, then it is possible to utilize titrimetry.

Alternatively, it would be necessary to employ suitable extraction procedures to eliminate possible interferences from the excipients and/or decomposition products before using titrimetry for monitoring stability of the product.

Organic polarography has been used for the analysis of products because it offers the desired specificity, but its use has been limited by several technical disadvantages (3). The advantages of polarography for determination of ethacrynic acid in the presence of its principle degradation product, a dimer, has been demonstrated (4). Polarography has also been found useful for studying acid and base hydrolyses and β-lactamase degradation of several cephalosporins (5). A wave believed to be due to reductive elimination of the C-3 position substituent is suitable for stability evaluation.

Electrometric detectors have been found to be very useful for a number of applications in HPLC and thus there is renewed interest in this technique (see Chapter 9).

8.1.3 Spectrophotometric Methods

Direct spectrophotometric methods commonly used in pharmaceutical analysis generally lack selectivity. These detectors constitute ultraviolet/visible spectrophotometry or fluorometry. Selectivity can be improved through chromatographic separations (see below) or by reaction of an appropriate functional group. For example, reactions that produce a colored product are generally measured in the visible region of the spectrum. Other reactions increase conjugation to permit measurement in the UV region. Details on methodology for spectrophotometric methods can be found in Chapter 4. A number of applications are discussed in Chapter 9.

Due to its limited sensitivity, infrared (IR) analysis is primarily used for identification of decomposition products and has found very few quantitative applications in stability evaluations. Nuclear magnetic resonance (NMR) spectroscopy is finding an increasing number of applications since it offers specificity along with simplicity of operation, but it too lacks sensitivity and precision. Mass spectrometry in combination with GC or HPLC has been found useful in some cases for monitoring impurities in pharmaceutical compounds (see Chapter 9).

8.1.4 Chromatographic Methods

A large number of stability-indicating methods entail some form of chromatography: thin-layer, gas (GLC), liquid (HPLC), or supercritical fluid (SFC). Of these methods HPLC has found the greatest applications (Table 8.1). The listing in the table shows a variety of methods that can be utilized, though the current trend favors reverse-phase HPLC.

Capillary electrophoresis (CE) is finding increasing applications in pharmaceutical analysis and is frequently included with the chromatographic methods, though it is not strictly a chromatographic method. Methodological information on this

technique is given in Chapter 4, and applications have been provided in Chapter 9.

Table 8.1 HPLC of Pharmaceuticals

COMPOUND	MODE OF HPLC
Prostaglandins A_2 and B_2	Anion exchange
Barbiturates	Anion exchange
Penicillin G potassium	Anion exchange
Ampicillin	Anion exchange
Tetracyclines	Cation exchange
Xanthines	Cation exchange
Trisulfapyrimidines	Cation exchange
Sulfa drugs	Cation exchange
Imidazolines	Cation exchange
Benzodiazepines	Adsorption
Riboflavin	Adsorption
Sulfacetamide sodium	Adsorption
Cholecalciferol	Adsorption
Canrenone	Adsorption
Carbamazepine	Adsorption
Phenytoin (diphenylhydantoin)	Adsorption
Phenobarbital	Adsorption
Aspirin, phenacetin, and caffeine	Adsorption
Corticosteroids	Partition
Sulfasalazine (salicylazosulfapyridine)	Partition (reverse phase)
Procaine	Partition (reverse phase)
Tetracyclines	Partition (reverse phase)
Synthetic estrogens	Partition (reverse phase)
Phenol	Partition (reverse phase)
Ergotamine	Partition (reverse phase)
Nortriptyline	Ion exchange and partition (reverse phase)
Androsterone (derivatized)	Ion exchange and partition (reverse phase)

Table 8.1 HPLC of Pharmaceuticals (continued)

COMPOUND	MODE OF HPLC
Dehydroepiandrosterone (derivatized)	Ion and partition (reverse phase)
Vitamins (water soluble)	Anion and cation exchange
Analgesics (aspirin, caffeine, acetaminophen, and salicylamide)	Anion and cation exchange

8.2 CLASSIFICATION OF STABILITY STUDIES

Stability studies can be broadly classified as follows:

- Accelerated studies
- Long-term studies

8.2.1 Accelerated Studies

Kinetic studies (see Chapter 7) can also be construed as accelerated studies. However, it should be pointed out that kinetic studies are generally conducted on the active ingredient, in a solid or solution state, where the effect of temperature, pH, oxygen, or light may be evaluated. They are rarely carried out on a formulation unless the formulation is very simple, e.g., an injectable aqueous solution of the drug of interest adjusted to a given pH.

Accelerated studies included here are those studies that are generally conducted on actual formulations for several weeks under accelerated conditions of temperature, humidity, or light. The primary objective of these studies is to obtain an early reading on whether a given active ingredient or formulation is likely to be sufficiently stable to deserve detailed exploration.

In performing these studies, an active ingredient or a formulation can be subjected to much higher temperatures, humidity, or light conditions than it is likely to be subjected to under normal transportation, storage, and usage conditions. For example, an active ingredient may be subjected to increasing

temperatures until decomposition is detected. In general, however, the active ingredient or formulation is subjected to sufficiently high temperature, humidity, or light exposure to provide some indication of potential instability.

8.2.2 Long-Term Stability

A modern pharmaceutical product is not only an optimum formulation, it is also the optimum formulation/package combination. Formulations are designed to maintain or enhance the stability of the active ingredients while allowing optimal delivery in the human body. The design is based on physicochemical properties of the active ingredient and its compatibility with the excipients. This information is generally derived from the kinetic or accelerated studies performed to develop optimum formulation (see Chapter 7).

Many approaches are used to stabilize or protect formulations, including lyophilization, microencapsulation, control of surface area, addition of chelating agents, preservatives, and antioxidants, physical separation of incompatible ingredients, coatings, and opaque coverings. The need and use of these approaches is intrinsic to the dosage form under consideration.

The stabilization of a dosage form-container combination is extrinsic to the stability of the dosage form. The container is an integral part of products such as topicals and parenterals. Wood (6) recommended that in addition to physical, chemical, bioavailability, and microbiological criteria, container interactions should be monitored in the evaluation of the stability of topicals. In many cases, it is not feasible for evaluation purposes to isolate the dosage form from the container. The container becomes an integral part of the product. Even though extensive studies may have been conducted on the dosage form, additional studies on the product in the container of choice are necessary to ensure the total stability characterization of the product.

It is essential that storage and end use be considered during the design of a product. Use tests may be indicated where the im-

mediate container is continually disturbed during use (e.g., in an elixir or syrup, the head space or the surface to volume ratio will change during use).

In addition, consideration should be given to possible requirements and restrictions on the storage of the product-container combination to provide adequate assurance that product performance is satisfactory throughout the determined shelf life. Establishment of packages, storage legends, and shelf life are at least as important as the basic efforts to determine the stability of the active ingredient as discussed above.

Selection of Container

Stability, safety, legal, and quality requirements primarily determine the selection of the container. Other considerations that are significantly important in the selection process are esthetics, economics, production, and quality control. Poor product stability in the chosen container affects all of the factors stated above. Hence it is of major importance.

For purposes of marketed product stability, the manufacturer container is defined to be all package components in intimate contact with the product or that provide a degree of protection, e.g., closure, seal, or overwrap.

Title 21, Section 314.1 of the Code of Federal Regulations requires that for any New Drug Application, stability data for the dosage form be provided "in container in which it is to be marketed." The significance of this requirement can be related to the problems experienced with nitroglycerin tablets that led to very explicit regulations (7). The enactment of this regulation followed the discovery that appreciable evaporation of nitroglycerin can occur when it is stored in plastic containers and certain strip packages. Much has been published on the stability of nitroglycerin tablets relative to the container in which they are stored or dispensed (8-12).

The United States Pharmacopeia (13) has provided definitions for various types of containers based on their capability to provide protection:

- Light-resistant containers
- Well-closed containers
- Tight containers
- Hermetic containers

A quantitative test to measure the permeation of the container's closure system can also be found in the USP.

Pharmaceutical packages are designed to provide not only a means of transport and band identification but to serve a more significant function of providing adequate protection of the product and to ensure the stability of the product while it is in distribution or storage. Products subject to hydrolysis or deleterious physical changes caused by moisture require containers that restrict moisture transmission. Light-sensitive products need protection from light. Formulations that are subject to oxidation can be protected by blanketing with an inert atmosphere, e.g., nitrogen for ampuls.

A basic understanding of the properties of package components, the container, and its closure is needed to make an appropriate package selection. The ultimate criterion of the suitability of a particular container or package combination is testing of the drug under normal and stress conditions for extended periods. Special consideration should be given to interactions between the product and the container that might not be tested under normal testing of the product itself. These interactions could include migration of one or more components of the package into the drug, absorption or adsorption of the drug into or onto the package, esthetic changes, and formation of a reaction product at the drug-container interface.

Dispensing and Repackaging

The USP includes an entire section entitled "Stability Considerations in Dispensing Practice." Included in this section are: an overall definition of stability; various aspects of stability including chemical, physical, microbiological, therapeutic, and toxicologic factors affecting stability, such as the nature of container; the

process by which the manufacturer selects the optimum formulation and container; and the responsibility of the pharmacist with regard to stability. Furthermore, this section directs the pharmacist to dispense pharmaceutical products in the proper container and closure.

As of April 1, 1977, the monograph requirements for tight or well-closed containers must be followed when dispensing a prescription. The responsibility of proper repackaging has been placed squarely on the pharmacist. Over the past few years there has been a growing concern about the stability of pharmaceuticals packaged for hospital use. The USP recommends against repackaging pharmaceuticals and advises pharmacists to take every precaution if repackaging is necessary. In general, products repackaged at the dispensing level are stored for relatively short periods, but during that time they can be exposed to harsh storage conditions in the hand of a patient. For example, it is not uncommon to leave these products in the bathroom, on a windowsill, or even in the glove compartment of a car. Hence, the pharmacist becomes a significant link in ensuring drug product stability.

Storage Conditions
An important part of stability evaluation is the determination of the effects of environmental conditions on the product. The factors commonly tested are heat, humidity, light, and air. Increased temperatures lead to acceleration of all reactions according to rate theory (see Chapter 7), and the other factors accelerate, catalyze, or mediate a variety of reactions such as hydrolytic, photolytic, oxidative, etc. (also see Chapter 9).

Data obtained from these studies form the basis for the establishment of an expiration date. Expiration dates, however, have real significance when they relate to specific storage conditions. The storage of pharmaceuticals can be controlled by the manufacturer through distribution to the wholesale and pharmacy level. Thereafter, the only means of control is the storage legend appearing on the product label. Temperature can vary significantly from one area to another (see the discussion later in this section)

therefore, it is best to relate laboratory storage of stability samples to actual market conditions. To obtain laboratory data more representative of field conditions, cycling storage conditions can be used to reproduce actual conditions.

Compendial monographs provide directions for "Packaging and Storage"; the storage conditions stipulated in individual monographs are defined as:

- Cold--Any temperature not exceeding 8°C.
- Cool--Any temperature between 8 and 15°C.
- Room temperature--Controlled room temperature is defined as a thermostatically controlled area between 15 and 30°C.
- Warm--Any temperature between 30 and 40°C.
- Excessive heat--Any temperature above 40°C.

The climatic conditions around the world can be classified into the following four categories (the calculated mean temperature and humidity are given in parentheses):

I   Temperate climate (20°C, 42% RH)
II   Subtropical climate (21.6°C, 52% RH)
III   Hot, dry climate (30°C, 40% RH)
IV   Hot, humid climate (30°C, 70% RH)

8.3 STABILITY PROGRAMS

Stability programs are devised to ensure that drug products retain their full efficacy up to the expiration date. During this program, stability testing has to be planned carefully. Such tests are carried out in development of a drug product from the experimental stage with the drug substance, formulation development, final dosage, and formula for commerce, and continue through production and any major changes. The whole program must be systematic and logical, so that the state of knowledge can be continuously

extended. As a result, the stability information is based on all of the results obtained during development.

Nearly all of the countries where drugs are produced have their own guidelines. Stability testing guidelines in the European Community (EC), Japan, and the United States show no significant differences in the basic principles listed below.

- Stability testing during drug development
- Selection of batches and samples
- Test criteria
- Analytical methods
- Specifications
- Storage conditions
- Test intervals
- Storage period
- Number of stability batches
- Packing materials
- Evaluation of data

However, formal requirements do vary. It would be very desirable to elaborate on the basic principles and demonstrate the importance of formal differences. On the basis of the discussions of these differences, harmonized guidelines can be developed that are acceptable to a large number of countries.

The aim of these guidelines is to present state-of-the-art stability testing, including the latest official requirements in the EC, Japan, and the United States, and to indicate future prospects in stability testing. The harmonized guidelines in these three areas will cover 83% of the world market for drug products. These guidelines will be adopted by Canada and Australia, and a number of other countries are sure to follow.

Stability Testing During Drug Development
Comprehensive and reliable stability information is based on a large number of individual results generated during development of a drug product. The individual stages during development should

be logically coordinated with each other to allow a rational formal statement on the stability of the product. The development process can be divided as follows:

- Tests on the active ingredient
- Preformulation studies
- Selection of clinical formulation and the final dosage form
- Stress and accelerated testing to select the final container
- Long-term testing
- Follow-up tests
- Tests to cover any major change

Each of these stages has a specific objective that determines its scope and level of effort. A stability program frequently concentrates mainly on long-term testing. The required scope of testing is accordingly extensive.

Selection of Batches and Samples
Data on test or experimental batches must be evaluated carefully, especially if the process has not been validated. The data on batches that are representative of the final product can provide a much better picture on product stability, since most of the variables have been minimized in the process validation stage. It is important at this stage that batches be homogeneous. The sampling is made as random as practically possible. Where possible, statistical considerations are used. The containers used are likely to be those that will be used commercially. The sample analyzed from a container should be representative of the container, and enough containers should be analyzed to ensure representation of the batch.

8.4 STABILITY GUIDELINES

An effort is being made to harmonize stability guidelines between the major (based on sales in dollars) drug-producing countries. However, these sales do not necessarily represent the units of drug products being produced in the world because of apparent wide

differences in currency values between the developed and developing countries. A short review of the guidelines from the EC, Japan, and the United States is given below to provide an overview of stability guidelines.

8.4.1 European Community (EC) Guidelines

The EC guidelines on stability testing of active substances and finished products were adopted by the Committee on Proprietary Medicinal Products (CPMP) in July 1988. It is envisaged that applications for certain categories of products (biotechnology and high-technology products) will be made through a centralized procedure. These products will be given a European marketing authorization, and this means that product labeling, including storage requirements and shelf life, must be uniform throughout the European Community.

The function of a stability test is to ascertain how the quality of a drug product varies as a function of time and under a variety of environmental conditions. The quality of a drug product is determined by:

- Percentage of active ingredient content (within specified limits)
- Purity (degradation products within reasonable limits)
- Good organoleptic properties
- Microbiological properties (within specified limits)

Active Ingredient
Studies are usually carried out during drug product development and are designed to show the effect of different solvents, effect of pH, effect of light, oxygen, etc.; and degradation routes are investigated. Where it is possible, degradation products are characterized.

The guidelines recommend that a minimum of two batches should be evaluated under a variety of temperature and stress conditions. The active ingredient is assayed using a validated

stability-specific method with identification and quantitation of the principal degradation products.

The retest date of the active ingredient is used to ensure the conformity of the batch to the specifications within the assigned period.

Stress Testing
These tests are invaluable for providing complementary data to support shelf-life requests if long-term real-time data is not available. They also show the effect of adverse storage conditions on the packaging and product in case special precautions are necessary.

The EC guidelines suggest a variety of possible test temperatures and conditions:

- Storage at three high temperatures
- Storage at low temperatures (e.g.,-15°C, 2 to 8°C, and freeze-thaw cycling)
- Storage at high humidity (RH not less than 75%)
- Storage at high temperature and RH
- Storage under light (artificial or natural)

Batches Tested
At least three batches should be studied except where the active ingredient and the product are known to be chemically stable, no decomposition products are formed, and other properties of the product do not change significantly.

Packaging
Sufficient data should be provided on the finished product as proposed for marketing. Supplementary data on batches stored in related packages may be used to augment these data.

Product Characteristics Evaluated
The product characteristics can be divided into the following three categories:

- Physical (tablet hardness or particle size of suspension)
- Microbiological (preservative efficacy at the end of storage)
- Chemical (assay of A.I., content of decomposition products, content of antimicrobial preservatives)

The following product characteristics should be evaluated: those in the finished product specifications that are likely to be affected by storage and those that are not necessarily monitored routinely in the finished product specifications, e.g., dissolution of vaginal products.

Validation of Test Methods
All test methods are to be described in detail and should be fully validated, and data from the validation studies should be provided in the application.

The analysis of the A.I. must be stability-specific. An expert report is filed which evaluates material balance (an accounting of total degradation products with the observed assay values).

The shelf life should be based on the least stable batch if differences between the batches have been observed. Shelf life of the volatile products has to be based on a when-first-opened basis. Shelf life of reconstituted products must be supported by physical stability and assay data.

Labeling
The guidelines demand that if there is evidence that defined storage temperatures are needed, then these must be stated on the label in degrees centigrade. If the product is stable at 25°C and withstands several weeks of exposure at 30°C (based on stress tests), no special storage instructions are necessary on the label.

Ongoing Stability Studies
If data on production batches are not provided, then ongoing studies should be carried out on the first two or three production batches and results provided to the authorities.

Toxicity of Degradation Products
Potential toxicity of degradation products should be reviewed and discussed in the Pharmaceutical Expert Report and Toxicological Expert Report.

8.4.2 Japanese Guidelines

The revised guidelines entitled *Handling of Data on Stability Testing Attached to Applications for Approval of Manufacture and Import of Drugs* were made public on February 15, 1991.

The purpose of stability studies, required in applying for approval of drugs, is to evaluate the stability of a drug's quality in order to ensure its efficacy and safety and to establish its storage requirements and shelf life. It is not always required to stick to the methods provided in the guidelines when there are rational reasons for altering test methods.

Sampling and data analysis should be made by statistically appropriate methods. Specimens should be chosen so that they are representative of all production lots, and test samples should be taken randomly from the specimens. The number of test samples and repetitive testing should be predetermined so that the reliability of testing is improved.

Stress Testing
Stress testing is conducted to examine the change in quality under severe conditions that may be encountered during distribution. The tests should be conducted with unpackaged specimens in principle, but they should be conducted with packaged specimens when necessary.

Storage conditions for the bulk drug substance and drug product should be selected so that effect of temperature, humidity, and light is clarified. When a drug product produces different degradation products from its bulk drug substance, the chemical structure, degradation mechanism, safety, and so forth should be discussed.

Long-Term Testing

This testing is conducted to ascertain the stability of a drug during a designated period of distribution.

One specimen of bulk drug substance is taken from each of three packed lots. Room temperature samples constitute 25 ⇧ 2°C for a period generally of three years unless a shorter expiration date is proposed.

Accelerated Testing

These studies are performed to estimate the stability of a drug during a designated period of distribution. Other storage conditions should be included in the evaluation in addition to the following, when necessary:

- 40°C (±1°C), 75% RH (±5%)
- Designed storage condition +15°C and 75% RH
- Storage duration not less than 6 months

Sampling should be made not less than at 4 time intervals, including zero time. Compatibility studies are to be conducted for combination products.

8.4.3 United States Guidelines

FDA guidelines cover the Investigational New Drug (IND), the New Drug Application (NDA), and the supplements that are required after marketing approval. It also contains a section dealing with Abbreviated New Drug Application (ANDA). In the United States, major changes need to be addressed by supplements to the approved drug application, which normally need prior approval. For example, changes in formulation, packaging, product manufacturing site, and in regulatory test methods/specifications normally need supplements.

Stability testing should be relevant to the stage of drug development. Studies conducted during development of a drug product do not necessarily follow a rigid separation into phases 1,

2, and 3, but should be adequate for the intended duration of stability tests. The emphasis in phase 3 is on final formulation in the probable market packaging and expiration dating.

Drug Substance
After the specifications and tests have been established for the drug substance, along with packaging, studies should be performed to determine the conditions of storage that will ensure quality up to the time of use. A retest date should be determined. This information should be determined from studies on three batches made by the same process at the same facility. The amount of degradation products, as well as the impurities, that will be tolerated, depends on the manufacturing process, packaging, amount of consumption, and the toxicity of the substances.

Drug Product
The U.S. Pharmacopeia defines controlled room temperature as 15 to 30°C. When a drug product is labeled for storage in this range, it is necessary to ensure that the product is stable at the extremes of the range.

For suspension metered-dose inhalation aerosols, one-time temperature cycling is recommended to evaluate the effect of temperature change on the quality of suspension in the container/closure system. Such a study may consist of 4 six-hour cycles per day between subfreezing temperature and 40°C for six weeks. In addition, containers must be stored upright and inverted to simulate potential storage and transportation conditions.

The sterility tests should be designed to demonstrate the ability of container, seals, closures, and other materials to maintain a barrier to microbial ingress throughout shipping, storage, and product handling.

Stability studies on reconstituted solutions, lyophilized products, and sterile drug substances need to be done, and the appropriate time period and temperature placed on the label.

For degradation products that arise during the expiration dating period, it is necessary to know the amount, structure, and toxicity.

If there are limits to be set on individual degradation products, it should be prepared for use as a reference standard.

