Analytical Profiles of Drug Substances and Excipients

Volume 26

edited by

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PREFACE

The comprehensive profile of drug substances and pharmaceutical excipients as to their physical and analytical characteristics continues to be an essential feature of drug development. The compilation and publication of comprehensive summaries of physical and chemical data, analytical methods, routes of compound preparation, degradation pathways, uses and applications, *etc.*, is a vital function to both academia and industry. It goes without saying that workers in the field require access to current state-of-the-art data, and the *Analytical Profiles* series has always provided information of the highest quality. For this reason, profiles of older compounds are updated whenever a sufficient body of new information becomes available.

Volume 25 proved to be an arduous compilation, since obtaining profile contributions seemed to be more difficult than ever in the past. Without the extraordinary work conducted by Professor A.A. Al-Badr and his colleagues, this volume might not have appeared until well into the coming millenium. It seems that sufficient author time for the production of profile articles is becoming harder and harder to come by, and many potential contributors are discouraged. The pace of modern drug development, its wide range of activities, and the continuing personnel down-sizing seems to conspire against professional activities such as profile writing. It is still ironic that the need for analytical profiles is as strong as ever, and yet potential authors are getting scarcer all the time. However, the contributors to the present volume have indeed found the resources to write their chapters, and I would like to take this opportunity to salute them for their dedication to this work.

As always, a complete list of available drug and excipient candidates is available from the editor. I continue to explore new and innovative ways to encourage potential authors, and welcome suggestions as to how to get people involved in the writing of analytical profiles. Communication from new and established authors is always welcome, and Email contact (address: hbrittain@earthlink.net) is encouraged. I will continue to look forward to working with the pharmaceutical community on the *Analytical Profiles of Drug Substances and Excipients*, and to providing these information summaries that are of such great importance to the field.

Harry G. Brittain

BENZOIC ACID

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Name

Benzoic acid

1.1.2 Nonproprietary Names

benzenecarboxylic acid; benzoate; carboxybenzene; diacyllic acid; benzene-formic acid; benzenemethonic acid; phenyl-carboxylic acid; oralyc acid; dracyllic acid; acidum benzoicum; benzoesaeure, [1-3]

1.1.3 Proprietary Names

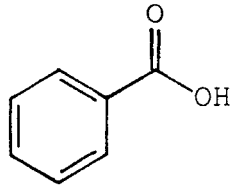
Aserbine; Malatex [4]

1.2 Formulae

1.2.1 Empirical

$C_7H_6O_2$

1.2.2 Structural



1.3 Molecular Weight

122.12

1.4 CAS Number

65-85-0

1.5 Appearance

Benzoic acid is a colorless or white powder, which can be odorless or with a slight characteristic odor [3].

1.6 Uses and Applications

Benzoic acid possesses anti-bacterial and anti-fungal properties. At a concentration of 0.1%, benzoic acid is a moderately effective preservative providing that the pH of the formulation (medicines, cosmetics, or foods) does not exceed 5.0. As ointment, benzoic acid is used for the treatment of fungal infections [3,5].

Benzoic acid can be used as a corrosion-inhibitor in emulsions and paints, as well as an anti-freeze formulation, plugging agent, and modifier in oil well applications. It has also found use as a textile dye carrier [6].

2. Methods of Preparation

A number of general methods are available for the preparation of benzoic acid, which represent applications of the general methods for the preparation of aromatic carboxylic acids.

2.1 Oxidation of a Side Chain

Toluene, ethylbenzene, styrene, *etc.*, produce benzoic acid when heated with strong oxidizing agents.

$$C_6H_5\text{-}CH_2CH_3 \rightarrow C_6H_5\text{-}COOH \quad (1)$$

The presence of a halogen atom in the side chain greatly facilitates the oxidation, so that substances such as benzyl chloride or benzyl alcohol are much more easily oxidized than are the corresponding hydrocarbons:

$$C_6H_5\text{-}CH_2Cl \rightarrow C_6H_5\text{-}CH_2OH \rightarrow C_6H_5\text{-}COOH \quad (2)$$

In addition, the presence of an oxygen atom in the side chain also promotes the reaction. For instance, the oxidation of acetophenone proceeds easily:

$$C_6H_5\text{-}CO\text{-}CH_3 \rightarrow C_6H_5\text{-}COOH + CO_2 + H_2O \quad (3)$$

2.2 Hydrolysis of a Trihalide

If toluene is chlorinated to benzo-trichloride, the substitution reaction has oxidized the carbon atom of the methyl group to the acid stage and no further oxidation is necessary. At that point, hydrolysis alone is sufficient:

$$C_6H_5\text{-}CCl_3 \rightarrow C_6H_5\text{-}C(OH)_3 \rightarrow C_6H_5\text{-}COOH \quad (4)$$

2.3. Hydrolysis of a Nitrile

Nitriles of the aromatic acids may be obtained from the sulfonic acids or from diazonium salts. Hydrolysis of the nitrile produces the carboxylic acid:

$$C_6H_5\text{-}CN + H_2O + HCl \rightarrow C_6H_5\text{-}COOH + NH_4Cl \quad (5)$$

2.4 Commercially Viable Methods

In the United States, benzoic acid is produced commercially by the liquid-phase oxidation of toluene [7,8]. A variety of cobalt catalysts (in the concentration range of 30–1000 ppm) are used for this purpose.

Benzoic acid can also be produced on the large scale by the chlorination of toluene [8]:

$$C_6H_5\text{-}CH_3 + 3\,Cl_2 \rightarrow C_6H_5\text{-}CCl_3 + 3\,HCl \quad (6)$$

$$C_6H_5\text{-}CCl_3 + C_6H_5\text{-}COOH \rightarrow 2\,C_6H_5\text{-}COCl + HCl \quad (7)$$

$$C_6H_5\text{-}COCl + H_2O \rightarrow C_6H_5\text{-}COOH + HCl \quad (8)$$

Reaction (7) requires the use of a $ZnCl_2$ catalyst.

Formerly, benzoic acid was produced by the decarboxylation of phthalic anhydride. Oxidation of acetophenon, benzyl bromide, and toluene with sulfur and water has been described in the literature, but are not commercially feasible routes of synthesis. Carboxylation of benzene with carbon dioxide is not practical due to the instability of benzoic acid at the required reaction conditions [8].

The world demand of benzoic acid increases approximately 3-5% each year. In 1995 230 million pounds were consumed, and in 1996 235 million pounds were used. By 2000 the projected amount is estimated to be about 275 million pounds [7].

3. Physical Properties

3.1 Particle Morphology

Benzoic acid is ordinarily obtained as a feathery, light crystalline powder. Certain habit modifications can cause the material to exist as needles or as plates.

When crystallized from an acetone-water mixture, benzoic acid is obtained as the crystalline needles shown in Figure 1A. On the other hand, a mixture of needles and irregular crystalline solid is obtained from methanol-water mixtures (Figure 1B). From pure water, aggregates of monoclinic crystals are obtained (Figure 1C).

3.2 Crystallographic Properties

3.2.1 Single Crystal Structure

The single crystal structure of benzoic acid has been reported [52]. In its crystal, the molecule is approximately planar, but distances of 0.042 Å and 0.068 Å displace the carboxylate carbon and one of its oxygen atoms, respectively, from the mean plane. The structure is characterized by the existence of hydrogen-bonded molecular dimers in the unit cell.

3.2.2 X-Ray Powder Diffraction Pattern

Benzoic acid is ordinarily obtained as a highly crystalline material, which aids in its purification and contributes to its use as a reference material. The x-ray powder diffraction pattern of benzoic acid was obtained on a Jeol JDX-3530 diffractometer system. The x-ray radiation was provided by copper target, operated at 40.0 kV and 30 mA. The powder pattern was obtained using a step angle of 0.040 degrees 2-θ, and each step was

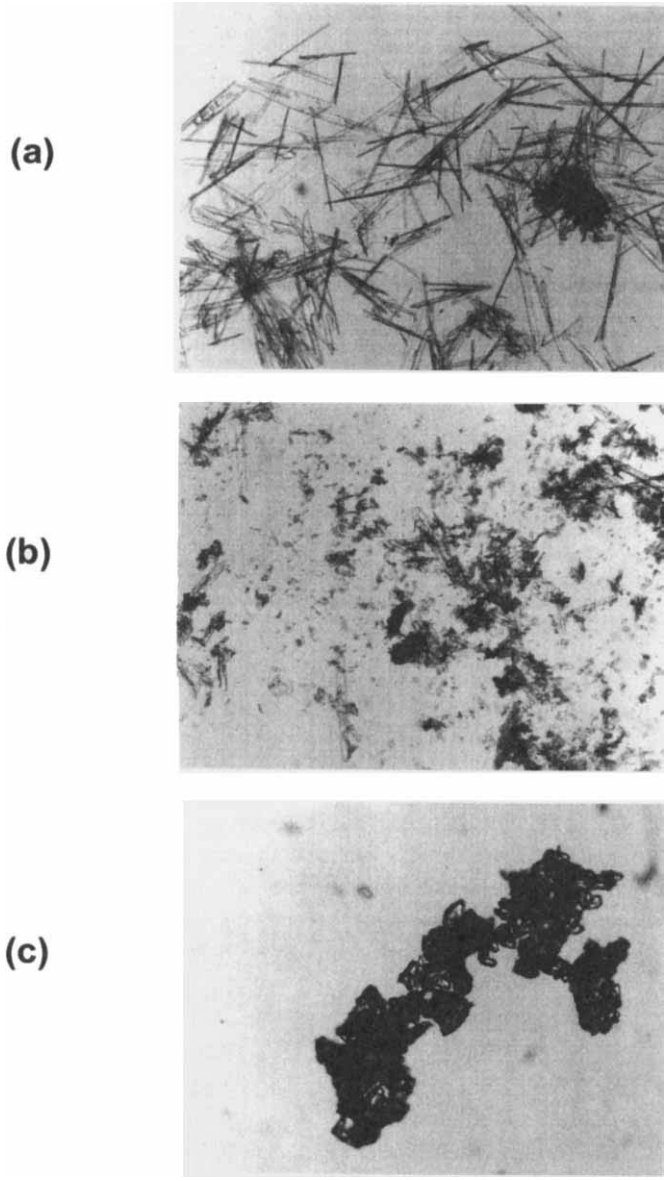


Figure 1. Optical photomicrographs of benzoic acid, obtained at a magnification of 100X, for product isolated from (a) acetone-water, (b) methanol-water, and (c) water.

averaged for 1.0 sec. The powder pattern thusly obtained is shown in Figure 2, and the crystallographic data associated with the pattern are listed in Table 1.

3.3 Thermal Characteristics

3.3.1 Melting Behavior

Benzoic acid of primary standard quality is used for the temperature validation of a variety of methods, and is known to exhibit a melting point of 122.375 ± 0.001 °C [10].

Benzoic acid will begins to sublime at around 100 °C [2], and will form a feathery sublimate near its melting point [5]. Other workers have noted that melted benzoic acid will congeal between 121°C and 123°C [9].

3.3.2 Thermal Analysis

The thermal properties of benzoic acid were evaluated using simultaneous differential thermal analysis (DTA) and thermogravimetric analysis (TGA). This work was performed on a Shimadzu DT-30 Thermal Analyzer system, which was calibrated using indium standard. Using a heating rate of 10°C/min, the thermograms presented in Figure 3 were obtained.

The DTA curve consists of a single sharp endothermic event having a maximum temperature at 122.4°C, which is identified as the melting endotherm. The DTA and TGA baselines begin to change above approximately 100°C, indicative of some compound decomposition had begun. The onset of sublimation was identified at 173.4°C, as indicated by the sudden change in the DTA curve and the large weight loss in the TGA thermogram.

3.3.3 Boiling Point

The boiling point of benzoic acid is 249°C [1].

3.3.4. Flash Point

The flash point of benzoic acid is 121°C [1].

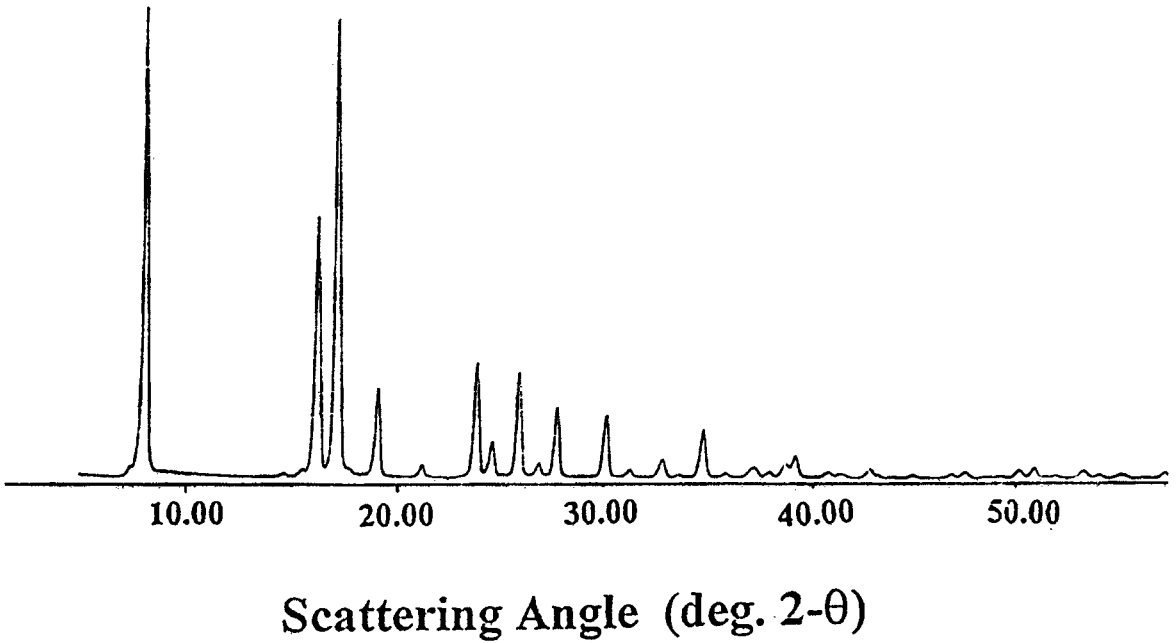


Figure 2. X-ray powder diffraction pattern of benzoic acid.

Table 1

Crystallographic Data from the X-Ray Powder Diffraction Pattern of Benzoic acid

Scattering Angle (degrees 2-θ)	d-spacing (Å)	Relative Intensity ($\%I/I_o$)
7.29	12.1622	4
8.13	10.8662	95
8.65	10.2141	4
15.45	5.7305	3
16.29	5.4368	56
17.20	5.1482	100
17.73	4.9984	4
19.09	4.6452	21
21.17	4.1933	4
23.81	3.7334	27
24.53	3.6260	9
25.85	3.4437	25
26.81	3.3227	5
27.77	3.2099	17
30.13	2.9636	16
32.89	2.7209	6
34.85	2.5723	12
38.77	2.3207	5
39.25	2.2934	6
50.93	1.7915	4

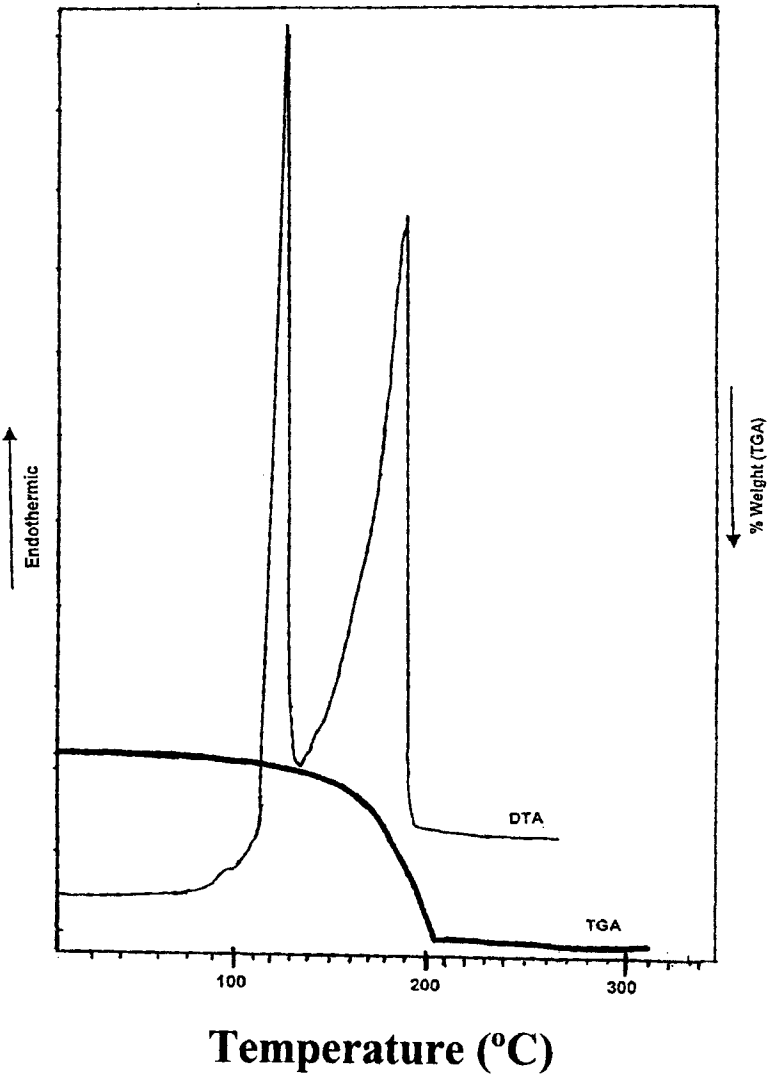


Figure 3. Thermogravimetric analysis (TGA) and differential thermal analysis (DTA) thermograms of benzoic acid.

3.4 Solubility Characteristics

The equilibrium solubility of benzoic acid at ambient temperatures in various nonaqueous solvents is presented in Table 2. The aqueous solubility was found to exhibit a strong pH dependence (Table 2), with the ionized form of the substance being much more soluble than the free acid.

The pH of a saturated solution of benzoic acid in water at 25 °C was found to be 2.8 [2].

3.5 Partition Coefficient

The *n*-octanol/water partition coefficient of benzoic acid is 1.9 [4].

3.6 Ionization Constant

The dissociation constant (pK_a) of benzoic acid in water at 25°C is 4.2 [4], or more exactly being equal to 4.199 [10].

3.7 Miscellaneous Physical Constants

3.7.1 Specific gravity

The specific gravity of benzoic acid is 1.08 [1].

3.7.2 Refractive index

The refractive index of solid benzoic acid (temperature = 15°C) is 1.53974, while the refractive index of liquid benzoic acid (temperature = 131.9°C) is 1.504 [8].

3.7.3 Viscosity

The viscosity of benzoic acid at 122.5 °C is 1.67 cP, and is 1.26 cP at 130 °C [8].

Table 2

Solubility of Benzoic acid in Various Solvents [8]

Solvent	Temperature of Measurement (°C)	Solubility (g/L)
Acetone	25	556
Benzene	25	121.7
Carbon tetrachloride	25	41.4
Chloroform	25	150.2
Ethanol absolute	25	584
Ethyl ether	25	408
Hexane	17	9.4
Methanol	23	715
Toluene	25	106
Water	0	1.7
Water	10	2.1
Water	20	2.9
Water	25	3.4
Water	30	4.2
Water	40	6.0
Water	50	9.5
Water	60	12.0
Water	70	17.7
Water	80	27.5
Water	90	45.4
Water	95	68.0

3.8 Spectroscopy

3.8.1 UV/VIS Spectroscopy

The ultraviolet absorption spectra of benzoic in 96 % ethanol (concentration = 8.3 ppm) and in 0.01 N HCl (concentration = 8.2 ppm) were recorded on a Shimadzu UV-265 spectrophotometer, and are shown in Figure 4. The spectra were not found to be greatly affected by the nature of the solvent used. An absorbance maximum of 227 nm was noted for the 96 % ethanolic solution, while a maximum at 229 nm was found in the 0.01 N HCl solvent. The absorbencies within these two solutions were found to be 0.815 and 0.768, respectively, so that the respective molar absorptivities are computed to be 12000 and 11400 liter/cm·mole.

The ultraviolet reflectance absorbance spectrum of a benzoic acid spot on a thin-layer chromatography place was recorded on a Shimadzu CS-930 TLC scanner, and is shown in Figure 5. The spectrum was obtained on a silica-gel F_{254} pre-coated TLC-plate (E. Merck) after elution with 7:2:2 acetone/ammonia 25 %/isopropanol. The absorbance maximum for this system was determined to be 225 nm.

3.8.2 Vibrational Spectroscopy

The infra red absorption spectrum of benzoic acid was obtained in a potassium bromide disc (using approximately 2 mg of benzoic acid dispersed in 200 mg KBr), and recorded on a Jasco 5300 FTIR spectrophotometer. The spectrum thusly obtained is shown in Figure 6, and the assignment of the characteristic bands is given in Table 3.

3.8.3 Nuclear Magnetic Resonance Spectrometry

3.8.3.1 ^{1}H-NMR Spectrum

The 90 MHz ^{1}H-NMR spectrum of benzoic acid shown in Figure 7 was obtained in deuterated chloroform using a Hitachi R-1900 FT-NMR spectrometer. Chemical shifts were measured relative to tetramethylsilane and assignments for the observed bands are found in Table 4. Due to the relatively low resolution of the 90 MHz NMR spectrometer, the only H-H

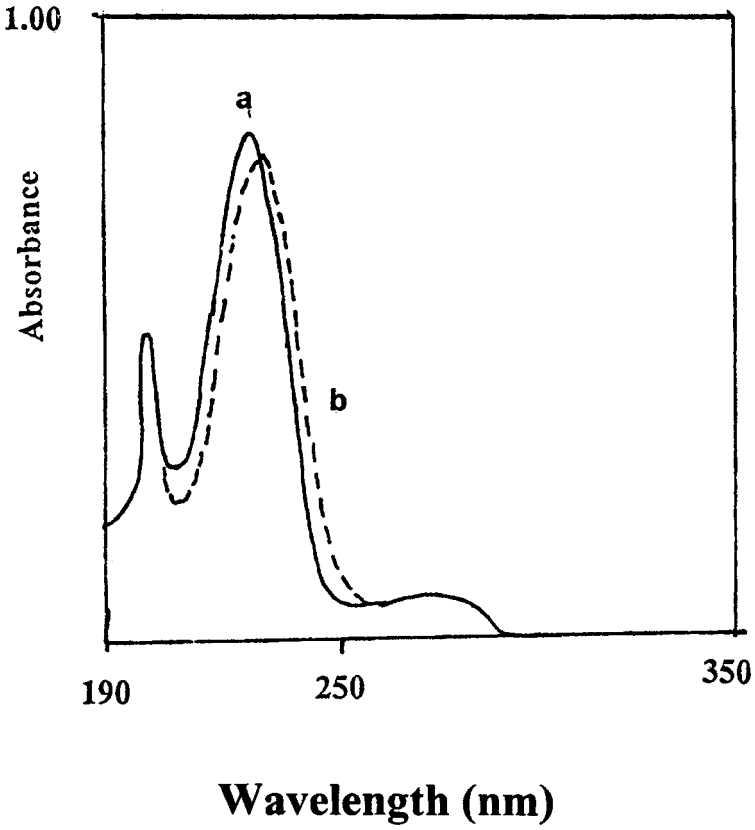


Figure 4. Ultraviolet absorption spectrum of benzoic acid in (a) 96 % ethanol and (b) 0.1 N HCl.

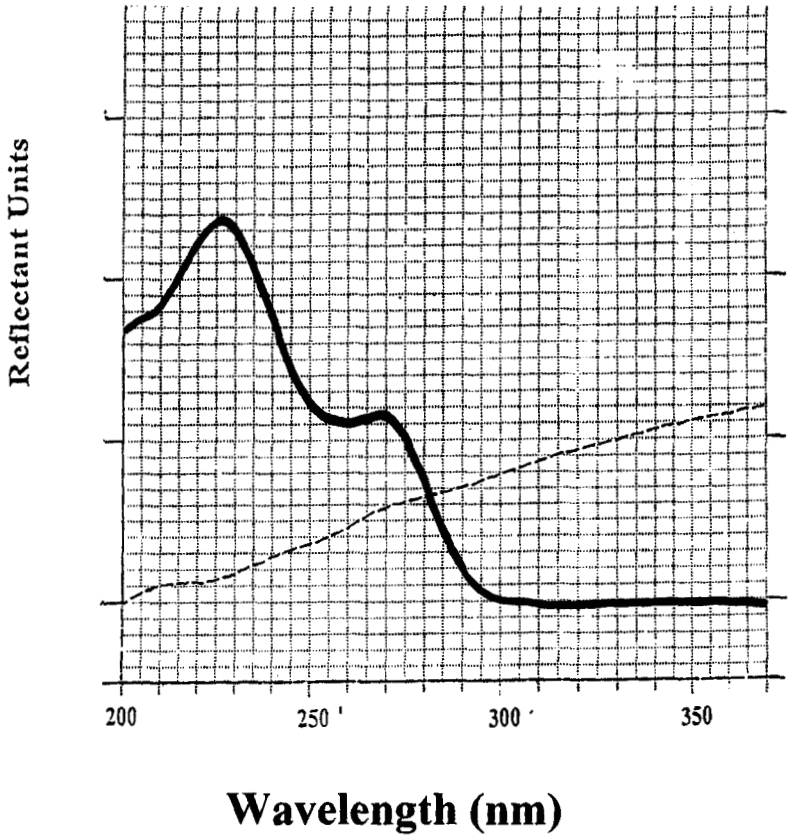


Figure 5. Reflectance ultraviolet absorption spectrum of benzoic acid spotted on a silica gel F254 pre-coated plate.

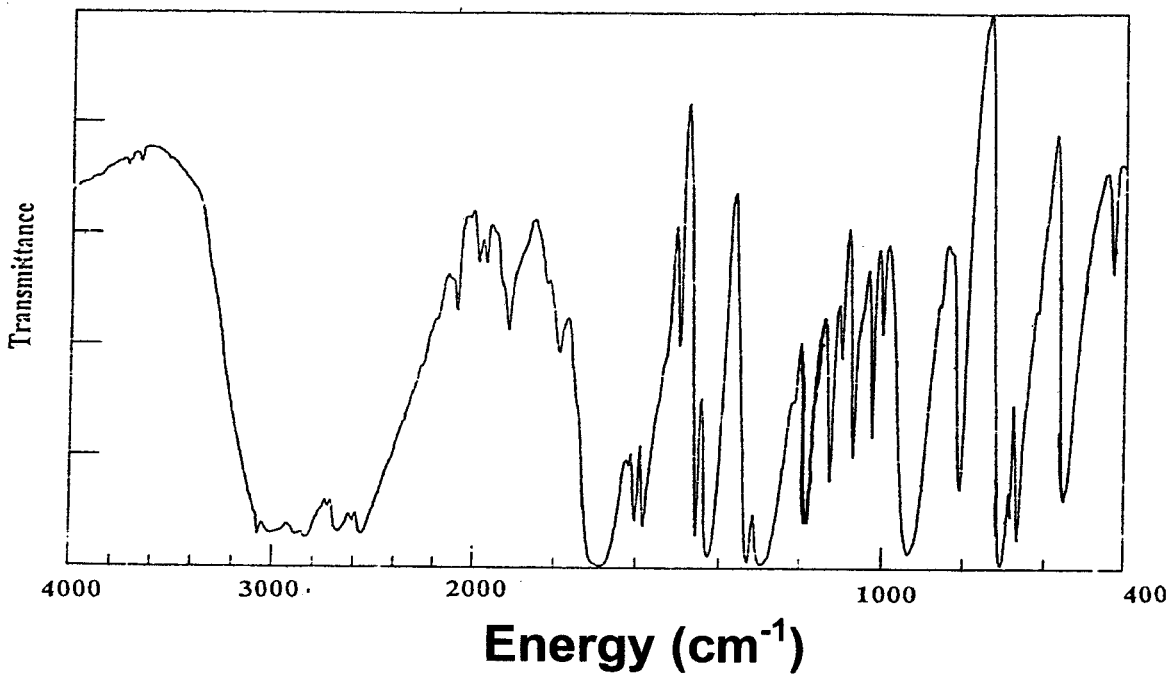


Figure 6. Infrared absorption spectrum of benzoic acid, obtained in KBr pellet.

Table 3

Assignment for the Characteristic Infrared Absorption Bands of Benzoic Acid

Energy (cm^{-1})	Band Assignment
3300-2500	Bonded –OH stretching mode
1687	Carbonyl -C=O stretching mode
2000-1667, 708, 667	Overtone bands of mono-substituted benzene ring
806, 708	Out of plane C-H bending mode
685	Out of plane C-H (benzene ring) bending mode

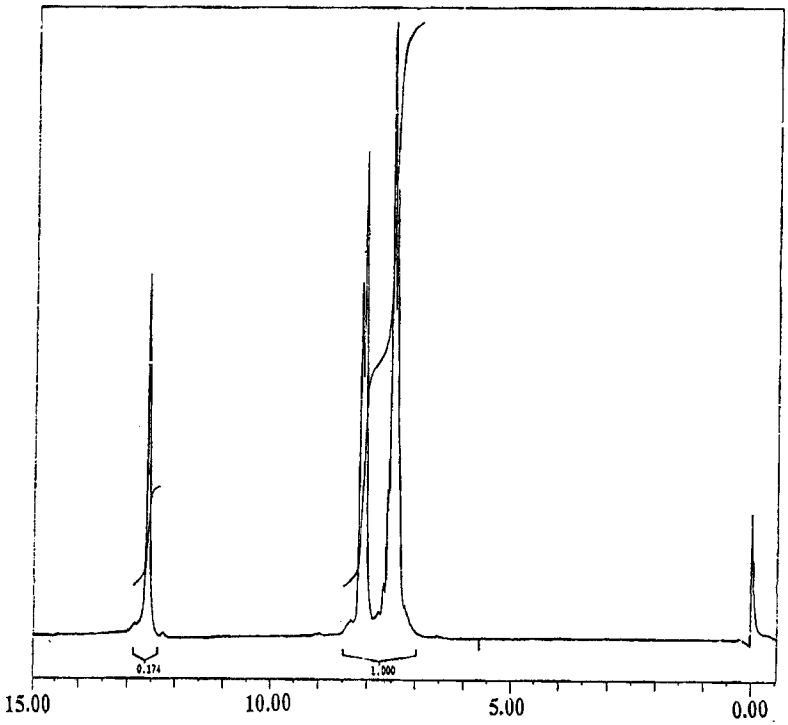


Figure 7. 90 MHz ^{1}H-NMR spectrum of benzoic acid in chloroform-d.

Table 4

Assignment for the Resonance Bands in the ^{1}H-NMR Spectrum of Benzoic Acid

Chemical Shift (ppm)	Multiplicity	Number of Proton	Assignment
12.56	Singlet	1	-COOH
8.11	Doublet (J=7.9 Hz)	2	H_2, H_6
7.60	Singlet	1	H_4
7.47	Doublet (J=7.1 Hz)	2	H_3, H_5

coupling observed was the coupling between *ortho* protons (H_2 and H_3; H_5 and H_6; J = 7-8 Hz). The coupling of *meta* protons was not observed.

3.8.3.2 ^{13}C-NMR Spectrum

The broad band decoupled ^{13}C-NMR spectrum of benzoic acid is shown in Figure 8. The spectrum was recorded at room temperature in chloroform-d on a Hitachi R-1900 FT NMR spectrometer operating at 22.6 MHz. Chemical shifts were measured relative to tetramethylsilane, and assignments for the observed peaks are found in Table 5.

3.8.4. Mass Spectrometry

The electron impact (Figure 9) and chemical ionization (figures 10 and 11) spectra of benzoic acid were obtained using a Finnigan Mat 8200 mass spectrometer. Methane and isobutane were used as the reactants for the CI methods. The studies were performed at 220°C (EI) and 130-140°C (CI), 0.05 mA, and 70 eV. The assignment of the main fragments is presented in Table 6.

4. Methods of Analysis

4.1 Compendial Tests

Benzoic Acid USP is specified to contain not less than 99.5% and not more than 100.5% of the substance, calculated on the anhydrous basis. It is to be preserved in well-closed containers. A number of compendial tests have been established by the USP [11].

4.1.1 Identification

The United States Pharmacopoeia 23 calls for the use of two identification tests [11]. Each of these begins with the preparation of a saturated solution of benzoic acid in water, which is filtered twice.

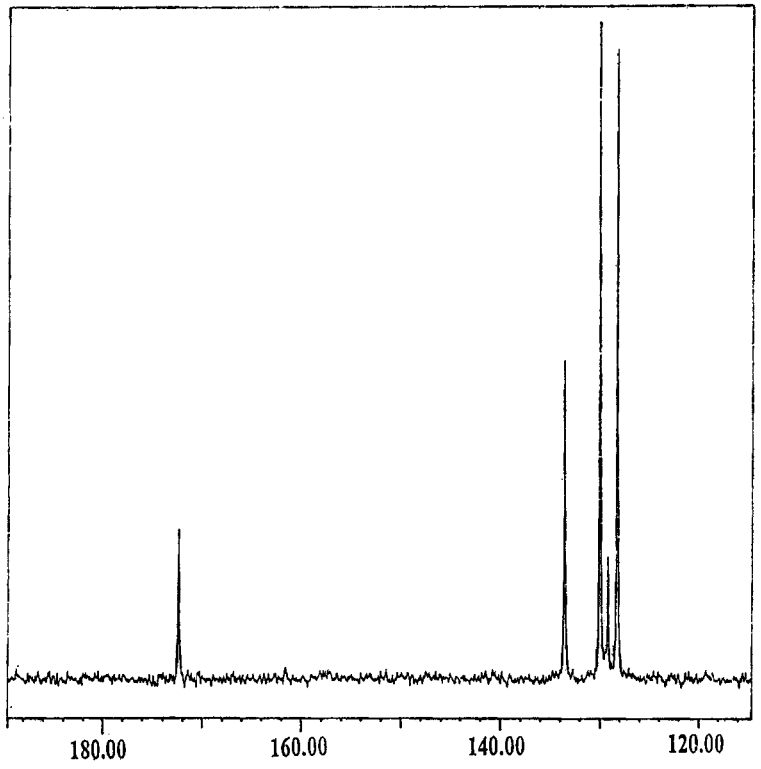


Figure 8. 22.6 MHz broad band decoupled ^{13}C-NMR spectrum of benzoic acid in chloroform-d.

Table 5

Assignment for the Resonance Bands in the ^{13}C-NMR Spectrum of Benzoic Acid

Chemical Shift (ppm)	Carbon Number
172.39	C_7
133.63	C_4
130.01	C_2, C_6
129.25	C_1
128.30	C_3, C_5

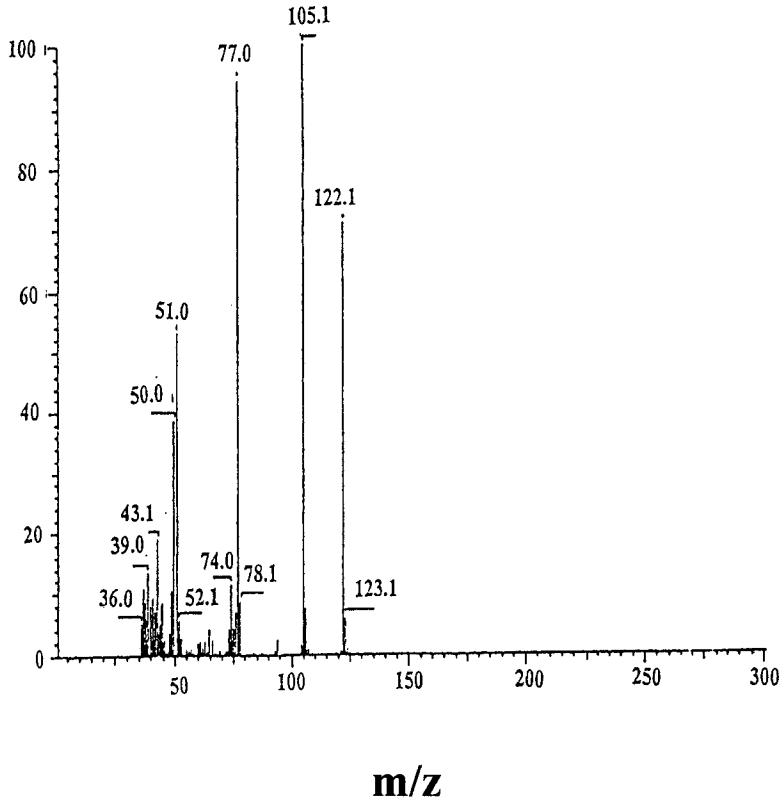


Figure 9. Electron-impact mass spectrum of benzoic acid.

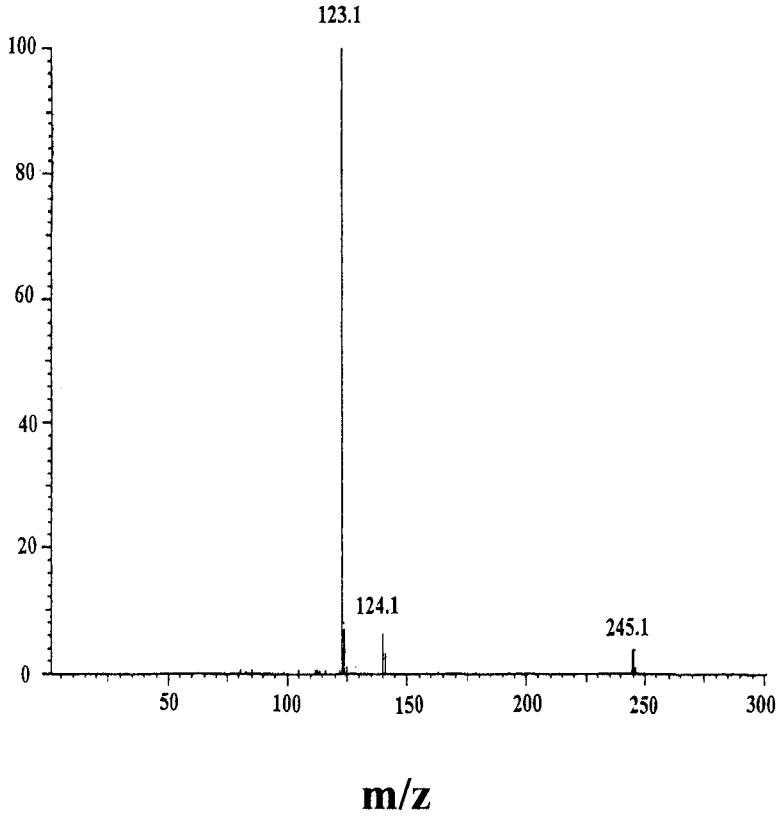


Figure 10. Chemical-ionization mass spectrum (obtained using isobutane) of benzoic acid.

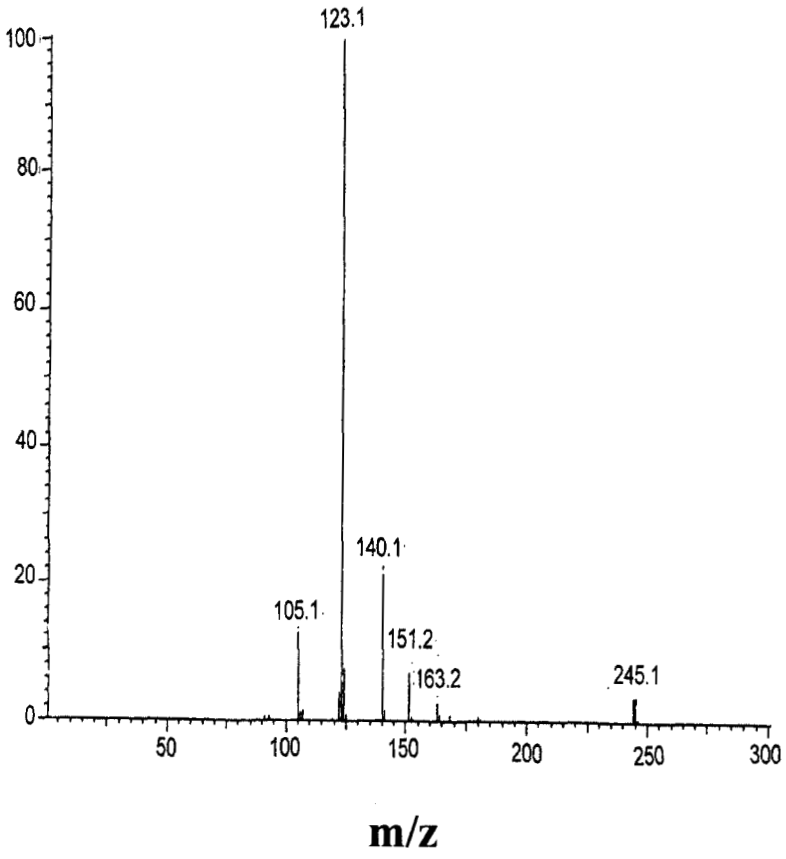
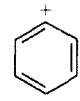


Figure 11. Chemical-ionization mass spectrum (obtained using methane) of benzoic acid.

Table 6

Electron Impact and Chemical Ionization Mass Spectral Data for Benzoic Acid

MS-Mode	m/z Ratio (% relative intensity)	Fragment assignment
EI	122.1 (70.5)	M^+
	105 (100)	$C_6H_5\text{-}C{\equiv}O^+$
	77 (93.3)	
	51 (52.4)	
CI (Isobutane)	245 (2.4)	$[2M + 1\ (H)]^+$
	123 (100)	$[M + 1\ (H)]^+$
CI (Methane)	245 (1.7)	$[2M + 1\ (H)]^+$
	163 (2.0)	$[M + 41\ (C_3H_5)]^+$
	151(6.9)	$[M + 29\ (C_2H_5)]^+$
	140 (21.4)	$[M + 1\ (H) + 17\ (CH_5)]^+$
	123 (100)	$[M + 1\ (H)]^+$
	105 (13.1)	$C_6H_5\text{-}C{\equiv}O^+$

4.1.1.1 Reaction with Ferric Chloride TS

To one portion of the filtrate add ferric chloride TS. A positive reaction is indicated by the formation of a salmon-colored precipitate.

4.1.1.2 Reaction with Sulfuric Acid

To a separate 10-mL portion of the filtrate, add 1 mL of 7 N sulfuric acid and cool the mixture. The formation of an ether-insoluble white precipitate in about 10 minutes is a second positive identification.

4.1.2 Congealing range

When tested according to USP general test <651>, melted benzoic acid should solidify in the range of 121 - 123°C.

4.1.3 Water Content

The water content of benzoic acid is tested according to USP general test <921>, Method I, using a 1 in 2 solution of methanol in pyridine as the solvent. Not more than 0.7% water can be present.

4.1.4 Readily Carbonizable Substances

When tested according to USP general test <271>, the resulting solution can have no more color than Matching Fluid Q.

4.1.5 Residue on Ignition

When tested according to USP general test <281>, the tested sample cannot yield more than 0.05%.

4.1.6 Arsenic

The arsenic content of benzoic acid is determined using USP general test <211>, Method II, and approved substances cannot contain more than 3 ppm.

4.1.7 Heavy Metals

The heavy metal content of benzoic acid is determined using USP general test <231>. In this procedure, 2.0 g of solid is dissolved in 25 mL of acetone, 2 mL of water is added, and followed by 10 mL of hydrogen

sulfide TS. Any color produced cannot be not darker than that of a control made with 25 m of acetone, 2.0 mL of the standard lead solution, and 10 mL of hydrogen sulfide TS.

4.1.8 Readily Oxidizable Substances

Any oxidizable materials present in a sample of benzoic acid are by the following procedure. A reagent is prepared by adding 1.5 mL of sulfuric acid to 100 mL of water, heating to boiling, and then adding 0.1 N potassium permanganate until the pink color persists for 30 seconds. 1.0 g of benzoic acid is dissolved in the hot solution, and titrated with 0.1 N potassium permanganate VS to a pink color that persists for 15 seconds. USP quality material requires that not more than 0.50 mL of permanganate reagent can be consumed.

4.1.9 Assay

Benzoic acid is assayed by titration with 0.1 N sodium hydroxide VS to a pink phenolphthalein endpoint. The procedure calls for the dissolution of about 500 mg of accurately weighed sample in 25 mL of diluted alcohol (previously neutralized with 0.1 N sodium hydroxide). Each milliliter of 0.1 N sodium hydroxide is equivalent to 12.21 mg of benzoic acid.

4.2 Colorimetric Identification Test Methods

4.2.1 Methods of the United States and Indonesian Pharmacopoeias

Both the of Indonesian Pharmacopoeia IV [9] and the United States Pharmacopoeia 23 [11] use the reaction with ferric chloride TS described in section 4.1.1 as an identity test.

4.2.2 Method of the British Pharmacopoeia

The British Pharmacopoeia 1993 calls for the use of two identification tests [12].

4.2.2.1 Reaction with Ferric Chloride

One milliliter of a 0.05 % w/v solution of benzoic acid is added to a 0.5 mL solution of ferric chloride, and a dull yellow precipitate is produced that is soluble in ether.

4.2.2.2 Sublimation Reaction

0.2 g of the substance being examined is moistened with 0.2 to 0.3 mL of sulfuric acid, and the test-tube containing these ingredients is gently warmed. A white sublimate deposit is formed on the inner wall of the tube.

4.2.3 Method of the Association of Official Analytical Chemists

An identification test has been established by the AOAC, 16th edition [14]. The solution is made alkaline with few drops of NH_4OH, excess ammonia is expelled, and the residue dissolved in few mL of hot water. The solution may be filtered if necessary. At that point a few drops of aqueous 0.5 % $FeCl_3$ solution is added, and a salmon color precipitate will form.

4.3 Elemental Analysis

The elemental analysis of benzoic acid should yield the following values [2]:

Carbon	68.84%
Hydrogen	4.95%
Oxygen	26.20%

4.4 Titrimetric Analysis

4.4.1 Methods of the United States and Indonesian Pharmacopoeias

Both the Indonesian Pharmacopoeia 1995 [9] and the United States Pharmacopoeia 23 [11] follow the same procedure. About 500 mg of benzoic acid (accurately weight) is dissolved in 25 mL of dilute alcohol that previously has been neutralized with 0.1 N NaOH, phenolphthalein TS is added, and that solution titrated with 0.1 N NaOH to a pink endpoint.

4.4.2 Method of the Europaeisches Arzneibuch

In the procedure of the Europaeisches Arzneibuch [16], 0.2 g of benzoic acid is dissolved in 20 mL of 96% ethanol, and titrated with 0.1 M NaOH. Using phenol red solution as the indicator, the endpoint is indicated when the color changes from yellow to violet.

4.4.3 Method of the Indian Pharmacopoeia

When following the procedure established by the Indian Pharmacopoeia 1996 [13], about 0.1 g of analyte is accurately weighed and dissolved in 15 mL of warm 95% ethanol (previously neutralized to phenolphthalein solution). One adds 20 mL of water and titrates with 0.5 M NaOH using phenolphthalein solution as the indicator. Each milliliter of 0.5 M NaOH titrant is equivalent to 0.06106 g of benzoic acid.

4.4.4. Determination of Benzoic Acid in the Presence of Salicylic Acid

The British P 1993 [15] describes a procedure to determine benzoic acid in benzoic acid ointment that also contains salicylic acid. In this method, 2 g of the ointment is added to 150 mL water, and warmed until melted. The solution is titrated with 0.1 M NaOH, using phenolphthalein solution as the indicator. Salicylic acid is assayed by a spectrophotometric procedure, and its content subtracted from the total acid value to obtain the benzoic acid content.

4.5 Spectrophotometric Methods of Analysis

The AOAC 16th edition describes a difference spectrophotometric method for the analysis of benzoic acid in various food preparations, such as jam, jellies, soft drinks, catsup, beverages, and fruit juices [14]. Before measuring any absorbance values, the sample is extracted four times with ether, and the ether extract purified by washing with solutions of HCl (1 + 1000) and 0.1 % of NH_4OH. The maximum absorbance (at ~272 nm) was subtracted by the average of the baseline absorbencies at ~267.5 and 276.5 nm.

4.6 Chromatographic Methods of Analysis

4.6.1 *Thin Layer Chromatography*

A large number of methods have been reported in the literature for the TLC separation of benzoic acid and various substances. The most important of these are presented in Table 7.

4.6.2 Gas Chromatography

The 16th edition of the AOAC handbook describes a gas chromatographic method for determining benzoic acid in food preparations [14]. After using ether to extract the analyte from the preparations, and partitioning with aqueous NaOH and CH_2Cl_2, the acid is converted to its tetramethyl-silane ester before being injected onto the chromatography system. A 1.8 m x 2 mm glass column with 3 % OV-1 ion 100-120 mesh Veraport 30 is used to effect the analytical separation. The operating temperatures are as follows: oven at 80 to 210°C, heating rate 8°C/min., injection port at 200°C, and the FID detector at 280°C. Nitrogen is used as the carrier gas at a flow rate of 20 mL/min.

Clark described another gas chromatographic method, which used a 2 m x 4 mm glass column with 2.5% SE-30 on 100 mesh Chromosorb G 80 [4]. An oven temperature of about 118°C and a nitrogen carrier flow rate of 45 mL/min. were used.

The Hewlett Packard company has mentioned a capillary column (HP-INNOWax, 30 m x 250 mm x 0.25 μm) that can be operated at an oven temperature of 80°C (1 min.) ramped to 250°C (2 min.) at 5°C/min. for the separation of 20 substances (including benzoic acid) in cologne [30]. A HP-5871 mass selective detector was used.

Supelco Inc. has reported the use of a capillary column (PTE-5, 30 m x 0.32 mm; 1 μm film) for the analysis of 25 semi-volatile acids, including benzoic acid [31]). The reported operating conditions were: oven at 35°C (1 min.) ramped to 320°C at 10°C/min., hold for 15 min., helium carrier gas at 35 mL/min, and a MS detector.

Table 7

Thin-Layer Chromatographic Methods for the Separation of Benzoic Acid and Other Substances

Layer	Mobile Phase	Visualization	Analyte	Reference
Silica gel	$CHCl_3$/EtOH (9/1)	UV 366 nm	Benzoic acid, Salicylic acid, Papaverine HCl	17
Silica gel	Petrol ether / acetic acid (9/1)	UV 223 and 295 nm	Benzoic acid, Salicylic acid	18
Silica gel	Toluene / acetone (40/1)		Benzoic acid, Sorbic acid, Salicylic acid	19
Silica gel	Toluene / EtOAc / NH_3	Fluorescence	Benzoic acid, Sorbic acid, Propionic acid	20
Poly-acrylamide	BuOH / NH_3/ EtOH (7/1/2)	0.04 % bromcresol purple	Benzoic acid, Sorbic acid	21
Silica gel	Hexane / ether / acetic acid (81/5/4)	Bromphenol blue	Benzoic acid, Sorbic acid, Salicylic acid	22
Silica gel	$CHCl_3$ / Acetic acid (90/3)		Benzoic acid, Parabens	23
Silica gel	Petrol ether / EtOAc / Acetic acid (20/1/1)	UV 230 nm	Benzoic acid	24
Silica gel / Kieselguhr (1/1)	$CHCl_3$	UV and Iodine	Benzoic acid	25
RP – 18	MeOH and 0.5M NaCl	UV 254 nm	Benzoic acid, Sorbic acid	26

Table 7 (cont.)

TLC Methods for Benzoic Acid

Layer	Mobile Phase	Visualization	Analyte	Reference
Silica gel	Hexane / $CHCl_3$/MeOH/ Diethyl amine (42/21/13/5)	UV 220 nm	Benzoic acid, Miconazole Nitrate	27
Silica gel	Petrol ether / dibutyl ether / formic acid / acetic acid (80/35/ 3/3)	Uv 366 NM	Benzoic acid, Sorbic acid, Salicylic acid	28
HPTLC NH_2, F_{254}	EtOH / NH_3 pH = 12 (6/4)	UV 254 nm	Benzoic acid, Pthalic acid, Benzene carboxylic acid	29
HPTLC RP-8, F_{254}	MeOH / H_2O (6/4), + 0.1 M $(C_2H_5)_4NBr$	UV 254 nm	Benzoic acid, Pthalic acid, Isopthalic acid	29
RP-8, F_{254}	Acetonitrile / H_2O (6/4)	UV 254 nm	Benzoic acid, Benzoin, Ethyl benzoate	29
RP-8, F_{254}	Acetone/ H_2O (4/6) + 0.005M $(C_3H_7)_4NBr$	UV 254 nm	Benzoic acid, Pthalic acid, ASA,2 Methyl benzoic	29
Silica gel GF254	Toluene / glacial acetic acid (80/20)	UV 254 nm	Benzoic acid, Salicylic acid	15
Silica gel	30/30/15/15/10 $CHCl_3$/acetone/ IPA /MeOH / NH_4OH	UV 254 nm	Benzoic acid, Salicylic acid	11

4.6.3 High Performance Liquid Chromatography

For the analysis and separation of benzoic acid, caffeine, aspartame, and saccharin in dietetic soft drinks, a HPLC system consisting of a Varian MCH-5N-CAP 150 x 4.6 mm column and a variable wave length UV/VIS detector was recommended [32]. The mobile phase is a gradient, beginning with 90% 0.01 M KH_2PO_4 (pH = 2) and methanol, and ending in 25 minutes with 60 % buffer / 40 % methanol.

Burton *et al.* reported the assay of benzoic acid, ethyl benzoate, and benzoyl peroxide in dermatological gels and lotions [33]. These workers used a Waters μ-Bondapak C_{18} column and UV detection at 254-nm. A mixture of 50% aqueous acetonitrile was used as the mobile phase.

Other benzoic acid derivatives (*i.e.*, nitrobenzoic acids, chlorobenzoic acids, and fluorobenzoic acids) were analyzed using Zorbax-C8, Zorbax-Phenyl, and Zorbax-CN columns. A mobile phase of 75% 25 mM sodium citrate (pH 2.5) and 25% methanol was used for the Zorbax-C8 method, while 65% 25 mM sodium citrate and 35% methanol were used for the Zorbax-Phenyl and Zorbax-CN methods. Detection was performed on the basis of the UV absorbance at 290 nm.

A HPLC system that separates various preservatives was described by Shodex [35]. The system consists of a Shodex Rspack DS-613 column, and a Shodex UV detector. A mobile phase mixture of 0.05 M K_2HPO_4 / 0.1 % H_3PO_4 / 40 % acetonitrile at a flow rate of 1 mL/min. was used, and UV detection at 210 nm was used. The method was used for the separation of benzoic acid, methylparaben, ethylparaben, propylparaben, saccharin, sorbic acids, and *p*-hydroxybenzoic acid.

An inter-laboratory study to determine benzoic acid in orange juices was reported, where a Hamilton PRP-1 column and a mobile phase mixture of 4 + 6 of acetonitrile / phosphate buffer was used [36]. In this work, the juices were fortified with 0.5-10 ppm of benzoic acid. Statistical analysis of the results showed a relative reproducibility between laboratories ranged in the range of 6.92% to 15.97% for juice fortified with 1-10 ppm and 27.90% for juice fortified with 0.5 ppm. Mean recoveries ranged from 94.5% to 114%.

Other methods for the HPLC analysis of benzoic acid, as provided by various column manufactures, are presented in Table 8.

4.6.4 Column Chromatography

Nelson reported the separation of benzoic acid, ethyl *p*-aminobenzoate, and benzoin using ion-exchange chromatography [41]. In this method, the analyte solution was passed over two sets of SPE tubes, one loaded with weak anion exchange resin in the neutral form, and the other loaded with a strong cation exchange resin in the protonated form. The weak anion exchange resin retained the acid, the cation exchange resin retained the amine, while the neutral compound passed through to the receiver. The acid was then eluted with a 1/1 mixture of EtOH/1 N HCl, while the amine was recovered by using a 1/1 mixture of EtOH /20 % NH_4OH. After separation, each compound could be subjected to a quantitative analysis.

The United States Pharmacopoeia 23 [11] and Indonesian Pharmacopoeia IV [9] describe the assay of benzoic acid and salicylic acid in ointments. Two chromatographic columns (20 x 2.5 cm) are used to effect the separation. One transfers a mixture of 1 g siliceous earth and 0.5 mL diluted phosphoric acid (3 in 10) to the first column (A), then packs above this a mixture of 4 g siliceous earth and ferric chloride-urea reagent. A mixture of 4 g siliceous earth and 2mL of sodium bicarbonate solution (1 in 12) is packed into the second column (B). For analysis, column A is mounted directly above column B. The sample solution is inserted onto column A, allowed to pass into the column, and then washed with 2-40 mL of chloroform. Benzoic acid can be eluted from column B by using a 3 in 100 solution of glacial acetic acid in chloroform. The benzoic acid content then can be determined by a spectrophotometric method such as that described earlier (section 4.5).

4.7 Determination in Body Fluids and Tissues

A GLC method for the determination of benzoic acid to concentrations as little as 10 ng/mL in plasma and urine was described by Sioufi and Pommier [42]. After addition of an internal standard (3-phenylpropionic acid), benzoic acid was extracted at acidic pH into diethyl ether, whereupon both compounds were derivatized with pentafluorobenzyl

Table 8

High-Performance Liquid Chromatographic Methods for the Separation of Benzoic Acid and Other Substances

Column	Detection	Mobile phase	Analytes	Reference
LiChrosorb Si 60	UV 235 nm	*n*-heptane/ diisopropyl ether/ glacial acetic acid (85/15/0.1,v/v)	Benzoic acid, Sorbic acid	29
LiChrosorb RP-8	UV 235 nm	MeOH / 0.01 M CH_3OONH_4 (60/30, v/v)	Benzoic acid, Sorbic acid	29
LiChrosorb RP-8	UV 254 nm	MeOH/TBA 1 g/L, gradient	Aminobenzoic, Benzoic, Salicylic, aminosalicylic acids	29
LiChrosorb RP-8	UV 280 nm	MeOH/10 mM Phosphate buffer (40/60)	Benzoic acid, Pyrogallol, Trihydroxy - benzophenone	37
LiChrosphr 100 DIOL	UV 254 nm	2,2,4-trimethyl pentane/1,4 dioxane (50/50)	Benzoic and gallic acids; Dodecyl-, Propyl-, Ethyl- ,Methylgallate	37
LiChrosorb RP-8	UV 254 nm	MeOH / acetic acid /H_2O (5/3/2)	Benzoic acid, Caffeine, Saccharin	37
LiChrosorb RP-8	UV-254 nm	MeOH/33 mM Phosphate buffer (25/75)	Benzoic acid, Caffeine, Aspartame	37
LiChrosorb RP Select B	UV 240 nm	MeOH/NaH_2PO_4 /Na_2HPO_4 (10/95/95)	Benzoic acid, Sorbic acid, Saccharin	37

Table 8 (cont.)

HPLC Methods for the Separation of Benzoic Acid

Column	Detection	Mobile phase	Analytes	Reference
Supelcosil LC-18	UV 254 nm	Acetonitrile/ 0.02 M sodium acetate (2/8)	Benzoic acid, Caffeine, Sorbic acid	31
Supelcosil LC-ABZ	UV 210 nm	Acetonitrile / 25 mM KH_2PO_4 (25/75); pH 2	Benzoic acid, Sorbic acid	31
Shim-pack CLC-ODS	UV 225 nm	10 mM sodium phosphate (pH 6) / acetonitrile (20/1)	Benzoic acid, Sorbic acid	38
Aminex HPX-87C	UV 210 nm	15 % acetonitrile in 0.01M H_2SO_4, containing 1 mM $CaSO_4$	Benzoic acid, Citric acid, Malic acid, Fumaric acid, etc.	39
Fast acid Analysis column	UV 233 nm	15% acetonitrile in 0.005 H_2SO_4	Benzoic acid, Sorbic acid	39
Aminex HPX-87H	UV 210 nm and RID	0.002 N H_2SO_4	Benzoic acid, Citric acid, Gluconic acid	39
Bio-Gel PRP 70-8	UV 254 nm	30 % acetonitrile in 0.02 M Sodium acetate	Benzoic acid, Saccharin, Caffeine, Aspartame	39
Capsel PAC C-18	UV 228 nm	MeOH/5 mM Phosphate Buffer, pH 2 (2/8)	Benzoic acid, hippurate in Serum	40

bromide. The derivatives were determined by GC using a Hewlett Packard model 18713A Ni electron capture detector. The stationary phase was 3% OV-17 on 100-120 mesh Gas-Chrom Q, operated at 148°C. The injector and detector temperature were maintained at 250°C and 300°C, respectively. A mixture of 9/1 argon/methane (at a flow rate of 60 mL/min) was used as the carrier gas.

Yamada *et al.* reported the analysis of serum benzoate and hippurate in normal human and patients with chronic liver disease by using a HPLC method [40]. The conditions for the analysis are given in Table 8.

Akira *et al.* reported the determination of deuterated benzoic acid in human urine by using high-field deuterium NMR [43]. The observed magnitudes of the ^{13}C and ^{2}H-2,6 resonances were used to calculate the quantities of 7-^{13}C and 2,6-^{2}H benzoic acid present in the samples. The lower limit of this method was approximately 60 nmol/mL.

5. Stability

5.1 Solid-State Stability

Benzoic acid has been found to be stable at normal temperature and pressure conditions, and hazardous polymerization will not occur under normal condition [44-46].

When benzoic acid is heated above its melting point in a sealed container, some formation of benzoic anhydride and water takes place [8]. When the acid is heated to 370°C, it is irreversibly decomposed to benzene and carbon dioxide, and a small portion (2-8%) decomposes into phenol and carbon monoxide. Copper and cadmium powder increase the reaction rate by factors of approximately 9-fold and 200-fold, respectively.

5.2 Solution-Phase Stability

Aqueous solutions of benzoic acid react with metals, and can generate hydrogen gas [46]. When a solution consisting of 1 mole of benzoic acid in 8 moles of water is heated in autoclave, a slight decomposition with gas

formation is noted at 200°C. Marked formation of benzene and carbon dioxide takes place within the range of 300 – 350°C [8].

5.3 Incompatibilities with Functional Groups

Benzoic acid is incompatible with strong bases, strong oxidizing agents, and strong reducing agents. Hazardous combustion or decomposition products resulting from these interactions are toxic fumes of carbon dioxide and carbon monoxide [45]. Benzoic acid must be keep away from heat, flame, ignition sources, and moisture [44].

In aqueous solution, sodium benzoate forms a 1:1 complex with xanthine derivatives such as theophylline and caffeine [47].

6. Drug metabolism, Pharmacology, and Pharmacokinetics

Benzoic acid is metabolized in the liver by conjugation with glycine, and is rapidly excreted in the urine as hippuric acid in most of animal species. It can also be excreted as benzoilglucoronic acid when ingested in a large dose. Benzoic acid can be found in urine as a metabolite of benzaldehyde and numerous other compounds [4, 48].

After oral administration of 7-^{13}C-2,6-^{2}H-benzoic acid in healthy human subject (4.2 mg/kg), the drug was found to be quantitatively biotransformed to hippuric acid and excreted in urine within 4 hours [43].

Rougier *et al.* reported that the percutaneous penetration of benzoic acid in human skin depended on the anatomical location of the skin. The rank order in skin permeability of benzoic acid appears to be arm < abdomen < postauricular < forehead [49].

Downard *et al.* reported that benzoic acid could induce cutaneous vasodilatation after topical application caused by the local formation of prostaglandin D_2 in the skin [50]. Benzoic acid can be clinically used to treat hyperammonemia caused by a genetic defect in urea metabolism [48].

Yamada *et al.* suggested that benzoate-metabolizing capacity, especially as indicated by the mean transit time value of the serum benzoate, appears to be a better index than the indocyanine green clearance rate for determining hepatic functional reserve in chronic lever disease [40].

Benzoic acid was found to be more sensitive to acidity changes than was anticipated from the Henderson-Hasselbach equation relating dissociated and non-dissociated fractions at pH 3 and pH 4 [51]. However, the sensitivity of preservative activity to dilution at both pH values remained similar.

7. References

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BRINZOLAMIDE

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(1) Analytical Chemistry

(2) Research Support/Pharmaceutics

(3) Research/Discovery Synthesis

(4) Pharmacokinetics/Drug Metabolism

(5) Toxicology

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Name

(*R*)-4-(Ethylamino)-3,4-dihydro-2-(3-methoxypropyl)-2*H*-thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide-1,1-dioxide

(*R*)-(+)-4-Ethylamino-2-(3-methoxypropyl)-3,4-dihydro-2*H*-thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide-1,1-dioxide

(*R*)-(+)-4-Ethylamino-2,3-dihydro-2-(3-methoxypropyl)-4*H*-thieno[3,2-*e*]thiazine-6-sulfonamide-1,1-dioxide

1.1.2 Nonproprietary Names [1, 2]

Brinzolamide (USAN, INN)

1.1.3 Proprietary Names

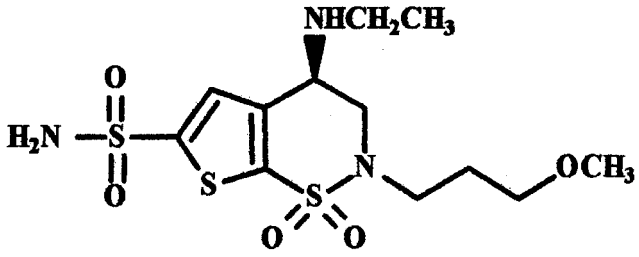
Azopt™ (Alcon)

1.2 Formulae

1.2.1 Empirical

$C_{12}H_{21}N_3O_5S_3$

1.2.2 Structural



1.3 Molecular Weight

383.51

1.4 CAS Number

138890-62-7

1.5 Appearance

White to off-white crystals or powder.

1.6 Uses and Applications

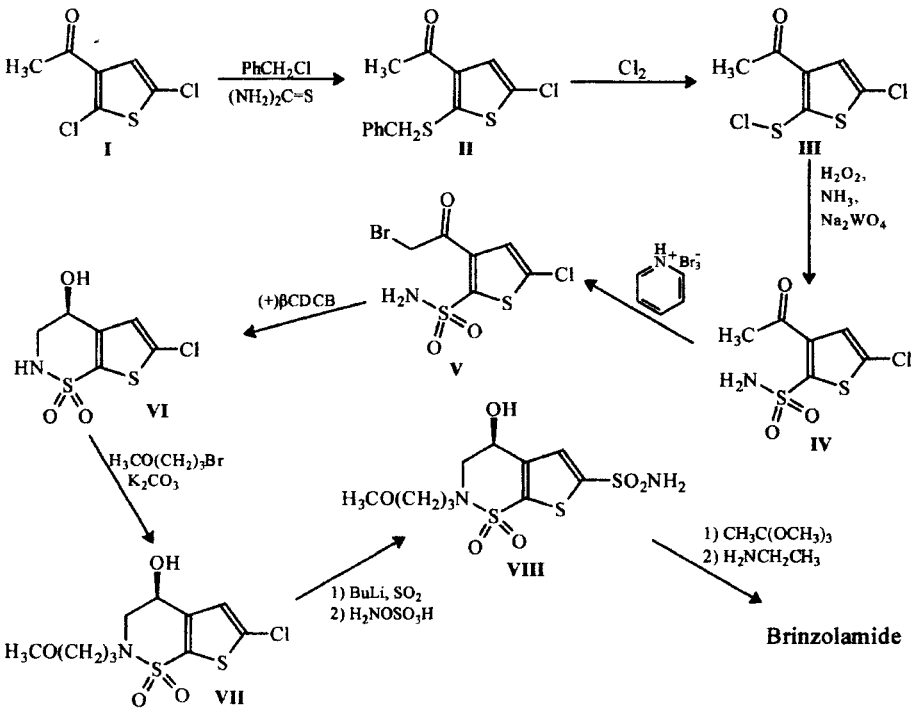
Azopt™ (brinzolamide ophthalmic suspension) 1% is a topical carbonic anhydrase inhibitor (CAI) indicated for the treatment of elevated intraocular pressure in patients with ocular hypertension or open-angle glaucoma [3-6]. In double-masked, controlled clinical trials, Azopt therapy was demonstrated to be safe, well-tolerated. In addition, it resulted in significant reductions in intraocular pressure, which were equivalent to Trusopt® (dorzolamide) 2% ophthalmic solution, the only other topical CAI on the market [3]. Formulated at a pH equivalent to that of human tears, Azopt is more comfortable than Trusopt, a benefit that may enhance patient compliance. In two clinical studies designed to evaluate ocular comfort, four times the number of glaucoma patients experienced burning and stinging with Trusopt than with Azopt [3, 4].

2. Method of Preparation

Several synthesis patents are reported in the literature for brinzolamide [7-10]. The process reported in references 8-10 represents an improvement over earlier processes and a schematic of this synthesis process is shown in Scheme 1. In this scheme, brinzolamide is produced in 8 steps starting from dichloroacetyl-thiophene(**I**). Chirality is introduced by enantioselective reduction of a bromoketone (VI) using a chiral reagent (+)-β-chlorodi-

Scheme 1

Synthesis of Brinzolamide



isopino-campheylborane((+)-CDPB)). This results in alcohols VII and IX, which have (*S*)-chirality. In the final step, the alcohol group is replaced by ethylamine with concomitant inversion of chirality.

3. Physical Properties

3.1 Crystallographic Properties

3.1.1 Single Crystal Structure

Unit cell parameters were obtained from a study conducted on a single crystal of the material. The crystal class was monoclinic within the $P2_1$ or $P2_1/m$ space groups. The unit cell was characterized by the following lattice parameters: $a = 9.686(2)$ Å, $b = 8.792(4)$ Å, $c = 10.085(6)$ Å, $\beta = 92.33(4)°$.

3.1.2 Polymorphism

Only one polymorphic form of the substance has been observed. A study was undertaken to attempt to produce different forms by flash cooling and slow evaporation from various solvents (*N,N*-dimethylformamide, acetonitrile, ethanol, dichloromethane, 2-propanol, water (neutral pH, pH 5, and pH 9), ethanol/water mixtures (1:1, neutral and at pH 5 and 9). No additional forms were observed upon examination of the resulting crystals by x-ray powder diffraction.

3.1.3 X-Ray Powder Diffraction Pattern

Diffraction data was obtained on a Philips diffractometer with a graphite diffraction beam monochromator using copper Kα radiation (1.54 Å). The crystallographic data (scattering angles, d-spacings, relative intensities and Miller Indices) are listed in Table 1, and the powder pattern is shown in Figure 1. The sample was ground to minimize preferred orientation effects.

3.1.4 X-ray Diffraction Study of Chiral Configuration

The absolute configuration of brinzolamide was confirmed by a crystallographic study on the di-*p*-toluoyl-*L*-tartaric salt. The diffraction data were collected with a Siemens P3 diffractometer, equipped with a Nicolet LT-2 low temperature (-85°C) device, using monochromatic Mo Kα radiation (0.71073 Å). The study confirmed that brinzolamide has the (*R*)-

Figure 1

X-Ray Powder Diffraction Pattern of Brinzolamide (Ground to Minimize Preferred Orientation Effects)

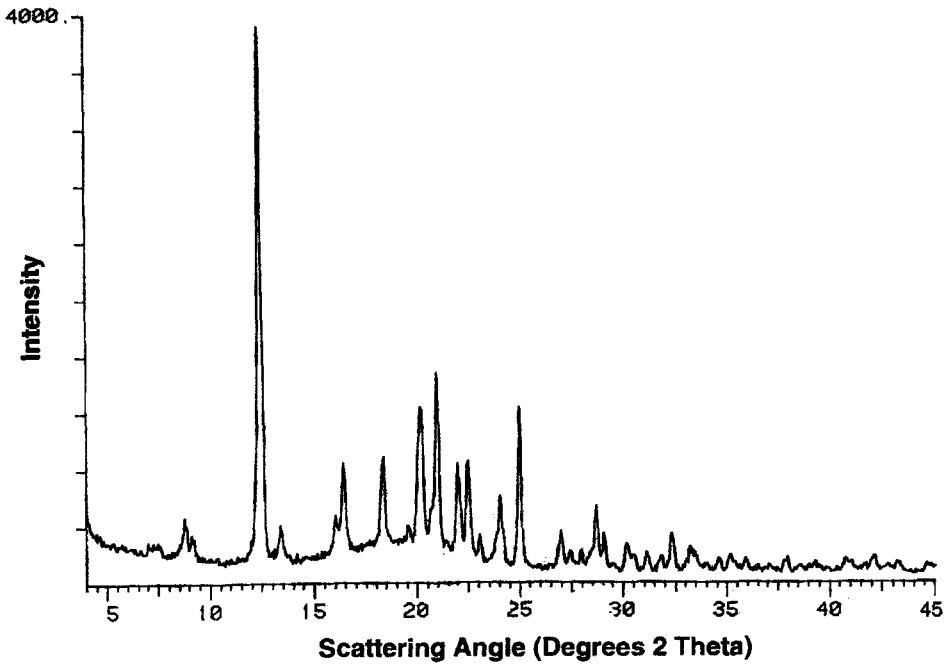


Table 1

X-Ray Powder Diffraction Data for Brinzolamide

Scatter Angle 2θ (deg.)	D Spacing (Å)	Relative Intensity (%)	Miller Indices		
			h	k	l
8.771	10.073	7.0	0	0	1
9.112	9.6971	4.5	1	0	0
12.422	7.1198	100.0	-1	0	1
13.371	6.6166	6.3	0	1	1
16.020	5.5279	7.2	-1	1	1
16.412	5.3965	16.8	1	1	1
18.329	4.8364	18.5	2	0	0
19.541	4.5391	6.8	-1	0	2
20.168	4.425	27.7	-2 1 0 0	0 0 2 1	1 2 0 2
~20.7 (not resolved)	4.29	~ 8	2	0	1
20.971	4.2327	34.4	2	1	0
22.011	4.0349	17.5	1 0 -1	2 2 1	0 1 2
22.484	3.9511	19.0	1 -2	1 1	2 1
23.045	3.8562	5.4	2	1	1
~23.8 (not resolved)	3.74	~ 5	-1	2	1
24.055	3.6964	12.4	1	2	1
24.980	3.5617	29.9	-2	0	2
~26.7 (not resolved)	3.33	~5	0	0	3
26.971	3.303	6.6	0 -2	2 1	2 2
28.715	3.1063	11.5	1 -2 -3	2 2 0	2 1 1
29.070	3.0692	6.7	2	2	1
30.190	2.9578	4.9	1	1	3
32.340	2.7659	6.9	-3 -2	0 2	2 2

configuration. The molecular conformation of brinzolamide as deduced from the x-ray study is shown in Figure 2.

3.1.5 X-ray Diffraction Study of Drug –Isozyme Binding

The molecular basis for the high affinity binding of brinzolamide to carbonic anhydrase II isozyme was studied using x-ray crystal diffraction [11]. Primary interaction occurs between the deprotonated sulfonamide nitrogen of brinzolamide and a zinc site on the isozyme, with several other features of the drug being important in fine-tuning the tightness of binding. An x-ray study has also been done to investigate the factors contributing to the nearly ten-fold selectivity of brinzolamide for carbonic anhydrase II relative to carbonic anhydrase IV isozyme [12].

3.2 Optical Activity

3.2.1 Optical Rotation

Brinzolamide has a positive optical rotation at the sodium D line (589.3 nm), defining this enantiomer as the (+)-isomer. The specific rotation (α_D, c=1.00, water) of the hydrochloride salt is +10.35° [7]. The optical rotation of brinzolamide, at the most sensitive wavelength examined (312.6 nm), was found to be -26.1° (1% solution in citrate buffer (pH 3, 0.2 M) at 25°C) [9].

3.3 Thermal Methods of analysis

3.3.1 Melting Behavior

The melting range of brinzolamide has been reported to be 125-127°C [9], although it has been found to be somewhat difficult to obtain a definitive melting range in our laboratories.

The melting range of the hydrochloride salt is 175-177°C [7].

3.3.2 Differential Scanning Calorimetry

A melt endotherm was observed for brinzolamide with an onset of 121°C and a maximum of 131°C. The DSC thermogram is shown in Figure 3.

Figure 2

X-Ray Structure of Brinzolamide Showing *R* Configuration at C4

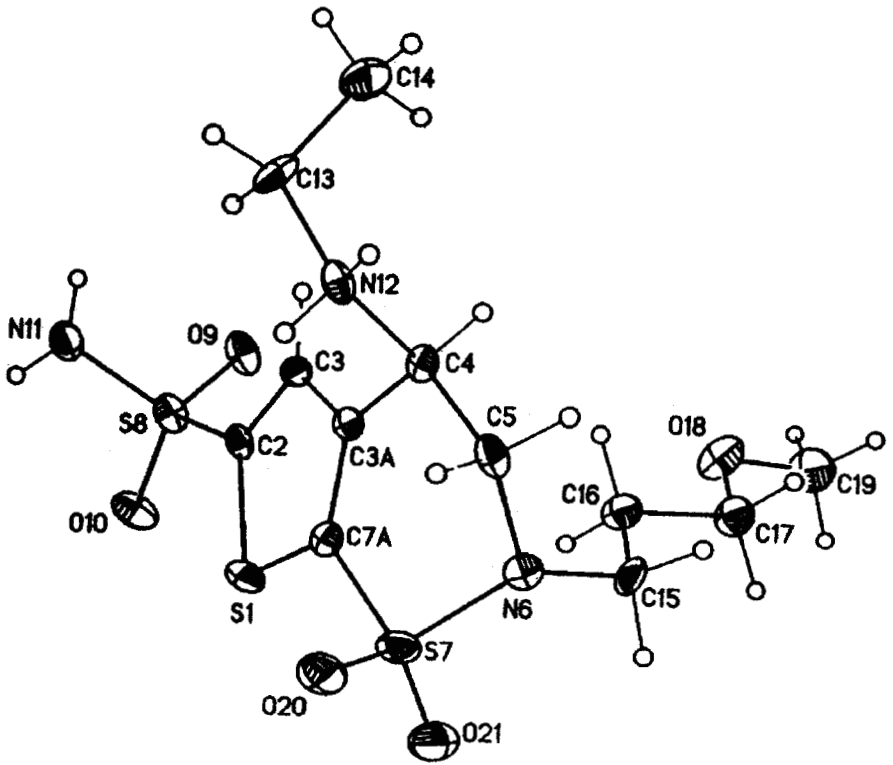


Table 2

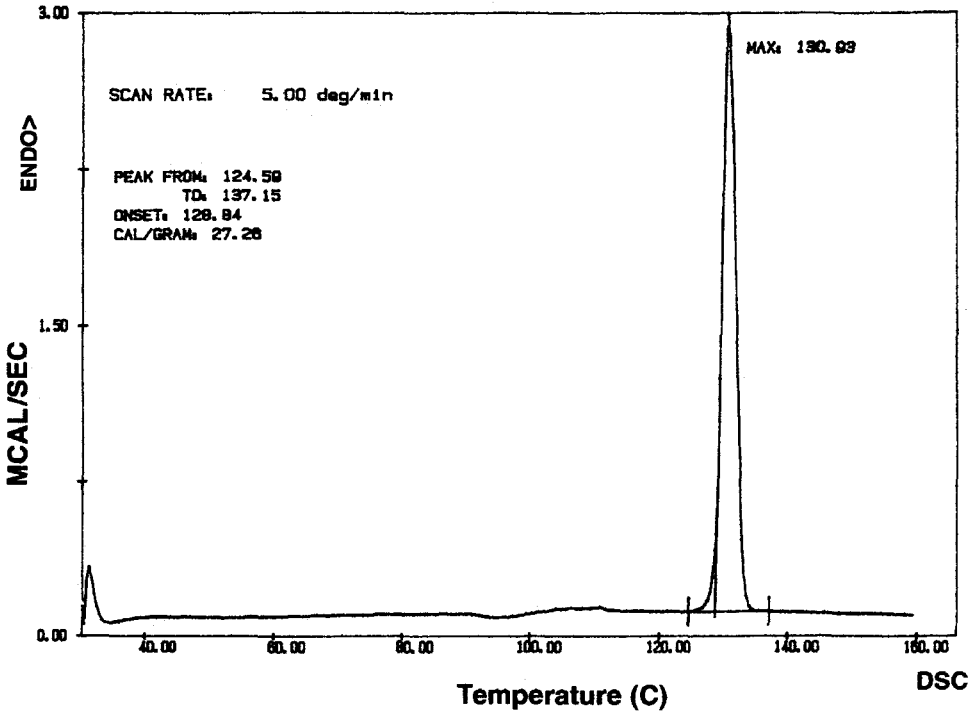
Solubility of Brinzolamide

Solvent	Solubility (mg/mL)
Methanol	> 20
Ethanol	8.0
2-Propanol	2.0
1-Octanol	0.50
Water	0.43
Water (pH 3.79)	63.8 30.4
Water (pH 5.10)	3.30
Water (pH 6.25)	0.59
Water (pH 7.37)	0.40
Water (pH 7.95)	0.47
Water (pH 9.03)	1.27
Water (pH 10.0)	12.30

The pH of water was adjusted with 1N NaOH and/or HCl

Figure 3

Differential Scanning Calorimetry Thermogram of Brinzolamide



3.3.3 Thermogravimetric Analysis

Brinzolamide is anhydrous and stable. Consequently, only thermally induced weight losses associated with residual moisture or solvent are observed until the region of thermal decomposition is reached (above 220°C). A representative thermogram is shown in Figure 4.

3.4 Hygroscopicity

Moisture uptake was not observed when brinzolamide was stored uncapped for 4 weeks in a 40°C / 75% relative humidity chamber. Thus the compound is essentially non-hygroscopic.

3.5 Solubility Characteristics

The aqueous solubility of brinzolamide is pH dependent, with minimal solubility existing at neutral pH and increased solubility at more acidic and basic pH values. In the alcohol series, maximal solubility is observed with methanol. Solubilities of brinzolamide observed in this laboratory are listed in Table 2.

3.6 Partition Coefficients

The partition coefficient of brinzolamide between 1-octanol and pH 5.0 phosphate buffer (0.1 M) was found to be 0.65. For a similar system where the buffer was at pH = 7.4, a value of 6.56 was obtained.

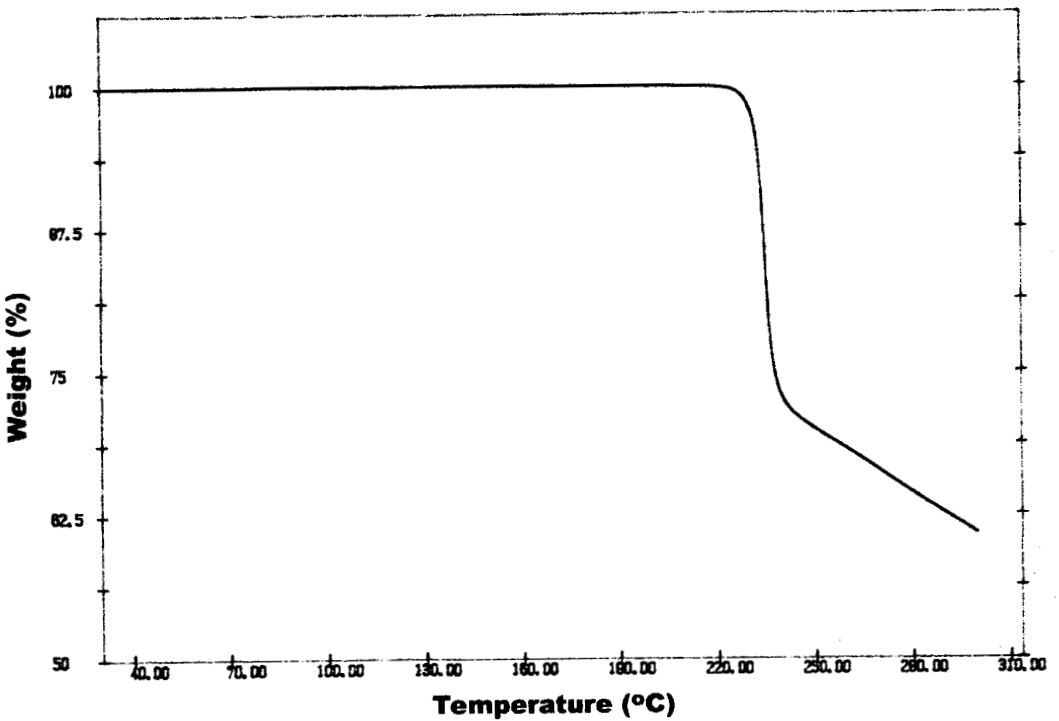
3.7 Ionization Constants

The dissociation constants of brinzolamide were determined by potentiometric titration of the hydrochloride salt with 0.05N NaOH, with two ionizations being observed. The first pKa, attributed to the deprotonation of secondary ammonium moiety, was observed at 5.88. The second pKa, attributed to deprotonation of the sulfonamide group, was at 8.48.

These constants are similar to those reported for the structurally similar carbonic anhydrase inhibitor, dorzolamide hydrochloride, (6.4 and 8.2,

Figure 4

Thermogravimetric Analysis of Brinzolamide (Heat Rate 10°C/minute)



respectively) [13], and explain the observed pH-solubility profile. Thus, brinzolamide exists as the relatively insoluble unionized species at neutral pH and is ionized at either higher or lower pH values, with a corresponding increase in aqueous solubility.

3.8 Spectroscopy

3.8.1 UV/VIS Spectroscopy

The ultraviolet spectrum of brinzolamide in reagent alcohol exhibited a maximum at 252 nm, with a molar absorptivity of 9,400 liters/mole. The spectrum is shown in Figure 5.

3.8.2 Infrared Spectroscopy

The infrared spectrum, for a sample prepared as a potassium bromide pellet, is highly characteristic of the compound. The spectrum is shown in Figure 6 and Table 3 lists the band assignments.

3.8.3 Nuclear Magnetic Resonance Spectrometry

NMR spectra were obtained using a Varian VXR-200 (200 MHz) spectrometer. In order to obtain unambiguous proton and carbon assignments, two different solvents (DMSO-d6 and $CDCl_3$) and several different NMR experiments (DEPT, COSY, HETCOR and long-range HETCOR) were required.

3.8.3.1 ^{1}H-NMR Spectrum

The proton, expanded proton and COSY (two-dimensional proton-proton correlation) spectra are shown in Figures 7, 8 and 9. The proton assignments are listed in Table 4. Due to the closely similar shifts of the protons on positions 9 (geminal), 11 and 12, it was difficult to determine the assignments. However, computer modeling of the assigned shifts, using second order spin simulation, produced a spectrum that closely matched the experimentally observed one.

