Analytical Profiles of Drug Substances

Volume 16

Edited by

Klaus Florey

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EDITORIAL BOARD

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The Squibb Institute for Medical Research
New Brunswick, New Jersey

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PREFACE

Although the official compendia define a drug substance as to identity, purity, strength, and quality, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. Such information is scattered through the scientific literature and the files of pharmaceutical laboratories.

I perceived a need to supplement the official compendial standards of drug substances with a comprehensive review of such information, and sixteen years ago the first volume of *Analytical Profiles of Drug Substances* was published under the auspices of the Pharmaceutical Analysis and Control Section of the APhA Academy of Pharmaceutical Sciences. That we were able to publish one volume per year is a tribute to the diligence of the editors to solicit articles and even more so to the enthusiastic response of our authors, an international group associated with pharmaceutical firms, academic institutions, and compendial authorities. I would like to express my sincere gratitude to them for making this venture possible.

Over the years, we have had queries concerning our publication policy. Our goal is to cover all drug substances of medical value and, therefore, we have welcomed any articles of interest to an individual contributor. We also have endeavored to solicit profiles of the most useful and used medicines, but many in this category still need to be profiled.

Klaus Florey

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BROMAZEPAM

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Analytical Profile of Bromazepam

1. History and Therapeutic Category

The last quarter century, and in particular the decade 1952-1962, has witnessed a break-through in the treatment and outlook of the mentally ill. Two groups of drugs were developed namely psychotic and psychotropic agents. Among the last named psychotropic agents, the benzodiazepines. These drugs such as diazepam, chlordiazepoxide and oxazepam have been shown to exert anxiolytic, sleep inducing, muscle relaxant and anticonvulsant effects in greater or lesser degree. Search continued for making more derivatives to produce suitable drug for specific cases or symptoms. In this way nitrazepam and more recently flurazepam have been found to be specially effective sleep inducers and clonazepam exerts even more potently the anticonvulsant activity known already for diazepam.

Search has continued and in 1974 bromazepam was marketed as a new psychotropic drug of the benzodiazepine series but belonging to a new class of pyridyl-benzodiazepines. It possesses certain biochemical features which distinguish it from other benzodiazepines. It has been shown to exert a more profound anxiolytic effect than other similar substances.

2. Description

2.1 Nomenclature

2.1.1 Chemical Names

7-Bromo-1,3-dihydro-5-(′2-pyridyl)-2H-1,4-benzodiazepine-2-one.

2.1.2 Generic Name

Bromazepam.

2.1.3 Trade Names

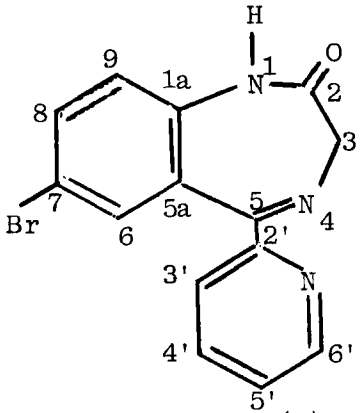
Lectopam; Lexomil; Lexotan; Lexotanil.

2.2 Formulae

2.2.1 Empirical

$C_{14}H_{10}BrN_3O$

2.2.2 Structural



2.2.3 Research Number (1)

Ro 5-3350.

2.2.4 Chemical Abstracts Registry Number

[1812-30-2]

2.3 Molecular Weight

316.16

3. Physical Properties

3.1 Appearance, Color, Odor and Taste

A pale yellow odorless crystalline solid (2).

3.2 Melting Range

237-238.5° (dec.).
247 (2).

3.3 Dissociation Constants

Bromazepam has three pKa values of 2.5, 5.2 and 11.8 corresponding to protonation at the azomethine and pyridine nitrogen atoms, and deprotonation at the nitrogen atom in position 1, respectively. These pKa values were determined by spectral and polarographic analysis (3).

3.4 Crystal Structure (4)

The crystal structure of bromazepam was determined using crystals from amyl acetate-ethanol obtained by slow evaporation. The intensity data were collected on an Enraf-Nonius CAD-4 diffractometer, crystal size 0.3 x 0.25 x 0.2 mm, cell dimentions from setting angles of 25 reflections and graphite monochromated MoKa radiation. Bromazepam is monoclinic with space groups $P2_1/c$ (non-centrosymetric). The cell dimentions are a = 10.304 (4), b = 15.897 (5), c = 8.122 (3) A°, β = 106.8 (3)°, U = 1273.6 A°, Z = 4, Dx = 1.649 Mg m^{-3}, μ(MoKa, λ = 0.71069 A°) = 3.13 mm^{-1}, F(000) = 632, room temperature, R = 0.040 for 1470 observed reflections.

In bromazepam the 7-membered ring is in a boat conformation. The angle between the benzo-moeity of the 1,4-benzodiazepine system and the heterocyclic ring system in the 5-position is 60.3 (6)°. Final atomic parameters for bromazepam are listed in Table 1; bond lengths, bond angles and selected torsion angles are listed in Table 2. The atomic numbering scheme is illustrated in Fig. 1. Bond lengths and angles generally agree well with values found in analogous molecules (5-7). The N(1)-C(2) formal single bond is shortened to 1.351 (5) A° in bromazepam and the disposition of bonds at N(1) is near planar so that the geometry of the bond resembles that of a normal double bond (cf. torsion angles in Table 2) (6). The N(4)-C(5) length in bromazepam corresponds to that of a C = N bond and the C(5)-C(1') and C(5)-C(11) lengths are within the accepted range (1.48-1.50 A°) for a $C(sp^2)$-$C(sp^2)$ single bond. There is, therefore, no evidence for any electron delocalization between the 5-pyridyl ring in bromazepam and the 1,4-benzodiazepine system.

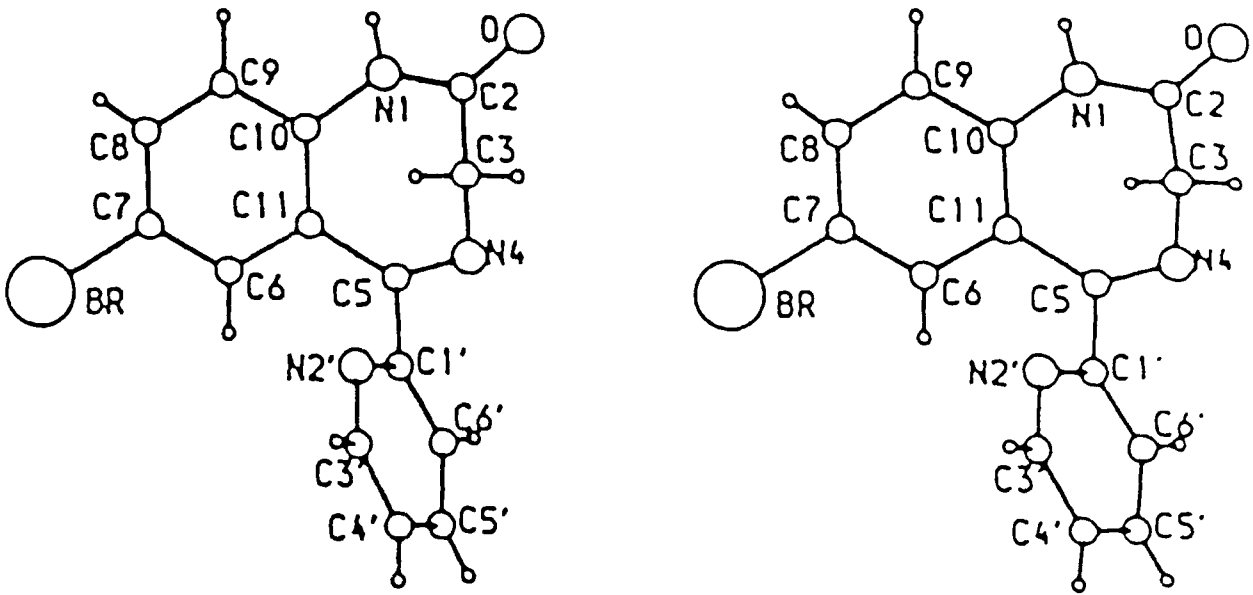


Fig. 1. The molecule of bromazepam viewed in a direction perpendicular to the mean plane through C(6)-C(11).

The seven-membered ring is in a cycloheptatriene-like boat conformation, C(10) and C(11) forming the 'stern' and C(3) the 'bow'. The bow angles, 60.3 (8°) in bromazepam and the stern angle 30.8 (8°).

Table 1. Fractional atomic coordinates ($\times 10^4$) with e.s.d.'s in parentheses and equivalent isotropic temperature factors ($A^2 \times 10^3$)

$$U_{eq} = 1/3\ (U_{11} + U_{22} + U_{33} + 2U_{13} \cos\beta).$$

	x	y	z	U_{eq}
Br	-1601 (1)	617 (1)	1023 (1)	184
O(2)	-8156 (3)	-1404 (3)	-6128 (5)	177
N(1)	-6566 (4)	-528 (2)	-4561 (5)	94
N(4)	-5071 (3)	-2169 (2)	-4171 (5)	102
N(2')	-2083 (3)	-1209 (2)	-4314 (5)	95
C(2)	-7119 (4)	-1298 (3)	-4969 (6)	110
C(3)	-6361 (5)	-2004 (3)	-3843 (8)	163
C(5)	-4128 (4)	-1626 (5)	-3632 (5)	86
C(6)	-3115 (5)	-520 (3)	-1495 (6)	101
C(7)	-3149 (4)	237 (3)	-701 (6)	104
C(8)	-4299 (5)	726 (3)	-1165 (6)	108
C(9)	-5411 (5)	449 (3)	-2404 (6)	119
C(10)	-5406 (4)	-324 (3)	-3221 (5)	78
C(11)	-4240 (4)	-821 (2)	-2761 (5)	75
C(1')	-2782 (4)	-1841 (3)	-3875 (5)	79
C(3')	-885 (4)	-1400 (3)	-4510 (6)	110
C(4')	-308 (5)	-2190 (3)	-4251 (6)	126
C(5')	-1025 (5)	-2820 (4)	-3779 (6)	125
C(6')	-2287 (5)	-2650 (3)	-3622 (6)	99

The major conformational difference between this compound and other known 5-phenyl-1,4-benzodiazepines is in the orientation of the 5-aryl ring. The angle between the mean plane of the 5-aryl ring and the 'benzo' plane is 60.3 (6°) in bromazepam and 75.5 (9°) in other benzodiazepines (8).

Table 2. Molecular dimensions

(a) Bond lengths (A°)			(b) Bond angles (°)		
N(1)-C(2)	1.351	(5)	C(12)-N(1)-C(2)	-	
C(2)-O(2)	1.214	(5)	C(12)-N(1)-C(10)	-	
C(2)-C(3)	1.453	(6)	H(1)-N(1)-C(2)	119	(3)
N(4)-C(5)	1.278	(5)	H(1)-N(1)-C(10)	113	(3)
C(5)-C(11)	1.483	(5)	C(10)-N(1)-C(2)	127.1	(4)
C(6)-C(11)	1.394	(6)	N(1)-C(2)-O(2)	122.1	(5)
C(6)-C(7)	1.371	(6)	N(1)-C(2)-C(3)	114.6	(4)
C(7)-C(8)	1.376	(7)	C(3)-C(2)-O(2)	123.3	(4)
C(8)-C(9)	1.362	(6)	C(2)-C(3)-N(4)	111.3	(4)
C(9)-C(10)	1.397	(6)	C(3)-N(4)-C(5)	117.4	(4)
C(10)-N(11)	1.395	(6)	N(4)-C(5)-C(11)	126.1	(4)
C(10)-N(1)	1.402	(5)	N(4)-C(5)-C(1')	116.3	(4)
Br-C(7)	1.892	(4)	C(11)-C(5)-C(1')	117.6	(3)
N(7)-C(7)	-		C(11)-C(6)-C(7)	121.3	(4)
N(7)-O(7A)	-		C(6)-C(7)-C(8)	120.3	(4)
N(7)-O(7B)	-		C(6)-C(7)-Br	119.8	(4)
C(12)-N(1)	-		C(8)-C(7)-Br	119.8	(3)
H(1)-N(1)	0.88	(5)	C(6)-C(7)-N(7)	-	
C(5)-C(1')	1.495	(6)	C(8)-C(7)-N(7)	-	
C(1')-N(2')	1.343	(5)	C(7)-N(7)-O(7A)	-	
C(1')-C(2')	-		C(7)-N(7)-O(7B)	-	
N(2')-C(3')	1.325	(6)	O(7A)-N(7)-O(7B)	-	
C(2')-C(3')	-		C(7)-C(8)-C(9)	119.5	(4)
C(3')-C(4')	1.380	(7)	C(8)-C(9)-C(10)	121.2	(4)
C(4')-C(5')	1.363	(7)	C(9)-C(10)-C(11)	119.5	(4)
C(5')-C(6')	1.370	(7)	C(9)-C(10)-N(1)	116.9	(4)
C(1')-(6')	1.378	(6)	C(11)-C(10)-N(1)	123.5	(4)
F-C(2')	-		C(10)-C(11)-C(6)	118.1	(4)
			C(10)-C(11)-C(5)	112.8	(4)
			C(6)-C(11)-C(5)	119.0	(4)
			C(5)-C(1')-N(2')	117.9	(4)
			C(5)-C(1')-C(2')	-	
			C(5)-C(1')-C(6')	120.7	(4)
			C(2')-C(1')-C(6')	-	
			N(2')-C(1')-C(6')	122.3	(4)
			C(2')-C(2')-C(3')	116.8	(4)
			C(1')-C(2')-C(3')	-	
			C(1')-C(2')-F	-	
			C(3')-C(2')-F	-	
			C(2')-C(3')-C(4')	-	
			N(2')-C(3')-C(4')	124.4	(5)
			C(3')-C(4')-C(5')	117.8	(5)
			C(4')-C(5')-C(6')	119.2	(5)
			C(5')-C(6')-C(1')	119.4	(5)

(c) Selected torsion angels (°); e.s.d.'s ca 0.6°

C(10)-N(1)-C(2)-C(3)	-1.1	C(11)-C(10)-N(1)-C(2)	39.8
N(1)-C(2)-C(3)-N(4)	-70.3	N(4)-C(5)-C(1')-N(2')	141.6
C(2)-C(3)-N(4)-C(5)	72.3	N(4)-C(5)-C(1')-C(2')	-
C(3)-N(4)-C(5)-C(11)	-2.7	C(11)-C(5)-C(1')-N(2')	-39.8
N(4)-C(5)-C(11)-C(10)	-37.6	C(11)-C(5)-C(1')-C(2')	-
C(5)-C(11)-C(10)-N(1)	-1.2		

4. Spectral Properties

4.1 Ultraviolet Spectrum

The ultraviolet spectrum of bromazepam in methanol is shown in Fig. 2. The spectra of bromazepam in 1N NaOH and 1N HCl are presented in Fig. 3 and Fig. 4, respectively. The $E^{1\%}_{1\,cm}$ and maximum wavelengths are given in Table 3 (9). These values agrees with the published data (10-12). Levillain (13), has studied the relationship of structure and the UV absorption characteristics of a series of 1,4-benzodiazepines, including bromazepam, considering the electronic distribution of the various substituents and the stereochemistry. The spectrum of bromazepam is consistent with that of other benzodiazepines with similar structure. Also bromazepam was identified in solid dosage forms by UV absorption spectrometry (14).

Table 3. UV Spectral characteristics

Solvent	λ_{max} (nm)	$E^{1\%}_{1\,cm}$
Methanol	320	54.54
	258	398.08
	233	1004.78
1N NaOH	350	59.33
	268	493.77
	238	794.25
1N HCl	348	39.23
	270	386.60
	238	552.15

The above values agrees with that of bromazepam.

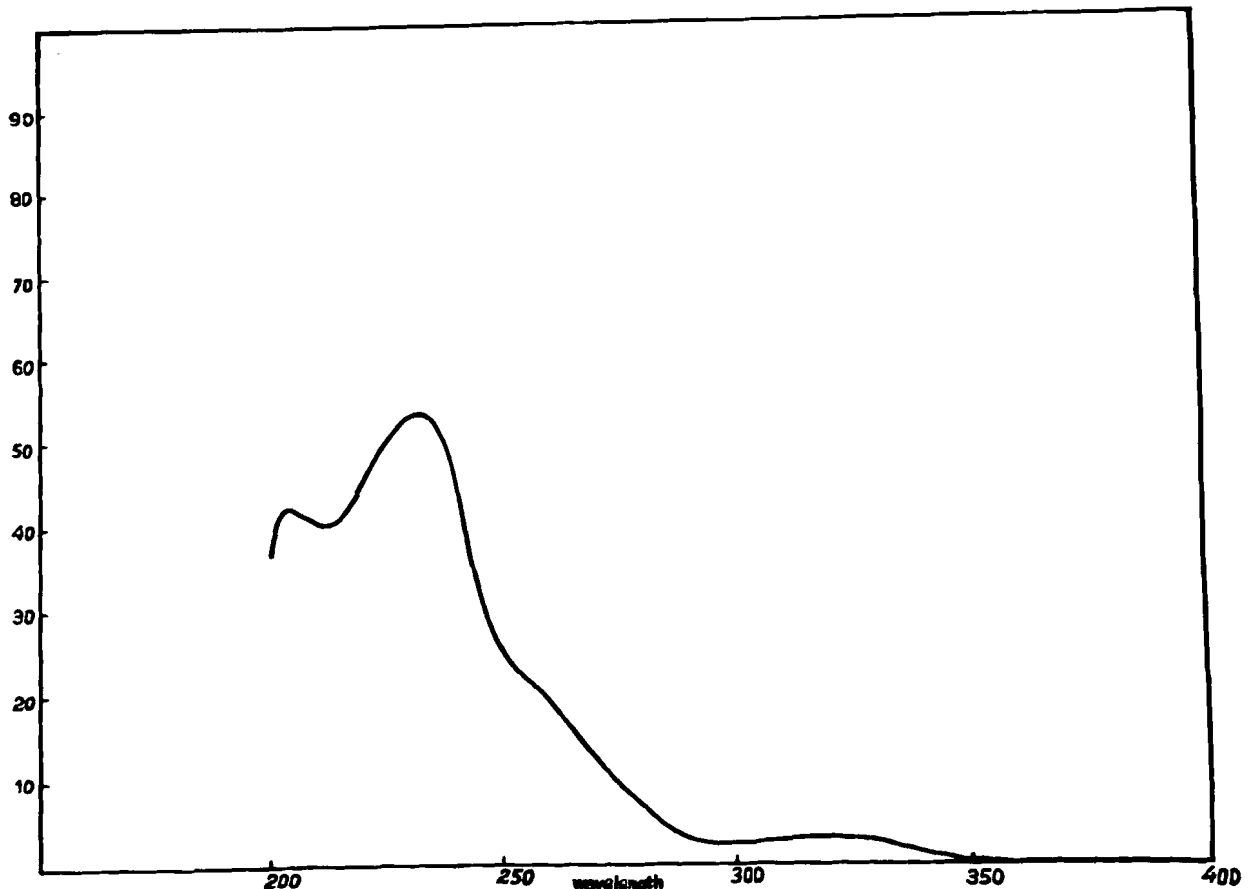


Fig. 2. The UV spectrum of bromazepam in methanol.

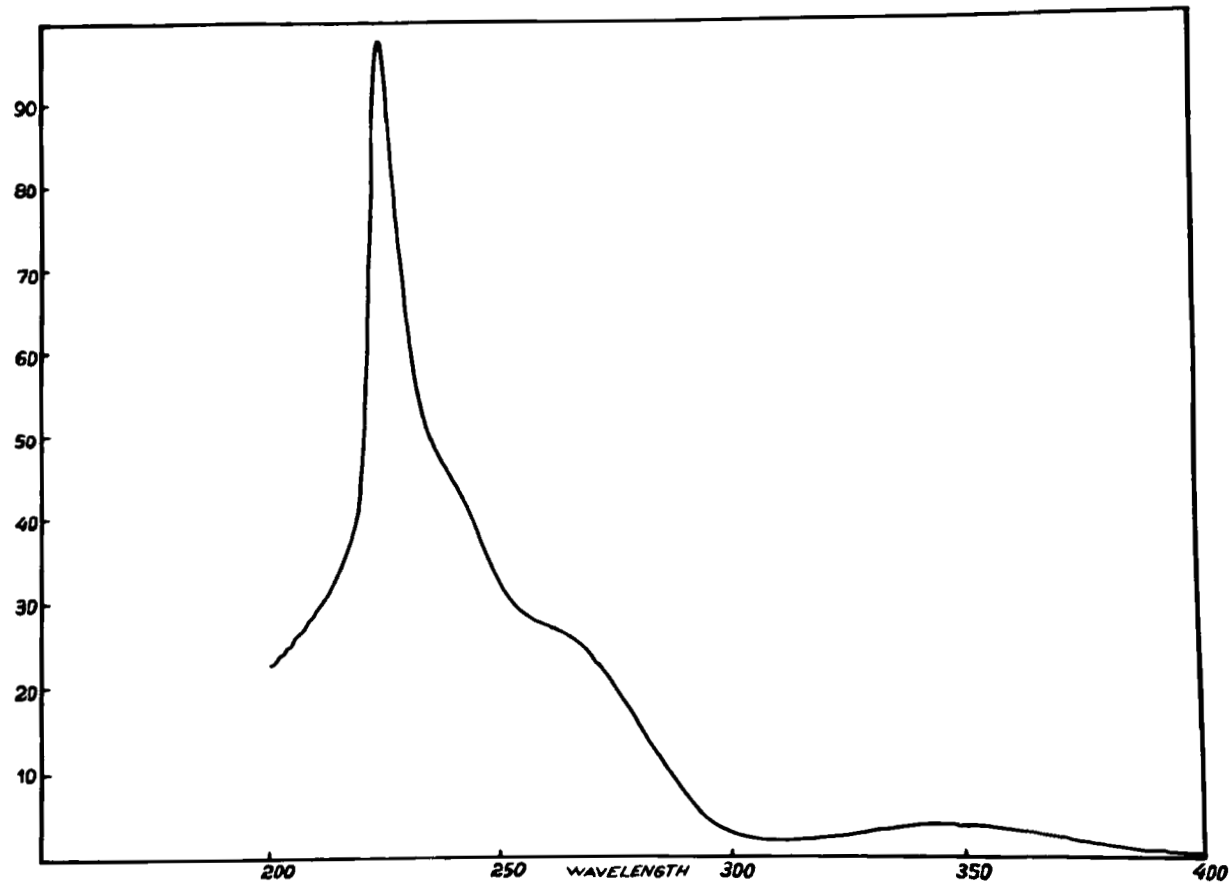


Fig. 3. The UV spectrum of bromazepam in 1N NaOH.

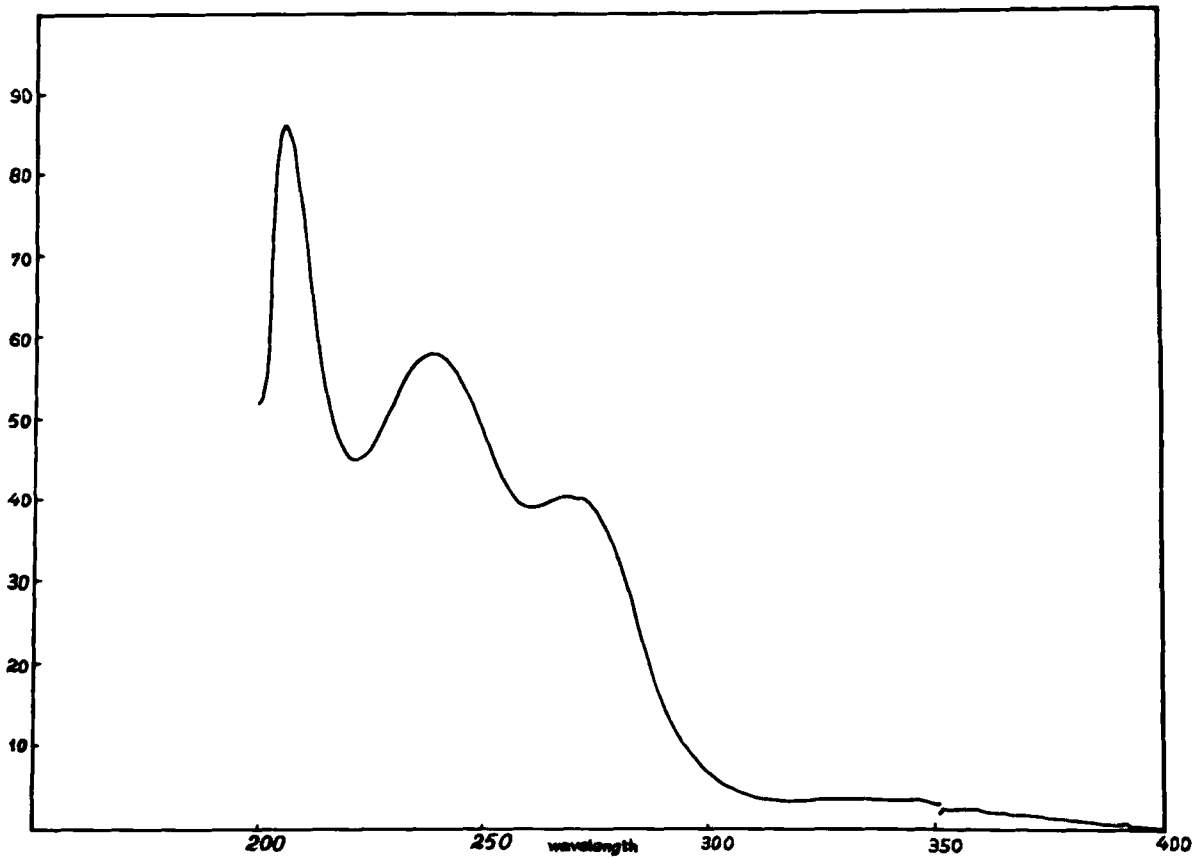


Fig. 4. The UV spectrum of bromazepam in 1N HCl.

4.2 Infrared Spectrum

An infrared absorption spectrum of potassium bromide dispersion of bromazepam (Roche - Reference Standard Lot 0401055) is shown in Fig. 5. The spectral band assignments (9,16,17) listed in Table 4.

Table 4. Infrared spectral assignments of bromazepam

Wave number (cm^{-1})	Assignment (Vibration mode)
3220,3150, 3080	N-H stretch
3000,2940,2860	C-H stretch
1695	C = O stretch
1615	C = N stretch of both benzodiazepine and pyridine.
1600,1590,1570,1470	Aromatic C = C stretch
815	Out of plane CH-deformation of 1,2,4-trisubstituted aromatic
715	Out of plane CH deformation of ortho-disubstituted aromatic
750-665	1,2,4-Trisubstituted aromatic and o-disubstituted aromatic. Also 2-substituted pyridine.

Other characteristic bands are 1440, 1385, 1340, 1320, 1230, 1200, 1090, 1040, 1000, 990, 890, and 790 cm^{-1}. Infrared has been used for identification of bromazepam with other psychotropic drugs (18). Bromazepam was identified in solid dosage forms by IR absorption spectrometry (14).

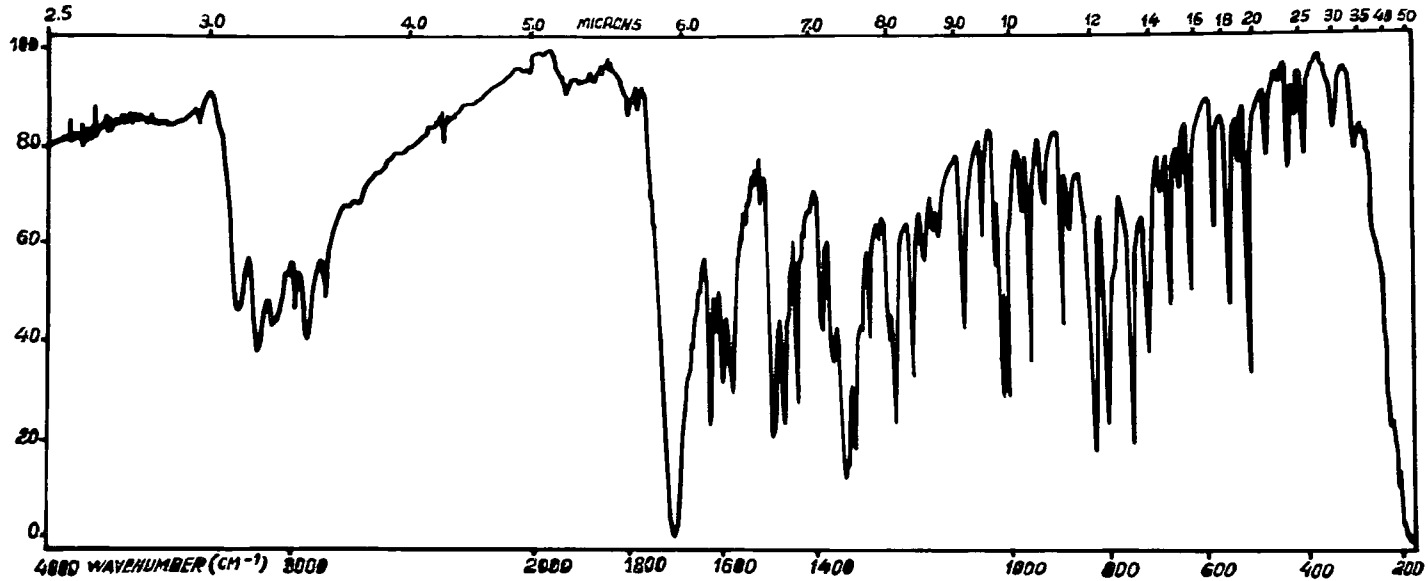


Fig. 5. The IR spectrum of bromazepam as KBr disc.

4.3 Nuclear Magnetic Resonance Spectra

4.3.1 ^{1}H-NMR Spectrum

A typical ^{1}H-NMR spectrum of bromazepam is shown in Fig. 6. The sample was dissolved in DMSO d6 and the spectrum was recorded on a 400 MHz NMR spectrometer, using TMS as an internal reference standard. NMR spectrum recorded on a Jeol-FX-100 90 MHz spectrometer is also shown in Fig 6a. The proton assignments are listed in Table 5. The spectrum after addition of D_2O is shown in Fig. 7.

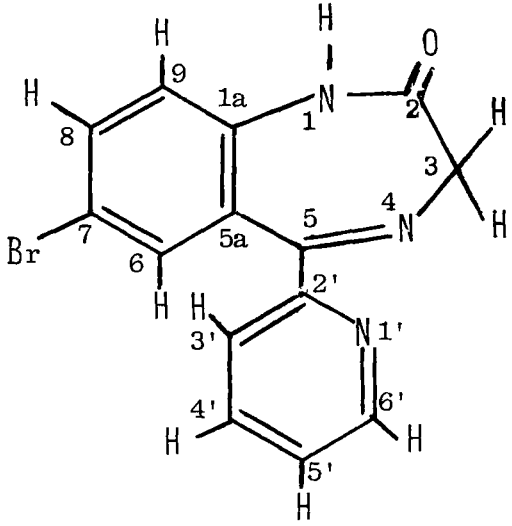


Table 5. PMR characteristics of bromazepam

Proton position	Group	Chemical shift ppm and splitting
C-3	$-CH_2$	4.22 (s)
C-9	-9H (Benzenoid)	7.23 (d)
C-6	-6H (" ")	7.44 (s)
C-5`	-5`H(Pyridine)	7.49 (m)
C-8	-8H (Benzenoid)	7.41 (m)
C-4`	-4`H(Pyridine)	7.95 (m)
C-3`	-3`H(Pyridine)	8.86 (d)
C-6`	-6`H(Pyridine)	8.57 (d)
N-1	-1H (Diazepine) (disappeared after D_2O exchange)	10.66 (s)

s = singlet d = doublet m = multiplet

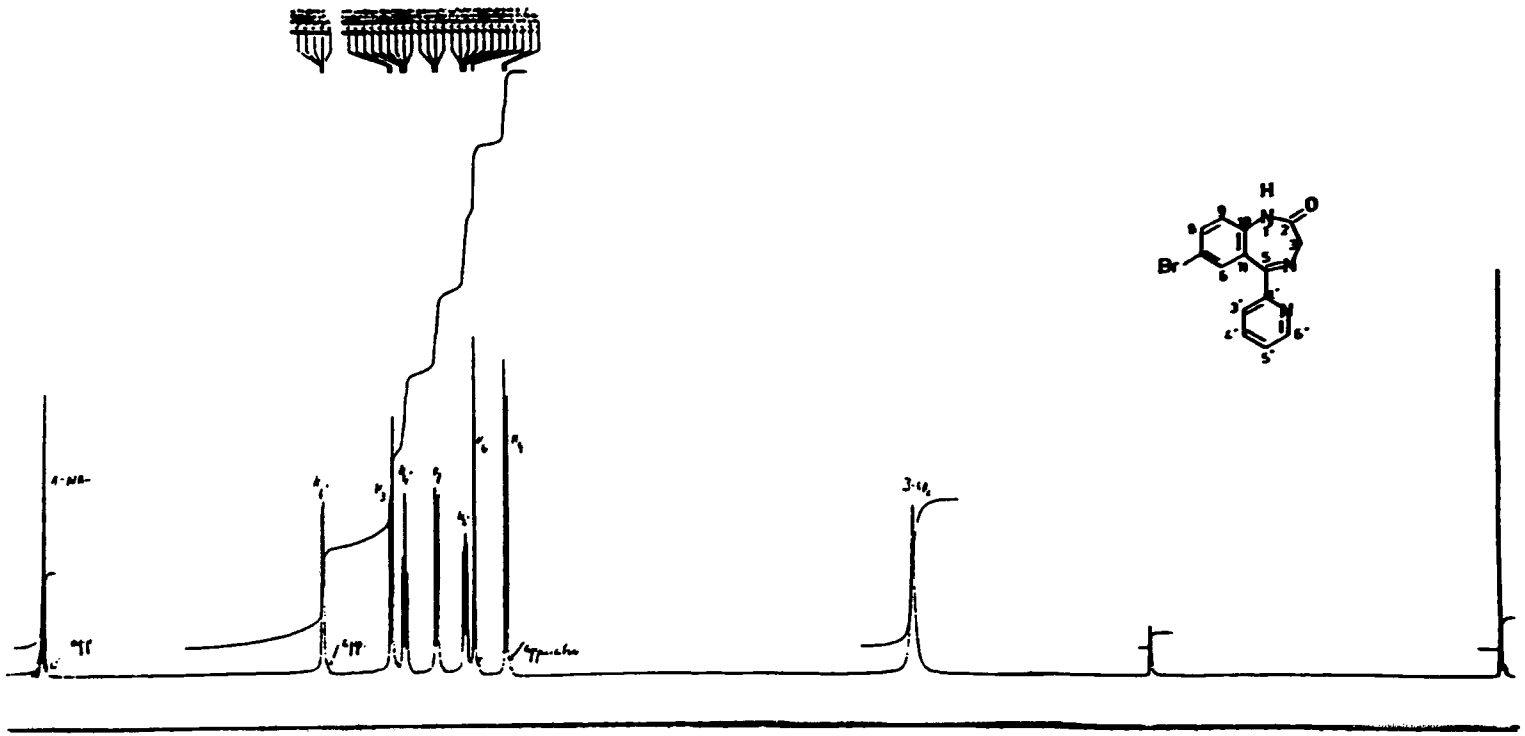


Fig. 6. ^{1}H-NMR spectrum of bromazepam in DMSO d_6 and TMS.

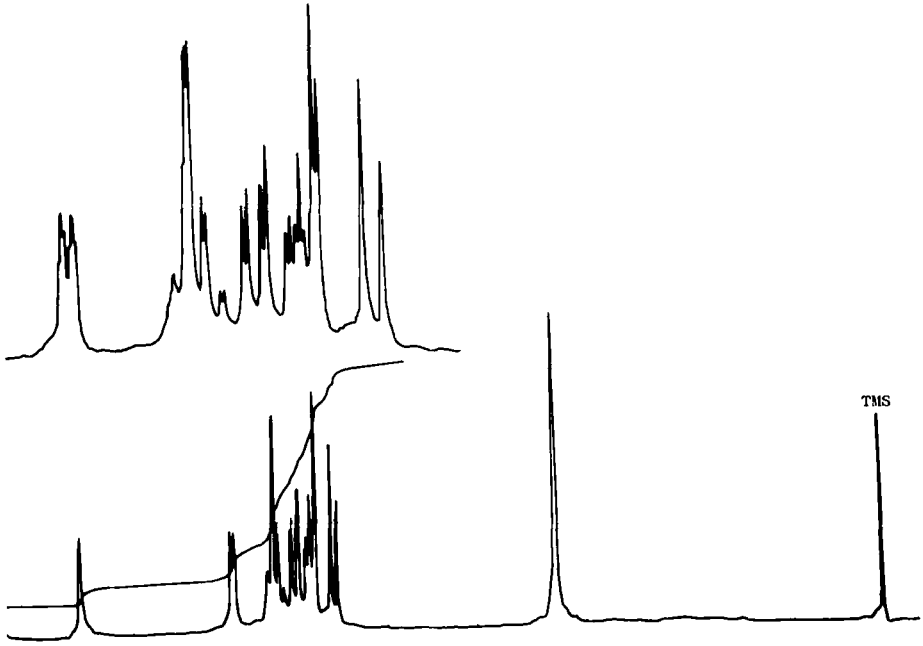


Fig. 6a. ^{1}H-NMR spectrum of bromazepam in DMSO d_6 and TMS.

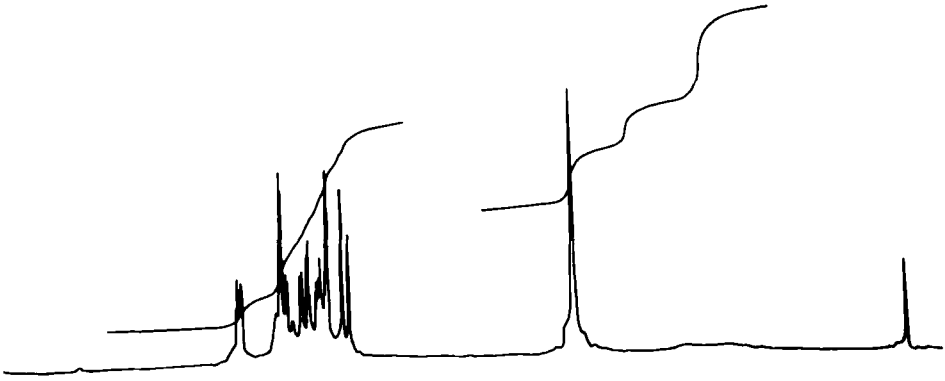


Fig. 7. ^{1}H-NMR spectrum of bromazepam in DMSO d_6 and TMS after D_2O exchange.

4.3.2 ^{13}C-NMR Spectra

^{13}C-NMR noise-decoupled and off-resonance spectra of bromazepam are shown in Fig. 8, and Fig. 9, respectively. Both were recorded over 5000 Hz range in deuterated dimethyl sulfoxide (DMSO d_6), (conc. 100 mg/ 1 ml), on Jeol FX-100 90 MHz instrument. Sample tube 10 mm and tetramethylsilane as an internal reference standard at 20°C were used. The carbon chemical shifts are assigned on the basis of the chemical shift theory and the off-resonance splitting pattern (Table 6).

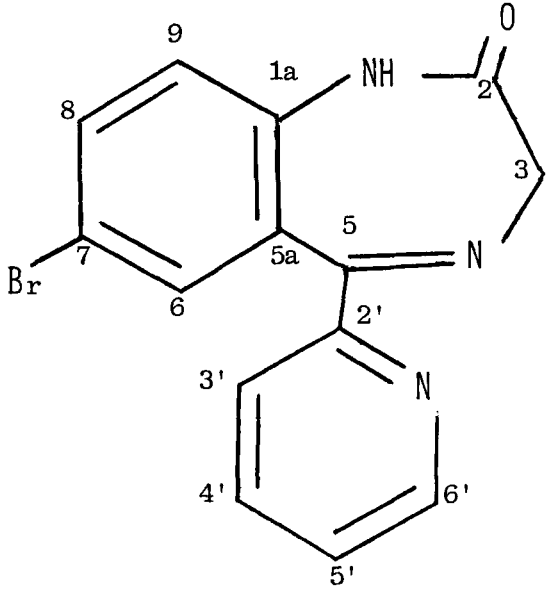


Table 6. Carbon chemical shifts of bromazepam

Carbon No.	Chemical shift δ (ppm)	Carbon No.	Chemical shift δ (ppm)
C-2	169.72 (s)	C-8	133.79 (d)
C-3	56.89 (t)	C-9	123.34 (d)
C-5	167.55 (s)	C-1a	138.49 (s)
C-5a	127.28 (s)	C-2`	155.63 (s)
C-6	133.50 (d)	C-6`	148.24 (d)
C-7	113.94 (s)	C-5`	122.87 (d)
		C-4`	136.96 (d)
		C-3`	124.81 (s)

s = singlet d = doublet t = triplet

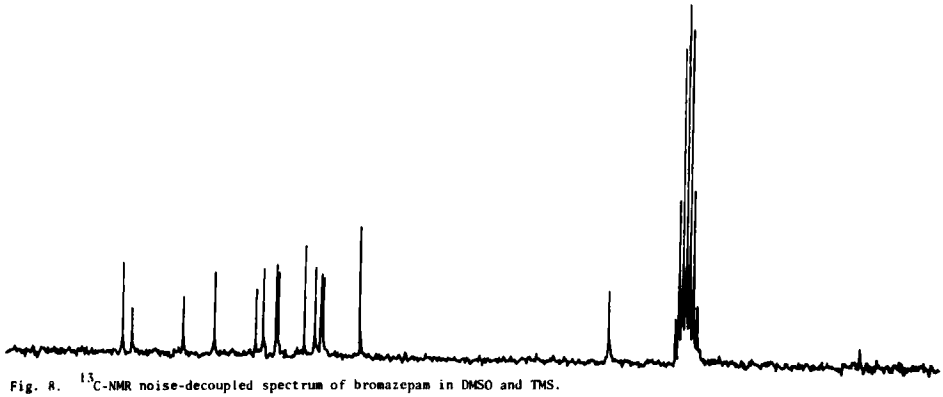


Fig. 8. ^{13}C-NMR noise-decoupled spectrum of bromazepam in DMSO and TMS.

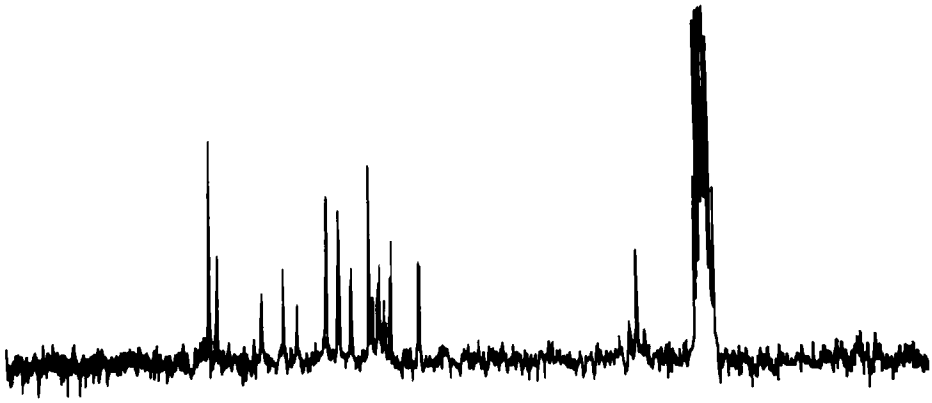


Fig. 9. ^{13}C-NMR off-resonance spectrum of bromazepam in DMSO and TMS.

4.4 Mass Spectrum

The EI mass spectrum of bromazepam (Roche Reference Standard, Lot 0401055) was obtained on a Finigan 1020 quadrupole mass spectrometer and shown in Fig. 10. Its GC trace is shown in Fig. 11. The ionizing electron beam energy was at 70 eV. It shows a molecular ion M^+ at m/e 316 (relative intensity 34.87) and $M^+ + 1$ at m/e 317 (relative intensity 63.88). Some of the most prominent ions are given in Table 7 (9).

Table 7. The most prominent fragments of bromazepam

m/e	Relative intensity	m/e	Relative intensity
317	63.88	236	100.00 (base peak)
316	34.87	208	43.56
315	60.70	207	30.37
314	26.84	206	23.33
289	33.24	179	30.89
288	59.95	104	21.09
287	35.95	90	35.54
286	52.84	79	19.69
261	19.28	78	31.90
259	19.36	77	24.08
		51	25.67

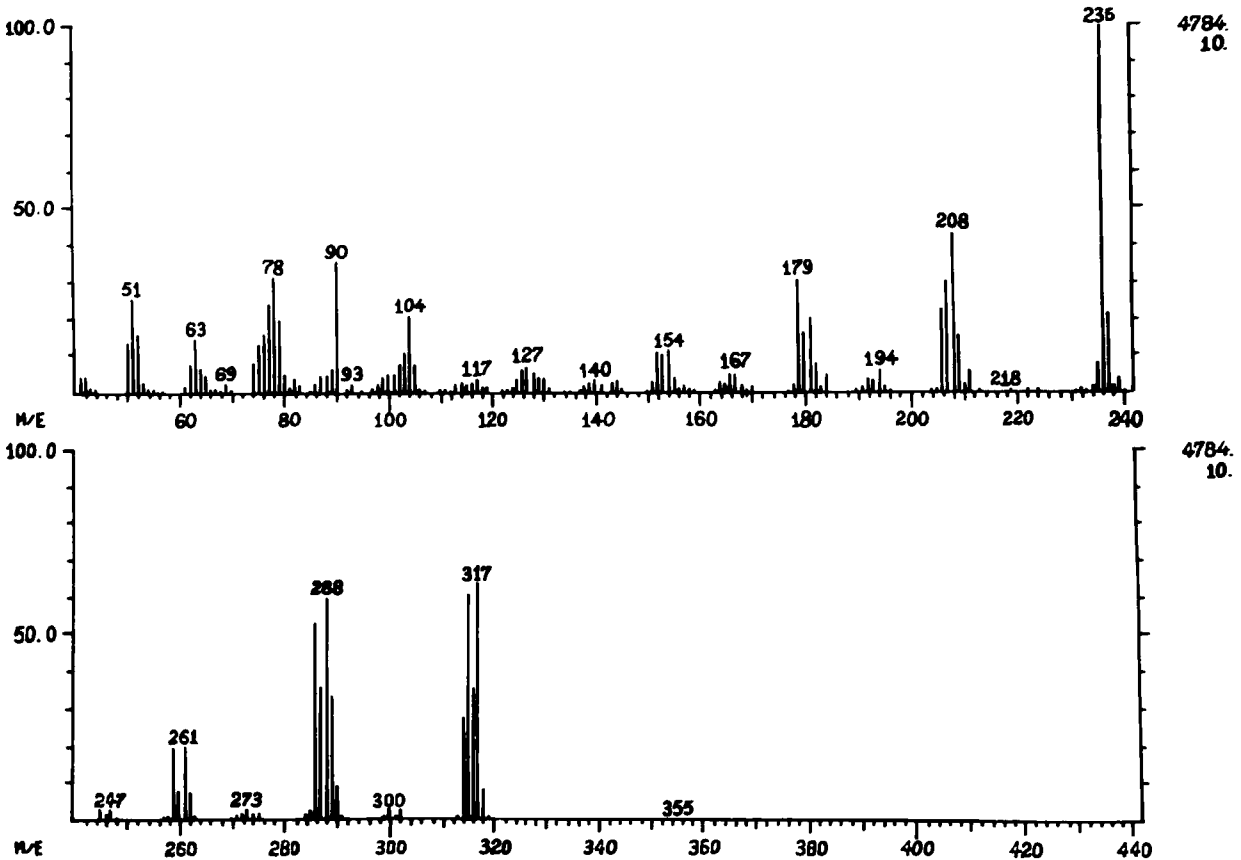
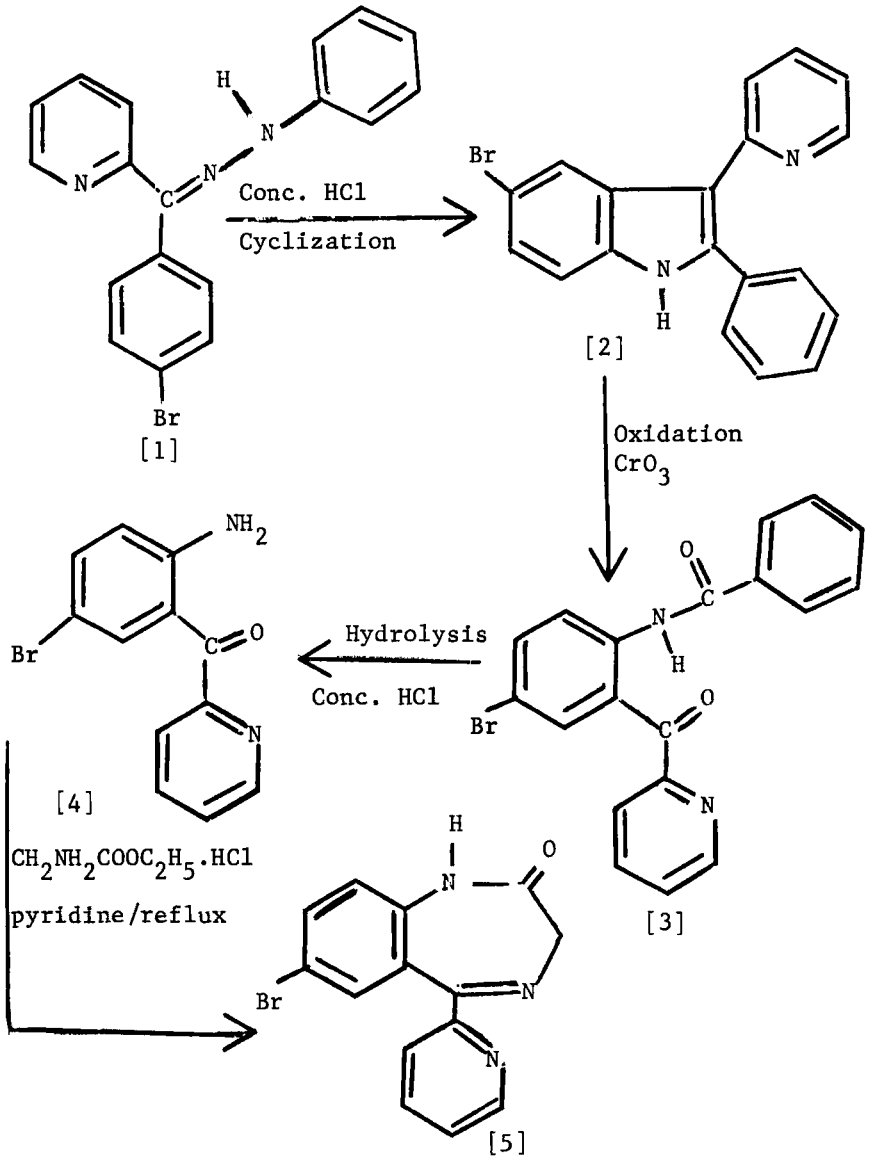


Fig. 10. EI-mass spectrum of bromazepam.

5. Synthesis

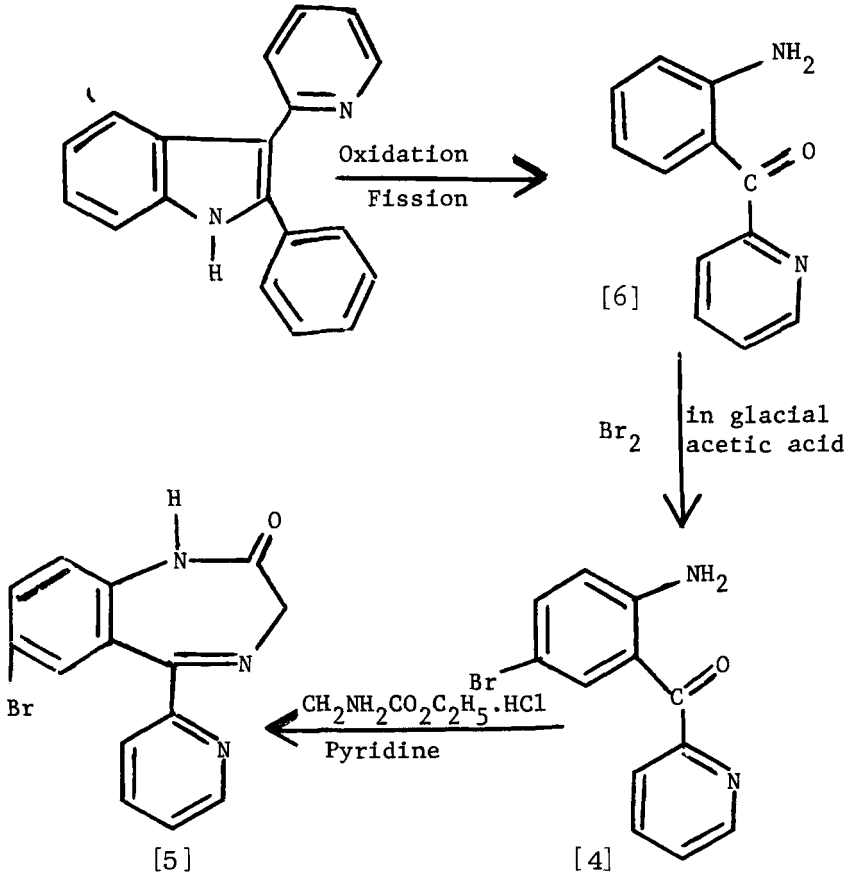
Different routes for the synthesis of bromazepam were reported (19-21).

Route I



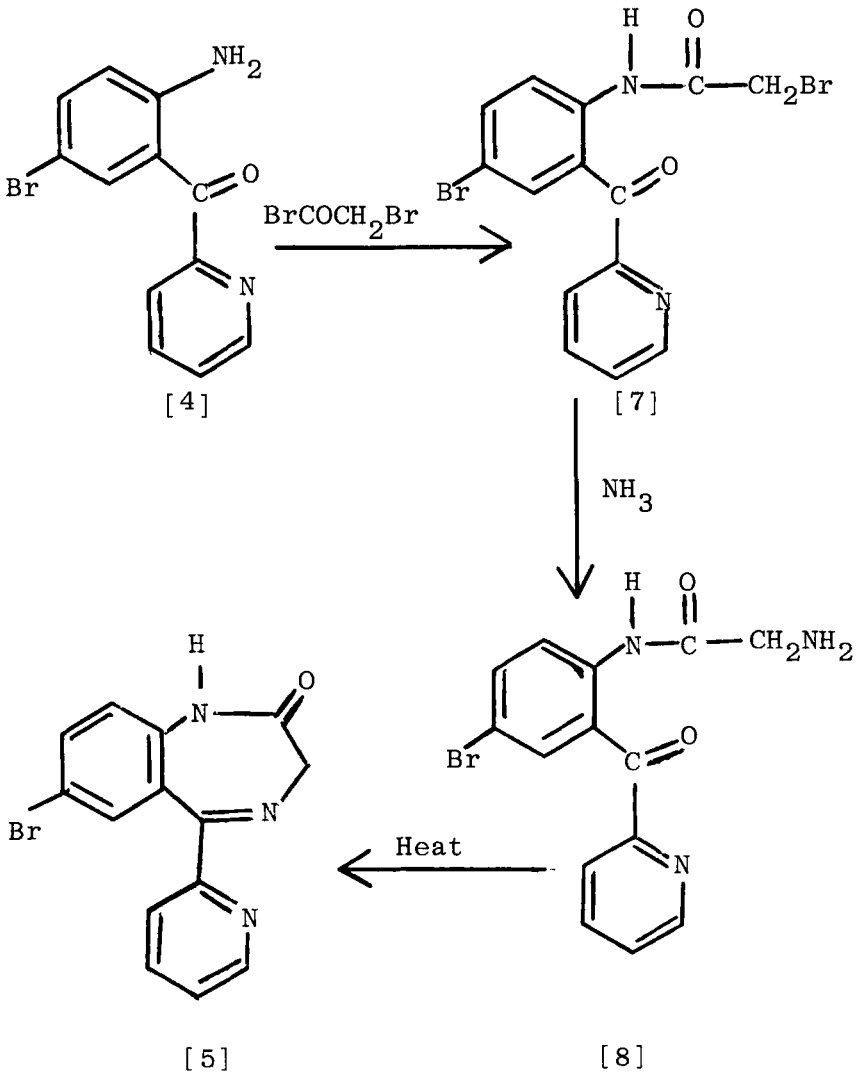
2-(2-Amino-5-bromobenzoylpyridine [4] was prepared from 2-phenacylpyridine p-bromophenyl hydrazone [1] through cyclization with concentrated hydrochloric acid to give 5-bromo-2-phenyl-3-(2-pyridyl)indole [2]. This compound was oxidized with chromium trioxide to give 2-(2-benzamido-5-bromobenzoyl)pyridine [3] (29,22) which on hydrolysis with concentrated hydrochloric acid afforded the desired compound 2(2-amino-5-bromobenzoyl)pyridine [4]. This compound is considered to be the starting material for the synthesis of 1,4-benzodiazepines. A mixture of [4], glycine ethyl ester hydrochloride and pyridine was refluxed to give bromazepam [5].

Route 2



Alternatively, the key intermediate compound [4] was prepared by bromination of 2-(2-aminobenzoyl)pyridine [6] with bromine and glacial acetic acid (23,24).

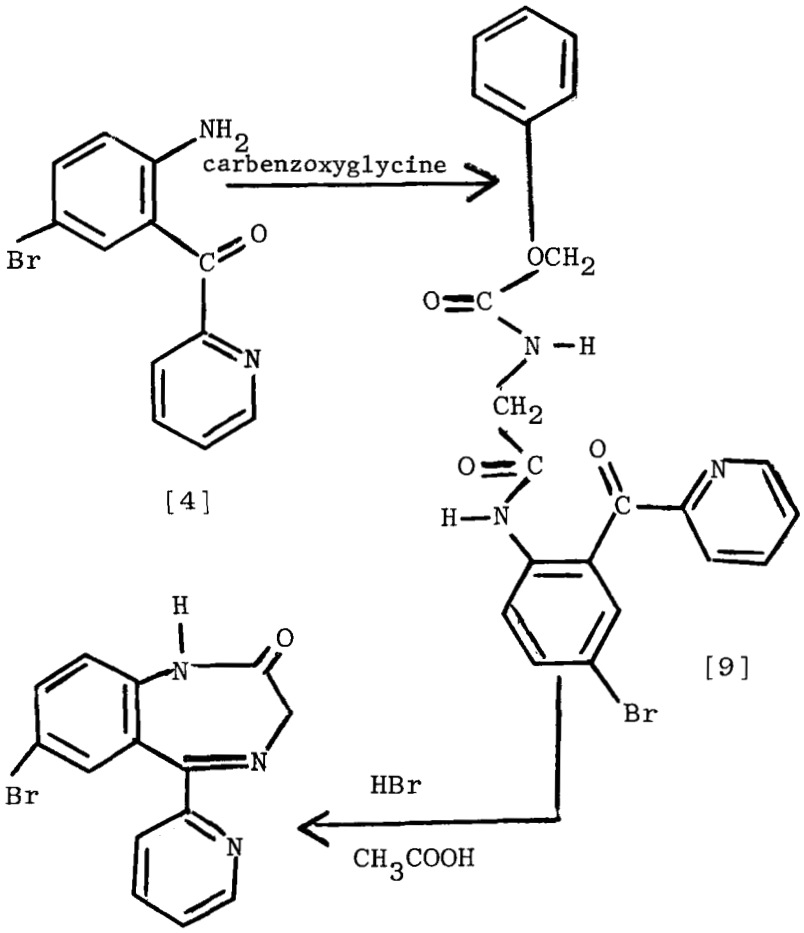
Route 3



Bromazepam

In this route 2-(2-aminobenzoyl)pyridine [4] was treated with bromoacetyl bromide in glacial acetic acid. The crude bromoacetyl derivative [7] was converted directly to bromazepam [5] by reaction with ammonia through the intermediate aminoacetamidobenzoyl pyridine [8] (20).

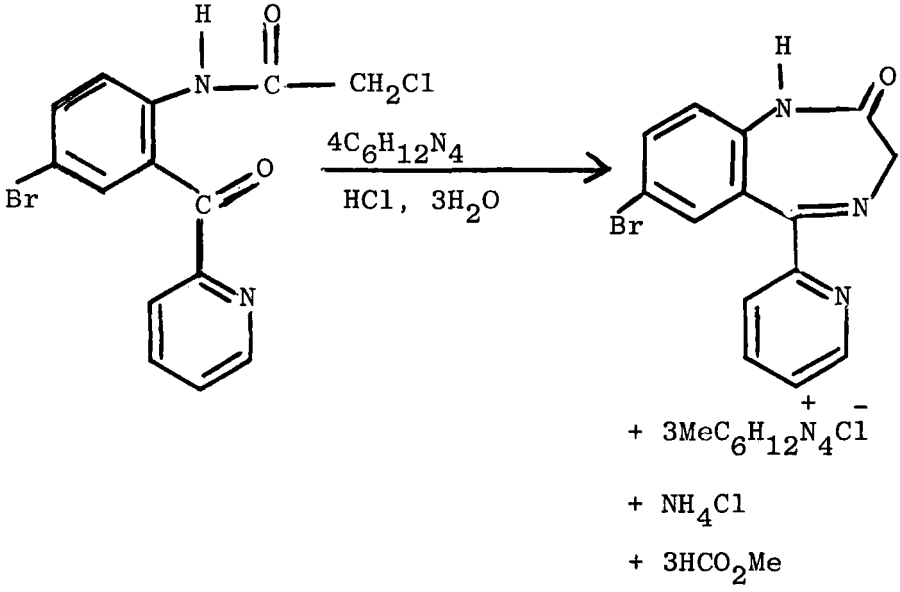
Route 4



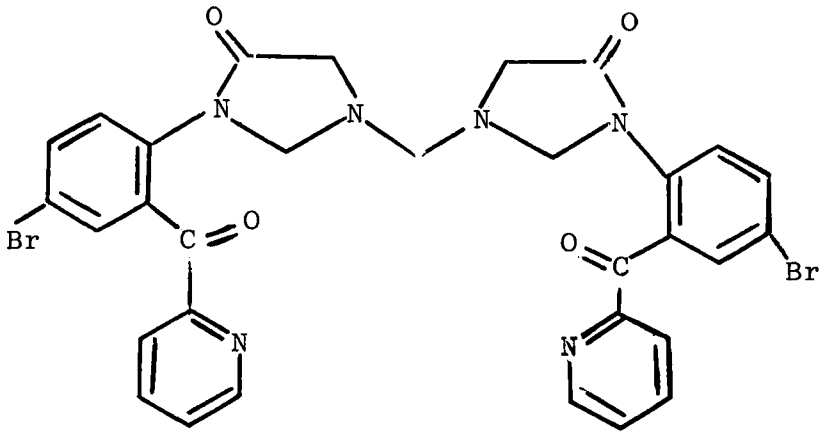
An alternative method (20) for the synthesis of bromazepam proceeded via the carbobenzoxyglyclyl derivative [9]. This compound is prepared according to the method of Sheehan and Hess devised for the synthesis of peptides (25,26). A mixture of the 2-(2-amino-5-bromobenzoyl)pyridine and carbobenzoxyglycine in methylene chloride was added. The methylene chloride solution after removal of N,N'-dicyclohexylurea was concentrated in vacuo at room temperature. The residue was dissolved in benzene and chromatographed to give [9]. Cleavage of the carbobenzoxy group with hydrogen bromide in glacial acetic acid (27) gave bromazepam [5] directly without isolation of the intermediate, 2-(2-aminoacetamido-5-bromobenzoyl)pyridine.

Route 5

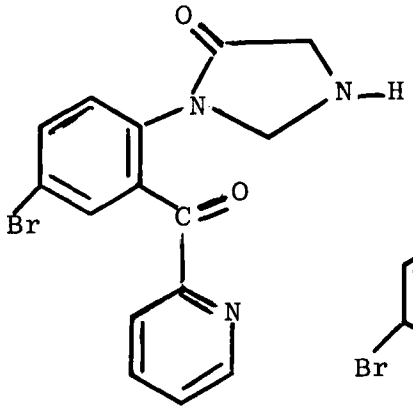
Clarke et al (1980) (28) have described in details the preparation of bromazepam [5] from the corresponding benzophenone by the use of hexamine and hydrochloric acid as outlined below:



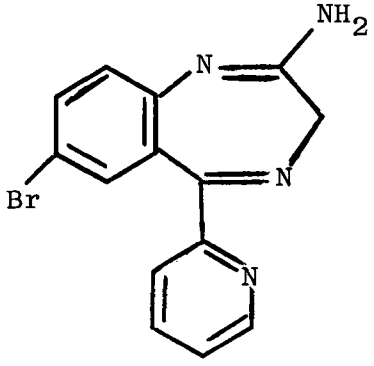
Three main by-product were identified : two imidazolidinones [10], [11] and an aminobenzodiazepine (12).



[10]



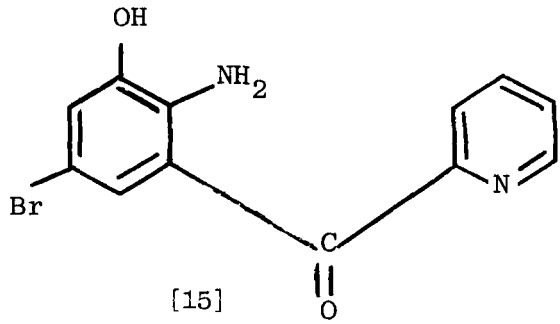
[11]



[12]

6. Metabolism

The first reported metabolic work on bromazepam was that published by Sawada in 1972 (29,30). This study was carried out both in dogs, rabbits and rats. The isolated urinary metabolite was assumed to be a 3-hydroxylated derivative of 2-amino-5-bromobenzoylpyridine [15]. The structural elucidation was based on elemental analysis, mass spectrometry and nuclear magnetic resonance studies.



[15]

The biotransformation of bromazepam, was studied in four species namely human, dog, rat and mouse (31). Four metabolites were identified and their excretion was quantitatively determined. The major urinary metabolites excreted by three humans given single 12 mg oral doses of bromazepam-5- ^{14}C were the conjugated forms of 3-hydroxy bromazepam [13] accounting for 13-30% of the dose, and 2-(2-amino-5-bromo-3-hydroxybenzoyl)pyridine [15] accounting for 4-25% of the dose. Conjugated [13] was also the major metabolite in dogs and mice administered ^{14}C-bromazepam, but its excretion by rats was very limited. Together with [13], metabolite [15] and 2-(2-amino-5-bromobenzoyl)pyridine [4] were detected in the excreta of the human, dog, rat and mouse. Although the pyridyl N-oxide [18] derivative of bromazepam was not detected as a metabolite in any speices, the N_4-oxide [14] was a minor metabolite in dog urine. This is the first reported instance of N_4-oxidation of a 1,4-benzodiazepine-2-one. This study has shown that the metabolism of bromazepam in human, dogs but not in rats fits the same pattern of other 1,4-benzodiazepine-2-one in affording conjugated 3-hydroxylated derivative (32). The identification and quantitation of the different metabolites were carried out by thin layer chromatography using several solvent systems.

de Silva et al, 1974 (33) have reported a sensitive and specific electron-capture GLC assay for bromazepam and its major metabolites (see under methods of analysis). Table 8 shows the chemical names and physical properties of bromazepam and its major metabolites.

Table 8. Chemical names and physical properties of bromazepam and metabolites

Compound No.	Chemical name	Molecular weight	Melting point
5	7-Bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one (bromazepam)	316.16	237-238.5° dec.
13	7-Bromo-1,3-dihydro-3-hydroxy-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one	332.2	198-200°
14	7-Bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one 4-oxide.	332.2	263° dec.
4	2-Amino-5-bromobenzoylpyridine.	277.12	97.5-99°
15	2-Amino-5-bromo-3-hydroxybenzoylpyridine.	293.12	190-196°
16	Bromazepam mercapturic acid methylester(7-bromo-1,3-dihydro-5-[2'-(6'-N-acetyl-L-cystein-S-yl)-pyridyl]-2H-1,4-benzodiazepin-2-one.	228	-
17	Methylthiobromazepam	-	-
18	Pyridyl N-oxide of bromazepam	-	-

Taleishi and Shimizu 1976 (34) has recently reported the isolation of a methylthio-containing metabolite which was identified as 7-bromo-1,3-dihydro-5-[2'-(6'-methylthio)pyridyl]-2H-1,4-benzodiazepin-2-one (methylthiobromazepam) [17]. Also they have shown that the methionine donor theory (34) is very unlikely in the case of the formation of methylthiobromazepam. Isolation of the mer-

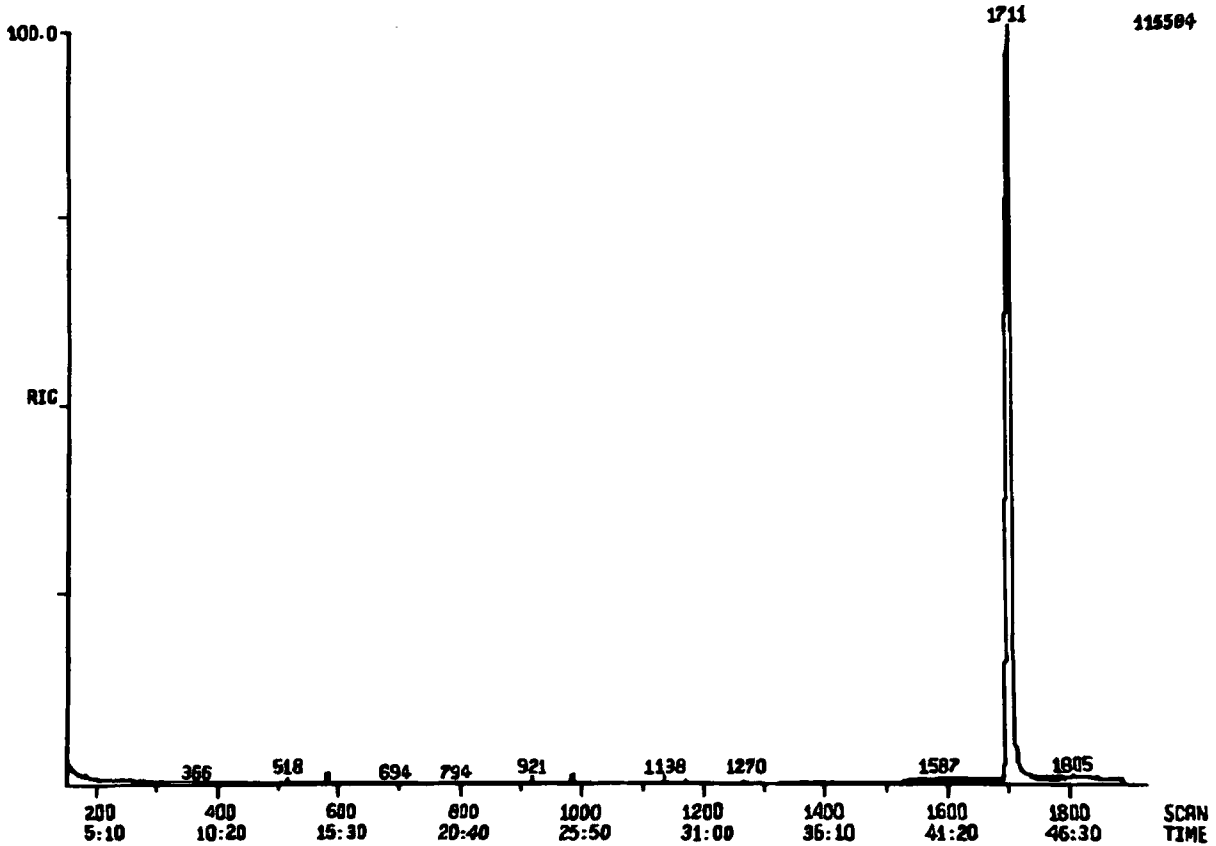
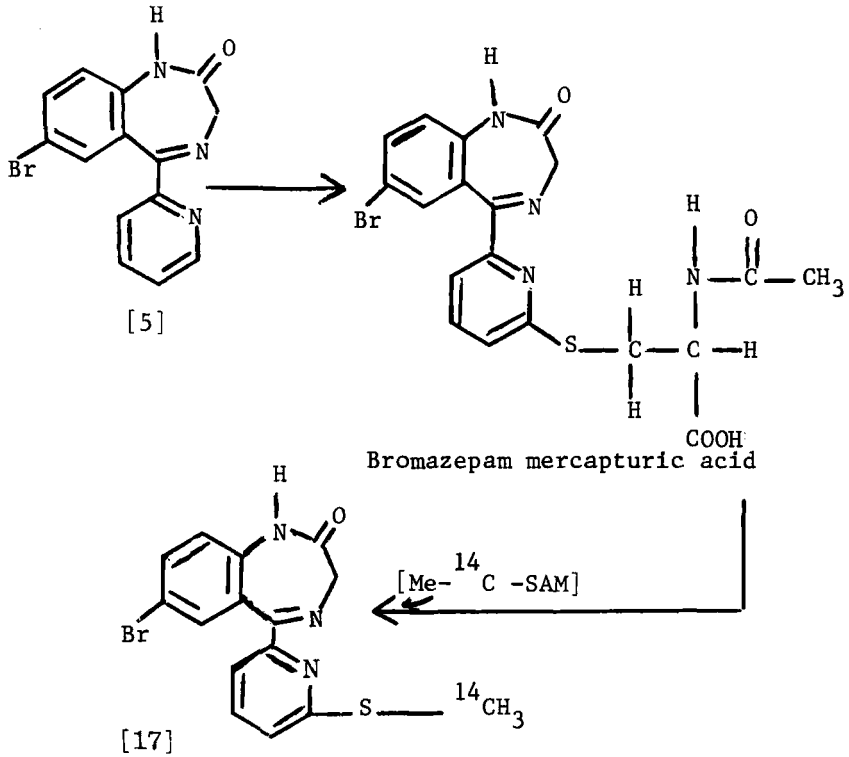


Fig. 11. GC trace of bromazepam.

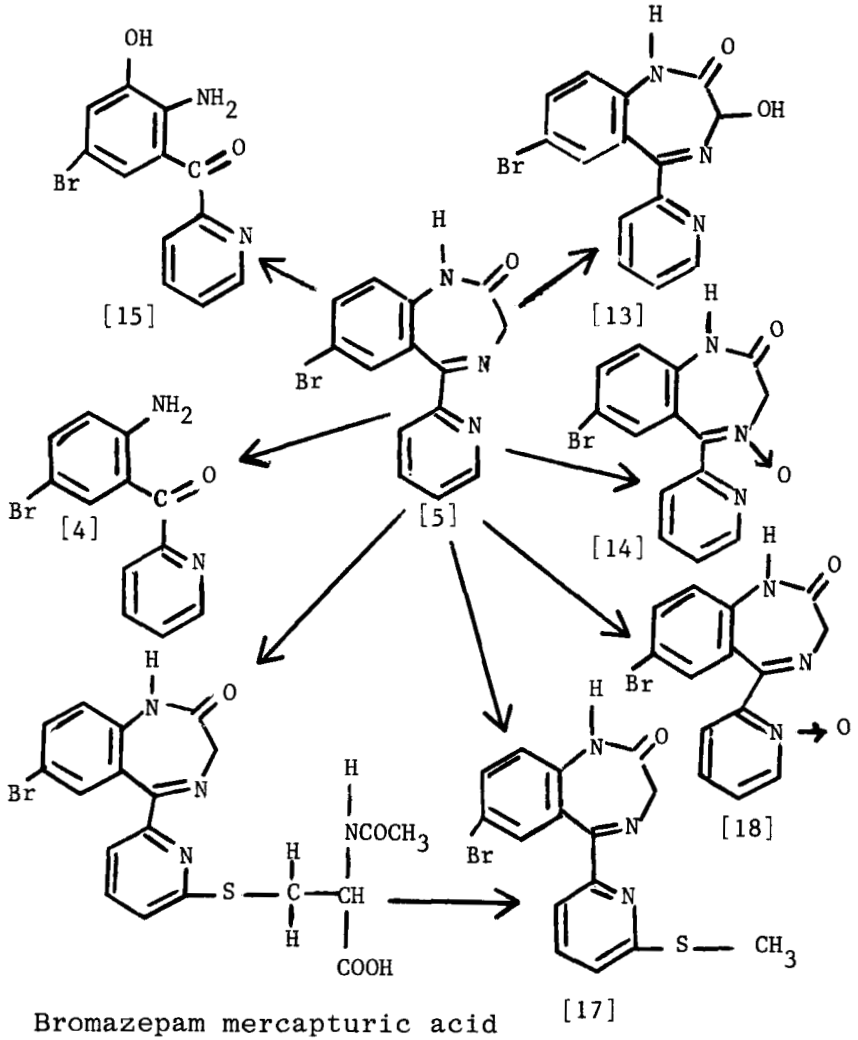
capturic acid conjugate of bromazepam from bile of rat given the drug, its identification and subsequent conversion of the mercapturic acid to methylthiobromazepam in rat liver preparation was reported (35). Scheme 1 shows a proposed pathway for the biotransformation of bromazepam to methylthiobromazepam in the rat.



Scheme I. A proposed pathway for the biotransformation of bromazepam to methylthiobromazepam in the rat.

In this study non-radioactive bromazepam, [5-^{14}C] bromazepam and bromazepam-N'-oxide (7-bromo-1,3-dihydro-5-[2'-(1'-oxo)-pyridyl]-2H-1,4-benzodiazepin-2-one were employed. 7-Bromo-1,3-dihydro-5-[2'-(6'-methyl,N-acetyl-L-cysteinate-5-yl)-pyridyl]-2H-1,4-benzodiazepin-2-one (bromazepam mercapturate methylester) was synthesised from bromazepam N'-oxide and methyl N-acetyl-L-cysteinate in acetic anhydride according to the pyridine-N-oxide

nucleophilic substitution described by Bauer and Dickerhofe (36). The results indicated that 6'-methylthiobromazepam isolated previously in the rat urine is formed at least in part via the mercapturic acid and that rat liver contains enzyme(s) capable of catalysing the conversion from the mercapturic acid to methylthiobromazepam. Chemical names and physical properties of bromazepam and its metabolites are listed in Table 8. Chemical reactions of bromazepam and its known metabolites are shown in Scheme 2.



Scheme 2. Chemical reactions of bromazepam and its known metabolites.

7. Pharmacokinetic Studies

Distribution and elimination of bromazepam has been studied in 4 human subjects following single oral and intravenous doses of 6 mg ^{14}C-bromazepam (37). Absorption and diffusion of the substance takes place rapidly, peak plasma levels being reached about an hour after oral administration. Bromazepam is mainly eliminated in the urine, and, unlike most other benzodiazepines, excretion follows a regular, rectilinear pattern which is practically completed after 120 hours. Excretion of the metabolites closely parallels that of the unchanged substance. Half-life for the elimination of bromazepam from the plasma is 20.1 hours following intravenous administration, compared with 22.5 hours for total ratioactivity (unchanged bromazepam plus metabolites). Fig. 12 and Fig. 13 for intravenous and oral administration are practically identical. Binding of bromazepam by plasma proteins was studied by equilibration dialysis, which showed that about 70% of the substance present is bound. This is considerably less than for diazepam and most other benzodiazepines, and is attributed to the increased polarity of the molecule due to the presence of the pyridyl radical (38).

Another work described the application of gas liquid chromatography for pharmacokinetic studies in man (39). The assay can be used also for routine plasma level monitoring. The important pharmacokinetic parameters have been calculated from the plasma concentration. $T\frac{1}{2}$ (B) = elimination half-life = 18.5 hours, $\overline{Cl}$ = total body plasma clearance = 347 ml/min and Vd_B = apparent volume of distribution = 0.91/kgm following single oral dose of 6 mg/day. However, using multiple dosing with 3 mg/day, it was found that $T\frac{1}{2}$ (B) = 19.5 hours $\overline{Cl}$ = 243 ml/min and Vd_B = 071/kg. Kaplan *et al*, 1976 (40) have reported plasma concentrations and time profiles which in agreement with the above mentioned work.

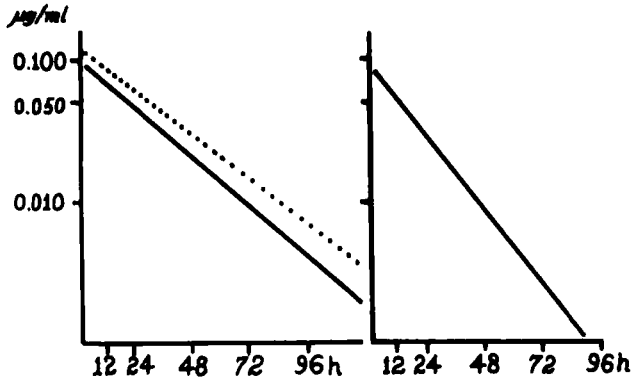


Fig. 12 Fig. 13

——— Bromazepam

····· Bromazepam + metabolites

Fig. 12. ^{14}C-Bromazepam and total radioactivity (µg/ml) in plasma after I.V. injection of 6.0 mg.

Fig. 13. ^{14}C-Bromazepam in plasma (µg/ml) after oral administration of 6.0 mg.

8. Methods of Analysis

8.1 Elemental Analysis

The elemental composition of bromazepam is

Element	% Theoretical
C	53.18
H	3.19
Br	25.28
N	13.29
O	5.06

8.2 Identification Tests

8.2.1 Color Reactions

Reported color reactions for bromazepam are as follows (41):

Compound	Diazo-coupling	Reaction with 1,3-dinitrobenzene $(CH_3)_4N$ OH	In 1M NaOH	In 2M HCl	In DMSO
Bromazepam	Orange red	Red violet	Yellow	Yellow	Orange brown

8.2.2 Thin-Layer Chromatogram (42)

Layer : Silica gel F_{254}, MERCK precoated plates 0.25 mm.

Mobile phase : Ethyl acetate - conc. ammonia (100 + 1) (prepare freshly) without saturation of the chamber atmosphere.

Application : 10 µl of each solution (sample and comparison)

Sample solution: Shake 400 mg of tablet powder with 5 ml of chloroform for approx. 3 min and filter.

Bromazepam comparison solution: Dissolve 60 mg of bromazepam in 25 ml of chloroform.

Front distance : 12 cm

Detection : 1. Observe under short-wave UV-light: decrease of the fluorescence through bromazepam.

2. Spray the dried plate with a freshly prepared solution of ferrous sulfate, dry for a short moment and spray then with an ammonia (1%): Bromazepam appears as a violet spot.

R_f-value : Bromazepam approx. 0.6

Ferrous sulfate solution : Dissolve 1 g of $FeSO_4 . 7H_2O$ in 100 ml of dist. water.

8.2.3 Microcrystal Tests

Bromazepam was identified in solution by microcrystal tests in microgram quantities (43).

A micro drop of the test solution (in 50% ethanol) was placed on the glass cover slip and a micro drop of the reagent was added. The drop being stirred with a slight scratching of the glass to promote the formation of crystals. The appearance of the crystals was recorded as follows:

1. With potassium cadmium iodide, crystals in the form of small tablets were obtained after one hour (Fig. 14a).

2. With potassium mercuric iodide, crystals in the form of round trifercate blackish structures, white on black back ground were formed after one hour (Fig. 14b).

3. With gold bromide hydrochloride, crystals in the form of flowers of dendrites were obtained instantly (Fig. 14c).

The sensitivities of the tests were 0.2, 0.5 and 0.1 microgram respectively.

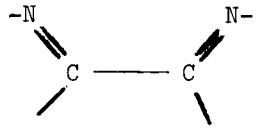
8.3 Titrimetric

8.3.1 Non-aqueous Titration

Among several 1,4-benzodiazepine derivatives, bromazepam was titrated with perchloric acid in dioxane or tetrabutylammonium hydroxide in benzene-methanol (43).

8.4 Spectrophotometric

Unlike other 1,4-benzodiazepines, the existence of the α, α'-dipyridyl type moiety enables bromazepam to form complexes with divalent metal ions. Sabatino *et al* (45) reported that ferrous ions form purple complexes with pyridyl benzodiazepin-2-ones; compounds having a basic dipyridyl type bond structure which has excellent metal complexing properties.



The method has been applied (46) to the determination of bromazepam in biological materials by adding methanol solution of ferrous chloride to the bromazepam obtained after extraction from blood or urine and measuring the absorbance of the solution at 580 nm. The method proved to be specific for bromazepam with no interference from 2-amino-5-bromobenzoylpyridine, one of its principal metabolites.

The procedure was also applied to the determination of bromazepam in rat blood (47).

