Analytical Profiles of Drug Substances

Volume 5

Edited by

Klaus Florey

The Squibb Institute for Medical Research
New Brunswick, New Jersey

Contributing Editors

Norman W. Atwater
Glenn A. Brewer, Jr.
Lester Chafetz
Boen T. Kho
Gerald J. Papariello
Bernard Z. Senkowski

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AFFILIATIONS OF EDITORS AND CONTRIBUTORS

H. Y. Aboul-Enein, USV Pharmaceuticals, Tuckahoe, New York, now: Riyadh University, Riyadh, Saudi Arabia

G. D. Anthony, Searle and Company, Chicago, Illinois

N. W. Atwater, Searle and Company, Chicago, Illinois

J. I. Bodin, Carter-Wallace Inc., Cranbury, New Jersey

G. A. Brewer, The Squibb Institute for Medical Research, New Brunswick, New Jersey

D. E. Cadwallader, School of Pharmacy, University of Georgia, Athens, Georgia

L. Chafetz, Warner-Lambert Research Institute, Morris Plains, New Jersey

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J. H. Johnson, Hoffmann-LaRoche, Inc., Nutley, New Jersey

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P. Lim, Stanford Research Institute, Menlo Park, California

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S. M. Miller, Wyeth Laboratories, Philadelphia, Pennsylvania

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C. E. Orzech, Ayerst Laboratories, Rouses Point, New York

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F. Russo-Alesi, The Squibb Institute for Medical Research, New Brunswick, New Jersey

B. Z. Senkowski, Hoffmann-LaRoche, Inc., Nutley, New Jersey

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E. H. Waysek, Hoffmann-LaRoche, Inc., Nutley, New Jersey

L. L. Wearley, Searle and Company, Chicago, Illinois

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 degredation 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 fifth.

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 physico-chemical 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 had made this venture possible. All those who have found the profiles useful are earnestly requested to contribute a monograph of their own. The editors stand ready to receive such contributions.

Klaus Florey

BENDROFLUMETHIAZIDE

Klaus Florey and Frank M. Russo-Alesi

CONTENTS

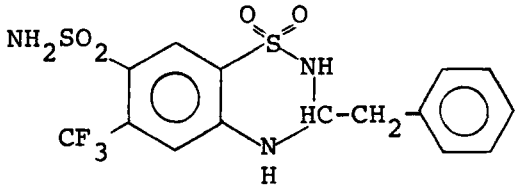
1. Description

1.1 History

Bendroflumethiazide belongs to the class of thiazide diuretics. Its synthesis was first reported by Holdrege, Babel and Cheney[1] in 1959 and its diuretic activity was first described in 1960 almost simultaneously by Lund and Kobinger[16] and by Kennedy, Buchanan and Cunningham[2].

1.2 Name, Formula, Molecular Weight

Bendroflumethiazide (also bendrofluazide, benydroflumethiazide, and benzylhydroflumethiazide) is 2H-1,2,4-Benzothiadiazine-7-sulfonamide, 3,4-dihydro-3-(phenyl methyl)-6-trifluoromethyl-1,1-dioxide or 3-Benzyl-3,4-dihydro-6-(trifluoromethyl)-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1 dioxide [73-48-3].



$C_{15}H_{14}F_3N_3O_4S_2$ Mol. Wt. 421.41

1.3 Appearance, Color, Odor

White or slightly off-white, uniform free flowing crystalline powder; slight floral odor.

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of bendroflumethiazide is given in Figure 1[1].

2.2 Nuclear Magnetic Resonance

The 60 MHz proton magnetic resonance spectrum in d_4-methanol containing tetramethylsilane as an internal reference (Figure 2) is assigned as follows[23]:

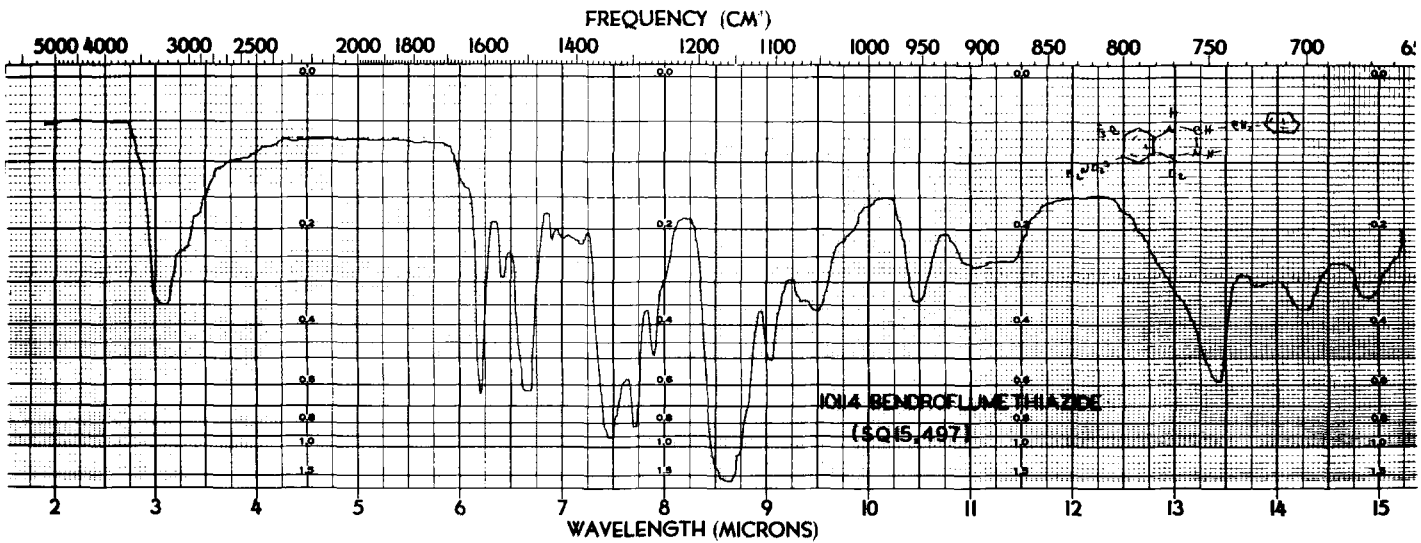


Figure 1. Infrared Spectrum of Bendroflumethiazide (batch #15); KBr, MeOH. Instrument: Perkin-Elmer 621.

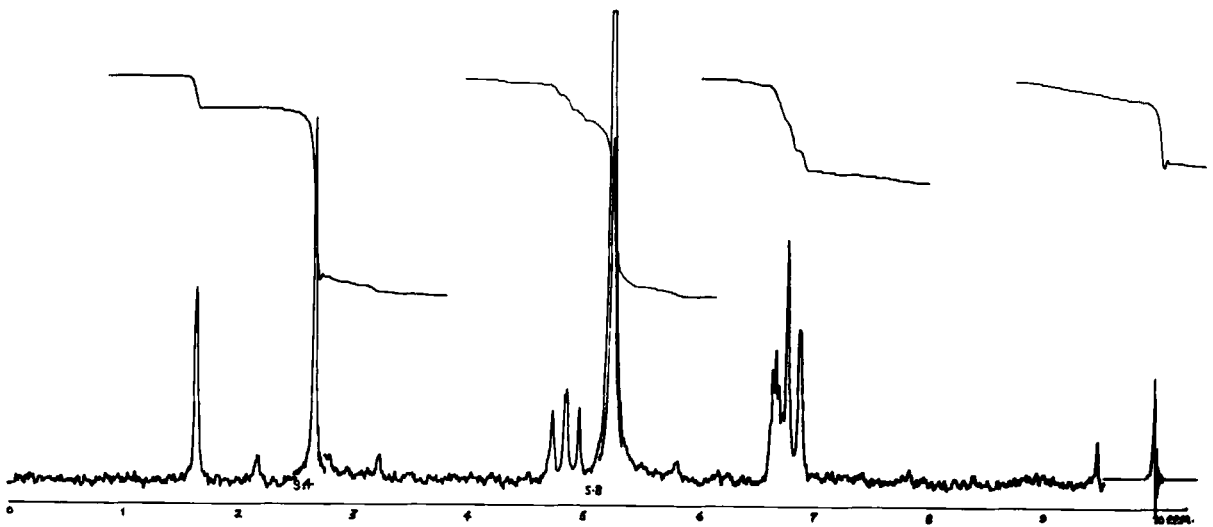
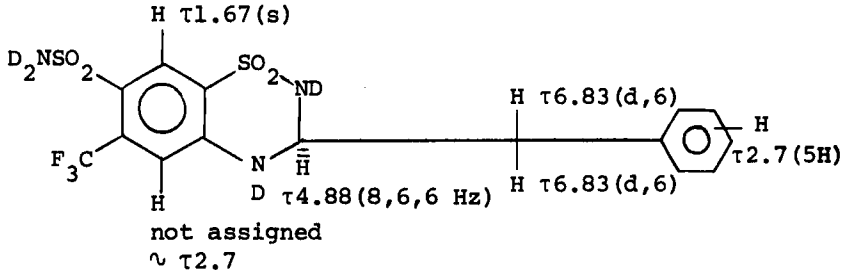


Figure 2. NMR Spectrum of Bendroflumethiazide (batch 15) in deuterated methanol (Instrument: Perkin Elmer R12B)



In d_6-DMSO (Figure 3), the NH proton resonances were observed near τ1.75 (singlet, superimposed with aryl proton), τ2 (broad), 2.45 (singlet) and 2.57 (singlet) (Figure 3) in addition to the other protons assigned from the d_4-methanol spectrum. The higher field singlets at τ2.45 and 2.57 are tentatively assigned to the sulfonamide $-NH_2$ protons. No preference of assignment is made of the other two -NH protons. On addition of D_2O, the -NH protons are rapidly exchanged for deuterium, resulting in the disappearance of the NH protons and sharpening of the proton resonance near τ5 which now appears like that of the d_4-methanol spectrum.

2.3 Ultraviolet Spectrum

Squibb House Standard (batch #15) in methanol exhibited three maxima at 208 mμ (E^1_1 745), 273 mμ (E^1_1 565) and 326 mμ (E^1_1 96)[13]. This agrees with measurements by Pilsbury and Jackson[15] and by Kracmar and Lastovkova[14] who discuss the U.V. spectrophotometry of benzothiadiazines and present spectra.

2.4 Mass Spectrum

The low resolution mass spectrum (Figure 4) shows only a very weak molecular ion (M+) of m/e 421 and a base peak of m/e 319 that could arise by a double proton rearrangement, which is not a common fragmentation pathway. The assignment of some of the fragment ions is shown below:

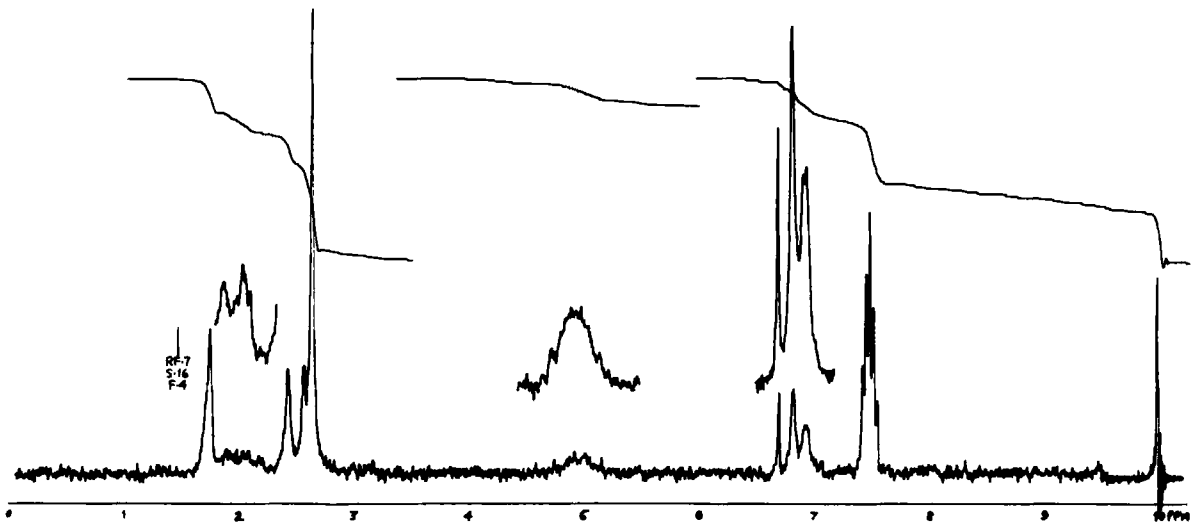


Figure 3. NMR Spectrum of Bendroflumethiazide (batch 15) in deuterated DMSO (Instrument: Perkin-Elmer R12B)

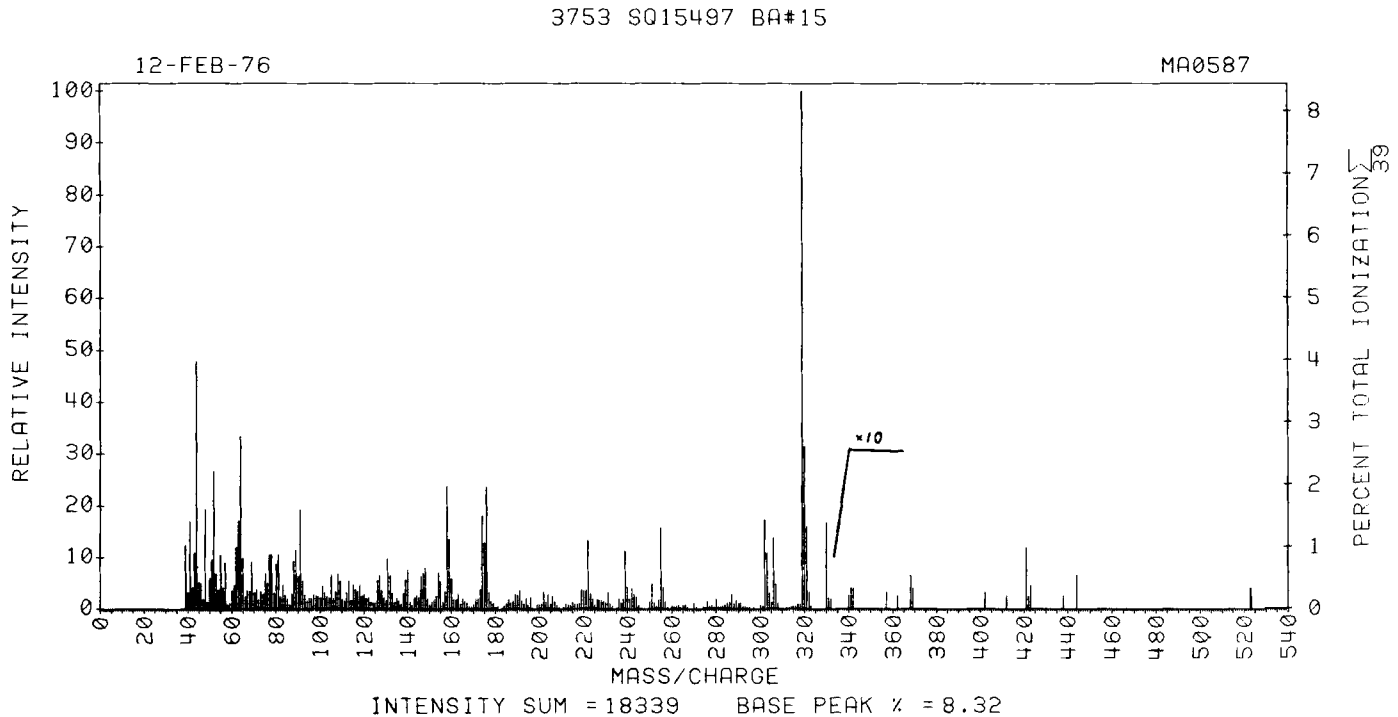
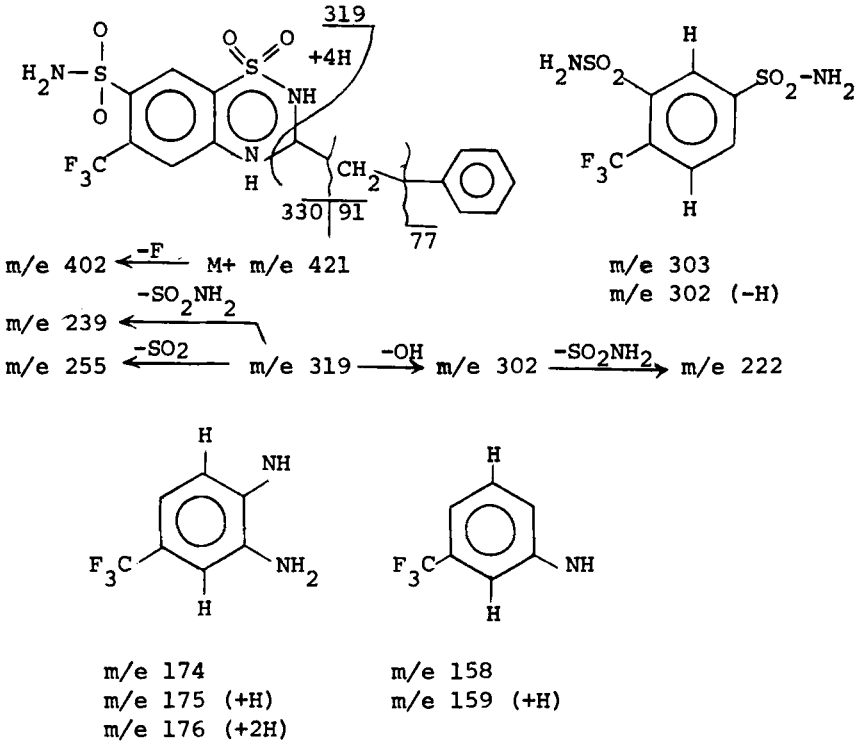


Figure 4. Low Resolution Mass Spectrum of Bendroflumethiazide (Instrument: AEI MS9)



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

2.5 Melting Range

The melting point does not occur sharply and depends on the rate of heating. The melting point of the Squibb House Standard (batch 15) was reported at 223.2° - 226.2° C. Literature values range from 220° - 228°.

2.6 Differential Thermal Analysis

Melting endotherm: 221° C. (17).

2.7 Solubility

Insoluble in water and chloroform.

Soluble in alkali, acetone and alcohol. Soluble in one equivalent of 0.1N NaOH. Slightly soluble in ether. Insoluble in acid, benzene, ligroin and petroleum ether[17]. Stable concentrated solution in polyethylene glycol, water and dimethyl-acetanilide or N-methyl-2-pyrolidinone have been claimed (18).

2.8 Crystal Properties

The powder x-ray diffraction pattern is presented in Table I and Figure 5[17].

TABLE I

d(A)*	I/I_o**
17.23	0.528
13.27	0.356
11.63	0.103
8.94	0.246
8.11	0.148
7.64	0.118
7.36	0.100
6.70	0.199
6.34	0.124
5.91	0.341
5.48	0.141
5.31	0.194
4.82	0.162
4.67	0.545
4.53	1.000
4.46	0.206
4.31	0.175
4.19	0.516
3.96	0.213
3.84	0.357
3.75	0.185
3.69	0.248
3.63	0.289
3.54	0.205
3.44	0.140
3.40	0.145
3.19	0.166
3.11	0.145
2.99	0.157

*d = interplanar distance

$$\frac{n\lambda}{2 \sin\theta}$$

λ = 1.539 A

Radiation: $K\alpha_1$ and $K\alpha_2$ Copper

** Relative intensity based on highest intensity of 1.00

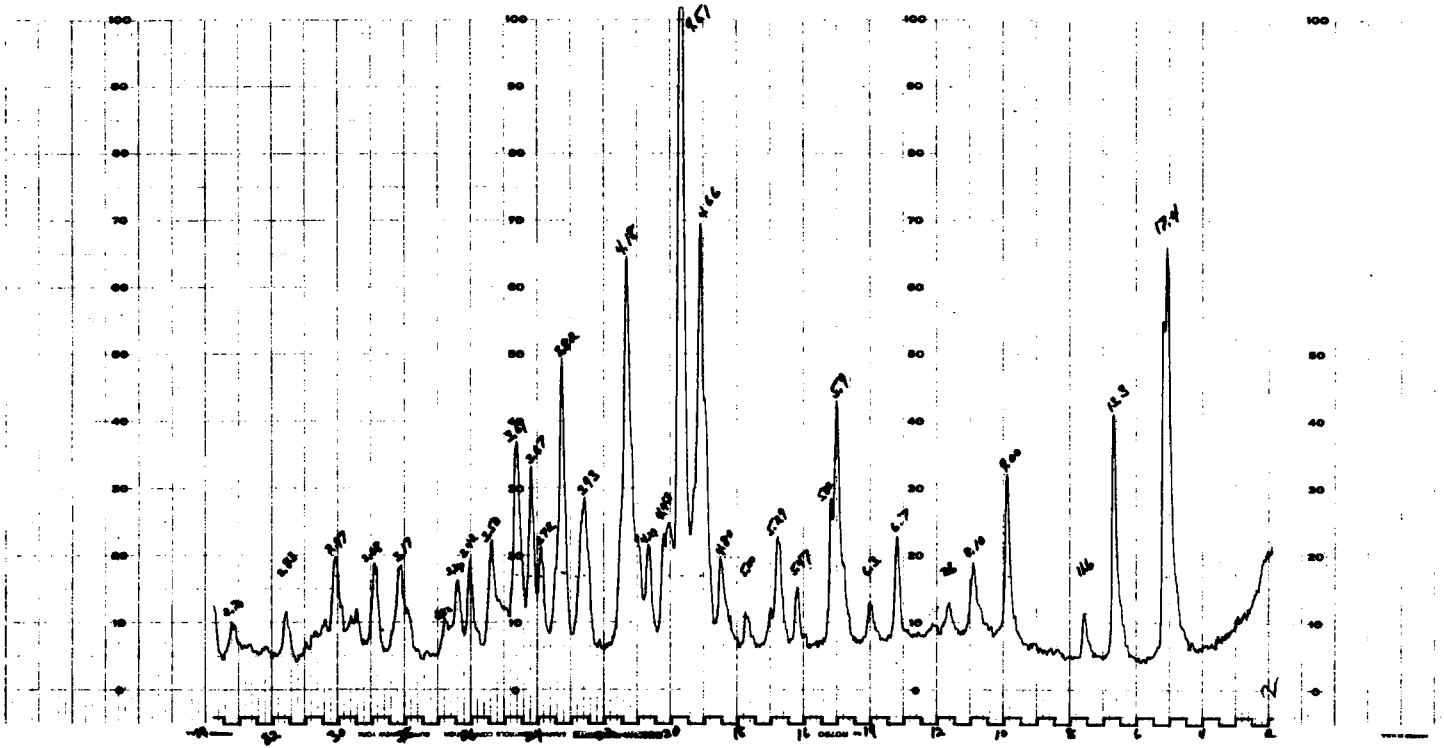


Figure 5. Powder X-Ray Diffraction Pattern of Bendroflumethiazide (Instrument: Norelco)

2.9 pKa

A pKa of 8.53 ± 0.05 was determined by using the solubility variation with pH method[45].

3. Synthesis

(see Figure 6)

The synthesis of bendroflumethiazide (I) by cyclization of 4-amino-6-trifluoromethyl-m-benzenedisulfonamide (II) with phenylacetaldehyde (III) was described by Holdrege, Babel and Cheney[1] and others[3,4,5,6,7]. Alternate approaches are condensation of 4-amino-6-trifluoromethyl-m-benzenedisulfonyl chloride (IV) with phenylacetaldehyde (III) in the presence of ammonia[8,9], condensation of III with phenylacetimine (V)[10], reaction of 4-amino-6-trifluoromethyl-m-benzenedisulfonamide (II) with acetoxystyrene (VI) in the presence of acetic acid[11] and by hydrogenation of 3-benzyl-6-trifluoromethyl-7-sulfamoyl-1,2,4-benzothiadiazine 1,1-dioxide (VII) with lithium aluminum hydride[12].

4. Stability and Degradation

Bendroflumethiazide, as a solid, appears to be very stable. The solid, exposed to 60° C. for a period of two weeks showed no decomposition as measured by I.R. and modified Bratton-Marshal reaction. In ethanol (1 mg/ml), it showed significant changes (25% decomposition) at the end of two weeks at 60° C. In aqueous suspension, almost complete breakdown to the disulfonamide (II, Figure 6) occurred at the end of one week at 60°C.[19].

In alkaline solution (pH 12) bendroflumethiazide undergoes complete hydrolysis to the disulfonamide (II) in one hour at 35° C.[20,22]. It is also unstable under certain acid conditions[21].

5. Drug Metabolic Products - Pharmacokinetics

No drug metabolites have been identified so far. The Pharmacokinetics have been studied in the rat[24] and in human beings[25]. In human beings, orally or intravenously administered doses of S^{35}-labeled bendroflumethiazide are excreted almost quantitatively in 24 hours[25]. The stability of the trifluoromethyl group was

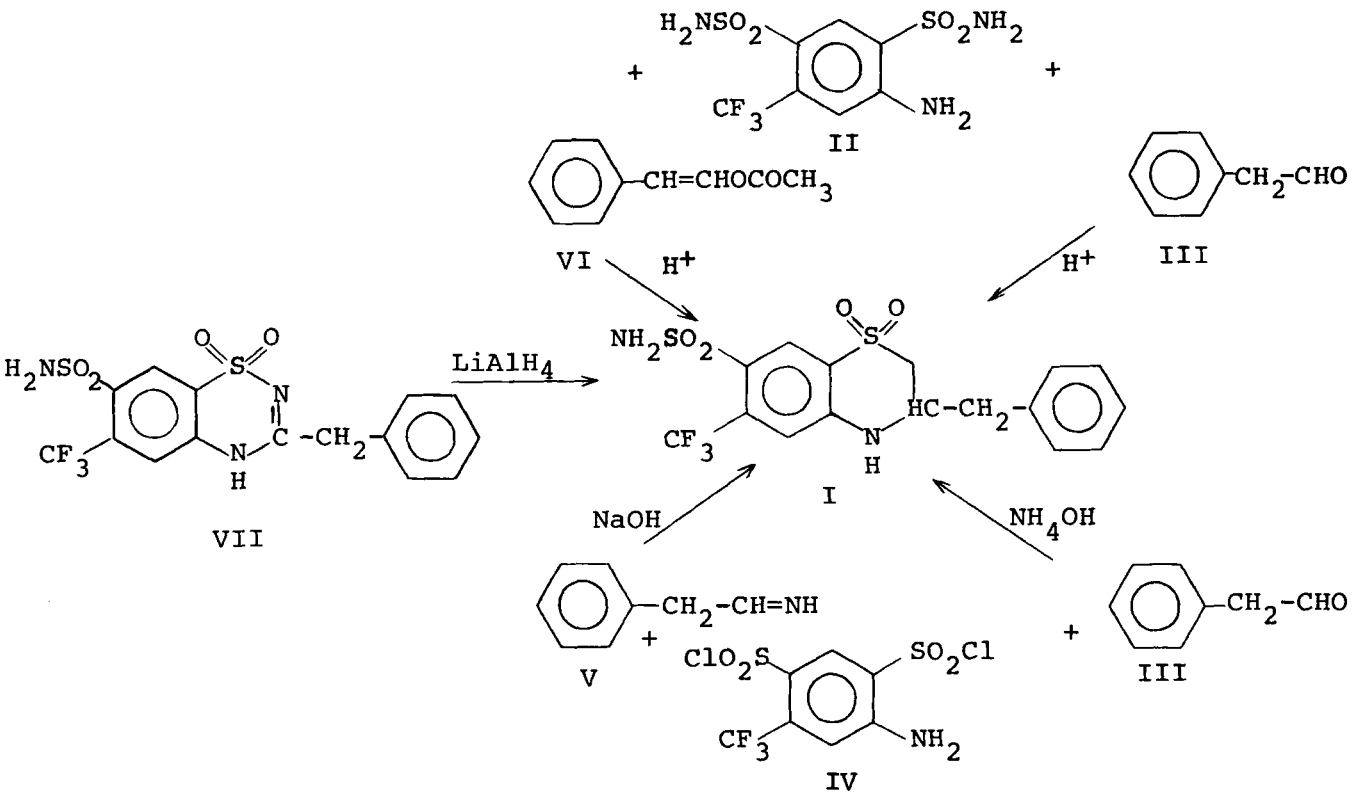


Figure 6. Synthetic Pathways to Bendroflumethiazide

studied in rats[26]. There was no detectable fluoride uptake in the teeth of rats on a carious diet. Bendroflumethiazide was found to be 94% protein bound[45].

6. Methods of Analysis

6.1 Elemental Analysis

	Calc. %	Found: (Squibb House Standard Batch 15)
C	42.75	42.63
H	3.35	3.27
F	13.53	13.83
N	9.97	9.92
O	15.21	-
S	15.22	15.60

6.2 Spectrophotometric Analysis

The ultraviolet absorption maximum at 273 mμ (E^{1}_{1} 565) can be used for the quantitation of bendroflumethiazide in dosage forms[23,27].

6.3 Colorimetric Analysis

Bendroflumethiazide can be quantitatively assayed in dosage forms by alkaline hydrolysis to the disulfonamide (II, Figure 6) diazotization, coupling with N-(1-naphthyl) ethylenediamine and determination of the absorption maximum at 515 nm[28,30]. Other coupling agents have been used[29,31]. This assay can also be used to determine the presence of the disulfonamide (II) in bendroflumethiazide[21,28].

6.4 Non-Aqueous Titration

An assay based on titration with sodium methoxide in dimethylformamide using p-nitro-benzene-azo-resorcinol as indicator has been developed[32]. Pyridine as solvent and azo-violet as indicator can also be used[28].

6.5 Chromatographic Analysis

6.51 Paper Chromatographic

Bendroflumethiazide (Rf 0.76) can be separated from the disulfonamide (II, Figure 6) using methylisobutylketone saturated with formamide as mobile phase and 30% formamide

in methanol as stationary phase. For quantitation, the spots are eluted with methanol and the concentration is determined spectrophotometrically (see 6.2)[33].

Identification and separation from other thiazide diuretics by paper chromatography has also been reported[15].

6.52 Thin-Layer Chromatography

Thin layer chromatographic systems are compiled in the following table.

Absorbent	Solvent System	Ref.	Rf
250 Silica Gel G	Benzene-Ethyl Acetate (2:8) and Ethyl Acetate-Methanol Ammonium Hydroxide (85:10:5)	34	0.91
250 Silica Gel G	Propanol-2/12N Ammonia (8:2)	34	0.88
" " " G	Propanol-1/12N Ammonia (8:2)	34	0.93
" " " G	Butanol-1/12N Ammonia (8:2)	34	0.70
" " " G	Pentanol-1/12N Ammonia (8:2)	34	0.54
" " " G	Ethyl Acetate/12N Ammonia (8:2)	34	0.98
" " " G	Chloroform/Methanol (8:2)	34	0.52
" " " G	Cyclohexane/Glacial Acetic Acid/ Acetone (4:1:5)	34	0.98
Silica Gel	Toluene/xylene/1-4-Dioxane/Isopropanol/ 25% Ammonia (50:10:30:10)	35	-
Silica Gel	Ethanol/Chloroform/Heptane (1:1:1)	36	0.98
Alumina GF-254 (Two Dimensional)	(1st) Ethanol; (2nd) Chloroform/ Butanol	37	-
Silica Gel	Ethanol containing 1.5% Water	37	0.71
Alumina G	Ethanol	38	-

Absorbent	Solvent System	Ref.	Rf
Silica Gel G	Ethanol/Benzene (80:20)	38	-
Silica Gel	Ethyl Acetate/Benzene (8:2)	39	0.70
" "	Benzene/Ethyl Acetate/25% Ammonia/ Methanol (20:80:1:10)	39	0.75
" "	Ethyl Acetate/Benzene/25% Ammonia/ Methanol (60:40:20)	39	0.40
Silica Gel	Ethyl Acetate/Benzene/Ammonia/ 25% Methanol (50:40:2:10)	39	0.68
Silica Gel	n-Hexane/Acetone/Ethylamine (60:30:10)	39	0.10
	n-Hexane/Acetone/Diethylamine (40:40:20)	39	0.46
Silica Gel	Chloroform/Methanol/Diethylamine (80:15:5)	39	0.75
Silica Gel G	Benzene/Ethyl Acetate (2:8)	40	0.82
" " G	Ethyl Acetate/Methanol/Ammonium Hydroxide (85:10:5)	40	0.82
" " G	Ethyl Acetate/Benzene (8:2)	41	0.98

Identification of oral hypoglycemic and diuretic drugs by TLC using metal ions has been described[42].

7. Identification and Determination in Biological Fluids

In plasma: Colorimetrically[12]

In urine: Colorimetrically[24]
Radioactivity[25]
TLC[39,40,41]

8. Miscellaneous

Pharmaceutical preparation of bendroflumethiazide have been patented[43,44].

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CEPHRADINE

Klaus Florey

TABLE OF CONTENTS

1. Description

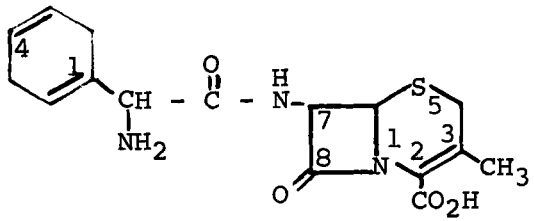
1.1 Names

Cephradine is 7-[D(-)-2-amino-(1,4 cyclohexadien-1-yl)acetamido-3-methyl-8-oxo-5-thia-1-azabicyclo-oct-2-ene-2-carboxylic acid; also 7-[2-amino-2-(1,4 cyclohexadienyl) acetamido]-desacetyl-cephalosporanic acid.

1.2 Definition

Cephradine, unless specified otherwise, is defined as a hydrated form containing 3-6% of water (for further discussion see Section 2.11).

1.3 Formula, Molecular Weight



$C_{16}H_{19}N_3O_4S$

Molecular Weights: 349.41 anhydrous
367.43 monohydrate
385.45 dihydrate

1.4 Appearance, Color, Odor

White crystalline powder, odorless to slightly sulphurous.

2. Physical Properties

2.1 Infrared Spectra

Spectra of cephradine (Batch #NN005NC) (Figure 1), cephradine dihydrate (house standard batch #NN005NB) (Figure 2), cephradine monohydrate recrystallized from acetonitrile-water (sample MSA 38719) (Figure 3) and cephradine monohydrate recrystallized from methanol (sample MSA 38680) (Figure 4) are presented[1].

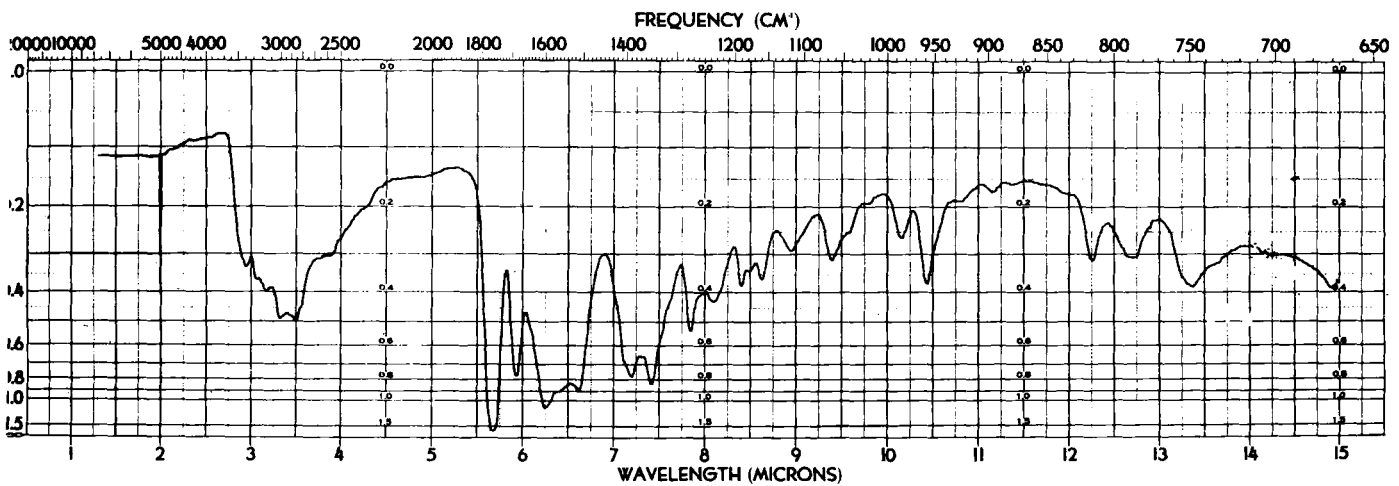


Figure 1. Infrared Spectrum of Cephradine Batch #NN005NC, KBr Pellet, Instrument, Perkin Elmer 21.

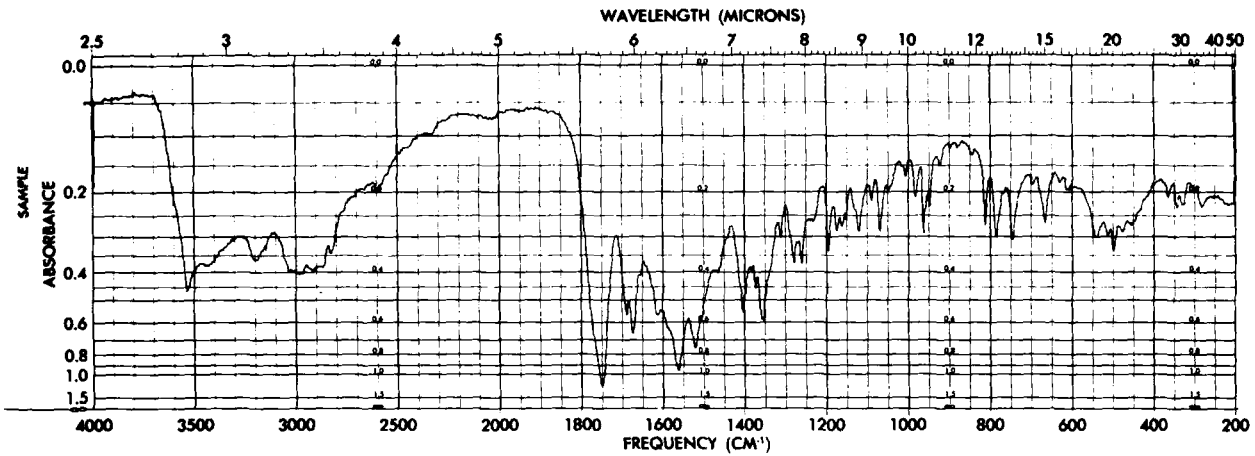


Figure 2. Infrared Spectrum of Cephradine Dihydrate(House Standard Batch # NN005NB) KBr Pellet, Instrument, Perkin Elmer 621.

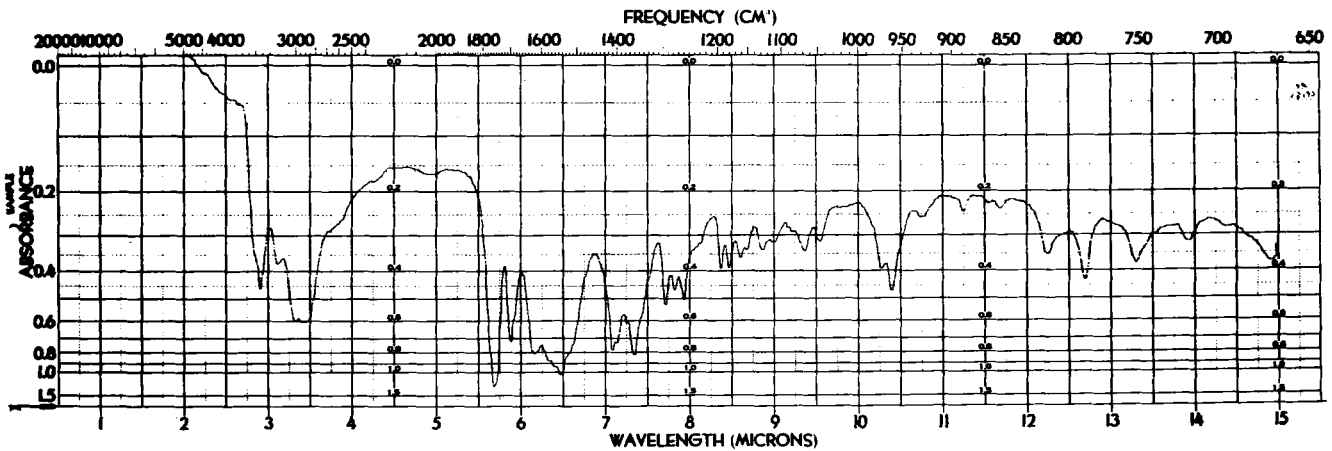


Figure 3. Infrared Spectrum of Cephradine Monohydrate Recrystallized From Acetonitrile(Water Sample MSA 38719) KBr Pellet, Instrument, Perkin Elmer 621.

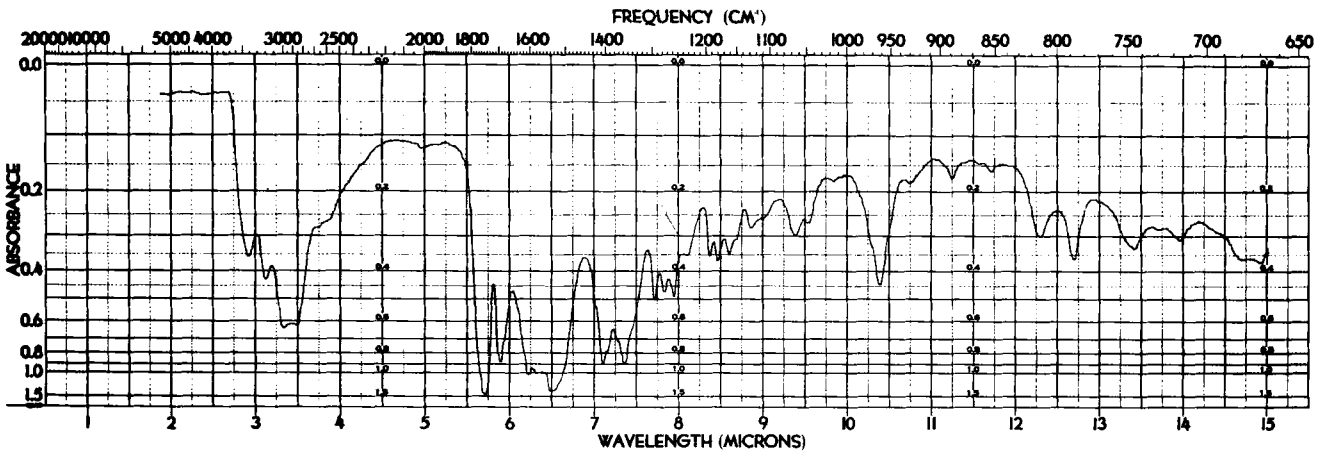


Figure 4. Infrared Spectrum of Cephradine Monohydrate, Recrystallized From Methanol (Sample MSA 38680) KBr Pellet, Instrument, Perkin Elmer 621.

2.2 NMR Spectra

NMR spectra in CF_3COOH (Figure 5) and D_2O (Figure 6) are presented[2].

In trifluoroacetic acid (TFA), the compound exists in protonated form with the NMR shift of the NH^+ at τ 2.44 with reference to internal tetramethyl silane(TMS). The imide NH proton appearing as a doublet (J = 9.0 Hz) at τ 2.00 is coupled to one of β-lactam ring protons, which appear as a quartet (J = 9.0,4.0 Hz) at τ 4.20. The second β-lactam proton resonance is a doublet (J = 4.0 Hz) at τ = 4.74. The $S-CH_2$ group protons appear as AB quartet (J = 18.0 Hz) at τ 6.36 and 6.54 while the methyl group appears at τ 7.61. In the dihydrophenyl ring, the olefinic protons appear at τ 3.62 (1 H) and 4.21 (2 H). The resonance at τ 7.13 is assigned to four protons of the dihydro ring. Finally the methine proton of the $CHNH_3^+$ group appears as a multiplet at τ 5.00. The spectrum obtained in deuterium oxide containing a drop of sodium deuterium oxide was recorded using 3-(trimethyl silyl)-1-propansulfonic acid sodium salt as an internal reference. The amine and NH group protons are exchanged with D_2O. In the dihydrophenyl ring, the chemical shifts are two olefinic hydrogens at τ 4.25, one olefinic proton at τ 4.15 and the other four hydrogens at τ 7.31. The β-lactam ring protons are a pair of doublets (J = 4.0 Hz) at τ 4.42 and 4.94. The $S-CH_2$ group protons appear as a AB quartet (J = 18.0) at τ 6.81 and 6.43. The methyl resonance is a doublet at τ 8.21 and may be a result of partial isomerization of the double bond in the basic solution. Finally, the methine($CHNH_2$) proton has a chemical shift of 6.00τ[2].

NMR can also be used to determine the amount of residual cephalexin by comparing the area under the phenyl protons (τ 2.50) and olefinic protons (τ 3.84)[2].

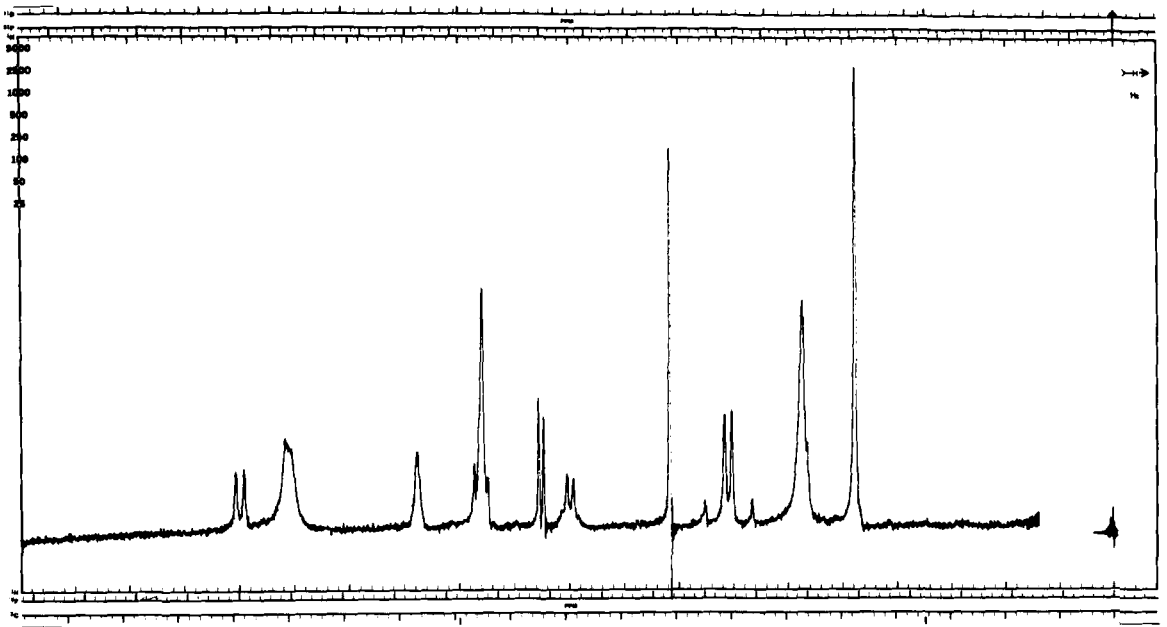


Figure 5. NMR Spectrum of Cephradine Batch NN005NC in CF_3COOH
Instrument: Varian XL-100.

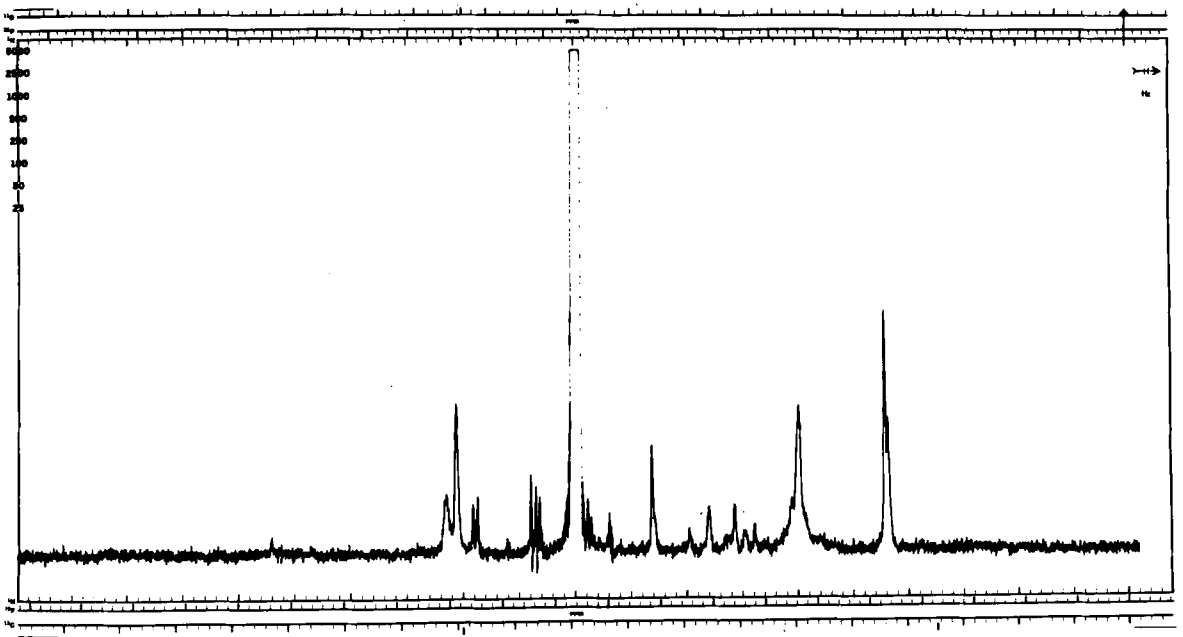
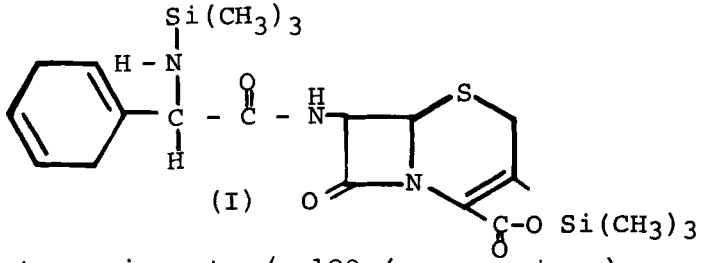


Figure 6. NMR Spectrum of Cephradine Batch NN005NC in D_2O
Instrument: Varian XL-100.

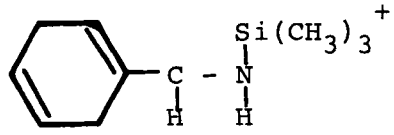
2.3 Mass Spectrum

Because of the low volatility of cephradine, a trimethyl silyl derivative was made using the reagent BSA (N,O-Bis-(Trimethyl silyl) acetamide). The low resolution mass spectrum (Figure 7) indicates that two and three trimethyl silyl groups were added. The compound having two trimethyl silyl groups was the predominant product with molecular ion at m/e 493. The loss of CH_3 from the molecular ion (typical of trimethyl silyl groups) yielded an ion at m/e 478[3]. The data support structure I.



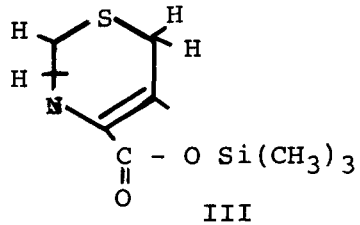
(I)

An intense ion at m/e 180 (see spectrum) corresponds to II,



II

another interval ion at m/e 230 corresponds to III.



III

which is a typical fragment obtained from β-lactam ring fragmentations[3].

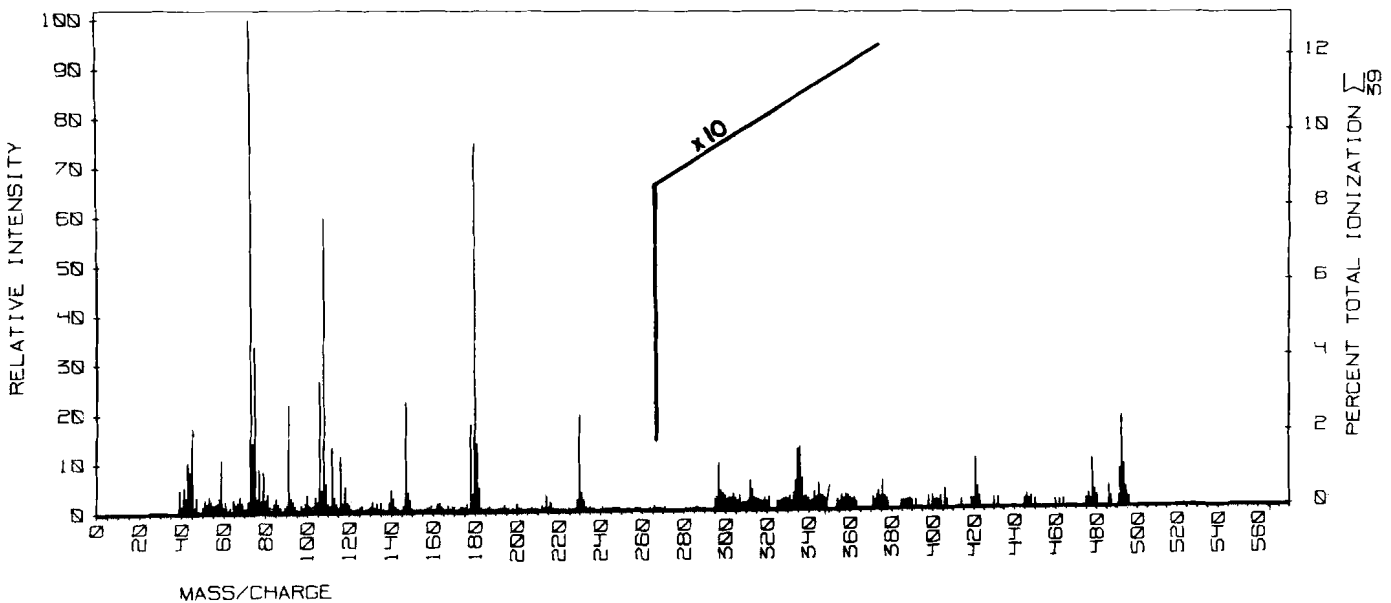


Figure 7. Low Resolution Mass Spectrum of Trimethyl Silyl Derivative of Cephradine. Instrument: AEI MS-902.

2.4 Ultraviolet Spectrum

Cephradine exhibits a single absorbance peak at 262 nm ($E^{1\%}_{1cm}$ = 220 for batch NN005NC)[4].

2.5 Optical Rotation

The specific rotation of the house standard (dihydrate, batch #NN005NB) in a pH 8 buffer was found to be + 88.3°(as is)[5]. The average specific rotation of seven batches of cephradine at a concentration of 0.5% in 0.1M acetate buffer (pH 4.6) and calculated on an anhydrous basis was found to be + 91.6° with a range from + 89.22° to 92.90°. The effect of pH on rotation was found to be slight, as can be seen from Table 1[5].

Table 1

$[\alpha]_D^{27^\circ}$ Conc. Approx. 1%

pH	Initial	1 Hour	3 Hours	5 Hours	24 Hours
4.01	+ 77.45°	+ 79.45°	+ 76.58°	+ 78.45°	+ 75.38°
5.00	+ 78.25°	+ 78.75°	+ 77.55°	+ 77.65°	+ 75.86°
6.03	+ 78.32°	+ 79.10°	+ 78.10°	+ 77.83°	+ 77.77°
7.00	+ 80.47°	+ 78.37°	+ 77.78°	+ 76.68°	+ 76.95°
8.20	+ 87.24°	+ 86.04°	+ 85.05°	+ 85.74°	+ 84.32°

2.6 Melting Range

Cephradine melts with decomposition. The melting range (USP) of cephradine dihydrate house standard (Batch NN005NB) was 183-185°.[5] The melting range of typical batches of cephradine has varied from 175-192°.

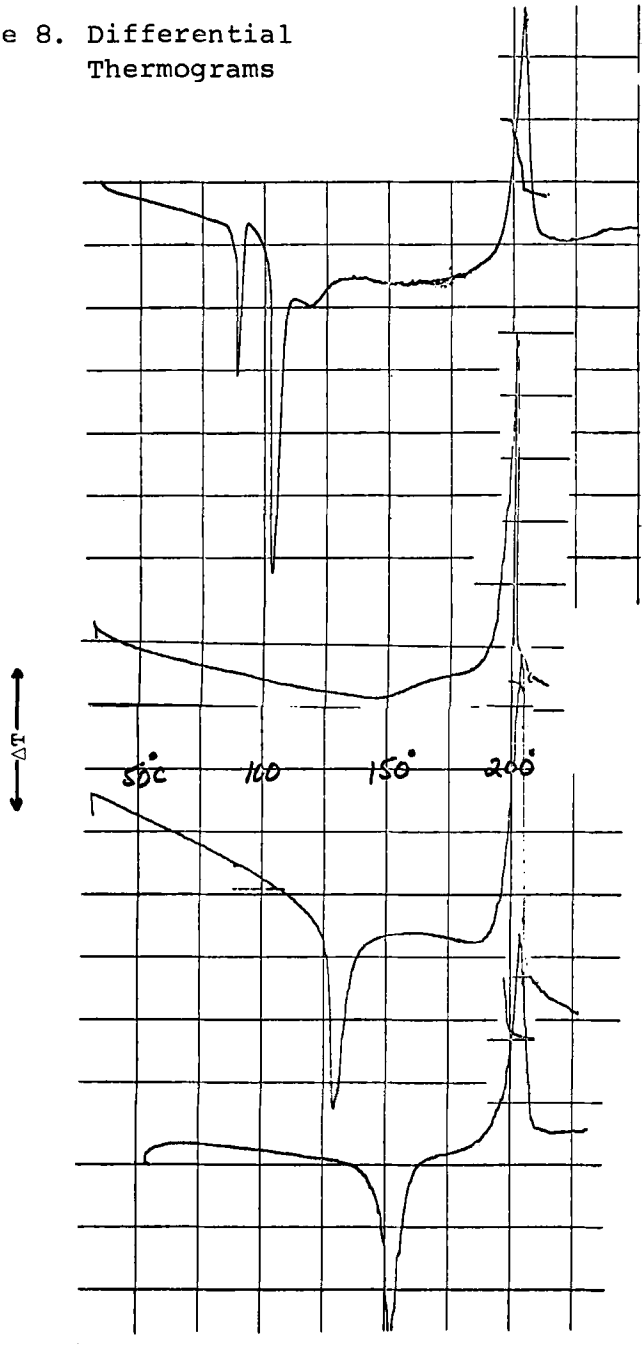
2.7 Differential Thermal Analysis

The thermogram of cephradine exhibits a single exotherm at approximately 200-203°, depending on heating rate. This exotherm indicates oxidative decomposition accompanied by melting[6]. On the other hand the thermogram of cephradine dihydrate exhibits two endothermic peaks at about 90° and at about 102° which are related to the hydration of the compound. A decomposition exotherm is observed at about 200°C. The difference in temperature between the two endothermic peaks is a measure of the stress (grinding, heating) leading to dehydration, to which the sample is subjected[6].

Thermograms of cephradine dihydrate, cephradine, cephalexin[7], and cephradine monohydrate (recrystallized from acetonitrile-water)[8] are presented in Figure 8[6]. The absence of an endotherm for cephradine suggests that cephradine is not a true hydrate and the water is not structurally bound in the crystal-lattice (see also section 2.11). On the other hand the dihydrate, the monohydrates obtained by recrystallization from acetonitrile-water[7](sharp endotherm at 150-152°) and methanol[8] (Endotherm at 152°)as well as cephalexin[7] are true hydrates.[6]

DTA has also been used as a screening technique to study the interaction of cephradine with potential adjuvants to a parenteral formulation[9].

Figure 8. Differential Thermograms



2.8 Thermogravimetric Analysis

By thermogravimetric analysis (TGA) of typical batches of cephradine from 3 to 6% volatiles (water), were found (theory for monohydrate water 4.93%). The dihydrate(batch B49988D)by TGA gave 9.8% volatiles (theory for dihydrate water 9.35%). VPC was used to confirm the volatile compound as water[48]. The 13% loss shown by TGA at about 200-210°(decomposition point) is assumed to be due to decarboxylation[6].

2.9 Ionization Constant, pK

By titration with sodium hydroxide a pK_1 of 2.63 and pK_2 of 7.27 was found[6].

2.10 Solubility

There is no difference in solubility of the various crystal forms. The solubility of cephradine in buffers at different pH values is reported in Table 2.

Table 2
Solubility of Cephradine at Different pH Values

pH of buffer	pH of Saturated Solution	Solubility mg/ml
4.00	4.02	35.8
water	4.91	21.3
60% sucrose	5.0	17.1
5.00	5.04	21.1
6.03	5.90	20.5
7.20	6.12	28.2
8.20	7.09	36.7
9.18	7.41	49.6

Cephradine is practically insoluble in ethyl ether, chloroform, benzene and hexane. The antibiotic is very slightly soluble in acetone and absolute ethanol. It is freely soluble in propylene glycol. The solubility terminology used is taken from USP XVIII.

The intrinsic dissolution rates of cephradine and its dihydrate as well as

cephalexin were found identical at an agitation intensity of 100 rpm[10]. The observed intrinsic dissolution rates (mg/ml/min/cm^2) are as follows:

Compound	Temperature	Intrinsic Dissolution Rate
Cephradine	22°C	0.08
	37°C	0.1
Cephradine dihydrate	22°C	0.08
	37°C	0.09
Cephalexin	22°C	0.08
	37°C	0.09

2.11 Crystal Properties

There is considerable evidence for polymorphism and four polymorphs or rather pseudopolymorphs have been characterized so far.

1.) Cephradine, hydrated. Although the water content varies from 3-6% it is not a stoichiometric hydrate since the water apparently moves freely in the crystal lattice(see section 2.7). It is obtained from aqueous solution. Anhydrous cephradine has been prepared and was found to be very stable and resistant to oxidation to cephalexin, when kept anhydrous(see section 4.1). However, it cannot be properly characterized, since it immediately hydrates on exposure to the atmosphere.

2.) Cephradine dihydrate. This compound which crystallizes from aqueous solution under controlled conditions[11], is a true dihydrate (see section 2.7). It is very stable and resistant to oxidation to cephalexin. However, on dehydration (loss of crystal structure) the dihydrate becomes very unstable (see section 4.1).

The structure of cephradine dihydrate was determined by single crystal x-ray diffraction in order to investigate the position of the water molecules in the crystal and the hydrogen

bonding of the water to various sites in the cephradine molecule[50]. The hydrogen bonding pattern is indicated in figure 9. There are two molecules in the unit cell. The projection of one is shown. The second is symmetry related by 180° rotation in the plane of projection, and translation of 1/2 of the b repeat. Atoms of the second molecule involved in hydrogen bonding are indicated with a prime notation.

The bonding may be summarized as follows: Water oxygens are numbered 69 and 71. Water oxygen #69 bonds with the β-lactam carbonyl of molecule 1, the amide nitrogen 13', of molecule 2, and with the second water molecule # 71. Water oxygen #71 bonds with one of the carboxyl oxygens, 64', of molecule 2, and with water molecule #69. The amino nitrogen, N5' of molecule 2 bonds with both carboxyl oxygens.

3.) Cephradine monohydrate recrystallized from acetonitrile water[8]. This is a true hydrate (see section 2.7).

4.) Cephradine recrystallized from anhydrous methanol[8] also appears to be a true monohydrate, although another one half mole of unbound water was also present. No residual methanol was found.

Cephradine, generally, seems to have a tendency to form solvates since acetonitrile and ethylene glycol solvates have been observed[12]. Powder x-ray patterns of the four crystal forms are presented in Tables 3-6[13].

The unit cell dimension, space group and cell content determinations of the four crystal forms were made by single x-ray crystallography[1] and are presented in Table 7.

For further discussion on these crystal forms, see sections on IR(2.1), DTA(2.7) and Bulk Stability (4.1).

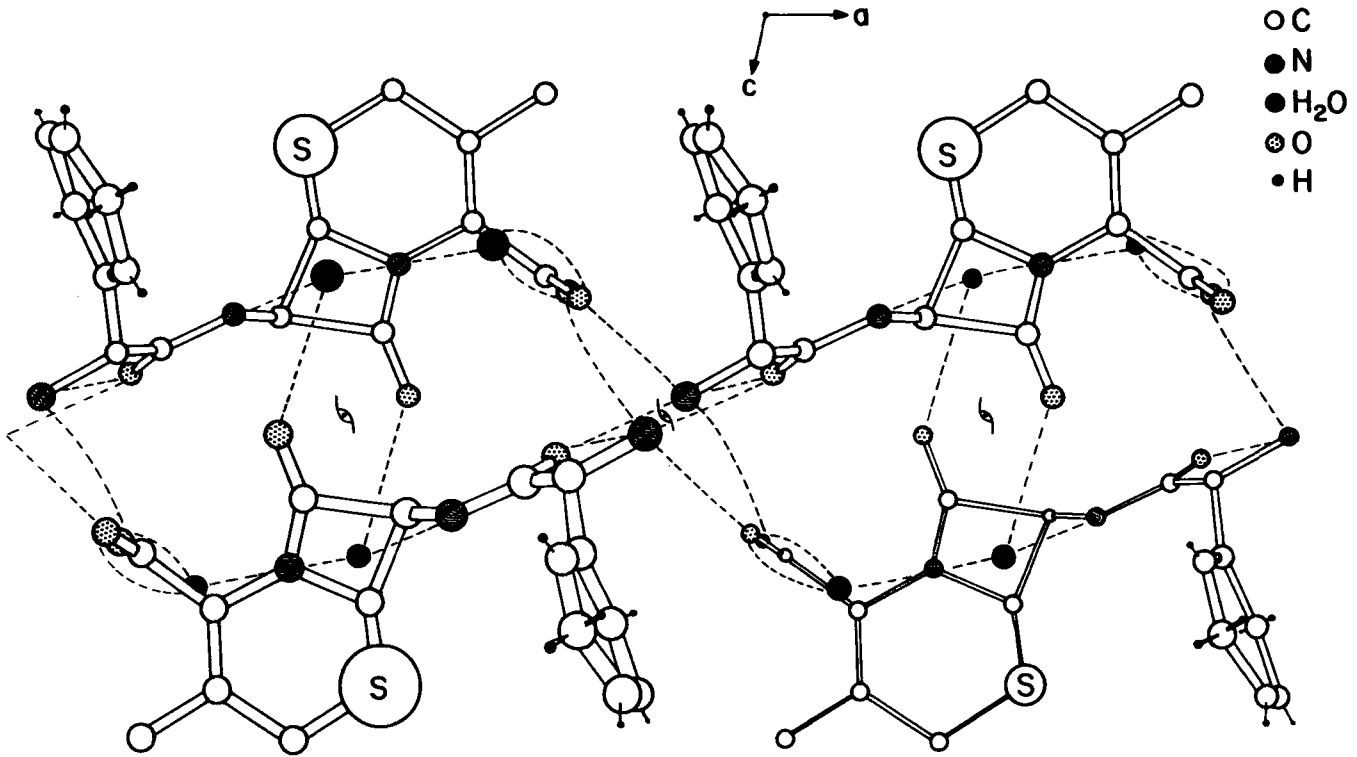


Figure 9. Distribution of water molecules in cephradine dihydrate.

Table 3
Powder X-Ray Pattern of Cephradine,Hydrated
Batch NN005NC

d	I/Io	d	I/Io
15.80	14.1	3.76	14.1
11.90	47.4	3.61	35.9
8.04	15.4	3.47	12.8
5.98	19.2	3.33	17.9
5.61	24.4	3.24	12.8
5.34	100.0	3.18	11.5
4.92	21.8	3.08	25.6
4.66	11.5	2.90	12.8
4.48	21.8	2.80	11.5
4.32	57.7	2.74	10.3
4.20	26.9	2.67	14.1
4.00	30.8	2.57	9.0
3.93	14.1	2.43	11.5

Table 4
Powder X-Ray Pattern of Cephradine Dihydrate
Batch NN005NB (House Standard)

d	I/Io	d	I/Io
11.60	35.4	3.76	22.9
10.50	15.6	3.67	26.0
8.75	10.4	3.55	100.0
7.05	19.8	3.45	43.8
6.20	37.5	3.42	46.9
6.00	17.7	3.19	28.1
5.80	24.0	3.08	30.2
5.61	56.3	2.92	54.2
5.23	20.8	2.80	10.4
5.10	37.5	2.68	15.6
4.68	7.3	2.61	15.6
4.55	9.4	2.57	13.5
4.45	26.0	2.49	14.6
4.25	17.7	2.46	17.7
3.94	9.4	2.39	7.3
3.87	12.5	2.31	12.5
3.80	28.1		

Table 5
Powder X-Ray Pattern of Cephradine Monohydrate
Recrystallized from Acetonitrile-Water
Sample #38720

d	I/Io	d	I/Io
9.92	100.0	3.05	17.0
8.83	94.0	3.02	18.0
6.96	82.0	2.96	25.0
6.45	24.0	2.90	14.0
5.64	80.0	2.80	17.0
5.43	35.0	2.77	35.0
4.92	15.0	2.75	14.0
4.84	22.0	2.70	13.0
4.74	91.0	2.65	18.0
4.39	45.0	2.60	26.0
4.23	100.0	2.47	11.0
4.00	45.0	2.42	26.0
3.91	35.0	2.38	23.0
3.64	21.0	2.35	31.0
3.41	32.0	2.24	25.0
3.36	29.0	2.21	17.0
3.22	24.0	2.14	25.0
3.10	61.0		

Table 6
Powder X-Ray Pattern of Cephradine Monohydrate
Recrystallized from Methanol
Sample # 38708

d	I/Io	d	I/Io
11.20	100.0	3.78	14.0
8.83	17.0	3.63	26.0
8.18	19.0	3.51	33.0
7.83	10.0	3.30	24.0
6.80	18.0	3.17	15.0
6.23	19.0	3.08	16.0
5.82	25.0	2.94	26.0
5.64	13.0	2.82	18.0
5.21	14.0	2.74	21.0
4.86	37.0	2.65	17.0
4.74	45.0	2.62	18.0
4.59	43.0	2.54	16.0
4.34	32.0	2.45	14.0
4.19	34.0	2.42	14.0
4.13	25.0	2.36	17.0
4.00	26.0	2.30	14.0
3.89	32.0		

Table 7

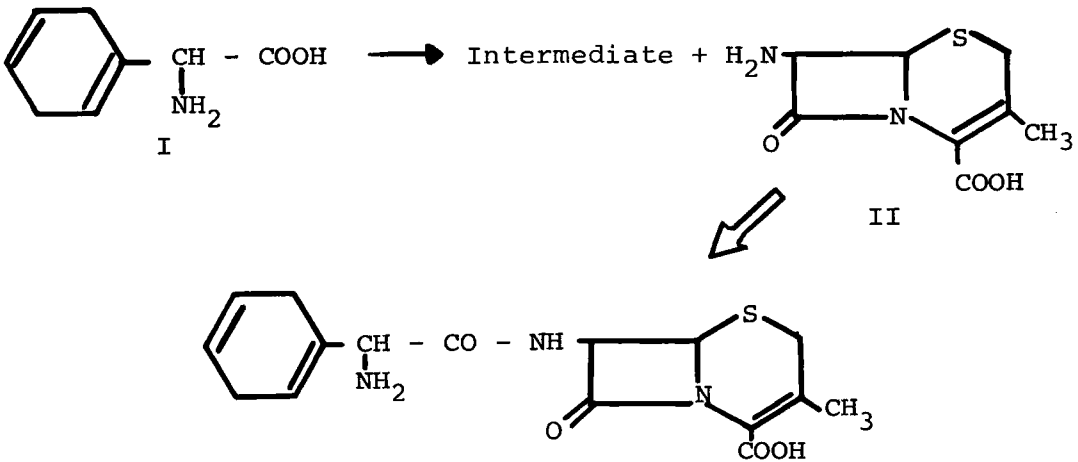
Sample	UNIT CELL CONSTANTS a	b	c	β	Vol.	SPACE GROUP	MEASURED DENSITY	CELL CONTENTS (molecules)
	Angstroms			Degrees	A^3			
Ceph-radine	27.06	12.05	16.89	109	5218	C2	1.37	12 cephradine 6 water
Cephradine dihydrate	10.72	7.31	11.87	102	908	$P2_1$	1.41	2 cephradine 4 water
Monohydrate Recrystallized from Acetonitrile	19.75	9.62	9.77	90	1856	$P2_12_12_1$	1.34	4 cephradine 4 water
Monohydrate Recrystallized from Methanol	17.58	9.4	21.6	90	3568	$P2_12_12_1$	1.33	8 cephradine 8 water

3. Synthesis

Cephradine (III, Figure 10) is synthesized by coupling 7-aminodesacetoxycephalosporanic acid (7-ADCA) (II) with a protected derivative of dihydrophenylglycine (I),

Figure 10

Synthetic Pathway to Cephradine



such as the tert.-butoxylcarbonyl derivative which can be converted to a mixed anhydride with ethylchloroformate and reacted with 7-ADCA[14]. Cephradine can also be made by forming the methyl acetoacetic ester enamine derivative of dihydrophenylglycine-which is converted to a mixed

anhydride with benzoyl chloride prior to coupling with 7-ADCA[12]. Cephradine is then crystallized from a biphasic MIBK-aqueous solution[12]. It also can be crystallized from water alone, as well as from other solvents (see section 2.11).

4. Stability-Degradation

4.1 Bulk Stability

Cephradine, when kept under dry and cool storage conditions, has shown reasonable bulk stability[12]. Like other 1,4 cyclohexadienes such as 2,5 dihydrophenylalanine[15], cephradine is prone to a slow rate of oxidation of the cyclohexadiene ring to the benzenoid ring. The exact mechanism of this reaction is not known, but in addition to oxygen and water, trace metals such as iron seem to have an accelerating effect.

The oxidation to cephalexin as well as degradation (loss of biopotency) can be prevented or significantly reduced by storage at low temperature and exclusion of oxygen, as well as removal of water. This last method, however, is impractical due to the extremely hygroscopic nature of anhydrous cephradine[12].

On the other hand cephradine dihydrate[11] being a true solvate (see section 2.11)where water cannot move freely in the crystal lattice exhibits remarkable resistence to cephalexin formation, loss of biopotency and color development during extended storage under air[12]. The difference between cephradine and its dihydrate is illustrated in Table 8[12].

However upon partial or full dehydration of cephradine dihydrate under a variety of conditions, drying at higher temperatures or certain kinds of milling this excellent stability is not maintained[12].

Table 8
Average Bulk Stability Data for Three Batches of Cephradine and for Three Batches of Cephradine Dihydrate After 9 Months of Storage Under Air

	Initial	5°C	RT	40°C	50°C
Cephradine ("as is")					
Bioassay, mcg/ml	949	947	942	924	849
Cephalexin, %	2.7	3.0	3.5	4.6	7.0
Cephradine Dihydrate ("as is")					
Bioassay, mcg/ml	909	870	870	897	906
Cephalexin,%	2.3	2.3	2.3	2.2	2.4

Cephradine is moderately light sensitive. When exposed to U.V. light, the solid turns yellow on the surface but no loss of bioactivity was noted[12]. The sensitivity of cephalosporin C to light has been previously noted[16].

Other than cephalexin, no degradation products of solid cephradine have been identified. TLC examination of degraded (loss of biopotency) samples revealed a multiplicity of U.V. absorbing and fluorescent spots[17].

4.2 Stability in Solution

In aqueous solution cephradine tends to be quite stable at pH 4.0 and below and much less stable at higher pH units. A pH-stability profile is presented in Table 9[6]. Cephradine was found to be fully potent for at least eight hours in a variety of parenteral infusion solutions[23].

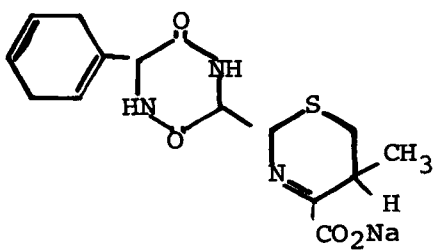
Although cephradine, like other cephalosporins is much more resistant to opening of the β-lactam ring by alkali than penicillins, the ring does open up with subsequent further degradation. Ring opening also leads the loss of U.V. absorbance at 262 nm.

Table 9
Stability of Cephradine in Phosphate Buffer
at Room Temperature

pH of Solution*	Percent of Remaining Bioactivity				
	2 days	4 days	7 days	10 days	14 days
4.0	95.9	105.1	99.3	99.3	95.2
6.0	73.0	86.9	69.5	30.2	18.5
8.0	67.1	43.1	24.5	13.4	10.9
10.0	64.0	41.1	25.2	13.3	11.7

*ph of samples at start of study. The pH of the high pH samples drifted toward lower pH values as the study progressed.

One of the alkaline degradation products precipitates from the solution and has been identified as 2-[6-(1,4 cyclohexadien-1-yl)-2,5-dioxo-3-piperazinyl]-5,6-dihydro-5-methyl-2H-1,3-thiazine-4-carboxylic acid, sodium salt[18].



The β-lactam ring of cephradine is quite resistant to penicillinase, but opens readily with cephalosporinase[19].

On the acid side NMR stability studies with a 2% solution at pH 1.6, held at 60°C, demonstrated that there is no β-lactam ring opening. However possible shifting of the double bond from carbons 2-3 to 3-4 was observed by NMR and confirmed by loss of U.V. absorbancy at 262 nm. After 93 hours NMR indicated a 50% double bond isomerization[2]. Very vigorous treatment with strong acid will lead at least in part to a split of the amide linkage to form 7-ADCA and dihydrophenylglycine[12]. This cleavage can also be achieved enzymatically with penicillin acylase[19]. In a phosphate buffer at pH 6 vigorous aeration or exposure to room light did not accelerate degradation[20]. However 10 hours of exposure to a Hanovia U.V. lamp of an aqueous solution (pH 5) of cephradine more than doubled the cephalexin content with little change in biopotency[12]. Cephradine was found to be stable for at least thirty days in frozen (-5°C) human serum and urine. No significant loss was detected by repeated thawing and refreezing[21]. On the other hand when incubated with serum at 37°C for 6 hours there was a 20% loss of activity. Incubation with whole blood under the same conditions caused little or no loss of biopotency[22].

It was found[51], that the activity of cephalosporins containing a phenylglycine moiety (cephaloglycin and to a lesser degree cephalexin and cephradine) is progressively lost in the presence of cupric ion. This degrading effect of cupric ion can be inhibited by d-penicillamine.

5. Drug Metabolism

Cephradine-^{3}H 250 mg dry filled capsules were administered to eight normal male subjects as a single oral dose in a bioavailability study[24]. Serum and urine samples were assayed in a double blind fashion for biological activity and in an open fashion for radiochemical activity.

The mean peak cephradine serum concentration (7.0 ± 1.0 μgm/ml - SE by bioassay and 7.8 ± 0.9 μgm/ml - SE by radioassay) occurred 55 minutes after dosing and decreased with a biological half-time of 40 minutes. The cumulative areas under the curves for cephradine were 161.5± 29 and 177.3 ± 34.7 min. μgm/ml for bioassay and radioassay curves respectively. Cephradine was rapidly excreted. Approximately 77% of cephradine was excreted within the three hour period following drug administration. After 24 hours approximately 87% of the administered dose of cephradine was recovered in the urine as measured by both bio and radiochemical assay[25]. Four healthy female subjects received a single intramuscular injection of 1 gram of cephradine-^{3}H[24]. The mean peak concentration of 10 ± 1.7 ug/ml - SE in plasma was reached at 2 hours after administration and then decreased with a biological half-life of 2 hours. Binding of cephradine to plasma protein was found to be 6% over the range of concentrations found in plasma during the absorptive and excretory phases.

Absorption, based on excretion of radioactivity in urine over the 24 hour period following administration, was 92 ± 6.6% ± SE for the four subjects. Another 1% was excreted in feces within the 72-hr period of the experiment. There was no significant difference in excretion based on the recovery of radioactivity or antimicrobial activity in urine. Excretion was 66 ± 6.9% ± SE at the end of 6 hr and 85 ± 6.5%

± SE at the end of 12 hours. Recovery of antimicrobial activity in urine and the area under the curve for concentration of antibiotic in plasma were in excellent agreement with those found when labelled cephradine was administered orally.

Cephradine appears to be slowly released from the site of injection to give levels of antibiotic in plasma which, though reaching a maximum at 1/3 those found after oral administration, provide the same amount of bioavailable antibiotic over a longer period of time[24].

No canine or human drug metabolites were found so far except trace amounts of cephalexin, as identified by mass spectrometry[26].

The above information was published (references 27-31).

6. Methods of Analysis

6.1 Elemental Analysis

%	C	H	N	S
Calc. for anhydrous	55.01	5.48	12.03	9.16
Calc. for monohydrate	52.30	5.76	11.44	8.73
Found for cephradine batch NN057NC	51.96	5.83	11.16	9.00
Calc. for dihydrate	49.85	6.01	10.90	8.31
Found for cephradine dihydrate batch NN005NB	49.77	6.06	10.95	8.39

6.2 Microbiological Assay

For bulk and formulated products a turbidimetric method using Streptococcus faecalis A.T.C.C. 10,541 is convenient. Alternatively agar plate methods using Sarcina lutea A.T.C.C. 9341, Bacillus pumilus A.T.C.C. 14,884 or Staphylococcus aureus, A.T.C.C. 6538P = FDA 209P, are also employed. For blood and body fluid samples an agar plate method is used employing Sarcina lutea as test organism because of its great sensitivity[32,46,47].

The minimum inhibitory concentration (MIC) of cephradine for the four test cultures is as follows:

	mcg/ml
Sarcina lutea	0.04
Staphylococcus aureus	0.40
Bacillus pumilus	0.40
Streptococcus faecalis	50.0

Cephradine is slightly more bioactive against Streptococcus faecalis and Sarcina lutea than cephalexin, while the reverse hold true for Staphylococcus aureus[33].

6.3 Iodometric Analysis

Cephradine can be determined by the iodometric assay. The β-lactam ring is opened with alkali or cephalosporinase followed by iodination at an acid pH (pH 4.5 phosphate buffer). About 4-5 moles of iodine are consumed[34]. It is interesting to note that penicillins under the same condition consume 8-9 moles of iodine. The precision of the iodometric assay for cephradine is not as good as that for penicillins when alkali is used for inactivation. The precision can be considerably improved by using cephalosporinase instead of alkali for ring opening[19]. Also, with the latter method much better agreement was obtained with the microbio-

logical assay (see Table 10) even with severely degraded bulk samples. It therefore can be considered stability indicating.

Table 10

Comparison of the cephradinase-iodometric, alkali-iodometric and bioassay methods for the determination of cephradine in bulk powders*

Cephradine potency = mcg/mg

Sample	Bioassay	Cephalosporinase-iodometric assay	Alkali-iodometric assay
NN054ND	812	813	979
NN059ND	854	843	991
NN061ND	778	846	980
NN054ND	578	578	744
NN059ND	424	466	643
NN061ND	405	464	639

*Results are the means of determinations on two consecutive days. The powders were stored at 50°C two years in bottles with varying volumes of head space.

The presence of 7-ADCA does not interfere with the iodometric assay[19].

It was found that when iodination was carried out at an alkaline rather than acid pH, 13 equivalents of iodine were consumed[34].

6.4 Spectrophotometric Analysis

The ultraviolet absorbance peak of cephradine at 262 nm (see section 2.4) can be used as an identify and homogeneity assay in formulations[35].

Opening of the β-lactam ring with alkali or preferably, with cephalosporinase abolishes the U.V. absorbance at 260nm. This has been made the principle of a quantitative assay which appears to be stability indicating in the "practical" range (less than 20% loss of bioactivity)[19].

6.5 Fluorometric Analysis

Cephradine can be assayed quantitatively in alkaline medium by fluorimetry, (excitation wave length 350 nm, emission wave length 495 nm). This method has been used for blood level studies but is not recommended for the determination in urine because of erratic results.

6.6 Colorimetric Analysis

The well known hydroxylamine method for penicillin has been adapted by FDA to cephradine as a batching assay. It is not stability indicating. Strongly alkaline reaction conditions and ferric nitrate were used[5]. When ferric ammonium sulfate was used at pH 7 only 15% of the response normal for penicillins was obtained[37].

A colorimetric method using 5,5'-dithiobis(2-nitrobenzoic acid) at pH 9.2 produces a yellow color which can be quantitated by measuring the peak absorbance at 412 nm[38].

6.7 Chromatographic Analysis

6.71 Paper

Cephalexin (slower moving component) can be separated from cephradine with a n-butanol-t-amyl alcohol-water(7:1:4)system and quantitated by bioautography using Streptococcus aureus 209P as the assay organism[29]. Dihydrophenlyglycine(faster moving component) can be separated from cephradine with a tert.-amyl alcohol-sec.-butanol-water(4:4:1) system and quantitated with a ninhydrin-copper complex[40].

6.72 Thin-Layer

There are two TLC methods by which cephalexin and other impurities can be separated from cephradine and both cephalexin and cephradine can be quantitated. Both methods give comparable results. In the first method[40], silica gel plates are impregnated with silicone fluid and developed for 2-1/2 hours in a pH 4.1 McIlvaine buffer and acetone(100:1.5). The zones are located with U.V. light, eluted and quantitated spectrophotometrically at 260 nm. The separation scheme of the known components is as follows:

	At 22°C	
Compound	Rf	Cephradine
Dihydrocephradine	0.31	0.72
Cephradine	0.43	1.00
Cephalexin	0.51	1.20
7-ADCA	0.65	1.51
7-ACA	0.65	1.51
Dihydrophenylglycine	0.74	1.72
Phenylglycine	0.80	1.88

To determine residual 7-ADCA quantitatively, it is advantageous to change to a solvent system of pH 6.5 McIlvaines buffer-acetone(50:1). The 7-ADCA zone is eluted with 0.24 M sodium bicarbonate and the absorbance at

260 nm is measured[41].

The second method[42], a modification of the first is preferred because of greater ease of performance. The plates are impregnated with tetradecane instead of silicone and since this interferes with the U.V. absorbance, quantitation is carried out with ninhydrin.

This method can also be used to determine dihydrophenlyglycine and 7-ADCA semi-quantitatively. Approximate Rf values are as follows:

Cephradine	0.2
Cephalexin	0.3
7-ADCA	0.6
Dihydrophenyl-glycine	0.7

6.73 Column Chromatography

High pressure liquid chromotography (HPLC) has been used to quantitate cephradine and residual cephalexin in cephradine. The mobile phase is a pH 4.3 glacial acetic-anhydrous sodium sulfate system, a Dupont strong cation exchange resin, at ambient temperature and a pressure of 1000 psig. Sulfamethazine is used as internal standard and the order of elution is sulfamethazine, cephalexin and cephradine[43]. A modification, uses an acetate-0.17M sodium sulfate buffer pH 4.7, Dupont Zipax cation exchange resin, n-(4-methoxy-methyl-6-methyl-2-pyrimidinyl sulfanilamide as internal standard and a pressure of 300 psig. The order of elution is cephalexin, cephradine, internal standard. 7-ADCA, when present, will appear in the void volume[44]. When adjusting the pH of the mobile phase to 3.70 and raising the pressure to 1800 psig, the system was able to separate cephradine (d-isomer) from the synthetically prepared l-isomer which peaks between cephalexin and cephradine. No l-isomer was

detected in regular cephradine samples[45]. A reverse phase system, useful for quantitation in formulation has also been described[49]. A 1 mm x 2.1 mm i.d. column, ODS-Sil-X-II packing and 7% methanol, 93% 0.05M ammonium carbonate as mobile phase were used. Pressure, 1000 psig; flow 0.6 ml/min; detector UV (254 nm); sensitivity, 0.08 AUFS.

7. Determination in Body Fluids and Tissues

Cephradine has been determined microbiologically (see section 6.2) in human serum, in human urine, human lung tissue, human eye tissue and in spinal fluid.

It has been determined fluorometrically (see section 6.5) in dog serum.

8. Determination in Pharmaceutical Preparation

In pharmaceutical preparations (capsules, oral suspensions and injectables) infrared has been used for identity tests, the hydroxylamine and iodometric assay for batching, the microbiological and cephalosporinase-iodometric assays for stability and chromatography for detection of impurities.

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CHLOROQUINE PHOSPHATE

Donald D. Hong

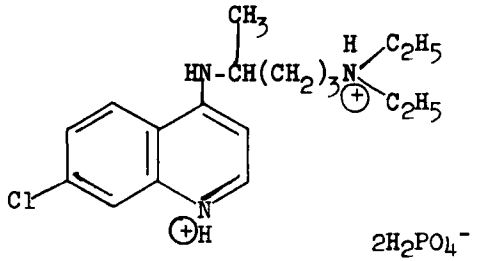
CONTENTS

Analytical Profile - Chloroquine Phosphate

1. Description

1.1 Name, Formula, Molecular Weight

The chemical name of chloroquine phosphate in Chemical Abstracts is found under the heading Quinoline and designated as 7-Chloro-[4-(4-diethylamino-1-methylbutylamino)]quinoline diphosphate. The hydrochloride and sulfate salts are also available.



$C_{18}H_{26}ClN_3 \cdot 2H_3PO_4$ Molecular Weight: 515.87

1.2 Appearance, Color, Odor

Chloroquine phosphate is a white, odorless, crystalline powder having a bitter taste; it discolors gradually on exposure to light.

2. Physical Properties

2.1 Ultraviolet Spectrum

A 10 γ/ml solution of chloroquine phosphate in 0.01 N HCl when scanned between 360 and 210 nm exhibits three maxima, three minima and several shoulders in the region from 270 to 225 nm, as shown in Figure 1. The maxima are located at 343 nm (a = 36.1), 328 nm (a = 32.6) and 222 nm (a = 59.9). The ratio of A_{343}/A_{328} is 1.11. Minima were observed at 335 nm, 280 nm and 243 nm.

2.2 Fluorescence Spectrum

Figure 2 shows the fluorescence spectrum of chloroquine phosphate obtained on a solution of 0.2 mg/ml pH 7.9 phosphate buffer using an Aminco-Bowman spectrophotofluorometer. Excitation at either 320 nm or 370 nm produced emission spectra with a maximum at 400 nm, the

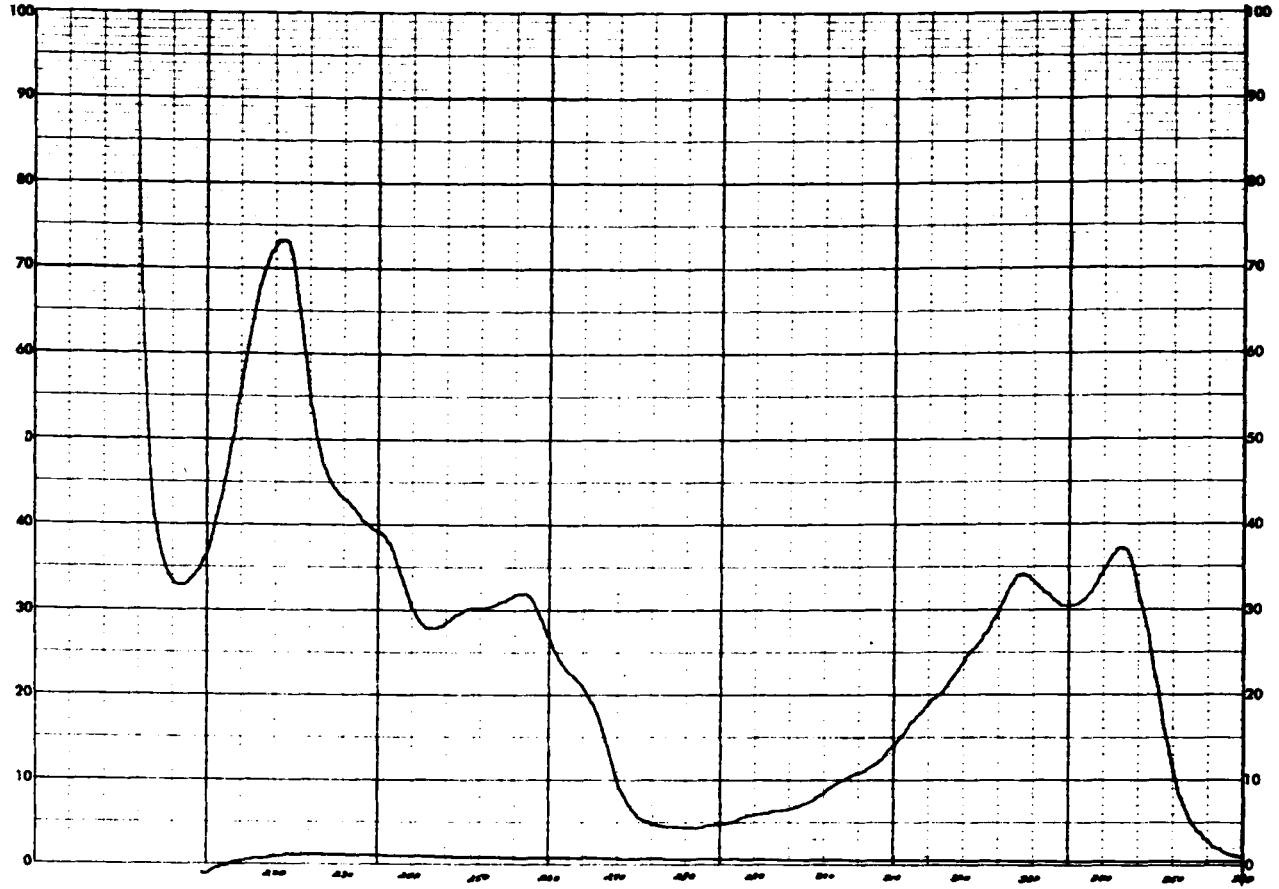


Figure 1. Ultraviolet Spectrum of Chloroquine Phosphate

Fluorescence

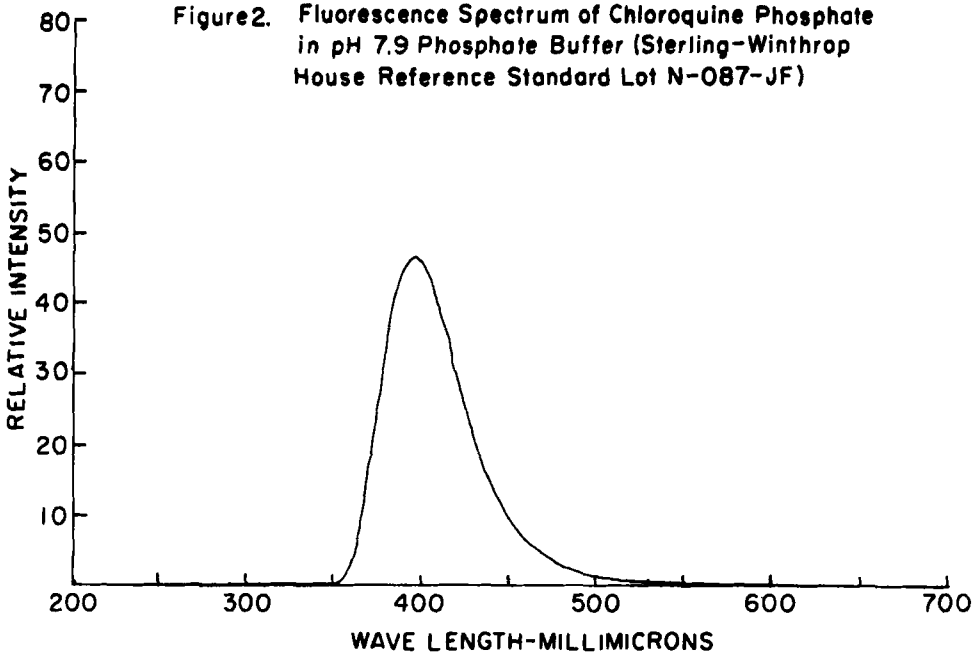
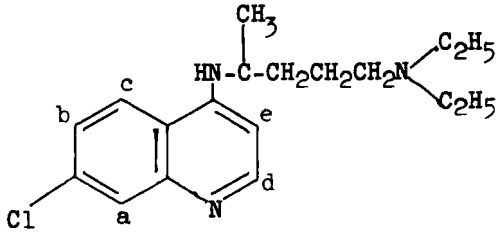


Figure 2. Fluorescence Spectrum of Chloroquine Phosphate in pH 7.9 Phosphate Buffer (Sterling-Winthrop House Reference Standard Lot N-087-JF)

latter excitation wavelength providing a higher emission response.