For ANDAs, there is usually data in the scientific literature, so accelerated data is considered acceptable for granting of tentative expiration date. The recommended accelerated conditions are as follows:

- 40°C and 75% RH for dosage forms proposed for controlled RT storage
- 25°C and ambient humidity for dosage forms proposed for refrigerated storage

The accelerated stability data should be generated at intervals of zero, 1, 2, and 3 months.

8.5 INTERNATIONAL HARMONIZATION

Comparable requirements for stability tests will allow companies to plan and implement tests whose results can be used for drug registration in every country in the world. In order to assure that a stability study is acceptable worldwide, it has to conform to a uniform, internationally accepted concept of product quality. In other words, tests should comply with international standards for composition, manufacture, and packaging, as well as with standardized drug product specifications.

An example of a reasonably logical stability-testing program follows (14).

Stability-Testing Program
1. Preformulation Studies

Type of study: Accelerated
Test sample: Laboratory batches
Objective: Evaluation of the following factors on the drug substance:

- Temperature
- Humidity
- Light
- Oxygen
- pH
- Compatibility with excipients
- Development of stability-indicating methods
- Identification of degradation products

2. Preliminary Studies

Type of study: Accelerated
Test sample: Laboratory/pilot-scale batches
Objective: Evaluation of the following factors on the drug product:

- Temperature
- Humidity
- Light
- Manufacturing procedure
- Interaction with packaging materials
- Establishment of stability-indicating methods
- Identification of degradation products
- Estimation of expiration date for clinical samples

3. Formal Studies

Type of study: Long-term/accelerated
Test ample: Pilot/production batches
Objective: Evaluation of the final formulation/container for the various climate zones

- In-use tests
- Confirmation of analytical methods
- Establishment of :
    Overages
    Specifications

Expiration date
Storage conditions

4. Ongoing Studies

Type of study: Comparative/long-term
Test sample: Production batches
Objective: Confirmation of stability profile on the first production batch:

- Assessment of stability profile after the following changes:
    Formulation
    Container
    Manufacturing procedure
    Synthesis route

5. Follow-up Studies

Type of study: Long-term
Test sample: Production batches
Objective: Monitoring of stability of retention samples:

An approach for harmonizing storage conditions worldwide is presented in the EC stability guidelines (Table 8.2). This approach states long-term studies under normal conditions should take place at $+25°C/$ 60% RH if the pharmaceutical product is intended to be stored and marketed in zones I and II, however, if the product is to be sold in zones III and IV (Tables 8.2 and 8.3), tests should be carried out at $+30°C/$ 70% RH. Long-term studies conducted at $25°C/60\%$ RH are to be performed primarily for products intended for EC countries, the USA, and Japan.

To obtain regulatory approval in other countries with climate conditions of zones III and IV, the long-term studies should be carried out at $+30°C/$ 70% RH.

Other storage conditions, e.g., $+5°C$ and $+15°C$, shown in Table 8.3 may be included if the drug product is very sensitive to heat.

Table 8.2 International Climate Zones and Climate Conditions (CPMP Stability Guidelines for Europe)

CLIMATE CONDITION	ZONE I [a]	ZONE II [b]	ZONE III [c]	ZONE IV [d]
Mean annual temperature	<20.5°C	20.5-24°C	>24°C	>24°C
Kinetic mean temperature (virtual temp.)	21°C	26°C	31°C	31°C
Mean annual relative humidity	45%	60%	40%	70%

a: Temperate
b: Mediterranean (subtropical)
c: Hot/dry or hot/moderate RH
d: Very hot/humid

Table 8.3 Storage Conditions for Long-Term Stability Testing

CLIMATE ZONE	STORAGE CONDITIONS TEMPERATURE/ HUMIDITY	TESTING INTERVALS (months) 1 3 6 9 12 18 24 30 36 48 60
I	+21°C/45%RH	× × × × × × × × × × ×
II	+25°C/60%RH	× × × × × × × × × × ×
III	+30°C/40%RH	× × × × × × × × × × ×
IV	+30°C/70%RH	× × × × × × × × × × ×
	+5°C*	× × × × × × × × × × ×
	+15°C*	× × × × × × × × × × ×

*For heat-sensitive products with corresponding storage directions.

Storage conditions, e.g., +40°C (elevated temperature only) and +40°C/75% RH (exposure to elevated temperature and humidity), can be regarded as internationally accepted (as shown in Table 8.4), based on regulations for accelerated studies in EC countries, the United States, and Japan.

To assess the effect of light, it is advisable to carry out the tests using both artificial sunlight and indoor light.

Table 8.4 Storage Conditions for Accelerated Stability Testing

| | STABILITY GUIDELINE REQUIREMENTS | | | PROPOSAL FOR INTERNATIONAL STUDIES | |
	JAPAN (MHW 1980)	USA (FDA 1984)	EUROPE (CPMP1988)		
Humidity	+40°C/ 75%RH (Acceleration test standard)	+40°C/ 75%RH	+40°C/ 75%RH	+40°C/ 75%RH	3,6,12 mo
Temperature	NS* (severe test)	NS*	NS*	+40°C/	3,6,12 mo
Light	NS* (severe test)	NS*	NS*	Artificial sunlight Indoor light	1,2,3 days 1,3,6 mo

*Not specified.

Sampling is performed at 3-month intervals during the first year of the study, at 6-month intervals during the second year, and once a year thereafter. For drug products that are expected to degrade rapidly, samples are taken more frequently.

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9
Applications

A large number of examples pertaining to impurities evaluation of pharmaceuticals have been discussed in the previous chapters. Special emphasis is given in this chapter to chiral compounds, where approaches to method development are also provided, in light of the great interest in this area.

Also discussed are a large number of additional examples for a variety of compounds that are not chiral. To enable the reader to find examples that may relate to the compound under investigation, the pharmaceutical compounds have been classified into the following categories:

1. Alkaloids
2. Amines
3. Amino acids
4. Analgesics
5. Antibacterials
6. Anticonvulsants, antidepressants, and tranquilizers
7. Antineoplastic agents
8. Chiral compounds
9. Local anesthetics
10. Macromolecules

11. Steroids
12. Miscellaneous

Since pharmaceutical chemists look at potential compounds from the standpoint of chemical structure and/or therapeutic category, this classification utilizes both approaches to provide ready access to compounds of interest. Selected examples have been included to provide an insight into methodology. Essential details of operating conditions, such as mobile phase, stationary phase, and detectors for the chromatographic methods, are provided to enable the reader to try a given method. Minimum detectability information has been included, when available, or a calculated value has been provided where data permits such a calculation. It is hoped that this will help the reader to gauge the usefulness of a published method for a particular application.

It is now well recognized that analytical methods that can control impurities to the ultratrace level are available (1,2). However, the level to which any impurity should be controlled remains open to discussion. Logically, this should be primarily determined by the pharmacological and toxicological effects of a given impurity. This should include all impurities: those originating out of synthesis and those arising from other sources such as degradation (3).

Specifications of impurities should be carefully established; however, as of to date specifications can vary between pharmacopeias. This needs to be rectified, especially when considering that a pharmaceutical compound can be prepared by a variety of methods that can lead to different impurity profiles. The need for methodologies suitable for controlling low levels of impurities and rational limits cannot be overemphasized.

The method development strategies should include considerations that relate mainly to the type of impurities. Table 9.1 has been prepared to assist in selection of a suitable methodology for separation of a given class of impurity.

Table 9.1 Selected Methodologies for Given Impurities

PARENT COMPOUND	METHODOLOGY	REFERENCE GROUP
Isomers		
Prostaglandin A_2	LC ($AgClO_4$)	Miscellaneous
Doxepine	GC	Antidepressant
Pilocarpine	RPLC	Alkaloid
Tetracycline	RPLC	Antibacterial
Carboprost	Adsorption, LC	Miscellaneous
Budesonide	RPLC	Steroid
Oxytetracycline	TLC	Antibacterial
Naproxen	Adsorption, LC	Analgesic
Diethylpropion	RPLC	Miscellaneous
Intermediates/by-products		
Levodopa	RPLC	Amino Acid
Levothyroxine	RPLC	Amino Acid
Liothyronine	RPLC	Amino Acid
Indomethacine	TLC	Analgesic
Meperidine	TLC	Analgesic
Oxytetracycline	TLC	Antibacterial
Griseofulvin	LC (Cyano)	Antibacterial
Chloramphenicol	RPLC	Antibacterial
Imipramine	TLC	Antidepressant
Desipramine	TLC	Antidepressant
Meprobamate	TLC	Antidepressant
Chlordiazepoxide	Adsorption, LC	Antidepressant
Prostaglandin E_2	GC/MS	Miscellaneous
Fludrocortisone	RPLC	Steroid
Hydrochlorothiazide	RPLC	Antibacterial
Folic acid	RPLC	Miscellaneous
Trihexyphenidyl	TLC	Miscellaneous
Methotrexate	Column, LC	Miscellaneous
Impurities arising from contaminants in solvents/intermediates		
Amphetamine	GC/MS	Amine
Trihexyphenidyl	TLC	Miscellaneous

Table 9.1 Selected Methodologies for Given Impurities (continued)

PARENT COMPOUND	METHODOLOGY	REFERENCE GROUP
Degradation products		
Aspirin	RPLC, TLC/HPLC	Analgesic
Penicillin G	RPLC	Antibacterial
Cephalexin	LC (Amino)	Antibacterial
Procarbazine	RPLC	Antineoplastic
Cyclophosphoramide	RPLC	Antineoplastic
Benzocaine	RPLC	Local anesthetic
Tetracaine	RPLC	Local anesthetic
Hydrocortisone	RPLC	Steroid
Dexamethasone	RPLC	Steroid
Prednisone	RPLC	Steroid
Digoxin	RPLC	Steroid
Chlorpropamide	Adsorption, LC	Sulfonamide
Tolbutamide	Adsorption, LC	Sulfonamides
Saccharin	RPLC, Ion-exchange, LC	Miscellaneous
Nitroglycerin	RPLC	Miscellaneous
Diethylpropin HCl	RPLC	Miscellaneous
Contaminants		
Ampicillin	GC	Antibacterial
Cephalosporin	GC	Antibacterial
Tetracycline	RPTLC/ Bioautography	Antibacterial
Penicillamine	RPTLC/ Bioautography	Antibacterial

9.1 ALKALOIDS

Derivatization of alkaloids such as cephaeline, codeine, emetine, ephedrine, morphine, and narcotine with dansyl chloride has been studied with the purpose of developing a sensitive and specific HPLC method for these substances in pharmaceutical dosage forms

(4). All these compounds, except codeine and narcotine, form derivatives that exhibit fluorescence. Direct derivatization is feasible for syrups and aqueous slurries of capsules. Earlier studies had reported detectability of several alkaloids by direct reaction with dansyl chloride (5-7) on the basis of reaction of the primary or secondary amino group or phenolic hydroxyl group of alkaloids. Similarly, cannabinoids in human urine and ephedrine have been determined on reaction with dansyl chloride (8, 9).

Substituted tetrahydroisoquinoline compounds have been determined by in situ oxidation in biological fluids by UV irradiation of the isoquinoline derivatives (10). Sennosides A, B, and C are analyzed after reaction with hydrazine. The reaction involves the formation of a hydrazone followed by an elimination step to form the condensed anthracene ring system that provides fluorescence.

Quaternization of the 3'-tertiary amino group of the methylimidazole ring of pilocarpine can be carried out with β-nitrobenzyl bromide. HPLC entails ion pairing with octane sulfonate on a C-18 reverse phase column (11).

Isopilocarpine is a commonly encountered impurity in pilocarpine, an alkaloid isolated from *Pilocarpus microphyllus*. This impurity is an inactive stereoisomer and can form as an artifact during the isolation process. It also occurs naturally in plants (12).

(+)-Pilocarpine, 3-ethyldihydro-4-[(1-methyl-1H-imidazol-5-yl)methyl]-2(3H)-furanone, can be prepared by stereoselective synthesis (13); however, in aqueous medium, pilocarpine (I) can hydrolyze to pilocarpic acid (III) and epimerize to isopilocarpine (II), which in turn can hydrolyze isopilocarpic acid (IV). Both epimerization and hydrolysis decrease pharmacological activity.
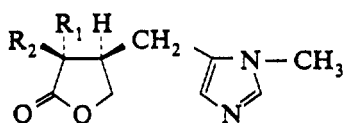
I: R_1 = H, R_2 = C_2H_5
II: R_1 = C_2H_5, R_2 = H
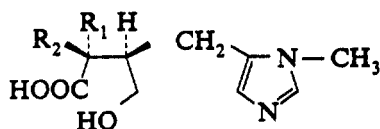
III: R_1 = H, R_2 = C_2H_5
IV: R_1 = C_2H_5, R_2 = H

The USP (14) requires a minimum purity of 98.5% for pilocarpine hydrochloride by titrimetric method and has a limit test for other alkaloids. An ion-exchange method provides significant improvement over the titrimetric method since it allows detection of 0.1 μg of isopilocarpine (15); however, this method does not resolve pilocarpic acid and isopilocarpic acid. The separation of all of these components has been obtained with reverse phase HPLC (16). Unfortunately, the detectability of isopilocarpine remains poor. This problem has been resolved to some extent (17,18) by RPLC on a Lichrosorb RP-18 column with a mobile phase composed of water-methanol (97:3) adjusted to pH 2.5 with potassium dihydrogen phosphate. A minimum detection limit of approximately 0.4 μg can be achieved by detection at 215 nm.

Codeine can be analyzed by HPLC (19) on a silica gel 10-μm particle-size column with methylene chloride-methanol-n-hexane-ammonium hydroxide (23.2/4.4/72.3/0.1) as a mobile phase. The method separates structurally related compounds such as naloxone hydrochloride, apomorphine hydrochloride, nalorphine hydrochloride, and morphine sulfate from codeine. The minimum detectable limit for these compounds except morphine sulfate is 0.1 μg.

Quinidine is commonly contaminated with isoquinidine. The USP (14) allows a dihydroquinidine concentration of up to 20%. Dihydroquinidine has a 40% higher oral LD_{50} (rats) and is less available in humans (20). It is possible to detect as little as 2.5 ng of dihydroisoquinidine by RPLC on a μBondapak C-18 column with methanol-water-acetic acid (25/4/71) as the mobile phase. Further improvements in minimum detectability are possible.

The hydroxide ion-catalyzed hydrolysis of securinine (Compound I in Scheme 1) involves the ring opening of the lactone moiety (21). Reaction kinetics have been monitored at either 330 or 290 nm since the reaction engenders a decrease in absorbance at 330 and 260 nm and an increase in absorbance during the course of the reaction at 290 and 220 nm. The rate of hydrolysis is insensitive to the ionic strength. The observed pseudo first-order rate constant reveals a decrease of approximately fourfold due to the increase in acetonitrile content from 4% to 50% (v/v) in mixed

aqueous solvent. The hydroxy carboxylate product of alkaline hydrolysis of securinine is shown to undergo cyclization in acidic medium to yield securinine. The observed pseudo-first-order rate constant for cyclization increase linearly with an increase in [H$^+$]. The change in content of acetonitrile from 3.8% to 47.2% (v/v) in mixed aqueous solvents does not show an effect on the rate of cyclization reaction.
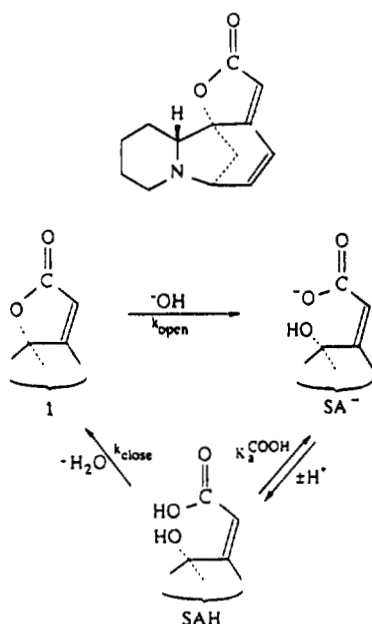
Scheme 1 (21).

The importance of analytical methodology in prediction of stability in pharmaceutical preparations has been demonstrated by following the kinetics of hydrolysis of cocaine degradation products (22). The kinetics of hydrolyses studied previously (Scheme 2), delineated rate constants k_1, k_2, and k_3 at various temperatures and pH values on the premise that rate-determining

steps for the loss of cocaine were k_1 going to benzoylecgonine and k_3 as the loss to nonchromophoric products.

$$\text{Cocaine} \xrightarrow{k_1} \text{benzoylecgonine} \xrightarrow{k_2} \text{ecgonine}$$
$$\downarrow\!\!\xrightarrow{k_3} \text{ecogonine methyl ester} \xrightarrow{k_4}\uparrow$$
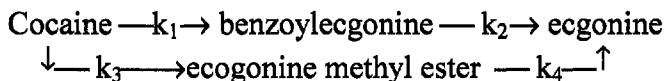
Scheme 2. Hydrolysis of cocaine.

Since no specific assay for nonchromophoric ecgonine methyl ester was available, the exact characterization of k_3 and k_4 were not possible at the time. Derivatization with phenyl isocyanate yields a derivative that can be chromatographed and quantitated by HPLC. This procedure allowed determination of k_4 and showed that there is no great build-up of ecgonine methyl ester from the solvolysis of cocaine due to pH factors alone since the rate of hydrolysis is greater than the rate of its formation.

9.2 AMINES

The USP monograph (14) on amphetamine sulfate requires a minimum purity of 98.0% based on a column chromatographic method. Since this is a low-resolution method, it is not capable of resolving impurities originating from a variety of sources. For example, α-benzylphenethylamine (X) has been found as an impurity in illicit amphetamine by TLC on silica gel plates that have been developed with ammonia-saturated chloroform-methanol (18/1) system, and detection is carried out by UV. Its origin can be postulated as follows (23):

The presence of traces of N-formylamphetamine (VI) in an illicit sample, detected by GC/MS, indicates that amphetamine (VII) has been prepared by reacting methyl benzyl ketone (V) with ammonium formate or formamide, thus providing Compound VI as an intermediate, as shown on the next page.

Dibenzylketone (VIII), a by-product in the synthesis of Compound V (see next page), can produce α-benzylphenethylamine (X) by the Leuckart procedure during the synthesis.

$$C_6H_5CH_2COCH_3 \longrightarrow \underset{\underset{CH_3}{|}}{C_6H_5CH_2CHNHCHO} \longrightarrow \underset{\underset{CH_3}{|}}{C_6H_5CH_2CHNH_2}$$

V	VI	VII

$$(C_6H_5CH_2)_2CO \longrightarrow (C_6H_5CH_2)_2CHNHCHO \longrightarrow (C_6H_5CH_2)_2CHNH_2$$

VIII	IX	X
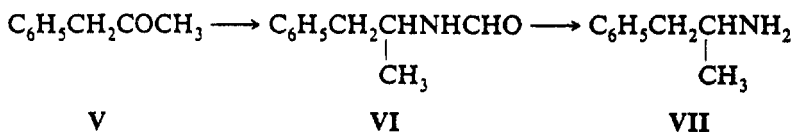
The formation of N-methyl-N-(α-methylphenylethyl)formamide during the synthesis of illicit methylamphetamine has been similarly reported (24). Information on the identification of precursors, intermediates, and impurities of 3,4-methylenedioxy-amphetamine has been published (25); however, no information is available on minimum detectabilities or the concentration of impurities found in this investigation.

HPLC analysis of an ophthalmic solution containing the active drugs naphazoline and antazoline revealed a degradation peak of unknown identity (26). To elucidate the identity of the degradant, the active drugs of each have been hydrolyzed by refluxing at high pH; their respective hydrolysis products are then isolated and spectrally characterized by NMR, FT-IR, and MS for conclusive structure elucidation. The degradant's identity has been confirmed by HPLC-MS analysis of the ophthalmic solution to be antazoline hydrolysis product N-[9N-benzylanilino)acetyl}ethylenediamine. The structures of the active drugs in the pthyhalmic solution with potential hydrolysis product of each are shown in Scheme 3.

A stability-indicating HPLC method has been developed to resolve N-[9N-benzylanilino)acetyl}ethylenediamine from the active drug. The mobile phase is comprised of methanol-water (57/43) containing 22 mM heptanesulfonic acid, 0.1% dibutyl-amine, and 1% acetic acid. It is pumped at a rate of 1 ml/minute through a Beckman Ultrasphere C-18 column (250 $\times$ 4.6 mm, 5μm particle size). The method utilizes detection at 280 nm and provides a detection limit of 2 μg/ml of N-[9N-benzylanil-

ino]acetyl) ethylenediamine. Analysis of an expired lot of the ophthalmic solution showed the concentration of N-[9N-benzylanilino] acetyl)ethylenediamine to be 0.002% (w/v).

