Analytical Profiles of Drug Substances

Volume 7

Edited by

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PREFACE

Although the official compendia list tests and limits for drug substances related to identity, purity, and strength, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. For drug substances important enough to be accorded monographs in the official compendia, such supplemental information should also be made readily available. To this end the Pharmaceutical Analysis and Control Section, Academy of Pharmaceutical Sciences, has undertaken a cooperative venture to compile and publish *Analytical Profiles of Drug Substances* in a series of volumes of which this is the seventh.

The concept of analytical profiles is taking hold not only for compendial drugs but, increasingly, in the industrial research laboratories. Analytical profiles are being prepared and periodically updated to provide physicochemical and analytical information of new drug substances during the consecutive stages of research and development. Hopefully, then, in the not too distant future, the publication of an analytical profile will require a minimum of effort whenever a new drug substance is selected for compendial status.

The cooperative spirit of our contributors has made this venture possible. All those who have found the profiles useful are requested to contribute a monograph of their own. The editors stand ready to receive such contributions.

Thanks to the dedicated efforts of Dr. Morton E. Goldberg, a long cherished dream has come to fruition with the publication of Volume I of *Pharmacological and Biochemical Properties of Drug Substances,* M. E. Goldberg, editor, published by APhA Academy of Pharmaceutical Sciences. This new series supplements the comprehensive description of the physical, chemical, and analytical characteristics of drug substances covered in *Analytical Profiles of Drug Substances* with the equally important description of pharmacological and biochemical properties.

Two drug substances, cefazolin and fenoprofen, have the distinction of being the first to be covered by monographs in both series, and beginning with this volume, the cumulative index will cross-reference drug substances appearing in the new series.

The goal to cover all drug substances with comprehensive monographs is still a distant one. It is up to our perseverance to make it a reality.

Klaus Florey

Analytical Profiles of Drug Substances, 7

ALLOPURINOL

Steven A. Benezra and T. Reney Bennett

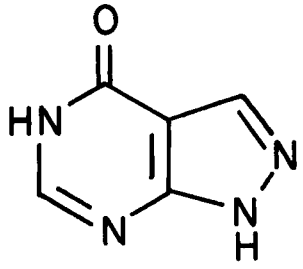
ISBN 0-12-260807-0

Table of Contents

1. Description

1.1 Name, Formula, Molecular Weight

Allopurinol is 1H-pyrazolo(3,4-d)pyrimidine-4-ol



$C_5H_4N_4O$ 136.11

1.2 Appearance, Color, Odor

Allopurinol is a white to off-white powder with a slight odor[1].

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of allopurinol is shown in Figure 1[2]. It was taken as a 0.2% dispersion of allopurinol in KBr with a Perkin Elmer model 457 infrared spectrophotometer. Table I gives the infrared assignments consistent with the structure of allopurinol[3].

Table I
Infrared Spectral Assignments for Allopurinol

Frequency (cm^{-1})	Assignment
3060	CH stretching vibrations of the pyrimidine ring
1700	CO stretching vibration of the keto form of the 4-hydroxy tautomer
1590	ring vibrations
1245	CH in-plane deformation

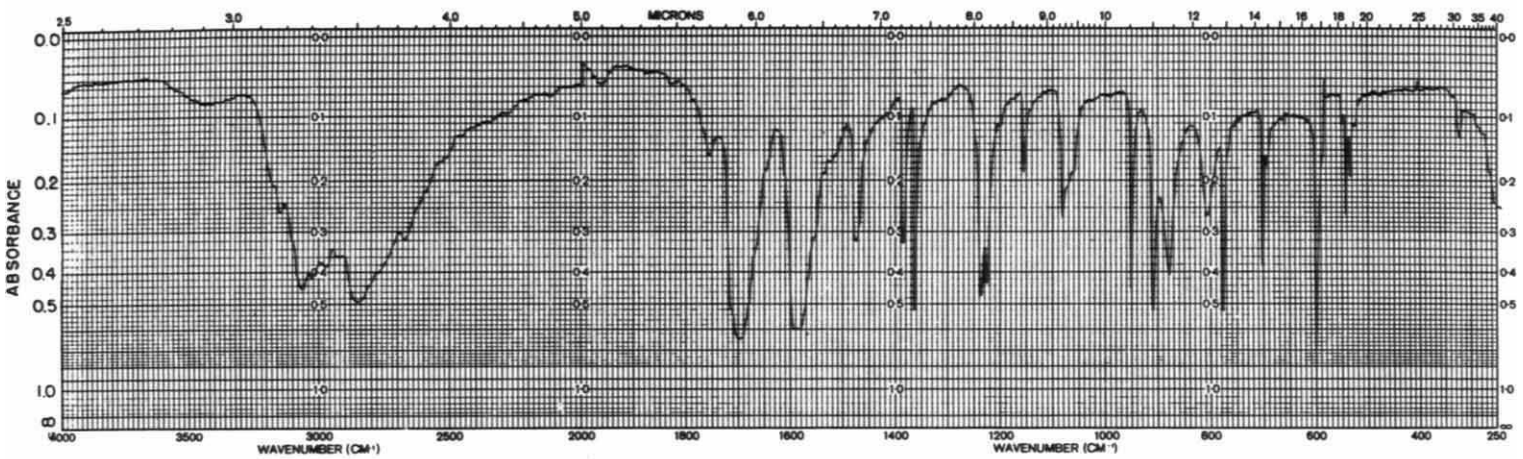


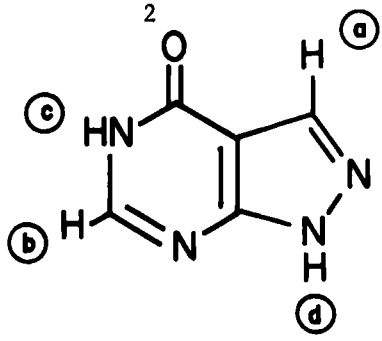
Figure 1- Infrared Spectrum of Allopurinol

790 CH in-plane deformation

2.2 Nuclear Magnetic Resonance (NMR) Spectrum

The NMR spectrum of allopurinol is shown in Figure 2[4]. The spectrum was obtained with a Varian model CFT-20 80 MHz NMR spectrometer. Deuterated DMSO was used as the solvent with tetramethylsilane as an internal standard. Based on the NMR spectrum, the following assignments for allopurinol can be made.

Proton	# of Protons	Chemical Shift (ppm)
b	1	7.95
a	1	8.07
c & d	2	11.75 (broad)



2.3 Ultraviolet Spectrum

The ultraviolet spectrum of allopurinol in 0.1N HCl was obtained with a Beckman ACTA$_5$CIII ultraviolet spectrophotometer and is shown in Figure 3[5]. Table II summarizes the ultraviolet spectral data for allopurinol.

Table II
Ultraviolet Spectral Data for allopurinol

Solvent	λmax (n)	ε
0.1N NaOH	257	7200
0.1N HCl	250	7600
Methanol	252	7600

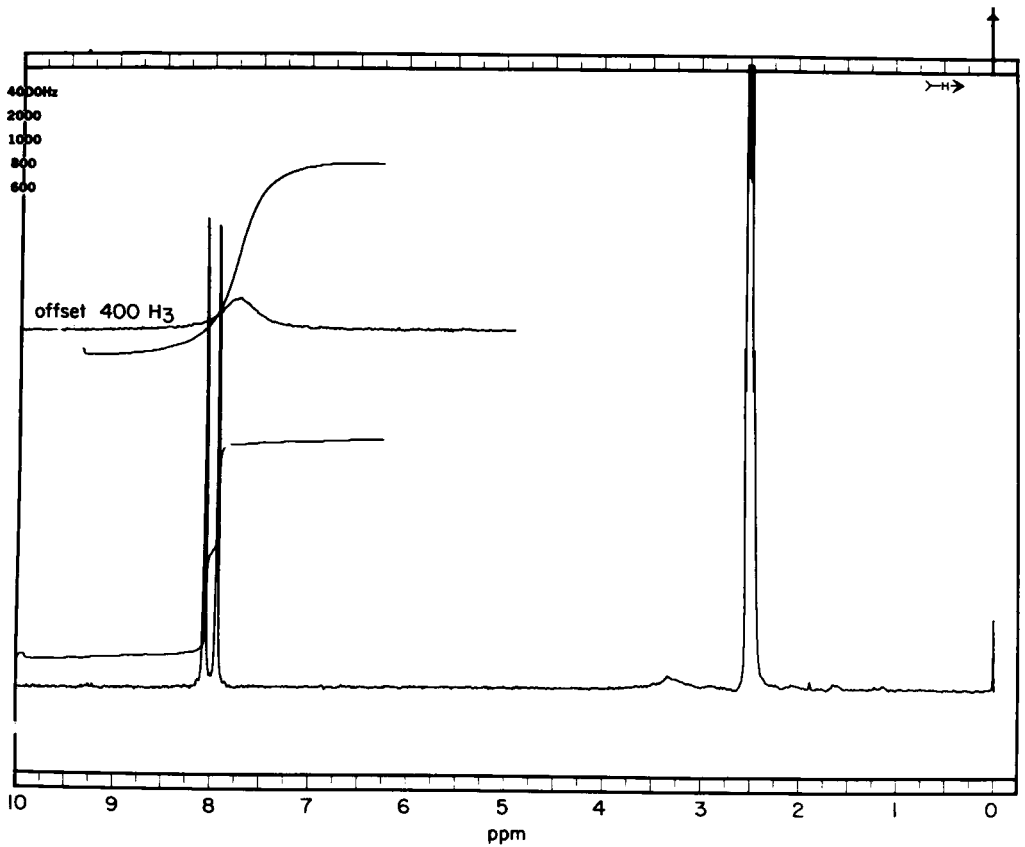


Figure 2- Nuclear Magnetic Resonance Spectrum of Allopurinol

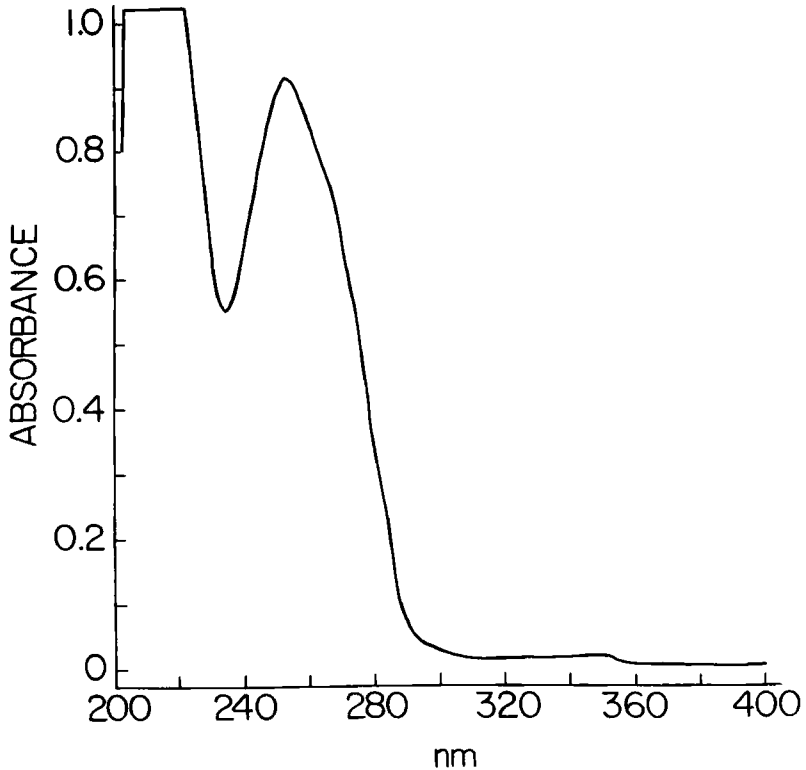
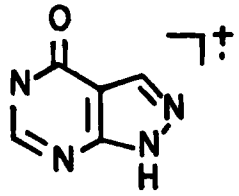


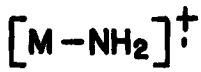
Figure 3- Ultraviolet Spectrum of Allopurinol

2.4 Mass Spectrum

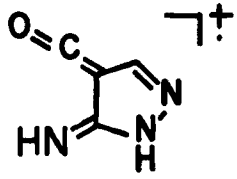
The low resolution electron impact mass spectrum of allopurinol is shown in Figure 4[6]. It was obtained with a Varian model CH5-DF mass spectrometer. Direct probe at 115°C into the source was used to obtain the mass spectrum. The electron energy was 70 eV. The assignments of the major ions formed in the mass spectrometer is given below.



m/e 136 (100%)



m/e 120 (4.2%)



m/e 109 (6.3%)

2.5 Melting Point

Allopurinol melts above 350°C[7].

2.6 Solubility

The solubility of allopurinol in various solvents at 25°C is given in Table III[7].

Table III
Solubility of Allopurinol at 25°C

Solvent	Solubility (mg/ml)
Water	0.48
n-octanol	<0.01
Chloroform	0.60
Ethanol	0.30
DMSO	4.6

2.7 Dissociation Constant

The pk_a of allopurinol determined spectrophotometrically is 10.2[7].

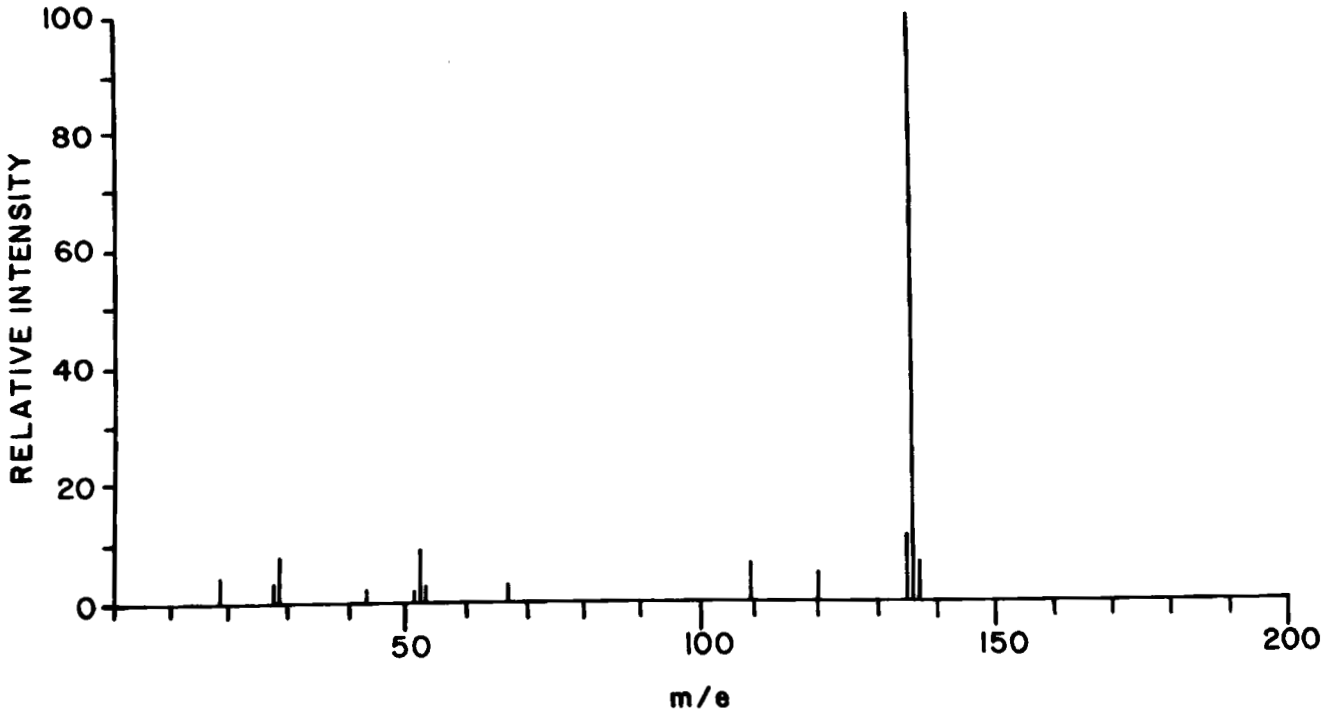


Figure 4- Mass Spectrum of Allopurinol

2.8 Partition Coefficient

The partition coefficients of allopurinol at 25°C in n-octanol/pH 1.2 and n-octanol/pH 6.0 are 0.24 and 0.33 respectively[7].

2.9 Crystal and Molecular Structure

Allopurinol crystals obtained by slow evaporation from acetone: water (1:1) and analyzed with a Picker FACS-1 x-ray diffractometer were found to be in the monoclinic space group $P2_1/c$. The cell constants were a=3.683A°, b=14.685A°, c=10.318A°, and β=97.47°.

The bases are hydrogen bonded in sheets parallel to the 102 plane. Layers of the sheets are stacked with a staggered overlap of the base rings. All ring nitrogens, atoms, oxygen, and C(2) are involved in hydrogen bonding[8].

3. Synthesis

Allopurinol can be prepared from a variety of synthetic procedures. Three of the synthetic routes to allopurinol are shown in Figure 5[9-11]. Synthetic route 1 uses malononitrile and triethylorthoformate to form ethoxymethylene malononitrile which reacts with hydrazine to give 3-amino-4-cyanopyrazole. The pyrazole is hydrolyzed to form 3-aminopyrazole-4-carboxamide. The carboxamide is reacted with formamide to give allopurinol. Synthetic route 2 uses formamidine hydrochloride and cyanoacetamide to form 3-amino-2-cyanoacrylamide. The 3-amino-2-cyanoacrylamide is reacted with hydrazine to give 3-aminopyrazole-4-carboxamide which is converted to allopurinol as in route 1. The third synthetic route uses cyanoacetamide, triethylorthoformate, and morpholine to prepare 3-morpholino-2-cyanoacrylamide. The 3-morpholino-2-cyanoacrylamide is reacted with hydrazine as in route 2 to give the carboxamide which is converted to allopurinol by reaction with formamide.

4. Stability

The primary decomposition product in acidic and basic solutions is 3-aminopyrazole-4-carboxamide. In basic solutions the 3-aminopyrazole-4-carboxamide decomposes to 3-aminopyrazole-4-carboxylic acid and 3-aminopyrazole. In acidic solutions the 3-aminopyrazole-4-carboxylic acid is not formed.

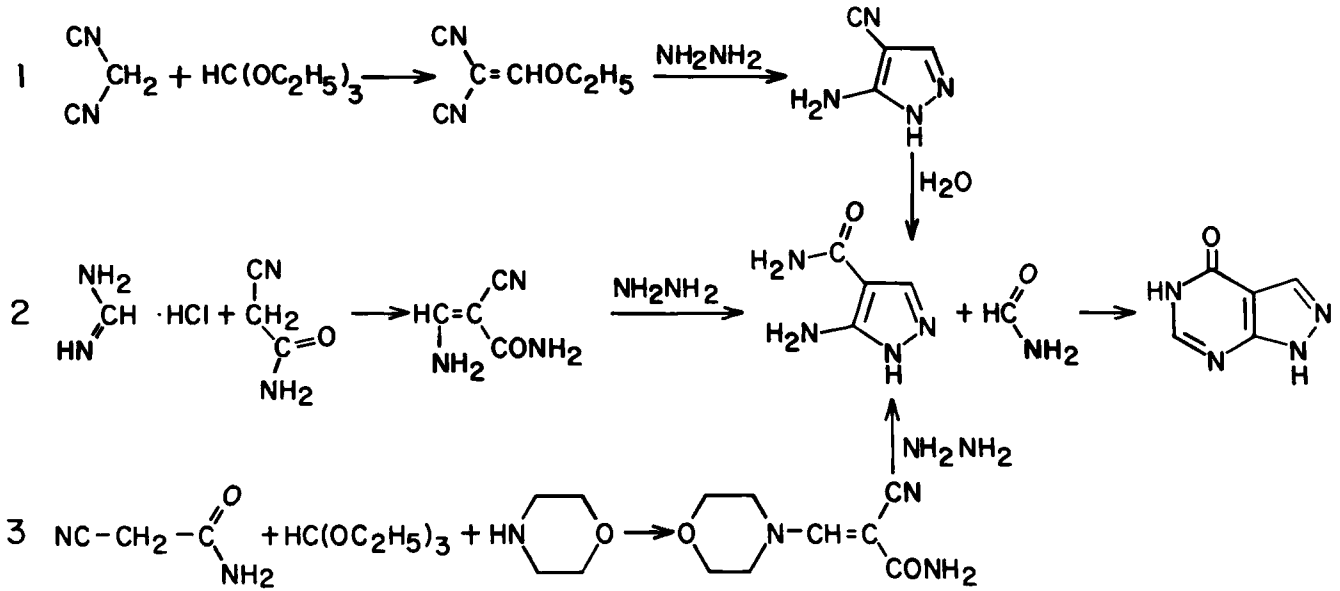


Figure 5- Synthesis of Allopurinol

At 105°C the maximum stability of allopurinol in solution occurs at pH 3.1-3.4. Rapid decomposition occurs at high pH. At 25°C the shelf life of a 2% suspension of allopurinol at pH 9.5 is predicted to be 5.4 years[12].

5. Methods of Analysis

5.1 Elemental Analysis

Element	% Calculated
C	44.13
H	2.96
N	41.17
O	11.76

5.2 Spectrophotometric Analysis

The official USP analysis of allopurinol and allopurinol in tablet form is a UV spectrophometric analysis[1]. A portion of the drug or powdered tablets is dissolved in sodium hydroxide solution and diluted stepwise with dilute hydrochloric acid to a concentration of approximately 10 µg/ml. The sample solution is compared against a standard solution at 250 nm to determine the purity of allopurinol or the labelled strength of the tablets.

5.3 Polarography

Dryhurst and De have analyzed allopurinol in the presence of uric acid by DC polarography[13]. The useful analytical pH range for the analysis of allopurinol was found to be pH 0-6. At pH values above 6 the height of the polarographic wave decreases. The half-wave potential for the reduction of allopurinol was found to be a function of pH and followed the equation $E_{1/2} = -1.118-0.067pH$. At low pH values of supporting electrolyte the polarographic wave is due to the 4 electron reduction of allopurinol to 2,3,6,7-tetrahydroallopurinol. At higher pH values of supporting electrolyte (pH 4.7-6.0) the reduction involves 2 electrons to give 6,7-dihydroallopurinol.

5.4 Chromatography

5.41 Thin Layer Chromatography

Various thin layer chromatographic systems for allopurinol are given in Table IV.

Table IV
Thin Layer Chromatography Systems for Alloprinol

Mobile Phase	Stationary Phase	R_f	Ref.
2-methoxyethanol:methyl ethyl ketone:ammonia 3:6:2	Cellulose	0.36	5
n-butanol:5% aqueous ammonia 2:1	Cellulose	0.21	5
chloroform:ethanol 3:2	Silica gel	0.64	14
chloroform:ethanol 3:1	Silica gel	0.14	14
acetone	Silica gel	0.57	14

5.42 Paper Chromatography

Paper chromatography has been used to separate allopurinol from metabolites and chemically related compounds[15]. The systems used for the separations are given in Table V.

Table V
Paper Chromatography Systems for Allopurinol

Mobile Phase	Paper	R_f
5% Na_2HPO_4:iso-amyl alcohol 2:1	Whatman 3MM	0.55
1-butanol:water 84:16	Whatman 3MM	0.44
1-propanol:water 7:3	Whatman 3MM	0.62
1-butanol:water 84:16(NH_3 atmosphere)	S&S 597	0.27

5.43 Column Chromatography

Column chromatography has been used to separate allopurinol from 3-aminopyrazole-4-carboxamide[14]. A Dowex 1xs (chloride form) anion exchange resin was used. The allopurinol was retained on the column using a mobile phase of pH 10 phosphate buffer. Phosphate buffer at pH 4 was used to quantitatively elute the allopurinol from the column. A Dowex-50(H^+) column has been used to separate allopurinol

from xanthine and uric acid[16]. Allopurinol was eluted with 1N hydrochloric acid.

5.44 High Performance Liquid Chromatography

High performance liquid chromatography has been utilized to separate allopurinol from various metabolites and analogues. Table VI gives the conditions and retentions times used for the separations.

Table VI
HPLC Conditions for Allopurinol Separations

Column	Mobile Phase	Flow Rate(ml/min)	R_t(min)	Ref
Spherisorb 10	CH_3CN:H_2O: NH_4OH 1000:80:4	10.0	0.7	17
Aminex A-27	NH_4OAC(pH 8.7), 70°C	1.0	6	18
Whatman PA-38	Potassium Phosphate 0.015M, pH 5.7, 40°C	0.25	10	19

5.45 Gas Chromatography

Allopurinol in tablets has been analyzed by gas chromatography. The allopurinol was extracted with 1.5N NaOH. The trimethylsilyl derivative was formed with BSTFA and injected onto a 2M, 3% OV-17 column maintained at 150°C. The retention index was 1723.6[20].

5.5 Mass Spectrometry

Multiple peak integrated current mass spectrometry has been used to analyze allopurinol in blood plasma[21]. Samples of plasma were dehydrated and 2-3 mg portions of the dehydrated plasma admitted directly into the mass spectrometer. The m/e 52.0187 peak of allopurinol was integrated over the lifetime of the peak. Allopurinol was determined in the 10-100 ppm level with an accuracy of $\pm$ 20%.

6. Metabolism and Pharmacokinetics

6.1 Metabolism

Allopurinol acts as a substrate for, and inhibitor of xanthine oxidase. It is converted primarily to 4,6-dihydroxypyrazolo(3,4-d)pyrimidine (oxypurinol). Allopurinol-1-riboside and oxypurinol-7-riboside are also metabolic products, although to a lesser extent than oxypurinol. Allopurinol-1-ribonucleotide, oxypurinol-1-ribonucleotide, and oxypurinol-7-ribonucleotide have been found in concentrations of 10^{-9}-10^{-6} M in rat liver and kidney. The amounts and relative proportions of the nucleotides were dependent upon dose, route of administration, and time[19].

6.2 Tissue Distribution

After 2 hours dose in mice, allopurinol is approximately equally distributed in the blood, liver, spleen, heart, and intestine. The brain and lung contain approximately half the concentration of allopurinol found in the blood[16].

6.3 Pharmacokinetics

Allopurinol has a half-clearance time in plasma of about 2 hours in man. This is due predominately to the rapid conversion of allopurinol to oxypurinol. Oxypurinol has a more prolonged half-clearance time than allopurinol, on the order of 28 hours. The rate of excretion of allopurinol was approximately 4 hours for half-clearance and on the order of 28 hours for oxypurinol. The half-clearance values vary, depending on whether the allopurinol was given as a single dose or over a prolonged administration[16].

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Analytical Profiles of Drug Substances, 7

AMOXICILLIN

Pranab K. Bhattacharyya and Winifred M. Cort

ISBN 0-12-260807-0

TABLE OF CONTENTS

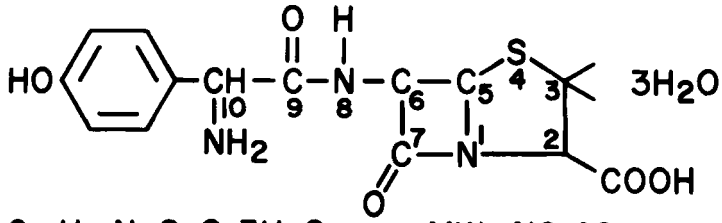
1. Description

1.1 Name: Amoxicillin

Chemical abstracts designates it by its complete chemical name; that is 4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid, 6-[(amino 4-hydroxyphenyl acetyl) amino]-3,3-dimethyl-7-oxo-[2S- [2α,5α,6β(S*)] 26787-78-0

Amoxicillin is generally referred to as 6-[D(-)-α-amino-p-hydroxyphenyl acetamido] penicillanic acid trihydrate and in early studies was called hydroxy-ampicillin.

1.2 Formula and Molecular Weight



$C_{16}H_{19}N_3O_5S.\ 3H_2O$ MW. 419.46

1.3 Isomers

In penicillins, the absolute configurations of the carbons 2,5,6, are S, R, R respectively. In addition the carbon at the 10 position adjacent to the aromatic ring has the R configuration formerly called D-(-). As in ampicillin, the S epimer at C10 was also synthesized and had inferior antibacterial activity. The synthesis was further extended to include isomers (6 in all) at the C10, with ortho, meta, and para-hydroxy on the aromatic ring, and the two most active ones in vitro had the R configuration on C10 and were meta and para-hydroxy substituted compounds. In vivo the R p-hydroxy isomer resulted in the highest blood levels, and, as a result, this has been the active amoxicillin (1).

1.4 Hydrates

Unlike ampicillin, the anhydrous forms have not been studied extensively, although produced (2).

1.5 Salts

Salts consisting of the hydrochloride, sulfate, β-napthalene sulfonate, sodium and potassium have been synthesized and identified (2). Similar to ampicillin the hetacillin type derivative, that is 6-(4-p-hydroxyphenyl-2,2-dimethyl-5-oxoimidazolidin-1-yl)penicillanic acid was synthesized (1). However, the form currently in use is the trihydrate.

1.6 Appearance, Color and Odor

Amoxicillin trihydrate is a white to off-white free flowing crystalline powder. One of the tests required for certification is the recognition of the crystals and observation of birefringence, typical of all penicillins (3). Amoxicillin similar to other penicillins also has the typical penicillin odor described as a sulfurous type.

2. Physical Properties

2.1 Ultraviolet Absorption:

The ultraviolet spectra (4) of amoxicillin trihydrate, which were recorded on a Cary Model 14 Spectrophotometer, are given in Fig. 1 & 2. The spectra represent a phenolic type chromophore.

Table I: UV spectral data

Ethanol			
Wavelength,nm:	230(max.)	274(max.)	∿281(Sh.)
ε :	10850	1400	1160
0.1 N HCl			
Wavelength,nm:	229(max.)	272(max.)	∿278(Sh.)
ε :	9500	1080	920
0.1 N KOH			
Wavelength,nm:	248(max.)	291(max.)	∿325(Sh.)
ε :	2200	3000	750

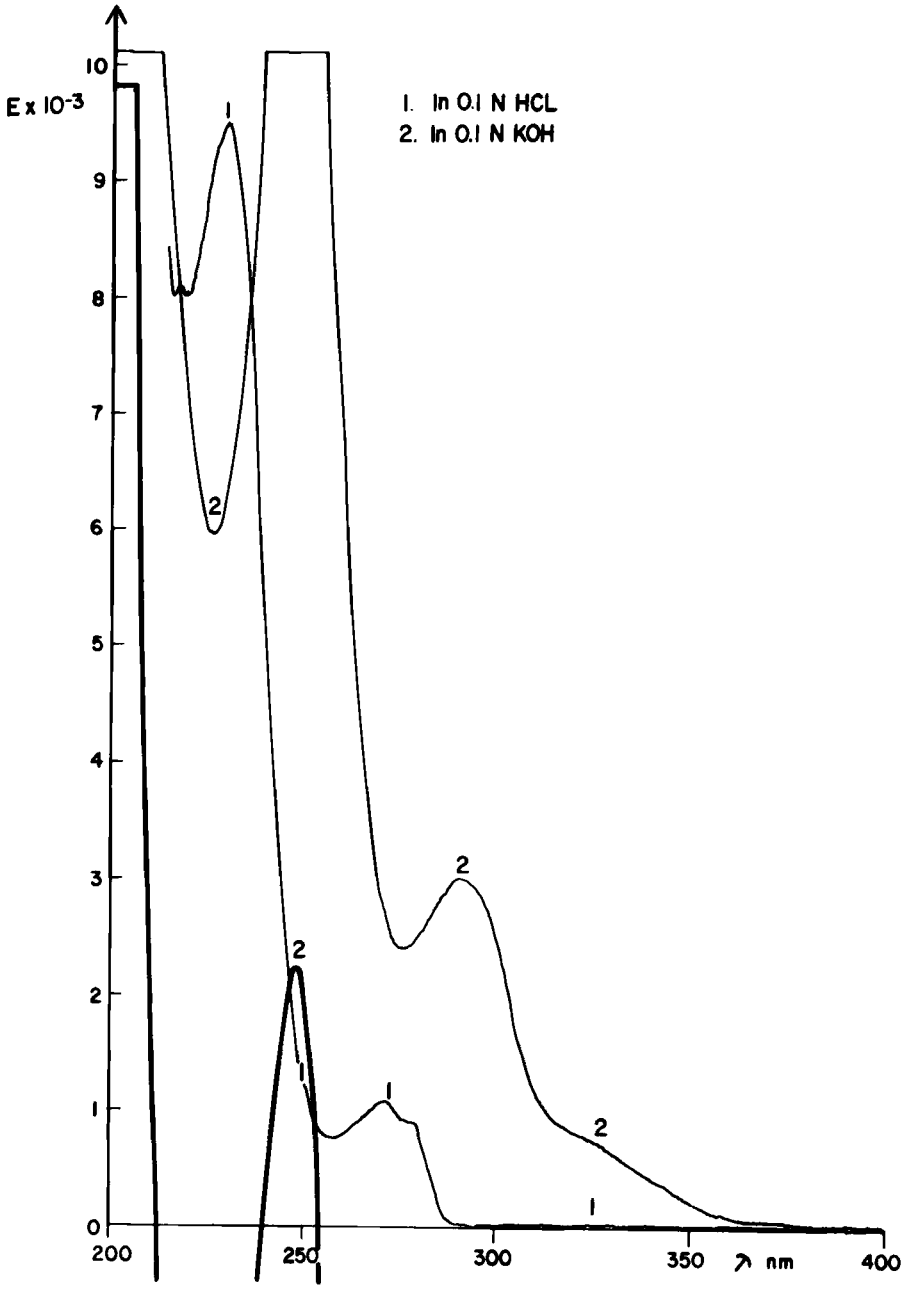


FIGURE 1

The UV Spectra of amoxicillin trihydrate in O.IN HCl and O.IN KOH respectively.

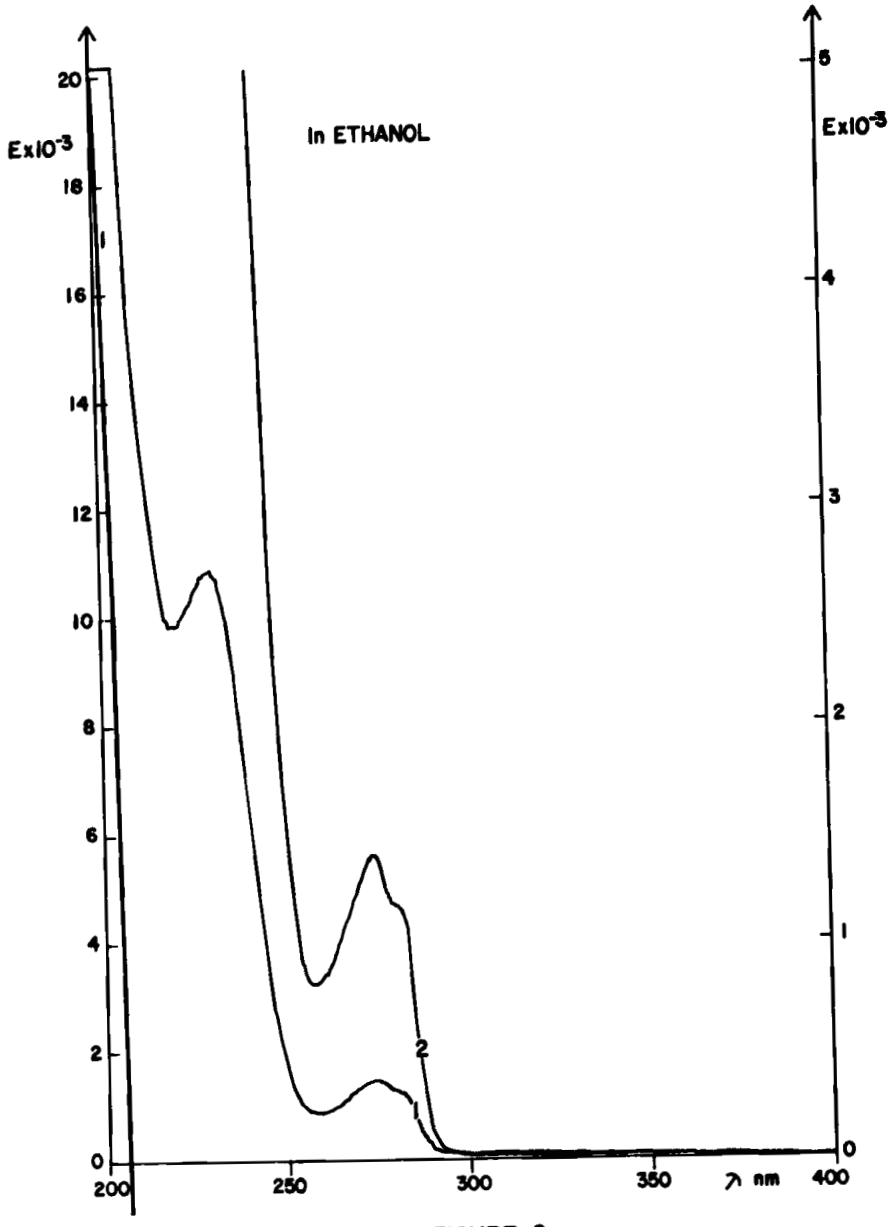


FIGURE 2

The UV Spectrum of amoxicillin trihydrate in ethanol.

2.2 ORD Spectrum:-

The optical rotatory dispersion spectrum was obtained on a JASCO Model J-20 Automatic Recording Spectropolarimeter. The ORD curve (see Fig.3) is in agreement with the structure of the compound.

Table II: Experimental data (4) from ORD Spectrum

Extrema	Wavelength (nm)	Rotation (ϕ)
Start	650	+880
NaD	589	+1,090
Hg	546	+1,320
Hg	436	+2,520
Hg	365	+4,240
Shoulder	290	+10,000
Peak	248	+38,400
Intersection	232/33	0
Trough	220	-23,200
Intersection	212	0
Peak	199/200	+130,000
Last	195	0

2.3 CD Spectrum:-

The CD spectrum was obtained on a JASCO Model J-20 Automatic Recording Spectropolarimeter.

The optical circular dichroism (see Fig.4) shows Cotton effects typical for a phenolic type chromophore.

Table III: Experimental data (4) from CD Spectrum

Extrema	Wavelength (nm)	Ellipticity (Θ)
Start	292	0
Inflection	280	-2,000
Maximum	273/74	-2,560
Inflection	268	-2,000
Intersection	263	0
Maximum	236/37	+45,200
Intersection	217	0
Maximum	207/09	-31,200
Intersection	203	0
Last	200	+32,000

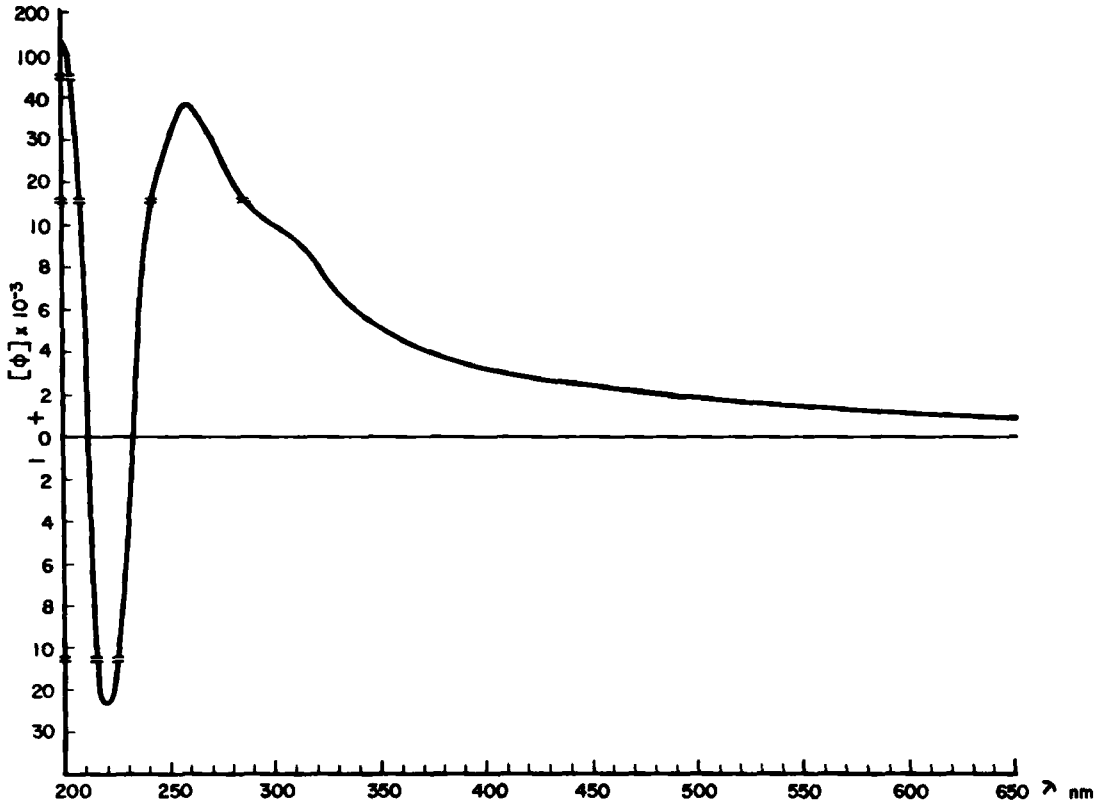


FIGURE 3

The ORD curve of amoxicillin trihydrate in water at room temperature.

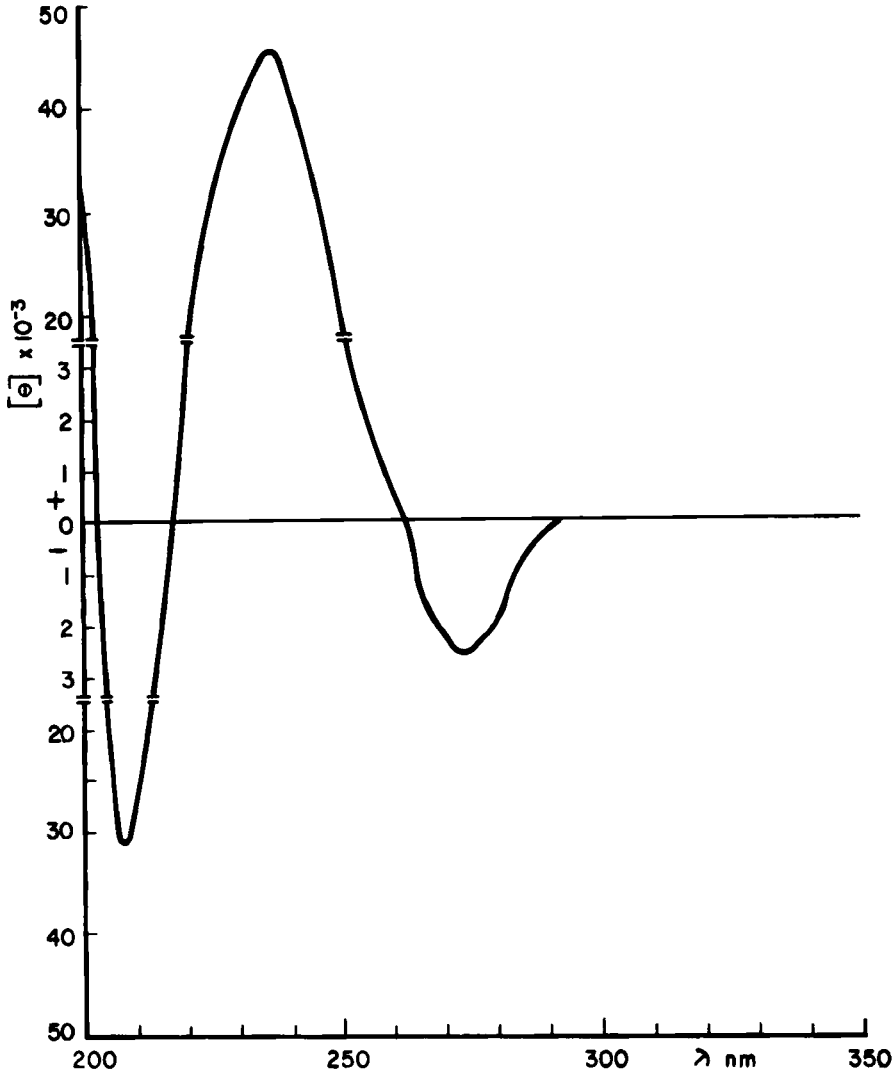


FIGURE 4

The circular dichroism of amoxicillin trihydrate in water at room temperature.

2.4 IR Spectrum:

The IR spectrum (5) (see Fig.5) of amoxicillin trihydrate was obtained on the Perkin Elmer Model 621 Double-Beam Spectrophotometer as a potassium bromide pellet. The frequencies and assignments are given in the following table.

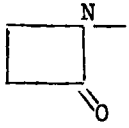
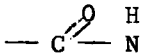
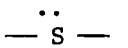
Table IV

Assignment	Frequency (cm^{-1})
OH stretching	3550-3400
NH_3^+	3200-2500
β-lactam C=O stretching	1776
Amide I C=O stretching	1688
COO^- asymmetric stretching	1582
Aromatic ring	1519

2.5 Raman Spectrum:

The Raman spectrum (6) (see Fig.6) of the compound in solid state was obtained on a Spex Model 1401 Double Spectrometer using the argon 4880 Å excitation derived from Model 164 Spectra-Physics Laser. A fluorescence background was also observed.

Table V: Raman spectral assigments

Assignment	Frequency(cm^{-1})
OH stretching	∿3500
NH stretching	3410
	1780
	1695
Aromatic C = C	1620, 1610 (sh)
	635

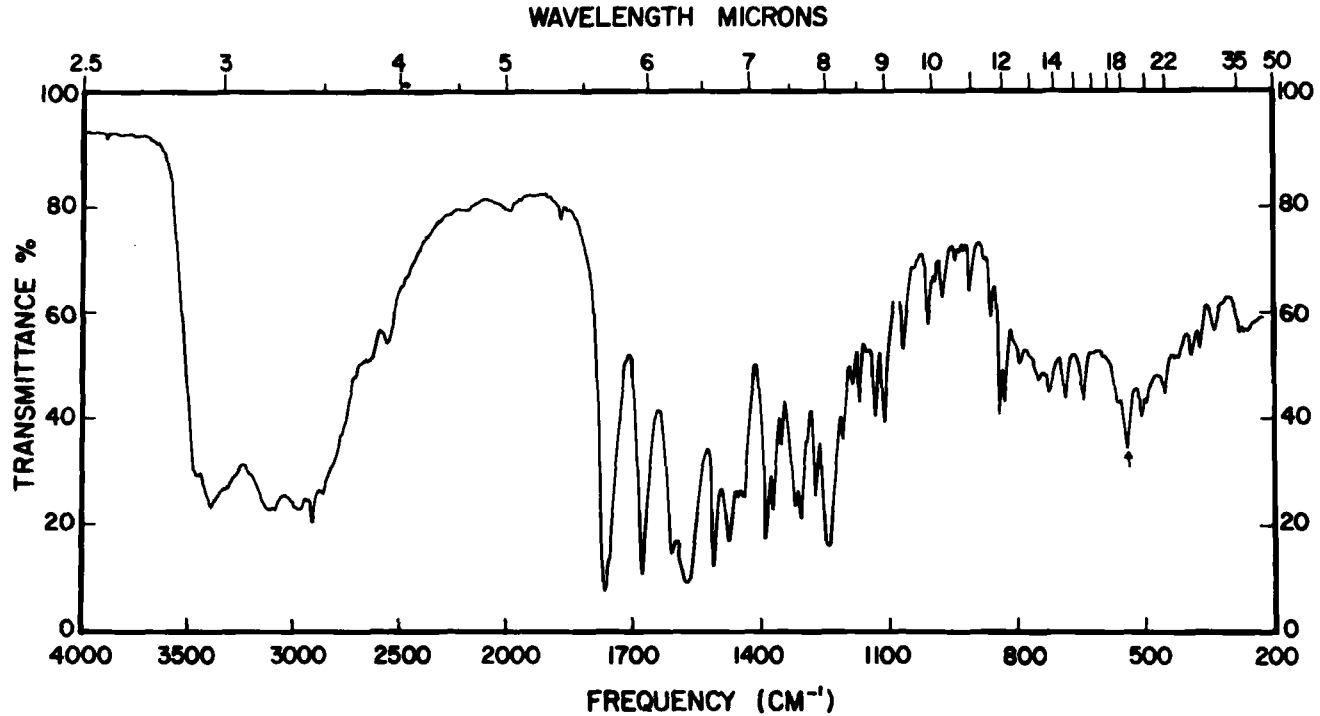


FIGURE 5

The infrared Spectrum of amoxicillin trihydrate.

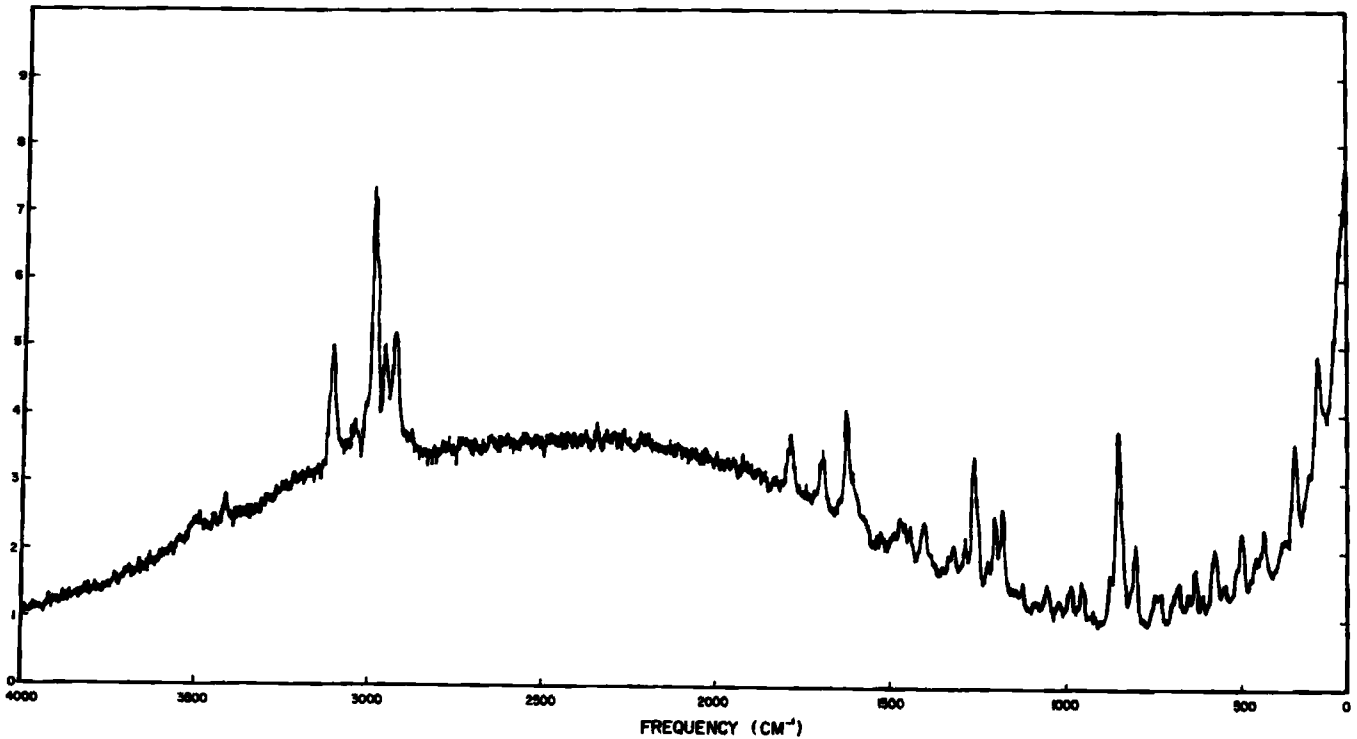
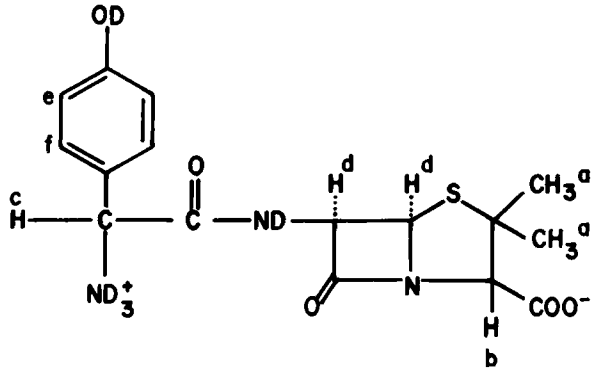


FIGURE 6

The Raman Spectrum of amoxicillin trihydrate.

2.6 NMR Spectrum:

The NMR spectrum (7) (see Fig.7) was obtained on a Varian XL-100 Spectrometer. The sample (about 50 mg) was dissolved into 0.5 ml of D_2O with about 2 mg of DSS (sodium 2,2 - dimethyl -2- silapentane-5 - sulfonate) added as an internal reference. Approximately 5 microlitres of DCL were also added for catalysing the rate of substitution of the exchangeable protons by deuterium nuclei.



Interpretation of the NMR spectrum of amoxicillin trihydrate has been reported (8).

Table VI: Proton NMR assignments

Proton Assignment	Chemical Shift(δ),ppm	Multiplicity
a	1.34,1.36	Singlet
b	4.40	"
c	5.14	"
d	5.46	"
e*	6.91	Doublet
f*	7.37	"

*Coupling constant J_{HH} = 9 Hz

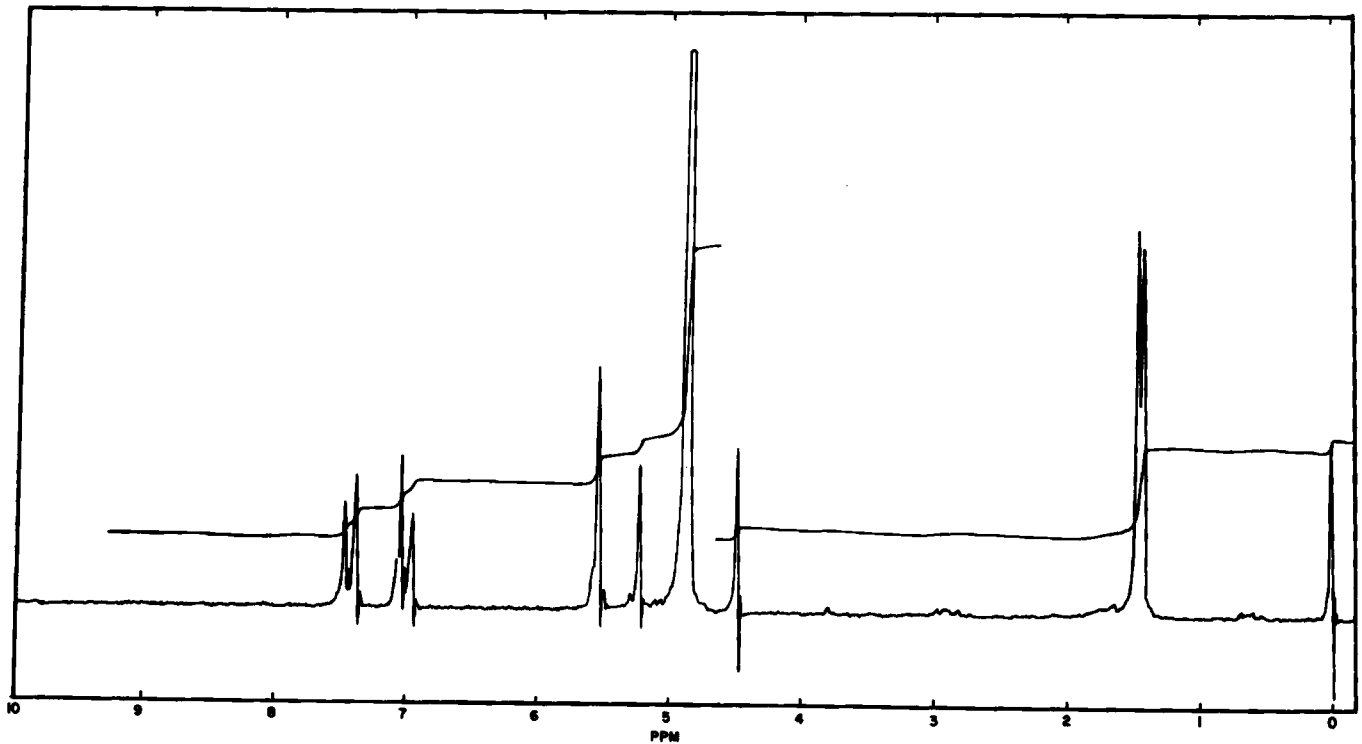


FIGURE 7

The NMR Spectrum of amoxicillin trihydrate in D_2O.

2.7 X-ray Diffraction Data:

The X-ray powder diffraction data are given in the following table (9).

Instrument Conditions

Instrument: GE Model XRD-6-Diffractometer operated at 50 KV, 12.5 mA. Target: Copper (CuKα = 1.5418Å). Beam slit: 3°. Detector Slit: 0.05. Soller: M.R. Filter: Ni. Detector: 1.75 KV and 0.95 KV. Pulse height selection: 5 volts. Window: out. At cps full scale: 1K. Time constant: 2.5. Focus: line. Take off angle: 4°. Scan and chart speeds: 1°/inch. Sample treatment: as is.

Table VII: X-ray Diffraction Results

2Θ(o°)	d(A°)	I/I_1
12.20	7.26	0.63
14.72	6.02	0.24
*15.13	5.86	0.54
*16.25	5.45	0.16
18.05	4.91	1.00
19.34	4.59	0.75
19.77	4.49	0.24
*20.20	4.40	0.12
*25.76	3.46	0.42
*26.68	3.34	0.51
*28.71	3.11	0.55

*Broad lines

I/I_1 = Relative Intensity

3. Amoxicillin Stability

Amoxicillin degradation is typical of all penicillin hydrolyses as reviewed by Ivashkiv (10). In alkali it decomposes first by opening the lactame to its penicilloic acid. This is the basis for the iodine absorption assay (11) as well as the hydroxylamine reaction. Penicilloic acid ultimately loses CO_2 and forms penilloic acid.

Amoxicillin in acid, again similar to other penicillins, hydrolyzes to form first amoxicillin penicillenic acid which absorbs at 320 nm. This hydrolysis has been the basis for determination of ampicillin (12) and amoxicillin (13). Neither 6-aminopenicillanic acid nor penicilloic acid forms penicillenic acid. Additional acid breakdown products are penillic acid, penaldic acid, penicillamine and ultimately penilloaldehyde.

Although the semi-synthetic penicillins are made from 6-amino penicillanic acid, (6-APA), it is not found as a degradation product. In fact, there are numerous patents on making (6-APA) from penicillins using penicillin amidases (14).

Amoxicillin in oral dosage forms is an insoluble emulsion at 125 and 250 mg/5 ml. These have at least 90% retention of activity after 2 weeks at room temperature (RT) or at $4^{\circ}C$. However, solubility at pH 6 is only 4.2 mg/ml (21 mg/5ml); of the solutions kept for 7 days at RT, only 50% of the amoxicillin remains intact although 100% is retained at $4^{\circ}C$. Since the insoluble amoxicillin is more stable than dissolved material, the stability of the oral dosage form is dependent on its lack of solubility. Dry bulk amoxicillin, capsules, and dry oral dosage powders have retained full activity for as long as five years. However, expiration dates are usually three years.

4. Solubility

Solubility data is somewhat meagre and is as follows (15).

Solvent	mg/ml
Water	∿4
Methanol	7.5
Ethanol (Absolute)	3.4
Acetone	1.3
Dioxane	0.8
Ethyl Acetate	Insoluble
Hexane	Insoluble
Acetonitrile	Insoluble
Benzene	Insoluble

Solubility in water depends on pH. However, at pH 4-8 this varies from 4.2 to 9.0 mg/ml.

5. Manufacturing Procedure

A number of synthetic procedures have been described (2). The method of choice (1) involves preparation of 6-(D-α-amino-p-hydroxyphenylacetamido) penicillanic acid by protecting the amino group as an enamine function (the hydroxyl did not have to be protected). The sodium salt of D-(-)-α-amino-p-hydroxyphenyl acetic acid I was heated with methyl acetoacetate in methanol to give the enamine II. This was crystallized by distilling off the methanol and replacing it with dry toluene. This was treated with ethyl chloroformate in cold dry acetone containing catalytic quantity of N-methylmorpholine to form the mixed Anhydride III which was then treated with the potassium salt of 6-APA in aqueous acetone for 30 minutes, then concentrated under reduced pressure. The aqueous concentrate was mixed with isobutyl methyl ketone pH 1.9 adjusted, solvent removed, aqueous phase adjusted to pH 5.1, concentrated and crystallized. Crystals are washed with acetone to dry. Summary of synthesis is presented in Figure 8.

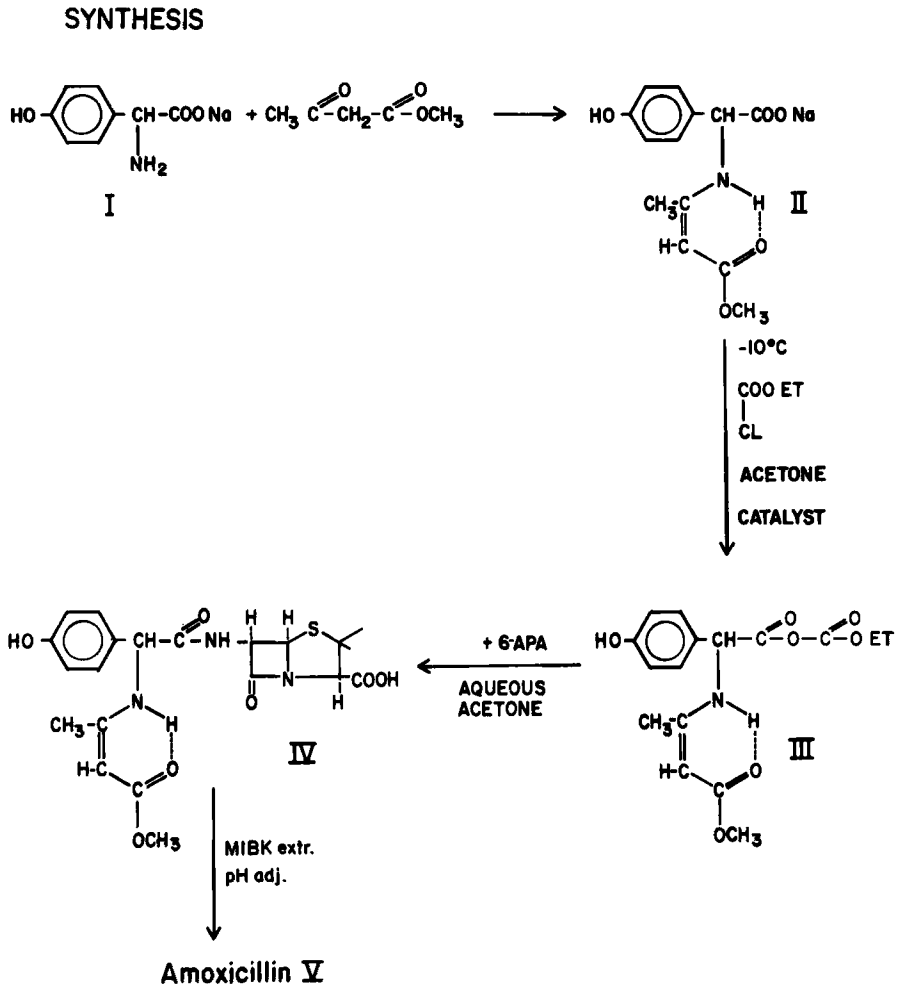


FIGURE 8
Synthesis of amoxicillin.

6. Methods of Analysis

The standard specifications in the Code of Federal Regulations requires (3) that the bulk drug be tested and certified. Testing includes potency 900-1050 mcg/mg on anhydrous basis, moisture 11.5-14.5%, pH 3.5-6.0, it must pass safety (436.33), it must be crystalline, give a positive IR identification, acid and base (lithium-methoxide and perchloric acid) titrations should not be less than 90% on an anhydrous basis. Assays may be performed iodometrically (436.204), colorimetrically by hydroxylamine, or microbiologically (436.105) using S. lutea in a horizontal agar diffusion procedure. Results of the iodometric procedure shall be conclusive. After the above tests, all bulk amoxicillin in the U.S. must be submitted to the FDA for certification. The marketed dosage forms, capsules and oral suspensions must use certified bulk amoxicillin, and all lots must be tested and submitted for certification. The tests for the capsules (440.103a) include moisture, assay and TLC and for the oral suspensions (440.1036) they include the same tests plus pH.

There have been other analytical procedures proposed for amoxicillin similar to published methods for other penicillins. These include measurement of U.V. absorption at 320 nm of the amoxicillin penicillenic acid formed in the presence of acid and Cu^{2+} (13). A spectrofluorometric method has also been proposed (16) using the reaction with formaldehyde and measuring at 430 nm after excitation at 366 nm. A fluorescamine assay (17) has been proposed for ampicillin which will determine 14 nanograms/ml at pH 6.5-7.5 and will also be applicable to amoxicillin. A method using high pressure liquid chromatography (HPLC) has been published for ampicillin (18). HPLC appears to be an excellent quantitative chromatographic procedure for amoxicillin (19,20) since it requires no heat (no destruction) and is readily quantitated by measurement of UV peak intensities. It is hoped that automated HPLC, eventually, will be an official procedure.

7. Pharmacological Evidence

Amoxicillin was synthesized and covered in a patent (21) several years after ampicillin. In the meantime many investigations (CF reference 10) were undertaken on ampicillin which became one of the major current penicillins. A series of papers presented in 1970 and published in 1971, focused attention on amoxicillin. Neu and Winshell (22) showed first that amoxicillin and ampicillin had equivalent antibacterial spectra. All four references (22, 23, 24, 25) reported that amoxicillin was better absorbed and produced higher serum levels than ampicillin. This information apparently stimulated Long, et. al. (1) to publish their work on the synthesis. Sutherland et. al. (26) further expanded on comparisons of ampicillin and amoxicillin and showed equivalent activity against Staph. aureus β-haemalytic streptococci, Strep pneumoniae S. faecalis, H. influenzae, N. gonorrhoeae, E. coli, Klebsiella, salmonellae, and S. marcescens. MIC values against 34 organisms were calculated. The degree of protein binding was 17% and thus equal to ampicillin and better than benzyl penicillin which binds to 50-60%. Serum levels 2 hours after oral administration were approximately twice as high as ampicillin. The blood level data were confirmed in clinical volunteers (27).

Blood levels of 6-10 mcg of penicillin immediately stimulated many clinicians to study amoxicillin. This resulted in three symposia, one in London (28), another held in New Orleans (29) and a third in Tokyo (30). Increased penicillin blood levels were reported by many investigators. May and Ingold (29) showed that amoxicillin penetrated deeper than ampicillin and all other penicillins into cell walls and was more effective against gram negative infections. They also reported that amoxicillin penetrated deeper into respiratory secretions and speculated that higher tissue levels were obtained.

In another review (31) 147 references on amoxicillin were quoted. Peak serum levels were twice those of ampicillin and generally more prolonged. Amoxicillin was more stable in gastric acid than ampicillin. Levels in sputum, sinus mucosa and interstitual fluid are higher than ampicillin. High levels were found in the liver of rats and kidneys of mice which confirmed higher tissue level speculations.

Further studies in healthy adults resulted in amoxicillin serum levels of 7.34 mcg/ml and in ampicillin 3.64 mcg/ml following administration of 500 mg oral dose. In patients with impaired renal function, levels as high as 20 mcg/ml were obtained.

Amoxicillin was also reported to be successfully used in various other infections (31) including biliary, gynaecological, and bone infections and in septic abortions. In hundreds of patients it was an effective antibacterial agent in urinary tract infections (70-93% cure), upper and lower respiratory infections, skin and soft tissue and G.I. tract infections. Eleven studies on uncomplicated gonorrhoeae resulted in 92% cure (31).

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Analytical Profiles of Drug Substances, 7

CHLORPHENIRAMINE MALEATE

Charles G. Eckhart and Thomas McCorkle

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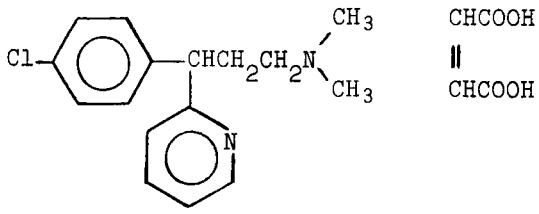
TABLE OF CONTENTS

1. Description

1.1 Formula, Name, Molecular Weight



$C_{16}H_{19}ClN_2 \cdot C_4H_4O_4$

Chlorpheniramine maleate is a racemic mixture of optical isomers and is known by the following chemical names (1).

i 2-pyridinepropanamine, γ-(4-chlorophenyl)-N,N-dimethyl-(Z)-2-butenedioate (1:1)
ii 2-[p-chloro- α-(2-dimethylaminoethyl)benzyl] pyridine maleate (1:1)

Molecular Weight: 390.87

1.2 Appearance, Color, Odor

Chlorpheniramine maleate is an odorless, white crystalline solid.

2. Physical Properties

2.1 Elemental Analysis

Analysis of chlorpheniramine maleate (Batch CM-6G-1001) was determined for carbon, hydrogen, nitrogen and chlorine. The carbon, hydrogen and nitrogen analysis was performed on a Perkin Elmer Model 240 instrument. Analysis for chlorine was performed by combustion of the sample and coulometric titration using an American Instrument Co. Chloride Titrator.

$C_{20}H_{23}N_2O_4Cl$

Theoretical Percentages - C: 61.45%, H: 5.93%,
N: 7.17%, Cl: 9.07%

Experimental Percentages - C: 61.34%, H: 6.02%,
N: 7.18%, Cl: 9.11%

2.2 Mass Spectrum

The mass spectrum of chlorpheniramine maleate (Batch CM-6G-1001) was obtained on a Varian MAT CH5 medium resolution single focusing magnetic sector instrument at 70 eV with a probe temperature of 110°C. Mass spectrum calibration was performed with perfluoroalkane-225. The results are tabulated in Table 1 and are in full support of the structure given.

TABLE 1

MASS	ION	LOSS
274	M^+	
230	M^+-44	$N(CH_3)_2$
216	M^+-58	$CH_2N(CH_3)_2$
203	M^+-71	$CHCH_2N(CH_3)_2$
167	M^+-107	$CH_2CH_2N(CH_3)_2$ + Cl
78		
72	$CH_2CH_2N^+(CH_3)_2$	
58	$CH_2-N^+(CH_3)_2$	
44	$N^+(CH_3)_2$	

The mass spectrum is shown in Fig. 1.

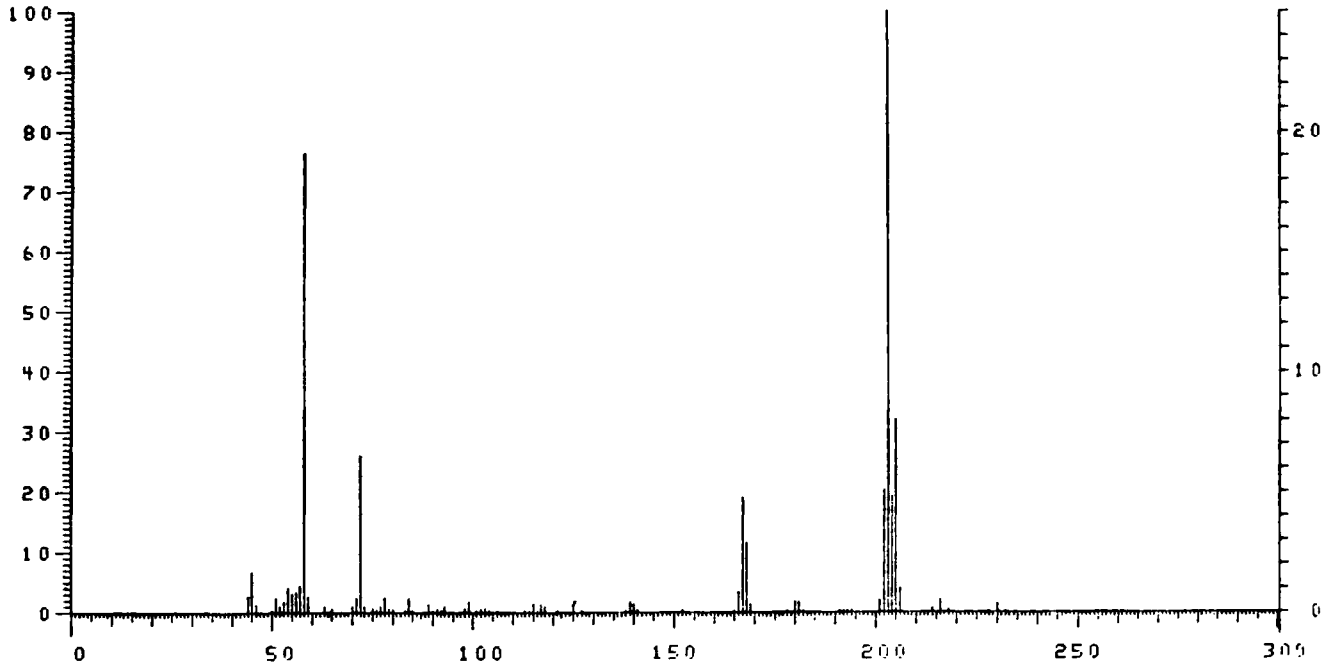


Fig. 1 Mass Spectrum of Chlorpheniramine Maleate.

Pattern recognition methods were used to differentiate drug classes (e.g. sedatives, tranquilizers) and to predict their pharmacologic activity based on their mass spectral fragmentation pattern (2). Chlorpheniramine maleate was used as an example.

2.3 Proton Magnetic Resonance Spectrum (PMR)

The 100 MHz proton nmr spectrum of chlorpheniramine maleate (Batch CM-6G-1001) was obtained as a 5% (W/V) solution in hexadeuteriodimethylsulfoxide (d^6-DMSO) with tetramethylsilane (TMS) as an internal reference. The spectrum was obtained on a Varian XL-100-15 Fourier Transform Spectrometer at ambient temperature. The assignments, chemical shifts and multiplicities are given in Table 2 and are in full support of the structure shown.

TABLE 2

PMR Spectral Assignments

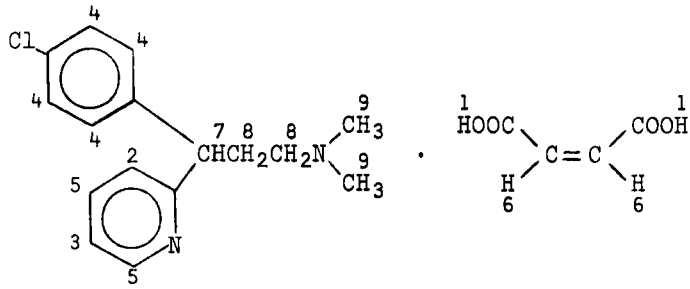


Table 2 (con't.)

Position of Proton	Chemical Shift (δ)	Intensity	Multiplicity
1	10.5	2 H	Singlet
2	8.7	1 H	Multiplet
3	7.8	1 H	Multiplet
4	7.4	4 H	Singlet
5	7.2-7.5	2 H	Multiplets
6	6.2	2 H	Singlet
7	4.2	1 H	Triplet
8	2.5-3.1	4 H	Multiplet
9	2.8	6 H	Singlet
10 DMSO/d_5H	2.5		

The PMR spectrum is shown in Fig. 2.

2.4 Carbon-13 Magnetic Resonance Spectrum (CMR)

The carbon-13 magnetic resonance spectrum was run on chlorpheniramine maleate (Batch CM-6G-1001). The spectrum was run on a Varian Associates XL-100-15, equipped with a 620-L100 disc-based computer. The spectrum was run in a saturated solution of d^6-DMSO. The field was locked to the deuterium resonance of the solvent. The data was zero filled to 16K prior to transformation; aquisition time was 0.8 sec. with no post delay used.

The spectrum (Fig. 3) represents 25.159 MHz with a 90^o pulse width in two micro seconds. The shifts shown in Table 3 are downfield of an internal TMS reference.

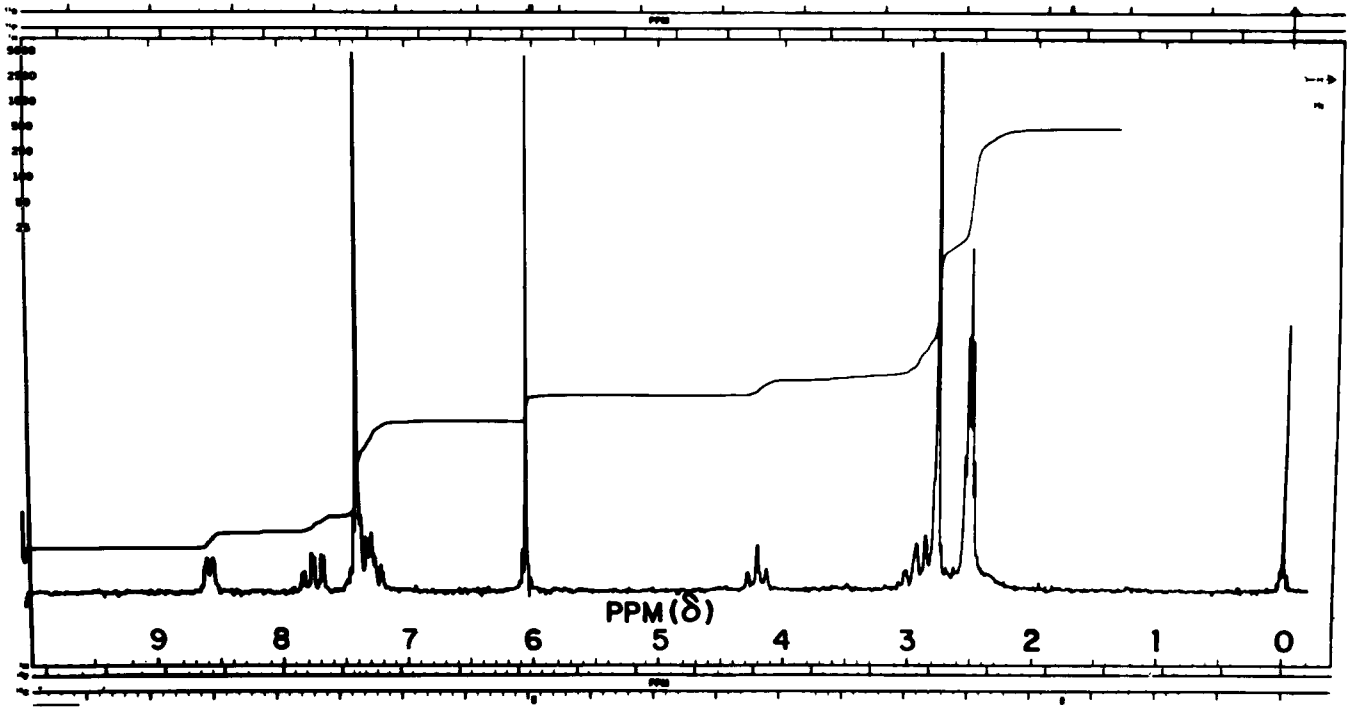


Fig. 2 100 MHz Proton Magnetic Resonance Spectrum of Chlorpheniramine Maleate in d^6- DMSO (5% w/v) with TMS as an Internal Reference.

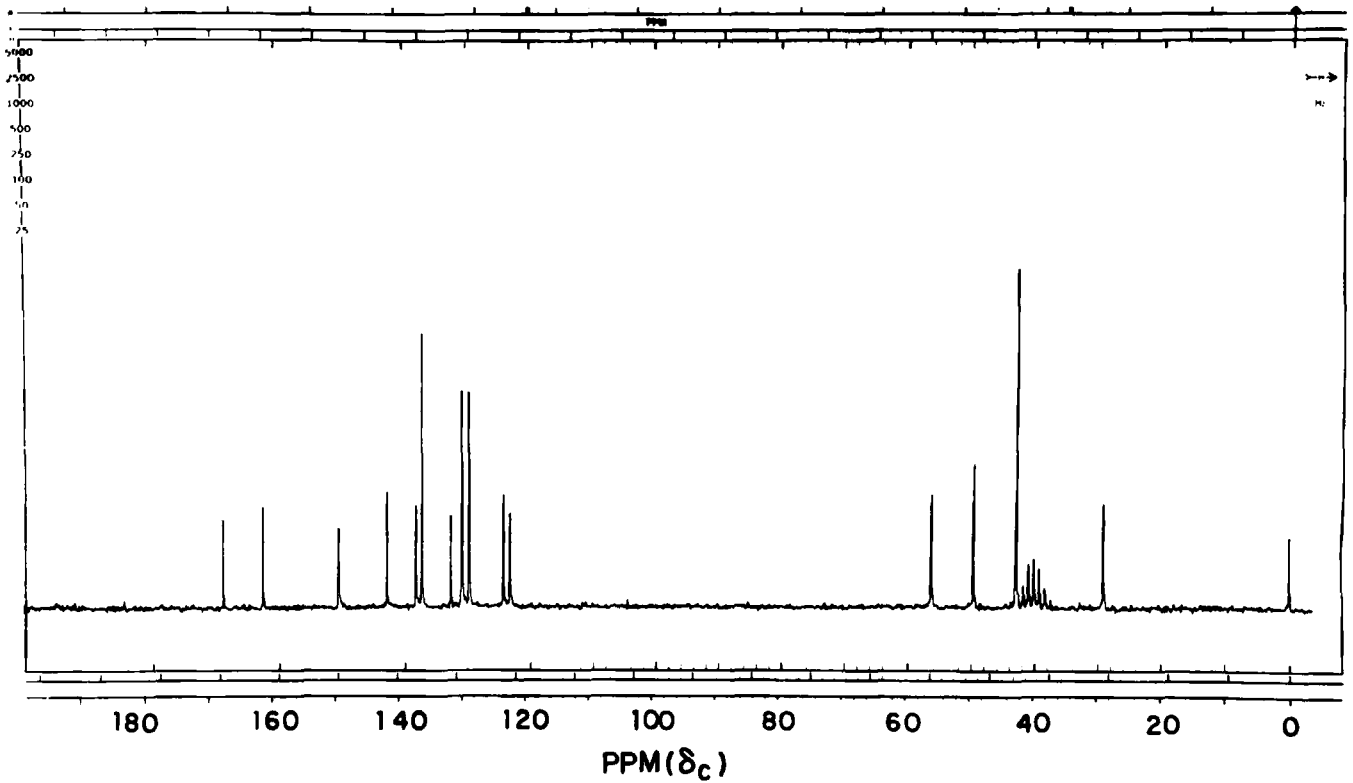
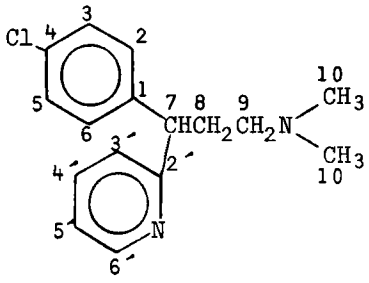
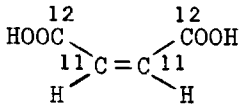


Fig. 3 C-13 Magnetic Resonance Spectrum of Chlorpheniramine Maleate in Saturated d^6- DMSO Solution with TMS as an Internal Standard.

TABLE 3

CMR Spectral Assignments





Position of Carbon	Chemical Shift (δ)
8	28.76
10	42.27
9	48.94
7	55.51
5′	122.00
3′	123.02
3, 5	128.49
2, 6	129.61
4	131.42
11	135.99
4′	136.97
1	141.56
6′	149.20
2′	161.20
12	167.55

2.5 Infrared Spectrum

The infrared spectrum of chlorpheniramine maleate (Batch CM-6G-1001) was obtained as a Nujol mull on a Perkin Elmer Model 180 grating infrared spectrophotometer. The major absorption bands are tabulated below in Table 4. These assignments are in full support of the given structure.

TABLE 4

Infrared Assignments

Wavenumber (cm^{-1})	Assignment
3090-3010 (w)	Aromatic and unsaturated C-H stretch
2700-2400 (m, vbr.)	N^{+}-H stretch
1705 (m)	C=O stretch
1620 (m), 1570 (s)	aromatic C=C, C=N stretch
1584 (s)	aromatic C=C, C=N, and carboxylate $C-O^{-}$ stretch
1359 (s)	carboxylate $C-O^{-}$ stretch
886 (m), 868 (s)	maleate

Notations: w-weak, m-medium, s-strong, vbr-very broad

The infrared spectrum is shown in Fig. 4.

2.6 Equilibrium Data

The pKa's of chlorpheniramine maleate are as follows:

$$pKa_1 = 9.2$$

$$pKa_2 = 4.0$$

The source of the pKa_1 data is reference (3). pKa_2 has been determined spectrophotometrically by Higuchi, et.al. (4) and Doye, et.al (5).

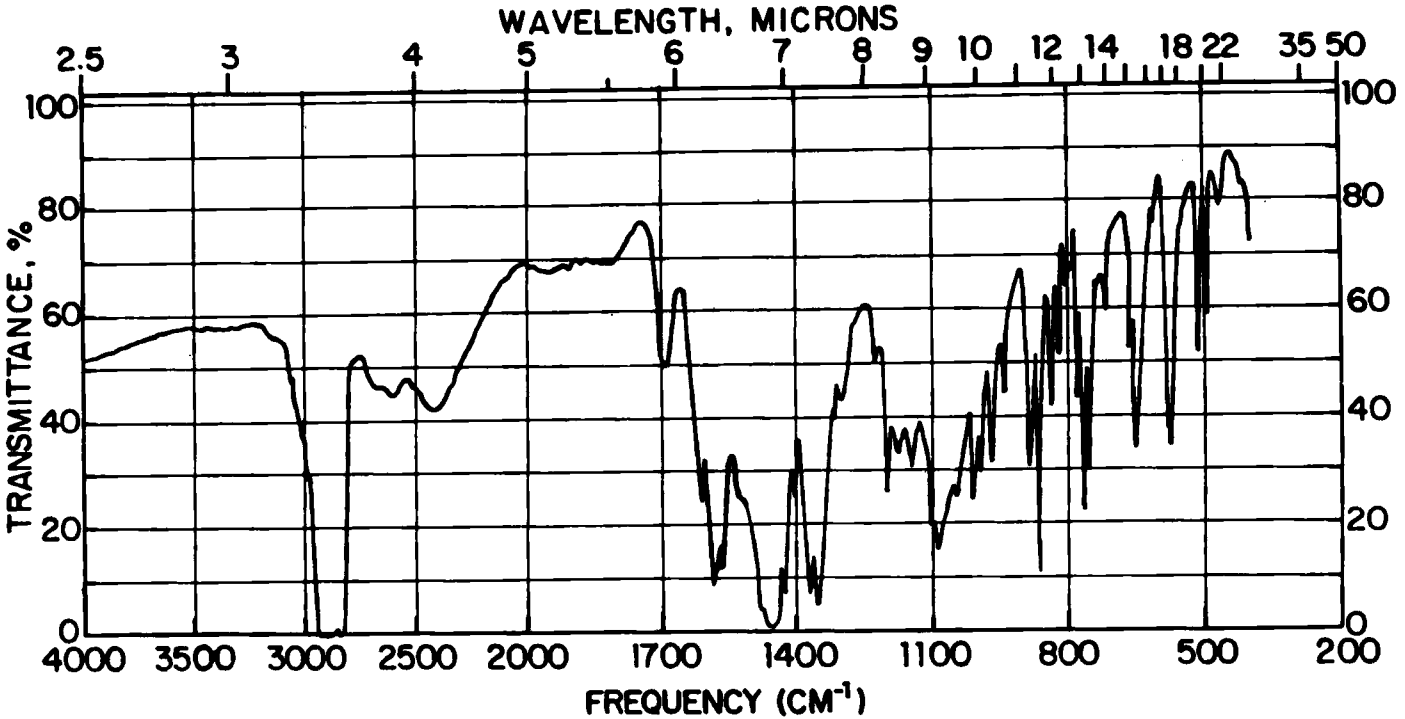


Fig. 4 Infrared Spectrum of Chlorpheniramine Maleate Obtained as a Nujol Mull.

An interesting application of ionization constants was made by Doyle and Proctor (6) in their study of selective elution parameters for several amine-containing drugs by partition chromatography. A graphical method using pKa's and extraction constants was used.

2.7 Ultraviolet Spectrum (UV)

The ultraviolet spectrum (Fig. 5) of chlorpheniramine maleate (Batch CM-6G-1001) was obtained in methanol on a Cary 118CX spectrophotometer. The spectral data is listed in Table 5.

TABLE 5

Ultraviolet Spectrum of Chlorpheniramine Maleate in Methanol

λmax, nm	$\varepsilon \times 10^{-3}$
250 shoulder	4.27
255	5.05
261	5.38
267	3.86
275 shoulder	1.17

Table 6 lists the spectral data of chlorpheniramine maleate obtained in various solvents.

TABLE 6

The UV Maximum and Molar Absorptivities in Various Solvents

Solvent	λmax	$\varepsilon \times 10^{-3}$
CH_3OH	261 nm	5.38
0.1N NaOH (CH_3OH)	261 nm	5.63
0.1N HCl (CH_3OH)	265 nm	8.48
H_2O	261 nm	5.76

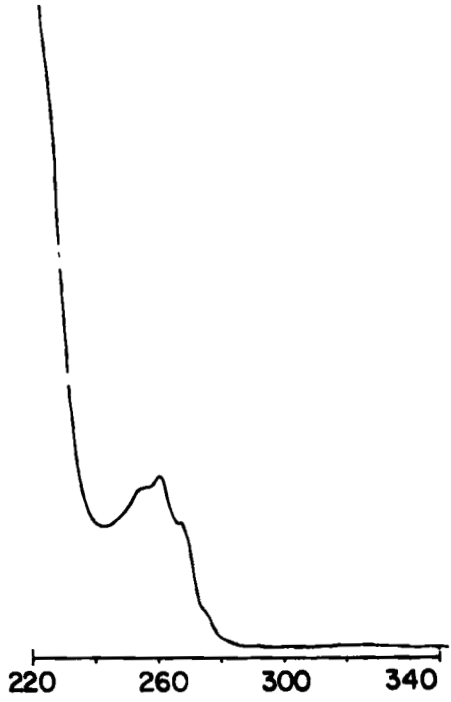


Fig. 5. Ultra-violet Absorption Spectrum of Chlorpheniramine Maleate in Methanol.

TABLE 6 (con't.)

Solvent	λmax	$\varepsilon \times 10^{-3}$
1N NaOH (aq.)	262 nm	5.77
1N HCl (aq.)	265 nm	8.39
$CHCl_3$	263 nm	5.77

It is found that the band maximum is relatively insensitive to solvent effects, but the fine structure is lost in acidic media.

2.8 Crystal Properties

According to Brandstatter-Kuhnert, et.al. (7) using the thermo-microscopic technique, chlorpheniramine maleate crystalizes near its melting point (129-134°C) to give plates, rhombs, grains and prisms. The supercooled melt will solidify to a glass on the microscope stage. At the present time no optical crystallography has been done since X-ray diffraction data is available.

There is optical crystallography on dexchlorpheniramine maleate (the (+) optical isomer). The National Formulary XII (1965) has given the following data (8):

nα	nβ	nγ
1.509	1.564	1.683

Optic sign: + Axial Angle: 2V = 70° (calc.)

The extinction is parallel to the crystal edge. These data are also reported in (9).

2.9 Thermal Analyses

Thermal Gravimetric Analysis (TGA) indicates no weight loss from ambient temperature to 140°C for chlorpheniramine maleate.

The Differential Scanning Calorimetry (DSC) Curve (Fig. 6) exhibits a single sharp melting endotherm with the onset temperature of 133°C. Grady, et.al. (10) report a H_F = 11,400 cal./mole on a 99.55 mole % pure sample of chlorpheniramine maleate.

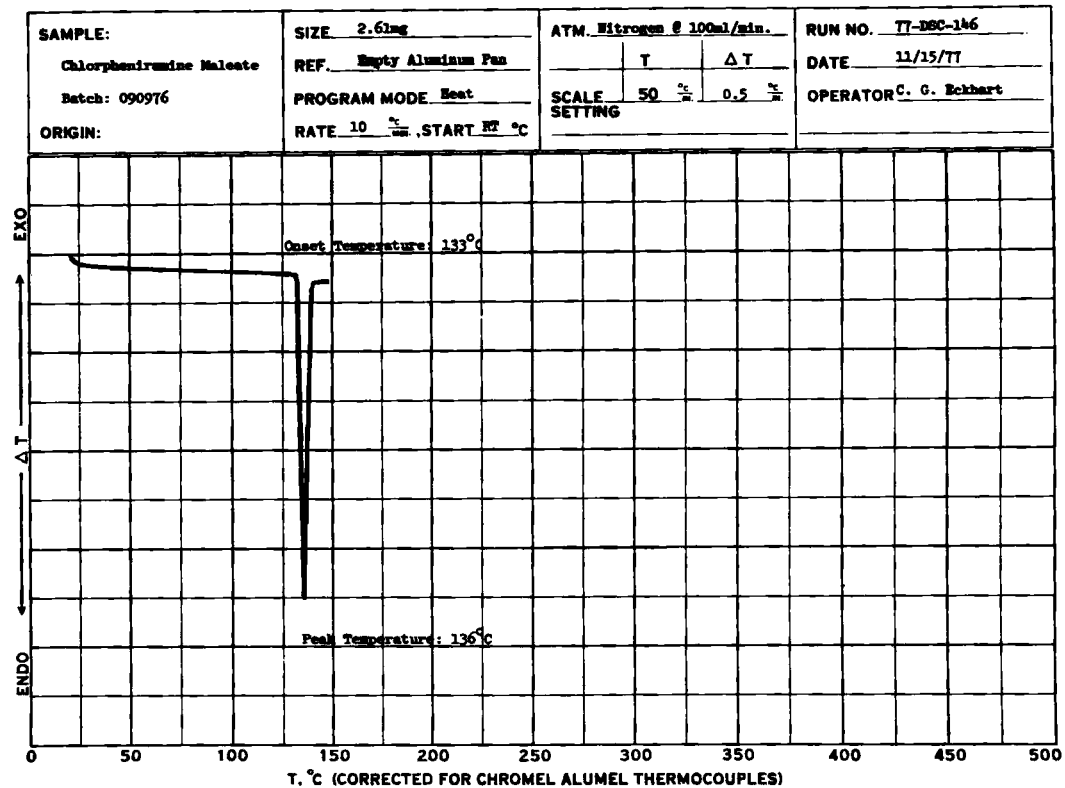


Fig. 6 DSC Curve of Chlorpheniramine Maleate
Onset Temperature 133° C; Peak Temperature 136° C.

2.10 X-Ray Diffraction

The X-ray powder diffraction pattern (Table 7) was obtained on chlorpheniramine maleate (Batch CM-6G-1001) with a Phillips ADP 3500 X-Ray Diffractometer utilizing CuK_α radiation (1.5418 Å).

TABLE 7

The X-ray Powder Diffraction Pattern of Chlorpheniramine Maleate

2Θ	d(Å)*	I/I**
12.214	7.246	9
12.957	6.832	25
16.943	5.233	6
17.011	5.212	6
18.262	4.857	7
18.543	4.784	14
18.613	4.766	13
18.805	4.718	14
19.214	4.619	100
19.536	4.543	5
19.661	4.515	7
20.215	4.392	90
20.561	4.319	11
20.777	4.275	19
20.965	4.237	14
21.368	4.158	23
21.836	4.070	25
24.058	3.699	59
24.636	3.613	29
24.959	3.568	21
25.599	3.480	17
26.214	3.399	29
28.059	3.180	20
29.960	2.982	15
30.618	2.920	7
32.161	2.783	20
33.622	2.666	8
33.697	2.660	8
33.918	2.643	9
34.722	2.583	11

*d (interplanar distance) = $n\lambda$ / 2 sin Θ

**I/I = relative intensity (based on highest intensity of 100)

2.11 Solubility and Partition Studies

The solubility of chlorpheniramine maleate in eighteen solvents was studied by gravimetric or ultraviolet spectral detection. The data is summarized in Table 8.

TABLE 8

Solvent	Solubility, mg/ml at 25°C	Measurement
Acetone	50.	Gravimetric
Benzene	0.7	Gravimetric
Carbon tetrachloride	$4. \times 10^{-2}$	Gravimetric
Chloroform	240.	Gravimetric
1, 2 Dichloroethane	47.	Gravimetric
Dioxane	6.0	Gravimetric
Ethyl acetate	4.0	Gravimetric
Ethyl alcohol	330.	Gravimetric
Glycerine	21.	Ultraviolet
Heptane	$8. \times 10^{-3}$	Gravimetric
0.1N HCl	47.	Gravimetric
Methyl alcohol	130.	Gravimetric
Methylene chloride	190.	Gravimetric
Petroleum ether	0.1	Gravimetric
Parenteral phosphate buffer soln.	190.	Ultraviolet
Saturated salt solution	28.	Ultraviolet
0.1N NaOH	180.	Ultraviolet
Water	160.	Ultraviolet

In order to facilitate extraction and chromatographic studies, liquid - liquid partitioning data were accumulated and are tabulated in Table 9.