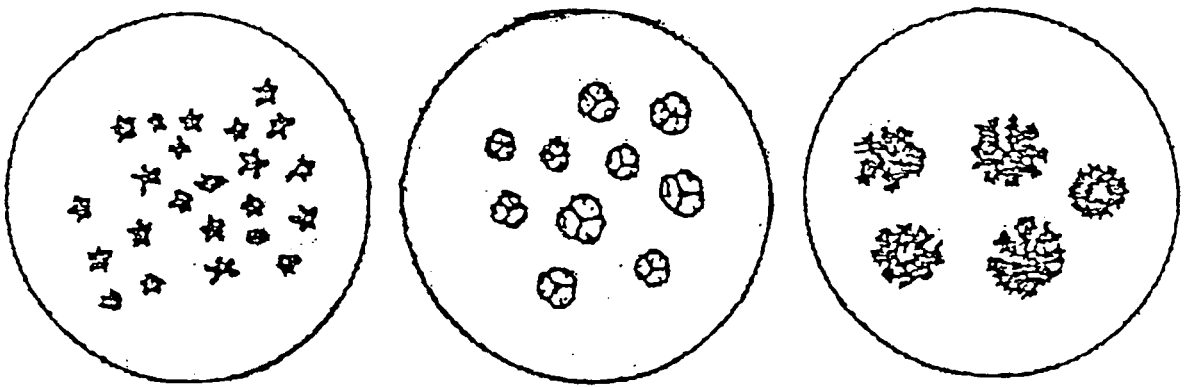


Fig. 14a Fig. 14b Fig. 14c

Fig. 14a. Microcrystals of bromazepam with potassium cadmium iodide.

Fig. 14b. Microcrystals of bromazepam with potassium mercuric iodide.

Fig. 14c. Microcrystals of bromazepam with gold bromide hydrochloride.

Smyth et al (48) studied the chelation of bromazepam with Fe(II), Cu(II), and Co(II) using spectroscopic techniques such as UV - vis. spectrophotometry.

Colorimetric determination of bromazepam, as well as some other benzodiazepines, was also performed after reaction with a modified Marquis's reagent (49). pKa values and mechanism of hydrolysis were also reported using UV spectrophotometry (3).

Another UV-spectrophotometric method used by F. Hoffmann - La Roche & Co Limited is as follows: (50)

Assay: The determination should be carried out within one hour under subdued day-light.

Pulverize 20 tablets and accuretely weigh approximately 1000 mg of tablet powder into a 250 ml-volumetric flask. Add about 200 ml of 0.1 N methanolic sulfuric acid and shake for 15 min or treat with ultrasonics. Dilute to volume with 0.1 N methanolic sulfuric acid. Filter a part of this solution (discard the first 15 ml) and dilute 10.0 ml of the clear filtrate to 100.0 ml with 0.1 N methanolic sulfuric acid (= test solution).

Method of Analysis: With a spectrophotometer measure the extinction of the test solution at 285 nm (max.) against 0.1 N methanolic sulfuric acid as a blank using 1 cm-quartz cells.

Calculation:

$$\text{mg of bromazepam tablet} = \frac{E \cdot 25000 \cdot A}{300 \cdot B}$$

E = extinction of the sample solution

A = average weight (mg)

B = weight of sample (mg)

300 = E(1%, 1 cm)-value of bromazepam at 285 nm (maximum) in 0.1 N methanolic sulfuric acid.

Methanolic sulfuric acid: Dilute 5.0 g of 98% sulfuric acid to 1000 ml with methanol. Prepare this solution at least 24 hours before use.

8.5 Polarographic

The ease of reduction of the azomethine ($> C_5 = N_4$) group of bromazepam and its 3-hydroxy metabolite, and the ($> C = O$) carbonyl group of its benzophenone metabolites, promotes their quantitation in the subnanogram range by differential pulse polarography.

De Silva et al (33) described a differential pulse polarographic method for the determination of bromazepam and its metabolites, 3-hydroxy-bromazepam, 2-amino-3-hydroxy-5-bromobenzoylpyridine and 2-amino-5-bromobenzoylpyridine in urine.

Polarography was carried out using phosphate buffer as the supporting electrolyte with pH of 5.5. The peak potential, Ep, due to the reduction of the azomethine group of bromazepam and its 3-hydroxy-metabolite occured at -0.535 and -0.555 V versus calomel electrode respectively, whereas the peak due due to the reduction of the carbonyl group of the metabolites 2-amino-5-bromobenzoylpyridine and 2-amino-3-hydroxy-5-bromobenzoylpyridine occured at -0.630 and -0.635 V versus calomel electrode, respectively (Fig. 15). Sensitivity limits ranged between 50 and 100 ng/5 ml urine.

Trace levels of bromazepam in blood was determined by differential pulse polarography (51). The current resulting from reduction of the 4,5-azomethine bond of bromazepam was measured at -0.610 V versus silver chloride electrode. The method has a recovery of 62.1% ± 7.8 in the range of 10-1000 ng bromazepam/1 ml of blood.

Polarography was also used to study acid-base and complexing behaviour of bromazepam (3), and for the identification of any one or more of several 1,4-benzodiazepines including bromazepam (52).

8.6 Atomic Absorption

Gonzales-Perez et al (53) described a method for the determination of bromazepam by atomic absorption spectrophotometry. The method is based on the extraction of the ion-pair formed by the bromazepam-nickel (II) cationic chelate and perchlorate from aqueous to methylisobutylketone (MIBK) solutions.

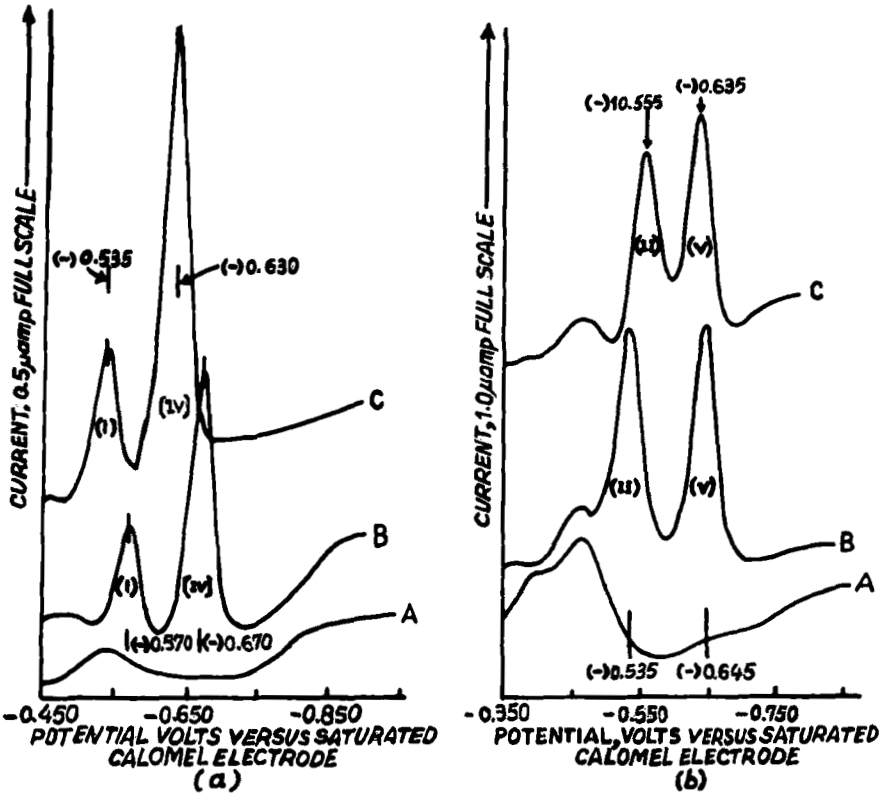


Fig. 15. Differential pulse polarograms of: (a) I and II (b) III and IV in 1.0 M pH 5.5 phosphate buffer as the supporting electrolyte. Key: A, control urine blank; B, authentic standard mixture; and C, authentic recovered from urine.

I. Bromazepam II. 2-Amino-5-bromobenzoylpyridine
III. 3-Hydroxymetabolite of bromazepam
IV. 2-Amino-3-hydroxy-5-bromobenzoylpyridine

The absorbance of nickel in the organic phase was determined at the nickel resonance a 232.0 nm line. The method that showed a detection limit of 2 X 10^{-6}M, was applied to the determination of bromazepam in drugs.

8.7 Radioimmunoassay

Robinson et al (54) described four radioimmunoassays for the determination of bromazepam and other benzodiazepines. In the most sensitive method, the antiserum from the Emit-tox serum benzodiazepine assay kit was used with [^{3}H]flunitrazepam and proved to be suitable for detecting sub-therapeutic levels of all benzodiazepines tested except one. The assay is particularly applicable to blood samples of forensic interest which may be hemolyzed or decomposing, and require only 75 ml of blood.

A radioreceptor assay for benzodiazepines including bromazepam in human blood, plasma, saliva and urine was developed (55). The method is based upon competition between [^{3}H]flunitrzepam and biologically active benzodiazepines in biological fluids for brain-specific receptors, prepared in a stable, dry form and easy to handle. The method is specific for biologically active benzodiazepines. Other method, described the determination of benzodiazepines in serum using the enzyme multiplied immuno assay-single test (EMIT-ot) drug detection system (56). The method is useful to clinical toxicology practice.

8.8 Chromatographic

8.8.1 Paper Chromatography

Bromazepam was separated from other benzodiazepines by using a thin layer system: Merck aluminium oxide F_{254} and $CHCl_3$ - PhMe - EtOH (40:60:2) complemented by a Whatman S.G. 81 silica gel loaded paper system developed in $CHCl_3$-EtOH (49:1). The spots were located by short UV wavelength and acidified potassium iodoplatinate (57).

8.8.2 Thin-Layer Chromatography

Haefelfinger (58) has reported a specific and sensitive method for the determination of bromazepam in plasma by quantitative thin-layer chromatography. Silica gel plates were used with methylacetate-ammonia 33% (100:1) as a solvent system. The quantitation of the spots was performed either by measuring the UV reflectance of the plate or by the hydrolysis of bromazepam to 2-amino-5-bromobenzoylpyridine on the plate, diazotization and coupling with N-(1-naphthyl)ethylenediamine to form an azo-dye, which was then evaluated densitometrically. The recovery from plasma was found to be over 90%. Bromazepam has an R_f value of 0.6-0.65.

Thin-layer chromatographic systems and detection methods described for the identification and determination of bromazepam are summarized in Table 9.

Table 9. Solvent systems and detection methods for bromazepam

Support	Solvent system	Detection	Ref.
Silica AR 7GF-5 (two dimentional TLC)	Heptane/ethyl acetate/ethanol/concentrated ammonia (50:50:3) and: Heptane/chloroform/ethanol/concentrated ammonia (50:50:20:1)	UV-light	59
Silica gel G	Chloroform/acetone (90:10), benzene/isopropanol/ammonia (85:15:10)	Bratton-Marshall, dragendorff and iodoplatinate reagents.	60
Silica gel 60 F_{254}	Diisopropylether-toluene-ethylacetate methanol - diethylamine (70:15:10:5:2)	UV-light (254 nm) 10% w/v H_2SO_4 heat at 120° for 10 minutes chamber with nitrous fumes Spray with α-naphthylethylene-diamine R.	50

Thin layer chromatographic detection and identification of bromazepam among other 1,4-benzodiazepines has also been reported (61-64).

8.8.3 Gas Chromatography

Numerous methods for the gas chromatographic analysis of bromazepam in biological fluids have been reported. Most of these methods described the determination of the compound either as the intact 1,4-benzodiazepine-2-one or as the hydrolyzed 2-amino-5-bromobenzoyl-pyridine (ABBP) (33, 65-69). Bromazepam has also been assayed as the N'-methyl derivative (70).

The presence of electronegative functional groups (the halogen in the 7-position and the 2-carbonyl) renders bromazepam amenable to sensitive determination by electron-capture gas-liquid chromatography (EC-GLC); therefore all (except one) the above mentioned methods applied (EC-GLC) to the determination of the drug. In principle, these methods are capable of detecting nanogram amounts of bromazepam in biological fluids where they compete with radioimmunoassays. Recently, it has been coupled with mass spectrometry (71). The experimental conditions used for the analysis of the drug in biological fluids are summarized in Table (10).

Table 10. Gas liquid chromatographic conditions for the determination of bromazepam and its metabolites

Drug or metabolite	Column packing	Carrier	Column temp.	R_T (mins)	Detectors	Ref.
2-Amino-5-bromo-benzoylpyridine (ABBP)	2% Carbowax 20M-TPA on silanized Gas ChromP (100-120 mesh)	N_2	220 ± 2°	6-7	EC	65
2-Amino-5-bromo-benzoylpyridine (ABBP)	3% OV 225 (methyl-phenylcyanopropyl-silicone) on Gas Chrom Q (60/80 mesh)	Ar-CH_4 (90:10)	214°	12.5	EC	66
Bromazepam	3% OV-17 on Gas Chrom Q (60/80 mesh).	Ar-CH_4 (90:10)	245°	6	EC	33
Bromazepam	3% OV-17 on Gas Chrom Q (60/80 mesh).	Ar-CH_4 (90-10)	240°	9.6	EC	67
Bromazepam	10% UCC-W-98 on Chromosorb W AW DMCS (80/100 mesh)	N_2	265°	4	FI	68
N'-Methylbromazepam	0.5% OV-17 Chromosorb G HP (100/200 mesh)	N_2	270°	3-4	EC	70
Bromazepam	4% SE-52 silica capillary column	He	Programmed 130-260°	3-4	EC	69
2(2'-Amino-5-bromobenzoyl)-pyridine	20% UCC-W on Chromosorb W AW DMCS (80/100 mesh)	He	Programmed 100-310°		Mass spectrometer	71

Ar = Air

8.8.4 High Pressure Liquid Chromatography

A high pressure liquid chromatographic procedure for the determination of bromazepam in human plasma has been described (72). After extraction from plasma, the extract was evaporated and the residue was suspended in acetonitrile/tetrabutylammonium hydroxide (60:40, v/v) before injection into the chromatograph. Analysis was performed on a μ Bondpack C_{18} column with mobile phase made from 20 ml MeOH, 30 ml MeCN, and 700 ml of solution containing 20 ml 10% tetrabutylammonium hydroxide in 1 L water. The internal standard was carbamazepine and detection was by UV spectroscopy.

In order to determine the lower therapeutic range of drug levels in serum (73), bromazepam, among other basic drug, was chromatographed on an RP18 Micropack MCH 10 column using UV detector and perchlorate/MeCN solution as eluent.

Hochmuth et al (74) described a simple and rapid method for the toxicological analysis of some benzodiazepines and antidepressants including bromazepam. After solvent extraction at pH 9, the analysis was performed by HPLC on a reverse-phase column with MeCN 0.6% KH_2PO_4 (1:1) adjusted to pH 3.

Heizmann et al (75) reported a HPLC method for the determination of bromazepam in plasma and of its main metabolites in urine. The unchanged drug was extracted from plasma with dichloromethane, and the residue was subsequently analysed by reversed phase HPLC with UV detection (230 nm). For the determination of the metabolites, the urine samples were incubated to effect enzymatic deconjugation before extraction with dichloromethane. The column used was a 15 cm Supelcosil LC18 and the mobile phase consisted of a mixture of methanol/phosphate buffer, pH 7.5, at a flow rate of 1 ml/min. The retention times ranging from 6 to 20 minutes.

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BUSULPHAN

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1. Foreword

Busulphan is a chemotherapeutic agent, which has been widely used as antineoplastic drug since its discovery in 1953 (1). This alkylating agent belongs to the series of alkanesulfonic acid esters, possessing significant cytotoxic activity and is the drug of preference in the treatment of chronic myelocytic or granulocytic leukaemia. It has recently been proposed as a drug of choice in polycythaemia and thrombocythemia (2-13). Busulphan has also been used for the treatment of bronchial carcinoma (18-19), myasthenia gravis (20) and chronic granulomatous disease in children (21). The drug is also used as reproductive sterilant for insect (22-23) and as a general immunosuppressant for organ transplant in the treatment of autoimmune disease (24-26).

2. Description

2.1 Nomenclature

2.1.1 Chemical Names

1,4-Butanediol dimethanesulfonate;
1,4-Bis(methanesulfonoxy)butane;
1,4-di(methanesulfonyloxy)butane;
1,4-di(methylsulfonoxy)butane;
1,4-Butanediylbismethane sulphonate;
Methanesulfonic acid tetramethylene ester;
Tetramethylene bis(methanesulfonate) (27-29).

2.1.2 Generic Name

Busulphan, CB 2041, GT 41, NSC 750, WR 19508
Busulfanum (29).

2.1.3 Trade Names

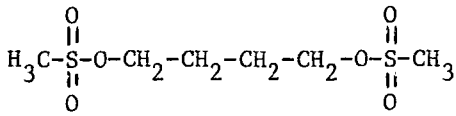
Myleran, Misulban, Mitosan, Myelosan, Myeleukon, Myeloleukon, Sulfabutin, Mielucin (27).

2.2 Formulae

2.2.1 Empirical

$C_6H_{14}O_6S_2$ (30).

2.2.2 Structural



2.2.3 CAS No.

[55-98-1] (27)

2.3 Molecular Weight

246.31 (31)

2.4 Elemental Composition

C, 29.26%; H, 5.73%; O, 38.98%; S, 26.03% (27).

2.5 Appearance, Color and Odor

A white almost odorless crystalline powder (29, 32).

3. Physical Properties

3.1 Melting Point

114-118°, 119°, 116°. (27).

3.2 Solubility

Soluble, at 20°, in 750 parts of water (in which it is slowly hydrolyzed) and in 25 parts of acetone; very slightly soluble in alcohol (27, 32).

3.3 Storage

Busulphan should be kept in a well-closed container, protect from light (30).

3.4 Spectral Properties

3.4.1 Ultraviolet Spectrum

The ultraviolet spectrum of busulphan in methanol, ethanol and in water was scanned from 200 to 400 nm using Varian DMS 90 Spectrophotometer. No absorbance has been noticed.

3.4.2 Infrared Spectrum

The infrared spectrum of busulphan as KBr disc is shown in Figure 1. It was recorded on a Pye-Unicam SP 1025 infrared spectrophotometer. The assignments of the characteristic bands in the infrared spectrum is shown below:

Frequency cm^{-1}	Assignments
2960-3060	CH stretch
1430-1415	$-CH_2-O$ vibration
1352	$S(=O)_2$ stretch (Assymm.)
1180	$S(=O)_2$ stretch (Symm.)
1000-770	S-O-C stretch

3.4.3 Proton Nuclear Magnetic Resonance (^1H NMR) Spectrum

The ^1H NMR spectrum of Busulphan is shown in Figure 2. The drug is dissolved in DMSO-d_6 and its spectrum determined on a Varian - T60 A NMR spectrometer using TMS as the internal standard.

Assignment of the chemical shifts to the different protons is shown below:

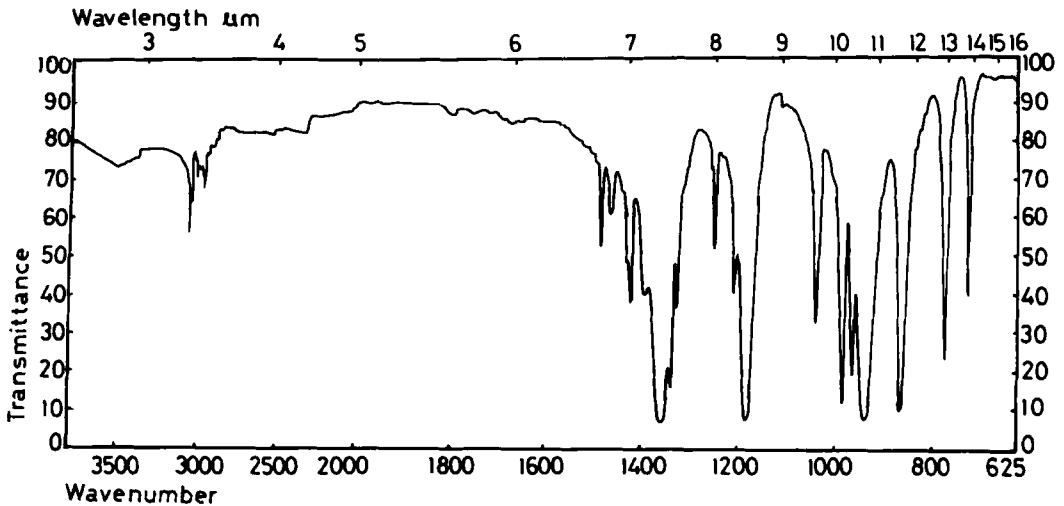


Figure 1: Infrared spectrum of Busulphan, KBr disc.

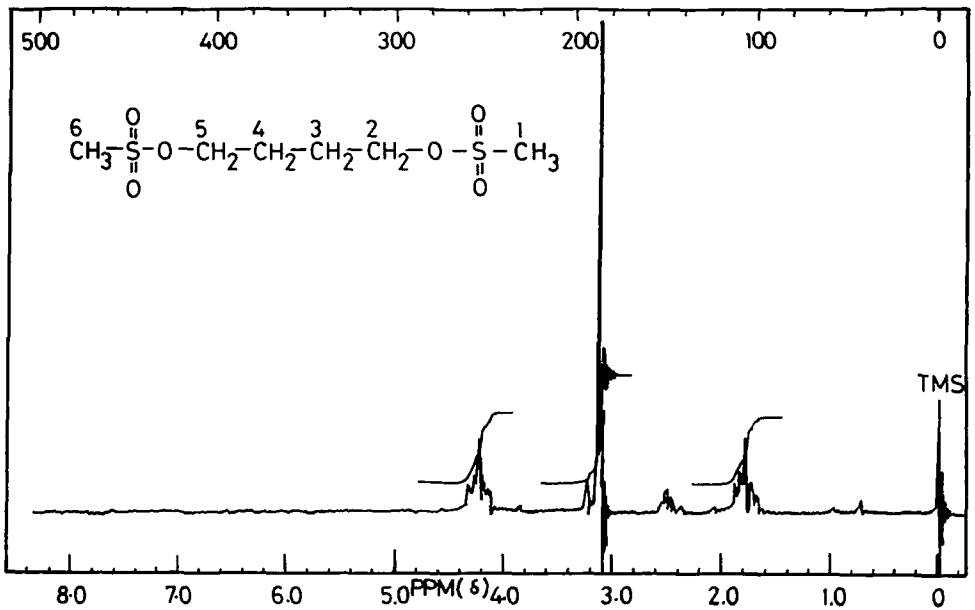


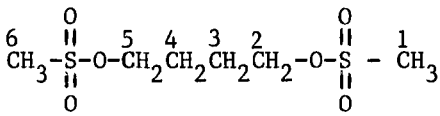
Figure 2: Proton nuclear magnetic Resonance spectrum of Busulphan in DMSO-d_6 using TMS as reference standard.

Chemical shift (δ)	Multiplicity	Proton assignment*
1.68-1.90	Multiplet	3 and 4 ($\underline{CH}_2$)
3.10	Singlet	1 and 6 ($\underline{CH}_3$)
4.1-4.40	Multiplet	2 and 5 ($\underline{CH}_2$)

*Please refer to Figure 2.

3.4.4 Carbon-13 Nuclear Magnetic Resonance Spectra (C-13 NMR)

The carbon-13 NMR spectra of busulphan in DMSO-d_6 using TMS as an internal reference are obtained using a Jeol FX 100 MHz spectrometer at an ambient temperature. Figure 3 and 4 represent the proton-decoupled and off-resonance spectra respectively.



The carbon chemical shifts are assigned on the basis of the chemical shift theory and the off-resonance splitting pattern and are shown below:

Chemical shift (δ)	Multiplicity	Carbon assignments
24.70	Triplet	3 and 4
36.52	Quartet	1 and 6
69.40	Triplet	2 and 5

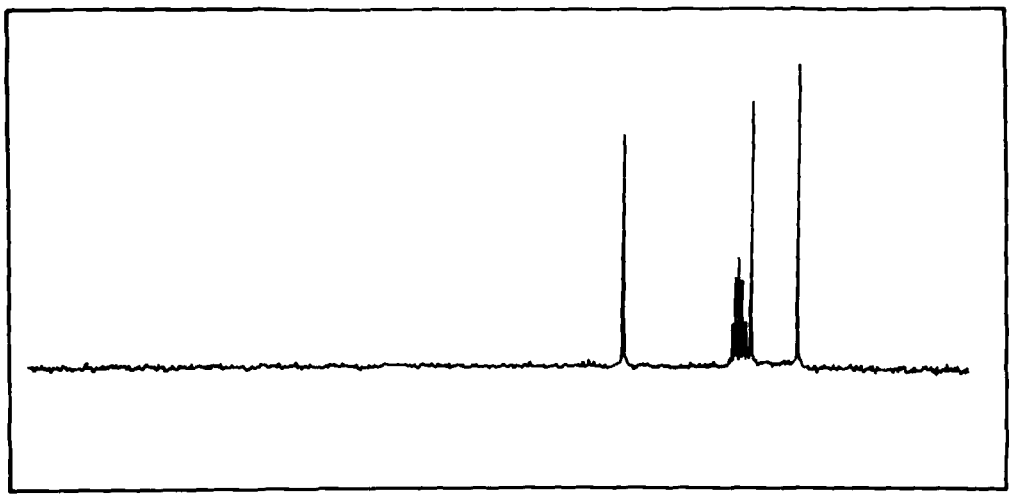


Figure 3 : Proton - decoupled carbon - 13 nuclear magnetic resonance spectrum of Busulphan in DMSO - d_6 using TMS as reference standard.

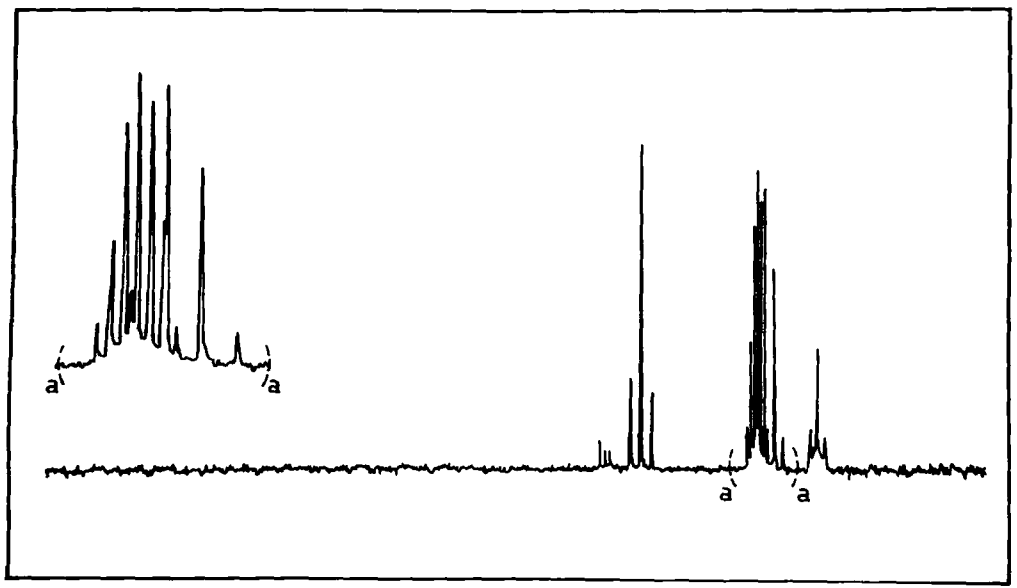


Figure 4: Off-resonance carbon-13 nuclear magnetic resonance spectrum of Busulphan in DMSO-d_6 using TMS as reference standard.

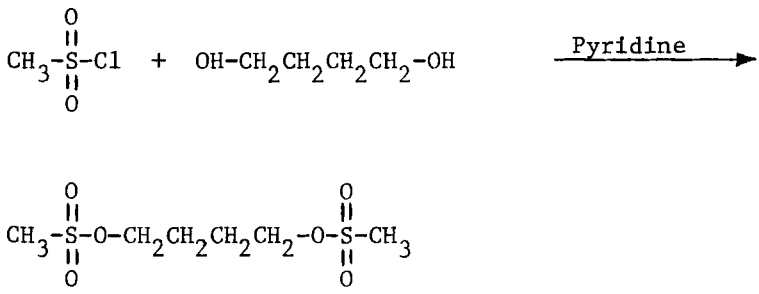
3.4.5 Mass Spectrum

The electron impact (EI) mass spectrum of busulphan at 70 eV recorded on Varian Mat 311 mass spectrometer and the direct chemical ionization (DCI) mass spectrum obtained with Finnigan 4000 mass spectrometer are shown in figures 5 and 6 respectively. The (EI) spectrum (Figure 5) shows a base peak at m/e 71, the molecular ion peak was not detected. Other major fragment are at m/e 175, 122, 111, 109, 97, 79, 55 and 42. A proposed mechanism of fragmentation of the EI fragments is shown in scheme 1.

The CI spectrum (Figure 6) is simple and shows a base peak at m/e 151 and at molecular ion peak at 247 corresponding to (M + 1) and minor peak at m/e 125.

4. Synthesis

By esterifying 1,4-butanediol [$HOCH_2CH_2CH_2CH_2OH$] with methanesulfonyl chloride [CH_3SO_2Cl] in the presence of pyridine (31, 33) as shown below:



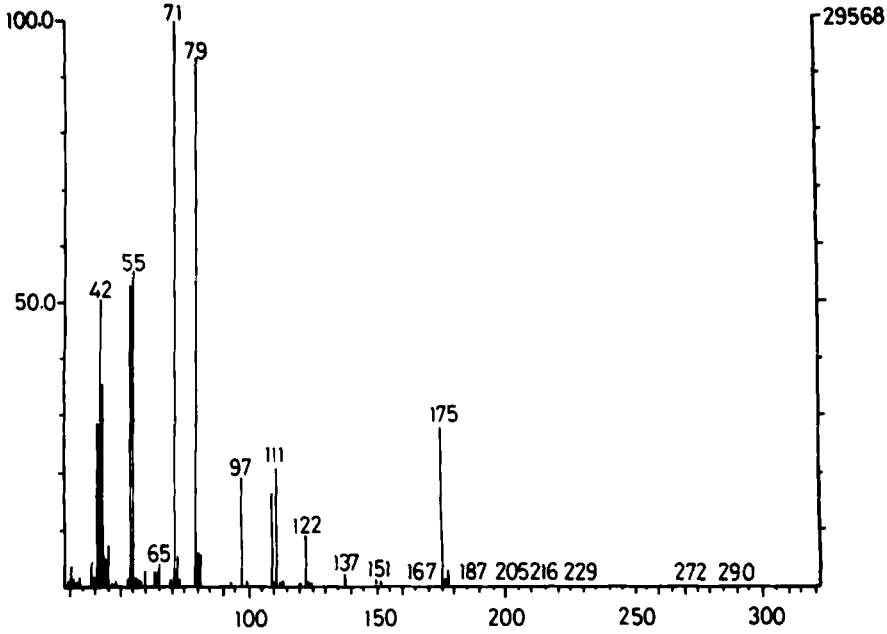


Figure 5 : Electron impact (EI) mass spectrum of Busulphan.

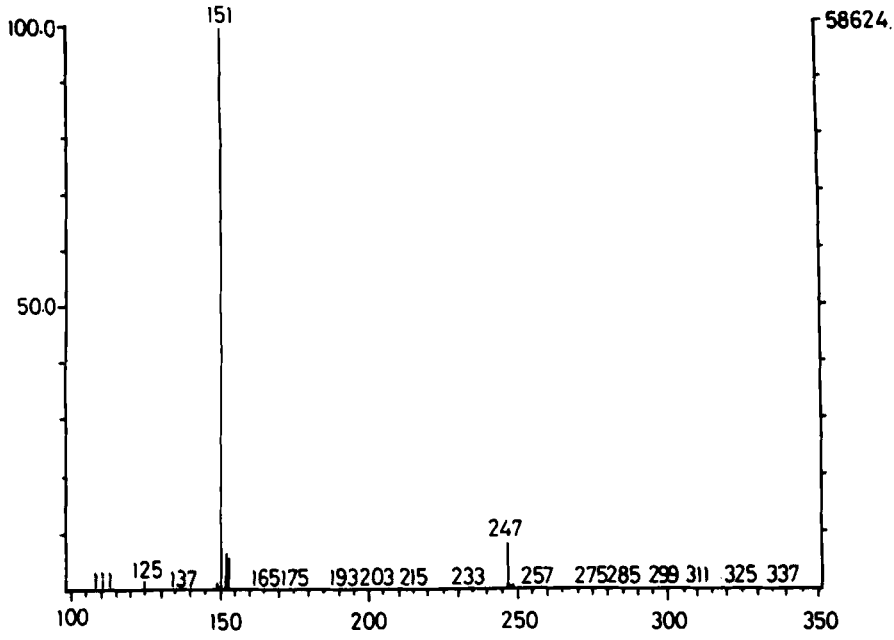
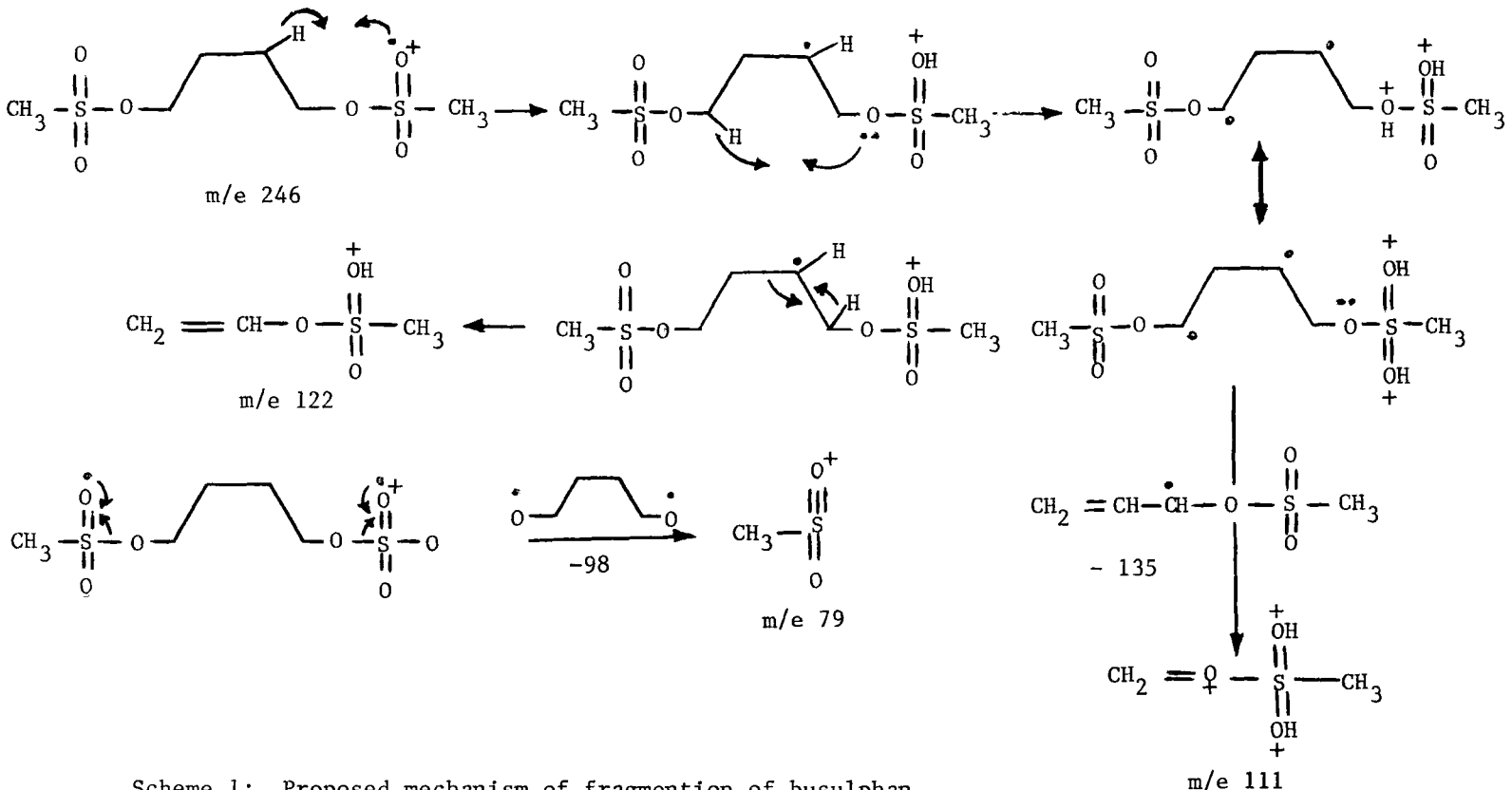
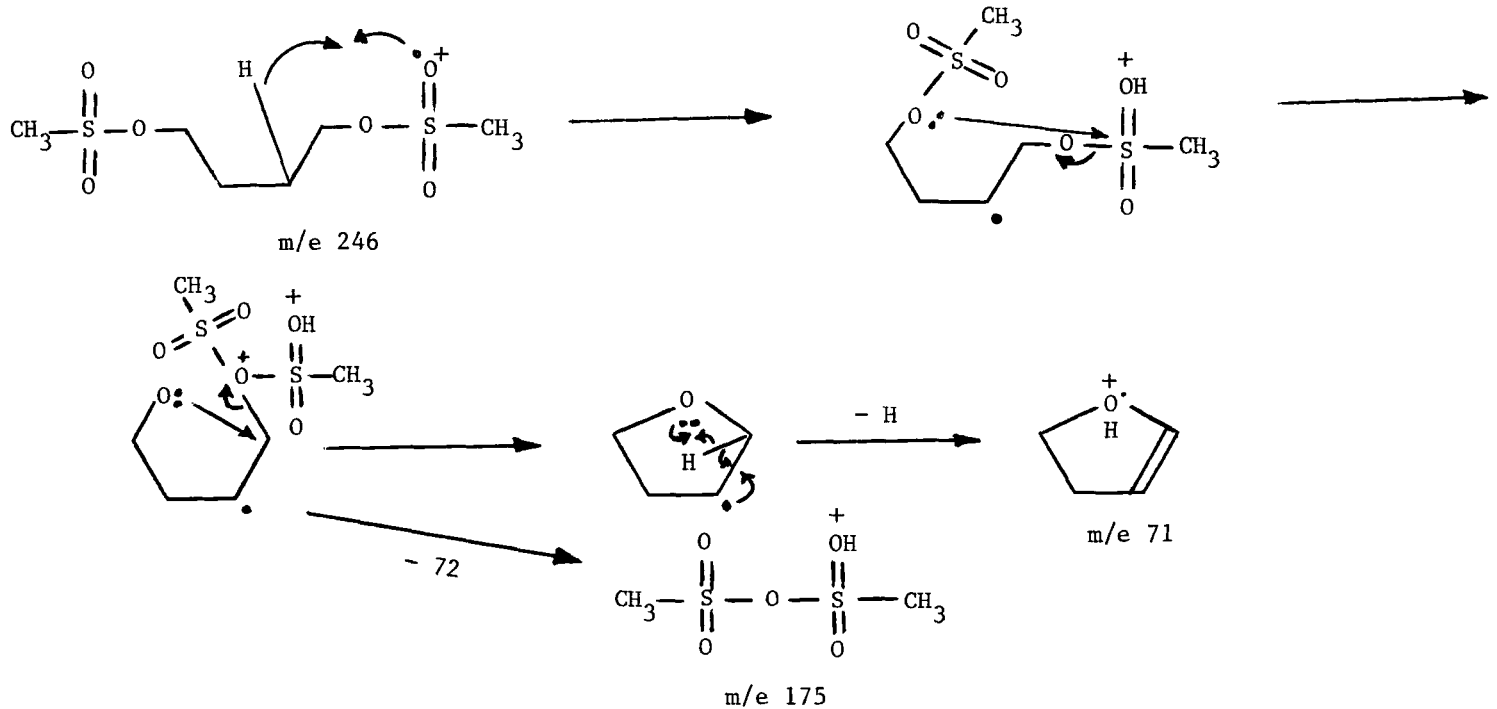


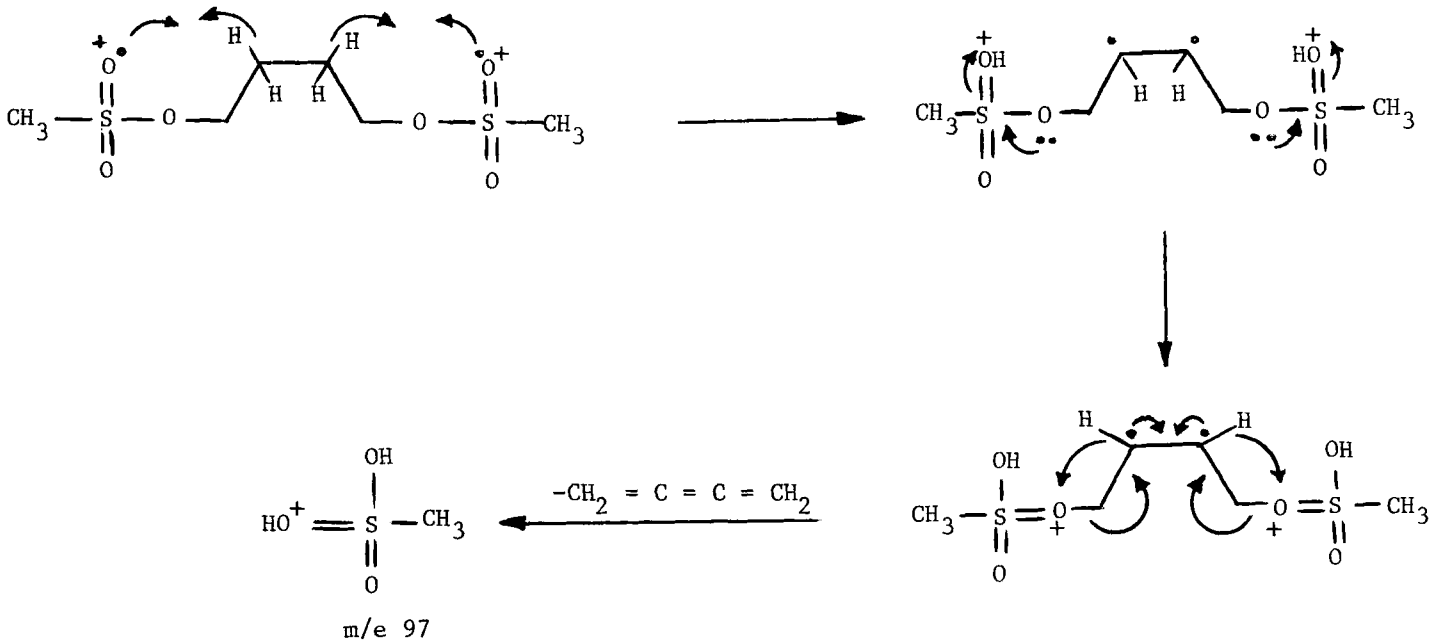
Figure 6: Chemical ionization(CI) mass spectrum of Busulphan.



Scheme 1: Proposed mechanism of fragmention of busulphan.



Scheme 1: Continued.



Scheme 1: Continued.

5. Pharmacokinetics

Absorption, Metabolism and Excretion

Busulphan is readily absorbed from the gastro-intestinal tract and rapidly disappears from the blood. It is largely excreted in the urine as sulphur - containing metabolite (29). Due to its alkylating activity, the compound might be bound both reversibly and irreversibly (covalently) to blood component (34). The metabolism takes place mainly in the liver. The clinical response usually begins within 1 to 2 weeks after initiation of therapy. It appears to be practically completely excreted in the urine as methane-sulphonic acid (35).

Nadkarni et al (4) using ^{14}C and ^{35}S-labeled busulphan found that, after intravenous dosing, the radioactivity in the blood rapidly decreased, and between 20% and 95% was removed within 3 to 5 minutes. After oral dosing an initial log phase of about 0.5 to 2 hours was observed before measureable blood levels were achieved. After oral ^{3}H-labeled busulphan, Vodopick et al, (36) found a prompt rise in the plasma level of radioactivity, which is peaked within the first hour, followed by a rapid fall and then a gradual rise in radioactivity. Busulphan is eliminated with $t_{\frac{1}{2}}$ of about 2.5 hour, for the initial phase of radio labeled drug in plasma. Only about 1% of a dose of busulphan is excreted as unchanged drug within 24 hours. The major portion of busulphan is probably eliminated by enzymatic activity rather than pure chemical degradation (36).

Ehrsson et al (37) have studied the busulphan kinetics and reported that kinetics were followed in patient with chronic myelocytic leukemia after oral dose of 2.4 to 6 mg. The plasma concentration-time data could be fitted to a zero-order absorption one-component open model. The elimination rate constant averaged 0.27 ± 0.05 hr^{-1} (SD). The plasma AUC was linearly related to the dose. The log time for the start of absorption, the time absorption ends, and the absorption rate constant showed

some inter-individual variation. About 1% of the drug is excreted unchanged in urine over 24 hours.

The fate of the drug in man has been studied after the administration of radiolabelled drug followed by measurement of total radioactivity in plasma and urine (4, 36, 37).

The drug is rapidly eleminated from the plasma and is reported to be extensively metabolized; twelve metabolites have been isolated, including methane-sulphonic acid and 3-hydroxytetrahydro-thiophene-1,1-dioxide, most of the metabolites have not been identified (38).

Wiygul et al (39) have studied metabolism of busulphan in the boll weevil. When one-day male boll weevils were force-fed ^{3}H- and ^{14}C-labelled busulphan, 34.34% of total radioactivity was recovered from the feces, about 1.09% as busulphan, at 72 hours after injection. Most metabolism of busulphan took place within 24 hours postingestion. 1,4-Butanediol, 2,3-butanediol, sulfolanes, malic acid, malonic acid, succinic acid, aminoacids, and methanessulphonic acid were identified as principal metabolites.

Jones and Campbell (40) have studied the metabolism, reactions and biological activities of some cyclic dimethanesulphonates. Relevance to the mechanism of action of busulphan (Myleran). The results obtained suggest that cycloalkylation of thiol groups by busulphan may represent a normal detoxification route rather than a mechanism of action.

Other studies on the metabolism of busulphan in boll weevils, are also reported (41, 42, 43).

6. Toxicity

Recently Bishop and Wassom (44) have published a detailed review article on the toxicity of busulphan incorporating around 290 references. The most important side effect of busulphan in high doses are thrombocytopenia and damage to haemotological tissues (45,53, 29).

Interstetial pulmonary fibrosis, known as "Busulphan lung", is as well recognised side effect busulphan therapy (54-64). A wasting or Addison - like syndrome has occurred in some patients receiving long term therapy with Busulphan (59, 65, 66). Other adverse effects include endocardial fibrosis (67), cataracts (68), cellular dysplasia of pancreas (69), adrenal insufficiency (70) and bone marrow damage (71-83). Laboratory studies in animals have shown significant immunosuppressive (48, 84, 85, 82, 86) and reproductive toxicity of busulphan (87 - 100).

Busulphan, administered during the third trimester of pregnancy, can adversely affect fetal growth (101). Teratogenic effect of this drug in animals has been reported by several workers (102 - 106). Busulphan is potentially mutagenic (104 - 114) and carcinogenic in man (115 - 123).

7. Methods of Analysis

7.1 Identification

The USP XX (1980) (32) describes the following identification tests for busulphan.

A) Fuse about 100 mg with about 100 mg of potassium nitrate and a pellet of potassium hydroxide weighing approximately 250 mg. Cool, dissolve the residue in water, acidify with diluted hydrochloric acid, and add a few drops of barium chloride TS; a white precipitate is formed.

B) To 100 mg add 10 ml of water and 5 ml of 1 N sodium hydroxide. Heat until a clear solution is obtained; an odor characteristic of methanesulfonic acid is perceptible.

C) Cool the solution obtained in Identification Test B, and divide it into two equal portions. To one portion add 1 drop of potassium permanganate TS; the purple color changes to violet, then to blue, and finally to emerald-green. Acidify the second portion of the solution with diluted sulfuric acid, and add 1 drop

of potassium permanganate TS: the color of the permanganate is not discharged.

British Pharmacopoeia (1980) (30) has also reported similar procedures for the identification of busulphan.

7.2 Titrimetric Methods

7.2.1 Aqueous

British Pharmacopoeia (1980) (30) described an aqueous titration procedure for the assay of busulphan as follows:

To 0.25 g of the drug add 20 ml of water and boil gently under a reflux condenser for thirty minutes. Wash the condenser with a small quantity of water, cool and titrate with 0.1 M sodium hydroxide VS, using phenolphthalein solution as indicator. Each ml of 0.1 M sodium hydroxide VS is equivalent to 0.01232 g of $C_6H_{14}O_6S_2$.

7.2.2 Gravimetric

Busulphan tablets are sugar coated and contain only milligram quantities, hence the flask combustion method is not applicable becuase of the large amount of organic matter. It is necessary to extract the busulphan with acetone and decompose it in a sealed tube with nitric acid as follows:

Triturate an accurately weighed quantity of powdered tablets equivalent to about 2 mg of busulphan with 10 ml of hot acetone, decant the liquid through a filter into a Carius tube and evaporate to small volume under a jet of warm air. Extract in the same way with two further 10-ml quantities of acetone, decanting and evaporating after each extraction and evaporating to dryness after the third extraction. Add 0.5 ml of fuming nitric acid, seal the tube and heat at 300° for two hours. After opening the

Carius tube transfer its contents to a small dish with 15 ml of water, evaporate to dryness, dissolve the residue in water and acidify with concentrated hydrochloric acid. Add an excess of 10 per cent barium chloride solution, heat on a water-bath for three hours, filter through a platinum filtering crucible, ignite at 800° for one hour, cool and weigh. Each mg of residue = 0.5276 mg of busulphan (124).

7.3 Chromatographic Methods

7.3.1 Gas Chromatographic Method

Hassan and Ehrrson (125) developed a gas chromatographic method for the determination of busulphan in plasma with electron capture detection. The method comprise a new derivatization technique for extraction of busulphan from plasma in a single step combined with high separation efficiency and sensitivity by using capillary column in combination with electron capture detection. The derivatization of busulphan was done directly in plasma by adding sodium iodide in acetone. Plasma sample (1.0 ml) was mixed with 0.1 ml of 1.0 µg/ml of 1,5-bis(methanesulfoxy)pentane in acetone and sodium iodide in water (1 ml acetone and 8 M Na^+ iodide). After addition of 0.4 ml n-heptane the reaction was carried out at 70°C for 70 minutes under stirring. The organic phase was separated for analysis on gas chromatography. 1-2 µl of solution was injected into Varian 3700 GC equipped with a constant current ^{63}Ni lectron-capture detector. An OV-I fused silica cappillary column (25 m x 0.3 mm i.d.) with a film thickness of 0.52 um (Hewlett-Packard) was used.

The instrument was operated isothermally with the oven, detector and injector port temperatures at 145°C, 250°C and 200°C respectively. The helium was used as carrier gas with a flow rate of 2 ml/min, the inlet pressure was

0.4 bar. Nitrogen was used as make up gas and added through the hydrogen inlet at a rate of 30 ml/min in the detector base. A split ratio of 1 : 10 was used. A high separation efficiency and sensitivity was obtained using this method, the retention time was 8 minutes. The standard curve using plasma was linear within the range of 5-300 ng/ml. The precision of this method ± 3.9% (c.v.) at the 10 ng/ml level (n = 5) and ± 2.3% (c.v.) at the 100 ng/ml (n = 5).

7.3.2 Gas Chromatography - Mass Spectrometry (GC-MS)

Ehrsson and Hassan (126) developed a chromatographic method for the determination of busulphan in plasma by GC-MS with selected ion monitoring. Busulphan extracted from plasma with methylene chloride and converted to 1,4-diiodobutane. 1.0 Ml of plasma was mixed with 0.1 ml of internal standard (1,5-pentanediol dimethanesulfonate 1.0 µg/ml) in acetone and extracted with 4 ml of methylene chloride using a mechanical shaker (100 strokes/min). The organic phase was separated and evaporated to dryness, derivatization was done by adding 0.1 ml of 1 M sodium iodide in acetone with a reaction time of 20 minutes at 70°C. After addition 0.05 ml of n-hexane and 0.1 ml of water the mixture was vigorously shaken. The aliquot (1-2 µg of n-hexane) was used for the analysis by GC-MS; LKB 2091 with an ionizing energy of 70 eV was used. The column (1.5 m x 2 mm i.d.) was packed with 10% SP-2401 on 100-120 Supelcoport and was operated at 140°using a helium flow rate of 20 ml/min. The injector and the ion source temperature were 230° and 270°C, respectively.

The minimum detectable concentration was 5.7×10^{-16} mole/sec. The standard curve was linear over a range of 10-400 ng/ml. The relative S.D. was 2.6% at 100 ng/ml and ± 4.3% at 10 ng/ml (n = 5).

7.4 NMR Spectrometric Methods

a) Truczan and Lau-Cam(127) described a simple NMR spectroscopic method for the assay of busulphan in tablets. 20 Gram of busulphan tablets are finely powdered, an accurately weighed quantity of powder equivalent to 6 mg of busulphan is transferred to a 15 ml vial. To this 1 ml of internal standard solution (11.68 mg of methenamine in 10 ml of chloroform) was added, the vial was closed with a septum and crimper sealed with an aluminium seal. 2 Ml of chloroform was added using a syringe by introducing the needle through septum. The contents were shaked vigorously. The insoluble matter is allowed to settle and 0.5 to 1 ml of upper layer is transferred to NMR tube. 1 Drop of reference standard (50 mg tetramethylsilane (TMS) in 10 ml chloroform) was added, caped and shaken. The NMR spectra were recorded with a 200 MHz, Wise bore, Fourier transform spectrometer equipped with 5 mm proton probe and computer with 24 K memory. The spectrometry conditions were: ambient temperature = 30°C, frequency 1 h = 200.06 MHz, Observation frequency range = 1600 H_2.

Pulse width	=	9.13 sec.
Pulse repetition time	=	14 sec.
FTD data points	=	8
Observation time	=	2.2 minutes.

Integrate the broad triplet at about 4.3 ppm due to the 4-methylene protons ($-CH_2-O-SO_2-$) of busulphan and the singlets and about 4.7 ppm due to the 12 methylene protons of the internal standard. The quantity of busulphan in mg is obtained from:-

$$(Au/As) \times (Ev/Es) \times C, \text{ where}$$

Au = Integral value of signal representing bulsulphan.
As = Integral value of signal represent methanamine.
Ev = Formula weight of busulphan /4, 61.58.
Es = Formula wieght of methenamine/12, 11.68.
C = Weight in mg of methenamine taken for the analysis.

b) Feit and Rastrup-Andersen (128) have investigated the hydrolysis of busulphan by means of NMR spectroscopy. The final product was found to be tetrahydrofuran (THF). The rather unstable intermediate, 4-methane-sulphonybutanol, was synthesized and proved to undergo cyclization to THF by intramolecular alkylation. The half-life of this first-order reaction in aqueous solution at 37° was determined to be approximately 12 minutes at pH 3 as well as at pH 7.4. From the data obtained, it is concluded that 4-methansulphony-loxybutanol is unlikely to be responsible for the biological action of busulphan.

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CHLORAMBUCIL

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1. History

This aromatic derivative of mechlorethamine was synthesized first at the Chester Beatty Research Institute in England. It was synthesized among other aromatic derivatives of the chloroethylamines by the British chemists, Everett, Roberts and Ross (1, 2). Chlorambucil has shown activity in a variety of human malignancies (3). These include chronic lymphocytic leukemia (4,5). Hodgkin's and non-Hodgkin's lymphoma (6), choriocarcinoma, ovarian carcinoma, and breast carcinoma (7). It is most often employed in the long-term maintenance management of chronic lymphocytic leukemia. Moore et al (8) found that a response rate of 19% was achieved in 52 advanced breast cancer patients.

2. Description

2.1 Nomenclature

2.1.1 Chemical Names

4-[Bis(2-chloroethyl)amino]benzenebutanoic acid;
4-[p-[Bis(2-chloroethyl)amino]phenyl] butyric acid;
N,N-di-2-chloroethyl-γ-p-aminophenylbutyric acid;
γ-[p-Di(2-chloroethyl)aminophenyl]butyric acid;
4-[4-Di-(2-chloroethyl)aminophenyl]butyric acid;
4-[4-(Bis(2-chloroethyl)amino)phenyl]butanoic acid;
p-Di(2-chloroethyl)aminophenyl butyric acid (9 - 12).

2.1.2 Generic Names

Chlorambucil, C.B. 1348. NSC 3088 Chlorbutinum (10-12).

2.1.3 Trade Names

Chlorambucil, Leukeran, Chloroambucil, (11).
Amboclorin; Chloraminophene; Linfolysin (10-12).

2.1.4 CAS Registry No.

(305-03-3).

2.1.5 Wiswesser Line Notation

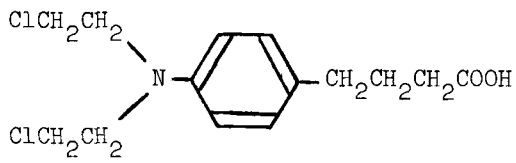
QV3R DN2G2G (13)

2.2 Formulae

2.2.1 Empirical

$C_{14}H_{19}Cl_2NO_2$ (14,15).

2.2.2 Structural



2.3 Molecular Weight

304.20 (10,15).

2.4 Elemental Composition

C 55.27%; H 6.30%, Cl 23.31%, N 4.60%, O 10.52% (10).

2.5 Appearance, Color and Odor

A white or off-white crystalline or granular powder with a slight odor (11).

2.6 Crystal Properties

Flattened needles from petroleum ether (10).

3. Physical Properties

3.1 Melting Point

Melts between 64° and 69° (12).

3.2 Extraction

Chlorambucil is extracted by organic solvents from aqueous acid solutions (12).

3.3 Solubility

Practically insoluble in water (14). Soluble at 20°C in 1.5 parts of alcohol, in 2.5 parts of chloroform, and in 2 parts of acetone, soluble in ether (10) and in dilute solution of alkali hydroxides (11).

3.4 Moisture Content and Hygroscopicity

Not more than 0.5%, determined by Fischer titration (14).

3.5 Dissociation Constant

pKa value 5.8 (16).

3.6 Storage

It should be stored in a cool place in airtight, light resistant containers (11).

3.7 Spectral Properties

3.7.1 Ultraviolet Spectrum

The ultraviolet spectrum of chlorambucil in methanol exhibits maxima at 258 nm (E 1%, 1 cm 650) and 302 nm (E 1%, 1 cm 85); and minima at 225 nm and 281 nm (12).

The UV spectrum of chlorambucil in water and in methanol which was scanned from 200 to 400 nm using DMS 90 Varian spectrophotometer. It exhibits absorption maxima at 257 nm and at 302 nm and minima at 224 and 280 nm. The UV spectra in water and in methanol are shown in Figs [1] and [2], respectively.

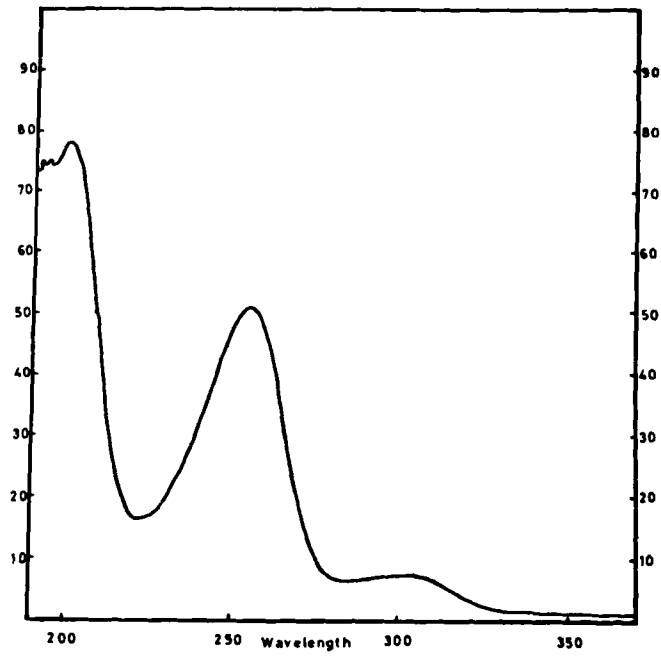


Figure 1 : Ultraviolet Spectrum of Chlorambucil in Water.

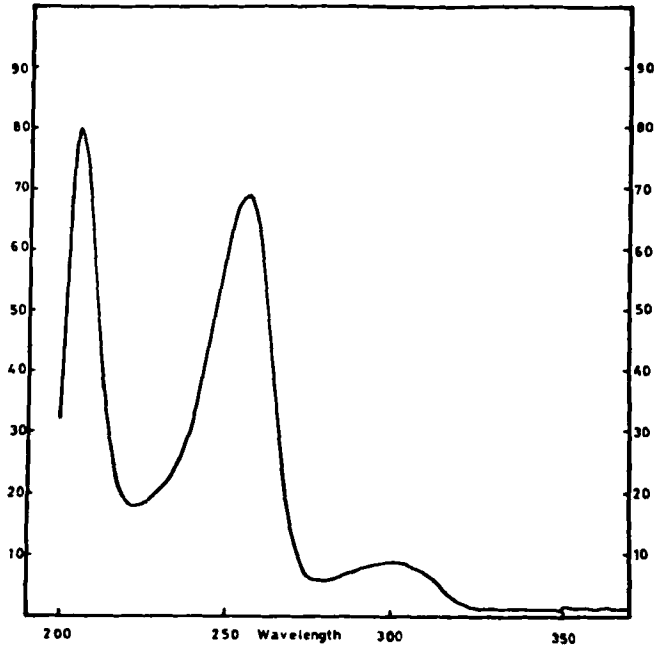


Figure 2 : Ultraviolet Spectrum of Chlorambucil in Methanol.

3.7.2 Infrared Spectrum

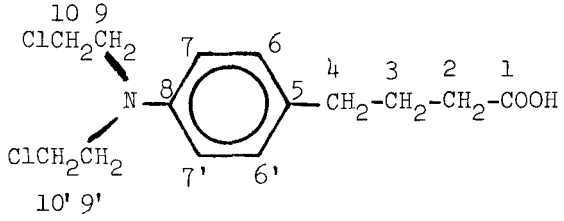
The infrared spectrum of chlorambucil is shown in Fig. [3]. The spectrum was obtained as KBr disc and is recorded in Pye-Unicam SP 1025 infrared spectrophotometer. The frequencies and their structural assignments are as follows:-

Frequency (cm^{-1})	Assignment
3100-2970	OH stretch and CH stretch
1720	C = O acid
1622	C = C aromatic
1450	CH deformation
730	C-Cl stretch

Other fingerprint bands are 1530, 1465 and 830 cm^{-1}. Clarke (12) reported the principal peak are 1695, 1520 and 1229 cm^{-1}.

3.7.3 Carbon-13 Nuclear Magnetic Resonance Spectra (C-13 NMR)

The C-13 NMR noise decoupled and off-resonance spectra are shown in Figs. [4] and [5], respectively. Both were recorded in deuterated chloroform on a Jeol-XL-100 MHz NMR spectrometer using tetramethylsilane as a reference standard. The spectral assignments are listed below:-



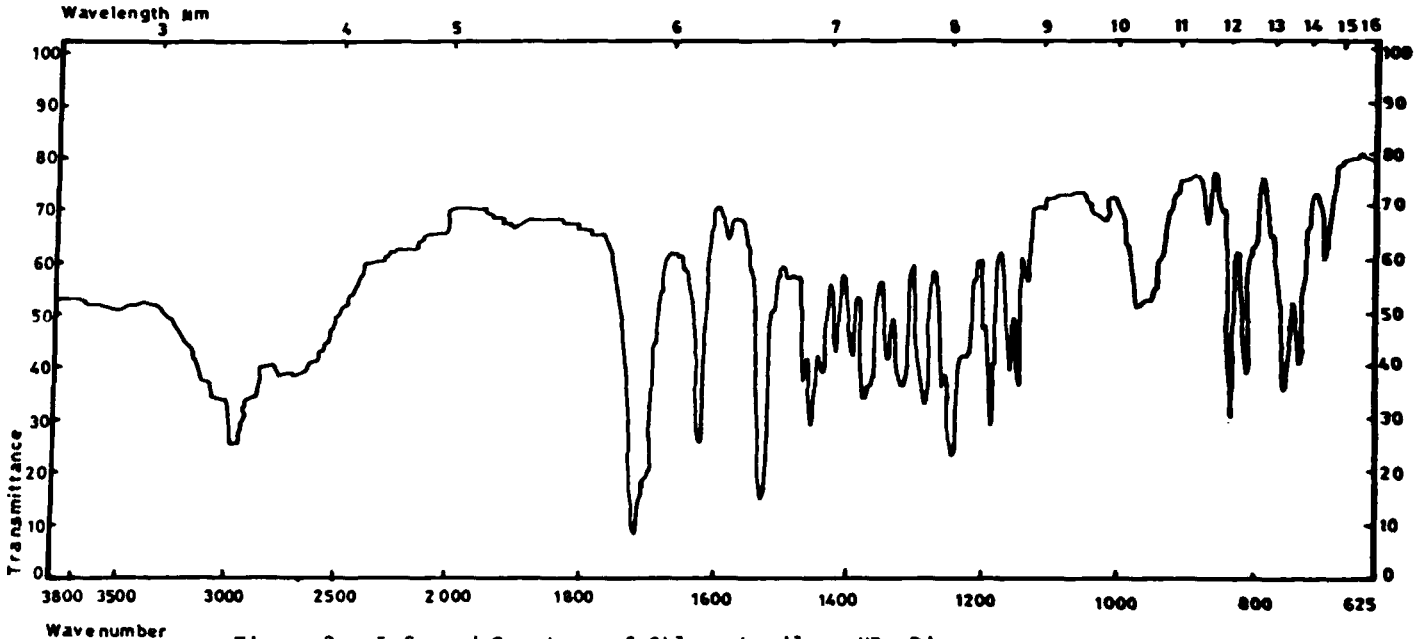


Figure 3 : Infrared Spectrum of Chlorambucil as KBr Disc.

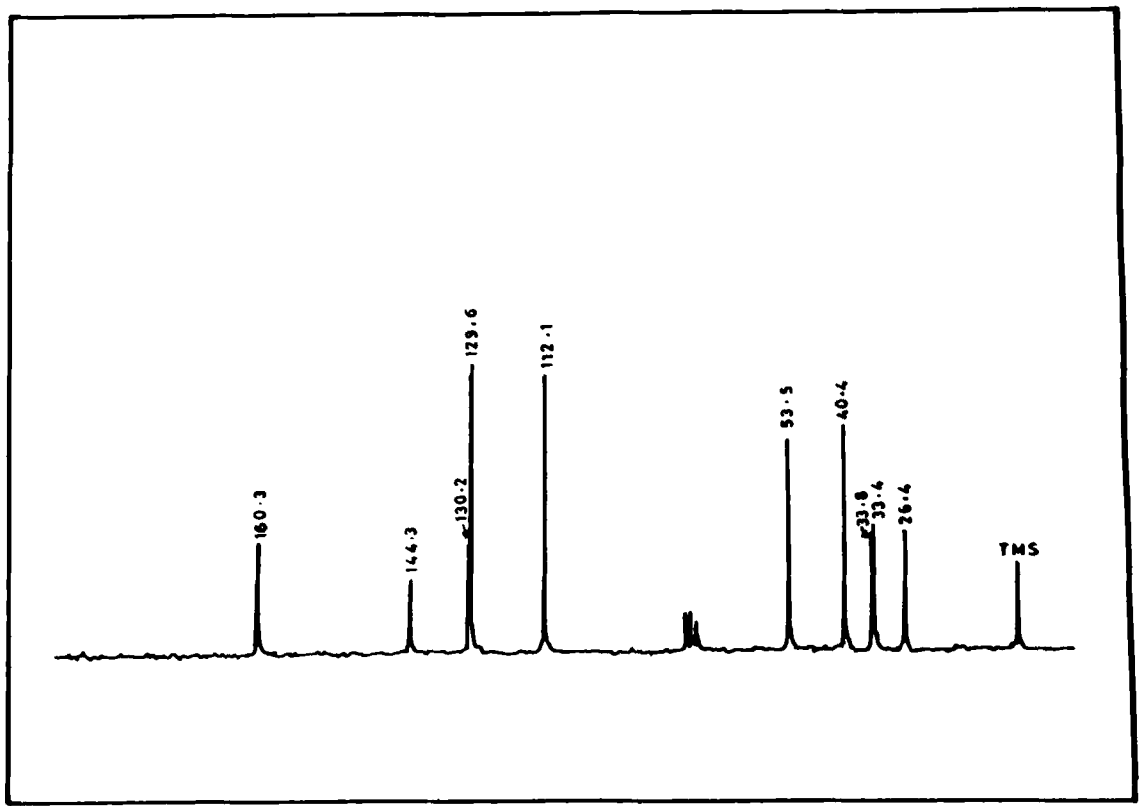


Figure 4 : ^{13}C-NMR Spectrum of Chlorambucil in $CDCl_3$ (Noise Decoupled).

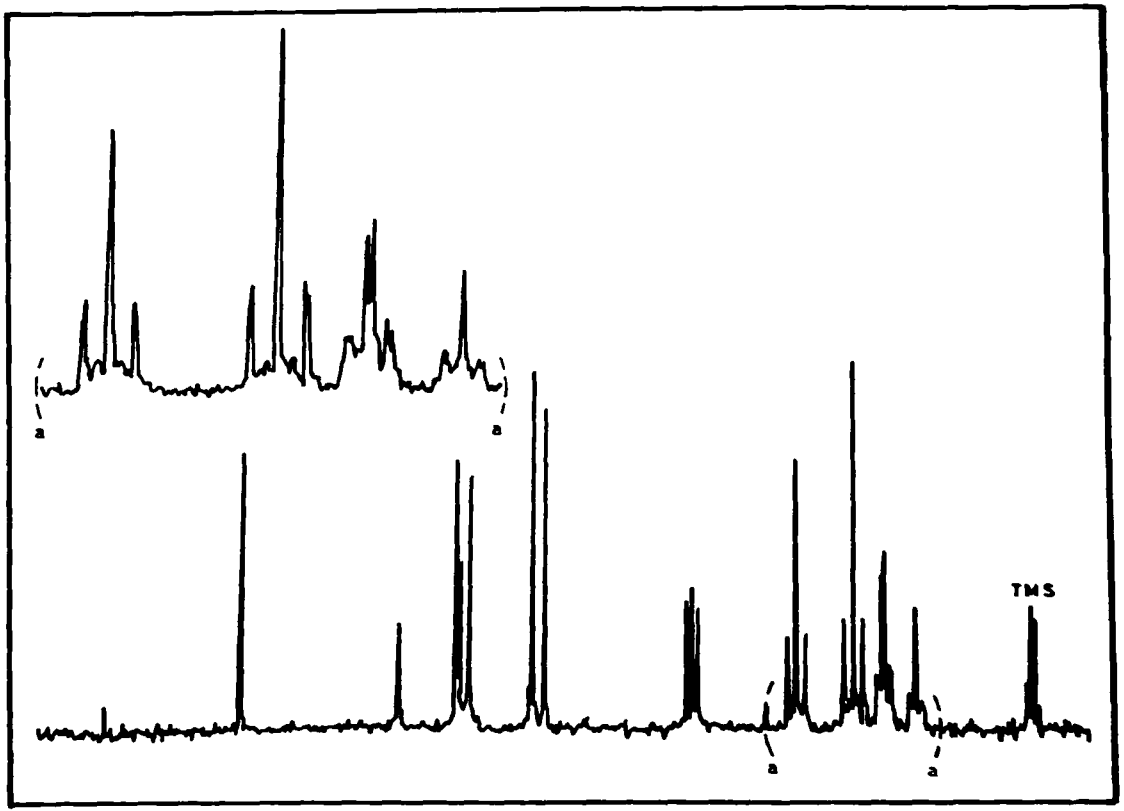


Figure 5 : ^{13}C-NMR Spectrum of Chlorambucil in $CDCl_3$ (Off-Resonance).

Carbon No.	Chemical shift (ppm)	Multiplicity
1	180.3	s
2	33.8*	t
3	33.4*	t
4	26.4	t
5	130.2	s
6 and 6'	129.6	d
7 and 7'	112.1	d
8	144.3	s
9 and 9'	53.5	t
10 and 10'	40.4	t

s = singlet d = doublet t = triplet

3.7.4 Proton Magnetic Resonance Spectra (^{1}H-NMR)

The 60 MHz ^{1}H-NMR spectrum of chlorambucil in deuterated chloroform is shown in Fig. [6]. The spectrum was recorded on Varian T60-A NMR spectrometer using tetramethylsilane (TMS) as the internal standard.

The following are the structural assignments:

Chemical shift (ppm)	Assignments
Multiplet at 1.8-2.8	$-(CH_2)_3-CO$ protons
Singlet at 3.60	$-N-CH_2CH_2$ protons
Multiplet at 6.6-7.08	Aromatic protons
Singlet at 11.5	OH (acidic) exchangeable with D_2O, Fig. [7].

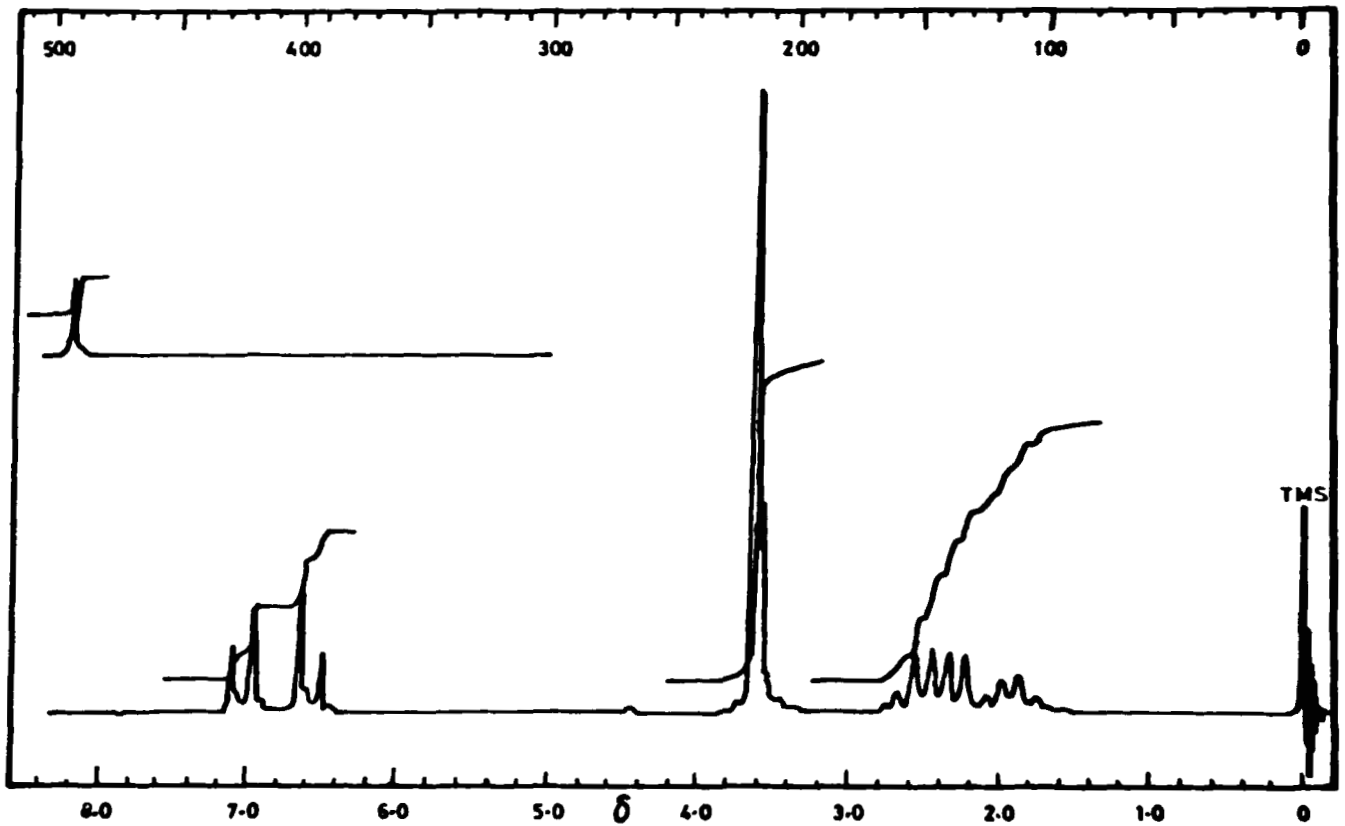


Figure 6 : ^{1}H-NMR Spectrum of Chlorambucil in Deuterated Chloroform.

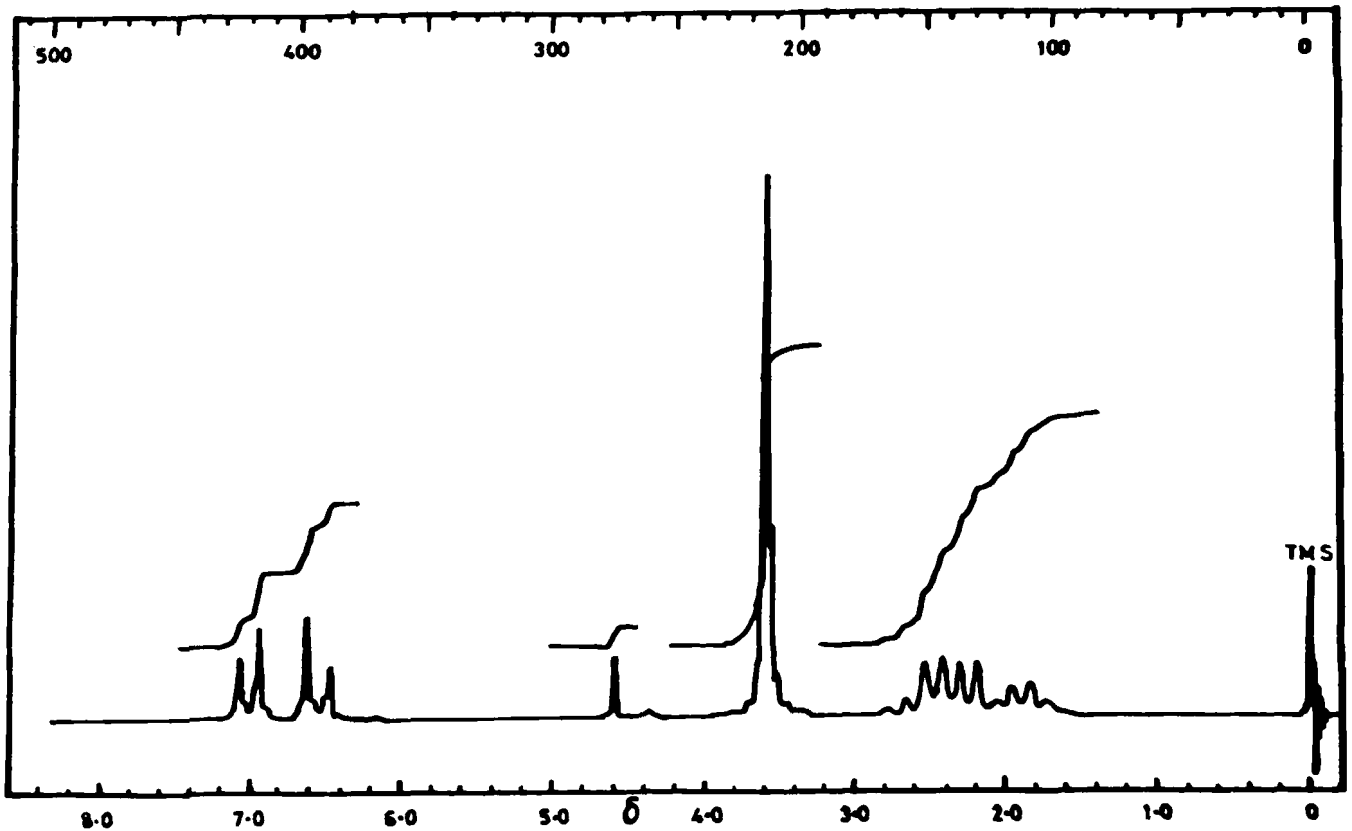


Figure 7 : ^{1}H-NMR Spectrum of Chlorambucil in Deuterated Chloroform + D_2O.

3.7.5 Mass Spectrum

The electron impact (EI) mass spectrum of chlorambucil at 70 eV, recorded on Varian Mat 311 mass spectrometer using ion source pressure of 10^{-6} Torr, ion source temperature of 180°C and an emission current of 300 μA. The spectrum is shown in Fig. [8]. The spectrum is dominated by m/e 254 ion (base peak) resulting from the loss of CH_2Cl and H. A proposed mechanism of fragmentation and the m/e of the major fragments is given in Scheme 1.

The chemical ionisation (CI) spectrum (Fig. [9]) is obtained on a Finnigan 4000 mass spectrometer with ion electron energy of 100 eV, ion source pressure of 0.13 Torr, ion source temperature of 150°C and emission current of 300 μA. The spectrum shows a pronounced peak resulting from the loss of HCl ($M^+ - 36$) constitute the base peak at m/e 268 and a molecular ion peak at m/e 304. A proposed fragmentation pathway for the fragment in the EI spectrum is shown in Schemes 1a and 1b. The mass spectral assignments of the prominant ions under CI conditions are given in the following

m/e	Fragment
306	$[M + 2H]+$
305	MH^+
304	M^+
303	$[MH - H_2]^+$
286	$[M - H_2O]^+$
268	$[M - HCl]^+$
270	$[M + 2H - H_2O]^+$
254	$[MH - (H_2 + CH_2Cl]^+$
232	$[M - 2HCl]^+$

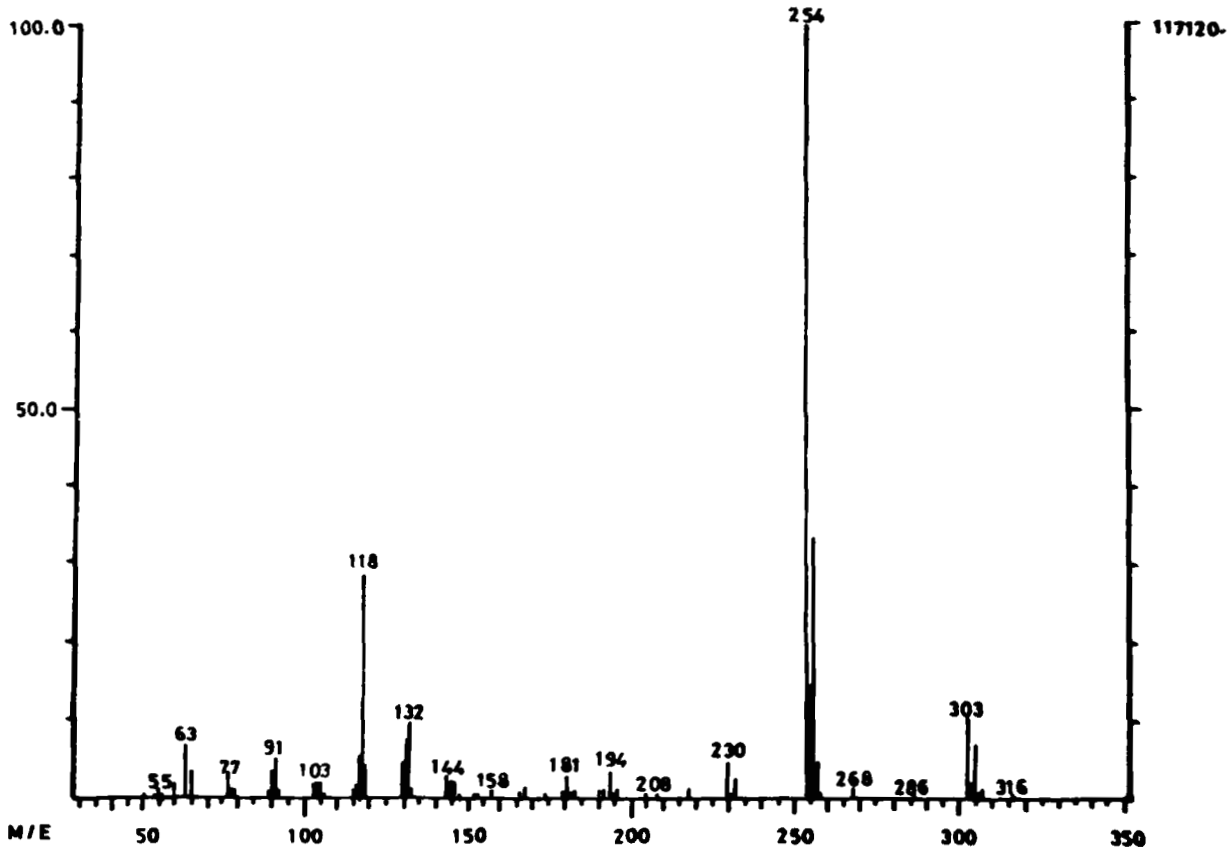


Figure 8 : Mass Spectrum of Chlorambucil (EI).