Naphazoline

Antazoline

Step 1 | 0.5M NaOH Heat

1-Naphthylacetylethylenediamine

N-Benzylanilinoacetylethylenediamine

Step 2 | 1M NaOH Heat

1-Naphthylacetic Acid

N-Benzylanilinoacetic Acid

+

$2\ H_2N-CH_2-CH_2-NH_2$
Ethylenediamine
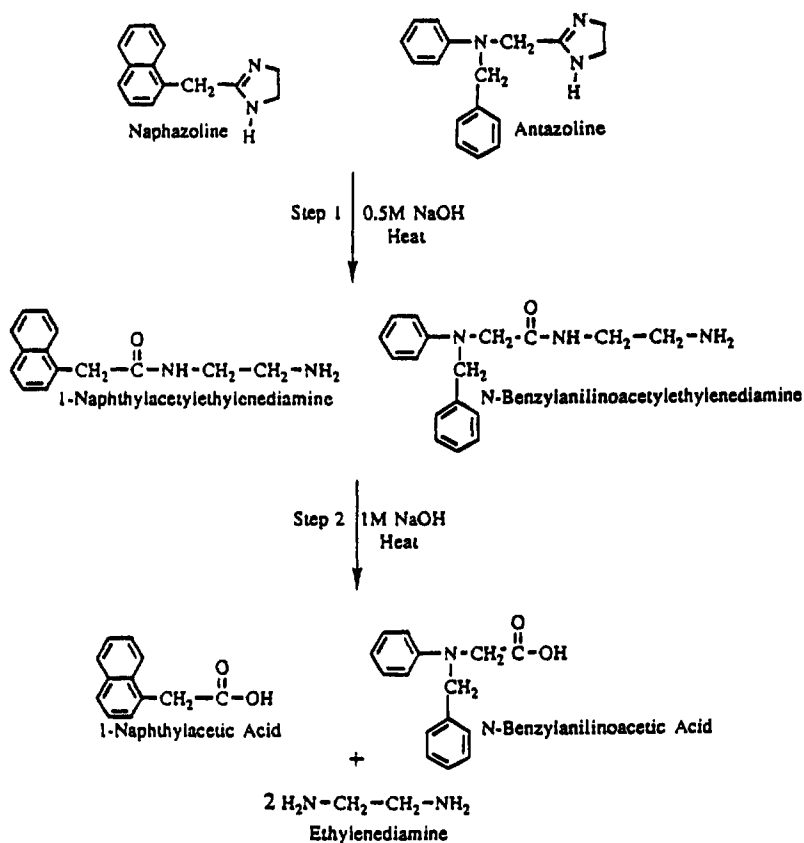
Scheme 3. Structures of the active drugs in ophthalmic solution with the potential hydrolysis path for each (26).

Acetate derivatives of some biogenic amines can be prepared to attain better chromatographic properties (27). Aromatic amines can generally be analyzed by HPLC with a UV detector--without any

prior derivatization; however, in the case of aliphatic amines, derivatization provides a valuable means of increasing UV detectability. A sensitive and selective method for the determination of an aliphatic amine, [tocainide 2-amino-N-(2,6-dimethylphenyl) propaneamide], is based on reaction with dansyl chloride (28). The fluorescent dansyl derivatives are separated on a μBondapak NH_2 column and quantitated at 0.1-5.0 μg/mL level in plasma. Derivatization with p-nitrobenzoyl chloride has been used to increase UV detectability of ketamine (29).

The parameters of the reaction of dansyl chloride with catecholamines, adrenaline, noradrenaline, and dopamine have been studied systematically and optimized (30). The derivatized catecholamines are separated by adsorption and reverse phase chromatography. Detectability of amines down to 50 pg is possible by using dansyl derivatives (31). Tri-dans adrenaline is unstable, and a plausible explanation for its instability has been reported (32).

HPLC has been used to separate and detect the fluorescent adducts formed as a result of the reaction of o-phthalaldehydes with catecholamines in the low picogram range (33). A reverse phase C-18 column with a methanol-acetic acid system as the mobile phase is used for HPLC separation.

9.3 AMINO ACIDS

The analysis of amino acids as it pertains to proteins and peptides may be found in macromolecules. Discussion here is limited to pharmaceutical compounds with amino acid structures that have been used as drugs. The USP (14) controls the concentration of related compounds in levodopa by TLC as follows: 3-hydroxy L-tyrosine (6-hydroxydopa) to 0.1% and 3-O-methyldopa to 0.5% of levodopa concentration. Tyrosine, a frequently observed impurity shows a spot with nearly the same Rf value as 3-O-methyldopa, and the 6-hydroxydopa spot is not easily visualized by this procedure (34). A reverse phase HPLC system that utilizes a μBondapak C-18 column with a mobile phase composed of 0.01 M NaH_2PO_4 solution adjusted to pH 2.5 with phosphoric acid, and

UV detection at 280 nm, is useful for determination of 6-hy-droxydopa, tyrosine, 3-O-methyldopa and other trace amino acid impurities. The trace impurities can be estimated at the ≤0.01% level, and the minimum detectability of the method is approximately 50 µg.

Determination of iodoamino acid contaminants such as 3,5-diiodothyronine, isoliothyronine, and levothyroxine at 50 ng per injection level has been performed by reverse phase HPLC (35). Comparable separations are obtained with Spherisorb (5 µm) ODS, LiChrosorb RP8 (5 µm), µBondapak C-18 (10 µm), and Chromegabond (5 µm) C-8 columns. The mobile phase is composed of 13.6 g potassium phosphate monobasic solubilized in 1 liter of water diluted to 2 liters with methanol. The pH is adjusted to 5.0 with orthophosphoric acid.

A reverse phase HPLC method has been reported in the literature for the analysis of baclofen tablets (36). Selectivity of separation has not been demonstrated for potential impurities. It may be recalled from Chapter 4 that baclofen is an amino acid with a structure similar to phenyl alanine with very low UV absorptivity (A1%, 1 cm = 11 at 266 nm). Investigations by Ahuja et al (37) have shown that lactam formed by the loss of a molecule of water is the primary degradation product.
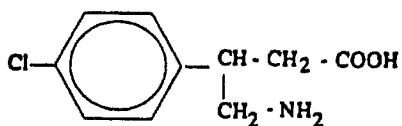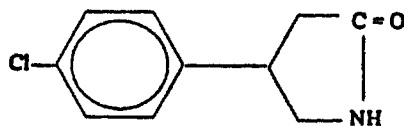
Baclofen   Lactam

Baclofen gives an asymmetric peak (Figure 9.1) when no ion-pairing (IP) reagent is used with a reverse phase µBondapak C-18 column with a mobile phase of methanol-water-acetic acid (44/56/1) at a flow rate of 0.6 ml/minute.

When baclofen is injected with an IP reagent such as pentane sulfonic acid in a stochiometric ratio of 1:1, a symmetric peak is produced (Figure 9.2).
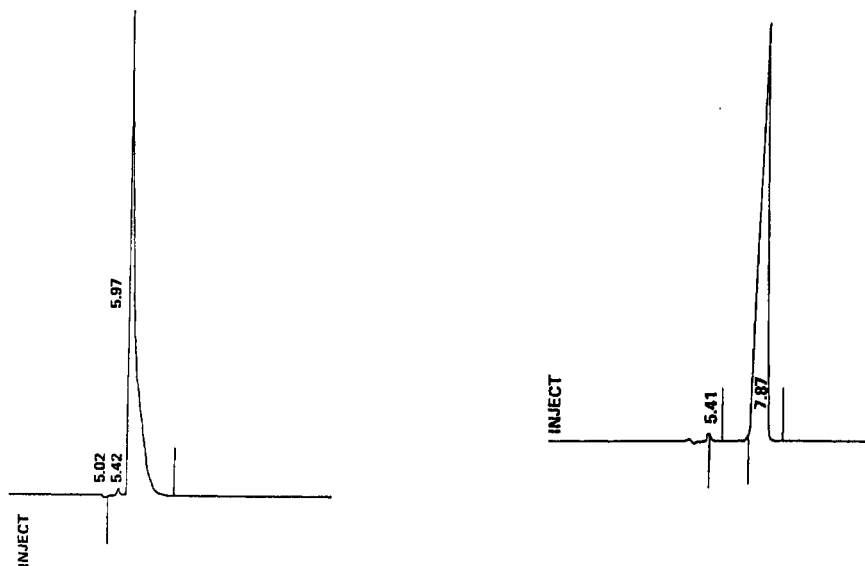
Figure 9.1 Chromatogram of baclofen without PSA.

Investigations on the optimal concentration of IP reagent suggest 0.007 M concentration is desirable for this separation (Figure 9.3). The retention time increases from approximately 6 to 10 minutes. The experimental conditions are as follows: A solution of sample in 4% HOAc in 30% methanol is chromatographed with 0.0073 M pentane sulfonic acid in MeOH-H$_2$O-HOAc (44/56/1) on a μBondapak C-18 column (30 cm × 3.99 cm i.d.) at a flow rate of 0.6 ml/ minute.

The lactam retention changes slightly, as anticipated, due to the presence of IP reagent, as it does not form an ion pair with the IP reagent. This method provides an excellent means of monitoring the stability of baclofen.
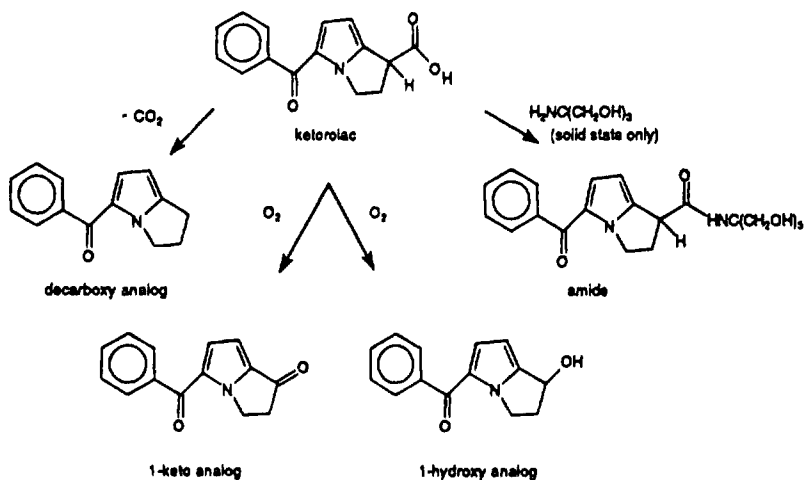
Figure 9.2 Chromatogram of baclofen injected with1:1
stoichiometric ratio of PSA.

Separation of several amino acid derivatives (DNP, dansyl, and PTH) by HPLC on a reverse phase of Amberlite and XAD-2, -4, and -7 copolymers has been reported (38). Amino acids react with o-phthalaldehyde in alkaline medium in the presence of a reducing agent to produce a fluorescent derivative (39). However, proline and hydroxyproline cannot be detected by the reaction. Reaction with pyridoxal in alkaline medium, followed by reduction, yields a stable pyridoxyl-amino acid that is fluorescent and can be detected at concentrations as low as 5×10^{-10} mol (40,41).

An easy, rapid, and sensitive method for the simultaneous determination of primary and secondary amino acids in different matrices (for example, protein hydrolysates, pharmaceutical formulations, plant extracts, food samples, and physiological fluids) has been described (42). After minimum sample preparation, amino

acids were derivatized with two different reagents, *o*-phthaldehyde-3-mercaptopropionic acid for primary amino acids and 9-fluorenylmethylchloroformate for secondary amino acids, by an automated precolumn derivatization technique.
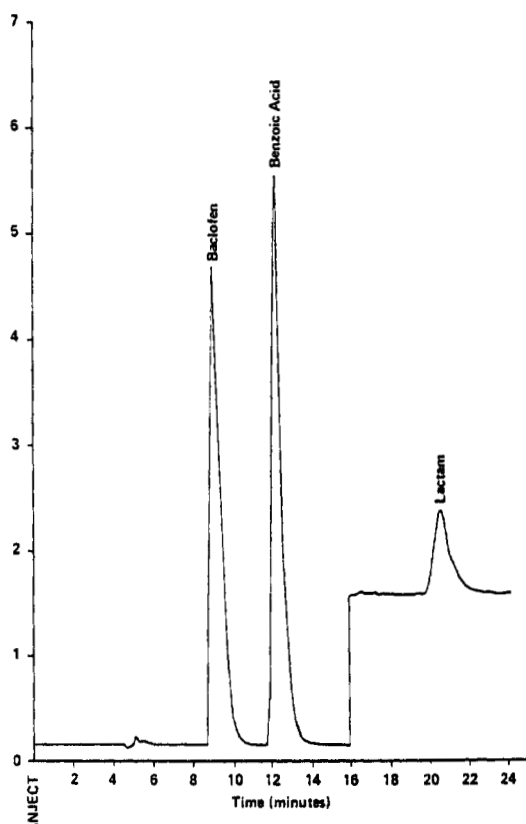
Figure 9.3 A typical chromatogram of baclofen.

With minor adjustments of separation parameters, this method can also be used to determine amino acids in different matrices.

Analysis time, including reactions, separation, and reconditioning, ranged from 20 minutes for hydrolysates to 60 minutes for physiological fluids. The separation is done on a reverse phase column with a gradient of acetate buffer-acetonitrile as the mobile phase. The precision for peak areas of the individual amino acids shows a relative standard deviation of 2% for the hydrolysate assay and 2-5% for the physiological assay, and for retention times better than 0.7%.

The detection limit with the diode array detector (UV-visible) was approximately. 2-5 pmol, measured at 338 nm for primary and 266 nm for secondary amino acids; with the fluorescence detector, 20-50 fmol were detectable at excitation and emission wavelengths of 230 and 455 nm for primary and 266 and 310 nm for secondary amino acids, respectively.

9.4 ANALGESICS

Classic analgesics are included here, as well as anti-inflammatory and antiarthritic compounds. A large number of publications have discussed the determination of salicylic acid in aspirin. Kirchhoefer (43) utilized a fluorescence detector to reduce the detection limits down to 3 μg for salicylic acid. A reverse-phase HPLC method with UV detection at 300 nm has been found useful for quantitating 0. 1 μg of salicylic acid in aspirin (44,45). A μBondapak C-18 column with a mobile phase composed of 0.1 M KH_2PO_4 solution containing 20% methanol adjusted to pH 2.3 has been used for this investigation.

Since the mobile phase is at pH 2.3, the decomposition of aspirin during the analysis has been minimized. LC on nonionic poly(methyl methacrylate) resin has been found useful for quantitating salicylic acid at 0.02% level of aspirin (46). The minimum detectability for salicylic acid is calculated to be 0.04 μg. An elaborate method that requires separation by silica gel TLC and reverse-phase HPLC has been used for quantitating salicylic acid down to 0.001% of the amount of aspirin present (47). A fluorometric method that is useful for the determination of salicylic

acid in salicylamide at the 10^{-7} mol level has also been reported (48).

The enantiomeric purity of naproxen [(s)-(+)-enantiomer of 2-(6'-methoxy-2'-naphthyl) propionic acid] has been determined by HPLC after derivatization with S-(+)-2-octanol (49). A chromatographic method is chosen over an NMR or direct rotation measurement method to obtain the desired specificity with a small sample size. To ensure that the ratio of diastereoisomers produced mirror exactly the ratio present in the original sample, the reagents used must be enantiomerically pure and there must be no kinetic resolution or racemization in the reaction. The mobile phase composed of 0.5'% hexane in ethyl acetate is used with a Spherisorb (5-μm) silica column for determination of enantiomeric purity.

The chemical structures of four impurities isolated from sulfasalazine, 5{[p-(2-pyridylsulfamoyl)phenyl]azo}salicylic acid, has been reported (50). Three of these impurities are by-products of the reaction process in drug synthesis:

- 2-{[p-(2-pyridylsulfamoyl)-phenyl]azo}hydroxybenzene
- 3-{[p(2-pyridylsulfamoyl)phenyl]azo}salicylic acid
- 5-{[p-[4-(2-pyridylanilino)]-N-phenyl]azo}salicylic acid

The fourth impurity is sulfapyridine, the starting material in the synthesis. Four lots of sulfasalazine from different manufacturers show all of the impurities described; however, no details regarding the content or minimum detectable limits have been provided.

The compendia do not include tests and limits for impurities in indomethacine even though it can be synthesized by several routes (51). TLC on silica gel GF with two different solvent systems (100 ether-3 acetic acid and 90 benzene-10 methanol-3 acetic acid) and UV detection have been used for the determination of several impurities down to the 0.1-μg level.

Two impurities (3-chlorobenzoic acid and 5-methoxy-2-methyl-3-indole acetic acid) have been found at less than 0.05% level in the batches of active ingredient tested for this study.

The following impurities, including three unknown compounds, have been found in meperidine (52):

- Ethyl 1-benzyl-4-phenyl-4-piperidine carboxylate
- Methyl-1-methyl-4-phenyl-4-piperidine carboxylate
- Ethyl 1-ethyl-4-phenyl-4-piperidine carboxylate

Two batches of the bulk drug contained three unknown impurities at low levels. Scheme 4 illustrates the structures and synthesis of several impurities.

Table 9.2 lists the minimum detection limits of impurities that have been detected by TLC (UV detector at 254 nm and spraying with dilute potassium iodobismuthate solution) on silica gel GF plates that were developed with a solvent system composed of ethyl acetate-cyclohexane-dioxane-water-conc. ammonium hydroxide (50:50:20:10:1).

Table 9.2 TLC of Meperidine and Its Synthetic Intermediates and Impurities (52)

COMPOUND	TLC Rf	MINIMUM DETECTION LIMIT[a] (μg)
XI	0.00	--
XX	0.00	0.50
XIX	0.10	--
XVI	0.20	--
XXI	0.39	0.50
XII	0.42	--
Meperidine	0.47	0.25
XVIII	0.60	0.25
XII	0.70	--
XV	0.71	--
XIV	0.73	--
XVII	0.75	0.25
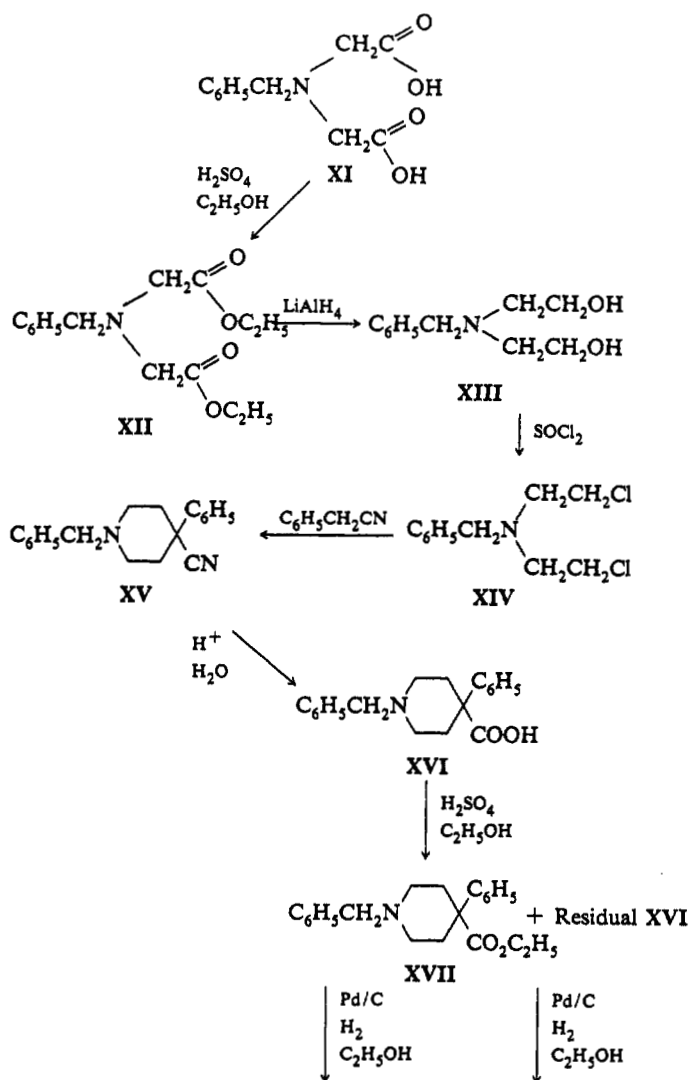
Scheme 4 (52).
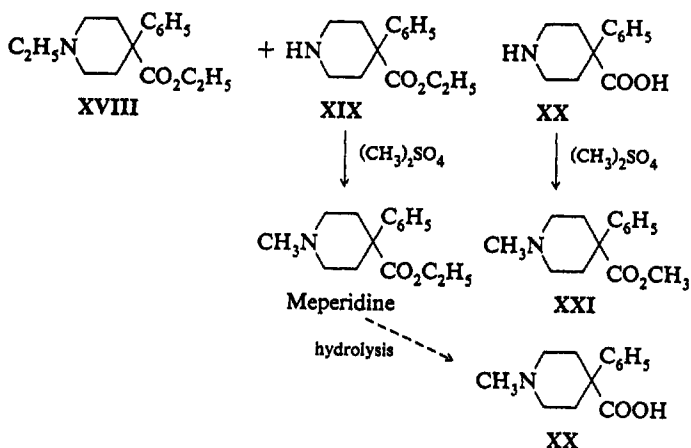
Scheme 4. (continued)

9.4.1 Anti-inflammatory Agents

Ketorolac tromethamine (toradol) is a nonsteroidal anti-inflam-matory drug that also exhibits analgesic and antipyretic effects. The degradation of ketorolac tromethamine in solution has been studied under thermal and photochemical stress conditions (53). Scheme 5 illustrates the solution degradation products (decarboxy, 1-keto, and 1-hydroxy analogue) that also form in the solid state. Condensation of ketorolac with tromethamine to form an amide also occurs. Methods for improving the stability of ketorolac powder blends under elevated humidity and temperature conditions have been investigated. The approaches that have been examined include: varying the salt ketorolac salt form, altering the excipient

ratio, and adding antioxidants or pH modifiers to the formulation. The most stable powder blends are achieved by substituting either the calcium salt or the free ketorolac for the tromethamine salt. This modification produces an extremely stable formulation due to low solubility of either form in water. The dry-blended propyl gallate (0.1% w/w) also stabilizes the drug. Also pH modifiers such as sodium carbonate, sodium bicarbonate, and citric acid increase the stability of ketorolac powder blends. Additionally, increasing the lactose: microcrystalline cellulose ratio and avoiding croscarmellose sodium improves the stability of powder blends.
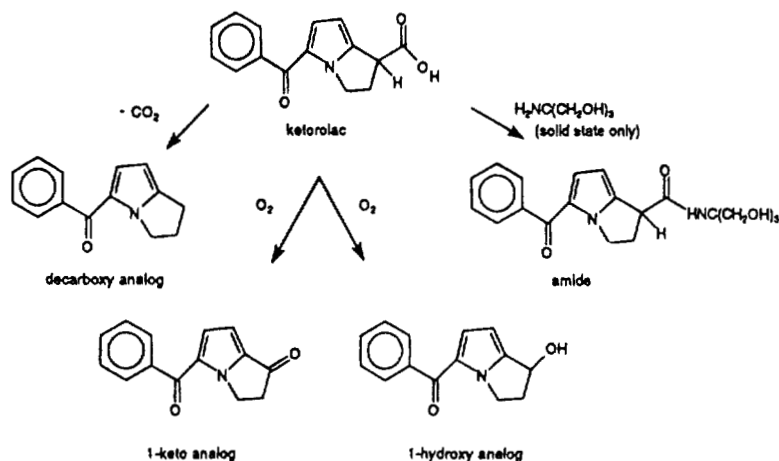
Scheme 5. Degradation pathways of ketorolac in solution and in the solid state (53).