Figure 5

Ultraviolet Spectrum of Brinzolamide (Alcohol)

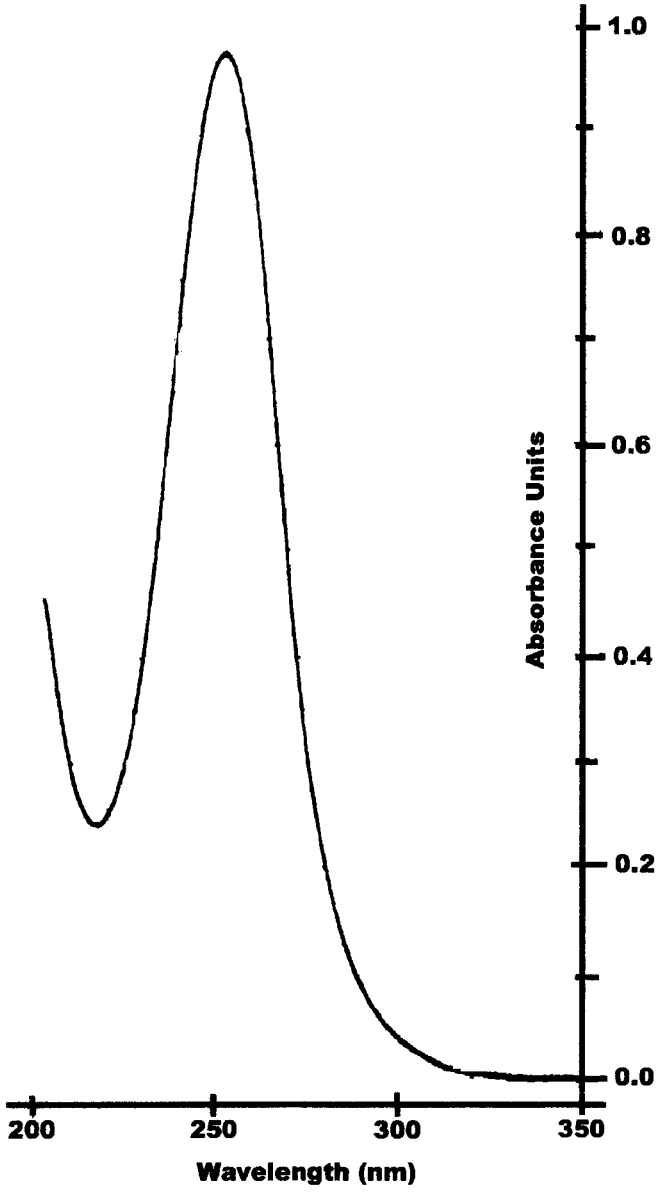


Figure 6

Infrared Spectrum of Brinzolamide (KBr Pellet)

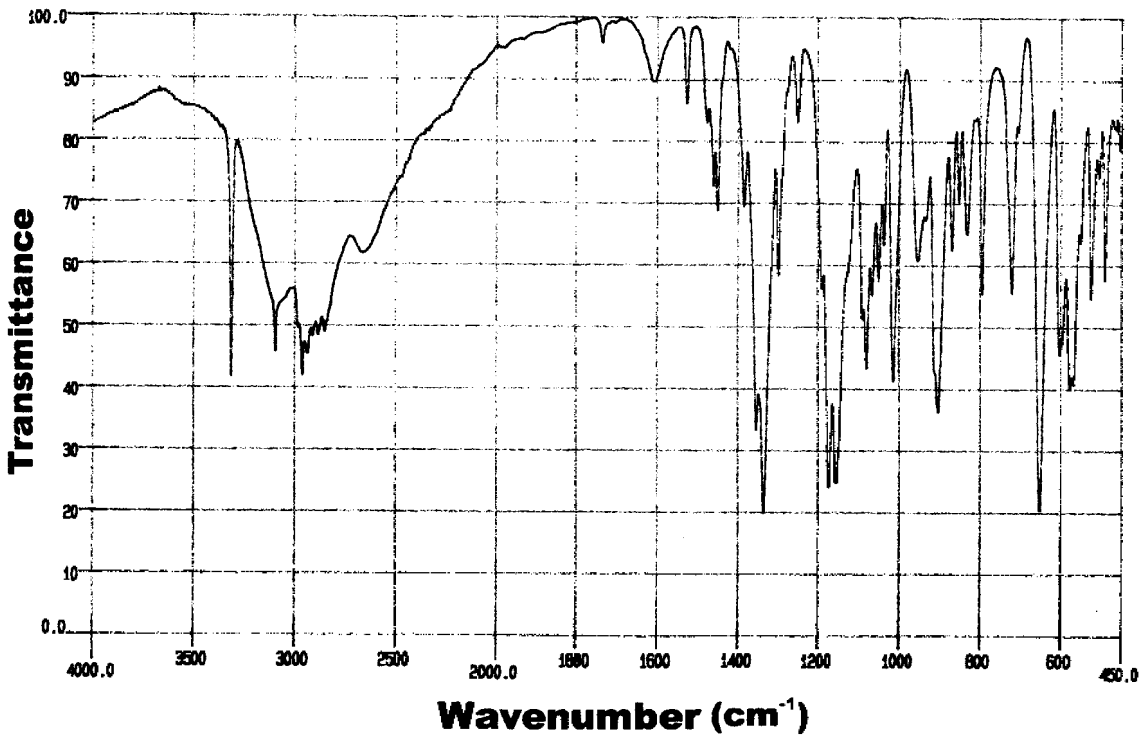


Table 3

Infrared Band Assignments for Brinzolamide

Band (cm^{-1})	**Assignment**
3313	N-H Stretch of sulfonamide
3095	Thiophene C-H Stretch
3000-2800	Aliphatic C-H stretch
1610	N-H deformation
1470-1440	CH_3 deformation
1400-1250	Ring Stretch
1355, 1338	Asymmetric stretch of O=S=O
1173, 1155	Symmetric stretch of O=S=O
904	S-N stretch of sulfonamide
651	Thiophene ring stretch

Figure 7

Proton NMR Spectrum of Brinzolamide (DMSO-d6)

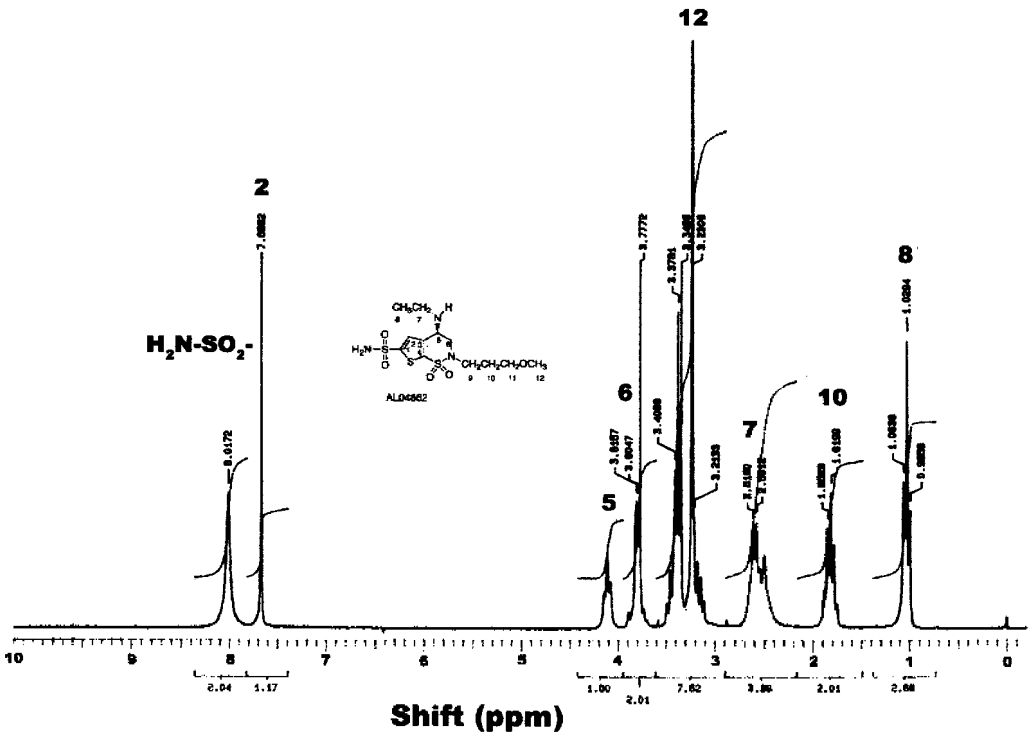


Figure 8

Expanded Proton NMR Spectrum of Brinzolamide (DMSO-d6)

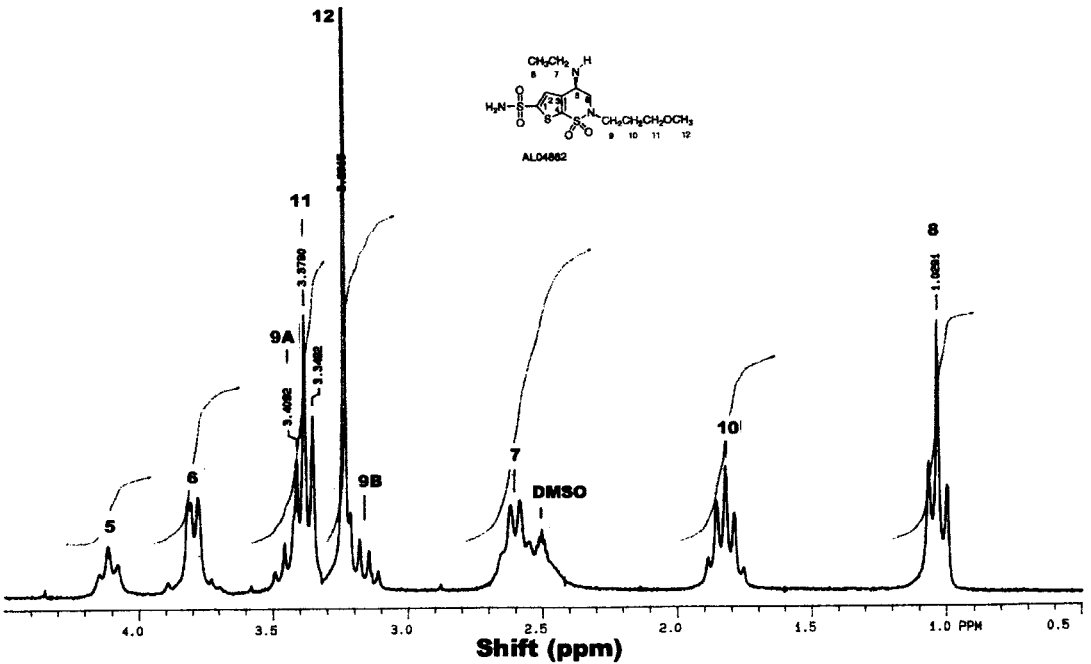


Figure 9

COSY NMR Spectrum of Brinzolamide (DMSO-d6)

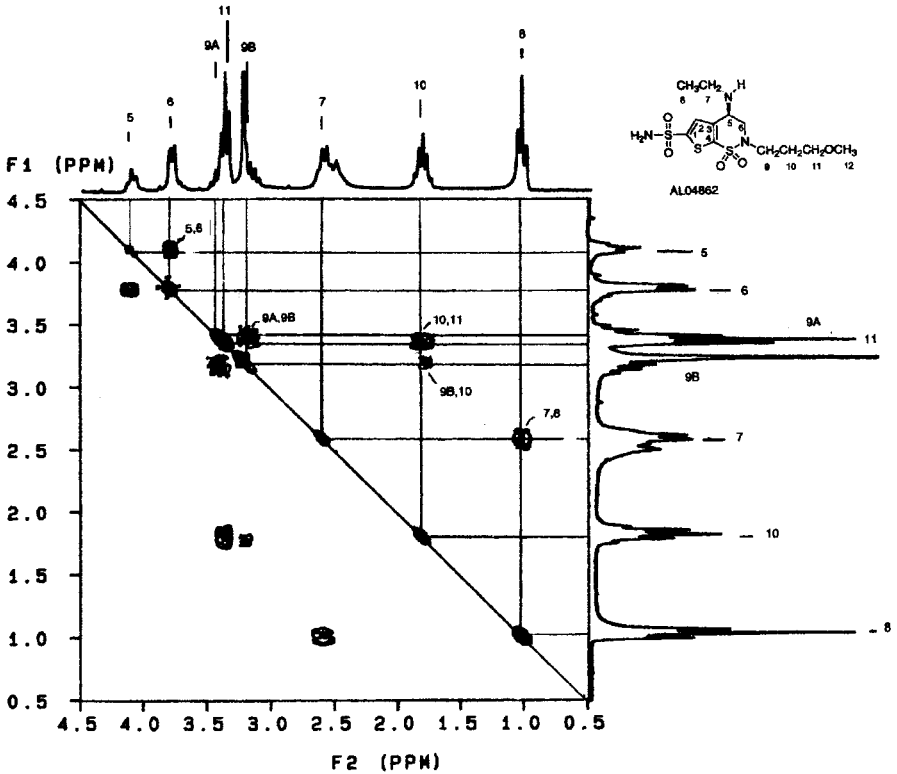
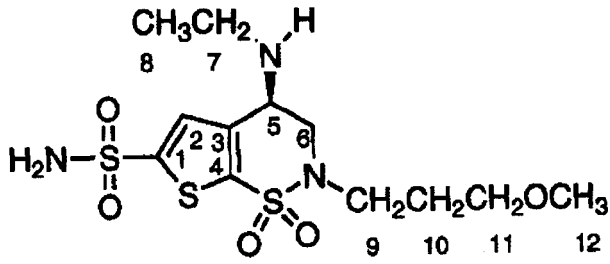


Table 4

Proton NMR Assignments for Brinzolamide

Shift (ppm)		Pattern	J Values, Hz (in $CDCl_3$)	Assignment
DMSO-d6	$CDCl_3$			
8.02	5.20	Singlet		$SO_2N\underline{H}_2$
7.67	7.62	Singlet		Proton on 2
------	7.26	------		$CDCl_3$
4.11	3.94	Triplet		Proton on 5
3.79	3.85	Multiplet		Protons on 6
3.44	3.55	Multiplet	$J_{9A, 9B} = 12.8$ $J_{9A, 10} = 9.4$	Proton A on 9
3.38	3.48	Triplet		Protons on 11
3.23	3.34	Singlet		Protons on 12
3.17	3.28	Multiplet	$J_{9B, 10} = 6.4$	Proton B on 9
2.60	2.77	Quartet	$J_{7, 8} = 7.1$	Protons on 7
2.50	------	----------		DMSO-d6
1.82	1.92	Multiplet	$J_{10, 11} = 6.4$	Protons on 10
1.03	1.14	Triplet		Protons on 8



3.8.3.2 ^{13}C-NMR Spectrum

The carbon and DEPT (distortionless enhanced polarization transfer) spectra are shown in Figure 10. The HETCOR (heteronuclear two-dimensional proton-carbon correlation) spectrum is shown in Figure 11. The carbon assignments are listed in Table 5. Long-range HETCOR experiments were used to make the assignments for the thiophene carbons.

3.8.4 Mass Spectrometry

A Finnigan MAT TSQ mass spectrometer was used to record the mass spectra of brinzolamide. The conditions were: 0.3 mA current, 1100-volt acceleration voltage and 10^{-7} preamp sensitivity. The ionization voltage was 70 eV for EI (electron impact) ionization mode. For CI (chemical ionization) mode, a positive ionization voltage of 100 eV and a pressure of 0.3 torr of methane were used. The EI and CI spectra are shown in Figures 12 and 13, respectively. Peak assignments for the EI and CI spectra are listed in Table 6. Both the CI and EI spectra have an MH^+ peak at m/z = 384.

4. Methods of Analysis

4.1 Identification

Identification of brinzolamide can be confirmed by comparison of the infrared spectrum (KBr pellet) with that of Figure 6.

4.2 Elemental Analysis

A typical elemental analysis of brinzolamide is listed in Table 7.

4.3 Titrimetric Analysis

Brinzolamide has been assayed by titration in glacial acetic acid using crystal violet T.S. (USP) as an indicator. A 500-mg sample is dissolved in 50 mL of glacial acetic acid and titrated with 0.1N perchloric acid to an emerald green endpoint.

Figure 10

Carbon and DEPT NMR Spectra of Brinzolamide (DMSO-d6)

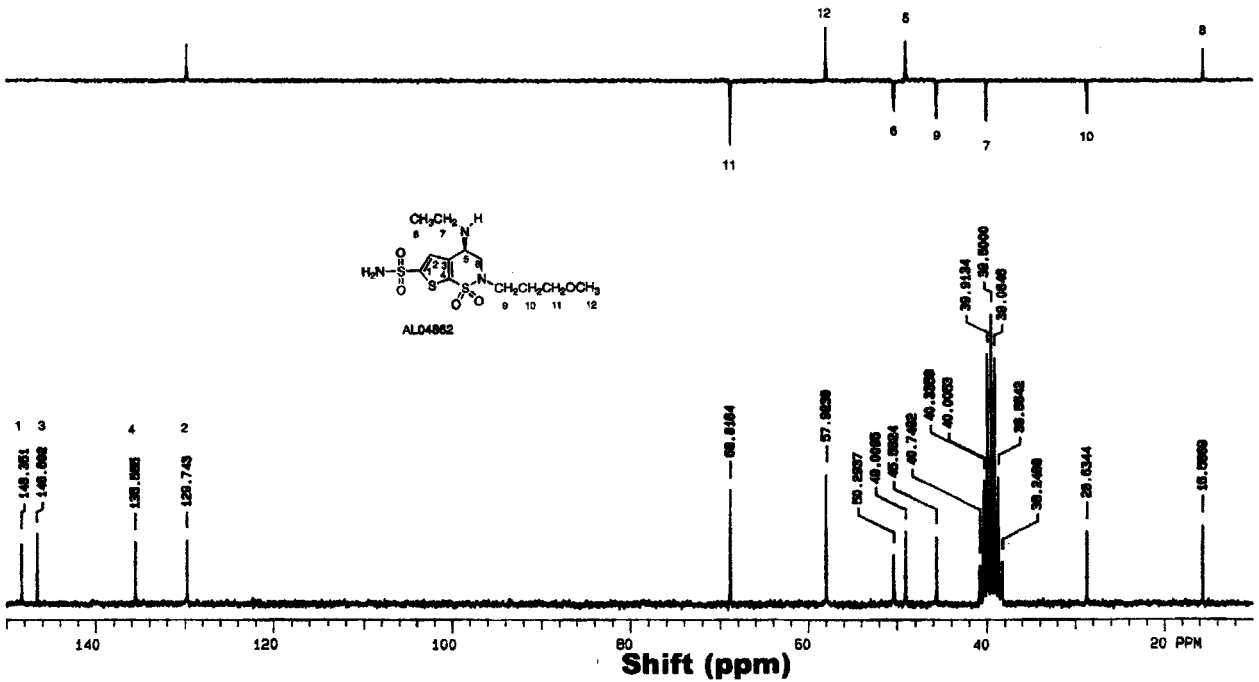


Figure 11

HETCOR NMR Spectrum of Brinzolamide (DMSO-d6)

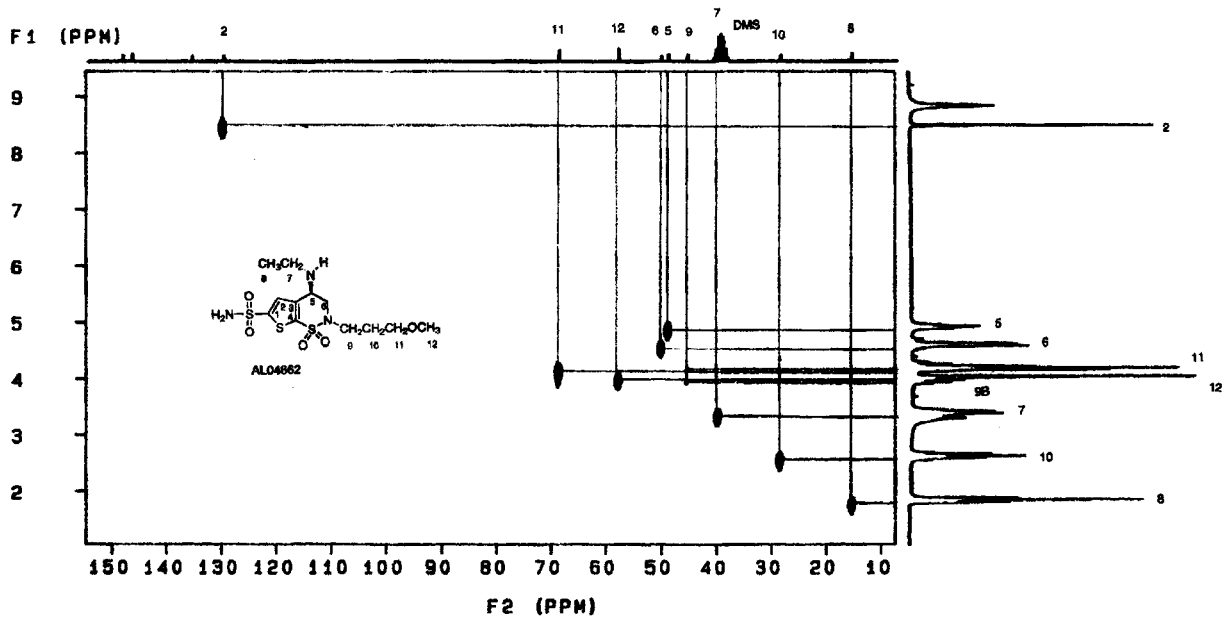


Table 5

Carbon NMR Assignments for Brinzolamide (in DMSO-d6)

Shift (ppm)	DEPT (No. of protons)	Assignment
148.4	None	Carbon 1
146.6	None	Carbon 3
135.6	None	Carbon 4
129.7	One	Carbon 2
68.8	Two	Carbon 11
57.9	Three	Carbon 12
50.3	Two	Carbon 6
49.0	One	Carbon 5
45.6	Two	Carbon 9
40.0	Two	Carbon 7
40.7-39.2	-------	DMSO
28.6	Two	Carbon 10
15.6	Three	Carbon 8

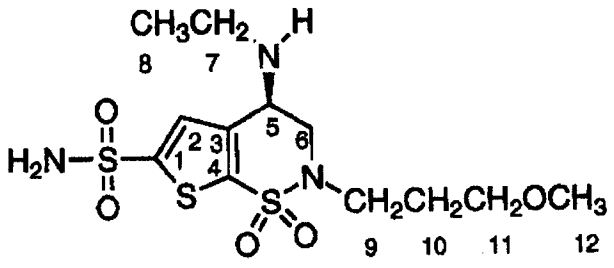


Figure 12

Electron Impact Mass Spectrum of Brinzolamide

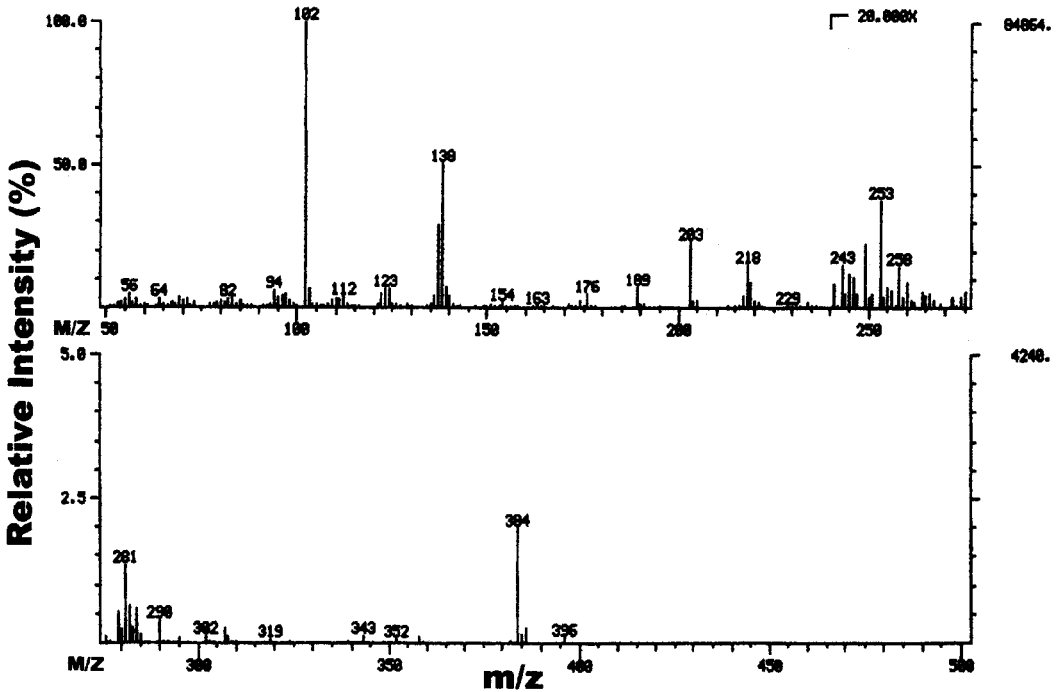


Figure 13

Chemical Ionization Mass Spectrum of Brinzolamide

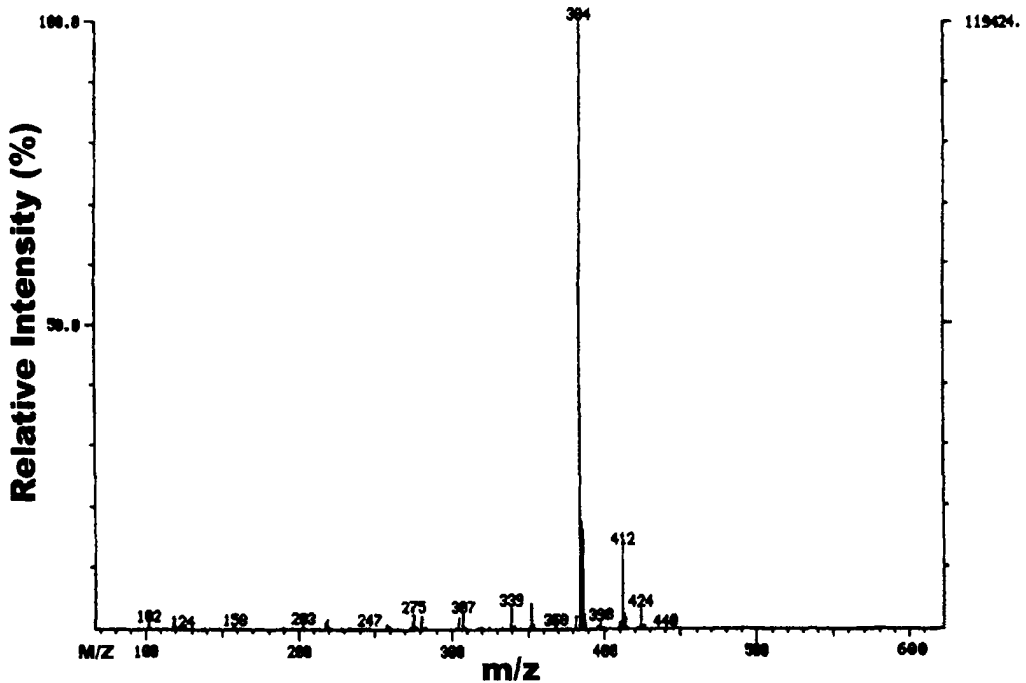


Table 6

Mass Spectral Assignments for Brinzolamide

Electron Impact		Chemical Ionization	
m/z	**Assignment**	**m/z**	**Assignment**
386	$[MH + 2]^+$ isotope peak consistent with 3 sulfur atoms (~15%)	424	$[M + C_3H_5]^+$
384	MH^+	412	$[M + C_2H_5]^+$
352	$MH^+ - CH_3OH$	384 (Base)	MH^+
319	$M^+ - SO_2$	352	$MH^+ - CH_3OH$
307	$MH^+ - \{CH_3CH_2NH_2 + CH_3OH\}$	339	$MH^+ - CH_3CH_2NH_2$
290	$MH^+ - CH_2SO_2NH_2$		
281	$MH^+ - CH_3NH(CH_2)_3OCH_3$		
258	$M^+ - \{SO_2NH_2 + CH_3CH_2NH_2\}$		
253	$MH^+ - \{CH_3NH(CH_2)_3OCH_3 + CH_2CH_2\}$		
249	$M^+ - \{CH_3CH_2NH_2 + NH_2(CH_2)_3OCH_3\}$		
243	$M^+ - \{SO_2NH_2 + CH_3CH_2OCH_3\}$		
218	$M^+ - SO_2N(CH_2)(CH_2)_3OCH_3$		
203	$MH^+ - \{SO_2NH_2 + H_2CN(CH_2)_3OCH_3\}$		
138	$[SO_2NH(CH_2)_3OH]^+$ or $[H_2NSO_2C(S)=CH_2]^+$		
102 (Base)	$[H_2C=NH(CH_2)_3OCH_3]^+$		

Table 7

Elemental Analysis of Brinzolamide

Element	**Theoretical (%)**	**Found [1] (%)**	**Found [2] (%)**
Carbon	37.58	37.63	37.66
Hydrogen	5.52	5.41	5.48
Nitrogen	10.96	10.93	10.96
Sulfur	25.08	25.14	-
Oxygen	20.86	21.40	-

(1) This work

(2) Reference [10]

4.4 Chromatographic Methods of Analysis

4.4.1 Thin Layer Chromatography

A normal phase TLC system has been developed for brinzolamide. The operating parameters for this method are summarized in Table 8.

4.4.2 High Performance Liquid Chromatography

Several different HPLC systems have been developed for brinzolamide by our laboratory. The operating conditions for these methods are summarized in Table 9. Figure 14 shows a chromatogram using the second set of conditions listed.

Two different chiral HPLC systems have been developed in this laboratory to evaluate the enantiomeric purity of brinzolamide. The operating conditions for these methods are also summarized in Table 9. Figure 15 shows a chiral HPLC chromatogram using the second set of conditions listed.

4.4.3 Capillary Electrophoresis

Capillary electrophoresis has also been used to examine the enantiomeric purity of brinzolamide. The system consisted of a Polymicro Technologies fused silica capillary column (50 cm x 50 μm), UV detection at 255 nm, pH 2.4 buffer (28 mM heptakis-(2,6-di-*o*-methyl)-β-cyclodextrin and 30 mM TRIS-(hydroxylmethylaminomethane),adjusted to pH with 85% H_3PO_4) and an applied voltage of 20 kV. Injection of a 200 μg/mL sample was accomplished with 50 mm gravity for 10 seconds.

4.5 Determination in Body Fluids and Tissues

Determination of brinzolamide and its 3 principal metabolites (the *N*-desethyl, *N*-desmethoxypropyl and *O*-desmethyl analogs) in whole blood and plasma from clinical and pre-clinical studies was performed using high performance liquid chromatography (HPLC) with UV detection. After addition of a known amount of internal standard (AL-5138, the 4-methoxybutyl analog of brinzolamide), the sample was acidified with 50 mM sodium phosphate buffer, pH 3.0 and extracted with ethyl acetate. The organic layer was extracted into 1:14 glacial acetic acid:water, and the aqueous layer adjusted to basic pH with 6N KOH and extracted with ethyl acetate. The organic layer from the latter extraction was evaporated and

Table 8

Thin-Layer Chromatographic Method for Brinzolamide

Plate	Whatman Silica Gel LHP-KF, 200 μm, 10 x 10 cm
Mobile Phase	1:1:1 (v/v/v) *n*-heptane – chloroform – ethanol
Target Concentration	5 μL of 20 mg/mL
Detection	UV absorbance at 254 nm
Relative Retention	0.4

Table 9

High Performance Liquid Chromatography Methods for Brinzolamide

Column	Mobile Phase	Flow	Target Concentration	Detection	Retention
Reversed Phase					
Phenomenex Shperisorb ODS (2), 5 μm, 4.6 x 250 mm	Buffer (0.4% triethanolamine adjusted to pH 3.0 with H_3PO_4)/acetonitrile, (75/25, v/v)	1.0 mL/min	20 μL of 0.1 mg/mL	UV at 254 nm	~ 5 min.
Phenomenex Prodigy ODS-2, 5 μm, 4.6 x 150 mm	Buffer (0.15 M ammonium acetate adjusted to pH 5.2 with glacial acetic acid)/methanol, (65/35. v/v)	1.0 mL/min	20 μL of 0.2 mg/mL	UV at 254b nm	~ 8 min.
Chiral					
Diacel Chiralcel OF, 10 μm, 4.6 x 250 mm	N-heptane/2-propanol/triethylamine, (50/50/0.1, v/v/v)	0.9 mL/min	5 μL of 1 mg/mL	UV at 254 nm	~21 min.
Diacel Chiralpak AD, 10 μm, 4.6 x 250 mm	Hexane/ethanol/methanol/diethyl amine, (40/55/5/0.2, v/v/v/v)	0.75 mL/min	5 μL of 0.4 mg/mL	UV at 254 nm	~ 8 min.

Figure14

HPLC Chromatogram of Brinzolamide

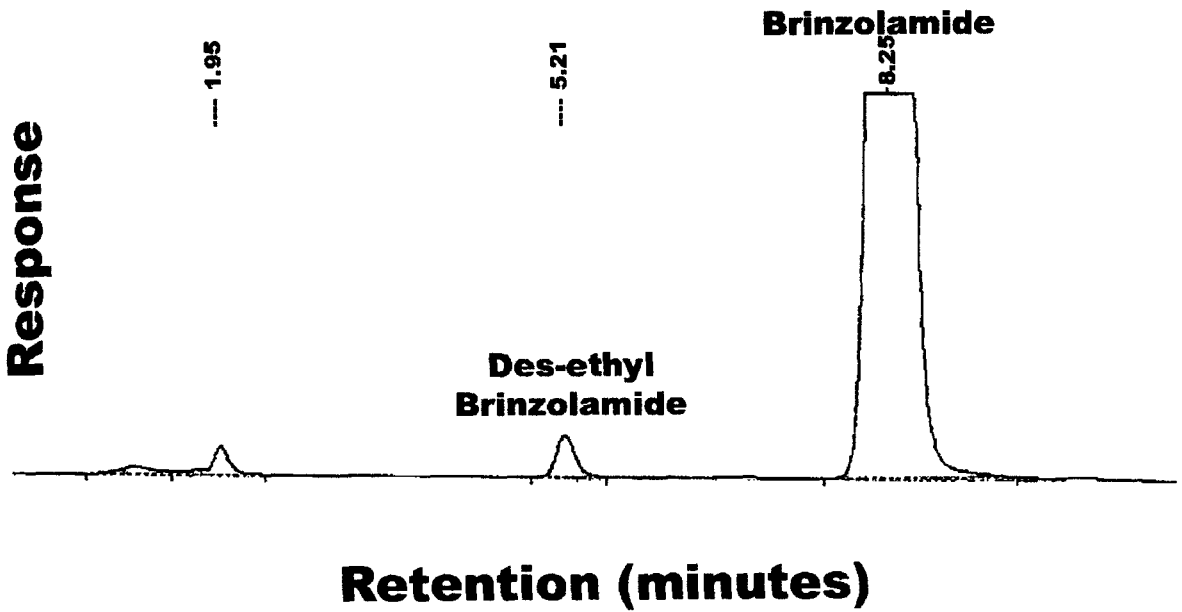
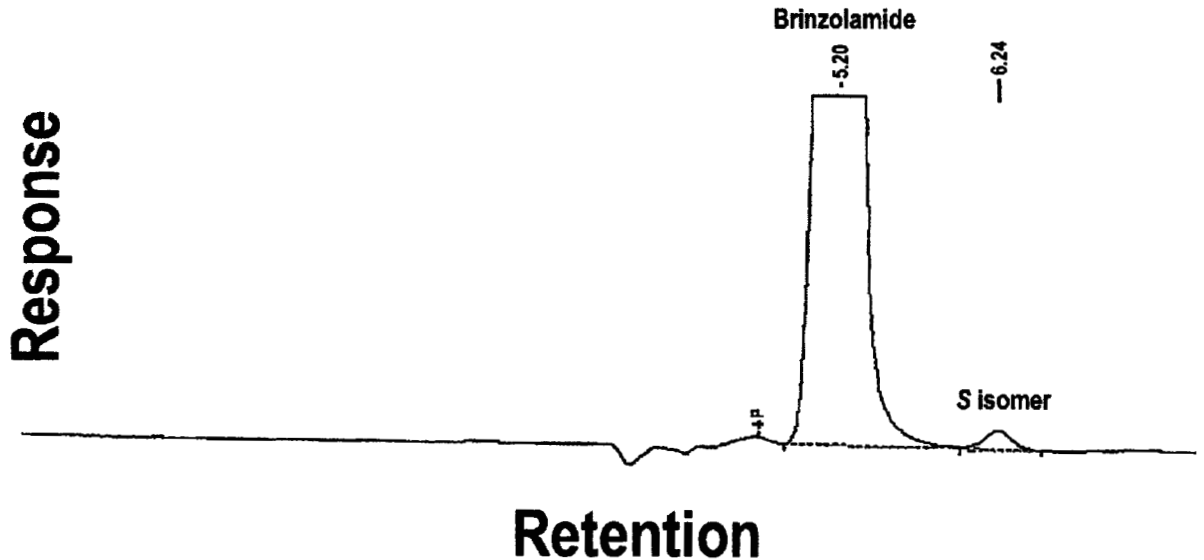


Figure 15

Chiral HPLC Chromatogram of Brinzolamide



reconstituted in mobile phase. The chromatographic conditions are summarized in Table 10. For whole blood, a known excess of another carbonic anhydrase inhibitor, AL-4457 (the 2-(*N*-propyl) analog of brinzolamide), was added prior to extraction. This served as a displacing agent to remove brinzolamide bound to carbonic anhydrase in red blood cells.

The procedure for simultaneous analysis of drug and active metabolites was validated in whole blood from cynomolgus monkeys and rats. The working range was 0.2 to 10.0 μg/mL for both species. The method was also validated in cynomolgus monkey plasma with a working range of 10.0 to 400 ng/mL. In addition, the procedure was validated in human whole blood and plasma. Working ranges in human whole blood were 0.35-9.0 μg/mL for parent drug and 0.2-9.0 μg/mL for metabolites. In human plasma, the working range was 7.5-500 ng/mL for all analytes except the *N*-desmethoxypropyl metabolite, for which the range was 10-500 ng/mL.

Concentrations of brinzolamide parent drug and metabolites (*N*-desethyl, *O*-desmethyl, *N*-desmethoxypropyl and the carboxylic acid analog of the *O*-desmethyl metabolite) were determined in human urine using electrospray HPLC/mass spectrometry. A 1 mL aliquot was spiked with approximately 20 ng of internal standard (AL-4623, the 2-methoxyethyl analog of brinzolamide) and acidified with 0.2 M HCl. The sample was then applied to an octadecylsilica solid phase extraction cartridge (Ansys SPEC.3ML.MP1) which was rinsed with methanol/water (20/80) to remove endogenous interferences. The components of interest were then eluted using methylene chloride/ethyl acetate/methanol/concentrated ammonium hydroxide (30/100/30/3). After the eluent was evaporated under nitrogen, the residue was reconstituted in HPLC mobile phase and chromatographed using the conditions shown in Table 10B with negative ion electrospray ionization. The M-H ions for each analyte and the internal standard were monitored. The working range for the procedure was 1–20 ng/mL for all analytes. Due to the widely varying concentrations of the different analytes typically found in human urine, samples were diluted into the appropriate range using control urine following an initial screening run.

Table 10A

High Performance Liquid Chromatographic Method for Brinzolamide and Metabolites in Whole Blood and Plasma

Column	Mobile Phase	Flow Rate	Injection Volume	Detection	Column Temperature
Octadecylsilica (Waters Symmetry) 100 x 4.6 mm with 5 mm guard cartridge	0.5% H_3PO_4, 0.7% triethylamine pH 3.0 buffer/acetonitrile (72/28 *) containing 6 mM 1-decanesulfonic acid	1.8 mL/minute	50 µL for whole blood, 60 µL for plasma	UV at 254 nm	45°C

* Whole blood mobile phase. For plasma, buffer/acetonitrile ratio is 75/25.

Table 10B

High Performance Liquid Chromatographic Method for Brinzolamide and Metabolites in Urine

Column	Mobile Phase	Flow Rate	Injection Volume	Detection	Column Temperature
Octadecylsilica (Phenomenex Prodigy ODS-3), 250 x 2 mm, with matching 30 x 2 mm guard column	Acetonitrile / 5mM ammonium acetate buffer, pH 3.5 (16/84)	0.3 mL/minute	30 µL	Negative ionization electrospray MS	30°C

5. Stability

5.1 Solid-State Stability

Brinzolamide has been found to be a highly stable drug in the solid state. Thus, no assay trend, evidence of degradation products, or other significant changes were noted on materials stored for 3 years at 25°C/65% relative humidity, 6 months at 40°C/75% relative humidity, four weeks at 65°C and under light cabinet (ICH) conditions.

5.2 Solution-Phase Stability

Brinzolamide has been found to be highly stable in solution when formulated at neutral pH (Azopt, pH 7.6) and stored under room temperature conditions. Figure 16 shows a pH degradation profile for brinzolamide solutions stored at 85°C and indicates that the drug degradation by heat is less rapid at acidic pHs. Brinzolamide was found to have a very slightly photosensitivity and to be impervious to oxygen in solution.

Degradation pathways are shown in Scheme 2. The major degradation products observed in heat studies were the *S* isomer and *N*-desethyl brinzolamide (also observed as a major metabolite). While racemization rate is essentially negligible at room temperature, it becomes significant at autoclave temperatures and is largely independent of pH. Desethyl brinzolamide forms with heat stress (rates higher at neutral and acidic pH) and also from light stress. These are the only degradation products observed during stability studies on Azopt.

5.3 Incompatibilities with Functional Groups

No incompatibilities with functional groups have been observed in our laboratories.

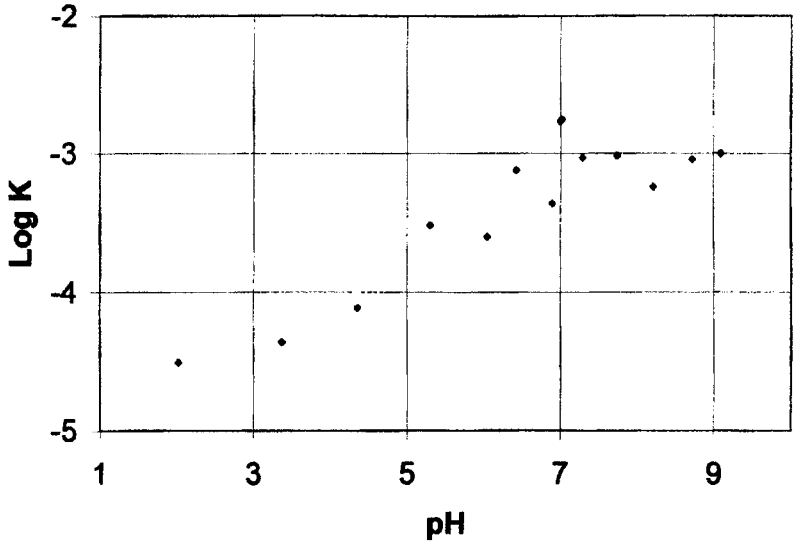
6. Drug Metabolism and Pharmacokinetics

6.1 Absorption and Bioavailability

Brinzolamide is well absorbed following oral dosing with absolute bioavailability in rats of about 70% from a 20 mg/kg dose. It was also found to be well absorbed in man. Due to saturable binding to carbonic

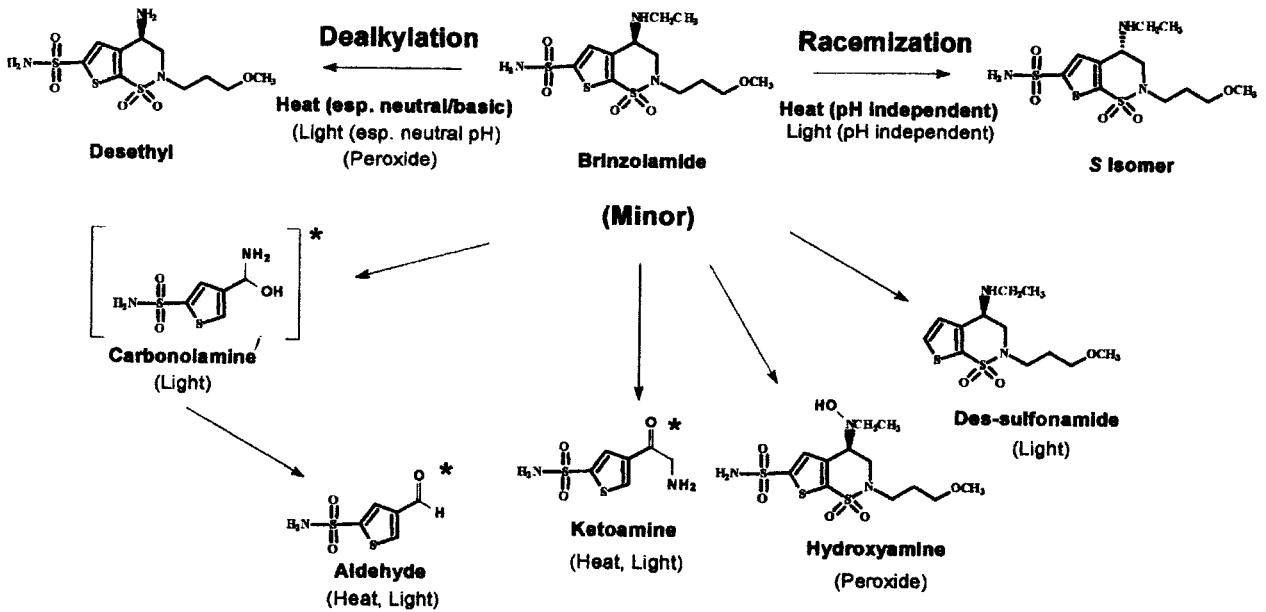
Figure 16

pH Degradation Profile of Brinzolamide (at 85°C)
K = Apparent First Order Rate Constant ($hours^{-1}$)
(Phosphate, acetate and borate buffers adjusted to 0.1 ionic strength)



Scheme 2

Solution Degradation Pathways of Brinzolamide



* Structures proposed based on LC/MS studies.

anhydrase (CA) in red blood cells, whole blood concentrations show minimal dose dependence and are much higher than corresponding plasma concentrations. Maximum blood and plasma concentrations are achieved within 1 hour of oral administration. Brinzolamide exhibits prolonged retention in whole blood due to CA binding with a long (greater than 100 hours) half-life, which is characteristic of this drug class [14].

Studies in rabbits, monkeys and humans showed brinzolamide to be absorbed into the systemic circulation following topical ocular administration. Substantial levels (several micrograms per mL in whole blood) were achieved upon long term repeated dosing.

6.2 Distribution

Radioactivity from ^{14}C-brinzolamide penetrated into rabbit ocular tissues following topical ocular dosing with highest concentrations in the conjunctiva and cornea. Radioactivity was eliminated fairly rapidly from the aqueous humor (approximately 3 hour half-life) while some other tissues showed much longer retention. Elimination half-lives in the iris-ciliary body (ICB), choroid, retina, and lens ranged from 34 to 294 days and are consistent with binding to CA, which is known to be present in these tissues. Comparison of ICB data from albino and pigmented rabbits indicates some propensity of brinzolamide to bind to melanin. However, CA binding is more important in determining overall kinetics.

Radiolabeled studies in rats demonstrated highest exposure to radioactivity from an i.v. ^{14}C-brinzolamide dose in tissues containing high CA concentrations. These included liver, kidneys, spleen, lungs and salivary glands. Radioactivity concentrations in fat, nerve, skeletal muscle, skin, eyes and testes were low. Experiments in pregnant and lactating rats demonstrated transfer of radioactivity across the placental barrier and secretion of low concentrations in milk.

Brinzolamide is moderately bound to plasma proteins (about 60% in humans, 25-30% in rats and 75-80% in monkeys).

6.3 Metabolism

Primary metabolic pathways of brinzolamide include formation of the *N*-desethyl, *N*-desmethoxypropyl and *O*-desmethyl analogs, all of which are

found in rats. The metabolic pathways known for brinzolamide are shown in Scheme 3.

In humans, only the *N*-desethyl metabolite is detected in whole blood, although trace amounts of the other two metabolites are found in urine. All three of the above exhibit binding to carbonic anhydrase and prolonged half-lives in whole blood. In rats, a carboxylic acid metabolite formed by oxidation of the *O*-desmethyl analog is the predominant urinary metabolite. Small amounts of this compound are also found in human urine.

No evidence for *in vivo* inversion of the chiral center of brinzolamide was found in rats, monkeys or humans.

6.4 Elimination

Following a single 1 mg/kg intravenous dose of ^{14}C-brinzolamide to rats, about 30% of the dose was excreted in urine over 14 days with a similar amount excreted in the feces over this time period. The remainder of drug-related material was found in the carcass, reflecting tight binding to carbonic anhydrase in whole blood and other tissues. Enterohepatic recirculation of brinzolamide in rats has been demonstrated. In humans, approximately 80% of the dose is eliminated in urine following oral administration to steady state. Most of this (about 60% of the dose) is unchanged drug.

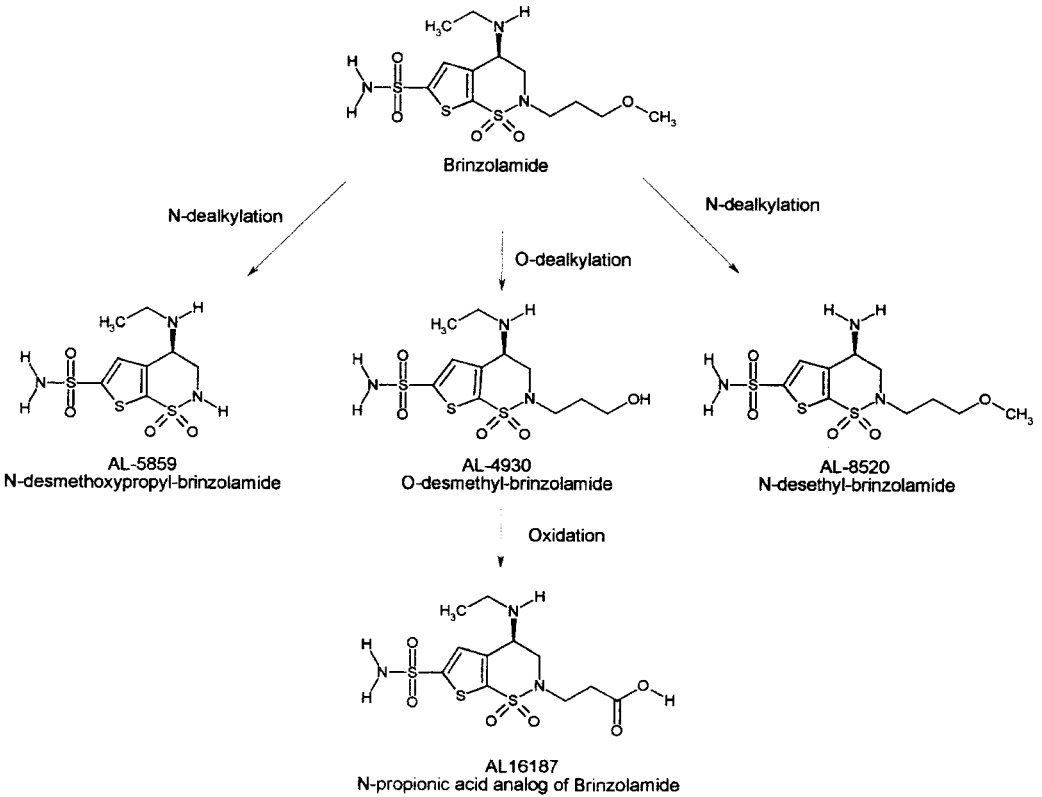
7. Toxicology

Adverse effects associated with brinzolamide are similar to those of other carbonic anhydrase inhibitors. In clinical experience with topical ocular administration of brinzolamide, events including transient, momentary blurred vision, bitter, and sour or unusual taste were reported in approximately 5 % - 10% of patients. Ocular discomfort, discharge or other ocular signs, and headache were reported at an incidence of 1-5% [15].

Preclinical studies employing brinzolamide as an ophthalmic aqueous suspension at concentrations of up to 4%, administered up to four times a

Scheme 3

Metabolic Pathways of Brinzolamide



day to rabbits or monkeys, and ranging in duration from one to 12 months, revealed no significant effects on the eyes or adnexa. Though corneal thickness was increased up to approximately 15% in treated eyes of rabbits, as compared with controls, corneal structures appeared healthy upon ophthalmic and histologic examination. Monkeys receiving brinzolamide ophthalmic suspension, up to 4%, three times a day for one year experienced no change in corneal thickness. Specular microscopy of the corneal endothelium of these monkeys confirmed corneal health. No systemic effects were observed on clinical laboratory values or on any tissues or organs examined microscopically.

Oral administration of brinzolamide in rats and mice resulted in findings consistent with other carbonic anhydrase inhibitors such as acetazolamide or dorzolamide. High doses (up to 180 mg/kg/day) of brinzolamide, administered by oral gavage as an aqueous solution at low pH (3.5), resulted in lesions (ulceration and proliferative change) of the forestomach. These gastric lesions appeared to be consistent with findings reported by Cho *et al.* [16] and Cho and Pfeiffer [17] following administration of acetazolamide to rats. Oral administration of an aqueous suspension at a neutral pH avoided gastric lesions. Rats treated with 0, 1, 3 and 8 mg/kg/day of brinzolamide orally for three or six months exhibited no significant signs of toxicity. Some mild changes in serum chemistry (K, Na, Cl) and increases in urine volume, with reduced urine specific gravity, urine sodium and potassium concentrations, were considered related to the pharmacologic action of the drug. Some degree of renal nephrosis, papillary granularity and mineralization, and urinary bladder hyperplasia were present, particularly among females, upon microscopic examination. Three mg/kg/day was considered the No Observed Effect Level (NOEL). These findings appear to be consistent with those reported for dorzolamide, though microscopic findings were more prevalent among males with that drug, and the incidence appeared to be dose related and present at doses of 0.05 mg/kg/day and above in the two year study [23].

Brinzolamide did not effect fertility or general reproductive capacity in male rats at administered doses up to 18 mg/kg/day, from 14 weeks prior to mating throughout the cohabitation period. No effect was seen in F0 females receiving the drug from two weeks prior to mating through day 20 of gestation, for approximately one-half of the dams, and through weaning for the remaining dams. Body weights of F1s were reduced slightly, as compared with controls among the C- section fetuses, but not the delivered

pups at day 1. However, F1 pup weights of the high dose group were slightly to moderately (up to13%) lower than controls during the weaning period. There were no effects on F1 fertility or reproductive capacity, or on F2 offspring.

Fetuses of rat dams, receiving brinzolamide orally at dose levels of up to 18 mg/kg/day during the period of major organogenesis, demonstrated slightly lower body weights, compared with controls, at the high dose only. Also an increase in the incidence of reduced ossification of sternebrae, hyoid and skull bone were observed at the high dose only. A teratology study in rabbits demonstrated no reduction in ossification in fetuses of does receiving up to 6 mg/kg/day, orally, by gavage, during the period of major organogenesis, though significant maternal toxicity was observed. These findings were not unexpected for a carbonic anhydrase inhibitor. Incomplete ossification and reduced fetal body weight was reported for dorzolamide at dose levels of 2.5 mg/kg/day and higher in rabbits, but not in rats at up to 10 mg/kg/day [15]. Oral carbonic anhydrase inhibitors, such as acetazolamide, dichlorphenamide, ethoxzolamide and benzolamide, have been associated with forelimb skeletal malformations in rats, mice, rabbits and monkeys [18, 19] and this effect has been postulated to be related to reduction in intracellular pH (20). A teratology study in rats at dose levels of up to 10 mg/kg/day administered to dams during days 6-17 of gestation had no significant effect on fetuses. Offspring of rat dams receiving brinzolamide at dose levels of up to 10 mg/kg/day, via oral administration to the mothers from gestation day 15 through lactation day 21, showed no differences in anatomic, behavioral or learning development, as compared with controls.

Sulfonamide derivatives possess the potential for immunologic sensitization. An *in vitro* study of glutathione reactivity conducted in order to predict the potential of brinzolamide for sensitization using the method of Graham *et al.* [21] indicated no reactivity; methazolamide yielded approximately 50% reactivity in the same test. A guinea pig maximization test was negative, confirming the *in vitro* glutathione reactivity results, and indicating little or no potential for delayed contact hypersensitization.

The genotoxic potential of brinzolamide was evaluated by the bacterial reverse mutation assay (Ames test) with *S typhimurium* and *E coli*, an *in vitro* mouse lymphoma forward mutation assay (MLFMA), an *in vivo*

mouse micronucleus assay and an *in vivo* sister chromatid exchange assay. A mild positive response in the MLFMA, at higher concentrations and in the presence of metabolic activation, was considered related to the high degree of cytotoxicity observed, and the low solubility of the drug at the pH tested. It was considered that the weight of evidence demonstrated no significant genotoxic risk.

Two-year carcinogenicity bioassays, in rats and mice, were conducted at oral dose levels of up to 8 and 10 mg/kg/day, respectively. No significant increase in tumor incidence was present at any dose level in rats. In mice, an increase in urinary bladder transitional cell hyperplasia and papilloma were apparent, especially among females. In addition, mesenchymal cell proliferative lesions of the urinary bladder were present at the high dose. One female mouse of the high dose had a urinary bladder carcinoma. Changes in the urinary bladder of mice were examined by an expert pathology working group. They were determined to be of a nature unique to the mouse (mesenchymal lesion) and/or secondary to crystalline material in the urine with associated irritation and inflammation [22-25].

TOPAMAX, a sulfamate-substituted monosaccharide approved for use as an antiepileptic drug at oral doses of up to 400 mg per day, exhibits carbonic anhydrase inhibition activity [15]. A 21-month dietary study in mice with TOPAMAX resulted in increased incidence of bladder tumors similar to those observed in the mouse carcinogenicity bioassay with brinzolamide at two years.

The aqueous solubility of brinzolamide is limited, and is highly pH dependent, as shown in Table 2. Urinary pH in rodents tends to be higher, more alkaline, than in man. Though urinary pH can vary widely, in these studies, the pH of rat urine was usually 6.5 – 7.0, whereas urine pH in man is generally 5.5 – 6.5. The solubility of brinzolamide drops precipitously from pH 5.1 (where it is 3300 ug/mL) to pH 7.37 (where it is 400 ug/mL) and high oral doses of brinzolamide will result in significant concentrations of drug and drug-related substances in the urine. It is likely that, upon repeated dosing, concentrations of drug exceeded solubility limits at rodent urinary pH [26].

Nine impurities were identified in brinzolamide raw material, with eight of these seen at concentrations of greater than or equal to 0.1%. Several of these were also identified as metabolites in one or more species, and it was

considered that exposure of animals to these as metabolites, at levels significantly greater than would occur with clinical use, was sufficient to qualify these for toxicological potential. Selected of the impurities were examined for toxicity potential, as required by ICH.

The (*S*)-isomer of brinzolamide (AL-7118) and *N*-desethylbrinzolamide (AL-8520) are also potential degradation products identified in the drug product. These were tested for genotoxicity (Ames test and mouse micronucleus assay), and were negative. Desethyl brinzolamide was also found to be a physiologic metabolite of brinzolamide (20% in rat urine), and was therefore considered toxicologically "qualified" through its presence in high dose systemic studies. In addition, the (*S*)-isomer was tested for topical ocular irritation potential in a formulation at 0.2% in order to ensure the safety of product through the shelf life. This concentration is 20% relative to the drug concentration of 1.0%, and is greater than 10 times the concentration of the degradant found in the most extreme samples.

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CHLORPROMAZINE

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Name

2-chloro-N,N-dimethyl-10*H*-phenothiazine-10-propanamine [3]

3-chloro-10-(3-dimethylaminopropyl)phenothiazine [3]

N-(3-dimethylaminopropyl)-3-chlorophenotbiazine [3].

[3-(2-chlorophenothiazine-10-yl)propyl]dimethylamine [4]

[3-(2-chlorophenothiazine-10-yl)propyldimethylamine [5]

10*H*-phenothiazine-10-propanoamine-2-chloro-N,N-dimethyl [6]

2-chloro-10-(3-dimethylaminopropyl)phenothiazine [7]

3-(2-Chlorophenothiazine-10-yl)-N,N-dimethylpropylamine [8]

Names of the salts formed upon condensation with an acid are added after the chemical names. For instance, [3-(2-chlorophenothiazine-10-yl)propyl]dimethylamine hydrochloride [4}.

1.1.2 Nonproprietary Names

Chlorpromazine; 2601-A, HL-5746, R-P-4560, SKF-2601-A [3]; chlorpromazine hydrochloride, aminazine, chlorpromazini hydrochloridum, cloridrato de clorpromazinea [5]

1.1.3 Proprietary Names

Amazin, Aminazine; Ampliactil; Amplictil; B-T 2569; Chlor-P2; Chlor-Promany; Chlorazin; Chlorderazin- Chloroactil; Chlorpromados; Clorazine; Clordelazin; Contomin; Elmarine; Esmind; Fenactil; Fleksin; Galmazin; Hebanil; Hibanil; Hibemal; Klorpromex; Largactil; Largaktyl; Marazine; Megaphen; Neurazine; Novomazina Ormazine; Plegomazin; Promacid; Promactil; Promapar; Promazil; Proma Propaphenin; Prozil, Sonazine; Sanopron; Taroctyl; Thorazine- Torazina; Wintermin [3,4,6]

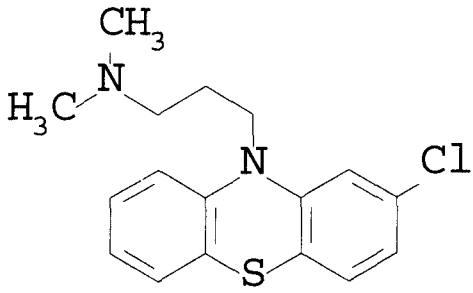
1.2 Formulae

1.2.1 Empirical

Chlorpromazine: $C_{17}H_{19}ClN_2S$ [3]

Chlorpromazine hydrochloride: $C_{17}H_{20}Cl_2N_2S$ [3]

1.2.2 Structural



1.3 Molecular Weights

Chlorpromazine: 318.86 [4]

Chlorpromazine hydrochloride: 355.32 [4]

1.4 CAS Numbers

Chlorpromazine: [50-33-3] [7]

Chlorpromazine hydrochloride: [69-09-0] [7]

1.5 Appearance

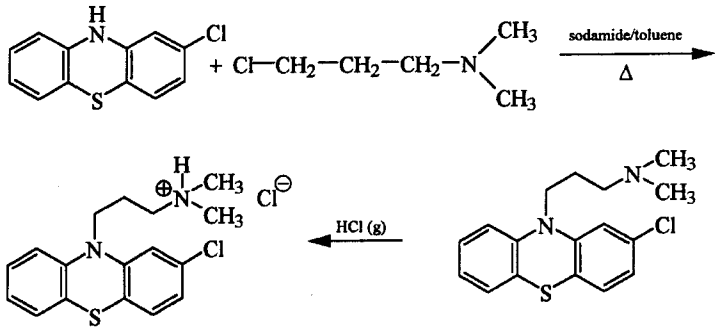
Chlorpromazine is a white to creamy-white powder or waxy solid, which darkens on prolonged exposure to light. It may have an amine odor [2,10].

1.6 Uses and Applications

Chlorpromazine is the prototype molecule of the series of substituted phenothiazine psychotherapeutic compounds. Chlorpromazine is used for the symptomatic management of schizophrenia and active acute psychoses. The drug is also used for the prevention and treatment of nausea and vomiting, and for the relief of restlessness and apprehension before surgery [1,2].

2. Method of Preparation [10-12]

Chlorpromazine is prepared by refluxing a toluene solution of 2-chloro-phenothiazine and (3-chloropropyl)-dimethylamine in the presence of sodamide for several hours, filtering the reaction mixture, and distilling off the toluene under reduced pressure. Then product is then purified by short-path distillation under high vacuum, and converted to the hydrochloride salt by passing hydrogen chloride gas into a solution of the base in a suitable organic solvent according to the following scheme:



3. Physical Properties

3.1 X-Ray Powder Diffraction Pattern

The x-ray powder diffraction pattern of chlorpromazine hydrochloride was obtained in our laboratory [13], using a Philips fully automated X-ray

diffraction spectrogoniometer fitted with PW 1730/10 generator. The incident radiation was provided by a copper target (Cu anode, operated at 2000 W, and yielding $\lambda = 1.5480$ Å). The high intensity X-ray tube was operated at 40 kV and 35 mA. The monochromator was a curved single crystal (PW 1752/00), and the divergence and receiving slits were 0 and 0.1 mm, respectively. The scanning speed of the goniometer (PW 1050/81) was 0.02 degrees-2θ per second, and the system was aligned using elemental silicon before use.

The powder pattern of chlorpromazine hydrochloride is presented in Figure 1, and the scattering angles, interplanar distances, and peak relative intensities are collected in Table 1.

3.2 Thermal Methods of analysis

3.2.1 Melting Behavior

The melting range reported for chlorpromazine free base is 56-58°C [5], while the melting range of chlorpromazine hydrochloride has been reported to be 195-198 [8].

The boiling range reported for chlorpromazine free base is 200-205°C [5].

3.2.2 Differential Scanning Calorimetry

The DSC thermogram of chlorpromazine hydrochloride shown in Figure 2 was obtained using Du Pont model 9900 differential scanning calorimetry system, using a sample size of 2.83 mg and a heating rate of 10°C/minute [13].

The strong exothermic peak has its maximum at 197.41°C C corresponds to the melting transition, and is characterized by a heat of fusion equal to 86.36 J/g. The equivalence of the baseline prior to and subsequent to the melting endotherm suggests that the compound melts without decomposition.

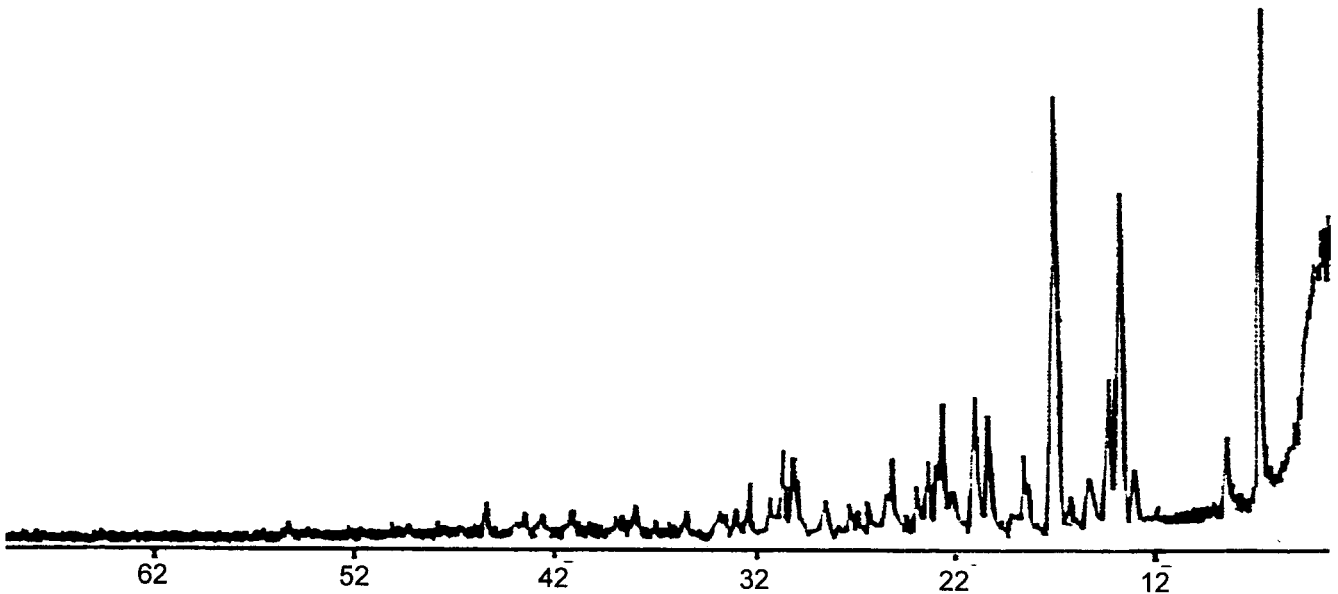


Figure 1. X-ray powder diffraction pattern of chlorpromazine hydrochloride.

Table 1

Crystallographic Data from the X-Ray Powder Diffraction Pattern of Chlorpromazine Hydrochloride

Scattering Angle (degrees 2-θ)	d-spacing (Å)	Relative Intensity (% I/I_o)
8.239	10.7310	100.00
9.830	8.9978	23.864
14.574	6.0777	18.523
15.526	5.7073	76.591
15.829	5.5986	35.284
16.726	5.3003	13.295
17.314	5.1215	7.898
17.586	5.0431	9.375
18.594	4.7719	91.193
18.913	4.6921	7.614
20.008	4.4378	13.352
20.179	4.4005	18.807
20.707	4.2895	9.660
21.474	4.1379	6.932
22.061	4.0291	27.557
22.590	3.9359	31.932
23.706	3.7531	12.500
24.330	3.6583	31.193
24.955	3.5681	19.716
25.507	3.4920	13.125
26.671	3.3422	22.045
26.693	3.3076	12.330
27.914	3.1962	13.693
28.386	3.1441	6.136
28.762	3.1039	8.068
29.658	3.0121	5.340
30.166	2.9625	10.284
31.710	2.8217	18.977

Table 1 (continued)

Crystallographic Data from the X-Ray Powder Diffraction Pattern of Chlorpromazine Hydrochloride

Scattering Angle (degrees 2-θ)	d-spacing (Å)	Relative Intensity (% I/I_o)
32.196	2.7802	20.23
32.823	2.7285	10.51
34.031	2.6344	14.261
34.712	2.5843	6.420
35.401	2.5355	8.580
37.059	2.4258	6.761
37.927	2.3722	4.773
38.673	2.3282	4.432
39.642	2.2735	7.955
40.310	2.2374	5.455
40.727	2.2154	6.250
42.855	2.1102	7.386
43.561	2.0776	3.580
44.334	2.0432	6.818
45.219	2.0053	6.250
45.619	1.9885	5.795
47.076	1.9303	7.216
47.620	1.9096	4.091
48.390	1.8809	4.148
51.078	1.7881	4.148
53.403	1.7156	3.352
56.118	1.6389	3.920
57.095	1.6131	5.625
61.869	1.4996	2.784
66.950	1.3976	1.932
75.791	1.2551	2.955
79.68	1.2033	1.761

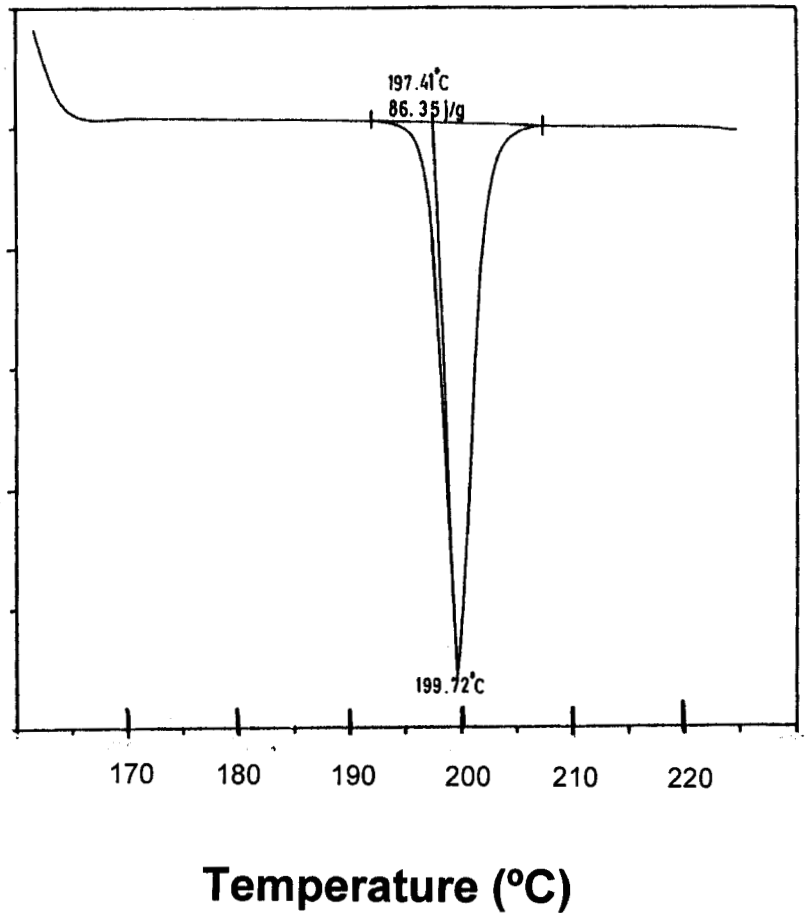


Figure 2. Differential scanning calorimetry thermogram of chlorpromazine hydrochloride.

3.3 Solubility Characteristics

Chlorpromazine is practically insoluble in water, soluble to the degree of 1 part in 2 parts of alcohol, 1 part in less than 1 part of chloroform, and 1 part in 1 part of ether [4].

Chlorpromazine hydrochloride is soluble to the degree of 1 part in 0.4 parts of water, 1 part in 1.3 parts of alcohol, 1 part in 1 part of chloroform, and practically insoluble in ether [4]. It has also been reported to be soluble in methanol, ethanol, and chloroform, and to be practically insoluble in ether and benzene [3].

3.4 Partition Coefficients

The partition coefficient between octanol and pH 7.4 buffer has been reported as log P = 3.4 [8].

3.5 Ionization Constant

The dissociation constant of chlorpromazine has been reported as pKa = 9.3 at a temperature of 20°C [8].

A 5% aqueous solution of chlorpromazine hydrochloride exhibits a pH in the range of 4.0-5.5, and is therefore slightly acidic to litmus [3].

3.6 Spectroscopy

3.6.1 UV/VIS Spectroscopy

UV spectroscopic studies of chlorpromazine hydrochloride were conducted in ethanol and water solutions, and the resulting spectra are shown in Figure 3. The data were obtained between 200 and 345 nm using a Shimadzu 1601 PC UV/VIS spectrophotometer (Figure 3).

In aqueous solution, the spectrum of chlorpromazine hydrochloride (Figure 3A) consists of two maxima. One band is located at 305.3 nm, and is characterized by $A_{1\%:1cm} = 111.3$, and a molar absorptivity of 396.

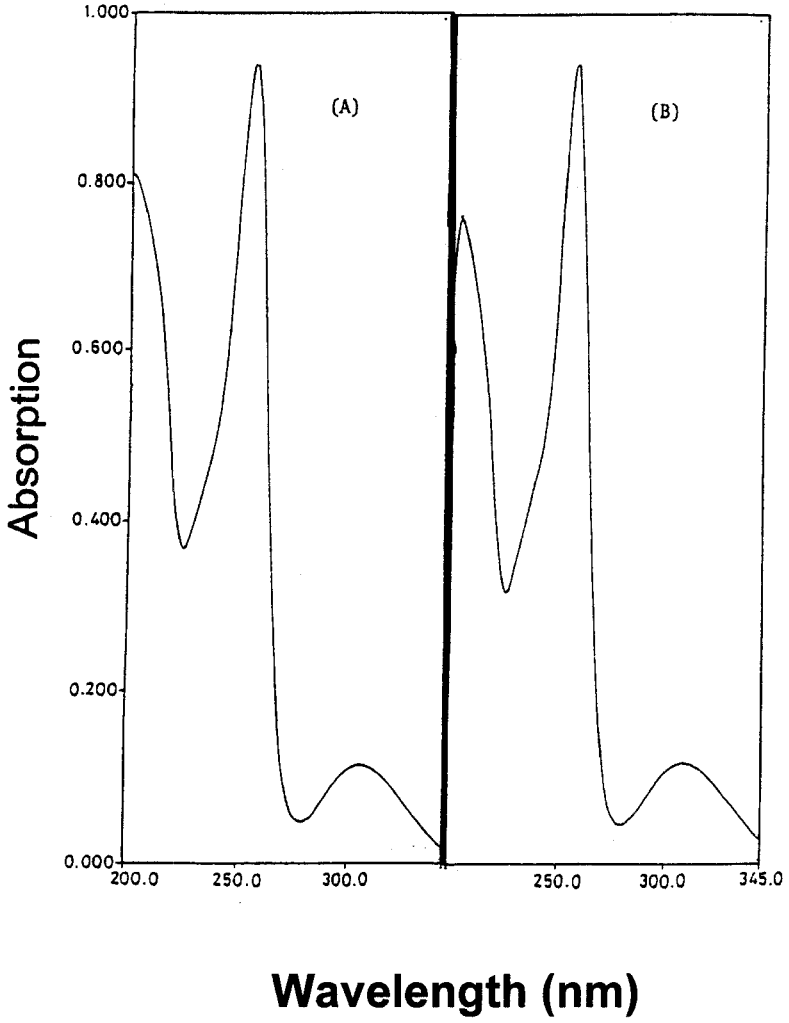


Figure 3. Ultraviolet absorption spectra of chlorpromazine hydrochloride dissolved in (A) water and in (B) ethanol.

The other band is located at 254.3 nm, and is characterized by $A_{1\%:1cm}$ = 897.2, and a molar absorptivity of 3450.

In ethanolic solution, the spectrum of chlorpromazine hydrochloride (Figure 3B) exhibited three absorption bands. These were located at 308.2 nm ($A_{1\%:1cm}$ = 124.7 and **a** = 443), 256.1 nm ($A_{1\%:1cm}$ = 970 and **a** = 3450), and 203.6 nm ($A_{1\%:1cm}$ = 782.3 and **a** = 2800.

The UV spectrum of chlorpromazine hydrochloride has also been obtained in aqueous acid, showed maximum a absorbance at 255 nm (for which $A_{1\%:1cm}$ = 1025) [8].

3.6.2 Vibrational Spectroscopy

Clarke has reported that the principal infrared absorption peaks of chlorpromazine hydrochloride are found at energies of 747, 1240, 1561, 1125, 1095, 1220 cm^{-1} [8].

The IR spectrum of chlorpromazine hydrochloride as a 1% dispersion in a KBr pellet was obtained in this laboratory using a Perkin-Elmer 580 B spectrometer. The spectrum is shown in Figure 4, and structural assignments have been correlated with group frequencies in Table 2 [13].

Table 2

Assignments for the Infrared Absorption Bands of Chlorpromazine Hydrochloride

Frequency (cm^{-1})	**Assignment**
3050-3000	Aromatic C-H stretching modes
2950-2850	Aliphatic C-H stretching modes
2650-2300	H_3C-$^+$NH stretching and combination modes
1585,1560	Phenyl ring vibrational modes
1450	C-H deformational modes
850,800,750,730	Aromatic C-H bending modes

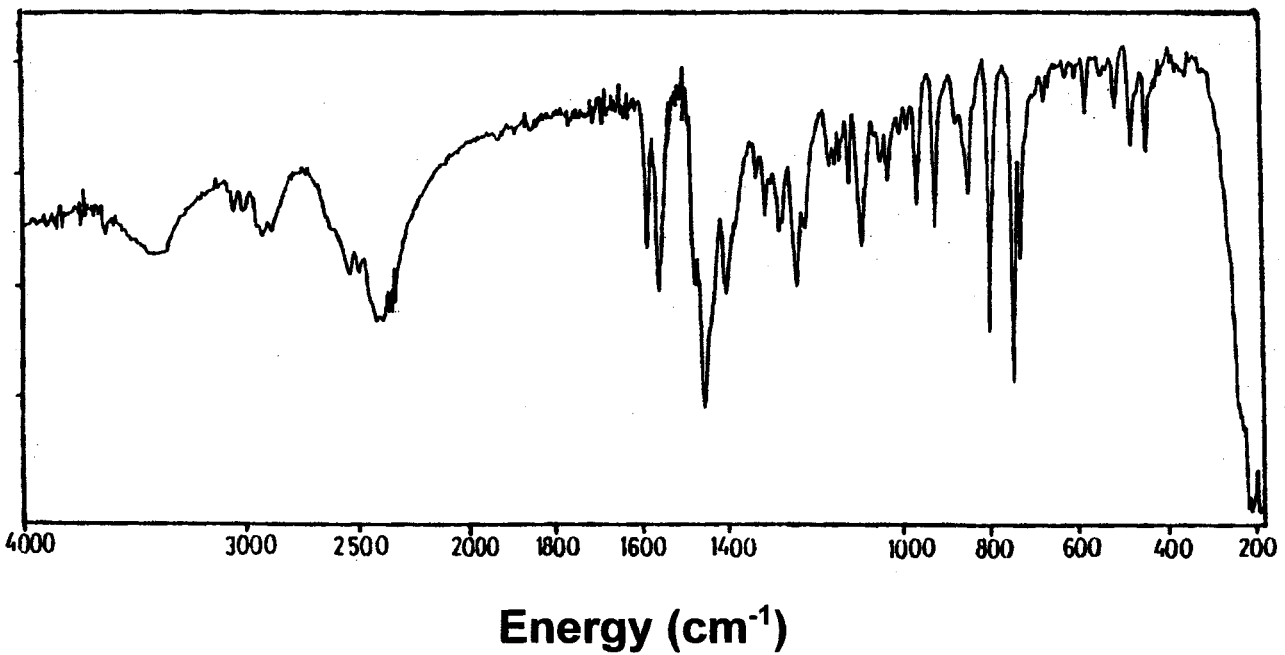


Figure 4. Infrared absorption spectrum of chlorpromazine hydrochloride.

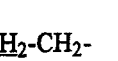
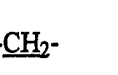
3.6.3 Nuclear Magnetic Resonance Spectrometry

3.6.3.1 ^{1}H-NMR Spectrum

The ^{1}H nuclear magnetic resonance spectrum of chlorpromazine hydrochloride in $CDCl_3$ is shown in Figure 5 [13]. The data were recorded on a Varian XL 200 MHz spectrometer using tetramethylsilane as the internal reference. Assignments for the observed resonance bands are presented in Table 3.

Table 3

Assignments for the ^{1}H-NMR Spectrum of Chlorpromazine Hydrochloride

Chemical Shifts δ [ppm] and (Multiplicity)	Number of Protons	Assignment
2.26 (m)	2	$-CH_2-\underline{CH_2}-CH_2-$
2.67 (s)	3	$-\underline{CH_3}$
2.70 (s)	3	$-\underline{CH_3}$
3.15 (m)	2	
4.00 (broad m)	2	
6.85-7.16 (m)	7	Aromatic
11.8 (broad s)	1	-NH

m = multiplet, s = singlet.

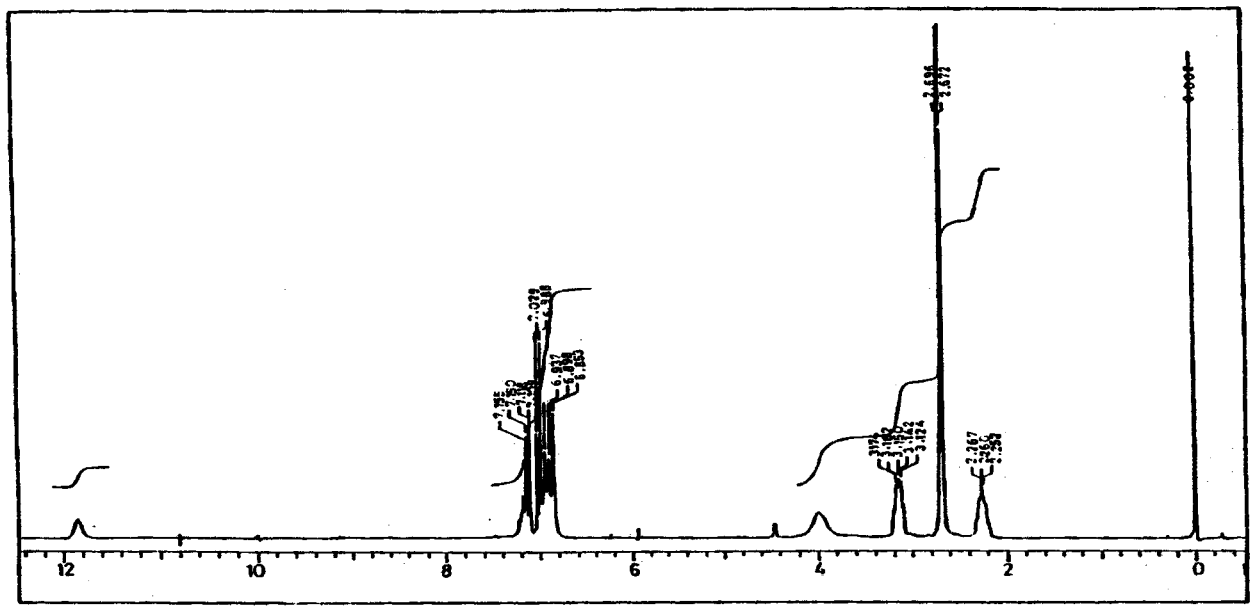


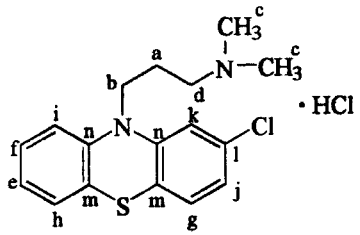
Figure 5. ^{1}H nuclear magnetic resonance spectrum of chlorpromazine hydrochloride.

3.6.3.2 ^{13}C-NMR Spectrum

The ^{13}C nuclear magnetic resonance spectrum of chlorpromazine hydrochloride in D_2O is shown in Figure 6 [13]. The data were recorded on a JEOL model Ex 400 MHz spectrometer using tetramethylsilane as the internal reference. Assignments for the observed resonance bands are presented in Table 4 together with an atom numbering system.

Table 4

Assignments for the ^{13}C-NMR Spectrum of Chlorpromazine Hydrochloride



Chemical shift δ (ppm)	Assignment
23.49	a
44.49	b
45.59	c
56.76	d
117.90	e
118.54	f
125.23	g
126.27	h
129.42	i
130.06	j
134.98	k
135.08	l
145.41	m
148.19	n

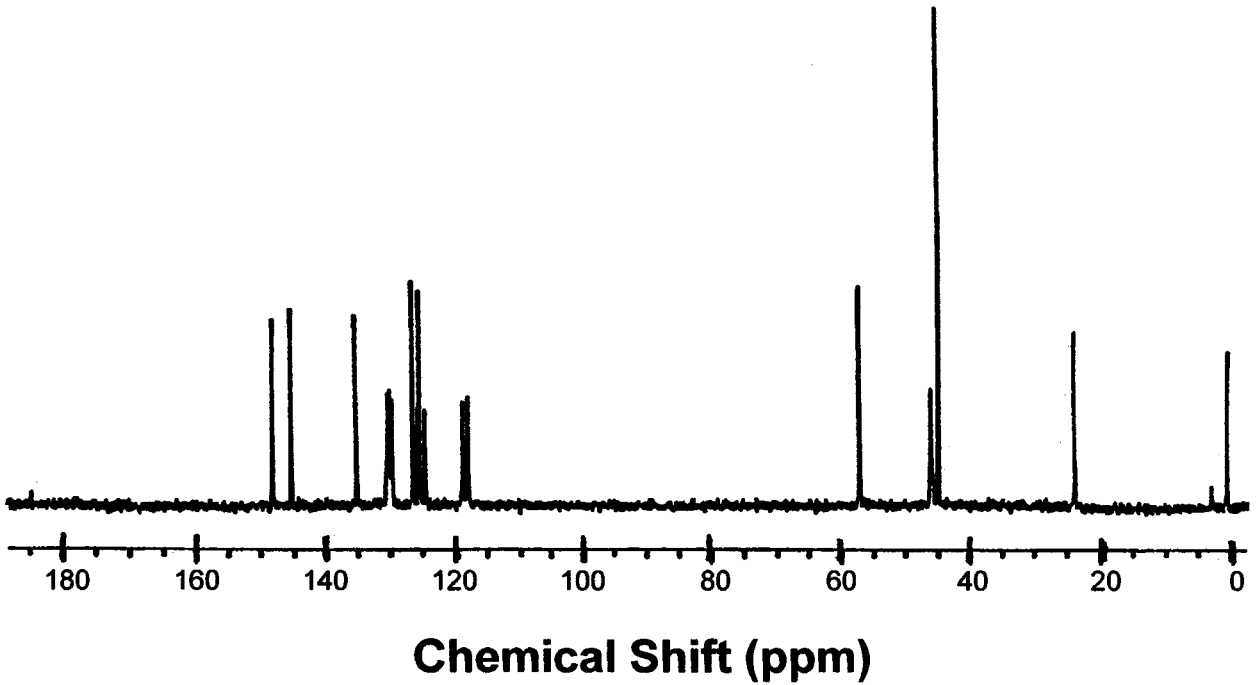


Figure 6. ^{13}C nuclear magnetic resonance spectrum of chlorpromazine hydrochloride.

3.6.4 Mass Spectrometry

The mass spectrum of chlorpromazine hydrochloride was obtained using a Shimadzu PQ-5000 mass spectrometer [13]. In this study, the parent ion was collided with helium as the carrier gas, and an ionization beam energy of 70 eV was used. The mass spectrum thusly obtained is presented in Figure 7. The molecular ion peak was observed at m/z = 318.2, and the base peak was at m/z = 58.2. Assignments for the fragmentation pattern, the peak relative intensities, and the proposed structures are found in Table 5.

Clarke has reported that the principle MS peaks are found at m/z values of 58, 86, 318, 85, 320, 272, 319, 273 [8].

4. Methods of Analysis

4.1 Identification

4.1.1 Chlorpromazine gives a red to violet color with formaldehyde / sulfuric acid, a red color with Forest's reagent, a red color with FPN reagent, a red to brown color with Lieberman's reagent, a green to violet color with Mandelin's reagent, and a violet color with Marquis' reagent [8].

4.1.2 An aqueous solution of chlorpromazine (0.1 mg/mL) gives a pink color when added to 5 mL of 1% $(NH_4)_2S_2O_8$ solution. Upon addition of 1 mL of 10% NaOH and extraction with ether (3 x 5mL), the organic phase is colorless. After evaporation of the organic phase to dryness on a steam bath, a white residue is obtained. If this residue is dissolved in 3 mL of concentrated HCl, and 5 mL of H_2O is added, a violet color is developed after 30 minutes[30].

4.1.3 In the presence of 0.1 M potassium hydrogen phthalate buffer (adjusted to pH = 4.5), $CHCl_3$, 0.05 N NaOH for extraction, and 0.04% methanolic bromphenol blue for color production, chlorpromazine gives a blue color [31].

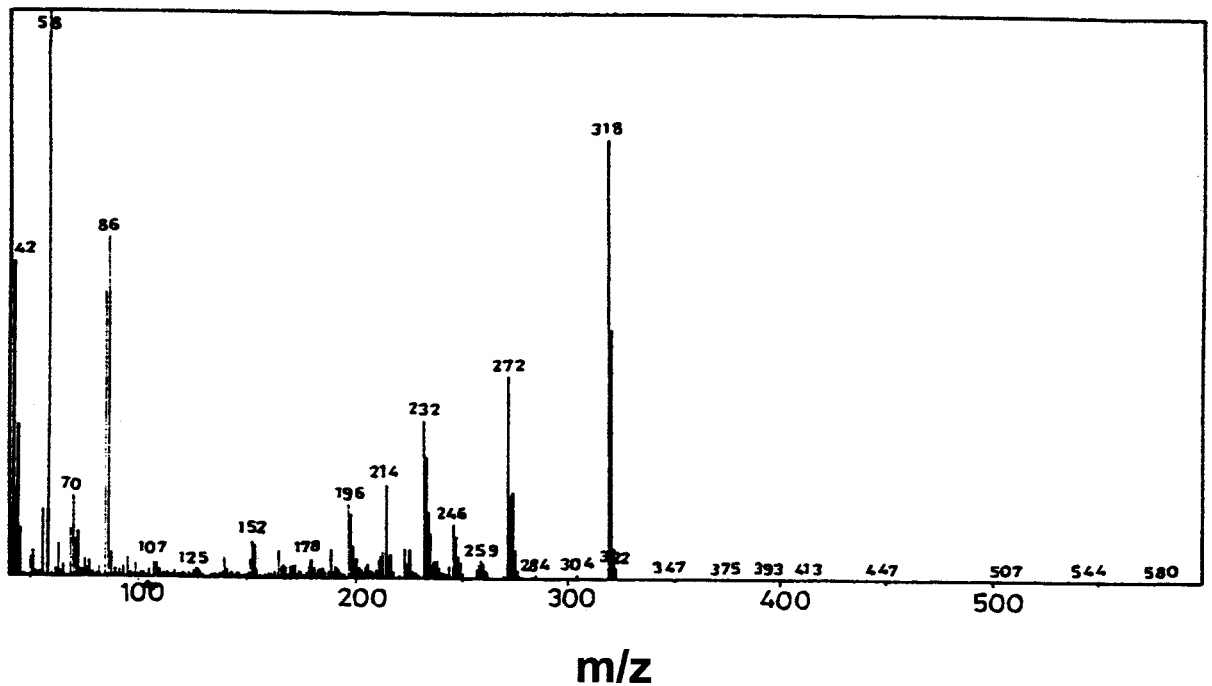
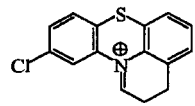
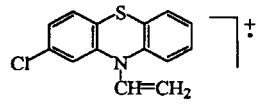
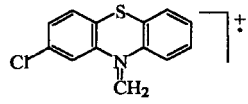
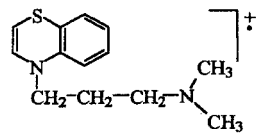
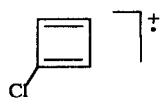
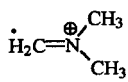
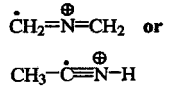


Figure 7. Mass spectrum of chlorpromazine hydrochloride.