TABLE 9

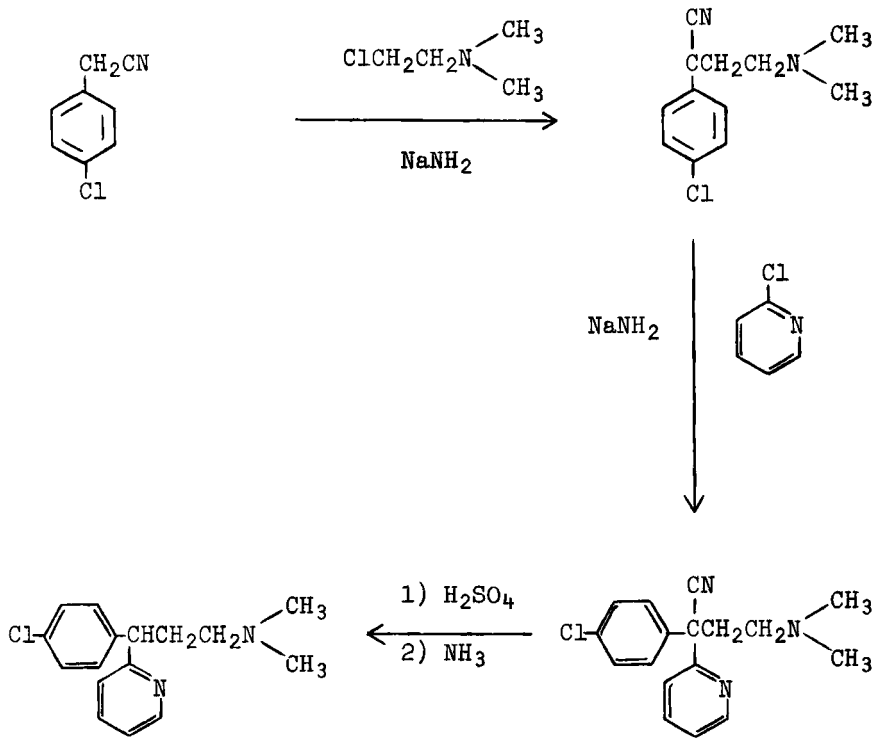
Liquid-Liquid Partitioning Data

Percent in Organic Phase

Aqueous pH	n-BuOH	$CHCl_3$	n-Heptane
0.1	-	0.6	-
1.3	-	-	0.8
1.7	-	1.0	-
2.7	35.4	-	-
2.6	-	-	7.1
3.5	73.8	44.0	-
3.6	-	-	8.5
4.3	79.2	70.0	-
5.8	-	-	74.7
6.2	83.3	91.2	-
6.7	-	-	81.5
7.1	92.7	99.0	-
7.7	-	-	94.9
8.0	99.4	-	-
8.7	-	99.6	97.4
9.4	-	-	99.4
10.8	-	99.7	-
11.9	-	99.6	-
12.5	-	99.6	99.8

3.0 Synthesis

The following synthesis has been used to produce chlorpheniramine maleate (11).



4. Stability

The stability of chlorpheniramine maleate has been studied in our laboratories using accelerated heat and light conditions on solutions of various pH values (Batch CM-6G-1001). The experimental conditions as well as the results are given in the following two paragraphs.

4.1 pH-Thermal Stability

This study was done using 30 mg of chlorpheniramine maleate in 10 ml of aqueous buffer over a wide pH range in sealed glass ampules. The samples were stored for 1 week at 95°C. They were then assayed by quantitative paper chromatography. Results are summarized as follows:

pH	Buffer Component	Percent Recovery
2	0.1 M citric acid	99
4	0.1 M sodium citrate	95
6	0.1 M sodium phosphate	100
7	0.1 M sodium phosphate	97
8	0.1 M sodium phosphate	96
10	0.1 M sodium borate	96
13	0.1 M sodium hydroxide	99

4.2 pH-Light Stability

This study was done with 15 mg of chlorpheniramine maleate in 5 ml of aqueous buffer, over the pH 2 to 8 range, in sealed glass ampules. Two sets of samples were prepared. One set was stored for three months at 25°C in a light box under a fluorescent light of about 350 candle power. The other set was stored for three months at 25°C in total darkness. The samples were then assayed by quantitative paper chromatography. Results of these assays are summarized as follows:

		Percent Recovery	
pH	Buffer Component	Light	Dark
2	0.1 M citric acid	101	101
4	0.1 M sodium citrate	103	101
6	0.1 M sodium phosphate	102	101
8	0.1 M sodium phosphate	102	100

4.3 Phase Solubility Analysis

Grady, et. al. (10) studied the purity of 115 drugs by several techniques including phase solubility analysis. Solubility and impurity data on chlorpheniramine maleate and dexchlorpheniramine maleate were included in the study.

5. Drug Metabolic Products

5.1 Metabolism of Chlorpheniramine Maleate in Man (12)

The pharmacokinetics and metabolism of ^{3}H chlorpheniramine maleate have been studied in man. After a p.o.

dose (12 mg), ^{3}H appeared rapidly in plasma and at two hours was equivalent to 32.48 ng of chlorpheniramine per ml; radioactivity persisted in plasma through 48 hours. At five minutes after an i.v. dose (4 mg), ^{3}H in plasma was equivalent to 20.88 ng of drug per ml and ^{3}H again persisted in plasma. This persistance of plasma ^{3}H was most probably due to tissue deposition of ^{3}H-drug (or metabolites), directly indicated by its large volume of distribution, 250% of body weight. The drug was 72 % bound to plasma protein at a concentration of 280 ng/ml. Unchanged drug as well as its metabolites were present in plasma, however, and in contrast to the persistant levels of ^{3}H in plasma, the levels of ^{3}H-chlorpheniramine declined steadily, although slowly, after the i.v. dose and after the two-hour peak after administration p.o. The plasma half-life of a p.o. dose was estimated at 12 to 15 hours and that of an i.v. dose, 28 hours. Excretion of the ^{3}H-drug was slow and only one-third of a p.o. or i.v. dose was recovered from the excreta during 48 hours after administration. The p.o. dose was completely absorbed, however, and only small amounts of the ^{3}H-drug were excreted into feces after its administration. Fecal excretion of ^{3}H of an i.v. dose indicated that ^{3}H-chlorpheniramine underwent an enterohepatic circulation. Chlorpheniramine was extensively metabolized and excreted in the urine as mono- and didesmethyl chlorpheniramine, two unidentified metabolites and small amounts of chlorpheniramine. The greatest portion of the drug was excreted as unidentified polar metabolite(s).

5.2 Metabolism of Chlorpheniramine Maleate in the Dog and Rat (13)

Studies of the pharmacokinetics and metabolism of ^{14}C and ^{3}H-chlorpheniramine maleate in the rat and dog have also been conducted. An oral dose of the drug was rapidly and extensively absorbed from the gastrointestinal tract. In the dog 75 per cent of the radioactivity of an oral dose was excreted in the urine and feces in 11 days and 86 per cent of an i.v. dose in 16 days. Most of the radioactivity was excreted in the urine in either case. In the rat 93 per cent of an oral dose was recovered from the excreta in 5 days; excretion of the drug was almost equally divided between urine and feces. Radioactivity of the ^{3}H drug disappeared quite slowly from the plasma of the dog, and the plasma half-life of radioactivity of an i.v. dose was approximately 10 days. Plasma radioactivityy was composed of chlorpheniramine metabolites as well as unchanged drug, however, and chlorpheniramine in contrast disappeared

rapidly from plasma having a half-life of 3 hrs. The drug was completely metabolized in the dog and rat and was excreted into the urine as relatively polar and non-polar products. N-dealkylation represented one pathway of metabolism and didesmethyl chlorpheniramine was the main N-dealkylated metabolite. Only 1 to 2 per cent of the dose was excreted unchanged.

6. Analytical Methods

6.1 Acid-Base Titration

Acid-base titrations were performed potentiometrically using non-aqueous solvents, with 0.1M $HClO_4$ as the titrant. A Beckman Model K automatic titrator was used to obtain the data which is tabulated in Table 10.

TABLE 10

Neutralization Equivalents of Chlorpheniramine Maleate By Non-Aqueous Titration

	Neutralization Equivalent	
Theoretical:	195.4	390.8
Experimental:		
Solvent		
Acetic Anhydride	191 (s)*	415 (w)
Acetonitrile	189 (m)	385 (m)
m-Cresol	182 (s)	-
chloroform	192 (m)	-
Nitromethane	-	394 (m)
Acetic Acid	195	-

* Symbol denoting relative magnitude of potentiometric inflection point:

s - sharp
m - medium
w - weak

Titration as an assay method is cited in the USP (1) where chlorpheniramine maleate is titrated with perchloric acid in an acetic acid media. An aqueous titration assay (14) has also been proposed that involves the back-titration of excess HCl that was not taken up by the free base.

6.2 Colorimetric Methods

Several colorimetric procedures for the assay of chlorpheniramine maleate have been proposed. Among the earliest (15, 16) was the formation of the Reinecke salt (ammonium tetrathiocynatodiamminechromate III) which was dissolved in acetone and the absorbance was read. The disadvantages of this method are that it lacks specificity, and that the colored complex can be decomposed by the presence of water.

Several variations of the Jones and Brady reaction (17) have been studied (18-22). The general reaction involves formation of an adduct through attack on the pyridine ring by cyanogen bromide, but further complexation with aniline is possible with many N-(2-pyridyl) antihistamines. The reaction is an adaptation of the Koenig reaction (23, 24).

Procedures for the complexation of chlorpheniramine maleate with sulfonephthalein dyes such as bromcresol green (25) bromcresol purple (26) or bromthymol blue (27) have been described. An automated procedure (28) that compared ethyl orange to bromphenol blue as complexing dyes has been developed using the Technicon automatic analyzer. Ethyl orange was the preferred dye since an orange residue developed on the glass junctions of the apparatus when bromphenol blue was used. Another automated technique (29) was developed that used bromthymol blue and bromcresol purple to selectively determine polar and non-polar classes of pharmaceutical amines in a mixture of the two classes. A single apparatus was used with two sets of dye reagents, depending on the class of compound to be analyzed.

6.3 Spectrophotometric and Fluorometric Methods

Analytical fluorescence techniques have been used for the analysis of chlorpheniramine maleate (30-31). One technique (31) describes the complexation of rose bengal dye. The second reference describes a comparison of two methods, oxidation by hydrogen peroxide and attack by cyanogen bromide. Both procedures show low sensitivity, and the rose bengal dye method shows sensitivity to solvent impurities (32-33).

Ultraviolet spectroscopy has been used for the determination of chlorpheniramine maleate in injectables (34) and in syrup (35). One of the drawbacks of these procedures is that they involve extraction of the free base from the sample and subsequent comparison of the UV spectrum

to that of the maleate. According to Hamilton, et.al. (36) this gives rise to a 2-3% negative bias, since the maleate salt gives a higher absorbance than the free base alone.

An interesting proposal for the use of orthogonal functions in deconvoluting UV spectra of chlorpheniramine maleate and its excipients is given by Abdine, et.al. (37).

6.4 Thin-Layer Chromatography (TLC)

Table 11 summarizes the TLC systems used, showing the type of adsorbant and the developing solvent used. The R_f values are also given. (R_f = Z solute / Z mobile phase), where Z is equal to the distance travelled on the plate.

TABLE 11

A Summary of Several Thin Layer Chromatography (TLC) Systems for Chlorpheniramine Maleate

Adsorbant	Solvent System	R_f	Reference
Silica gel dipped or prepared with 0.1M KOH	cyclohexane:benzene: diethylamine (75:15:10)	0.38	38, 39
Silica gel dipped or prepared with 0.1M KOH	methanol	0.19	38
Silica gel dipped or prepared with 0.1M KOH	acetone	0.06	38
Silica gel dipped or prepared with 0.1M $KHSO_4$	methanol	0.08	38, 39
Silica gel (0.5 mm.)Merck GF	ammonia:methanol (1.5:100)	-	43
Silica gel Merck GF	benzene:methanol: conc. aq. ammonia (40:10:1)	0.60	40

TABLE 11 (con't.)

Adsorbant	Solvent System	R_f	Reference
Silica Gel G	ethyl acetate: methanol :conc. aq. ammonia (170:20:10)	0.88	41[a]
0.25mm Silica Gel F_{254} Merck	ethyl acetate: methanol:water: conc. aq. ammonia (85:10:3:1)	0.33	42[b]
0.25 mm Polygram Silica Gel Sheets Machery, Nagel & Co.	chloroform: methanol:conc. aq. ammonia (90:10:1)	0.67	42
0.5 mm Silica Gel GF_{254} Merck	ammonia (s.g. = 0.91) methanol (1.5:100)	-	43[c]
0.25 mm Silica Gel Glass Microfiber sheet I.T.L.C. Type SA Gelman	ethyl acetate: cyclohexane: p-dioxane:methanol: water:ammonium hydroxide (50:50:10:10:1.5:0.5)	0.44	44
0.25 mm Silica Gel Glass Microfiber sheet I.T.L.C. Type SA Gelman	ethyl acetate: cyclohexane: p-dioxane:methanol: water:ammonium hydroxide (50:50:10:10:0.5:1.5)	0.58	44
0.25 mm Silica Gel Glass Microfiber sheet I. T.L.C. Type SA Gelman	ethyl acetate: cyclohexane: ammonium hydroxide: methanol:water (70:15:2:8:0.5)	0.70	44
0.25 mm Silica Gel Glass Microfiber sheet I. T.L.C. Type SA Gelman	ethyl acetate: cyclohexane: methanol: ammonium hydroxide (70:15:10:5)	0.91	44

Notes: a. This reference gives TLC systems for 90 drug substances.
b. This reference shows how drugs of abuse may be identified by way of their different R_f's as well as their reaction to different spray reagents.
c. The author of this reference directly scrapes the compound of interest from the TLC plate and introduces it into a mass spectrometer.

6.5 Paper Chromatography (PC)

Table 12 summarizes paper chromatographic systems for chlorpheniramine maleate.

TABLE 12

Paper Chromatographic Systems for Chlorpheniramine Maleate

Support	Solvent System	R_f	Reference
Whatman No. 1 paper dipped in 5% dihydrogen citrate	butanol:water:citric acid 870:130:4.8g.	0.45	38
Whatman No. 1 paper dipped in 10% tributyrin in acetone	acetate buffer pH 4.58, 95°C	0.69	38
Whatman No. 1 paper dipped in 10% tributyrin in acetone	phospate buffer pH 7.4, 86°C	0.13	38
Whatman No. 4 paper dipped in 0.1 N HCl in acetone	n-butanol saturated with 0.5 N HCl	0.35	40
Whatman 3MM paper impregnated with 2% $(NH_4)_2SO_4$ (pH 5.3)	isobutanol: acetic acid :water (100:10:24)	0.52	67*

TABLE 12 (con't.)

Support	Solvent System	R_f	Reference
Whatman 3MM paper impregnated with 0.5M KNa_2PO_4 (pH 4.2)	isobutanol: acetic acid :water (100:10:24)	0.87	67*
Whatman No. 1 paper impregnated with 5% monosodium citrate	n-butanol: water:citric acid (50:50:3g)	0.78	46
Whatman No. 4 paper	n-BuOH saturated with 1.0 N HCl	0.65	47

* Note: This reference contains R_f data on 270 drug substances, using two paper chromatographic systems.

6.6 Separation by Countercurrent Distribution

Onishi, et.al. (48) separated chlorpheniramine maleate quantitatively from several other drug substances using Craig-type counter-current distribution. Fifty transfers were needed to a 100% recovery of chlorpheniramine maleate from a mixture.

6.7 Gas-Liquid Chromatography (GLC)

The following table summarizes the recent GLC systems showing the liquid phase used, the column length and the oven temperature.

TABLE 13

Gas-Liquid Chromatographic Systems for Chlorpheniramine Maleate

Column	Length	Temperature	Reference
SE-30	2 m	350°C	49
Apiezon L/KOH	2 m	300°C	49
OV17	1 m	350°C	49

TABLE 13 (con't.)

Column	Length	Temperature	Reference
Carbowax 20M/KOH	1 m	230°C	49
Carbowax 20M	1 m	230°C	49
CDMS	2 m	230°C	49
DEGS/KOH	1 m	190°C	49
DEGS	2 m	190°C	49
Chromosorb W	2 m	250°C	50
3% OV-17 on Gas Chrom Q	-	230°C	51
1.2% Carbowax 20M on Gas Chrom Q	2 m	215°C	52
1.2% Carbowax 20M on chromosorb WHP	1.3 m	200°C	52
4% Carbowax 20M on Anakrom ABS	0.76 m	130 - 150°C (10°/min)	53 53
0.25% Uncon oil 50-HB-5100 on glass beads	2 m	130 - 150°C (10°/min)	53
3% Carbowax 20M on Anakrom HBS	0.76 m	180°C	54
2% SE-30 on Chromosorb W (HP) acid washed and silanized	2.44 m	200°	55
methylvinyl silicone gum rubber	2 m	250°	56
2% Carbowax 20M on 60/80 chromosorb W	5 ft.	190°C	45
2% SE-30 and 2% Carbowax 20M on 80/90 Anakrom ABS	4 ft.	185°C	57

TABLE 13 (con't.)

Column	Length	Temperature	Reference
5% silicone gum rubber (SE-30) on Chromosorb G 80/100 acid washed amd HMDS treated	1 m	215°C	58
2.5% SE 30 on 80/100 Chromosorb G	2 ft.	200°C	59[a]
1% cyclohexanedi-methanol succinate and 10% SE-5S (silicone gum rubber) on 80/100 mesh Gas Chrom Q	1.8 m	200-250°C	60
0.07% SE-30 on 120/170 Glass Beads	6 ft.	175°C	61[b]
0.08% PDEAS on 120/170 Glass Beads	6 ft.	175°C	61
1.07% XF-1150 on Chromosorb W-HMDS 100/120 mesh	6 ft.	175°C	61
1.08% Carbowax 20M on 100/120 Gas Chrom P	6 ft.	175°C	61
3% OV-17 on 60/80 Gas Chrom Q	6 ft.	195°C	62
10% Dexsil 300 on 80/100 Chromosorb W (HP)	6 ft.	220, 258°C	63
17% Dexsil 300 on 80/100 Chromosorb W (HP)	8 ft.	222°C	63
1% Carbowax 20 M on Gas Chrom Q	3 ft.	205°C	64

TABLE 13 (con't.)

Column	Length	Temperature	Reference
1.2% Carbowax 20 M on KOH treated Gas Chrom Q	6 ft.	215°C	64

Notes: a. This reference gives gas chromatographic data on 600 drug substances.

b. This system was judged by the authors to give the best peak shapes and shortest retention times for the 16 antihistamines studied on the four systems.

6.8 High Performance Partition and Ion-Exchange Liquid Chromatography (HPLC)

Several high performance liquid chromatographic procedures have been developed in recent years for the purpose of assaying chlorpheniramine maleate dosage forms and separating the antihistimine from excipients or other drugs. Table 14 summarizes the HPLC systems used along with some earlier open column ion-exchange work.

TABLE 14

Liquid Chromatographic Systems for Chlorpheniramine Maleate

Column	Mobile Phase	Reference
Permaphase ODS 37-44 microns pellicular DuPont	0.2 M KCI 0.2M boric acid 0.2 ml conc. ammonia in 390 ml water 610 ml ethanol pH 8.8	65
Corasil/C-18 37-50 microns pellicular packing Waters	50-90% acetonitrile 50-10% 0.1% ammonium carbonate - pH 8.5-8.9	66
Corasil/phenyl 37-50 microns pellicular packing Waters	60-80% acetonitrile 40-20% 1.0% ammonium acetate pH 7.4-7.6	66

TABLE 14 (con't.)

Column	Mobile Phase	Reference
Corasil/phenyl 37-50 microns pellicular packing Waters	50-90% acetonitrile 50-10% 0.1% ammonium carbonate pH 8.5-8.9	66
Corsil/C-18 37-50 microns pellicular packing Waters	60-80% acetonitrile 40-20% 1.0% ammonium acetate pH 7.4-7.6	66
Zipax SCX 25-37 microns pellicular packing DuPont	0.01 M - 0.04M $(NH_4)_2HPO_4$ with 32% dioxane in water	68
μBondapak Phenyl ca. 10 microns porous packing Waters	45% methanol 55% water 0.005 M heptane sulfonate (sodium salt) 1% acetic acid	69
μBondapak Phenyl ca. 10 microns porous packing Waters	33% methanol 67% 0.01M sodium acetate (pH 4.0)	69
μBondapak CN ca. 10 microns porous packing Waters	60% methanol 40% 0.01 M $(NH_4)_2HPO_4$ (pH 7.5)	69
Zorbax CN ca. 5 microns porous spherical packing Dupont	75% acetonitrile 25% 0.01 M KH_2PO_4	70

Partition chromatography using Celite 545 (Johns-Manville Corp.) and several 25 x 200 mm columns have been used for the selective elution of chlorpheniramine (71-73). The Hudanick (18) colorimetric technique is used.

An ion-exchange elution technique has been developed and evaluated by Smith (74, 75) who uses a sulfonated polystyrene resin column to hold chlorpheniramine maleate prior to elution. Chlorpheniramine was subsequently determined colorimetrically, by the method of Hudanick (18). A good review of the ion-exchange chromatography of nitrogen-containing bases is given by Jandera and Churacek (76); 171 references are given.

6.9 Polarography

Chlorpheniramine Maleate exhibited (77) a 2-electron irreversible reduction that requires two protons be consumed. The procedure recommends that the assay be run in 0.2 M sulfuric acid (pH 0.7) and a sample concentration from 10^{-5} to 10^{-3} M. A d.c. polarogram is run from -0.4V to -0.8V. The diffusion current is read at -0.7V and the concentration is obtained from a plot of the diffusion current vs. various concentrations of standards.

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Analytical Profiles of Drug Substances, 7

DIHYDROERGOTOXINE METHANESULFONATE

W. Dieter Schoenleber, Allen L. Jacobs, and Glenn A. Brewer, Jr.

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Contents

Ergot Alkaloids General Introduction

Ergot is the sclerotial stage of fungi of the genus Claviceps that grow parasitically in the pistils of different grasses. These sclerotia produce alkaloids of the ergot group, the most important being Secale cornutum, ergot of rye, which is produced by the strain Claviceps purpurea.[1,2,3,4]

Both the toxic properties and the medical use of crude ergot alkaloids have been known for hundreds of years[5], but the first physiologically active, crystalline alkaloid, ergotoxine, was not isolated before 1906.[6,7,8,9] In 1918, Stoll found a second active compound, ergotamine.[10] The third active alkaloid, ergonovine, was isolated in 1935 by four groups of workers.[11]

Stoll and Hofmann[12,13] later discovered that ergotoxine was not a single compound, but consisted of three alkaloids, ergocornine, ergocristine and ergokryptine, which could be separated by fractional crystallization of their di-(4-toluyl)-L-tartrates.

Recently,[14,15,16] the application of a special paper chromatographic technique has shown that one of the components, ergokryptine, consists of two closely related isomers, designated as α- and β- isomers, the first containing L-leucine, the latter containing L-isoleucine in the peptide part of the molecule.

The naturally occurring alkaloids cause strong uterine contractions and are circulatory stimulants.[17,18] Stoll and Hofmann[19] prepared the 9,10-dihydrogenated derivatives of ergotoxine and its components. These alkaloids no longer have action on the uterine muscle, but possess sympathicolytic-adrenolytic activity accompanied by suppression of the vasomotor center.[20] Dihydroergotoxine, in the form of its methanesulfonic acid salt (mesilate), is now widely used for the treatment of symptoms of mild to moderate impairment of mental function in the elderly.[21] Special studies have been made of its action on metabolism in the brain, in particular.[22-26]

This analytical profile has been prepared for 9,10-dihydroergotoxine, and additionally for its components 9,10-dihydroergocornine, 9,10-dihydroergocristine, 9,10-dihydro-α-ergokryptine, and 9,10-dihydro-β-ergokryptine in the form of their mesilates.

1. Description

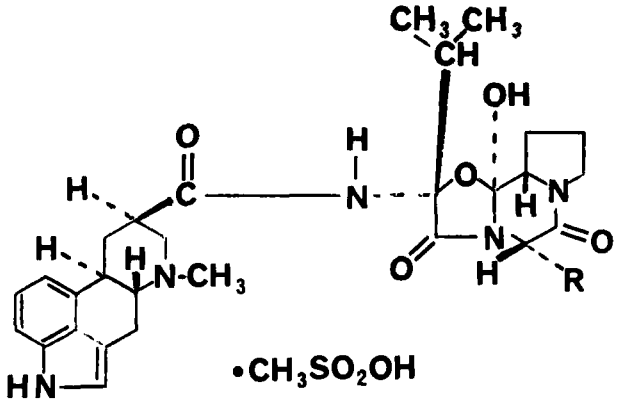
1.1 Name, Definition

Dihydroergotoxine mesilate is the salt of the hydrogenation product of the naturally occurring ergotoxine. Hence, it comprises the four compounds dihydroergocornine, dihydroergocristine, dihydro-α-ergokryptine, and dihydro-β-ergokryptine.

The drug substance contains equal amounts of dihydroergocornine, -cristine and -kryptine, the ratio of the α- and β-isomers of dihydroergokryptine being approximately 2:1. These proportions correspond to amounts found in natural ergotoxine isolated from selected and cultivated strains of *Claviceps purpurea*.

1.2 Formula, Molecular Weight

1.21 Dihydroergotoxine mesilate



R = $-CH_2-C_6H_5$, $-CH(CH_3)_2$,

$-CH(CH_3)-C_2H_5$, $-CH_2-CH(CH_3)_2$

Chemical Abstracts Registry Numbers:

base : 11 032-41-0

mesilate: 52 655-92-2

Average Molecular Weights:

base : 584.40

mesilate: 680.51

Average Formulas:

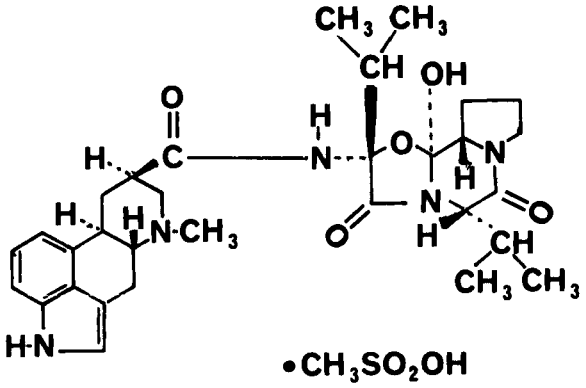
base : $C_{32.67}H_{42.67}N_5O_5$

mesilate: $C_{33.67}H_{45.67}N_5O_8S$

1.22 Components of Dihydroergotoxine

1.221 Dihydroergocornine mesilate

Dihydroergocornine mesilate is the salt of 9,10α-dihydro-12'-hydroxy-2',5'α-bis (1-methylethyl)-ergotaman-3',6',18-trione.



Chemical Abstracts Registry Numbers:

base : 25 447-65-8

mesilate: 29 261-94-7

Molecular Weight: base : 563.70

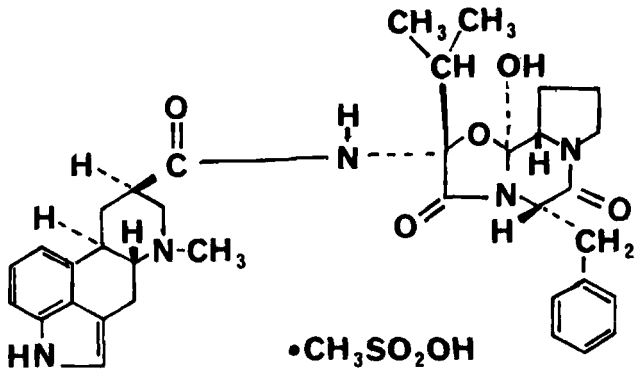
mesilate: 659.81

Formula: base : $C_{31}H_{41}O_5N_5$

mesilate: $C_{32}H_{45}O_8N_5S$

1.222 Dihydroergocristine mesilate

Dihydroergocristine mesilate is the salt of 9,10α-dihydro-12'-hydroxy-2'-(1-methyl-ethyl)-5'α-(phenyl-methyl)-ergotaman-3',-6',18-trione.



Chemical Abstracts Registry Numbers:

base : 17 479-19-5

mesilate: 24 730-10-7

29 261-92-5

Molecular Weight: base : 611.74

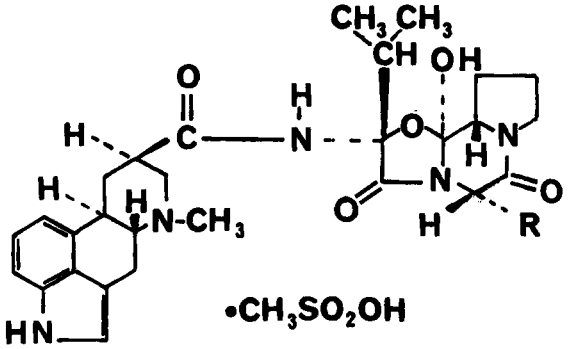
mesilate: 707.85

Formula: base : $C_{35}H_{41}O_5N_5$

mesilate: $C_{36}H_{45}O_8N_5S$

1.223 Dihydroergokryptine mesilate

Dihydroergokryptine mesilate is the salt of the α- and β-isomers in the molar ratio of 2:1.



R = $-CH_2-CH(CH_3)_2$

or $-CH(CH_3)-CH_2-CH_3$

Molecular Weight: base : 577.73

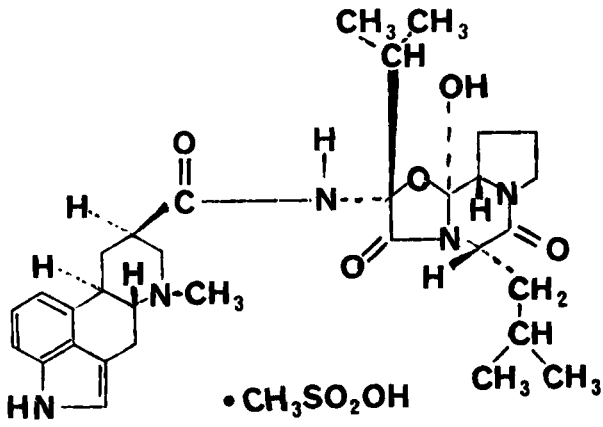
mesilate: 673.85

Formula: base : $C_{32}H_{43}O_5N_5$

mesilate: $C_{33}H_{47}O_8N_5S$

1.2231 Dihydro-α-ergokryptine mesilate

Dihydro-α-ergokryptine mesilate is the salt of 9,10α-dihydro-12'-hydroxy-2'-(1-methylethyl)-5'α-(2-methylpropyl)-ergotaman-3',6',18-trione.



Chemical Abstracts Registry Numbers:

base : 25 447-66-9

mesilate: 29 261-93-6

Molecular Weight: base : 577.73

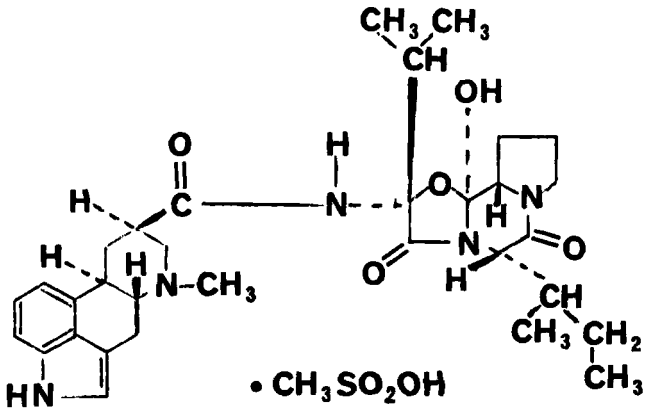
mesilate: 673.85

Formula: base : $C_{32}H_{43}O_5N_5$

mesilate: $C_{33}H_{47}O_8N_5S$

1.2232 Dihydro-β-ergokryptine mesilate

Dihydro-β-ergokryptine mesilate is the salt of 9,10α-dihydro-12'-hydroxy-2'-(1-methylethyl)-5'α-(1-methylpropyl)-ergotaman-3',6',18-trione.



Chemical Abstracts Registry Numbers:

base : 19 467-62-0

mesilate: not yet assigned

Molecular Weight: base : 577.73

mesilate: 673.85

Formula: same as α-isomer

1.3 Appearance, Colour

Dihydroergotoxine mesilate and the mesilates of its components are white to off-white crystalline powders.

2. Physical Properties

2.11 Infrared Spectra

Of the infrared spectra of the 9,10-dihydrogenated alkaloids of the ergotoxine group, only those of the dihydro-α-ergokryptine and dihydro-β-ergokryptine bases have so far been reported.[15]

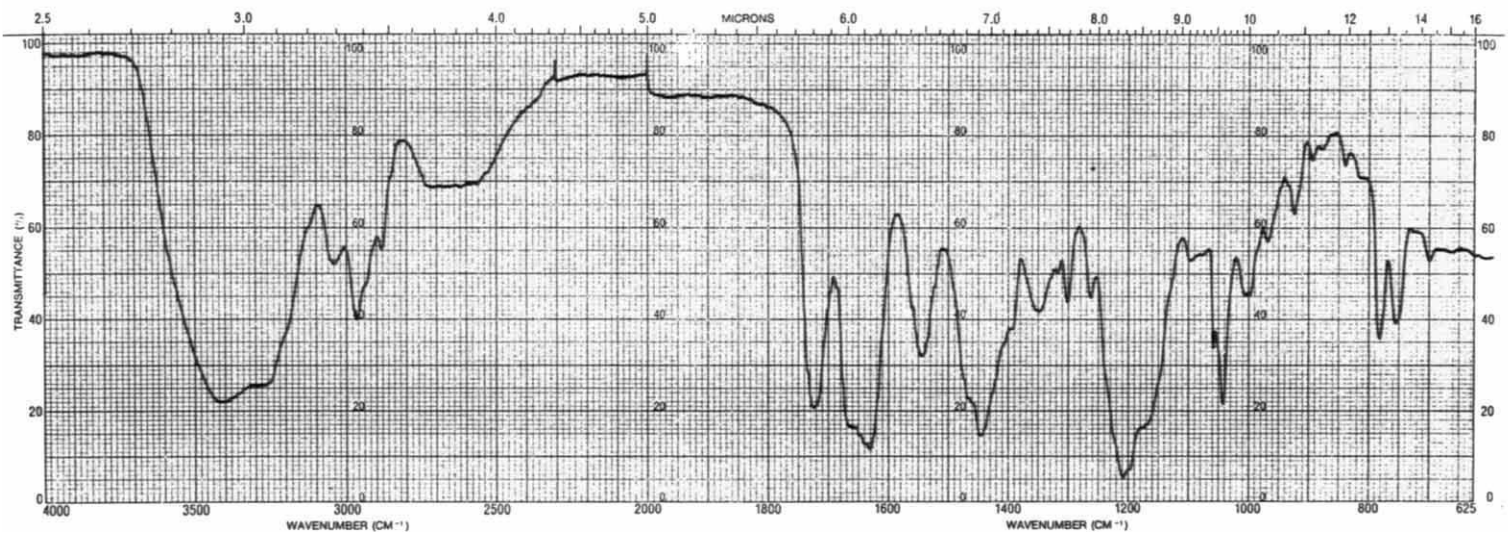


Figure 1. Infrared Spectrum of Dihydroergotoxine mesilate
KBr pellet, instrument: Perkin Elmer 257

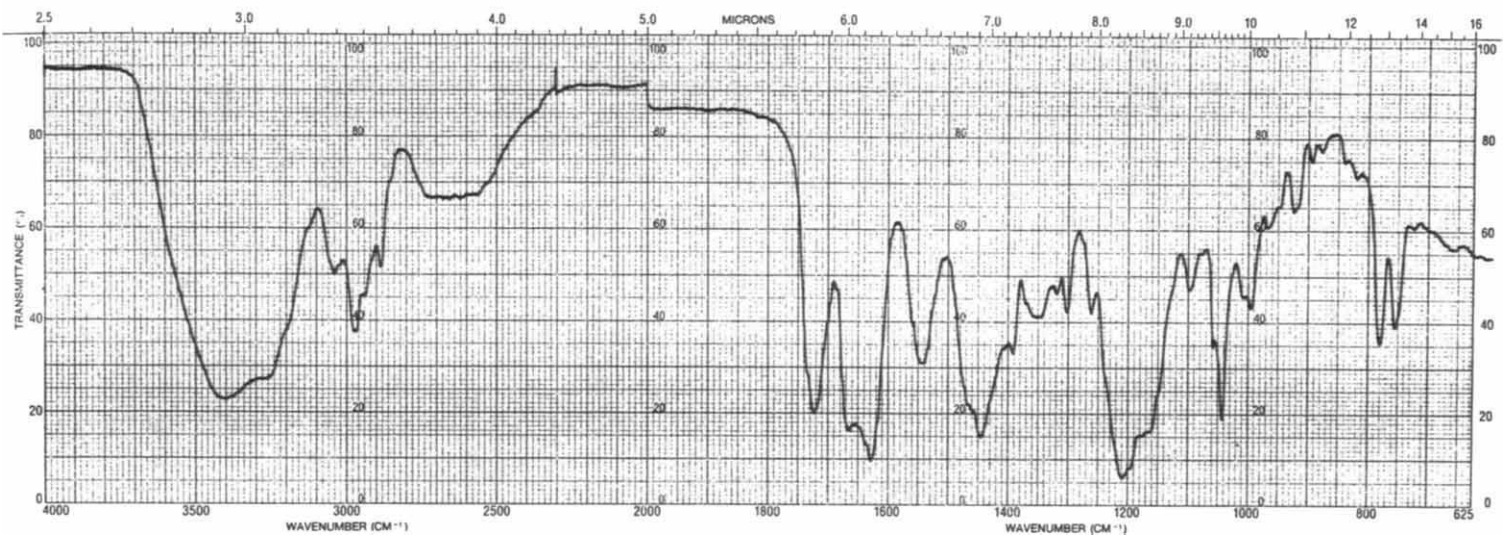


Figure 2. Infrared Spectrum of Dihydroergocornine mesilate
KBr pellet, instrument: Perkin Elmer 257

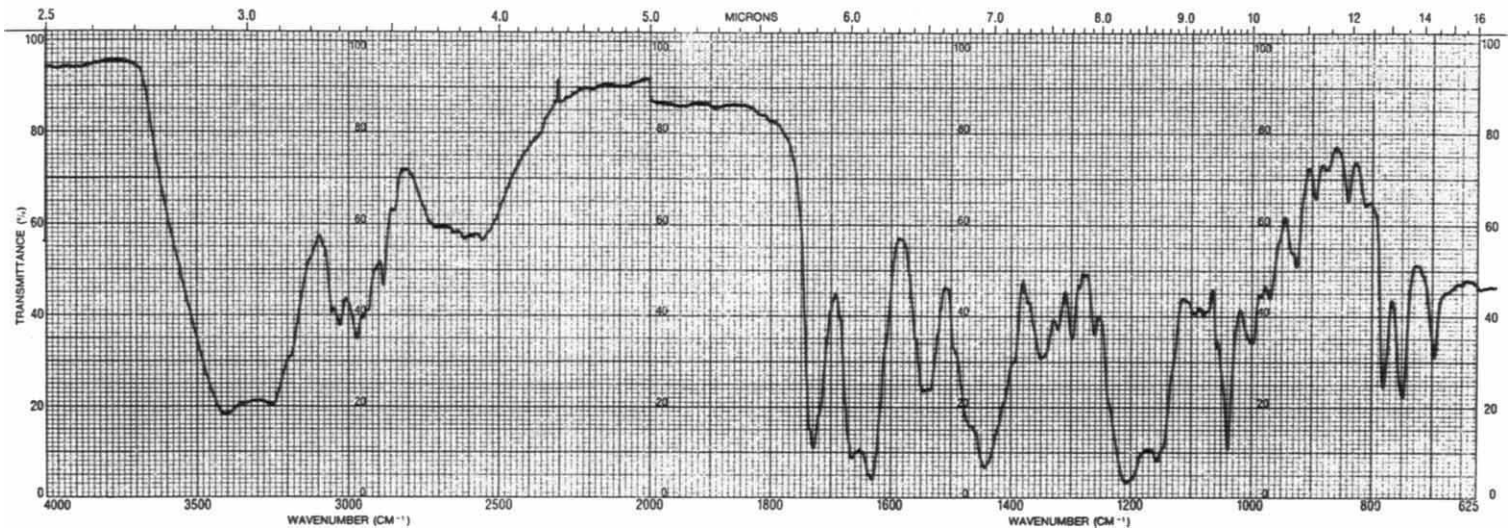


Figure 3. Infrared Spectrum of Dihydroergocristine mesilate
KBr pellet, instrument: Perkin Elmer 257

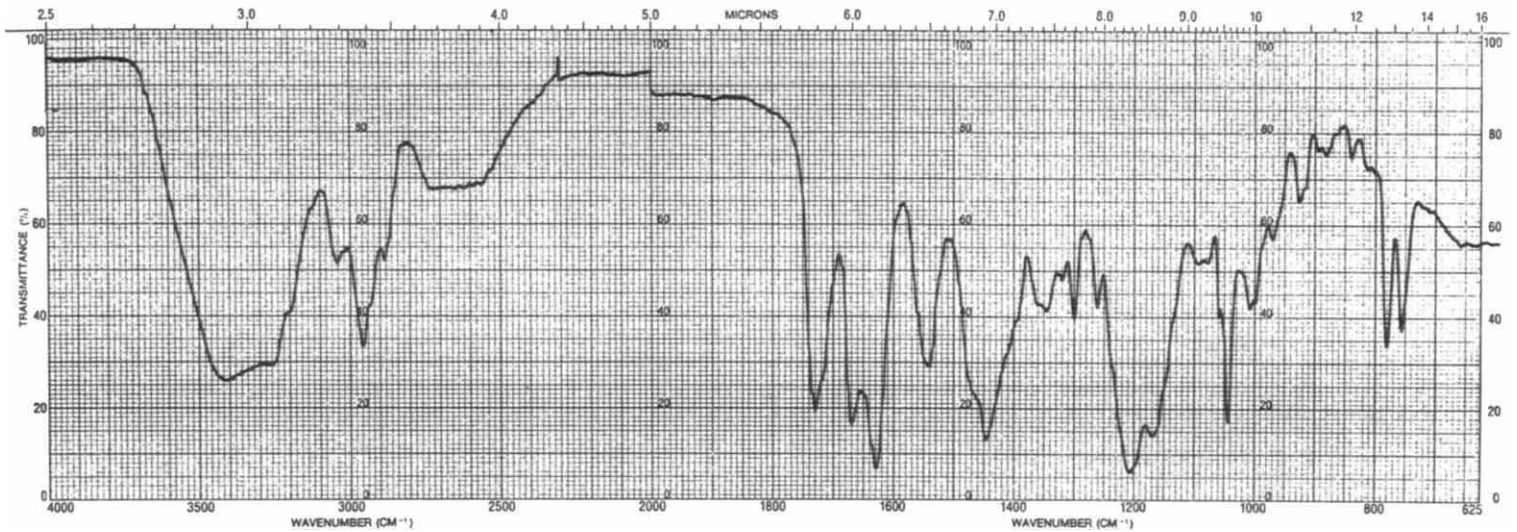


Figure 4. Infrared Spectrum of Dihydroergokryptine mesilate
KBr pellet, instrument: Perkin Elmer 257

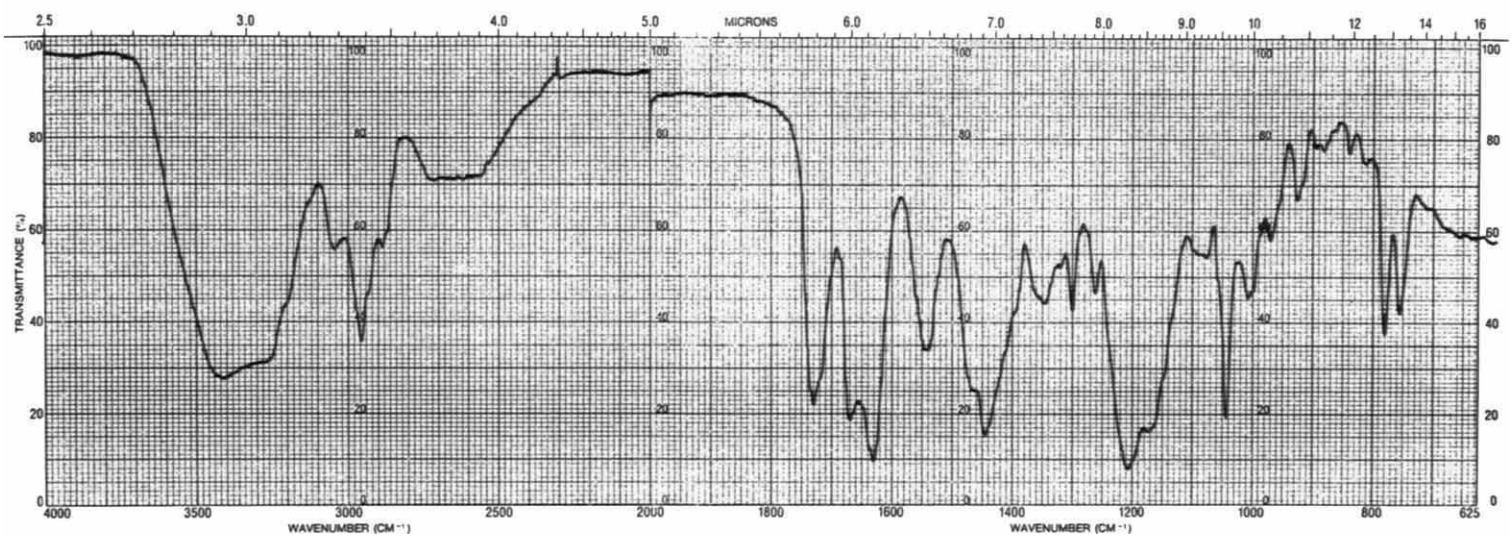


Figure 5. Infrared Spectrum of Dihydro-α-ergokryptine mesilate
KBr pellet, instrument: Perkin Elmer 257

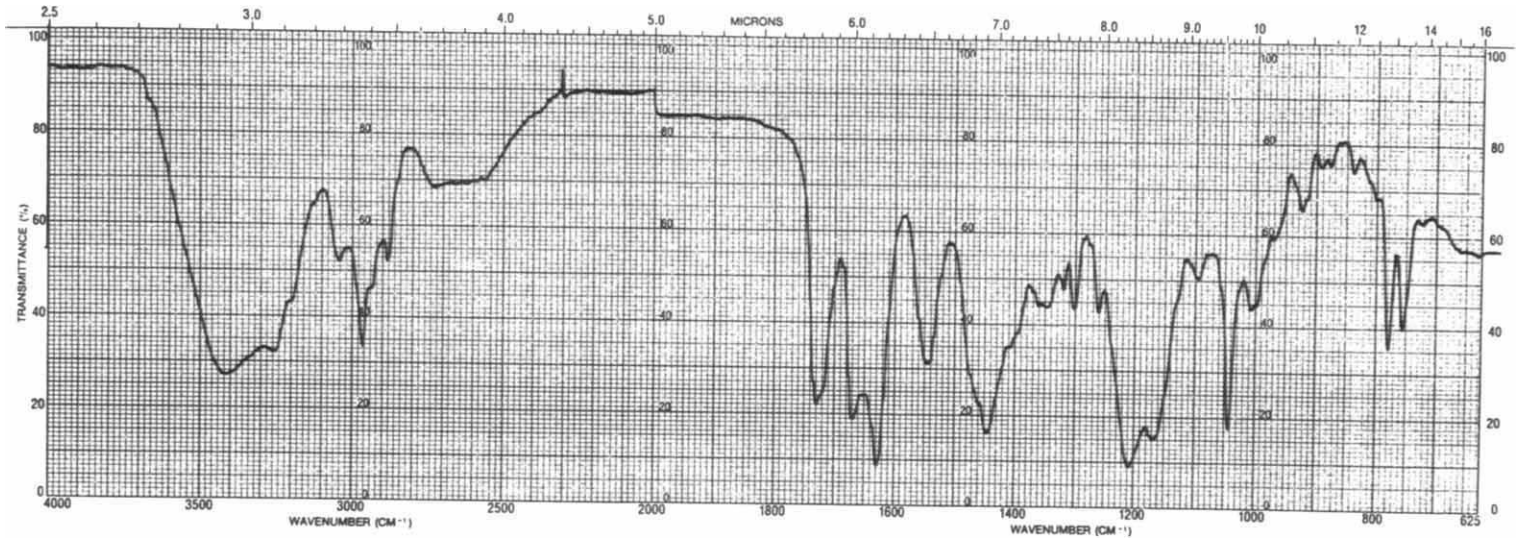


Figure 6. Infrared Spectrum of Dihydro-β-ergokryptine mesilate
KBr pellet, instrument: Perkin Elmer 257

The spectra of the drug substance itself and of its constituents are presented in figures 1 - 6.

Conditions were: pellet made from 1.5 mg of each compound plus 400 mg of KBr. Instrument: Perkin-Elmer 257.

2.12 Ultraviolet Spectra

The absorbance of dihydroergotoxine mesilate was measured in several solvents[27] as follows:

solvent	max, nm		$E^{1\%}_{1cm}$
95% ethanol	Sh	275	94.8
		281	101
		291	85.6
0.01 N HCl in 95% ethanol	Sh	275	95.1
		281	101
		291	87.1
0.01 N NH_4OH in 95% ethanol	Sh	277	93.2
		281	98.6
		292	81.0
0.01 M tartaric acid	Sh	274	98.0
		279	93.3
		288	79.3

The ultraviolet spectra of the 3 components, dihydroergocornine, dihydroergocristine and dihydroergokryptine, have been reported by M. Prosek and co-workers[28] measured on a cellulose plate using a chromatogram spectrophotometer.

2.13 Fluorescence

Quite in contrast to their parent compounds, dihydroergotoxine and its components do not fluoresce in the visible region of the spectrum.[5,29-31]

A yellowish green fluorescence can however be induced by intensive irradiation with ultraviolet light.[30-32] This effect has been utilized for the identification and partly for the quantitation of dihydroergotoxine (see also section 6.32).

2.14 Proton Nuclear Magnetic Resonance Spectra

A comprehensive investigation of the PMR spectra of peptide alkaloids has so far not been reported. A recent publication by Floss, Wenkert and co-workers[33] deals primarily with clavine alkaloids.

The spectra of the dihydroergotoxine alkaloid mesilates in deuterated dimethyl sulphoxide are presented in figures 7 - 12.

Common features: The $\overset{+}{H}N-CH_3$ shows a singlet for methyl at δ = 2.3 ppm and a broad, uncharacteristic signal for H at 10 ppm. The mesilate methyl group shows up at δ = 3 ppm. The pattern of 9,10-dihydrolysergic acid, with the very characteristic signals for the indole moiety (pyrrolic NH at 11, the aromatic protons at about 7 ppm) and the adjacent rings C and D in the aliphatic region, is present in the spectra of all four components. The amide proton at position 19 exhibits a neat singlet at 9.3 - 9.4 ppm. Moreover, the peptide fraction leads to signals superimposing the already complex aliphatic part of the spectrum.

Individual features: The PMR pattern is characteristically altered by the type of 5' substitution. Thus, in the spectra of dihydro-α-ergokryptine and of dihydroergocristine mesilate, the protons at C-5' appear as neat triplets at δ = 4.3 and 4.6 ppm, respectively. For dihydroergocornine and dihydro-β-ergokryptine there is a doublet at 4.3 ppm. The signals of the individual C-5' substituent (2-propyl-, 1-methylpropyl, 2-methylpropyl, and benzyl) add to the corresponding region of aliphatic or aromatic signals, respectively, yielding a characteristic pattern for each alkaloid.

The conspicuous, additional singlet emerging on top of the broad multiplet at 3.35 ppm in the dihydroergocristine mesilate spectrum stems from the water content of the substance (see also thermogram, section 2.23).

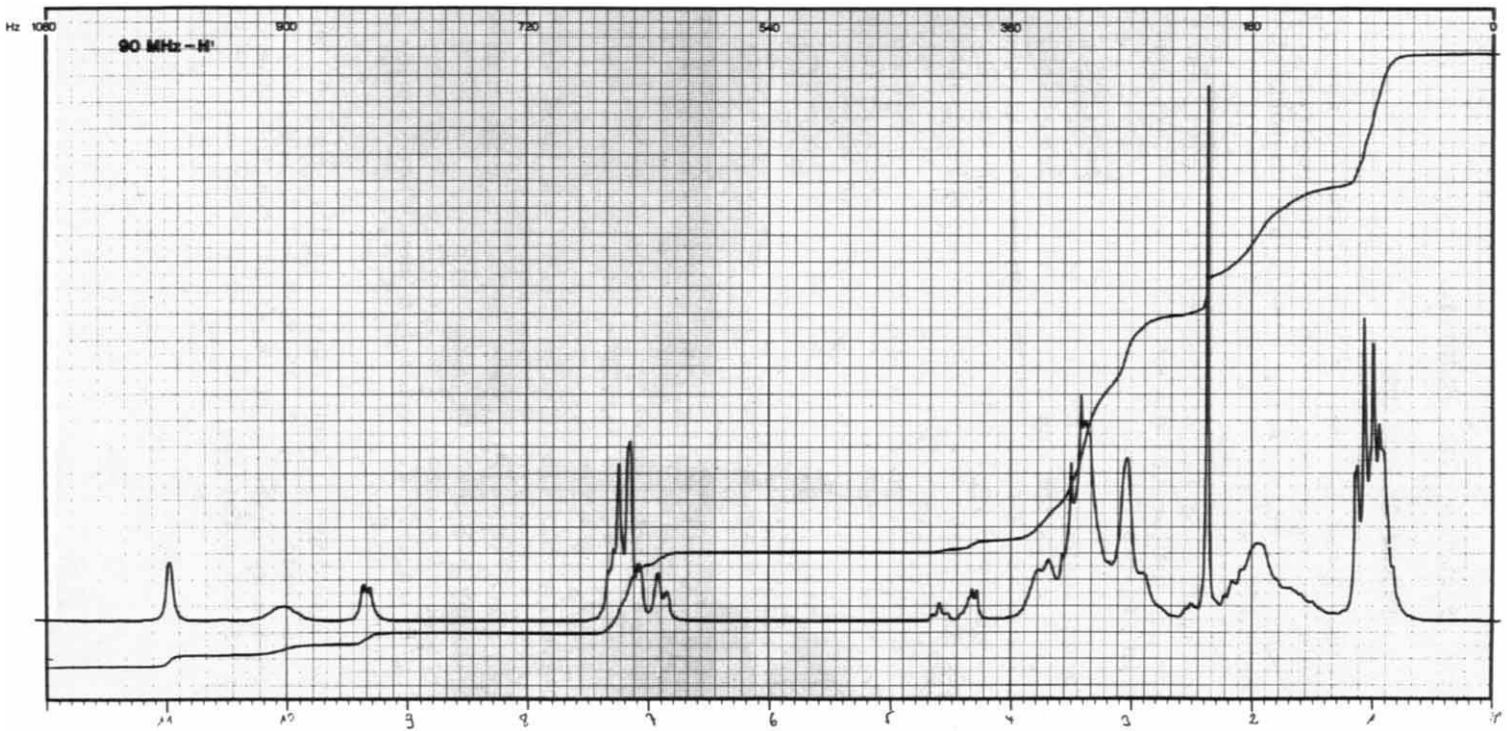


Figure 7. PMR Spectrum of Dihydroergotoxine mesilate in $(CD_3)_2SO$, instrument: Bruker HX-90

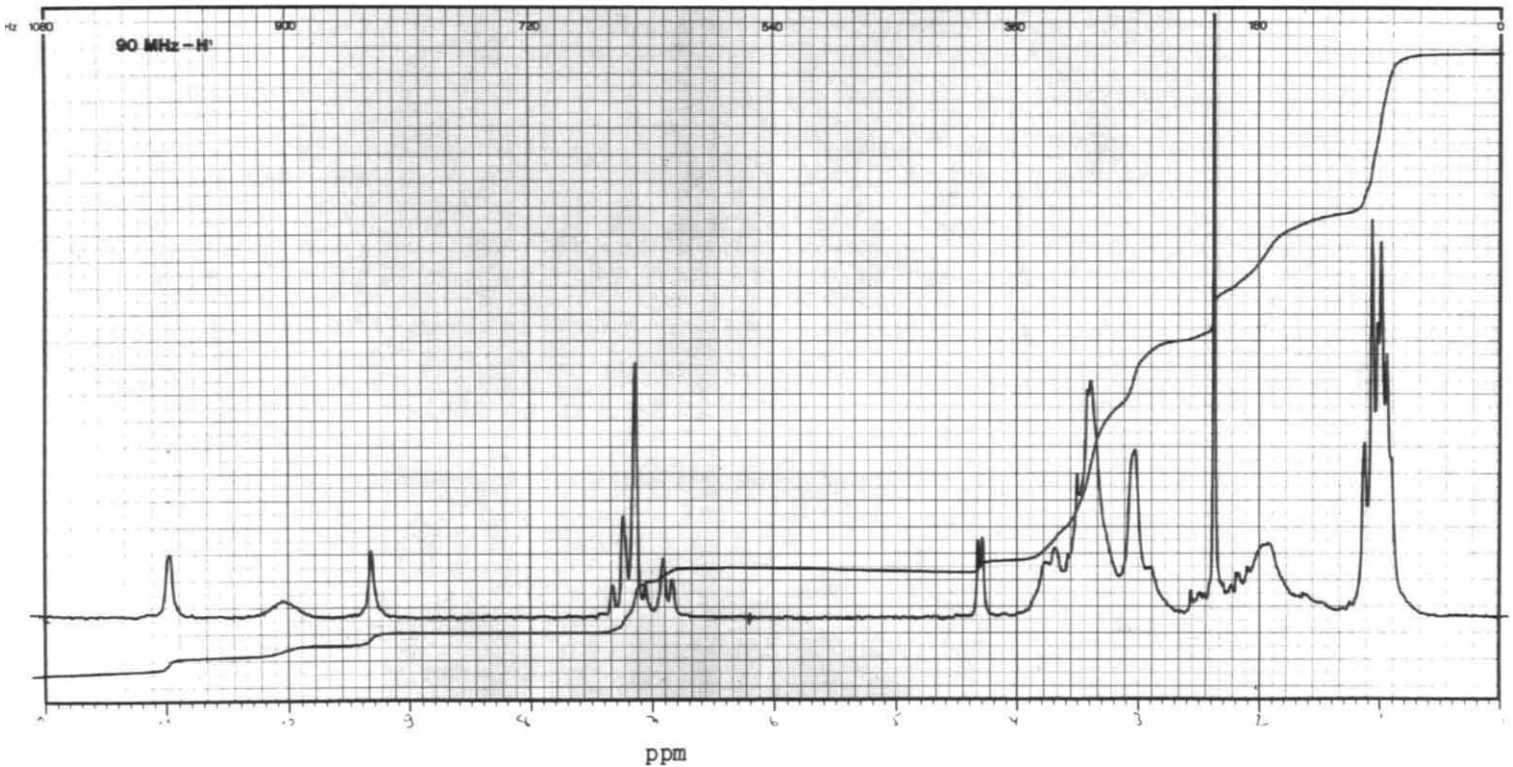


Figure 8. PMR Spectrum of Dihydroergocornine mesilate in $(CD_3)_2SO$, instrument: Bruker HX-90

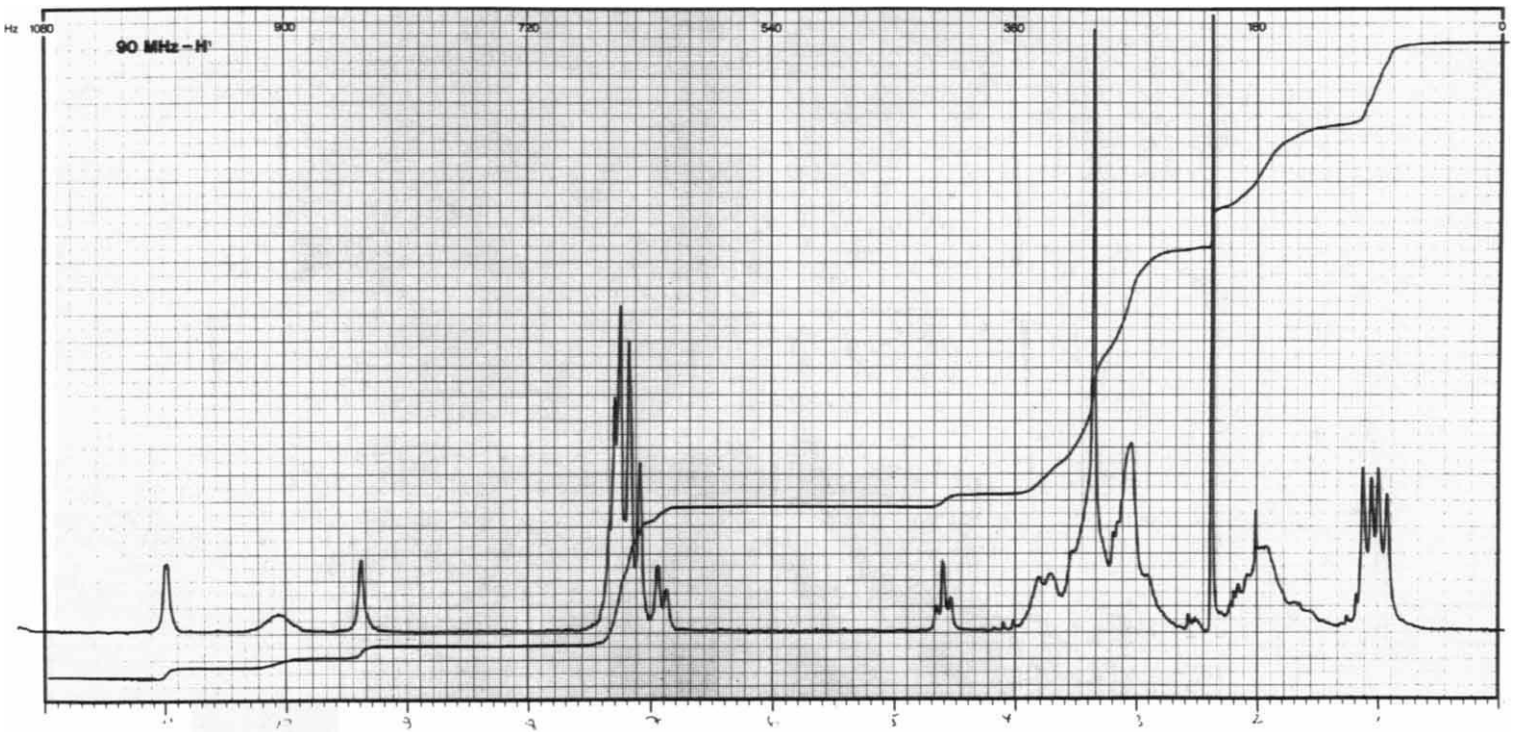


Figure 9. PMR Spectrum of Dihydroergocristine mesilate in $(CD_3)_2SO$, instrument: Bruker HX-90

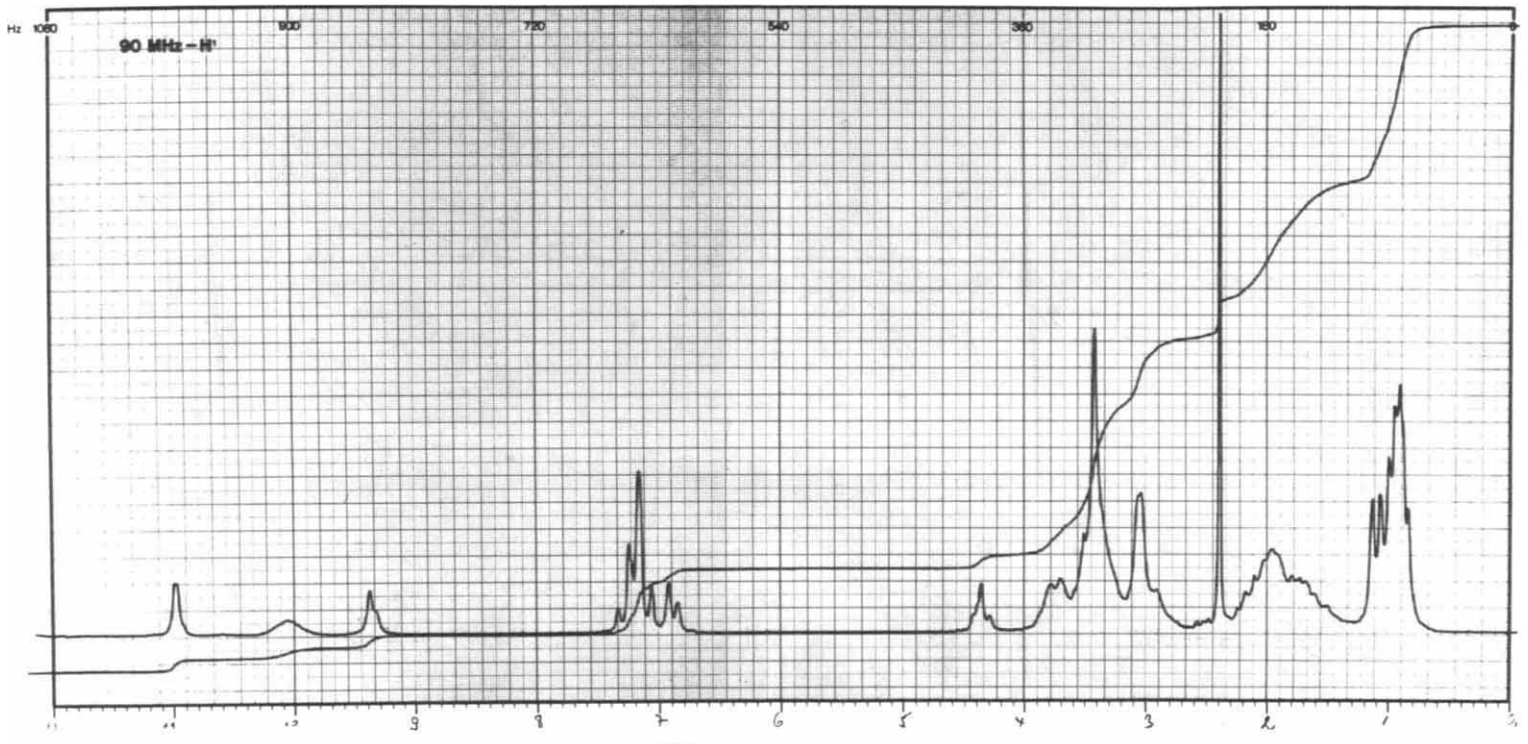


Figure 10. PMR Spectrum of Dihydroergokryptine mesilate in $(CD_3)_2SO$, instrument: Bruker HX-90

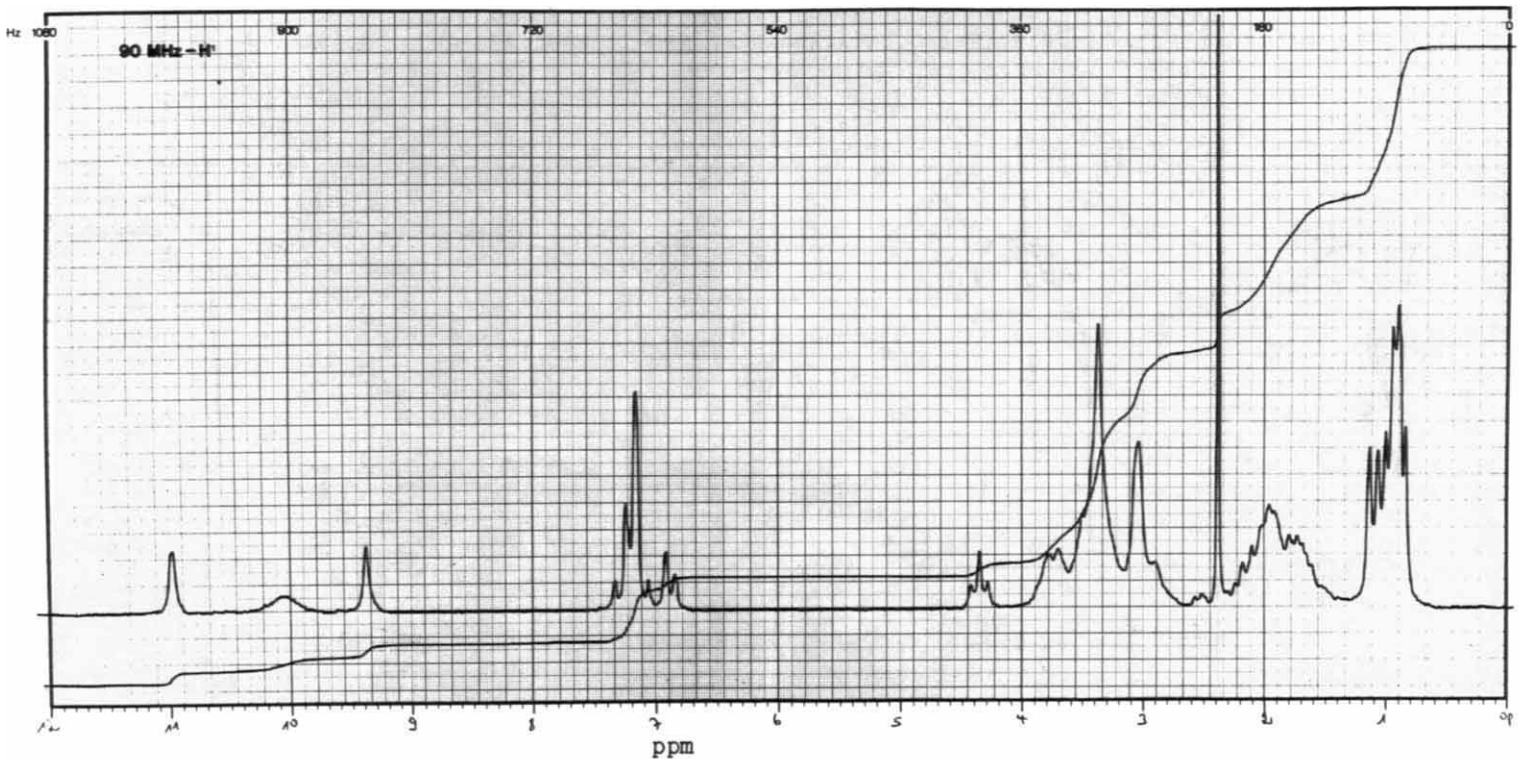


Figure 11. PMR Spectrum of Dihydro-α-ergokryptine mesilate in $(CD_3)_2SO$, instrument: Bruker HX-90

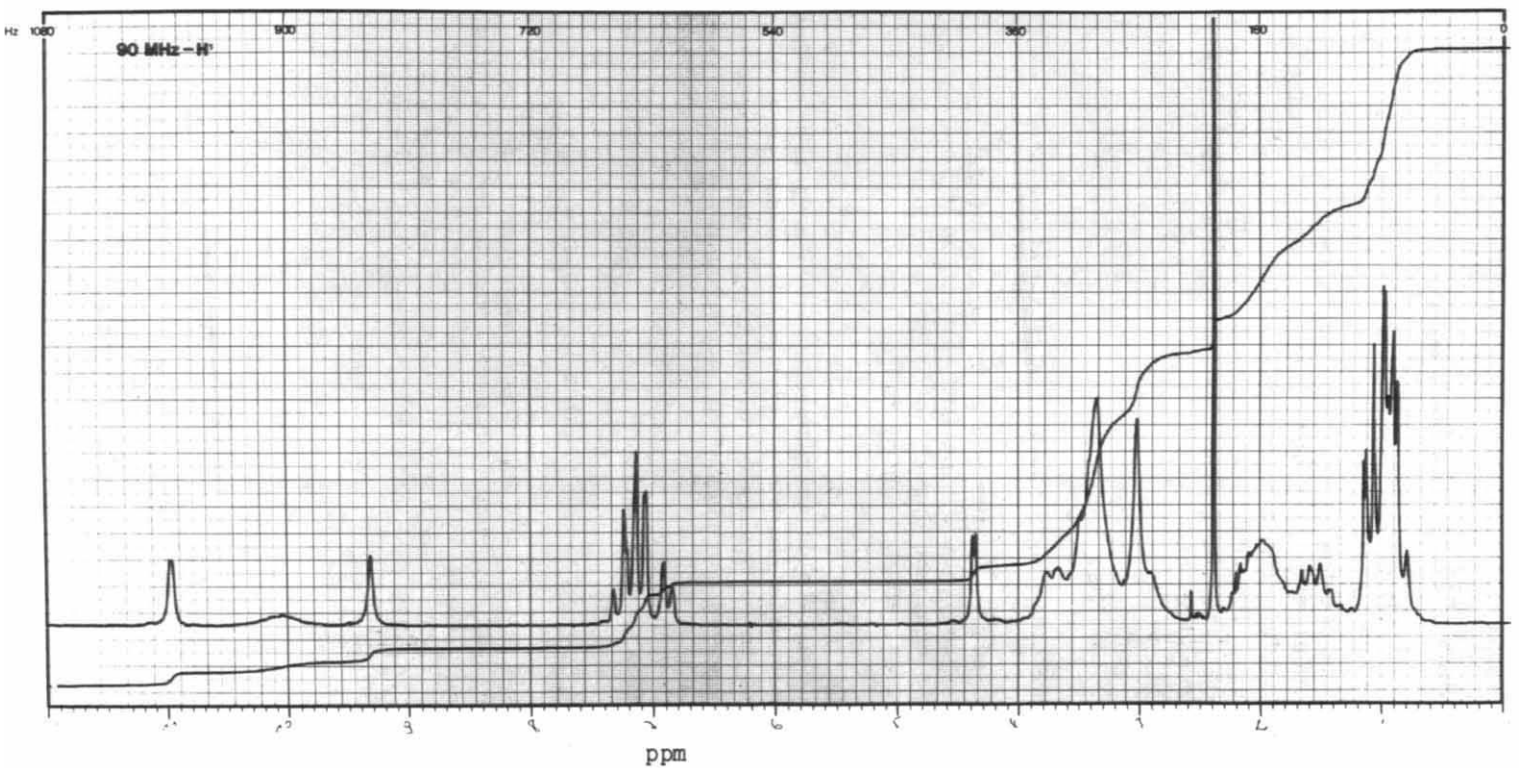


Figure 12. PMR Spectrum of Dihydro-β-ergokryptine mesilate in $(CD_3)_2SO$, instrument: Bruker HX-90

2.15 ^{13}C Nuclear Magnetic Resonance Spectra

The spectra of dihydroergotoxine mesilate and the individual alkaloids have been recorded[34] in dimethylsulphoxide using a Bruker HX-90 MHz NMR spectrophotometer. The chemical shifts of the four constituents are presented in figure 13. As expected, the shift difference in the spectra is a maximum for the C-5' signal.

2.16 Mass Spectra

Although mass spectra of non-hydrogenated ergot alkaloids and that of dihydroergotamine[35] have been discussed,[36,37] dihydroergotoxine has so far not been investigated. The low resolution mass spectra of this compound and those of its components, as mesilates, are presented in figures 14 - 19.

General: Due to the low volatility of the ergot peptide alkaloids in general, molecular peaks are not always present in the spectra, their occurrence being subject to carefully optimized instrument parameters and to a certain portion of good luck.

Common fragmentation pattern: Ergot alkaloids in general are known to undergo cleavage between their peptide and ergine fractions[36] at the site of the 19 - 2' bond accompanied by a hydrogen abstraction. This leads to a common pattern for the lysergic acid part[38] for all ergot alkaloids. In the case of the 9,10-dihydrogenated ergotoxine alkaloids, this species is the dihydrolysergamide ion fragment at m/e = 269 (along with its decay peaks at m/e = 223, 224, 225 for individuals with split-up ring D)

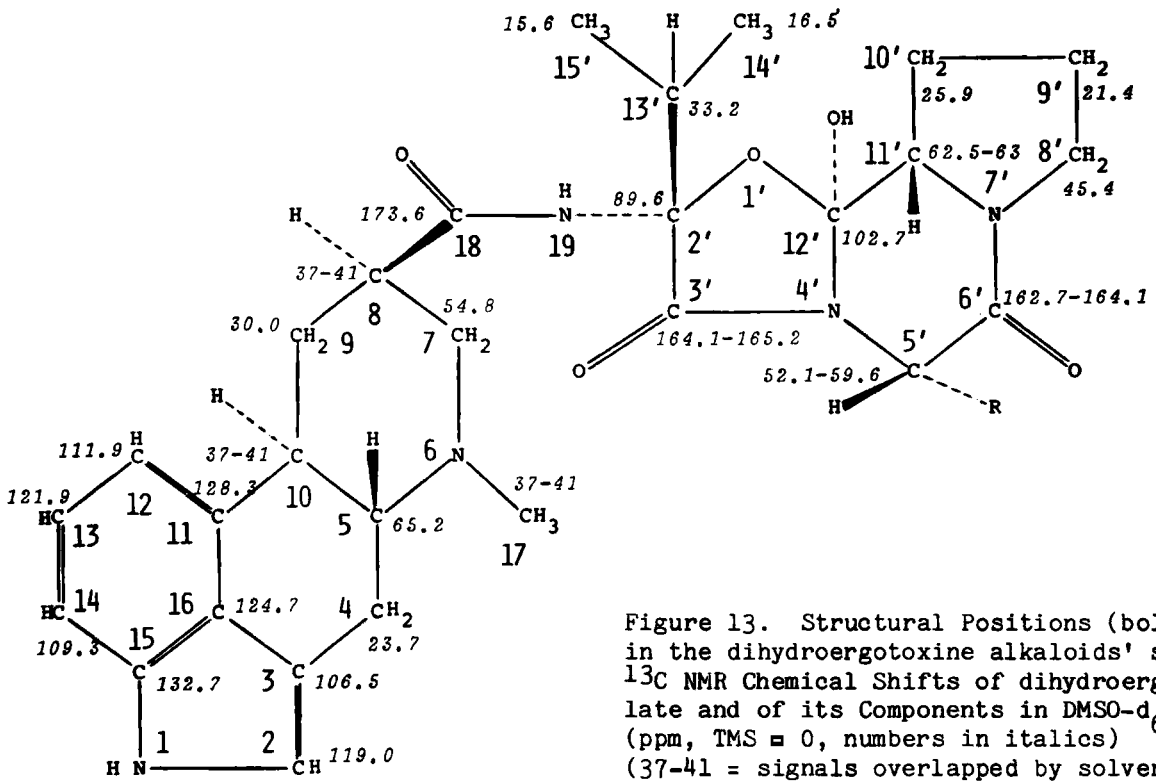


Figure 13. Structural Positions (bold numbers) in the dihydroergotoxine alkaloids' skeleton and ^{13}C NMR Chemical Shifts of dihydroergotoxine mesilate and of its Components in DMSO-d_6 (ppm, TMS ≡ 0, numbers in italics) (37-41 = signals overlapped by solvent)

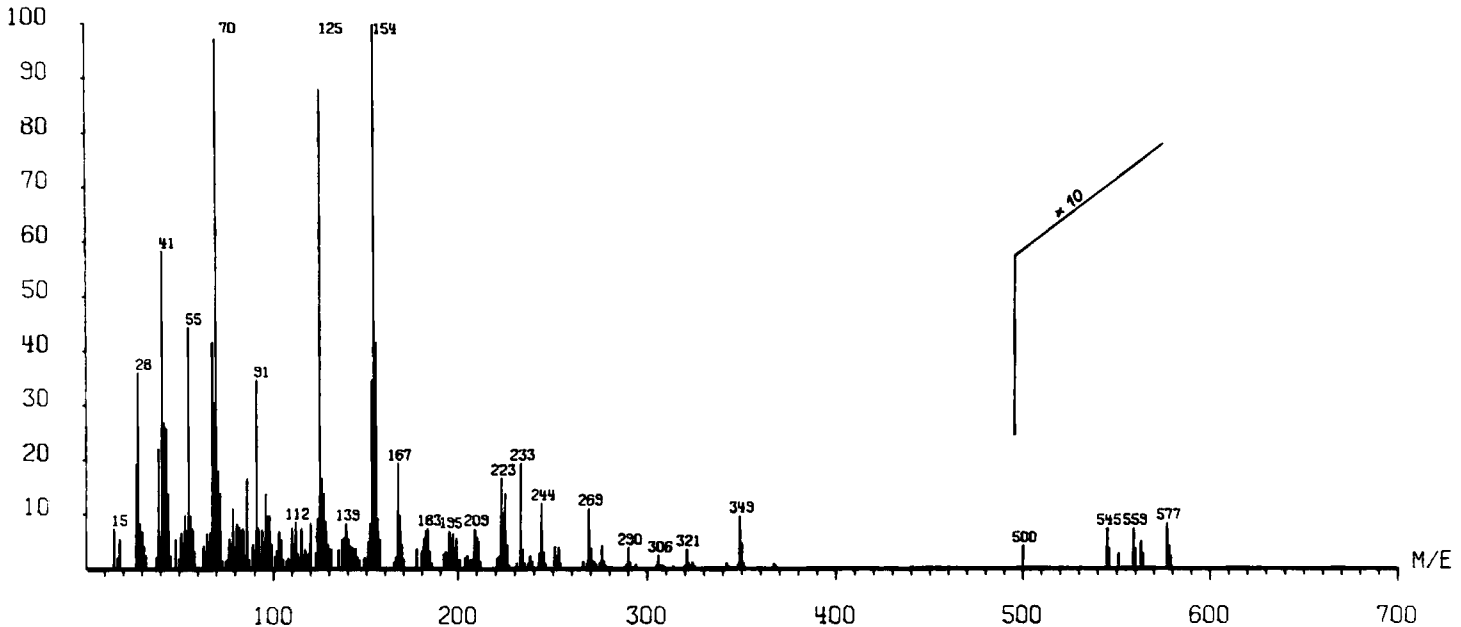


Figure 14. Low Resolution Mass Spectrum of Dihydroergotoxine mesilate
Instrument: CEC 21-110 B

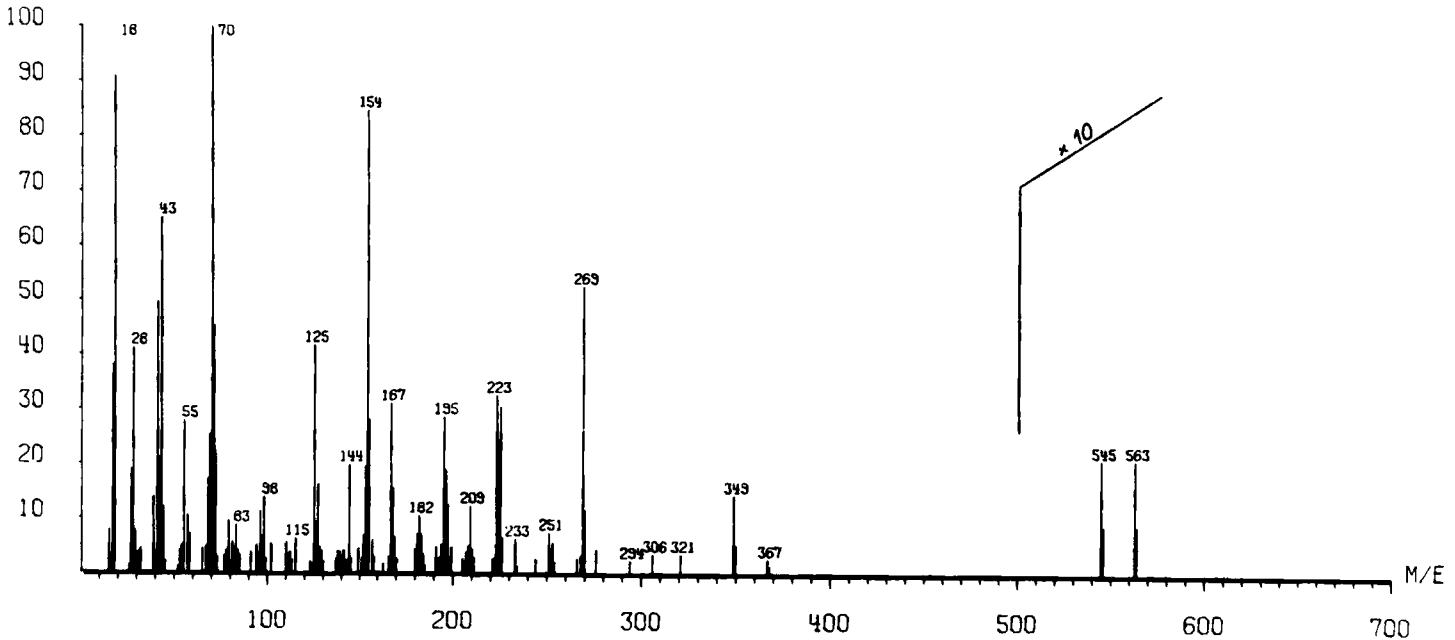


Figure 15. Low Resolution Mass Spectrum of Dihydroergocornine mesilate
Instrument: CEC 21-110 B

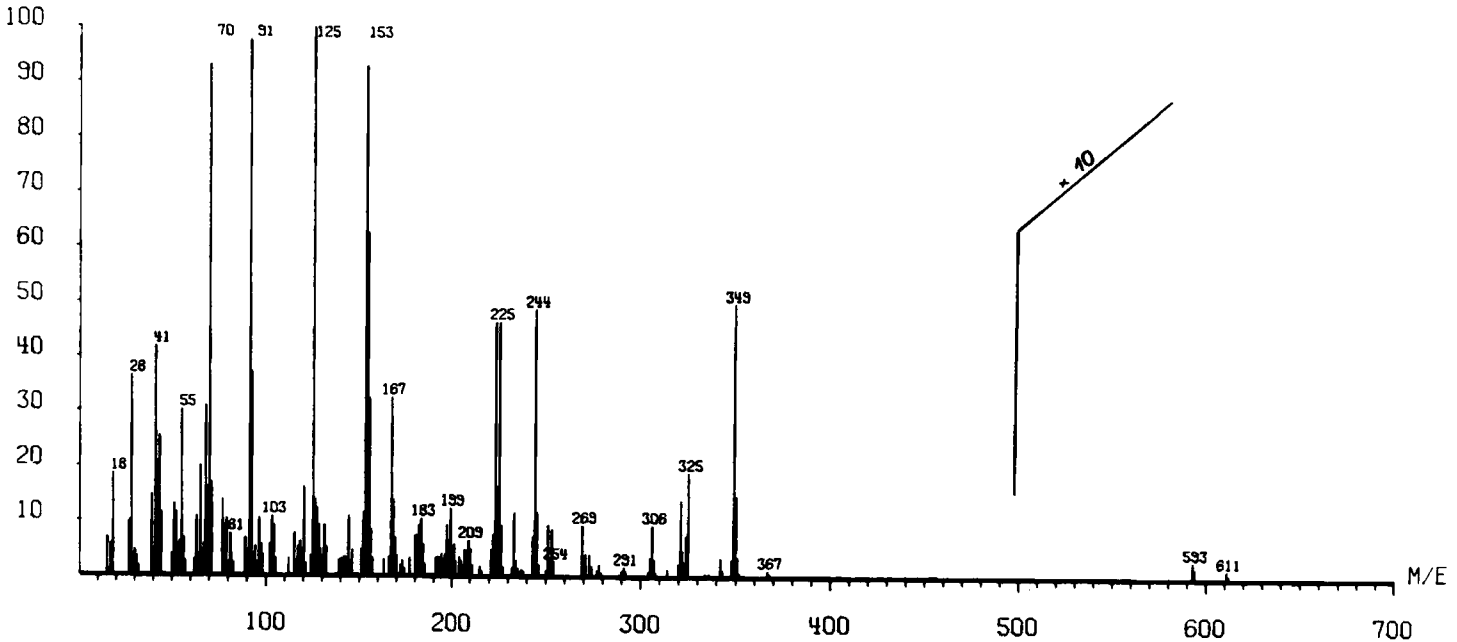


Figure 16. Low Resolution Mass Spectrum of Dihydroergocristine mesilate
Instrument: CEC 21-110 B

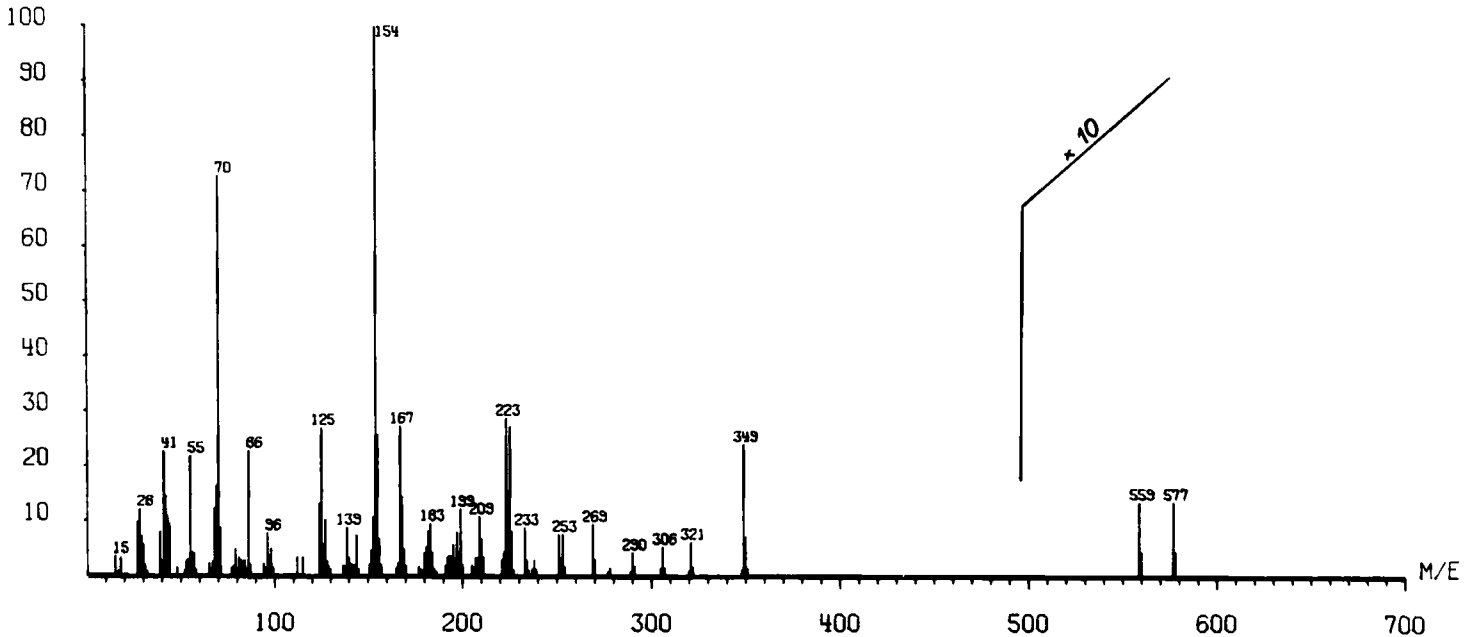


Figure 17. Low Resolution Mass Spectrum of Dihydroergokryptine mesilate
Instrument: CEC 21-110 B

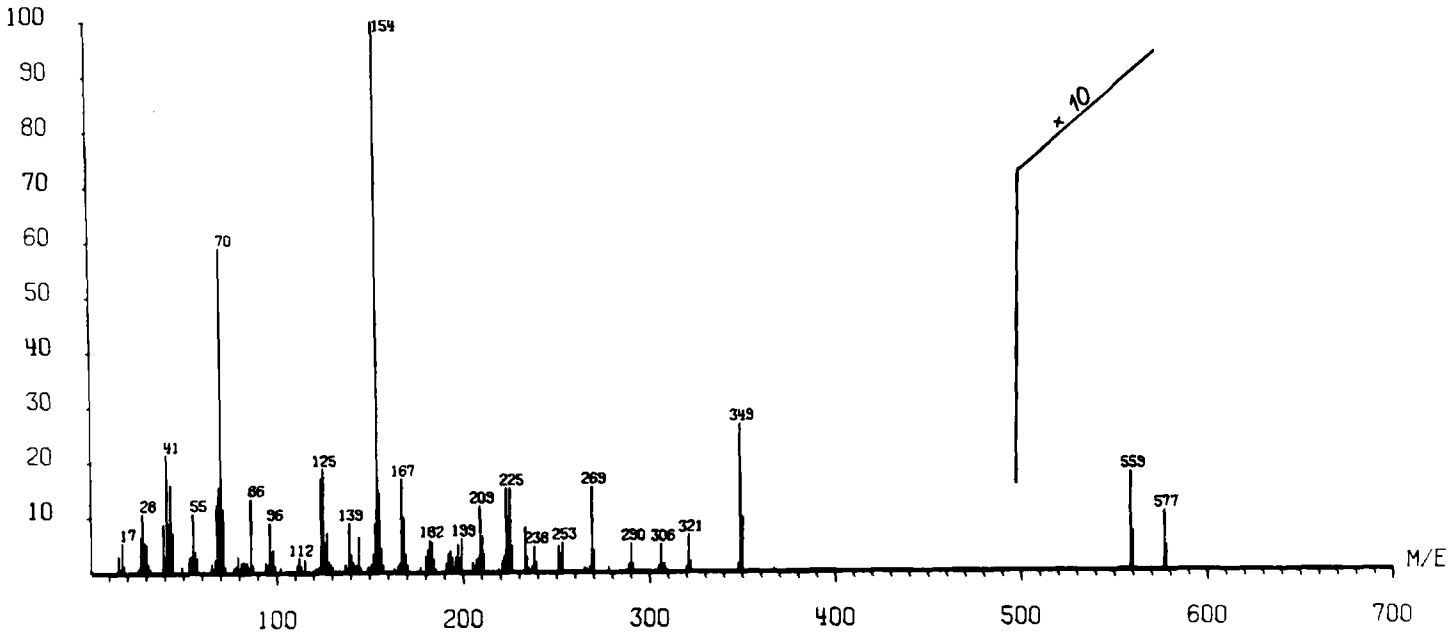


Figure 18. Low Resolution Mass Spectrum of Dihydro-α-ergokryptine mesilate
Instrument: CEC 21-110 B

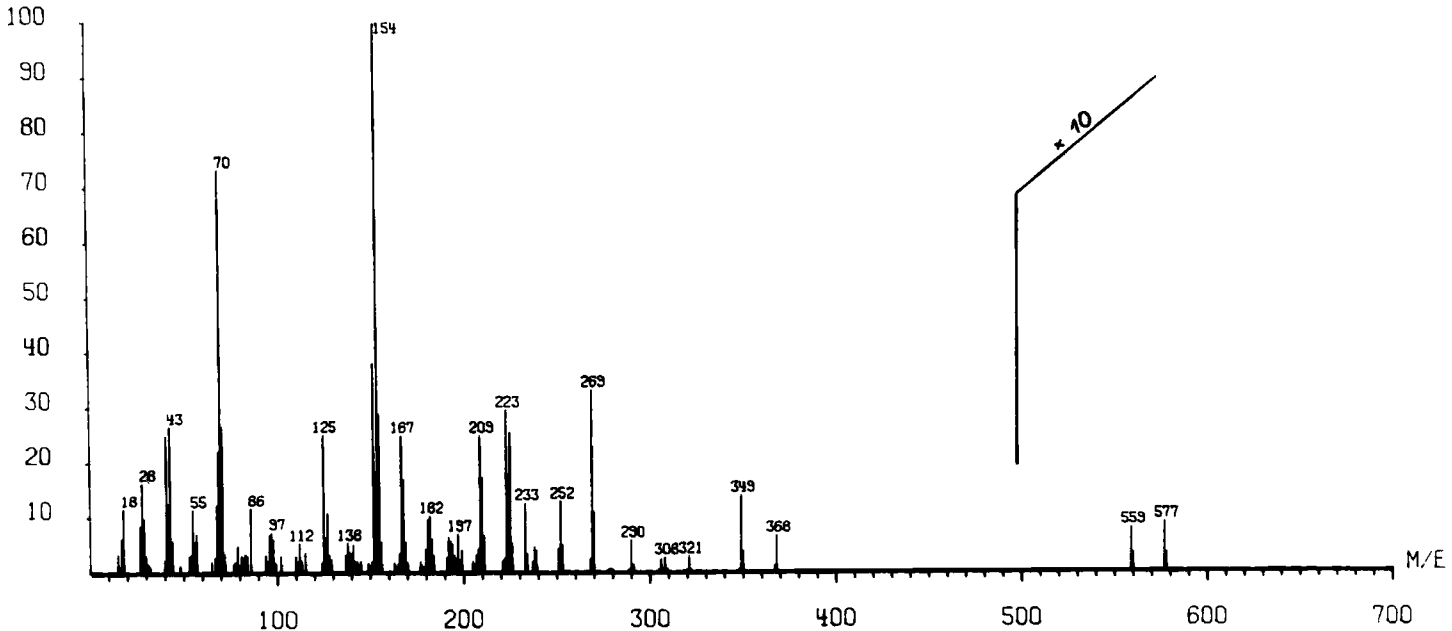
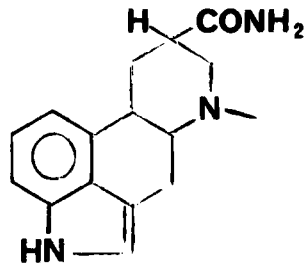
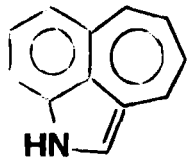
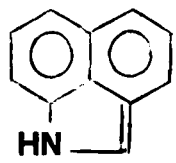


Figure 19. Low Resolution Mass Spectrum of Dihydro-β-ergokryptine mesilate
Instrument: CEC 21-110 B

at m/e = 167 and 154 for the fragments presented below.