2.3 Nuclear Magnetic Resonance Spectrum (NMR)

The spectrum in Figure 3 was obtained with a Varian A60 NMR Spectrometer using a 20% solution in D_2O containing TMS as an external standard. The spectral assignments are summarized below (1).



Protons	No. Protons Derived from Integration	σ Chemical Shift	Multiplicity
CH_3-CH_2	6	1.68-1.80	doublet
CH_3-CH	3	1.90-1.98	doublet
CH_2-CH_2	4	2.35	broad singlet
CH_2-N	6	3.55-3.91	quintet
CH-N	1	4.38-4.65	broad singlet
NH	exchanged	5.39	sharp singlet
e	1	7.15-7.28	doublet
b	1	7.58-7.75	multiplet
a	1	7.75	singlet
c	1	8.33-8.40	doublet
d	1	8.48-8.55	doublet

2.4 Mass Spectrum

The mass spectrum is shown in Figure 4 and was obtained using a Joel JMS-01SC mass spectrometer with an ionizing energy of 75 eV. The highest mass observed at m/e 319 is a thermal breakdown product where two phosphoric acid moieties were lost from the parent compound. The base peak at m/e 86 is due to the N,N,N-diethylmethylene fragment (1).

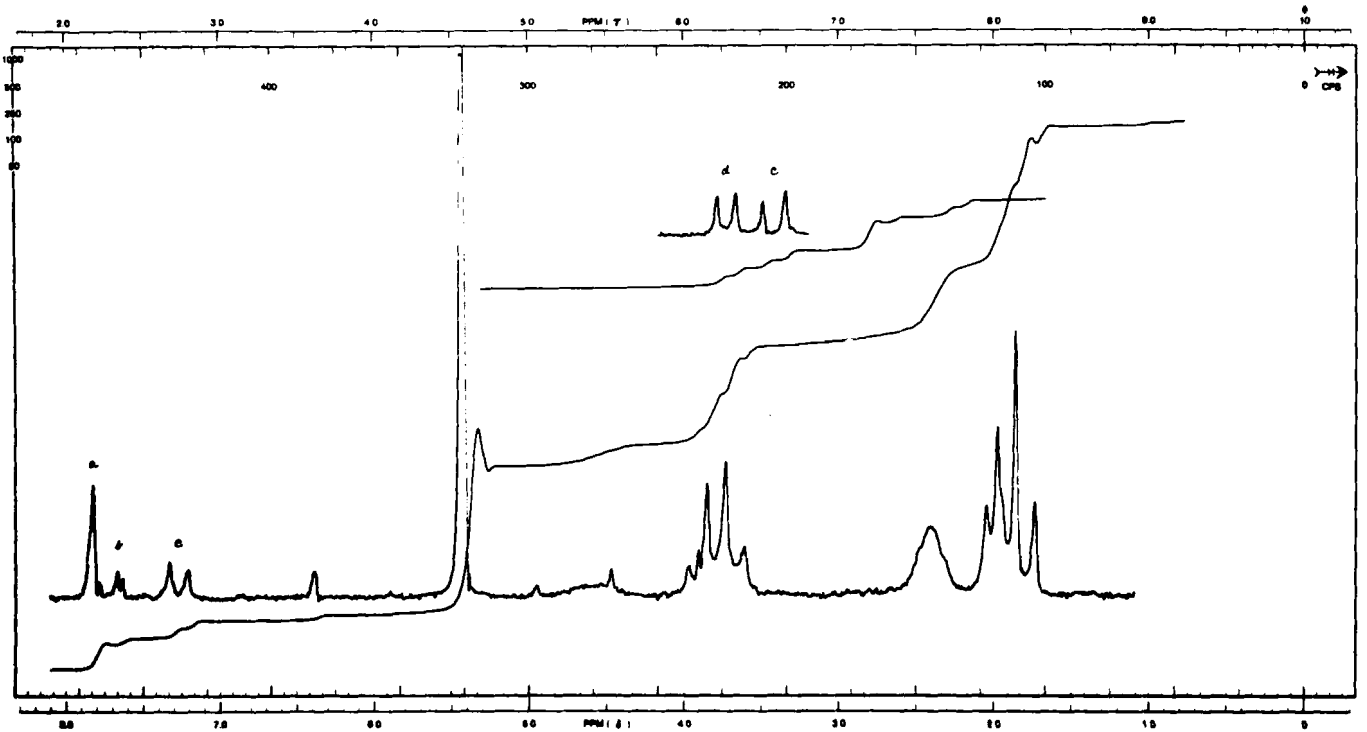


Figure 3. NMR Spectrum of Chloroquine Phosphate. Instrument: Varian A60

Intensity

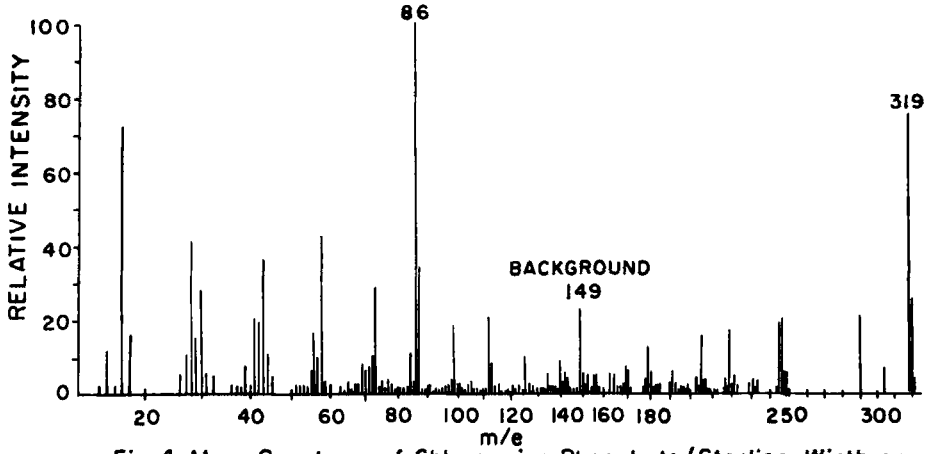
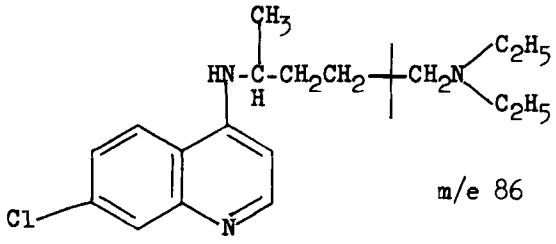


Fig. 4 Mass Spectrum of Chloroquine Phosphate (Sterling-Winthrop House Reference Standard Lot N-087-JF)



2.5 Optical Rotation

Chloroquine phosphate exhibits essentially no optical activity, existing as a racemic mixture. Riegel and Sherwood (2) have shown that neither of the optically active enantiomorphs showed any significant differences in antimalarial activity in birds and for toxicity in dogs.

2.6 Polymorphism and Melting Range

Chloroquine phosphate exists in two polymorphic forms giving rise to two melting ranges. USP XIX (3) reports one melting between 193° and 195° and the other between 210° and 215°. Mixtures of the forms melt between 193° and 215°. It is possible to obtain one form selectively by regulating the rate of crystallization (4).

2.7 Dissociation Constant

The pKa's for chloroquine phosphate by the titrimetric method were found to be 8.10 and 9.94 (5).

2.8 pH

A 1% aqueous solution has a pH of about 4.2.

2.9 Freezing Point Depression

Cryoscopic measurements were made on 10% and 20% (w/v) solutions of the drug.

	Freezing Point Depression		Calculated
	10%	20%	Isotonic
Chloroquine phosphate	0.73°	1.343°	7.0%

The value for "calculated isotonic" solution was obtained by graphic interpolation to FPD of 0.550°, representing 0.9% sodium chloride solution (5).

2.10 Solubility

Chloroquine phosphate is freely soluble in water; practically insoluble in alcohol, in chloroform and in ether (3).

2.11 Differential Scanning Calorimetry (DSC)

Two polymorphic forms of chloroquine phosphate are exhibited by DSC. A mixture of the two crystal forms may be demonstrated also by the transition temperatures (6). The DSC thermogram of a chloroquine phosphate standard shown in Figure 5 was obtained on a Perkin-Elmer DSC-1B differential scanning calorimeter at a heating rate of 10°C per minute under nitrogen. This is an example of the low melting form. Figure 6 shows another sample of chloroquine phosphate containing a mixture of the low and high melting forms.

Both the low and high melting polymorphs may be obtained from the same aqueous solution of chloroquine phosphate by selective crystallization. The high melting form usually occurs as small crystals while the low melting polymorph crystallizes as significantly larger crystals. The two forms exhibit slight differences in their IR curves from a KBr matrix.

3. Synthesis

Elderfield (7) and Kenyon is collaboration with Wiesner and Kwartler (8) and more recently Basu, et al (9) have summarized the American effort to develop an antimalarial drug necessitated by World War II. This need was compounded when Japan seized control of the East Indies, effectively cutting off the natural sources of quinine, which was the drug of choice for malaria at the time. Chloroquine was one of the fruits of this concerted effort.

The synthesis of chloroquine was first reported by the German chemists Andersag, Breitner and Jung (10, 11). Since the patent literature lacked the detail information required to prepare the necessary intermediates, a research program was started at Winthrop Chemical Company resulting in a method of synthesis for chloroquine by Surrey and

Figure 5. DSC Thermogram of Chloroquine Phosphate (Sterling-Winthrop House Reference Standard Lot N-087-JF) Low Melting Form

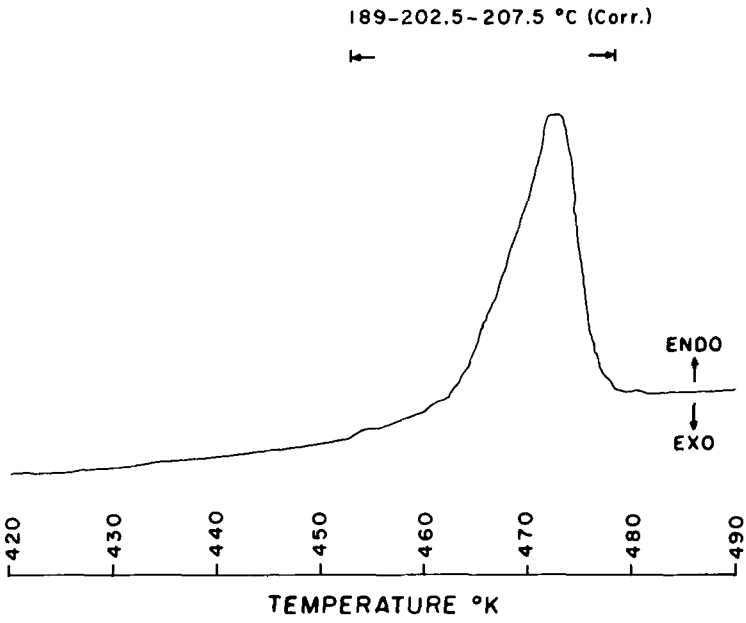
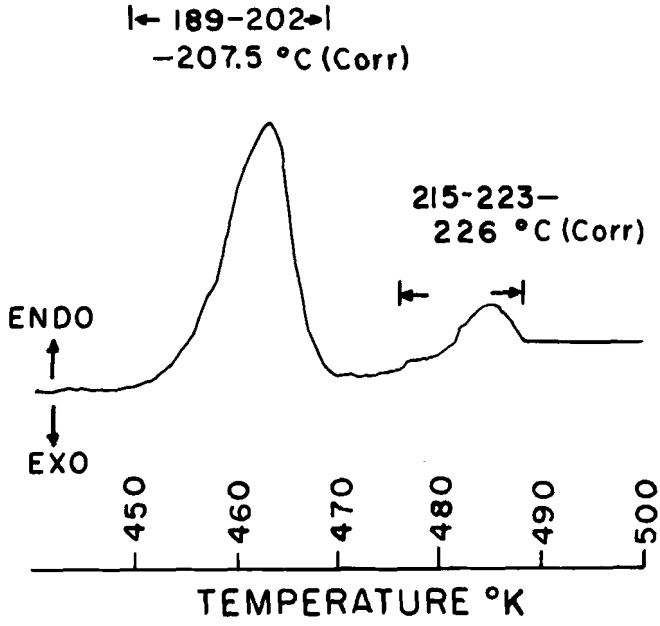


Figure 6 DSC Thermogram of Chloroquine Phosphate (Lot An-K-67) Mixture of Low and High Melting Forms



Hammer (12).

The two key intermediates required in the synthesis are 4,7-dichloroquinoline and 4-diethylamino-1-methylbutylamine ("novol diamine"). The synthetic scheme is shown in Figure 7.

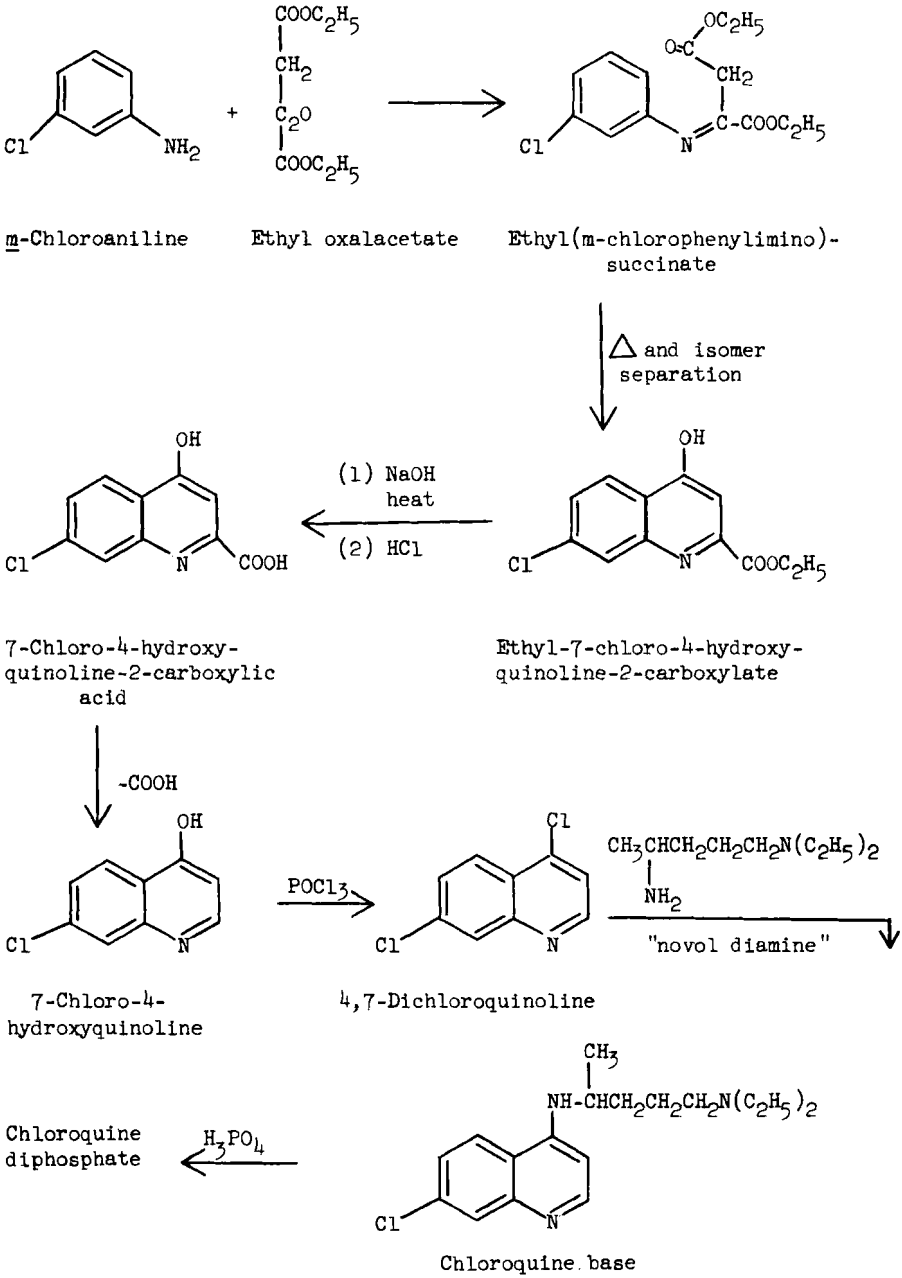
4. Drug Metabolic Products

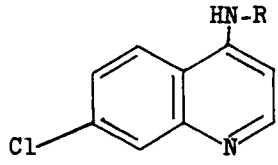
4.1 Biotransformation Products

Several metabolites in addition to the unchanged drug have been isolated from human tissues and urine. Titus, et al (13) used counter-current distribution to isolate the desethyl compound (A) from the urine of human volunteers. Kuroda (14) isolated four metabolites of chloroquine from human tissues using a combination of paper chromatography and UV spectroscopy. In addition to the desethyl compound (A) of Titus, they were the bisdesethyl (B), the carbinol (C) and the 4-amino-7-chloroquinoline (D) derivatives.

Similar observations were seen from urine of human subjects but no trace of the 4-hydroxy-7-chloroquinoline was found. The unchanged drug was always found to be the major compound. McChesney, et al (15, 16) using a fluorescence technique confirmed the above findings and in addition found traces of the 4'-aldehyde (E) and the 4'-carboxy (F) derivatives. They listed the amount of determinable excretory products as 70% chloroquine, 23% desethylchloroquine, 1-2% bisdesethylchloroquine and the others as trace degradation products. In addition they reported that about one-third of the administered chloroquine was unaccounted and remains obscure in this very complex biotransformation of the drug.

Figure 7: Synthesis of Chloroquine Phosphate





Compound	R
Chloroquine	$-CH(CH_3)CH_2CH_2CH_2N(C_2H_5)_2$
A	$-CH(CH_3)CH_2CH_2CH_2NHC_2H_5$
B	$-CH(CH_3)CH_2CH_2CH_2NH_2$
C	$-CH(CH_3)CH_2CH_2CH_2OH$
D	H
E	$-CH(CH_3)CH_2CH_2CHO$
F	$-CH(CH_3)CH_2CH_2COOH$

4.2 Distribution in Human Tissues

Chloroquine is predominantly localized in the liver and to lesser degrees in the spleen, heart, kidney, lung, brain, leucocytes and skin. Chloroquine was originally used to treat malaria. Subsequently it was found to be effective against other parasitic disorders. According to Rubin, et al (17) traces of the drug and its metabolites were found in the blood and urine of subjects up to five years after discontinuing long-term therapy.

5. Methods of Analysis

5.1 Phase Solubility Analysis (PSA)

PSA is probably not promising for chloroquine phosphate because the multiple pKa's would allow substantial disproportionation (18).

5.2 Identification of Chloroquine Phosphate by Spot Tests

In the approximate period of two decades following World War II where there was increasing use of chloroquine phosphate for mass prophylaxis of malaria it became necessary to have a relatively simple method to insure that the drug was taken regularly. Tests of this type usually measure the drug in urine. Other tests utilize various reagents to react either with the pure drug or the drug in dosage form. Some of these tests are summarized in Table 1.

5.3 Non-Aqueous Titration

Chloroquine phosphate can be titrated with acetous 0.1 N perchloric acid. The titration may be carried out manually with crystal violet as indicator or determined potentiometrically. The titration is rapid although non-selective. This non-specificity is no drawback, however, so long as good identification tests are also adopted (30). Wu, et al (31) have reported the determination of eleven antimalarial drugs using this titrimetric procedure.

Each ml of 0.1 N $HClO_4$ is equivalent to 25.79 mg of chloroquine phosphate.

5.4 Spectrophotometric Assay

The UV spectra of chloroquine base and phosphate salt are similar in 0.01 N HCl. Absorption maxima are observed at 343, 328, 256 and 222 nm. Measurements are most favorably made at 343 nm where absorption is most intense and least affected by interfering substances in the biological sample.

The chloroquine base is obtained by ether or chloroform extraction of an alkaline homogenate of the biological sample. After separation of interfering

Table 1 - Spot Tests

Test	Form	Color	Sensitivity	Ref.
1. Complex with Copper	Tablet	Pale green	NA	19
2. Complex with Cobalt	Tablet	Violet	NA	19
3. Dimethylaminobenz-aldehyde (Ehrlich's reagent)	Tablet	Yellow	NA	20
4. Styphnic acid	Pure drug	Rosettes of plates	0.5γ	21
5. Nitroprusside and Piperazine (Lewin's reagent)*	Pure drug	NA	50γ	22
6. Eosin yellowish (Dill & Glazko)	Urine	Yellow to violet-red	NA	23
7. Mercuric iodide/KI (Mayer-Tanret's reagent)	Urine	**	2.5-9.5γ per ml urine	24
8. Methyl orange	Urine	Yellow	2 mg/liter	25
9. Complex with $HClO_4/AuCl_3$	Pure drug or biol. extract	Rosettes and dendrites	0.4γ	26
10. Aconitic acid/ acetic anhydride/ ethylene dichloride	Pure drug	Red	5γ	27
11. $H_2SO_4/KClO_3$	Biol. extract	Red-violet	5γ	28
12. $HCl/KClO_3$	Biol. extract	Yellow	10γ	28
13. 25% H_2SO_4/Chlorinated lime	Biol. extract	Yellow	10γ	28
14. BPB/boric acid	Free base	Blue-violet to blue-green	0.8 mg%	29

* Specific for N-ethyl group
** Turbidity is measured

materials, the base is in turn extracted into a solution of 0.1 or 0.01 N HCl and quantitatively determined by measuring its UV absorption. Alternatively the acid solution can be made alkaline and the base extracted with ether or chloroform. The organic phase is evaporated to dryness and the residue further examined by IR and paper or TLC.

A brief summary of the spectrophotometric procedures for the quantitative determination of chloroquine and metabolites is summarized in Table 2.

Table 2 - Spectrophotometric Methods

Method of Analysis	Isolation from Human	Reference
UV	Tissues	32
UV	Blood	33
UV, IR, TLC, GLC	Tissues	34
UV, IR, PC	Tissues*	14
UV	Urine	35

* Includes identification of chloroquine metabolites also

5.5 Fluorometric Analysis

Fluorometric procedures have been extensively utilized for the quantitative determination of chloroquine in biological materials. In the early days of fluorescence when instrumentation was not sufficiently advanced, an additional irradiation step was required to convert the chloroquine to a more intense fluorophore (36). With the advent during the mid 1950's of the highly sensitive spectrophotofluorometers utilizing the xenon arc source and monochromators, it is now possible to measure the chloroquine fluorescence directly (15, 16, 37, 38).

Brodie, et al (36) found that the fluorescence of chloroquine in pH 9.5 borate buffer after isolation from biological specimen may be stabilized by the addition of cysteine. The sample is then irradiated with UV light and the measurement made using a suitable fluorometer. The sensitivity of the procedure is about 0.1 mcg.

5.6 Gravimetric Analysis

Parikh and Mukherji (39) reported the quantitative formation of chloroquine-silicatungstate [SiO_2-

$12WO_3 \cdot 2(C_{18}H_{26}N_3Cl) \cdot 2H_2O$] precipitate from chloroquine phosphate and silicatungstic acid. By use of the appropriate gravimetric factor the various salts of chloroquine could be determined. USP XVI (10) also contained a gravimetric method whereby the chloroquine base is measured.

5.7 Chromatographic Analysis

5.71 Paper Chromatographic Analysis

A number of paper chromatographic systems for chloroquine phosphate and its base are summarized in Table 3. Goldbaum and Kazyak (41) use iodoplatinate reagent to visualize the chloroquine which in turn may be eluted off the paper and the spot quantitated by other means.

5.72 Thin-Layer Chromatographic Analysis

The following TLC systems (Table 4) are useful as an identity test and in the evaluation of the purity of the drug substance. The nature of the impurities present is also helpful in that it tells indirectly, for example, similarity or dissimilarity of the manufacturing process.

All of the systems utilize precoated silica gel containing a fluorescent indicator.

5.73 Gas-Liquid Chromatographic Analysis (GLC)

The following procedures have been demonstrated to be applicable to the GLC examination of chloroquine base. Vanden Heuvel, et al (46) Viala, et al (47) and Kazyak and Knoblock (48) have reported the detection of the drug in microgram amounts from biological samples after solvent extraction. Holtzman (49) has shown that as little as 5 nanogram quantities of chloroquine base could be detected using electron capture. The conditions reported, however, are for pure drug and may be of potential value in the analysis of the substance in biological materials.

The following conditions have been used for the GLC determination of chloroquine base (50).

Column: 3.8% Silicone Gum SE 30, 4 ft, glass
Support: Diatoport S
Detection: FID

Table 3 - Paper Chromatographic Systems

	Solvent System	Species	Paper	Detection	R_f	Reference
1.	n-Butanol sat'd with buffer	base	Whatman No. 2 sat'd with pH 3.0 MacIlvaine's buffer	UV	0.15	41
2.	n-Butanol sat'd with buffer	base	Whatman No. 2 sat'd with pH 5.0 MacIlvaine's buffer	UV	0.16	41
3.	n-Butanol sat'd with buffer	base	Whatman No. 2 sat'd with pH 6.5 Sorensen's buffer	UV	0.26	41
4.	n-Butanol sat'd with buffer	base	Whatman No. 2 sat'd with pH 7.5 Sorensen's buffer	UV	0.89	41
5.	Ethanol-Water-Conc ammonia (35-63-2)	base	Whatman No. 1 sat'd with petroleum (195-220° fraction)	UV	0.36	42
6.	As No. 5 (45-53-2)	base	as above	UV	0.60	42
7.	As No. 5 (55-43-2)	base	as above	UV	0.79	42
8.	As No. 5 (65-33-2)	base	as above	UV	0.87	42
9.	As No. 5 (75-23-2)	base	as above	UV	0.88	42
10.	As No. 5 (85-13-2)	base	as above	UV	0.89	42
11.	As No. 5 (95-3-2)	base	as above	UV	0.89	42

Table 4 - TLC Systems

System	Spotting Soln	Rf x 100	Detection	Reference
Methanol-water-conc ammonia (72:25:3)	Chloroform[1]	28	A, B	43
Benzene-methanol-isopropylamine (87:10:3)	Chloroform[1]	41	A	43
Chloroform-methanol-isopropylamine (94:3:3)	MeOH-H_2O (7:3)	40	A	43
Chloroform-isopropylamine (97:3)	Chloroform[2]	15	A	43
Ether-hexane-isopropylamine (90:7:3)	H_2O/MeOH/$CHCl_3$/ isopropylamine	28	A, C	43
n-Butanol-conc ammonia-water (85:4:11)	Water	59	A, D, E	43
Ethylacetate-conc ammonia-abs. alcohol (5:2:2)	Water	60	A, C, D	44
25% ammonia-benzene-dioxane-ethanol (1:10:8:1)	Water	28	A, C, D	44
Chloroform-cyclohexane-diethylamine (5:4:1)	Water	40	A, C, D	44
25% ammonia-methanol (3:200)	Water	20	A, C, D	44
Acetone-water-ammonia (90:40:1)	abs. alcohol[3]	15	-	45

Spotting solution

1. The drug is extracted into chloroform after basifying with 10% Na_2CO_3.
2. Similar to above but from human plasma.
3. Spotted as the base.

Detection

A - UV-254
B - Iodine vapor/20% H_2SO_4
C - Dragendorff's reagent
D - UV-360
E - Iodoplatinate reagent

Temperature:

Inj. port	275 °C
Column	240 °C
Detector	250 °C

Flow Rate: 30 ml/min helium
Retention Time: 7.0 min

5.8 Miscellaneous Methods of Analysis

Roushdi and Shafik (51) reported the determination of chloroquine phosphate by titration with an anionic surfactant, dioctyl sodium sulfosuccinate (Aerosol O.T.), using dimethyl yellow as indicator.

A complexometric method using bismuth complexonate to precipitate the chloroquine base and titrating the liberated EDTA with zinc sulfate was also reported by these authors (52).

Various quinine salts and chloroquine phosphate have been determined using ammonium reinckate (53). For chloroquine phosphate the insoluble reinckate salt is formed at pH 1, removed, and the amount of excess reagent in the filtrate is measured colorimetrically. The difference in absorbance between the sample and blank and the standard and blank represent the absorbances of the sample and standard solutions, respectively.

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DAPSONE

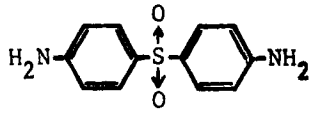
Chester E. Orzech, Norris G. Nash, and Raymond D. Daley

CONTENTS

1. DESCRIPTION

1.1 Name, Formula, Molecular Weight

Dapsone is 4,4'-diaminodiphenyl sulfone, also known as p,p'-sulfonyldianiline and bis(4-aminophenyl) sulfone. Chemical Abstracts indexes dapsone as benzenamine, 4,4'-sulfonylbis-, starting with Volume 76; previously the index name was aniline, 4,4'-sulfonyldi-. The CAS Registry Number is [80-08-0].



$C_{12}H_{12}N_2O_2S$ Mol. Wt.: 248.31

1.2 Appearance, Color, Odor

Dapsone is a white or creamy white odorless crystalline powder.

2. PHYSICAL PROPERTIES

2.1 Infrared Spectra

Infrared spectra of two crystal forms of dapsone (designated Form I and Form II) are shown in Figures 1 and 2. The samples were prepared as mineral oil mulls between potassium bromide plates. A Beckman Model IR-12 spectrophotometer was used. The spectrum of Form I is similar to those published by Pouchert (1) and by Hayden et al (2). The spectrum of a hydrate of dapsone has also been observed; since it is different from the spectra of the anhydrous forms, samples should be dried at 105°C before obtaining spectra. Some of the absorption bands may be assigned as follows (3):

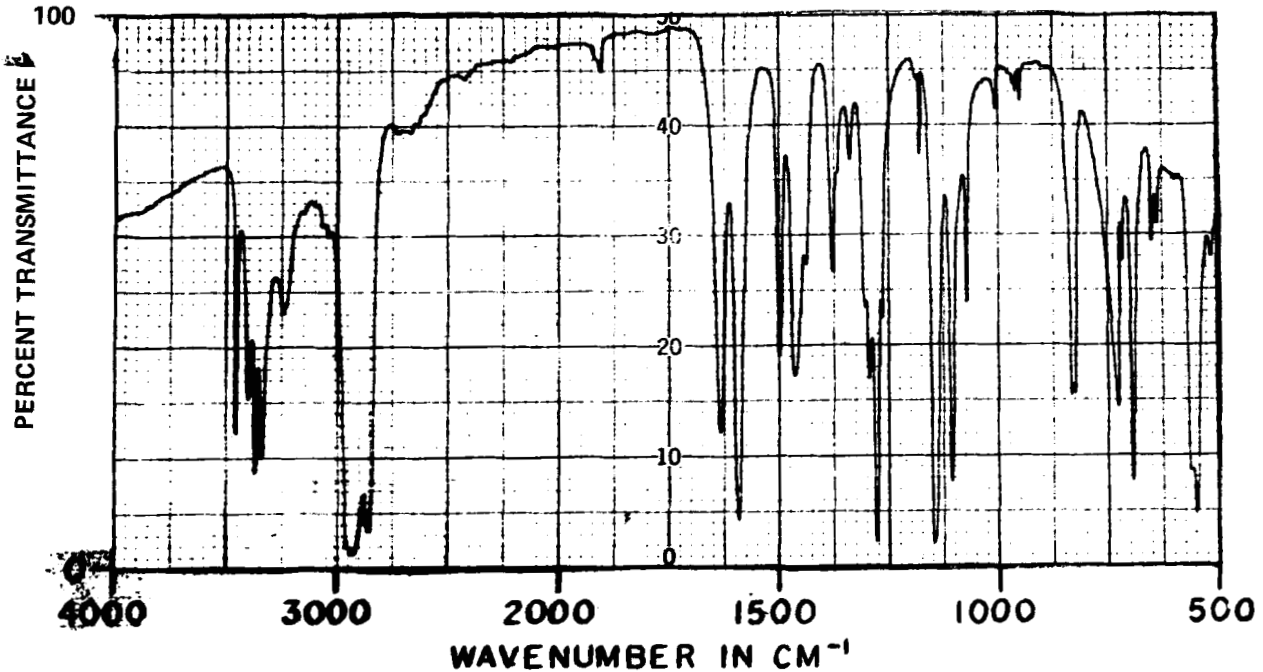


Figure 1. Infrared Spectrum of Dapsone (Form I), Mineral Oil Mull

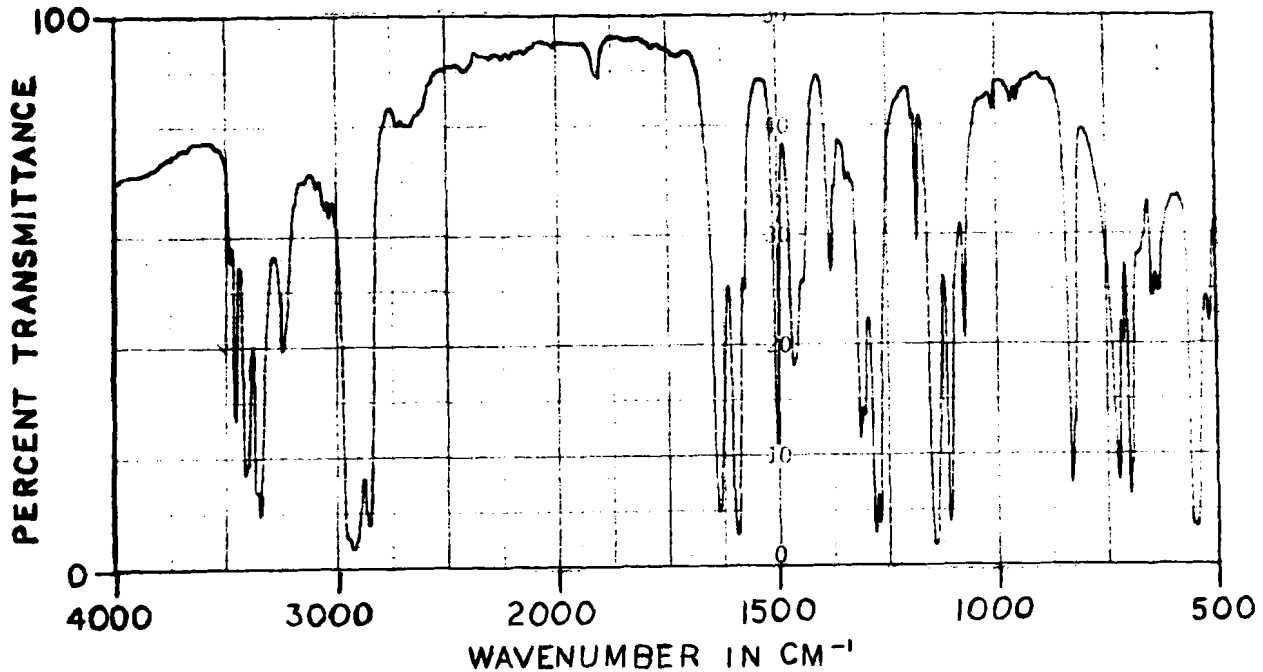


Figure 2. Infrared Spectrum of Dapsone (Form II), Mineral Oil Mull

Band frequency, cm^{-1}	Assignment
3200-3500	N-H Stretch
3000-3100	Aromatic C-H Stretch
2800-3000	Mineral Oil
1635	N-H Deformation
1590, 1500, 1440	Aromatic C=C Stretch
1460, 1380	Mineral Oil
1300 Region	Asymmetric $-SO_2-$ Stretch
1150	Symmetric $-SO_2-$ Stretch
830-840	2 Adjacent H on aromatic ring
550	$-SO_2-$ Scissoring

2.2 Nuclear Magnetic Resonance Spectrum

The NMR spectrum shown in Figure 3 was obtained by dissolving USP reference standard dapsone in acetone-d_6 containing tetramethylsilane as internal reference. The spectrum was produced using a Varian EM-360 NMR spectrometer. The series of peaks centered at 7.2 ppm is an A_2B_2 pattern that is typical of para substitution and the broad singlet at 5.4 ppm is due to the amine protons. The peaks at 2 ppm and 3 ppm are due to the solvent. These values correlate well with those previously reported (4).

2.3 Ultraviolet Spectra

Figure 4 is the ultraviolet absorption spectrum of dapsone in methanol solution, run on a Cary Model 14 spectrophotometer. The solution contained 8.0 mg of dapsone per liter of methanol, and was run against methanol (1 cm cells). The spectrum exhibits peaks at 295 nm and 260 nm with absorptivities of 30,100 and 18,300 l./mole cm respectively. The ultraviolet identity test of the British Pharmacopoeia (5) specifies peaks at

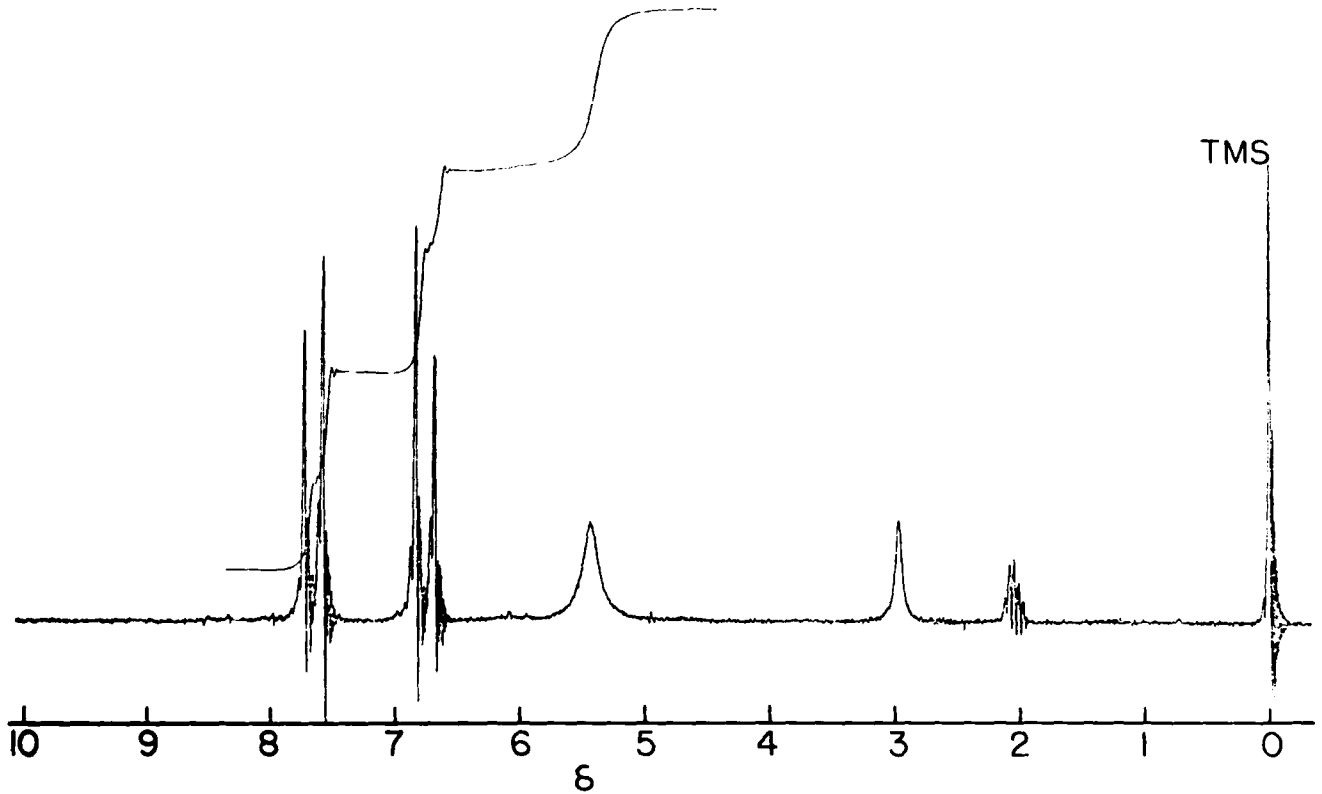


Figure 3. Nuclear Magnetic Resonance Spectrum of Dapsone

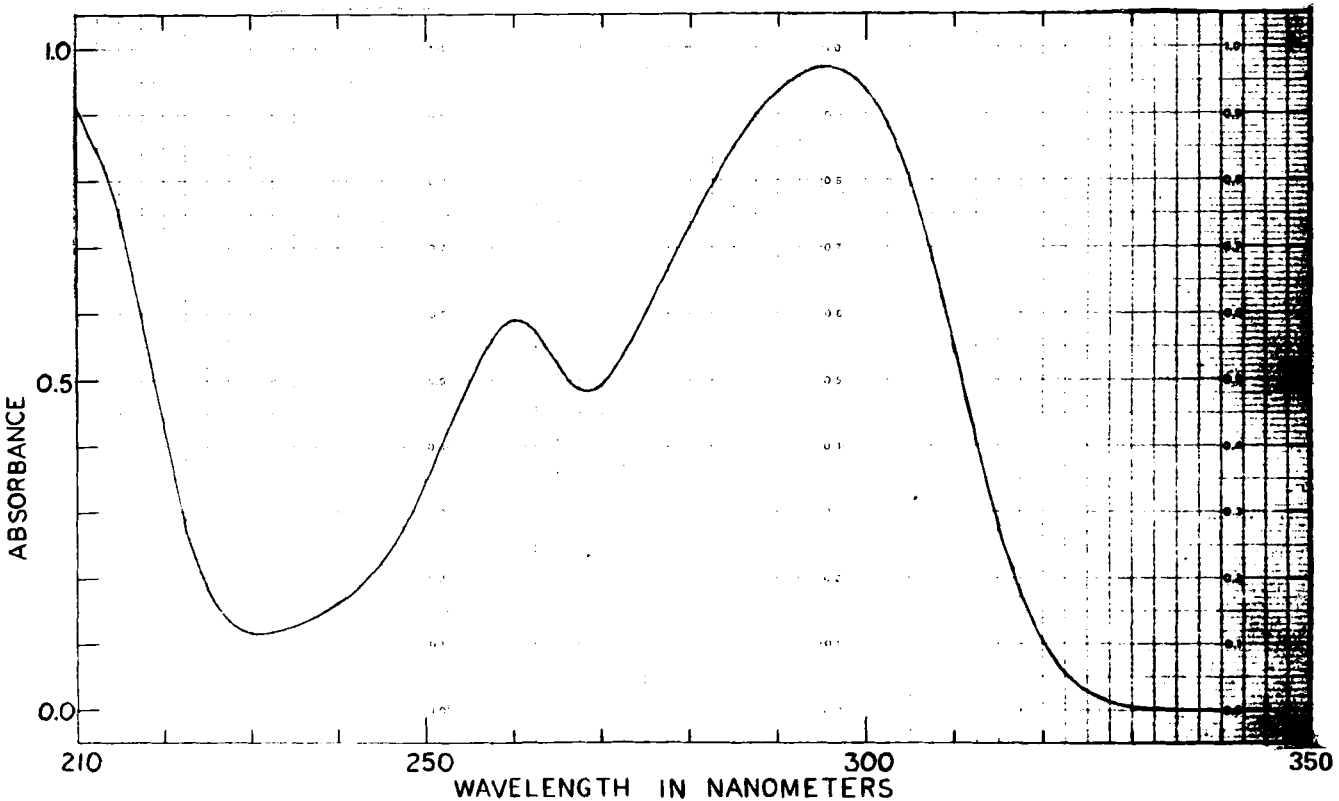


Figure 4. Ultraviolet Spectrum of Dapsone, 8 mg/l., in Methanol, 1 cm Cells

295 and 260 nm with absorptivities of about 30,000 and 18,100 l./mole cm respectively.

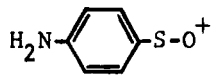
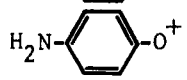
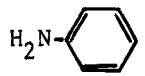
A similar spectrum is obtained in 95% ethanol solution, except that the absorptivities are apparently lower. Baliah and Ramakrishnan (6) report peaks at 295 and 261 nm with absorptivities of 27,000 and 17,800 l./mole cm. Hayden et al (2) report peaks at 295 and 260 nm.

Maschka et al (7) reported ultraviolet spectra of dapsone at various hydrogen ion concentrations; the data are as follows:

Condition	Wavelength of Maxima, nm	Molar Absorptivity, l./mole cm
1. pH 11.0	291.0	19,820
	258.5	12,190
2. pH 6.8	291.0	17,950
	259.0	10,810
3. pH 1.8	289.5	13,490
4. 2N HCl	273.5	1,850
	264.5	2,000
	234.5	10,790

2.4 Mass Spectrum

Figure 5 shows the low resolution mass spectrum of dapsone. The data was obtained with an LKB 9000S mass spectrometer, with an ionization voltage of 70 eV, source temperature 250°C. Some of the peaks may be assigned as follows (8):

m/e	Assignment
248	M^+
140	
108	
92	

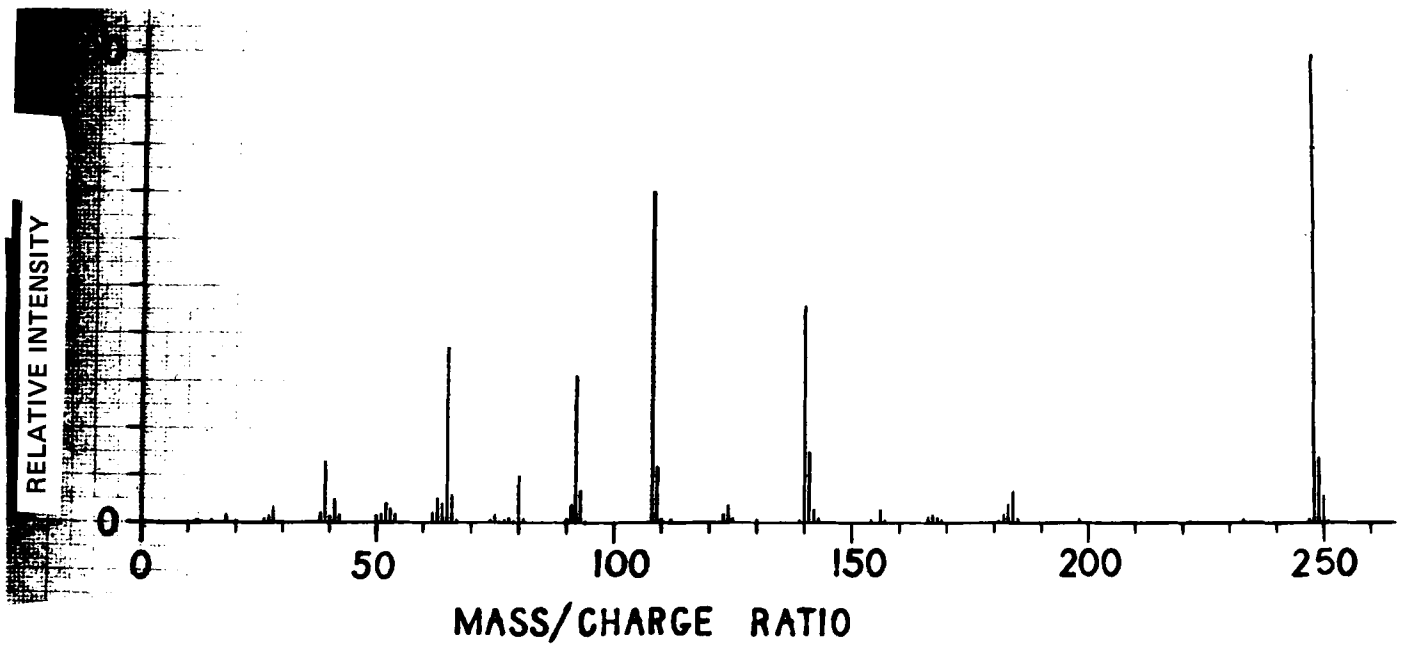


Figure 5. Mass Spectrum of Dapsone

2.5 Differential Thermal Analysis (DTA)

The DTA curve in Figure 6 was obtained with a DuPont Model 900 instrument. The curve shows a sharp endothermic solid-solid phase transition at 84°C and a melting endotherm at 178°C.

2.6 Differential Scanning Calorimetry (DSC)

The DSC melting thermogram of dapsone crystallized from methanol and water is shown in Figure 7. The thermogram was obtained with a Perkin-Elmer DSC-1B differential scanning calorimeter at a heating rate of 1.25°C/minute in a nitrogen atmosphere. The purity of samples of this compound can be determined by analysis of the melting thermogram (9).

2.7 Crystal Properties

Butt (10) reported that dapsone can be obtained in at least two crystal forms, with different melting points. Infrared spectra (see Section 2.1) and x-ray powder diffraction patterns also indicate that dapsone occurs in at least two crystal forms, and that it forms a crystalline hydrate. The powder diffraction patterns are given in Table 1. These patterns were obtained with a Norelco diffractometer, using nickel-filtered copper $K\alpha$ radiation.

The crystal and molecular structure of dapsone has been determined by Alleaume and Decap (11), and by Dickinson et al (12, 13). Both groups report 4 molecules in an orthorhombic unit cell, symmetry $P2_12_12_1$, with similar dimensions (Å):

$a=25.57 \pm 0.01$, $b=8.07 \pm 0.01$, $c=5.77 \pm 0.01$ (11)

$a=8.065 \pm 0.005$, $b=25.57 \pm 0.02$, $c=5.760 \pm 0.001$ (12)

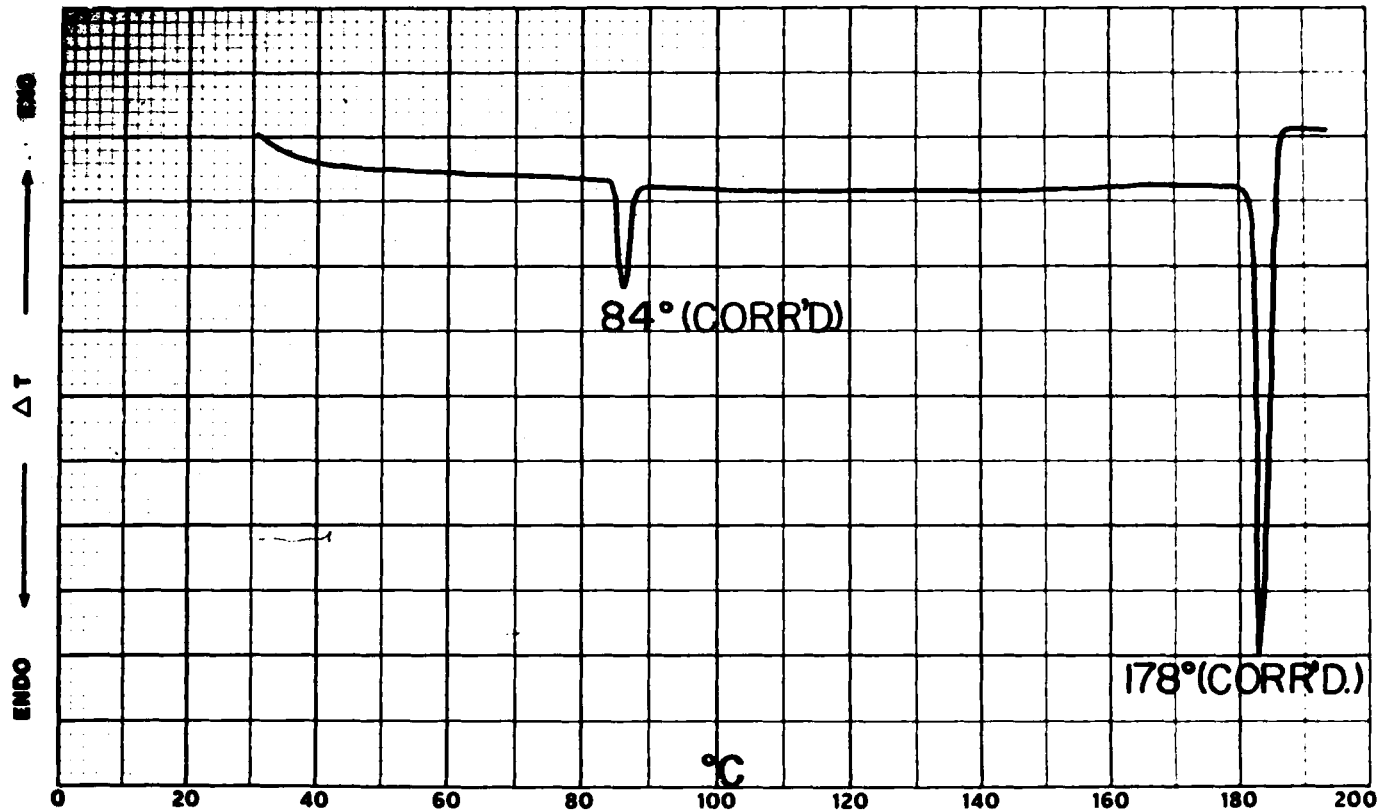


Figure 6. Differential Thermal Analysis Curve of Dapsone

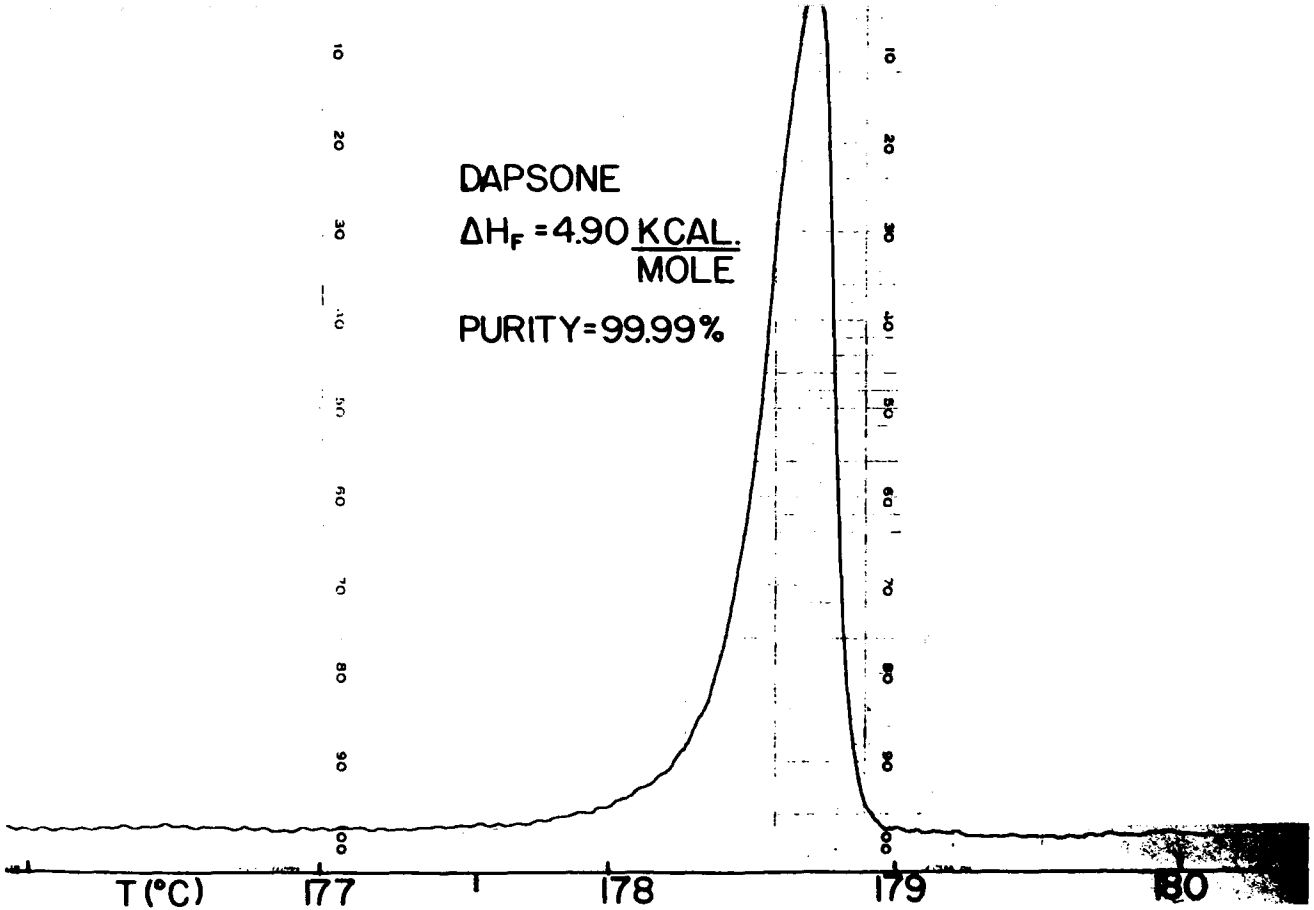


Figure 7. Differential Scanning Calorimeter Curve of Dapsone

TABLE 1
DAPSONE X-RAY POWDER DIFFRACTION PATTERNS

Form I		Form II		Hydrate	
d	I/Io	d	I/Io	d	I/Io
12.79	4	13.07	5	12.55	1
7.74	8	10.78	2	12.25	4
6.86	32	9.21	1	11.13	3
6.42	53	7.41	7	8.54	16
5.88	4	6.85	8	7.73	29
5.66	9	6.66	89	7.62	13
5.28	60	6.57	18	7.54	47
5.03	34	6.40	18	7.45	28
4.71	17	5.80	3	6.82	1
4.64	100	5.68	3	6.39	7
4.42	49	5.45	27	6.25	7
4.30	62	5.35	54	5.98	4
4.12	1	5.01	23	5.74	28
4.00	48	4.66	97	5.68	35
3.84	22	4.57	20	5.62	19
3.78	44	4.37	96	5.53	17
3.65	2	4.27	9	5.45	11
3.46	1	3.99	8	5.32	4
3.42	10	3.86	21	5.12	3
3.32	2	3.82	100	5.00	21
3.21	10	3.52	23	4.93	58
3.16	4	3.50	25	4.86	9
3.09	46	3.42	8	4.79	2
2.98	2	3.37	9	4.68	100
2.94	9	3.33	17	4.59	39
2.88	4	3.28	2	4.55	75
2.80	6	3.20	8	4.45	6
2.78	4	3.13	22	4.39	27
2.74	4	3.09	14	4.31	13
2.64	7	3.02	26	4.27	54
2.56	3	2.89	9	4.22	31
2.51	2	2.86	17	4.18	12
2.44	5	2.73	3	4.09	87
2.40	5	2.68	3	4.05	19
2.35	3	2.52	6	4.01	13
2.31	2	2.43	2	3.95	4
2.25	5	2.40	2	3.86	15
2.20	3	2.34	5	3.83	19
2.17	4	2.28	2	3.77	4
2.13	8	2.21	2	3.72	3

2.08	5	2.17	3	3.65	1
2.06	1	2.10	7	3.58	14
1.96	2			3.46	11
				3.42	9
				3.37	13
				3.35	6
				3.30	15
				3.28	8
				3.23	30
				3.20	10
				3.14	21
				3.09	14
				3.02	8
				2.96	6
				2.87	5
				2.84	3
				2.82	3
				2.79	6
				2.72	6
				2.69	5
				2.65	7
				2.62	7
				2.58	1
				2.50	3
				2.45	3
				2.42	2
				2.27	6
				2.15	5
				2.11	2
				2.10	2
				2.07	3
				1.96	3

2.8 Solubility

The solubility at room temperature is as follows:

Solvent	Approximate Solubility, mg/ml
Methanol	52
Ethanol (95%)	28
2-Propanol	6
Ethyl Acetate	34
Ethyl Ether	0.9
Chloroform	3
Benzene	0.5
Water	0.2 (14)
2,2,4-trimethylpentane	<0.2

Dapsone is very soluble in acetone and acetonitrile; one gram of dapsone dissolves in 1.8 ml of acetone or 5 ml of acetonitrile. It is also soluble in dilute mineral acids; one gram dissolves in about 10 ml of 1N hydrochloric acid.

2.9 Fluorescence Spectra

Peters et al (15) found the excitation maximum at 285 nm and the fluorescence maximum at 350 nm, in ethylene dichloride solution. Ellard and Gammon (16) reported an excitation maximum at 298 nm and the fluorescence maximum at 345 nm, in ethyl acetate. Glazko et al (73) and Cucinell et al (17) reported the excitation maximum at 297 nm and the fluorescence maximum at 340 nm, in ethyl acetate.

2.10 Melting Point

A range of melting points has been reported for dapsone:

172°C	(18)
172-173°C	(19)
172-174°C	(20)
174°C	(21)
174-176°C	(22)
175°C	(23,24)
175-176°C	(25)
176°C	(26,27)
176.3-177.5°C	(28)
178-179°C	(29)
179-180°C	(30)

Butt (10) reported that dapsone can be obtained in two forms, one melting at about 178.5°C, the other at about 180.5°C.

3. SYNTHESIS

Dapsone has been prepared by various procedures, starting with 4,4'-dinitrodiphenyl sulfide (21, 22, 31, 32) prepared from p-chloronitrobenzene (33), or by oxidation of 4,4'-diacetylaminodiphenyl sulfide (19, 23, 25) prepared from 4-nitro-4'-aminodiphenyl sulfide (19, 23, 24) or 4,4'-diaminodiphenyl sulfide (25). It has also been made from a sulfinate and a halonitrobenzene (24, 26, 29, 35); from 4-acetylaminobenzenesulfonyl chloride and acetanilide (20, 36, 37, 38); from acetanilide and thionyl chloride (27, 39, 40); from 4,4'-dichlorodiphenyl sulfone (18, 28, 30, 41, 42, 43); from the diphthaloyl derivative of 4,4'-diaminodi-

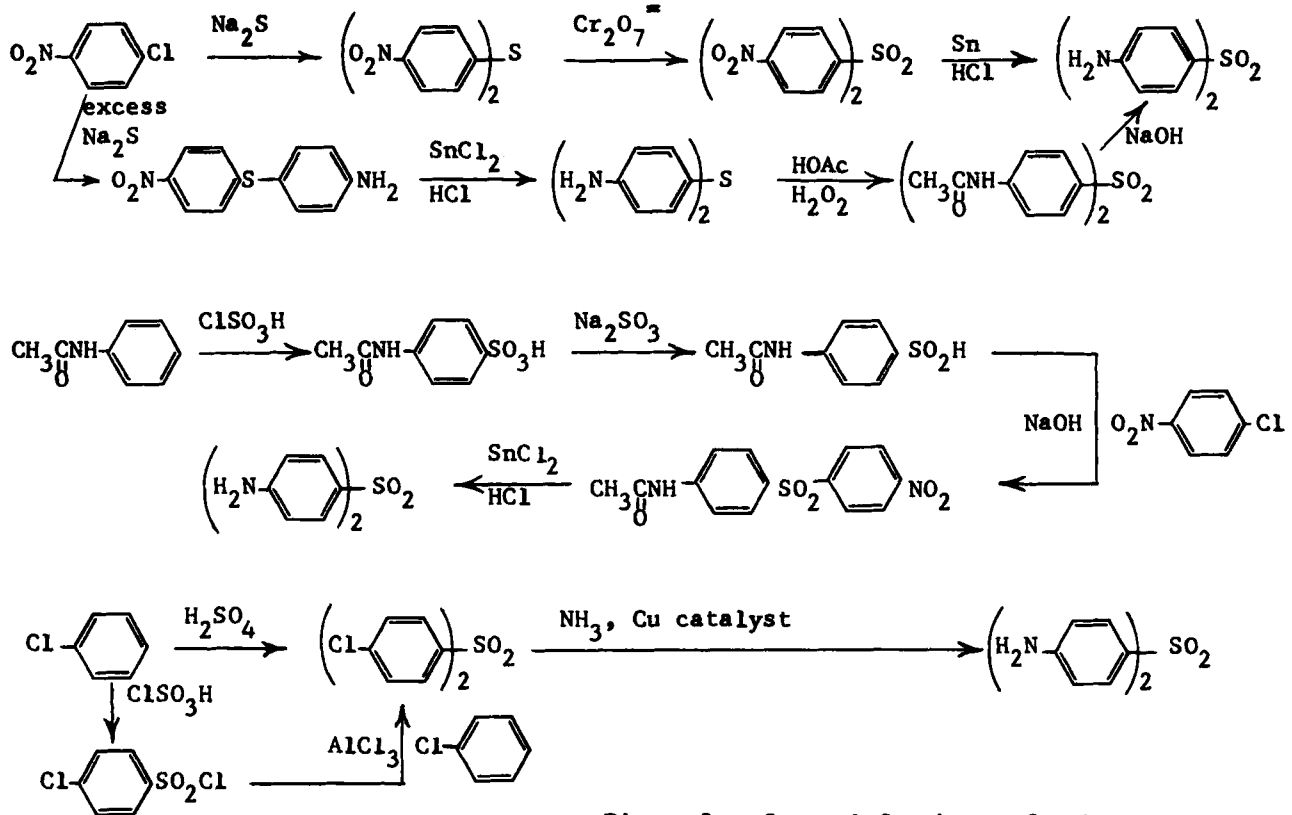


Figure 8. Several Syntheses for Dapsone

phenyl sulfide (44, 45); and from 4,4'-dimethyldiphenyl sulfone by oxidation to the dicarboxylic acid and Hofmann degradation of the diamide (46). The 4,4'-diacetylaminodiphenyl sulfone obtained by any of these methods can easily be hydrolyzed to the diamino compound (23). Sanghavi (47) has reviewed the various methods of synthesis. A few of these procedures are outlined in Figure 8.

4. STABILITY-DEGRADATION

No reports of stability studies or degradation of dapsone were found.

5. DRUG METABOLIC PRODUCTS

Dapsone metabolic products have been reported as follows:

Metabolite	Species	References
Dapsone glucuronide	Man	(48,49,50)
	Monkey	(51,52)
	Rabbit	(48,51,53, 54,55)
Dapsone sulfamate	Man	(48,50)
	Rat	(51,52)
Dapsone monohydroxylamine	Man	(56,57,58)
	Dog	(56,58)
Monoacetyl dapsone	Man	(15,51,57, 59,60,61)
	Monkey	(51,52,62)
	Rabbit	(51,61,62)
Dapsone monohydroxylamine sulfamate	Man	(58)
Dapsone monohydroxylamine glucuronide	Man	(58)
Monoacetyl dapsone sulfamate	Man	(50)
	Monkey	(51,52)

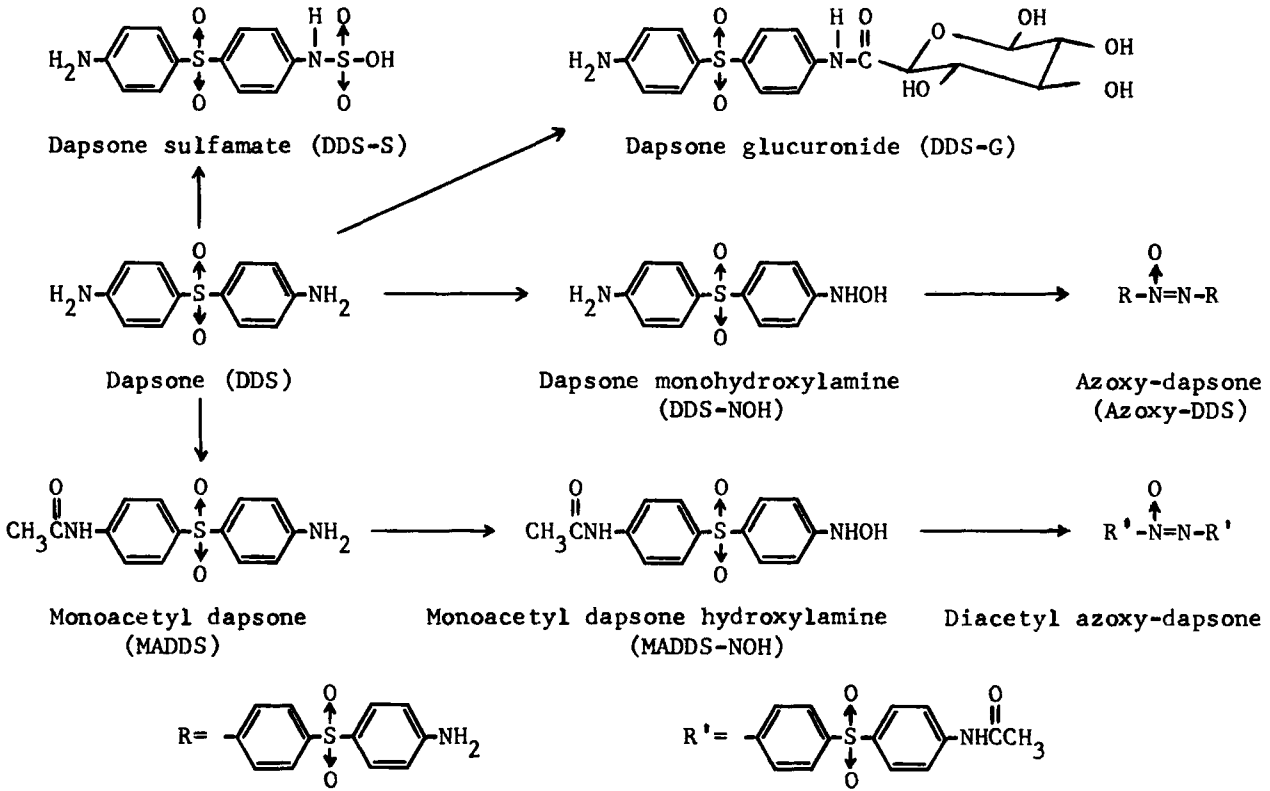


Figure 9. Some Dapsone Metabolites

Monoacetyl dapsone glucuronide	Man	(50,51)
	Monkey	(52)
	Rabbit	(51)
	Rat	(51)
Monoacetyl dapsone hydroxylamine	Man	(57)
Azoxy-dapsone	Man	(57)
	Rat	(57)
Diacetyl azoxy-dapsone	Man	(57)

The structures of some of these metabolites are shown in Figure 9.

6. METHODS OF ANALYSIS

6.1 Identification Tests

Dapsone is easily identified by the physical properties described in section 2 above. Where identification of dapsone in formulations is necessary, it can be extracted by various organic solvents such as methanol, chloroform, ethylene dichloride, etc. If the identification of very small amounts of dapsone is necessary, the colorimetric method of Peters et al (63), the colorimetric tests described in section 6.3, or the fluorometric tests described in section 6.5 may be useful.

6.2 Elemental Analysis

The elemental composition of dapsone is as follows:

Element	%Theory
Carbon	58.04
Hydrogen	4.87
Nitrogen	11.28
Sulfur	12.91
Oxygen	12.89

6.3 Colorimetric Methods

There are two basic colorimetric methods for dapsone. The first and most widely used is the method of Bratton and Marshall (64). Dapsone is diazotized, then coupled with N-(1-naphthyl)-ethylenediamine to form an azo dye. Other investigators have used this basic method but have used different coupling reagents: Nitti et al (65), dimethyl-α-naphthylamine; Rose and Bevan (66), N-β-sulfa-

toethyl-m-toluidine; Schoog (67), 1-sulfomethylaminonaphthalene-8-sulfonic acid; Merland (68), N-naphthyl-N',N'-diethylpropylenediamine.

The second type of method, reported by Levy and Higgins (69), is based on the formation of a Schiff base between dapsone and 4-dimethylaminobenzaldehyde. This method is reported to be 2.4 times more sensitive than the Bratton-Marshall technique and, in addition, it is reported to be specific for dapsone in the presence of its metabolites.

6.4 Titration Methods

Tomicek (70) titrated dapsone potentiometrically with perchloric acid in glacial acetic acid. Wojahn brominated dapsone with bromide-bromate and back-titrated the excess bromate with sodium thiosulfate solution (71). The USP method of assay is titration of the cooled, strongly acid sample solution with sodium nitrite solution to an electrometric endpoint (72).

6.5 Fluorometric Methods

Glazko (73) reported a fluorometric method for dapsone in plasma and urine samples. Complete details of sample preparation are given. The fluorescence of the ethyl acetate extract is determined by activating at 297 nm and measuring the emission at 340 nm. Ellard and Gammon (16) developed an extraction scheme to determine dapsone and two possible metabolites, MADDS and DADDS, fluorometrically in plasma and urine samples. Peters et al (15) modified and expanded the above procedure. They extracted with ethylene dichloride instead of ethyl acetate and thus eliminated the problem of quenching caused by small quantities of water in the ethyl acetate extract.

6.6 Paper Chromatography

Longenecker (74) reported procedures for partition chromatography of dapsone by both ascending and descending chromatography on paper and string. Bushby and Woiwood (54, 55) used paper chromatography to isolate and identify dapsone and some diazotizable metabolites by paper chromatography and electrophoresis. Wadia et al (75) reported R_f values for dapsone and twenty five other diaminodiphenyl sulfides, sulfoxides, and sulfones using four different solvent systems. Jardin and Stoll (76), Khosla et al (77), and Tsutsumi (48) all used paper chromatography in their studies on the metabolism of dapsone.

6.7 High Pressure Liquid Chromatography

Gordon and Peters (78) separated dapsone, monoacetyl dapsone (MADDS), and diacetyl dapsone (DADDS) at the 1 to 20 μg level on a silica gel column with ethyl acetate as solvent. The effluent was monitored at 280 nm. Murray et al (79) used a fluorometric detector and increased the sensitivity of the determination to the 10 ng level. This procedure was modified by Murray et al (80) to increase the sensitivity to 0.1 ng in 0.5 ml. Ribi et al (81) used pressure accelerated chromatography through microparticulate silica gel columns, packed by centrifugation, to separate dapsone, MADDS, and DADDS, with chloroform-methanol (97:3) as solvent and ultraviolet absorption at 254 nm for detection.

Gordon et al (82) used a silica gel column with chloroform-carbon tetrachloride (7:3) solvent to examine dapsone for impurities. Column effluent was monitored by the absorption at 280 nm. Fractions were collected and evaporated, and the residues were dissolved in ethylene dichloride. The fluorescence of the re-dissolved fractions was measured. Impurities determined were 2,4'-diaminodiphenyl sulfone and 4-aminodiphenyl sulfone.

Krol and Mannan (83) developed a method for the analysis of dapsone using a commercially available silica gel column (μ-Porasil®), a solvent containing isopropyl alcohol, acetonitrile, ethyl acetate, and pentane (1:1:1:7), and detection at 254 nm. A 30 cm by 4 mm I.D. column was suitable either for assay for dapsone in tablets or for analyzing for impurities in dapsone. The following related compounds can be separated from dapsone: (a) 2,4'-diaminodiphenyl sulfone; (b) 4-aminodiphenyl sulfone; (c) 4-amino-4'-chlorodiphenyl sulfone; (d) 4,4'-diacetamidodiphenyl sulfone (DADDS); (e) 4-amino-4'-acetamidodiphenyl sulfone (MADDS); (f) 4-amino-4'-hydroxydiphenyl sulfone; (g) 4,4'-diacetamidodiphenyl sulfoxide; 4,4'-diaminodiphenyl sulfide (90).

6.8 Gas Chromatography

Burchfield et al (84, 85) reported that dapsone can be chromatographed directly. Because their later work required greater sensitivity, and, in addition, the determination of MADDS and DADDS, the iodo derivatives were prepared by diazotization and an electron capture detector was used. Using a 4 foot by 0.25 inch O.D. glass U-tube packed with 3% Poly-A-103 on 100-120 mesh Gas Chrom Q at 285°C with nitrogen, 280 pg of iodo derivative was easily detectable.

Chang et al (52) investigated the metabolism of dapsone by chromatographing trimethylsilyl derivatives of the metabolic products on 3% OV-17 (Gas Chrom Q) at 275°C.

6.9 Thin Layer Chromatography

Thin layer chromatography systems have been compiled in Table 2.

7. ACKNOWLEDGMENTS

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TABLE 2

Adsorbent	Solvent System	Rf	Ref.
Silica gel	Chloroform-Butanol-Petroleum Ether (1:1:1)	0.62	86
Silica gel DF-5	Toluene-Ethyl acetate (1:1)	0.30	87
Silica gel G	Chloroform-Heptane-Ethanol (1:1:1)	0.60	88
"	Acetone-Chloroform (1:9)	0.20	89
"	Chloroform-Ethyl Ether (85:15)	0.11	"
"	Toluene-Ethyl acetate (1:1)	0.27	"
"	Chloroform-Methanol (95:5)	0.47	"
"	Chloroform-Methanol (9:1)	0.48	"
"	Chloroform-Ethanol (9:1)	0.65	"
"	Chloroform-Acetone-Diethanolamine (5:4:1)	0.69	"
"	Methanol-Acetone-Diethanolamine (50:50:1.5)	0.78	"
"	Acetone-Chloroform (9:1)	0.84	"
"	Isopropyl alcohol-cyclohexane-25% Ammonia (65:25:10)	0.85	"
"	Dimethyl formamide-Diethylamine-Ethanol-Ethyl acetate (1:1:6:12)	0.88	"

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Literature surveyed through June, 1975.

FLUCYTOSINE

Edward H. Waysek and James H. Johnson

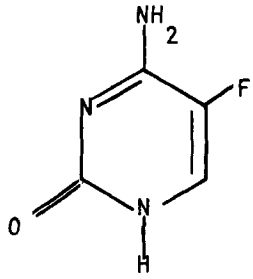
INDEX

Analytical Profile - Flucytosine

1. Description

1.1 Name, Formula, Molecular Weight

Flucytosine is 4-amino-5-fluoro-2(1H)-pyrimidone.



$C_4H_4FN_3O$ Molecular Weight: 129.1

1.2 Appearance, Color, Odor

Flucytosine is a white, odorless, crystalline powder.

2. Physical Properties

2.1 Infrared Spectrum (IR)

The infrared spectrum of flucytosine is presented in Figure 1. The spectrum was recorded with a Perkin-Elmer Model 621 Grating Infrared Spectrophotometer as a Fluorolube suspension from 4000 to 1350 cm^{-1} and as a mineral oil suspension from 1350 to 400 cm^{-1}. The assignments for the characteristic bands in the IR spectrum are listed in Table 1 (1).

FIGURE 1

Infrared Spectrum of Flucytosine

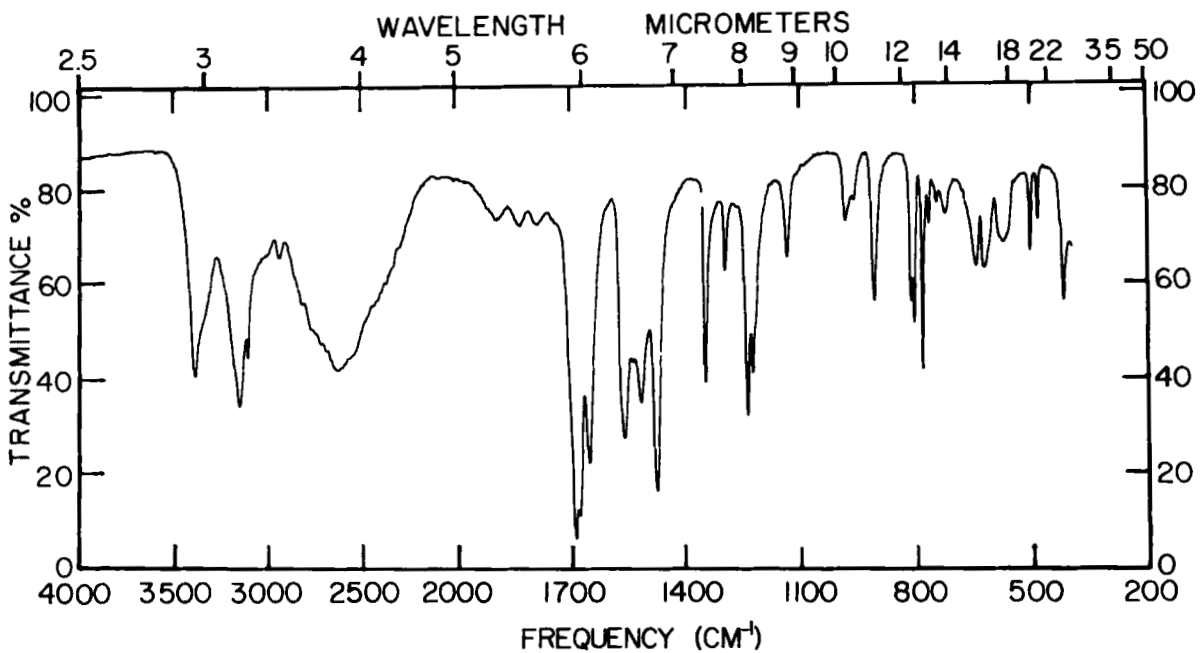


Table I

Infrared Assignments For Flucytosine

Frequency (cm^{-1})	Characteristic Of
3374, 3138	Bonded NH
2800-2200 (broad)	Enolic OH (associated)
1684, 1672	C=O stretch
1647	N-C=H (amidine)
1554	C=C (extended conjugation)

2.2 Nuclear Magnetic Resonance Spectrum (NMR)

The NMR spectrum of flucytosine in Figure 2 was determined on a JEOL C-60 HL Spectrometer at ambient temperature (ca. 25°). The sample was dissolved in 0.1N deuterium chloride containing sodium 2,2-dimethyl-2-silapentane sulfonate as an internal reference (MeSi = 0 ppm). The C-6 proton exhibited a doublet (J_{h-f} = 5.5 Hz) at 8.03 ppm.

2.3 Ultraviolet Spectrum

The ultraviolet spectrum of flucytosine in 0.1N hydrochloric acid in the region 340 to 210 nm exhibits a maximum at 283-287 nm ($\varepsilon = 9.2 \times 10^3$) and a minimum at 243-247 nm. The spectrum presented in Figure 3 was obtained from a reference standard solution of flucytosine at a concentration of 0.85 mg per 100 ml of 0.1N HCl (2).

2.4 Fluorescence Spectrum

Figure 4 shows the excitation and emission spectra of a solution of reference standard flucytosine from 250 to 650 nm. The spectra measured in a methanol solution of flucytosine (0.025 mg/ml using a Farrand MK-1 spectrofluorometer, showed one excitation maximum at 290 nm and one emission maximum at 366 nm.

FIGURE 2

NMR Spectrum of Flucytosine

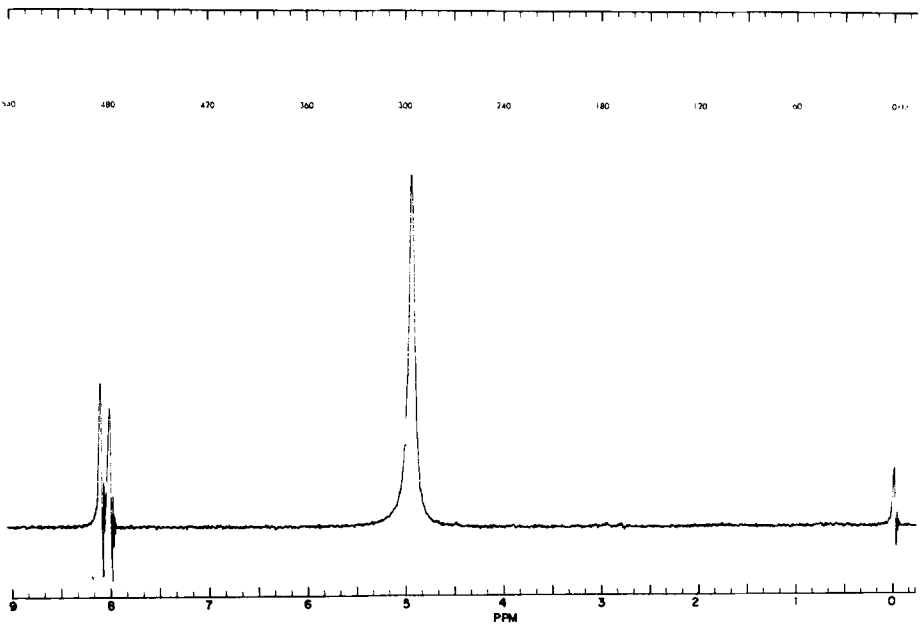


FIGURE 3

Ultraviolet Spectrum of Flucytosine

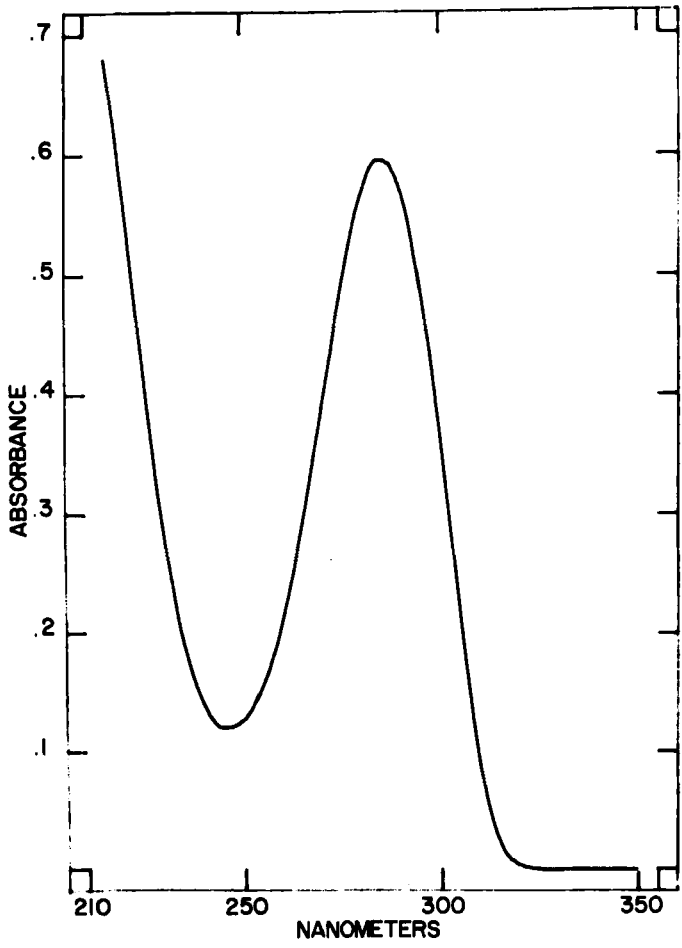
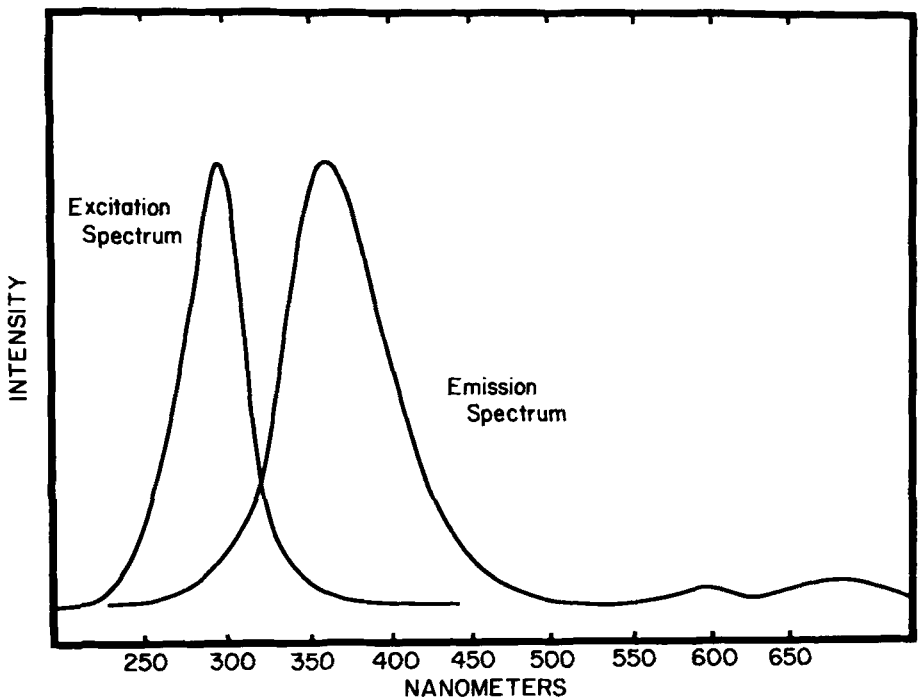


FIGURE 4: Fluorescence Spectra of Flucytosine



2.5 Mass Spectrum

The mass spectrum of flucytosine was obtained using a CEC 21-110 mass spectrometer with an ionizing energy of 70 eV. The output from the mass spectrometer was analyzed and presented in the form of the bar graph, shown in Figure 5, by a Varian 100 MS dedicated computer system (3).

The spectrum shows a strong molecular ion peak at m/e 129. The peak at m/e 101 is due to the loss of CO from the parent mass and the fragment of m/e 86 is due to the loss of HNCO, also from the molecular ion.

2.6 Optical Rotation

Flucytosine exhibits no optical activity.

2.7 Melting Range

A sharp melting point is not observed with flucytosine. The melting range depends on the rate of heating. When the USP Class Ia procedure (4) is used, the melting point lies between 292° and 298°C. Melting is accompanied by melt decomposition.

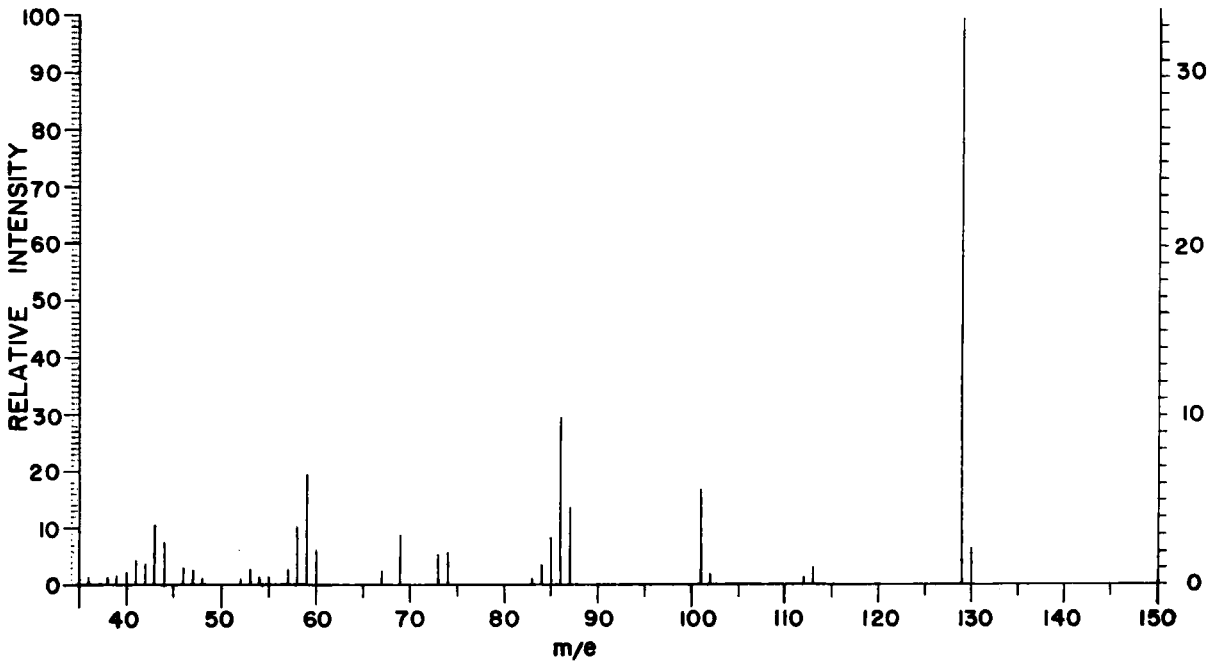
2.8 Differential Scanning Calorimetry (DSC)

When scanned at a programmed rate of 10°C per minute using nitrogen as the inert gas, decomposition occurs before any observed transition, therefore, DSC is not useful for characterization of this compound (5).

2.9 Thermogravimetric Analysis (TGA)

No weight loss was observed by TGA between ambient temperature and 210°C at a heating rate of 10°C per minute. A weight loss began at about 210°C and amounted to about 64% of the sample weight at 500°C (6).

FIGURE 5: Mass Spectrum of Flucytosine



2.10 Solubility

The solubility data for flucytosine obtained at 25°C is given in Table II (7). The solubilities were measured after an equilibration period of three hours.

Table II

Flucytosine Solubility

Solvent	Solubility (mg/ml)
3A alcohol	1.3
Absolute ethanol	0.5
Acetone	<0.1
Benzene	<0.1
Chloroform	<0.1
Diethyl ether	<0.1
Ethyl acetate	<0.1
Hexane	<0.1
2-propanol	1.1
Methanol	5.0
USP alcohol	1.7
Water	14.2

2.11 X-Ray Crystal Properties

The x-ray powder diffraction pattern of flucytosine is presented in Table III (8). The instrumental conditions are given below.

Instrumental Conditions

General Electric Model XRD-6 Spectrogoniometer

Generator	50 KV, 12.5 mA
Tube Target	Copper
Radiation	Cu (Cu Kα = 1.5418 Å)
Optics	0.1° Detector slit
	M.R. Soller slit
	3° Beam slit
	0.0007" Ni filter
	4° take off angle

Goniometer	Scan at 0.2°/minute 2θ Amplifier gain - 16 coarse 8.17 fine Sealed proportional counter tube and D.C. voltage at plateau, pulse height selector E_L; 8.0 volts, Eu; out Rate meter T.C. 4, 2000 c/s full scale
Recorder	Chart speed 1" per 5 min.
Sample	Prepared by grinding at room temperature

Table III

X-Ray Powder Diffraction Pattern of Flucytosine

2θ	d(Å) (1)	I/I_1 (2)
13.74	6.45	0.4
14.76	6.00	.39
15.14	5.85	.76
17.26	5.14	.09
18.76	4.73	.22
20.18	4.40	.81
21.81	4.08	.98
22.75	3.91	.16
23.94	3.72	1.00
25.94	3.44	.29
26.44	3.37	.67
29.24	3.06	.72
29.94	2.99	.14
30.65*	2.92	.15
32.01*	2.80	.55
33.48	2.68	.22
37.57	2.39	.22
39.98	2.26	.07
40.70	2.22	.09
42.74	2.12	.15
51.75	1.77	.15

*Broad Peaks

(1) d-(interplanar distance) $\frac{n\lambda}{2 \sin \theta}$

(2) I/I_1 = relative intensity

2.12 Dissociation Constant

The apparent pKa values for flucytosine have been determined spectrophotometrically to be 2.90 ± 0.05 and 10.71 ± 0.05 (9). The apparent dissociation constants were calculated from the UV spectral data in water at various pH values. pKa_1 (2.90) represents the protonation of N-3 and pKa_2 (10.71) represents deprotonation at the amide moiety. These values are in good agreement with the pKa's reported by Ueda and Fox for 3-methylcytosine (10).

3. Synthesis

Flucytosine may be prepared by the reaction scheme shown in Figure 6. 5-Fluorouracil is refluxed with dimethylaniline and phosphorous oxychloride. The 2,4-dichloro-5-fluoro-pyrimidine formed is reacted with ammonia and ethanol to give 2-chloro-4-amino-5-fluoropyrimidine which yields flucytosine hydrochloride upon being treated with concentrated hydrochloric acid. Flucytosine hydrochloride is neutralized with ammonium hydroxide forming 5-fluorocytosine (11).

4. Stability

Flucytosine stored at room temperature for five years was stable when tested by ultraviolet absorption and thin-layer chromatography using ethyl acetate: methanol:conc. NH_4OH (50:50:2) as the solvent system (12). The stability of 0.1% buffered solutions is shown in Table IV (13).