Table 5

Assignments for the Prominent Fragments Observed in the Mass Spectrum of Chlorpromazine Hydrochloride

m/e	Relative intensity %	Fragment
318	75%	$M^{+\bullet}$
272	30%	
259	5%	
246	12%	
232	20%	
86	60%	
58	100%	
42	52%	 or

4.1.4 Chlorpromazine and other phenothiazines can be identified by their reactions with benzene-, toluene-*o*-, and toluene *p*-stilbinic acids, and with *p*-hydroxy-, *m*-nitro-, *m*-amino-, and *p*-nitrobenzene stilbinic acids in an HCl medium. The reaction products are initially colorless masses but rapidly oxidize to yield colored products [32].

4.1.5 A chloroform solution of chlorpromazine can be subjected to chromatography on Whatman No. 1 paper, using a mobile phase consisting of 1:1 5% aqueous $(NH_4)_2SO_4$ solution and isobutyl alcohol. When H_2PtCl_6 – 0.1 N iodine solution is used as the detecting agent, a blue-violet spot at Rf = 0.44 indicates the identification of chlorpromazine [33].

4.1.6 Treatment of chlorpromazine hydrochloride solution in H_2O or in 0.5 N H_2SO_4 with concentrated H_2SO_4 (cooling in ice-cold water during the reaction), will give a pink color if the mixture is subsequently heated on a boiling water-bath and then re-cooled [34].

4.1.7 Many authors have described the identification of chlorpromazine using a thin-layer chromatographic separation, and followed by detection of the analyte spot with different reagents [35-53]. A summary of these methods is provided in Table 6.

4.2 Elemental Analysis [3]

	Chlorpromazine	Chlorpromazine hydrochloride
carbon	64.03%	57.47%
hydrogen	6.01%	5.67%
chlorine	11.12%	19.95%
nitrogen	8.79%	7.88%
sulfur	10.06%	9.02

Table 6: A summary of TLC methods adopted for the identification of chlorpromazine.

Plate	Mobile phase	Detection	Ref.
Kieselgel G	Butanol-acetic acid-water (88:5:7 or 13:3:4).	H_2SO_4 and U.V. light examination	35
Silica gel G	Butanol-conc. HCl (5:1) saturated with H_2O.	U.V. light. R_f value of chloropromazine is 0.7 to 0.72	36
Silica gel G activated at 110°C for 45 minutes.	Ethyl ether-acetone-diethylamine (90:19:1). Benzene-dioxan-diethylamine (400:95:6).	Dil. H_3PO_4 containing 2 ml of 0.3% $KClO_3$ solution per 100 ml. Iodoplatinate reagent followed by dil. H_2SO_4, followed by iodoplatinate.	37
Kieselgel G/UV_{254} mixed with 0.1N Na_2CO_3 (0.25 mm thick) activated at 105°C	Methanol-acetone (3:22). H_2O-ethanol (1:24). Isopropylalcohol-isopropyl ether (163:837). Methanol-methyl acetate (177:823). Methanol-methyl acetate-cyclohexane (89:93:168).	Dragendorff and UV visualisation	38
Kieselgel G activated at 110°C for 30 minutes	Cyclohexane-benzene-diethylamine (15:3:2)	Bromine vapours	39
Kieselgel GF_{254} activated at 120°C for 90 minutes	0.3% solution of iodine in dry $CHCl_3$.	I_2 (brown spot)	40

Table 6: A summary of TLC methods adopted for the identification of chlorpromazine. (Continued) ...

Kieselgel G (0.5 mm)	2-D development methanol followed by methanol-N- HCl (1:1)	Dragendorff and iodine	41
—	—	Fe^{3+}-1,10-phenanthroline. Fe^{3+}-2,2'-bipyridyl. Cu^{2+}-2,2-biquinolyl. Cu^{2+} - Na 2,2'-bicinchoninate.	42
Silica gel G	Methanol-aq. 25% ammonia (200:3)	60% $HClO_4$-nitromethane (1:1).	43
Silica gel prewashed with toluene-acetone (1:1)	Toluene-acetone-aq. ammonia (125:125:6).	365 nm.	44
Silica gel 60 F_{254} preactivated at 110°C.	Acetone-methanol-aq. NH_3 (70:30:1). Acetone-aq. ammonia (50:1). Ammonium acetate-water-methanol (3:20:100).	Diazotized sulfanilic acid	45
Silica gel 60 F_{254}	Chloroform-methanol-benzene-aq. ammonia (90:5:5:1).	$BiCl_3$ reagent	46
Silica gel GF_{254}	Chloroform-methanol-benzene-aqueous 25% NH_3 (90:5:5:1). Dichloromethane-propan-2-ol-H_2O (6:3:1); butanol-1N ammonia (5:1). chloroform-acetone-aq. 25% ammonia (50:50:1).	Five reagents	47

Table 6: A summary of TLC methods adopted for the identification of chlorpromazine. (Continued) ...

Silica gel sintered TLC plates pretreated with $K_2Cr_2O_7$ and activated at 100°C for 2 hours.	—	4-dimethylamino-benzaldehyde	48
Silica gel GF_{254}	Cyclohexane-acetone-diethylamine (8:1:1).	254 nm.	49
—	2-D development with acetone-CH_2Cl_2-aq. 25% NH_3 (40:60:1) followed by acetone.	Dragendorff reagent	50
Silica gel 60 F_{254}, alumina 60 F_{254} E; silanized silica gel GF_{254}	Cyclohexane-benzene-diethylamine. Cyclohexane-acetone-diethylamine. methanol-aqueous 25% ammonia for silica gel. Benzene-acetone for alumina. Acetone-water-aq. 25% NH_3 for silanized silica gel.	—	51
PHTLC Si 60	—	254 nm.	52
Silica gel G	Cyclohexane-acetone diethylamine (3:1:1).	253 nm.	53

4.3 Titrimetric Analysis

The British Pharmacopoeia has adopted the assay of chlorpromazine in acetone, using a non-aqueous titration with perchloric acid and methyl orange as the indicator [4]. According to the described procedure, each mL of 0.1 M perchloric acid is equivalent to 0.03189 g of drug substance. For chlorpromazine hydrochloride, the BP has described its titration with NaOH in a 1:10 solution of 0.01 M HCl / ethanol. Determining the end point potentiometrically, each mL of 0.1 M sodium hydroxide is equivalent to 0.03558 g of substance.

The United States Pharmacopoeia has used a method similar to that of the BP for chlorpromazine, but which uses crystal violet as the indicator [7]. For chlorpromazine hydrochloride, the USP has described its titration with 0.1 M perchloric acid in glacial acetic acid (containing Hg(II) acetate), and a potentiometric determination of the end point.

Non-aqueous titration methods have been described for the rapid determination of chlorpromazine and its hydrochloride salt by titration with perchloric acid [54]. The titration is performed after the proscribed extraction from suppository, tablet, and ampoule dosage forms is completed.

The oxidative titration of chlorpromazine with ceric sulfate or $KBrO_3$-KBr in acid solution has been described, with the end point being determined by a dead-stop end point technique [55]. A similar method involving visual or potentiometric detection of the end point was also reported [56].

Chlorpromazine hydrochloride was assayed in neutral solvents such as acetone or acetonitrile with a glacial acetic acid solution of perchloric acid. The combination of methyl violet and bromcresol green served as a mixed indicator [57].

A precipitation titration method has been reported where chlorpromazine hydrochloride is precipitated from solutions using excess standard CdI_2 in the presence of KI or HI, and the excess CdI_2 in the filtrate is then determined complexometrically using xylenol orange as the indicator [58].

The determination of chlorpromazine and other phenothiazines with aqueous NaOH solution in a dimethylformamide medium has been successfully applied to their powder dosage forms [59]. The amperometric determination of chlorpromazine with $Ce(SO_4)_2$ titrant was also described [60]. The drug was also determined by the titration with sodium lauryl sulfate in an imiscible phase [61].

Blazek determined chlorpromazine in H_2O and H_2SO_4 media by titration with 0.05N $Ce(SO_4)_2$ [62]. The endpoint consisted of the complete color discharge that appeared during the nitration step. When determining the drug in tablets or drops, the use of ferroin indicator was recommended. A blank titration must be performed to achieve proper results.

Chlorpromazine was determined by diluting 5 mL of a 0.01M solution with 25 mL of H_2O and 20 mL of ethanol, and titrating to a conductimetric endpoint with 0.05N acid or 0.05 N NaOH [63]. Chlorpromazine was determined by titration with $KBrO_3$, $NaNO_3$, or $Ce(SO_4)_2$, the determination being made with the use of a molybdenum anode and a graphite, platinum, or tungsten cathode [64].

The chloride ion in the hydrochloride anion of chlorpromazine can be obtained with high precision using potentiometric titration with Ag(I) [65]. 05 M $Ce(SO_4)_2$ was used for the determination of chlorpromazine in dilute H_2SO_4 solution to a colorless end-point [66].

Chlorpromazine was determined by precipitation with an excess of 0.2% ammonium reineckate solution, and titration of the unconsumed reagent bromatometrically after alkaline hydrolysis [67]. Excess hexathiocyanato-chromate was used as a precipitating reagent for chlorpromazine, whereupon the unconsumed reagent was determined with $KBrO_3$ in the filtrate [68].

Chlorpromazine hydrochloride in 25-mg tablets has been was determined according to the following scheme [69]. 10 tablets are dissolved in H_2O, 5 mL of HNO_3 is added, and the solution set aside until a yellow color develops. At that point, one adds 25 mL of 0.1N $AgNO_3$, and lets the solution stand for 15 minutes. The solution is then diluted to 100 mL and filtered. To 50 mL of the filtrate is added 1 mL of 10% aqueous $Cu(NO_3)_2$

and 1 mL of starch solution, and then the mixture titrated with 0.1N KI solution [69].

Chlorpromazine formed an insoluble 1:1 complex with lead picrate, and 5:3 complexes with the picrates of cadmium, copper, and zinc [70]. The sample (0.1 g) was dissolved in 15 mL of 95% ethanol, and the solution adjusted to pH 9 with 0.1 N NaOH. After adding 25 mL of a 0.02 M picrate reagent (30 mL of Pb), the solution was set aside for 2 hours. The precipitate was collected on a sintered glass funnel, and the unconsumed metal in the filtrate was titrated directly with 0.02M EDTA at pH 10.4 (after adding 0.5 g of potassium sodium tartrate for Pb). Eriochrome black T was used as the indicator.

A 200 μL sample of chlorpromazine hydrochloride injection solution, or of a chloroform extract of powdered tablets, was applied to a layer of Kieselgel GF254 [71]. Development was carried out in the dark using a mobile phase of 80:20:1 benzene-ethanol-concentrated ammonia. The zone containing chlorpromazine (at Rf 0.66) was scraped from the plate, whereupon 30 mL of water, 30 mL of $CHCl_3$, and dimethyl yellow solution (the indicator) were added to the mixture. This solution was titrated with sodium dodecyl sulfate solution (accompanied by vigorous shaking) until the color of the chloroform layer changed from yellow to orange. A similar method, but without chromatographic separation, was also described [72].

A 300 mg sample of chlorpromazine hydrochloride can be determined by titration with 0.1N $HClO_4$ in anhydrous acetic acid medium, in the presence of 5% Hg(II) acetate (10 mL) and ascorbic acid (1 g) using crystal violet as the indicator [73]. Ascorbic acid prevents the formation of a red color which would otherwise interfere.

Chlorpromazine hydrochloride was determined by titration of 15-50 mg samples with 0.02M $NaVO_3$ in the presence of H_3PO_3 and H_2SO_4 at pH 2 [74]. The end point was shown by decolorization of the drug solution, or by potentiometric means.

Direct titration of chlorpromazine hydrochloride with $HClO_4$ in acetic anhydride (with naphtholbenzein as the indicator) was described [75]. A test solution containing about 1 mg/mL of chlorpromazine in 10% HCl

was treated with a known excess amount of 5 mM 1,3-dibromo-5,5-diphenylhydantoin, or with 0.01M N-bromosuccinimide. The mixture was shaken occasionally, and after 30 minutes the unconsumed reagent was determined iodometrically [76].

A mixture consisting of 25 mL of an aqueous (0.2 to 1.0 mM) solution of chlorpromazine hydrochloride and 5 mL of 0.1M acetate buffer (pH 3.3) was titrated to a potentiometric endpoint with 0.01M sodium tetraphenylborate [77]. The titrant was added at a rate of 0.36 mL/min with continuous stirring, and the temperature of the medium was maintained at 22 ± 2°C. The end point was detected by a tetraphenylborate-selective electrode.

The drug substance has been determined by direct potentiometric titration using chlorpromazine-sensitive electrodes [78}. These consisted of an electroactive material in the membrane being either the chlorpromazine complex with either tetraphenylborate or dinonyinaphthalene sulfonate set in a PVC matrix. 2-nitrophenyl octyl ether was used as the plasticizer.

Zakhari *et al.* have described the titration of chlorpromazine hydrochloride (in anhydrous acetic acid-acetic anhydride) with 0.1M-trifluoromethane sulfonic acid or with 0.1 M $HClO_4$ [79]. The titrations were conducted in the presence or absence of Hg(II) acetate, with the end point being detected either visually (using crystal violet as the indicator) or potentiometrically.

Another method adopted the addition of 2% bismuth oxyacetate in anhydrous acetic acid to a chlorpromazine hydrochloride solution in anhydrous acetic acid (with stirring) until any precipitate re-dissolved. This was followed by titration with 0.1N trifluoromethyl sulfonic acid, and the end-point was detected with the use of a glass Ag/AgCl combination electrode [80].

Yao *et al.* have described an oscillographic titration of chlorpromazine hydrochloride in a non-aqueous medium [81].

4.4 Electrochemical Analysis

4.4.1 Polarographic Methods

A 20-50 mg sample of chlorpromazine was polarographically titrated with 0.01M tungstosilicic acid in 3.5% HCl [146]. Alternatively, a solution or extract of the analyte in 0.5N HCl (25 mL, containing 25-200 μg of chlorpromazine) can be shaken with 2 drops of saturated aqueous bromine solution, set aside for 1 minute, flushed with nitrogen, and examined with a cathode ray polarograph [147].

Chlorpromazine was quantitatively oxidized to either a free radical by 12N H_2SO_4, or to a sulfoxide by 1N H_2SO_4 [148]. Electro-reduction of the free-radicals occurred at -0.25 V *vs.* S.C.E. on platinum wire.

Chlorpromazine sulfoxide can be determined in the presence of chlorpromazine using cathodic polargraphy [149]. Since the bromination products of these compounds are indistinguishable, the total chlorpromazine and sulfoxide content in a sample can be determined by reductive polarography after bromination.

Bulk chlorpromazine can be dissolved in 15 mL of H_2O (or injectable solutions diluted to 15 mL), to which is added 10 mL of 2.5% aqueous acetic acid. This solution is titrated with 0.05M sodium tetraphenylborate reagent at a constant potential of 0.12V (dropping mercury electrode and external S.C.E.), and the polarogram recorded [150].

Bhatt *et al.* have described a method based on the complex formation between chlorpromazine and $K_3Fe(CN)_6$ [151]. The latter substance yielded a reduction wave at zero applied potential, and addition of chlorpromazine decreased the wave height in an amount directly proportional to the amount added. Optimum conditions for the determination were reported to be pH 7.4-6.2, use of 0.1M KCl as the supporting electrolyte, and 0.001% methyl red solution as the maximum suppressor. In this system, chlorpromazine can be determined up to concentrations of 1.4 μg/mL.

Chlorpromazine can be oxidized by aqueous bromine solutions, and after removal of any unconsumed bromine by sparging the solution with nitrogen, the sulfoxide oxidation product is determined by polarography

[152]. In this method, the peak current was found to be directly proportional to the drug concentration over the range of 1-20 mg/dL at a half-wave potential of –0.8 V *vs.* S.C.E. A derivative polarogram was reported for chlorpromazine.

Powdered chlorpromazine hydrochloride (50 mg) was dissolved in 50 mL of water, a portion of the solution treated with 0.5N KCl and 0.2% gelatin solution, and diluted. Nitrogen was passed through the solution before polarography was performed out at 20°C. The polarographic scan initiated at –1.2V, and used an internal calomel compression electrode and a dropping mercury electrode. The method was used for the determination of chlorpromazine in injectable solutions and tablets [153].

4.4.2 Voltammetric Methods

Jarbawi *et al.* have described a thin-layer differential pulse voltammetric method for the determination of chlorpromazine in biological fluids [154].

The use of hydrodynamic modulation voltammetry was reported [155] and modified [156]. A simple treatment of the data transforms the voltammetric current-voltage waves into peak shaped curves, which gave improved resolution in the analysis of mixtures.

Jarbawi and Heineman have used differential pulse voltammetry and chronocoulometry to study the effect of immersing a wax-impregnated graphite electrode in an aqueous solution of chlorpromazine [157]. Extraction of substance into the electrode and absorption at the interface were both found to occur.

Chlorpromazine was pre-concentrated by its adsorption onto a disc working electrode, and voltammetry was conducted using a graphite-liquid paraffin paste with a Ag/AgCl reference electrode and a graphite counter electrode [158]. Chlorpromazine in aqueous media, background and analytical stripping voltammograms, were recorded by scanning from 0 to 1 V at 5 mV/sec, followed by cleaning at +0.8V.

Wang *et al.* have reported a catheter microelectrode assembly for *in vivo* and *in vitro* voltammetric analyses of chlorpromazine in biological fluids [159].

Wang and Dewald have investigated the possibility of using rapid scan differential pulse voltammetry for the detection of chlorpromazine in flow-injection analysis [160]. The measurement was made with the use of a flow-cell equipped with a carbon paste or a vitreous-carbon disc electrode, a carbon rod auxiliary electrode, and a silver-silver chloride reference electrode. Potential scanning was effected at 2 V/min.

Compensation for background currents due to evolution of hydrogen, reduction of oxygen, solvent oxidation, or surface processes, was made by recording the voltammogram while the sample and carrier solution followed through the cell. The difference between these was taken as the net response for the sample [161].

Bishop and Hussein have reported an anodic voltammetric method for the determination of chlorpromazine at gold and platinum electrodes [162]. Solution (freed from chloride ion by passage through washed amberlite IRA, S042 form and eluted with water) was deaerated with nitrogen and examined by anodic voltammetry using high-precision platinum or gold rotating-disc electrodes (scan speed 5 mV/s). The use of clean discs was reported to be essential, but under those conditions the drug gave three well defined and reproducible anodic waves.

Wang and Freiha have designed a carbon-paste electrode, based on a piston principle that enables an outer layer of paste to be extruded and removed, and used this in investigating possible extractive accumulation and further characteristics of the pre-concentration step in voltammetric analysis [163]. Chlorpromazine was used as a model compound.

Takamura *et al.* have reported an electrochemical method for the determination of chlorpromazine with an anodically pretreated vitreous carbon electrode [164]. Optimal conditions for the pre-treatment were attained by the anodic oxidation of vitreous carbon electrodes in 0.5 mM phosphate buffer (pH 6.7) at 1.6 V *vs.* S.C.E. for 2 minutes. This was found to enhance the oxidation peak of the cyclic voltammogram for chlorpromazine by a factor of simeq 30. The peak current at +0.75 V was directly proportional to the concentration of chlorpromazine over the range of 0.2-40 μM and the detection limit was 0.1 μM.

Wang *et al.* have studied the extractive and/or adsorptive behavior of chlorpromazine at the carbon paste electrode [165]. The method used a voltammetric cell equipped with a carbon paste working electrode, a platinum wire auxiliary electrode, and a silver-silver chloride reference electrode. 0.05M phosphate buffer (pH 7.4) was used as the supporting electrolyte. Pre-concentration was carried out at 0.0 V or, for experiment involving medium exchange before stripping, on open circuit. Differential pulse and cyclic voltammograms were recorded with scanning at 5 and 50 mV/sec, respectively.

Ebel *et al.* have used a microliter vessel in the voltammetry and polarographic determination of small sample volumes of chlorpromazine [166]. The concentration of cells in glass or PTFE was described for use with a dropping-mercury electrode (sample volume 180 μL), a rotating disc electrode (sample volume 1 mL), or a stationary vitreous-carbon electrode (sample volume 80 μL). Chlorpromazine was determined using oxidative voltammetry at a 3 mm vitreous-carbon or a rotating electrode.

Takamura *et al.* have reported a voltammetric method for the determination of chlorpromazine using an anodically oxidized carbon electrode [167]. A vitreous-carbon electrode was maintained at +1.6 V *vs.* S.C.E. for 2 minutes (in 0.5 M phosphate buffer at pH 6.8). Under these conditions, chlorpromazine gave an oxidation peak current on cyclic voltammograms that varied linearly with concentration over the range of 50 nM to 1 μM.

Zimova *et al.* have determined chlorpromazine by differential pulse voltammetry in an acetonitrile medium [168]. The method involves oxidation of the derivative to the radical cation, with the reaction taking place in acetonitrile that is also 0.03M in perchloric acid. Maximum sensitivity was achieved with a scan rate of 2 mV/sec, a modulation amplitude of 50 mV, and a clock time of 40 seconds.

Song *et al.* have reported the determination of chlorpromazine by adsorptive stripping voltammetry [169]. Phosphate buffer saline solution (pH 7.4), consisting of 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl, 8 g NaCl, and 0.047 g $MgCl_2$, was used as the supporting electrolyte. Two methods were reported for the determination of chlorpromazine:

Method 1 - Anodic differential pulse voltammetry, involving voltage scanning from +0.3 to +1.0 V and recording the peak current, was found to yield an instrumental response proportional to the concentration of chlorpromazine in the range 9.6 to 340 μM.

Method 2 -An adsorptive stripping method also involving pulse voltammetry, which yielded a peak current that was proportional to the concentration of chlorpromazine in the range of 83 to 2000 nM.

Takamura *et al.* have determined chlorpromazine by the use of differential pulse voltammetry incorporating rotating glassy-carbon disc electrodes [170]. The determination was carried out after pre-treatment of vitreous carbon by anodic oxidation for two minutes in 0.5 M phosphate buffer (pH 6.3) at 1.6 V *vs.* Ag/AgCl reference electrode. The determination was made with the use of 50 mV pulses at 2 seconds intervals, a rotation rate of 2500 rpm, and a scan rate of 5 mV/sec.

The effectiveness of glassy-carbon electrodes coated with cellulose acetate for adsorptive stripping voltammetry was evaluated to alleviate interference from co-adsorbed species [171]. The carbon electrode was coated using a 5% cellulose acetate solution, with the coating being hydrolyzed with 0.07M potassium hydroxide. Repetitive measurements of 1 μM chlorpromazine in the presence of 20 mg/L albumin resulted in peak currents with residual standards of deviation equal to 1.4 and 1.3%, respectively.

Farsang *et al.* have reported a comparative study of a thin layer cell for the analysis of stationary and streaming solutions [172]. The analytical parameters were compared with laboratory-built Kissinger-type twin electrode thin layer cells, either filled with stationary solution or used as a flow through detector. In both modes, the cell was polarized by using a slow linear potential sweep, and the current voltage curves exhibited a limiting current region. Sensitivities and detection limits for chlorpromazine was similar for both modes.

The electrochemical behavior of chlorpromazine hydrochloride in 0.2M H_2SO_4 was studied by cyclic and linear sweep voltammetry at an oxidized and a non-oxidized ruthenium wire electrode [173]. Preparation of a stable and permanent coating of RuO_2 on the electrode was very time-consuming, but the resulting curves were highly reproducible. The

oxidized electrode was not suitable for determination of chlorpromazine. Calibration graphs were linear from 0.1 to 0.8 mM chlorpromazine, with the use of the non-oxidized electrode and a scan rate of 10 mV/sec.

A twin electrode thin layer Kissinger cell was designed in which the channel volume could be varied through the use of PTFE spacers [174]. The working and counter electrodes were carbon paste (3.14 m^2) and the reference electrode was Ag/AgCl. The performance of the cell was tested on 50 μL portions of chlorpromazine solutions in 0.01 M HCl, and the three cited methods were compared. Linear sweep voltammetry was found to be the simplest to apply and showed moderate sensitivity.

For the simultaneous determination of chlorpromazine and other phenothiazines, drug tablets were dissolved and diluted to 50 mL with ethanol. The supernatant solution was analyzed by stripping voltammetry, at a carbon paste electrode. The method used an electrolytic cell equipped with a carbon paste piston electrode containing 40% Nujol as the working electrode, a Ag/AgCl reference electrode, and a platinum wire auxiliary electrode. The stripping curve was recorded by differential pulse voltammetry from 0 to +1.0 V, at a scanning rate of 10 mV/sec, and an amplitude of 100 mV. The stripping peak for the drug was observed at +0.72 V. The method was applied to the determination of chlorpromazine in pharmaceutical and biological fluids [175].

Lu *et al.* have described a single sweep voltammetric method for the determination of chlorpromazine at carbon paste electrodes [176]. Powdered tablet was dissolved in and diluted to 100 mL with water, whereupon a 10 mL portion of the solution was mixed with 10 mL of acetate buffer solution (pH 5.3). This solution was then diluted to 100 mL. The solution was analyzed using an electrolytic cell equipped with a carbon paste working electrode, a reference saturated calomel electrode, and a platinum counter-electrode. The voltammogram was recorded by single sweep scanning from 0 to 1 V, with a scanning rate of 10 mV/sec.

Wang *et al.* have detected chlorpromazine and other phenothiazine drugs in Langmuir-Blodgett films of tyrosine hydroxylase [177]. The drug was pre-concentrated *in situ* by a receptor binding process, and determined (and removed) by stripping voltammetry. The technique was illustrated through the determination of phenothiazines using of multiply compressed

films applied to a polished gold electrode. Differential-pulse voltammetry was carried out in a cell equipped with a Ag/AgCl reference electrode and a platinum wire auxiliary electrode, using 0.1 M phosphate buffer (pH 7.4) as the supporting electrolyte.

4.5 Spectrophotometric Methods of Analysis

4.5.1 UV/VIS Absorption Methods

The quantitative determination of chlorpromazine can be performed on the basis of its UV absorption in dilute H_2SO_4 medium [82]. Chlorpromazine exhibits an E(1%, 1-cm) of 1520 at 278 nm. Chlorpromazine and its sulfoxide were assayed in the injectable dosage form using absorbance values obtained at 240 and 255 nm, respectively [83].

Chlorpromazine hydrochloride in injectable solutions can be measured spectrophotometrically upon formation of the charge-transfer complex with iodine [84]. The complex is formed in an aqueous medium in the presence of excess I_2, and is extracted into chloroform for a spectrophotometric determination at 292 and 362 nm. This method is unaffected by exposure to direct light.

Tablets containing chlorpromazine and promethazine were powdered and dissolved in 95% ethanol. The absorbance of a 6 μg/mL solution was measured at 6 wavelengths, and the amount of each component calculated by a least squares method [85]. A spectrophotometric method for the simultaneous determination of 1-18 mg of chlorpromazine hydrochloride and 3-27 mg of promethazine hydrochloride (in 20 mM HCl) was presented, where the absorbance was measured over a spectral region of 275-240 nm [86].

In injectable dosage forms, chlorpromazine was diluted with 0.1M HCl to a concentration of 0.05 mg/mL, and the absorbance measured at 306 nm [87]. The recovery was found to be 99.37%, and the method proved to be linear in the range of 20-80 μg/mL. There was no interference from ascorbic acid or other stabilizers.

Ultraviolet spectral characteristics, such as wavelengths of maxima and minima, were used to identify and distinguish chlorpromazine and some other phenothiazines in biological materials [88].

First-derivative spectrophotometry was used to identify chlorpromazine in the presence of other phenothiazines, while second derivative spectrophotometry enabled the direct determination of chlorpromazine in spiked blood samples [89]. In addition, third derivative spectroscopy was used to determine chlorpromazine and its sulfoxide decomposition product in pharmaceutical dosage forms [90].

Dual-wavelength spectrophotometric assay was used for the determination of chlorpromazine hydrochloride and promethazine hydrochloride in chlorpromazine hydrochloride composite tablets [91]. Stable regression analysis was used in the simultaneous spectrophotometric determination of chlorpromazine hydrochloride and promethazine hydrochloride in tablets without preliminary separation [92].

4.5.2 Colorimetric Methods

Many colorimetric methods have been reported for the determination of chlorpromazine or chlorpromazine hydrochloride [93-133]. The following summarizes the main colorigenic reagent and the wavelength of maximum absorbance of the colored product:

Pb(II) at 500 nm [93]

Bi(III) at 488 nm [94]

KIO_3 at 525 nm [95]

$KBrO_3$ at 525 nm [96]

Bi(III), Sb(III), Cd(II), Hg(II) at 460 nm [97]

Titan yellow at 405 nm [98]

Co(II) + SCN^- at 620 nm [99]

$K_2Cr_2O_7$ at 400 nm [100]

$FeCl_3$ at 525 nm [101]

Fe(III) + 2,2'-bipyridine at 525 nm [102]

$SbCl_3$ at 438 nm [103]

$\{Cr(SCN)_6\}^{3+}$ at 540 nm [104]

Tungstosilicic acid at 540 nm [105]

4-dimethylaminobenzaldehyde at 520 nm [106]

Molybdoarsenic acid at 540 nm [107]

Qlizarin red S at 428 nm [108]

Solochrome black T, solochrome dark blue at 520 nm [109]

W(VI)-catechol violet at 650 nm [110]

o-Hydroxy-quinolphthalein at 525 nm [111]

Vanadoboric acid at 495 nm [112]

Tungstophosphonic acid at 540 nm [113]

Eosin at 540 nm [114, 120]

Sodium cobaltinitrite at 520 nm [115]

Amaranth at 520 nm [116]

$FeCl_3$ at 488 nm and at 525 nm [117, 101]

5M H_2SO_4 at 490 nm [118]

2,3-dichloro-5,6-dicyano-*p*-benzoquinone at 460 nm [119] and 533 nm [126]

Ce(IV) at 525 nm [121]

KIO_4 at 525 nm [122]

$\{Co(SCN)_6\}^{3+}$ at 625 nm [123]

3-methylbenzothiazolin-2 hydrazone at 720 nm [124]

Malachite green at 621 nm [125]

$Fe(NO_3)_3$ at 525 nm [127] and at 540 nm [130]

2-Iodoxybenzoate at 510 nm [128]

Ge(IV) and catecholviolet at 500 nm [129]

N-bromophthalimide at 500 nm [131]

Bromphenol blue at 410 nm [132]

Ce(IV) in 35 mM H_2SO_4 at 525 nm [133]

4.6 Fluorimetric Methods of Analysis

A fluorimetric method for the determination of chlorpromazine after extraction from blood and urine samples (protected from light) has been described [134]. The fluorimetric determination of chlorpromazine with tetrabromosulphone fluorescein was also reported [135].

Chlorpromazine reacts with 9-bromomethylacridine in acetonitrile to give a quaternary ammonium derivative, which on subsequent photolysis yields fluorescent products [136]. The fluorescence is linear over the analyte range of 0.05 to 1 μg. Lumogallion {5-chloro-3-(2,4-dihydroxy-phenylazo)-2-hydroxybenzenesulfonic acid} forms a fluorescent ion-pair complex with chlorpromazine in the presence of Al(III) which is extracted (without interference from primary or secondary amines) up to equimolar concentrations [137, 138].

White *et al.* have reported a rapid fluorimetric determination of chlorpromazine by an *in situ* photochemical oxidation [139]. Variable-angle synchronous scanning fluorescence spectroscopy has also been applied to the determination of chlorpromazine and its sulfoxide [140].

Individual and simultaneous stopped-flow fluorimetric methods for the determination of chlorpromazine and perphenazine were reported, with the fluorescence being monitored at 380 nm after excitation at 340 nm [141]. The technique was used to determine chlorpromazine and perphenazine in mixture, tablet, and liquid formulations.

The determination of chlorpromazine after formation of a ternary complex with eosin and Pd(II) was reported [142]. The method employs excitation at 462 nm, and detection of the emission at 545 nm.

A simultaneous fluorimetric determination of chlorpromazine and promethazine has been reported [143]. The method was based on the fact that in media of different pH, the photochemical conversion of chlorpromazine and promethazine into a fluorescent product varies.

Photooxidation at 254 nm of chlorpromazine hydrochloride in 0.146 mM HCl yielded a fluorescent product suitable for emission work [144].

One fluorimetric method for the determination of chlorpromazine hydrochloride in bulk and in various dosage forms begins with the mixing of 0.5 mL of a 60 μg/mL solution with 1 ml of pH 7.2 buffer solution. To this is also added 1 mL of 0.2 mg/mL *N*-bromosuccinimide, whereupon the mixture is diluted to 10 mL with methanol. After 30 minutes of reaction time, the fluorescence intensity is measured at 378 nm (excitation at 280 nm). The method was found to be linear over the range of 0.005 - 10 μg/mL [145].

4.7 Chromatographic Methods of Analysis

4.7.1 Thin Layer Chromatography

Many thin-layer chromatographic methods have been used for the identification of chlorpromazine [35-53], and for its assay [52, 53, 185-187]. The operating conditions associated with these methods are found in Tables 6 and 7, respectively.

4.7.2 High Performance Liquid Chromatography

HPLC has been used by several authors to determine chlorpromazine in various dosage forms and in biological materials [203-239]. Table 10 summarizes some of the HPLC conditions used by various authors.

4.8 Determination in Body Fluids and Tissues

Chlorpromazine was analyzed in different body biological fluids by a variety of gas chromatography procedures [188-198], the operating conditions for some of which are given in Table 8.

Curry has described analysis on a glass-coil column (6 ft x 3.5 mm) containing 3% of OV-17 on Chromosorb W B-P (80-100 mesh) at 265°C [195]. The method uses argon as the carrier gas (at a flow rate of 50

Table 7: Summary of conditions used for TLC analysis of chlorpromazine.

Plate	Developing system	Detection	Remarks	Ref.
HPTLC Si 60	—	Scanning at 254 nm	Detection limit was 1 μg/ml and coeff. of variation was 4.38%	52
Silica gel G	Cyclohexane-acetone-diethylamine (3:1:1)	Scanning at 253 nm	Coeff. of variation was 1.7%	53
HPTLC silica gel 60 plates (Merck) washed with 2 developments of hexane-acetone-diethylamine (80:20:3) and dried for 1 hour	Hexane-acetone-diethylamine (80:20:3)	Scanning at 254 nm	Chlorpromazine could be determined over the concentration range of 5-200 ng/ml in the serum	185
Silica gel	Methanol-acetone-aq. NH_3 (50:50:1)	Fluorimetry after oxidation with H_2O_2	The detection limit was 10 nM	186
HPTLC on silica gel (Merck) washed with toluene-acetone (1:1)	Toluene-acetone-aq. NH_3 (125:125:6)	Densitometry at 365 nm	Detection limit was 10 ng/ml in plasma	187

mL/min) and electron capture detection. As little as 10 ng/mL chlorpromazine in plasma can be determined.

Dahl and Jacobsen have adopted a glass column (180 cm x 2 mm) containing 3% OV-17 on Supelcoport (80-100 mesh) [196]. The column is treated with silyl-8 prior to use, and operated at 245 or 255°C with nitrogen as the carrier gas (25 mL/min). The use of flame ionization detection was found suitable for chlorpromazine after its extraction from plasma.

Holzbecher and Ellenberger have assayed chlorpromazine in liver by the use of enzyme digestion and GLC [197]. Sparagli *et al.* have assayed chlorpromazine after its extraction from plasma using GLC on a 3 % OV-17 or 3 % SP-2250 column [198]. In the latter method, chlorpromazine was converted to its *N*-trifluoroacetyl derivative prior to analysis, and a nitrogen-phosphorus detector was used in the analysis.

Chlorpromazine has been analyzed in different body biological fluids using gas chromatography - mass spectrometry procedures [199-202]. The conditions for these reported methods are given in Table 9.

4.9 Miscellaneous Analysis Methods

A nuclear magnetic resonance method for chlorpromazine has been reported [178]. For tablets, capsules, and bulk chemical, the sample is shaken with $CHCl_3$ containing cyclohexane or piperanol as an internal standard. For injectable solutions, tetramethylammonium bromide was used as the internal standard. The NMR spectrum was recorded between 0 and 7.0 ppm, and the drug resonance at 2.7 ppm (relative to TMS) measured. The signals for the respective internal standards were at 1.5, 6.0, and 3.3 ppm.

A gravimetric assay method based on the precipitation of chlorpromazine hydrochloride in bulk and in various dosage forms has been reported [179]. The method uses a KBrO-KBr mixture in the presence of 15% HCl, and after standing for 30 minutes, 10% NaOH is added. After an additional 15 minutes a precipitate is formed which can be extracted with

Table 8: Gas chromatography methods of chlorpromazine.

Column	Length	Detector	Column temp. (°C)	Carrier gas & flow rate (ml/min)	Detection limit	% Recovery	Sample	Ref.
5% SE-30	2 ft	H_2-flame ionization	270	—	—	80±15%	Urine	188
3% OV-17	1.8 m	N-P detector	255	N (40 ml/min)	—	55%	Serum	189
1.5% OV-17	2 m	ECD	—	Ar (60 ml/min)	—	85%	Blood	190
3% OV-17	2 m	N-P detector	275	N (22 ml/min)	—	—	Plasma	191
5% SE-30	1 m	—	—	—	—	> 90%	Blood or liver	192
3% OV-17	1.8 m	ECD	260	N (50 ml/min)	2 ng/ml	—	Blood	193
Fused-silica	30 m	Surface-ionization	120-280	He	250-500 pg/ml	79.9%	Blood or urine	194

Table 9: Gas chromatography - mass spectrometry methods of chlorpromazine.

Column	Length	Detector	Column temp. °C	Carrier gas	Sample	Ref.
3% OV-17	1.22 m	EI-ionization	280	He	Plasma	199
1% OV-17	2 m	EI-selected ion mode	—	He	Liver, erythrocyte and plasma	200
SE 54	—	EI-70 eV	30-270	He	Plasma	201
1.5% OV-1	2 m	EI-70 eV	220	—	Blood or urine	202

Table 10: HPLC method of chlorpromazine.

Column	Mobile phase	Detection	Remarks	Ref.
Lichrosorb NH_2 or μ Bondapak NH_2	Acetonitrile-benzene-H_2O (16:4:1) H_2O contains 0.01% $Na_2S_2O_5$ and 0.01% (+)-araboascorbic acid.	Fluorescence excit. 280 nm emission 450 nm.	Rapid assay of chlorpromazine and products, in pharmaceutical sample.	203
25 cm x 5 mm of Hypersil ODS (10 μM)	Aqueous 90% methanol containing 0.2% ethanol amine	At 257 nm.	Assay of the drug in pharmaceutical formulation.	204
Spherisorb CN (5 μm) 25 cm x 3.2 mm	0.015 M acetate buffer of pH 6.5-acetonitrile (1:1)	At 254 nm.	Results were in good agreement with the R.I.A.	205
μ Bondapak C18	Aqueous acetonitrile containing 0.1% of H_3PO_4	—	The method was considered to be unsuitable for use with urine.	206
—	—	—	Analysis of the drug and metabolites in biological samples by ion-pair HPLC	207
25 cm x 3.2 mm of Lichrosorb RP-2	Aqueous 0.04 to 0.01% ammonium carbonate-acetonitrile (1:1)	At 254 nm.	Sensitivity, selectivity and reproducibility were adequate for the analysis of single tablet.	208

Table 10: HPLC method of chlorpromazine. (Continued) ...

Stainless steel column 12.5 cm x 4.6 mm packed with Hypersil ODS (5 μm)	Aqueous 50% ethanol plus 2% decylamine actfusted to pH 9.5 with $HClO_4$	U.V. detection.	Chlorpromazine was not extracted from plasma under the conditions used. Detection limits were 1 to 3 ng.	209
25 cm x 5 mm of Spherisorb 5	70% methanol containing 0.1% trifluoroacetic acid.	254 nm.	Assay of the drug and its sulphoxide by ion-pair HPLC, in plasma.	210
—	—	—	Comparison with a GC-MS method, in plasma with electrochemical detection	211
30 cm x 4 mm of 10 μm silica	0.2% Ethanolamine in aqueous 40% methanol	At 254 nm.	Extraction and analysis and metabolites in post-mortem material by enzymic digestion and HPLC	212
6.5 cm x 5 mm of TSK 410 ODS silica gel (20 to 32 μm)	Acetonitrile-0.1M phoshate buffer of pH 5 (1:1)	At a vitreous-carbon electrode.	Direct plasma sample injection.	213
25 cm x 4.6 mm of Sherisorb CN (10 μm)	0.1M Ammonium acetate-acetonitrile (1:9) (4 ml/min).	Electrochemical.	Subnanogram analysis, in plasma.	214

Table 10: HPLC method of chlorpromazine. (Continued) ...

25 cm x 4.6 mm of Supelcosil C8.	Acetonitrile-water-diethylamine-85% H_3PO_4 (533:451:10:4) at pH 7.2	At 254 nm.	Rapid method for the measurement of six tricyclic antidepressant.	215
Micropak CN-10 30 cm x 4 mm	55% Acetonitrile in 0.01 M $NH_4H_2PO_4$	Electrochemical	Automated HPLC, in human plasma	216
Stainless steel column 15 cm x 4.6 mm of Zorbax CN	0.025 M sodium acetate buffer (pH 4.8)-acetonitrile-methanol. (4:7:9), (2.5 ml/min).	At 254 nm.	Identify, assay and content uniformity of antidepressants.	217
—	—	—	Therapeutic of drug monitoring, pitfalls in plasma analysis.	218
—	—	—	Review on coupling of micro HPLC with mass.	219
Sherisorb CN	—	—	Therapeutic monitoring, pitfalls in whole blood.	220
Sherisorb CN	—	—	Limit of detection was 1 ng ml^{-1}	221
10 cm x 4 mm of Lichrosorb Alox T (10 μm)	Saturated solution of acetonitrile in heptane (1.08 ml/min).	At 254 nm.	Analysis in blood.	222
12.5 cm x 4.9 mm Sperisorb S5 CN	Acetonitrile-phosphate buffer of pH 6 (13:7) (2 ml/min.)	Electrochemical.	The HPLC method was more sensitive than the R.I.A. method	223

Table 10: HPLC method of chlorpromazine. (Continued) ...

50 cm x 2.1 mm of Zipax-SCX	(7:13) of methanol and 0.2 M borate buffer (pH 9)-0.5 M sodium nitrate (1 ml/min.)	Electrochemical.	Analysis in human serum acid in pharmaceuticals.	224
30 cm x 3.9 mm of μ Bondapak C18	0.1 M ammonium acetate, 20 mM citric acid and 100 μl of butylmine in aqueous 38% acetonitrile (1.5 ml/min.)	Coulometric.	Analysis of drug and metabolite in human brain.	225
25 cm x 2 mm of Ultrasphere CN (6 μm)	1.5 ml/min 0.05 diethylamine solution in acetonitrile-0.06 M sodium acetate-0.06 M ammonium acetate (23:1:1)	At 248 nm.	The HPLC method requires less separation steps than the R.I.A. method	226
25 cm x 4.6 mm of Spherisorb ODS (5 μm)	0.02 M Na_2HPO_4 of pH 7.8 containing 70 to 80% of methanol.	At 254 nm.	Evaluation of enzymic digestion procedure in release of basic drugs from tissue.	227
5 cm x 5 mm of TSK Gel Toyopearl HW 65T	–	At 254 nm.	Analysis of the drug in blood or plasma by direct injection.	228
25 cm x 4.6 mm of C8 bonded silica (40 μm)	2 ml/min of aqueous 55% methanol containing 275 mM methylammonium chloride and 25 mM Na_2HPO_4 (pH 6.75)	At 254 nm.	Analysis of drug and 13 metabolites, in plasma.	229
25 cm x 4 mm of LiChrosorb NH_2 (5 μm)	Acetonitrile-0.1 mM HCl (pH 4, 83:17) containing 0.01% each of $Na_2S_2O_5$ and isoascorbic acid and 0.06% of NaCl (1 ml/min.)	Fluorimetric at 450 nm.	Analysis of drug and degradation products, in dosage forms, a stability indicating assay.	230

Table 10: HPLC method of chlorpromazine. (Continued) ...

30 cm x 3.9 mm of μ Bondapak C18 and gard column 1 cm x 2.1 mm of same material	Acetate buffer of pH 6.5-acetonitrile (9:1) (1.2 ml/min).	U.V. at 240 nm or fluorimetry at 351 mm (excitation at 246 nm).	Analysis of drug in Swine kidney.	231
15 cm of Lichrosorb RP 18 (5 μm)	Methanol-water-aqueous ammonia (88:11:1)	At 254 nm	The range of application was 3.6-36 μg ml^{-1}	232
—	—	—	Analysis in plasma	233
15 cm x 4.6 mm of SAS Hypersil (5 μm) (1 cm x 3 mm precolmn).	2 ml/min of aqueous 44.5% Acetonitrile containing 0.01 M ammonium acetate	At 235 nm.	Analysis in pig kidneys.	234
25 cm x 4.6 mm of Supelcosil C8 (5 μm) at 40°C. C8 gard column.	275 mM methylamine-methnaol (9:11; pH 6.7)	At 214 nm.	Optimization of HPLC for the drug and 13 metabolites.	235
LiChrosorb C18 (10 μm)	Acetonitrile-water (20:1) containing NH_4ClO_4 (2 ml/min.)	At 254 nm.	Analysis of drug from controlled-release suspension	236
15 cm x 6 mm of Shim Park CLC ODS 18 (5 μm)	Methanol-water-tetramethylethylene diamine-anhydrous acetic acid (670:362:4.4:3.52) (1.5 ml/min).	At 254 nm.	Analysis in plasma, urine, stomach in case of poisoning.	237

Table 10: HPLC method of chlorpromazine. (Continued) ...

30 cm x 3.9 mm μ Bondapak C18	10 mM KH_2PO_4 – methanol (1:3) (0.8 ml/min.)	At 249 nm.	Dual wavelength detection for the analysis of chlorpromazine in composite tablets	238
15 cm x 4.6 mm of Develosil C8 (5 μm)	0.5% Potassium dihydrogen phosphate-acetonitrile (13:7).	At 254 nm	Analysis in human breast milk and serum.	239

$CHCl_3$. The organic layer was evaporated to dryness and the residue dried at 105°C for 3 hours. Chlorpromazine was weighed as its brominated derivative.

Other gravimetric methods involve precipitation with tungstosilic acid [180], tungstophosphoric acid [181], and $\{CdI_4\}^{2-}$ [182], and sodium or ammonium vanadate [183].

A radioimmunoassay method has been reported for the monitoring of therapeutic concentration of chlorpromazine and its 7-hydroxy metabolite in plasma by [184]. The limit of detection for chlorpromazine was reported to be 0.1 ng, and its calibration curve linear up to 2 ng. The limits of detection for the 7-hydroxy metabolite was reported as 0.1 ng, and the calibration curve linear up to 1.2 ng.

5. Stability and Storage

Chlorpromazine and chlorpromazine hydrochloride darken on prolonged exposure to air and light [2, 10].

Consequently, commercially available preparations of chlorpromazine and its hydrochloride salt should be protected from light. Chlorpromazine hydrochloride oral solutions, tablets, extended-release capsules and injections should be stored at a temperature less than 40°C, preferably between 15-30°C and in well-closed containers [2].

6. Drug Metabolism and Pharmacokinetics

Chlorpromazine hydrochloride is rapidly absorbed from the gastrointestinal tract and from parenteral sites of injection. Following oral administration, the drug undergoes considerable metabolism during absorption in the gastrointestinal mucosa and first pass through the liver, and there is also evidence of enterohepatic recycling [2,5]. Owing to the first-pass effect, plasma concentrations following oral administration are much lower than those observed following intramuscular administration. There is a very wide inter-subject variation in the plasma concentration of

chlorpromazine following oral administration [14-17]. Following administration of chlorpromazine hydrochloride in a tablet formulation, the onset of pharmacologic action is 30-60 minutes, and the duration of action is 4-6 hours. Following oral administration of extended-release formulations, the onset time is 30-60 minutes and the duration of action is 10-12 hours [2,18]. Following rectal administration, the onset of pharmacologic action is much slower than following oral administration, and the duration of action is 3-4 hours [2,19].

Chlorpromazine is 92 to 97% bound to plasma proteins, principally albumin [5,20]. It crosses the blood-brain barrier, and concentrations of the drug in the brain are higher than those in plasma [17]. The relationship of plasma concentration to clinical response and toxicity has not been clearly established. Chlorpromazine and its metabolites cross the placenta and are distributed into milk [21]. About 10-12 metabolites of chlorpromazine in humans have been identified. In addition to hydroxylation at positions 3 and 7 of the phenothiazine nucleus, the N-dimethylaminopropyl side chain of chlorpromazine undergoes demethylation and is metabolized to an N-oxide or sulfoxide derivative. These metabolites may be excreted as their 0-glucouronides, with small amounts of ethereal sulfates of the mono- and dihydroxy derivatives. The major metabolites found in urine are the monoglucouronide of N-demethylchlorpromazine and 7-hydroxychlorpromazine [2]. Although the plasma half life of chlorpromazine itself has been reported to be few hours, the elimination of metabolites may be very prolonged [8, 22-24].

7. Adverse Effects

Chlorpromazine may cause or be associated with a variety of undesirable side effects, which are summarized in the following categories:

CNS effects: Drowsiness, extrapyramidal reactions.

Cardiovascular effects: Postural hypotension, bradycardia, faintness and cardiac arrest.

Hematologic disorders: Agranulocytosis, eoisnophilia, leukopenia, hemolytic anaemia, pancytopenia, thrombocytopenic purpura.

Liver: Jaundice.

Allergic reactions: Urticaria or dermatitis.

Endocrine effects: In the female, ovulation block, infertility, breast enlargement and pseudo-pregnancy lactation. In the male, gynecomstia or changes in libido have been observed.

Other reported reactions have included increases in cholesterol level, dry mouth, nasal congestion, constipation, urine retention, mydriasis, weight gain, fever, and suppression of the cough reflex [5,9,20,25].

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CORTISONE ACETATE

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

1.1.1.1 Cortisone

17α, 21-dihydroxypregn-4-ene-3,11,20-trione

17α, 21-dihydroxy-4-pregnene-3,11,20-trione

Δ^4-pregnene-17α, 21-diol-3,11,20-trione

pregn-4-ene-3,11,20-trione, 17α, 21-dihydroxy

11-dehydro-17α-hydroxycorticosterone

17α-hydroxy-11-dehydrocorticosterone

11-dehydrocortisol

1.1.1.2 Cortisone acetate

17α, 21-dihydroxypregn-4-ene-3,11,20-trione 21-acetate (IUPAC)

pregn-4-ene-3,11,20-trione, 21-(acetyloxy)-17α-hydroxy (CAS Index)

21-Acetyloxy-17α-hydroxy-pregn-4-ene-3,11,20-trione

17α, 21-dihydroxypregn-4-ene-3,11,20-trione 21-acetate

1.1.2 Nonproprietary Names

1.1.2.1 Cortisone

Kendall's compound E

Wintersteiner's compound F

Reichstein's substance Fa

11-Dehydrocortisol

1.1.2.2 Cortisone acetate

Cortisone C_{21}, acetate

Cortisone monoacetate

Cortisone 21-acetate

1.1.3 Proprietary Names

1.1.3.2 Cortisone acetate

Incortin, Cortone, Cortadren,
Corlin, Scheroson, Cortogen

1.1.3.2 Cortisone acetate

Cortistab, Cortelan, Cortisyl Artriona
Adreson, Cortone acetate

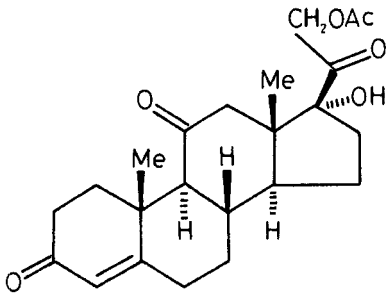
1.2 Formulae

1.2.1 Empirical

Cortisone: $C_{21}H_{28}O_5$

Cortisone acetate: $C_{23}H_{30}O_6$

1.2.2 Structural



1.3 Molecular Weights

Cortisone: 360.46

Cortisone acetate: 402.49

1.4 CAS Numbers

Cortisone: 53-06-5

Cortisone acetate: 50-04-4

1.5 Appearance

When obtained from 95% ethanol, cortisone occurs as rhombohedral platelets [1].

Cortisone acetate can be obtained as either clusters of radiating rods from chloroform [1], or as a white crystalline powder [2,3].

1.6 Uses and Applications

Cortisone is a natural corticosteroid secreted by the adrenal cortex along with many steroidal hormones. It has mainly glucocorticoidal activity and some degree of mineralo-corticoid activity.

The drug is commercially available as the acetate ester, being esterified at the hydroxy methyl group (at C_{21}). It is formulated as oral tablets (dosage strengths of 5 mg, 10 mg, and 25 mg), or as a parenteral sterile suspension (50 mg/mL) for intra-muscular administration.

Cortisone acetate and hydrocortisone are usually the corticoids of choice for replacement therapy in patients with primary adrenocortical insufficiency (such as Addison's disease), or after adrenalectomy where both glucocorticoid and mineralo-corticoid replacement is needed. In secondary adrenal insufficiency, associated with inadequate corticotrophin (ACTH) secretion, glucocorticoid replacement alone is usually adequate [62].

Cortisone and other glucocorticoids (having an oxygen function at C_{11}) are used medicinally as anti-inflammatory and immuno-suppressant drugs in a large number of disorders. Cortisone is rapidly effective when given by mouth, and is slowly effective when given by intramuscular injection. Unlike hydrocortisone acetate, the drug is ineffective when injected into joints or applied to the skin.

Glucocorticoids are also used in conjunction with antineoplastic agents in regimens for the management of malignant diseases. They are also given to reduce immune responses after organ transplantation, often in conjunction with azathioprine or cyclosporin.

2. Methods of Preparation

2.1 Isolation from Natural Sources

During the 1930's, several scientists isolated cortisone among other adrenocortical hormones from the suprarenal glands of cattle. Kendall *et al.* [12-14] isolated 8 steroidal hormones (cortisone being Kendall's compound E), while Wintersteiner *et al.* [15,16] isolated 4 compounds (Wintersteiner's compound F was cortisone). Reichstein [17] isolated 31 substances, with his compound Fa being cortisone. Kuizenga and Cartland [18] isolated 5 substances, and Wettstein *et al.* [19] isolated 18 hormones.

Further methods of isolation were later developed [20-23]. The isolation was mostly effected by the use of Girard's reagent T to separate the keto steroids from the non-keto compounds, and each fraction was then separated by adsorption or partition chromatography.

It was found that eight of these isolated steroids were highly active physiologically, and cortisone became the first hormone to be available for therapeutic uses. The demand for cortisone therapy became enormous after the findings of Hench *et al.* that cortisone was effective in relieving symptoms of rheumatoid arthritis [24].

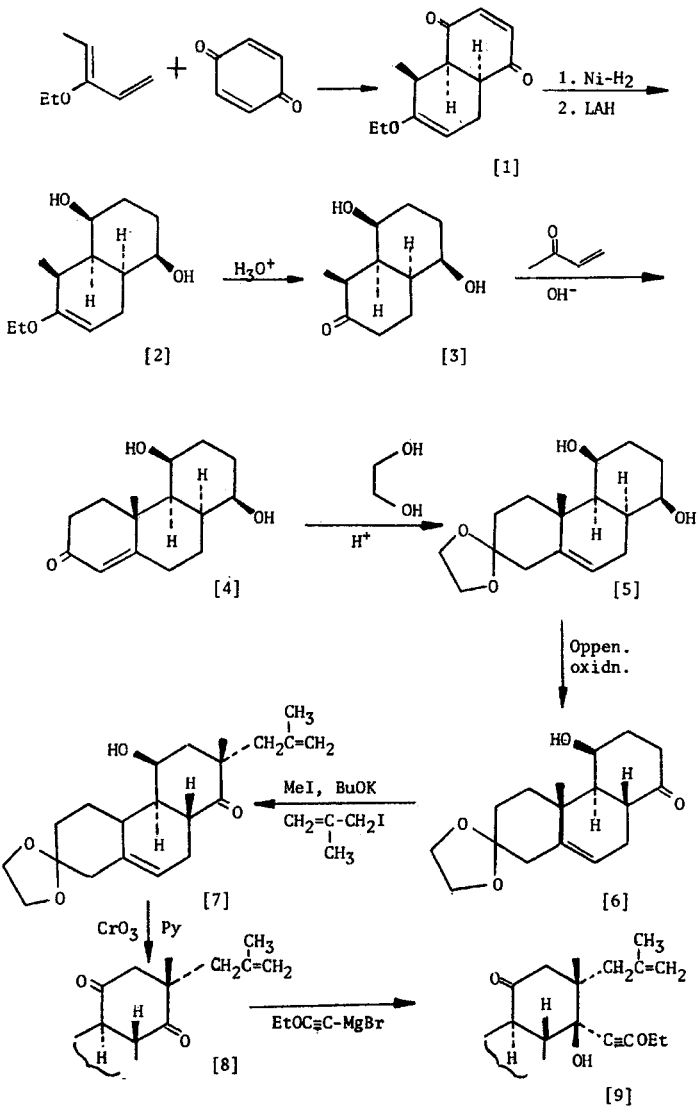
As the natural abundance of these hormones in animal adrenal glands is extremely small, very large quantities of glands are therefore required to isolate minute quantities of such hormones. As a result, isolation from natural sources is no longer viable to prepare steroidal hormones in larger or commercial quantities.

2.2 Total Synthesis of Cortisone

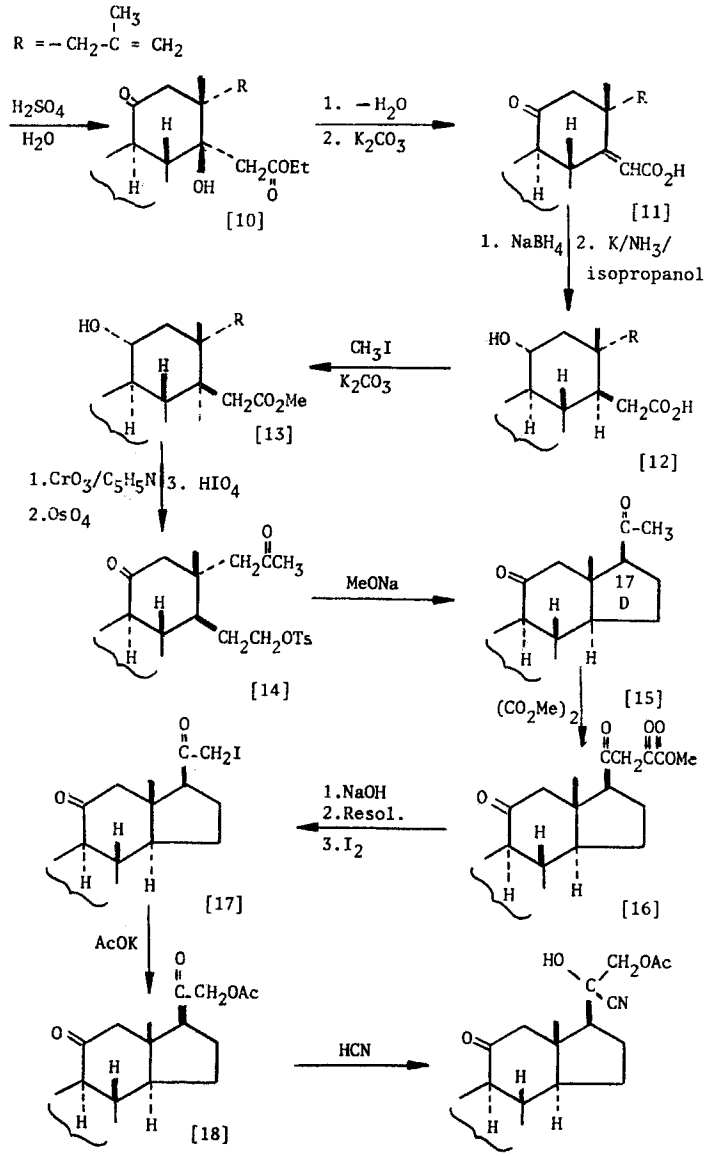
Several total syntheses of cortisone have been described [25-32]. One of these is the highly stereospecific synthesis of Sarett *et al.* [30], which is described below and outlined in Scheme 1. This synthesis is of the type C → BC → ABC → ABCD, where one constructs ring-C at first, followed by conversion to rings BC, then rings ABC, and finally to rings ABCD.

3-Ethoxypenta-1,3-diene underwent the Diels-Alder reaction with *p*-benzoquinone to give the cis-fused diketone **l**. Selective catalytic hydrogenation (Ni) of **l**, followed by reduction with lithium aluminum

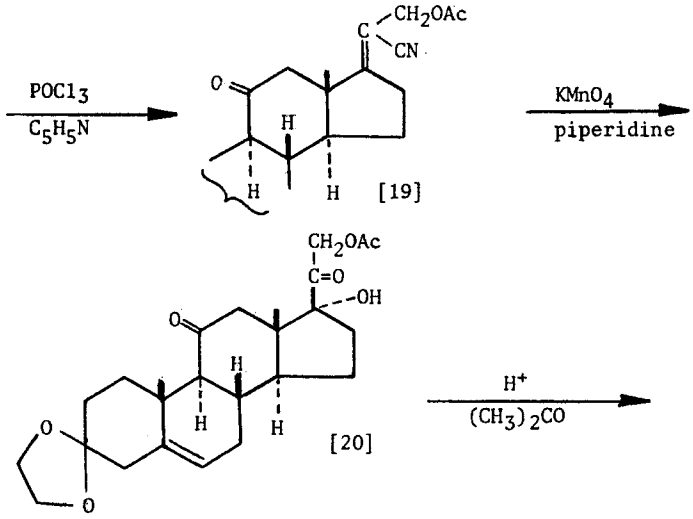
Scheme 1. Stereospecific synthesis of cortisone, as reported by Sarett *et al.* [30].

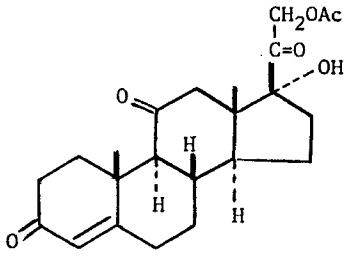


Scheme 1 (continued). Stereospecific synthesis of cortisone [30].



Scheme 1 (continued). Stereospecific synthesis of cortisone [30].





Cortisone Acetate
[21]

hydride, formed the diol enol ether **2**. When treated with acid, this compound was hydrolyzed to **3**. On treatment of compound **3** with methyl vinyl ketone in the presence of alkali, it underwent addition (Michael condensation) at the less hindered side followed by cyclization to produce the tricyclic ketone **4**. This was converted into the ethylene ketal **5** (protection of the oxo group; this protection was kept until the final step of the synthesis). Oppenauer oxidation of **5** yielded attack at the less hindered hydroxyl group to give **6**. Treatment of **6** with methyl iodide and potassium *t*-butoxide produced the 2-methyl derivative, which was alkylated in turn with methallyl iodide to furnish **7**. Oxidation of **7** with chromium trioxide-pyridine complex yielded the corresponding 1,4-diketone derivative **8**. Condensation of **8** with ethoxyacetylene magnesium bromide gave the ethoxyethinyl derivative **9**.

Treatment of **9** with dilute sulfuric acid afforded the carbethoxymethylene derivative **10**, which was dehydrated followed by hydrolysis to give the free keto acid **11**. Double reduction of **11** first with sodium borohydride (the keto group) followed by potassium-ammonia-isopropylalcohol (the exocyclic double bond) gave **12**, which was converted to the carboxymethyl derivative **13**. Successive oxidation of the monotosylate of **13** with the chromium trioxide-pyridine complex, with osmium tetroxide, and with periodic acid afforded **14**. Upon treatment of **14** with sodium methoxide, cyclization occurred with the formation of ring D and a C_{17} acetyl group **15** (The side chain was initially α-oriented but epimerization was performed to give the desired product). 3-Ethylene dioxy-Δ^5-11-ketoprogesterone **15** was converted into the oxalyl derivative **16**, which was then resolved via the strychnine salt. The (*d*)-form of **16** was transformed by iodination to give the 21-iododerivative **17**, which was subjected to acetoxylation and cleavage to afford the ketol acetate derivative **18**.

Substance **18** was treated with hydrocyanic acid to produce the 20-cyanhydrin derivative, which in turn was dehydrated to the $\Delta^{5,17}$-20-cyanopregnadiene **19**. Oxidation with potassium permanganate in piperidine gave 3-ethylenedioxy-Δ^5-pregnene-17α, 21-diol-11,20-dione

acetate **20**. Acid hydrolysis of the latter yielded cortisone acetate **21**, which was found to be identical with natural cortisone.

2.3 Commercial-Scale Synthesis of Cortisone

Presently, cortisone is commercially produced by the microbiological transformation of progesterone, which serves as starting substance to prepare most (if not all) steroid hormones. Progesterone itself is commercially prepared from several naturally available steroids. Thus, progesterone can be prepared from stigmasterol, as initially described by Butenandt *et al.* [33], and improved by other workers [34-36]. It can also be synthesized from pregnane-diol [37], cholesterol [38], diosgenin [39,40], and from ergosterol [41].

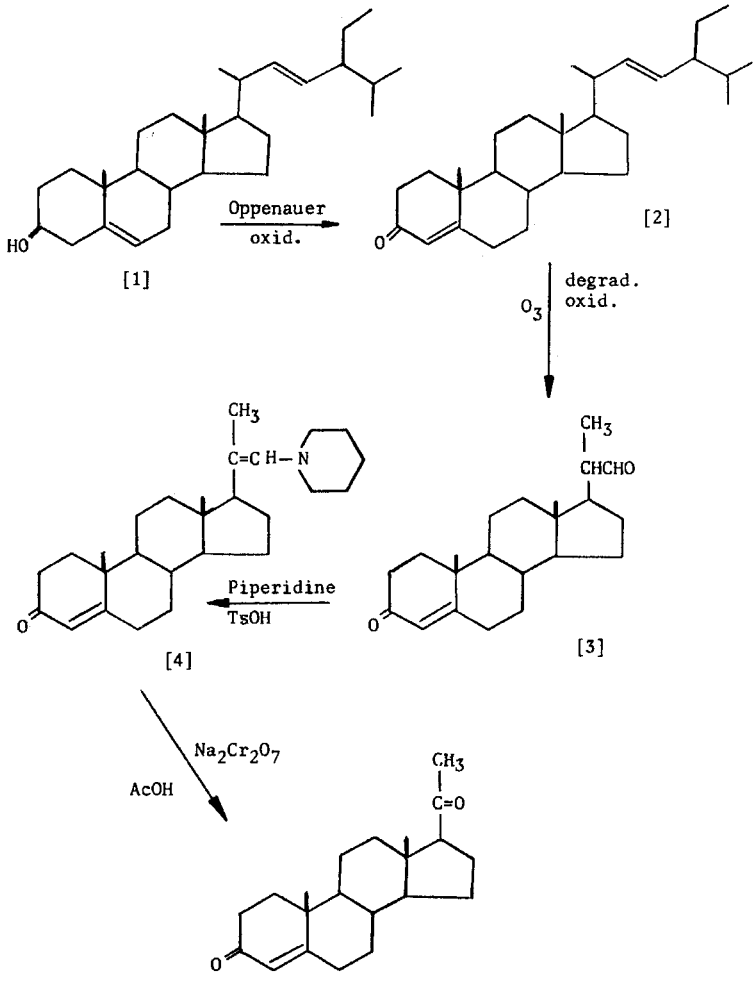
2.3.1 Synthesis of Progesterone from Stigmasterol

A highly efficient route for the synthesis of progesterone from stigmasterol has been described by Heyl *et al.* [34], and which is illustrated in Scheme 2. The Oppenauer oxidation of stigmasterol **1** afforded stigmastadienone, **2**. This compound was subjected to ozonolysis, where oxidation and degradation took place to produce the bisnoraldehyde, **3**. Slomp and Johnson found that when pyridine was added at the 1% level to a solution of **2** in methylene chloride, a marked increase in yield to 90% was noted for production of **3** [35]. Compound **3** was condensed with piperidine in *p*-toluene sulfonic acid to give the enamine derivative **4**. This was oxidized to progesterone **5** with sodium dichromate in anhydrous acetic acid-benzene medium in 88% yield.

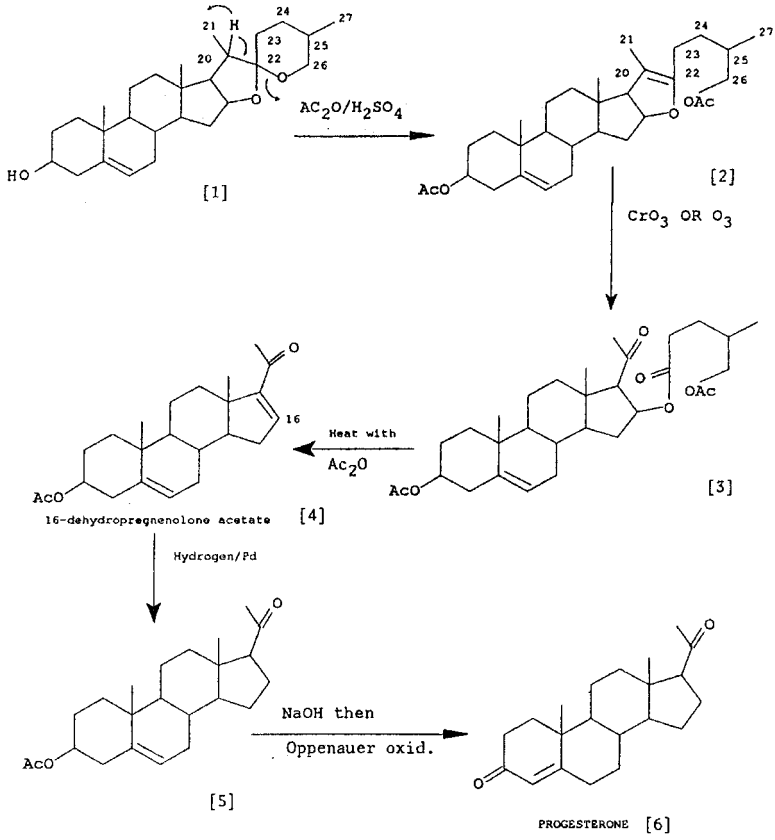
2.3.2 Synthesis of Progesterone from Diosgenin

Progesterone has been synthesized from diosgenin by Marker's degradation [39,40], and the procedure is illustrated in Scheme 3. Diosgenin (**1**) can be obtained from the steroidal saponin "dioscin ", which is the active constituent of the *Dioscorea* species. Of particular use are two high-yielding Mexican species, *D. composita* and *D. macrostachya*. Both species commonly called the Mexican yam. Compound **1** was acetylated with acetic anhydride in the presence of sulfuric acid to give the pseudo-diosgenin acetate, **2**. This compound was oxidized either with

Scheme 2. Synthesis of progesterone from stigmasterol [34].



Scheme 3. Synthesis of progesterone from diosgenin [39,40].



chromic acid or with ozone to produce diosone acetate, **3**. Upon heating of **3** with acetic acid, degradation occurred at the side chain to yield 16-dehydropregnenolone acetate **4**. Substance **4** was hydrogenated over palladium catalyst to yield pregnenolone acetate **5**. This was in turn subjected to treatment with sodium hydroxide solution, followed by Oppenauer oxidation, to produce progesterone **6** in high yield.

2.3.3 Synthesis of Progesterone from Ergosterol

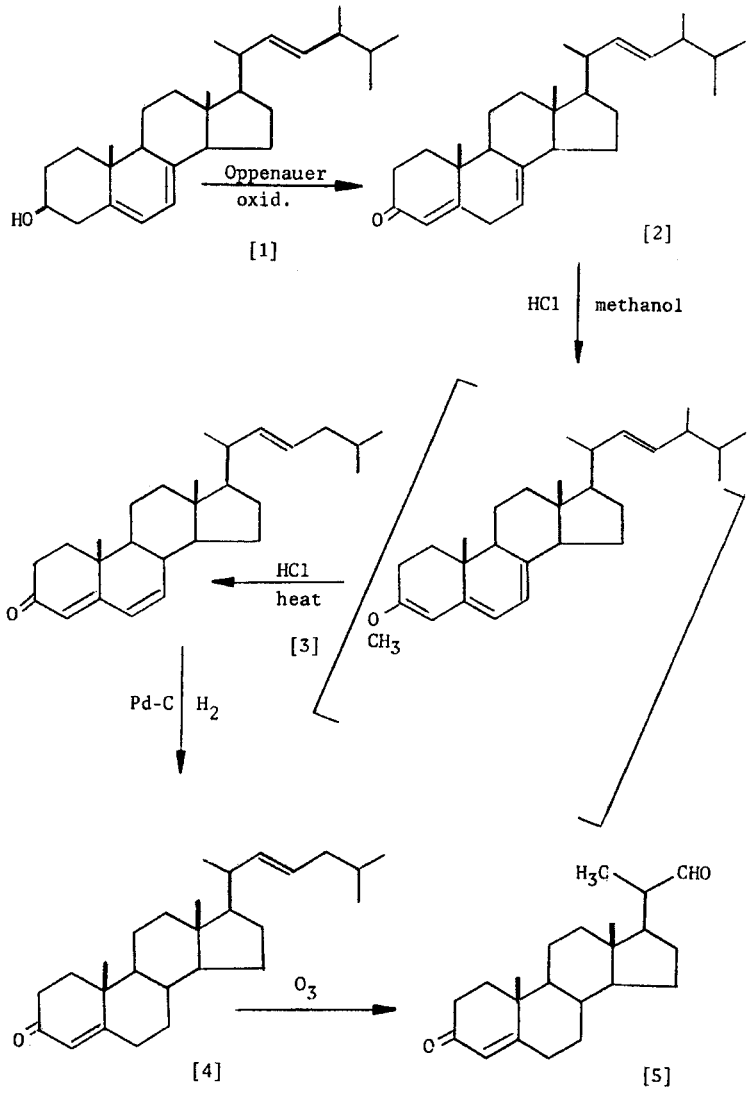
As illustrated in Scheme 4, progesterone has also been synthesized from ergosterol, and this is one of the most practical syntheses of progesterone [41]. Ergosterol, **1**, was subjected to Oppenauer oxidation to afford ergosterone, **2**. This compound was treated with hydrochloric acid in methanol to give the methyl-ether intermediate, which upon heating with hydrochloric acid yielded isoergosterone, **3**. Reduction of **3** with palladium on carbon afforded **4**. This was subjected to ozonization, where oxidation and degradation both occurred to produce the aldehyde derivative **5**. Product **5** was condensed with piperidine in *p*-toluene sulfonic acid to give the enamine adduct **6**, which was oxidized to progesterone **7** with sodium dichromate in acetic acid.

2.3.4 Conversion of Progesterone to Cortisone

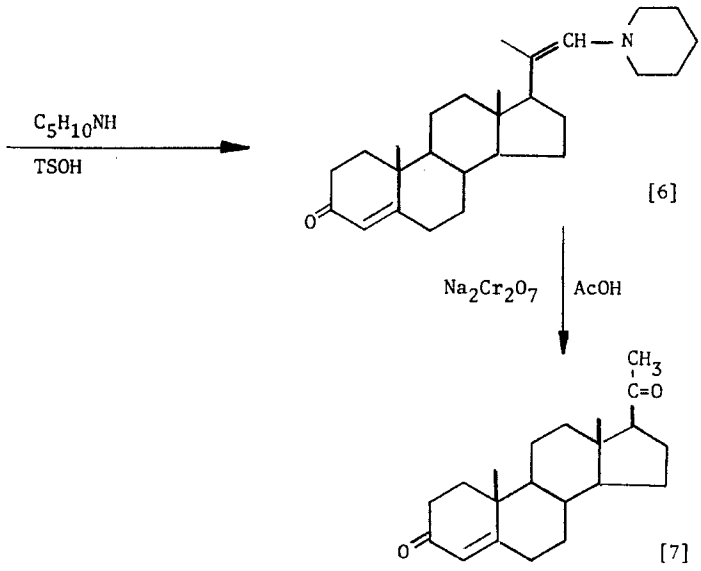
It proved quite difficult to react progesterone and introduce an oxygen functionality at C_{11} by a chemical reaction. Finally, in 1952, Peterson and Murray managed to introduce this group by microbiological hydroxylation [42-44]. Their procedure is shown in Scheme 5. These workers demonstrated that the microorganism, *Rhizopus arrhizus*, and other fungi of the order *Mucorales*, could convert progesterone **1** into 11α-hydroxyprogesterone, **2**, in high yield (80 to 90%). Substance **2** could be successively reacted through compounds **3** to **7** to achieve the commercially viable conversion to cortisone or cortisone acetate, **8**.

A vast amount of research resulted in the improvement of this bioconversion, as well as the development of many other microbiological reactions. Some of these include C_1-dehydrogenation, C_{16}-α-hydroxylation, and C_{11}-β-hydroxylation of steroids [45-52]. Other

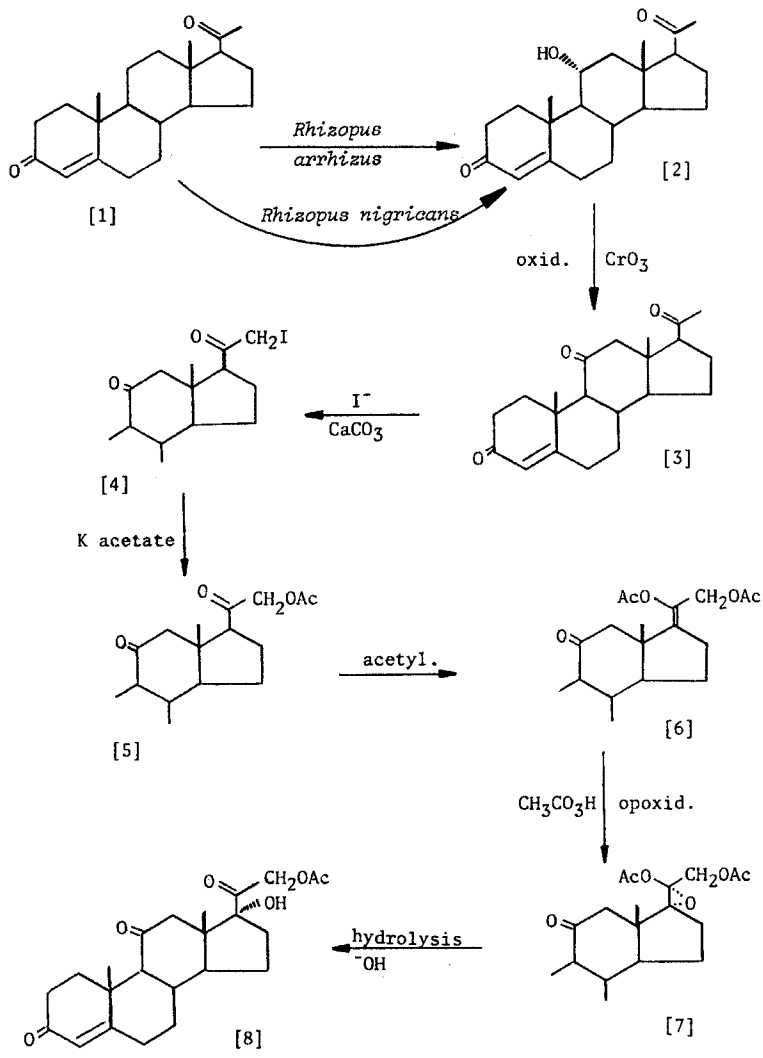
Scheme 4. Synthesis of progesterone from ergosterol [41].



Scheme 4 (continued). Synthesis of progesterone from ergosterol [41].



Scheme 5. Commercial synthesis of cortisone acetate.



researchers have patented high yield microbiological processes for converting cholesterol, sistasterol, stigmasterol or other C_{17}-alkyl sterols into C_{17}-ones as steps to produce steroidal hormones [53-55].

2.4 Biosynthesis of Cortisone

It is well known that the steroid hormones in mammals are biosynthesized from cholesterol. This compound is derived from the acetate-mevalonate pathway through the monoterpene geranyl pyrophosphate, which undergoes several enzymatic reactions to form the triterpene squalene. This substance undergoes degradation, oxidation, and cyclization reactions to form the first steroid "lanosterol", which is transformed into cholesterol after several steps. Schematic outlines of these biosynthetic pathways are well illustrated in the literature [56-59].

The biosynthesis of cortisone from cholesterol is presented in Scheme 6. The initial steps in the biosynthesis of adrenocortical hormones involve the enzymatic hydroxylation of cholesterol **1** to produce C-20,22-α-dihydroxy cholesterol **2**. Enzymatic cleavage of the C-20—C-22 bond affords pregnenolone **3**. This precursor undergoes either hydroxylation by the enzyme 17α-hydroxylase to give 17α-hydroxypregnenolone, **4**, or oxidation by oxidase to form progesterone, **5**. Dehydrogenation of **4** and hydroxylation of **5** by the enzyme 17α-hydroxylase affords the 17α-hydroxy progesterone, **6**. Oxidation of **6** with the enzyme 21-hydroxylase produces cortexolone (11-deoxycortisone), **7**. This is hydroxylated by 11-β-hydroxylase to form hydrocortisone (cortisol), **8**, which is in turn dehydrogenated at C-11 to yield cortisone **8**.

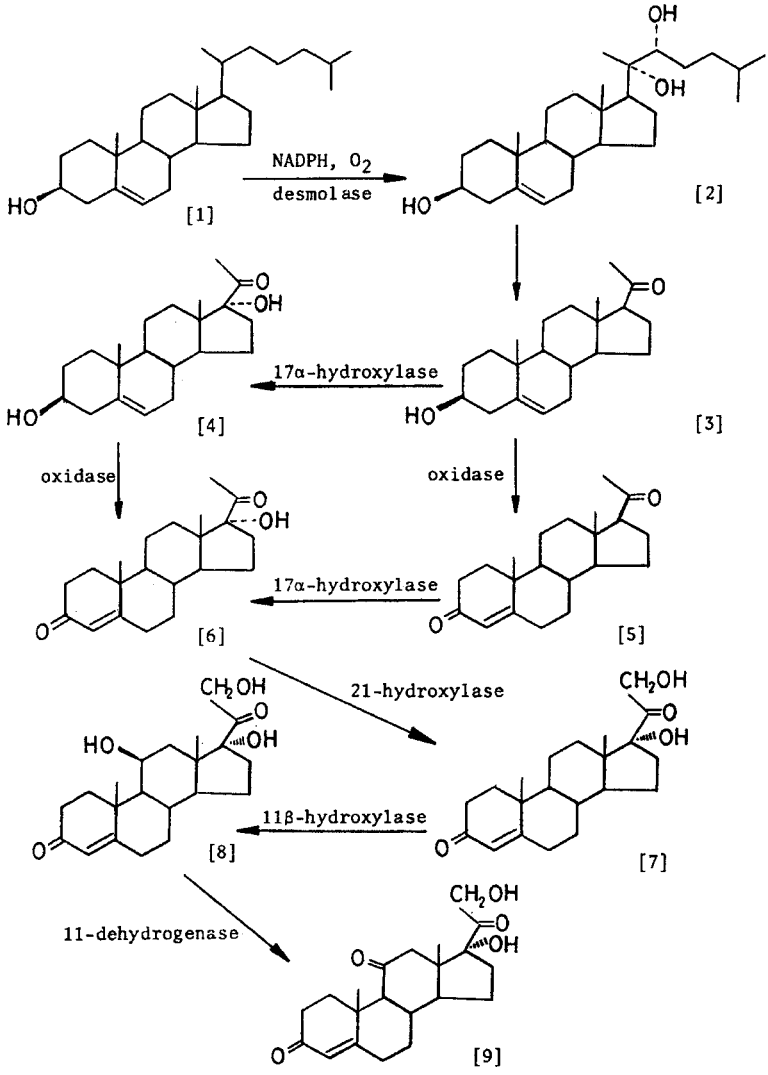
3. Physical Properties

3.1 Crystallographic Properties

3.1.1 Single Crystal Structures and Polymorphism

Cortisone acetate has been obtained in at least five distinct crystalline forms. Four of these forms are unstable in presence of water, and change to the stable form I [5]. The crystallographic information gathered from

Scheme 6. Biosynthesis of cortisone from cholesterol.



single crystal studies is summarized in Table 1, and additional characterization information is available in the literature [5].

Through the use of differential scanning calorimetry in conjunction with infrared spectral data, it was shown that cortisone acetate occurs in three anhydrate and two hydrated crystal forms [6].

3.1.2 X-Ray Powder Diffraction Pattern

The x-ray powder diffraction pattern of cortisone acetate was obtained using a Siemens D 5000 x-ray diffractometer system. The incident radiation was provided by a copper tube, yielding $\lambda = 1.5480$ Å. The powder pattern thusly obtained is presented in Figure 1, and the scattering angles, interplanar distances, and peak relative intensities are collected in Table 2 for diffraction lines whose relative intensity exceeds 4%.

The x-ray powder diffraction patterns for the crystal forms of cortisone acetate have been reported and indexed [5].

3.2 Optical Activity

As is the case for steroid molecules of natural origin, cortisone is optically active and exhibits optical rotation. The following specific rotation results have been measured at the sodium-D line (546 nm) at 25C:

Cortisone:	+ 209° (c = 1.2 in 95% alcohol)
	+ 269° (c = 0.125 in benzene
	+ 248° (c = 0.1 to 0.2 in alcohol) [1]
Cortisone acetate:	+ 164° (c = 0.5 in acetone)
	+ 208 - 217° (dioxane) [1]
	+ 208 - 219° (1% in dioxane) [2]
	+ 209 - 219° (1% w/v in dioxane) [3]

Table 1

Crystallographic Date Reported for the Polymorphs of Cortisone Acetate

Constant	Form I	Form II	Form III	Form IV	Form V
shape	Monoclinic	orthorombic	orthorombic	orthorombic	monoclinic
m.p.	241-245°	235-238°	251-253°	245-247°	
a	15.68	11.21	12.50	9.76	9.65
b	7.52	27.14	20.95	30.59	7.46
c	26.58	7.11	7.96	7.58	16.4
β	97.0	-	-	-	98.0
V	3112.04	2165.58	2084.52	2263.07	1163.76
Z	6	4	4	4	2
Sp	$P2_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_1$
D_{obs}	1.250	1.210	1.250	1.260	1.250
anhydr D_{cal}	1.288	1.234	1.282	1.181	1.148
hydr D_{cal}	-	-	-	1.280	1.251

a, *b*, *c* = cell constants in A°. β = cell angle indegrees.
V = cell volume in $A^{\circ 3}$. Z = number of molecules in unit cell.
Sp = space group. D_{obs} = density observed in g/ml. anhydr D_{cal} = density calculated with hydration. hydr D_{cal} = density calculated assuming two molecules of water per molecule of cortisone acetate.

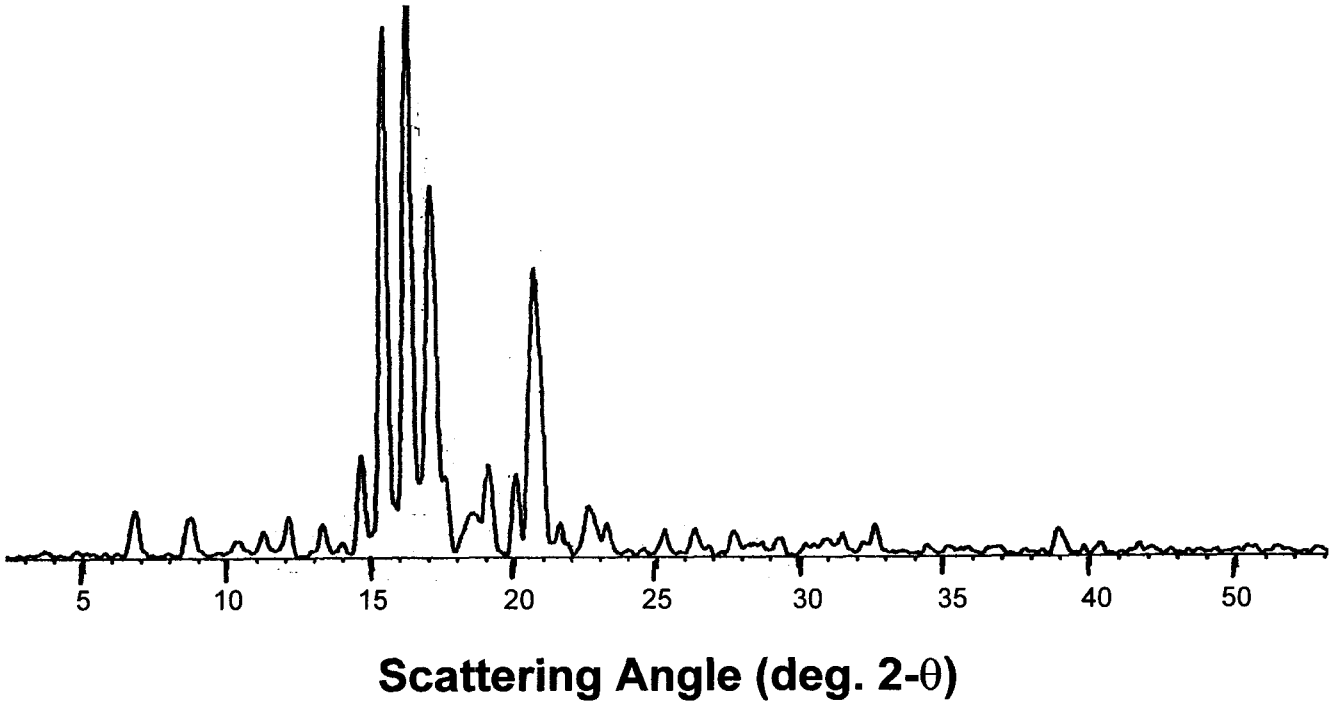


Figure 1. X-Ray powder diffraction pattern of cortisone acetate.