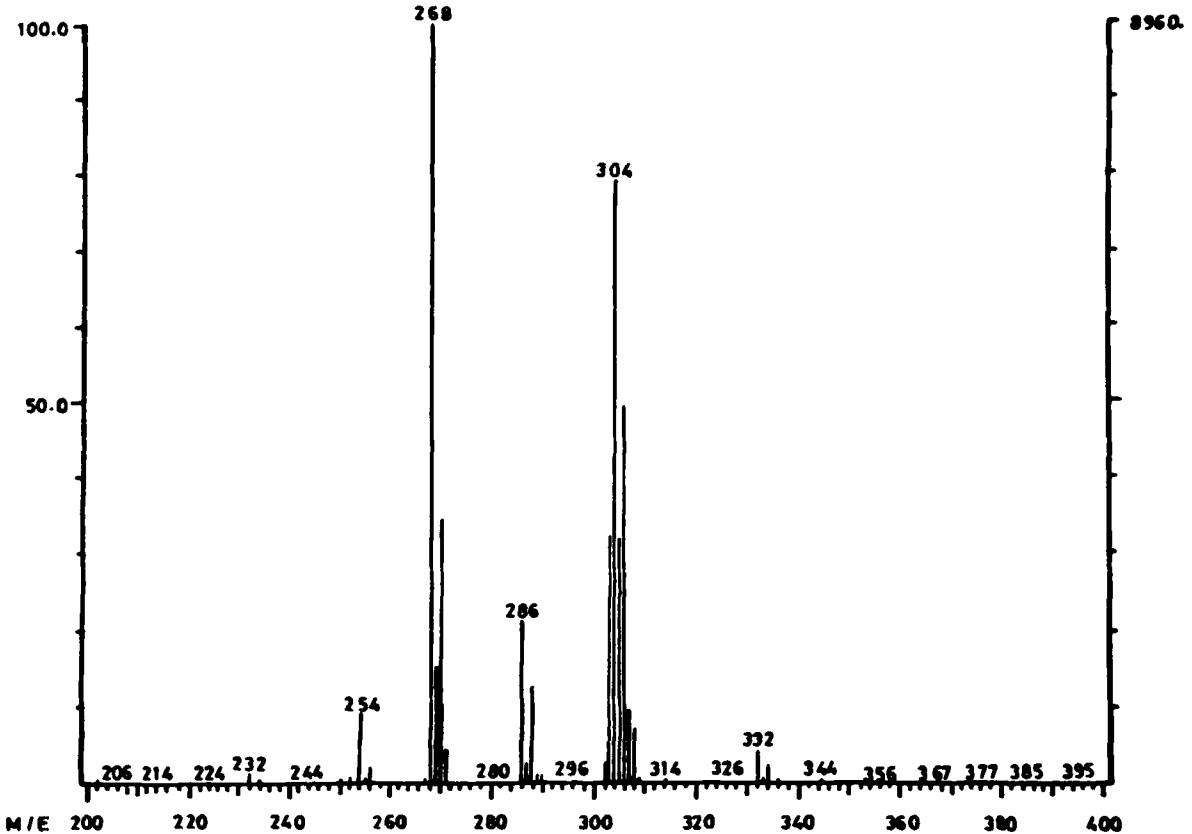
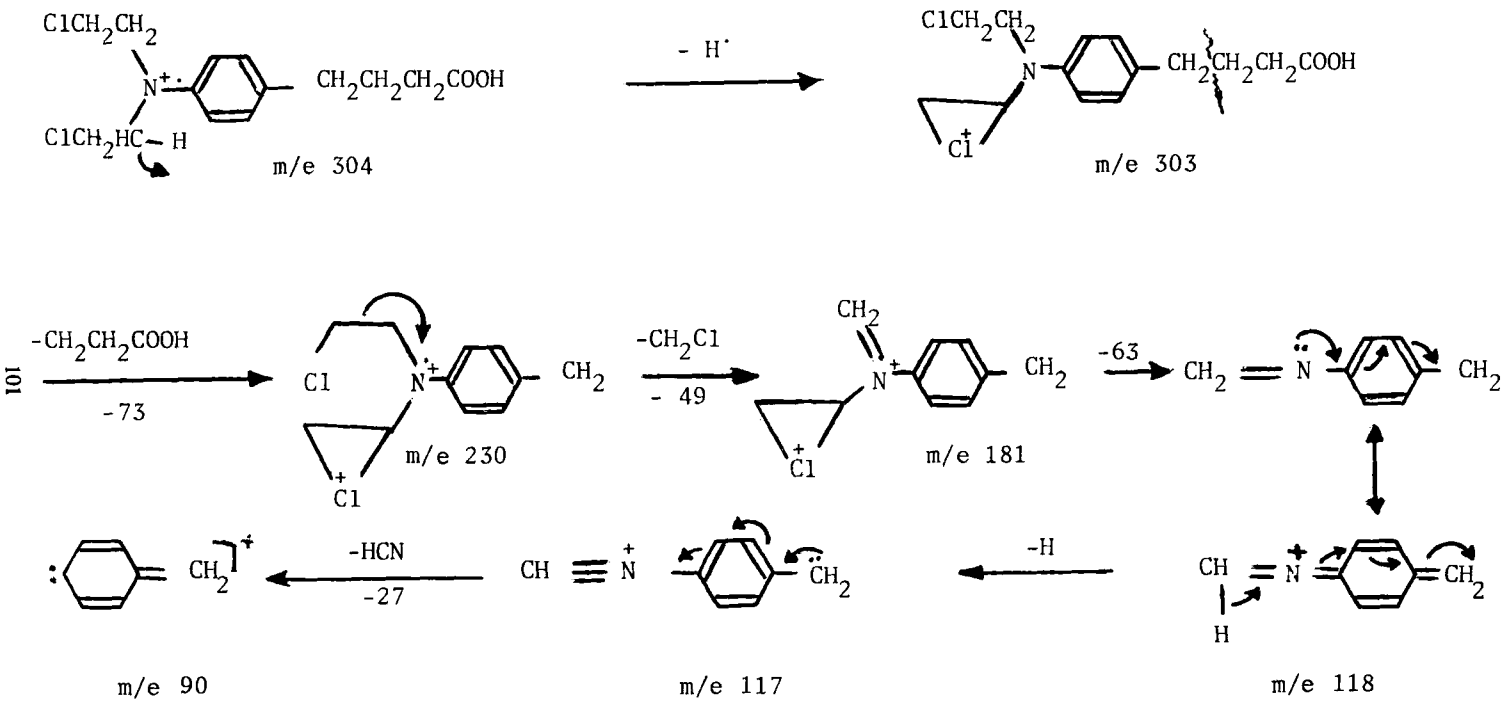
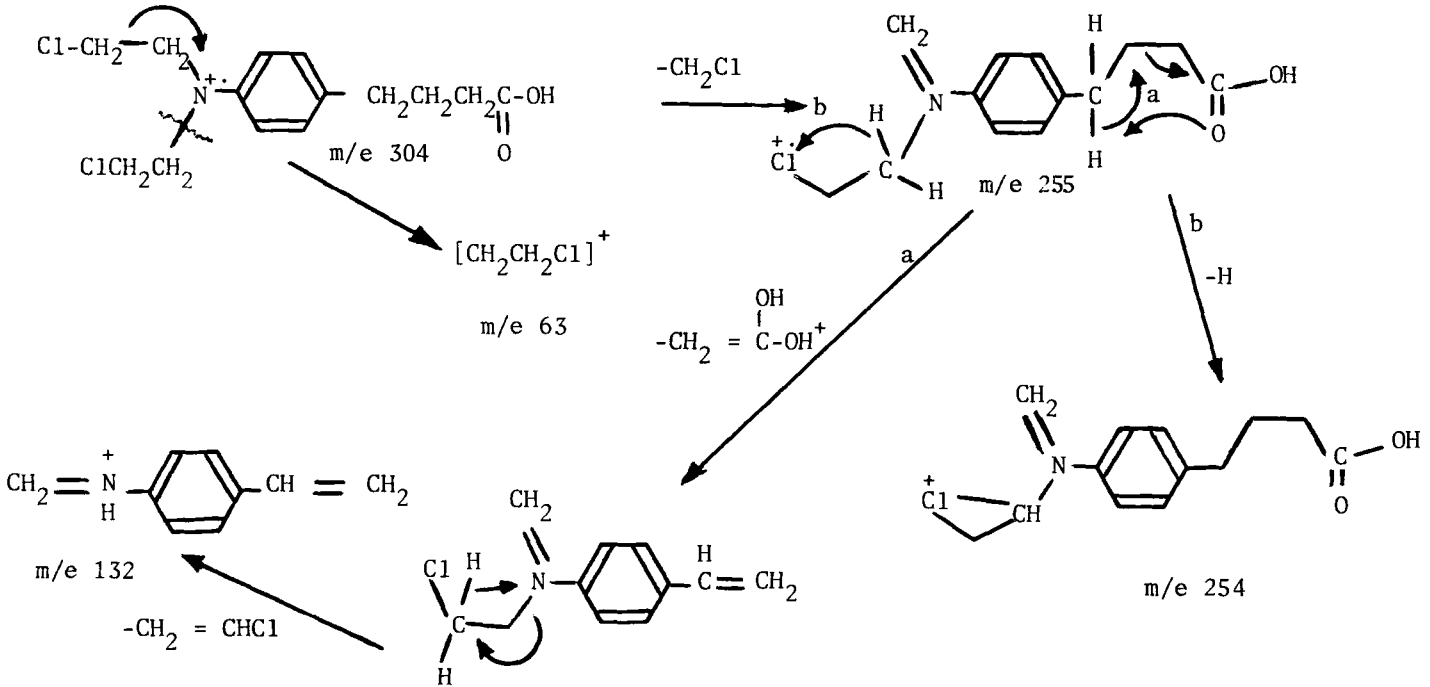


Figure 9 : Mass Spectrum of Chlorambucil (CI).



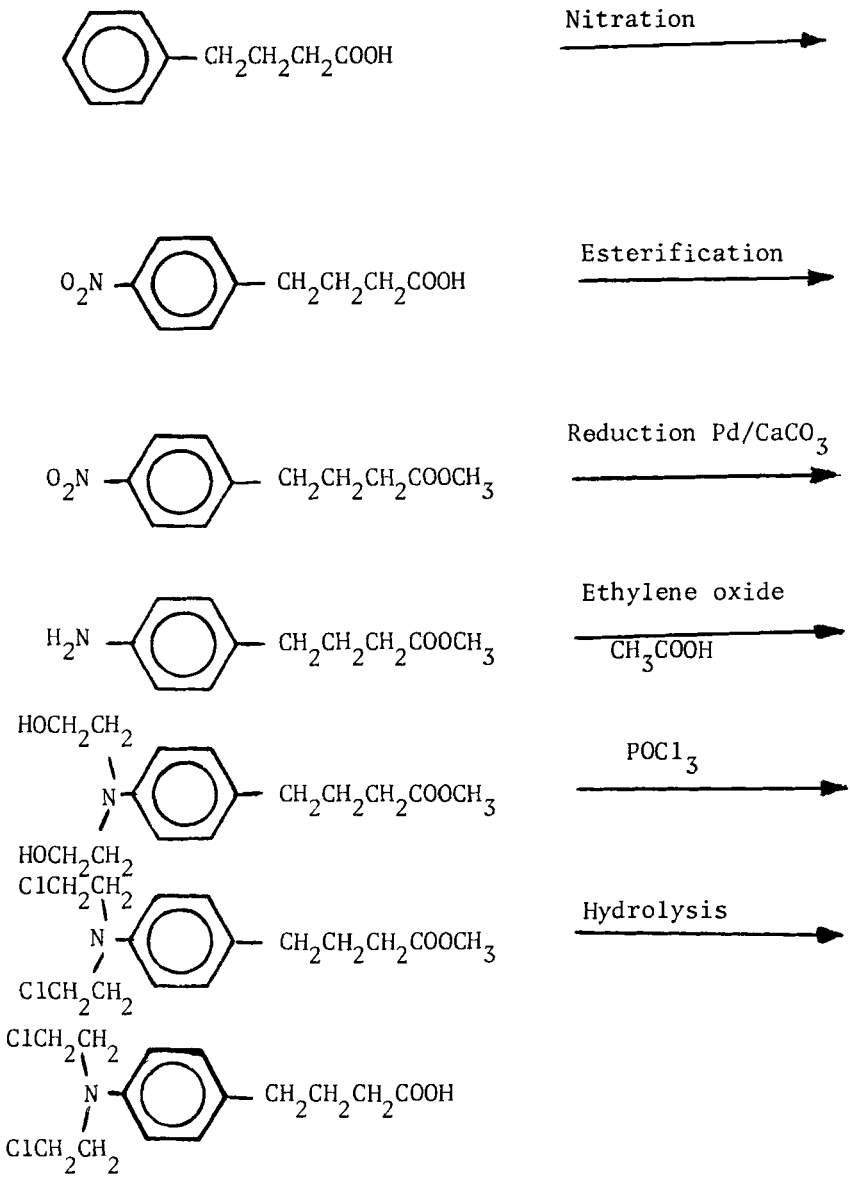
Scheme 1a: Proposed mechanism of fragmentation of chlorambucil.



Scheme 1b: Proposed mechanism of fragmentation of chlorambucil.

4. Synthesis

Everett et al (1) have synthesized chlorambucil and some other nitrogen mustard. The synthesis have been reported (17,18) as follows:



5. Pharmacokinetics

5.1 Absorption, Distribution, Metabolism and Excretion

Oral absorption of chlorambucil is adequate and reliable. The drug has a half-life in plasma of approximately 90 minutes, and it is almost completely metabolized (19). Although the intravenous route produced higher concentrations, absorption from the gastro-intestinal tract was consistently rapid and appeared to be complete; peak-plasma concentrations were achieved in 40 to 70 minutes. An hour after administration by either route the rate of metabolism was similar and sufficient to make a contribution to the activity of the drug (20).

There appears to be extensive metabolic degradation of the drug and a major metabolite, an aminophenylacetic acid derivative which has a half-life of about 2.5 hr in man, has been identified. The ultimate metabolite(s) of chlorambucil in man has yet to be defined and further work is required to confirm these initial pharmacokinetic observations (21).

McLean et al (20) reported two metabolites one was identified as the β-oxidation product of chlorambucil; 2[4-N,N-bis(2-chloroethyl)aminophenyl] acetic acid, also called phenylacetic mustard. This metabolite is known to have an alkylating action in animals.

Dorr and William (21) extensively reviewed the disposition of chlorambucil in human being and experimental animals. In rats, radioactive drug appear to be cleared from the blood rapidly, and at one hr the highest tissue concentration is found in the liver. However, fairly homogeneous tissue distribution was noted. The drug has also been shown to distribute well into ascitic fluid after subcutaneous administration. Sixty percent of drug radioactivity was excreted into the urine by 24 hr, with the majority of the remainder existing as tissue as tissue-bound drug (22). Significant drug binding to gamma globulin proteins has been observed. Apparently no drug is excreted in the feces. Because a portion of the drug molecule has lipophilic

properties, some fat (depot) drug storage may occur. This could explain the prolonged clinical effect of chlorambucil occasionally observed in man.

6. Therapeutic Uses

Clinical trials of chlorambucil were instituted in 1954. During the subsequent 5 years the substance was studied in quite some detail. The therapeutic uses of chlorambucil have been discussed by Larionov (23). The drug is given orally in tablet form in doses from 0.1 to 0.4 mg/kg, i.e. 6 to 25 mg daily. The course of treatment usually lasts 3-9 weeks. The total dose on average is about 400 mg per course but in individual patients it varies greatly with the form of the disease, the stage, the condition of the organism, previous treatment, etc. The therapeutic effect does not come at once; but usually in the 2nd to 3rd week after starting the drug. At these doses side effects in the gastrointestinal tract are rarely observed. Towards the end of the course, neutropenia and thrombocytopenia may appear, thus necessitating withdrawal of the preparation. The maintenance dose of the compound is from 0.03 to 0.1 mg/kg, i.e. 2-6 mg daily.

Chlorambucil gives the best effect in chronic lymphoid leumaemia both in the leukaemic and aleukaemic form and, in particular, in that variant known as follicular lymphoma Galton and Till (24). Altman et al (25), Israels et al (26) Doan et al (27). In chronic lymphoid leukaemias, chlorambucil is given in doses of 0.1 - 0.2 mg/kg, i.e. 6-12 mg daily for 4-8 weeks. The effect consists remission expressed, in particular, in reduction of the lymph nodes, spleen and liver, increased haemoglobin, fall in lymphocytosis and sometimes increase in neutrophils in the blood. The remission lasts from 3 months to 1-3 years. After the onset of a relapse, remission can again be obtained in some patients by the same drug (23).

Moore et al (8) used a continuous daily dose of 0.1 - 0.2 mg/kg. The drug may also be administered on an intermittent basis (0.4 mg/kg every four weeks (28). Both regimens commonly include prednisone. On the intermittent schedule, monthly pulse doses as high as 1.5 - 2.0 mg/kg were well tolerated and did not produce undue marrow toxicity. An effective biweekly dosing schedule

with lessened hematologic toxicity is also reported: 0.4 mg/kg every 2 weeks, increasing by 0.1 mg/kg increments to toxicity or remission (5).

7. Toxicity

According to Dorr and William (21) chlorambucil is one of the best tolerated oral alkylating agents. Bone marrow depression, including neutropenia, thrombocytopenia, and lymphopenia, occurs with prolonged use. Irreversible bone marrow damage, however, may occur. Thus myelosuppression is the common dose-limiting toxicity with chlorambucil. Gastrointestinal distress with larger doses occurs but is usually not serious. Rare hepatitis and disturbances of liver function have also been reported.

Central nervous system stimulation has occurred with chlorambucil but is uncommon unless large doses are used. Inadvertent toxic ingestions in children of 1.5 - 5 mg/kg have occurred without fatalities but with moderate toxicity characterized by vomiting, agitation, irritability, and hyperactivity followed by lethargy and eventual mild pancytopenia (29,30). Chromosomal damage is well documented for this agent although the exact mechanism is not well known (31). Lerner (32) has further associated chronic chlorambucil therapy with a high incidence of secondary acute myelogenous leukemia.

Similar to some other alkylating agents, chlorambucil can also cause alveolar dysplasia and pulmonary fibrosis with long-term use (33). A good clinical response to drug discontinuance and corticosteroids was noted in this case. Spermatogenesis is also depressed while the patient is on chlorambucil therapy (34). LD_{50} in rats in a single administration is 21-23 mg/kg (35).

8. Methods of Analysis

8.1 Identification

British Pharmacopoeia 1980 (9) describes the following identification tests:

1) Mix 0.4 g of chlorambucil with 10 ml of 2 M hydrochloric acid and allow to stand for thirty minutes, shaking occasionally. Filter, wash the

residue with two quantities, each of 10 ml, of water, and reserve the mixed filtrates and washings for test for identification 2. Melting point of the residue, after drying over phosphorous pentoxide at a pressure not exceeding 0.7 KPa (about 5 torr) for three hours, about 146°.

2) To 10 ml of the mixed filtrate and washings reserved in test for identification, add 0.5 ml of potassium mercuriiodide solution; a buff-colored precipitate is produced. To a further 10 ml add 0.5 ml of potassium permanganate solution the purple color is discharged.

3) Mix 0.6 g with 0.2 ml of phenylhydrazine, heat in a water bath for ten minutes, stirring occasionally, add 2 ml of absolute ethanol, heat for twenty seconds, filter immediately, and allow to stand for thirty minutes. Crystals of phenylhydrazinium chloride are formed, which, after washing with two quantities, each of 1 ml, of absolute ethanol and drying at 105°, have a melting point of about 245°, with decomposition.

U.S. Pharmacopeia XX (14) describe the following tests for the identification of chlorambucil:

1) The infrared absorption spectrum of a 1 in 125 solution in carbon disulfide, in a 1 mm cell, exhibits maxima only at the same wavelength as that of a similar solution of USP Chlorambucil Reference Standard.

2) Dissolve 50 mg in 5 ml of acetone, and dilute with water to 10 ml. Add 1 drop of 2 N sulfuric acid, then add 4 drops of silver nitrate (test solution): no opalescence is observed immediately (absence of chloride ion). Warm the solution on a steam bath: opalescence develops (presecne of ionisable chlorine).

8.2 Titrimetric Methods

U.S. Pharmacopeia XX (14) describes the following method:

Dissolve about 200 mg of chlorambucil, accurately weighed, in 10 ml of acetone, add 10 ml of water,

and titrate with 0.1 N sodium hydroxide VS, using phenolphthalein TS as the indicator. Each ml of 0.1 N sodium hydroxide is equivalent to 30.42 mg of $C_{14}H_{19}Cl_2NO_2$.

British Pharmacopoeia 1980 (9) describes the following method:

To 0.5 g of chlorambucil, add 20 ml of M sodium hydroxide and boil under a reflux condenser for one hour. Cool, transfer the mixture to a 100-ml graduated flask with the aid of water, add 50 ml of 0.1 M silver nitrate VS and 5 ml of nitric acid, mix, and add sufficient water to produce 100 ml. Filter and titrate the excess of silver nitrate in 50 ml of the filtrate with 0.1 M ammonium thiocyanate VS, using 3 ml of ammonium iron (III) sulfate solution as indicator. Each ml of 0.1 M silver nitrate VS is equivalent to 0.01521 g of $C_{14}H_{19}Cl_2NO_2$.

8.3 Spectrophotometric Methods

8.3.1 Colorimetric Method

Petering and Van Giessen (36) described a colorimetric procedure for determination of nitrogen mustards including Chlorambucil, Dopan, Uracil Mustard and Mustargen in plasma. Mix the drug (10 to 70 µg), dissolved in a 5% solution of dimethyl-acetamide in either ethanol or 0.9% NaCl solution, with phthalate buffer solution (pH 4.0) (1 ml), 5% 4-(p-nitrobenzyl)-pyridine solution in acetone (1 ml) and 0.9% NaCl solution (1 ml), and dilute with ethanol to 4 ml. Heat at 80° for 20 min., cool in ice, add N-KOH in 90% ethanol (0.1 ml), dilute with ethanol at 600 mµ within 2 or 3 min. Carry out a blank determination.

8.3.2 Infrared Spectrometric Method

Kozlov and Sbezhneva (37) reported an infrared spectroscopic analysis of chloram-bucil in pharmaceutical preparations. Specially to determine the amount of active

ingredients in carcinostatic preparations containing chlorambucil. 0.1 g of sample of the preparation is extracted in 100 ml of acetone and the absorbance of aliquot is measured at 770 cm^{-1}. The error does not exceed ± 1%.

8.3.3 Ultraviolet Spectrometric Method

El-Tarras et al (38) developed a simple method for the determination of chlorambucil in pharmaceutical preparations. The powdered tablets are extracted into ethanol (4 X 10 ml). The filtered extract was diluted to 50 ml with ethanol. A portion of this solution was further diluted as required with ethanol and the absorbance measured at 250 nm vs ethanol. The concentration of the drug is calculated using a calibration curve.

8.3.4 Mass Spectrometric Methods

Chang et al (39) developed a sensitive and specific method for the determination of chlorambucil and its metabolites in biological samples. The method is based on selected ion monitoring detection following simple extraction of the parent compound, its metabolite and internal standard (Chlorambucil-d8) from plasma and urine samples. To determine chlorambucil and 4-[bis-(2 chloroethyl)amino]phenyl acetic acid in 0.5 ml plasma or urine add 0.5 ml of 4% $HClO_4$ (perchloric acid) and [$^2H_8$] chlorambucil as internal standard. The mixture is extracted with ethyl acetate and hexane (1:1). The organic layer was dried with purified N_2 at room temperature and several drops of methylene chloride were added to dry residue to form trimethyl silyl derivative. This is submitted to GLC column (50 cm X 2 mm) of 3% of OV-17 on gas Chrom Q (100 to 120 mesh) after 40 seconds at 190°C the column temperature is rapidly raised to 310°C; 70 eV-m.s detection is used. Trimethylsilylated chlorambucil is

monitored at m/e 326 and 328, the derivative of the metabolite of chlorambucil at m/e 298, and that of the internal standard at m/e 383 and 385. The mean recovery and standard deviation were 94.3 ± 1.3%.

Jakhammer et al (40) described an analytical procedure for simultaneous determination of chlorambucil and prednimustine in plasma. The method involves extraction and separation followed by derivatization to chlorambucil methyl ester and mass fragmentography. The drugs are extracted into chloroform, hexane (3:7) and separation is done by partition after adding 0.1 M phosphate - 0.1 M borate buffer (pH 9.0). Chlorambucil in the aqueous phase add predenimustine in organic phase are each transesterified with methanol-BF_3 diethyletherate (4:1). The compounds are determined using fragmentography. A Varian MAT mass spectrometer in combination with Varian Aerograph 1400 gas chromatograph with a column of 0.5% of Carbowax 20 M on Chromosorb W-HP, are used. Analogues, prepared by introducing eight 2H atoms into the bis-(2-chloroethyl)amino group of both compounds are used as internal standards and carriers. The detection limit is about 8 ng/ml for both determinations and the relative standard deviation 3% for chlorambucil and 5% prednimustine at 30-60 ng/ml level.

8.4 Chromatographic Methods

8.4.1 Thin Layer Chromatograph (TLC)

Norpoth et al (41) described a TLC method for the determination of alkylating cytostatic agents on a thin layer plate with isonicotinaldehyde benzothiazol-2-yl-hydrazone. The drug samples are dissolved in methanol. The spot is applied on a sheet or precoated plates. After developing and drying the chromatogram, it is sprayed with 3% solution of reagent in acetophenone - dimethylformamide (7:3) containing 0.01 ml of concentration HCl per 100 ml. It is then

placed over a bath of acetophenone and bath and plate are heated at 200°C for 20 minutes; the cooled plate is then sprayed with triethylamine. Chlorambucil produces red spot. For quantitative determination the color intensity of the spot is measured using chromatogram spectrophotometer.

8.4.2 Gas Liquid Chromatography (GLC)

Ehrsson et al (42) described a GLC technique for determination of chlorambucil in plasma by gas-liquid chromatography with selected ion-monitoring. 2 ml of plasma sample containing 8 µg of chlorambucil per ml was mixed with hydrochloric acid, phosphoric acid, or phosphate buffer (0.2 ml). The mixture was extracted with methylene chloride (2 ml) for 30 minutes. An aliquot of organic phase was separated and evaporated to dryness with nitrogen gas. The residue was dissolved in methanol-water 0.1 M, acetic acid (75:15:10) and analysed on gas chromatography. Alternatively the plasma samples were adjusted to pH 3 with 1 M phosphoric acid and extracted with ethylene dichloride for 30 minutes. The organic phase was separated and extracted with 0.5 ml of 0.1 M sod. sulphide for 5 minutes. The aqueous phase was separated and heated for 15 minutes at 80°C to convert chlorambucil into tetrahydrothiazine derivative, then concentrated phosphoric acid (0.91 ml) was added, and H_2S removed with N_2 for 5 minutes. To this 0.05 ml of 12 M NaOH, 0.2 ml of 0.5 M tetrabutyl ammonium, 0.2 ml methylene chloride and 0.05 ml allylbromide were added and the mixture was shaken for 30 min at 25°C. The organic phase (1-2 mcg) was injected in GLC (at 250°C) on a column of 5% of OV-17 on gas-Chrome-Q operated with 70 eV.m.s. detection. The internal standard used was [$^2H_8$] chlorambucil. The peaks were monitored at m/e 305 and 313 for chlorambucil and

[$^{2}H_{8}$] chlorambucil respectively. The coefficient of variation for 10 ng/ml of chlorambucil was 5%.

8.4.3 High-Pressure Liquid Chromatography (HPLC)

Newell et al (43) developed a HPLC method for estimation of chlorambucil, phenylacetic mustard and predenimustine in human plasma. One ml plasma was extracted with 2 ml of ethyl acetate, the emulsion formed by vigorous agitation on a Whirlimixer was frozen by immersing the tubes in a methanol carbondioxide bath at - 68°C. The tubes were centrifuged at 600 µg for 10 minutes at 4°C until the thawed aqueous phase could be separated. The procedure was repeated with further 2 ml of ethylacetate, the pooled ethyl acetate extracts were dried over anhydrous sodium sulphate for 1 hour at room temperature. 2 ml of dried ethyl acetate extract was evaporated to dryness in a stream of nitrogen at 45°C. The residue was dissolved in 100 ml of ethyl acetate for chromatographic analysis. 50 µl of the extract was injected on a µ Bondapak C_{18} column. Linear gradient or elution was done at 2 ml per minute with aqueous 0.175 M - acetic acid (from 40 to 0%) in methanol during 10 minutes followed by isocratic elution with methanol. This method gave a good separation of chlorambucil in less than 12 minutes. The absorbance of elute was monitored at 254, and 280 nm simultaneously. Detector response was rectilinear over a range of 5 to 1000 ng of the compound. The recovery of chlorambucil was constant over the range of 0.05 to 10 µm.

Leff and Bardsley (44) have reported pharmacokinetic of chlorambucil in ovarian carcinoma using a new HPLC assay. The apparatus consisted of a Waters Associates M6000 pump, a 10 X 0.46 cm steel column packed with S 5 ODS, a Waters Associates Model U6K injector, a Pye-Unicam LC3 variable

wavelength UV detector operating at 254 nm. The mobile phase was water: methanol 20:80 (v/v) with 0.1% ammonium acetate (w/v). The flow rate used was 1 ml min^{-1} (≡ 500 psi).

Zakaria and Brown (45) reported a rapid assay for plasma chlorambucil and its metabolite (phenylacetic mustard) using reversed-phase liquid chromatography. Plasma (20 or 30 µl) is injected directly in the chromatographic system, which incorporates a 5 cm Co: Pell ODS pre-column for sample clean up. A partisil PXS-10/25 ODS column (25 cm X 4.6 mm) or, for faster elution, a chromegabond MC-18 column (15 cm X 4.6 mm) is used for the analysis. The mobile phase being methanol - 0.02 M - KH_2PO_4 (1:1 or 11:9, respectively) at 1.5 or 1.0 ml min^{-1}, respectively. Calibration graphs are rectilinear over the range of interest. Simultaneous detection at 280 and 254 nm allows picomole amounts to be determined.

Chatterji et al (46) have studied kinetics of chlorambucil hydrolysis using high-pressure liquid chromatography. The high-pressure liquid chromatograph was equipped with a fixed-volume loop injector and a fixed wavelength detector. A 250 X 4.6 mm i.d. reversed-phase column was used. Separation was performed on a reversed-phase column (Zorbax C-8, 6 µm average particle size, Dupont Instruments, Willington, Del.) and methanol-acetonitrile-0.01 M acetate buffer, pH 4.5 (65:5:30), at a flow rate of 1.6 ml/min was used as the mobile phase. A 10 µl full loop volume was quantitatively injected, and the recorder was set at 0.32 aufs (254 nm detector).

Ehrsson et al (47) described a reverse phase HPLC method to study degradation of chlorambucil in aqueous solution. Chlorambucil and its degradation products, 4-(4-(2-chloroethyl-2-hydroxyethylamino)-phenyl) butyric acid, are determined in aqueous methanol. The chlorambucil solution is

prepared by adding 10 mg of compound in 0.5 ml methanol and the volume is made upto 100 ml with distilled water. At different time intervals extraction is done with ethyl acetate, the solvent is evaporated to 0.2 ml under a nitrogen atmosphere. Reversed-phase HPLC analysis is done on a column (15 cm X 4 mm) of Li-Chrosorb RP-18 (5 μm). Methanol/water containing 0.01 M phosphoric acid is used as mobile phase. A UV detector adjusted at 253.7 nm was used for determination of the concentration.

Ahmed et al (48) described a quantitative method for determination of chlorambucil in plasma by reversed-phase high-performance liquid chromatography. The plasma samples are deproteinised by adding 4 volumes of acetonitrile at pH 3. The tubes were centrifuged for 2 minutes and the supernatent was rapidly frozen in solid carbondioxide-acetone to complete deproteinisation. The tubes are centrifuged for 2 minutes. The aliquot so obtained was analysed on Water's Radial-PAK C_{18} (10 μm) cartridge, with acetonitrile-0.2% acetic acid (13:7) as mobile phase. Absorbance was monitored at 263 nm and recorded on a Data Module. A standard curve was developed using acetonitrile solution of chlorambucil standard.

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CHLORZOXAZONE

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1. Foreword, History, Therapeutic Category

Chlorzoxazone is a centrally active muscle relaxant for the treatment of painful muscle spasms associated with muscloskeletal disorders, such as fibrositis, bursitis, myositis, spondylitis, sprains, and muscle strains. The skeletal muscle relaxant properties were first discovered by Marsh at McNeil Laboratories in the late 1950s (1). Onset of therapeutic activity is observed within one hour and the duration usually lasts up to 6 hours. The drug was reported to be useful in the treatment of painful spasm of skeletal motor muscles by acting at spinal levels of the central nervous system (2). The drug exhibits minimal adverse effects and almost no gastrointestinal irritation. Chlorzoxazone was ranked among the top 100 most widely prescribed drugs for many years (3).

2. Description

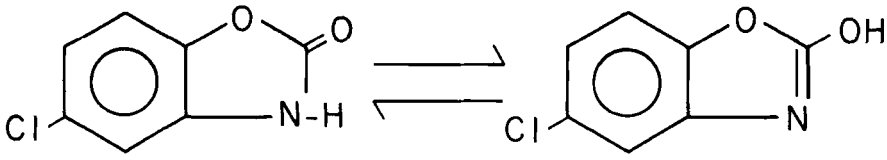
2.1 Names, formula, molecular weight

Generic name - Chlorzoxazone

Trade name - Marketed by McNeil Pharmaceutical under the trade name, Paraflex.

Chemical names - 5 Chloro-2-(3H)-benzoxazolone, 5-Chloro-2-benzoxazolol, 5-Chloro-2-hydroxybenzoxazole, 2-Hydroxy-5-Chlorobenzoxazole, 5-Chlorbenzoxazolin-2-one, 5-Chlorobenzoxazolidone.

Chemical Abstracts Registry No. 95-25-0

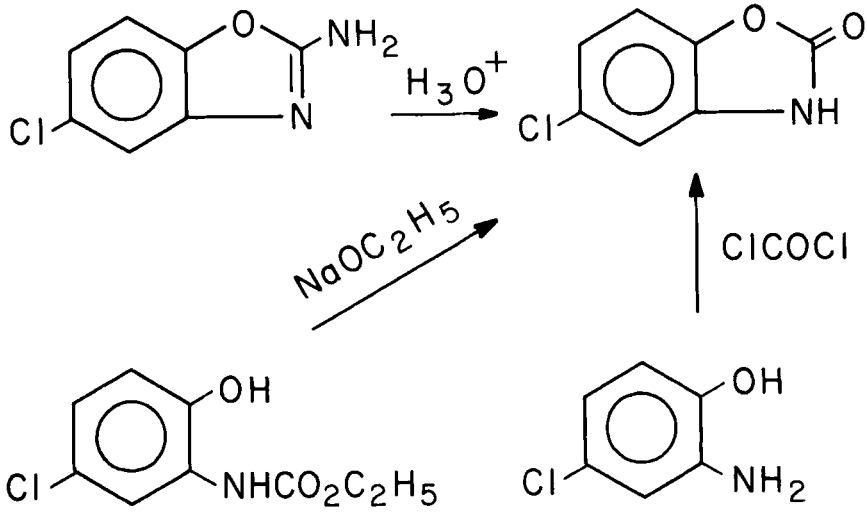


$C_7H_4ClNO_2$ Mol. Wt. 169.58

2.2 Appearance, Color, Odor, Taste - Odorless colorless crystals or a white to creamy white crystalline powder with a bitter taste.

3. Synthesis

Chlorzoxazone is synthesized by either the acid hydrolysis of 2-amino-5-chlorobenzoxazole (4) or by treating 2-amino-4-chlorophenol with phosgene in ethyl acetate (5). It can also be prepared from the reaction of sodium ethoxide with 2-ethoxycarbonylamino-4-chlorophenol in tetralin (6).



4. Physical Properties

4.1 Infrared Spectrum - An FTIR spectrum of chlorzoxazone is shown in Figure 1.

cm^{-1}	% T	cm^{-1}	% T	cm^{-1}	% T
3470	72.6	1367	43.8	732	52.8
3155	36.3	1330	53.6	713	32.2
3117	39.7	1300	27.3	600	45.8
3087	37.3	1262	26.2	591	41.8
3057	35.2	1153	21.1	552	49.0
2978	46.4	1101	58.7	417	67.6
2282	69.1	1064	54.8	394	70.2
1924	67.4	964	16.4	382	79.1
1885	70.2	923	33.1	352	66.1
1772	3.2	867	46.1	234	8.8
1621	30.5	845	23.7	216	5.0
1482	13.5	805	19.2	210	6.7
1463	34.3				

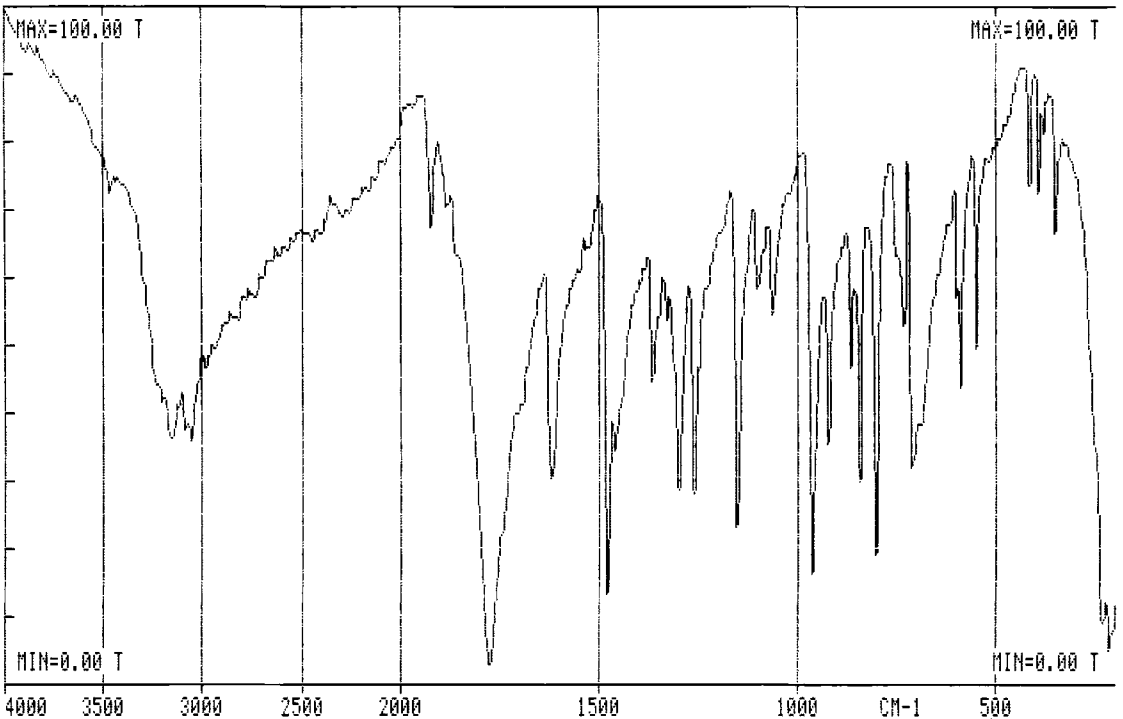
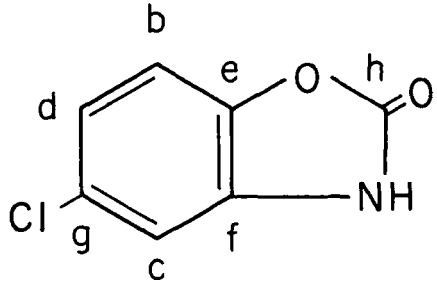


Fig. 1 FTIR spectrum of chlorzoxazone

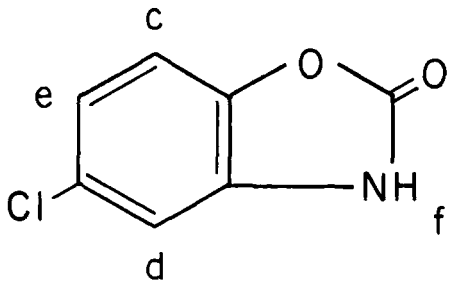
4.2 Ultraviolet Absorption Spectrum - A solution of the drug in absolute methanol shows a maximum at 282 ± 2 nm and a minimum at 248 ± 2 nm. There is an inflection at about 228 nm (see Figure 2).

4.3. Nuclear Magnetic Resonance Spectra - ^{13}C and proton nuclear magnetic resonance spectra are shown in Figures 3 and 4.



^{13}C NMR

Carbon	δ(ppm)	
a	39.4	DMSO-d_5
b	109.8	
c	110.8	
d	121.4	
e	127.7	
f	131.7	
g	142.1	
h	154.2	



Proton NMR

Proton	δ(ppm)	
a	2.49	DMSO-d_5
b	3.36	HDO
c	7.10	
d	7.13	
e	7.28	
f	11.8	

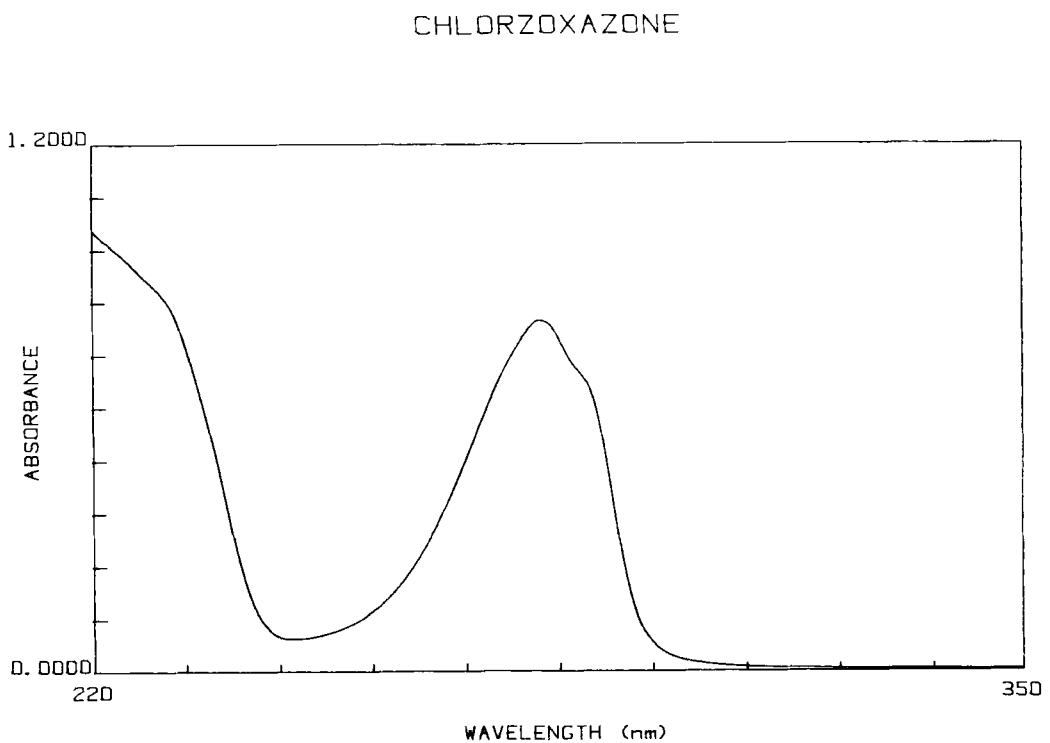


Fig. 2 UV spectrum of chlorzoxazone in methanol

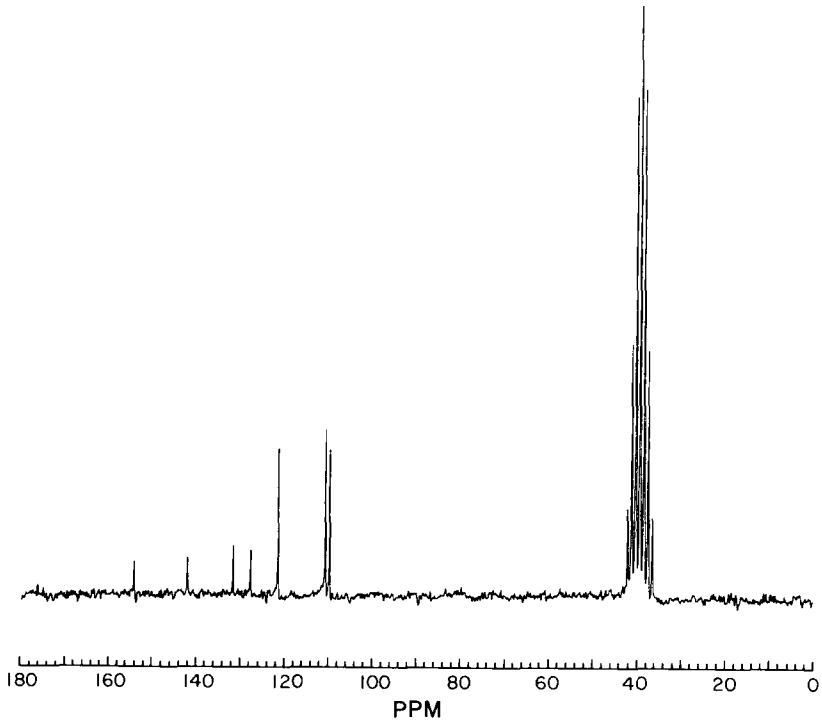


Fig. 3 ^{13}C-NMR spectrum of chlorzoxazone in deuterated dimethylsulfoxide

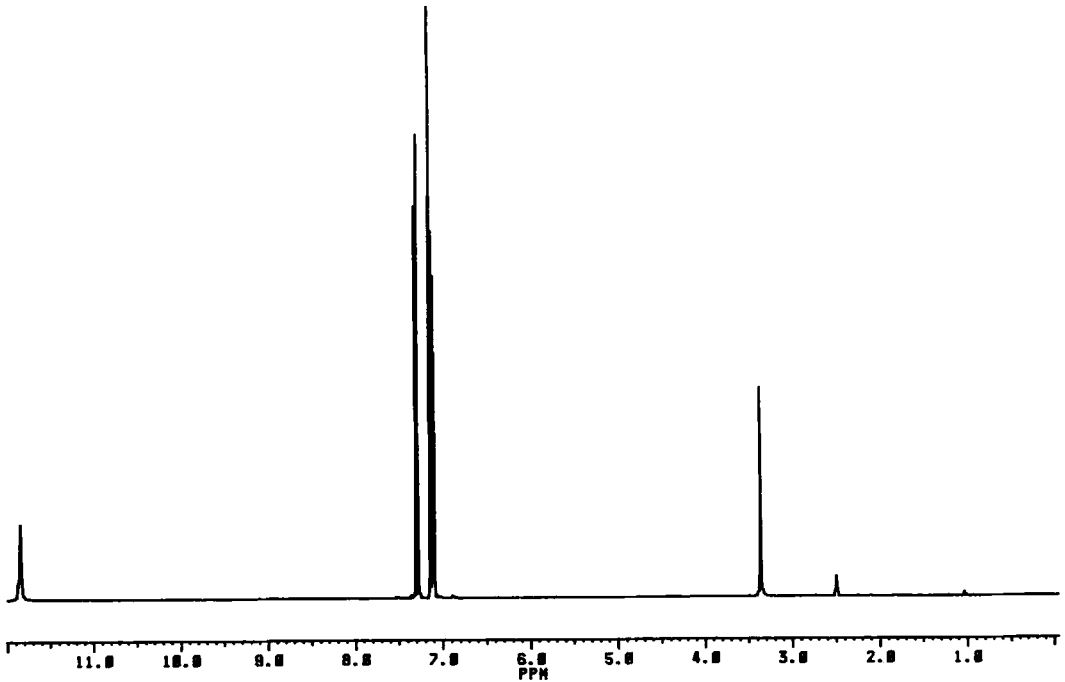


Fig. 4 ^{1}H-NMR spectrum of chlorzoxazone in deuterated dimethylsulfoxide

4.4 Mass Spectra - Electron impact (EI) and chemical ionization (CI) mass spectra are shown in Figures 5 and 6.

4.5 X-Ray Diffraction - Figure 7 shows an X-ray diffraction pattern for chlorzoxazone powder.

4.6 Thermal Analysis - A DSC thermogram of chlorzoxazone is shown in Figure 8.

4.7 Melting Range - The melting point range is 189-194°C.

4.8 Solubility - Chlorzoxazone is very slightly soluble in water (0.2 - 0.3 mg/ml), sparingly soluble in ethanol and methanol, soluble in chloroform (1 g in 250 ml) and ether (1 g in 60 ml). It is freely soluble in aqueous solutions of ammonia and alkali hydroxides.

4.9 pKa - The pKa of chlorzoxazone in 8.3.

5. Methods of Analysis

5.1 Fluorescence Spectrophotometry - Stewart and Chan have studied the reaction of chlorzoxazone with dansyl chloride, fluorescamine, 2,4-dihydroxybenzaldehyde, and salicylaldehyde to form fluorophores (7). Fluorescamine gave the highest intensity product and was used to analyze chlorzoxazone in the 0.27 - 3.4 µg/ml range. The same authors also reported that chlorzoxazone exhibits native fluorescence in chloroform using excitation and emission wavelengths of 286 and 310 nm, respectively (8).

5.2 Ultraviolet Spectrophotometry - Conney et al utilized a back-extraction approach to quantitate chlorzoxazone at 289 nm (9). The drug can also be directly determined at 282 nm in absolute methanol (10).

5.3 Iodometric Titrimetry - Beral et al titrated chlorzoxazone (after treatment with boiling 10% sulfuric acid, potassium bromide and potassium bromate) with 0.1 N sodium thiosulfate after potassium iodide was added to a cooled solution (11).

5.4 Gas Chromatography - Kaempe has chromatographed chlorzoxazone on a packed 15% Dexsil 300 on HP

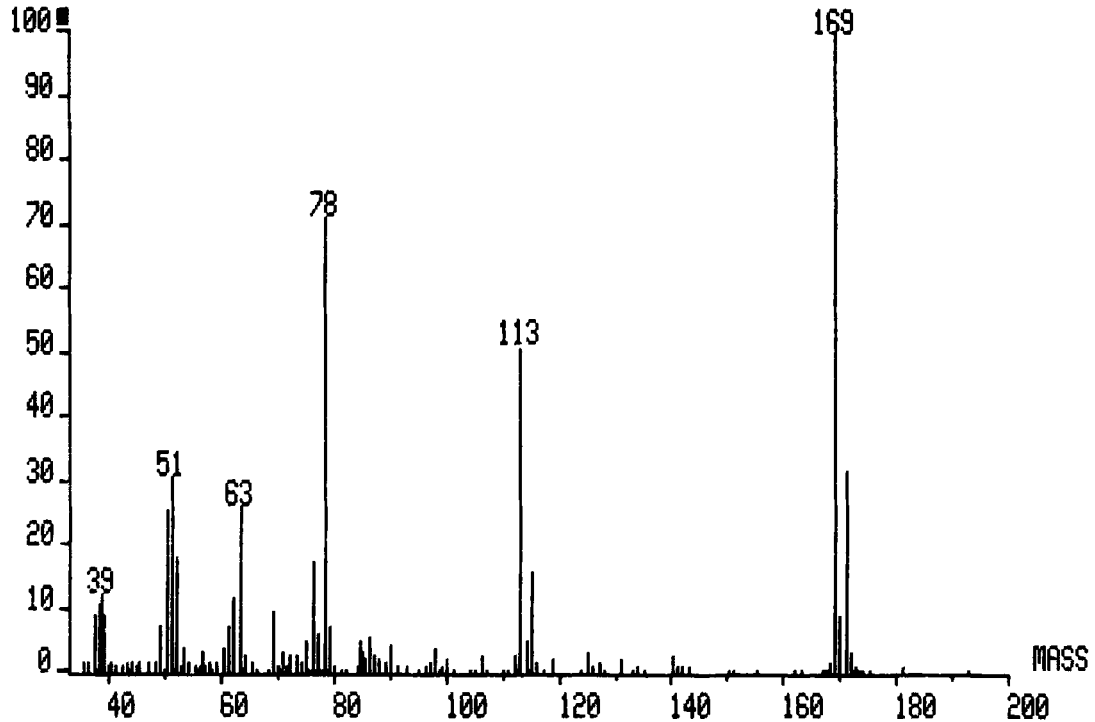


Fig. 5 Electron impact (EI) mass spectrum of chlorzoxazone

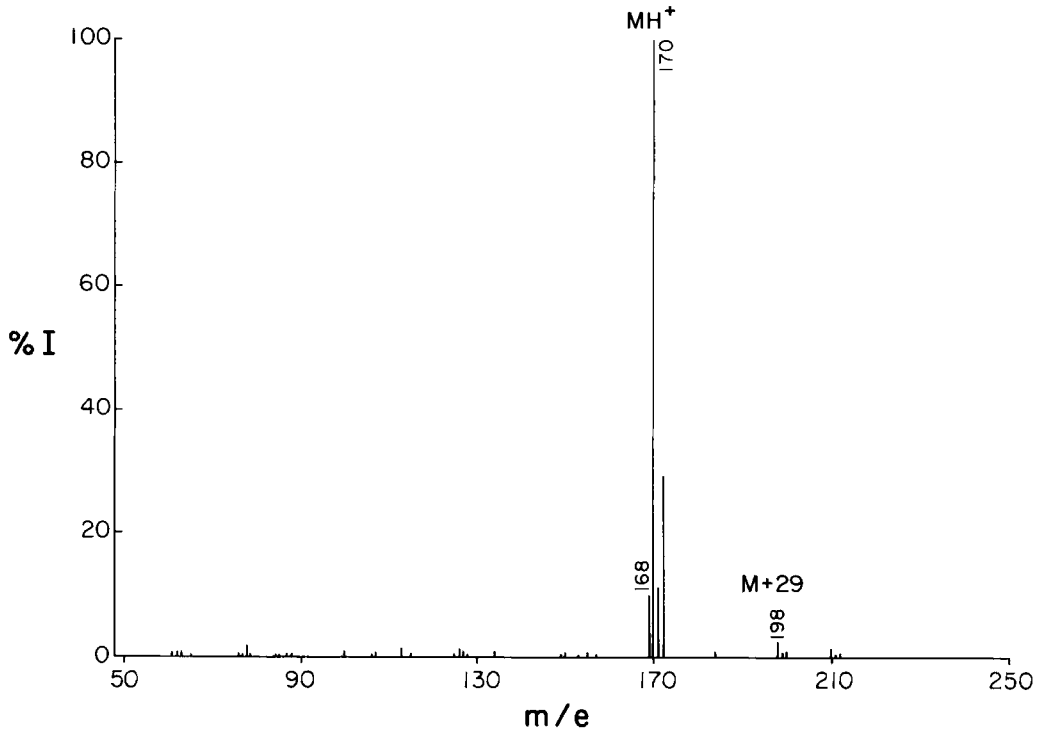


Fig. 6 Chemical ionization (CI) mass spectrum of chlorzoxazone

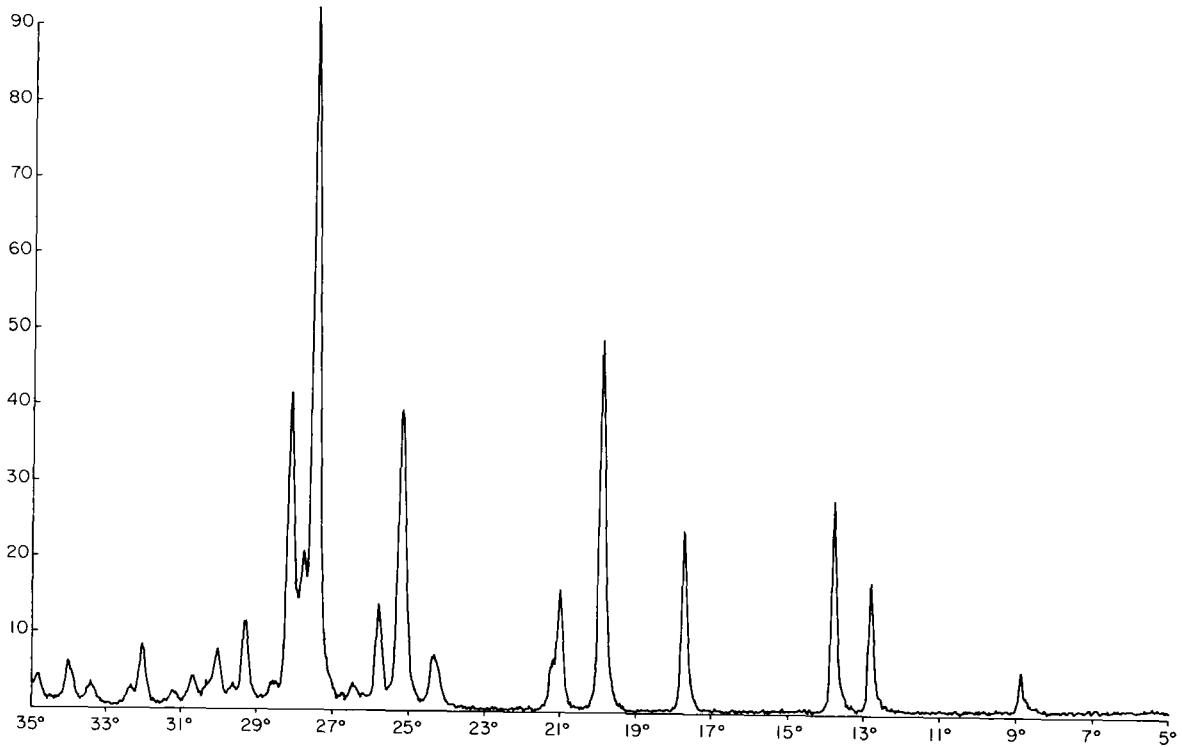


Fig. 7 X-Ray diffraction pattern of chlorzoxazone

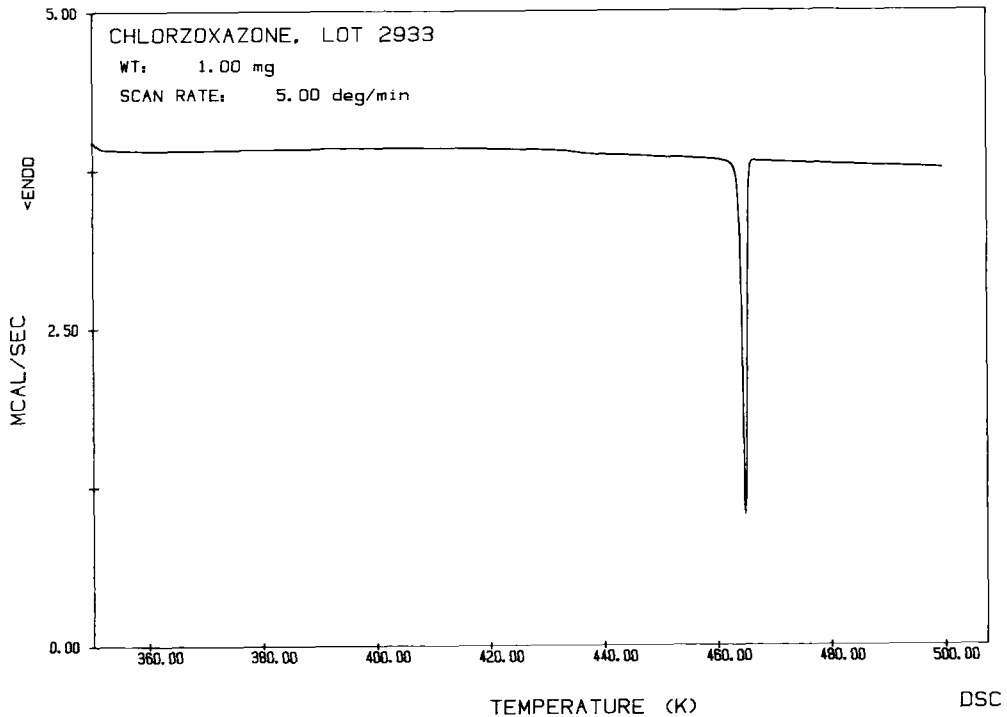


Fig. 8 DSC thermogram of chlorzoxazone

Chromosorb W 80/100 mesh glass column (6 ft x 6.4 mm i.d.). The retention time was 0.62 relative to caffeine using a column temperature of 220°C (12). Parker et al attempted to chromatograph the drug on SE-30 (0.05% on glass microbeads 60/80 mesh) at 150 and 165°C column temperatures with no success (13). Desiraju et al chromatographed chlorzoxazone on a 6 ft x 4 mm i.d. glass column packed with 3% OV-1 on 60/80 mesh Gas Chrom Q (14). Column temperature was 130°C and retention time was 3.25 minutes using a column flow rate of 30 ml/minute.

Beckett et al reported that chlorzoxazone can be chromatographed on a 2.5% SE-30 on 80/100 mesh Chromosorb W acid-washed HMDS treated 5 ft x 4 mm i.d. glass column at a column temperature of 225°C (15). Retention time was 0.69 relative to diphenhydramine. Nitrogen was used as carrier gas at 50 ml/min and FID detection was utilized. Ardrey and Moffat also reported that a 2 m x 4 mm i.d. glass column containing 2.5% SE-30 on 80/100 mesh Chromosorb G acid-washed HMDS treated will separate chlorzoxazone using a column temperature of 173°C (16). Retention time is given relative to n-alkanes.

Pedroso and Moraes have chromatographed chlorzoxazone on 2.5% SE-30 and 3% OV-17 at 200 and 220°C column temperatures, respectively (17). They used the retention data to perform qualitative analysis of the drug based on Kovat's Retention Indices.

5.5 Thin-Layer Chromatography

Stationary Phase	Mobile Phase	R_f
Silica Gel HF_{254}	Chloroform-methanol 19:1	0.32[18]
	Chloroform-methanol 9:1	0.58
	Chloroform-methanol 4:1	0.87
	Chloroform-methanol 7:3	1.00
	Chloroform-methanol 3:2	1.00
Kieselgel $60F_{254}$	Toluene - ethylacetate - 85% formic acid (50:45:5)	0.69[19]

Kieselgel $60F_{254}$	Toluene - Isopropanol - concd. ammonium hydroxide (70:29:1)	0.68[19]
Kieselgel $60F_{254}$	Toluene-dioxane - methanol - ammonium hydroxide (20:50:20:10)	0.55[19]
Silica Gel $60F_{254}$	Toluene - Acetone - 2N Acetic Acid - (30:65:5) -	0.83[20]
Silica Gel $60F_{254}$	Toluene - Isopropanol - Ethyl Acetate - 2N Acetic Acid (10:35:35:20)	0.95[20]
Bonded C18F	Methanol - 0.5% Phosphoric Acid 3% Sodium Chloride (60:10:30)	0.32[20]
Bonded C18F	Isopropanol - 0.5% Phosphoric Acid - 3% Sodium Chloride (40:10:50)	0.35[20]
Bonded C18F	Isopropanol - Methanol - 0.5% Phosphoric Acid - 3% Sodium Chloride (23:23:10:44)	0.33[20]
Bonded C18F	Tetrahydrofuran - Methanol - 0.5% Phosphoric Acid - 3% Sodium Chloride (28:28:10:34)	0.32[20]
Bonded C18F	Methanol - 2N Ammonia - 3% Sodium Chloride (60:10:30)	0.60[20]

Bonded C18F	Isopropanol - 2N Ammonia 3% Sodium Chloride (40:10:50)	0.47^{20}
Bonded C18F	Isopropanol - Methanol - 2N Ammonia - 3% Sodium Chloride (23:23:10:44)	0.43^{20}
Bonded C18F	Tetrahydrofuran - Methanol - 2N Ammonia - 3% Sodium Chloride - (28:28:10:34)	0.55^{20}
Bonded C18F	Acetonitrile - 3% Sodium Chloride (50:50)	0.46^{20}
Bonded C18F	Methanol - 3% Sodium Chloride (60:40)	0.32^{20}
Bonded C18F	Acetone 3% Sodium Chloride (50:50)	0.25^{20}
Bonded C18F	Tetrahydrofuran - 3% Sodium Chloride (43:57)	0.18^{20}
Bonded C18F	Isopropanol - 3% Sodium Chloride (40:60)	0.35^{20}
Bonded C18F	Methanol - Isopropanol - Tetrahydrofuran - 3% Sodium Chloride (17:17:17:49)	0.36^{20}

5.6 Paper Chromatography - Clarke reported that chlorzoxazone gave an R_f of 0.93 using a mobile phase of 87:13 n-butanol-water containing 0.48% citric acid (21).

5.7 High Performance Liquid Chromatography - Stewart et al have chromatographed chlorzoxazone on an octadecylsilane column using varying proportions of absolute methanol-distilled water (22). Chlorzoxazone was effectively separated from acetaminophen in a mixture with 50:50 methanol-water at a 2.0 ml/min flow rate using refractive index detection. The drug has also been chromatographed on an octadecylsilane column using 60:40 water-methanol (23), and a 1:1 acetonitrile-water mixture (10). These methods utilized ultraviolet detection at 280 nm.

5.8 Non-Aqueous Titrimetry - Chlorzoxazone is titrated with 0.1 N sodium methoxide in dimethylformamide using thymol blue as indicator (24). The drug can also be titrated in dimethylsulfoxide using 0.1N propanolic potassium hydroxide as titrant and metanil yellow as visual indicator (25).

5.9 Colorimetry - Sanghoi reported a colorimetric procedure for chlorzoxazone based on base hydrolysis of the drug followed by diazotization of the product with nitrous acid formed in situ (26). The color measured at 405 nm is stable for 60 minutes and obeys Beer's Law in the 4-32 μg/ml range.

5.10 Color Test - The Koppanyi-Zwikker test gives a violet color (27).

5.11 Polarizing Microscopy - Watanabe et al., reported that polarizing microscopy can be used as a qualitative identification tool for chlorzoxazone (28).

5.12 Potentiometry - A potentiometric method for the determination of chlorzoxazone, based on the use of a carbon dioxide gas-sensing electrode, is described by Tagami and Muramoto (29). Upon refluxing the drug with 3N sodium hydroxide, aminophenol and sodium carbonate are formed.

Acidification of the reaction mixture yields carbon dioxide which is sensed by the gas-permeable membrane electrode. A linear calibration plot was obtained within the 3×10^{-4} to 5×10^{-3} M range of chlorzoxazone.

6. Stability

Chlorzoxazone is stable in the solid state for up to 5 years at room temperature. Storage at temperatures for

up to 80°C and in artificial sunlight conditions (1000 foot candles) for up to 4 weeks did not cause significant degradation.

Tablets of chlorzoxazone have also been found to be stable for up to 5 years at room temperature. Temperatures for up to 60°C, humidity conditions of 40°C/80% relative humidity and artificial sunlight (1000 foot candles) for up to 4 weeks have not caused degradation of the product.

An aqueous suspension of the drug in distilled water or acidic media is generally stable but in solutions with a pH greater than 7, the solution is generally not stable. Chlorzoxazone yields 2-amino-4-chlorophenol as its major degradation product.

7. Drug Metabolic Products, Pharmacokinetics, and Bioavailability

7.1 Drug Metabolic Products

7.11 Urine metabolism - The metabolic product of chlorzoxazone was isolated from urine of human subjects administered an oral dose of the drug. Ether extraction of urine yielded a residue which was identified as 6-hydroxy-chlorzoxazone (9). Studies showed that the metabolite is eliminated as the glucuronide conjugate to the extent of 74% of the dose. 6-Hydroxychlorzoxazone had little or no muscle relaxant activity when tested in mice and rats. In 1982, Twele and Spiteller identified two additional chlorzoxazone metabolites from human urine, a 5-chloro-2,4-dihydroxy-acetanilid produced from 6-hydroxychlorzoxazone by ring cleavage and acetylation of the amino moiety, and 6-hydroxybenzoxazolone, produced by substitution of hydrogen for chlorine and hydroxylation of a neighbor ring-carbon atom (30).

7.12 In-vitro metabolism - Chlorzoxazone was incubated with fortified homogenates of mouse and rat liver. The reaction was stopped by addition of 3N hydrochloric acid. Spectrophotometric assay of a sample preparation identified 6-hydroxy-chlorzoxazone as the major metabolite (31). It has been established that the liver is the principal site of metabolism.

7.2 Pharmacokinetics and Bioavailability - The following pharmacokinetic data were obtained following a 750 mg oral dose of chlorzoxazone to human subjects (14):

Peak plasma concentration	36.3 ± 2.3 μg/ml
Peak time	38 ± 3.3 minutes
AUC (0-10 hr)	4084 ± 284 μg min/ml
Apparent Volume of Distribution (V/F)	13.70 ± 5.07 liters
Elimination Rate Constant (K)	0.73 ± 0.29 hr^{-1}
Absorption Rate Constant (k_a)	2.28 ± 2.08 hr^{-1}
Elimination Half Life ($t_{\frac{1}{2}}$)	1.12 ± 0.48 hr
Apparent Clearance (KV/F)	148.0 ± 39.9 ml/min

There is rapid absorption and elimination of chlorzoxazone. Chlorzoxazone is widely distributed in plasma and tissues. Less than 1% of the drug is excreted unchanged in urine. The drug concentration in fat is twice the plasma concentration, while liver, muscle, brain and kidney concentrations are one-half or less that found in plasma. The apparent volume of distribution of approximately 14 liters suggests that the drug is not widely distributed and that it would be confined to the circulatory system and possibly the extracellular fluid.

8. Identification and Determination in Body Fluids and Tissues

8.1 Ultraviolet Spectrophotometry - Conney et al reported the extraction and estimation of chlorzoxazone in urine (9). Petroleum ether containing 1.5% isoamyl alcohol was shaken with the urine sample for 60 minutes. The organic phase was separated and the drug was back extracted into 0.5 N sodium hydroxide. Absorbance read at 289 nm followed Beers Law. Recovery of drug from urine was 93 ± 2%.

8.2 Fluorescence Spectrophotometry - Stewart and Chan reported the quantitation of chlorzoxazone from plasma and/or urine samples (8). Chlorzoxazone was extracted from the biological sample at pH 2.5 with petroleum ether containing 1.5% isoamyl alcohol. The organic phase was re-extracted with strong base, the pH adjusted to pH 6.8 with hydrochloric acid, and the drug extracted into chloroform for the measurement step. Excitation and emission wavelengths were 286 and 310 nm, respectively.

Drug concentration and fluorescence were linear from 0.06 - 3.2 µg/ml and 0.13 - 3.0 µg/ml for plasma and urine, respectively. Limits of detection were 60 and 130 ng/ml for plasma and urine, respectively. Percent recoveries of chlorzoxazone from plasma and urine were 86.63 ± 1.66 and 95.37 ± 2.42%, respectively.

Stewart and Chan also reported a procedure for the analysis of chlorzoxazone based upon solvent extraction of the drug from human plasma or urine followed by chemical derivatization with fluorescamine (7). Linear detection range was 2-10 µg/ml using excitation and emission wavelengths of 370 and 505 nm, respectively.

8.3 High Performance Liquid Chromatography - Honigberg, Stewart, and Coldren reported an HPLC assay for chlorzoxazone and 6-hydroxy-chlorzoxazone in plasma based on ether extraction of the compounds from acidic plasma (22). The separation was achieved on an octadecylsilane column (30 cm x 3.9 mm, i.d.) at a flow rate of 2 ml/min with UV detection at 280 nm and a mobile phase of 40:60 absolute methanol-distilled water. Retention times for the hydroxy compound and drug were approximately 4 and 8 minutes, respectively. The limit of detection for each compound was calculated to be 80 ng at signal/noise = 2.

Another HPLC assay for chlorzoxazone in plasma reported by Ng at McNeil Pharmaceutical (32) used at 1:1 acetonitrile-distilled water mobile phase, an octadecylsilane column (25 cm x 4.6 mm, i.d.), a 2.0 ml/min flow rate, and UV detection at 280 nm. Retention time for the drug is 2.9 minutes. Ethyl 5-chloro-2-hydroxycarbanilate was used as internal standard (retention time of 4.5 minutes). A plasma sample was made acidic with hydrochloric acid and extracted once with ethyl acetate. The ethyl acetate was evaporated to dryness and the residue dissolved in methanol prior to injection into the HPLC.

Stewart and Carter reported modifications in the above mentioned HPLC assay by Honigberg, Stewart and Coldren in which an octadecylsilane solid-phase extraction column was used to separate chlorzoxazone and 6-hydroxychlorzoxazone from human serum (33). Percent recoveries were 90.24 ± 4.28 and

86.06 ± 3.58% for drug and metabolite, respectively. The separation was achieved on an octadecylsilane column using a mobile phase of 50:50 acetonitrile-aqueous 0.05 M sodium dihydrogen phosphate, pH 4.5 with phenacetin as internal standard. The two compounds were detected at the glassy carbon electrode using a cell potential of + 1300 mV. These modifications halved the original HPLC analysis time and increased detection for each compound to 2.5 ng, 30 times the previous limit of detection using 280 nm.

8.4 Gas Chromatography - Desiraju et al reported a packed column method for the quantitation of chlorzoxazone in plasma samples (14). The drug is extracted from acidified plasma with ethyl acetate containing the internal standard, n-hexadecane. The ethyl acetate is separated and evaporated to dryness. Pyridine and acetic anhydride are added to the residue, the tube is capped, and the reaction allowed to proceed at 42°C for 20 minutes. An aliquot of the mixture is injected into the gas chromatograph.

The GC parameters were flame ionization detection (30 and 300 ml/min flow rates for hydrogen and air, respectively), carrier gas (nitrogen at 30 ml/min), 6 ft x 4 mm i.d. glass column packed with 3% OV-1 on 60/80 mesh Gas Chrom Q, and column temperature of 130°C. The retention times of chlorzoxazone and internal standard were 3.25 and 4.5 minutes, respectively. The limit of detection was 0.5 µg/ml using 1 ml of plasma. The calibration curve was linear in the 0.5 - 25 µg/ml range. Extraction efficiency for chlorzoxazone was 88 ± 8% (n = 6).

9. Identification and Determination in Pharmaceuticals

9.1 Colorimetry - Twenty tablets were weighed and powdered in a dry mortar and pestle. An aliquot of the powder equivalent to 40-60 mg of chlorzoxazone was hydrolyzed with 20% sodium hydroxide solution, cooled, and reacted with nitrous acid to yield the diazonium salt, whose absorbance measured at 405 nm obeyed Beer's Law in the 4-32 µg/ml range (26). Acetaminophen and indomethacin were shown to interfere with the method. Recovery of chlorzoxazone in bulk powder and tablet samples was in the 98.3 - 99.9% range utilizing the assay procedure.

9.2 Ultraviolet Spectrophotometry - Kirschner of McNeil Pharmaceutical reported an assay for the drug in tablets as follows (10):

Standard Preparation: Dissolve a suitable quantity of USP Chlorzoxazone RS, accurately weighed, in methanol, and dilute quantitatively and stepwise with methanol to obtain a solution having a known concentration of about 20 µg/mL.

Assay Preparation: Weigh and finely powder not less than 20 Chlorzoxazone tablets. Transfer an accurately weighed portion of the powder, equivalent to about 100 mg of chlorzoxazone, to a 100-ml volumetric flask. Add about 80 ml of warm methanol, and shake by mechanical means for about 15 minutes. Dilute with methanol to volume, and mix. Filter, discarding the first 20 mL of the filtrate, and pipet 2 ml of the filtrate into a 100-ml volumetric flask. Dilute with methanol to volume, and mix.

Procedure: Concomitantly determine the absorbances of the Assay and Standard Preparations at 282 nm, with a suitable spectrophotometer, using methanol as the blank. Calculate the quantity, in mg, of chlorzoxazone in the portion of tablets taken by the formula 5C (Au/As), in which C is the concentration, in µg per ml, of USP chlorzoxazone RS in the Standard Preparation and Au and As are the absorbances of the Assay and Standard Preparations, respectively.

9.3 Fluorescence Spectrophotometry - Stewart and Chan used the intrinsic fluorescence of chlorzoxazone to assay for the drug in commercial tablets containing acetaminophen (8). Recovery of drug was in the 98-101% range. These same authors also demonstrated that chlorzoxazone could be successfully analyzed in a tablet dosage form via chemical derivatization with fluorescamine to form a fluorophore (7).

9.4 Potentiometry - Tagami and Muramoto described a method based on the use of a carbon dioxide gas-sensing electrode (29). Twenty tablets were powdered and a portion equivalent to about 424 mg of drug was weighed and extracted with 20 ml of acetone. After stirring and centrifugation, the supernatant acetone solution was removed and

diluted with additional acetone. This extraction procedure was performed four times. The collected acetone fractions were evaporated to dryness. The residue was refluxed for 2 hr with 50 ml of 3N sodium hydroxide and after acidification, the carbon dioxide was determined using a gas permeable electrode. Mean recovery of chlorzoxazone was 99.6% with a standard deviation of 0.24.

9.5 High Performance Liquid Chromatography - The USP XXI method for Chlorzoxazone with Acetaminophen tablets is a gradient HPLC method (34). In the method, m-chloroaniline, p-aminophenol (a degradation product of acetaminophen), p-chlorophenol and 2-amino-4-chlorophenol are separated from chlorzoxazone, acetaminophen and the internal standard phenacetin.

An example of the separation is as follows:

Component	Retention time, minutes
Acetaminophen	2.3
2-Amino-4-chlorophenol	6.0
p-Aminophenol	7.1
m-Chloroaniline	7.5
Internal Standard (phenacetin)	8.3
p-Chlorophenol	9.2
Chlorzoxazone	10.1

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CYCLOSPORINE

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Cyclosporine

1. History and Therapeutic Category

It was from Hardanger Vidda, a bleak treeless plateau in the South of Norway, that a soil sample was obtained in the early seventies. The fungus isolated from this sample was called Tolypocladium inflatum Gams (1), formerly (2) designated as Trichoderma polysporum (Link ex Pers.) Rifai or Cylindrocarpon luciderm Booth (3). Several metabolites of this fungus have been isolated of which cyclosporins A, C and G to have so far been shown to possess strong immunosuppressive activity. The main component in normal fermentation broths was named cyclosporin A (4), now known as cyclosporine. As reported by Borel et al (5-9), cyclosporine is a selective immunosuppressive drug with antifungal and antiinflammatory properties.

Today cyclosporine can be regarded as the most important recent innovation in the field of organ transplantation.

2. Description

2.1 Nomenclature

2.1.1 Chemical Names

Cyclosporin A; Cyclosporine.

2.1.2 Generic Names

Cyclosporine (USAN) and is adopted for the basic structure, meanwhile the following names has been accepted in other countries. England, cyclosporin (BAN name); France, cicylosporine; Switzerland and others, ciclosporin (INN name, WHO).

2.1.3 Trade Names

Sandimmune, Sandimmun

2.2 Formulae

2.2.1 Empirical

$C_{62}H_{111}N_{11}O_{12}$

2.2.2 Structural

Cyclosporine (Fig. 1) is a neutral, hydrophobic cyclic peptide composed of 11 amino acid residues, all having the S-configuration of the natural L-amino acids, except for the D-Ala in position 8, which has the R-configuration, and Sar in position 3. Seven acids are N-methylated. Ten are known acids, they are α-Abu in position 2, Sar-in position 3, N-MeLeu in position 4,6,9 and 10, Val in position 5, Ala in position 7, D-Ala in position 8, N-MeVal in position 11 and MeBmt in position 1.

2.2.3 Degradation and Structural Elucidation

The structure of cyclosporine [1] (Fig. 1) was determined by chemical degradation (4) and x-ray crystallographic analysis (10,11). Also with an x-ray crystalographic analysis of the iodo derivative[1a]. It has the molecular formula $C_{62}H_{111}N_{11}O_{12}$ as deduced by NMR and mass spectra. Hydrolytic cleavage of cyclosporine furnished 11 amino acids as fragments, among them an artifact of a new C_9-amino acid. The amino acid sequence was determined by Edman degradation of isocyclosporin, a basic rearrangement product formed from cyclosporine by N,O-acyl migration. On the basis of the chemical, spectroscopic and crystallographic evidence, the structure of cyclosporine was elucidated as the neutral cyclic oligopeptide It is composed of eleven amino acid residues, all having the L-configuration of the natural amino acids, except for the D-alanine in position 8 and the non-chiral sarcosine (N-methylglycine) in position 3. Seven amino acids - in positions 1, 3, 4, 6, 9, 10 and 11- are N-methylated. Ten of the

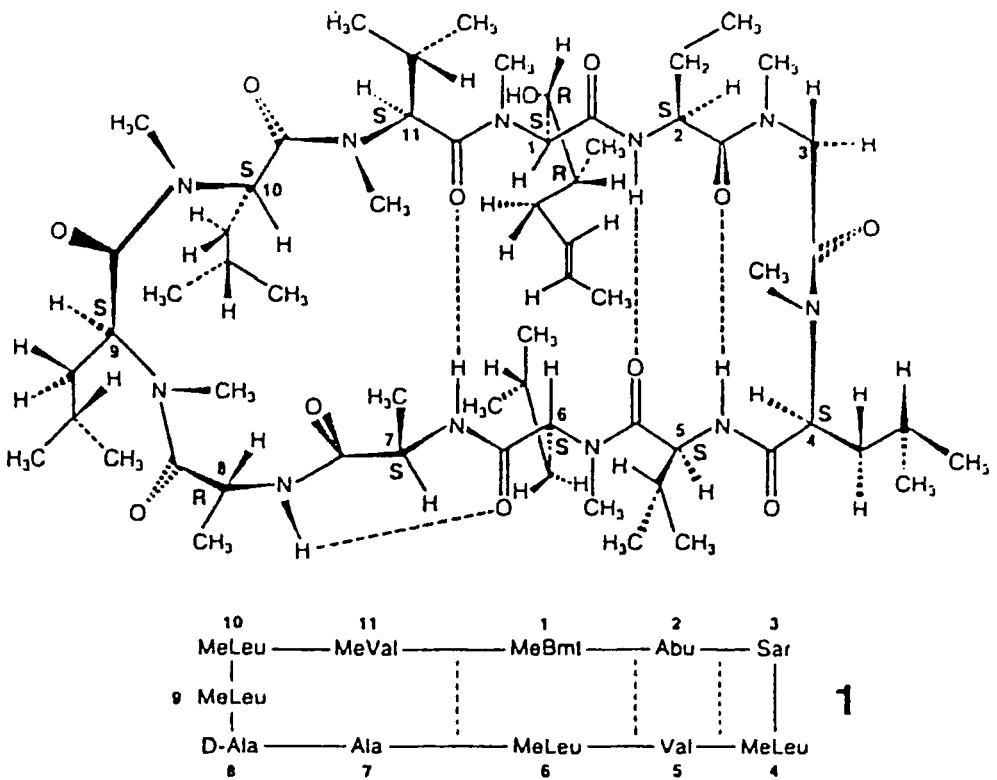
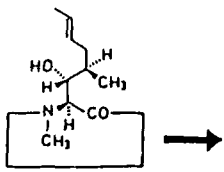
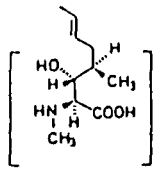


Fig. 1. Structure of cyclosporine 1 (schematic). MeBmt = (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine (see text).



cyclosporine

1



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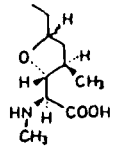
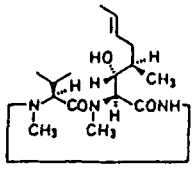


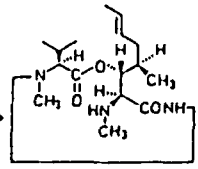
Ib

cyclic derivative of the N-methyl-C9-amino acid



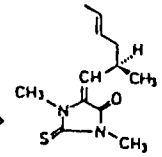
cyclosporine

1



ISOCYCLOSPORINE

Ic

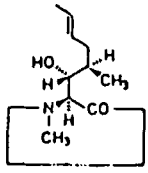


Id

anhydro-methylthio hydantoin derivative

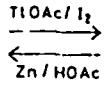
⟹ Edman degradation with CH_3-NCS under carefully chosen mild conditions

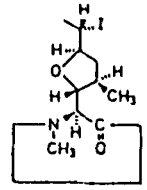
➡ peptide hydrolysis with 6N HCl followed by ion exchange chromatography.



cyclosporine

1





Iodo-derivative

Ia

eleven ring members are derivatives of known aliphatic amino acids:
α-aminobutyric acid (Abu) in position 2,
Sarcosine (Sar) in position 3,
N-methylleucine (MeLeu) in positions 4, 6, 9 and 10,
Valine (Val) in position 5,
Alanine (Ala) in position 7,
D-Alanine (D-Ala) in position 8, and
N-methylvaline (Me Val) in position 11.
The amino acids were readily characterised following acid hydrolysis of cyclosporine.

The novel amino acid was found to have the composition (2S, 3R, 4R, 6E)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoic acid, and in accord with amino acid nomenclature, is now designated as (4R)-4-[(E)-2-butenyl]-4-N-dimethyl-L-threonine and abbreviated MeBmt. Thus the novel amino acid has the polar features of an N-methyl-L-threonine which is substituted at the end of the carbon chain by butenyl and methyl groups. This amino acid was hitherto not known in the free form, since only antifacts and derivatives were obtained from degradation experiments on cyclosporine(4).

Hydrolysis of cyclosporine followed by ion exchange chromatography afforded a cyclic derivative [1b] of the MeBmt aminoacid. Acidic treatment of cyclosporin [1] in the absence of water effects an N, O-acyl-migration of the methylvalyl moiety and furnishes isocyclosporine [1c]. A modified Edman degradation of isocyclosporine [1] using methyl isothiocyanate produced an anhydrothiohydantoin-derivative [1] and thus established MeBmt as the first amino acid in the peptide sequence. The complete sequence was established by repetitive Edman degradation.

2.2.4 Conformation of Cyclosporine

The conformation of cyclosporine in the crystal and in solution was reported (11). The

conformational analysis was based on X-ray crystallography in the crystal and on NMR spectroscopy in solution using different lipophilic solvents. Cyclosporine molecule is highly lipophilic, therefore the solid state conformation and that deduced from NMR studies in apolar solvents are closely similar. It assumes a rather rigid conformation, both in the crystalline state and in solution (Fig. 2). The only differences are the side-chain conformation of MeBmt (a major change between the chain folded in upon the molecule in the solid state, and pointing out into solvent in the NMR study), the side-chain conformation of MeLeu-10, and the detailed backbone conformation in the region of D- Ala-8 (Fig. 3). Here the X-ray structure clearly shows one H-bond from D-Ala-8 (NH) to MeLeu-6 (CO), whereas in solution a bifurcated H-bond to both the carbonyl O-atom of MeLeu-6 and to the carbonyl O-atom of D-Ala-8 itself. The conformation change needed to convert the X-ray backbone into the NMR backbone is small, and can be brought about by relatively small rotation in the backbone torsion angles in the region MeLeu-6 to MeLeu-9, principally around the 7- and 8-residues.