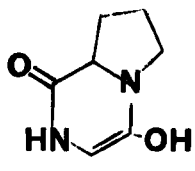


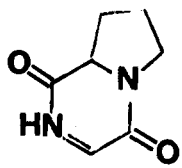


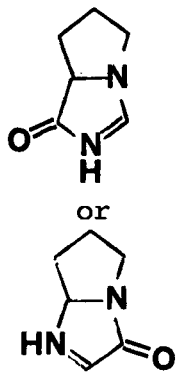


m/e = 269 m/e = 167 m/e = 154

In dihydroergotoxine and its constituents, the peptide moiety also leads to a common fragmentation pattern in those pathways where the 5'-substituent is not involved, such as the following fragments:







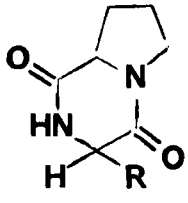
m/e = 154 m/e = 153 m/e = 70 m/e = 71
base peak

m/e = 125

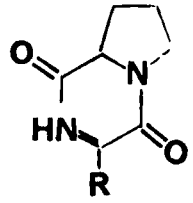
A further common fragment is that at m/e = 349 that contains the dihydrolysergamide fraction plus an 80 mass units part presumably from the dimethyl pyruvic acid section of the peptide moiety.

Individual fragmentation: The type of substitution at the 5' position leads to the observed differences in the mass spectra of the alkaloids in question, expressed not only in the correspond-

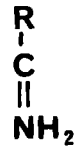
ing molecular peak, if observable, but also in the fragments of the peptide fraction. Typical fragments are listed below.



I



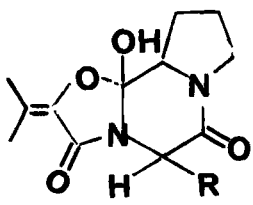
II



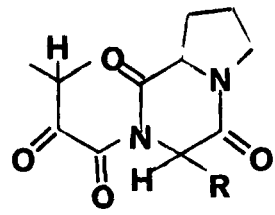
III

Component	I m/e	II m/e	III m/e
dihydroergocornine	196	195	72
dihydroergocristine	244	243	108
dihydro-α-ergokryptine	210	209	86
dihydro-β-ergokryptine			

In the spectrum of dihydroergocristine, another fragment shows up which is obviously stabilized by the benzylic radical at C-5'. In analogy to the quoted literature, its structure is likely to be the following:



or



m/e = 344

The presence of the benzyl group in dihydroergocristine is also confirmed by the occurence of a fragment at m/e = 91, corresponding to the tropylium ion.

2.17 Optical Rotation

The optical properties of the single bases have been reported by Stoll and Hofmann.[19] Those of their mesilates are given below.

Optical Rotation of dihydroergotoxine alkaloids

$[\alpha]_D^{20}$ in 50 % ethanol, c=1 %

dihydroergotoxine mesilate	+ 13.5°
dihydroergocornine mesilate	+ 14.0°
dihydroergocristine mesilate	+ 3.1°
dihydroergokryptine mesilate	+ 21.4°
dihydro-α-ergokryptine mesilate	+ 18.6°
dihydro-β-ergokryptine mesilate	+ 28.1°

2.2 Physical Properties of the Solid

2.21 Melting Characteristics

As dihydroergotoxine mesilate decomposes at about 200 degrees, a melting point cannot be given. The melting of three components as their bases have been reported by Hofmann:[5]

dihydroergocornine 187°, decomposition
dihydroergocristine 180°, decomposition
dihydroergokryptine 235°, decomposition

2.22 Differential Scanning Calorimetry

The thermogram was obtained with a Perkin Elmer scanning calorimeter at a heating rate of 10 /min. in a nitrogen atmosphere in a loosely covered vessel. Dihydroergotoxine mesilate and its components exhibit endotherms for the melting/decomposition between 180° and 200 °. Minor and broad endotherms indicate the loss of solvent and water (see figures 20 - 25).

2.23 Thermogravimetry

The thermogravimetric analyses were carried out on a Perkin Elmer TGS-1 thermobalance. The samples were heated at a rate of 10°/min. under a nitrogen atmosphere. Weight losses, as indicated in figures 20 - 25, were observed below melting which are attributed to solvent and water losses. Massive sample decomposition obviously begins after melting.

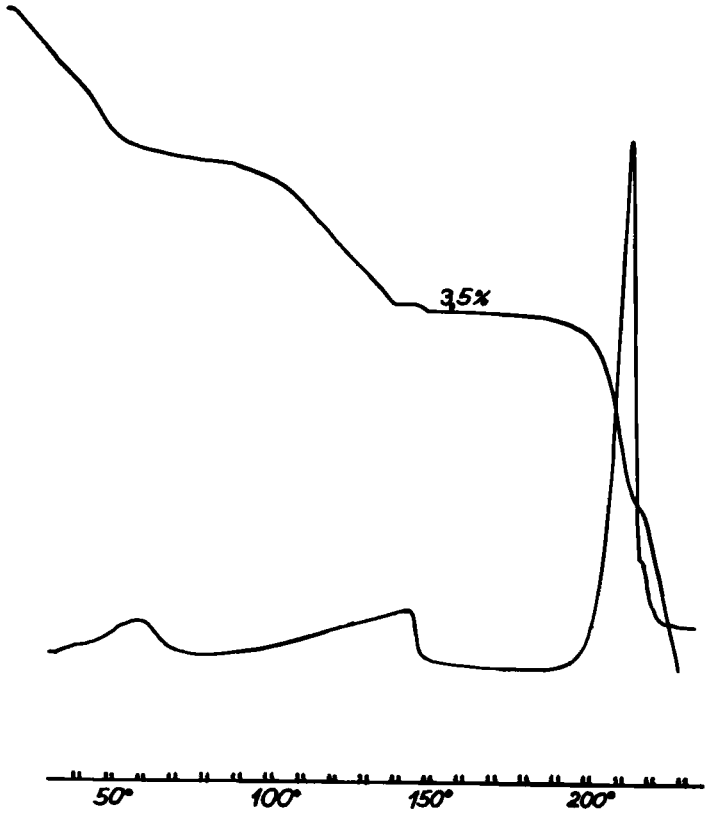


Figure 20. Differential Scanning Calorimetric and Thermogravimetric Curves of Dihydroergotoxine mesilate

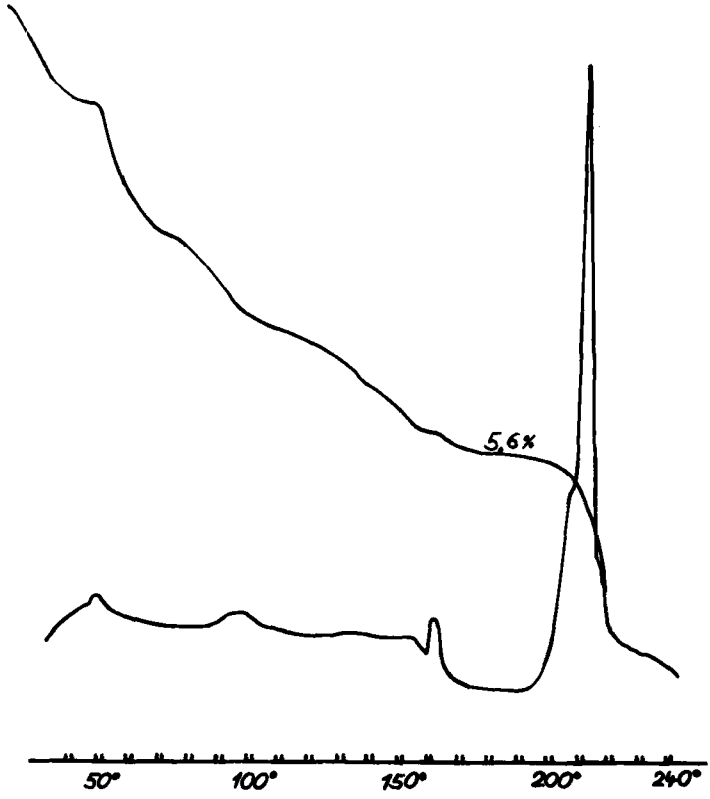


Figure 21. Differential Scanning Calorimetric and Thermogravimetric Curves of Dihydroergocornine mesilate

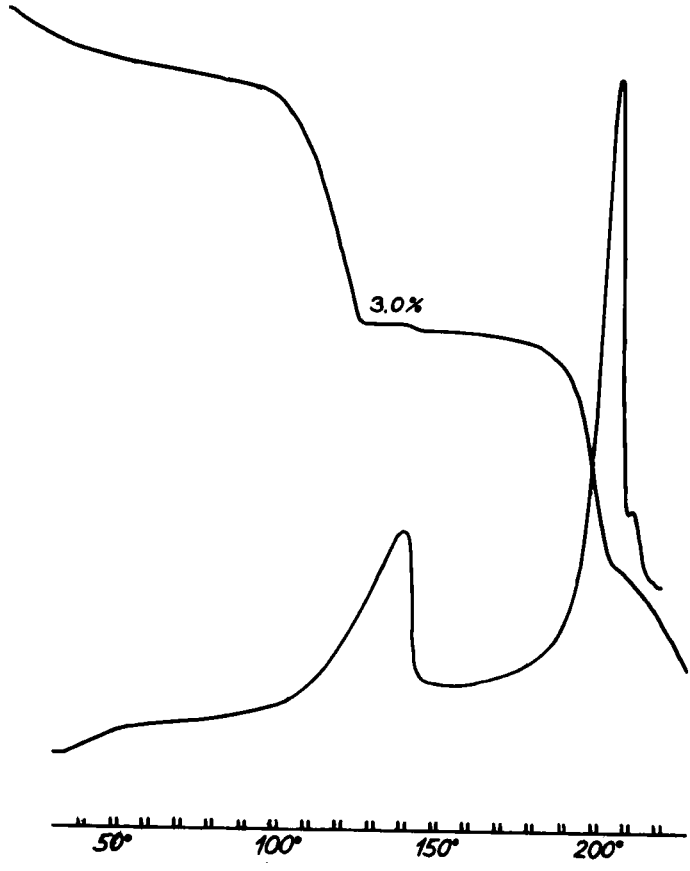


Figure 22. Differential Scanning Calorimetric and Thermogravimetric Curves of Dihydroergocristine mesilate

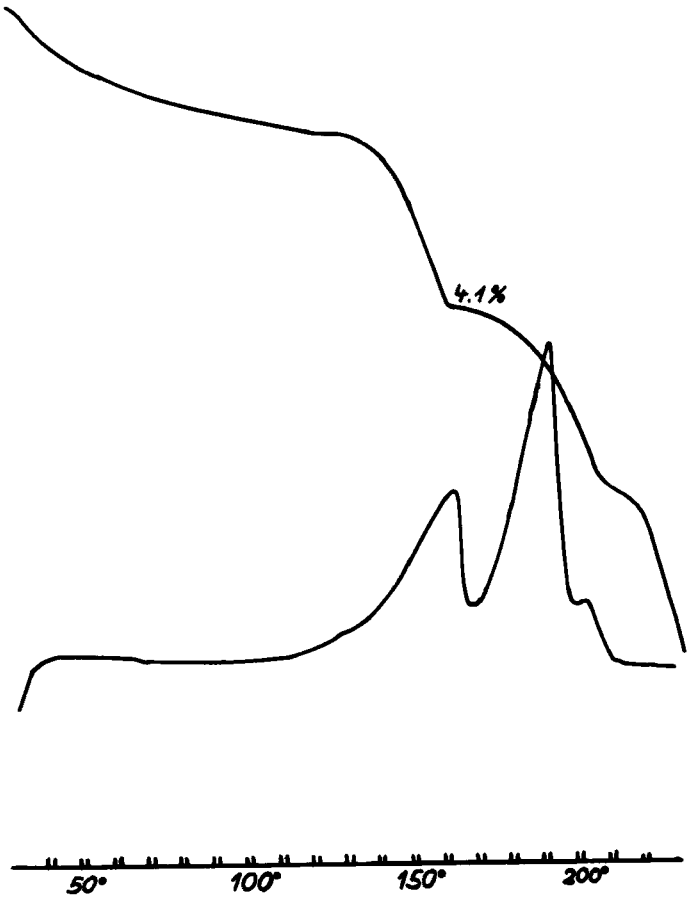


Figure 23. Differential Scanning Calorimetric and Thermogravimetric Curves of Dihydroergokryptine mesilate

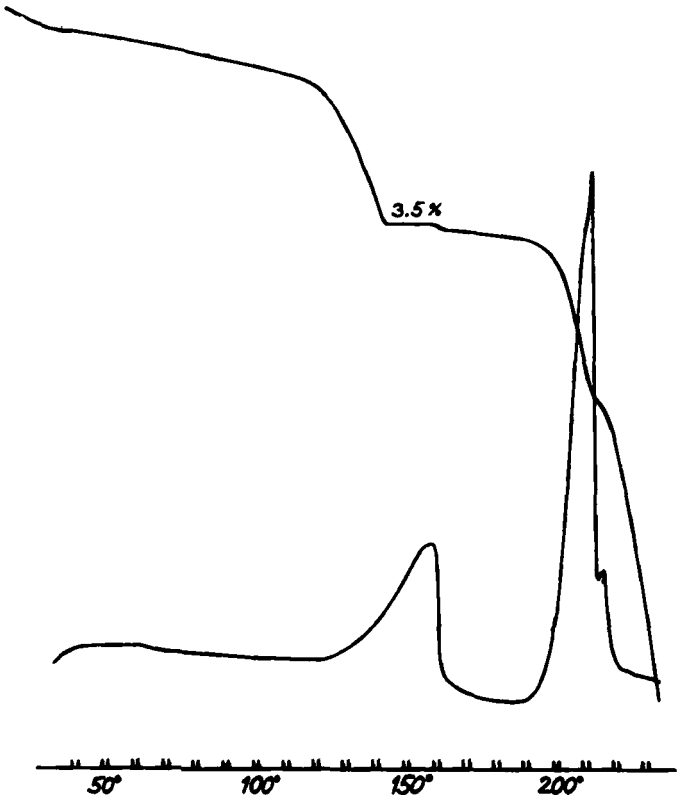


Figure 24. Differential Scanning Calorimetric and Thermogravimetric Curves of Dihydro-α-ergokryptine mesilate

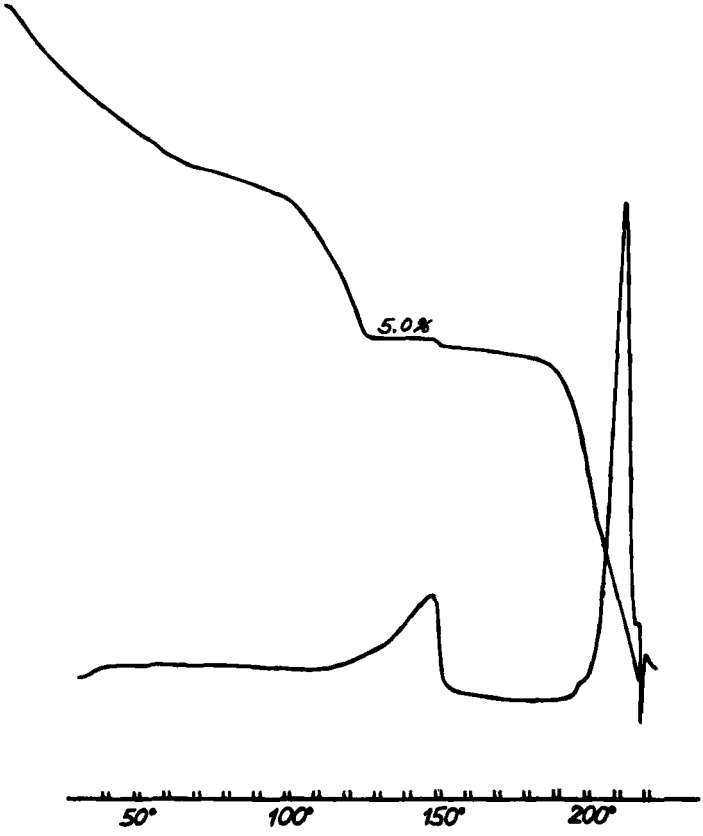


Figure 25. Differential Scanning Calorimetric and Thermogravimetric Curves of Dihydro-β-ergokryptine mesilate

2.24 Ionization Constants

Maulding and Zoglio[39] have reported the ionization constants for the dihydroergotoxine components at 24° in water as

6.91 ± 0.07 for dihydroergocornine
6.89 ± 0.07 for dihydroergocristine
and 6.89 ± 0.07 for dihydroergokryptine

The pK_{MCS} values (MCS = methyl cellosolve) according to the method of Simon[40] have been determined[41] as

5.78 for dihydroergocornine,
5.83 for dihydroergocristine,
5.81 for dihydro-α-ergokryptine,
and 5.84 for dihydro-β-ergokryptine.

2.25 Partition Coefficient

The partition equilibrium of dihydroergotoxine mesilate between water of pH 1.2 and chloroform, on the one hand, and water pH 1.2 and n-octanol, on the other, have been investigated at 37.0 ± 0.5°.

partition system	equilibrium distribution ratio
water pH 1.2/$CHCl_3$	0.12 : 1
water pH 1.2/n-octanol	1 : 22.9

2.26 Solubility

The solubilities of dihydroergotoxine mesilate in different solvents have been determined as follows: in water, 95% ethanol, 50% ethanol, methanol, and acetone: > 2 g/100 ml., in chloroform: 0.9 g/100 ml., in dichloromethane: < 0.1 g/100 ml., in ether and petroleum ether: insoluble.

3. Production

The ergot alkaloids are mainly isolated from field cultivated sclerotia which are produced after artificial infection of rye ears with conidia of *Claviceps purpurea*.[5,17,43,44]

After defatting with benzene, the suspension is made alkaline with ammonia, the alkaloids are extracted with benzene and subsequently crystallized and fractionated into the different alkaloid groups as their salts.[5]

Another more recent method of production consists of the cultivation of selected strains of Claviceps in large vessels under submerged conditions.[42,45-52]

The selective catalytic hydrogenation of the 9,10-double bond in the ergot alkaloids leading to the dihydrogenated species in question has been investigated and described in detail.[19,53]

4. Stability and Degradation

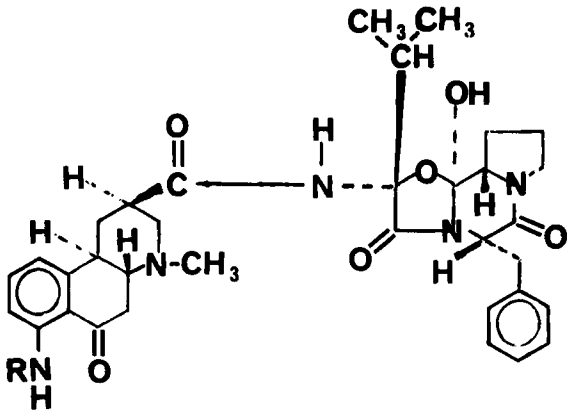
4.1 Bulk Stability

Dihydroergotoxine mesilate has been shown to be stable at room temperature for up to ten years when stored in the absence of light. Irradiation by artificial daylight leads to a slightly yellowish aspect of the powder accompanied by a lowered drug content.[54]

4.2 Stability in Solution

Like all ergot and most indole alkaloids,[35] dihydroergotoxine mesilate and its components are prone to light enhanced oxidative degradation in aqueous solutions.[55] Therefore, such formulations must be kept in amber glass vials and in an inert atmosphere, or they have to be stabilized by an adequate excipient.[56]

In the case of dihydroergocristine, the main oxidation products have been identified by spectroscopy and synthesis[57] as N-formyl-B-seco-dihydroergocristine and its deformylation product, B-seco-dihydroergocristine, the structures of which are given in figure 26. They fluoresce very intensively but do not react with van Urk's reagent (see section 6.2). The pathway has been proposed via autoxidation at the 3 position and subsequent rupture of the 2-3 bond. The remaining components of dihydroergotoxine undergo the same type of autoxidative degradation in solution, however, the structures of the corresponding products have not yet been elucidated.



R = -CHO N-formyl-B-seco-dihydroergocristine

= -H B-seco-dihydroergocristine

Figure 26. Autoxidation Products of Dihydroergocristine

4.22 Acid and Base Catalyzed Isomerizations

In hydroxyl-containing solvents, nonhydrogenated ergot peptide alkaloids are readily epimerized at carbon 8 to an equilibrated mixture of the lysergic and isolysergic acid series, called the -ine/-inine forms.[20,58] The double activation of the hydrogen at C-8 is a prerequisite for this conversion, therefore it is rendered impossible by the 9,10-dihydrogenation. Consequently, dihydroergotoxine alkaloids do not exhibit this behaviour.

However, at elevated temperatures another type of acid catalyzed rearrangement takes place in both nonhydrogenated and hydrogenated ergot peptide alkaloids: the so-called aci-isomerization at C-2' in the peptide moiety[20,59,60] leading to more acidic epimers. These show almost no pharmacological activity and can readily be separated from their starting material due to their - as their name reveals - enhanced acidity.

4.23 Solvolysis

In alcoholic solutions and under acid catalysis[61] the hydroxy group at C-12' in the peptide portion is slowly replaced by the corresponding alkoxy group.[35]

In aqueous solutions dihydrolysergamide and dihydrolysergic acid occur as hydrolysis products. The two breakdown products can therefore be observed in small quantities in aged liquid pharmaceutical preparations.[20]

5. Drug Metabolism and Pharmacokinetics

Tritium labelled dihydroergotoxine mesilate (see section 6.4) administered orally in man at 1.0 mg was rapidly absorbed with an absorption half-life of 0.52 hours,[62] the peak plasma concentration of 0.5 ng-equivalent/ml. was reached after 2.3 hours after administration.

The elimination occurs in two phases with half-lives of 4.1 and 13 hours respectively. The main pathway for the excretion of the dihydroergotoxine alkaloids is the biliary route, as shown in rats for

dihydroergocristine by Nimmerfall and Rosenthaler.[63,64] Only a very small percentage of the administered dose appears in the urine.

Maurer[65] succeeded in isolating and identifying 16 metabolites of dihydro-β-ergokryptine from rat bile. The major metabolites stem from the oxidative biotransformation at the 8' position in the proline part of the peptide moiety: A series of stereoisomeric 8' and 9' as well as 8' and 10' dihydroxylated compounds could be isolated, accompanied by products of further oxidation at the 8' position such as glutamic and hydroxy glutamic acid derivatives.

As minor metabolites, glucuronides of the mono-8'-hydroxylated and of the 8',9'-dihydroxylated metabolic products were isolated together with a small amount of dihydrolysergamide and a 9'-hydroxylated derivative conjugated with glutathione. A similar biotransformation has been observed for the other components of dihydroergotoxine in rats.[66]

6. Methods of Analysis

6.1 Elemental Analysis

6.11 Dihydroergotoxine mesilate

Element	C	H	N	S
calculated %	59.4	6.8	10.3	4.7
found %	59.0	6.8	10.5	4.8

6.12 Components of Dihydroergotoxine mesilate

The elemental analyses of the bases were reported by Stoll and Hofmann:[15,19]

	Element	C	H	N
Dihydroergocornine	calc. %	66.03	7.33	12.43
	found %	66.04	7.56	12.66
Dihydroergocristine	calc. %	68.70	6.76	11.46
	found %	68.78	6.86	11.40
Dihydroergokryptine	calc. %	66.51	7.50	12.13
	found %	66.36	7.59	12.12
Dihydro-β-ergokryptine	calc. %	66.51	7.50	12.13
	found %	66.2	8.0	12.0

6.2 Identity Tests

6.21 General Tests

Some general non-specific identity tests for ergot alkaloids are Mayer's reagent (mercuric potassium iodide), which gives a precipitate in dilutions of 1 part per million of the alkaloids. Iodine in potassium iodide also yields precipitates from dilute solution. Other alkaloidal reagents give precipitations with ergot alkaloids (phosphomolybdic acid, phosphotungstic acid, cadmium-potassium iodide, potassium iodobismuthate, etc.).

Since ergot alkaloids, including their 9,10-dihydro derivatives, possess an indole nucleus, unsubstituted at C-2, they give various indole colour tests.[5] Some of these are given below:

I. Keller's test - The alkaloid is dissolved in glacial acetic acid to which has been added a trace of anhydrous ferric chloride. After carefully layering in concentrated sulfuric acid, an intense blue-violet colour is produced at the interface.

II. Glyoxylic acid reagent - A test based on the presence of glyoxylic acid in acetic acid as an impurity has been developed:[67] The reagent gives a blue colour with the alkaloid in the presence of concentrated sulfuric acid. This reaction has been used, for example, for the visualization of dihydroergotoxine and several other alkaloids after thin layer chromatography on silica gel by Puech, Duru and Jacob.[68] The spraying agent contained glyoxylic acid monohydrate (0.5 g), ferric chloride hexahydrate (0.5 g), acetic acid (60 ml) and sulfuric acid (30 ml) plus 10 ml of distilled water. The method is sensitive to 0.1 microgram of alkaloid.

III. Van Urk's test - Ergot alkaloids, including dihydrogenated ones, yield a deep blue colour with 4-dimethylaminobenzaldehyde reagent (0.125 g 4-dimethylaminobenzaldehyde,

0.1 ml 5% $FeCl_3$ diluted in 65% sulfuric acid to give 100 ml) with an absorption maximum at about 585 nm. This test has been widely used for the colorimetric assay of indole alkaloids (see section 6.33). It has also found application in a field test for halucinogens.[69]

A modified van Urk reagent containing ethanolic hydrochloric acid (Ehrlich's reagent) has often been used for the visualization of alkaloidal compounds on thin layer plates.[70]

6.22 Specific Identification Tests

6.221 Infrared Spectrum

- see section 2.11

6.222 Chromatographic Behavior

Rf value, visualization in thin layer and k'-values in high performance liquid chromatography both for the dihydroergotoxine group alkaloids as a whole and for the single components are generally considered to be very specific identification criteria (see section 6.36).

6.223 Amino Acid Analysis

The amino acids present in the peptide moiety of the ergot alkaloids can be used for indirect identification, but this method does not allow distinction between epimers. The alkaloids are hydrolyzed with concentrated hydrochloric acid in a sealed tube at 100° for sixteen hours. The generated amino acids are identified after separation by thin layer, paper or ion exchange chromatography according to familiar methods.

Valine occurs only in dihydroergocornine, phenylalanine in dihydroergocristine, leucine results from the hydrolysis of dihydro-α-ergokryptine, and isoleucine from dihydro-β-ergokryptine. Consequently, dihydroergotoxine mesilate will contain these four amino acids, together with proline.

This method has been utilized for the quantitation of the components' ratio (section 6.38).

6.3 Quantitative Methods

6.31 Ultraviolet Spectrometry

A spectrophotometric method used to determine nonhydrogenated alkaloids in dihydroergotoxine has been described,[71,72] measuring the unhydrogenated parent compound at 318 nm which shows a molar absorptivity of 8000 compared to 80 for dihydroergotoxine.

6.32 Fluorometric Methods

Fluorometric methods to measure nonhydrogenated ergot alkaloids as impurities in dihydroergotoxine have been described[71] (see also section 2.13).

The quantitative uv-fluorodensitometric determination of the hydrogenated alkaloids of the ergotoxine group was reported by Prosek and co-workers.[28] The excitation wavelength was 230 nm, the emission was a maximum between 250 and 320 nm. The utilization of an adequate cut-off filter is essential.

6.33 Colorimetric Analysis

The van Urk reaction[20,73-75] has been used by many workers to determine the ergot alkaloids both in bulk substance and after chromatographic separation (see section 6.21).

For lack of the intact indole system, the oxidation products of dihydroergotoxine mesilate do not give the van Urk reaction, so that they will not interfere with the assay.[35,56,76]

6.34 Gravimetric Analysis

Gravimetric methods for alkaloids are quite nonspecific but can be used to measure the total alkaloid content of preparations that do not contain other basic substances. In most methods a precipitant such as phosphomolybdic acid, potassium iodide, potassium iodobismuthate, picric acid, phosphotungstic acid, silicotungstic acid, picrolonic acid, ammonium reineckate and trichloroacetic acid is utilized. Details of some typical methods for various alkaloids are described by Higuchi and Bodin.[77]

6.35 Volumetric Analysis

Since the ergot peptide alkaloids are basic, various titrimetric methods have been developed for their assay. The methods are non-specific and cannot be used to measure the stability of the alkaloids, for example. A general view of volumetric methods has been presented.[77]

6.36 Chromatography

The history of the ergot alkaloid chemistry as a whole and that of dihydroergotoxine in particular is tightly connected to the development of the separation techniques. Before 1943, chromatographic methods described dihydroergotoxine mesilate as a "single" compound. Afterwards, three-component separations are reported, and finally, the four-component separation has been achieved. In the following chapter, all three separation modes will be quoted.

6.361 Paper Chromatography

A number of paper chromatographic systems have been used for the separation of dihydroergotoxine alkaloids.

Stoll and Rüegger[78] utilized a reverse phase system with dimethylphthalate as the stationary phase and formamide-water pH 4 (1:4) as the mobile phase.

Adamanis and co-workers[79] used a modification of the Stoll system to achieve separation. The paper was impregnated with 10% diethylphthalate in ether and developed in circular, as a time-saving technique, with formamide-water (1:4) at pH 4.0 containing a small amount of diethylphthalate. The Rf of dihydroergocornine, dihydroergocristine and dihydroergokryptine in the system were 0.66, 0.54 and 0.47, respectively.

The alkaloids of the dihydroergotoxine group (3 components) have been separated from each other[31] and from their autoxidation products[56] using paper impregnated with 40% formamide in ether and developed with benzene-chloroform (1:1) for 5 hours. The spots were visualized by induced fluorescence via UV irradiation.

The separation of the dihydroergotoxine alkaloids from their light degradation products has been described[80] using paper impregnated with formamide-ethanol (2:3) and development with benzene-chloroform (1:1).

Gawrych and Wilczynska[71] have described two systems for the separation of ergotoxine and dihydroergotoxine:

1. Paper impregnated with formamide, elution with formamide-saturated benzene-chloroform (1:1).

2. Paper impregnated with formamide, elution with formamide-saturated chloroform-cyclohexane-diethylamine (2:7:1).

A pc separation without preparation of the paper has been reported by Margasinski and co-workers[81] developing with the same mobile phase as Adamanis[79] (formamide-water 1:4), leading to Rf values of 0.14, 0.22 and 0.34 for dihydroergocristine, dihydroergokryptine and dihydroergocornine, respectively.

Reio[77] has listed six paper chromatographic systems for the separation and identification of dihydroergotoxine and various other alkaloids, the best suited for dihydroergotoxine being:

A. Methyl isobutyl ketone-formic acid-water (10 parts ketone saturated with 1 part 4% formic acid)

F. Methyl ethyl ketone-acetone-formic acid-water (40:2:1:6).

The spots were visualized with van Urk's, Ehrlich's, or Dragendorff's reagent (Potassium bismuth iodide/water).

Hofmann[20] has described two pc systems leading to three-component separations.

I. Dimethylphthalate-impregnated paper, development with 20% formamide/80% pH 4.4 citrate buffer (Soerensen) and

II. Paper impregnated with formamide-benzoic acid, development with diethyl ether.

The van Urk reaction served as a means of detection and for colorimetric quantitation after elution of the alkaloids from the paper.

6.362 Thin Layer Chromatography

Zinser and Baumgärtel[83] have used a TLC system to separate dihydroergotoxine (as its then-known components) from the parent compounds and several other alkaloids (see also[84]). Benzene-chloroform-ethanol (2:4:1) was applied on neutral silica gel. Quantitation was carried out by elution with 40% methanol containing 1% tartaric acid and subsequent colorimetry after van Urk-reaction.

Wichlinski[85] has reported a TLC method with an acetone-benzene-petroleum ether (4:1:1) system for the separation of dihydroergotoxine and some other ergot alkaloids on silica gel G.

The same purpose can be accomplished by applying ethyl acetate-dichloromethane-methanol-conc. ammonia (50:50:3:1) on Merck F 254 silica gel. Dihydroergotoxine exhibits an Rf value of 0.45 under these conditions.[86]

Several indole alkaloids, including dihydroergotoxine, have been separated by Puech, Duru and Jacob[68] on thin layer silica gel G plates with either butanol-acetic acid-water (3:1:1) or chloroform-acetone-diethylamine (5:4:1). The alkaloids were visualized by spraying with glyoxylic acid reagent.

Dihydroergocristine has been included in a multi-supports/multi-solvents screening project for the identification of lysergic and dihydrolysergic acid derivatives yielding a variety of applicable TLC systems.[29,87]

Hohmann and Rochelmeyer[30] used a cellulose plate impregnated with dimethylformamide and developed with ethylacetate-heptane-diethylamine (5:6:0.02) to separate the dihydroergotoxine alkaloids into the components dihydroergocornine, dihydroergocristine and dihydroergokryptine. The spots were visualized by exposing the plate to the light of a mercury lamp to form the blue fluorescent UV degradation products of the alkaloids (see also 2.13). A modification of this method was applied by Prosek and co-workers[28] in densitometric studies.

Zarchska and Ozarowsky[88] have used talc as the adsorbent in a TLC system. The formamide-impregnated plate was dried for 1 hour at 25°, a mixture of n-heptane-tetrahydrofuran-toluene (5:4:1) was used for development. The three alkaloids were visualized with dimethylaminobenzaldehyde (modified van Urk reagent).

Röder, Mutschler and Rochelmeyer[89] reported two TLC systems with homogeneous azeotropic solvent mixtures for the separation of dihydroergocornine and dihydroergocristine, namely, dichloromethane-methanol (92.7:7.3), and chloroform-ethanol (92:8), respectively.

Reichelt and Kudrnac[90] used silica gel impregnated with formamide as a support. The TLC plates were developed with formamide-saturated diisopropyl ether-tetrahydrofuran-diethylamine (80:20:0.2) or with dibutyl ether-dichloromethane-diethylamine (60:20:0.2), each yielding a three-component separation.

Quite recently, Reichelt[91] reported on the three-component separation of dihydroergotoxine mesilate from bulk and pharmaceutical form samples on formamide-saturated thin layer prefabricated plates (Silufol "Kavalier", Merck or Schleicher + Schuell) with diisopropyl ether-tetrahydrofuran-diethylamine (90:10:0.7) as a mobile phase. The dihydrogenated alkaloids were detected with van Urk's agent.

Dihydroergocornine, -cristine, and -kryptine separate readily in the system ether-dimethylformamide-conc. ammonia (85:15:2) on Merck silica gel F 254 prefab plates resulting in optimal Rf values of 0.50, 0.44 and 0.55, respectively.[86]

Another neat three-component separation - via reversed phase - can be accomplished on dimethyl phthalate impregnated AVICEL®/silica gel (9:1) plates with dimethyl phthalate-equilibrated pH 2 citrate buffer.[86] The components are eluted with ethanol-water-acetic acid (9:9:2) and allowed to react with van Urk reagent. Spectrophotometry at 585 nm yields quantitative results.

6.363 Gas Chromatography

Not being volatile without decomposition, the dihydroergotoxine alkaloid mesilates are not susceptible to GLC. However, recently an interesting GLC approach has been reported by Szepesi and Gazdag.[92] The mesilates are converted to bases by ion exchange. Subsequently, those are decomposed at 235° in the stainless steel injection port of a gas chromatograph in a surprisingly reproducible manner. Hence the method lends itself not only to the identification but also to the quantitation of dihydroergocornine, dihydroergocristine and dihydroergokryptine. The nature of the chromatographing species, derived from the alkaloids' peptide fraction, is still under investigation.

GLC Conditions:

Column: 2% Dexsil 300 on 80/100 mesh Gas Chrom Q in a 1 m x 3.2 mm stainless steel tube.

Carrier gas: Nitrogen, 11.5 ml/min.

Injection port temperature: 235°

Column temperature: 180 - 280°C/heating rate 5°/min.

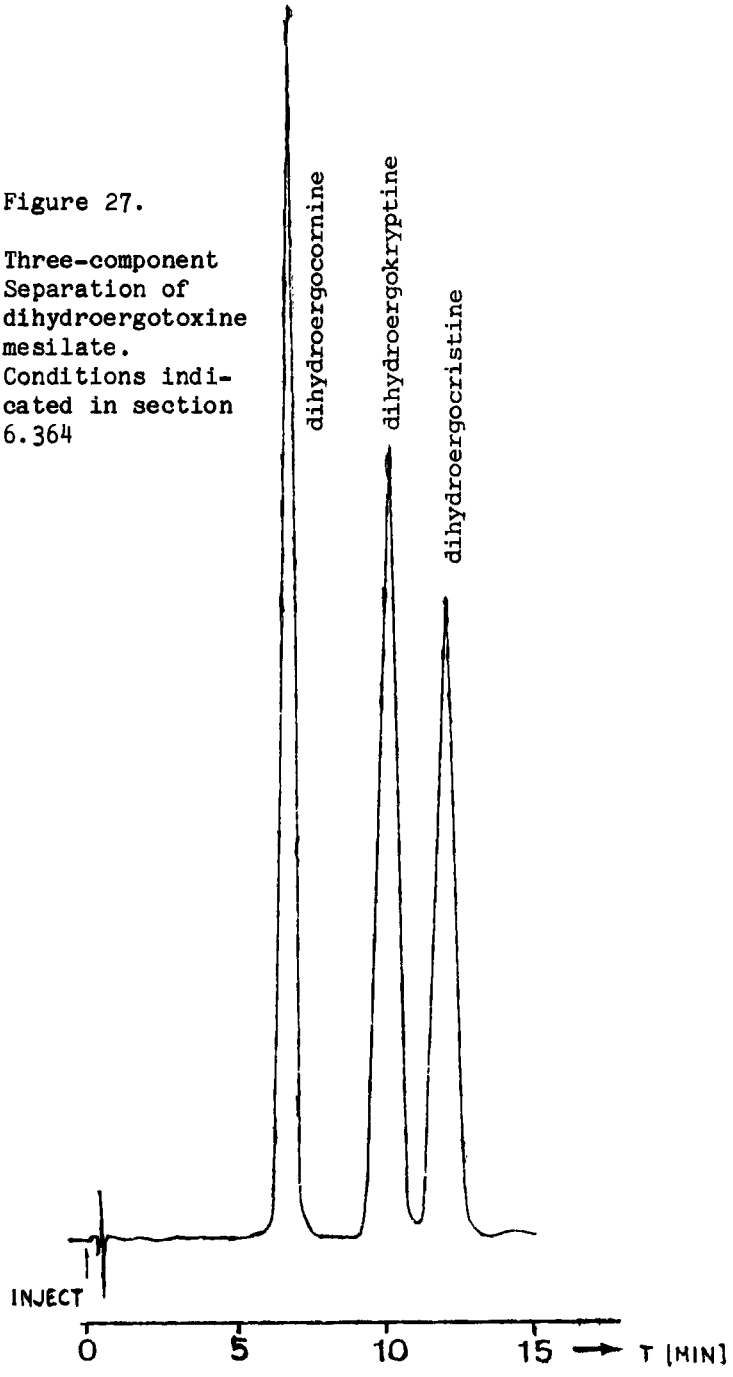
6.364 High Performance Liquid Chromatography

Wittwer and Kluckhohn[93] have utilized two HPLC systems in the separation of various alkaloids. Dihydroergocristine served as an example for the 9,10-dihydrogenated ergot peptide alkaloids. Its retention relative to ergotamine was reported as 0.81 in the system acetonitrile-isopropyl ether (40:60) on Sil-X (Perkin-Elmer) as the stationary phase and as 0.73 in the system acetonitrile-isopropyl ether (25:75) on Corasil-II (Waters).

Jane and Wheals[94] have reported three different relative retention times for dihydroergocornine, dihydroergocristine and dihydroergokryptine, as the individual alkaloids on Corasil C 18 (Waters). The method has been improved by Vivilecchia and co-workers[95] using

Figure 27.

Three-component Separation of dihydroergotoxine mesilate. Conditions indicated in section 6.364



the corresponding microparticles, μ-Bondapak-C 18 (Waters), separating these three alkaloid components of dihydroergotoxine.

In all the above cases, the detection was carried out at 254 nm with a fixed wavelength UV detector.

Quite recently, Hartmann, Rödiger, Ableidinger and Bethke[96] reported the HPLC separation of dihydroergotoxine mesilate. The splitting-up of dihydroergotoxine mesilate into dihydroergocornine, dihydroergocristine and dihydroergokryptine (3 component separation) was accomplished using prepacked as well as self-made columns with 5 or 10 μm C-8 or C-18 reversed phase material. The mobile phase was acetonitrile-0.02 M ammonium carbonate in water (0.54:1) or acetonitrile-1/30 M phosphate buffer, pH adjusted to 7.5 (0.54:1) (see fig. 27).

According to the same authors, the base line separation of dihydroergokryptine into its two isomers, resulting in a four component separation of dihydroergotoxine, can be accomplished by the application of the following HPLC systems:

I. LiChrosorb RP 18 (Merck) 5 μm (12.5 cm, 4 mm I.D.) or 10 μm (25 cm, 4 mm I.D.)/ water-acetonitrile-triethyl amine (32:8:1) (v/v/v)

II. LiChrosorb RP 8 (Merck) 7 μm (25 cm, 3 mm I.D.)/water-acetonitrile-triethyl amine (75:40:1) (v/v/v)

III. μ-Bondapak C-18 (Waters) 10 μm (30 cm, 4 mm I.D.)/water-acetonitrile-triethyl amine (22.8:11.4:1) (v/v/v)

See figure 28.

A spectrophotometer set at the absorption maximum of about 280 nm was used as a detector.

This method has been utilized for identification and quantitation purposes as well as for the determination of the relative ratios of the four dihydroergotoxine components.

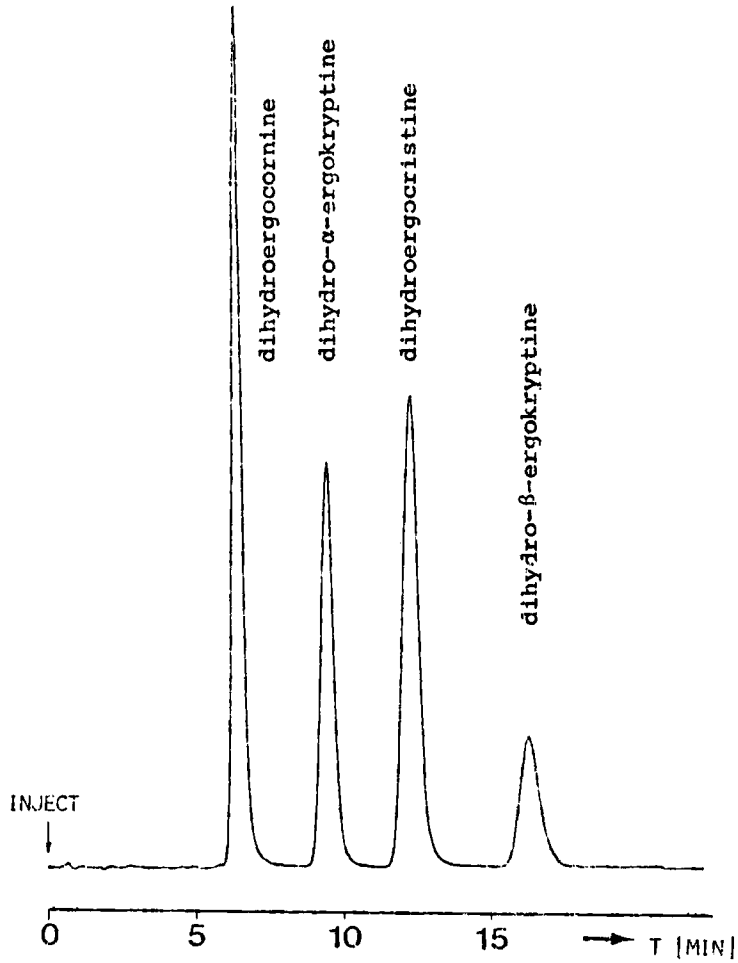


Figure 28. Four-component HPLC Separation of dihydroergotoxine mesilate. Conditions indicated in section 6.364

6.37 ^{13}C-Nuclear Magnetic Resonance

Quite recently Ernie and Loosli[34] have shown that this powerful technique can be used to determine the proportions of the four components of dihydroergotoxine mesilate. Dissolved in deuterated dimethylsulphoxide, the four alkaloids show discrete resonance signals for C-5' thus enabling convenient (but admittedly time-consuming) component ratio quantitation, figure 29 (see also section 2.15).

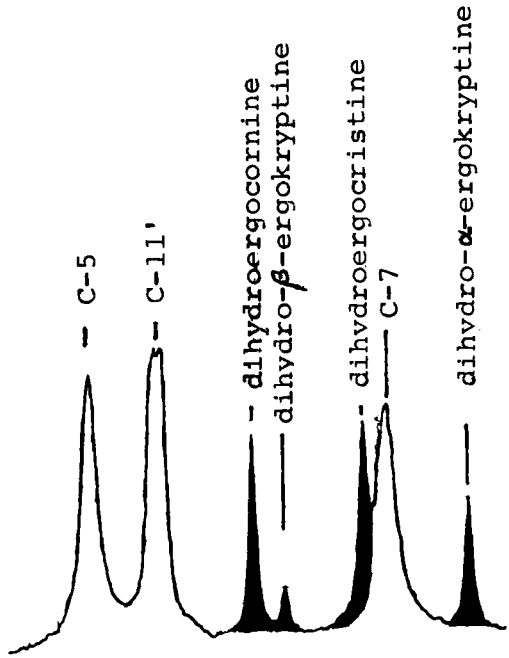


Figure 29. 50 to 70 ppm Section of the ^{13}C NMR Spectrum of dihydroergotoxine mesilate (500 mg in 1.5 ml dimethylsulfoxide-d_6). C-5' Signals in black.

6.38 Amino Acid Analysis

Amino acid analysis (see also section 6.223) has been utilized for the determination of the proportions of the four dihydroergotoxine components. The drug substance is subjected to hydrolysis with hydrochloric acid leading to the breakdown of the peptide fraction. The resulting amino acids are then chromatographed by ion exchange and detected after reaction with ninhydrine according to common procedures. From the peak areas of leucine, isoleucine, phenylalanine and valine, the relative proportions of the four components can be calculated.

6.4 Countercurrent Distribution

Galeffi and Delle Monache[97] have utilized countercurrent distribution to separate dihydroergotoxine into 3 components. A solvent system of 1 part of chloroform and 1 part of carbon tetrachloride was equilibrated with phosphate buffers of various pH values. After 270 transfers at pH 4.8 and 250 at pH 4.5, dihydroergocornine could be separated from dihydroergocristine and dihydroergokryptine. The latter two could be separated after more than 750 additional transfers at pH 4.5.

6.5 Determination in Body Fluids and Tissues

Due to their strong biopotency, the dihydroergotoxine alkaloids are administered in very small dosages of a few milligrams per day, leading to minute concentrations in body fluids and tissues. A sensitive fluorometric derivatization method has been developed,[98] but it is still not sensitive enough to cover the extremely low concentration range for the alkaloids. As mentioned in section 6.363, gas chromatography and GC-MS coupling, inclusively, cannot be utilized readily because of the low volatility and the thermal instability of the compounds in question. Therefore, the only methods presently available for biopharmaceutical studies are the radiotracer (^{3}H, ^{14}C) and the radio immuno assay techniques.[63] These methods on the other hand require a very high level of sophistication concerning standard stability and

purity for the first one, high specific radioactivity and accurately established test specificity for the latter. It is due to these crucial experimental conditions that successful biopharmaceutical studies have only recently been reported.[63-65]

6.6 Determination in Pharmaceutical Forms

Dihydroergotoxine mesilate can be identified in the various pharmaceutical forms by spectrophotometry, photometry and colorimetry following the van Urk reaction, or by its paper, thin layer or high performance liquid chromatographic behaviour.

Quantitation in dosage forms has been accomplished by colorimetry,[31,56,75,76] thin layer chromatography plus colorimetry,[30,86] densitometry[28] and high performance liquid chromatography.[96]

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Analytical Profiles of Drug Substances, 7

DIPHENOXYLATE HYDROCHLORIDE

Donald D. Hong

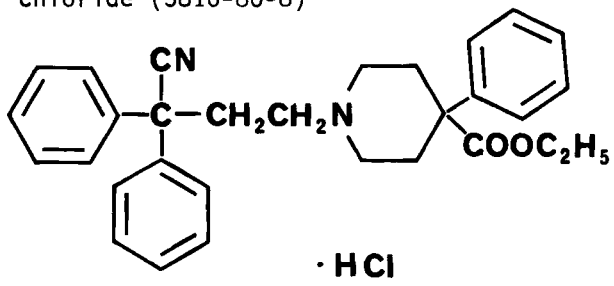
ISBN 0-12-260807-0

CONTENTS

1. Description

1.1 Name, Formula, Molecular Weight

The chemical name of diphenoxylate hydrochloride in Chemical Abstracts is found under Isonipecotic acid, designated as Isonipecotic acid, 1-(3-cyano-3,3-diphenylpropyl)-4-phenyl-, ethyl ester, monohydrochloride (3810-80-8)



$C_{30}H_{32}N_2O_2$ HCl MW 489.06

1.2 Appearance, Color, Odor

Diphenoxylate hydrochloride is a white, odorless, microcrystalline powder having a bitter taste. A saturated aqueous solution of the chemical is colorless.

2. Physical Properties

2.1 Bulk Density and Specific Gravity

The bulk density of the compound is about 0.2 g/ml with a specific gravity of 0.832 - 0.836 (1).

2.2 Differential Scanning Calorimetry

The DSC thermogram of diphenoxylate hydrochloride obtained on a Perkin-Elmer DSC-2 at a heating rate of 10^{o} C/minute is shown in Figure 1. The endothermic range corresponds to 219.2 - 223.5 - 228^{o} C (onset of melt - point of maximum melting rate - end of melt).

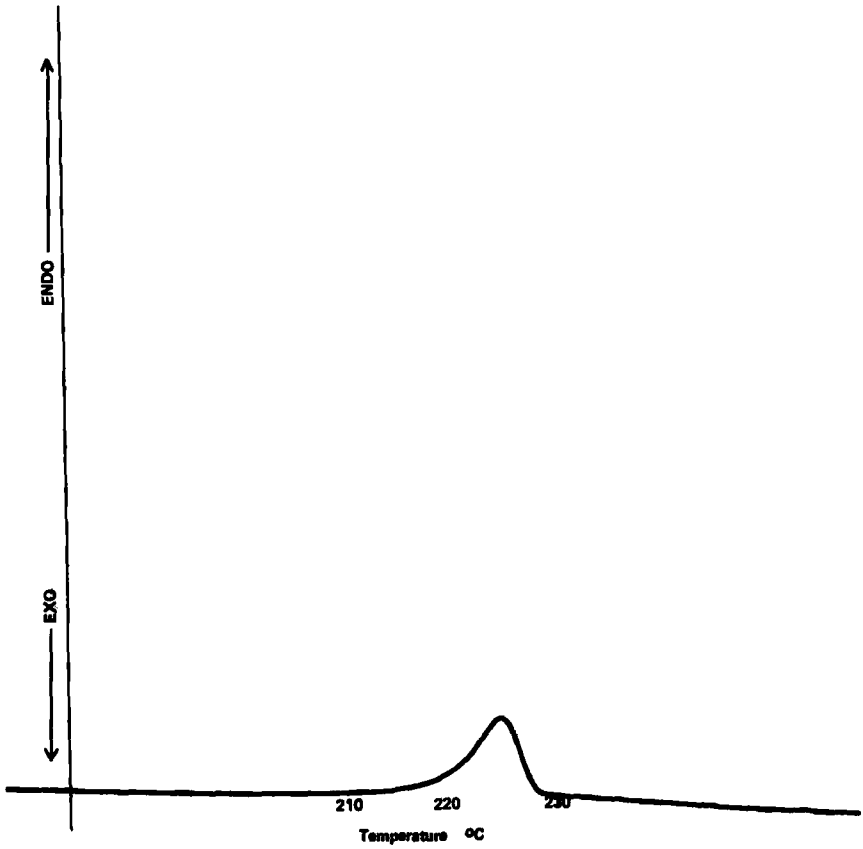


FIGURE 1: DSC THERMOGRAM OF DIPHENOXYLATE HYDROCHLORIDE (Lot RER 4–103A)

2.3 Dissociation Constant

The pKa of diphenoxylate hydrochloride by the titrimetric method was found to be 7.1 (1).

2.4 Fluoresence

Diphenoxylate hydrochloride exhibits no natural fluorescence when observed as 0.001 - 0.100% w/v solutions in MeOH (1).

2.5 Infrared Spectrum

The infrared absorption spectrum of diphenoxylate hydrochloride in a KBr dispersion is shown in Figure 2. Table 1 lists the major absorption band assignments (2).

Table 1. Infrared Band Assignments

Band (cm^{-1})	Assignment
2800-3140	CH_3 stretch
2320	$R_3\overset{+}{N}$-H, C≡N stretch
1770-2000	Aromatic overtone bands
1730	C=O stretch
	O
1605,1450-1500	Aromatic C=C stretch
1025	O-CH_2CH_3 (O-C) stretch
855	Characteristic of ethyl ester

2.6 Mass Spectrum

The mass spectrum of diphenoxylate is shown in Figure 3. The peaks of interest at m/e 246, 377 and 452 are as indicated.

2.7 Nuclear Magnetic Resonance (NMR)

The spectrum in Figure 4 was obtained with a Varian A60 NMR Spectrometer using a 10.6% solution of diphenoxylate hydrochloride in deuterated chloroform. Band assignments are relative to TMS internal standard and summarized in Table 2 (2).

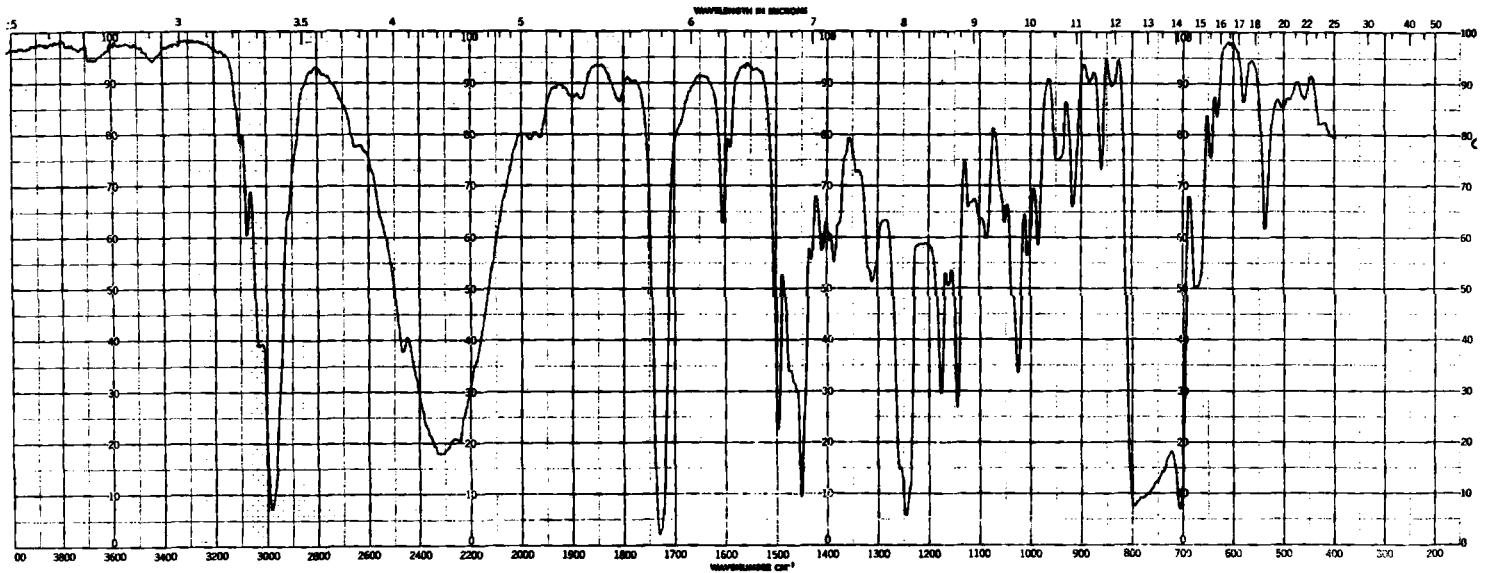


Figure 2: Infrared Spectrum of Diphenoxylate Hydrochloride

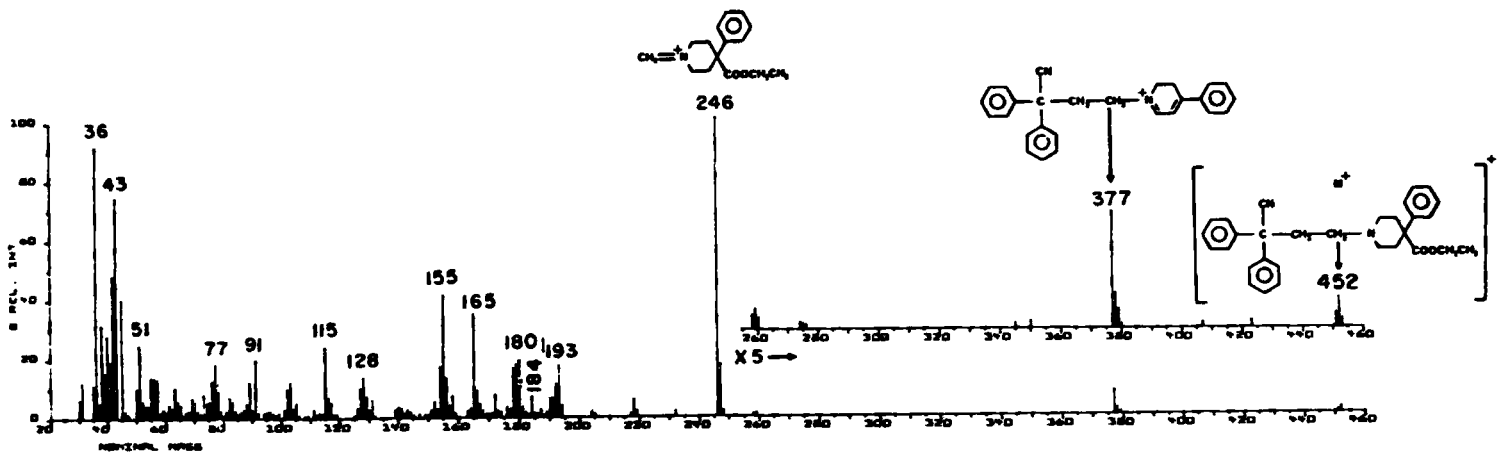
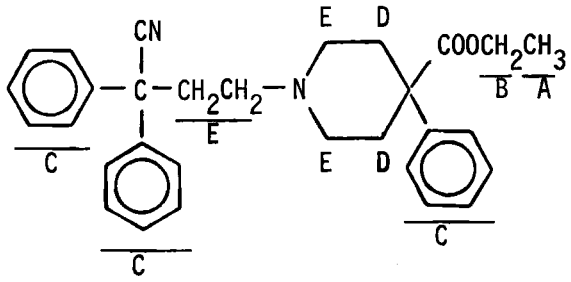


Figure 3. Mass spectrum (electron impact) of diphenoxylate
(After Karim and Heykants, ref. 3)

Table 2. NMR Spectral Assignments



Protons	PPM Chemical Shift	Multiplicity
A	1.19	Triplet
B	4.21	Quartet
C	7.4	Multiplet
D	2.7	Broad Singlet
E	2.5 - 3.8	Broad Bands

2.8 Melting Range

The melting range of diphenoxylate hydrochloride given in USP XIX, p. 158, is 220° to 226° C.

2.9 Optical Rotation

Diphenoxylate hydrochloride exhibits no optical activity (1).

2.10 Partition Coefficient

$$K = \frac{C_{CHCl_3}}{C_{H_2O}\ (pH\ 6.8)} = 473$$

There is little, if any, variation in K between $CHCl_3$ and water as a function of pH (1).

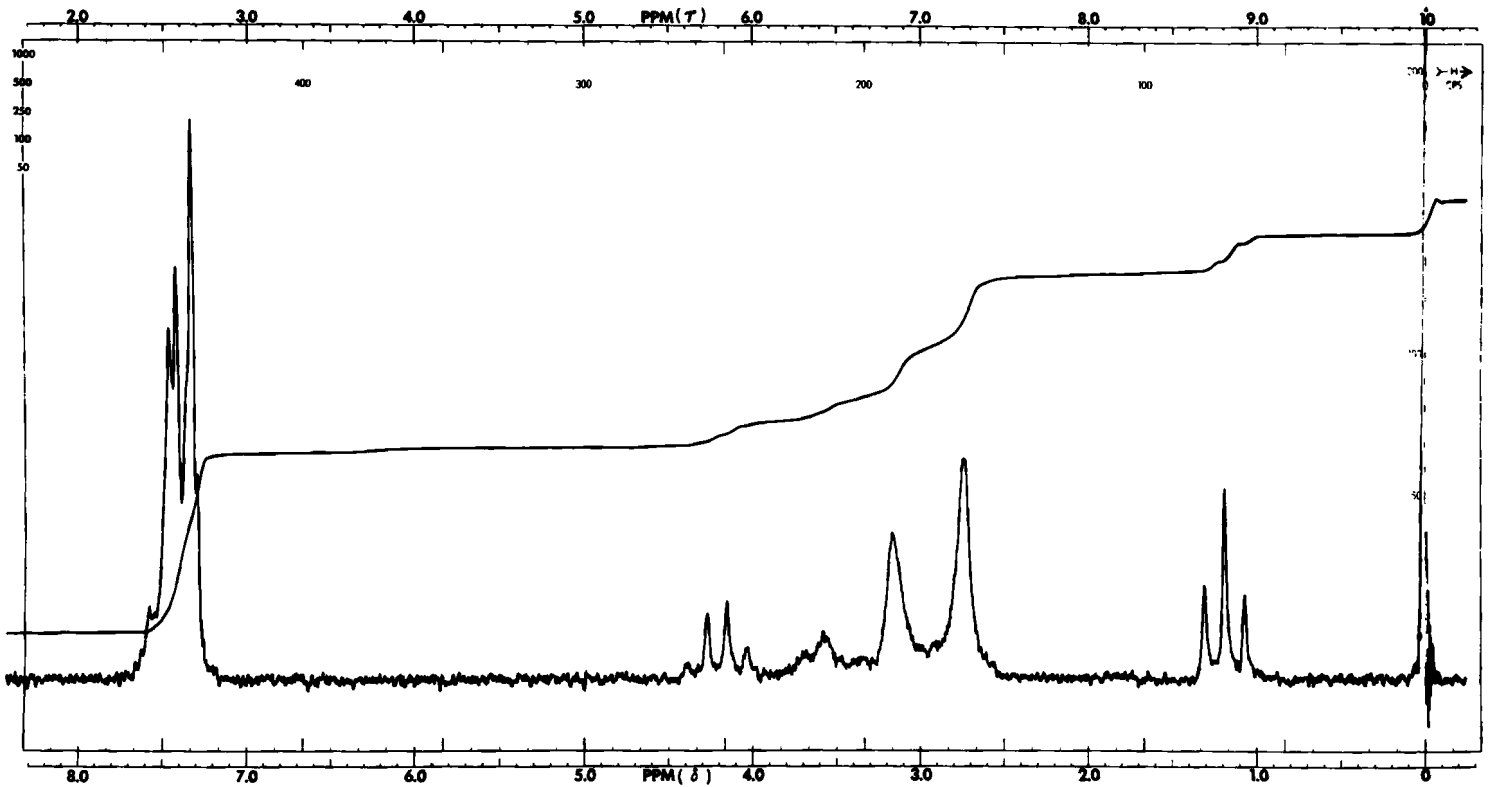


FIGURE 4. NUCLEAR MAGNETIC RESONANCE SPECTRUM OF DIPHENOXYLATE HYDROCHLORIDE

2.11 pH

A saturated solution of the compound in water has a pH of about 3.3 (4).

2.12 Solubility

Solubilities in various solvents at 25° C are tabulated in Table 3.

Table 3. Solubility Data for Diphenoxylate HCl

Solvent	Solubility, mg/ml	Reference
Methanol	>50	5
Chloroform	>50	
Acetone	6	
Benzene	3	
Ethanol	3	
Ethyl Acetate	2.3	
Water	0.8	
Heptane	<0.1	
Ethyl Ether	<0.1	
Simulated gastric fluid	<0.1	
Acetic Acid	500	1
Dimethylformamide	500	
Chloroform*	360	
Dioxane	46	
Tetrahydrofuran	21	
i-Propanol	2.5	
Carbon Tetrachloride	1	
Hexane	0.5	
0.1N HCl	<0.1	
0.1N NaOH	<0.1	
0.1N NH_4OH	<0.1	

*The free base in $CHCl_3$ has a solubility of ~420 mg/ml

2.13 Ultraviolet Spectrum

The UV spectrum of diphenoxylate hydrochloride in methanol is shown in Figure 5. The spectrum is an aromatic multiplet having maxima at 264 nm (a = 1.06), 258 nm (a = 1.32) and 252 nm (a = 1.09).

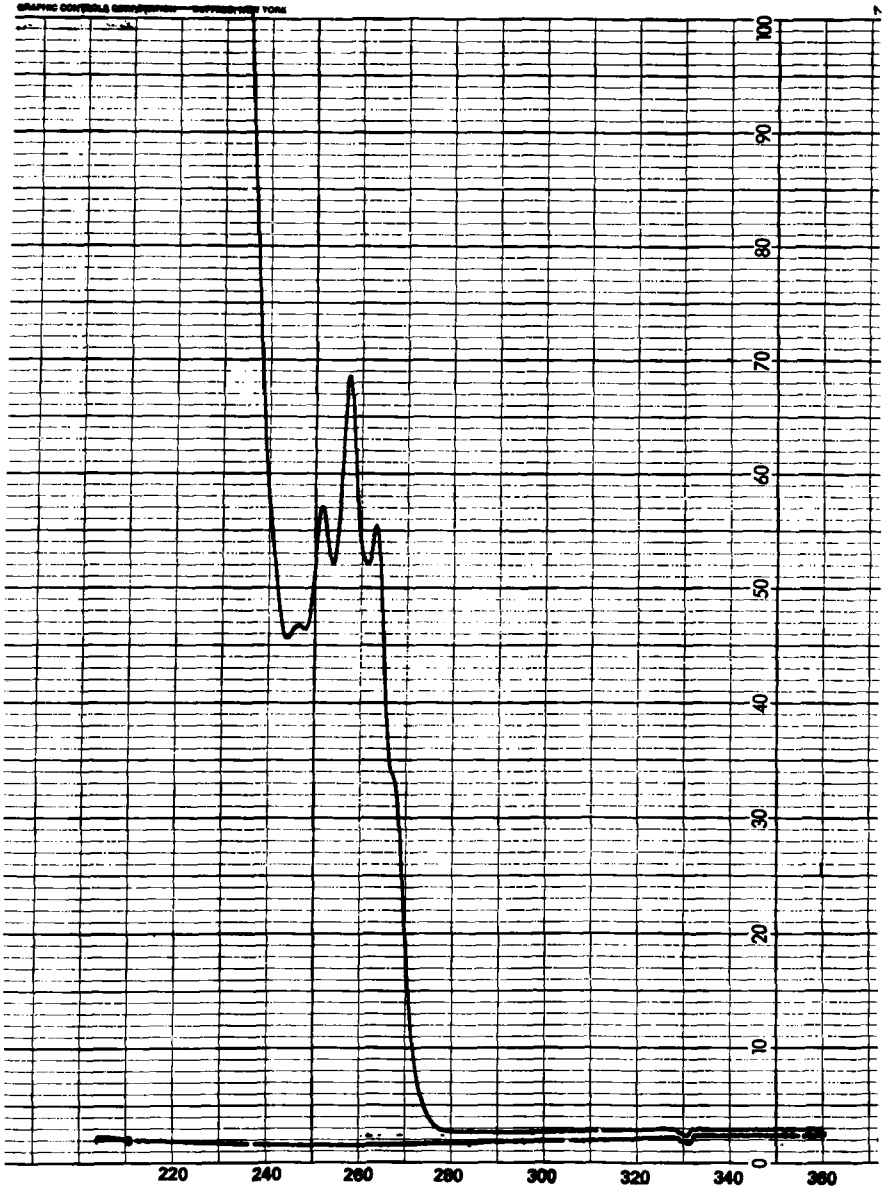


FIGURE 5: ULTRAVIOLET SPECTRUM OF DIPHENOXYLATE HYDROCHLORIDE IN METHANOL

3. Synthesis

W. Van Bever, et al (6) have reviewed the chemistry of synthetic antidiarrheal agents as pertained to diphenoxylate, difenoxin, loperamide and related compounds.

Specifically diphenoxylate hydrochloride can be synthesized (7) as presented in Figure 6. Diethanolamine is condensed with p-toluenesulfonyl chloride in aqueous sodium carbonate solution and N,N-bis-(2-hydroxyethyl)-p-toluenesulfonamide (I) is isolated. The compound is reacted with thionyl chloride to form N,N-bis-(2-chloroethyl)-p-toluenesulfonamide (II). The amide in turn is treated with sodium hydride and benzyl cyanide in toluene to give N-(p-toluenesulfonyl)-4-cyano-4-phenylpiperidine (III). The piperidine is then hydrolyzed in aqueous sulfuric acid to form 4-carboxy-4-phenyl piperidine tosylate salt (IV) which crystallizes upon diluting and cooling the mixture with water. The tosylate is isolated and treated with ethyl alcohol to form 4-carbethoxy-4-phenylpiperidine (V). The reaction mixture is made alkaline and the piperidine is separated from the sodium tosylate by solvent extraction. The solvent is then stripped.

Diphenylacetonitrile, ethylene bromide and sodium hydride are reacted in toluene to give 2,2-diphenyl-4-bromobutyronitrile (VI) and crystallized.

A mixture containing (V) and (VI) along with sodium carbonate, potassium iodide and methyl isobutyl ketone is reacted. The mixture is then treated with HCl/toluene to give the final product, 2,2-diphenyl-4-[(4-carbethoxy-4-phenyl)piperidine] butyronitrile hydrochloride (VII) which is recrystallized from ethyl alcohol.

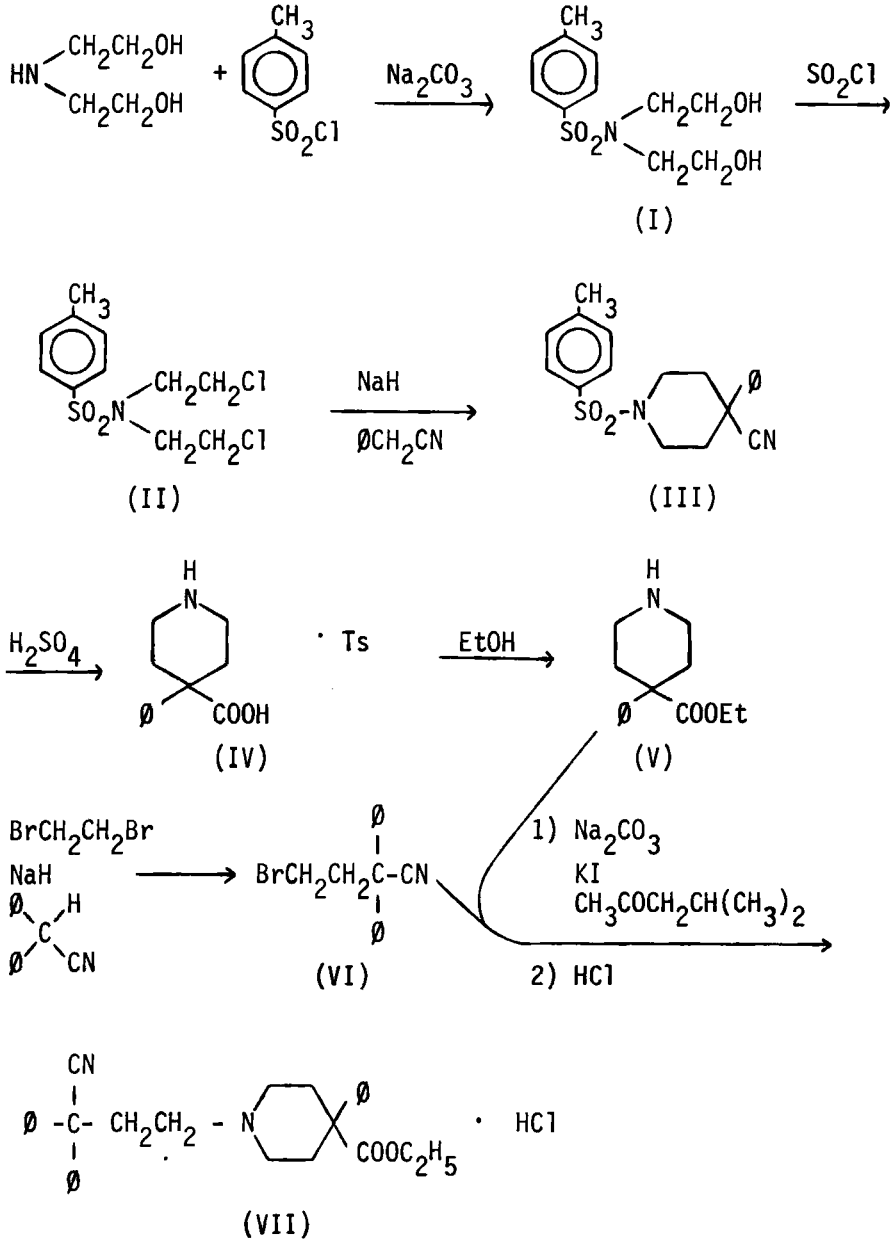
4. Drug Metabolism

4.1 Biotransformation Products

Karim, et al (8, 9), using ^{14}C-labelled diphenoxylate hydrochloride, reported that the major portion of the administered label in man was found in the feces, followed by urine and plasma. After oral administration approximately 10% of the dose was found in the urine and about 40% in the feces. Even after intravenous administration about 70% of the dose was recovered in the feces.

In all three routes of elimination the major metabolic product of diphenoxylate hydrochloride was diphenoxylic acid. Hydroxydiphenoxylic acid and

Figure 6. Synthesis of Diphenoxylate Hydrochloride



3-cyano-3,3-diphenylpropionic acid have also been identified (10). In the urine both free and conjugated metabolites were isolated. Some unchanged drug was recovered in the plasma along with a compound that was more polar than the acid.

A summary of the metabolites of diphenoxylate hydrochloride, their mass spectral data and the species from which they were found is shown in Figure 7.

In man the identity of the metabolite from feces following oral administration of ^{14}C-labelled diphenoxylate HCl is dependent on the dosage form of the drug. Karim, et al (11) found that when the drug was administered in ethanolic solution, the major metabolites were diphenoxylic acid and compounds more polar than the acid. When the drug was given as a tablet or as a capsule more than 50% of the metabolite was as the unchanged drug. These studies suggest that the drug is better absorbed in ethanol solution than as a solid dosage form. The reason for this is that diphenoxylate is not very soluble in water and the dissolution rate of the drug in the tablet/capsule would be a rate-limiting step.

4.2 Pharmacokinetics

In a study involving three normal male subjects, Karim, et al (9) reported the pharmacokinetic analysis of ^{14}C-diphenoxylate hydrochloride using a one-compartment open model showed a rapid absorption ($t_{\frac{1}{2}}$ = 19.7 minutes) and a rapid elimination ($t_{\frac{1}{2}}$ = 2.5 hours). The plasma half-life ($t_{\frac{1}{2}}$ = 4.4 hours) of the major metabolite, diphenoxylic acid, was higher than the diphenoxylate.

In the same study these investigators showed that within 4 days approximately 49% of the administrated label was recovered in the feces. The fecal radioactivity in the 0 to 24 hour sample was very low. Most of the activity was recorded in the 24 to 48 and 48 to 72 hour samples. Approximately 14% of the administered label was recovered in urine within 3 days, with about 11% being found in the first 24 hours.

5. Stability

Diphenoxylate hydrochloride, under ordinary conditions of air and light, is an extremely stable compound. Its major degradation product is diphenoxylic acid.

To study the degradation pattern of diphenoxylate hydrochloride (1), the compound was subjected to the following conditions, extracted with chloroform and compared with known degradation products by TLC. The

Figure 7: Mass Spectral Data of Diphenoxylate and Its Metabolites

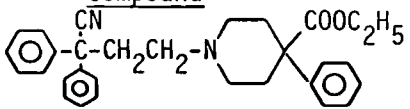
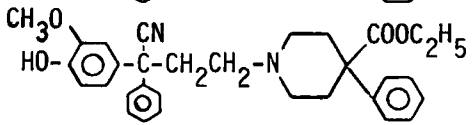
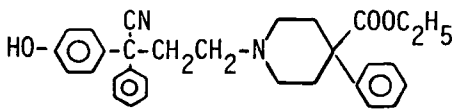
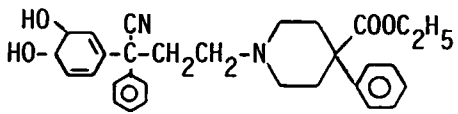
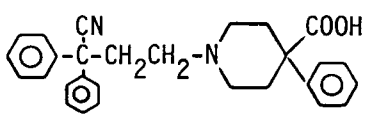
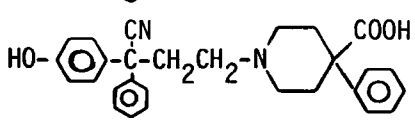
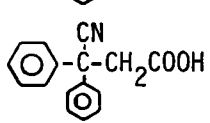
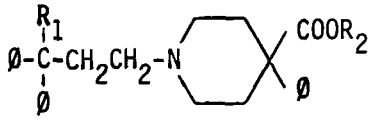
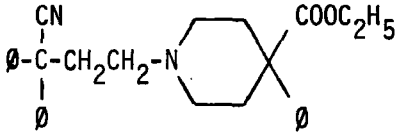
Compound	M^+	Molecular Formula	Base Peak	Species
	452	$C_{30}H_{32}N_2O_2$	246	Man
	498	$C_{31}H_{34}N_2O_4$	246	Man Dog Rat
	468	$C_{30}H_{32}N_2O_3$	246	Man Dog Rat
	486	$C_{30}H_{34}N_2O_4$	246	Dog
	424	$C_{28}H_{28}N_2O_2$	218	Man Dog Rat
	-	$C_{28}H_{28}N_2O_3$	-	Man Dog Rat
	-	$C_{16}H_{13}NO_2$	-	Man

Table 4. Degradation Products of Diphenoxylate Hydrochloride

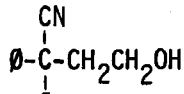


Diphenoxylate: R_1=CN; R_2=C_2H_5

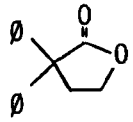
Condition	Degradation Product
1. 24 hr reflux in 1M H_2SO_4/ MeOH (1:1)	Diphenoxylic Acid (0.5%) R_1=CN; R_2=H
2. 24 hr reflux in 1M NaOH/ MeOH (1:1)	Diphenoxylic Acid (major) Amide (1%) and Acid (0.2%) of Diphenoxylic Acid Amide: R_1=$CONH_2$; R_2=H Acid: R_1=COOH; R_2=H
3. 24 hr reflux in H_2O/MeOH (1:1)	No degradation found
4. 105° C for 4 weeks	No degradation found
5. 24 hr reflux in 1M H_2SO_4/ Peroxide	3-Cyano-3,3-diphenylpropanol (A) 4-Phenyl-4-piperidinecarboxylic acid (B) 2,2-Diphenylbutyrolactone (C) 4-Phenylpiperid-3-ene (D)



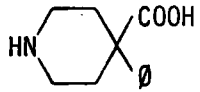
Diphenoxylate



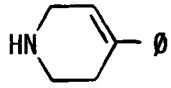
(A)



(C)



(B)



(D)

results are summarized in Table 4.

6. Methods of Analysis

6.1 Elemental Analysis

Table 5 shows the results of an elemental analysis of diphenoxylate hydrochloride (Lot RER 4-103-A) (12).

Table 5. Elemental Analysis of Diphenoxylate Hydrochloride

Element	Theory	Found
C	73.68	73.99
H	6.80	6.79
Cl	7.25	7.20
N	5.73	5.73
O	6.54	6.33

6.2 Microchemical Identification

E.G.C. Clarke (13) has summarized the identification tests and R_f values of diphenoxylate along with various other drugs. They are classified under type of precipitation with 27 alkaloidal reagents, type of crystals, and sensitivity with selected reagents.

6.3 Chromatographic Analysis

6.31 Thin Layer Chromatography. Most of the development work on diphenoxylate hydrochloride using TLC evolved around one basic system. The systems are summarized in Table 6.

6.32 Gas Liquid Chromatography. A GLC procedure has been developed to measure diphenoxylic acid, the major metabolite of diphenoxylate hydrochloride in urine (16). The procedure involves base hydrolysis to liberate diphenoxylic acid from compounds conjugated in urine and then removal of interfering substances by alumina column chromatography. Quantitation was carried out using p-chlorodiphenoxylic acid as an internal standard. To increase volatility, it was necessary to methylate diphenoxylic acid and its derivatives with diazomethane. Sensitivity is about 200 ng/ml.

To further increase the sensitivity of the method, the authors (17) used GC/MS to detect the diphenoxylic acid in plasma using a deuterium labelled internal standard and monitoring the base peak of the metabolite.

Table 6. TLC Systems

Solvent	Adsorbent	Dosage Form	Detection	R_f	Ref	Note
Chloroform:Methanol: Acetic Acid (92:3:5)	A	Chemical	I	0.7	1	a
Chloroform:Methanol: Acetic Acid (92:3:5)	A	Tablets	II	0.63	14	b
Chloroform:Methanol: Acetic Acid (94:5:1)	A	Chemical	III	0.8	15	-
n-Butanol:Acetic Acid: Water (10:2:8)	A	Chemical	IV,V	0.73	5	-

Adsorbent

A. Silica Gel 60 F254 (250 u)

Detection

I. Potassium iodobismuthate
II. Iodine vapors
III. Zonal scraping (radioactive counting)
IV. Dragendorff
V. t-Butylhypochlorite/KI starch

Note: a. This system is capable of resolving diphenoxylate hydrochloride from its possible degradation products and impurities. These include bromobutyronitrile (R_f 0.90), tetraphenyldicyanobutane (R_f 0.82), amide of diphenoxylate (R_f 0.42), diphenoxylic acid (R_f 0.38), amide of diphenoxylic acid (R_f 0.23), dipethadine (R_f 0.13) and piperidine ester (R_f 0.03).

b. Active extracted with chloroform.

A second GLC method has been developed that is capable of measuring diphenoxylate hydrochloride chemical directly (18). Cholesteryl propionate is used as the internal standard. The method is described briefly as follows:

Instrumental Conditions

Instrument: Gas chromatograph with FID detector
Column: 1% Dexsil 300 GC on 80-100 Chromosorb Q, 2 ft x 4 mm id, glass
Carrier Gas: Nitrogen, 62 ml/min. (Hydrogen and air flow rates should be optimized for maximum sensitivity and stability.
Temperatures: Column 260°C
Injector 290°C
Detector 290°C
Retention time: Cholesteryl propionate 6.2 min.
Diphenoxylate hydrochloride 8.0 min.

6.4 Colorimetric Analysis

Diphenoxylate hydrochloride can be analyzed by the dye-salt procedure (19). This involves extraction of the compound from a basic aqueous solution with chloroform. A portion of the organic extract is transferred to a centrifuge tube to which bromocresol green and an aqueous phase buffered to pH 5.6 are added. The resulting yellow colored complex in the organic phase is back extracted to a basic aqueous phase. The absorbance of the resulting blue aqueous phase is then read.

6.5 Spectrophotometric (UV) Analysis

Diphenoxylate hydrochloride may be determined spectrophotometrically in 0.01N HCl in methanol. The UV absorption maximum is at about 258 nm.

6.6 Titrimetric Analysis

6.61 Non-Aqueous Titration. Being an amine, diphenoxylate hydrochloride can be titrated with acetous 0.1N perchloric acid. The titration may be performed manually using crystal violet as indicator or determined potentiometrically. Mercuric acetate T.S. is added to overcome the acidity of the HCl which causes an early end-point. A blank determination

should be carried out and any corrections made.

Each ml of 0.1N perchloric acid is equivalent to 48.91 mg of diphenoxylate hydrochloride (20).

6.62 Semi-microtitrimetry Using Sodium Lauryl Sulfate. F. Pellerin et al (21,22) have described the titration of diverse nitrogenous compounds including diphenoxylate with sodium lauryl sulfate in a 2-phase system of water/chloroform. The method depends upon the reaction of the quaternary ammonium cation, in 1:1 ratio, with the sodium lauryl sulfate which is an anionic detergent. The salt that is formed is insoluble in water but soluble in chloroform and methyl yellow is used as the indicator.

7. Acknowledgments

The author wishes to express his appreciation to Dr. G. Anthony for reviewing the manuscript; to Ms. P. Finnegan for the IR and NMR data; to Dr. A. Karim for the information on the mass spectrum and for the section on drug metabolism; to Mr. G. Lee for the data on GC; to Corporate Information Service for the literature search; to Ms. M. LLorente for technical assistance; and to Ms. A. Fenton for typing the monograph.

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Analytical Profiles of Drug Substances, 7

DROPERIDOL

Casimir A. Janicki and Roger K. Gilpin

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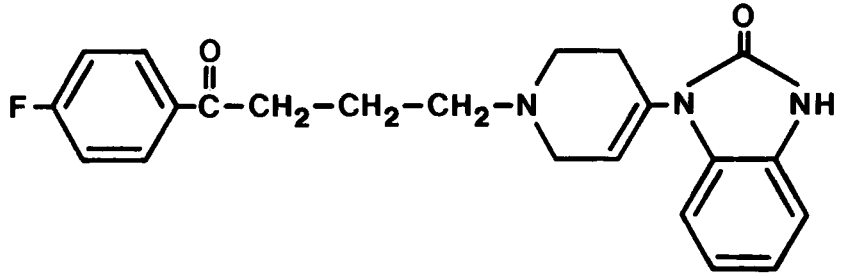
TABLE OF CONTENTS

1. Description

1.1 Name, Formula, Molecular Weight

Droperidol is 1-{1-[4-(4-Fluorophenyl)-4-oxobutyl]-1,2,3,6-tetrahydro-4-pyridinyl}-1,3-dihydro-2H-benzimidazol-2-one. The parenteral product is known as INAPSINE® injection. It is also a component of INNOVAR® and THALAMONAL® injection.



1.2 Appearance, Color, Odor

White to light tan, odorless crystalline powder.

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum is presented in Figure 1. The spectrum was obtained from a potassium bromide dispersion using a Perkin-Elmer 521 grating IR spectrophotometer. A list of the assignments made for some of the characteristic bands is given in Table I (1).

Table 1

IR Spectral Assignment for Droperidol

3060	-CH-
2950 2895	$-CH_2-$

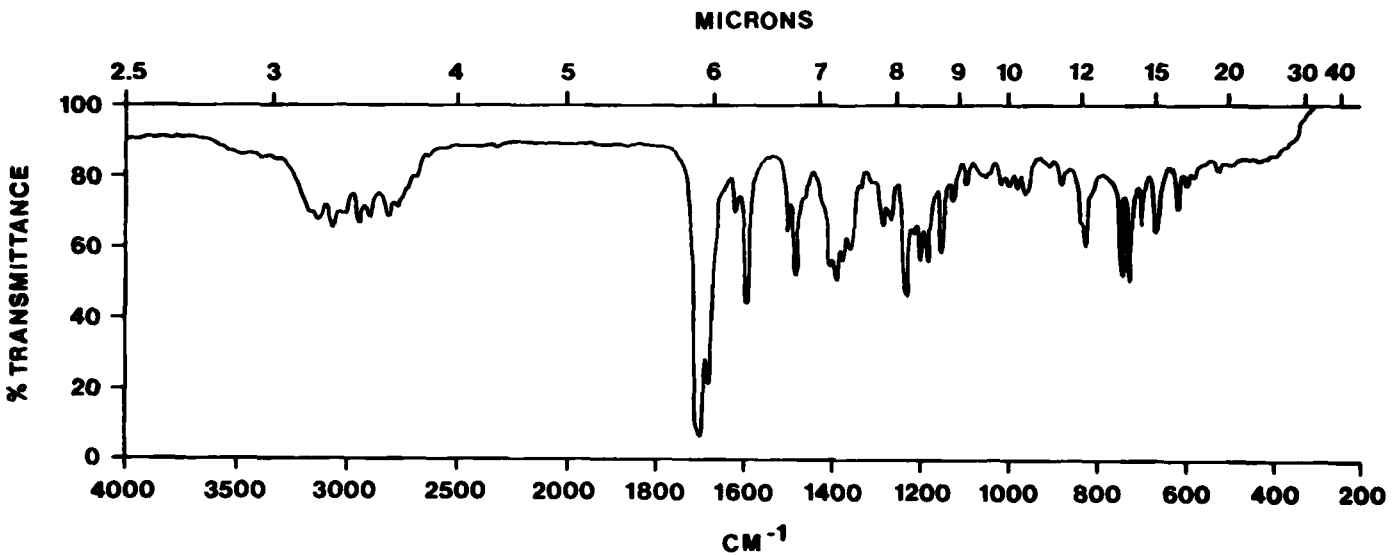


Figure 1. The Infrared Spectrum of Droperidol, KBr Dispersion. Instrument; Perkin-Elmer 521

1700	C=O ring
1685	C=O ring
1595 1500 1485	aromatic substitution
1235	p-substitution
828	p-substitution
745	o-substitution

2.2 Nuclear Magnetic Resonance Spectrum

The 90 mHZ spectrum of droperidol presented in Figure 2 was obtained in DMSO d6 at an approximate concentration of 98 mg/ml with tetramethylsilane as the internal standard. Spectral assignments are listed in Table II (1).

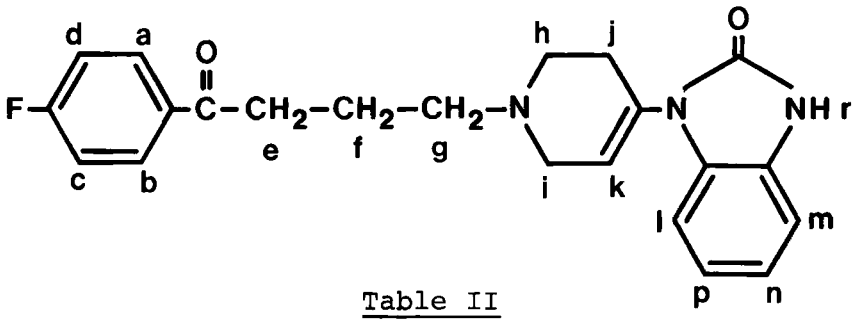


Table II

NMR Spectral Assignments for Droperidol

Chemical Shift (ppm)	Multiplicity	Characteristic of Proton
8.0	multiplet	a,b
7.25	multiplet	c,d
6.8-7.2	multiplet	l,m n,p
5.9	multiplet	k
2.4-3.1	multiplet	e,f g,h

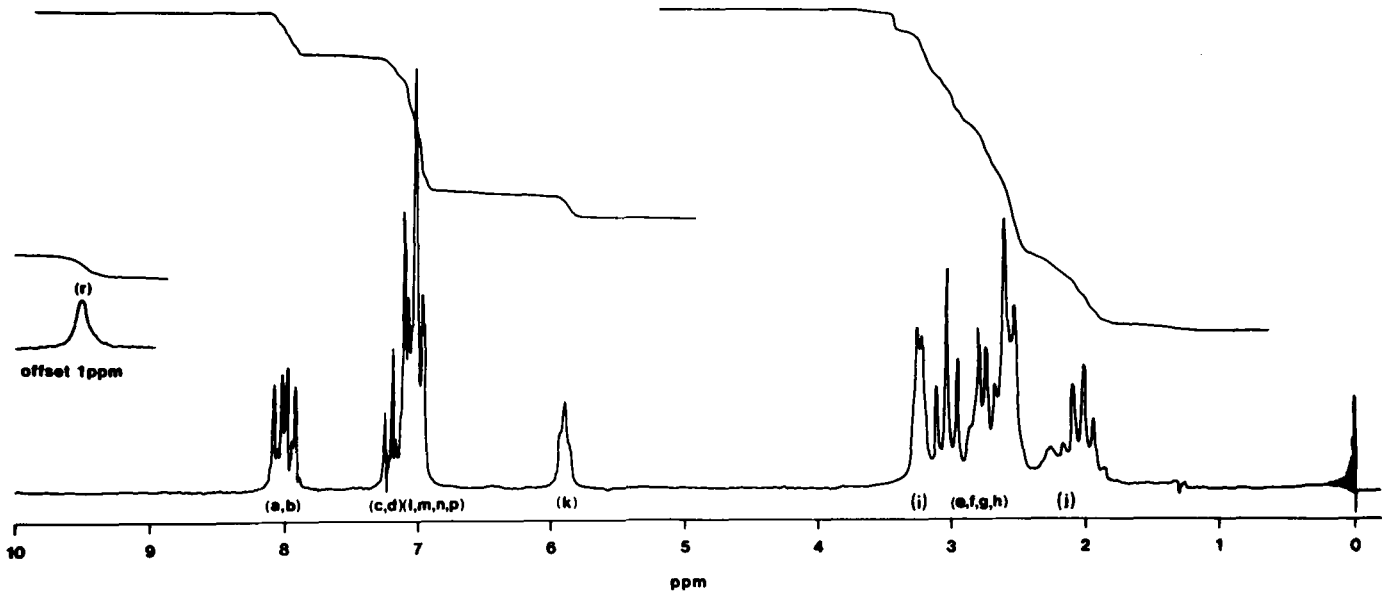


Figure 2. The NMR Spectrum of Droperidol in $CDCl_3$ with TMS as Internal Standard. Instrument; Perkin-Elmer R-32

3.25	multiplet	i
1.8-2.2	multiplet	j
10.5	singlet	r

2.3 Ultraviolet Spectrum

The ultraviolet absorption spectrum of droperidol obtained from a 9:1, 0.1 M hydrochloric acid: methanol solution is shown in Figure 3. Droperidol exhibits two maxima, one about 245 nm (ε = 15,600) due to the butyrophenone part of the molecule and the second about 280 nm (ε = 7500) due to the benzimidazolinone part of the molecule. The UV spectrum of droperidol obtained from a 9:1, 0.1 M Citric acid: methanol solution is very similar to the spectrum in Figure 3.

2.4 Mass Spectrum

The mass spectrum obtained on a Finnigan model 1015D EI mass spectrometer is presented in Figure 4. The fragmentation patterns are discussed in a paper by Blessington (3).

2.5 Melting Range*

A dried droperidol sample melts between 144° and 148°C, NF XIV Class 1 procedure. The existence of polymorphic forms plus a hydrated form is partially demonstrated by a melting point determination. The most stable polymorphic form melts between 146.5° - 148.5°C, with the least stable form between 139.8° - 142.5°C. The hydrate melts at approximately 115°C followed by recrystallization and then remelting at approximately 140°C (4).

2.6 Differential Scanning Calorimetry (DSC)

The DSC of droperidol is shown in Figure 5. A melting endotherm is observed at 421°K (148°C) using a temperature program of 10°/minute. The DSC

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*Data given as uncorrected values appearing in listed references.

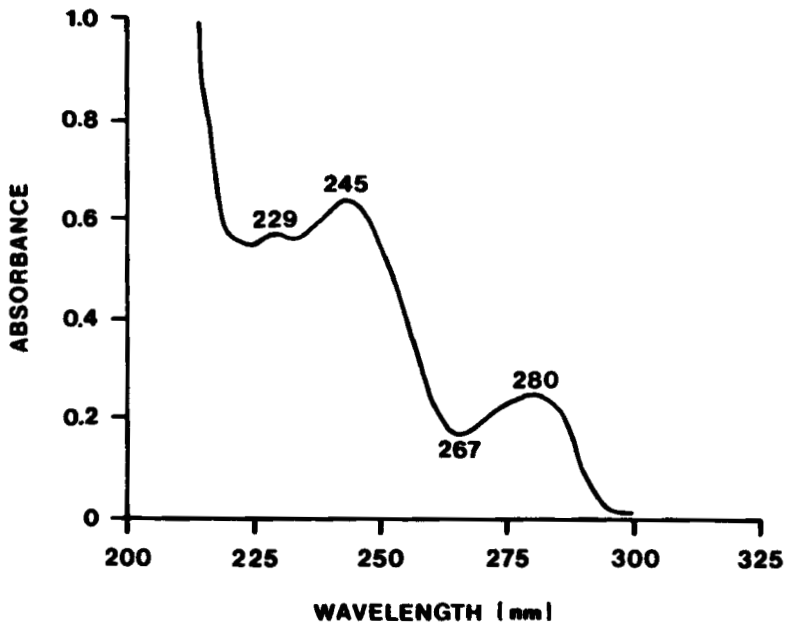


Figure 3. The UV Absorption Spectrum of Droperidol in 9:1 0.1M Hydrochloric Acid:Methanol.