3-Carboxy-5-methyl-N[4-(trifluoromethoxy)phenyl]-4-isoxazolecarboxamide (XXI) is a potential prodrug for the antiarthritic agent 2-cyano-3-hydroxy-N-[4-(trifluoromethoxy)phenyl]-2-butenamide, XXIV (54). It metabolizes similarly to the established prodrug leflunomide. Stability studies of XXI in solution have been investigated to support the toxicology formulations. Also, by monitoring the concentration of XXIV as a function of time, the kinetics and products of the active form have been elucidated (Scheme 6), so that these studies would provide insight into the transformation of the active form in vivo.
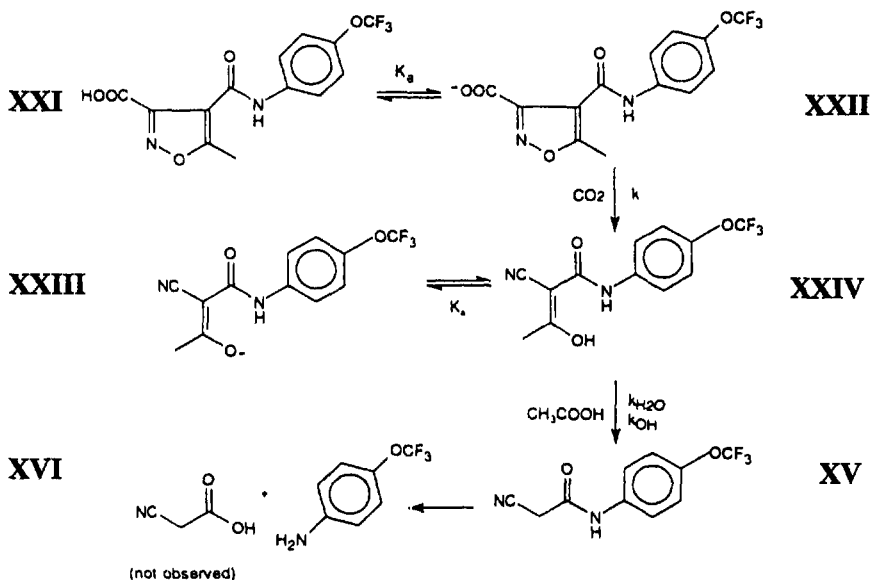
Scheme 6 (54).

Degradation product XXIV has been prepared by reacting a solution of XXI at pH 10.0 and 100°C for 90 min. Upon cooling a white precipitate formed, which is collected by filtration. Degradation product XXV is prepared by dissolving XXI in 0.1 N HCl solution in aqueous ethanol and heating at 80°C for 72 hours. The ethanol is then removed by evaporation, resulting in a precipitate. Both structure and purity are confirmed by proton NMR, C-13 NMR, MS, and HPLC.

The stability of XXI has been studied in aqueous buffer solutions from pH 1 to 10 at 40, 50, and 60°C. The degradation kinetics are found to follow first-order kinetics for >2 half-lives. The pH-dependence of the degradation rate at 40, 50, and 60°C is shown in Figure 9.4. When data is extrapolated to 25°C, a t_{90} of approximately 3 months is predicted from pH 3 to pH 10. The reactivity of XXI in the pH region studied ($t_{1/2}$ = 70 days at 37°C) cannot explain the in vivo conversion to the active form,

suggesting that the reaction is mediated by biological catalysts (55).

The degradation products from the decomposition of XXI in aqueous solution have been identified by using HPLC and coinjecting authentic samples.

40°C (▲), 50°C (●), and 60°C (■)
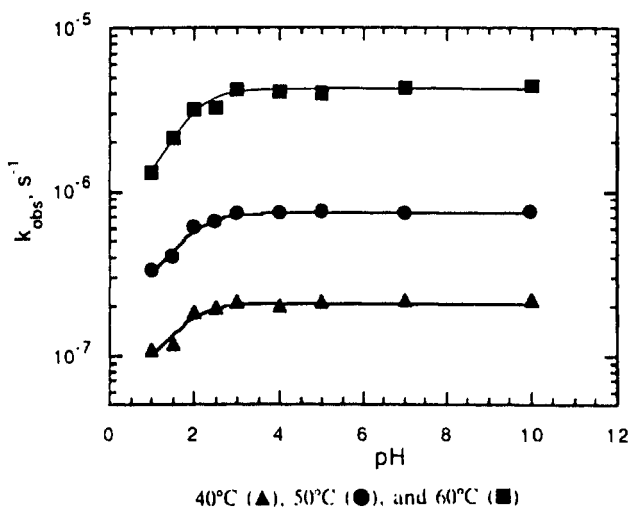
Figure 9.4 A pH-rate profile for the decomposition
of XXI in aqueous solution (54).

9.5 ANTIBACTERIALS

For the purpose of this discussion, antibacterials have been divided into two groups: antibiotics and sulfonamides.

9.5.1 Antibiotics

Lam and Grushka (56) have reported on the formation of phenacyl esters of natural non-UV-absorbing penicillins. The derivatives formed are separated on a reverse phase column. Lawrence and Frei (57) have suggested that antibiotics such as ampicillins, kanamycin, neomycin, polymycin B, and streptomycin react with fluorescamine to produce fluorescent derivatives on the basis of

reaction of a primary amino group. HPLC of these derivatives should be possible.

A mixture of antimycins A has been separated into eight hitherto unreported subcomponents--A_1a, A_1b, A_2a, A_2b A_3a, A_3b, A_4a, and A_4b (58)--by using a reverse phase HPLC technique. Although a baseline resolution of the known four major antimycins A_1, A_2, A_3, and A_4 is readily achieved with mobile phases containing acetate buffers, the separation of the new antibiotic subcomponents is highly sensitive to variation in mobile-phase conditions. The type and composition of organic modifiers, the nature of buffer salts, and the concentration of added electrolytes has profound effects on capacity factors, separation factors, and peak resolution values. Of the numerous chromatographic systems examined, a mobile phase consisting of methanol-water (70:30) and 0.005 M tetrabutylammonium phosphate at pH 3.0 yielded the most satisfactory results for the separation of the subcomponents. Reverse-phase gradient HPLC separation of the dansylated or methylated antibiotic compounds produces superior chromatographic characteristics, and the presence of added electrolytes is not a critical factor for achieving separation. Differences in the chromatographic outcome between homologous and structural isomers can be interpreted based on a differential solvophobic interaction rationale.

A wide variety of HPLC methods for assaying many antimicrobial agents have been summarized in Table 9.3 (59).

A GC method can be used for the determination of residual N, N-dimethylaniline as a contaminant in ampicillin, cephalosporin, and other penicillins (60). The method involves dissolution of the sample in aqueous alkali, extraction of the organic base with cyclohexane, and injection onto a phenylsilicone column. Dimethylaniline can be determined down to levels of 0.1 ppm (minimum detectability is in the ng range). The residual levels of ampicillin and penicillin G in various tetracycline and penicillamine have been detected by reverse-phase TLC, followed by bioautography (by measuring inhibition of growth of *Sarcina lutea*) (61). The lower limit of detection is <1 ng with bioautography on

Table 9.3 Summary of HPLC Methods for Trace/Ultratrace Concentrations of Antimicrobial Agents

AGENT	STATIONARY PHASE	MOBILE PHASE	DETECTOR	SENSITIVITY
Aminoglycosides				
Amikacin	Silica gel	Methanol-water-acetonitrile	Fluorescence	1.0
Gentamicin	C-18	Acetonitrile-water	Fluorescence	0.2
Netilmicin	C-18	Acetonitrile-water	Fluorescence	0.5
Tobramycin	C-18	Methanol-water-diethylamine-acetonitrile	UV	0.5
Bacitracin	C-18	Methanol-acetonitrile-phosphate buffer	UV	--
Cephalosporins				
Cefamendole	C-18	Methanol-sodium acetate	UV	0.3
Cefazoline	C-18	Methanol-ammonium carbonate	UV	0.1
Cefmenoxime	C-18	Acetonitrile-acetate buffer	UV	0.05
Cefoperazone	Phenyl	Acetonitrile-tetrabutyl-ammonium buffer	UV	1.0
Cefatetan	C-18	Acetonitrile-phosphate buffer	UV	0.7
Cefoxitine	C-18	Methanol-phosphoric acid	UV	0.3

Table 9.3 Summary of HPLC Methods for Trace/Ultratrace Concentrations of Antimicrobial Agents (continued)

AGENT	STATIONARY PHASE	MOBILE PHASE	DETECTOR	SENSITIVITY
Cefsulodin	C-18	Acetonitrile-acetate buffer	UV	0.2
Cephalexin	C-18	Methanol-water	UV	0.5
Chloramphenicol	C-18	Acetic acid-methanol-water	UV	0.5
Erythromycin	C-18	Acetonitrile-acetate-water	Fluorescence	0.1
Imipenem	C-18	Borate buffer-sodium hydroxide	UV	0.5
Metronidazole	C-18	Acetonitrile-phosphate buffer	UV	0.5
Nitrofurantoin	C-18	Methanol-sodium acetate	UV	0.02
Penicillins				
Amoxicillin	C-8	Methanol-phosphate buffer	UV	0.5
Ampicillin	C-18	Methanol-phosphate buffer	UV	0.5
Benzyl penicillin	C-18	Methanol-phosphate buffer	UV	0.1
Cloxacillin	C-18	Acetonitrile-water-ammonium acetate	UV	0.5
Piperacillin	C-18	Methanol-acetate buffer	UV	0.05
Sulfonamides				
Sulfacetamide	C-8	Methanol-phosphate buffer	UV	0.5
Sulfadiazine	C-18	Acetonitrile-acetic acid	UV	1.0

Sulfadimidine	C-8	Methanol-phosphate buffer	UV	0.5
Sulfamerazine	C-18	Acetonitrile-acetic acid	UV	1.0
Sulfanilamide	C-8	Methanol-phosphate buffer	UV	0.5
Sulfapyridine	C-18	Chloroform-methanol-ammonia	UV	0.0007
Sulfathiazole	C-8	Methanol-phosphate buffer	UV	0.5
Tetracyclines				
Tetracycline	C-18	Methanol-EDTA	UV	0.3
Trimethoprim	C-8	Methanol-phosphate buffer	UV	0.75
Vancomycin	C-18	Acetonitrile-phosphate buffer	UV	0.5
Antiviral agents				
Adenine arabinoside	Ion exchange	Sodium borate-sodium acetate	UV	0.05
Antifungal agents				
Amphotericin-B	C-18	Methanol-EDTA	UV	0.02
Ketoconazole	C-18	Acetonitrile-phosphate buffer	Fluorescence	0.1
Griseofulvin	C-18	Acetonitrile-water	Fluorescence	0.05

silica gel GF plates impregnated with silicone and developed with barbital acetate buffer (pH 7.4)-acetone (94:6). An HPLC method has been reported for the separation of penicillin G potassium, and six decomposition products (62). The method utilizes a C-18 column and acetonitrile-phosphate buffer (1:4) adjusted to pH 4.1 as mobile phase. No minimum detectability information is available on this method.

The analysis of tetracycline epimers in tetracycline has been accomplished by HPLC on a microparticulate phenyl column with a step gradient of 12-22% acetonitrile in 0.2 M phosphate buffer at pH 2.2 (63). This method is suitable for resolving chlortetracycline, 4-epitetracycline, 4-epianhydrotetracycline, and anhydrotetracycline. The lowest quantitation limit for some of these impurities is 0.4µg. The bulk samples have been found to be approximately 2% of chlortetracycline and 0.04% 4-epianhydrotetracycline. A TLC fluorometric method has been found suitable for the determination of several impurities such as anhydroxy-tetracycline, epioxytetracycline, α-apooxytetracycline, and β-apooxytetracycline in oxytetracycline (64). Kieselguhr G plates (at pH 9) are developed with a solvent system composed of ethylene glycol-water-acetone-ethyl forinate-ethyl acetoacetate (3:6:45:30:15) and quantitation is achieved by fluorometry (λexcit 365 nm). The minimum detectability of the method is 10^{-2} µg.

An HPLC system consisting of methanol-water (3:2) and a Zorbax CN column has been utilized for the evaluation of impurities in eight batches of griseofulvin obtained from worldwide sources. Deschlorogriseofulvin is the major impurity, and it is common to all of the batches tested. The USP standard contains 0.6% of this impurity; however, some of the batches show as high as 3% of this impurity (65). The minimum detectability of this method is 0.24 µg.

The FDA spectrophotometric assay for certifying chloramphenicol succinate does not differentiate between chloramphenicol and its 3- or 1-succinate esters (66). An HPLC method has been developed to carry out these separations at 275 nm on a Lichrosorb RP-18 column with a mobile phase composed of

22% solution of acetonitrile in 0.05 M sodium acetate (adjusted to pH 5.7).

An HPLC method that can be used for monitoring potential degradation products of cephalexin as contaminants is based on the separation of its trinitrophenyl derivative on an amino Sil-X-1 column with a mobile phase composed of 1% citric acid in a methanol-water mixture (5:40) and detection at 425 nm (67).

A kinetic study on the alkaline hydrolysis of cefotaxime at pH 10.5 and 37°C has been carried out by using HPLC and ^{1}HNMR superscript (68). The main resulting products have been isolated and identified. The products include the well-known deacetyl-cefotaxime, the exocyclic methylene derivative, the 7-epimer of cefotaxime, and the 7-epimer of deacetylcefotaxime.

The kinetic constants involved in the process have been determined, and according to the experimental results, the attack of the hydroxyl group on the ester function bonded to the 3'-carbon is the fastest step in the proposed kinetic scheme. It should be emphasized that the base-catalyzed epimerization of the hydrogen at the 7 position clearly depends on the presence of a good electron-withdrawing group at C3'. On the other hand, no hydrolysis of the amide at the 7 position is detected.

9.5.2 Sulfonamides

A simple TLC method for determination of sulfamethoxyzole, trimethoprim, and impurities of sulfanilamide and sulfanilic acid has been developed and validated (69). A chloroform-n-heptane-ethanol (3:3:3) solvent system is used for quantitative evaluation of chromatograms on the silica gel plates. The chromatographic zones, corresponding to the spots of sulfamethoxazole, tri-methoprim, sulfanilamide, and sulfanilic acid are scanned in the reflectance/absorbance mode at 260 nm. The limit of detection for sulfanilamide and sulfanilic acid are 4.5 and 4.1 ng/μl, respectively. This equates to 0.01 % for the limit of detection.

The degradation kinetics of a new potential tripanocidal and antibacterial agent, 3-bromo-2-hydroxy-N-(3,4-dimethyl-5-isoxa-

zolyl)-1,4-naphthoquinon-4-imine (XXVIII), in 95% ethanol, has been investigated between 35 and 50°C under room light and light-protected conditions (70). The decomposition product is isolated and identified as 2-hydroxy n-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthaquinon-4-imine (XXVII). A simple, rapid stability-indicating method for the determination of XXVIII in the presence of I using "zero crossing" first-derivative spectrophotometry has been utilized for these studies. Pseudo-first-order rate constants for the degradation reaction of II are obtained from the linear plot of the residual concentration logarithms vs. time.
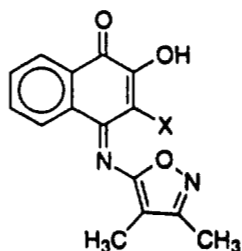
XXVII: X = H
XXVIII: X = Br

HPLC has been used for the determination of chlorpropamide, tolbutamide, and their respective hydrolysis products, p-chlorobenzene sulfonamide and p-toluene sulfonamide (70a). The separations are performed on a LiChrosorb Si-60 column with a mobile phase with 4% ethanol, 9% tetrahydrofuran, and 0.06% acetic acid in n-hexane. These impurities can be quantitated down to 0.2 μg.

The current USP (14) has replaced the old titrimetric method for hydrochlorothiazide (a diuretic, included here because of its sulfonamido character) with an HPLC method because it could not distinguish hydrochlorothiazide from the degradation and in-process impurities such as chlorothiazide and 4-amino-6-chloro-1,3-benzenedisulfonamide (70b). However, this is possible with an HPLC method that entails separation on a μBondapak C-18, column with 5% methanol adjusted to pH 4.5 with acetic acid as the mobile phase. Minimum detectability at the 0.01-μg level is possible with this method. Analyses of eight batches from two suppliers have shown a significant difference in purity as

determined by the titrimetric and HPLC methods. Two batches that had been analyzed at 100% by the titrimetric method showed a purity of 96.4% and 97.3% by the HPLC method.

9.6 ANTICONVULSANTS, ANTIDEPRESSANTS, AND TRANQUILIZERS

TLC has been used for monitoring impurities of imipramine and desipramine (71). TLC plates (silica gel G with 254-nm fluorescent indicator) are used with the following solvent systems:

- Imipramine--benzene-ethyl acetate-absolute ethanol-conc. ammonium hydroxide (50:50:15:3)
- Desipramine--benzene-ethyl acetate-absolute ethanol-conc. ammonium hydroxide (50:50:20:1)

The Rf and minimum detectability of the impurities are given in Table 9.4.

Table 9.4. TLC of Imipramine and Desipramine (71)

Compound	TLC/Rf	(μg)
Iminodibenzyl	0.81	0.06
Imipramine[a]	0.67	0.03
Compound (XXIX)	0.26	0.03
Desipramine[b]	0.36	0.03

a: as an impurity in desipramine
b: as an impurity in imipramine

Compound (XXIX), an unknown prior to this investigation, has been synthesized and has the following structure:
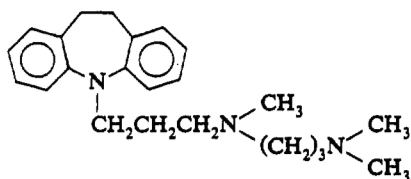
XXIX

Iminodibenzyl is a synthetic precursor and may also result from breakdown of the drug.

The drug doxepin, 11-(3-dimethylaminoethylpropylidene)-6H-di-benz[b,e]-oxepin is structurally related to imipramine and amitriptyline. A 15:85 mixture of the cis and trans isomers of doxepin is used as an antidepressant agent (72). The conversion of the cis isomer into the less active trans isomer can occur. A GLC method with an OV-17 capillary column and a nitrogen detector has been used for the determination of nanogram amounts of the cis and trans isomers of both doxepin and desmethyl doxepin. While doxepin gives a symmetrical peak underivatized, desmethyl-doxepin, a more polar secondary amine, tailed under these conditions. The peak shape can be improved by formation of N-trifluoroacetyl derivative.

Two reported degradation products of amitriptyline hydro-chloride, 3(propa-1,3-dienyl)-1,2:4,5-dibenzocyclohepta-1,4-diene and dibenzosuberone, have been analyzed by HPLC on μBondapak CN columns with a mobile phase of a 50:50 mixture of acetoni-trile-0.02 M ammonium acetate-0.01 M methanesulfonic acid (73).

The USP (14) controls other impurities by TLC in meproba-mate to 2% and methyl carbamate to 0.5% by HPLC. BP (74), on the other hand, controls related substances to 1% by TLC on silica gel GF with chloroform-ethanol (90:10), followed by spraying with furfural and concentrated HCl has been used for detection of impurities (75). Several synthetic impurities have been detected by this method, and it has been theorized that an impurity (XXX) may form by reaction of (XXXI) with the semichlorinated derivative of (XXXII), which, upon treatment with ammonia, yields (XXX).
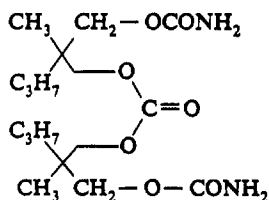
XXX
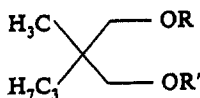
XXXI:R,R'= COCl
XXXII: R'= H, R = COCl

Two common impurities of chordiazepoxide, 7-chloro-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepine-2-one-4-oxide and 2-amino-5-chlorobenzophenone, have been quantitated at 6.3 and 0.9 ng, respectively, by adsorption HPLC (76). A LiChrosorb SI 60 (5μm) column with two different mobile phases is used for these separations:

- System A: 5% ammonical ethanol and 30% tetrahydrofuran in n-hexane
- System B: 25% tetrahydrofuran in n-hexane.

The investigations show that two systems are preferable over gradient elution where low precision, major baseline shifts, and longer re-equilibration time are necessary.