FIGURE 6: Synthesis of Flucytosine

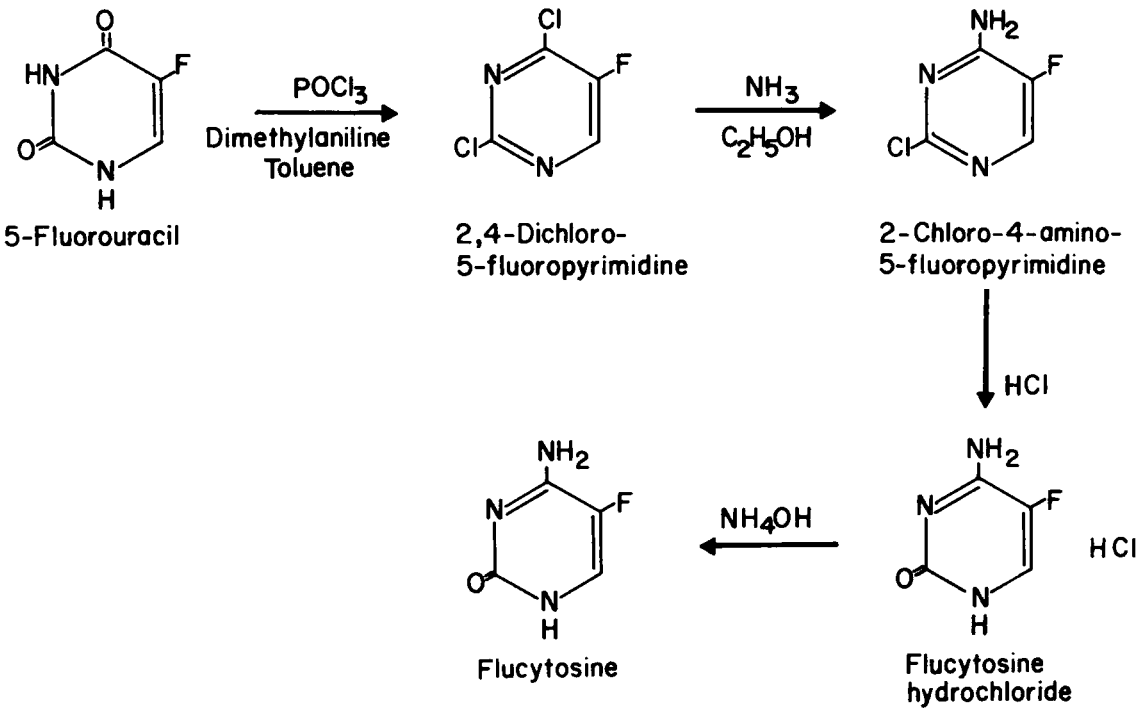


Table IV

Stability of 0.1% Buffered Solution

Before Heating			After 1 Hour at 100°C			
pH	APHA Color	Clarity	pH	APHA Color	Clarity	% Recovery
3.10	0-10	Clear	3.15	0-10	Clear	92.9
5.50	0-10	"	5.60	0-10	"	95.9
6.90	0-10	"	6.90	0-10	"	99.0
9.40	0-10	"	9.40	0-10	"	98.5

Method Used: Ultraviolet absorption and thin-layer chromatography.

The stability of flucytosine has also been studied in its dosage forms (14). In ampuls flucytosine was stable to the extent of 2% or less breakdown at room temperature for 36 months. No degradation products, urea, uracil, and 5-fluorouracil, were detected in capsules after six months at 37°C and twelve months at 25°C.

5. Drug Metabolic Products

It has been reported that flucytosine did not undergo significant biotransformation in humans after oral dosage and 90% of the flucytosine administered was excreted unchanged in the urine (15). Koechlin, et al. have studied the metabolism of flucytosine in the rat and in man using drug labeled in the 2-position with carbon-14. Rats given a parenteral dosage did not metabolize flucytosine and the dosage was recovered quantitatively, mostly in the urine. Flucytosine was deaminated to 5-fluorouracil to the extent of 20-30% after oral administration to rats, which was in turn metabolized further to α-fluoro-β-ureido-propionic acid, urea, and carbon dioxide.

No metabolic degradation of flucytosine in humans was

found after a single oral 2 g dose (16). Polak and Scholer (17, 18) using radiolabeled flucytosine in Candida albicans, determined that flucytosine enters the cell by means of the enzyme cytosine permease and is deaminated to 5-fluorouracil by cytosine deaminase. The 5-fluorouracil thus formed is incorporated into the RNA in place of uracil by means of the following pathway (19).

5-fluorocytosine→5-fluorouracil→5-fluorouridine monophosphate
→5-fluorouridine diphosphate→5-fluorouridine triphosphate
→RNA (up to 50% of uracil replaced by 5-fluorouracil)

5.1 Toxicology

The following excerpt on the toxicology of flucytosine was taken from Drug Evaluation Data (15):

"The LD_{50} for flucytosine has been studied in rats and mice. The LD_{50} in rats is reported as 8 g/kg orally and 100 mg/kg intravenously. The LD_{50} for mice is reported to be 2 g/kg orally and subcutaneously, 1.2 g/kg intraperitoneally and 500 mg/kg intravenously."

6. Methods of Analysis

6.1 Elemental Analysis

The results from the elemental analysis are listed in Table V (20).

Table V

Elemental Analysis of Flucytosine

Element	% Theory	% Found
C	37.22	37.24
H	3.12	3.13
N	32.55	32.40
F	14.72	14.60

6.2 Phase Solubility

Phase solubility analysis of flucytosine may be carried out using methanol as the solvent. Figure 7 shows a typical example and lists the conditions of the analysis (21).

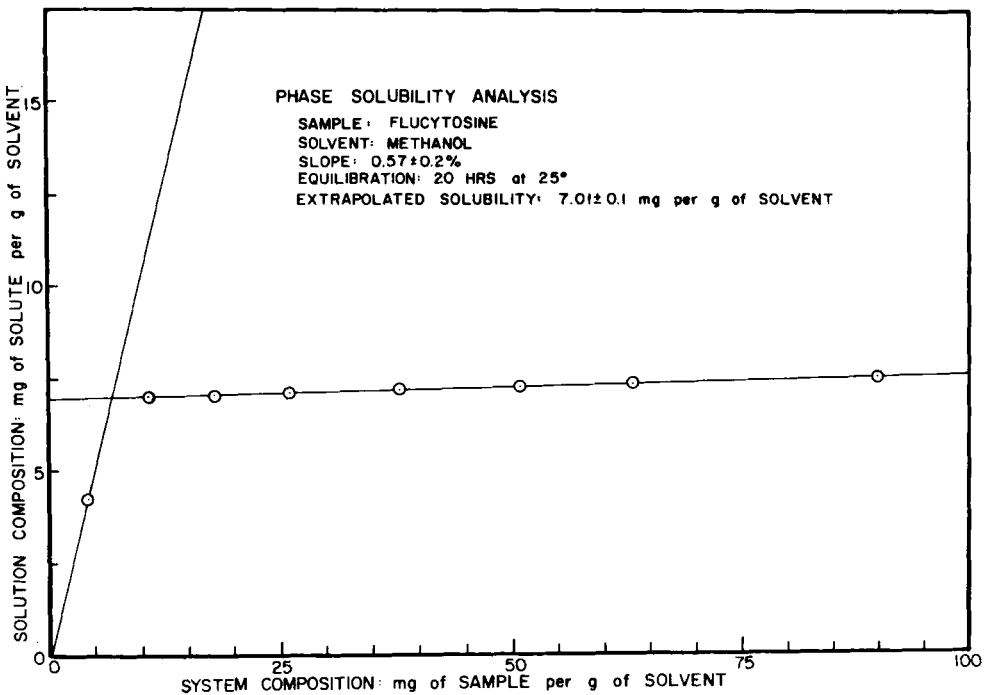
6.3 Thin-Layer Chromatographic Analysis (TLC)

The thin-layer chromatography of flucytosine and other fluoropyrimidines has been extensively studied by Hawrylyshyn, Senkowski, and Wollish (22). The R_f values presented in Table VI were obtained using various solvent systems with 10 µg of sample spotted on a silica gel plate and developed for at least 10 cm. The plates were air dried and viewed under shortwave ultraviolet radiation.

One and two-dimensional TLC systems are given by Koechlin, et al. (16) for the separation of flucytosine, α-fluoro-β-ureido-propionic acid, 5-fluorouracil, and urea in urine. The solvent systems used were ethyl acetate:formic acid: water (60:5:35), 2-propanol:ammonia (20:1), and methanol:water (85:15).

The following TLC system is used for the detection of fluorouracil in flucytosine drug substance (23). Twenty µl of a 25 mg/ml solution of flucytosine in a mixture of glacial acetic acid and water (8:2) is spotted on a silica gel GF plate and developed by ascending chromato-

FIGURE 7


PHASE SOLUBILITY ANALYSIS
SAMPLE: FLUCYTOSINE
SOLVENT: METHANOL
SLOPE: 0.57±0.2%
EQUILIBRATION: 20 HRS at 25°
EXTRAPOLATED SOLUBILITY: 7.01± 0.1 mg per g of SOLVENT
SOLUTION COMPOSITION: mg of SOLUTE per g of SOLVENT
0
5
10
15
0
25
50
75
100
SYSTEM COMPOSITION: mg of SAMPLE per g of SOLVENT

graphy in a mixture of chloroform:glacial acetic acid (65:35). After development for at least 14 cm, the air-dried plate is viewed under shortwave ultraviolet radiation. Flucytosine has an approximate R_f value of 0.3 with this system and fluorouracil 0.45 (24).

Table VI

R_f Values for Flucytosine in Various Solvent Systems (22)

	Solvent System	R_f Value
1.	Ethyl acetate:acetone:water (70:40:10)	0.1
2.	Ethyl acetate:methanol:conc. ammonium hydroxide (75:25:1)	0.3
3.	Acetone	0.02
4.	Ether	0.0
5.	Ethyl acetate	0.0
6.	Methanol	0.6
7.	2-propanol	0.1
8.	Ethyl acetate:methanol (80:20)	0.2
9.	Ethyl acetate:methanol (75:25)	0.2
10.	Ethyl acetate:water (100:1)	0.0
11.	Ethyl acetate:water (100:3)	0.0
12.	Ethyl acetate:methanol:acetic acid (75:25:1)	0.4

6.4 Non-Aqueous Titration

Flucytosine may be titrated in a mixture of glacial acetic acid:acetic anhydride (2:1) using acetous perchloric acid with a glass/calomel electrode system (23).

6.5 Fluorometric Analysis

A spectrofluorometric method of assaying flucytosine in biological fluids has been reported by Wade and Sudlow (25). Flucytosine is isolated from protein-free biological fluids by TLC and eluted from the silica gel with water. After making the solution alkaline, the

fluorescence is measured. Excitation was carried out at a wavelength of 300 nm and the emission was measured at 365 nm.

6.6 Direct Spectrophotometric Analysis

Direct spectrophotometric analysis can be used to obtain a quantitative assay of flucytosine in capsules (23). In dilute hydrochloric acid (1 in 100) the reported maximum is 283-287 with $\varepsilon = 9.2 \times 10^3$.

6.7 Fluorine Analysis

6.71 Free Fluoride Analysis

The determination of free fluoride in flucytosine drug substance can be carried out by direct measurement with a fluoride-specific ion electrode (23). Electrode potential measurements are made in a citrate containing acetate buffer solution (pH between 5.0 and 5.5). The potential measurements are converted to free fluoride concentration by reference to a calibration curve.

6.72 Organically Bound Fluorine Analysis

Bound fluorine may be determined by Schöniger combustion followed by measurement with a fluoride-specific ion electrode (26).

6.8 Biological Assays

Shadomy, et al. (27) indicated that the activity of flucytosine should be studied in a completely synthetic medium. In later studies (28) Shadomy described a cylinder plate bioassay for the determination of flucytosine in sera, urines, cerebrospinal fluids, and tissue homogenates. The indicator organism used was S. cerevisiae ATCC 9763 on yeast nitrogen base agar supple-

mented with L-asparagine and dextrose. For sera and other biological fluids the lower sensitivity limit was 0.4 to 0.5 µg flucytosine/ml. Block and Bennett (29) reported a cylinder plate bioassay that permitted the determination of flucytosine in biological fluids in the presence of amphotericin B which otherwise would interfere. Blaker and Doutt (30) described a disc diffusion method for assaying flucytosine in serum and other body fluids using a strain of Saccharomyces cerevisiae as the test organism on yeast nitrate base agar. The method was useful in the concentration range 2 to 6 µg/ml. Holt and Newman (31) also reported a method for the routine assay of flucytosine in biological fluids using C. albicans (Carshalton 2606) as the indicator organism. The method readily detected 0.1 µg flucytosine/ml. Marks and Eickhoff (32) studied the antifungal activity of flucytosine using broth-dilution, microtiter dilution, agar-dilution, and disc susceptibility tests. Their investigation indicated that the disc susceptibility test may be applicable in diagnostic microbiology laboratories for yeasts such as Candida and Torulopsis.

7. Acknowledgements

The authors wish to acknowledge M.V. Go, Dr. K. Blessel, E. Kohler, and the Scientific Literature Department of Hoffmann-La Roche Inc. for their assistance in the preparation of this analytical profile.

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GLUTETHIMIDE

Hassan Y. Aboul-Enein

CONTENTS

Analytical Profile - Glutethimide

CONTENTS (cont'd)

1. Description

1.1 Nomenclature

1.11 Chemical Names:

a. (±) 2-Ethyl-2-phenylglutarimide.

b. α-ethyl-α-phenylglutarimide.

c. 3-ethyl-3-phenyl-2,6-piperidinedione.

d. 3-ethyl-3-phenyl-2,6-dioxopiperidine.

e. 3-ethyl-3-phenyl-2,6-diketopiperidine.

1.12 Generic Name:

Glutethimide

1.13 Trade Names:

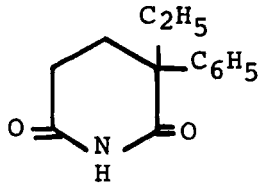
Doriden, Noxyron, Elrodorm.

1.2 Formulae:

1.21 Empirical:

$C_{13}H_{15}NO_2$

1.22 Structural:



1.3 Molecular Weight:

217.26

1.4 Elemental Composition:

C, 71.86; H, 6.96; N, 6.45; O, 14.73

1.5 Appearance, Color, Odor:
Glutethimide is a colorless crystalline white solid, odorless, has a bitter taste.

2. Physical Properties:

2.1 Crystal Properties:

2.11 Crystallinity

Glutethimide is a crystalline solid. A typical photomicrograph of glutethimide obtained by sublimation, recrystallization from 50% aqueous ethanol and recrystallization from concentrated ammonia is shown in Fig. (1). These crystalline structures can be used as a microscopic means of identification of glutethimide (1). The behavior of glutethimide crystals extracted from the tablet formulations was discussed by Penprose and Biles (1).

2.12 X-Ray Diffraction:

Analytical x-ray diffraction data for glutethimide, its anhydrous, hydrous forms and optical active antipodes were studied in detail by Bonamico et al. (2) along with other analogs. The elemental crystal structure for these forms are shown in Table I.

Further information regarding conformation of the chemical structure of glutethimide through analysis of x-ray diffraction patterns are given by Bonamico et al. (2).

The optical crystallographic properties for glutethimide crystals are as follows (3):

α 1.572; β 1.585, γ 1.590
Optic Sign: Negative 2V. large
Extinction: parallel and inclined
Elongation: positive
System: 6-sided rods and plates

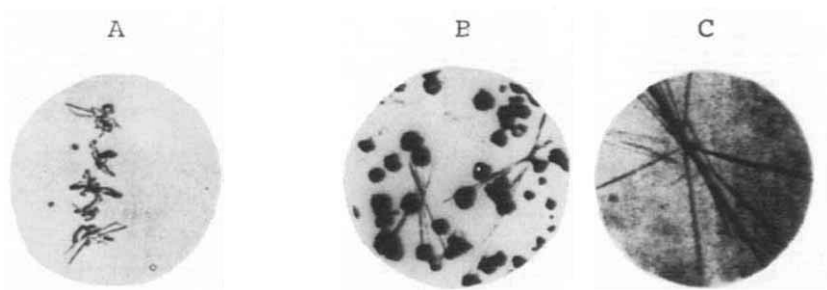


Fig. 1 - Photomicrographs showing variation in crystal structure of glutethimide (1). A-sublimation, B-from 50% EtOH, C-from conc. NH_4OH.

Table I
X-Ray Diffraction Data for Glutethimide

Form	a, Å	b, Å	c, Å	Z	V	B	Special Group
Glutethimide hydrous rhombic prism	7.53±0.02	30.50±0.09	10.83±0.03	8	2487 Å^3		$D_2^{15} - P_{bca}$
Glutethimide anhydrous monoclinic prisms	20.40±0.06	11.39±0.03	20.60±0.06	16	4791 Å^3	92° 40′ ±20′	$P_{2/c}$ or P_c - C_{2h}^4 - C_2^s
Glutethimide (+)or (-) antipode rhombic prisms	6.87±0.02	25.75±0.07	6.90±0.02	4	1221 Å^3		$P2_12_12_1$

2.12 X-ray Diffraction (continued)

The characteristic diffraction patterns were obtained by subjecting glutethimide powder to CuK γ-radiation from x-ray spectrometer and recording the diffracted radiation on a chart, using a modified GM-tube with a recording potentiometer (1). The D-distances were calculated as shown in Table II.

Table II

10.3
7.23
6.22[a]
6.01
5.11[a]
4.59
3.78[a]
3.52
3.27
3.20

a - Strong

2.13 Melting Range

The National Formulary XIV (4) specifies a melting range for glutethimide between 86° and 89° as a criteria of acceptability. Furthermore, it was reported that glutethimide showed a m.p. range of 85°-88° with residual crystals growing sluggishly only below 80° into grains and prisms (5). The melt solidifies to a glass. The glassy powder showed n_D 1.5403.

The eutectic temperature of glutethimide with azobenzene and with benzil was reported to be 53° and 61°, respectively.

Table III shows the melting range of glutethimide reported in the literature.

Table I
X-Ray Diffraction Data for Glutethimide

Form	a, Å	b, Å	c, Å	Z	V	B	Special Group
Glutethimide hydrous rhombic prism	7.53±0.02	30.50±0.09	10.83±0.03	8	2487 Å^3		$D_2^{15} - P_{bca}$
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Glutethimide (+)or (-) antipode rhombic prisms	6.87±0.02	25.75±0.07	6.90±0.02	4	1221 Å^3		$P2_12_12_1$

Table III

m.p., C°	Reference
91-92	1
85-87, $b_{0.3}$ 168°	6
82-83 (isopropanol)	7
86-88 anhydrous glutethimide	8
80-85 (EtOAc-pet. ether)	9
83-85	10
68.5.70.5 (dil EtOH)	11

2.14 Differential Scanning Calorimetry

Reubke and Mollica (12) reported the application of the differential scanning calorimetry in the quantitative estimation of purity of glutethimide and the detection of polymorphism. They reported ΔH value for glutethimide to be 28.7±1 cal/gm. at 86.8° - 87°.

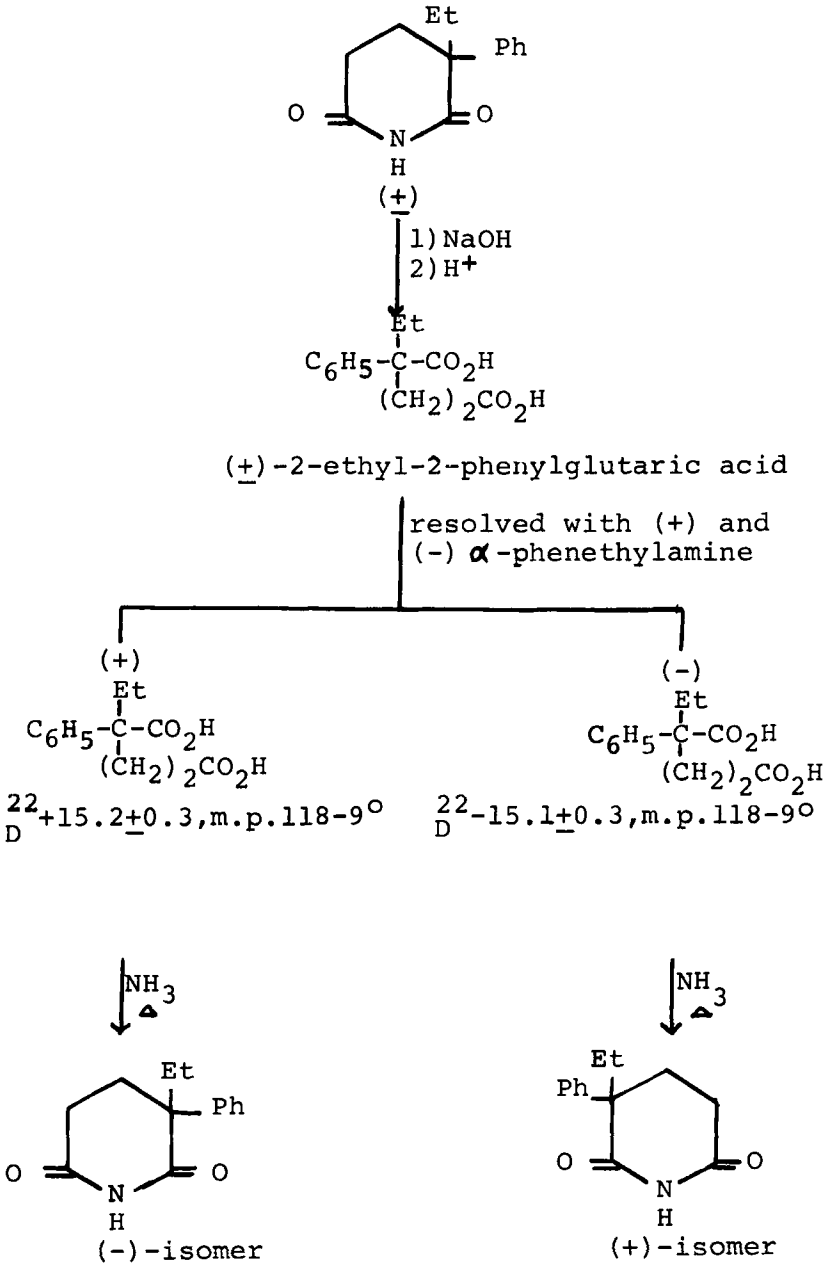
2.2 Solubility:

Insoluble in water (1.0 mg/ml), soluble 1 in 5 of ethanol, 1 in 12 of ether and 1 in less than 1 of chloroform, dichloromethane. Soluble in acetone, ethyl acetate. A saturated solution in water is acidic to litmus.

Recently, it was reported that the tetramethyl-substituted amides of pimelamide, suberamide, azelamide and sebacamide markedly enhance the solubility of glutethimide in aqueous solution (13). The solubility of glutethimide was increased significantly above the critical concentrations and from the nature of the solubility curves, a micellar type of solubilization appears to be dominant.

2.3 Optical Activity

Glutethimide is marketed as the racemic mixture of 2-ethyl-2-phenylglutarimide. Keberle et al. (14) describes a procedure for resolution of the racemic mixture to the pure optical antipodes as shown in Scheme 1. The sedative-hypnotic activity of the (+) isomer is 2-3 times more potent than the (-) isomer (15).


Et
Ph
O
O
N
H
(±)
1) NaOH
2) H+
Et
C6H5-C-CO2H
(CH2)2CO2H
(±)-2-ethyl-2-phenylglutaric acid
resolved with (+) and
(-) α-phenethylamine
(+)
Et
C6H5-C-CO2H
(CH2)2CO2H
22D +15.2±0.3, m.p. 118-9°
(-)
Et
C6H5-C-CO2H
(CH2)2CO2H
22D -15.1±0.3, m.p. 118-9°
NH3
Δ
Et
Ph
O
O
N
H
(-)-isomer
NH3
Δ
Et
Ph
O
O
N
H
(+)-isomer

Scheme 1

Table IV

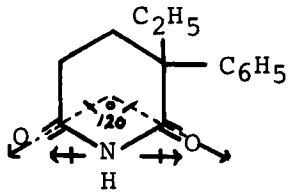
	Specific rotation	m.p., C°	Reference
(+)-isomer	$[\alpha]_D^{25}$ + 183 (EtOH)	104	14
	$[\alpha]_D^{25}$ + 186 ± 2 (EtOH)	104	16
	$[\alpha]_D^{25}$ + 184 (C=1, EtOH)	103-104	17
	$[\alpha]_D^{20}$ + 176 ± 2 (C=1, MeOH)	102.5-103	18
(-)-isomer	$[\alpha]_D^{25}$ - 183 ± 1 (EtOH)	104	14
	$[\alpha]_D^{25}$ - 186 ± 2 (EtOH)	104	16
	$[\alpha]_D^{25}$ - 184	103-104	17
	$[\alpha]_D^{20}$ - 181 ± 2 (C=1, MeOH)	102-103	18

2.3 Optical Activity (continued)

The specific rotations, melting points for the (+) and (-) isomers are given in Table IV as recorded in the literature.

2.4 Molecular Orbital Calculations and Dipole Moment

The molecular orbital calculations of glutethimide was discussed by Andrews (19) using two methods, extended Huckel theory (EHT) and complete neglect of differential overlap $CNDO/_2$. Calculated dipole moment (D) of glutethimide was found to be 8.67 using EHT, while the experimental dipole moment (D) was reported by Lee and Kumler (20) to be 2.83. Andrews showed that $CNDO/_2$ method is more suitable for assessing net atomic charges than the EHT.



2.83D

Furthermore, Lee and Kumler (20) concluded from their study that of the dipole moments of several six-membered cyclic imides the imide groups in these compounds are in a cis-cis conformation.

2.5 Acid Dissociation Constant

Doornbos and De-Zeeuw (21) measured the "proton lost" dissociation constant K_2H and the "proton gained" K_3H dissociation constant for glutethimide by a previously described potentiometric titration method at 20°. K_2^H and K_3^H for glutethimide at ionic strength of μ=0.10 was found to be 4.518.

2.6 Identification

The following identification tests are published in B.P. 1973 (22) as a part of the identification of glutethimide.

1. The light absorption, in the range 230 to 350 nm, of a 2-cm layer of a 0.03 per cent w/v solution in dehydrated alcohol exhibits three maxima, at 252 nm, 258 nm, and 264 nm; extinction at 252 nm, about 1.0, at 258 nm, about 1.1, and at 264 nm, about 0.86.

2. Heat 1 g with 5 ml of sodium hydroxide solution and 15 ml of water on a water-bath for thirty minutes, cool and acidify to litmus paper with dilute hydrochloric acid; filter; melting point of the precipitate, after washing with water and drying at 100°, about 159°.

Furthermore, Imaoka and Ogura (23) published a procedure for identification of glutethimide in tablets by spot test. A powdered sample (2-3 mg) on Whatman's Test Paper is wetted with 2 drops acetone, 1 drop 1% copper acetate or 1% cobalt acetate solution, kept for 30 seconds then 1 drop 10% isopropylamine solution in acetone was added, glutethimide gives a clear violet colored spot.

Most bulking agents, e.g. lactose, starch, sucrose, gun arabic, glucose or talc with the exception of alginic acid does not interfere with the test.

2.7 Spectral Properties

2.71 Ultraviolet

Glutethimide in solution absorbs ultraviolet radiation over a broad range to produce a spectrum with maximum at 257, 251, 263 nm (typical spectrum, Fig. 2), with $A^{1\%}_{1cm}$ = 19 for the wave length 257 nm (24-26).

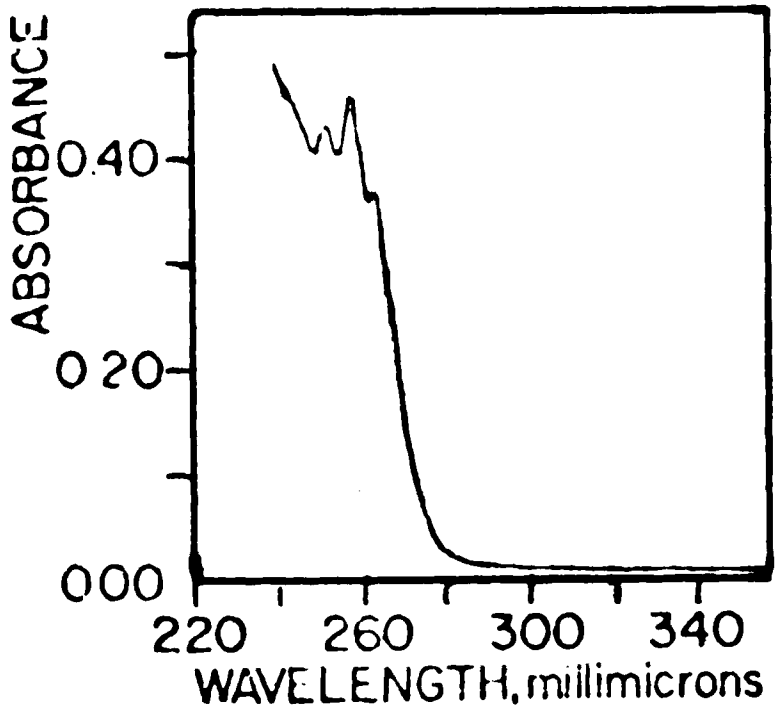


Fig. 2 -

Ultraviolet spectrum of glutethimide in methanol (24).

2.72 Infrared Spectrum

The infrared spectrum of glutethimide is shown in Fig. 3. The spectrum was obtained on a Perkin-Elmer 621 spectrophotometer from a KBr pellet.

The structural assignments have been correlated with the following band frequencies:

Frequency (cm^{-1})	Assignment
3190-3100	NH stretching imide
1710	C=O imide carbonyl
1680	C=O imide carbonyl next to the α-phenyl group
1200	C-O stretching
760-700	Monosubstituted phenyl

Further information with regard to the infrared spectra of glutethimide is given by several authors (27-29).

2.73 Nuclear Magnetic Resonance Spectrum

A typical NMR spectrum of glutethimide is shown in Fig. (4). The sample was dissolved in carbon tetrachloride. The spectrum was determined on a Varian T-60 NMR Spectrometer with TMS as the internal standard.

The following structural assignments have been made for Fig. (4).

Chemical Shift (δ)	Assignment
Triplet 0.80	$-CH_2-\overline{CH_3}$
Multiplet 1.8 to 1.97	$-CH_2-\overline{CH_3}$ and two $-\overline{CH_2}-$ of glutarimide ring
Singlet 6.97	Aromatic phenyl protons
Broad singlet 8.27	NH imide, exchangeable with D_2O.

Further information concerning the interpretation of the NMR spectrum of glutethimide and related cyclic imides can be obtained

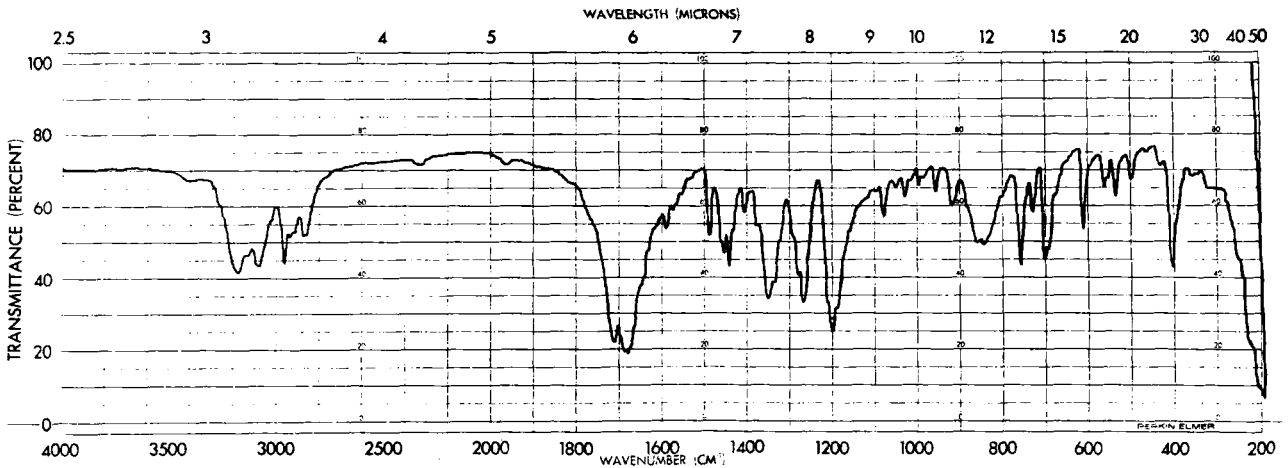


Fig. 3 - Infrared spectrum of glutethimide, KBr pellet.

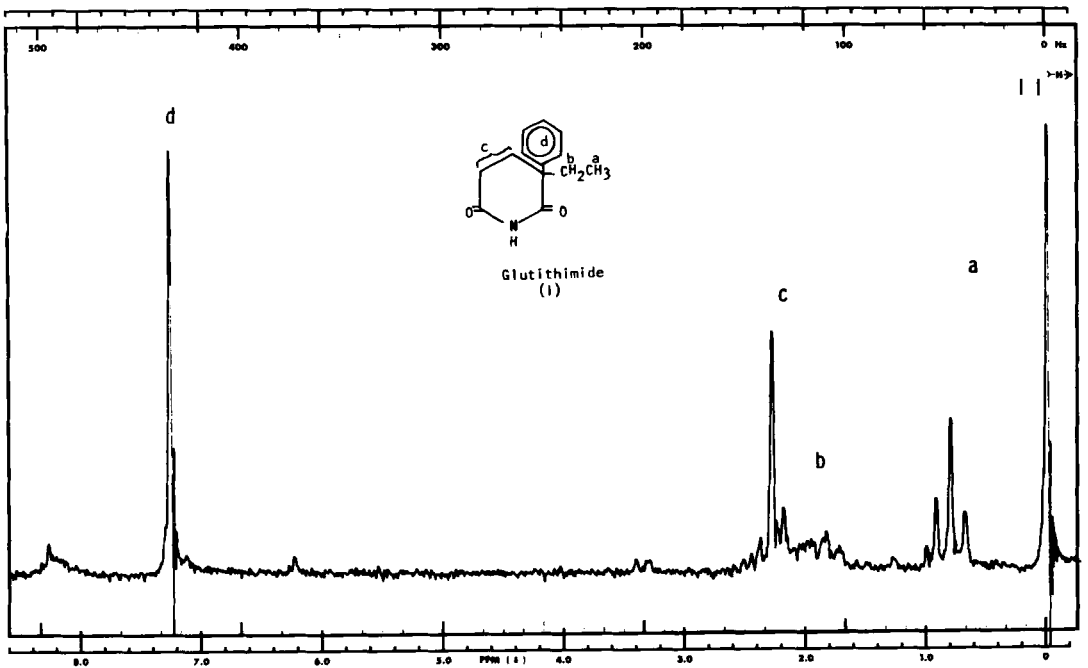


Fig. 4 - NMR spectrum of glutethimide in CCl_4 containing TMS as internal standard.

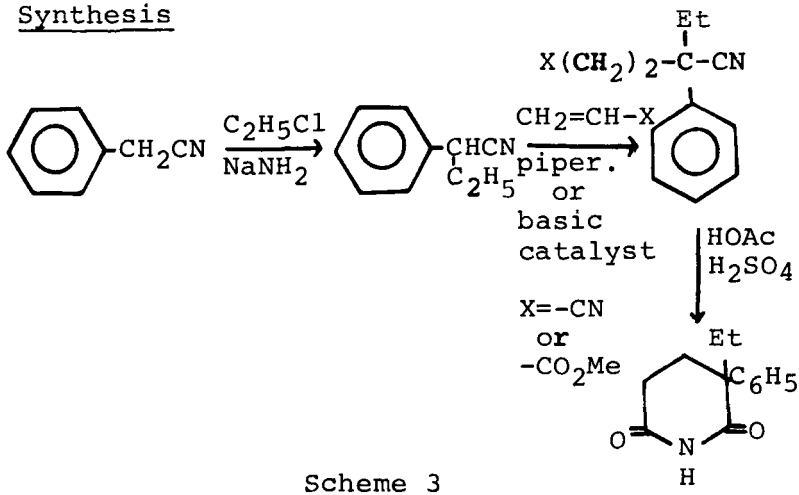
from Casini and Salvi's (29) study of the NMR of a series of cyclic imides and also by Defay and Dorlet (30).

2.74 Mass Spectra

The mass spectrum of glutethimide obtained by conventional electron impact ionization shows a molecular ion M^+ at m/e 217. The M^+ ion peak is only 5% (Fig. 5). The base peak is at m/e 117. The mass spectral fragmentation mechanism was discussed by Rücker (31) as shown in Scheme 2. Mass spectrometry was also used as an analytical tool for rapid, accurate identification of and diagnosis of glutethimide overdoses and poisoning cases (32-33).

The chemical ionization (CI) mass spectrometry of glutethimide was recently reported by Saferstein and Chao (34). The ionization was accomplished by the reaction of the drug with either ionized gaseous methane or isobutane. The resulting spectrum was less complex than that produced by conventional EI ionization and usually results in the formation of a molecular ion plus one ($M^+ + 1$) i.e., 218 for glutethimide.

3. Synthesis



Scheme 3

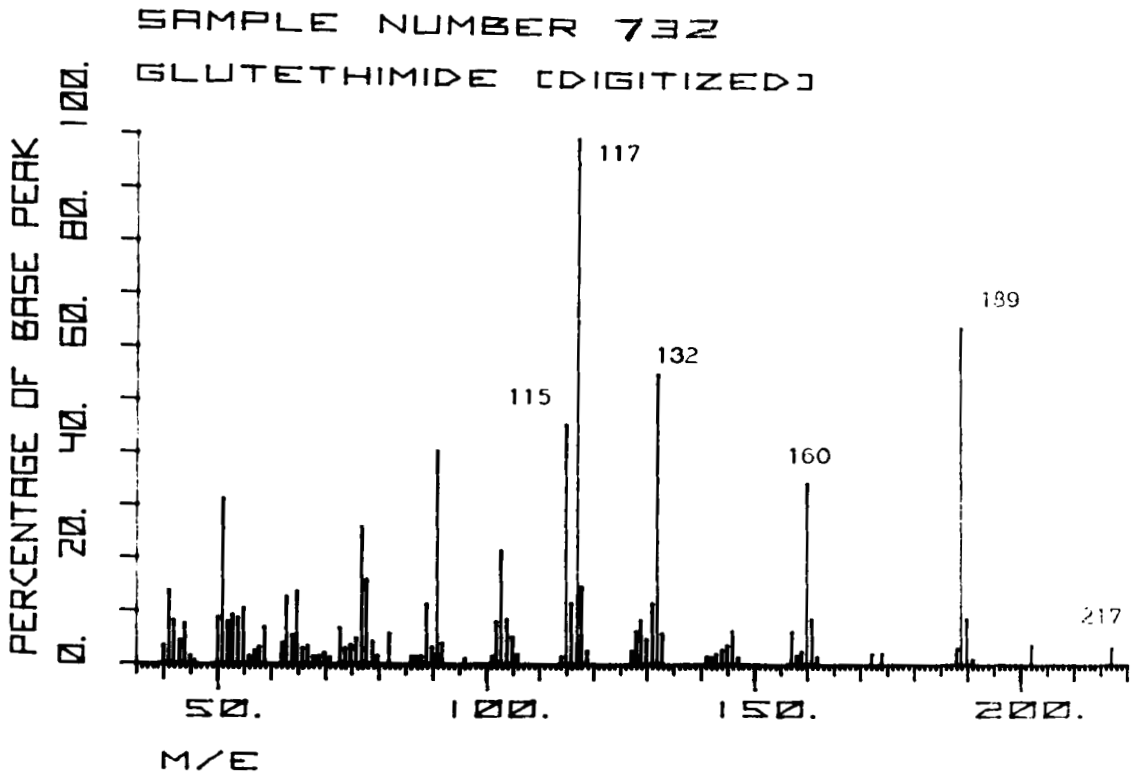
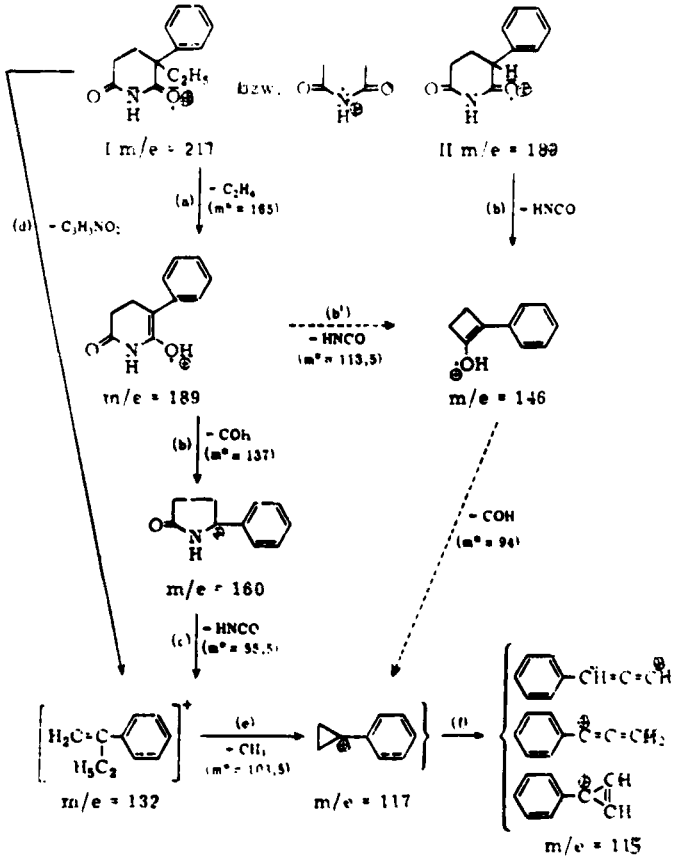


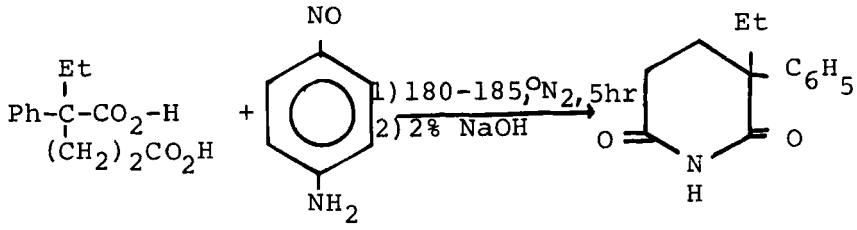
Fig. 5 - Mass Spectrum of Glutethimide (EI).



Scheme 2 - Mass spectral fragmentation mechanism of glutethimide (31).

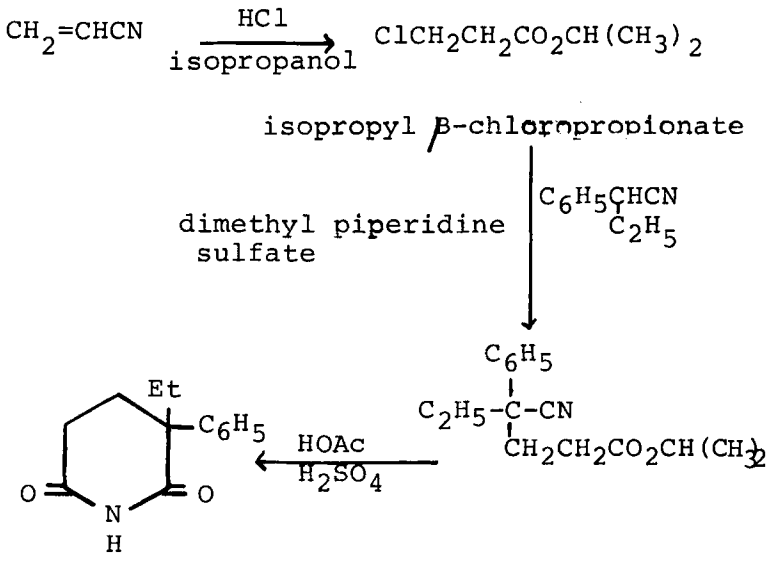
Glutethimide is synthesized as shown in Scheme 3. Starting with benzylcyanide which is treated with ethyl chloride or bromide in the presence of sodium amide to yield α-ethyl benzylcyanide. This is then allowed to react with methyl acrylate or acrylonitrile (Michael condensation) under the catalytic influence of piperidine or another suitable basic catalyst, thus, forming methyl 4-cyano-4-phenylhexanoate or its corresponding dinitrile derivative, respectively. The latter intermediate was purified by distillation and cyclized in the presence of acetic acid and 90% sulfuric acid to yield glutethimide (6, 35-37).

Another synthetic route for glutethimide is described by Iwase et al. (9) shown in Scheme 4. A mixture of 1-phenyl-1-ethylpropane-1,3-dicarboxylic acid and p-nitrosoaniline was treated at 180-185° under nitrogen atmosphere for 5 hrs., refluxed with 2% sodium hydroxide for 3 hrs., acidified, washed with ether, neutralized with sodium carbonate, extracted with chloroform to yield glutethimide.



Scheme 4

Other route (38) for glutethimide synthesis is shown in Scheme 5.



Scheme 5

4. Stability & Decomposition Products

Several reports showed that glutethimide is hydrolyzed to 4-ethyl-4-phenylglutaramic acid in alkaline solution (36, 39). Yamaha and Mitzukami (40) reported that this decomposition occurs in sodium hydroxide and sodium carbonate solutions by second order reaction but not in sodium bicarbonate solution. The degradation rate constants at 20°, 30° and 40° in 0.01N sodium hydroxide were 7.66×10^{-1}, 1.67×10^{-1} and 3.56×10^{-1} and those in 0.01M sodium carbonate were 3.56×10^{-1}, respectively. The activation energies in 0.01N sodium hydroxide and 0.01M sodium carbonate were 14.3 and 14.6 K cal., respectively. Furthermore, Wesolowski et al. (41) studied the kinetics of degradation of glutethimide in buffered aqueous solutions in the pH range 1.5-8.0. They supported the previous reports that the degradation of glutethimide is a base-catalyzed reaction since the contribution of ionized species to the reaction rate in the pH range studied is very small. The rate is first order with respect to the concentration of glutethimide. The apparent energy of activation Ea, for the degradation of glutethimide at pH8 is found to be in the order of 25.86 K cal./mole. However, the energy of activation corrected for heat of ionization of water at 50°, 60°, and 65° was found to be 13.47 K cal., 13.92 K cal., and 14.15 K cal./mole, respectively.

The mechanism proposed for the glutethimide degradation is shown in Fig. 6 and involves direct attack by a hydroxyl ion on the unhindered carbonyl of the glutarimide ring followed by the cleavage of the ring to 4-ethyl-4-phenyl-glutaramic acid.

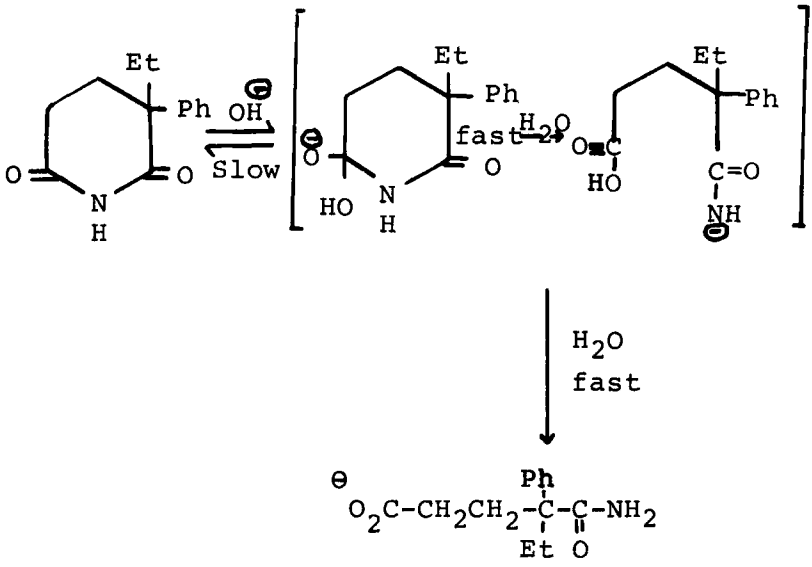


Fig. 6
The Mechanism of Glutethimide Degradation (41).

A plot of log $t_{½}$ versus pH (Fig. 7) shows that below pH5, glutethimide is very stable since at pH 1.0 and 5.0 the rate is independent of hydrogen ion concentration.

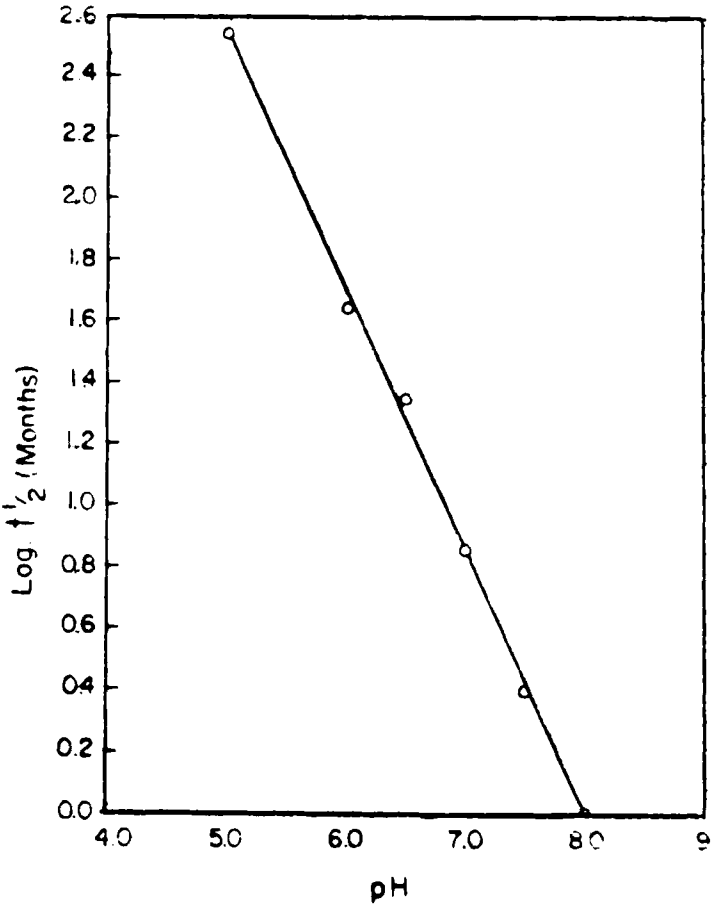


Fig. 7 -

Log $t_{\frac{1}{2}}$, the half-life of the reaction in months against pH at 25° (41).

5. Metabolism

The distribution and metabolism of glutethimide in the dog and rat were extensively studied and summarized by Keberle et al. (42). The metabolic fate in man was studied by Butikofer et al. (43). Both of these studies indicated that the (+)-isomers are metabolized via two different biochemical routes. The (+)-isomer was hydroxylated on the glutarimide ring at the 4-position to yield 4-hydroxy-2-ethyl-2-phenylglutarimide, a small percent of which undergoes dehydration to give 2-ethyl-2-phenylglutaconimide. The (-)-isomer predominantly hydroxylated on the ethyl side chain to form 2-(1-hydroxyethyl)-2-phenylglutarimide, of which a small percent lose a moleculae of acetaldehyde to form 2-phenylglutarimide, Fig. (8). In 1969, Post and Schütz (44) identified a phenolic metabolite which they assumed to be 2-(4-hydroxyphenyl) glutarimide.

Recently Stillwell et al. (45) identified more polar metabolites present in the urine of rats and guinea pigs, (Fig. 9). They characterized these metabolites by combined GC/MS as follows:

a. 4-hydroxy-2-ethyl-2-phenylglutarimide
b. 3-hydroxy-2-ethyl-2-phenylglutarimide
c. 2-(1-hydroxyethyl)-2-phenylglutarimide
d. 2-ethyl-2-(4-hydroxyphenyl)glutarimide
e. 2-ethyl-2-(3,4-dihydroxyphenyl)glutarimide
f. 2-ethyl-2-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)glutarimide
g. 2-ethyl-2-phenylglutaconimide
h. 2-phenylglutarimide

They demonstrated that the hydroxylation of the aromatic ring in the rat and guinea pig occurs via an epoxide pathway. These authors also supported the fact that the rat and guinea pig, like the dog and man, metabolize the (-)-isomer of glutethimide by hydroxylation of the ethyl side chain to form 2-(1-hydroxyethyl)-2-phenylglutarimide. However, the metabolism of the (+)-

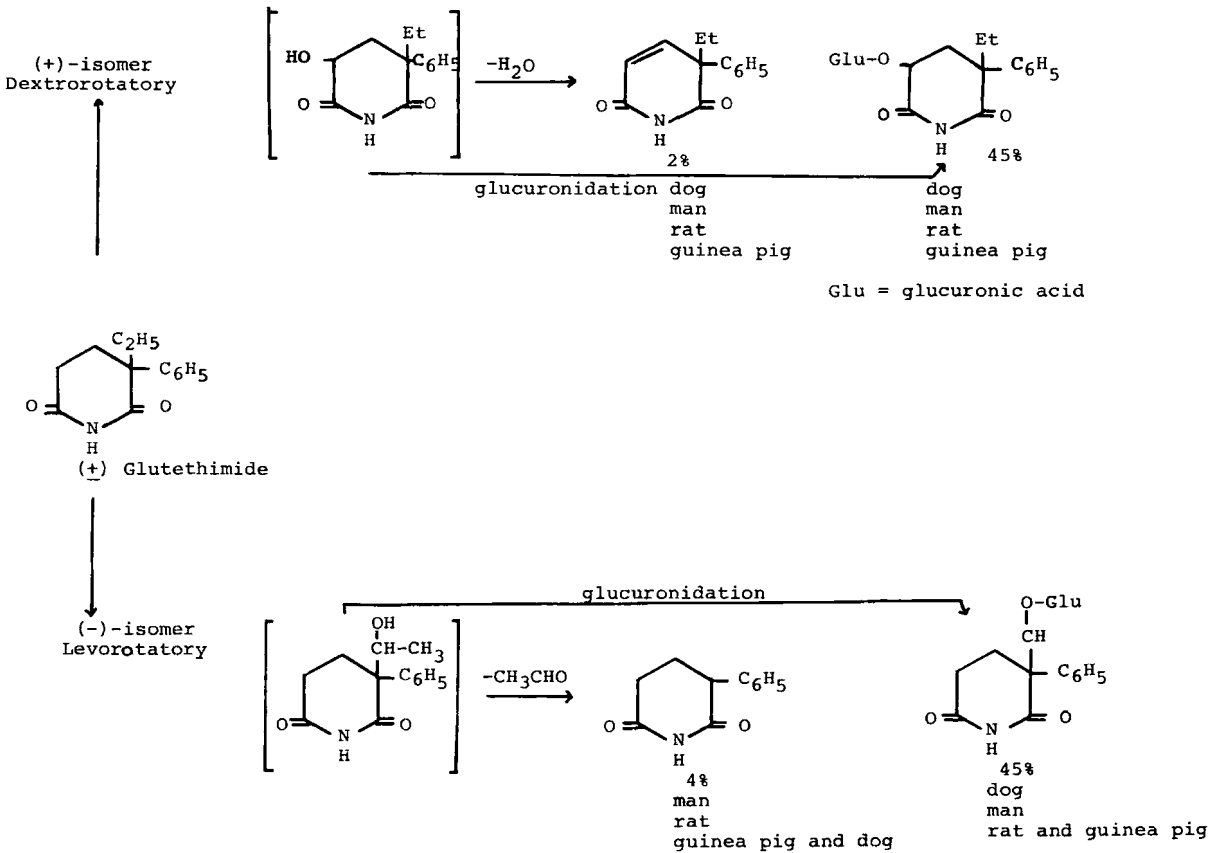


Fig. 8 Metabolism of Glutethimide

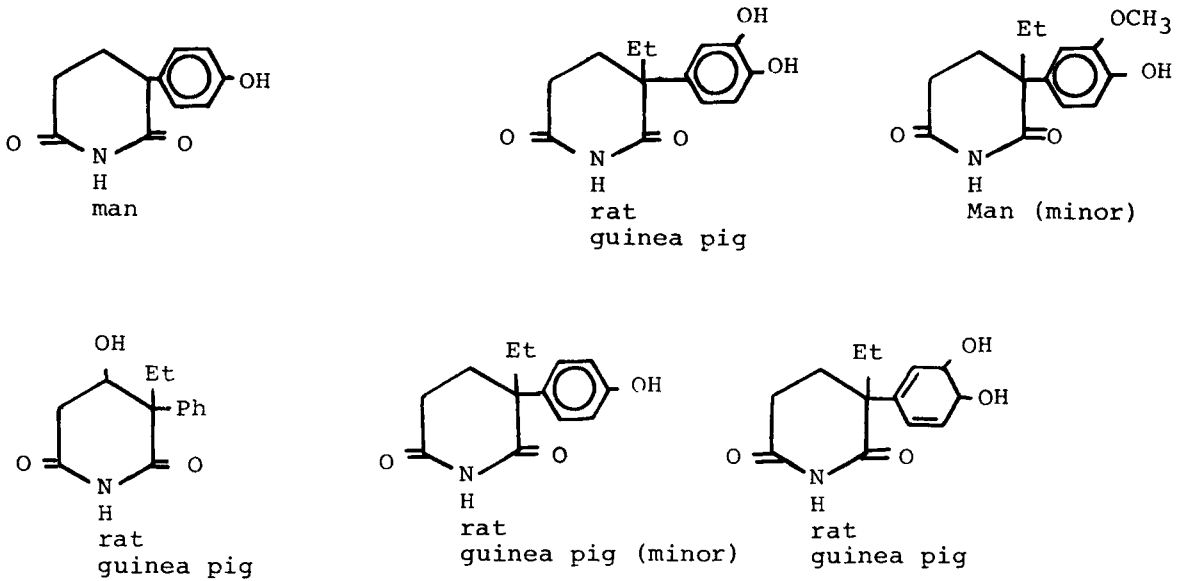


Fig. 9 Polar Metabolites of Glutethimide

isomer seems to be species dependent. In contrast to man and dog, the rat apparently metabolized the (+)-isomer of glutethimide by hydroxylating the aromatic as opposed to the heterocyclic ring to form 2-ethyl-2-(4-hydroxyphenyl)-glutarimide, however, some hydroxylation of the heterocyclic ring occurs. The guinea pig hydroxylates both the aromatic and the heterocyclic rings and these metabolites are presumably formed from the (+)-isomer. Further studies on the metabolism of the (-) and (+) isomers by the rat and guinea pig are needed to validate these conclusions.

Ambre and Fischer (46) showed that monohydroxylated metabolite of glutethimide accumulated in the plasma of humans intoxicated with glutethimide overdose. This metabolite was subsequently isolated from urine of dogs given large doses of glutethimide and was chemically identified as 4-hydroxyglutethimide (47). This metabolite was found to possess twice the sedative-hypnotic activity of glutethimide (47).

Recently, it was shown that the concentration of 4-hydroxyglutethimide is highest when the respiratory status of the patient was near its lowest (48, 49). This led to the conclusion that 4-hydroxyglutethimide accumulation plays an important role in acute glutethimide poisoning. 4-Hydroxyglutethimide and other potential glutethimide metabolites were chemically synthesized and pharmacologically tested by Aboul-Enein et al. (50).

Among other metabolites previously identified in animals and man, Andreson et al. (51) recently reported the identification and chemical characterization of 2-ethyl-2-(4-hydroxyphenyl) glutarimide in human urine from patients overdosed with glutethimide as a major metabolite. They also characterized 2-ethyl-2-(3-methoxy-4-hydroxyphenyl)-glutarimide as a new metabolite of glutethimide.

The toxic effects possibly caused by the metabolites of glutethimide has interested many research laboratories to continue investigating this problem.

6. Methods of Analysis

6.1 Titrimetric Methods

6.11 Aqueous

A titrimetric method was developed for analysis of glutethimide. The procedure involves the alkaline hydrolysis of glutethimide with standard alcoholic potassium hydroxide and subsequent back titratation of the unconsumed alkali with standard hydrochloric acid using phenolphthalein as indicator (52), a method adapted by the British Pharmacopeia (22).

6.12 Non-Aqueous

Ellert et al. (53) described a method for determination of glutethimide by non-aqueous titration with 0.1N sodium methoxide in methanol-benzene in a solution of ethylenediamines (against 0-nitroaniline) or in pyridine (against Azo Violet). Glutethimide can also be quantitated in tablet formulation by non-aqueous titration in dimethyl formamide and titrating with propanol-benzene 0.1N potassium hydroxide (using metanil yellow as indicator) (54).

6.2 Colorimetric

6.21 Qualitative

The following color reaction is published in the B.P. 1973 (22) as part of an identification scheme.

Shake 10 mg with 2 ml of water, 0.1 g of hydroxylammonium chloride and 1 ml of sodium hydroxide solution, allow to stand for ten minutes and add 2 ml of dilute hydrochloric acid and 1 ml of ferric chloride test-solution; a deep brownish-red color is produced.

6.22 Quantitative

Sheppard (39) and associates utilized a method based on the formation of the colored complex between ferric ion and the hydroxamate resulting from the reaction of glutethimide with alkaline hydroxylamine for determination of the drug in urine. The color was read within five minutes at 510 nm. The presence of lactose and anions which complex with ferric ion or salts which form precipitates with ferric ion interfere with this method. Phang et al. (55) introduced a modification to Sheppard's procedure so the fatty impurities do not interfere with the quantitation of glutethimide in vomitus material.

Belova and Zinakova (56) reported a similar colorimetric method in which the color developed was measured at 490 nm with a no. 5 light filter and claimed that the presence of lactose does not interfere with the result of glutethimide determination.

This procedure, however, is not very sensitive or applicable to blood specimens and is relatively non-specific (57).

6.3 Polarographic Analysis

Glutethimide failed to have polarographic wave on a.c. and d.c. polarograms using 0.1M lithium chloride, or tetramethylammonium chloride as supporting electrolyte (58).

Recently, a detailed study of an indirect polarographic determination of glutethimide after nitration was reported by Lauermann (59). The method can be used to quantitate glutethimide in post-mortum tissues and biological fluids, with sensitivity of 0.8 mg/100g of material. The conversion of the phenyl group to nitrophenyl group after nitration makes it possible to utilize the polarographic technique in analysis of glutethimide.

6.4 Ultraviolet Spectrophotometric

The ultraviolet absorption of glutethimide in methanol at λ_{max} 257 nm (60) is used as a sensitive criteria for its analysis. This method is sensitive to a concentration range of 50-500 μg/ml (61).

Ultraviolet spectroscopy has been extensively applied to glutethimide determination in biological fluids such as (62-64) serum and urine and pharmaceutical preparations (54, 65) with high degree of sensitivity.

6.5 Fluorometric Analysis

A fluorometric procedure was described for the quantitative determination of glutethimide in table formulations (66). The method is based on the fluorogen resulting from the reaction of glutethimide with conc. sulfuric acid containing formaldehyde. The fluorescent intensity is a straight line function of concentration over a wide range. The excitation maxima were observed at 280 and 365 nm and the corresponding maximum fluorescent emission occurred at 450 nm. The procedure is applicable for the determination of glutethimide in the presence of its degradation product 4-ethyl-4-phenylglutaramic acid, since the latter yields only 1/10 the fluorescence of glutethimide. The fluorescent reaction does not occur with compounds lacking a phenyl group or containing a substituted phenyl ring and there is no interference with the tablet excipients.

6.6 Chromatographic Analysis

6.61 Paper

The chromatographic behavior of glutethimide and related analogs was established by Davies and Nicholls (67) without interference from the chemically related barbiturates by ascending paper chromatography. Whatman No. 1 paper was used and some paper was impregnated with liquid paraffin (4% in hexane), olive oil

(20% in acetone) or tributyrin (10% in acetone). All compounds were applied in amounts of 100 µg by the window technique.

Several reagents were used to detect glutethimide after chromatography. These include the hypochlorite reagent by Creig and Leaback (68), alkaline, hydroxylamine spray of Sheppard et al. (39), and 1% $HgNO_3$ solution (69). Some of the most useful solvent systems are shown in Table V.

Table V

Solvent System		Reference
PhMe-HOAc-H_2O	10:5:4	67
CCl_4-HOAc-H_2O	1:2:1	67
aq. NaCl	10% w/v	67
0.066M Na_3PO_4(pH 7.3 on tributyrin paper		67

A method for rapid detection of glutethimide and other hypnotic was described (70) using pigment impregnated paper, and a combination of 2 dimensional chromatographs. Solvent system used in this technique were piperidine-petroleum ether (1:9) followed by cyclohexane, $CHCl_3$ (1:3) or solvent system piperidine-petroleum ether (1:9) followed by benzene: $CHCl_3$ (1:5).

The applicability of pigment impregnated paper for the resolution of hypnotics in cadaveric material was also discussed.

Kloecking (69) described the use of wedge-strip procedure to achieve better separation of glutethimide and other barbiturates in chemical-toxicological analysis than the usual ascending descending chromatograms. A good separation was obtained by using ascending chromatograms with dioxane-xylene-toulene-isopropanol-25% NH_3 (1:2:1:4:2) upper phase on Schleicher and Schnell 2045 Bgl paper for 24

hours at 20°. Other pertinent information in this regard is to be found in references (71-74).

6.62 Thin Layer

Several thin layer chromatographic systems have been developed to study various aspects of glutethimide. Several procedures were described for its detection, identification in biological fluids, forensic and toxicological analysis, (82-90). Several solvent systems used for its identification and semiquantitation of glutethimide are shown in Table VI.

6.63 Gas Chromatography

Many investigators have used gas chromatography to analyze glutethimide. Although most of the published work is concerned almost entirely with analysis of glutethimide and its metabolites in biological fluids and tissues, many of the methods could be modified easily for analysis of glutethimide in pharmaceutical dosage forms.

A number of the systems used for GC are listed in Table VII. Berry (91) conducted in-depth study for the gas chromatographic behavior of glutethimide in presence of several barbiturates on 12 GC columns. OV-225 and CDMS were the most useful of the columns tested for analysis of the barbiturates, glutethimide and interfering drugs at therapeutic and overdose levels in plasma. The method was sensitive, rapid and suitable for emergency toxicological cases.

Bloomer et al. (92) developed a rapid method for diagnosis of several acidic and neutral sedative-hypnotics using GC in intoxicated patients. The procedure permits simultaneous identification and measurements of a

Table VI

Solvent System	Developer	R_f	Reference
$CHCl_3$:Et_2O 85:15	KI-benzidine	0.66	75
$CHCl_3$:EtOH 90:10	KI-o-tolidine HCl (after treatment with gaseous Cl_2)	0.96	75
EtOAc:MeOH:NH_3 85:5:2.5	0.1% $KMnO_4$ and 1% Ag NO_3	0.89	76
$CHCl_3$:Me_2CO 9:1	1% $HgSO_4$	R_f were reptd. on various commerc. avail. silica gel tlc, plates, sheets, films.	77
EtOAc:MeOH:NH_4OH 85:10:5			77
Cyclohexane:C_6H_6:Et_2NH 15:3:2			77
$CHCl_3$:Me_2CO 9:1	0.2% aq. solution $CuSO_4$ with pyridine (50:4)-grey color developed.		78
IsoPrOH:$CHCl_3$:25%NH_4OH 45:45:10		0.84	79

Table VI (cont'd)

Solvent System	Developer	R_f	Reference
EtOAc:cyclohexane-dioxane: MeOH:H_2O:NH_4OH 50:50:10:10: 1.5:0.5 50:50:10:10:0.5:1.5			79
EtOAc-cyclohexane:MeOH:NH_4OH 56:40:0.8:0.4 70:15:10:5	0.1% diphenyl carbazone (orange pink-spot); 1% AgOAc (blue-purple spot; $HgSO_4$ (purple fades away).	0.89	79
EtOAc:cyclohexane:NH_4OH 50:40:0.1			79
EtOAc:cyclohexane:NH_4OH: MeOH:H_2O 70:15;2:8:0.5			79
$CHCl_3$:BuOH:HCO_2H 70:40:3.5 (chamber saturated with NH_4OH)	1% diphenyl carbazone; Hg $(NO_3)_2$		80
EtOAc (redistilled) Dioxane: CH_2Cl_2:H_2O 1:2:1 $CHCl_3$:Me_2CO 9:1	UV at 254 nm Cl_2 and starch KI solution	0.68 0.96 0.55	81

Table VII
Gas Chromatographic Systems Used for Glutethimide Analysis

All systems listed used flame ionization detectors.

Column	Carrier Gas	Column Temp, C°	Ref.
3% OV-17 on Supelcoport (100-120 mesh) (derivatized with dimethylformanide dimethyl acetal).	55 ml/min He	160-220° at 7°/min	95
3% OV-1 on 100-120 mesh Gas Chrom Q	30 ml/min He	120-220° at 10°/min	96
4% OV-17 on 80-100 mesh Chromosorb WHPAW-DMSC	15 psi N_2	183	97
3.8% SE-30 on 80-100 mesh Chromosorb WHPAW-DMSC	25 psi N_2	183	97
10% Dexsil 300 on 800-100 mesh Chromosorb WHP	N_2	220	98
17% Dexsil 300 on 80-100 mesh Chromosorb WHP	N_2	220	98
1% Carbowax 20M*	50-60 ml/min N_2	205	91

* on 80-100 mesh Chromosorb W, HP.

Table VII (cont'd)

Column	Carrier Gas	Column Temp, C°	Ref.
3% Poly A-103*	50-60 ml/min N_2	215	91
3% NGA+0.07% trimer acid*	50-60 ml/min N_2	230	91
1% SP-1000*	50-60 ml/min N_2	230	91
3% PPE-20*	50-60 ml/min N_2	200	91
10% Apiezon L*	50-60 ml/min N_2	190	91
4% CDMS*	50-60 ml/min N_2	220-240	91
3% OV-1*	50-60 ml/min N_2	155	91
5% OV-17*	50-60 ml/min N_2	200	91
3% OV-25*	50-60 ml/min N_2	165	91
4% OV-210*	50-60 ml/min N_2	160	91
4% OV-225*	50-60 ml/min N_2	205	91
3% OV-225 on 80-100 mesh Chromosorb W	40 ml/min N_2	200	99

Table VII (cont'd)

Column	Carrier Gas	Column Temp, C°	Ref.
8% X F 1112 on 60-80 mesh Chromosorb WHMDS	25 ml/min N_2	195	100
7% DC-200 on Gas Chrom Q (80-100 mesh) derivatized as N methyl derivative	50 ml/min He	190	101
5% SE-30 on 70-80 mesh AW Chromosorb W	60 ml/min N_2	195	102

* on 80-100 mesh chromosorb, W, HP.

variety of sedative agents. By virtue of acid-base extraction with chloroform, the acidic and neutral agents are separated. The chloroform extracts were dried, evaporated and the residue dissolved in isopropanol. Column used was 3% SE-30 on 80-100 mesh Chromosorb W.

Gel filteration using Sephadex LH-20 was used to remove impurities from chloroform extracts of acidified blood, urine and homogenized tissue sample. Quantitative determination was made by GC using 15% SE-30 in Chromosorb W at column temperature of 150^{o}-250^{0} (93).

Fischer and Ambre (94) showed that analysis of urine from patients intoxicated with glutethimide on columns containing SE-30, OV-1 and OV-17 lead to an overestimation of the unchanged drug in urine. These columns were considered non-selective. However, 3% OV-225 on 80-100 mesh Supelcoport and 2% Carbowax 20M on 100-120 mesh Supelcoport were selective to separate the drug from its potentially interfering metabolites and thus, eliminates the possible overestimation of glutethimide in biological samples.

Several gas chromatographic methods for measurement of glutethimide in biological fluids are reported employing a variety of extraction procedures and gas chromatographic conditions (103-123).

6.7 High Voltage Electrophoresis

Hoffman et al. (124) described a method for the separation, detection and semiquantitation of glutethimide and other barbiturates from urine and other biological fluids using high voltage electrophoresis. It was reported that glutethimide migrates at the cathode side at a distance of 0.22 relative to crotylbarbital.

6.8 Biological Assay

Glutethimide can be detected by hemagglutination inhibition method described by Valentour et al. (125). Antisera from rabbits injected with glutethimide-bovine serum albumin (10 mg/ml) were sensitive enough to detect 50 ng glutethimide /0.1 ml plasma or urine.

The method was used to analyze urine and plasma patients suspected of glutethimide intoxication.

6.9 Nuclear Magnetic Resonance

Aboul-Enein (126) has reported a method for quantitative determination of glutethimide by NMR both in powder and tablet formulations. The method offers a rapid, accurate procedure for analysis of the drug. Furthermore, it provides a confirmatory identification for glutethimide.

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LEVODOPA

Ralph Gomez, Robert B. Hagel, and Edward A. MacMullan

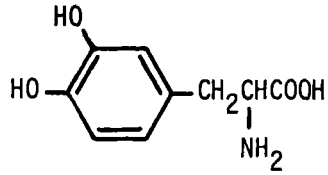
INDEX

Analytical Profile - Levodopa

1. Description

1.1 Name, Formula, Molecular Weight

Levodopa is (-)-3-(3,4-Dihydroxyphenyl)-L-alanine.



1.2 Appearance, Color, Odor

Levodopa is an odorless white to off-white crystalline powder.

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of levodopa is shown in Figure 1 (1). The spectrum was recorded on a Perkin-Elmer Model 621 Grating Infrared Spectrophotometer and was measured in a KBr pellet which contained 1 mg of levodopa in 300 mg of KBr.

The following absorptions have been assigned for Figure 1:

a. OH stretching (bonded): 3375 cm^{-1}, 3210 cm^{-1}
b. NH_3^+: 3070 cm^{-1}, 2700-2300 cm^{-1} (broad)
c. COO^-: 1656 cm^{-1}, 1569 cm^{-1}
d. Aromatic CH out of plane bending of two adjacent free H's: 821 cm^{-1}, 816 cm^{-1}

2.2 Nuclear Magnetic Resonance Spectrum (NMR)

The NMR spectrum of levodopa, recorded on a JEOL C60HL spectrometer, is shown in Figure 2 (2). The spectrum was recorded using a solution of 60 mg of levodopa/0.4 ml D_2O + 0.1 ml DCL. The spectral assignments are listed in Table I.

FIGURE 1

Infrared Spectrum of Levodopa

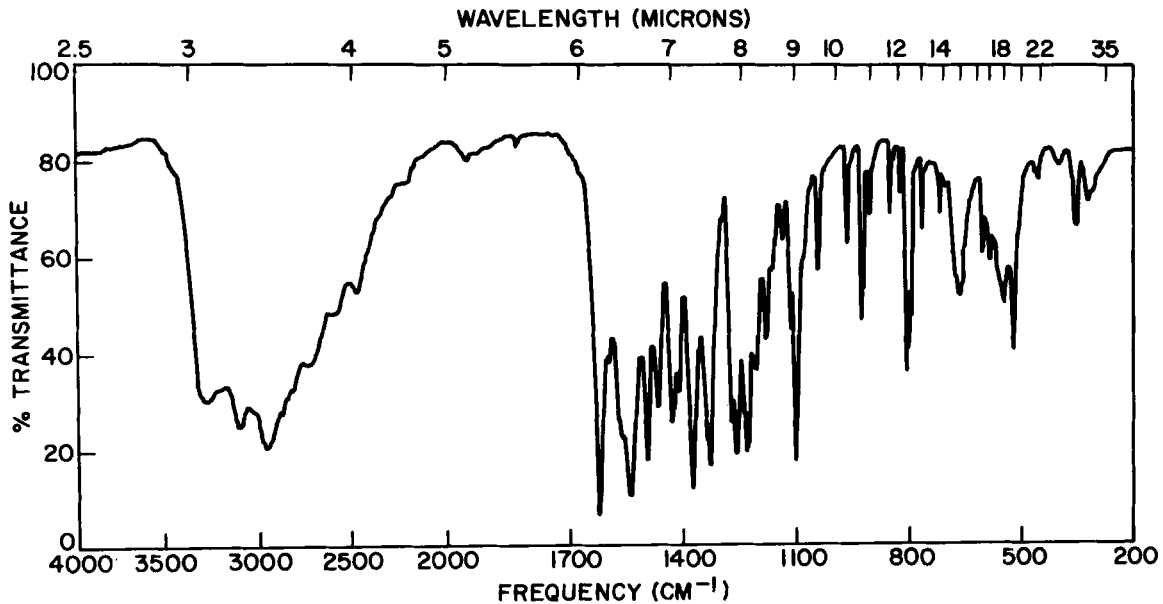


FIGURE 2

NMR Spectrum of Levodopa

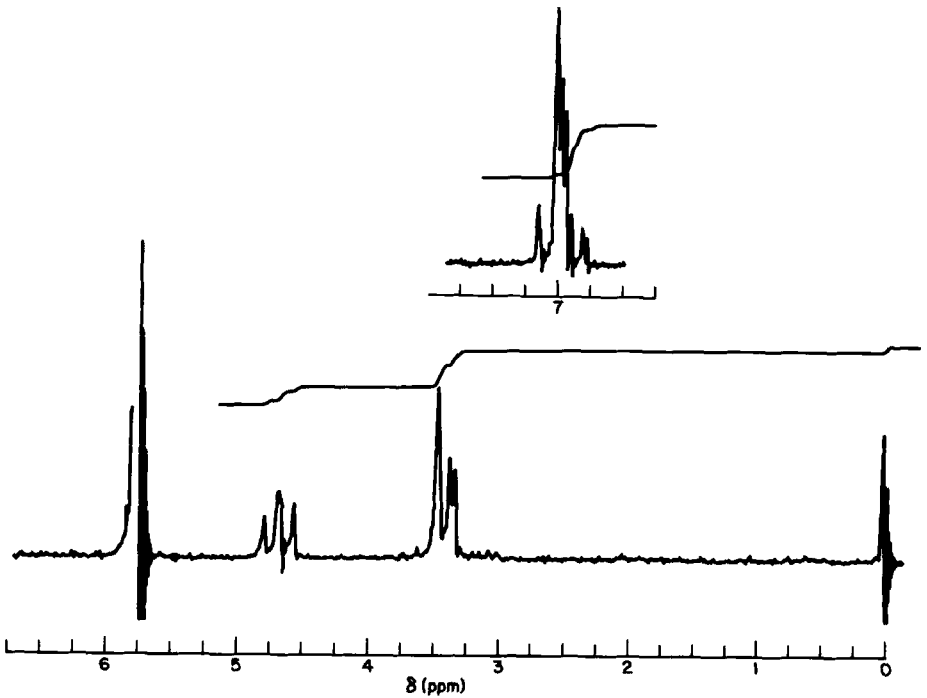
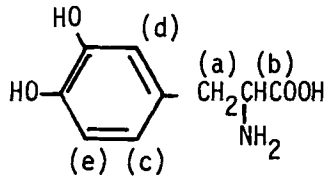


TABLE I

NMR Spectral Assignments for Levodopa



Proton	Chemical Shift (δ)	Spectral Structure (J in Hz)
a	3.22 ppm	Multiplet (7.5)
b	4.44 ppm	Triplet
c	6.80 ppm	Doublet (2 sets; 2.0, 8.0)
d	6.92 ppm	Doublet
e	7.05 ppm	Doublet

The remaining protons exchange in the deuterated solvent used.

2.3 Ultraviolet Spectrum

The ultraviolet spectrum of levodopa (4 mg of levodopa/100 ml of 0.1N hydrochloric acid) in the region of 230 to 370 nm exhibits one maximum at 280 nm (ε = 2.8×10^3) and one minimum at 250 nm. The spectrum is shown in Figure 3 (3).

2.4 Fluorescence Spectrum

The excitation and emission spectra of levodopa are presented in Figure 4 (4). The sample was dissolved in methanol at a concentration of 1.43 mg of levodopa/ml and the spectra were recorded using a Farrand MK-1 recording spectrofluorometer. Levodopa exhibits excitation maxima at 236 nm and 286 nm and emission peaks at 320 nm and 620 nm.

2.5 Mass Spectrum

The low resolution mass spectrum of levodopa is shown in Figure 5 (5). The spectrum was obtained using a

FIGURE 3

Ultraviolet Spectrum of Levodopa

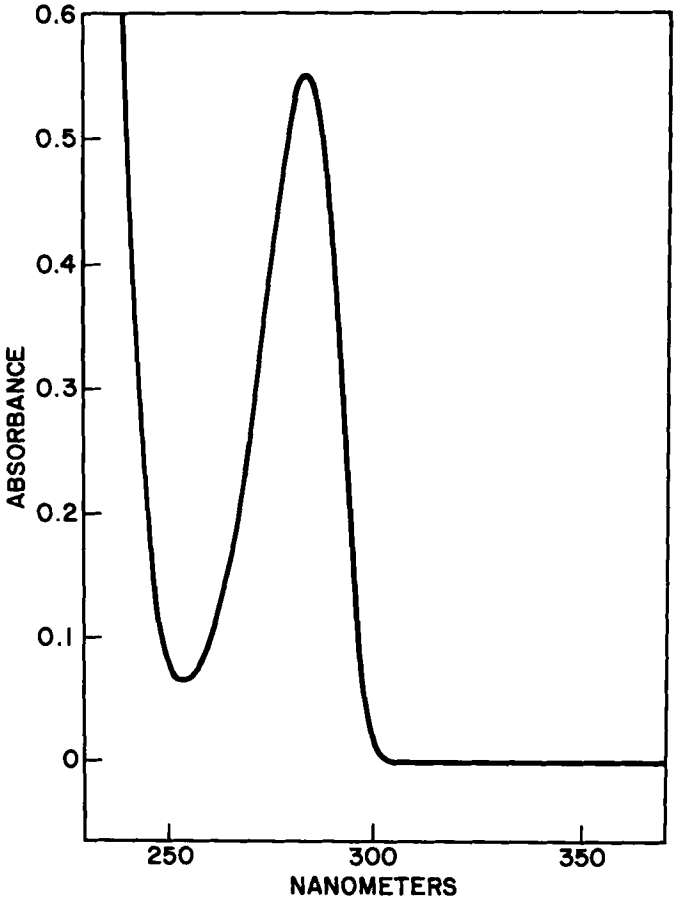


FIGURE 4

Fluorescence Spectrum of Levodopa

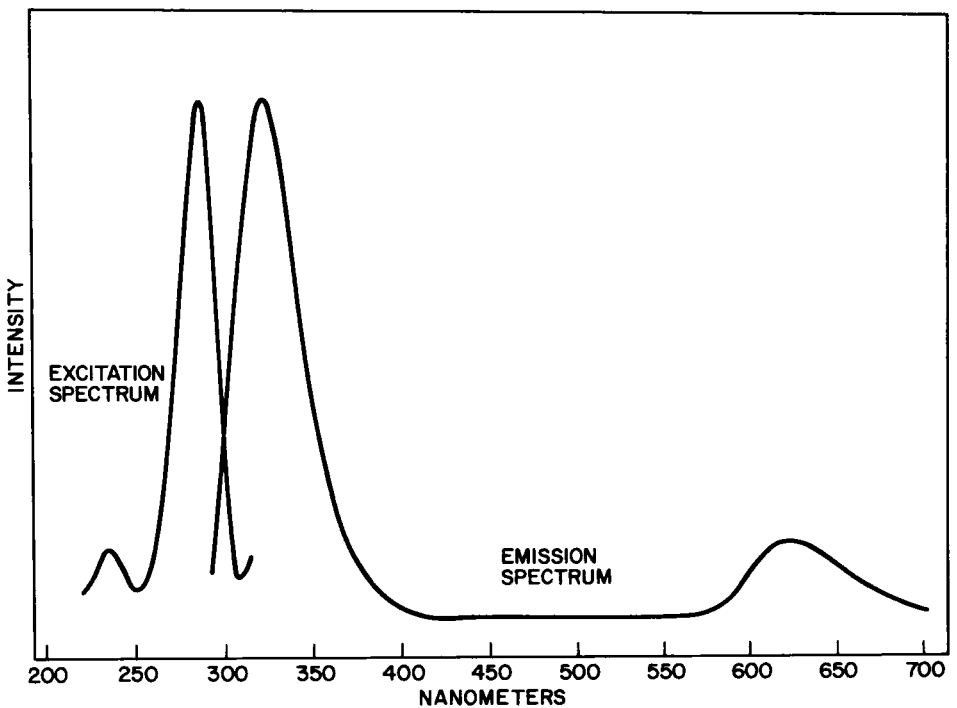
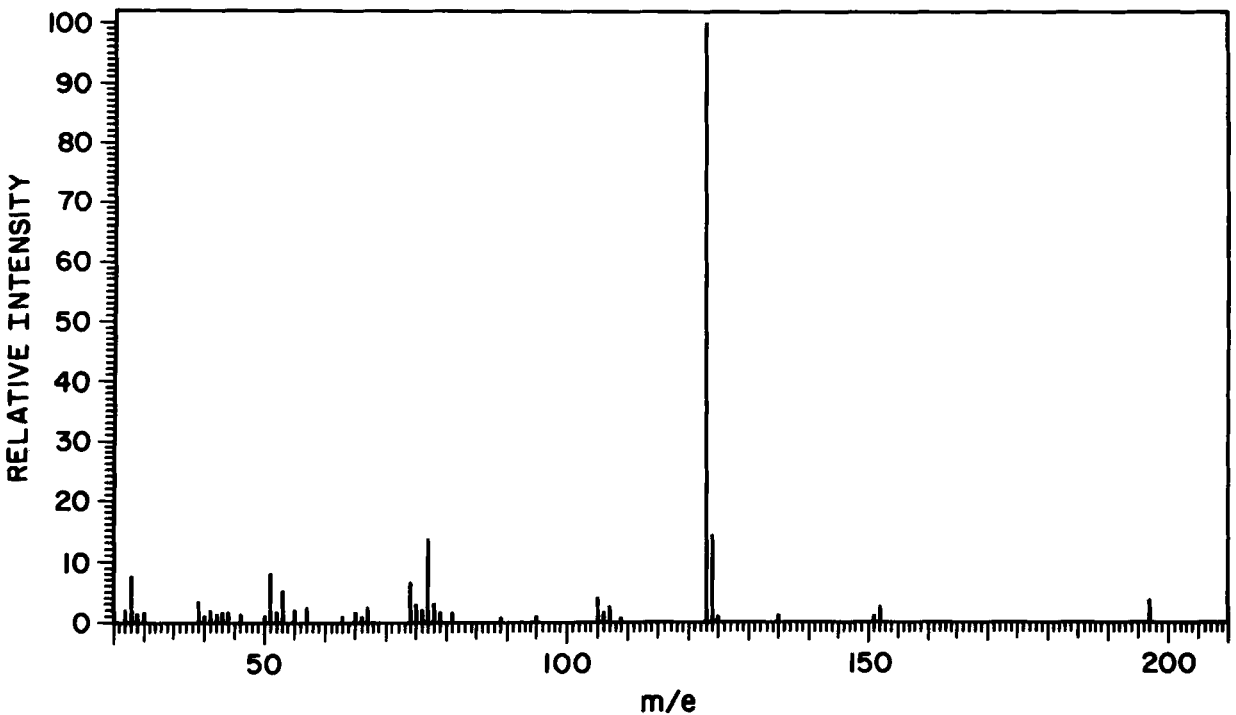


FIGURE 5

Mass Spectrum of Levodopa



Varian MAT spectrometer with an ionizing voltage of 70 eV, which was interfaced with a Varian data system 620I. The data system accepts the output of the spectrometer, calculates the masses, compares the intensities to the base peak and plots this information as a series of lines whose heights are proportional to the intensities.

The molecular ion for levodopa was measured at m/e = 197. Other characteristic masses were observed at m/e = 179 corresponding to the loss of H_2O from the molecular ion, m/e = 152 which corresponds to the loss of COOH from the parent mass, and m/e = 123, the base peak, which is the 3,4 dihydroxyphenyl moiety. A high resolution scan confirmed the results of the low resolution spectrum. Table II lists the elemental compositions for the ions as determined by high resolution mass spectrometry.

TABLE II

High Resolution Mass Spectrum of Levodopa

Found Mass	Calcd. Mass	C	H	N	O
197.0678	197.0689	9	11	1	4
179.0540	179.0583	9	9	1	3
177.0137	177.0189	9	5	0	4
175.9964	175.9984	8	2	1	4
165.0149	165.0188	8	5	0	4
161.0472	161.0477	9	7	1	2
152.0722	152.0713	8	10	1	2
151.0633	151.0634	8	9	1	2
139.0207	139.0270	6	5	1	3
136.0537	136.0525	8	8	0	2
134.0612	134.0580	5	10	0	4
132.0446	132.0423	5	8	0	4
130.0032	130.0055	8	2	0	2
127.0162	127.0184	9	3	0	1
123.0415	123.0447	7	7	0	2

2.6 Optical Rotation

The specific rotation of an aluminum complex of levodopa in an acetate buffer at 25°C is plotted versus

wavelength in Figure 6 (6). The specific rotation observed at 589 nm was approximately -41° while the rotation at 365 nm was approximately -122°. In 0.1N hydrochloric acid the specific rotation of levodopa at 589 nm has been reported to be -11° (7).

Chafetz and Chen (8) have reported the specific rotation of an acidified solution of levodopa containing methenamine to be approximately -165° at 589 nm.

2.7 Melting Range

Levodopa melts with decomposition above 270°C (9).

2.8 Differential Scanning Calorimetry

An endotherm was obtained in the 290°-300°C region where melting, accompanied by sample decomposition, occurred. The temperatures observed for the decomposition transitions are dependent on instrumental conditions as well as sample size and cannot be considered characteristic for the compound (10).

2.9 Thermogravimetric Analysis (TGA)

A TGA scan showed no loss of weight as the temperature increased from 30° to 270°C at a rate of 10°C/minute (10).

2.10 Solubility

The approximate solubility data obtained for a sample of levodopa at 25°C is listed in Table III (11). The equilibration time was 20 hours for each system.

TABLE III

Solubility Data for Levodopa

Solvent	Solubility (mg/ml)
Water	3.0
95% Ethanol	0.3
3A Alcohol	0.15

FIGURE 6

Specific Rotation Versus Wavelength for Levodopa

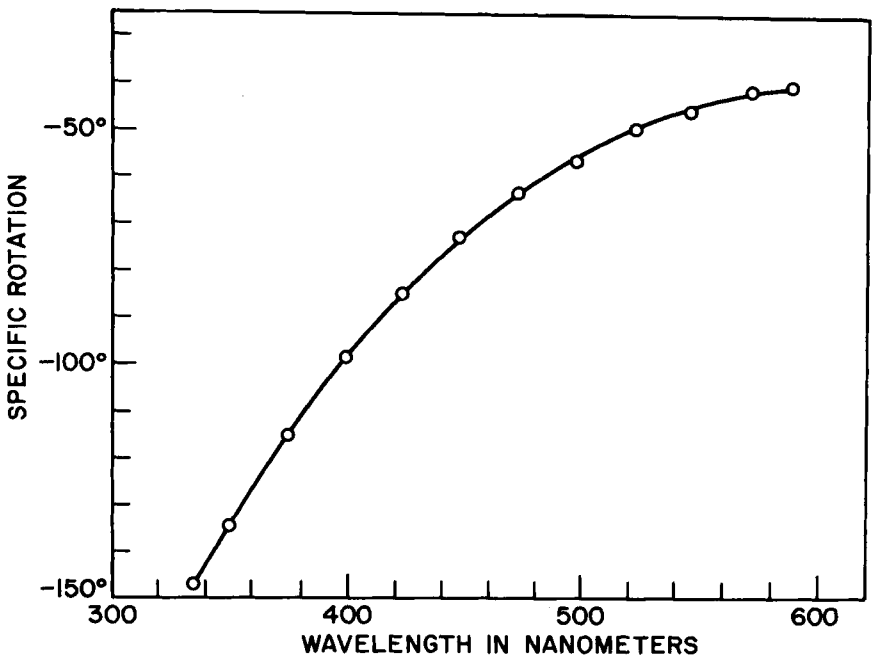


TABLE III (cont.)

Solubility Data for Levodopa

Solvent	Solubility (mg/ml)
Methanol	0.1
2-Propanol	<0.01
Chloroform	0.1
Diethyl Ether	<0.01
Petroleum Ether (30°-60°C)	<0.01
Benzene	<0.01
Dimethylacetamide	<0.01
Propylene Glycol	0.2
Benzyl Alcohol	<0.01
Acetone	0.03
Acetonitrile	0.07

2.11 Crystal Properties

2.111 X-Ray Diffraction

The X-ray powder diffraction data for levodopa are presented in Table IV (12); instrumental conditions are given below.

Instrument and Operating Conditions

Instrument	GE Model XRD-6 Spectrogoniometer
Generator	50 KV, 12.5 mA
Tube Target	Copper (Cu $K\alpha$ = 1.5418 Å)
Optics	0.1° Detector slit M.R. Soller slit 3° Beam slit 0.0007" Ni Filter 4° Take off angle
Goniometer	Scan at 0.2° 2θ per minute
Detector	Amplifier gain-16 coarse; 8.7 fine. Sealed proportional counter tube and DC voltage at plateau. Pulse height selection E_1 5 volts, Eu out. Rate meter T.C. 4, 2000 c/s full scale.

Recorder	Chart speed 1"/5 minutes.
Samples	Prepared by grinding at room temperature.

TABLE IV

Levodopa Powder Diffraction Data

2θ	d (Å)*	I/Io**
6.54	13.52	4
13.08	6.77	2
14.75	6.01	4
15.31	5.79	2
16.85	5.26	20
17.91	4.95	4
18.45	4.81	53
19.75	4.50	14
21.21	4.19	100
22.38	3.97	21
22.73	3.91	42
23.05	3.86	38
23.85	3.73	3
24.91	3.58	60
25.86	3.45	55
26.35	3.38	6
26.92	3.31	28
28.57	3.13	10
29.61	3.02	9
31.25	2.86	21
33.19	2.70	8
33.64	2.66	12
34.31	2.61	6
35.43	2.53	5
36.15	2.49	6
36.28	2.48	21
37.25	2.41	9
37.74	2.38	25
38.34	2.35	8
39.35	2.29	5
40.04	2.25	10

TABLE IV (cont.)

Levodopa Powder Diffraction Data

2Θ	d (Å)*	I/Io**
40.80	2.21	6
41.24	2.19	8
41.82	2.16	8

*d = $\frac{n \lambda}{2 \sin \Theta}$ (interplanar distance)

**I/Io = percent relative intensity (based on maximum intensity of 1.00)

2.112 Crystal Structure

Levodopa has been observed to exist as needles and plates. Rapid recrystallization from water at low temperatures without agitation produced the needle-like crystal habit. The needles were found to exist in two crystalline forms (13).

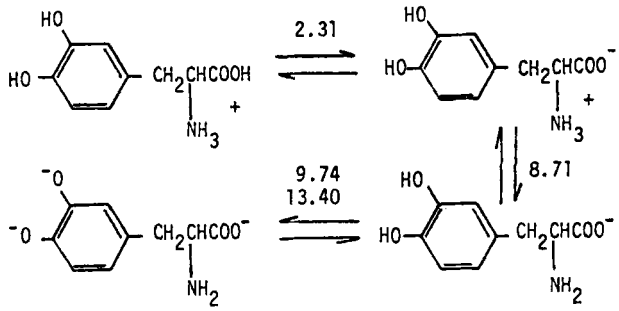
Mostad, et al. (14) have determined the structures of two monoclinic crystal forms of levodopa. A stable form was produced by the slow diffusion of absolute alcohol into a half-saturated solution of levodopa in formic acid. The cell dimensions are as follows: a = 13.629 Å; b = 5.308 Å; c = 6.049 Å; and β = 97.53°.

A less stable form of levodopa was obtained as thin needles by using ethyl ether as a precipitating agent. This form is not stable when separated from the mother liquor. Its cell dimensions are: a = 16.9 Å; b = 5.88 Å; c = 9.0 Å; and β = 99°.

Additional data on crystal structures have been reported (15, 16).

2.12 Dissociation Constants

The dissociation constants for levodopa have been determined titrimetrically and are reported as follows (17):



3. Synthesis

Yamada, et al. (18) report the synthesis of levodopa starting with three different compounds: 3-(3,4-methylenedioxyphenyl)-L-alanine (I); N-acetyl-3-(3,4-methylenedioxyphenyl)-L-alanine (II); or N-acetyl-3-(3,4-methylenedioxyphenyl)-L-alanine *l*-menthyl ester (III). The starting material, red phosphorous and a mixture of HI and acetic anhydride are refluxed for several hours to give (-)-3-(3,4 dihydroxyphenyl)-L-alanine. The reaction scheme is shown in Figure 7.