The side-chain conformations of MeVal and Val and three of the four MeLeu-residues (MeLeu -4, -6 and -9) are identical in crystal and in solution. Only that of MeLeu-10 is rotated by 120°. The side-chain conformation of MeBmt in solution can be derived from the X-ray conformation simply by means of 120° rotation about the α,β-bond. This rotation which is energetically allowed, could be the result from the formation of an intramolecular H-bond between the B-OH and the carbonyl O-atom of MeBmt replacing the intermolecular H-bond found in the crystal. A large portion of the backbone (residues 1-6) adopts an antiparallel β-pleated sheet conformation which contains three transannular H-bonds

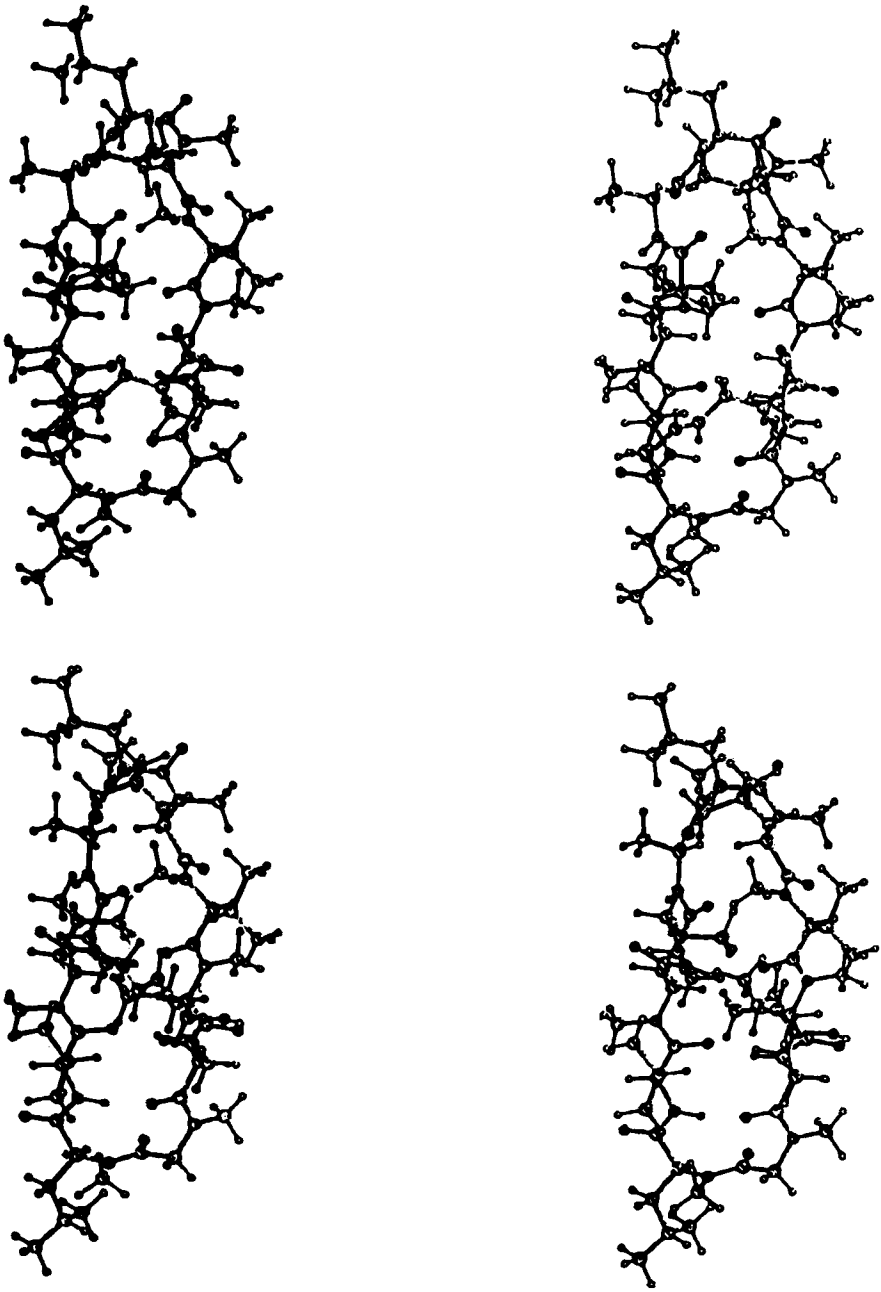


Fig. 2. Stereoview of cyclosporine. a) Solid-state conformation, b) Computer-generated conformation in apolar solvents (in solution the distal atoms of Abu-2 and MeBmt-1 have a high flexibility).

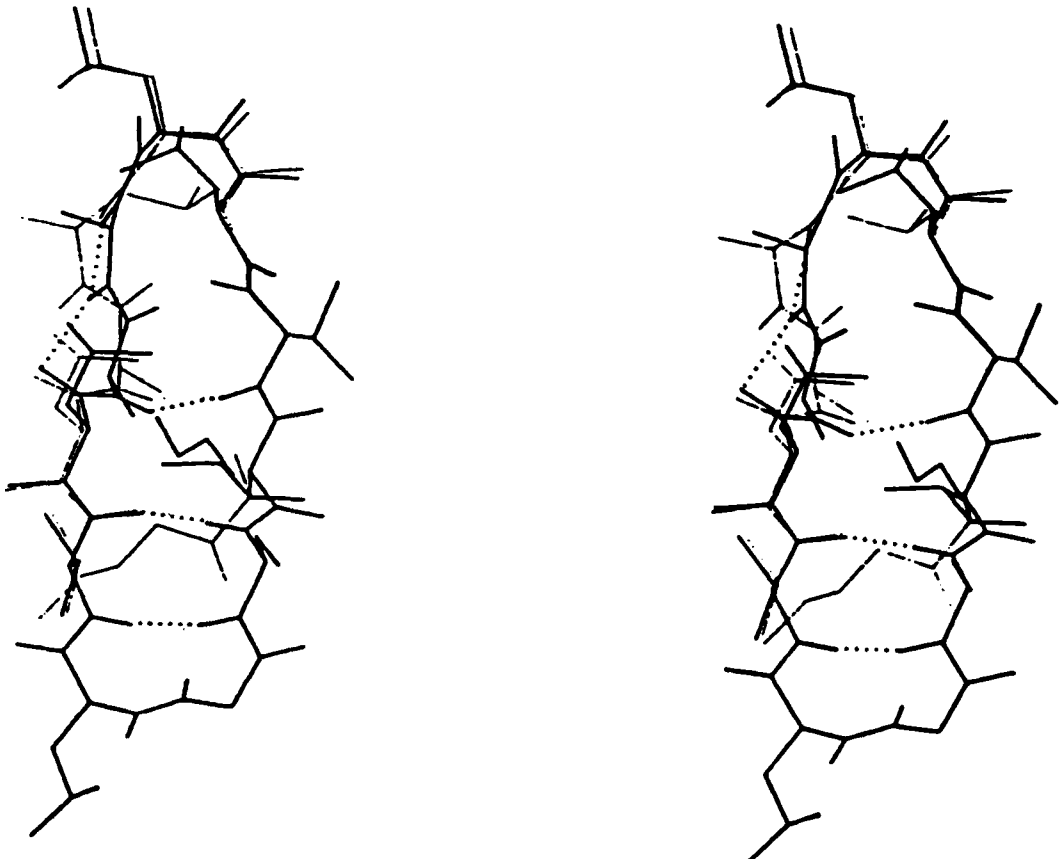


Fig. 3. Superposition of the solid-state conformation (thin lines) and the computer-generated conformation of cyclosporine in apolar solvent (thick lines).

and is markedly twisted. The sarcosine in position 3 and N-methylleucine in position 4 participate in a type 11β-turn (12-14). This means that, in the orientations chosen in Figures 1 and 2, the CO group of Abu-2 and the N-CH_3 group of MeLeu-4 are up and the CO group of Sar-3 and NH group of Val-5 are down - pro -S proton of the methylene group of Sar 3 is axial, and the side-chain of MeLeu-4 is requatorial. The remaining residues 7-11 form an open loop. This loop contains the only cis- amide linkgage in the molecule between the two adjacent N-methylleucine residues 9 and 10. The remaining H-bond of a γ-type and serves to hold the backbone in a folded L-shaped.

As a consequence of this rather rigid conformation of the cyclosporine skeleton, six amino acids have their side-chains directed quasi-perpendicular to the plane of the peptide ring; in Abu-2, Val-5 and MeLeu-6 they are pointing upwards, in MeBmt-1 and MeLeu-6 they are pointing downwards. The side-chains of the remaining aminoacids lie more or less in the plane of the peptide ring.

The resulting overall molecular shape in apolar solution is that of the previously postulated "butterfly" (10), in which the MeBmt side-chain, known to be important for immunosuppressive activity, stands out, probiscis - like, in a manner suggestive of special function (Fig. 4).

2.2.5 CAS Registery Number

Cyclosporine [79217-60-0]
Cyclosporine A [59865-13-3]

2.2.6 Clinical Code

CyA_1OL 27-400

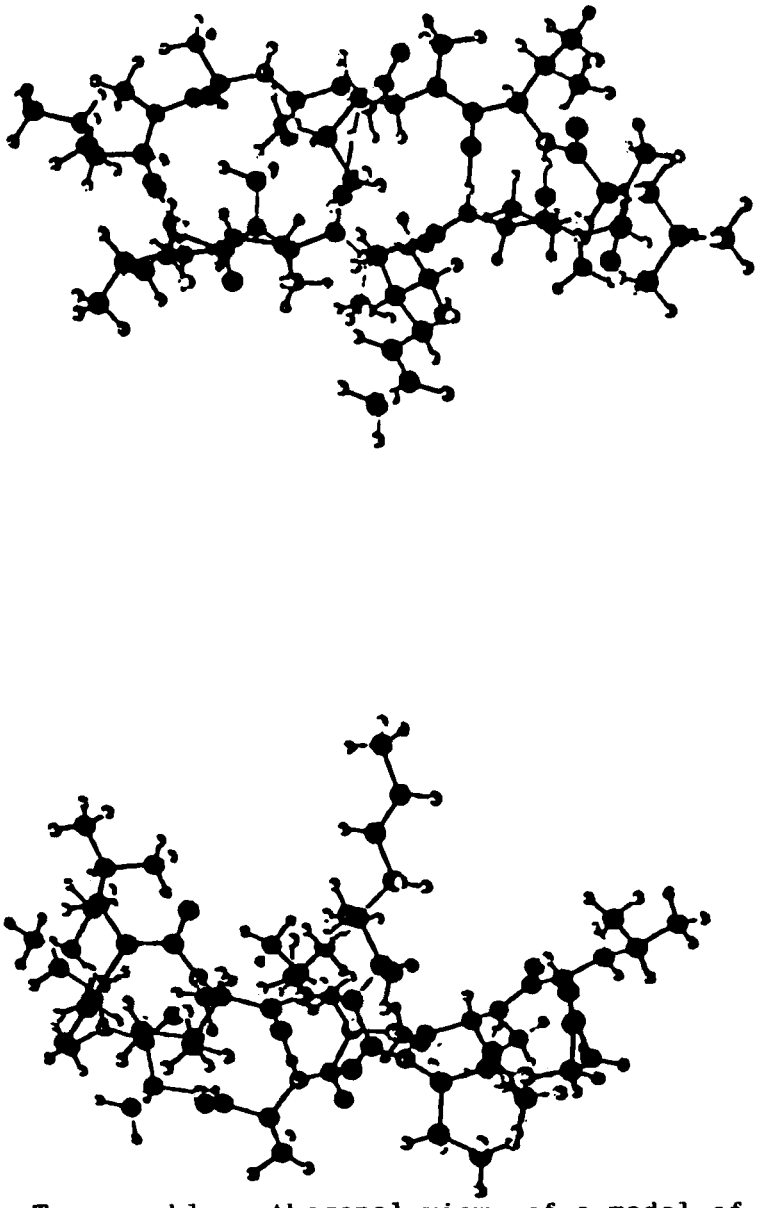


Fig. 4. Two roughly orthogonal views of a model of native cyclosporine in the conformation suggested as probable. The backbone conformation corresponds to that of iodocyclosporine.

2.3 Molecular Weight

1202.64

2.4 Appearance, Color, Taste, Odor

White prismatic crystals.

3. Physical Properties

3.1 Crystal Properties

3.1.1 X-ray Diffraction

Crystal Data

X-ray crystal structures of cyclosporine and its iododerivative [Ia] were determined (10,11). A summary of the crystal data and refractometry for cyclosporine is given in Table 1 (11).

Table 1. Crystal and Diffraction Data

Molecular formula	$C_{62}H_{111}N_{11}O_{12}$	Molecules per cell	Z = 4
Molecular weight	1202.5	Diffractometer	CAD4 (Enraf-Nonius)
Crystallisation	from acetone	Radiation	CuK_α (Graphite monochromator)
Crystal form	colourless, prismatic	Intensity scans	$\omega/2\theta = 1.0$; $\Delta\omega = 1.0° + 0.3 \tan\theta$
Crystal size	ca. 0.2 x 0.2 x 0.3 mm		$\sigma(I)/I = 0.02$ ($t_{max} = 120s$)
Space group	$P4_1$ (No 76)	Sphere of reflexion	$\sin\theta/\lambda \leq 0.51$ (36508 reflexions)
Cell dimensions	a = b = 13.837 (2), c = 41.242(3) A; V = 7896A^3	Intensities measured	5057 (unique)
		Intensities significant	4272($I \geq 2.5\sigma(I)$; $(I)=\{\Sigma i_n+0.02I\}^{\frac{1}{2}}$
Crystal density (calc.)	d_c = 1.042 g. cm^{-3}		

The crystal data for iodocyclosporine (10), $C_{62}H_{110}N_{11}O_{12}$ ± , M = 1327, colorless prisms from n-heptene, 2.5:1 monoclinic, a = (10.475(5), b = 19.60(1), c = 21.05(1)A°, β = 99.35 (2°), U = 4264 A°³, Dc = 1.03, Z = 2, space group $P2_1$, (C_2^2, No. 4). CuK_α radiation, λ = 1.54187 A°, graphite monochromator. A crystal of approximate dimensions 0.3 x 0.7 x 0.4 mm was used on an Enraf-Nonius CAD4-F automatic diffractometer.

A stereoview of iodocyclosporine molecule is shown in Fig. 5a, and that of cyclosporine in Fig. 5b.

The iodocyclosporine showed the antiparellel β-pleated sheet conformation of residues 1-6, the open loop of residues 7-11, and the rather large thermal vibrations of some of the side-chain atoms, particularly those of MeLeu-9 are apparent. The absolute configuration was not determined as part of the structure analysis, since sufficient of the hydrolysis products could reliably be identified as L-amino-acids. It became apparent during the analysis, however, that Ala-8 has the D-configuration (15).

The conformation of cyclosporine observed in the crystal (Fig. 5b) with 50% probability vibrational ellipsoids of the atomic thermal motion (16) which display the relatively small, isotopic vibrations of the backbone atoms - indicating conformational rigidity - in contrast to the more flexible side-chain atoms whose vibrational amplitudes increase with the distance from (α).

Fig. 6a shows the backbone of the cyclic peptide with the ϕ-, ψ-, and ω- angles and some structural features. Three H-bonds bridge the two short β- strands adding to the stability of β-fragment.

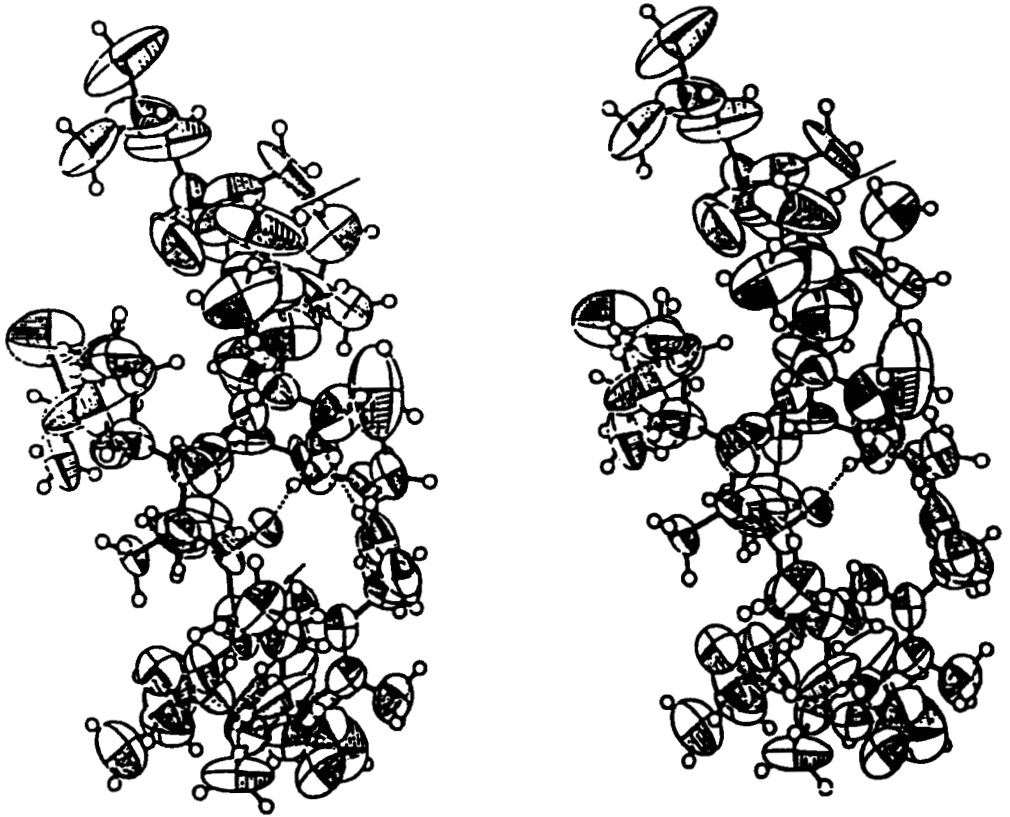


Fig. 5a. A stereoscopic view of the iodocyclosporine molecule showing 50% probability ellipsoids of thermal vibration. The C_9 amino acid is at centre left on the forward side of the molecule and the conventional sequence then extends upwards at the front through α-aminobutyric acid, glycine etc. One of the hydrogen bonds of the β-pleated sheet is indicated (dotted) in the centre and the interesting hydrogen bond Ala-8 → MeLeu-6 appears at centre right.

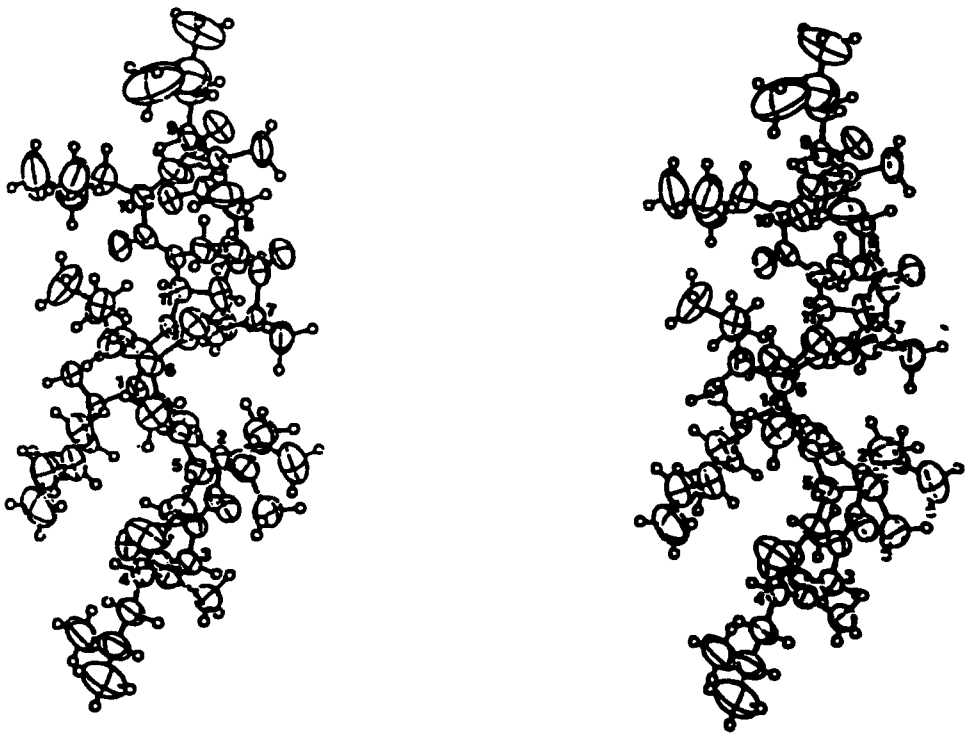


Fig. 5b. ORTEP drawing of the crystal structure of cyclosporine. Anisotropic atomic vibrations are represented by 50% - probability ellipsoids. The numbers shown are residue numbers.

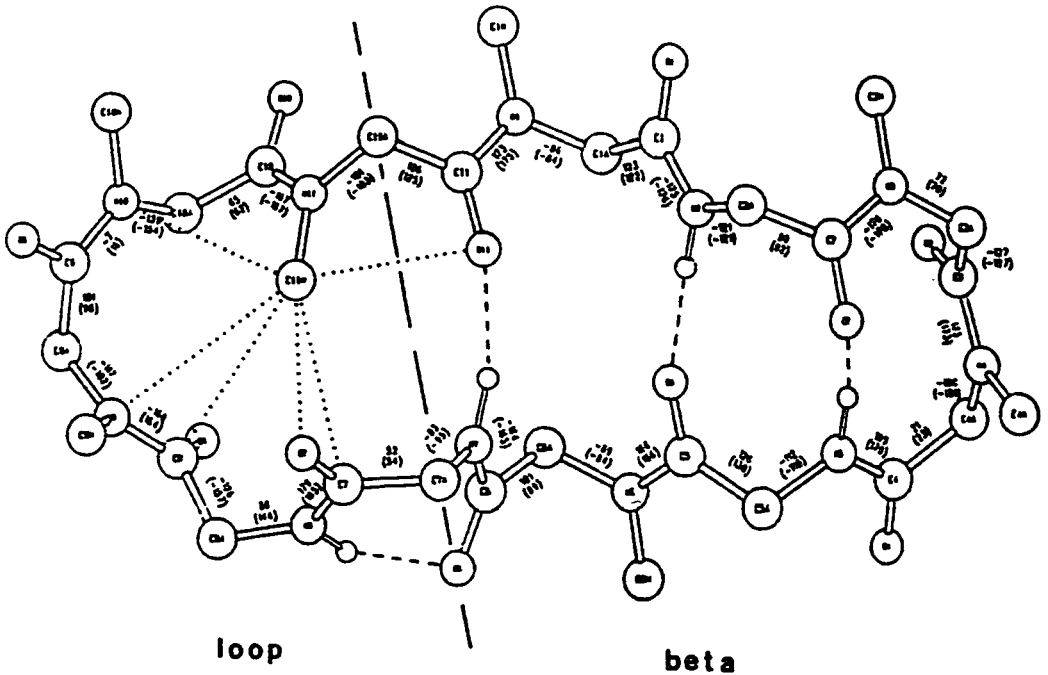


Fig. 6a. Backbone conformation of crystalline cyclosporine with indication of the loop and β-fragment. The torsion angles ϕ, ψ, and ω and in brackets the computer-modeled values for the NMR conformation are given. The dashed lines indicate H-bonds, the dotted lines close contacts from CH_3N of MeVal to different atoms (O 11:3.04 A^o; C 7:3.36 A^o; O 7:3.26 A^o; C 8:3.41 A^o; N 9:3.54 A^o; N 10:3.12 A^o).

Fig. 6b. **Stereoview of a space-filling model of the crystal conformation of cyclosporine showing the side chain of MeBmt-1 (shaded) folded between MeLeu-6 and MeLeu-4 into the ply of the β-fragment**

The side chains of the Abu, Val, and MeLeu residues are in the staggered conformation, the MeLeu side chains being extended. Rather unexpected is the conformation of the C_8-alkylene side chain of MeBmt-1; it is neatly folded into the ply of the β-pleated sheet which allows the molecule to adopt a globular compact shape (Fig. 6b).

3.2 Melting Range

White prismatic needles from acetone at - 15°, m.p. 148-151° (17). The synthetic cyclosporine m.p. 149-150° (18).

3.3 Solubility

The compound is neutral, rich in hydrophobic amino acids, insoluble in water and n-hexane, but very soluble in all other organic solvents. It is very soluble in methanol, ethanol, acetone, ether and chloroform.

3.4 Optical Rotation (17)

$[\alpha]_D^{20}$ - 244° (C = 0.6 in chloroform).

$[\alpha]_D^{20}$ - 189° (C = 0.5 in methanol).

3.5 Spectral Properties

3.5.1 Ultraviolet Spectrum

The UV spectrum of cyclosporine in methanol was recorded on a Beckmann-DK2 spectrophotometer shows only an end absorption at 200 nm (4)

3.5.2 Infrared Spectrum

The infrared characteristics in methylene-chloride (CH_2Cl_2) and in carbontetrachloride (CCl_4) were reported (4,11). The IR spectrum in methylene chloride recorded on a Perkin Elmer 21.IR spectrophotometer is shown in Fig. 7. It should be noted that solution studies, indicated the NH groups

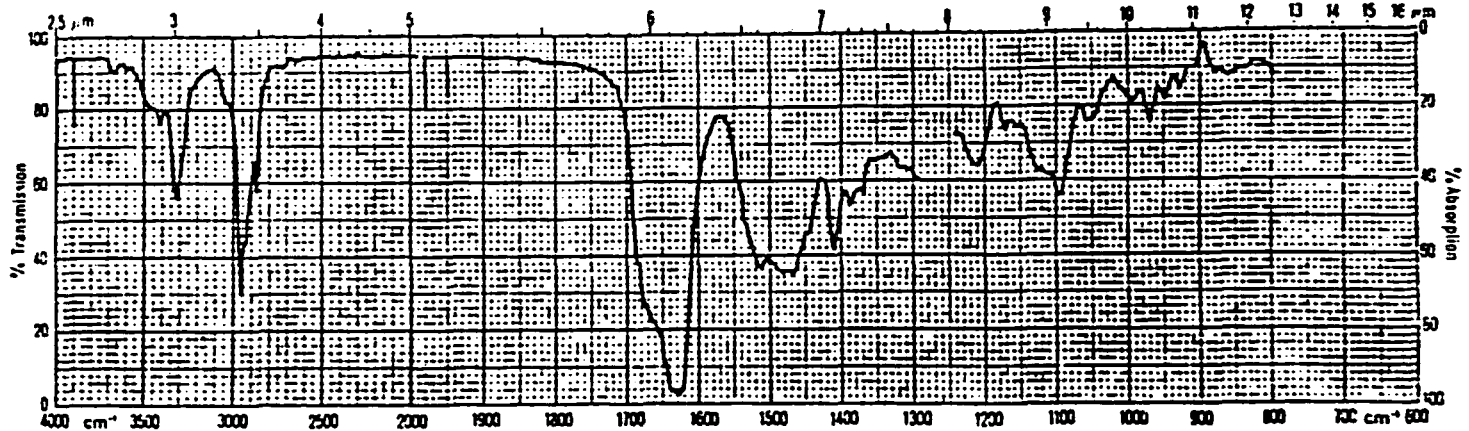


Fig. 7. IR-spectrum of cyclosporine in CH_2Cl_2.

are involved in H-bonding. Their absorption bonds (3330, 3290 cm^{-1} in CCl_4 solution and 3400-3330 cm^{-1} in CH_2Cl_2 solution) are independent of the concentration in the range of 4.10^{-2} to 4.10^{-4} M in CCl_4 solution. The spectrum shows also an intensive amidecarbonyl bond at 1630-1670 cm^{-1}.

3.5.3 Nuclear Magnetic Resonance Spectra

^{1}H-NMR, ^{13}C-NMR and ^{1}H-NMR-NOE-difference spectra of cyclosporine in deuterochloroform and deuterobenzene were reported (4,18, 11). However, the complete assignment of ^{1}H-NMR, ^{13}C-NMR and ^{15}N-NMR spectra in deuterochloroform and deuterobenzene by a combination of homonuclear and heteronuclear two dimentional techniques were described by Kessler et al (19).

3.5.3.1 ^{1}H-NMR Spectra

The ^{1}H-NMR spectrum of cyclosporine (Fig. 8) in $CDCl_3$ and TMS was recorded on Bruker HX-90-E, 90 MHz instrument (4). A part of ^{1}H-NMR spectrum of cyclosporine in C_6D_6 (Fig. 9) was recorded on Bruker HX-360, 360 MHz instrument. This has proved the E-configuration of the double bond in cyclosporine by using the double resonance technique ($J\varepsilon$, ζ = 16 Hz) (4). From these spectra, identification of spin systems were obtained. It is evident from the spectra that one conformation strongly dominates. Some minor peaks specially in the N-methyl region (2.5-3.5 ppm) are visible indicating the presence of another conformation in slow exchange. A completely different situation is observed in DMSO d_6 solution, where a mixture of at least seven conformations leads to a spectrum of high complexity. Roughly

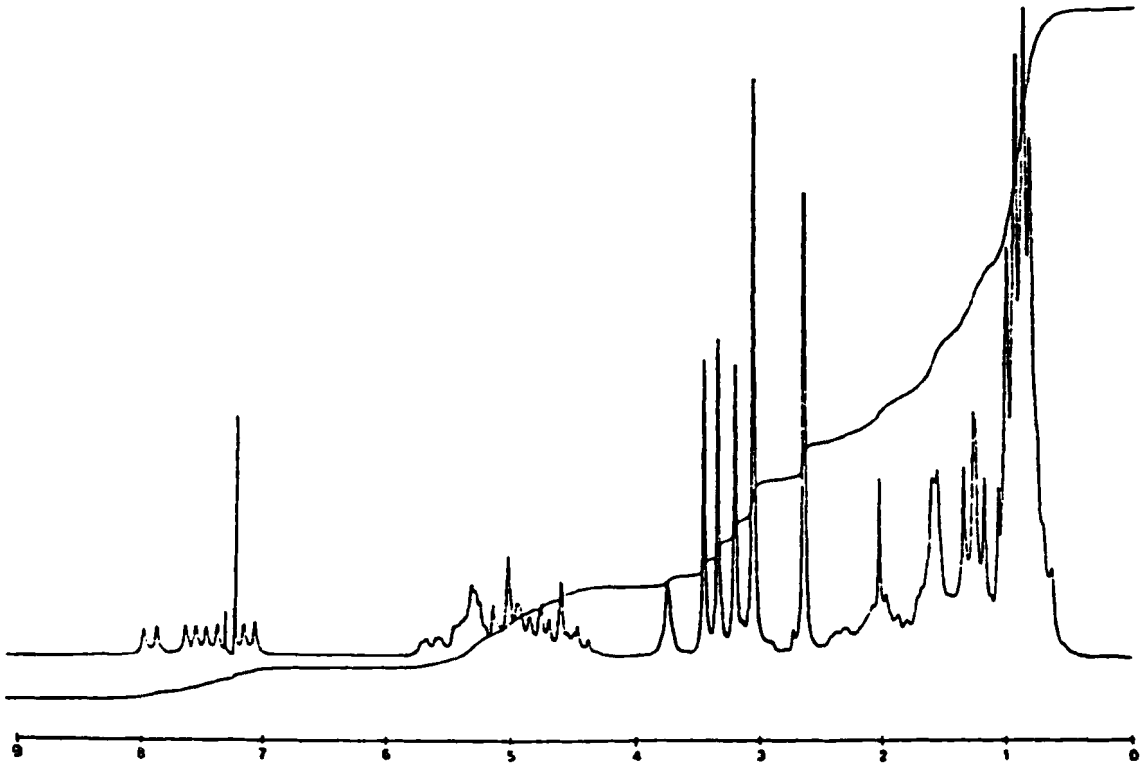


Fig. 8. ^{1}H-NMR-spectrum of cyclosporine by 90 MHz in $CDCl_3$, TMS = 0 ppm.

speaking, five more or less separated spectral regions are distinguishable:

NH protons	7 - 8.3 ppm
α-protons as well as the vinylic protons	4.5 - 6 ppm
N-methyl groups	2.6 - 3.8 ppm
C-methyl groups	0.6 - 1.8 ppm
Aliphatic β-, γ- and δ protons	1.1 - 2.7 ppm

The proton chemical shifts for cyclosporine in $CDCl_3$ and C_6D_6 are listed in Table 2.

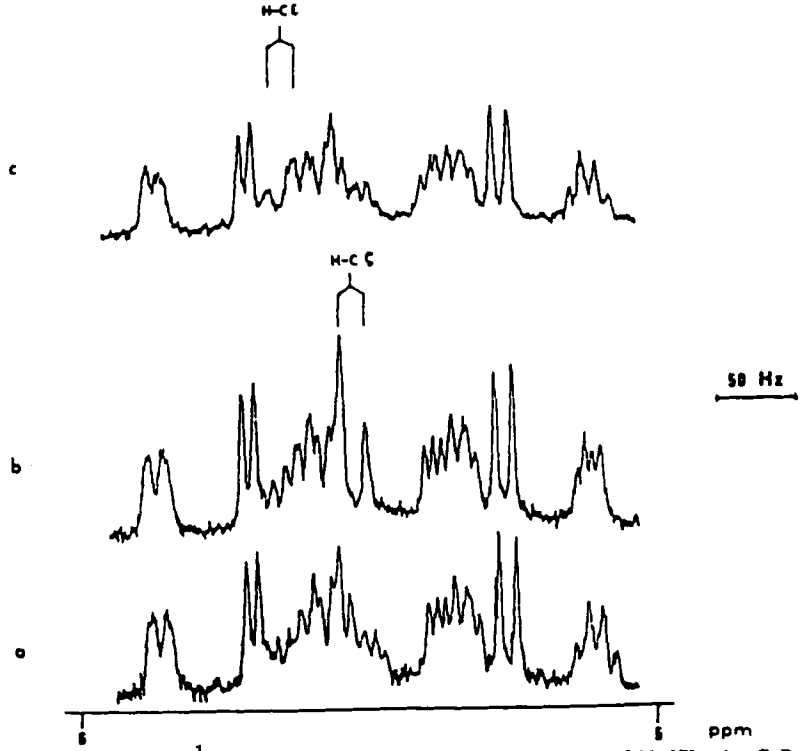


Fig. 9. Part of the ^{1}H-NMR-spectrum of cyclosporine by 360 MHz in C_6D_6.
a) Undecoupled spectrum.
b) Decoupled snectrum keeping lock on H-C(η) of C_9 As
c) Decoupled spectrum keeping lock on H-C(δ) of C_9 As

Table 2. ^{1}H- and ^{13}C-NMR chemical shifts of cyclosporine [1] in $CDCl_3$ and C_6D_6[a])

Residue No.	Amino-acid residue	Group	^{1}H-NMR				^{13}C-NMR	
			$CDCl_3$		C_6D_6		$CDCl_3$	C_6D_6
			2K	32K	2K	32K		
1	MeBmt	CH_3N	3.52	3.51		3.73	33.97 q	34.33
		CO	-	-	-	-	169.65 s	170.73
		H-C(α)	5.45	5.47	5.7	5.72	58.75 d	59.96
		H-C(β)	3.82		4.21	4.20	74.74 d	74.95
		OH	3.87			3.5	-	-
		H-C(γ)	1.63		2.07		35.99 d	36.30
		H^1-C(δ)	2.41		2.65		35.63 t	36.04
		H^2-C(δ)	1.73		2.13			
		CH_3(δ)	0.72	0.71	1.15		16.76 q	18.30
		H-C(ε)	5.36		5.64		129.68 d	131.38
		H-C(ζ)	5.35		5.52		126.32 d	126.76
		CH_3(η)	1.62		1.75		17.96 q	18.69
2	Abu	NH	7.93	7.96		8.26	-	-
		CO	-	-	-	-	173.04 s	174.29

Table 2. (Continued)

Residue No.	Amino-acid residue	Group	^{1}H-NMR $CDCl_3$ 2K	^{1}H-NMR $CDCl_3$ 32K	^{1}H-NMR C_6D_6 2K	^{1}H-NMR C_6D_6 32K	^{13}C-NMR $CDCl_3$	^{13}C-NMR C_6D_6
		H-C(α)	5.03		5.12	5.12	48.86 d	49.54
		H-C(β)	1.6-1.74		1.79		25.06 t	26.11
		H-C(γ)	0.87		0.89		9.93 q	10.73
3	Sar	CH_3N		3.40		3.08	39.40 q	39.58
		CO	-	-	-	-	170.50 s	171.80
		H-C(α)	4.76	4.74		4.01	50.37 t	50.10
4	MeLeu	CH_3N		3.11		2.59	31.32 q	31.39
		CO					169.35 s	170.21
		H-C(α)	5.34		5.60		55.51 d	56.20
		H^1-C(β)	2.00		2.27		35.99 t	37.01
		H^2-C(β)	1.64		1.58			
		H-C(γ)	1.44		1.42		24.90 d	25.79
		$CH_3(\delta_1)$	0.95		0.98		23.49 q	24.28
		$CH_3(\delta_2)$	0.88		0.91		21.18 q	22.70[b])
5	Val	NH	7.47	7.48		7.46	-	-

Table 2. (Continued)

Residue No.	Amino-acid residue	Group	^{1}H-NMR $CDCl_3$ 2K	^{1}H-NMR $CDCl_3$ 32K	^{1}H-NMR C_6D_6 2K	^{1}H-NMR C_6D_6 32K	^{13}C-NMR $CDCl_3$	^{13}C-NMR C_6D_6
		CO	-	-	-	-	173.07 s	174.76
		H-C(α)	4.67	4.66		4.89	55.39 d	56.05
		H-C(β)	2.41		2.61		31.17 d	32.19
		$CH_3(\gamma_1)$	1.06		1.15		19.81 q	20.59
		$CH_3(\gamma_2)$	0.90		0.93		18.48 q	19.06
6	MeLeu	CH_3N		3.25		3.23	31.53 q	32.13
		CO	-	-	-	-	170.87 s	172.28
		H-C(α)	5.02		5.39	5.39	55.31 d	56.05
		H^1-C(β)	2.06		2.27		37.41 t	38.33
		H^2-C(β)	1.41		1.45			
		H-C(γ)	1.76		2.12		25.40 d	26.11
		$CH_3(\gamma_1)$	0.94		1.13		23.87 q	24.16[b])
		$CH_3(\gamma_2)$	0.85		1.05		21.93 q	21.93
7	Ala	NH	7.75	7.68		8.00	-	-

Table 2. (Continued)

Residue No.	Amino-acid residue	Group	^{1}H-NMR				^{13}C-NMR	
			$CDCl_3$		C_6D_6		$CDCl_3$	C_6D_6
			2K	32K	2K	32K		
		CO	-	-	-	-	170.44 s	171.84
		H-C(α)	4.52	4.52		4.81	48.69 d	49.54
		H-C(β)	1.36	1.36		1.68	16.07 q	16.61
8	D-Ala	NH	7.18	7.17		7.63	-	-
		CO	-	-	-	-	172.87 s	174.80
		H-C(α)	4.84	4.83		4.84	45.20 d	45.83
		H-C(β)	1.26	1.26	1.06		18.19 q	18.46
9	MeLeu	CH_3N		3.12		2.93	29.65 q	29.95
		CO	-	-	-	-	169.75	171.04
		H-C(α)	5.70	5.70	5.88	5.87	48.30 q	48.95
		H^1-C(β)	2.13		2.19		39.04 t	40.25
		H^2-C(β)	1.25		1.26			
		H-C(γ)	1.32		1.28		24.70 d	25.48
		$CH_3(\delta_1)$	0.97		0.91		23.74 q	25.07[b])

Table 2. (Continued)

Residue No.	Amino-acid residue	Group	^{1}H-NMR				^{13}C-NMR	
			$CDCl_3$		C_6D_6		$CDCl_3$	C_6D_6
			2K	32K	2K	32K		
		$CH_3(\delta_2)$	0.89		0.84		21.86 q	24.81
10	MeLeu	CH_3N		2.70		2.85	29.83 q	30.47
		CO	-	-	-	-	169.41 s	170.98
		H-C(α)	5.10		5.35		57.54 d	58.36
		H^1-C(β)	2.13		2.42	2.42	40.73 t	42.06
		H^2-C(β)	1.24		1.29			
		H-C(γ)	1.49		1.79		24.55 d	25.62
		$CH_3(\delta_1)$	0.98		1.17		23.85 q	24.34
		$CH_3(\delta_2)$	0.98		1.16		23.38 q	22.64[b])
11	MeVal	CH_3N		2.71		2.98	29.81 q	30.96
		CO	-	-	-	-	172.85 s	174.61
		H-C(α)	5.15	5.14	5.27		57.93 d	58.84
		H-C(β)	2.17		2.27		29.05 t	29.99

Table 2. (Continued)

Residue No.	Amino-acid residue	Group	^{1}H-NMR				^{13}C-NMR	
			$CDCl_3$		C_6D_6		$CDCl_3$	C_6D_6
			2K	32K	2K	32K		
		$CH_3(\gamma_1)$	1.01		0.96		18.75 q	19.38
		$CH_3(\gamma_2)$	0.86		0.66		20.26 q	20.59

a) At 296 K in ppm from internal TMS. The ^{1}H-NMR data were obtained from a conventional 32K spectrum or from the cross-sections of a ^{1}H,^{1}H-COSY with 2K data points in f_2 at 300 MHz

b) The signals of MeLeu-4(δ_2) and MeLeu-9(δ_1) as well as those of MeLeu-6(δ_1) and MeLeu-10(δ_2) in C_6D_6 may be exchanged).

3.5.3.2 ^{13}C-NMR Spectra

In the broadband-decoupled ^{13}C-NMR spectra in $CDCl_3$ or C_6D_6 were reported (19). Almost all 62 C-signals are resolved and only few signals overlap, but these split when the solvent is changed. All carbonyl C-atoms resonate in the range between 169 and 175 ppm. Part of the ^{13}C-NMR spectrum (the carbonyl region) of cyclosporine in $CDCl_3$ measured on a Bruker-WH-360, 360 MHz instrument is shown in Fig. 10 (11). The two olefinic C-atoms of MeBmt are also in a typical range 126-132 ppm, whereas 49 C-signals are in the aliphatic region. The number of attached protons were obtained via the usual DEPT technique (20,21). A heteronuclear 2D-J,δ spectrum (22-26) was also performed in $CDCl_3$. The chemical shift for all carbons and mutliplicities in $CDCl_3$ and in C_6D_6 are given in Table 2.

3.5.3.3 ^{15}N-NMR Spectra

AH-decoupled ^{15}N-NMR spectrum shows eleven N-signals (Table 3).

Table 3. ^{15}N-NMR chemical shifts of 1. δ ppm from $NH_4{}^{15}NO_3$ in an external capillary.

in $CDCl_3$	in C_6D_6	in $CDCl_3$	in C_6D_6
- 248.8 A7	- 248.1 A7	- 259.2	- 260.4 Val
- 255.5 Abu	- 255.1 Abu	- 260.1	- 261.8
- 256.9	- 256.7	- 261.8	- 263.1
- 257.3 A8	- 256.9 A8	- 264.2	- 263.9
- 258.0	- 258.3	- 265.2	- 265.1
- 258.6 Val	- 259.2		

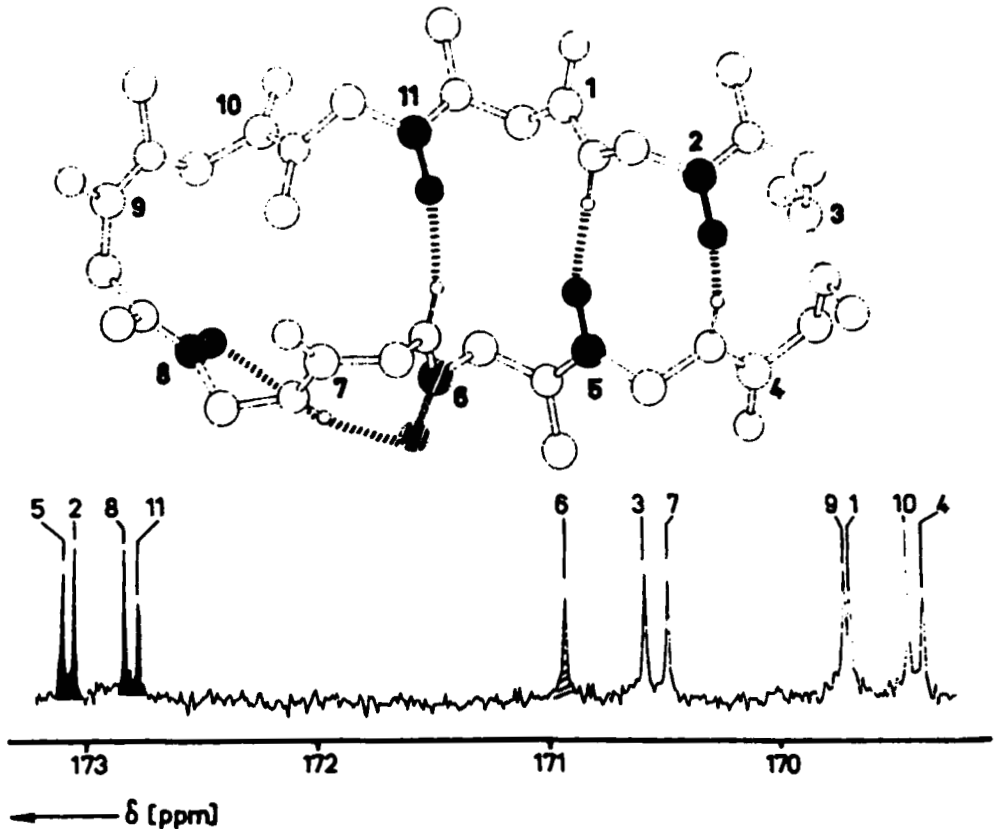


Fig. 10. ^{13}C-NMR spectrum of cyclosporine: CO region. Those CO groups whose signals are shifted downfield are marked in black. The CO signal of residue 6 (shaded) is lowest from those attached to NH groups (residues 1,4,6, and 7).

3.6 Mass Spectrum

3.6.1 EI Spectrum

The electron impact spectrum of cyclosporine was measured on a CEC-mass spectrometer 21-110B (4). The electron energy was 70 eV, the acceleration rate 4.6-8 KV, the temperature of the source 280-300°C and a pressure of 10^{-5} - 10^{-6} Torr. The molecules was unstable giving a molecular ion peak at m/e 1201 which vanishes quickly. As the M^+ vanishes a peak at m/e 1183 increases in intensity and the spectrum remains stable. The peak at m/e 1183 is formed by elimination of a molecule of water. The exact mass measurement were made at 1183.831 ± 0.003 and 1201.842 ± 0.003. The molecular formula obtained by this measurement was $C_{62}H_{111}N_{11}O_{12}$ (physical molecular mass 1201.841). This was in agreement with that deduced from NMR and other findings. Other mass spectral data will be presented under metabolism.

3.6.2 FD Spectrum

The field desorption mass spectrum was measured on a MAT 711 mass spectrometer (4). The acceleration energy rate 6 KV, the temperature of the ion source 90° and the power factor for multiplier 10^6. A molecular ion peak M^+ of m/e 1201.8 was obtained and m/e 1203.6 for the dihydrocyclosporine. Another FD spectrum was reported (18). A molecular ion peak M^+ at m/e 1202, NH^+ at m/e 1203 and $(M + Na^+)$ at m/e 1225.

4. Isolation of Cyclosporine

Dreyfuss *et al* (2) and others (4, 27-30) have reported the isolation of cyclosporine from other fungal metabolites of the fungi *Tolypocladium inflatum* Gams. This fungus was previously known as *Trichoderma polysporum* (Link ex Pres. Rifai (2).

The strain used is Tolypocladium inflatum designated No. N RRL 8044. This was grown in an aerobic submerged culture.

A reported method of isolation of cyclosporine from fermentation media was as follows (4):

The fermentation medium was extracted with n-butylacetate. Then after the separation of the organic layer it was concentrated under vacuum. The crude residue obtained was defatted with 90% methanol and petroleum ether. The product obtained was dissolved in chloroform and applied to a column chromatography of silica gel. The polarity of the eluting solvent was gradually increased by adding methanol to chloroform. The fractions obtained by chloroform-methanol (98:5:1.5) contained cyclosporin A. Fractions contained cyclosporin -C was obtained with chloroform-methanol (97:3). The fractions containing cyclosporin A were subjected to gel filtration with Sephadex LH-20 with methanol. The top fractions were dissolved in toluene and applied to column chromatography of aluminium oxide using toluene-ethylacetate as the eluent. The fractions obtained were evaporated and dissolved in alcohol, then treated with charcoal. The filtrate was evaporated to give cyclosporin A as an amorphous powder.

Another reported procedure (27) was as follows:

The fermentation broth was separated into the mycelial cake and the culture filtrate. The former was homogenized three times with methanol-water (9:1) and the combined filtrates were concentrated under vacuum to remove the methanol. The water solution was extracted three times with 1,2-dichloroethane and the organic layers were evaporated to dryness. Working-up of the culture filtrate by extraction with 1,2-dichloroethane resulted in an additional portion of crude material. The combined extracted were submitted to gel filtration on Sephadex LH-20 with methanol. Those fractions which contain the cyclosporine (as a mixture) were pooled and then separated into the components by silica gel column chromatography (Merck, 0.063-0.2 mm) with ethyl acetate saturated with water. In accordance to their polarity, the cyclosporines were eluted in the following order: D, G, A, B and C. The crude cyclosporines were further purified by crystallization (cyclosporine G from ether/petroleum ether at room temperature; cyclosporines A, C and D from acetone at - 15°C). The

resulting pure cyclosporines A, B, C, D and G were characterized by analytical methods (thin layer chromatography, melting point, optical rotation) and spectral data (U, IR, ^{1}H- and ^{13}C-NMR, mass spectrum). They were found to be identical in all respects with authentic samples isolated from fermentation in complex nutrient media as published by Dreyfuss et al (2), Ruegger et al (4) and Traber et al (28,30).

5. Biosynthesis of Cyclosporine

Kobel et al (31) and others (27,32,33) have reported the biosynthesis of cyclosporine and other fungal metabolites. In the initial biosynthetic studies of Kobel et al (31), ^{3}H- and ^{13}C-labeled precursors were fed to the culture and the position of incorporation of the label in cyclosporine was determined by NMR spectroscopy. It was demonstrated that the N-methyl groups of the molecule and the methyl group in the γ-position of the unsaturated amino acid MeBmt are introduced as intact methyl groups from methionine and that the remaining carbons of the MeBmt moiety are derived from the head-to-tail condensation of four acetate units. The ^{13}C-NMR spectrum of the enriched cyclosporine derived from [1-^{13}C]acetate showed four enhanced signals corresponding to the carbon atoms 1, 3, 5 and 7 of the MeBmt unit and no ^{13}C incorporation into any other amino acid.

In enniatin, an analogous example of non-ribosomal cyclic peptide synthesis by fungi, Zocher and Kleinkauf (32) demonstrated that a multifunctional enzyme carries out the N-methylation of constituent amino acids following an activation step when the acids are bound to the enzyme as thioesters. Supporting evidence for a similar mechanism in cyclosporine biosynthesis was provided by the observation of Zocher et al (33) that [^{14}C] sarcosine was not incorporated and that the radioactivity from [Me-^{14}C]methionine incorporated into each N-methylated amino acid was directly proportional to the number of the corresponding amin acid residues in cyclosporine, thus indicating that N-methylation of the amino acids occurred simultaneously.

From the results of Kobel et al (31) the site of biosynthesis of the Bmt unit remains unclear. However, the results obtained by the Zocher group (33) with short-term feeding experiments using labeled acetate and methionine, in which probably only incorporation of

label in the N-methyl group occurred, suggest that the amino acid Bmt is not biosynthesized on the enzyme matrix but at some other site before incorporation into cyclosporine.

6. Synthesis

6.1 Cyclosporine

Winger (18, 34-38) has reported the total synthesis of cyclosporine and analogues.
The strategy of the total synthesis of cyclosporine has involved the following steps:-

I. The synthesis of the N-methyl-C-9-amino acid (Scheme 1) (36, 39-44).

II. Building of the tetrapeptide BOC-D-Ala-MeLeu-MeLeu-MeVal-benzylester (37).

III. Preparation of the dipeptide BOC-Abu-Sar-benzylester (18,37,46).

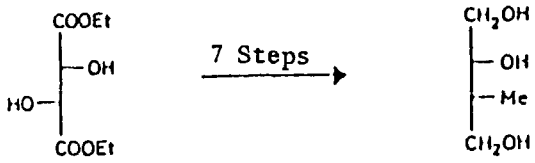
IV. Building of the tetrapeptide BOC-MeLeu-Val-MeLeu-Ala-benzylester (18).

V. The synthesis of the hexapeptide BOC-Abu-Sar-MeLeu-Val-MeLeu-Ala-benzylester (18).

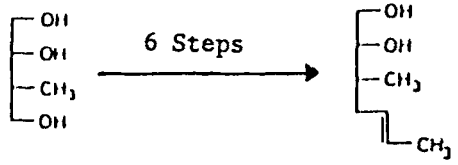
VI. Synthesis of the heptapeptide H-Me-C9-Abu-Sar-MeLeu-Val-MeLeu-Ala-benzylester (18,47).

VII. Formation of the undecapeptide BOC-Ala-MeLeu-MeLeu-MeVal-Me-C_9-Abu-Sar-MeLeu-Val-MeLeu-Ala-benzylester (18,48).

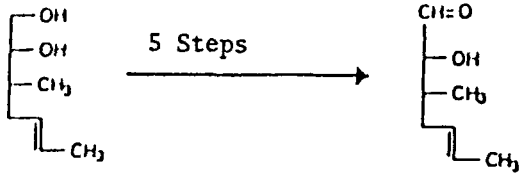
VIII. Cyclization of the undecapeptide with a free amino group and free carboxyl group to produce cyclosporine (48,50,51).



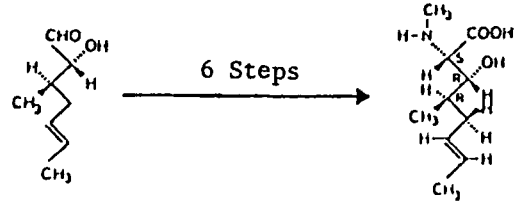
(2R,3R)-3-methyl-1,2,4,-butane-triol



(2R,3R,5E)-3-methyl-5-heptene-1,2-diol



(2R, 3R, 5E)-2-hydroxy-3-methyl-5-heptenal



(2S, 3R, 4R, 6E)-3-hydroxy-4-methyl-2-(methylamino)-6-octenoic acid (MeBmt)

Scheme 1. Synthesis of MeBmt

VIII. Total Synthesis of Cyclosporine (18, 34, 35, 37)

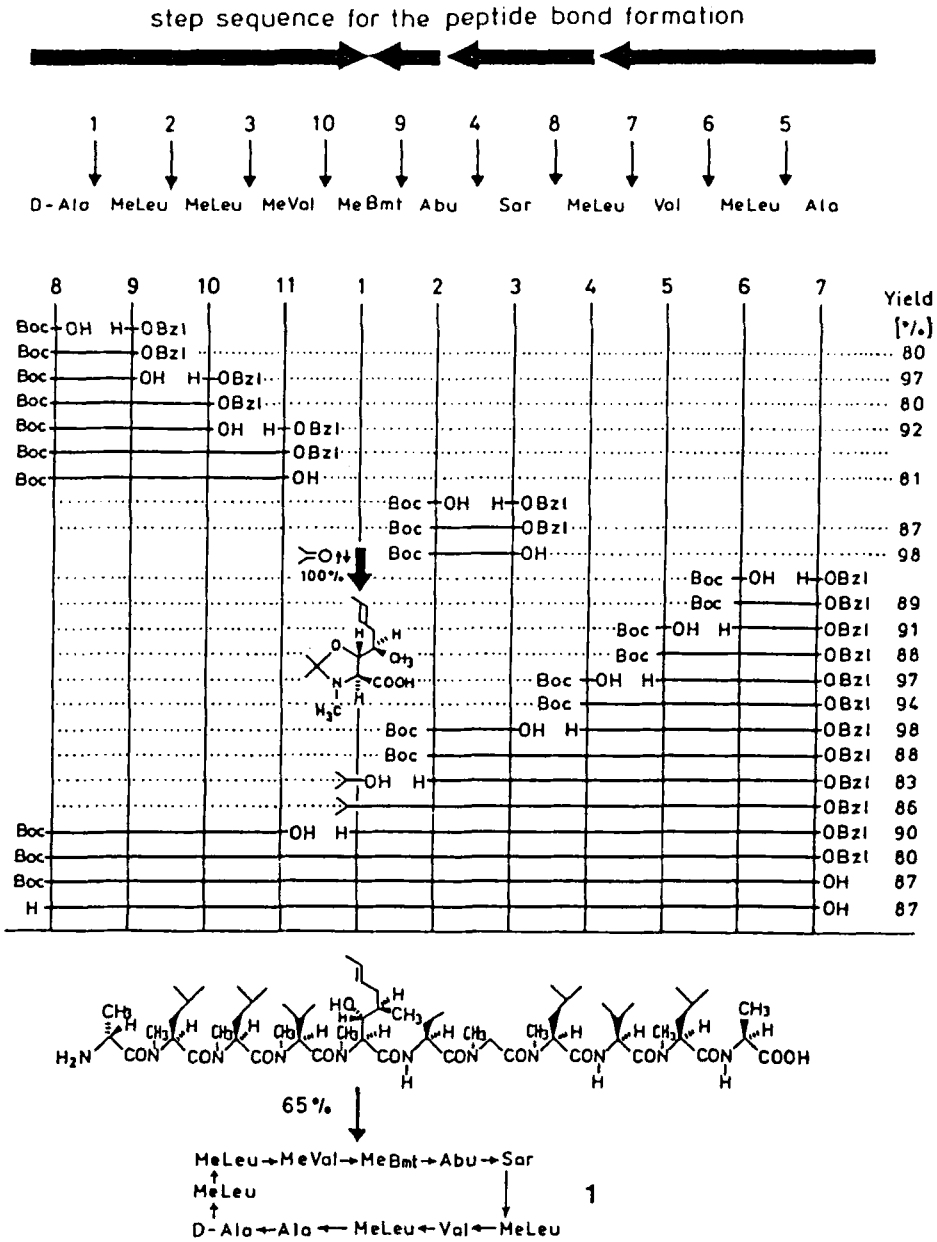
Strategy Used for the Synthesis of Cyclosporine

For the synthesis of cyclosporine[1](Fig. 1) the peptide bond between the L-alanine in position 7 and the D-alanine in position 8 was chosen for the cyclization step. There were two main reasons for choosing this strategy: 1. The intramolecular hydrogen bonds between the amide groups of this linear peptide could operate so as to stabilize the open chain in folded conformations approximating the cyclic structure of cyclosporine and thus assist cyclization. 2. The bond formation between N-methylated amino acids is more difficult than between non-methylated amino acids (37,45); therefore, bond formation between the only consecutive pair of non-methylated amino acids in cyclosporine appeared the logical choice for the cyclization step.

For the synthesis of the undecapeptide, a fragment-condensation technique introducing the amino acid MeBmt at the end of the synthesis was used. In this way, the number of steps after the introduction of this amino acid was minimized. Scheme 5 shows the step sequence used for joining the (protected) peptide fragments. The carboxy groups were generally activated using a variation of the mixed pivalic anhydride method reported by Zaoral (46) and adapted for the N-methylamino acid derivatives (37). This strategy allowed slow anhydride formation in chloroform at - 20°C with pivaloyl chloride in the presence of 2 equivalents of a tertiary base such as N-methylmorpholine before adding the amino acid or peptide esters to be coupled in the form of their free bases. The free bases were obtained by removal of the Boc-protecting groups with trifluoroacetic acid at - 20°C and subsequent

neutralization with $NaHCO_3$; the benzyloxy-protecting groups of the peptide intermediates were removed hydrogenolytically with palladium/carbon in ethanol.

The tetrapeptide Boc-D-Ala-MeLeu-MeLeu-MeVal-OBzl was synthesized from the left to the right by forming bonds 1, 2 and 3 (Scheme 2). Bond 4 was formed on synthesis of the dipeptide Boc-Abu-Sar-OBzl. The tetrapeptide Boc-MeLeu-Val-MeLeu-Ala-OBzl was synthesized from the right to the left by forming bonds 5, 6 and 7 in that order. Subsequent coupling (bond 8) with the previously synthesized dipeptide then furnished the hexapeptide Boc-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl. During incorporation of the amino acid MeBmt, the hydroxy- and N-methylamino functions were protected in the form of a dimethyloxazolidine derivative. This isopropylidene-protecting group was readily introduced by refluxing the amino acid in acetone and has the advantage of avoiding epimerization of the amino acid MeBmt during peptide-bond formation. This means that the oxazolidine ring retains the thermodynamically more stable trans-configuration of substituents during carboxy activation and peptide formation. On preparation, but before activation, the protected MeBmt amino acid was stabilized by addition of N-methylmorpholine (1 equiv.). To prepare the protected heptapeptide from the protected MeBmt amino acid and the hexapeptide, the dicyclohexylcarbodiimide (DCCI) coupling method was used in presence of N-hydroxybenzotriazole (47). After removal of the isopropylidene-protecting group (1 equiv. of 1 N HCl/MeOH), the final amide linkage 10 was made by coupling Boc-D-Ala-MeLeu-MeLeu-MeVal-OH with the heptapeptide in presence of N-methylmorpholine in CH_2Cl_2 at room temperature using the reagent (1H-1,2,3-benzotriazol-1-yloxy)tris (dimethylamino)-phosphonium hexafluorophosphate ($BtOP(NMe_2)_3^+ PF_6^-$) developed by Castro *et al* (48). After hydrolysis of the



Scheme 2. Synthesis of cyclosporine 1. Bold arrows: strategy for the synthesis of the peptides. For details see text. Boc = *tert*-butoxycarbonyl, Bzl = benzyl, >—OH = isopropylidene-protected MeBmt.

ester group (NaOH at 0°C) and removal of the Boc group (CF_3COOH at - 20°C), the unprotected undecapeptide was cyclized at room temperature to cyclosporine 1 (0.0002 M CH_2Cl_2, 1 equiv. $BtOP(NMe)_3^+ PF_6^-$ (48), N-methylmorpholine). The yield of crystalline cyclosporine was 62%. By using the mixed phosphonic anhydride method described by Wissmann and Kleiner (49) or the pentafluorophenol-DCCI complex of Kovacs (50) to effect cyclization of the unprotected undecapeptide, the yield of cyclosporine could be increased to 65%. Using the fragment-condensation technique described here it is now possible to synthesize cyclosporine very efficiently in 27.5% yield with respect to the amino acid MeBmt. Thus, 1.6 g of cyclosporine can be produced starting from 1 g of the amino acid MeBmt.

7. Metabolism of Cyclosporine

Maurer et al (52) have reported the disposition of cyclosporine in man and several animal species and also structural elucidation of its metabolites. The metabolites were isolated from dog and human urine and from rat bile and faeces. The structures of the identified metabolites were used to establish the biotransformation processes. ^3H-labeled cyclosporine with specific activities ranging from 25 to 80 µCi/mg was prepared biosynthetically by feeding submerged cultures of a selected strain of the fungus T. inflatum Gams with [methyl-H^3] methionine as labeled precursor (31). The radiochemical purity of the labeled substance was better than 95%. ^3H-NMR analysis revealed the incorporated tritium to be located in the seven N-methyl groups and in the methyl group at position 4 (C_6) of amino acid (Fig. 11).

The separation of metabolites was performed by HPLC. Nine ether-extractables of cyclosporine were isolated from urine of dog and man and from rat bile and faeces (Fig. 12).

Fig. 11. Site and type of biotransformation in cyclosporine

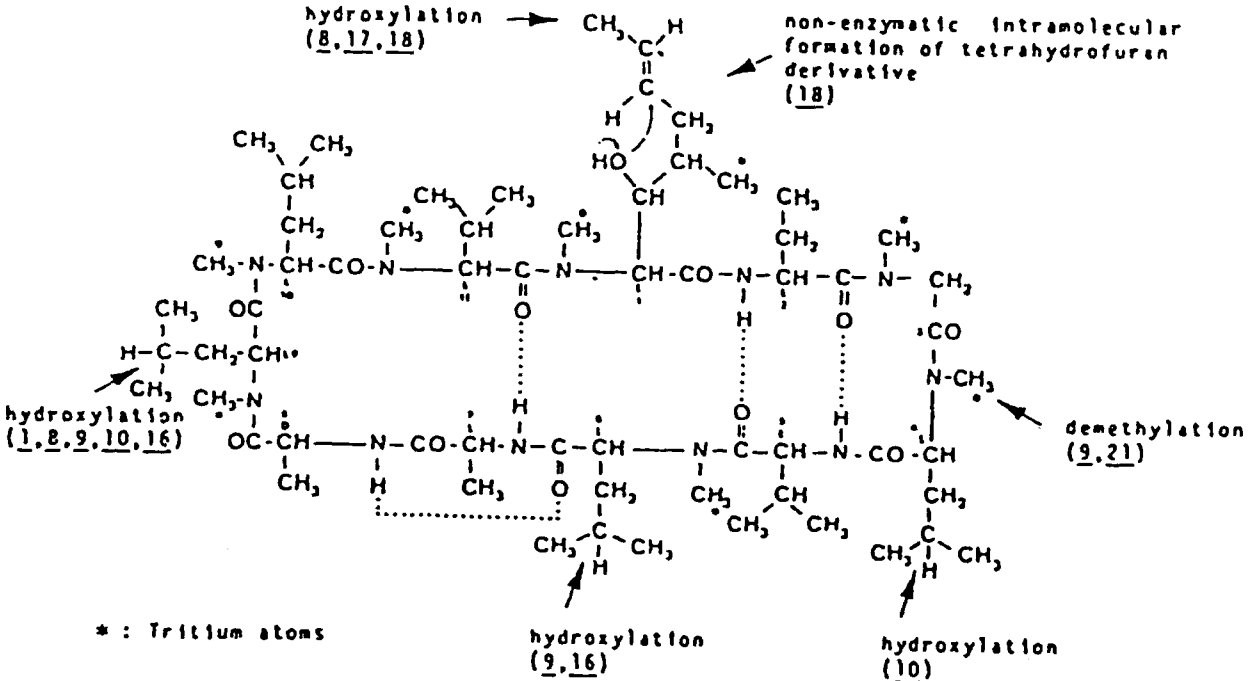
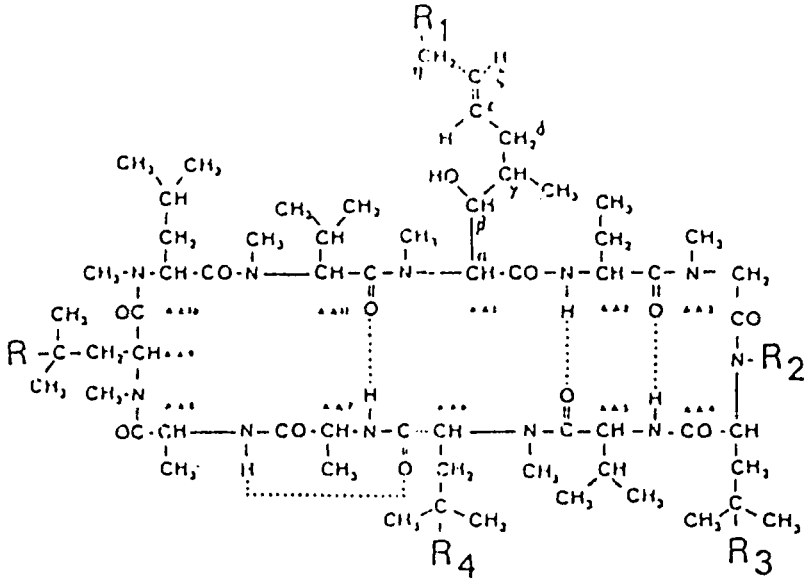


Fig. 12. Structure of cyclosporine and its metabolites.



Metabolite No.	R	R_1	R_2	R_3	R_4	Other modification	Molecular weight
Cyclosporine	H	H	CH_3	H	H		1202.64
1	OH	H	CH_3	H	H		1218.64
8	OH	OH	CH_3	H	H		1234.64
9	OH	H	H	H	OH		1220.62
10	OH	H	CH_3	OH	H		1234.64
16	OH	H	CH_3	H	OH		1234.64
17	H	OH	CH_3	H	H		1218.64
18	H	OH	CH_3	H	H	CH(OH)-CH_2 of AA1	1218.64
21	H	H	H	H	H		1188.62
13	hydroxylated and N-demethylated derivative of cyclosporine						1204.62

Despite the complex structure of cyclosporine, the reactions involved in the biodegradation of the molecule are limited and produce only a relatively small number of metabolites. The resulting products are lipophilic as indicated by their occurrence in the ether phase of the liquid-liquid extraction.

Site and type of biotransformation in cyclosporine are summarized in Fig. 11. The cyclic oligopeptide structure of cyclosporine is preserved in all identified metabolites. Metabolism mainly consists of oxidative processes (phase I reactions). Typical phase II reactions like conjugation with sulfuric acid or glucuronic acid could not be detected. Only a limited number of the cyclosporine subunits, namely four of 11 amino acids, undergo regiospecific oxidative modifications. Hydroxylation reactions appear to be restricted to the η-position of amino acid 1 (C_9-amino acid) and the γ-position of the N-methylleucines 4, 6, and 9. Oxidative N-demethylation, so far, seems to occur only on the N-methylleucine 4. The N-methylleucines 6 and 9 are subject of a single reaction (hydroxylation) whereas amino acid 1 and the N-methylleucine 4 are substrates for two types of transformation: hydroxylation and intramolecular ether formation or hydroxylation and N-demethylation.

The proposed pathways for the biotransformation of cyclosporine (Fig. 13) are based on the structure of the isolated metabolites. The regioisomeric monohydroxylated cyclosporines 1 and 17 and the N-demethylated cyclosporine 21 are primary metabolites. Metabolites 1 and 17 result from hydroxylation of either the N-methylleucine 9 at the γ-position or the amino acid 1 at the η-position. Metabolite 21 represents the product of N-demethylation on the N-methylleucine 4. Further oxidation of metabolite 1 on either one of the N-methylleucines 4 and 6 or on the C_9-amino acid 1, and of metabolite 17 on the N-methylleucine 9 generates dihydroxylated derivatives of cyclosporine (metabolites 8, 10, and 16). The identification of a dihydroxylated and N-demethylated derivative of cyclosporine (metabolite 9) indicates further oxidation to occur on either the dihydroxylated metabolite 16 by N-demethylation of the N-methylleucine 4 or the primary N-demethylcyclosporine 21 by hydroxylation of both N-methylleucines 6 and 9.

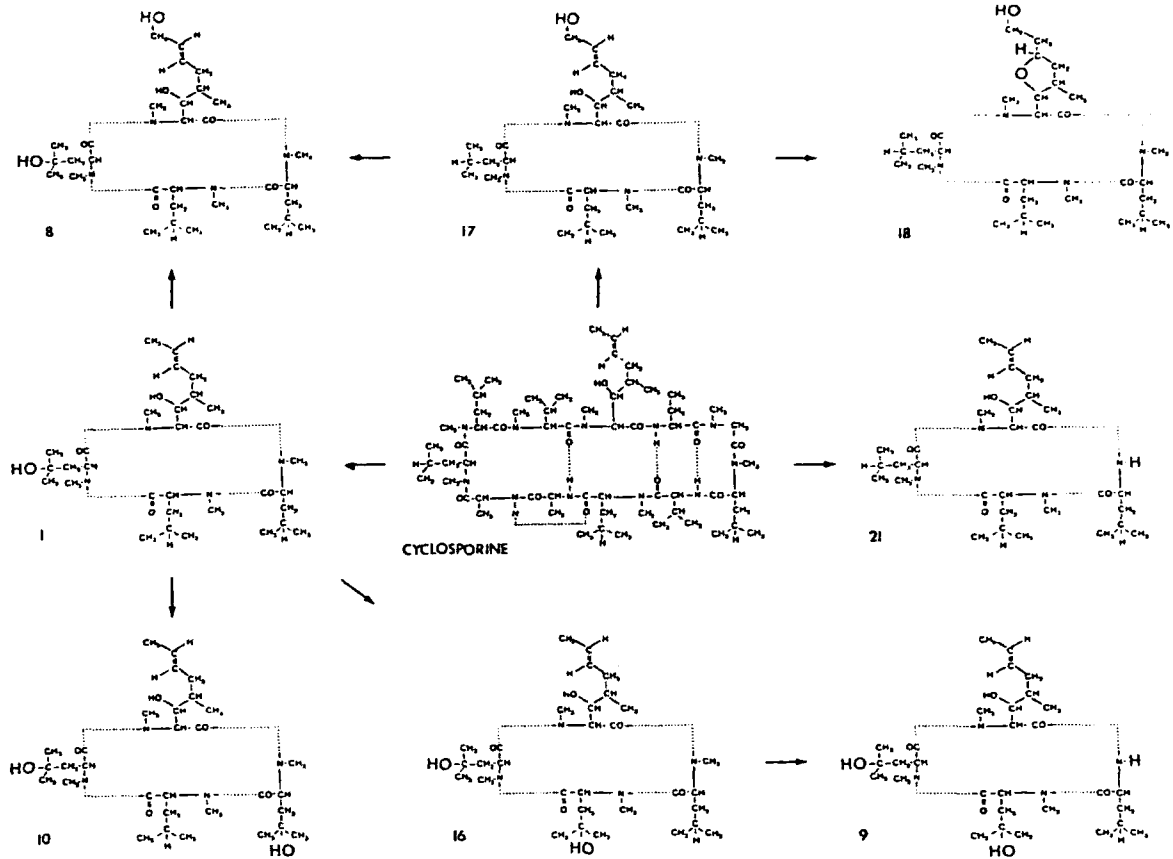


Fig. 13. Proposed pathways for the biotransformation of cyclosporine.

Metabolite 18, containing a cyclic ether moiety (tetrahydrofuran), may be derived from 17 (monohydroxycyclosporine) by intramolecular addition of the β-hydroxyl group to the double bond of the C_9-amino acid 1. This cyclization is suggested to proceed by a nonenzymatic mechanism. An artifact with a similar cyclic ether structure has previsouly been observed among the acid hydrolysis products of cyclosporine.

Three other metabolites possessed the basic cyclic structure of the parent drug. The spectroscopic data of two of them were compatible with hydroxylated and N-demethylated derivatives of cyclosporine, whereas those of the third indicated a dihydroxylated derivative. Their structures remain incompletely determined.

It should be noted that metabolites 10 and 16 were not found in man (53).

8. Pharmacokinetics of cyclosporine

The absorption, distribution, metabolism and elimination of cyclosporine have been investigated by many authors (52-57).

The pharmacokinetic data on cyclosporine in man as follows:

Absorption	
Absolute oral bioavailability	20-50% (mean 34%)
Plasma peak concentration time	1-6 hours
Distribution	
Binding to plasma proteins	90% (20°C)
Apparent volume of distribution	3.5 l/Kg
Metabolism	
Extent of metabolism	Ca. 99%
Number of components	10-12 (9 of which have been characterised as metabolites).

Elimination

Terminal elimination half-life	14-26 hours
Total body clearance	0.27-0.47 l/h/Kg

because of its very low water-solubility, the oral dose form of cyclosporine in an olive-oil-based solution. The absolute oral bioavailability from this solution (in steady state) is 20-50% (mean 34%).

In whole blood, about 50% of cyclosporine is taken up by erythrocytes and 10-20% by leucocytes. Of the total amount of drug in the plasma, approximately 90% is protein-bound, mostly to lipoproteins.

In animal experiments using ^{3}H-cyclosporine, the highest tissue concentrations of radioactivity were found in liver, kidney, adrenals, pancreas, thymus, thyroid and renal fat. From blood concentration-time data following intravenous administration in man, the drug was found to be distributed according to a three-compartment distribution model. The total apparent volume of distribution was 3.5l/Kgm.

Cyclosporine is slowly but extensively metabolised by liver. In addition to the parent drug, 10-12 metabolites have been detected in human plasma. Nine metabolites have been isolated and identified; all contained the intact cyclic oligopeptide structure of the parent molecule. Structural modifications consisted of mono- and di-hydroxylation and N-demethylation at various positions on the cyclosporine.

Terminal elimination half-lives from blood or plasma of 14-26 hours have been reported.

The drug and its metabolites are excreted mainly via the liver and only to a slight extent via the kidneys. In man the total urinary excretion of radioactivity in the period up to 96 hours following a single dose of ^{3}H-cyclosporine was 6% of the dose; unchanged cyclosporine accounted for only 0.1% of the oral dose.

9. Pharmacological Activity and Clinical Application

As reported by Borel et al (5-9), cyclosporine is a selective immunosuppressive drug with antifungal and antiinflammatory properties. It has been shown to strongly suppress the production of haemagglutinating antibodies against sheep erythrocytes in mice and to be effective in inhibiting both humoral and cell-mediated immune responses. Its effect is reversible, and specific for T-lymphocytes. The hemopoietic tissues in immunosuppressed animals are not affected. Bueding et al (58) demonstrated an antishistosomal activity and Thommen-Scott (59) an antimalarial effect of cyclosporine, both antiparasitic activities being independent of immunosuppression. The first transplantation in humans with the aid of cyclosporine were reported in 1978 by Calne et al (60) for kidney and by Powles et al (61) for bone marrow. Today cyclosporine is used successfully to prevent graft rejection following bone marrow and organ transplantations.

Other possible applications, such as in the treatment of autoimune and other diseases, are currently under investigations.

10. Pharmaceutical Forms

Cyclosporine (Sandimmun) is available as:

a) Drinking Solution: 100 mg/ml cyclosporine dissolved in olive oil and labrafil.

b) Intravenous Preparation: Ampoules of 5 ml (250 mg cyclosporine) and 1 ml (50 mg cyclosporine).

11. Methods of Analysis

11.1 Elemental Composition

Cyclosporine ($C_{62}H_{111}N_{11}O_{12}$):

C	H	N	O
61.90	9.30	12.80	16.00

11.2 Introduction to Analysis

Several methods have been developed for the analysis of cyclosporine in plasma and or blood. The first method reported was the radioimmunoassay (RIA) of Donatsch (62) which is now available as a kit and has been used extensively during the clinical development for cyclosporine. The method was initially developed for plasma, and as shown later, is also applicable to blood samples. The main criticism of the RIA method has been the cross-reactivity of the antisera with some of the circulating metabolites of cyclosporine. Other RIA procedures were reported (54,55, 63-67).

Subsequent to the introduction of the RIA method, Niederberger et al (68) developed a high pressure liquid chromatography (HPLC) method based on gradient elution. The technical complexity of this method encouraged several investigators, (69-75) to reinvestigate the method. The results of these activities are shown in Table 4.

11.3 Radioimmunoassay (RIA)

Several radioimmunoassay procedures have been developed for both the analysis and immunopharmacological monitoring of cyclosporine (54,55, 63-67).

One method described recently by Randall et al (64) is as follows:

Sample Preparation: Venous blood was collected in heparinized tubes from 38 post-transplant patients receiving cyclosporine, just before their next dose and 12 h after their last.

Non-temperature-standardized protocol: About 0.5 to 3 h after collection, an aliquot of each sample for whole-blood analysis was set aside and centrifuged the remaining specimen at room temperature to remove cells. The plasma and whole-blood specimens were both then stored at - 20°C.

Temperature-standardized protocol: After collection, samples were refrigerated for no longer than 4 h, re-equilibrated to 37°C by incubation in a water bath for 30 min, then centrifuged without delay (1100 X g, 10 min, room temperature). The resulting plasma was stored frozen at -20°C until analysis.

Time and temperature experiments: Aliquots of whole blood from patients receiving cyclosporine were incubated for 0.25, 0.5, 1, 2, 4, 6, and 24 h at 4°C, room temperature (about 22°C), and 37°C. At these specified times, samples stored at room temperature or 4°C were centrifuged at room temperature; those stored at 37°C were centrifuged at 37°C and the plasma was decanted and frozen.

In addition, the treated aliquots of samples stored at room temperature and 4°C for the above times by re-equilibrating to 37°C by incubating for 15 min in a 37°C water bath, followed by centrifugation at 37°C and prompt removal of the plasma.

Equilibration time experiment: Aliquots of whole blood from patients receiving cyclosporine were maintained at 4°C for 4 h after collection, then equilibrated in a 37°C water bath for 0, 10, 15, 30, 60, 120, and 180 min. All specimens were centrifuged at 37°C, and the plasma was immediately decanted and stored at -20°C until analysis.

Measurement of cyclosporine: Cyclosporine was measured by radioimmunoassay, using tritiated cyclosporine as tracer and an antibody in a kit supplied by Sandoz Ltd., Basle, Switzerland. Duplicate 5 µL patient's samples were mixed with 100 µL of tracer, 700 µL of Tris buffer (0.5 mol/L, pH 8.5), and 100 µL of sheep antiserum to cyclosporine, then incubating for 2 h at room temperature. Free and bound fractions were separated by extraction with charcoal for min at 4°C. The radioactivity was measured in each specimen with a liquid scintillation

counter, using a quench-corrected counting program. The analytical recovery of cyclosporine in plasma and whole blood supplemented with the drug was about 75%.

Correction for hematocrit : Hematocrits of samples for cyclosporine analysis were measured as part of the routine blood count on a Coulter S Plus (Coulter Instruments, Hialeah, FL). The plasma cyclosporine results were corrected for hematocrit by multiplying the uncorrected plasma cyclosporine results by the patient's hematocrit/0.39, the normal value for the hematocrit.

11.4 High Performance Liquid Chromatography

Several HPLC procedures were reported for the estimation of cyclosporine in blood and or plasma as well as immunopharmacological monitoring of cyclosporine (53,54, 67, 69-75). These methods were developed to overcome the drawbacks of the RIA methods particularly the overestimation of cyclosporine in biological fluids. This is because, the antibodies cross react with the metabolites. The HPLC conditions used for the analysis of cyclosporine are listed in Table 5. A recent HPLC method was reported by Kates and Latini (75) and is as follows:

Chemicals and reagents: Cyclosporine and the internal standard, cyclosporin D (Fig. 14), are obtained from Sandoz (Basle, Switzerland). Glass-distilled diethyl ether, acetonitrile, η-hexane, and methanol are obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Tris(hydroxymethyl)aminomethane is purhcased from Aldrich (Milwaukee, WI, U.S.A.)

Instrumentation and chromatographic conditions: A Waters Instrument (Milford, MA, U.S.A.) Model 6000A HPLC pump, Model 480 variable-wavelength UV detector, Model 710B sample injector and Model 730 data module are employed. The column used is a Brown-Lee Labs. (Santa Clara, CA, U.S.A.) RP-8 MLPC analytical cartridge

(10 cm X 4.6 mm I.D.; particle size 10 μm). A 3-cm RP-8 guard cartridge is positioned at the head of the column. The column is maintained at 70°C with an Eldex (Menlo Park, CA, U.S.A.) column heater. The heater is left on at all times and a constant flow is maintained through the column. The flow-rate of the mobile phase is 0.6 ml/min which produces a precolumn pressure of about 34 bars (500 p.s.i.). The effluent from the column is monitored at a wavelength of 215 nm.

Mobile phase: The mobile phase consists of a simple mixture of acetonitrile-water (72:28). This mixture is filtered and degassed by vacuum and sonication. The mobile phase is continuously recyclsed and replaced about every two weeks.

Preparation of extraction columns: The extraction of cyclosporine involves the use of a Baker-10 SPE extraction system (J.T. Baker, Phillipsburg, NJ, U.S.A.). The columns used are the 3 ml cyano disposable extraction columns. These columns are prepared by washing with 6 ml of methanol and then 6 ml of water under vacuum. They are left approximately one half to three quarters full of water until the sample is added.

Extraction procedure: One ml of whole blood or serum is added to PTFE-lined, screw-capped tubes. To the blood or serum is added 450 ng of internal standard, 3 ml of 0.1 M Tris buffer (pH 9.8) and 10 ml of diethyl ether. They are rocked for 20 min on a labquake rocker (Lab. Industries, Berkeley, CA, U.S.A.) and centrifuged for 20 min to separate the ether and aqueous layers. The diethyl ether is pipetted into a clean tube and the blood or serum residue is discarded. To the diethyl ether is added 200 μl of 75% methanol in water. The diethyl ether is then evaporated at room temperature with a gentle stream of nitrogen. Only the diethyl ether is evaporated, leaving 150-200 μl of the methanol-water remaining. To this are added another 100 μl of methanol. The

methanol-water is then transferred to a 3 ml Baker extraction column. The residue is eluted onto the column with water. The column is then washed with 3 ml of 25% acetonitrile in water and 6 ml of n-hexane. The columns are then dried by drawing through air for 4 min. Cyclosporine and cyclosporin D are then dluted off the column with three washings of 200 µl of methanol. The methanol is collected into disposable tubes and evaporated to dryness with a stream of nitrogen at 50°C. The resulting residue is dissolved in 200 µl of the mobile phase. An aliquot of 100 µl is then injected onto the column (Fig. 14).