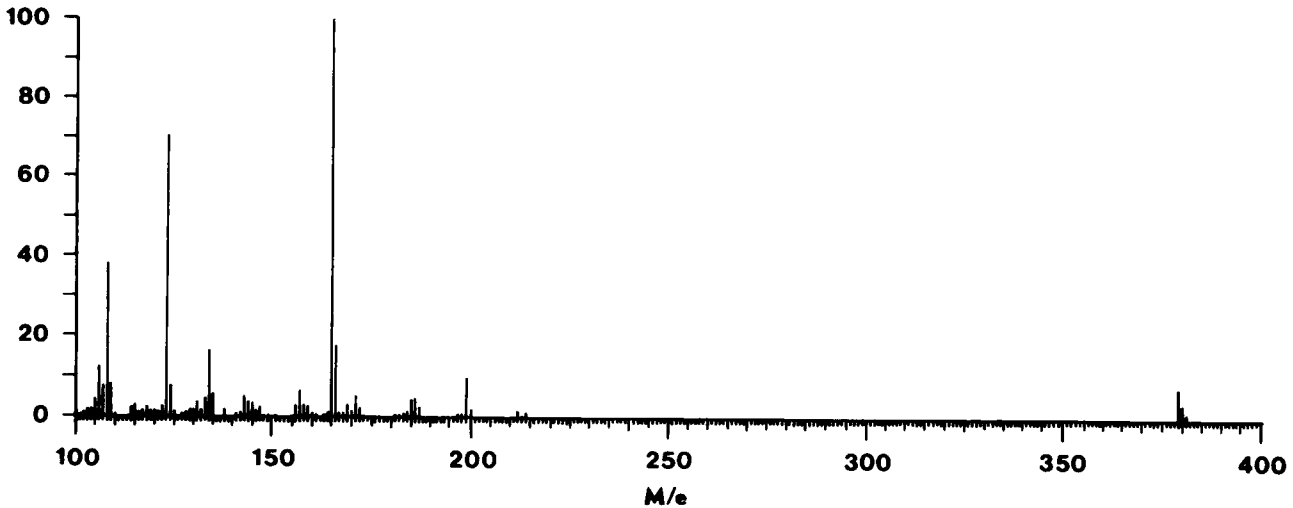


Figure 4. The Mass Spectrum of Droperidol, Electron Impact Ionization. Instrument; Finnigan Model 1015D

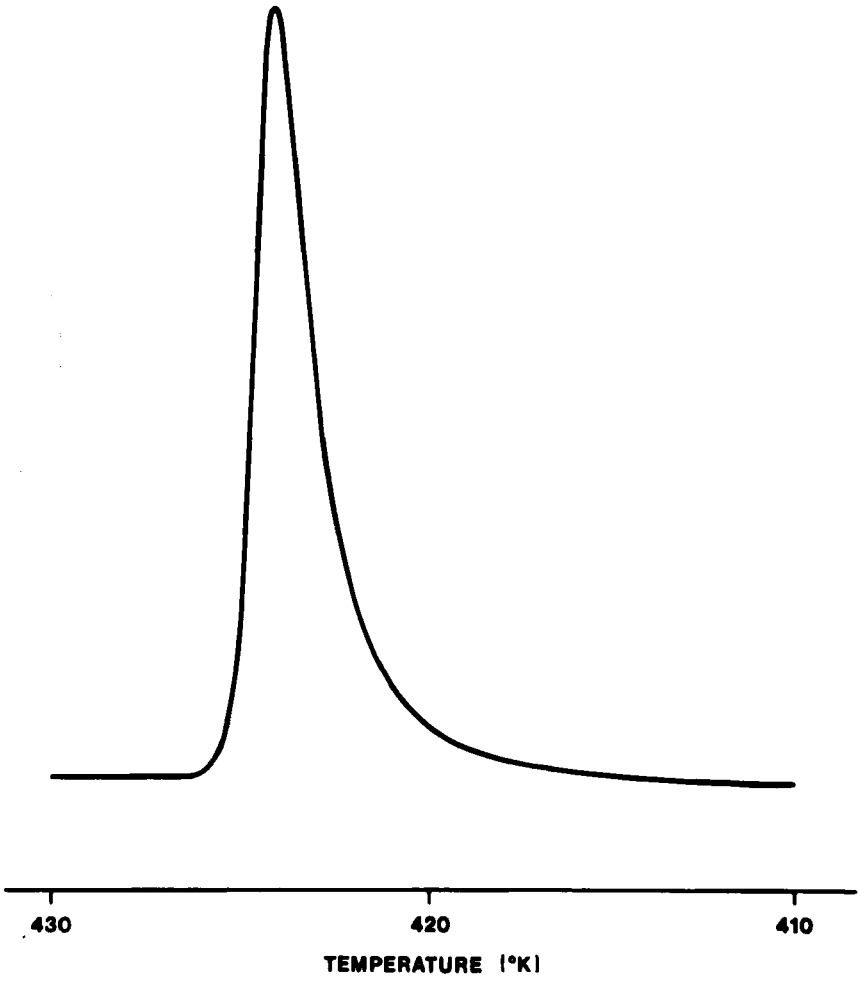


Figure 5. The DSC of Droperidol.
Instrument; Perkin-Elmer DSC-2.
Heating Rate: 10° per min.
ATM.: Nitrogen

shows different metling peaks for the other polymorphic forms. The least stable polymorph has one peak about 416°K and the hydrate has two peaks at about 394°K and 418°K (4).

2.7 Solubility

The approximate solubilities obtained at room temperature are listed in Table III (1).

Table III

Approximate Solubilities of Droperidol

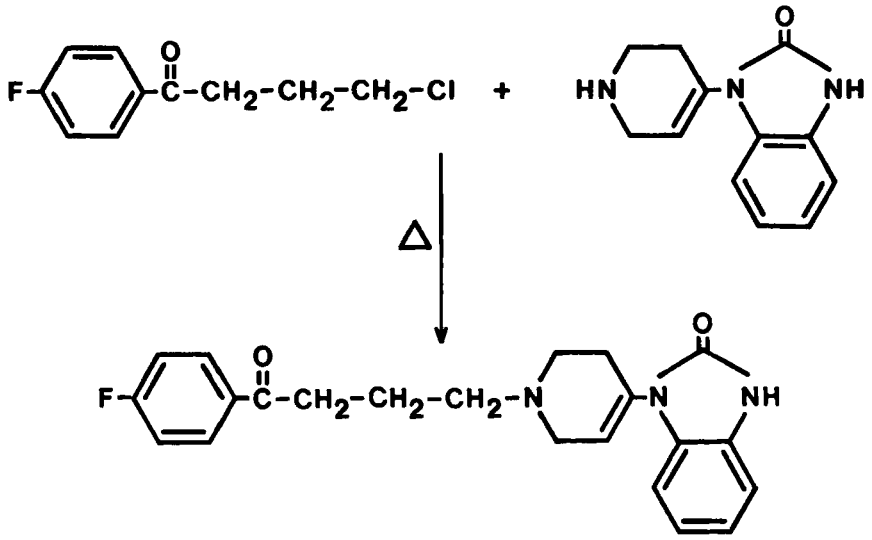
Solvent	Solubility (g/100 ml)
Acetic acid (0.1 M)	1.3
Acetone	1.5
Benzene	0.55
Chloroform	40
Dimethylformamide	17
Ether	0.24
Ethanol	0.34
Hydrochloric Acid (0.1 M)	0.15
Lactic Acid (0.1 M)	2.3
Methanol	0.41
2-Propanol	1.1
Propylene Glycol	1.2
Tartaric Acid (0.1 M)	2.4
Water	<0.001

2.8 pKa

The pK_a of droperidol is 7.64 determined titrimetrically (1).

3. Synthesis

Droperidol is synthesized by refluxing a mixture of 4-Chloro-1-(4-fluorophenyl)-1-butanone, sodium carbonate, 1-[1,2,3,6-tetrahydro-4-pyridinyl]-1,3-dihydro-2H-benzimidazol-2-one, and potassium iodide in a solvent of 4-methyl-2-pentanone. The droperidol is recrystallized from 1:1 ethyl acetate: diisopropyl ether or alcohol (5).



4. Stability - Degradation

Although droperidol is sensitive to heat and light (6), it is stable for up to 5 years when stored in the dark at room temperature (1). Droperidol is slightly hygroscopic (1).

When droperidol was refluxed overnight in 1M hydrochloric acid, it was found to undergo complete hydrolysis (2). The hydrolysis is given in Figure 6. The major products are 1-[4-(4-fluorophenyl)-4-oxobutyl]-4-piperidinone (I) and 1,3-dihydro-2H-benzinidazolin-2-one (II).

A solution of droperidol in monoglyme was refluxed overnight in 2M sodium hydroxide, and droperidol was recovered unchanged.

Droperidol solutions between pH 2.5 and 4.0 in Type I glass ampuls (tartaric acid of lactic acid) are very stable and have shown no loss of potency after a 5 year period at room temperature. These solutions were stable even after 7 months at 60°C

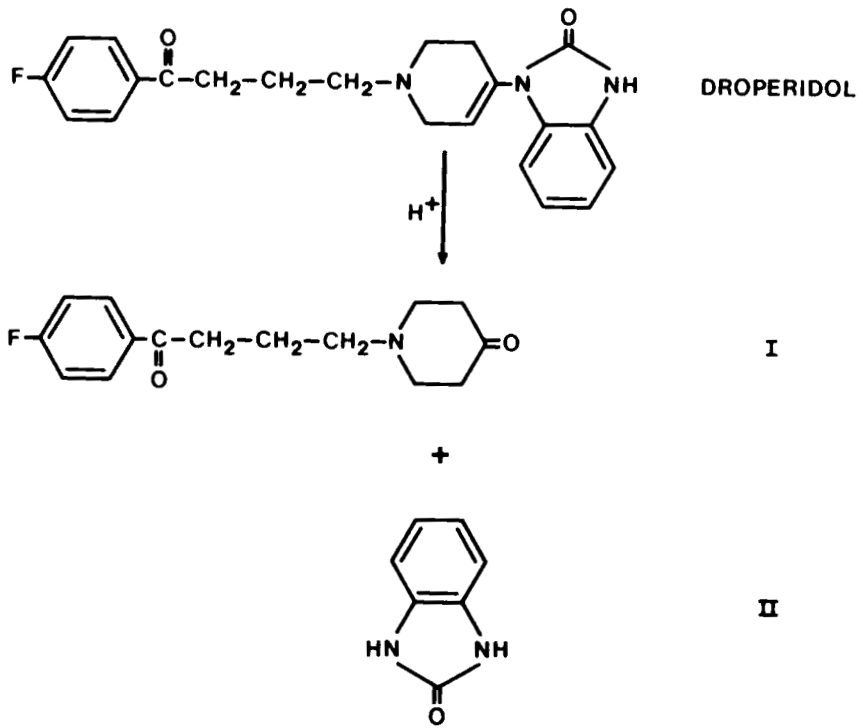


Figure 6. The Acid Hydrolysis of Droperidol.

and even when exposed to sunlight. Solutions of droperidol containing fentanyl citrate in a 50:1 ratio, with or without parabens, have been found to be stable for up to three years at room temperature when stored in Type I glass. In addition, solutions of droperidol containing fentanyl citrate in a 50:1 ratio have been found to be compatible for up to 3 months with 1 mg/ml atropine sulfate (6).

5. Drug Metabolic Products and Pharmacokinetics

The butyrophenones used clinically are neuroleptics in the sense of Delay and Deniker (7). Droperidol, an important member of the butyrophenones series of compounds, is characterized by its short onset of activity (8,9) and its rapid metabolism.* The metabolism of droperidol has been studied by a number of investigators in several species. These studies are catagorized below:

Data presented	Species	References
A	Rat, Dog & Man	7
D	Sheep, Goat & Rat	10
D	Mice, Dog & Cat	10
A,B,C	Rat	11
B,D	Dog & Rat	9
A,C	Rat	12
A,B	Man	13
A,B,C	Man	8

A = Metabolic
B = Pharmacokinetic
C = Distribution
D = Other (pharmacological, toxicological, etc.)

---

*Although it is rapidly metabolized, droperidol has a relatively long duration of activity.

The most commonly reported pathway for metabolism of droperidol is oxidative N-dealkylation giving rise to 4-fluoro-γ-oxobenzenebutanoic acid and 1-(1,2,3,6-tetrahydro-4-pyridinyl)-1,3-dihydro-2H-benzimidazol-2-one, followed by rapid metabolism of 4-fluoro-oxobenzenebutanoic acid to 4-fluorobenzeneacetic acid and its glycine conjugate, N-(4-fluorobenzoyl) glycine (7,8,11,12), the most important of the urinary metabolites (7,12). These proposed metabolic pathways are summarized in Figures 7 and 8.

6. Methods of Analysis

6.1 Elemental Analysis

Element	% Theory
Carbon	69.64
Hydrogen	5.84
Nitrogen	11.08
Oxygen	8.43
Fluorine	5.01

6.2 Nonaqueous Titrimetric Analysis

The nonaqueous titration procedure of analysis is the official method listed in the National Formulary XIV (14). An accurately weighed 300 mg sample of droperidol is dissolved in 30 to 40 ml of glacial acetic acid. After addition of α-naphtholbenzeine indicator, the solution is titrated with a previously standardized 0.1M $HClO_4$ solution to an end point.

6.3 Colorimetric Analysis

Haemers and Van Den Bossche (15) have described a general procedure for the quantitative determination of several butyrophenones, one of which was droperidol. Their procedure involves the reaction of butyrophenones with 3,5-dinitrobenzoic acid in an alkaline medium, resulting in the formation of a red complex. Using this procedure, the amount of droperidol in an injectable (2.5 mg/ml) form was determined.

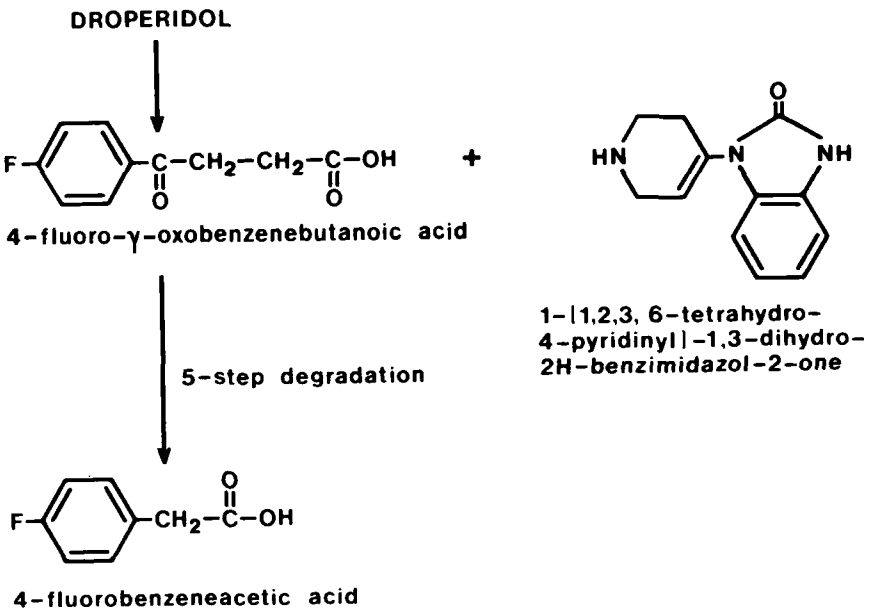


Figure 7. Pathway in the Metabolic Disposition of Droperidol.

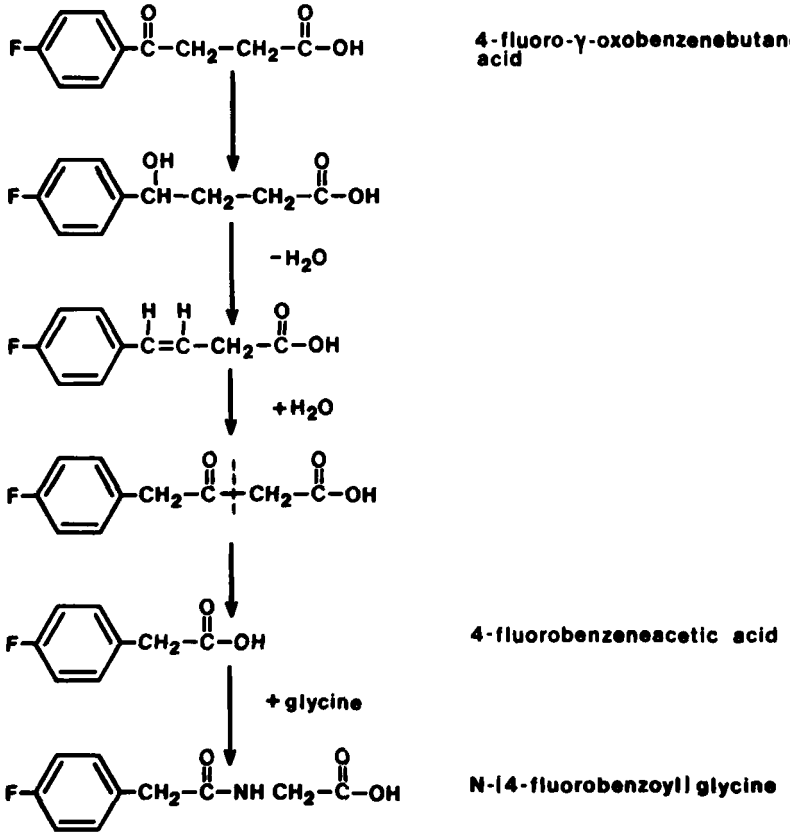


Figure 8. Pathway in the Metabolic Disposition of Droperidol.

The method was found to be useful for both aqueous and alcoholic solution.

6.4 Spectrophotometric Analysis

Spectrophotometric determination of droperidol by a non-specific procedure involving the extraction of the compound into 0.1M hydrochloric acid and reading it at 246 nm has been reported (16). The effect of the amount of ether and extraction time on the purified drug was reported.

A specific assay method for determining droperidol by itself or in the presence of fentanyl citrate (2) involves a double extraction procedure prior to recording the UV spectrum. The initial droperidol solution is diluted with water to contain 0.15 mg/ml droperidol. Fifteen ml of the diluted droperidol solution is added to 10 ml of a pH 6 phosphate buffer, 10 ml of a 1% sodium bisulfite solution, and extracted with 15 ml of chloroform. Five ml of the chloroform layer are re-extracted into 50 ml of 0.1M citric acid and the UV spectrum recorded from 230 to 300 nm.

6.5 Thin Layer Chromatographic Analysis

Solvent System	Absorbent	Detection Technique	R_f	Ref
A	Silica Gel[a]	3, 4	.75	17
B	Silica Gel[a]	3, 4	.44	17
C	Silica Gel[a]	3, 4	.45	17
D	Silica Gel[a]	3, 4	.80	17
E	Silica Gel 60F[b]	1, 2	.46	1
F	Silica Gel G[b]	3	.78	1
G	Silica Gel G[c]	2	.58	6
H	Silica Gel G[c]	1, 2	.25	2
I	Silica Gel Q4F[d]	4	.59	18

Solvent Systems

A Acetone (100)
B Benzene: acetone: petroleum ether: ammonium hydroxide (10:10:10:2)

C Acetone: petroleum ether (7:3)
D n-Butanol: isopropanol: acetic acid: water (3:3:2:4)
E Ethyl acetate: chloroform: methanol: 0.1M sodium acetate buffer, pH=4.7 (54:23:18:5)
F Chloroform: methanol: ammonium hydroxide (91:8:1)
G Methanol: 0.1M sodium acetate buffer, pH=4.7 (95:5)
H Chloroform: methanol (95:5)
I Ethyl acetate: methanol: ammonium hydroxide (85:10:3)

Detection Techniques

1 Ultraviolet light
2 Iodine staining
3 Dragendorff Reagent
4 Iodoplatinate Reagent

Adsorbents

a Unspecified
b Merck
c Analtech
d Quantum Industries

6.6 Gas Chromatographic Analysis

Droperidol has been reported to be unsuited for gas chromatographic analysis due to on-column decomposition (17).

7. Determination in Biological Fluids

Presently, there are no published chemical methods for determining droperidol in blood or urine. However, a radiochemical assay method has been published (13). Tritium-labeled droperidol is synthesized according to the method of Soudijn et al (19). Total radioactivity in urine or plasma samples containing tritium-labeled droperidol is determined by counting directly in a 20% ethanol-toluene scintillator or a 20% Beckman BBS-3-toluene scintillator, respectively. The intact droperidol from blood or urine is obtained by

extracting an alkaline solution of the urine or plasma with freshly seeded chloroform (25 µg of unlabeled droperidol per ml of chloroform). A portion of the chloroform layer is transferred to a scintillator vial, evaporated to dryness, and the radioactivity determined using a 20% ethanol-toluene scintillator. The remainder of the chloroform layer is evaporated to dryness, and the residue redissolved in a small volume of chloroform. The entire chloroform sample is spotted onto a Silica G plate and developed with 91:8:1 chloroform: methanol: ammonium hydroxide. The droperidol spot (R_f between 0.6-0.7) is scraped and placed into a scintillation vial. Twenty percent ethanol-toluene scintillator is added and the radioactivity determined. The counting efficiency is not affected by the variation in the amounts of silica gel present in the counting vials.

8. Determination in Pharmaceuticals

Chekova proposed a method for solutions containing parabens, which involved the dilution of the dosage form with 0.1M hydrochloric acid and removing the parabens with an ether extraction. The UV spectrum of the hydrochloric acid solution is recorded. Haemers and Van den Bossche reacted droperidol with 3,5-dinitrobenzene to form a colored complex, which is used for the quantitative determination of droperidol in dosage forms. Both these methods suffer from the fact that they are not specific for intact droperidol.

The specific ultraviolet assay method for droperidol, described in section 6.4, is the preferred assay. The assay is modified slightly when the dosage form contains parabens. After the droperidol is extracted into citric acid, the citric acid is washed with diethyl ether. This removes any UV interferences in the citric acid due to the parabens (6).

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Analytical Profiles of Drug Substances, 7

EPINEPHRINE

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CONTENTS

1. General Information

1.1 Nomenclature

1.11 Chemical Names

1-α-3,4-dihydroxyphenyl-β-methylaminoethanol; 1-1-(3,4-dihydroxyphenyl)-2-methylaminoethanol; 3,4-dihydroxy (1-hydroxy-2-methylaminoethyl)benzene; 1-methylaminoethanol-catechol; 3,4-dihydroxy-α-(methylaminomethyl)benzyl alcohol; (R)-(-)-3,4-Dihydroxy-α[(methylamino)methyl]benzyl alcohol; 4-[1-Hydroxy-2-(methylamino)ethyl]-1,2-benzendiol.

1.12 Generic Name

Epinephrine

1.13 Trade Names

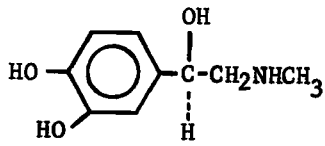
Many trade names (1) exist for this drug. Some of the more commonly used trade names in the United States are Adrenalin (Parke, Davis), Suprarenalin (Armour & Co.), and Suprarenin (Sterling Drug, Inc.).

1.2 Formula

1.21 Empirical

$C_9H_{13}NO_3$; $(OH)_2C_6H_3CHOHCH_2NHCH_3$

1.22 Structural and Stereochemical



1.3 Molecular Weight

1.31 Free Base

183.21

1.32 Bitartrate (1:1 salt)

333.30 ($C_9H_{13}NO_3 \cdot C_4H_6O_6$)

1.4 Elemental Composition (base)

C 59%, H 7.1%, N 7.7%, O 26.2%

1.5 Description

The USP XIX (2) describes epinephrine as "white to nearly white, odorless, microcrystalline powder or granules, gradually darkening on exposure to light and air. With acids, it forms salts that are readily soluble in water, and the base may be recovered by the addition of ammonia water or alkali carbonates. Its solutions are alkaline to litmus."

1.6 Forms of Epinephrine Recognized in Official Compendia.

The USP XIX (3) contains epinephrine as the free base, the bitartrate salt, and the following formulations:

Formulation	Category
Epinephrine	Bronchodilator
Epinephrine Injection	Adrenergic
Epinephrine Nasal Solution	Adrenergic
Sterile Epinephrine Oil Susp.	Bronchodilator
Epinephrine Bitartrate Ophthalmic Solution	Adrenergic
Epinephrine Bitartrate for Ophthalmic Solution	Adrenergic

2. Physical Properties

2.1 Associated with the Solid State

2.11 Crystallinity

Although epinephrine was the first hormone to be obtained in crystalline form, crystallographic information with regard to crystalline structure is not available. Winchell (4) describes epinephrine as colorless or light brown crystals or powder which darkens on exposure to air and quotes refractive indices as determined by Keenan (5) as being $N_1 = 1.555$, $N_2 = 1.733$

2.12 X-ray Diffraction

Powder X-ray diffraction data (6) for epinephrine are given in Table 1.

2.13 Melting Point

Kirk-Othmer (7) report the melting point of epinephrine as 211 - 212°C (215°C when heated rapidly).

TABLE I. Powder X-ray Diffraction Data

d					
4-0227	*5.13*	*7.84*	*4.23*	*7.84*	$C_9H_{13}NO_3$
I/I					
4-0232	*100_b*	*70*	*70*	*70*	*Adrenaline*

$CH_3\text{-}NH\text{-}CH_2\text{-}CH\ OH$

Rad., CuKa λ = 1,5418 Filter,Ni
Dia. 19 cm Cut off, 9.3 Coll.
I/I_1, Visual d corr. abs.? No
Ref., See below

Sys. S.G.
a_o b_o c_o A C
α β γ Z
Ref.

ξ α nωβ ξ γ Sign
2V D mp 216d. Color White
Ref.

Taken for the Institute of Physics at University College, Cardiff.
Average temperature - 17.4°C.
Mol. wt., 183.2

dÅ	I/I_1	hkl	dÅ	I/I_1	hkl
7.84	70		2.05	40	
5.13	100_b		2.00	5	
4.86	5		1.95	40	
4.58	50		1.91	10	
4.23	70		1.87	5	
3.91	40		1.82	10	
3.75	40		1.77	20	
3.60	5		1.70	10_b	
3.38	70		1.55	10	
3.27	20		1.49	5	
3.13	70		1.41	10	
2.99	10				
2.85	30				
2.69	10				
2.57	30				
2.51	30				
2.42	30_b				
*2.29	10_{bb}				
2.18	10				
2.12	20				

**Probably more than one line.*

2.2 Solubility

Solvent	Temperature	Solubility	Reference
Water	20°C	1 part in 3000	7
Ethanol	25°C	1 part in 2000	7
Ethyl Ether	--	insoluble	2
Chloroform	--	insoluble	2

The solubility of epinephrine in water is dependent on pH. It has minimal solubility at pH 9.4 and its solubility increases as pH deviates from this value due to formation of water soluble species bearing net positive or negative charges. (See section on ionization in aqueous solution.) Aqueous alkaline systems are seldom employed because of stability considerations whereas at pH's of pharmaceutical or therapeutic importance the intrinsic solubility of epinephrine in water is adequate so as to not cause complications.

2.3 Spectral

2.31 Ultraviolet

Epinephrine is reported (8,9) to have the following ultraviolet absorption characteristics in aqueous systems:

λ_{max}, n m (ε)

anion (net charge-1)		cation (net charge +1)		
295(4400)	243(7000)	Sh285(2500)	280(2750)	224(6000)

Isobestic points

λ, n m (ε)

281(2750)	267(1500)	232(4900)

2.32 Infrared - The infrared spectrum of epinepharine is given in Fig. 1.

2.33 Mass

The mass spectrum of epinephrine(10) as obtained on a mass spectrometer with an electron beam energy of 70 eV using the direct heated inlet system is shown in Figure 2. As discussed by the authors, phenylalkylamines such as epinephrine, noradrenalin, and isoprenaline which posess 2 phenolic hydroxyl groups give an observable

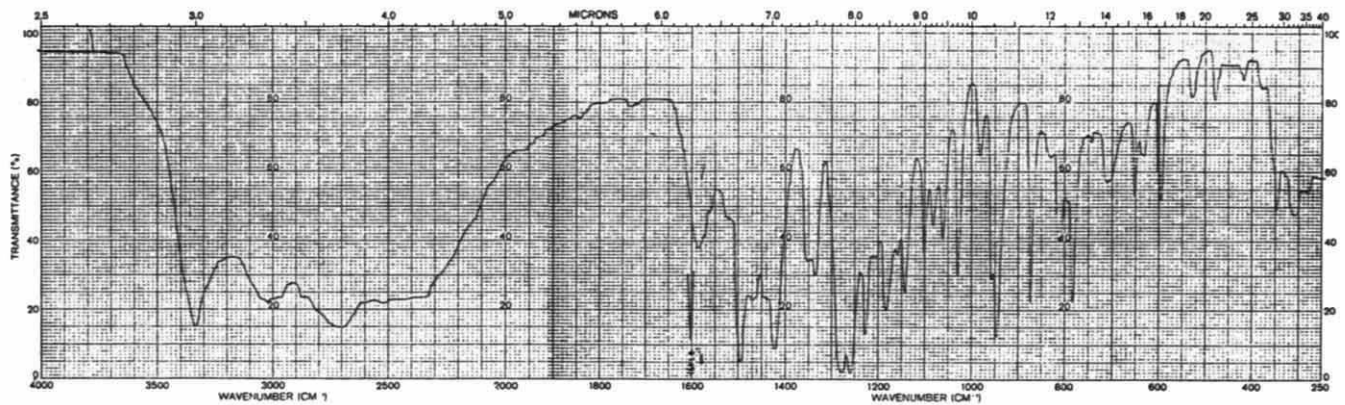


Figure 1 - Infrared Spectrum of Epinephrine, USP. Synthetic, KBr Pellet

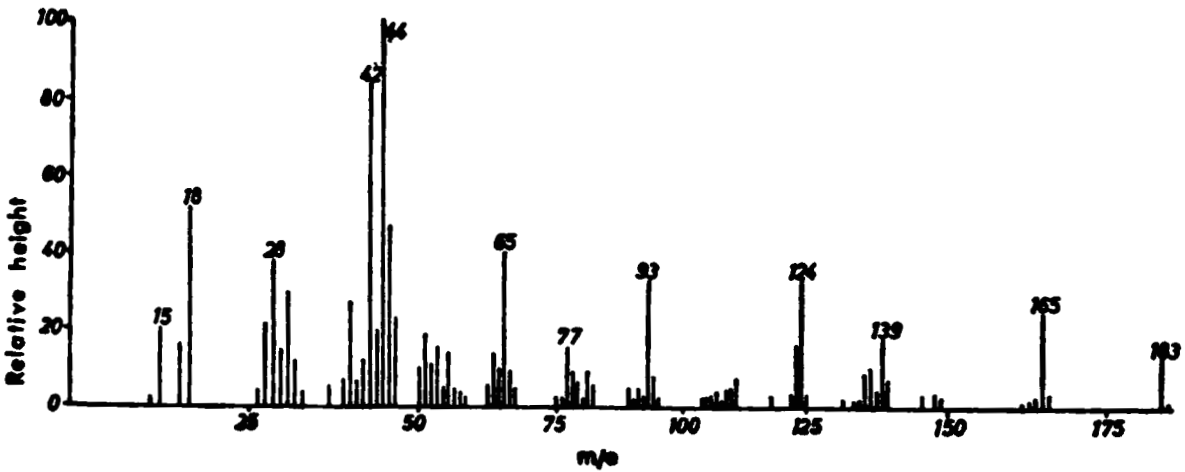


Figure 2 - Mass spectrum of epinephrine

molecular ion. The 100% peak (m/e = 44) arises from β cleavage with production of the $CH_2NHCH_3^+$ ion. For a thorough discussion on the topic of the mass spectra of phenylalkylamine derivatives, the original article should be consulted. Further information pertinent to the identification of microquantities of epinephrine can be obtained by mass spectroscopy of the 1-dimethylamino-5-naphthalenesulfonyl (Dansyl) derivative (11). Characteristics of the mass spectrum obtained for the tridansyl derivative of epinephrine are shown in Table II.

2.34 Optical Rotation and Dispersion

The USP XIX (2) uses optical rotation as a criterion of acceptability and specifies it as follows: "The optical rotation of epinephrine as determined at 25°C on a 2% solution in 0.5N HCl using sodium D light should not be less than -50° nor more than -53.5°."

The optical rotatory dispersion curve of epinephrine was determined by Lyle (12) and also by Manna and Ghislandi (13) who extended rotatory dispersion studies to a series of epinephrine derivatives. The dispersion spectrum is a plain curve over the region 700 to approximately 325 n m. Dispersion follows the Drude equation (14,15).

$$[\Phi] = A/(\lambda^2 - \lambda_0^2)$$

with the constant A having a value of -32.3 (absolute ethanol, 0.5N with respect to HCl as solvent and temperature of 22°C). The dispersion curve obtained under these conditions is shown as Figure 3.

These dispersion studies indicate that levorotatory epinephrine (that which is used medicinally and is contained in the USP XIX [2]) has the D configuration.

2.35 NMR

The nuclear magnetic resonance spectrum of epinephrine is shown in Figure 4 (16). The peak assignments as per Jardetsky follow:

Chemical shifts (in cps) of 0.1M epinephrine in D_2O with respect to benzene as external standard

ring	CH	CH_2	CH_3
-24.4	94.2	195.1	224.5

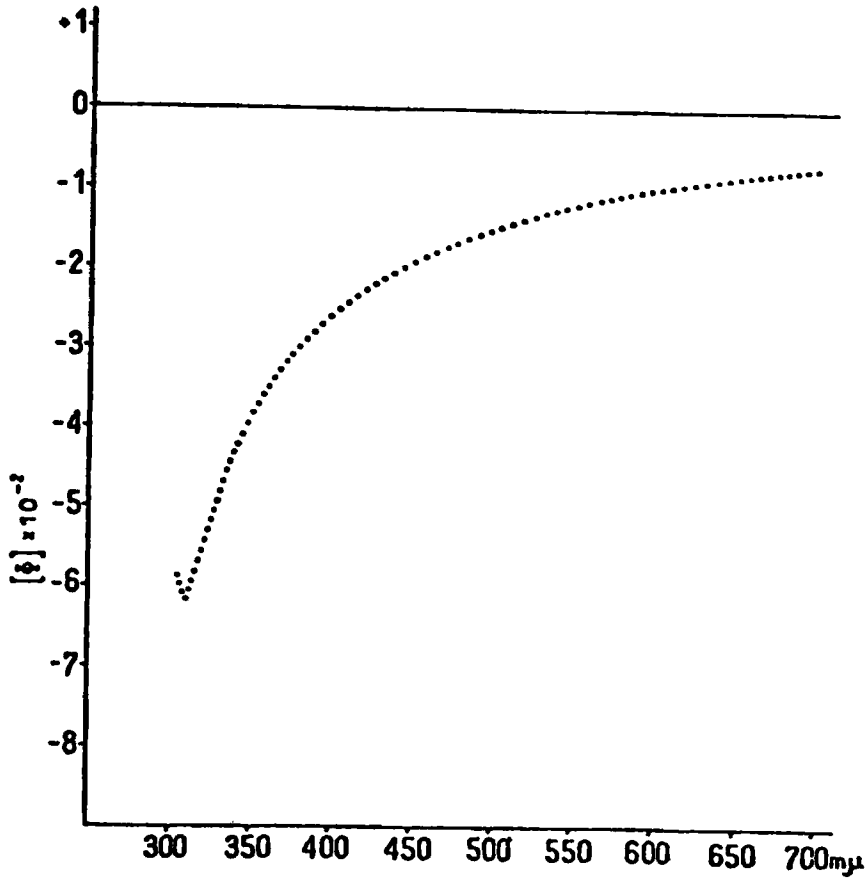


Figure 3 - Optical rotary dispersion curve of epinephrine

Table II

Molecular weight and relative abundance of peaks for the dansyl derivative of Epinephrine. The relative abundance of the peaks was established by comparison to m/e 170 (or 171) = 100%. Instrument CH5, Varian Mat, electron beam energy 70 eV.

Empirical Formula	No. of Danyl Groups	MW M^+	Relative Abundance of Peaks					Other Characteristic Peaks MW (% Relative Abundance)	Temperature of Inlet System °C
			M^+	234	250	263	277		
$C_{45}H_{46}N_4O_9S_2$	3	882	5	40	1	1	30	868(0.5); 864(0.5); 648(2); 630(0.5); 605(3); 441.5(0.3); 398(4); 384(2); 371(2); 350(2).	340

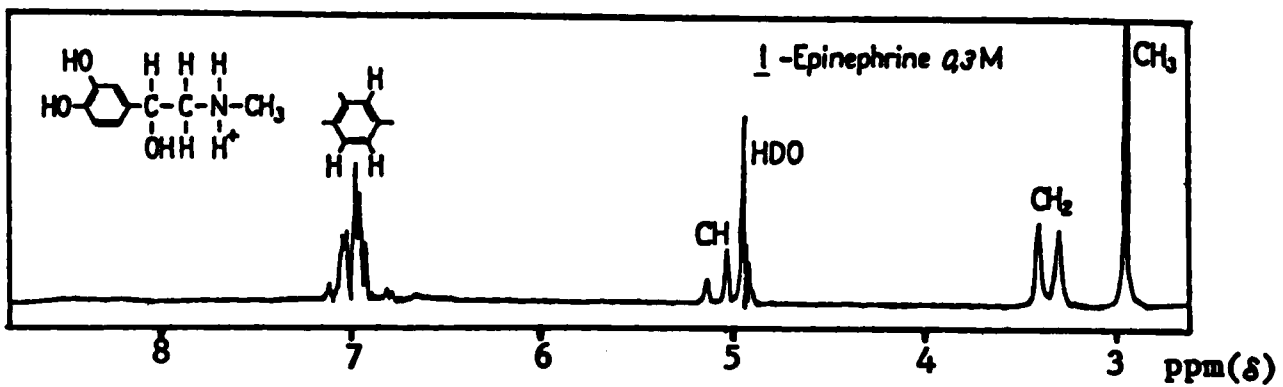


Figure 4 - NMR spectrum of 0.3M l-epinephrine in D_2O. Temperature 27° pH 5.6

The NMR spectrum of epinephrine and other biogenic phenylalkamines was studied by Reisch et al (17) and found to be of value for their characterization.

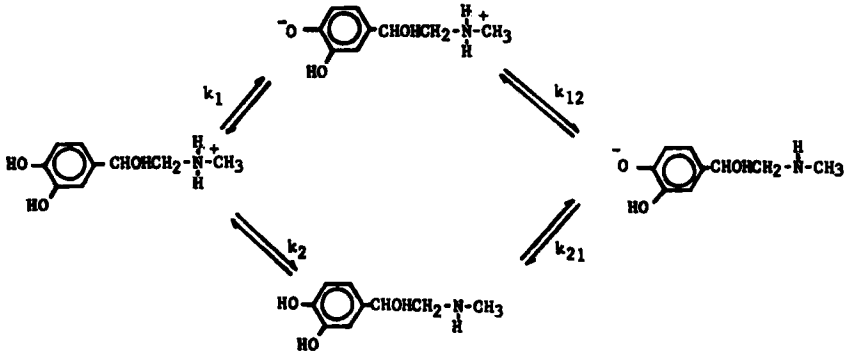
2.4 Distribution

Epinephrine is not easily extracted from aqueous solution. Changes in pH do not significantly benefit extraction since epinephrine free base, like other catecholamines, is quite hydrophillic (18). Polar water immiscible solvents (n-butanol, etc.) have been used to extrace epinephrine from an aqueous phase.

3. Ionization in Aqueous Solution

Ionization characteristics of epinephrine and related compounds have been the subject of numerous studies (19-26). Among these investigations that of Martin's (24) is most definitive since complications which arise from the presence of substituted ammonium and phenolic moieties having comparable acidities are considered.

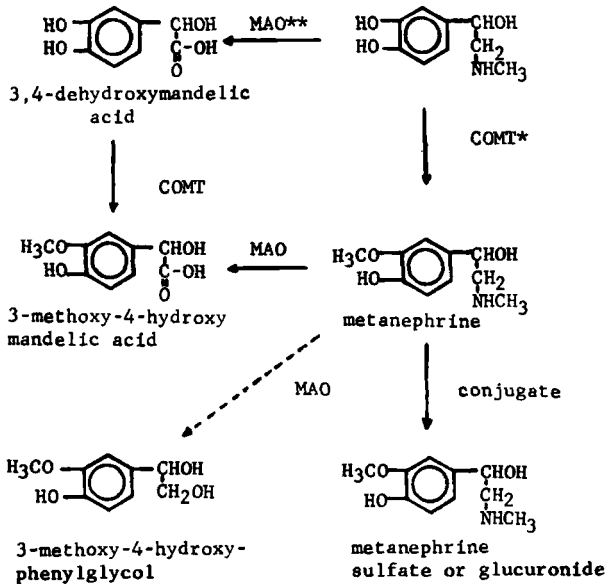
The first deprotonation reaction of fully protonated epinephrine involves proton loss from both ammonium and phenolic groups and generates a mixture of zwitterionic and neutral molecules. This reaction is associated with a macroscopic pK_a of 8.69 (25°C, μ = 0.1). The second deprotonation, associated with proton loss from the zwitterionic and neutral forms previously generated results in formation of a single specie bearing a net charge of -1 and is associated with a macroscopic pK_a of 9.90 (conditions as before). The ionization sequence for epinephrine on a molecular level with corresponding microconstants follows:



The ratio of tautomeric molecules having no net charge, i.e., zwitterion/nonzwitterion, is approximately 4 at 25°C. This then means that the first deprotonation primarily involves proton loss from one of the phenolic hydroxyls, whereas the second is mainly loss from the amino group present in the zwitterion. The distribution of various species present in an aqueous solution of epinephrine as a function of pH is given in Figure 5. The ionization constants used in calculating this distribution were taken from Martin's work (24) as tabulated in Table III. The isoelectric point of epinephrine is 9.4.

4. Biochemical Considerations

Epinephrine is not active orally due to destruction in the gastrointestinal tract and conjugation and oxidation which occur in the liver (27). Normally the drug is administered parenterally, although preparations suitable for inhalation are available to provide largely for a local effect in the lungs. Epinephrine is metabolized as in the following scheme (27).



* Catechol-O-methyltransferase
**Monoamine oxidase

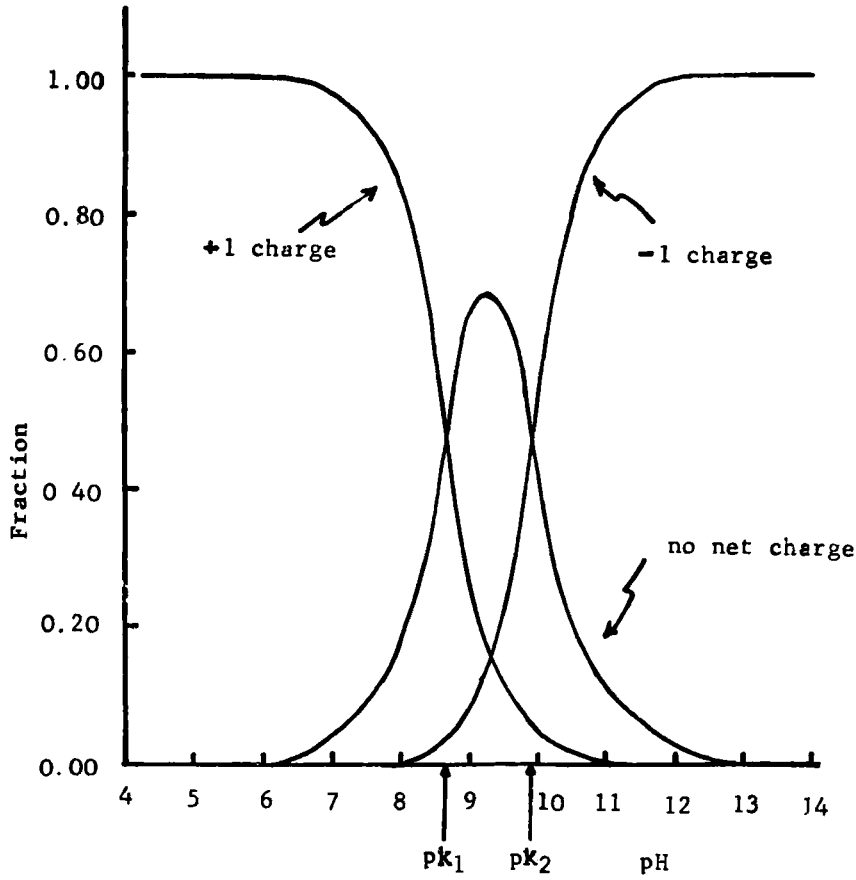


Figure 5 - Species distribution diagram for epinephrine

Table III

	pK_1	pK_2	pk_1	pk_2	$R = k_1/k_2$	$pk_{21} - pk_1$	T °C	μ
Epinephrine	8.79	10.10	8.88	9.51	4.3	0.50	25	~ 0
	8.66	9.95	8.72	9.57	7.1	0.32	25	0.10
	8.71	9.90	8.81	9.39	3.8	0.41	20	0.10

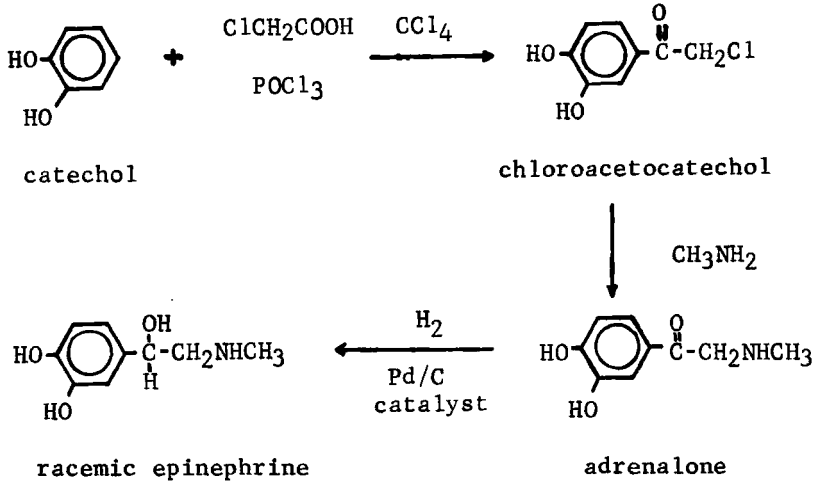
5. Synthesis

A crystalline compound was first isolated from suprarenal medulla extracts by Takamine (28) and Aldrich (29) which showed the same pharmacological action as extracts from this gland. This compound was later characterized as l-α-3,4-dihydroxyphenyl-β-methyl-aminoethanol primarily through the work of Pauly (30) and Jowett (31). Resolution of racemic epinephrine as first synthesized by Stolz and Dakin (32,33) was effected by Flacher (34) and it was found that the synthetic l-isomer was identical with that isolated from the natural source.

Many catechol sympathomimetic amines are synthesized on a commercial scale by essentially the same process. This process (35) can be divided into 4 stages, i.e.,

1. production of chloracetocatechol,
2. conversion of chloracetocatechol to adrenalone hydrchloride by reaction with methylamine,
3. purification of the adrenalone as produced in 2 and reduction of the salt to racemic epinephrine,
4. resolution of the resulting racemate to yield the desired levo isomer which is generally accomplished by crystallization from methanol as the tartrate.

A flow diagram for this process (35) follows:



6. Stability and Behavioral Chemistry

6.1 Solid State

Epinephrine is a relatively unstable compound and is susceptible to decomposition in the solid state. Special precautions must be observed during the synthesis of the free base, and material so obtained should be stored under regulated conditions, i.e., N_2 atmosphere, amber bottles, etc. (35). In regard to the stability of the solid base, moisture content appears to be a significant factor since thoroughly dried material is more stable than that which has a significant moisture content.

6.2 Aqueous Solutions

6.21 Racemization

Epinephrine racemizes at an appreciable rate even at near ambient temperature. Data obtained by Kisbye and Schroeter (36,37,38) indicate that racemization of epinephrine is acid catalyzed. This is an important consideration in dosage form design since an acidic environment is desirable in regard to prevention of discoloration. Estimates of the rate of racemization at near ambient temperatures are given in Table IV (38).

At pH 3.5 or less, racemization occurs by an SN type reaction. This acid catalyzed reaction is thought to occur by rapid protonation of the secondary alcoholic oxygen atom followed by rate determining elimination of this hydroxyl as water. Attack of a second molecule of water on the shielded incipient carbonium ion then results in production of the optical isomer. Since the same reaction occurs on the d-isomer, a racemic mixture results. The d-isomer of epinephrine has little pharmacological activity as compared to the l-isomer.

6.22 Reaction with Bisulfite

Closely allied with epinephrine's racemization reaction is its reaction with anions of sulfurous acid. Bisulfite or metabisulfite salts are frequently used as antioxidants in aqueous formulations of epinephrine where they function to retard color formation.

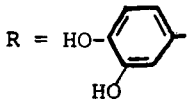
Initial studies (39,40) demonstrated that the potency of solutions of epinephrine could be lost without discoloration or true racemization being the cause. Further, it was shown that at pH 4.7 or higher, epinephrine was lost more rapidly in the presence of bisulfite than in its absence.

TABLE IV - Predicted Rates and Times of Maintenance of a Minimum 95 and 90 Per Cent Optical Activity

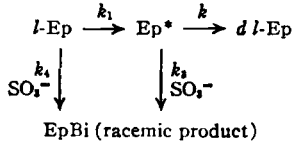
	Predicted Rate Constant, Min.$^{-1}$	*Time of Minimum 95% Optical Activity*[a]	*Time of Minimum 90% Optical Activity*[b]
pH 2.5, 25°	2.63×10^{-7}	1.9×10^{5} min. (ca. 4½ mo.)	4.0×10^{5} min. (ca. 9 mo.)
pH 2.5, 35°	9.34×10^{-7}	5.4×10^{4} min. (ca. 1 mo.)	1.1×10^{5} min. (ca. 2 mo.)
pH 3.0, 25°	6.92×10^{-8}	7.3×10^{5} min. (ca. 17 mo.)	1.5×10^{6} min. (ca. 35 mo.)
pH 3.0, 35°	2.45×10^{-7}	2.0×10^{5} min. (ca. 5 mo.)	4.3×10^{5} min. (ca. 10 mo.)
pH 3.5, 25°	1.91×10^{-8}	2.6×10^{6} min. (ca. 60 mo.)	5.5×10^{6} min. (ca. 120 mo.)
pH 3.5, 35°	6.77×10^{-8}	7.5×10^{5} min. (ca. 17 mo.)	1.6×10^{6} min. (ca. 36 mo.)

Subsequent investigation (41) demonstrated that anions of sulfurous acid actually react with epinephrine to produce 1-(3,4-dihydroxyphenyl)-2-methylaminoethane sulfonic acid according to the following equation:

$$R\text{-}CHOHCH_2NH_2CH_3^{\oplus} + SO_3^{\ominus} = R\text{—}\underset{\displaystyle SO_3^{\ominus}}{\underset{|}{CH}}\text{—}CH_2\overset{\displaystyle \oplus}{N}H_2CH_3 + OH^{\ominus}$$



The kinetic behavior of epinephrine's reaction with bisulfite is explicable on the basis of the following reaction scheme (41).



Rate measurements were consistent with and indicative of the following expression which describes the rate of loss of epinephrine from bisulfite containing aqueous solution. (41)

$$\frac{-d(Ep)}{dt} = \frac{k_3(SO_3^-)\, k_1(Ep)}{k_2 + k_3(SO_3^-)} + k_4(Ep)(SO_3^-)$$

The preceding studies showed that since sulfite is so much more reactive than water, epinephrine does not racemize when bisulfite is present. In the presence of bisulfite, optical and physiological activity is lost through formation of the sulfonate.

6.23 Oxidation

Since epinephrine is an o-diphenol containing a hydroxyl group in the α position, it is a strong reducing agent (42). As such, it is easily oxidized by such oxidizing agents as molecular oxygen, iodine, potassium ferricyanide, potassium persulfate, and manganese dioxide. Oxidation of epinephrine is thought to occur through the transient formation of epinephrine quinone with formation, under proper conditions, of adrenochrone (43,44,45,46). Oxidation of epinephrine by molecular oxygen can also result in formation of a brownish insoluble material of indefinite structure. An investigation of the oxidation of epinephrine by molecular oxygen (47) indicated that the reaction involved is extremely complex. Data obtained suggests that oxidation

of epinephrine in aqueous solution can occur in the absence of heavy metal ions and likely involves free radical sequences. Oxidative discoloration of epinephrine solutions occurs more rapidly as pH is increased.

6.24 Miscellaneous Reactions

In addition to the preceding reactions which are relevant to dosage form design, manufacture, and storage, epinephrine can undergo a variety of other reactions which have relevance to other areas. Reaction products of these reactions and conditions are summarized in table 5.

6.25 Dosage Form Stability

Available information indicates that oxidation of epinephrine in aqueous dosage forms is an extremely important consideration in regard to dosage form design, manufacture, and storage. This is the situation because oxidation produces both inactivation and discoloration. Discolored solutions of epinephrine are considered to be unusable (2) regardless of potency.

Published reports (51,52,53) indicate that the kind of container (ampoule vs. multiple dose vial) significantly influenced shelf life. Epinephrine solutions formulated with metabisulfite contained in ampoules did not show significant formation of 1-(3,4-dihydroxyphenyl)-2-methylaminoethane sulfonic acid even after 7 years storage at 15°C. Racemization of the epinephrine contained in these ampoules was likewise insignificant. Results of stability studies of epinephrine ampoules using various analytical probes are given in Table VI (52).

7. Analytical Methods

7.1 Fluorometric

Measurement of native or induced fluorescence provides a useful means of assaying epinephrine. While the scope of this analytical approach is limited, in that stereoselectivity is not attainable, sensitivity and a fair degree of analytical specificity are provided.

Naturally occurring catecholamines such as epinephrine, norepinephrine, dopa, and dopamine possess native fluorescence (54,55). All of these compounds are maximally excited at 285 nm and fluoresce at 325 nm. Since several catecholamines which occur in living tissue possess essentially the same fluorescent characteristics, native fluorescence is not

Table V

Products Formed from Epinephrine

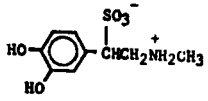
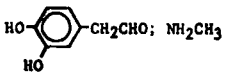
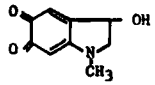
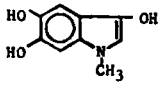
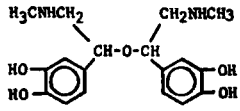
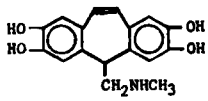
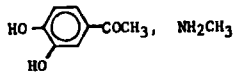
Reaction Product	Reaction Conditions	Reference
 1-(3,4-dihydroxyphenyl)-2-methylaminoethane sulfonic acid	Mild conditions of pH and temperature. Presence of HSO_3^- or $SO_3^=$.	41
 3,4-dihydroxyphenyl acetaldahyde; methyl amine	Strong acid and high temperature.	48
 Adrenochrome	Mild conditions, oxidation (I_2, O_2 etc)	43-46
 Adrenolutine	Mild temperature conditions, oxidation in basic environment	43-46
d-isomer of Epinephrine	Variable pH, reaction occurs more rapidly at low pH, relatively mild conditions	36,37,39
 Diadrenaline ether	Elevated temperature, strong acid	49
 Adnamine	Elevated temperature, strong acid	49
 3,4-dihydroxyacetophenone, methyl amine	Elevated temperature, strong base	50
Complex ions containing epinephrine and boron	Mild conditions. Presence of anions of boric acid	106,107

TABLE VI

The Stability of Adrenaline Injections[a]
(pH adjusted to 3·0 by addition of hydrochloric acid)

Heat treatment	Storage period (months)	pH	Color	Adrenaline recovery in per cent of original content: Colorimetric method	Fluorimetric method	Rat b. p. method	Rat uterus method
Unheated	0	3.0	colorless	100	-	-	-
	6	3.0	light red	99	102	98 (96-104)[c]	102 (86-116)
	20	3.0	light red	99	100	94 (95-106)	90 (92-109)
100° 20 min.	0	3.0	colorless	101	106	99 (98-103)	99 (87-115)
	6	3.0	light brown	99	96	99 (97-103)	98 (89-112)
	20	3.0	light brown	98	97	97 (94-106)	104 (92-109)
120° 20 min.	0	3.0	light red	99	100	92 (95-105)	94 (94-106)
	6	3.0	brown, dark ppt.	99	95	93 (97-104)	88 (90-111)
	20	3.0	brown, dark ppt.	96	95	92 (91-110)	83 (88-113)

The Stability of Adrenaline Injections[b]
(pH adjusted to 3.6 by adding sodium metabisulphite)

Heat treatment	Storage period (months)	pH	Color	Adrenaline recovery in per cent of original content: Colorimetric method	Fluorimetric method	Rat b. p. method	Rat uterus method
Unheated	0	3.6	colorless	99	102	-	-
	6	3.4	-	100	97	100 (95-105)[c]	101 (92-109)
	20	3.4	-	100	100	100 (93-108)	87 (89-113)
100° 20 min.	0	3.4	colorless	99	101	96 (94-106)	97 (93-108)
	6	3.3	-	100	96	100 (97-103)	96 (93-108)
120 20 min.	0	3.4	colorless	98	94	97 (93-108)	100 (84-120)
	6	3.3	-	99	95	98 (94-106)	98 (89-113)
	20	3.3	-	99	98	97 (95-106)	94 (83-121)

[a] *Adrenaline bitartrate 1.82 gm, sodium chloride 8.0 gm, hydrochloric acid q.s., pH 3.0, Water for injection 1000 ml.*

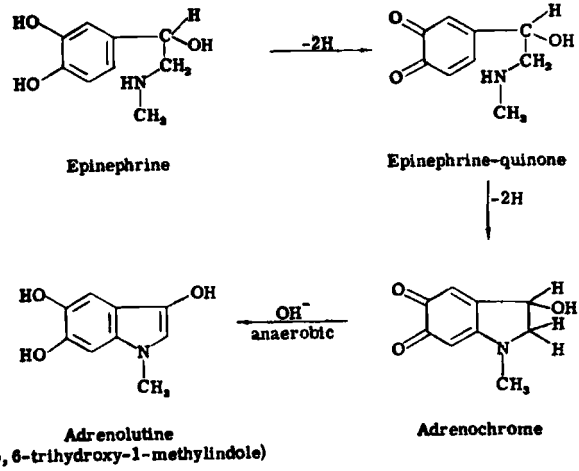
[b] *As above except hydrochloric acid omitted and 0.5 gm sodium metabisulfite added. pH 3.6.*

[c] *Fiducial limits, in parenthesis, are expressed as percentages (P = 0.05).*

commonly employed in the assay of a particular hormone or compound in body fluids or tissue. Application of this phenomenon, however, has been made to dosage form analysis (56).

Primarily because epinephrine's native fluorescence occurs in the ultraviolet range, need existed for an analytical approach which would produce fluorescence in the visible region. Two general schemes evolved to provide this. The first of these involves production of a highly fluorescing hydroxyindole by oxidative cyclization of epinephrine under basic conditions. The second involves production of a fluorescent quinoxaline.

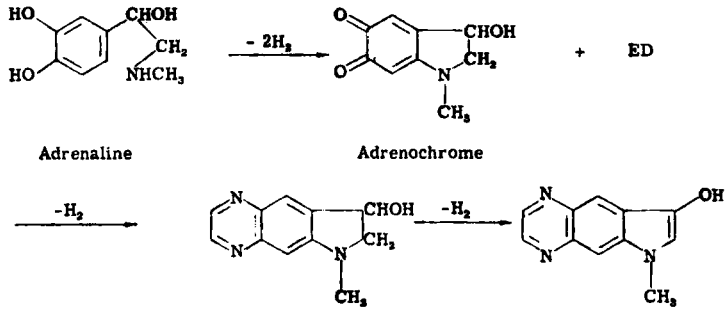
As was observed by Loew (57), epinephrine produces a highly fluorescent material when oxidized in a basic aqueous environment. Initial investigations (58-61) indicated that assay of epinephrine on this basis was feasible. The nature of the reaction involved in producing the fluorescing material was studied, and it was determined that the fluorescing material produced is a hydroxyindole formed as in the following scheme (43-46).



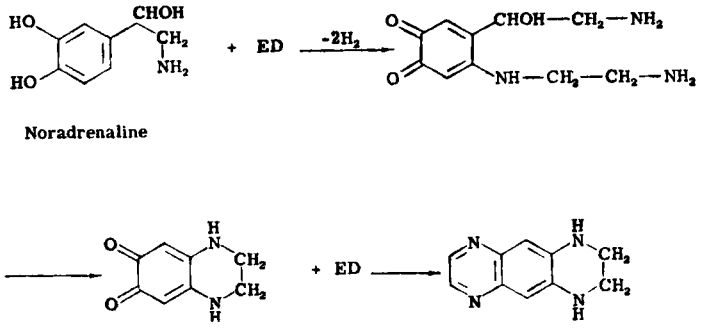
The chemistry of adrenochrome has been reviewed (62). The fluorometric analysis of epinephrine, based on production of the trihydroxyindole derivative, has undergone extensive modification and is still being modified (63) to provide for a more reliable or selective procedure. The method has been used extensively for analysis of catecholamines in body

fluids (54) as well as for analysis of epinephrine in dosage forms (63). For a thorough review of this procedure in regard to analysis of epinephrine in body fluids or tissues, a basic text should be consulted (54).

The second approach used in fluorimetric assay of epinephrine and other catechol amines involves condensation with ethylenediamine. While this has been applied quite extensively to analysis of epinephrine and norepinephrine in biological systems, no examples of applications to dosage form analysis have appeared. Investigations reported thus far (64-69) suggest that epinephrine reacts with ethylenediamine in the following manner.



A different product is produced from norepinephrine's reaction, i.e.,



The quinoxaline produced from norepinephrine has different fluorescent characteristics than the hydroxyindole produced by epinephrine permitting simultaneous determination of both compounds in biological samples. Details of this procedure can be found in a basic text (70).

7.2 Colorimetric

Colorimetric procedures which have been applied to analysis of epinephrine are based on the presence of the catechol or phenolic groups and hence have a variable degree of selectivity. Some of the more recently developed colorimetric procedures are reasonably selective; however, since none are stereoselective, their application is limited to those situations where enantiomorphic composition is determined independently or the much less potent d-isomer is known to be absent. Other common sources of analytical interference include biogenic catecholamines (norepinephrine) and the sulfonated product which results from reaction of bisulfite and epinephrine.

Barker et al (71) in a critical survey of colorimetric procedures for epinephrine indicated that a colored material could be produced from this hormone by oxidation with potassium persulfate. The procedure was confirmed by Rees (72) and shown to give results which were in accord with those obtained pharmacologically. Details of this procedure are given in Garratt's text on the quantitative analysis of drugs (73). Other oxidizing agents such as iodine (74) have been used to generate a colored product and provide differentiation between epinephrine and norepinephrine in biological samples. One complication which arises in analytical colorimetric schemes involving oxidation is that bisulfite is known to interfere. Doty's (75) method which employs ferrous ion to generate color copes with this problem in the sense that interference from anions arising from sulfurous acid is no longer apparent; however, epinephrine sulfonate may interfere. A more recent approach to colorimetric determination of epinephrine (76) involves reaction of thiosemicarbazide with epinephrine under basic conditions. Under the conditions of this procedure, apparently only catechols yield a colored product.

Other color producing reactions which involve epinephrine are known. Some of these are useful for identification of the hormone (50), while others serve as means of detection in paper and thin layer chromatography. (42,83)

7.3 Polarimetric

Methods which involve measurement of optical rotation have been proposed and are used to assay epinephrine in dosage forms. Several factors restrict the direct application of polarimetric methods and require that they be used in conjunction with other procedures. A primary consideration in this regard is that loss of optical acticity can be associated with either complete or only partial loss of pharmacological activity. Unless an ancillary method is used which assays total (d + l) epinephrine content, correlation of observed rotation and l-epinephrine content is not possible.

This problem was recognized by Rosenblum, Goldman, and Feldman (77) as well as Hellberg (78) who used concurrent polarimetric and colorimetric measurements to determine epinephrine content. Unfortunately the colorimetric procedures used are not selective and interference from other chromogenic compounds associated with epinephrine is possible. Nevertheless their approach is valid and if a more selective procedure (e.g., Prasad's fluorometric method [63]) is used to estimate total (d + l) epinephrine, accurate results may be expected.

Another analytical approach based on independent determinations of total epinephrine and enantiomorphic distribution is that proposed by Welsh (79) and modified by Higuchi (80). This scheme involves acetylating the mixture to be analyzed, isolating the acetyl derivative produced, and determining total epinephrine by gravimetric or spectrophotometric means. Enantiomorphic composition is then determined by polarimetric measurement of the isolated acetyl derivative. This is the procedure which has received official recognition by the USP.

7.4 Titrimetric

Epinephrine can be titrated using a nonaqueous system. Usually the titration is carried out in glacial acetic acid using perchloric acid as titrant and crystal violet as indicator. The purity of bulk epinephrine and epinephrine tartrate U.S.P. is established in this manner (2). Photometric microtitration procedures have been developed and applied (81).

7.5 Chromatography

7.51 Thin-layer and Paper

Thin layer and paper chromatography have been used extensively for analysis of epinephrine and other adrenergic compounds contained in body fluids or pharmaceutical formulations. This subject is reviewed in several books

devoted to the subject of paper and thin layer chromatography (82,83). Summaries of chromatographic behavior and detection reagents are given in Tables VII.

As discussed by Macek (83), multiple spot formation is a considerable problem in both paper and thin layer chromatographic analysis of epinephrine. Additional spots may be as the result of formation and differential migration of free base and various complex salts formed during isolation or the chromatographic process or, as indicated by Roberts and Broadley (49), may be products of the reaction of epinephrine in the presence of hydrochloric acid.

7.52 Gas Chromatography

Since epinephrine is a polar, nonvolatile compound, derivitization is a requirement for analysis by gas chromatography. Information relevant to derivatization and the gas chromatographic behavior of the derivatives has been summarized in several review articles and one text (89, 90, 91).

Silylation has been found to be a useful means of converting epinephrine to a derivative with suitable gas chromatographic characteristics. Sen and McGeer (92) found that such a derivative could be produced by treating epinephrine with hexamethyldisilazane (HMDS). This silylated product was characterized by Horning (93) as the N-trimethylsilyl derivative of epinephrine. Horning (94) further demonstrated that preferential O-silylation could be accomplished using trimethylsilylimidazole (TMSI) in acetonitrile. Under these conditions, N-silylation does not occur. The chromatogram shown as Fig. 6 was obtained after derivatization in this manner (95).

When desired the basic secondary amino moiety can be blocked by N-acylation of the O-trimethylsilyl derivative using N-acetyl or N-heptafluorobutyrylimidazole reagents (96).

Table VII

PC and TLC R_F Values for Epinephrine (82)

Systems:
PC - S1 n-Butanol-acetic acid-water 4:1:5 (84)
S2 Isobutanol-formic acid-water 100:12:10 (85)
S3 Paper impregnated with citrate buffer pH 4; n-butanol saturated with water (86)
TLC - S4 Silica Gel G (pH 4 buffer)/n-butanol saturated with water (treated with SO_2) (87)
S5 as above except pH 4 buffer + .01 M borax/ n-butanol saturated with water (87)
S6 Cellulose/phenol-0.1N HCl (85:15) (88)

	PC			TLC		
	S1	S2	S3	S4	S5	S6
Epinephrine	38	09	15	27	15	62
Adrenalone	44	--	--	38	07	--

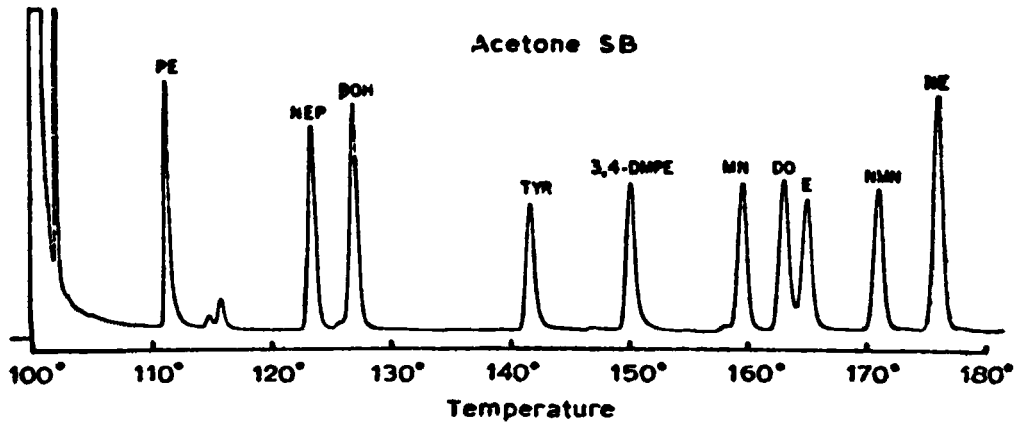


Fig. 6 Chromatogram of a mixture of catecholamines after their trimethylsilylation and conversion into enamines[91]. PE = β-Phenylethylamine; NEP = norephedrine; βOH = β-hydroxy-β-phenylethylamine; TYR = tyramine; 3,4-DMPE = β-(3,4-dimethoxyphenyl)ethylamine; MN = metanephrine; DO = dopamine; E = epinephrine; NMN = normetanephrine; NE = norepinephrine. Conditions: 10% F-60, temperature programming at 1.5°/min.

Various chromatographic conditions have been employed for the chromatography of epinephrine and related compounds. Columns packed with such liquid phases as siloxane polymer SE-30 or methysiloxane polymer F-60 are useful. Column temperatures in the region 170-220°C appear to be appropriate.

7.53 Liquid Column Chromatography

Liquid column chromatography has been applied to the analysis of epinephrine and related compounds. Since the underivatized hormone does not easily partition into water immiscible liquids, liquid column chromatography as associated with epinephrine is usually conducted using ion exchange resins or adsorbents.

Starch columns were employed to separate epinephrine, norepinephrine, and related catechol amines (97, 98), however, lengthy elution times were involved. Good separation of epinephrine, norepinephrine, and hydroxytyramine was achieved more quickly by using columns packed with Amberlite IRC-50 in the ammonium form and 0.4 molar ammonium acetate buffer, pH 5.0 as eluent (99). Masuoka and others used chromatography on alumina to accomplish group separations of noncatechols, catechol amines, and catechol acids as contained in biological samples (100,101). Mattock and Wilson (102-104) made use of complex formation which occurs between epinephrine and borate ions to accomplish group separation of methylated catechol amine metabolites, i.e., metanephrine etc., from the catechol amines epinephrine and norepinephrine. Group separation of epinephrine and norepinephrine from DOPA was found by McDonald and Murphy (105) to be easily accomplished using columns containing Sephadex G-10. Liquid column ion exchange chromatography was applied to analysis of epinephrine containing pharmaceuticals by Smith (106) who used a sulfonated polystyrene resin to adsorb the hormone and aqueous HCl for elution.

Higuchi (107) used partition chromatography to analyze epinephrine as part of work done on the reaction of epinephrine and bisulfite. In this case the hormone was acetylated to render it more lipophillic. The procedure separated intact d, l epinephrine from degradation products.

Fu and Sibley (108) developed a high efficiency liquid chromatographic method for epinephrine in pharmaceutical formulations, using a cationic exchange resin column and an aqueous phosphate buffer as the mobile phase. The method allows separation of epinephrine from its oxidative and other

degradation products and hence is stability-indicating. Recently, Holcomb (112) developed a method for the analysis of Epinephrine Injection using paired-ion chromatography on C_{18} Bondapak® column. The mobile phase used was methanol:water (2:3) containing EDTA, acetic acid, and dioctyl sodium succinate. Although high efficiency liquid chromatography has become widely used, very little data for epinephrine are found in the literature. Perhaps as the technique of paired-ion chromatography (109) becomes better known, applications will appear.

7.6 Spectrophotometric

Analysis of epinephrine through direct spectrophotometric measurement is subject to many of the same considerations as is colorimetric analysis of the hormone. Epinephrine absorbs ultraviolet radiation due to the presence of the catechol chromophore. Other related biogenic catechol amines (norepinephrine) and inactive compounds which are associated with epinephrine (l-(3,4-dihydroxyphenyl)-2-methylaminoethane sulfonic acid, d-epinephrine, etc.) likewide contain this chromophore and hence have similar spectra. Further complications, arising from spectral interference due to the presence of colored material, are possible. These factors limit the application of direct ultraviolet measurement in the analysis of epinephrine to those cases in which supplemental information, regarding absence of interfering materials, is available.

In lN mineral acid (HCl or H_2SO_4), epinephrine has an absorption maximum near 280 nm. The British Pharmacopia (110) specifies that the ultraviolet spectrum of a given sample show a maximum at only this wavelength. Adrenalone, a possible process-related impurity, is detected and monitored by UV measurement at 310 nm (49,110). At this wavelength epinephrine does not have significant absorption but adrenelone absorbs maximally (111).

8. Acknowledgments

The authors wish to express special thanks to Mrs. Pohl for her secretarial assistance in preparing this manuscript.

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Analytical Profiles of Drug Substances, 7

ETHAMBUTOL HYDROCHLORIDE

Ching-San Lee and Leslie Z. Benet

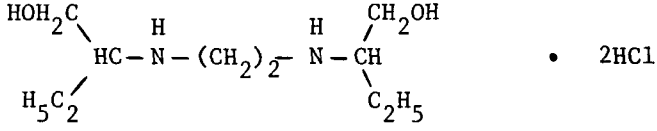
ISBN 0-12-260807-0

CONTENTS

1. Description

1.1 Name, Formula, Molecular Weight

While screening selected compounds, Thomas and coworkers[1] found that N, N'-di-isopropylethylenediamine was effective in the treatment of experimental tuberculous infections in mice. A number of congeners of this compound were examined; the one that eventually proved to be the most tuberculostatic was d-2, 2'-(ethylenediimino)-di-1-butanol. The summary formula for the dihydrochloride salt of ethambutol is $C_{10}H_{24}O_2N_2(2HCl)$, with a corresponding molecular weight of 277.5. The commonly used trade name is Myambutol (Lederle).



$C_{10}H_{24}O_2N_2(2HCl)$ M.W. = 277.5

1.2 Appearance, Color, Odor

Ethambutol is a white, scentless, bitter tasting, thermostable crystalline powder.

2. Physical Properties

2.1 Infrared Spectrum

According to Wilkinson et al.[2], the principal infrared bands, identical for the (+) and (-) bases, are: 3310, 3180, 2990, 2900, 1475, 1385, 1374, 1360, 1228, 1148, 1095, 1068, 1055, 1015, 990, 885, 846, 839, 816, and 768 cm^{-1}.

An IR spectrum of ethambutol hydrochloride was also obtained on a KBr disc and is presented in Fig. 1. The IR spectra for both free base and hydrochloride salt indicate the absence of free -OH in the ethambutol molecular hydrogen bonding. Assignment of some of the absorption bands is given in Table I.

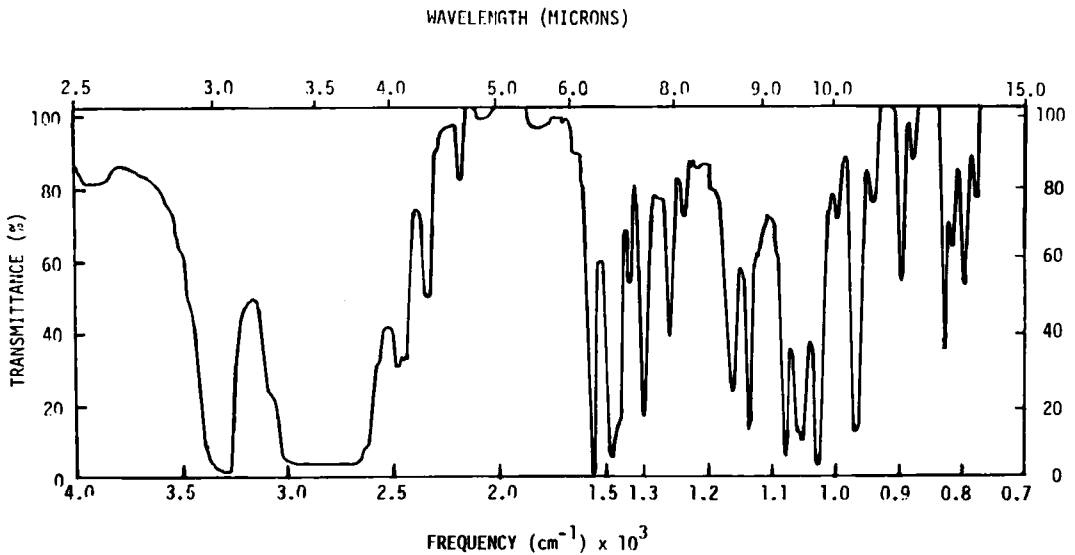


Fig. 1 Infrared spectrum of ethambutol hydrochloride in KBr pellet. Instrument: Perkin-Elmer Model 337.

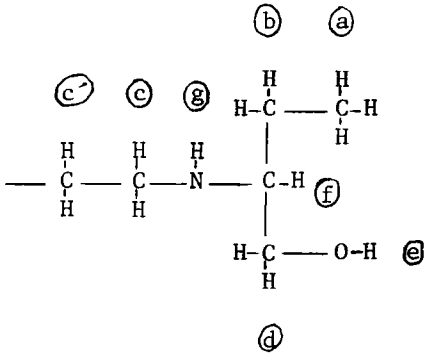
TABLE I

IR Spectral Assignments for Ethambutol HCl

Wavelength of Absorption (cm^{-1})	Vibration Mode
3450-3200	intermolecular H-bonded, polymeric association
3100-2550	combination of intramolecular H-bonded, -OH in chelate form and $-CH_2$, $-CH_3$ stretching
1500-780	the fingerprint region

2.2 Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance (NMR) spectrum (Fig.2) was obtained by preparing a 2 mg/ml solution of ethambutol free base in deutero chloroform containing tetramethylsilane as the internal reference. A Varian XL-100 NMR Spectrometer was used. The NMR proton spectral assignments are given in Table II.



2.3 Mass Spectrum

Chemical ionization mass spectra of ethambutol were obtained using a quadrupole mass spectrometer. Source pressures were maintained at 15 torr (isobutane). The source temperature was 150-170°. Samples (1 $\pm$ .5 mcg) were placed into a glass capillary by a direct-insertion probe, which was then gradually heated above 200° to effect volatilization. The major peak at m/e 205 in Fig. 3 corresponds to the M+1 molecular ion of the ethambutol free base.

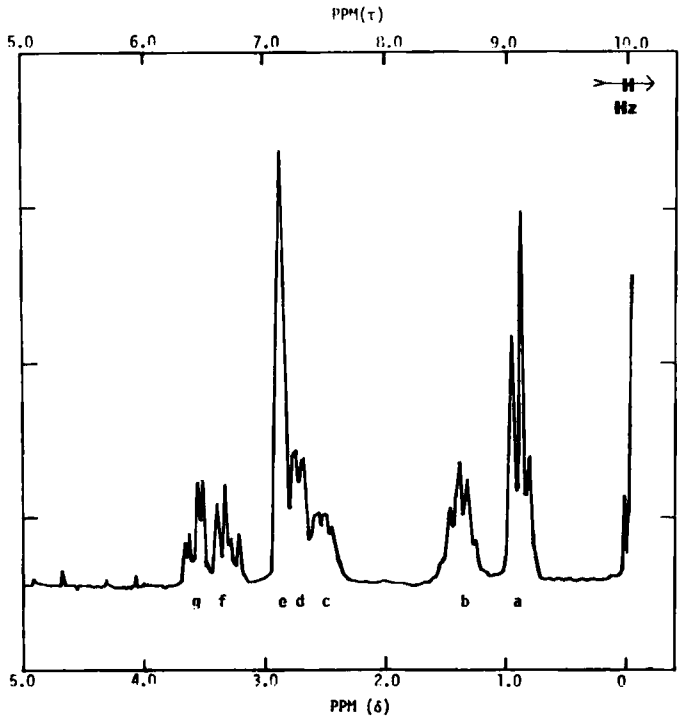


Fig. 2 NMR spectrum of ethambutol in deuterated chloroform. Instrument: Varian XL-100.

TABLE II

NMR Spectral Assignments for Ethambutol

Chemical shift (ppm)	Protons	Splittings
.83 - .98	a	triplet
1.29 - 1.56	b	quintet
2.48 - 2.60	c	sextet
2.70 - 2.78	d	quartet
2.91	e	singlet
3.24 - 3.44	f	- -
3.56 - 3.70	g	- -

2.4 Ultraviolet Spectrum

The ethanolic solution of ethambutol shows only end absorption in the UV region starting at 220 nm. No absorption maximum is observable in the visable light field. The UV spectrum was scanned using a Cary 15 Spectrophotometer.

2.5 Optical Rotation

The specific rotations of the optically active isomers in water[2] are (+)-base $[\alpha]_D^{25}$ + 13.7°, (+)-dihydrochloride $[\alpha]_D^{25}$ + 7.6°

2.6 Melting Range

The following melting point temperatures (°C) have been reported:

	M.P.	Reference
(+)-$C_{10}H_{24}N_2O_2$	87.5 - 88.8	2
	82.0 - 83.5	3
(+)-$C_{10}H_{24}N_2O_2 \cdot 2HCl$	201.8 - 202.6	2
	198.5 - 200.3	4
	200.0 - 202.0	3

2.7 Solubility

The dihydrochloride salt of ethambutol is easily soluble in water and dimethylsulfoxide; soluble in glycol; sparingly soluble in ethanol and very difficultly soluble in acetone and chloroform [5]. On the contrary, the free base of ethambutol is very soluble in chloroform, methylenechloride, and ethylenedichloride; less soluble in benzene and carbon tetrachloride; and sparingly soluble in water [6].

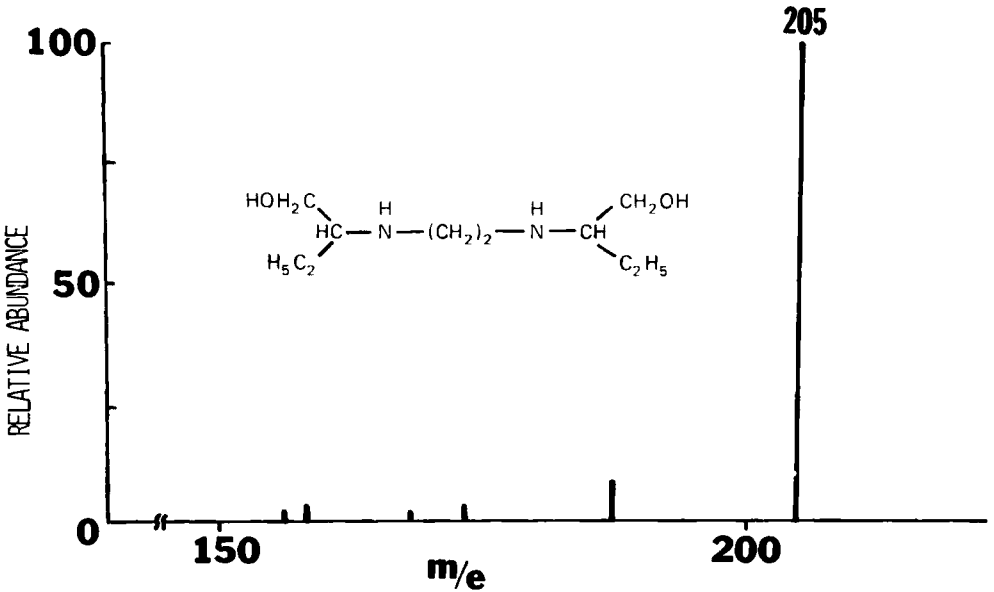


Fig. 3 Chemical ionization mass spectrum of ethambutol using isobutane as the reagent gas. Instrument: Finnigan quadrupole mass spectrometer - Model 2700.

2.8 Dissociation Constant

Ethambutol is a weak base with reported pKa values [7] of 6.6 and 9.5. The first pKa value of 6.6 was confirmed using an aqueous titration technique [3].

3. Synthesis

The highly active d-isomer was prepared by brief heating of ethylene dichloride with excess d-2-amino-1-butanol [8]. A 42% yield of purified d-2,2'- (ethylenediimino)-di-1-butanol was obtained after removal of the less soluble meso isomer corresponding to approximately the amount of levo impurity in the d-2-amino-1-butanol. Ethylene bromide or ditosyl glycol may be used as substitutes for ethylene chloride.

An interesting alternative synthesis [9], reductive alkylation of 2-aminobutanol with glyoxal using sodium borohydride as the reducing agent, gave good yields. Bernardi et al. [10] have reported a new synthesis of ethambutol based on the key intermediate, 2-chloro-3-buten-1-ylchloroformate, easily obtainable in high yield by the reaction of phosgene with 3,4-epoxy-1-butene.

4. Pharmacokinetics

Large differences in pharmacokinetic parameters have been reported in the literature. This is best exemplified by the variation found in peak plasma levels reported following oral and intravenous dosing of comparable dose levels as shown in Table III. For example following oral dosing of 25-30 mg/kg, reported peak values range from 1 to 6 mcg/ml. Likewise plasma concentrations following a 2 hr intravenous infusion of a 20 mg/kg dose are reported to range between 1 and 45 mcg/ml. Similar differences have been noted in pharmacokinetic parameters as shown in Table IV. The various studies have utilized microbiological, colorimetric, radiochemical and most recently gas liquid chromatographic assays, some of which may be measuring metabolites of ethambutol as well as unchanged drug. The majority of the studies reported in Tables III and IV were done on tuberculous patients with normal kidney function. The large variations in kinetic parameters among tuberculous patients may be attributed to their disease histories and age scatterings in addition to the insensitive and non-specific assays used.

A recent study [18] with well controlled normal volunteers indicates only minor variation among individuals. The peak values from this oral study tend to be higher than those reported by others. The data over the first 12 hrs post dosing favor longer half-life values than those previously reported. Twenty-four hr plasma samples and 24 to 72 hr urine samples indicate that ethambutol possesses an even longer terminal half-life of about 10 hrs. Another study [21] in the same group

TABLE III

Variation in Reported Peak Plasma Levels of Ethambutol Following Oral and Intravenous Dosing

Reference	Dose (mg/kg)	Oral Dosing Peak Conc. (mcg/ml)	Peak Time (hr)	Assay Method
11	4	.7	2-4	microbiol.
	8	1.3		
	12.5	2.0		
	25	4.2		
	50	8.6		
12	25	2-4	2-4	microbiol.
13	25	2-5	3	colorimet.
14	25	2	2	microbiol.
15	25	1-6	2	colorimet.
16	15	2-2.5	2-4	microbiol.
	20	3.1-3.5		
	25	4-5		
	50	8.6		
17	20	3.4	3	microbiol.
18	15	3.3-6.0	1.5-4.0	GLC
Reference	Dose (mg/kg)	I.V. Dosing Peak Conc. (mcg/ml)	Infusion Time (hr)	Assay Method
19	15-20	35-45	2	microbiol.
20	15-25	5-23	1.5	colorimet.
16	20-25	1-10	2	microbiol
17	20	2.4-26	2	microbiol.
21	15	11.6-15.4	1	GLC

TABLE IV

Summary of Ethambutol Pharmacokinetic Parameters Reported in Man

Reference	Elimination Half-Lives in Normals (hrs)	Fraction Excreted Unchanged	Renal Failure Half-Lives (hrs)	Volume of Distribution L/kg	Plasma Clearance (ml/min)	Renal Clearance (ml/min)	Assay
11	4	0.6-0.8 (oral)					microbiol.
14	4.2	0.46 (oral)	7.2				microbiol.
15	12-15		12-15			variable[f]	colorimet.
22	3.0[a] 1.5[b]	0.84 (iv)	7.4	.8 [c]	532	446	radiochem.
23	2.5			.3 [d]	100	GFR	microbiol.
18	3.6-5.5	0.54-0.67 (oral)	6.9-9.6[e]			412	GLC
21	2.5-3.6	0.75-0.80 (iv)		1.65 [c]	530	415	GLC

[a] Estimated from Fig. 3 in reference 22.

[b] Calculated from clearance data in reference 22.

[c] Two compartment model was used for data analysis.

[d] Probably corresponds only to the central compartment value.

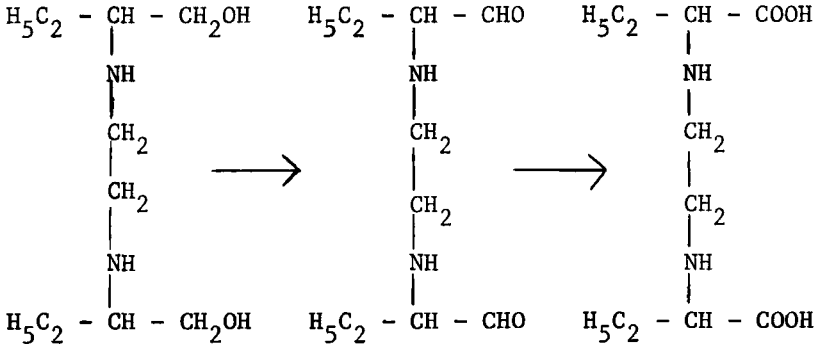
[e] Reference 3.

[f] No correlation with inulin clearance.

of human subjects with ethambutol injection indicates very minor variation in peak serum levels following intravenous infusion over a one hour period. A much larger volume of distribution, which approximates 165% of body weight, was estimated for ethambutol in the intravenous study.

5, Drug Metabolism

Initial metabolism of ethambutol results from the oxidation of the primary alcohol groups to an aldehyde, this is followed by a further oxidation to a dicarboxylic acid to produce the end metabolite [24]. The catalytic enzyme responsible for ethambutol metabolism is alcohol dehydrogenase. These extremely polar metabolites are pharmacologically inactive and excreted by the kidneys readily without further conjugation. The biotransformation of ethambutol is shown below:



The total urinary metabolic pattern over 24 hrs following ethambutol administration was obtained using countercurrent distribution of radioactive material in the urine of four patients [24]. Following an intravenous dose of ^{14}C-ethambutol in one subject, 5% of the dose was excreted as dialdehyde, 3% as diacid, and 65% as unaltered ^{14}C-ethambutol. Similarly, of the 54% of administered ^{14}C excreted into the urine in the 24 hrs following oral dosing, 8% of the dose was excreted as dialdehyde, 7% as diacid and 39% as ethambutol.

The diacid end metabolite was also identified using ion exchange liquid chromatography [3]. The reaction of ninhydrin with the diamino dicarboxylic acid metabolite yields a purple color that absorbs maximally near 570 nm. An automatic amino acid analyzer (Beckman Model 116C) was used for separation and identification. The ion exchange resin used was Beckman UR-30. Using norleucine as an internal standard, it is possible to quantitate the metabolite in the urine [3]. Figure 4 shows the liquid chromatogram of 0.1ml of human urine spiked with the diacid metabolite and norleucine as the internal standard. Table V lists the conditions applied for the metabolite analysis.

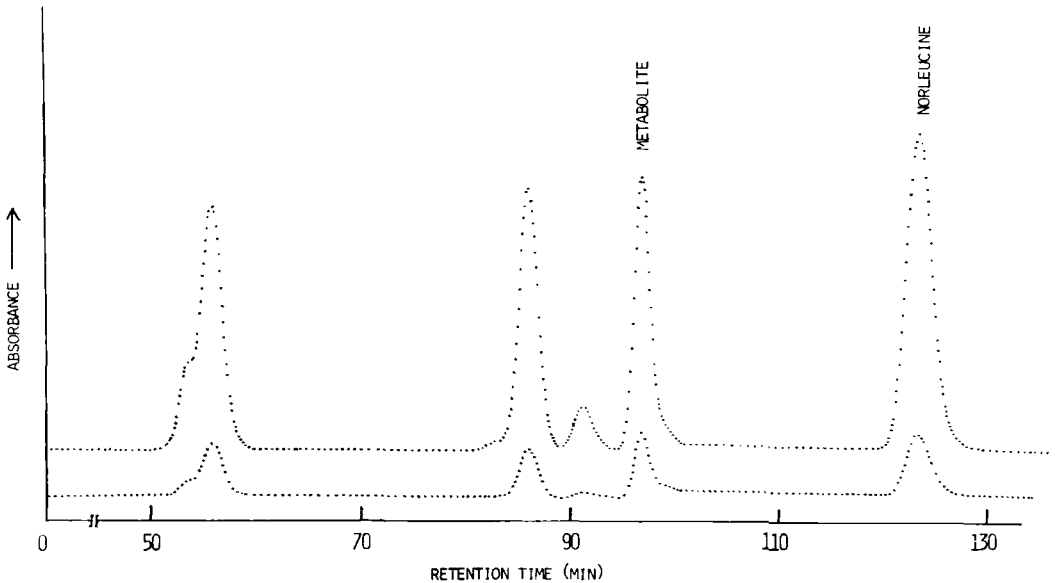


Fig. 4 Chromatogram of 0.1 ml of human urine spiked with 0.25 µM of ethambutol diacid metabolite and 0.15 µM of internal standard (norleucine). Instrument: Beckman Amino Acid Analyzer - Model 116 C.

TABLE V

Conditions Applied for the Analysis of Ethambutol Metabolite

Methodology	2 hour physiological fluid on the model 116C
column size	0.9 X 69 cm
resin type	Beckman UR-30
height of resin column	56.0 cm
column flow rate	68 ml/min
column back pressure	250 psi
first buffer	3.25 ± .01 (0.2N)
second buffer	4.25 ± .02 (0.2N)
buffer change time	55 min
column temperature	55.5° C

6. Methods of Analysis

6.1 Elemental Analysis (as $C_{10}H_{24}O_2N_2 \cdot 2HCl$)

Element	% Theory	Reported [4]
C	43.3	43.5
H	9.5	9.7
N	10.1	10.4
Cl	25.6	25.6

6.2 Microbiological Analysis

This technique consists of measuring the diameter of the zone of inhibition of disks impregnated with the sample to be tested, or of cylinders containing the sample to be tested, on a homogeneous culture of Mycobacterium smegmatis [25] Mycobacterium aurum [26], Mycobacterium parafortuitum [26] or Mycobacterium dierhoferi [17].

6.3 Colorimetric Analysis

This method is based on the formation of a complex of bromothymol blue and ethambutol, a complex whose yellow color may be quantitatively measured by means of spectrophotometry. The technique was first described by Kelly and de la Huerga[27], modified by Strauss and Erhardt [13] and by Froseth [28]. According to Strauss and Erhardt [13], the sensitivity for the determination of ethambutol in serum is 0.5 mcg/ml.

6.4 Reineckate Analysis

The Reineckate assay procedure [29] is a gravimetric determination. The prepared test sample solution of ethambutol is precipitated by adding saturated ammonium reineckate solution. Each gram of the precipitate dried at 105° is equito 0.3292 gm of ethambutol hydrochloride. The washed precipitate can be further dissolved in methanol and the absorbance read at 525 nm. The chelation of ethambutol with reineckate produces a stable color with low absorptivity, and small amounts can not be determined with sufficient accuracy.

6.5 Gas-Liquid Chromatographic Analysis

6.51 Electron Capture Detection

Developed by Lee and Benet [30], this method involves the use of an internal standard, dextro-2,2'-(ethylenediimino)di-1-propanol and derivatization with trifluoroacetic anhydride (TFA). The method allows micro determination of ethambutol in biologic fluids such as plasma, blood, dialysate and urine. Briefly described: 0.2 ml samples, to which 5 mcg of internal standard [dextro-2,2'-(ethylenediimino)-di-1-propanol] has been added, are extracted with 8 ml of chloroform under alkaline conditions (3-4 drops 4N NaOH). Two to three drops of 1 N HCl are added before chloroform aliquots are evaporated to dryness under nitrogen. Methylene chloride (0.5 ml) is added and evaporated to dryness. Residues are dissolved in 1 ml of benzene and made alkaline by addition of 3-4 drops of diluted pyridine (1:4 in benzene). Derivatization with 20 µl trifluoracetic anhydride added to a closed tube is completed in 2 hr at room temperature. Excessive derivatization agent is washed into an aqueous phase with 3 ml of 0.01 M HCl. Appropriate aliquots of the benzene layer (2-3 µl) are injected into a GC equipped with a scandium tritide electron capture detector under the following conditions: glass column, 6 ft x 1/8 in, 3% OV-17 on Gas-Chrom Q, 100-200 mesh; carrier gas (nitrogen) flow-rate, 20 ml/min; injector temperature, 210°; oven temperature, 157°; detector temperature, 230°.

Under the chromatographic conditions described above, the internal standard and ethambutol have retention times of 2.5 and 4 min, respectively. The electron capture detector response is linear between 0.1 and 1 mcg where therapeutic concentrations can be conveniently interpolated. Frozen ethambutol samples and the TFA derivatives of ethambutol and internal standard have been shown to be stable up to seven days [30].