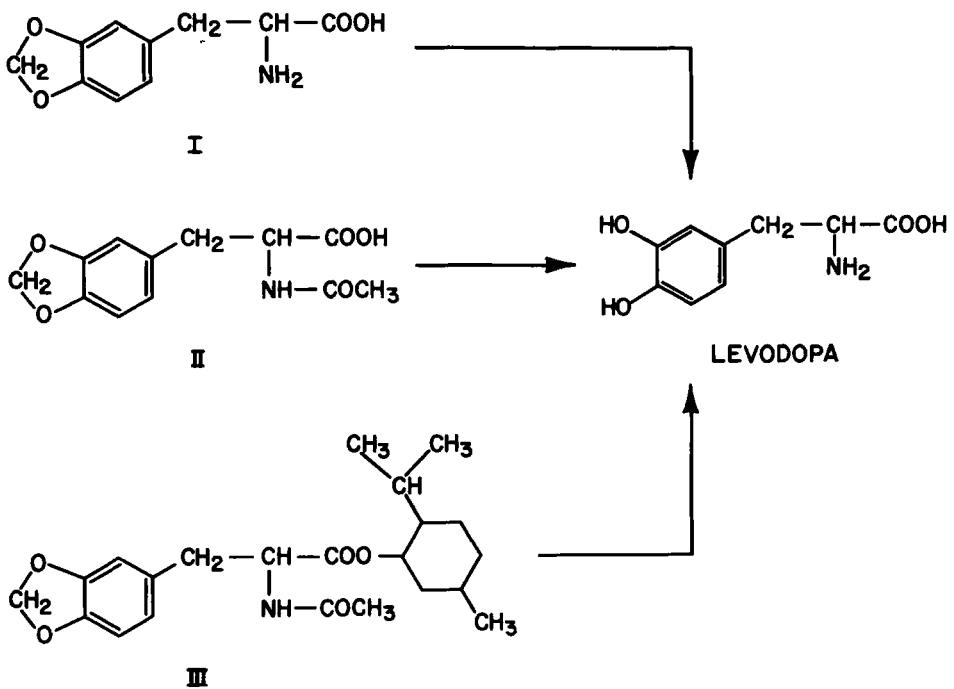
4. Separation of Racemates

Methods described in the literature for the separation of DL-3-(3,4-dihydroxyphenyl)alanine into its optical isomers generally involve the resolution of an intermediate in the synthesis. The optically pure intermediate is then reacted further to give pure levodopa or D-Dopa (19-24).

A procedure has been described which was used to resolve DL-3-(3,4-dihydroxyphenyl)alanine into its optically active antipodes (25). A supersaturated solution of the racemic mixture was seeded with crystals of one pure enantiomorph which crystallized pure enantiomorph. The mother liquor was transferred to a separatory vessel, heated and treated with fresh finely ground racemic mixture; the amount added was approximately twice the amount of pure enantiomorph obtained in the first recrystallized stage. A preferential solution of the deficient enantiomorph occurred which left the antipode behind as a crystalline product. The method

FIGURE 7

Synthesis of Levodopa



is applicable only when the optically active antipodes form a racemic mixture and not a compound.

5. Stability and Degradation

Levodopa, in the presence of moisture, is rapidly oxidized by atmospheric oxygen and turns green (9). The oxidation of levodopa in basic solution results in the formation of melanin and related intermediates (26). The stability of levodopa in the bulk form was studied under conditions of elevated temperature by storing samples at 105°C for varying periods of time. The samples were examined for evidence of degradation by measuring the color of a 10% w/v solution in 10% hydrochloric acid and by thin-layer chromatography. The color of the solutions showed some darkening after 24 hours, and increased with increasing heating time. However, no degradation was detected in these solutions by thin-layer chromatography (27).

6. Drug Metabolic Products

The major metabolites of levodopa in humans have been reported to be 3-methoxy-4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, dopamine, and 3-methoxytyrosine (28-33). Barbeau (34) has reported that patients given levodopa show an increase in the excretion of methylated derivatives such as o-methyldopa, 3-methoxytyramine and 5-hydroxyindoleacetic acid. Wada and Fellman (35) have presented evidence that 2,4,5-trihydroxyphenylacetic acid is a metabolic product of 3,4-dihydroxyphenylpyruvate, a levodopa metabolite. Gjessing and Borud (36) and O'Gorman, et al. (30) have also studied the metabolic fate of levodopa in humans. Figure 8 shows the metabolism of levodopa as described by them. The chemical names of the compounds in Figure 8 are listed in Table V.

TABLE V

Metabolites of Levodopa Referred to in Figure 8

I.	3,4-dihydroxyphenylalanine
II.	3,4-dihydroxyphenylethylamine
III.	3,4-dihydroxyphenylpyruvic acid
IV.	3,4-dihydroxyphenyllactic acid
V.	3,4-dihydroxyphenylacetic acid

FIGURE 8

Metabolism of Levodopa

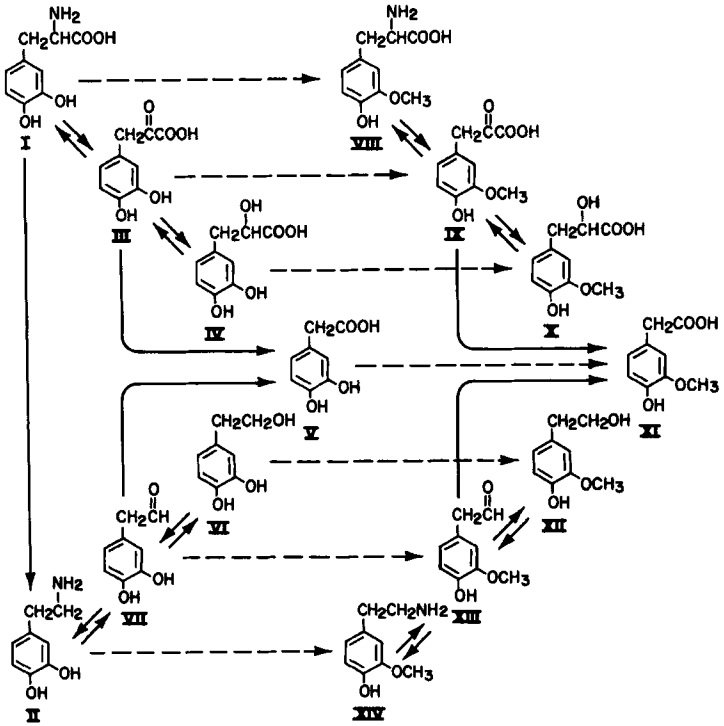


TABLE V (cont.)

Metabolites of Levodopa Referred to in Figure 8

VI.	3,4-dihydroxyphenylethanol
VII.	3,4-dihydroxyphenylacetaldehyde
VIII.	3-methoxy-4-hydroxyphenylalanine
IX.	3-methoxy-4-hydroxyphenylpyruvic acid
X.	3-methoxy-4-hydroxyphenyllactic acid
XI.	3-methoxy-4-hydroxyphenylacetic acid
XII.	3-methoxy-4-hydroxyphenylethanol
XIII.	3-methoxy-4-hydroxyphenylacetaldehyde
XIV.	3-methoxy-4-hydroxyphenylethylamine

7. Methods of Analysis

7.1 Elemental Analysis

A typical elemental analysis of a sample of levodopa is presented in Table VI (37).

TABLE VI

Elemental Analysis of Levodopa

Element	% Theory	% Found
C	54.82	54.82
H	5.62	5.74
N	7.10	7.10
O	32.46	32.34 (by difference)

7.2 Phase Solubility Analysis

Phase solubility analysis has been carried out for levodopa using 1:1 methanol:water as the solvent. An example is presented in Figure 9 along with the conditions under which the analysis was performed (38).

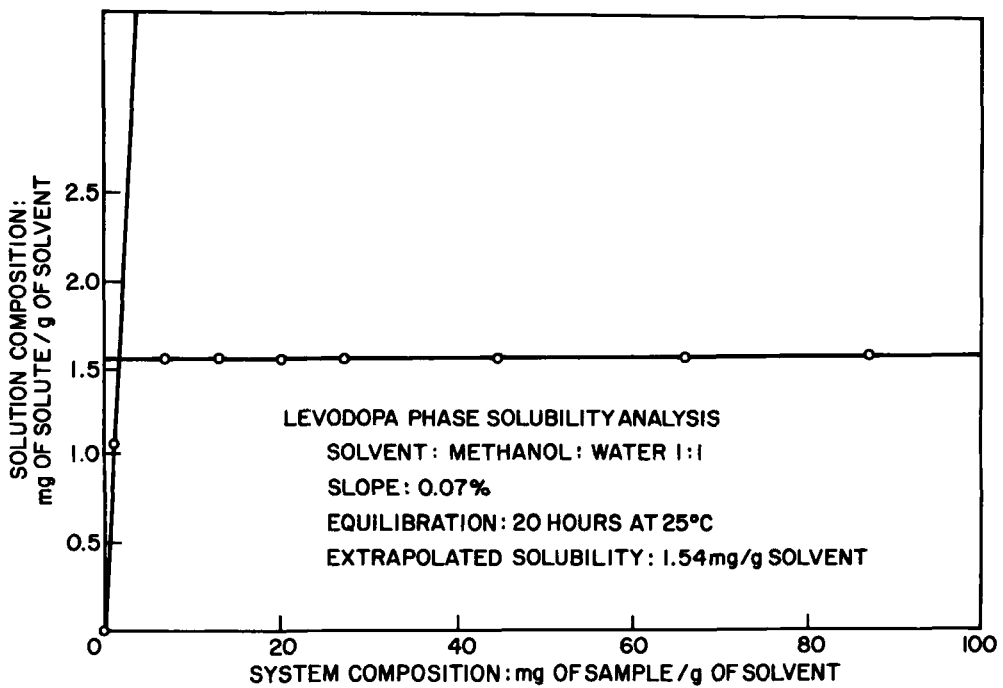
7.3 Chromatographic Methods

7.31 Thin-Layer Chromatography (TLC)

The following TLC procedure appears in USP XIX and is useful for separating levodopa from 3-methoxytyrosine and 3,4,6-trihydroxyphenylalanine (6). An Avicel plate, previously predeveloped in the developing

FIGURE 9

Phase Solubility Analysis of Levodopa



solvent, is spotted with 0.1 mg of levodopa from 9:1 acetone:4% hydrochloric acid. The plate is subjected to ascending chromatography in *n*-butanol:glacial acetic acid: double distilled water:methanol (150:75:75:15). After development of at least 15 cm, the plate is air dried and sprayed with 2:1 10% w/v ferric chloride:5% w/v potassium ferricyanide. The approximate R_f values are summarized in Table VII.

TABLE VII

Summary of TLC Data

Compound	Approximate R_f
3,4,6 Trihydroxyphenylalanine	0.25
Levodopa	0.4
3-Methoxytyrosine	0.5

Additional TLC separations of levodopa from related compounds or metabolites have been reported (39-42). A summary of these methods is found in Table VIII.

TABLE VIII

Thin-Layer Chromatographic Systems for Levodopa

Adsorbent	Solvent	R_f Value of Levodopa	Reference
Cellulose MN 300	Amyl Alcohol:Formic Acid:Water (40:40:20)	0.50	39
Cellulose MN 300	*n*-Propanol:Water (65:25)	0.39	39
Cellulose MN 300	*n*-Heptane:Carbon Tetrachloride:Methanol (70:40:30)	0.02	39
Polyamide	Isobutanol:Glacial Acetic Acid:Cyclohexane (80:7:10)	0.18	40

TABLE VIII (cont.)

Thin-Layer Chromatographic Systems for Levodopa

Adsorbent	Solvent	R_f Value of Levodopa	Reference
Cellulose	*n*-Butanol:Glacial Acetic Acid:Water (5:1:3)	0.28	41
Cellulose	Ethyl Acetate: Glacial Acetic Acid:Water (5:1.5:3)	0.23	41
Cellulose	Ethyl Acetate:*n*-Butanol:Glacial Acetic Acid: Water (3:2:1:3)	0.33	41
Cellulose	Methyl Ethyl Ketone:Acetone: 2.5N Glacial Acetic Acid (40:20:20)	0.16	42

7.32 Paper Chromatography

Paper chromatographic separations of levodopa have been used for identification as well as for its separation from related compounds (41, 43, 44, 45). Table IX is a summary of some of the paper chromatographic methods employed for levodopa. Whatman number 1 paper was used in each case.

TABLE IX

Paper Chromatographic Systems for Levodopa

Solvent	Development	R_f Value of Levodopa	Reference
n-Butanol:Glacial Acetic Acid:Water (5:1:3)	Descending	0.27	41

TABLE IX (cont.)

Paper Chromatographic Systems for Levodopa

Solvent	Development	R_f Value of Levodopa	Reference
Ethyl Acetate: Glacial Acetic Acid:Water (5:1.5:3)	Descending	0.21	41
Methanol:Water: Quinoline (160: 40:8)	Descending	0.42	43
n-Butanol:Pyridine: 0.2N Sodium Acetate (1:1:1)	Descending	0.47	44
n-Butanol:Pyridine: 1M Sodium Acetate (1:1:2)	Descending	0.68	44
n-Butanol:Pyridine (1:1) saturated with 1M Sodium Acetate	Descending	0.40	44
n-Butanol:Pyridine: Water (1:1:1)	Descending	0.54	44
Benzene:Methanol: *n*-Butanol:Pyridine: Water (1:2:1:1:1)	Descending	0.36	44
Methanol:Water: Pyridine (20:5:1)	Descending	0.46	44
Methanol:Water: Pyridine (20:5:1)	Ascending	0.37	44

TABLE IX (cont.)

Paper Chromatographic Systems for Levodopa

Solvent	Development	R_f Value of Levodopa	Reference
Toluene:Ethyl Acetate:Pyridine: Water:Methanol (1:1:1:1:1)	Descending	0.59	44
Toluene:Ethyl Acetate:Methanol: Water (1:1:1:1)- Aqueous Phase	Descending	0.62	44
Water saturated with methyl ethyl ketone	Descending	0.05	44
Water saturated with methyl ethyl ketone	Ascending	0.02	44
n-Butanol:Ethanol: Water (2:1:1)	Descending	0.23	44
Methanol:*n*-Butanol: Benzene:Water (2:1: 1:1)	Descending	0.37	44
Methanol:*n*-Butanol: Benzene:Water (4:3: 2:1)	Descending	0.21	44
Methanol:*n*-Butanol: Benzene:Water (4:3: 2:1)	Ascending	0.20	44
Toluene:Ethyl Acetate:Methanol: 0.1N HCl (1:1:1:1)	Descending	0.75	44

TABLE IX (cont.)

Paper Chromatographic Systems for Levodopa

Solvent	Development	R_f Value of Levodopa	Reference
Methanol:Amyl Alcohol:Benzene: 2N HCl (37:17.5: 35:12.5)	Descending	0.51	44
n-Butanol saturated with 1N HCl	Descending	0.19	44
n-Butanol saturated with 1N HCl	Ascending	0.18	44
tert.-Butanol: Acetone:Formic Acid: Water (160:160:1:39)	Descending	0.08	44
tert.-Butanol: Acetone:Formic Acid: Water (160:160:1:39)	Ascending	0.06	44
Chloroform:Glacial Acetic Acid:Water (2:1:1)	Descending	0.80	44
n-Butanol:Glacial Acetic Acid:Water (4:1:1)	Descending	0.21	44
tert.-Butanol: Acetone:Propionic Acid:Water (160: 160:1:39)	Descending	0.06	44
Benzene:Propionic Acid:Water (2:1:1)	Descending	0.83	44

TABLE IX (cont.)

Paper Chromatographic Systems for Levodopa

Solvent	Development	R_f Value of Levodopa	Reference
tert.-Butanol: Methyl Ethyl Ketone:Formic Acid:Water (40:30:15:15)	Ascending	0.42	45
n-Butanol: Glacial Acetic Acid:Water (50: 25:25)	Ascending	0.44	45
Phenol-Water (Lower Phase)	Ascending	0.38	45
Water:*sec.*-Butanol:*tert.*-Butanol (48.4:43: 8.6)-Upper Phase	Ascending	0.33	45
2-propanol:Water: Concentrated HCl (65:18.4:16.6)	Ascending	0.40	45
sec.-Butanol: Water-Upper Phase	Ascending	0.17	45
Ethyl Acetate: Formic Acid:Water (70:20:10)	Ascending	0.30	45
Ethyl Acetate: Water:Formic Acid (60:35:5)-Upper Phase	Ascending	0.00	45

TABLE IX (cont.)

Paper Chromatographic Systems for Levodopa

Solvent	Development	R_f Value of Levodopa	Reference
tert.-Butanol: Methyl Ethyl Ketone:Water: Formic Acid: (44:44:11: 0.26)	Ascending	0.10	45

7.33 Gas-Liquid Chromatography

A gas-liquid chromatographic procedure for the determination of levodopa purity and detection of possible impurities has been developed (46).

Gas-liquid chromatography permits the qualitative identification of the impurities by relating the retention times relative to an added internal standard. Derivatization was carried out by the reaction of levodopa and/or proposed impurities with bis-trimethylsilyl-acetamide reagent (BSA). Because levodopa is virtually insoluble in most non-acidic or non-aqueous solvents, the conversion to the trimethylsilyl (TMS) derivative was accomplished without the use of a solvent. Direct addition of the BSA reagent to the compound followed by moderate heating was sufficient for complete derivative formation.

The TMS derivatives were successfully chromatographed on a column packed with 20% SE-30 on Gas Chrom Q under isothermal conditions and detected using a thermal conductivity detector. In order to compensate for column characteristics, instrumental variations, and sample introduction technique, an internal standard, docosane, was employed for relative retention time data and response data. Assay values calculated by area normalization yielded a precision of $\pm$ 0.5% for five degrees of freedom.

An absolute determination using an internal standard yielded a precision of ± 1.2% for five degrees of freedom.

Gehrke and Stalling (47) reported the quantitative gas-liquid chromatographic separation of the N-trifluoroacetyl-n-butyl ester of levodopa from 14 other non-protein amino acids. The temperature programmed chromatography was carried out on columns of 60/80 mesh acid-washed Chromosorb W coated with 5% w/w DC-550. Yields of 98 ± 3% were obtained.

Atkinson, Brown and Gelby (48) separated the trimethylsilyl derivatives of levodopa, tyrosine, phenylalanine, N-acetyltyramine, tyramine, N-acetyldopamine and dopamine isothermally on various columns. By using the column in conjunction with a flame ionization detector, levodopa was detected at levels as low as 1-2 μg.

7.34 Column Chromatography

As part of a study of the biosynthesis and metabolism of catecholamines, Masuoka, et al. (49) developed a separation of the constituents of tissue extracts by column chromatography. The extracts were separated into three fractions on an alumina column by eluting successively with 0.5M (pH 6.1) ammonium acetate buffer, 0.01M (pH 4.0) ammonium acetate buffer and 2N acetic acid. The third fraction was then passed through a Dowex 50 column which separated levodopa from the other constituents. The fractions were monitored by measuring the absorbance at 279 nm.

Spiegel and Tonchen (50) described a method for the separation of levodopa from catecholamines found in plasma. The sample was adsorbed on alumina, eluted with 0.1N hydrochloric acid, then adsorbed on and eluted from an AG50W-X4 cation-exchange column.

A method for the separation of catechol derivatives, including levodopa, from guinea pig brains by column chromatography with Duolite C-25 was described by Nakajima (51).

Rolland, Lasry and Lissitzky (52) separated levodopa from L-tyrosine and other amino acids contained in protein or natural extracts on Dowex 50. The limit of detection of levodopa was reported to be 50-200γ.

7.4 Direct Spectrophotometric Analysis

Levodopa, in tablets or capsules, can be assayed directly by an ultraviolet absorption procedure. The tablets are finely ground or the contents of the capsules are mixed. A portion of the powder is weighed and quantitatively diluted with 0.4N hydrochloric acid. The contents are mixed, filtered and the absorbance of an appropriately diluted solution is measured at 280 nm. The amount of levodopa in tablets or capsules is determined by comparing the absorptivity of the sample at 280 nm with the absorptivity of a solution of levodopa reference sample similarly prepared and measured (6).

7.5 Colorimetric Analysis

An automated method utilizing the Doty reaction has been successfully applied to the quantitative determination of levodopa in tablets (53). The method is specific for the catechol configuration and will indicate any decomposition due to the oxidation of this moiety. The tablets are dissolved in 1N sulfuric acid, homogenized with distilled water, and quickly combined with sodium bisulfite solution to prevent oxidation. A ferrous citrate solution is introduced into the system followed by a strongly basic buffer which produces a stable purple color measureable at 545 nm. The amount of levodopa is calculated by comparison with a calibration curve prepared from pure levodopa, similarly treated (54).

7.6 Non-Aqueous Titration

A potentiometric titration with perchloric acid in glacial acetic acid is the method of choice to assay levodopa. The sample is dissolved in formic acid, glacial acetic acid is added, and the titration is carried out with 0.1N perchloric acid in glacial acetic acid. Each ml of 0.100N perchloric acid is equivalent to 19.72 mg of levodopa (6).

7.7 Determination of D-Dopa

Coppi, Vidi and Bonardi (55) described a method for the determination of D-Dopa in levodopa. The method is based on the quantitative reaction of a levodopa decarboxylase, present in a *Streptococcus frecalis* suspension, which converts levodopa to dopamine without affecting D-Dopa. D-Dopa was separated from dopamine by eluting a solution of the mixture with 0.05M pH 6.0 phosphate buffer on an Amberlite IRC50 ion-exchange column. The eluate was assayed fluorimetrically for D-Dopa according to Anton and Sayre (56).

8. Acknowledgements

The authors wish to acknowledge the assistance of the Scientific Literature Department and the Research Records Office of Hoffmann-La Roche Inc. for their literature searches.

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SODIUM LEVOTHYROXINE

Alex Post and Richard J. Warren

CONTENTS

CONTENTS (continued)

CONTENTS (continued)

1. Description

Sodium levothyroxine is a physiologically active material being the levo-isomer of thyroxine. The data presented in this analytical profile, unless otherwise stated, will refer to the levo-isomer. References to data obtained for the dextro-isomer, sodium dextrothyroxine, the DL-form, or for the free amino acid of either stereoisomer will be so designated.

1.1 Nomenclature

1.11 Chemical Names

Several chemical names have been used to describe sodium levothyroxine and sodium dextrothyroxine:

(a) Sodium derivative of 3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]-L-alanine[1]

(b) L-3,3',5,5'-Tetraiodothyronine, sodium salt, pentahydrate[2]

(c) β-[(3,5-Diiodo-4-hydroxyphenoxy)-3,5-diiodophenyl]-alanine, sodium salt, pentahydrate[2]

(d) D-Tyrosine, O-(4-hydroxy-3,5-diiodophenyl)-3,5-diiodo-, monosodium salt, hydrate[3]

(e) L-Tyrosine, O-(4-hydroxy-3,5-diiodophenyl)-3,5-diiodo-, monosodium salt, hydrate[4]

1.12 Generic Names

Sodium dextrothyroxine (D-T4, Na); sodium levothyroxine (L-T4, Na); and L-thyroxine, sodium (L-T4, Na).

1.13 Trade Names

Choloxin, Synthroid, Letter, Cytolen, Levoid, Eltroxin, Laevoxin, Levaxin, Oroxine, and Synthroid Sodium.

1.2 Formula, Molecular Weight, Structure

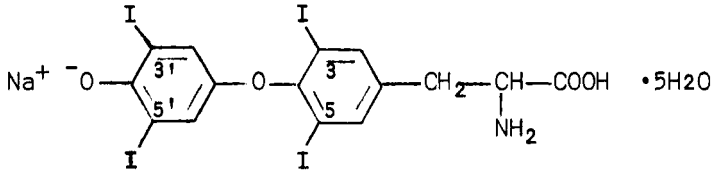
1.21 Empirical Formula, Molecular Weight

(a) Sodium Salt, Pentahydrate:
$C_{15}H_{10}I_4NNaO_4 \cdot 5H_2O$ 888.96

(b) Anhydrous Sodium Salt:
$C_{15}H_{10}I_4NNaO_4$ 798.86

(c) Free Acid:
$C_{15}H_{11}I_4NO_4$ 776.93

1.22 Structure



1.3 Appearance, Color, Odor

The sodium salt, pentahydrate, is an odorless, white to pale buff powder or crystalline powder[1]. The anhydrous powder is light yellow to buff colored and hygroscopic[3].

2. Physical Properties

2.1 Spectral Properties

2.11 Ultraviolet Absorption Spectra

Gemmill[5] reported λ max 325 (ε = 6207) in 0.4 N KOH and λ max 295 (ε = 4160) in 0.4 N HCl. Evidence has been presented that the shift in the maxima is due to the dissociation of the phenolic hydroxyl group. Edelhoch[6] also reported λ max 325 (ε = 6180) in 0.1 N NaOH.

The ultraviolet spectrum of thyroxine in acidified ethanol (pH 2.0)[7] is shown in Figure 1. A maximum at 300 nm (ε = 4600) and a shoulder at 290 nm are bands characteristic of the conjugated aromatic system.

Figure 2 is the ultraviolet spectrum in alkaline ethanol (pH 13.0)[7] which produces the sodium salt of the

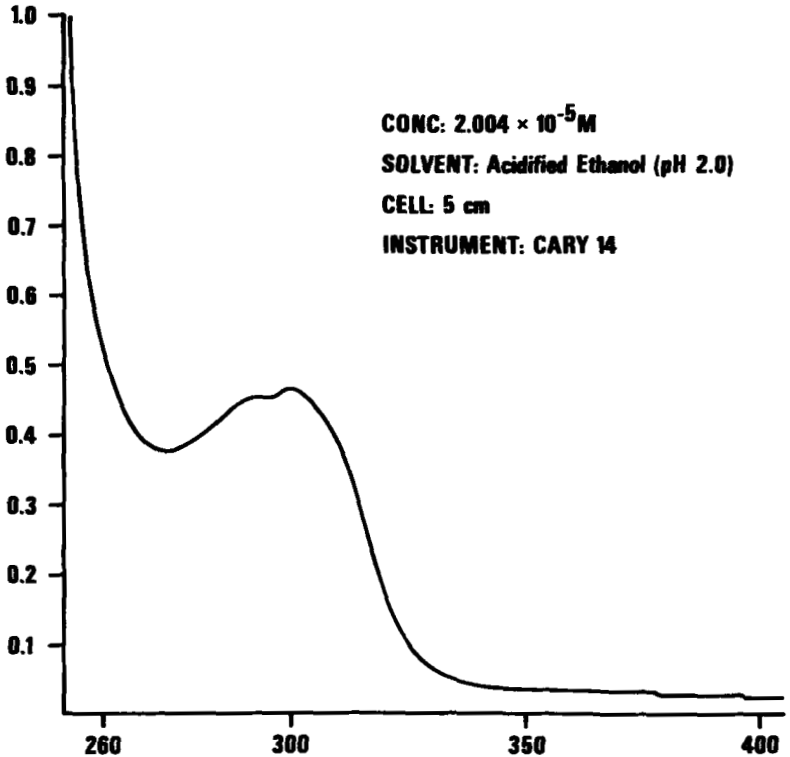

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
260
300
350
400
CONC: 2.004 × 10⁻⁵M
SOLVENT: Acidified Ethanol (pH 2.0)
CELL: 5 cm
INSTRUMENT: CARY 14

Figure 1

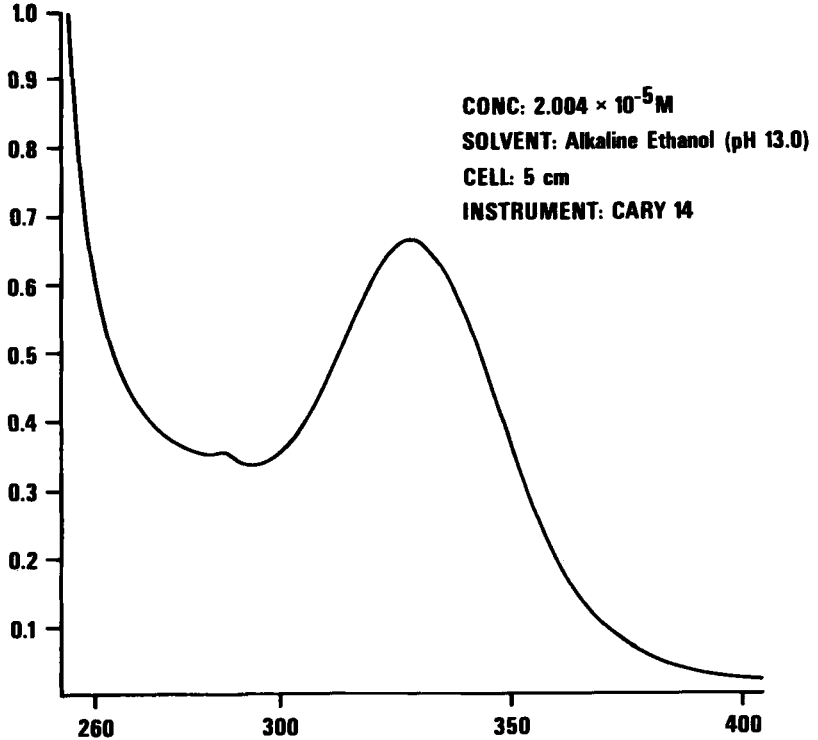

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
260
300
350
400
CONC: 2.004 × 10-5M
SOLVENT: Alkaline Ethanol (pH 13.0)
CELL: 5 cm
INSTRUMENT: CARY 14

Figure 2

phenolic hydroxyl on the aromatic system. The spectrum shows the expected shift in maximum to 328 nm (ε = 6580) with corresponding increase in intensity.

2.12 Infrared Spectrum

Figure 3 is the infrared spectrum of thyroxine, USP, taken as a mineral oil dispersion from 4000-625 cm^{-1} on a Perkin-Elmer Model 457 infrared spectrophotometer. The following absorption bands are assigned:[7]

Table 1
Infrared Spectral Assignments

Wavelength (cm^{-1})	Assignment
3600	OH
3500-3200	broad, NH_2 + H_2O
1610, 1415	COO^-
1245	R—O—R

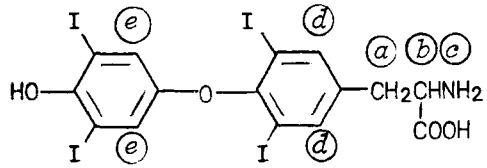
The infrared spectrum of L-thyroxine has been reported by Hagen, *et al.*[8]

2.13 Nuclear Magnetic Resonance Spectra

2.131 Proton NMR Spectrum

The proton NMR spectrum (Figure 4) was obtained in a DMSO-d_6 solution which contained about 100 mg thyroxine/ml and tetramethylsilane as the internal reference. The spectrum was obtained on a Perkin-Elmer Model R32 90 MHz spectrometer. The assignments are as follows:

Table 2
Proton NMR Spectral Assignments



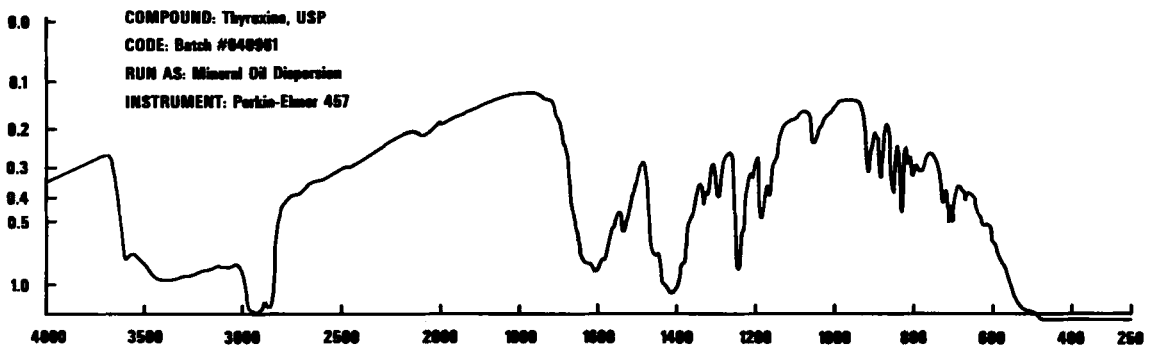

COMPOUND: Thyroxine, USP
CODE: Batch #848961
RUN AS: Mineral Oil Dispersion
INSTRUMENT: Perkin-Elmer 457
0.0
0.1
0.2
0.3
0.4
0.5
1.0
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
400
250

Figure 3

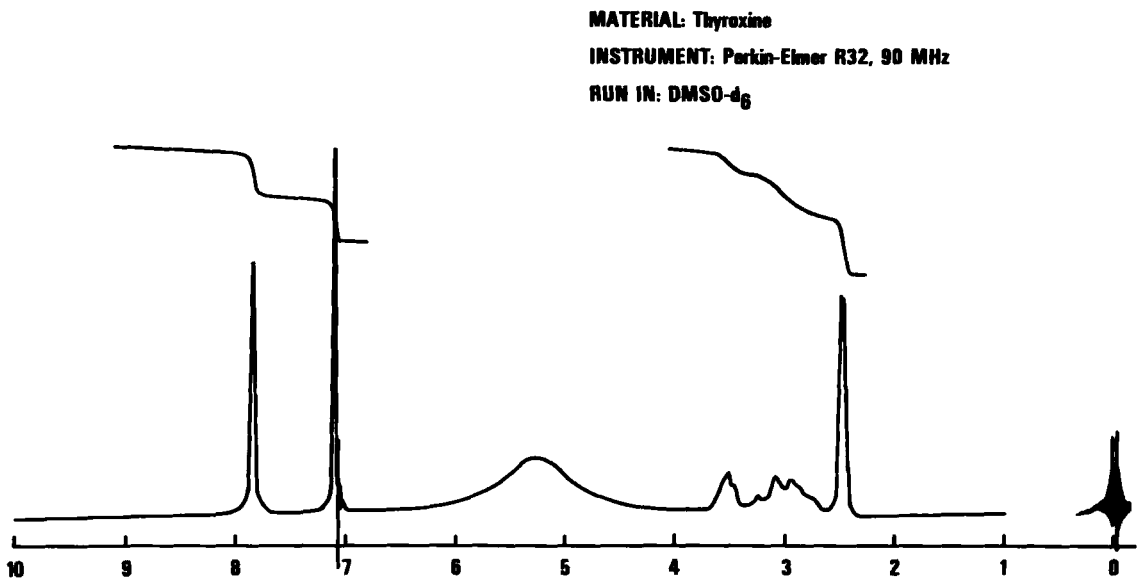

MATERIAL: Thyroxine
INSTRUMENT: Perkin-Elmer R32, 90 MHz
RUN IN: DMSO-d_6
10
9
8
7
6
5
4
3
2
1
0

Figure 4

Table 2 (continued)

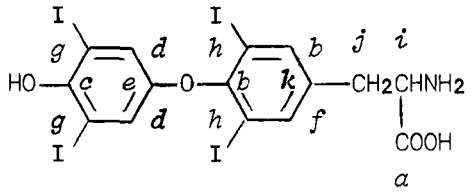
Proton Position	Signal Appearance	Chemical Shift (ppm)
a	**broad** multiplet	3.00
b	broad multiplet	3.51
c	broad, NH_3^+ + H_2O	5.30
d	singlet	7.09
e	singlet	7.83

2.132 Carbon-13 NMR Spectrum

The carbon-13 NMR spectrum of thyroxine was taken in DMSO-d_6 solution on a Varian CFT-20 spectrometer. The spectrum is shown in Figure 5. The assignments are as follows:[7]

Table 3

Carbon-13 NMR Spectral Assignments



Carbon-13 Position	Chemical Shift (ppm)
a	169.30
b	151.27
c	149.80
d	140.90
e	139.06
f and *k*	125.00
g	91.50
h	87.59
i	54.78
j	overlapped by solvent

2.14 Mass Spectrum

The field desorption mass spectrum of

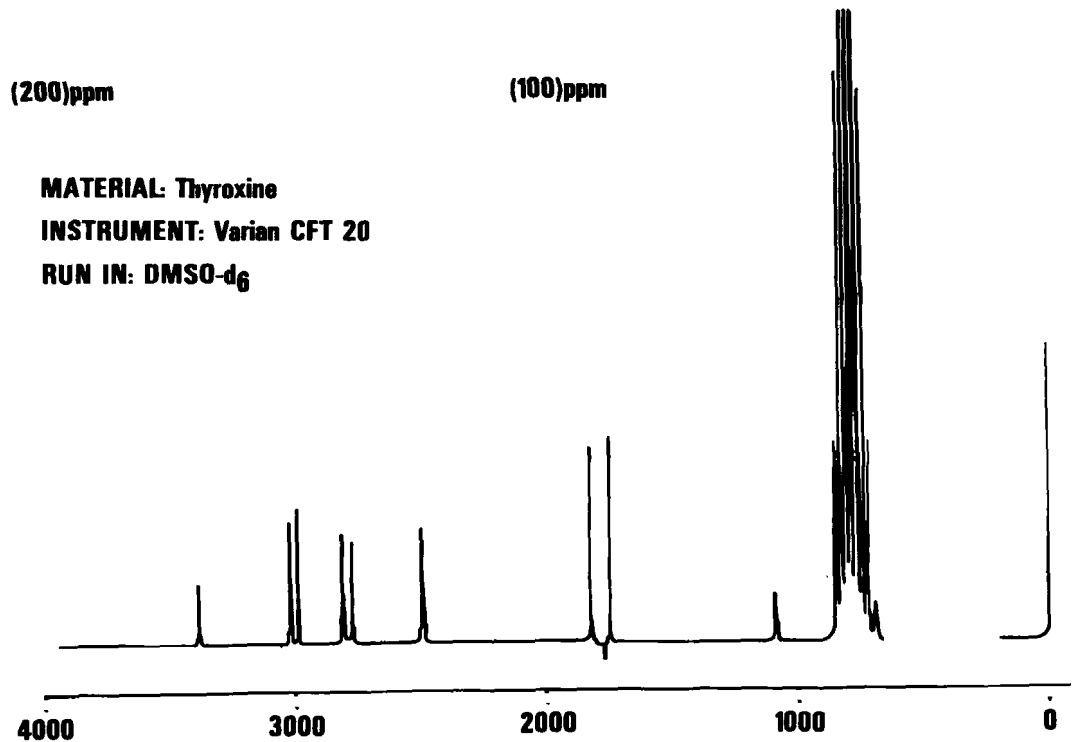

(200)ppm
(100)ppm
MATERIAL: Thyroxine
INSTRUMENT: Varian CFT 20
RUN IN: DMSO-d6
4000
3000
2000
1000
0

Figure 5

L-thyroxine was obtained on a Varian MAT CH-5 spectrometer.[9] The results are presented as a bar graph in Figure 6. The presence of a trace of triiodothyronine is evidenced by fragments at m/e 577, 607 and 651. Mass spectrometric studies of thyroxine trimethylsilyl derivatives have been reported.[10]

2.15 Specific Optical Rotation

As both the levo and dextro isomers are pharmacologically important substances, their respective specific rotation values have been listed in several compendia as criteria of acceptability. The following are several reported values, together with references (R#):

Isomer	Concentration	Solvent	°C	λ max	[α]	R#
Levo[a]	0.66 g	6.07 g 0.5 N NaOH + 13.03 g C_2H_5OH	21	546	-3.2	11
Levo[a]	0.66 g	6.07 g 0.5 N NaOH + 13.03 g C_2H_5OH	25	546	-3.2	2
Levo[a]	3%	0.13 N NaOH in 70% C_2H_5OH	20	D	-4.4	12
Levo[a]	2.2%	1 N NaOH : C_2H_5OH (1:2)	20	D	-5.7	12
Levo[a]	3.28%	1 N NaOH : C_2H_5OH (1:2)	21	D	-5.4	13
Levo[a]	3.25%	24 g 0.5 N NaOH + 56 g C_2H_5OH	17.5	D	-4.6	13
Levo[a]	5%	1 N HCl : C_2H_5OH (1:2)	25	D	+15	14
Levo[a]	3%	0.2 N NaOH in 70% C_2H_5OH	20	D	-4.5	14
Levo[b]	2%	1 N HCl : C_2H_5OH (1:4)	20	D	+16 to +20	1
Levo[b]	3%	1 N NaOH : C_2H_5OH (1:2)	20	D	-5 to -6	4
Levo[a]	2%	1 N HCl : C_2H_5OH (1:4)	20	D	+19.1	15
Dextro[a]	2%	1 N HCl : C_2H_5OH (1:4)	20	D	-19.2	15
Dextro[b]	3%	1 N NaOH in C_2H_5OH	25	D	+5 to +6	3

[a]Anhydrous free amino acid [b]Anhydrous sodium salt

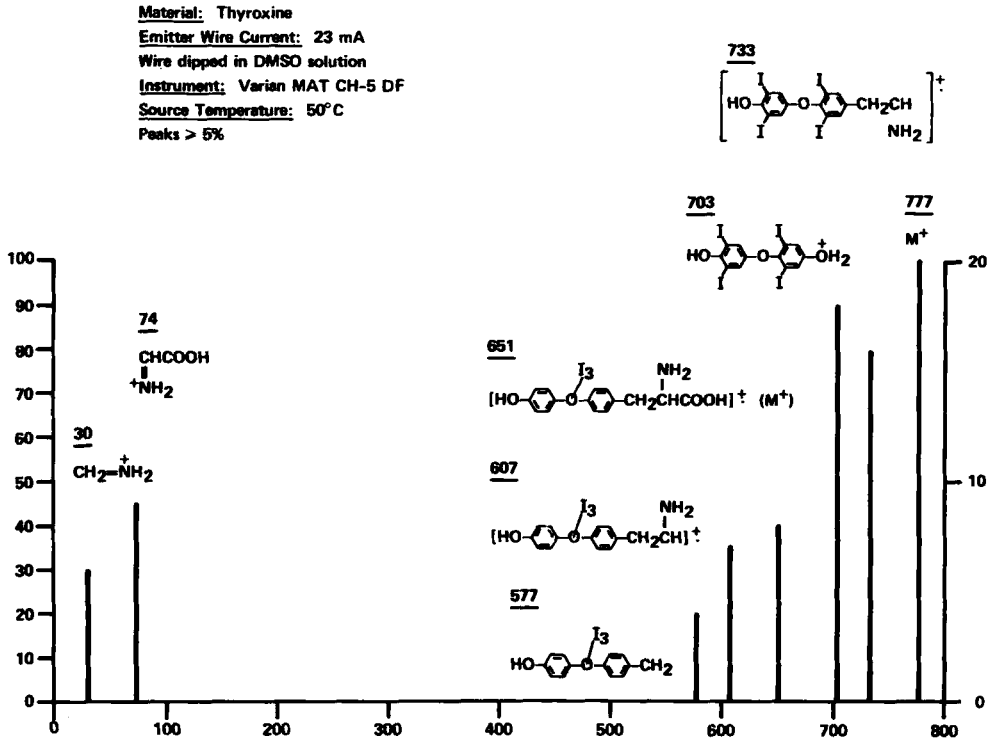

Material: Thyroxine
Emitter Wire Current: 23 mA
Wire dipped in DMSO solution
Instrument: Varian MAT CH-5 DF
Source Temperature: 50°C
Peaks > 5%
733
703
777
M+
74
30
651
607
577
100
90
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
700
800
20
10
0

Figure 6

Specific rotations of L-thyroxine (C = 2%, 0.2 N HCl-ethanol) at several wavelengths and temperatures have been reported:[15]

Temp (°C)	[α] 589 nm	578 nm	546 nm	436 nm	364 nm
10	+19.5	+20.3	+23.3	+42.2	+71.0
20	+19.1	+20.1	+23.2	+42.6	+70.1
30	+18.9	+19.7	+22.8	+42.0	+64.5
40	+18.4	+19.3	+22.1	+41.5	+62.7

Equivalent specific rotation values, but of opposite sign, were also reported for D-thyroxine.[15]

Using the above data, Felder, et al[15] obtained the optical rotatory dispersion curves and established the L- configuration of the sample of L-thyroxine.

2.2 Solubility

Table 4

L-Thyroxine Solubility

Solvent	g/100 ml	Ref #
H_2O	0.14	3
95% ethanol	0.3, 0.4	1
alkali hydroxides	soluble	1
chloroform	almost insoluble	1
ethyl ether	almost insoluble	1
pH 7.4 buffer	0.022-0.044	16

Evert[16] used a phosphate buffer at pH 7.2-7.8 at 38°C and a range of ionic strength from 0.032-0.162. He postulated that the increase in ionic strength by the addition of sodium chloride produced a change in the ionic atmosphere of the central ion resulting in a 'salting-in and salting-out' effect. Ultraviolet absorption analysis was used to establish the solubilities. Evert has presented solubility curves which show the effect of temperature (25° and 38°C) and of ionic strengths on the solubility of L-thyroxine sodium. Solubility is significantly increased above pH 7.4.

2.3 Crystal Properties

2.31 Crystallinity

Crystal data on L-thyroxine, sodium salt, pentahydrate, have been reported by Cody, et al.[17]

2.32 X-Ray Diffraction

The crystal and molecular structures of L-thyroxine hydrochloride, monohydrate have been determined by X-ray crystallography and reported in the literature.[18] The crystal system is monoclinic, and the sample crystallized in space group C2 with a = 17.23, b = 5.14, C = 25.15 Å; β = 90.47°; Z = 4.

2.4 pKa, Ionization Constant

The apparent pKa of the phenolic hydroxyl, carboxyl and amino functions has been reported:

Function	pKa	Ref #	pKa[a]	Ref #
carboxyl	2.2	19	3.832	20
phenolic hydroxyl	6.7	5	8.085	20
amino	10.1	19	9.141	20

[a] In 75% dimethylsulfoxide-water and 0.1 M KNO_3
Titrant: potentiometric with sodium hydroxide

2.5 Melting Range

The following melting ranges have been reported for thyroxine:

Isomer	Melting Range (°C)	Reference #
L-T4	233-235 (decomp)	12
L-T4	235-236 (decomp)	2
D-T4	237 (decomp)	2
L-T4	236 (corr)	8

The melting range of L-thyroxine (SK&F reference BB-2746-227) employing the USP XIX, Class I procedure, was 235-236°C (decomp).[21]

2.6 Differential Thermal Analysis

A differential thermal analysis curve of L-thyroxine (SK&F reference BB-2746-227), obtained on a DuPont Model 900 Differential Thermal Analyzer from room temperature to 250°C at a heating rate of 10°C per minute under nitrogen sweep, is shown in Figure 7. A sharp exothermic change occurred from 230-235°C, indicating decomposition with no observable prior melting (endotherm).[22]

2.7 Thermogravimetric Analysis

The thermogravimetric analysis of L-thyroxine sodium, pentahydrate, was obtained on a DuPont Thermogravimetric Analyzer (see Figure 8).[21] The compound was heated at a rate of 20°C per minute under nitrogen sweep to 475°C. A weight loss of ~9% was observed. As expected, inception of rapid decomposition appeared to occur at approximately 200°C. The result obtained by the loss on drying procedure of the USP XIX[4] was 8.75%.

3. Synthesis

3.1 Chemical Synthesis

3.11 L-Thyroxine, Monosodium Salt

The synthetic route to L-thyroxine, and subsequently to its monosodium salt, pentahydrate, was described by Chalmers, et al[12] and is presented in Figure 9. Evidence was also presented showing the stereospecificity of this synthesis. The overall yield was 26%.

To a cooled suspension of L-tyrosine (I) in sulfuric acid was added nitric acid which on neutralization yielded 3,5-dinitro-L-tyrosine (II). An alkaline solution of (II) acetylated with acetic anhydride yielded the amide (III). Esterification of (III) to (IV) was effected using ethyl alcohol and p-toluenesulfonic acid and then azeotroping the water with chloroform. The diphenyl ether (V) was prepared from (IV) by treatment with p-toluenesulfonylchloride and

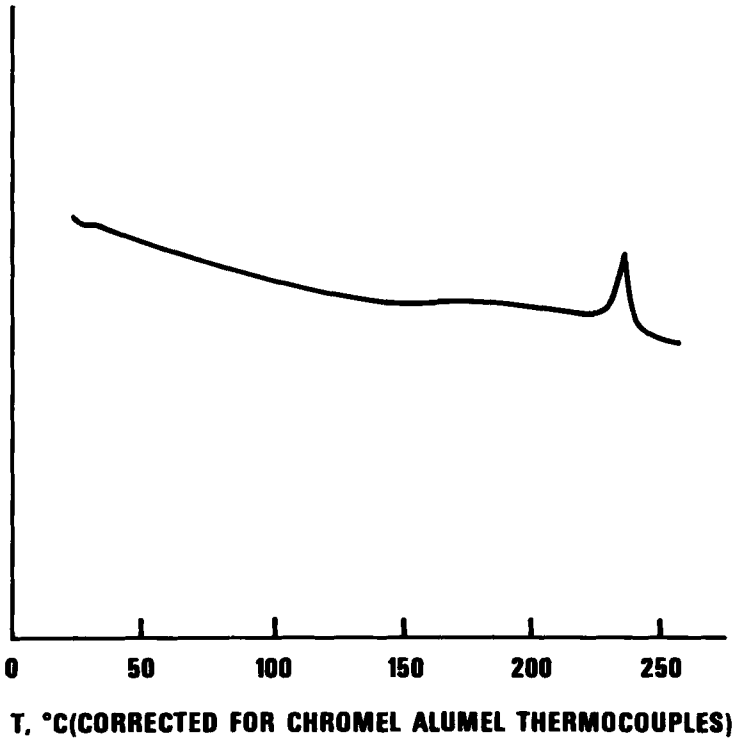

0
50
100
150
200
250
T, °C(CORRECTED FOR CHROMEL ALUMEL THERMOCOUPLES)

Figure 7

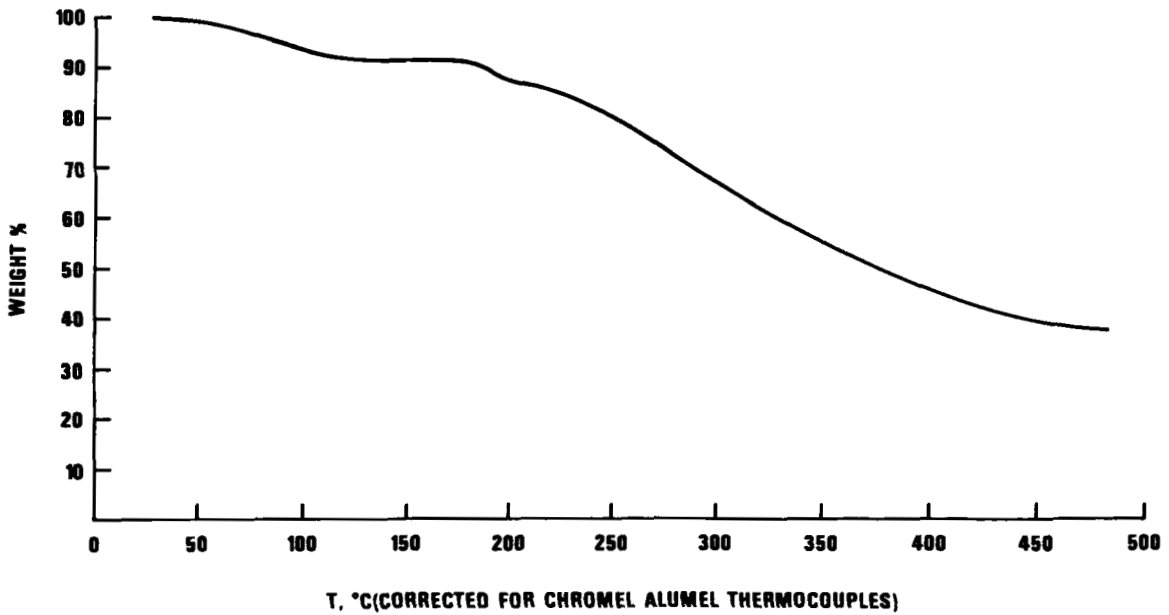
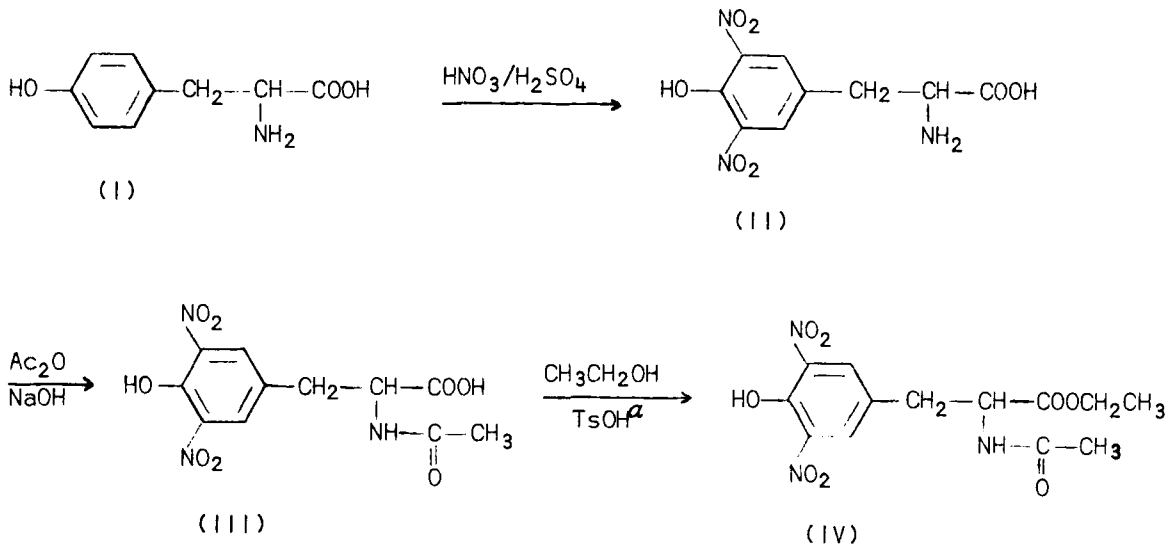

100
90
80
70
60
50
40
30
20
10
WEIGHT %
0
50
100
150
200
250
300
350
400
450
500
T, °C(CORRECTED FOR CHROMEL ALUMEL THERMOCOUPLES)

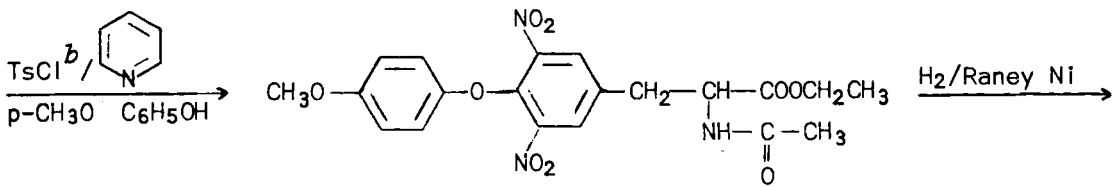
Figure 8

Figure 9

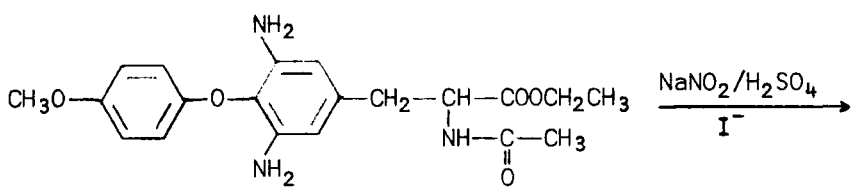


[a] p-toluenesulfonic acid

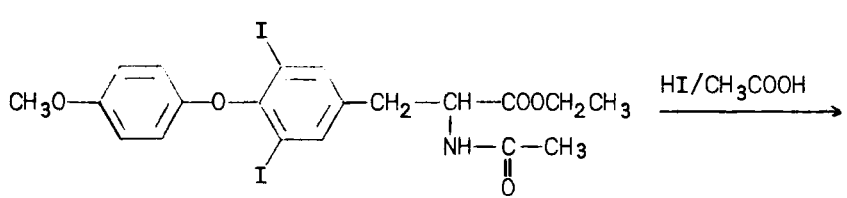
Figure 9 (continued)



(V)



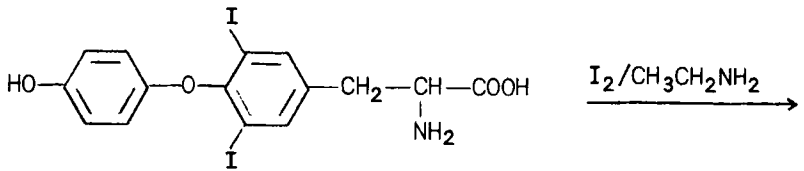
(VI)



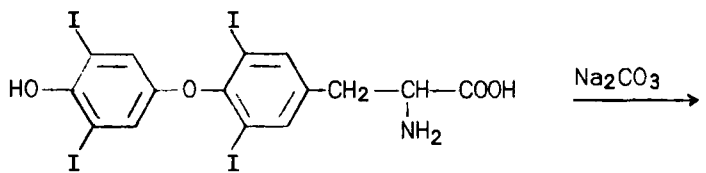
(VII)

b p-toluenesulfonylchloride

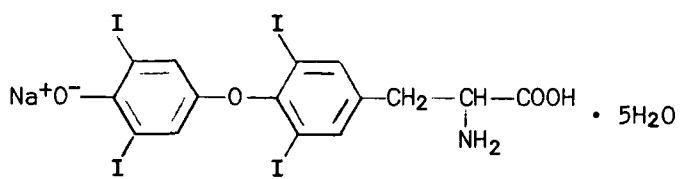
Figure 9 (continued)



(VIII)



(IX)



(X)

p-methoxyphenol. Reduction of (V) to the diamine (VI) was readily obtained by treating an alcohol solution of (V) with Raney Ni.

Replacement of the amino groups was brought about via tetrazotisation and Sandmeyer procedures. Tetrazotisation was carried out by the slow addition of a solution of (VI) in a mixture of acetic and sulfuric acids to one of sodium nitrite in the mixture of the same acids. The diazonium groups were removed by the addition of a solution of iodine in aqueous sodium iodide.

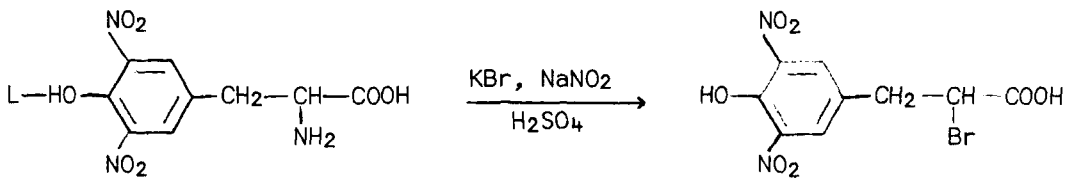
All the protective groups present in (VII) were removed by treatment with a mixture of hydroiodic acid and glacial acetic acid. Iodination of (VIII) with a solution of iodine in ethylamine yielded L-thyroxine (IX). The addition of L-thyroxine to a boiling 2 N sodium carbonate solution produced the desired product (X).

3.12 D-Thyroxine, Monosodium Salt

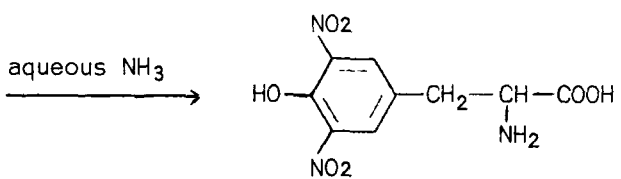
Elks and Waller[23] prepared the D-isomer by first inverting the 3,5-dinitro-L-tyrosine (II-from Figure 9) to the D-form (XII-Figure 10) by treatment of (II) with nitrosylbromide which, after ammonolysis of (XI-Figure 10), yielded the 3,5-dinitro-D-tyrosine (XII-Figure 10). The D-thyroxine was then prepared in a series of reactions essentially equivalent to that used by Chalmers, et al[12] [see (III)-(X) in Figure 9].

3.2 Nonenzymic Synthesis of L-Thyroxine

In an attempt to explain the biosynthesis of thyroxine from 3,5-diiodo-L-tyrosine, Shiba, et al[24] proposed a nonenzymic model for this pathway (Figure 11). At neutral pH and at room temperature and in the presence of sodium glyoxylate (II), cupric acetate, and oxygen, the reaction proceeded rapidly through a transamination via a metal chelate of the Schiff base of diiodotyrosine and glyoxylic acid (III). Oxidative coupling of 4-hydroxy-3,5-diiodophenylpyruvic acid (IV) with tyrosine yielded the L-thyroxine (V). The analytical data presented established the stereospecificity of this series of reactions. The overall yield was 8%.



(II - from Figure 9) (XI)



(XII)

Figure 10

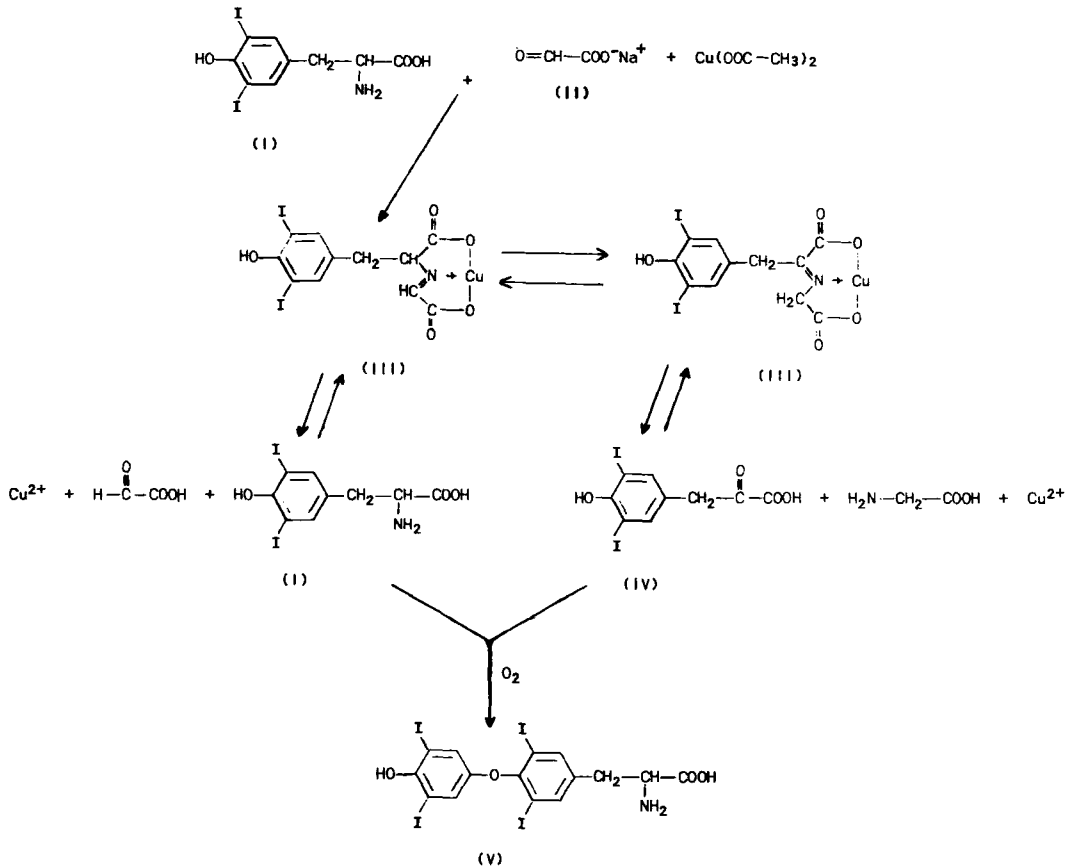


Figure 11

3.3 Synthesis of Radiolabeled L-Thyroxine

Weeke and Orskov[25] have developed a rather facile procedure for the synthesis and purification of monolabeled ^{125}I-LT4 with a very high specific activity for use in radioimmune assay.

Five mCi of ^{125}I (specific activity > 14 mCi/µg) were added to 50 µl of 50 mmol/l phosphate buffer, pH 7.5, for buffering the alkaline ^{125}I solution. Addition of 2 µg (20 µl) of 3,5,3'-triiodo-L-thyronine and 90 µg (25 µl) of chloramine-T, mixing for 15 seconds and halting the reaction with 240 µg (100 µl) of sodium metabisulfite, yielded a ^{125}I-LT4 of very high specific activity (~3000 mCi/mg T4). The ^{125}I label is presumably in the 5' position.

A more general and simple procedure for the synthesis of radioactive L-thyroxine, carrying the label ^{131}I or ^{14}C either in the phenolic ring or in the non-phenolic ring and the side chain, was reported by Shiba and Cahnmann.[26] It is based on the coupling of 4-hydroxy-3,5-diiodophenylpyruvic acid and diiodotyrosine. Labeled 4-hydroxy-3,5-diiodophenylpyruvic acid was prepared by the condensation of iodinated p-hydroxybenzaldehyde with acetylglycine and the hydrolysis of the azlactone. Depending on the specific labeled compound prepared, yields ranged from 12-20%.

4. Stability

The USP,[4] NF,[3] and BP[1] state that levothyroxine and dextrothyroxine sodium are to be protected from light. On exposure to light these compounds may assume a pink color. In addition, the USP[4] states that levothyroxine is stable in dry air.

Several investigators have studied the stability of thyroxine as a function of the deiodination process, which yields free iodine and triiodothyronine. Stanbury[27] has reviewed the pathway and mechanism of this process by both in vitro and in vivo experimentation. He indicated that the major factor in the deiodination process is involved with the facile ionizability of the hydroxyl group. Because of this, one can expect that the iodines in the 3' and 5' positions are more labile than those in the 3 and 5 positions

(refer to §1.22). The released iodide is then oxidized to free iodine. That this appears to be the in vivo mechanism has subsequently been borne out by other investigators.

High energy gamma radiation has been shown to cause rapid deiodination and the formation of other iodinated organic molecules.[28]

The rationale and explanation for the spontaneous deiodination of ^{131}I labeled thyroxine is still of concern. Taurog[29] has shown that the spotting of dilute aqueous buffer solutions on filter paper and drying for 10-20 minutes led to a significant and variable loss of ^{131}I from the ^{131}I-thyroxine. This effect was also observed when glass paper and thin layer silica gel were used. Reduction in the amount of deiodination occurred when ethanol or propylene glycol was added to the sample. Shortwave ultraviolet light greatly enhanced the rate of deiodination.

These spontaneous deiodination effects were also studied by Jolin, et al.[30] They indicated that this effect arises when one of the components of the solution under study becomes an active deiodination agent during the analytical procedure. Thus, a strict adherence to procedure must be followed.

Reviczky and Nagy[31] found that under ultraviolet radiation deiodination occurred to a greater extent and more rapidly in aqueous solutions than in butanol, the release of iodide is proportional to the decrease in pH, and chromatography showed that in addition to the presence of free iodide triiodothyronine was the prime decomposition product.

5. Drug Metabolism

5.1 Biological Half-Life

In a study similar to that of Sterling, et al,[32] Demeester-Mirkine showed that the half-life of ^{131}I-thyroxine is 7 days in serum and 0.4 days in tissues.[33]

5.2 Metabolic Products

Thyroxine is converted to triiodothyronine in hypothyroid human subjects maintained on synthetic sodium L-

thyroxine administered orally.[34] This was confirmed by Sterling, et al,[32] who injected purified thyroxine labeled with ^{14}C in ring A and in the alanine side chain. Methods for determining this conversion have been reviewed by Surks and Oppenheimer.[35]

6. Elemental Analysis

The elemental composition of L-thyroxine as the monosodium salt pentahydrate, the anhydrous monosodium salt, and the anhydrous free acid is as follows:

Element	% (Theory) Monosodium (Pentahydrate)	Monosodium (Anhydrous)	Amino Acid (Anhydrous)
Carbon	20.27	22.55	23.19
Hydrogen	2.27	1.26	1.43
Nitrogen	1.58	1.75	1.80
Oxygen	16.20	8.01	8.24
Sodium	2.59	2.88	-
Iodine	57.10	63.54	65.34

The theoretical percentage of water in the monosodium pentahydrate form is 10.13%.

6.1 Determination of Organically Bound Iodine

As the criterion of acceptability in both the USP[4] and the NF[3] is the assay value for iodine, the methods of analysis employed to determine this element have been carefully investigated.

6.11 Oxygen Flask Combustion[36] + Iodometric Titration[37]

This method is described in both the USP XIX and NF XIV monographs for sodium levothyroxine and sodium dextrothyroxine.

Apparatus - The apparatus consists of a heavy-walled conical, deeply lipped or cupped 500-ml flask (unless a larger flask is specified), fitted with a ground-glass stopper to which is fused a sample carrier consisting of

heavy-gauge platinum wire and a piece of welded platinum gauze measuring about 1.5 x 2 cm.

Procedure - Weigh accurately about 25 mg of sample on a piece of halide-free filter paper measuring about 4 cm square, and fold the paper to enclose it. Place the sample, together with a filter paper fuse-strip, in the platinum gauze sample holder. Place the absorbing liquid, consisting of a mixture of 10 ml of sodium hydroxide solution (1 in 100), in the flask and moisten the joint of the stopper with water. Flush the air from the flask with a stream of rapidly flowing oxygen, swirling the liquid to favor its taking up oxygen. [NOTE--saturation of the liquid with oxygen is essential for the successful performance of the combustion procedure.] Ignite the fuse-strip by suitable means. If the strip is ignited outside the flask, invert the flask so that the absorption solution makes a seal around the stopper, and hold the stopper firmly in place. When the combustion is complete, place a few ml of water around the stopper and allow the flask to stand for about 15 minutes. Loosen the stopper, and rinse the stopper, sample holder, and sides of the flask with about 20 ml of water, added in small portions. Add 1 ml of an oxidizing solution prepared by adding 5 ml of bromide to 100 ml of a 1 in 10 solution of sodium acetate in glacial acetic acid. Insert the stopper in the flask, and shake vigorously for 1 minute. Add 0.5 ml of formic acid, replace the stopper, and shake vigorously for 1 minute. Remove the stopper, and rinse the stopper, the sample holder, and the sides of the flask with several small portions of water. Bubble nitrogen through the flask to remove the oxygen and excess bromine, add 500 mg of potassium iodide, swirl to dissolve, add 3 ml of diluted sulfuric acid, swirl to mix, and allow to stand for 2 minutes. Titrate with 0.02 N sodium thiosulfate, adding 3 ml of starch TS as the endpoint is approached. Each ml of 0.02 N sodium thiosulfate is equivalent to 0.1057 mg of iodine.

6.12 Ashing + N-Bromosuccinimide Titration

Bakarat, et al,[38] ashed the sample in the presence of potassium carbonate and, after dilution with water, titrated the solution with 0.02 N N-bromosuccinimide. Accuracy of greater than 99% was obtained for samples containing 5-10 mg of thyroxine.

6.13 Oxygen Flask Combustion + Coulometric Titration

A modification[39] of the oxygen flask combustion method has been used prior to the coulometric titration[40] of the iodide:

To a 500-ml oxygen flask filled with oxygen is added 10.00 ml of 0.4 N potassium hydroxide and several milligrams of hydrazine sulfate. The filter paper tab is ignited, and after combustion is complete the flask is cooled for a few seconds in a stream of cold water and set aside for at least 30 minutes for complete absorption of the combustion products. The absorbing solution is acidified with 10.00 ml of a solution of 0.6 N nitric acid in 20% glacial acetic acid. The flask is swirled to remove some of the carbon dioxide evolved, stoppered and vigorously shaken. An aliquot of 4.00 ml is titrated coulometrically on a suitable coulometric titrator, such as the Aminco-Cotlove Automatic Titrator.[41,42]

6.14 Specific Ion Electrode

The specific ion electrode sensitive to iodide has been used by Paletta and Panzenbeck[43] for samples of L-T4. The iodine is stripped from the compound with activated aluminum foil at pH 11 at 60°C in 10 minutes. After neutralization with dilute hydrochloric acid, the potential is measured using the specific ion electrode[44] versus the calomel reference electrode. The amount of iodide present is determined by comparing the potential reading with those obtained for a series of standard solutions containing 10^{-8} to 10^{-7} grams of iodide per ml. The assay results compared favorably with those obtained by the conventional titrimetric procedure using a sodium thiosulfate titrant.

6.2 Determination of Water

L-thyroxine monosodium is generally prepared as a hydrate. Thus, in order to compare samples on an anhydrous basis, the moisture content is determined.

6.21 Gravimetric Method (USP XIX, Method III, p.668)

From the monograph for levothyroxine sodium

the determination is as follows:

'Dry about 500 mg, accurately weighed, over phosporous pentoxide at 60°C and at pressure not exceeding 10 mm of mercury for 4 hours.

6.22 Thermogravimetric Analysis (TGA)

The procedure described in 2.7 has been found to yield equivalent results to the USP XIX procedure. A sample (Batch #27) analyzed by the USP XIX procedure showed the presence of 8.75% moisture, and by thermogravimetry it showed 8.85%.[7]

6.3 Chromatographic Analysis

As thyroxine is often contaminated with triiodothyronine, and in thryoid powders with a series of iodinated thyronines and/or tyrosines, and in sera with similar compounds, it is important that the chromatographic system employed be capable of separating these related compounds. Thus, in the several chromatographic techniques used to evaluate thryoxine and thryoxine-containing materials, the R_f, R_T, etc. of these related compounds are also reported when available.

The following abbreviations of thryoxine and related compounds will be used:

T4	thyroxine
T3	3,3',5-triiodothyronine
T2	3,5-diiodothyronine
T1	3-iodothyronine
T	thyronine
I^-	inorganic iodide
Ty	tyrosine
MIT	3-iodotyrosine
DIT	3,5-diiodotyrosine

6.31 Paper Chromatography (PC)

A significant amount of literature is available concerning the application of paper chromatography to the separation of the iodoamino-acids. A few of the relevant publications have been cited.[45-53]

Table 5

Rf Values of Thyroxine, Analogs, and Related Compounds (Paper Chromatography)

Compound	Mobile Phase 1	2	3	4	5	6	7	8	9	10	11	12
	Rf											
T4	0.89	0.47	0.45	0.45	0.51	0.43	0.86	0.70	0.28	0.43	0.22	0.07
T3	0.91	0.70	0.63	0.65	0.63	0.58	0.86	0.85	0.36	0.78	0.33	0.25
T2	0.69	0.06	0.11	0.11	0.68	0.62	0.82	0.85	0.47		0.42	
TI							0.87	0.62			0.35	
T											0.26	
I^-	0.26		0.40	0.90								0.89
T	0.46		0.26	0.25	0.17	0.17						
MIT	0.58	0.39	0.18	0.28								0.72
DIT							0.67					0.59

Mobile Phase: — Reference #

1. n-Butanol:2N acetic acid (1:1) — *46*
2. n-Butanol:ethanol:0.5N NH_4OH (5:1:2) — *47*
3. n-Butanol:dioxane:2N NH_4OH (4:1:5) — *48*
4. Collidine (conc. NH_4OH vapor) — *46*
5. n-Butanol:ethanol:2N NH_4OH (5:1:2) — *49*
6. n-Butanol:2N NH_4OH (5:2) — *49*
7. n-Butanol:2N formic acid — *50*
8. n-Butanol:6N NH_4OH — *50*
9. Isoamyl alcohol:tert-amyl alcohol:6N NH_4OH (1:1:2-upper phase) — *45*
10. tert-amyl alcohol:2N NH_4OH:hexane (5:6:1) — *53*
11. 1,1-dimethyl propanol-1 saturated with 6N NH_4OH — *52*
12. 3% sodium chloride — *53*

Table 6

Detection Methods
Used in Paper Chromatography of Thyroxine

A comprehensive description of the following detection methods is listed in Reference *46* - they are only briefly described here:

1. Ceric-arsenite reagent:

   This method of detection depends upon the liberation of iodine from the compounds by oxidation with ceric sulfate. The iodine-containing compounds appear as white spots on a yellow background. As little as 0.1 µg of L-T4 and L-T3 and 0.01 µg of I^- could be detected. A detailed account of the application of this method can be found in Reference *139*.

2. Ferrichloride-ferricyanide-arsenious acid reagent:

   Gmelin and Virtanen[140] demonstrated that the catalytic acceleration by I^- of the simultaneous reduction of ferrichloride and ferricyanide to yield a mixture of Turnbull's blue and Prussian blue was a sensitive detection method for iodothyronines and iodotyrosines (0.002 µg for each of the amino acids and 0.001 µg for I^-.)

3. Starch:

   Datta, et al.[49] showed the use of a starch spray, an overspray with KIO_3, and exposure to UV light to be a sensitive detection reagent. A transient blue color from as little as 0.05 µg of an iodoamino-acid can be detected.

Less sensitive detection reagents are:

4. Diazotized sulfanilic acid:

   10-20 µg of thyroxine can be detected. A range of colors from reddish purple (L-T4) to orange (L-T1) is obtained.

Table 6 (continued)

5. Diazotized N,N-diethylsulfanilamide:

   This reagent is only slightly more sensitive than the above, and the colors are in the purple range.

6. Ninhydrin:

   A less specific reagent, since it reacts with amino acids. L-T4 appears as a purplish brown color rather than the typical purple. The limit of detection of L-T4, L-T3, and L-T2 is 1 µg.[45]

7. Ultraviolet light:

   These compounds absorb strongly in UV light and can be detected if 20-30 µg are present.

A unique reagent (Emerson's)[141] was used by Eisdorfer and Post[45] to qualitatively and quantitatively determine the presence and amount of L-T2 and L-T3 in L-T4. The reagent is prepared by dissolving 4-aminoantipyrine hydrochloride in aqueous sodium carbonate solution. The reagent is sensitive in detecting 1 µg of each of the iodoamino acids forming a range of colors: L-T2 (orange), L-T3 (red), and L-T4 (purple).

Detection of labeled iodoamino acids can be accomplished by:

1. Neutron activation analysis

2. Radioautography (X-ray film)

3. Automatic chromatographic scanners fitted with a Geiger-Muller detector

Table 7

Rf Values of Thyroxine, Analogs, and Related Compounds

(Thin Layer Chromatography)

Compound	Rf (Mobile Phase (see notes))									
	1	2	3	4	5	6	7	8	9	10
T4	0.55	0.25	0.38	0.48	1.28	0.89	0.14	0.18	0.24	0.58
T3	0.73	0.36	0.46	0.59	1.76	1.13	0.34	0.50	0.30	0.49
T2			0.50	0.66			0.48	0.58	0.27	0.38
T										0.27
I^-	0.87	0.75	0.70	0.53			0.90	0.62	0.31	
Ty										0.12
MIT	0.18		0.29	0.23	0.67	0.39	0.83	0.07	0.14	0.18
DIT	0.73		0.24	0.19	0.15	0.17	0.72	0.04	0.09	0.30

NOTES

Mobile Phase	Adsorbent	Detection	Ref #
1. n-Butanol:chloroform (4:7) saturated with NH_4OH atmosphere	Kieselguhr G	Radioscan	55
2. 30% NH_4OH (w/v):methanol:chloroform (0.1:2:2, v/v)	Silica Gel H	Ceric sulfate: sodium arsenite: methylene blue	56 57

Table 7 (continued)

Mobile Phase	Adsorbent	Detection	Ref #
3. Tert-amyl alcohol:acetone:NH_4OH (25:8:7, v/v)	Kieselgel G	Ninhydrin, $PdCl_2$	*58*
4. Tert-butanol:tert-amyl alcohol: NH_4OH:methylethylketone:H_2O	Eastman Sheets #6060 (Silica Gel)	Ninhydrin, $PdCl_2$	*59*
5. *(Rf given as relative Rf of I^-, Rf of I^- not reported)* Tert-amyl alcohol:dioxane:1N NH_4OH (2:2:1)	20% Silica Gel G+ 80% Cellulose MN 300	Diazotized sulfanilic acid, $PdCl_2$	*60*
6. *(Rf given as relative Rf of I^-, Rf of I^- not reported)* Ethanol:methylethylketone:2N NH_4OH (1:4:1)	Same as 5	Same as 5	*60*
7. Formic acid:H_2O (1:5)	Cellulose Powder	Ferric chloride: ferricyanide: arsenious acid	*61*
8. Tert-butanol:2N NH_4OH:chloroform (376:70:60)	Same as 7	Same as 7	*61*
9. Ethylacetate:methanol:2N NH_4OH (100:40:60, v/v)	Silica Gel G	Ceric sulfate: sodium arsenite	*62*
10. Chloroform:methanol:formic acid (70:15:15, v/v)	Silica Gel G	*(not reported)*	*66*

Table 5 lists the Rf of several of the iodoaminoacids in different systems. Detection methods are listed in Table 6. Several Whatman papers have been used: Whatman 1, 3, 4, 3MM being the most popular. In addition, the procedure has been carried out in the ascending, descending and circular modes. In each case, it has been the experience of the authors that, under the proper conditions of temperature, equilibration time, and length of run, the Rfs obtained with any of these modes gave essentially equivalent resolution from day to day and laboratory to laboratory. Deiodination effects during paper chromatography have been evaluated and described in Section 4.

Paper chromatography, using four different solvent systems on Whatman #1 paper, was used to establish the chromatographic purity of the L-thyroxine sodium reference substance prior to its addition to the British Pharmacopoeia.[54] $R_{thyroxine}$ values are reported for the compounds listed in Table 7.

6.32 Thin Layer Chromatography (TLC)

Thin layer chromatography offers some advantages to paper chromatography in that better separations are generally obtained with higher loadings. However, reproducibility of Rf values is oftentimes difficult to obtain because of the batch to batch differences in adsorbents, temperature variations within a laboratory, and equilibration times used by different investigators. Thus, the data presented in Table 7 should be considered in light of these variables; and the analyst should be expected to alter the mobile phase, although slightly, to effect a suitable separation.

The references cited[55-62] indicate the variety of systems available for the separation of these compounds. Chapters from several texts[46,63,64] contain additional information.

A critical thin layer chromatographic analysis of L-thyroxine sodium was made by a joint committee of the Pharmaceutical Society of Great Britain and the British Pharmacopoeia prior to establishing the sample as a reference substance.[54] Five different mobile phases on five different adsorbants were used to establish its purity.

Schorn and Winkler[65] systematically investigated more than two dozen solvent systems on Silica gel G plates in the separation of L-T4, L-T3, L-T2, L-TI and I^-. The results clearly showed the viability of this technique to the separation of the iodoaminoacids.

6.33 Column Chromatography

As column chromatography is a rather non-specific term to describe a separation procedure, we have delineated this technique into four specific types: ion exchange, gel filtration, gas liquid, and high performance liquid chromatography. Their individual applications are described in following sections.

6.331 Ion Exchange Chromatography (IEC)

Resin column chromatography has been evaluated and employed by many investigators in the separation and quantitaiton of iodoamino acids and iodothyronines isolated from biological materials.[67-78] Although these procedures are amenable to assaying thyroxine and thyroid powders, the new separation techniques described in Sections 6.332, 6.333, and 6.334, have, in general, supplanted ion exchange chromatographic separations.

Anionic resins, Dowex I-X2 and 50-X4 (Dow Chemical Co., Midland, Mich.), using ammonium acetate, sodium acetate or ammonium formate buffers at pH rnages from 3.2-5.6, *without or containing up to 30% ethanol*, have been used to separate several of the iodothyronines. Automated procedures for detecting the components in effluents have also been reported.[68,73,74,77,78] The conventional ceric-arsenite reaction determination for iodine is the most frequently used detection method.

The application of cation exchange resins to separate the iodothyronines has been reported.[71, 77,78] The cited references deal almost exclusively with two iodoaminoacids and two iodothyronines, MIT, DIT, T3 and T4, respectively. Recently, Sorimachi and Ui[79] reported the separation of eight different iodothyronines on (1.0 x 15 cm) cation exchange resin, AG 50W-X4 (30-35 µm), equilibrated with o.04 M ammonium acetate buffer, pH 4.7, containing 30% (v/v) ethanol at 50°C and a gradient of increasing pH. The

gradient consisted of starting buffer and 0.65 N NaOH. Detection was by the iodine catalyzed ceric-arsenite reaction. Table 8 lists the elution volumes of a series of iodoaminoacids and iodide obtained by this procedure.

Table 8

Elution Volumes of Iodoaminoacids

Compound	Approximate Elution Volumes (ml)
Iodide	6
MIT	37
DIT	50
T1	80
T2	80
T3	106
T4	91
3'-T1	96
3,3'-T2	113
3',5'-T2	88
3,3',5'-T3	96

6.332 Gel Filtration Chromatography (GFC)

The application of gel filtration separation of the iodothyronines has been primarily used to determine their individual contents in biological samples. The information provided in Table 9 indicates the chromatographic systems used to separate the iodoaminoacids isolated from these samples. Each of the cited references describes the important steps required to prepare these columns (e.g., their dimensions, mesh size of the gel particles, etc.), the detection systems used (generally the ceric-arsenite reaction, ultraviolet absorption, liquid scintillation counting of tagged isolates, etc.), and the precision and accuracy of the particular variation employed by the respective invesitgator.

Blasi and DeMasi[80] have listed the partition coefficients, K_d, of several tyrosines and thyronines as obtained from the Sephadex G-25 column and their elution conditions. From these data, relationships between the structure of the compound and the elution volume can be established. The K_d values are listed in Table 10.

Table 9

Gel Filtration Chromatographic Separation Systems

Column Material	Eluent	Compounds Separated	Ref #
Sephadex G-25[a]	0.01 N NaOH	DIT, T3, T4	81
Sephadex G-25	tert-amyl alcohol saturated with 2 N NH_4OH	T3, T4	82
Sephadex G-25	0.02 N NaOH	Ty, MIT, DIT, T, TI,[b] T2, T3, T4	80
Sephadex LH-20[a]	ethyl acetate: methanol: 2 N NH_4OH (100:25:10, v/v)	MIT, DIT, T3, T4	83
Sephadex G-25	0.1 N NaOH 0.005 N NaCl	I^-, T3, T4	84
Sephadex G-15[a]	0.02 N NaOH	T3, T4	85

[a] Pharmacia Fine Chemicals, Inc., Piscataway, NJ

[b] 3-iodothyronine. In addition 3',5'-diiodothyronine and 3,3',5'-triiodothyronine were also separated.

Table 10

K_d Values of Iodothyronines and Related Compounds

Compound[a]	K_d
Ty	0.32
MIT	0.36
DIT	0.52
T	0.52
3-Iodothyronine	0.93
T2	1.13
3',5'-diiodothyronine	1.95
T3	2.35
3,3',5'-triiodothyronine	4.40
T4	5.20

[a] 1.5 mg in 0.5 ml 0.02 N NaOH

Table II

Gas Liquid Chromatographic Separation Systems

Compound Separated	Derivative	Column	Column Temperature	Detector	Amount Injected	Ref #
T4, T3 MIT, DIT, T	N,O-dipivalyl methyl ester	0.5% SE-30 on Gas Chrom Q	Program 130-305°C 10°/min	FID[a]	µg	66
T4, T3, T2 MIT, DIT	N,O-bistrifluoro acetyl methyl ester	3.8% SE-30 on Diatoport S	250°C	FID	0.01 µmol	86
T4, T3, MIT, DIT	N,O-dipivalyl methyl ester	1% polysulfone on Gas Chrom Q	232°C	ECD[b]	ng	87
T4, T3, MIT, DIT	N,O-dipivalyl methyl ester	3% OV-17 on Gas Chrom P	282°C	ECD	pg	87
T4, T3, MIT, DIT	TMS[c]	3% OV-1 on Gas Chrom Q	285°C	ECD	50-150 ng	88
T4, T3, T2, MIT, DIT, T	TMS	0.5% SE-30 on DMSC-treated[d] Chromosorb G	75-250°C @ 4.6°/min	FIC	20 ng	89
T4, T3, T2	N,O-dipivalyl methyl ester	5% OV-17 on Gas Chrom Q	225-235°C @ 5°/min	FID	<1 µg	90
T4, T3, T2, MIT, DIT, T	N,O-dipivalyl methyl ester	5% OV-17 on Gas Chrom Q	285°C	ECD	3 ng	90

Table II (continued)

Compound Separated	Derivative	Column	Column Temperature	Detector	Amount Injected	Ref #
T4, T3, T2 DIT, Ty	TMS	2% SE-33 on Gas Chrom Q	150-280°C @ 10°/min	FID	50 ng	*91*
T4, T3, T DIT, MIT, Ty	TMS	3% OV-17 on Gas Chrom Q	165°C @ 3 min to 265° @ 10 min	FID	3-15 µg	*92*
T4, T3, T2, T, MIT DIT, Ty	TMS	1% OV-1 on Chromosorb WHP	135-255°C @ 5°/min	FID	~4 µg	*93*
T4, T3, T2, T, MIT, DIT, Ty	TMS	1% OV-1 on Chromosorb WHP	180-225°C @ 25°/min	ECD	0.3-1.5 ng	*93*
T4, T3, T2	TMS	3% OV-17 on Diatomite CQ	*(e)*	FID	5-20 ng	*94*
T4,*(f)* T3, T2, DIT	N,O-dimethyl methyl ester	3% OV-1 on Gas Chrom Q	250°C	FID	12 µg	*95*
T4, T3, T2	TFAA*(g)* methyl esters	2.3% OV-1 on Gas Chrom Q	*(e)*	ECD	1.4-2.5 ng	*96*
T4, T3, T2	N,O-dipivalyl methyl esters	2.3% OV-1 on Gas Chrom Q	290°C	ECD	6-8 ng	*96*
T4, T3,	TMS	1% OV-1 on Gas Chrom Q	165-285°C @ 10°/min	FID	4-16 µg	*97*

Table II (continued)

Compound Separated	Derivative	Column	Column Temperature	Detector	Amount Injected	Ref #
T4, T3	N,O-dipivalyl methyl ester	3% DEXSIL 300 GC on Chromosorb WHP	305°C	ECD	0.25-3 ng	98

(a) FID = Flame Ionization Detector

(b) ECD = Electron Capture Detector

(c) Trimethylsilyl derivative

(d) Dimethylchlorosilane

(e) Variable, depending on which compounds are to be separated

(f) As the sodium salt, hydrate

6.333 Gas Liquid Chromatography (GLC)

Since the iodoaminoacids are not volatile and thus not amenable to a gas chromatographic analysis, the preparation of suitable stable volatile derivatives prior to analysis is a prerequisite.

The first successful gas chromatographic separation of T4, T3, DIT, MIT, and Ty, as their N,O-dipivalyl methyl ester derivatives, was reported by Stouffer, et al.[66] Since then this techniques has been critically evaluated because of its inherent sensitivity, speed of analysis, and applicability to the quantitation of these compounds in biological preparations. As it is beyond the scope of this monograph to provide precise details of the gas chromatographic methods, a tabulation of the various reports is listed in Table II. It is recommended that the cited references be referred to for the preparation of the volatile derivatives, the use of internal standards for quantitative analysis, and for the preparation of the column substrates.

From the information in Table II it is apparent that the two favored derivatives are the N,O-dipivalyl methyl ester and the TMS. The advantage of the former is that the esters have greater stability. However, they require a two-step synthesis, whereas the latter can be prepared in a single step but are sensitive to moisture.

6.334 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) has been used by Karger, et al.[99] to separate T4, T3 and T2 in about two minutes in a mobile phase of butanol and methylene chloride on a silica gel column coated with a mixture of perchloric acid and sodium perchlorate. On a similar system T4, MIT and DIT separated in less than eight minutes. DuPont Instruments[100] reported the separation of T4 and T3 on a strong cation exchange (SCX) column. Both of these methods showed excellent sensitivity, in that nanogram quantities could be readily detected using highly sensitive UV detectors at 254 nm.

Thyroxine and triiodothyronine have also been separated in less than 12 minutes on a Micropak

C-18 (reverse phase) column using a methanol-ammonium citrate mobile phase.[101] Waters Associates[102] reported a similar separation on a C_{18}/Corasil column using a mobile phase consisting of acetonitrile-n-butanol:0.005 M sodium perchlorate. A clear separation was effected in less than 20 minutes.

6.4 Neutron Activation Analysis

Neutron activation analysis was used by Alsos[103] to determine the L-thyroxine sodium content in tablets. In this procedure, the tablet was irradiated for 5 minutes in a neutron flux of $\approx 2.5 \times 10^{12} n/cm^2/sec$ and quickly transferred to a counting tube for measurement of ^{128}I. Comparison with standards of potassium iodide irradiated for the same time yielded the content of L-thyroxine sodium. Interfering nuclear reactions were negligible, and the error was less than 2%.

Neutron activation analysis was also used by Globel, et al.[104] to determine the relative amounts of L-thyroxine (T4) and 3,5,3'-triiodothyronine (T3) in serum. These hormones were removed from the proteins by passing 20 ml of serum (at pH 11) through a Dowex 1X-2 ion exchange column in the acetate form. The eluate was concentrated and chromatographed on a cellulose thin layer plate to separate the T4 and T3 from the inorganic iodide. The isolated T4 and T3 fractions were irradiated in a thermal neutron flux of $5 \times 10^2 n/cm^2/sec$ using a Triga Mark I reactor, followed by identification and measurement of induced ^{128}I activity with a germanium (Li) solid state detector. The limits of detection were 5 ng.

Schmoelzer and Mueller[105] used a similar approach but separated the T4 and T3 on a QAE Sephadex A-25 column prior to activation analysis.

6.5 Polarographic Analysis

Polarography was employed by Wacholz and Pfeifer to assay thyroxine[106] and to determine the thyroxine content of thyroid powders and tablets.[107]

In the assay of thyroxine, 5-50 µg of sample in 1 ml of 2 N nitric acid is heated at 60°C for 1 hour. After cooling and the addition of 5 ml of 0.06 N sodium hydroxide, the

solution is deoxygenated with nitrogen. The thyroxine content is determined by comparison of wave height of the sample at $E_{\frac{1}{2}}$ -0.6 to -0.7V with that of standards. The method is sufficiently sensitive to determine the thyroxine content of spots isolated by thin layer chromatography.

In the assay for thyroxine in tablets and powders, prior extraction procedures are required to remove the thyroxine and other iodinated amino acids. After separation by thin layer chromatography, elution of the thryoxine spot, concentration and nitration,[106] the wave height at the previously specified $E_{\frac{1}{2}}$ is obtained.

6.6 Kinetic Methods of Analysis

The application of kinetic measurements of the iodine catalyzed ceric arsenite reaction[108,109] has been utilized for the determination of thyroxine iodine in chromatographic eluates.[76,110,111] The reported methods are rapid, have high precision and are sensitive, generally detecting less than 1 ng of T4.

6.7 Double-Isotope Dilution Analysis

A double-isotope derivative assay for serum iodothyronines[112] (L-T4 and L-T3) has been modified and improved upon by Hagen, et al.[8] In this procedure, the unknown thyroxine is labeled by formation of an acetyl derivative[113] using tritium-labeled acetic anhydride. As the specific activity of the tritiated derivative is known, the thyroxine content of the sample can be calculated. Losses in the complex purification steps are accounted for by the addition of a high specific activity ^{131}I-labeled thyroxine.

6.8 Determination of Stereoisomeric Purity

Information presented by the Joint Committee of the Pharmaceutical Society of Great Britain and the British Pharmaceutical Committee[54] indicates that all D-thyroxine contains trace amounts of the L-isomer. A method for determining the amount of L-T3, an intermediate in the synthesis of D-T4, has been reported.[45] An adaptation of this method has been applied to D-T4 containing less than 1% of the L-isomer.[114]

6.9 Equilibrium Dialysis

Several investigators[115-117] have used equilibrium dialysis to determine the free thryoxine in serum. A critical study of this procedure was made by Lee and Pileggi.[115] The effects of pH, incubation time and temperature, buffer composition and concentration, protein concentration, and specimen dilution were studied. Using ^{131}I-L-thyroxine and a reusable plastic dialysis cell, recoveries of 92-96% were obtained when the dialysis was run against 0.05 M phosphate pH 7.6 buffer at 37°C for 18 hours. Within-run and between-run precisions were 10.6% and 14.2%, respectively.

Fang and Selenkow,[116] using the conventional dialysis bag and ^{125}I-L-thyroxine, determined the free thyroxine content after dialysis at 4°C, against pH 7.4 phosphate buffer for 18-24 hours.

Bird and Abiodun[117] employed equilibrium dialysis and ion exchange chromatography to determine free thyroxine. Ion exchange chromatography was used to separate the ^{125}I iodide from the ^{125}I-L-thyroxine prior to determining the free thyroxine content.

The literature is replete with a significant number of publications describing modifications of equilibrium dyalysis or a combination of this procedure with other separation techniques.[118-122] The cited papers offer a good basic background to the application of this method to the study of the thryoxine-protein binding phenomenon.

7. Methods of Analysis - A Compilation

The following tables (12, 13, 14 and 15) include the references to analytical procedures for the analysis of chemicals, tablets, powders, and serum and tissue. Several additional references which were not cited in the specific sections are included in the following compilations. Two review articles, by Cahnmann[123] and by Rall, et al.[124], are also noted.