Preparation of calibration standards: Stock solutions of cyclosporine and cyclosporin D are prepared in methanol and stored in amber bottles at room temperature. A stock solution of cyclosporin D is prepared in a concentration of 30 ng/µl. The stock solution of cyclosporine is prepared in a concentration of 10 ng/µl. This solution is used to prepare standards for the calibration curve. The calibration curve samples are prepared by adding amounts of cyclosporine (50-800 ng) to whole blood or serum from a normal volunteer. These are then treated as described in the extraction procedure. This procedure has a lower limit of sensitivity below 50 ng/ml.

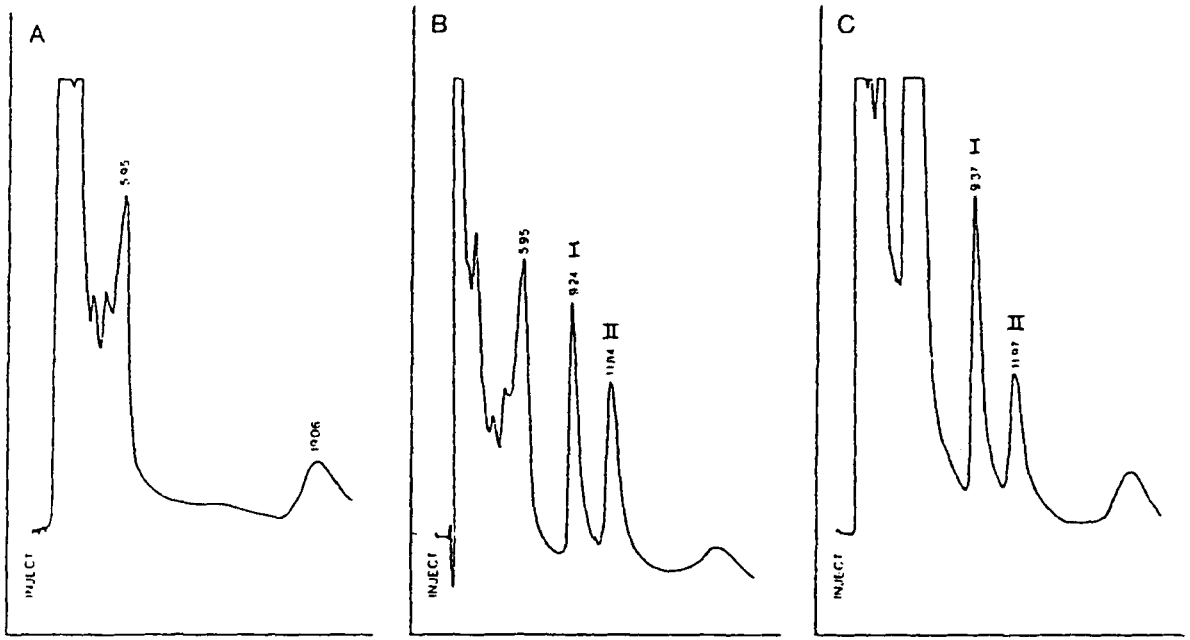


Fig. 14. Chromatograms of extracted whole blood samples. (A) Blood from normal healthy volunteer, not taking cyclosporine; (B) blood to which has been added 200 ng/ml of cyclosporine and the internal standard; (C) blood from a patient who was taking cyclosporine (the concentration in this patient sample is 304 ng/ml). Peaks: I = cyclosporine; II = cyclosporin D, internal standard.

Table 4. Comparison of published methods of analysis of cyclosporine

Typle of analysis	Sample type	Sample* preparation	Detection limit (µg/litre)	Specificity	Standar-dization	Chromato-graphy Time(min)	Ref.
RIA**	Blood/plasma	-	1-20	Metabolite x-reactivity	Ext.	-	54,55, 63-67
HPLC-Grad.	Plasma	Long	20	High	Int.	30	68
HPLC-Isocr.	Serum	Long	100	-	Ext.	5	69
HPLC-Isocr.	Blood/plasma	Very long	25	High	Int.	10	70
HPLC-Col.Swit.	Blood/plasma	Short	20	High	Int.	30	71
HPLC-Isocr.	Plasma	Long	100	High	Int.	20	72
HPLC-Isocr.	Blood/plasma	Long	100	High	Int.	45	63
HPLC-Grad.	Serum	Long	30-50	High	Int.	15	73
HPLC-Isocr.	Plasma	Long	31	High	Int.	30	74
HPLC-Col.Swit.	Blood/plasma	Short (semiautoma-ted)	8	High	Ext.	15	67
HPLC-Isocr.	Blood/plasma	Long	< 50	High	Int.	19	75

*Short: protein precipitation and injection; Long: extraction & evaporation; Very Long: additional sample manipulation.

**RIA: Radioimmunoassay. Ext. = External; Int. = Internal; Grad. = Gradient; Isocr. = Isocratic; Col-Swit. = Column switching.

Table 5. HPLC conditions used for the analysis of cyclosporine

Sample	Instrument	Column	Mobile phase	Flow rate (ml/min)	Retention time (mins)	Detection	Ref.
Blood/serum	Waters Model 6000A	RP-8 MLPC analytical cartridge, 10 cm X 4.6 mm I.D. 3-cm RP-8 guard cartridge, at 70°C.	Acetonitrile/ water 72:28	0.6	9.2	UV at 215 nm	75
Blood/ plasma	Technicon HPLC system	LC-8 Supelcosil, 5 µm, supelco, Inc., Bellefonte, PA., at 75°C.	Acetonitrile/ water 55:45 v/v	3.0	-	UV at 202 nm	67
Blood/ plasma	Altex pumps AX-110. UV detector LCD 725 Uvikon. Recorder Tarkan 600 W+W. Altex microprocessor 420. Water injector WISP710B and Rheodyne 7125	Column I; Altex perisorb RP-8, 40 X 4.6 mm I.D., 30-40 µm. Column II; Lichrosorb RP-18, 150 X 4.6 mm I.D., 5 µm, at 70°C. Alternative column; Beckman Ultrasphere ODS, 150 X 4.6 mm I.D., Guard column; Altex perisorb RP-2, 40 X 3.2 mm I.D., 30-40 µm.	Consists of 6 solvents. 1. Acetonitrile/ methanol/water 35:20:45 v/v/v 2. Acetonitrile/ water 55:45 v/v 3. Acetonitrile/ water 72:28 v/v 4. Tetrahydrofuran 5. Acetonitrile/ water 90/10 v/v 6. Methanol	1.7	-	UV at 210 nm	71

Table 5. (Continued)

Sample	Instrument	Column	Mobile phase	Flow rate (ml/min)	Retention time (mins)	Detection	Ref.
Serum	Varian Vista HPLC system.	Beckman ultrasphere ODS, 25 X 0.46 cm, 5 µm. Precolumn Vydac C_{18}, 4 X 0.4 cm. Both columns at 70°C.	Starting with trifluoroacetic acid/acetonitrile 35:65 and increasing proportions until 5:95 at 15 mins.	1	14.1	UV at 215 nm	73
Serum	Waters system controlled with M-660 solvent programmer.	Beckman ultrasphere-octyl, 25 cm X 4.6 mm, 5 µm. Maintained at 72°C.	Acetonitrile/ methanol/water 47:20:33 by Vol.	1.5	20.46 and 20.53	UV at 210 nm	74

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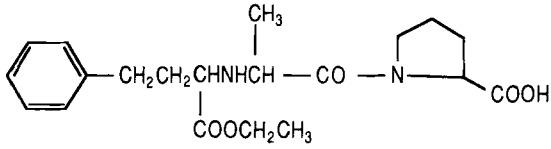
ENALAPRIL MALEATE

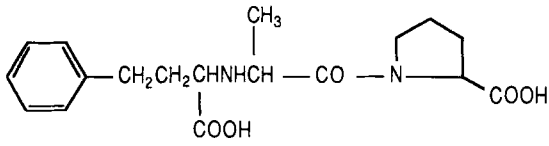
Dominic P. Ip and Gerald S. Brenner

1. History and Therapeutic Properties

Enalapril is the ethyl ester of a long-acting angiotensin converting enzyme inhibitor, enalaprilat. Enalapril maleate, discovered and developed by the Merck Sharp and Dohme Research Laboratories (1), is indicated for the treatment of essential and renovascular hypertension. Enalapril is a pro-drug; following oral administration, it is bioactivated by hydrolysis of the ethyl ester to enalaprilat, which is the active angiotensin converting enzyme inhibitor.



Enalapril



Enalaprilat

Since the oral absorption of enalapril is superior to that of enalaprilat, it is the compound that is used in oral dosage forms. Enalaprilat, the deesterified parent diacid of enalapril is the form of the drug that is administered intravenously. Several review articles give a detailed account of the history, design, chemistry and pharmacology of the drug. (2-6).

2. Description

2.1 Nomenclature

2.1.1 Chemical Name

(S)-1-[N-[1-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl]-L-proline, (Z)-2-butenedioate(1:1)salt

2.1.2 Generic Name

Enalapril Maleate

2.1.3 Laboratory Codes

L-154,739-001D, MK-0421

2.1.4 Trade Names

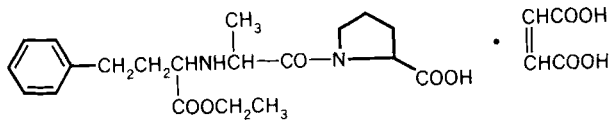
Vasotec, Renitec, Innovace, Pres, Xanef

2.1.5 CAS Registry Number

76095-16-4

2.2 Formula and Molecular Weight

$C_{20}H_{28}N_2O_5 \cdot C_4H_4O_4$ mol wt 492.53



2.3 Appearance, Color, Odor

Enalapril maleate is a white to off-white, crystalline, odorless powder

3. Synthesis

Enalapril maleate has been prepared by the scheme outlined in Figure 1 (7). Ethyl 2-oxo-4-phenylbutanoate (1) is condensed with L-alanyl-L-proline (2) to give Schiff base 3, as a mixture of syn and anti isomers. Reduction of the imine bond in 3 results in formation of the diastereomeric mixture 4 (SSS and RSS). The more biologically active diastereomer (SSS) is isolated

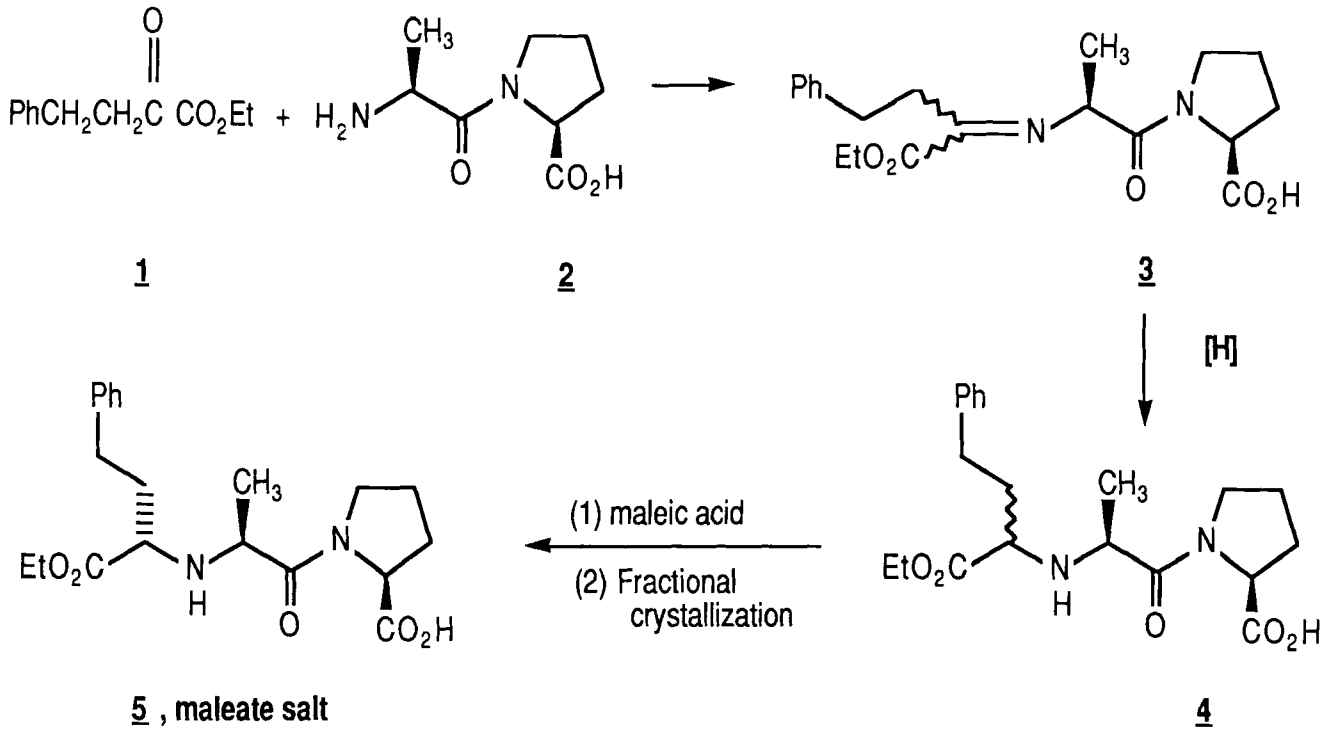


Figure 1. Synthesis of Enalapril Maleate

by fractional crystallization of the maleate salts to yield enalapril maleate (5) of greater than 99% purity in 32 - 34% overall yield. In addition to this synthetic route, others have also been described in the literature (1, 8, 9).

4. Physical Properties

4.1 Infrared Spectrum

The infrared spectrum is shown in Figure 2 (10). The spectrum was obtained as a potassium bromide pellet using a Perkin-Elmer Model 281B spectrophotometer. Assignments for the characteristic bands in the spectrum are listed in Table I.

Table I

Wavenumber, cm^{-1}	Assignment
3100-3010	Aromatic C-H Stretch
2970 and 2920	Methyl and methylene C-H asymmetric stretch
2800-2200 (Broad, structured)	$\rangle NH_2^+$ Stretch
1750	Acid carbonyl stretch
1727	Ester carbonyl stretch
1645	Amide carbonyl stretch
1570	Monohydrogen maleate carboxyl stretch
1500-1390	Mainly methylene bending
1375	Methyl symmetric bending
1355	Methylene bending
1187	Ester C-O stretch
750	In-phase, out-of-plane hydrogen bending (5-adjacent aromatic hydrogens)
695	Phenyl out-of-plane ring bending

4.2 ^{1}H-Nuclear Magnetic Resonance Spectrum

The proton magnetic resonance spectrum is shown in Figure 3 (11). The spectrum was obtained using a Varian Associates Model SC300 spectrometer at a concentration of 0.55% w/v in deuterium oxide (D_2O). Active protons exchange with D_2O in

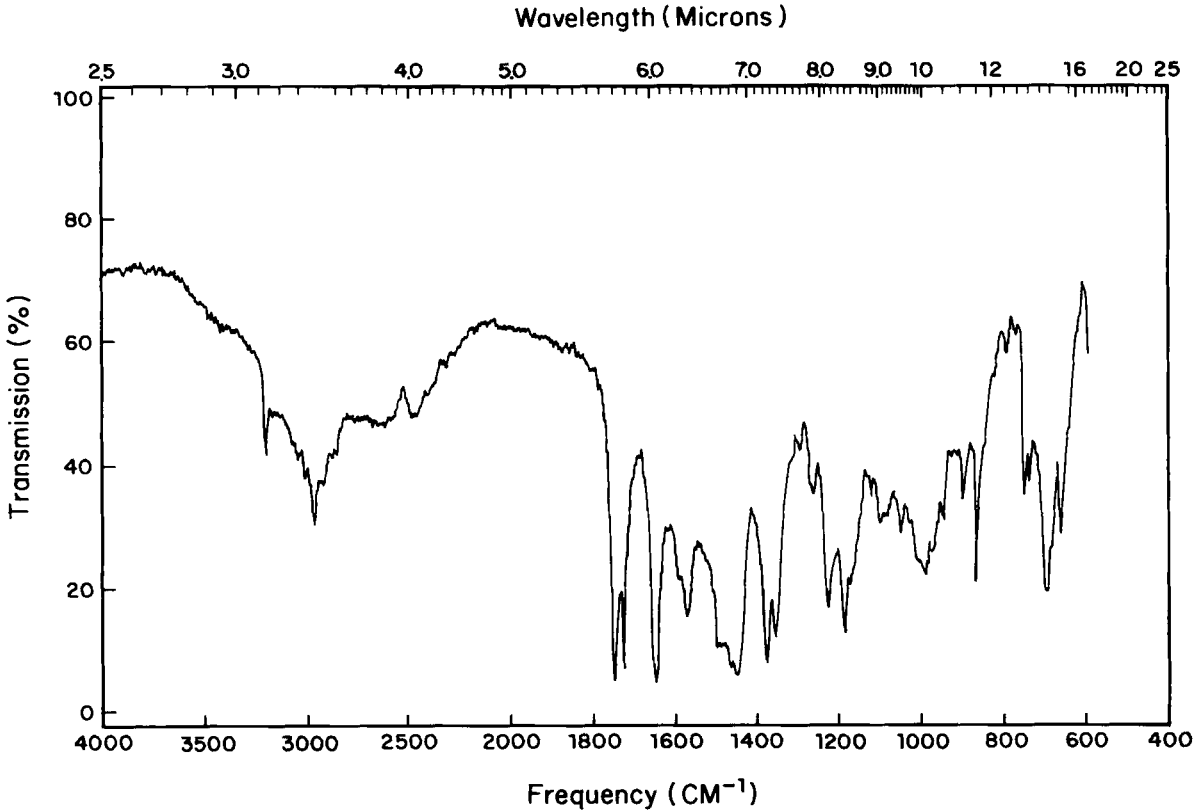


Figure 2. Infrared Absorption Spectrum of Enalapril Maleate

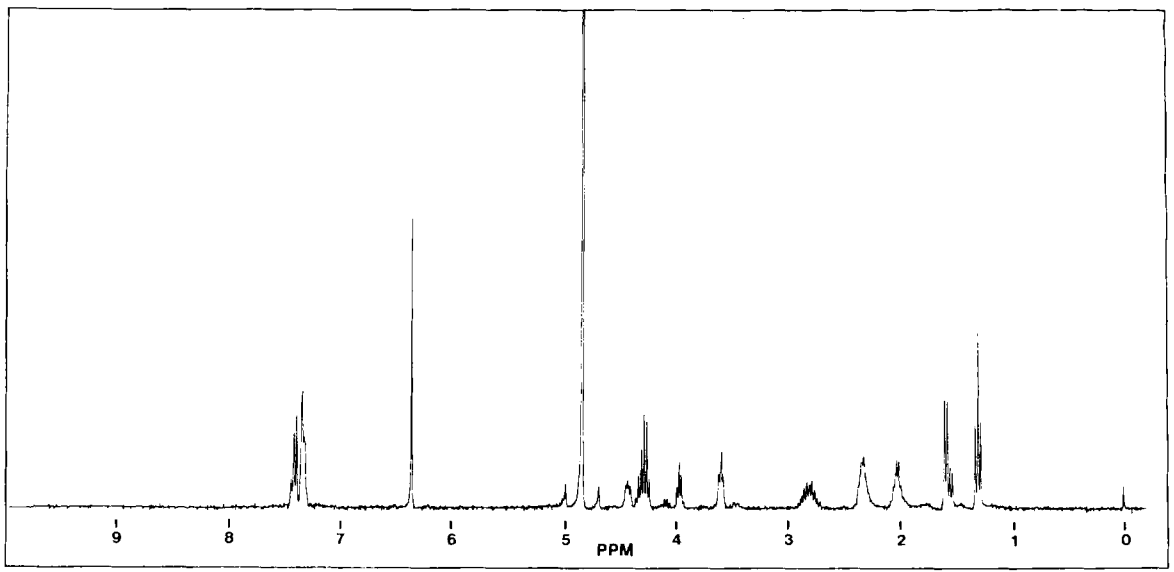


Figure 3. The Proton Magnetic Resonance Spectrum of Enalapril Maleate

solution and appear in the HOD signal at approximately 4.8-4.9 ppm. Figure 4 shows an expanded scale spectrum which more clearly illustrates the signals pattern.

For several protons in the molecule, slow rotation about the proline amide C-N bond gives rise to paired signals. The phenomena has been attributed to the existence of rotational conformers as in the case of captopril in aqueous solution (12). A tabulation of the chemical shifts and assignments for the numbered structure below is shown in Table II. The notations 'major' and 'minor' denote the relative abundance of the two rotamers relative to each other in the spectrum.

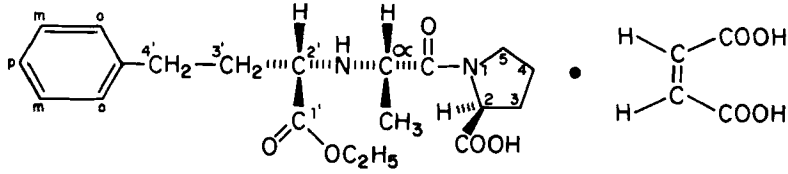


Table II

^{1}H, δ (ppm)	Multiplicity	Assignment
1.30	t	CH_3CH_2O-
1.54	d	Alanyl CH_3 (minor)
1.59	d	Alanyl CH_3 (major)
1.75	m	Proline C-4 proton (minor)
2.02	m	Proline C-4 proton (major)
2.02	m	Proline C-3 protons (minor)
2.33	m	Proline C-3 protons (major)
2.33	m	C-3' protons
2.80	m	C-4' protons
3.60	t	Proline C-5 protons
3.95	t	C-2' proton (minor)
3.97	t	C-2' proton (major)
4.27	q	CH_3CH_2O-
4.09	q	C_α proton (minor)

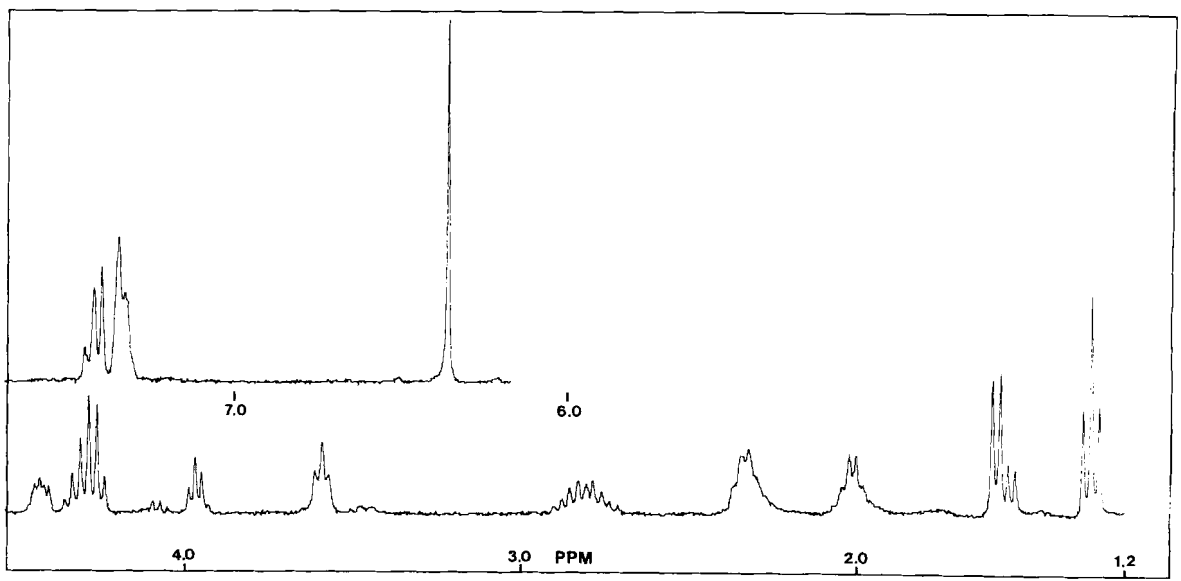
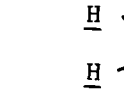


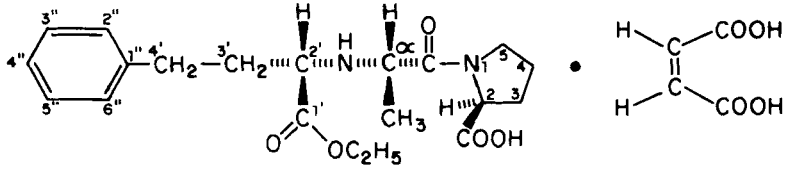
Figure 4. The Proton Magnetic Resonance Expanded Spectrum of Enalapril Maleate

Chemical Shift ^1H, δ (ppm)	Multiplicity	Assignment
4.33	q	C_α proton (major)
6.33	s	
7.37	m	Aryl protons - m
7.41	m	Aryl protons - o & p

4.3 ^{13}C Nuclear Magnetic Resonance Spectrum

The ^{13}C magnetic resonance spectrum shown in Figure 5 was run using a Varian Associate Model XL-200 spectrometer at a concentration of 8.4% w/v in deuterated methanol (13). The internal standard was tetramethylsilane. The pulse width was 4 μs, acquisition time was 0.9 s.

The assignments for the numbered structure below are shown in Table III. As discussed above, the two rotamers are designated 'major' and 'minor' in order of their relative abundances.



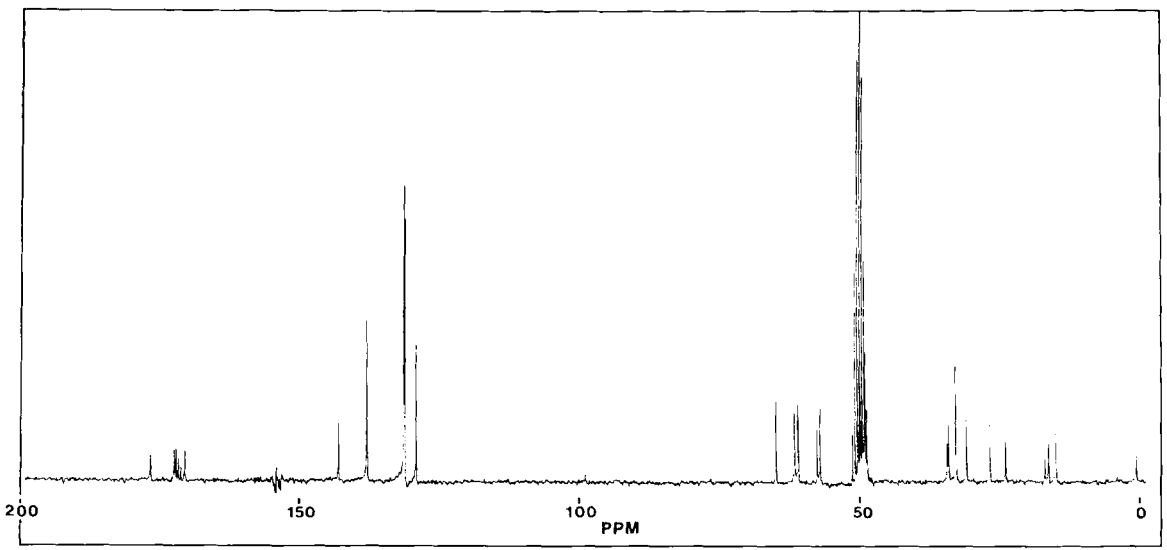


Figure 5. The Carbon-13 Magnetic Resonance Spectrum of Enalapril Maleate

Table III

^{13}C, Chemical δ (ppm)	Assignment
16.2	$-OCH_2\underline{C}H_3$
17.4	Alanyl $\underline{C}H_3$ (major)
18.1	Alanyl $\underline{C}H_3$ (minor)
24.9	The signals at 24.9-35.2 ppm
27.7	arise from the four carbons -
31.8	C-3', C-4' and C-4, C-3
33.7	proline including signals
35.0	corresponding to rotamers. An
35.2	exact assignment of the signals in this region was not made.
49.8	Proline C_5
57.9	The signals at 57.9-65.7 ppm
58.4	arise from the four carbons -
61.7	Proline C-2', Alanyl C-α, C-2'
61.8	and $-O\underline{C}H_2CH_3$ including
62.3	signals attributable to
62.4	rotamers. An exact assignment
65.6	of the signals in this region
65.7	was not made.
129.3	Phenyl C-4"
131.2	Phenyl C-2", C-6", and phenyl
131.4	C-3", C-5"
138.0	Maleate $-H\underline{C}=\underline{C}H-$
142.9	Phenyl C-1"
170.5	The signals at 170.5-176.7
171.2	arise from alanyl, proline,
171.6	ester and maleate carbonyl
172.0	carbons. However, definitive
172.4	assignments were not made
176.5	for the carbonyl carbons.
176.7	

Carbon-13 NMR may also distinguish enalapril maleate and its RSS diastereomer. Major differences between ^{13}C spectra of the two compounds occur between 31.8 and 34.0 ppm, between 54.7 and 60.8

ppm and between 168.6 and 170.7 ppm. However, this technique is not preferred for accurate measurement of small quantities of either compound in the other.

4.4 Ultraviolet Spectrum

Enalapril maleate is characterized by inflections or "shoulders" at about 251 and 263 nm and barely discernible maxima at about 257 nm ($A^{1\%}_{1\,cm}$, about 15) and about 267 nm ($A^{1\%}_{1\,cm}$, about 8.6). Figure 6 shows the ultraviolet absorption spectra of enalapril maleate in N/10 methanolic hydrochloric acid at concentrations of 0.991 mg/ml and 0.0991 mg/ml.

The low intensity and lack of well-defined maxima in the ultraviolet absorption spectrum of enalapril maleate, typical of an unconjugated phenyl moiety, does not make it useful for characterization or quantitation of the compound.

4.5 Mass Spectrum

The direct probe electron impact (EI) mass spectrum of enalapril maleate as shown in Figure 7 was obtained on the VG MM7035 mass spectrometer employing 70 eV electron energy and a probe temperature of 160°C (14).

Under the condition of analysis the maleic acid moiety is pumped off before the peptide derivative is vaporized. Molecular ion information for enalapril base is also absent in the spectrum obtained by direct probe EI at 70 eV and 20 eV, respectively. Such information is only possible when a gross sample overload is employed. Under field desorption (FD)/MS however, good molecular ion and pseudomolecular ion $[M+H]^+$ formation (376, 377) is observed (Figure 8).

The low resolution EI 70 eV spectrum (Figure 7) shows a base peak at m/e at 91 (benzylic ion), $[M-H_2O]^+$ at m/e 358 and other major fragment ions. Accurate mass measurements (high resolution, 70 eV, 160°C) of the diagnostic fragment ions at m/e 358, 313, 285, 257, 254, 234, 208,

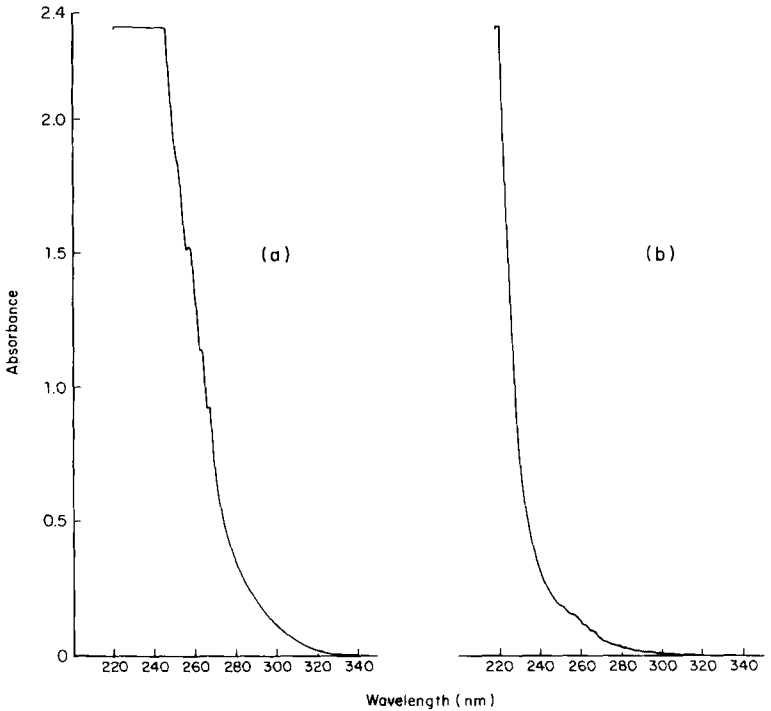


Figure 6. The Ultraviolet Absorption Spectrum of Enalapril Maleate in (a) N/10 Methanolic Hydrochloric Acid; Concentration: 0.991 mg/ml and (b) N/10 Methanolic Hydrochloric Acid; Concentration: 0.0991 mg/ml

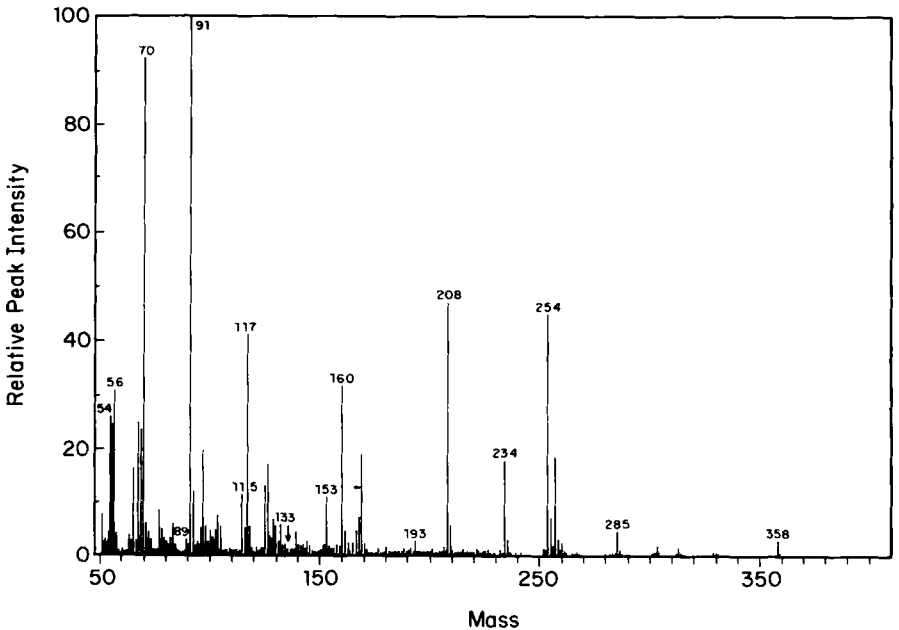


Figure 7. The Low Resolution (EI) Mass Spectrum of Enalapril Maleate

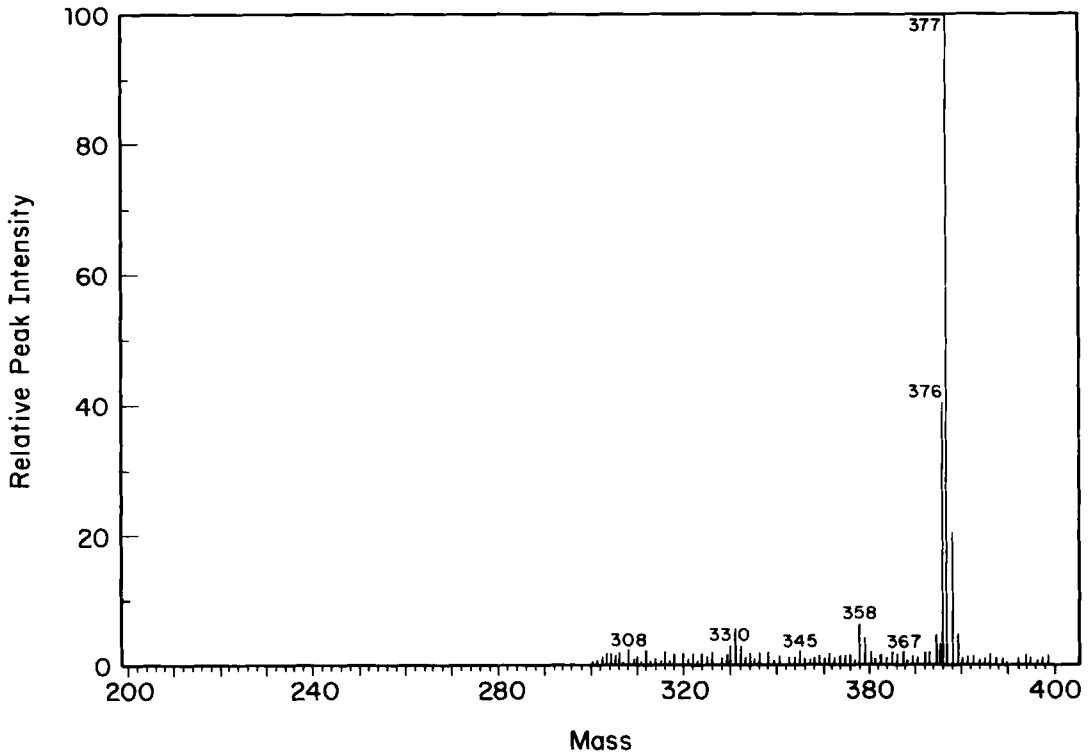


Figure 8. The Field Desorption (FD) Mass Spectrum of Enalapril Maleate

169, 168, 160, 126, 91 and 70 facilitated a proposed fragmention pattern as shown in Figure 9.

4.6 Optical Rotation

The optical rotation, $[\alpha]_D^{25}$, of enalapril maleate (three asymmetric centers) in methanol is, -42.3° (c=1%).

4.7 Thermal Behavior

Enalapril maleate exhibits a single melting endotherm by differential thermal analysis (DTA) under a stream of nitrogen. The peak temperature of the endotherm varies with heating rate, indicating accompanying decompostion during melting of the compound (~ 140°C at 2°C/min. and ~ 149°C at 20°C/min.) A DTA curve for enalapril maleate obtained on a DuPont 1090 equipped with a DTA head is given in Figure 10.

By capillary melting point determination (USP Class Ia method) enalapril maleate melts at ~ 143-144°C (15).

4.8 Solubility

The following approximate solubility data (Table IV) have been obtained at ambient temperature.

Table IV

Solvent	Solubility (mg/ml)
Water	25
Methanol	200
Ethanol	80
Isopropanol	1.4
Acetone	9.1
Acetonitrile	3.3
Dimethylformamide	>400
Chloroform	0.6
Diethyl ether	< 0.1
Hexane	< 0.1
Polyethylene glycol 300	< 0.1

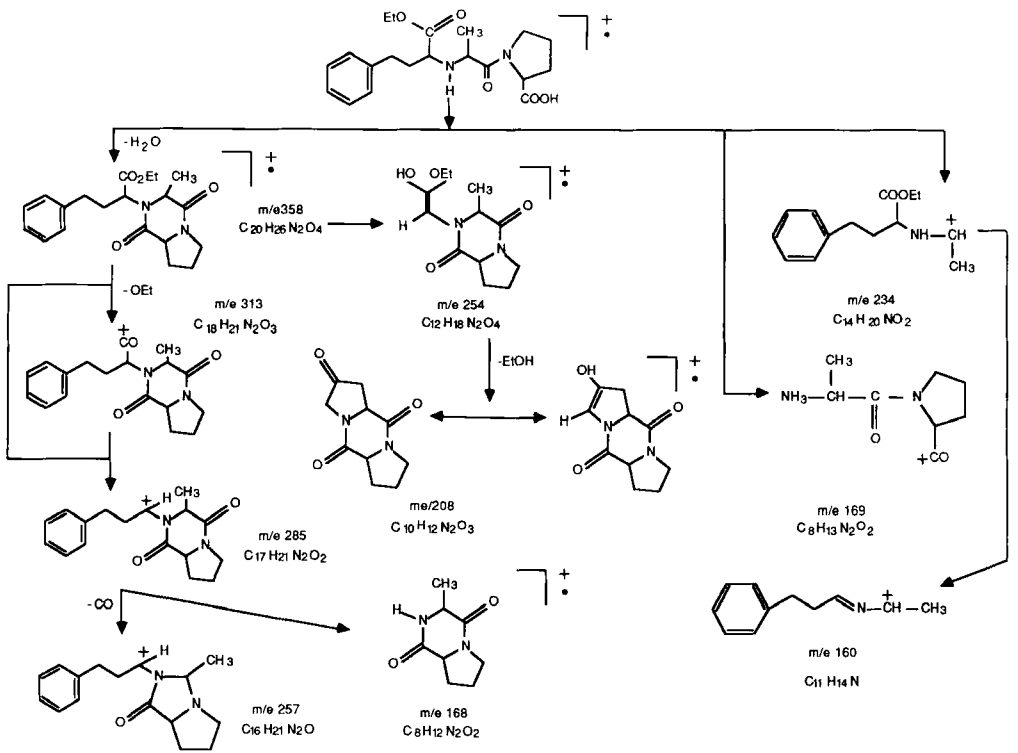


Figure 9. A Proposed Fragmentation Pattern to Explain the Mass Spectrum of Enalapril Maleate

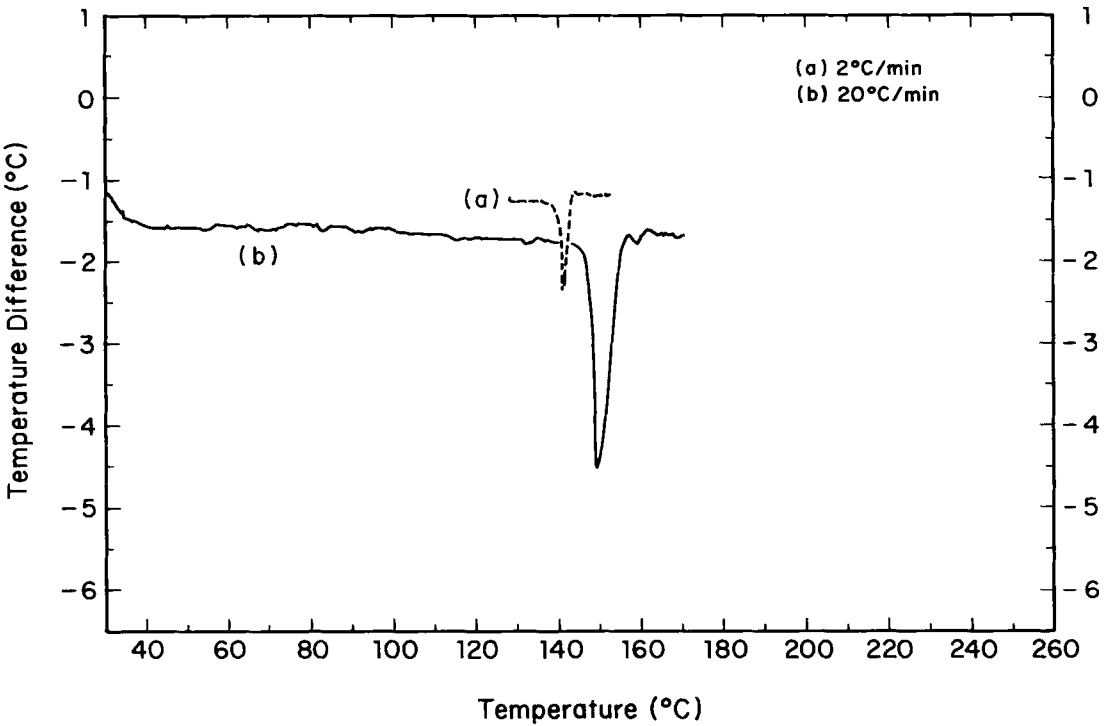


Figure 10. DTA Thermal Curve of Enalapril Maleate

The solubility of enalapril maleate increases with pH. A pH versus solubility profile constructed to compare theoretical points to actual data is shown in Figure 11 (16).

4.9 Crystal Properties

Enalapril maleate is a white to off-white crystalline compound. The compound is polymorphic. Two nonsolvated polylmorphs have been detected and characterized by high resolution spectroscopic techniques (FTIR, Raman, solid state ^{13}C NMR) and solution calorimetry (17). However, these polymorphs, referred to as Form I and Form II are very similar in energy, differing only by 0.6 Kcal/mol. The X-ray powder diffraction spectra for Form I and Form II of enalapril maleate are shown in Figures 12 and 13, respectively. The spectra were obtained using a Philips Electronics X-ray diffractometer using CuKα irradiation.

4.10 Dissociation Constants

Aqueous acidic/basic potentiometric titration yielded pKa values of 2.97 and 5.35 at 25°C for enalapril (18).

4.11 Partition Behavior

At room temperature, enalapril maleate does not partition out of the the aqueous media listed below into mineral oil or n-octanol (19).

Solvent Pairs
n-Octanol/pH 7 buffer
mineral oil/pH 3.5 buffer
mineral oil/pH 4.5 buffer
mineral oil/pH 5.5 buffer

4.12 Conformational Isomers

Proton NMR and carbon-13 NMR spectra of enalapril maleate are characterized by several paired signals attributed to slow rotation about the proline amide bond, resulting in a slow equilibrium between two rotational conformers (cis and trans). The two rotamers are distinguishable on the NMR

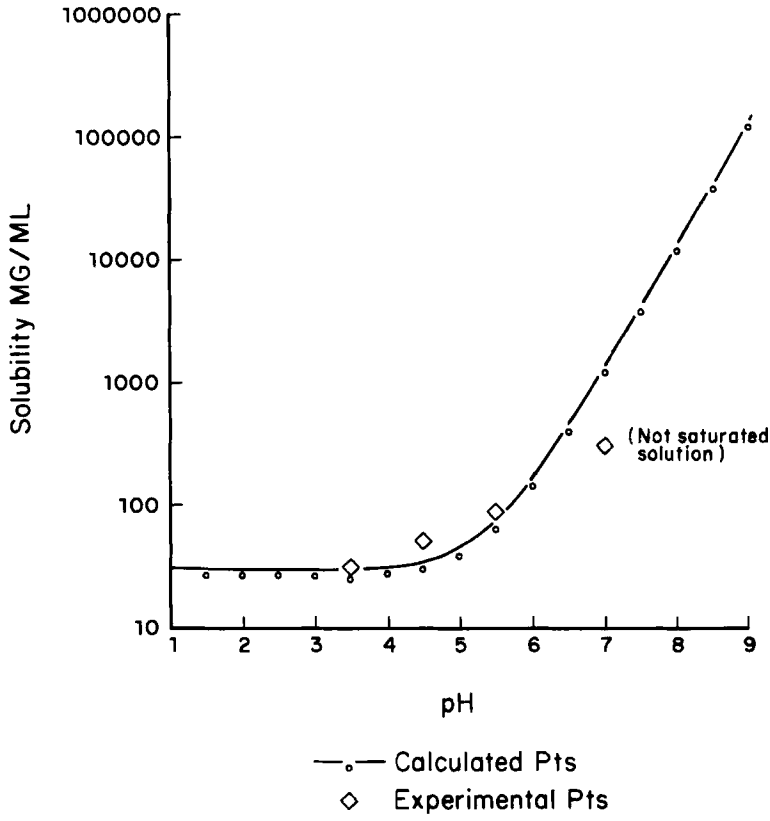


Figure 11. pH - Solubility Profile of Enalapril Maleate

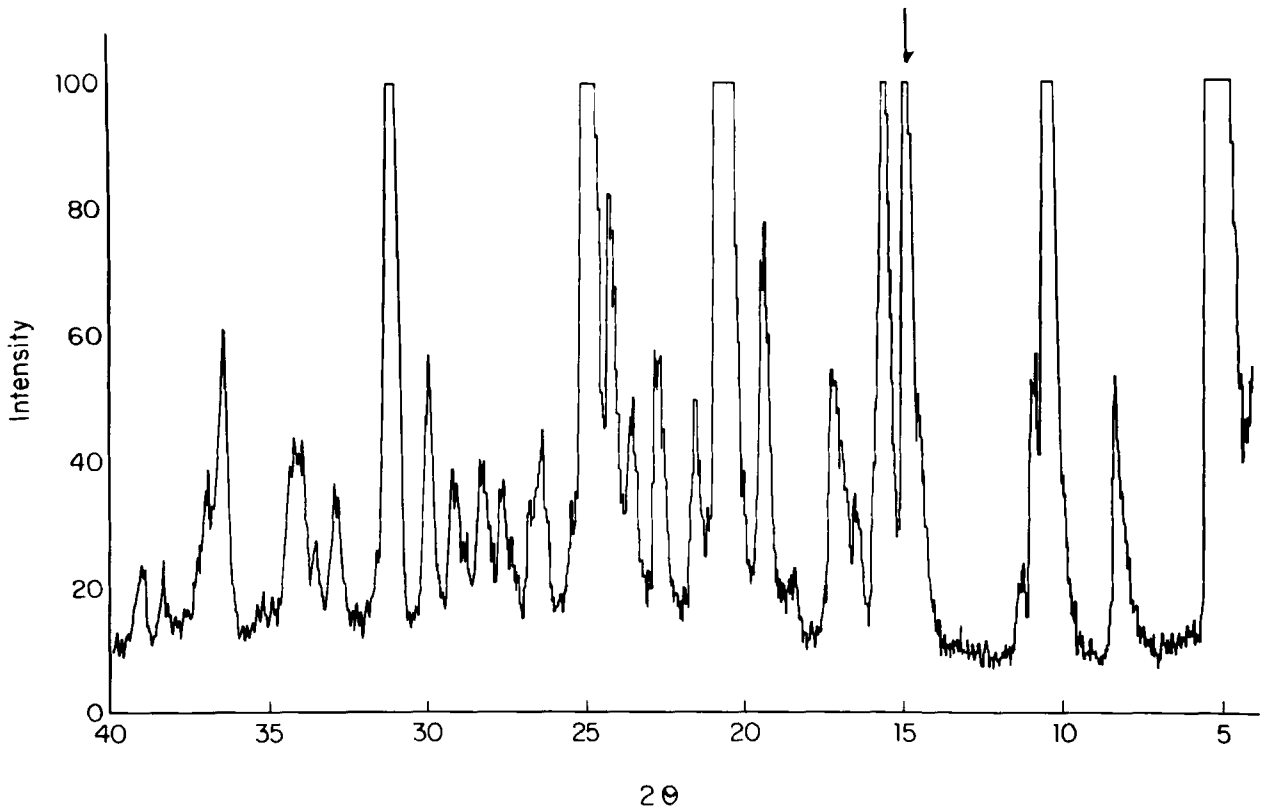


Figure 12. Powder X-Ray Diffraction Pattern of Enalapril Maleate, Form I

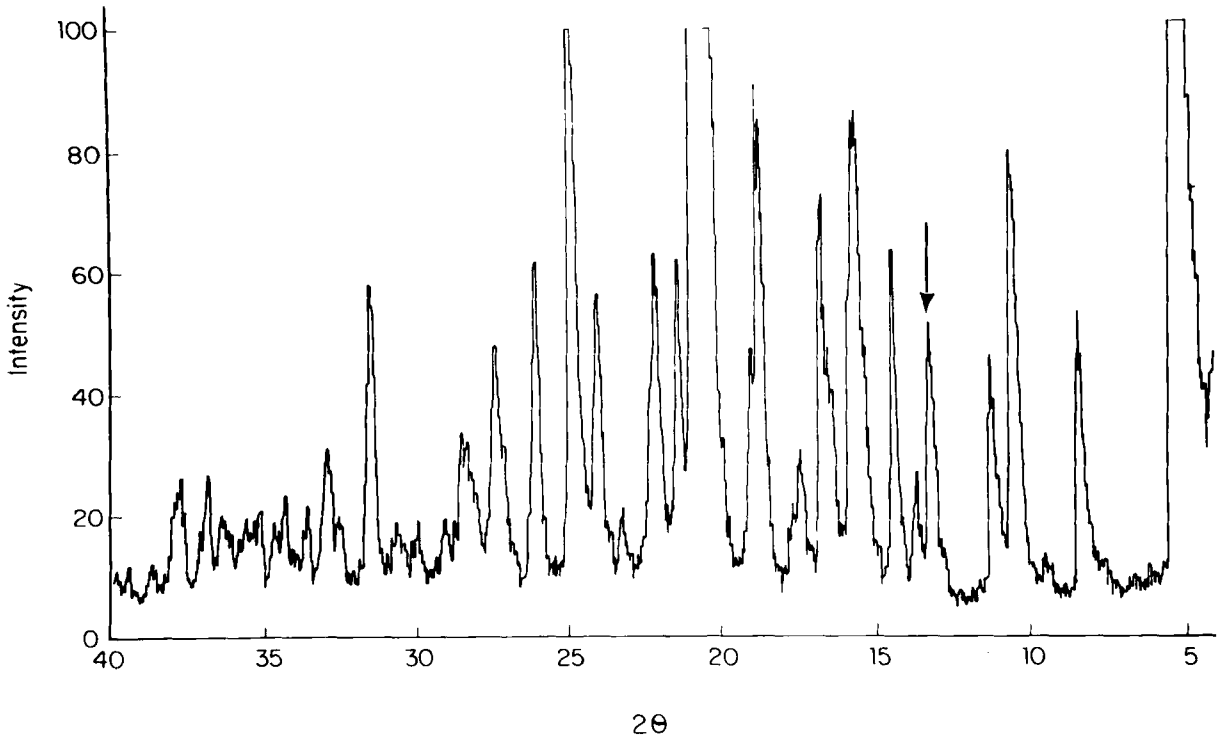


Figure 13. Powder X-Ray Diffraction Pattern of Enalapril Maleate, Form II

time scale. In addition, HPLC has provided firm evidence for their existence by demonstrating the chromatographic behavior of enalapril maleate at various temperatures (20). Figure 14 shows chromatograms obtained by reverse-phase HPLC at column oven temperatures of 10°C and 80°C. At 10°C, in addition to the early eluting maleic acid peak (~ 1.2 minute), the enalapril base peak is resolved into two distinct rotamer peaks. At 80°C, the rotamer peaks have coalesced to yield a single symmetrical peak.

5. Methods of Analysis

5.1 Elemental Analysis

Analysis of Merck Sharp & Dohme reference lot, L-154,739-001D22 for carbon, hydrogen and nitrogen was reported as follows:

Element	% Calculated	% Found
C	58.53	58.53
H	6.55	6.75
N	5.68	5.82

5.2 Colorimetric/Spectrophotometric

Due to the lack of strong UV absorption maxima, more sensitive ways for the analysis of enalapril maleate are needed. One approach has been an autoanalyzer colorimetric assay procedure (21). In this approach enalapril maleate forms a yellow dye complex with bromothymol blue in acidic aqueous, pH 2/$CHCl_3$ mixture and is then assayed spectrophotometrically at 415 nm after extraction with chloroform. This method is suitable for content uniformity and dissolution assays of enalapril maleate capsules and tablets.

Analysis of enalapril in pharmaceutical dosage forms via flow injection analysis has recently been reported (22). Enalapril is determined by extraction as an ion-pair with bromothymol blue in an unsegmented flow system. The procedure is stability indicating; the degradation products of enalapril and excipients in the dosage forms do not interfere.

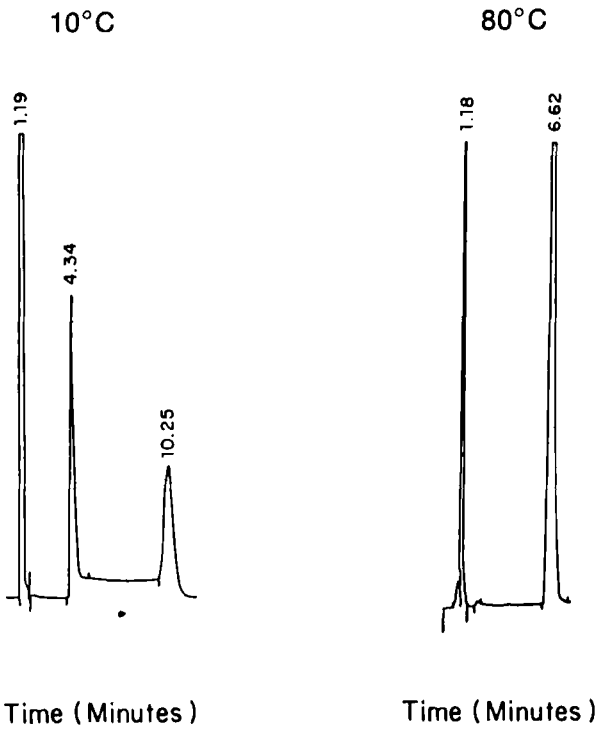


Figure 14. HPLC Chromatograms of Enalapril Maleate at 10° and 80°C

5.3 Chromatographic

5.3.1 Thin-Layer Chromatography

Three solvent systems (23) for the thin-layer chromatography of enalapril maleate are given below. The systems separate enalapril base from its salt forming acid (maleic acid) and its hydrolysis product (enalaprilat - see Section 6.1)

Solvent System	Rf (Approximate)
A (chloroform/methanol/ acetic acid 90:10:1)	0.6 (enalapril base) 0.2 (maleic acid) 0 (enalaprilat)
B (n-Butanol/water/ acetic acid 3:1:1)	0.7 (enalapril base and maleic acid) 0.35 (enalaprilat)

Solvent System	Rf (Approximate)
C (n-Butanol/toluene/ water/acetone/acetic acid 1:1:1:1:1)	0.55 (enalapril base) 0.45 (maleic acid and enalaprilat)

These solvent systems use a silica gel adsorbent containing an ultraviolet-fluorescence quench indicator (Analtech GF or Whatman K1F or E Merck Silica Gel G60 type plates). Detection of components is accomplished by visualization of dried plates under short wavelength (254 nm) ultraviolet light. Iodine vapor yields a more intense spot for the enalapril base but does not visualize the maleic acid.

In the systems reported above, the low sensitivity of the method (detection limit 1 to 2%) renders it unsuitable for the

detection and quantification of low levels of impurities in enalapril maleate bulk substance.

5.3.2 High Performance Liquid Chromatography (HPLC)

Several HPLC methods have been developed which are suitable for: (a) quantification of enalapril maleate and process impurities in the bulk substance (24), (b) analysis of enalapril maleate and degradates in tablets (25), (c) content uniformity assays of enalapril maleate in tablets (26) and (d) dissolution of enalapril maleate in tablets (27). Reverse-phase HPLC conditions are employed. Detection is by uv at 210 or 215 nm. Flow rate is normally 1.8 or 2.0 ml/min. A mobile phase of aqueous phosphate buffer and organic modifier (acetonitrile or a mixture of acetonitrile and methanol) and an oven temperature of 70°C or 80° provide the selectivity and sensitivity required for satisfactory chromatography.
The significance of specifying a high column temperature has been discussed in section 4.12. A summary of these HPLC systems is given in Table V.

5.4 Identification Tests

Identification of enalapril maleate can be carried out by infrared absorption (section 4.1), HPLC (section 5.3.2) and thin-layer chromatography (section 5.3.1).

Supportive evidence for identification can be obtained by differential thermal analysis (section 4.7) and complexation with bromothymol blue (section 5.2).

5.5 Titration

Enalapril maleate can be determined by potentiometric titration with aqueous sodium hydroxide and perchloric acid. With respect to the sodium

Table V

HPLC Systems for Enalapril Maleate

Sample	Column	Mobile Phase	Temperature
Bulk Drug	Ultrasphere C8, 250 x 4.6 mm, 5 μ (Altex)	Isocratic 60% A, 40% B A = 0.01M KH_2PO_4(pH 4.0) B = 80% of 50:50 CH_3CN:CH_3OH + 20% A	70°C
		Gradient 70:30 A:B to 30:70 A:B in 40 min. (linear gradient)	70°C
Tablets	Lichrosorb C8, 200 x 4.6 mm, 10μ (Hewlett Packard)	68% A, 32% B A = 0.001M KH_2PO_4(pH 2.0) B = CH_3CN	80°C
Tablets	Lichrosorb C8, 200 x 4.6 mm, 10μ (Hewlett Packard)	70% A, 30% B A = 0.02M KH_2PO_4(pH 4.0) B = CH_3CN	80°C
Tablets	Endcapped C8 50 x 4.6 mm, 5μ (IBM)	80% A, 20% B A = 0.02M KH_2PO_4(pH 4.0) B = CH_3CN	70°C

hydroxide titration, 0.1N sodium hydroxide is the titrant and a combination pH electrode is employed as the electrode system. The perchloric acid titration employs 0.1N perchloric acid in glacial acetic acid as the titrant and an anhydrous electrode system, e.g. Metrohm #EA 441/5 silver-silver chloride electrode filled with 0.1N lithium perchlorate in glacial acetic acid (or acetic anhydride), versus a glass electrode is used.

6. Stability and Degradation

6.1 Solid State Stability

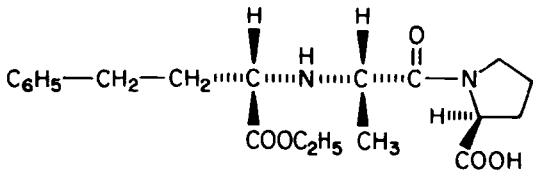
There are two major potential modes of degradation for enalapril maleate: (a) hydrolysis of the ethyl ester to II, enalaprilat, the free acid and (b) cyclization to a diketopiperazine derivative III. The structure of these compounds are shown in Figure 15.

Crystalline enalapril maleate is a very stable solid. The compound stored at room temperature (amber glass) for four years shows no evidence of degradation by HPLC analysis. Less than 2% of degradation can be induced by storage at 80°C for 3 weeks (in screw-cap vials) and < 1% of decomposition at 40°C and 75% RH (open vial) for 16 weeks.

Enalapril maleate tablets are stable when protected from elevated temperatures and high humidity. Degradation can be induced, however, if tablets are stored at 40°C and 75% RH in open containers, resulting in enalapril maleate loss of about 10% or greater for three months. The major degradation product is II. The mass balance between loss of enalapril maleate and formation of II and III has been demonstrated.

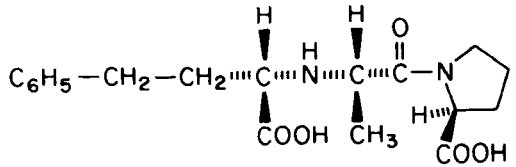
6.2 Solution Stability

The stability of enalapril maleate in aqueous buffer solutions has been studied as a function of pH in the range of 2-7 (28). Maximum solution stability occurred around pH 3. The rate of enalapril loss and the mode of degradation are dependent upon the solution pH. At room temperature, t_{90}



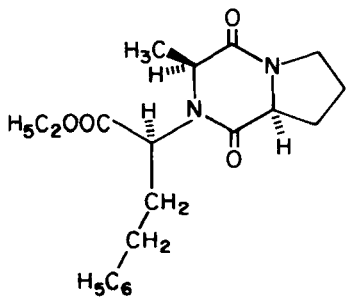
Enalapril (SSS)

(I)



Enalaprilat (SSS)

(II)



(III)

Figure 15. Chemical Structures of Enalapril and Related Compounds

(time required for 10% loss of starting material) values are 262 days and 114 days for 0.5 mg/ml at pH 2 and 5, respectively. For up to 20% loss at pH 2, III was the major degradation product while II represented only a minor fraction of the loss. At pH 3, III was the major degradate but the fraction of II was increased. At pH 5 and above, II accounted for the bulk of enalapril loss with III present only in trace quantities.

7. Pharmacokinetics and Metabolism

The pharmacokinetics and metabolism of enalapril maleate following oral administration have been the subject of a number of investigations and reviews (4, 29-32).

Following oral administration of enalapril maleate to man (33) peak serum concentrations of enalapril and enalaprilat occur within 0.5 to 1.5 and 3 to 4 hours respectively. Based on urinary recovery of the total drug (enalapril plus its active metabolite, enalaprilat) absorption is at least 61%. The primary route of excretion of the drug is renal. Approximately 94% of the dose is recovered in the urine and feces as enalaprilat or enalapril.

Enalapril, when administered orally, is rapidly absorbed and bioactivated extensively to enalaprilat. Bioactivation appears to be largely post-absorptive and there is no evidence for metabolism of enalapril beyond bioactivation to enalaprilat in man. The in vitro conversion of enalapril to enalaprilat in human autopsy tissues has been examined (34). Human cadaver liver was the only tissue in which significant conversion was demonstrated.

Enalapril absorption is not influenced by the presence of food in the gastrointestinal tract (35). The drug was administered to normal volunteers both in the fasting state and with a normal meal. The area under the serum concentration-time curves for enalaprilat and urinary recoveries for enalaprilat and the total drug did not differ significantly between the fed and fasted conditions.

The pharmacokinetics of enalapril maleate was determined in normal volunteers after repeated single daily

doses for eight days (36). Serum profiles show little accumulation of enalprilat over the course of the study. An average effective half-life for accumulation of approximately 11 hours was calculated from urine data. An average steady state recovery of enalaprilat is achieved by the third or fourth dose. The serum concentration profile of enalaprilat is characterized by a prolonged terminal phase, apparently representing a small fraction of the administered dose that has been bound to angiotensin converting enzyme. The amount bound does not increase with dose indicating a saturable site of binding.

8. Determination in Biological Fluids

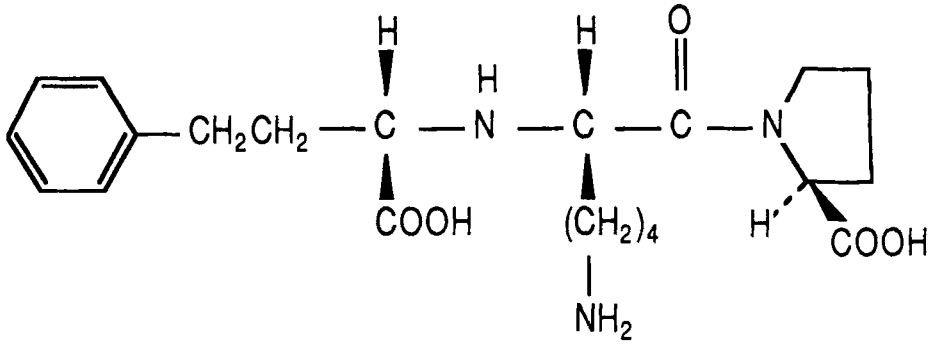
A number of procedures have been developed for the analysis of enalapril and enalaprilat in biological fluids. Those of primary utility include radiometric, enzyme inhibition and radioimmuno-methods.

The physiological disposition and metabolism of enalapril maleate in laboratory animals has been studied with both radiolabeled drug and an enzyme-inhibition assay (37). Following administration of the radioactive drug; urine, feces or plasma samples may be counted by liquid scintillation techniques for total radioactivity. Urinary radioactivity may be separated into enalapril and enalaprilat by thin-layer chromatography.

Enalaprilat can also be determined by its in vitro inhibition of angiotensin converting enzyme (37). Plasma, urine and homogenates of feces are placed on XAD-4 columns, washed and then eluted with methanol to yield enalapril and enalaprilat. The enalaprilat component of the mixture is determined by the enzyme assay which uses as a substrate, the tripeptide carbobenzoxycarbonyl-phenylalanyl-histidyl leucine and converting enzyme isolated from porcine plasma. The product, histidyl-leucine is then quantified by fluorescence after reaction with o-phthalaldehyde (38). For the determination of the total drug (enalapril plus enalaprilat) samples are hydrolyzed at high pH and then reassayed for enalaprilat. Enalapril levels may then be calculated as the difference between the total drug and enalaprilat prior to hydrolysis. An enzyme inhibition assay for enalaprilat has also been developed

which uses radiolabeled substrate (39) thereby eliminating derivatization with o-phthalaldehyde and fluorescence detection of the enzyme products. The radiolabeled substrate employed is [^{3}H] hippuryl-L-glycyl-L-glycine. The product, [^{3}H] hippuric acid is extracted into a water-immiscible scintillation cocktail and then quantified by counting in a liquid scintillation spectrometer.

Enalaprilat has also been determined in serum and urine samples by radioimmunassay procedures. (40, 41). In one such procedure (40) antibodies for the radioimmunoassay are raised in rabbits using an immunogen prepared by coupling the lysine analog of enalaprilat (lisinopril) to albumin with difluoro-dinitrobenzene. The radiolabeled component is obtained by iodination of the



Lisinopril

amidine derived from the reaction of lisinopril and methyl p-hydroxybenzimidate. The total drug is measured as enalaprilat after sample hydrolysis by rat liver homogenate. Enalapril can then be calculated as the difference between enalaprilat before and after hydrolysis.

Acknowledgements

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9. References

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HOMATROPINE HYDROBROMIDE

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1. Description

1.1 Nomenclature

1.1.1 Chemical Names

- α- Hydroxybenzene acetic acid 8-methyl-8-azabicyclo [3.2.1] oct-3-yl ester, hydrobromide.
- 1 α H, 5αH-tropane-3 α -ol mandelate (ester) hydrobromide.
- Benzeneacetic acid, α-hydroxy-, 8-methyl-8-azabicyclo [3.2.1] oct-3 yl ester hydrobromide, endo-(±)-.

1.1.2 Generic Names

Homatropine Hydrobromide, DL-Homatropine Hydrobromide, The hydrobromide of tropine mandelate, Tropine mandelate Hydrobromide, Mandelyltropine Hydrobromide.

1.1.3 Trade Names

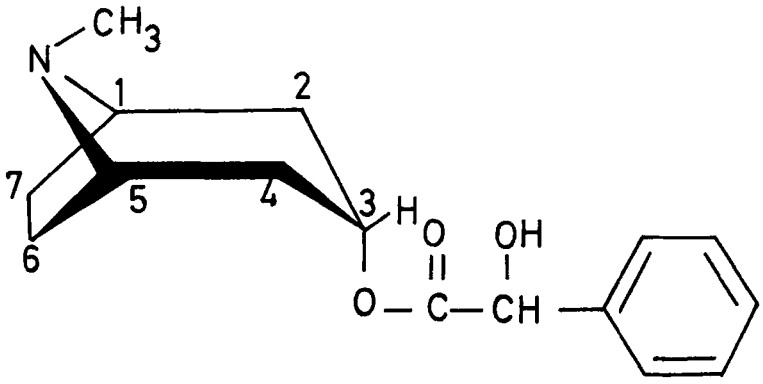
Homatrisol, Bufopto, Homatrocel.

1.2 Formulae

1.2.1 Empirical

$C_{16}H_{21}O_3N$. HBr

1.2.2 Structural



1.2.3 CAS Registry No.

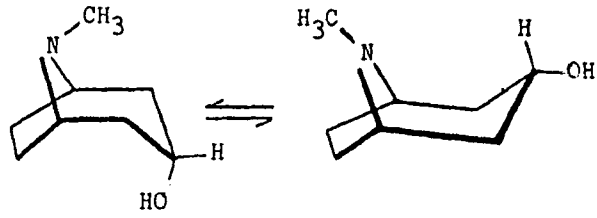
[51-56-9] $C_{16}H_{21}O_3N$. H Br

1.2.4 Wiswesser Line Notation

T56 A ANTJ A _GOVYGR & EH (1)

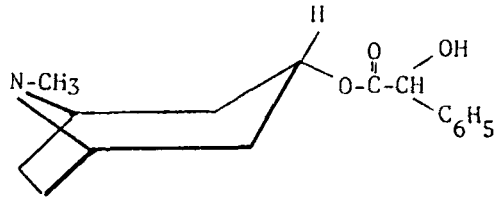
1.2.5 Stereochemistry

Examination of the NMR spectra of some tropane deuterohalides has shown that the N-substituent in tropanes is predominantly equatorial (2). X-ray analysis of tropine hydrobromide has shown the presence of chair conformation (3). Study of the dipole-moment and Kerr-constant measurements of a number of tropane derivatives has shown that the piperidine ring is in the chair form with the N-methyl equatorial (4). Another study of the dipole-moments and NMR spectra of some tropane derivatives have confirmed that the piperidine ring is in the chair conformation with the N-methyl group predominantly equatorial (5). In tropine, however, the predominant conformation is the piperidine ring in a deformed chair form together with a minor amount in the boat form (6).



Tropine

In atropine, the α-3-substituent is of greater bulk than the hydroxyl, and the boat form may will be favored because of the increased interactions involving the dimethylene bridge in the chair conformation (7).
Homatropine would be the same as atropine.

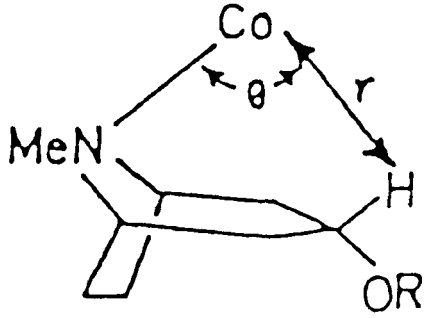


A detailed review is available for the boat or chair conformation in tropines (8).

Other PMR study suggested a preference for the boat conformation in several tropane derivatives. This study showed strong cross-ring intramolecular interactions of the type N---C=O and N---H-O were indicated by the broadening of the proton signal due to the coupling between 1(5)-H and 2(4)-H protons in the boat conformer compared with the chair. This broadening arises as a consequence of eclipsing of these protons in the boat conformer (9).

Application of ^{1}H-NMR contact shifts using nickel and cobalt diacetylacetonates, to tropines has demonstrated that the six-membered ring of tropines is in a nearly semiplaner form. It is suggested that the piperidine ring of tropanes is somewhat different in conformation from the normal chair form (10).

Carbon-13 magnetic resonance study has also suggested a non-chair conformations in tropane derivatives (11).



1.3 Molecular Weight

275.33 (Homatropine)
356.3 (Homatropine HBr)

1.4 Elemental Composition

C, 69.79%; H, 7.69%; N, 5.09%; O, 17.43% (Homatropine).

C, 53.94%; H, 6.22%; Br, 22.44%; N, 3.93%; O, 13.47% (Homatropine HBr).

1.5 Appearance, Color, Odor and Taste

Colorless crystals or a white crystalline powder, odorless, with a bitter taste (12).

Orthorhombic, bipyramidal prisms (from water), odorless (13).

1.6 Dissociation Constant

pKa 9.9(20°) (14)

1.7 pH Range

A 2% solution in water has a pH 5.5 to 7 (12), a 5.67% solution in water is iso-osmotic with serum. pH (1% aqueous solution) : 5.4 (13).

2. Physical Properties

2.1 Melting Point

214 - 217° (with decomposition) (12)
Other melting points were also reported:
215 (with decomposition) (15)
217-218 (with decomposition) (1)

2.2 Solubility

One gram dissolves in 6 ml water, 40 ml alcohol, 12 ml alcohol at 60°, 420 ml chloroform. Insoluble in ether. The solubility increases rapidly as the temperature rises (12).

2.3 Crystal Structure

The crystal structure of both homatropine and homatropine hydrobromide were reported (16). Crystals of homatropine were obtained by recrystallization from ethanol, whereas the crystals of homatropine hydrobromide ($C_{16}H_{21}NO_3$. HBr) were obtained by recrystal-

lization from water.
Oscillation and Weissenberg photographs were used to determine the space group (Table 1).
In both homatropine and homatropine hydrobromide the nitrogen atom may be optically active, since the space groups are centrosymmetric, the unit cells must be assumed to contain equal numbers of L-and D-molecules (16).

Table 1. The Crystal Structure of Homatropine and of Homatropine HBr.

Compound	a(A°)	b(A°)	c(A°)	Density ($g.cm^{-3}$) observed	Density ($g.cm^{-3}$) calculated	Z	Space group
Homatropine	14.5	15.1	6.97	1.21	1.22	4	$P2_1/c$
Homatropine HBr	10.3	16.4	19.25	1.48	1.46	8	$Pcab$

2.4 X-ray Powder Diffraction

The X-ray diffraction pattern of homatropine hydrobromide was determined with a Philips X-ray diffraction spectrogoniometer equipped with PW 1730 generator.
X-ray radiation was provided by a copper target (Cu-anode 2000 W). High intensity X-ray tube operated at 40 KV and 20 mA. The monochromator was a single curved crystal (γ=1.5918A°). The unit was equipped with Philips PM 8210 printing recorded digital printer. Divergance slit and the receiving slit were 1°. The scanning speed of the goniometer used was 0.02 2θ/sec., recorder full scale was 10,000 counts at recorder speed of 4 mm/2θ. The lower level and the upper level of the signal control was 35 & 75 respectively.
The unit was aligned and examined, using a Solicon standard, to attain maximum operation conditions. The X-ray pattern of homatropine hydrobromide is presented in Fig. 1. Interplannar distance and relative intensity are tabulated in table 2.

Table 2 : X-Ray Powder Diffraction Pattern of Homatropine Hydrobromide

d (A°)	I/Io	d (A°)	I/Io
6.98	17.8%	2.87	11.7%
6.41	17.3%	2.83	24.1%
6.00	22.1%	2.72	19.5%
5.15	15.7%	2.64	10.7%
5.04	21.1%	2.61	14.2%
4.84	73.5%	2.58	20.0%
4.64	12.6%	2.52	22.9%
4.48	14.4%	2.50	12.1%
4.35	39.0%	2.42	22.0%
4.28	85.9%	2.35	13.5%
4.19	100.0%	2.32	25.9%
3.96	36.3%	2.29	13.7%
3.57	48.7%	2.26	13.1%
3.50	46.1%	2.15	11.9%
3.43	11.2%	2.07	11.3%
3.30	38.5%	2.03	16.4%
3.23	21.3%	1.98	10.7%
3.17	20.2%	1.93	13.0%
3.12	22.7%	1.87	11.2%
3.07	20.7%	1.80	11.0%
3.00	20.8%	1.77	13.5%
2.97	17.8%		

d = interplanar distance, I/I_o = relative intensity (based on the highest intensity of 100).

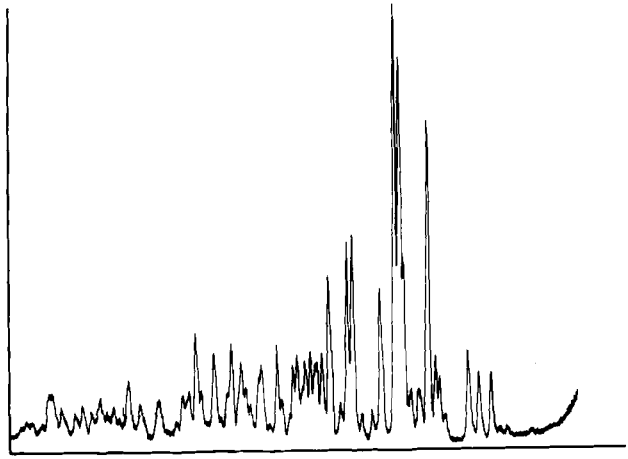


Fig. 1 : X-RAY DIFFRACTION PATTERN OF HOMATROPINE HYDROBROMIDE.

2.5 Spectral Properties

2.5.1 Ultraviolet Spectrum

The UV spectrum of homatropine hydrobromide in methanol (Fig. 2) was scanned from 200 to 350 nm using DMS 90 Varian Spectrophotometer. It exhibits the following UV data (Table 3).

Table 3. UV Characteristics of Homatropine

λmax. at nm	ε	(A 1%, 1cm)
252	224.28	6.3
258	267	7.5
264.5	228.5	6.42
273	78.32	2.2

Other reported UV spectral data for homatropine in 0.1 N sulfuric acid (17).

λmax. at 252 mμ (E 1%, 1 cm 4.8), 258 mμ (E 1%, 1 cm 6.1) and 264 mμ (E 1%, 1 cm 5).

λmax. in 0.1 N hydrochloric acid at 254 nm (E 1%, 1 cm = 5); at 259 nm (E 1%, 1 cm =5.2) and 266 nm. (18).

2.5.2 Infrared Spectrum

The IR spectrum of homatropine hydrobromide as KBr-disc was recorded on a Perkin Elmer 580 B Infrared Spectrometer to which infrared data station is attached (Fig. 3).
The structural assignment have been correlated with the following frequencies (Table 4).

Table 4. IR Characteristic of Homatropine

Frequency cm^{-1}		Assignment
Base	HBr	
3350	3270	OH (stretch)
2940,3050	2965	CH (stretch)
-	2800-2585	$\overset{+}{N}H$
1735	1755	$-O-\overset{O}{\overset{\|}{C}}-$(ester)

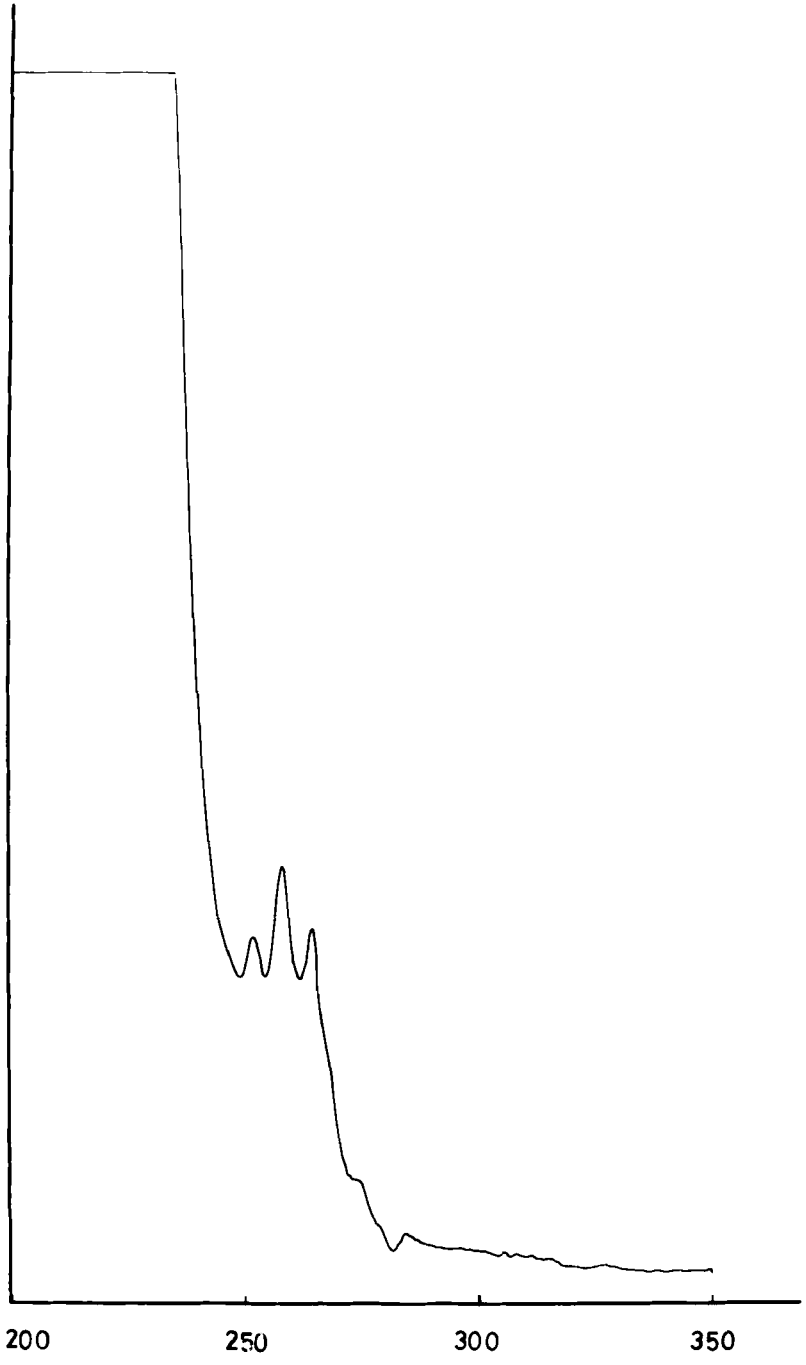


Fig. 2 : THE UV SPECTRUM OF HOMATROPINE HYDROBROMIDE IN METHANOL

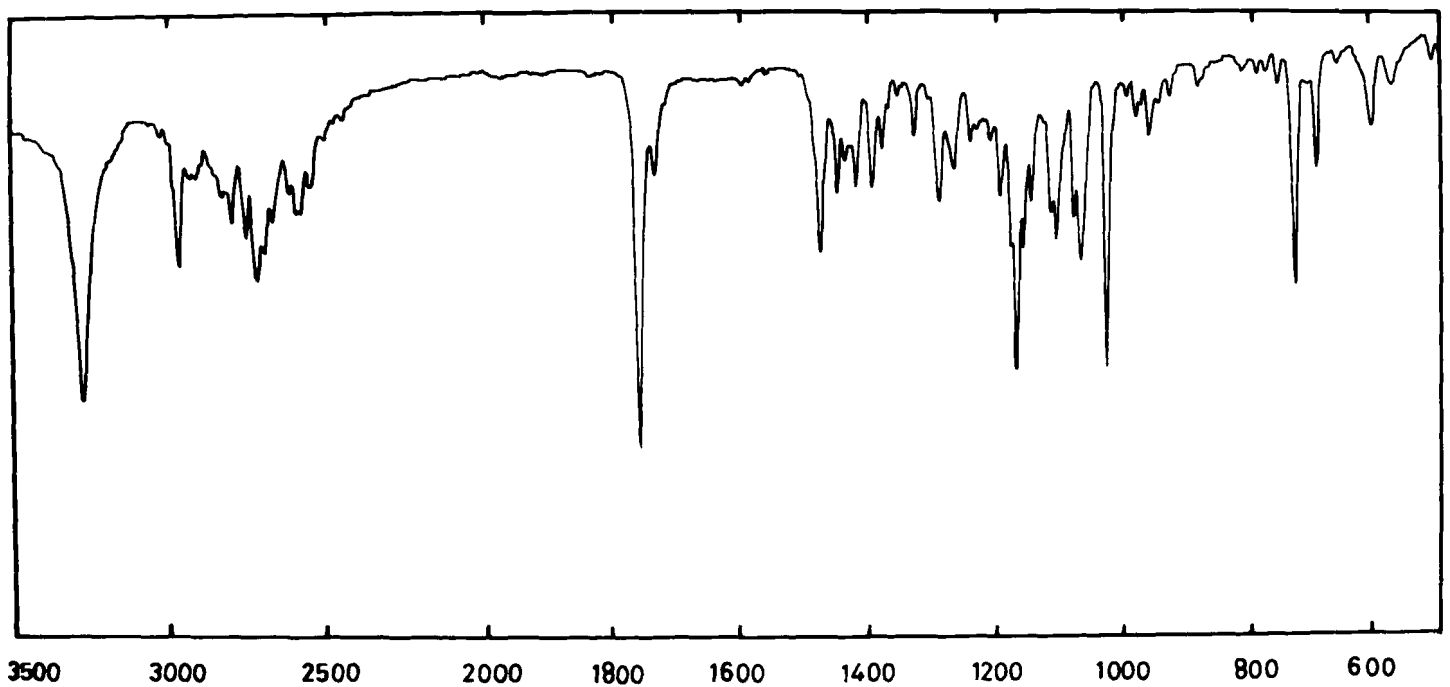


Fig. 3 : THE IR SPECTRUM OF HOMATROPINE HYDROBROMIDE AS KBr DISC.