The structural changes of adinazolam mesylate, 8-chloro-1-[(dimethylamino)methyl]-6-phenyl-4H-s-triazolo[4,3-a][1,4]benzodiazepine in aqueous solution under various pH conditions by NMR spectroscopy has been examined (77). By plotting the signal integration and chemical shifts of the side chain, pKa values for the imine hydrolysis and the side chain are determined. The pKa values of the ring-opening reaction of the main compound, a common reaction in most benzodiazepines under acid conditions, and of the side chain amine are determined by NMR spectroscopic titration.
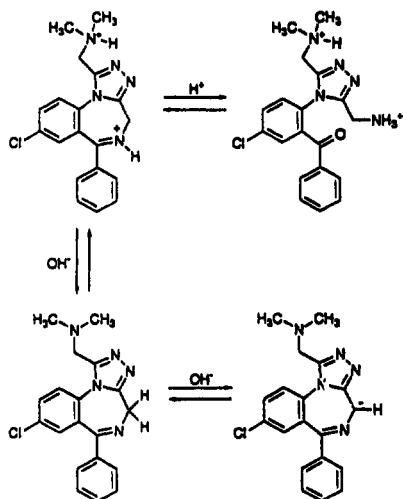
Scheme 7. pH-dependent structural change of adinazolam mesylate (77).

The conformation of the main compound is determined by measurement of isotope-induced shift effects, NOE, and T_1. Hydrogen/deuterium exchange of I has also been revealed by NMR spectroscopy. The pH-dependent structural changes are given in the scheme.

The acidic degradation of diazepam in methanolic aqueous solution has been investigated (78). Besides the known degradation products, 2-(N-methylamino)-5-chlorobenzophenone and glycine, produced by the hydrolytic cleavage of the benzodiazepinone ring, five novel products have been isolated and fully characterized by their spectroscopic features and independent synthesis. The formation of substituted 2-amino-3,5-dichlorobenzophenones and 2,4-dichloroacridinones in a methanol-aqueous solution of HCl at 50°C is mechanistically intriguing.

Benzodiazepines when taken concurrently with ethanol can encounter a pharmacological action that can potentiate the central nervous system depression produced by either drug. A study has been conducted to describe a novel chemical reaction between temazepam (a 3-hydroxy-1,4-benzodiazepine) and ethanol under acidic conditions similar to those found in vivo, resulting in a 3-ethoxylated product (79). The results raise the possibility that the ethanolysis reaction may occur in the stomach of people who consume alcohol and 3-hydroxy-1,4-benzodiazepine on a regular basis.

Temazepam (TMZ) undergoes hydrolysis in simulated gastric fluid (SGF; pH 1.2) (80). The hydrolysis reaction of TMZ in acetonitrile:SGF (1:19, v/v) at 37°C is an apparent first-order reaction with a half-life of 5.47 hours, i.e., 12% of the remaining TMZ is hydrolyzed per hour. The predominant hydrolysis product (2'-benzoyl-4'-chloro-N-methyl-2-amino-2-hydroxyacetanalide) and a minor hydrolysis product [2-9 methylamino)-5-chloro-benzophenone], derived from acid-catalyzed reaction of TMZ in aqueous solution have been characterized by UV-visible absorption mass, IR, and NMR spectra analyses. The kinetics of the hydrolysis reaction are studied as a function of acid concentration, temperature, and ionic strength in deuterated solvent. The results

indicated that the predominant hydrolysis reaction at pH $\neq$ pKa (1.46) is caused by protonation at N4, followed by a nucleophilic attack by water at C5 of the C5-N4 imium ion and a subsequent ring-opening reaction. The results of this study suggest that a fraction of an orally taken TMZ may be inactivated by the hydrolysis in the highly acidic gastric fluid.

2-Oxoquazepam [7-chloro-1-(N-2,2,2-trifluoroethyl)-5-(2'-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one], a major pharmacologically active metabolite of quazepam, has been hydrolyzed in sodium hydroxide solution to form sodium salt of 2 -(N-2,2,2-trifluoroethyl)amino-5-chloro-α-(2'-fluorophenyl-benzylidene) glycine (81). The hydrolysis product is formed via a rapidly established acid-base equilibrium, followed by a rate-determining, ring-opening reaction involving two negatively charged ions. Following neutralization, the hydrolysis product is isolated by RPLC and subsequently identified by its UV-visible absorption and MS analyses. The kinetics of hydrolysis reaction in acetonitrile/water mixture has been studied by RPLC analysis as a function of water content, NaOH concentration, temperature, and ionic strength.

A gradient HPLC method has been developed to determine degradation products of adinazolam mesylate in a sustained-release tablet formulation (82). Sample preparations are chromatographed on a YMC basic column using formate buffer/acetonitrile gradient with absorbance detection at 254 nm. Adinazolam mesylate is found to degrade at high relative humidity and temperature to form a major product, the 6-aminoquinoline analogue, plus numerous other compounds. Five of these compounds have been identified, and their structures indicate that the solid-state degradation of adinazolam, in the presence of sufficient moisture, involves not only a hydrolytic mechanism but also an oxidative mechanism (Schemes 8 and 9).

Potential process impurities are resolved from the drug and degradation products. The average mass balance obtained for a sample that were an average of 9.5% degraded is 95.0 $\pm$ 1.5%. The decrease in mass balance with increase in percentage degradation

may be explained by the formation of many components at trace
levels due to degradation by various permutations of hydrolytic and
oxidative reaction pathways.
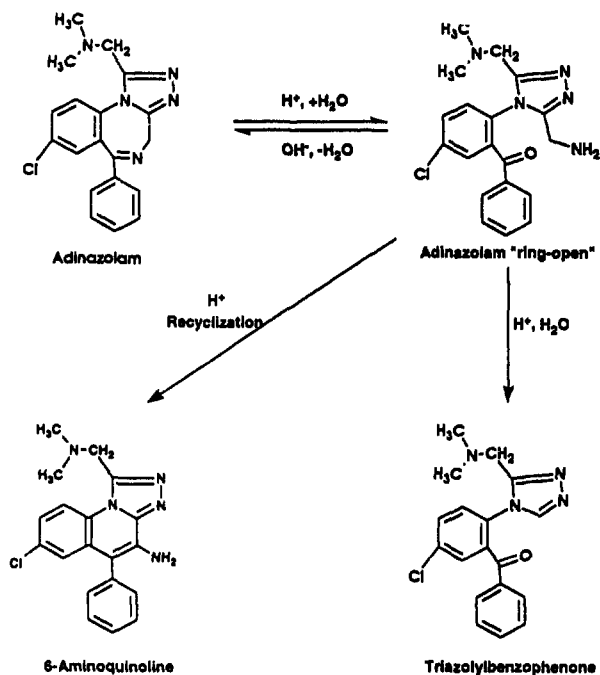
Scheme 8. The proposed acid-catalyzed hydrolytic
decomposition pathway of adinazolam in the
sustained-release formulation (82).

-(4-Chlorophenyl)-5-(4-pyridyl)-Δ^2-1,2,3-triazoline is one rep-
resentative member of a novel class of triazoline anticonvulsants in
the preclinical development stage (83). A study of the chemical
stability and degradation kinetics of this compound in aqueous
solution indicates that it is highly unstable and undergoes rapid
degradation with increasing temperature and decreasing pH,
yielding aziridine as one of the major products, with the loss of
nitrogen.
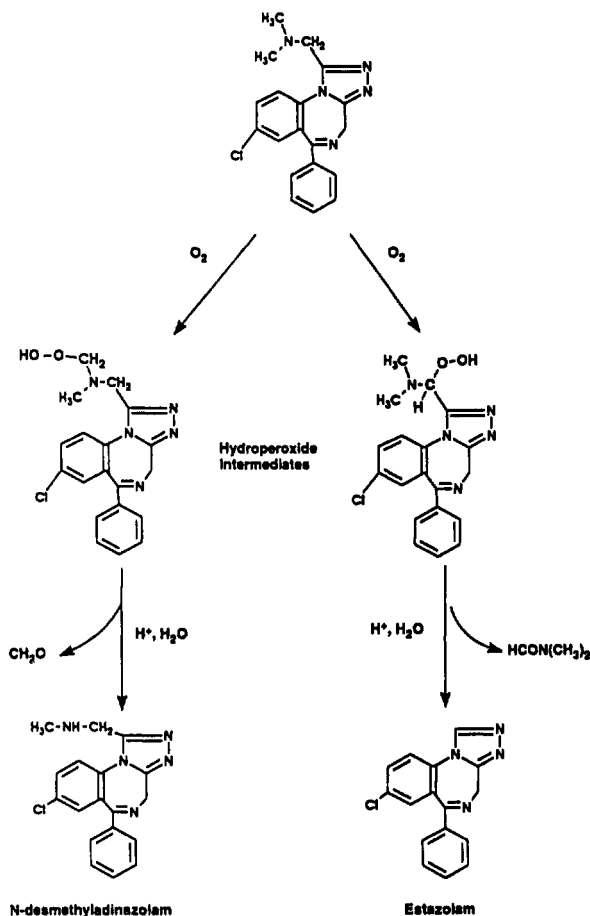
Scheme 9. The proposed oxidative decomposition pathway of adinazolam in the sustained-release formulation (82).

The observed degradation of this compound, particularly at a pH within the acidic and neutral range (pH 2 to 7), has posed some intractable problems in the formulation of this compound to be employed in preclinical studies. Regardless of oral or intravenous route of administration, the compound must be capable of

withstanding degradation not only at the gastric and intestinal pH in the gastrointestinal tract, but also at the physiological pH (7.4) in the blood. The degradation of triazolines is shown in Scheme 10.
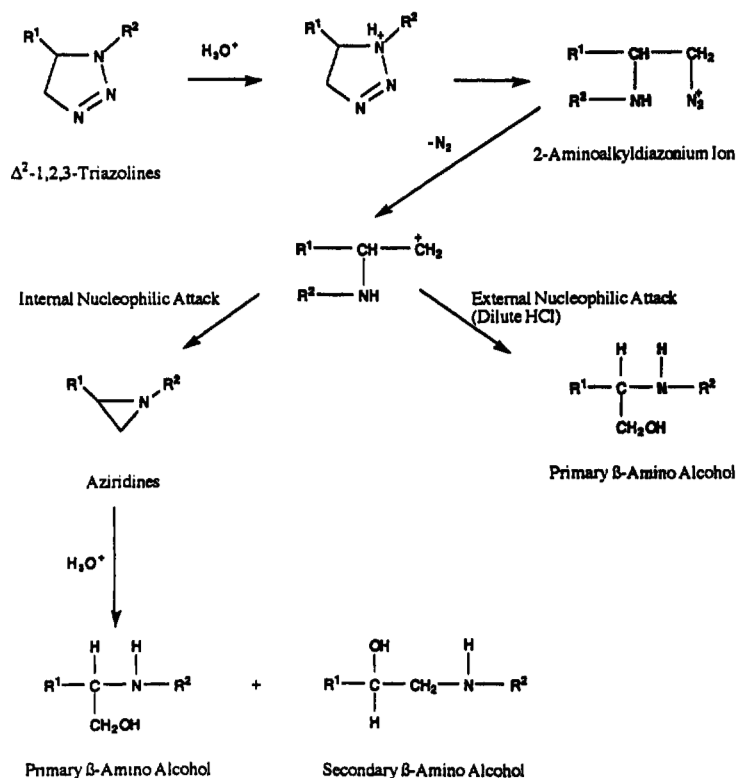
Scheme 10. Chemical degradation of triazolines (83).

This study demonstrates the important role of para substituents on the phenyl group in maintaining the stability of triazolines. However, metabolic lability, to afford the putative pharmacologically active primary β-amino alcohol metabolite, needs to be

designed in these prodrugs so that the active ingredient is readily generated metabolically when the prodrug reaches the target tissues.

9.7 ANTINEOPLASTIC AGENTS

Several degradation products of procarbazine hydrochloride (N-(1-methyl-ethyl)-4-[(2-methylhydrazino)methyl]benzamide) have been resolved by HPLC. A Partisil PXS 10/25 ODS-2 column with a 44% methanol solution buffered to pH 5 allows determination of degradation products at the 0.02% level (83a). A reverse phase HPLC method suitable for determination of degradation products has been used for kinetic studies on 2,5-diaziridinyl-3,6-bis (carboethoxyamino)-1,4-benzoquinone (84). A Spherisorb ODS (5 μm) column with 15% acetonitrile and UV detection at 345 nm provides detectability of 3 ng for the main compound. An impurity found in the bulk drug has been identified as 2,5-diamino-2,6-dichloro-1,4-benzoquinone.

A reverse-phase HPLC method [LiChrosorb C-18 (10-μm) column and 45% methanol as mobile phase and UV detector set at 200 nm] has been used for studying the purity of cyclo-phosphamide (N,N-bis(2-chloroethyl)tetrahydro)-2H-1,3,2-oxaza-phorin-2-amine-2-oxide) (85). The degradation products are eluted with the solvent; hence no minimum detectability information is available. Since methotrexate has been used mainly as a folic acid antagonist, the information on its impurity analysis has been given in the Miscellaneous group.

The anti-tumor agent gemcitabine hydrochloride, a β-difluo-ronucleoside, is remarkably stable in solid state. In 0.1 N HCl solution at 40°C, deamination of gemcitabine occurs, yielding its uridine analogue (86). Under these conditions, after 4 weeks approximately 86% of the initial gemcitabine remains. Cleavage of its N-glycocidic bond or conversion to its α-anomer in 0.1 N HCl solution is not observed over a 4-week period. However, it anomerizes in 0.1 N NaOH at 40°C. Approximately 72% of the initial gemcitabine remains after 4 weeks under the basic

conditions used. Uridine hydrolysis products are also formed under these conditions. The anomerization reaction, which is unusual under basic conditions, has been confirmed by characterization of the chromatographically isolated α-anomer by NMR and mass spectrometry. A mechanism involving an acyclic intermediate has been proposed.

The important role of albumin in determining relative human blood stabilities of camptothecin has been investigated (87). These agents exert their antitumor activities by producing topoisomerase I-mediated DNA damage. Camptothecin and several of its water-soluble derivatives contain an α-hydroxy-δ-lactone ring that hydrolyzes under physiologic conditions, i.e., pH 7 or above, with the lactone moiety readily opening up to yield the carboxylate form. It is known that biological activity of camptothecins both in vitro and in vivo are significantly greater for the lactone than for the carboxylate species. The results of this study indicate that HSA plays an important role in determining the relative human blood stabilities of camptothecins. In the case of camptothecin and 9-amino camptothecin, the protein acts as a sink for the carboxylate drug form, binding the opened-ring species and thereby shifting the lactonecarboxylate equilibrium to the right.

9.8 CHIRAL COMPOUNDS

Prior to development of chiral separation methods, it is important to have a basic understanding of stereochemistry. The stereochemistry of a molecule, i.e., the difference in spatial arrangements of atoms, is intimately related to its biological activity. Pronounced differences in pharmacologic activity have been observed, based on chirality, i.e., the molecules differing from each other only as an object and its mirror image. This explains why regulatory requirements demand detailed investigations on chiral molecules. Basic information on stereochemistry is provided here to prepare the reader for a better understanding of separation mechanisms that come into play in chiral separation methods. The choice of a method may be influenced by molecular structure or the individual

chemist's preference for an achiral or chiral stationary phase. Logical reasons for selecting a given approach are discussed below.

9.8.1 Stereoisomerism

Molecules that are isomeric but have a different spatial arrangement are called stereoisomers. Symmetry classifies stereoisomers as either enantiomers, as defined above, or diastereomers. It is a good idea to remember:

- A pair of enantiomers is possible for all molecules containing a single chiral carbon atom (one with four different groups attached).
- Diastereoisomers, or diastereomers, are basically stereoisomers that are not enantiomers of each other.
- A chiral molecule can have only one enantiomer, however, it can have none or several diastereomers.
- Two stereoisomers cannot be both enantiomers and diastereomers of each other simultaneously.
- Stereoisomerism can result from a variety of sources other than the single chiral carbon (stereogenic or chiral center) mentioned above, i.e., a chiral atom that is a tetrahedral atom with four different substituents.
- It is not necessary for a molecule to have a chiral carbon in order to exist in enantiomeric form, but it is necessary that the molecule, as a whole, be chiral.
- There are two simple molecular sources of chirality: molecules having a stereogenic center and those having a stereogenic axis.

Detailed discussions on these topics may be found in several books and review articles (2, 88-94).

9.8.2 Stereochemistry and Biological Activity

The importance of determining the stereoisomeric composition of chemical compounds, especially those of pharmaceutical im-

portance, is well recognized (95-97). About half of the drugs listed in the *U.S. Pharmacopeial Dictionary of Drug Names* contain at least one asymmetric center, and 400 of them have been used in racemic or diastereomeric forms (98).

The differences in the pharmacologic properties between enantiomers of these racemic drugs have not been examined in many cases, probably because of difficulties of obtaining both enantiomers in optically pure forms. Some enantiomers may exhibit potentially different pharmacologic activities, and the patient may be taking a useless or even undesirable enantiomer when ingesting a racemic mixture. To ensure the safety and effect of currently used and newly developing drugs, it is important to isolate and examine both enantiomers separately. Furthermore it is necessary, in at least three situations, to measure and control the stereochemical composition of the drugs. Each situation presents a specific technical problem during: (a) manufacture, where problems of preparative scale separations may be involved; (b) quality control (or regulatory analysis), where analytical questions of purity and stability predominate; and (c) pharmacologic studies of plasma disposition and drug efficacy, where ultratrace methods may be required (2).

Accurate assessment of the isomeric purity of substances is critical since isomeric impurities may have unwanted toxicologic, pharmacologic, or other effects. Such impurities may be carried through a synthesis and react preferentially at one or more steps, yielding an undesirable level of another impurity. Frequently one isomer of a series may produce a desired effect, while another may be inactive or even produce an undesired effect. Large differences in activity between stereoisomers point out the need to accurately assess isomeric purity of pharmaceuticals. These differences in therapeutic activity frequently exist between enantiomers, which are the most difficult stereoisomers to separate.

In 1987 the FDA issued a set of initial guidelines on the submission of NDA, where the question of stereochemistry was approached directly in the guidelines on the manufacture of drug substances (99, 100). The finalized guidelines require a full

description of the methods used in the manufacture of the drug, including testing to demonstrate its identity, strength, quality, and purity. Therefore, submissions should show the applicant's knowledge of the molecular structure of the drug substance. For chiral compounds, this includes identification of all chiral centers. The enantiomer ratio, although 50:50 by definition for a racemate, should be defined for any other admixture of stereoisomers. The proof of structure should consider stereochemistry and provide appropriate descriptions of the molecular structure. An enantiomeric form can be considered an impurity, and therefore, it is desirable to explore potential in vivo differences between these forms.

It is now recognized that chromatographic methods offer distinct advantages over classic techniques in the separation and analysis of stereoisomers, particularly for the more difficult class of enantiomers. Chromatographic methods show promise for moderate-scale separations of synthetic intermediates as well as for final products. For large-scale separations and in consideration of the cost of plant-scale resolution processes, the sorption methods offer substantial increases in efficiency over recrystallization techniques. The latter are still more commonplace because of the limited extent of current knowledge about stereospecific reactions required to tailor such separations. Of various stereoisomers, diastereomers specifically are inherently easier to separate because they already possess differences in physical properties. In recent years many significant advances have occurred that allow the chromatographic resolution of enantiomers.

There are basically two approaches to the separation of an enantiomer pair by chromatography. In the indirect approach, the enantiomers may be converted into covalent, diastereomeric compounds by a reaction with a chiral reagent, and these diastereomers are typically separated on a routine, achiral stationary phase. In the direct approach, several variations can be tried: (a) the enantiomers or their derivatives are passed through a column containing a chiral stationary phase; or (b) the derivatives are passed through an achiral column using a chiral solvent or,

more commonly, a mobile phase that contains a chiral additive. Both variants of the second case depend on differential, transient diastereomer formations between the solutes and on the selector to bring about the observed separation.

9.8.3 Separation Methods

It is well recognized that chromatographic methods (TLC, GLC, and HPLC) offer distinct advantages over classic techniques in the separation and analysis of stereoisomers, especially enantiomers which are generally much more difficult to separate (90, 101-105). Chromatographic methods show promise for moderate-scale separations of synthetic intermediates as well as for final products. For large-scale separations and in consideration of the cost of plant-scale resolution processes, the sorption methods offer substantial increases in efficiency over recrystallization techniques. Most of the discussion in this chapter is on HPLC, as it offers the greatest promise.

Derivatization of a given enantiomeric mixture with a chiral reagent, leading to a pair of diastereomers (indirect method), allows separation of samples by chromatography. On the other hand, using the chiral stationary or mobile-phase systems in chromatography (direct method) can provide a useful alternative procedure. This approach has been examined rather extensively by many research scientists. Early successful results did not attract much interest; the technique remained relatively dormant and little was done to develop this approach into a generally applicable method. Approximately twenty years ago systematic research was initiated for the design of chiral stationary phases functioning to separate enantiomers by gas chromatography. Molecular design and preparation of the chiral phase systems for liquid chromatography have been examined since then. More recently, efforts have been directed toward finding new types of chiral stationary and mobile phases on the basis of the stereochemical viewpoint and the technical evolution of modern liquid chromatography.

Since HPLC is now one of the most powerful separation techniques, resolution of enantiomers by HPLC is expected to move rapidly with the availability of efficient chiral stationary phases. Large-scale, preparative liquid chromatographic systems have already been put on the market as process units for isolating and purifying chemicals and natural products. Chiral HPLC is ideally suited for large-scale preparation of optical isomers.