6.52 Flame Ionization Detection

In 1968, Calo et al.[31] published a gas chromatographic method for the separation of three antitubercular drugs, isoniazid, iproniazid and ethambutol. The benzene solution of trimethylsilyl ethambutol, injected along with an alcohol solution of isoniazid and an ethereal solution of iproniazid was chromatographed using a programmed temperature system. The retention time for the trimethylsilyl ethambutol was 12 min. In 1970, Richard et al.[32] reported a gas chromatographic determination of ethambutol involving derivatization with N-trimethylsilylimidazole. The method made use of a single step derivatization, isothermal temperature and a multipurpose column. The retention time for the trimethylsilyl ethambutol was 4 min. No internal standard was used.

Lee and Benet [33] modified this method by the inclusion of an internal standard 1,10-decanediol and the use of a different derivatizing agent, bis(trimethylsilyl)trifluoroacetamide. The use of an internal standard presents an improvement over the other published chromatographic techniques [31,32]. The flame ionization method is suitable for urine samples or for dosage form analysis in which high levels of ethambutol are anticipated. The flame ionization method avoids the tedious and possibly erroneous dilution of the samples necessary for the GLC assay using an electron capture detector.

Briefly described [33]: appropriate aliquots of sample, to which 10 mcg of decanediol had been added, were extracted with 8 ml of chloroform for 10 min under alkaline conditions. Portions of the chloroform were transferred to another tube. Two to three drops of 1 N HCl were added before the chloroform aliquots were evaporated to dryness under nitrogen. Residues were dissolved in 0.1 ml of spectroquality chloroform. Derivatization was initiated by adding 10 µl of bis(trimethylsilyl)trifluoroacetamide and was completed at room temperature in 30 min. Aliquots (2-3µl) of the reaction mixture were injected into the gas chromatograph.

Nitrogen was used as the carrier gas at a flow rate of 66.6 ml/min measured at the flame ionization detector base. The air flow rate was 300 ml/min and hydrogen flow 27 ml/min. Other chromatographic conditions are the same as those described in 6.51. The trimethylsilyl derivative of ethambutol exhibited a retention time of 6 min, and the 1,10-decanediol internal standard had a retention time of 4 min. The response of the flame ionization detector is linear in the 10-100 mcg range.

Using a dual column and dual detector gas liquid chromatograph, simultaneous micro and macro determinations of ethambutol become possible using both the electron capture and flame ionization procedures described. Details such as derivative formation, assay recoveries and choice of internal standard were discussed [33].

6.6 GC-CIMS Analysis

The method [34] involves the use of a tetradeuterated ethambutol standard and derivatization with trimethylsilyimidazole prior to injection onto a GC-CIMS. Mass fragmentography was monitored at m/e 349 and 353, the M+1 ions for the di-TMS derivatives of ethambutol and internal standard respectively, and at m/e 333 and 337, corresponding to the M-CH_3 ions for ethambutol and ethambutol-d_4. The nominal sensitivity on a 0-20 mcg/ml range is 50 ng/ml.

7. Acknowledgments

The authors express there appreciation to Lederle Laboratories for their aid and support in our ongoing ethambutol studies. Special thanks to Dr. Raymond G. Wilkinson for supplying us withsamples of dextro 2,2'-(ethylenediimino)di-1-propanol, Dr. Robert G. Kelly for radiolabelled ethambutol and John B. Hill for his continuing help in obtaining oral and intravenous dosage forms. The authors thank Ms. Irene Beck for her help in preparation of the final typed form of this monograph.

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Literature surveyed through July, 1977.

Analytical Profiles of Drug Substances, 7

FLUOXYMESTERONE

Joel Kirschbaum

ISBN 0-12-260807-0

CONTENTS

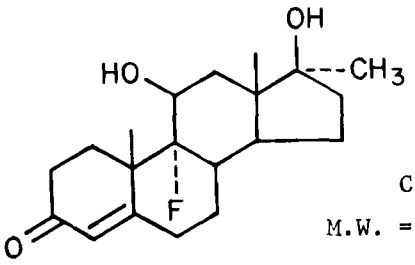
1. History, Description, Precautions and Synthesis

1.1 History

Fluoxymesterone is an androgen used in replacement therapy and for inoperable mammary cancer.[1] It originated as a derivative of cortisone and hydrocortisone in which the 9α-hydrogen atom is replaced by halogen[2], leading to greatly increased glucocorticoid activity.

1.2 Names, Formula and Molecular Weight

Fluoxymesterone is the United States adopted name[3] (USAN). The preferred chemical name[4] is 9-fluoro-11β, 17β-dihydroxy-17-methylandrost-4-en-3-one. Other chemical names are: 11β, 17β-dihydroxy-9α-fluoro-17α-methyl-4-androsten-3-one, 9α-fluoro-11β-hydroxy-17α-methyltestosterone, 9α-fluoro-11β, 17β-dihydroxy-17α-methyl-4-androsten-3-one, 9α-fluoro-17α-methyl-Δ^4-androstene-11β-17β-diol-3-one, and 9α-fluoro-11, 17-dihydroxy-17-methyl-(11β-17β)-androst-4-en-3-one. The chemical abstracts systematic number is 76-43-7. The National Service Center (National Cancer Institute) number is 12165.



$C_{20}H_{29}FO_3$

M.W. = 336.45 daltons

Fluoxymesterone has also been called, Androsterolo, Androfluorene, Androfluorone, Fluotestin, Halotestin, Oratestin, OraTestryl, Testoral and Ultandren.

1.3 Appearance, Color and Odor

Fluoxymesterone is a white or practically white, odorless powder consisting of free flowing crystals or crystalline material.

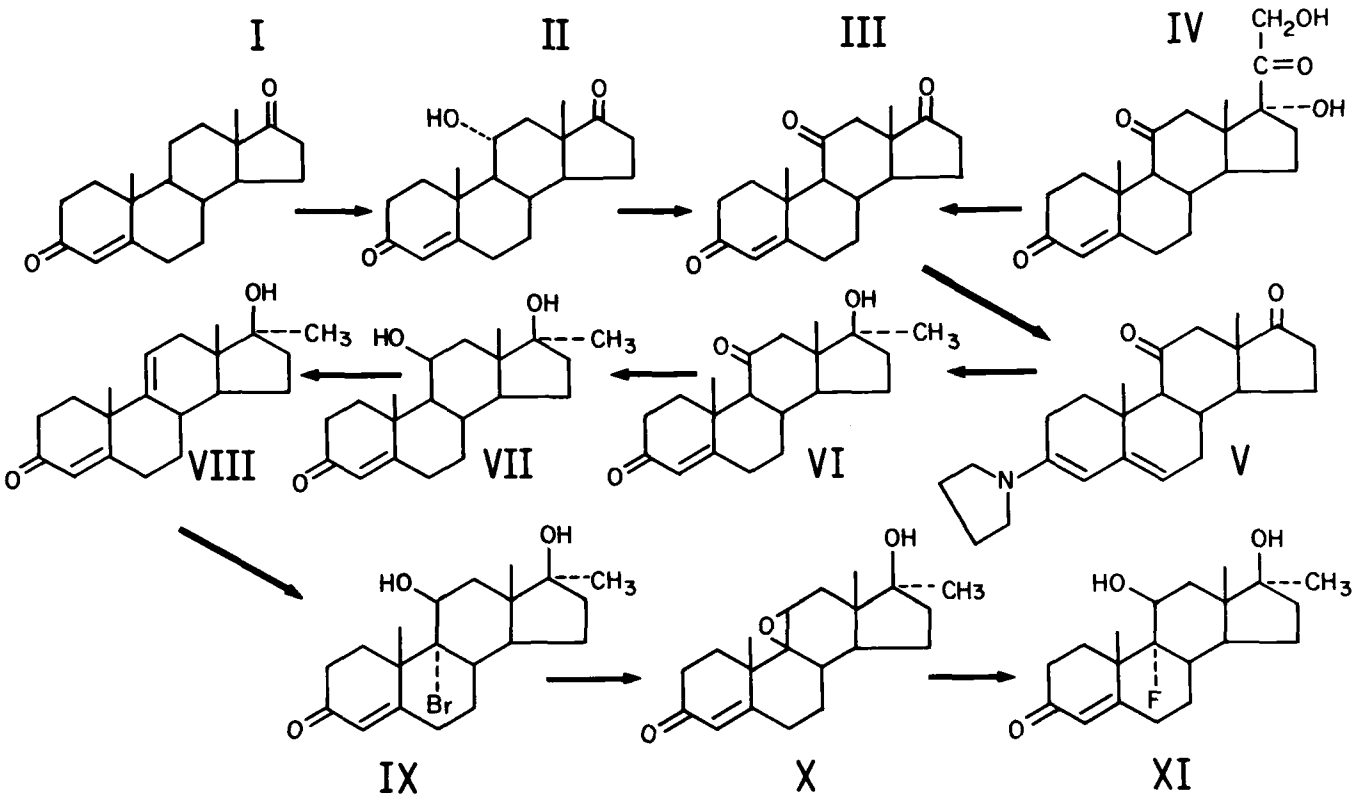
1.4 Precautions

Since the human dose is 1 to 30 mg daily, care should be taken to avoid inhaling the powder.

1.5 Synthesis and Stereochemistry

The synthesis[5,6] of fluoxymesterone is summarized in Figure 1. Microbiological oxidation[7] of androstenedione (I) yields 11α-hydroxyandrostenedione (II), which may be

Figure 1: Synthesis of Fluoxymesterone



oxidized with chromium trioxide to the 11-keto derivative adrenosterone (III). Scission of the dihydroxyacetone side chain of cortisone (IV) with sodium bismuthate also yields III. Careful reaction of III with pyrrolidine gives eneamine formation[8] (V) at the readily accessible 3-ketone position, without derivatization of the 11-ketone. Grignard reaction with methylmagnesium bromide, followed by removal of the eneamine, leads to the 17α-methyl, 17β-hydroxyl derivative (VI). Reconversion of the 3-ketone to the eneamine, followed by reduction with lithium aluminum hydride, and then hydrolysis of the eneamine, gives the 11β, 17β-dihydroxy-3-ketone derivative (VII). The secondary 11-hydroxy is selectively converted to the toluenesulfonate ester, which, on basic hydrolysis, gives the 9(11) olefin (VIII). Reaction of VIII with aqueous N-bromoacetamide gives the 9α-bromo-11β-hydroxy derivative (IX). Essentially this is the addition to the double bond of hypobromous acid, with the initial bromonium ion forming on the less hindered side. Bromine is subsequently displaced by alkoxide ion to give the 9, 11-epoxide (X). The synthesis of fluoxymesterone is completed by opening the oxirane with hydrogen fluoride[9] to give the 9α-fluoro-11β-hydroxy derivative (XI). References 10 to 14 are additional sources.

Although the x-ray structure of fluoxymesterone was not determined, the structure of the related steroid 9α-bromo-17β-hydroxy-17α-4-androstene-3, 11-dione was elucidated.[15] The optical rotatory dispersion spectra are similar. The 3-keto, 9α-fluoro and 17-methyl substituents are on one side of the molecule. The 11-keto, 17-hydroxy and 18- and 19-dimethyl groups are on the other side.

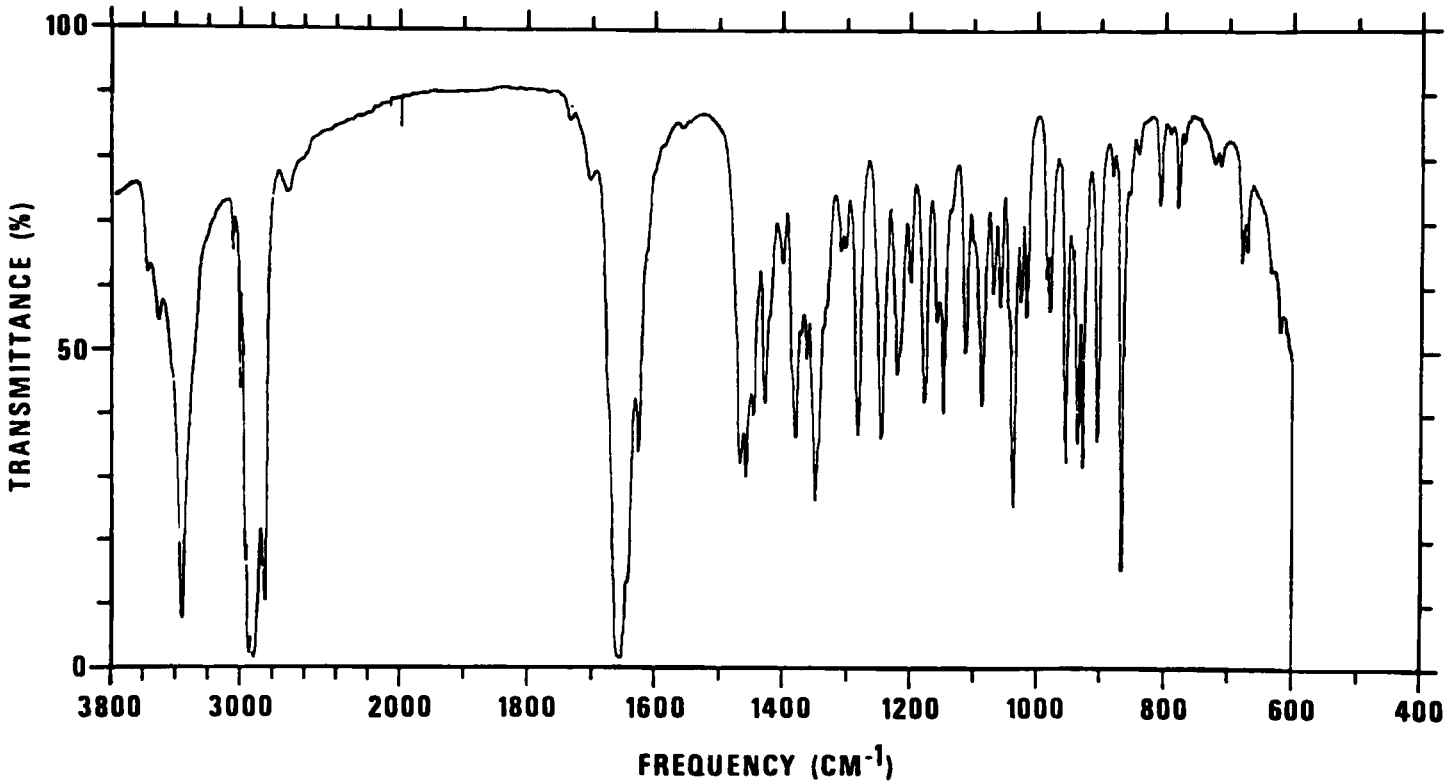
2. Physical Properties

2.1 Infrared Spectrometry

Figure 2 shows the infrared spectrum of fluoxymesterone, run as a mineral oil mull, using a Digilab Model 14D Fourier transform spectrophotometer. Below are the interpretations of various absorbances.[16]

Absorption (cm^{-1})	Structural Feature	Assignment
3580 3520 3370	alcohols	O-H stretch
3050 3010	alkene	C-H stretch

Figure 2: Infrared Spectrum of Fluoxymesterone, Mineral Oil Mull, as Recorded on a Digilab Model 14D Fourier Transform Spectrophotometer.



Absorption (cm^{-1})	Structural Feature	Assignment
1650	Δ^4-3-one	C=O stretch
1626	Δ^4-3-one	C=C stretch
1349, 1282, 1245, 1219, 1175	skeletal modes	C-C stretch and C-H deformation
1146, 1085 1035, 953, 933, 926 903	alcohols and aliphatic fluorine	C-O stretch and C-F stretch

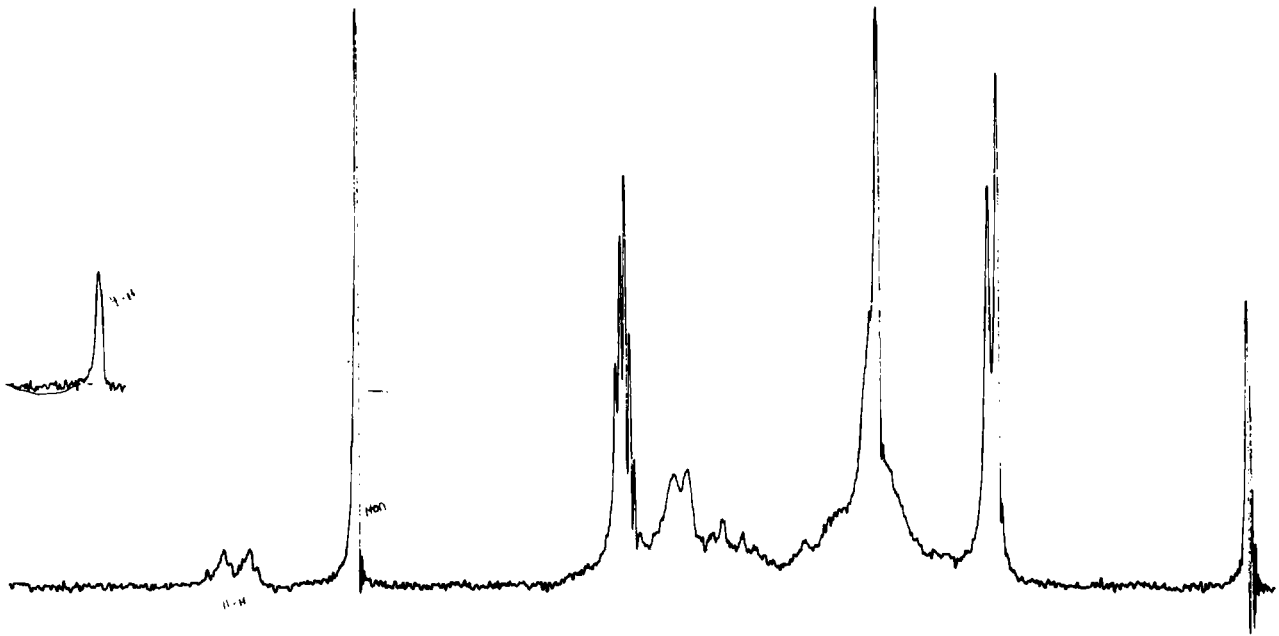
The C_{17} alcohol may be responsible for absorptions at 1146, 953, 933 and 926 cm^{-1}, and the C_{11} alcohol for the bands at 1085 and 1035 cm^{-1}. Peaks at 2960, 2920, 2870, 2850, 1457, and 1370 cm^{-1} are due, in part, to the aliphatic C-H vibrations of mineral oil.

Infrared spectra were previously determined in liquid paraffin and examined[17-19] during a futile search for polymorphism.

2.2 Nuclear Magnetic Resonance Spectrometry (NMR)

Figure 3 is the 100 MHz NMR spectrum of fluoxymesterone in deuterodimethylsulfoxide containing tetramethylsilane as internal reference at 0 Hz. The instrument was a Varian Associates, Inc., Model XL-100 NMR spectrometer equipped to perform Fourier transform spectrometry. Below is an interpretation of the various resonances.[20]

Figure 3: Fourier Transform, Nuclear Magnetic Resonance of Fluoxymesterone in Deuterodimethyl-Sulfoxide, as Recorded on a Modified Varian XL-100 NMR Spectrometer.



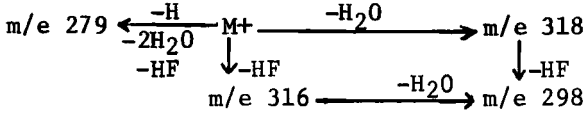
Chemical Shift (ppm)	Relative Area	Multiplicity	Type	Assignment
1.00	3	Singlet	CH_3 group	H,as 20β-CH_3
1.04	3	Singlet	CH_3 group	H,as 17β-CH_3
1.3-1.8	13	Overlapping Multiplets	Aliphatic CH_2's	1,7,12,15,16-H's
1.5		Singlet	CH_3 group	H,as 19β-CH_3
1.8-2.4	6	Overlapping Multiplets	Allylic CH_2's and methines	2,6,8,14-H's
4.05[a]	1	Singlet	Exchangeable H ($\omega_{\frac{1}{2}}H$ = 20 Hz)	17-OH
4.10	1	Doublet of quartets	Carbinol H	11-H
4.90[a]	1	Doublet of doublets	Exchangeable H	11-OH
5.65	1	Singlet	Vinyl hydrogen	4-H

[a]Concentration dependent.

Changing solvents to such aromatic ones as deuteropyridine produces changes in the chemical shifts of protons on the tertiary-substituted methyl groups. This shows the influence of π-electron bonding to hydroxyl groups.[21]

2.3 Mass Spectrometry

Figure 4 is the low resolution mass spectrum of fluoxymesterone as obtained on an AEI Scientific Apparatus, Ltd., Model MS902 mass spectrometer. The sample was inserted into the source at a temperature of approximately 180°. The molecular ion of m/e 336 is observed, along with several mass fragment ions representing the loss of small molecules, hydrogen fluoride and water in several pathways.[22]



The weak ions at m/e 123 and 124 are representative of Δ^4-3-ones ($C_8H_{11}O$, $C_8H_{12}O$). $\Delta^{1,4}$-3-Ones usually have very intense ions at m/e 121 and m/e 122.

Although these appear to be reasonable and typically steroidal assignments, they should be considered tentative since the assignments are not confirmed by high-resolution mass spectrometry.[23]

2.4 X-ray Powder Diffraction

To observe x-ray diffraction patterns, a Philips powder diffraction unit emitting Cu$K\alpha$ radiation at 1.54Å was used with a scintillation counter detector. Four samples from different lots were scanned and recorded from ∿2 to 40 degrees, (2θ).

The table below is the sorted data corresponding to the x-ray diffraction pattern of fluoxymesterone, Figure 5. The data[24] agree well with prior powder data.[25] Lack of significant differences indicates no apparent polymorphism.

Figure 4: Mass Spectrum of Fluoxymesterone. Instrument: AEI-MS902. See Text For Details.

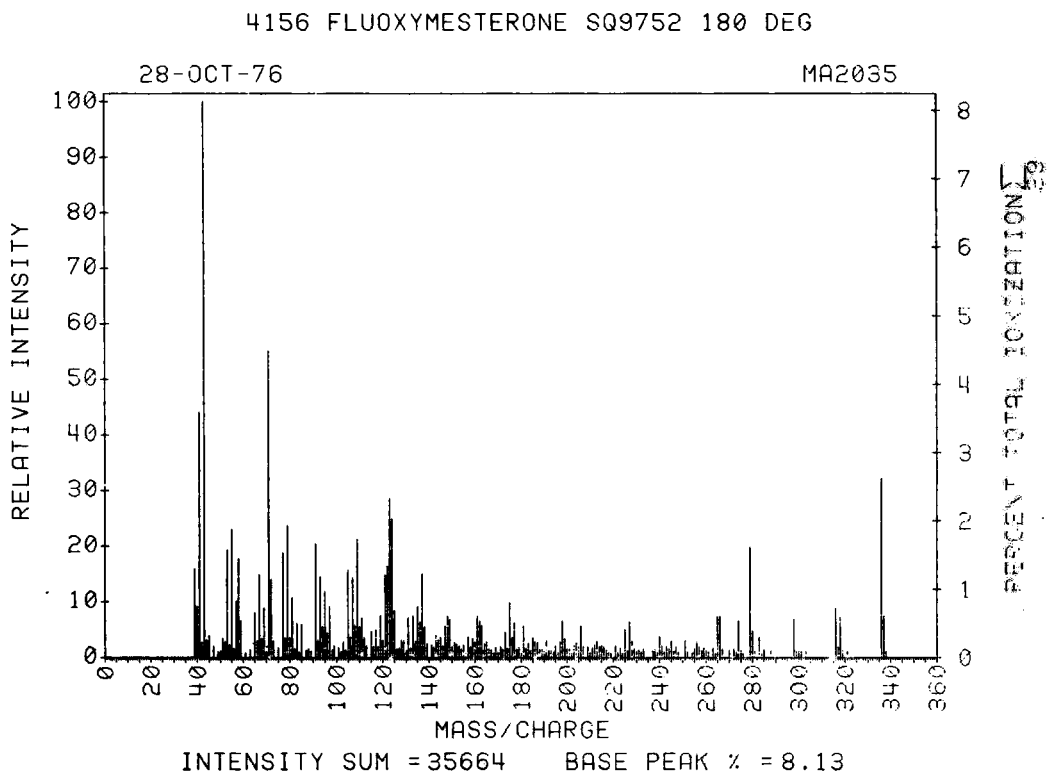
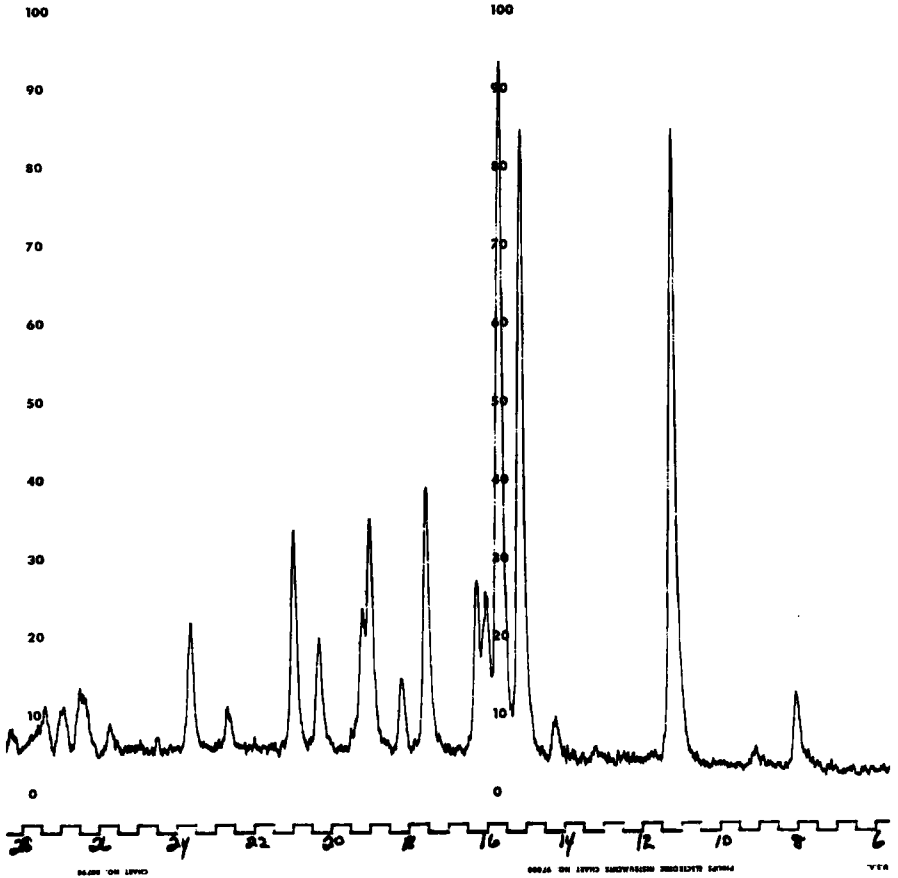


Figure 5: Powder X-ray Diffraction Pattern of Fluoxymesterone.
See text for details.



2θ(Degrees)	'd'(Angstroms)[1]	Relative Area[2]
15.71	5.66	1.000
11.29	7.84	0.995
15.12	5.86	0.961
18.94	4.69	0.758
17.50	5.07	0.581
20.90	4.25	0.425
20.22	4.39	0.353
26.25	3.39	0.330
29.40	3.04	0.291
23.45	3.79	0.268
16.05	5.52	0.262
16.22	5.46	0.256
29.91	2.99	0.233
22.51	3.95	0.225
30.25	2.95	0.218
27.19	3.28	0.209
8.15	10.85	0.199
32.46	2.76	0.195
14.27	6.21	0.188
18.09	4.90	0.187
30.84	2.90	0.181
26.76	3.33	0.174
27.95	3.19	0.173
33.14	2.70	0.172
35.94	2.50	0.167
25.49	3.49	0.162
34.75	2.58	0.148
38.07	2.36	0.135
28.55	3.13	0.121
23.96	3.71	0.121
21.92	4.05	0.104
27.53	3.24	0.087
23.11	3.85	0.079
21.32	4.17	0.077

[1]Interplanar distance.

[2]Relative area, or intensity, is based on highest intensity of 1.00 using Cu$K\alpha$ radiation.

2.5 Ultraviolet Spectrometry

In ethanol, the λ_{max} was reported[14] to be 240 nm (ε = 16,700). Spectral shifts occur depending on the solvent. At a λ_{max} of 239 nm, using traditional nomenclature, the $E^{1\%}_{1cm}$ value for fluoxymesterone, is 495 (reference 26). In 0.1*M* hydrochloric acid, the $E^{1\%}_{1cm}$ value is 430.

2.6 Optical Rotatory Dispersion and Optical Rotation

The rotatory dispersion curves of the various 11β-hydroxy-17α-methyl testosterones are similar to that of testosterone. For fluoxymesterone, specifically,[27] $[\alpha]_{700}$ +77°, $[\alpha]_{589}$ +114°, $[\alpha]_{275}$ +3410°, "max" $[\alpha]_{410-430}$ +199°, "min" $[\alpha]_{367.5}$ -165°, "max" $[\alpha]_{360}$ -79°, "min" $[\alpha]_{353}$ -198°, "inflection" $[\alpha]_{340}$ +484°, "inflection" $[\alpha]_{325}$ +1450°; c = 0.11.

The specific rotations in alcohol[28] were determined[29] to be as follows,

Wavelength (nm)	Specific Rotation
589	+110°
576	+116°
546	+131°
436	+211°

2.7 Fluorescence Spectrometry

Fluoxymesterone was reported to give a yellow-green fluorescence[30] at a concentration of 100 μg/mL in 37% hydrochloric acid. This thousand-fold higher concentration than is usual for fluorometry indicates that either an unstable species or an impurity is responsible for fluorescence.

2.8 Melting Range

Following the U.S.P. procedure[31] for class 1A compounds, the melting range of fluoxymesterone is 262.7-263.7°, corrected.[26] This value is in fair agreement with the results of differential thermal analysis (*cf.* Section 2.9, below).

2.9 Differential Thermal Analysis

A Dupont Model 900 Differential Thermal Analyzer shows fluoxymesterone to have a sharp endotherm[32] at 269°. Decomposition on melting precludes differential scanning calorimetry studies for purity.

2.10 Thermal Gravimetric Analysis

Fluoxymesterone, contains less than 0.1% total volatile material.[32] A Perkin-Elmer Model TGS-2 thermogravimetric analyzer was used. The temperature was increased by 20°/min. up to 260°, under a nitrogen atmosphere.

2.11 Microscopy and Crystal Type

One lot of fluoxymesterone consists of rectangular crystals of different sizes.[33] Typical small crystals are 100 by 7μ, medium crystals are 325 by 20μ and typical large crystals are 750 by 60μ. Hot stage microscopy was performed at a rate of 3°/min., using a Mettler FP 52 temperature controller. This lot showed some crystals exhibiting the first signs of melting at 253.1°, and all crystals were completely melted at 280.8°. Another lot consisted of small, fine birefringent crystals 1 to 2μ square. Occasional larger slabs 25 to 35μ square are found. This latter lot began melting at 273.4° and was completely melted at 285.1°. These studies show evidence of possible polymorphism. Additional evidence is from another hot stage microscopy study[34] in which rods, grains, prisms and stems were visible, again indicating multiple crystal forms.

2.12 Polymorphism

There is no evidence for polymorphism from infrared spectroscopy, powder x-ray diffraction, differential thermal analysis, and only weak data from microscopy. However, it is anticipated that, like many other compounds, further investigation will disclose distinct crystal forms with markedly different physical and spectroscopic properties.

2.13 Hydration

The crystals are not solvated with water, based on a water content of less than 0.1%, (*cf.*, thermal gravimetric analysis Section 2.10 above).

2.14 Surface Area

As measured by gas adsorption,[35] the surface area of three lots of fluoxymesterone, were 0.7, 1.06 and 1.4 m^2/g.

3. Solution Properties

3.1 Intrinsic Dissolution Rate

The intrinsic dissolution rate was determined after compressing powder under a 1500 P.S.I.G. pressure using 3/8" diameter disc-shaped dies. In one liter of 0.1*M* hydrochloric acid at 37°, agitated at a rate of 50 rpm, the intrinsic dissolution rate of fluoxymesterone is 2.55×10^{-4} mg $min.^{-1}$ cm^{-2}, using ultraviolet spectrometry.[36]

3.2 Solubilities in Aqueous and Nonaqueous Solvents

Solubilities of fluoxymesterone were determined in various solvents.[33] Results are reported using the U.S.P. definitions.[37]

Solvent	Solubility
Distilled water	Practically insoluble
0.1*M* Hydrochloric acid	Practically insoluble
0.1*M* Sodium hydroxide	Practically insoluble
Methanol	Sparingly soluble
Acetone	Slightly soluble
Acetonitrile	Slightly soluble
Chloroform	Slightly soluble
Diethylether	Practically insoluble
Benzene	Practically insoluble
Hexanes	Practically insoluble
Pyridine	Soluble

3.3 Partition Coefficients

Fluoxymesterone was partitioned between water and equal volumes of either hexanes, diethylether or chloroform. After one hour of mixing, the steroid content was determined by ultraviolet spectrometry of both phases, wherever possible. The following partition ratios[33] were found for the compound in [aqueous phase/organic phase]; hexanes, 7.1; diethylether, 0.27; and chloroform, 0.016.

3.4 Phase Solubility Analysis

Using previously described methodology,[38] fluoxymesterone at a concentration of 14.4 mg/g in 95% ethanol was determined[29] graphically to be 99.7% pure.

4. Methods of Analysis

4.1 Elemental and Inorganic Analyses

The elemental analysis of fluoxymesterone is: carbon, 71.68% (71.4%, theoretical); hydrogen, 8.71% (8.69%, theoretical), and fluorine, 5.23% (5.65%, theoretical).[39]

Emission Spectrochemical analysis for metals was performed[40] using a Spec Industries carbon arc A.C. unit with a Bausch and Lomb dual grating spectrograph. Data, recorded on glass plates, were interpreted by means of a microphotometer. Fluoxymesterone contained the following metallic impurities, in μg/g; iron, 2; manganese, 0.3; calcium, 5; magnesium, 95; copper, 0.4; aluminum, 3, and sodium, 68. The

magnesium may be from the Grignard reaction in the synthesis. Residue-on-ignition[41] of the same lot[42] is 0.13%. Heavy metals content (ref. 43, method II) is less than 0.001%. After 3 hours at 105° in vacuum, the loss-on-drying[31] value[42] is 0.07%. A portion of the same lot of fluoxymesterone was dissolved in pyridine and, after retention on a precolumn, water content was determined by vapor phase (gas) chromatography by comparison with authentic standards. The value[44] of 0.1% agrees well with the loss-on-drying data.

4.2 Identification, Ultraviolet and Colorimetric Analyses

Compendial identification tests[45] involve comparing either the infrared or ultraviolet absorption spectra of sample fluoxymesterone with that of an authentic sample. To this author, a chromatographic method is preferable since elution time depends on much of the molecule interacting, via weak bonding forces, with the solid and mobile phases. Ultraviolet absorption (*cf.* Section 2.5) depends principally only on the 3-one-4-ene, A and B ring region being intact.

Fluoxymesterone has been quantitated by a differential borohydride reduction assay.[46] This differential assay involves measuring the ultraviolet absorbance of an aliquot of methanolic steroid solution, containing sodium borohydride decomposed prior to the addition of steroid. Its absorbance is determined against a methanolic reference solution of steroid reduced by sodium borohydride. The utility of this procedure is that many interferences from excipients and other unconjugated steroids can be eliminated in the assay of a formulation.

The addition of fluoxymesterone to various colorimetric reagents gives results typical of steroids with its substituents.[47,48] As tested in our laboratories[33], fluoxymesterone reacts with acidic ethanolic 4-nitrophenylhydrazine[49], after heating, cooling and the addition of sodium hydroxide, to give a brilliant violet color. With methanolic isoniazid[50], fluoxymesterone gives a yellow-green color. Fluoxymesterone added to 4-aminoantipyrine[51] in methanolic hydrochloric acid gives a pale green color. Fluoxymesterone added to ethanolic tetramethylammonium hydroxide[52], and heated, gives a cloudy amber color. If added to ethanolic tetramethylammonium hydroxide[53] and picric acid, an orange-red (tea colored) solution results with fluoxymesterone. In concentrated sulfuric acid[54] fluoxymesterone gives a deep yellow color. Adding water slowly causes a deep violet color

to form at the interface. Fluoxymesterone gives a royal purple color with blue tetrazolium,[55] and a yellow-brown color with aluminum chloride in nitromethane.[55]

4.3 Chromatographic Analysis

4.31 Gas Chromatography

Bulk fluoxymesterone, fluoxymesterone in tablets and fluoxymesterone in urine can be quantitated[56] using glass columns packed with 3% or 4% XE-60 phase on Gas Chrom Q support. The temperature was approximately 225° and the nitrogen pressure from 10 to 24 P.S.I.G. Typically, fluoxymesterone (retention time 36.8 min.) can be separated from testosterone (9.4 min.), epitestosterone (8.5 min.), 9α-fluoro-17β-hydroxy-17-methyl-4-androstene-3,11-dione (16.8 min.), and several other steroids. The peaks are Gaussian.

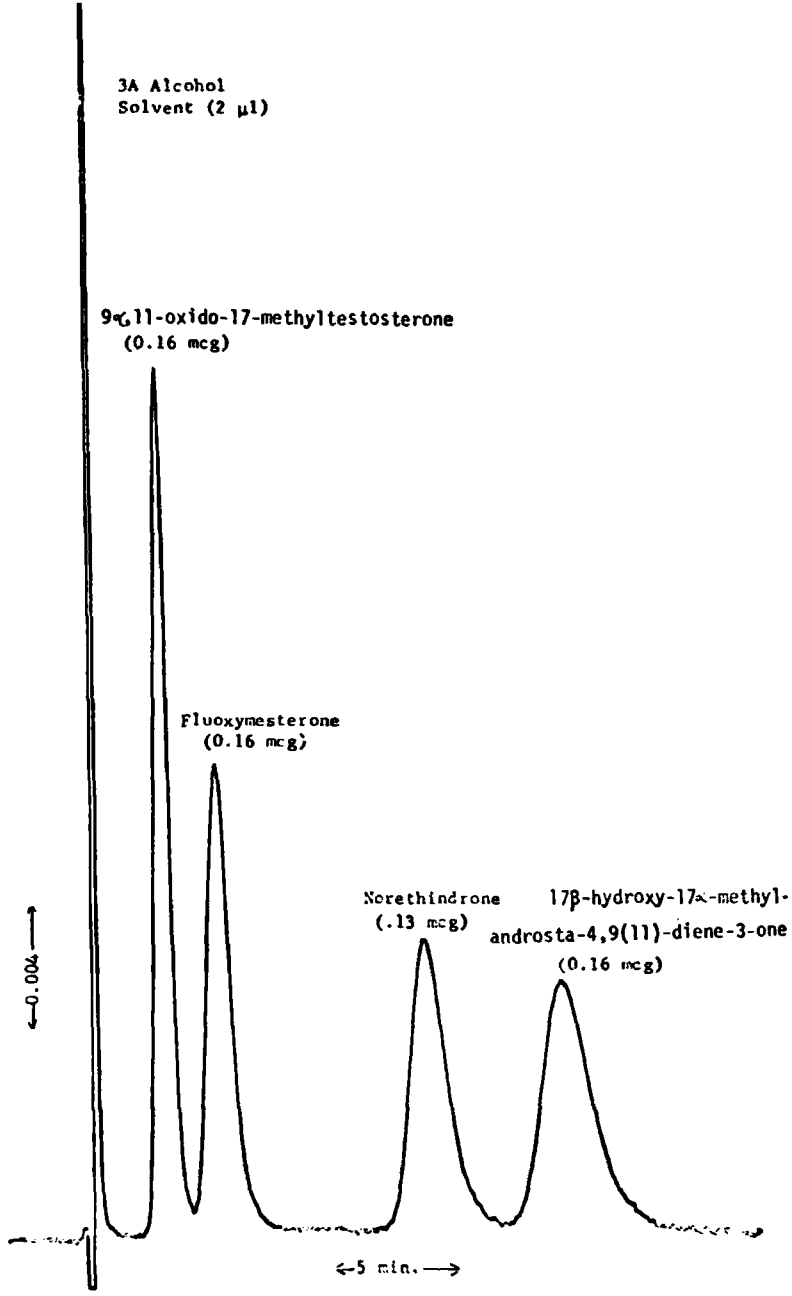
Residual solvents can also be determined by gas chromatography[44] (*cf.* Section 4.1). Methanol content of fluoxymesterone was less than 0.1%.

4.32 High Pressure Liquid Chromatography

Reverse phase high pressure liquid chromatographic methods have been developed to separate and quantitate bulk and formulated fluoxymesterone, using absorbance measurements at 254 nm. A compendial procedure[45] utilizes an octadecylsilane column and aqueous methanol mobile phase (4:1) to separate and quantitate fluoxymesterone (retention time 4-7 min.) with norethindrone as the internal standard (elution in 8-14 min.). Figure 6, courtesy of W. Beyer, Upjohn Co., Inc., shows the results of this procedure using a flow rate of 0.4 mL/min. The response is linear, electronically measuring peak areas of fluoxymesterone and norethindrone, from 0.05 to 0.50 mg fluoxymestrone per mL. The coefficient of variation is 1.4%.

A μBondapak, prepared, octadecylsilane column, with aqueous acetonitrile (3:1) flowing at 3 mL/min., gave a retention time for fluoxymesterone of 3 to 7 min. and for norethindrone of 6 to 10 min.[57] The coefficient of variation is 0.32%. Another assay[58] utilizing aqueous acetonitrile (3:2) uses a reverse-phase octadecylsilane prepared Partisil column. With a flow rate of 0.8 mL/min., the retention time for fluoxymesterone is 12 min. Repetitive injections with a precision loop injector gave a coefficient of variation of 0.41%. Injecting higher concentrations of steroid, using a more sensitive detector, permits the quantitation or estimation of trace amounts of ultraviolet-absorbing impurities that do not elute either with fluoxymesterone or at the void volume.

Figure 6: Reverse Phase High Pressure Liquid Chromatographic Separation of Fluoxymesterone, Norethindrone (Internal Standard) and Related Compounds. See text for details.



4.33 Thin Layer Chromatography

Fluoxymesterone chromatographed[59] on silica gel, G.F., as support, with a mobile phase of butyl acetate-acetone (8.2) gave a R_f of 0.48. Visualization was with short wavelength ultraviolet light. The purity of one lot is 99.7%, with one impurity, R_f 0.57, accounting for 0.3%. With benzene-ethyl acetate, (CAUTION), 1:1, fluoxymesterone remained at the origin, but a trace impurity migrated. The steroid is quantitated after elution from the plate with ethanol, by ultraviolet spectrometry.

Kieselguhr G plates, impregnated with 10% propylene glycol solution in acetone, were used to chromatograph fluoxymesterone with toluene as mobile phase.[60] Visualization utilized a 20% solution of *p*-toluenesulfonic acid in 94% ethanol as spray, followed by heating at 120° for 10 minutes, and then examination under visible and long wavelength ultraviolet light.

Fluoxymesterone was chromatographed on silica gel, F-254, plates and visualized either by ultraviolet light or spraying with 40% methanolic sulfuric acid. Fluoxymesterone gave one spot in four mobile phases[29]: benzene-acetone, 4:1 (CAUTION), R_f 0.22: chloroform-methanol (93:7), R_f 0.41; ethanol-chloroform-benzene (CAUTION) (2:2:1), R_f 0.89, and methanol-ethanol-chloroform-acetone (3:3:3:1), R_f 0.70.

Model studies for gas-liquid chromatography[56] involved thin layer chromatography to determine polarity. With benzene-ethyl acetate, 1:1 (CAUTION), the R_f of fluoxymesterone is 0.12. Testosterone and epitestosterone have an R_f of 0.27 and androsterone has an R_f of 0.38. In ligroin-toluene-methanol-water (5:5:7:3) fluoxymesterone, testosterone and epitestosterone are not separated, R_f 0.42. Androsterone has an R_f of 0.39. In benzene-ether (1:1), fluoxymesterone remains at the origin, testosterone has an R_f of 0.16, epitestosterone 0.15, and androsterone 0.23.

Reverse-phase thin layer chromatography, using silica gel plates impregnated with silicone oil, was utilized to study the lipophilic character of 101 steroids,[61] including fluoxymesterone. Relative mobilities (R_m) in 15 to 65% acetone were determined and extrapolated to 0% acetone. For fluoxymesterone R_m is -0.5%. R_m values, using 45% acetone as the mobile phase, generally correlate with steroidal partition coefficients in ether-water, hemolytic activity and membrane and protein binding. The effect on ΔR_m of substituting or derivatizing groups could also be estimated.

4.34 Paper Chromatography

Various hormonal steroids were spotted on Whatman[62] No. 1 paper. After being dipped into 50% methanolic propylene glycol, chromatography was with either heptane saturated with propylene glycol (46 hours) or heptane-methanol-water, 10:8:2 (3 hours). Visualization was with either 20% antimony trichloride in chloroform, 20% phosphoric acid, or sulfuric acid-acetic acid (1:9), under normal and ultraviolet light. Relative to testosterone, the mobility of fluoxymesterone is 0.0 in both systems.

Whatman No. 1 paper has also been used in two other systems[63] to determine the homogeneity of fluoxymesterone. Twenty percent formamide in methanol as stationary phase, and methyl isobutyl ketone-formamide (20:1) as mobile phase; 25% propylene glycol in chloroform as stationary phase, and toluene saturated with propylene glycol, as mobile phase, were once routinely used for determining purity.

4.35 Column Chromatography

A diatomaceous earth column was used to separate fluoxymesterone from excipients.[64] Aqueous methanol was the solvent for the formulation and chloroform was used to elute the steroid. Quantitation involved ultraviolet spectrometry.

4.4 Fluoxymesterone in Tissues and Body Fluids

Biological assays for fluoxymesterone in lower animals include effects on seminal vesicle weight,[65] sperm mobility[66] and rate of mounting and ejaculation.[67] In man, these effects can be evidenced by increases in fertility.

A radioimmunoassay has been developed[68] in which the 3-[O-(carboxymethoxime)] derivative of fluoxymesterone is conjugated to bovine serum albumin. Hapten is prepared in rabbits with the aid of Freund's Adjuvant. The resulting antibody binds 75% of 6,7-^{3}H-fluoxymesterone at a 1:5000 dilution. Twenty-five picograms of fluoxymesterone can be detected in 0.1 ml of unextracted serum. No endogeneous human steroids cross react appreciably with the primary antiserum. Specificity is derived mainly from the 11β-hydroxyl and 17-methyl group, since there is considerable cross reaction with 1-dehydrofluoxymesterone, 17-methyltestosterone and 11β-hydroxy-17-methyltestosterone. Injecting 2, 5 or 10 mg of fluoxymesterone into dogs gave peak concentrations, after 1-2 hours, of 120, 195 and 505 ng/mL, respectively.

Using gas chromatography[56] (*cf.* Section 4.31), less than 5% of injected fluoxymesterone (plus closely related compounds) was found in human urine.

5. Stability: Effects of Temperature, Time and Light

Fluoxymesterone, formulated into tablets, was stored at 22° or 40° for 28 months. After extraction with chloroform, drying, and redissolution in ethanol, the steroid was assayed with isonicotinic acid hydrazide (nydrazid) and blue tetrazolium reactions (*cf.* Section 4.2) or by paper chromatography followed by nydrazid. Results within 6% of label were found,[42] indicating stability.

Storage of solid fluoxymesterone, at 50° for 3 weeks, showed neither a loss in content nor the appearance of new peaks, on comparison with refrigerated fluoxymesterone. Analyses utilized high pressure liquid chromatography[58] (*cf.*, Section 4.32).

Exposure of fluoxymesterone to approximately 900 footcandles of light for 3 weeks caused a 2% loss in content, as determined by reverse-phase high pressure liquid chromatograph.[58] A new peak eluted prior to fluoxymesterone and was equivalent to 0.3%, assuming similar absorbtivity as fluoxymesterone, and correcting for diffusion. Photolytic degradation of the A-ring is expected since hydrocortisone and prednisolone undergo rearrangements[69-71] when alcoholic solutions are exposed to ultraviolet radiation or ordinary fluorescent laboratory lighting.

6. Acknowledgements

The author gratefully acknowledges the assistance of the many contributors cited as "personal communication." In many instances, the information was especially obtained for this summary. Special thanks go to R.M. Johnson and W. Beyer of the Upjohn Company, Kalamazoo, Michigan, for their help; to R. Poet, G. Brewer and S. Perlman of E.R. Squibb, New Brunswick, New Jersey, for their critical reading of this MS., and to my family, for their patience and understanding shown during the compiling of this analytical profile.

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Literature reviewed to January, 1978.

Analytical Profiles of Drug Substances, 7

HEXETIDINE

Gerhard Satzinger, Wolfgang Herrmann, and Friedrich Zimmermann

ISBN 0-12-260807-0

TABLE OF CONTENTS

1. Introduction

Hexetidine (1,3-bis(2-ethylhexyl)-5-amino-5-methyl-hexahydropyrimidine) can be prepared in pure state with difficulty only. For both the preparations Hexoral[R] [1]) and Hexoral[R]-Spray[1]) a purified hexetidine is used which is prepared from commercial hexetidine according to a process (1) patented to Goedecke Company, Berlin. Structurally, hexetidine occupies a special position among the chemotherapeutics; even among the heterocycles with biocidal activity there is no chemically comparable substance.

Hexetidine is a highly substituted derivative of hexahydropyrimidine. As is well known, the parent compound "pyrimidine" possesses an extraordinary biological importance as structural element of the purines, thiamine, uric acid, uracil and others. Therefore, it is of special interest that the "synthobiotic" activity of hexetidine could be traced back to its interaction with metabolic processes of vital importance for the growth of pathogenic organisms (2). The broad spectrum of antibacterial and fungicidal activity of hexetidine is a result of this property. Another striking characteristic of hexetidine is its unusual tissue affinity. Topically applied, hexetidine is not displaced too early from the active site by either physiological or pathological secretions.

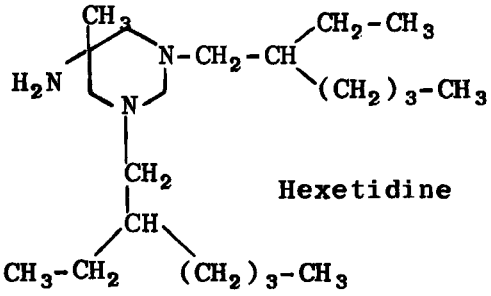
Purified hexetidine differs from commercial hexetidine (3) mainly by its thin-layer chromatographic and gas chromatographic purity. As yet, only a few analytical data of commercial hexetidine have been reported in the literature (4,5). In the following, we report mainly on physico-chemical properties of purified hexetidine and on spectroscopic data pertaining to its structure.

2. Description

Hexetidine (I) is 1,3-bis(2-ethylhexyl)-5-amino-5-methyl-hexahydropyrimidine.

Purified hexetidine is a clear, colorless, oily liquid of weak, amine-like odor.

[1]Manufacturer: Goedecke AG, Berlin



(I) Hexetidine

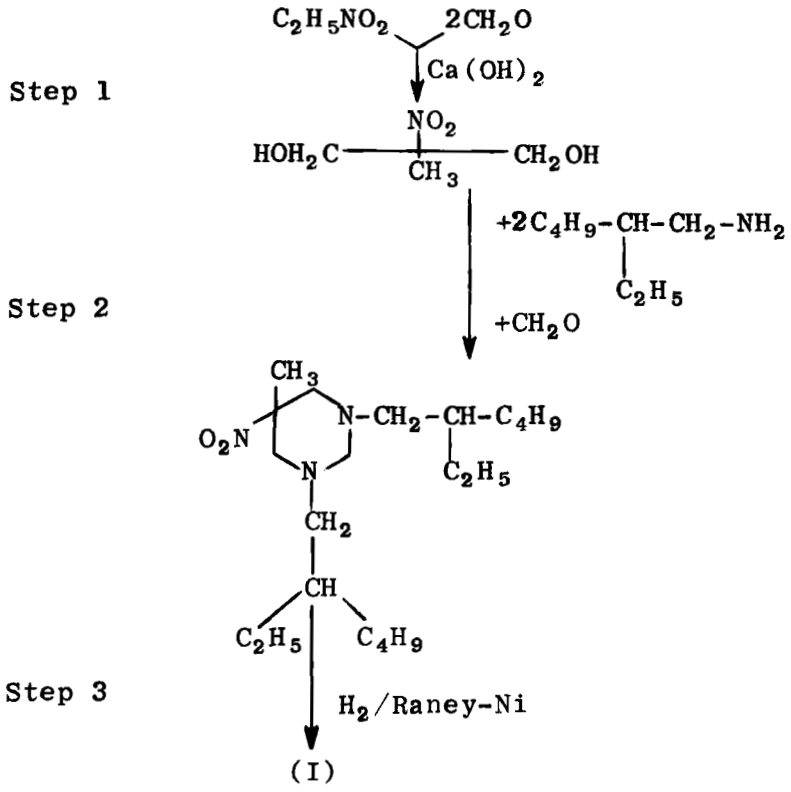
Empirical formula: $C_{21}H_{45}N_3$
Molecular weight: 339.6

Hexetidine can be distilled in high vacuum (b.p. 160° C/0.4 Torr (4); 140° C/0.3 Torr (6)), but purification cannot be achieved in this way.

Hexetidine is readily soluble in organic solvents such as acetone, ethanol, benzene, chloroform, n-hexane and methanol, but is practically insoluble in water ($\sim 10^{-4}$ g/ml).

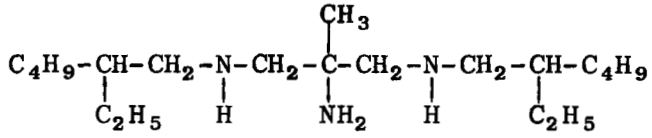
3. Preparation and Stability

Hexetidine is synthesized technically according to a three-step process (see Formula Scheme 1) starting from nitroethane, formaldehyde and 2-ethylhexylamine (6).



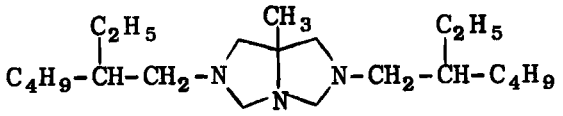
Formula Scheme 1

During the course of our studies on hexetidine, some new findings as to the chemical behavior of I and related compounds were obtained. Thus, we have found, for example, that contrary to the literature (4) the hexahydropyrimidinering system in hexetidine is not particularly stable; it can be cleaved thermally and hydrolytically giving rise mainly to the open-chain compound II and to the condensated heterocycle III. This process is some kind of disproportionation which can be reversible under certain conditions.



II

N_1,N_3-Bis-(ß-ethylhexyl)-2-methyl-propane-triamine-(1,2,3)("triamine")



III

2,6-Bis-(ß-ethylhexyl)-hexahydro-7α-methyl-1H-imidazo[1,5-c]imidazole ("hexedine")

II and III are formed also under the conditions of the technical synthesis and subsequent distillation according to the process of the Commercial Solvents Corporation so that commercial hexetidine always is a mixture of about 75% of hexetidine and of about 10% of II and III each. Moreover, additional amines can be detected in smaller amounts by thin-layer chromatography.

With the aim of using a drug as pure as possible for Hexoral, we have developed a process that allows to remove the by-products contained in commercial hexetidine. This process essentially consists of selectively separating I from the mixture of the amines with the aid of an aromatic disulfonic acid at low temperatures. On the subsequent recovery, I is obtained with a purity of about 99%. The amines II (7) and III (8) (both colorless oils of slight amine odor) have not been characterized in detail in the literature up to now. Their most important data are listed in Table 1.

Table 1

Physical Properties of Triamine (II) & Hexedine (III)

	Triamine (II)	Hexedine (III)
Empirical formula	$C_{20}H_{45}N_3$	$C_{22}H_{45}N_3$
Mol. wt.	327.6	351.6
B.p.	130-132°C/0.05 Torr	125°C/0.02 Torr
n_D^{20}	1.4580	1.4660
d_4^{20}	0.884	0.853
pK_a	9.1	6.0

Table 1 (continued)

	Triamine (II)	Hexedine (III)
Titration	corresp. to I eq.wt.=$^1/_3$mol.wt.	corresp. to I eq.wt.=$^1/_2$mol.wt.
IR (neat)	3360 cm^{-1}(NH_2)str.)	
	3300 cm^{-1} (NH_2, NH str.)	
	2810 cm^{-1} (N-CH_2 str.)	2790 cm^{-1} (N-CH_2 str.)
	1570 cm^{-1}(NH_2 d.)	
	1115 cm^{-1}(C-N str.)	1115 cm^{-1}(C-N str.)

4. Physical Properties

4.1 Refractive Index and Density

Table 2

Purified Hexetidine		Commercial Hexetidine		
n_D^{20}	1.4640	1.4668	1.4653	1.4686
d_2^{20}	0.8697	0.8889	0.868	0.8832

4.2 Partition Coefficients (purified hexetidine)

$$K = \frac{c_{H_2O}}{c_{CHCl_3}} = 5 \times 10^{-3}$$

$$K = \frac{c_{buffer\ 7.4}}{c_{CHCl_3}} = 1 \times 10^{-3}$$

$$K = \frac{c_{H_2O}}{c_{n\text{-octanol}}} = 9 \times 10^{-3}$$

$$K = \frac{c_{buffer\ 7.4}}{c_{n\text{-octanol}}} = 1 \times 10^{-2}$$

The partition coefficients were determined according to a method described in detail in the literature (9) ($c = 1.5 \times 10^{-3}$ mole/l).

4.3 Dissociation Constant

$pK_a = 8.3$ (purified hexetidine)

Due to the sparing solubility of the bases in water (see also II, III) the dissociation constants were determined by titration with 0.1 M HCl in 50% aqueous ethanol ($c = 8$ mMole/l).

Method: $pK_a = pH - \log \frac{[\text{undiss.base}]}{[\text{base cation}]}$; if $[B]=[BH^+]$, then $pK_a = pH$ [10].

4.4 IR Spectrum

The IR spectrum of purified hexetidine (Fig. 1) exhibits the characteristic bands of the nitrogen groups (see Table 3).

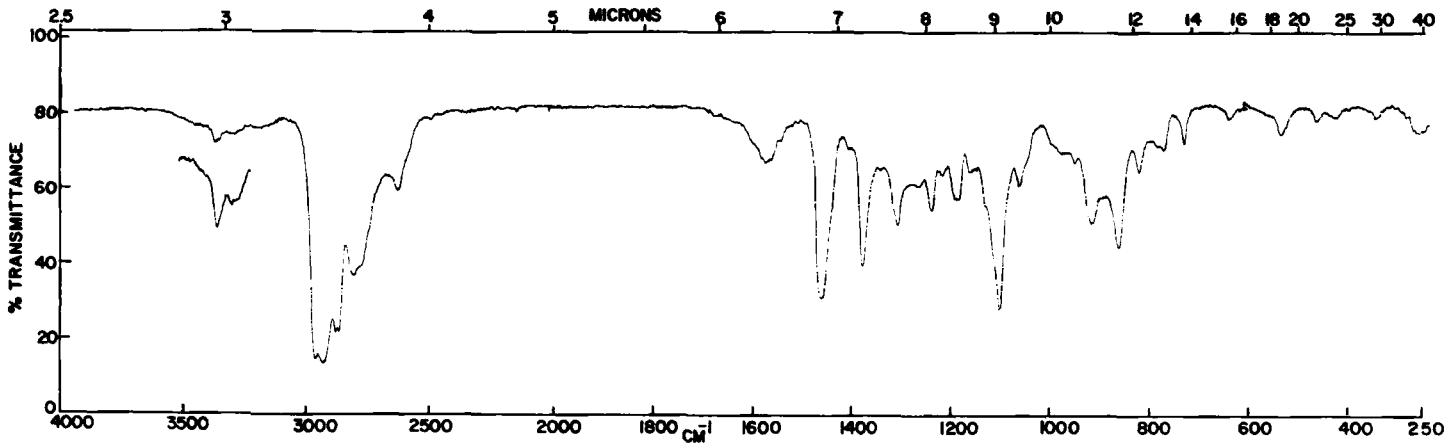


Figure 1: IR Spectrum of Purified Hexetidine (neat on CsJ).

Table 3

Band Positions and Vibrational Modes of the IR Spectrum of Purified Hexetidine

Band (cm^{-1})	Vibrational mode
3360	asym. N-H stretching of NH_2
3300	sym. N-H stretching of NH_2
2920	asym. C-H stretching of CH_2 and CH_3
2860	sym. C-H stretching of CH_2 and CH_3
2800	C-H stretching of N-C(H)H
1570	N-H deformation of NH_2
1460	asym. C-H deformations of CH_2 and CH_3
1380	sym. C-H deformations of CH_2 and CH_3
1100	C-N stretching
920-860	N-H deformations of NH_2

The IR spectrum of commercial hexetidine shows an additional band at 1600 cm^{-1} which is to be assigned predominantly to the dehydrohexetidine according to our investigations.

4.5 NMR Spectrum

The NMR spectrum (Fig. 2) confirms the structural formula. The assignment of the signals (chemical shift δ in ppm) to the individual molecular groups (for numbering, see structural formula) is given in Table 4. A complete analysis including 270-MHz proton spectrum and ^{13}C-spectrum gives a number of information concerning structural details. After completion, these studies will be the subject of a separate publication.

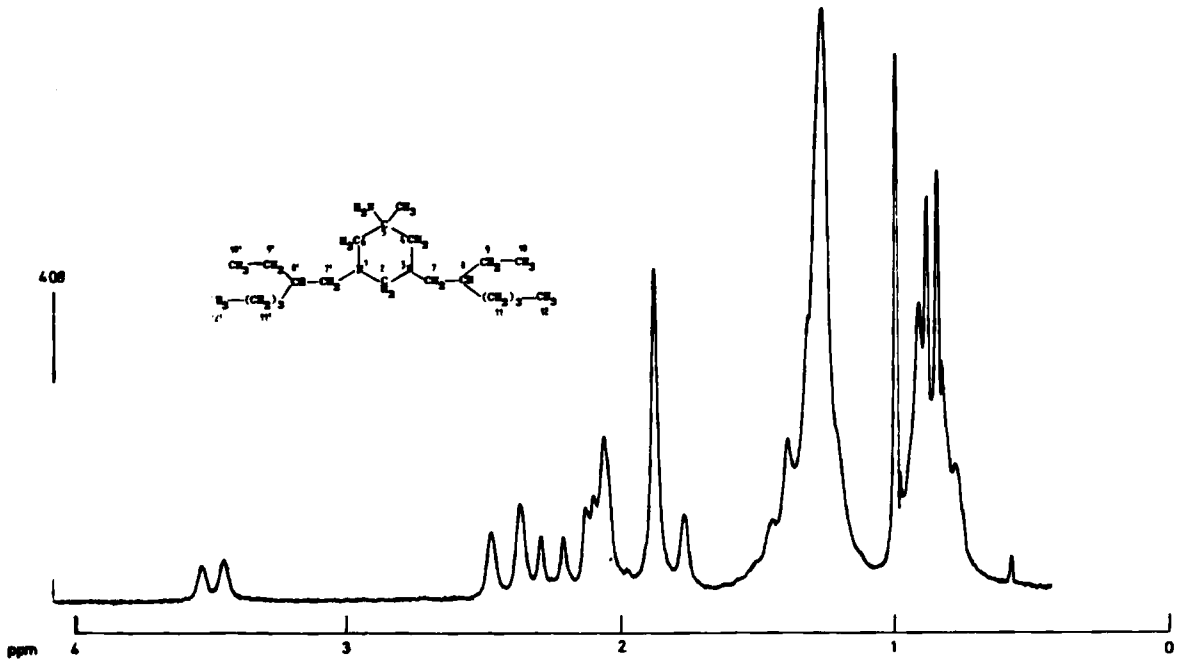


Figure 2. 100 MHz NMR spectrum of purified hexetidine in $CDCl_3$ with TMS as internal standard.

Table 4

Assignments in the NMR Spectrum

δ (ppm)	Group(no.)	δ (ppm)	Group(no.)
3,51; 2,28	CH_2(2)		CH (8,8')
2,45; 1,85	CH_2(4,6)	1,6-1,3	CH_2(9,9')
2,2-2,0	CH_2(7,7')		$(CH_2)_3$(11,11')
1,91	NH_2(5)	1,03	CH_3(5)
		0.95-0,80	CH_3(10,10', 12,12')

4.6 Mass Spectrum

The mass spectrum of purified hexetidine (Fig. 3) shows along with the molecular ion at m/e 339 at M-1 peak at m/e 338 and characteristic ions at m/e 226, 197 and 142 (base peak) which can be accounted for by the fragmentations as represented in Formula Scheme 2.

5. Analytical Methods

5.1 Elemental Analysis (Purified Hexetidine)

	C	H	N
Calc.:	74.28	13.35	12.37
Found:	74.06	13.29	12.44

5.2 Chromatographic Analysis

5.2.1 TLC-Analysis

The following experimental conditions are especially suitable for the purity control of I:

Layer:	Silica gel F 254 (Merck pre-coated plate) 10 x 20 cm (Pretreatment with ammonia vapor)
Solvent system:	Benzene/methanol (8:2) (saturated atmosphere)
Running distance:	ca. 15 cm
Running time:	ca. 1 h

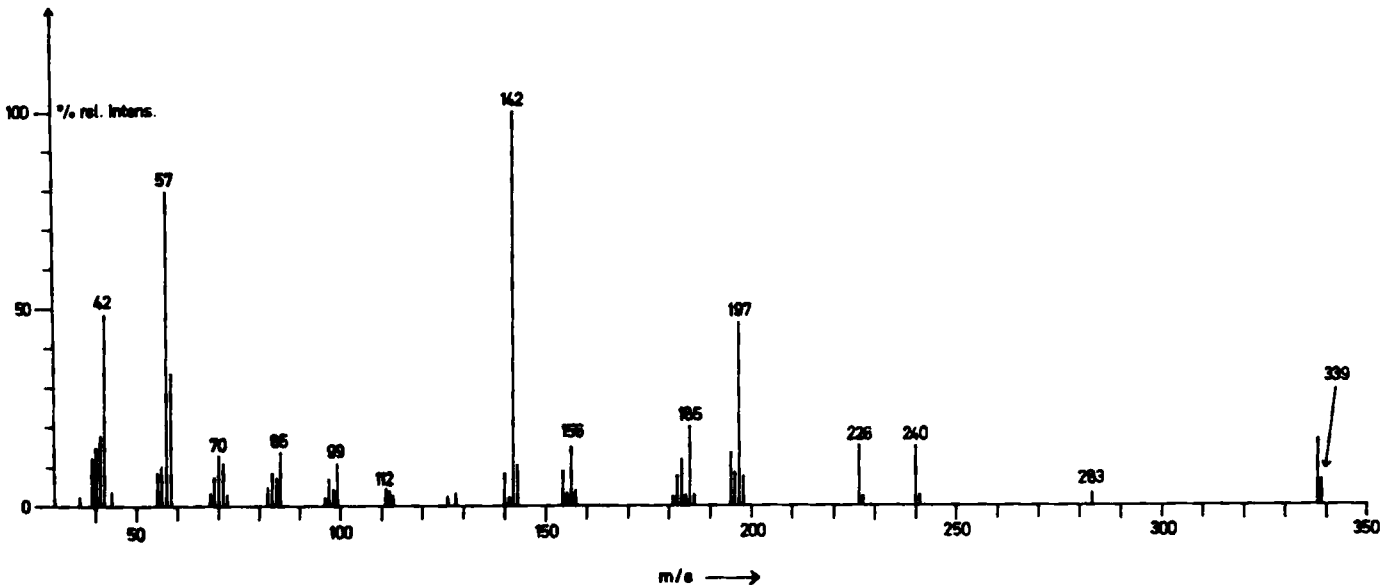
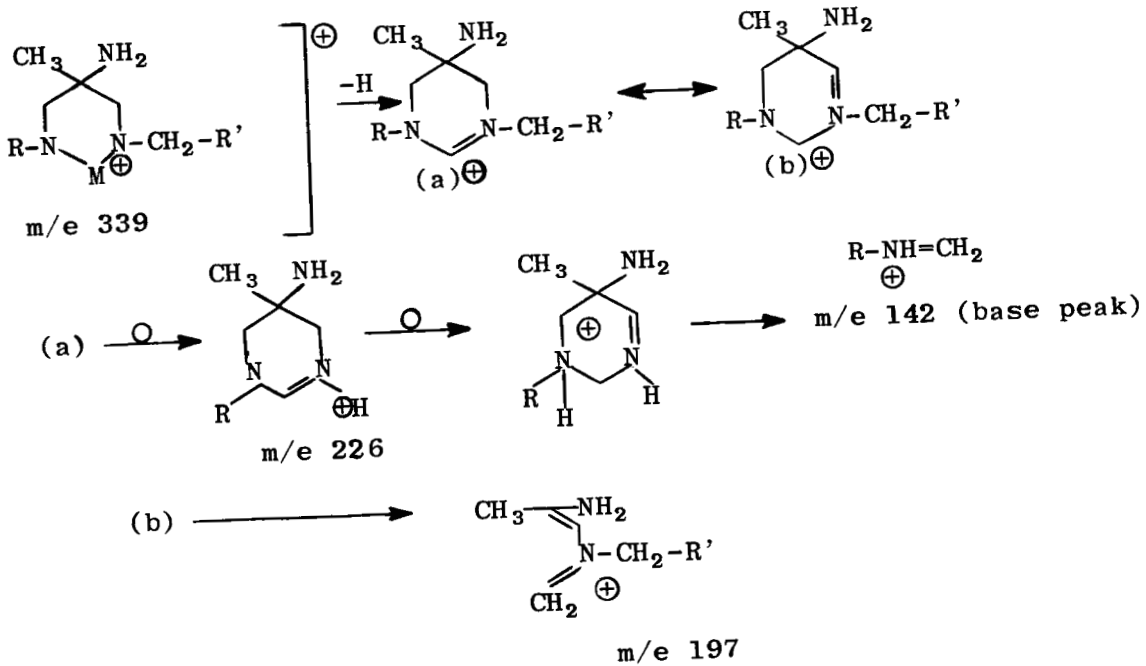


Figure 3. Mass spectrum of purified hexetidine, 70 eV.



Formula Scheme 2

Detection:	Iodine vapor (ca. 30 min)
Sample solution:	100 mg of hexetidine/ml of n-hexane
Sample size:	1 µl (corresp. 500 µg)

According to the thin layer chromatogram (cf. Fig. 4) commercial hexetidine contains at least 3 additional accompanying compounds (amines), each in amounts of more than 1%. These were isolated and their structure, unless known, was elucidated. As may be seen from the TLC, the spots at hR_f 65 and at hR_f 15 correspond to the described amine III (hexedine) and to the base II (triamine), respectively. The spot at hR_f 35 could be assigned to an oxidation product of hexetidine, named by us dehydrohexetidine (IV) the properties and structure of which will be reported on in a separate publication.

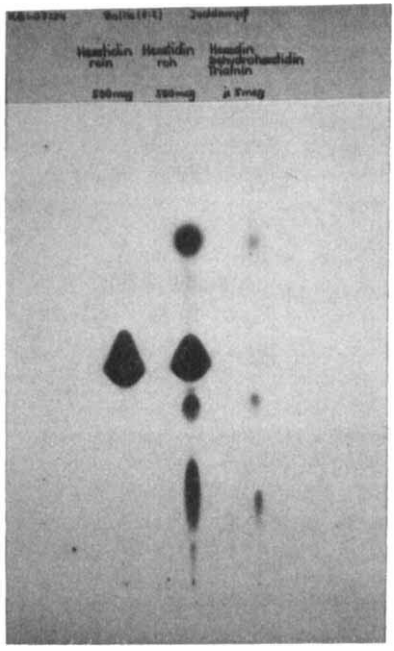


Fig. 4: Thin layer chromatogram of purified hexetidine and commercial hexetidine as compared to 1%-spots of II, III and dehydrohexetidine (IV) each.

The concentrations of spots present in the TLC in addition to hexetidine (I) can be estimated semiquantitatively as follows:

hR_f 65: (ca. 12%), III
hR_f 45: (ca. 70%), I
hR_f 35: (ca. 5%), IV
hR_f 15: (ca. 10%), II
hR_f 75-70 and 10: (each 1%), unknown.

5.2.2 GLC Analysis

The gas chromatographic separation of amines II and III and of additional unknown accompanying compounds of hexetidine and the reproducible quantitative determination of the components without thermal isomerisation and without formation of artifacts can be achieved on a few stationary phases and on glass columns only. The following operative GLC conditions proved advantageous (see Fig. 5):

Apparatus:	FID detector and temperature programming
Column:	1.0 m, glass; 2 mm internal diameter.
Packing material:	1% OV 7 on 80-100 mesh Chromosorb G HP
Carrier gas:	40 ml N_2/min.
Hydrogen:	30 ml/min.
Synth. air:	350 ml/min.
Oven temperature:	Program: 0-15 min: 150°C. isothermal, 15-27 min: 4°C/min 27-38 min: 198°C.
Injection port:	240°C.
Detector:	240°C.
Chart speed:	20 cm/h.
Sample injected:	0.5 µl of a solution in n-hexane (≙ 5 µg)

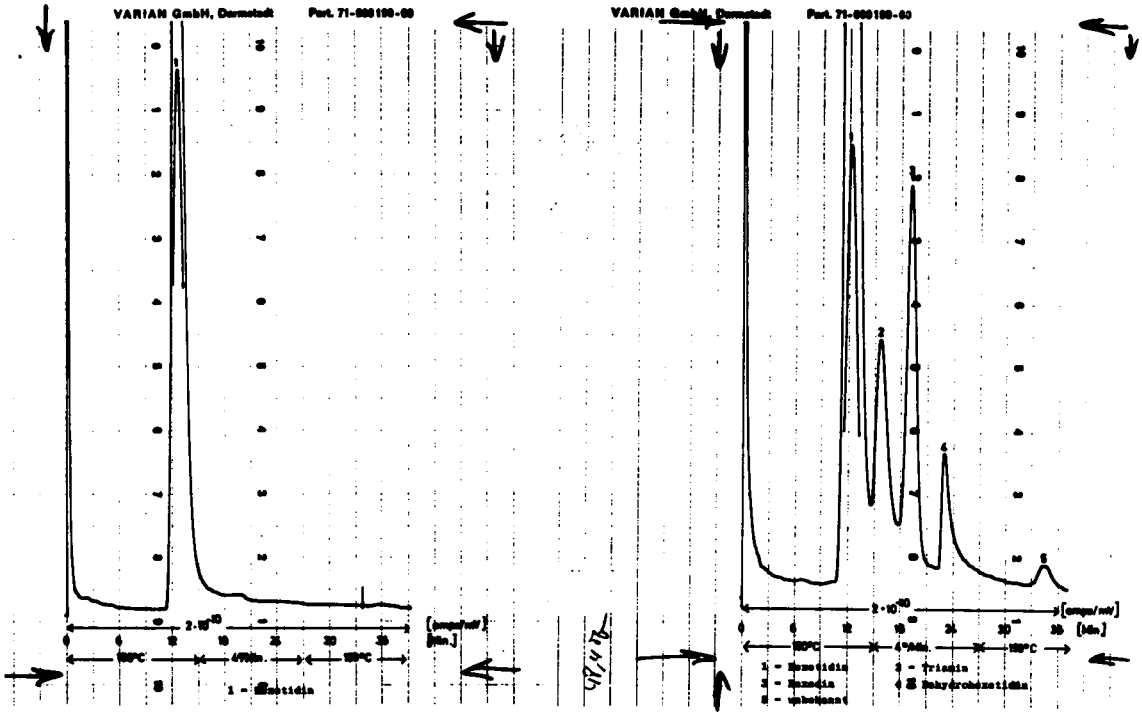


Figure 5. Gas chromatogram of purified hexetidine (top) in comparison with commercial product (bottom) (in n-hexane). 1 = hexetidine (I), 2 = triamine (II), 3 = hexedine (III), 4 = dehydrohexetidine (IV), 5 = unknown.

R_t-times		Commercial hexetidine	
		Values of one batch (see Fig.5)	Variation
Hexetidine (I):	755 sec	(72.2%)	70-85%
Triamine (II):	961 sec	(8.5%)	5-10%
Hexedine (III):	1173 sec	(14.4%)	5-15%
Dehydro-hexetidine(IV):	1382 sec	(3.7%)	1-5%
Unknown	2076	(1.2%)	max. 2%

5.3 Titration in nonaqueous medium

Contrary to the literature (5), the content of hexetidine can be determined without difficulties by nonaqueous titration in glacial acetic acid with perchloric acid either potentiographically or with quinaldine red or crystal violet as indicator.

Equivalent weight = 1/2 mol. wt., i.e. 1 ml of 0.1 N $HClO_4$ is equivalent to 16.98 mg of hexetidine.

6. Experimental

Spectra and chromatograms were recorded using the following instruments:

UV:	Beckman Acta V
IR:	Perkin-Elmer 457
NMR:	Varian HA 100
MS:	Varian MAT CH4
GLC:	Varian Aerograph 1840
Titration:	Metrohm Potentiograph E 436

7. Acknowledgement

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Analytical Profiles of Drug Substances, 7

HYDROFLUMETHIAZIDE

Chester E. Orzech, Norris G. Nash, and Raymond D. Daley

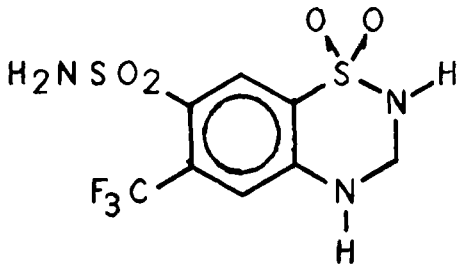
ISBN 0-12-260807-0

CONTENTS

1. DESCRIPTION

1.1 Name, Formula, Molecular Weight

Hydroflumethiazide is 3,4-dihydro-6-(trifluoromethyl)-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide, also known as 6-trifluoromethyl-3,4-dihydro-7-sulfamoyl-2H-1,2,4-benzothiadiazine 1,1-dioxide and as 3,4-dihydro-7-sulfamyl-6-trifluoromethyl-1,2,4-benzothiadiazine 1,1-dioxide. Chemical Abstracts indexes it as 2H-1,2,4-benzothiadiazine-7-sulfonamide, 3,4-dihydro-6-(trifluoromethyl)-, 1,1-dioxide. The CAS Registry Number is [135-09-1].



$C_8H_8F_3N_3O_4S_2$ Mol. Wt.: 331.29

1.2 Appearance, Color, Odor

Hydroflumethiazide is white to cream colored, odorless, powder or crystals.

2. PHYSICAL PROPERTIES

2.1 Infrared Spectra

The infrared spectrum of hydroflumethiazide NF Reference Standard Lot No. 69178 is shown in Figure 1. The spectrum was obtained in a potassium bromide dispersion, using a Beckman Model IR-12 spectrophotometer. A similar spectrum is observed when a mineral oil or other dispersing medium is used, except for absorption bands of the medium. The spectrum is similar to those already published (1,2). In addition to the absorption bands shown here, hydroflumethiazide exhibits weak but sharp bands at 382, 338, and 315 cm^{-1}, and very weak bands at 267, 242, 230, and 218 cm^{-1}, when the spectrum is obtained as a fairly thick mineral oil dispersion. Some of the absorption bands may be assigned as follows (3):

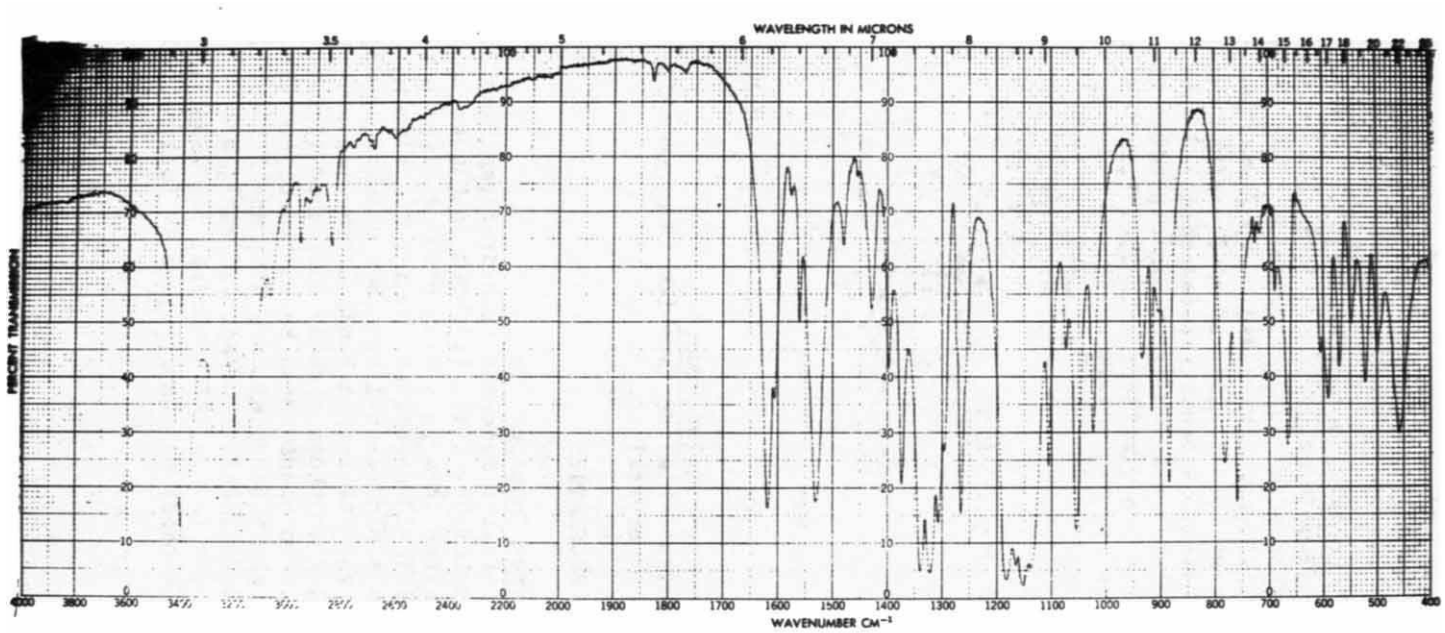


Figure 1. Infrared Spectrum of Hydroflumethiazide, Potassium Bromide Pellet.

Absorption Bands, cm^{-1}	Assignment
3380, 3260, 3170	N-H stretch
1300 to 1350	$-SO_2-$ stretch; $-CF_3$ deformation
1140 to 1200	$-SO_2-$ stretch; $-CF_3$ deformation

2.2 Nuclear Magnetic Resonance Spectrum

The NMR spectrum shown in Figure 2 was obtained by dissolving Ayerst house reference standard hydroflumethiazide #59-1 in acetone-d_6 containing tetramethylsilane as internal reference. The spectrum was produced using a Varian EM-360 NMR spectrometer. The peaks at 7.42 ppm and 8.37 ppm are due to the aromatic protons. The methylene protons are at 5.00 ppm and the -NH protons are the broad peaks at 6.70, 6.90, and 7.45 ppm. The series of peaks at 2.10 and 3.07 ppm are due to the solvent.

2.3 Ultraviolet Spectra

Figure 3 is the ultraviolet absorption spectrum of NF Reference Standard hydroflumethiazide, Lot No. 69178, in methanol. The spectrum was obtained on a Cary Model 14 spectrophotometer at a concentration of 19.3 mg per liter. Discontinuities in the spectrum are due to changes in the absorbance range. The 277 to 267 nm and the 222 to 210 nm regions of the scan are on the 1.0 to 2.0 absorbance range; the remainder of the scan is on the 0.0 to 1.0 range.

Pilsbury and Jackson (4) compare ultraviolet spectra of hydroflumethiazide with those of other thiazides. They report E (1%, 1 cm) for hydroflumethiazide of 450 at 276 nm at pH 10, 580 at 273 nm at pH 2.

Kracmar and Lastovkova (5,6) compare ultraviolet spectra of hydroflumethiazide in methanol, ethanol, 0.1N hydrochloric acid, and 0.1N potassium hydroxide to the spectra of other thiazides. The absorptivity ($\log \epsilon$) of hydroflumethiazide is reported as 4.182 at 275 nm in 0.1N potassium hydroxide, 4.290 at 273.5 nm in 0.1N hydrochloric acid, 4.229 at 272.5 nm in ethanol, and 4.286 at 272.5 in methanol.

Other ultraviolet spectral data are those of DePaulis and Dipietromaria (7), who report E (1%, 1 cm) of 560 at 274 nm in 70 percent ethanol; Fazzari (8), reporting an absorptivity of 45.4 liter/g cm at 273 nm in 0.2N sodium hydroxide; and Sunshine (9), with spectra in 0.1N hydrochloric acid and in 0.1N sodium hydroxide solution. The British Pharmacopeia

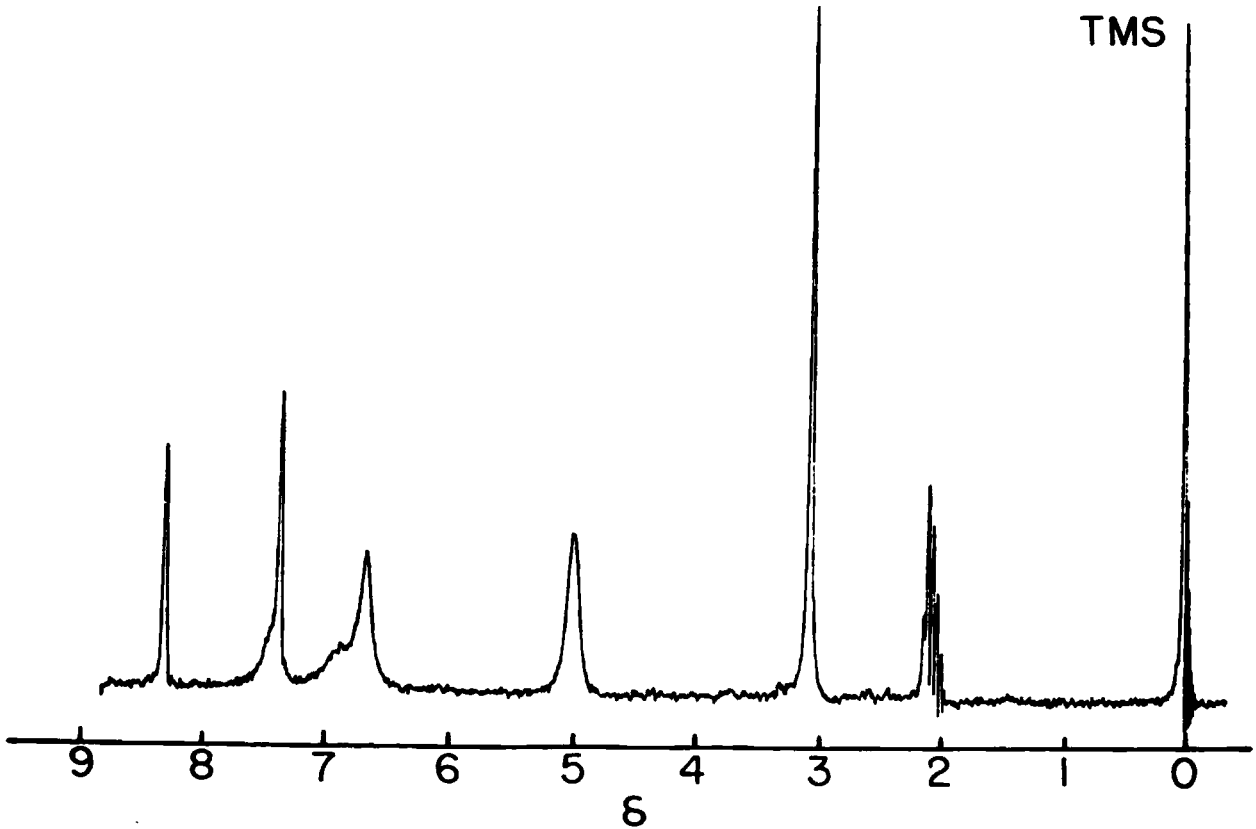


Figure 2. Proton Magnetic Resonance Spectrum of Hydroflumethiazide, in Deuterated Acetone.

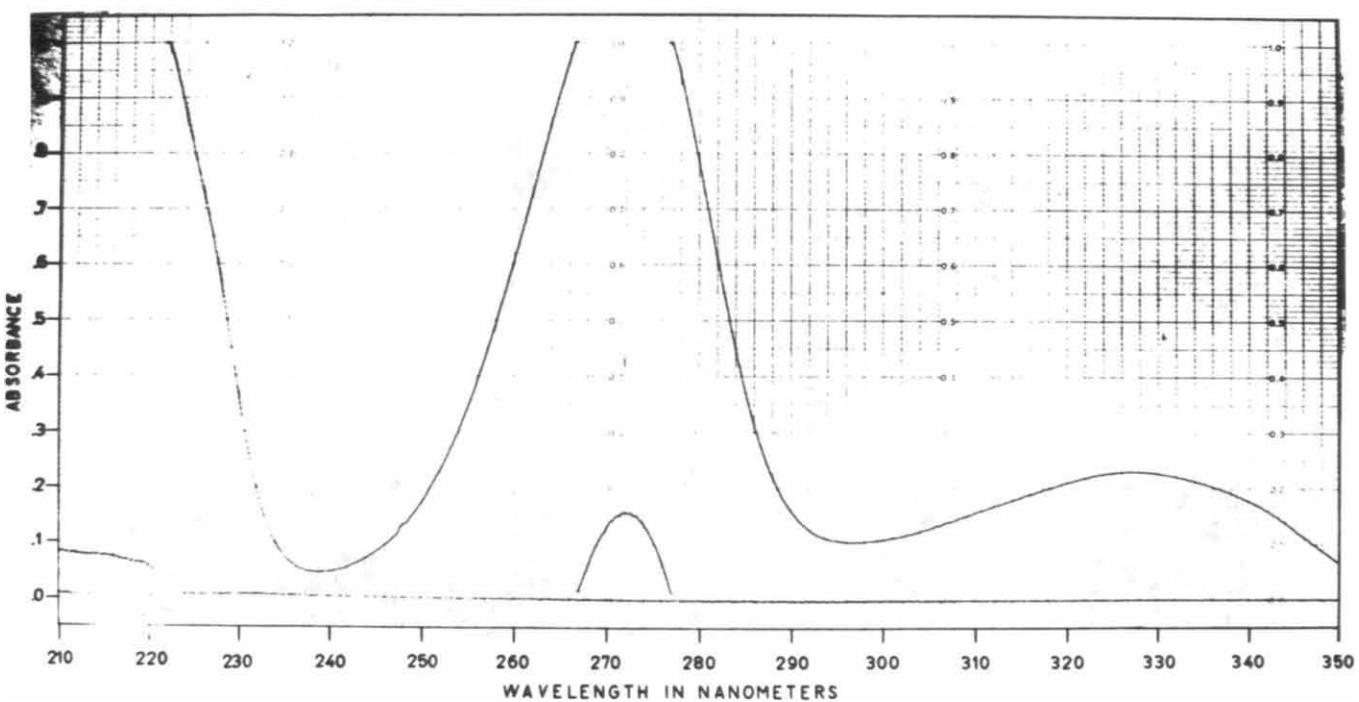


Figure 3. Ultraviolet Spectrum of Hydroflumethiazide, 19.3 mg/liter, in Methanol.

1973 ultraviolet identity test (10) corresponds to an absorptivity of about 46 liter/g cm at 274 nm in 0.01N sodium hydroxide.

2.4 Mass Spectrum

Figure 4 is the low resolution mass spectrum of hydroflumethiazide NF Reference Standard Lot No. 69178, obtained with an LKB 9000S mass spectrometer, ionization voltage 70 eV, source temperature 250°C. Some of the peaks may be assigned as follows (11):

m/e 331		Molecular ion M+
m/e 331	$\xrightarrow{-CHNH}$	m/e 303 (*277.3)
m/e 303	$\xrightarrow{-SO}$	m/e 255
m/e 303	$\xrightarrow{-SO_2}$	m/e 239
m/e 239	$\xrightarrow{-NH_3}$	m/e 222 (*206)
m/e 222	$\xrightarrow{-SO}$	m/e 174
m/e 222	$\xrightarrow{-SO_2}$	m/e 158
m/e 69		CF_3

2.5 Differential Thermal Analysis (DTA)

The DTA curve in Figure 5 was obtained with a DuPont Model 900 instrument. The curve shows only a melting endotherm at 274°C with subsequent decomposition.

2.6 Crystal Properties

The powder diffraction pattern is given in Table I. This pattern was obtained with a Norelco diffractometer, using nickel-filtered copper K_α radiation. The pattern is similar to one published by Corrigan and Timoney (12); they also report the existence of a 1:1 crystalline ethanol solvate.

Kuhnert-Brandstätter et al give information for the microscopic characterization of hydroflumethiazide (13), as does Groenewegen (14).

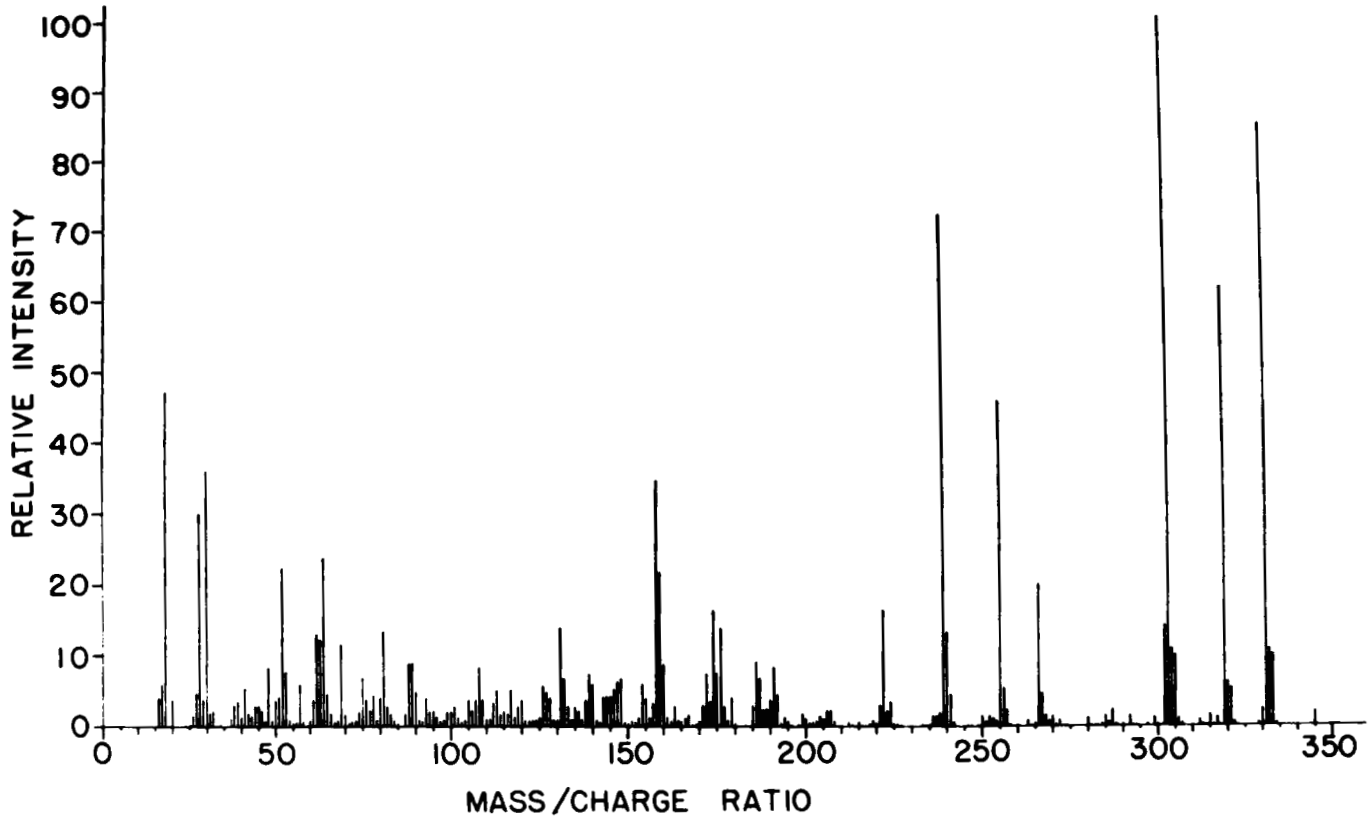


Figure 4. Mass Spectrum of Hydroflumethiazide.