Table 2

Crystallographic Data Obtained from the X-Ray Powder Diffraction Pattern of Cortisone Acetate

Scattering Angle (°2 theta)	*d*-Spacing (A°)	Relative Intensity (I/I max)
16.296	5.4348	100.00
15.441	5.7338	93.64
17.097	5.1820	65.62
20.696	4.2883	51.20
14.630	6.0500	18.07
19.067	4.6508	16.31
20.032	4.4289	14.74
17.545	5.0505	14.36
22.588	3.9331	9.10
6.790	13.0077	8.51
18.493	4.7939	7.89
12.089	7.3150	7.36
8.703	10.1514	7.18
21.574	4.1156	6.07
23.213	3.8287	6.06
13.288	6.6574	5.90
32.624	2.7425	5.68
26.305	3.3852	4.97
38.908	2.3128	4.95
25.250	3.5241	4.88
11.236	7.8681	4.57
27.697	3.2181	4.56
31.478	2.8397	4.16

3.3 Thermal Methods of analysis

3.3.1 Melting Behavior

The two literature reports for cortisone report a melting range of either 220-224°C (accompanied by some decomposition) [1], or 217-224°C (also with decomposition) [2].

For cortisone acetate, one reported melting range is 235-238°C (with slight sintering at 230°C) [1], and the other is about 240°C with decomposition [2,3].

3.3.2 Differential Scanning Calorimetry

The differential scanning calorimetry thermogram of cortisone acetate was obtained using a DuPont model TA 9900 thermal analyzer system, at a heating rate of 10°C/minute under a nitrogen flow. The DSC thermogram thusly obtained is shown in Figure 2, and consists of a single melting endotherm. The melting point of the substance studied was determined to be 247.7°C, and the enthalpy of fusion to be 45.6 kJ/mole. Using the DSC purity program (version V1.1A), the purity of the cortisone acetate sample was determined to be 97.48%.

3.4 Solubility Characteristics

Cortisone is slightly soluble in water (0.28 mg/mL at 25°C), fairly soluble in methanol, ethanol, and acetone, and is much less soluble in ether, benzene, and chloroform [1].

Cortisone acetate is sparingly soluble in water (0.022 mg/mL at 25°C), more soluble in propylene glycol (0.44 mg/mL), and soluble in chloroform (182 mg/g) [1].

The British Pharmacopoeia specifies the following solubility data for Cortisone acetate: practically insoluble in water, soluble in 4 parts of chloroform, and in 300 parts of 96% ethanol, soluble in 1,4-dioxan, sparingly soluble in acetone, and slightly soluble in both ether and methanol [3,4].

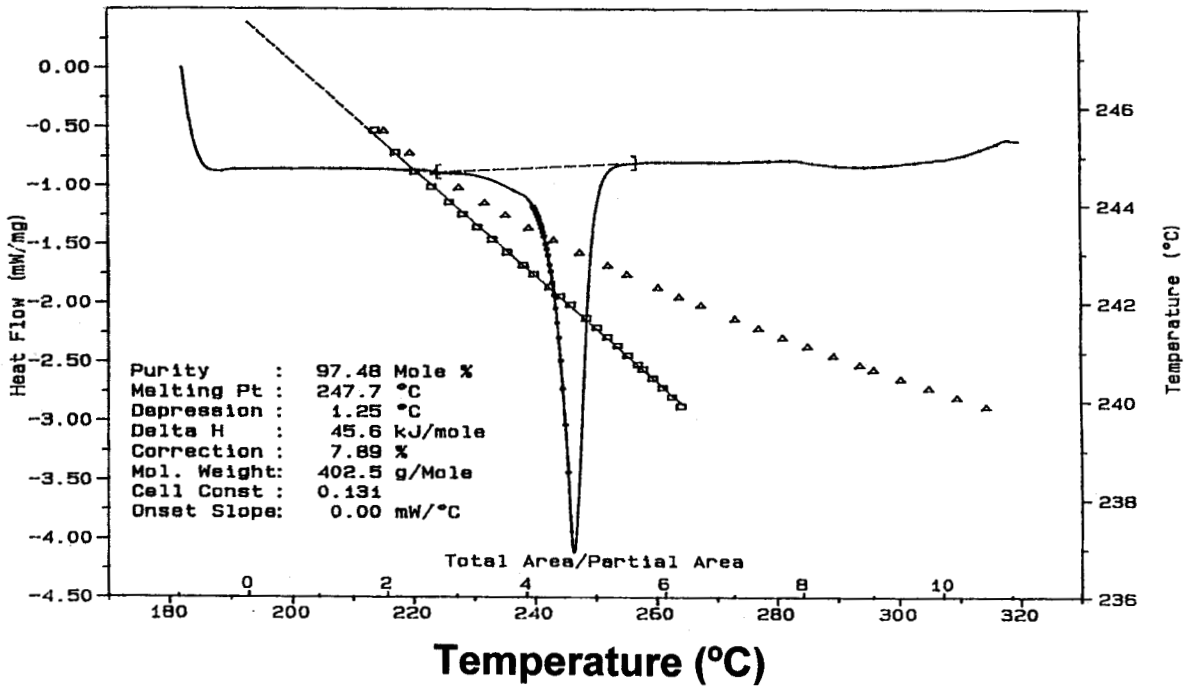


Figure 2. Differential scanning calorimetry thermogram of cortisone acetate.

3.5 Partition Coefficients

The partition coefficient of cortisone was calculated using the predictive program of Advanced Chemistry Development Laboratories, yielding a value of log P = 1.27 ± 0.52 [183]. Similarly, a theoretical log P value of 1.42 ± 0.55 was calculated for cortisone acetate.

3.6 Spectroscopy

3.6.1 UV/VIS Spectroscopy

The UV absorption spectrum of cortisone acetate dissolved in ethanol was recorded on a Shimadzu UV 1601 PC spectrometer over the range of 200 to 400 nm. The spectrum is shown in Figure 3, and consisted of a single absorbance maximum at 328 nm. The (1%,1cm) absorbance value of this band was determined to be 393, and the molar absorptivity to be 15818.

Clarke reported that in ethanolic solution, cortisone acetate exhibits a maximum at 240 nm [2]. For this band, the A(1%,1cm) value was approximately 390.

3.6.2 Vibrational Spectroscopy

The infrared absorption spectrum of cortisone acetate was obtained as a KBr pellet (approximately 2% loading) using a Perkin Elmer 1310 IR spectrophotometer. The spectrum itself is shown in Figure 4. The functional groups of the molecule are correlated with the observed frequencies in Table 3.

Table 3
Assignments for the Major Observed Infrared Absorption Bands

Frequency (cm^{-1})	Assignment
3420	-OH stretching modes
2960-2880	-CH stretching modes
1740, 1230	Acetate -C=O stretching mode
1690	C= 0 stretching mode
1650	C=C stretching mode
1130	C-0 tertiary OH mode

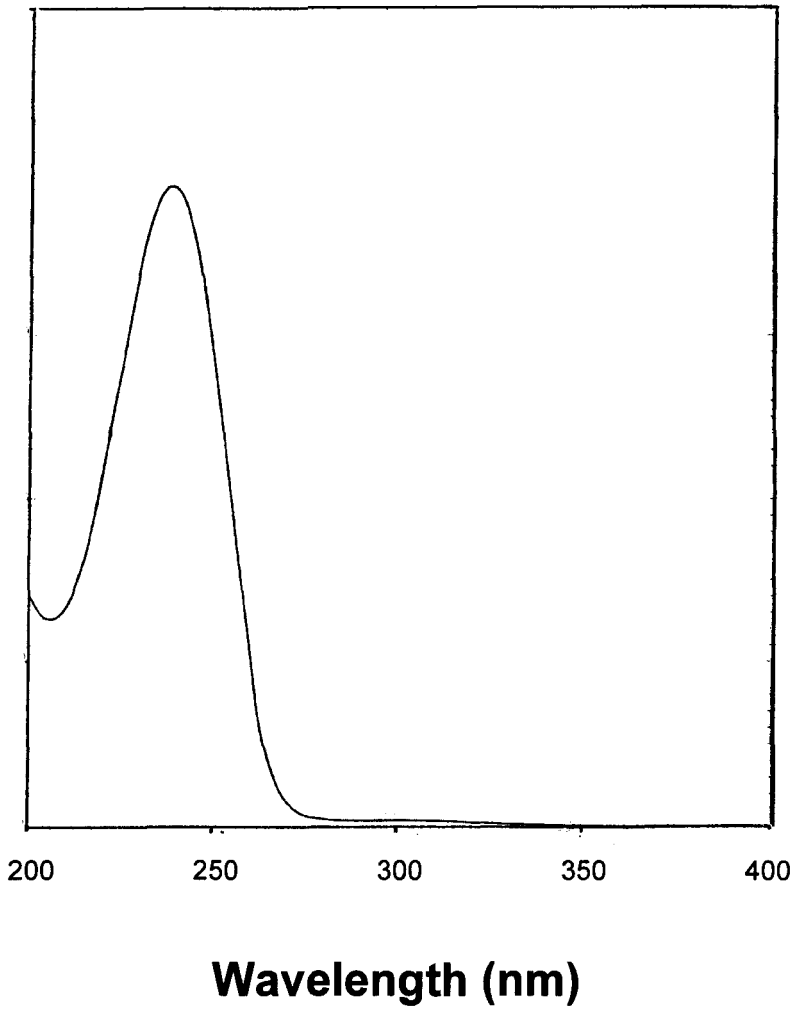


Figure 3. Ultraviolet absorption spectrum of cortisone acetate.

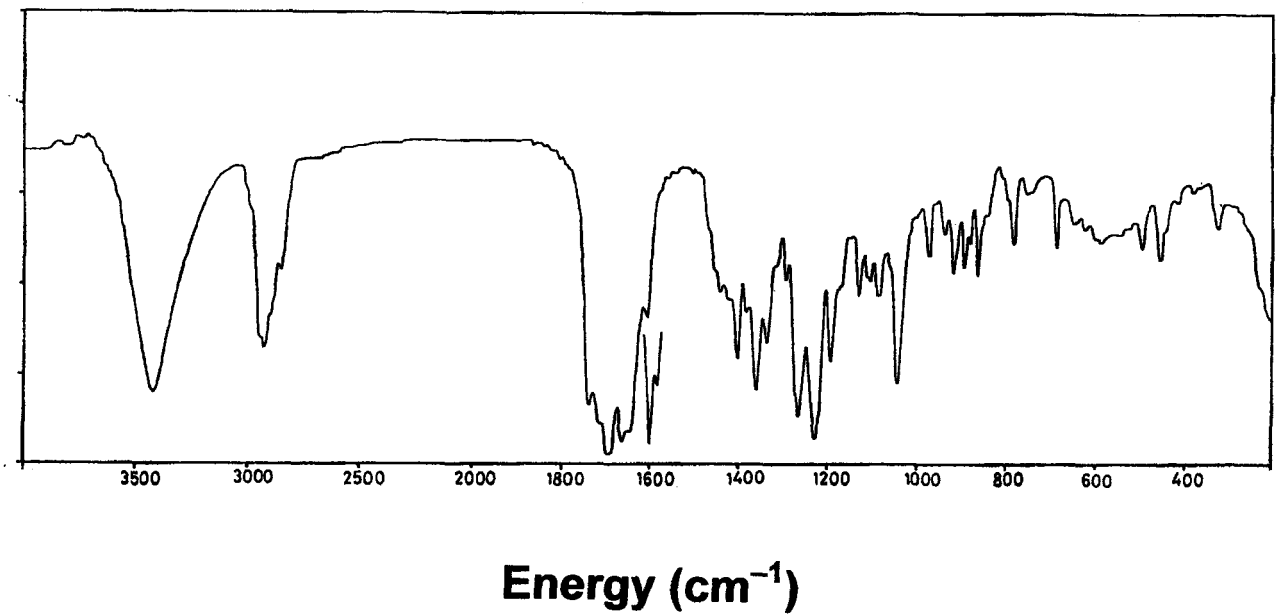


Figure 4. Infrared absorption spectrum of cortisone acetate.

Principal infrared absorption bands of cortisone acetate (obtained in a KBr disc) have been reported at 1700, 1660, 1235, 1720, 1275, and 1750 cm^{-1} [4].

3.6.3 Nuclear Magnetic Resonance Spectrometry

NMR spectral data for cortisone, as well as many other steroids, have been reported in the literature [7-11].

3.6.3.1 ^{1}H-NMR Spectrum

The ^{1}H nuclear magnetic resonance spectrum of cortisone acetate was obtained on a Joel FT 400 MHz spectrometer system, using tetramethylsilane as the internal reference. To obtain the spectrum, the substance was dissolved in $CDCl_3$. The full ^{1}H-NMR spectrum is shown in Figure 5, while expansions of various regions are given in Figures 6 and 7. Assignments for the observed resonance bands are provided in Table 4.

3.6.3.2 ^{13}C-NMR Spectrum

The ^{13}C nuclear magnetic resonance spectrum of cortisone acetate was obtained on a Joel FT 100 MHz spectrometer system, using tetramethylsilane as the internal reference. To obtain the spectrum, the substance was dissolved in $CDCl_3$. The full ^{13}C-NMR spectrum is shown in Figure 8, and assignments for the observed resonance bands are provided in Table 5.

3.6.4 Mass Spectrometry

The electron impact ionization mass spectrum of cortisone acetate was obtained using solid probe introduction, using a Shimadzu QP-Class-5000 gas chromatography mass spectrometer system. The most prominent ions observed, and their relative intensities, are shown in Table 6.

Fast-atom-bombardment (FAB)-Mass spectrometry has been used for the pharmaceutical analysis of corticosteroids [182]. The FAB-mass spectra of 24 corticosteroids (such as cortisone, prednisolone, hydrocortisone, and betamethasone) in were recorded in glycerol and/or thioglycerol. The

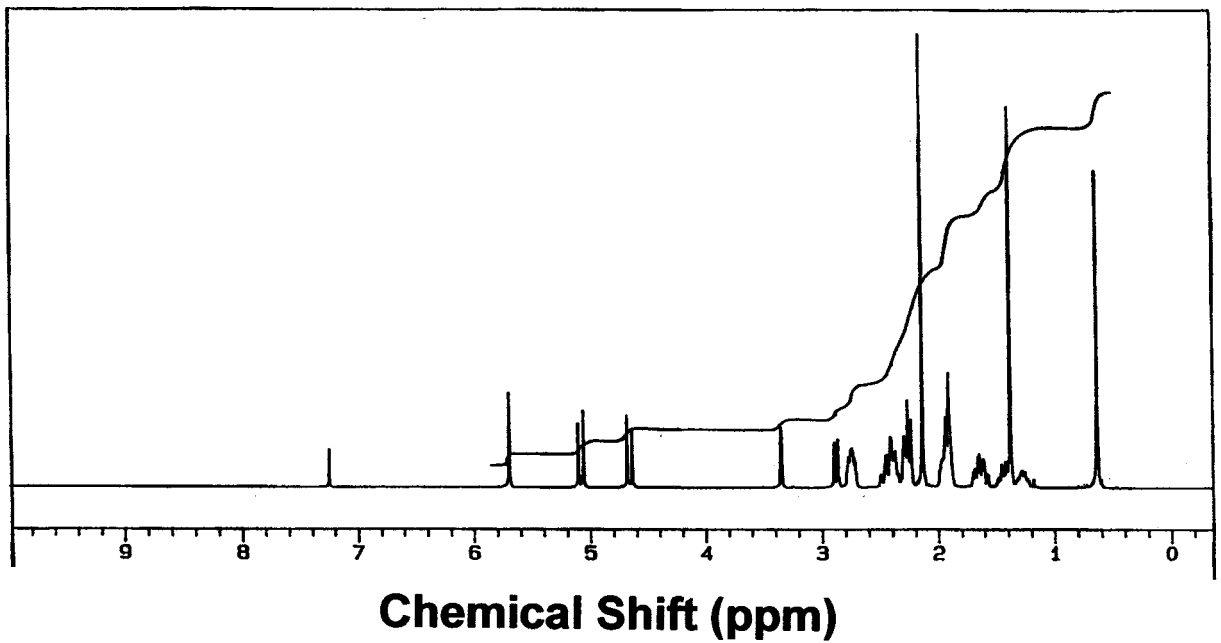


Figure 5. Full ^{1}H nuclear magnetic resonance spectrum of cortisone acetate.

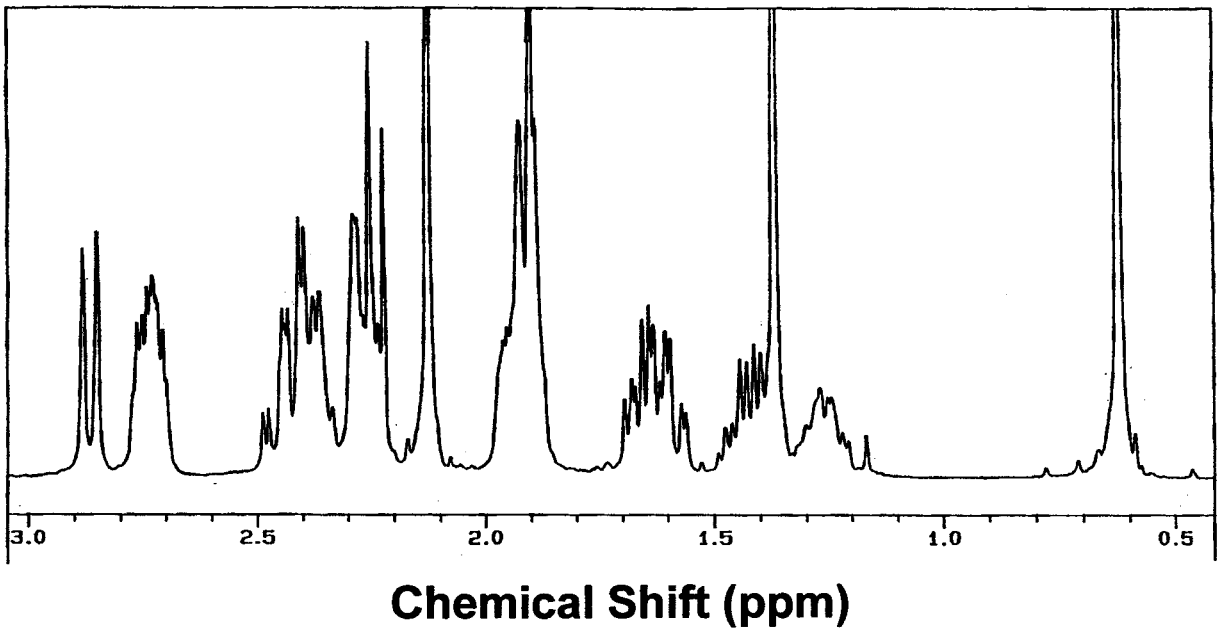


Figure 6. Expanded ^{1}H nuclear magnetic resonance spectrum of cortisone acetate.

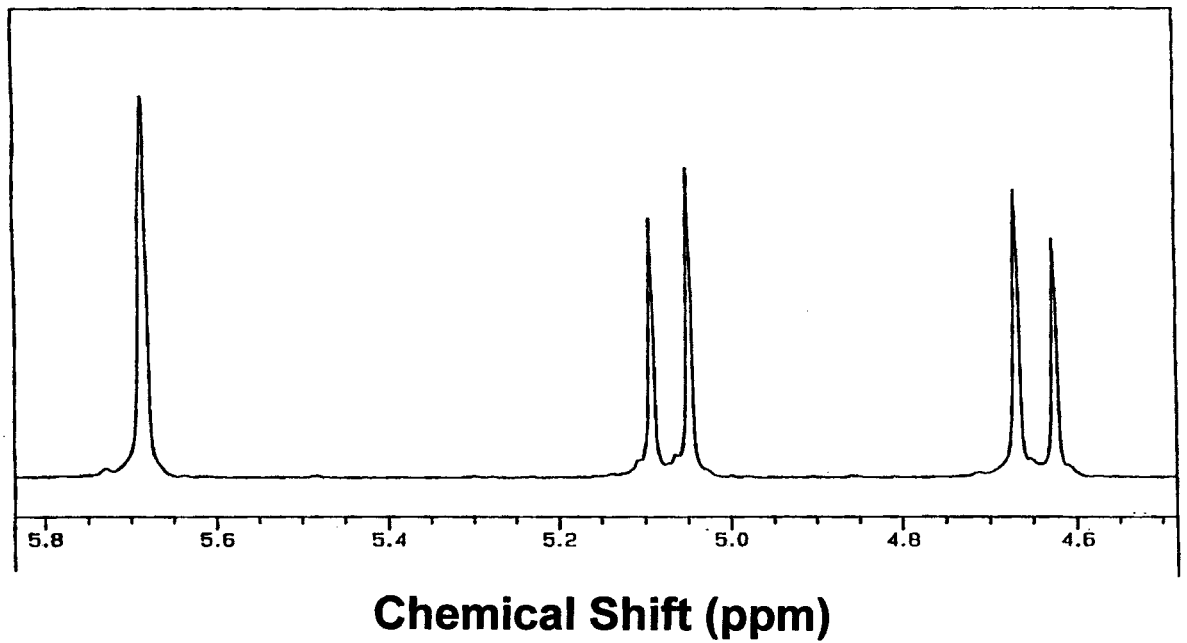


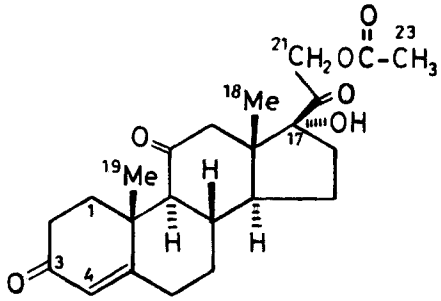
Figure 7. Expanded ^{1}H nuclear magnetic resonance spectrum of cortisone acetate.

Table 4

Assignments for the Resonance Bands Observed in the ^{1}H-NMR Spectrum of Cortisone Acetate

Proton Assignment	Chemical Shift δ (ppm)
C_{18} - CH_3	0.620 (s)
C_{19} - CH_3	1.366 (s)
C_{23} - CH_3 (OAc)	2.125 (s)
C_{17} - OH	3.335 (D_2O, exchangeable)
C_{21} - CH_2O_	Two pairs of doublets centered at δ 5.0696 and at δ 4.647 J= 17.6 Hz (geminal coupling)
C_4 - H	5.685 (s)

s = singlet.



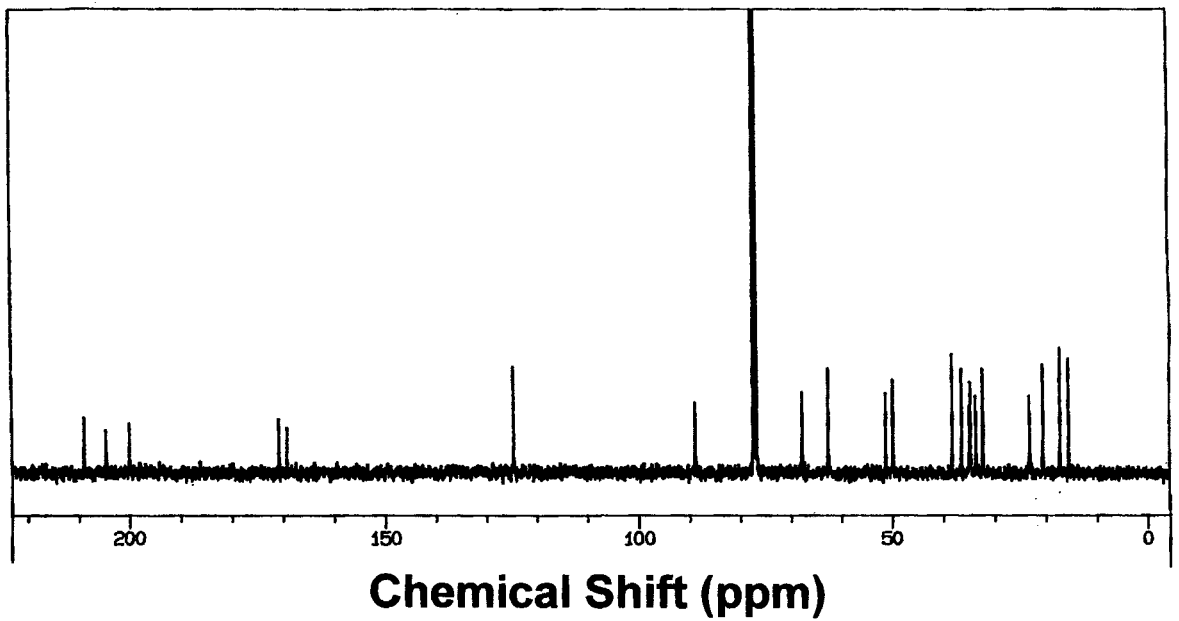
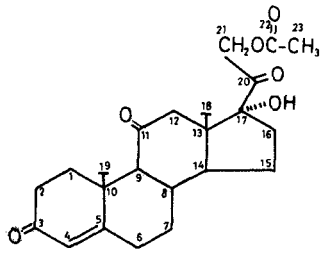


Figure 8. ^{13}C nuclear magnetic resonance spectrum of cortisone acetate.

Table 5

Assignments for the Resonance Bands Observed in the ^{13}C-NMR Spectrum of Cortisone Acetate

Carbon Assignment	Chemical shift (ppm)
C_1	36.40
C_2	34.85
C_3	200.08
C_4	124.44
C_5	169.06
C_6	33.64
C_7	32.27
C_8	32.21
C_9	49.73
C_{10}	38.18
C_{11}	204.76
C_{12}	62.44
C_{13}	49.86
C_{14}	51.18
C_{15}	23.16
C_{16}	34.65
C_{17}	88.80
C_{18}	15.37
C_{19}	20.47
C_{20}	209.08
C_{21}	67.66
C_{22}	170.62
C_{23}	17.14



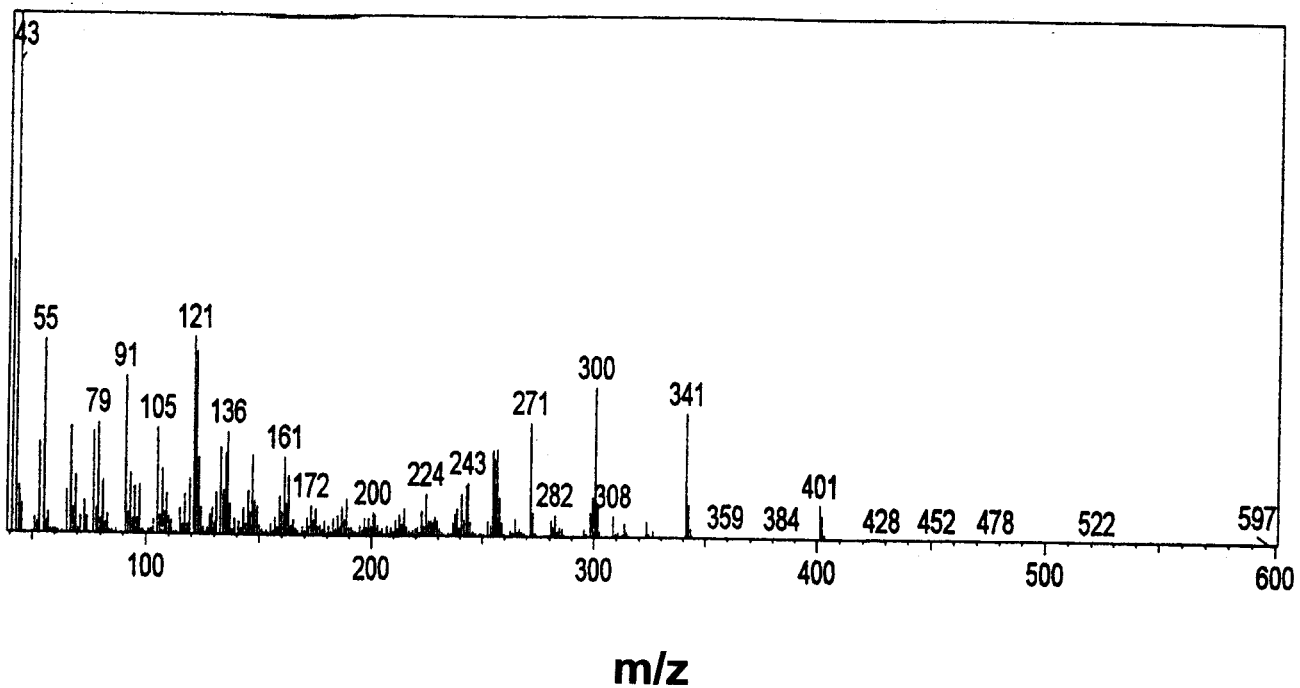


Figure 9. Mass spectrum of cortisone acetate.

Table 6

Fragment Ions Noted in the Mass Spectrum of Cortisone Acetate

m/z	Relative Intensity %	m/z	Relative Intensity %
402	6.1 M^+	121	35.0
341	23.8	120	39.2
300	27.7	118	10.5
271	19.3	107	12.3
256	16.2	105	19.7
255	14.5	93	12.2
254	16.7	91	30.7
243	10.2	79	27.1
162	11.3	68	11.2
161	14.8	66	20.9
146	15.3	55	37.0
136	19.3	53	17.6
134	15.9	43	100.0
122	14.9	41	52.1

spectra were recorded on a Finnigan NIAT 312 instrument, using xenon as the bombardment gas (energy = 8 kV) and detection in the mass range of m/z 100 to 1300.

4. Methods of Analysis

4.1 Identification

A number of identification tests are described for cortisone acetate in the British Pharmacopoeia [3].

4.1.1 The infrared absorption spectrum is concordant with the spectrum of cortisone acetate EPCRS. If the spectra are not concordant, prepare new spectra using 50% w/v solutions in chloroform IR.

4.1.2 Dissolve 10 mg in sufficient 96% ethanol to produce 100 mL, and dilute 10 mL to 100 mL with the same solvent. The A(1%,1 cm) of the resulting solution at the maximum of 240 nm is 375 to 405.

4.1.3 A 1% w/v solution in 1,4-dioxan gives a specific optical rotation of +211° to +220°.

4.1.4 Add 2 mg to 2 mL of sulfuric acid and shake to dissolve; a faint yellow color is produced within 5 minutes. Add the solution to 10 mL of water and mix; the color is discharged and a clear solution is produced.

The following identification tests are mentioned under cortisone acetate in the USP [71].

4.1.5 Dissolve a portion of cortisone acetate in methanol in a beaker, evaporate the solvent on a steam bath with the aid of a current air, then dry the residue at 105°C for 30 minutes. The infrared absorption spectrum of a potassium bromide dispersion of the residue so obtained exhibits maxima only at the same wavelengths as that of a similar preparation of USP cortisone acetate RS.

4.1.6 The ultraviolet absorption spectrum of a 1 in 100,000 solution in methanol exhibits maxima and minima at the same wavelengths as that of a similar solution of USP

cortisone acetate RS concomitantly measured. The respective absorptivities, calculated on the dried bases as the wavelength of maximum absorbance at about 238 nm do not differ by more than 3.0%.

4.1.7 The specific rotation shall be between +208° and +217°, calculated on the dried basis when determined in a dioxane solution containing 100 mg in each 10 mL.

Other identification tests are as follows:

4.1.8 To a warm 1% solution in alcohol, add an equal volume of potassium cupri-tartrate solution. A red precipitate is produced.

4.1.9 Dissolve about 200 μg in 1 mL of 95% alcohol, evaporate to dryness *in vacuo*, add 5 mL of 1N sodium hydroxide, and heat at 700°C for 30 minutes. A yellow solution is produced that has a strong absorption at 370 nm, which permits a distinction from prednisone.

4.1.10 To about 15 mg in a test-tube, add 3 drops of phosphoric acid and close the tube with a stopper through which passes a smaller test-tube filled with water, and on the outside of which hangs a drop of lanthanum nitrate solution. Heat in a water-bath for 5 minutes (or if necessary bring slowly to the boil over a flame). Mix the drop of lanthanum nitrate solution with 1 drop of 0.02 N iodine on a white tile, and place 1 drop of dilute ammonia solution at the edge of the mixture. A blue color slowly appears at the junction of the two liquids indicating the presence of an acetyl group.

4.2 Elemental Analysis

Cortisone:	C = 69.98%
	H = 7.83%
	O = 22.19%
Cortisone acetate:	C = 68.64%
	H = 7.51%
	O = 23.85%

4.3 Colorimetric Methods of Analysis

The various functional groups present in corticosteroid structures enable specific reactions to be conducted that facilitate the determination of these compounds. The most important functional groups allowing such reactions are the -3-keto group, the Δ^4-3-keto groups (or other Δ-3-keto groups such as the Δ^1-3-keto groups), and the 21-hydroxy-20-keto group (ketol function). Some of the more specific reaction methods [72] will be described in the following sections. Additional colorimetric methods for the determination of corticosteroids are found in the literature [105-111].

4.3.1 The Isoniazid Method

Condensation of isoniazid (isonicotinic acid hydrazide) with any Δ^4-3-ketosteroid in acidic medium affords a yellow color conjugate, allowing the determination of 10-40 μg of the steroid [73]. The reagent is prepared by dissolving 0.8 g of isoniazid in 100 mL of methanol containing 1 mL of concentrated HCl, and then 12.5 mL of this solution is further diluted to 100 mL with methanol. To a 2 mL methanolic sample solution of the Δ^4-3-ketosteroid is added 2 mL of the reagent. The mixture is allowed to stand at room temperature for an hour, and then the absorbance of the resulting colored solution is measured at 380 nm. In a 1 cm spectrophotometer cell, an absorbance of 0.3 will be produced by 42 μg of cortisone, 34 μg of prednisolone, 29 μg of prednisone, or 32.5 μg of dexamethasone [72].

The isoniazid method was applied to the determination of corticosteroids in urine [74] and to the determination of some steroids in pharmaceutical formulations [75].

4.3.2 The *p*-Dimethylaminoaniline Method

A yellow-orange color is developed upon the condensation of *p*-dimethylaminoaniline with Δ^4-3-ketosteroids under the action of perchloric acid in methanol [76]. The condensation reaction is performed at room temperature.

To 0.5 mL of sample solution in 0.01 N methanolic perchloric acid, 1 mL of 0.1% methanolic dimethyl-*p*-phenylenediamine oxalate is added. The resulting solution is left to stand for 30 minutes in the dark, 2.5 mL of

methanol is added, and the absorbance at 420 nm determined. In a 1 cm spectrophotometer cell, an absorbance of 0.3 will be produced by 62 μg of cortisone, 62 μg of prednisolone, or 62 μg of 16α-methylprednisone [72].

4.3.3 The 2,6-Di-tertbutyl-*p*-Cresol Reaction [77]

The reaction of Δ^4-3-ketosteroids with 2,6-di-tertbutyl-*p*-cresol is specific, and different analytes yield various colors. In alkaline media and in the presence of an oxidant (*e.g.*, hydrogen peroxide), Δ^4-3,11-diketo steroids (such as cortisone and prednisone, at levels of 5-25 μg) develop yellow-orange colors with this reagent.

The reagents needed for this reaction are a 1% ethanolic solution of 2,6-di-tertbutyl-*p*-cresol, and a 0.03% aqueous solution of hydrogen peroxide (equivalent to 0.1 volume oxygen). The reaction is performed by adding 1 mL of the 2,6-di-tertbutyl-*p*-cresol reagent and 0.2 mL of the hydrogen peroxide reagent to 2 mL of the sample solution in ethanol. The resulting solution is heated at 80°C for 30 minutes, cooled to 18-20°C in a water bath, and the absorbance then determined at 460 nm. An absorbance of 0.3 (in a 1 cm cell) will be produced from 26 μg of cortisone [72].

This reaction was applied (with various modifications) to the estimation of corticosteroids in pharmaceutical preparations [78, 79], and also for the estimation of hydrocortisone and cortisone in admixtures of the two [80].

The 2,6-di-tertbutyl-*p*-cresol reaction also produces fluorescent products with certain corticosteroids, which allows the development of fluorimetric methods for their determination. These will be discussed in a subsequent section.

4.3.4 Reaction with Sodium Molybdate

Reactions based on the presence of the 21-hydroxy-20-keto functionality (ketol group) are much more specific for corticosteroids, and these methods have been used for the determination of corticosteroids in pharmaceutical formulations or in biological fluids [72]. For instance, only 21-unesterified corticosteroids react with sodium molybdate in acetic acid medium [81]. The blue color obtained by reducing the ketol group allows the determination of 40-200 μg of these steroids.

The reagent is prepared by adding 40 mL of glacial acetic acid to 0.5 mL of a 25% aqueous solution of sodium molybdate. The reaction is carried out by adding 4 mL of the reagent to 3 mL of the sample solution. The resulting solution is allowed to stand for 2 hours and then the absorbance is determined at 650 nm. In a 1 cm cell, an absorbance of 0.3 is produced by 123 μg of cortisone, 187 μg of hydrocortisone, 172 μg of prednisolone, or 119 μg of prednisone.

4.3.5 Reaction with Cu(II) and *o*-Phenylenediamine

In neutral medium, and in the presence of air and cupric acetate, the ketol group is first oxidized into a ketoaldehyde that then condenses with *o*-phenylenediamine to produce a quinoxaline derivative. This derivative absorbs strongly in the near-UV range [82], with all of the corticosteroids affording equivalent sensitivity levels [72].

The cupric acetate reagent is prepared by diluting 10 mL of a 3% aqueous solution to 100 mL with methanol. The *o*-phenylenediamine reagent is prepared by adding 1 g of solid to 5 ml of 1N HCl and diluting to 100 mL with water. To conduct the reaction, 0.1 mL of the Cu(II) reagent is added to 1 mL of a methanolic sample solution, and the solution heated at 50°C for 5 minutes. The mixture is cooled in a water bath for a few minutes, 0.5 mL of the *o*-phenylenediamine reagent is added, and the solution heated again at 50°C for 5 minutes. Finally, the solution is cooled in a water bath, 2 mL of 5N HCl is added with mixing, and read the absorbance determined at 330 nm. In a 1 cm cell, an absorbance of 0.3 is produced by 33.5 μg of cortisone, 39.5 μg of deoxycorticosterone, 40 μg of prednisolone, 40 μg of prednisone, 43 μg of hydrocortisone, 172 μg of dexamethasone, or 53 μg of triamcinolone [72].

4.3.6 Tetrazolium Methods

In the presence of the C-20-ketogroup at the side chain, tetrazolium salts are reduced to the corresponding strongly colored formazans. Mader and Buck were the first to apply tri-phenyl tetrazolium chloride to assay pharmaceutical steroids [83], but it was later found that air affects the procedure leading to poor reproducibility [84,85]. More stable reagents such as 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium bromide [86] and 2,5-diphenyl-3-(*p*-strylphenyl)tetrazolium chloride [87] were employed successfully. Good results were also obtained with blue tetrazolium, or 3,3'-(3,3'-dimethoxy-4,4'-biphenylene) bis {2,5-diphenyl-

2H-tetrazolium chloride} [88]. The blue tetrazolium method is now widely used and has been adopted by the USP [89].

21-Esters of corticosteroids can also be determined. Studies with cortisone acetate showed that hydrolysis of the ester is a prerequisite to the reaction with a tetrazolium salt [83].

4.3.6.1 The Triphenyltetrazolium Chloride Reaction

The 1980, 1988 and 1993 editions of the British Pharmacopoeia have adopted the triphenyltetrazolium chloride method, but with certain modifications as suggested by Johnson *et al.* [85]. The method described in the following section is that recommended by the B.P. of 1988 [3]. It should be noted that all solutions must be protected from light: throughout the assay.

A quantity of cortisone acetate (or any official corticosteroid) is dissolved in sufficient aldehyde-free ethanol so as to produce a solution containing between 340 and 360 μg in 10 mL. 10 mL of this solution is transferred to a 25-mL graduated flask, 2.0 mL of triphenyltetrazolium chloride solution (0.2 g in 20 mL of aldehyde-free dehydrated ethanol) is added, and the air displaced from the flask with oxygen-free nitrogen. Immediately, 2.0 mL of dilute tetramethylammonium hydroxide solution is added, and any aid is displaced with oxygen-free nitrogen. The flask is stoppered, the contents mixed by gently swirling, and the mixture allowed to stand in a water-bath at 35°C for one hour. After that, the contents are cooled rapidly, sufficient aldehyde-free absolute ethanol is added to produce 25 mL, and the solution mixed. The absorbance of the resulting solution is determined in a closed 1-cm cell at a wavelength of 485 nm, using 10 mL of aldehyde-free absolute ethanol treated in the same manner as the blank. The content of cortisone acetate is calculated from the measured absorbance by repeating the operation using cortisone acetate EPCRS or EPCRS instead the substance being assayed.

4.3.6.2 The Tetrazolium Blue Reaction

The tetrazolium blue method was first recommended by Chen *et al.* [90], and was modified by several authors [91-94]. It has been the most widely used procedure in the routine determinations of corticosteroids. The following procedure has been recommended [88].

The main reagent is a 0.1% solution of tetrazolium blue in ethanol, but the method also requires diluted tetramethylammonium hydroxide. The latter reagent is obtained by diluting 5 mL of a 10% aqueous solution to 50 mL with ethanol. 0.5 ml of the tetramethylammonium hydroxide reagent, 0.5 mL of the blue tetrazolium reagent, and 2.0 mL of chloroform are added to 1 mL of an ethanolic sample solution (containing 11-37 μg). The resulting mixture is allowed to stand at room temperature for 5 minutes when assaying for free ketols, 10-15 minutes for esterified ketols, or 20 minutes for triamcinolone (only exposed to subdued light). The absorbance is then read at 525 nm. In a 1 cm cell, an absorbance of 0.3 is produced by 17 μg of cortisone, 18 μg of hydrocortisone, 21 μg of hydrocortisone acetate, 11 μg of triamcinolone, or 37 μg of prednisolone m-sulfobenzoate. 21-corticosteroid phosphates do not react [72].

A modification of the tetrazolium blue method for the determination of urinary adrenocortical steroids has been reported [95]. Good recoveries of cortisone and cortisone acetate in urine were obtained by this modification. Corticosteroids have also been separated by chromatographic means and then assayed by a UV or colorimetric determination [123).] In one report, a known quantity of analyte was separated using thin-layer chromatography, and the plate dried. The corticosteroids were located by spraying the plates with 0.1% aqueous tetrazolium blue / 2N NaOH (1:9) reagent. After that, the areas corresponding to spots were removed, suspended in water, and extracted with chloroform. The residues from the washed and dried extracts were dissolved in absolute ethanol (at a concentration of 5-10 μg/mL) and determined by the UV absorption between 238-243 nm. In another study, adrenal cortical hormones were determined after separation by gradient elution chromatography [124]. The fractions were assayed either by measuring the direct absorption at 240 nm, or by treatment with tetrazolium blue and measurement at 520 nm.

4.3.7 The *p*-Nitroso-dimethylaniline Reaction

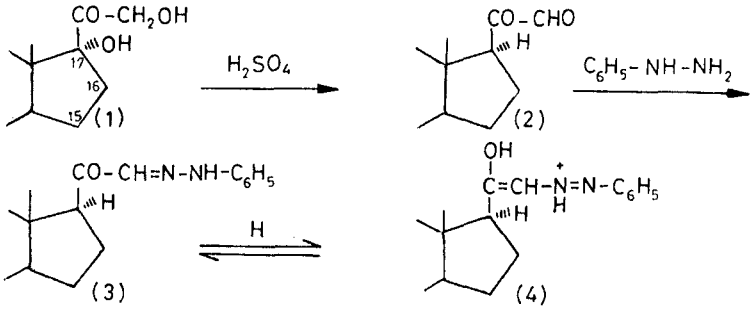
In alkaline medium, *p*-nitroso-*N,N*-dimethylaniline is reduced by the ketol group to *p*-dimethylaminoaniline, which is then developed to a green color with phenol in the presence of ferricyanide ion [96,97].

Four reagents are required for performance of this reaction. The first is a 0.1% ethanolic solution of *p*-nitroso-*N,N*-dimethylaniline. The second is pH 9.8 Clark and Lubs buffer, prepared by adding 40.8 mL of 0.2 M sodium hydroxide (carbon dioxide-free) to 50 mL of a 0.2M aqueous solution of both boric acid and potassium chloride. The third reagent is a 0.1% solution of phenol in ethanol, and the fourth reagent is a freshly prepared 1% aqueous solution of potassium ferricyanide.

To execute the procedure, one adds 0.5 mL of the *p*-nitroso-*N,N*-dimethylaniline reagent solution to 1 mL of an ethanolic sample solution. The tube containing this solution is immersed in ice water for 5 minutes, then 0.5 mL of 0.1N sodium hydroxide is added. The reaction tube is plugged with cotton-wool and allowed to stand at 0°C in the dark for 1 to 5 hours according to the identity of the compound tested. This is generally 1 hour for cortisone and its esters, 2 hours for hydrocortisone, prednisolone and their esters, and 4 hours for dexamethasone. After this, 2 mL of the Clark and Lubs buffer, 5 mL of the phenol reagent, and 0.5 mL of the potassium ferricyanide reagent are added. The resulting mixture is allowed to stand in a water bath at 20±2°C for 10 minutes, and the absorbance at 650 nm determined. In a 1 cm cell, an absorbance of 0.3 is produced by 120 μg of cortisone (1 hour reaction time), 134 μg of cortisone acetate (1 hour reaction time), 116 μg of corticosterone (5 hour reaction time), 120 μg of hydrocortisone (2 hour reaction time), 134 μg of hydrocortisone acetate (2 hour reaction time), 131 μg of prednisolone (2 hour reaction time), 144 μg of dexamethasone acetate (4 hour reaction time), or 173 μg of dexamethasone hemisuccinate (4 hour reaction time) [72].

4.3.8 The Porter-Silber Method

This method is specific for the 17,21-dihydroxy-20-keto corticosteroids (such as cortisone and hydrocortisone). The method depends upon the action of sulfuric acid with the 17,21-dihydroxy-20-keto groups to give rise to a glyoxal side chain. Subsequent condensation with phenylhydrazine yields a stable yellow color. This reaction was developed by Porter and Silber [98], and its mechanism was elucidated by Lewbart and Mattox [99] as follows:



The reagent is prepared by dissolving 0.065 g of phenylhydrazine hydrochloride in 100 mL of a diluted sulfuric acid solution (prepared by mixing 310 mL of concentrated acid and 190 mL of water, and allowing to cool). The reaction is performed by adding 8.0 mL of the reagent to 1 mL of a methanolic sample solution (containing 12-70 μg of analyte). The solution is heated at 60°C for 20 minutes, allowed to cool, whereupon the absorbance at 410 nm is determined. Esters at the 21-position, such as acetates, hemisuccinates, or *m*-sulfobenzoates, also react.

This method was used for the determination of the glucuronide esters of 17-hydroxycorticosteroids in urine [100]. An absorbance of 0.3 was attained in a 1 cm cell for 40 μg of cortisone, 46 μg of cortisone acetate, 55 μg of cortisone hexahydrobenzoate, 40 μg of prednisone, 70 μg of hydrocortisone, 91 μg of hydrocortisone acetate, 108 μg of hydrocortisone hemisuccinate, 60 μg of prednisolone, 60 μg of dexamethasone, or 70 μg of dexamethasone acetate.

It has been found that triamcinolone [101] and the 21-phosphate esters [102] do not under go the Porter-Silber reaction. It was also shown that an increased sensitivity for this procedure can be obtained using the *p*-hydrazino-benzenesulfonic acid-phosphoric acid reagent [103].

4.3.9 The 3-Methylbenzothiazoline-2-one Hydrazone Reaction

The reaction occurs with the 17,21-dihydroxy-20-keto functionalities of the side chain, and is also specific for these corticosteroids [104].

One reagent is a 0.5% aqueous solution of 3-methylbenzothiazolin-2-one hydrazone hydrochloride, and the other is a 0.25% aqueous solution of ferric chloride hexahydrate. To run the procedure, one introduces 1 mL of the sample solution in chloroform into a tube, and carefully evaporates to dryness on a steam-bath. No trace amount of the solvent should remain in the tube since it inhibits the reaction. 1 mL of water is added to the tube, then 0.5 mL of the 3-methylbenzothiazolin-2-one hydrazone reagent and 0.5 mL of 0.1N sodium hydroxide. This mixture is heated at 100°C for 10 minutes, and cooled for 5 minutes in a 15°C water bath. After that, one adds 0.5 mL of 1N hydrochloric acid and 2 mL of the ferric chloride reagent. The resulting solution is allowed to stand for 1 hour at room temperature, and the absorbance read at 630 nm. In a 1 cm cell an absorbance of 0.3 was obtained for 21 μg of cortisone, 18 μg of hydrocortisone, 17 μg of prednisone, or 19 μg of prednisolone.

4.4 Fluorimetric Methods of Analysis

4.4.1 Reaction with Sulfuric Acid

Fluorescent products are produced upon reacting any corticosteroid with concentrated sulfuric acid [16], and Bernstein and Lenhard have reported the spectra of 220 steroids dissolved in this reagent acid [112]. The solutions, generally containing a concentration of about 7 μmole of steroid per 100 mL of sulfuric acid, were allowed to stand for 2 hours prior to determination. Cortisone showed a significant maximum at 419 nm and an inflexion at 480 nm.

The relation between fluorescence intensity and structure was studied by Goldzieher *et al.* [113]. For this work, 0.2 mL of an ethanolic steroid solution was added to 1 mL of 90% (v/v) sulfuric acid, the mixture heated at 80°C for 10 minutes, and finally diluted with 4.0 mL of 65% (v/v) sulfuric acid. The resultant solution contained 25-250 μg/ml, and was measured at an excitation wavelength of 436 nm. No correction was made for self-absorption of the solutions.

Sweat [114] studied the fluorescence of sulfuric acid reacted corticosteroids, and adopted the following procedure. To 1 mL of an ethanolic steroid solution, 1 mL of concentrated sulfuric acid was added rapidly. The solution was thoroughly mixed, and allowed to stand at room temperature for 20 minutes. The relative intensities of observed

fluorescence followed the series hydrocortisone (100 units/μg), corticosterone (87 units/μg), cortisone (7 units/μg), deoxycorticosterone (4.5 units/μg), and dehydrocorticosterone (0.8 units/μg).

The fluorimetric determination of corticosteroids in sulfuric acid-ethanol mixtures has given rise to numerous studies, particularly with the aim of estimating these compounds in urine or plasma. These all require prior methods of fractionation to permit a more or less selective separation of the various corticosteroids. The purified sample solution is then evaporated to dryness, and the residue treated with the reagent to yield fluorescence which can be measured [72].

4.4.2 Reaction with Zinc dust and Sulfuric Acid

When Δ^4-3-ketosteroids or $\Delta^{1,4}$-3-ketosteroids are treated with zinc dust in 40% sulfuric acid, a fluorescent product is produced. This product can be extracted by butyl ether and measured quantitatively [115]. The relative order of fluorescence intensities was prednisolone (100), triamcinolone (95), triamcinolone acetonide (60), cortisone (3.7), hydrocortisone (3.5), and progesterone (5.2).

4.4.3 Reaction with 2,6-Di-tertbutyl-*p*-cresol

Δ^4- and $\Delta^{1,4}$-3,11-diketosteroids (such as cortisone and prednisone) develop fluorescence when reacted with 2,6-di-tertbutyl-*p*-cresol, allowing the determination of 1-5 μg of the former and 40-200 μg of the latter [116].

To run the reaction, two reagents are required. One of these is a 1% solution of 2,6-di-tertbutyl-*p*-cresol in ethanol, and the other is a 0.03% aqueous solution of hydrogen peroxide. The reaction is initiated by mixing 2 mL of an ethanolic sample solution, 1 mL of the 2,6-di-tertbutyl-*p*-cresol reagent, 0.2 mL of the hydrogen peroxide reagent, and 2.0 mL of 1N sodium hydroxide solution. The mixture is heat in subdued light at 80°C for 30 minutes, and then cool in ice water for 2 minutes. Finally, the resulting fluorescence is measured using an excitation wavelength of 436 nm and an emission wavelength of 520 nm.

4.4.4 Periodate Oxidation

17-ketolsteroids and 17-hydroxy-17-ketol-steroids are first subjected to oxidation by periodic acid, forming 17-carboxysteroids and formaldehyde. The liberated formaldehyde is condensed with ammonia and ethyl acetoacetate, whereupon the fluorescence of the condensate is measured [117). Using this method, one can determine 6-40 μg of 21-unesterified ketol-steroids.

Three reagents are required to perform the periodate oxidation method. The first of these is a 0.01M aqueous solution of sodium metaperiodate. The second is prepared by dissolving 0.25 g of stannic chloride decahydrate in 100 mL of 1N hydrochloric acid (this reagent must be prepared fresh before use). The third reagent is a 4% solution of ethyl acetoacetate in 20% aqueous ammonium acetate. To run the method, 0.2 mL of the sodium metaperiodate reagent is mixed with 1 mL of the sample dissolved in 2% ethanol, and allowed to let stand at room temperature for 20 minutes. After that, one adds 0.8 mL of the stannic chloride reagent, 0.8 mL of 1N sodium hydroxide, 1.2 mL of water, and 1 mL of the ethyl acetoacetate reagent. The mixture is heat at 60°C for 20 minutes, allowed to cool, and filtered. The fluorescence of the reaction produce is determined using an excitation wavelength of 366 nm and an emission wavelength of 470 nm. A linear relationship was found to hold over the range of 6-30 μg for deoxycorticosterone, cortisone, and prednisone, and over the range of 8-40 μg for prednisolone.

A variety of other fluorimetric methods have also been reported [118-122].

4.5 Chromatographic Methods of Analysis

4.5.1 Paper Chromatography

Clarke described a chromatographic system for the identification of corticosteroids including cortisone [2]. The method uses Whatman paper #1, impregnated with a 40% v/v solution of formamide in methanol, which must be prepared immediately before use. A saturated solution of formamide in chloroform was used as the solvent system [125]. The paper was equilibrated for 2 1/2 hours in a tank that had been saturated with the solvent for 24 hours. Development was continued in a descending manner until the solvent front had advanced about 35 cm. To locate the analytes,

the chromatogram was first heated at 105° for 40 minutes, then rapidly passed through a shallow layer of DPST solution [a solution of 2,5-diphenyl-3-(4-styryl-phenyl) tetrazolium chloride in ethanol, with 9 volumes of 2N sodium hydroxide]. The reagent produced a violet spot on a pale yellow background, which can be directly examined in subdued light. The reported R_f values were cortisone = 0.62, hydrocortisone = 0.26, prednisone = 0.55, and prednisolone = 0.15.

The system just described has been modified to identify corticosteroidal salts, including cortisone acetate. The solvent is a saturated solution of formamide in a 1:1 mixture of benzene and chloroform. Development is descending, until the solvent front has advanced about 22 cm [2,125], and the spot location is the same as described for the preceding method. The reported R_f values were cortisone acetate = 0.80, hydrocortisone acetate = 0.59, prednisone acetate = 0.74, and prednisolone acetate = 0.49.

Corticosteroids have been identified using strips of glass-fiber paper [126]. The strips were soaked in 0.1M KH_2PO_4, dried, and stored in a desiccator. The solvent consisted of various amounts of dimethylformamide (0-3% v/v) in benzene, which was placed in covered glass jar. The chromatograms were developed by the ascending technique, and a 20 cm strip was completed in 15 minutes. Spot location was effected by spraying with concentrated sulfuric acid, whereupon the charred steroids appeared as gray-black spots on white background.

A variety of other paper chromatographic techniques have also been described for the determination of cortisone acetate [127-129].

4.5.2 Thin Layer Chromatography

Many TLC systems have been recommended for the identification cortisone and cortisone acetate. The details of several of these systems are presented in Tables 7 and 8.

Clarke has described two TLC systems for the identification of cortisone acetate [2]. Both use glass plates coated with silica gel G (0.25 mm thick) as the separating agent, and each is spotted with a 1 μL aliquot of a corticosteroid solution in 1:1 chloroform-methanol. In both systems, the analytes were located using the DPST spray reagent. This visualizing

Table 7

Thin-Layer Chromatography Systems for Cortisone and Cortisone Acetate

	Chromatogram	The Mobile Phase	hRf		Remarks	Ref.
			Cortisone	Cortisone Ac.		
1.	Silica gel G	1,2-dichloroethane-methanol-water (475:25:1)	51	125	hRf in these systems are relative to hydrocortisone acetate = 100	(130)
2.	" "	1,2-dichloroethane-2-methoxy-ethyl acetate-water (80:20:1)	63	145		(130)
3.	" "	cyclohexane-ethyl acetate-water (25:75:1)	53	102		(130)
4.	" "	heptane-chloroform-acetic acid (1:1:2)	-	82		(131)
5.	" "	chloroform-acetic acid (9:1)	-	76		(131)
6.	" "	ethyl acetate-cyclohexane (17:3)	-	88		(131)
7.	" "	1,2-dichloroethane-dioxane-water (2:1:1)	137	188	hRf relative to hydrocortisone =100	(132)
8.	" "	dichloromethane-diethylether-methanol-water (385:75:40:6)	47	82		(133)
9.	" "	dichloromethane-dioxane-water (2:1:1)	42	70		(133)

Table 8

Thin-Layer Chromatography Systems for Cortisone

Chromatogram	The Mobile Phase	hRf	Remarks	Ref.
1. Silica gel G plates	benzene-ethanol (4:1)	43	chromatograms 1 to 7	
2. " " "	benzene-ethanol (9:1)	17	are recommended for	
3. " " "	cyclohexane-ethylacetate-		the identification of	
	ethanol (9:9:2)	21	natural corticosteroids	(134)
4. " " "	chloroform-ethanol (9:1)	34	including cortisol and	
5. " " "	chloroform-ethanol (19:1)	13	cortisone, their meta-	
6. " " "	ethylacetate-hexane-acetic		bolites as well as many	
	acid-ethanol (144:27:20:9)	47	androstane derivatives.	
7. " " "	ethylacetate-hexane-acetic			
	acid (15:14:1)	29		
8. Polygram Sil G	1,2-dichloroethane-ethanol		chromatograms 8 to 10	
sheets	(7:1)	36	are reported for the	
9. " "	1,2-dichloroethane-acetone		identification and	(135)
	(67:33)	41	separation of natural	
10. " "	diisopropyl ether-acetone		corticosteroids.	
	(7:3)	34		

Table 8 (continued)

Thin-Layer Chromatography Systems for Cortisone

Chromatogram	The Mobile Phase	hRf	Remarks	Ref.
11. Silica gel G 250 µm thick plates	methylene chloride-ether-methanol-water (77:15:8:1.2)	72	chromatograms 11 to 13 are recommended for the identification of cortisone acetate.	(136)
12. " "	dichloroethane-methanol-water (95:5:0.2)	28		
13. Kieselguhr 250 µm thick, impregnated in a mixture of acetone-formmadide (9:1)	toluene-chloroform (3:1)	55		
14. Silica gel G plates	chloroform-methanol-water (90:11:1)	66		(137)
15. " " "	ethylacetate-methanol-water (90:10:1)	89		(137)
16. " " "	diethylether-benzene-acetic acid (95:5:1)	21		(137)
17. " " "	diethylether-ethyl acetate (1:1)	44		(134)
18. " " "	chloroform-ethyl acetate-acetone (9:2:1)	19		(138)

agent is prepared by taking 5 mL of a 0.5% w/v solution of 2,5-diphenyl-3-(4-styrylphenyl) tetrazolium chloride in ethanol, and diluting to 50 mL with 2 N sodium hydroxide.

In the first system described by Clarke, the solvent consists of the lower layer obtained after the thorough equilibration of 50:25:25 dichloroethane-methyl acetate-water. Cortisone acetate was run as the control. In the second system, hydrocortisone was used as the reference substance. The solvent consists of the lower layer obtained after the thorough equilibration of 50:25:25 methylene chloride-dioxane-water. The R_f values in the second system are cortisone acetate = 1.88, prednisone = 1.21, prednisolone = 0.83, and triamcinolone = 0.59 (all relative to hydrocortisone).

A TLC technique has been adopted by the British Pharmacopoeia of 1988 [3] and of 1993 [139] to test related substances in cortisone acetate. The chromatography is carried out on plates coated with silica gel GF_{254} using a mobile phase consisting of a 77:15:8:1.2 v/v mixture of dichloromethane-ether-methanol-water. The method was set up by separately applying 5 μL each of the following solutions in a 9:1 mixture of chloroform-methanol. Solutions (1), (2), (3), and (4) contain 1.0, 0.10, 0.020, and 0.010 % w/v, respectively, of the substance being examined. Solution (5) contains 0.10% w/v of cortisone acetate reference standard. Solution (6) contains 0.010% w/v of each of the substances being examined and the hydrocortisone acetate reference standard).

Analytes are detected upon air-drying of the plate, and subsequent examination under UV light (254 nm). Any secondary spot in the chromatogram obtained with solution (1) must not be more intense than the spot in the chromatogram obtained with solution (3) and not more than one such spot can be more intense than the spot in the chromatogram obtained with solution (4). The test is not valid unless the chromatogram obtained with solution (6) shows two clearly separated principal spots.

4.5.3 Gas Chromatography

Many GC systems have been reported for the identification and quantitation of steroid hormones, including cortisone and its acetate ester.

The following represent selected examples of these systems. Numerous other GC techniques have also been reported [146-149].

System (1) has been reported for the quantitative determination of cortisone and hydrocortisone [140]. The column uses a packing of 1% QF-1 containing 1% XE-60 on HP Chromosorb W AW DMCS (80-100 mesh). The system is operated at 250°C, uses helium as the carrier gas, and detection is effected using flame ionization. It was found that solutions containing approximately 1% of analyte could be analyzed with an error less than 5%.

System (2) has been described for the assay of corticosteroids (cortisone, hydrocortisone, prednisone, and prednisolone) in urine [141]. Prior to introduction into the GC system, the sample was eluted with 2:1 ethyl acetate-methanol, the extracts evaporated to dryness, and then oxidized with sodium bismuthate. Used in the method was a silanized column (132 cm x 5 mm) containing 2,2-dimethylpropane-1,3-diol adipate (0.65%) supported on celite, and operated at 230°C. The carrier gas was argon, and the detector used strontium 90-ionization. The standard deviation was 3.5 % (based on 47 determinations).

System (3) has been recommended for the separation and determination of 17,21-dihydroxy-20-oxosteroids, including cortisone [142]. Prior to introduction into the GC system, treatment with sodium bismuthate in acetic acid at room temperature was performed, followed by addition of aqueous NaOH. The analytes were then extracted with chloroform. The column used was glass column (6 ft x 0.25inch), containing 3% QF-1 on GasChrom Q (100 to 120 mesh), operated at 225°C. Argon (150 mL/min) was used as the carrier gas, and detection was based on a P-ionization detector. It was reported that results obtained with a 1:1 mixture of cortisone and hydrocortisone were accurate to within approximately 1%.

System (4) has been reported for the quantitative determination of steroids in health and disease [143]. Prior to analysis, free steroids were removed from the sample. Conjugated steroids were enzymatically hydrolyzed, and the liberated products extracted into 1:3 tetrahydrofuran-ethyl ether. The extract was evaporated, the residue dissolved in acetic acid, and then oxidized with sodium bismuthate. The final product was acetylated or formulated, and subjected to GC analysis along with the standard. A stabilized column of 4% LAC-2R-446 and 1% 85% phosphoric acid on

GasChrom Z was used, operated at 270°C. The recoveries of tetrahydrocortisol and cortisone (in the range of 25 to 200 μg added to urine) exceeded 91% and 96%, respectively.

System (5) has been used for the direct analysis of steroids, including cortisone and cortisone acetates, as the trimethylsilyl ethers of their methoxyimmino derivatives [144]. A glass column (180 cm x 4 mm) containing 1.5% SE-30 on 60 to 80 mesh Chromosorb W, operated at 235°C, is used for the separation. Nitrogen is used as the carrier gas (flow rate of 35 mL/min), and detection is by flame ionization.

System (6) has been described for the simultaneous determination of sub-microgram amounts of 17α-deoxy and 17α-hydroxy corticosteroids [145]. Prior to analysis, the corticosteroids were treated with periodic acid in 50% aqueous dioxane, and extracted with dichloromethane. Acylation of the 3- and 17α-hydroxyl groups was carried out using 1:1 butyric anhydride and pyridine. {4-^{14}C}-cholesterol was added as the tracer, and cholesterol isobutyrate as the internal standard. The column used was glass (8 ft x 5 mm) packed with 1% SE 30 or QF-1 on GasChrom Q (100-120 mesh), and was operated at 250-260°C. Argon (at a flow rate of 30 mL/min) was used as the carrier gas, and detection was by a ^{98}Sr detector. It was reported that the corticoids could be determined with a precision of approximately 15% down to levels as low as 0.1-0.2 μg.

4.5.4 High Performance Liquid Chromatography

Numerous HPLC systems have been reported for the identification and quantitation of corticosteroids, including cortisone. Several selected systems are presented in the following sections, while other HPLC procedures are summarized in Table 9.

System (1) has been adopted by the USP [71] for the assay of cortisone acetate and its official pharmaceutical formulations (either sterile suspension or tablets). The standard is prepared by transferring about 12 mg of cortisone acetate RS, accurately weighed, into a glass-stoppered 50-mL conical flask. 25.0 mL of internal standard solution is added, and the solution sonicated for 5 minutes. Approximately 1 mL of this solution is combined with 3 mL of mobile phase to obtain the standard preparation. The column is any 25 cm x 4.6 mm column that contains packing L3. The

Table 9

Miscellaneous High Performance Liquid Chromatographic Methods

Description	Reference
Simultaneous determination of serum cortisol and cortisone by reversed-phase liquid chromatography.	(157)
Serum steroid hormonal profiles by reversed-phased liquid chromatography in patients with 17α-hydroxylase deficiency.	(158)
Determination of serum cortisone by reversed-phase liquid chromatography using pre-column sulfuric acid-ethanol fluorescence derivatization and column switching.	(159)
Automated direct HPLC assay for esterol, estriol, cortisone and cortisol in serum and amniotic fluid.	(160)
Differential measurement of cortisol and cortisone in human saliva.	(161)
Ultra-sensitive differential measurement of cortisol and cortisone in biological samples using fluorescent ester derivatives in normal phase HPLC.	(162)
Isocratic reversed-phase HPLC determinations of twelve natural corticosteroids in serum with online UV and fluorescence detection.	(163)
Quantitation of corticosteroids as common adulterants in local drugs by HPLC.	(156)
Reversed-phase liquid chromatographic determination of dexamethasone acetate and cortisone acetate in bulk drugs & dosage forms.	(164)

Table 9 (continued)

Miscellaneous High Performance Liquid Chromatographic Methods

Description	Reference
Use of HP micro-column LC to identify and to assess quality of drugs of the cortisone group.	(165)
Identification and determination of corticosteroids in adrenal gland extracts for pharmaceutical use.	(166)
Determination of glucocorticoids by HPLC III. Application to ointments & a cream containing cortisone acetate, dexamethasone acetate, fluorometholone and betamethasone valerate.	(167)
Computer-aided techniques for the analysis of steroids by HPLC with rapid-scanning UV detection.	(168)
Fluorimetric determination of free glucocorticoids in human urine by HPLC.	(169)
New fluorimetric determination of 17-hydroxy-corticosteroids after HPLC using post-column derivatization with benzamidine.	(170)
Simultaneous determination of serum cortisol and cortisone.	(171)
Quantitative determination of corticosteroids in human biological fluids.	(172)
Determination of plasma corticosteroids by HPLC combined with radio-immunoassay.	(173)
Quantitative determination of corticosteroids from plasma.	(174)

mobile phase is 95:95:14:7:6 butyl chloride-water saturated butyl chloride-tetrahydrofuran-methanol-glacial acetic acid, and is eluted at a flow rate of 1 mL/min. Detection is on the basis of the UV absorbance at 254 nm.

System (2) has been employed for the determination of cortisol and cortisone in urine [150]. Urine containing 6α-methyl prednisolone as an internal standard was applied to a Sep-Pak C_{18} cartridge, eluted with aqueous 90% acetonitrile, and the eluent analyzed by HPLC. The analytical column (25 cm x 4.6 mm) is packed with Altex Ultrasphere C_{18} bonded silica (5 μm), and requires the use of a (5 cm x 5 mm) Hypercil ODS (5 μm) pre-column. The mobile phase used gradient elution with less than 100% acetonitrile, and detection is by UV absorbance at 260 nm.

System (3) is a specific method for simultaneously measuring cortisone, hydrocortisone, prednisone, and prednisolone as endogenous glucocorticoids in plasma [151]. A 1 mL sample is extracted with 9 mL of 2:1 dichloromethane-ethyl ether. The extract is washed once with 2 mL of 0.1M HCl, twice with 2 mL of 0.1M NaOH, and dexamethasone is added as the internal standard. The column is 5 μm silica gel (25 cm x 3.2 mm), and the mobile phase is 1937:20:42:1 dichloromethane-tetrahydrofuran-methanol-anhydrous acetic acid eluted at a flow rate of 1.3 mL/min. Detection is by UV absorbance UV at 254 nm, and a limit of detection of 10 μg/L has been reported.

System (4) has been described for the analysis of corticosteroids in human adrenal tissues [152]. Prior to the HPLC analysis, the corticosteroids were extracted from adrenal tissues by acetone. The permissible columns were 20 cm x 2.4 mm, consisting of Zorbax-SIL (for more polar compounds) or Zorbax-CN (for less polar compounds). The mobile phase was 9:4:1 cyclohexane-dichloromethane-ethanol or aqueous methanol, and eluted at a flow rate of 0.4 mL/min. Using a UV detection wavelength of 254 nm, sample recoveries were 78.5-99.5%, and the relative standard deviation was 2-5%.

System (5) has been reported for the determination of C_{21}-hydroxy corticosteroids in human urine, and uses prednisone as the internal standard [153]. The column (25 cm x 4.6 mm) was packed with TSK gel ODS-120 T (5 μm). The mobile phase consisted of a stepwise gradient elution at 1 mL/min with methanol-1M ammonium acetate-acetonitrile

(49:38:13), then (33:14:3), and finally (41:5:4) for 27, 22, and 21 minutes, respectively. Fluorescence detection was used, exciting at 350 nm and observing the emission at 390 nm.

System (6) was recommended for the analysis of hydrocortisone, cortisone, and their acetates in pharmaceutical preparations, as well as their separation from a number of impurities and decomposition products [154]. The sample (creams, ointments, lotions, or suppositories) was extracted with hot ethanol prior to introduction into the HPLC system. The column was stainless-steel (100 cm x 2.1 mm) packed with 1% ANH on Zipax, and the mobile phase was water containing 1% methanol. A flow rate of 0.5 mL/min was used, and the analytes detected by their UV absorbance at 254 nm.

System (7) is designed to separate hydrocortisone, cortisone and aldosterone [155]. The column stationary phase was Sil-X (R-P) with octadecyltrichlorosilane, and the mobile phase was either 100:4 or 100:5 chloroform : 1,4-dioxane. Detection was based on the UV absorbance of the analytes.

System (8) has been described for quantitation of corticosteroids as common adulterants in local drugs [156]. The sample is extracted from its matrix by methanol, and the resulting supernatant layer subjected to the HPLC analysis. The column used was an ODS-Zorbax column (25 cm x 4.6 mm), and the mobile phase 7:2:11 acetonitrile-methanol-aqueous 1% phosphoric acid. An eluent flow rate of 0.8 mL/min was used, and the analyte detection was performed using the UV absorbance at 240 nm. The calibration graph was found to be linear in the ranges of 1-15 μg/mL for betamethasone, 0.5-20 μg/mL for prednisolone, and 1-30 μg/mL for cortisone acetate.

4.6 Determination in Body Fluids and Tissues

Corticosteroids have been determined by several GC-MS methods in various body fluids [179-181]. The following represent some selected examples of this methodology.

System (1) has been described for the analysis of corticosteroids in urine, and uses HPLC and thermospray LC-MS technology. The method can be

used in testing urine for steroid abuse [175]. The procedure is performed by mixing 3 mL of a urine sample with betamethasone (the internal standard), and adjusting to pH 9.0 with solid K_2HPO_4 (0.1 g) and Na_2SO_4 (0.5g). The corticosteroids were extracted with 5 mL of ethyl ether, centrifuged, and the ether layer then evaporated to dryness under vacuum. The residue was dissolved, filtered and a 15 μL aliquot was analyzed by a HPLC system interfaced with a mass spectrometer via thermospray probe detection. The HPLC column (10 cm x 4.6 mm) contained of Hypersil ODS (5 μm) maintained at 40°C, and the mobile phase consisted of aqueous acetonitrile eluted using a gradient program initially equal to 4%. The reported limits were 10 to 50 ng in scan mode, or 1 to 5 ng in selected ion-monitoring mode.

System (2) has been reported for the simultaneous quantitation of endogenous levels of cortisone and cortisol in human nasal and bronchoalveolar lavage fluids, as well as in plasma [176]. The method is based on GC-negative ion chemical ionization mass spectrometry. To run the procedure, one mixes either plasma (2.5 nL) or bronchoalveolar nasal lavage fluid (1.5 mL) with 15-20 ng each of $\{^2H_3\}$-cortisone and $\{^2H_4\}$-cortisol (the internal standards) and 1 mL of 0.5M H_3PO_4. The solution was applied to a C_{18}-Plus Sep-Pak cartridge to separate the analytes. After washing the column with water, cortisone and cortisol were eluted with methanol and sequentially derivatized with 2% carboxymethoxylamine hemichloride in pyridine. After drying, the residue was extracted with hexane, evaporated to dryness under nitrogen, re-dissolved in heptane, and analyzed by GC-MS. This system used a column (1.5 m x 0.25 mm) coated with methylsilicone (0.05 μm), and was operated using a temperature programmed of 200 to 300°C at 3°C/min. Helium was used as the carrier gas, and 70 eV CIMS detection was employed. Linear calibration graphs for cortisone and cortisol were reported.

System (3) described the use of stable-isotope-dilution mass spectrometry for the simultaneous determination of cortisol, cortisone, prednisolone, and prednisone in plasma [177]. Cortisol, cortisone, prednisolone, and prednisone were simultaneously extracted from acidified plasma on a Sep-Pak C_{18} cartridge. The column was eluted with methanol, the eluent evaporated, and the residue compounds reacted to their bismethylenedioxy-3-heptafluoro-*n*-butyryl derivatives by treatment with formaldehyde and heptafluoro-*n*-butyric anhydride. The derivatives were

separated on a fused-silica column (15 m x 0.25 mm) using helium as the carrier gas, and a temperature program of 100 to 280°C combined with MS detection at 70 eV. Linear calibration graphs were obtained for 4-254 ng of cortisol, 3-123 ng of cortisone, 12-957 ng of prednisolone, and 12-824 ng of prednisone. The detection limits for these compounds were reported to be 250, 10, 100, and 250 pg, respectively. An inter-assay coefficient of variation of 4.5% was reported.

System (4) has been reported for the simultaneous determination of cortisol and cortisone in human plasma by stable-isotope dilution-MS [178]. For the determination, capillary GC-MS with $\{^2H_5\}$-cortisol and $\{^2H_5\}$-cortisone (as internal standards) was used. The method used a SPB-1 fused-silica capillary column (7 m x 0.25 mm) and helium as the carrier gas (at 29.4 Pa), with 70 eV EIMS being used for selective-ion monitoring. The concentrations were determined from the peak height ratios of the [M-31] fragment ions of the methoxime-TMS derivatives of cortisol, cortisone, and their internal standards. The sensitivity of the method was 200 pg per injection for both cortisol and cortisone.

5. Stability

5.1 Solid-State Stability

Cortisone acetate tablets and suspensions should be stored in well-closed containers at a temperature less than 40°C, preferably at 15-30°C. Cortisone acetate preparations should be protected from light.

5.2 Solution-Phase Stability

Cortisone acetate injectable formulations have been reported to be incompatible with various drugs, but the compatibility depends on several factors (such as drug concentrations, resulting pH, temperature, *etc.*). Specialized references should be consulted for specific incompatibility information [61].

5.3 Stability of Suspensions

Freezing of suspension formulations should be avoided. Suspensions of cortisone acetate are heat labile and must not be autoclaved [61]. It has

been also stated that mixing or diluting cortisone acetate suspensions with other substances may affect the state of suspension, may change the rate of absorption, or can reduce the effectiveness of the drugs [61].

Since cortisone acetate exists in several polymorphic forms, when aqueous suspensions are prepared, all particles should be converted to the most stable form to prevent undesirable crystal growth [64].

6. Drug Metabolism and Pharmacokinetics [60-70]

6.1 Absorption

Most corticosteroids are rapidly and completely absorbed from the gastrointestinal tract. However, the naturally occurring compounds (such as cortisone) are so rapidly destroyed as they pass through the liver that they are poorly effective by the oral route. To achieve systemic effects, they must be given parenterally. Esterification with certain organic acids (acetic acid, cypionic acid, *etc.*) decreases the solubility and slows absorption from sites of injection. Following intramuscular (IM) administration, absorption of the water-soluble sodium phosphate and sodium succinate salts is rapid, while the rate of absorption of the lipid-soluble acetate and acetonide esters is much slower. When the most rapid onset of action is desired, a water soluble glucocorticoid ester should be administered intravenously (IV).

All the glucocorticoids are absorbed from the skin, and are effectively are used as anti-inflammatory drugs. Following oral inhalation, glucocorticoids are absorbed from the gastrointestinal and respiratory tracts.

The normal endogenous plasma concentrations of cortisol and cortisone are 4-30 μg/dL and 1-2 μg/dL, respectively.

6.2 Distribution

Most glucocorticoids are rapidly removed from the blood and distributed to muscles, liver, skin, intestines, and kidneys. Corticosteroids in circulation are extensively bound to plasma proteins, mainly to globulin,

and less so to albumin. They vary in the extent to which they are bound to plasma proteins.

Glucocorticoids cross the placenta and may be distributed into milk.

6.3 Blood Levels

After an oral dose of 200 mg of cortisone acetate, the peak plasma concentration of cortisone is attained at 2 hours and reaches a concentration of about 20 μg/mL. At this dosing, the 2-hour plasma concentrations for the metabolites, hydrocortisone, tetrahydrocortisol, and tetrahydrocortisone, are approximately 60, 59, and 67 μg/mL, respectively. In cirrhosis, the plasma concentrations of hydrocortisone are increased as a result of impaired ring A reduction.

Hepatic degradation of glucocorticoids reduces plasma concentrations fairly rapidly. After 8 hours, only 25% of the peak concentration value remains, and the active drug disappears completely in about 12 hours. The plasma half-life is about 30 minutes.

6.4 Metabolism

Pharmacologically active glucocorticoids are metabolized in most tissues (but principally in the liver) to biologically inactive metabolites.

Cortisone and hydrocortisone (cortisol) are enzymatically interconvertible, and so one finds metabolites from both. Glucocorticoids having a ketone group at C_{11} (*e.g.*, cortisone, prednisone, and meprednisone) must be reduced in the liver to their corresponding 11β-hydroxyl analogs (hydrocortisone, prednisolone, and meprednisolone) in order to be pharmacologically active.

The primary routes of catabolism are as follows:

a) Reduction of the carbonyl groups at positions C_3, C_{11}, and C_{20} to the corresponding 3α-hydroxy, 11β-hydroxy, and 20α-hydroxy metabolites, respectively.

b) Reduction of the C_4 double bond (Δ^4) to yield inactive 5β-pregnanes (5β-hydro metabolites).

c) Cleavage of the side chain to form C_{19} androsterone metabolites (17-ketosteroids) which lack corticosteroid activity.

Most of the resulting metabolites are conjugated with glucuronic or sulfuric acids. The primary metabolites are tetrahydrocortisol and tetrahydrocortisone, and their conjugates.

6.5 Elimination

Corticosteroid metabolites are excreted in the urine by the kidneys mainly as glucuronides and sulfates, but also as non-conjugated products. Small amounts of non-metabolized drugs are also excreted in the urine, while negligible amounts of most of the drugs are excreted in the bile.

When radioactively labeled steroids are injected intravenously in man, most of the radioisotope is recovered in the urine within 72 hours. Neither biliary nor fecal excretion is any of quantitative importance in man [63].

6.6 Contra-Indications

Care should be taken in treating diabetic patients, as insulin requirements are usually increased during administration of cortisone. The risk of peptic ulceration is real and caution is necessary when treating patients with past symptoms of peptic ulceration, as relapse and hemorrhage or perforation may occur.

Cortisone should be used with caution in pregnancy, especially during the first 14 weeks, as it may give rise to fetal damage. The drug should also be administered with caution to nursing mothers [64].

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DILOXANIDE FUROATE

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ANALYTICAL PROFILES OF DRUG SUBSTANCES AND EXCIPIENTS—VOLUME 26
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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Names [5-8]

2,2-dichloro-4-hydroxy-*N*-methylacetanilide-2-furoate

2,2-dichloro-*N*-(4-hydroxyphenyl)-*N*-methylacetamide-2-furoate

N-dichloroacet-4-hydroxy-*N*-methylanilide-2-furoate

4-hydroxy-*N*-methyldichloroacetanilide-2-furoate

dichloro-acet-4-hydroxy-*N*-methylanilide-2-furoate

4-(N-methyl-2,2-dichloroacetamido)-phenyl-2-furoate

1.1.2 Nonproprietary Names

Diloxanide furoate

1.1.3 Proprietary Names [5]

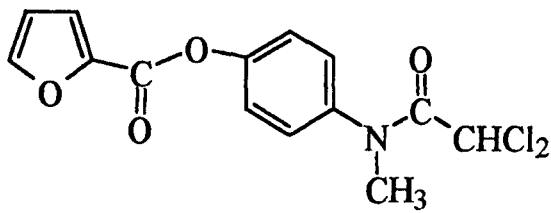
Furamide, Histomibal, Miforon

1.2 Formulae

1.2.1 Empirical

$C_{14}H_{11}Cl_2NO_4$

1.2.2 Structural



1.3 Molecular Weight [5]

328.15 Daltons

1.4 CAS Number

3736-81-0

1.5 Appearance

A white or almost white, crystalline powder, which is odorless or almost odorless, and tasteless [6].

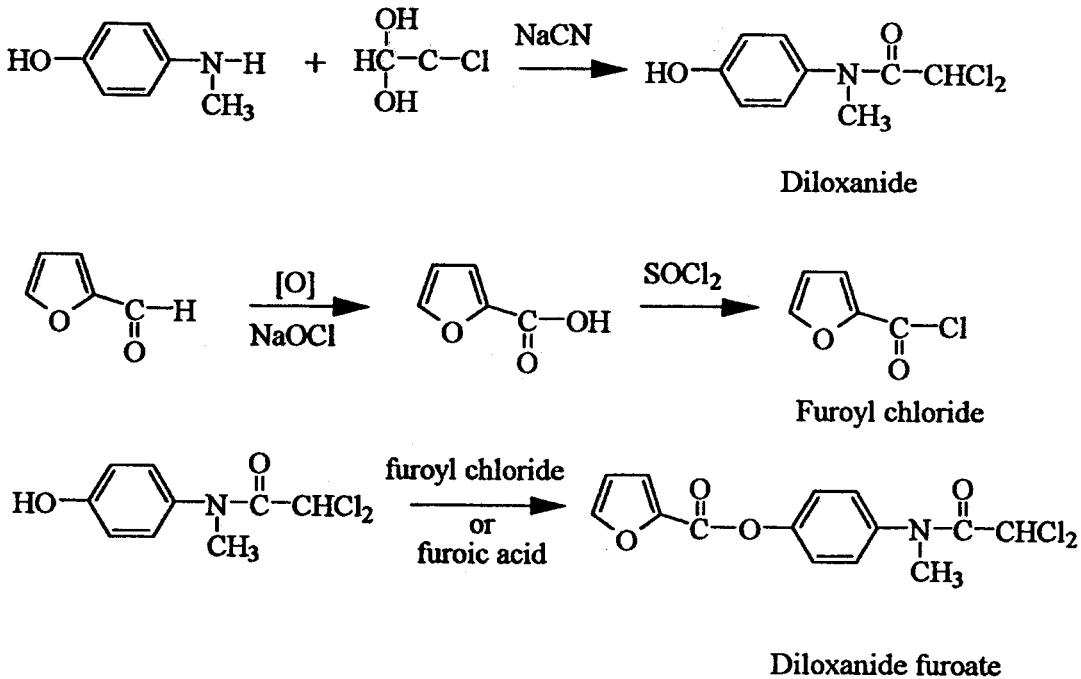
1.6 Uses and Applications

Diloxanide furoate is the furoate ester of 2,3-dichloro-4-hydroxy-*N*-methyl acetanilide. This antiamoebic drug was developed as a result of the discovery that various α,α-dichloroacetamides possessed an amoebicidal activity [1]. Diloxanide furoate is considered as a safe and effective drug for the treatment of asymptotic or mildly symptomatic persons who are passing cysts of *Entameba histolytica* [2,3]. It acts principally in the bowel lumen, and is used in the treatment of the intestinal amoebiasis. It is less effective in amebic dysentery than in asymptotic infection, but the furoate gives high intestinal concentrations and is possibly more effective than metronidazole in the treatment of cyst passers [4].

Diloxanide furoate has been used in the treatment of the asymptotic carriers of *Entameba histolytica* [1,3,47], and is excellent amoebicide for cyst passers [48,49]. The combination of diloxanide furoate and metronidazole in tablets is widely used for the acute and the chronic amoebiasis and giardiasis [21]. The drug is given orally in a dose of 500 mg, three times daily, for a period of ten days. Children may be given 20 mg/kg body weight, daily in divided doses, for ten days [4, 8]. The drug is remarkably free of side effects, but occasional flatulence, abdominal distension, anorexia, nausea, vomiting, diarrhea, pruritis, and urticaria may occur [1].

2. Method of Preparation

Diloxanide was first prepared by the reaction of 4-hydroxy-*N*-methyl aniline with sodium cyanide and chloral hydrate in the presence of a base [9]. Furoic acid was prepared by the hypochlorite oxidation of



Scheme 1 Synthesis of diloxanide furoate.

furfuraldehyde at low temperature [10]. Furoyl chloride was then prepared by the reaction of furoic acid with sulfonyl chloride. The ester was in turn obtained by the condensation of diloxanide with furoyl chloride [11].

The synthetic method just described was modified by Pant *et al.* [12], in order to eliminate the handling of furoyl chloride, and to allow the interaction of furoic acid with diloxanide. The entire procedure is illustrated in Scheme 1.

3. Physical Properties

3.1 X-Ray Powder Diffraction Pattern

The x-ray powder diffraction pattern of diloxanide furoate is shown in Figure 1. This pattern was obtained using a Philips PW-1710 diffractometer, equipped with a single crystal monochromator and which used copper Kα radiation. Values of the observed scattering angles, computed d-spacings, and relative intensities were automatically obtained on a Philips digital printer, and are given in Table 1.

3.2 Thermal Methods of analysis

3.2.1 Melting Behavior

The melting point of diloxanide furoate has been reported to be 114-116°C [6].

3.2.2 Differential Scanning Calorimetry

The differential scanning calorimetry thermogram of diloxanide furoate is shown in Figure 2. The data were obtained using a DuPont TA-9900 thermal analyzer system interfaced with the DuPont data unit. The thermogram was recorded using a heating rate of 10°C/minute, over a temperature interval of 100-250°C.

A single melting endotherm was observed having an onset temperature of 113.6°C, and a peak maximum of 114.6°C. Integration of this thermal feature indicated an enthalpy of fusion equal to 105.6 J/g.