9.8.4 Separation Mechanisms

It is important to understand the mechanism of chiral separation before one can develop optimum methods. The chromatographic process is considered a series of equilibria and the equilibrium constant describes the distribution of a compound between the stationary and the mobile phases. The separation occurs because of differences in retention of various compounds on the stationary phase. It is customary to distinguish between partition and adsorption chromatography, where the stationary phase is a liquid for the former and a solid for the latter. With the introduction of bonded organic phases in HPLC, this distinction is not very clear. Therefore primary importance should be given to the types of molecular interactions with the stationary phase that lead to retention.

Our understanding of chiral separations with some of the systems is quite good, while it remains poor for protein and cellulose stationary phases. A number of chiral recognition models have been proposed to account for optical resolutions by HPLC, which are often based on the three-point interaction rule advanced by Dalgliesh (106) in 1952. He arrived at his conclusions from paper chromatographic studies of certain aromatic amino acids. He assumed that the hydroxyl groups of the cellulose were hydrogen-bonded to the amino carboxyl groups of the amino acid. A third interaction was caused, according to these views, by the aromatic ring substituents. It is postulated that three simultaneously operating interactions between an enantiomer and the stationary phase are needed for chiral discrimination. However, this is not

always necessary. Steric discrimination could also result from steric interactions.

In adsorption chromatography, some kind of bonding interaction with the sorbent must occur. This may arise through a non-covalent attachment that is possible under the prevailing conditions. For example, hydrogen bonding as well as ionic or dipole attraction is enhanced by non-polar solvents, whereas hydrophobic interactions are more important in aqueous media.

Chiral separations are also possible through reversible diastereomeric association between achiral environment introduced into a column and an enantiomeric solute. Since chromatographic resolutions are possible under a variety of conditions, one can conclude that the necessary difference in association can be obtained by many types of molecular interactions. The association, which may be expressed quantitatively as an equilibrium constant, will be a function of the magnitudes of the binding as well as the repulsive interactions involved. The latter are usually steric, although dipole-dipole repulsions may also occur, whereas various kinds of binding interactions may operate. These include hydrogen bonding, electrostatic and dipole-dipole attractions, charge-transfer interactions, and hydrophobic interactions (in aqueous systems).

At times, a single type of bonding interaction may be sufficient to promote enantiomer differentiation. For example, hydrogen bonding as the sole source of attraction, is sufficient for optical resolution in some HPLC modes of separation.

The fact that enantiomeric solutes bearing only one hydrogen bonding substituent can be separated under such conditions points to the conclusion that only one attractive force is necessary for chiral discrimination in this type of chromatography. Taking a one-point binding interaction as a model, we may envision a difference in the equilibrium constant of the two enantiomers at the chiral binding site as due to effects from the site forcing one of the enantiomers to acquire an unfavorable conformation. This resembles a situation often assumed to be present in enzyme-substrate interactions to account for substrate specificity.

It may be possible to construct chiral cavities for the preferential inclusion of only one enantiomer. Molecular imprinting techniques are very interesting in this respect (107). The idea is to create rigid chiral cavities in a polymer network in such a way so that only one of two enantiomers will find the environment acceptable. Other types of CSPs, where steric fit is of primary importance, include those based on inclusion phenomena, such as cyclodextrin and crown ether phases.

9.8.5 Modes of Separation in HPLC

The chromatographic separation of enantiomers can be achieved by various methods; however, it is always necessary to use some type of chiral discriminator or selector (108, 109). Two different kinds of selectors can be distinguished: a chiral additive in the mobile phase (see p. 238) or a chiral stationary phase (see p. 242). Another technique is to use precolumn derivatization (see Table 9.6) of the sample with chiral reagents to produce diastereomeric molecules that can be separated by chiral chromatographic methods.

Some discussion on the mechanism of separation is provided for each mode of separation; however, as pointed out earlier, detailed mechanisms for chiral separations have not been worked out. The proposals made by certain scientists appear attractive; however, vigorous differences prevail, so an attempt has been made to avoid highlighting any single proposal. Discussed below are various approaches that can be used for chiral separations.

Chromatography of Diastereomeric Derivatives
This is the oldest and most widely used chromatographic approach to the resolution of enantiomers (93). The precolumn derivatization of an optically active solute with another optically active molecule depends on the ability to derivatize the target molecule. A large number of functional groups and derivatives have been investigated including amino groups (derivatized to amides, carbamates, ureas, thioureas, and sulfonamides), hydroxyl groups

(esters, carbonates, and carbamates), carboxyl groups (esters and amides), epoxides (isothiocyanate), olefins (chiral platinum complexes), and thiols (thioesters).

This method has been used with a wide variety of HPLC columns and mobile phases, including normal and reverse phase approaches. At present there is no definitive way of determining which chromatographic approach will work. The advantages of derivatization are:

- The methodology has been extensively studied, making the application relatively easy and accessible.
- It is possible to use readily available standard HPLC supports and mobile phases.
- Detectability can be improved by appropriate selection of a derivatizing agent with a strong chromophore or fluorophore.

The main limitations are:

- The synthesis of the diastereomeric derivatives requires the initial isolation of the compounds of interest prior to their derivatization. This hinders the development of an automated procedure for large numbers of samples.
- The application to routine assays often is limited by enantiomeric contamination of the derivatizing agent, which can lead to inaccurate determinations. The problem of enantiomeric contamination of the derivatizing agent has been encountered in a number of studies. Silber and Riegelman (110), for example, use (-)-N-trifluoroacetyl-L-prolyl chloride (TPC) in the determination of the enantiomeric composition of propranolol in biological samples. They found that commercial TPC was contaminated with 4% to 15% of the (+)-enantiomer and that the reagent rapidly racemized during storage.
- Enantiomers can have different rates of reaction and/or equilibrium constants when they react with another chiral molecule. As a result, two diastereomeric products may be

generated in proportions different from the starting enantiomeric composition (111).

Enantiomeric Resolution Using Chiral Mobile-Phase Additives

The resolution of enantiomeric compounds has been accomplished through the formation of diastereomeric complexes with a chiral molecule(s) added to the mobile phase. The chiral resolution is due to differences in the stabilities of the diastereomeric complexes, solvation in the mobile phase, or binding of the complexes to the solid support. A general overview of this method has been published by Lindner and Pettersson (112).

There are three major approaches to the formation of diastereomeric complexes: transition metal ion complexes (ligand exchange), ion pairs, and inclusion complexes. Each method is based on the formation of reversible complexes and uses an achiral chromatographic packing.

Ligand Exchange

Chiral ligand-exchange chromatography is based on the formation of diastereomeric complexes involving a transition metal ion (M), a single enantiomer of a chiral molecule (L), and the racemic solute (d and l). The diastereomeric mixed chelate complexes formed in this system are represented by the following formulas: L-M-d and L-M-l. The most common transition metal ion used in these separations is Cu, and the selector ligands are usually amino acids such as L-proline. The chromatography is most often carried out using an achiral HPLC packing (such as C-18) with these compounds added to the mobile phase.

The efficiency and selectivity of a chiral ligand-exchange system can be improved by binding the selector ligand to the stationary phase. Some examples of this approach are the L-proline-containing stationary phase, an L-(+)-tartaric-acid-modified silica reported by Kicinski and Kettrup, and a chiral phase composed of a C-18 column dynamically coated with (R,R)-tartaric acid mono-n-octylamide (92).

A number of chiral molecules have been resolved by ligand-exchange chromatography. However, resolution is possible for only those molecules that are able to form coordination complexes with transition metal ions. This method is most often utilized with free and derivatized amino acids and similar compounds. There has been some success with other classes of compounds, including carboxylic acids, amino alcohols (as Schiff bases), barbiturates, hydantoins, and succinimides (112). The mobile phases employed with chiral ligand exchange are aqueous, with the metal ions and selector ligands added as modifiers.

Chiral ligand exchange is an excellent method for the resolution of amino acids and amino acid-like compounds. The molecules need not be derivatized and the aqueous mobile phases are compatible with automated column-switching techniques.

The major disadvantage of chiral ligand exchange is the small number of compounds that can be resolved by this approach. Many of the cationic and anionic molecules of pharmacologic interest have not been resolved by this method.

Ion Pairing

Ion-pair chromatography is a liquid chromatographic method commonly used with charged solutes. The method is based on the formation of a neutral complex (ion pair, SC) between a charged solute (S) and a counterion of opposite charge (C).

When both the solute and the counterion are optically active, diastereoisomeric ion pairs are formed. These ion pairs often can be separated by differences in their solvation in the mobile phase or in their binding to the stationary phase. A number of different counterions have been employed in this approach, including (+)-10-camphor-sulfonic acid, quinine, quinidine, cinchonidine, (t)di-n-butyltartrate, and the protein, albumin. This method has been reviewed by Pettersson and Schill (113).

The solutes resolved by chiral ion-pair chromatography have included amino alcohols such as alprenolol, carboxylic acids such as tropic acid and naproxen, and amino acids such as tryptophan.

The composition of the mobile phase depends on the chiral agent used. When a chiral counterion is used, a mobile phase of low polarity such as methylene chloride is used to promote a high degree of ion-pair formation. The retention of the solute can be decreased by increasing the concentration of the counterion or by the addition of a polar modifier such as 1-pentanol (113). The latter approach usually results in a decrease in the stereoselectivity. The water content of the mobile phase also appears to be important, and a water content of 80 ppm to 90 ppm has been recommended.

With serum albumin as the chiral agent, aqueous mobile phases containing phosphate buffers are used. The retention and stereoselectivity can be altered by changing the pH. Both aqueous and nonaqueous mobile phases can be used when (t)di-n-butyltartrate is the chiral modifier.

In some cases it appears that the modifier is retained by the stationary phase when the column is equilibrated with an aqueous mobile phase. The system then can be used with an organic mobile phase (113).

The chiral ion-pair systems are not stable. The chromatography can be affected by the water content of the mobile phase, temperature, pH, and a number of other variables. This makes routine applications difficult. In addition, the counterions often absorb in the UV region, reducing the sensitivity of the system. Indirect photometric detection (114) or other detection methods must be used.

Inclusion

Cyclodextrins are cyclic oligosaccharides composed of d-α-glucose units linked through the 1,4 positions. The three most common forms of this molecule are α-, β-, and γ-cyclodextrin, which contain 6, 7, and 8 glucose units. Cyclodextrin has a stereospecific, doughnut-shaped structure. The interior cavity is relatively hydrophobic and a variety of water-soluble and insoluble compounds can fit into it, forming inclusion complexes. If these compounds are chiral, diastereoisomeric inclusion complexes are formed.

β-Cyclodextrin has been used by Sybilska et al (115) as a chiral mobile-phase additive in the resolution of mephenytoin, methylphenobarbital, and hexobarbital. They attribute the observed resolution to two different mechanisms. The resolution of mephenytoin is due to a difference in the adsorption of the diastereoisomeric complexes on the achiral C-18 support. For methylphenobarbital and hexobarbital, the relative stabilities of the diastereoisomeric complexes are responsible for the resolution of these compounds.

In addition to the compounds listed above, mobile phases modified with β-cyclodextrin can resolve mandelic acid and some of its derivatives (116, 117).

Aqueous mobile phases modified with a buffer such as sodium acetate are commonly utilized. Alcoholic modifiers, such as ethanol, can be added to the mobile phase to reduce retention.

Automation is possible for the direct measurement of biological samples. Sybilska et al. (115) have used it for preparative separations of mephenytoin, methylphenobarbital, and hexobarbital.

The applications of this approach seem limited. For example, unlike hexobarbital and methylphenobarbital, the chiral barbiturates secobarbital, pentobarbital, and thiopental are not resolved when chromatographed with a β-cyclodextrin contained in a mobile phase (113). Additional information on solutions involving cyclodextrin inclusion complexes is discussed in the following section.

Enantiomeric Resolution with Chiral Stationary Phases

Enantiomers can be resolved by the formation of diastereomeric complexes between the solute and a chiral molecule that is bound to the stationary phase. The stationary phase is called a CSP, and the use of these phases is the fastest growing area of chiral separations. The first commercially available HPLC-CSP was introduced by Pirkle in 1981 (118). Currently a large number of chiral phases are commercially available. Separation of enantiomeric compounds on CSP is due to differences in energy be-

tween temporary diastereomeric complexes formed between the solute isomers and the CSP; the larger the difference, the greater the separation. The observed retention and efficiency of a CSP is the total of all the interactions between the solutes and the CSP, including achiral interactions.

Selection Procedure for CSPs

A classification of chiral stationary phases is provided in Table 9.5 and a flow diagram for the selection procedure is given in Figure 9.5. The chromatographic analysis of entantiomeric compounds can also be broadly divided into normal phase or reverse phase (119).

Table 9.5 Classification of Chiral Stationary Phases

TYPE	DESCRIPTION	EXAMPLES	USUAL MODE
1	Pirkle-type (donor-acceptors)	DNB-Glycine DNB-Leucine Naphthylalanine	Normal phase (polar modifier)
2	Cellulose triesters or carbamates on silica	Chiralcel-OA, -OB, -OF, -OJ	Normal phase (polar modifier)
3	Inclusion CSPs Cyclodextrins Polyacrylates Polyacrylamides Crown ethers	Cyclobond 1-3 Chiralpak-OP, -OT; Chiralcel-CR	Reverse phase (aqueous MeCN or MeOH)
4	Ligand exchangers	Proline HO-proline	Reverse phase (aq. buffer)
5	Proteins	Albumin Glycoprotein	Reverse phase (aq. buffer)

Normal Phase Analyses: Type 1 or 2 CSPs are preferable for normal phase analyses. Derivatization by an achiral reagent can be used to increase analyte-CSP interactions and hence improve resolution of enantiomers. It can also bring other benefits, such as increased detectability by UV or fluorescence detectors, or enhanced solubility in normal phase eluents.
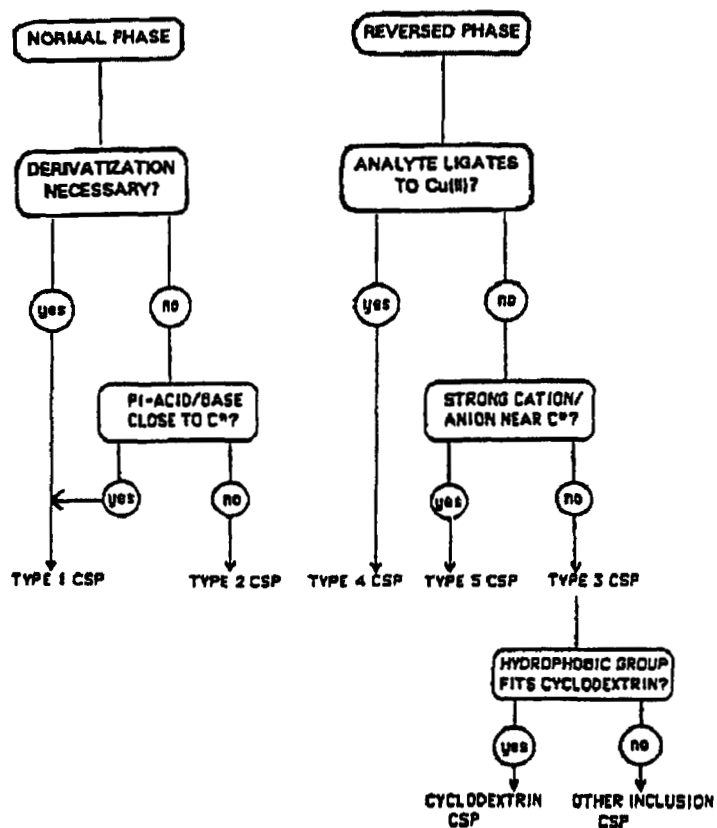
Figure 9.5 Selecting a CSP.

If derivatization is possible, a type 1 CSP (donor-acceptor) is favored. Otherwise, a type 2 CSP (silica-supported cellulose triesters, etc.) is selected except when a π-acid or a π-base is

already located close to a chiral center in the analyte. For example, an arylsulfoxide is better separated on a type 1 CSP.

The analytes identified as candidates for separation with a type 1 CSP can be further divided into those with π-donor groups or π-acceptor groups. When there is a π-donor group (i.e., phenyl or naphthyl) close to the asymmetric center, a π-acceptor type 1 CSP such as the dinitrobenzoyl (D)-phenyl-glycine phase may be initially selected. Otherwise a π-donor type 1 CSP (e.g., naphthylvaline) would be indicated if a π-acceptor group were similarly located in the analyte or its derivative. These requirements are not overriding, however, since type 1 CSPs also operate by dipole-dipole, H-bonding, and steric interactions.

Reverse Phase (RP) Analyses: If bidentate ligation to a metal ion such as Cu(II) is possible, type 4 CSPs (ligand exchange) are indicated. In the absence of such potential ligation, the selection process centers around CSPs of types 3 and 5.

For non-ligating RP analytes, if a strongly cationic or anionic site is located close to the asymmetric center, the analyte may be resolvable on a protein-based CSP (type 5). Alternatively, inclusion CSPs (type 3) should be tried first.

Inclusion Analyses: If a non-polar includable group (ideally 5-9 A across) is present, which does not lie too far from an asymmetric center, the selection of one of the Cyclobond range of cyclodextrin CSPs is indicated. The absence of a non-polar includable group may lead to selection of other types of inclusion CSPs, such as the chiral acrylate polymers currently marketed by Merck and Daicel.

Macrocyclic antibiotics have been investigated as a new class of chiral selectors for liquid chromatography (120). These stationary phases appear to be multimodal in that they can be used in both normal and reverse phase modes.

Since there are such a large number of HPLC-CSPs available to the chromatographer, it is difficult to determine which is most suitable to solve a particular problem. This difficulty can be partially overcome by grouping the CSPs for chiral separations

according to a common characteristic. The first step, that is, the formation of the solute-CSP complexes, is more readily adaptable to the development of a classification system. Using this criterion for the division of CSP groups, the current commercially available CSPs can be divided into five categories (119).

(a) Pirkle-Type and Related Phases (Type 1)
These phases are often based on an amino acid derivative. The columns are available on π-acid or π-base columns (amide, urea, or ester moiety in CSP). They show the following solute-stationary phase interactions:

- dipole-dipole
- hydrogen bonding
- π-π interactions

The mobile phase usually contains a nonpolar organic solvent with varying amounts of a polar modifier, e.g., hexane/isopropyl alcohol.

Polar functional groups, e.g., amino, carboxyl, and some hydroxyl, require derivatization to less polar derivatives (Table 9.6).

Table 9.6 Derivatizations with Achiral Reagents

GROUP	REAGENT	PRODUCT
-NH$_2$	COOH deriv.	amide
	isocyanate	urea
	chloroformate	carbamate
-COOH	alcohol	ester
	aniline	anilide
	amine	amide
-OH	COOH deriv.	ester
	isocyanate	carbamate

Recently a hybrid column of both π-acid (3,5-dinitrobenzoyl) and π-base (naphthyl) has been developed; it resolves a variety of compounds, including underivatized COOH (121).

(b) Derivatized Cellulose and Related CSPs (Type 2)

Derivatized cellulose Cellulose is a crystalline polymer composed of β-D glucose units. Crystalline cellulose is unable to withstand HPLC pressures and therefore has to be modified. Several approaches have been reported, which are based on derivatizing the available hydroxyl groups:

- cellulose triacetate (CTA)
- cellulose tribenzoate (CTB)

Alcohols are needed as the mobile phase for CTA, whereas, hexane-2-propanol is commonly used for CTB. A variety of analytes can be resolved. CTA prefers the phenyl group, which is also well suited for preparative work. Multiple interactions are involved in chiral recognition, including inclusion into channels and cavities.

Table 9.7 Derivatized Cellulose Columns*

NAME	SEPARATION	MOBILE PHASE
Chiralpak-OT(+), -OP(+)	Compounds with an aromatic group	Methanol or hexane-2-propanol
Chiralcel-OA, -OB, -OC, -OK, -OD	Compounds with nitro, aromatic, carbonyl, hy-droxyl, or cyano group	Hexane-2-propanol ethanol (and water)
Chiralcel-CA-1	Compounds with nitro, aromatic, carbonyl, hy-droxyl, or cyano group	Ethanol (and water)
Chiralpak-WH, -WM, -WE	dl-Amino acid or its derivative	Aqueous $CuSO_4$

*Source: Daicel Chemical Industries

Derivatized cellulose on silica gel Cellulose is depolymerized to shorter units, then derivatized to an ester or carbamate derivative,

followed by coating onto silica, i.e., a non-covalent attachment (Table 9.7).

The mobile phase is usually hexane-isopropyl alcohol and a base or acid additive, depending on the analyte.

The chiral recognition mechanism is not well known; multiple mechanisms, including fit into cavities, are possible and successful in many applications. Many compounds are resolved without derivatization; some require derivatization.

The mobile phase is usually hexane-isopropyl alcohol and a base or acid additive, depending on the analyte.

The chiral recognition mechanism is not well known; multiple mechanisms, including fit into cavities, are possible and successful in many applications. Many compounds are resolved without derivatization; some require derivatization.

(c) Cyclodextrins and Other Inclusion-Complex-Based CSPs (Type 3)

Cyclodextrins are cyclic oligosaccharides of α-D-glucose formed by the action of *Bacillus macerans* amylase on starch. Six, seven, or eight glucose units are possible, namely, α-, β-, and γ-cyclo-dextrin, respectively.

They have toroidal form, and the interior is relatively hydro-phobic. The size of the interior cavity varies: $\alpha < \beta < \gamma$. The naphthyl group can fit into a β-cyclodextrin cavity. Chiral rec-ognition is based on entry of the solute or a portion of the solute molecule into the cavity to form an inclusion complex stabilized by hydrogen bonds and other forces.