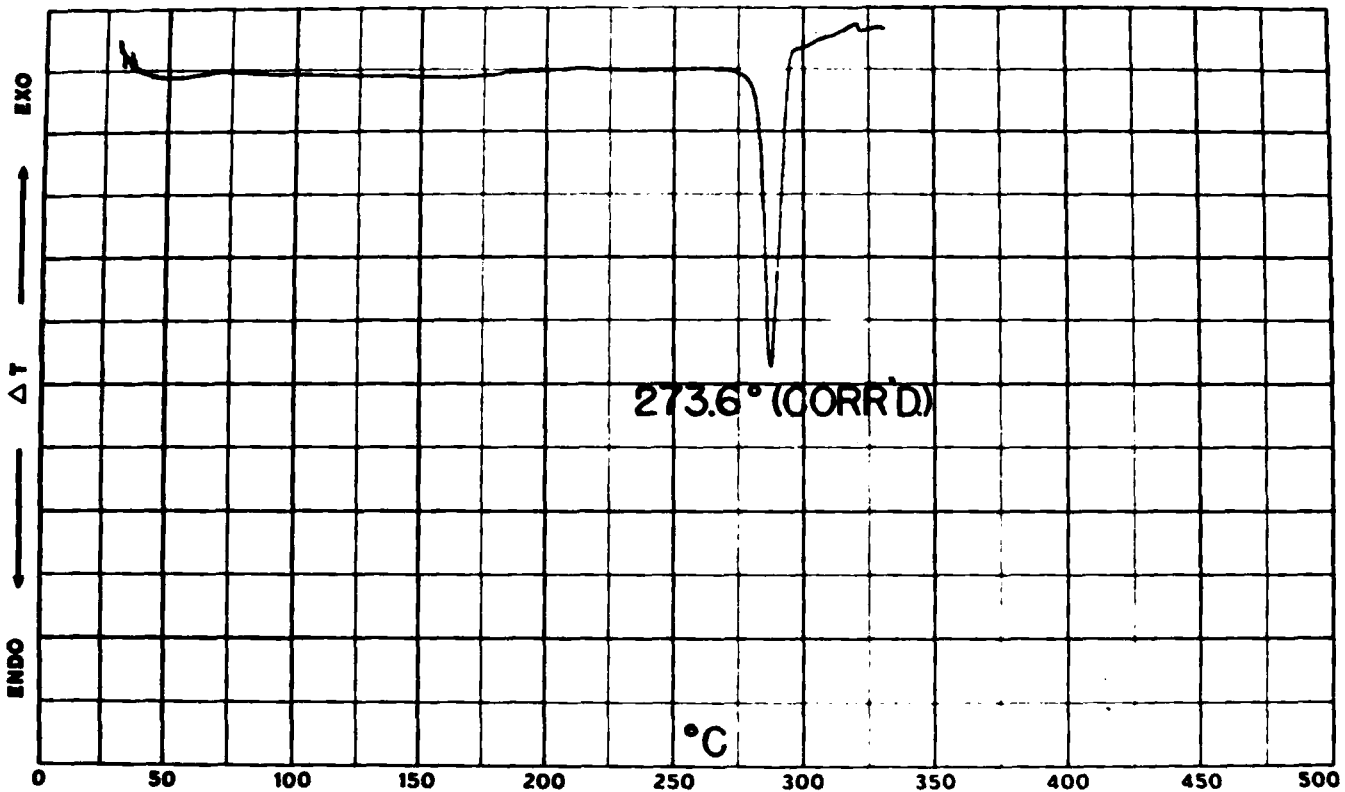


Figure 5. Differential Thermal Analysis Curve of Hydroflumethiazide.

TABLE I

X-RAY POWDER DIFFRACTION PATTERN OF HYDROFLUMETHIAZIDE

d(A)	I/I	d(A)	I/I
5.44	30	2.59	1
5.08	1	2.54	1
4.90	4	2.50	3
4.66	41	2.44	10
4.58	100	2.41	2
4.34	17	2.35	4
4.27	22	2.31	2
4.05	5	2.25	1
3.93	2	2.22	4
3.69	16	2.13	5
3.60	3	2.08	2
3.48	5	2.05	1
3.37	5	2.02	2
3.32	3	2.01	1
3.25	6	1.96	1
3.23	5	1.94	1
3.14	14	1.92	1
3.06	11	1.90	2
3.01	6	1.87	2
2.88	2	1.83	4
2.77	2	1.79	2
2.72	3	1.77	1

2.7 Solubility

Some room temperature solubilities are as follows:

Solvent	Approximate Solubility, mg/ml
Methanol	58.
Ethanol (95%)	21.
Ethyl Acetate	11.
Ethyl Ether (anhydrous)	0.2
Chloroform	0.1
Benzene	<0.1
Water	0.3 (15)
Acetonitrile	43.
Acetone	>100.

Kobinger and Lund report that hydroflumethiazide is easily soluble in the lower alcohols and ketones, tetrahydrofuran, and pyridine. The partition coefficient between ethyl acetate or methyl isobutyl ketone and water is about 30. It is also easily soluble in bases (15).

Hydroflumethiazide is soluble in a mixture of polyethylene glycol 400, N-methyl-2-pyrrolidinone, and water (40:5:55), or in a mixture of polyethylene glycol 400, dimethylformamide, and water (40:5:55); the solubilities are 11.2 and 15.8 mg/ml, respectively (16).

2.8 Ionization Constant

Kobinger and Lund report a pK_1 of 8.9 and a pK_2 of 10.7 at room temperature (15). Smith et al report a pK_1 of 8.73, determined by spectrophotometry at 273 nm, and of 8.79, determined by fluorometry with excitation at 333 nm and emission at 393 nm (17).

2.9 Fluorescence Spectra

Hydroflumethiazide fluorescence has an excitation maximum at 333 nm and an emission maximum at 393 nm. Fluorescence is much more intense at pH 8 or less (17).

2.10 Melting Points

Reported melting points range from about 200° to 275°C (18-36, 38). The melting point is specified as 270° to 275°C in NF XIV.

3. SYNTHESIS

Hydroflumethiazide has been prepared by various procedures from trifluoromethyl-5-aminobenzene-2,4-disulfonamide (18-28, 30, 32, 34-38), trifluoromethyl-5-aminobenzene-2,4-disulfonyl chloride (31, 33), or 6-(trifluoromethyl)-7-sulfamyl-1,2,4-benzothiadiazine 1,1-dioxide (29). A few of these procedures including syntheses of the starting materials are outlined in Figure 6.

4. STABILITY-DEGRADATION

Kobinger and Lund (15) reported 1 percent loss in 24 hours in 0.1N hydrochloric acid at 38°C, and 0.5 percent in 24 hours in 0.1N sodium hydroxide at room temperature. However, boiling in 3N sodium hydroxide for 2 hour quantitatively converted hydroflumethiazide to 2,4-disulfamyl-5-trifluoromethylaniline.

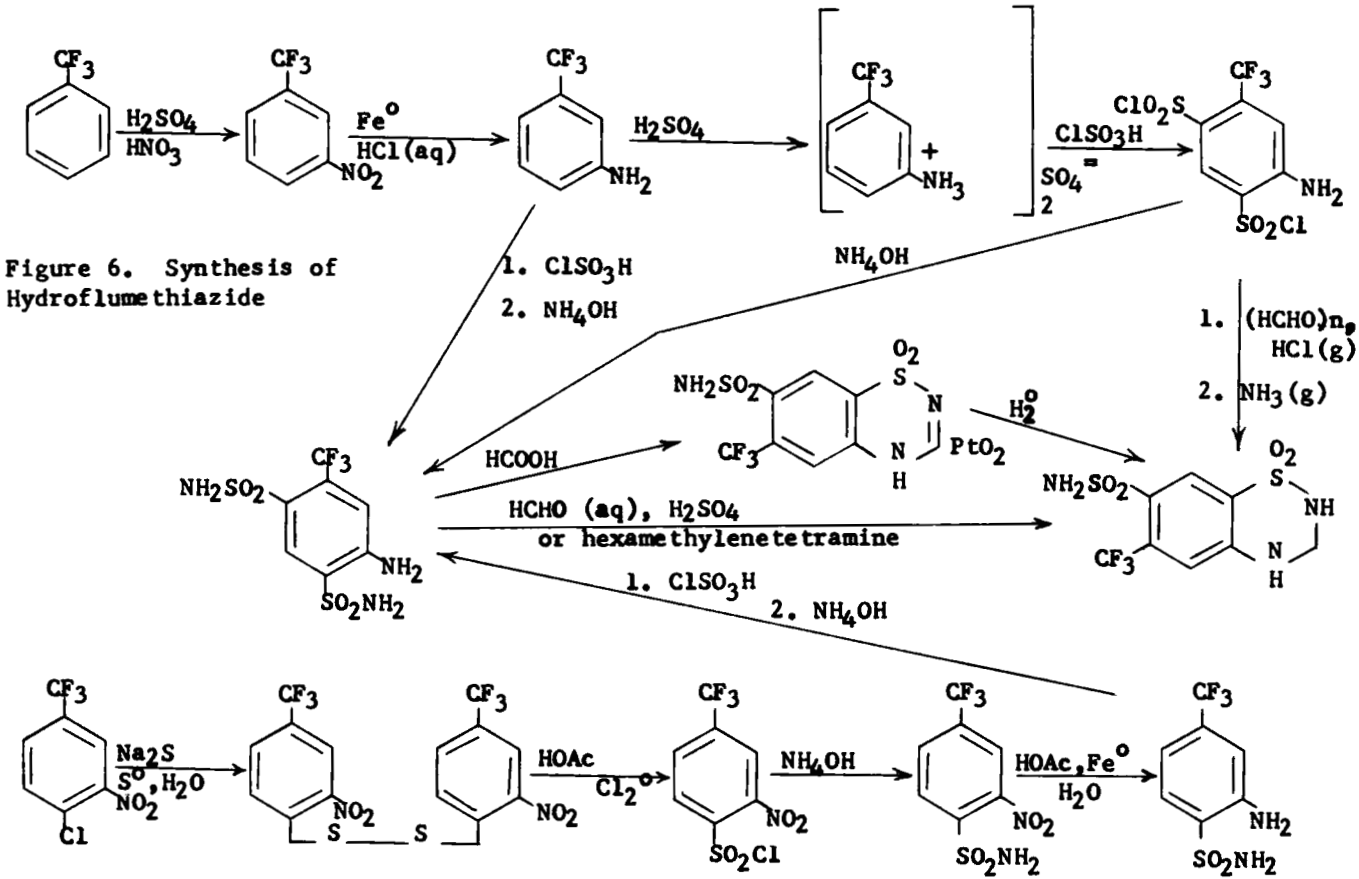


Figure 6. Synthesis of Hydroflumethiazide

Fazzari (8) reported that less than 0.04 percent of the disulfonamide formed when hydroflumethiazide was stored for 5 hours in 0.2N sodium hydroxide at room temperature.

5. METABOLISM

No reports of metabolic studies on hydroflumethiazide were found.

6. METHODS OF ANALYSIS

6.1 Identification Tests

Hydroflumethiazide is easily identified by the properties described in section 2 above. Where identification of hydroflumethiazide in solid formulations is necessary, it can be extracted with a small volume of sodium hydroxide solution; the extract is made slightly acid to precipitate the hydroflumethiazide, which can be washed with water and dried before further testing. Hydroflumethiazide can be extracted from acidic aqueous solution with diethyl ether and recovered by evaporating the ether extract; the recovered material can be identified from its infrared spectrum, as reported by Fazzari (8) and as directed in NF XIV (39). Ethyl acetate and methyl isobutyl ketone are also suitable for extraction of hydroflumethiazide (15).

If the identification of small amounts of hydroflumethiazide is necessary, thin layer chromatography procedures are quite specific. Several procedures for identifying thiazide diuretics, using thin layer chromatography (40, 41, 42, 43, 44, 45) or paper chromatography (4), with various detection methods, have been reported. Ultraviolet absorption spectra in acid and alkaline solution show comparatively small shifts in wavelength maxima in the case of hydroflumethiazide (4), but may help to differentiate it from some other thiazides.

Microscopic tests for hydroflumethiazide are described by Groenewegen (14) and by Kuhnert-Brandstätter et al (13).

6.2 Elemental Analysis

The elemental composition of hydroflumethiazide is as follows:

Element	% Theory
Carbon	29.00
Hydrogen	2.43
Fluorine	17.21
Nitrogen	12.68
Oxygen	19.32
Sulfur	19.36

6.3 Colorimetric Method

Bermejo (46) determined hydroflumethiazide by hydrolyzing it to 2,4-disulfamyl-5-trifluoromethylaniline, diazotizing the hydrolysis product and coupling to chromotropic acid or to N-(1-naphthyl)ethylenediamine dihydrochloride. Kobinger and Lund (15) also indicate that this technique can be used for quantitation of hydroflumethiazide.

6.4 Titration Methods

DePaulis and Dipietromaria (7) titrated hydroflumethiazide in anhydrous ethylenediamine solution with sodium methoxide in benzene-methanol (85:15), using a saturated solution of azo violet in benzene as indicator.

Chiang (47) titrated hydroflumethiazide in dimethylformamide with sodium methoxide in benzene-methanol solution, using a saturated solution of p-nitrobenzene-azo-resorcinol in benzene as indicator; thymol blue indicator gave unsatisfactory endpoints. Sugar and lactose interfered, however.

6.5 Paper Chromatography

Adam and Lapiere (40) used two systems for paper chromatography of hydroflumethiazide: (a) Whatman No. 1 paper, with 1-pentanol, 12N ammonia, and water (80:20:60) as mobile phase; (b) Whatman No. 1 paper impregnated with formamide, with chloroform and 1-pentanol (80:20) as mobile phase. The spots were detected using short wavelength ultraviolet radiation.

Pilsbury and Jackson (4) also used two systems to differentiate hydroflumethiazide from other thiazide diuretics: (a) Whatman No. 3 paper impregnated with tributyrin, developing for 20 minutes at 90°C with pH 7.4 phosphate buffer; (b) Whatman No. 1 paper, with amyl alcohol and ammonia (9:1) as mobile phase. The spots were located using short wavelength ultraviolet radiation, and also by spraying with 0.1N sodium hydroxide solution followed by a saturated solution of sodium 1,2-naphthoquinone-4-sulfonate in ethanol and water (1:1); the latter treatment develops an orange-red color

with thiazides.

6.6 Column Chromatography

Fazzari (8, 48) used a basic Celite® column to purify tablet extracts prior to ultraviolet quantitation. Sohn et al (43) used non-ionic resin columns to recover and clean up thiazides in urine prior to thin layer chromatography.

Hydroflumethiazide can be separated and quantitated by column chromatography with a gravity fed eluant as follows (49):

Column: Sephadex® LH-20, 23 cm x 1.2 cm I.D.
Eluant: Methanol-cyclohexane-chloroform-acetic acid (40:30:30:1 vol/vol)
Elution Volume: 72 to 117 ml
Quantitation: Fluorescence - 333 nm excitation, 380 nm emission
Sample: About 100 μg of hydroflumethiazide

Pressurized liquid chromatography can also be used to determine hydroflumethiazide. The following system is suitable (49):

Column: Waters μ-Bondapak® C_{18}, 30 cm x 4 mm I.D. pre-packed column
Solvent: Methanol - 0.02M pH 7.4 phosphate buffer (2:8 vol/vol)
Pressure: 1500 psi
Flow Rate: 1 ml/minute
Sample: About 1 μg
Detection: Schoeffel Variable Wavelength Ultraviolet Detector, 280 nm

6.7 Ultraviolet Methods

Identification of hydroflumethiazide by ultraviolet absorption was discussed in sections 2.3 and 6.1.

Fazzari (8) developed a partition chromatography-ultraviolet method for hydroflumethiazide in drug formulations. The compound is held on an alkaline Celite® column, which is washed with chloroform and ether, then eluted with acetic acid in ether. The eluate is then extracted with 0.2N sodium hydroxide and the strength of the sample is determined by the absorptivity at 273 nm. This method is now the NF assay procedure for hydroflumethiazide tablets (39).

Other investigators (4, 5, 6, 7) have described the use of ultraviolet absorption measurements to determine hydroflumethiazide.

6.8 Polarography

Polarographic studies on the reduction of hydroflumethiazide have been reported (50, 51, 52, 53). These are primarily studies of the reduction of the trifluoromethyl group or sulfonamide group, although Lund (51) reports the assay of hydroflumethiazide in urine by polarography.

6.9 Thin Layer Chromatography

Table II lists thin layer chromatography systems for hydroflumethiazide. Hydroflumethiazide can be located on the plate by examination under an ultraviolet lamp, or by any of the spray reagents described (42, 43, 44, 45, 54).

Garceau et al (55) reported a quantitative thin layer chromatography procedure with fluorometric detection, for the determination of hydroflumethiazide in urine and plasma. The limit of detection was reported to be 10 ng of drug per ml of plasma.

ACKNOWLEDGMENTS

The writers wish to thank Dr. B. T. Kho for his review of the manuscript, Dr. G. Schilling of Ayerst Research Laboratories for his mass spectral data and interpretation, the library staff for their literature search, and Mrs. B. Juneau for typing the profile.

TABLE II

THIN LAYER CHROMATOGRAPHY SYSTEMS FOR HYDROFLUMETHIAZIDE

Absorbent	Solvent System	Rf	Reference
Alumina G	Ethyl Acetate	0.36	40
Silica Gel G	Ethyl Acetate-Benzene (80:20)	0.41	40
Silica Gel G	Ethyl Acetate	0.46	40
Alumina GF254	Ethyl Acetate-Water (98:2)	0.52	41
Silica Gel DSF5	Ethyl Acetate-Water (197:3)	0.53	41
Silica Gel GF	Toluene-Xylene-1,4-Dioxane-2-Propanol-25% Ammonia (1:1:3:3:2)	0.43	42
Silica Gel G	Benzene-Ethyl Acetate (2:8)	NR*	43
Silica Gel G	Ethyl Acetate-Methanol-Ammonia (85:10:5)	NR*	43
Silica Gel G	Ethyl Acetate-Benzene (8:2)	0.78	54
Silica Gel F254	Ethyl Acetate	0.45	44
Silica Gel F254	Ethyl Acetate	0.45	45
Silica Gel G25-22	Ethyl Acetate-Methanol-0.1N Ammonia (96:2:2)	0.52	55

*Not Recorded

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Literature surveyed through March, 1976.

Analytical Profiles of Drug Substances, 7

HYDROXYZINE DIHYDROCHLORIDE

Josef Tsau and Nicholas DeAngelis

ISBN 0-12-260807-0

Contents

1. Introduction

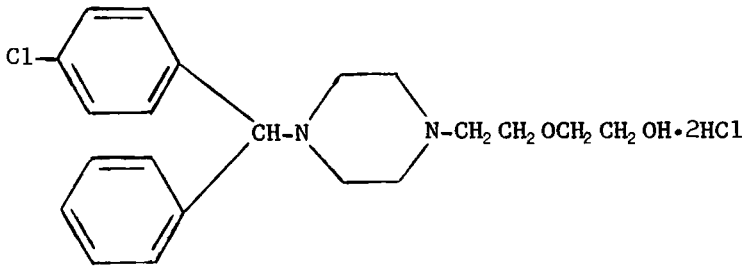
Hydroxyzine is classified as an antihistaminic drug which has several pharmacological effects: sedation, relaxation of muscles, and hypnotic properties. It is comparatively non-toxic and chemically unrelated to phenothiazine, reserpine, and meprobamate drugs.[1,2]

Hydroxyzine is mainly used to reduce anxiety and tension, for skeletal muscular relaxation or as an antiemetic drug. Its antispasmodic effect is probably due to its interference with the mechanism that responds to spasmogenic agents such as serotonin, acetylcholine, and histamines.[3]

2. Description

2.1 Name, Formula, Molecular Weight

Hydroxyzine dihydrochloride is designated as ethanol, 2-[2-[4-[(4-chlorophenyl) phenylmethyl]-1-piperazinyl] ethoxy]-, dihydrochloride in Chemical Abstracts and NF XIV[4]. The commonly used trade names are Atarax, Ataraxoid, Cartrax, Enarax, Marax, and Vistaril.



$C_{21}H_{27}ClN_2O_2 \cdot 2HCl$ Mol. Wt. = 447.83

2.2 Appearance, Color, Odor

Hydroxyzine dihydrochloride is a white, odorless crystalline powder.

3. Physical Properties

3.1 Infrared Spectrum

The infrared (I.R.) spectrum of hydroxyzine dihydrochloride is given in Figure 1, and is comparable to that published by Hayden et al.[5] The spectrum was

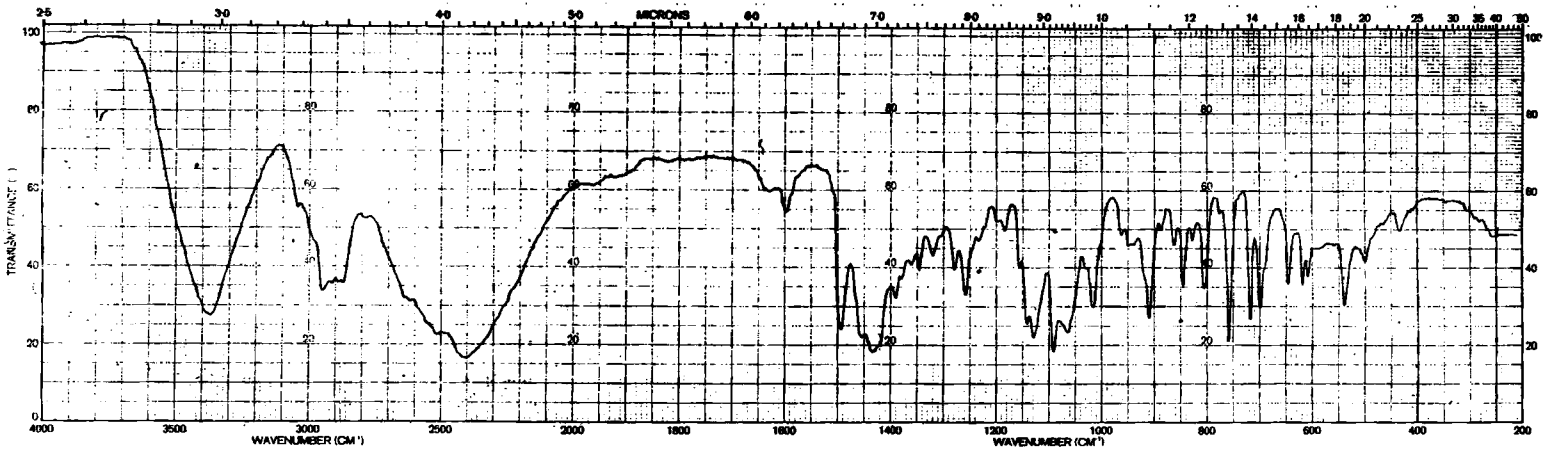


Figure 1-I.R. Spectrum of Hydroxyzine Dihydrochloride, NF Reference Standard LOT NO. 6818.
1% KBr Pellet-Instrument: Perkin Elmer Model 467

recorded with a Perkin-Elmer 467 Grating I.R. Spectrophotometer and was obtained in a 1% KBr pellet. Structural assignments of some of the significant bands are given in Table I.

Table I

Infrared Assignments for Hydroxyzine Dihydrochloride

Frequency range (cm^{-1})	Assignment
3100-3700	O-H stretching
2800-3100	C-H stretching
2410	tertiary amine salt[6]
1596, 1495, 1455	aromatic C=C in plane skeletal vibrations
1090	C-O-C stretching
700, 760, 805	aromatic CH out-of-plane bending

3.2 Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance (NMR) spectrum of hydroxyzine dihydrochloride in deuterated dimethylsulfoxide (d_6) containing tetramethylsilane as the internal standard is shown in Figure 2. This spectrum was obtained on a Varian A60 NMR spectrometer. The spectral peak assignments are given below:

Chemical Shift (ppm)	protons
3.0-4.4	all methylene protons (16)
6.0-6.4	methine proton (1)
7.2-8.3	all aromatic protons (9)

The NMR spectrum after D_2O exchange indicates that there are 3 exchangeable protons (1 from OH and 2 from HCl groups) which are likely broad and weak peaks hidden under aromatic proton peaks and methylene proton peaks.

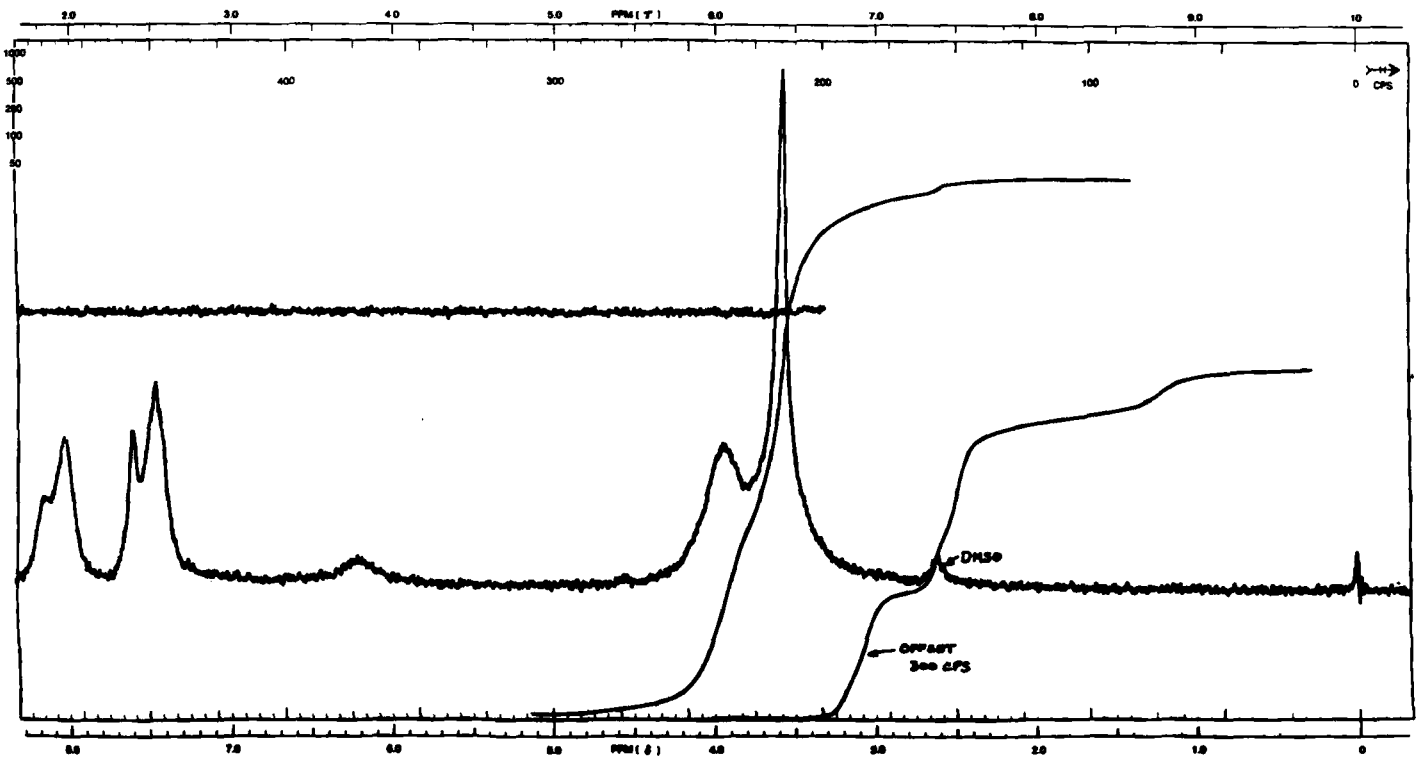


Figure 2-NMR Spectrum of Hydroxyzine Dihydrochloride, N.F. Reference Standard LOT NO. 6818. Solvent: Deuterated Dimethylsulfoxide, Internal Standard: tetramethylsilane. Instrument: Varian A 60.

3.3 Ultraviolet Spectra

The ultraviolet (UV) absorption spectra of hydroxyzine dihydrochloride in acetate buffer at pH 4.5, in dilute NaOH solution at pH 10.3 and pH 1.8 in dilute HCl solution are given in Figure 3. The spectra were recorded on a Cary 14 Spectrophotometer. The UV spectra all have a λ max at 230 nm with an "a" of 33.0 at pH 4.50, 33.5 at pH 10.3 and 35.2 at pH 1.83. These values agree reasonably well with that previously reported[7] ("a" = 35.0). The overall UV spectrum of hydroxyzine dihydrochloride is characteristic of disubstituted benzenes and some biphenylmethyl derivatives.[8] We found that that part of the spectrum due to the benzenoid groups is pH dependent, Figure 4. The UV spectrum of hydroxyzine dihydrochloride in ethanol is similar to those shown in Figure 3[5].

3.4 Ionization Constant

The first stage ionization constant of hydroxyzine dihydrochloride was determined potentiometrically[9] to be

$$pK_a = 1.834 + \left[1.018\sqrt{I}/(1 + 0.517\sqrt{I})\right] + 0.160\ I$$

where I is the ionic strength adjusted with NaCl. The authors also determined the pK_a spectrophotometrically to be 2.0.

3.5 Melting Range

Hydroxyzine dihydrochloride melts at about 200°C with decomposition.[4] Its melting range is reported elsewhere to be 192-3°C.[2,10,11]

3.6 Thermal Analyses

The DSC thermogram of hydroxyzine dihydrochloride obtained at a scan rate of 10°C/min. exhibited a wide range endotherm from 190°C to 225°C, where melting was accompanied by decomposition. The TGA thermogram obtained at a scan rate of 10°C/min. showed no weight loss from room temperature to 150°C, about 10% loss from 150°C to 200°C and over 80% loss above 250°C.

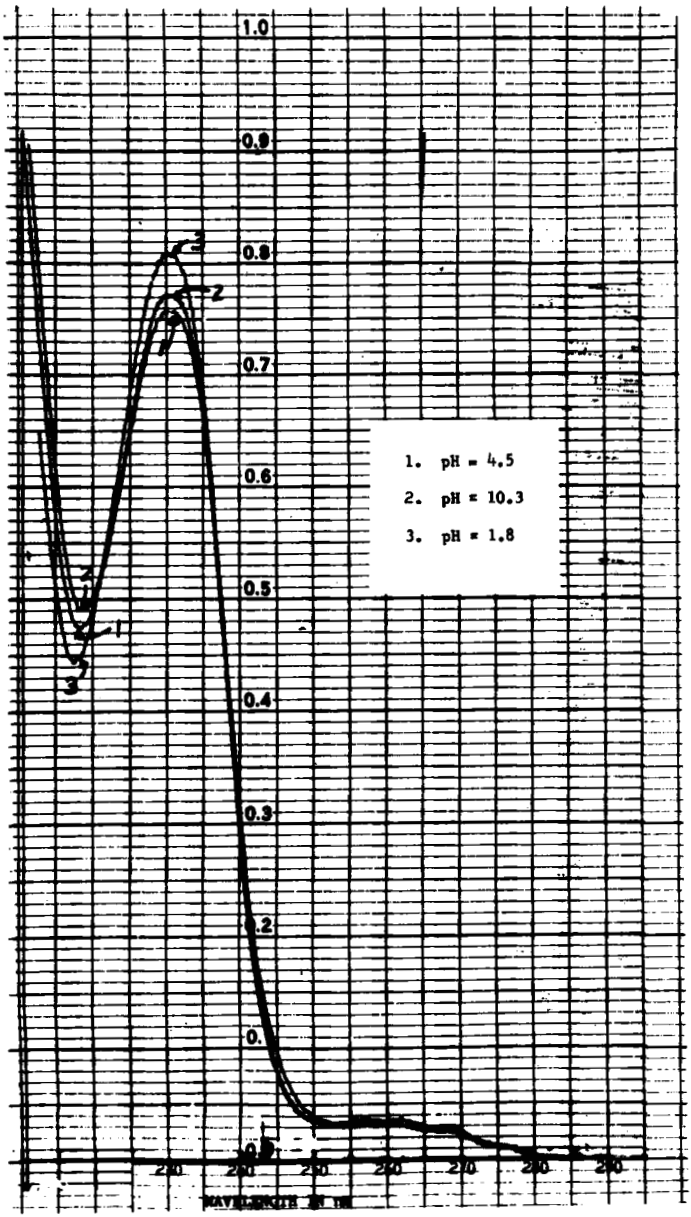


Figure 3-UV Spectra of Hydroxyzine Dihydrochloride,
Instrument: Cary 14

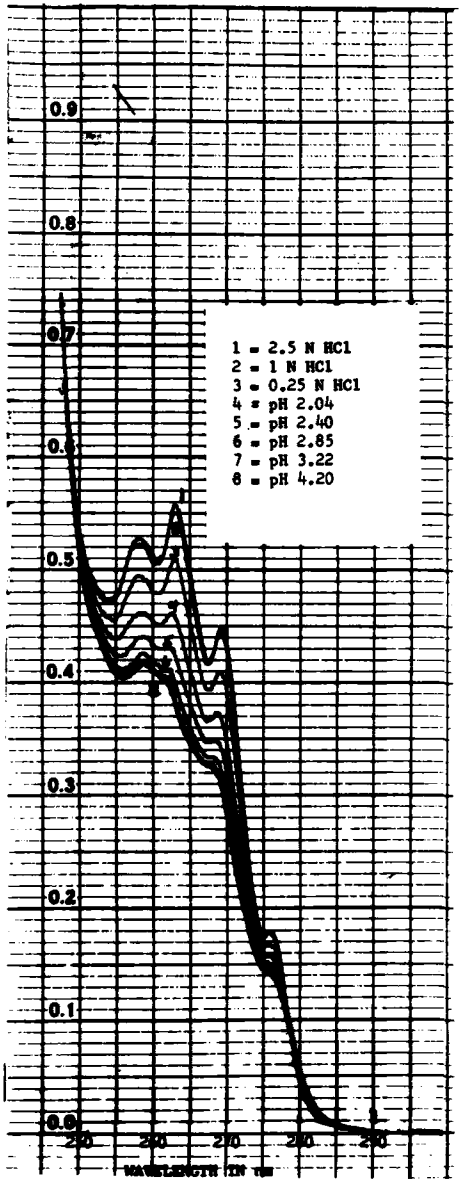


Figure 4-UV Spectra of Hydroxyzine Dihydrochloride, Instrument: Cary 14

3.7 Crystal Properties and X-Ray Powder Diffraction

The crystal habit of hydroxyzine obtained from both sublimation and recrystallization from 95% alcohol is needle-like crystals.[12]

The X-ray powder diffraction pattern of hydroxyzine dihydrochloride obtained with a Norelco Phillips diffractometer using CuK_{α} radiation is shown in Figure 5. The calculated d-spacings are given in Table II, and agree well with those previously published.[38]

Table II

X-Ray Powder Diffraction Pattern For Hydroxyzine Dihydrochloride

2 θ (degree)	d (Å)	I/Io
6.2	14.28	19
8.2	10.79	14
11.8	7.49	6
12.4	7.13	6
13.4	6.59	45
14.5	6.09	43
15.3	5.78	46
16.8	5.26	50
17.2	5.13	100
19.5	4.53	18
20.2	4.37	56
21.4	4.13	30
22.3	3.96	46
23.5	3.76	39
24.7	3.58	64
26.9	3.28	89
27.6	3.20	54
29.1	3.04	31
30.0	2.94	26

d (interplanar distance) = $\frac{n\lambda}{2 \sin \theta}$

I/Io = relative intensity

3.8 Solubility

The authors determined the solubility of hydroxyzine dihydrochloride to be $>$700 mg/ml in water, 60 mg/ml in chloroform, 2 mg/ml in acetone and $<$0.1 mg/ml in ether.

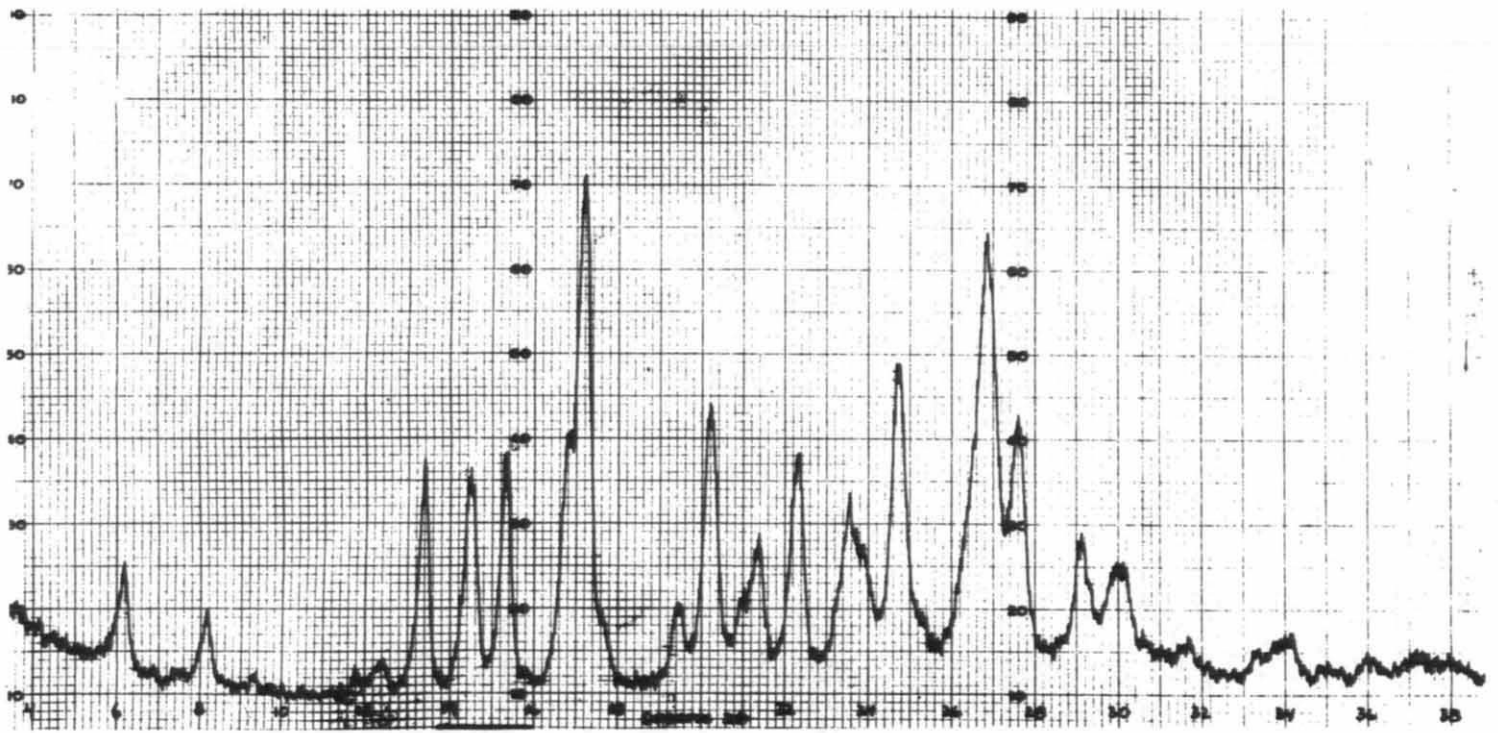


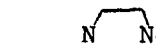
Figure 5-X-Ray Diffraction Pattern of Hydroxyzine Dihydrochloride, NF Reference Standard Lot No. 6818. Radiation-CuK_{α} , Instrument: Norelco Philips Diffractometer.

3.9 Mass Spectrum

The electronic impact (E.I.) mass spectrum of hydroxyzine dihydrochloride shown in Figure 6 (spectrum is of the hydroxyzine base) was obtained with a MS-902 double focusing, high resolution mass spectrometer. The probe temperature was 200°C and the ionization electron beam energy was 70 eV. Structural assignments of the major peaks are given in Table III for E.I. and chemical ionization (C.I.).

Table III

High Resolution Mass Spectrum of Hydroxyzine

M/e	Assignment	% Relative Intensity
375 (C.I.)	MH^+	90
374.1732 (E.I.) (Calc. 374.1761)	M^+	6
299	$M^+ - CH_2OCH_2CH_2OH$	18
201		100
173		10

4. Synthesis

The synthesis of piperazine ethers including hydroxyzine dihydrochloride were first reported by Morren.[10,11] There are five major synthetic methods depending on the availability of starting material and intermediates.[11] A typical reaction scheme is given in Figure 7.[13]

5. Stability

Hydroxyzine dihydrochloride solutions are unstable when irradiated with intense UV light.[14,15] The authors obtained the results given below on hydroxyzine dihydrochloride solutions (10 mg/ml) stored in sealed ampules under N_2 atmosphere for 15.5 months.

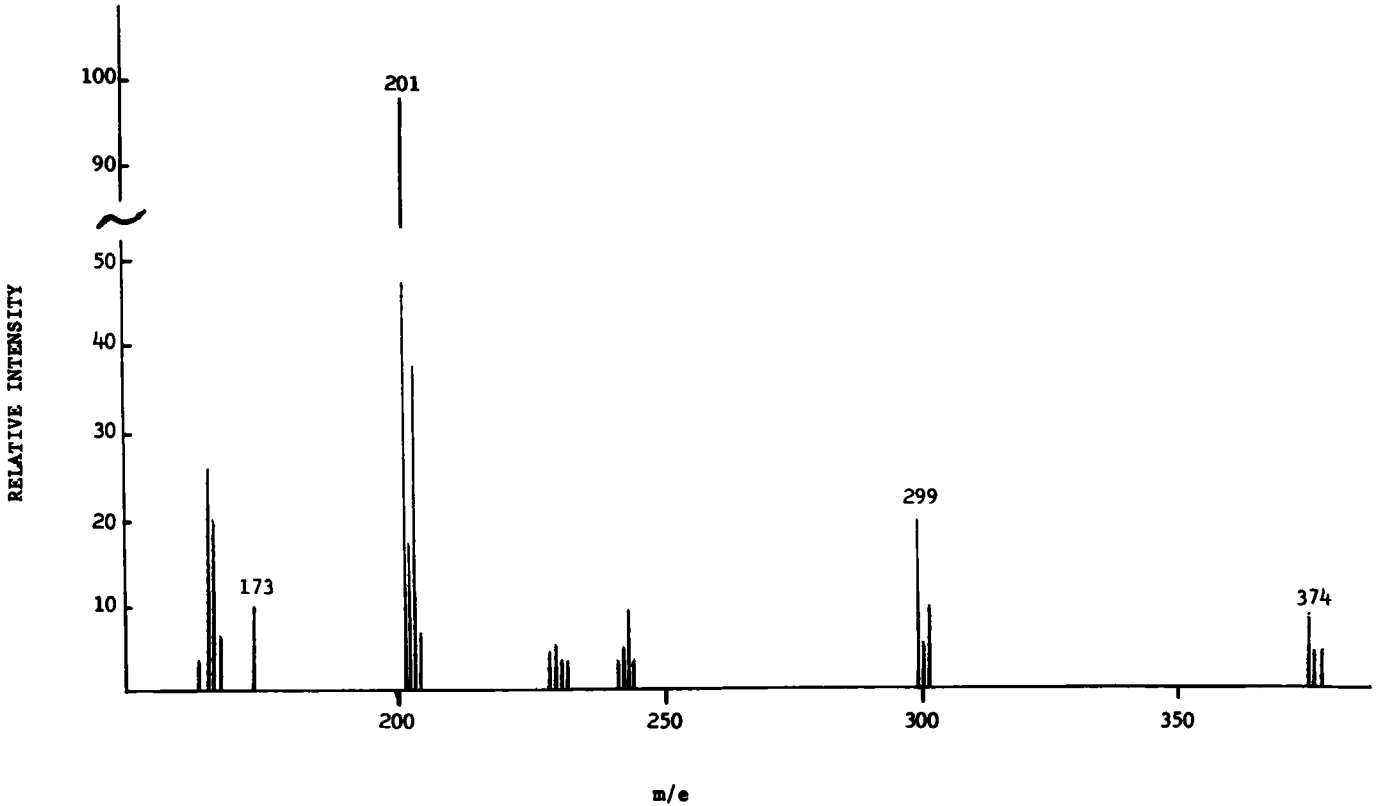
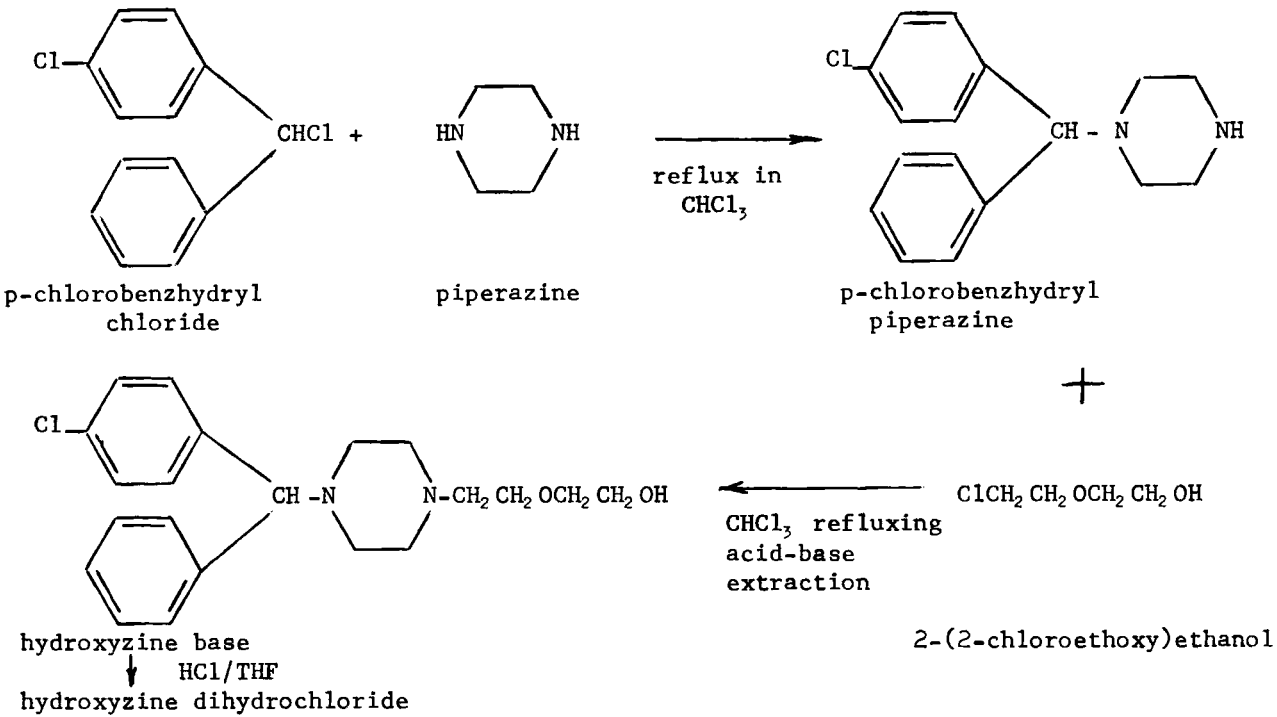


Figure 6-Mass Spectrum (Electron Impact) of Hydroxyzine Dihydrochloride, NF Reference Standard Lot No. 6818. Instrument: MS 902.

Figure 7

A Synthetic Route for Hydroxyzine Dihydrochloride



pH	Percent of initial concentration		
	RT	60°C	75°C
1	101	0	0
3	101	51	9
5	100	93	72
7	94	51	42

This study indicates that hydroxyzine solution is most stable at a pH of approximately 5.

6. Distribution, Excretion, and Metabolism

In rats after intraperitoneal administration, hydroxyzine distributes rapidly to the organs with the highest concentration in lungs, followed by fat, liver, spleen and kidneys.[16] Hydroxyzine is also rapidly absorbed in the gastrointestinal tract.[3]

Hydroxyzine is excreted mainly within the first day, in feces via the bile. It is completely metabolized in bile and urine principally to the glucuronides of the diphenylmethane derivatives.[16]

The following metabolites were identified in rats[16,17,18]; p-chloro-p'-hydroxybenzophenone,p-chlorobenzhydrol and its glucuronide, p-chlorobenzophenone, piperazine, 2-[2-(1-piperazinyl) ethoxy] ethanol, norchlorcyclizine and hydroxyzine N-oxide.

7. Identification and Elemental Analysis

Hydroxyzine dihydrochloride can be identified by means of its characteristic I.R. and U.V. spectra or by means of the many chromatographic methods given in Section 8.

The theory elemental analysis is: C=56.3%, H=6.53%, N=6.26%, Cl=23.75%, O=7.14%.

8. Methods of Analysis

8.1 Non-Aqueous Titration

The NF XIV[4] describes non-aqueous titration methods to determine hydroxyzine dihydrochloride drug substance as well as injection, tablet, and syrup dosage forms. The procedure involves dissolving about 150 mg of hydroxyzine dihydrochloride drug substance, or for dosage forms chloroform extraction of hydroxyzine base from alkaline solution. Glacial acetic acid and mercuric acetate are added and the solution is titrated with 0.1N perchloric acid using quinaldine red as the indicator.

Two procedures which are very similar to the above NF method were described by Ciaccio et al,[19] and Pasich and Stasiewska.[20]

In another method[21] hydroxyzine was precipitated by sodium tetraphenylborate from acidic solution. This precipitate was redissolved in 50% acetone, acidified with acetic acid and potentiometrically titrated with $AgNO_3$ solution.

8.2 Formation of Color Derivatives

With concentrated H_2SO_4 hydroxyzine develops a yellow color with a maximum absorbance at 460 nm.[22,23]

Hydroxyzine dihydrochloride when treated with cobalt thiocyanate reagent and extracted with $CHCl_3$ saturated with ammonium thiocyanate produces a color which has a maximum absorbance near 600 nm.[24]

8.3 Conductometric Titration

Hydroxyzine dihydrochloride drug substance and tablet dosage forms were analyzed by titrating conductometrically using picric acid as titrant.[25]

8.4 Chromatographic Methods

8.4a. Gas Liquid Chromatography (GLC)

The following GLC methods have been reported.
Method 1- For biological fluids[26]

column: 1/8" x 7' stainless steel packed with 3% OV-17 on Gas Chrom. Q, 100-120 mesh.
carrier gas: N_2, 60 ml/min
temperatures: column 275°C, detector 300°C, inlet block 300°C
detector: FID
retention time: 5 minutes
sample preparation: two extractions from pH 7.2 buffer into ether. Evaporate to dryness. Reconstitute in 100 µl acetone containing internal standard griseofulvin
comment: GLC-mass spectrometry recommended by the authors to increase sensitivity.

Method 2-Cardini et al[27] showed that by using 2% GE-SE30 on Aeropak (Varian Aerograph), 80-100 mesh, hydroxyzine was well separated from other tranquilizers, such as glutethimide, methaqualone and carisoprodol.

Method 3-Method 1 was adapted to determine hydroxyzine in an injection formulation, and was proven to be stability indicating.[28]

column: 2 mm i.d. x 70 cm glass column packed with 3% OV-17 on Gas Chrom Q, 100-120 mesh.
carrier gas: N_2
temperatures: column 275°C, detector, 300°C, injection port 300°C
detector: FID
retention time: 3 minutes
sample preparation: 3X extraction of the equivalent of about 100 mg of hydroxyzine from NaCl saturated alkaline solution into chloroform and then add internal standard (n-dotriacontane).

8.4b. High Performance Liquid Chromatography (HPLC)

The authors have developed a HPLC method to determine hydroxyzine dihydrochloride in injection formulations. This method is stability indicating. The chromatographic conditions are given below.

column: Bondapak phenyl/corasil, 35-50 µm, 4 mm i.d. x 50 cm (Waters Assoc.)
mobile phase: 0.25% $(NH_4)_2CO_3$ in water: acetonitrile (5:3)
flow rate: 1.5 ml/min

detector wavelength: 230 nm
sample and standard solutions: prepare and properly dilute with distilled water to about 0.1 mg/ml.

8.4c. Paper Chromatography

Sybirska and Gajkzinska[29] reported a cation exchange paper chromatographic method to quantify hydroxyzine. Several other paper chromatographic methods have been developed to systematically identify tranquilizing drugs including hydroxyzine.[30,31]

8.4d. Thin Layer Chromatography (TLC)

TLC was used to study hydroxyzine dihydrochloride and its metabolites in biological fluids and tissues.[16,32,33] The chromatographic behavior of hydroxyzine dihydrochloride in several TLC systems is given in Table IV and various detection methods are given in Table V.

Table IV

TLC Methods for Hydroxyzine Dihydrochloride

Eluent	Adsorbent	Impregnated with	R_f	Ref
methanol:12N NH_4OH(100:1.5)	Silica Gel G	-	0.68	32
benzene:ethanol:12N NH_4OH (95:15:5)	"	-	0.4	"
cyclohexane:diethylamine: benzene (75:20:15)	"	0.1N NaOH	0.22	"
acetone	"	"	0.33	"
chloroform:methanol (90:10)	"	"	0.59	"
cyclohexane:benzene:diethyl-amine (75:15:10)	Silica Gel	0.1M KOH	0.08	34
methanol	"	"	0.59	"
acetone	"	"	0.39	"
methanol	"	0.1M $NaHSO_4$	0.56	"
95% ethanol	"	"	0.25	"
chloroform:acetone:NH_4OH (80:20:1)	Silica Gel-gypsum	-		35
methanol:acetone(12:88)	Silica Gel G UV254	0.1N Na_2CO_3	0.45	33
ethanol:carbon tetra-chloride (16:84)	Silica Gel G UV254	0.1N Na_2CO_3	0.40	"
methanol:benzyl alcohol(31.7:68.3)	"	"	0.48	"
ethanol:toluene (68:32)	"	"	0.53	"

Table IV

TLC Methods for Hydroxyzine Dihydrochloride

Eluent	Adsorbent	Impregnated with	R_f	Ref
methanol:cyclohexane:methyl acetate (17.8:33.6:48.6)	Silica Gel G UV254	0.1N Na_2CO_3	0.49	33
acetone:methanol: ammonium hydroxide (50:50:1)	Silica Gel G	-	0.64	36
cyclohexane:benzene: diethylamine (75:15:10)	Silica Gel G	-	0.41	16
methanol:acetone(1:1)	"	-	0.8	"
95% ethanol	"	-	0.68	"
n-butanol:ethanol:water (5:2:2)	"	-	0.65	"
isoamyl alcohol (water saturated): acetic acid (85:15)	"	-	0.30	"
chloroform:cyclohexane (1:1)	"	-	0	"

Table V

Detection of Hydroxyzine Dihydrochloride on Thin-Layer Plates

Detecting reagent	Color		Fluorescence		Ref
	Room temp	100°C	Room temp	100°C	
A	white	bluish	-	-	32
B	-	yellow	-	gold	"
C	-	flesh	-	violet	"
D	yellow	-	-	-	37
E	brown	-	-	-	"
F	violet	-	-	-	"
G	brown	-	-	-	-
-	-	-	green-yellow	-	32

A. Folin-Ciocalteau reagent (Fischer Scientific Co., Cat. NO. SO-P-24) diluted 1:1 with distilled water prior to use.

B. FPN reagent

5% $FeCl_3$ solution: 70% perchloric acid-water (1:5): concentrated HNO_3-water (1:1)-1:9:10

C. Mandelin reagent

10 mg/ml of ammonium vanadate in concentrated H_2SO_4

D. K_2MnO_4 solution (1%)

E. Dragendorff reagent

F. Iodoplatinate reagent

G. Iodine chamber

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Analytical Profiles of Drug Substances, 7

6-MERCAPTOPURINE

Steven A. Benezra and Penelope R. B. Foss

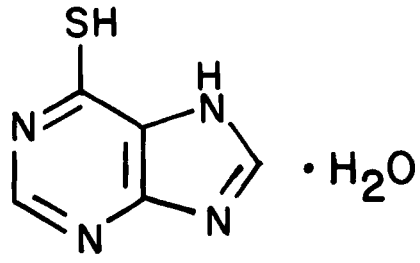
ISBN 0-12-260807-0

Table of Contents

1. Description

1.1 Name, Formula, Molecular Weight

Mercaptopurine is purine-6-thiol monohydrate



$C_5H_4N_4S \cdot H_2O$ 170.19

1.2 Appearance, Color, Odor

Mercaptopurine is a yellow, practically odorless, crystalline powder[1].

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of mercaptopurine (anhydrous) is shown in Figure 1[2]. It was taken as a 0.2% dispersion of mercaptopurine in KBr with a Perkin Elmer model 457 infrared spectrophotometer. Table I gives the infrared assignments consistent with the structure of mercaptopurine[3].

Table I
Infrared Spectral Assignments for Mercaptopurine

Band (cm^{-1})	Assignment
3420, 3490	Aromatic NH stretch
3120, 3040, 2780	Aromatic CH stretch
1200	C=S stretch
930	CH bend

2.2 Nuclear Magnetic Resonance (NMR) Spectrum

The NMR spectrum of mercaptopurine is shown in Figure 2[4]. It was obtained with a Varian CFT-20 80 MHz NMR spectrometer. Deuterated DMSO was used as the solvent with tetramethylsilane as an internal standard. Based on the NMR spectrum, the following proton assignments for mercaptopurine can be made.

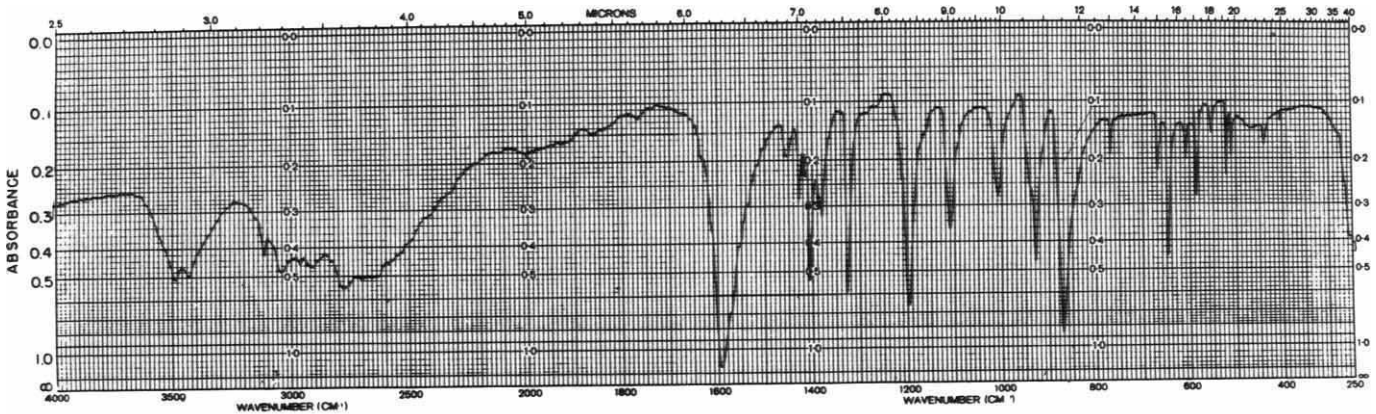


Figure 1- Infrared Spectrum of 6-Mercaptopurine

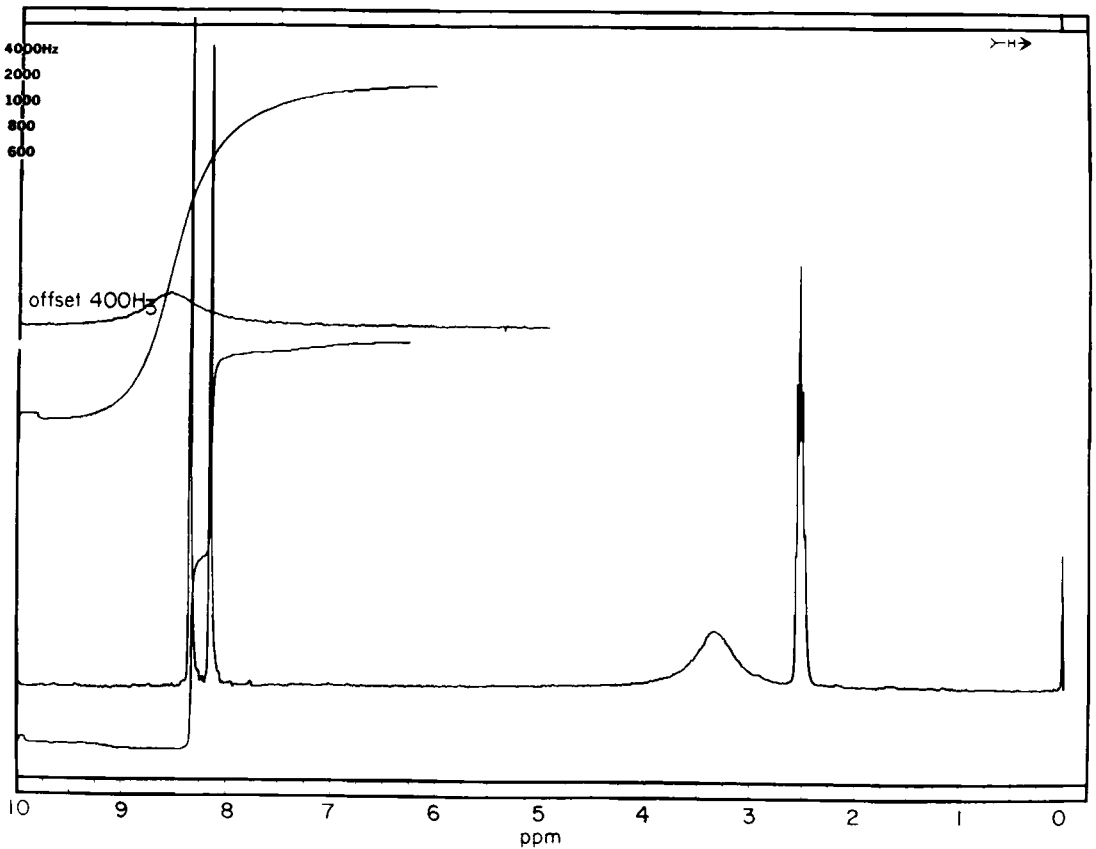


Figure 2- Nuclear Magnetic Resonance Spectrum of 6-Mercaptopurine

Proton	Chemical Shift (ppm)
S-H	2.75-3.75 broad singlet
C-H (aromatic)	8.15 singlet
C-H (aromatic)	8.35 singlet
N-H	11.0-12.0 broad singlet

2.3 Ultraviolet (UV) Spectrum

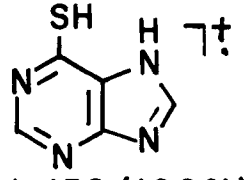
The UV spectra of mercaptopurine in 0.1N NaOH, 0.1N HCl, and methanol were taken with a Beckman ACTA CIII UV spectrophotometer and are shown in Figure 3[3]. Table II summarizes the UV data.

Table II
UV Spectral Data for Mercaptopurine

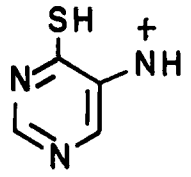
Solvent	λ max (nm)	ε
0.1N NaOH	230	14000(3)
	312	19600(5)
0.1N HCl	222	9240(3)
	327	21300(5)
Methanol	216	8940(3)
	329	19300(3)

2.4 Mass Spectrum

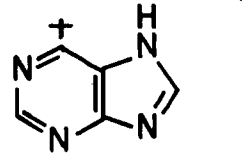
The low resolution mass spectrum of mercaptopurine is shown in Figure 4[6]. It was obtained with a Varian-MAT model 731 mass spectrometer. Direct probe at 145°C into the electron impact source was used. The electron energy was 70eV. The assignment of fragment ions is given below.



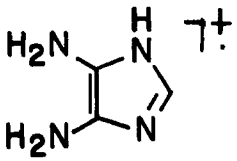
m/e 152 (100%)



m/e 125 (18%)



m/e 119 (16%)



m/e 98 (10%)

C_2NSH^+

m/e 71 (11%)

$(CN)^+$

m/e 26 (20%)

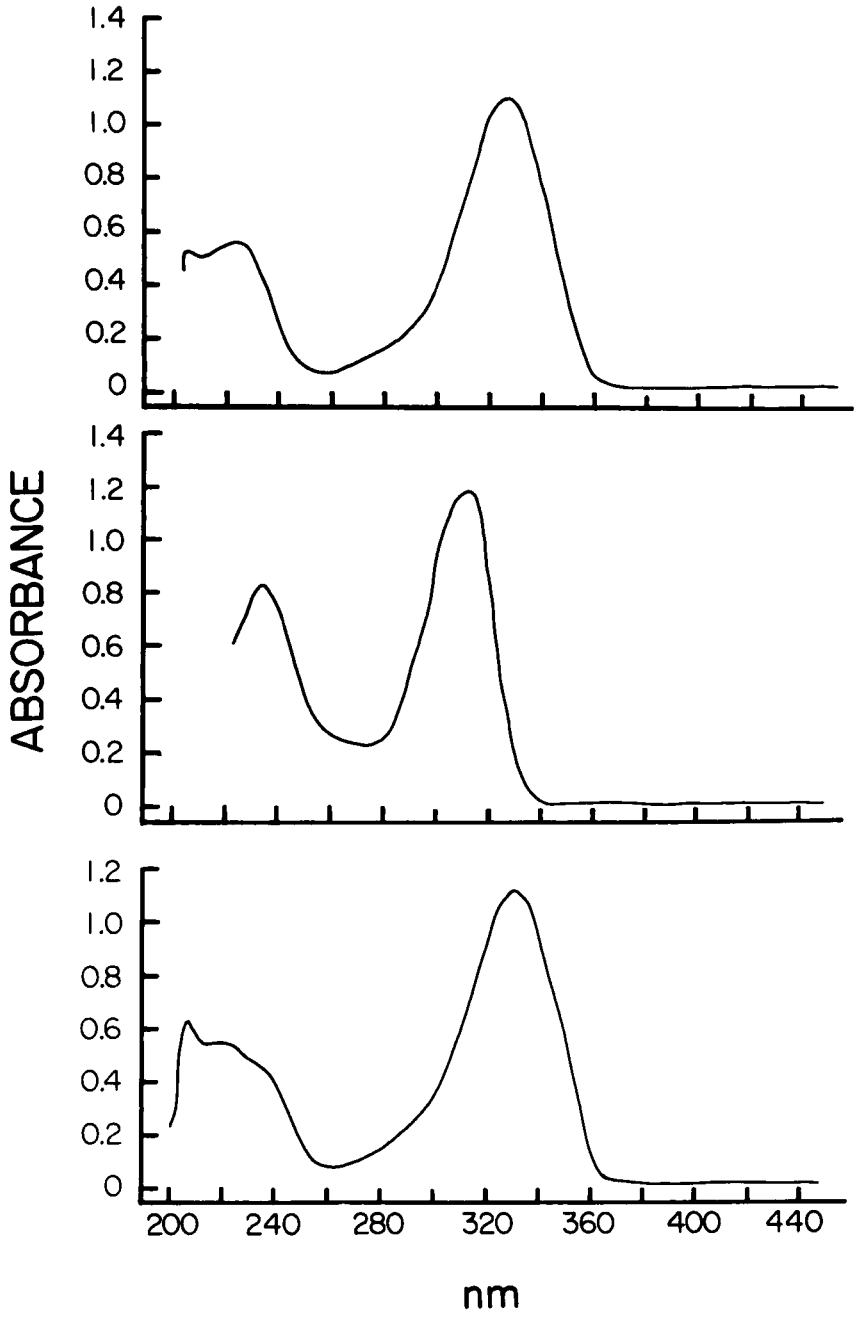


Figure 3- Ultraviolet Spectra of 6-Mercaptopurine
Top-0.1N HCl, Middle-0.1N NaOH, Bottom-Methanol

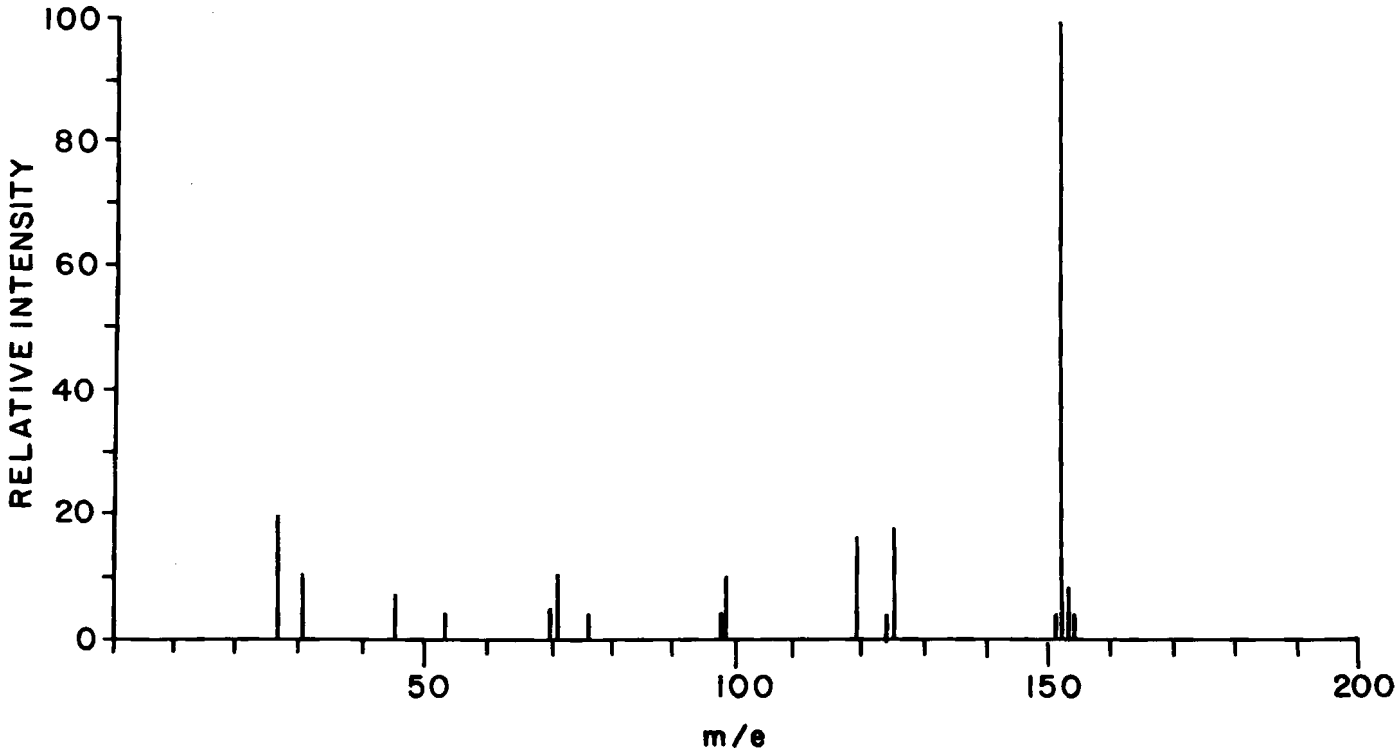


Figure 4- Mass Spectrum of 6-Mercaptopurine

2.5 Melting Point

Mercaptopurine decomposes above 308°C[1].

2.6 Solubility

Mercaptopurine is insoluble in water, acetone, and ether. It is soluble in hot ethanol and dilute alkali solutions[1]. It is slightly soluble in dilute sulfuric acid[1].

2.7 Dissociation Constant

The pK_{a1} and pK_{a2} of mercaptopurine determined potentiometrically is 7.77 and 11.17 respectively[7].

3. Synthesis

Mercaptopurine has been prepared by a variety of synthetic procedures[8-11]. These procedures are outlined in Figure 5. Process 1 involves treatment of 4-amino-5-formamido-6-hydroxypyrimidine with phosphorous pentasulfide, decomposition of excess phosphorous pentasulfide with base and acidification to pH 4-5. Mercaptopurine precipitates from the acidic solution. Process 2 treats hypoxanthine with phosphorous pentasulfide. The solid formed is treated with NH_4OH to pH 5. Mercaptopurine precipitates from the solution.[4] Process 3 utilizes 4-amino-6-chloro-5-nitropyrimidine as the starting material. Treatment of this compound with potassium hydrosulfide gives 4,5-diamino-6-mercaptopyrimidine. The 4,5-diamino-6-mercaptopyrimidine is heated in concentrated formic acid to yield 7-amino-thiazolo(5,4-d)pyrimidine. Mercaptopurine is precipitated after treatment of 7-amino-thiazolo(5,4-d)pyrimidine with NaOH and adjusted to pH 5 with acetic acid. Process 4 is a varient of process 3. The 4,5-diamino-6-mercaptopyrimidine is treated with 50% formic acid to give 4-amino-5-formamido-6-mercaptopyrimidine, which in turn can be treated as the 7-amino-thiazolo(5,4-d)pyrimidine in process 3 to give mercaptopurine. Process 5 treats 4-aminoimidazole-5-carboxamide with phosphorous pentasulfide. The product, 4-aminoimidazole-5-thiocarboxamide, is heated with formamide to form the six-membered ring. Removal of the formamide and recrystallization of the residue gives mercaptopurine.

4. Stability

The decomposition of mercaptopurine in 0.1N NaOH, 0.1N HCl, distilled water, and photolytically has been studied[12]. When mercaptopurine is refluxed for 6 days in 0.1N NaOH, 4-aminoimidazole-5-thiocarboxamide and 4-amino-5-cyanoimidazole are the major products formed. When refluxed for 4 days in 0.1N HCl and 7 days as an aqueous suspension in distilled water, mercaptopurine decomposes primarily to 4-aminoimidazole-5-thiocarboxamide. Mercaptopurine in 0.1N NaOH and as an

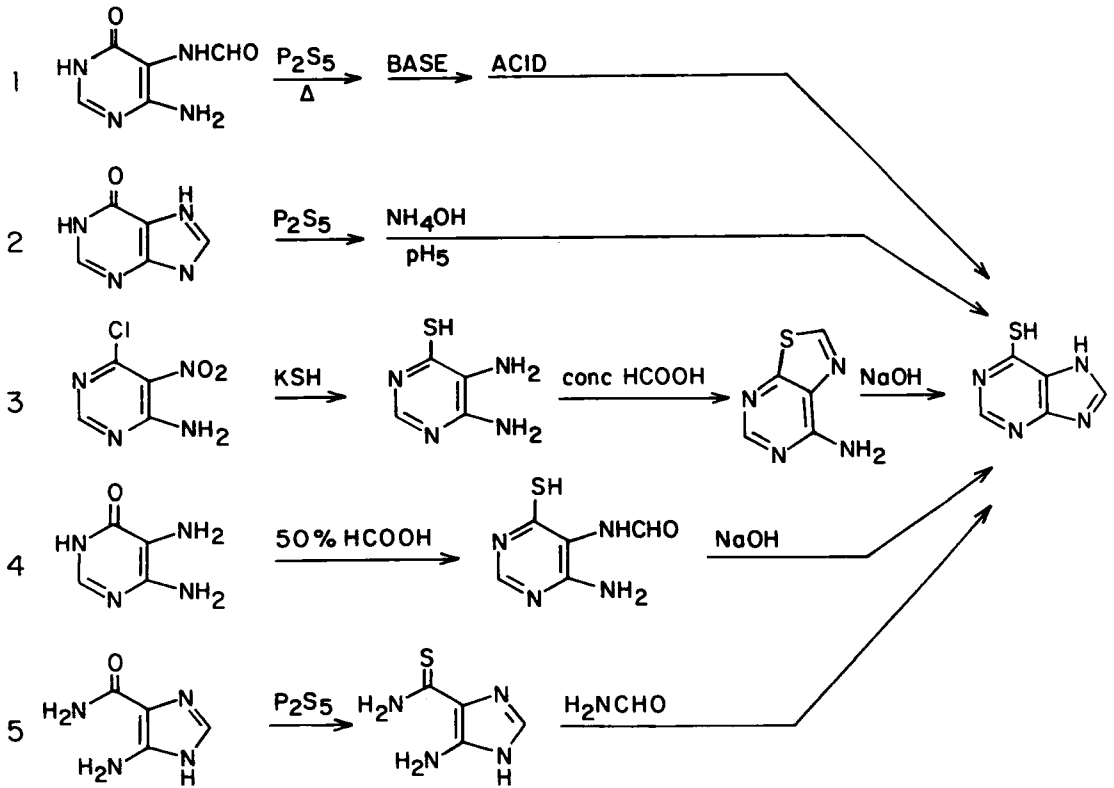


Figure 5- Synthesis of 6-Mercaptopurine

aqueous suspension in distilled water forms hypoxanthine when irradiated for 72 hours with a medium pressure mercury lamp.

5. Methods of Analysis

5.1 Elemental Analysis (As the Hydrate)

Element	C	H	N	S
% calculated	35.28	3.55	32.92	18.83

5.2 Nonaqueous Titrimetric Analysis

Nonaqueous titration is the official method of analysis in the USP for mercaptopurine[1]. An accurately weighed sample of mercaptopurine is dissolved in dimethylformamide. The solution is titrated with standardized 0.1N sodium methoxide using thymol blue as an indicator. Precautions must be taken against absorption of atmospheric carbon dioxide.

5.3 Spectrophotometric Analysis

The official USP analysis of mercaptopurine in tablets is a spectrophotometric analysis. A portion of powdered tablets is weighed. Twenty ml distilled water, and 1.5 ml NaOH TS is added to the powder in a 100 ml volumetric flask. The flask is brought to volume with distilled water, filtered, and a portion of the filtrate diluted with dilute hydrochloric acid. The solution is compared against a Reference Standard prepared in a similar manner at 325 nm in 1 cm cells.

5.4 Polarography

Alternating current polarography has been used to determine decomposition kinetics of mercaptopurine[13]. A catalytic wave with Q=0.45 was observed for mercaptopurine in 1N H_2SO_4.

5.5 Mass Spectrometry

Quantitative analysis of mercaptopurine in plasma has been accomplished with GC/MS[14]. Mercaptopurine was extracted from plasma, derivatized with methyl iodide, and separated by gas chromatography using a 1.83 M, 3% OV-225 column at 200°C, and detected with a mass spectrometer equipped with a peak monitor. Limit of detection was 20 ng/ml of mercaptopurine in plasma.

5.6 Chromatography

5.61 High Performance Liquid Chromatography

High performance liquid chromatography was used to determine mercaptopurine and metabolites in cultured cells and animal tissues[15]. A 0.18 x 100 cm column packed with Beckman M71 strong cation exchange resin was eluted with 0.4M

ammonium formate (pH 4.6) at 8 ml/hr. The column was kept at 50°C. The retention time of mercaptopurine under these conditions was 39 minutes. Detection was accomplished with a UV detector at 322 nm.

5.62 Column Chromatography

Column chromatography has been used to separate mercaptopurine from its metabolites in urine[16]. A cation exchange resin, Zeo-carb 225, eluted with 20% (v/v) ammonium hydroxide separated mercaptopurine and other 6-thiopurines from a concentrated urine sample.

5.63 Gas Chromatography

A gas chromatographic analysis of mercaptopurine in serum has been described[17]. A 1.5M x 6.3 mm o.d. column packed with 10% w/w SE-30 maintained at 135°C was used. Mercaptopurine derivatized with trimethylanilinium hydroxide had a retention time of 22 minutes.

5.64 Thin Layer Chromatography

The separation of mercaptopurine from mixtures of purines and pyrimidines has been accomplished by thin layer chromatography using anion exchange ECTEOLA cellulose plates[18]. The plates were developed in acetone:0.1M H_2SO_4: ethyl acetate (45:10:45). A second development was done in deionized water:acetone (8:2). The R_f value of mercaptopurine was 0.36. Cellulose plates developed in 0.1N HCl, H_2O, and isopropanol:methanol:H_2O:NH_4OH (60:22:20:1) gave R_f values for mercaptopurine of 0.43, 0.26, and 0.55 respectively[19].

6. Pharmacokinetics and Metabolism

6.1 Excretion Rate

In the rat, by the end of 48 hours after injection, 55% of mercaptopurine was excreted in the urine[20]. The largest proportion was excreted in the first 24 hours. For an intraperitoneal injection in the mouse of 1 mg of S^{35}-6-mercaptopurine, 43.5% of the radioactive material was excreted in the urine in the first four hours. At the end of 2 days over 60% of S^{35} was excreted. Approximately the same urinary excretion rate was found with oral doses, but some radioactive CO_2 was detected in the expired air[21].

6.2 Tissue Distribution

In the mouse the concentration of radioactive mercaptopurine was highest in the gut, almost twice as high as in the blood, and lowest in the brain. Mercaptopurine has some difficulty passing the blood-brain barrier. The brain concentration is one-tenth the concentration in the blood[21]. The presence of a tumor in different sites in rats, as well as in mice, causes lower blood levels of mercaptopurine when compared to non-tumor bearing animals. The apparent volume of

distribution is markedly increased in the presence of a tumor[22].

6.3 Metabolism

The pathway of metabolism of mercaptopurine is by hydroxylation *via* the enzymes xanthine oxidase and aldehyde oxidase. Mercaptopurine is transformed into mercaptopurine riboside, 6-thiouric acid, sulfates, and nucleotide metabolites in the liver[21,22,23].

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Analytical Profiles of Drug Substances, 7

PHENOBARBITAL

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Contents

1. General Information

1.1 Nomenclature

1.11 Chemical Names[1]

5-ethyl-5-phenyl-2,4,6(1H,3H,5H)-pyrimidinetrione; 5-ethyl-5-phenylbarbituric acid. The compound is listed in Chemical Abstracts under the heading pyrimidinetrione. CAS registry number[2] for this compound is 50-06-6 and for the sodium salt is 57-30-7.

1.12 Generic Names[1]

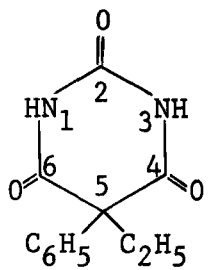
Phenobarbital, phenobarbitone, phenylethylmalonylurea.

1.13 Trade Names[1,3]

Luminal, Gardenal, Barbenyl, Barbiphenyl, Dormiral, Euneryl, Neurobarb, Barbipil, Lubrokal, Lubergal, Phenyral, Cratecil, Nunol, Phenonyl, Noptil, Phenobal, Agrypnal, Eskabarb, Etilfen, Gardepanyl, Somonal.

1.2 Formulas and Molecular Weight[1]

$C_{12}H_{12}N_2O_3$



Molecular Weight
232.24

1.3 Description

White, odorless, glistening, small crystals or white crystalline powder which exhibits polymorphism[2].

1.4 Forms in Official Compendia

The USP[2] contains Phenobarbital as the free acid, the sodium salt and the following formulations:

Phenobarbital Elixir
Phenobarbital Tablets
Phenobarbital Sodium Injection
Sterile Phenobarbital Sodium
Phenobarbital Sodium Tablets

and the USP reference standard for phenobarbital is available.

2. Physical Properties

2.1 Spectra

2.11 Infrared

The infrared spectrum[4] of phenobarbital is presented in Figure 1. The band assignments are summarized in Table 1.

Table 1
Infrared Band Assignments for Phenobarbital[4]

Wave No. (cm^{-1})	Assignment
3080-3220	Inter bonded N-H stretching.
1710, 1770	C=0 stretching.
1500, 1590	Aromatic ring skeletal vibration.
1250-1450	Amide III Bond (mainly C-N stretching).
830	N-H Wagging.
720, 770	C-H out of plane deformation of mono-substituted phenyl.

Two other spectra of phenobarbital were obtained from the same lot of sample but using different preparation techniques. Figure 2 represents the spectrum of which the sample is dissolved in a small amount of chloroform and mixed with KBr, after it is vacuum dried the resulting powder is pressed to a disc[4]. Figure 3 is the spectrum obtained from the similar procedure except using methanol as the solvent. The literature[5,6] presents other spectra obtained from different disc

Figure 1. Infrared Spectrum of Phenobarbital (Lot 597821) in KBr.
Instrument: Digilab FTS14

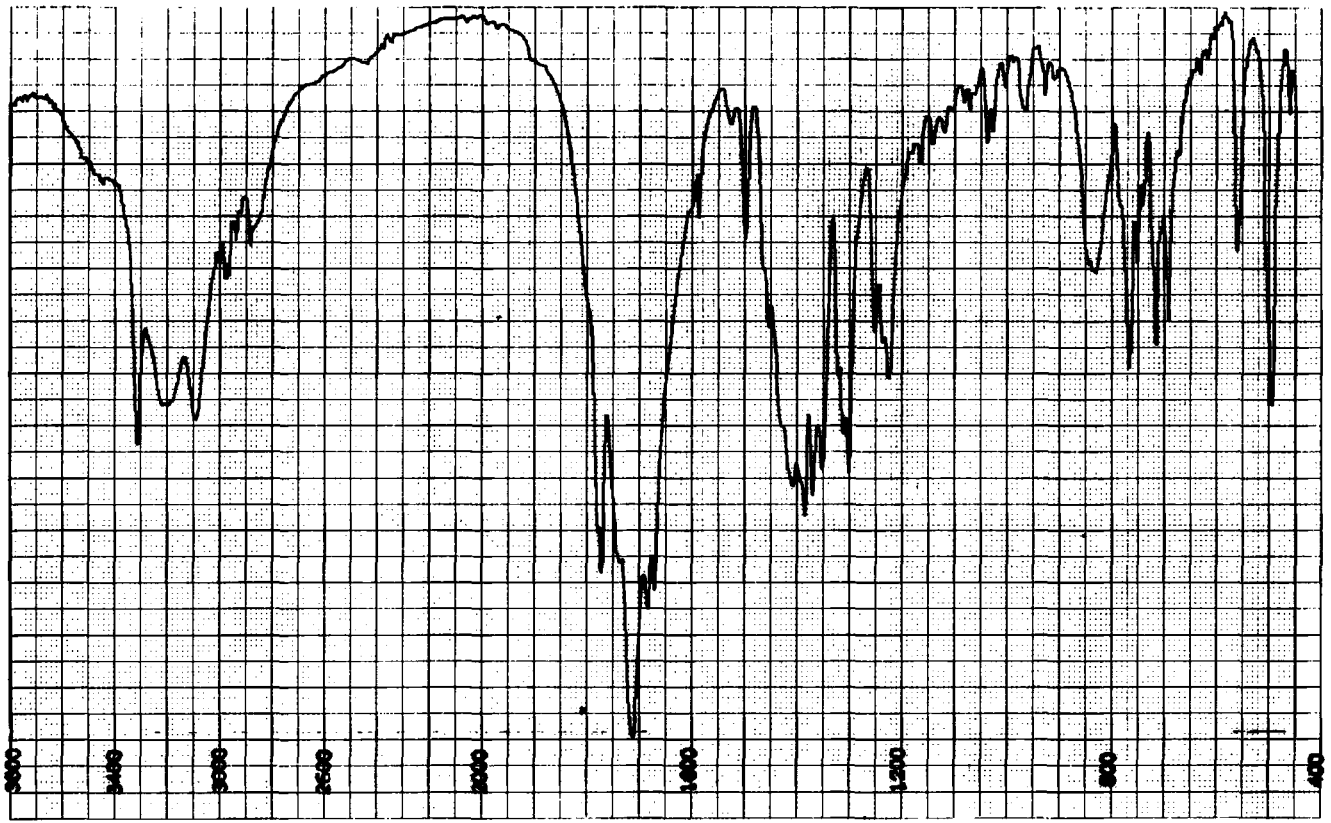


Figure 2. Infrared Spectrum of Phenobarbital (Lot 597821) from chloroform in KBr. Instrument: Digilab FTS14

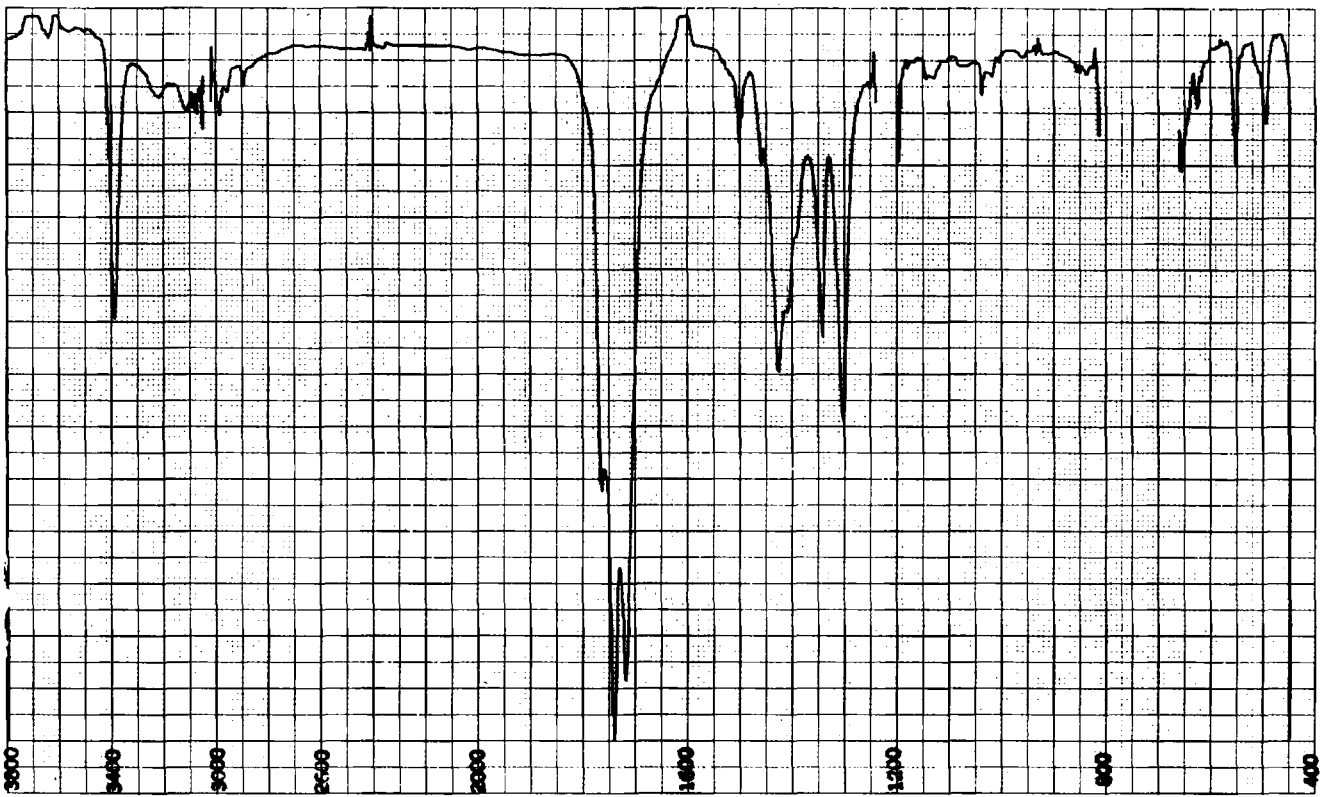
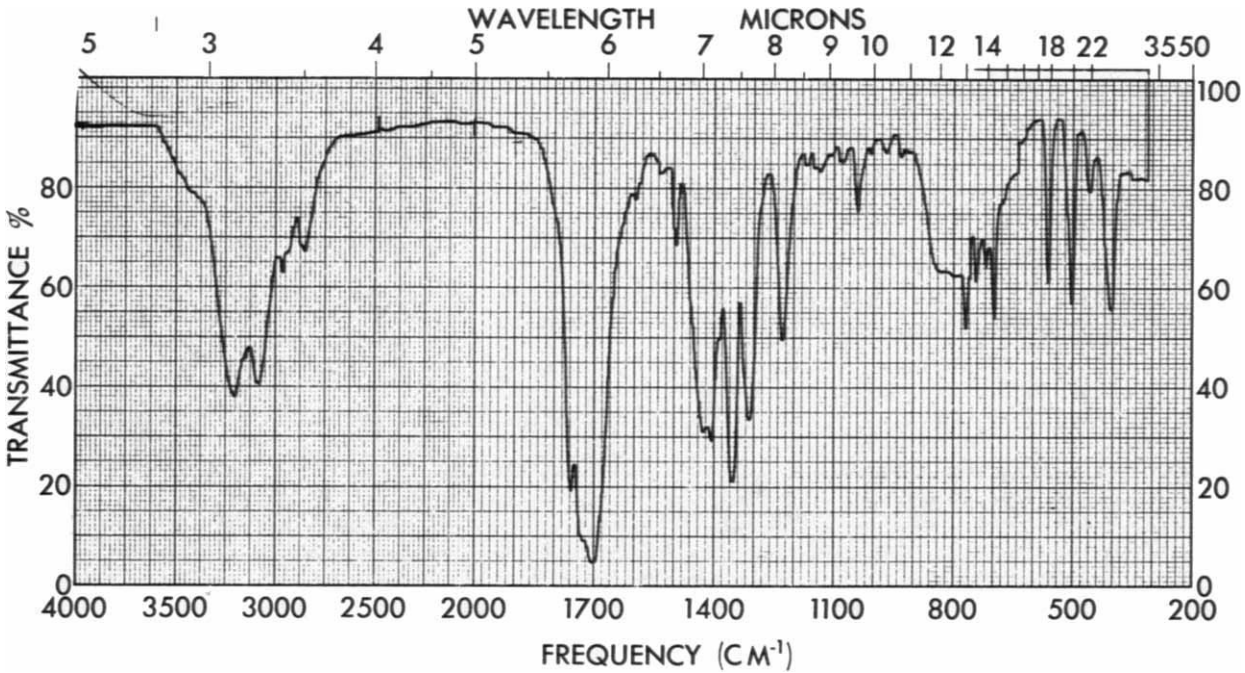


Figure 3. Infrared Spectrum of Phenobarbital (Lot597821) from methanol in KBr. Instrument: Perkin-Elmer 621



preparation methods such as (C_6H_6-C_7H_{16}-KBr) and (C_6H_6-CH_3OH-KBr). The obvious different appearance of these spectra confirms that phenobarbital forms different crystals depending on the solvent used. Mesley and Clements[7] evaluated the infrared identification of phenobarbital with particular reference to the occurrence of polymorphism.

2.12 Raman

The Raman spectrum[8] is shown in Figure 4. The band assignments are listed in Table 2.

Table 2
Raman Band Assignments for Phenobarbital[8]

Wave No. (cm^{-1})	Assignment
1690, 1740	C=0 stretching.
630	Ring "breathing" vibration.
620, 1000, 1040, 1590	Mono-substituted phenyl group.

2.13 Nuclear Magnetic Resonance

The NMR spectrum[9] of phenobarbital in DMSO at 90 Meg. Hz. is shown in Figure 5 and the spectral assignments are in Table 3.

Table 3
Nuclear Magnetic Resonance Spectral Assignments for Phenobarbital

Chemical Shift (σ)	(τ)	Relative Intensity	Multiplicity	Assignment
0.8	9.2	3	triplet	$-CH_3$
2.2	7.8	2	quartet	$-CH_2-$
7.3	2.7	5	singlet	$-C_6H_5$
11.6	-1.6	2	singlet	$2(>NH)$

2.14 Mass

The mass spectrum[6] of phenobarbital is shown in Figure 6. The parent peak is at m/e 232 and the base peak at m/e 204 where the pattern is

Figure 4. Raman Spectrum of Phenobarbital. Courtesy of Analytical Chemistry 44, 1229, 1972.