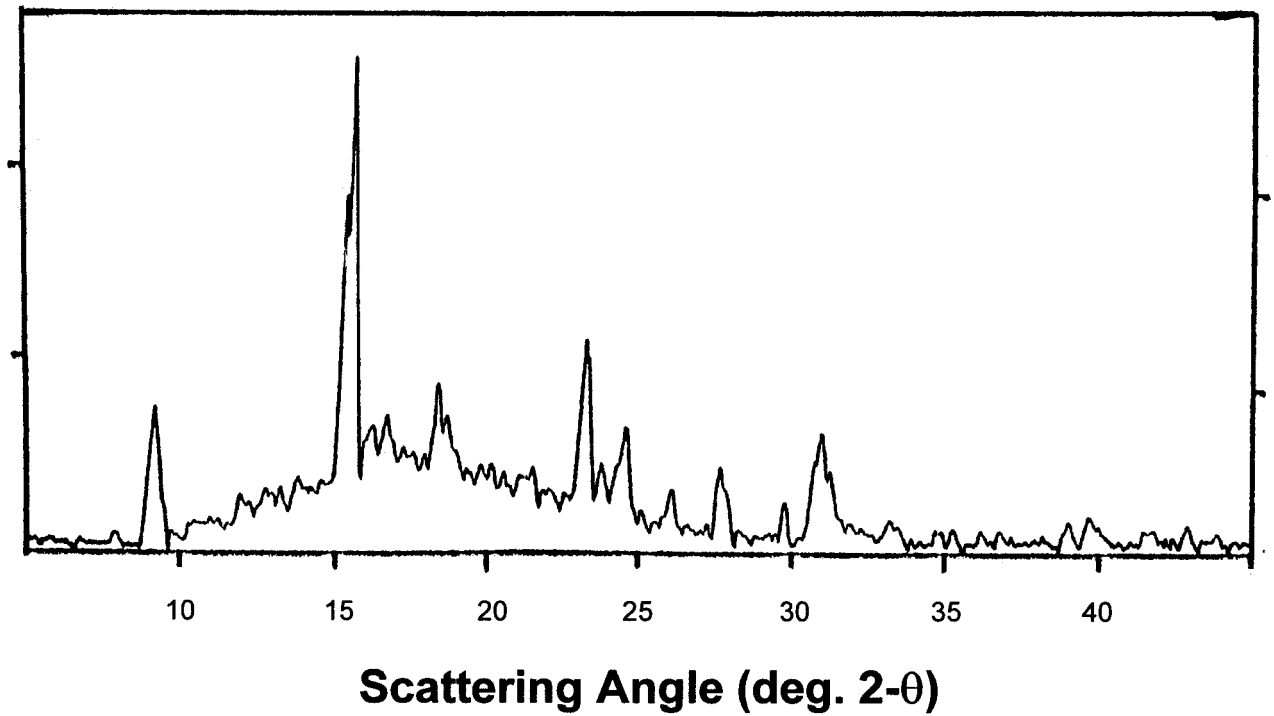


Figure 1. X-Ray powder diffraction pattern of diloxanide furoate.

Table 1

Crystallographic Data from the X-Ray Powder Pattern of Diloxanide Furoate

Scattering Angle (degrees 2θ)	d-Spacing (Å)	Relative Intensity (I/Imax * 100)
6.613	13.3550	1.97
10.114	8.7382	8.09
11.194	7.8980	17.26
13.072	6.7673	2.16
13.520	6.5438	9.66
13.860	6.3844	39.83
14.989	5.9056	2.36
16.093	5.5029	11.96
17.520	5.0578	8.20
17.839	4.9682	33.49
18.804	4.7151	4.85
19.393	4.5734	5.05
20.011	4.4335	100.00
21.525	4.1249	3.06
22.189	4.0029	9.15
23.031	3.8585	5.11
23.720	3.7479	25.39
24.053	3.6968	94.46
24.707	3.6004	2.26
25.088	3.5467	2.58
25.454	3.4841	19.64
25.812	3.4487	14.43
27.195	3.2764	3.26

Table 1 (continued)

Crystallographic Data from the X-Ray Powder Pattern of Diloxanide Furoate

Scattering Angle (degrees 2θ)	d-Spacing (Å)	Relative Intensity (I/Imax * 100)
27.750	3.2121	34.83
28.506	3.1287	7.55
28.916	3.0852	28.67
29.436	3.0319	4.02
30.223	2.9547	5.44
30.799	2.9007	9.62
32.230	2.7751	9.78
32.628	2.7422	9.58
33.664	2.6601	13.20
34.158	2.6227	12.68
34.772	2.5779	1.37
35.357	2.5365	5.50
35.812	2.5053	5.76
36.265	2.4751	3.26
36.893	2.4344	6.71
37.679	2.3854	3.00
38.649	2.3277	1.31
39.169	2.2980	3.89
40.612	2.2196	4.16
41.460	2.1762	3.53
43.690	2.0701	2.89
44.866	2.0186	1.38

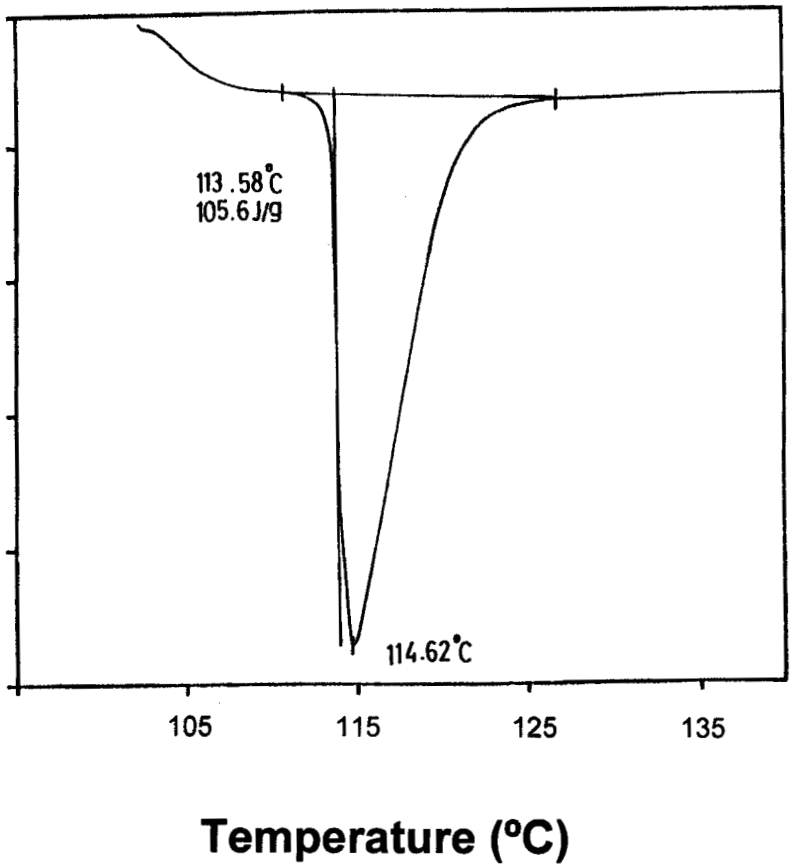


Figure 2. Differential scanning calorimetry thermogram of diloxanide furoate.

3.3 Solubility Characteristics

Diloxanide furoate is very slightly soluble in water, soluble to the extent of 1 in 100 in alcohol, 1 in 25 of chloroform, and 1 in 130 of ether [7].

3.4 Partition Coefficient

No partition coefficient data have been reported for diloxanide furoate, but a theoretical log P value of 1.42 ± 0.55 has been obtaining using the predictive program of Advanced Chemistry Development Laboratories [50].

3.5 Spectroscopy

3.5.1 UV/VIS Spectroscopy

The ultraviolet absorption spectrum of diloxanide furoate was recorded on a Shimadzu model 1601 PC UV/VIS spectrophotometer, and is shown in Figure 3. In aqueous solution, the spectrum exhibited a single absorption maximum located at 260 nm. For this band, the $A_{1\%:1cm}$ value was 700, and the molar absorptivity equal to 22970.

Clarke [7] has reported that in aqueous, the absorption maximum is observed at 262 nm, and that $A_{1\%:1cm} = 224$. In ethanol, the absorption maximum is slightly shifted to 258 nm, and $A_{1\%:1cm} = 705$.

3.5.2 Fluorescence Spectroscopy

The fluorescence spectrum of diloxanide furoate in aqueous methanol (concentration of 8 μg/mL) was recorded using a Perkin Elmer NPF-44B fluorimeter system. As shown in Figure 4, the excitation maximum was noted at 240 nm, which agrees well with the absorption maxima observed in the UV/VIS studies. The emission maximum was found at 335 nm.

3.5.3 Vibrational Spectroscopy

The infrared absorption spectrum of diloxanide furoate is shown in Figure 5, and was obtained as a KBr disc using a Perkin Elmer infrared spectrophotometer. The principal peaks were observed at energies of

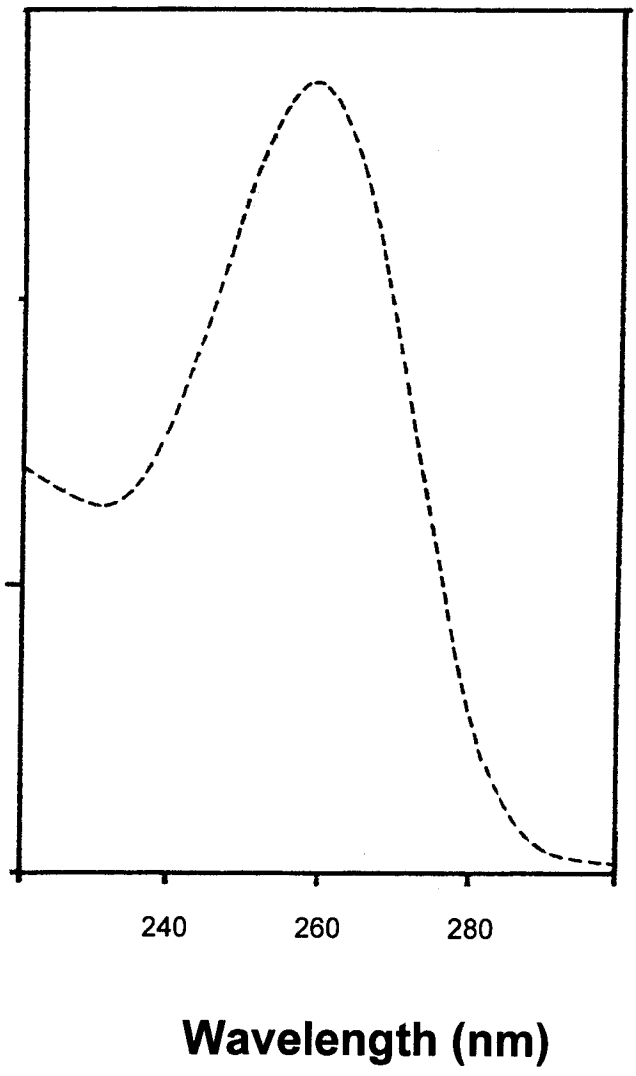


Figure 3. Ultraviolet absorption spectrum of diloxanide furoate.

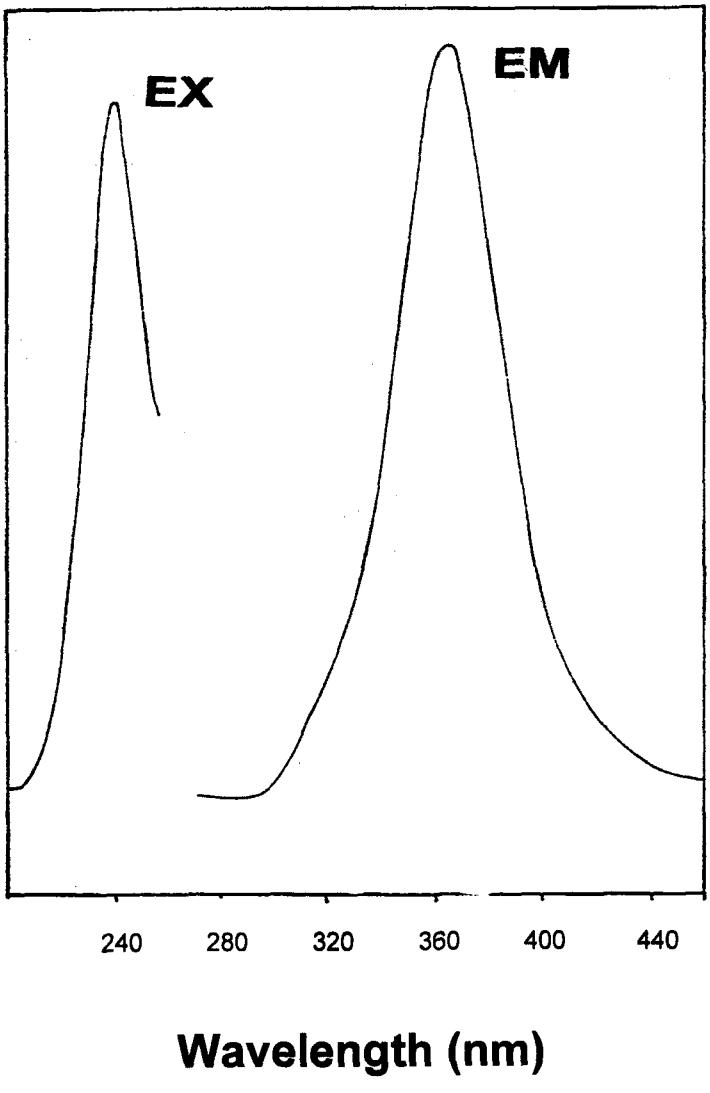


Figure 4. Excitation (EX) and emission (EM) fluorescence spectra of diloxanide furoate.

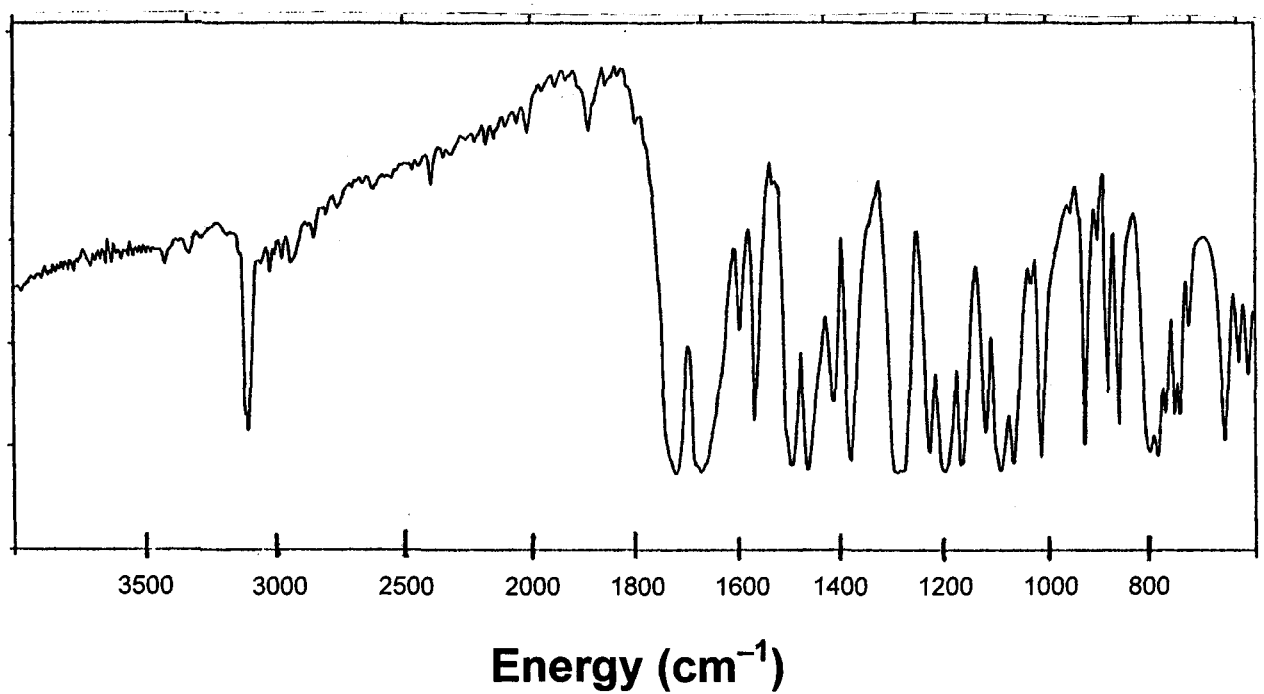


Figure 5. Infrared absorption spectrum of diloxanide furoate.

1678, 1197, 1093, 1290, 1727, and 1167 cm^{-1}. The same absorption bands were reported by Clarke [7].

3.5.4 Nuclear Magnetic Resonance Spectrometry

3.5.4.1 ^{1}H-NMR Spectrum

The proton NMR spectrum of diloxanide furoate was obtained using a Bruker system operating at 300, 400 or 500 MHz. Standard Brucker software was used to execute recording of DEPT, COSY, and HETCOR spectra. The sample was dissolved in CH_3OH-d_4, and all resonance bands were referenced to the tetramethylsilane internal standard.

The ^{1}H-NMR spectra are shown in Figures 6 and 7, while assignments for the observed resonance bands are given in Table 2 together with an atom numbering system.

3.5.4.2 ^{13}C-NMR Spectrum

The ^{13}C-NMR spectrum of diloxanide furoate was obtained using a Bruker system operating at 75, 100 or 125 MHz. The sample was dissolved in CH_3OH-d_4, and all resonance bands were referenced to the tetramethyl-silane internal standard.

The ^{13}C-NMR spectrum is shown in Figure 8, and assignments for the observed resonance bands are given in Table 3 along with an appropriate atom numbering system.

3.5.5 Mass Spectrometry

The mass spectrum of diloxanide furoate was obtained using a Shimadzu PQ-5000 mass spectrometer, with the parent ion undergoing collision with helium carrier gas. The mass spectrum is shown in Figure 9, and features a base peak at m/z = 95, a molecular ion peak at m/e = 328, as well as numerous other fragments. Table 4 summarizes the proposed mass fragmentation pattern.

Clarke reported that the principal MS peaks are found at m/z values of 95, 327, 39, 329, 96, 122, 244, and 67 [7].

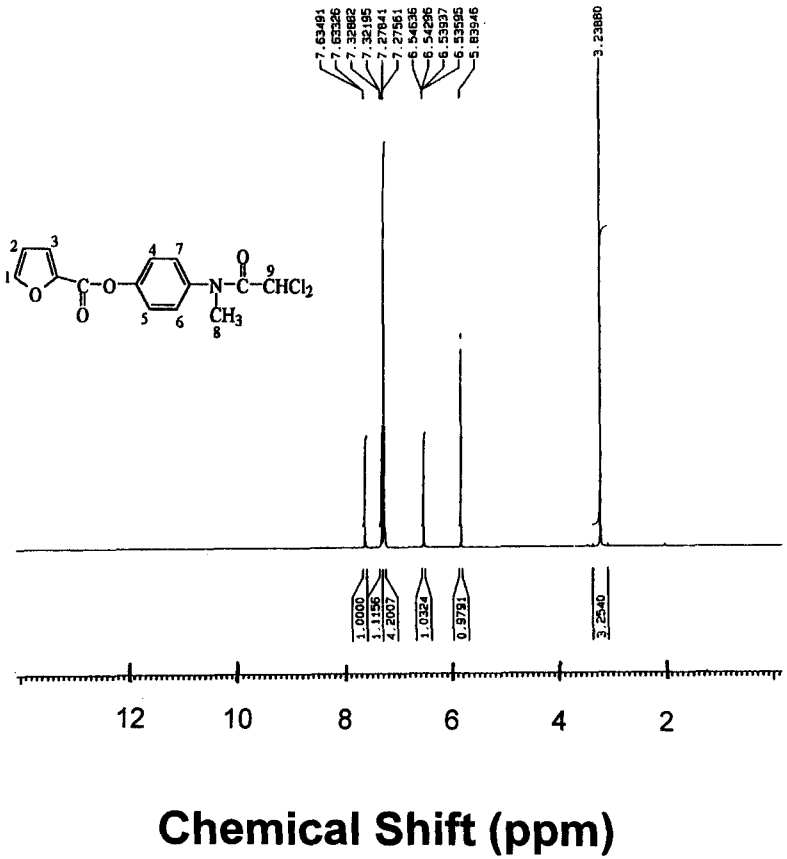


Figure 6. Full ^{1}H nuclear magnetic resonance spectrum of diloxanide furoate.

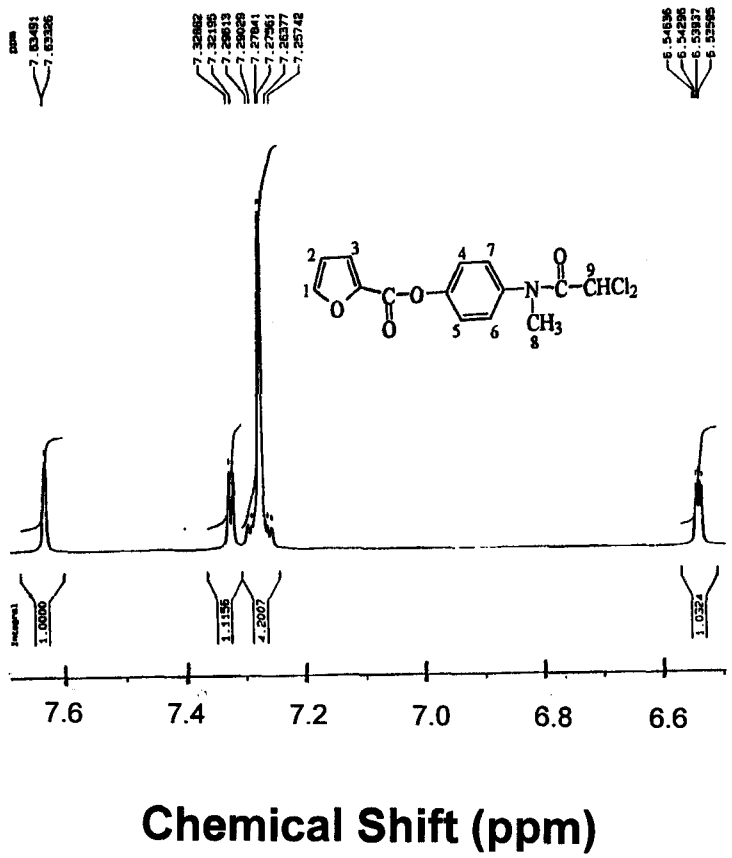


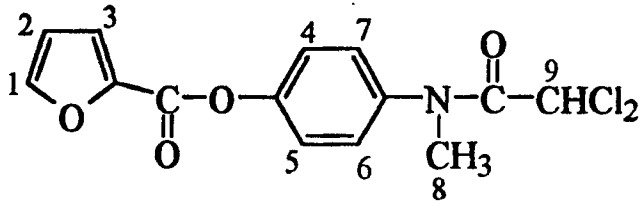
Figure 7. Expanded ^{1}H nuclear magnetic resonance spectrum of diloxanide furoate.

Table 2

Assignments for the ^{1}H-NMR Resonance Bands of Diloxanide Furoate

Proton ID	Multiplicity	Chemical shift (ppm)
H1	doublet	7.630 – 7.634
H2	multiplet	6.535 – 6.546
H3	doublet	7.321 – 7.328
H4 and H5	two doublets	7.275
H6 and H7	two doublets	7.278
H8	singlet	3.238
H9	singlet	5.838

Atom numbering scheme for the ^{1}H-NMR spectral assignments:



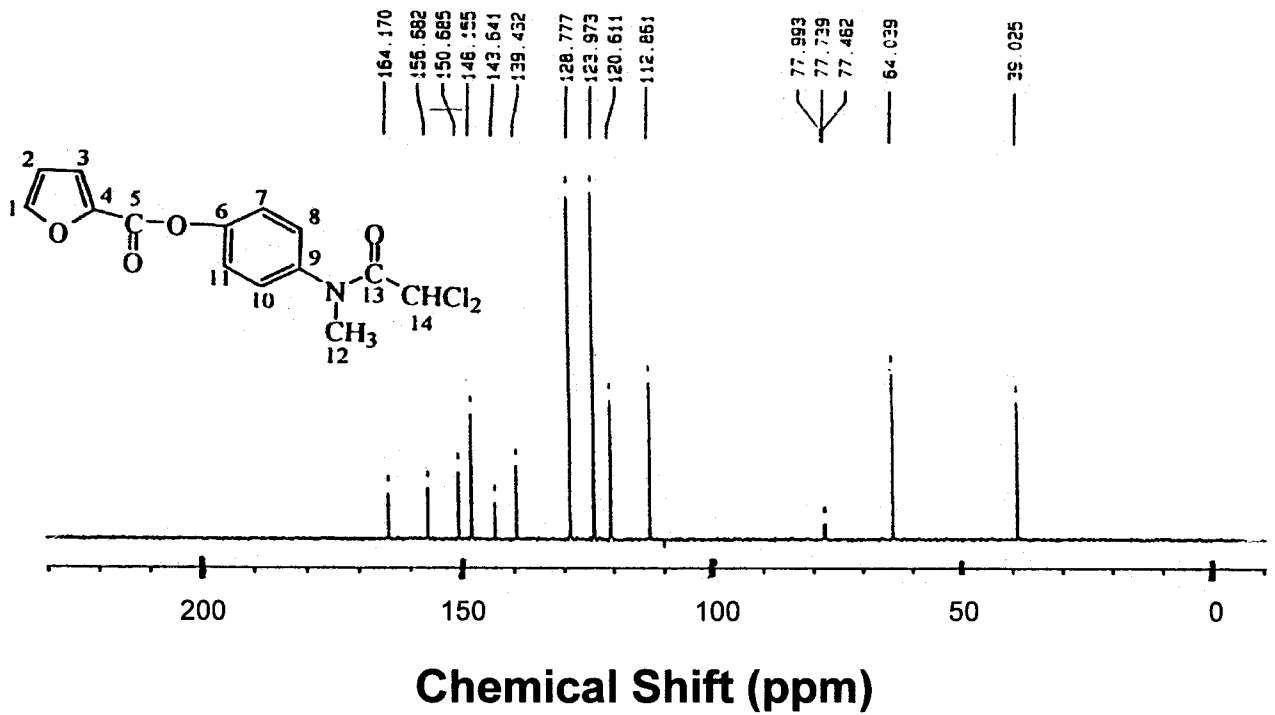


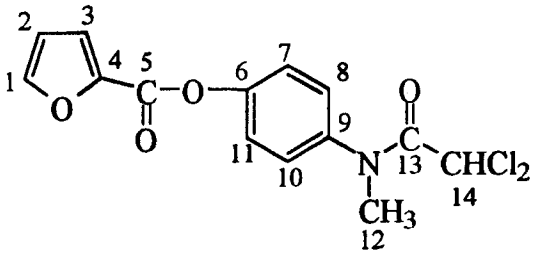
Figure 8. ^{13}C nuclear magnetic resonance spectrum of diloxanide furoate.

Table 3

Assignments for the ^{13}C-NMR Resonance Bands of Diloxanide Furoate

Carbon ID	Chemical shift (ppm)
C1	148.15
C2	139.43
C3	143.63
C4	150.68
C5	164.17
C6	128.77
C7	120.61
C8	112.86
C9	123.97
C10	112.86
C11	120.61
C12	39.03
C13	156.68
C14	64.04

Atom numbering scheme for the ^{13}C-NMR spectral assignments:



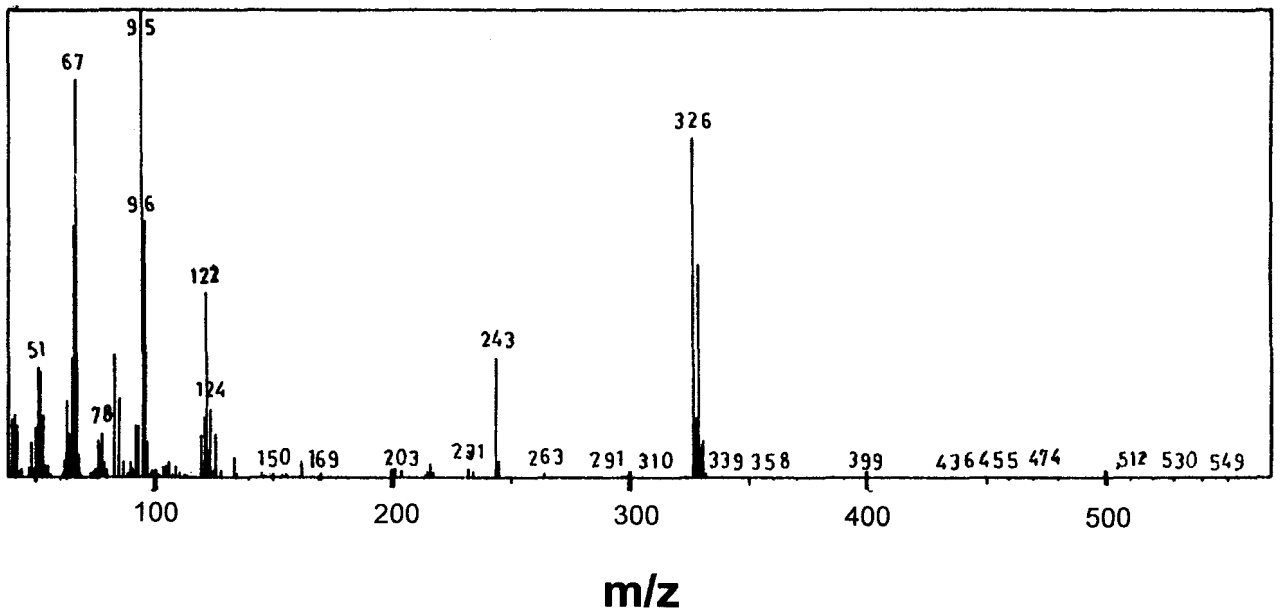
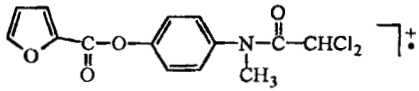
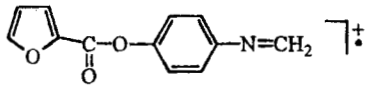
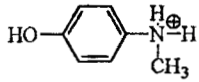
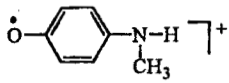
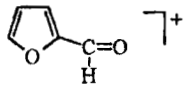
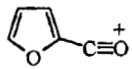
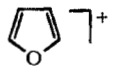


Figure 9. Mass spectrum of diloxanide furoate.

Table 4

Fragmentation Pattern in the Mass Spectrum of Diloxanide Furoate

m/z	Relative intensity	Fragment
328	5%	
243	25%	
124	12%	
122	32%	
96	55%	
95	100%	
67	82%	

4. Methods of Analysis

4.1 Identification

Clarke has reported that diloxanide furoate gives a blue color with the Folin-Ciocalteu Reagent, and also yields a yellow color when subjected to Libermann's test [7].

The British Pharmacopoeia has described three identification tests [6].

a. The infrared absorption spectrum of the substance is in agreement with the reference spectrum of diloxanide furoate.

b. The light absorption of the substance in the range 240 to 350 nm of a 0.0014% w/v ethanolic solution exhibits a maximum only at 258 nm. The absorbance at this maximum is about 0.98.

c. 20 mg of the substance is burned by the method for oxygen-flask combustion, using 10 mL of 1 M sodium hydroxide as the absorbing liquid. Upon completion of the process, the liquid is acidified with nitric acid and silver nitrate solution is added. A white precipitate is produced.

4.2 Elemental Analysis [5]

C: 46.18%
H: 3.88%
Cl: 30.29%
N: 5.98%
O: 13.67%

4.3 Titrimetric Analysis

The British Pharmacopoeia describes a non-aqueous titration method for the determination of diloxanide furoate [6]. To perform the test, one dissolves 0.3 g of diloxanide furoate in 50 mL of anhydrous pyridine, and titrates to a potentiometric end point using 0.1 N tetrabutylammonium

hydroxide. Each milliliter of tetrabutylammonium hydroxide titrant is equivalent to 0.03282 grams of diloxanide furoate.

4.4 Electrochemical Analysis

Roy and Prakash have developed a conductimetric method for the determination of diloxanide furoate [13]. The drug undergoes hydrolysis on heating with aqueous sodium hydroxide, and the excess sodium hydroxide is then determined conductimetrically. The method is reported to be useful for the analysis of the raw material, as well as for the analysis of tablets.

4.5 Spectrophotometric Methods of Analysis

4.5.1 Ultraviolet Absorption Spectrometry

Srinath and Bagavant reported a spectrophotometric method for the analysis of binary mixtures of diloxanide furoate and tinidazole or metronidazole [14]. The mixtures were analyzed at 258 and 310 nm, and it was found that Beer's law was obeyed over the range of 10 to 25 μg/mL of tinidazole and diloxanide furoate. Analyte recoveries were in the range of 98.2 to 101.9%.

Talwar *et al.* reported a simultaneous spectrophotometric determination of diloxanide furoate and metronidazole in dosage forms [15]. The drug substances were extracted from tablets with methanol, and the extract diluted with 0.01 M sodium hydroxide. The absorbance of the solution was measured at 247 and 320 nm against 0.01 M sodium hydroxide, and the concentration of each individual drug was calculated by the Vierordt method. Drug recoveries were in the range of 99 to 100%, and the method was satisfactorily applied to the analysis of commercial samples.

Sethi *et al.* reported the assay by two methods of diloxanide furoate and tinidazole in combined dosage forms, [16]. One of these was a dual-wavelength spectrophotometric method, and the other a difference spectrophotometric method. In the first method, the absorbance of sample solution was measured at 259 and 311 nm. The concentration of tinidazole was calculated from absorbance at 311 nm, and the concentration of diloxanide furoate was calculated with the use of a given equation. In the second method, the absorbance of an aqueous solution of

diloxanide furoate was measured at 267 nm against an alkaline solution of the drug, and also at 320 nm against an acidic solution of the drug. The analyte concentrations were then calculated from given equations.

Galal *et al.* determined diloxanide furoate and metronidazole in two-component tablets using first (D_1) and second (D_2) derivative spectrophotometric methods [17]. The methods were based on the direct measurement of diloxanide furoate in 0.1 N hydrochloric acid solution at 262 nm (D_1 method) and at 248 nm (D_2 method), without any interference from the co-existing component. The methods were applied for the determination of diloxanide furoate in the laboratory-made mixtures and in tablets, and the authors reported a relative standard deviation less than 2%.

Daabees determined the drug and its degradation product by second (D_2) and third (D_3) derivative spectrophotometry [18]. The methods were based on measuring the D_2 and D_3 amplitudes at 260 and 270 nm, respectively, for analytes dissolved in 0.1 N hydrochloric acid. No interference with the degradation product was noted.

Das and Haider described a simultaneous spectrophotometric method for the analysis of binary dosage form mixtures of diloxanide furoate with metronidazole or with tinidazole [19]. Powdered tablets or suspension, equivalent to 50 mg of the drug substances, were dissolved in 50 mL of dimethylformamide with shaking. After 15 minutes, the solution was diluted to 100 mL with water and filtered. A 1 mL portion of the filtrate was diluted to 50 mL with water, and the absorbance of the resulting solution measured at 320 and 262 nm for metronidazole and diloxanide furoate simultaneously. Alternatively, readings were taken at 318 and 262 nm for the simultaneous determination of tinidazole and diloxanide furoate. Recoveries were reported to be quantitative.

Parimoo and Umapathi used difference spectroscopy for the simultaneous quantitative determination of the drug and tinidazole in tablets [20]. The method comprised measurement of the absorbance at 282 and 240 nm of a solution of the tablet extract in pH 2 buffer solution relative to that of an equimolar solution in pH 13 buffer.

Parimoo *et al.* have also reported the use of difference spectroscopy for the simultaneous quantitative determination of diloxanide furoate and

metronidazole in a tablet preparation [21]. This study described the pH-induced difference spectrophotometric method for the quantitation of the drugs and their excipients in the presence of each other. The difference absorption of the drug solution showed maximum values of ΔA at 297 and 243 nm, and a minimum value of ΔA at 268 nm.

Sanghavi and Kulkarni estimated the drug in the presence of its degraded products by differential spectroscopy [22]. Sastry *et al.* determined diloxanide furoate by a spectrophotometric method [23].

Two differential spectrophotometric methods were used by Chatterjee *et al.* for the simultaneous analysis of diloxanide furoate and metronidazole in pharmaceutical formulations [24]. The first method involved measurement of the absorbance of a methanolic solution of the two drugs at 259 and 311 nm. Since the absorbance of diloxanide furoate at 311 nm is zero, the concentration of metronidazole is directly measured, and a simple equation based on absorbance ratios is used to calculate the concentration of diloxanide furoate. The second method was a differential spectrophotometric determination based on pH-induced spectral changes, on changing from an acidic to an alkaline solution. A marked bathochromic shift was exhibited by metronidazole, while diloxanide furoate showed a slight hypsochromic shift. The wavelength of maximum absorption difference for diloxanide furoate was 267 nm, where metronidazole did not absorb. Similarly, diloxanide furoate did not interfere with metronidazole at when measured at 322 nm.

Podder *et al.* used a simple and convenient spectrophotometric method for the determination of tinidazole and diloxanide furoate in combined pharmaceutical formulations [25]. The absorbance of a methanolic solution of a sample containing 20 to 40 μg/mL of each drug was measured between 254 to 310 nm. Diloxanide furoate was reported to exhibit an absorption maximum at 254 nm.

Sadana and Gaonkar described a simultaneous derivative spectroscopic method for the determination of diloxanide furoate and tinidazole in pharmaceutical dosage forms [26]. Drugs were powdered and dissolved in methanol, and the solution set aside for 30 minutes with frequent shaking. After filtration, the filtrate and washings were diluted with methanol. A suspension equivalent to 150 mg of diloxanide furoate was extracted with chloroform. The filtered extract was evaporated to dryness, and the

residue diluted to 100 mL methanol. The extract was further diluted to 1:10 and 1:25 with methanol, and the second derivative spectra recorded from 190 to 400 nm.

4.5.2 Colorimetry

Shah and Mehta described a calorimetric method for the estimation of diloxanide furoate in pharmaceutical formulations [27]. The method is based on its interaction with hydroxylamine in alkaline solution. This method was used for the determination of the drug either alone or when combined with other agents.

Diloxanide furoate was determined by Sane *et al.* using a simple spectrophotometric method [28]. The drug was extracted from tablets with ethanol, or was filtered from a suspension and dissolved in ethanol. The resulting solution was mixed with 6% aqueous sodium hydroxide and Folin-Ciocalteu reagent, or with a 1% solution of sodium nitroprusside in aqueous 10% sodium hydroxide. The complexes formed had absorbance maxima at 650 nm, or at 675 nm, respectively.

Sastry and Aruna described the use of 3-methyl-2-benzothiazolinone hydrazone hydrochloride as a new technique for the spectrophotometric determination of diloxanide furoate and other anthelmentic and antiamoebic agents [29]. Aliquots of sample solutions containing 10-100 μg of drug substance were transferred into a series of 10 mL graduated test tubes, and the volume adjusted to 3 mL with the respective solvent blank. 3-Methyl-2-benzothiazolinone hydrazone hydrochloride and 1 mL of Cr(VI) were added, and the mixture diluted with methanol. The absorbance at 500 nm was measured against a reagent blank.

Sastry *et al.* used iodine and isonicotinic acid hydrazide for the spectrophotometric determination of diloxanide furoate in tablets and in syrups [30]. Powdered tablets or syrup were dissolved in methanol and hydrolyzed under reflux with dilute hydrochloric acid. The mixture was cooled, and excess HCl removed under vacuum. The hydrolysate was dissolved in and diluted with water. Iodine solution and the isoniazid solution were added at two minute intervals to a potassium hydrogen phthalate/HCl buffer solution (pH 3), and diluted with water. The solution was set aside for 10 minutes, whereupon the absorbance was measured at 630 nm against a reagent blank.

Sanghavi *et al.* reported a colorimetric method for the estimation of diloxanide furoate [31].

4.6 Miscellaneous Non-Chromatographic Methods of Analysis

Mohamed reported the use of proton nuclear magnetic resonance spectrometry for the assay of diloxanide furoate in bulk and in tablet formulations [32]. A solution containing 100 to 130 mg of the drug in 2 mL of trichloroethylene (also containing 12 to 14 mg/mL of tetramethylsilane internal reference) was subjected to NMR analysis. The resonances bands at 0.00 ppm (reference) and at 3.23 ppm (analyte) were each integrated three times. One gram of powdered tablets was extracted with chloroform (4 x 5 mL), the combined extracts diluted to 25 mL with chloroform, and a 15 mL portion of this solution evaporated on a water-bath. The residue was dried *in vacuo* over P_2O_5 and then dissolved as before, but this time in 1.5 mL of solvent, for the NMR analysis. The average drug recovery was reported to be 98%. The method of standard additions could also be used if necessary.

Mohamed *et al.* reported the use of a flow injection procedure for the analysis of the drug among other antheimentics and antiprotozoal compounds [33]. The ethanolic sample solution (24 μL), prepared from tablets or suspensions, was injected into a carrier stream of ethanol and the absorbance measured in an 80 μL flow cell having a path length of 1 cm. The paper reports the analyzing wavelength, flow rate, and other parameters are reported.

4.7 Chromatographic Methods of Analysis

4.7.1 Thin Layer Chromatography

Clarke described three thin layer chromatography (TLC) systems for the separation of diloxanide furoate [7].

Method 1 [34]

Plate: Silica gel G, 250 μm, dipped in or sprayed with 0.1 M methanolic potassium hydroxide, and dried

Mobile Phase:	100: 1.5 methanol : strong ammonia solution
Developing Agent:	Acidified iodoplatinate spray
R_f:	66
Reference Compounds:	diazepam (R_f = 75), chlorprothexine (R_f = 56), codeine (R_f = 33), atropine (R_f = 18)

Method 2 [34]

Plate:	Silica gel G, 250 μm, dipped in or sprayed with 0.1 M methanolic potassium hydroxide, and dried
Mobile Phase:	75:15:10 cyclohexane : toluene : diethylamine
Developing Agent:	Acidified iodoplatinate spray
R_f:	16
Reference Compounds:	dipiponone (R_f = 66), pethidine (R_f = 37), desipramine (R_f = 20), codeine (R_f = 06)

Method 3 [34]

Plate:	Silica gel G, 250 μm, dipped in or sprayed with 0.1 M methanolic potassium hydroxide, and dried
Mobile Phase:	90: 10 chloroform : methanol
Developing Agent:	Acidified iodoplatinate spray
R_f:	74
Reference Compounds:	meclozine (R_f = 79), caffeine (R_f = 58), dipipanone (R_f = 33), desipramine (R_f = 11)

4.7.2 Gas Chromatography

Clarke has described a gas chromatography system for the determination of diloxanide furoate [7]. The column is a 2 m x 4 mm internal diameter glass column containing 2.5% SE 30 on 80-100 mesh Chromosorb G (acid washed and dimethyldichlorosilane-treated). It is essential that the column

be used only for performance of this method. The column temperature is 242°C, and the reference compound can be an *n*-alkane having an even number of carbon atoms. The retention index was reported to be 2420 [35].

Sane *et al.* reported the determination of diloxanide furoate in pharmaceuticals by gas chromatography [36]. A sample of powdered tablets equivalent to 250 mg of drug was dissolved in chloroform and diluted to a concentration of 5 mg/mL. A mixture of 2 mL of the sample solution and 1 mL of bromhexine hydrochloride solution (the internal standard) was diluted to 5 mL with chloroform, was used for the analysis. The injection volume was 400 nL, which was analyzed at 265°C on a stainless steel column (3 m x 2 mm) containing 3% OV-13 on Chromosorb W-HP (80-100 mesh). Nitrogen was used as the carrier gas at a flow rate of 50 mL/min, and analyte detection was effected using a dual flame ionization detector.

Sadana and Gaonkar have simultaneously determined diloxanide furoate and tinidazole in pharmaceutical dosage form by gas liquid chromatography [37]. Powdered tablets or suspension formulations were dissolved in chloroform, the solution filtered, and then diluted to 25 mL with chloroform. The solution also contained metronidazole as an internal standard. A 600 nL aliquot was analyzed on a stainless steel column (1 m x 3.2 mm) containing 3% of OV-17 on Chlorosorb W-UP (100-120 mesh). The GC system was operated at 200°C, using nitrogen as the carrier gas (45 mL/min). Flame ionization detection was used to observe the analytes.

4.7.3 Supercritical Fluid Chromatography

The separation and estimation of diloxanide furoate and metronidazole in solid dosage forms was reported by Bhoir *et al.*, using packed column supercritical fluid chromatography [38]. A JASCO C_{18} column (10 μm particle size, 25 cm x 4 mm) was used at 40°C, with an injection volume of 20 μL. The mobile phase consisted of 26% methanol in CO_2 (flow rate of 2 mL/min), and operated at a pressure of 17.6 MPa. When detected on the basis of its ultraviolet absorbance at 230 nm, the retention time for the drug was 1.6 minutes. The linear region of the calibration graph was reported to be 20-70 μg/mL.

4.7.4 High Performance Liquid Chromatography

Ray used high performance liquid chromatography to estimate diloxanide furoate and tinidazole in single and combined dosage forms [39]. Tablets were dissolved in the mobile phase, and 20 μL was injected on to a stainless steel column (30 cm x 3.9 mm) of μ-Bondapak C_{18}. 8:3 methanol : 0.05 M phosphoric acid (pH 3) was used as the mobile phase (flow rate of 2.5 mL/min), and detection was on the basis of the UV absorption at 254 nm.

El-gizawy reported the analysis of diloxanide furoate in its dosage forms by a HPLC method [40]. Furazol tablets containing 200 mg of metronidazole and 250 mg of diloxanide furoate were treated with 50 mL of methanol, sonicated for 10 minutes, and diluted to 100 mL with methanol. A portion of the resulting solution was centrifuged, and a 20 μL portion of the clear supernatant solution diluted to 10 mL with the mobile phase. This process yielded a final analyte concentration equivalent to 5 μg/mL. 20 μL aliquots of the solution were annualized by HPLC using a stainless steel column (10 cm x 4.6 mm) packed with Cyclobond I. The mobile phase consisted of 13:7 0.05 M phosphate buffer (pH 7) : methanol (flow rate of 1 mL/min), and detection was performed at 254 nm.

Rao *et al.* reported a high performance liquid chromatographic method to determine diloxanide furoate and metronidazole in single and in combined dosage forms [41]. A 30 mg equivalent of diloxanide furoate and 25 mg of metronidazole (either as the bulk drug substances or in powdered tablets) was dissolved in methanol, amidopyrine added as the internal standard, and the mixture analyzed by HPLC at room temperature. The analytical column (30 cm x 3.9 mm) consisted of μ-Bondapak C_{18}, with 9:9:1:1 methanol : water : 0.05 M KH_2PO_4 : 0.05 M NaH_2PO_4 as the mobile phase. The flow rate was 1 mL/min), and detection was performed at 254 nm.

5. Pharmacology

Diloxanide is used as the furoate ester, which is more effective as an antiamoebic agent than is diloxanide itself. The ester is hydrolyzed in the intestine, and only diloxanide appears in the plasma from which it is

rapidly cleared [42]. The drug is well absorbed, and is present in serum largely as the glucuronide conjugate that is almost entirely excreted in the urine [1, 43]. The drug has a direct amoebicidal action, affecting the ameba before encystment [2]. Diloxanide is considered as a luminal amoebicide acting in the bowel lumen [4,[44-46]. The drug is directly toxic to *Entameba histolytica* when tested *in vitro*, but its mechanism of action is still not known.

6. Acknowledgement

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7. References

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DORSOLAMIDE HYDROCHLORIDE

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Name

(4-S-*trans*)-4-ethylamino-5,6-dihydro-6-methyl-4*H*-thieno-[2,3-*b*]thiopyran-2-sulfonamide 7,7 dioxide monohydrochloride

1.1.2 Nonproprietary Names

Dorzolamide
MK-0507

1.1.3 Proprietary Names

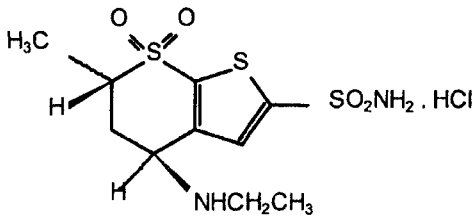
TRUSOPT

1.2 Formulae

1.2.1 Empirical

$C_{10}H_{16}N_2O_4S_3 \cdot HCl$

1.2.2 Structural



1.3 Molecular Weight

360.91

1.4 CAS Number

130693-82-2

1.5 Appearance

Dorzolamide hydrochloride is a white to off-white crystalline solid.

1.6 Uses and Applications

Carbonic anhydrase plays an important role in the secretion of aqueous humor [1,2]. This enzyme was first demonstrated to be present in the ciliary processes of the rabbit, and its presence was later confirmed in human ciliary processes [3,4]. Carbonic anhydrase is responsible for the generation of bicarbonate anions which are secreted from the ciliary process into the posterior chamber, with sodium being the counter ion. Inhibition of carbonic anhydrase in the ciliary processes of the eye decreases aqueous humor secretion, presumably by slowing the formation of bicarbonate ions with subsequent reduction in sodium and fluid transport. The role of the enzyme in aqueous humor secretion has been reviewed in detail by Maren [1].

Orally administered carbonic anhydrase inhibitors lower the intraocular pressure of glaucoma patients, however they induce a number of intolerable side effects associated with extraocular inhibition of the enzyme [5,6]. Thus, much research has been directed towards the search for a topically effective agent. Several compounds have been synthesized since the 1980's in Merck Sharp & Dohme Research Laboratories, and have been found to be topically active in man [7]. Unfortunately, many of these compounds were not very soluble. Attempts to obtain an active carbonic anhydrase inhibitor with good solubility resulted in the synthesis of Dorzolamide hydrochloride [8,9], which was first made available for pharmacological evaluation in 1987. Like other carbonic anhydrase inhibitors sulfonamides (such as acetazolamide, ethoxzolaniide, and methazolamide) dorzolamide is an inhibitor of human carbonic anhydrase isoenzymes I, II, and IV. In contrast to the other sulfonamides, dorzolamide is a potent inhibitor of isoenzymes II and IV, and a weak inhibitor of isoenzyme I [10]. Isoenzyme II is thought to play a major role in aqueous humor secretion.

Dorzolamide hydrochloride, marketed as a 2% ophthalmic solution, was approved for the treatment of elevated intraocular pressure in patients having ocular hypertension or open-angle glaucoma.

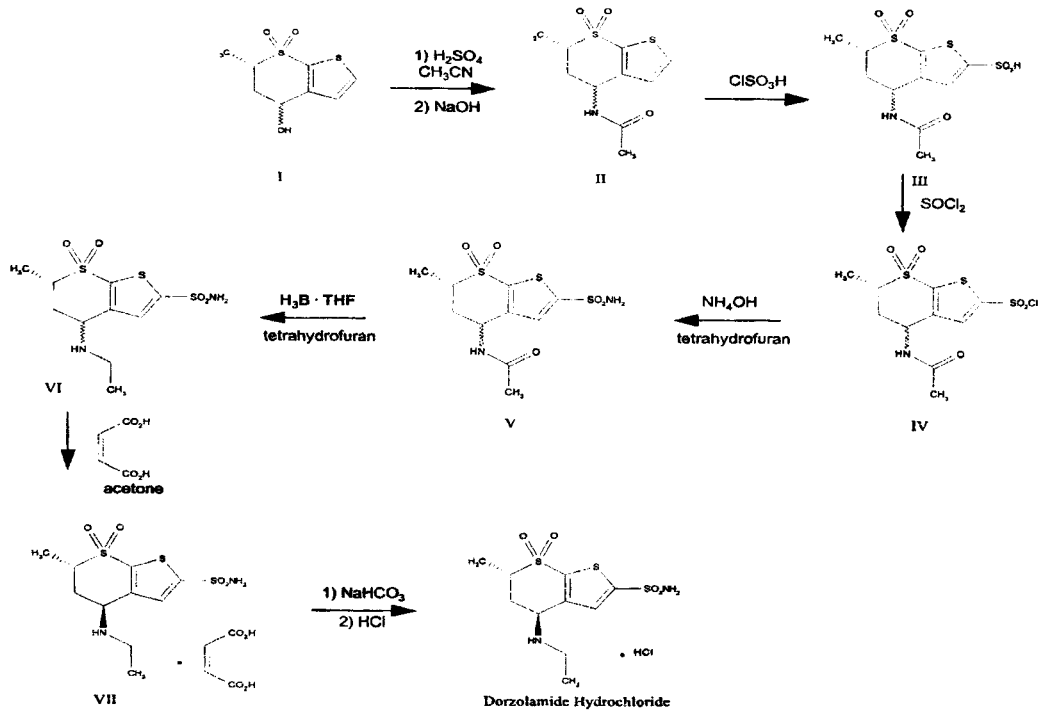
2. Method of Preparation

Dorzolamide is prepared according to the scheme outlined in Scheme 1. The hydrochloride salt is prepared by a multi-step synthesis from (6*S*)-5,6-dihydro-6-methyl-4*H*-thieno-[2,3-*b*]-thiopyran-4-ol-7,7-dioxide. The starting material establishes the absolute stereochemistry and the required *S*-configuration at the C6 position. Electrophilic substitution of the 4-hydroxyl group with acetonitrile in the presence of concentrated sulfuric acid provides a 4-acetamido group, and establishes the absolute configuration at the C4 position predominately in the desired *S*-configuration. The resulting diastereomers, 4*S*,6*S*-*trans* (less than 75%) and 4*R*,6*S*-*cis* (less than 25%) are carried forward in subsequent reactions.

Introduction of the C2 sulfonamide is accomplished via sulfonylation with chlorosulfonic acid, conversion to the sulfonyl chloride using thionyl chloride, and amidation using concentrated ammonium hydroxide in tetrahydrofuran. Reduction of the 4-acetamido compound using borane-tetrahydrofuran complex provides the 4-ethylamino derivative. The 4*S*,6*S*-*trans* diastereomer is selectively crystallized as its maleate salt from acetone in the presence of the unwanted 4*R*,6*S*-*cis* diastereomer. Neutralization of the maleate salt and extraction of the free base in ethyl acetate, followed by formation of the hydrochloride salt, yields crude dorzolamide hydrochloride.

Crude dorzolamide hydrochloride is purified by dissolution in water with heating, and treatment with activated carbon. The final crystallization step provides dorzolamide hydrochloride in the desired form.

Dorzolamide contains two chiral centers, and is therefore capable of existing in four diastereomers. The stereochemistry at the C-6 position of the starting material is preserved during the various chemical reactions which take place during the synthesis. The stereochemistry at the C-4 position (absolute configuration being *S*) results from the Ritter substitution reaction (Scheme 1, Steps I-II) used to transform the alcohol to an acetamide. The Ritter reaction yields mostly the *trans*-diastereomer, and the *cis*-diastereomers are easily separated as their maleate salts. The potential sulfonamide positional isomer (3-sulfonamide) has not been observed at levels greater than 0.1% in HPLC analyses.



Scheme 1. Multi-step synthesis of dorzolamide hydrochloride.

3. Physical Properties

3.1 X-Ray Powder Diffraction Pattern

Dorzolamide has been found to exist in two non-solvated polymorphic forms, which are distinguishable on the basis of their characteristic x-ray powder diffraction patterns. Typical XRPD patterns for Forms I and II are shown in Figures 1 and 2, respectively. Form I has been shown to be the more thermodynamically stable form at ambient temperature.

Form I is the polymorph typically produced by the synthetic process described in section 2.0 of this profile. Isolation of dorzolamide hydrochloride from organic solvents (i.e., *n*-propanol) can result in the formation of either Form I or Form II, while crystallization from water leads to Form II.

3.2 Optical Activity

Dorzolamide is optically active and has two chiral centers at positions C-4 and C-6. The specific rotation ($[\alpha]_D^{25}$) for a 1 mg/mL solution in water has been found to be -17°.

3.3 Differential Scanning Calorimetry

The DSC thermogram for Form I was obtained at a heating rate of 20°C/minute under nitrogen, and is shown in Figure 3. The sole thermal event noted was the melting endotherm which began with an onset temperature of 259°C, and exhibited a peak maximum at 275°C. The endothermic heat of fusion was determined to be 248 J/g.

The DSC thermogram of Form II is characterized by a its melting endotherm at 264°C, with an onset temperature of 253°C. The endothermic heat of fusion was also found to be 248 J/g.

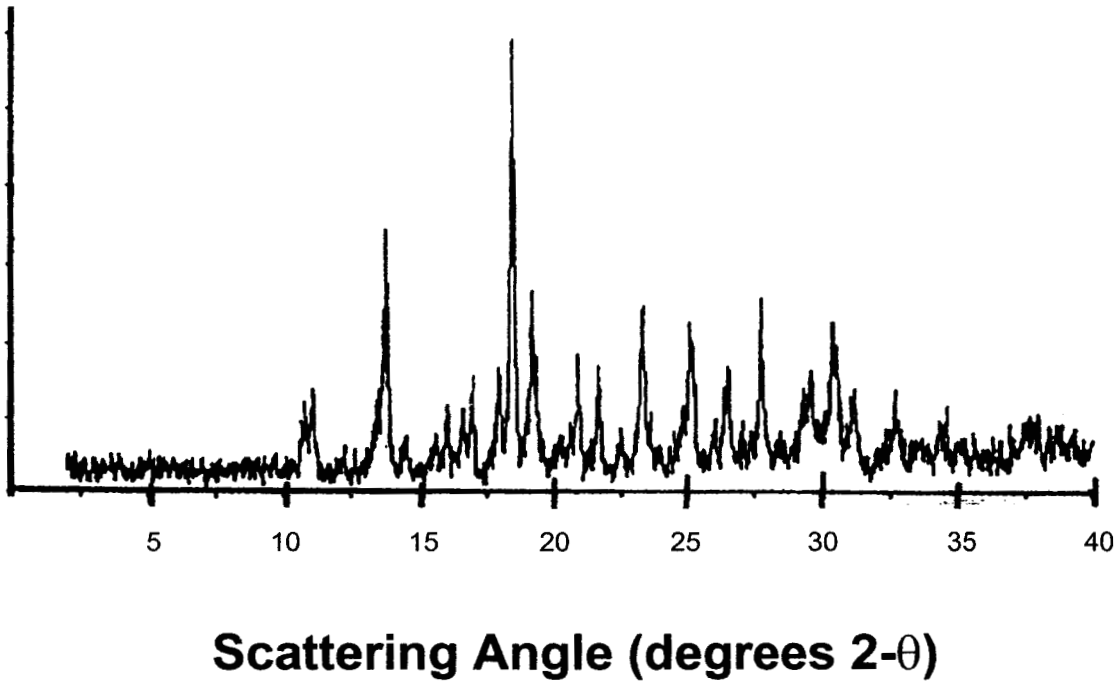


Figure 1. X-ray powder diffraction pattern of dorzolamide hydrochloride, Form I.

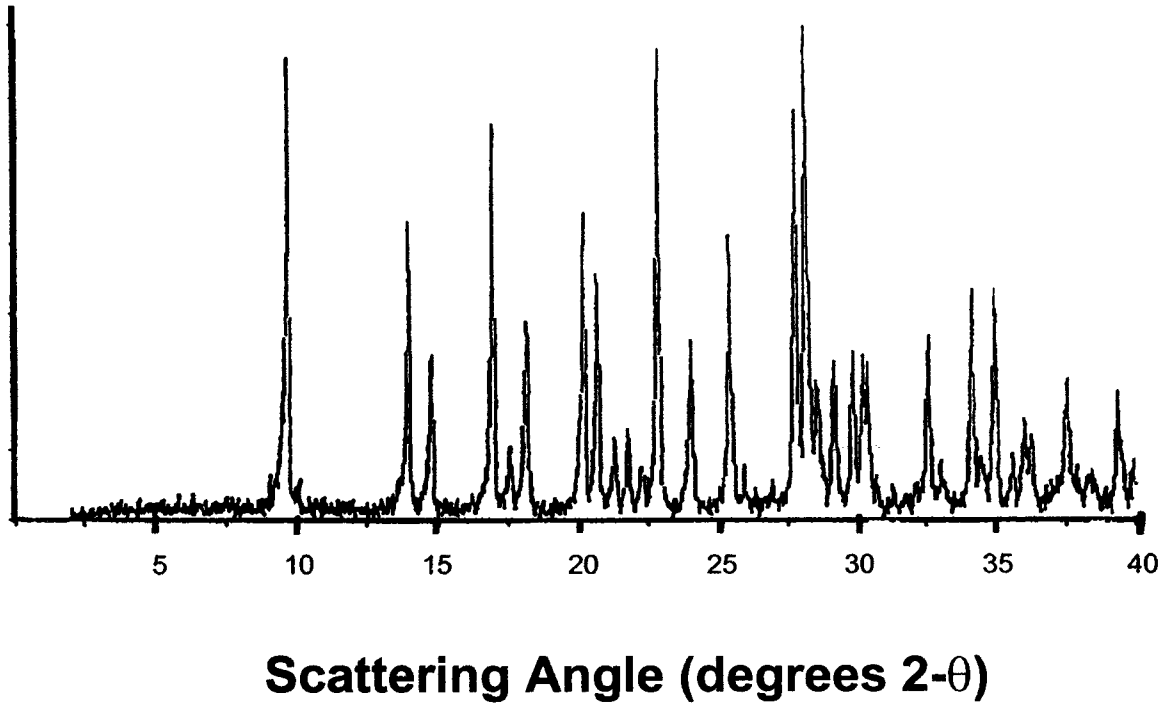


Figure 2. X-ray powder diffraction pattern of dorzolamide hydrochloride, Form II.

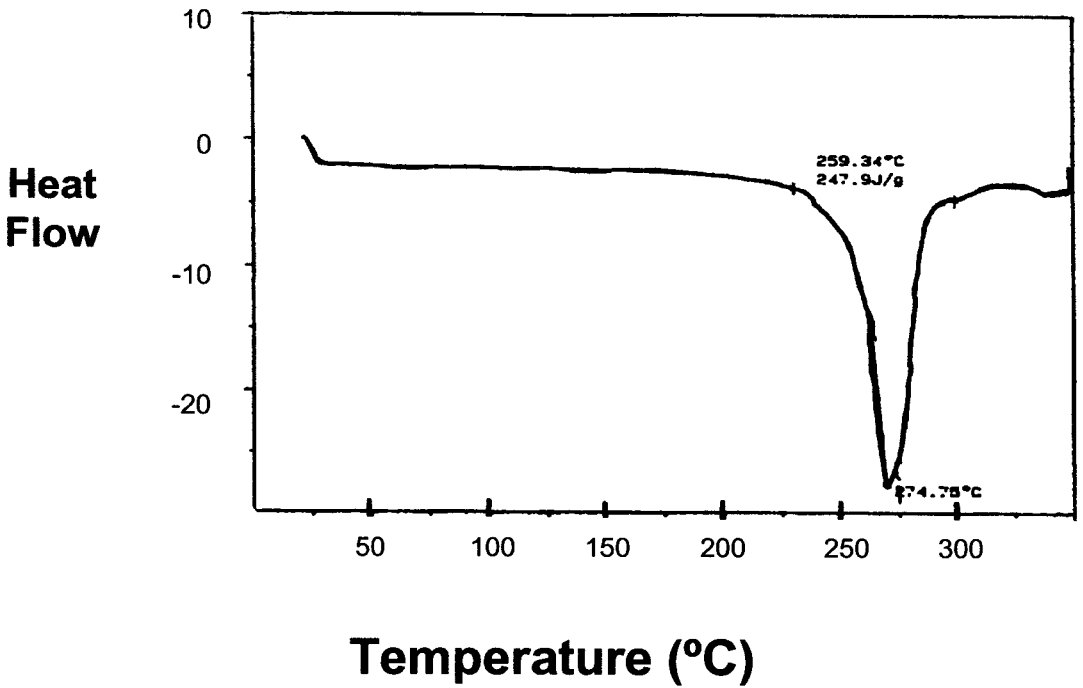


Figure 3. Differential scanning calorimetry thermogram of dorzolamide hydrochloride.

3.4 Solubility Characteristics

Solubilities of dorzolamide Forms I and II at room temperature are presented in Table 1. The pH of a saturated aqueous solution is found to be approximately 3.7.

The pH dependence of dorzolamide solubility was also determined between pH 4.0 and 7.0, using acetate, citrate, and phosphate buffer solutions to set the desired pH. These data are collected in Table 2 (also plotted in Figure 4), and show a maximum solubility of approximately 40 mg/mL at pH 5.6. The equilibrium solubility decreases to approximately 13 mg/mL at pH 6 and 4 mg/mL at pH 7.0. These data indicate that in order to have a stable 2% solution for an ophthalmic formulation, the solution pH should be maintained below 5.8. At pH values exceeding 5.8, precipitation of the free base could occur.

The existence of a second dissociation is indicated by the rise in solubility that takes place above pH 8.5, and which is associated with pK_{a2}.

3.5 Partition Coefficients

The octanol-water ($P_{o/w}$) partition coefficient was found to be 1.96 for the *n*-octanol/pH 7.4 McIlvaine buffer system, and 0.48 for the chloroform/pH 7.4 McIlvaine buffer system. These determinations were conducted at 33°C to mimic the temperature of the cornea.

3.6 Ionization Constants

Potentiometric titration studies were used to obtain values for the two ionization constants of dorzolamide hydrochloride. The first of these yielded $pK_{a1} = 6.35$, corresponding to the secondary amino functional group. The second ionization corresponded to sulfonamido group, for which $pK_{a2} = 8.50$.

Table 1

Equilibrium Solubilities of the Two Polymorphs of Dorzolamide Hydrochloride in Various Solvents

Solvent	Solubility (mg/mL)
Form I	
Water	41.1
Methanol	9.6
Acetonitrile	0.13
Ethyl ether	0.04
Ethyl acetate	0.04
Dimethylformamide	Greater than 100
Dichloromethane	0.03
Form II	
Water	43.6
Methanol	13.6
Ethanol	1.0

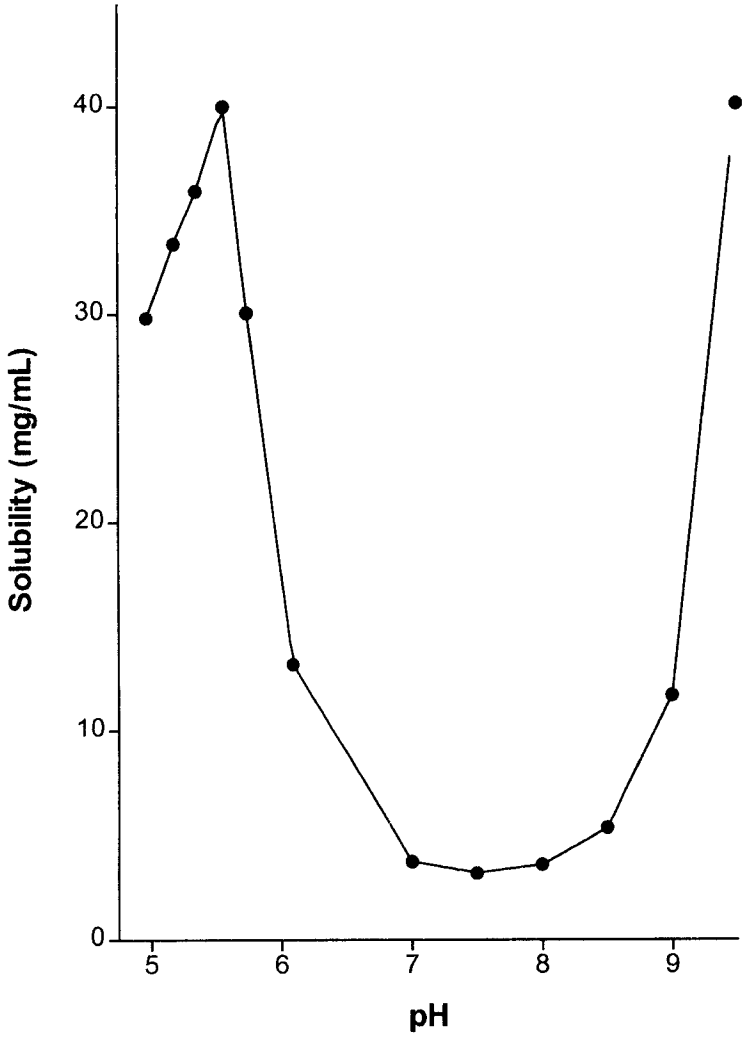


Figure 4. pH dependence of the equilibrium solubility of dorzolamide hydrochloride, Form I.

Table 2

pH Dependence of the Equilibrium Solubility of Form I Dorzolamide Hydrochloride

pH	Solubility (mg/mL)
4.97	29.79
5.18	33.35
5.35	35.92
5.56	40.00
5.74	30.06
6.09	13.21
7.00	3.74
7.50	3.19
8.00	3.59
8.50	5.33
9.00	11.71
9.50	40.10

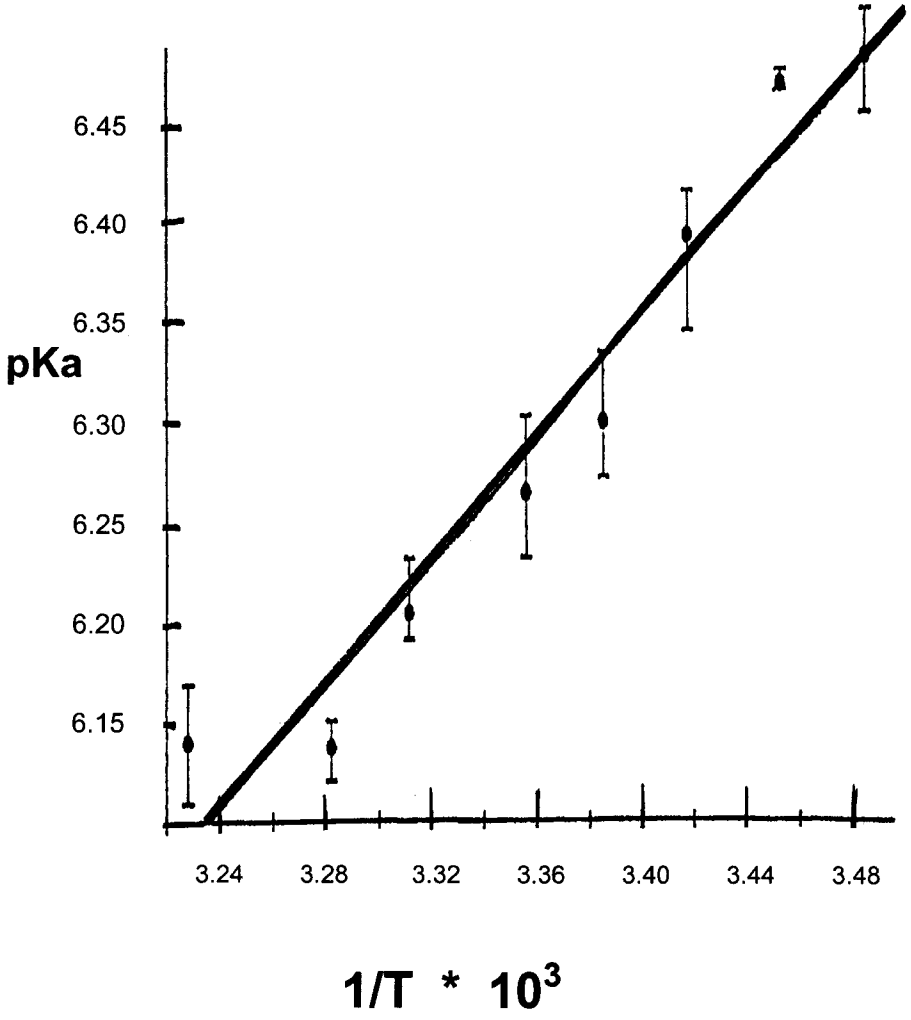


Figure 5. Temperature dependence of the amine pK_a values of dorzolamide hydrochloride.

The pK_a of the basic nitrogen of dorzolamide is very temperature-dependent, and the nitrogenous base becomes weaker as the temperature increases. Consequently, the measured pH of any given solution will vary with temperature. As illustrated in Figure 5, an increase in pH with decreasing temperature is accompanied by a change in the free base concentration.

3.7 Spectroscopy

3.7.1 UV/VIS Spectroscopy

The ultraviolet absorbance spectrum of dorzolamide hydrochloride was obtained using an AVIV Model 118DS UV/VIS spectrophotometer, with the compound being dissolved in 0.1N methanolic hydrochloric acid. As evident in Figure 6, the spectrum is characterized by an absorbance maximum at 255 nm. The $A^{1\%}$ value for this band was calculated to be approximately 357.

3.7.2 Vibrational Spectroscopy

The infrared absorption spectrum of dorzolamide was obtained in a Nujol mull using a Nicolet 510p spectrophotometer, and is shown in Figure 7. Assignments for the characteristic absorption bands in the spectrum are provided in Table 1.

3.7.3 Nuclear Magnetic Resonance Spectrometry

3.7.3.1 ^{1}H-NMR Spectrum

The proton nuclear magnetic resonance spectrum of a 4.7% (w/v) solution of dorzolamide hydrochloride was obtained in deuterated dimethyl sulfoxide, and is shown in Figure 8. The spectrum was recorded using a Brucker model AM-400 NMR spectrometer. The band assignments were referenced relative to dimethyl sulfoxide-d_5 (at 2.5 ppm), and the hydrogen atom assignments are tabulated in Table 4. The assignments make use of the numbering system given on the following page.

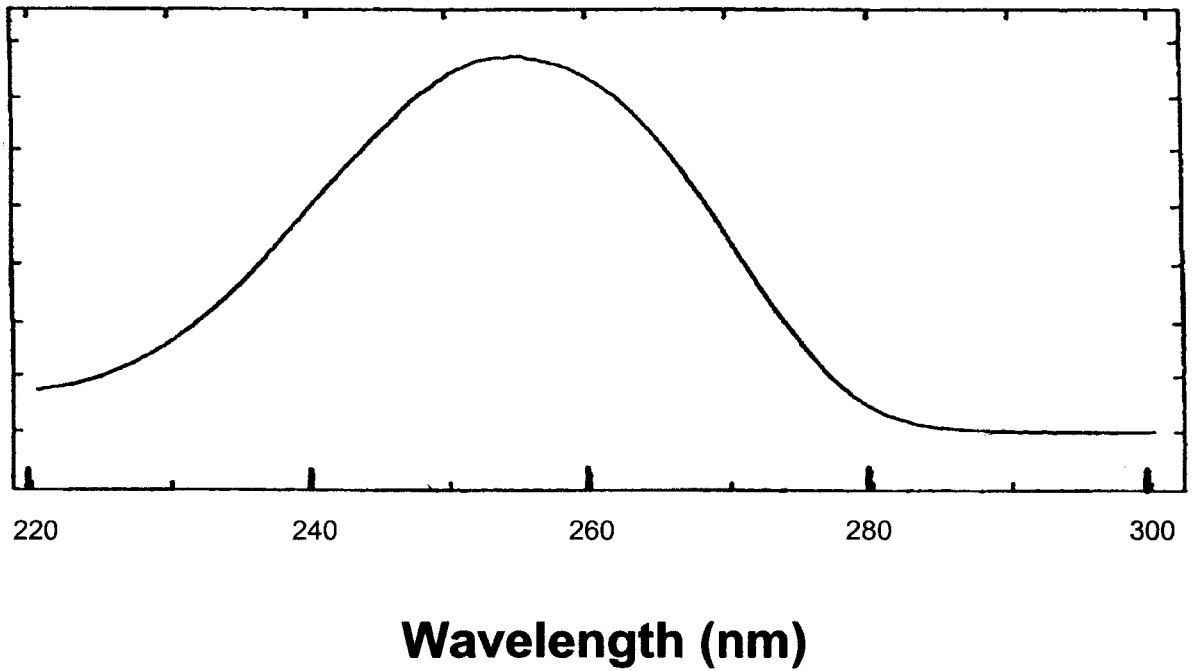


Figure 6. Ultraviolet absorption spectrum of dorzolamide hydrochloride.

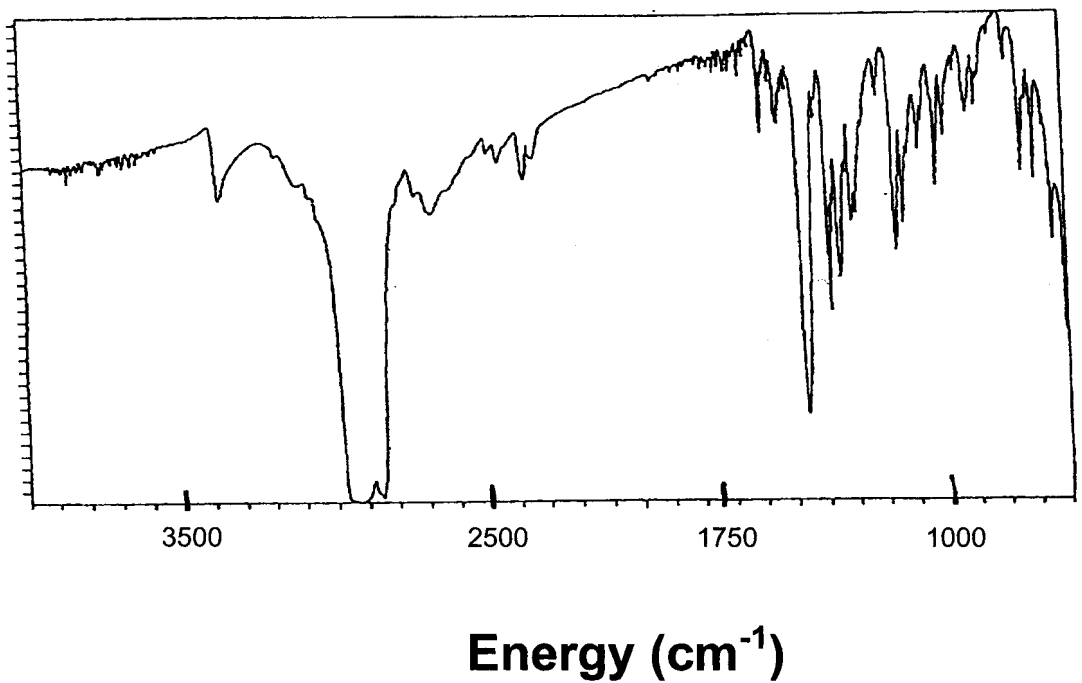


Figure 7. Infrared transmittance spectrum of dorzolamide hydrochloride.

Table 3

Assignments for the Major Absorptions of Dorzolamide Hydrochloride in the Infrared Spectral Region

Frequency (cm^{-1})	Assignment
3372	NH stretching mode of SO_2NH_2
~3040	C-H stretching mode of thiophene
2300-2800, 2689	NH_2^+ stretching mode
1589, 1536	C=C stretching mode
1345	SO_2 asymmetric stretching mode, sulfonamide
1306	SO_2 asymmetric stretching mode, sulfone
1159	SO_2 symmetric stretching mode, sulfonamide
1132	SO_2 symmetric stretching mode, sulfone

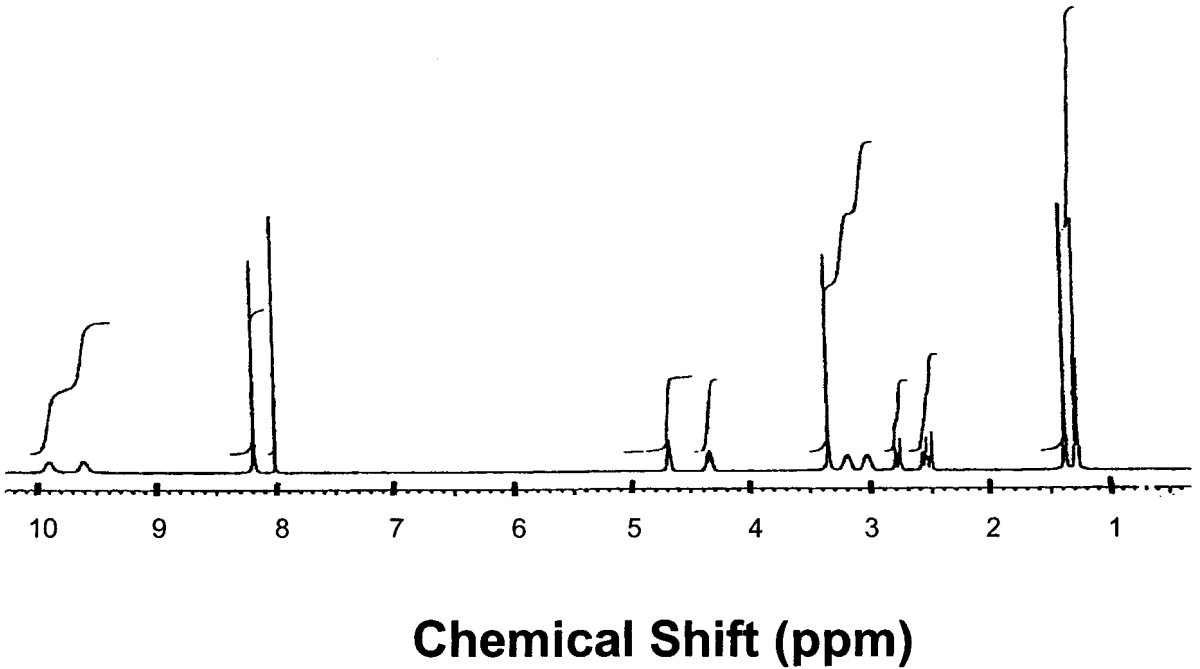


Figure 8. ^{1}H-NMR spectrum of dorzolamide hydrochloride.

Table 5

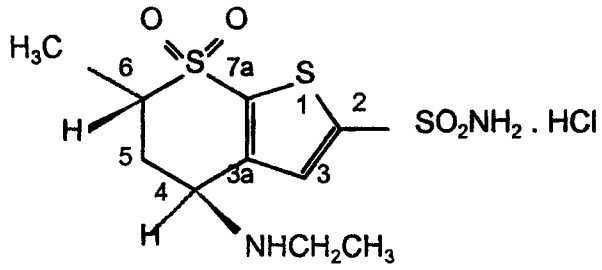
Assignments for the Resonance Bands observed in the ^{1}H-NMR Spectrum of Dorzolamide Hydrochloride

Chemical Shift (ppm)	Multiplicity[1]	Number of Protons	Assignment
9.92, 9.63	broad s	2	NH_2^+ Cl^-
8.19	s	2	SO_2NH_2
8.01	s	1	carbon-3 proton
4.68	broad	1	carbon-4 proton
4.36	m	1	carbon-6 proton
3.35	s		H_2O
3.20, 3.04	broad	2	CH_3-C**H**$_2$-N-
2.79	m	1	carbon-5 proton
2.55	m	**1**	carbon-5 proton[2]
1.38	d	3	[carbon-6]-C**H**$_3$
1.29	t	3	C**H**$_3$-CH_2-N

Notes:

(1) Multiplicity: s = singlet, d = doublet, t = triplet, and m = multiplet

(2) The isotopic impurity of the DMSO-d_6 solvent is under this carbon-5 proton multiplet.



Numbering system for dorzolamide hydrochloride

3.7.3.2 ^{13}C-NMR Spectrum

The carbon-13 nuclear magnetic resonance spectrum of a 4.7% (w/v) solution of dorzolamide hydrochloride was obtained in deuterated dimethyl sulfoxide, and is shown in Figure 9. The spectrum was recorded using a Brucker model AM-400 NMR spectrometer. The band assignments were referenced relative to dimethyl sulfoxide-d_5 (39.5 ppm), and the carbon atom assignments (using the same numbering system as just described) are collected in Table 5.

3.7.4 Mass Spectrometry

The low-resolution electron ionization spectrum of dorzolamide hydrochloride is shown in Figure 10, and was obtained using a JEOL SX102 with probe temperature ramped from ambient temperature to 400°C at a rate of 64°C/min. The mass spectrum is in accord with the structure of dorzolamide. The major fragment ions observed in the mass spectrum are assigned in the figure provided on the following page.

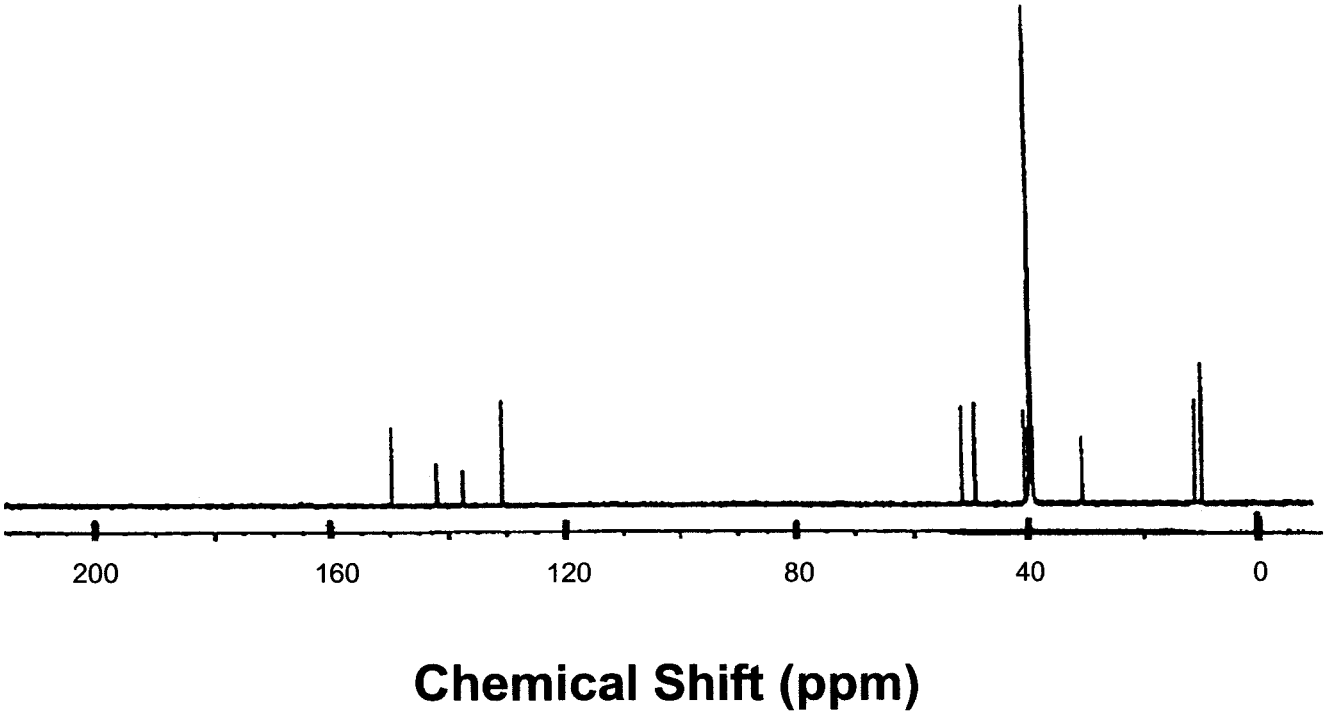


Figure 9. ^{13}C-NMR spectrum of dorzolamide hydrochloride.

Table 5

Assignments for the Resonance Bands observed in the ^{13}C-NMR Spectrum of Dorzolamide Hydrochloride

Chemical Shift (ppm)	**Assignment**
149.6	carbon-2
141.8	carbon-7a
137.3	carbon-3a
130.6	carbon-3
51.5	carbon-6
49.1	carbon-4
40.7	CH_3-<u>C</u>H_2-N-
30.6	carbon-5
11.1	<u>C</u>H_3-CH_2-N-
9.9	[carbon-6]-<u>C</u>H_3

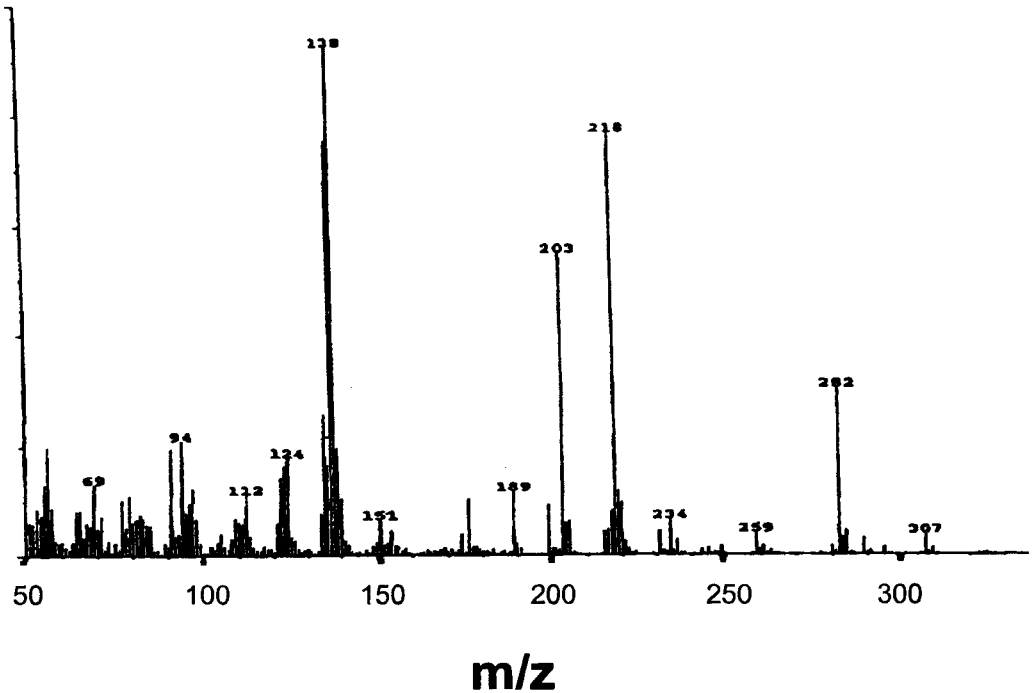
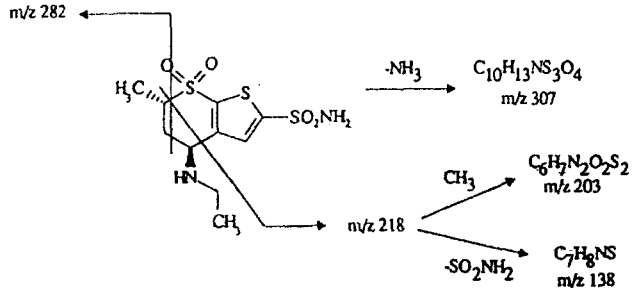


Figure 10. Mass spectrum of dorzolamide hydrochloride.