Frequency cm^{-1}		Assignment
Base	HBr	
1600	1600	C=C (aromatic)
1450,1500	1475	C=C (aromatic)
1100,1065	1170,1070	C-O-C (ether)
755,700	735,700	5 H (monosubstituted aromatic)

Other characteristic bands are 2750, 2710, 1450, 1420, 1395, 1378, 1328, 1288, 1265, 1195, 1105, 1025, 960, 885, 820 and 615 cm^{-1}.
Other IR data for homatropine and the hydrobromide salt have been reported (1,14,17).

2.5.3 Nuclear Magnetic Resonance

2.5.3.1 ^{1}H-NMR Spectra

The ^{1}H-NMR spectra of homatropine hydrobromide in DMSO-D_6 and in D_2O were recorded on a Varian T-60A, 60 MHz NMR spectrometer using TMS (tetramethylsilane) as an internal reference. These are shown in Fig. 4 and 5 respectively. The following structural assignment have been made (Table 5).

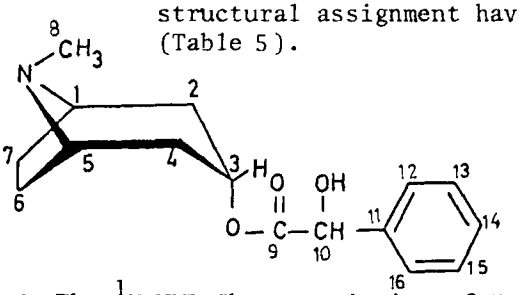


Table 5. The ^{1}H-NMR Characteristics of Homatropine

Group	Chemical Shift (pmm) DMSO-d_6	D_2O
5 aromatic protons (12,13,14,15,16 H)	7.33 (s)	7.4 (s)
10 H (OH)	6.03 (d)	
10 H	5.13 (d)	5.33 (s)

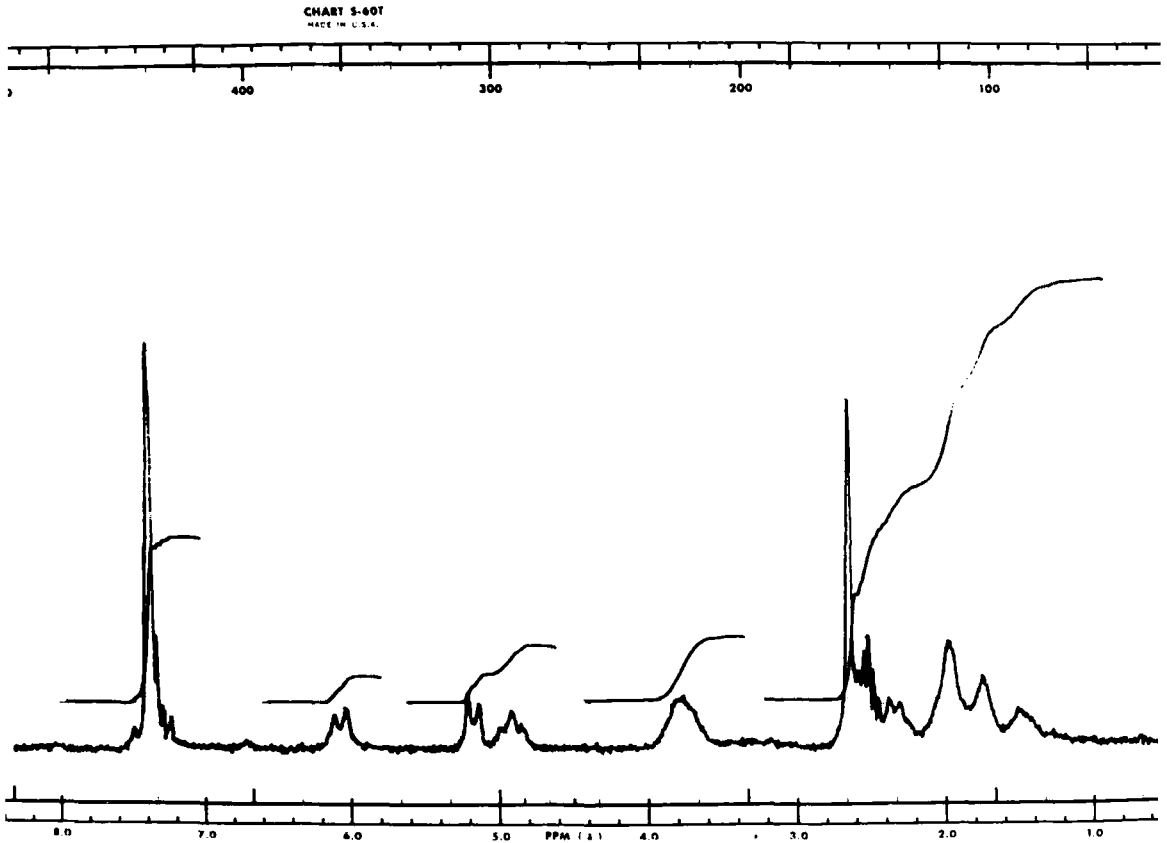


Fig. 4 : THE ^{1}H-NMR SPECTRUM HOMATROPINE HYDROBROMIDE IN DMSO-D_6

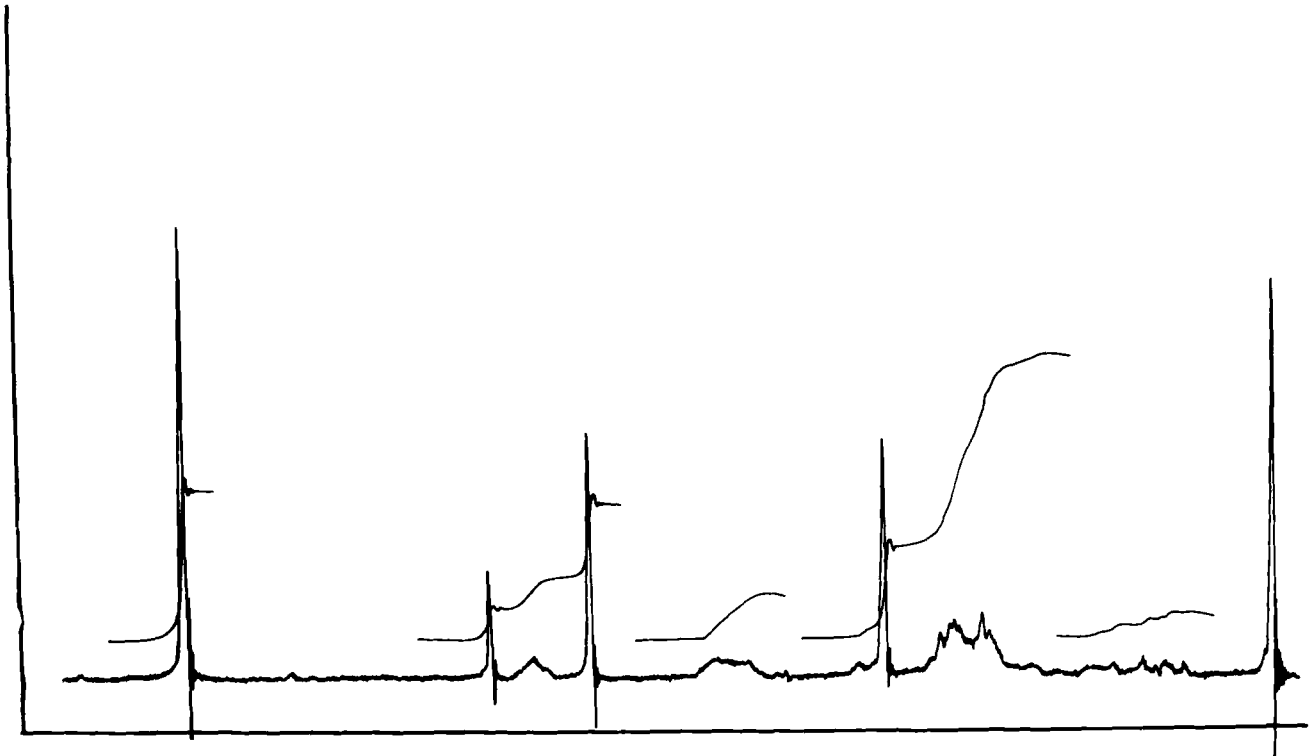


Fig. 5 : THE ^{1}H-NMR SPECTRUM OF HOMATROPINE HYDROBROMIDE IN D_2O.

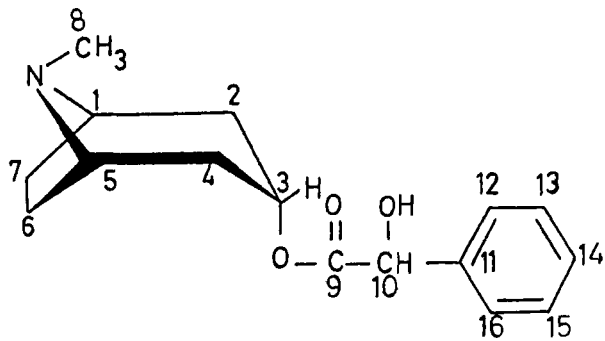
Group	Chemical Shift (pmm) DMSO-d_6	D_2O
3H	4.70 (t)	5.03 (t)
1 H and 5 H	3.73 (bs)	3.66 (bs)
8-N-Me	2.63 (s)	2.67 (s)
2,4,6,7 H	2.4-1.27 (m)	2.4-1.8 (m)

s=singlet, d=doublet, t=triplet, bs=broad singlet

Other ^{1}H-NMR data for homatropine hydrobromide was reported (1). Ohashi et al (10) have reported ^{1}H nuclear magnetic resonance contact shifts of some tropanes including homatropine in $CDCl_3$ at 220 MHz. The contact shifts used were nickel and cobalt diacetylacetonates.

2.5.3.2 ^{13}C-NMR

The ^{13}C-NMR completely decoupled and off resonance spectra of homatropine hydrobromide are presented in Fig.6 and Fig.7 respectively. Both were recorded over 5000 Hz range in a mixture of DMSO-D_6 and $CDCl_3$ (conc. 100 mg/ml), on a Joel FX-100-90 MHz spectrometer. A sample tube 10 mm and TMS as internal standard at 20° were used. The carbon chemical shifts are assigned on the basis of the chemical shift theory and the off-resonance splitting pattern (Table 6).



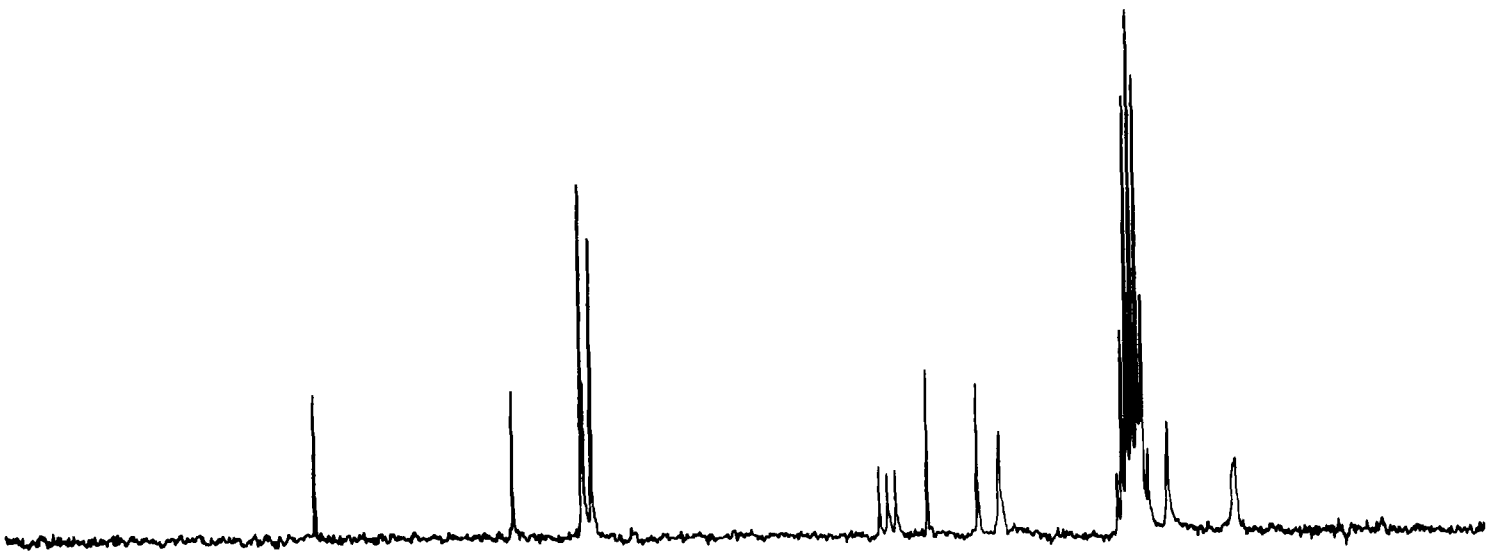


Fig. 6 : THE ^{13}C-NMR COMPLETELY DECOUPLED SPECTRUM OF HOMATROPINE HYDROBROMIDE.

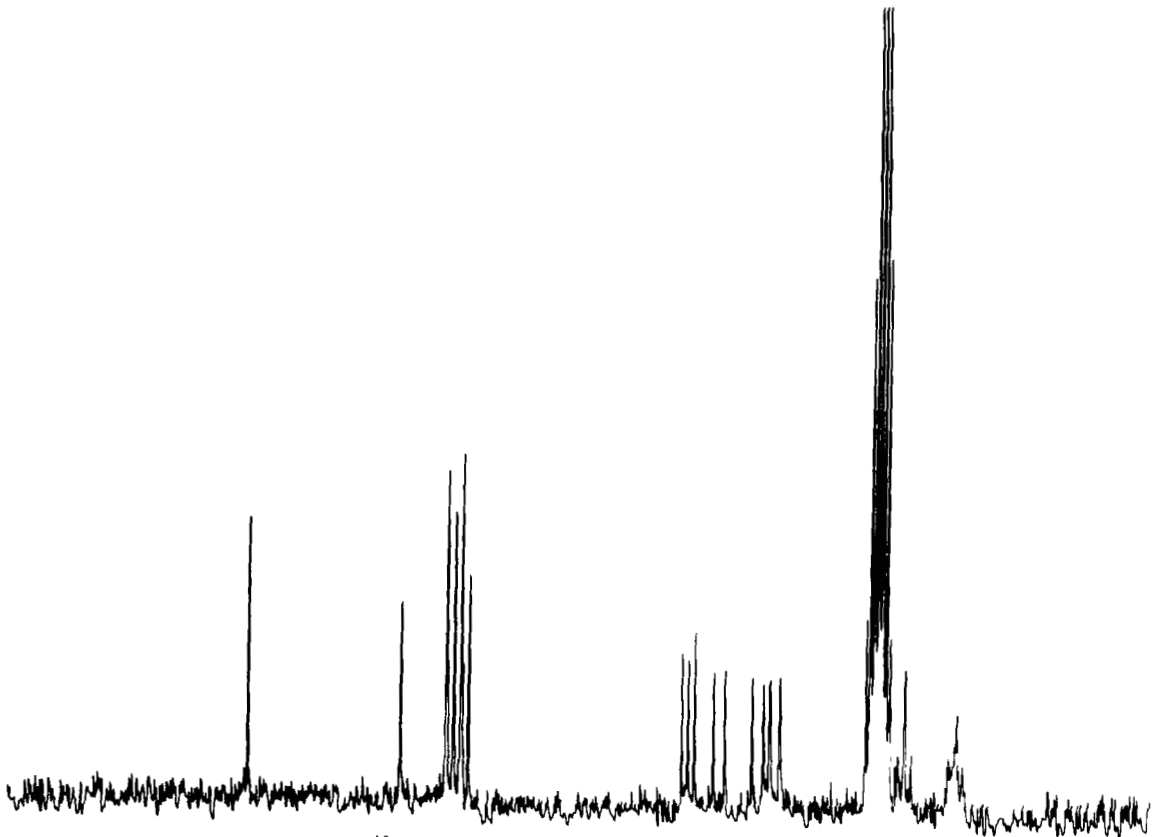


Fig. 7 : THE ^{13}C-NMR OFF-RESONANCE SPECTRUM OF HOMATROPINE HYDROBROMIDE.

Table 6. Carbon Chemical Shifts of Homatropine

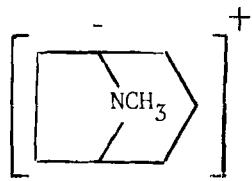
Carbon No.	Chemical Shift [ppm]	Carbon No.	Chemical Shift [ppm]
C_1	59.18 (d)	C_9	169.38 (s)
C_2	32.11 (t)	C_{10}	62.76 (d)
C_3	70.74 (d)	C_{11}	137.38 (s)
C_4	32.11 (t)	C_{12}	126.34 (d)
C_5	59.18 (d)	C_{13}	124.76 (d)
C_6	21.19 (t)	C_{14}	125.99 (d)
C_7	21.19 (t)	C_{15}	124.76 (d)
C_8	40.21 (q)	C_{16}	126.34 (d)

s = singlet, d = doublet, t = triplet, q = quartet.

2.5.4 Mass Spectrum

The mass spectrum of homatropine obtained by electron impact ionization at 70 eV, was recorded on a Finigan-Mat 5100 Mass Spectrometer. The spectrum (Fig. 8) shows a molecular ion peak M^+ at m/e 275. The base peak is at m/e 124.
The most prominent fragments, their relative intensities and some proposed ion fragments are shown in table 7.

Table 7. Mass Fragements of Homatropine

m/e	Relative Intensity %	Ions
275	4.4	M^+
234	1.2	-
193	1.2	-
165	1.3	-
142	6.4	-
140	4.6	-
125	10.5	

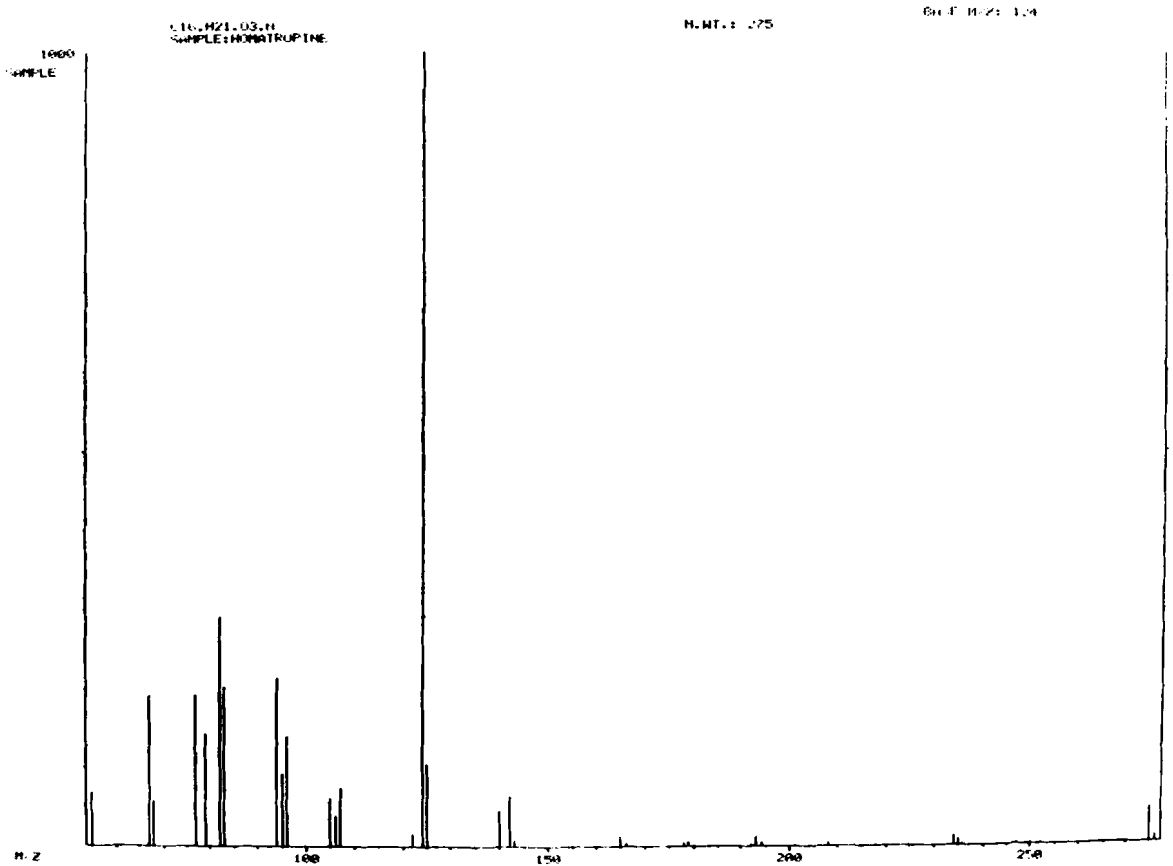
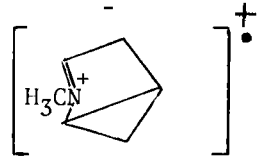
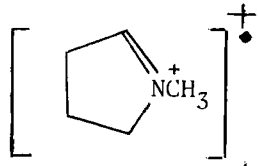
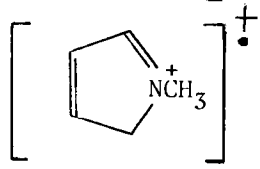


Fig. 8 : THE MASS SPECTRUM OF HOMATROPINE.

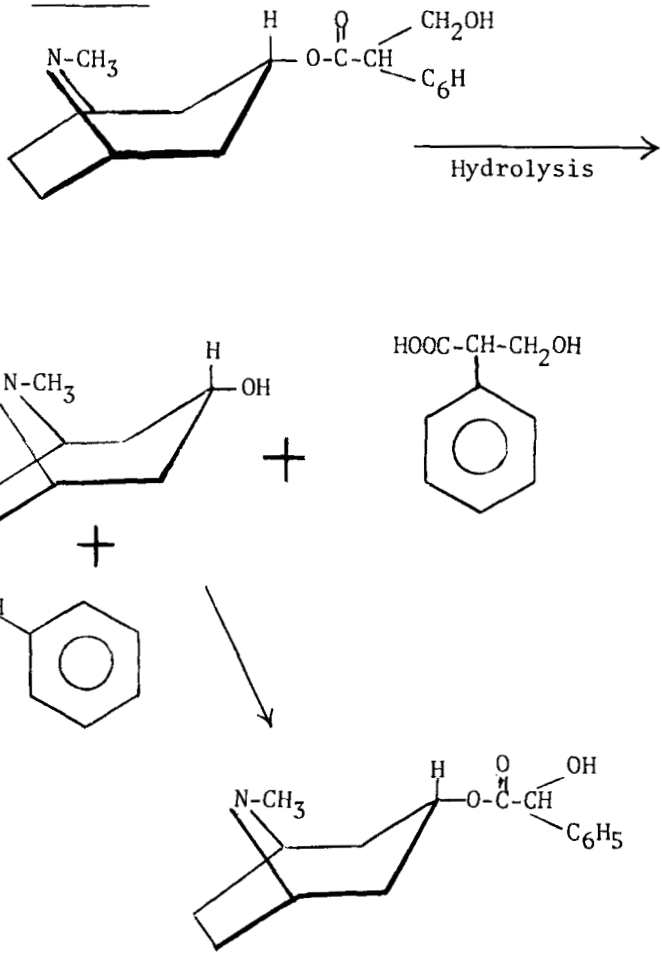
m/e	Relative Intensity %	Ions
124	100	125-H
122	1.6	-
107	7.5	-
106	3.8	-
105	6.1	-
96	14	
95	9.3	96-H
94	21.5	95-H
83	20.3	
82	29.2	
79	14.3	-
77	19.2	-
68	5.9	-
67	19.1	68-H
55	6.7	$[CH_2=\overset{+}{N}=CH-CH_2]^{+\bullet}$

Other mass spectral data of homatropine have been reported (19,20).

3. Preparation of Homatropine Hydrobromide

Atropine is hydrolyzed with barium hydroxide solution to give tropine and tropic acid.
The tropine is heated with mandelic acid in the presence of hydrogen chloride. Ammonia is added, and the liberated homatropine is extracted with chloroform. The chloroform solution is evaporated to dryness. The residue is treated with hydrobromic acid and the homatropine hydrobromide obtained is purified by recrystallization (21, 22). The preparation is outlined in Scheme I.

Scheme I.



Homatropine

An alternative method of hydrolyzing atropine was achieved microbically by the action of *Arthrobacter atropini (23)*.

4. Total Synthesis

Since Homatropine is the tropine ester of mandelic acid, schemes for the total synthesis of tropine and of mandelic acid were reported.

4.1 Total Synthesis of Tropine

Four schemes for the total synthesis of tropine are known. Scheme II was also modified to give a much better yield.

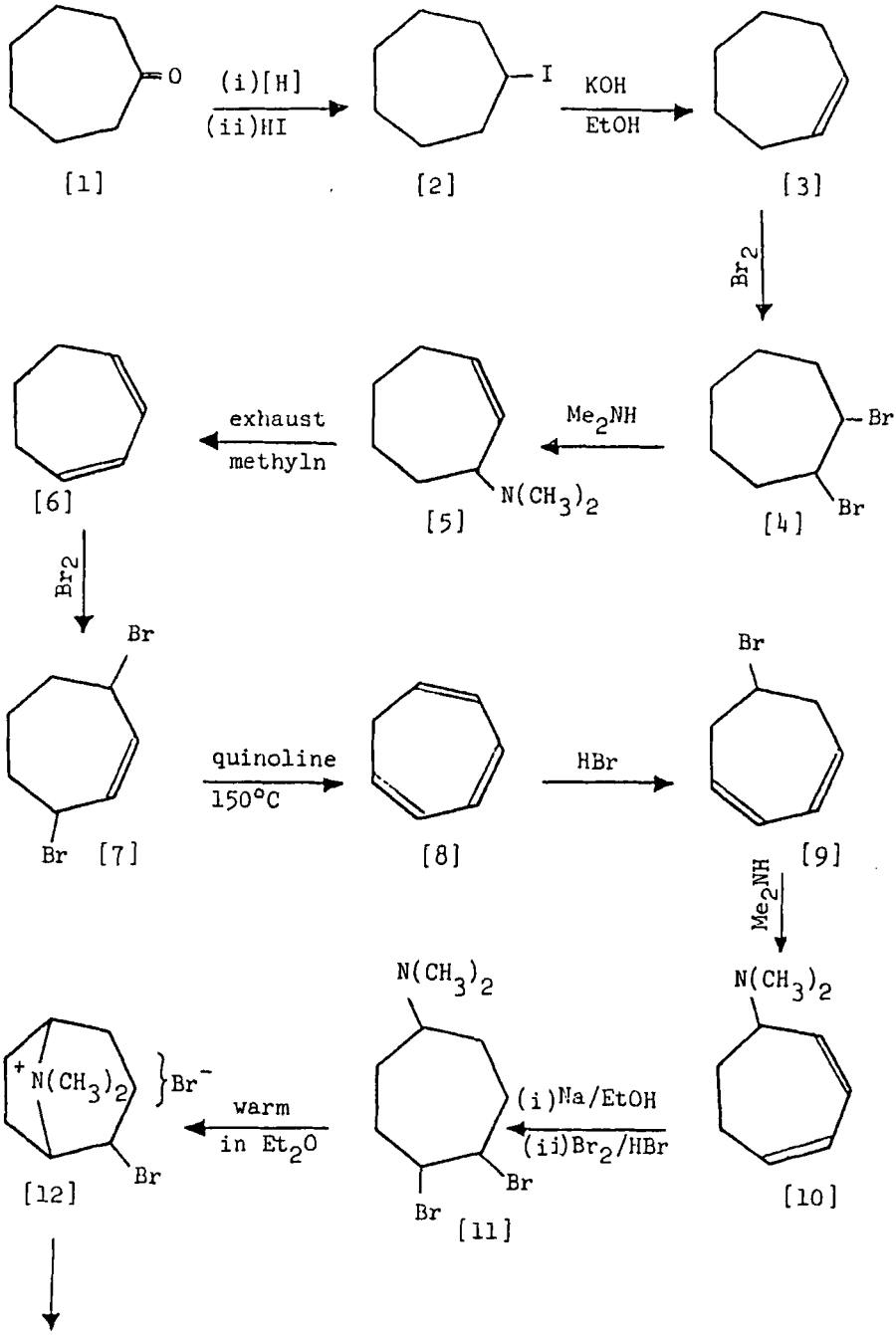
Scheme II: Willstätter's total synthesis of tropine (24).

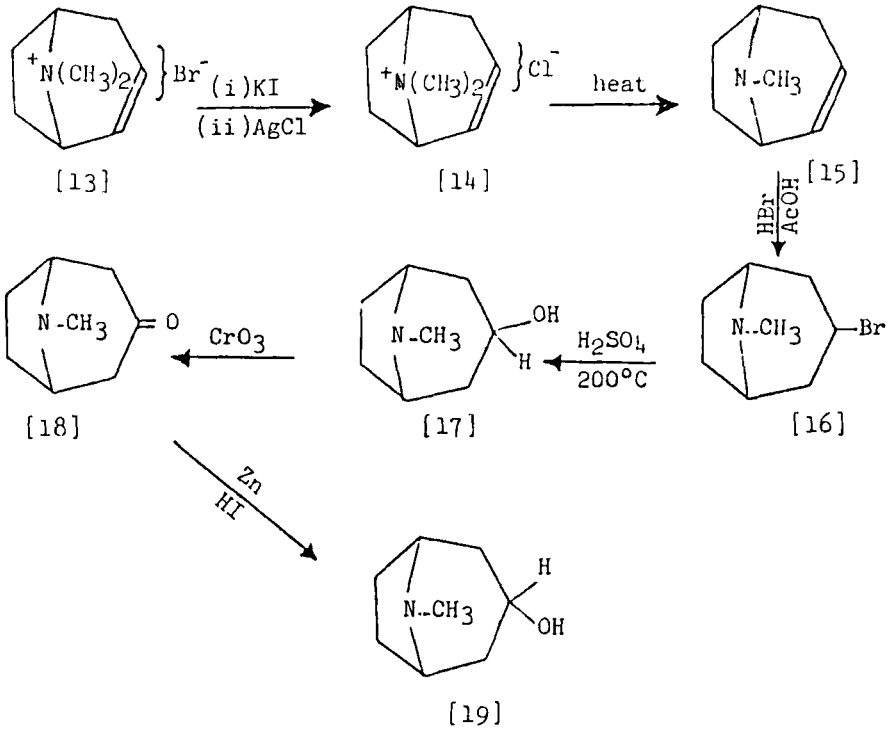
Suberone (cycloheptanone) [1] is reduced to suberol which is treated with hydrogen iodide to give suberyl iodide [2]. This is treated with potassium hydroxide in ethanol to give cycloheptene [3]. Cycloheptene is brominated to give 1,2-dibromocycloheptane [4] which is treated with dimethylamine to yield dimethylaminocyclohept-2-ene [5]. The latter is converted to cyclohepta-1,3-diene [6] by exhaustive methylation. [6] is brominated at 1,4-positions to give 1,4-dibromocyclohept-2-ene [7]. Elimination of two moles of the hydrogen bromide of [7] is effected by quinaline to give cycloheptatriene [8].

Substance [8] is treated with hydrogen bromide to give bromocyclohepta-3,5-diene [9] which is reacted with dimethylamine to give dimethyl aminocyclohepta-2,4-diene [10]. The latter is treated with sodium in ethanol followed by bromination to give 1,2-dibromo-5-dimethylamino-cycloheptane [11]. This is warmed in ether when intramolecular alkylation occurs to give 2-bromotropane methobromide [12]. Hydrogen bromide is eliminated from [12] by the action of alkali to yield tropidine methobromide [13]. This is transformed to tropidine methochloride [14] by the action of potassium iodide followed by the action of silver chloride. Substance [14] is pyrolized to give tropidine [15].

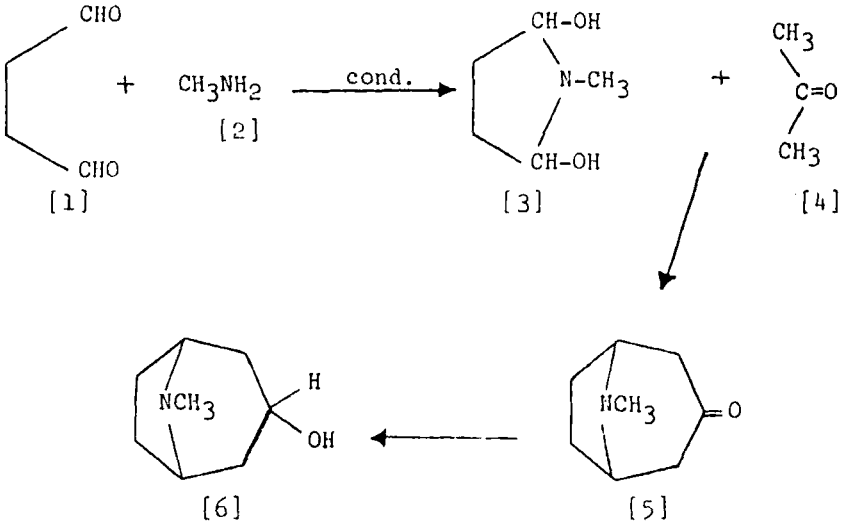
Hydrogen bromide is added to an acetic acid solution of tropidine [15] to yield 3-bromotropane [16] which is hydrolysed with 10% sulfuric acid at 200-210° to give pseudotropine [17]. ψ-tropine [17] is oxidized with chromium trioxide to give tropinone [18]. This ketone is reduced with zinc and hydriodic acid to tropine [19].

Scheme II: Willstatter's total synthesis of atropine





Scheme III: Robinson's total synthesis of atropine



Scheme III: Robinson's synthesis (25)

Succindialdehyde [1] is condensed with methylamine [2] to give the condensate biscarbinolamine [3]. This in turn condensed with acetone [4] to give tropinone [5] (This mixture is allowed to stand in water at ordinary temperature for half an hour).
Tropinone [5] is reduced with zinc and hydriodic acid to tropine [6].

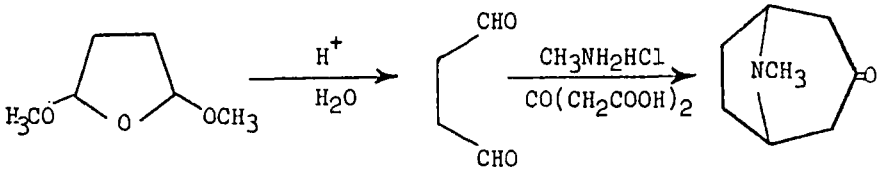
The yield can be improved by substitution of the more reactive acetone dicarboxylate or its ester for acetone.
Succindialdehyde [1] is condensed with methylamine [2] to give biscarbinolamine [3]. [3] is condensed with calcium acetonedicarboxylate [4] to afford the condensate [5]. This is warmed with hydrochloric acid to give tropinone [6]. Tropinone [6] is reduced with zinc and hydriodic acid to tropine [7].

Scheme IV: Willstatter's second synthesis(26)

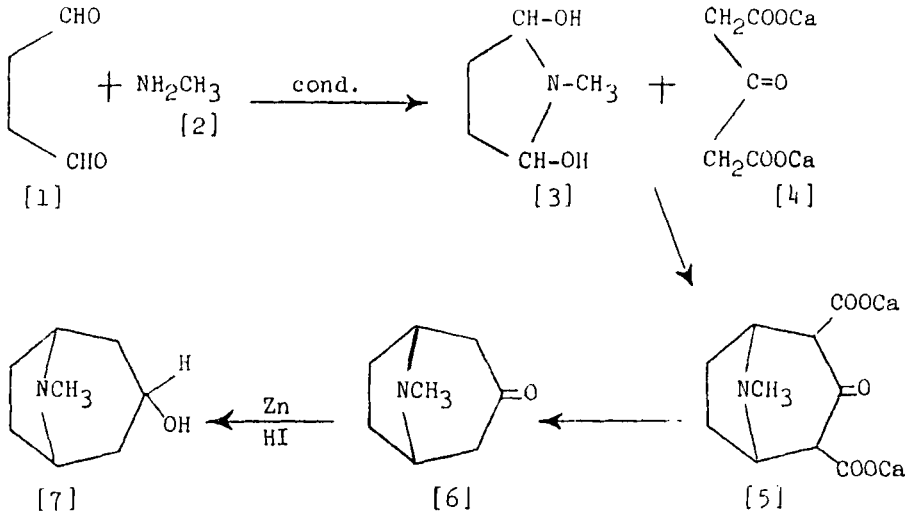
Succinyldiacetic ester [1] is condensed with methylamine [2] to give diethyl-N-methylpyrrolediacetate [3]. This is reduced (H_2+Pt) to afford diethyl-N-methylpyrrolidinediacetate [4]. The cis form of [4] is cyclized in the presence of Na and p-cymene to give ethyltropinone-2-carboxylate [5]. Hydrolysis of [5] with 10% sulfuric acid gives ethyltropinone-2-carboxylic acid [6]. The latter is heated to yield tropinone [7] which is reduced with zinc and hydriodic acid to tropine [8].

Scheme V:

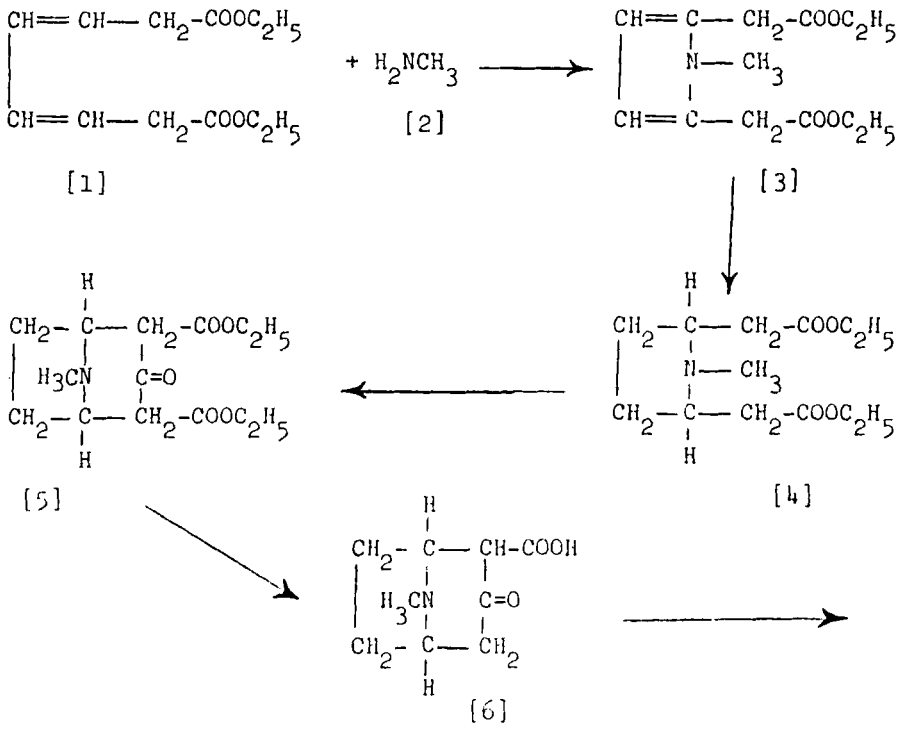
Tropinone can also be synthesized (27) using methylamine hydrochloride acetondicarboxylic acid and generating succindialdehyde in situ by the action of acid on 2,5-dimethoxy tetrahydrofuran as follows:

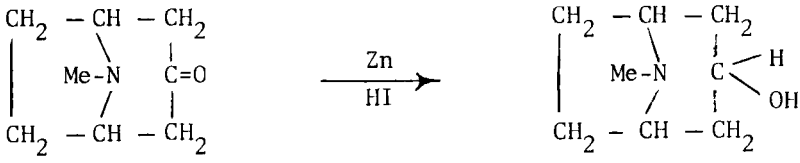


Scheme III: Robinson's synthesis (yield improvement)



Scheme IV: Willstatter's second synthesis





[7] [8]

4.2 Total Synthesis of Mandelic Acid

Several schemes for the total synthesis are known (28-30).

Scheme I

Benzaldehyde [1] is treated with a mixture of a saturated solution of sodium hydrogen sulfite and aqueous sodium cyanide to give mandelonitrile [2]. [2] is hydrolyzed with cold concentrated hydrochloric acid to yield mandelic acid [3] (28).

Scheme II

Acetophenone [1] is converted into dichloroacetophenone [2] by the action of chlorine in acetic acid. [2] is treated with sodium hydroxide to afford the sodium salt of mandelic acid, which is treated with hydrochloric acid to give mandelic acid [3] (29).

Scheme III

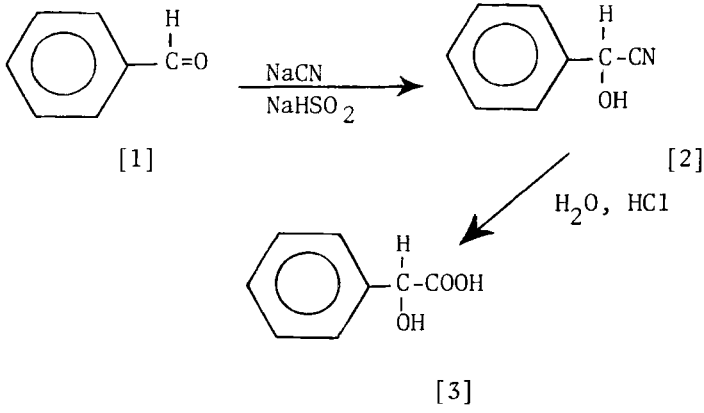
Amygdalin [1] is treated with fuming hydrochloric acid to produce mandelonitrile [2] which is then hydrolyzed with hydrochloric acid to give mandelic acid [3] (30).

4.3 Total Synthesis of Homatropine

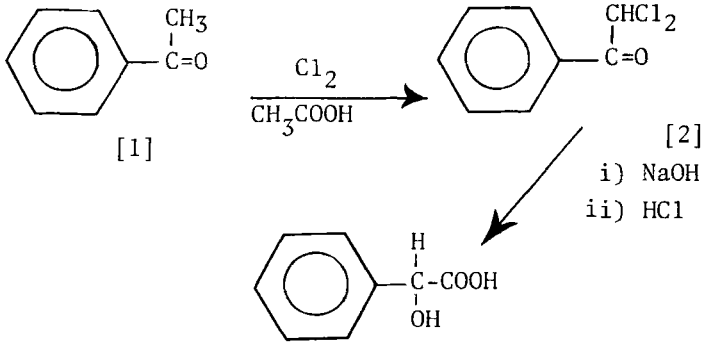
Tropine is then esterified with (±)-mandelic acid in the presence of hydrogen chloride (Fischer-Speier esterfication) to give (±)-homatropine.

Total Synthesis of Mandelic Acid

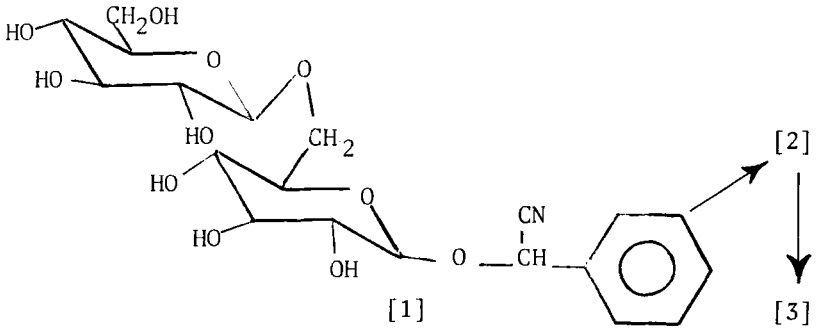
Scheme I



Scheme II



Scheme III



5. Therapeutic Uses and Pharmacological Effects

Homatropine is used as a mydriatic and cycloplegic drug in ophthalmology. It is preferred to atropine for diagnostic purposes because its action is more rapid, less prolonged and is more readily controlled by physostigmine (12). The effect of homatropine is exerted in 15 to 30 minutes and passes off in 12 to 24 hours. It is sometimes used with cocaine which enhances its mydriatic action (12).

Homatropine has also less tendency than atropine to increase the intra-ocular pressure (18), but it produces a less satisfactory mydriasis in children (18). It is seldom used internally (12).

Homatropine is an anticholinergic drug with effects similar to but much weaker (about one-tenth) than those of atropine (12,31), generally the belladonna alkaloids are absorbed rapidly from the gastrointestinal tract. They also enter the circulation when applied locally to the mucosal surfaces of the body (31). Some of these compounds (atropine and homatropine) when applied locally to the eye can cause mydriasis and cycloplegia (31).

Homatropine is used as a 2% solution in castor oil, but more often in form of 2 to 5% homatropine hydrobromide ophthalmic solutions (12,31).

It has been reported (32) that homatropine in doses of 0.5 to 4.0 mg, induces bradycardia in man, when it is administered subcutaneously

6. Drug Stability

Homatropine hydrobromide in the dry state,in air tight container at room temperature and protected from light, showed no decomposition after five years when examined according to the B.P. (15).

In aqueous solution, homatropine hydrolyses to tropine and mandelic acid. Hydrolysis is catalyzed by hydrogen ions and hydroxide ions (18). At 25°, the rate of hydrolysis is a minimum at pH 3.7.

A solution containing homatropine hydrobromide 1% and boric acid 1.55%, lost 2% of its homatropine content after autoclaving for 20 minutes at 120° (12).

The pH of this solution fell from 4.5 to 3.8 (33,34).

7. Methods of Analysis

7.1 Identification Tests

The following identification tests are mentioned in the B.P. (15).

- Dissolve 10 mg of homatropine hydrobromide in 1 ml of water, add a slight excess of 10M ammonia and shake with 5 ml of chloroform. Evaporate the chloroform layer to dryness on a water bath and add 1.5 ml of a 2 per cent w/v solution of mercury (II) chloride in ethanol (60%); a yellow color develops which turn to red on warming.
- A 2 percent w/v solution yields the reaction characteristic of alkaloids and the reactions characteristic of bromides.
- The following test is mentioned in the U.S.P. (35). The infrared absorption spectrum of a potassium bromide dispersion of it, exhibits maxima only at the same wavelengths as that of a similar preparation of USP Homatropine Hydrobromide Reference Standard.
- A simple, rapid and sensitive test for the detection of homatropine and other alkaloids in physiological fluids such as saliva and urine is reported (36) To the solution of alkaloids, a methanolic bromophenol blue is added to give blue stable color.
- Another color test was reported as follows (37): Homatropine is evaporated to dryness with a mixture of fuming nitric acid-acetic anhydride (4:3) and 5% methanolic tetramethylammonium hydroxide solution is added to a solution of the residue in acetone, the color develops and remains constant for 6 to 8 minutes. It is possible to determine the alkaloid quantitatively by measuring the color at 570 nm.
- Other identification tests have been also mentioned (38).

7.2 Microcrystal Tests

Homatropine hydrobromide was dissolved in water (10 mg in 10 ml), 1 to 2 drops of this solution was treated with the reagent on a microscopical glass slide. After specific time, the crystals were microscopically examined (39).

7.2.1 Wagner's reagent was added to the test solution, minute irregular brown blades were formed after 2-3 minutes (Fig. 9).

7.2.2 Picric acid was added to the test solution, radiating rods and irregular blades were formed after 10 minutes (Fig. 10).

7.2.3 Dragendroff's reagent was added to the test solution, minute irregular orange crystals were formed after 1-3 minutes (Fig. 11).

Other microcrystal tests have also been reported (17,40,41).

7.3 Titrimetric Determinations

7.3.1 Aqueous

The following assay is mentioned in U.S.P (35)

- Dissolve about 400 of Homatropine Hydrobromide, accurately weighed, in water to make 50 ml and mix.
  Transfer 10 ml of this solution to a beaker, add 5 ml of sodium hydroxide TS, and heat the solution just to boiling. Add 10 ml of dilute nitric acid (1 in 15), add water to make 50 ml, and cool in an ice bath. Concomitantly add 5 ml of dilute nitric acid (1 in 15) to a second 10 ml portion of the solution of Homatropine Hydrobromide, add water to make 50 ml, and cool in an ice bath. Add 1 drop of nitrophenanthroline TS to each solution and while keeping the solutions cold, titrate with 0.05N ceric ammonium nitrate until the pink color is discharged.
  Each ml of the difference in volumes of 0.05N ceric ammonium nitrate required is equivalent to 8.907 mg of $C_{16}H_{21}NO_3.HBr$.

- Homatropine was determined by titration with aqueous 0.01 M-tetraphenylborate, with tetrabromophenolphthalein ethyl ester as an indicator in 1,2-dichloroethane (42).

- A potentriometric titration of homatropine hydrobromide in galenical preparations with sodium tetraphenylboron was described (43).

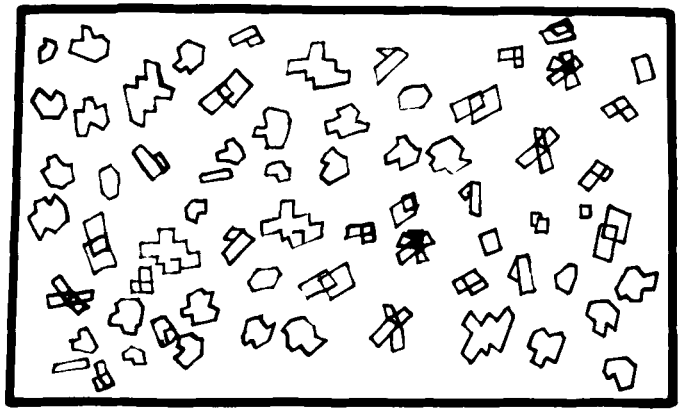


Fig. 9. Homatropine HBr with Wagner's reagent

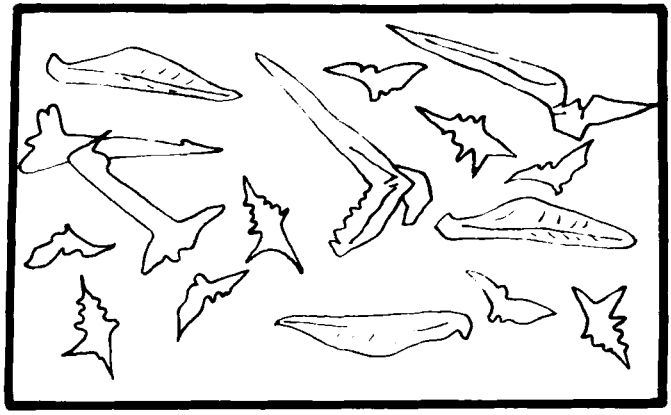


Fig. 10. Homatropine HBr with picric acid reagent

Fig. 11 Homatropine HBr with Dragendorff's reagent

- Homatropine hydrobromide was titrated in dimethyl sulphoxide medium with 0.1 M $AgNO_3$. End-point was determined by conductometric, potentiometric and polarimetric techniques, and the results were satisfactory by all three techniques. Amounts of 0.1 mmol (44) could not be determined.

7.3.2 Non-aqueous

- The following assay is mentioned in the B.P. (15).
  Dissolve 0.3 gm in 20 ml of anhydrous glacial acetic acid, add 10 ml of mercury (II) acetate solution and carry out Method I for non-aqueous titration, Appendix VIII A, determining the end-point potentiometrically. Each ml of 0.1 M perchloric acid VS is equivalent to 0.03563 gm of $C_{16}H_{21}NO_3$.HBr.
- An alternative method involves direct titration of homatropine hydrobromide with perchloric acid in acetic anhydride medium using naphtholbenzein or Sudan red β as indicator (45).
- Other non-aqueous titration has been also reported (46).

7.4 Complexometry

- Precipitation of homatropine as a thalium complex using one part 0.1 N thallium sulfate and 3 parts 0.1 N iodine in 0.15 M potassium iodide solution. The precipitation in a neutral medium is most sensitive. The use of this precipitation reagent for an indirect determination of small amounts of homatropine is based on the determination of thallium in the precipitate (47).
- Another method utilizing merceuric-potassium iodide reagent in 0.3 sodium hydroxide solution was reported (48). The determination is based on the extraction of the precipitate formed with chloroform. The combined extracts are evaporated and water and 0.1M-silver nitrate are added. Methylthymolblue is used as indicator and sufficient 20% hexamethylenetetramine solution is added to give a blue color. The mercury in the solution is then titrated with 0.05M EDTA to a yellow end point.

7.5 Polarographic

- The oscillopolarographic behaviour of homatropine and other alkaloids was reported (49). A dropping mercury electrode served as a polarizable electrode, and a graphite one as a reference. The depolarizing potentials were referred to the potentials of Tl ions. Semiquantitative determination from the depth of the characteristic Cuts-in on the curve is possible in alkaline solutions.
- Another oscillopolarographic method for identification of homatropine hydrobromide in alkaloidal mixtures was also described (50).

7.6 Spectrophotometric methods

7.6.1 Colorimetry

- A colorimetric method for the determination of homatropine and other related alkaloids was reported (51). The method is based on the nitration of the drug with a solution of 20% potassium nitrate in concentrated sulfuric acid at 50-60° for 30 minutes. The product is made alkaline with hot 20% sodium hydroxide and the color which develops is measured.
- A stability indicating assay for the determination of homatropine hydrobromide and methobromide and their degradation products in galenical preparations was reported (52). The method is as follows:-
  To 5 ml of prepared solution (≡ ≃3 mg of alkaloid) in a 50 ml flask placed in an ice-water bath, add saturated aqueous hydroxylammonium chloride solution (1 ml) and 10.5 M potasium hydroxide (1 ml), mix and set aside for 1 hr. Add 4 M hydrochloric acid (2 ml) to give pH 1.2 to 1.4, mix, add 0.37 M Ferric chloride solution in 0.1 M hydrochloric acid (1 ml) and mix again. Remove the flask from the bath, allow the evolution of gas to subside and measure the extinction of the solution at 540 nm.
- Absorptiometric determination of homatropine hydrobromide in eye drops (53):
  Dilute 2.5 ml of sample to 25 ml and to 1 ml of the resulting solution [0.25 to 2 mg of

homatropine hydrobromide] add 2.5 ml of water, 3 ml of 5% sodium hydroxide solution and after 5 minutes, 3.5 ml of Folin reagent. After 10 minutes, measure the extinction of the blue solution with use of a red filter. The sensitivity of the method is 0.25 mg in the final solution the mean error is ±3%.

- Another colorimetric determination of homatropine hydrobromide has been also reported (54). The method is as follows: Dissolve 1 mg of sample in 100 ml of water and to 1 ml of the solution add 3 ml of mM-bromocresol purple and 2 ml of buffer solution (pH 4). Extract the colored 1:1 product into chloroform (2x5 ml); dilute the combined extracts to 10 ml with chloroform and measure the absorbance at 405 to 410 nm. Hydrolysis products of the drug does not interfere unless present in three fold amounts. The relative error of the method does not exceed ± 1%.

- Other colorimetric determination of homatropine by cis-aconitic anhydride has been described (55,56). It is as follows: The sample (0.05 gm) was dissolved in 50 ml toluene and from this solution, the standards at 20,50 and 100 γ/ml were prepared. Each standard (2 ml) was diluted with 2 ml toluene and 1 ml reagent prepared by dissolving 0.25 gm cis-aconitic anhydride in 100 ml redistilled acetic anhydride. The solutions were heated for 45 minutes on a steam bath and cooled for 15 minutes in the dark. Then were diluted to 10 ml. with 20:80 acetic anhydride-toluene mixture and absorbancy measured at 535 nm. Aqueous solutions of the salt was basified with ammonia and extracted with toluene.

- Homatropine was determined using tetrabromophenolphthalein ethyl ester at pH 8-10.5 and the addition compound formed was extracted with 1,2-dichloroethane and extinction is measured at 560 nm (57).

- Colorimetric determination of the thalium complex of homatropine hydrobromide using crystal violet was also reported (58).

7.7 Chromatographic Methods

7.7.1 Paper chromatography

Homatropine can be detected by paper chromatography. Table 8 includes paper chromatographic conditions used for homatropine.

Table 8. Paper Chromatography of Homatropine

Solvent system	Conditions	Detecting reagent	R_f value	Reference
- 5% aqueous ammonia saturated with octanol	Whatman No. 1 paper impregnated with 7% methanolic octanol. Descending technique. Drying the paper in air for 5 to 10 minutes.		-	(59)
- 4.8 gm of citric acid in a mixture of 130 water and 870 of n-Butanol	Paper Whatman No.1 dipped in 5% solution of sodium dihydrogen citrate and dried	U.V. or Iodoplatinate	0.3	Clark (17)
- acetate buffer (pH 4.58)	Whatman No.1 or No.3 impregnated by dipping in 10% solution of Tributyrin in acetone and drying in air.	Location under U.V. Light (254 mμ) or Iodoplatinate spray	0.96	Clark (17)
- BuOH-AcOH-H_2O (40:10:50) BuOH-HCO_2H-H_2O (10:1:10) BuOH-$C_2H_5CO_2H$-H_2O (10:1:10)	Descending technique	0.2% Iodine solution spray		(60)

Other paper chromatography has also been reported (61).

7.7.2 Thin layer chromatography (TLC)

Homatropine can be detected by thin layer chromatography. Table 9 includes thin layer chromatographic conditions used for homatropine.

Table 9. Thin layer chromatography of Homatropine

Solvent system	Conditions	Detecting reagent	R_f value	Reference
Strong ammonia solution: methanol (1.5:100)	silica gel G	acidified iodoplatinate or under U.V. (254 nm)	0.15	Clark (17)
Ethanol-pyridine-water (1 : 6 : 4)	alumina	iodoplatinate spray	0.87	(62)
Chloroform-acetone-diethylamine (5 : 4 : 1)	Kieselgel GF 254	Dragendorff spray	-	(63)
Acetone-water-25% ammonia solution (90 : 4 : 6)	Kieselgel G	Dragendorff ethylacetate reagent	-	(64)
The upper phase of Butanol-acetic acid-water (10 : 1 : 5)	Cellulose	Dragendorff spray	-	(65)

Other T.L.C. for homatropine has been also reported (66).

7.7.3 Electrophoresis

- Electrophoresis of homatropine was performed on Whatman No.1 paper (18x46 cm) at 8 v./cm for 3 hrs. in the presence of universal buffer. The relative displacements at pH 2.3, 4.3, 6.4, 8.2, 10.5 and 11.4 were 63,84, 104,105,100,16 respectively (67,68).
- Other electrophoresis for several alkaloids have been reported (69).

7.7.4 Column chromatography

Chromatographic assay of homatropine hydrobromide and other related alkaloids are quantitatively eluted with 95% acetone from a column packed with basic alumina (70).

The purity of homatropine hydrobromide can be determined on alumina column as follows (71).
Homatropine hydrobromide (0.1 gm) was eluted over a column packed with basic alumina (5 gm) by using aqueous acetone as solvent. After elution, the eluate was diluted with water and homatropine hydrobromide is titrated with 0.1 N hydrochloric acid using bromophenol blue as an indicator.

7.7.5 Gas chromatography (GC)

The gas chromatography for homatropine are listed in table (10).

Table 10. The GC of Homatropine

Column condition	Carrier gas	Detector	Retention time	Ref.
2.5% SE-30 on 80-100 mesh chromosorb W (5 Ft x 4mm internal diameter). Column temperature 225°.	Nitrogen (50 ml/min.)	F.I.D. hydrogen (50 ml/min.) air (300 ml/min.)	0.36 relative to codeine	Clark (17)
3% XE-60 silicone nitrile polymer on 100-120 mesh Chromosorb W. Column temperature 225°.	Nitrogen (50 ml/min.)	F.I.D. hydrogen (50 ml/min.) air (300 ml/min.)	0.39 relative to codeine	Clark (17)
5% SE-30 on 60-80 mesh Chromosorb W AW (5 ft x 1/8 diameter) Column temperature 230°.	Nitrogen (30.7 ml/min.)	F.I.D. hydrogen (22 ml/min.)	0.44 relative to codeine	Clark (17)
3% JXR on silanised G Chrom P on 100-120 mesh (1.8m x 2mm) Column temperature 250°.	Nitrogen (50 ml/min.)	F.I.D.	-	(72)
5% SE-30 on 60-80 mesh acid washed Chromosorb W at 190,210,230, 250-270°	-	F.I.D.	-	(73)
15 % QF-1 on 60-80 mesh, Gas Chrom(R)Q., (1.5m x 3.2 mm) column temperature 160°.	Nitrogen (50 ml/min)	F.I.D.	4.2 (min)	(74)

Other gas chromatographic analysis have been reported (75-78)

7.7.6 High Pressure Liquid Chromatography (HPLC)

Fell et al. (79) have reported an analysis of homatropine hydrobromide by reversed-phase high-pressure liquid chromatography. Homatropine hydrobromide was determined on a column of Hypersil ODS (5 μm) with 50 mM. Sodium acetate in 10 mM-tetrabutylammonium sulfate (pH 5.5) - acetonitrile (3:1) as the mobile phase and detection at 254 nm. The internal standard was p-toluic acid. Rectilinear calibration graph was obtained for homatropine hydrobromide (in eye drops) in the range 0 to 4 mg ml^{-1}.

Stutz and Sass (80) have described a high-speed, high pressure liquid chromatography of tropane alkaloids including homatropine. The compound was separated on a stainless-steel column (1 meter x 4.6 mm) packed with sil-X absorbent with 28% aqueous ammonia tetrahydrofuran (1: 100) as the solvent and with a column inlet pressure of 500 lb per sq. inch. A differential refractive index detector and a UV detector operating at 254 nm were used to monitor the eluate.

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MEBENDAZOLE

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1. Foreword, History

Mebendazole is a broad spectrum anthelmintic agent synthesized and developed by Janssen Pharmaceutica, Research Laboratody, Beerse, Belgium. After its introduction there in 1972, the drug became available in numerous countries around the world, including the United States and Canada (1). It produces high cure rates in infestations by Ascaris, threadworms (Enterobius), hookworms (Ancylostoma and Necator Spp.), and Whipworms (Trichuris). It is also active in some (about 40%) Dwarf tapeworm (Hymenolepis) infestations (2-5).

2. Description

2.1 Nomenclature

2.1.1 Chemical Names

(5-Benzoyl-1H-benzimidazol-2-yl)-carbamic acid methyl ester.
5-Benzoyl-2-benzimidazolecarbamic acid methyl ester.
Methyl 5-benzoyl-2-benzimidozolecarbamate.
Methyl-5-benzoylbenzimidazol-2-ylcarbamate.
Methyl-(5-benzoyl-1H-benzimidazol-2-yl) carbamate.
Carbamic acid, 5-benzoyl-1H-benzimidzol-2-yl, methyl ester. (6)

CAS Registry Number

[31431-29-7] (6)

2.1.2 Generic Name

Mebendazole; R 17,635 (7).

2.1.3 Trade Names

Propretary Names

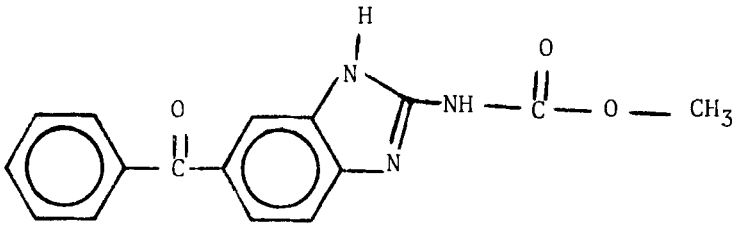
Bendrax, Besantin, Esadirase, Equivuarm plus Forverm, Gendal, Granverm, Lomper, Mebendacin, Mebendozol MK, Mebendazole, Mebenix, Mebutar, Nemasole, Oxiben, Pantelmin, Parelmin, Sirben, Telmin, Trotil, Vermirax, Vermox, and Zakor (1,6, 8-11).

2.2 Formulae

2.2.1 Empirical

$C_{16}H_{13}N_3O_3$

2.2.2 Structural



2.3 Molecular Weight

295.30 and 295 (7 & 9).

2.4 Elemental Composition

C, 65.08%, H, 4.44%, N, 14.23%; O, 16.25%

2.5 Appearance, Color and Taste

Off-white to slightly yellow amorphous powder and not unpleasant to taste. (10-12)

3. Physical Properties

3.1 Melting Point

Melts at about 290^{o}, 288.5^{o}, above 280^{o} with decomposition (6-8 & 10).

3.2 Solubility

Almost insoluble in water, ethanol, ether, chloroform, and dilute mineral acids; readily soluble in formic acid (9 & 11).

3.3 Hygroscopicity

Mebendazole is not hygroscopic and it is stable in air.

3.4 Extraction

Mebendazole is extracted by chloroform from aqueous alkaline solutions (11).

4. Spectral Properties

4.1 Ultraviolet Spectrum

The ultraviolet absorption spectrum of mebendazole obtained from a solution in neutral methanol in the region of 200 to 400 nm using DMS 90 spectrophotometer is shown in Figure 1. Three absorption maxima at about 210, 247 and 310 nm and two minima at 228 and 273 nm were observed.

Clarke (11) reported the following:

Mebendazole in 0.05% formic acid in isopropanol; maxima at 248 nm (E1%, 1 cm 1005) and 313 nm (E1%, 1 cm 518), minima at 230 nm and 275 nm.

Mebendazole in 0.01 N sodium hydroxide in isopropanol, maxima at 270 nm (E1%, 1 cm 802) and 355 nm (E1%, 1 cm 653), minima at 245 nm and 302 nm. Mebendazole in 0.1 N hydrochloric acid in isopropanol, maxima at 234 nm (E1%, 1 cm 1000) and 288 nm (E1%, 1 cm 524), minima at 215 nm and 266 nm.

4.2 Infrared Spectrum

The infrared spectrum of mebendazole is presented; in Figure 2. The spectrum was obtained from KBr disc using a Pye Unicam SP 1025 infrared spectrophotometer. The spectral assignments are presented in the following table.

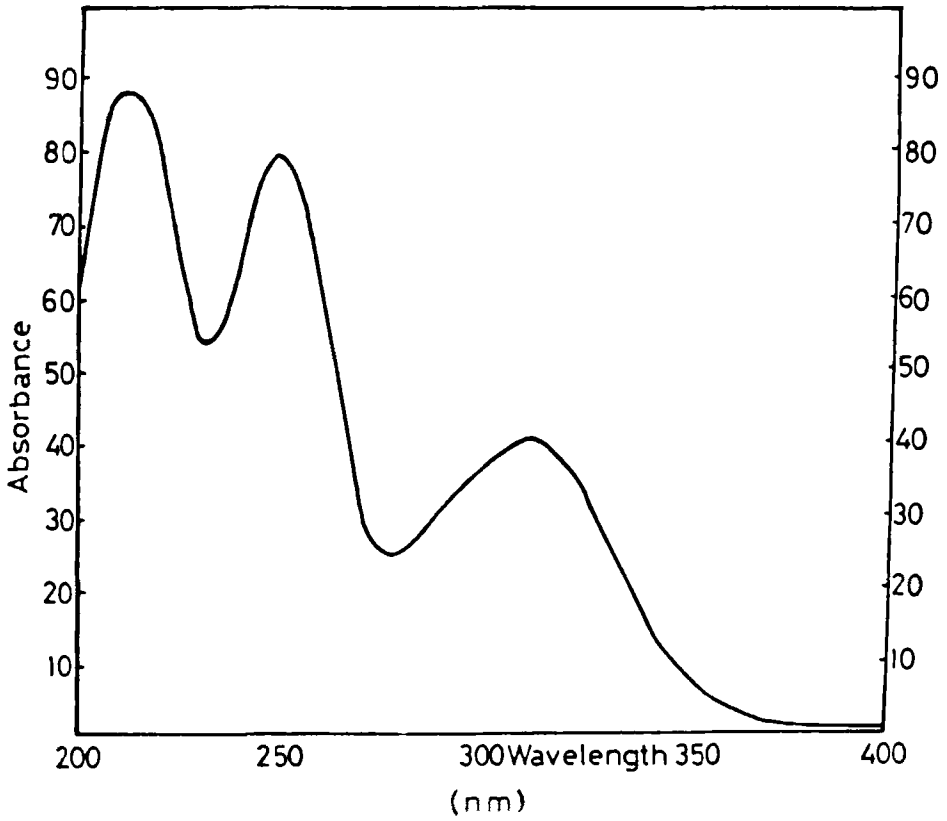


FIGURE 1: ULTRAVIOLET SPECTRUM OF MEBENDAZOLE IN METHANOL

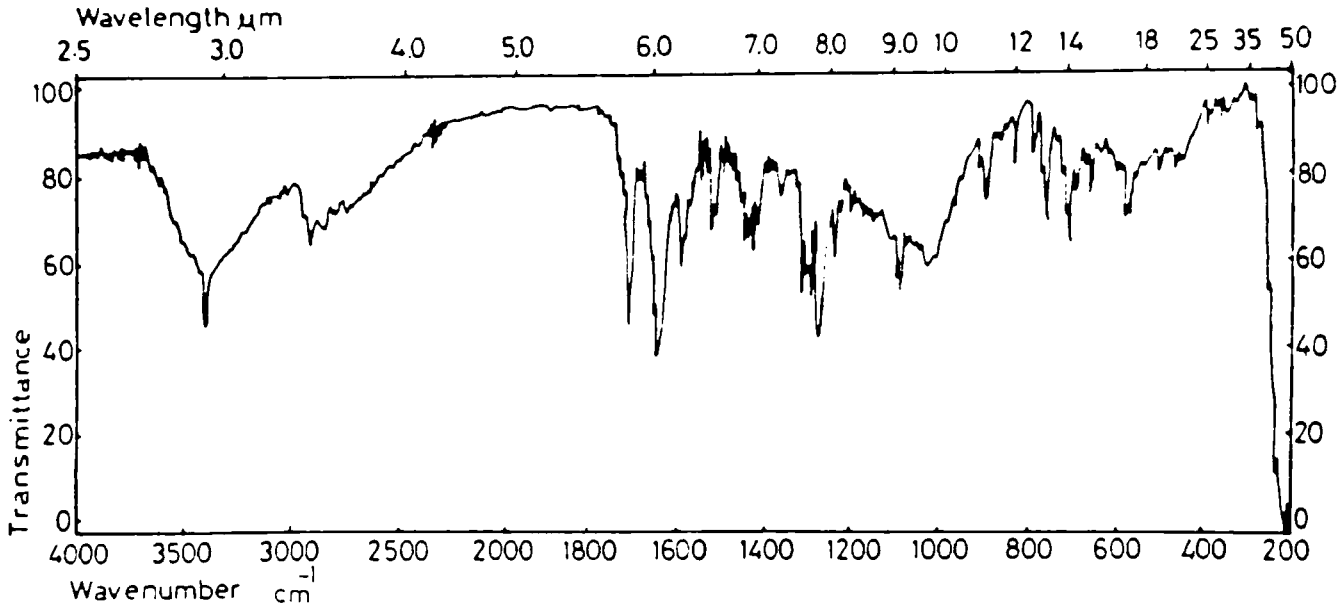


FIGURE 2: INFRARED SPECTRUM OF MEBENDAZOLE (KBr disc)

Frequency cm^{-1}	Assignment
3415	NH stretch
2500-2950	CH stretch
1720	C = O (amide) stretch
1650	C = O stretch
1600 1530	C = C stretch
1410-1460	CH_3-O (C-O) stretch
700-765	Monosubstituted benzene
878 900	1,2,4 Trisubstituted benzene

Clarke (11) reported the principal peaks (KBr disc) are 705, 1260, 1590, 1635 and 1730 cm^{-1}.

4.3 ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) Spectrum

The ^{1}H NMR spectrum of mebendazole is shown in Figure 3. The drug is dissolved in Trifluoroacetic acid (TFA) and its spectrum determined on a Varian T60 A NMR spectrometer using TMS (tetramethylsilane) as the internal standard. Assignment of the chemical shifts to the different protons is shown below:

Chemical Shift PPM (δ)	Multiplicity	Proton Assignment
7.5 - 8.3	multiplet	Aromatic
4.08	singlet	methyl

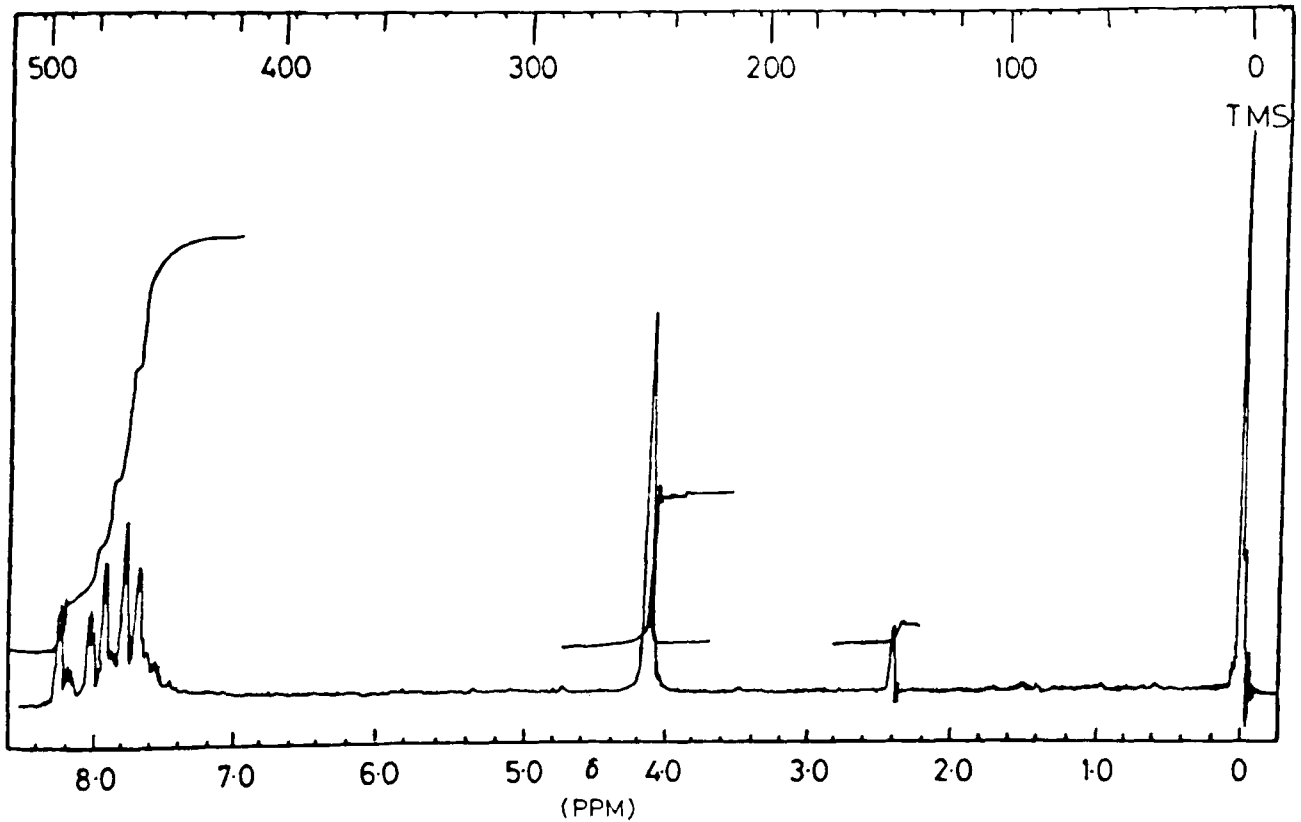
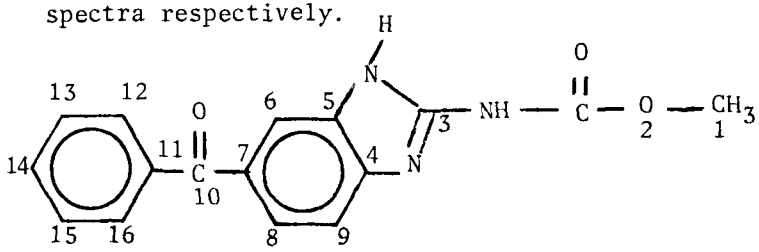


FIGURE 3: PROTON MAGNETIC RESONANCE SPECTRUM OF MEBENDAZOLE IN TRIFLOUROACETIC ACID (TFA)

4.4 ^{13}C Nuclear Magnetic Resonance (^{13}C NMR) Spectrum

The ^{13}C NMR spectra of mebendazole in formic acid using TMS (tetramethylsilane) as an internal standard are obtained using a Joel FX 100 MHz spectrometer at an ambient temperature. Figures 4 and 5 represent the ^{1}H-decoupled and off-resonance spectra respectively.



The carbon assignments are based on chemical shift values and by comparison with model compounds and are shown below:

Chemical Shift PPM (δ)	Multiplicity	Carbon Assignment
57.5	quartet	C_1
155.8	singlet	C_2
148.4	singlet	C_3
139.2	singlet	C_4
134.4	singlet	C_5
118.2	doublet	C_6
130.5	singlet	C_7
130.9	doublet	C_8
115.8	doublet	C_9
200.5	singlet	C_{10}
136.7	singlet	C_{11}
132.9	doublet	C_{12} and C_{16}
131.3	doublet	C_{13} and C_{15}
136.3	doublet	C_{14}

FIGURE 4: PROTON DECOUPLED CARBON-13 NMR SPECTRUM OF MEBENDAZOLE IN FORMIC ACID.

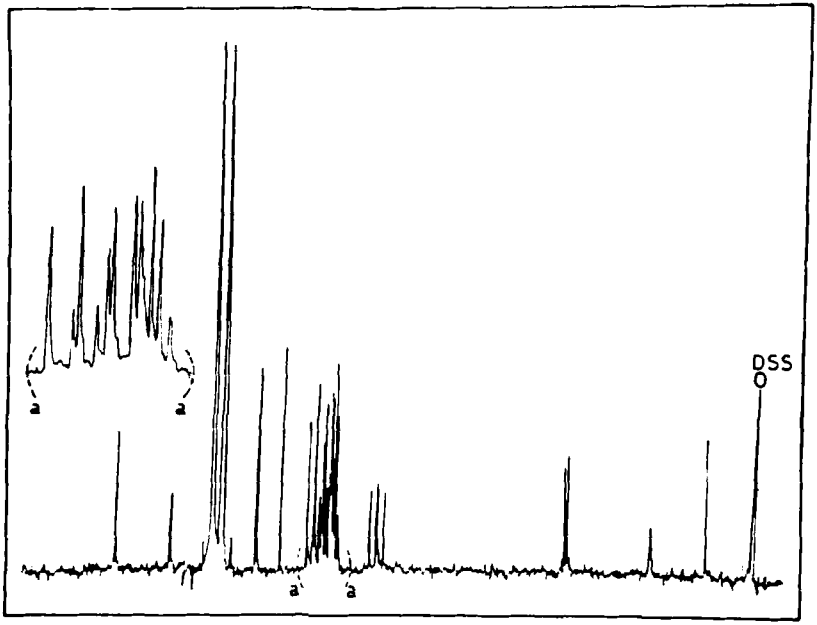


FIGURE 5: OF-RESONANCE CARBON-13 NMR SPECTRUM OF MEBENDAZOLE IN FORMIC ACID

4.5 Mass Spectrum and Fragmentometry

Figure 6 shows the 70 eV electron impact (EI) mass spectrum obtained on Varian MAT 311 mass spectrometer using ion source pressure of 10^{-6} Torr, ion source temperature of 180°C and an emission current of 300 μA. The spectrum is dominated by m/e 186 ion (base peak) resulting from the loss of CH_3OH and C_6H_5 fragments. A proposed mechanism of fragmentation and the mass/charge ratios of the major fragments is given in Scheme 1 (a and b).

Chemical ionisation (CI) spectrum is presented in Figure 7 and is obtained on a Finnigan 4000 mass spectrometer with ion electron energy of 100 eV, ion source pressure of 0.3 Torr, ion source temperature of 150°C and emission current of 300 μA.

The mass spectral assignment of the prominent ions under CI conditions is given below:

m/e	Species
296	$M^+ + 1$
295	M^+
218	$M^+ - C_6H_5$

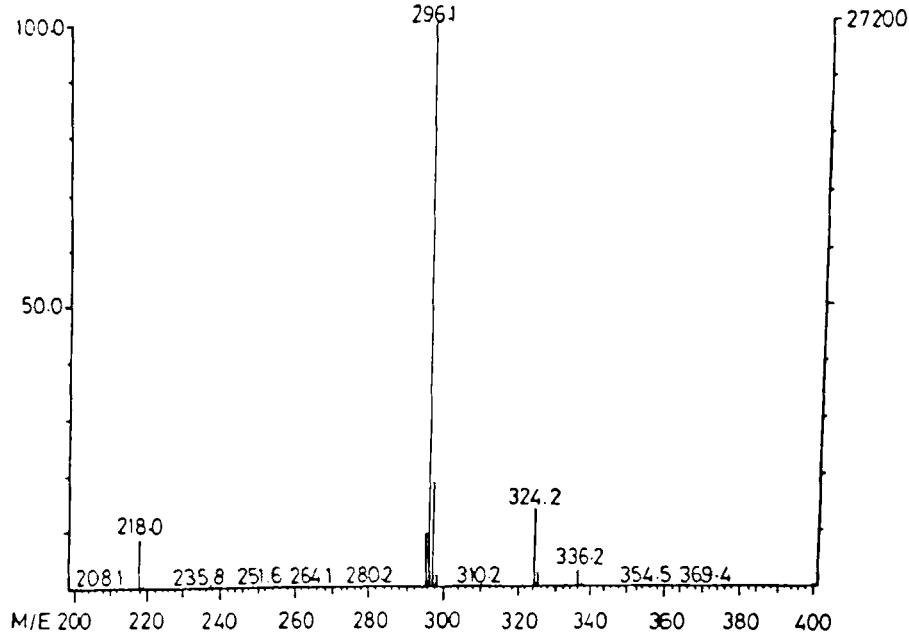


FIGURE 6 : MASS SPECTRUM OF MEBENDAZOLE (EI).