Mobile phases are generally composed of aqueous (often buffered) methanol, ethanol, or acetonitrile mixtures. Polar organic acetonitrile (ACN) mobile phase (with TEA/HOAc) has been used recently.

Derivatized forms of cyclodextrin Several CSPs are obtained by derivatizing OH groups, to produce acetylates or other esters and carbamate derivatives, e.g., N-1-(l-naphthyl)ethylcarbamate deriva-tives (R, S, RS). A chiral recognition mechanism(s) does not appear to be an inclusion complex.

These CSPs can be used with organic mobile phases, e.g., hexane/isopropyl alcohol.

(d) Ligand Exchangers (Type 4)
As discussed before, this technique is based on complexation to metal ions, e.g., Cu(II). The chiral ligand immobilized on the column is usually an amino acid, e.g., proline. Solutes must be able to form coordination complexes with metal ions. Examples are amino acids and derivatives, hydantoins, and amino aicohols.

The mobile phase contains the metal ion, typically added as copper sulfate. Retention and resolution can be manipulated with concentration of the metal ion, pH adjustment, addition of modifiers, and change in temperature.

(e) Protein CSPs (Type 5)
Proteins are polymers composed of chiral units (L-amino acids) and are known to be able to bind small organic molecules. The protein can be immobilized on solid support by a variety of bonding chemistries; the choice of bonding method affects selectivity of CSP obtained. The following protein CSPs have been utilized:

- α acid glycoprotein (AGP)
- ovomucoid (a glycoprotein from egg white)
- human serum albumin
- bovine serum albumin

The retention and chiral recognition mechanisms may be unrelated to drug-protein binding by free protein (i.e., in vivo or in vitro). The retention is most likely based on hydrogen bonding, ionic interactions, etc. Recent investigations with commercial chicken ovomucoid indicates that chiral recognition ability may be due to an ovoglycoprotein that is present as an impurity (122).

The mobile phases are generally aqueous buffers within a limited pH range and with limited organic modifiers. To manipulate retention and stereoselectivity, one can vary mobile

phase composition, pH, and temperature. The capacity of protein CSPs is limited; however, they enjoy broad applicability.

9.9 LOCAL ANESTHETICS

HPLC has been found useful for simultaneous analysis of benzocaine and its primary degradation product, p-aminobenzoic acid. A μBondapak C-18 column with methanol-water-acetic acid (33:4:63) mobile phase gives detectability of 3 ng at 254 nm with a UV detector, which can be further enhanced by detection at 294 nm and increasing the volume of injection (123). Similarly, p-n-butylaminobenzoic acid has been separated from tetracaine by reverse phase HPLC on a μBondapak C-18 column and a mobile phase containing 0.067 v/v H_2SO_4, 0.5% w/v Na_2SO_4, and 0.02% w/v sodium heptane sulfonate in water-acetonitrile-methanol (60:20:20). Detectability is in the ng range (124).

A highly sensitive GLC method with nitrogen-phosphorous sensitive detector has been reported for the analysis of mepiv-acaine, bupivacaine, etiocaine, lidocaine, and tetracaine (125). The minimum detectability by this method is in the low-ng range. The method can be also used for purity determination of these compounds.

Dyclonine hydrochloride, a local anesthetic, is known to degrade in aqueous media (126). Two major degradation products, formed by heating an aqueous solution of dyclonine hydrochloride for 2 weeks at 50°C have been isolated and characterized (Scheme 11).

The degraded solution is extracted with diethyl ether and then the ethereal layer is concentrated under vacuum. The resulting semisolid residue is chromatographed by preparative TLC on silica gel 200-mm thick plates with a mobile phase of ethyl acetate-hexane (1/1).

Two products, compounds A and B, are detected at Rf 0.76 and 0.39 respectively. The proton and carbon-13 NMR, IR, and MS data are utilized to conclusively show that the two products are 1-(4-butoxyphenyl)-2-propen-1-one and 1-(4-butoxyphenyl)-3-hy-

droxy-1-propanone. Other degradation products can also be detected in small amounts but these have not been isolated and characterized.
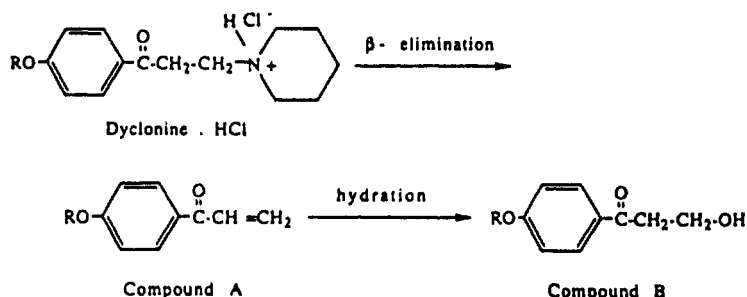
Scheme 11. Degradation pathways of dyclonine hydrochloride (126)
$(R = CH_3CH_2CH_2CH_2)$.

9.10 MACROMOLECULES

A study has been conducted to extend our understanding of the stability of heparin (127). Heparin is a highly sulfated, linear polysaccharide comprised of alternating hexuronic acid and glucosamine residues. It is biosynthesized. Despite its widespread use, heparin has low bioavailability when administered by routes other than intravenously and causes a number of side effects. These problems are related in part to heparin's relatively high molecular weight.

The stability study entailed incubating sodium heparin, derived from porcine intestinal mucosa in 0.1 N HCl and 0.1 N sodium hydroxide at 30 and 60°C and sampling at times ranging from 0 to 1000 h. The absorbance spectra of the products formed under basic conditions showed a UV maxima at 232 nm associated with chemically catalyzed β-elimination at the uronic acid residues. The products formed under acidic conditions showed a decreased staining intensity consistent with desulfation and a decrease in molecular weight corresponding to the hydrolysis of glycosidic

linkages when analyzed by gradient polyacrylamide gel elec-
trophoresis. Heparin samples in 10 mM sodium phosphate buffer at
pH 7.0 in sealed ampuls that are flushed with nitrogen and
incubated at 100°C and sampled at 0 to 4000 h, showed that
heparin is relatively stable over the first 500 h, after which it
degrades rapidly. Heparin assays, using both anti-factor Xa and
anti-factor IIa amidolytic methods, showed 80-90% of its activity
over the first 500 h, but these activities dropped precipitously, to
approximately 6% and 0.5% of the initial activity at 1000 h and
2000 h, respectively. The proposed mechanism of heparin under
acidic, basic, and neutral conditions is given in Scheme 12 on the
next page.

The rapid decomposition begins only after the buffering
capacity of the solution is overwhelmed by acidic degradants,
which causes pH to decrease. Decomposition processes observed
under these conditions include the endolytic hydrolysis of glyco-
sidic linkages and loss of sulfation, particularly N-sulfate groups,
and are similar to degradation processes observed in 0.1 N HCl.

Investigations have been conducted to study oxidation of
methionine residue of antiflammin 2 (HDMNKVLDL, AF2) as a
function of pH, buffer concentration, ionic strength, and
temperature, using different concentrations of hydrogen peroxide
to determine the accessibility of methionine residues to oxidation
(128). RPLC has been utilized for peptide purification on a
Beckman ODS column (10×250 mm, 10-μ m particle size) and all
other analytical measurements are conducted on a Vydac ODS
column (4.6×250 mm, 5-μm particle size). The mobile phase is
composed of 20% acetonitrile in water with 0.1% TFA, and
detection is performed at 220 nm.

Met(O)-3-AF2 is the only oxidation product detected at pH 3.0
to pH 8.0. The oxidation rates are independent of buffer con-
centrations, ionic strength, and pH from 3.0 to 7.0. However, there
is an acceleration of rates in the basic pH range, and small amounts
of degradation products other than Met(O)-3-AF2 are observed in
alkaline region. These results led to the conclusion that oxidation
of methionine in AF2 does not cause biological inactivation.
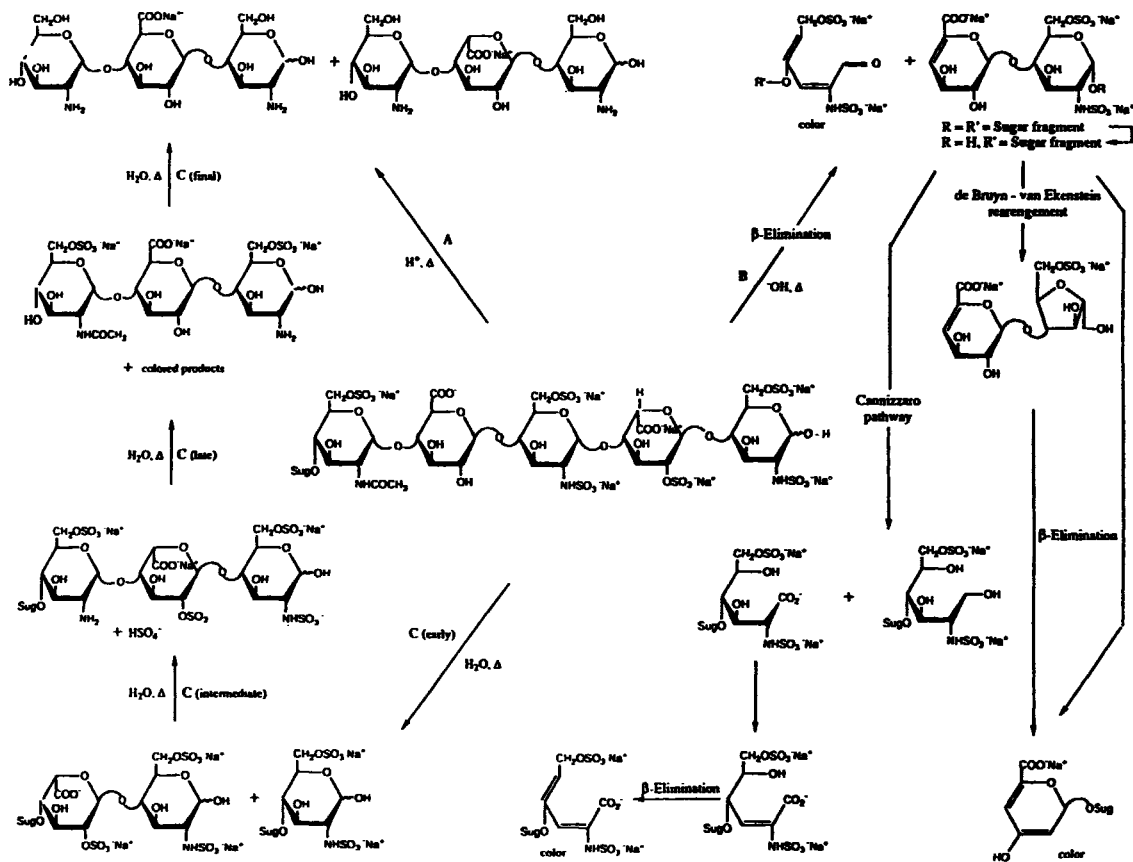
Scheme 12. Proposed mechanism for heparin degradation under acidic (A), basic (B), and neutral (C) conditions.

Human granulocyte-colony stimulating factor (G-CSF) is a hematopoietic growth factor that regulates proliferation and differentiation of neutrophilic granulocytes (129). Recombinant human G-CSF (rhG-CSF) has been found useful in treating myelosuppression associated with cancer chemotherapy treatments.

The liquid stability of rhG-CSF has been investigated after polyethylene glycol (PEG), with an average molecular weight of 6000 daltons, is covalently attached to the N-terminal methionine residue. The conjugation methods chosen for modifying the N-terminal residue are alkylation and acylation. The N-terminally PEGylated rhG-CSF conjugates are purified by cation exchange chromatography. The physical characterization methods of SDS-PAGE endoproteinase peptide mapping, circular dichroism, and in vivo bioassays are used to test the difference between the PEG-rhG-CSF molecules. Physical characterization indicated no apparent differences in the rhG-CSF molecules that are conjugated with either method. Stability of these conjugated molecules, in liquid at elevated temperatures, indicates that the primary pathway of degradation is aggregation. Conjugation through alkylation offers the distinct advantage of decreasing, by approximately 5 times, the amount of aggregation present as compared to acylation.

RMP-7 triacetate (Arg-Pro-Hyp-Gly-Thi-Ser-Pro-4-MeTyr Ψ (CH$_2$NH)Arg) is a bradykinin B$_2$-receptor agonist, which has been evaluated for its ability to facilitate entry of therapeutic agents into the brain for a number of diseases (130). The degradation pathways of RMP-7 and pH/buffer stability profiles have been investigated. The results show two major pathways of degradation of RMP-7. A portion of RMP-7 drug substance is transformed into an acetylated adduct, presumably by acylation directly or indirectly from the acetate counterion, which indicates that the choice of counterion can affect stability. The predominant degradation pathway for the drug substance and a number of aqueous solutions involves the formation of an N-terminal diketopiperazine and a C-terminal heptapeptide, which is a result of the sequence of RMP-7 (like bradykinin) containing proline at position 2. Such a cyclization can

be minimized in solution by avoiding general base catalysts, such as phosphate, or acetate buffers.

9.11 STEROIDS

Hydrocortisone can yield at least three unknown degradation products under accelerated storage conditions (131). These impurities can be quantitated at submicrogram levels by HPLC on a Partisil-10 ODS column with acetonitrile:water (36:65) as the mobile phase. Budesonide is a glucocorticoid that is composed of a 1:1 mixture of two isomers. This compound has been evaluated by HPLC with respect to epimer distribution and impurities (132). It is possible to resolve the two epimers of budesonide,16-a-hydroxyprednisone (starting material), a mixture (1:1) of two epimers [lba,]7a,(22S)- and 16a-17a(22R)-methylmethylene dioxy-pregna-1,4-diene-11,6,21-diole-3,20-dione], and several unknown impurities on a μBondapak C-18 column with an ethanol:water (48:52) mobile phase (the 43:57 mixture is used for epimers and foreign steroids).

The method is suitable for quantitation of known impurities down to 0.05% of budesonide concentration. A significant difference was found between the impurities determined by the above method and a "normal-phase method" in eight small-scale analyzed batches. The "normal-phase method" generally shows much lower levels of these impurities (50 to 100%).

A mixture of 16a and 16,8 methyl epimers of 9-fluoro-I I-,6-hydroxy 16 methylandrosta-1,4-diene-3,17-dione has been found as an impurity in dexamethasone sodium phosphate (133). The separations are obtained on a μBondapak C-18 column with 50% methanol as the mobile phase. The method is also suitable for resolving 17 ketone impurity. The presence of 17-oxo product as an impurity suggests that oxidation can occur under acidic or basic conditions favorable to epimerization of a-ketone.

A reverse phase RPLC method can be used to separate the following known degradation products and impurities at the submicrogram level in fludrocortisone acetate:
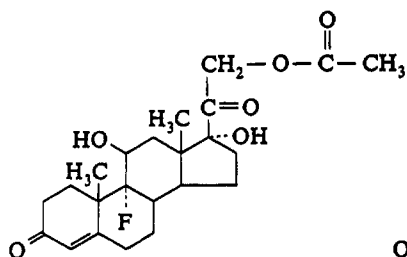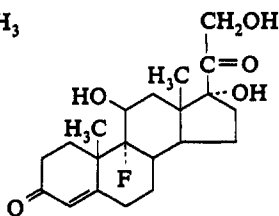
Fludrocortisone acetate

Fludrocortisone
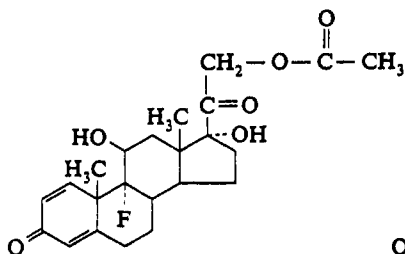
Δ¹,⁴-Diene system of
fludrocortisone acetate
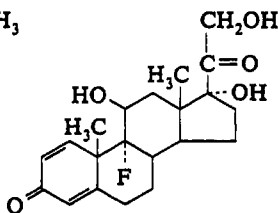
Δ¹,⁴-Diene system of
fludrocortisone

The method requires the use of a μBondapak C-18 column and a mobile phase with equal volumes of methanol and water (134).

Contamination of prednisone with prednisolone can occur in aqueous solution, as the latter changes to the former on standing (135). An HPLC method that utilizes a μBondapak C-18 column and a mobile phase containing 0.01% m KH_2PO_4 in 50% methanol can resolve submicrogram amounts of these steroids.

Digoxin rapidly converts to digoxigenin, bisdigitoxoside, digitoxigenin monodigitoxoside, and digitoxigenin in acidic solution. These impurities can be monitored down to submicrogram levels by HPLC on a LiChrosorb (5-μm) RP-18 column with 25% acetonitrile at 220 nm (136).

Ketosteroids have been analyzed as their 2,4-dinitrophenyl-hydrazone derivatives by HPLC (137,138). This method is sensitive and has been used for determination of these compounds in urine and other biological fluids. Fluorescent hydrazones can be formed on reaction with 5-dimethylamino-naphthalene-1-sulfonyl-hydrazine.

Benzoyl derivatives have been prepared for non-UV absorbing hydroxy steroids (139). HPLC of the derivative is achieved on a reverse phase column, such as Permaphase ODS or Corasil C-18, with methanol-water as the mobile phase.

The reaction of 1-ethoxy-4-(dichloro-sym-triazinyl)naphthalene with the hydroxyl group of corticosteroids has been used for detection of small amounts of these compounds (140). The reagent does not react with compounds such as testosterone, which has secondary aliphatic groups.

Estrogens form UV-absorbing derivatives with azobenzene-4-sulfonyl chloride (141). Dansylation of estrogen on the basis of reaction with the phenolic groups has been used for the analysis of these compounds in urine (142-144). Estradiol and ethinyl estradiol can be derivatized with dansyl chloride, and the dansyl estrogens are chromatographed on a microparticulate silica column with chloroform-n-heptane mobile phase. The detection limits for these estrogens is between 0.04 and 0.05 ng (145).

Conjugated estrogens are defined as a mixture of sodium estrone sulfate and sodium equilin sulfate obtained synthetically or from equine urine. They may contain other conjugated estrogenic substances, such as equilenin, estradiol, 17-dihydroequilin, or 17-dihydroequilenin. Conjugated estrogens contain 50-63% of sodium estrone sulfate and 22.5 to 32.5% sodium equilin sulfate. GC has been used for the analysis of conjugated estrogens after hydrolysis. This method is very time consuming and can be replaced with HPLC. The procedure can be used for direct analysis of estrogens without any hydrolysis, and the results are comparable to those obtained after application of a sulfatase hydrolysis (146).

A new isomeric impurity has been found in danazol (146a). The impurity designated as isodanazol is detected by RPLC and

TLC. Its structure is determined after separation by preparative HPLC on a silica column (irregular-shaped particles of 10-μm particle size packed in 250×21.2 mm column). The eluent consists of hexane-2-propanol-tetrahydrofuran (95.5/4/0.5) and is pumped at a flow rate of 7 ml/minute, and the chromatogram is monitored at 254 nm.

MS revealed the isomeric nature of the impurity while the UV spectrum indicated profound differences in the isoxazole moieties. The structure of the isomeric isoxazole ring in isodanazol has been determined by NMR spectroscopy. The difference between the UV spectra of danazol and isodanazol (Figure 9.6) is explained on the basis of differences between the aromaticites of their isoxazole rings, supported by quantum calculations. The quantitative determination of the impurity down to 0.05% level can be performed by HPLC, GC, and TLC densitometry.
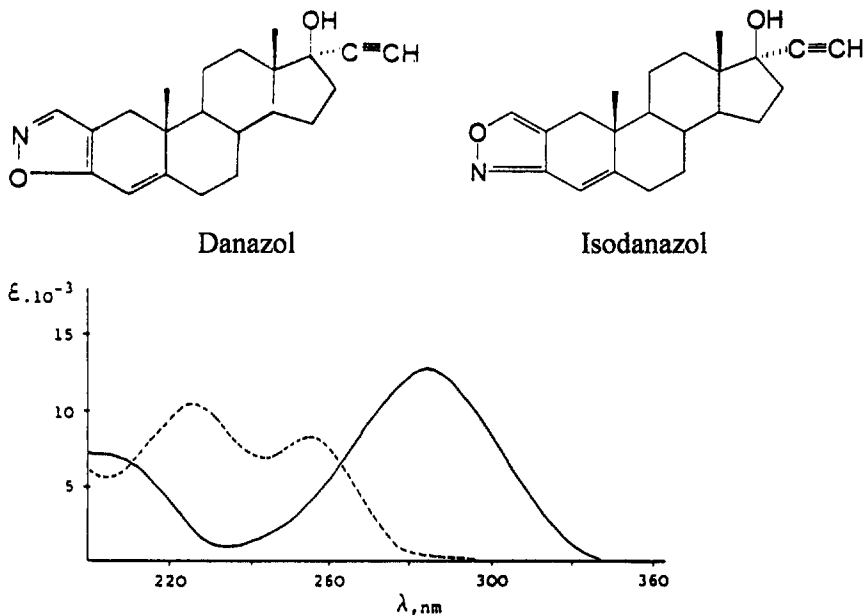
Figure 9.6 UV spectra of danazol and isodanazol (146a)
(dotted and solid line respectively).

9.12 MISCELLANEOUS

A high-performance liquid chromatographic method can resolve nitroglycerin from its degradation products, glyceryl mononitrate and dinitrate (147). The detectability of the method for nitroglycerin is in the ng range; however, no detection limits for degradation products are available. The method requires detection at 200 nm, with separations performed on a μBondapak C-18 column with 60% methanol.