Table 12

Analysis of Thyroxine Chemicals

Analysis:	See Reference #:
Identification	*1, 3, 4, 54, 125, 126*
Titrimetry:	
Iodometric	*1, 3, 37, 107*
N-Bromosuccinimide	*38*
Coulometric	*39, 40*
Specific Ion Electrode	*43*
Cerimetry	*129*
Chromatography:	
PC	*45, 54, 130, 131, 132, 133*
TLC	*54, 55, 58, 59, 61, 62, 107, 130, 131, 134*
IEC	*68, 69, 77, 79, 136*
GFC	*110, 127, 137*
GLC	***86***, *87, 89, 90, 91, 92, 93, 94, 97, 98, 138*
HPLC	*101, 142, 143*
Neutron Activation	*103*
Polarography	*97, 106, 107*
Kinetic	*76, 78, 111*
Spectrophotometry	*5, 6, 7, 107, 144*
Automation	*68, 77, 78, 110, 136, 145, 146, 147*
Electrophoresis	*54*
Phase Solubility	*54*

Table 13

Analysis of Thyroxine Tablets

Analysis	See Reference #
Titrimetry:	
Iodometric	*1, 3, 4*

Table 13 (continued

Chromatography:	
PC	*148*
TLC	*107, 144, 149*
Polarography	*107*
Spectrophotometry	*107, 144, 148, 149, 150, 151, 152, 153*

Table 14

Analysis of Thyroxine Powders

Analysis	See Reference #
Cerimetry	*61*
Chromatography	
PC	*154*
TLC	*59, 61, 149, 155, 156*
IEC	*154*
GLC	*94, 97, 157*
Spectrophotometry	*149, 150, 156*
Titrimetry	*1*

Table 15

Analysis of Sera and/or Tissues for Thyroxine and Analogs

Analysis	See Reference #
Cerimetry	*68, 69, 71, 73, 74, 75, 76, 77, 78, 79, 110, 133, 145, 146, 158, 159, 160, 161*
Chromatography:	
IEC	*67, 69, 70, 71, 72, 73, 74, 76, 77, 110, 117, 136, 145, 146,*
GFC	*147, 159, 163, 164*
GFC	*85, 110, 137, 165, 166, 167*
GLC	*87, 90, 98, 138*
Neutron Activation	*104, 105*

Table 15 (continued)

Kinetic	*111*
Dialysis	*115, 116, 117, 118, 119, 168, 169, 170, 171, 172*
Spectrometry	*163, 169, 173*
Automation	*73, 74, 78, 136, 145, 146, 147, 159, 170*
Radioimmunoassay	*174, 175, 176*
Competitive Protein Binding	***105***, *128, 170, 171, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187*
Nephelometry	*188*
Electrophoresis	*70, 135, 172*
Fluorometry	*162*

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METHOTREXATE

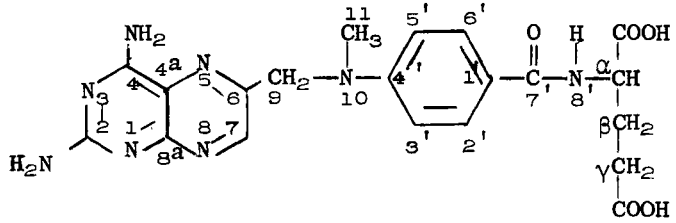
Arthur R. Chamberlin, Andrew P.K. Cheung, and Peter Lim

Contents

1. Description

1.1 Name, Structural and Empirical Formulae, Molecular Weight

Methotrexate is N-[4-{[(2,4-diamino-6-pteridinyl)-methyl]methylamine}benzoyl]glutamic acid. Frequently, the name is abbreviated to MTX. Methotrexate also is known as 4-amino-10-methylfolic acid and amethopterin and is identified by the National Cancer Institute code number NSC-740.



$C_{20}H_{22}N_8O_5$ Mol. wt. 454.46

1.2 Isomeric Forms

The presence of an asymmetric carbon in the glutamic acid moiety provides for optical isomerism. Unless specified, commercially available methotrexate is prepared from L-glutamic acid. Recently, Lee and co-workers[1] prepared methotrexate starting with D-glutamic acid. The D-enantiomer of methotrexate was active against L-1210 in the mouse and had less toxic effects than methotrexate itself, the L-enantiomer.

1.3 Appearance, Color, Odor

Methotrexate is a bright yellow-orange, odorless powder. It generally is hydrated to the extent of 8 to 10% water. It also has been prepared as the hydrochloride (1:0.3) and hydrate (1:2.5)*.

* Private communication from Dr. H.B. Wood, Jr., of the National Cancer Institute and Mr. D.F. Worth of Parke, Davis and Co.

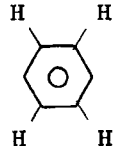
2. Physical Properties

2.1 Infrared Spectrum

Recorded as a suspension in mineral oil, the spectrum in Figure 1 shows relatively broad structures, indicating the complexity of the molecule and suggesting the lack of crystallinity of the sample. Table I gives the assignments to the major bands.

TABLE 1

Infrared Assignments for Methotrexate

IR Absorption Band (μ)	Interpretation
2.90-3.10	H_2O, $-NH_{(2)}$
3.00-4.00	$-COOH$
5.90-6.10	$-\overset{O}{\overset{\|}{C}}-(COOH, -\overset{O}{\overset{\|}{C}}-\overset{H}{\overset{\|}{N}}-)$
6.20, 6.50-6.60	Aryl systems
6.50-6.60	Amide II
11.9	

2.2 Proton Magnetic Resonance Spectrum (pmr)

The pmr spectrum in Figure 2 was recorded on a Varian A60-A spectrometer with the sample as a solution in DMSO-d_6. The chemical shifts are in ppm relative to TMS designated as 0.00.

Table II gives the structural assignments to the resonances in Figure 2.

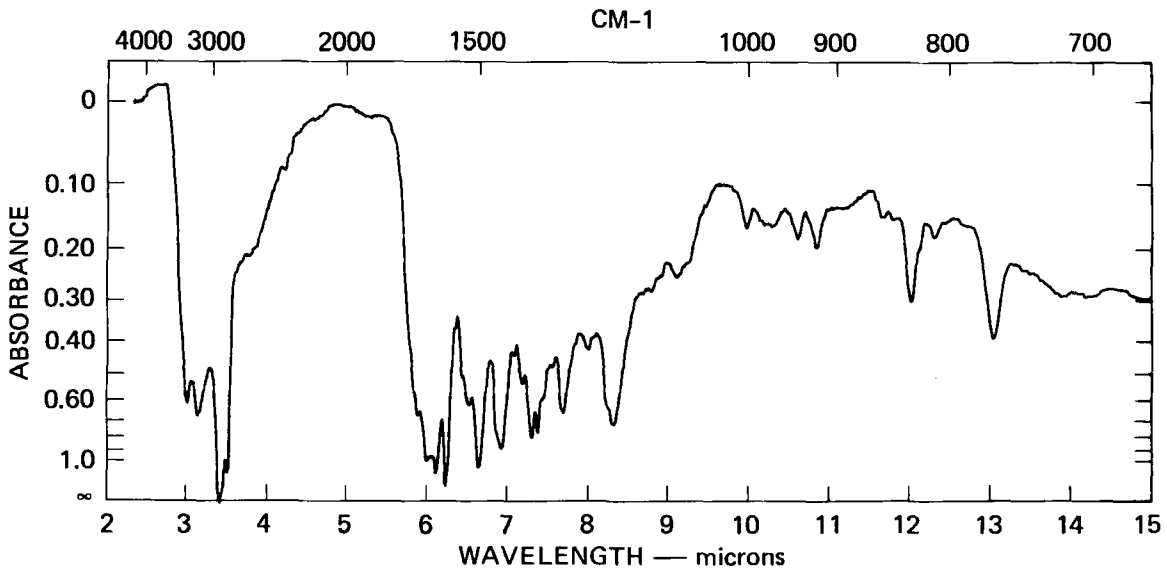


FIGURE 1 INFRARED SPECTRUM OF METHOTREXATE

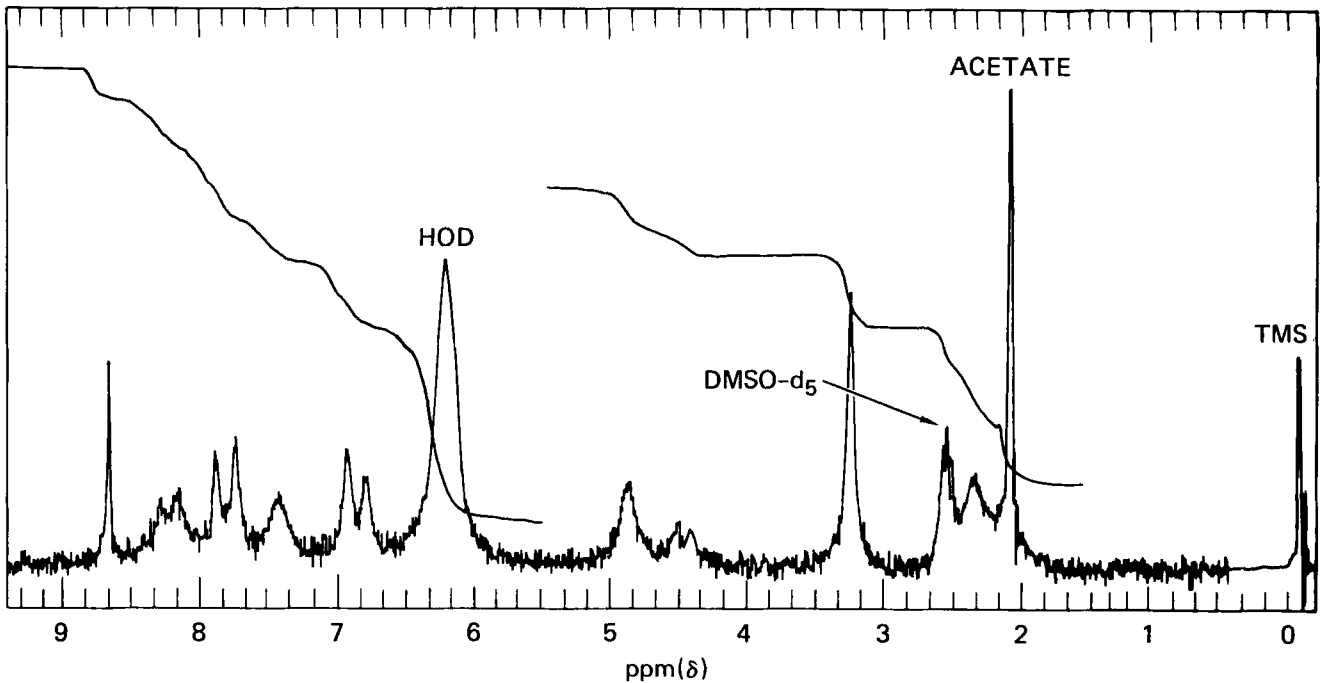


FIGURE 2 PMR SPECTRUM OF METHOTREXATE

TABLE II

pmr Assignments for Methotrexate

Assignments	Chemical Shifts (ppm)	Multiplicity	J(Hz)
H_2N-2,4	7.42	s(broad)	--
H-7	8.64	s	--
H-9	4.81	s(broad)	--
H_3C-11	3.21	s	--
H-2',6',3',5'	6.81,7.78	d	8.2
H-8'	8.21	d	8.0
H-α	4.43	q	7.0
H-β,γ	1.70-2.60	m	--
H(COOH, HOH)	6.21	s(broad)	--

The values agree reasonable well with those reported by Pastore[2] who studied the pmr spectra of methotrexate in solutions at pH 7.5.

2.3 Carbon-13 magnetic spectrum (cmr)

The ^{13}cmr spectrum in Figure 3 was recorded on a Varian XL-100 spectrometer with the sample as a solution in DMSO. The assignments given in Table III for Figure 3 are in general agreement with those reported by Ewers and co-workers,[3] but minor differences exist.

TABLE III

^{13}C Assignment For Methotrexate (TMS 0.00 ppm)

Chemical Shift (ppm)	Assignment
162.67 }	C-2
160.01	C-4
148.31 }	C-6
148.96 }	C-7
150.94	C-8a
121.66	C-4a
~ 40 (amid DMSO)	C-9
51.96	C-11
121.24	C-1'
128.94	C-2', C-6'
111.12	C-3', C-5'
150.85	C-4'
166.41	C-7'
54.82	C-α
26.17	C-β
30.55	C-γ
173.96 }	α-COOH
174.11 }	γ-COOH

2.4 Ultraviolet Spectrum*

Figure 4 is the UV spectrum of methotrexate in 0.1 N NaOH. The longest wavelength maximum appears at 372 nm and is ascribable to the diaminopteridine moiety. The intermediate wavelength maximum occurs at 303 nm and is due primarily to the aminobenzoyl group. The short wavelength maximum is at 258 nm and is attributed to both chromophores. In 0.1 N HCl methotrexate experiences a hypsochromic shift resulting in a UV spectrum (Figure 5) that exhibits

* UV spectra were recorded on a Cary Model 14 and the molar absorptivities are based on anhydrous methotrexate.

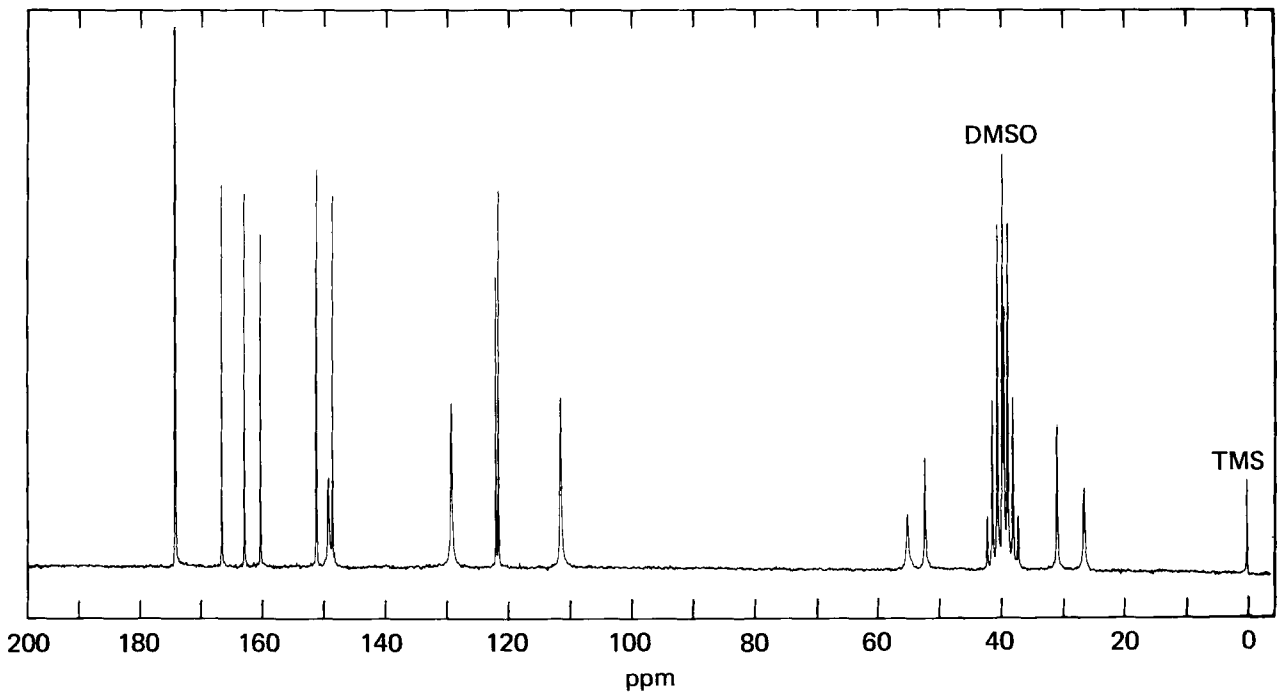


FIGURE 3 $C^{13}mr$ SPECTRUM OF METHOTREXATE

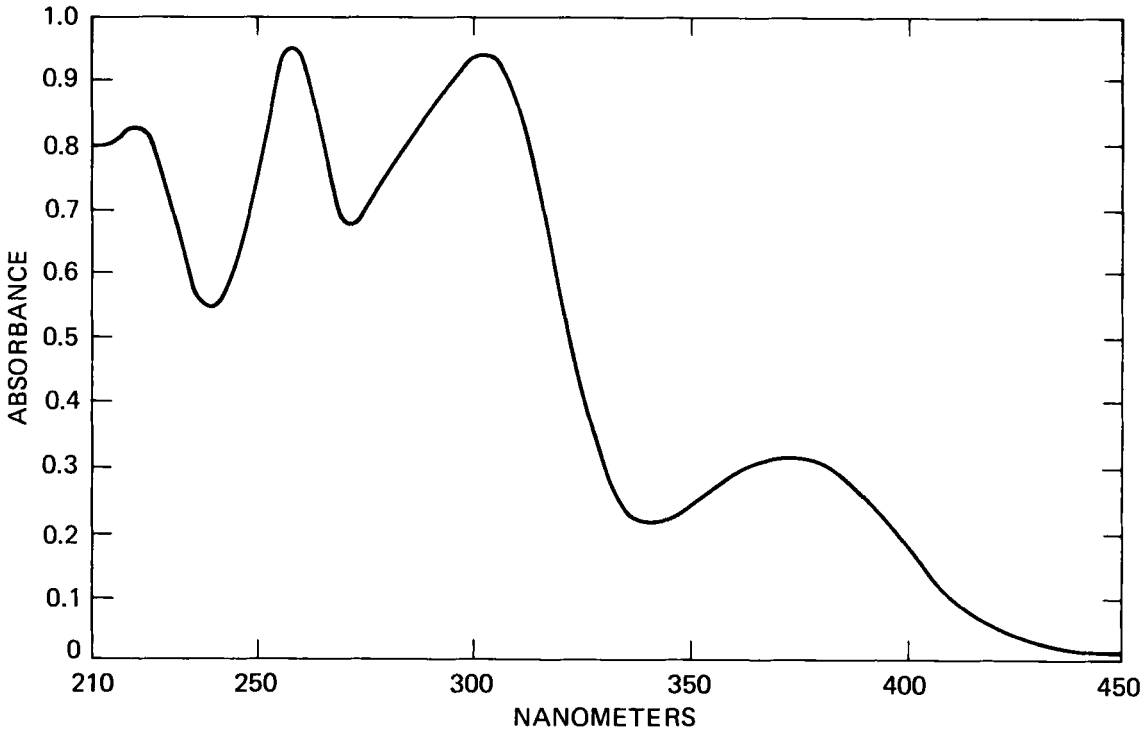


FIGURE 4 ULTRAVIOLET SPECTRUM OF METHOTREXATE SOLVENT—0.1N NaOH

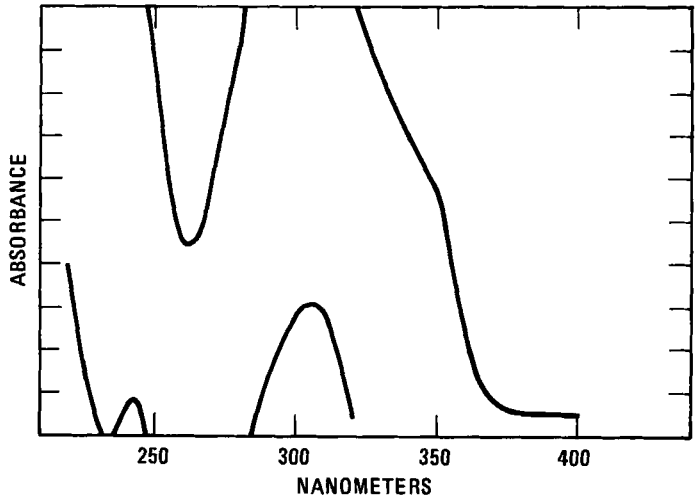


FIGURE 5

maxima at 307 and 243 nm. The UV data are summarized in Table IV and in general are in agreement with those reported by Seeger and co-workers[4].

TABLE IV

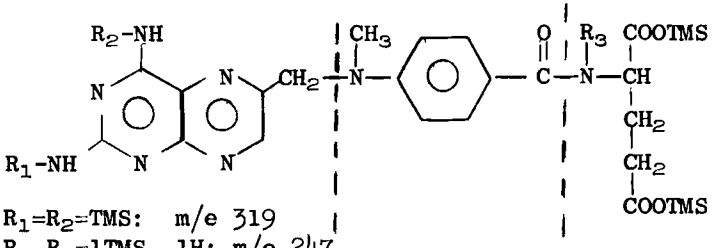
Absorption Spectra of Methotrexate

Solvent	λ_{max} nm ($\varepsilon_{max} \times 10^{-3}$)
0.1 N HCl	307 (21.6), 243 (18.1)
0.1 M pH6.7 Tris buffer	370 (7.45), 302 (24.8), 257 (23.3)
0.1 N NaOH	372 (8.05), 303 (24.8), 258 (24.7)

2.5 Mass Spectrum

Methotrexate itself does not yield a satisfactory mass spectrum because of non-volatility. However, treatment of methotrexate with a TMS reagent affords a mixture of tri-, tetra-, and penta-TMS derivatives that does give useful mass spectral data. That several TMS-containing derivatives are formed is not surprising, considering the number and variety of functional groups involved.

The mass spectral fragmentation pattern obtained for the tri-, tetra-, and penta-TMS derivatives is summarized as follows:



R_1=R_2=TMS: m/e 319
R_1,R_2=1TMS, 1H; m/e 247

R_1=R_2=TMS m/e 452
R_1,R_2=1TMS, 1H m/e 380

M"→R_1=R_2=R_3=TMS m/e 814
M→ R_1=R_2=TMS, R_3=H m/e 742
M'→R_1,R_2=1TMS, 1H, R_3=H m/e 670

M-CH_3 m/e 727
M-HOTMS m/e 652
M-HOTMS-CH_3 m/e 637

2.6 Optical Rotation

$$\alpha]^{21}_{589} = 20.4 \pm 0.6^{\circ} \quad (\underline{C}\ 1,\ N/10\ NaOH)$$
$$\phantom{\alpha]^{21}}_{546} = 26.9 \pm 0.8^{\circ}$$

*

2.7 Dissociation Constants

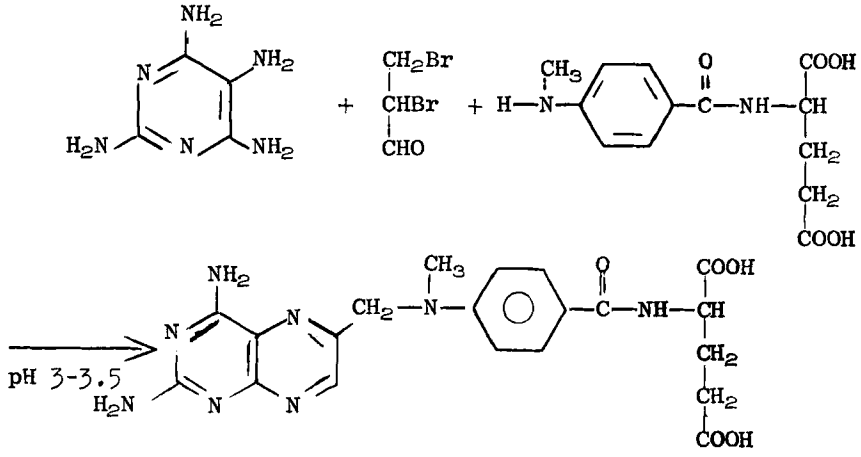
Neither the acidic nor the basic pKa for methotrexate has been reported. However, Albert and co-workers[5] reported that the basic pKa values for 2,4-diaminopteridine were < 0.5 and 5.32. Kallen and Jencks[6] reported the acidic pKa values for p-aminobenzoylglutamic acid as 4.83 and 3.76. By spectrometry, we found a pKa of 5.60 ± 0.03, which is assignable to the diaminopteridinyl moiety.

2.8 Solubility[7]

Methotrexate is practically insoluble in water, alcohol, chloroform, and ether. It is freely soluble in dilute solutions of alkaline and carbonates; it is slightly soluble in dilute hydrochloric acid (1 in 2).

3. Synthesis

The first reported synthesis of methotrexate, that by Seeger and co-workers[8] is as follows:



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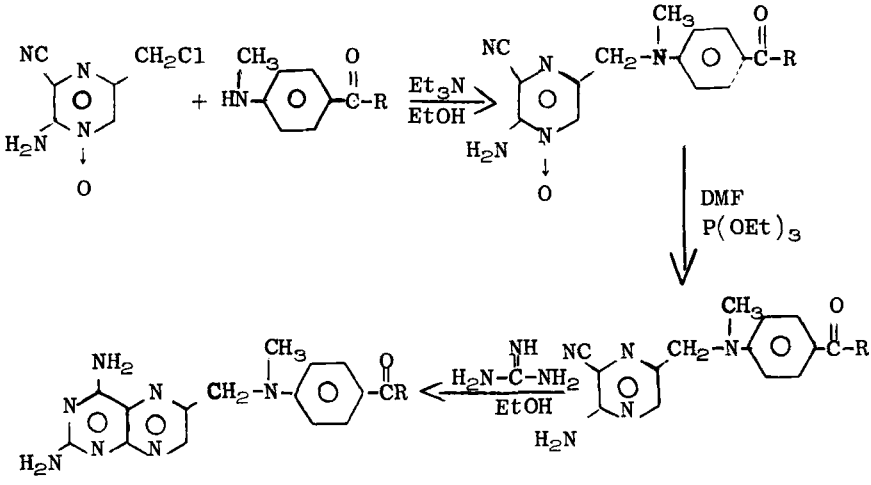
* These specific rotation values are based on anhydrous methotrexate.

This reaction often is referred to as the Waller reaction, because Waller initially prepared pteroylglutamic acid by an analogous method.

Theoretically, the substitution on the pyrazine ring can be at the C-6 or the C-7 position. Proof of structure is based on the pterine carboxylic acid obtained from an alkaline permanganate oxidation of methotrexate. Of the two acids possible, only the C-6 has been found. Distinction between the two possible acids has been based on comparative paper chromatography, although pmr or cmr also could be used.

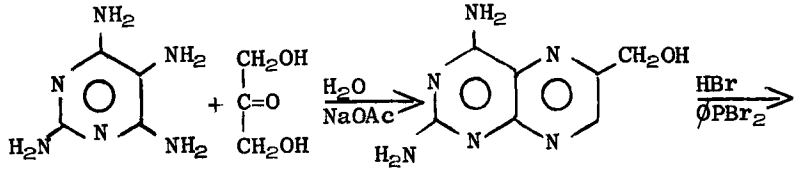
Because the Waller reaction generally yields impure products that are very difficult to purify, many researchers have attempted to improve the synthesis of methotrexate. Among the more successful attempts are those by Taylor[9] and by Piper and Montgomery[10]. The respective methods are outlined as follows:

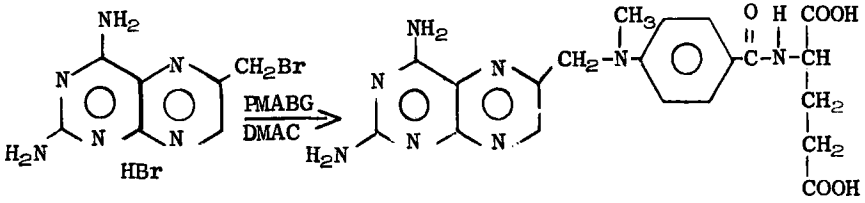
Taylor



R=N-(glutamyl)

Piper and Montgomery





PMABG = N-(p-methylaminobenzoyl)glutamic acid
DMAC = N,N-dimethylacetamide

These improved procedures not only yield products that are easier to purify, but the substitution on the pyrazine is unambiguous.

4. Stability

4.1 Bulk

When stored in a capped, brown bottle at room temperature, samplings removed over a twelve-month period yielded indistinguishable UV and paper chromatographic data. These results indicate that methotrexate is stable for at least one year under these conditions.

4.2 Solution

As dilute solutions (0.5 mg and 0.05 mg/ml) in pH 7.0 aqueous sodium bicarbonate maintained at room temperature in darkness and under laboratory illumination, aliquots removed over a 24-hour period yielded identical papergrams, within experimental errors. On this basis, these solutions were considered stable under these conditions.

Under strongly acidic aqueous conditions, the amide is subject to hydrolysis, yielding N^{10}-methyl-4-amino-4-deoxypteroic acid and glutamic acid. Under highly alkaline aqueous conditions, especially at elevated temperatures, the principal decomposition products are N^{10}-methylfolic acid, N^{10}-methylpteroic acid, and glutamic acid--all as the carboxylate ions.

Photodecomposition of certain pterines is well documented.[11] However, no specific report on the photochemistry of methotrexate has been found in the literature.

5. Metabolism

In man, a very large portion of the methotrexate administered is excreted unchanged,[11a] indicating that little metabolism of this drug occurs.

Johns and Loo[12] reported that methotrexate is metabolized rapidly by rabbit liver aldehyde oxidase, and the metabolite was identified as 4-amino-4-deoxy-7-hydroxy-N^{10}-methylpteroylglutamic acid (7-hydroxy-MTX).

Johns and Valerino[13] have observed that, when methotrexate is incubated with cecal contents from the mouse *in vitro*, 4-amino-4-deoxy-N^{10}-methylpteroic acid is produced. Levy and Goldman[14] have demonstrated that this metabolite can be produced from methotrexate by action of carboxypeptidases from strains of *Psudomonas*.

6. Methods of Analysis

6.1 Elemental Analysis

Table IV presents the results from a representative elemental analysis of methotrexate (reference standard).

TABLE V

Elemental Analysis of Methotrexate

Element	% Theory	% Found*
C	52.86	52.72
H	4.88	4.85
N	24.66	24.51

* Adjusted for the found water

6.2 Equivalent Weight Determinations

6.21 Nonaqueous Titration

De Carnevale et al.[15] described an equivalent weight determination based on a sodium methoxide titration in pyridine to an azo-violet end point. Because the basis of the titration is an acid/base neutralization, it lacks specificity. Consequently, the method has not been employed widely as an assay for methotrexate.

6.22 Complex-Formation Titration

Guerello has described[16] a second titration by which equivalent weights of methotrexate samples can be obtained. The method is based on the complexation between the Ca^{++} and the glutamyl moiety and thereby affords higher specificity than an acid/base neutralization. Because glutamic acid containing impurities are commonly found in methotrexate samples, results from this titration must be interpreted carefully.

6.3 Biological Assay

6.31 Microbiological Assay

Several microbiological assays are described in the literature.[17] All are relatively nonspecific and very time consuming and thus have little usefulness for routine analyses.

6.32 Enzymic Assay

Werkheiser and co-workers[18] have developed an enzymatic assay for methotrexate using folic acid reductase. Methotrexate binds very tenaciously and stoichiometrically to this enzyme and is determined colorometrically by titration of the drug with the enzyme.

6.4 Polarographic Assay

Asahi[19] has reported polarographic reduction of methotrexate. He attributes the first wave as being the reduction to dihydromethotrexate, the second wave as being the reductive cleavage of the CH_2-N bond, and the third wave as being the reduction to 2,4,-diamino-6-methyl-5,6,7,8-tetrahydropteridine.

6.5 Spectrophotometric Analysis

6.51 Fluorometric Analysis

Fluorometric methods[20] have been used widely in the analysis of methotrexate. The methods are based on an oxidative transformation of methotrexate to the presumed pterine carboxylic acid which fluoresces intensely. Because many of the impurities commonly found in methotrexate also undergo the same reaction, these methods lack specificity unless the oxidation is preceded by a separation scheme in which methotrexate is isolated.

6.52 Ultraviolet/Visible Analysis

Because commercial methotrexate samples are impure, and because the organic impurities have UV absorption characteristics that are similar to those of methotrexate, direct uv/vis spectrophotometric analysis is entirely too nonspecific to be of any value in the quantitative analysis of methotrexate. Practically all the contaminants likely to be present in a sample of methotrexate--N^{10}-methylpteroic acid, and so forth--would interfere with a direct UV or visible method.

6.6 Chromatography

6.61 Paper

Nichol and co-workers[21] have reported the separations of methotrexate from related compounds on Whatman No. 1 with pH 7.0, 0.1 M sodium phosphate and with pH 5.0, 0.1 M sodium acetate. In our laboratory, we have used 0.5% $NaHCO_3$ and 0.5% Na_2CO_3 with the same paper.

Balazs and co-workers[22] reported a rapid assay for methotrexate based on paper chromatographic separation followed by UV measurement of the isolated methotrexate component. The limitations of this assay are the accuracy of the reference UV value and the recovery of methotrexate from the papergram. The molar absorptivity at 303 nm for methotrexate in N/10 NaOH reported by Balazs and co-workers is in error; it should be 24,800.

The assay procedure for methotrexate cited in USP XVIII is similar to the assay reported by Balazs et al except that the reference standard is USP methotrexate

which itself is impure.

6.62 Thin-layer

Copenhaver and O'Brien[23] have used ion-exchange thin-layer chromatography to separate a number of folic acid analogs including methotrexate. The cation-exchange resin was AG50W-X4, and the developing solvent was 15% $Na_2HPO_4 \cdot 12\ H_2O$, pH 8.5 buffer containing 0.1 M mercaptoethanol.

6.63 Column

Heinrich and co-workers[24] reported the use of Dowex 1-chloride with very dilute HCl or NaCl to separate folic acid and related compounds. Noble[25] described a purification procedure based on the use of a Dowex 1-acetate with pH 3.2, M acetate buffer. Oliverio[26] cited the use of DEAE cellulose and pH 8 phosphate buffer gradient to separate folic acid analogs. Gallelli and Yokoyama[27] developed a methotrexate assay procedure entailing a DEAE cellulose separation followed by a spectrophotometric measurement of the isolated component.

The use of DEAE cellulose to separate folic acid analogs is well established. The principal limitations of this method are the time required to pack the column and the time needed to develop the chromatogram.

6.64 High Speed Liquid

In our work with other folic acid antagonists, cation exchange high speed liquid chromatography (HSLC) has been very effective in separating closely related pteridines. Taking advantage of this selectivity, we have developed a HSLC method of analysis for methotrexate that is specific, fast, and sensitive. We use it to assay bulk and formulated methotrexate. The procedure requires a reference methotrexate sample of known purity, which is used as an external reference or in conjunction with an internal reference that, in our laboratory, has been 2-amino-4-methylpyridine. The column, 1m x 3mm O.D. Vydac Cation Exchange Packing, is held at 55° during the development with pH 4.30, 0.1 M KH_2PO_4 at a flow rate of 1.0 ml/min. The effluent is monitored by a UV-detector set at 254 nm. With the detector set at its highest sensitivity, solutions as diluted as 1 μg methotrexate/ml can be injected

directly and detected.

A second procedure employing a 1m x 3mm O.D. column packed with reverse-phase phenyl and a mobile phase of 5% MeOH in 0.05 M KH_2PO_4, pH 7.0, buffer also has been developed and found useful. The reverse-phase system is less sensitive to minor pH variations resulting from sample impurities. For this reason, this column produces high precision more readily.

6.7 Proton Magnetic Resonance Assay

Methotrexate has been assayed by quantitative pmr on a Varian A-60A spectrometer. Accurately weighed portions of methotrexate and of an internal standard (2,4-dimethoxy-5-methylpyrimidine) were dissolved in DMSO, and the spectrum was recorded. The percentage of anhydrous methotrexate in the sample then was calculated according to the formula

$$\% \text{ MTX} = \frac{Wr}{Ws} \times \frac{As}{Ar} \times \frac{454.4}{154.2} \times P$$

where Wr = weight of internal standard used

Ws = weight of methotrexate sample

Ar = integrated area of internal standard H_6 singlet (8.02 δ)

As = integrated area of methotrexate H_7 proton (8.64 δ)

154.2 = molecular weight of internal standard

454.4 = molecular weight of anhydrous MTX

P = purity of the internal standard

Although the pmr method may lack sensitivity and possibly specificity in complex mixtures, it is one method of assay that does not require a methotrexate reference sample of known purity. Thus, it may be useful in cases in which no reference material is available.

7. Acknowledgments

Supported by Contract NO1-CM-33723 from the division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare. The opinions expressed are those of the authors and not necessarily those of the National Cancer Institute.

The authors wish to acknowledge the technical assistance rendered by Ms. Leslie Pont, Ms. Barbara Senuta, Ms. Florence Yoshikawa, Mr. Martin Struble and Mr. John Jee of Stanford Research Institute.

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METHYCLOTHIAZIDE

James A. Raihle

Contents

Methyclothiazide

1. Description

1.1 Nomenclature

1.11 Chemical Name

Methyclothiazide is 6-chloro-3-(chloromethyl) -3,4-dihydro-2-methyl-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide. (1) It is also known as 6-chloro-3-chloromethyl-3,4-dihydro-2-methyl-7-sulfamoyl-1,2,4-benzothiadiazine 1,1-dioxide; 6-chloro-3-chloromethyl-2-methyl-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxide (2) and by many slight variations of the particular nomenclature. The CAS Registry No. is [135-07-9].

1.12 Generic Name

Methyclothiazide

1.13 Trade Names

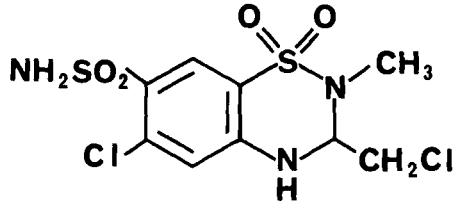
Enduron® and Aquatensen®

1.2 Formulae

1.21 Empirical

$C_9H_{11}Cl_2N_3O_4S_2$

1.22 Structural



1.3 Molecular Weight

360.23

1.4 Elemental Composition

C - 30.00; H - 3.08; Cl - 19.68; N - 11.66; O - 17.77; S - 17.80.

1.5 General

Methyclothiazide occurs as a white to practically white crystalline powder which is principally odorless.

2. Physical Properties

2.1 Infrared Spectrum (IR)

The infrared spectrum of methyclothiazide (NF Reference Standard, Lot No. 69196) is presented in Figure 1. The spectrum of a KBr pellet is taken on a Perkin-Elmer Model 521 Spectrophotometer. The assignments for the characteristic bands in the IR spectrum are listed in Table I. (3)

Table I

Characteristic Bands in the IR Spectrum of Methyclothiazide

Wavelength (cm^{-1})	Characteristic of
3270 and 3360	NH stretching vibration of sulfonamide group
1595 and 1502	C = C stretch of aromatics
1155 and 1330 (doublet)	S = O stretching vibrations of sulfonamide groups

This spectrum is consistent with that published by Fazzari and co-workers. (4)

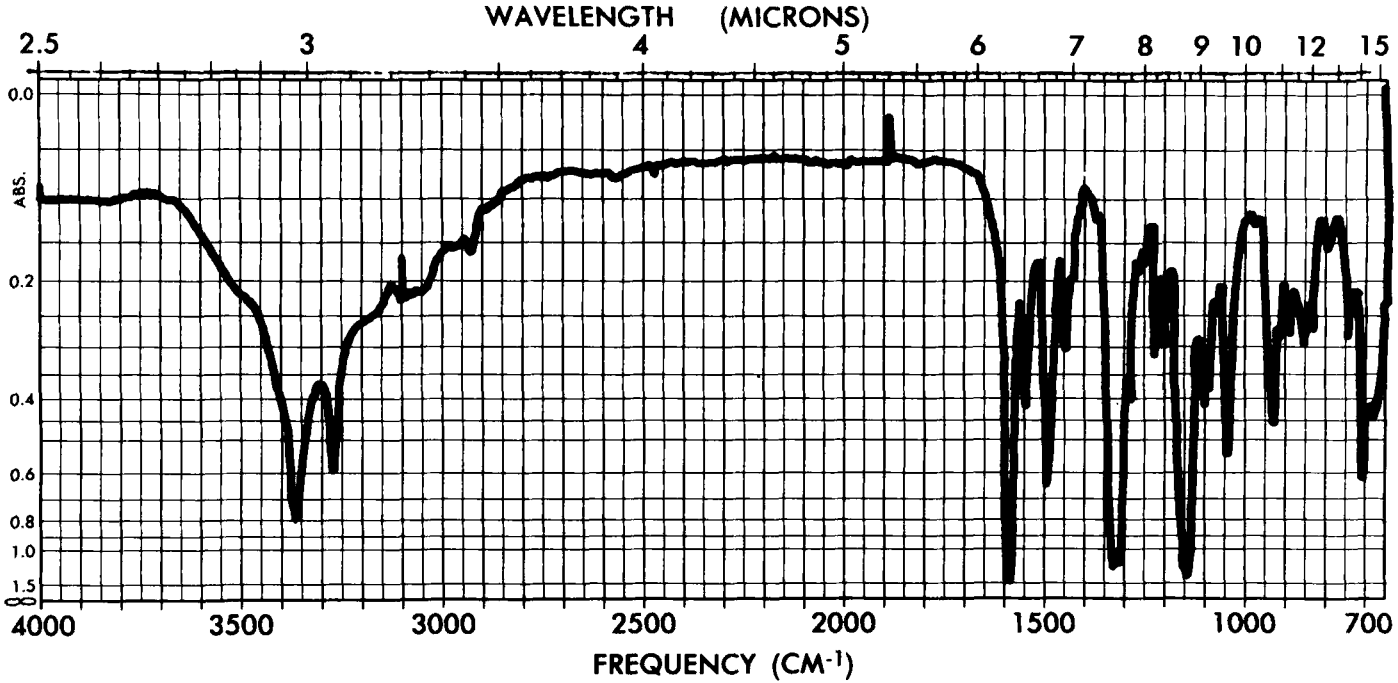


FIGURE 1 - INFRARED SPECTRUM OF METHYCLOTHIAZIDE

2.2 Raman Spectrum

The raman spectrum of the methyclothiazide reference standard was determined in the solid phase on a Cary Model 83 Spectrophotometer. The assignments of the characteristic bands as shown in Figure 2 are listed in Table II. (3)

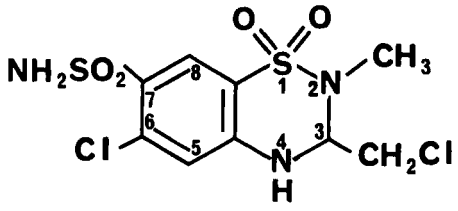
Table II

Characteristic Bands in the Raman Spectrum of Methyclothiazide

Wavelength (cm^{-1})	Characteristic of
3270 and 3363	NH stretching vibration of sulfonamide group
1600	C = C stretch of aromatics
1160	S = O stretching vibrations of sulfonamide groups

2.3 Nuclear Magnetic Resonance Spectrum (NMR)

The 60 MHz NMR spectrum of methyclothiazide is presented in Figure 3. The spectrum was determined in deuterated acetone (d_6) on a Varian T-60 Spectrometer. Spectral assignments are given in Table III. (5)



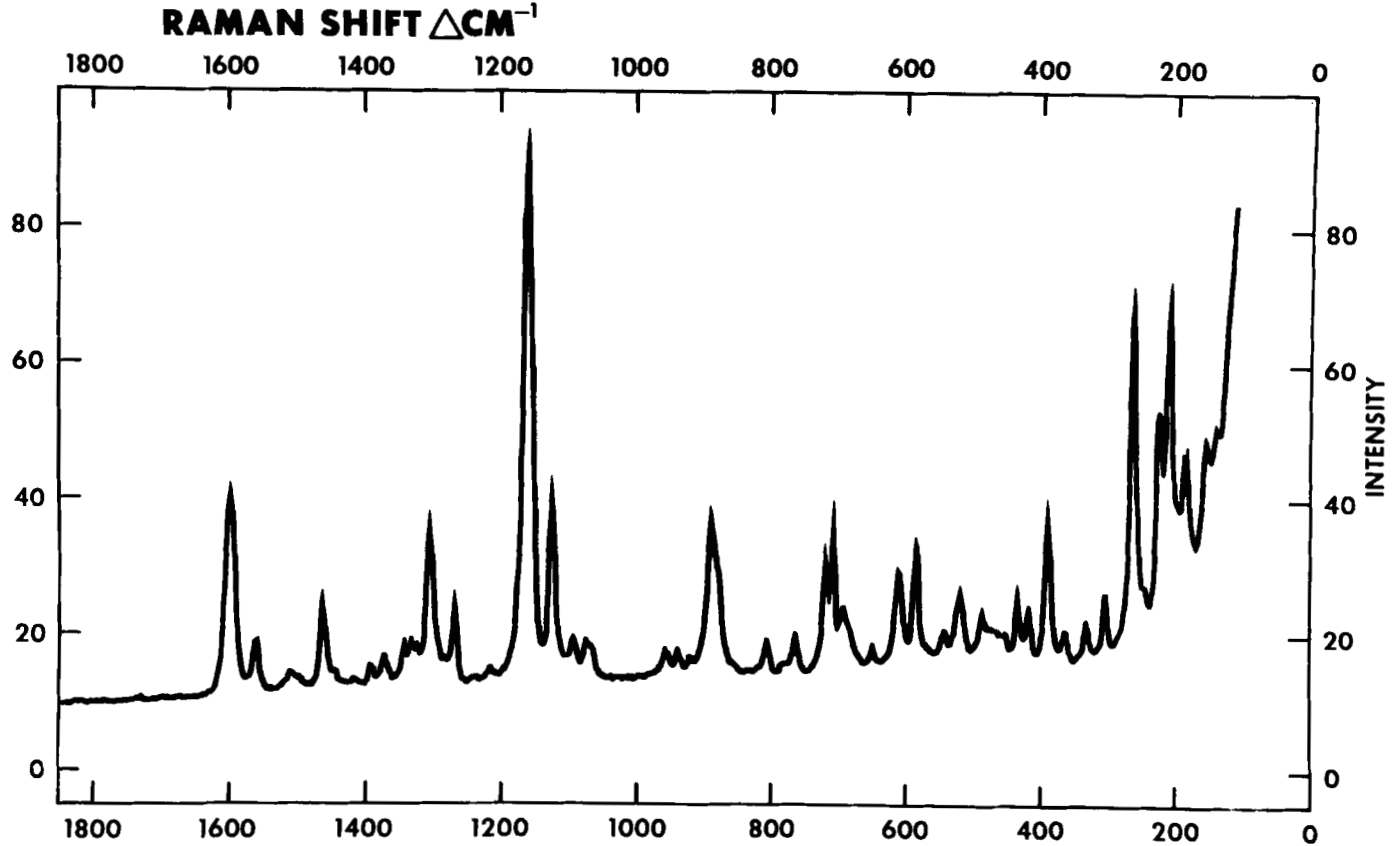


FIGURE 2 - RAMAN SPECTRUM OF METHYCLOTHIAZIDE

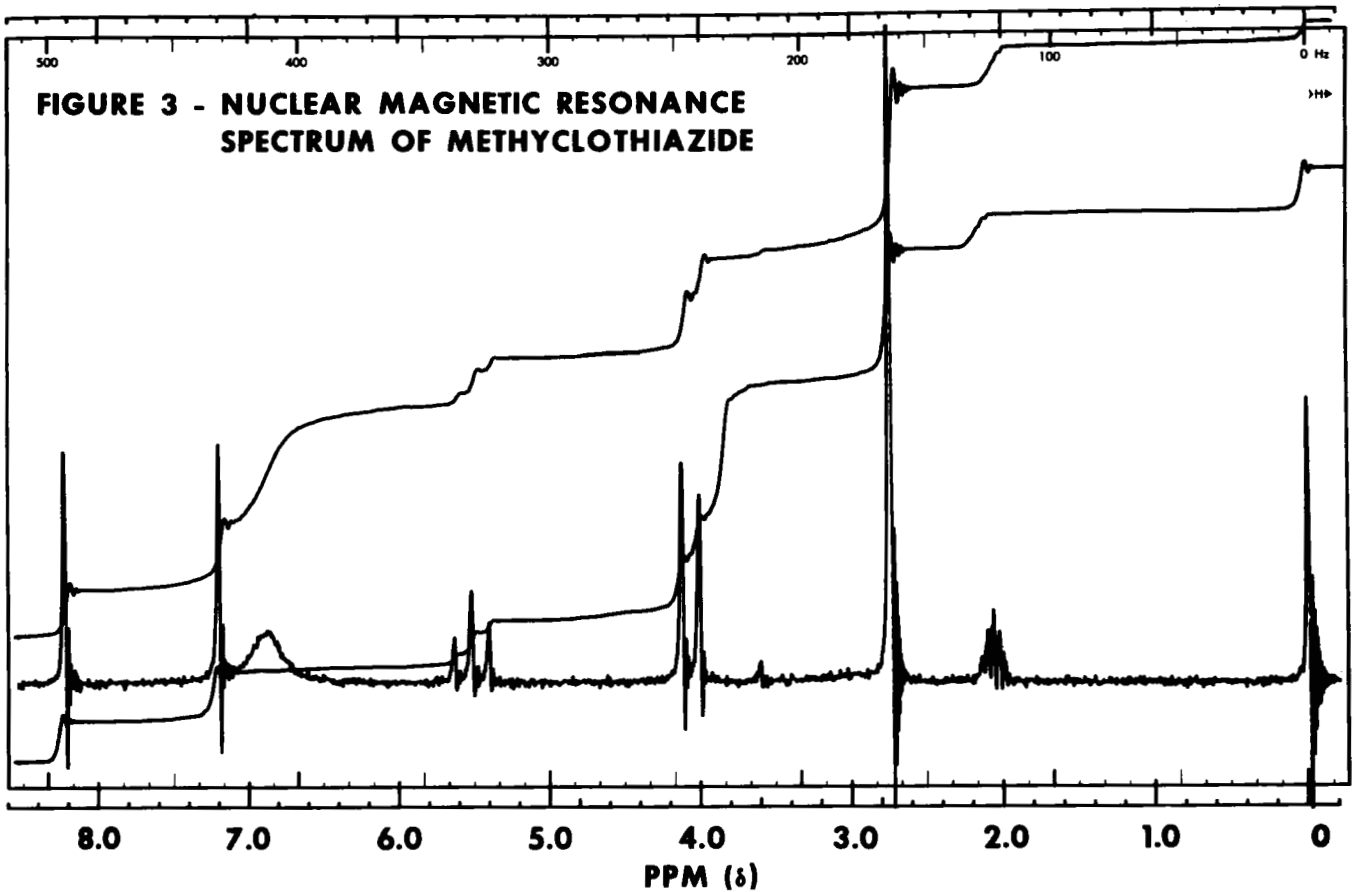


FIGURE 3 - NUCLEAR MAGNETIC RESONANCE SPECTRUM OF METHYCLOTHIAZIDE

Table III

NMR Assignments for Methyclothiazide

Assignment	Number of Protons	Chemical Shift (ppm)	Multiplicity
Aromatic proton at carbon 8	1	8.23	S
Aromatic proton at carbon 5	1	7.22	S
Exchangeable protons: NH, NH_2	3	6.87	M(b)
Methyne proton at carbon 3	1	5.53	T
Methylene protons of chloromethyl group at carbon 3	2	4.07	D
Methyl protons of 2-methyl group	3	2.77	S

2.4 Ultraviolet Spectrum (UV)

The UV spectrum of methyclothiazide prepared as a 1 in 100,000 solution in methanol is shown in Figure 4. The spectrum exhibits three maxima and two minima characteristic of substituted benzothiadiazines. The maxima are at 226 nm (ϵ_m = 39,300), 267 nm (ϵ_m = 21,250) and ca 311 nm (ϵ_m = 3,300). Minima were observed at 240 nm and 290 nm. The spectrum is consistent with previously published reports by Furman (6) and Fazzari, et. al. (4)

2.5 Mass Spectrum

The mass spectrum shown in Figure 5 was obtained using an Associated Electrical Industries Model 902 Mass Spectrometer with an ionizing energy of 50 eV and a temperature of 150°C. Methyclothiazide yields a spectrum with a base peak at m/e 359. Subsequent fragments, Table IV,

FIGURE 4 - ULTRAVIOLET SPECTRUM OF METHYCLOTHIAZIDE

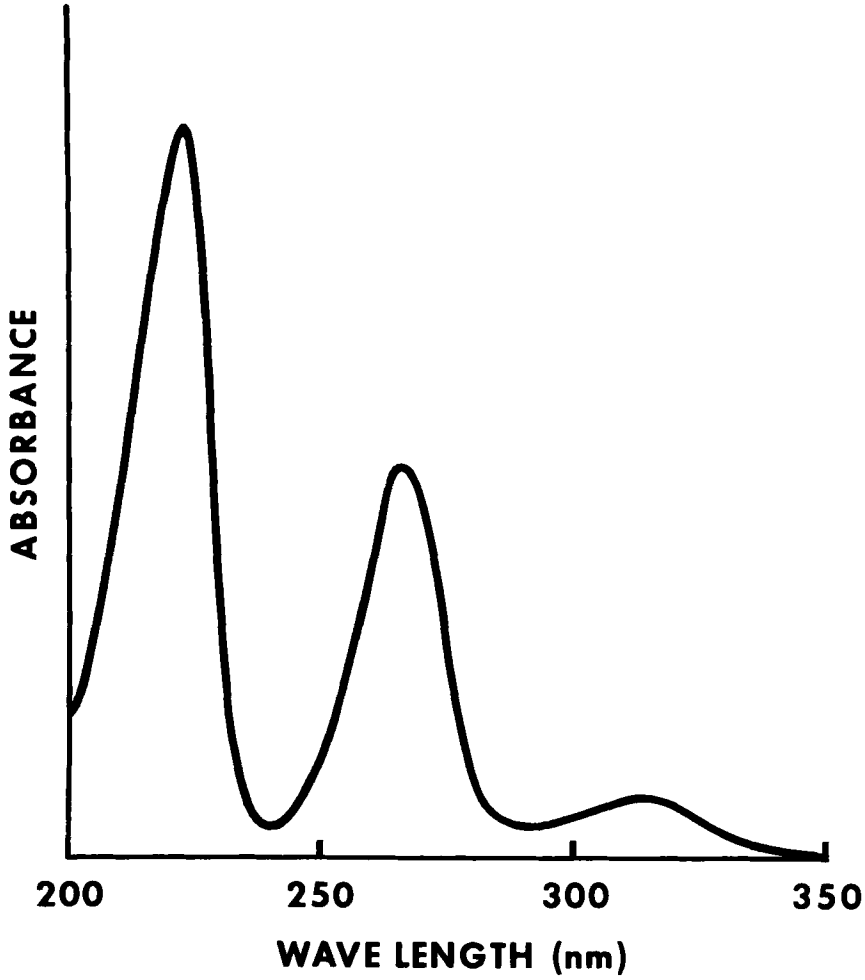
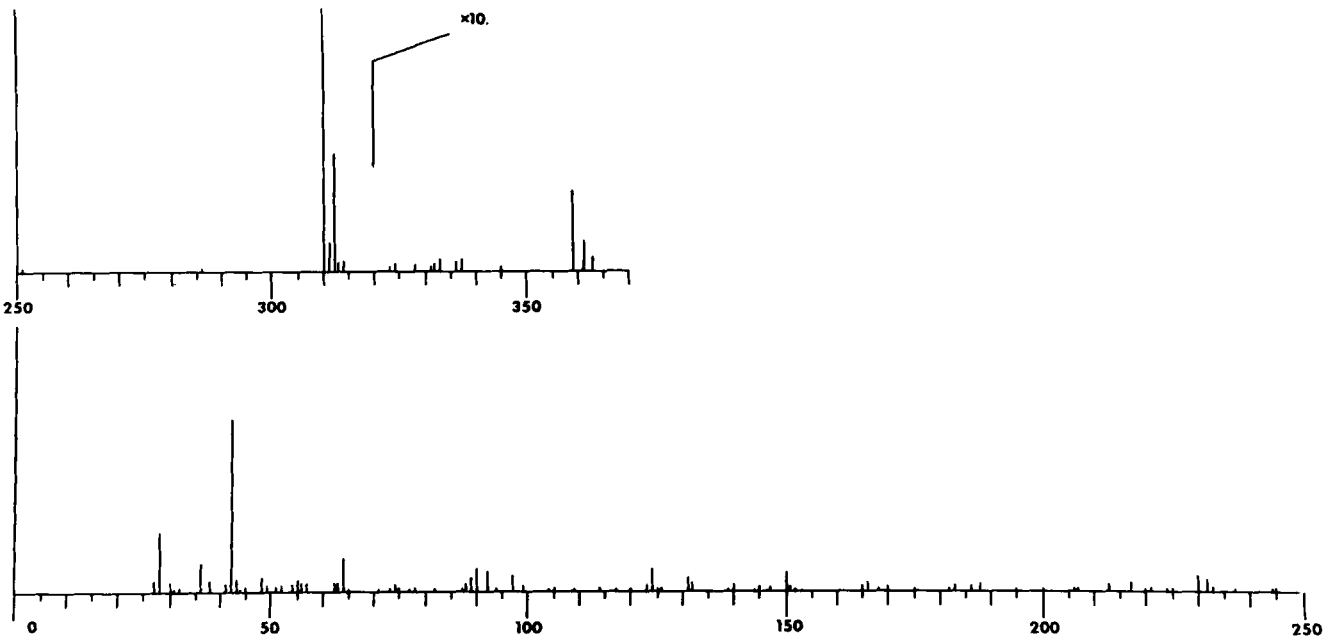


FIGURE 5, MASS SPECTRUM OF METHYCLOTHIAZIDE



reflect the loss of the chloromethyl and sulfonamide groups as well as the fragmentation of the ring system. (7)

Table IV

High Resolution Mass Spectrum of Methyclothiazide

Mass Found	Relative Intensity	Error (mmu)	Composition					
			C	H	N	O	S	Cl_{35}
358.9565	3.06	-0.32	9	11	3	4	2	2
309.9711	100.00	-1.21	8	9	3	4	2	1
291.9629	0.56	1.18	8	7	3	3	2	1
229.9914	6.02	-0.26	8	7	2	2	1	1
166.0280	3.52	-1.78	8	7	2	0	0	1
131.0615	5.42	0.57	8	7	2	0	0	0
123.9952	7.89	-0.22	6	3	1	0	0	1
96.9847	5.95	0.16	5	2	0	0	0	1
90.0113	6.77	0.25	3	5	1	0	0	1
42.0345	63.94	0.13	2	4	1	0	0	0

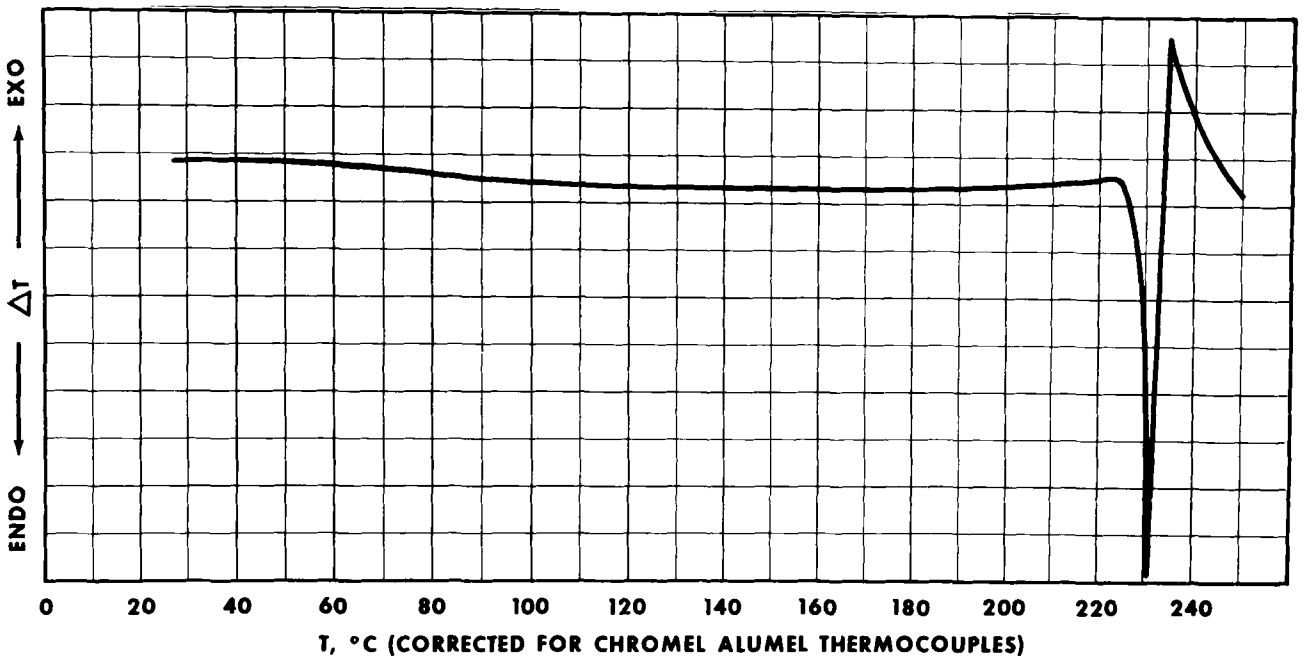
2.6 Melting Range

Methyclothiazide melts with rapid decomposition between 225°C and 227°C. Slight discoloration of the solid may be observed at about 215°C.

2.7 Differential Thermal Analysis

The thermogram depicted in Figure 6 shows a large endothermic melt between 224°C and 231°C. The thermogram shows a subsequent exothermic response confirming the visual observation of rapid decomposition.

FIGURE 6 - DIFFERENTIAL THERMAL ANALYSIS CURVE OF METHYCLOTHIAZIDE



2.8 Dissociation Constant

The pKa of methyclothiazide was determined by the titrimetric method by extrapolation of data from acetone-water mixed solvents to 100% water. The pKa is 9.4 (proton lost).

2.9 Solubility

The following solubilities have been determined for methyclothiazide at room temperature.

Solvent	Solubility	National Formulary Descriptive Term(1)
Water	0.06 mg/ml	Very Slightly Soluble
Chloroform	0.03 mg/ml	Very Slightly Soluble
Benzene	< 1 mg/ml	Very Slightly Soluble
n-Butanol	1 mg/ml	----
PEG-600	1 mg/ml	----
Ethanol	11 mg/ml	Slightly Soluble
Acetonitrile	5 mg/ml	----
Methanol	> 10 mg/ml	Sparingly Soluble
Acetone	$\simeq$ 200 mg/ml	Freely Soluble
Pyridine	$\simeq$ 200 mg/ml	Freely Soluble

2.10 Crystal Properties

The X-ray powder diffraction pattern of methyclothiazide was determined by visual observation of a film obtained with a 143.2 mm Debye-Scherrer Powder Camera. An Enraf-Nonius Diffractis 601 Generator; 38 KV and 18 MA with nicker filtered copper radiation; λ = 1.5418 was employed. A listing of d-spacings and intensities is presented in Table V. (8)

Table V

X-Ray Powder Diffraction Pattern
d-Spacings and Intensities

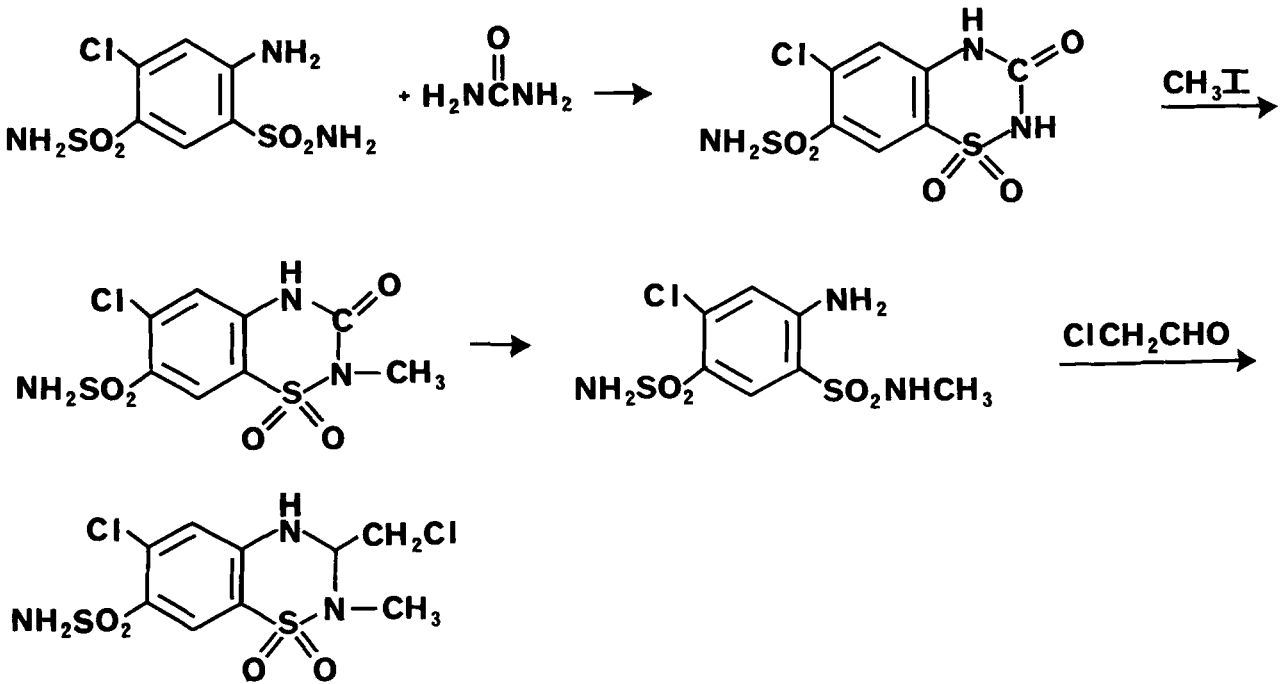
dA	I/I_1	dA	I/I_1
9.8	5	2.95	5
7.75	50	2.90	10
7.5	30	2.81	60
7.2	5	2.72	3
6.25	20	2.68	5
5.75	50	2.62	15
5.3	5	2.51	10
5.1	10	2.42	3
4.85	100	2.27	15
4.56	80	2.24	10
4.42	20	2.15	3
4.3	15	2.11	5
4.11	20	1.99	8
4.00	2	1.88	3
3.90	2	1.85	5
3.75B	20	1.81	8
3.6	3	1.77	2
3.52	20	1.75	3
3.44	5	1.73	3
3.32	5	1.69	2
3.30	8	1.64	4
3.12	5	1.5 B	4
3.07	8	1.35B	3
3.00	5	1.3 B	3

3. Synthesis

Methyclothiazide is synthesized by the reaction sequence shown in Figure 7. Sprague (9) described the reaction of 4-amino-6-chloro-1,3-benzenedisulfonamide with urea to form the 3-keto derivative. Close, et. al. (10) have preferentially alkylated the more acidic cyclic sulfonamide with methyl iodide to form 6-chloro-2-methyl-3-oxo-7-sulfamyl-3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxide. This intermediate is readily ring opened by alkaline hydrolysis and then re-cyclized with chloroacetaldehyde to form the desired product.

Figure 7

Synthesis of Methyclothiazide



4. Stability-Degradation

Methyclothiazide is stable in the solid state and under ordinary ambient conditions. It is rapidly decomposed in boiling acidic solutions to 2-methylsulfamyl-4-sulfamyl-5-chloroaniline. In alkaline solution it rapidly loses one chlorine atom to form the 3-hydroxymethyl analog. This product has been shown to degrade further under severe conditions, however none of the alkaline degradation products contain primary aromatic amine centers. Solutions buffered at pH 4.0 show 22% hydrolysis after 7 days at 60°C, 10% after 28 days at 40°C, but only 2% after 28 days at 25°C. Solutions buffered at pH 6.0 gave 1.6% hydrolysis after 7 days at 60°C, and less than 1% after 28 days at 40°C or 25°C.

5. Drug Metabolic Products

No report of metabolic products related to methyclothiazide has been published.

6. Methods of Analysis

6.1 Titrimetric Methods

6.11 Argentimetric Titration

The compendial procedure for the purity determination of the drug substance is based upon the argentimetric titration of the chloride liberated after reflux in methanolic potassium hydroxide. (1) The equivalent weight is 360.23 since only the chlorine from the 3-chloromethyl group is liberated during reflux. The method is specific since this group is added during the final step of the synthesis and a limit of 0.02% free chloride is imposed on the drug substance.

6.12 Potentiometric Titration

Methyclothiazide can be titrated as an acid using either tetra-butylammonium hydroxide in chlorobenzene (11) or potassium hydroxide in isopropanol (12) as the titrant. The solvents are pyridine and acetone, respectively. Methyclothiazide consumes two equivalents of base per

mole of drug substance. The first equivalent is from the neutralization of the free sulfamyl group. The exact location for the reaction of the second equivalent has not been determined, however, it may result from rapid hydrolysis of the 3-chloromethyl function.

6.2 Chromatographic Methods

6.21 Column Chromatography

Fazzari (13) has published a collaborative study on a column chromatographic method for the analysis of methyclothiazide from tablets. The drug is eluted from a 0.1 M $NaHCO_3$-Celite column with chloroform and measured directly at 267 nm by UV spectrophotometry. The precision of the method was 99.8 $\pm$ 1.64% on a commercial preparation of 2.5 mg tablets.

6.22 Paper and Thin-Layer Chromatography

Paper chromatography has been applied by Pilsbury and Jackson (14) for the rapid detection and identification of thiazide diuretics in tablets, gastric fluid, and urine. Identification is accomplished by ascending reverse phase chromatography using tributyn treated paper and developing for 20 minutes at 90°C with a phosphate buffer (pH 7.4). The thiazides are located by ultraviolet light (254 nm) and confirmed by the red color given by alkaline sodium 1,2-naphthaquinone-4-sulfonate spray reagent. Paper chromatography can also monitor the extent of manufacturing by-products. Ascending chromatography using butanol saturated with 3% ammonium hydroxide and descending chromatography using butanol:acetic acid:water (50:15:60) have been employed. Visualization is by ultraviolet light.

Thin-layer chromatography using the system chloroform:methanol:ammonium hydroxide (170:30:2) on 0.25 mm silica gel GF_{254} plates and ultraviolet detection rapidly isolates and identifies the common manufacturing intermediates.

6.3 Spectrophotometric Assays

Methyclothiazide can be determined after acid hydrolysis to 2-methylsulfamyl-4-sulfamyl-5-chloroaniline by a modified Bratton-Marshall procedure. This procedure without prior acid hydrolysis also monitors diazotizable substances in the drug substance. (1) Ultraviolet absorption at 267 nm is seldom directly employed since the intermediates and degradation products have similar absorption spectra. The ultraviolet procedure has been utilized after prior separation by chromatography (13) or for non-specific content uniformity measurements. (1)

6.4 Polarographic Method

The current compendial assay for methyclothiazide tablets is a polarographic assay in an aqueous system containing 6% v/v dimethylformamide and 0.1 M tetra-n-butylammonium chloride as the supporting electrolyte. A mercury anode is used in conjunction with the DME since the half-wave potential of methyclothiazide occurs in the region where potassium ions from classical agar electrodes would interfere.

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METRONIDAZOLE

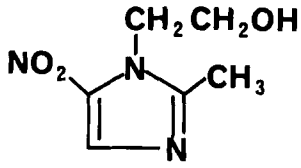
Lorraine L. Wearley and Gaylord D. Anthony

Contents

1. Description

1.1 Name, Formula, Molecular Weight

Metronidazole is 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole



$C_6H_9N_3O_3$ Molecular Weight: 171.16

1.2 Appearance, Color, Odor

Metronidazole is a white to pale yellow, odorless crystalline powder.

2. Physical Properties

2.1 Infrared Spectrum

The infrared absorption spectrum of a metronidazole reference standard compressed in a KBr pellet is shown in Figure 1. The following assignments have been made for absorption bands in Figure 1.[1]

Band (cm^{-1})	Assignment
3230	OH stretch
3105	C=CH; C-H stretch
1538 & 1375	NO_2; N-O stretch
1078	C-OH; C-O stretch
830	C-NO_2; C-N stretch

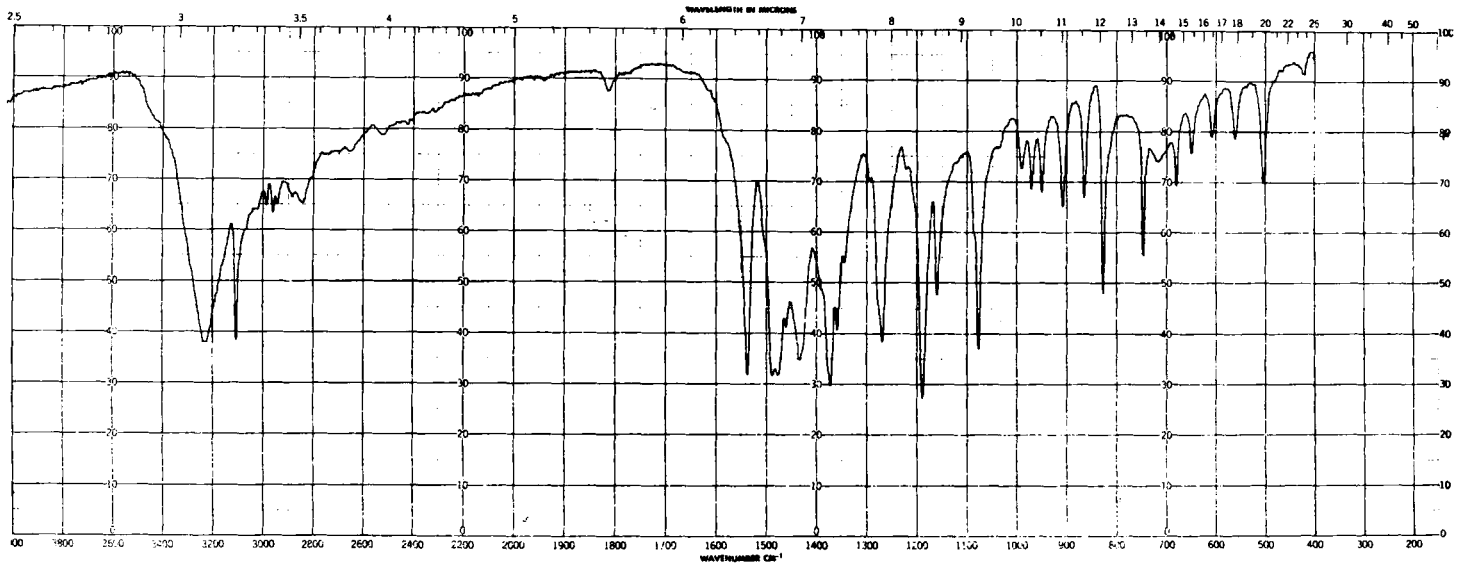


Figure 1

Infrared Spectrum of Metronidazole

2.2 Nuclear Magnetic Resonance Spectrum

The NMR spectrum of metronidazole in deuterated acetic acid is shown in Figure 2. Below are the assignments of the major signals. Positions of absorption bands are reported as shifts downfield from the signal of the protons in tetramethylsilane which was used as internal standard.[1]

Band (H_2)	Assignment
155 singlet	$\diagup\!\!\diagdown C - \underline{CH_3}$
241 (triplet)	$CH_2 - \underline{CH_2} - OH$
274 (triplet)	$\underline{CH_2} - CH_2 - OH$
481 (singlet)	$\diagup\!\!\diagdown \underline{C} - H$

2.3 Ultraviolet Spectrum

Metronidazole exhibits an absorption maxima at about 274 nm. using 0.1 N sulfuric acid in methanol as solvent. The molar absorptivity in this solvent is 6333. The ultraviolet absorption spectrum is shown in Figure 3.[2]

2.4 Mass Spectrum

The low resolution mass spectrum of metronidazole is shown in Figure 4.[3] Structure assignments are as follows:

M/e	Assignment	% Relative Intensity
171	$M\cdot^+$ (molecular ion)	12.5
154	M-OH	4.0
125	$M - NO_2$	22.1
125	$M - NO_2 - H$	25.7
41	$M - CH_3CN$	100

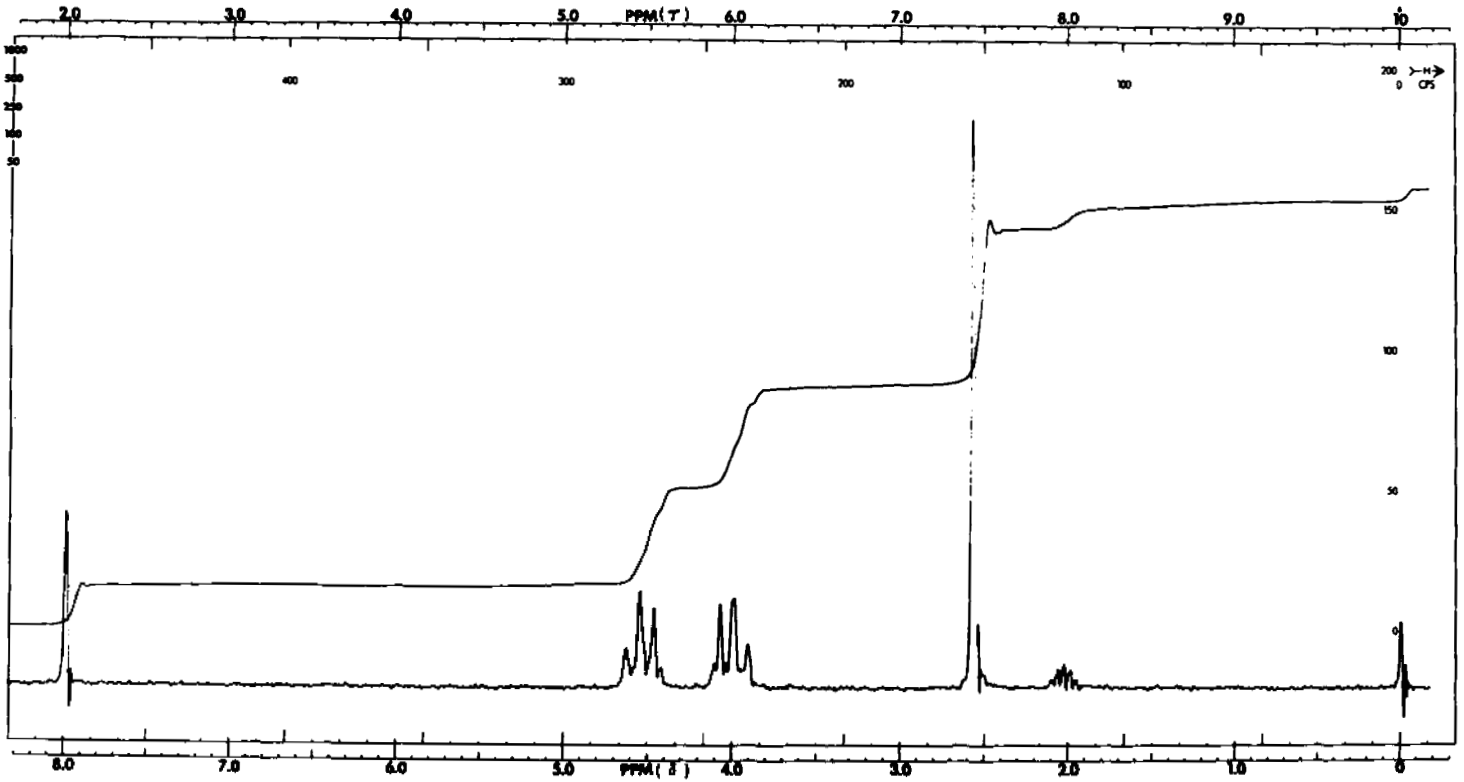


Figure 2

Nuclear Magnetic Resonance Spectrum of Metronidazole

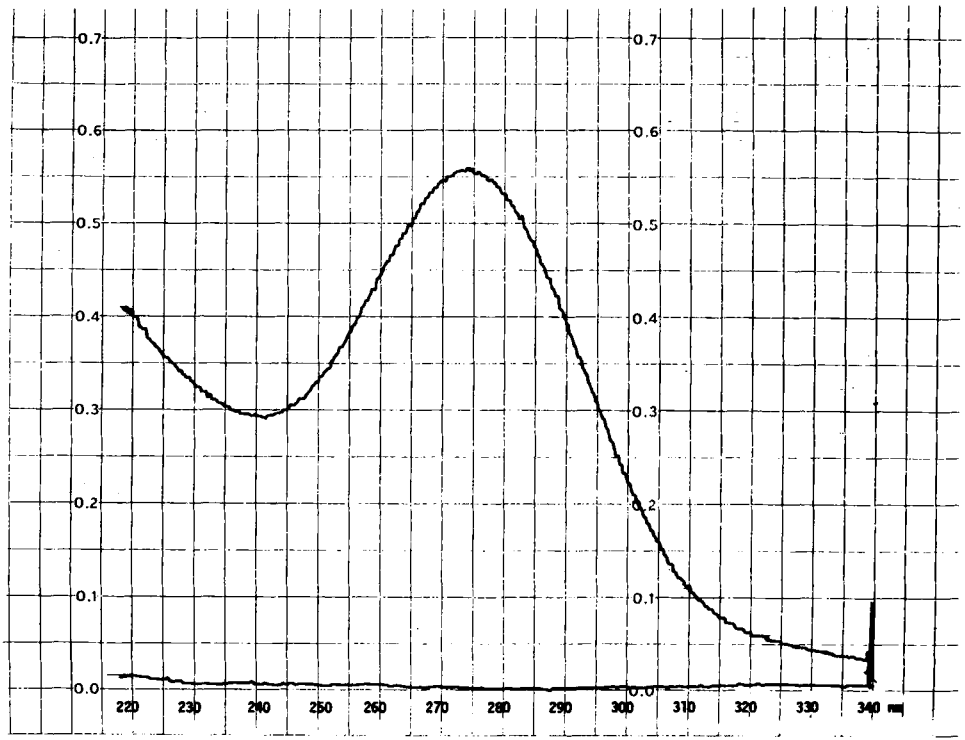


Figure 3

Ultraviolet Spectrum of Metronidazole in 0.1 N Sulfuric Acid in Methanol

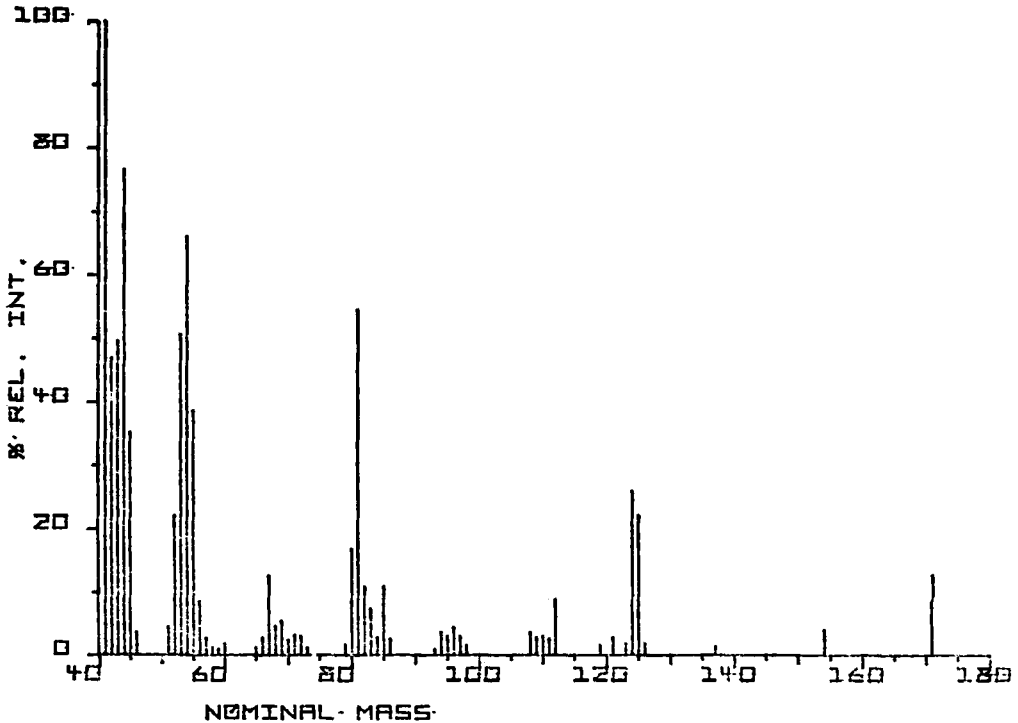


Figure 4

Mass Spectrum of Metronidazole

2.5 Optical Rotation

Metronidazole exhibits no optical activity.

2.6 Melting Range

The melting range given in the USP XIX is 159° to 163°C.

2.7 Differential Scanning Calorimetry

The DSC thermogram of metronidazole obtained at a heating rate of 20°C/minute is shown in Figure 5. The endothermic change observed at about 162°C corresponds to the melting of the compound.[4]

2.8 Thermogravimetric Analysis

The TGA of metronidazole obtained at a heating rate of 10°C/minute is shown in figure 6.[4]

2.9 Solubility

Solubilities in various solvents at 25°C are given in the following table.[5]

Solvent	Solubility, mg/ml
Water	10.5
Methanol	32.5
Ethanol	15.4
Chloroform	3.8
Heptane	<0.01

3. Metabolism

Stambaugh et al.[6] found that the major urinary excretion products of metronidazole in man and CD-1 mice were unchanged metronidazole, 1-(2-hyroxyethyl)-2-hydroxymethyl-5-nitroimidazole and their ether glucuronides.

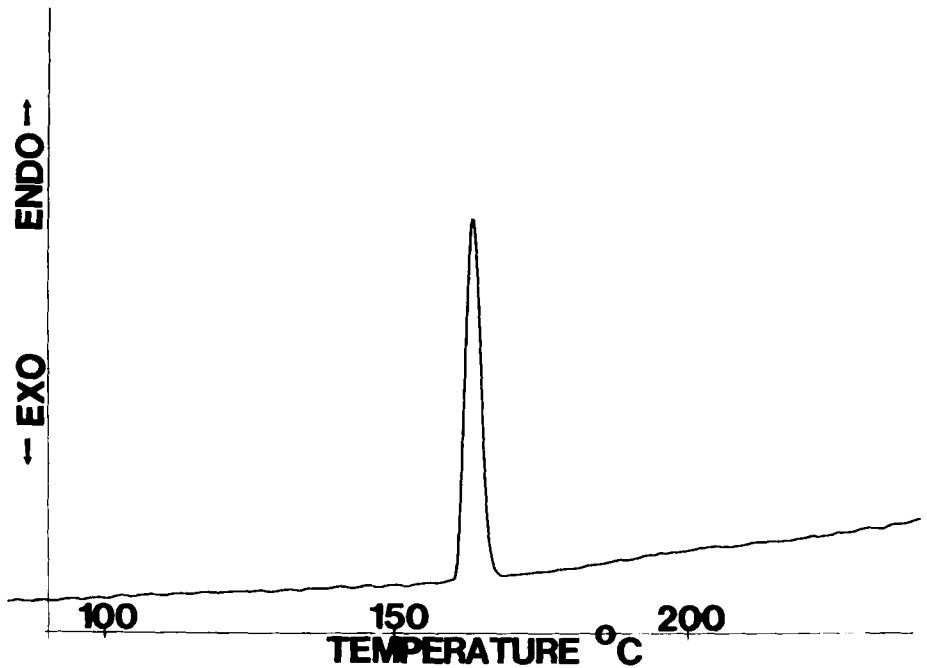


Figure 5

DSC Thermogram of Metronidazole

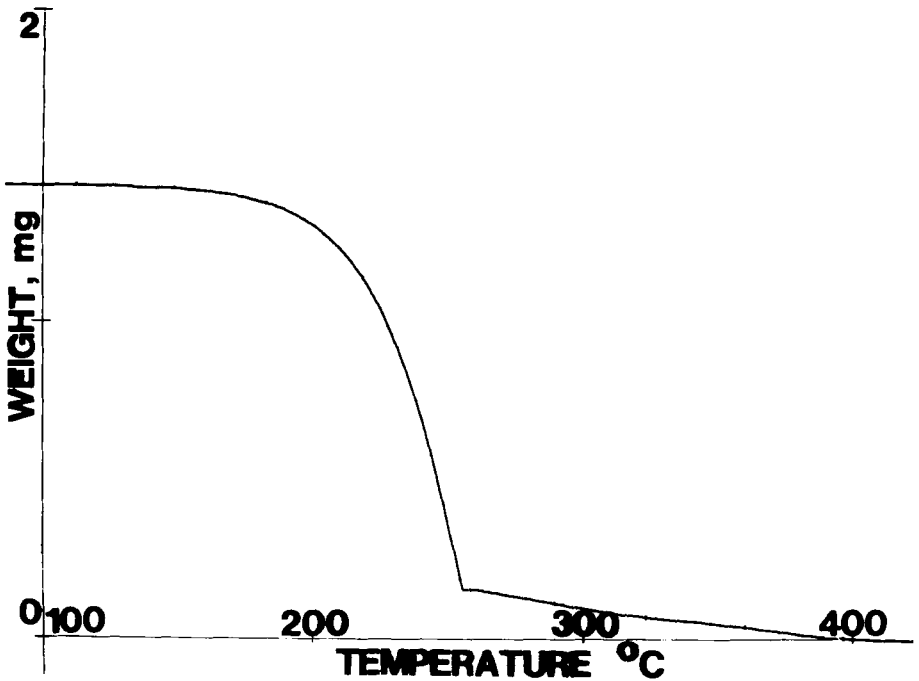


Figure 6

TGA of Metronidazole

These products represented 70-90% of the total urinary nitro fraction. Minor metabolites were reported to be 1-(2-hydroxyethyl)-2-carboxylic acid-5-nitroimidazole and 1-acetic acid-2-methyl-5-nitroimidazole (see Fig. 7). Oxidation of the methyl group of metronidazole appears to occur more facilely than the hydroxyethyl group. Nitro reduction products have not been found in animal or human urine.

The major vaginal products in females given oral doses of drug were found by Manthei et al to be unchanged drug, and 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitro-imidazole. These products were also present in the urine. In addition a fluorescent lipophilic product thought to be a cyclized lactone was found.

4. Pharmacokinetics

In a study on healthy females receiving single and multiple doses of 200 mg metronidazole tablets, Welling and Monro reported the biological half-life to be 6.2 hr. Serum concentration data fit a one compartment open model. Steady state serum concentrations of metronidazole on a regimen of 200 mg twice daily averaged 7.07 ug/ml maximum and 2.47 ug/ml minimum.

Ings et al.[9] studied the distribution of [^{14}C]metronidazole in rats. They found that oral doses were rapidly absorbed from the gastrointestinal tract; and rapidly equilibrated between blood and most tissues. Radioactivity was found to concentrate in the liver, kidney, gastro-intestinal tract and vaginal secretions. The half life of clearance was longest for the gastro-intestinal tract. I.V. doses showed similar distribution.

5. Methods of Analysis

5.1 Titrimetric Analysis

The titration with perchloric acid is the method of choice to assay metronidazole. The sample is dissolved in acetic anhydride, and warmed slightly to effect solution. After cooling, one drop of malachite green T.S. is added, and the titration with 0.1 N perchloric acid to a yellow-green endpoint is carried out. A blank determination is

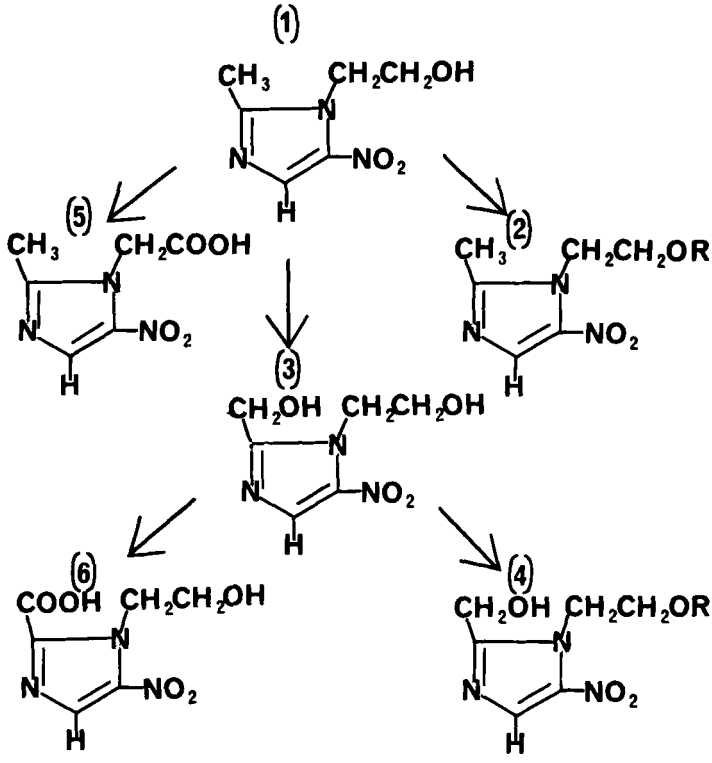


Figure 7: Pathways proposed by Stambaugh et al. for the metabolism of metronidazole in man.
(1) metronidazole (2) the corresponding ether glucuronide (R = glucuronide) (3) 1 - (2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (4) its corresponding glucuronide (5) 1-acetic acid-2-methyl-5-nitroimidazole (6) 1-(2-hydroxyethyl)-2-carboxylic acid-5-nitroimidazole.

performed and any necessary correction is made. One equivalent of the compound is titrated.[10]

5.2 Spectrophotometric Analysis

Spectrophotometric analysis of metronidazole may be carried out using 0.1 N sulfuric acid in methanol as the solvent. The ultraviolet absorption maxima is at about 274 nm.

5.3 Colorimetric Analysis

5.31 Metronidazole can be analyzed colorimetrically by reducing the nitro group to the corresponding amine, which is subsequently determined by diazotization and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride (Bratton-Marshall Reagent).[11]

5.32 A variation of the above method involves the alkaline hydrolysis of the nitro group of metronidazole. The nitrous acid produced diazotizes sulfanilamide in acidic medium to form a diazonium salt. After coupling with Bratton-Marshall reagent the concentration is determined spectrophotometrically by comparison to nitrite standards which have been carried through the colorimetric procedure.[12]

5.4 Chromatographic Analysis

5.41 Thin Layer Chromatography - Several TLC systems and corresponding R_f values are summarized below.[13]

Solvent System	Absorbent	Detection	R_f
Acetone	Silica Gel	1	0.65
Chloroform:Methanol: Water:Acetic Acid 74:20:4:2	Silica Gel	1	0.76
Benzene:methanol: ammonium hydroxide 79:20:1	Silica Gel	1	0.36
Chloroform:methanol: water:acetic acid 70:24:4:2	Silica Gel	1, 2	0.66

1. Spray with 1% aqueous titanium trichloride; heat at 130° C for 3 minutes. Spray with 1% Dimethylaminobenzaldehyde in 2 N HCl.

2. Saturate plate with t-butyl hypochlorite vapors. Spray with aqueous 1% starch/1% potassium iodide solution. (This spray has been found to be much more sensitive than #1.)

5.42 Gas-Liquid Chromatography - Metronidazole can be chromatographed as the trimethyl silyl derivative. The silyl derivative is prepared by dissolving metronidazole in a 1:1 mixture of dimethylformamide and bis(trimethylsilyl)-trifluoroacetamide. The silylation reaction is complete in 30 minutes at room temperature.[14]

Instrumental Conditions

Column: 6 ft. glass column packed with 3% OV-1 on Gas Chrom Q

Column Temp.: 160° C

Carrier: Nitrogen at 70 ml/min

Detector: Hydrogen Flame Ionization

Retention Time: 4.1 minutes

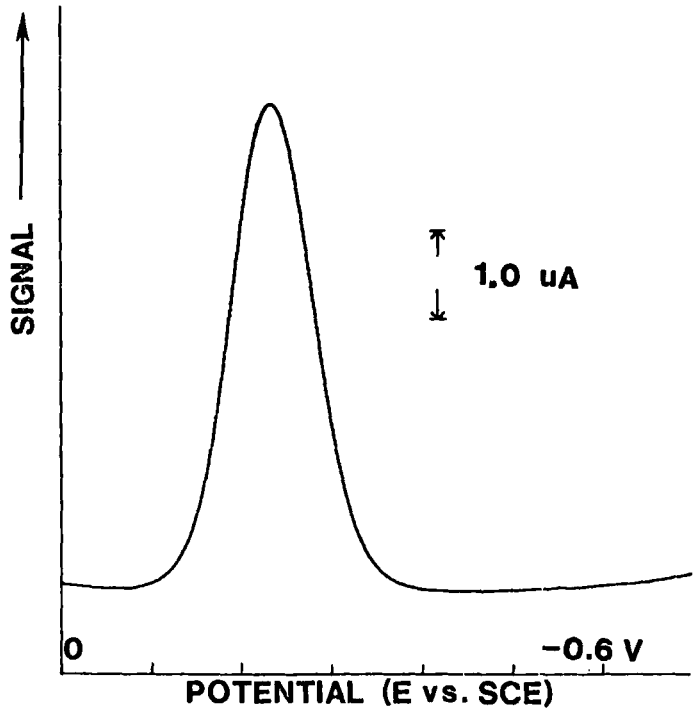


Figure 8

Polarograph of Metronidazole,
2% Solution in pH 3.8± 0.2 Buffer

5.5 Polarographic Analysis

A suitable polarographic analysis of metronidazole may be carried out at a concentration of approximately 5 ug/ml in a 2% solution of pH 3.8± 0.2 buffer. The scan shown in figure 8 was obtained on such a solution, using differential pulse mode, 3 electrode system. For less concentrated solutions addition of a maxima suppressant may be necessary. Peak maximum value is approximately -0.23 volts vs saturated calomel electrode.[4]

6.0 Synthesis

2-methyl-imidazole (I) is nitrated by reacting with nitric acid in the presence of sulfuric acid catalyst. The resulting 5-nitro product (II) can then be reacted with either chloroethanol or ethylene oxide to produce 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole (III). See figure 9.