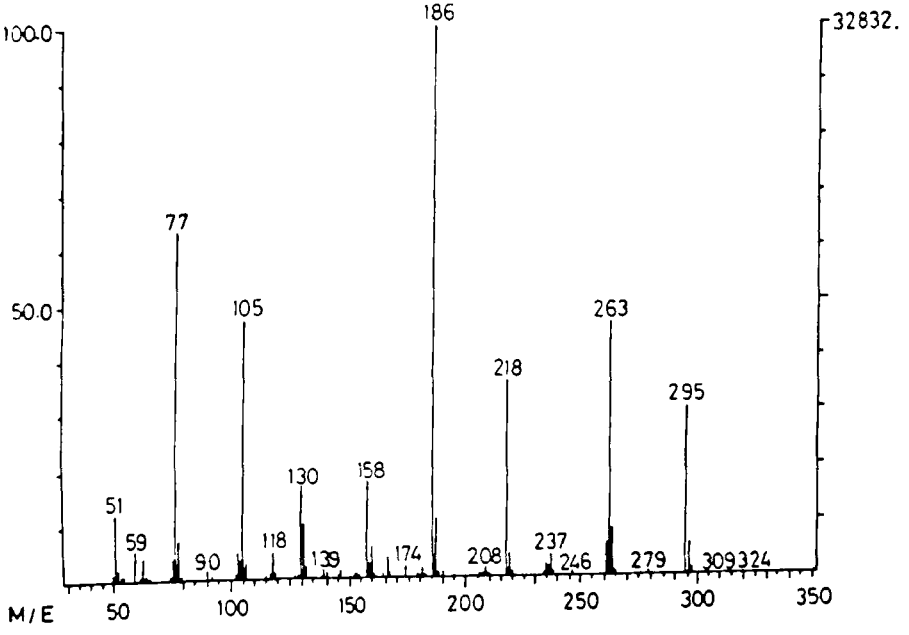
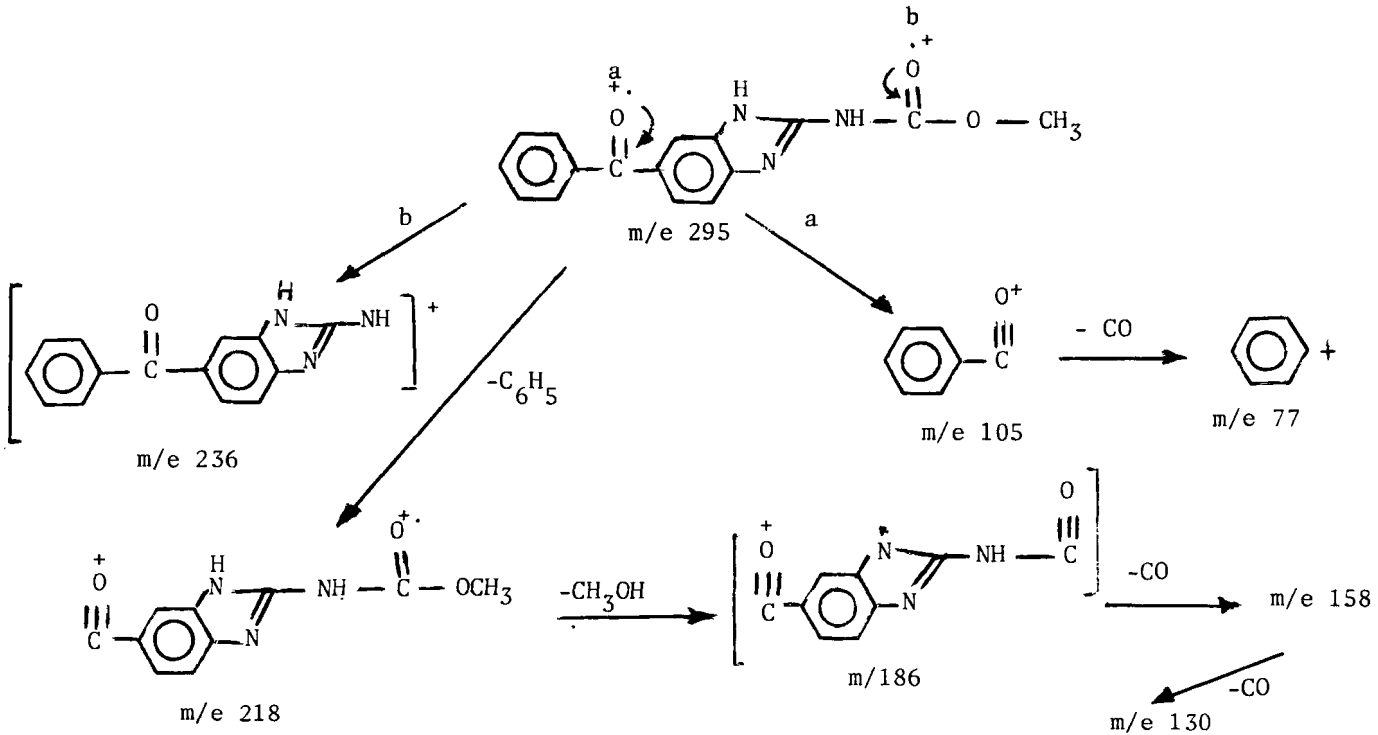


FIGURE 7: MASS SPECTRUM OF MEBENDAZOLE (CI).



Scheme 1a: Proposed mechanism of fragmentation of mebendazole.

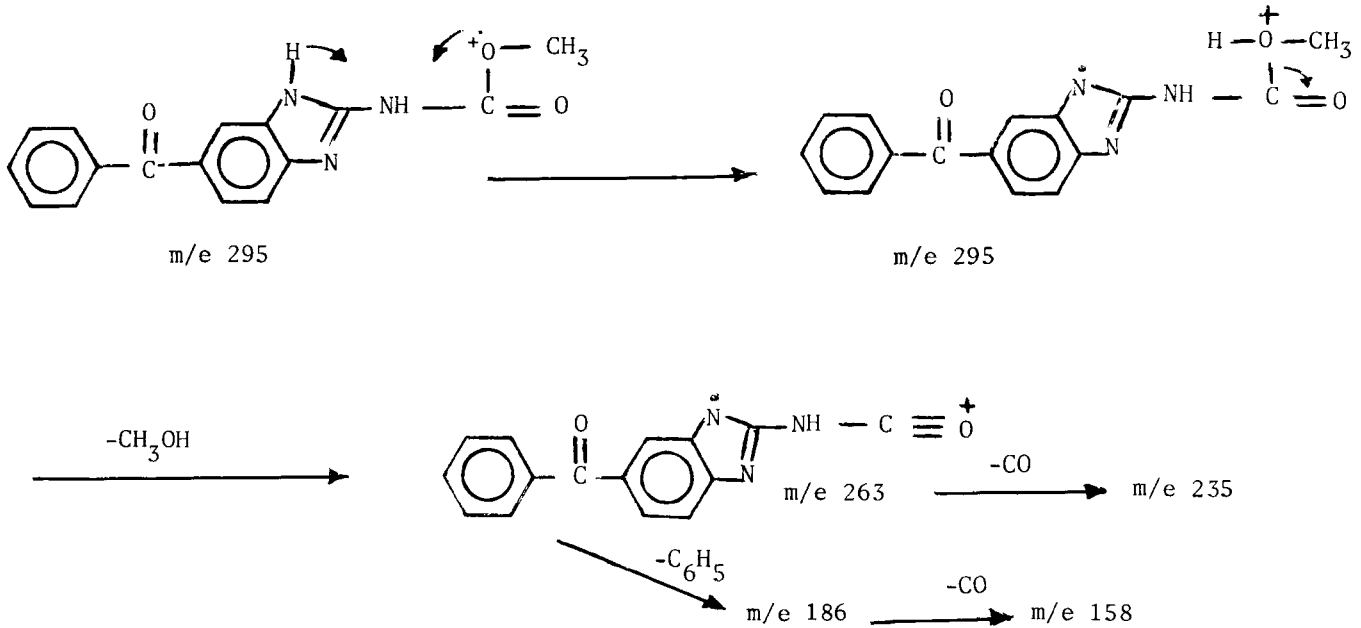
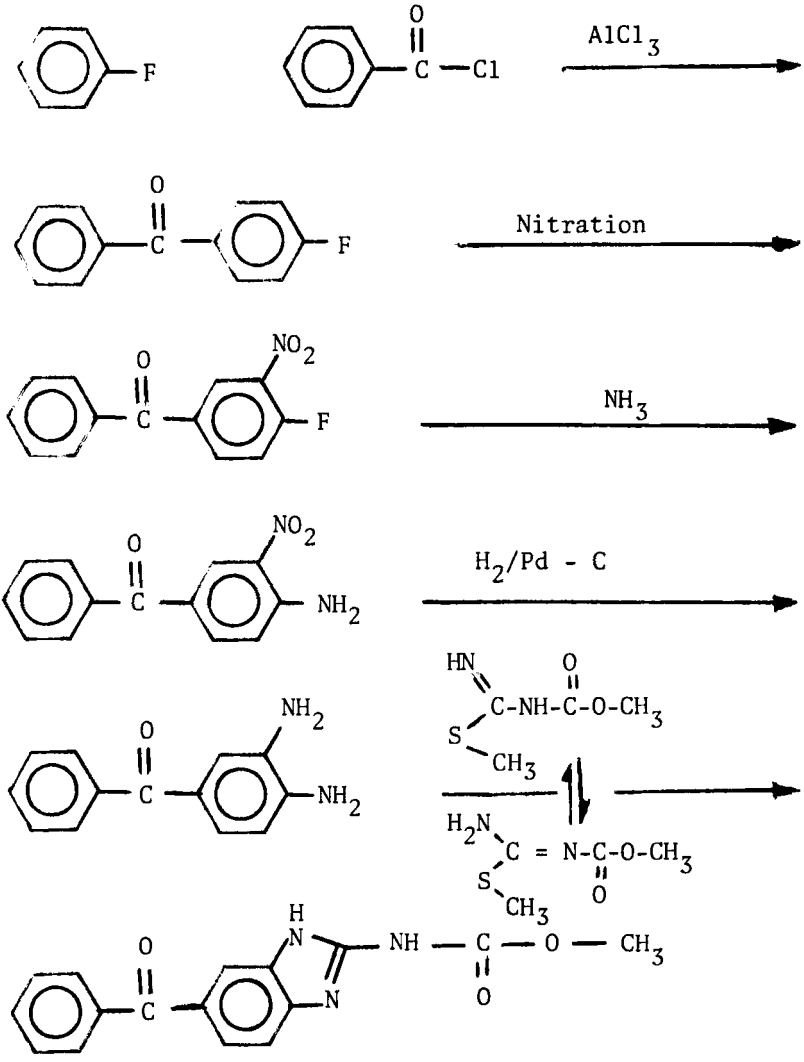


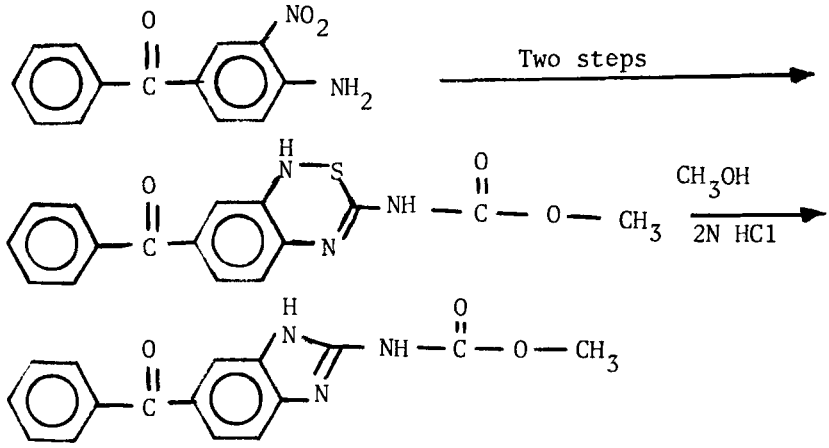
Figure 1b: Proposed mechanism of fragmentation of mebendazole.

5. Synthesis

1- Mebendazole was prepared (13) from 3,4-$(NH_2)_2C_6H_3COC_6H_5$ and $NH_2(CH_3S)C = N - COOCH_3$ according to the following scheme.



2- Mebendazole was also prepared in 52% yield by decomposition of methyl 7-benzoyl-1H-2,1,4-benzothiadiazine-3-yl carbamate in methanol with 2 N HCl. The latter compound is prepared in two stages from 4-benzoyl-2-nitroaniline (14).



6. Pharmacokinetics

6.1 Absorption, Distribution, Metbolism and Extraction

Mebendazole is poorly absorbed by gastrointestinal tract after oral administration (10). Only 5-10% of the orally administered frug is found in the blood (1). The poor intestinal absorption of mebendazole is of therapeutic advantage in the treatment of intestinal helminthis. However, for tissue dwelling organism high oral doses are required (15).

The absorption of drug in man is markedly enhanced when the drug is used together with the meals, as concomitent fat ingestion ehances its intestinal absorption. Following oral administration peak plasma level reach in 2-4 hours (16-18).

Munst et al (19) reported a significant variation in plasma mebendazole level in human subjects following oral ingestion. According to this study the plasma half-life was ranging between 1.5 and

5.5 hours. The peak concentration following oral administration were detected at 30 minutes to 2 hours of the dose. Mebendazole is metabolized primarily in liver by decarboxylation (20), the major metabolite was 2-amino-5(6-benzimidazolyl phenylketone. Braithwaite et al (21) reported that mebendazole is more lipophilic than any of its metabolites. The distribution study by same author showed that 63.3% of mebendazole was distributed in plasma and 36.7% in cellular fraction. Most of the drug in plasma (95%) was present in the protein bound form. The concentration of mebendazole in liver was considerably higher as compared to the fatty tissue. Gilbaldi and Perrier (22) reported that steady state AUC of mebendazole is independent of absorption rate in human subjects. Approximately 90% of the orally administered dose of mebendazole is eliminated primarily as unchanged drug in faeces. About 5-10% of the administered drug may be recovered in the form of its conjugates, and metabolites in the urine (11).

In rats about 85% of an I.V. dose was eliminated with the bile and remainder with the urine. The majority of the dose was recovered as conjugated metabolites (23-24). In another study (25), three metabolites of mebendazole were isolated from the bile of rats and identified as : methyl-5-(α-hydroxybenzyl)-2-benzimidazole carbamate; 2-amino-5-(α-hydroxybenzyl)benzimidazole and 2-amino-5-benzoylbenzimidazole.

7. Toxicity

Gastrointestinal symptoms including constipation have been reported infrequently following use of mebendazole (26). High doses may on occasions be associated with mild colic pain or diarrhoea as reported by Chavarria (27) using doses of upto 300 mg BID in the treatment of taeniasis.

Acute and chronic toxicity studies in animals indicate a wide range between therapeutic and toxic doses. The LD_{50} (mg/kg orally) was more than 640 mg/kg in rabbits and dogs and more than 1280 mg/kg in mice and rats (11). Significant haematologic, biochemical, or pathologic abnormalities were not found in animals given 40 mg/kg daily for 13 weeks but were noted in rats

given a daily dosage of 130 mg/kg. Mebendazole is contraindicated in pregnant women because it has shown embryotoxic and teratogenic activity in single per oral dose as low as 10 mg/kg (18, 28, 29). Rare cases of leukopenia in human subjects following the administration of mebendazole was also reported (30). In high doses 40-50 mg/kg/day, the drug has been associated with severe irreversible neutropenia. No significant CNS effects in the patients taking mebendazole therapy has been reported except slight headache and dizziness (31).

8. Methods of Analysis

8.1 Identification

a) Infrared Spectrum

The infrared absorption spectrum of a potassium bromide dispersion of it, previously dried, exhibit maxima only at the same wavelength as that of a similar preparation of USP mebendazole RS (32).

b) X-ray Diffraction

The X-ray powder diffraction data for identifying mebendazole and other anthelmintics have been obtained by diffractometer and Debye - Scherrer camera techniques (33). The data were tabulated in terms of the lattice spacings and the relative intensities of the lines. Patterns using three different X-ray wavelengths with the camera method are compared with each other and with the diffractometer pattern.

8.2 Titrimetric Method

USP XX (1980) (32) described the following titrimetric method for the assay of mebendazole:

Dissolve about 225 mg of mebendazole, accurately weighed, in 30 ml of glacial acetic acid. Titrate with 0.1 N perchloric acid VS, determining the end-point potentiometrically, using a calomel-glass electrode system. Perform a blank determination, and make any necessary correction. Each ml

of 0.1 N perchloric acid is equivalent to 29.53 mg of $C_{16}H_{13}N_3O_3$.

Wahbi and Onsy, (34) developed the two titrimetric methods to determine the mebendazole in pharmaceutical preparations. Direct titration and back titration methods are mentioned as follows:

a) Direct Titration Method

200 Mg of mebendazole was taken in dry conical flask and dissolved in 2 ml of 99% formic acid followed by the addition of 60 ml of glacial acetic acid. This solution was titrated with 0.1 N perchloric acid in glacial acetic acid using crystal violet as indicator, development of a persistent green color was designated as end point. Blank determination was performed and the necessary corrections were made. The calculation was done by using the equivalence factor which is 1 ml of 0.1 N perchloric acid is equal to 29.53 mg of mebendazole.

b) Back-Titration Method

200 Mg of mebendazole was taken in dry conical flask, the powder was dissolved in 20 ml of 1 M perchloric acid in glacial acetic acid. This solution was titrated with 0.1 N sodium acetate in glacial acetic acid, crystal violet was used as indicator, development of bluish green as indicator. These methods are reproducible with standard deviation of 0.3%.

Mebendazole (in amounts upto 250 mg) in anhydrous acetic acid medium can be titrated with 0.1 M - perchloric acid (also in acetic acid) using 0.5% crystal violet solution as indicator. Results agree with those obtained by spectrophotometry at 313 nm (35).

8.3 Spectrophotometric Methods

8.3.1 Ultraviolet Spectrometric Methods

Patel et al (36) reported two spectrophotometric methods for the estimation of

mebendazole in raw material. A solution of the sample in formic acid was diluted with a mixture of chloroform : methanol (17:3). Thin-layer chromatography was run on silica gel in chloroform : ethyl acetate; acetone : formic acid (70:80:9.5:0.5), developed for 15 cm and dried for 30 minutes at 115°. The drug is then extracted into chloroform and measured at 313 nm.

In another methods, a solution of the drug in formic acid is diluted with isopropanol and treated with MeOH and 30% potassium hydroxide. After 30 min. the absorbance was measured at 420 nm.

Wahbi and Osny (34) reported a spectrophotometric method of analysis of mebendazole in tablets with a standard deviation of 1.4%. Powdered tablets equivalent to 50 mg of mebendazole were taken into 50 ml standard flask followed by 10 ml of 70% perchloric acid. The mixture was shaken for 10 minutes and filtered through a dry filter paper. 1 Ml of filtrate taken to a 100 ml flask and diluted to volume with distilled water. The absorbance of this solution was measured in a 1 cm cell at 288 nm wavelength using a spectrophotometer. 0.2 Ml of 70% perchloric acid diluted with 100 ml of distilled water was used as blank. Calculation of concentration was done by taking 566 as the (E 1%, 1 cm) value at 288 nm or by using a suitable calibration curve.

8.3.2 Phosphorescence Method

Mebendazole has been reported to show good phosphorescence at room temperature when determined on Whatman 42 filter paper using Pb(IV) and Tl(I) as phosphorescence enhancing agents. In tablets, this method gave a recovery of 97.6% of mebendazole (37).

8.3.3 Fluorimetric Method

Abdel Fattah et al (38) have reported a fluorimetric determination method of mebendazole in pharmaceutical dosage forms after alkaline hydrolysis. In this method 2 to 5 mg of mebendazole is dissolved in 10 ml of 1 M sodium hydroxide and heated for 1 hr on a boiling water bath. After dilution with methanol, 1 M sodium hydroxide (49:1), the solution was spotted on to Whatman 42 filter paper and dried at 65^{o} for 5 to 10 minutes. The fluorescence was measured at 460 nm (excitation at 365 nm). The limit of detection was 0.1 for mebendazole with rectilinear calibration graphs upto 10 and 50 ppm (in the final solution). For 8 ng of mebendazole per spot, the coefficient of variation was 9.5% (n = 19). For pharmaceutical formulations, recovery of mebendazole ranged from 99.04 to 100.56%, with coefficient of variation of 2.27 to 2.97%.

8.3.4 Colorimetric Method

Rana et al (39) have described a method for colorimetric determination of mebendazole in tablets and suspension by adding hydroxyl amine hydrochloride, dicyclohexylcarbodiimide and ferric chloride to a solution of tablets (or a dilution of a suspension) in isopropanol and formic acid and measuring the absorbance at 520 nm. The calibration graph was linear for 0.4 to 2.0 µg/ml of mebendazole .

8.4 Chromatographic Methods

8.4.1 Thin-Layer Chromatography (TLC)

Clarke (11) has described the following TLC system for the identification of mebendazole.

Solvent system: Chloroform : methanol : formic acid (90:5:5).

Absorbent: Silica gel HF 254 (Merck).

Visualizing agent: Iodine vapour; dragendorff spray (Location under ultraviolet light). R_f : 0.53.

United States Pharmacopeia "USPXX 1980 (32) have used TLC to determine the purity of mebendazole. The method is described as follows:

Dissolve 50 mg in 1.0 ml of 98 percent formic acid in a 10-ml volumetric flask, add chloroform to volume, and mix. Similarly prepare a solution of USP Mebendazole RS in the same medium having a concentration of 5 mg per ml. Transfer 1.0 ml of this Standard solution to a 200-ml volumetric flask, add a mixture of chloroform and 98 percent formic acid (9:1) to volume, and mix (diluted Standard solution). On a suitable thin-layer chromatographic plate, coated with a 0.25-mm layer of chromatographic silica gel mixture, spot 10-µl portions of the test solution, the Standard solution, and the diluted Standard solution. Allow the spots to dry, and develop the chromatogram in a solvent system consisting of chloroform, methanol, and 98 percent formic acid (90:5:5) until the solvent front has moved about three-fourths of the length of the plate. Remove the plate from the developing chamber, mark the solvent front, allow the solvent to evaporate, and examine the plate under short-wavelength ultraviolet light. The R_f value of the main spot obtained from the test solution corresponds to that obtained from the Standard solution, and no spot, other than the main spot, in the chromatogram of the test solution is larger or more intense than the main spot obtained from the diluted Standard solution.

8.4.2 High-Pressure Liquid Chromatography (HPLC)

A sensitive method for separation and simultaneous determination of mebendazole and hydroxymebendazole in human plasma or cyst liquid sample using flubendazole as

internal standard by high-performance liquid chromatography with electrochemical detector is described (40).

The chromatographic conditions: The column (4.6 mm X 5 cm) was packed with Spherisorb 5 μm ODS (Phase Sep. Queensferry Clwyd, U.K.). The mobile phase consisted of 20% 2-propanol in a pH 7.0 phosphate buffer. The eluent was delivered by Kipp 9208 high-pressure liquid chromatography pump, at a flow rate of 2.5 ml/min. The electrochemical detector equipped with a rotating disc working electrode was used. The limit of detection for mebendazole and hydroxymebendazole was ~ 5 and 2.5 ng/ml respectively.

Allan et al (41) reported two high-performance liquid chromatographic methods for determination of mebendazole and its metabolites in human plasma using a rapid Sep Pak C18 extraction. The methods eliminate the need for solvent extraction as such. One method includes isocratic elution and other, gradient elution. The gradient elution system provides superior resolutions.

Plasma samples (5 ml) was spike with major metabolites of mebendazole, methyl 5-(α-hydroxybenzyl)-2-benzimidazole carbamate, 2-amino-5-benzoyl-benzimidazole and 2-amino-5-(α-hydroxybenzyl)-benzimidazole and with 5.0 μg of ethyl-5-benzoylbenzimidazole carbamate as internal standard in the range of 10 ng - 30 μg. Samples were adjusted to pH 6 with dilute hydrochloric acid or sodium carbonate solution and extracted by passing through a Sep Pak C18 cartridge fitted to a luer lock glass syringe. After the spiked plasma was passed through the cartridge, it was washed with 20 ml of distilled water, 0.5 ml of 40% methanol in water and 0.4 ml of methanol. The next 1.6 ml of methanol eluted the five compounds from cartridge, this 1.6 ml of methanol was evaporated to dryness in

pointed and the residue was dissolved in 100 µl DMSO. Aliquotes of 20 µl were injected into HPLC system. The instrument used was Altex Model 322 MP HPLC (Altex Scientific, Berkeley, CA, USA) equipped with fixed wavelength detector (254 nm, 8-µl flow cell), a Rheodyne (Berkeley, CA, USA) Model 7120 injector and Hewlett-Packard (Avondale, PA. USA) Model 3380 A integrator recorder. For mebendazole the determination limits are 20 ng/ml (isocratic system) and 10 ng/ml (gradient system).

Chromatographic Conditions:

Column	:	(250 x 4.6 mm I.D.) LiChrosorb, RP-8 10-µm reversed-phase packing. A precolumn (35 x 3.2 mm I.D.) dry packed with Corasil C18 was also used.
Mobile phase	:	Methanol-water (55:45 pump A) and methanol-aqueous ammonium phosphate 0.05 M pH 5.5 (55:45 pump B).
Flow rate	:	1.7 ml/min.
Pressure	:	1200-1300 p.s.i.

Karlaganis et al (42) developed an HPLC method for the determination of mebendazole in biological samples. 2 Ml plasma of the patients was alkalinized with 8 ml of sodium carbonate buffer (0.05 M, pH 11.3) and was extracted with 10 ml of chloroform by shaking in a glass tube for 15 minutes. The contents were centrifuged and aqueous phase was aspirated and the chloroform layer was evaporated under reduced pressure. The residue was dissolved in 200 µl mobile phase, 150 µl were injected. Analysis using Waters Model M6000A High-Pressure Liquid Chromatograph equipped with Wisp Model 710 injector and a Perkin-Elmer UV detector Model LC55.

Chromatographic Conditions:

Column	:	25 cm x 3 mm (i.d.) LiChrosorb Sl 60 5 m.
Mobile phase	:	Acetonitrile/water-saturated chloroform/25% (weight/ volume) ammonia in water (75/92.5/0.1, by volume), pH 6.5.
Flow rate	:	0.8 ml/min
Pressure	:	400 psi
Column temperature	:	Ambient
Detector wavelength	:	307 nm
Recorder	:	5 mV (0.02 absorption units full scale).

Calibration curve was prepared using 40, 80, 200 and 400 ng of mebendazole. 1 Mg of ciclobendazole was used as a internal standard, detection rate in the range of 6.117 ng/ml.

Alton et al (43) developed a simple, rapid and specific HPLC method for quantitative determination of mebendazole in plasma. 2 Ml of plasma was diluted with 2 ml of 0.05 M potassium hydrogen phosphate buffer (pH 7.0) and extracted with 7 ml of ethyl acetate. The organic layer was removed and dried, the residue was dissolved in 0.001 N HCl and re-extracted with 6 ml of petroleum ether and centrifuged, supernatent was discarded the aqueous fraction was alkalinized with 2 ml 0.01 N NaOH and extracted twice with ethyl acetate. The solvent was dried and dissolved in 60 µl of ethyl acetate and 20 µl was injected in chromatograph (Model ALC 202/204 Waters Associates) for analysis.

Chromatographic Conditions:

Column	:	μ Bondapak C-18 300 x 3.0 mm (i.d.) reversed-phase.
Mobile phase	:	0.05 M KH_2PO_4-NaOH buffer (pH 6.0)-acetonitrile (73:27 v/v).
Flow rate	:	2.5 ml/min
Pressure	:	2500 psi
Column temperature	:	Ambient
Detector wavelength	:	UV (313 nm).

Flubendazole was used as internal standard in the quantity of 0.15 μg/ml for each sample. This method can be used to measure plasma mebendazole level as low as 10 ng/ml.

A simple and rapid method to detect the presence of different benzimidazole compounds in drug preparations have been reported (44). The procedure involves two different chromatography systems:

i- Reversed-phase gradient.

ii- Normal partition chromatography with isocratic elution.

Three different mixtures A, B, and C were used. The benzimidazoles were dissolved in pure formic acid (5 ml) then in concentrated hydrochloric acid (5 ml) and in 50% (%) aqueous ethanol (90 ml). Mixtures A and C contain mebendazole. The solutions A and B were separated by reversed-phase gradient elution with the solvent containing acetonitrile - aqueous 1% H_2SO_4 with the programme: 10% acetonitrile for 2 minutes then from 10% acetonitrile to 30% at a rate of 5% per minute. Mixture C was eluted

isocratically on a amino phase chemically bonded to silica gel (Type NH_2) with the solvent: methylene chloride + isopropyl alcohol (90 + 10) - acetonitrile (50:50). Initially for determination of retention time each of the benzimidazoles was separately chromatographed.

Chromatographic Conditions:

Chromatograph	:	Varian LC 8500 HPLC with UV detector.
Column	:	LiChrosorb RP 8 - micro-particulate (15 cm 4.7 mm i.d.) LiChrosorb NH_2 (Merck) (15 cm x 4.7 mm i.d.)
Mobile phase	:	Methylene chloride + isopropyl alcohol (90 + 10) - acetonitrile (50:50).
Flow rate	:	80 ml h^{-1}
Detector sensitivity	:	0.5 AUFS
Column pressure	:	500 psi
Detector wavelength	:	(UV) 254 nm

This method would be useful in the quality control of raw materials in the quantification of benzimidazoles in foodstuffs of animal origin, and in the detection of residues.

A simple, accurate and reproducible HPLC method has been described (45) for determination of mebendazole in tablets. 20 Tablets were taken and ground in fine powder, equivalent to about 10 - 20 mg of mebendazole was accurately measured out for each determination. The powder was extracted with 5 ml of formic acid. 5 Ml of salicylamide solution (8.5 mg/ml) was added to each

extract as internal standard. The mixture was centrifuged and the supernatent was used for HPLC determination.

Chromatographic Conditions:

Chromatograph: Waters Associates Model 440

Column : μ Bondapak C-18

Mobile phase : Tetrahydrofuran-0.5% formic acid (30:60).

Flow rate : 1.9 ml/min.

Temperature : 25-28^{o}C.

Pressure : 1500 psi.

Detector wavelength : (UV) 254 nm

The recovery was between 99.58 - 100.15%.

An HPLC method for the estimation mebendazole, its prodrug, 4-amino-3-(3'-methoxycarbonyl 2'-thioureido)benzophenone, and their known or expected metabolites and degradation products in aqueous media and rat blood was developed (46). After oral administration of the prodrug, the prodrug was rapidly converted to mebendazole and the area under the blood level vs time curve of mebendazole, in rats dosed with prodrug, was more than twice that obtained after giving rats an equimolar amount of mebendazole. Only the prodrug, mebendazole and known metabolites of mebendazole were detected in rats given the prodrug.

Behm et al (47) have measured the concentration of mebendazole and its major metabolites by HPLC in sheep plasma at 12.5, 25, 50 or 100 mg/kg. At 12.5 mg/kg the peak plasma concentrations occured between nine and 24 hours for all dose rates and decline rapidly. Two major metabolites were detected; their

concentrations exceeded that of mebendazole at all dose rates.

8.5 Polarographic Method

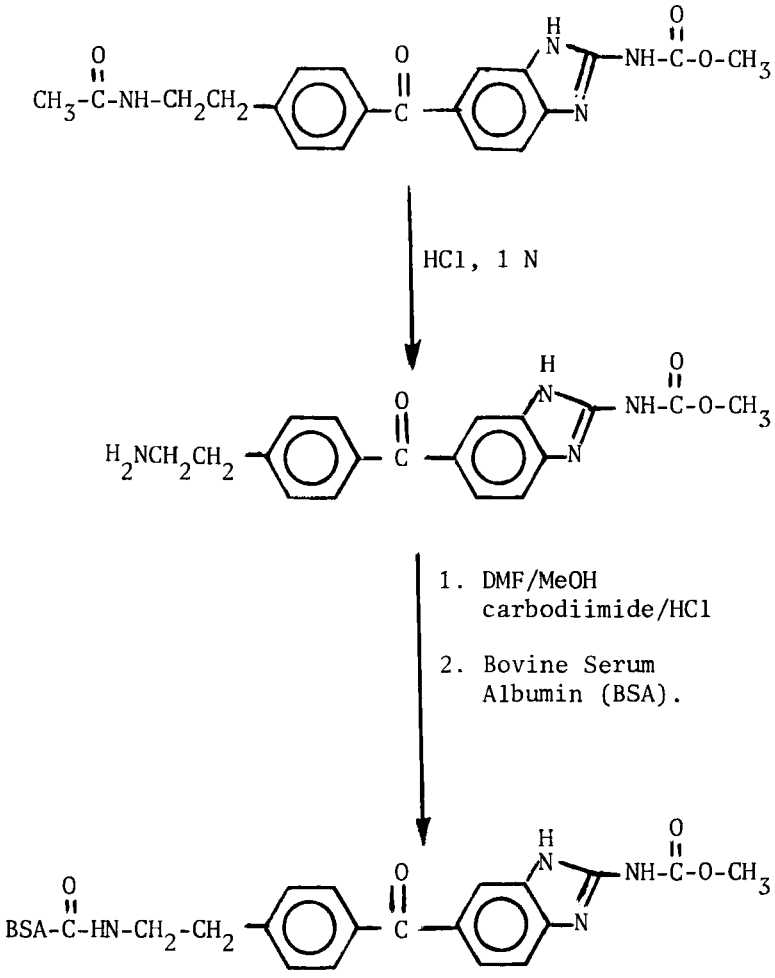
Pinzauti et al (48) described a direct-current polarographic reduction method of mebendazole at the dropping-mercury electrode, and established the optimum conditions for the determination of this drug in dosage form. Six tablets of mebendazole (about 0.3 g) were ground. A quantity of the powder equivalent to 50 mg of mebendazole was transferred to 100 ml volumetric flask. To this powder 8.6 ml of 70% perchloric acid was added and the mixture stirred for 10 minutes and diluted to volume with water. The suspension was filtered under suction through a fine porsity glass crucible. 2 Ml portion of the filtrate was transferred to a 50 ml volumetric flask, 3 ml of 1 M perchloric acid was added and the solution diluted to volume with the McIlvaine buffer pH 2.6 (2.18 vol. 0.2 M sodium phosphate with 17.82 vol. 0.1 M citric acid).

A 20 ml of this solution (corresponding to 0.4 mg of mebendazole) was transferred into polarographic cell (Metrohm E506 recording polarograph equipped with polarography stand).

Deaeration and polarography were performed on using Metrohm EA 290 hanging mercury drop system. The number of electrons involved in the reduction was calculated at a mebendazole concentration of 1×10^{-4} M using a solid state control potential coulometric apparatus. A calibration curve was prepared using 5 to 50 µg/ml mebendazole solution prepared in McIlvaine buffer, using this method mebendazole analysis could be performed with a limit of detection 100 ng/ml (48).

8.6 Radioimmunoassay Method

Michiels et al (49) developed an extremely highly specific radioimmunoassay procedure for the determination of mebendazole in plasma. Mebendazole was converted to methyl [5-[4-(2-aminoethyl) benzoyl]-1H-benzimidazol-2-yl]carbamate. This hapten was coupled to bovine serum albumin using a water soluble carbodiimide as follows:



Synthesis of the hapten and the mebendazole-protein conjugate used for the immunization of the rabbits (49).

The resulting conjugate was used to immunize rabbits according to conventional procedures. The anti-serum elicited in this way was tested for its ability to bind specifically mebendazole. Blood was collected on EDTA and plasma was obtained after centrifugation of the blood at 2200 g for 15 minutes. Unmetabolized mebendazole was measured by direct radioimmunoassay using anti-bodies. Under the standardized assay condition, the rabbit serum bound specifically nearly 50% of added 0.2 ng H^3-Mebendazole at 1/100 serum dilution. Unspecific adsorption to plasma constituents did not exceed 2%. The method is quite sensitive to assay mebendazole in plasma at concentration as low as 100 picogram/ml. Metabolites did not interfere with the binding of parent drug to antibodies.

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METOCLOPRAMIDE HYDROCHLORIDE

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1. Description

1.1 Nomenclature

1.2 Chemical Names

Benzamide, 4-amino-5-chloro-N-[2-(diethylamino) ethyl]-2-methoxy,
monohydrochloride, monohydrate CAS 54143-57-6
anhydrous CAS 7831-21-5
4-Amino-5-chloro-N-[2-(diethylamino)ethyl]o-anisasamide

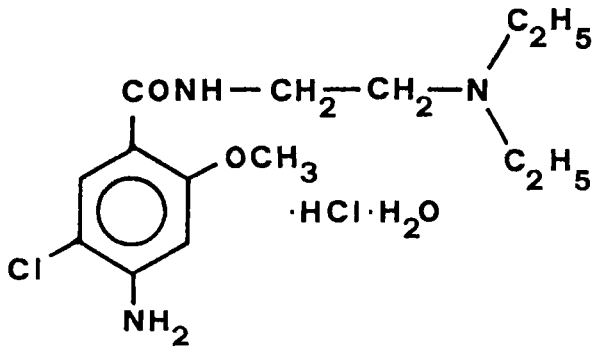
1.3 Generic Names

Metoclopramide Hydrochloride USAN-BP 1980-F.U.IX
Metoclopramidhydrochloride DAC 1979
Metoclopramidum hydrochloricum 2.AB-DDR

1.4 Trade Names

Cerucal (Arzneimittelwerk-Dresden)
Elieten (Nippon Kayaku, Tokyo-Japan)
Emperal (Neofarma-Helsinki)
Maxeran (Delagrange-Paris)
Plasil (Lepetit-Milan)
Peraprin (Taiyo, Gifuken-Japan)
Primperan (Delagrange-Paris)
Reglan (Robins, Richmond, USA)

1.5 Formula and Molecular Weight



$C_{14}H_{22}ClN_3O_2.HCl.H_2O$ Mol.Wt. = 354.3

1.6 Appearance, Color, Odor, Taste

A white or almost white crystalline powder, odorless (1)

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum of metoclopramide hydrochloride, is presented in Fig. 1. The spectrum was recorded with a solid sample disc composed of 1 mg of compound/200 mg KBr on a Perkin Elmer Model 1310.
The following bands (cm^{-1}) were assigned and are reported in Table 1.

Table 1

Wave Number	Assignments
3200, 3300, 3340, 3400, 3460	νNH , νOH
2860, 2950, 2980	$\nu C_{sp^3}H$
3030	$\nu C_{sp^2}H$
2100, 2500, 2660, 2710	$\nu \overset{+}{N}H$
1600	νC=O
1540	δNH (Amide)
1270	νC-O-C (Asymmetric)
700	νC-Cl

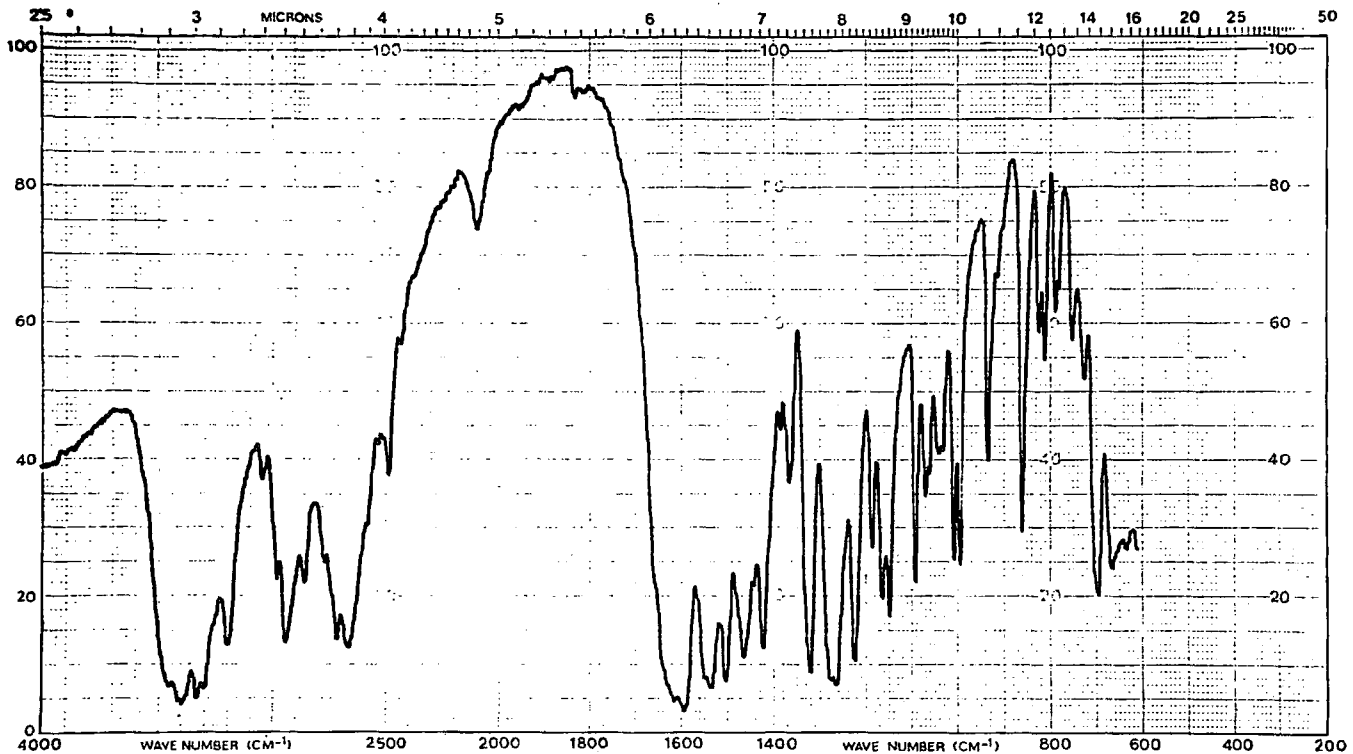


Fig. 1 - Infrared Spectrum of Metoclopramide Hydrochloride

2.2 Nuclear Magnetic Resonance Spectra

2.2.1 ^{1}H-NMR

^{1}H-NMR spectrum of metoclopramide hydrochloride, shown in Fig. 2, was obtained in DMSO-d_6 with a Varian spectrometer EM-390 operating at 90 MHz. The chemical shifts are listed in the Table 2.

Table 2

H (ppm, TMS)	Moltepli city	Number of protons	Assignments
1.33	t	6	$N-(CH_2-\underline{CH}_3)_2$
3.0-3.2	m	6	$\underline{CH}_2-N-(\underline{CH}_2-CH_3)_2$
3.72	q	2	$NH-\underline{CH}_2$
3.95	s	3	OCH_3
6.03	broad s	2,exch.	NH_2
6.60	s	1	H_3 (arom.)
7.72	s	1	H_6 (arom.)
8.44	t	1,exch.	CONH
10.85	broad s	1,exch.	$\overset{+}{N}H$

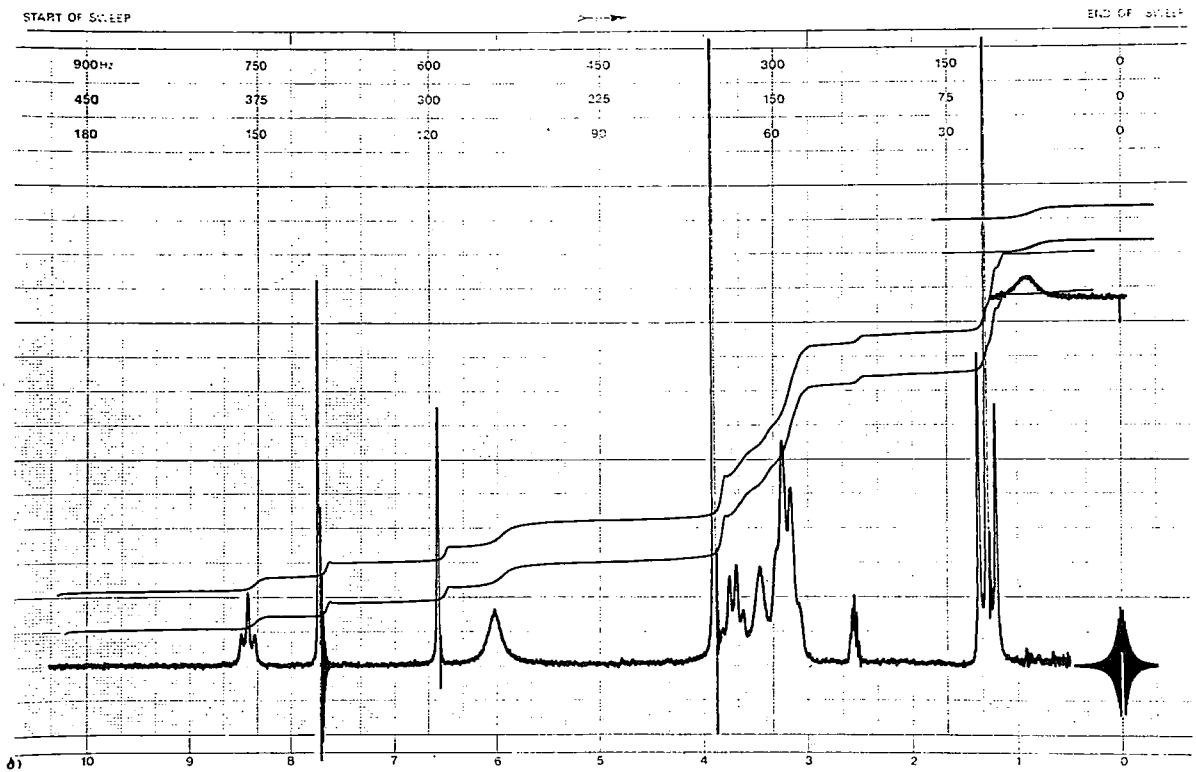


Fig. 2 - ^{1}H-NMR (90 MHz) Spectrum of Metoclopramide hydrochloride in DMSO-d_6

2.2.2 ^{13}C-NMR

The ^{13}C-NMR spectrum of metoclopramide hydrochloride is shown in Fig. 3. The spectrum was recorded in D_2O with Varian XL.200 spectrometer operating at 50.391 MHz. The chemical shifts are listed in Table 3.

Table 3

Line N.	(p.p.m)	Multiplicity	Assignments
1	8.38	q	$N(CH_2.\underline{CH}_3)_2$
2	35.15	t	$\underline{CH}_2\text{-}N(C_2H_5)_2$
3	48.27	t	$N(\underline{CH}_2\text{-}CH_3)_2$
4	51.40	t	$NH.\underline{CH}_2$
5	55.89	q	OCH_3
6	98.00	d	C_3 (aromatic)
7	109.18	s	C_1 or C_5
8	110.61	s	C_5 or C_1
9	131.43	d	C_6
10	148.57	s	C_4
11	158.07	s	C_2
12	167.46	s	C=O

2.3 Ultraviolet Spectrum

The ultraviolet spectrum of metoclopramide hydrochloride in water ($c = 5.763\ 10^{-5}$ m/l) was obtained (60) with a Beckman Mod. 24 spectrophotometer and it is shown in Fig. 4

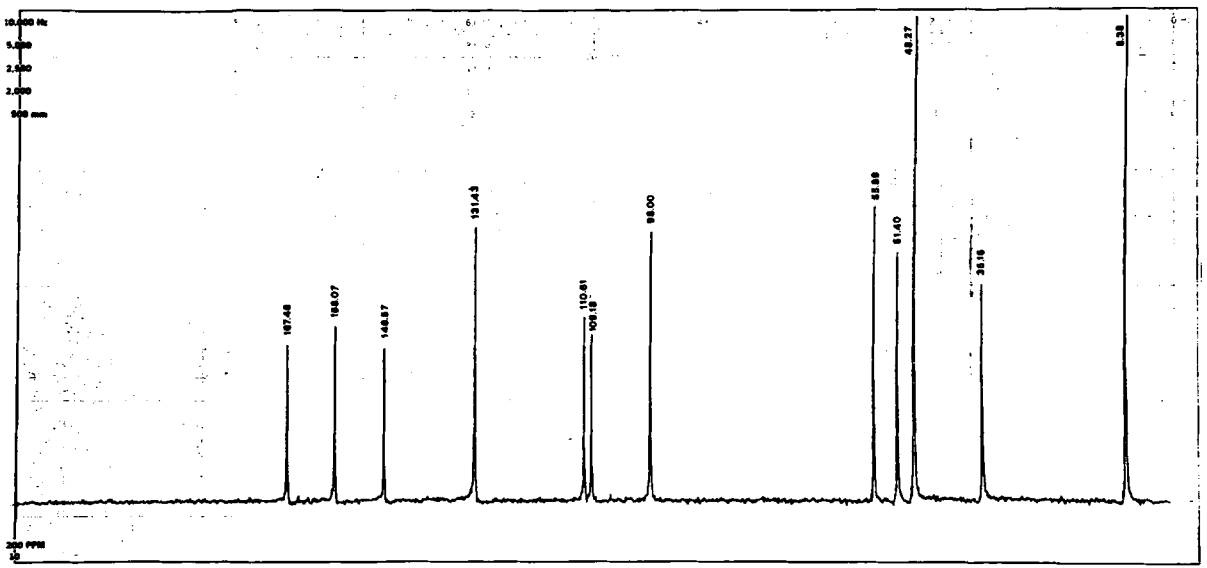


Fig. 3 - ^{13}H-NMR (90 MHz) Spectrum of Metoclopramide hydrochloride in DMSO-d_6

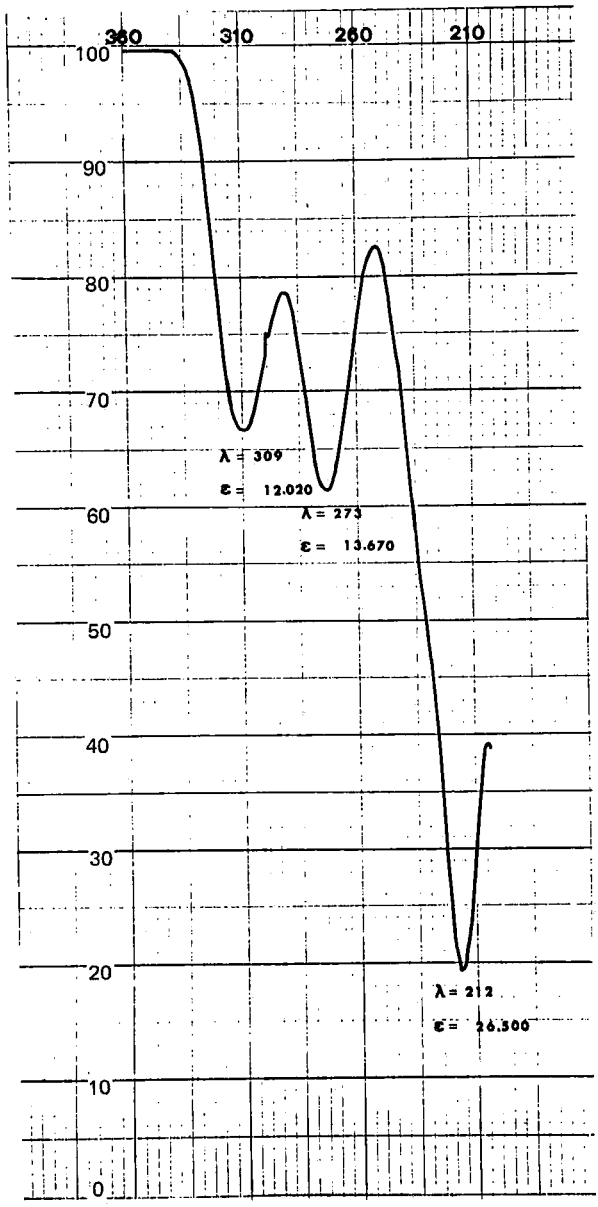
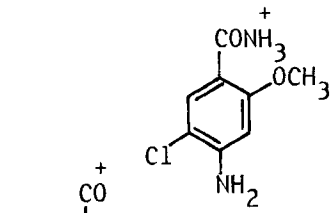
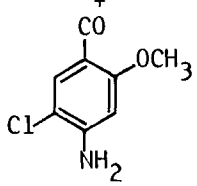
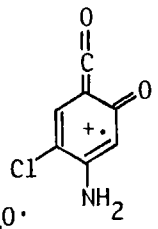
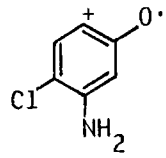
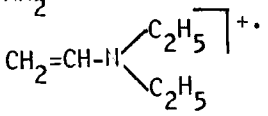
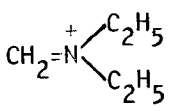


Fig. 4 - Ultraviolet Spectrum of Metoclopramide hydrochloride in Water

2.4 Mass Spectrum

The mass spectrum of metoclopramide hydrochloride was recorded (Fig.5) on Finnigan 1020 spectrometer by conventional electron impact ionisation at 70 eV. Prominent fragments and their relative intensity are shown in Table 4.

Table 4

m/e	Intensity	Attribution
229	0.23	$[M]^{+\cdot}$
227	0.90	$[M - N(C_2H_5)_2]^{+}$
201	7.60	
181	6.43	
169	0.66	
141	1.78	
99	27.96	
86	100	

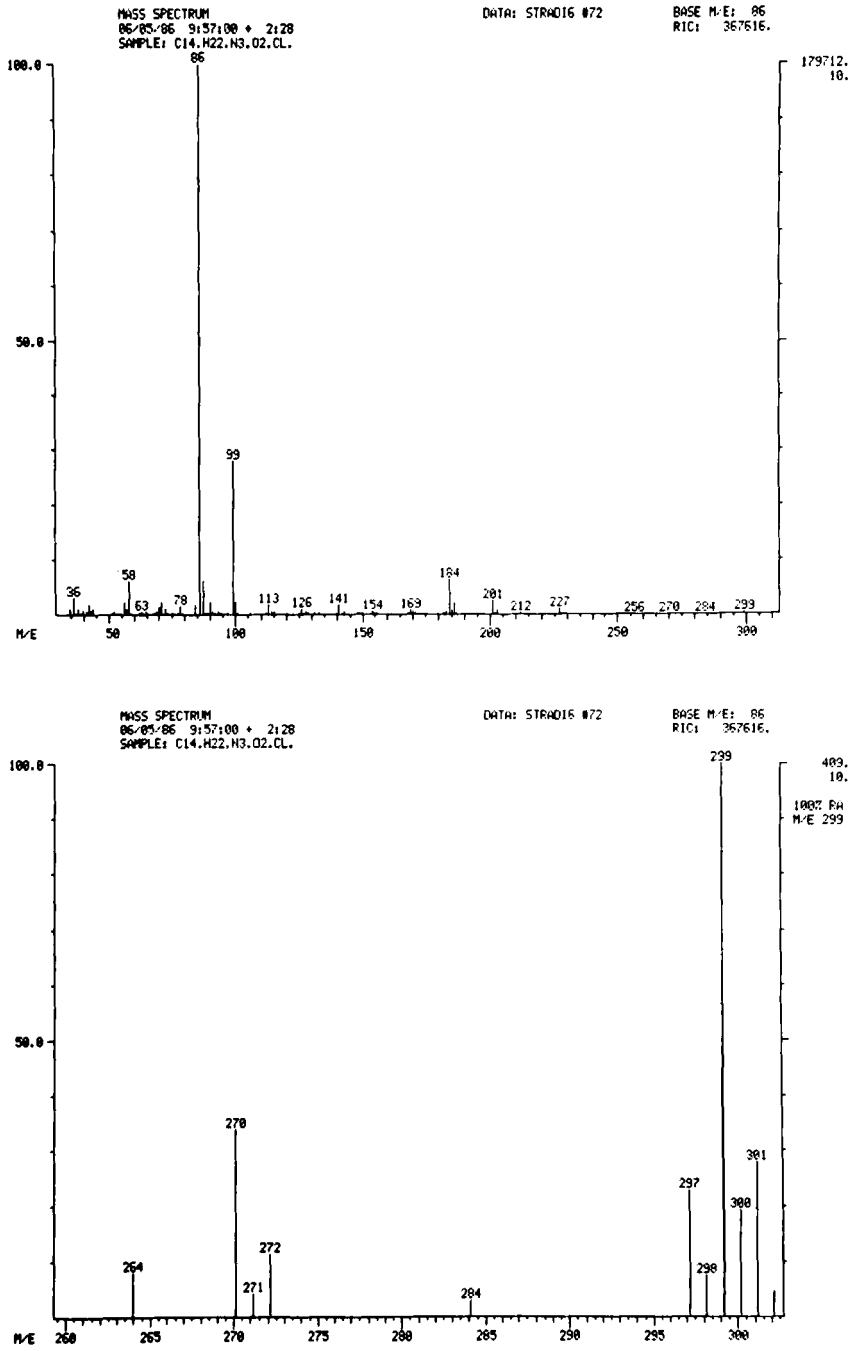


Fig. 5 - Electron impart Mass Spectrum of Monoclopramide hydrochloride

2.5 Fluorescence Spectrum

A solution of metoclopramide hydrochloride in water exhibits fluorescence when excited with ultraviolet light. When excited at 353 nm, it shows a sharp peak at 353 nm and a second peak at about 273-276 nm.
The emission spectrum of the compound consists of a single peak at 355 nm. The spectra obtained in water (5 mcg/ml), on a spectrophotometer Perkin-Elmer MPE 44A,are given in Fig.6.

2.6 X-Ray Powder Diffraction

The X-ray powder diffraction data of metoclopramide hydrochloride was determined (2) by a Philips Powder Diffractometer PW 1710 with nichel-filtered copper radiation (1.54051 A) with the following instrumental conditions:

TUBE: BF type, CU/Ni, 40 KV, 40 mA; SLITS: 1°-0.1 mm-1°; DETECTOR:PW1711 proportional counter + discriminator; SCALE: $2x10^3$ cps; TIME CONSTANT: 1"; SCANNING SPEED: 0.008°x1"; PAPER SPEED 1 cmx1°; SPECIMEN HOLDER: Niskanen.
The X-ray diffraction data are given in Table 5.

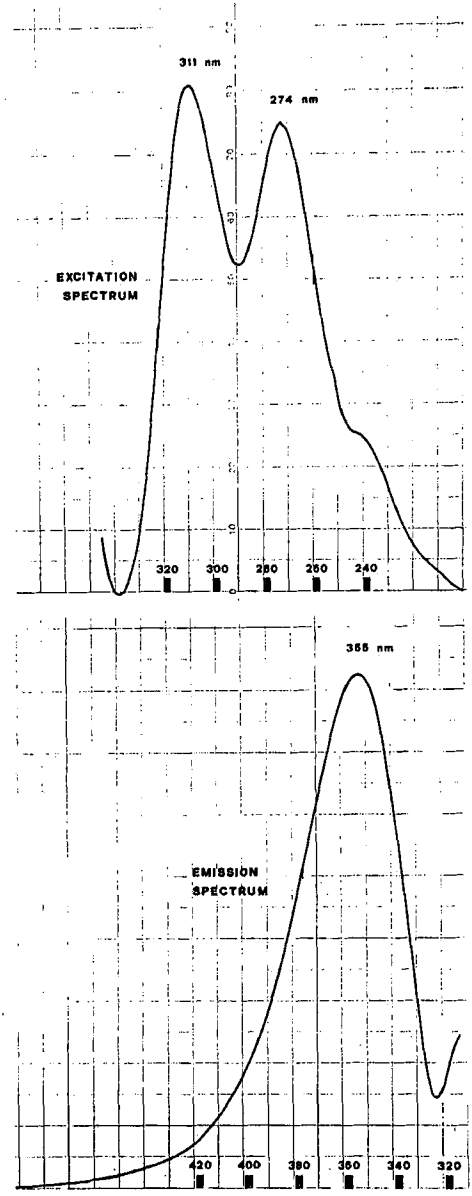


Fig. 6 - Fluorescence Spectrum of Metoclopramide hydrochloride in water

Table 5

N.	d(A)*	I/I_o**	N.	d(A)*	I/I_o**
1	9.23	5	27	3.15	12
2	8.46	25	28	3.07	8
3	7.64	20	29	3.04	6
4	6.98	22	30	2.99	11
5	6.64	13	31	2.95	12
6	6.28	65	32	2.92	10
7	6.02	8	33	2.86	20
8	5.26	20	34	2.82	13
9	5.18	10	35	2.78	8
10	4.87	38	36	2.74	21
11	4.71	45	37	2.69	11
12	4.60	30	38	2.59	10
13	4.48	31	39	2.53	9
14	4.27	10	40	2.51	13
15	4.23	20	41	2.43	18
16	4.03	20	42	2.33	10
17	3.95	6	43	2.31	8
18	3.82	47	44	2.29	10
19	3.78	35	45	2.26	11
20	3.71	17	46	2.16	11
21	362	10	47	2.14	10
22	3.50	58	48	2.05	8
23	3.47	30	49	2.01	9
24	3.41	75	50	1.81	8
25	3.36	100	50	1.81	8
26	3.32	11	51	1.71	7
			52	1.63	9

PLUS OTHER LINES <5

* Interplanar distances = 1.54051/2sin diam.

** Relative intensities are based on highest intensity of 100

2.7 Crystal Structure

The metoclopramide hydrochloride monohydrate is monoclinic, space group $P2_1/n$ with a = 12.138(3), b = 8.553(2), c = 17.120(4) A and β = 98.06(7) Z = 4. The structure was solved by direct methods and refined to R = 0.055 (3).

The metoclopramide free base (CAS 364-62-5) is triclinic, space group P1 with a = 13.310 b = 8.728, c=7.477 A, α = 114.20, β = 81.13 and γ = 101.23°, Z = 2. A study of structure-activity relations shows an analogy between metoclopramide and apomorphine(4).

2.8 Melting Range

Employing the USP method the metoclopramide monohydrochloride monohydrate melts at 182-185°(5) and with termal microscopy the melting begins at 181° and completes at 183°(6). The dihydrochloride monohydrate melts in the range of 148° with decomposition. The melting points for metoclopramide free base are about 148°(7) or m.p. 146-148°(8). For dihydrochloride is also reported an eutectic melting point of 160° with phenacetin and of 117° with benzanilide(7).

2.9 Solubility

At 25° metoclopramide monohydrochloride is soluble in 0.7 g of water, 3 g of ethanol (96 per cent) and 55 g of chloroform, whereas it is practically insoluble in ether (10). Solubility of the base and of dihydrochloride are reported in Table (6). (11).

Table
Solubility of metoclopramide g/100 ml at 25°

Solvent	Base	Dihydrochloride
Water	0.02	48
Ethanol 95	2.90	9
Ethanol	1.90	6
Benzene	0.10	0.10
Chloroform	6.60	0.10

2.10 pKa

Metoclopramide hydrochloride shows two ionisation costants; pK_1 = 9.71 and pK_2 = 0.42. The determination was carried out spectrometrically in aqueous solution and the values are a mean of 16 determinations with a standard deviation of 0.03 and 0.02 (12).

2.11 pH Range

The pH of a 10% water solution must be between 4.6 and 6.5 according to BP 1980.

2.12 Partition Coefficient

The partition coefficient in octanol/water has been determined(13) by reversed-phase HPLC at 20°. The hydrochloride gives

Experimental	LogP = 2,667
Calculated from R.A. Deckker (14)	LogP = 2.76

3. Manufacturing Procedures

3.2 Synthesis

Among the synthetic methods leading to metoclopramide (I) it seems suitable to report only the ones of greatest interest that are those using p-aminosalicilic acid (II), as starting product, a compound whose properties are economically excellent. The two main processes are listed in Fig 7.

In Scheme A, referring to the first invention(15), the acid (II)is acetylated to (III) and then treated with methyl sulfate to give the ether-ester (IV). This compound is reacted with $(C_2H_5)_2N-CH_2CH_2-NH_2$ to the corresponding amide. The subsequent desacetylation leads to metoclopramide(1).

In scheme B the first step in the synthesis is the ether-ester (VI). The protection of the amino aromatic group was not considered necessary (16). The direct chlorination (18) of (VI) with sodium ipochloride gives (VII) which is transformed in excellent yields into (I).

4. Stability

Aqueous solution

The maximum stability of metoclopramide, investigated at different pH values, was found at pH = 7.6 while the maximum degradation was found at pH = 2. One of the degradation products was identified as N,N-diethylethylendiamine(17).

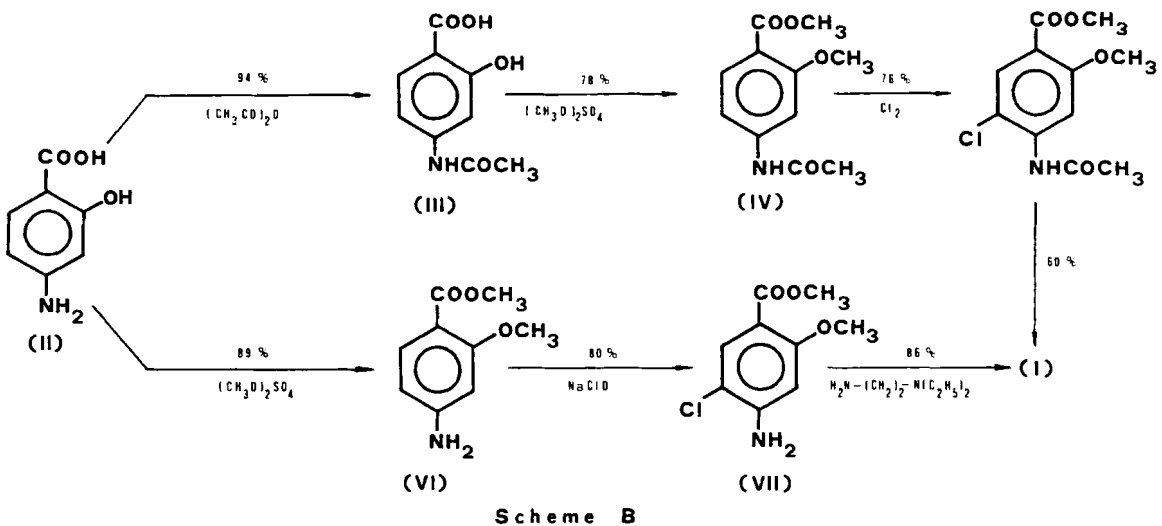


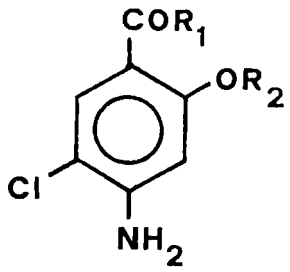
Fig. 7 - Synthesis of Metoclopramide

5. Metabolism and Pharmacokinetics

5.1 Metabolism

The metabolites identified in the in vitro and in vivo studies are listed in Table 7, in which metoclopramide is indicated as (I).

Table 7
Non conjugated metabolites found



N.	R_1	R_2	REFERENCES
I	$-NHCH_2CH_2N(C_2H_5)_2$	$-CH_3$	(18) (19)
II	$-NHCH_2CH_2NHC_2H_5$	$-CH_3$	(18) (20) (21)
III	$-NHCH_2CH_2NH_2$	$-CH_3$	(18) (19)
IV	$-OH$	$-CH_3$	(19) (20) (21)
V	$-NHCH_2CH_2N(C_2H_5)_2$	$-H$	(19) (20)
VI	$-NHCH_2CH_2N(C_2H_5)_2$	$-H$	(19)
VII	$-NH_2$	$-CH_3$	(20)
VIII	$-NHCH_2COOH$	$-CH_3$	(20)
IX	$-NHCH = CH_2$	$-CH_3$	(20)
X	$-NHCH_2CH_2NHCOCH_3$	$-CH_3$	(20)

By studies of the in vitro metabolism of (I) using hepatic fractions from several animal species (1) (2) five metabolites (II) (III) (IV) (V) (VI) were detected.
Identification studies of the metabolites in urinary excretes of rat and dog (20) after enzimatic hydrolysis evidenced some compounds already known from the in vitro studies and some new ones corresponding to (VII) (VIII) IX) (X). In particular in rat urine, were found metabolites (II) (IV) (V) (VII) (VIII), while in dog urine were present metabolites (V) (VI) (VIII) (IX) (X). In human urine the excretion was found to be 60% in the first 24 hours. After enzimatic hydrolisis was identified metabolite (VIII)(CAS 52702.04.2), but the main metabolite was found to be unchanged metoclopramide originated from the sulfate and glucuronide. Metabolic products were quantified in rabbit urines (21) (II) and (IV) but also unchanged (I),as N^4-glucoronide and N^4-sulfonate were identified. Another product of N-oxidation of (I) was also found. Metoclopramide N^4-sulfonate (CAS 27260-42.0) is the main metabolite in human urine.

5.2 Pharmacokinetic

The studies of pharmacokinetics of ^{14}C-metoclopramide in rats, dogs and humans show that the drug is distributed within a few minutes after oral administration and it is eliminated mainly in the urine of the first 24 hrs. (20).

Table 8
Excretion of ^{14}C-metoclopramide in rats, dogs, and humans

Dosage p.o. mg/kg	100		20		10	
hours	Rats		Dogs		humans	
	U	F	U	F	U	F
0-24	71.9	5.7	65.3	----	77.8	20
24-48	8.5	4.4	6.9	18.3	8.7	1.0
48-72	1.0	1.7	1.0	0.8	0.8	1.6

U = urinary excr. F = fecal excr. % of the administered dose

The pharmacokinetic of metoclopramide HCl was studied in normal male volunteers after i.v. dosage of 10 mg. The shape of the plasma level curve in these subjects fits a two-compartment model with a distribution of $T1_{/2}$ α = 4.6 min. and an elimination of $T_{1/2}$ β = 165.7 min. The body plasma clearance was 10.9 ml/min/g (22) (23). G. Berner and co-workers (24) reported the study of bioavailability of commercial formulations of metoclopramide in male healthy volunteers. The following results were obtained: for tablets 57.6-80.3%, for solutions 49.7-76.7% and for capsules 54.8%.

5.3 Acute Toxicity

The acute toxicity i.v. (LD_{50}) of the metoclopramide dihydrochloride is 85.7 mg/kg for the rat and 71 mg/kg for the mice (25).

6. Methods of Analysis

6.1 Elemental Composition

C, 47.4; H, 7.12; Cl, 20.0; N, 11.9; H_2O, 5.1 for the monohydrate

C, 50.01; H, 6.89; Cl, 21.08; N, 12.5; O, 9.50 for the anhydrous form

C, 56.9; H, 7.40; Cl, 11.83; N, 14.02; O, 10.67 for the base.

6.2 Identification Tests

a) Infrared Spectroscopic Test

BP 1980 (10) cites the use of the infrared absorption spectrum of a potassium chloride dispersion of the metoclopramide hydrochloride in accordance with the reference spectrum reproduced in the appendix.

b) Ultraviolet Spectroscopic Test

The light absorption, in the range 230 to 350 nm of a 2-cm layer of a 0.001 per cent w/v solution in 0.01M hydrochloric acid exhibits two maxima, at 273 nm and 303 nm; absorbance at 273 nm about 0.79 and at 309 nm about 0.69. This is another identification test recommended by B.P.1980 (10).

c) Color Tests

With a solution of 4-dimethylaminobenzaldehyde - yellow-orange(1); ammonium vanadate test - light brown (sensitivity : 1.0 ug), (7).
Vitali's test: pale-yellow/light brown (sensitivity : 1.0 ug) (7).

d) Crystal Tests

Gold Cyanide solution - needles, sometimes in rosettes (sensitivity: 1 in 1000).
Lead iodide solution - feathery rosettes (sensitivity: 1 in 1000) (7).

6.3 Nonaqueous Titration

The metocloropramide hydrochloride may be titrated (10) potentiometrically in glacial acetic acid containing mercuric acetate with perchloric acid in glacial acetic acid as titrant.

6.4 Complessometric Analysis

The method is based on the use of a column of amberlite IR 120 in the Zn^{2+} form. The released quantity, corresponding to the absorbed metoclopramide, is titrated with EDTA using Eriochromo Black T as indicator (26).

6.5 Colorimetric Analyses

The colorimetric assays have been the most widely used especially to determine metoclopramide in pharmaceutical preparations. They are listed in the following table 9.

Table 9
Colorimetric assays of Metoclopramide

N.		max	Bear's law /ug/ml	Ref.
1	Citric acid in Ac_2O	600	2-14	27
2	NH_4SCN + Co $(NO_3)_2$	620	--	28
3	Z α, β-dinitrostilbene	390	--	29
4	Diazotization+N(1-naphthyl) ethylendiamine	525	0.4-4	30-11
5	HNO_2 to form the nitrous derivative	375	10-60	31
6	Diazotization + α-Naphthyl-amine	510	0.1-11	32
7	Diazotization + R-Salt	500	1-14	33
8	Dragendorff's	475	20-120	34
9	Diazotization + NH_4 Sulfamate	495	1-11	35
10	Diazotization + 2,4-dihydroxybenzoic acid	440	0.8	36

6.6 Ultraviolet Spectrophotometric Analysis

B.P 1980 (10) has described a spectrophotometric assay for the quantitative determination of metoclopramide tablets. After the extraction of the base in chloroform, the metoclopramide can be determined by measurement of absorbance at 305 nm. E(1%, 1cm) = 265.

6.7 Fluorimetric Analysis

Fluorimetric measurements are carried out in pH=2 buffer solution on the basis of intense emission at 360 nm that occurs when the sample is excited at 310 nm. The method, with detection limit of 3×10^{-2} ug ml^{-1}, was successfully applied to analyses of pharmaceutical formulations with a recovery of 100.8-101.8% and a coefficient of variation of 0.9-1.9% (37).

Chromatography

6.8 Paper Chromatography

Several chromatographic assays are summarized in the following table 10.

Table 10

Solvent system	Paper	Detection	Rfx100	Ref.
4.6 g of citric acid in 130 ml H_2O and 870 ml BuOH	A	U.V. Iodoplatinate spray	45	(7)
Acetate buffer pH = 4.58	B	"	77	(7)
Phosphate buffer pH = 7.4	A	"	39	(7)
Toluene: Methanol: conc. NH_4OH	C	1% of chloranyl in Bz	-	(38)

Paper:

A - Whatman N 1, sheet 11 x 6 cm buffered by dipping in a 5% solution of sodium citrate, blotting and drying at 25° for 1 hour.

B - Whatman N 1 N 3, sheet 17x 19 cm impregnated by dipping in a 10% solution of tributyrin in acetone and drying in air.

C - Whatman N 1

6.9 Thin-Layer Chromatography

The methods for separation and detection of metoclopramide are summirized in table 11.

Table 11

Solvent system	Plate	Rf x 100	Reference
I	A,B	40	(7)(38)
II	C	10	(39)
III	C	46	(39)
IV	C	16	(39)
V	C	54	(39)
VI	C	29	(40)(41)
VII	C	55	(41)
VIII	F	-	(11)
IX	C	98	(21)
X	C	68	(21)
XI	F	47	(42)
XII	F	13	(42)

Solvent Sytem

I Strong ammonia solution: methanol (1,5:100)
II Methanol-chloroform (1:4)
III 1,2-Dichloroethane-ethanol-ammonia solution (sp.gr.0.88) (70: 15:2)
IV n-Butanol-acetic acid-water (4:1:1)
V Isopropanol-ammonia solution (sp.gr. 0.88) (80:4:5)

VI Butanol-acetic acid-water (4:1:1)
VII Isopropanol-NH_4OH-water (80:4:5)
VIII Dioxan-NH_4OH-benzene (8:1:1)
IX Chloroform-methanol-28% ammonia (10:4:1)
X Chloroform-methanol-dioxane-28% ammonia (90:14:10:3)
XI Methanol-ammonia (100:1.5)
XII Acetone

Detection

- Acidified iodoplatinate spray
- Spray solution: 1% solution sodium nitrite in HCl N and then with 0.4% of N-(1-naphthyl)-ethylaminediammonium dichloride as coupling agent in methanol.
- Spray solution: 0.2% solution of chloranil in acetonitrile followed by heating the plate at 100-105° for 2 min.
- Spray solution: 2 g of iodic acid dissolved in 10 ml H_2O and made up to volume of 100 ml with 90% (w/v) sulfuric acid (5).
- Ultraviolet light (250 nm).

Plate

A. Glassplate, 20 x 20 cm coated with silica gel
B. Pre-coated silica gel G (Merck)
C. Silical gel 60 F_{254} (Merck)
D. GF Plate. Analtech (6)
E. Kodak Silice Fluorescente K301
F. Silica dipped in 0.1N KOH and dried

6.10 High Performance Thin-Layer Chromatography

HPTLC was performed on metoclopramide base using HPTLC Fertiplatten Kiegelgel 60F(Merck)and MeOH:$CHCl_3$: NH_4OH 25% (80:85:0.2) as solvent. The compound may be quantified by means of excitation fluorimetry at 312 nm and emission at 365 nm integrating the peak area with a videointegrator. The results are linear from 160 /ug to 20/ug with a standard deviation of 2-5% (43).

6.11 Gas Liquid Chromatography

T. Daldrup and co-workers (44) described a GLC assay method on a glass-column (6 ft x 2 inc.) packed with 3% OV-1 on Chromosorb W-HP (100 to 120 mesh) and operated at 150°-250° (10°C/min), with N_2 as carrier gas (50 ml min^{-1}) and a flame ionization detector. Internal standard: 2-amino-5-chlorobenzophenone. Detector and injection temperature were 350° and 250°C. Retention time: 1.87.

GLC has been used as the method for the determination of the drug and its 15 metabolites in biological fluids (45) (46) (47) (48) and Y.K. Tamond, J.E. Axelson (49) have been determined the metoclopramide in picogram quantities in plasma with a ^{63}Ni-electron capture detector. The procedure involved the extraction of the drug from an alkalinized aqueous solution in benzene and derivatization with heptafluorobutirric anhydride.

The determination of the derivative was done using diazepam as internal standard. Working conditions: glass column (1.2 m x 2 inc. packed 3% OV-17, coated with 80-160 mesh, chromosorb W. Injection 258°; oven 250 and detection 350.

A mixture of argon-methane (95:5) was employed with a flow rate of 40 ml/min^{-1}. The retention times were 3.76 min for the metoclopramide and 9.1 min for diazepam.

6.12 High-pressure Liquid Chromatography

Teng et al.(1) used a column 15 cm x 0.5 mm packed with silica gel M 171; flow rate 2.0 ml/min^{-1} and CH_3OH--$CHCl_3$-NH_4OH conc. (30:70:0.5) at room temperature. Detection 280 nm. The retention times of metoclopramide is 2.8 min and 4.5 min for the internal standard (4-amino-5-chloro-N-/2-(propylamino)ethyl/-2-methoxybenzamide).
W. Block et al (50) determined metoclopramide using reverse--phase HPLC with an RP-18 column under isocratic condition (CH_3OH:H_2O:NH_4OH conc.,75:24.9:0.1). Flow rate 1.2 ml/min^{-1}. Detection at 308 nm. Elution after 5 min.

7. Determination of metoclopramide in Body Fluids and Tissues

The determination of metoclopramide in biological fluids of man and of various animal species is the object of many communications as reported below:

Blood (Plasma, Serum)

Colorimetry: (11)
TLC : (3) (41) (40) (52) HPLC: (24)
GC : (45) (46) (47) (53) (48)
HPLC : (54) (55) (20) (50) (53) (56) (57)
GC-Mass Spectrometry: (15)

Urine

Colorimetry : (21) (52)
TLC : (21) (40) (39)
HPCL : (20) (56)
GC : (54) (19)
GLC-Mass Spectrometry: (58)

Saliva
HPLC : (59)

Bile
Colorimetry : (21)

Feces
Radioactivity with ^{14}C-metoclopramide : (20)

Microsomal fraction of liver homogenate
HPLC : (39)

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MITOMYCIN C

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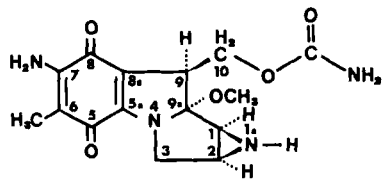
1. HISTORY

In 1956 Hata and coworkers (1) first reported about the isolation of a new group of antitumour antibiotics which they named mitomycins. These pigmented compounds were obtained by extraction and chromatographic purification of the fermentation broth filtrate of Streptomyces caespitosus. In 1958 another Japanese group, led by Wakaki, succeeded in the isolation of a mitomycin derivative, called mitomycin C, from the same actinomyces strain (2). Several mitomycin derivatives have since been isolated from Streptomyces species (3-8). Extensive chemical degradation studies, X-ray diffraction crystallography, chromatographic and spectroscopic analysis and biosynthesis studies have contributed to the elucidation of the structure and absolute stereochemistry of mitomycin C and related mitomycins (9-23a). Mitomycin C was selected for further development after it had been established that mitomycin C possessed profound antineoplastic activity and that the therapeutic index was more favourable than that of related mitomycin congeners. The drug has activity against an array of solid tumours such as adenocarcinomas of the gastrointestinal tract. Unfortunately, clinical experience with mitomycin C has shown that it causes severe toxicity in particular a delayed, cumulative bone marrow suppression. Mitomycin C is rarely used as a single agent except for the treatment of superficial cancer of the urinary bladder, intravesically administered. This administration mode has been shown to be very effective and devoid of serious haematological toxicities. For more specific information about the clinical usefulness of mitomycin C combinations in lung, breast and stomach cancer the reader is referred to review articles and books (24-31).

2. DESCRIPTION

2.1 Name, Formula, Molecular Weight

The generic name is mitomycin C (50-07-7). The drug is marketed under the trade names of Mutamycin®, Mitomycin-C Kyowa® and Ametycine®. The molecular formula for mitomycin C is $C_{15}H_{18}N_4O_5$. The molecular weight is 334.13.



2.2 Appearance, Colour, Odour

Blue-violet crystalline powder which is odourless.

3. BIOSYNTHESIS, CHEMICAL SYNTHESIS

By using ^{13}C, ^{14}C, ^{3}H and ^{15}N-labeled precursors in feeding experiments, investigators have studied the biosynthetic pathways of mitomycins (20, 21, 32-35). Although considerable progress has been made in elucidating the biosynthesis routes the exact pathway remains to be established. Feeding studies have revealed that D-glucosamine, L-methionine, L-citrulline, L-arginine, pyruvate and D-erythrose are candidates as biosynthetic precursors of the mitomycines (21). The total chemical synthesis of mitomycin C has been accomplished from 2,6-dimethoxytoluene as starting material by Kishi and collaborators (36). The synthesis route is lengthy (47 steps) and complex.

4. PHYSICAL PROPERTIES

4.1 Ultraviolet-Visible Spectrum

The ultraviolet spectrum of mitomycin C ($3x10^{-5}M$) in water is depicted in Figure 1. The spectrum was recorded by using a Shimadzu UV-200 double beam spectrophotometer in 1 cm quartz cell. In the visible region a broad band of low intensity is present. At pH values over 10 the absorption maximum at 360 nm shifts to 295 nm due to keto-enol tautomerization and deprotonation of the 7-amino quinone part of the mitomycin C molecule (Figure 2)(37). Molar absorptivities (ε) and $A^{1\%}_{1\ cm}$ values are reported in Table I (9).

Table I UV-VIS Spectral Data for Mitomycin C in Methanol(9)

λ_{max} (nm)	$A^{1\%}_{1\ cm}$	ε ($l.mol^{-1}.cm^{-1}$)
217	736	24,600
360	689	23,000
555	6.3	209

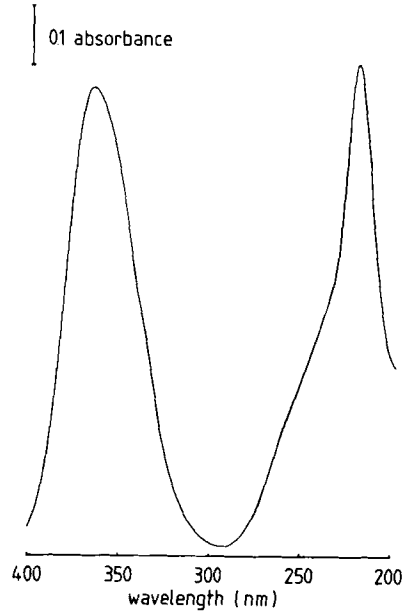


Figure 1.
UV spectrum of mitomycin C

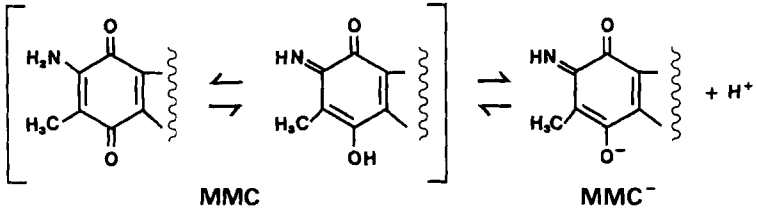


Figure 2.
Keto-enol tautomerization and deprotonation of mitomycin C (MMC)

4.2 Infrared Spectrum

The infrared spectrum of mitomycin C in a potassium bromide pellet is presented in Figure 3. The spectrum was recorded with a Jouan-Jasco IRA-grating infrared spectrophotometer. Assignments of a number of prominent bands is listed in Table II.

Table II Infrared Assignments for Mitomycin C

wavenumber (cm^{-1})	assignment
3460^{s}, 3310^{s}, 3260^{s}	ν(NH) and $\nu(NH_2)$ of aziridine, carbamate and 7C-NH_2
1735^{s}	ν(C=O) of carbamate function
1600^{s}, 1558^{s}	ν(C=O) of quinone

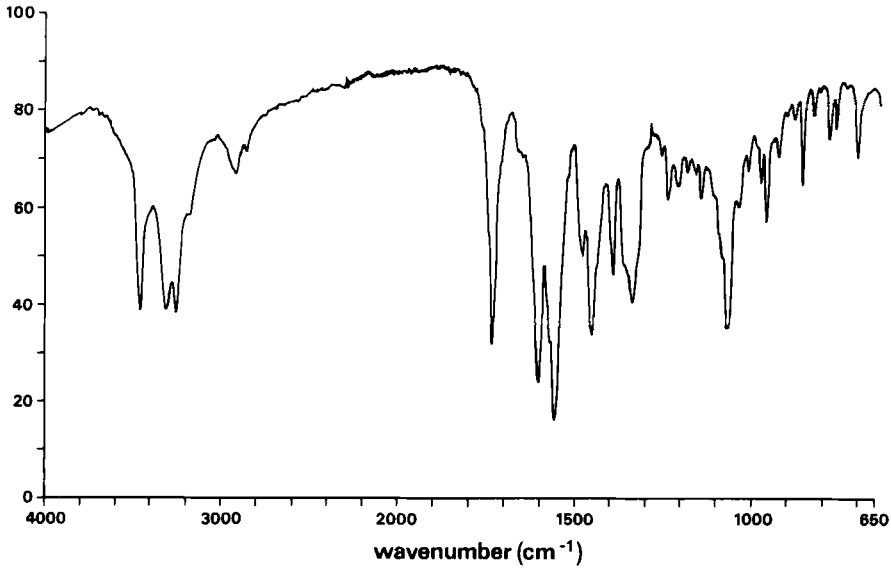


Figure 3.
Infrared spectrum of mitomycin C

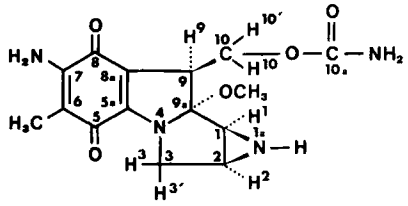
4.3 Nuclear Magnetic Resonance (NMR) Spectrum

The proton NMR spectrum was recorded in pyridine-d_5 containing tetramethylsilane as internal reference and with the use of a Bruker WM-300 spectrometer at frequency 300.13 MHz (37a). The spectrum (detail) is presented in Figure 4 and refers to the situation after proton exchange. The spectral assignments are presented in Table III (in pyridine-d_5) and are derived from Lown and Begleiter (38). Irrigularities in intensity comparison of the signals make the assignments tentative. Proton exchange with deuterium oxide results in disappearance of NH, NH_2 and H-1 signals, while the H-3, H-9, H-10 and H-10' signals shift 0.10 to 0.20 to lower ppm values (spectrum Fig. 4).