Mass spectrum fragmentation pattern of dorzolamide hydrochloride

4. Methods of Analysis

4.1 Identification

Three methods are routinely used to identify dorzolamide:

1. The characteristic infrared spectrum
2. The formation of a precipitate with $AgNO_3$
3. The characteristic chromatographic retention time.

4.2 Elemental Analysis

Elemental analysis for carbon, hydrogen, sodium, sulfur, and chloride yielded values that are in good agreement with the calculated values.

	Calculated (%)	Found (%)
Carbon	32.28	33.33
Hydrogen	4.75	4.70
Sodium	7.76	7.67
Sulfur	26.66	26.60
Chloride	9.84	9.77
Oxygen (by difference)	18.71	17.93

4.3 Titrimetric Analysis

Dorzolamide can be titrated to a potentiometric endpoint using 0.1N $AgNO_3$ as the titrant. For the analysis, the sample is dissolved in 20:20:1 (v/v/v) water : methanol : concentrated nitric acid.

4.4 Chromatographic Methods of Analysis

4.4.1 Thin Layer Chromatography

Dorzolamide can be chromatographed using silica gel TLC plates, and a mobile phase of 80:20:1 methylene chloride : methanol : ammonia. Visualization can be effected by either viewing the developed plate under ultraviolet light, or by exposing the developed plate to iodine vapor. The R_f of dorzolamide is approximately 0.62.

4.4.2 High Performance Liquid Chromatography

A variety of gradient and isocratic reverse phase HPLC systems-have been used to chromatograph dorzolamide, and the salient characteristics of these are summarized in Table 6.

4.5 Determination in Body Fluids

A procedure for the determination of dorzolamide and its desethyl metabolite in biological fluids has been described using HPLC and UV detection at 252 nm [13]. The limit of detection for both compounds was 5 ng/mL in whole blood, plasma, and urine.

Since dorzolamide possesses two chiral centers, an indirect chiral separation has been developed [18]. The procedure employs the chemical derivatization of the secondary amino group of the inhibitor, formation of diastereomeric urea derivatives (each with three chiral centers in the molecule), and their separation under non-chiral HPLC conditions. Using

Table 6: High Performance Liquid Chromatographic Systems

Application	System #	Column	Mobile phase	Detection nm	Reference
Drug Substance Potency	I	Perkin-Elmer CR C-8 8.0 cm x 3.0 mm - 3μm	Acetic acid: triethylamine:water (pH=4.5) 1:1:998 (v/v/v)	254 nm	Unpublished Data
Drug Substance Purity	II	Perkin-Elmer CR C-8 8.0 cm x 3.0 mm - 3μm	**Solvent A** Acetic acid: triethylamine:water (pH=4.5) 1:1:998 (v/v/v) **Solvent B** Acetonitrile **100% A for 10min then linear gradient to 50%A, 50% B over 20 min.**	254 nm	11.
Drug Substance Chiral Purity	III	Zorbax silica 25 cm x 4.6 mm - 3μm	**Solvent A** Methyl tert-butyl ether/ actonitrile/ water 967:30:3 (v/v/v) **Solvent B:** Heptane 65% solvent A:35% solvent B	254 nm after derivatization	12.
Measurement of Dorzolamide in Plasma	IV	Beckman RP-8 (250 x 4.6 mm, 5μm)	**Solvent A:** Acetonitrile **Solvent B:** Sodium salt of octanesulfonic acid in 0.085% phosphoric acid in water 25% solvent A:75% solvent B	252 nm	13.
Measurement in Eye-drops	V	Zorbax RX-C8 (25 cm x 4.6 mm, 5 μm)	**Solvent A:** Orthophosphoric acid solution 0.2% + triethylamine pH=3 **Solvent B:** Acetonitrile: 95% solvent A:5% solvent B.	253 nm	15.

this method, it was established that there was no inversion of configuration and that only the 4*S*,6*S* isomers of both dorzolamide and its metabolite were present in the post-dose whole blood and urine samples from human subjects.

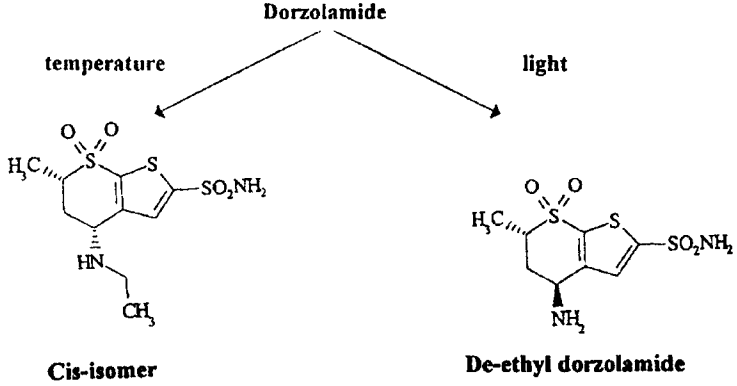
5. Stability

5.1 Solid-State Stability

Bulk dorzolamide drug substance was found to be stable under accelerated thermal and photolytic conditions. When kept in double polyethylene bags inside cardboard shipping containers, the substance showed no evidence of degradation after 51 months of storage at ambient temperature and relative humidity.

The stability of material stored at elevated temperatures and increased relative humidities was also evaluated. No changes were evident after 3 months of storage at 80°C, or after 3 months of storage at 90% of relative humidity. When kept for 3 months under 2000 lux or 3 days under UV light, the bulk drug substance was unchanged and no degradants were observed.

Structures of the two main degradation products are as follows:



5.2 Solution-Phase Stability

Dorzolamide (which is the (-)-*trans* diastereomer having the 4*S*,6*S* configuration) was found to be very stable in acidic solutions. In neutral and alkaline solutions, however, the *cis* diastereomer (having the 4*R*,6*S* configuration) was formed. The rate of formation of this degradant was found to increase with the temperature of storage, and with the alkalinity of the solution. At extreme alkaline conditions (*i.e.*, exceeding pH 10), the sulfonic acid derivative was observed to form as an additional degradant.

When the stability of dorzolamide hydrochloride was studied in 2% ophthalmic solutions over two years, photodegradation was not observed. However, when solutions were exposed to daylight, a degradant identified as the 4*S*,6*S* de-ethylated analog was formed.

When the pH of a solution formulation was maintained between 4.5 to 6.0, formation of the thermal degradant increased with increasing pH. The percentage of the degradant formed after 2 years of storage at 25°C as a function of pH is illustrated in Figure 11. The data indicate that the amount formed at pH 6.0 was approximately 7-fold higher than that formed at pH 4.5. This finding, together with other considerations (such as clinical efficacy and tolerability by the patient), led to the selection of the pH of the finished product as 5.65.

6. Drug Metabolism and Pharmacokinetics

Dorzolamide Hydrochloride is an inhibitor of human carbonic anhydrase II. The inhibition of carbonic anhydrase in the ciliary processes of the eye decreases the secretion of aqueous humor, with a resulting reduction in intraocular pressure. The drug is formulated as an ophthalmic solution, and administered topically as eyedrops.

6.1 Absorption and Distribution

Dorzolamide hydrochloride is commercially available as a 2% ophthalmic solution. In man, it is well tolerated, and topical administration into the

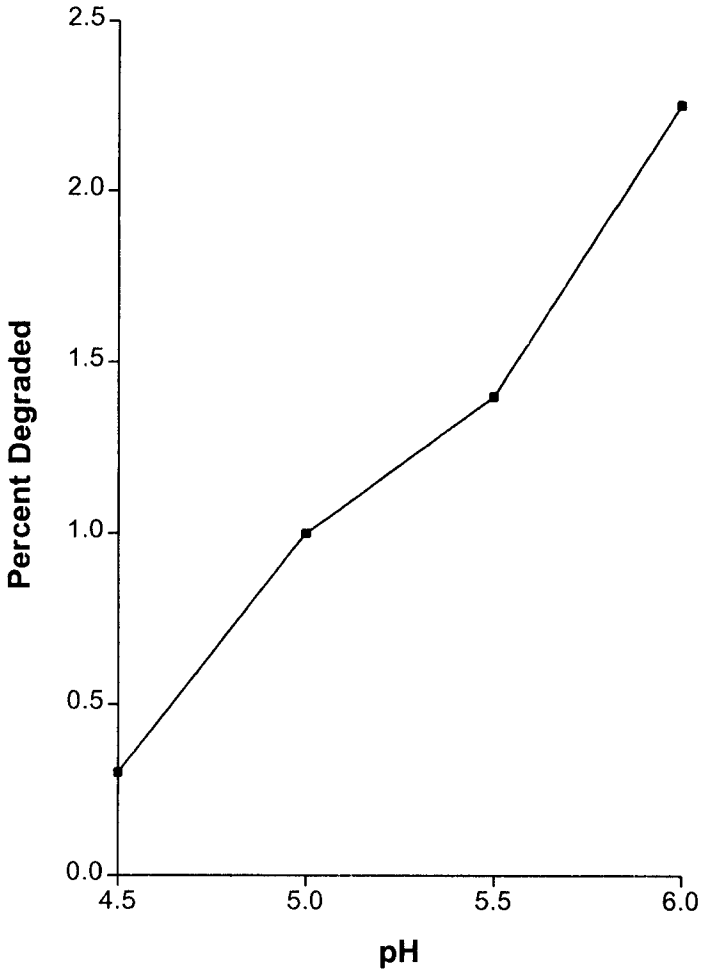


Figure 11. Degradation of dorzolamide hydrochloride as a function of pH after 2 years of storage at 25°C.

conjuctival sac results in the expected lowering of intra-ocular pressure [10,14]. Following instillation, dorzolamide reaches the systemic circulation. The binding of dorzolamide and its metabolite in the red blood cells and other tissues represents the major influence on systemic pharmacokinetics and pharmacodynamics. Plasma concentrations of the drug and its metabolite are generally below assay limits of detection (15 nM), indicating the presence of almost no free drug or metabolite [15,16].

In vitro studies indicated that dorzolamide binds strongly to erythrocyte carbonic anhydrase CA II and very weakly to CA I. The desethyl metabolite also binds to both CA I and CA II, but has less affinity for CA II and is considerably less selective for CA II than dorzolamide.

The inhibition of CA II and total carbonic anhydrase activities was found below the degree of inhibition anticipated to be necessary for a pharmacological effect on renal function and respiration when dorzolamide was administered orally at 2 mg b.i.d for up to 20 weeks [14].

6.2 Metabolism and Excretion

In man, dorzolamide is metabolized by *N*-deethylation. Sensitive HPLC assays for both entities in whole blood, plasma, and urine have been developed to support clinical pharmacokinetic studies [13]. Both the parent compound and *N*-desethyl-dorzolamide are excreted in the urine. At steady state, renal excretion was 1.3 mg/day of the total daily dose of 4 mg, and the renal clearance was 90 mL/min. Unchanged dorzolamide accounts for about 80 % of the total urinary excretion, and *N*-desethyl-dorzolamide accounts for the remaining 20% [16].

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INDINAVIR SULFATE

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ANALYTICAL PROFILES OF DRUG SUBSTANCES AND EXCIPIENTS—VOLUME 26
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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Name

[1(1*S*, 2*R*), 5(*S*)]-2,3,5-trideoxy-*N*-(2,3-dihydro-2-hydroxy-1*H*-inden-1-yl)-5-[2-[[(1,1-dimethylethyl) amino]carbonyl]-4-(3-pyridinylmethyl)-1-piperazinyl]-2-(phenylmethyl)-D-*erythro*-pentonamide sulfate (1:1) salt

1.1.2 Nonproprietary Names

U.S. Adopted Name: Indinavir Sulfate

International Non-Proprietary Name: Indinavir

International Non-Proprietary Name modified: Indinavir Sulfate (This nomenclature will be used throughout to designate the sulfate salt (ethanol solvate) unless otherwise indicated.)

Laboratory Codes: MK-0639, L-735,524-001J

1.1.3 Proprietary Names

CRIXIVAN™

1.2 Formulae

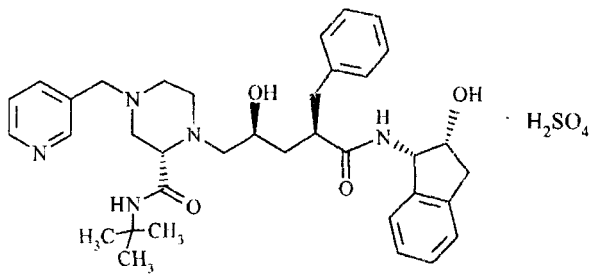
1.2.1 Empirical

Free base: $C_{36}H_{47}N_5O_4$

Sulfate Salt: $C_{36}H_{47}N_5O_4 \cdot H_2SO_4$

Ethanol Solvate: $C_{36}H_{47}N_5O_4 \cdot H_2SO_4 \cdot C_2H_5OH$

1.2.2 Structure (Figure 1)



1.3 Molecular Weight

Free base: 613.80

Sulfate Salt: 711.88

Ethanol Solvate: 757.94

1.4 CAS Number

157810-81-6

1.5 Appearance

Indinavir sulfate is a white to off-white, free-flowing powder.

1.6 Uses and Applications

The viral protease enzyme, human immunodeficiency virus type 1 (HIV-1), is a causative agent of AIDS. HIV-1 mediates the processing of the viral precursor polyproteins. This process is essential for the production and maturation of infectious viruses [1,2]. CRIXIVAN™ is a specific and potent inhibitor of the HIV-1 protease, and is used in the treatment of AIDS.

2. Method of Preparation

The synthetic commercial synthetic route to produce indinavir sulfate is shown in Figure 2 [3-6]. The synthesis of indinavir sulfate involves the formation of a 4-hydroxy-5-piperazino-pentonamide backbone via a convergent coupling of an optically active mono-substituted epoxide (Compound **V**) and an optically active, commercially obtained, piperazine-2-carboxamide with 4-*t*-butylcarbamate (BOC) protection on the amino group (Compound **VI**). The epoxide is prepared by treatment of *cis*-aminoindanol (Compound **I**) with hydrocinnamoyl chloride (Compound **II**), followed by conversion to the corresponding acetonide derivative (Compound **IV**) and epoxide formation via an allylation / iodohydrin formation / epoxidation reaction sequence. Following the coupling and after simultaneous acid-mediated removal of the protecting groups (acetonide and *t*-butylcarbamate), the resulting Compound **VIII** is subjected to alkylation of the 4-piperazine nitrogen with 3-picolyl chloride producing crystalline indinavir free base monohydrate (Compound **IX**).

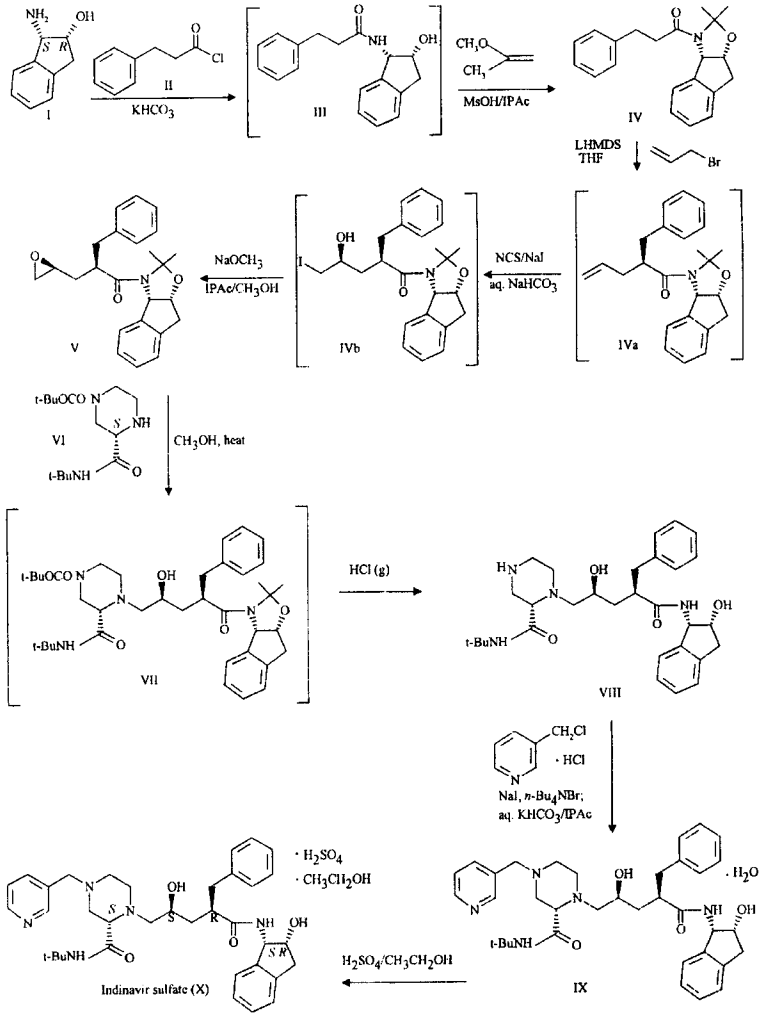


Figure 2. Synthetic commercial route for the production of indinavir sulfate.

Indinavir sulfate (Compound **X**) as the crystalline ethanolate is then prepared by treatment of the free base monohydrate with sulfuric acid in anhydrous ethanol.

3. Physical Properties

3.1 Particle Morphology

The crystals of indinavir sulfate exist as a mixture of anisotropic rods and needles [5].

3.2 Crystallographic Properties

3.2.1 X-Ray Powder Diffraction Pattern

Indinavir sulfate, produced as the ethanolate using the synthetic process described in Section 2, is crystalline as determined by the x-ray powder diffraction (XRPD) method. The XRPD pattern and associated data, obtained using a Phillips Model APD 3720 x-ray diffractometer, is shown in Figure 3 and Table 1 [7].

3.2.2 Single Crystal Structure

Crystallographic quality crystals of indinavir sulfate salt were grown by slow diffusion of methanol into an ethanol/water solution. As confirmed by TG/IR results, the crystals obtained were a mixed mono-methanol / mono-ethanol solvate. The compound crystallized in the $P2_1$ space group, (monoclinic crystal system) with 2 molecules per unit cell. The cell constants were found to be: a = 14.321(1)Å, b = 10.091(1)Å, c = 15.192(1)Å, β = 95.50(1)°, and V=2185.5Å^3. The calculated density was 1.200 g/cm^3. A view of the crystallographic unit cell packing is shown in Figure 4, with the solvent molecules omitted [7].

As shown in Figure 5, the XRPD pattern calculated from the crystal structure results was found to be very similar to the experimental XRPD obtained for the bulk product. The dissymmetry of all chiral centers

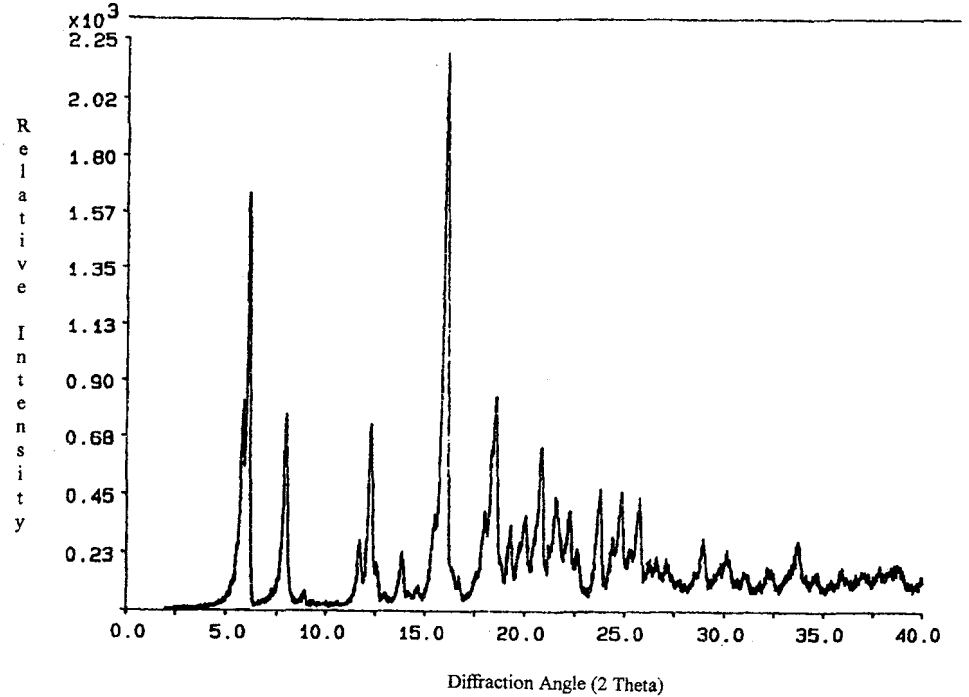


Figure 3. X-ray powder diffraction pattern of indinavir sulfate.

Table 1

X-ray Powder Diffraction Pattern of Indinavir Sulfate

Scattering Angle (degrees 2θ)	d-Spacing (Å)	Relative Intensity (I/Imax * 100)
5.8375	15.1277	38.72
6.1200	14.4300	77.83
7.9175	11.1576	33.13
7.9950	11.0496	34.68
8.9075	9.9196	2.49
11.7425	7.5303	12.02
12.2600	7.2136	32.87
12.3200	7.1786	30.86
12.5525	7.0461	8.35
12.9675	6.8216	2.22
13.8900	6.3705	8.22
14.6150	6.0561	3.16
15.4325	5.7371	14.61
16.0550	5.5160	100.00
16.6875	5.3083	4.46
16.7675	5.2832	3.16
17.5100	5.0608	4.46
18.0100	4.9214	16.72
18.3300	4.8362	28.44
18.5700	4.7742	37.89
19.3225	4.5900	14.44
20.0575	4.4234	15.65
20.8325	4.2606	27.50
21.5500	4.1203	18.39
22.2450	3.9931	16.18

Table 1 (Continued)

X-ray Powder Diffraction Pattern of Indinavir Sulfate

Scattering Angle (degrees 2θ)	d-Spacing (Å)	Relative Intensity (I/Imax * 100)
22.6525	3.9222	9.13
23.7750	3.7395	20.35
24.3950	3.6458	10.10
24.8575	3.5790	18.78
25.2975	3.5178	9.27
25.7700	3.4543	17.83
26.2775	3.3888	6.19
26.6600	3.3410	6.76
27.1850	3.2777	5.87
28.9225	3.0846	9.82
30.1925	2.9577	6.76
31.0850	2.8748	3.57
32.2650	2.7723	4.46
33.6925	2.6580	9.13
33.8025	2.6496	7.72
34.7250	2.5813	2.78
35.9025	2.4993	3.65
36.9675	2.4297	3.48
37.7900	2.3787	3.48
38.8750	2.3148	3.91

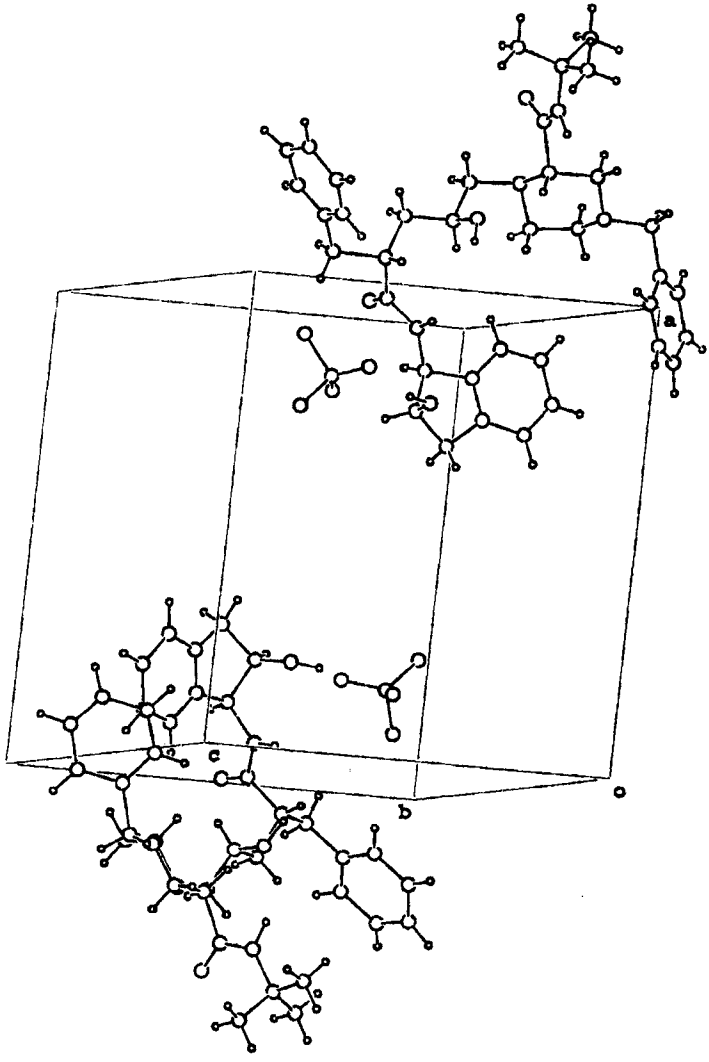


Figure 4. View of the unit cell of indinavir sulfate, as determined from the single-crystal structure.

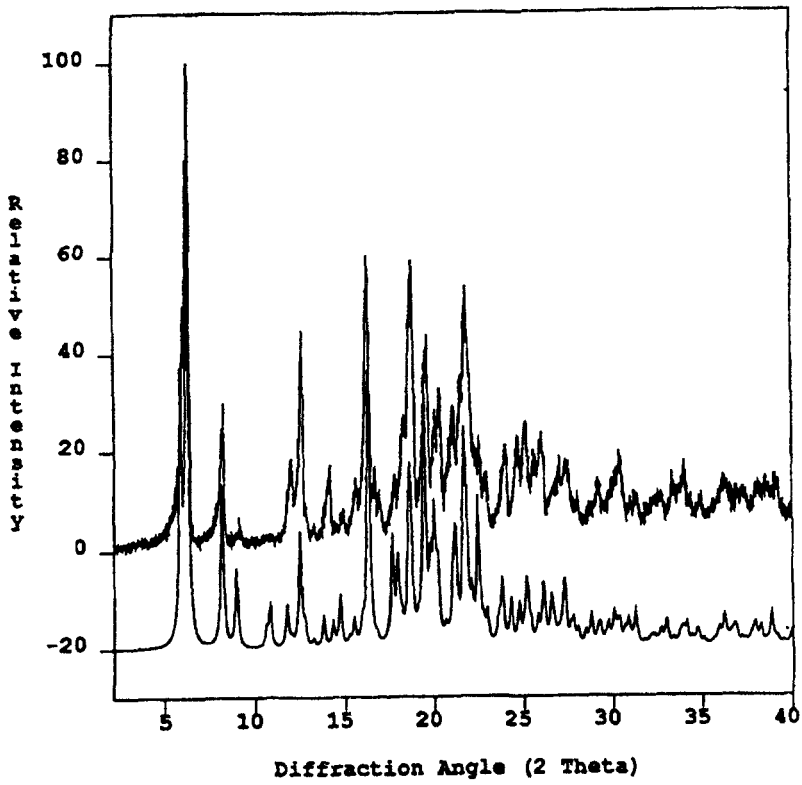


Figure 5. Experimental x-ray powder diffraction pattern of indinavir sulfate (upper trace), and the XRPD pattern calculated from the single-crystal structure results (lower trace).

agreed with the known crystal structure, confirming the absolute configuration of the molecule [7].

The deduced bond angles and lengths are shown in Tables 2 and 3, and a computer generated perspective drawing of the molecule is shown in Figure 6. Neither the disordered solvent molecules nor the nitrogen protonation site were determined crystallographically, so these are not included in the figure [7].

3.2.3 Polymorphism

Both a hydrated crystal and amorphous form of indinavir sulfate have been observed following prolonged exposure of the ethanolate to moisture [7].

3.3 Optical Activity

Indinavir sulfate has 5 chiral centers, and is obtained as an optically active form. The specific rotation, $[\alpha]_{365}^{25^\circ}$, is ~ +125° (c = 1% in water) [8].

3.4 Thermal Methods of analysis

3.4.1 Differential Scanning Calorimetry

The differential scanning calorimetry (DSC) curve for indinavir sulfate, shown in Figure 7, was obtained at a heating rate of 10°C/min under nitrogen flow in an open cup. The thermogram shows an endothermic transition having an onset temperature of 144°C, a peak temperature of 152°C, and a corresponding transition heat equal to 60 J/g. This thermal feature is due to the combination of melting with decomposition, and the simultaneous loss of ethanol [7].

3.4.2 Thermogravimetry

The thermogravimetric (TG) profile for indinavir, shown in Figure 8, was obtained under nitrogen flow with a heating rate of 10°C/min. The thermogram consists of a low temperature weight loss of 0.4% between ambient and 60°C, followed by a high temperature weight loss of 5.1%

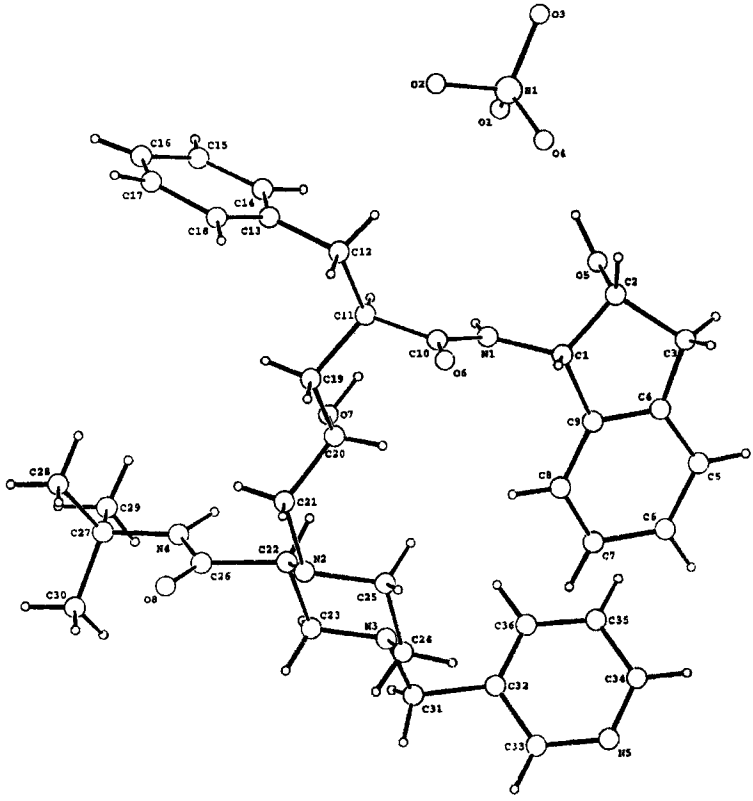


Figure 6. Conformation of the indinavir molecule, as determined from the single-crystal structure results.

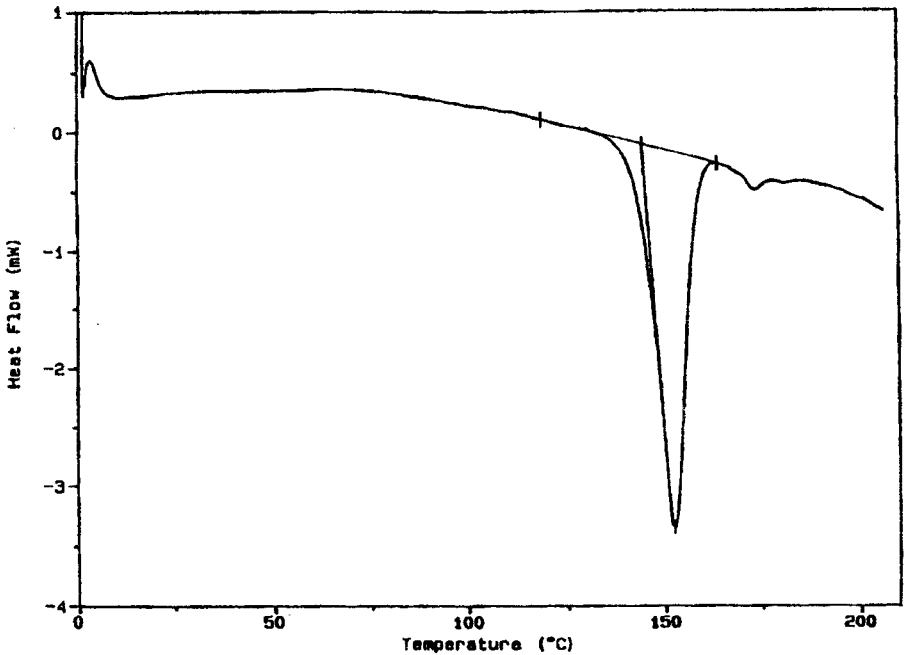


Figure 7. Differential scanning calorimetry thermogram of indinavir sulfate.

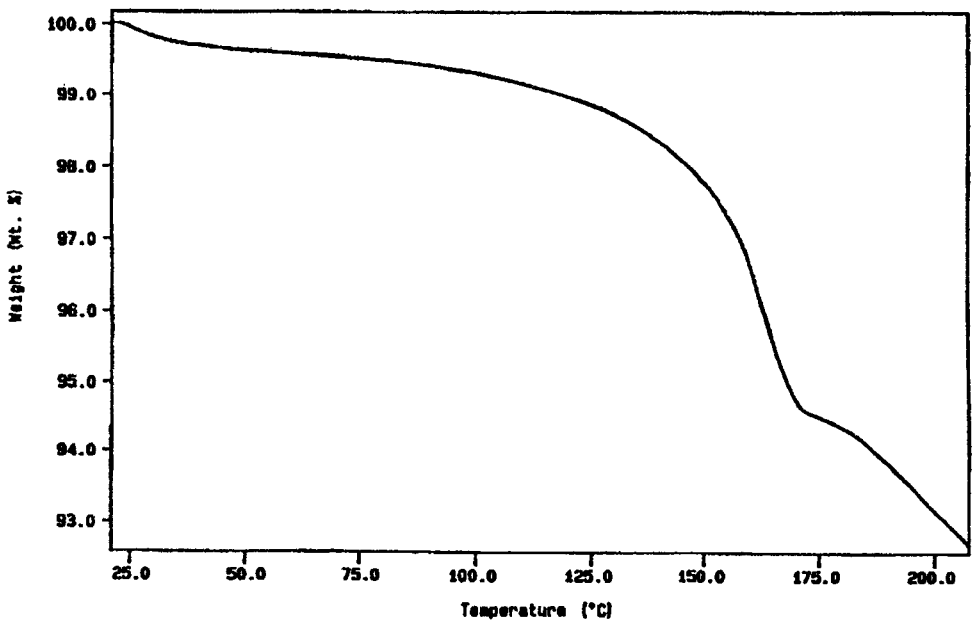


Figure 8. Thermogravimetric profile of indinavir sulfate.

between 60°C and the decomposition onset at approximately 174°C. Based on independent Karl Fischer titration and gas chromatographic results for this sample (water = 1.0% and ethanol = 6.3%), the low temperature weight loss is attributed to the loss of water and the higher temperature loss is attributed to loss of ethanol [7].

3.5 Hygroscopicity

Indinavir sulfate is hygroscopic, and will convert to either an amorphous state or a hydrated form [7].

3.6 Solubility Characteristics

As demonstrated by the pH - solubility plot generated with citric acid / citrate buffered solutions of indinavir free base monohydrate, the aqueous solubility of indinavir free base monohydrate increases with decreasing pH. The data points of this study are given in Table 4, and plotted in Figure 9.

Table 4.

pH Solubility Profile Data

pH	Solubility (mg/mL)
3.4	61.2
3.5	31.7
3.6	20.3
4.0	5.13
4.9	0.312
5.9	0.037
6.9	0.019
7.0	0.023
8.0	0.017

Consistent with these findings, the aqueous solubility (unbuffered) of the indinavir sulfate salt exceeds 100 mg/mL. The solubility of indinavir

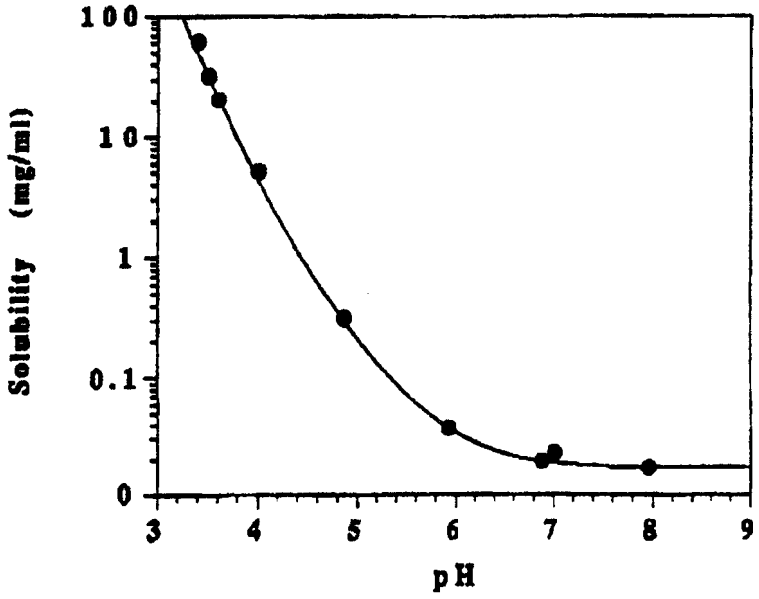


Figure 9. pH – solubility profile of indinavir sulfate.

sulfate in methanol has been estimated to be between 50 and 100 mg/mL, the solubility in acetonitrile was estimated at approximately 0.1 - 1 mg/mL, and the solubility in hexane is less than 0.1 mg/mL [7].

The pH of a 1% (1 g dissolved in 100 mL solvent) solution of indinavir sulfate in water is about 3.0 [8].

3.7 Partition Coefficient

Partition coefficients for indinavir sulfate, as determined using buffered solutions of indinavir free base monohydrate, have been obtained in *n*-octanol / aqueous buffer systems at various pH values. The results, expressed as log of the ratio of the concentrations of the compound in the organic phase versus the concentrations in the aqueous phase (log P), are shown in Table 5 [7].

Table 5

Partition Coefficients for Indinavir Sulfate

pH (Buffer)	log P
1.20 (0.1 *M* HCl)	-2.14
2.49 (0.1 *M* citric acid)	-1.83
3.22 (0.1 *M* citrate)	-0.701
4.06 (0.1 *M* citrate)	0.467
5.00 (0.1 *M* citrate)	1.70
6.00 (0.1 *M* citrate)	2.45
7.02 (0.1 *M* citrate)	2.66
7.95 (0.1 *M* citrate)	2.67

3.8 Ionization Constants

The apparent pKa value for indinavir sulfate, calculated from the pH/aqueous solubility profile for the free base compound, is 6.2. A

second apparent pKa value of 3.8 was determined from absorption spectroscopic studies conducted at various pH values [7].

3.9 Micromeritic Properties

3.9.1 Particle Size Distribution

The particle size distribution of indinavir is typically in the range of 5 to 300 μm, with a mean particle size of 20 μm [7].

3.9.2 Surface Area

A surface area analysis conducted on Merck & Co., Inc., reference standard lot L-735,524-001J011, yielded a value of 2.9 m^2/g [9].

3.9.3 Density

The loose bulk density of indinavir sulfate is typically 0.2 g/cc, while the tapped bulk density is typically 0.2 and 0.4 g/cc [8].

3.10 Spectroscopy

3.10.1 UV/VIS Spectroscopy

The ultraviolet absorbance spectrum of indinavir sulfate shown in Figure 10 was obtained on a Shimadzu UV 2101PC UV/VIS spectrophotometer, using a 0.14 mg/mL methanolic solution. The spectrum is characterized by an absorbance maximum at 260 nm, having an $A^{1\%}_{1cm}$ value of approximately 53, as calculated on a water- and solvent-free basis [8].

3.10.2 Vibrational Spectroscopy

The infrared transmittance spectrum of indinavir sulfate, shown in Figure 11, was obtained as a mineral oil (Nujol®) dispersion using a Nicolet Model 510 Fourier transform infrared (FTIR) spectrometer [8]. The spectrum is consistent with the structure assigned to the compound. Assignments for the most characteristic bands are tabulated in Table 6 [10].

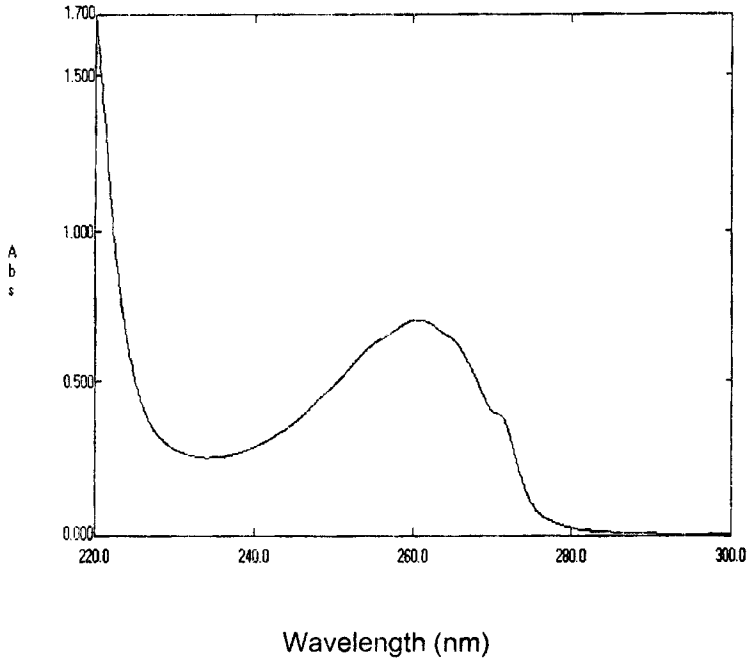


Figure 10. Ultraviolet absorption spectrum of indinavir sulfate in methanol.

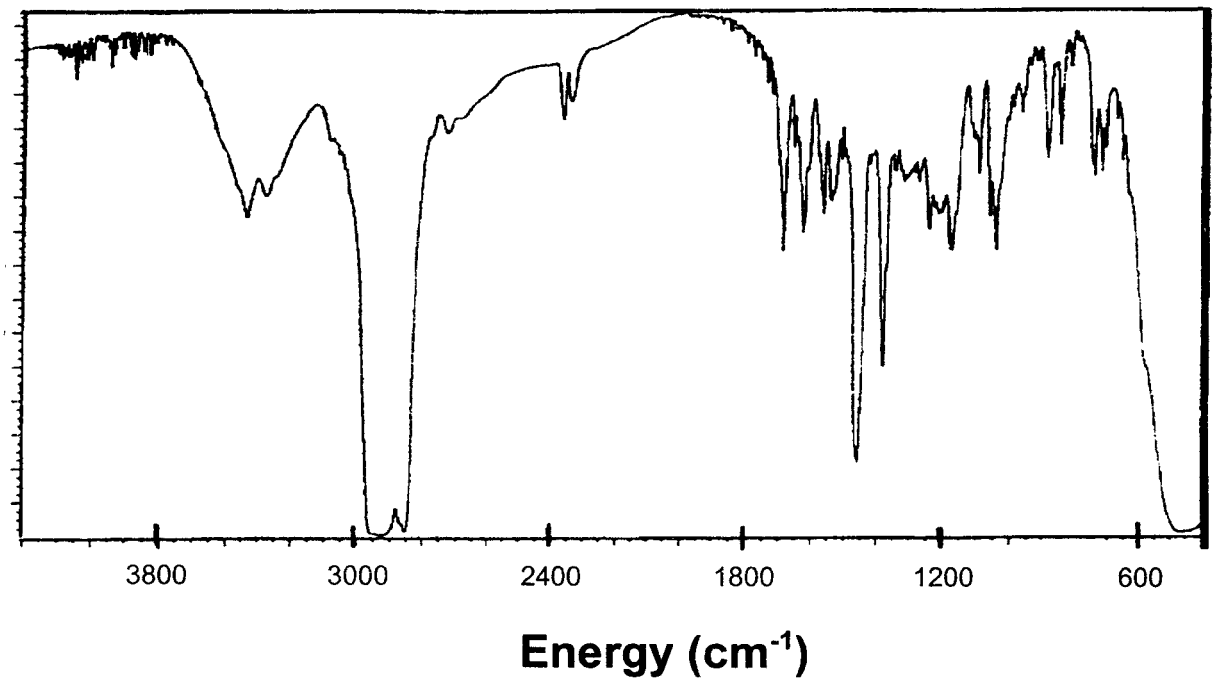


Figure 11. Infrared absorption spectrum of indinavir sulfate.

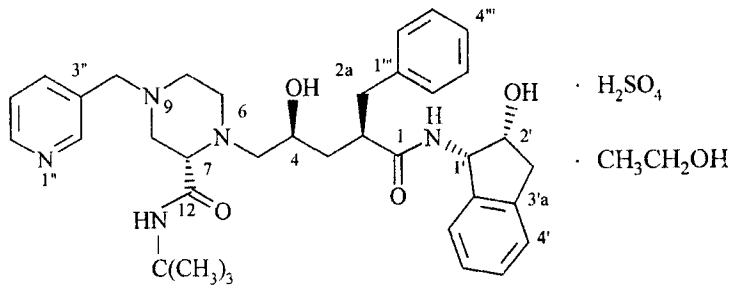
Table 6

Indinavir Sulfate Infrared Peak Assignments

Frequency (cm^{-1})	Assignment
3335 – 3277	OH and NH stretch
2926 – 2855	CH stretch (Nujol®)
1684	Amide carbonyl stretch
1624	Amide carbonyl stretch
1458	Nujol®
737	Aromatic CH deformation

3.10.3 ^{1}H-Nuclear Magnetic Resonance Spectrum

The proton magnetic resonance spectrum of indinavir sulfate shown in Figure 12 was obtained using a Bruker Instruments model AMX-400 NMR spectrometer operating at a frequency of 399.87 MHz as an approximate 4.16 % w/v solution in deuterium oxide. The HOD reference (chemical shift equal to 4.8 ppm) was used as the reference. Signal assignments are tabulated Table 7, following the numbered structural formula below [11].



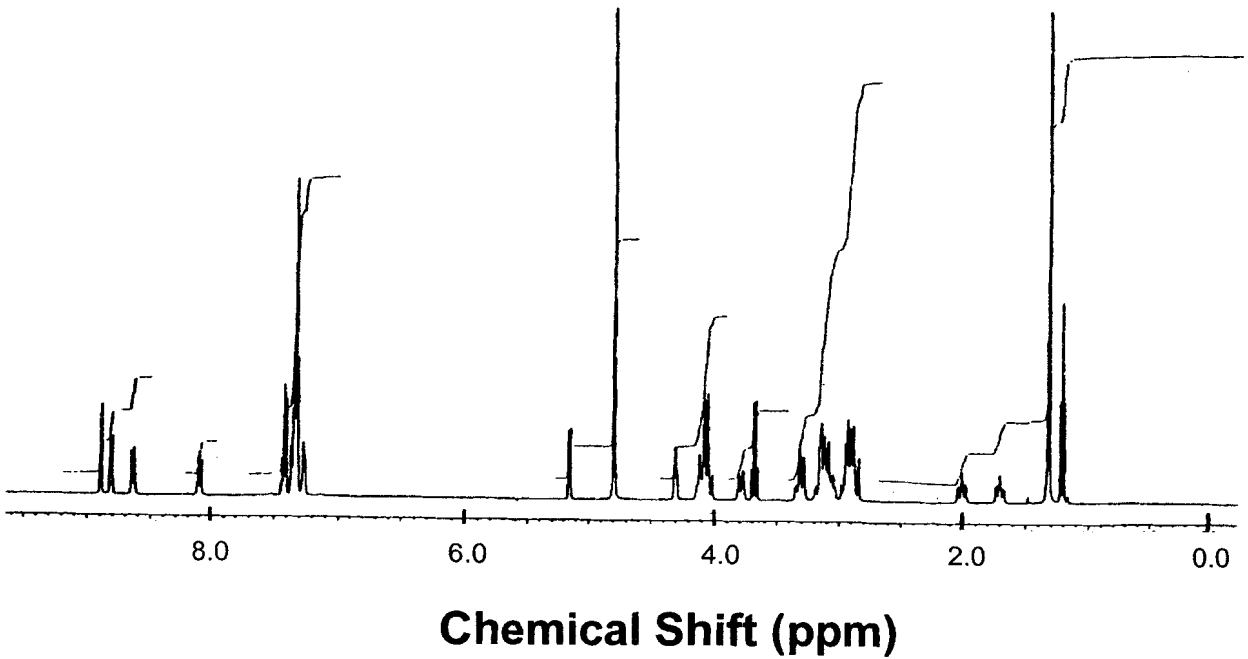


Figure 12. ^{1}H nuclear magnetic resonance spectrum of indinavir sulfate.

Table 7

Indinavir Sulfate ^{1}H-NMR Resonance Peak Assignments

Chemical Shift (ppm)	Multiplicity[1]	Assignment[2]
8.89	br s	$C_{2''}H$
8.82	br d, (J=5.8)	$C_{6''}H$
8.65	d, (J=8.0)	$C_{4''}H$
8.10	dd, (J=8.0, 5.8)	$C_{5''}H$
7.43	M	$C_{3'''}H$, $C_{5'''}H$
7.38-7.24	om	C_4H, C_5H, C_6H, C_7H, $C_{2'''}H$, $C_{6'''}H$, $C_{4'''}H$
5.16	d, (J=4.8)	C_1H
4.31	td, (J=4.8, 1.2)	C_2H
4.16-4.01	om	C_4H, N_9-CH_2, C_7H
3.80	M	$C_{11}H$
3.69	q, (J=7.1)	$HOC\underline{\mathbf{H}}_2CH_3$
3.32	om	C_8H, $C_{11}H$
3.22 - 2.82	om	C_2H, C_5H_2, C_8H, $C_{10}H_2$, $C_{2a}H_2$, C_3H_2
2.01	m	C_3H
1.71	m	C_3H
1.33	s	$C(CH_3)_3$
1.22	t, (J=7.1)	$HOCH_2C\underline{\mathbf{H}}_3$

Notes: (1) Multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, om = overlapping multiplet, br = broad. Coupling constants (J) are given in Hertz.

(2) The peak assignments refer to numbered structure. The active hydrogens (NH and OH) are exchanged by the D_2O solvent.

3.10.4 ^{13}C-Nuclear Magnetic Resonance Spectrum

The ^{13}C-NMR spectrum of Indinavir sulfate, shown in Figure 13, was obtained using a Bruker Instruments model AMX-400 nuclear magnetic resonance spectrometer operating at a frequency of 100.55 MHz as an approximate 4.16 % w/v solution in deuterium oxide. The 67.4 ppm resonance of dioxane was used as an external reference standard. Peak assignments are found in Table 8, and make use of the numbered structural formula given previously [11].

3.10.5 Mass Spectrometry

The low-resolution mass spectrum of Indinavir sulfate was measured using fast atom bombardment mass spectrometry on a JEOL HX110A mass spectrometer set at a resolution of 5000. The sample was ionized from a 5:1 dithiothreitol: dithioerythritol matrix using xenon as the FAB gas. The low-resolution mass spectrum [12] of indinavir is shown in Figure 14, while the structures of the structurally significant fragment ions are illustrated in Figure 15.

4. Methods of Analysis

4.1 Identification

Three methods are routinely used to identify indinavir sulfate:

a. The characteristic infrared absorption spectrum

b. The chromatographic retention time

c. The formation of a white precipitate upon titration of an aqueous solution of the substance with $Pb(ClO_4)_2$.

4.2 Elemental Analysis

The carbon, hydrogen, nitrogen and sulfur analyses conducted on Merck & Co., Inc., reference lot L-735,524-001J007 Yielded the values given in Table 9 [13], which compare favorably to the theoretical values.

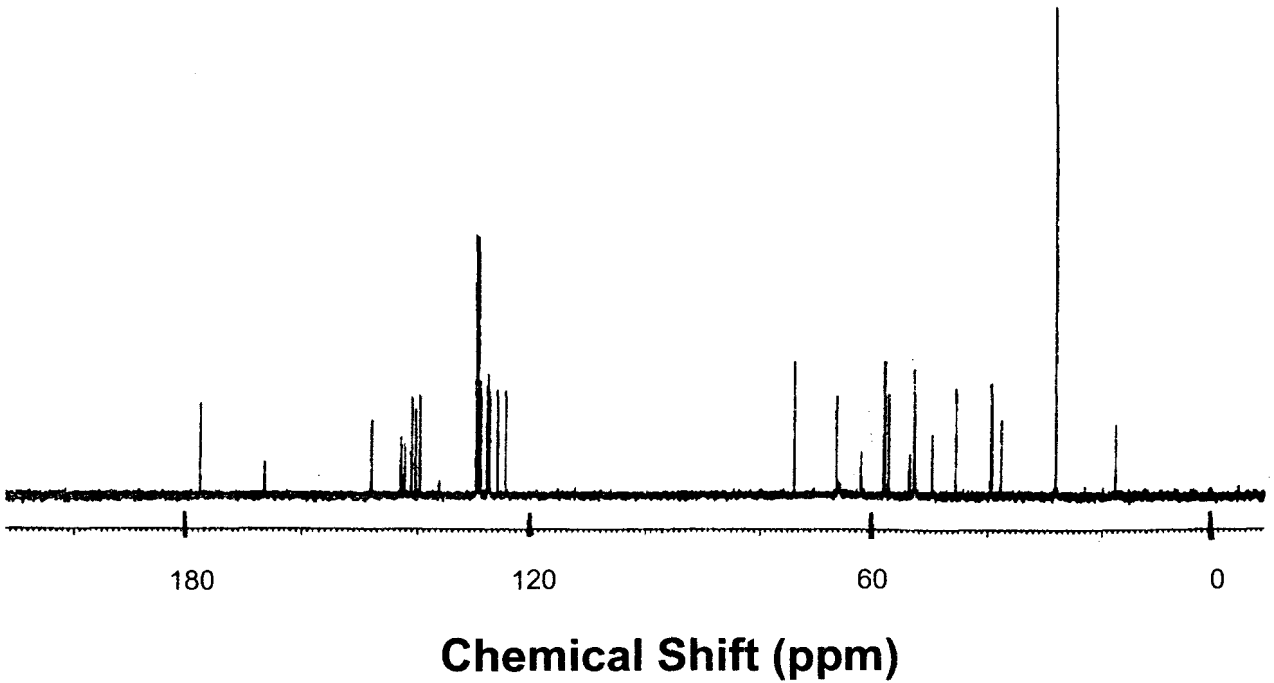


Figure 13. ^{13}C nuclear magnetic resonance spectrum of indinavir sulfate.

Table 8

Indinavir Sulfate ^{13}C-NMR Resonance Peak Assignments

Chemical Shift (ppm)	Assignment[1]	Chemical Shift (ppm)	Assignment[1]
177.8	C_1	65.1 (broad)	C_7
166.6	C_{12}	62.2	C_5
148.0	$C_{4''}$	58.3	$HO\underline{C}H_2CH_3$
143.0	$C_{2''}$	58.1	$C_{1'}$
142.3	$C_{6''}$	57.5	N_9-CH_2
141.2, 140.5	$C_{3'a}$, $C_{7'a}$	53.8	C_8
139.8	$C_{1''}$	53.0	$NH\underline{C}(CH_3)_3$
136.4	$C_{3''}$	52.8 (broad)	C_{11}
129.9	$C_{2''}$, $C_{6''}$	49.8	C_{10}
129.6	$C_{3''}$, $C_{5''}$	45.7	C_2
129.1, 127.8, 126.3, 124.9	$C_{4'}$, $C_{5'}$, $C_{6'}$, $C_{7'}$	39.7	C_{2a}
128.0	$C_{5''}$	39.5	$C_{3'}$
127.7	$C_{4''}$	37.8	C_3
73.8	$C_{2'}$	28.3	$NHC(\underline{C}H_3)_3$
66.5	C_4	17.6	$HOCH_2\underline{C}H_3$

Note: Assignments were grouped where unequivocal assignments could not be made.

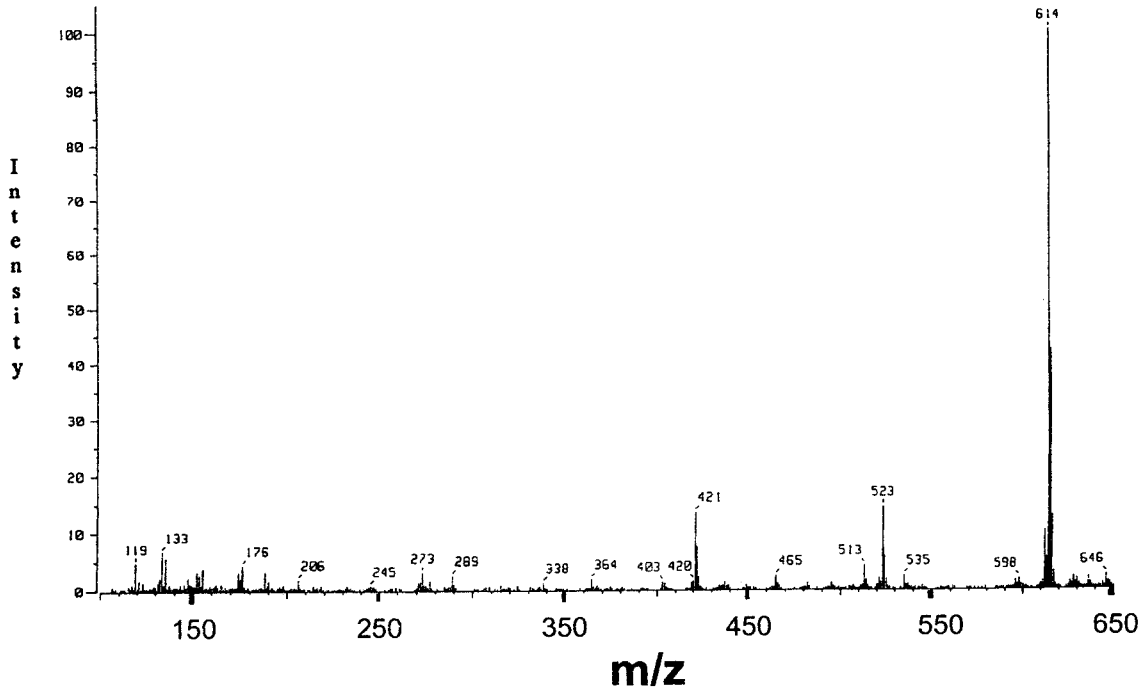


Figure 14. FAB mass spectrum of indinavir sulfate.

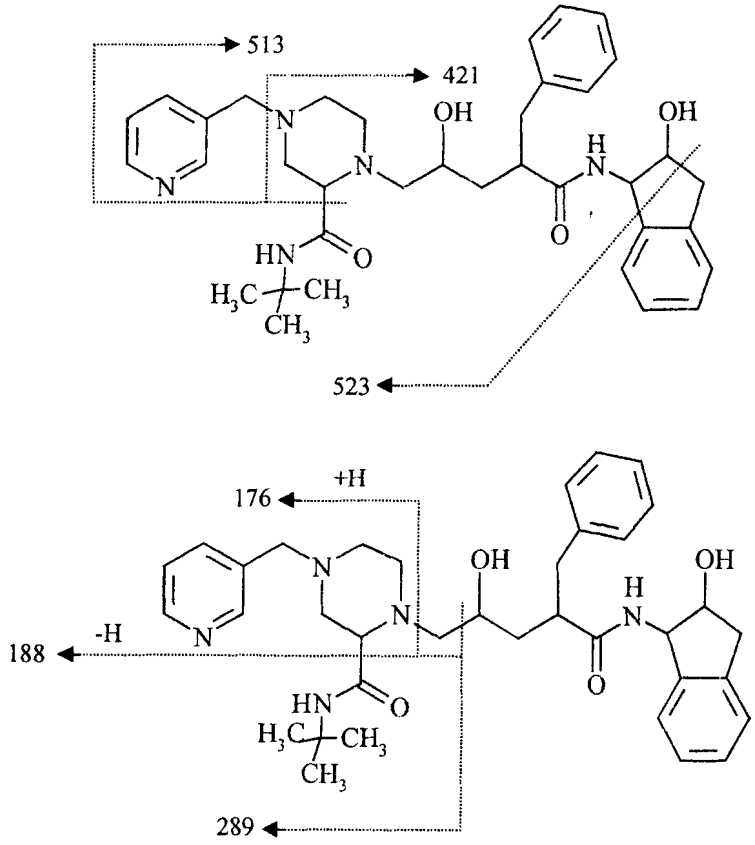


Figure 15. Structurally significant fragment ions found in the FAB mass spectrum of indinavir sulfate.

Table 9
Elemental Analysis of Indinavir Sulfate

	Found (%)	Theoretical (%)
Carbon	60.14	60.21
Hydrogen	7.24	7.26
Nitrogen	9.15	9.24
Sulfur	4.38	4.23

4.3 Titrimetric Analysis

Total sulfate may be determined in a 50:50 water-methanolic formaldehyde solution by titration with standardized 0.1*N* lead perchlorate. Endpoint detection is effected using a combination lead ion-selective electrode, and the level of sulfate is typically 13.8 wt % [14].

4.4 Solvent Analysis

4.4.1 Ethanol

Total ethanol may be determined by gas chromatography using a Stabilwax (polyethylene glycol) column with helium carrier gas under isothermal (35°C) conditions [8]. Analyte detection is performed using with a flame ionization detector [8]. The level of ethanol is typically 6 % w/w.

4.4.2 Water

Total water may be determined by the Karl Fischer method, using pre-titrated methanol as the vessel solvent [8]. The level of water is typically 0.5 % w/w.

4.5 Chromatographic Methods of Analysis

4.5.1 Thin Layer Chromatography

Indinavir sulfate may be analyzed by TLC using E. Merck silica gel 60F-254 high performance thin layer chromatographic plates, and eluted with a mobile phase of 8:1:1 (v/v/v) ethyl acetate: methanol: ammonium hydroxide [8]. Visualization was performed by developing the plate with iodine stain, and viewing under short-wave UV (254 nm) light. When using this method, the Rf of indinavir sulfate is approximately 0.6 [8].

4.5.2 High Performance Liquid Chromatography

A variety of chromatographic systems have been used to chromatograph indinavir sulfate and its metabolites, and the essential points of these have been summarized in Table 10.

5. Stability

5.1 Solid-State Stability

The solid state stability of indinavir sulfate has been evaluated under a variety of storage conditions and containers. For materials stored in open dishes or in double polyethylene liners within fiber containers, changes in crystallinity (*i.e.*, conversion of the crystalline ethanolate to amorphous material or to a hydrate crystal form) have been detected using XRPD or KF methods [7]. Changes in chemical purity (*i.e.*, formation of degradation products) have been detected using GC and HPLC methods [7].

In addition, chemical degradation products form when the drug substance is stored in open dishes or in polyethylene bags [8]. These chemical degradation products include the lactone (Figure 16) and *cis*-aminoindanol (Compound I). Degradation is prevented by storage of the material in a tightly sealed container impermeable to ethanol/moisture transport, under an inert nitrogen atmosphere at a controlled room temperature of 15 to 30°C.

Table 10

High Performance Liquid Chromatographic Systems

Application and Reference	Column Type	Mobile Phase	Detection Mode
Drug Substance Purity [8]	Inertsil ODS-2	Gradient: A = aqueous 10 mM potassium phosphate pH 7.5; B = acetonitrile	UV 220 nm
Drug Substance Purity [8]	Zorbax RX-C8	Gradient: A = aqueous 100 mM potassium phosphate pH 7.2; B = acetonitrile	UV 220 nm
Drug Substance Potency [8]	Zorbax RX-C8	Isocratic: (45:55) A = aqueous 10 mM dibutylammonium phosphate pH 6.5; B = acetonitrile	UV 260 nm
Drug Substance Chiral Assay [15]	Cyclobond: native gamma cyclodextrin	Isocratic: (98:2) A = methylene chloride; B = methanol	UV 260 nm
Drug Substance Chiral Assay [15]	Cyclobond: gamma-cyclodextrin	Isocratic: (98:2) A = methylene chloride; B = methanol	UV 260 nm
Drug Substance Chiral Assay [16]	Chiral-pak AD	Isocratic (SFC): (75:25) A = carbon dioxide; B = methanol	UV 254 nm
Measurement in Capsules [17]	Zorbax RX-C8	Isocratic: (60:40) A = aqueous 20 mM citrate buffer pH 5.0; B= acetonitrile	UV 260 nm

Table 10 (continued)

High Performance Liquid Chromatographic Systems

Application and Reference	Column Type	Mobile Phase	Detection Mode
Measurement in Body Fluids and Tissues [18-21]	Zorbax RX-C8	Isocratic: (76:24) A = aqueous 15 mM phosphoric acid pH 3.2; B = acetonitrile	UV 220 nm
Measurement in Body Fluids and Tissues [22]	BDS Hypersil C8	Isocratic: (60:40) A = aqueous 7 mM ammonium acetate pH 4.9; B = acetonitrile	ion-spray MS
Measurement in Body Fluids and Tissues [23]	Zorbax SB-CN or Inertsil ODS-2	Isocratic: (66:34) or (62:38) A = aqueous 10 mM phosphoric acid pH 7.5; B = acetonitrile	UV 210 nm
Metabolites [24,25]	DuPont Zorbax C8	Gradient: A = aqueous 1 mM ammonium acetate pH 6.1; B = acetonitrile	UV and radiochem
Metabolites [26]	Supelcosil LC-18	Gradient: A = aqueous 10 mM potassium phosphate pH 5.2 and 5 mM hexyltriethylammonium phosphate; B = acetonitrile	radiochem
Metabolites [27]	Symmetry C8	Isocratic: (60:40) A = aqueous 3.3 mM ammonium acetate; B = acetonitrile	APCI-MS-MS
Metabolites [28]	multiple	multiple	UV, radiochem and ion-spray MS

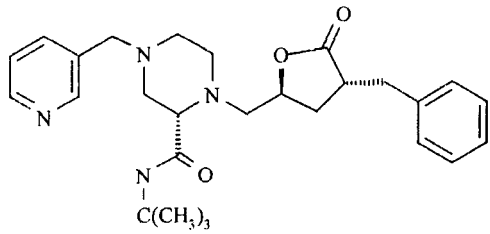


Figure 16. Lactone degradant of indinavir sulfate.

5.2 Solution-Phase Stability

A 80:20 v/v aqueous acetonitrile solution of indinavir sulfate (where the aqueous phase is 2 mM KH_2PO_4 + 8 mM K_2HPO_4, at pH 7.5) stored under ambient room temperature/light is stabile for up to 2 days. After 2 days, chemical degradation was noted by the appearance of the lactone degradation product and another polar degradant [8].

6. Drug Metabolism and Pharmacokinetics

The pharmacokinetic and metabolic profile of indinavir sulfate has been described in detail [19, 20, 24-27, 29].

6.1 Absorption and Distribution

Indinavir was rapidly absorbed in the fasted state, with a time to peak plasma concentration (T_{max}) of 0.8 ± 0.3 hours (mean ± S.D.) (n=11). A greater than dose-proportional increase in indinavir plasma concentrations was observed over the 200-1000 mg dose range. At a dosing regimen of 800 mg every 8 hours, the steady-state area under the plasma concentration time curve (AUC) was 30,691 ± 11,407 nM·hour (n=16), peak plasma concentration (C_{max}) was 12,617 ± 4037 nM (n=16), and plasma concentration eight hours post dose (trough) was 251 ± 178 nM

(n=16) (K). Indinavir was approximately 60% bound to human plasma proteins over a concentration range of 81 nM to 16,300 nM [28].

6.2 Metabolism

Following a 400-mg dose of ^{14}C-indinavir, 83 ± 1% (mean ± S.D.) (n=4) and 19 ± 3% (n=6) of the total radioactivity was recovered in feces and urine, respectively. The radioactivity due to parent drug in feces and urine was 19.1% and 9.4%, respectively. Seven metabolites have been identified, one glucuronide conjugate and six oxidative metabolites. *In vitro* studies indicate that cytochrome P-450 3A4 (CYP344) is the major enzyme responsible for formation of the oxidative metabolites [28].

6.3 Elimination

Less than 20% of indinavir is excreted unchanged in the urine. The mean urinary excretion of unchanged drug was 10.4 ± 4.9% (mean ± S.D.) (n=10) and 12.0 ± 4.9% (n=10) following a single 700-mg and 1000-mg dose, respectively. Indinavir was rapidly eliminated with a half-life of 1.8 ± 0.4 hours (n=10). Significant accumulation was not observed after multiple dosing at 800 mg every 8 hours [28].

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ISOXSUPRINE HYDROCHLORIDE

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ANALYTICAL PROFILES OF DRUG SUBSTANCES AND EXCIPIENTS—VOLUME 26
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Contents

1. Description [1]

1.1 Nomenclature

1.1.1 Chemical Names [2]

4-hydroxy-α-[1-[(1-methyl-2-phenoxyethyl)-amino]ethyl] benzenemethanol

p-hydroxy-α-[1-[(1-methyl-2-phenoxyethyl)-amino]ethyl]benzyl alcohol

p-hydroxy-*N*-(1-methyl-2-phenoxyethyl)norephedrine

1-(*p*-hydroxyphenyl)-2-(1-methyl-2-phenoxyethylamino)-1-propanol

2-(3-phenoxy-2-propylamino)-1-(*p*-hydroxyphenyl)-1-propanol

1.1.2 Nonproprietary Name

Generic: Isoxsuprine hydrochloride

Synonym: phenoxy isopropyl norsuprifen [3]

1.1.3 Proprietary Names

Dilavase, Duvadilan, Duviculine, Isolait, Novilax, Suprilent, Vadosilan, Vasodilan, Vasoplex, Vasotran

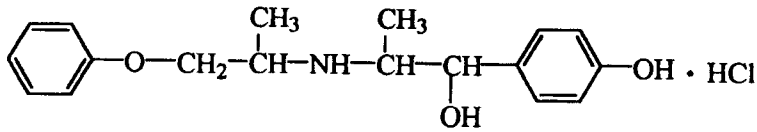
1.2 Formulae

1.2.1 Empirical

Isoxsuprine: $C_{18}H_{23}NO_3$

Isoxsuprine HCl: $C_{18}H_{24}NO_3Cl$

1.2.2 Structural



1.3 Molecular Weight

Isoxsuprine: 301.4

Isoxsuprine HCl: 337.8

1.4 CAS Numbers

Isoxsuprine: 395-28-8.

Isoxsuprine HCl: 579-56-6

1.5 Appearance

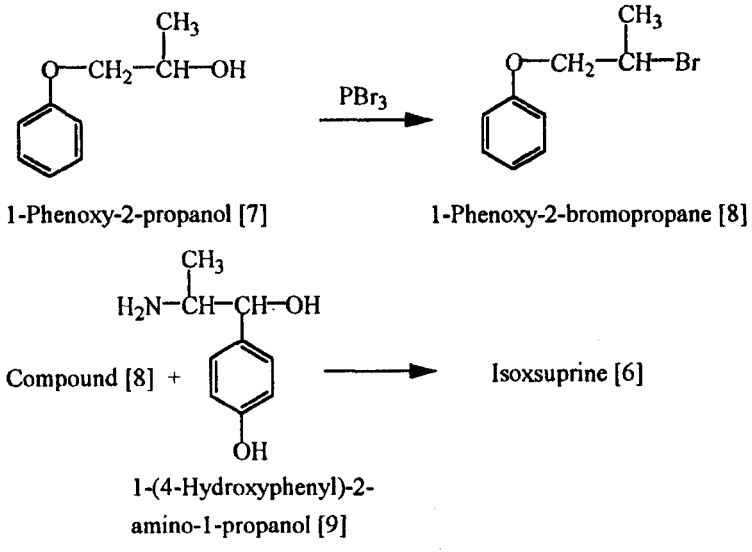
Isoxsuprine hydrochloride is a white crystalline powder, being odorless but with a bitter taste.

1.6 Uses and Applications

Isoxsuprine HCl is a β-agonist with a slight selectivity for the β_2-adrenoreceptors. However, a considerable portion of the vasodilator actions is attributable to a non-selective depression of vascular smooth muscles. It is considered to be "possibly effective" in Raynaud's disease, thromboangilitis daliterans, obliterative arteriosclerosis, and cerebrovascular insufficiency. In the management of premature labor, it has been superseded by more selective β-agonists [1].

2. Methods of Preparation

One method for the preparation of isoxsuprine is given in Scheme 1 [5]. A mixture of 1-(4-hydroxyphenyl)-1,2-propandione **(1)**, 1-methyl-2-phenoxyethylamine **(2)**, and elemental Pt catalyst in ethanol was hydrogenated to give the Schiff base intermediate **(3)**. This was further reduced to afford a racemic mixture of 1-(4-hydroxyphenyl)-2-(1-methyl-2-phenoxyethylamino)propanol **(6)**, m.p. 102.5-103.5°C. Compound **(6)** could also be obtained using an alternate route, where 1-(4-hydroxy-phenyl)-1-hydroxy-2-propanone **(4)** is reacted with compound **(2)**, to yield



Scheme 1. Alternate synthetic method for the manufacture of Isoxsuprine hydrochloride.

the intermediate Schiff base **(5)**. Further reduction of compound **(5)** without isolation yielded the racemate product **(6)**.

Another alternate pathway for the synthesis of racemate product **(6)** is shown in Scheme 2 [6]. 1-phenoxy-2-bromopropane **(8)** was obtained in 38% yield by the bromination of 1-phenoxy-2-propanol **(7)** with PBr_3. Compound **(8)** was refluxed in butanol with 1-(4-hydroxyphenyl)-2-amino-1-propanol **(9)** to yield racemate product **(6)**.

Compound **(6)** contains 3 centers of dissymmetry, and its resolution into separated enantiomers could effected using (±)-mandelic acid [7]. The least soluble diastereomer was found to be the (-)-mandelate salt (m.p. 164.5-164.8°C). Formation of the hydrochloride salts of both enantiomers gave (+)-isoxsuprine•HCl (m.p. 196-196°C) and (-)-isoxsuprine•HCl (m.p. 195-196°C). The two asymmetric centers at C-1 and C-2 were correlated with those of the *erythro* (*p*-OH-C_6H_4-CH(OH)-$CH(CH_3)$-NH-) residue.

3. Physical Properties

3.1 X-Ray Powder Diffraction Pattern

The x-ray powder diffraction pattern of isoxsuprine HCl has been measured using a Philips PW-1710 diffractometer, equipped with a single crystal monochromator and using copper Kα radiation. The pattern thusly obtained is shown in Figure 1, and the table of scattering angles, interplanar d-spacings, and relative intensities are found in Table 1.

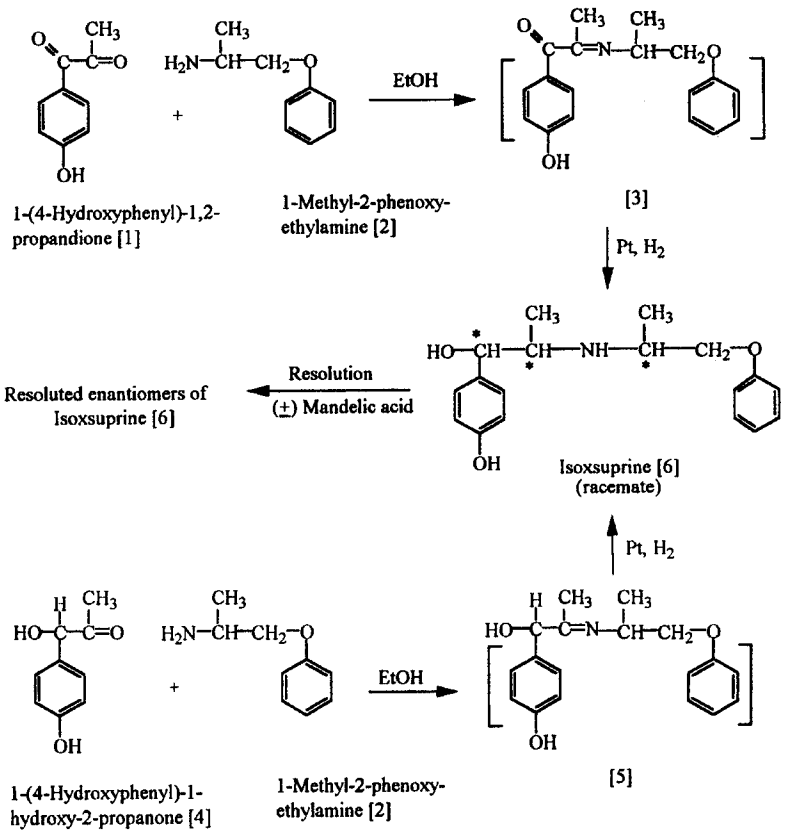
3.2 Thermal Methods of analysis

3.2.1 Melting Behavior

Isoxsuprine HCl exhibits a melting point range of 203-204°C [1], and is found to thermally decompose around 200°C [3].

3.2.2 Differential Scanning Calorimetry

The DSC thermogram of isoxsuprine HCl was obtained using a DuPont TA-9900 thermal analyzer system, interfaced to the DuPont data collection system. The curve shown in Figure 2 was recorded from 100 to 250°C,



Scheme 1. Synthetic method for the manufacture of Isoxsuprine hydrochloride.

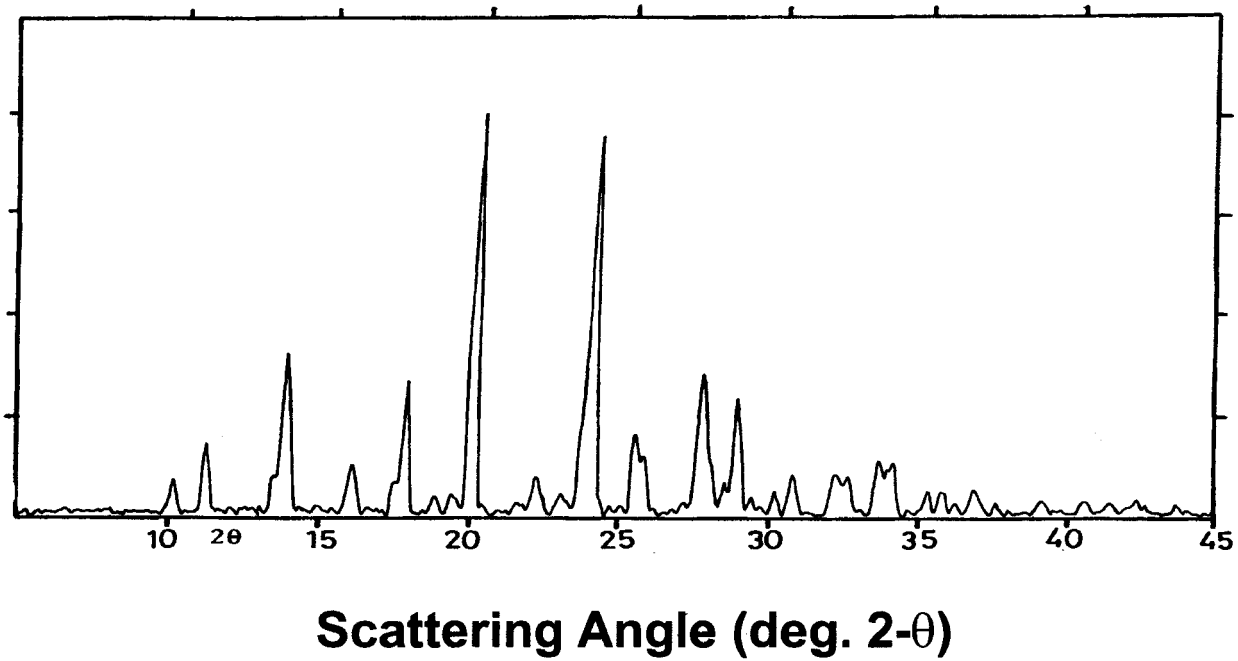


Figure 1. Powder x-ray diffraction pattern of Isoxsuprine hydrochloride.

Table 1

X-ray Powder Diffraction Pattern of Isoxsuprine Hydrochloride

Scattering Angle (degrees 2θ)	d-Spacing (Å)	Relative Intensity (I/Imax * 100)
8.002	11.0126	3.84
9.020	9.7959	15.91
9.233	9.5709	28.65
11.968	7.3885	10.82
13.848	6.3895	13.82
15.395	5.7508	72.03
15.626	6.6664	100.0
16.250	5.4502	23.92
16.737	5.2925	26.2
18.415	4.8139	33.37
21.548	4.1206	15.64
23.266	3.8200	42.38
23.802	3.7352	16.30
24.593	3.6168	24.25
26.077	3.4143	11.59
27.650	3.2235	14.72
27.880	3.1974	11.29
29.807	2.9949	9.75
30.960	2.8860	23.37
31.239	2.8609	15.24
34.736	2.5804	3.33
35.276	2.5422	3.89
36.139	2.4834	3.20
39.037	2.3054	5.36
39.689	2.2691	6.49
41.100	2.1944	2.58
41.793	2.1596	4.10
42.837	2.1093	4.23
43.762	2.0669	2.93

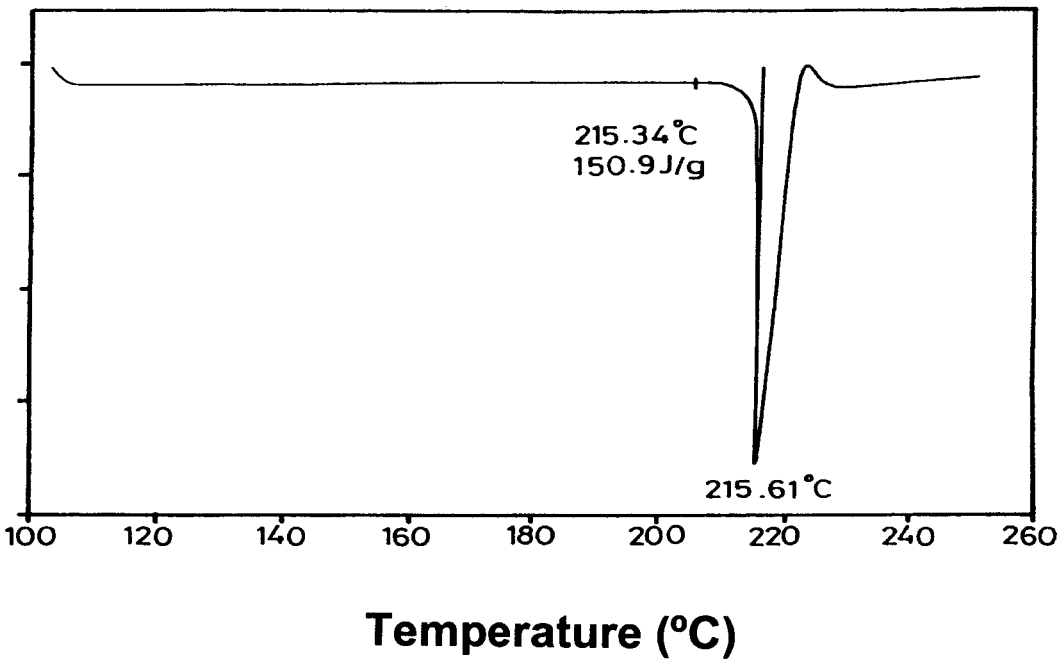


Figure 2. Differential scanning calorimetry thermogram of Isoxsuprine hydrochloride.

using a heating rate of 10°C/minute. It was found that the compound melted at 215.34°C, with an enthalpy of fusion equal to 150.9 J/g.

3.3 Solubility Characteristics

Isoxsuprine HCl is soluble to the extent of 1:500 in water, 1:100 in ethanol and dilute sodium hydroxide solution, and 1:2500 in dilute hydrochloric acid. The compound is practically insoluble in either chloroform or ether [3].

3.4 Ionization Constants

Isoxsuprine HCl has been found to exhibit two acid ionization constants, with pKa values of 8.0 and 9.8 being reported [3].

The pH of a 1% aqueous solution has been reported to be in the range of 4.5 to 6.0 [4].

3.5 Spectroscopy

3.5.1 UV/VIS Spectroscopy

The ultraviolet absorption spectrum of isoxsuprine HCl dissolved in 0.1 N HCl is shown in Figure 3, and was recorded using a Shimadzu UV-VIS model 1601 PC spectrophotometer. The transition characteristic of a hydroxy-substituted phenyl group was found to split into two maxima, with the first being noted at 274.7 nm (A1% @ 1cm = 66, and molar absorptivity 2230 L/mole•cm), and the other at 268.9 nm (A1% @ 1 cm = 67, and molar absorptivity 2265 L/mole•cm). The far more intense singlet→singlet transitions of the phenyl systems are evident in the rapidly rising absorption noted below 240 nm.

3.5.2 Fluorescence Spectroscopy

Isoxsuprine HCl has been found to be naturally fluorescent. The fluorescence spectrum shown in Figure 4 of a 5 μg/mL aqueous solution was recorded on a Perkin-Elmer NPF-44B spectrofluorimeter, using excitation and emission slit widths of 10 and 6 mm, respectively. The

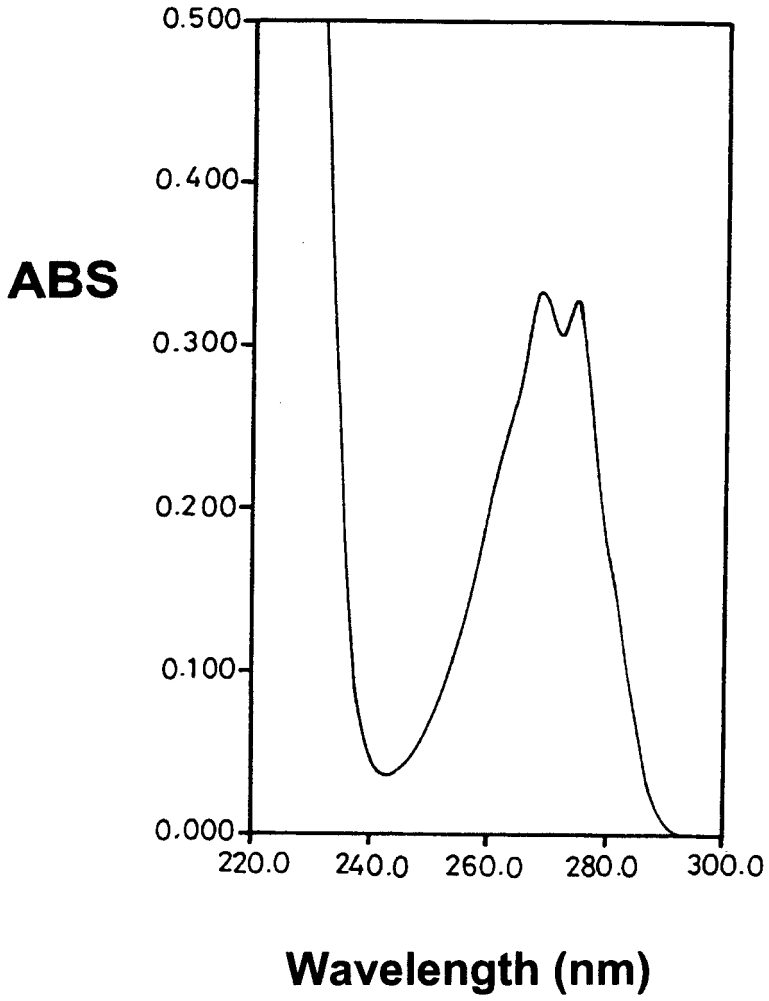


Figure 3. Ultraviolet absorption spectrum of Isoxsuprine hydrochloride in 0.1 N HCl.

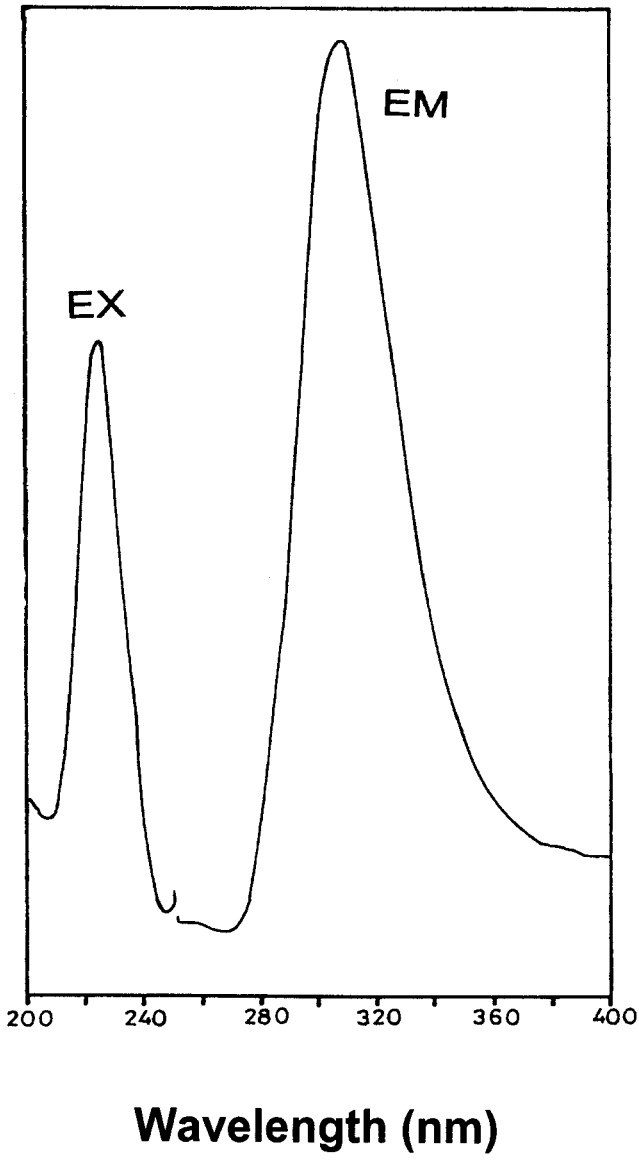


Figure 4. Fluorescence excitation (EX) and emission (EM) spectra of Isoxsuprine hydrochloride.

excitation maximum was found to be 227 nm, which most likely represents excitation into the singlet→singlet transitions of the hydroxy-substituted phenyl group. The fluorescence peak exhibited a maximum wavelength of 306 nm, again characteristic of a hydroxy-substituted phenyl group.