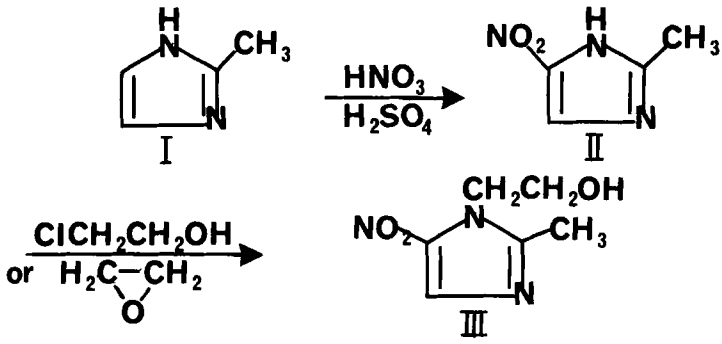


Figure 9

Synthesis of Metronidazole

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8. Acknowledgments

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NITROFURANTOIN

Donald E. Cadwallader and Hung Won Jun

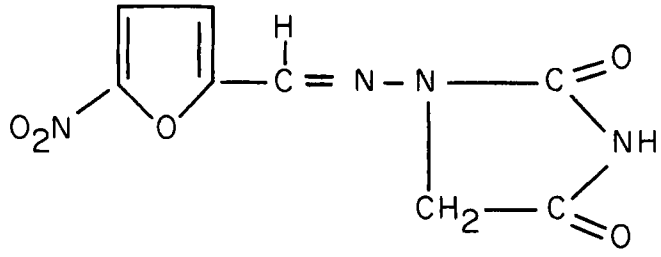
Table of Contents

Table of Contents, continued.

1. Description

1.1 Name, Formula, and Molecular Weight

Nitrofurantoin is N-(5-Nitro-2-furfurylidene)-1-aminohydantoin; 1-(5-Nitro-2-furfurylideneamino) hydantoin. Furadantin® and Macrodantin® are the most commonly used trade marks; 11 additional are listed in the Merck Index (1).



$C_8H_6N_4O_5$ Mol. wt.: 238.16

1.2 Appearance, Color, Odor and Taste

Nitrofurantoin is described as lemon-yellow, odorless crystals or fine powder, having a bitter taste (1,2).

2. Physical Properties

2.1 Ultraviolet Spectra

Values of E (1%, 1cm) in water at 367.5 and 265 nm are found to be 760 and 540, respectively (3). The molar extinction coefficients, ε at 367 and 265 nm are 13,100 and 17,300, respectively (4).

When the UV spectrum of nitrofurantoin in 2% dimethylformamide (DMF) was scanned from 260 to 400 nm, two maxima occurred at 265 and 367 nm. The spectrum shown in Figure 1 was obtained from a solution of 10.00 mg of nitrofurantoin/liter of 2% DMF in water (3).

Figure 2 shows the effect of pH on maximal wavelength and E (1%, 1cm) values of nitrofurantoin (3). KH_2PO_4 - NaOH and boric acid - NaOH buffer systems were employed. The spectral shift was not due to hydantoin ring - opening in the alkaline solution since the shift was reversible upon reacidification (3).

2.2 Infrared Spectrum

The infrared spectrum of nitrofurantoin (Norwich Pharmacal Reference Standard Purity) as a mineral oil mull is shown in Figure 3. Interpretation of the spectrum was made by Michels (3) using Cross, Stevens and Watts (5) as a reference.

Table I

IR Band Assignments for Nitrofurantoin

Wavelength (microns)	Assignment
3.05	NH
5.6-5.75	hydantoin C = O
6.6-7.45	α-nitrofuran
10.4	2,5-disubstituted furan
8.05	asymmetrical C-O-C
9.8	symmetrical C-O-C

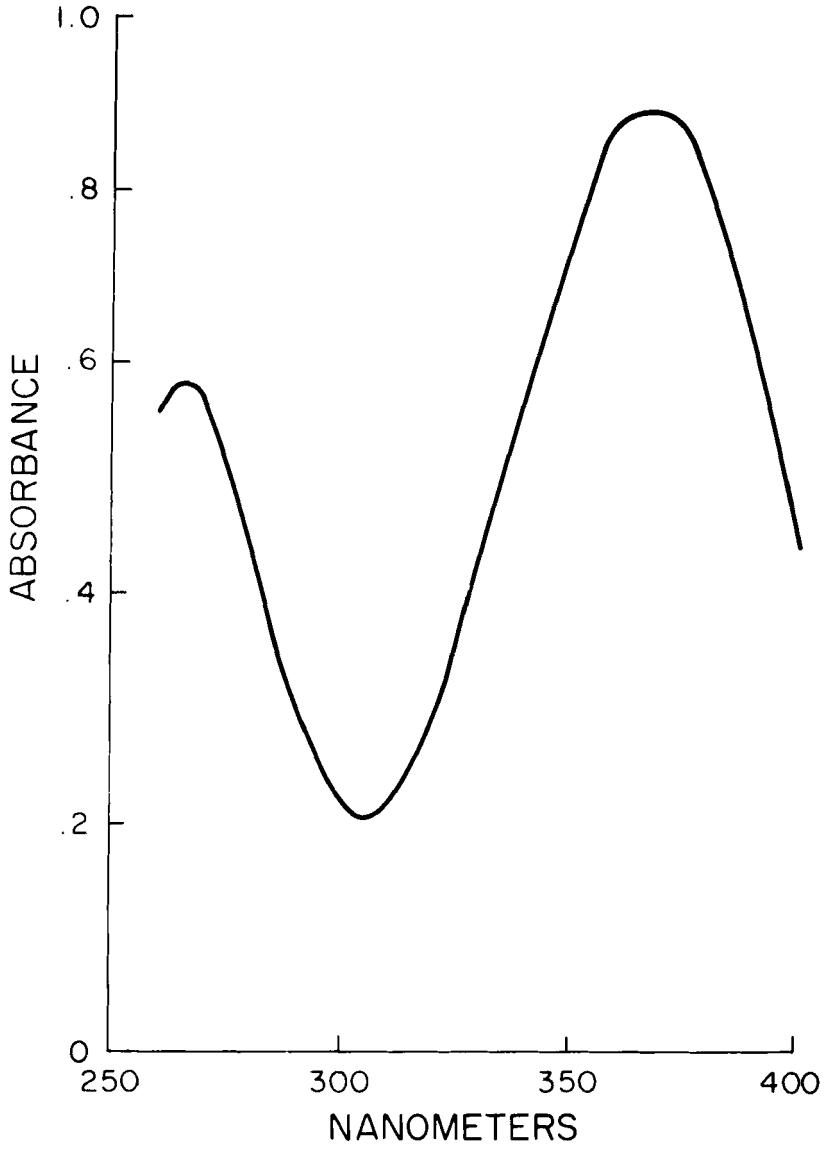


Figure 1. Ultraviolet Spectrum of Nitrofurantoin (Coleman 124)

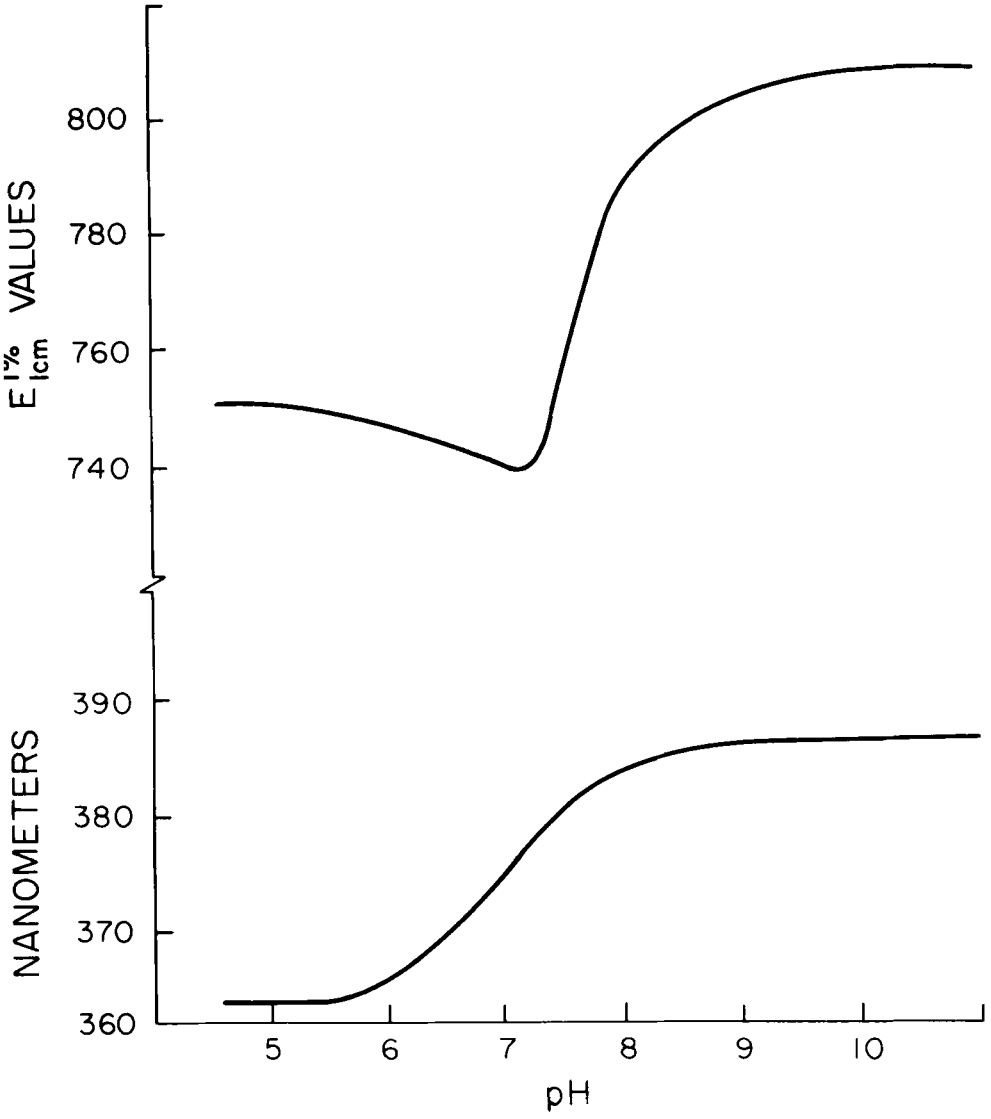


Figure 2. Ultraviolet Absorption of Nitrofurantoin as a Function of pH

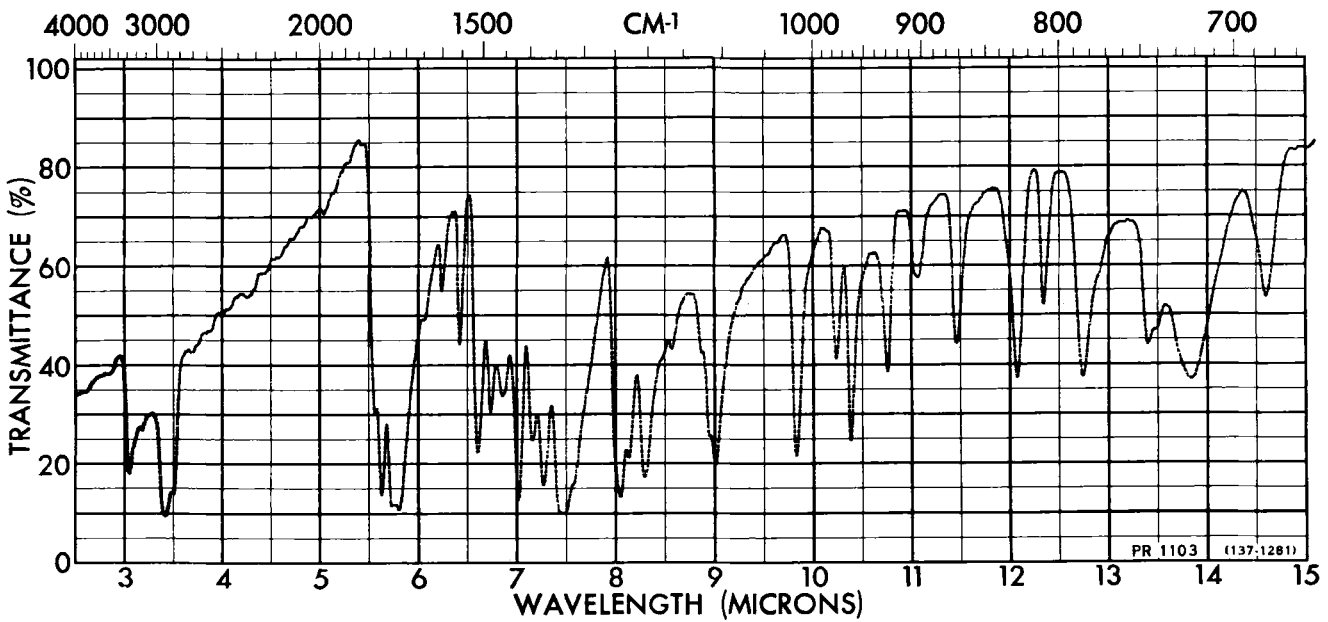


Figure 3. Infrared Spectrum of Nitrofurantoin (Perkin Elmer 467)

2.3 Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum for nitrofurantoin (Norwich Pharmacal Reference Standard Purity) in DMSO - d_6 containing a tetramethylsilane as the internal reference is shown in Figure 4 (3). The spectral assignments are presented in Table II (3).

Table II

NMR Spectral Assignments for Nitrofurantoin

Band (ppm, δ)	No. of Protons	Assignment
Singlet 4.40	2	hydantoin CH_2
Doublet 7.15 (J=4)	1	furan 3H
Doublet 7.62 (J=4)	1	furan 4H
Singlet 7.83	1	-CH=N-
Broad Singlet 11.4 (exchangeable)	1	hydantoin NH
2.5	-	solvent

2.4 Dissociation Constant

Michels (3) determined the pKa of the free acid to be 7.0 using the method described by Stockton and Johnson (6). A pKa of 7.2 is also reported for nitrofurantoin (1).

2.5 Melting Range

Nitrofurantoin melts at 270-272° C. (1, 7).

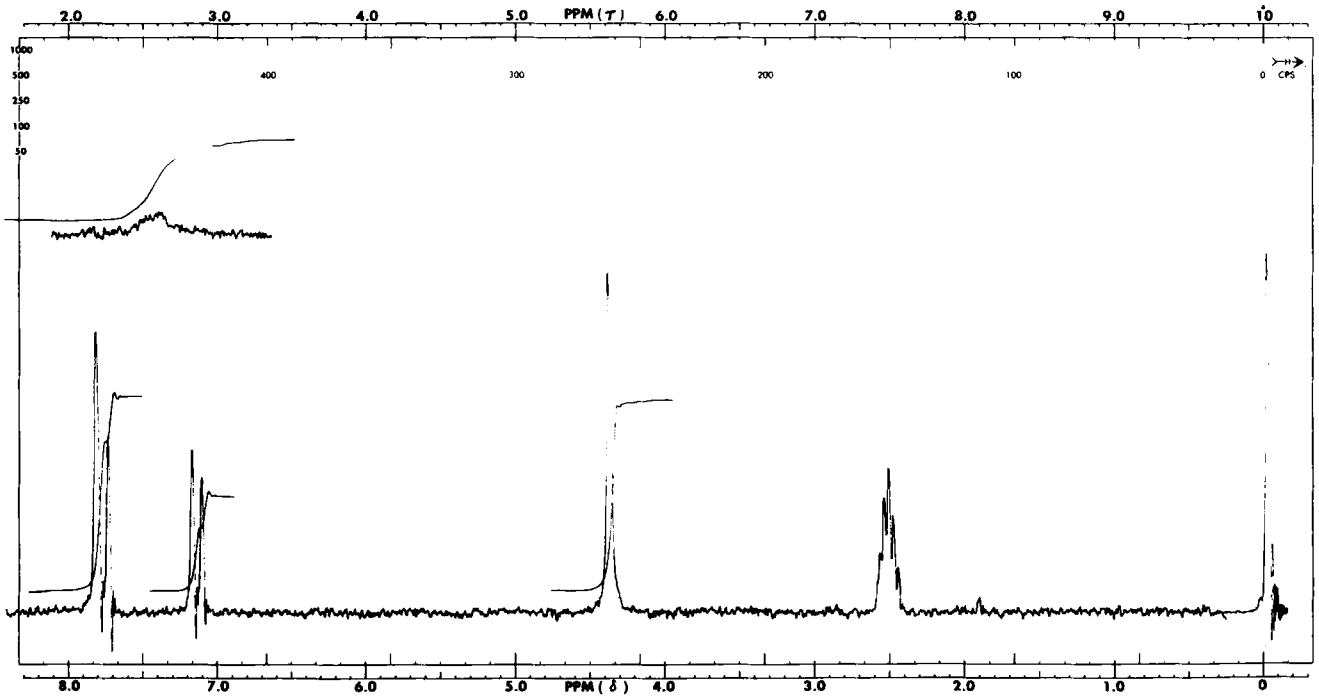


Figure 4. NMR Spectrum of Nitrofurantoin (Varian A60A)

2.6 Crystal Properties

2.61 Crystal Shape

Crystallization from diluted acetic acid yields needle-like crystals (1).

2.62 Crystal Size

The crystal size of nitrofurantoin has been found to affect the degree of emesis and the rates of gastrointestinal absorption and urinary excretion following oral administration (8). The different crystal sizes of nitrofurantoin were prepared by recrystallization from nitromethane.

2.63 Hydrates

It has been found that nitrofurantoin can exist in anhydrous and monohydrate form (9). Anhydrous nitrofurantoin and previously dried nitrofurantoin monohydrate become hydrated only at very high humidity (> 92% R.H.). Nitrofurantoin monohydrate does not lose or gain moisture upon storage at various relative humidities in the range of 31-92% R.H. Monohydrate form is very stable in terms of retaining water at 50°C.

2.7 Salts

The sodium salt of nitrofurantoin is available and is used to prepare parenteral formulations (10). Aqueous solutions of the salt are very unstable.

2.8 Solubility

2.81 Solubility in Aqueous Media

Solubilities of nitrofurantoin in aqueous media have been reported for various temperature and pH conditions. These solubility values are presented in Table III.

Table III

Solubilities of Nitrofurantoin in Aqueous Media

Temperature, °C	pH	Solubility (mg/l)	References
24	5	76.3	11
24	7	131.1	11
24	d. H_2O	79.5	11
25	7	190	1, 12
30	d. H_2O	113.4	11
37	1.12	154	13
37	4.8	125	14
37	5	167.8	11
37	d. H_2O	174.1	11
37	7	312.1	11
	7.2	374	13
45	d. H_2O	251.2	11

2.82 Solubility in Organic Solvents

The solubilities of nitrofurantoin in various organic solvents are presented in Table IV.

Table IV

Solubilities of Nitrofurantoin in Organic Solvents*

Solvent	Solubility (mg/l)
Acetone	5,100
Dimethylformamide	80,000
Ethanol 47.5%	189
70%	712
95%	510
Glycerin	600
Peanut Oil	20.7
Polyethylene glycol 300	15,100
Propylene glycol 20%	1,560

*The solubility values were reported in References (1) and (15). The temperature was not indicated.

3. Synthesis

Nitrofurantoin can be prepared by the reaction of 1-aminohydantoin as the sulfate (16) and as the hydrochloride (17) with 5-nitrofurfural diacetate in isopropyl-alcohol-water media. Details of the production of nitrofurantoin were described by Sanders et al. (18). The reaction scheme is shown in Figure 5. Nitrofurantoin was also prepared by the condensation of 1-aminohydantoin with 5-nitro-2-furaldehyde (19). Another method for the synthesis of nitrofurantoin was described by Jack (20).

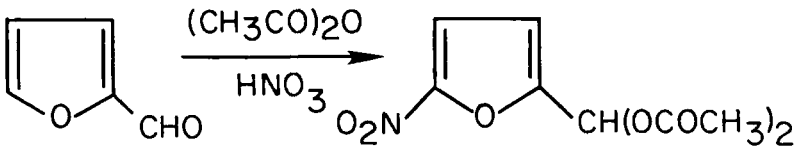
4. Stability

4.1 Stability to Light and Metal

Nitrofurantoin crystals and its solutions are discolored by alkali and by exposure to light, and are decomposed upon contact with metals other than stainless steel and aluminum (2). Since nitrofurantoin solutions are photosensitive, all analytical operations must be conducted under subdued light. Nitrofurantoin solutions are also extremely sensitive to alkali; therefore, all glassware must be analytically clean and dry for assay procedures.

4.2 Shelf-Life and Storage Conditions

Storage conditions of nitrofurantoin and oral suspensions are recommended to be in tight, light-resistant containers (2). Recommended shelf-life of tablets and suspensions is five years when stored at room temperature and in regular glass containers (21).



5-Nitrofurfural Diacetate

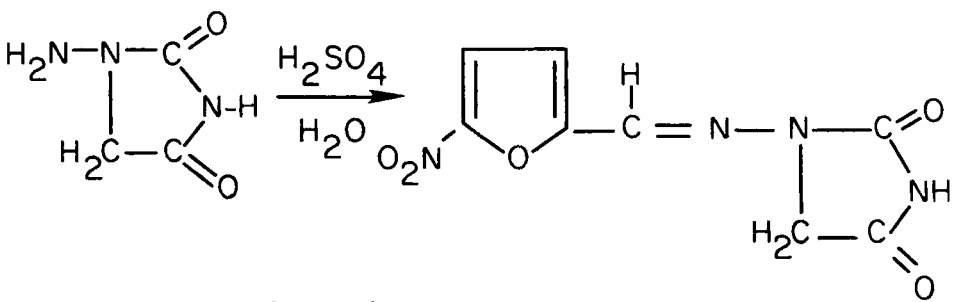


Figure 5. Synthesis of Nitrofurantoin

Nitrofurantoin

When the products are packaged in light-resistant containers and stored at room temperature, the drug showed negligible loss of potency over a five-year period of time. The nitrofurantoin products should not be stored where temperatures are expected to exceed 86°F.

5. Metabolism

Reckendorf et al. (22) reported that large amounts of nitrofurantoin (30-50%) of an orally and intravenously administered dose were recovered intact in the urine of rat, dog and man. Beutner et al. (23) also found a similar recovery in urine after oral administration. These studies suggest that nitrofurantoin undergoes metabolic transformation in the body to a significant extent. The possible metabolic pathways of nitrofurantoin are not completely elucidated in the literature. However, nitrofurantoin would follow somewhat similar pathways in metabolism to that for nitrofurazone which undergoes reduction in the nitro group and hydrolysis in the azomethane linkage.

6. Methods of Analysis

6.1 Identification

Nitrofurantoin may be identified by its melting point (270-272°C) and by means of its characteristic infrared spectra (see Figure 3).

6.2 Color Reaction Test

A number of color reaction tests for the identification of nitrofurantoin has been presented in a publication (24).

6.3 Elemental Analysis

The results of an elemental analysis of nitrofurantoin (Norwich Pharmacal Co., Lot. No. E3769) are presented in Table V (25).

Table V

Elemental Analysis of Nitrofurantoin

Element	% Theory	% Found
C	40.34	40.22
H	2.54	2.57
N	23.53	23.53
O	33.59	

6.4 Chromatographic Systems

6.41 Thin Layer Chromatography

The following TLC systems have been found suitable for detection of possible postulated impurities (26).

Solvent System I

Acetone:	Glacial Acetic Acid:	Methanol
90	5	5 (v/v)

R_f = 0.81 on Brinkman Silica Gel plate without fluorescent indicator as visualized by short-wave ultraviolet light. It was found that the linear dynamic range was 0.25-1.10 μg/spot.

Solvent System II

Acetone:	Benzene:	Glacial acetic acid
80	20	1 (v/v)

R_f = 0.95 on Brinkman Silica Gel plate without fluorescent indicator as visualized by short-wave ultraviolet light and H_2SO_4 charring.

Other TLC procedures for the identification and separation of nitrofurantoin have been described (27).

6.42 Paper Chromatography

The use of paper chromatography in the analysis of nitrofurantoin and other nitrofuran compounds has been reported by Breinlich (28), who examined several solvent systems and methods for identification of the spots.

6.43 Column Chromatography

Bender et al. (29) used column chromatographic procedures to separate nitrofurantoin from other components of urine samples and subsequently determined the drug concentrations by spectrophotometric assay.

6.5 Quantitative Analysis

6.51 Assay of Dosage Forms

The methods of analysis that are used for the determination of nitrofurantoin depend on its ultraviolet absorption characteristics. The U.S.P. XIX (30) describes a spectrophotometric determination of nitrofurantoin in an acidic 2% dimethylformamide-water solution. This general method is applicable to nitrofurantoin in tablet, capsule and suspension dosage forms where the excipients are UV non-absorbing. Under conditions where separations are not made, any drug degradation products are reasonably expected to be reflected in the value of the UV ratio; conversely, constancy of the UV ratio as compared to the initial formulation value can be taken as evidence for stability (31). A polarographic (32) and gravimetric (33) determination of nitrofurantoin in tablets have been described.

6.52 Quantitative Determination in Biological Samples

Paul et al. (34) utilized the ultraviolet absorption of nitrofurantoin to determine drug concentrations in rat urine followed by extraction with ether, but the absorption maximum was pH dependent as indicated by Stoll and co-workers (35).

In 1965 Conklin and Hollifield (36) introduced the nitromethane-Hyamine procedure for the determination of nitrofurantoin in urine, and this method has been established as the preferred assay for nitrofurantoin in biological samples. The original procedure has been modified to assay the drug in whole blood or plasma (37). This procedure has a sensitivity of 2 μg/ml and is specific for nitrofurantoin. However, this method was inadequate for quantitating nitrofurantoin blood levels in subjects who received normal therapeutic doses. Mattock, McGilveray and Charette (38) improved the nitromethane-Hyamine method for the determination of nitrofurantoin in blood samples so that smaller volumes are required (0.8 ml) and the sensitivity is greater (0.2 μg/ml). A modified nitromethane-Hyamine method was described by Hollifield and Conklin (39) for the assay of nitrofurantoin in urine in the presence of phenazopyridine and its metabolites.

Buzard et al. (40) developed a colorimetric method for the analysis of nitrofurantoin in plasma or serum of the rat after various oral doses.

Jones et al. (41) described a polarographic method for the determination of nitrofurantoin in urine. The sensitivity limit of assay is 1 μg/ml. Several authors (42, 43) have reported polarographic procedures for the assay of the drug.

A microbiological procedure for the assay of nitrofurantoin in urine was also described by Jones et al. (41). Gang and Shaikh (44) used an indicator organism in a turbidimetric method for the assay of nitrofurantoin and other nitrofuran derivatives in serum and urine samples.

A column chromatographic method for the determination of nitrofurantoin in urine was reported by Bender et al. (29).

Stone (45) and Puglisi (46) have measured a trace of nitrofurantoin in milk by the colorimetric and spectrophotometric methods, respectively. Both procedures are based on the conversion of nitrofurantoin to 5-nitrofurfuraldehyde phenylhydrazone and are followed by the extraction and concentration on a chromatographic column. Final determinations depend on the development of a blue color by the addition of Hyamine base.

7. Biopharmaceutics and Pharmacokinetics

7.1 Absorption

Nitrofurantoin is efficiently and rapidly absorbed after oral administration (47). Limited drug absorption occurs when nitrofurantoin is administered rectally (48).

The first evidence that absorption and excretion were affected by differences in particle size of the drug was published in 1967 (8). This paper reported on the relationship of particle size of nitrofurantoin to emesis in dogs and to absorption and urinary

excretion in man and rats. It was found that larger crystals of the drug caused less emesis in dogs and slower absorption in man and rats. Thus, it was concluded that the use of large crystals of nitrofurantoin (Macrodantin®) could minimize adverse effects of this drug such as nausea and vomiting by slowing the rate of absorption in the gastrointestinal tract.

Nitrofurantoin occasionally causes nausea, vomiting, drowsiness, headache and skin rashes (49). Incidence of these reactions may be reduced to some degree by administration of the drug with food (50, 51).

Bates and co-workers (52) studied the effect of food on the absorption of nitrofurantoin from commercial dosage forms. They found considerably increased absorption in nonfasting as compared to fasting subjects.

7.2 Distribution

After absorption into the blood circulation, nitrofurantoin is rapidly distributed into most body fluids (53). During normal oral therapeutic regimen, blood or plasma levels of the drug are usually very low, in the neighborhood of 1 μg/ml. However, nitrofurantoin levels about 3-5 times greater than this are usually found in these fluids when drug is administered intravenously or intramuscularly. Detailed studies of the absorption and distribution of nitrofurantoin have been carried out by Buzard et al. (54) in small animals.

7.3 Elimination

The biological half-life of nitrofurantoin in man appears to be 30 min. or less (55, 56, 57, 22). In clinical studies in human subjects, urinary drug concentration of 200 to 400 mg/liter have been reported (50, 58). Renal excretion involves glomerular filtration and active tubular secretion. Schirmeister et al. (59) found that the clearance of nitrofurantoin in human subjects was lower in acid urine than in alkaline urine. During renal impairment, nitrofurantoin urinary excretion was significantly reduced (60).

Conklin and Wagner (61) and Conklin et al. (62) reported that as much as 20% of the intravenous dose of nitrofurantoin sodium was excreted in hepatic bile in the dog.

7.4 Bioavailability

Bioavailability of nitrofurantoin has received a considerable attention in recent years. Cadwallader (63) presented a monograph on the bioavailability of nitrofurantoin in the special APhA Bioavailability pilot project. The general characteristics and experimental criteria for bioavailability testing of nitrofurantoin were discussed. More recently, Meyer and co-workers (64) evaluated the bioavailabilities of 14 commercially available nitrofurantoin preparations by in vivo and in vitro procedures. They found that some products that met the official Compendial requirement, were less bioavailable than other products tested. Earlier studies by various authors

(65, 66, 67) also reported bioavailability problems associated with the use of commercial nitrofurantoin tablets. An updated monograph on the bioavailability of nitrofurantoin was recently presented by Cadwallader (68).

7.5 Pharmacokinetics

In man, plasma levels of nitrofurantoin appear to decline exponentially with a half-life value of about 20 to 30 minutes (56, 57, 22). Urinary excretion and biotransformation appear to be mainly and equally responsible for the elimination of nitrofurantoin. The one-compartment model appears to be adequate for describing the kinetics involved in nitrofurantoin absorption and elimination.

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PIPERAZINE ESTRONE SULFATE

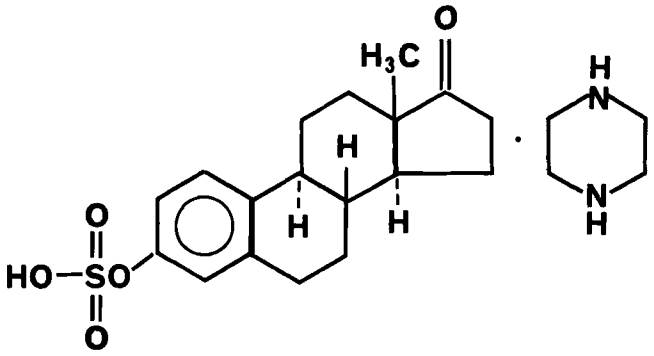
Zui L. Chang

Contents

1. Description

1.1 Name, Formula, Molecular Weight

Piperazine estrone sulfate is estra-1,3,5 (10)-trien-17-one, 3-(sulfooxy)-, compound with piperazine (1:1).[1,2]



Piperazine Estrone Sulfate

$C_{18}H_{22}O_5S \cdot C_4H_{10}N_2$ Molecular Weight 436.56

1.2 Appearance, Color, Odor

Piperazine estrone sulfate is a white to yellowish white, fine crystalline powder. It is odorless.

1.3 Elemental Composition

C-60.53; H-7.39; N-6.42; O-18.32; S-7.34.

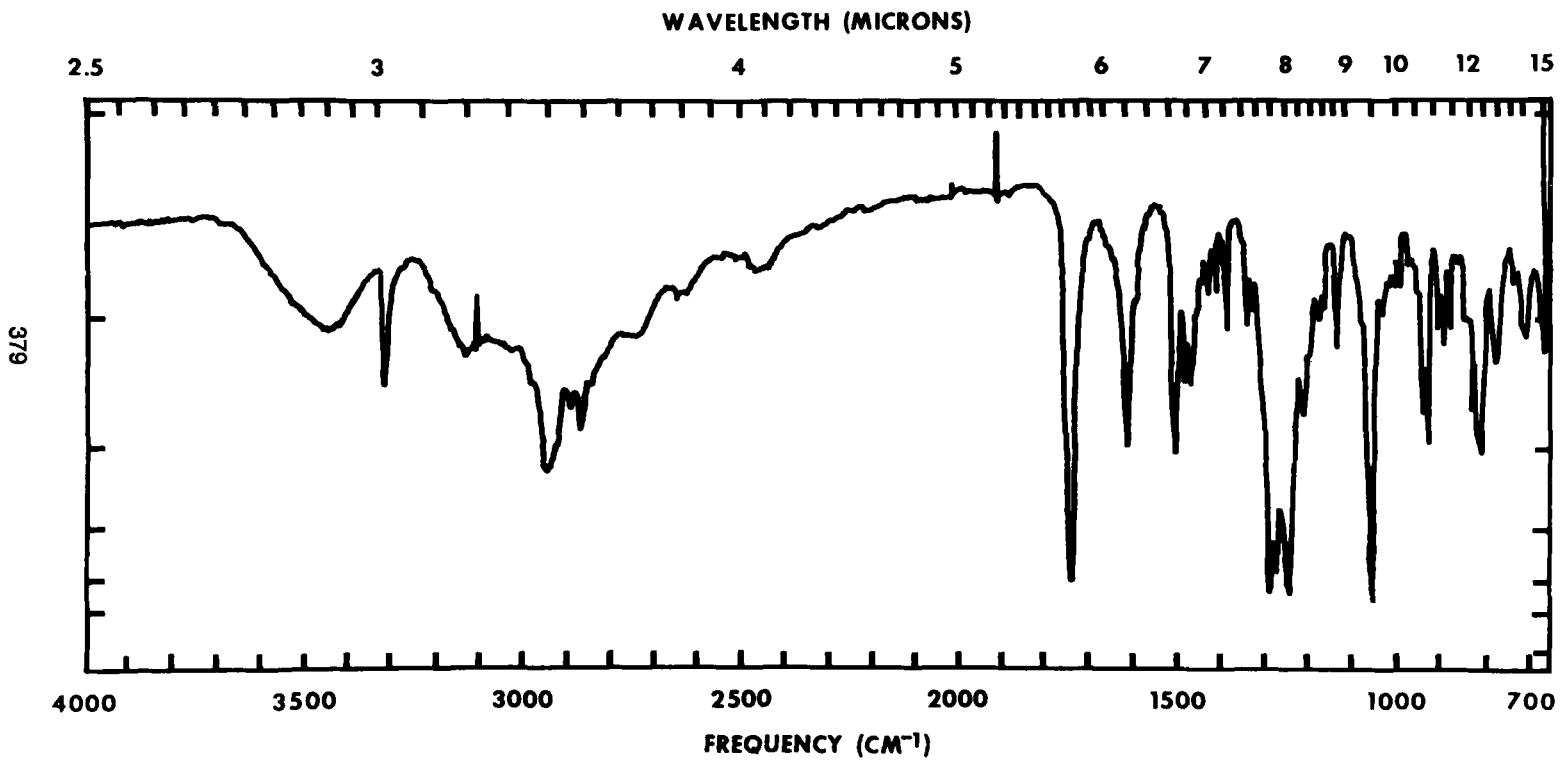
2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of piperazine estrone sulfate is presented in Figure 1. The spectrum was measured in the solid state as a potassium bromide dispersion. The following bands (cm^{-1}) have been assigned for Figure 1.[3]

a. 3400-2300 cm^{-1} broad complex of bands due mainly to N-H stretching vibrations of the amine salt.

FIGURE 1 - INFRARED SPECTRUM OF PIPERAZINE ESTRONE SULFATE



b. 1730 cm^{-1} characteristic C=O stretching vibration of the 17-keto group.

c. 1600 and 1490 cm^{-1} characteristic skeletal stretching vibrations of the aromatic ring.

d. 1040 cm^{-1} due to S-O linkage

2.2 Nuclear Magnetic Resonance Spectrum (NMR)

The nuclear magnetic resonance spectrum of piperazine estrone sulfate as shown in Figure 2 was obtained on a Varian HA-100 NMR Spectrometer in deuterated dimethylsulfoxide (d_6) containing tetramethylsilane as the internal standard. The spectral peak assignments[4] are presented in Table I.

2.3 Ultraviolet Spectrum (UV)

When the UV spectrum of 0.1% solution of piperazine estrone sulfate in 0.04% sodium hydroxide solution was scanned from 400 to 210 nm, two maxima and two minima were observed (Figure 3).[1] The maxima are at 275 nm (ε = 838) and 268 nm (ε = 851). The minima occur at 272 nm and 239 nm. The spectrum was obtained with a Cary Model 14 Recording Spectrophotometer.

2.4 Mass Spectrum

The mass spectrum shown in Figure 4 was obtained using an Associated Electrical Industries Model MS-902 Mass Spectrometer with an ionizing energy of 70 eV. The mass spectrum of piperazine estrone sulfate indicates the presence of estrone, piperazine, and a sulfur-oxygen constituent, but it does not yield a molecular ion for the complete chemical entity. This is attributed to the following behavior:

(a) Dissociation of the amine salt into the free amine (piperazine) and the free acid (estrone hydrogen sulfate).

(b) Possible thermal decomposition of estrone hydrogen sulfate to estrone, SO_2, OH^-, etc.

FIGURE 2 - NUCLEAR MAGNETIC RESONANCE SPECTRUM OF PIPERAZINE ESTRONE SULFATE

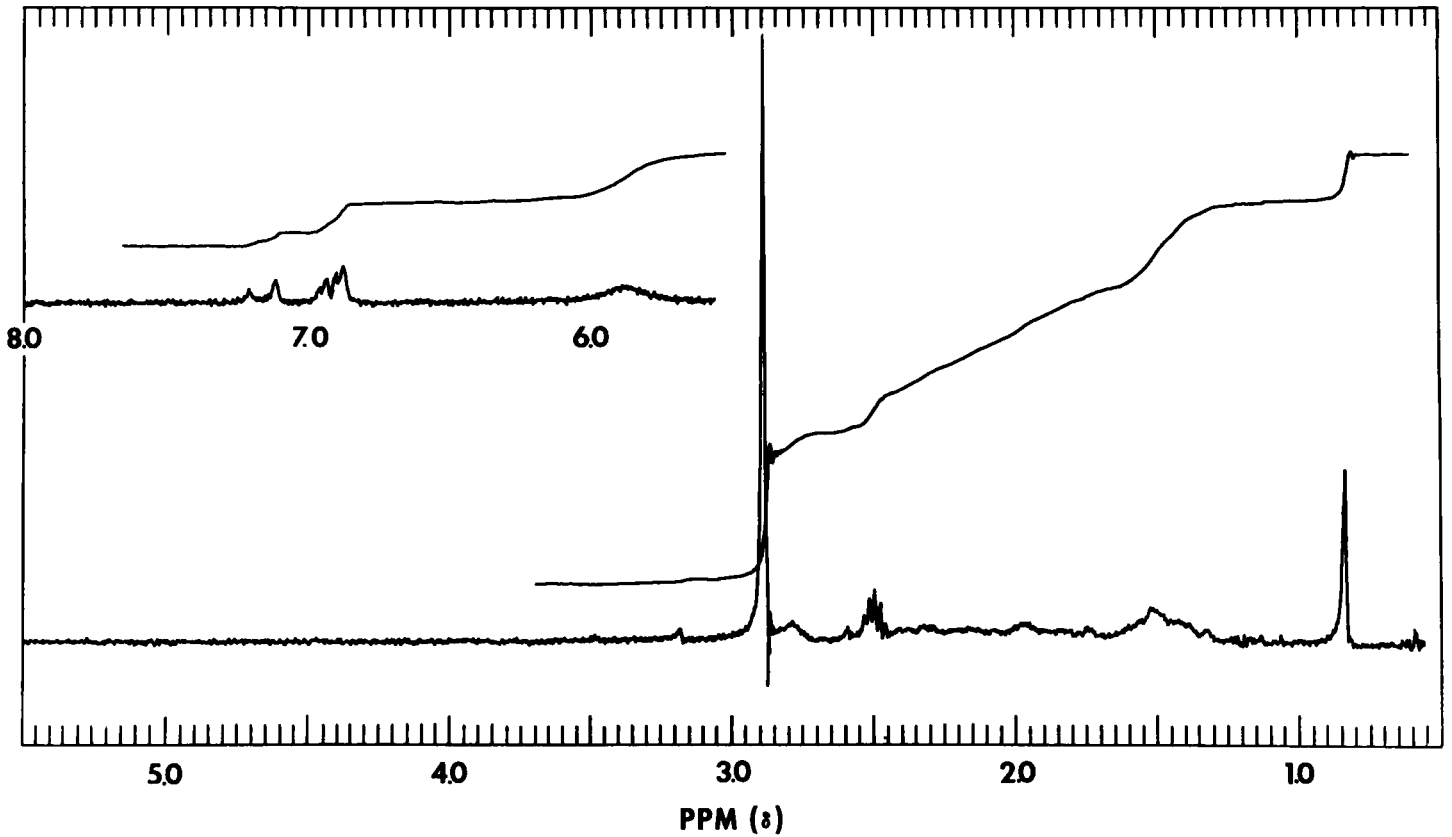


Table I

NMR Spectral Assignments for Piperazine Estrone Sulfate

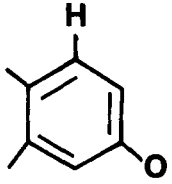
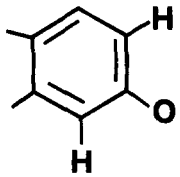
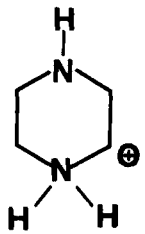
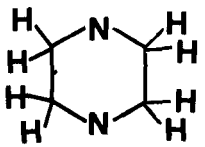
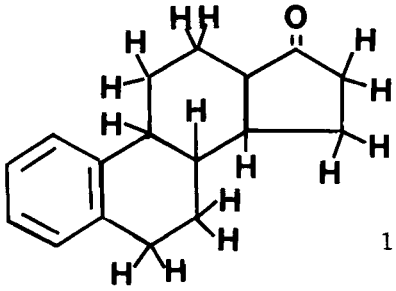
Proton Assignment	Chemical Shift (ppm)	Multiplicity
	7.17	doublet $J=9.5H_z$
	6.92	Multiplet
	5.5	Broad
	2.89	Singlet

Table I Cont.

Proton Assignment	Chemical Shift (ppm)	Multiplicity
	1.2 - 3.0	Complex
C — CH_3	0.84	Singlet

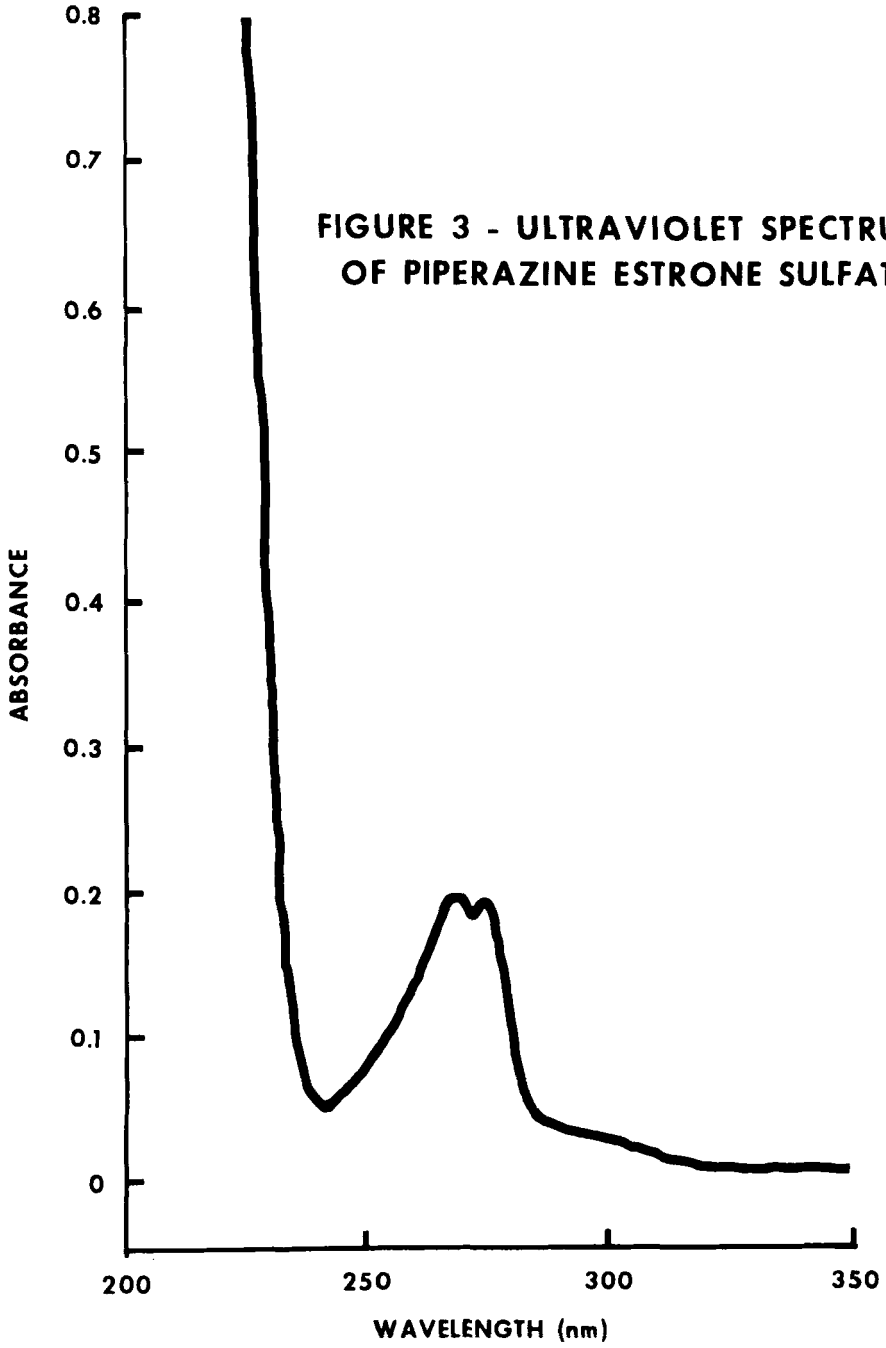
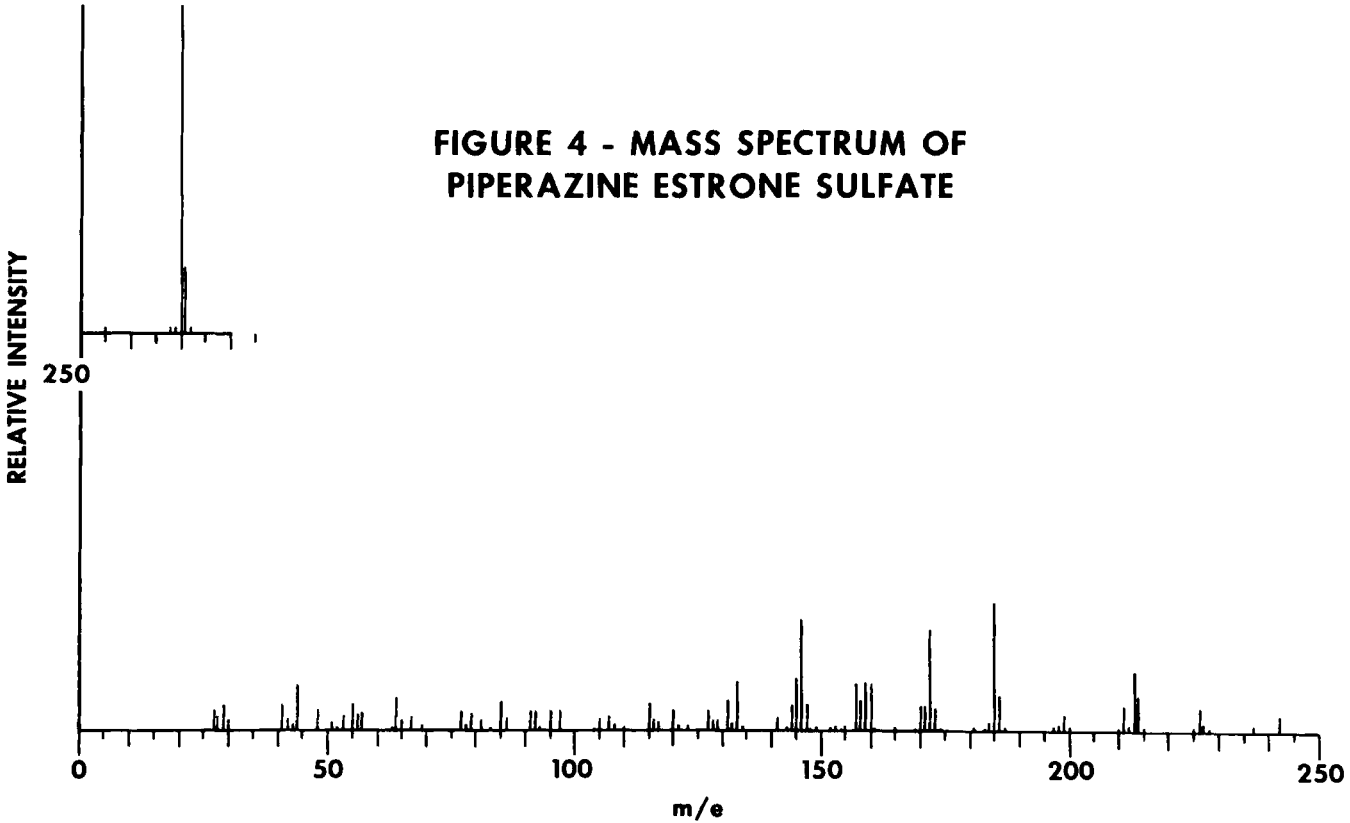


FIGURE 3 - ULTRAVIOLET SPECTRUM OF PIPERAZINE ESTRONE SULFATE

FIGURE 4 - MASS SPECTRUM OF PIPERAZINE ESTRONE SULFATE



The mass spectrum assignments of the prominent ions and subsequent fragments are shown in Table II and Figure 5.[5]

2.5 Raman Spectrum

The Raman spectrum of piperazine estrone sulfate as shown in Figure 6, was obtained in the solid state on a Cary Model 83 Spectrometer. The following bands (cm^{-1}) have been assigned for Figure 6.[3]

a. 1733 cm^{-1} due to the C=O stretching of the 17-keto group.

b. 1608 cm^{-1} due to skeletal stretching mode of the aromatic ring.

c. 1050 cm^{-1} due to S-O linkage

2.6 Optical Rotation

A 1% solution of piperazine estrone sulfate in 0.4% sodium hydroxide solution exhibited a rotation of $[\alpha]_D^{25}$ + 87.8° when determined on a Perkin-Elmer Model 141 Polarimeter.[6]

2.7 Melting Range

Piperazine estrone sulfate melts at about 190°C to a light brown, viscous liquid which re-solidifies, on further heating, and finally melts at about 245°C with decomposition.[1]

2.8 Differential Thermal Analysis (DTA)

The DTA curve obtained on a Dupont Model 900 Analyzer as shown in Figure 7 confirms the observed melting characteristics described in section 2.7.

2.9 Solubility

Approximate solubility data obtained at room temperature are given in the following table:[7,8]

Table II

High Resolution Mass Spectrum of Piperazine Estrone Sulfate

Found Mass	Calculated Mass	C	H	N	O	S
270.1620	270.1620	18	22	0	2	0
213.1287	213.1279	15	17	0	1	0
185.0960	185.0966	13	13	0	1	0
172.0887	172.0888	12	12	0	1	0
146.0725	146.0732	10	10	0	1	0
86.0843	86.0844	4	10	2	0	0
85.0771	85.0766	4	9	2	0	0
63.9618	63.9619	0	0	0	2	1
47.9669	47.9670	0	0	0	1	1

FIGURE 5 - FRAGMENTATION PATHWAYS OF PIPERAZINE ESTRONE SULFATE

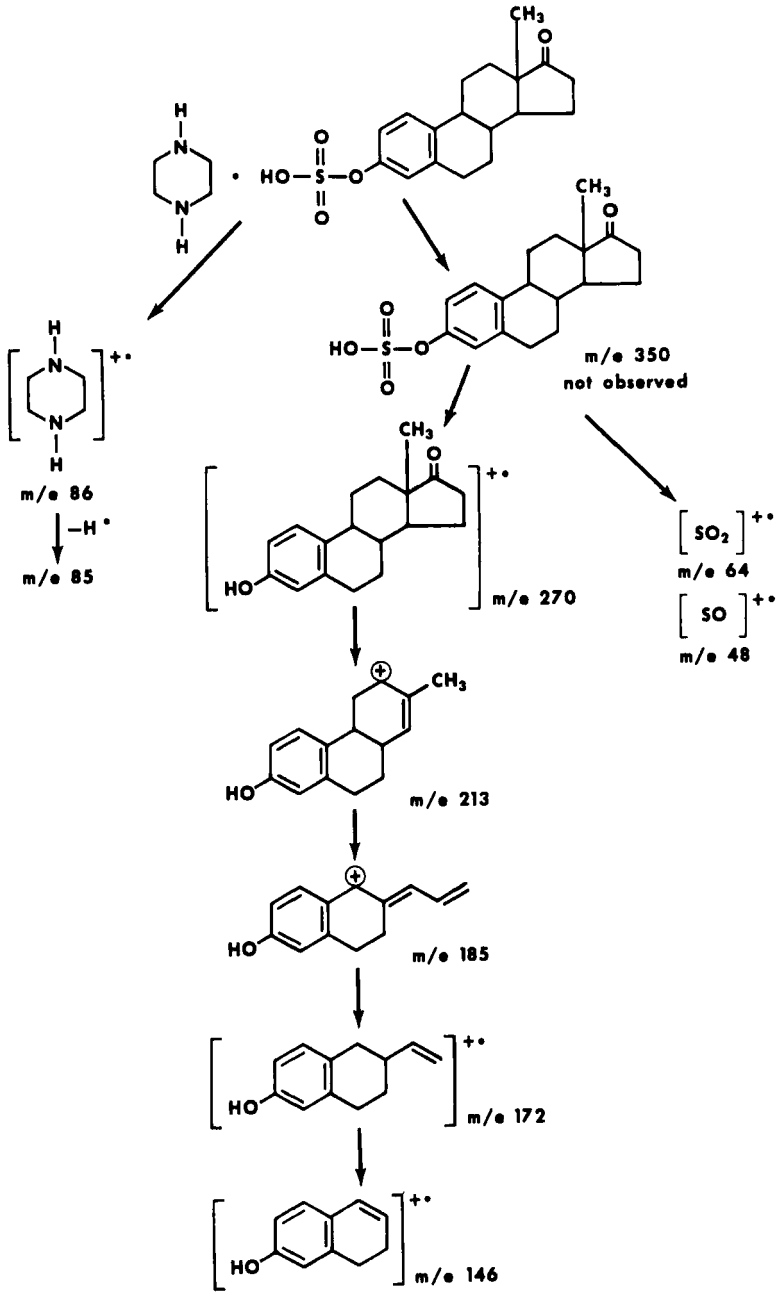


FIGURE 6 - RAMAN SPECTRUM OF PIPERAZINE ESTRONE SULFATE

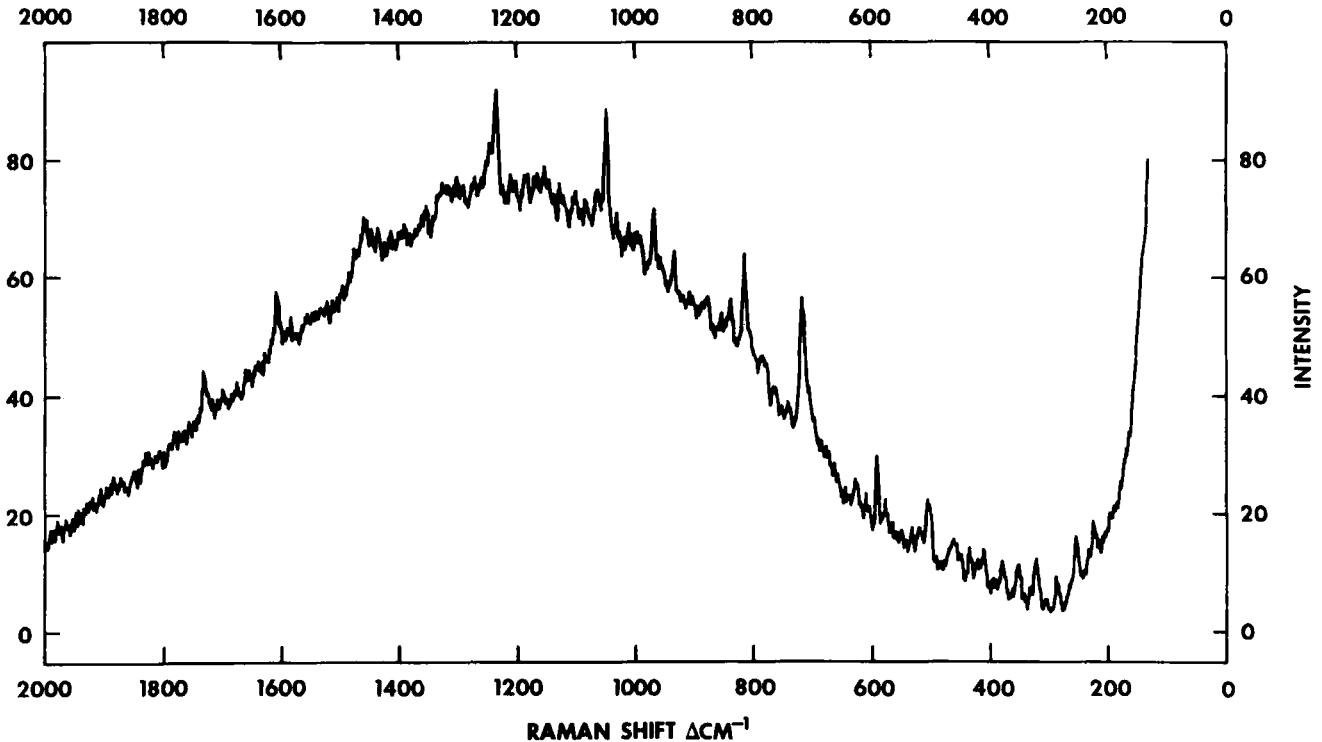
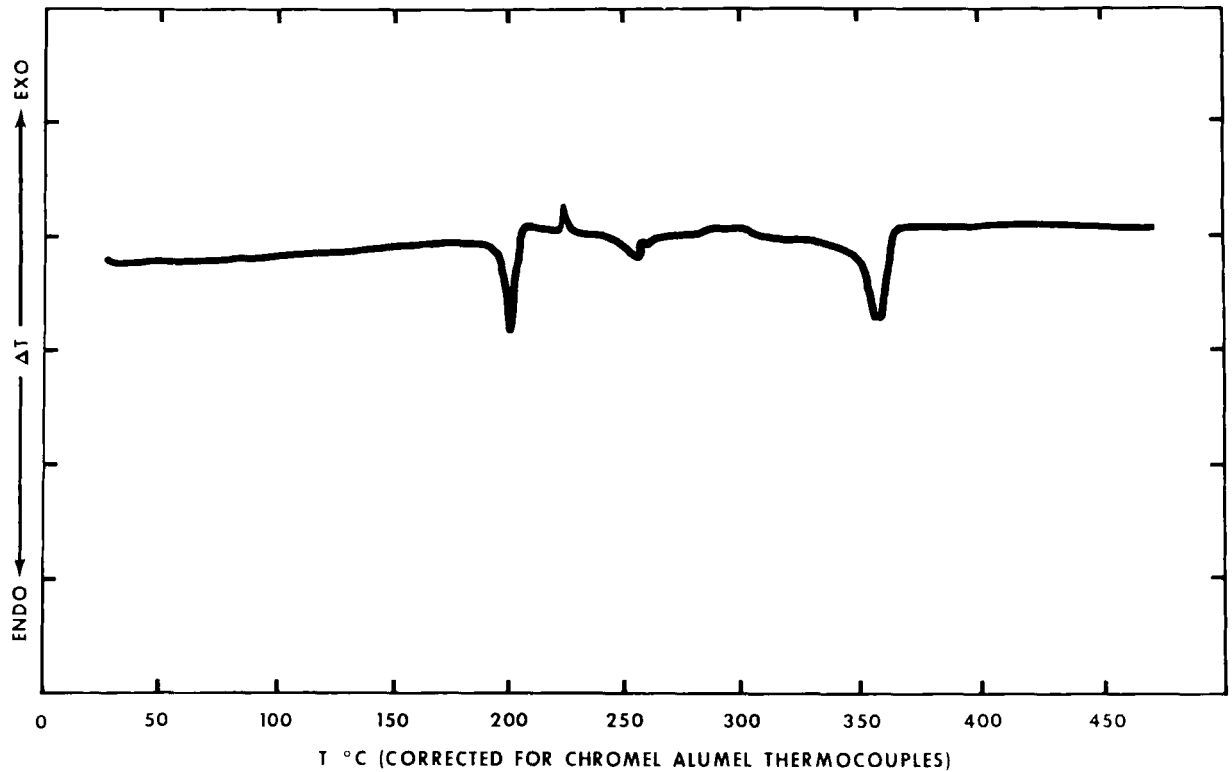


FIGURE 7 - DIFFERENTIAL THERMAL ANALYSIS CURVE OF PIPERAZINE ESTRONE SULFATE



Solvent	Solubility (mg/ml)
Water	8
95% Ethanol	7.4
Chloroform	1
Ether	Practically insoluble
Acetone	0.2
Benzene	Practically insoluble
Methylene dichloride	1.6
Isopropanol	Practically insoluble
0.1 N Sodium hydroxide	57
Propylene glycol	35
Mineral oil	2
Sesame oil	2

2.10 Crystal Properties

The X-ray powder diffraction pattern of piperazine estrone sulfate was determined by visual observation of a film obtained with a 143.2 mm Debye-Scherrer Powder Camera (Table III). An Enraf-Nonius Diffractis 601 Generator; 38 KV and 18 MA with nickel filtered copper radiation; λ = 1.5418, were employed.[9]

2.11 Dissociation Constant

The apparent pKa value of the unprotonated piperazine nitrogen (proton gained) was found to be 3.6, by titration in acetonitrile-water (80/20, v/v) with aqueous sodium hydroxide. Attempts to find systems to extrapolate the pKa to 100% water were unsuccessful.

The pKa value of the protonated piperazine nitrogen (proton lost) was found to be 9.7 by titration in pyridine-water mixtures with methanolic KOH, and extrapolation to 100% water.[8]

Table III

X-Ray Powder Diffraction Pattern
d-Spacings and Intensities

dA	I/I	dA	I/I
16.5	10	2.98	2
7.7	10	2.92	2
7.4	40	2.86	5
6.6	40	2.73	10
6.0	20	2.53	5
5.67	30	2.46	8
5.23	40	2.37	1
4.55B	20	2.32	5
4.38	80	2.28	5
4.22	60	2.21	1
4.05	5	2.16B	5
3.86	30	2.11	1
3.77	100	2.07B	8
3.61	5	1.95	2
3.54	2	1.89	2
3.40B	20	1.85B	3
3.22	5	1.75B	5
3.05	1	1.70	3

2.12 Fluorescence

Piperazine estrone sulfate does not exhibit fluorescent properties in either methanol or in an alkaline aqueous solution. However, it does exhibit fluorescence at 488 nm when excited at 465 nm in 65% sulfuric acid solution.[6] The strong sulfuric acid converts the piperazine estrone sulfate to estrone which reacts with sulfuric acid to yield the fluorescent species.

2.13 Hygroscopic Behavior

Piperazine estrone sulfate was not hygroscopic when exposed to a relative humidity of 40-50% for three weeks.[8]

Piperazine estrone sulfate does not absorb moisture at 79% relative humidity, and is only very slightly hygroscopic (0.89%) at 100% relative humidity.[7]

2.14 Sublimation

Piperazine estrone sulfate did not sublime when it was stored at 105°C for one month.[8] No evidence of sublimation was noted when it was heated with a hot stage to 204°C.[7]

3. Synthesis

Piperazine estrone sulfate was first prepared by Hasbrouck in 1951.[10]

The compound can also be prepared from estrone by a fast, complete conversion reaction using a dimethylformamide/sulfur troxide complex as the sulfating reactant. Excess dimethylformamide is the solvent. The reaction is completed by the addition of piperazine.[11]

4. Stability-Degradation

Piperazine estrone sulfate was found to be stable when refluxed in water for 3 hours. But it degrades completely after 3 hours in refluxing 1 N hydrochloric acid to yield estrone and piperazine sulfate. It degrades slightly in refluxing 1 N sodium hydroxide after 3 hours to yield less than 10% free estrone.[12]

Piperazine estrone sulfate yields about 10% free estrone when heated at 105°C for one month.

The rate of hydrolysis of piperazine estrone sulfate to estrone at 90°C was studied over the pH range of 2.5-9.1. The extent of degradation was determined by a spectrophotometric measurement in 0.1 N sodium hydroxide solution.[13]

The hydrolysis of piperazine estrone sulfate to estrone follows first order kinetics with respect to the piperazine estrone sulfate concentration remaining. Futh :more, the degradation is first order with respect to t e hydrogen ion concentration, resulting in a 10-fold rate increase with each pH unit decrease.[13] Some rate cons ants at different pH and temperature values are shown in T. bles IV and V. The activation energy of the degradati: n reaction, Ea (obtained from the slope of the plot of l: K, as a function of 1/T where T is the absolute temp rature), for three pH levels is shown in Table V.

5. Drug Metabolic Products and Pharmacokinetics

The drug substance is hydrolyzed to estrone in acidic media. The known metabolic interconversions of the estrone are summarized in Figure 8.[14]

Purdy's[15] work suggests that estrone, as the sulfate conjugate, is an important transport form of estrone in human plasma.

Urinary excretion studies of sodium estrone sulfate have been performed by Twombly and Levitz[16], and Brown.[17]

Biliary excretion was also studied by Twombly and Levitz.[16]

A very exhaustive study of urinary and biliary metabolites was described by Jirku and Levitz.[18]

Quantitative determination of plasma estrogens by radioimmmunoassay has been developed by Vega.[19]

Table IV

First Order Rate Constants of Piperazine Estrone Sulfate as a Function of pH at 90°C

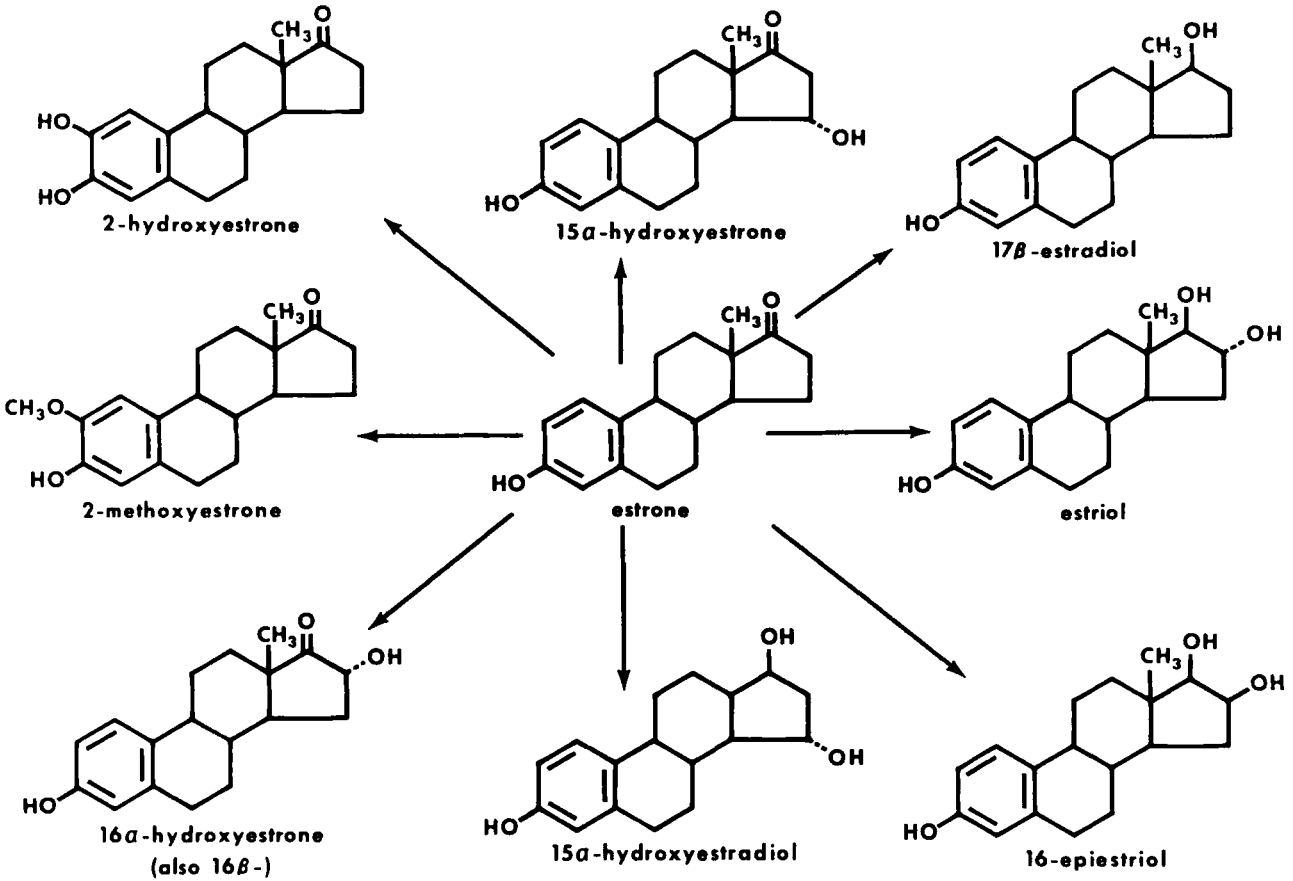
pH	Initial Concentration mg./ml.	No. of Samples	$k_1 \pm$ 95% Conf. Lim. $\times 10^4$, hour^{-1}
2.50	0.3	8	1,830 ± 250
3.03	0.3	8	480 ± 25
3.07	1.5	12	396 ± 16
3.40	1.5	13	201 ± 14
3.57	0.3	7	130 ± 16
3.88	1.5	15	59.1 ± 2.9
4.26	1.5	15	22.4 ± 1.5
4.86	1.5	15	7.51 ± 1.03
5.16	1.5	15	3.63 ± .68
5.98	1.5	13	1.85 ± .77
6.11	1.5	14	1.12 ± .44
6.48	1.5	15	.68 ± .30
6.88	1.5	14	.72 ± .17
7.50	1.5	13	.42 ± .12
7.90	1.5	15	.14 ± .11
8.43	1.5	13	.22 ± .08
9.10	1.5	12	.18 ± .11

Table V

First Order Rate Constants and Activation Energies of Piperazine Estrone Sulfate at Three pH levels

	pH 2.50	pH 3.03	pH 3.57
$k_1 \times 10^4$, hr.$^{-1}$			
90°C.	1,830 ± 250	480 ± 25	130 ± 16
80°C.	695 ± 31	150 ± 4.6	44.8 ± 4.0
70°C.	262 ± 15	52.2 ± 2.2	14.6 ± 2.3
Arrhenius Relationship			
Linear Correlation Coeff.	1.000	.999	1.000
Activation Energy, Ea	24,100	27,500	27,100

FIGURE 8 - METABOLIC PATHWAYS OF PIPERAZINE ESTERONE SULFATE



6. Methods of Analysis

6.1 Identification

The presence of piperazine may be identified with quinone T.S.[1,20] or thin-layer chromatography (Section 6.21).

The presence of estrone hydrogen sulfate may be identified by thin-layer chromatography (Section 6.21).

The presence of free estrone may be identified with a 2.5% solution of β-naphthol in sulfuric acid[20] or thin-layer chromatography (Section 6.21).

6.2 Chromatographic Analysis

6.21 Thin-Layer Chromatography

A number of thin-layer chromatographic systems on silica gel have been described for the separation of hydrolysis products of piperazine estrone sulfate from the parent substance.[1,6,21,22] Piperazine estrone sulfate chromatographs as the piperazine and estrone sulfate moieties. Various systems, methods of detection and R_f valves are summarized in Table VI.

6.22 Gas-Liquid Chromatography

Piperazine estrone sulfate can not be directly chromatographed. However, the principal degradation product, estrone, may be silylated with BSA and chromatographed using 3% QF-1 on Gas Chrom Q[12], or it may be silylated with a silylating mixture containing N-trimethylsilylimidazole, BSA and trimethylchorosilane in the ratio 1:30:10, and chromatographed using 10% SE-30 on Chromosorb W-AW.[21]

6.3 Spectrophotometric Analysis

Direct spectrophotometric analysis of piperazine estrone sulfate is applicable provided significant quantities of interferring contaminents are not present. The drug substance may be examined directly in a methanol solution at 269 nm (ε = 860) or in 0.1 N sodium hydroxide solution.[6]

Table VI

TLC Systems for Piperazine Estrone Sulfate

Solvent System	Detection	R_f Piperazine	R_f Estrone Sulfate	R_f Estrone	Reference
Chloroform: Methanol (5:4)	Arsenomolybdate Spray	Origin	0.64	0.82	6
n-Propanol: Ethanol: Conc. Ammonia (2:1:2)	Short wave UV or 10% Sulfuric Acid in Ethanol	0.42	0.78	0.92	21
Chloroform: Methanol (3:1)	Short wave UV or 10% Sulfuric Acid in Ethanol	0.03	0.35	0.72	21
n-Butanol: Water: Conc. Ammonia (1:1:1)	Short wave UV or 10% Sulfuric Acid in Ethanol	0.10	0.28	0.72	21
Chloroform: Methanol: Stronger Ammonia TS (85:15:1)	Iodine Vapor	----	----	----	1, 22

The estrone content of piperazine estrone sulfate can be determined with a suitable spectrophotometer at 238 nm after the conversion of piperazine estrone sulfate to estrone with the use of hydrochloric acid.[1]

The degradation product, estrone, may be quantitated in the drug substance by a liquid-liquid extraction into chloroform and comparison to an estrone reference standard at 280 nm. Alternately, the chloroform extract may be evaporated and the residue dissolved in 65% sulfuric acid solution. The estrone is dehydrated to a species which fluorescence at 488 nm with excitation at 465 nm.[6]

6.4 Colorimetric Analysis

Piperazine estrone sulfate may be determined as dehydrated estrone using a phenol-sulfuric acid mixture as the color development reagent. The chromophore absorbs at 522 nm.[20]

Piperazine estrone sulfate may also be determined using 65% sulfuric acid as the reagent. The reaction product with sulfuric acid has a yellow chromophore at 450 nm.[6]

These colorimetric methods may be used for the analysis of piperazine estrone sulfate in tablets or creams. The primary degradation product, estrone, can be removed by prior chloroform extraction.

6.5 Nitrogen Analysis

The amount of nitrogen present in piperazine estrone sulfate may be determined by converting the nitrogen to ammonia and titrating with 0.05 N sulfuric acid.[20]

6.6 Titration

Piperazine estrone sulfate may be potentiometrically titrated in pyridine-water mixtures using methanolic potassium hydroxide and glass-calomel electrodes.[8]

7. Acknowledgements

The author wishes to thank Mr. V. E. Papendick and Mr. J. A. Raihle for their review of the manuscript, and Miss Sandra Hudson for typing and drawing of the manuscript.

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PROCARBAZINE HYDROCHLORIDE

Richard J. Rucki

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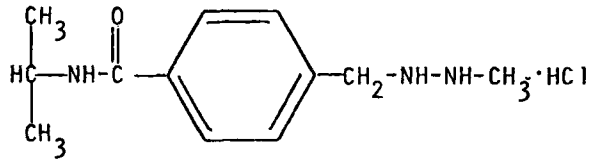
Analytical Profile - Procarbazine Hydrochloride

1. Description

1.1 Name, Formula, Molecular Weight

Procarbazine hydrochloride is N-isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride.



Procarbazine Hydrochloride

$C_{12}H_{19}N_3O \cdot HCl$ Molecular Weight: 257.76

1.2 Appearance, Color, Odor

White to pale yellow crystalline powder with a slight characteristic odor.

2. Physical Properties

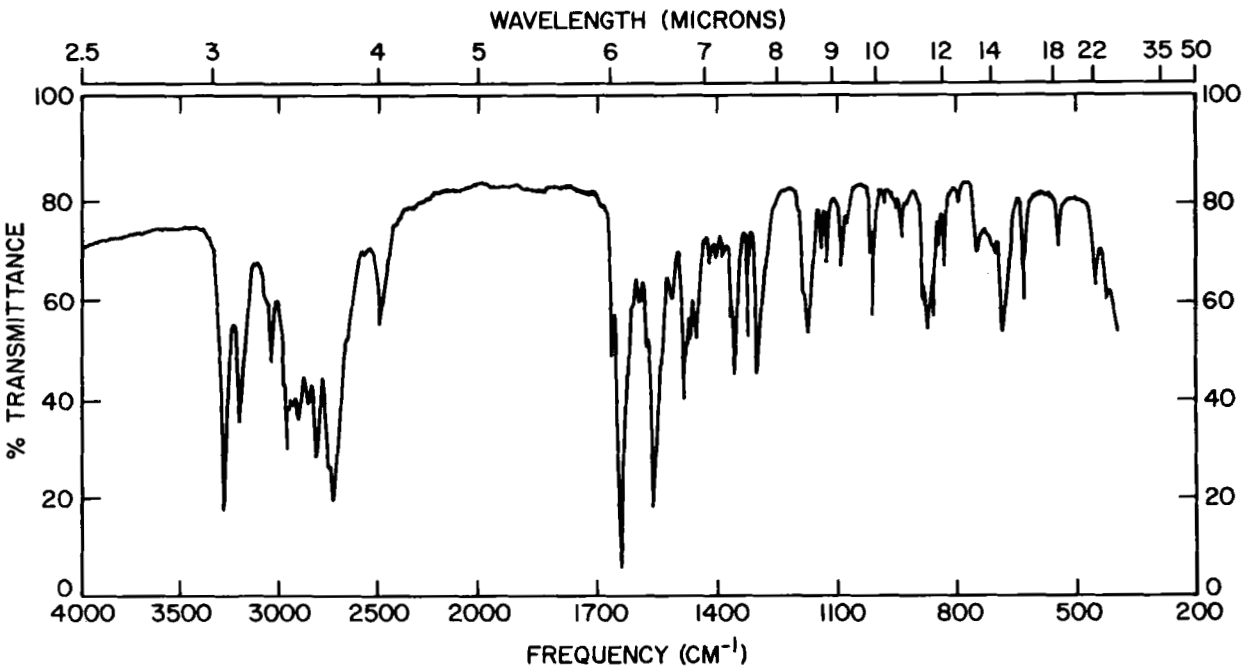
2.1 Infrared Spectrum (IR)

The infrared spectrum of procarbazine hydrochloride is presented in Figure 1 (1). The instrument used was a Perkin-Elmer Model 621 Grating Spectrophotometer. The sample was dispersed in Fluorolube^R to record the spectrum in the region of 4000-1340 cm^{-1} and in mineral oil for the region of 1340-400 cm^{-1}. The following assignments have been made for the bands in Figure 1 (1).

Band (cm^{-1})	Assignment
3277 and 3200	NH stretch
3035	Aromatic CH stretch
2961 and 2853	Aliphatic CH stretch
2760-2300, main band at 2725	NH_2^+

FIGURE 1

Infrared Spectrum of Procarbazine Hydrochloride



1660 and 1636	C=O stretch (Amide I)
1556	Amide II
1299	Amide III
1361 and 1351	CH_3 / CH deformation / CH_3
857	Aromatic CH out-of-plane bending

2.2 Nuclear Magnetic Resonance Spectrum (NMR)

The NMR spectrum shown in Figure 2 was obtained by dissolving 50.4 mg of procarbazine hydrochloride in 0.5 ml of DMSO-d_6 containing tetramethylsilane as internal reference. The spectral assignments are shown in Table 1 (2).

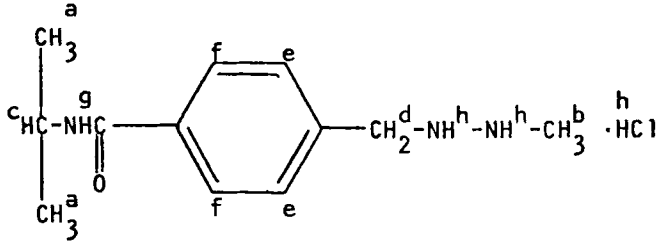
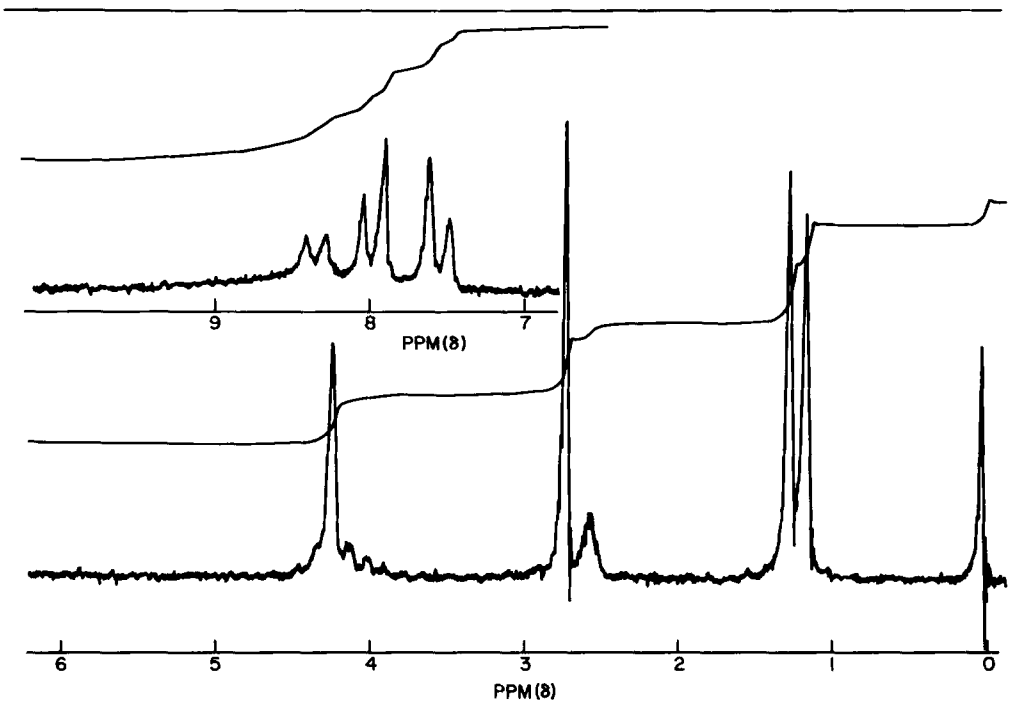


Table 1

Protons	Chemical Shift δ (ppm)	Multiplicity	Coupling Constant, J (in Hz)
a	1.20	Doublet	6.0
b	2.72	Singlet	--
c	4.18	Multiplet	6.0
d	4.23	Singlet	--
e	7.55	Doublet	8.5
f	7.99	Doublet	8.5
g	8.36	Doublet	7.0
h	6.33-10.33	Singlet (broad)	--

FIGURE 2

NMR Spectrum of Procarbazine Hydrochloride



2.3 Ultraviolet Spectrum (UV)

The ultraviolet spectrum of procarbazine hydrochloride in the region of 350 to 200 nm is shown in Figure 3 (3). The spectrum exhibits one maximum at 232 nm ($\varepsilon = 1.3 \times 10^4$) and a minimum at 213 nm. The solution concentration was 0.01 mg/ml in 0.1N hydrochloric acid and the quartz cell width was 1 cm.

2.4 Fluorescence Spectrum

Excitation and emission scans were carried out for procarbazine hydrochloride in solutions of 0.1N hydrochloric acid, 0.1N sodium hydroxide and water. No fluorescence, however, was observed under these conditions (2).

2.5 Mass Spectrum

The low resolution mass spectrum shown in Figure 4 was obtained using a Varian MAT CH5 mass spectrometer, interfaced with a Varian data system, with an ionizing energy of 70 eV (4). The data system accepted the output of the spectrometer, calculated the masses, compared their intensities to the base peak and plotted this information as a series of lines whose heights were proportional to the intensities.

The molecular ion of the free base was observed at m/e 221. The HCl moiety was observed at m/e 36. The ions at m/e 191, 177 and 163 correspond to a loss from the free base of $NHCH_3$, $NHNHCH_3$, and $NHCH(CH_3)_2$, respectively. The ions at m/e 149 and 135 correspond to the loss of C_3H_6 by McLafferty rearrangement from m/e 191 and 177, respectively. The base peak at m/e 118 results from the loss of $NHNHCH_3$ from m/e 163.

A high resolution scan confirmed the results of the low resolution spectrum. Table II lists the elemental compositions for the ions as

FIGURE 3

Ultraviolet Spectrum of
Procarbazine Hydrochloride

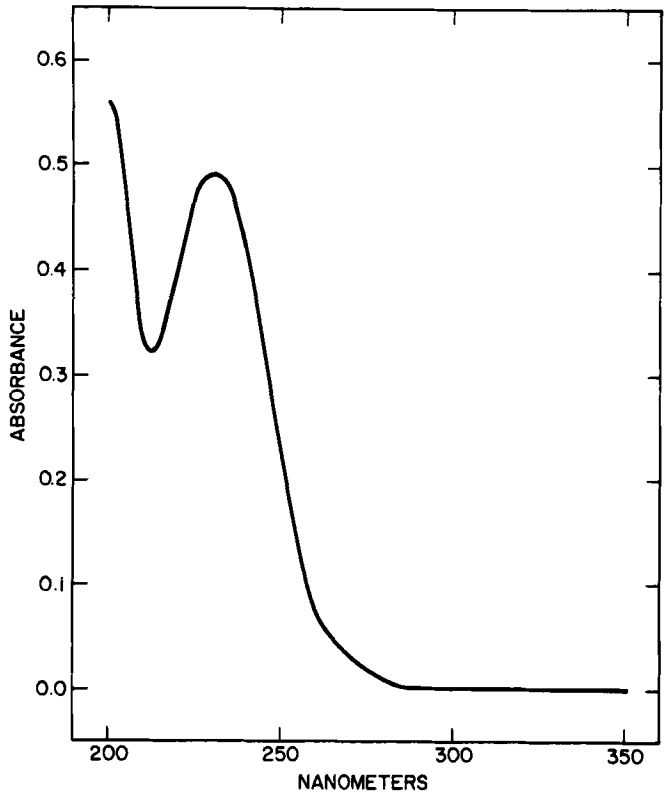
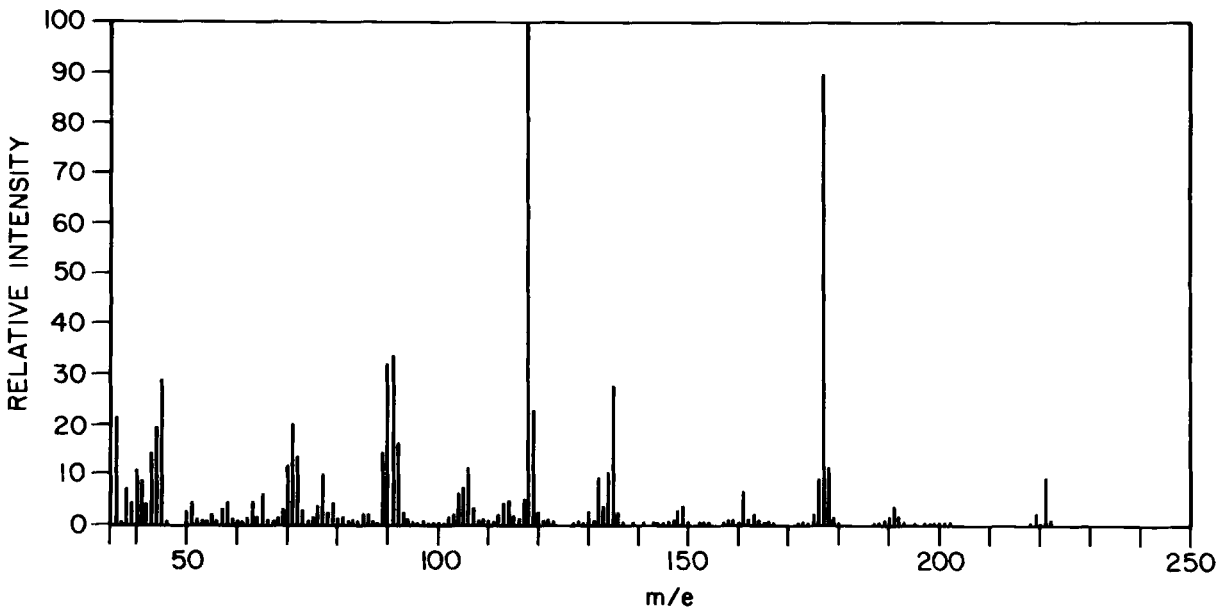


FIGURE 4

Mass Spectrum of Procarbazine Hydrochloride



determined by high resolution mass spectroscopy (4).

Table II

High Resolution Mass Spectrum of Procarbazine Hydrochloride

Found Mass	Calcd. Mass	C	H	N	O
118.0428	118.0419	8	6	0	1
135.0698	135.0684	8	9	1	1
149.0753	149.0715	8	9	2	1
163.0870	163.0872	9	11	2	1
177.1170	177.1154	11	15	1	1
191.1288	191.1311	12	17	1	1
221.1533	221.1529	12	19	3	1

2.6 Optical Rotation

Procarbazine hydrochloride exhibits no optical activity (3).

2.7 Melting Range

According to USP XIX, procarbazine hydrochloride melts at about 223°C, with decomposition (5).

2.8 Differential Scanning Calorimetry (DSC)

DSC spectra for procarbazine hydrochloride at a scan rate of 10°C/min. exhibited an endotherm at about 230°C, where melting was accompanied by sample decomposition. The endotherm was followed immediately by a slow exotherm (continuing decomposition). The observed temperature of the melting/decomposition endotherm is dependent upon instrumental conditions and, therefore, is not characteristic of the compound.

2.9 Thermogravimetric Analysis (TGA)

A thermogravimetric analysis performed on

procarbazine hydrochloride exhibited no loss of weight from 30-150°C. At about 150°C, decomposition weight losses began and continued to 500°C (upper limit of instrument).

2.10 Solubility

Approximate solubility data obtained after 3 hours at 25°C are given in Table III (6,7).

Table III

Solubility of Procarbazine Hydrochloride

Solvent	Solubility (mg/ml)
Water	200
95% Ethanol	27
Absolute Ethanol	8
Methanol	59
Acetone	<0.5
Diethyl Ether	<0.1
Petroleum Ether (30-60°)	<0.1
Chloroform	2
Benzene	<0.5
3A Alcohol	12
Isopropyl Alcohol	1
Cyclohexane	<0.5
Ethyl Acetate	<0.5
Dioxane	0.7
Acetonitrile	<0.5
Carbon Tetrachloride	<0.5
Methylene Chloride	<0.5

2.11 Crystal Properties

The x-ray powder diffraction pattern of procarbazine hydrochloride is presented in Table IV (8).

Instrument Conditions

Instrument	GE Model XRD-6 Spectrogoniometer
Generator	50 KV, 12.5 mA

Tube Target	Copper (Cu Kα = 1.5418 Å)
Optics	0.1° Detector slit M.R. Soller slit 3° Beam slit 0.0007" Ni Filter 4° Take off angle
Goniometer	Scan at 0.2° 2θ/minute
Detector	Amplifier gain-16 coarse 8.7 fine Sealed proportional counter tube and DC voltage at. plateau. Pulse height selection El 5 volts, Eu out. Rate meter T.C. 4, 2000 c/s full scale.
Recorder	Chart speed 1"/5 minutes
Samples	Prepared by grinding at room temperature.

Table IV

Procarbazine Hydrochloride

2θ	d (Å)*	I/I_o**
11.240	7.8719	0.24
12.400	7.1380	0.46
18.020	4.9225	0.48
18.640	4.7601	0.23
19.800	4.4838	0.17
20.370	4.3596	0.40
21.550	4.1235	0.61
22.550	3.9428	0.35
23.930	3.7185	0.30
25.190	3.5352	1.00
25.800	3.4530	0.10
28.800	3.2090	0.14
28.410	3.1415	0.04
29.550	3.0228	0.22
29.650	3.0128	0.23
33.740	2.6564	0.07

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

**I/I_o = relative intensity (highest intensity = 1.00)

2.12 Dissociation Constant

The dissociation constant for procarbazine hydrochloride was determined titrimetrically using 0.1N sodium hydroxide and a solution ionic strength of 0.1. The value for the pKa determined by this method was 6.8 (9).

3. Synthesis

Procarbazine hydrochloride may be prepared by the reaction scheme shown in Figure 5. 4-Formylbenzoic acid isopropylamide (I) is combined with methylhydrazine in dimethylformamide to prepare the corresponding methylhydrazone (II), which is then reduced, using palladium charcoal catalyst, to N-isopropyl-α-(2-methylhydrazino) -p-toluamide (III). Hydrogen chloride is added to the reaction mixture of III converting it to the hydrochloride of N-isopropyl-α-(2-methylhydrazino)-p-toluamide (IV).

4. Stability and Degradation

Procarbazine hydrochloride, in the presence of moisture or in aqueous solution, undergoes oxidation by atmospheric oxygen. This autoxidation has been reported to be catalyzed by metal ions such as Mn^{+2} and Cu^{+2} (10,11). The major products of this oxidation are N-isopropyl-α-(2-methylazo)-p-toluamide (II) and N-isopropyl-α-(2-methylhydrazono)-p-toluamide (III). To a much lesser extent, toluic acid isopropylamide (IV) and 4-formylbenzoic acid isopropylamide (V) can also be formed (12,13). The oxidative degradation scheme is shown in Figure 6. In addition, procarbazine hydrochloride is very sensitive to ultraviolet light (14), necessitating the use of low-actinic glassware during analyses. In closed, amber bottles at room temperature, degradation of procarbazine hydrochloride in the solid state is slow, and the compound remains suitably stable for at least three years (15). A nitrogen atmosphere has been found to retard degradation.