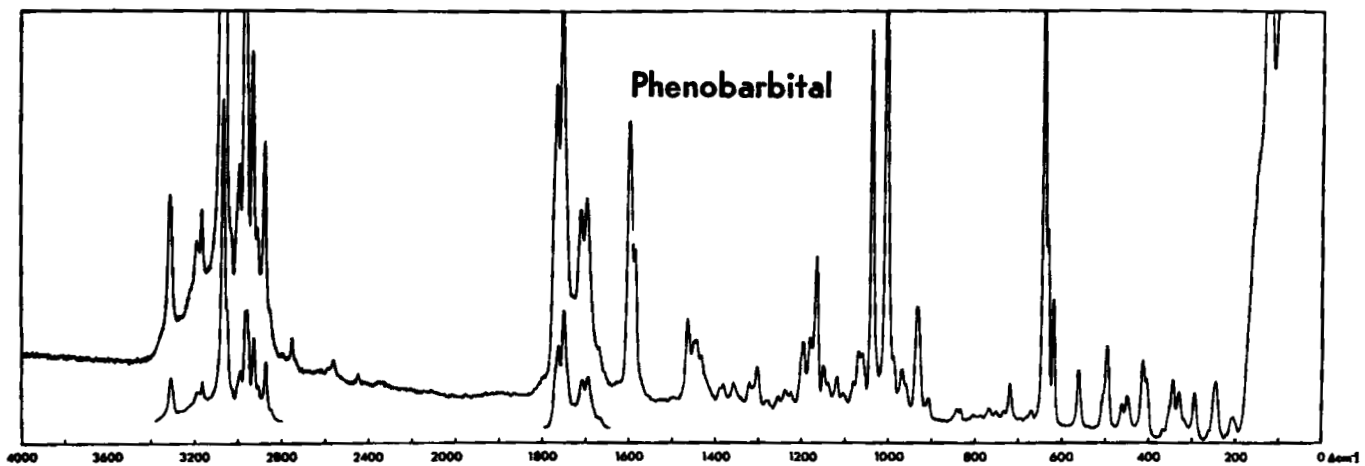


Figure 5. Nuclear Magnetic Resonance Spectrum of Phenobarbital (Lot 597821) in DMSO. Instrument: Bruker WH-90

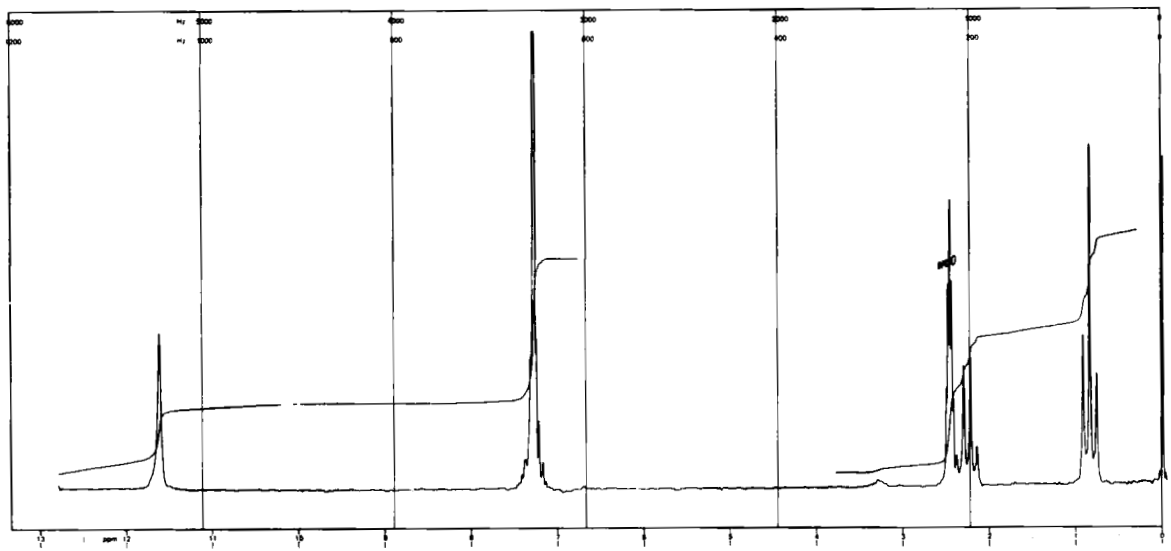
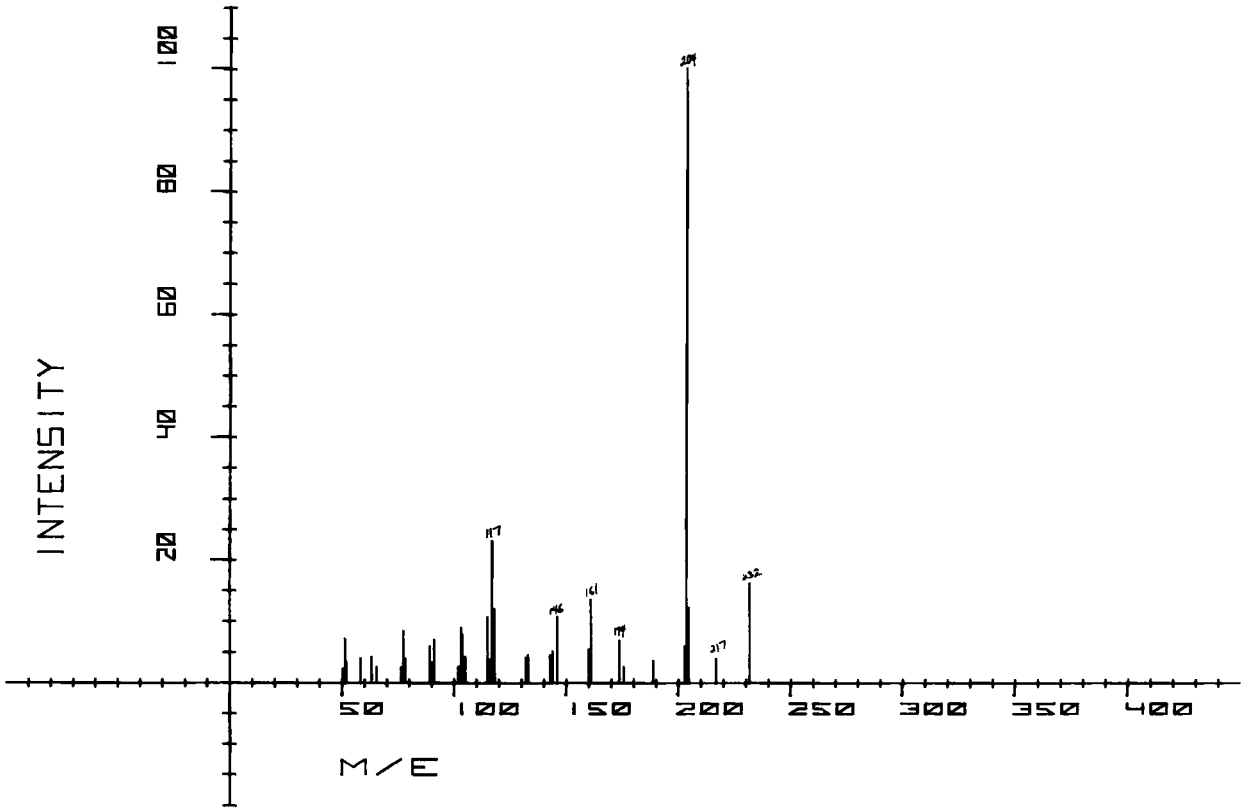


Figure 6. Mass Spectrum of Phenobarbital (Lot 597821) Instrument: Finigan 1015



consistent with a progressive loss of CO. The structural assignments are listed in Table 4.

Table 4
Mass Spectrum Assignments for Phenobarbital

Nominal Mass	Relative Intensity	Structure Assignment
232	16.2	M^+
217	4.0	M-(CH_3)
204	100.0	M-(CO)
189	4.0	M-(HNCO)
174	6.9	M-(HNCONH)
161	13.5	M-(CONHCO)
146	10.8	M-(CONHCONH)
117	23.2	M-(C_2H_5)-(CONHCONH)
77	8.4	$C_6H_5^+$

2.15 Ultraviolet Spectrum

The UV spectra of phenobarbital in 0.01 N HCl (unionized form), pH 10 buffer (singly ionized form) and 0.1 N NaOH (doubly ionized form) are presented in Figure 7. The spectra are in good agreement with those reported in the literature[10]. The absorption maximum of pH 10 and pH 13 solutions are at 240 nm and 256 nm, a(1%, 1 cm) values are 431 and 314 respectively.

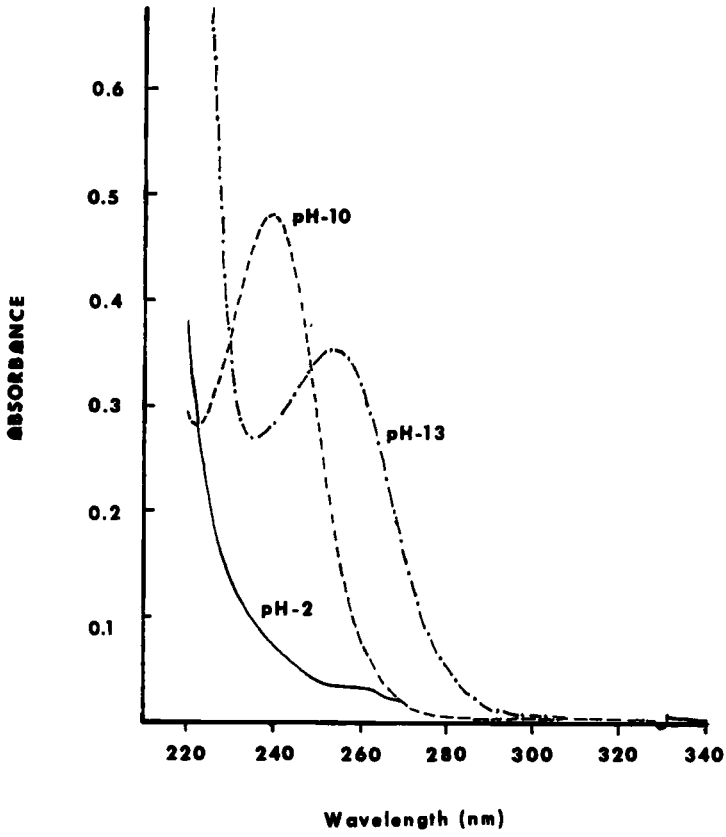
2.2 Crystal Properties

2.21 Crystal Morphology

Phenobarbital exhibits polymorphism[11,12]. The stable form[13] exists as rhomboids with angles of 61° and a leveling plane at 94°. A metastable phase forms long acicular crystals which are monoclinic needles. Another metastable phase is monoclinic hemimorphic.

The refractive indexes of phenobarbital have been reported[14] α=1.447, β=1.620 and γ=1.667.

Figure 7.
Ultraviolet Absorbance Spectrum of Phenobarbital (Lot 597821) in solutions with different pH. Instrument: Perkin-Elmer 124



2.22 X-Ray Diffraction

Williams[15] presented the x-ray diffraction patterns of phenobarbital which is crystallized from diethyl ether. Huang[16] reported five polymorphic forms of phenobarbital with characteristic x-ray diffraction patterns. More recently, Mesley and co-workers[12] have isolated thirteen crystal forms and documented the diffraction data in the literature.

2.23 Melting Range

Phenobarbital[1] reportedly melts at 174-178°C. Williams[15] determined the melting points of three crystal phases of phenobarbital; the stable phase at 174°C; one metastable phase at 156-157°C; and another metastable phase at 166-167°C. Mesley[12] summarized the melting points for eleven possible crystal forms of phenobarbital.

2.3 Solubility

One gram of phenobarbital dissolves in about one liter of water, 8 ml of alcohol, 40 ml of chloroform, 13 ml of ether and about 700 ml benzene. The compound is soluble in alkaline hydroxides and carbonates. A saturated aqueous solution is acid to litmus (about pH 5)[2].

The solubility of phenobarbital in water at various temperatures have been determined: 20°C (0.97 g/l)[17]; 25°C (1.2 g/l)[18]; 37°C (1.42 g/l)[11]. Aldrich and Watson[19] studied the solubility of phenobarbital at different pH values in aqueous solution: pH 5.2 (1.1 g/l); pH 7.1 (1.75 g/l); pH 8.1 (9.57 g/l); pH 8.5 (16.4 g/l); pH 9.1 (55.3 g/l). Also, the effect of pH upon the solubility of phenobarbital in alcohol-aqueous solution has been investigated by Urdang[20]. A study of the solubility of phenobarbital in alcohol-glycerin-water systems at various composition has been presented[18].

2.4 Dissociation Constants

The first and second ionization constants, pK_1 and pK_2, of phenobarbital have been determined

to be 7.3 and 11.8 by Krahl[21] and Butler[22] respectively. Biggs[23] confirmed the pK_1 value by ultraviolet spectrophotometry and it is in good agreement with that reported by Krahl.

2.5 Distribution Coefficients

Bush[24] determined a number of distribution coefficients (K) for phenobarbital between a few selected organic solvents and aqueous solution. The results are tabulated as follows:

Organic Solvents	K
1-Chlorobutane	0.40
Benzene	1.0
Dichloromethane	3.0
Diethyl Ether	50

$$\text{Where } K = \frac{\text{Concentration in organic phase}}{\text{Concentration in aqueous phase}}$$

More information of the distribution coefficients (K) of phenobarbital between a few organic solvents and aqueous solutions in different pH values ranged from 3 to 12 have been reported. Riedel and co-workers[25] measured the K values for three organic solvents and the results are presented below:

K Values in Various Solvents at Different pH

pH	CH_2Cl_2	$CHCl_3$	DCE
4	19.0	3.6	10.1
5	11.5	4.0	8.1
6	4.9	3.6	5.7
7	3.0	2.7	4.0
8	1.10	0.54	1.13
9	0.35	0.14	0.18
10	0.01	0.08	0.04

DCE = 1,2-dichloroethane

Meijer[26] obtained additional data on K values of phenobarbital as listed below:

K Values in Various Solvents at Different pH

Solvent	pH 3.4	pH 7.4	pH 12.4
Hexane	0.006	0.005	0.0004
Toluene	0.538	0.552	0.0006
Chloroform	4.16	2.35	0.004
1,2-Dichloroethane	5.26	2.85	0.0014
Ether	16.4	17.4	0.003
Ethyl Acetate	57.3	52.0	—
Acetone	—	762	—

3. Synthesis

Two synthetic routes of general applicability have been used for phenobarbital. One method[27,28] is based on the condensation of α-ethylbenzenepropanedioic ester (I) with urea (II) in the presence of sodium ethoxide. The synthetic route is shown in Figure 8, scheme 1.

The other synthetic method[29] includes the following steps: (1) condensing benzeneacetonitrile (III) with diethyl carbonate (IV) in ether solution to form α-cyanobenzeneacetic ester (V); (2) ethylating this ester to α-cyano-α-ethylbenzeneacetic ester (VI) which is further condensed with urea yielding the iminobarbituric acid (VII); (3) hydrolyzing compound (VII) to phenobarbital. The complete reaction is presented in Figure 8, scheme 2.

4. Stability and Degradation

Phenobarbital is quite stable in aqueous solutions of low pH, but is readily hydrolyzed at high pH. Figure 9 shows graphically the essential features of the hydrolytic decomposition of phenobarbital in alkaline solutions[30,31]. The barbituric acid ring is ruptured between the 1-6 (or 3-4) bond yielding the intermediate compound which loses carbon dioxide to form N-(aminocarbonyl)-α-ethylbenzeneacetamide. Under more severe conditions, especially on heating, the second peptide bond is broken with the formation of the α-ethylbenzeneacetic acid and urea as major products. Kapadia and co-workers[32] separated and identified these decomposed products by paper chromatography. The kinetics and mechanism of phenobarbital degradation

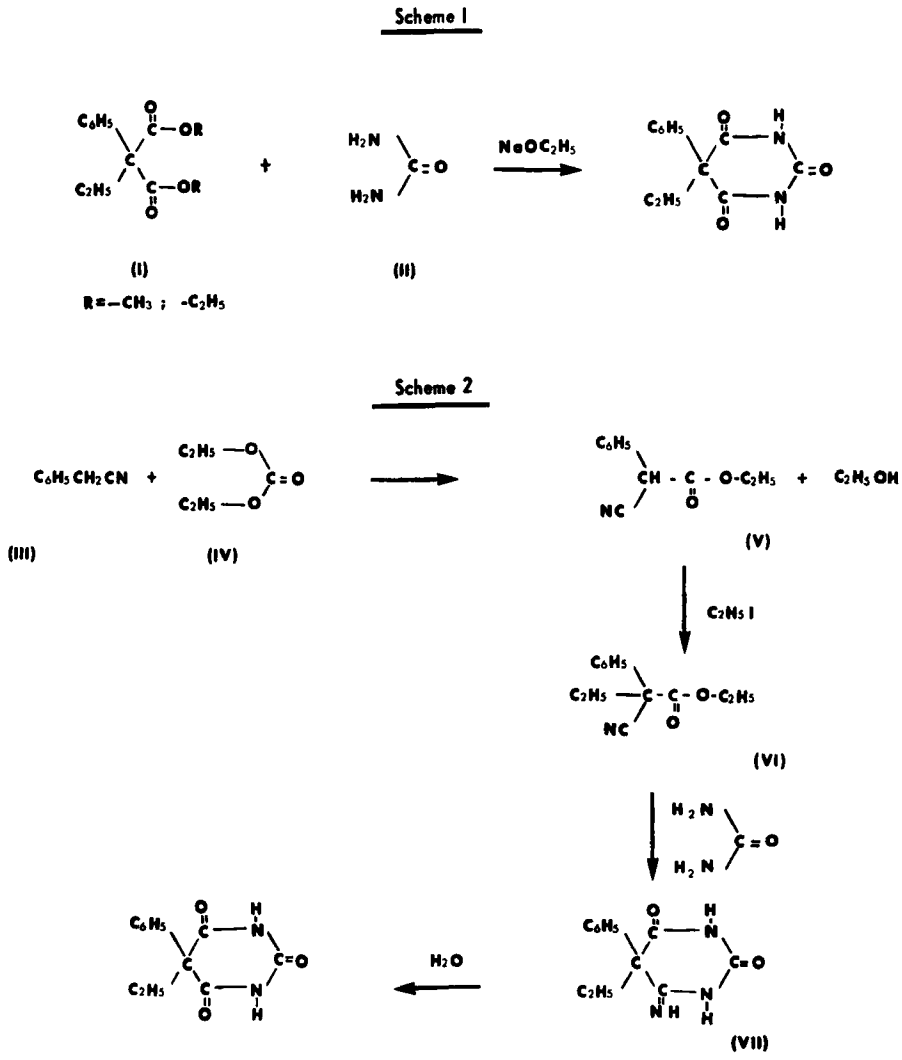


Figure 8. Synthetic Procedure for phenobarbital

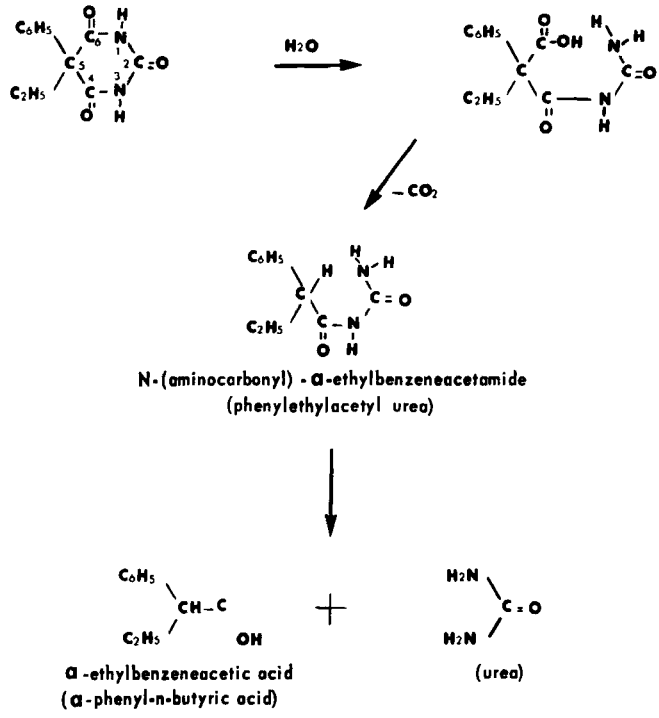


Figure 9. Hydrolytic decomposition of phenobarbital in alkaline solution

have been studied by Goyan[33] and Tishler[34]. Bush[24] measured the half-life of phenobarbital in 0.1 N KOH at 25°C as 46 hours. Maulding[35] obtained the apparent first order rate constants for decomposition of phenobarbital at 80°C in 0.1 N NaOH (pH 11.5) as 3 x 10^{-4} sec^{-1} and in 0.033 N NaOH (pH 10.99) as 1.5 x 10^{-4} sec^{-1}. Schmitz and Hill[36] reported the stability of phenobarbital in hydro-alcoholic and glycero-hydro-alcoholic solutions.

Phenobarbital is stable to strong oxidizing reagents such as permanganate and dichromate[24]. This provides a simple "clean-up" procedure to separate phenobarbital from other, easily oxidized, compounds.

5. Metabolism and Pharmacokinetics

Phenobarbital is partially metabolized and partially excreted unchanged in the urine. Raun-Jansen and co-workers[37] reported that about 11 to 25% of the dose of phenobarbital was found in the patient's urine in the form of unchanged drug.

Butler[38] found that the p-hydroxyphenobarbital (I) is the major metabolite of phenobarbital. On this evidence, Maynert[39] indicated that the primary pathway for the metabolism of phenobarbital is hydroxylation of the phenyl ring. The possible metabolic scheme is shown in Figure 10, scheme 1. It is reported that some other possible metabolites such as ortho- or meta-hydroxyphenobarbital and dihydrodiol derivatives have not been found in the biotransformation products of phenobarbital. However, Harvey, et. al.[40] isolated and identified 5-(3,4-dihydroxy-1,5-cyclohexadin-1-yl)-5-ethyl-barbituric acid (II), 5-(1-hydroxyethyl)-5-phenyl-barbituric acid (III) and 5-(3,4-dihydroxyphenyl)-5-ethylbarbituric acid (IV) as the metabolites in the urine of humans using gas chromatography-mass spectrometry. Figure 10, scheme 2 suggests the other possible metabolic pathway with the formation of metabolites (II), (III) and (IV) from phenobarbital[41].

Maynert[39] studied the absorption, distribution and excretion of phenobarbital in man and determined

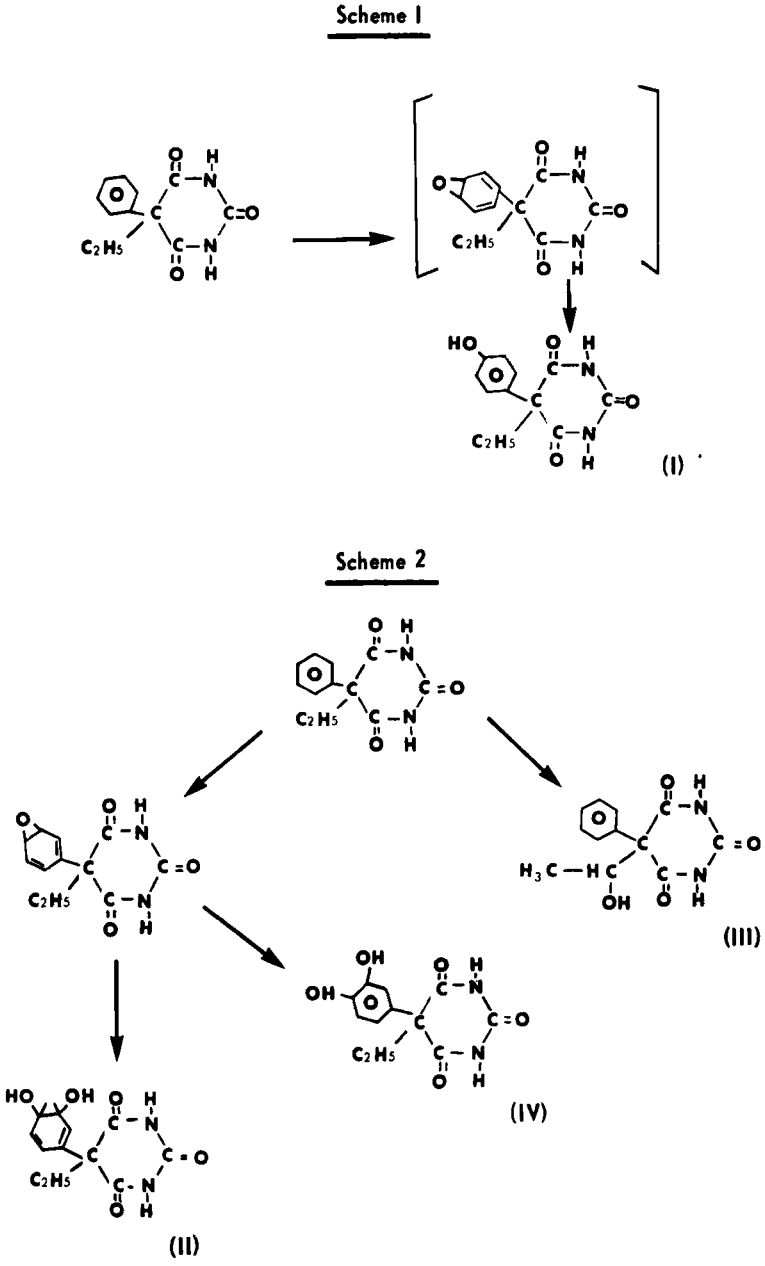


Figure 10. Metabolic Pathways

the half-life for the disappearance of the drug from the plasma ranges from 53 to 140 hours. Studies on the biological half-life of phenobarbital in human babies[42] and children[43] have been reported. The value of blood and plasma level assays for determining the dosage of phenobarbital has been the subject of recent reviews[44,45,46]. Vajda and co-workers[47] measured the phenobarbital concentrations in human brain, cerebrospinal fluid and plasma. Lin[48] studied the pharmacokinetic aspects of elimination from plasma and distribution to brain and liver of phenobarbital in rats.

McArthur[49] and co-workers studied the binding of phenobarbital to human whole blood in vitro. A study of the application of two dimensional immunoelectrophoresis to investigate the binding of phenobarbital to human serum proteins has been reported[50].

6. Identification - Microchemical Tests

Phenobarbital reacts with iodide-potassium iodide reagent solution to form characteristic crystals in little dark grains with a few large red blades and dark splinter-rods in clusters[51]. Also mentioned is a method using ammoniacal nickel acetate as the reagent with which phenobarbital forms in single rectangular crystals. Davis[52] developed a procedure for the identification and differentiation of twenty barbiturates using a silver reagent which is composed of 10% aqueous silver nitrate and ethylenediamine (85:15, v/v).

7. Methods of Analysis

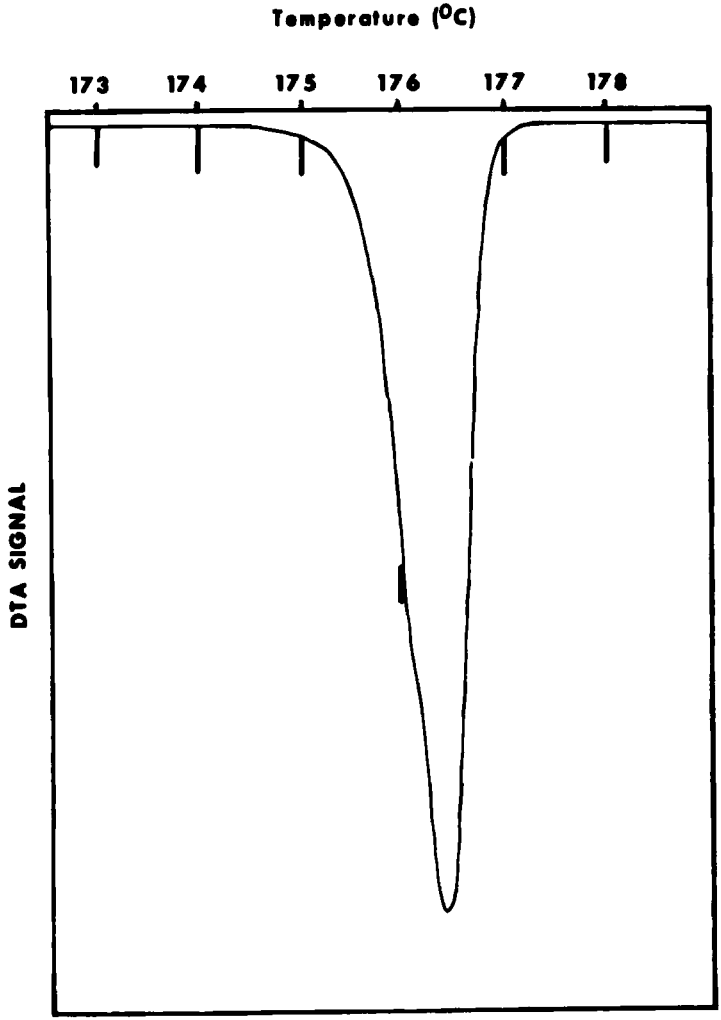
7.1 Differential Thermal

As phenobarbital exhibits polymorphism, a recrystallization step is recommended prior to a differential thermal analysis in order to ensure the presence of a single crystalline phase. Figure 11 shows a thermogram of a recrystallized sample which contains 0.17% impurity.

7.2 Elemental

The data[9] as shown in the following table

Figure 11.
Thermogram: Differential Thermal Analysis of Phenobarbital
(Lot 597821) Instrument: Mettler TA 2000



is obtained for Phenobarbital Lot 597821.

Element	% Found	% Theory
C	62.54	62.06
H	5.30	5.21
N	11.97	12.06

7.3 Colorimetric

Colorimetric methods based on the formation of a complex of phenobarbital with cobalt or copper salts in the presence of a base have been well studied[11,53]. Pfiel and Goldbach[54] determined phenobarbital by precipitating it with mercury and spectrophotometrically measuring the mercury complex with dithizone. A few laboratories[55,56,57] modified the reaction of dithizone with Hg (II)-phenobarbital complex and applied the method to determine phenobarbital in biological fluids.

7.4 Spectrophotometric

7.41 Ultraviolet

Curry[58] reviewed the use of ultraviolet spectrophotometric methods for assaying phenobarbital either by directly measuring the absorbance at 250-255 nm in an alkaline solution at pH 13 or by determining the difference in absorption at 240 nm in solutions of pH 10 and pH 2. Jain and Cravey[59] also indicated that the concentration of phenobarbital is proportional to the difference in absorbance readings between an alkaline solution in pH 13 at 240 nm and a buffer solution in pH 10 at 260 nm. The potential interference with the differential ultraviolet spectrophotometric analysis of phenobarbital has been studied by Jatlow[60]. Phenobarbital in elixir and tablet products is assayed by extraction and subsequent measurement of ultraviolet absorbance at 240 nm in pH 9.6 borate buffer[2].

7.42 Phosphorimetric

Weinfordner and Tin[61] developed a procedure for the phosphorimetric measurement of phenobarbital. The excitation and emission maximum

wavelengths are at 240 and 380 nm; the detection limit is 0.1 µg/ml.

7.43 Fluorometric

A fluorometric method[62] is also available for the determination of phenobarbital. The excitation and emission maximum wavelengths are at 265 nm and 400 nm in a buffer at pH 13. The sensitivity is reported to be 0.5 parts per million in the final solution.

7.5 Polarographic

A differential pulse polarographic procedure[63] was developed for the determination of phenobarbital. The sample is nitrated with potassium nitrate in sulfuric acid and the nitro-derivative is analyzed by differential pulse polarography.

7.6 Titrimetric

Phenobarbital can be determined by potentiometric titration[2] with 0.1 N NaOH. Cheronis and Ma[64] listed titrimetric methods for phenobarbital determination including an acidimetric titration in non-aqueous media. argentiometry, use of precipitation agents. iodimetry and the Kjeldahl method. A review of non-aqueous titrimetric methods for phenobarbital was presented by Kucharsky[65]. In addition, Agarwal[66] described a spectrophotometric titration of phenobarbital in acetonitrile using triethyl-n-butylammonium hydroxide as a titrant.

7.7 Gravimetric

Gravimetric determination was one of the main methods used in earlier days for assaying phenobarbital in different dosage forms[67] and tissues[68]. The procedure involves extraction with chloroform followed by evaporating the solvent and dissolving the residue in ethyl ether. The ether solution is again evaporated to dryness and the amount of phenobarbital is measured gravimetrically.

7.8 Chromatographic

7.81 Paper

Paper chromatography was one of the early methods used for the identification of phenobarbital. Curry[58] documented different detection methods for phenobarbital on the developed chromatogram. Clark[69] summarized a number of chromatographic systems and R_f values for phenobarbital. The identification of barbiturates from biological specimens by paper chromatography have been reviewed by Jain and Cravey[70] and deZeeuw[71].

7.82 Thin-Layer

Thin-layer chromatography has been widely used as a rapid, simple screening method for phenobarbital. Clark[69], deZeeuw[71], Kirchner[72] and Stahl[73] reported many systems for phenobarbital and its corresponding R_f values. Gardner-Thorpe and co-workers[74] evaluated a number of procedures and found that a system using chloroform-acetone (90:10) as the mobile phase and ultraviolet absorption at 254 nm as the detection method would be suitable for routine clinical work, R_f value for phenobarbital is 0.5. Itiaba, et. al.[75] used a fiberglass sheet impregnated with silica gel as the stationary phase, Rhodamine B dye as the spray reagent, and detected phenobarbital under ultraviolet light. By this method R_f values determined for three different mobile phases are: petroleum ether-ethyl acetate (100:14), R_f = 0.41; benzene-acetone (100:2), R_f = 0.28; carbon tetrachloride-acetone (100:5), R_f = 0.28. Dünges and Peter[76] described a new procedure in which a derivative formed from the reaction of phenobarbital with a dimethylaminonaphthalenesulphonic acid is applied directly to thin-layer plates and the amount of phenobarbital is determined fluorometrically. Jain and Cravey[70] presented a review article including more than ten recently developed TLC systems for the determination of phenobarbital. Atwell[77] reported a list of R_f values for phenobarbital on silicic acid gel thin layer plate using 17 various mobile phases. A quantitative thin-layer chromatography using scanning of remission technique was applied to phenobarbital determination in capsules[78].

7.83 Liquid

Phenobarbital is separated from phenytoin on a reversed phase (silanized celite) column using 25% amyl alcohol in chloroform as the stationary phase and 0.05 M borate buffer pH 9.5 as the mobile phase. After the separation, phenobarbital is determined from its ultraviolet absorption in 0.1 N NaOH at 253 nm[79]. Another system[14] employing a column packed with a mixture of acid washed celite 535 (2 g) and 12% $BaCl_2$ solution (1 ml) and using a water-saturated solution of 15% amyl alcohol in chloroform as the mobile phase was also presented for the separation of phenobarbital from phenytoin. Kullberg and co-workers[80] described a procedure using Amberlite XAD-2 as the adsorbent resin to separate phenobarbital from urine samples, using a mixture of ethyl acetate-1,2-dichloroethane (3:2) as the mobile phase. A similar procedure was presented by Hetland, et. al.[81] where a mixture of ethyl acetate-methanol-ammonium hydroxide (170:20:10) was used as the mobile phase.

7.84 Gas

Brochmann-Hanssen[82] reviewed some early studies on gas chromatographic separation and identification of barbiturates. Clark[69] and Gudzinowicz[83] summarized a number of systems for phenobarbital and its corresponding retention time. A number of gas chromatographic studies of phenobarbital determination were collected in the book edited by Meijer, et. al.[84]. Jain and Cravey[70] thoroughly reviewed the development and application of gas chromatography to determine phenobarbital in biological samples. The article includes detailed information about derivatization, column packing materials, stationary phases and internal standards. Berry[85] critically examined twelve gas chromatographic systems for phenobarbital measurement in plasma and urine samples.

Recently, Ehrsson[86] suggested an extractive methylation procedure in carbon disulfide for barbiturate determination and compared this procedure with other methylation methods. Various detectors have been used for the determination of

phenobarbital. The application of a nitrogen-specific detector has been studied[87,88]. Gyllenhaal[89] and Walle[90] have developed similar procedures converting phenobarbital to its bispentafluorobenzyl derivative and measuring it with an electron-capture detector. Phenobarbital in the range of nanograms to picograms from a sample size of 0.1-1 ml of plasma or 1-5 ml of urine can be detected by a mass spectroscopy-computer system[91] where an internal standard labeled with stable isotopes is used.

The more recent gas chromatographic work on the determination of phenobarbital is summarized in Table 5.

7.85 Modern Liquid

Modern liquid chromatography provides a new technique for the separation, identification and quantitative determination of phenobarbital in pharmaceutical dosages or in biological samples. The method is accurate, simple, rapid and sensitive. Results and working conditions reported from different laboratories are summarized in Table 6.

A high-speed molecular-size exclusion technique[111] has been applied to analyze phenobarbital in blood plasma without any clean-up preparation. The column used is a 2.6 mm, i.d. x 1 ml stainless steel tube packed with Vit-X-328 (Perkin-Elmer); the mobile phase is double distilled water; flow rate at 0.5 ml/min. and UV detection is used.

7.9 Ion Selective Electrode

Carmark and Freiser[112] developed a coated-wire selective electrode for the assay of phenobarbital based on the ion pair complex between phenobarbital anion and the quaternary ammonium cation, tricaprylylmethylammonium ion. The electrode is sensitive for the concentration range from 0.1 M to 10^{-5} M of phenobarbital at pH 9.6, and the results obtained are in agreement with the standard USP method. The assay using the ion selective electrode can be accomplished within twenty minutes where the USP method needs four hours. The useful lifetime of the electrode is about three months.

TABLE 5

DETERMINATION OF PHENOBARBITAL BY GAS CHROMATOGRAPHY

Derivatization	Column Length	Column Base	Stationary Phase	Support	Detector	Ref.
N.M.	2 m	3.175 mm	4% SE-30 6% QF-1 on acid washed DMCS	Chromosorb W 80/100	F.I.	92
TMAH (Flash Heater)	200 cm	3 mm	2% QF-1	Chromosorb W 60/80	F.I. E.C.D.	93
TAAH (Flash Heater)	6 ft.	0.25 in.	3% QV-17	Gas Chrom Q 100/120	F.I.	94
TMAH (On Column)	5 ft.	0.25 in.	5% OV-17	Chromosorb W 60/80	F.I.	95
TMAH (Pre Column)	N.M.	N.M.	3% XE-60	Gas Chrom Q 100/120	N.M.	96
TMPAH	200 cm	2 mm	3% SP2250	Supelcoport methyl-phenyl (50:50) 100/120	F.I.	97

TABLE 5 (Continued)

Derivatization	Column Length	Column Base	Stationary Phase	Support	Detector	Ref.
N.M.	6 ft.	2 mm	3% OV-17 or SP2250	Deactivated Chromosorb 750 100/120	E.C.D.	98
TBAH (On Column)	1.8 m	2 mm	3% OV-101	Chrom G HP 100/120	F.I.M.	99
TMAH	6 ft.	1/8 in.	10% UV-W98	Chromosorb W HP/80/100	—	100
N.M.	4 ft.	4 mm	1% OV-17	High Performance Chromosorb G	F.I.	101
N.M.	213 cm	4 mm	3% OV-17	Celite 545 100/120	F.I.	102

TAAH = Tetraalkylammonium Hydroxide
TMAH = Trimethylanilinium Hydroxide
F.I. = Flame Ionization Detector
E.C.D. = Electrolytic Conductivity Detector
N.M. = None Mentioned
TBAH = Tetrabutylammonium Hydroxide
TMPAH = Trimethylphenylammonium Hydroxide

TABLE 6

DETERMINATION OF PHENOBARBITAL BY MODERN LIQUID CHROMATOGRAPHY*

Column	Mobile Phase	Flow Rate (ml/hr)	Retention Volume (ml)	Remark	Ref.
Anion Exchange, Pellicular Type LSF (0.04 in. i.d. x 120 in. l)	NaCl	26	11	80°C (a)	103
SAX (Du Pont) (2.1 mm i.d. x 1 m l)	0.01 M Na_3BO_3 0.01 M $NaNO_3$	28.8	9.36(c)	(b)	104
	0.01 M Na_3BO_3 0.05 M $NaNO_3$	28.8	2.25(c)	(b)	
	0.01 M Citric Acid	21.6	4.59(c)	(b)	
ETH Permaphase (Du Pont) (2.1 mm i.d. x 1 m l)	d.d. Water	24	1.17(c)	(b)	104

*UV detector (254 nm) is used for the measurement unless otherwise mentioned in the remark.

TABLE 6 (Continued)

Column	Mobile Phase	Flow Rate (ml/hr)	Retention Volume (ml)	Remark	Ref.
Micro Pak 10 μ (Varian) (2 mm i.d. x 50 cm l)	CH_2Cl_2-CH_3OH-NH_4OH (92:7:1 v/v/v)	45	3	(d)	105
μBondapak C_{18} (Waters) (4 mm i.d. x 30 cm l)	CH_3OH-Buffer pH 3 (60:40)	120	4.4	(e) UV detection at 230 nm	106
Silica Gel[h]	d.d. Water	50	3	(f)	107
Bondapak C_{18} on Corasil II (1/8 in. i.d. x 2 ft. l)	CH_3OH-H_2O-HOAc (25:75:0.1)	120	4.4	30°C (g)	108
Corasil C_{18} (Waters)[h]	Absolute Methanol-1% $(NH_4)H_2PO_4$ (40:60)	84	2.15	(e)	109
Corasil Phenyl (Waters)[h]	Absolute Methanol-1% $(NH_4)H_2PO_4$ (40:60)	84	1.6	(e)	109

TABLE 6 (Continued)

Column	Mobile Phase	Flow Rate (ml/hr)	Retention Volume (ml)	Remark	Ref.
μ Porasil (Waters) (4 mm i.d. x 30 cm l)	Chloroform-dioxane-2-propanol-acetic acid (310:9.7:1.0:0.1)	90	4.5	(i)	77
SAX (Permaphase AAX) (2.1 mm i.d. x 50 cm l)	4 mM $(NH_4)_2HPO_4$ pH 6.2	24	1.7	(i)	110

(a) Separation of four barbiturates and their metabolites.
(b) Separation of phenobarbital from fifteen other barbiturates.
(c) The retention volume has been corrected for the column dead volume.
(d) Simultaneous measurement of phenobarbital and phenytoin in serum.
(e) Data obtained from mobile phases with different mixture composition have also been reported.
(f) The system has been applied to the separation of phenobarbital from a mixture of six antiepileptic drugs and to the determination of phenobarbital in serum extract as well.
(g) Simultaneous measurement of phenobarbital and phenytoin in dosage form.
(h) Column size is not mentioned.
(i) Simultaneous determination of phenobarbital and phenytoin in plasma.

7.10 Immunoassay

Recently, a homogeneous immunoassay method[113] (Enzyme Multiplied Immunoassay Technique, EMIT) has been applied to the quantitative determination of phenobarbital in biological samples. The method employs three reagents antibody, enzyme and enzyme substrate. The drug to be analyzed combines with the enzyme to form a drug-enzyme conjugate. When the antibody complexes with the conjugate, the active site of the enzyme is blocked and the enzyme is inactive. However, in the presence of the free drug, the drug conjugate competes with the free drug for the antibody binding site. If the free drug is bound to the antibody, the conjugate would be uncomplexed and the enzyme active site is available for the enzymatic reaction with the enzyme substrate. The level of enzyme activity is directly proportional to the concentration of free drug present in the sample. Pippenger[114,115] and Schneider[116] evaluated the method for the quantitation of phenobarbital in serum and plasma.

Another immunoassay technique, radioimmunoassay, was also performed on the measurement of phenobarbital in saliva and plasma[117]. The principle of the technique is that the drug present in the sample equilibrates and displaces a proportional quantity of radioactive-labeled drug which is bound to the specific antibody, the uncomplexed radioactive-labeled drug is then estimated in a liquid scintillation spectrophotometer or a gamma counter. The method is reported to be sensitive to saliva and plasma levels of phenobarbital and it only requires 10 µl sample for the measurement. Chung[118] and Satoh[119] described a highly specific procedure using phenobarbital at picomole levels.

8. Automated Analytical System

Jain and Cravey[70] reviewed the automated analysis of barbiturates. Blackmore and co-workers[120] described an automated method for screening large numbers of urine samples for drugs using the Technicon Auto Analyzer. The method utilizes differential ultraviolet spectrophotometry for the determination of phenobarbital. Adams[121]

reported an automated gas chromatographic technique for assaying phenobarbital in biological samples. The method employs an automated capsule system of injection, the Perkin-Elmer MS-41 and an automatic data processor, Perkin-Elmer PEP-1.

An auto analyzer method has been used to determine phenobarbital in compressed tablets and elixir[122]. In this method, the sample solution is dialyzed into a recipient stream of pH 9.5 borate buffer followed by ultraviolet spectrophotometric measurement at 240 nm.

Literature surveyed through September, 1977.

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Analytical Profiles of Drug Substances, 7

SULFAMETHAZINE

Constantin Papastephanou and May Frantz

ISBN 0-12-260807-0

SULFAMETHAZINE

Contents

SULFAMETHAZINE

1. Description

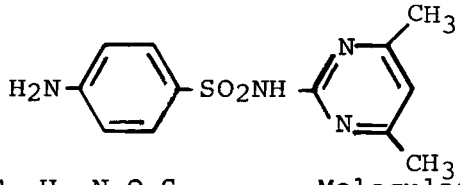
1.1 Name, synonyms, formula, molecular weight:

4-Amino-N-(4,6-dimethyl-2-pyrimidinyl) benzenesulfonamide;

N^4-(4-6-dimethyl-2-pyrimidyl)sulfanilamide;

4,6-Dimethyl-2-sulfanilamidopyrimidine;

Sulfamezathine, sulfadimerazine, sulfadimidine, sulfamidine, sulfadimethylpyrimidine.



$C_{12}H_{14}N_4O_2S$ Molecular weight: 278.33

1.2 Appearance, color, odor:

White to yellowish white powder, which may darken on exposure to light. Has a slightly bitter taste and is odorless.

2. Physical Properties

2.1 Infrared spectrum:

The infrared spectrum of a USP reference standard sulfamethazine is shown in Figure 1. The spectrum, obtained on a Perkin Elmer Model 621 spectrometer as a KBr pellet, indicates the presence of the absorptions shown in Table I (1).

Table I

Infrared Assignments for Sulfamethazine

Frequency (cm^{-1})	Characteristic of
3420	
3320	N-H stretch (NH_2 + NH)
3220	
1635	
1590	Aromatic C = C and
1505	C = N stretch
1500	
1475	
1300	
1140	SO_2
860	p-substituted phenyl ring

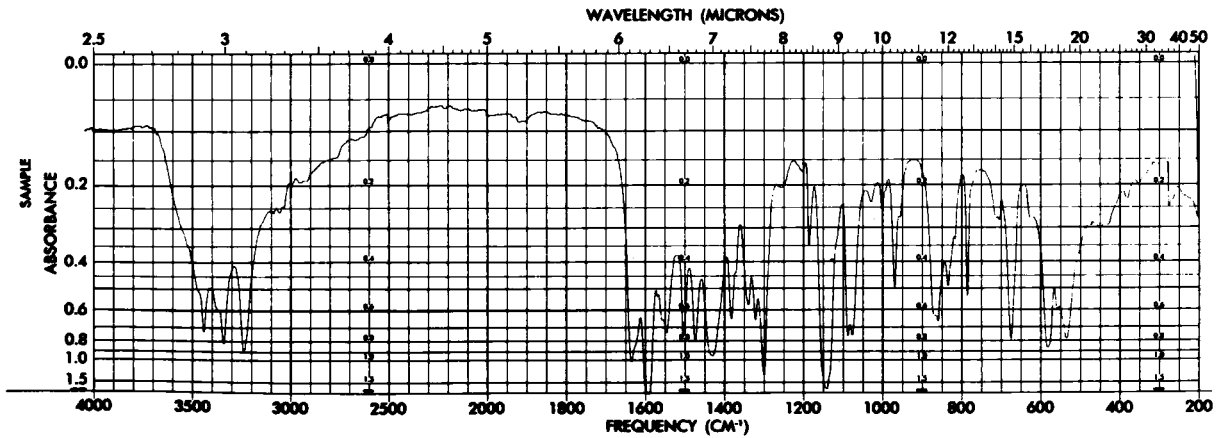
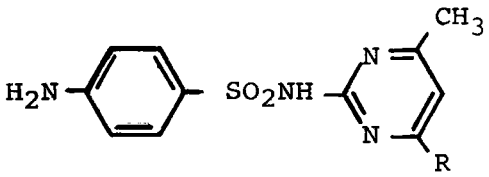


Figure 1. Infrared spectrum of sulfamethazine (KBr pellet).

2.2 Nuclear Magnetic Resonance Spectrum:

The 60 MHz NMR spectrum (Figure 2) obtained in dimethyl sulfoxide-d_6 containing TMS as internal reference, utilizing the Perkin-Elmer R12B spectrometer, indicates the presence of resonances at δ6.57 (d, J = 9.0 Hz, 2H ortho to NH_2) and 7.67 (d, J = 9.0 Hz, 2H ortho to SO_2)assigned to p-substituted benzene ring protons, δ6.70 s (1H) assigned to the heterocyclic proton, the amine protons resonance at δ 5.90, the SO_2NH proton resonance at δ 11.05 and the methyl protons resonance as a singlet at δ 2.15. The data are consistent with the assigned structure 1.

The comparison of NMR data of sulfamethazine, 1 with that of sulfamerazine, 2 indicates the absence of two vicinally coupled heterocyclic protons resonance as the result of methyl substitution in the former compound. The presence of methyl resonance at δ 2.15 (6H) is indicative of a symmetrical substitution pattern consistent with structure 1. The chemical shift of the remaining proton of the heterocyclic ring is only mildly affected by the presence of the additional methyl group (δ6.70 (1) vs δ 6.86 (2)),(2).



1- Sulfamethazine: R = CH_3

2- Sulfamerazine: R = H

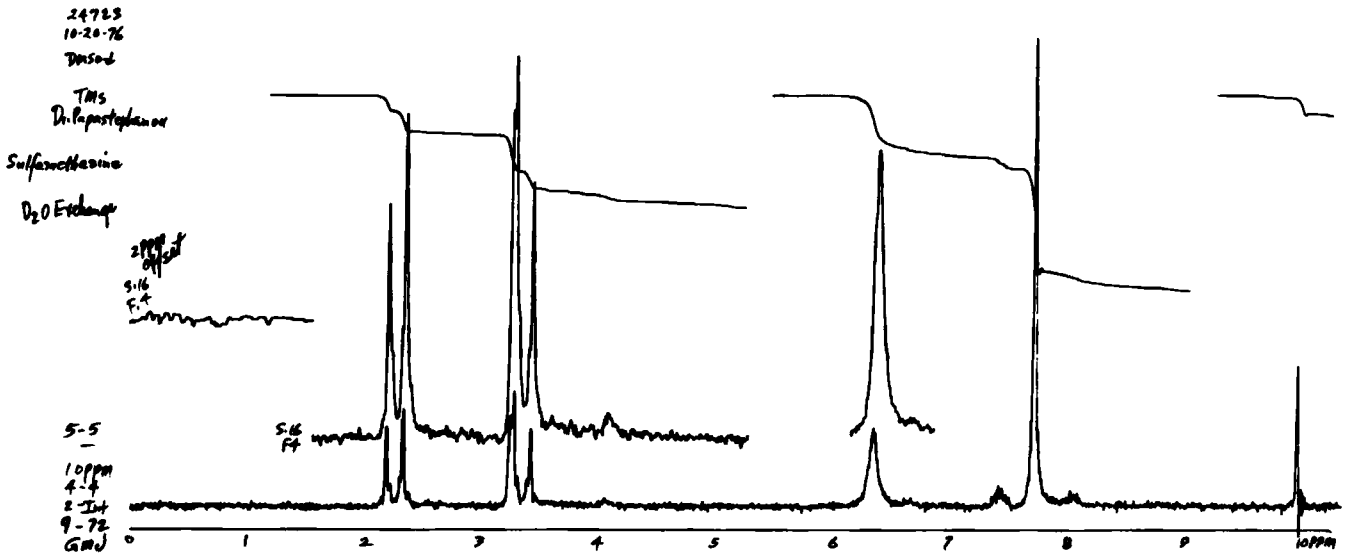
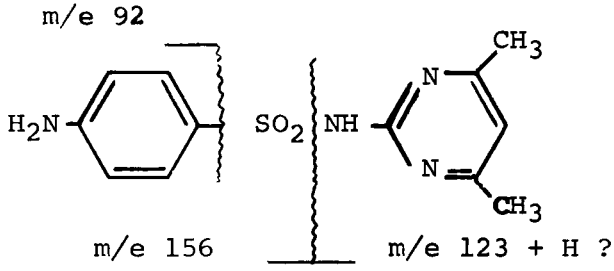


Figure 2. NMR spectrum of sulfamethazine.

2.3 Mass Spectrum:

The mass spectrum (Figure 3) was obtained on an AEI-MS902 mass spectrometer by direct sample introduction into the source (165°C). The spectra were recorded on magnetic analog tape and processed by in-house programs using a PDP-11 computer.

The mass spectrum shows no molecular ion but an interesting rearrangement ion at m/e 214 arising from the loss of SO_2 from the molecular ion. Other fragment ions consistent with the structure are depicted below, (4).



2.4 Ultraviolet Spectrum

Figure 4 shows the UV spectrum of aqueous solutions of sulfamethazine in water (pH 6.6), 0.01N HCl and 0.01N NaOH.

At pH 6.6, one peak at 241 nm is observed ($E^{1\%}_{1cm}$ = 670) with a shoulder at 255 nm.

Sulfamethazine in 0.01N NaOH shows 2 peaks at 243 and 257 nm with $E^{1\%}_{1cm}$ of 765 and 776 respectively.

When the drug is dissolved in 0.01N HCl, two peaks are observed at 241 and 297 nm with $E^{1\%}_{1cm}$ of 561 and 266 respectively.

2.5 Optical Rotation

Sulfamethazine exhibits no optical activity since it has no center of asymmetry.

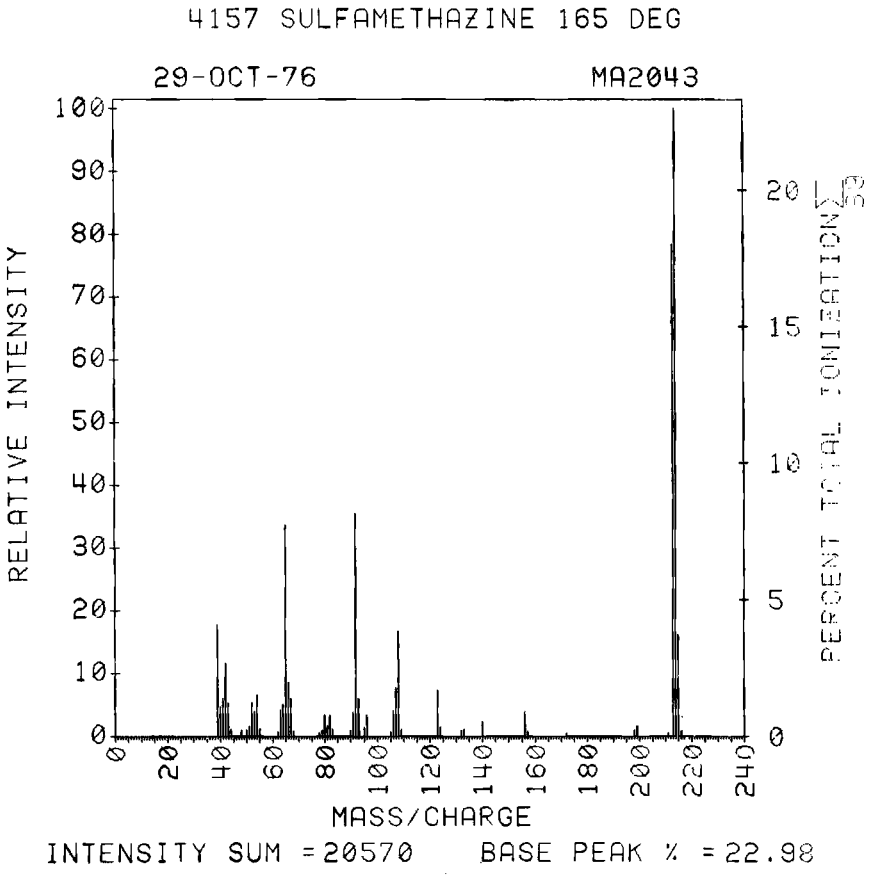


Figure 3. Mass spectrum of sulfamethazine.

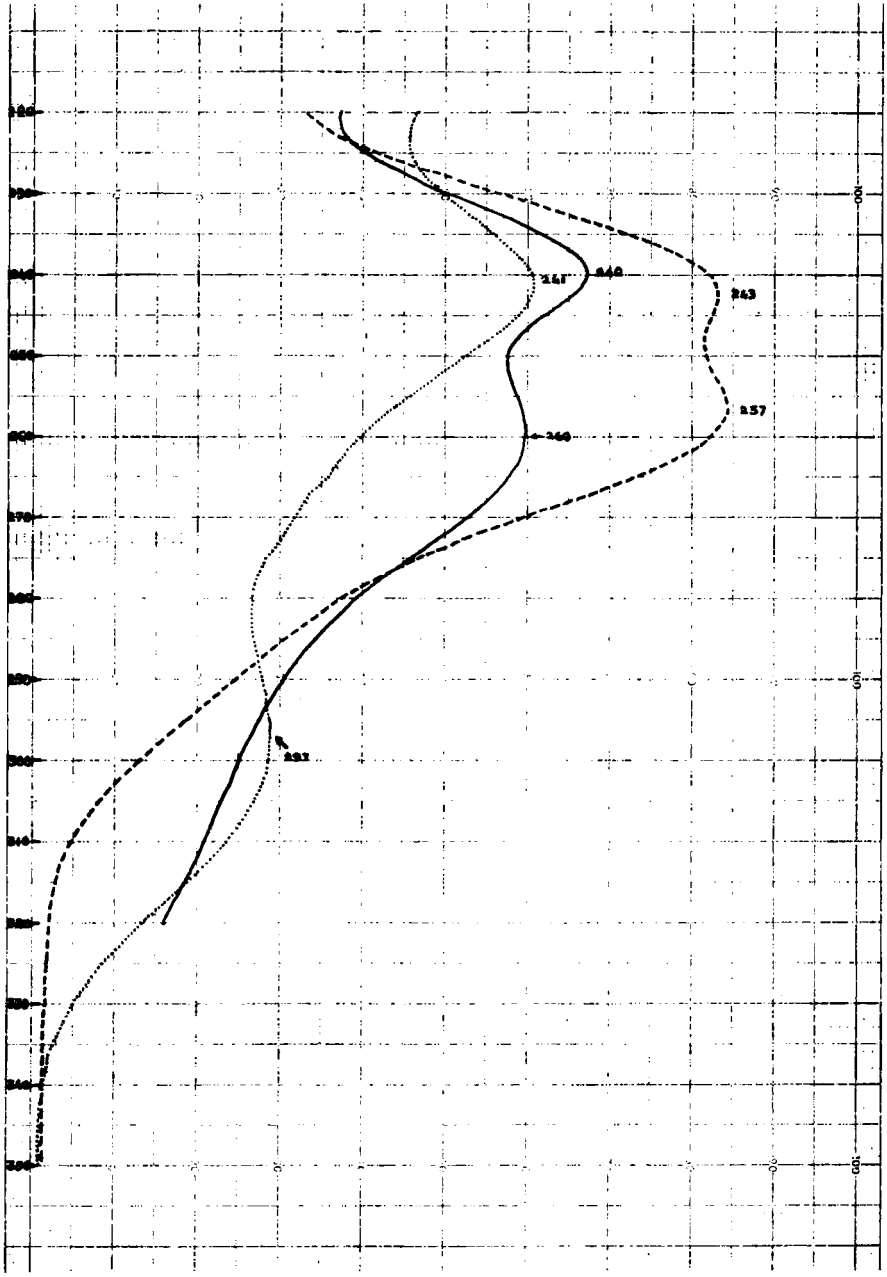


Figure 4. UV spectrum of aqueous solutions of sulfamethazine (———) in water, (....)0.01N HCl, (---) 0.01N NaOH.

2.6 Luminescence Properties:

Sulfamethazine has no fluorescence activity in aqueous solutions at any pH. It does, however, show a phosphorescence peak at 410 nm when excited at 310 nm at 77°K. The lifetime is 0.8 seconds, (5).

2.7 Solubility:

Sulfamethazine is very slightly soluble in water and chloroform; slightly soluble in ethanol, acetone and 0.1N HCl; sparingly soluble in 0.1N NaOH and insoluble in benżene (7).

2.8 Melting Range:

The melting range reported in the USP XIX for sulfamethazine is between 197° and 200° using the Class I procedure (6).

2.9 Differential Thermal Analysis

The only thermal event in the differential thermal analysis curve of this compound is a sharp endotherm at 197°C (8). Sunwoo and Eissen (9)have calculated the heat of fusion to be 7438 ± 170 cal/mole.

2.10 Powder X-ray Diffraction

The x-ray powder diffraction pattern of sulfamethazine is shown in Table II and Figure 5 (10).

There has been considerable speculation as to the presence of 2 or 4 forms of sulfamethazine. Shiang Yang and Guillory (11) and Mesley and Houghton (12) report the presence of two forms of sulfamethazine. The data shown in these two papers, however, indicates very small differences in fusion temperature, IR spectra and x-ray powder diffraction data.

Kuhner-Brandstätter and Wunsch (13) reported that they were able to detect four forms of sulfamethazine by thermomicroscopic methods. No IR spectra of these forms were published in subsequent reports.

Table II

X-ray powder diffraction pattern
of sulfamethazine

2 Θ	d (Å)	Relative Intensity
9.68	9.14	1.000
10.70	8.27	0.045
15.21	5.83	0.101
17.16	5.17	0.075
18.44	4.81	0.083
19.71	4.50	0.571
21.33	4.17	0.088
24.64	3.61	0.338
25.92	3.44	0.103
27.70	3.22	0.049
28.81	3.10	0.303
29.23	3.06	0.052
30.25	2.95	0.046

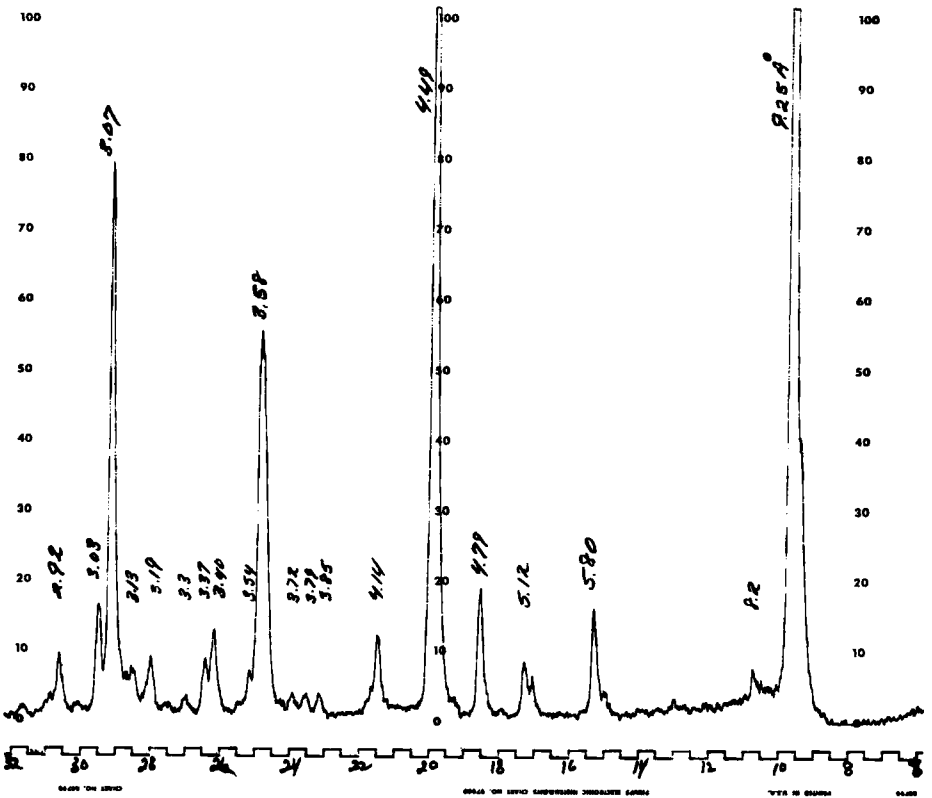
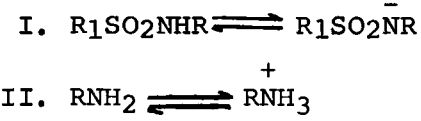


Figure 5. X-ray diffraction pattern of sulfamethazine.

2.11 Dissociation Constants

The pK was found to have a value of 7.4 ± 0.2 for equilibrium I and a value of 2.65 ± 0.2 for equilibrium II (14).



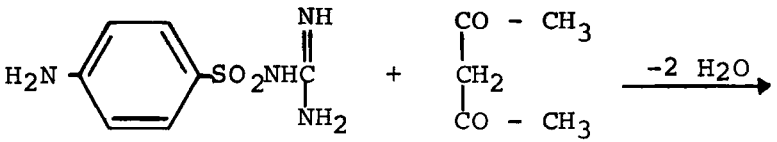
I. $R_1SO_2NHR \rightleftharpoons R_1SO_2\bar{N}R$

II. $RNH_2 \rightleftharpoons RNH_3^+$

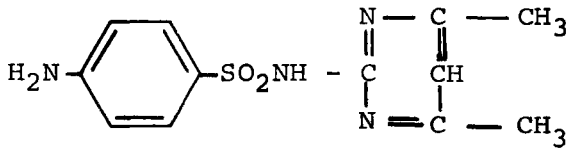
3. Synthesis

Sulfamethazine is prepared by reacting acetylsulfanilyl chloride with 2-amino-4,6-dimethylpyrimidine suspended in dry pyridine or in acetone and pyridine, followed by alkaline hydrolysis of the 2-(N^4-acetylsulfanilamido)-4,6-dimethylpyrimidine; the resulting salt being neutralized with SO_2. The 2-amino-4,6-dimethylpyrimidine is prepared by condensing acetylacetone with guanidine carbonate in toluene (15). Alternatively it can be prepared by condensation of equimolar amounts of sulfanilylguanidine and acetylacetone directly. It is precipitated in the presence of water in the form of very pale yellow crystals (16) Figure 6.

Figure 6
Synthesis of Sulfamethazine



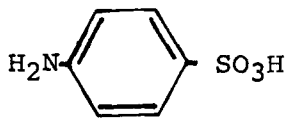
Sulfanilylguanidine Acetylacetone



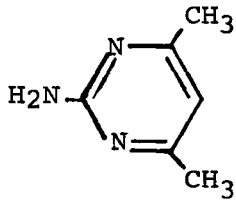
Sulfamethazine

4. Stability and Degradation

Venturella (17) reports that refluxing 10 g of sulfamethazine in 135 ml of 2N HCl for 6 hours yielded sulfanilic acid (I) and 2-amino-4,6-dimethyl-pyrimidine (II).



(I)



(II)

5. Drug Metabolism

Sulfamethazine is rapidly absorbed from the gastro-intestinal tract so that regular oral administration of the drug will produce blood concentrations of 5 to 10 mcg %. Sulfamethazine is highly acetylated, about 30% in the blood and 60% in the urine being in the form of the acetyl derivative. A correlation appears to exist with the acetylator phenotype as determined with procainamide/N-acetylprocainamide and isonicotinic acid hydrazide. Sulfamethazine is firmly bound to plasma proteins (18,19).

The metabolism of sulfamethazine has been studied in various species:

Species	References
Human	18,19,20,21,22
Cattle	23,24,25,26
Hen	27
Rabbit	28,29
Swine	30
Horse	31
Goat	32
Monkey	33
Rat	34,35

6. Method of Analysis:

6.1 Elemental analysis

The results from the elemental analysis of sulfamethazine are listed in Table III (36).

Table III
Elemental Analysis of Sulfamethazine

Element	% Theory	% Found
C	51.78	51.53
H	5.07	5.00
N	20.13	19.90
S	11.52	11.42

6.2 Volumetric methods

Various titrimetric methods can be used for the routine assay of sulfonamides of pharmaceutical importance. Titration with sodium nitrite solution, to determine the aromatic amine function, is the most widely used assay procedure (37,38).

Agarwal et al (39) describe a spectrophotometric titration procedure for the analysis of a number of sulfonamide drugs. The drug dissolved in a mixture of concentrated hydrochloric acid and glacial acetic acid (2:8) is titrated with a 0.1N bromate-bromide mixture. The end-point is determined spectrophotometrically at 345 nm.

Thermometric titration methods have been used for the determination of sulfonamides in aqueous solutions. The reactions employed include diazotization with 0.1M sodium nitrite (40), oxidation with 0.5M sodium hypochlorite (41), the formation of silver derivatives with 0.3M silver nitrate (42), and catalytic thermometric titrimetry in which the acidic function of sulfamethazine is determined by titrating a solution of the drug in dimethylformamide with tetra-n-butylammonium hydroxide. The heat evolved during the alkali-catalyzed anionic polymerization of acrylonitrile (indicator) is used to establish the end-point (43).

6.3 Colorimetric Analysis

One of the most widely used methods for the determination of sulfamethazine is the Bratton-Marshall test (44) which is based on diazotization and coupling with N-(1-naphthyl ethylenediamine dihydrochloride).

Davis et al describe a method for the determination of sulfonamides by the formation of an indophenol dye and measuring the absorbance at 725 nm, (45).

6.4 Column Chromatography

Banes and Riggleman describe a chromatographic method for the assay of trisulfapyrimidine tablets and oral suspensions (46). Total sulfonamides are measured colorimetrically by the Bratton-Marshall procedure. Sulfamethazine is separated from its homologs by column partition chromatography and is determined by UV spectrophotometry.

Rader (47) describes a method for the operation and determination of individual sulfonamides in mixtures. These procedures are based on the ion-pair formation of the sulfonamides with tetrabutylammonium ion, followed by separation on partition chromatographic columns. The separated sulfonamides are then determined by UV spectrophotometry.

6.5 High-Pressure Liquid Chromatography

Sharma et al (48) describe a method for the separation and quantitative determination of sulfamethazine, sulfamerazine, sulfathiazole, and their N^4-acetylated metabolites on an amino-bonded reversed-phase column. The method is suitable for the analysis of these compounds in pure solutions as well as cattle urine.

Poet and Pu (49) and Kram (50) also describe methods for the separation of sulfa drugs using HPLC.

Karger et al (51) have developed a method combining HPLC and high selectivity ion-pair

partition to separate mixtures of sulfa drugs.

6.6 Gas Chromatography

Sulfapyrimidines have been analyzed by gas chromatography after an acid hydrolysis to obtain sulfanilic acid and their respective heterocyclic amines using a column packed with 5% SE-30 and 5% Carbowax 20M on chromosorb W with a column temperature of 150°(52).

Sulfamethazine present in feeds has also been analyzed by GLC (53, 54).

6.7 Paper Chromatography

Sulfamethazine and other sulfonamides have been analyzed by paper chromatography in six solvent systems and the spots located with a variety of visualizing reagents (55).

The solvent systems used were:

A: Methyl isobutyl ketone-formic acid-water (10 parts ketone saturated with 1 part 4% formic acid)

B: Chloroform-methanol-formic acid-water (10 parts chloroform saturated with a mixture of 1 part methanol and 1 part 4% formic acid)

C: Benzene-methyl ethyl ketone-formic acid-water (a mixture of 9 parts benzene and 1 part ketone saturated with 1 part 2% formic acid)

D: Benzene-formic acid-water(10 parts benzene saturated with 1 part 2% formic acid)

E: Methyl ethyl ketone-acetone-formic acid-water (40:2:1:6)

The chromatography was performed on Whatman No. 1 filter paper. Sulfamethazine gave R_f values of 0.91, 0.46, 0.88, 0.83, 0.54 and 0.22 with solvent systems A to F respectively.

The spots were localized with the use of either:

1) UV light
2) Potassium permanganate spray reagent

(1% aqueous solution)
3) Bromophenol blue spray reagent (0.05% in ethanol)
4) Ehrlich reagent spray reagent (1% p-dimethylaminobenzaldehyde in 1N HCl)
5) p-Dimethylaminocinnamaldehyde spray reagent (0.1% in 1N HCl)

6.8 Thin-Layer Chromatography

Sulfamethazine has an R_f of 0.65 when spotted on silica gel plates and developed with a mixture of ethyl acetate and methanol (90:10). The spot can be located using the following reagents: (1) copper acetate in methanol (saturated): yellowish green color; (2) 5% copper sulfate in water:orange color; (3) 2% ceric sulfate in water using 5 ml conc. sulfuric acid:yellowish purple(56).

A TLC system based on polyamide or silica gel G layers and an aqueous mobile phase was also used for the separation of a series of sulfonamides, (57). The mobile phase was veronal acetate buffer at pH 7.4, alone or mixed with different amounts of acetone. Localization was obtained by spraying with p-dimethylaminobenzaldehyde (0.1% in ethanol)/ concentrated hydrochloric acid (99:1). Sulfamethazine gave an R_M value of -0.18 on the silica gel G plates (0% acetone) and R_M: 0.26 and 0.13 on polyamide plates (0% acetone and 10% acetone respectively). ($R_M = \log \left[\frac{1}{RF} - 1\right]$).

The chromatographic behavior of sulfamethazine and other sulfonamides has also been investigated on strong and weak cation and anion exchange TLC plates with polystyrene, paraffin and cellulose matrices using aqueous and aqueous-organic eluents (58).

A method has also been described for the analysis of mixed sulfonamides by TLC and UV spectrophotometry (59). The samples are spotted on fluorescent silica gel H plates and developed in

chloroform-methanol (88:12). The developed spots are delineated under UV light, scraped from the plate and extracted with 1N NaOH. The UV absorptions of the centrifuged extracts are read with a recording spectrophotometer.

Cieri (60) describes a method for the detection of sulfamethazine and other sulfonamides in animal feeds. The sulfonamides are extracted from animal feed samples with alcohol or acetone and cleaned up by a celite column chromatographic technique. The eluate is spotted on a neutral Absorbosil-1 TLC plate, which is developed in chloroform-methanol (95:5) and then sprayed with an alcoholic solution of p-dimethylaminobenzaldehyde.

6.9 Microbiological Assay

Sulfa drugs and antibiotics have been detected in milk using Bacillus megaterium ATCC 9855 as the test organism and Mueller-Hinton agar as the test substrate. Incubation was at 37° for 4 to 5 hours.

6.10 Assay in Body Fluids and Tissues

Several methods have been used for the determination of sulfamethazine in serum, urine and tissues. The references mentioned in section 5 (Drug Metabolism) describe the most commonly used procedures.

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Analytical Profiles of Drug Substances, 7

THIOSTREPTON

Klaus Florey

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TABLE OF CONTENTS

1. Introduction

1.1 History

Thiostrepton, a polypeptide antibiotic of yet not fully known structure, was isolated and characterized by scientists of the Squibb Institute for Medical Research[1-3] in 1954. It was first obtained from a fermentation broth, that had been inoculated with Streptomyces azureus, isolated from a soil sample collected in New Mexico. Thiostrepton is active against gram-positive bacteria and has found use in veterinary medicine.[6] The antibiotics thiactin and bryamcin were found to be identical with thiostrepton.[4]

1.2 Formula and Molecular Weight

No exact formula or molecular weight can be given. The formula probably ranges between $C_{69}H_{91}N_{18}O_{22}S_5$ and $C_{72}H_{83}N_{19}O_{17}S_5$ and the molecular weight is approximately 1600[5] (see section 3).

1.3 Appearance, Color, Odor

White to light yellow, odorless crystals.

1.4 Salts, Complexes and Esters

Thiostrepton is a weak base which forms salts. The hydrochloride,[7,8] phosphate[8] and sulfate[8] are described as are complexes with calcium[7,8] and sodium chloride.[8] The hemisuccinate ester has also been disclosed.[9]

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of thiostrepton is presented in figure 1. The following assignments can be made:[2,8]

cm^{-1}	Assignment
3400 (broad)	OH,NH stretch
1650, 1550	Amide, C=O, NH
2920, 2970	C=N stretch

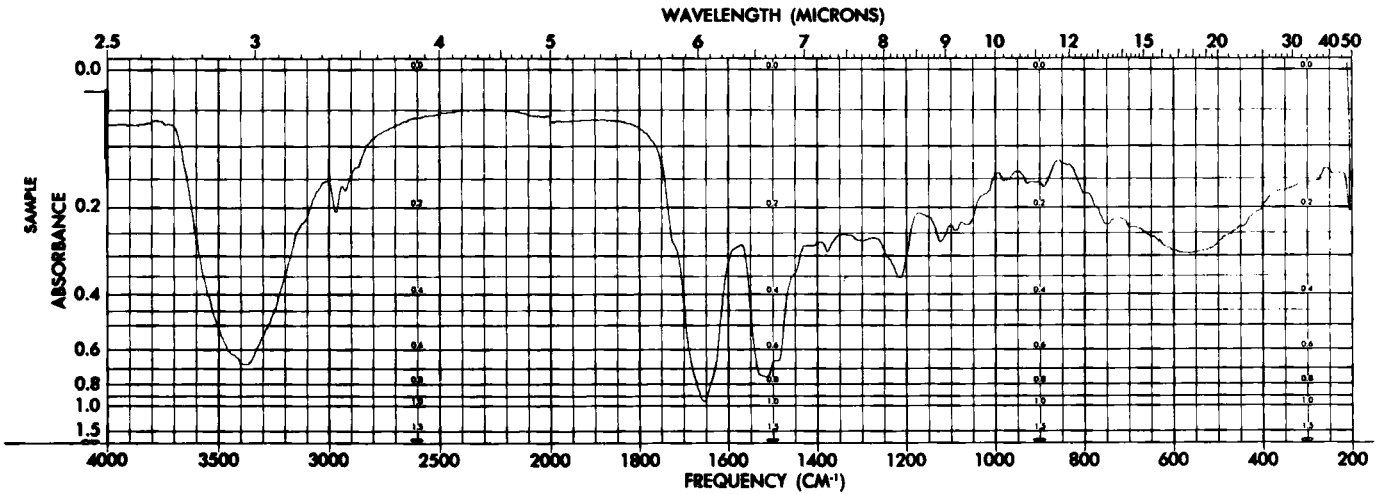


Figure 1. Infrared Spectrum of Thiostrepton (batch #2148A) KBr pellet.
Instrument: PE 621

2.2 NMR Spectra

The 100 MHz NMR spectrum of thiostrepton was studied[32] in DMSO-d_6 containing TMS as internal reference utilizing a Varian XL-100-15 NMR spectrometer (Figures 2 and 3). For the purposes of assignment, the NMR spectrum may be divided into three parts:

a) 0-3 ppm consisting of methyl and methylene protons,
b) 3-6 ppm consisting of methylene and methine protons attached to the heteroatoms, and
c) 6-10 ppm consisting of aromatic, heterocyclics and exchangeable protons.

The addition of D_2O exchanged several resonances at δ 7.70, 8.20, 9.20, 9.45 and 9.95 (Figure 3). It has not been possible to make specific assignments.

The ^{13}C NMR spectrum of thiostrepton (Figure 4) was obtained at 60° in $CDCl_3$-CD_3OD (8:2 v/v) utilizing Varian XL-100-15 spectrometer, equipped with Fourier Accessories obtained from Nicolet Corporation. The system was locked to ^2H frequency of 15.4 MHz and operated at a frequency of 25.2 MHz. After the expansion, it was possible to account for 71 carbons out of a total of 72. The chemical shifts (Table 1) are comparable to ones known in the literature.[33]

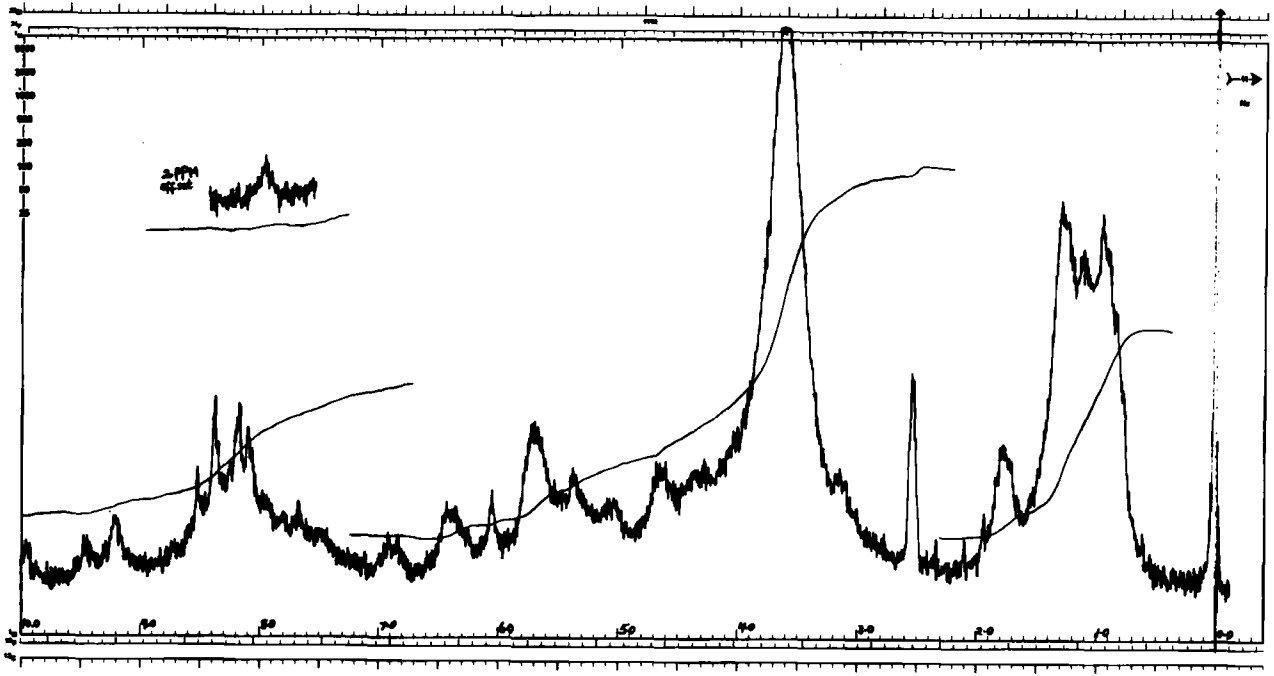


Figure 2. Proton NMR Spectrum of Thiostrepton in DMSO-d_6.
Instrument: Varian XL-100

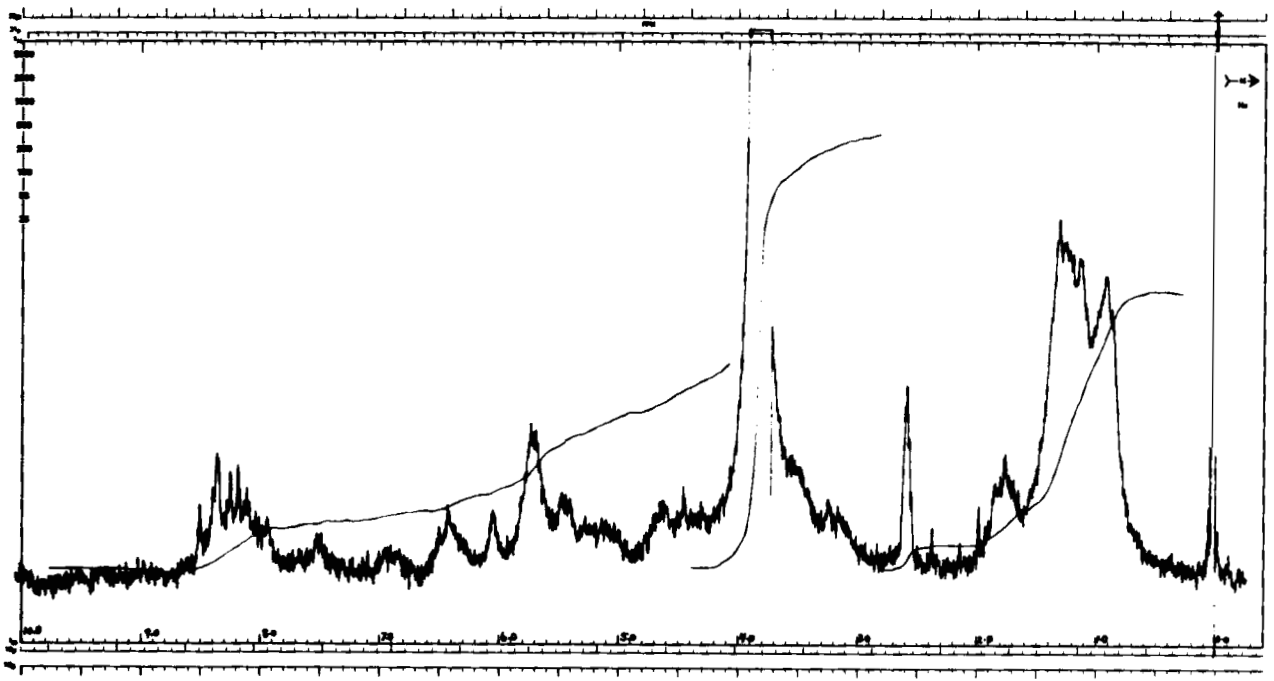


Figure 3. Proton NMR Spectrum of Thiostrepton in DMSO-D_2O.
Instrument: Varian XL-100

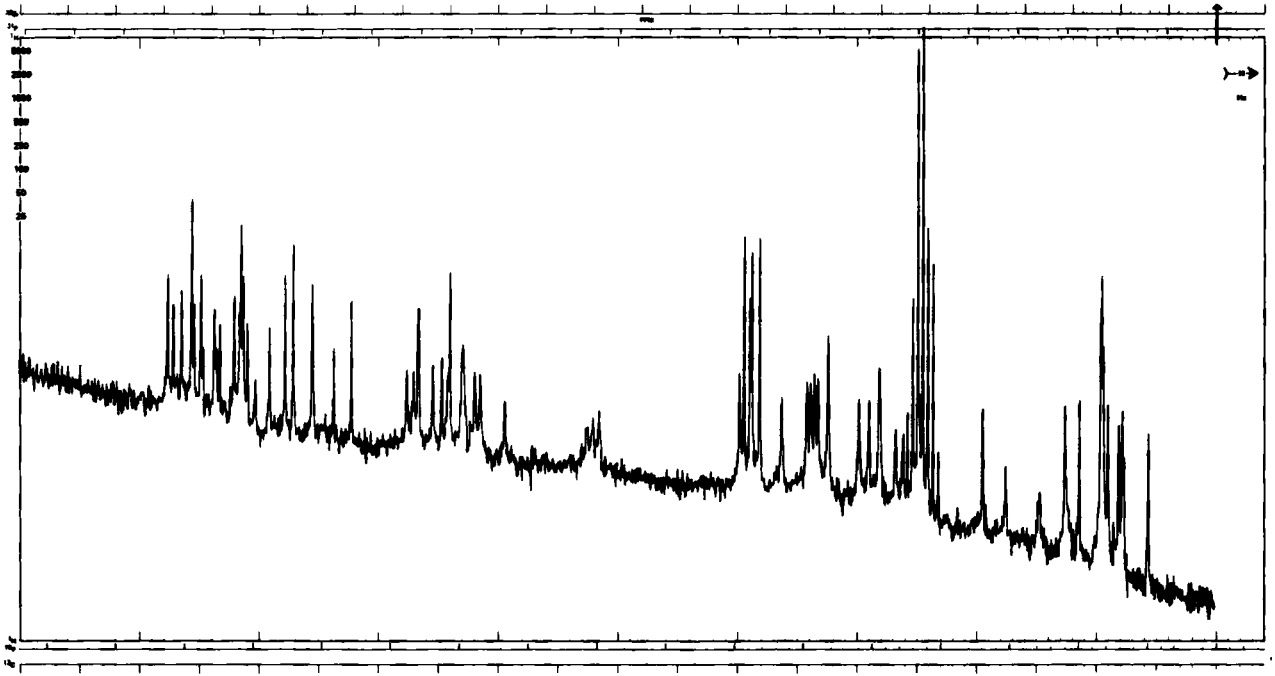


Figure 4. ^{13}C NMR Spectrum of Thiostrepton in $CDCl_3$-CD_3OD (8:2)
Instrument: Varian XL-100

Table 1

^{13}C Chemical Shift and Tentative Assignments

C-CH_3	11.0, 15.1, 15.3, 15.9, 17.7 22.4, 18.5, 18.8, 18.9
C(S)-CH_2	24.6, 24.8, 29.1
C-CH	34.7, 38.5
N(O)CH	49.1, 51.7, 51.8, 53.0, 55.8, 57.4, 59.1 64.2, 66.0, 67.2, 67.7, 71.9, 79.0, 66.5
(N(O)-C	57.4, 77.2
C=CH_2	102.3, 103.3, 104.3
C=CH	118.0, 122.1, 123.0, 125.0, 125.1, 127.3, 128.4
C=C	127.0, 129.9
C=C-N	132.3, 133.1, 134.4, 143.4
N=C	146.4, 150.0, 153.1, 154.5, 157.1, 159.5
CO	160.8, 161.5, 161.9, 162.1
N=C-S	163.0, 165.4, 166.0, 166.3, 168.2, 168.5 169.7, 170.1(2), 171.8, 173.1, 174.0

The chemical shifts are relative to δ $CDCl_3$ = 76.9 ppm from TMS.

2.3 Mass Spectrum

Attempts to obtain a mass spectrum of thiostrepton or silanized derivatives were unsuccessful.[26]

2.4 Ultraviolet Spectrum

The ultraviolet spectrum presents no maxima but shows characteristic shoulders at 225(E_1^1 520), 250 (E_1^1 380) and 280 (E_1^1 225) nm.[2]

2.5 Optical Rotation

The specific rotation varies with the solvent as follows:[2]

	$[\alpha]_D^{23}$; c = 1.0
glacial acetic acid	-98.5°
dioxane	-61.0°
pyridine	-20.0°

2.6 Melting Range

Thiostrepton melts with decomposition over the range 246 to 256° C.[2,7]

2.7 Differential Thermal Analysis

DTA was performed on a sample of thiostrepton (house standard) under static air at a heating rate of 15° C/min, using capillaries (Figure 5).

A broad endotherm was observed with a peak temperature of 120° C., probably representing the dehydration. A relatively sharp exotherm at 232° C. probably represents an oxidative melting and degradation process.[34]

2.8 Phase Rule Analysis

The purity of thiostrepton can be determined by phase rule analysis, using chloroform-carbon tetrachloride 3:1 as solvent system and equilibrating for 168 hours at 25° C. The purity of one sample was found to be 98.6%.[35]

2.9 Solubility

The solubility of thiostrepton was determined as follows,[25] expressed as mg/ml:

Water 0.088	Methanol 0.285
Ethanol 0.990	Isopropanol 0.595
Isoamyl Alcohol 5.250	Cyclohexane 0.030
Benzene 1.352	Petroleum Ether 0.018
Isooctane 0.0	Carbon Tetrachloride 0.055
Ethyl Acetate 0.685	Isoamyl Acetate 0.285
Acetone 0.492	Methyl Ethyl Ketone 0.720
Diethyl Ether 0.032	Ethylene Chloride 0.182

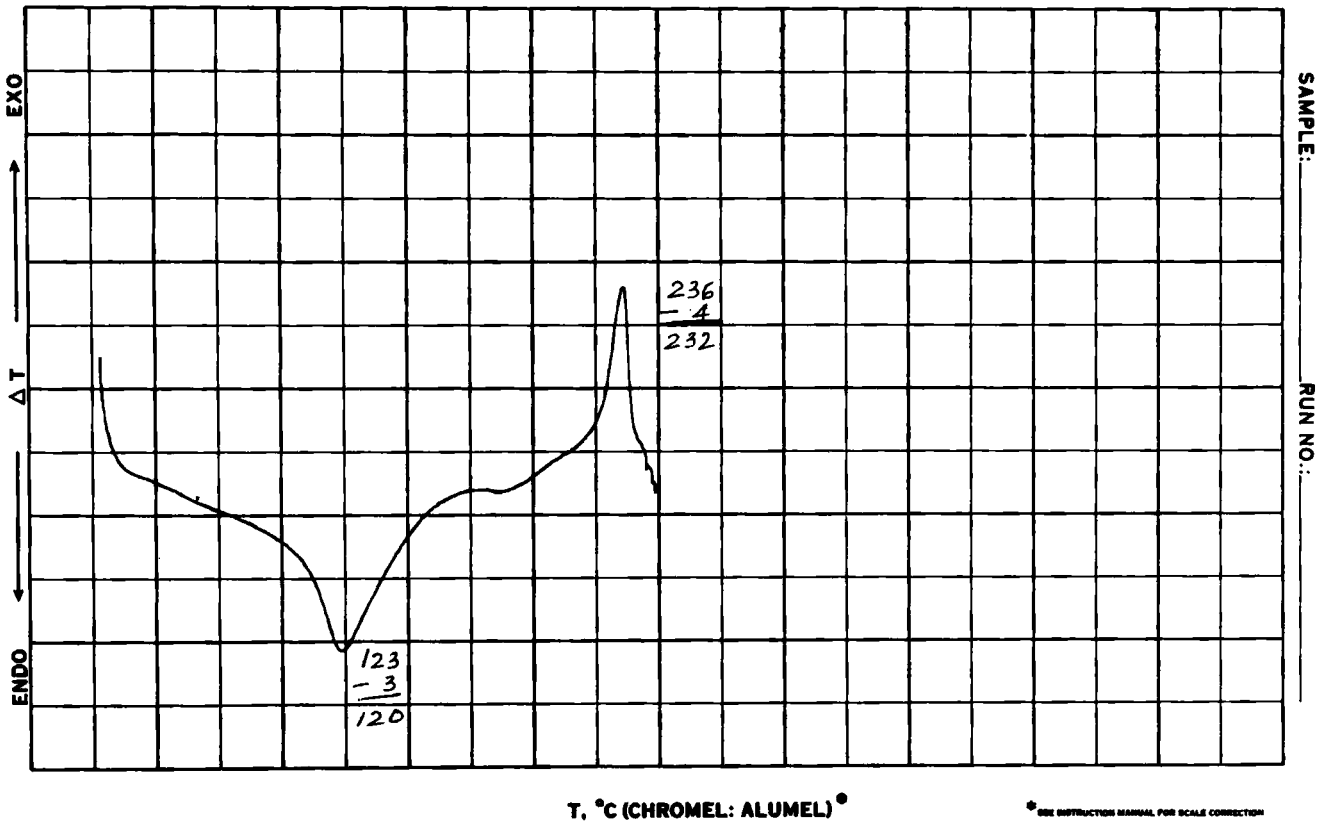


Figure 5. DTA of Thiostrepton (house standard) Heating Rate: 15° C/min.
Instrument: DuPont 900

1,4 Dioxane >20	Chloroform >20
Carbon Disulfide 0.032	Pyridine >20
Formamide >20	Ethylene Glycol 5.840
Propylene Glycol 17.360	Dimethylsulfoxide >20
0.1 N NaOH 4.986	0.1 N HCl 10.055

In contrast to the last two values, Vandeputte and Dutcher[2] claim that thiostrepton is not dissolved in dilute aqueous acid or base, but is dissolved in methanolic acid or base. The latter solvent, however, rapidly inactivates the antibiotic.

2.10 Crystal Properties

The powder x-ray diffraction pattern of thiostrepton is presented in Table 2 and Figure 6.[31] No polymorphs of thiostrepton are known.

Table 2

Powder X-Ray Diffraction Pattern of Thiostrepton
Batch #7H10-1

2θ	'd'Val-Å	Relative Intensity	2θ	'd'Val-Å	Relative Intensity
4.7	18.7	.2836	16.4	5.40	.6268
5.7	15.5	1.0000	17.5	5.05	.5074
6.7	13.1	.3134	18.4	4.81	.6418
7.5	11.8	.3880	19.7	4.50	.3433
8.1	10.8	.5373	20.7	4.27	.6866
9.5	9.3	.2388	21.5	4.13	.4179
10.2	8.7	.1045	22.8	3.89	.3283
10.7	8.3	.1343	23.9	3.72	.5074
12.5	7.1	.2985	25.0	3.50	.2537
13.8	6.4	.3134	26.1	3.42	.1940
14.4	6.15	.3283	26.6	3.35	.2388
15.1	5.87	.4328	27.2	3.28	.2388
15.3	5.77	.3880	28.0	3.08	.1045
16.0	5.53	.3731			

3. Synthesis

3.1 Fermentation, Isolation

Thiostrepton is produced by fermentation[1] using the micro-organism Streptomyces azureus.[7] The yield can be improved by the addition of heavy metal cations to the fermentation medium.[10,11]

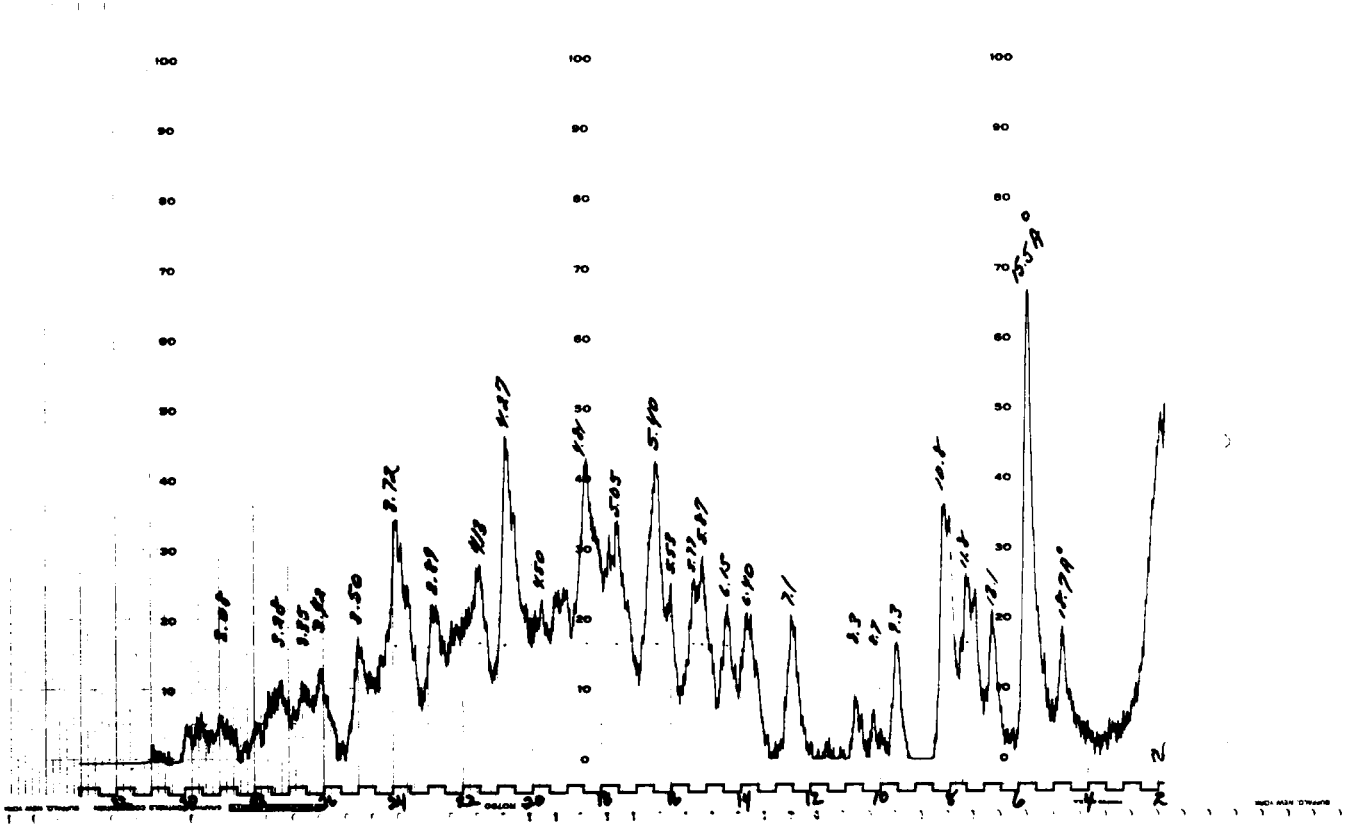


Figure 6. Powder X-Ray Diffraction Pattern of Thiostrepton
Instrument: Norelco

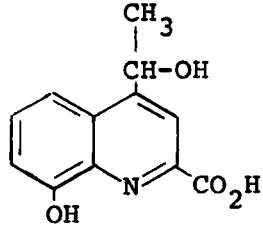
Actinomyces species also produce thiostrepton.[12] Thiostrepton is found mainly in the mycelium while at least two other antibiotics are found in the supernatant broth. One of the companion antibiotics has been identified as siomycin.[24] It is chemically very similar to thiostrepton; however, one isoleucine unit is replaced by valine, and it also has one less alanine unit. Thiostrepton is extracted from the mycelial cake by suitable organic solvents such as chloroform, dioxane, dimethyl formamide or benzyl alcohol.[7] Further purification is carried out by carbon treatment and repeated crystallization from chloroform or dioxane-methanol.[2]

The preparation of sulfur labeled thiostrepton has also been described.[13,14]

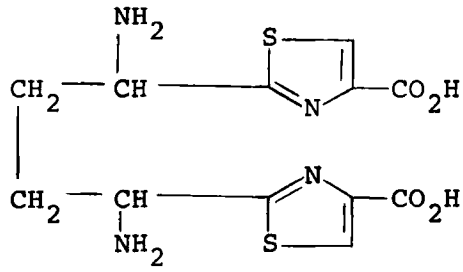
3.2 Structure

The structure of thiostrepton is not yet fully known. Pioneering studies in the elucidation of structure have been carried out by Bodanszky[15-21] and Kenner[22-23] with their collaborators. A significant contribution to clarify the structure was made by Anderson, Hodgkin and Viswamitra[5] who examined thiostrepton by single crystal x-ray diffraction.