3.5.3 Vibrational Spectroscopy

The infrared absorption spectrum of isoxsuprine HCl is shown in Figure 5, and was obtained in a KBr pellet using a Perkin Elmer infrared spectrophotometer. The principal peaks were noted at 745, 1043, 1220, 1240, 1500, 1514, and 1601 cm^{-1}.

3.5.4 Nuclear Magnetic Resonance Spectrometry

The ^{1}H-NMR and the ^{13}C-NMR spectra of isoxsuprine HCl were obtained using a Bruker Instruments system operating at 300, 400, or 500 MHz (proton NMR), or at 75, 100, or 125 MHz (carbon NMR). Standard Bruker software was used to obtain DEPT, COSY, and BETCOR spectra. All measurements were obtained with the compound being dissolved in deuterated methanol, using tetramethylsilane as the internal standard.

3.5.4.1 ^{1}H-NMR Spectrum

The entire ^{1}H-NMR spectrum of isoxsuprine is shown in Figure 6, with various regions being expanded in Figures 7-9 for easier viewing. Assignments for the resonance bands are given in Table 2. Since the spectral data were obtained in MeOH-d_4, none of the exchangeable protons were detectable.

Figure 7 shows two doublet absorptions at chemical shifts of 1.13 and 1.53 ppm (J = 6.8 Hz), which could be correlated to the two methyl groups at positions 3' and 3.

Based on chemical shift assignments and coupling constants, the two methine functions at positions 2 and 2' could be correlated to the two multiplets at chemical shifts of 3.61 - 3.65 and 3.94 - 3.98 ppm,

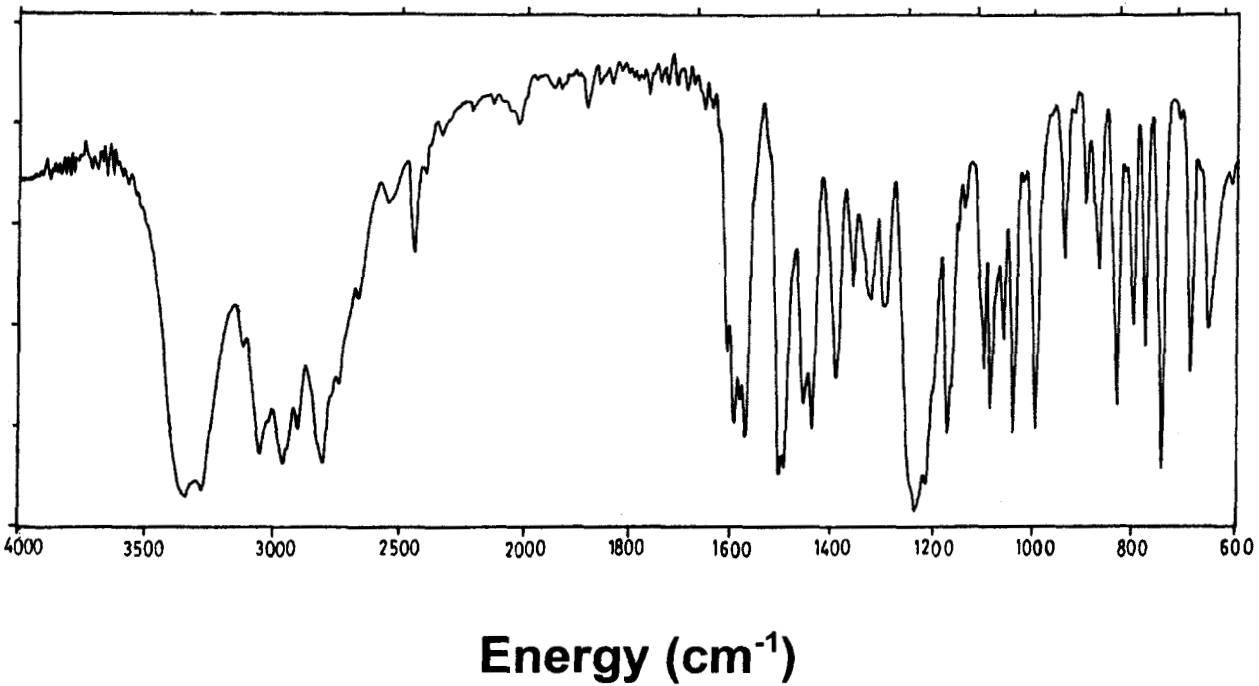


Figure 5. Infrared absorption spectrum of Isoxsuprine hydrochloride.

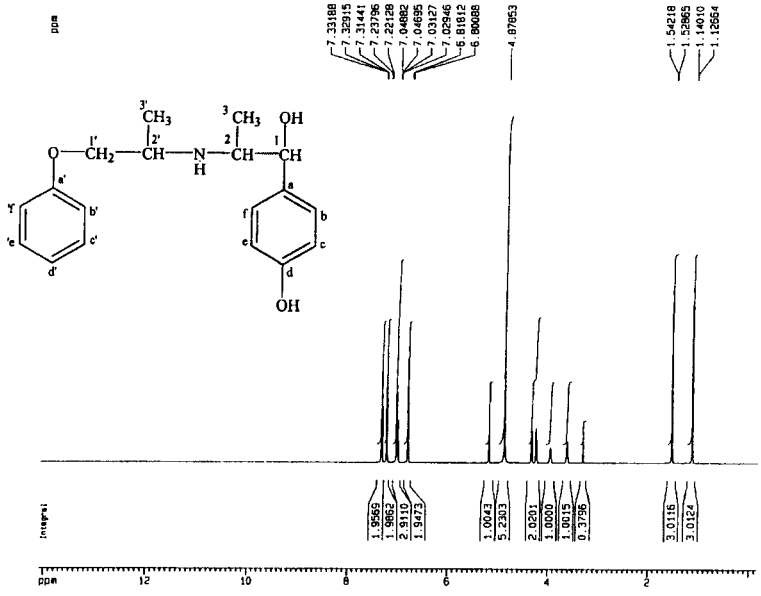


Figure 6. Full ^{1}H-NMR spectrum of Isoxsuprine hydrochloride.

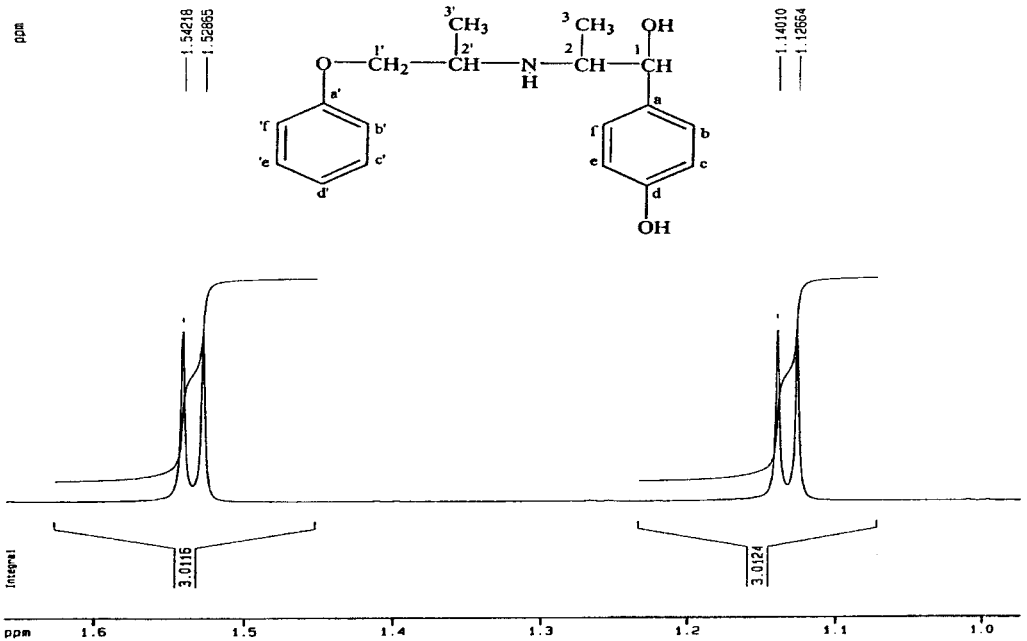


Figure 7. Expanded ^{1}H-NMR spectrum of Isoxsuprine hydrochloride.

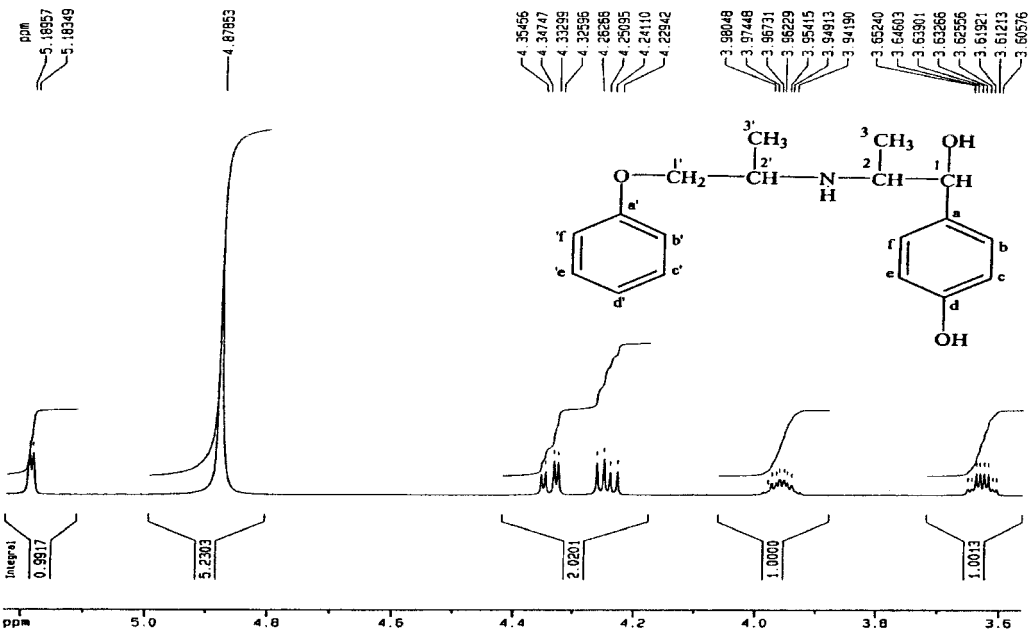


Figure 8. Expanded ^{1}H-NMR spectrum of Isoxsuprine hydrochloride.

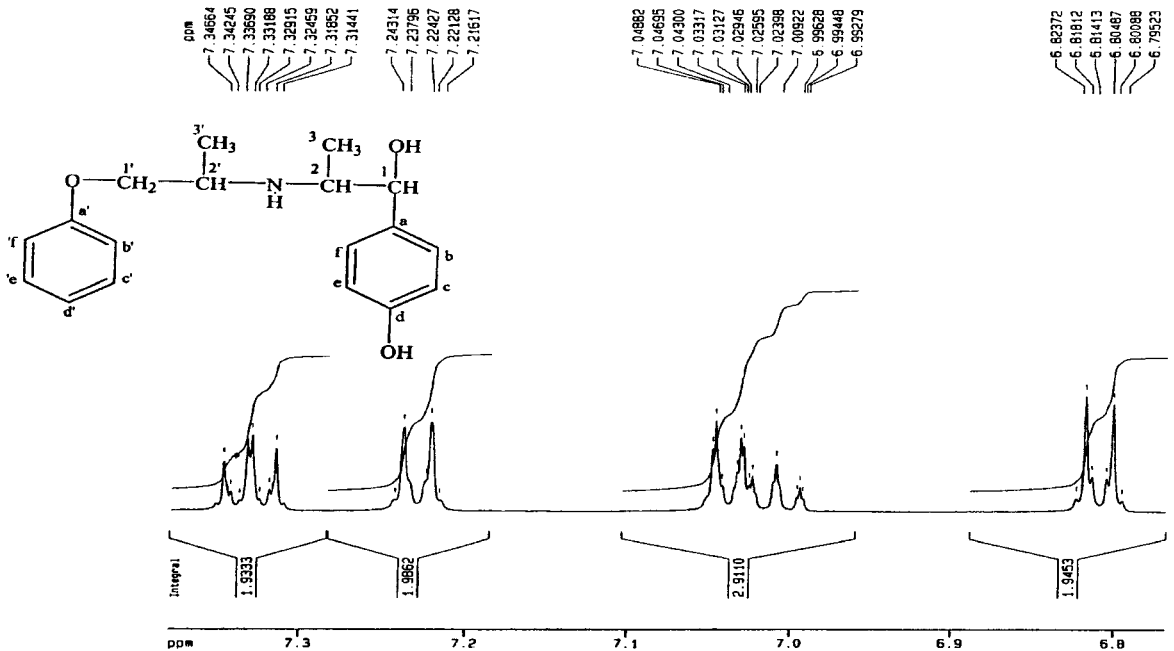


Figure 9. Expanded ^{1}H-NMR spectrum of Isoxsuprine hydrochloride.

Table 2

Assignments for the Resonance Bands Observed in the ^{1}H-NMR spectrum of Isoxsuprine Hydrochloride

Chemical Shift (ppm)[a]	Number of Protons	Multiplicity[b] and Coupling Constant (J)	Assignment
1.13	3	d (6.8 Hz)	CH_3 (3 or 3')
1.53	3	d (6.8 Hz)	CH_3 (3' or 3)
3.61 – 3.65	1	m (3.0, 6.8 Hz)	CH at position 2-
3.94 – 3.98	1	m (3.5, 4.0, 6.0, 6.8 Hz)	CH at position 2'-
4.23 – 4.35	2	ddd (3.5, 6.0, 11.0 Hz)	CH_2 at position 1'
5.18	1	d (3.0 Hz)	CH at position 1
6.8	2	d (10.0 Hz)	b and f ArH[c]
6.99 – 7.05	3	m	Phenoxy ArH[c]
7.24	2	d (10.0 Hz)	e and c ArH[c]
7.31 – 7.35	2	m	Phenoxy ArH[c]

Notes:

a. Chemical shifts are measured relative to tetramethylsilane

b. d = doublet, ddd = doublet of doublet of doublets, m = multiplet

c. ArH signifies the aromatic protons

respectively (see Figure 8). Both multiplets contain a J value of 6.8 Hz, proving their coupling with methyl groups at positions 3 and 3', respectively. The resonance bands in the chemical shift range of 4.23 - 4.35 ppm (also shown in Figure 8) exist as doublet of doublet of doublets correlated to the methylene group at position 1', which contains J values of 3.5 and 6.0 Hz. This suggests its coupling with the neighboring 2'-methine containing the same coupling values. The doublet at a chemical shift of 5.18 ppm (Figure 8) is assigned to the benzylic methine at position 1 (coupling constant equal to 3.0 Hz), and the multiplet at 3.61 - 3.65 ppm previously assigned to CH at position 2- confirms this assignment.

The aromatic portion of the ^{1}H-NMR spectrum is shown in Figure 9, and shows a doublet of doublets at 6.80 and 7.24 ppm (J = 10.0 Hz) representing the 1,4-disubstituted aromatic pattern of the phenol residue. The two multiplets at 6.99 -7.05 and at 7.31 - 7.35 ppm integrated for 3 and 2 protons, respectively, and are correlated to the phenoxy moiety.

3.5.4.2 ^{13}C-NMR Spectrum

The ^{13}C-NMR spectrum of isoxsuprine HCl is shown in Figure 10. Singlet absorption resonance bands were displayed at chemical shifts of 11.20 (CH_3), 15.30 (CH_3), 52.87, 59.09, 69.69, 17.51, 116.17, 116.70, 123.33, 128.58, 131.21, 132.57, 158.74, and 159.70 ppm. This finding is consistent with the 18 carbon content of isoxsuprine.

3.5.5 Mass Spectrometry

The mass spectrum of isoxsuprine HCl was obtained utilizing a Shimadzu PQ-5000 mass spectrometer, where the parent ion was collided with helium as the carrier gas. Figure 6 shows the detailed mass fragmentation pattern, where a base peak appeared at m/z = 178 along with a molecular ion peak at m/z = 301. The mass fragmentation pattern of the compound is detailed in Table 3.

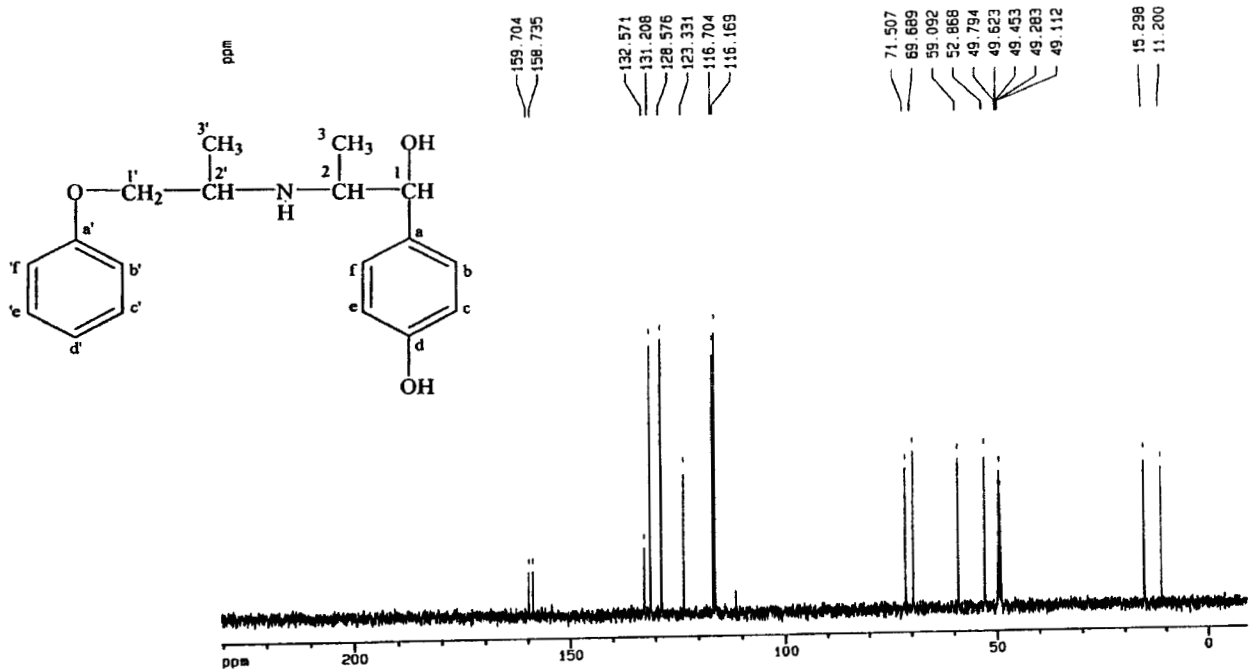


Figure 10. ^{13}C-NMR spectrum of Isoxsuprine hydrochloride.

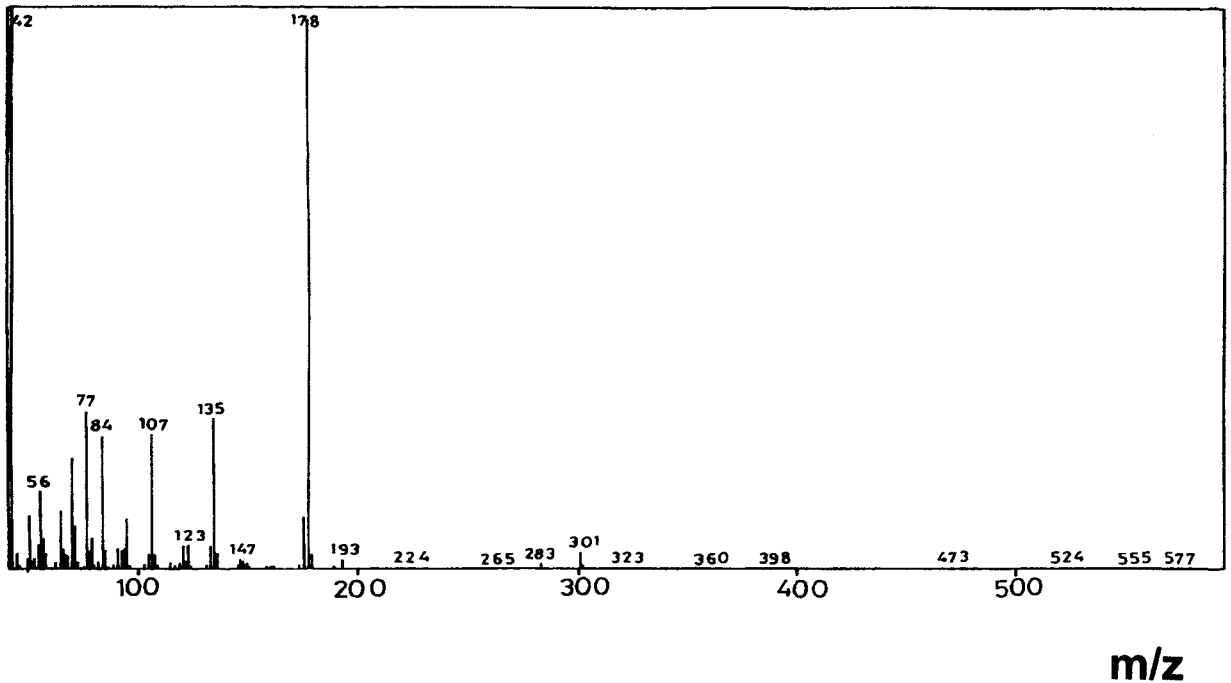
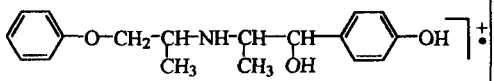
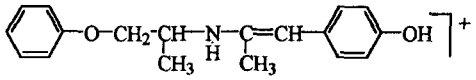
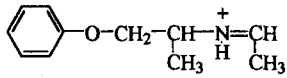
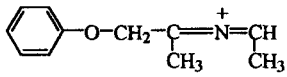
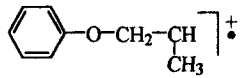
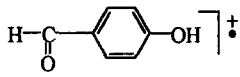
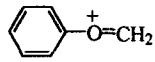
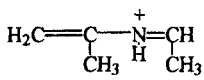
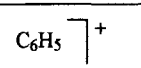
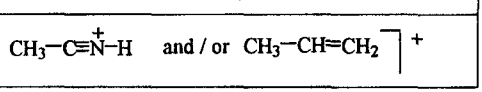


Figure 11. Mass spectrum of Isoxsuprine hydrochloride.

Table 3

Mass Fragmentation Pattern of Isoxsuprine Hydrochloride

M/z	Relative intensity	Fragment
301	5%	
283	2%	
178	100%	
176	10%	
135	33%	
122	8%	
107	28%	
84	29%	
77	28%	
42	95%	

4. Methods of Analysis

4.1 Compendial Tests

4.1.1 Bulk Drug Substance

The United States Pharmacopoeia describes a spectrophotometric method for the bulk drug substance [4]. The method is based on measuring the difference in absorbance at 269 nm and 300 nm for the sample and a reference standard solution:

$$C_{UNK} = C_{STD} * \{ (A_{UNK(260)} - A_{UNK(300)}) / (A_{STD(260)} - A_{STD(300)}) \}$$

where $A_{UNK(260)}$ and $A_{UNK(300)}$ are the absorbencies of the analyte solution at 269 and 300 nm, $A_{STD(260)}$ and $A_{STD(300)}$) are the absorbencies of the reference standard solution at 269 and 300 nm, C_{STD} is the concentration of the standard solution in units of μg/mL, and C_{UNK} is the concentration of the analyte solution in the same units.

The British Pharmacopoeia on the other hand, recommends the use of a non-aqueous titration method for the bulk drug substance [12]. The method calls for the titration of isoxsuprine HCl against 0. 1 M perchloric acid (after dissolving the solid in acetic anhydride), and using 1-naphtho-benzein solution as the indicator.

4.1.2 Dosage Forms

The USP recommends a column partition chromatographic method for the assay of tablets and ampoules. The analyte is separated using a chromatographic separation, and the isoxsuprine concentration determined by spectrophotometry.

The BP calls for the use of a spectrophotometric method for the assay of tablets and injections. The method is based on measuring the difference in absorbance at 274 and 300 nm, taking 73 as the value of ΔA (1% @ 1 cm).

4.2 Identification

4.2.1 Mandelin's Test

When reacted with ammonium vanadate / H_2SO_4 reagent, isoxsuprine yields a green color [3].

4.2.2 Marquis Test

When reacted with 1:9 formaldehyde / H_2SO_4 reagent, isoxsuprine yields a red-violet color [3].

4.2.3 Copper Sulfate Test

To a 1% solution of isoxsuprine HCl, add 0.5 mL of copper sulfate solution and 1 mL of 5 M NaOH solution. A positive reaction is indicated by the formation of a blue color. Add 1 mL of ether and shake, and the ether layer should remain colorless [12].

4.2.4 Sodium Nitrite Test

To 1 mL of 1% solution of isoxsuprine HCl, add 3 mL of a 1:15 solution of sodium nitrite in 2 M H_2SO_4. Add NH_4OH dropwise, whereupon a yellow precipitate is formed which will dissolve upon addition of 1:5 NaOH solution [4].

4.2.5 Phosphomolybdic Acid Test

To 1 mL of a 1% solution of isoxsuprine HCl, add 1 mL of 1:100 phosphomolybdic acid solution. A positive reaction is indicated by the formation of a pale yellow to white precipitate [4].

4.3 Elemental Analysis

The theoretical elemental composition of isoxsuprine HCl is as follows [2]:

carbon	71.73 %
hydrogen	7.69 %
nitrogen	4.65 %
oxygen	15.93 %

4.4 Spectrophotometric Methods of Analysis

Being a phenolic compound, isoxsuprine has been determined by a diversity of colorimetric methods. A colorimetric method was described for its determination in dosage forms, based on treatment with the nitrating mixture composed of 1:1 sulfuric and nitric acids [13]. This mixture is heated at 100°C for 20 minutes, followed by the addition of acetone and NaOH. The yellow color produced is quantitated on the basis of its absorbance at 384 nm.

An ion-pairing spectrophotometric method has also been described [14]. This method involves the use of bromphenol blue, bromcresol purple, bromcresol green, bromthymol blue, or methyl orange, and extracting the ion-pair species into chloroform. The absorbance of the yellow product is determined at 420 nm.

Numerous other chromogenic agents have been used for the spectrophotometric determination of isoxsuprine in dosage forms. These include diazotized sulfanilic acid at 440 nm [15], diazotized benzocaine at 460 nm [16], diazotized *p*-nitroanilline at 480 nm [17], Folin-Ciocalteu reagent at 650 nm [18], phosphomolybdic acid at 660 nm [19]), Fe(III) / 2,4,6-tris(2-pyridyl)-*s*-triazene [20], 2,6-dichloroquinone-4-chlorimide at 610 nm [21], sodium cobaltinitrite at 375 nm [22], and 3-methylbenzo-thiazolin-2-one hydrazone at 510 nm [23]. Other methods use copper chelation at 525 nm [24], *N,N*-dimethyl-*p*-phenylenediamine at 510 nm [25], *p*-aminophenol at 635 nm [26], metanile yellow at 407 nm [27], fast green at 630 nm or orange II at 495 nm [28], 4-aminophenazone / potassium ferricyanide at 502 nm [29], neutral red at 517 nm [30], and 2,2-dipyridine / ferric chloride at 520 nm [31].

4.5 Chromatographic Methods of Analysis

4.5.1 Gas Chromatography

A gas chromatographic method was described for the determination of isoxsuprine after its extraction from plasma [32]. The method is based on the reaction of the analyte with trifluoroacetic anhydride to form the trifluoroacetyl derivative. The GLC analysis was carried out a 3% OV-17

column using an electron capture detector. The working range was reported to be 0.5-20 ng/mL, with a limit of detection equal to 0.5 ng/mL.

A capillary GC method was also described for the determination of isoxsuprine and its metabolites in the urine of horses receiving a single dose of the drug [33].

4.5.2 High Performance Liquid Chromatography

A stability-indicating HPLC method has been described for isoxsuprine [11]. The mobile phase consisted of methanol / pH 7 phosphate buffer, and detection was effected on the basis of the absorbance at 276 nm.

Another reversed phase HPLC method reported the use of a Spherisorb column to effect the analytical separation [34]. The mobile phase consisted of methanol, to which perchloric acid was added at a level of 100 μL/L. The k value was reported to be 0.8.

A HPLC method that used electrochemical detection was developed for the determination of isoxsuprine in the plasma of horses after intravenous administration [35].

4.5.3 Thin Layer Chromatography

Isoxsuprine was quantitatively determined after separation on HF-254 silica gel plates using a mobile phase consisting of 60:37:3 chloroform: methanol: ammonia [30]. Quantitation of the analyte was carried out at 223 nm.

Several TLC methods have been described for the identification of isoxsuprine [36]. In all systems, silica gel G, dipped in sodium hydroxide solution or methanol, was used as the stationary phase. Either 100:1.5 methanol: strong ammonia, 75:15:10 cyclohexane: toluene: diethylamine, or 90:10 chloroform: methanol was successfully used as the mobile phase. In all systems, the spray reagent used to develop the places was acidified potassium permanganate solution.

4.5.4 Flow Injection Analysis

An automated flow injection spectrophotometric determination of isoxsuprine based on its oxidative condensation with 1-nitroso-2-naphthol has been described [37]. Oxidation was effected with either Ce(IV) or Pb(IV), and the resulting absorbance monitored at either 540 nm or 510 nm, respectively.

A kinetic flow injection analytical method was also described for isoxsuprine, which was based on the use of a fluoride-selective electrode for detection after reaction with 1-fluoro-2,4-dinitrobenzene [38].

5. Stability

Sevgi and Guneri used thin-layer chromatography to study the stability of isoxsuprine HCl in aqueous media and in marketed ampoules [8]. The solutions and ampoules were heated at 40, 60, and 80°C, with the TLC spots being visualized under UV light at 250 nm. Quantitation was performed using the fluorescence observed at 272 nm. The authors found that under these conditions, the compound was stable in aqueous solutions for 323 days and in ampoules for 389 days.

Sevgi and Guneri then studied the stability of aqueous solutions between pH 2.29 and 5.49 under daylight, in colored and colorless bottles, and in bottles protected from daylight for periods up to 150 days [9]. They found out that the degradation in solutions of different pH values stored in daylight obeyed first-order kinetics. The authors concluded that isoxsuprine is much more stable in solutions having pH = 3.27, and that protection from light is necessary.

In a further study, Sevgi and Guneri investigated the thermal degradation of isoxsuprine at 80°C [10]. It this study, they learned that degradation at this temperature follows first-order kinetics, and suggested that oxidative deterioration was a possible degradation mechanism. *p*-hydroxy-benzaldehyde and phenoxy-propylamine were identified as two degradation products.

Volpe *et al.* studied the photothermal degradation of isoxsuprine on TLC plates irradiated with intense UV light (the heat also emitted from the UV

lamps contributed to the photoreaction) [11]. The major degradation products were found to depend in type and amount on the exposure time to the UV lamp. In this work, the authors identified *p*-hydroxy-benzoic acid, *p*-hydroxy-benzaldehyde, *p*-hydroxy-benzyl alcohol, *p*-hydroxy-acetophenone, *p*-hydroxy-ephedrine, and 1-methyl-3-phenylpropylamine as degradation products.

6. Drug Metabolism and Pharmacokinetics

6.1 Pharmacological Action

Isoxsuprine is a vasodilator that also stimulates β-adrenergic receptors. It causes relaxation of vascular and uterine smooth muscles, and its vasodilating action is greater on the arteries supplying skeletal muscles than on those supplying skin. The drug also produces positive inotropic and chronotropic effects [39].

6.2 Pharmaceutical Applications

Isoxsuprine has been used to arrest premature labor, where it is given in a loading dose of 0.2-1 mg/min for 10 minutes and then reduced to 0.1-0.3 mg/min [40, 41]. It has been also used in the treatment of cerebral and peripheral vascular diseases [42], intermittent claudication [43], and obliterative arterial diseases [44].

The usual oral dose for adults is 10-20 mg, administered 3 or 4 times daily. The intramuscular dose for adults is 5-10 mg, administered 2 or 3 times daily [1]. Isoxsuprine may cause transient flashing, hypotension, tachycardia, rashes, and gastrointestinal disorders [39]. It has also been reported to induce pulmonary edema [46].

In veterinary medicine, isoxsuprine is used to treat navicular disease, which is a debilitating disease affecting the lower limbs of horses [45].

6.3 ADME Profile

Isoxsuprine is well absorbed from the gastrointestinal tract, with the peak plasma concentration occurring about 1 hour after oral administration. A

plasma half-life of 1.5 hours has been reported, and isoxsuprine is excreted in the urine mainly as glucouronoide conjugates [39].

7. Acknowledgement

The authors would like to thank Mr. Tanvir A. Butt, Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, for typing this manuscript.

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PROCAINE HYDROCHLORIDE

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Names [9,10]

4-aminobenzoic acid, 2(diethylamino)ethyl ester

p-aminobenzoyldiethylaminoethanol

2-diethylaminoethyl- *p*-aminobenzoate

benzoic acid, 4-amino-2-(diethylamino)ethyl ester

2-diethylaminoethyl-4-aminobenzoate

1.1.2 Nonproprietary Names [9]

Procaine borate: Borocaine

Procaine hydrochloride: Novocain, Ethocaine

1.1.3 Proprietary Names [9]

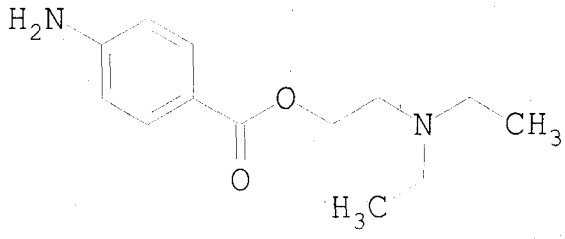
Allocaine, Alocaine, Aminocaine, Anesthesol, Anestil, Atoxicocaine, Bemacaine, Cetain, Chlorocaine, Enpro, Ethocaine, Eugerase, Gero H3 Aslan, Gero, Irocaine, Isocaine-Asid, Isocaine-Heisler, Jenacain, Juvocaine, Kerocaine, Medaject, Naucaine, Neocaine, Novocain, Omnicaine, Paracain, Planocaine, Resocaina, Rocain, Scurocaine, Sevicaine, Syncaine, Topokain, Westocaine

1.2 Formulae

1.2.1 Empirical and Molecular Weight; CAS Numbers

Procaine	$C_{13}H_{20}N_2O_2$	236.31	[59-46-1]
Procaine borate	$C_{13}H_{25}B_5N_2O_{12}$	455.40	[149-13-3]
Procaine butyrate	$C_{17}H_{28}N_2O_4$	324.42	[136-55-0]
Procaine hydrochloride	$C_{13}H_{21}N_2O_2Cl$	272.77	[51-05-8]
Procaine nitrate	$C_{13}H_{21}N_3O_5$	299.33	[6192-92-3]

1.2.2 Structure (Procaine base)



4.2 Elemental Analysis

The elemental composition of procaine and various salts is as follows:

	Carbon	Hydrogen	Nitrogen	Oxygen	Other
Procaine	66.07%	8.53%	11.86%	13.54%	
Procaine borate	34.29%	5.53%	6.15%	42.16%	boron: 11.87%
Procaine butyrate	62.94%	8.70%	8.63%	19.73%	
Procaine HCl	57.24%	7.76%	10.27%	11.73%	chlorine: 13.00%
Procaine nitrate	52.16%	7.07%	14.04%	26.73%	

1.3 Appearance [9]

Procaine	Hygroscopic, anhydrous plates
Procaine borate	Small, monoclinic, tabular crystals
Procaine hydrochloride	Six-sided plates, monoclinic or triclinic habit, benumbing taste

1.5 Uses and Applications

Procaine is a derivative of *p*-aminobenzoic acid, and is a one of the oldest used ester-type local anaesthetic agents [1]. The compound was originally developed by Einhom [2,3], and later with and Uhlfelder [4]. This anti-arrhythmic drug itself has a short half-life, but is able to form salts with other drugs which causes an increase in the duration of action [5].

Procaine is used for a wide variety of clinical nerve blocks, and is devoid of the severe local and systemic toxicity of cocaine [6]. Adrenaline constricts blood vessels in the vicinity of the procaine injection, thus preventing procaine from being washed away into the blood supply and prolonging its duration of action [7]. The drug remains the prototype molecule with which subsequent analogues (having the suffix *-caines*) are compared. Procaine exhibits considerably lower degrees of toxicity and irritation, is well-tolerated, and is characterized by superior stability in the solution phase [8].

Procaine is ineffective when administered through surface application, and is used only by injection. The onset of action for the drug is 2 to 5 minutes, and its duration of action is short. Vasoconstrictors are usually co-administered with this vasodilator drug to delay its absorption and to increase the duration of action. The drug is used for infiltration anesthesia, peripheral nerve block, and spinal anesthesia.

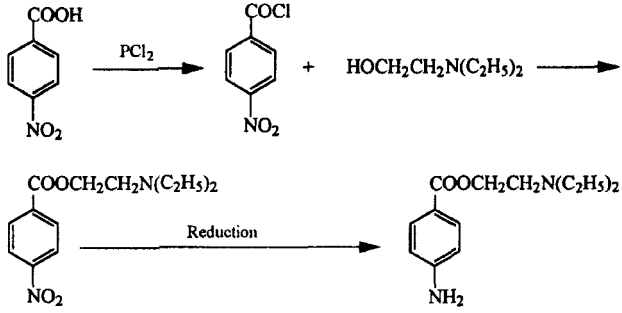
Procaine forms poorly soluble salts or conjugates with some drugs [12], and was reported to be a strong prostaglandin antagonist and a weak agonist [13]. It is used in the treatment of malignant hyperpyrexia [14-17]. Procaine hydrochloride was also used by intravenous injection for the relief of pain in acute pancreatitis [18-21].

2. Methods of Preparation

Procaine can be prepared by one of three main routes, the basic pathways of which are illustrated in the three Schemes which follow [1-4,11].

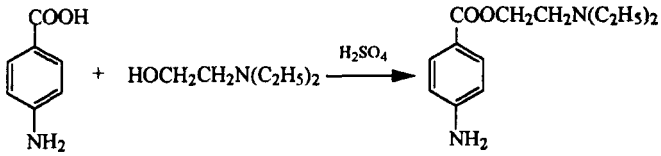
Scheme 1:

In one method, *p*-nitrobenzoic acid is converted to the corresponding *p*-nitrobenzoyl chloride, which is allowed to interact with 2-dimethylaminoethanol. The product of this reaction is 2-diethylaminoethyl-*p*-nitrobenzoate, which is reduced to yield procaine.



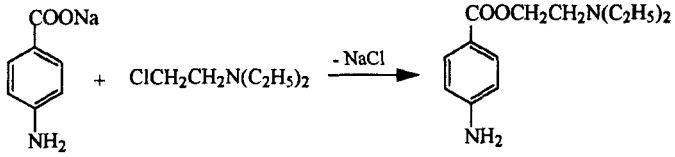
Scheme 2:

Another method entails the esterification of *p*-aminobenzoic acid with 2-diethylaminoethanol using concentrated sulfuric acid as a catalyst.



Scheme 3:

A third method of synthesis employs alkylation of the sodium salt of *p*-aminobenzoic acid by 2-chlorotriethylamine.



3. Physical Properties

3.1 X-Ray Powder Diffraction

The x-ray powder diffraction pattern of procaine hydrochloride is presented in Figure 1, and was determined using a Philips fully automated x-ray diffraction system equipped with a PW 1730/10 generator. The source was a copper target (Cu anode 2000 w, $\lambda = 1.548$ Å) within a high intensity x-ray tube operated at 40 kV and 35 mA. The monochromator was a curved single crystal (PW 1752), with the divergence and receiving slits being set at 1° and 0.1°, respectively. The scanning speed of the goniometer (PW 1050/81) was 0.2 degrees-2θ per second, and the goniometer was aligned using elemental silicon before use. A summary of scattering angles, interplanar d-spacings, and relative peak intensities are shown in Table 1.

Owen *et al.* reported x-ray powder diffraction data for procaine and 16 other anesthetics, as obtained using the Debye-Scherrer technique [55]. The data for the drugs was tabulated in the usual terms of lattice spacing (d-spacing) and relative intensities. In addition, a classification scheme was provided which was based on the 3 most intense scattering lines of each drug, with their relative intensities arranged in descending numerical order with regard to the d-spacing of the most intense line from each pattern.

3.2 Thermal Methods of analysis

3.2.1 Melting Behavior

The following melting points have been reported for procaine and its salts [9]:

Procaine:	61°C
Procaine borate:	165 - 166°C
Procaine hydrochloride:	153 - 156°C

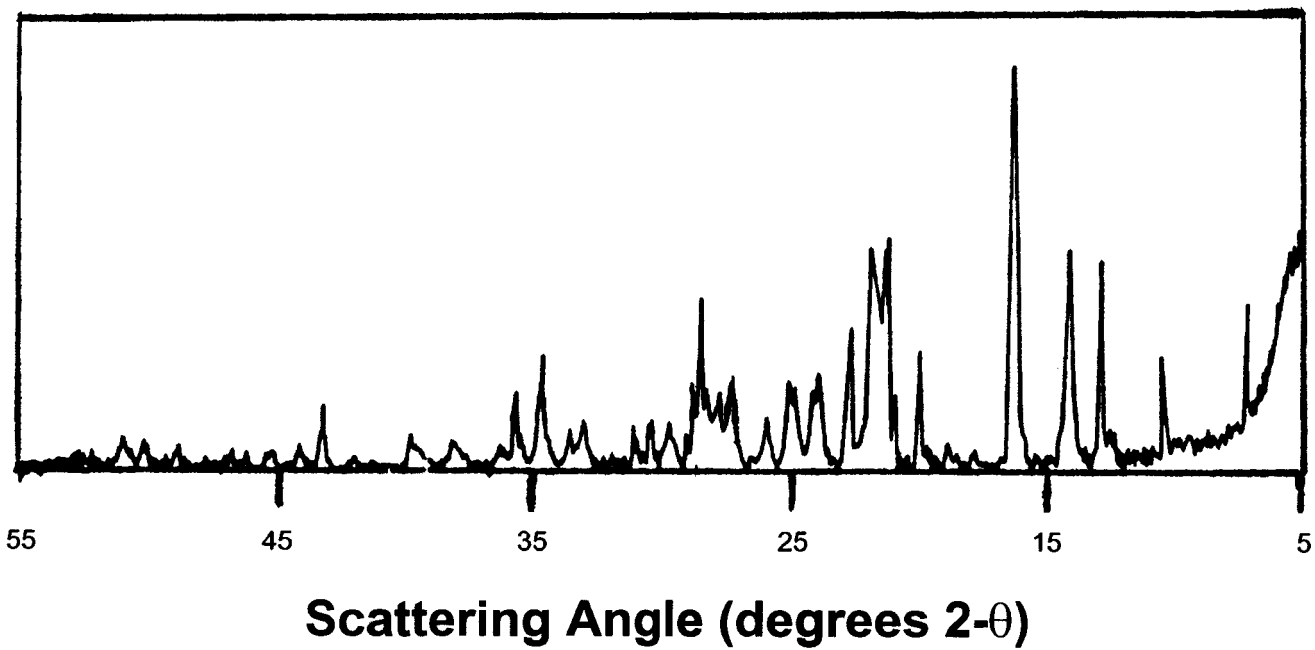


Figure 1. X-ray powder diffraction pattern of procaine.

Table 1

X-ray Powder Diffraction Pattern of Procaine Hydrochloride

Scattering Angle (degrees 2θ)	d-Spacing (Å)	Relative Intensity (I/Imax * 100)
7.06	12.53	27.93
12.86	6.88	38.58
14.71	6.25	96.37
16.36	5.42	92.97
17.92	4.95	6.80
18.93	4.69	7.68
20.06	4.43	25.74
21.44	4.15	50.09
21.92	4.05	100.00
22.77	3.91	30.62
24.10	3.39	23.65
25.06	3.55	30.84
26.03	3.42	10.07
27.38	3.26	19.65
28.04	3.18	40.99
28.64	3.12	31.61
30.64	2.92	10.37
32.10	2.78	3.56

Table 1 (continued)

X-ray Powder Diffraction Pattern of Procaine Hydrochloride

Scattering Angle (degrees 2θ)	d-Spacing (Å)	Relative Intensity (I/Imax * 100)
33.26	2.69	12.56
33.86	2.65	11.86
34.85	2.56	25.85
35.94	2.50	17.28
36.46	2.46	5.21
38.31	2.35	9.16
39.87	2.26	8.06
42.18	2.14	5.16
43.45	2.08	14.98
44.36	2.04	8.14
45.46	2.00	6.86
46.46	1.95	5.43
47.05	1.93	4.61
49.14	1.85	5.76
50.35	1.81	6.80
51.14	1.79	9.65
56.32	1.63	3.23

3.2.2 Differential Scanning Calorimetry

The DSC thermogram of procaine hydrochloride was obtained using a DuPont model TA 9900 computer / thermal analyzer system. The analysis was carried out between 60 and 240°C, and the resulting thermogram is found in Figure 2. The compound was found to exhibit a clear and well-defined melting endotherm, whose temperature inflection was observed at 159°C.

Since procaine hydrochloride melts without decomposition, the DSC method can be used to establish the absolute purity of the material. The DSC purity of the tested sample was found to be 100.14 mole%, and the enthalpy of fusion for this sample was determined to be 31.5 kJ/mole (7.52 Kcal/mole).

3.3 Solubility Characteristics

The following solubility data have been reported for procaine and its salts [9]:

Procaine:	One gram dissolves in 200 mL water. Also soluble in alcohol, ether, benzene, and chloroform.
Procaine borate:	Soluble in water and alcohol, slightly soluble in chloroform, and almost insoluble in ether.
Procaine hydrochloride:	Soluble in water and alcohol, slightly soluble in chloroform, and almost insoluble in ether.

3.4 Ionization Constant

A pKa value of 9.0 has been reported for procaine at 20°C [10].

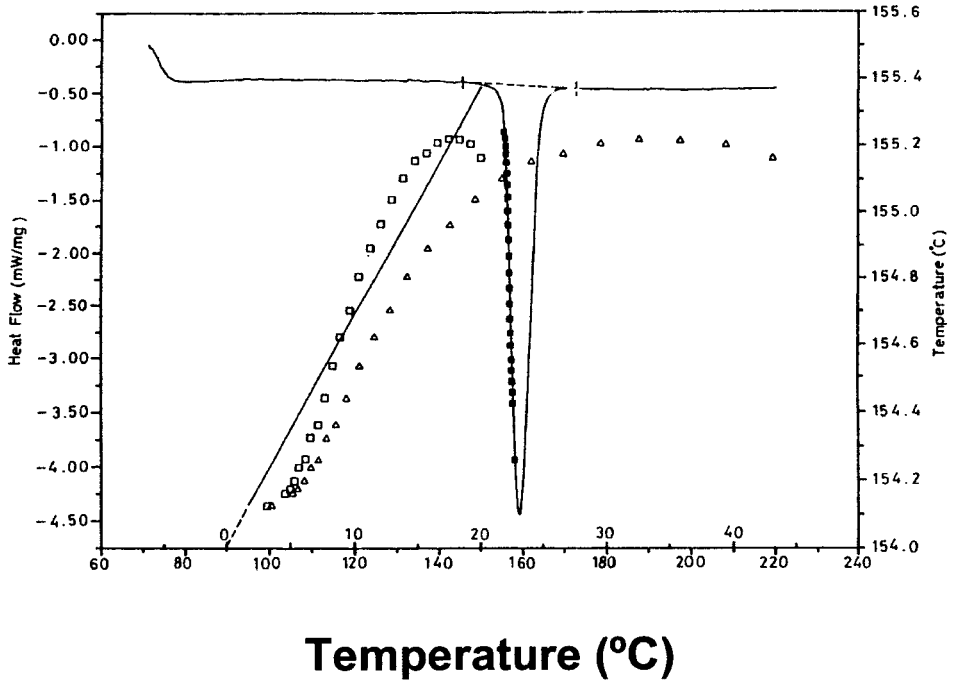


Figure 2. Differential scanning calorimetry thermogram of procaine.

3.5 Spectroscopy

3.5.1 UV/VIS Spectroscopy

The ultraviolet absorption spectra of procaine hydrochloride were obtained in ethanol and water on a Shimadzu 1601 PC UV/VIS spectrophotometer, and are shown in Figure 3.

In ethanol, the spectrum is characterized by the presence of absorption maxima at 299.9 nm {A(1%,1cm) = 767.6, and molar absorptivity = 2010}, and at 223.6 nm {A(1%,1cm) = 269.8, and molar absorptivity = 736}.

The spectrum of procaine hydrochloride in water is characterized by absorption maxima at 290.5 nm {A(1%,1cm) = 652, and molar absorptivity = 1780}, and at 220.5 nm {A(1%,1cm) = 309.1, and molar absorptivity = 842}.

Clarke reported the observation of an absorption maximum at 279 nm for procaine in aqueous acid, and a maximum at 296 nm in methanol [10].

3.5.2 Vibrational Spectroscopy

The infrared spectrum of procaine hydrochloride, presented in Figure 4, was obtained in a KBr pellet and recorded on a Pye-Unicam model SP 1025 infrared spectrophotometer. The assignments of the characteristic bands in the infrared spectrum is given in Table 2:

Clarke reported principal peaks at frequencies of 1690, 1274, 1605, 1174, 1116, and 772 cm^{-1} [10].

3.5.3 Nuclear Magnetic Resonance Spectrometry

3.5.3.1 ^{1}H-NMR Spectrum

The ^{1}H-NMR spectra of procaine hydrochloride in DMSO-d_6 were recorded on a Varian XL 200 (200 MHz spectrometer), with tetramethyl-silane being used as the internal standard. The simple proton (Figure 5) and HOMCOR (Figure 6) spectra were used to determine the exact chemical shifts, and the proton coupling scheme. The HETCOR spectrum (Figure 7) was used to assign the protons to their respective carbons in the

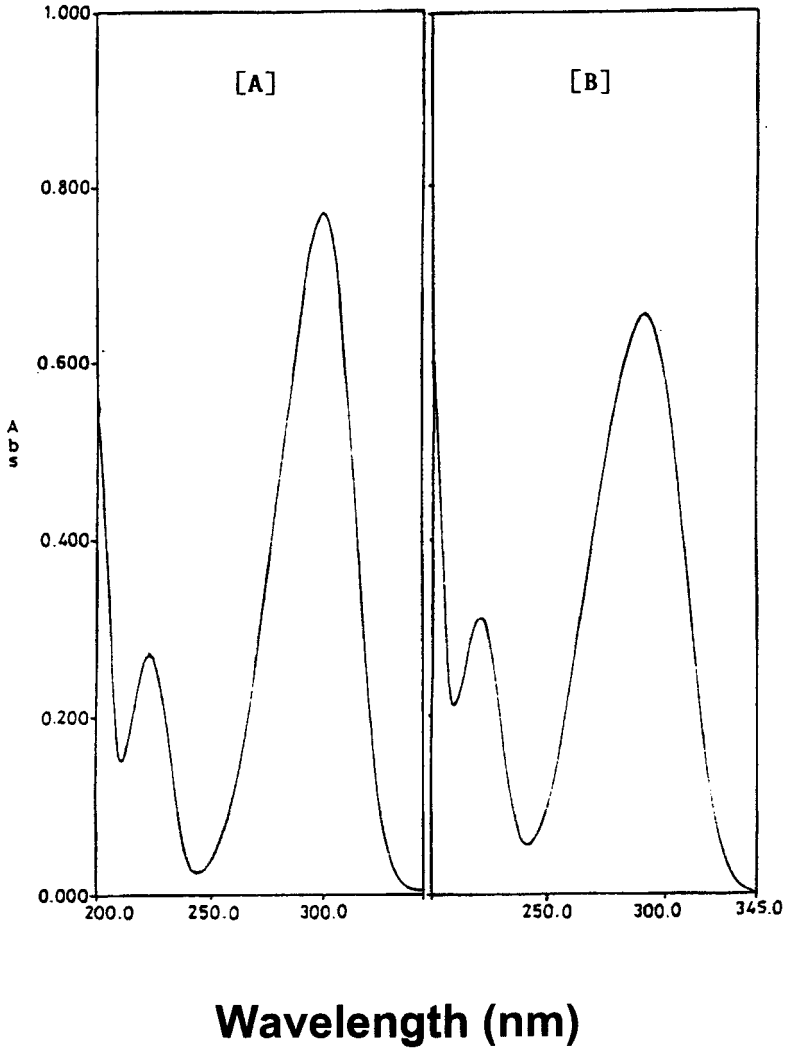


Figure 3. Ultraviolet absorption spectrum of procaine in [A] ethanol and in [B] water.

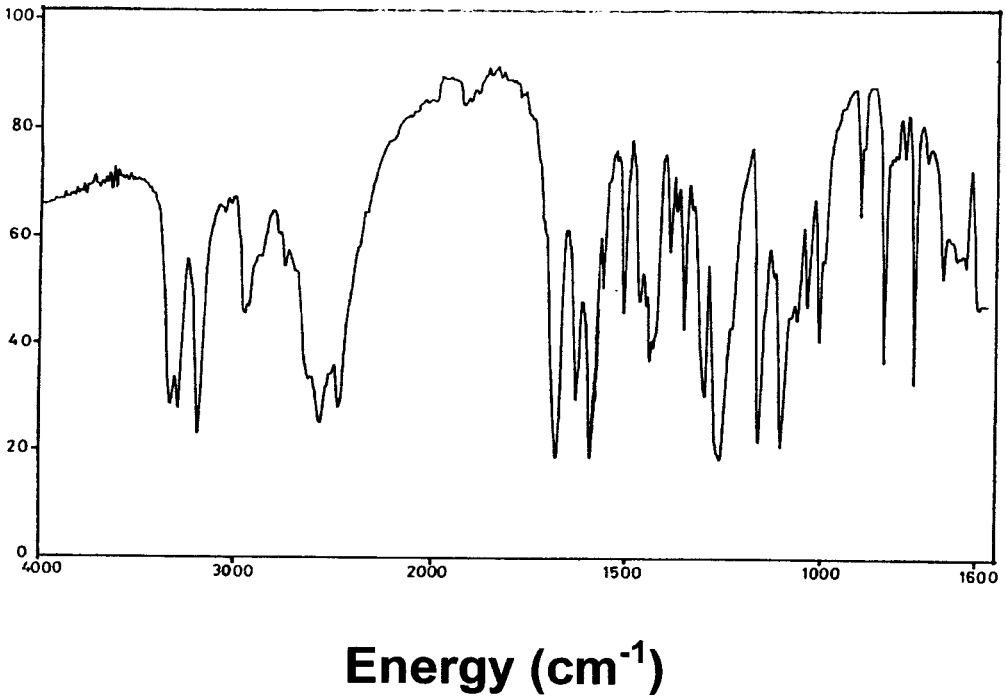


Figure 4. Infrared absorption spectrum of procaine.

Table 2

Assignments for the Characteristic
Infrared Absorption Bands of Procaine HCl

Frequency (cm^{-1})	Assignment
3180-3100	N-H stretching mode
2950	Aromatic C-H stretching mode
1690	Conjugated C = O stretching mode
1600	Aromatic C = C stretching mode
1210-1280	C-0 and C-N stretching mode

Table 3

Assignments for the Resonance Bands in the ^{1}H-NMR Spectrum of Procaine HCl

Chemical Shift (ppm)	Multiplicity	Number of Protons	Assignment
1.3	Triplet	6	$-CH_2—C\mathbf{H}_3-$
3.3	Multiplet	8	$-CH_2—C\mathbf{H}_2-$
6.0	Singlet	2	Exchangeable $-N\mathbf{H}_2$
6.6	Doublet	2	$C_3—\mathbf{H}$
7.8	Doublet	2	$C_2—\mathbf{H}$

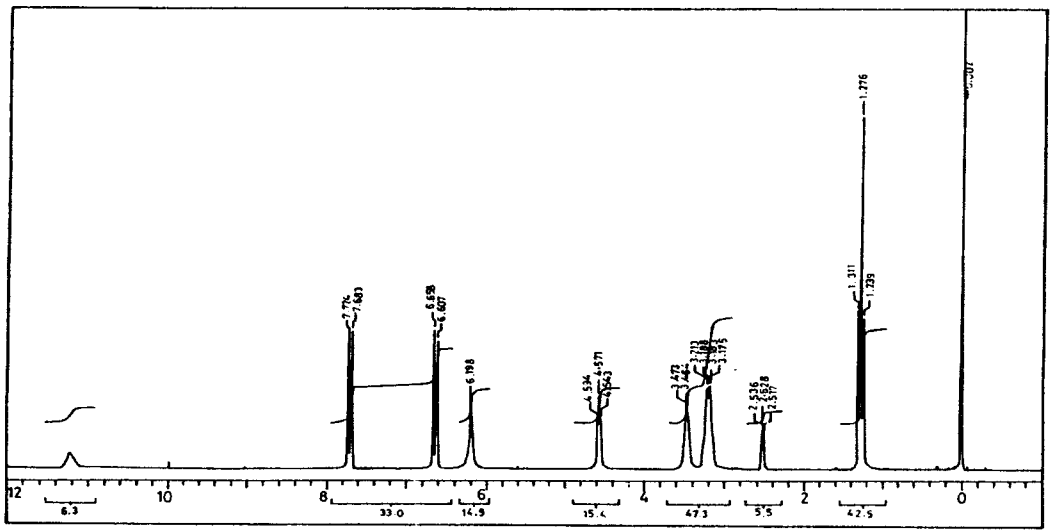


Figure 5. ^{1}H nuclear magnetic resonance spectrum of procaine (TMS used as the internal standard).

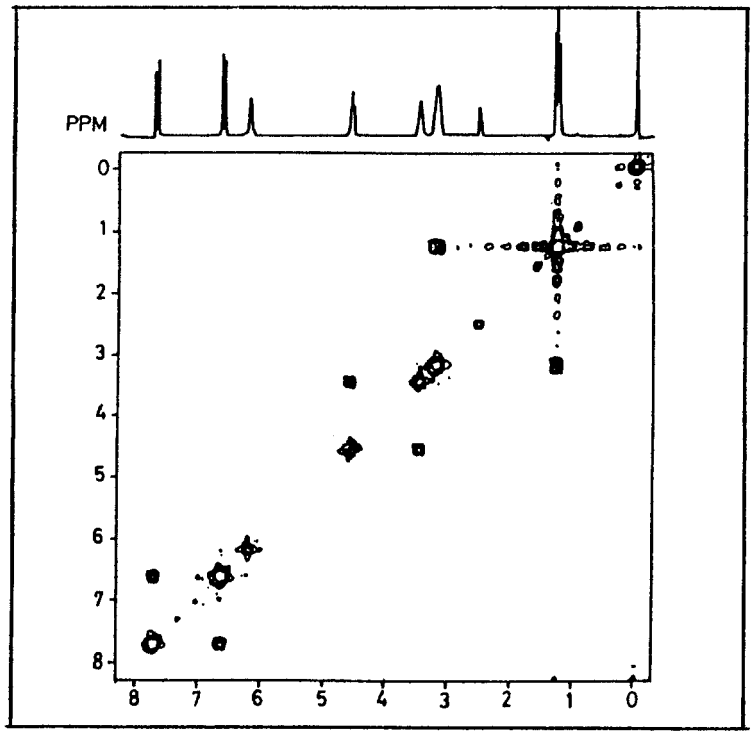


Figure 6. The HOMOCOR nuclear magnetic resonance spectrum of procaine.

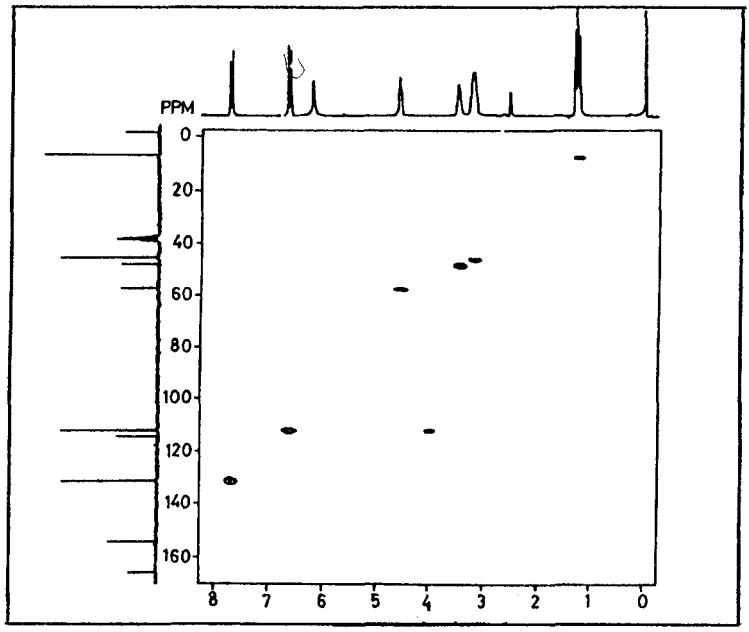


Figure 7. The HETCOR nuclear magnetic resonance spectrum of procaine.

molecule. The assignment of the chemical shifts to the different protons is presented in Table 3:

3.5.3.2 ^{13}C-NMR Spectrum

The ^{13}C-NMR spectra of procaine hydrochloride in DMSO-d_6 were recorded on a Varian XL 200 (200 MHz spectrometer), using tetramethylsilane as the internal standard. The APT and DEPT spectra are presented in Figures 8 and 9, respectively. Assignments for the observed resonance bands are provided in Table 4, together with the carbon numbering scheme.

3.5.4 Mass Spectrometry

The mass of procaine hydrochloride spectrum was obtained utilizing a Shimadzu model PQ-5000 mass spectrometer, with helium being used as the carrier gas. As shown in Figure 10, the mass spectrum shows a base peak at m/z = 86. The proposed fragmentation pattern of procaine hydrochloride is fully outlined in Table 5.

Clarke reported principal peaks at m/z = 86, 99, 120, 58, 87, 30, 92, and 71, and peaks for *p*-aminobenzoic acid at m/z = 137, 120, 92, 65, 39, 138, 121, and 63 [10].

4. Methods of Analysis

A review of the methods which facilitates the rapid choice of an optimum procedure to be used for the determination of procaine and other medicinals derived from aminocarboxylic acids has have been published [28]. This review covers volumetric, optical, electrochemical and polarographic methods.

Frangopol and Morariu have edited a seminar on procaine and related drugs, methods of analysis, and effects on cell membranes [29]. Items covered include studies on Romanian drugs by mass spectrometry and gas chromatography-mass spectrometry, quantitative and qualitative determination of procaine in biological samples, separation and quantitative thin layer chromatography determination of procaine

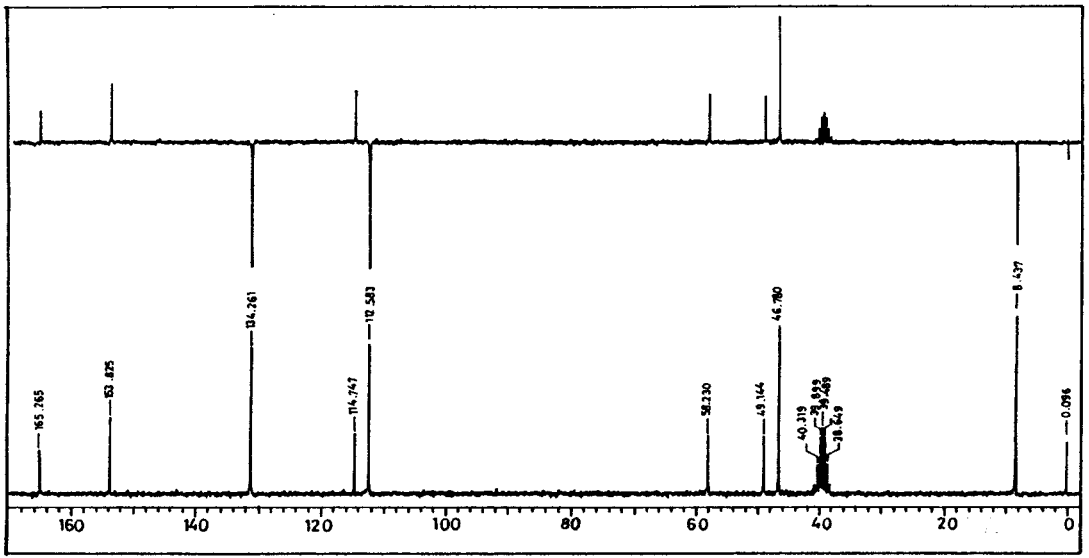


Figure 8. ^{13}C nuclear magnetic resonance spectrum of procaine (APT).

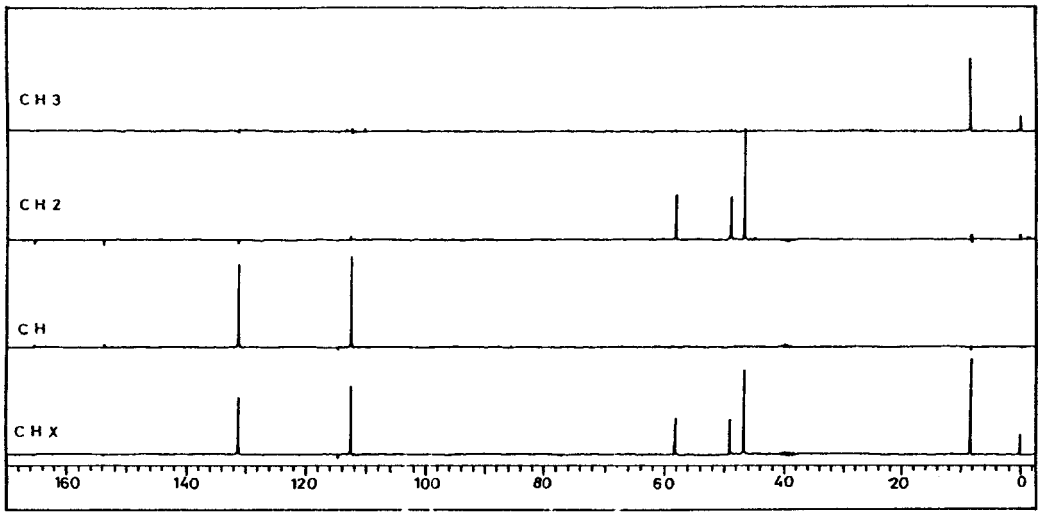
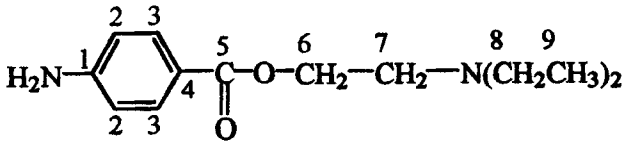


Figure 9. ^{13}C nuclear magnetic resonance spectrum of procaine (DEPT).

Table 4

Assignments for the Resonance Bands in the ^{13}C-NMR Spectrum of Procaine HCl



Chemical Shift (ppm)	Carbon Number
8.44	9
46.77	8
49.12	7
58.23	6
112.58	3
134.26	2
114.74	1
153.82	4
165.26	5

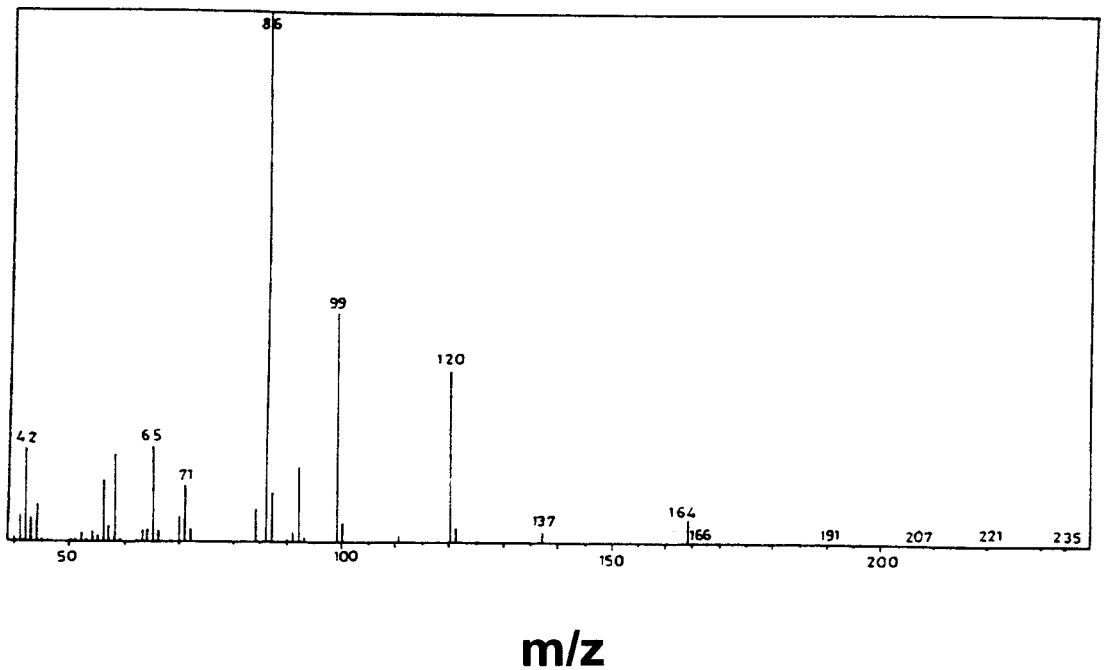
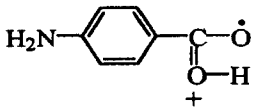
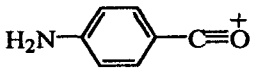
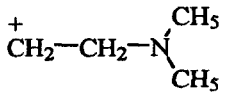
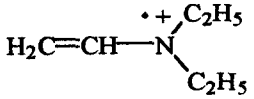
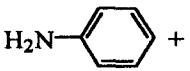
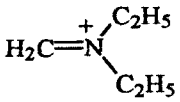
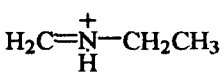
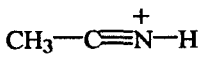


Figure 10. Mass spectrum of procaine.

Table 5

Mass Spectral Fragmentation Pattern for Procaine HCl

m/z	Relative Intensity	Fragment
137	6%	
120	20%	
100	7%	
99	21%	
92	10%	
86	100%	
65	6%	
58	18%	
42	13%	

hydrochloride from Gerovital tablets, and gas chromatographic analysis of some constituents of Gerovital H3.

4.1 Identification

A simple spot test was used for the identification of procaine, and other drugs [30]. The method uses one or more of the following reagents: 0.4% Alizarin red S (C.I. Mordant Red 3) in 20% acetic acid, 0.3% indigo carmine (C.I. Acid Blue 74), 0.4% indigo carmine in 30% acetic acid, 0.4% indigo carmine in 15% hydrochloric acid, 0.2% methylene orange, or 0.2% methylene orange in O.IN sodium hydroxide. The optimum reagents for each drug have been tabulated together with reaction time, form of crystalline product (characteristics are also given), detection limit, and limiting dilution. In addition, sixteen drugs which did not react with the reagent were also listed.

The United States Pharmacopoeia describes the following two identification tests for procaine [31]. In one, the infrared absorption spectra of standard and sample in KBr pellets are compared, and require to exhibit maxima only at the same frequencies. In another test, 10 mg of drug is dissolved in 1 mL of water, to which is added 1 drop each of hydrochloric acid and 1:10 sodium nitrite solution. One then adds 1 mL of a solution prepared by dissolving 0.2 g of 2-naphthol in 10 mL of 1:10 sodium hydroxide solution, shakes, and obtains a scarlet-red precipitate as a positive reaction.

The British Pharmacopoeia describes another identification test [32]. To 0.2 mL of a 10 % w/v solution, one adds 2 mL of water and 0.5 mL of 1 M sulfuric acid. The mixture is shaken, and then 1 mL of a 0.1 % w/v solution of potassium permanganate is added. A positive reaction is where the color is immediately discharged.

Clarke has described yet another identification test for procaine [10]. The sample is first dissolved in 2 M hydrochloric acid, and then 1 drop of this solution is placed on a white tile. One then adds 1 drop of a 1% sodium nitrite solution and 1 drop of a 4% 2-naphthol solution in 2M sodium hydroxide, and observes a red-orange color as a positive reaction.

4.2 Titrimetric Analysis

4.2.1 Potentiometry

The potentiometric method has been used for the determination of pure procaine base or its hydrochloride salt, and for procaine in pharmaceutical formulations. Lemahieu and Resibois reported the potentiometric determination of procaine hydrochloride with silver nitrate in dimethyl sulfoxide [64]. Procaine was potentiometrically analyzed using a procaine-selective membrane electrode [65]. Abou-Ouf *et al.* have used potentiometry to determine procaine and other drugs in ointments and creams with dibromohydantoins [66].

Selig reported a potentiometric titration method for the analysis of procaine and some other organic cations precipitated by tetraphenylborate [67]. The development of ion selective coated-wire electrodes, and their application in the titration of procaine and other pharmaceutically important substances, was reported [68].

Shoukry *et al.* have prepared plastic membrane ion-selective electrodes for the determination of procaine and other anaesthetic compounds [69]. The electrode selective for procaine was prepared with the use of a membrane containing 15% of the procaine tetraphenylborate ion pair with 40% of dioctyl phthalate and 45% of poly vinyl chloride (PVC). The membrane was attached as a disc (12 mm diameter, 0.3 mm thick) to the polished PVC cap of the electrode tube, which contained an internal solution of 0.1 M sodium chloride made 1 mM in the same drug, and in contact with a Ag-AgCl wire. Linear response ranges were determined to be 20.0 μM to 16 mM for procaine over the pH range of 3.1 to 7.9. The electrodes could also be used in the potentiometric titration of the drug with 0.01 M sodium tetraphenylborate.

The direct potentiometric determination (using a cation-selective membrane electrode) of procaine and some physiologically active amines in pharmaceuticals has been reported [70]. The sensing membrane was formed from PVC plasticized with dibutyl phthalate, and contained 0.1 mM trioctyloxybenzene-sulfonic acid in dibutyl phthalate. The reference solution was a mixture of 1 mM solution of the organic base and hydrochloric acid. Response was found to be linear over a wide concentration range, and the method was highly selective.

Liu *et al.* prepared a procaine-sensitive FET transducer by coating a suitable electrode with sodium tetraphenylborate-procaine active material, 5% of poly(vinylchloride), and dibutyl phthalate in tetrahydrofuran [71]. The optimum operating pH was determined to be between 2 and 7. The transducer was applied in the potentiometric titrimetric determination of procaine in injection solution, where the recovery was 100.7% and the coefficient of variation (n = 2) was 1.4%.

New modified polymeric electrodes selective to procaine and other local anaesthetic compounds were reported [72]. The electrode was constructed by incorporating the ion-pair complex of procaine with tungsto-phosphoric acid into ethylene-vinyl acetate copolymer. Best results were obtained with 1:1 nitrobenzene-dioctyl phthalate as a plasticizer. The calibration graph was linear from 18 μ to 10 mM of procaine. When these electrodes were applied to the determination of the drug in pharmaceutical formulations, the recoveries were found to be quantitative.

Satake *et al.* reported the use of a coated wire electrode sensitive to procaine and other local anesthetic cations, and their application to potentiometric determination [73]. Electrodes were constructed from a copper wire (0.8 mm diameter), coated with a PVC membrane comprising a mixture of the drug-tetraphenylborate ion-pair, dioctyl phthalate, polyvinyl chloride, and tetrahydrofuran. Potential measurement was made with respect to a Ag-AgCl reference electrode. The electrodes showed linear responses with a Nernstian slope for procaine over the concentration range investigated. The method was used for analyses of the drug in pharmaceutical preparations.

A poly (vinylchloride) membrane electrode was described for local anesthetics, based on dibenzo-24-crown-8 as the electroactive material, and di(2-ethyl)hexyl phthalate as the plasticizer [74]. It was reported that the electrode exhibited a Nernstian response to procaine, and other electrode properties were also presented. The analysis was performed at pH 6 to 6.5 *vs.* S.C.E., with a 0.2 M lithium acetate agar bridge. Less efficient crown ethers studied at this time were benzo-15-crown-5, dibenzo-18-crown-6, and dibenzo-30-crown-10.

The application of another ion-selective electrode in the determination of procaine hydrochloride was reported [75]. Sample solutions containing

procaine (1 mL of a 1% solution) were diluted to 25 mL, and acidified with 2 mL of 0.1 M sulfuric acid. The solution was titrated to a potentiometric endpoint with 1 mM molybdophosphoric acid, with the aid of an ion-selective electrode (based on a plasticized PVC membrane) and a saturated potassium chloride supporting electrolyte. The working range was found to be from 10 mM to 0.1 M, and the coefficient of variation was 12%.

Hamada *et al.* used a poly (vinyl chloride) matrix membrane ion-selective electrode for the analysis of procaine [76]. Procaine flavianate (10 mg, prepared by precipitation from an equimolar mixture of procaine hydrochloride and flavianic acid), was mixed with PVC powder (150 mg), dioctyl phthalate (370 mg), and tetrahydrofuran (4 mL). This mixture was used to produce membranes (3 cm diameter), from which discs were cut to prepare ion-selective electrodes. The electrodes were used in conjunction with a double-junction Ag-AgCl (KNO_3) reference electrode for the potentiometric determination of procaine hydrochloride at 25°C.

4.2.2 Aqueous Titration

Krishna *et al.* reported a nitritometric method, using aminoanthraquinone dyes as indicators, for the determination of procaine, other 4-amino-benzenesulfonamides, and 4-aminobenzoic acid derivatives [77]. Using sodium dioctyl sulfosuccinate, Faicao and Vianna determined procaine hydrochloride [78].

Veinbergs *et al.* have reported a comparative study of the methods used for the determination of procaine in Celnovocaine by titrimetry and photometry [79]. Tsubouchi *et al.* have reported the application of single-phase endpoint change systems in two-phase titrations to the analysis of procaine and some other amines [80].

Wang has determined citric acid and procaine in Xiaozhi injectable (an anti-hemorrhoid agent) by two-phase titration [81]. With constant shaking, a mixture of 1:1:1:2 Xiaozhi solution - water - chloroform - ethanol was titrated against 0.1 M sodium hydroxide to the phenolphthalein end point, thus obtaining the citric acid content. Dilute nitric acid was then added to the solution until the pink color disappeared, and the solution titrated against 0.1 M silver nitrate (with 5 drops of

K_2CrO_4 solution as the indicator, which turns brick-red at the endpoint). This latter titration yields the procaine hydrochloride content.

Wang has determined procaine hydrochloride in the presence of citric acid [82]. To the sample (prepared as 5 mL of aqueous solution) were added 5 mL of water, 1 mL of 0.1 M hydrochloric acid, and 200 mg potassium bromide. The resulting solution was titrated with 0.1 M sodium nitrite, ultimately yielding the total content of procaine hydrochloride and citric acid.

Medvedovskii *et al.* have developed a method of alkalimetric two-phase titration for the determination of procaine hydrochloride and other organic base salts [83].

The method of Koval'chuk *et al.* was applied in the determination of procaine [84]. These authors have also developed another alkalimetric two-phase titration method for determination of the salts of organic bases, including procaine hydrochloride [85]. A solution of the base salt was mixed with 2 mL chloroform and 2 drops of 0.15% methylene blue solution, and the mixture titrated with 0.02 to 0.1 M sodium hydroxide with shaking. At the endpoint, the chloroform layer becomes pink-violet.

A linear titration method was reported by Feng *et al.* to determine procaine hydrochloride, and the hydrochlorides of other drugs [86]. This method was purported to be a new approach for the replacement of non-aqueous titration. For the titration of procaine, a sample containing 0.5 to 0.6 mmol of the hydrochloride was treated with 10 to 40 mL of 95% ethanol and 10 mL of 1 M KCl, and diluted to 100 mL with water. The solution was titrated potentiometrically with 0.1 M sodium hydroxide, using a glass electrode and a S.C.E. as the indicator and the reference electrodes, respectively. Multi-linear regression analysis was used to calculate the results, and agreement was obtained with results obtained by Ingman and Still [87] who used a non-aqueous titration method. The method was reported to be suitable for the titration of organic base hydrochlorides for which the conjugate acid stability constants ranged from 103 to 1010.

The United States Pharmacopoeia (3 1) describes the following titrimetric assay method:

Transfer about 0.5 g of procaine hydrochloride, accurately weighed, to a beaker, add 100 mL of cold water, 5 mL of hydrochloric acid, and 100 mg of potassium bromide, and stir until dissolved. Cool to about 15°C, and slowly titrate with 0.1 M sodium nitrite VS that previously had been standardized against USP sulfanilamide RS. Determine the end point electrochemically, using a suitable electrode combination (platinum-calomel or platinum-platinum). Place the buret tip below the surface of the solution to eliminate air oxidation of the sodium nitrite, and stir the solution gently, using a magnetic stirrer, without putting a vortex of air under the surface, maintaining the temperature at about 15°C. When the titration is within 1 mL of the endpoint, add the titrant in 0.1 mL portions, allowing not less than 1 minute between additions. The instrument needle deflects and then returns to approximately its original position until the end point is reached. Perform a blank determination and make any necessary corrections. Each mL of 0.1 M sodium nitrite is equivalent to 27.28 mg of procaine hydrochloride.

4.2.3 Non-Aqueous Titration

A non-aqueous method for the titration of procaine and other drugs using trifluoromethanesulfonic acid was reported by Zakhari and Kovar [88]. Solutions of procaine hydrochloride in anhydrous acetic acid, acetic anhydride, their mixture, or in acetone, was titrated with an acetic acid solution of 0.1 M trifluoromethanesulphonic acid or 0.1 M perchloric acid. Titration was effected in the presence or in the absence of mercurous acetate. The end point was detected visually (using crystal violet as indicator) or potentiometrically.

Asahi *et al.* have determined procaine hydrochloride by potentiometric titration with perchloric acid in a mixture of acetic anhydride and anhydrous acetic acid [89]. Procaine was determined by titration in acetic anhydride.

Potentiometric non-aqueous titration (using bismuth oxyacetate and perchloric acid or trifluoromethylsulfonic acid) was used by Zakhari *et al.* (90) for the determination of procaine and other drugs (halides and nitrogen bases) [90]. Procaine hydrochloride was dissolved in 5:1 acetic

acid - acetic anhydride by heating under reflux for 2 minutes before completing the titration. The mixture was treated with a 2% solution of bismuth oxyacetate in anhydrous acetic acid. The mixture was then stirred until any formed precipitate re-dissolved, then titrated potentiometrically with 0.1 N trifluoromethylsulfonic acid. The endpoint was detected with the use of a glass Ag-AgCl combination electrode.

4.2.4 Complexiметric Titration

Kosheleva *et al.* have reported a complexiметric method for the determination of procaine in the presence of its hydrolysis products [91]. In solution, the drug may hydrolyze to yield diethylaminoethanol and 4-aminobenzoic acid. For the analysis, the sample solution is treated with 0.2 M $(NH_4)_2Zn(SCN)_4$, heated to the onset of boiling, and cooled with slow rotational mixing for 4 minutes. This process yields clear solution and an oily precipitate, which is filtered through cotton wool. The filter is washed with 10% NH_4SCN solution, and cooled to 17°C. The precipitate is dissolved in acetone or dimethylformamide. Water, ammonia buffer solution (pH unspecified), and acid chrome black special indicator are added, and the mixture titrated with 10 mM EDTA to determine Zn(II), and hence procaine.

4.2.5 Gravimetry

Zalkowska and Piotrowska have reported a gravimetric method for the determination of procaine and other organic compounds by the Buerger method [92]. The accuracy of the gravimetric results for carbon and hydrogen were reported to depend on variation in the balance readings.

4.3 Electrochemical Analysis

4.3.1 Electrochemical Devices

Liu *et al.* have reported the development and applications of the commonly used local anaesthetic sensitive field-effect transistor(FET) [56]. The ion-pair complexes of procaine with silicotungstate, tetraphenylborate, or reineckate were prepared as electroactive materials for a drug sensor. These active materials were coated onto the platinum draw wire of a MOSFET to make a local anaesthetic-sensitive FET that

exhibited Nernstian response in the range of 2 μM to 10 μM. These local anaesthetic-sensitive FET devices have the advantage of fast response (5 to 10 sec.), small size, and good stability. Conditions for the determination of procaine in its pure form and in injection solutions have been established.

Shen *et al.* have reported studies on procaine-selective electrodes embodying PVC membranes [57]. Various ion-pair complexes (procaine derivatives with tetraphenylborate, dipicrylamine, tetraiodomecurate, and reineckate) were incorporated into platinized PVC membranes, and with dinonyl phthalate as the solvent mediator, formed procaine selective electrodes. The efficiency and performance of these were compared, and it was found that procaine picrylamine and procaine tetraphenylborate were the best electroactive materials. The procaine picrylamine electrode exhibited a Nernstian response over the range 10 μM to 0.1 M, and was used as the indicator electrode for the potentiometric determination of procaine. The method recovery was found to be 99.8%, with a standard deviation of 0.9%.

4.3.2 Polarography

Procaine has been determined by polarographic [58-60] and oscillopolarographic titration [61,62] in drug forms, eye drops, injections, suppositories, and in the commercial product "Menovasin".

Bezuglyi *et al.* have described an indirect polarographic method for the determination of procaine in eye drops and injection solutions [58]. Ogurtsov *et al.* have used polarography to determine procaine hydrochloride in some drug forms [59]. In this latter method, the sample solution was suitably diluted, a portion made 0.1 M in LiCl, the solution deaerated with nitrogen, and the polarogram was recorded at 25°C from -1.20 V *vs.* S.C.E. The drug content was determined at the reported half-potential of 1.75 V using a calibration graph. The method was applied either to bulk drug substance, or to a 2% solution for injection.

Ogurtsov and Yavors'ka have used polarography to determine procaine in suppositories and in "Menovasin" preparation [60]. A portion of an aqueous extract of the suppository, or a portion of the liquid "Menovasin" preparation, was mixed with 10 mL of 0.1 M LiCl, and diluted to 25 mL

with water. Beginning at –1.2 V, the polarogram was recorded using a cell equipped with a dropping-mercury electrode (period 3 sec., mercury column, 65 cm) and a S.C.E. reference. The procaine concentration was found to from a calibration graph constructed with standard solution.

Chen and Gao studied the use of oscillopolarographic titration, and reported a direct titration of the salts of weak organic bases (or acids) in aqueous solution [61]. In their work, they included the determination of procaine by an oscillopolarographic titration method.

In their applications of A.C. oscillopolarographic titration for pharmaceutical analysis, Huang *et al.* reported a method for the titration of procaine hydrochloride with sodium tetraphenylborate [62]. Procaine hydrochloride was mixed with sodium tetraphenylborate in acetate buffer (pH 4.6). The precipitate was filtered off, and the unconsumed tetraphenylborate titrated with thallium sulfate by A.C. oscillo-polarography. The recovery was found to be 99.9 to 100.0%, and the coefficient of variation (n = 10) was 0.19%. The method could also be used to identify outdated samples of procaine hydrochloride injection solution, as its loss of water solubility is indicated by an incision in the titration curve.

4.3.3 Coulometry

Nikolic *et al.* reported the preparation and coulometric determination of quaternary ammonium iodides of procaine and of other local anesthetics [63]. After extraction from 0.33 M NaOH, the quaternary iodide salts were prepared by precipitation with methyl iodide in ethyl ether. The quaternary iodides were then coulometrically determined with the use of a Radiometer titrator. The method used a silver cathode and anode (in electrolytes of 2 M and 0.4 M H_2SO_4, respectively), and a reference mercurous sulfate electrode. For drug determinations in the range of 0.12 to 0. 96 mg, the standard deviations were typically found to be 4 to 8 μg.

4.4 Spectrophotometric Methods of Analysis

Assay methods for Procaine have been reported which make use either of its direct ultraviolet absorption, or which are based on colorimetric reactions of the drug entity.

4.4.1 Ultraviolet Spectrometry

An ultraviolet spectroscopic method was presented, and used for the assay of procaine and nitrofural in a multicomponent collagen sponge without prior separation of the drugs [33]. Crushed Collagen Sponge (0.1 g) was dissolved in 70 mL of 1 mM HCl, and heated for ten minutes. The solution was cooled, diluted to volume, mixed, filtered, whereupon the first 20 mL was discarded. The absorbance of the analyte solution was then measured at 290 and 373 nm (against 1 mM HCl) for procaine and nitrofural, respectively.

Santoni *et al.* have developed and used an ultraviolet spectroscopic method for the simultaneous assay of procaine and antipyrine [34]. The method allowed for a rapid and accurate determination of such mixtures over the tested concentration range of 2-9 μg/mL for procaine.

Carmona *et al.* described a simple and rapid kinetic spectrophotometric method for the determination of procaine in pharmaceutical preparations [35]. The method was applied to the determination of the drug in various pharmaceutical samples.

Huang and Guan have reported an ultraviolet spectrophotometric and coefficient-multiplied method for the determination of procaine hydrochloride in compounded zinc sulfate eye drops [36]. 3 mL of sample was shaken with 1.5 mL of ethyl ether and water, and after removal of the organic phase, water was added to achieve a final volume of 50 mL. The absorbance of this solution was measured at 291 and 344.5 nm. The recovery ($n = 6$) for procaine was found to be 99.9%, with a relative standard deviation equal to 0.44%.