Utilizing spectrophotometric (16) and polarographic time studies, it was determined that procarbazine

FIGURE 5

Synthesis of Procarbazine Hydrochloride

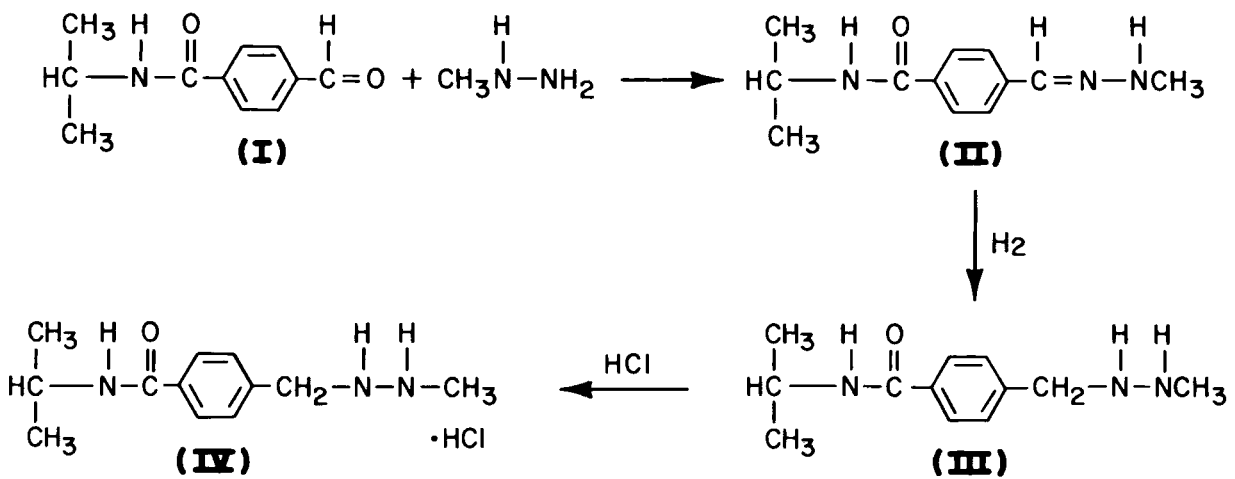
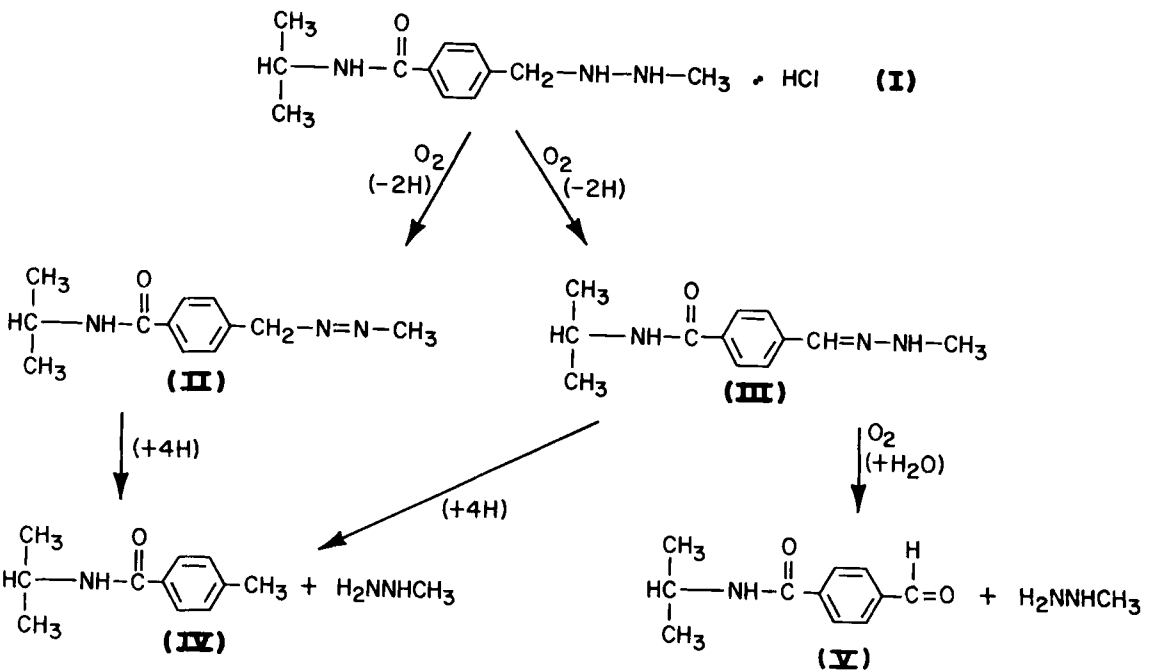


FIGURE 6

Degradation of Procarbazine Hydrochloride



hydrochloride degrades rapidly in alcoholic media (including acidified and deaerated alcohol) and more slowly in aqueous media. Stability was best in aqueous acid and decreased with increasing pH (13,16).

5. Drug Metabolic Products

The metabolism of the tumor-inhibiting procarbazine hydrochloride proceeds according to a similar pattern in man, dog and rat (17). The major metabolites of procarbazine hydrochloride are N-isopropyl-α-(2-methylazo)-p-toluamide (AZO), N-isopropyl terephthalamic acid (TAC), methylhydrazine and carbon dioxide (17-24). A large portion of the drug is excreted in urine as the inactive TAC, while both TAC and the active metabolite AZO have been detected in blood (17, 20, 21).

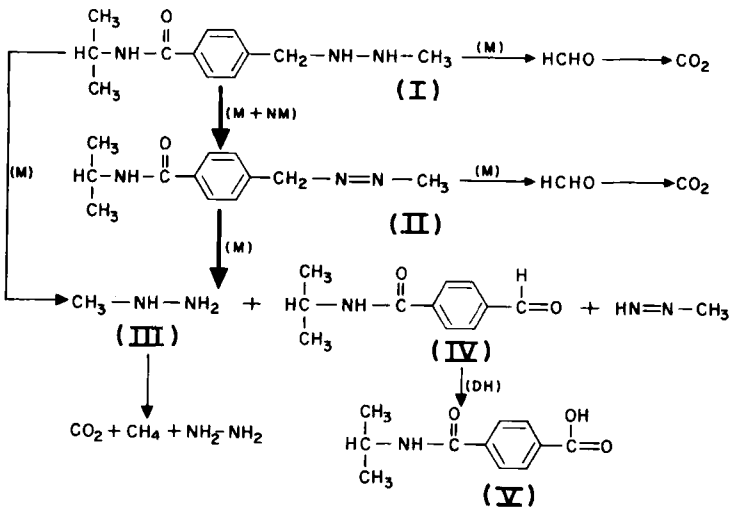
A photometric method for the quantitative determination of procarbazine hydrochloride in blood plasma, utilizing extraction with ethanol/chloroform and oxidation of the hydrazine group with ferricyanide, is described by Raaflaub (17, 25).

The major metabolic pathways consist of rapid oxidation (microsomal and non-microsomal) of procarbazine to AZO followed by N^2-C cleavage of AZO to 4-formylbenzoic acid isopropylamide and methylhydrazine. While several authors (17, 20, 26) suggest that cleavage occurs after AZO is isomerized to N-isopropyl-α-(2-methylhydrazono-p-toluamide (HYDRAZONE), Baggiolini, et al. (18) present data which suggests that the major pathway is direct cleavage of AZO. The metabolic scheme proposed by the latter is presented in Figure 7. The chemical names of the compounds in Figure 7 are listed in Table V.

Although the mode of cytotoxic action of procarbazine hydrochloride has not yet been clearly defined, there is evidence that the drug acts by inhibition of protein, RNA and DNA synthesis (27-29). The N-methyl group of procarbazine hydrochloride and its azo metabolite, possibly in the form of a methyl free radical (30), has been found to be essential for

FIGURE 7

Metabolic Products of Procarbazine Hydrochloride



(M) = Oxidation by microsomal hydroxylase

(NM) = Oxidation other than by (M)

(DH) = Oxidation by NAD-linked dehydrogenase

⟶ = Most important steps

biological activity as a tumor inhibitor (30-32). It has also been suggested that hydrogen peroxide, produced during procarbazine oxidation to the azo compound, may be responsible for the cytotoxic effect (33, 34).

Table V

Metabolites of Procarbazine Hydrochloride Referred to in Figure 7

I. N-isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride (procarbazine hydrochloride)
II. N-isopropyl-α-(2-methylazo)-p-toluamide
III. methylhydrazine
IV. 4-formylbenzoic acid isopropylamide
V. N-isopropyl terephthalamic acid

6. Toxicity

The major drug toxicities in acute and chronic animal studies were hematologic with granulocyte depression, thrombocyte depression and anemia. Reticuloendothelial system lymphocytic depletion, marrow cell depression, testicular atrophy and mucous membrane ulceration were further evidence of in vivo cytotoxity (35). Leukemia and pulmonary tumors in mice, and mammary adenocarcinomas in rats, have been observed subsequent to procarbazine hydrochloride administration in high doses. The oral LD_{50} in mice and rats was determined to be 1320 ± 66 mg/kg and 785 ± 34 mg/kg, respectively. In rabbits the oral LD_{50} was 147 ± 11.5 mg/kg (35).

7. Methods of Analysis

7.1 Elemental Analysis

A typical elemental analysis of a sample of procarbazine hydrochloride is presented in Table VI (36).

Table VI

Elemental Analysis of Procarbazine Hydrochloride

Element	% Theory	% Found
C	55.91	56.17
H	7.82	7.90
N	16.30	16.29
Cl	13.75	14.00

7.2 Thin-Layer Chromatographic Analysis

The following TLC procedure is useful for separating N-isopropyl-α-(2-methylazo)-p-toluamide and N-isopropyl-α-(2-methylhydrazono)-p-toluamide from procarbazine hydrochloride (37). The procedure is used to evaluate the stability of the dosage form. Using silica gel GF plates (about 300 μ thick) and ethyl acetate as the developing solvent, the equivalent of 1 mg of procarbazine hydrochloride in 2.5% (w/v) cysteine hydrochloride in methanol is spotted on the plate and subjected to ascending chromatography. After the solvent front has ascended about 15 cm, the plate is air dried and observed under shortwave ultraviolet radiation. The plate is then sprayed either with modified Ehrlich's reagent or with glacial acetic acid followed by 10% aqueous ferric chloride:5% aqueous potassium ferricyanide (1:1). The approximate R_f values are as follows:

Procarbazine hydrochloride	0.0
N-isopropyl-α-(2-methylhydrazono)-p-toluamide	0.6
N-isopropyl-α-(2-methylazo)-p-toluamide	0.7

7.3 Direct Spectrophotometric Analysis

The procarbazine hydrochloride content (as base equivalent) in capsules may be determined spectrophotometrically by using the following procedure. An amount of capsule fill is mixed and a portion of the powder equivalent to approxi-

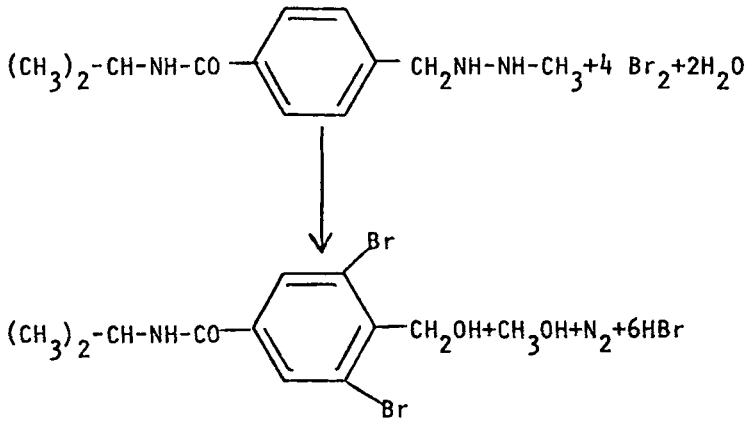
mately 25 mg of procarbazine base is weighed. The powder is mixed with 100 ml of 0.1N hydrochloric acid and the mixture is filtered through dry filter paper, discarding the first 15 ml of filtrate. A suitable aliquot of the remaining filtrate is diluted with 0.1N hydrochloric acid to give a solution containing about 12.5 µg/ml. The absorbance of the solution is measured at 232 ± 2 nm using 0.1N hydrochloric acid as reference. The amount of procarbazine base is calculated by comparison to the absorbance of a solution of procarbazine hydrochloride reference standard similarly prepared and measured (38).

7.4 Coulometric Analysis

Procarbazine hydrochloride can be assayed both as the drug substance and in the dosage form by coulometric titration. Using a platinum generating system and a polarized platinum indicating system, a constant current of 96.5 mA is applied to the sample in solution with 0.5M potassium iodide solution, buffered at pH 8.4 ± 0.1.

The titration results in the quantitative oxidation of the hydrazo moiety of procarbazine hydrochloride to the azo group, making the method quite specific. Each coulomb of electricity is equivalent to 1335 µg of procarbazine hydrochloride (39).

A coulometric titration with electrolytically generated bromine has been used to determine procarbazine hydrochloride in capsules (40). This method is not as specific as the above method of Oliveri-Vigh, et al., since electrochemically generated bromine not only oxidizes the hydrazine moiety but also substitutes into the phenyl ring as follows (39):



7.5 Polarographic Analysis

Polarographic analysis of procarbazine hydrochloride is utilized as both an identity and an assay method for the dosage form (5), an identity for the drug substance (41), and a stability testing procedure for the dosage form (42, 43) and drug substance. In pH 12 Britton-Robinson buffer, the halfwave potential for the oxidation of procarbazine hydrochloride occurs at about -0.16V versus a saturated calomel reference electrode and the diffusion current is proportional to concentration. The polarographic wave is attributed to the oxidation of the hydrazine moiety (-NH-NH-) to the azo moiety (-N=N-) and is, therefore, specific for the intact drug substance. A polarographic procedure utilizing an electrolyte of aqueous sodium acetate:absolute ethanol (2:1 v/v) has also been reported (13, 44).

7.6 Titrimetric Analysis

Procarbazine hydrochloride is assayed by dissolving the sample in water and titrating with 0.1N sodium hydroxide. The endpoint is determined potentiometrically, using a glass-calomel

electrode system. Each ml of 0.1N sodium hydroxide is equivalent to 25.78 mg of $C_{12}H_{19}N_3O \cdot HCl$ (5). Alternatively, the endpoint may be determined visually using phenolphthalein T.S. as indicator (41, 45). Titrimetric assay of procarbazine hydrochloride has also been reported using titrants of aqueous silver nitrate solution and perchloric acid in glacial acetic acid (13, 46). Bromide-bromate, potassium ferricyanide, and nitrite titrations have been attempted with limited success (13). Titration with 0.1N iodine led to unfavorable stoichiometry, caused by localization of excess titrant during the analysis (13, 39).

8. Acknowledgements

The author wishes to acknowledge the assistance of the Research Records Office and the Scientific Literature Department of Hoffmann-La Roche Inc.

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PROMETHAZINE HYDROCHLORIDE

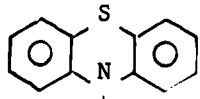
Charles M. Shearer and Susan M. Miller

CONTENTS

1. Description

1.1 Name, Formula, Molecular Weight

Promethazine hydrochloride is 10H-phenothiazine-10-ethanamine, N,N,α-trimethyl-, monohydrochloride, or 10-[2-(dimethylamino)propyl] phenothiazine, monohydrochloride (1). Additional names are 10-(2-dimethylamino-2-methylethyl) phenothiazine hydrochloride and N-(2'-dimethylamino-2'-methyl)ethylphenothiazine hydrochloride. The most commonly used trade name is Phenergan. The empirical formula is $C_{17}H_{20}N_2S \cdot HCl$ with a molecular weight of 320.88.



$CH_2\,CH(CH_3)N(CH_3)_2 \cdot HCl$

The CAS Registry number is 60-87-7 for 10H-phenothiazine-10-ethanamine, N,N,α-trimethyl and 58-33-3 for the hydrochloride salt of this compound.

1.2 Appearance, Color, Odor

White to faint yellow, practically odorless, crystalline powder. Slowly oxidizes, and acquires a blue color, on prolonged exposure to air (1).

2. Physical Properties

2.1 Infrared Spectrum

An infrared spectrum of a mineral oil dispersion of promethazine hydrochloride (USP Reference Standard Material) was obtained on a Perkin Elmer Model 467 grating infrared spectrophotometer (3). This spectrum is shown in Figure 1. The spectral band assignments are listed in Table I. This spectrum compares favorably with that presented by Sunshine and Gerber (4) in which the promethazine (presumably the hydrochloride salt) is dispersed in a potassium bromide pellet. A mineral oil dispersion is the preferable technique for this material because the possibility exists for salt interchange between the bromide and chloride if potassium bromide is used.

2.2 Ultraviolet Spectra

The ultraviolet spectra of USP Reference Standard promethazine hydrochloride in water is presented (7) as Figure 2. Table II gives ultraviolet spectral data obtained on the USP Reference Standard in various solvents. The Merck Index (2) describes the ratio of absorbance by $10(A_{249}/A_{298}) = 8.0\text{-}8.8$.

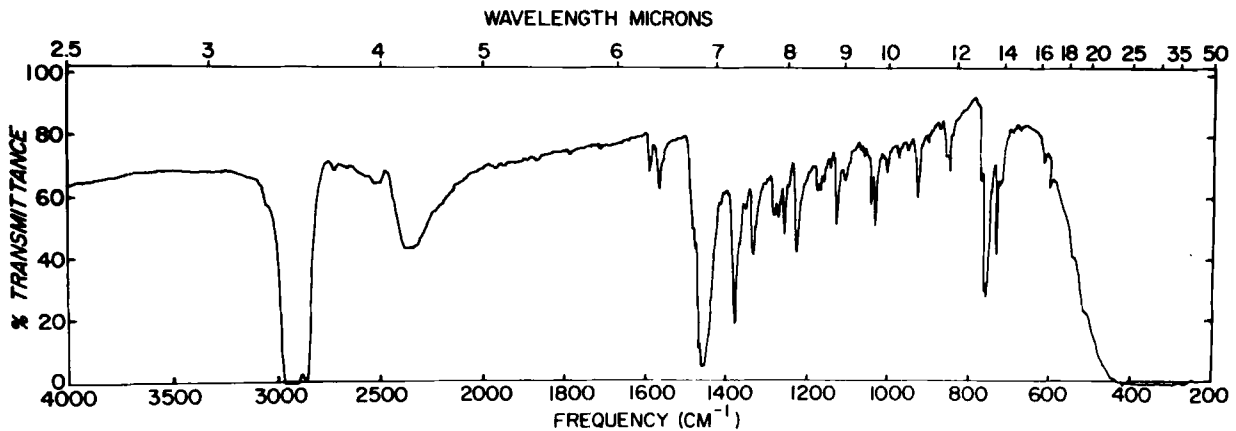


Figure 1 - Infrared Spectrum of Promethazine Hydrochloride (USP Reference Standard), Mineral Oil Dispersion

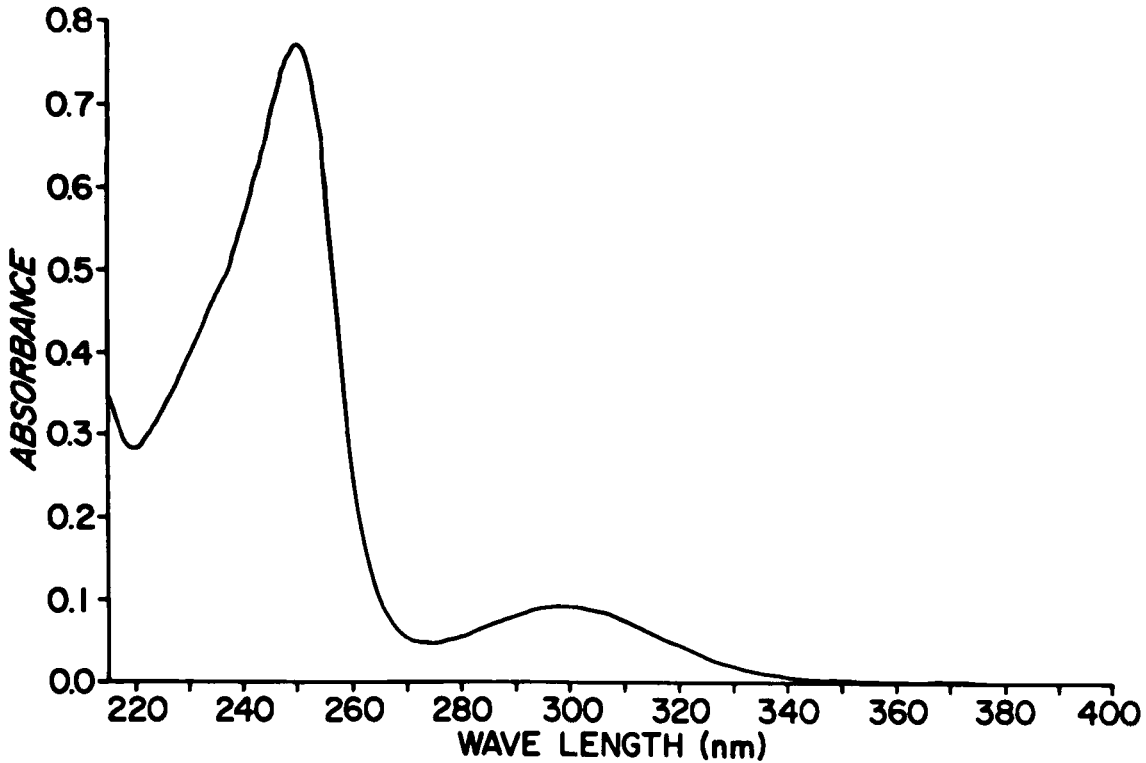


Figure 2 - Ultraviolet Spectrum of Promethazine Hydrochloride (USP Reference Standard)
Solvent - water

Table I
Infrared Spectral Assignments for Promethazine Hydrochloride (5, 6)

Wave Number (cm^{-1})	Vibration Mode
2800-3000	CH stretching (Nujol)
2200-2480	NH^+ stretching
1591	aromatic C=C stretching
1430-1470	CH_3 and CH_2 bending
1378	CH_3 bending
1334	C-N stretching of tertiary amine
850-860, 757 and 731	out of plane CH bending of disubstituted aromatic

Table II
Ultraviolet Spectral Characteristics for Promethazine Hydrochloride (7)

Solvent	max (nm)	a	ϵ
water	249	89.9	28,770
	297	10.6	3,400
0.1 N HCl	249	89.4	28,690
	297	10.6	3,400
USP alcohol	253	95.5	30,640
	302	11.4	3,660

2.3 Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum of promethazine hydrochloride dissolved in deuterochloroform containing tetramethylsilane as the internal standard is presented (8) as Figure 3. The spectral peak assignments are listed in Table III.

Table III
NMR Spectral Assignments of Promethazine Hydrochloride

Chemical Shift (δ)	Protons	Splitting
1.50	$CH\text{-}\underline{CH_3}$	doublet
2.85	$N\text{-}\underline{(CH_3)_2}$	singlet
3.5-4.3	$N\text{-}\underline{CH_2}\text{-}CH$	multiplet
4.6-5.0	$N\text{-}\underline{CH_2}\text{-}\underline{CH}$	multiplet
6.8-7.5	aromatic	---
11.5-12.5	NH^+	broad

2.4 Mass Spectra

The mass spectrum of promethazine hydrochloride (USP Reference Standard Material) was obtained with a MS-902 double focusing, high resolution mass spectrometer (9). The

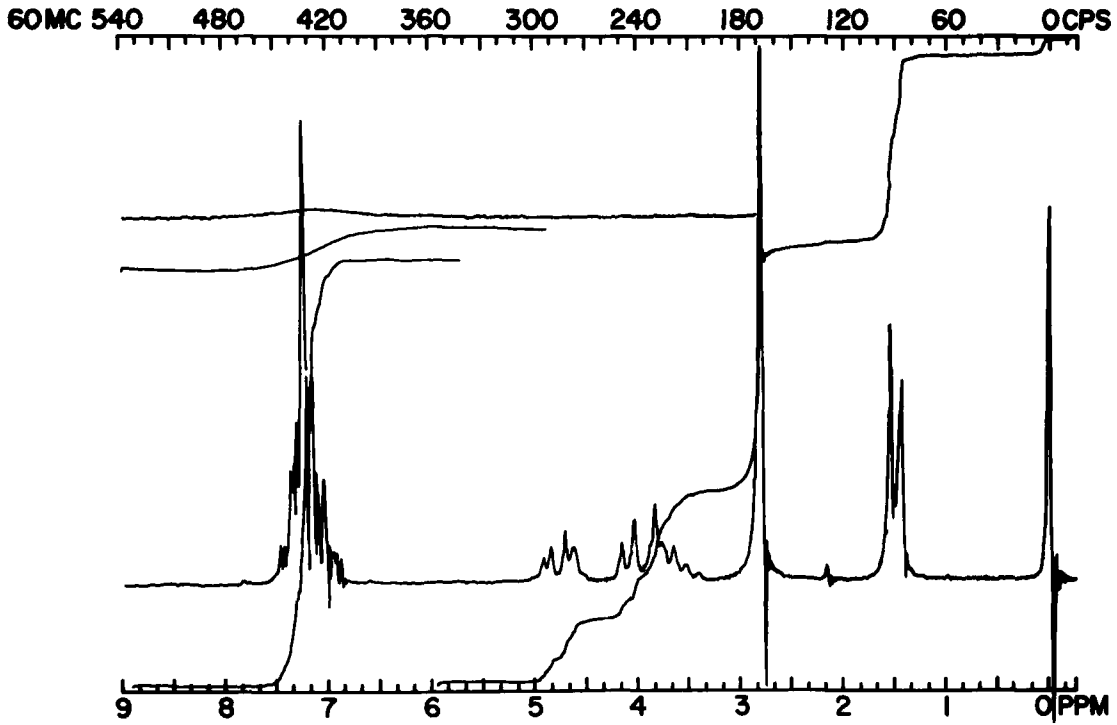


Figure 3 - Nuclear Magnetic Resonance Spectrum of Promethazine Hydrochloride (USP Reference Standard) Solvent - deuterochloroform

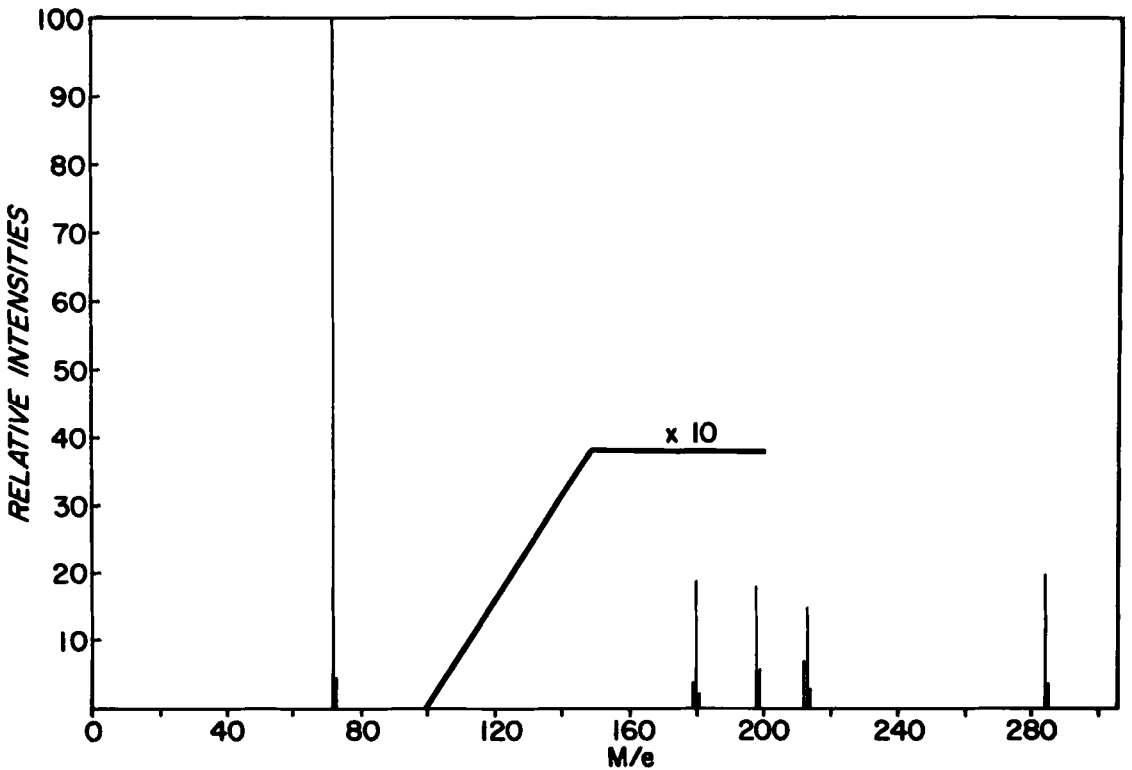


Figure 4 - Mass Spectrum of Promethazine Hydrochloride (USP Reference Standard)

probe temperature was 150°C and the ionization electron beam energy was at 70 eV. High resolution data were compiled and tabulated with the aid of the PDP-8 Digital computer. These data showed the molecular ion (formula $C_{17}H_{20}N_2S$) to have a measured mass of 284.1359 compared to a calculated mass of 284.1347. Figure 4 is a bar graph of the mass spectrum, with the molecular ion at m/e 284. The base peak at m/e 72 ($C_4H_{10}N$) arises from the side chain by cleavage of the C-C bond which is β to both the side chain nitrogen and the heterocyclic nitrogen. Loss of this fragment creates the peak at m/e 212, and a proton transfer before cleavage generates m/e 213. Loss of the complete side chain results in m/e 198, and elimination of sulfur from the 212 fragment gives the other predominant peak at m/e 180. This spectrum and route of fragmentation is in agreement with that presented by Gilbert (10) and Audier (11) and Fales (12). A chemical ionization spectrum showed the parent peak at M + 1, that is, m/e 285 (9).

2.5 Melting Range

The observed melting range (13) for the USP Reference Standard promethazine hydrochloride is 219.5°C-220.5°C with decomposition using the USP Class Ia procedure. The melting range is increased with increased heating rates. The Merck Index (2) reports a value of 230°C with decomposition.

2.6 Differential Scanning Calorimetry (DSC)

The DSC thermogram (14) of promethazine hydrochloride (USP Reference Standard) is shown as Figure 5. The thermogram was obtained at a heating rate of 10°C/minute in a nitrogen atmosphere utilizing a Perkin-Elmer DSC-2. The thermogram exhibits no endotherms or exotherms other than that associated with the decomposition melt.

2.7 Solubility

The following solubility data were obtained at uncontrolled room temperature (7).

Solvent	Solubility mg/ml
ethyl acetate	1
absolute ethanol	85
isopropanol	9
USP ethanol	150
methanol	320
chloroform	335
water	500

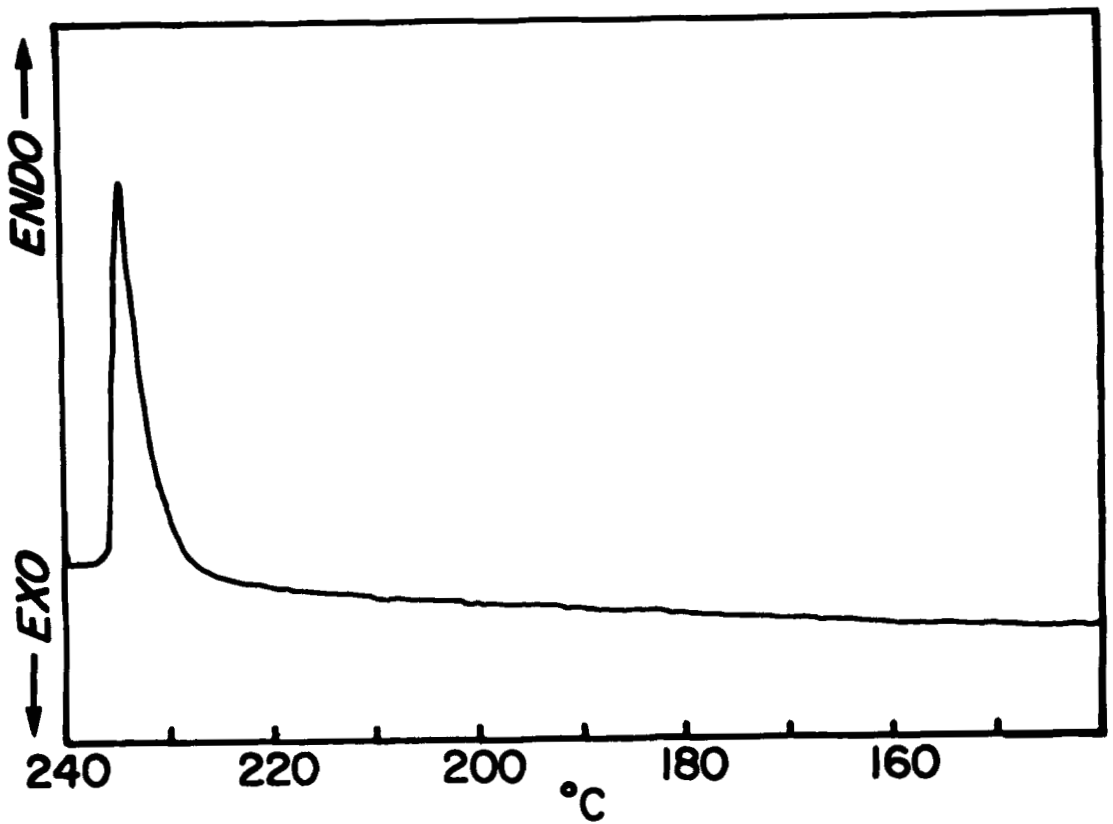


Figure 5 - Differential Scanning Calorimetry Curve of Promethazine Hydrochloride (USP Reference Standard)

2.8 Crystal Properties

2.81 X-Ray Diffraction

The X-ray powder diffraction pattern of promethazine hydrochloride (USP Reference Standard), obtained with a Phillips diffractometer using CuKα radiation is presented (14) in Figure 6. The calculated "d" spacings (Table IV) are in good agreement with those obtained by Rajeswaran and Kirk (15).

Table IV

X-Ray Diffraction Pattern of Promethazine Hydrochloride

d(A°)	I/Io	d(A°)	I/Io
7.85	0.28	3.40	0.12
7.11	0.59	3.36	0.10
6.93	0.32	3.25	0.62
6.61	0.63	3.18	0.30
5.72	0.47	3.10	0.38
5.60	0.54	3.06	0.20
5.23	0.61	3.03	0.08
4.89	0.94	2.96	0.06
4.39	1.00	2.85	0.12
4.23	0.25	2.80	0.12
3.99	0.17	2.73	0.12
3.86	0.52	2.57	0.15
3.80	0.16	2.51	0.16
3.65	0.47	2.35	0.07
3.53	0.46	2.27	0.09
3.45	0.10		

2.82 Optical Crystal Properties

Promethazine hydrochloride has been described as colorless rods and irregular fragments. In parallel polarized light the extinction is parallel and the sign of the elongation is positive. The refractive indices are: $\alpha = 1.617$, $\beta = 1.691$, $\gamma = 1.733$ all $\pm$ 0.002 (16). Escobar (17) describes the crystalline structure of promethazine hydrochloride. The crystalline parameters are given. The space group is P2(1)-C with four molecules per unit cell. Projections of the structure along the OZ and OY axis are illustrated.

2.9 Micelle Formation

Florence and Parfitt (18, 19) have determined the critical micelle concentration of promethazine hydrochloride by the use of nuclear magnetic resonance and by pH measurements. Ionic strength effects are also discussed. Stevens (20) discusses the

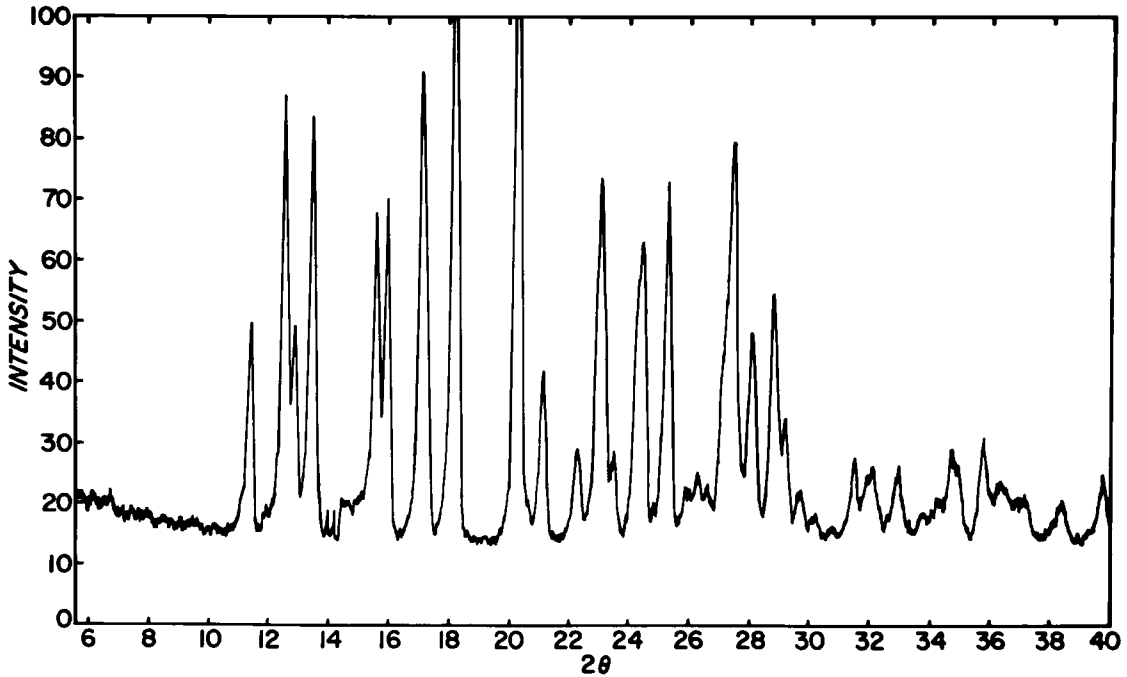


Figure 6 - X-Ray Diffraction Pattern for Promethazine Hydrochloride (USP Reference Standard)

effects of pH and temperature upon the critical micelle concentration. Zografi (21) and Vilallonga (22) discuss the surface activity of promethazine hydrochloride at the air-solution interface.

2.10 Ionization Constant

The ionization constant for promethazine hydrochloride has been reported as 9.1 using the solubility method in water (23) and as 9.1 using a potentiometric titration with varying amounts of methanol in water as the solvent and extrapolating the values obtained to zero percent methanol (24).

2.11 Metal Complex and Charge Transfer Complex Formation

Promethazine hydrochloride has been shown to complex with Fe(III), Co(III) and Mn(III) to produce rose-red colored solutions (25). The ultraviolet and visible spectra of the cobalt complex has been published (26). The complex with Pd(II) has been used as a method of analysis (27). The cobaltous thiocyanate complex with promethazine has been isolated and characterized (28).

In the presence of promethazine the polarographic wave corresponding to the reduction of oxygen disappears, and a new wave appears at a more negative potential. The latter apparently represents the reduction of the phenothiazine oxygen complex (29).

Charge transfer complexes of bromine and iodine with promethazine which form in acetonitrile have been detected by the use of conductometric titrations (30). Infrared spectra of the iodine complex showed a new band at 1700-1650 cm^{-1} (30). Association constants of promethazine with 1,4 dinitrobenzene in carbon tetrachloride or chloroform were measured by nuclear magnetic resonance (31). Charge transfer spectral studies of promethazine in chloroform, acetonitrile and ethanol-acetone, with bromanil, chloranil, p-benzoquinone, m-dinitrobenzene and tetracyanoethylene have been reported (32,33). These electron acceptors have also been used as spray reagents for detecting promethazine on thin-layer chromatography plates (34).

2.12 Adsorption and Protein Binding Properties

Promethazine hydrochloride is adsorbed from aqueous solution onto talc, kaolin, and activated charcoal (35,36). It is bound by carrageenan, furcellaren, carboxymethyl cellulose, sodium alginate, pectin, gum tragacanth, gum arabic, locust bean gum, gum agar and quince seed mucilage (37). It can also bond to surface active agents such as polysorbate 80 (38) and sodium polyethylenesulfonate (39). The bonding of

promethazine hydrochloride to bovine serum albumin has been studied (40, 41).

2.13 Optical Activity

Promethazine as normally synthesized is a racemic mixture. The optical isomers have been separated by use of dibenzoyl-D-tartaric acid (42). Both the (+) and (-) forms obtained in this manner decompose at 220-221°C. The optical activity $[\alpha]_{D}^{20}$ of (+) promethazine is +7.6°: that of the (-) form is -7.6°. The toxicity, antihistaminic activity and central nervous action of the separated optical isomers and the racemic promethazine are the same (42).

2.14 Dipole Moment

Barbe (43) reports the dipole moment for promethazine base at 25°C as $\mu(D) = 2.05 \pm 0.01$. A second paper by the same author (44) discusses the conformation of the side chain with respect to the ring portion of the molecule.

2.15 Partition Coefficients

Burger (45) has studied the distribution of promethazine hydrochloride between 0.5 N HCl and various organic phases. The results he obtained are given in Table V. Doyle (46) discusses the effect of solvent composition on the partition of amines in general.

Table V

Partition Coefficients of Promethazine Hydrochloride between 0.5 N HCl and Various Organic Phases (45)

$$K = \frac{\text{conc. in 0.5 N HCl}}{\text{conc. in organic phase}}$$

organic phase	K
butanol	0.07
chloroform	0.15
amyl acetate	1.14
ethyl acetate	2.70
benzene	5.25
ethyl ether	15.20
hexane	18.20

3. Synthesis

The most direct synthesis of promethazine involves the reacting of phenothiazine with 2-chloro-1-dimethylaminopropane in the presence of various bases such as sodium hydroxide (47, 48, 49), sodamide (47, 50, 51), potassium hydroxide (48), phenyl lithium (52) and calcium carbonate (53). This synthetic route is illustrated in Figure 7. The salt can then be prepared by treating the promethazine base with hydrogen chloride.

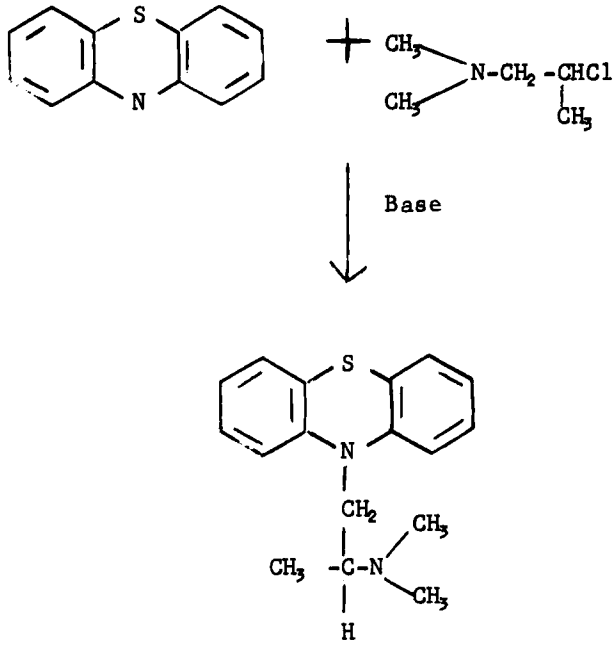


Figure 7

Synthesis of Promethazine

A second synthesis is via the Grignard reaction (54, 55). Phenothiazine is refluxed with methyl iodide and magnesium. To this is then added 2-chloro-1-dimethylaminopropane to give promethazine.

1-Phenothiazine-2-hydroxypropane p-toluenesulfonate when heated with dimethylamine gave promethazine (52).

Promethazine can also be synthesized by first reacting 2-bromo-2'-amino-diphenylsulfide with 1-dimethylamino-2-chloropropane in xylene solution with sodamide present, to give 2-bromo-2'-(2"-dimethylaminopropyl)-amino-diphenyl-sulfide. To bring about cyclization, this product is heated with potassium carbonate and copper powder in dimethylformamide (56).

4. Stability and Degradation

Promethazine is decomposed primarily by oxidation and/or photolysis. Oxygen has been shown by polarography to form a complex with promethazine (57, 29).

Promethazine hydrochloride when refluxed in an aqueous solution produced 10-methyl phenothiazine, acetaldehyde and dimethylamine (58). It was concluded that the cleavage of the promethazine was probably due to a free radical mechanism and oxygen in some way caused an activation of the molecule. The same products were formed when a promethazine hydrochloride (pH 5) solution was placed in an ampule, sealed under nitrogen and irradiated with a fluorescent light (59). However, Yamamoto found no degradation in a similar solution of promethazine under nitrogen when exposed to a light intensity of 5000 luxes at 35° (60). Aqueous solutions of promethazine hydrochloride stored at room temperature in diffused daylight for 2 days to six months decomposed to promethazine sulfoxide, 9, 9 dioxopromethazine, N-demethyl-promethazine and several more unidentified compounds (61, 62). Phenothiazine was identified as a major degradation product when a solution of promethazine hydrochloride was exposed to sunlight (63).

A kinetic study was reported by Stevens (64). The pH was controlled with Sorenson citrate buffer containing 0.1% EDTA and adjusted to constant ionic strength. The sample flask was held at constant temperature in the dark and the solution was bubbled with O_2 at a flow rate of 10 ml/min. The first order rate constants are given for the pH range 1.2 to 5.0. Within this range promethazine is most unstable

at a pH around 4.3. At pH's between 5.5 and 7 the degradation no longer followed first order kinetics.

The stability of promethazine hydrochloride in solution is improved by the addition of ascorbic acid (65, 66, 67, 68), cysteine (65), sodium metabisulfite (69) or rongolite (68, 69). In several of these publications the effect of pH upon the stability of the material was discussed.

5. Metabolism and Pharmokinetics

Hansson and Schmiterlow (70) investigated the excretion and metabolism of S^{35} labeled promethazine in rats. Maximum excretion takes place during the first 24 hours. The drug was readily metabolized, primarily to the sulfoxide. Two other unidentified metabolites were also found. The sulfoxide was found as the only metabolite in the brain and liver. Rusiecki and Wysocka-Paruszewska (71) in another study on rats found similar excretion patterns - most intensive during the first 72 hours after administration and lasting no more than 5 days. Six or seven, depending upon the dose, metabolites were found in the urine. These metabolites were not identified chemically, but characterized by R_f on thin layer chromatography. In the organs (kidney, spleen, lungs and stomach) 24 hours after the drug was administered, only traces of promethazine and its metabolites were found. These metabolites were the same as found in the urine. In the blood 24 hours after administration, no unchanged promethazine and only traces of two metabolites were found. Metabolites of promethazine in rats were characterized as being produced by sulfoxylation, aromatic hydroxylation and N-demethylation. In human volunteers promethazine was found to be excreted primarily as the glucuronide conjugate (72). Robinson (73, 74) has shown that hydroxylation (two different products), dealkylation and demethylation of promethazine occur in rat liver homogenate.

6. Identification

Several color reactions for the identification of promethazine are given in Table VI. Kirk (75) describes the preparation of many of the lesser known reagents. Bradford (76) presents a systematic procedure for the isolation of promethazine from biological material and dosage forms. The identification of the isolated material is by ultraviolet spectrophotometry. DeLeenheer (77) describes a system for combining preparative gas-liquid chromatography with micro-infrared spectroscopy for the identification of drugs. Infrared spectroscopy can be used directly on the raw material for

Table VI

Identification Color Tests for Promethazine Hydrochloride

Reagent	Response	Reference
Palladium chloride	red-purple precipitate	142
Ferric chloride	red color	142
Conc. H_2SO_4	fuschia color	14, 143
Conc. HNO_3	magenta to yellow-orange	14, 143
Mandelin Reagent	pink color	14, 143
Marquis Reagent	magenta color	14, 143
Buckingham's Reagent	brilliant red to magenta	14
Frahde's Reagent	pink color	14, 75
Chloroplatinic acid	flat purple crystals	14
Bromine water 1%	red fading to colorless	14, 143
Phosphomolybdate acid	violet precipitate	143
10% $K_4Fe(CN)_6$	yellow precipitate	143
10% $K_3Fe(CN)_6$	yellow precipitate	143
H_2SO_4-HNO_3 (1:1)	pink turning yellow	143
Mecke's Reagent	purple color	75
Reickard's Reagent	pink color	75
Fluekkiger's Reagent	red color	75
Vitalis Reagent	yellow-pink color	75
Schneider's Reagent	brownish-green color	75
0.3 M Periodic acid in 1 N H_2SO_4	red color	144
Ammonium cerric nitrate in 5% nitric acid	brownish-red color	145
Conc. H_2SO_4, then 0.1% $NaNO_3$	red color	146
Ehrlich's Reagent	orange color	147
10% Chloramine plus $CHCl_3$	violet color	147
Fuming HNO_3	red color	143, 147
Vanillin with H_2SO_4	pink, red color	147

its identification (1). Edge and Wragg (78) discuss the identification of promethazine and isopromethazine. Several identification tests are described by Clarke (79). He also gives a microchemical test to distinguish promethazine hydrochloride from promethazine 8-chlorotheophyllinate.

Several authors describe microcrystalline identification tests for promethazine. Andres (80) gives a description of the crystals and also photomicrographs of the products of promethazine reacted with platinum bromide, ammonium reineckate and gold chloride. Promethazine from tablets was identified by these tests. An amorphous precipitate was obtained with picric acid. Bogs (81) obtained a crystalline product (mp 159-160°C) by treating promethazine hydrochloride with sodium 4,4'-dichlorodiphenyldisulfimide.

7. Methods of Analysis

7.1 Elemental Analysis

The elemental analysis of promethazine hydrochloride USP Reference Standard is presented below.

Element	% Calculated	% Reported (8)
C	63.59	63.61
H	6.59	6.59
N	8.73	8.68
Cl	11.05	10.76
S	9.99	9.90

7.2 Phase Solubility Analysis

Phase solubility analysis was carried out using acetone as the solvent (13). Experience in this laboratory has shown that 24 hours with vibration at 31.2°C is sufficient for equilibration to be attained. A purity of 99.7 $\pm$ 0.5% was obtained.

7.3 Direct Spectrophotometric Analysis

Promethazine may be assayed by ultraviolet spectrophotometry at 249 nm in water or at 256 nm in ether (82). Ultraviolet spectrophotometric methods are used (1) for the analysis of promethazine hydrochloride syrups, injections and tablets after separation from the inert ingredients by a biphase extraction or a partition column. Pellerin (83) describes a method which involves first, forming ion pairs of promethazine with lauryl sulfate or dioctylsulfosuccinate, second, extracting these from an aqueous into a non-miscible organic solvent and third, determining the concentration of promethazine by ultraviolet spectrophotometry. Separation prior to spectrophotometric determination by partition column chromatography (63, 84), ion exchange chromatography (85, 86) and reverse phase partition column

chromatography (87) have been reported. Salvesen (88) describes a quantitative infrared spectrophotometric determination of promethazine.

7.4 Colorimetric Analysis

Cavatora (89) describes methods for determining promethazine in the presence of promazine and chlorpromazine by reacting it with mercuric sulfate to form a colored species.

Promethazine hydrochloride can be determined by forming a colored product with sodium 1,2 naphthoquinone-4-sulfonate in 50% sulfuric acid (90). Many common inert ingredients in pharmaceutical preparations do not interfere with the assay.

Promethazine hydrochloride can be oxidized by $FeNH_4(SO_4)_2$ and nitric acid to form a product which can be estimated spectrophotometrically (91).

Ryan (27) describes a colorimetric assay for promethazine by forming a colored complex of it with palladium chloride. The method is stability indicating with respect to the oxidation of promethazine to the sulfoxide. A similar method is discussed by Cavorta (89). Mercaldo and Gallo (92) have automated this method of analysis using Technicon AutoAnalyzer equipment.

Hetzel (93) describes a colorimetric assay for compounds having a phenothiazine moiety by forming a yellow colored nitration product. The method was used for assays of phenothiazine drugs in biological samples.

Oxidation of promethazine hydrochloride by heating it with ammonium persulfate gave a colored product with absorption maxima at 520 nm (94). The method was applied to physiological saline and to syrups by extracting the promethazine base from a basic solution into ether and then extracting the hydrochloride salt into 0.1 N HCl.

Sodium chlorite reacts with promethazine hydrochloride to produce a rose-violet color which can be measured at 533 nm (95).

The reaction between promethazine hydrochloride and p-benzoquinone can be applied to the determination of promethazine. The method was used for biological samples (96).

Beer's law is followed in the range 2.0-3.2 mg/50 ml for promethazine hydrochloride reacted with 2-nitro-1,3-indandione in acetic acid, when measured at 540 nm (97).

The reaction between antimony pentachloride and promethazine in dichloroethane can be used for the quantitative determination of the drug in solutions, or in biological media (98).

Eriochrome Black T with promethazine forms salts which can be extracted into chloroform and measured at 510-520 nm (99).

Promethazine hydrochloride can be determined photometrically after extraction as ion-pairs with methyl orange (100). An automated application of the method is presented.

Promethazine hydrochloride has been analyzed by the formation of the photooxidation products which are free radicals characterized by their strong absorption in the visible region. An automated system for the generation and determination of the free radicals is described (101).

Promethazine can be quantitatively precipitated as the reineckate salt and redissolved in acetone and determined spectrophotometrically (102). Promethazine can be determined by precipitating it with molybdophosphoric acid (103) or tungstophosphoric acid (104) and then redissolving it in dimethylformamide-methanol solution and measuring it spectrophotometrically.

7.5 Electrochemical Analysis

Kabaskalian and McGlotten (105) studied the polarographic oxidation of promethazine and other phenothiazine tranquilizers. A gold micro-electrode was used. The effects of pH, concentrations and temperature on the position and magnitude of the anodic wave were investigated.

Meckle and Discher (106) found that controlled potential electrolysis is suitable for the coulometric determination of promethazine. In 14 N H_2SO_4 promethazine is quantitatively oxidized to the free radical at + 0.7V vs SCE and to the sulfoxide at + 0.98V. Controlled potential electrolysis at + 0.70V was suitable for the analysis of the raw material.

Hynie (107) and Dusinsky (108) investigated the use of oscillographic polarography for analysis of promethazine.

Promethazine can be nitrated with conc. nitric acid and the resulting product determined by polarography (109).

7.6 Titrimetric Analysis

Promethazine hydrochloride can be titrated by the Pifer-Wollish method using crystal violet as the endpoint indicator (1). Mainville and Chatten (110) describe a similar titration using acetonitrile as the solvent, 0.1 N perchloric acid in dioxane as the titrant and endpoint determination by either the color change of crystal violet or by potentiometry using a glass-calomel electrode combination. This procedure was satisfactory for the drug substance but not for promethazine hydrochloride tablets. Kerney (111) used acetone as a solvent in a Pifer-Wollish type titration.

Promethazine hydrochloride can be determined by a two phase (chloroform and acid) titration using sodium lauryl sulfate as the titrant and methyl yellow as the indicator (112, 113, 114). This method has been applied to promethazine hydrochloride tablets (115). Sodium dioctylsulfosuccinate has also been used as the titrant (116).

Oxidimetric titrations of promethazine hydrochloride with cerric sulfate and with potassium bromate (potassium bromide added) have been studied (117, 118, 119, 120). The endpoint can be determined by potentiometry, visually or by the dead stop method. Similar methods using electrogenerated cerric ion were described by Patriarche (121, 122). Lead tetraacetate has been used as the oxidative titrant for promethazine hydrochloride with endpoint determination employing platinum foil as indicator and a calomel electrode as the reference (123).

Promethazine hydrochloride can be titrated with silicotungstic acid determining the endpoint either conductometrically (124) or by the color change of congo red (125). The method has been applied to drug dosage forms.

Promethazine base after extraction from a sodium bicarbonate solution into chloroform has been titrated with an arylsulfonic acid in dioxane using dimethyl yellow as the endpoint indicator (126).

The chloride portion of promethazine hydrochloride has been determined by the use of a chloride ion selective electrode (127). Promethazine hydrochloride has been titrated with 0.1 N sodium hydroxide for the proton of the acidic portion of the salt by dissolving it in dimethylformamide and

using thymol blue for endpoint determination (128).

Danek (129) precipitated promethazine reineckate, redissolved it in acetone-water and determined the amount of the reineckate present by titrating it with silver nitrate. Promethazine can be quantitatively precipitated with mercuric chloride and the excess mercury determined by a complexometric titration (130). Dilute solutions of promethazine hydrochloride in water, when treated with a known equal volume of 0.15% reineckate salt solution gives a precipitate which is removed by filtering and the excess reineckate determined by potassium bromate titrations (131).

Promethazine hydrochloride in dosage forms was determined by using an anion exchange resin and titration of the eluted promethazine free base (132, 133).

7.7 Gravimetric Analysis

Gravimetric analysis of promethazine hydrochloride can be carried out by precipitating it as the styphnate (134), reineckate (135), molybdophosphone complex (103), or silicotungstate (136). A different type of gravimetric determination of promethazine involves the oxidation and subsequent bromination of it. The resulting compound was extracted from base with chloroform, the organic layer evaporated and the residue weighed (137).

7.8 Fluorometric Analysis

Promethazine hydrochloride can be oxidized in 50% glacial acetic acid with hydrogen peroxide and heat to give a stable product which can be quantitatively determined by its fluorescence (138). This procedure can be used to determine promethazine in blood samples (139). Another fluorometric procedure utilizes the fluorescent product produced by diluting into dimethylsulfoxide a solution of promethazine in sulfuric acid. The procedure has also been used for analysis of promethazine hydrochloride in biological samples.

7.9 Microbiological Analysis

Solution of greater than 0.5 mg/ml promethazine hydrochloride can be assayed by a microbiological method using β. anthracin (141).

7.10 Separation Methods of Analysis

7.101 Paper Chromatography

Promethazine has been chromatographed on

Table VII

Paper Chromatography of Promethazine

Eluent	Paper	R_f	Reference
Butyl alcohol:amyl alcohol:acetic acid: water, 25:75:12:15½	S & S 2045b	0.66	148
Butanol:water:citric acid, 50:50:1	Whatman No. 1	0.7	149
Acetone:1 M sodium acetate:1 M acetic acid, 10:20:5	Whatman No. 1	0.83	150
Butanol:acetic acid:water, 4:1:2	S & S 2043	0.85	151
Butanol:acetic acid:water, 4:1:5	Whatman No. 1		70
n-Butanol:acetic acid:water, 60:15:25	Munktell OB		152
Phosphate buffer (pH 6.5)	CMC paper CM-82, ion exchange	0.31	153
0.1 M NaCl:methanol, 5:1	CM-82	0.40	153
0.1 M NaCl:formamide, 5:1	CM-82	0.51	153
Dichloroethane:acetic acid:water, 20:8:2	S & S 2043 or 2045	0.69	154
5% aqueous $(NH_4)_2SO_4$:isobutanol, 1:1	Whatman No. 1	0.62	155

Table IX

TLC Systems Using Silica Gel as Adsorbent

Eluent	R_f	Reference
Ethanol:water:acetic acid; 20:20:1	0.41	159
0.3% I_2 in chloroform	0.29	164
Cyclohexane:acetone; 50:50	0.15	165
Cyclohexane:diethylamine; 9:1	0.62	166
Methanol:ammonia; 100:1.5	0.61	166
Chloroform:methanol; 50:50	0.44	167
Benzene:ethanol:ammonia; 95:15:1	0.41	168
Methanol:acetone; 12:88	0.26	169
Water:ethanol; 4:96	0.21	169
Isopropanol:isopropyl ether; 16:84	0.16	169
Methanol:methyl acetate:cyclohexane; 18:49:33	0.47	169
Ethyl acetate:isopropanol:ammonia; 70:25:5	0.73	170
Ethyl acetate:dichloroethane:ammonia; 80:20:5	0.56	170
Benzene:dioxane:ammonia; 10:80:10	0.77	171
Acetone:methanol:ammonia; 50:50:1	0.53	172
95% Ethanol[1]	0.23	173, 174
Methanol[1]	0.45	173, 174
Cyclohexane:benzene:methanol; 75:15:10[2]	0.46	173, 174
Methanol[2]	0.47	173, 174
Acetone[2]	0.37	173, 174

[1]Support prepared with 0.1 M $NaHSO_4$ or 0.1 M $KHSO_4$

[2]Support prepared with 0.1 N KOH or 0.1 N NaOH

paper under various conditions. Solvent systems and other pertinent data are given in Table VII. Methods of detection are given in Table VIII.

Several of these systems (148, 152, 154) have been used in analyzing urine and other biological materials for metabolic products of promethazine.

Table VIII
Spray Reagents for Detection of Promethazine on Paper Chromatography

Reagent	Color	Reference
Dragendorff's Reagent	orange	148
sulfuric acid-ethanol	red	148
sulfuric acid	red	156
nitric acid	rose	156
palladium chloride	orange	156
ferric chloride	rose	156
cerric sulfate	rose	156
Ehrlich's reagent	rose	156

7.102 Thin Layer Chromatography

The various eluent systems for thin layer chromatography of promethazine using Silica Gel as adsorbent are given in Table IX. Systems using other adsorbants are included in Table X. Table XI lists spray reagents and other detection methods successfully used in analyzing promethazine. Direct conversion of promethazine to the sulfoxide on the plate, and subsequent analysis by ultraviolet spectroscopy has also been described (157, 158). Separation of phenothiazine was achieved using a pH gradient from 7.0 to 8.5 on Silica Gel layers (159), and on alumina (160).

Table X
TLC Systems Using Adsorbents Other Than Silica

Eluent	Adsorbent	R_f	Reference
	alumina		
benzene:ethanol; 98:2		.34	161
benzene:ethanol; 95:5		.66	161
benzene:ethanol; 90:10		.73	161
chloroform:ethanol; 98:2		.74	161
chloroform:ethanol; 95:5		.80	161
chloroform:n-propanol:25% ammonia; 98:1:1		.88	161
acetone:chloroform; 15:85		.70	161
ether:petroleum ether; 1:1		.55	161
cyclohexane:ethanol; 85:15		.67	161

Table X
TLC Systems Using Adsorbents Other Than Silica

Eluent	Adsorbent	R_f	Reference
	cellulose		
isobutyl alcohol saturated with 5% $(NH_4)_2SO_4$		.54	162
chloroform:acetone:25% ammonia; 50:50:1		.96	163
chloroform:ethyl acetate:25% ammonia; 50:50:1		.90	163

7.103 Gas Chromatography

Gas chromatography has been used to analyze promethazine and to separate it from other phenothiazines. The column conditions and necessary data for the various methods are given in Table XII. The various phenothiazines have been characterized by gas chromatography of their pyrolysis products (180).

Gas chromatography of promethazine has been used in combination with micro-infrared spectroscopy (181).

Table XI
Detection Methods Used in TLC of Promethazine

Reagent	Color	Reference
Aniline vapors, then bromine vapors	brown changing to green	175
Iodoplatinate spray	violet	176
Iodine vapors	brown	176, 162
1.0% w/v selenious acid in conc. H_2SO_4	red-purple	168
Concentrated H_2SO_4	fuschia	170
Folin's reagent	red-violet	170
Ceric sulfate in sulfuric acid	red	170
Marquis reagent	yellow-red	177
Palladium chloride	red	172, 178
Dragendorff's reagent	orange	176, 178
Dimethylaminobenzaldehyde	rose	176
Furfural in sulfuric acid	rose	176
$KMnO_4$	yellow	176
Sulfuric acid:ethanol; 1:9	rose	179
Perchloric acid	red	179
Phosphomolybdic acid	rose	179

7.104 Column Chromatography

Promethazine can be separated from other dosage form components and drugs by partition chromatrography (63, 84), ion exchange chromatography (85, 86, 132, 133) and reverse phase chromatography (87).

Table XII

Gas Chromatography of Promethazine Hydrochloride

Column	Temperature	Retention Time	Reference
6 ft x 3 mm i.d.; 0.07% SE-30 on 120-170 mesh glass beads	175°	7.2 min.	182
6 ft x 3 mm i.d., 0.08 PDEAS on 120-170 mesh glass beads	175°	21.7 min.	182
6 ft x 3 mm i.d., 1.1% XF-1150 on 100-120 mesh Chromosorb W-HMDS	175°	27.9 min.	182
6 ft x 3 mm i.d.; 1.08% CW-20M on 100-120 mesh Gas Chrom P	175°	17.1 min.	182
4 ft x 4 mm i.d.; 5% Apiezon L on 100-110 mesh Anachrom ABS	240°		183
1.8 meter x 4 mm i.d.; 3.8% SE-30 on 80-100 mesh Diatoport S	220°		184
6 ft x 5 mm i.d.; 2% SE-30 on 80-100 mesh Gas Chrom S	205°		185

Pound and Sears (186) discuss the use of high pressure liquid chromatography for the analysis of promethazine hydrochloride.

7.105 Electrophoresis

Promethazine was separated from other phenothiazines by paper electrophoresis (Whatman No. 2 paper) using a glycol-HCl electrolyte (pH 3.34). Visualization was by Dragendorff, iodoplatinate, palladium chloride or cerric sulfate spray (187).

7.106 Counter Current Partition

Promethazine was separated from promazine and chlorpromazine using counter-current partition between chloroform and hydrochloric acid (188).

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RIFAMPIN

Gian G. Gallo and Pietro Radaelli

TABLE OF CONTENTS

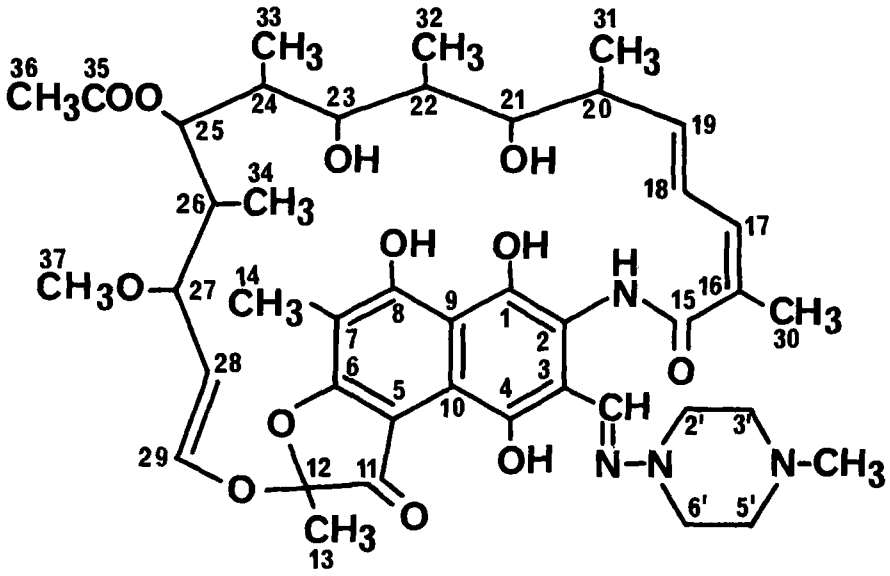
1. DESCRIPTION

1.1 NAME

Rifampin (1) is designated by IUPAC rules as 2,7-(Epoxypentadeca[1,11,13]trienimino)naphtho[2,1-b]furan-1,11(2H)-dione,5,6,9,17,19,21-hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[N-(4-methyl-1-piperazinyl)formimidoyl]-21-acetate. However, in the literature, rifampin has been preferentially known as 3-[[(4-methyl-1-piperazinyl)imino]methyl]rifamycin SV, according to the original nomenclature of rifamycins (2-4).

Rifampin (5,6) is the USAN name for the compound, rifampicin is the international nonproprietary name (7) and other trivial names used are rifamycin AMP and rifaldazine (8,9).

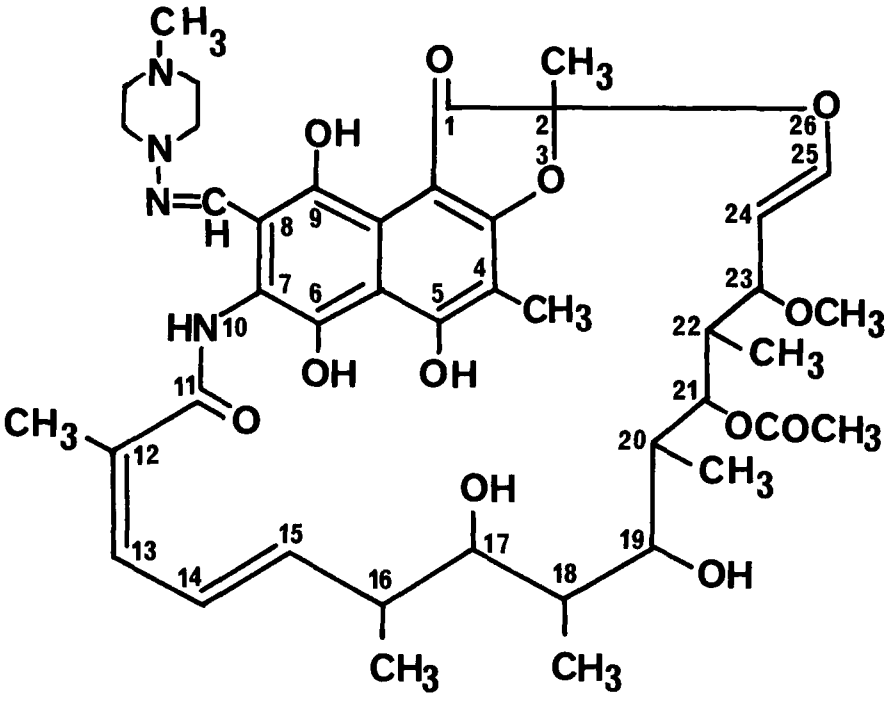
1.2 FORMULA AND MOLECULAR WEIGHT



$C_{43}H_{58}N_4O_{12}$ Mol.Wt.= 822.95

This numbering system, according to the special nomenclature of rifamycins, is adopted in the present profile.

The numbering system according to IUPAC rules is:



The molecule of rifampin is usually described as consisting of a naphthohydroquinone chromophoric part spanned by an aliphatic chain called the ansa with a piperazine side chain attached.

1.3 POTENTIAL STEREOISOMERISM

Rifampin, a semi-synthetic antibiotic (8), even though it contains 9 asymmetric carbon atoms and 3 double bonds, is found only as one isomer because all the isomerogenic centers belong to the natural part of the molecule and only one isomer is specifically produced by the microorganism (4,10).

1.4 APPEARANCE, COLOR AND ODOR

Rifampin is a red-orange, odorless, crystalline powder.

2. PHYSICAL PROPERTIES

2.1 SPECTRA

2.11 Ultraviolet Spectra

UV-VIS spectrophotometry has been used for structural determination of various rifamycins to obtain specific information on the chromophoric part of the molecule. In particular, the VIS maximum, which undergoes a hypochromic effect and a small hypsochromic shift with strong acids, is characteristic of the naphthohydroquinone form carrying the acidic ionizable function (11-13), and the auxochromic effect on the same VIS maximum depends on the nature of the substituents in position 3 (14-17).

The UV spectrum of rifampin, recorded on a Perkin Elmer model 4000-A spectrophotometer in aqueous phosphate buffer pH 7.38 (8), exhibits the absorption maxima given in Table I.

Table I
Ultraviolet absorption of rifampin

λ_{max}, nm	ε
237	33,200
255	32,100
334	27,000
475	15,400

The variation of the UV-VIS spectrum of rifampin with pH (figure 1) indicates the presence of an ionizable function, attributed to the acidic 8-hydroxyl group in peri position to the hydroquinonic hydroxyl (13,18,19).

2.12 Infrared Spectra

The infrared spectra of various rifamycins usually have been reported in the literature but only

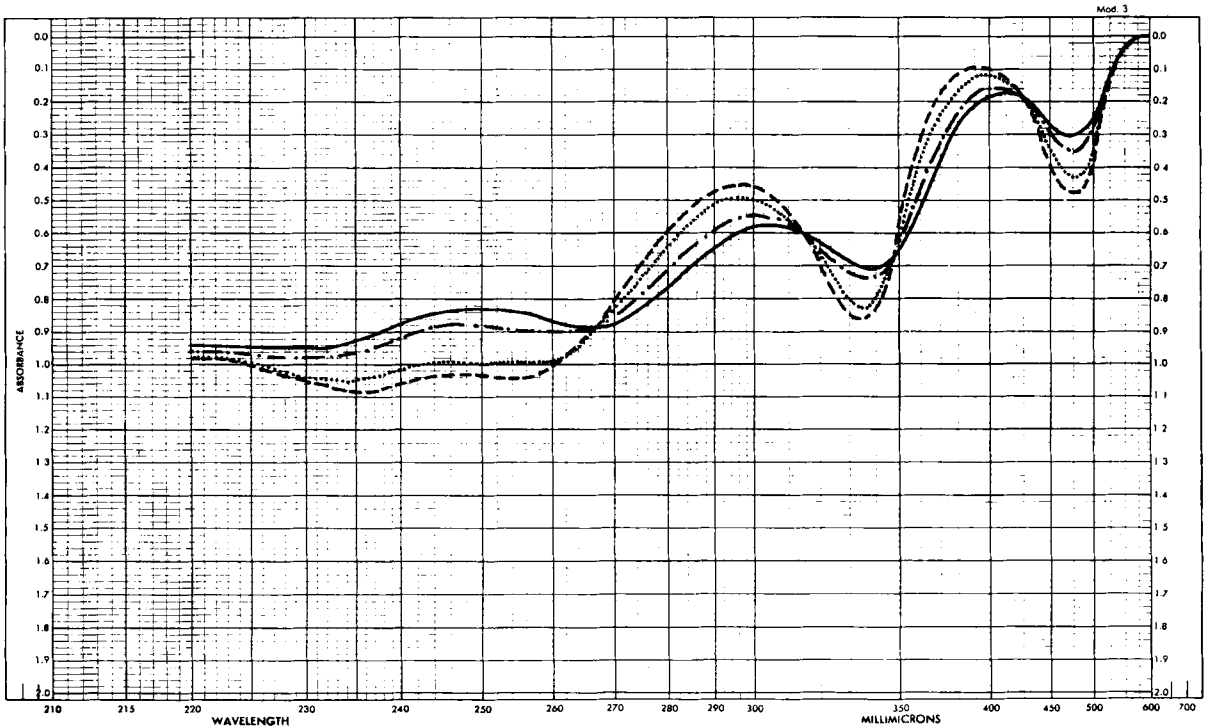


Figure 1 - UV spectra of rifampin in methanol-water (2:3) solution at different pH's

———— pH 0.5; -.-.-.-.-.-.- pH 1.8;

..... pH 2.5; ---------- 3.5-11.0

occasionally have been discussed (19,20). Exhaustive spectral assignments have only recently been made (21).

The infrared spectrum of rifampin in chloroform solution was reported by Maggi et al. (8). Figures 2 and 3 are the spectra of rifampin recorded as mineral oil mull and in deuterochloroform solution, respectively, with a Perkin-Elmer mod.421 spectrophotometer. Interpretation of the spectrum of rifampin (21) is given in Table II. In $CDCl_3$ solution, rifampin does not exist as the zwitterion, while in solid state it seems to.

Table II
Infrared spectra of rifampin

IR absorption band, cm^{-1}		Interpretation
Mineral oil mull	$CDCl_3$ solution	
3500-3300	3480	νOH-bonded
*	2970, 2930 and 2880	νCH_3
*	2820	νCH_3O
*	2800	νCH_3-N
3200-2300	3300-2300	νNH and νNH-bonded and νOH-bonded
1715	1715	νCO acetyl
1735	1640	νCO furanone (in solution intra H-bonded)
1670 and 1610	1620	amide I
1570	1570	νC=C
1540 and 1520	1540 and 1520	amide II
1255,1050 and 1020	1255,1040 and 1020	νC-O-C acetyl

* Non-transparent region of the mineral oil

2.13 Nuclear Magnetic Resonance Spectra

2.131 Proton Magnetic Resonance Spectra

^{1}H-NMR spectroscopy has been widely used as a fundamental tool for structural determination of various rifamycins (18,19,22-24).

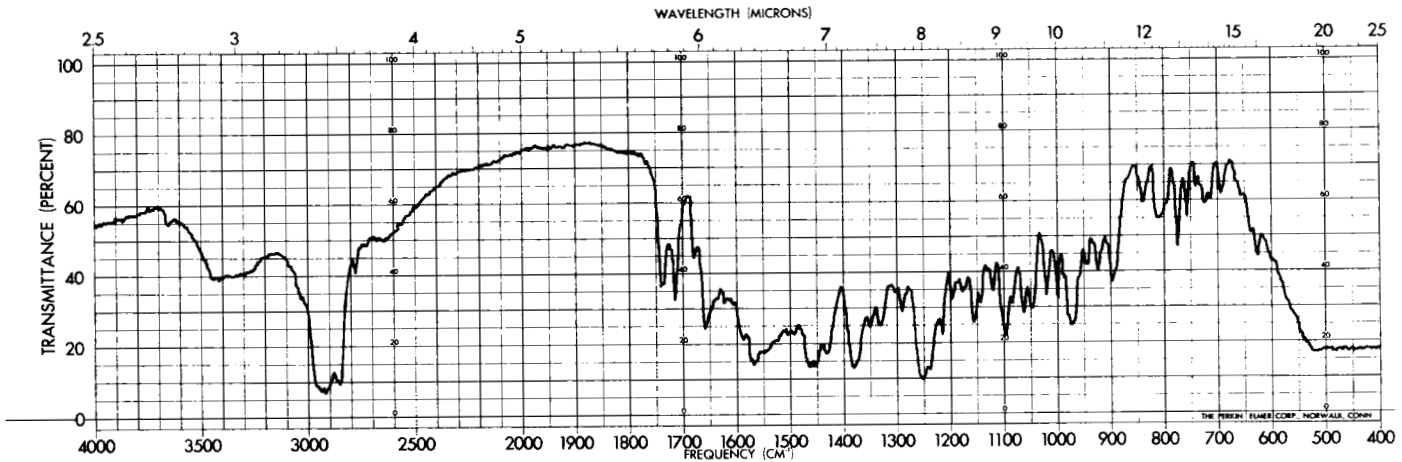


Figure 2 - IR spectrum of rifampin in mineral oil mull.

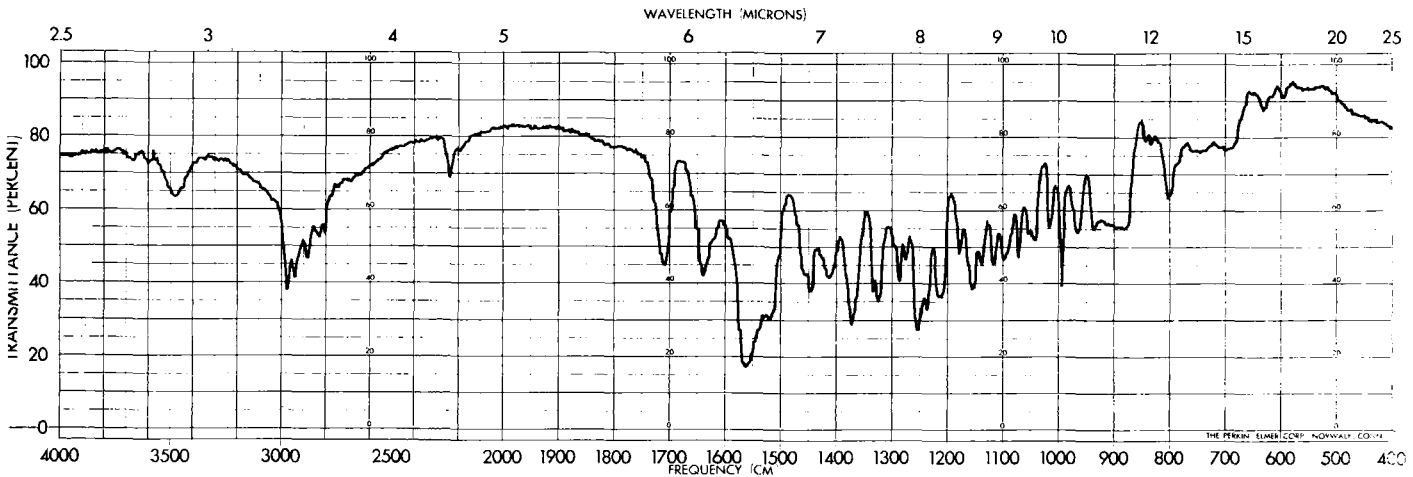


Figure 3 - IR spectrum of rifampin in $CDCl_3$ solution.

The ^{1}H-NMR spectrum of rifampin in $CDCl_3$ at 60 MHz has been reported and some assignments given (8). Figure 4 is the spectrum of rifampin in $CDCl_3$ recorded at 100 MHz with a Varian XL-100-15 NMR spectrometer. Complete interpretation of the spectrum has been made (25) and is given in Table III.

2.132 Carbon Magnetic Resonance Spectra

^{13}C-NMR spectroscopy has been recently used for structural determination in the field of rifamycins (24,26-28).

Figure 5 is the FT ^{13}C proton-noise-decoupled NMR spectrum of rifampin in $CDCl_3$ recorded at 25.2 MHz with a Varian XL-100-15 NMR spectrometer, equipped with an FT accessory. Interpretation of the spectrum has been made (25) and is given in Table IV.

2.14 Mass Spectra

The mass spectra of some rifamycins have been studied and fragmentation patterns have been interpreted (29). In particular, the most characteristic peaks correspond to $M^{\cdot+}$, $[M-CH_3OH]^{\cdot+}$ and to the chromophoric ions.

Figure 6 is the spectrum of rifampin obtained under 70eV electron impact in DIS at 200°C with a Perkin Elmer 270 spectrometer. Interpretation of this spectrum has been published (30). The spectrum was only partially characteristic as the compound decomposes in the ion source and $M^{\cdot+}$ and $[M-CH_3OH]^{\cdot+}$ were absent, while the peak at m/e 398 corresponded to the chromophoric ion.

The mass spectrum of rifampin was also obtained under field desorption ionization conditions at wire current 12 mA with a Varian MAT 731 spectrometer (31). It exhibited the molecular ion at m/e 822 and the [M+1] peak corresponded to the isotopic contribution. No fragmentation peaks were observed.

2.2 IONIZATION CONSTANTS

The ionization properties of rifamycins have been used in conjunction with UV-VIS spectrophotometry (see Section 2.11) to obtain information on the chromophoric

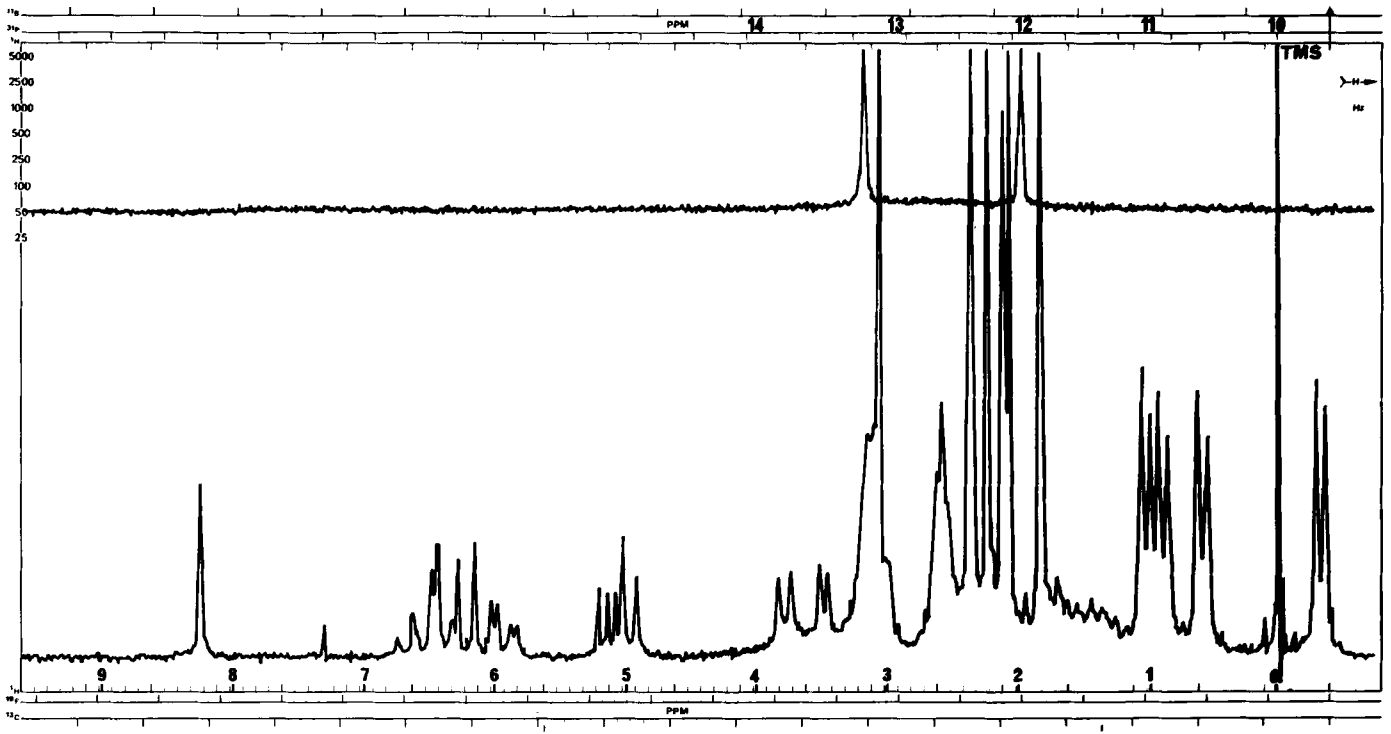


Figure 4 - ^{1}H-NMR spectrum at 100 MHz of rifampin in $CDCl_3$ solution.

Table III

^{1}H-NMR data of rifampin in $CDCl_3$ solution [Multiplicity, chemical shifts (δ, ppm) and vicinal interproton coupling constants (J,Hz)].

Proton	Multipl.[a]	δ	J
NH	s	11.96[b]	-
CH=N	s	8.22	-
CH_2-2',6'	m	2.9-3.3	c)
CH_2-3',5'	m	2.4-2.8	c)
N-CH_3	s	2.34	-
OH-1, OH-8	bs	11.4-14.0[b]	-
OH-4	s	13.16[b]	-
CH_3-13	s	1.82	-
CH_3-14	s	2.22	-
H-17, H-18	m	6.3-6.8	c)
H-19	dd	5.92	15 and 5
H-20	ddq	2.26	5 and 9 and 7
H-21	dd	3.78	9 and 1
OH-21, OH-23	bs	3.2-4.2[b]	-
H-22	ddq	1.70	1 and 1.5 and 7
H-23	dd	3.04	1.5 and 10
H-24	ddq	1.52	10 and 1 and 7
H-25	dd	4.96	1 and 10
H-26	ddq	1.22	10 and 1.5 and 7
H-27	dd	3.58	1.5 and 7
H-28	dd	5.00	7 and 13
H-29	d	6.20	13
CH_3-30	s	2.10	-
CH_3-31	d	0.88	7
CH_3-32	d	1.01	7
CH_3-33	d	0.58	7
CH_3-34	d	-0.33	7
CH_3-36	s	2.06	-
CH_3-37	s	3.05	-

a) s=singlet; d=doublet; dd=doublet of doublets; ddq=doublet of doublets of quartets; m=multiplet; bs=broad signal;
b) it exchanges with D_2O
c) not determined

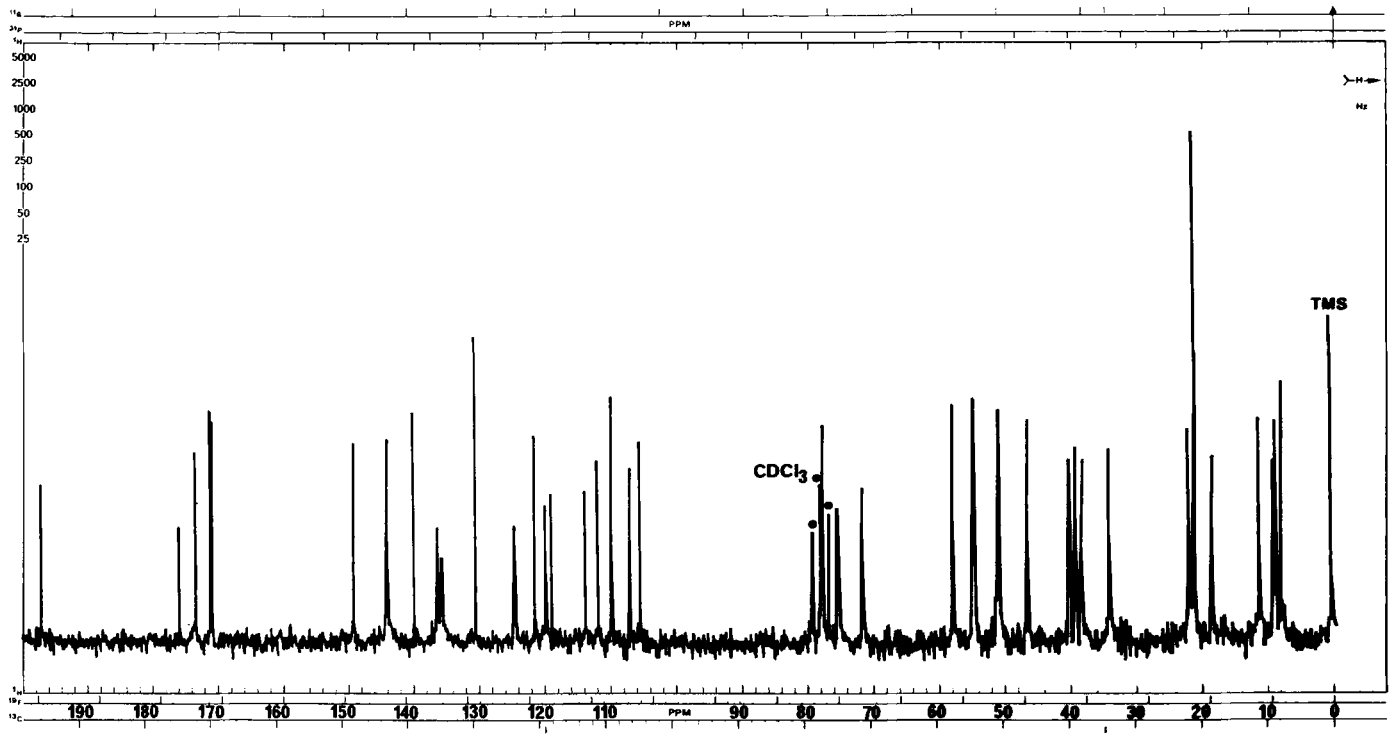


Figure 5 - FT ^{13}C proton-noise-decoupled NMR spectrum at 25.2 MHz of rifampin in $CDCl_3$ solution.

Table IV

^{13}C-NMR data of rifampin in $CDCl_3$ solution. [Chemical shifts (δ, ppm) and one-bond coupling constants (^1J, Hz)].

carbon	δ	^1J
1	138.6	-
2	105.9[a]	-
3	110.8[a]	-
4	147.8	-
5	112.8[a]	-
6	174.3	-
7	120.3[a]	-
8	169.3	-
9	104.4[a]	-
10	117.8[a]	-
11	195.3	-
12	108.7	-
13	21.5	130
14	7.6	130
15	169.6	-
16	129.4	-
17	135.0	150
18	123.2	150
19	142.6	150
20	38.6	125
21	70.7	140
22	33.4	125
23	76.7	140
24	37.6	125
25	74.4	140
26	39.5	125
27	76.7	140
28	118.7	155
29	142.6	190
30	20.7	130
31	17.8	130
32	10.9	130
33	8.5	130
34	8.8	130
35	171.9	-
36	20.7	130
37	57.0	140
CH=N-	134.4	170
C-2'-	50.2	130
C-3'-	53.9	130
C-5'-	53.9	130
C-6'-	50.2	130
CH_3-N-	45.8	130

a) These assignments may be interchanged

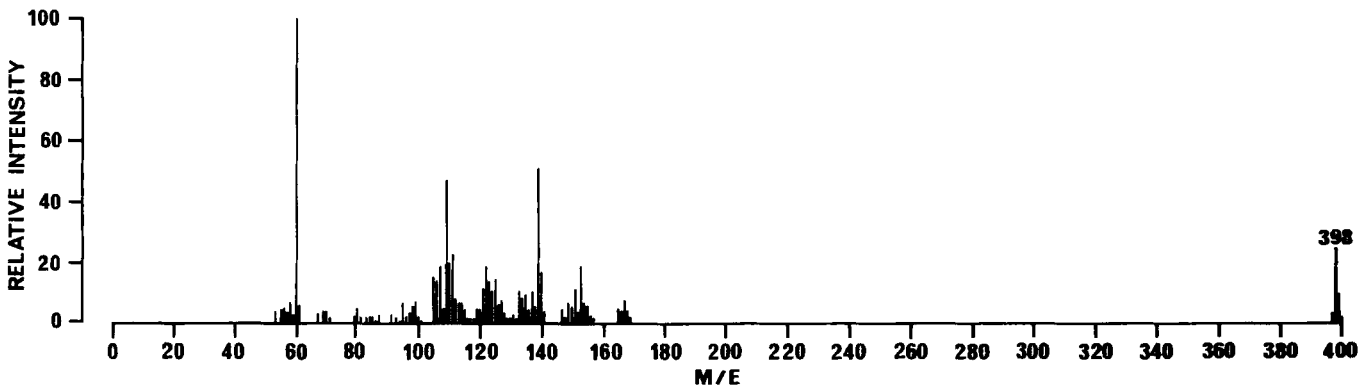


Figure 6 - MS spectrum of rifampin at 70 eV in DIS at 200°C.

part of the molecule and as a quantitative method of analysis (11-13, 18).

The pK values for rifampin have been determined spectrophotometrically (see fig.1) and potentiometrically in solution in water and in methylcellosolve-water (4:1) and are reported in Table V. Rifampin exists in water solution as the zwitterion with isoelectric poin equal to 4.8 (8,13).

Table V
Ionization constants of rifampin

	pKa	pK_{MCS}	Attribution	Reference
proton lost	1.7	3.6	hydroxyl at C-8	13,18,19
proton gained	7.9	6.7	piperazine N-4	13

Rifampin ionizes in non-aqueous solvents, i.e., in glacial acetic acid the basic piperazine nitrogen can be titrated with perchloric acid (32).

2.3 POLAROGRAPHY

The polarographic behaviour of rifamycins has been described and the presence of an oxidation or reduction wave at about 0 volts vs. SCE indicates the hydroquinone or quinone (14,33-35) system, respectively. These polarographic properties permit amperometric titration of rifamycins (36).

Rifampin in methanol-acetate buffer solution, pH 5.9, shows an oxidation wave with $E^{1}/2$ = +0.10 volt vs. SCE, attributed to the hydroquinone system (8), and in aqueous phosphate buffer, pH 6.88, there is also a reduction wave with $E^{1}/2$ = -1.66 volts vs. SCE, not attributed (37). Sano et al.(38) reported an oxidation potential of $E^{1}/2$ = +0.06 volt vs. SCE, without other details.

2.4 OPTICAL ROTATION

The optical rotation for rifampin was reported by Sano et al. (38): $[\alpha]_D^{25^\circ}$ = +10.6° (C=0.5% in $CDCl_3$).

2.5 CRYSTAL PROPERTIES

2.51 X-ray Diffraction

Single-crystal X-ray diffraction was used to deduce the structure of the p-iodoanilides of rifamycin B and rifamycin Y (10,39-42). The single-crystal X-ray diffraction method was applied to rifampin crystallized with 5 water molecules in the orthorombic system (43).

The X-ray powder diffraction pattern of rifampin, is reported in Figure 7 (44) and Table VI gives the values according to the ASTM rules. The grinding causes the crystallinity of rifampin to disappear and an amorphous form to originate, as shown in Figure 8.

2.52 Thermal Analysis

2.521 Melting Range

Rifampin melts with decomposition at 183-188°C (open capillary glass tube).

2.522 Differential Scanning Calorimetry

Untill now, DSC has not been applied to the field of rifamycins.

The heating curve of rifampin has been obtained on a Du Pont Differential Thermal Analyzer mod.990 with a temperature rise of 10°C/min and is reported in fig.9 (45). It shows an endotherm at 193°C corresponding to the melting, immediately followed by an exotherm corresponding to the recrystallization of the melt, which then decomposes exothermically at about 240°C.

2.6 DISTRIBUTION PROPERTIES

2.61 Solubility

The approximate solubilities of rifampin in various solvents have been determined at room temperature (32). The results are reported in Table VII, according to the definition used by the US Pharmacopeia XIX . Rifampin is soluble in acidic and alkaline water (8).

Quantitative solubility data for rifampin were reported, as shown in Table VIII.