The hydrolysis product, I-phenyl- 1,2-propanedione and the enol tautomer of diethylpropion hydrochloride can be resolved by HPLC (148). The method requires a μ Bondapak C-18 column and a mobile phase containing 80% methanol and 20% pH 7.8 phosphate buffer. It appears that minimum detectability is in the submicrogram range.

Three impurities have been found in a potential anti-hypertensive, 4-acetyl-2-(2'-hydroxyethyl)-5,6-bis(4-chlorophenyl)-2H-pyridazine-3-one (149). These impurities can be isolated for individual determination by combining LC [silica gel column with toluene-ethyl acetate-acetonitrile (2:1:1) mobile phase] and preparative TLC [silica gel plate with chloroform-ethyl acetate (17:3) solvent system]. The results of these investigations show that one of these impurities [(4-acetyl-3-(2'-hydroxyethoxy)-5,6-bis(4-chlorophenyl)pyridazinel had a higher LD_{50} than the compound of interest and the other two lacked antihypertensive activity.

Several potential impurities (XXXIII, XXXIV, XXXV) of trihexyphenidyl (Scheme 13) have been screened by TLC, and their detectability varies between 0.25 and 2.5 μg (150). TLC is performed on silica gel GF plates with a chloroform-methanol-ethanol-ethyl acetate (20:20:20:40) system. Spots are visualized with UV (254 nm) and with Dragendorff spray. An impurity (XXXV) has been found at the 1.6% level in a batch of bulk drug. This impurity may result from the presence of ammonium chloride in the piperidine hydrochloride used as the starting material in the synthetic process. Compound (XXXIII) is an intermediate in the

synthesis and compound (XXXIV) may originate from de-
composition of (XXXIII).
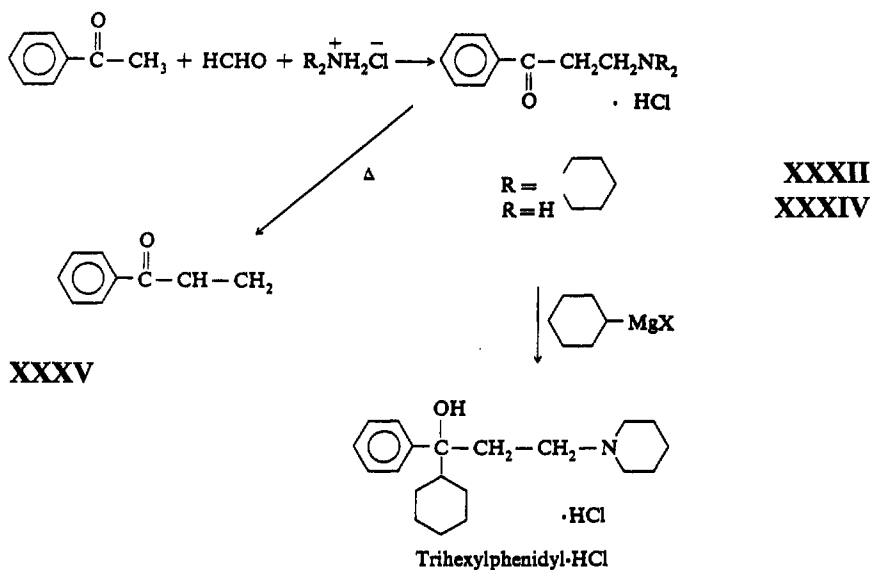
Scheme 13 (150).

Two impurities in bulk methotrexate have been chromato-
graphically separated by linear gradient elution with 0.01-0.04 M
ammonia-ammonium bicarbonate buffer (pH 8.3) on a dieth-
ylaminoethylcellulose column (151). Besides known impurities,
two hitherto unknown impurities, 4-amino-N^{10}-methylpteroyl-N-
methylglutamine and 2,4-diamino-N^{10}-methylpteramide, have been
identified. The USP grade reference material gives a purity figure
of 86.0%, and the concentration of identified and unidentified
impurities is 7.6% and 1%, respectively.

The photodecomposition of furnidipine [2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-tetrahydrofurfuryl 5-methyl diester] has been studied by voltametric, UV-vis spectrophotometric, and HPLC techniques with three different light conditions (artificial daylight, UV light, and room daylight) (152). The artificial daylight photodecomposition of furnidipine follows 0.5-order kinetics as assessed by the above-mentioned techniques. Furthermore, the daylight photoderivative has been isolated and identified by NMR and IR as 2,6-dimethyl-4-(2-nitrosophenyl)pyridine-3,5-dicarboxylic acid 3-tetrahydrofurfuryl 5-methyldiester.

Quantitative kinetic data for the UV photodecomposition of furnidipine cannot be obtained due to both the high rate of degradation (<1 min) and intermediate reactions. However, polarographic, spectroscopic, and chromatographic evidence permits identification of this product as 2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylic acid 3-tetrahydrofurfuryl 5-methyl diester. Polarography is the most useful technique to assess the photodegradation of this drug from the qualitative point of view, and quantitative kinetic data is similar to that obtained by HPLC and UV-vis spectroscopy.
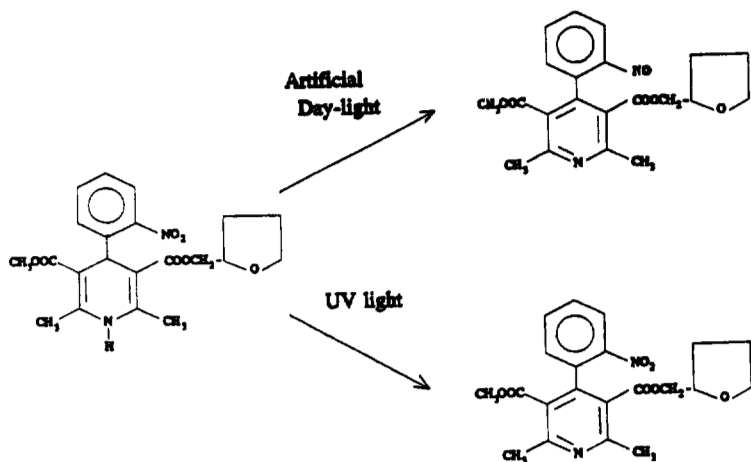
New photodegradation products of nifedipine XXXVI have been isolated (153). They are encountered in tablets dispensed in pulverized form by hospitals. XXXVI decomposed concurrently into six components after storage of 30 days under exposure to normal light. The main photoproduct is a nitroso derivative, XXXVII, and others are minor products. Preparative TLC has been used to isolate six degradation products. The chemical structures of these isolated compounds have been identified or estimated by comparison with the authentic samples and/or using melting point, elemental analysis, UV, IR, proton NMR, and MS. From these analyses, it has been found that XXXVI is converted into a cis-azoxy derivative, a trans-azoxy derivative, a N,N'-dioxide derivative, and a lactam derivative in addition to XXXVII, and a nitro derivative XXXVIII. Furthermore, it has been proposed that XXVII is mainly responsible for the formation of these new products by photochemical condensation.
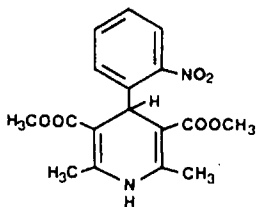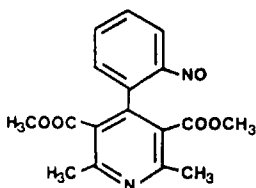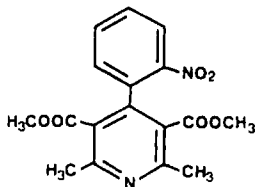
The photostabilty of the fluoroquinolones ciprofloxacin (CPX), ofloxacin (OFX), and fleroxacin (FLX) toward UV radiation (UVA) and room light has been investigated in dilute aqueous solutions (154). A series of photooxidation products is observed by HPLC for all three drugs. As little as 1 hour of exposure to room

light is enough for the formation of detectable amounts of CPX photoproducts. The major CPX photoproduct is characterized as a dimer by liquid secondary ion mass spectrometry, but its structure has not been determined. Since irradiation of CPX results in loss of antibacterial activity and since all substances, parent as well as their photoproducts, are potential candidates for undesired drug effects, quinolone drugs should be strictly protected from all light during storage and administration.

CPX

OFX

FLX
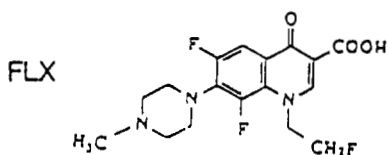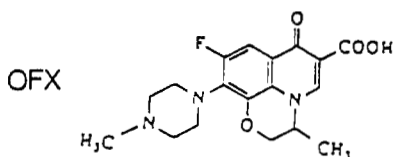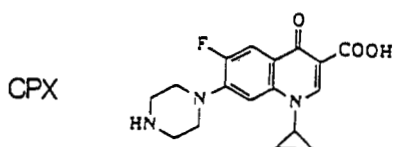
The degradation kinetics of N-(5-methyl-4-isoxazolyl)-4-amino-1,2-naphthoquinone (XXXIX) has been studied in aqueous solution over a pH range of 0.65-7.50, at 35°C, and at constant ionic strength of 0.5 (155). The degradation rates are determined by HPLC and have been observed to follow pseudo-first-order kinetics with respect to the concentration of XXXIX.

The pH-rate profile is linear with slope-1 under acidic pH, becoming pH independent from 3.50 to 7.50. Good agreement between the theoretical pH-rate profile and the experimental data support the proposed degradation process. The catalytic rate

constants of hydrogen ion and water are $k_H = 0.901$ m^{-1} h^{-1} and k_0 = 1.34×10^{-3} h^{-1}, respectively. The degradation reaction is shown in Scheme 14.

XXXIX
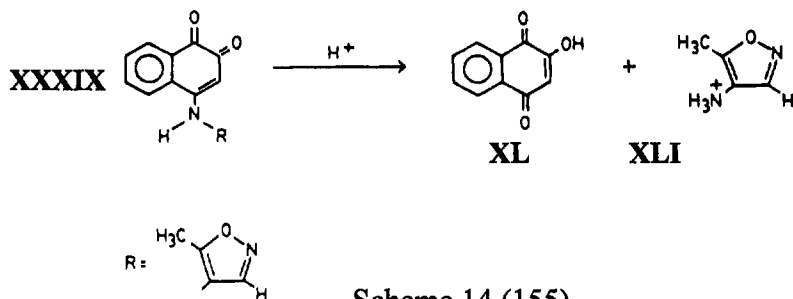
Scheme 14 (155).

No systematic decrease in hymercromone (4-MU) spectral absorbance has been observed at constant pH value ranging from 2 to 11 over 20 minutes (156). A 36.7 µM solution of 4-MU at pH 12.2 and 23.9 µM solution of 4-MU in 0.1 and 1.0 N NaOH (calculated pH values of 12.88 and 13.81, respectively) have been monitored spectrophotometrically at 19.5°C, and their absorbances have isobestic points (wavelengths of invariant absorbances while 4-MU degrades) of 314-316 and 324 nm, respectively. The maximal 360-nm absorbances of the mono-ionized 4-MU decreases with respective half lives of 63, 74.5, and 2.54 min. This is probably due to the formation of the disodium salt of the open ring hymercromone from the hydrolysis of the coumarin ring to open lactone (Scheme 15).

Coumarin   Disodium salt of coumarinic acid
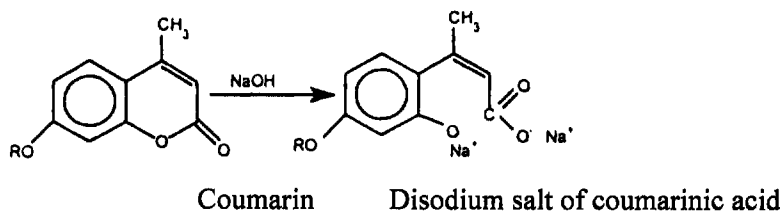
Scheme 15 (156).

4-MU and 4-MUG can be analyzed by HPLC with great sensitivity and specificity using fluorescent detection; however, this is not feasible with insignificantly fluorescent 4-MUS, the direct detection of which is limited to spectrophotometry. Since MUS can be readily degraded to 4-MU in acidic media that do not affect 4-MU or 4-MUG, 4-MUS can be analyzed by the difference in the sensitive fluorometric assays of 4-MU before and after acid treatment.

Stereoselective hydrolysis of two ester prodrugs of propanolol, isovaleryl propanolol (IV-PL) and cyclopropanoyl propanol (CP-PL) have been studied in tris-HCl buffer (pH 7.4) containing 0.15 M KCl, skin and liver homogenates, 5% plasma in tris-HCl buffer, skin cytosol and microsomes, and liver cytosol and microsomes (157). The hydrolysis rate constants of (R)isomers of the prodrugs are 1.1 to 30.3 times greater than those of the respective (S)-isomers in tissue preparations. The hydrolyzing capabilities of buffer and different tissue preparations per milligram of protein content are in increasing order as follows: buffer, skin homogenate, plasma, and liver homogenate.

A derivatization procedure prior to HPLC has been used for the separation of the 15R-epimer and the 5-trans-isomer of carboprost [(15S)-15-methyldinoprost] (157). The UV-absorbing naphthacyl ester derivatives are resolved on a silica gel column (micro-Porasil) with a mobile phase consisting of methylene chloride-1,3-butanediol-water (496:40:0.25). The derivatization procedure has been developed to obtain greater sensitivity for the assay of 15R-epimer in carboprost samples. It also provides better chromatography in that baseline separations are obtained.

Prostaglandin A_2 can occur as cis- or trans-configuration at the 5,6 double bond; however, the cis form is more common. The HPLC method is based on resolution of cis-trans isomers, using silver-ion complex formation. The thermodynamics of the reaction suggest that temperatures near 25°C are required for appreciable complexation. This requirement is easily met by HPLC. The 5,6-cis-trans isomers of prostaglandin A, have been resolved on a C-18 column (37-μm) with 0.5 M $AgClO_4$, dissolved in 20%, methanol

(158). The elution of the cis isomer before the trans isomer suggests that complexation takes place in the mobile phase and that the silver cis isomer complex is more stable.

A GC procedure that may be useful for the determination of low-level impurities of 11-methyl-16,16-dimethyl prostaglandin E_2 requires preparation of trimethylsilyl derivative by reaction with hexamethyldisilazane-trimethylchlorosilane in acetonitrile. The chromatographic procedure entails temperature programming (0-290°C) on a 3% OV-1 Chromosorb column and detection with a hydrogen flame ionization detector (159).

Picomole detection of 2,4-dimethoxyanilides of prostaglandins has been reported by HPLC with electrochemical detection (160).

Chromatographic methods (TLC and HPLC) have been used to resolve p-aminobenzoic acid, N-(p-aminobenzoyl)-L-glutamic acid, 2-amino-1,4-dihydro-4-oxo-6-pteridine-carboxylic acid, 2-amino-4(IH)-pteridinone, and several unidentified impurities of folic acid (161). The HPLC method utilizes a μBondapak C-18 column and a mobile phase buffered to pH 7.2 containing 4% methanol and 3.51% sodium perchlorate monohydrate. The method is useful for the determination of the above-mentioned impurities at submicrogram levels.

An HPLC method that is suitable for the determination of several degradation products of ergocalciferol requires a Micropak MCH-10 column and a mobile phase composed of 50 methanol/50 acetonitrile (162). However, aside from the fact that these products have higher polarity, no information on their structure or content is provided in this investigation.

Chromatographic analysis of fat-soluble vitamins in clinical chemistry has been reviewed (163). Factors controlling the separation of seven water-soluble vitamins on reverse phase columns have also been systematically evaluated. Factors studied include both mobile-phase constituents and column parameters (164). Data showed that a mobile phase containing hexanesulfonate (5 mM), methanol (15%), acetic acid (1%), and triethylamine (0.10% to 0.13%) yield excellent separations with several C-8 and C-18 columns. Lowering the methanol con-

centration in the mobile phase enhanced the resolution of early-eluting peaks, while the triethylamine level controlled the peak shape and retention of thiamine.

Saccharin is included in this section because of its potential of being used in pharmaceutical formulations. HPLC has been used for the determination of saccharin in the presence of its most likely impurities and degradation products (165). These compounds have been identified as follows: 1,5-naphthalenedisulfonic acid, ammonium-o-sulfobenzoate, o-sulfamoylbenzoic acid, p-carboxybenzene sulfonamide, and o- and p-toluene sulfonamides. The method requires a UV detector set at 280 nm, a μBondapak C-18 column, and a mobile phase composed of 10 mL of acetic acid and 600 mL of methanol diluted to 2 L with water. No minimum detectability information on impurities is provided; however, it is anticipated that submicrogram detections are possible. Quantitation of o- and p-sulfamoylbenzoic acid residues in saccharin and its sodium salt has been performed by high-pressure ion-exchange chromatography (166). A 1 m-long, strong cation-exchange column with a mobile phase composed of 0.01 M sodium borate (pH 9.2) and 0.008 M $NaNO_3$ was used for these separations. The minimum detectability for these acids is 0.05-0.16 μg.

Packed column SFC is ideally suited to the separation of moderately polar compounds, including many families of basic drugs (167). The effect of mobile phase composition, temperature, and pressure on the retention and selectivity of stimulants has been investigated.

A wide range of these compounds is rapidly and efficiently separated with symmetrical peaks with tertiary supercritical mobile phase and cyanopropyl liquid chromatography column. Compared with other drug families in this series, the stimulants exhibit a much broader range of retention. Several strong bases, including primary aliphatic amines, are easily eluted with a modified mobile phase. The retention data on a number of stimulants studied is given in Table 9.8.

As one might anticipate, the modifier concentration proved to be the most effective means of changing retention and selectivity.

Table 9.8 SFC Retention Data of Stimulants

SOLUTE	T_R (minutes)
Cocaine	3.20
Amphetamine	2.15
Methamphetamine	6.00
Benzphetamine	2.44
Phenmetrazine	3.77
Methylphenidate	3.18
Ephedrine	7.62
Phenylephrine	12.55
Hydroxyamphetamine	14.62
Nylidrine	3.27
Phenylpropanolamine	6.67
Mephentermine	6.68
Naphazoline	11.34

Note: Operating parameters: mobile phase; 10% methanol
with 0.5% v/v isopropylamine in carbon dioxide; 2ml/min.
flow rate; 40°C oven temp. and 200 bar outlet pressure;
detection at 220 nm

Fourteen antipsychotic drugs, mostly phenothiazines, have been chromatographed on a cyanopropyl column with tertiary mobile phase of carbon dioxide, methanol, and isopropylamine(168). The retention data is given in Table 9.9.

Table 9.9. SFC Retention Data of Antipsychotic Drugs

DRUG	T_R (minutes)
Triflupromazine HCl	2.15
Carphenazine maleate	4.07
Methotrimeprazine	2.72
Promazine HCl	3.32
Compound 3.41	3.41
Perphenazine	3.60
Chloroprothixine	2.48
Deserpidine	4.31
Thiothixene	4.73

**Table 9.9. SFC Retention Data of
Antipsychotic Drugs (continued)**

DRUG	T_R (minutes)
Reserpine	4.85
Acetophenazine dimaleate	4.33
Ethopropazine	2.70
Promethazine HCl	2.49
Propriomazine HCl	2.48
Triflupromazine HCl	2.15

Column: 4.6×250 mm, 5μm Lichrosphere cyanopropyl column;
mobile phase: 10% methanol (containing 0.5% isopropylamine)
in carbon dioxide; oven 50°C and 200 bar outlet pressure.

An arbitrary mixture of the first 10 components listed in Table 9.9 can be baseline resolved in very short time. Detection limits are as low as 125 ppb, with a feasible tenfold decrease. Without the isopropylamine in the mobile phase, none of the solutes eluted. Changing the modifier concentration is the most effective physical parameter for changing retention. Temperature change provided surprisingly large changes in selectivity, with numerous peak reversals occurring over only a 30°C temperature range. Additionally, temperature programming is the most effective means to optimize resolution of the 10-component mix.

A pharmaceutical agent has been quantified in rat feed matrix wherein sample preparation is achieved by SFC extraction. Spiking levels ranged from 0.0335% to 1.12% (169). Pure carbon dioxide, solid phase trapping on stainless steel with acetonitrile and HPLC assay yielded recoveries greater than 90% with relative standard deviations of less than 5% in all cases except for the lowest spiking level. In this case recovery is 89.6% with an RSDF of 9.6%.

An approach for capillary electrophoresis (CE) method development has been applied to small-molecule pharmaceutical separations, using analgesics as examples (170). The effects of various parameters on selectivity have been evaluated. Parameters such as linearity, sensitivity, and reproducibility have also been examined. This knowledge has been extended to other application areas, and CE separations of compounds such as penicillins,

steroids, water-soluble vitamins, and enantiomeric compounds have been developed. It is shown that the selectivity, sensitivity, and reproducibility of CE are adequate for routine use in the pharmaceutical laboratory.

Terbutaline and propanolol have been resolved using capillary electrophoresis (171). The effect of the type and amount of cyclodextrins added to the background electrolyte on the migration time and resolution of their enantiomers has been studied. Good resolution of racemic terbutaline has been obtained using phosphate buffer at pH 2.5, containing either 5 mM heptakis(2,6-di-O-methyl)-β-cyclodextrin or 15 mM β-cyclodextrin. The background electrolyte, 50 mM phosphate buffer (pH = 2.5)-4 M urea-40 mM β-cyclodextrin in 30% (v/v) methanol, on the other hand, gave the best resolution of propanolol enantiomers.

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