Chemova *et al.* have reported a photometric procedure for the analysis of procaine hydrochloride [37].

4.4.2 Colorimetry

A colorimetric assay procedure for the quantitative analysis of procaine hydrochloride was developed by Tan and Shelton [38]. The method is based on the interaction of procaine with *p*-dimethylamino-

cinnamaidehyde in the presence of trichloroacetic acid. Using absolute methanol as the reaction medium, a red Schiff base is formed. The method was applied to the analysis of injectable procaine formulations.

Sakai *et al.* determined procaine by a colorimetric method that was proposed for the assay of procaine on the basis of solvent extraction [39]. Tetrabromophenolphthalein ethyl ester anion was added to an aqueous solution containing procaine, and the extract took on a red color (absorbance maximum of the extract at 580 nm). The optimal pH range for extraction of the drug from the aqueous solution was found to be 8-9. Procaine was found to form a 1:1 associated ion pair compound with the reagent in 1,2-dichloroethane.

Salama and Omar have described a rapid, specific, and convenient colorimetric method for the determination of procaine hydrochloride in pharmaceutical preparations [40]. The method is based on an intensity measurement of the orange-red color developed when the drug is allowed to react with 1,2-naphthoquinone-4-sulfonic acid (sodium salt) in an aqueous solution. The method is suited for routine analysis of official preparations of procaine.

Novakovic presented a colorimetric method for the determination of procaine hydrochloride, either in its bulk form or in various pharmaceutical formulations [41]. The method utilizes conversion of the drug to a hydroxamic acid, and subsequent complexation with iron. The reaction product is violet in color.

Minka *et al.* have reported an absorption spectroscopic method for the determination of procaine [42]. The drug (base or hydrochloride) was determined after a diazotization coupling reaction with 0.1% 3-(1,2-dicarboxyethyl)-rhodamine solution. After addition of 20% sodium hydroxide solution, the absorbance of the red solution was obtained using a blue filter. Sample values were evaluated by means of a calibration graph, and Beer's law was obeyed over the range of 1 - 10 μg/mL of procaine.

El-Kommos and Emara described a rapid method utilizing 3-methylbenzo-thiazoline-2-one hydrazone coupling for the determination of procaine

hydrochloride in pure form and in dosage form [43]. Procaine has been determined in complex dosage forms by colorimetry [44].

Comparative values for several physicochemical analytical methods for establishing stability of the drug solutions and detecting impurities (including colorimetry) was reported [45].

Syoyama and Nogima reported a colorimetric method for the detection of procaine in urine, where the drug and other phenethylamines were extracted [46]. The extraction involved ion association with Chrome Azurol S (C.I. Mordant blue 29), and was applied to a screening test for the drug and other related amines.

Fayez *et al.* have reported the colorimetric analysis of procaine and other local anesthetics by the acid-dye technique [47].

5-(*p*-dimethylamino-phenyl)-penta-2,4-dienal was used by Nakatsuji *et al.* as an analytical reagent [48]. These authors described a simple method for preparation of the reagent, and its application to the colorimetric determination of procaine and other primary aromatic amines.

4.4.3 Atomic Absorption

Procaine was indirectly determined by Minami *et al.* using atomic absorption spectrometry [51]. Nerin *et al.* also used indirect atomic spectrometry to determine procaine, with their method involving the formation of an ion-pair with $Co(SCN)_4^{2-}$ and extraction of the ion pair into 1,2-dichloroethane [52}. Quantitation of the Co response was effected using the atomic absorption at 241 nm, and optimal pH conditions and the linear regions of the calibration graphs were reported.

Montero *et al.* reported an indirect atomic absorption spectrometric method combined with a flow-injection precipitation technique for the determination of procaine in pharmaceutical preparations [53]. The precipitate formed by the injection of Co(II) into a sample stream containing 10 to 110 μM procaine at pH 8.0 to 9.1 was retained on a stainless steal filter, and analyzed at 240.7 nm by an online atomic

absorption spectrometer. The amount of precipitated Co(II) was found to be proportional to the concentration of drug in the sample.

Lei *et al.* reported a method for the indirect determination of trace amounts of procaine in human serum by atomic absorption spectrophotometry [54]. The sample was mixed with $HClO_4$, heated at 85°C for 30 minutes, diluted to a known volume with water, and centrifuged. 1 mL of the supernatant solution was buffered with 0.1 M sodium acetate-acetic acid to pH 3.86, and mixed with 0.2 M $Zn(SCN)_2$ reagent to a final concentration of 0.1 M. After dilution to 50 mL with water, the solution was shaken for 1 minute with 10 mL of 1,2-dichloroethane, whereupon the zinc extracted into the organic phase was determined by air-acetylene flame atomic absorption spectrometry for the indirect determination of procaine. The detection limit was found to be 0.1 μg/g, with a recovery of 89-98% and a coefficient of variation (n = 10) equal to 3.2%.

4.5 Fluorimetric Methods of Analysis

de Silva and Strojny have presented a systematic examination of fluorimetric assays for procaine and other drugs [49]. The compounds in question were reacted with fluorescamine in solution and on thin layer chromatographic plates. The analytical parameters for the optimal reaction and the sensitivity were presented, and could be utilized for the determination of the drug in biological fluids.

Wang *et al.* have described a fluorimetric method for the determination of serum procaine concentrations [50].

4.6 Chromatographic Methods of Analysis

4.6.1 Paper Chromatography

Clarke has described three systems based on paper chromatography for the identification of procaine [10a].

System 1 [97, 98]

Paper: Whatman No. 1 (14 x 6 in.), buffered by

dropping in a 5% solution of sodium hydrogen citrate, then blotting and drying at 25°C for 1 hour. It can be stored indefinitely.

Sample: 2.5 μL of a 1% solution in 2N acetic acid if possible, otherwise in 2N hydrochloric acid, 2N sodium hydroxide, or ethanol.

Solvent: 4.8 g of citric acid in a mixture of 130 mL of water and 870 mL of n-butanol.

R_f: 0.31

Visualization: Blue fluorescence when viewed under UV light.

Development reagents: Iodoplatinate spray; strong reaction.

System 2 [99]

Paper: Whatman No. 1 or 3 sheet (17 x 19 cm) impregnated by dipping in a 10% acetone solution of tributyrin, and drying in air.

Sample: 5 μL to 1 to 5% solution in ethanol or chloroform.

Solvent: Acetate buffer (pH 4.58).

R_f: 0.89

Equilibration: The beaker containing the solvent is equilibrated in a thermostatically controlled oven at 95°C for about 15 minutes.

Development: Ascending. The paper is folded into a cylinder and clipped. The cylinder is inserted in the beaker containing the solvent, which is not removed from the oven (a plate glass disk thickly smeared with silicon grease may serve as a

cover). The run time is approximately 15 minutes.

Visualization: Examination under UV light (254 nm).

System 3 [100,101]

Paper: Whatman No. 1 or 3 sheet (17 x 19 cm) impregnated by dipping in a 10% acetone solution of tributyrin, and drying in air.

Sample: 5 μL to 1 to 5% solution in ethanol or chloroform.

Solvent: Phosphate buffer (pH 7.4).

R_f: 0.27

Equilibration: The beaker containing the solvent is equilibrated in a thermostatically controlled oven at 86°C for about 15 minutes.

Visualization: Examination under UV light (254 nm).

4.6.2 Thin Layer Chromatography

Several thin layer chromatographic methods have been reported that were used for the analysis of procaine [102-114], and selected examples are presented in Table 6.

Clarke [10] reported the following four systems:

System 1 [113]

Plate: Silica gel (thickness 250 μm), dipped in or sprayed with 0.1 M methanolic potassium hydroxide, and dried.

Mobile Phase: 100: 15 methanol : strong ammonia solution.

Table 6

Thin-Layer Chromatography Systems Used in the Analysis of Procaine

Adsorbent	Solvent System	Comment and Visualizing agent	Ref.
Silica gel	Methanol-aq. 25% ammonia (200:3).	Spray with 10% $SnCl_2$ solution in 8% HCl, heating at 50°C to 60°C for 1 to 2 min. and treating with 1% 4-dimethylaminobenzaldehyde solution in 8% HCl. UV at 288 nm. R_f 0.55.	106
Silica gel	Benzene-ethanol (9:1)	Stability of injections. Iodine vapor.	107
Silica gel $60F_{254}$	Cyclohexane-diethylamine (7:3).	UV at 309 nm.	108
Silufol	Five mobile phases, and combination for two dimensional TLC.	Dragendorff, iodoplatinate and ninhydrin reagents. In eye lotions and infusion solutions.	109
—	—	TLC-UV densimetry at 254 nm. In ointments.	110
—	Cyclohexane-benzene-ethylenediamine (25:3:2)	UV at 223 nm	111
Silica gel G	Toluene-anhydrous acetic acid-acetone-methanol (14:1:1:4).	UV at 284 nm	112

Reference Compounds: Diazepam $R_f = 75$; chlorprothixene, $R_f = 56$; Codeine $R_f = 33$; atropine $R_f = 18$.

Analyte R_f: 54

System 2 [113]

Plate: Silica gel (thickness 250 μm), dipped in or sprayed with 0.1 M methanolic potassium hydroxide, and dried.

Mobile Phase: 75:15: 10 cyclohexane : toluene : diethylamine.

Reference Compounds: Dipipanone R_f 66; Pethidine R_f 37; desipramine $R_f = 20$; codeine $R_f = 06$.

Analyte R_f: 06

System 3 [113]

Plate: Silica gel (thickness 250 μm), dipped in or sprayed with 0.1 M methanolic potassium hydroxide, and dried.

Mobile Phase: 90 : 10 chloroform : methanol.

Reference Compounds: Meclozine R_f 79; caffeine R_f 58; dipipanone R_f 33; desipramine $R_f = 11$.

Analyte R_f: 31

System 4 [114]

Plate: Silica gel G (thickness 25 μm), dipped in or sprayed with 0.1 M methanolic potassium hydroxide, and dried.

Mobile Phase: Acetone.

Visualization: Dragendorff spray, positive, acidified iodoplatinate solution positive,

	ninhydrin spray, positive.
Reference Compounds:	Meclozine R_f 70; mepivacaine R_f 48; procaine R_f 30; amitriphylene R_f = 15.
Analyte R_f:	30

4.6.3 Gas Chromatography

Several gas chromatographic methods have been reported that were used for the analysis of procaine [115-124].

Clarke [10] reported the details of two gas chromatography systems.

System 1 [115]

Column:	2.5% SE-30 on 80-100 mesh chromosorb G (Acid-washed and dimethyldichlorosilane-treated), 2 m x 4 mm internal diameter glass column. It is essential that the support is fully deactivated.
Column Temperature:	Between 100 and 300°C. As an approximate guide, the temperature to use is the retention index ÷ 10.
Carrier Gas:	Nitrogen at 45 mL/min.
Reference Compounds:	*n*-alkane with an even number of carbon atoms.
RI:	2018

System 2 [116]

Column:	3% Poly A103 on 80-100 mesh Chromosorb W HP, 1 m x 4 mm internal diameter glass column.
Column Temperature:	200°C.
Carrier Gas:	Nitrogen at 60 mL/min.

Reference Compounds: *n*-alkane with an even number of carbon atoms.

RI: 2580

Other gas chromatography assay methods reported in the literature [117-123] are shown in Table 7.

4.6.4 High Performance Liquid Chromatography

Several high performance liquid chromatography systems that were used for the analysis of procaine have been reported [125-143].

Clarke [10] has provided descriptions of two systems:

System 1 [125]

Column: (Spherisorb S5W silica, 5 μm, 12.5 cm x 4.9 mm internal diameter.

Eluent: A solution containing 1.175 g (0.01 M) of ammonium perchlorate in 1000 mL of methanol, adjusted to pH 6.7 by the addition of 1 mL of 0.1 M methanolic sodium hydroxide.

k': 1.9

System 2 [126]

Column: ODS-Hypersil (ODS-Silica), 5 μm, 12.5 cm x 4.9 mm internal diameter.

Eluent: 3:70:100:1.4 solution of methanol : water : 1% v/v phosphoric acid : hexylamine.

k': 0.00

Gupta and Shek reported on the use of high pressure liquid chromatography, ultraviolet spectrometry, and colorimetry as stability-

Table 7

Gas Chromatography Systems Used in the Analysis of Procaine

Column	Support	Carrier Gas & Detection	Ref.
Glass column (20 cm × 2.5 mm)	2% OV 17 on Chromosorb W (80 to 100 mesh) at 195°C.	Nitrogen gas (25 ml/min) and e.c.d.	117
Capillary column	SE-54	Hydrogen gas and f.i.d.	118
25 m Glass capillary column	SE-54	Hydrogen gas and f.i.d.	119
120 cm × 4 mm column	SE-30 (2, 4 and 6%) supported on Chromosorb W AW-DMCS (60 to 80 mesh)	Nitrogen gas and f.i.d.	120
Stainless steel (1 m × 2 mm) containing steel beads (0.25 to 0.4 mm diam.)	SE-30 (0.2%)	Electrically generated hydrogen and f.i.d.	121
Column (2 m × 3 mm)	10% of PEG 20 M on Chromosorb W AW-DMCS at 30°C.	Nitrogen gas and f.i.d.	122
Glass column (15 m × 0.3 mm)	SE-30 (0.2 μm)	Hydrogen gas and f.i.d.	123

indicating assay methods [127]. Using studies of Procaine and other compounds, they found that HPLC is the most reliable method.

Khalil and Sheiver have determined procaine in pharmaceuticals by a high speed liquid chromatography method [128]. Maurich *et al.* reported the determination of procaine in ear drops by an HPLC procedure [129].

Other HPLC assay methods reported in the literature [130-143] are shown in Table 8.

4.6.5 Column Partition Chromatography

Wang and Li have reported the determination of procaine hydrochloride injections, and the quality control of 4-aminobenzoic acid [144]. The column packing used for this work consisted of 8 g of silanized siliceous earth support with 5 mL of hexanol as the stationary phase, previously percolated with 20 mL of 0.05 M sodium carbonate. The drug injection solution (containing 10 mg of procaine hydrochloride) was applied to the column, and eluted with 30 mL of 0.05 M sodium carbonate. The eluent was diluted to 50 mL with water, and 4-aminobenzoic acid was determined by an absorbance measurement at 266 nm. Procaine was then eluted from the column using 60 mL of 0.1 M hydrochloric acid. This eluent was treated with 10 mL of acetate buffer (pH 6), and diluted to 100 ml with water. The analyte was determined on the basis of its absorbance at 290 nm. Equations for the computation of procaine and 4-amino-benzoic acid concentrations were presented.

4.6.6 Liquid Chromatography

Mikami *et al.* have described a rapid liquid chromatography method for the determination of procaine and other drugs in pharmaceutical preparations [145]. The separation is effected using a Wakosil-5 C_{18} column. The mobile phase consisted of aqueous acetonitrile - triethylamine in various ratios, or of 30% or 50% aqueous acetonitrile. Detection was based on the ultraviolet absorption at 225 to 265 nm, and method recoveries from dosage forms were in the range of 98.5 to 100.3%.

Table 8

High-Performance Liquid Chromatography Systems Used in the Analysis of Procaine

Column	Mobile phase and flow rate	Detection	Sample and comments	Ref.
(30 cm × 4 mm) μ-Bondapak C18 at 25° to 28°C	Methanol-aq. 1% acetic acid (2:3) of pH 4.7 (1.1 ml/min).	UV at 254 nm.	Used for stability in the aqueous system.	130
(50 cm × 8 mm) of Shodex D-814	Aq. 70% methanol at 40°C	UV at 270 nm and by differential refractometry	In horse urine.	131
Stainless steel column (30 cm × 4 mm) of μ Bondapak C_{18}	Phosphate buffer (pH 3) – methanol (2:1), (2 ml/min).	UV at 254 nm and 280 nm.	Analyses in sample containing procaine and cocaine and other amines.	132
(10 cm × 4.6 mm) ODS-Hypersil (5 μm) at 34°C.	Acetonitrile and aq. phosphate with sodium heptane-1-sulphonate	UV at 258 nm	Reverse phase ion-pair HPLC. Formulation containing penicillin procaine	133
(30 cm × 2.6 mm) ODS Sil-X-1 at 35°C	Aq. 0.1 mM H_2SO_4 (1 ml/min)	UV at 205 nm	Reversed phase HPLC Injections containing procaine.	134
—	Tetra-alkylammonium.	—	Reversed phase HPLC	135
(30 cm × 4.6 mm) Varian Micropak MCH-10 (10 μm)	Acetonitrile and 0.1M H_2SO_4 (1 ml/min)	UV 220 nm	IM injected procaine penicillin G from tissues of swine.	136

Table 8 (continued)

High-Performance Liquid Chromatography Systems Used in the Analysis of Procaine

10 cm × 5 mm Radial-Pack C_{18} (5 μm)	Methanol-water (1:1 or 2:3) containing 0.7% (v/v) butylamine, adjusted to pH 3 with H_2SO_4 (1 ml/min.)	Electrochemical vitrous-carbon electrode	Allows detection of procaine in illicit cocaine preparation.	137
(25 cm × 4.6 mm) Spherisorb A5Y.	Acetonitrile-methanol-0.01M tetramethylammonium hydroxide buffer (17:28:55) pH 6 (1 ml/min.).	UV 254 nm	Photoiodide-array detector. Used for forensive purposes	138
(25 cm × 5 mm) Spherisorb S5 ODS.	Aq. 80% methanol + ammonium acetate and potasium nitrate (1.2 ml/min.)	Amperometric	Reversed phase HPLC	139
(25 cm × 2.6 mm) Nucleosil 10 C_{18} at 20°C.	Methanol-0.1M acetate buffer of pH 4.5 (2:3) (0.2 ml/min.)	UV 285 nm.	Procaine and metabolite in injections	140
(25 cm 4.6 mm) Alcoa Unisphere alumina (8 μm).	Aq. 0.01M 4-morpholinopropane sulphonate (pH 7.4)-methanol (7:3) (2 ml/min.).	UV 220 nm.	Comparison of stationary phases for lipophilicity estimation.	141
(25 cm × 4.88 mm) Bondapak C_{18} (10 μm)	75% Acetic acid (0.8 ml/min.).	UV at 254nm	Plasma, the metabolite PABA is also determined.	142
Radial pak C_{18} (10 μm)	Acetonitrile-0.0165M triethylamine (17:3; adjusted to pH 3). (2 ml/min.).	UV at 288 nm.	Equine plasma and urine.	143

4.6.7 Electrophoresis

Pesakhovych reported the use of 1,3,5-trinitrobenzene as an electroosmosis and rheophoresis indicator in the analysis of electrophoretic spectra [146]. In experiments on the electrophoresis of procaine and other nitrogen-containing drugs (using tetraethylammonium iodide as a standard), it was shown that 1,3,5-trinitrobenzene and dextran blue were equally effective as electroosmosis and rheophoresis indicators. Since the former gives a circular zone, it was judged to be preferred. Reagents for the development of the various zones were given.

Jin and Zhou have determined 4-aminobenzoic acid in procaine injection solutions by electrophoresis on paper [147]. A 5 μL portion of the injection solution was diluted to 2 mg/mL in procaine hydrochloride, and applied to No. 1 paper (25 cm x 24 cm) for electrophoresis in a JMDY-WI apparatus. The system used a pH 3 buffer solution (9.76 g of citric acid and 1.03 g of sodium citrate in 100 mL), and a potential gradient of 20 V/cm applied for 20 minutes. The paper was then dried and sprayed with a solution containing 100 mL of ethanolic 2% *p*-dimethylaminobenz-aldehyde and 5 mL of anhydrous acetic acid. A standard solution containing 0.03 μg/μL of 4-aminobenzoic acid was used for comparison.

Hoyt and Sepaniak have used capillary zone electrophoresis to determine procaine in pharmaceuticals as a cation of benzylpenicillin [148]. A benzylpenicillin potassium tablet (250 mg) was treated with 20 mL of a 0.2% phenol solution (the internal standard), and dispersed in water. The solution was diluted to 500 mL, and samples were introduced into the fused silica capillary tube (70 cm x 50 gm) by siphoning. With 10 mM Na_2HPO_4-6mM $Na_2B_4O_7$ buffer as the mobile phase, the samples were subjected to electrophoresis at 30 kV (25 to 30 μA), and the emerging analytes detected at 228 nm within 10 minutes.

4.6.8 Capillary Isotachophoresis

Klein reported the use of a quantitative and qualitative determination of procaine in pharmaceutical preparations by capillary isotachophoresis [149].

Fanali *et al.* have described a capillary isotachophoresis method for the determination of procaine in pharmaceuticals [150]. The drug was determined in a 6 μL sample of solution (Spofa product, obtained from Czechoslovakia, and diluted 180-fold) by cationic isotachophoresis in the single column mode. The system used a PTFE capillary column (20 cm x 0.3 mm) and a conductivity detector. The separation was carried out at room temperature, at 50 μA (but switched to 25 μA during detection). The leading electrolyte was 2.8 μM ammonium acetate - acetic acid buffer (pH 4.9) containing 0.3% Triton X 100. 5 μM acetic acid served as the terminator.

Procaine and other local anesthetics were separated by Chmela *et al.* with a VLD isotachophoresis apparatus, equipped with coupled PTFE separatory (23 cm x 0.8 mm) and analytical columns (23 cm x 0.8 mm) [151]. In one system, the leading cation was K^+ (0.01 M) containing 0.05 poly(vinylalcohol), with acetate as the counter-ion. The pH of the leading electrolyte was 4.75. The terminating electrolyte was 0.03 M beta-alanine. Two other systems were also reported.

Kostelecka and Haller have determined procaine in mass-produced and extemporaneous pharmaceuticals by capillary isotachophoresis [152]. The method was carried out using pH 4.85 acetate buffer solution, and 0.01 M formic acid as leading and terminating electrolytes, respectively. Detection was effected with a conductivity detector. The method was used to determine procaine in mass-produced suppositories and ointments, and in locally prepared pharmaceutical solutions. The results were found to agree with those obtained using either spectrophotometry or polarography.

Isotachophoresis in open tubular fused silica capillaries, with on-column multi-wavelength detection, has been reported [153]. The system consisted of a 75 μm fused silica capillary of approximately 90 cm in length, together with a Model UVIS 206 PHD fast-scanning multi-wavelength detector with an on-column detector cell toward the capillary end. Cationic analysis was carried out on a mixture of procaine, ephedrine, and cycloserine, with scanning performed in the high-speed polychrome mode from 195 to 320 nm at 5 nm intervals. Purity control was carried out on the synthetic peptide *L*-histidyl-*L*-phenylalanine. It was found that sophisticated characterization of isotachophoric zones,

identification of compounds in these zones, investigation of zone purity, and confirmation of separation in isotachophoresis were all possible.

4.7 Determination in Body Fluids and Tissues

Seno *et al.* (93) have reported both the positive and the negative ion mass spectrometry, and the rapid isolation with Sep-Pak C_{18} cartridges of procaine and other local anesthetics [93]. The drug was isolated from blood and cerebrospinal fluid using solid-phase extraction and 9:1 chloroform-methanol as the eluent. The drug was separated by gas chromatography on a SPB-1 column (15 m x 0.53 mm, particle size of 1.5 μm), using a temperature program of 100°C to 300°C at 10°C / minute. Alternatively, a HP-17 column (10 m x 0.53 mm, particle size of 2 μm), with a temperature program of 140°C to 280°C at 10°C/minute was used. In either case, nitrogen was used as the carrier gas, and detection effected by means of flame ionization and mass spectroscopy. Mass spectrometry was obtained in the positive ion electron impact (EI), the positive ion chemical ionization (CI), and the negative ion chemical ionization (CI), modes.

Culea *et al.* reported a quantitative GC-MS analysis of procaine and some neurotransmitters in rat brain tissue [94]. Procaine was extracted from brain homogenates by the ultrasonication method of Sundlof *et al.* [95], and was determined in its underivatized form on a 24 m glass capillary column coated with Silar 10C (temperature programmed from 120°C to 225°C at 12°C/min with pyrene as the internal standard). It was found necessary to wash the injector liner and the GC-MS interface stainless steel tubing with 1:1 0.1 M KOH-methanol so that the interface tubing could be coated with a film of OV-17 (from acetone solution), and to condition the apparatus by injecting bis-(trimethylsilyl)-acetamide and triethylamine.

Culea *et al.* have described an isotope dilution mass spectrometric method for the determination of procaine in biological samples [96]. 1 mL of Liver homogenate was mixed with 0.2 mL of 1 M hydrochloric acid, 20 μL ^{15}N-procaine internal standard solution, and 2 mL ethyl acetate for 30 seconds. The mixture was centrifuged for 3 minutes and the supernatant solution discarded. The pH was adjusted to 11 by mixing with sodium

carbonate - potassium carbonate in benzene (2 mL) for 90 seconds, and then centrifuging the mixture. The organic phase was concentrated to 30 μL at 50°C. under argon. The concentrate (3 μL) was placed in the crucible of the direct inlet probe and the solvent evaporated. Electron impact mass spectrum (EIMS, 70 eV) was carried out with an ion-source temperature of 200°C, and ion-selective detection at m/e 86 and 87. The calibration graph was linear up to 40 μg/mL procaine. The detection limit was 10 μg/mL of procaine, and coefficient of variation was 5%.

5. Stability

Loucas *et al.* studied the stability of procaine hydrochloride in a buffered cardioplegic solution [154]. The time required for procaine to degrade to the lower shelf-life limit of 90% of its initial concentration was extrapolated to be approximately two days at room temperature, and eleven days under refrigeration.

Synave *et al.* (reported that the degradation of procaine in a cardioplegic solution containing magnesium, sodium, potassium, and calcium salts was temperature dependent [155]. At a storage temperature of 6°C, the shelf-life of the solution was 5 weeks. This increased to 9 weeks when the storage temperature was -10°C. Using carbon dioxide instead of nitrogen in the head space did not affect the stability of procaine [12].

Varea *et al.* described a study of the stability of procaine hydrochloride in cardioplegic solutions, prepared from Ringer's solution and electrolytes (both un-buffered, or buffered with sodium bicarbonate) [156]. The content of the drug was measured by ultraviolet spectrophotometry, and the was found to follow pseudo-first order kinetics. The stability of the drug entity in buffered solutions was estimated to be approximately 5-7 days.

6. Drug Metabolism and Pharmacokinetics

6.1 Absorption and Fate

Procaine is readily absorbed and is rapidly hydrolyzed in plasma by cholinesterase to *p*-aminobenzoic acid and diethylaminoethanol [12]. Some of the drug is metabolized in the liver, and about 80% of *p*-amino-

benzoic acid is excreted unchanged. The serum half-life of procaine was prolonged in newborn infants, patients with liver disease, and in some uremic patients [22]. Maximal hydrolysis of the drug is less in plasma from patients with renal failure than in a control group, due to the low concentration of plasma cholinesterase [23, 24].

6.2 Mechanism of Action

Procaine and the other local anaesthetic drugs prevent the generation and the conduction of the nerve impulses. Their main site of action is the cell membrane, since conduction block can be demonstrated in giant axons from which the axoplasm has been removed [25].

Local anesthetics block conduction by decreasing or preventing the large transient increase in the permeability of excitable membrane to that is produced by a slight depolarization of the membrane [26]. This local anaesthetic action is due to their direct interaction with voltage-sensitive Na^+ channels. As the anaesthetic action progressively develop in a nerve, the threshold for electrical excitability gradually increases. The rate of rise of the action potential declines, the impulse conduction slows, and the safety factor for conduction decreases. These factors decrease the probability of propagation of the action potential, and nerve conduction fails [25].

Raising the concentration of Ca^{2+} in the medium pathing, a nerve may relieve conduction block produced by local anesthetics. Relief occurs because Ca^{2+} alters the surface potential on the membrane, and hence the transmembrane electrical field. This, in turn, reduces the degree of inactivation of the Na^+ channels and the affinity of the latter for the local anaesthetic molecule [25, 27].

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SORBITOL

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Name

D-glucohexane-1,2,3,4,5,6-hexaol

1.1.2 Nonproprietary Names

D-glucitol
D-sorbitol
L-gulitol

1.1.3 Proprietary Names

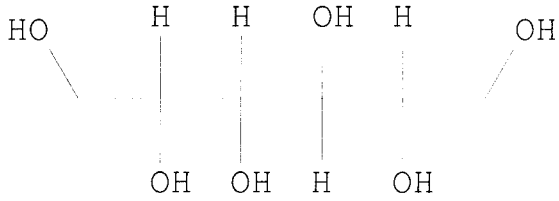
Sorbit	Cystosol
Resulax	Sorbilax
Sorbitur	Sorbo
Sorbostyl	Sorbilande

1.2 Formulae

1.2.1 Empirical

$C_6H_{14}O_6$

1.2.2 Structural



1.3 Molecular Weight

182.17 Daltons

1.4 CAS Number

50-70-4

1.5 Appearance

Sorbitol is an odorless powder that is white or almost colorless. Various grades based on particle size are available, and it is obtained in various forms such as flakes, granules, pellets, or powder.

1.6 Uses and Applications

Sorbitol is a hexahydric alcohol that is isomeric with mannitol, and which has been described as a humectant, plasticizer, sweetening agent, and tablet and capsule diluent. It is used extensively in the pharmaceutical, cosmetics, and food industries, since it has a sweet taste and approximately 50-60% the sweetness of sucrose.

In pharmaceutical applications, sorbitol is used as a tablet diluent in wet granulation or dry compression formulations. It is commonly used in chewable tablets because of its sweet taste, and it is also used as a plasticizer for gelatin in capsule formulations. Sorbitol is utilized in sugar-free liquid preparations and as a stabilizer for drug, vitamin, and antacid suspensions. When it is used in syrups, crystallization around bottle caps is prevented.

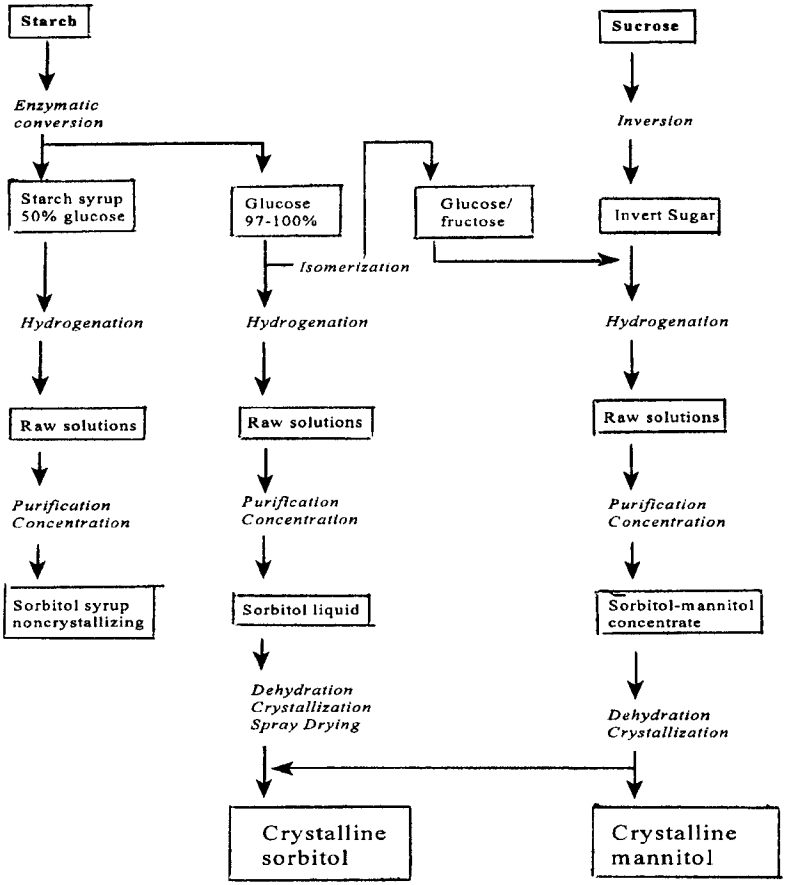
Sorbitol has also been used in injectable and topical products, and has a therapeutic use as an osmotic laxative.

2. Methods of Preparation

Sorbitol is a naturally occurring sugar found in a wide range of fruits. It was first isolated in 1872 from mountain ash berries, and the berries of the *Sorbus* genus are still the richest source of this chemical [3].

Commercial manufacture of sorbitol uses catalytic hydrogenation of glucose using either batch or continuous hydrogenation procedures. The manufacturing scheme is outlined in Scheme 1. The source of glucose is corn sugar or hydrolyzed starch [4]. The glucose is then hydrogenated

Scheme 1. Manufacturing procedure for sorbitol production.



above 120°C with hydrogen pressures of approximately 100 atmospheres employing a catalyst. Supported nickel or Raney nickel catalysts are usually used for the hydrogenation. The spent catalyst is collected on a filter and is separately regenerated for reuse. Reaction conditions can vary widely depending on the final grade of sorbitol solid or syrup being produced [3,5,6].

The sorbitol solution produced from hydrogenation is purified in two steps [4]. The first involves passing the solution through an ion-exchange resin bed to remove gluconate and other ions. In the second step, the solution is treated with activated carbon to remove trace organic impurities. The commercial 70% sorbitol solution is obtained by evaporation of the water under vacuum. The solid is prepared by dehydration until a water-free melt is obtained which is cooled and seeded. The crystals are removed continuously from the surface (melt crystallization). The solid is sold as flakes, granules, pellet, and powder forms in a variety of particle size distributions.

Instant sorbitol is made by spray drying a concentrated aqueous solution of sorbitol [2].

3. Physical Properties

To acquire information on the physical properties of various grades of sorbitol, a variety of materials were characterized. The characteristics of these, as supplied by the vendor, are summarized in Table 1. Grades with various particle size distributions were chosen for comparison.

3.1 Particle Morphology

Various particle morphologies have been reported using scanning electron microscopy (SEM) [7,25,26]. The γ-form can exhibit morphologies of dense irregularly shaped particles, or of needle-like particles that result in a more loose-knit matrix [7,25]. Instant sorbitol was found to be an

Table 1

Identity and Vendor Characteristics of the Sorbitol Samples Analyzed in this Profile

Type of Sorbitol	**Vendor**	**Vendor Particle Size Specifications**	**Lot**
Instant sorbitol	Pharma		M770303
Crystalline sorbitol fines, NF	ICI Polyols	minimum of 95% through 100 mesh	4405K5
Crystalline sorbitol 712, NF	ICI Polyols	maximum 0.5 % on 30 mesh maximum 5% on 40 mesh maximum 30% through 200 mesh	5131D3
Crystalline sorbitol 834, NF	ICI Polyols	maximum 0.5% on 30 mesh maximum 5% on 40 mesh maximum 12% through 200 mesh	5024A5
Crystalline sorbitol 2016, NF	ICI Polyols	maximum 0.5% on 30 mesh maximum 5% on 40 mesh maximum 12% through 200 mesh	5220G5
Crystalline sorbitol 1162, NF	ICI Polyols	maximum 5% on 16 mesh maximum 25% on 20 mesh maximum 5% through 200 mesh	4370K5

Note: The crystalline sorbitol samples are listed from finest grades at the top of the table to the coarsest grades at the bottom of the table.

agglomeration of irregular particles characterized by the presence of rough surfaces [26].

SEM images collected on the sorbitol samples of this study were found to be in agreement with the literature reports, with selected lots being shown in Figure 1. At the magnification used, the differences in particle size are apparent for the various lots. For instant sorbitol (lot M770303), the agglomeration of the material is obvious. In contrast, the crystalline sorbitol samples exhibit dense, irregularly shaped particles with semi-smooth surfaces.

Photomicrographs taken at higher magnifications of representative surfaces of instant sorbitol and crystalline sorbitol particles are presented in Figure 2. For the instant sorbitol lot, the surface appears porous and comprised of needle-like particles. The surface of crystalline sorbitol, however, exhibits some roughness and some fine particles, but the overall surface is compact and non-porous.

3.2 Crystallographic Properties

3.2.1 Polymorphism and Single Crystal Structure

Sorbitol exhibits a number of different crystal forms and there is little agreement in the literature concerning the number of polymorphs, the existence of hydrated species, melting points of the various forms, and even nomenclature [7-14]. Much of the data in the literature was collected on commercial powders that were not fully described, or whose history is not fully known. Table 2 attempts to summarize the various forms based on reports by DuRoss [7] and Quinquenet *et al.* [8].

It has been reported that the γ-form, which melts at approximately 101°C, is the thermodynamically most stable form at room temperature and that the sorbitol system is monotropic [8]. Studies of the various forms show that upon standing or upon stress conditions, sorbitol will convert to the γ-form [7]. However, a solution calorimetry study performed on selected crystal forms of sorbitol reports that sorbitol hydrate is the most stable form followed by the γ-form [18].

Figure 1a. Scanning electron microscopy images, obtained at 50X magnification, for sorbitol lots (a) M770303, (b) 4405K5, and (c) 5131D3.

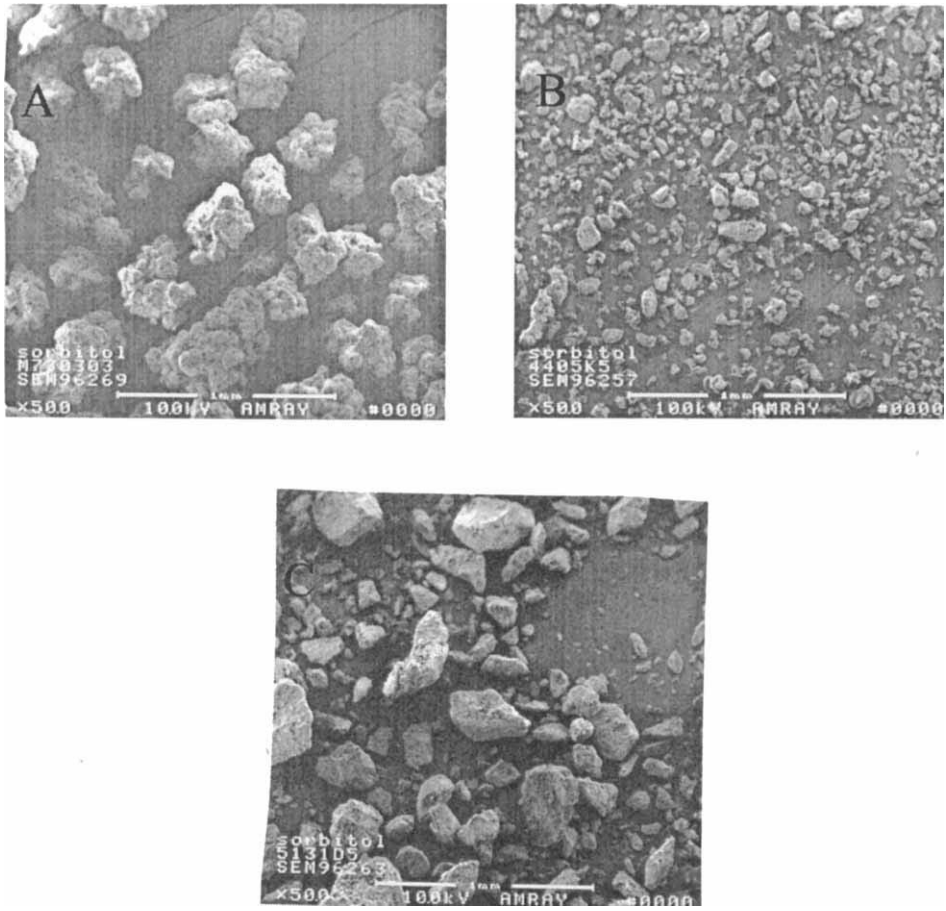


Figure 1b. Scanning electron microscopy images, obtained at 50X magnification, for sorbitol (d) 5024A5, (e) 5220G5, and (f) 4370K5.

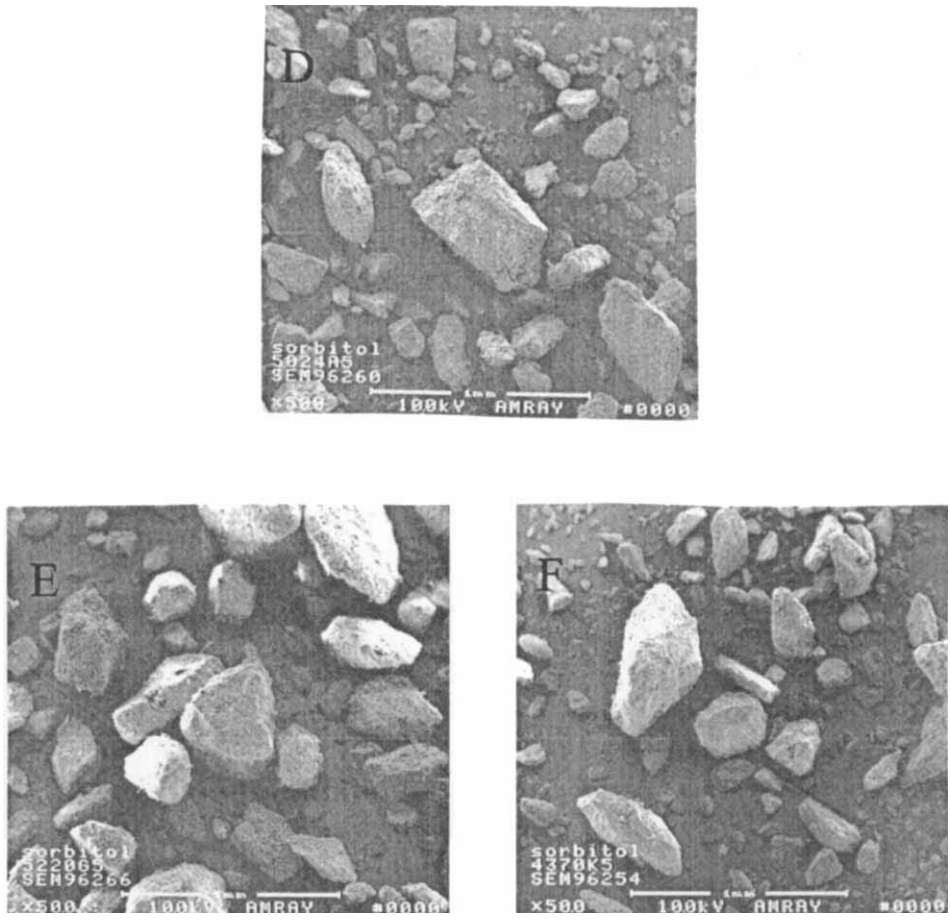


Figure 2. Scanning electron microscopy images, obtained at 400X magnification, for sorbitol lots M770303 (upper) and 4370K5 (lower).

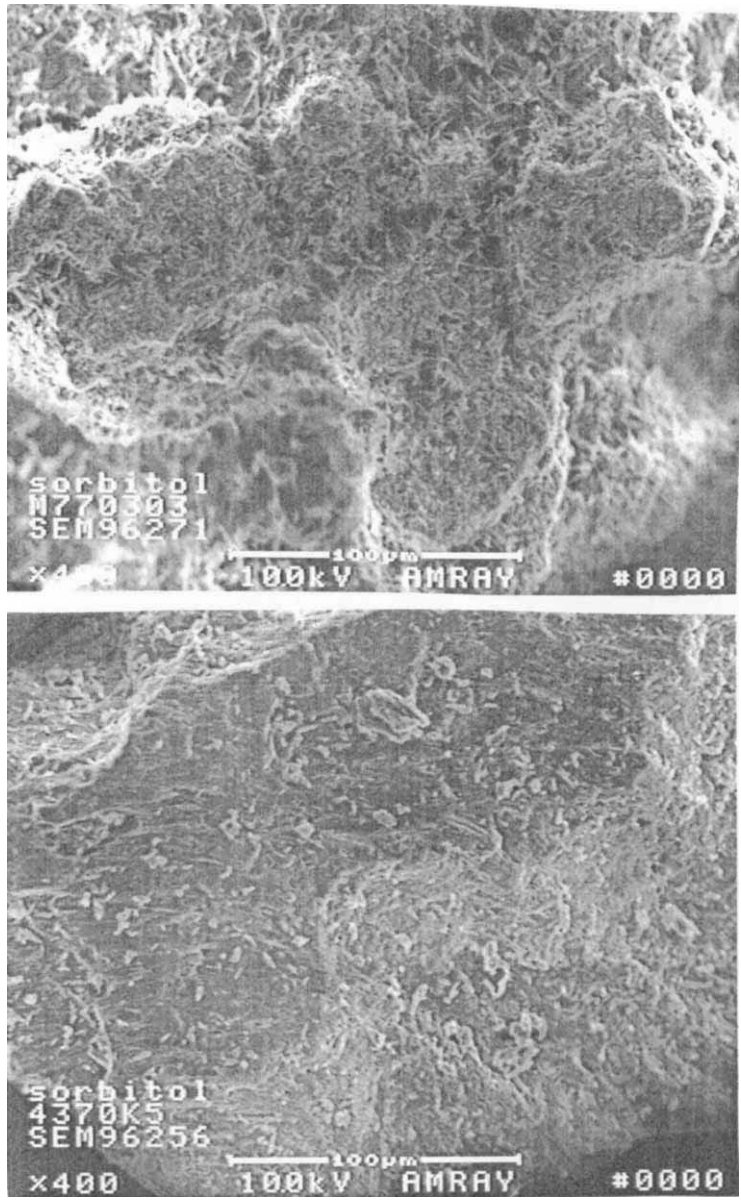


Table 2. Summary of Sorbitol Crystal Forms [7,8]

Property	**Form**													
	E		**α**		**δ**		**β**		**γ**		**solidified melt**		**hydrate**	
DSC peak (°C)														
maximum	75/83		88		89		98		101		nd		nd	
onset	68		85		86		95		99		47/67		50	
Melting range (°C)	77		90-92		87-89		94-95		99-102		47		50	
Density (g/mL) flotation	nd[c]		1.550		1.460		1.481		1.527		nd		nd	
Water solubility[a]	252		nd		252		216		211		nd		nd	
X-ray powder diffraction[b]	d	I/I_o[b]	d	I/I_o	d	I/I_o	d	I/I_o	d	I/I_o	d	I/I_o	d	I/I_o
	4.73	100	4.22	100	4.23	100	3.95	100	4.73	100	4.02	100	5.26	100
	3.50	67	3.93	75	3.71	66	5.95	80	3.49	67	4.49	94	3.92	98
	4.04	60	4.33	70	4.77	64	4.66	74	4.04	61	3.70	43	4.11	89
	6.00	45	2.65	51	2.12	53	4.98	50	3.91	53	5.16	40	4.31	60
	7.57	40	3.78	41	2.64	44	4.24	42	4.33	50	2.70	40	8.48	48

[a] water at 20(°C (parts per 100 parts water)

[b] d-spacings (Å) for 5 strongest peaks in powder pattern, I/I_o = relative intensity

[c] nd-not determined

A single crystal structure has been reported only for form A, which was crystallized from ethanol [9]. The space group was $P2_12_12_1$, and contained 4 molecules in the unit cell. The unit cell parameters were reported as a= 8.677(5), b=9.311(8), and c=9.727(4), with 1 molecule in the asymmetric unit. The molecule has a bent chain conformation with one carbon out of the plane of the other five carbon atoms, which are coplanar. The molecules stack with their chain axes approximately parallel.

3.2.2 X-Ray Powder Diffraction Pattern

The commercial crystal form produced after 1975 is the γ-form. XRPD powder patterns collected on the various sorbitol samples listed in Table 1 are in agreement with the reported γ-form [15], and are shown in Figure 3. The listing of peak positions, d-spacings, and relative intensities for this crystal form is given in Table IV.

3.3 Optical Rotation

The specific rotation ($[\alpha]^{25}_D$) of sorbitol is -1.9° for an aqueous solution having a concentration of in 0.1g/ mL [2].

The optical rotation of aqueous solutions will increase to + 4.0 to +7.0° by the addition of complexing salts, such as borax or ammonium molybdate.

3.4 Thermal Methods of analysis

3.4.1 Melting Behavior

Thermal analysis, such as differential scanning calorimetry (DSC) and differential thermal analysis (DTA), has been widely used to characterize the various forms of sorbitol [7,8,12,14,16-18], although some of the reported forms lack substantiating data such as x-ray powder diffraction. Selected melting points from the literature are summarized in Table 2. Mixtures of selected forms are easily distinguished using DSC [7], and sorbitol can be distinguished from other sugars and sweetening agents based on its thermal properties [14,16].

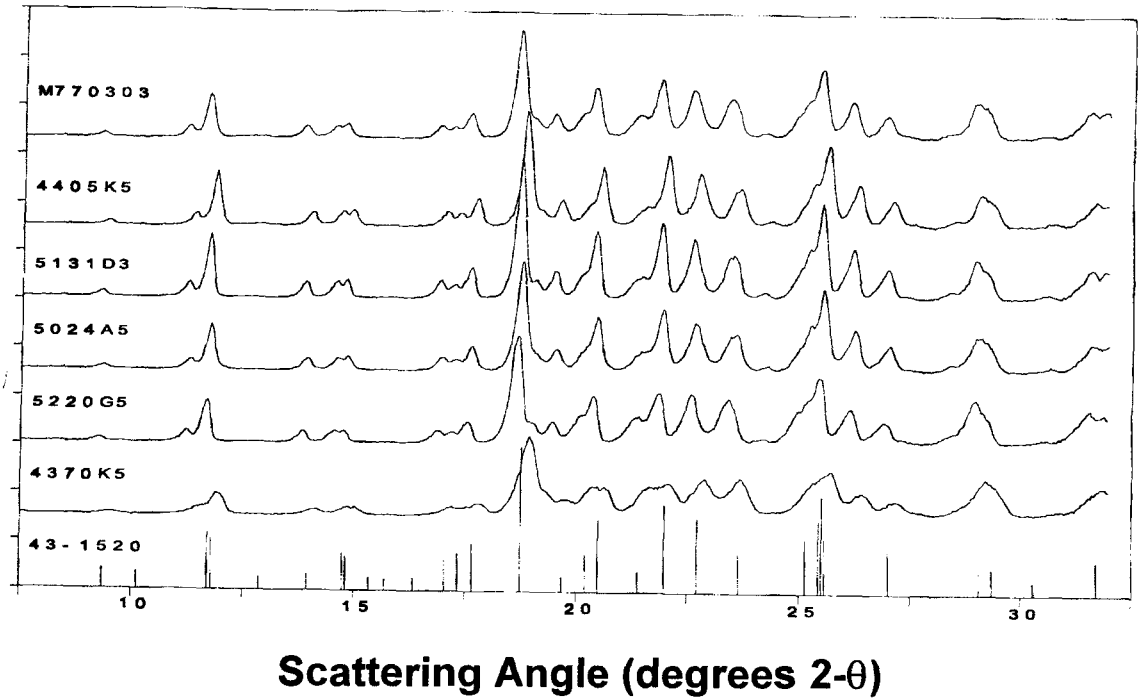


Figure 3. X-ray powder diffraction patterns of various sorbitol lots. The stick plot at the bottom of the figure represents the peak positions and intensities for γ-sorbitol from ICDD card 43-1520 (as listed in Table 3).

Table 3
X-Ray Powder Diffraction Data for the γ-Form of Sorbitol [15]

Peak Position (2-θ)	d-spacing (Å)	I/I_0 [a]
9.36	9.44	14
10.14	8.72	12
11.71	7.55	39
12.87	7.49	35
13.96	6.87	9
14.75	6.34	11
15.34	6.00	26
15.70	5.97	23
16.34	5.77	9
17.03	5.64	8
17.34	5.42	9
18.75	5.20	21
19.67	5.11	26
20.21	5.02	33
20.50	4.73	100
21.39	4.51	11
21.98	4.39	26
22.72	4.33	50
23.64	4.15	15
25.14	4.04	61
25.43	3.91	53
25.50	3.76	30
25.58	3.54	38
26.99	3.50	50
29.06	3.49	67
29.36	3.48	38
30.27	3.30	30
30.32	3.07	18
31.70	3.04	18
33.89	2.95	9
35.05	2.946	6

[a] Relative intensity for fixed slit

3.4.2 Differential Scanning Calorimetry

DSC data for the γ-form samples analyzed in this study are summarized in Table 4. The DSC curves for the materials, collected at 10°C/min, exhibited a major endothermic transition maxima ranging from 96.9 to 102.6°C, which is in agreement with literature values for this form [7,8, 17]. A representative DSC curve is presented in Figure 4. This thermal event was confirmed as a melting transition for all samples using hot stage microscopy. A partial explanation for the somewhat wide temperature range is the variation in particle size for the sorbitol samples, which is known to shift the melting endotherm [19]. For the majority of samples, the smallest particle size lots melted at lower temperatures, with the melt temperature increasing with larger size materials.

3.4.3 Glass Transition Temperature

The glass transition temperature of sorbitol has also been studied using thermal analysis, and temperatures ranging from 0 to -55°C have been reported [8,20-24]. The temperature values were dependent on heating rate, history of the sample, and pressure. The extensive variation in conditions explains the wide range of temperatures reported for this parameter.

3.5 Hygroscopicity

The γ-form is known to be anhydrous, and contains minimal water. The USP water specification for sorbitol requires that a sample contain less than or equal to 1.0% water in the sample [1]. The thermogravimetry (TG) and Karl Fisher (KF) data for the γ-form sorbitol samples analyzed in this study are summarized in Table 4. TG analysis indicated the existence of minimal volatile content in the samples, indicating that the materials were anhydrous. The volatile component was identified as water, based on the KF values obtained for the samples.

The hydrated form of sorbitol has been reported to contain 6-7% water, which is equivalent to 0.6 moles of water per sorbitol molecule [8,18].

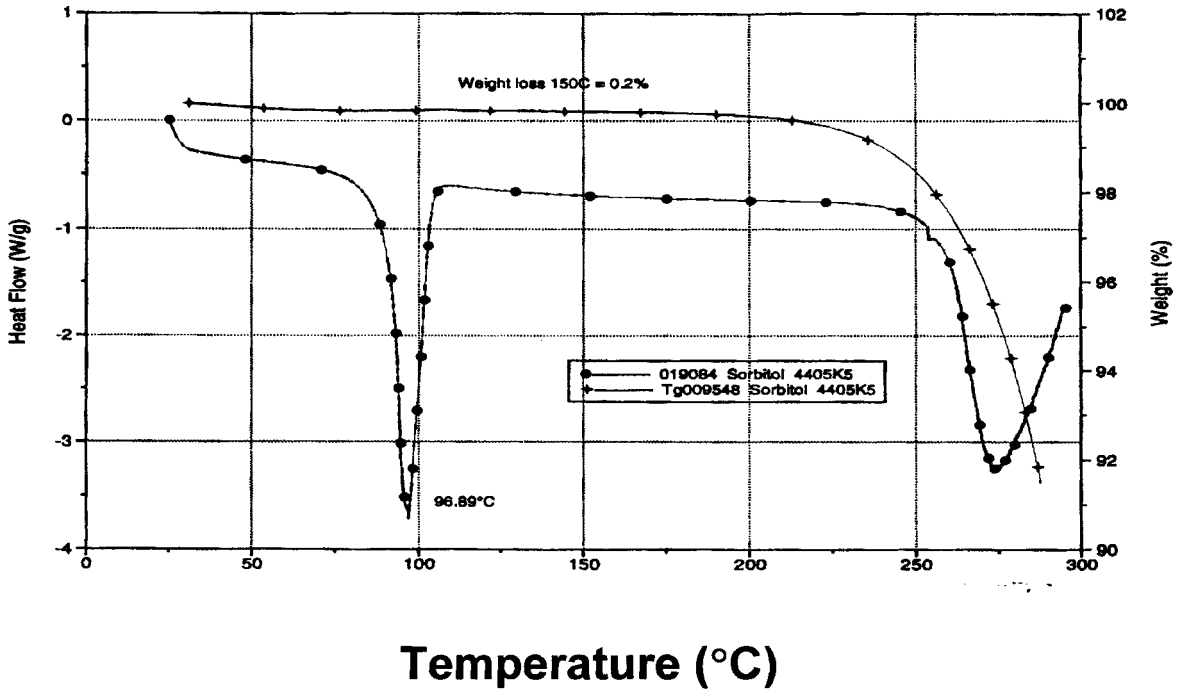


Figure 4. Representative TG (top) and DSC (bottom) curves for sorbitol, lot 4405K5.

Table 4

DSC and TG Data for Sorbitol Samples

Type of Sorbitol	Lot	KF Titration Value (%)[a]	Total Volatile Content by TG (%)	DSC peak maximum (°C)
Instant sorbitol	M770303	0.43	0.42	101.2
Crystalline sorbitol fines, NF	4405K5	0.49	0.23	96.9
Crystalline sorbitol 712, NF	5131D3	0.56	0.43	98.9
Crystalline sorbitol 834, NF	5024A5	0.51	0.39	101.3
Crystalline sorbitol 2016, NF	5220G5	0.45	0.25	102.6
Crystalline sorbitol 1162, NF	4370K5	0.38	0.33	99.0

[a]Data collected by BMS Analytical R&D

The amount of water adsorbed by a solid can be expressed in a number of ways. Many investigators report the amount of water sorbed, but do not taken into account the amount of water initially in the sample. The calculation of percent moisture uptake relative to the dry weight of the sample normalizes samples to the same initial point, and makes data from various samples comparable. The percent sorbed relative to dry weight is calculated from the following:

$$Weight\ \% = \frac{W_1 - [W_0 - (W_0 x \frac{A}{100})]}{[W_0 - (W_0 x \frac{A}{100})]} x\,100$$

where: W_1 = weight of sample at equilibration
W_0 = original weight of sample
A = percent moisture in original sample

A second method of reporting data is the equilibrium moisture content (EMC) [1,31], which is calculated using the following equation:

$$EMC = \frac{P}{P + 100} x\,100$$

where:

$$P = \frac{[W_0 x \frac{A}{100}] \pm B}{W_0 - [W_0 x \frac{A}{100}]} x100$$

and:
P = % moisture dry basis
B = weight change at equilibrium

The hygroscopicity of the commercial sorbitol samples was investigated with an automated moisture balance system, where the relative humidity (RH) could be varied over the range of 0 to 90% [32]. EMC sorption values were calculated from the raw data at each relative humidity and are summarized in Table 5, and moisture uptake curves for the sorbitol

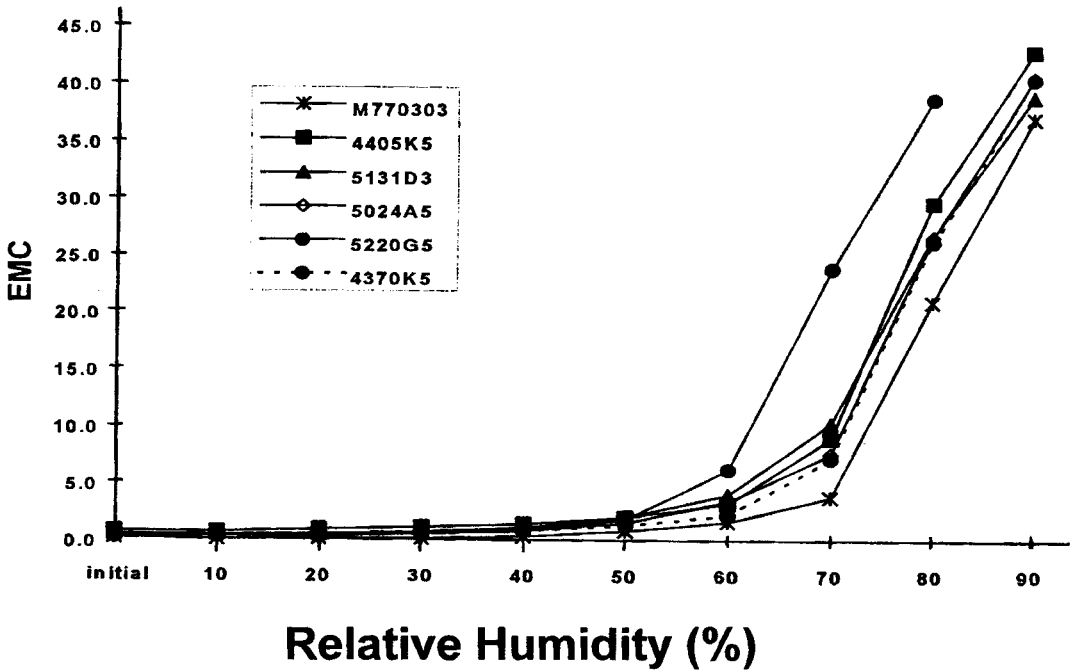


Figure 5. Moisture uptake isotherms for sorbitol samples.

Table 5

Sorption EMC Values for Sorbitol at Various Relative Humidities

Lot	**Approximate Relative Humidity (%)**									
	initial	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
M770303	0.1	0.0	0.1	0.1	0.3	0.8	1.6	3.8	20.9	37.0
4405K5	0.7	0.7	0.9	1.1	1.4	2.0	3.2	9.0	29.6	42.8
5131DS	0.4	0.4	0.5	0.7	1.1	2.0	4.0	10.3	26.7	38.9
5220G5	0.2	0.2	0.3	0.4	0.6	0.9	1.8	6.2	23.8	38.7
5024A5	0.3	0.3	0.3	0.5	0.8	1.5	3.4	7.6	26.6	40.5
4370K5	0.3	0.3	0.4	0.5	0.8	1.3	2.2	7.2	26.2	40.4

samples are given in Figure 5. Significant moisture sorption was evident by 60% RH for all samples. Above this humidity value, the material continually sorbs water until deliquescence takes place. These findings are in agreement with reported hygroscopicity data for sorbitol materials [1,33].

3.6 Solubility Characteristics

Sorbitol is readily soluble in water, exhibiting a solubility of 2.56 g/mL H_2O at 25°C [1]. It is practically insoluble in chloroform and ether and slightly soluble in methanol [1]. Solubilities in water, and in various ethanol / water mixtures, are given in Table 6.

Sorbitol is stable in cold dilute acids and bases, but will form water soluble chelates with many divalent and trivalent metal ions in strongly acidic and basic media. Solutions of sorbitol will react with iron oxide and become discolored.

3.7 Partition Coefficient

No partition coefficient data have been reported for sorbitol, but a theoretical log P value of -4.96 ± 0.38 has been obtaining using the predictive program of Advanced Chemistry Development Laboratories [51]. This finding indicates the extreme hydrophilic nature of the compound.

3.8 Micromeritic Properties

3.8.1 Particle Size Distribution

Various grades of sorbitol are available that differ in their particle size distribution. Most of the particle size measurements reported for sorbitol are by sieve methods [1,7,26,27], and permit direct comparison with the vendor's specifications. For the samples used in this study, the vendor specified sieve limits for the various grades are listed in Table 1.

Table 6

Solubility of Sorbitol in Water and Ethanol [1]

Solvent	Solubility at 25°C
Ethanol (95%)	1 in 2.5
Ethanol (82%)	1 in 8.3
Ethanol (62%)	1 in 2.1
Ethanol (41%)	1 in 1.4
Ethanol (20%)	1 in 1.2
Ethanol (11%)	1 in 1.14
Water	1 in 0.5

Particle size was also measured on the various lots using optical microscopy (25X magnification) and an image analysis system, and the data are summarized in Table 7. The instant sorbitol (lot M770303) exhibited the largest particles while the crystalline sorbitol fines (lot 4405K5) exhibited the smallest particles. The particle sizes measured for the remaining crystalline sorbitol lots did not vary substantially for the primary particles used in the analysis. The particle size distribution of the fine particles measured with image analysis, given in Figure 6, showed minimal variations in the distribution of the fine particles below 90 μm in length. The instant sorbitol lot contained significantly fewer particles in this particle size range when compared to the other sorbitol lots.

3.8.2 Surface Area

Surface area measurements reported for sorbitol powders have been obtained using the BET method of nitrogen sorption [7,26,28]. Values ranged from 0.4 to 1.5 m^2/g can be obtained depending on the vendor and grade of material.

Surface area measurements collected on the sorbitol samples in this study are summarized in Table 7. A 5-point BET method was used, with the samples being outgassed under vacuum at ambient temperatures. The surface areas range from 0.9 to 2.2 m^2/g. The highest surface area value was obtained for the crystalline fines (lot 4405K5), which also exhibited the smallest particle size. The lowest surface area was found for the sample for which 95% of the particles were less than 66.3 x 105.6 μm, which is the largest value particle size of any of the crystalline sorbitol samples. The other three crystalline sorbitol lots showed similar surface area values, which agree with the mean particle sizes as measured by optical microscopy. The instant sorbitol exhibited a high surface area of 1.2 m^2/g considering the large particle size of the material. This would indicate the existence of a more porous material, which was noted in the SEM data.

Table 7

Particle Size and Surface Area Data for the Sorbitol Lots

Type of Sorbitol	**Lot**	**Mean Particle Size (μm)**	**Particle Size 95% Less Than (μm)**	**Surface Area (m^2/g)**
Instant sorbitol	M770303	233.6 x 321.3	506.7 x 696.9	1.2
Crystalline sorbitol fines, NF	4405K5	20.3 x 32.9	46.6 x 75.3	2.2
Crystalline sorbitol 712, NF	5131D3	26.3 x 40.8	63.4 x 98.2	1.2
Crystalline sorbitol 834, NF	5024A5	25.1 x 40.0	66.3 x 105.6	0.9
Crystalline sorbitol 2016, NF	5220G5	21.9 x 34.7	55.1 x 87.5	1.6
Crystalline sorbitol 1162, NF	4370K5	23.0 x 36.7	55.7 x 88.7	1.4

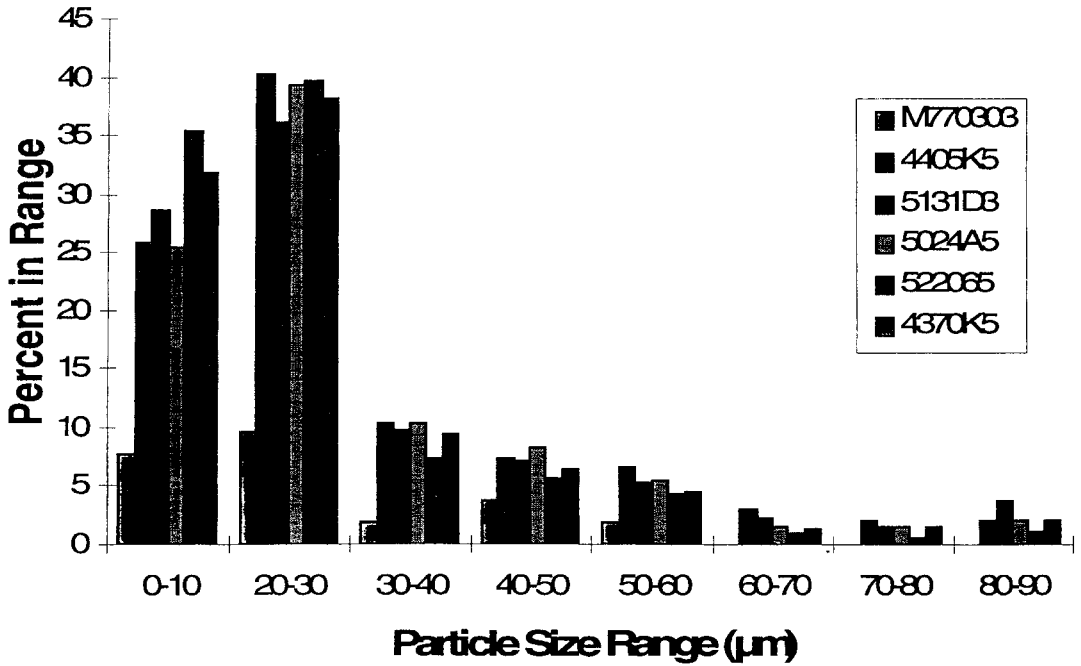


Figure 6. Particle size distributions of sorbitol samples obtained using optical microscopy and image analysis.

3.8.3 Bulk Powder Properties

Bulk powder properties are important in understanding the handling properties of an excipient or a granulated product. Common parameters measured are bulk and tap density. From these values the compressibility can be calculated using the following:

$$100 \times \frac{(tap\ density - bulk\ density)}{tap\ density} = \%\ compressibility$$

The classification system introduced by Carr [29,30] was used to evaluate the flow properties of the sorbitol powders. In Carr's system, a flowable powder is defined as free flowing and will tend to flow steadily and consistently. This is to be contrasted with a floodable powder, which will exhibit an unstable, discontinuous, and gushing type of flow. The parameters in Carr's system include the angle of repose, angle of spatula, compressibility, cohesion, and dispersibility. Based on these parameters, flowability and floodability indices are calculated to determine the handling properties of bulk solids.

The sorbitol samples were characterized with Carr's system, using a Hosokawa Powder Characteristics Tester to acquire the data summarized in Table 8. The parameters used to obtain the flowability index are compressibility (from bulk and tap density), angle of repose, and angle of spatula. The flowability indices ranged from 41 to 64, with the highest flowability index for the instant sorbitol, indicating that it should exhibit the best handling properties. The lowest index was calculated for crystalline sorbitol lot 4405K5, indicating that this lot will would exhibit more difficult handling properties. This lot also contained the highest number of fine particles, which contributed to the poor flow properties.

The floodability index was calculated from the dispersibility, cohesion, angle of fall, and flowability index parameters. The values for this property ranged from 55 to 76. The most floodable material was lot 4405K5, and the least floodable material lot was the instant sorbitol, which is in agreement with the flow index and the particle size data. The

Table 8

Bulk Powder Characteristics Data for Sorbitol Lots

Property	**M770303**	**4405K5**	**5131D3**	**5024A5**	**5220G5**	**4370K5**
True Density (g/cc)	1.47	1.48	1.47	1.47	1.48	1.47
Bulk Density (g/cc)	0.46	0.31	0.59	0.61	0.60	0.69
Tap Density (g/cc)	0.48	0.53	0.81	0.80	0.76	0.81
Compressibility (%)	4.5	41.9	26.7	23.5	21.3	15.2
Dispersibility	9	7	9	9	9	9
Cohesion	88	4	42	40	38	58
Angle of Repose (°)	33	48	42	46	44	45
Angle of Spatula (°)	40	63	67	59	52	55
Angle of Fall (°)	30	31	29	29	33	34
Angle of Difference (°)	3	17	13	17	11	11
Flowability Index	64	41	52	54	60	56
Floodability Index	55	76	67	70	63	58
Mass Flow Rate (g/sec)	4.4	0.1	2.5	3.5	5.3	5.5

remaining crystalline sorbitol lots exhibited similar flowability and floodability properties.

The mass flow rates of the materials were also measured, and ranged from 0.1 to 5.5 g/sec. Lot 4405K5 was found to exhibit the poorest flow rate, as would be expected from the flowability index, floodability index, and particle size data. The four crystalline sorbitol lots (5131D3, 5024A5, 5220G5, and 4370K5) showed an increase in the flow rate as the grade became more coarse, indicating a dependence on particle size. The instant sorbitol exhibited a flow rate of 4.4 g/sec.

3.9 Spectroscopy

3.9.1 Vibrational Spectroscopy

3.9.1.1 Infrared Absorption Spectrum

Diffuse reflectance infrared spectra were acquired for the sorbitol samples. As shown in Figure 7, identical IR spectra were observed for all materials. The IR spectral band assignments are presented in Table 9, and the measured absorbance bands are consistent with the structure of sorbitol.

3.9.1.2 Raman Spectrum

The Raman spectra obtained for the sorbitol samples are shown in Figure 8, with identical spectra being obtained for each. The Raman spectral assignments are presented in Table 10, and the measured Raman bands are consistent with the sorbitol structure.

3.9.2 ^{13}C Solid-State Nuclear Magnetic Resonance Spectrometry

Solid-state ^{13}C variable-amplitude cross polarization magic-angle spinning (VACP/MAS) nuclear magnetic resonance (NMR) spectra were acquired for the sorbitol samples. Proton decoupling was achieved by a two-pulse phase modulation (TPPM) sequence. Identical ^{13}C spectra were measured for the γ-form sorbitol samples, and a representative spectrum is shown in Figure 9.

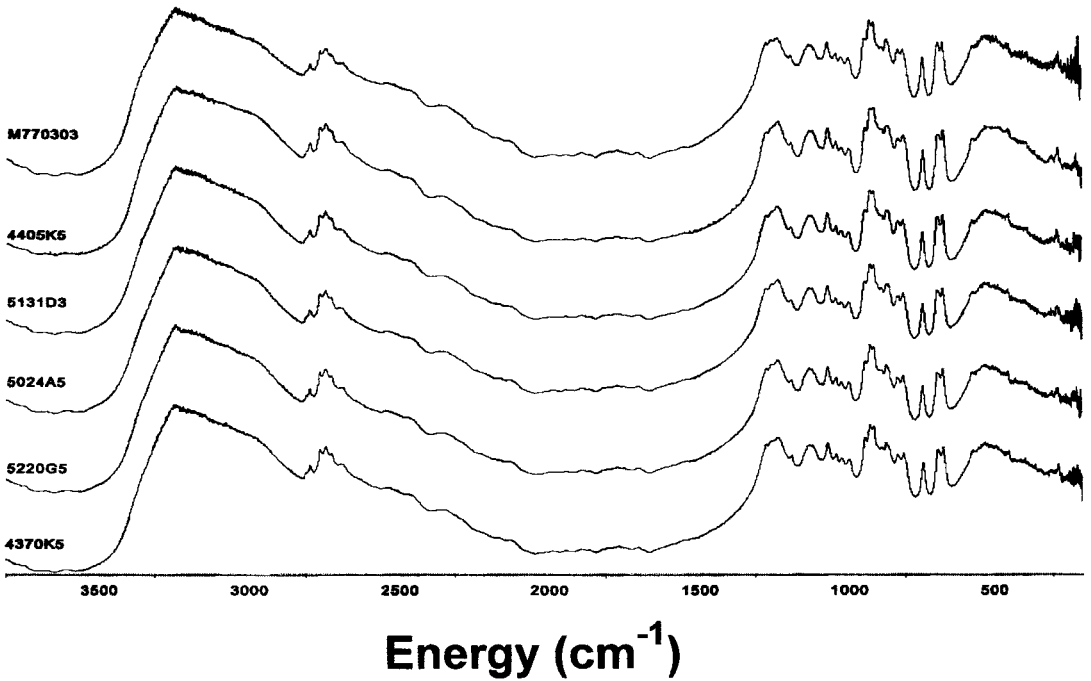


Figure 7. Infrared spectra, obtained using diffuse reflectance, of various sorbitol samples.

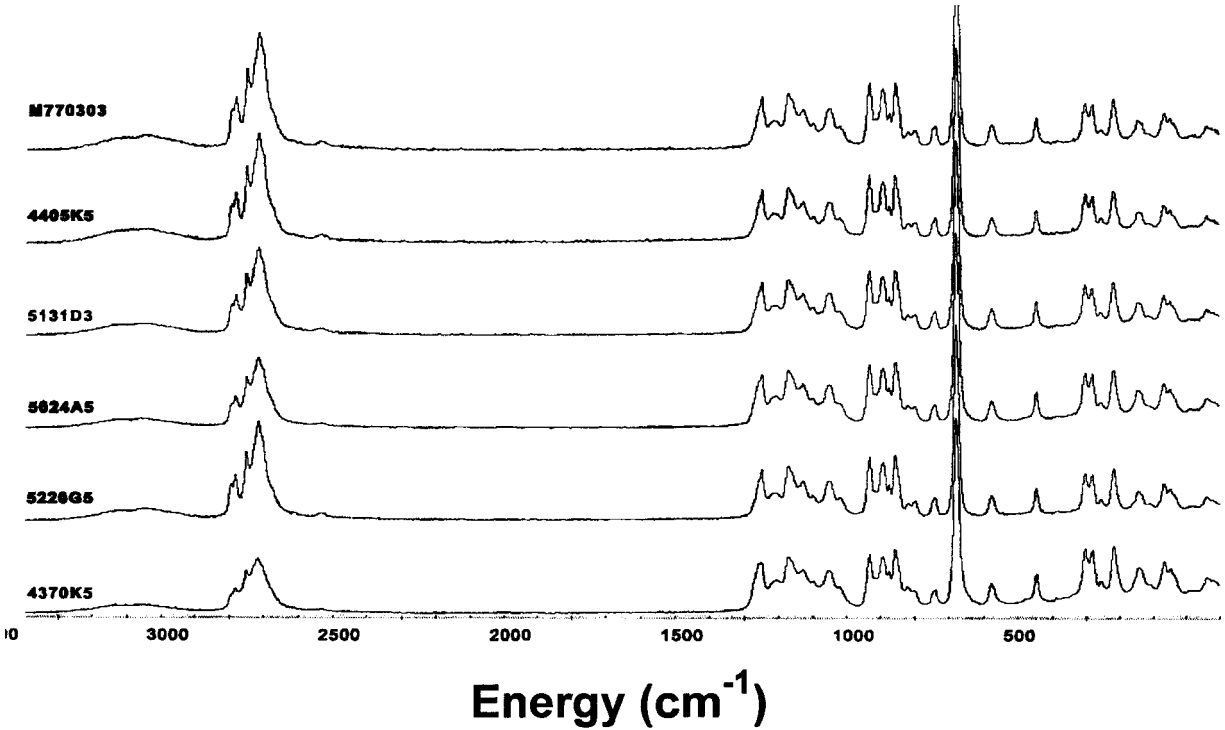


Figure 8. Raman spectra of various sorbitol samples.

Table 9

Assignments for the Major Peaks Noted in the Infrared Absorption Spectrum of Sorbitol

Energy (cm^{-1})	Structural Assignment
3430	OH stretch
2985, 2952, 2933	CH stretch
1422, 1378	CH bend
1116, 1105	C-O-C stretch
873	C-O stretch

Table 10

Assignments for the Major Peaks Noted in the Raman Spectrum of Sorbitol

Raman shift (cm^{-1})	Structural Assignment
~3240	OH stretch
2984, 2952, 2917	CH stretch
1449, 1371, 1328	CH bend
1132, 1093, 1056	C-C stretch
879	C-O stretch

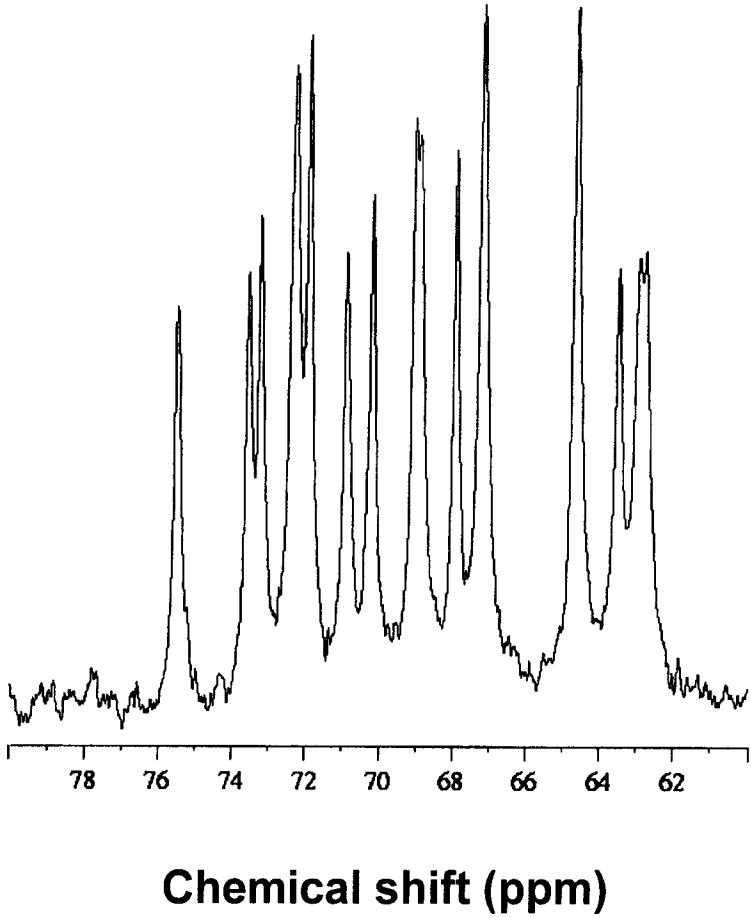


Figure 9. ^{13}C VACP/MAS nuclear magnetic resonance spectrum of sorbitol, lot 4370K5.

Even though there is no single structure available for the γ-form, solid-state NMR clearly identifies the presence of at least 3 molecules per asymmetric unit. This is a consequence of observing at least 15 distinct lines, which can be integrated into 18 peaks (3 molecules/asymmetric unit with 6 carbons/molecule). The resonance positions and structural assignments for the solid-state ^{13}C-NMR spectrum of sorbitol have not yet been fully assigned.

4. Methods of Analysis

4.1 Compendial Tests

The compendial tests given here are for sorbitol solid. Separate tests for sorbitol solution USP are listed separately in The National Formulary [34].

Sorbitol should contain not less than 91.0%, and not more than 100.5%, of $C_6H_{14}O_6$ calculated on an anhydrous basis. The sample may contain small amounts of other polyhydric alcohols.

The National Formulary [34] contains the following assays for sorbitol solid:

> **Identity:** The infrared absorption spectrum of a potassium bromide (KBr) dispersion exhibits maxima only at the same wavelengths as that of a similar preparation of USP sorbitol reference standard.
>
> **Water:** Following test method I <921>, the sample contains not more than 1.0%.
>
> **Residue on ignition:** Following test method <281>, the weight of the residue can not exceed 0.1%.
>
> **Chloride:** Following test method <221>, a 1.5 g sample shows not more than 0.0050% which corresponds to 0.10 mL of 0.020 N hydrochloric (HCl) acid.

Sulfate: Following test method <221>, a 1.0 g sample shows not more than 0.010% which corresponds to 0.10 mL of 0.0220 N sulfuric (H_2SO_4) acid.

Arsenic: Following method II <211>, the limit is 3 ppm arsenic.

Heavy metals: Following test method <231>, the limit is 0.001% when 2.0 g of sorbitol is dissolved in 25 mL of water.

Reducing sugars: Accurately weigh 7 g of sorbitol and transfer to a 400 mL beaker with 35 mL of water and mix. Add 50 mL of alkaline cupric tartrate test solution, and cover the beaker. Heat so that it boils in approximately 4 minutes and boil for 2 minutes. Collect the precipitated cuprous oxide in a tared filtering crucible previously washed with hot water, alcohol, and ether, and then dried at 105°C for 30 minutes. Wash the cuprous oxide on the filter with hot water, then with 10 mL of alcohol, and then 10 mL of ether. Dry at 105°C for 30 minutes. The weight of the cuprous oxide should not exceed 50 mg.

Total sugars: In a 250 mL flask fitted with a ground glass joint, place 2.1 g of sorbitol and add 40 mL of 0.1 N HCl. Attach a reflux condenser and reflux for 4 hours. Transfer the solution to a 400-mL beaker, rinsing the flask with about 10 mL of water and neutralize with 6 N sodium hydroxide (NaOH). Go to test for **Reducing sugars** and begin with "add 50 mL of alkaline cupric tartrate test solution". The weight of the cuprous oxide should not exceed 50 mg.

Assay:

Mobile phase: use degassed water.

Resolution solution: dissolve mannitol and USP sorbitol reference standard in water to obtain a solution having a concentration of approximately 4.8 mg/mL of each.

Standard preparation: make a solution of USP sorbitol reference standard having a known concentration of approximately 4.8 mg/mL.

Assay preparation: Transfer approximately 0.24 g of sorbitol to a 50 mL volumetric flask, dissolve in a 10 mL of water, dilute with water to volume and mix.

Chromatographic system (follow Chromatography test method <621>): the liquid chromatograph (LC) is equipped with a refractive index detector maintained at a constant temperature. A 7.8 mm X 30 cm column with L19 packing maintained at 30 ± 2°C is used with a flow rate of approximately 0.2 mL/min. Chromatograph the standard preparation by separately injecting equal volumes (approximately 20 µL) of the assay preparation and the standard preparation into the chromatograph and measure the responses for the major peaks. Calculate the quantity, in mg, of $C_6H_{14}O_6$ in the sorbitol using the following formula:

$$50C\ (r_u/r_S)$$

where C is the concentration in mg/mL of USP sorbitol reference standard in the standard preparation and r_u and r_S are the peak responses obtained from the assay preparation and standard preparations, respectively.

4.2 Chromatographic Methods of Analysis

A number of chromatographic methods have been reported for sorbitol [35-38], and will be summarized in this section.

A novel chromatography column of a silica based iminodiacetic acid bonded phase has been used for the HPLC analysis of sugars, sugar alcohols, and amino sugars [35]. The column was developed to perform carbohydrate analysis under the conditions used in ligand-exchange chromatography. Baseline separation of xylose, glucose, sorbitol, sucrose and maltose was obtained.

Electrochemical detection of carbohydrates at nickel-copper and nickel-chromium-iron alloy electrodes has been reported for sorbitol, and has been used as a detector for HPLC analysis [36]. Oxidation of various carbohydrates at the electrodes was used for detection, and baseline separation was achieved for mixtures of sorbitol, rhamnose, glucose, arabinose, and lactose.

Gas chromatography-mass spectrometry has also been used to detect sorbitol [37]. The components were converted to the trimethylsilyl derivatives and separated on a capillary column. Sorbofuranose, sorbopyranose, fructofuranose, fructopyranose, sorbitol, and 5-keto-sorbose were able to be identified in sorbose using the technique

Thin-layer chromatography of sorbitol has also been reported on silica gel plates impregnated with cupric ion [38]. The plates were impregnated with Cu(II) by immersing them into copper solutions. Developer solutions of distilled water, aqueous ethanol, or aqueous *n*-propanol resulted in good separation of sucrose and glucose.

5. Stability

5.1 Solid-State Stability

Sorbitol is not flammable, corrosive, or volatile. In the absence of catalysts, sorbitol is stable in air, but the bulk material is hygroscopic and should be stored in an airtight container. Sorbitol does not darken or decompose at elevated temperatures or in the presence of amines.

5.2 Compatibility Studies

The compatibility of sorbitol in various solid [49,50] and solution [45-47] formulations has been investigated. In a study conducted using DSC as a stability screening tool, sorbitol was found to be incompatible with salbutanol sulfate [49] and cephalexin [50]. Sorbitol has been found to be

incompatible with ampicillin [45] and amoxicillin [47] in the solution phase.

The photostability of (*L*)-ascorbic acid was found to be enhanced in a solution formulation containing sorbitol [46].

6. Excipient Studies

6.1 Use as a Tableting Agent

A number of studies have been performed using sorbitol to investigate various aspects of tableting [7,26-28,39,40-43]. Modeling studies of bonding in tablets suggests that bond formation requires two components, one of which is a ductile mechanism, and the other of which is a brittle fracture mechanism [39]. It was shown that sorbitol operates under both mechanisms, exhibiting ductile behavior at low compressions forces and brittle behavior at high compression forces. Another study found that sorbitol did not undergo fracture at the compression forces used, suggesting only a ductile mechanism [40].

It has also been reported that the tableting characteristics of instant sorbitol result from its unique particle morphology [28]. Disintegration of instant sorbitol model tablets was found to be slower than tablets prepared using lactose, but this parameter can be optimized by proper disintegrant selection.

A number of other studies have also shown that altering the physical, physicochemical, and mechanical properties of the material [7,26,27,40] can improve various sorbitol characteristics. Improvements in tableting characteristics, compressibility, and texture have been obtained by modifying the morphology and solid properties of the sorbitol powders.

The influence of sorbitol in specific formulations has also been reported [41-44]. In a paracetamol formulation consisting of sorbitol, sodium lauryl sulfate, and Aerosil, it was found that an increase in sorbitol and sodium lauryl sulfate content caused a decease in tablet hardness. This

was accompanied by a corresponding increase in the friability, disintegration, and dissolution rate of the tablets [41].

The effects of mixing time and lubricant system on tableting properties of sorbitol based lozenges has been studied [42]. The lubricating system contained magnesium stearate and polyethylene glycol 6000. Using statistical analysis, it was found that the optimal formulation contained 1% polyethylene glycol 6000 with a mixing time of 7.5 minutes. The magnesium stearate concentration was a compromise between the positive effect on dissolution and the negative effect on hardness. Since the hardness was a more critical parameter for lozenges, the magnesium stearate concentration was chosen to optimize the hardness.

Wet granulated formulations were used to determine if hygroscopic excipients, such as sorbitol, impacted the disintegration properties of selected super-disintegrants [43]. The super-disintegrants used in the investigation included sodium starch glycolate, crospovidone and croscarmellose sodium. The other formulation ingredients included lactose, calcium phosphate dibasic, sorbitol, and naproxen sodium. It was found that highly soluble and/or hygroscopic materials decreased the effectiveness of the disintegrant.

Another study examined the influence of hygroscopic ingredients on the sorption characteristics of tablets in a standard tablet formulation [33]. The three hygroscopic excipients used were citric acid anhydrous, sorbitol, and maltodextrin. It was found that hygroscopic materials do not markedly increase the adsorption of atmospheric moisture by a tablet until high relative humidities were attained.

6.2 Use as a Solution Excipient

Various studies of sorbitol in solution formulations have been reported [44-48]. Sorbitol is readily soluble and compatible with alcohol, syrup, and other polyols. It can be used with sugar solutions and artificial sweeteners to improve body, mouth feel, and sweetness characteristics. It is used with glycerin or propylene glycol to alleviate undesirable tastes,

and is commonly used in elixirs containing antihistamines, barbiturates, and alkaloids. Sorbitol is regularly used in syrup preparations of antitussive agents, vitamins, or minerals and suspensions of analgesics, antacids, and antibiotics s [44].

Specific studies include the use of sorbitol with solutions of ampicillin [45], (*L*)-ascorbic acid [46], and amoxicillin [47]. It has also been found to influence the bioavailability of theophylline when compared to water [48].

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