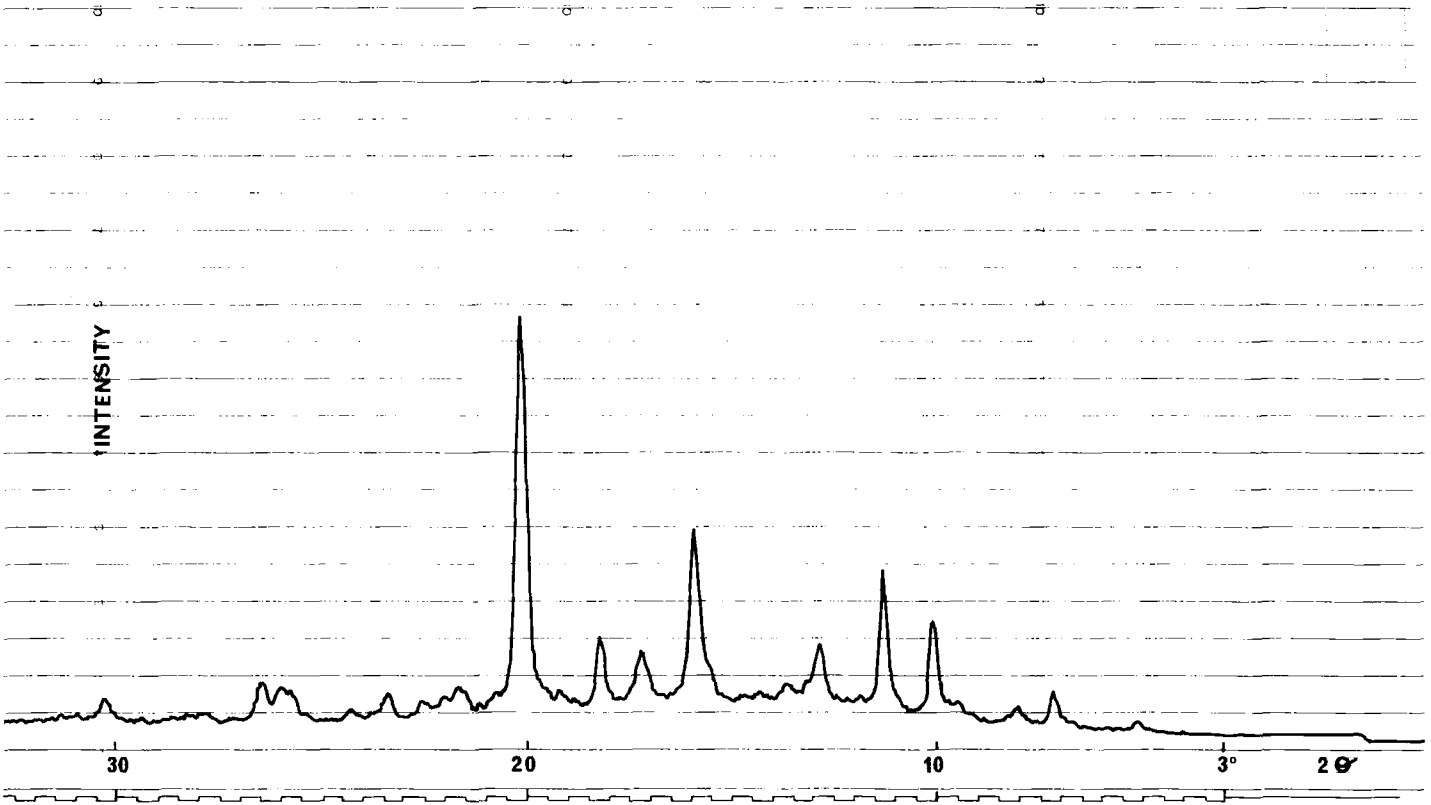


Figure 7 - X-ray powder diffraction pattern of rifampin before grinding.

Table VI

X-ray powder diffraction pattern of rifampin before grinding, according to ASTM rules

d(Å)	4.43	5.60	7.86	17.16
I/I_1	100	44	35	2

Rad. Cukα $\bar{\lambda}$ = 1.5418 Å
Filter Ni

I/I_1 Spectrometer

Rifampin (sample before grinding)					
d(Å)	I/I_1	hkl	d(Å)	I/I_1	hkl
17.16	2		5.18	13	
12.43	8		4.90	17	
11.11	3		4.63	2	
10.76	1		4.43	100	
9.40	1		4.12	5	
8.80	23		4.05	2	
7.86	35		3.95	3	
7.51	2		3.82	6	
6.91	14		3.67	3	
6.51	4		3.44	8	
6.21	2		3.38	8	
5.60	44		2.96	5	

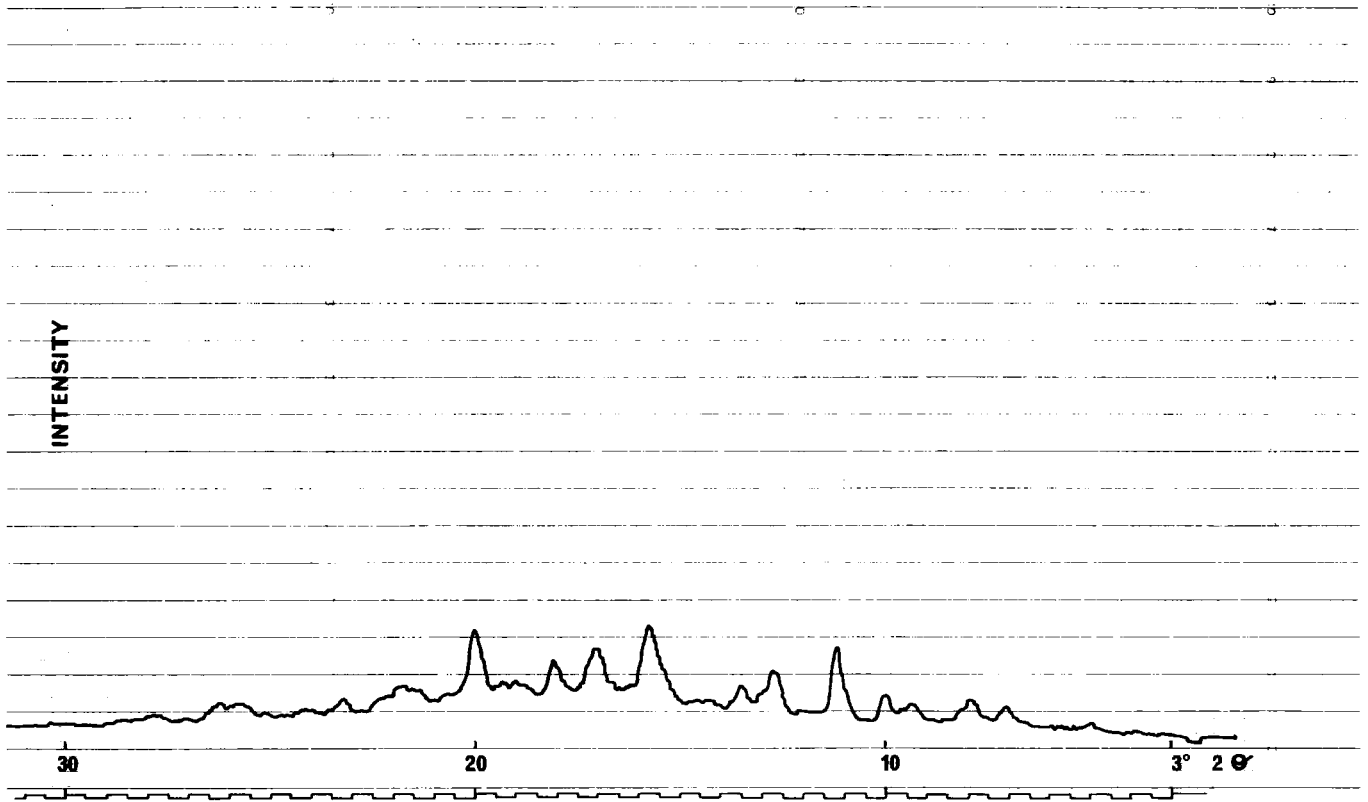


Figure 8 - X-ray powder diffraction pattern of rifampin after grinding

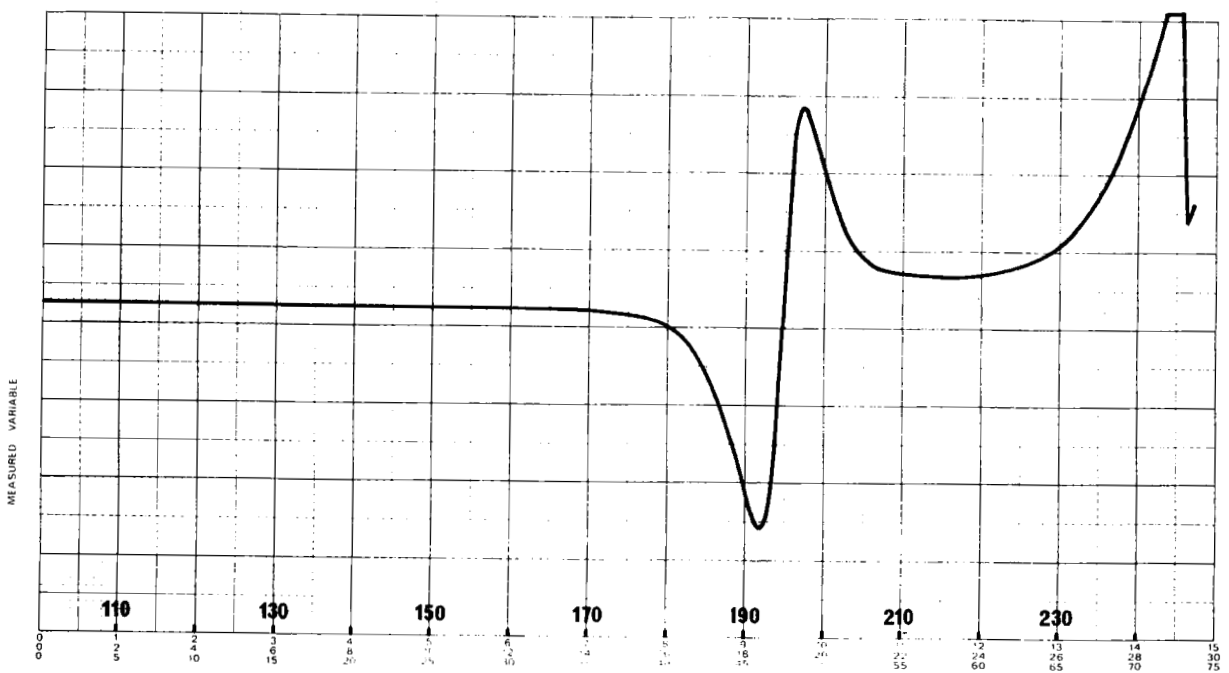


Figure 9 - Heating curve of rifampin

Table VII

Approximate solubilities of rifampin

Solvent	Parts of solvent required for 1 part of rifampin	Descriptive term
chloroform	from 1 to 10	freely soluble
methanol	from 10 to 30	soluble
dimethylformamide	from 10 to 30	soluble
dimethylsulphoxide	from 10 to 30	soluble
ethanol 95 per cent	from 100 to 1,000	slightly soluble
acetone	from 100 to 1,000	slightly soluble
benzene	from 1,000 to 10,000	very slightly soluble
carbon tetrachloride	more than 10,000	practically insoluble
n-hexane	more than 10,000	practically insoluble
cyclohexane	more than 10,000	practically insoluble
n-butanol	more than 10,000	practically insoluble
propyleneglycol	more than 10,000	practically insoluble
glycerol	more than 10,000	practically insoluble
carbowax 400	more than 10,000	practically insoluble

Table VIII

Solubilities of rifampin

Solvent	mg/ml	Temperature	reference
chloroform	349		
dichloromethane	216		
ethyl acetate	108		
dioxane	39	25°C	38
methanol	16		
acetone	14		
n-hexane	0.43		
petroleum ether	0.33		
water pH 7.3	2.5		
water pH 4.3	1.3		
water pH 7.5	2.8	room	46
water pH 2.0	99.5		
0.1N HCl	200.0	37°C	47
phosphate buffer pH 7.4	9.9		

2.62 Lipid-water partition

2.621 Organic solvent-water partition

The study of the partition between water and organic solvents as an indirect measure of the lipid-water partition has not been generally applied to the field of rifamycins. The partition of rifampin in the system n-octanol/aqueous phosphate buffer, pH 7.4 was determined by Seydel (47) to be K=15.6, while Curci et al. (48) have measured it in the system benzene/aqueous buffers in the range pH 5.5-7.0, and K equaled 9.7; at pH 7.5, K=9.0; at pH 8.0, K=4.6.

2.622 Silicon oil-water partition

The lipid-water partition has been obtained for various rifamycins from R_f values in partition reverse-phase thin layer chromatography on Silicagel plates impregnated with silicon oil as stationary phase and water-acetone as mobile phase (49-51). The free energy parameter $R_M=\log(\frac{1}{R_f}-1)$ (52), was used for quantitative correlations between structure and antibacterial (49,50) and antiviral (51) activities.

R_M values were obtained for rifampin and are reported in Table IX.

Table IX
R_M values for rifampin

R_M	mobile phase	stationary phase	Ref.
0.029	30% acetone in water (v/v)	silicon oil	49
-0.293	40% acetone in water (v/v)	silicon oil	50

2.623 Surface activity

Rifamycins have been shown to have surface activity, from the variation of the surface tension of buffer water solutions with concentration (53,54).

The surface properties of rifampin depend on the pH of the medium. In the alkaline to neutral range, rifampin is a weak surfactant and no associations are observed; in the acidic range (pH 4-0) a pronounced lowering of the surface tension with concentration is observed, and micelle formation is apparent at a concentration of about 10^{-5} mole/liter (55).

3. STABILITY

3.1 STABILITY AS POWDER

Rifampin is very stable in the solid state in sealed containers at room temperature, as described in Table X (56). Rifampin in the solid state is stable also at temperatures up to 70°C, as reported by Sano et al. (38).

3.2 STABILITY IN SOLUTION

The stability of rifampin in aqueous solution has been widely investigated and the conditions and the transformation products are reported in Table XI. Like other rifamycins, rifampin undergoes desacetylation on alkaline treatment, yielding the corresponding 25-desacetyl derivative without substantial loss of antibacterial activity (57).

In mildly alkaline aqueous solutions and in the presence of atmospheric oxygen at room temperature, rifampin transforms into rifampin quinone; this oxidation can be prevented by sodium ascorbate. Under the same conditions and at 60-70°C, rifampin yields three main transformation products, which were identified by Maggi et al.(22) as 25-desacetyl-rifampin, 25-desacetyl-23-acetyl-rifampin and 25-desacetyl-21-acetyl-rifampin.

Sano et al. (38) have studied the stability of rifampin at 25° in aqueous solutions at different pH's: it decomposes rapidly in acidic or alkaline conditions, but slowly in neutral ones. 3-Formylrifamycin SV is the main decomposition product of rifampin in aqueous

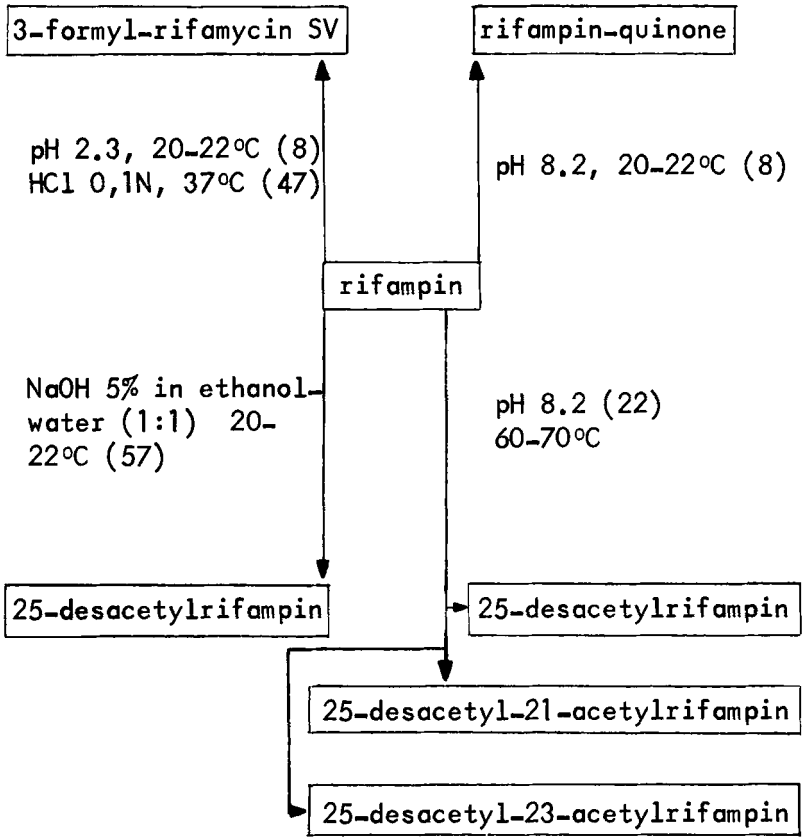
Table X

Stability of rifampin in the solid state at room temperature (56)

	starting		12 months		21 months		30 months		41 months	
	UV Assay %	Microbiological Assay %	UV Assay %	Microbiological Assay %	UV Assay %	Microbiological Assay %	UV Assay %	Microbiological Assay %	UV Assay %	Microbiological Assay %
Rifampin	99.5	98.6	101.1	96.4	99.0	100.0	100.2	95.4	97.3	95.9
	TLC		TLC		TLC		TLC		TLC	
3-formylrifamycin SV	traces		traces		traces		traces		traces	
rifampin quinone	absent		1-1.5%		1.5-2%		1.5-2%		2.5-3%	
rifampin N-oxide	traces		1-1.5%		1-1.5%		1-1.5%		1-1.5%	
25-desacetyl-21 acetyl-rifampin	absent		traces		traces		traces		0.5-1%	
25-desacetyl rifampin	0.5 - 1%		1.5 - 2%		1.5 - 2%		1 - 1.5%		1-1.5%	
25-desacetyl-23 acetyl-rifampin	absent		absent		absent		traces		traces	

Table XI

Stability of rifampin in aqueous solutions



acidic medium (8). Seydel (47) has determined the decomposition rate of rifampin in 0.1N HCl vs. temperature and, from the Arrhenius plot, calculated the activation energy, ΔHa=19.2 Kcal/mole/degree.

Rifampin does not lower its microbiological activity in the various conditions reported in Table XII.

Table XII

Stability of the antibacterial activity of rifampin in solution.

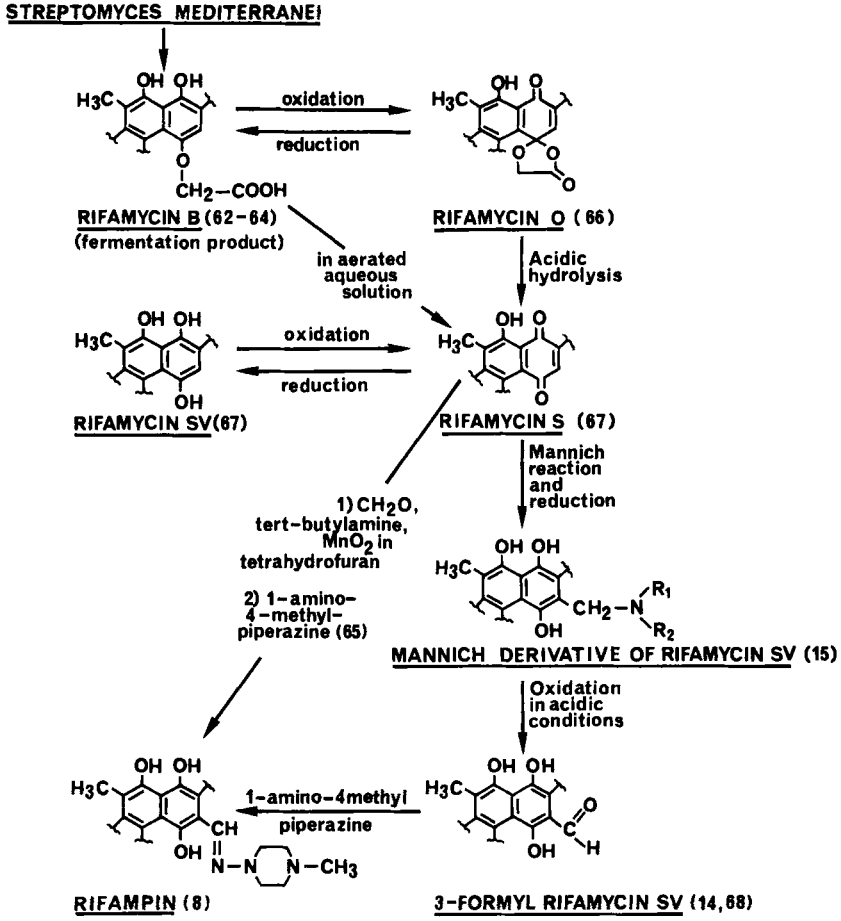
Conditions	Concentration(mg/ml)	Time	Ref.
water-dimethylformamide (95:5) at 5°C	1	90 days	58
dimethylsulphoxide at 15°	10	8 months	59
water-ethanol (8:2) at 4°C or 20°C	1	8 weeks	60,61

4. SYNTHESIS

Rifampin is a semi-synthetic rifamycin, obtained by condensation of 1-amino-4-methylpiperazine with 3-formyl-rifamycin SV in peroxide-free tetrahydrofuran at 10°-15°C. The 3-formyl-rifamycin SV (14) was obtained from rifamycin B, the fermentation product (62-64), by the chemical procedure reported in table XIII. Gianantonio et al. (65) have patented the production of rifampin by reacting rifamycin S directly with formaldehyde, tert-butylamine and MnO_2 and condensing with 1-amino-4-methylpiperazine (see table XIII).

Table XIII

Synthesis of rifampin



5. METHODS OF ANALYSIS

5.1 ELEMENTAL ANALYSIS

Element	Theoretical %
C	62.76
H	7.10
N	6.81
O	23.33

5.2 IDENTIFICATION TESTS

Rifampin is identified and differentiated from the other rifamycins by infrared and nuclear magnetic resonance spectroscopy, field desorption mass spectrometry, potentiometric titration, differential scanning calorimetry and elemental analysis. In order to define the homogeneity of the rifampin sample, chromatographic procedures (paper and thin layer) and thermal analysis are used.

Colorimetric tests based on oxidation were described. To 5 ml of aqueous rifampin solution (about 1 mg/ml), 1 ml of 10% (w/v) ammonium persulphate in phosphate buffer, pH 7.38, is added: the color turns from orange-yellow to violet-red (69). A similar method, employing ferric nitrate as oxidizing agent, was described by Eidus et al. (70,71), who used it to identify the presence of rifampin and desacetyl rifampin in biological fluids.

5.3 SPECTROPHOTOMETRIC METHODS

5.31 Spectrophotometric assay on bulk product

The VIS maximum at 475 nm in aqueous phosphate buffer solution pH 7.38 with an absorptivity (g/l) value of 18.7 (see Section 2.11) enables rifampin to be quantitatively assayed.

The main impurities of rifampin are 3-formyl rifamycin SV and rifampin-quinone. The two products can be spectrophotometrically quantitated: the first one by an extraction method using n-hexane:ethylacetate (2:1) (13) and the second one by a differential spectrophotometric method which takes advantage of the reduction by sodium ascorbate of the quinone (13).

5.32 Spectrophotometric assay on pharmaceutical preparations

Rifampin can be determined in pharmaceutical preparations by using the differential spectrophotometric method (72), modified by Pasqualucci et al. (73).

5.33 Spectrophotometric assay in biological fluids

Numerous spectrophotometric methods for determining rifampin in biological fluids have been described in the literature, but owing to their low sensitivity they were satisfactorily applied only to the determination of relatively high levels (more than 5μg/ml). The methods were based on the extraction of rifampin and its metabolites with organic solvents and on the determination of the VIS absorbance of the organic extract. The experimental conditions are summarized in Table XIV.

5.4 FLUOROMETRIC DETERMINATION

Rifamycins do not exhibit natural fluorescence. A quantitative method was developed for rifamycin B (82), based on its transformation into the triacetyl derivative.

Rifampin was determined fluorometrically by transforming it with hydrogen peroxide (83) into a fluorescent product. The maximum fluorescence develops in an aqueous carbonate-bicarbonate buffer, pH 9.2 at 480 nm, when the excitation wavelength is 370 nm. The relative fluorescence intensity is linear with concentrations of rifampin in the range 0.1 to 10 μg/ml.

5.5 ANALYSIS BY COMPLEX FORMATION

Rifamycins complex with metallic ions, as reported for rifamycin L (34) and for rifamycin S (84).

Rifampin in methanol solution gives a complex when treated with an aqueous solution of $AlCl_3$ in the ratio 2:1 (85); the instability constant in water-methanol (1:1) is $3.4 \cdot 10^{-8}$. Complex formation was demonstrated to take place also with Hg^{++}, Cu^{++}, Ag^{+} and Fe^{+++} (86).

Table XIV

Spectrophotometric methods for the determination of rifampin and its metabolites in body fluids.

Body fluid	Extraction solvent	Compound Assayed as stated in the reference	Analytical wavelength (absorptivity, g/l)	Reference
bile, urine	benzene	R+DA	475nm(15.6)	74
urine, serum	iso-amyl alcohol	R+DA	475nm and 335nm (calibration curve)	75
urine	benzene-hexane (1:1)	R	335nm (calibration curve)	
urine, serum, bile and tissues	butanol-hexane (4:1)	R+DA	478nm (calibration curve)	76
urine	butanol-hexane (4:1)	R	478nm (19.6)	77
teeth	butanol-hexane (4:1)	R	478nm (calition curve)	78
serum	butanol-heptane (4:1)	R	482nm (15.6)	79
serum	ethylalcohol-ethylacetate (1:1)	R	475nm (15.5)	80
urine	cyclohexane-butyl acetate (1:1)	R	blue light filter (calibration curve)	81

R = rifampin

DA = 25-desacetyl rifampin

The formation of the complex with $AlCl_3$ was employed for quantitative determination of rifampin in bulk, in pharmaceutical formulations and in urine (87), by measuring the cherry-red color at 507nm in methanol-water (49:1) or in methanol-water-butanol-hexane (19:1:4:1).

5.6 VOLUMETRIC METHODS

Rifampin in capsules was determined by a volumetric method (88): the antibiotic was oxidized with an excess of ferric chloride, which was then titrated iodometrically.

5.7 CHROMATOGRAPHIC METHODS

5.71 Thin layer chromatography

TLC has been widely used in the field of rifamycins (89,90) and the colored spots are directly located.

Many TLC procedures were developed for the analysis of rifampin and its metabolites in body fluids. The Rf values were shown to be dependent on the concentrations (91). The spots were quantitated by using the bioautographic technique (92) or the densitometric one (93) or by visual estimation (94) or, finally, by the elution method followed by microbiological assay (95). A reverse-phase partition TLC procedure has been described for the determination of rifampin (96):silanized Silicagel plates were used as stationary phase, with phosphate buffer, pH 7 containing 0.1% sodium ascorbate as mobile phase (other mobile phases are reported). The colored spot was eluted and measured spectrophotometrically. Reverse-phase partition TLC has been used for structure-activity correlations (see Section 2.622).

5.72 Column chromatography

The content of rifampin and its metabolites in urine, bile and serum, was determined by extraction with chloroform and by column chromatography, followed by spectrophotometry (97). The liquid-solid chromatography was carried out using a glass column 7 cm long, 4 mm I.D., packed with Silicagel G, buffered at pH 6, and chloroform and chloroform-methanol in progressively

increasing amounts (5%, 10% and 16.6%) as solvent, at a flow rate of 0.15 ml/min. Rifampin and its metabolites were then quantitated by reading the absorbance of the eluates at 475 nm.

5.73 Paper chromatography

A paper chromatographic technique for determination of rifampin and 25-desacetylrifampin was used for urine and bile (74). Descending chromatography was carried out on Whatman 3MM paper using methanol: n-octanol (4:1) as stationary phase and aqueous buffer, pH 6, as mobile phase for 6 hr. The intensity of the red-orange spots was measured by an Analytrol photodensitometer (mod.R13-450 nm filter) or the materials determined bioautographically.

Akimoto et al. (98) reported a paper chromatographic method for the separation of rifampin and its metabolites, using ethyl acetate-water-dimethylformamide (10:10:1).

5.74 High pressure liquid chromatography

Rifampin has been frequently cited as an example of the usefulness of HPLC (99-102). Rifampin, 25-desacetylrifampin, rifampin quinone and 3-formyl rifamycin SV were separated (99) under the following conditions: DuPont 820 Chromatograph equipped with UV detector, ODS Permaphase column at 50°C and 1,000 psi, water to methanol with linear gradient 8%/min as mobile phase and 1ml/min flow rate.

5.8 MICROBIOLOGICAL METHODS

Microbiological methods have been widely described for the determination of rifampin potency in bulk products, in pharmaceutical formulations and in body fluids, as listed in the review by Binda et al. (103). These methods can be classified as a) diffusion plate methods, b) serial tube dilution methods and c) turbidimetric methods.

5.81 Diffusion plate assay methods

These methods were used to determine rifampin in serum, bile and urine as well as in other body fluids and organs (fragments of organs and tissues were

appropriately treated for extracting rifampin). They can be divided in different classes according to technical aspects as reported in Table XV, where other experimental details are summarized. The cylinder-plate technique described by Grove et al. (104) has been adopted in Lepetit S.p.A. (105)

5.82 Serial tube dilution methods

This method, first applied by Clark et al. (115) to rifamide and rifampin, has been used for the determination of rifampin in serum by various labs. Some details are reported in Table XVI.

5.83 Turbidimetric methods

A turbidimetric microbiological assay of rifampin using an automatic analyzer was developed by Simoncini et al. (120). The method is based on the measurement of the optical density of the bacterial suspension (Klebsiella Pneumoniae ATCC 10031 or Escherichia coli ATCC 10536 as test microorganism) in a Difco culture medium containing rifampin, after incubation at 37° for 3.5 hr.

6. PROTEIN BINDING

The binding of rifampin to blood serum and plasma proteins in man and other animal species was widely studied. The data obtained cover a relatively large range, probably because of the different methodologies used and of the many parameters that can influence the phenomenon (121).

In vitro experiments were carried out by Curci et al. with a dialysis technique, and they reported that at the concentrations reached *in vivo*, about 75-85% of rifampin binds with serum proteins (48,122-125). Interestingly, they found that PAS (para-amino-salicylate) in concentrations from 100 to 200 μg/ml decreases the binding of the antibiotic (122). By an ultracentrifugation technique, Seydel (47) obtained a percentage of rifampin bound to bovine albumin *in vitro* in the range from 50 to 70%. From these data, Keberle et al. (126) derived that each albumin molecule has two binding sites for rifampin. Mashimo (127) has found the protein

Table XV

Microbiological assays of rifampin by diffusion plate assay methods

Technical aspects	Medium	Microorganism	Reference
metal cylinders containing the liquid to be assayed	Agar (Difco 0263-02)	Sarcina lutea (ATCC 9341)	105
wells (10mm) containing the liquid to be assayed	Agar (Difco) + KH_2PO_4	Sarcina lutea (ATCC 9341)	106
wells (9mm)	1% Agarose + 0.8% Difco + 10% aqueous phosphate buffer pH 7.2	Staphylococcus aureus	107
disks (6mm) soaked in the liquid to be assayed	Agar	Staphylococcus aureus	108
disks (9mm)	Agar (Difco 0263-02)	Bacillus brevis (IP 5122)	109
disks	Agar (Difco 0263-02)	Streptococcus (strain 8668)	110
disks	Agar (Difco 0270-02)	Sarcina lutea, sensitive to 0.1 µg/ml Bacillus subtilis, sensitive 1µg/ml Staphylococcus aureus, resistant to other antibiotics	111
disks (9mm)	Salt-glucose-peptone broth (Pasteur Inst. Codex)	Sarcina lutea (IP 5345)	112
disks (9mm)	Casein peptone-soya peptone-agar	Staphylococcus coagulase negative	113
disks (6mm)	Agar (Difco 0263-02)	Sarcina lutea (ATCC 9341)	114

Table XVI

Microbiological assays of rifampin by serial tube dilution methods.

Medium	Microorganism	Inoculum	Reference
non-specified broth	Sarcina lutea	0.03ml of a 1:250 dilution of 18h culture	115
Difco sucrose broth with phenol red	Staphylococcus aureus (ATCC 6538P)	0.05ml of a 1:100 dilution of 18h culture	116
mannite and phenol red selective broth	Staphylococcus aureus (Oxford, sensitive to 0.02µg/ml)	0.05ml of a 1:200 dilution of 24h culture	117
Youmans	Mycobacterium tuberculosis ($H_{37}Rv$)	$5\text{-}100 \cdot 10^4$ viable units	118
Lockemann	Mycobacterium tuberculosis ($H_{37}Rv$ or Fuhrmann	-	119

binding of rifampin by dialysis, ultrafiltration and ultracentrifugation, at a concentration of 20µg/ml, to be 10%, 90% and 70%, respectively. No clear interpretation was given for the differing data.

In vivo experiments of protein binding were carried out using ^{14}C-rifampin (128) and about 75% of rifampin was found to be bound to human serum protein. Pilheu et al. (129) found ratios of 15 to 40% between cerebrospinal fluid (considered as a protein-free fluid) and plasma levels. Aoyagi has reported (130) the binding of rifampin to the serum proteins of various animals: in the horse 40-46%, in the rabbit 13-17% and in man 17-38%. A recent study by dialysis using ^{14}C-rifampin (131) showed that 86.1% and 88.9% of rifampin were bound to the plasmas of tubercular patients and

healthy volunteers, respectively. Yokosawa et al. (132) have suggested, on the basis of electrophoresis and microbiological assay, that rifampin associates with α_1-, α_2- and β-globulins of human serum.

7. PHARMACOKINETICS AND METABOLISM IN MAN

The pharmacokinetics of rifampin has been widely studied and the serum levels of rifampin after single and repeated administration of different doses were determined by various investigators and have been reviewed (103). To summarize, after oral administration, rifampin is well-absorbed with the maximum plasma levels at 1.5-3 hr over a wide range of single dose, i.e., 0.1 to 1.2 g. The levels are still appreciable (5-10% of the peak level) after 12 hr only at the high doses (103).

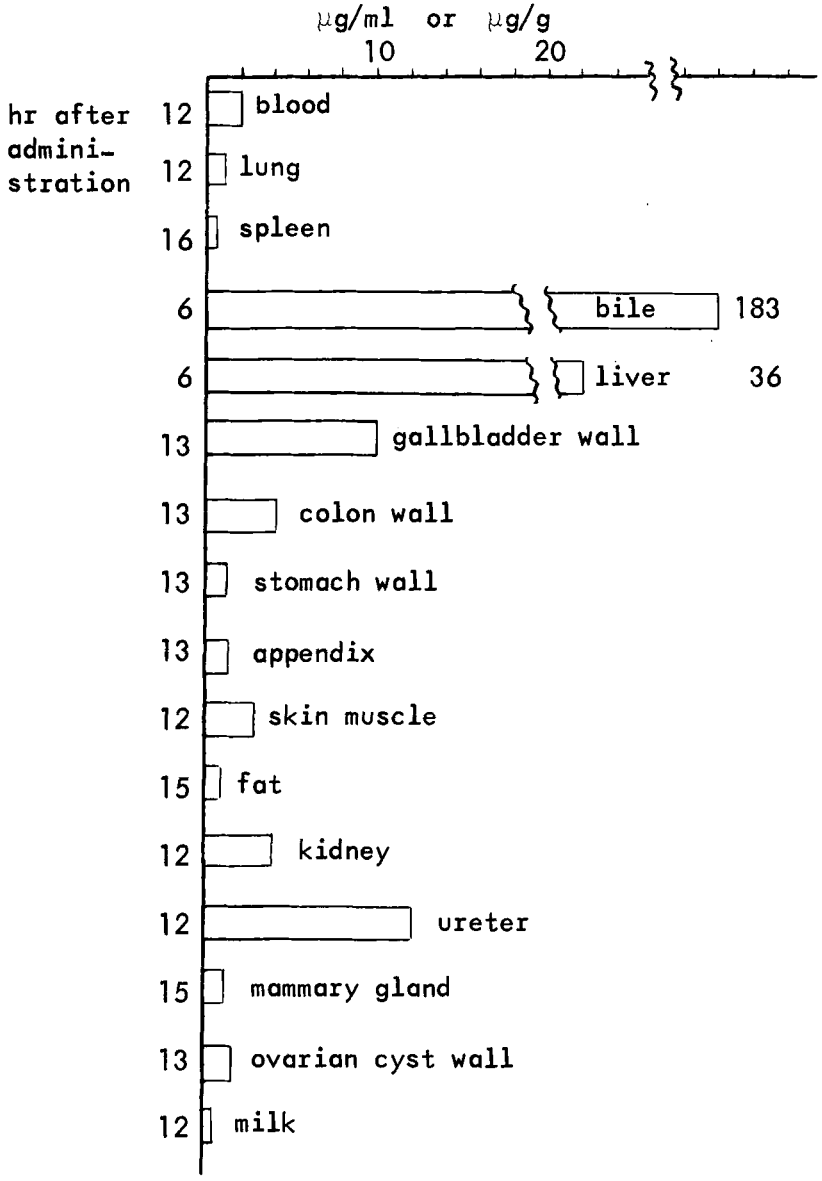
Rifampin is widely diffusible in the tissues and in the various body fluids (105), as indicated in Table XVII. This is related to the high lipotropism of rifampin (47,48).

Rifampin elimination, which must be considered slow, occurs mainly through the bile but also through the urine (103,128,133). The total amount of rifampin eliminated in the bile is not proportional to the dose administered (48,116,134) while the urinary elimination increases with the dose (77,105,109,116,133-136). In an investigation carried out over 6 days (105), about 22% was eliminated in the faeces and about 26% in the urine. In another study, however, Riess(128) found that 96 hrs after administration of 300 mg of labeled rifampin, 94% of the total radioactivity had been recovered in equal percentages in the faeces and urine.

The main metabolite of rifampin in man was identified, after isolation from bile and urine, as 25-desacetylrifampin (74,92,94,137). This compound is much less lipophilic than rifampin and it is easily excreted in urine (46) and not reabsorbed (138,139). Sano et al. (93) reported that in addition to 25-desacetylrifampin, a second metabolite is 3-formyl rifamycin SV.

Table XVII

Distribution of rifampin in human tissues and fluids after oral administration of a single 450 mg dose (105)



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SULFASALAZINE

J. Patrick McDonnell

CONTENTS

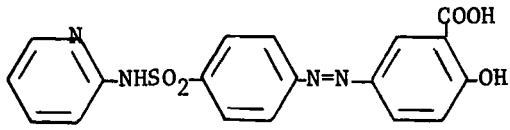
Analytical Profile - Sulfasalazine

SULFASALAZINE

1. Description

1.1 Name, Formula, Molecular Weight

Sulfasalazine is known chemically as benzoic acid, 2-hydroxy-5-/ /4-/(2-pyridinylamino)sulfonyl/phenyl/azo/-; 5-/ /p-(2-pyridylsulfamoyl)phenyl/azo/salicylic acid (1) or 4-(pyridyl-2-amidosulfonyl)-3'-carboxy-4'-hydroxyazobenzene (2). Common names for this drug substance are salazosulfapyridine and salicylazosulfapyridine (2).



$C_{18}H_{14}N_4O_5S$ Molecular Weight 398.39

1.2 Appearance, Color, Odor

The compound is a bright yellow to brownish-yellow, odorless, fine powder.

2. Physical Properties

2.1 Infrared Spectrum

The infrared absorption spectrum of sulfasalazine (Salsbury Reference Standard XP-2) is presented in Figure 1. The spectrum was taken in a potassium bromide pellet with a Perkin-Elmer, Model 735B Infrared Spectrophotometer. The assignments are as follows:

2500 - 3400	OH stretch (bonded)
1670 - 1680	C = O vibration
1580 - 1630	C = C or N = N vibrations
1360	CO_2NH vibration
1280 - 1290	SO_2 asymmetrical
1260 - 1278	C - O stretching vibration or OH deformation
1170 - 1195	SO_2NH
1135	SO_2 group - symmetrical
770, 790, 800	CH deformation (aromatic ring)

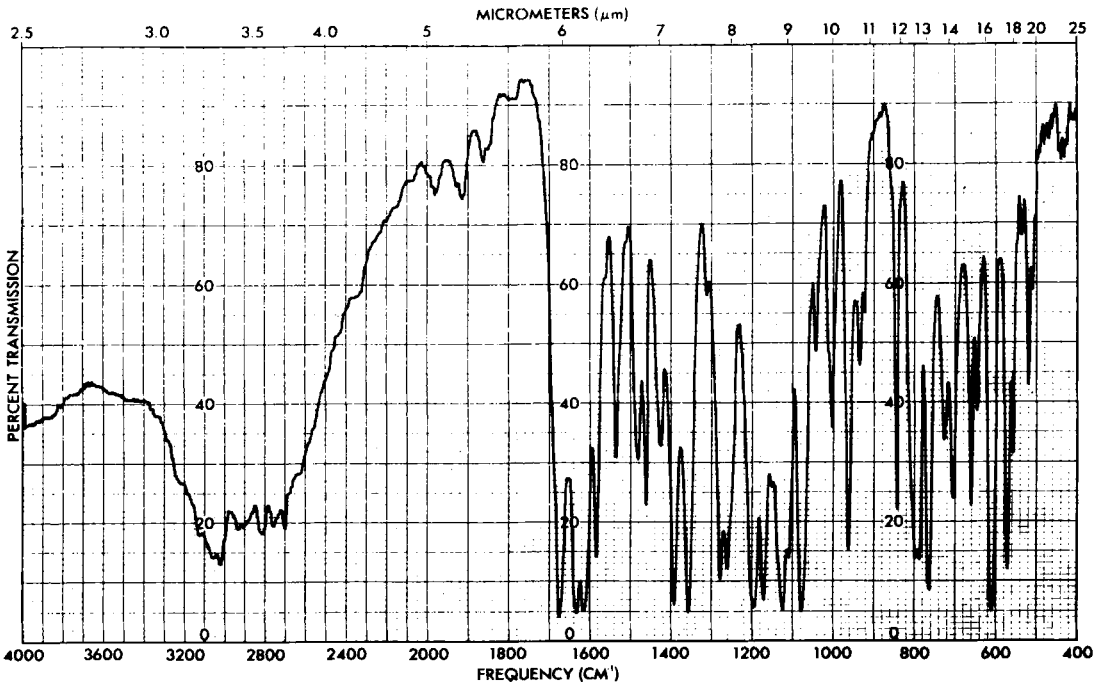


Figure 1 Infrared Spectrum - Sulfasalazine

2.2 Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum of sulfasalazine (U.S.P. LN231547, Lot F) is shown in Figure 2. The spectrum was run on a Varian, Model HA-100 as 100 MHZ proton spectrum using tetramethylsilane as the reference lock signal. Sweepwidth was 1000 cycles in the field sweep mode. Deuterated N, N-dimethylformamide (d_7 - DMF) was the solvent. The spectral pattern arises from aromatic protons on different rings. These are found in the region of 6.8 ppm to 8.8 ppm. Each of the small separated groups corresponds to one proton. Three groups lie upfield of the region of mixed absorptions and one lies downfield (3).

2.3 Ultraviolet-visible Spectrum

The ultraviolet-visible spectrum of sulfasalazine (U.S.P. LN231547, Lot F) is shown in Figure 3. It was made using a Cary, Model 15 Spectrophotometer on a 1.9×10^{-5}M solution of the compound. The maximum and minimum absorptions are similar to those reported in the literature (4, 5). In the pH range of 1 to 10 aqueous solutions of sulfasalazine (concentration 1.9×10^{-5}M) exhibited spectral absorption maxima at 235-240 nm and 350-360 nm. Absorption minimum was shown at 285-290 nm. At pH above 11.6 absorption maxima were exhibited at 450 nm, 235-240 nm and about 290 nm (4).

2.4 Mass Spectrum

The low resolution mass spectrum of sulfasalazine (U.S.P. LN231547, Lot F) was obtained using a Varian/MAT CH_4 Mass Spectrometer. The sample temperature was 360° C (See Figure 4).

The primary fragmentation pattern indicates elimination of SO_2H (m/e = 65) from the molecule (M - 65 = 333) followed by elimination of CO_2 (m/e = 45) from the molecule (m - 109 = 289). This may indicate an original molecular structure involving cyclization of the sulfonamide, pyridine nitrogen and the 2 position of the neighboring phenyl group (6).

2.5 Melting Range

It melts at about 255° C with decomposition (1).

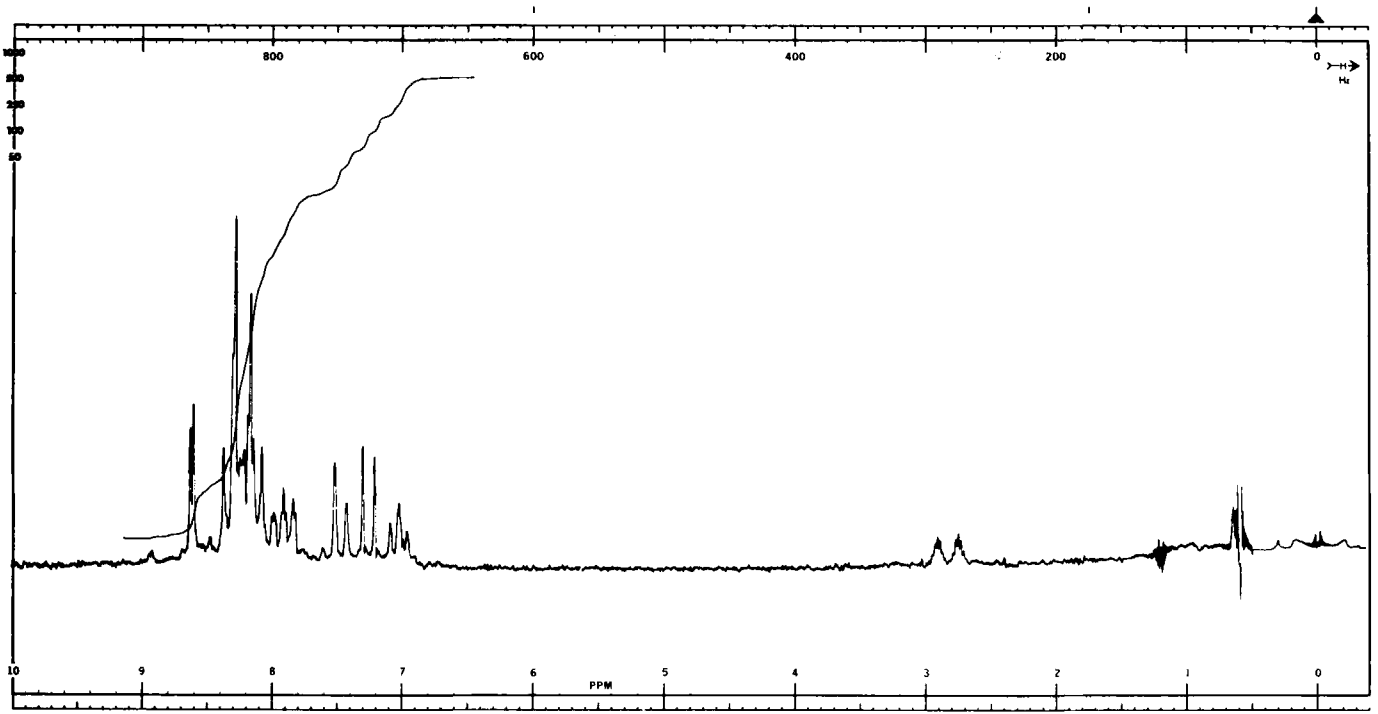
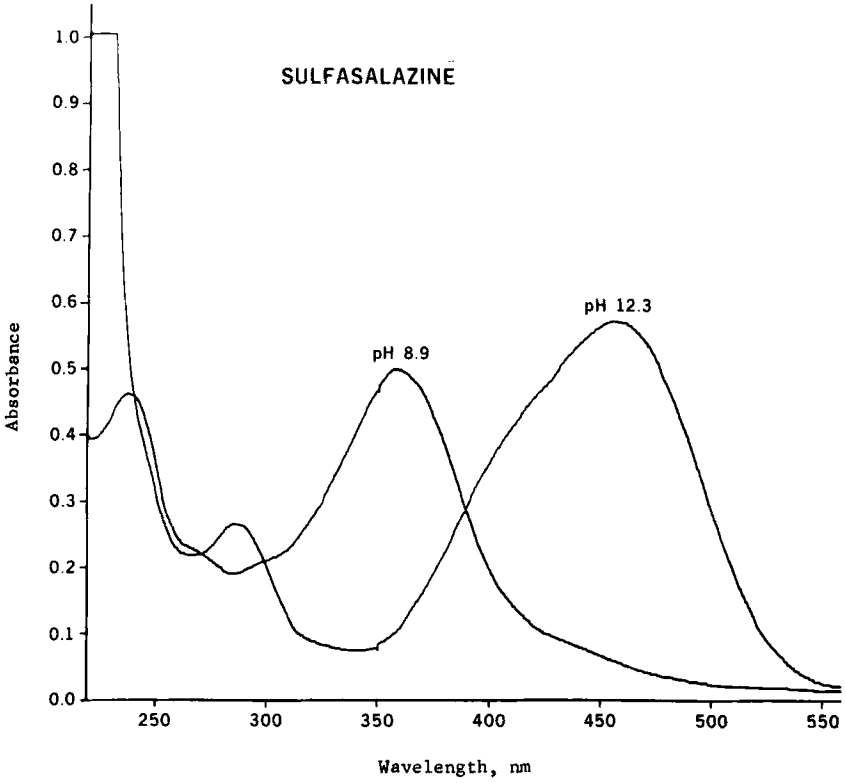


Figure 2 Nuclear Magnetic Resonance Spectrum - Sulfasalazine

Figure 3 Ultraviolet-visible Spectrum



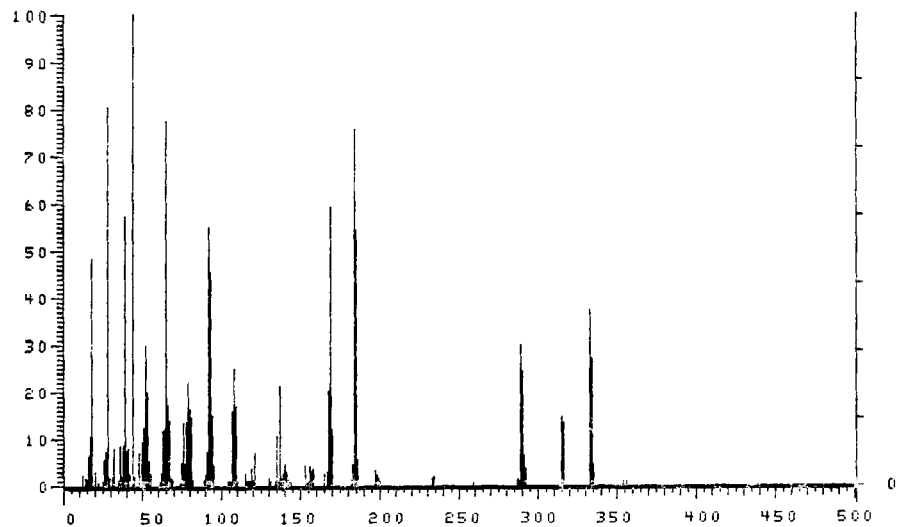


Figure 4 Mass Spectrum - Sulfasalazine

2.6 pK Values (Dissociation Constants)

The four dissociation constants of sulfasalazine determined spectrophotometrically are as follows: pK_1 = 0.6; pK_2 = 2.4; pK_3 = 9.7 and pK_4 = 11.8 (4).

2.7 Solubility

The solubility of sulfasalazine is as follows (1):

Practically insoluble in water
Practically insoluble in ether
Practically insoluble in chloroform
Practically insoluble in benzene
Very slighly soluble in alcohol
Soluble in aqueous solutions of alkali hydroxides

3. Synthesis

Sulfasalazine is prepared by diazotizing sulfapyridine and reacting this with an alkaline solution of salicylic acid (7, 8). Other reported methods of preparation are: a) reacting 5-nitrososalicylic acid with sulfapyridine, and b) reacting nitroso-sulfapyridine with 5-aminosalicylic acid (8) (See Figure 5).

4. Impurities - Stability

Partial characterization of impurities in commercial sulfasalazine was reported by Stone et al (9). Of the three impurities studied, one was proposed to be a positional isomer of sulfasalazine; the second to be polymeric resulting from the formation of a benzyne intermediate; and the third an undiazotized sulfamide (See Figure 6). The compound did not degrade when dissolved in dimethylformamide and was subjected to thermal stress at 80° C for 196 hours (10).

5. Drug Metabolic Products

In man, sulfasalazine is metabolized by reductive cleavage, presumably by the gut flora, to sulfapyridine and 5-aminosalicylic acid. The sulfapyridine portion is then subject to N^4-acetylation or pyridine ring hydroxylation, followed by conjugation to glucuronic acid, or both. The 5-aminosalicylic acid moiety is N-acetylated (11) (See Figure 7). In rats, sulfasalazine is metabolized similar to that in man (12, 13).

Figure 5 Synthesis of Sulfasalazine

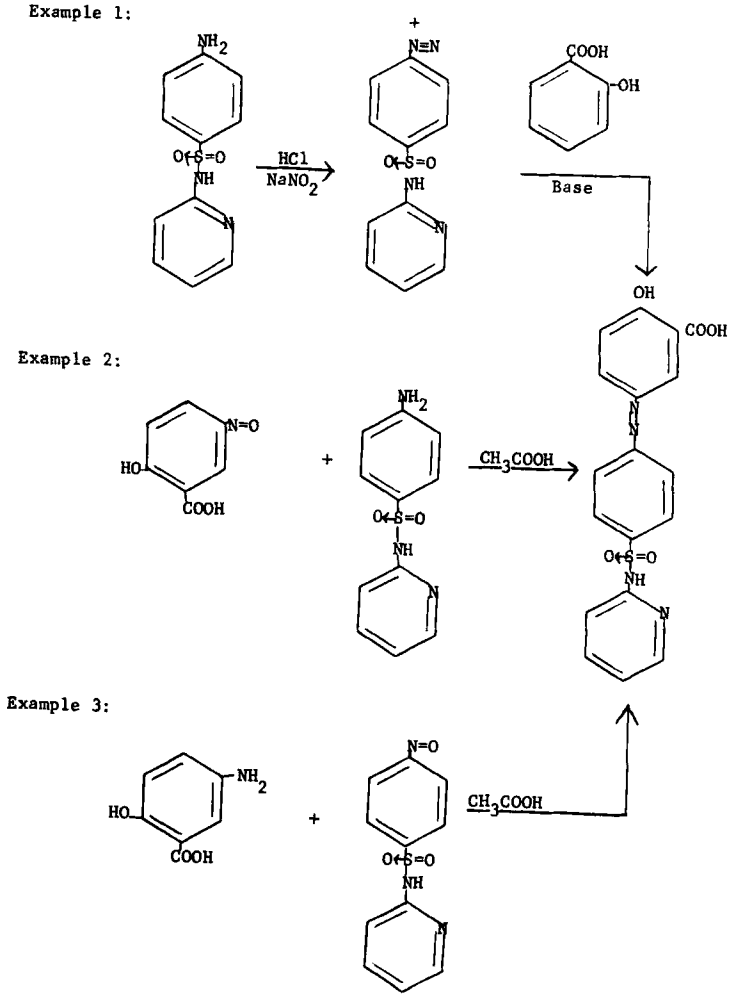
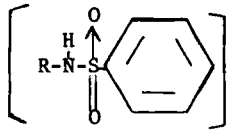
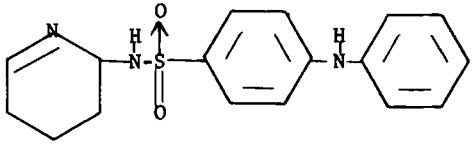


Figure 6 Sulfasalazine Impurities

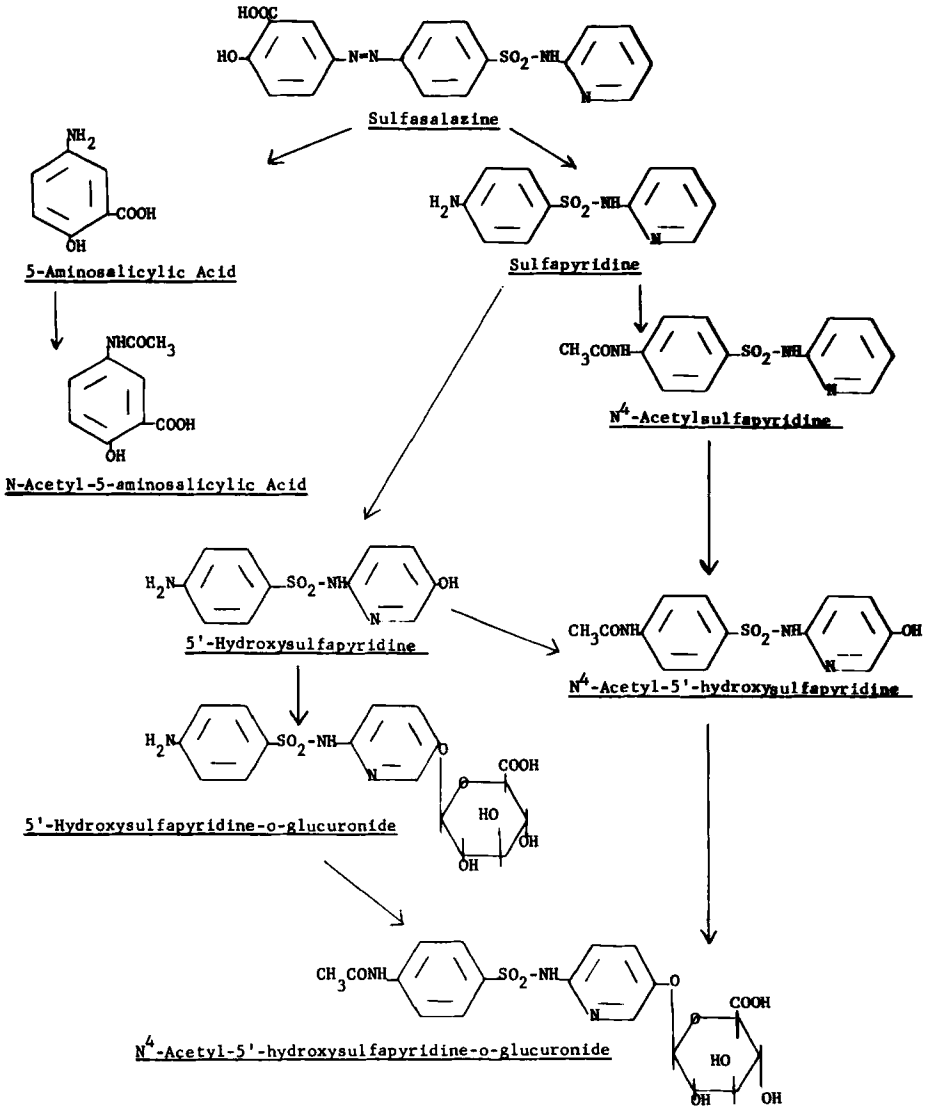


Benzyne Intermediate



Undiazotized Sulfamide

Figure 7 Sulfasalazine Metabolic Pathway



6. Methods of Analysis

6.1 Ultraviolet Spectrophotometry

Berggren et al (5) developed a spectrophotometric procedure for sulfasalazine which consisted of dissolving the sample in 0.1 N sodium hydroxide, adjusting the pH to between 4 and 5 with 0.1 N acetic acid and determining the absorbance in a 1 cm quartz cell at 359 mu. This method was also reported in the 1953 Edition of Tests and Standards for New and Nonofficial Remedies (14).

6.2 Quantitative Thin-Layer Chromatography (TLC)

Two methods of quantitative TLC analysis of sulfasalazine have been published (1, 15). Both use the same developing solvent reported by Kiger et al (16) and are similar in the method of preparing and conditioning the developing tank. The Powell et al method (15) consists of dissolving the sample and reference standards in dimethylformamide/methanol solution, spotting the solutions on a TLC plate, developing the plate, cutting the sulfasalazine spot (Rf. 0.6-0.7) from the plate and eluting it in a 0.016 N sodium hydroxide solution. The solution is filtered and an aliquot combined with a 0.1 N acetic acid solution. The absorbance of the reference standard and sample solutions are compared at 360 nm using water as a blank.

The method provided in the Fourteenth Edition of the National Formulary (1) involves dissolving the sample and reference standard in dimethylformamide, spotting the solution on a TLC plate, developing the plate, scraping the sulfasalazine spot (Rf. about 0.6) and eluting it with dimethylformamide. The resultant standard and sample solution absorbancies are compared at 406 nm.

6.3 Column Chromatography

Stone et al (9) reported an assay procedure for sulfasalazine using column chromatography. The sulfasalazine sample is dissolved in pyridine then separated on a silicic acid column. The developing solvent system is methyl ethyl ketone/acetone/water (16:16:1) and the flow rate is 1.2 ml per minute. The second of the three bands to elute is sulfasalazine which is collected and the solvent removed by evaporation. The resultant sample is taken up with 0.1 N sodium hydroxide and to this is added

0.1 N acetic acid and diluted to volume with water. The absorbance of this solution is determined at 359 nm using water as a blank.

6.4 Polarography

A polarographic method of analysis for sulfasalazine has been preliminarily outlined by Nygard (17) and independently also by Lastovkova et al (18). Based on the work of these investigators, Nygard et al (19) developed a polarographic assay method which they used to determine sulfasalazine purity.

6.5 Titrimetric Analysis

Berggren et al (5) also developed a titrimetric method of analysis which was adopted in the 1953 New and Nonofficial Remedies (14). The sample is dissolved in 2 N ammonium hydroxide and diluted to volume with water. The solution is heated to 70° C and carbon dioxide is passed through it. Hydrochloric acid and 0.1 N titanium trichloride are added and the mixture is maintained at 70° C until all the precipitate has gone into solution. It is cooled to 15° C and the excess titanium trichloride is titrated with 0.1 N ferris chloride using 2 ml of 10% ammonium thiocyanate as the indicator.

6.6 High Speed Liquid Chromatography

High speed liquid chromatography methods of analysis have been used to determine the purity of sulfasalazine bulk powder and to quantify the level of sulfasalazine in tableted dosage forms (10, 20). Bighley et al (10) used a reverse phase Corasil C_{18} column and 10% 2-propanol in pH 7.7 phosphate buffer as the mobile phase to accomplish analysis. The sulfasalazine bulk drug or tablet was dissolved in dimethylformamide and propyl paraben added as the internal standard. Quantitation was effected by peak height or peak area using a UV photometric detector (254 nm radiation using a low pressure mercury source) as the detection system.

A Corasil C_{18} column packing was used by Frahm et al (20). Their mobile phase was 13% acetonitrile in pH 7.3 phosphate buffer and the internal standard was methyl meta-nitrobenzoate. The sulfasalazine was dissolved in dimethylformamide. Quantitation was effected by peak height measurement and the detection made at 254 nm using

a low pressure mercury source.

7. Distribution in Fluids and Tissues

Following intravenous injection in mice, sulfasalazine attached to connective tissue and was present also in high concentrations in peritoneal, pleural and synovial fluids, in the liver, and in the intestinal lumen (21, 22).

8. Pharmacokinetics

The steady-state serum concentrations in humans for sulfasalazine and its metabolites obtained by Day 5 (dose 4 grams daily) were as follows:

	Range	Median
Sulfasalazine	4.7 to 45 ug/ml	12 ug/ml
Total Sulfapyridine Metabolites	37 to 92 ug/ml	50 ug/ml
Total 5-Aminosalicylic Acid	Less than 2 ug/ml	-

The urinary excretion of unmetabolized sulfasalazine ranged from 1.7% to 10% of the dose. Approximately 80% of the dose was excreted in the urine as metabolites of sulfapyridine and one-third of the dose was excreted as the metabolite of 5-aminosalicylic acid. Feces did not contain any sulfasalazine, but about 5% of the dose was present as metabolites of sulfapyridine, and an unknown amount as metabolites of 5-aminosalicylic acid (11).

9. Determination in Body Fluids and Tissues

9.1 Spectrophotometry

von Porat (23) published two colorimetric methods for the determination of sulfasalazine in serum. One was a direct measurement of the drug color in alkaline serum. The serum blank, however, was high, corresponding to 14 ug/ml of sulfasalazine. In addition, hemolyzed and icteric sera interferred considerably with the measurement. The other method involved a combined precipitation and extraction method. The serum blank decreased to 5 ug/ml and the interference from icteric sera diminished.

Bottiger (24) reported a direct colorimetric method similar to von Porat's. He used an acidified portion of the serum sample as a reference. The effect of slight hemolysis and icteric sera were thus, to a large extent, eliminated.

Sandberg (25) presented a spectrophotometric method for determining sulfasalazine in serum and urine by extracting the compound intact and measuring spectrophotometrically at 455 nm. In bile and feces the drug was reduced to sulfapyridine which was extracted and then determined with a slightly modified Bratton-Marshall procedure. After the extraction step, serum, urine, bile and feces gave low blank values corresponding to 0.3 and 1 ug/ml sulfasalazine in serum and urine and less than 1 ug/ml in bile and feces.

9.2 Polarography

Nygard (17) described a polarographic method for analyzing sulfasalazine in serum. Due to the formation of a polarographic, non-reducible adduct between serum proteins and sulfasalazine, 1,2-diphenyl-3,5-dioxo-4-n-butyl pyrazolidine (Butazolidin^{R}) was added to displace sulfasalazine from its molecular adduct with albumin.

9.3 Paper Chromatography

Hanngren et al (21, 22) used paper chromatography in conjunction with autoradiograms of the chromatograms to semiquantitatively determine sulfasalazine and its metabolic by-products in urine, liver extracts and intestinal extracts. The developing system was a mixture of pyridine/iso-amyl alcohol/water (35:35:30). Whatman No. 1 filter paper was used and the chromatograms were developed by descending chromatography.

10. Acknowledgments

The author wishes to acknowledge the assistance of Dr. Lyle D. Bighley in reviewing this analytical profile, Mary Lou Bullard for typing the manuscript and Harold E. Haus for preparing the art work.

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In the preparation of this Analytical Profile, the literature was reviewed through September, 1975.

TESTOLACTONE

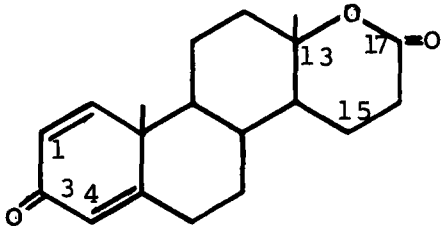
Klaus Florey

CONTENTS

1. Description

1.1 Name, Formula, Molecular Weight

Testolactone is also known as Δ'-testololactone, 1-dehydro-testololactone, 13,17 secoandrosta-1,4-dien-17-oic acid, 13-hydroxy-3-oxo-ʃ-lactone and D-homo-17α-oxaandrosta-1,4-diene-3,17-dione. SQ 9538.



$C_{19}H_{24}O_3$ M.W. 300.38

1.2 Appearance, Color, Odor

Testolactone is a white to off-white, odorless crystalline powder.

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum (KBr pellet) of testolactone (batch 36B) is presented in Figure 1. It supports the presence of a lactone with a carbonyl stretching frequency of 1703 cm^{-1} and an A ring dienone with a carbonyl stretching frequency at 1650 cm^{-1} and C=C stretching frequencies of 1620 and 1595 cm^{-1} [24]. This agrees with data presented by Fried et.al.[1,12] and the spectrum given by Gual, Dorfman and Rosenkrantz[25]. The same authors also reported frequencies in the fingerprint region[26].

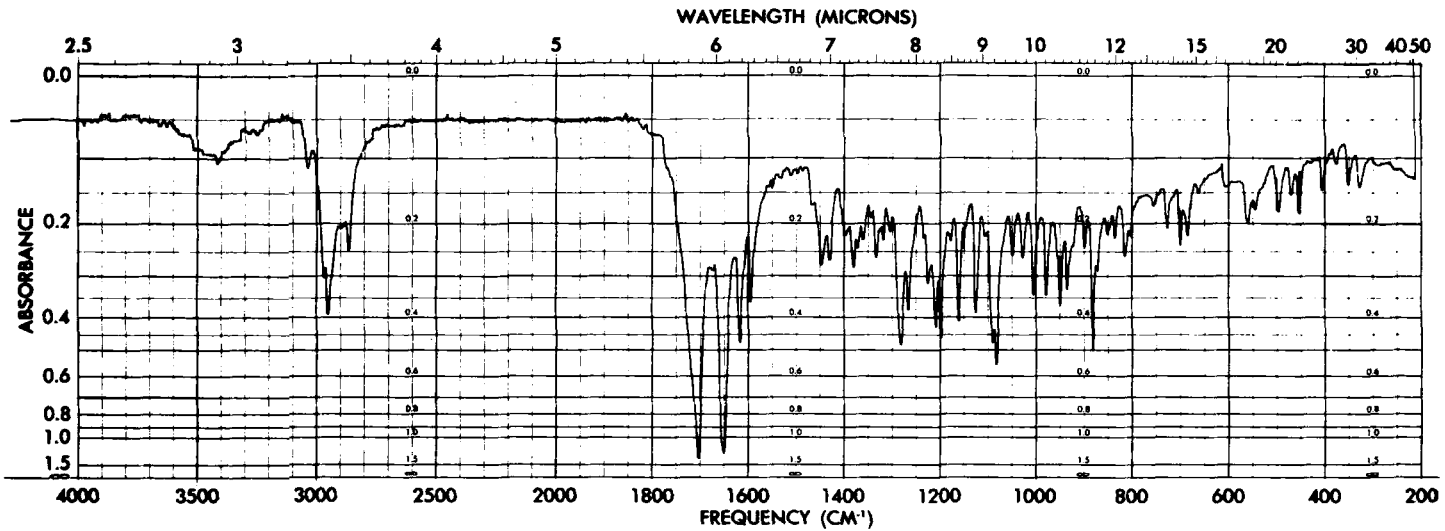


Figure 1. Infra Red Spectrum of Testolactone (batch 36B). KBr Pellet. Instrument: Perkin-Elmer 621.

2.2 Nuclear Magnetic Resonance Spectrum

The 60 MHz proton spectrum is shown in Figure 2. There are 24 protons present. The C-18 and C-19 protons occur as 3-proton singlets, the C-1 proton is a doublet, the C-2 proton is a quartet and the C-4 proton appears as a broad multiplet (see Table 1)[24].

2.3 Ultraviolet Spectrum

Fried et.al.[1] reported λ_{max} 242 nm (ε = 15,800). An $E^{1cm}_{1\%}$ of 545 at the same wavelength was reported for Squibb House Standard (Lot # 41040-102)[27].

Table I

60 MHz NMR Data in $CDCl_3$*

Group	SQ 9,538 Batch #36B
C-1	7.05 d; $J_{1,2}$ = 10
C-2	6.28 q; $J_{2,4}$ = 1.5, $J_{1,2}$ = 10
C-4	6.13 m
C-18	1.25 s
C-19	1.40 s

*The chemical shifts are in delta, with internal reference TMS.

s = singlet; d = doublet; m = multiplet; q=quartet

J = coupling constant in Hz.

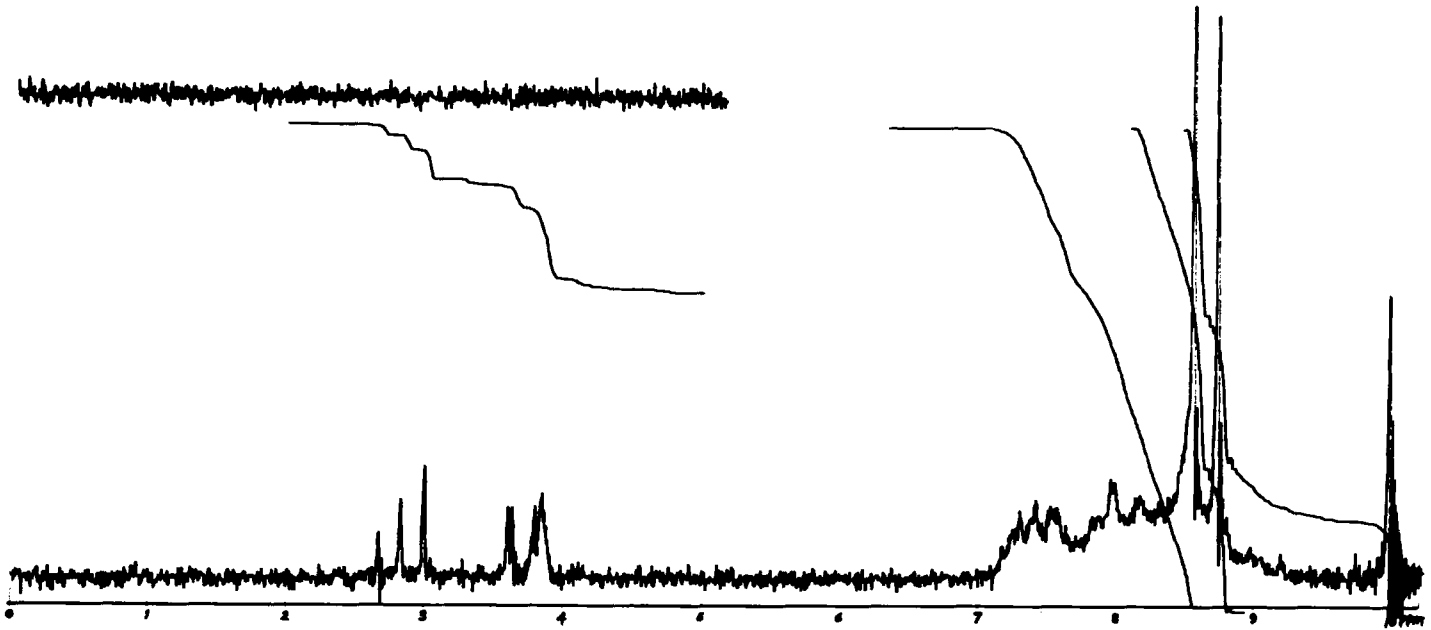
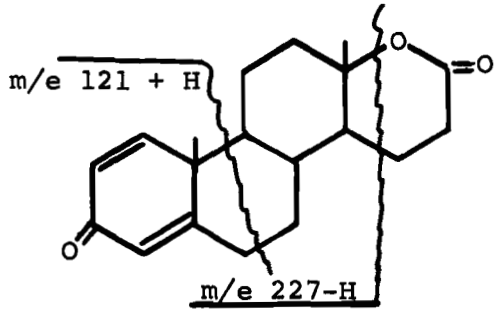


Figure 2. NMR Spectrum of Testolactone (batch 36B) in $CDCl_3$.
Instrument: Perkin-Elmer R12B(60 MHz)

2.4 Mass Spectrum

The low-resolution mass spectrum of testolactone (Figure 3) was obtained by direct insertion of the sample into a 180°C source of an AEIMS-902 spectrometer. The M+ of m/e 300 corresponds to the formula of $C_{19}H_{24}O_3$. The two fragment ions shown below are diagnostic for this structure[28].



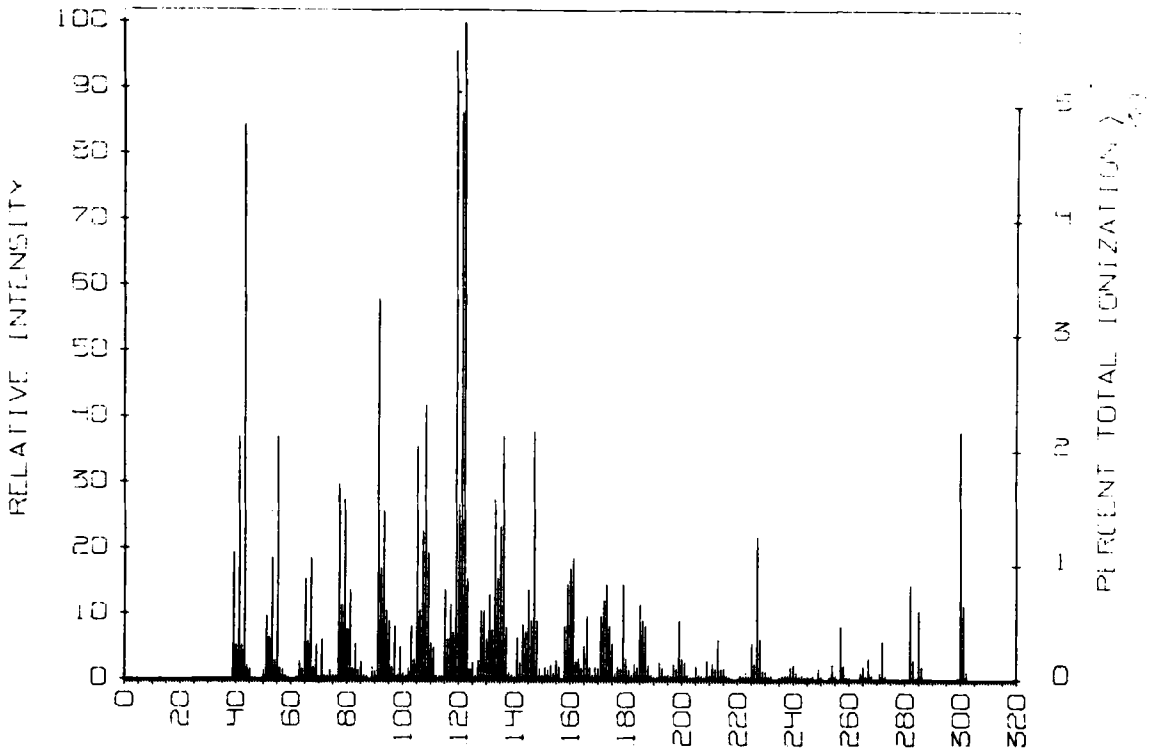


Figure 3. Low-resolution mass spectrum of Testolactone (Batch 36B). Instrument: AEI-MS-902.

2.5 Optical Rotation

Fried et.al.[1] reported $[\alpha]_D^{23}$ -44° (c = 1.29 in $CHCl_3$). $[\alpha]_D$ - 49.2 was recorded for Squibb House Standard Lot 41040-102[27].

2.6 Melting Range

A melting range of 218-219° was reported by Fried et.al.[1] The melting behavior on the Kofler hot stage has been described[35].

2.7 Differential Thermal Analysis

D.T.A. of house standard Lot 41040-102 showed a large single endotherm at 219°C[27].

2.8 Solubility

Testolactone is slightly soluble in water and in benzylalcohol, is soluble in alcohol and in chloroform and is insoluble in ether and in solvent hexane[29].

2.9 Crystal Properties

No polymorph of testolactone have been described so far except that two modifications were observed on the Kofler hot stage[35].

The powder x-ray diffraction pattern of testolactone is presented in Table 2, corresponding to the pattern in Figure 4.

Table 2

'd'*	Relative Intensity**
7.75	0.74
6.52	0.40
6.20	0.11
5.60	0.11
5.30	1.00
4.85	0.72
4.62	0.09
4.34	0.86
4.05	0.15
3.95	0.14
3.88	0.32
3.85	0.17
3.71	0.57
3.45	0.43
3.29	0.15
3.22	0.14
3.16	0.41
3.10	0.16
2.94	0.17
2.86	0.06
2.67	0.04
2.57	0.09
2.44	0.05
2.32	0.04

*d in Å = interplanar distance

**based on highest intensity of 1.00

Radiation: $K\alpha_1$ and $K\alpha_2$ Copper

Instrument: Phillips

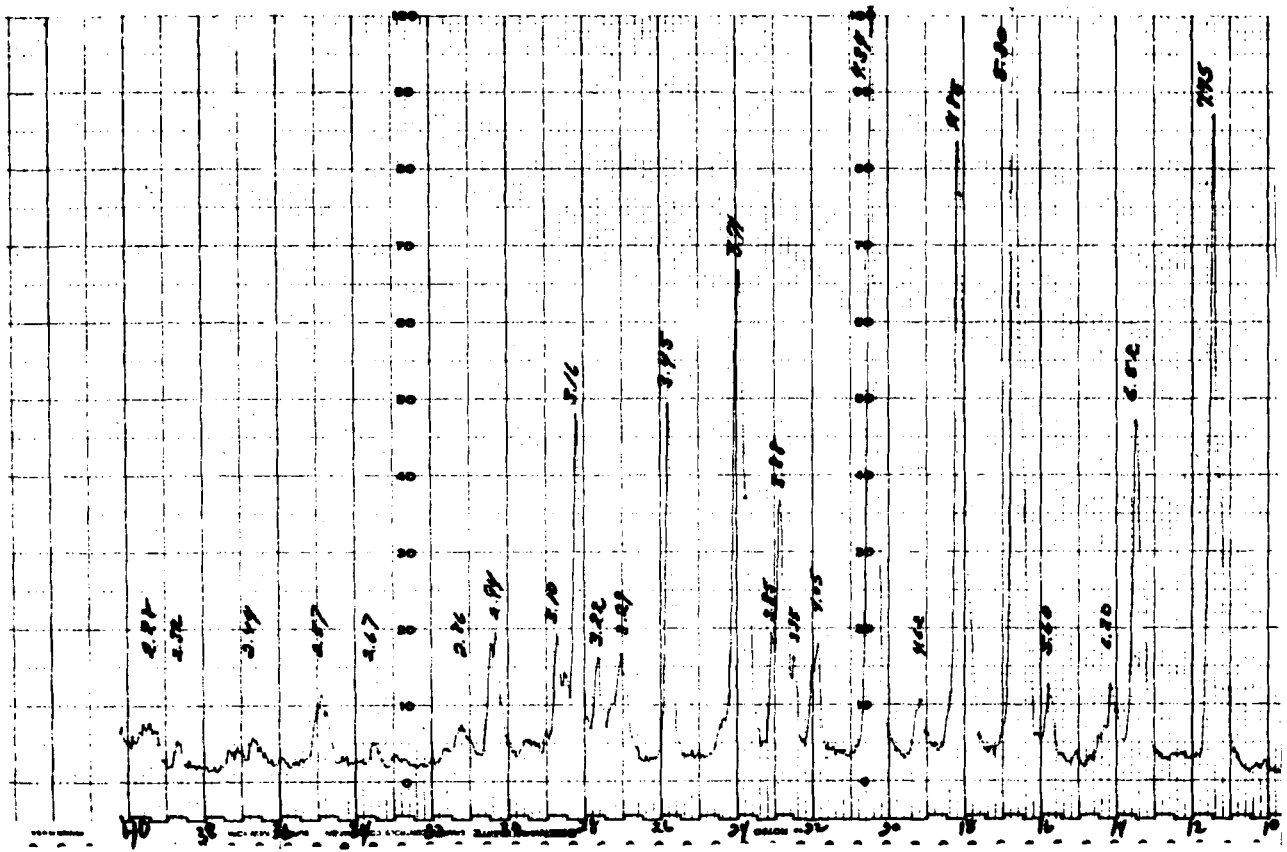


Figure 4. Powder X-ray diffraction pattern of testolactone (batch 41040-102) Instrument: Phillips

3. Synthesis

Testolactone(I) was first isolated and identified as a bio-oxidative product of the fermentation of progesterone(II), with Cylindrocarpon radicola by Fried, Thoma and Klingsberg[1], (see Figure 5). Peterson, Thoma, Perlman and Fried[2] were able to show that 1-dehydro-testosterone(III) and $\Delta^{1,4}$-androstadiene-3,17-dione(IV) are intermediates in this conversion. Conversion of testololactone(V) to testolactone(I) by the same microorganism has been observed[5]. Other microorganisms have also been used for the production of testolactone[6-11,19]. Patents also have been issued[12-17]. A chemical synthesis of testolactone starting with epiandrosterone acetate via epiandrololactone, and dihydrotestololactone has been reported[18].

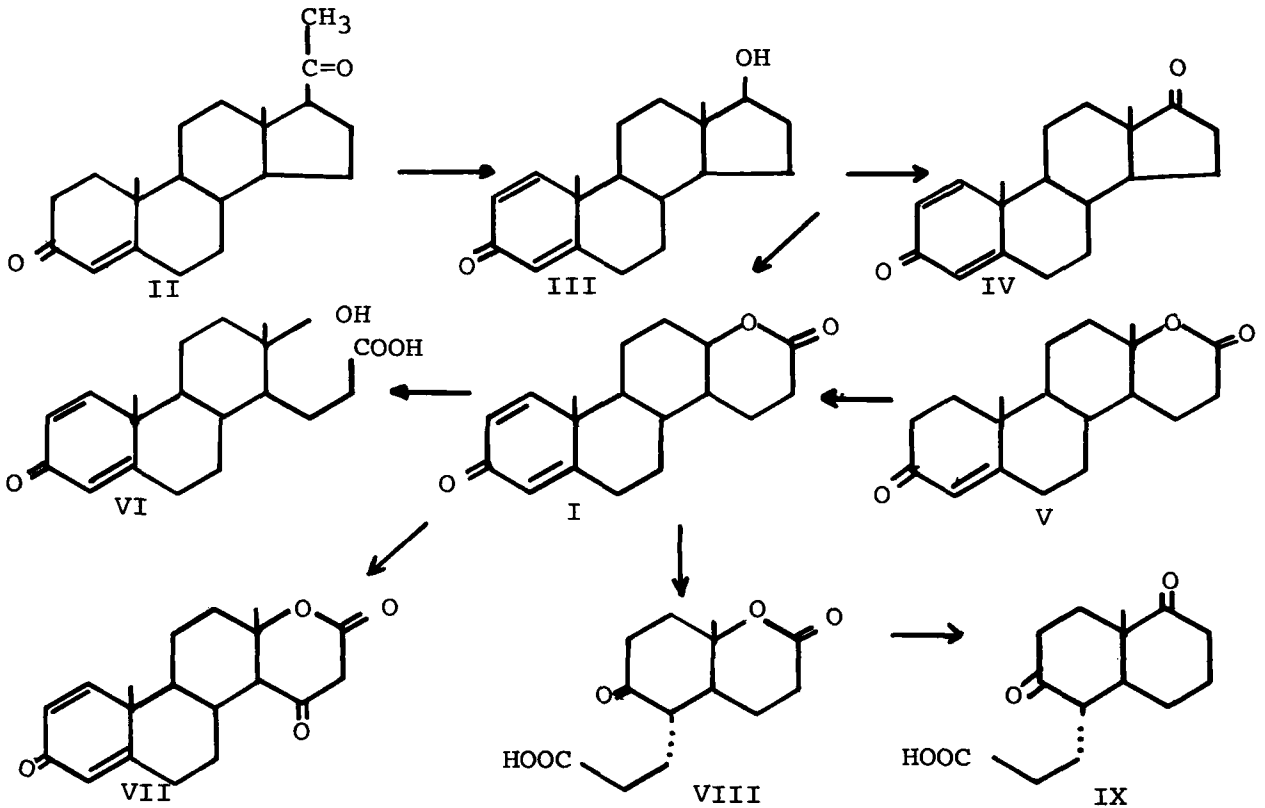


Figure 5. Synthesis and Degradation.

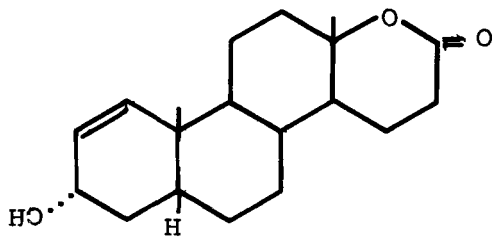
4. Stability, Degradation

Testolactone is very stable as a solid. In strongly alkaline solution the lactone ring will open to Δ^1-testolic acid[20](VI). This can also be accomplished by microbiological degradation. Other microbiological transformation products are 15-keto-testo-lactone(VII)[21] 1-methyl-cyclohexan-1-ol-4-keto 2,3-diproprionic acid lactone (VIII) and surprisingly 7α-methylhexahydrindan-1,5-dione-4α-(3-propionic acid) (IX)[22] (see Table 4).

It has been reported[23] that hydrocortisone and prednisolone when exposed to ultraviolet radiation or ordinary fluorescent laboratory lighting in alcoholic solutions, undergo photolytic degradation of the A-ring. Since testolactone has the same A-ring as prednisolone it probably also is labile under these conditions.

5. Drug Metabolism

In human subjects the urinary metabolites found were unchanged testolactone and 3α, 13α-dihydroxy-13,17-seco-5β-androsta-1-ene-17-oic acid lactone the latter both in the free form and as the glucuronide.



Bovine blood albumin contains a Δ^4-5β-hydrogenase system which also reduces testolactone[4].

6. Methods of Analysis

6.1 Elemental Analysis

	Calc for $C_{19}H_{24}O_3$	Found	
		Fried, et. al[1]	House Std.[27]
C	75.97	76.29	75.89
H	8.05	7.81	8.23

6.2 Phase Solubility Analysis

The purity of Squibb House Standard lot 41040-102 was found to be 99.6% pure by phase solubility analysis[27]. The solvent system used was n-propanol-methanol (2:1). Equilibration was carried out over 24 hours at 25°C. The extrapolated solubility was determined at 25.5 mg/g of solvent.

6.3 Colorimetric Analysis

The reaction of the 3-keto-$\Delta^{1,4}$-diene system of testolactone with isonicotinic acid hydrazide to form a hydrazone with an absorption maximum at 415 nm[30] has been made the basis for the compendial assay of testolactone itself and its dosage forms[29].

As other steroid lactones, testolactone forms a pink chromogen when subjected to the action of potassium hydroxide, hydroxylamine and ferric chloride[29,31]. This has been used for a compendial identity test[29].

6.4 Fluorometric Analysis

When testolactone is heated in 85% phosphoric acid at 100°C for 30 minutes a fluorogen is formed which on dilution with methanol exhibits excitation and fluorescent spectra with maxima at 275 nm and 375 nm, respectively. This can be used for the determination of testolactone in fermentation broth.

Progesterone and 1-dehydro-progesterone do not interfere[31].

6.5 Nonaqueous Titration

It has been reported[32] that testolactone after precipitation with sodium phenylborate can be titrated with cetyl pyridinium chloride and thymol blue indicator with acceptable accuracy.

6.6 Chromatographic Analysis

6.61 Paper Chromatographic Analysis

The following paper chromatographic solvent systems have been reported:

1.) Methycyclohexane-carbitol[2].
2.) Toluene-propylene glycol[2].
3.) Propylene glycol-cyclohexane-chloroform[7].
4.) Toluene saturated with propylene glycol[33].
5.) Impregnation with formamide-methanol(20:80) then methyl isobutyl ketone-formamide (20:1)[33].
6.) Methylcyclohexane-chloroform(4:1)[33].

All systems separate testolactone from progesterone, $\Delta^{1,4}$-androsta-diene-dione and testolactone. In system 4 progesterone and testololactone have greater mobilities than testolactone. In system 5 Δ^{14}- and Δ^{15}-testolactone (both with an Rf of 0.68) can be separated from testolactone (Rf 0.60). In system 6 the order of separation (with increasing Rf) is as follows: testolactone(Rf 0.23) testololactone, testosterone, and progesterone. System 4, 5 and 6 have also been used for quantitation following the general procedure of Roberts and Florey[34]. In systems 4, 5 and 6 elution with an acidified methanolic solution of isonicotinic acid hydrazide was used, followed by quantitation (see section 6.3). Alternately in system 6 elution with 95% ethanol and measure-

ment of absorption at 242 nm can also be used[33].

6.62 Thin Layer Chromatographic Analysis

The following thin-layer chromatographic solvent systems have been reported:

1.) The method of Belic and Socic[19] is summarized in Table 3:

Table 3. Rf-values and color reaction of steroids with 50% sulphuric acid on silica gel developed with cyclohexane/ethylacetate (1:2).

Steroid	Rf	Daylight Color	Relative Intensity	Fluorescence
Progesterone	0.63	yellow	weak	greenish
Δ^4-Androstene-3,17-dione	0.52	blue-green	strong	pale-blue
Testosterone	0.44	blue-green	strong	blue
$\Delta^{1,4}$-Androstadiene-3,17-dione	0.45	bright-red	intense	orange
1-Dehydrotestosterone	0.36	ochre-red	strong	orange
Testololactone	0.24	blue-green	medium	green
1-Dehydrotestololactone	0.18	ochre	medium	orange
1-Dehydroprogesterone	0.53	pink	weak	ochre
$\Delta^{1,4}$-Androstadien-3,17-dione[a]	0.45	bright-red	intense	orange
Testosterone[a]	0.40	blue-green	strong	blue

[a]developed with benzene/ethylacetate(1:1).

2.) Ethyl-acetate-hexane(5:5) or chloroform-methanol(97:3) on silica gel HF coated glass plates. Detection by U.V. light[5].

3.) Chloroform-acetone (94:6) on silica gel SF. Detection by U.V. light or sulfuric acid spray. The following Rf values were found: testolactone 0.22; Δ^1-testosterone, 0.27; androstadienedione, 0.49; Δ^1-progesterone, 0.58; progesterone, 0.61[11].

4.) Butylacetate-acetone(4:1) on silica gel. Detection by U.V. light[29].

6.63 Column Chromatographic Analysis

Column chromatography on alumina[1] or silica gel[3,14] has been used to separate testolactone from other steroids and impurities in fermentation extracts. Petroleum ether-chloroform (1:1)[3] and ethyl acetate[5] have been used as elution solvents.

6.64 Vapor Phase Chromatographic Analysis

Testolactone has been quantitated together with its bioconversion precursors on a 6-ft glass column containing 3% SE-30 on 80-100 mesh Diatoport. Column and flame-ionization detector temperatures were 250°C and the carrier gas was helium at 50 ml/min[11].

7. Determination in Pharmaceutical Preparations

In addition to the compendial isonicotinic acid hydrazide assay[20] (see section 6.3), the paper chromatographic systems (see section 6.61) 2 and 6 have been used as a stability indicating assay for testolactone in suspension and tablets[33] following the general procedure of Roberts and Florey[34].

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ADDENDA AND ERRATA

Diatrizoic Acid
Volume 4, p. 151

Under 5.2, Free Iodine and Free Halide, replace sentence starting with, "An alternate procedure....." with the following:

For detection of free iodine and iodide, the filtrate is acidified, chloroform and sodium nitrite are added, and the reddish color in the chloroform layer is compared to a standard solution (4,12).

Isosorbide Dinitrate
Volume 4, p. 225

The correct name of the first author is Sivieri, not Silvieri.

Phenformin Hydrochloride
Volume 4, p. 319

To the embarrassment and regret of the editor, the full list of authors was inadvertently not presented by him for this Profile. The correct title is:

Phenformin Hydrochloride

Michael J. O'Hare, Hridaya Bhargava, Ruth Wasserman and Joseph E. Moody

5. Chemical ionization mass spectrometry was used for the determination of Phenformin Hydrochloride in biological fluids by S. B. Matin, J. K. Karam, P. H. Forsham and J. B. Knight, Biomedical Mass Spectrometry 1, 320 (1974).

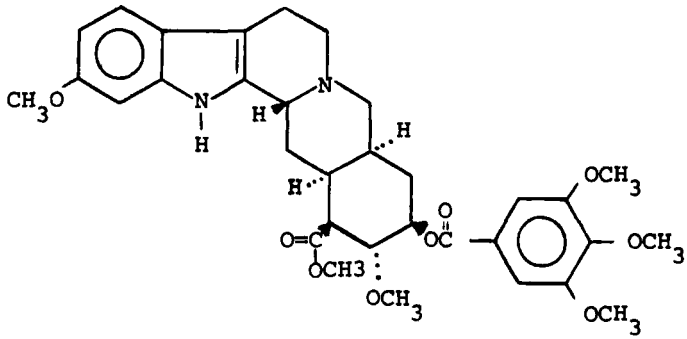
6.5 An HPLC method for the determination of Phenformin Hydrochloride has been developed by R. K. Gilpin, J. A. Korpi and C. A. Janicki, J. of Chromat. 107, 115 (1975).

Reserpine

Volume 4, p. 386

It was pointed out by Prof. H. Vanderhaeghe of Leuven, Belgium that in the structural formula on p. 386 the substituent on C15 should be H (see formula on p. 400). Also, since the absolute configuration is known (see Klyne and Buckingham, "Atlas of Stereochemistry," Chapman and Hall (1974)), the structure given is that of the inactive enantiomer.

The correct structural formula is:



Tolbutamide

Volume 3, p. 513

5. Solid probe chemical ionization mass spectrometry and gas chromatography has been used for the determination of Tolbutamide and its metabolites in human plasma by S. B. Matin and J. B. Knight, Biomedical Mass Spectrometry 1, 323 (1974).

Triflupromazine Hydrochloride

Volume 2, p. 523

2.5 pKa

The pKa of 6.5 as listed in Volume 2, p. 533 is in error. Reexamination by Dr. H. Jacobson (The Squibb Institute) gave a pKa value of 9.45, in reasonable agreement with values determined by

references 8 and 9.

2.10 The crystal structure of Triflumpromazine Hydrochloride has been determined by x-ray diffraction by D. W. Phelps and A. W. Cordes, Acta Cryst. B30, 2812 (1974).

CUMULATIVE INDEX

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