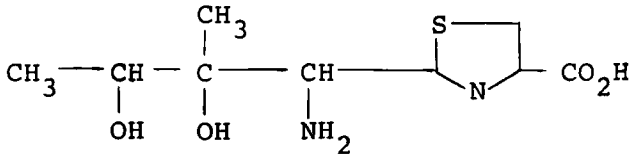
The principal fragments obtained on hydrolysis of thiostrepton are (-)-alanine, (-)-threonine, (-)-isoleucine, (+)-cysteine, pyruvic acid, also



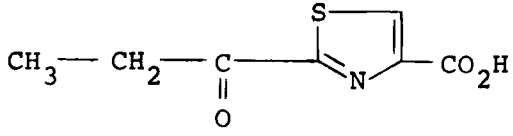
4-(α-Hydroxyethyl)-8-hydroxyquinaldic acid.



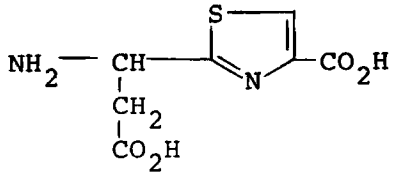
Thiostreptoic acid.



Thiostreptine.



2-Propionylthiazole-4-carboxylic acid.



2-(1-Amino-2-carboxyethyl)thiazole-4-carboxylic acid.

All these fragments fit in the structure as determined by single crystal x-ray diffraction (Figure 7).[5] However, Figure 7 does not yet represent the total structure. The side chain (top of Figure 7) probably contains two additional dehydroalanine and one α,α-diaminopropionic acid residue.[20,33] The structure as presented in Figure 7 also does not fully disclose the conformation of the molecule. As a whole, the molecule appears roughly globular with the two large rings folded over one another and the long side chain protruding from their junction. The conformation is maintained by a number of intramolecular hydrogen bonds.[5]

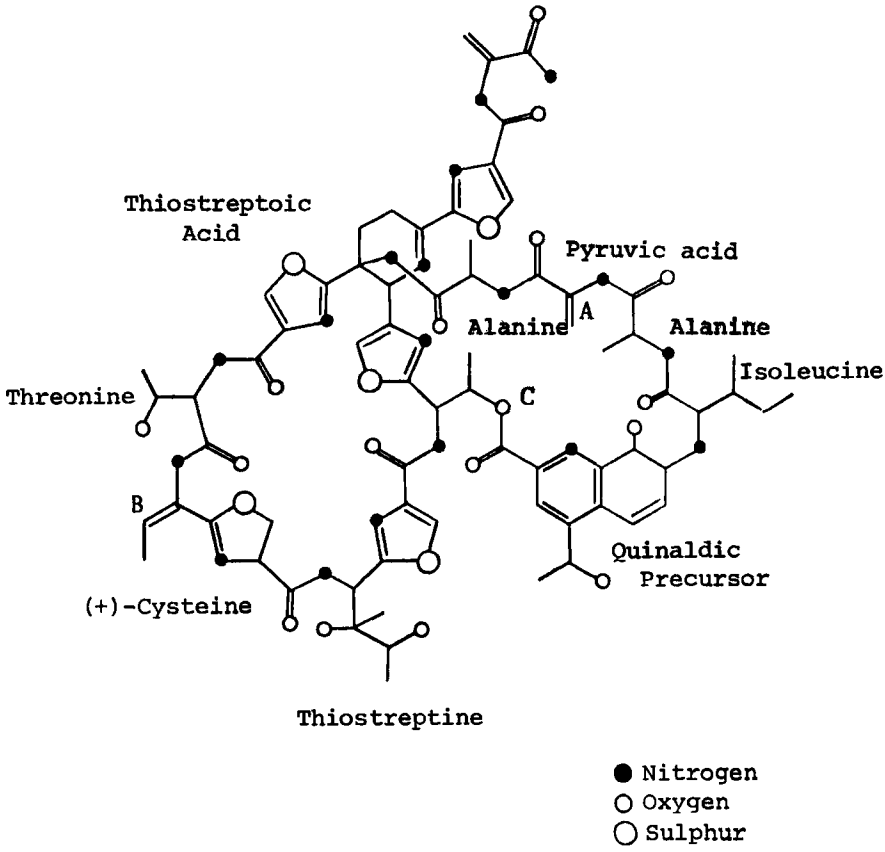


Figure 7. Structure of Thiostrepton, as Determined by Single Crystal X-ray Diffraction.[5]

4. Stability-Degradation

Thiostrepton is stable as a crystalline powder. In N,N -dimethyl acetamide, it retains its potency for at least 16 days at 37° C. and for at least three months under refrigeration. The antibiotic is also stable at 30° C. for at least one week in 50% aqueous dioxane at a pH of 7, although it rapidly loses potency in the same solvent systems at a pH of 2 or 11.[41] For hydrolytic degradation products, see section 3.2.

The acid sulfate and hydrochloride salts of thiostrepton in alcoholic solution soon lose their antibiotic activity. Thiostrepton base is precipitated when water is added to these alcoholic solutions.[41]

5. Drug Metabolism, Pharmacokinetics

No drug metabolites are known. Apparently, the antibiotic is not absorbed from the intestinal tract since systemic activity cannot be demonstrated following oral administration of the drug. Parenterally, however, thiostrepton is quite active. Following intramuscular administration, thiostrepton may lie in the local tissues as a depot. No significant blood or urine levels could be demonstrated.[27]

6. Methods of Analysis

6.1 Elemental Analysis

	Found %		
Element	Ref. 2	Ref. 7	Ref. 17
C	51.3	51.75	
H	5.4	5.3	
N	14.6		
S	7.4	9.22	9.2-10.2%

Due to the capacity of the molecule to retain solvents tenaciously and to absorb moisture from the air after careful drying, the elemental analysis is difficult to perform.[17]

6.2 Microbiological Analysis

A microbiological agar diffusion assay, using Micrococcus pyogenes var. aureus (ATCC6538P) as the test organism, has been described in detail.[29] This assay can be used to determine thiostrepton in

ointments and milk.

An automated turbidimetric assay system, using streptococcus faecalis (ATCC10541) has also been used.[30]

The activity of thiostrepton is measured in units. One microgram of thiostrepton is equal to 1.1 units.[29]

6.3 Nonaqueous titration

Nonaqueous titration with perchloric acid in glacial acetic acid gives an equivalent weight of 420.[2]

6.4 Color Reactions

Thiostrepton gives the following color reaction:[36]

Folin-Ciocalteau:	Intense blue color
Ehrlich reagent: (p-dimethylamino-benzaldehyde in dilute acid)	Strong violet color (see also 6.5)
Alkaline hypoiodite:	Small amount of iodoform
Ninhydrin:	Negative (positive after hydrolysis)
Biuret:	Positive[2]

6.5 Colorimetric Analysis

Thiostrepton in dimethylformamide can be reacted with p-dimethylaminobenzaldehyde in the presence of alcoholic hydrochloric acid. The resulting color formation can be used for quantitation, by reading the absorption at 535 nm.[36] This method can be used for the determination of thiostrepton in mycelial cake[36] and ointments.[37]

6.6 Chromatographic Analysis

6.61 Paper

The following systems have been used in the ascending mode:[1]

	Dev. time (Hrs.)	R_f
Acetone:water (70:30)	5	0.50
Acetone:propanol:water (40:40:20)	7	0.80
0.2N Acetic acid	6	0.08

Paper chromatography, followed by bioautography has also been used with the following conditions:[38]

Stationary phase:	Ethanol washed Whatman #54 paper impregnated with propylene glycol
Mobile phase:	Benzene saturated with propylene glycol
Development time:	Sixteen hours, descending
Drying conditions:	20 minutes at 90°C.
Detection:	a. U.V. light b. Bioautography with Staph. aureus 209P. Exposure of strips to culture: 15 min. Incubation for 18 hours at 37°C.

6.62 Thin-Layer

The following methods separated thiostrepton from siomycin:

a.[38] Analtech Silica Gel G.F.
Developing Solvent: Chloroform-methanol (90:10)
R_f Thiostrepton 0.36
R_f Siomycin 0.43
Visualization: Short-wave U.V.

b.[39] Neutral grade IV alumina
Chloroform-methanol (97:3)

6.7 Counter Current Distribution

When thiostrepton was subjected to 16-transfer counter current distribution in a system of toluene-chloroform-methanol-water (1:2:2:1),

thiostrepton was found in tubes 0-5. Redistribution in a toluene-chloroform-methanol-water (5:5:8:2) system and a 350 tube transfer concentrates thiostrepton in a band with K=0.3.[40]

7. Determination in Pharmaceutical Preparations

Thiostrepton is determined in pharmaceutical preparations (ointments) by microbiological[29] or colorimetric[37] assay.

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Analytical Profiles of Drug Substances, 7

TRIMETHOPRIM

Gerald J. Manius

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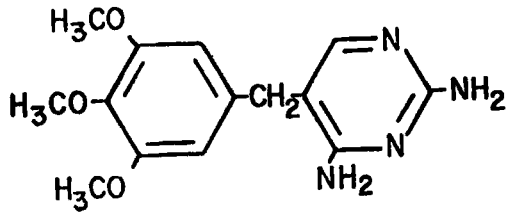
INDEX

1. Description

1.1 Name, Formula, Molecular Weight

Trimethoprim is 2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine



Trimethoprim

$C_{14}H_{18}N_4O_3$ Molecular Weight: 290.32

1.2 Appearance, Color, Odor

White to pale yellow, odorless, crystalline powder.

2. Physical Properties

2.1 Infrared Spectrum (IR)

The infrared spectrum of trimethoprim is presented in Figure 1 (1). The spectrum was obtained on a Perkin-Elmer Model 621 Grating Infrared Spectrophotometer as a solution in chloroform in a concentration of 14.8 mg/ml. The pathlength was 0.1 mm NaCl. The spectrum was run under survey conditions.

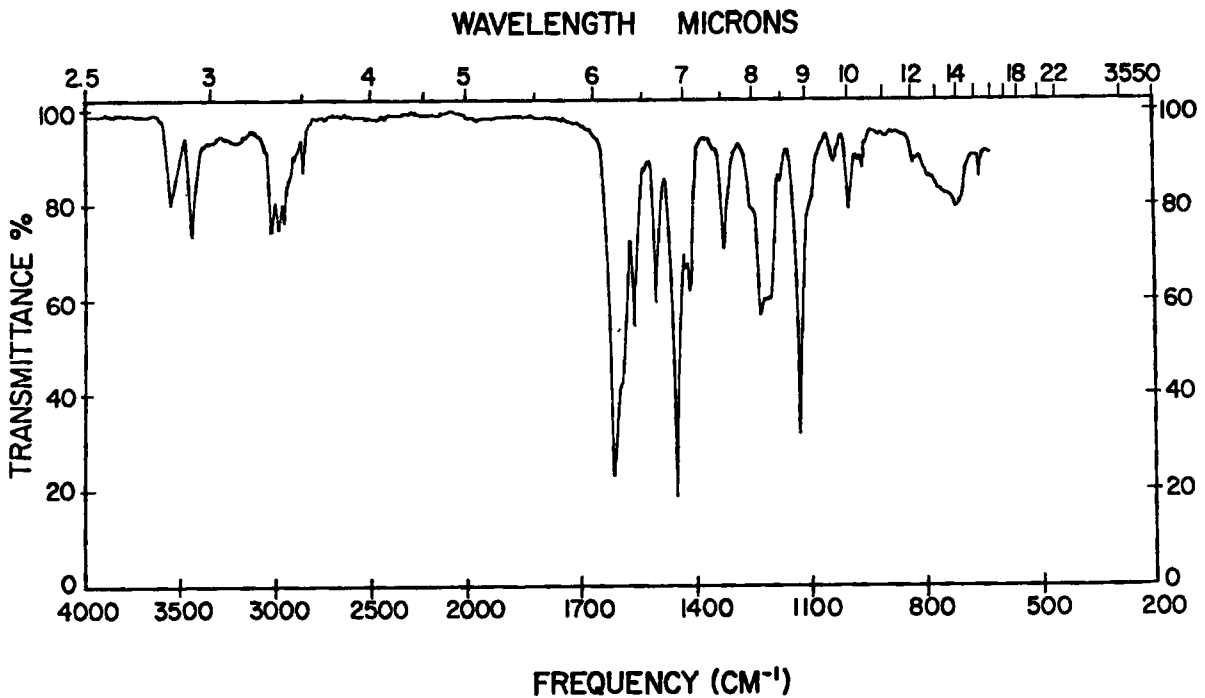


FIGURE 1

Infrared Spectrum of Trimethoprim

Table I

IR Spectral Assignments for Trimethoprim

Characteristic of	Frequency (cm^{-1})
Asymmetric NH_2 stretching	3516
Symmetric NH_2 stretching	3414
Aromatic CH stretching	3010
Aliphatic CH stretching	2966, 2940, 2840
NH_2 deformation overlapped with aromatic ring	1616, 1593
Aromatic ring	1506
CH deformation	1452
Aromatic methoxy	1236, 1129

2.2 Raman Spectrum

The Raman spectrum of trimethoprim is shown in Figure 2. It was obtained as a neat solid on a Spex 1401 Double Spectrometer at 4880 Å using an Ar^+ laser. A fluorescence background was observed. Band assignments are listed in Table II (2).

Table II

Raman Spectral Assignments for Trimethoprim

Band	Intensity	Assignment
3500 cm^{-1}	Strong	NH stretching
1610 cm^{-1}	Very Strong	Aromatic C=C stretching
1000 cm^{-1}	Medium	1.2.3.5 - benzene ring breathing
785 cm^{-1}	Very strong	 breathing

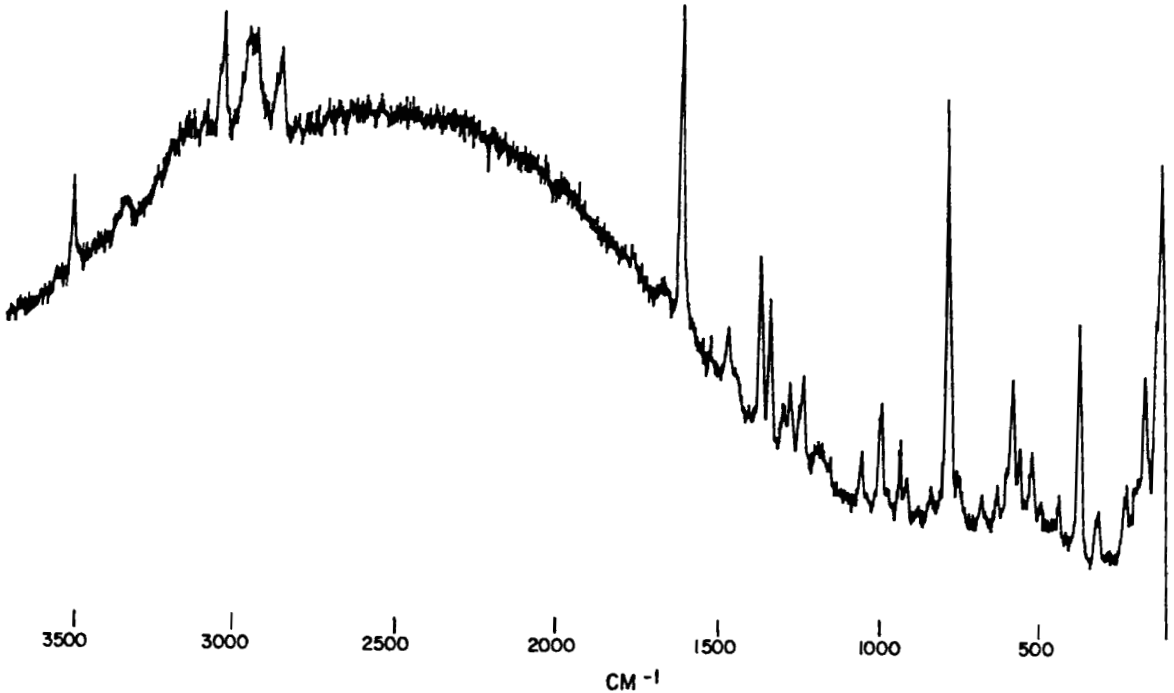


FIGURE 2

Raman Spectrum of Trimethoprim

2.3 Nuclear Magnetic Resonance Spectrum (NMR)

The NMR spectrum shown in Figure 3 was obtained by dissolving 50 mg of trimethoprim in 0.5 ml of DMSO-d_6 containing tetramethylsilane as an internal reference. The NMR spectrum was determined on a Varian XL-100 Spectrometer at probe temperature ca. 31°C. Proton assignments are shown in Table III (3).

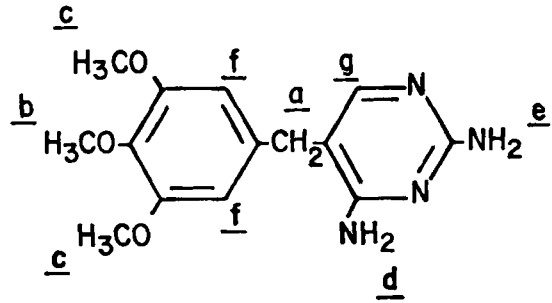


Table III

NMR Spectral Assignments for Trimethoprim

Protons	Chemical Shift δ (ppm)	Multiplicity	Coupling Constant J (in Hz)
a	3.54	Singlet	--
b	3.63	Singlet	--
c	3.94	Singlet	--
d	5.80	Singlet	--
e	6.16	Singlet	--
f	6.58	Singlet	--
g	7.56	Singlet	--

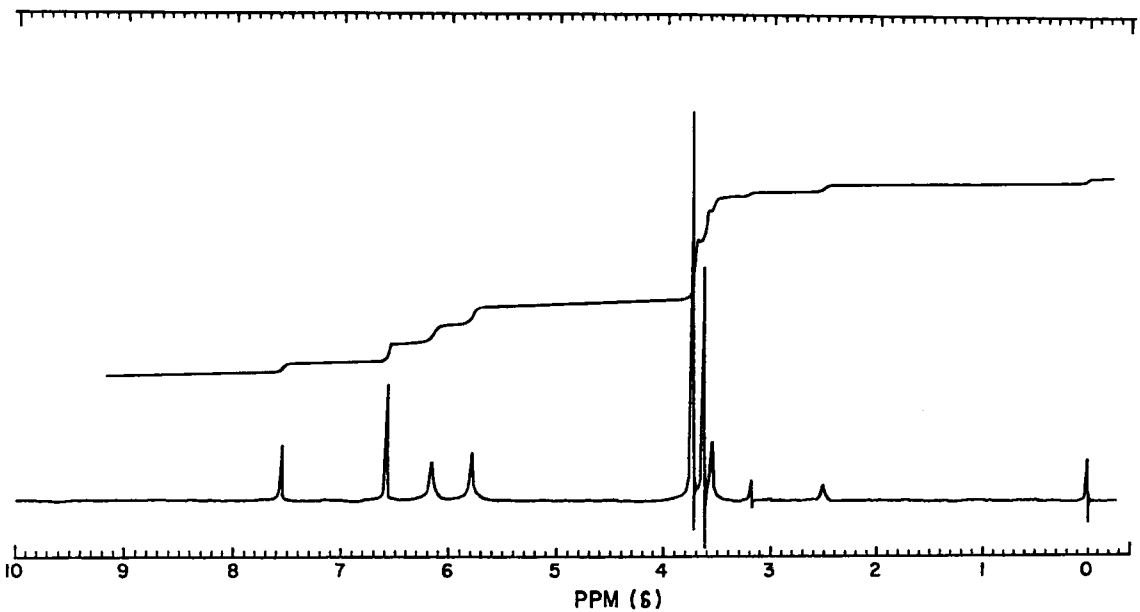


FIGURE 3

NMR Spectrum of Trimethoprim

2.4 Ultraviolet Spectrum (UV)

The ultraviolet spectrum of trimethoprim in the region of 400 to 230 nm is shown in Figure 4 (4). The spectrum exhibits one maximum at 287 ± 2 nm ($\varepsilon = 7.4 \times 10^3$, absorptivity = 25.5) and a minimum at approximately 257 nm. The solution is prepared by dissolving approximately 100 mg of trimethoprim in 25 ml of USP ethanol and diluting to 100 ml volume with 0.4% NaOH. Subdilutions are made in 0.4% NaOH to a final concentration of 0.02 mg/ml. The reference cell contains 0.4% NaOH. The spectrum was obtained on a Cary 14 Spectrophotometer.

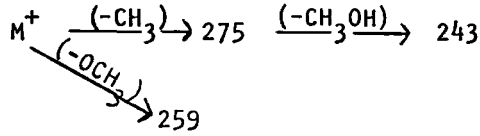
2.5 Fluorescence Spectrum

Trimethoprim exhibits slight fluorescence in relatively concentrated solutions of chloroform (10 mg/ml) and USP ethanol (5 mg/ml). Neither acidification nor basification of the alcohol solution enhanced the fluorescence (5). Air oxidation over several days standing may intensify the fluorescence.

2.6 Mass Spectrum

The low resolution mass spectrum shown in Figure 5 was obtained using a Varian MAT CH 5 Mass Spectrometer, interfaced with a Varian data system. The ionizing energy was 70 eV, source temperature, 150°C and the probe temperature was 30 → 100°C. The following fragmentations were observed (6).

Molecular Ion (M^+) at m/e 290



A high resolution scan was run on the CEC-110 Mass Spectrometer at 70 eV, ionizing energy and at a source temperature of 210°C. Table IV lists the fragmentation ions (6).

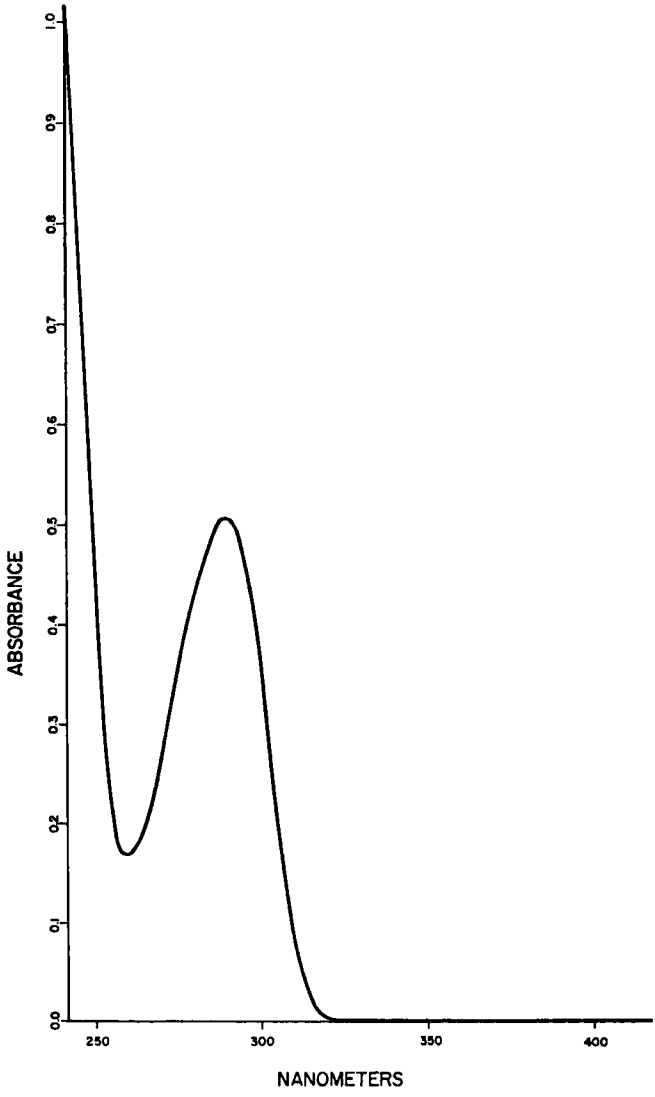


FIGURE 4
UV Spectrum of Trimethoprim

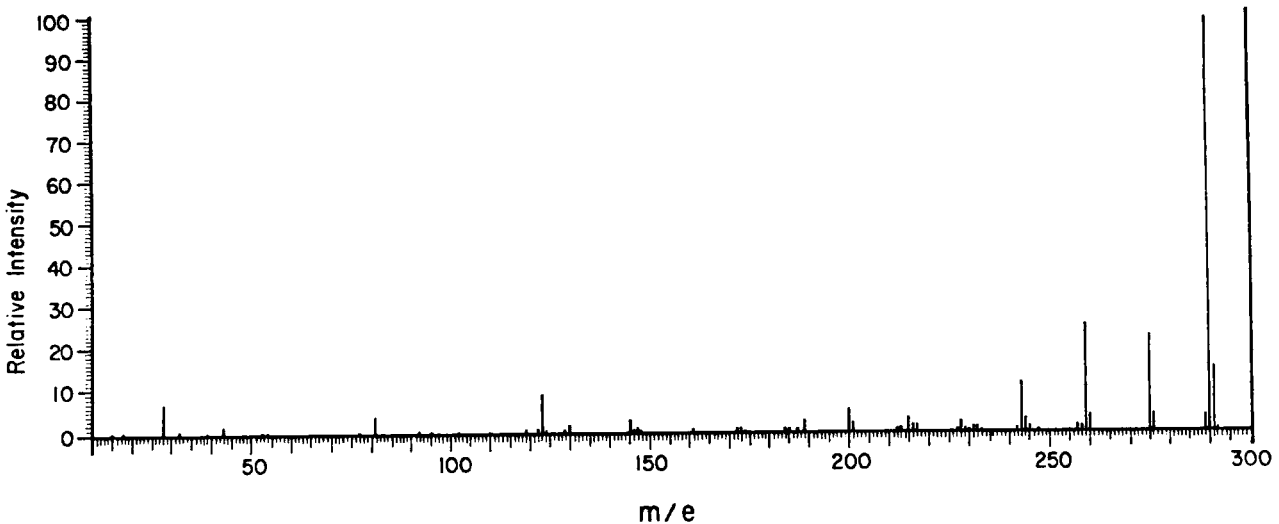


FIGURE 5

Mass Spectrum of Trimethoprim

Table IV

High Resolution Mass Spectrum of Trimethoprim

Observed Mass	Theoretical Mass	Error*	C	H	N	O	Ion
243.0916	243.0882	3.4	12	11	4	2	$275\text{-}CH_3OH$
258.0921	258.0879	4.2	13	12	3	3	$275\text{-}NH_3$
259.1221	259.1196	2.5	13	15	4	2	$M^+\text{-}OCH_3$
275.1198	275.1145	5.3	13	15	4	3	$M^+\text{-}CH_3$
289.1327	289.1301	2.6	14	17	4	3	$M^+\text{-}H$
290.1437	290.1379	5.8	14	18	4	3	M^+

*Mass Difference = Observed Mass - Theoretical Mass in Millimasses

2.7 Optical Rotation

Trimethoprim exhibits no optical activity.

2.8 Melting Range

According to USP XVII, Class I Method, trimethoprim melts at approximately 201°C (7).

2.9 Differential Scanning Calorimetry (DSC)

DSC spectra showed a single endothermic transition at 199.6 ± 0.2°C to 201.0 ± 0.2°C, extrapolated onset to peak (8). Figure 6 represents the DSC spectrum obtained.

2.10 Thermogravimetric Analysis (TGA)

Trimethoprim exhibited a single weight loss as follows (9):

	145°C	225°C	285°C	325°C	500°C*
% Loss	0	2.4	36.0	69.0	80.0

*Limit of the instrument

The TGA spectrum is shown in Figure 7.

ΔH_f = 9.6 KCal/mole

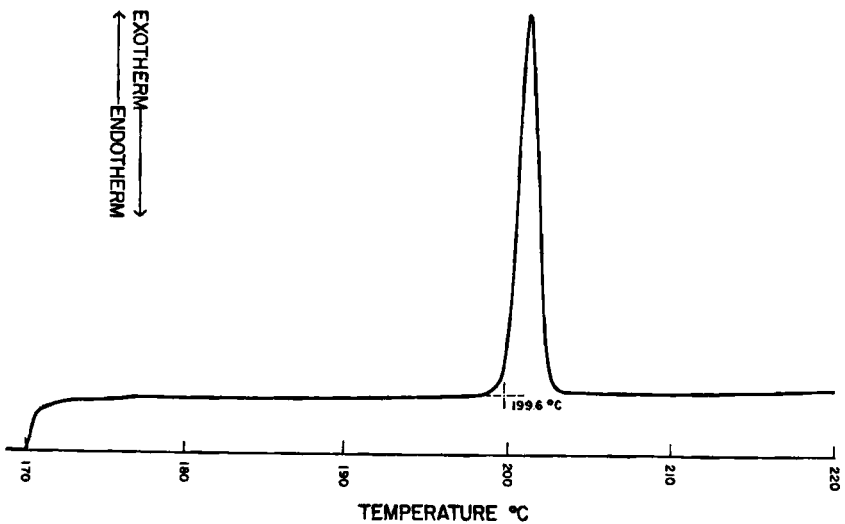


FIGURE 6
DSC Curve For Trimethoprim

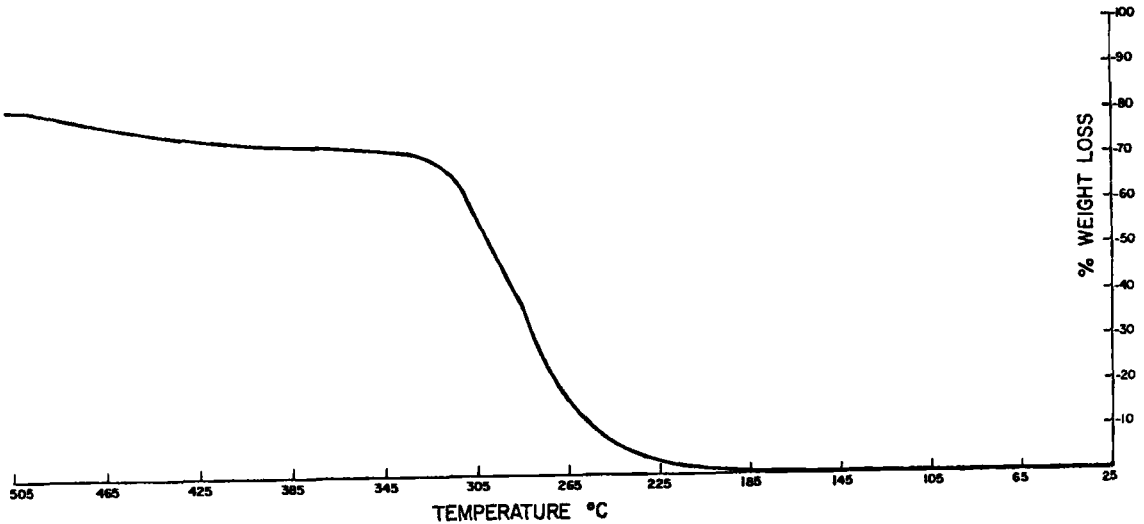


FIGURE 7
Thermogravimetric Analysis of
Trimethoprim

2.11 Solubility

Solubility data for trimethoprim at 25°C (7,10).

Table V

Solvent	Solubility (g/100 ml)
Water	0.04
95% Ethanol	0.81
3A Alcohol	0.35
Methanol	1.21
Isopropanol	0.12
Chloroform	1.82
Ethyl Ether	0.003
Carbon Tetrachloride	0.002
Petroleum Ether	0.02
Benzene	0.002
Acetone	0.35
Benzyl Alcohol	7.29
Dimethylacetamide	13.86
Propylene Glycol	2.57

2.12 Crystal Properties

The X-ray powder diffraction pattern of trimethoprim is presented in Table VI (11).

Instrument Conditions

Instrument	GE Model XRD-6 Diffractometer
Generator	50 KV, 12.5 mA
Tube Target	Copper (CuKα = 1.5418 Å)
Optics	0.05° Detector Slit M.R. Soller Slit 3° Beam Slit Ni Filter 4° Take Off Angle Scan at 1°/inch
Detector	1.75 KV and 0.95 KV Pulse Height Selection 5 Volts Window Out

At cps FullScale 1K
Time Constant 2.5
Sample Treatment As is

Table VI

Trimethoprim

2θ (°)	d (Å)	I/I_1
11.89	7.44	0.44
15.22	5.82	0.94
*16.22	5.47	0.21
17.55	5.05	0.74
18.65	4.76	0.48
*22.28	3.99	1.00
22.91	3.88	0.36
23.11	3.85	0.39
*23.81	3.74	0.75
25.37	3.51	0.34
25.85	3.45	0.69

*Lines broadened

I/I_1 = Relative Intensity

2.13 Dissociation Constant

Due to its low solubility in water, trimethoprim solutions were prepared in ethanol-water mixtures at various concentrations and then titrated with aqueous sodium hydroxide. The apparent pK_a was plotted versus concentrations of ethanol resulting in a straight line whose intercept is the value of pK_a in water. The pK_a in water was found to be 6.6 (7). This value was confirmed by others (12) who postulated a relationship between dissociation constants and physiological activity, in this case the ability to inhibit the enzyme dihydrofolate reductase.

3. Synthesis

Trimethoprim may be prepared by the reaction scheme shown in Figure 8. A mixture of methanol, sodium, 3,4,5-trimethoxybenzaldehyde and β-methoxypropionitrile is allowed to reflux for several hours. The cooled product, 3,4,5-trimethoxy-2'-methoxymethylcinnamonitrile, is refluxed overnight with methanol in the presence of sodium. The

TRIMETHOPRIM SYNTHESIS

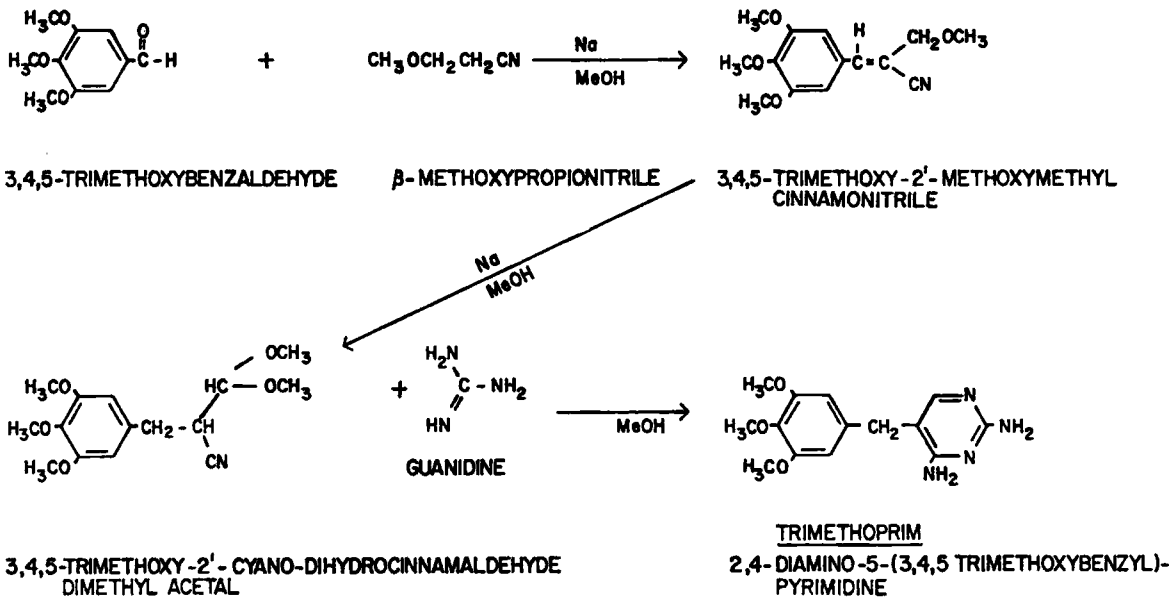


FIGURE 8

Synthesis of Trimethoprim

product is extracted with toluene and recrystallized to form the dimethylacetal. Trimethoprim is then produced by refluxing the dimethylacetal with guanidine in methanol solution. The dried crystals are slurried with methanol, filtered and dried again (13).

4. Stability, Degradation and Dissolution

Trimethoprim, 100 mg tablets, packaged in both amber glass and opaque high density polyethylene bottles with metal caps were stored at room temperature for 58 months. No changes in physical appearance were reported, no degradation products were detected by thin-layer chromatography and the purity was approximately at claim, spectrophotometrically (14). Dissolution studies performed in simulated gastric fluid on the stored tablets show that at least 60% is dissolved within 30 minutes and 100% is dissolved within 60 minutes. The *in vitro* dissolution procedure is spectrophotometric (15).

Disintegration times determined initially and after 58 months at room temperature show no significant change (14).

5. Drug Metabolic Products

The metabolic pathways of trimethoprim are illustrated in Figure 9. Five major metabolites have been isolated: Metabolite I = 2,4-diamino-5-(4-hydroxy-3,5-dimethoxybenzyl)-pyrimidine; Metabolite II = 2,4-diamino-5-(α-hydroxy-3,4,5-trimethoxybenzyl)-pyrimidine; Metabolites III (a and b), a = 2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine-1-oxide, and b = 2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine-3-oxide, and Metabolite IV = 2,4-diamino-5-(3-hydroxy-4,5-dimethoxybenzyl)-pyrimidine. Studies by Schwartz (16) postulated four metabolites, to which a fifth, the N-3 oxide was later added (17). Metabolite IV is excreted twice as much as Metabolite I. The N-1 and N-3-oxide forms of Metabolite III are produced equally (18). Metabolite II is a minor metabolite barely detectable in the urine of some individuals (19). Metabolites II and III are unconjugated in both plasma and urine, whereas Metabolites I and IV are conjugated and excreted in the urine as glucuronides (16).

As alluded to in the section on dissociation constants (12) trimethoprim's activity appears related to its pKa. It was postulated that protonated pyrimidines interact with the dihydrofolate coenzymes with the binding energy being pH dependent.

METABOLITES OF TRIMETHOPRIM

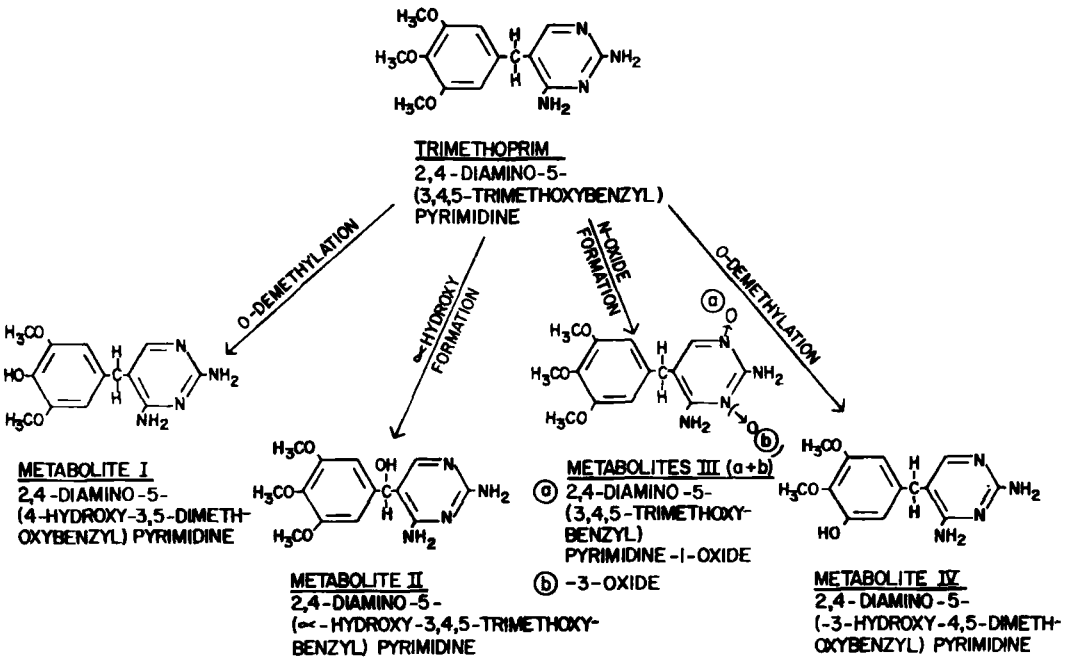


FIGURE 9
Metabolic Pathways of Trimethoprim

Trimethoprim is a sulfonamide potentiator which together act synergistically to deprive bacteria of folate coenzymes (20). They work in different parts of the bacterial protein synthesis chain to block purine and thymine production, and hence RNA and DNA formation (21).

6. Toxicity

Trimethoprim's mode of action, as indicated, is to have an inhibitive effect on the folate metabolism of microorganisms. For most individuals having no deficiency in folic acid, trimethoprim therapy is apparently quite safe, considering of course, length of treatment, amount of dose and potentiation by other drugs. Those who might be under folate stress such as the elderly, pregnant women, the chronically ill and the malnourished, must be treated more carefully (22).

7. Methods of Analysis

7.1 Elemental Analysis

A typical elemental analysis of a trimethoprim sample is presented in Table VII (23).

Table VII

Elemental Analysis of Trimethoprim

Element	% Theory	% Found
C	57.92	58.06
H	6.25	6.31
N	19.30	19.48

7.2 Thin-Layer Chromatographic Analysis

Trimethoprim, and its immediate precursor 3,4,5-trimethoxy-2'-cyano-dihydrocinnamaldehyde dimethyl-acetal have been separated by thin-layer chromatography using the following set of conditions (7):

Adsorbent	Silica Gel GF
Solvent System	Benzene:diethyl ether:methanol: conc. NH_4OH (50:50:10:1)
Sample	Dissolve 100 mg in 10 ml of methanol. Apply 0.05 ml (equivalent to 500 µg of trimethoprim)

Development	Ascending for at least 15 cm. Chromatography commences directly after the solvent has been poured into the tank.
Detection	(a) Shortwave ultraviolet light (b) Modified Dragendorff Reagent Solution A: Dissolve 0.8 gm of $Bi(NO_3)_2$ in 10 ml of glacial acetic acid and dilute to 40 ml with water. Solution B: Dissolve 4 gm of KI in 10 ml of water. Spray Reagent: Mix 5 ml each of the 2 solutions and dilute to 50 ml with 10% H_2SO_4. (c) Spray the plate with 10% H_2SO_4 and then with a 1:1 mixture of 10% w/v ferric chloride and 5% w/v potassium ferricyanide.

Table VIII

Thin-Layer Chromatography of Trimethoprim

Compound	Approximate R_f	Sensitivity of Detection (a) µg	(b) µg	(c) µg
Trimethoprim	0.25	0.1	0.05	0.05
Dimethylacetal	0.66	10	20	0.1

A thin-layer chromatographic method has been reported (24) which separates trimethoprim and four metabolites as fluorescence spots on non-fluorescing silica gel plates. Metabolite II by this method is inseparable from Metabolite III b, the N-3-oxide metabolite, whose presence has been quantitated by an updated method employing development in ethanol/benzene (3:7) prior to the chloroform/n-propanol/28% NH_4OH system below (19). Extractions are made from biological fluids and tissue and 2 aliquots are drawn; one for the determination of the unconjugated metabolites and trimethoprim and the other for the determination of the conjugated metabolites. The latter is treated with Glusulase and is developed using a solvent system of chloroform, n-propanol and 28% NH_4OH (100:10:1) Trimethoprim and Metabolites II and III are

developed on separate plates from subdilutions of the aliquot taken. The same combination of developing solvents is used but the volume ratios differ: 80:20:1 for trimethoprim: 25:20:1 for M III(a) and 100:10:1 followed by 80:20:1 for M II. Each plate as it air dries shows a light-blue fluorescent spot visible at 360 nm.

7.3 High Performance Liquid Chromatographic Analysis

A high performance liquid chromatographic method has been developed (25) for the quantitative determination of trimethoprim. The column was steel, 0.5 m x 3 mm I.D., and packed with Merckosorb SI 60, whose particle size was 5 μm. The mobile phase was 10% methanol, 89.9% chloroform (containing 0.6-1% ethanol) and 0.1% concentrated ammonia, at a flow rate of 0.6 ml/min., and a pressure of 1150 psi. A UV detector was used at a wavelength of 290 nm. As an internal standard, 2-amino-pyrimidine was found suitable. A 2 μl methanolic solution containing 1 to 2 μg of each is injected. Figure 10 shows the separation of the internal standard (eluting first) and trimethoprim at a retention time of about 10 minutes.

7.4 Gas-Liquid Chromatographic Analysis

A gas-liquid chromatographic method has been reported for quantitation of trimethoprim in tissues (26). A 5% OV-101 on Gas Chrom Q, pyrex column was used at 250°C in conjunction with a nitrogen thermionic detector. Acetone as a dissolution solvent was found to reduce trimethoprim adsorption onto the glass. N,N-di-n-butyl-4-n-hexyloxy-1-naphthamidine was used as an internal standard.

A gas-liquid chromatographic separation of trimethoprim from its immediate precursors, the cinnamonitrile and the dimethylacetal can be achieved on a 4 ft x 2 mm I.D. glass column packed with 5% QF-1 on 60/80 mesh Supelcoport. The column was programmed from 150°C to 250°C at 8°C/min. The carrier gas was nitrogen at a flow rate of 30 ml/min (27). Figure 11 shows the separation.

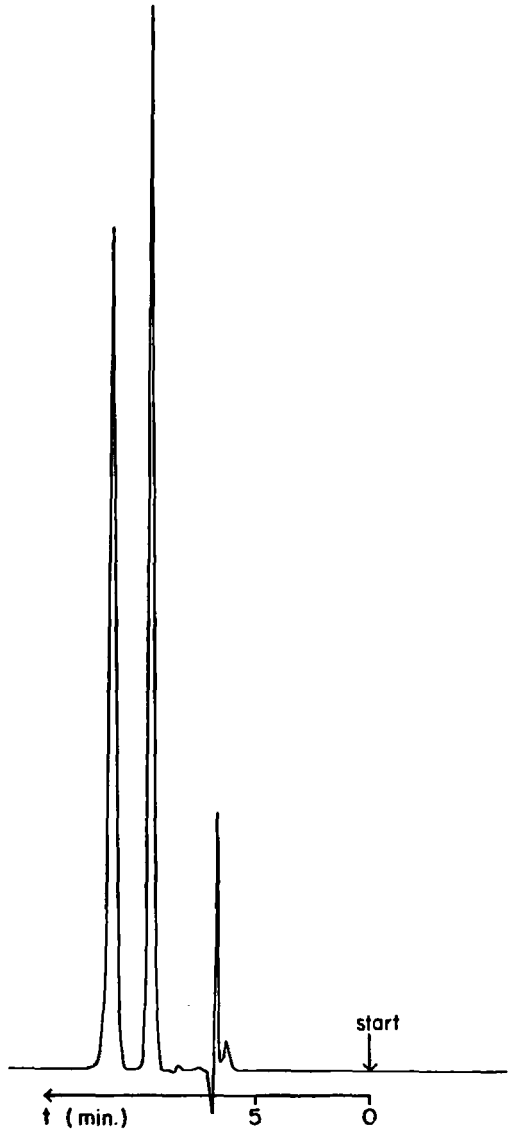


FIGURE 10
HPLC Analysis of Trimethoprim

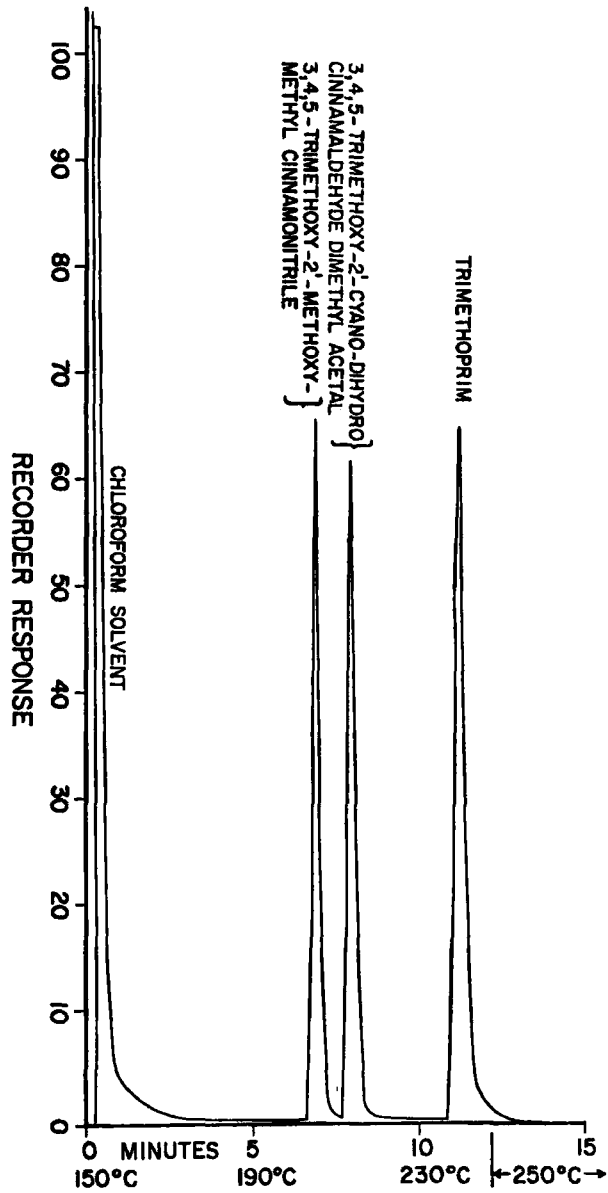


FIGURE 11
GLC Separation of Trimethoprim and Precursors

7.5 Spectrophotofluorometric Analysis

A semiautomated spectrophotofluorometric method of analysis of trimethoprim in biological fluids and tissue has been reported (28). The method requires oxidation with alkaline potassium permanganate to the fluorescent trimethoxybenzoic acid. The fluorescence is measured at an activation wavelength of 275 nm, and a fluorescence wavelength of 345 nm. A Technicon Sample II is used with a Perkin-Elmer model 204 Fluorescence Spectrophotometer with 150-W Xenon light source.

7.6 Direct Spectrophotometric Analysis

A direct spectrophotometric analysis of trimethoprim can be done by ion-pair formation with bromcresol green and measurement for the primary amine at 415 nm. Trimethoprim has been assayed in trimethoprim tablets and in combination with sulfamethoxazole in tablets (29).

7.7 Polarographic Analysis

Trimethoprim has been analyzed on the PAR Model 174 Polarographic Analyzer using the following conditions. Sample concentration was 0.090 µg/10 ml 0.1N H_2SO_4. The temperature was 25°C. The voltage range was -0.8 to -1.5 vs. Ag/AgCl reference with the E_p = -1.02V. The current range was 5 µa full-scale; the scan rate 5 mv/sec over a chart range of 0-30 cm. The modulation amplitude was 50 mv. The Hg column height was 60 cm, and the drop time was 1/2 second. The operation mode was differential pulse polarography using a low pass filter of 0.3. Figure 12 shows the results of the analysis (30). A DPP method has also been developed for the analysis of the two N-oxide metabolites (18).

7.8 Titrimetric Analysis

A titrimetric analysis of trimethoprim can be performed potentiometrically by titrating 0.8 - 0.9 grams of trimethoprim in 100 ml of 90:10 acetic anhydride:glacial acetic acid with 0.1N perchloric acid in glacial acetic acid (7). One ml of 0.1N perchloric acid is equivalent to 29.03 mg of trimethoprim.

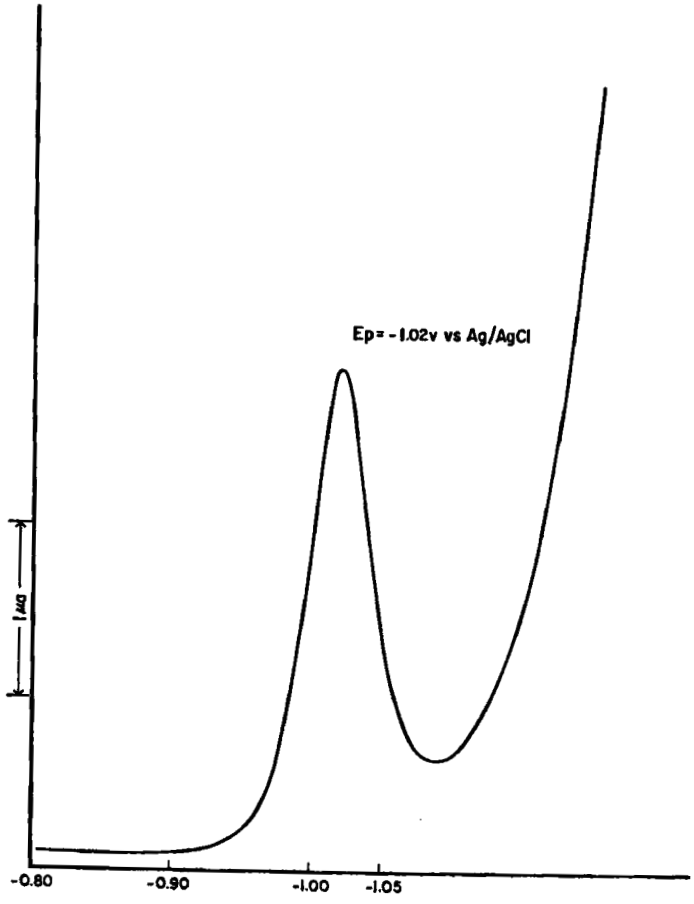


FIGURE 12
Polarographic Analysis of
Trimethoprim

$$\frac{\text{ml } HClO_4 \times \text{N } HClO_4 \times 0.2903 \times 100}{\text{Sample Wt. (g)}} = \% \text{ Trimethoprim}$$

Figure 13 shows a typical titration curve (31) A Mettler DK 10 was used with a combination glass-calomel electrode at a range of 500 mv.

7.9 Microbiological Analysis

A microbiological analytical method for trimethoprim has been reported (32). An agar medium was prepared containing Evans peptone 0.5%, Lab-Lemco beef extract 0.3%, New Zealand agar 1.5% in distilled water, pH 7.5. The test organism was Bacillus pumilus. The test biological fluids were spiked with 0.5 to 0.005 µg/ml of trimethoprim to produce the standards. The plates were incubated at 28°C and were measured for size of inhibition as soon as growth was observed. A graph was constructed on semi-log paper of the concentrations of the standards versus zone sizes and the sample levels were then picked off from there.

7.10 Phase Solubility Analysis

Phase solubility analysis may be carried out using a 1:1 mixture by volume of water and methanol as the solvent. A typical example for trimethoprim is shown in Figure 14 which also lists the conditions under which the analysis was performed (33).

8. Acknowledgements

The author wishes to thank the members of the Research Records Department and Scientific Literature Department of Hoffmann-La Roche for their efforts. Sincere thanks also to Dr. S. Moros and Mr. R. Tscherne for their helpful advice. Finally, warm appreciation to Dr. P. Manchand for his contributions of some of the compounds necessary for this monograph.

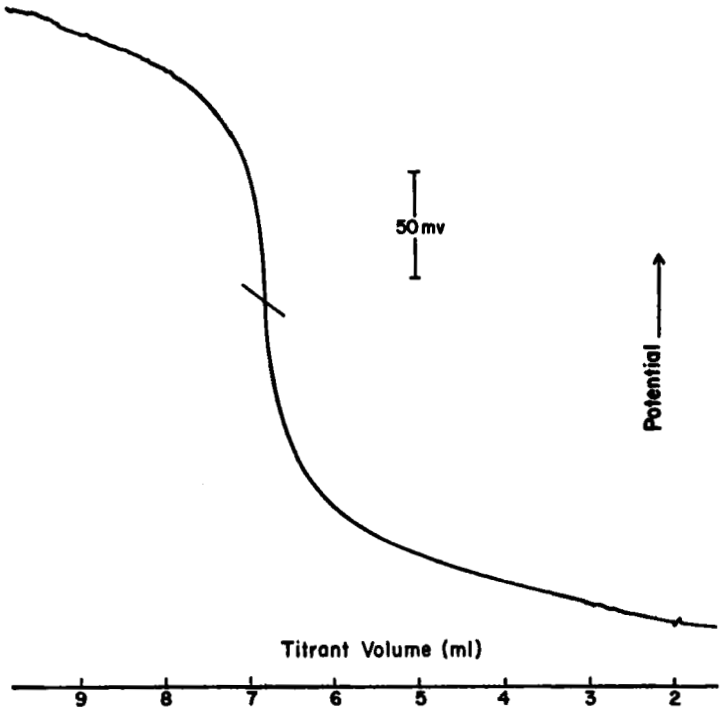


FIGURE 13
Titrimetric Curve for Trimethoprim

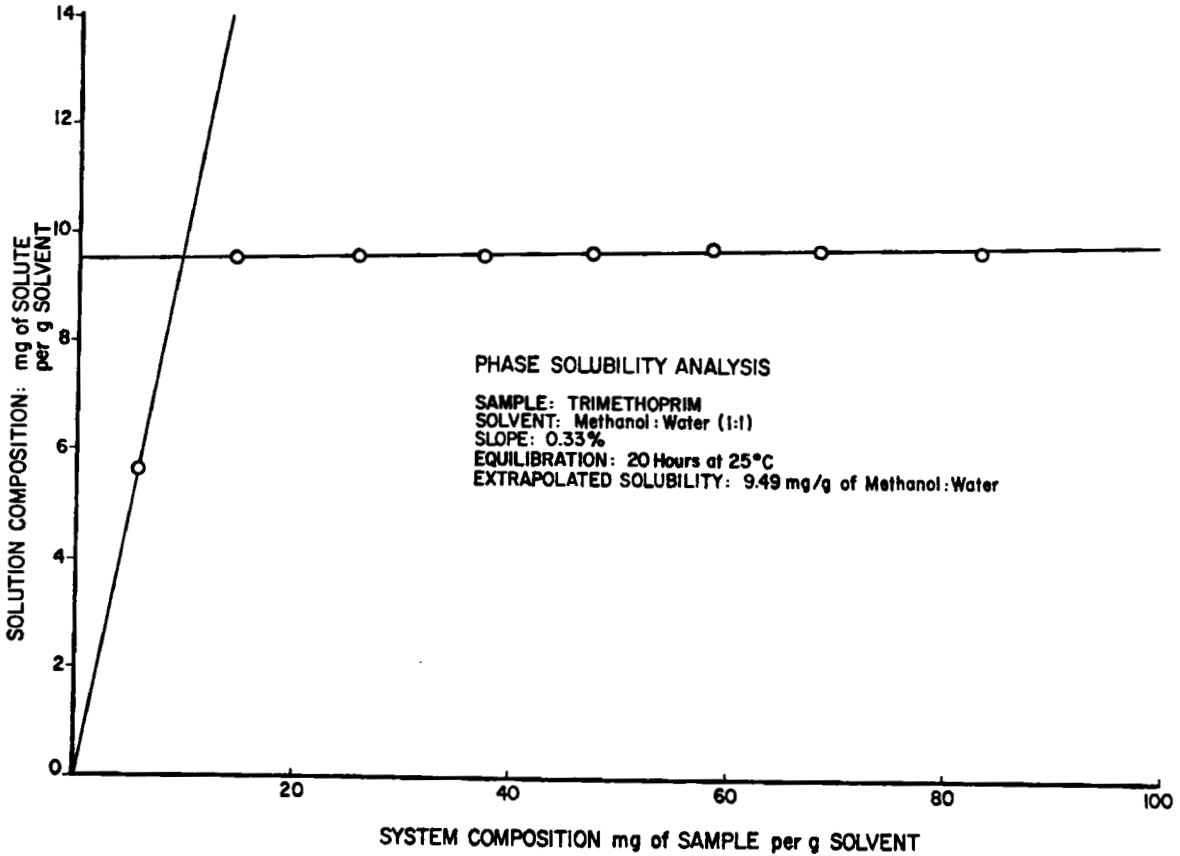


FIGURE 14
Phase Solubility Analysis of Trimethoprim

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Analytical Profiles of Drug Substances, 7

TUBOCURARINE CHLORIDE

Constantin Papastephanou

ISBN 0-12-260807-0

TUBOCURARINE CHLORIDE

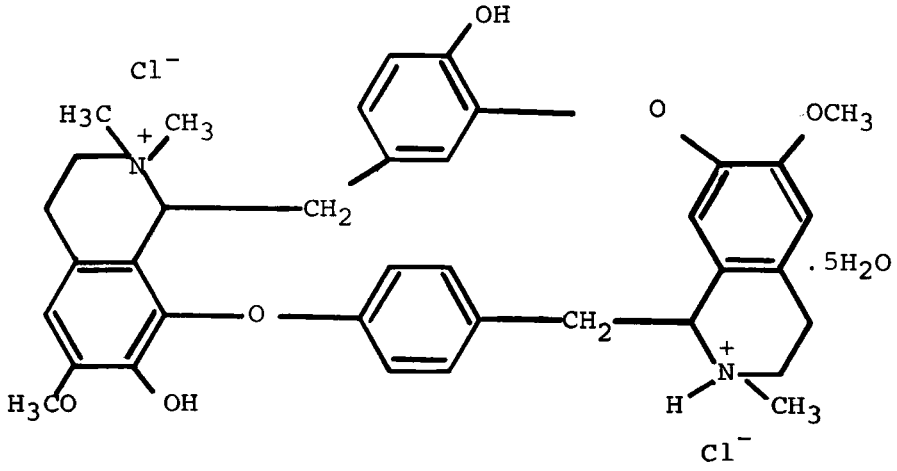
Contents

TUBOCURARINE CHLORIDE

1. Description

1.1 Name, formula, molecular weight

Tubocurarium, 7',12'-dihydroxy-6,6'-dimethoxy-2,2',2'-trimethyl-, chloride, hydrochloride, pentahydrate. (+)-Tubocurarine chloride hydrochloride pentahydrate.



$C_{37}H_{42}Cl_2N_2O_6 \cdot 5\ H_2O$ Molecular weight: 771.73

1.2 Appearance, odor, color

White or yellowish white to grayish white, crystalline odorless powder.

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of U.S.P. reference standard tubocurarine chloride is shown in Figure 1. The spectrum obtained as a KBr pellet using a Perkin Elmer 621 spectrometer, indicates the following frequencies (1).

Frequency cm^{-1}	Assignment
3410, 3330	O-H, N-H stretch
2930	C-H stretch
2500 (broad)	H-Cl
1610, 1590, 1505, 1220	Aromatic C = C stretch = C - O ether and hydroxyl

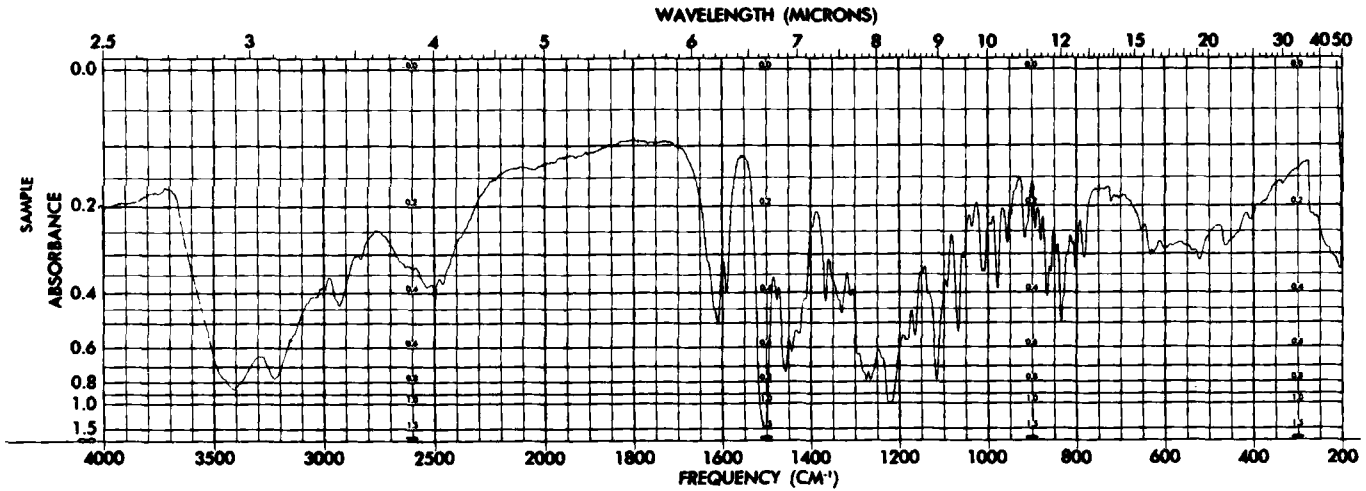
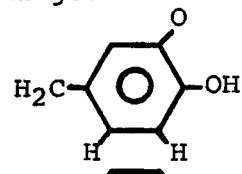


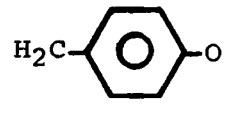
Figure 1. Infrared spectrum of tubocurarine chloride.

2.2 Nuclear Magnetic Resonance Spectrum

The 100 MHz proton magnetic resonance spectrum of d-tubocurarine (Figure 2) studied in DMSO-d_6 containing TMS as internal reference, and utilizing a Varian XL-100-15 NMR spectrometer, indicates the presence of the following resonances (2):

Chemical Shifts (δ ppm)	Assignments
9.63	s, 1H, OH, D_2O Exchanged
8.01	s, 1H, OH, D_2O Exchanged
7.18	d, J=8.0 Hz, 1H
6.18	d, J=8.0 Hz, 1H
7.00	d, J=8.5 Hz, 2H
6.60	d, J=8.5 Hz, 2H
6.86, 6.92, 7.08	other aromatic singlets
5.20	m, J=7.5 Hz, 1H
4.86	s, 1H,
	two  groups
3.96	s, 6H, OCH_3
3.36	s, 10H, 5 H_2O
3.12	s, 3H, $\overset{\oplus}{N}-CH_3$
2.94	s, 3H, $\overset{\oplus}{N}-CH_3$
2.75	broad, s, $\overset{\oplus}{N}H-CH_3$





s = singlet, d = doublet

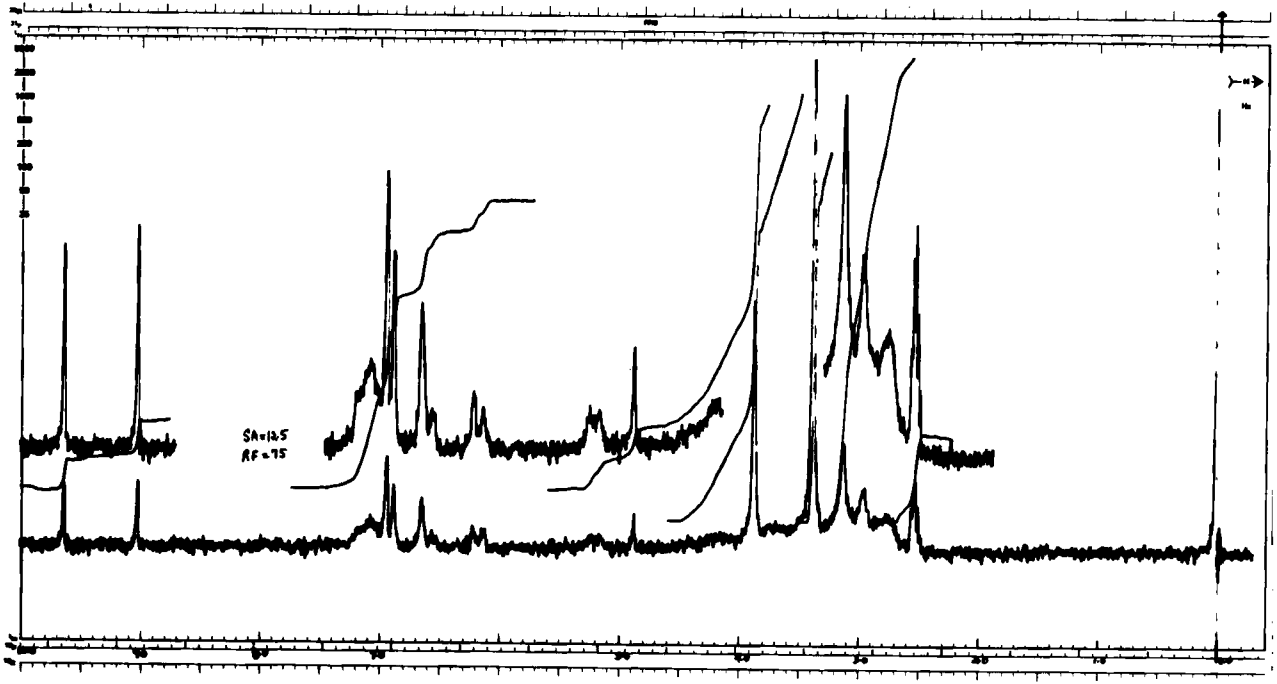


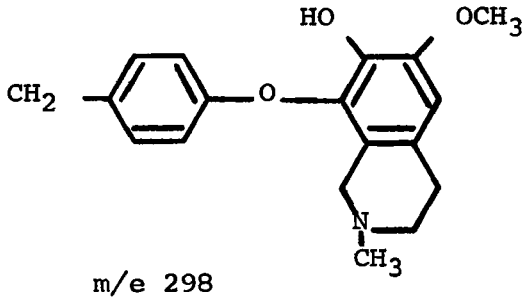
Figure 2. NMR spectrum of tubocurarine chloride.

Some of the salient features of the NMR spectrum are changes, on D_2O exchange, in the resonances due to water (5 moles) and N-methyl protons; the former moved downfield while the latter moved slightly upfield. The resonance at δ 2.75 also became significantly sharper.

2.3 Mass Spectrum

The mass spectrum of tubocurarine chloride (Figure 3) yields a molecular ion of m/e 594, which is consistent for the free base due to elimination, by electron-impact and/or thermolysis of the elements of methyl chloride and HCl. The molecular ion at m/e 50 suggests that CH_3Cl is eliminated thermally. In addition a peak at m/e 608 could be due to elimination of two molecules of HCl either thermally and/or by electron-impact.

Fragmentation at the bonds β-to the nitrogen with the formation of a tropylium ion structure leads to the intense ion at m/e 298, depicted below and arising from the molecular ion at m/e 594.



m/e 298

The other half of the molecule would thus be represented by m/e 296.

The reaction of tubocurarine chloride with trimethyl silyl imidazole reagent yields molecular ions at m/e 738 and m/e 752 analogous to the underivatized compound. The m/e 370 fragment ion corresponds to m/e 298 plus substitution of one silyl group confirming the presence of only one hydroxyl group in this portion of the molecule.

Likewise m/e 368 corresponds to one silyl group substituted on the m/e 296 fragment.

A large m/e 58 ion in both the spectra of the compound and its silyl derivative arises from the amino portion of the molecule and probably has the elements C_3H_8N.

A weak peak at m/e 622 in the spectrum of the underivatized compound as well as higher mass ions at m/e 796, 810, 812 and 826 in the spectrum of the silyl derivative suggests that a trace impurity is present or thermal reactions occur in the mass spectrometer. The origin of these ions has not been established (2).

2.4 Ultraviolet Spectrum

Figure 4 shows the UV spectrum of a solution of tubocurarine chloride in water. One peak at 280 nm with an $E^{1\%}_{1cm}$ of 107 is seen.

The same peak and $E^{1\%}_{1cm}$ are obtained for a solution of tubocurarine chloride in 0.01N HCl. The spectrum of the drug in 0.01N NaOH however, changes to one with a peak at 291 nm and an $E^{1\%}_{1cm}$ of 156.

The spectra in these three solvents remained unchanged after 2-1/2 hours (3).

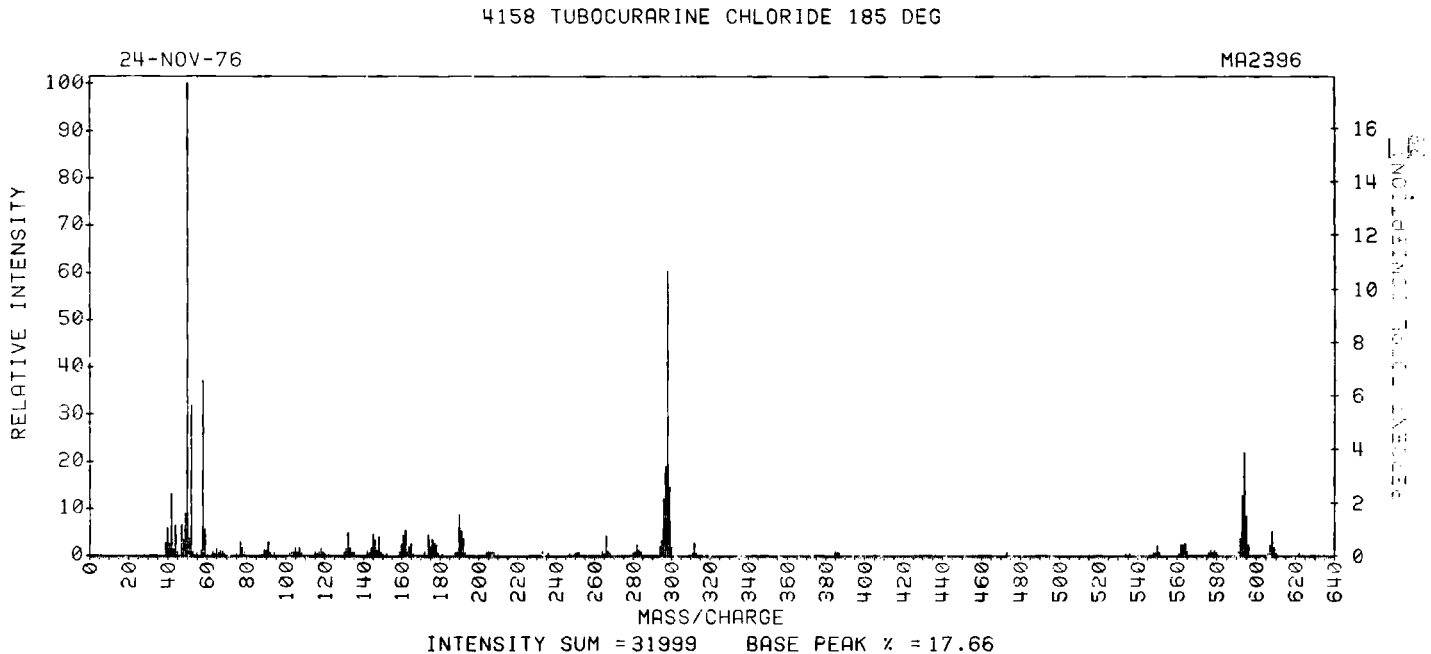


Figure 3. Mass spectrum of tubocurarine chloride.

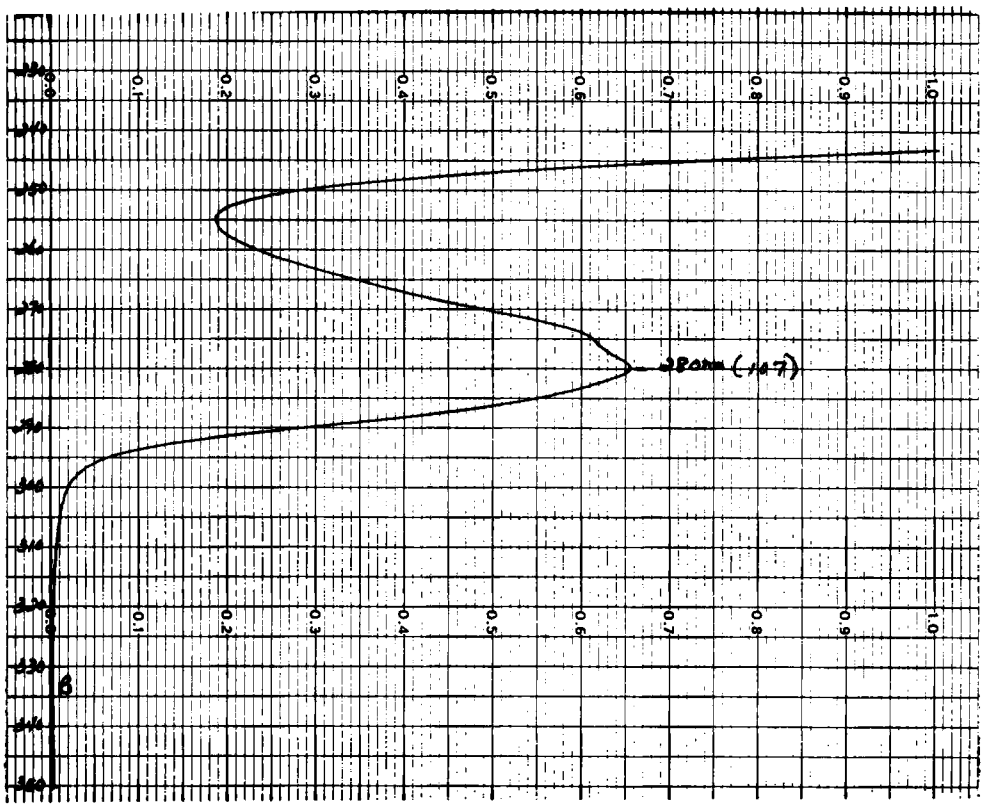


Figure 4. UV spectrum of tubocurarine chloride in water.

2.5 Optical Rotation

The U.S.P. XIX gives a specific rotation value of + 210° to + 224° for tubocurarine chloride, calculated on an anhydrous basis, and determined in a solution containing 100 mg in 10 ml of water (4).

2.6 Luminescence Properties

The uncorrected excitation and emission spectra for tubocurarine chloride (10 mg/ml water) are shown in Figure 5. The maximum excitation wavelength is 375 nm and the maximum emission wavelength is 425 nm (5).

2.7 Solubility

Tubocurarine chloride is soluble in water; sparingly soluble in ethanol; and almost insoluble in ether and chloroform (6).

2.8 Melting Range

The melting point reported in the U.S.P. XIX for tubocurarine chloride is about 270° with decomposition (4).

2.9 Differential Thermal Analysis

The principal features of the differential thermal analysis are a sharp endotherm at 125° and a decomposition endotherm at 273° (7). The endotherm at 125° represents the loss of water of hydration.

2.10 Powder X-ray Diffraction

The powder x-ray diffraction pattern of tubocurarine chloride is shown in Table I and Figure 6 (8).

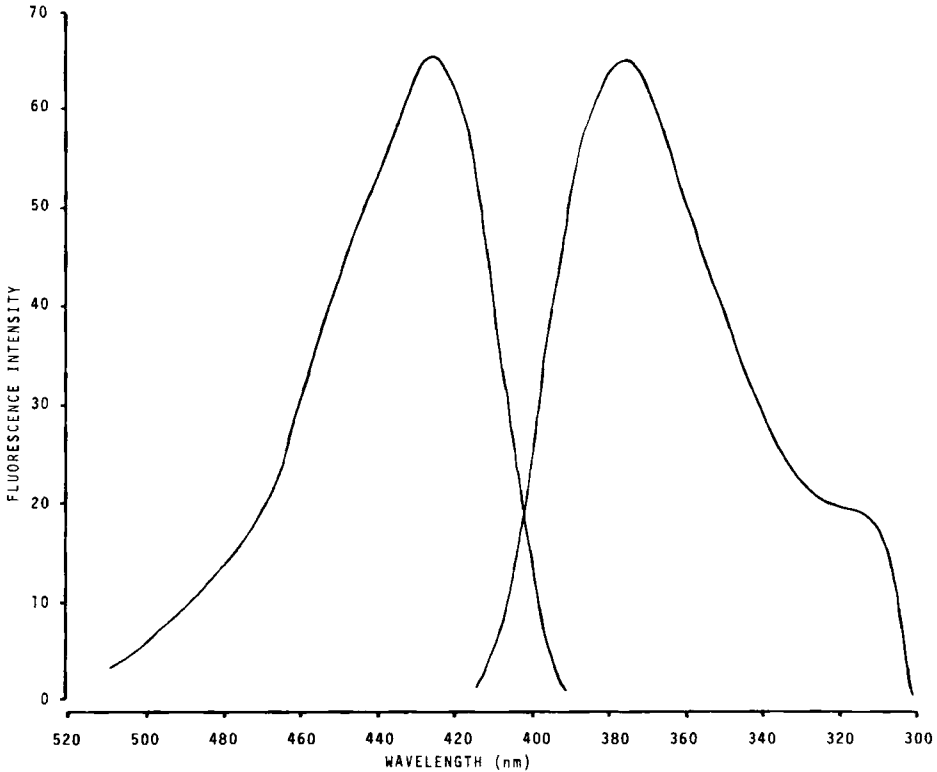


Figure 5. Fluorescence spectrum (uncorrected) of tubocurarine chloride in water.

Table I
Powder X-ray Diffraction Pattern of
d-tubocurarine

2 θ	d(Å)	Relative Intensity
4.58	19.29	1.000
11.80	7.50	0.224
12.31	7.19	0.125
13.25	6.68	0.197
14.52	6.10	0.194
16.90	5.25	0.368
17.33	5.12	0.101
17.84	4.97	0.267
19.37	4.58	0.205
19.96	4.45	0.506
20.73	4.28	0.215
21.07	4.22	0.088
21.58	4.12	0.325
22.51	3.95	0.353
23.28	3.82	0.395
24.81	3.59	0.312
25.74	3.46	0.139
26.00	3.43	0.179
27.02	3.30	0.498
27.70	3.22	0.141
28.80	3.10	0.202
30.84	2.90	0.278
33.73	2.66	0.155

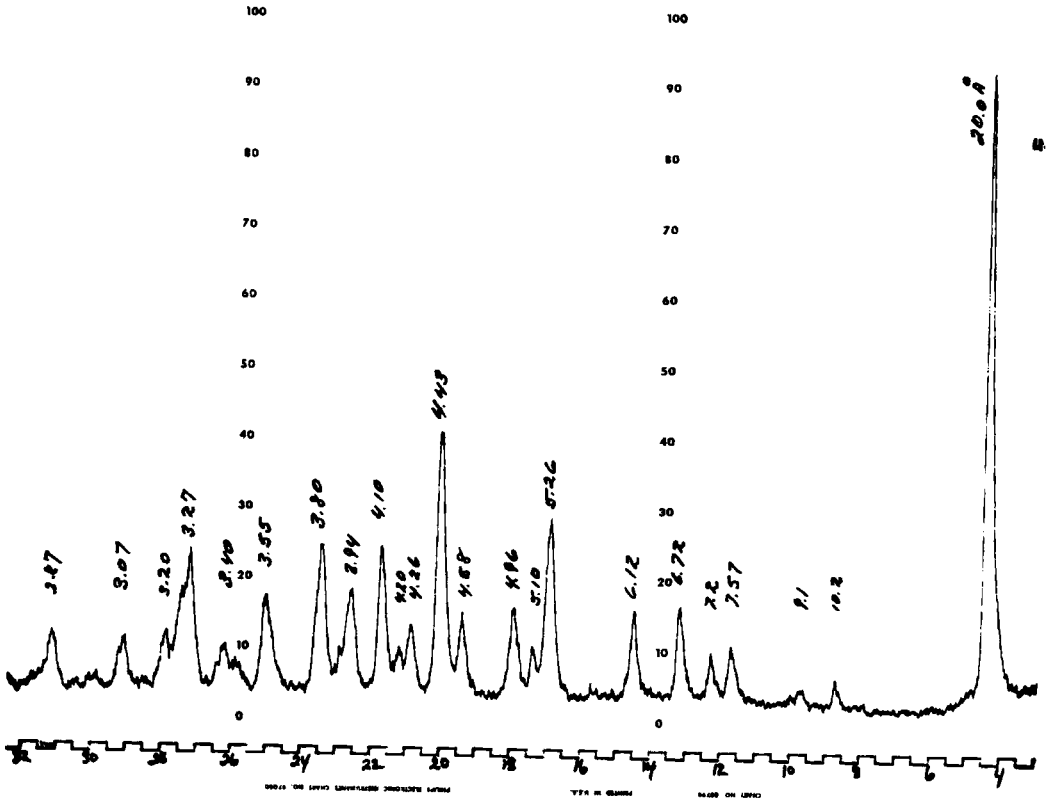


Figure 6. Powder X-ray diffraction pattern of tubocurarine chloride.

2.11 Dissociation Constant

The pK was found to be 7.4 (6).

3. Isolation and Purification

The South American arrow poisons known as curare were first described in writing at the beginning of the sixteenth century. The first attempt to isolate the active components of curare was made in the early nineteenth century by Boussingault and Roulin (9).

It was recognized early that the poisons were of several varieties and that, in general, the containers used by native groups for the preparation of the poisons were fairly diagnostic of the curare in them. Curares were therefore classified into three categories: calabash- or gourd-curare, pot-curare, and tube-curare (tubocurarine).

d-Tubocurarine chloride was first isolated from a specimen of tube curare in 1935 by King(10) and later isolated from Chondodendron tomentosum (also spelled Chondrodendron) Ruiz and Pavon by Dutcher in 1946 (11). It was later obtained in pure crystalline form also by Dutcher in 1952 (12) by recrystallization from 0.1N HCl.

4. Stability and Degradation

Pure tubocurarine chloride appears to be very stable when stored in a tight container at 5°C. A Squibb House Standard of d-tubocurarine chloride was reassayed after ten years of storage and the potency of this standard was found to be identical to that of the original standard (13).

One report in the literature claims that pharmaceutical preparations of tubocurarine chloride may degrade after several years of storage at room temperature (14).

5. Drug Metabolism

In man, about 50% of the injected dose of tubocurarine is excreted unchanged in the urine in the first 10 hours (15, 16). There is a species variation in the urinary excretion of the drug(17). The elimination of tubocurarine in feces or by

other routes is negligible.

Cohen et al (18) have shown in a study of the metabolism of tubocurarine-H^3 in dogs that this drug does not undergo significant degrees of biotransformation. The kidneys normally provide the major avenue of elimination, with the liver contributing an alternate route. In the absence of normal renal function, the ability of the liver to eliminate tubocurarine via the bile is greatly increased.

Investigation of the serum protein pattern of 52 patients undergoing upper abdominal surgery showed a highly significant correlation between the level of gamma globulin in serum and the dose of tubocurarine required to maintain adequate muscular relaxation. Baraka and Gabali (19) attributed this to the ability of gamma globulin to bind tubocurarine.

Olsen et al (20) studied the binding of tubocurarine di [methyl-^{14}C] ether iodide (TCE) and other radio labelled amines to bovine nasal septum, chondroitin sulfate and human plasma protein. They found that 30-40% TCE was bound to plasma protein and that each gram of cartilage could bind about 10^{-7} mol. TCE. They concluded that cartilage may represent a measurable distribution pool for tubocurarine.

6. Methods of Analysis

6.1 Identity Tests

To 20 ml of a solution of tubocurarine hydrochloride (1 in 2,000 in water) are added 0.02 ml sulfuric acid and 2 ml potassium iodate solution (1% in water). After mixing and warming on a steam bath for 30 minutes, a yellow color is produced (4,21).

When 5 ml of mercuric nitrate solution (3 ml Hg in 27 ml HNO_3) are added to 0.5 ml of a solution of tubocurarine hydrochloride (1% w/v), a cherry red color slowly appears (21,22,23).

When a few drops of ferric chloride solution are added to 1 ml of a solution of tubocurarine hydrochloride (1% w/v) a green color is

produced which then changes to brown after warming the mixture in a water bath (23).

6.2 Elemental Analysis

The results from the elemental analysis of tubocurarine are listed in Table II(2).

Table II
Elemental Analysis of d-Tubocurarine Chloride

Element	% Found	% Calculated
C	58.08	57.58
H	6.65	6.79
N	3.62	3.63
Cl	8.99	9.19

6.3 Ultraviolet Absorption

According to the British Pharmacopoeia (22) pure tubocurarine can be measured by its absorption at about 280 nm. The concentrations of d-tubocurarine in a solution may be calculated by taking 105 as the value of $E^{1\%}_{1cm}$ at the maximum of about 280 nm.

The European Pharmacopoeia lists the same assay but gives the $E^{1\%}_{1cm}$ of tubocurarine at 280 nm as 118.

Cohen et al have reported a method based on UV absorption for the determination of tubocurarine in plasma (24,25). Extraction of the curare from the plasma is based on its solubility in ethylene dichloride at an alkaline pH, and then the salt is reformed in 0.01N HCl. Satisfactory measurements can be made to concentrations of less than 1 µg/ml of plasma.

6.4 Colorimetric Analysis

Momot (26) describes a colorimetric quantitative determination of d-tubocurarine in human blood. Plasma, 3 ml, is shaken for 5 minutes with 10 ml ethylene dichloride and 0.5 ml glycine buffer. The mixture is centrifuged, the aqueous phase is discarded and the solvent phase is trans-

ferred to another test tube containing 3 drops of citrate buffer and some methyl orange crystals. The solution is mixed, centrifuged, and after removal of the excess dye, 0.5 ml ethanol is added and the sample is read in a colorimeter.

6.5 Fluorescence Assay

Tubocurarine can be extracted from body fluids and tissues with ethylene dichloride at alkaline pH in the presence of excess potassium iodide and then back-extracted into hydrochloric acid. The drug is then analyzed by complexing with rose bengal in potassium hydrogen phosphate and the resulting tubocurarine-rose bengal complex is extracted in a chloroform-phenol mixture(25% phenol in chloroform). The chloroform layer is then carefully removed, mixed thoroughly with acetone and measured fluorometrically (27,28).

6.6 Thin-Layer Chromatography

A rapid TLC method for the determination of tubocurarine is described by Crone and Smith(29) using microscope slides coated with Silica Gel H and a drug sample of 5μg in 1 μl 1N HCl. The chromatograms are developed using 1N HCl for about 5 minutes (Solvent I), or 1N HCl: ethanol (1:1) for 15-20 minutes (Solvent II). The slides are then dried with a hair-dryer and sprayed with Dragendorff's reagent(30). Tubocurarine has an Rf of 0.12 and 0.54 in solvents I and II respectively.

d-Tubocurarine chloride can be separated from d-chondrocurarine chloride (a naturally occuring compound,similar in structure to tubocurarine) using Silica Gel GF plates in a solvent system consisting of a 80:10:10 mixture of methyl ethyl ketone, water and formic acid. The plate is sprayed with iodoplatinate spray reagent (30). Using this system d-tubocurarine chloride has an Rf of 0.30 and d-chondrocurarine chloride has an Rf of 0.17(32).

Tubocurarine and other skeletal muscle relaxants have been extracted from biological material and analyzed qualitatively and quantita-

tively by TLC (33). Extracts have been analyzed using three systems: (I), alumina Woelm, acid (chloroform:methanol, 80:20); (II), alumina Woelm, basic (methanol:chloroform, 75:25) and (III), alumina G Merk (methanol:acetic acid:water,92:3:3).

Qualitative analysis of the test drugs on developed chromatograms was affected by examination under UV light and by spraying with iodoplatinate solution (31). Quantitation of drugs was also accomplished by transferring the area containing the spot to a centrifuge tube containing phosphate buffer pH 6.5. After shaking and centrifuging, an aliquot was removed and mixed with methylene dichloride and sodium 9,10 dimethoxy-anthracene-2-sulfonate solution. This mixture was again shaken, centrifuged and an aliquot of the extract removed. To this was added tetrabutyl ammonium hydroxide and the resulting fluorescence determined (ex. 368 nm, em. 448 nm). Tubocurarine gave Rf values of 0.83, 0.65 and 0.72 in systems I, II, and III, respectively.

Fiori and Marigo (34) have described a method for the detection of d-tubocurarine, gallamine, decamethonium, and succinylcholine in biological material. The method is based on the purification of extracts from body fluids and organs by ion exchange separation on Amberlite IRC 50 resin. The curares are eluted and precipitated as reineckates, which are then regenerated to allow their identification by means of paper and thin-layer chromatography. Thin-layer chromatography with acid alumina layers and chloroform:methanol (80:20) as the solvent system gives a good resolution (Rf = 0.22 for tubocurarine). The spots are revealed by the iodoplatinate reagent (31). The eluates of untreated spots are examined spectrophotometrically in the UV to detect tubocurarine and finally are injected into the tail vein of mice for a biological test.

6.7 High-Pressure Liquid Chromatography

Twitchett and Moffat (35) have shown that tubocurarine can be effectively separated from other

drugs and quantitatively measured using octadecylsilane as the stationary phase with aqueous methanol eluents.

6.8 Gas Chromatography

A paper by Vidic (36) described a gas chromatographic method for the determination of choline and its esters, neostigmine, tubocurarine methylcurarine and scopolamine Bu bromide.

6.9 Electrophoresis

Marini-Bettolo and Coch Frugoni (37) have studied the electrophoretic separation of some 70 alkaloids on paper at pH 2-12.

Mazzei and Lederer (38) have also studied the paper electrophoretic behavior of tubocurarine and other quaternary ammonium compounds.

Wan (39) has described a procedure for the thin-layer electrophoresis of alkaloids on glass plates coated with cellulose powder. Electrophoresis was carried out in acid and alkaline electrolytes at 500 V and 3,000 V.

6.10 Bioassay

The only assay procedure described in the U.S.P. XIX (4) is the rabbit "head drop" method (40). In the assay the reference standard and test sample of tubocurare are each injected intravenously into a number of rabbits in a cross-over pattern. The endpoint of the test, "head-drop", is relaxation of the neck muscles to such a degree that the animal's head cannot be raised or turned in response to a physical stimulus.

6.11 Radioimmunoassay

Horowitz and Spector (41) have developed a radioimmunoassay that depends upon a competition between unlabelled d-tubocurarine and a standard of labelled ^{3}H-d-tubocurarine for the specific antibodies present in rabbit antisera. The assay is extremely sensitive measuring as little as 5 ng/ml and requires only 10 µl of serum or

urine sample. The advantage of this type of assay is the fact that no extraction from biological materials is required.

6.12 Assays in Body Fluids and Tissues

The following methods have been used for the assay of tubocurarine in body fluids and tissues:

Method	Reference
Ultraviolet absorption	24,25
Colorimetric	26
Fluorescence	27,28
Thin-layer chromatography	33,34
High-pressure liquid chromatography	35
Radioimmunoassay	41

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ADDENDA AND ERRATA

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Affiliations of Editors and Contributors

Volume 6, p. vii

Correct affiliation:
S. A. Benezra, Burroughs Wellcome Co.,
Research Triangle Park, North Carolina

Amphotericin B

Volume 6, p. 26

4.3 Mass Spectrometry
(TMS = trimethyl-silane)

Cyclizine

Volume 6, p. 83

The name of the co-author was inadvertently omitted.

Correct title: Cyclizine
Steven A. Benezra and
Chen-Hwa Yang

Triamcinolone Acetonide

Volume 1, p. 416

7. Determination in Body Fluids and Tissues.

A radioimmunoassay to determine plasma levels after topical application has been developed by M. Ponec, M. Frolich, A. DeLijster, A. J. Moolenaar, Arch. Dermatol. Res., 259, 63 (1977).

CUMULATIVE INDEX

Italic numerals refer to volume numbers

* Monographs in "Pharmacological and Biochemical Properties of Drug Substances, Volume 1" M. E. Goldberg, D. Sc., Editor American Pharmaceutical Association, 1977.
Cefazolin, 155
Fenoprofen, 183