Table III ^{1}H NMR Assignments for Mitomycin C in pyridine-d_5

Chemical shift δ (ppm)	Multiplicity	Number of atoms	Assignment
2.00	singlet	3	CH_3(C-6)
2.10	triplet	1	NH (C1-C2)
2.72	quartet	1	H-2
3.13		1	H-1
3.19	singlet	3	OCH_3(C-9)
3.57	doublet	1	H-3'
4.02	quartet	1	H-9
4.53	doublet	1	H-3
5.11	triplet	1	H-10
5.43	quartet	1	H-10'
7.6	broad	4	NH_2(C-7) and $CONH_2$

The natural abundance carbon 13 NMR spectrum was recorded under the same experimental conditions except that the frequency was 75.46 MHz (37a). The proton-noise decoupled spectrum is presented in Figure 5 and the spectral assignments are summarized in Table IV. The assignments of Keller and Hornemann are followed (39).



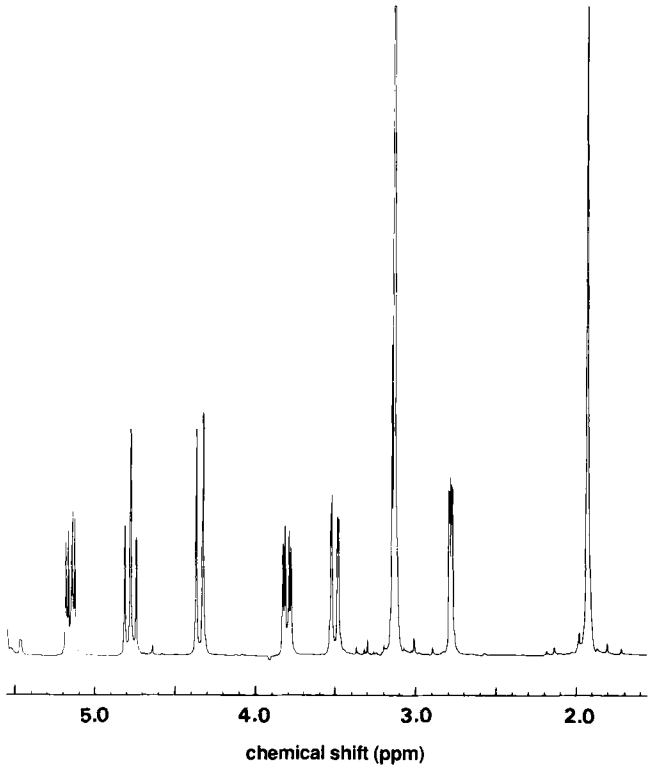


Figure 4.
Proton NMR spectrum of mitomycin C (detail)

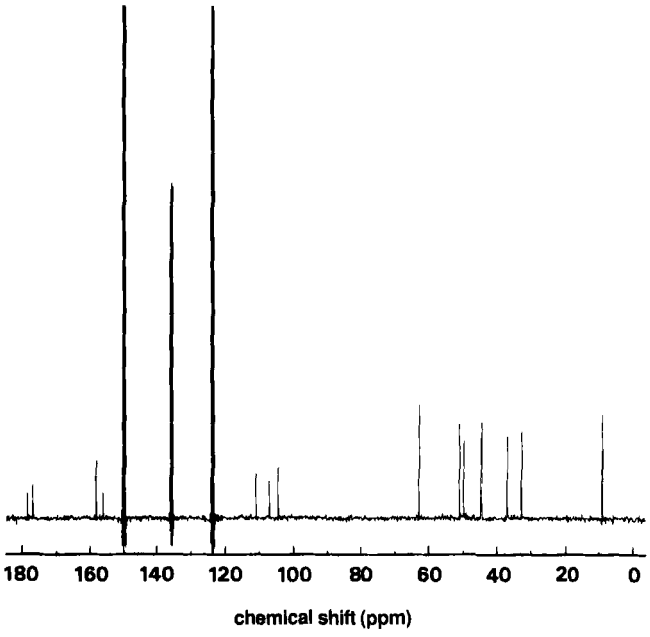


Figure 5.

^{13}C NMR spectrum of mitomycin C

Table IV ^{13}C NMR Assignments for Mitomycin C in pyridine-d_5

Chemical shift δ(ppm)	Assignment
8.8	CH_3(C-6)
32.6	C-2
36.7	C-1
44.2	C-9
49.5	OCH_3(C-9a)
50.6	C-3
62.5	C-10
104.3	C-6
106.8	C-9a
110.7	C-8a
149.6[a]	C-7
156.1	C-5a
158.1	C-10a
176.7	C-8
178.3	C-5

[a] obscured by solvent

4.4 Mass Spectrum

Electron impact (EI) mass spectroscopy of mitomycins, including mitomycin C, has been investigated in detail by Van Lear (40). His observations were confirmed in later studies (41, 42). For this analytical profile electron impact (EI), chemical ionization (CI), field desorption (FD) and fast atom bombardment (FAB) in the positive and negative ion mode, spectra of mitomycin C (lot nr. 010783) were recorded. EI mass spectrometry was performed by direct probe analysis on a Kratos MS-80 mass spectrometer. Source conditions were: 200 °C source temperature, 70 eV electron energy and 100 μA ionising current. Direct insertion probe CI mass spectra were obtained by using a Finnigan 3200 quadrupole mass spectrometer combined with a Finnigan 6000 data system. Methane was used as the ionising gas. The emission current was 0.20 mA and the multiplier voltage 1800 V. FD mass spectra were obtained with a Varian MAT 711 double focussing mass spectrometer equipped with a MAT 100 data acquisition unit. 10 μm tungsten wire FD emitters containing carbon microneedles

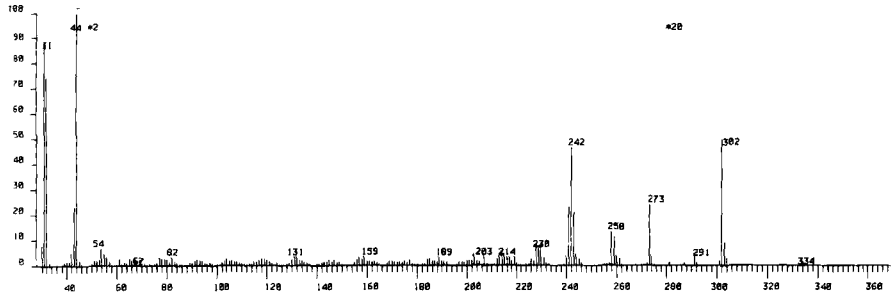


Figure 6.
EI mass spectrum of mitomycin C

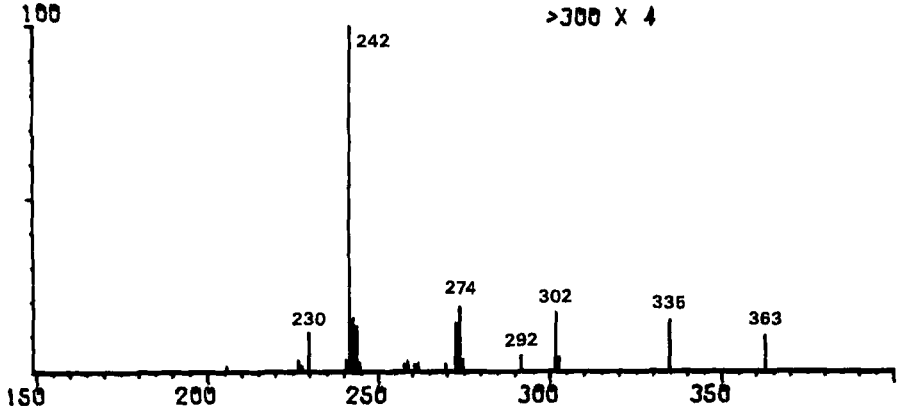


Figure 7.
CI mass spectrum of mitomycin C

with an average length of 30 μm were used. The mitomycin C sample was dissolved in methanol and then loaded onto the emitters by the dipping technique. An emission current of 12 mA was used to desorb the sample. The ion source temperature was 70 °C. FAB mass spectrometry was carried out using a V.G. micromass ZAB-2F mass spectrometer, an instrument with reverse geometry and fitted with a high field magnet and coupled to a V.G. 11-250 data system. The sample was loaded in thioglycerol solution onto a stainless steel probe and bombarded with Xenon atoms having a 8 keV energy. The recorded spectra are depicted in Figures 6, 7, 8, 9 and 10 and the major ion assignments are given in Table V.
A high resolution mass spectrum exhibited the molecular ion at m/z 334.1280 with composition $C_{15}H_{18}N_4O_5$ (calculated mass 334.1277).

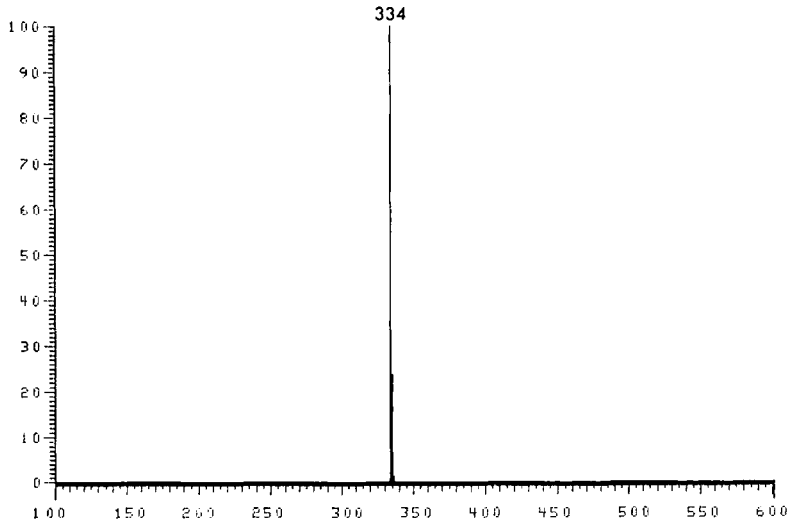


Figure 8.
FD mass spectrum of mitomycin C

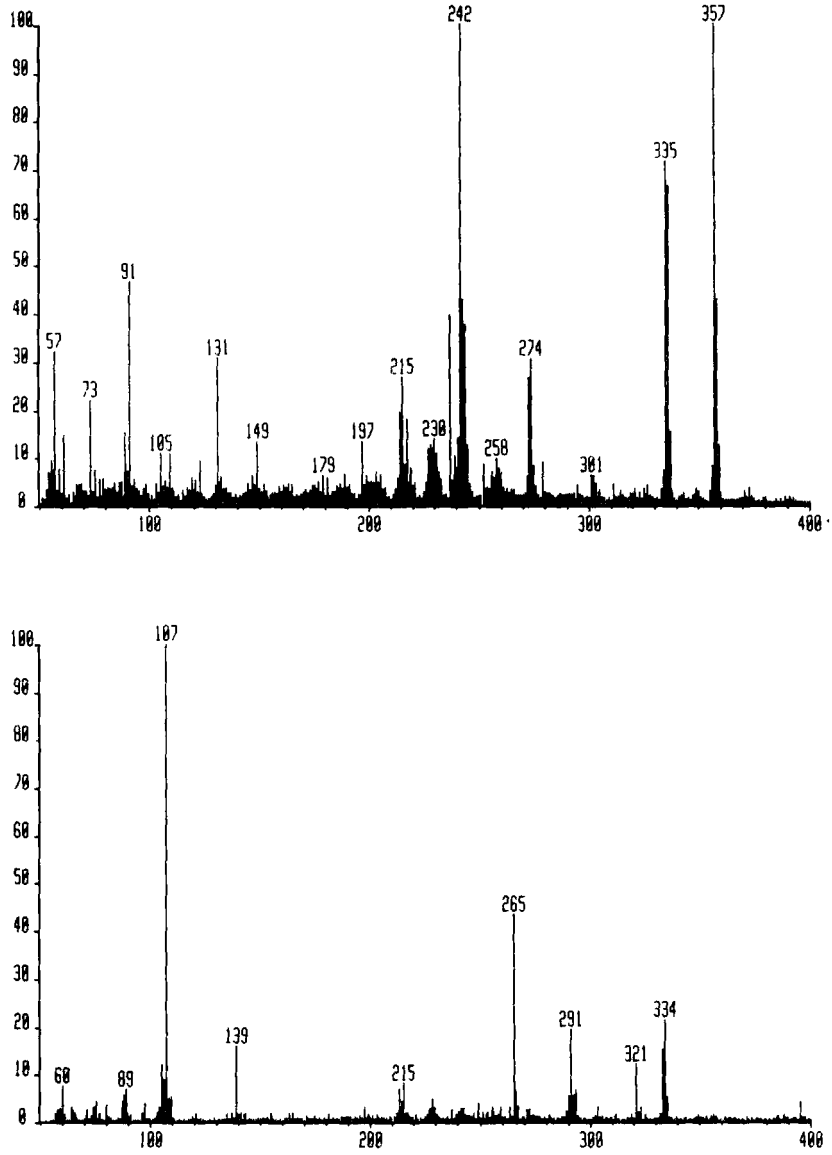


Figure 9/10.
FAB(+) mass spectrum (upper spectrum) and FAB(-) mass spectrum (lower spectrum) of mitomycin C

Table V Mass Spectral Data for Mitomycin C

	m/z	assignment
FD	334	$M^{+\cdot}$
	335	$(M+H)^+$
FAB(+)	358	$(M+H+Na)^+$
	357	$(M+Na)^+$
	336	$(M+2H)^+$
	335	$(M+H)^+$
	274	$((M+H)-O_2CNH_3)^+$
	242	$(((M+H)-H_3COH)-O_2CNH_3)^+$
m/z 57, 91 and 215 signals come from the thioglycerol matrix		
FAB(-)	334	$M^{-\cdot}$
	333	$(M-H)^-$
	291	$(M-CONH)^-$
m/z 107, 215 and 321 signals come from the thioglycerol matrix		
m/z 139 and 265 signals cannot be assigned with certainty		
CI	363	$(M+C_2H_5)^+$
	335	$(M+H)^+$
	302	$(M-H_3COH)^+$
	274	$(M-O_2CNH_2)^+$
	242	$((M-H_3COH)-O_2CNH_2)^+$
EI	334	$M^{+\cdot}$
	302	$(M-H_3COH)^+$
	291	$(M-OCNH)^+$
	273	$(M-O_2CNH_3)^+$
	259	$((M-O_2CNH_2)-CH_3)^+$
	242	$((M-H_3COH)-O_2CNH_2)^+$

4.5 Circular Dichroism (CD) Spectrum

The chiral centers at C1, C2, C9a and C9 in the mitomycin C molecule are responsible for the CD Cotton effects at 355 nm and 395 nm. The CD spectrum of mitomycin C in a methanolic solution (c=0.001%), determined by using a Jobin Yvon Dichrograph III, is shown in Figure 11.

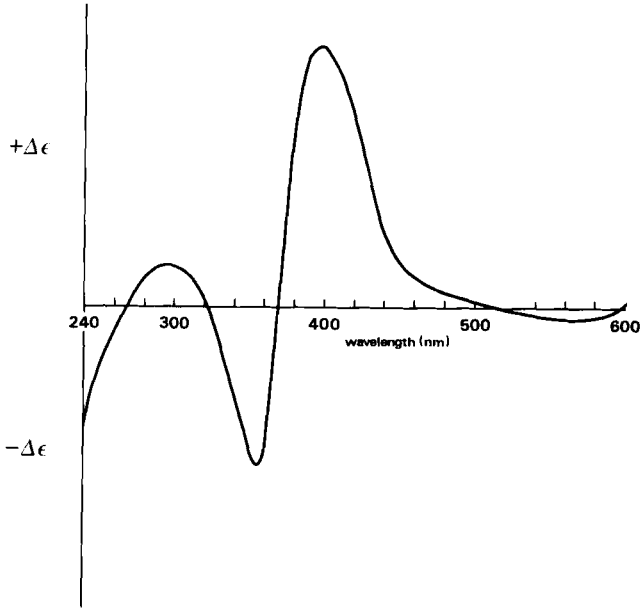


Figure 11.
CD spectrum of mitomycin C

4.6 Optical Rotation

The optical rotation $(\alpha)_D^{25}$ of mitomycin C in methanol (c=0.1%) was determined to be +232°. This analysis was performed with a Thorn NPL Automatic Polarimeter Type 243 at 589 nm. The optical rotatory dispersion (ORD) spectrum of mitomycin C has been reported by Hornemann et al. (43).

4.7 Melting Point

In the literature no melting point for mitomycin C is mentioned except for the statement that it should be over 300 °C (11).

4.8 Differential Scanning Calorimetry

In nitrogen atmosphere no transitions were recorded up to 330 °C in a closed (air tied) sample holder. In an open sample holder an undefined large exothermic decomposition takes place from 215 °C. The applied apparatus was a Feteram 111, the heating rate was 5 K/min and the sample size was 3 mg (44).

4.9 Solubility

Mitomycin C is readily soluble in methanol, acetonitrile normale saline and water, but is nearly insoluble in hydrocarbon solutions. The following solubility data have been reported (45), determined by suspending an excess amount of mitomycin C in a solvent, followed by filtration and HPLC analysis.

Table VI Solubilities of Mitomycin C

solvent	solubility (mM)	temperature (°C)
water	2.73	25
sesame oil	0.0180	25
isopropylmyristate	0.0193	37
hexane	0.0234×10^{-3}	25

4.10 Partition Coefficient

The partition coefficient for mitomycin C has been measured in various organic phase/distilled water systems. The results have been combined in Table VII.

Table VII Partition Coefficients for Mitomycin C

solvent	partition coefficient	reference
chloroform	0.26; 0.27	(45; 46)
n-octanol	0.41; 0.42[a]	(45; 47)
ethyl acetate	0.51	(46)
1-pentanol	2.32	(46)
chloroform/2-propanol (90+10)	0.50	(46)
chloroform/2-propanol (50+50)	1.52	(46)
chloroform/1-pentanol (90+10)	1.12	(46)
chloroform/1-pentanol (80+20)	1.85	(46)
dichloromethane/2-propanol (50+50)	1.04	(46)

[a] the aqueous phase was 0.07M phosphate buffer pH = 7.4

The organic solvents chloroform (1,2) and ethyl acetate (7, 48) have been used with success for the isolation of mitomycins from the aqueous fermentation broths of Streptomyces species.

4.11 Dissociation Constants

The mitomycin C molecule contains several prototropic functions. Basic groups are the 7-amino group, the N-4 nitrogen and the aziridine nitrogen. Due to rapid degradation the pKa values of the conjugated acid concerning the 7-amino group and N-4 nitrogen can not be deduced from titration experiments with intact mitomycin C. Therefore the acid dissociation constants for these functions were derived from titrations with stable analogs (37). Results are listed in Table VIII. The pKa of the aziridine nitrogen has been determined titrimetrically (11) and kinetically from the pH-rate relationships in degradation studies (49-51) although the titrimetrical determination also has been influenced by degradation. Mitomycin C also has acidic properties with an (apparent) pKa 12.44 at room temperature. These acidic properties emerge after keto-enol tautomerization of the 7-amino quinoid moiety which precedes deprotonation in alkaline medium (Figure 2)(37).

Table VIII Prototropic Functions of Mitomycin C

function	determination method	pKa(25°C)	reference
N-4	spectrophotometry	-1.2	(37)
7-amino	spectrophotometry	-1.3	(37)
aziridine	potentiometric titration	3.2	(11)
	via pH-rate profile	2.8	(49)
	via pH-rate profile	2	(50)
	via pH-rate profile	2.74	(51)
	via pH-rate profile	2.50(49.5°C)	(49)
7-amino	spectrophotometry	12.44	(37)

4.12 Electrochemistry

Due to the presence of the quinone ring of the mitomycin C molecule the compound can be reduced and transformed into its hydroquinone form. This conversion triggers a complicated pattern of chemical consecutive reactions. Although much progress has been made in the elucidation of the processes occurring during and following electrochemical reduction of mitomycin C the exact complicated mechanisms have not yet been unravelled (50). This is due to:

- rapid degradation of mitomycin C at pH < 3 and pH > 12;
- the extremely high reactivity of the hydroquinone form of mitomycin C;
- the fact that chemical degradation products as well as electrochemically generated degradation products of mitomycin C can be reduced within the region of reduction potentials of mitomycin C itself (50).

Electrochemistry of mitomycin C has been studied in aqueous solutions (50, 52-54) and aprotic solvents (55), by using polarography (50, 52-54) and cyclic voltammetry (50, 52, 53, 55). Direct current polarographic curves for mitomycin C are shown in Figure 12. Rapid degradation of mitomycin C within the time scale of recording a direct current polarographic curve at pH < 3 and pH > 12 strongly influences the shape and heights of these curves (50).

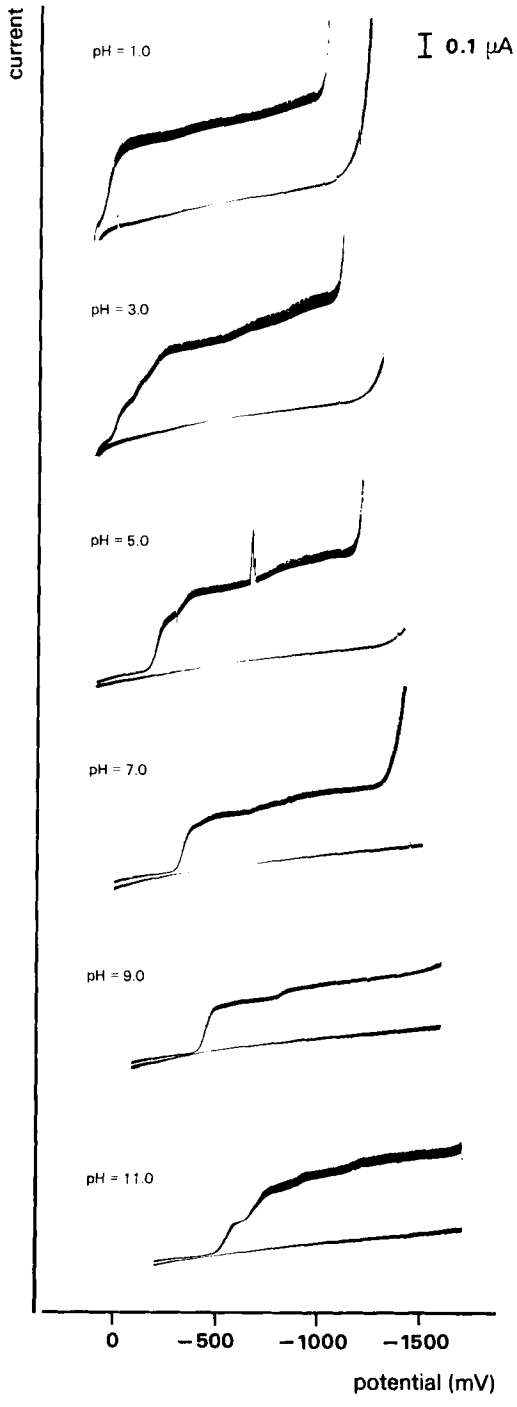


Figure 12.
Direct current polarographic curves of mitomycin C (10^{-4}M)

5. METHODS OF ANALYSIS

5.1 Elemental Analysis

The elemental analysis of a purified mitomycin C sample for C, H, N and O was reported to be as follows:

Table IX Elemental Analysis of Mitomycin C (11)

element	% theory	% found
C	53.83	53.55
H	5.43	5.57
N	16.76	16.86
O	23.98	24.13

5.2 Ultraviolet Spectrophotometry

Mitomycin C has been determined by using UV spectrophotometry at 360 nm in methanol solution. This assay is linear in the range 2×10^{-6} to 4×10^{-5} M, with a precision of ± 1%.

5.3 Thin Layer and Paper Chromatography

A diverse array of thin layer chromatographic systems have been described in the literature for analysing mitomycin C and derivatives (55). Some representative systems have been summarized in Table X.

The mitomycin C spot can be located by:

- irradiation with UV light of 254 nm;
- examination under daylight (mitomycin C has a blue-violet colour);
- spraying with a 1 in 100 solution of ninhydrin in alcohol, followed by heating the plate at 110°C for 15 minutes by which mitomycin C appears as a pink spot (56).

Table X Thin Layer and Paper Chromatography of Mitomycin C

plate	solvent (v/v)	Rf	reference
silica-gel	butanol (sat. with water)	0.57	(12)
	ethyl acetate (sat. with water)	0.56	(12)
	methanol-benzene-water (10:5:5)	0.02	(12)
	isopropylalcohol-1%NH_4OH (2:1)	0.88	(57)
	methanol	0.80	(57)
	1-octanol-acetone-ligroin (90-115°C)(2:5:5)	0.11	(58)
	chloroform-methanol (9:1)	0.12	(59)
cellulose paper	isobutyric acid-0.5M NH_4OH (10:6)	0.75	(57)
	butanol-water (84:16)	0.57	(60)
	methanol-benzene-water (1:1:2)	0.02	(60)
	ethyl acetate (sat. with water)	0.56	(60)

5.4 Gel Filtration Chromatography

For the separation of mitomycin C from its degradation products and alkylation adducts Tomasz et al (57, 61-64) utilized gel filtration chromatography on Sephadex G-25 columns and 0.02 M NH_4HCO_3 solution as eluent. By using a 1.5x 41.5 cm Sephadex G-25 (fine) column the elution volume of mitomycin C is 96 ml (57).

5.5 High Performance Liquid Chromatography

HPLC procedures reported in the literature have been developed mainly for the analysis of mitomycin C and derivatives in decomposition mixtures (51, 59, 65-71) in biological matrices (plasma, urine, bile, tissues)(46, 72-86) and other systems such as (enzyme containing) culture media (62, 69, 87-90). Detection by UV absorbance measurements at 365 nm is usually preferred (55, 75, 76) because of the high specificity and sensitivity, but detection at 254 nm, which is less sensitive, is also reported (66, 91, 92). The polarographic electrochemical detection mode is an elegant alternative for the detection of mitomycin C (50, 75, 93), but demands extensive experimental precautions to be taken, such as removal of oxygen from the mobile phase and damping of pulsating flow of the solvent delivery system (93). Important HPLC systems have been enumerated in Table XI.

Table XI High Performance Liquid Chromatography for Mitomycin C

column	mobile phase	reference
	Reversed Phase HPLC	
μ-Bondapak C18 (10 μm)	methanol-water (35:65)	(77-80)
μ-Bondapak C18 (10 μm)	methanol-0.01 M phosphate buffer (pH 6.0)(30:70;w/w)	(46)
Lichrosorb RP18 (10 μm)	methanol-water (30:70;w/w) + 0.5% (v/w) phosphate buffer pH 7.0	(67)
Lichrosorb RP8 (5 μm)	methanol-water-phosphate buffer pH 7.0 (20:70:10)	(82)
Ultrasphere ODX	acetonitrile-0.03M potassium phosphate buffer (12.5:87.5)	(91)
Hypersil-MOS (5 μm)	acetonitrile-0.05M phosphate buffer pH 7 (10:90)	(75)
Hewlett-Packard C8 (10 μm)	acetonitrile-water; gradient from 5% to 41% acetonitrile	(94)
Radial Pak C18 (10 μm)	methanol-0.01M phosphate buffer pH 7; gradient from 0% to 50% methanol	(42)
	Ion Pair HPLC	
Lichrosorb RP18 (10 μm)	methanol-water (40:63;w/w) + 0.5% (v/w) glacial acetic acid (pH*=4.3)[a]	(65)
Lichrosorb RP18 (10μm)	methanol-water (30:70;w/w) + 0.5% (v/w) phosphate buffer pH 7.0 + 0.5% (v/w) tetrabutylammoniumbromide solution (20%, w/w)	(59)
Hypersil ODS (5 μm)	acetonitrile-water (25:75) + 0.2% sodium laurylsulphate + 0.025M sodium acetate	(66)
Hypersil ODS (5 μm)	acetonitrile-water (20:80) + 0.2% cetrimide + 0.0125M sodium acetate	(66)
Resolve C18 (5 μm)	methanol-1 mM octanesulfonicacid sodium salt solution (30:70;v/v) (pH* 4.5)[a]	(85)
	Normal Phase HPLC	
μ Porasil P (10 μm)	tetrahydrofuran-methanol-isooctane-dichloromethane (3:7:15:75)	(95)
μ Porasil (10 μm)	chloroform-methanol (9:1)	(92)
	ethyl acetate-methanol (95:1)	(92)
Silica Si-60	ethyl acetate-methanol-water-dichloromethane (97:2:1:1)	(75)

[a] pH values of organic modifier-water mixtures

5.6 Identification and Purity Tests in Official Compendia

In the United States Pharmacopeia XXI (56) the monographs "Mitomycin" and "Mitomycin for Injection" have been included. In these monographs the identification of mitomycin C is based on IR and UV spectroscopy and chromatography by which the sample should exhibit the same spectroscopic and chromatographic properties as a similar preparation of a USP Mitomycin Reference Standard. The pH of a solution containing 5 mg/ml should be between 6.0 and 8.0. The water content of the raw material may not exceed 5%. Furthermore Mitomycin and Mitomycin for Injection (a dry mixture of mitomycin C and mannitol) must meet the requirements of specific tests described in the USP XXI such as: Safety Tests, Depressor Substances Tests, Pyrogen Tests and Sterility Tests (56).

6. STABILITY, DEGRADATION

6.1 Stability in Aqueous Solutions

Mitomycin C and related mitomycins are not stable when stored as aqueous solutions (11-13, 51, 55, 57, 59, 65-68, 82, 96-108). Degradation is accelerated by acid, alkali, (buffer) ions and high temperatures (108). At pH < 7 mitomycin C is converted into 1-hydroxy-2,7-diaminomitosenes and at pH > 7 it is hydrolysed into 7-hydroxymitosane. The overall degradation scheme is given in Figure 13. In the mitosene compounds the C9a methoxy group has been cleaved, a double bond between C9 and C9a has been introduced and the aziridine moiety has been opened. Aziridine ring opening occurs with configurational retention at C2 and water attachment at C1 with inversion as well as retention of the C1 stereochemistry yielding 1,2-trans- and 1,2-cis-1-hydroxy-2,7-diaminomitosenes, respectively. Under drastic acidic conditions progressive degradation reactions lead to hydrolysis of the 7-amino group and the C10 carbamate side chain (9-11, 13). The mechanism of the initial degradation step of mitomycin C in acid is shown in Figure 14 (107, 108). In the cascade of reactions an intermediate is proposed, having a positive charge at C1 (49, 107-110). This electrophilic center serves as target for attacking (nucleophilic) water molecules resulting in the formation of 1-hydroxy-2,7-diaminomitosenes. The degree of protonation of the 2-amino function in the proposed intermediate species (pKa:2.8(65)) determines the C1 stereochemistry of the resulting mitosenes. A protonated 2-amino function may direct incoming water molecules to a cis configuration. Whenever the amino function in the intermediate is not protonated the directing electrostatic power is absent and a reacting water molecule may approach the C1 carbonium ion from both

sides with roughly equal chances. This explains the remarkable influence of pH on the C1 stereochemistry of the resulting mitosenes. At pH=1 the cis/trans ratio is about 4 while at pH=6 the ratio approaches 1 (65). When mitomycin C degrades in acidic medium in the presence of nucleophiles such as acetate, phosphate or even DNA parts, 1-nucleophile-2,7-diaminomitosenes are generated (57, 103, 111-114). These are important observations which lend credibility to the hypothesis that acid activation may play a role in the in vivo mitomycin C DNA alkylation.

In alkaline solution (pH 7 to 13) the 7-amino group of the mitomycin C molecule is substituted by a hydroxy function (59, 100-102). Under prolonged severe alkaline treatment the quinone chromophore is destroyed (59).

In buffered media the degradation kinetics of mitomycin C can be described adequately by (pseudo) first order kinetics. Some kinetic data are summarized in Table XII. For further detailed kinetic information is referred to the literature (49, 51, 55, 67, 107, 108).

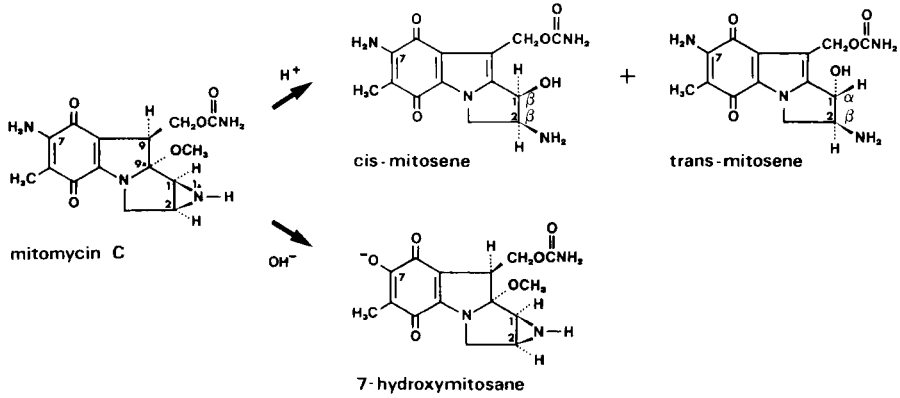


Figure 13.
Overall degradation scheme of mitomycin C

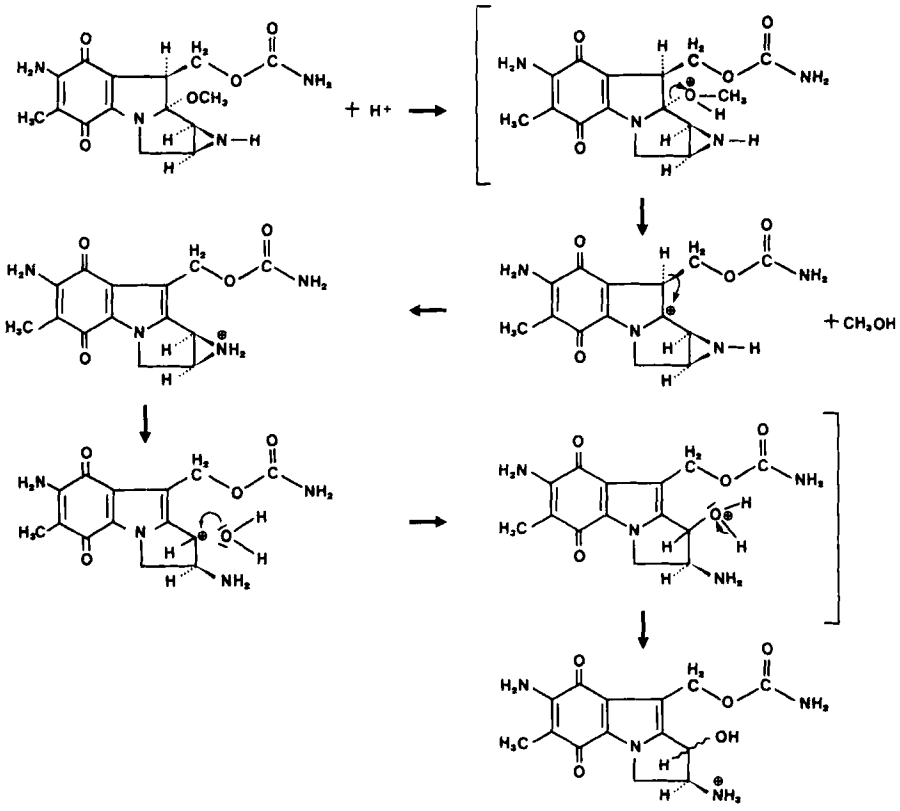


Figure 14.
Acid degradation mechanism of mitomycin C

Table XII Degradation Kinetics for Mitomycin C

conditions/method	$k_{obs}(s^{-1})$	reference
pH 0.87; perchloric acid solution; 20°C/ HPLC	$1.1x10^{-3}$	(65)
pH 2.86; 0.001M acetate buffer; 20°C/ HPLC	$4.0x10^{-3}$	(65)
pH 3.0; 1.0M phosphate buffer; 25°C/ HPLC	$1.2x10^{-3}$	(114)
pH 4.64; 0.1M phosphate buffer; 40°C/ spectrophotometry	$1.4x10^{-4}$	(107)
pH 7.4; 0.1M phosphate buffer; 37°C/ HPLC	$4.1x10^{-7}$	(105)
pH 9.0; 0.1M phosphate buffer; 37°C/ spectrophotometry	$1.8x10^{-6}$	(104)
pH 11.1; 0.1M phosphate buffer; 25°C/ HPLC	$5.3x10^{-5}$	(67)

6.2 Stability in Pharmaceutical Formulations

Mitomycin C is commercially available in a lyophilized form containing mannitol (Mutamycin® : 5 mg mitomycin C + 10 mg Mannitol) or sodium chloride (Mitomycin C-Kyowa® :10 mg mitomycin C + 240 mg sodium chloride; Ametycine 10® : 10 mg mitomycin C + 240 mg sodium chloride) as excipients. In this state the drug is stable for at least two years if kept dry and in well closed containers at room temperature in the dark (115). After reconstitution the drug has only limited stability, depending strongly on the pH of the final solution (68). An overview of stability data of mitomycin C in infusion fluids is given in Table XIII. More specific and extended data are described in the references concerned (68, 70, 71, 116-120).

Table XIII Stability of Mitomycin C in Infusion Fluids at Room Temperature and -30°C

infusion fluid	concentration	stability	reference
5% dextrose	50 µg/ml	t_{90} = 3.0 h; t_{50} = 78 h	(68)
0.9% sodium chloride	50 µg/ml	93% remained after 5 days	(68)
sterile water	2 mg/ml	stable for 7 days	(118)
5% dextrose	50 µg/ml	26% remained after 12 hours	(70)
0.9% sodium chloride	50 µg/ml	t_{50} = 12 h	(70)
phosphate buffer pH 7.7	50 µg/ml	t_{90} = 15 days	(70)
5% dextrose	0.4 mg/ml	t_{90} = 1 h	(116)
0.9% sodium chloride	0.4 mg/ml	t_{90} = 24 h	(116)
0.9% sodium chloride	0.6 mg/ml	99% remained after 4 weeks storage at -30°C	(71)

Further research is required to resolve the problem of conflicting stability data on mitomycin C in 0.9% sodium chloride solution (68, 70, 116, 119, 120).

6.3 Stability in Biological Media

Chemical instability of mitomycin C in biological fluids and tissues may be a complicating factor in the bioanalysis of the drug. Ignorance of the chemical stability of the drug during sampling and sample pre-treatment procedures may lead the researcher to misinterpretations. Recently, den Hartigh et al. (86) published an extensive study concerning the handling of mitomycin C biological samples. The following recommendations and guidelines were given by these authors (86):

- whole blood samples should be placed in ice immediately after sampling; separation of plasma and red blood cells should take place within half an hour after collection of the samples;
- mitomycin C samples should be protected from long-term exposure to daylight in order to prevent analyte degradation;
- the pH of urine samples should always be checked and, if necessary, adjusted to neutral values, as a low pH will induce mitomycin C decomposition and the formation of precipitates upon which mitomycin C may be adsorbed;
- plasma and urine samples need not necessarily be placed in the refrigerator or freezer immediately after preparation, provided analysis is carried out within a period of 2 h;
- plasma and urine samples may be stored in a freezer at -20°C; however, analysis should take place within 3 weeks as longer storage at this temperature may induce considerable reduction of the mitomycin C concentration;
- repeated freezing and thawing of plasma and urine samples does not influence the analytical results of the mitomycin C assay;
- extracts of biological samples may be kept for at least 24 h, in the dark, either dry or dissolved in methanol at 4°C or 20°C prior to analysis.

The in vitro half life of mitomycin C was determined to be > 14 days under conditions of the clonogenic assay (121). Mitomycin C is fairly stable (70-80% of the drug remained) when incubated for 5 days at 37°C in Medium E, RPMI, Fischer's or MEM culture media, supplemented with serum (69). In Mark's M-20 culture medium with fetal calf serum, Proctor and Gaulden (90) observed that the amount of mitomycin C was reduced by 29% after 30 minutes incubation.

7. PHARMACOLOGY

7.1 Mechanism of Action

Mitomycin C induced cytotoxicity is believed to be mediated by DNA binding. The drug as such, in its oxidized form, is not cytotoxic although some interaction with nucleic acids may exist (122). Mitomycin C must be activated before it is capable of alkylating DNA (123-128). The alkylating capacity is unmasked by reductive or acidic activation. An outline of the reductive activation pathway is given in Figure 15. Reduction can be mediated by enzyme systems (87-89, 129-132) or by chemical reducing agents (58, 126). The conversion of mitomycin C into its reduced form destabilizes the compound drastically and almost instantaneously the C9a methoxy group is lost (Figure 15). A subsequent rearrangement in species C leads to the formation of the quinone methide D which acts as target for nucleophilic nucleic acid attack, by which a monofunctional alkylation adduct is formed. An intramolecular SN^2-displacement of the carbamate moiety leads to the bifunctional cross-linked adduct F (Figure 15)(128). The interaction of mitomycin C with DNA is more than 90% of the monofunctional type. Chemical structures of monofunctionally linked mitomycin C adducts with DNA, nucleotides and nucleosides have been reported (62-64, 133-136). Reduction of mitomycin C may follow either one or two electron reduction steps. The semiquinone radical is also believed to be capable of producing interstrand cross-links between complimentary strands of DNA (87, 137). Under aerobic conditions, the semiquinone radical formed after a one electron reduction can transfer its electron to oxygen to generate superoxide anion under regeneration of the oxidized quinone (138-142). Reactive oxygen species can cause DNA strand scission and membrane lipid peroxidation, leading to cell damage and can contribute in this manner to mitomycin C induced cytotoxicity. In the absence of any reducing agent or enzymes mitomycin C is also capable of coupling covalently with DNA or nucleic acid parts, but then, activation by acid is a requisite (57, 103, 111-113, 143, 144). Acid catalyzed degradation of mitomycin C (Figure 14) occurs through reaction intermediates possessing an electrophilic C1 center which is the target for nucleophilic nucleic acid attack.

More studies are necessary in order to elucidate which of the proposed mechanisms or combination is actually responsible for mitomycin C induced cytotoxicity in vivo.

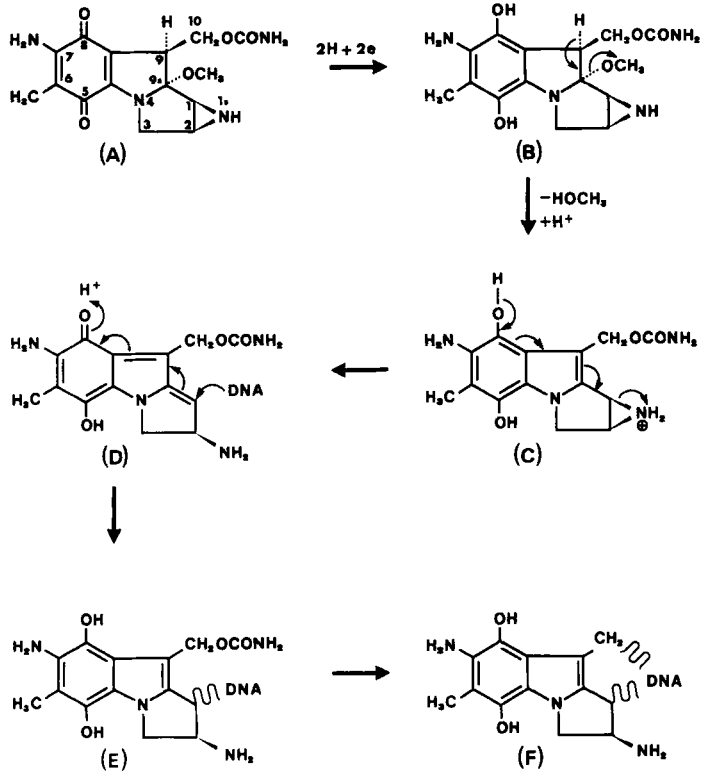


Figure 15.
Reductive activation mechanism of mitomycin C

7.2 Clinical Antitumour Activity

Single agent activity of mitomycin C has been demonstrated against various solid neoplasms in humans such as breast cancer (29, 145), stomach cancer (146), pancreatic cancer (147), colorectal cancer (29), non-small cell lung cancer (148), squamous cell carcinoma of the uterine cervix (28) and head and neck cancer (28). Encouraging results have been obtained on the treatment of superficial transitional cell carcinoma of the bladder when mitomycin C is administered intravesically (28-30, 149). Mitomycin C is usually used in combination with other cytostatics (28-30).

7.3 Clinical Toxicity

Delayed cumulative myelosuppression is the most important toxicity. Others include nausea, vomiting, anorexia, diarrhea, stomatits, skin rashes and alopecia (24, 28, 150). Extravasation of mitomycin C causes severe cellulitis accompanied with chronic pain and ulceration (151). Interstitial pneumonitis, pulmonary fibrosis, hemolytic-uremic syndrome and renal failure have occasionally also been documented as due to the drug. Following topical intravesical mitomycin C therapy for bladder cancer dermatological toxicity has been encoutered in some cases (154).

7.4 Pharmacokinetics

Following intravenous bolus injection of mitomycin C the plasma concentration-time curves show a bi-phasic, exponential decline (72, 155). Mitomycin C pharmacokinetics (at clinically relevant doses) is not dose dependent (72, 155). Major routes of elimination are liver metabolism and urinary excretion. Metabolites have never been identified. Absorption after oral administration is very irregular(156). After intravesical instillation, limited absorption of mitomycin C into the systemic circulation occurs (30, 157). Pharmacokinetic data after intra-arterial administration are available (73).

8. DETERMINATION IN BODY FLUIDS

The analysis of mitomycin C in biological samples is hampered by its instability, low concentrations and the polar nature of the mitomycin C molecule (55, 76). The following techniques have been used: microbiological assays (74, 95, 104, 158-161, 166), bioassay by using a repair deficient mutant of Chinese hamster ovary cells (162), immunological assay (163, 164), high-performance differential pulse polarography (54) and HPLC assays (46, 72-86, 93-96, 155). The HPLC assay designed by Den Hartigh et al. (46, 72) has shown to be very suitable for pharmacokinetic studies (72-74). The following procedure is followed for plasma samples: 0.2, 0.5 or 1.0 ml samples is mixed with the internal standard porfiromycin (about 500 ng) and with 2.0, 5.0 or 10.0 ml, respectively, of chloroform-2-propanol (1+1, w/w). After shaking for 1 minute and centrifugating for 5 minutes (2500 g), the clear supernatant is transferred into a conical tube and evaporated to dryness at 30-40°C under nitrogen. The residue is dissolved in 50-100 µl of methanol with the aid of a vortex-type mixer. Aliquots of 10-20 µl are injected into the chromatograph. HPLC: column: µ Bondapak C18 (30 cm x 3.9 mm i.d., particle size 10 µm); mobile phase: 0.01 M phosphate buffer pH 6.0-methanol (70+30, w/w); flow rate 1.0 ml/min; detection: 365 nm.

An overview of published determination methods of mitomycin C in body fluids is given in Table XIV.

Table XIV Determination Methods for Mitomycin C in Body Fluids

matrix	sample pretreatment [a]	determination limit(ng/ml) [b]	test organism	reference
		microbiological assays		
blood, tissues, urine, bile	no	2	E.coli B	(159)
plasma, urine	no	10	E.coli B	(95)
plasma, urine	no	500	B.subtilis	(160)
serum, urine	no	60	E.coli	(165)
		bioassay with hamster ovary cells		
plasma	no	1		(162)
		immunological assays		
serum, urine	no	4		(163)
		polarography		
plasma, urine	no	200		(54)
plasma, urine	1.s	25		(54)

Table XIV (continued)

matrix	high performance liquid chromatography: sample pre-treatment [a]	internal standard [c]	determination limit (ng/ml) [b]	chromatographic mode [d]	reference
serum, urine, ascites	1.1.	no	40 (serum)	RP	(77)
plasma	1.1.	no	1	RP	(46)
plasma	1.1.	no	1	RP	(73)
plasma, urine, bile, ascites	1.1.	yes	1	RP	(72)
serum, plasma, urine	1.s.	yes	5 50 (urine)	RP	(75)
plasma, urine	1.s.	yes	4 (plasma)	RP (gradient)	(94)
plasma, urine	1.s.	no	2 (plasma) 500 (urine)	RP	(82)
plasma, urine	1.1.	yes	1 (plasma) 200 (urine)	NP	(95)
plasma, urine	1.1. (plasma)	no	1 (plasma)	RP	(74)
serum	1.s.	no	10	RP	(78)
serum	deproteination with methanol	no	10	RP	(80)
plasma, urine	1.s.	yes	25	RP	(84)
plasma, urine	1.1.	yes	10	RP	(85)

[a] 1.1.: sample pre-treatment by liquid-liquid extraction; 1.s.: sample pre-treatment by liquid-solid extraction.
[b] determination limits for 25 µl (80), 50 µl (85, 163), 100 µl (84), 0.2 ml (160), 1.0 ml (46, 72-74, 77, 78, 82, 162), 2.0 ml (54, 75, 95) and 2.5 ml (94) samples
[c] the internal standard is porfiromycin
[d] RP: reversed phase chromatography; NP: normal phase chromatography.

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NEOSTIGMINE

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1. History

As a result of the basic research of Stedman and associates (1,2,3) in elucidating the chemical basis of the activity of physostigmine, Aeschlimann and Reinert (4) systematically investigated a series of substituted phenyl esters of allylcarbamic acids. Neostigmine (Prostigmin), a most promising member of this series, was introduced into therapeutics in 1931 for its stimulant action on the intestinal tract. It was reported independently by Remen (5) and Walker (6) to be effective in the symptomatic therapy of myastheniagravis which is due to defect in synaptic transmission at neuromuscular junction. When the patient is given an appropriate dose of neostigmine, the response to titanic stimulation is improved along with symptomatic improvement in muscle strength (7). Neostigmine was also found useful in paralytic illeus, atony of urinary bladder and in glaucoma (8,9).

2. Description

2.1 Nomenclature

2.1.1 Chemical Names

3-(Dimethylcarbamoyloxy)-N,N,N-trimethylanilinium bromide.
3-(Dimethylcarbamoyloxyphenyl)trimethylammonium bromide.
Benzenaminium, 3-[[(dimethylamino)carbonyl]oxy]-N,N,N-trimethyl bromide.
(m-Hydroxyphenyl)timethylammonium bromide dimethyl carbamate.
3-[[(Dimethylamino)carbonyl]oxy]-N,N,N, trimethylbenzenaminium bromide
3-[[(Dimethylamino)carbonyl]oxy]-N,N,N-trimethylbenzaminium methyl sulphate.
(m-Hydroxyphenyl)trimethylammonium methyl sulphate dimethylcarbamate.
(3-Dimethylcarbamoxyphenyl)trimethylammonium methyl sulphate (10-13).

2.1.2 Generic Names

Neostigmine bromide; Neostigmine DCF; NFN; Neoeserine; Proserine; Synstigmine; Eustigmine; Philostigmine; Neostigmini bromidum; Neostigminii bromidum; Neostigmium bromatum (13,14).

2.1.3 Trade Names

Neostigmine bromide

Prostigmin; Juvastigmine; Konstigmin; Metastigmin; Normastigmin; Prostigmin; Leostigmin (13,14).

Neostigmine methyl sulphate

Intrastigmina; Juvastigmin; Mestastigmin; Neoesserin; Normastigmin; Prostigmin; Hodostin; Stiglyn; Stigmosan (13,14).

2.1.4 CAS Registry Number

59-99-4 Neostigmine
114-80-7 Neostigmine bromide
51-60-5 Neostigmine methyl sulphate (14)

2.1.5 Wiswesser Line Notation

1N1 & VOR CK-& E (Neostigmine bromide) (15)

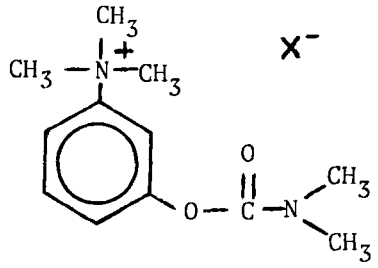
2.2 Formulae

2.2.1 Empirical

$C_{12}H_{19}BrN_2O_2$ Neostigmine bromide

$C_{13}H_{22}N_2O_6S$ Neostigmine methyl sulphate

2.2.2 Structural



X = Br^- Neostigmine bromide

X = $CH_3SO_4^-$ Neostigmine methyl sulphate

2.3 Molecular Weight

Neostigmine bromide	303.2
Neostigmine methyl sulphate	334.4

2.4 Elemental Composition

C 47.53%, H 6.32%, N 9.24%, Br 26.36%, O 10.55% (bromide).
C 46.69%, H 6.63%; N 8.38%, S 9.59%, O 28.71% (methyl sulphate).

2.5 Appearance, Color, Odor and Taste

Odorless, colorless crystal or a white crystalline, slightly hygroscopic powder with a bitter taste (14).

3. Physical Properties

3.1 Melting Point

Neostigmine bromide

Crystals from alcohol and ether melt at 167° with decomposition (13).

Neostigmine methyl sulphate

Crystals from alcohol melt between 142°C and 145°C (13).

3.2 Extraction

Neostigmine salts are quaternary ammonium compounds and are soluble in water. The aqueous solution is acidified with dilute acetic acid, evaporated to dryness and extracted with methanol. The methanol extract will contain most of the quaternary ammonium compound and may be purified by paper chromatography (16).

3.3 Solubility

Neostigmine salts are freely soluble in water. Moderately soluble in chloroform and ethanol. Insoluble in ether (11).

3.4 Acidity

Dissolve 0.20 g in 20 ml carbon dioxide-free water and titrate at pH 7.0 with 0.02 N sodium hydroxide (carbonate-free) not more than 0.2 ml is required (17).

3.5 Moisture Content and Hygroscopicity

Not more than 1%, determined by drying at $105^{o}C$ (10).

3.6 Storage

It should be stored in airtight containers protected from light (10).

3.7 Spectral Properties

3.7.1 Ultraviolet Spectrum

The UV spectrum of neostigmine bromide in ethanol is given in Figure 1. It shows two maxima at 260 nm and 266 nm. The spectrum was recorded on Varian spectrophotometer model DMS 90.

Clarke (11) reported the following: Neostigmine methyl sulfate in 1 N H_2SO_4; maxima at 260 nm (E 1%, 1 cm 20) and 266 nm (E 1%, 1 cm 18).

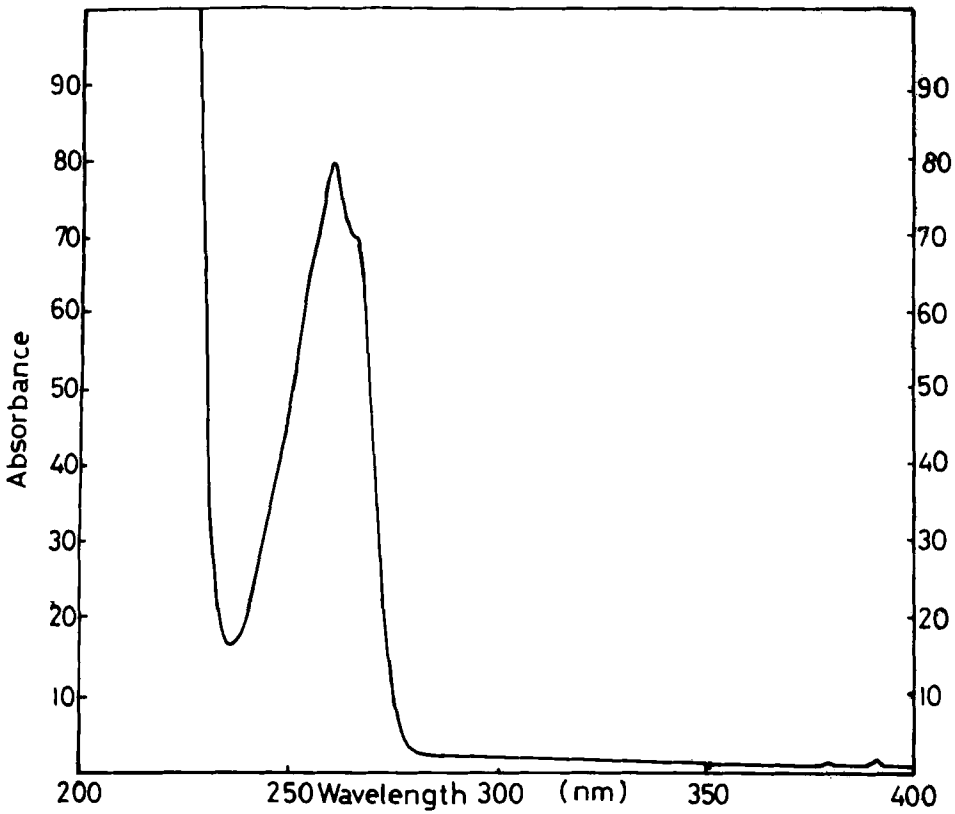


Figure 1: Ultraviolet spectrum of neostigmine bromide in methanol.

3.7.2 Infrared Spectrum (IR)

The IR spectrum of neostigmine bromide in KBr disc is presented in Figure 2, and was recorded on Pye-Unicam IR spectrophotometer model SP 1025. The frequencies and the structural assignments as shown below:

Frequency cm^{-1}	Assignment
3020-2900	CH stretch
1730	C = O (amide) stretch
1610, 1595	C = C stretch (aromatic)
1490-1450	CH bending vibrations
1030, 1070	C - N vibration (aliphatic)
780 and 895	CH (aromatic) bending vibrations

Clarke (11) reported the following:

Neostigmine bromide, potassium bromide disc the principal peaks are 1711, 1215 and 1154 cm^{-1}.

3.7.3 Proton Nuclear Magnetic Resonance (^1H NMR) Spectrum

The ^1H NMR spectrum of neostigmine methyl sulphate is shown in Figure 3. The drug was dissolved in deuterium oxide (D_2O) and its spectrum was determined on a Varian - T60 A NMR spectrometer using sodium 2,2-dimethyl 2-silapentane-5-sulphonate (DSS) as the internal standard.

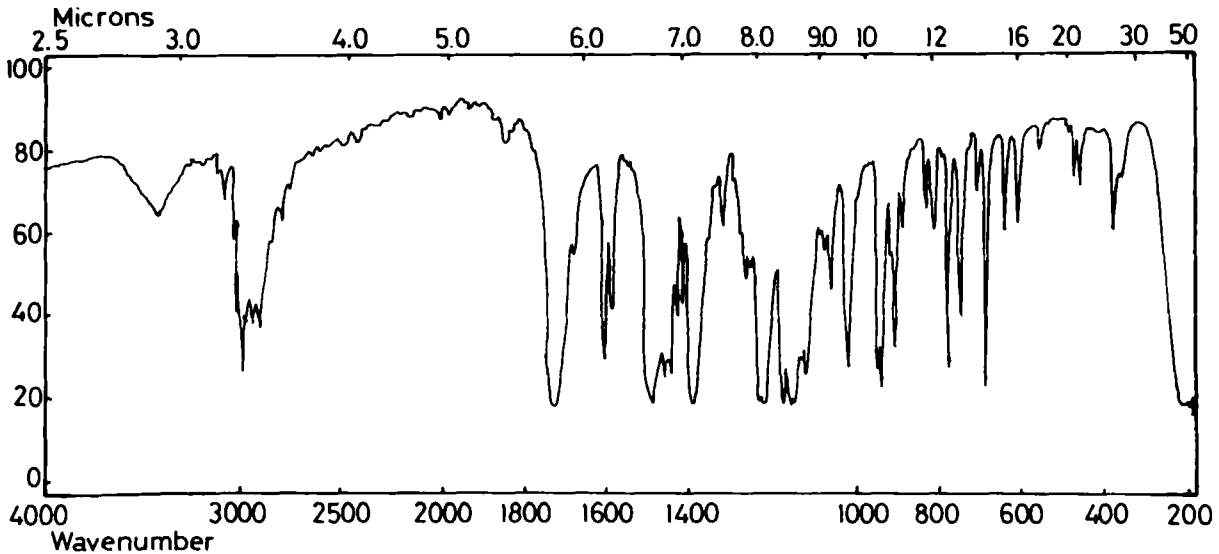


Figure 2: Infrared spectrum of neostigmine bromide, KBr disc.

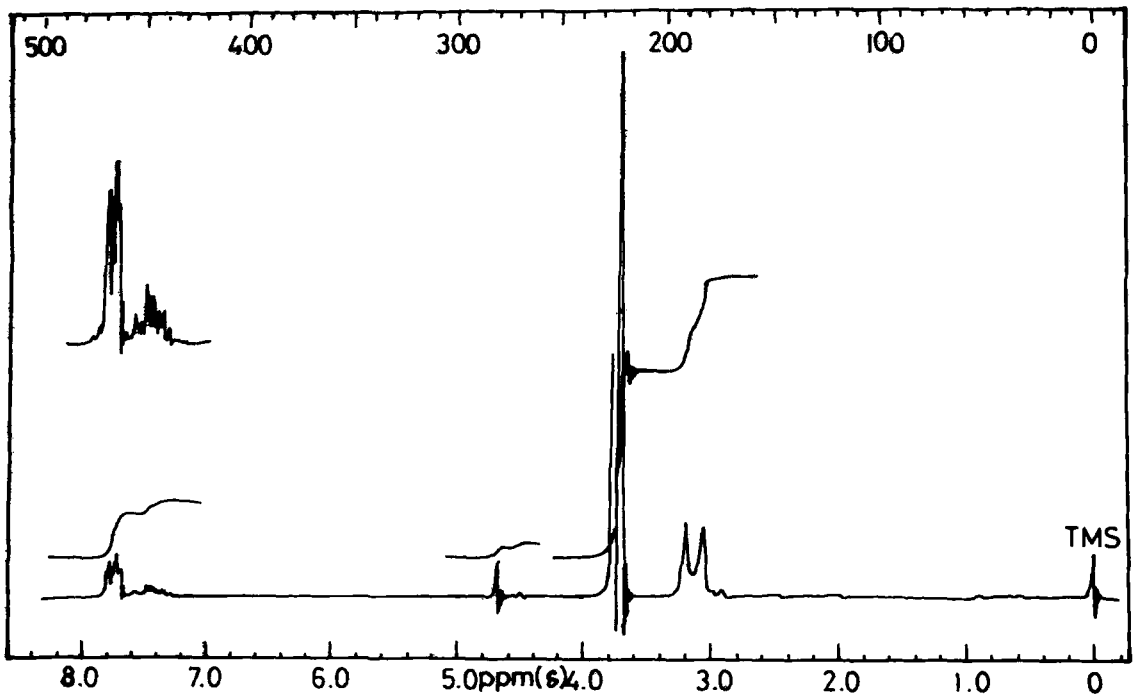


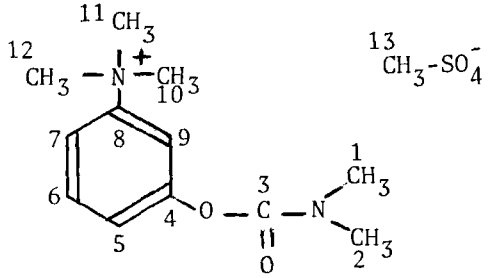
Figure 3: Proton NMR spectrum of neostigmine methyl sulphate in D_2O with TMS as internal reference.

Assignment of the chemical shifts to the different protons is shown below:

Chemical shift (ppm)	Multiplicity	Proton assignment
3.04, 3.12	singlets	$-\overset{O}{\overset{\|\|}{C}}-N(\underline{CH_3})_2$
3.72	singlet	$-\overset{+}{N}-(\underline{CH_3})_3$
3.80	singlet	$\underline{CH_3}-SO_4$
7.15-7.60, 7.66-8.30	multiplets	Aromatic protons

3.7.4 Carbon-13 Nuclear Magnetic Resonance (C-13 NMR) Spectrum

The C-13 NMR spectra of neostigmine methyl sulphate in deuterium oxide (D_2O) using DSS (sodium 2,2-dimethyl 2-silapentane-5-sulphonate) as an internal reference were obtained using a Jeol FX 100 MHz spectrometer at an ambient temperature. Figures 4 and 5 represent the proton-decoupled and off-resonance spectra respectively.



The carbon chemical shifts were assigned on the basis of the chemical shift theory and the off-resonance splitting pattern and are shown below:

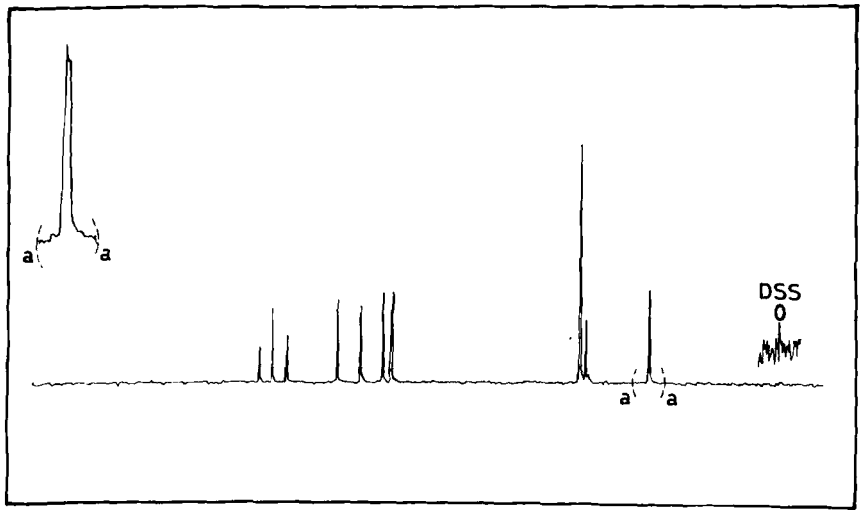


Figure 4: Proton-decoupled carbon-13 NMR spectrum of neostigmine methyl sulphate in D_2O with DSS as internal reference.

Figure 5: Off — Resonance carbon-13 NMR spectrum of neostigmine methyl sulphate in D_2O with DSS as internal reference.

Chemical shift (ppm)	Multi-plicity	Carbon assignment
38.9	quartet	1
38.8	quartet	2
158.3	singlet	3
149.8	singlet	4
126.9	doublet	5
134.0	doublet	6
119.8	doublet	7
154.5	singlet	8
117.1	doublet	9
59.6	quartet	10, 11 and 12
57.9	quartet	13

3.7.5 Mass Spectrum

The electron impact (EI) mass spectrum at 70 eV was recorded on Varian MAT 311 mass spectrometer is shown in Figure 6. In the spectrum an ion at m/e 428 was observed which most probably arises from a recombination of fragments. The spectrum shows a base peak at m/e 72. The most prominent ions are shown below:

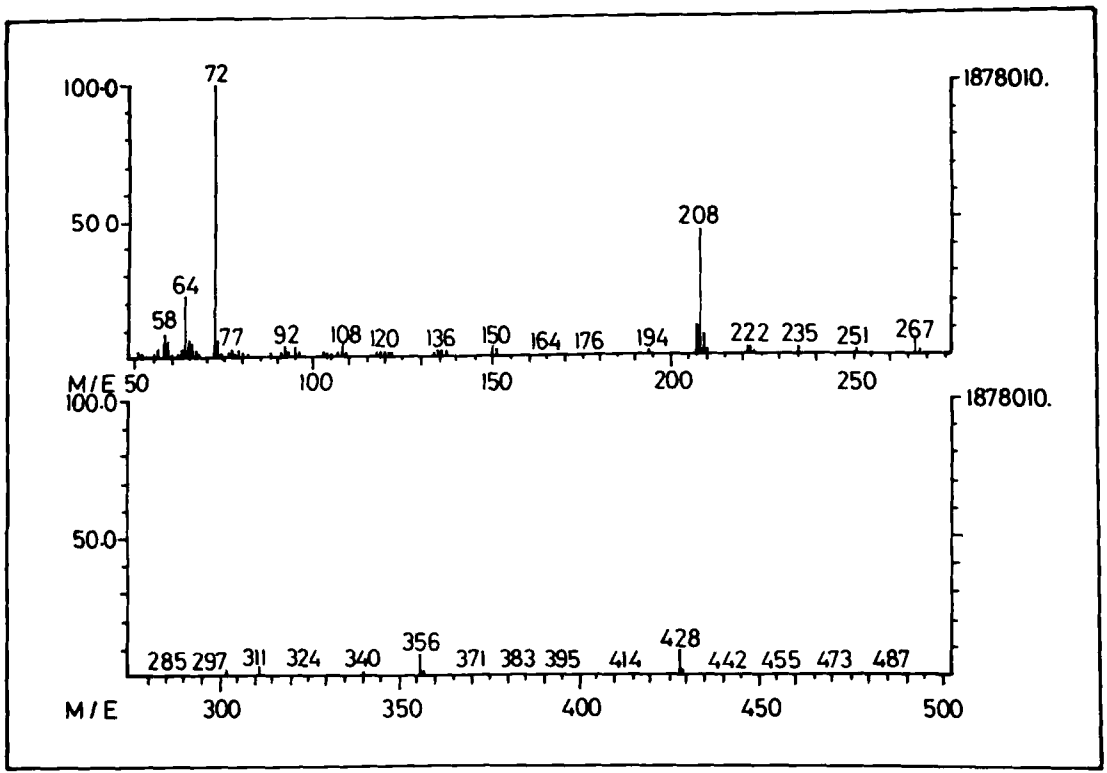
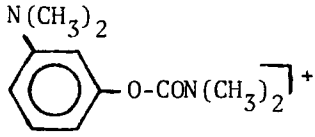
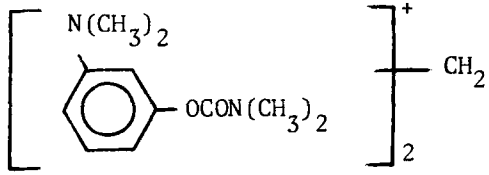


Figure 6: Electron impact (EI) mass spectrum of neostigmine methyl sulphate.

m/e	Fragment
72	$CON(CH_3)_2^+$
208	
356	$428 - CON(CH_3)_2^+$
428	

The chemical ionisation (CI) mass spectrum was obtained on Finnigan 4000 mass spectrometer and is shown in Figure 7. The spectrum shows ions at m/e 127 (the base peak) and at m/e 303 and both are probably arised from a recombination of fragments. The most prominent ions are shown below:

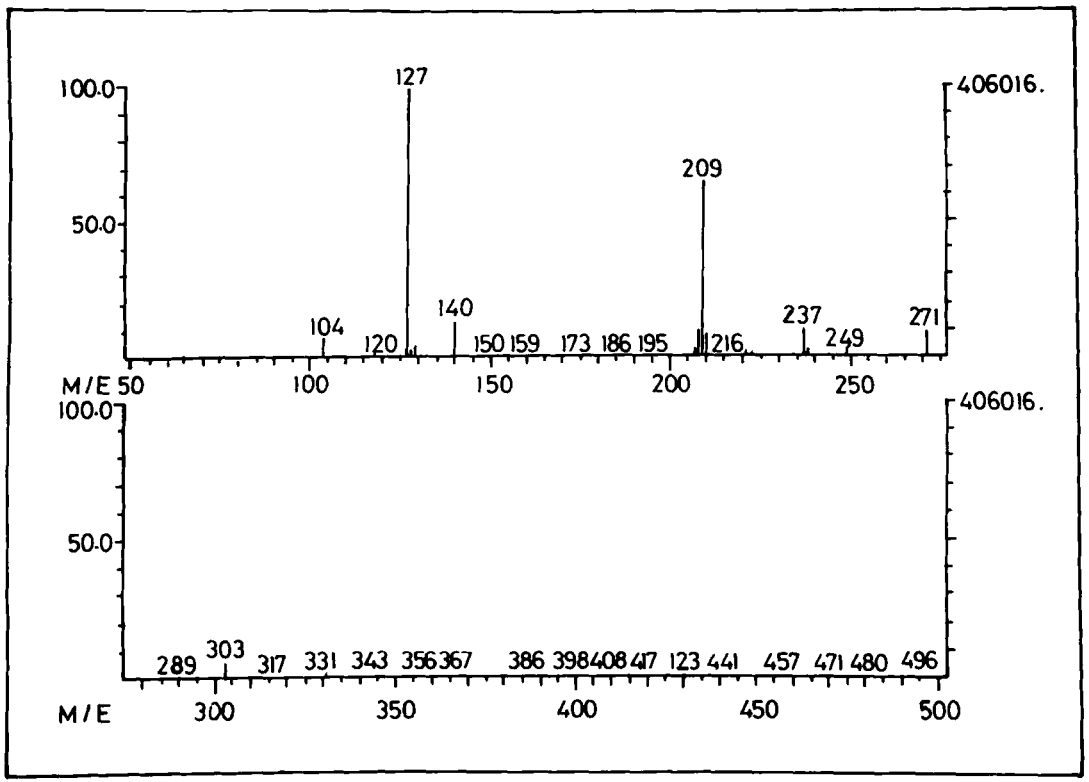
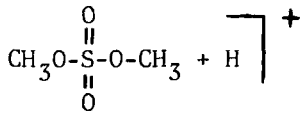
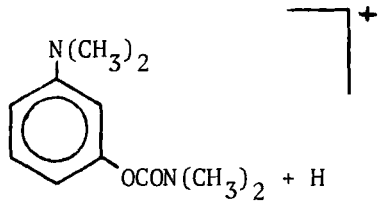
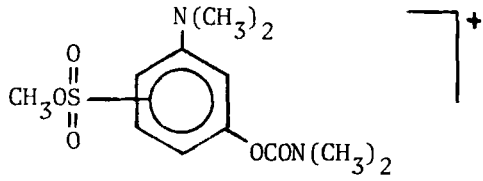
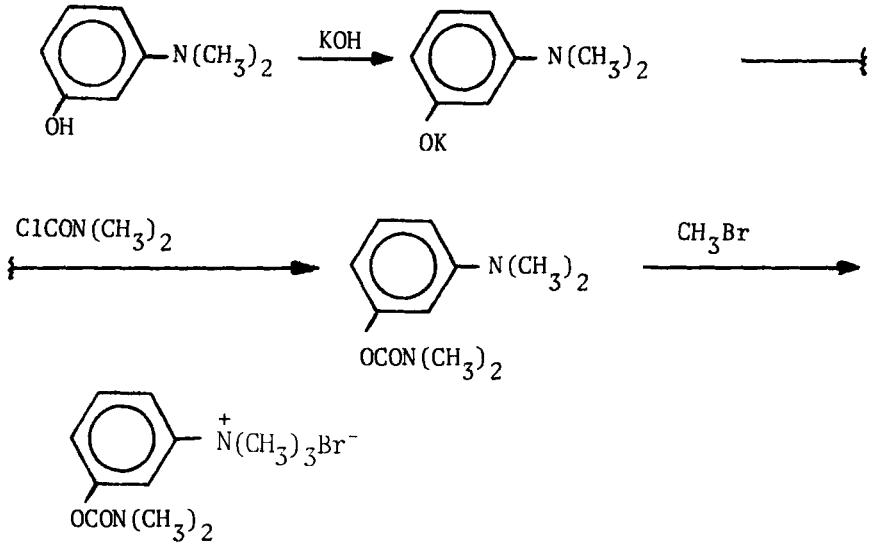


Figure 7: Chemical ionization (CI) mass spectrum of neostigmine methyl sulphate.

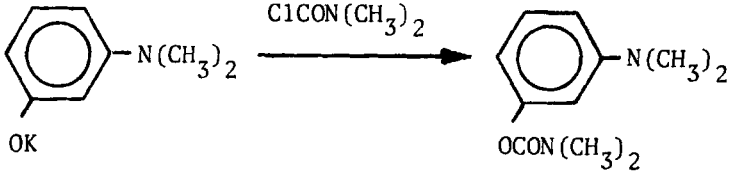
m/e	Fragment
127	
209	
303	

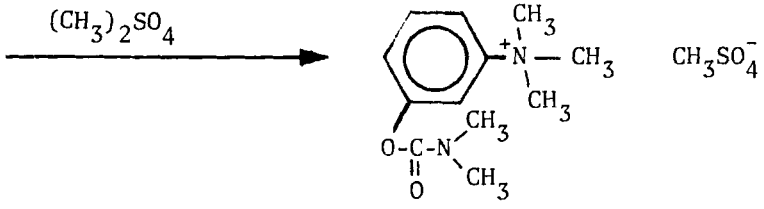
4. Synthesis

Neostigmine bromide can be prepared from 3-hydroxydimethylaniline, which is made from 3-nitroaniline by ordinary synthetic methods. The 3-hydroxydimethylaniline is dissolved in an alcoholic solution of the calculated amount of potassium hydroxide, and the solution of the potassium hydroxide, and the solution of the potassium derivative so formed is treated with dimethylcarbamoyl chloride, $Me_2N.COCl$ (made from dimethylamine and carbonyl chloride). This gives a tertiary base which, by combination with methyl bromide, yields the required quaternary salt - i.e. the dimethylcarbamic ester of 3-hydroxy-NNN-trimethylanilinium bromide: (18, 19).

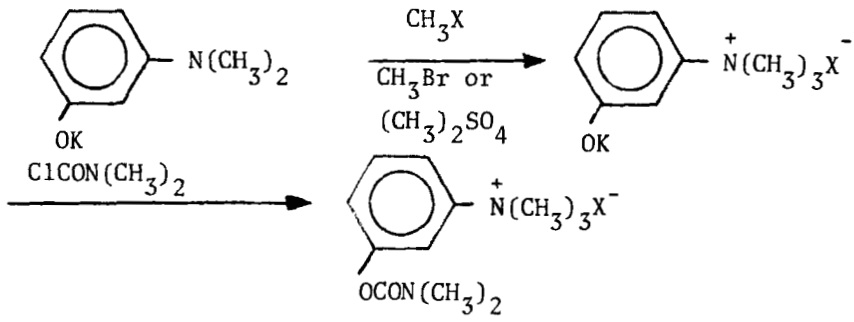


Neostigmine methylsulphate can be made in the same way, except that in the last stage the secondary amine is combined with methyl sulphate to form the salt $[(R\text{-}\overset{+}{N}Me_3)MeSO_4^-]$ (18).

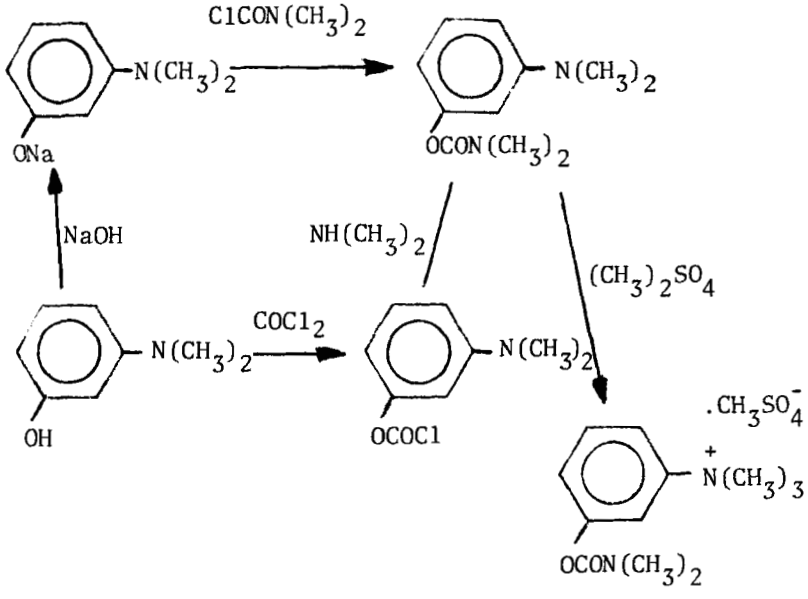




A modification of this route is to convert the hydroxydimethylaniline into the quarternary salt (by combination with methyl bromide or methyl sulphate), and then to treat the salt with dimethylcarbamoyl chloride (18).



Neostigmine can be synthesized either directly from 3-hydroxydimethylaniline with phosgene to give the carbonyl chloride and then with dimethylamine to give 3-dimethylaminophenyldimethylurethane or the latter may be prepared from the sodium salt of the starting material and dimethylcarbamic chloride. In either case the product is converted to the methylsulphate by treatment with dimethyl sulphate (20).



5. Pharmacokinetics

Neostigmine is absorbed poorly after oral administration, such that much larger doses are needed than by the parenteral route. Whereas the effective parenteral dose of neostigmine in man is 0.5 to 2.0 mg, the equivalent oral dose may be 30 mg or more. Large oral doses may prove toxic if intestinal absorption is enhanced for any reason and the quaternary alcohol and parent compound are excreted in the urine. Pyridostigmine and its quaternary alcohol are also the predominant entities found in urine after administration of this drug to man (21).

Neostigmine was rapidly eliminated from the plasma of 5 patients to whom 5 mg of the methylsulphate had been given to antagonise residual neuromuscular block. The plasma concentration of neostigmine declined to about 8% of its initial value after 5 minutes with a distribution half-life of less than one minute. Elimination half-life ranged from about 15 to 30 minutes. Trace amounts of neostigmine could be detected in the plasma after one hour (14).

Of neostigmine that reaches the liver, 98 per cent is metabolized in 10 minutes. (22) Its transfer from plasma to liver cells and then to bile is probably passive in character. Since cellular membranes permit the passage of plasma proteins synthesized in liver into the blood stream through capillary walls or lymphatic vessels, they may not present a barrier to the diffusion of quaternary amines such as neostigmine. Possibly the rapid hepatic metabolism of neostigmine provides a downhill gradient for the continual diffusion of this compound (23). A certain amount may be hydrolyzed slowly by plasma cholinesterase. When neostigmine was given by mouth to 2 patients with myasthenia gravis less than 5% was found unchanged in the urine (14). Following intramuscular administration about 65% of a dose is excreted in the urine unchanged (10). 20% of an oral dose is excreted in the urine and 50% in the faeces; less than 5% of the oral dose is excreted in the urine unchanged.

6. Therapeutic and Other Uses

Neostigmine is a quaternary ammonium anticholinesterase. It acts at the esteratic site of the enzyme to form the inactive dimethylcarbamoyl enzyme. Neostigmine has widespread actions, but fortunately its effects are more prominent on certain structures than on others, being particularly effective on the bowel, urinary bladder, and skeletal muscle, the pupil, the heart, blood pressure, and secretions being affected to a much lesser extent in doses that are ordinarily effective on related structures (19).

The drug inhibits cholinesterase activity and prolongs and intensifies the muscarinic and nicotinic effects of acetylcholine. It probably also has direct effects on skeletal muscle fibres. The anticholinesterase actions of neostigmine are reversible. It is used mainly for its action on skeletal muscle, and less frequently to increase activity of smooth muscle (14).

Neostigmine methylsulphate is used in the treatment of myasthenia gravis in usual doses of 1 to 2.5 mg daily given in divided doses by subcutaneous, intramuscular, or intravenous injection according to the severity of the condition (14).

Neostigmine is used in conditions of urinary bladder atony due to postanesthetic depression or to neurological disorders (19). It is also used in the treatment of paralytic ileus postoperative urinary retention, in doses of 0.5 to 1 mg. For expelling intestinal flatus prior to radiography of the gall-bladder, kidneys, or ureters, a single dose of 500 µg has sometimes been used (14).

Neostigmine can be employed as a diagnostic test, especially after symptoms have been purposely accentuated by the administration of quinine. Neostigmine is used as diagnostic test agent in myotonia congenita, in which condition neostigmine aggravates the symptoms. It may be used as a diagnostic agent for early pregnancy or to treat delayed menstruation: given intramuscularly on three successive days it will induce menstruation within 72 hours after the last dose unless the patient is pregnant. Neostigmine is used topically to treat primary open-angle glaucoma and in

the emergency treatment of primary acute-angle glaucoma. Miosis lasts 12 to 36 hours. Longer-acting anticholinesterases are preferred for the treatment of accommodative esotropia (19).

7. Toxicity and Side Effects

Adverse and side effects of neostigmine include salivation, anorexia, nausea and vomiting, abdominal cramps and diarrhoea. Symptoms of overdosage include sweating, lachrymation, watery nasal discharge, eructation, involuntary defaecation and urination flushing, miosis, conjunctival congestion, cilia spasm, brow ache, nystagmus, restlessness, agition, fear, excessive dreaming, increased brochial secretion combined with bronchoconstriction, bradycardia and hypotension, muscle cramps, scattered fasiculations and eventual severe weakness and paralysis, convulsions, and coma. It has also been stated that paradoxical effect could occur due to interaction between nicotinic and muscarinic actions; a consequence of this could be some evidence of an acceleration pulse-rate and elevation of blood pressure. Death may follow due to cardiac arrest or central respiratory paralysis and pulmonary oedema. The major symptom of overdosage in myasthena gravis is increased muscular weakness (14).

It is contraindicated in asthmatic patients. It should not be employed along with choline esters except for ophthalmologic use. Quinidine interferes with the action of neostigmine (19).

Neostigmine methyl sulphate, by increasing intestinal motility, may cause disruption of intestinal suture lines (10).

8. Methods of Analysis

8.1 Identification

a) To 0.1 ml of a 1 per cent w/v solution, add 0.5 ml of sodium hydroxide solution and evaporate to dryness on a water-bath. Heat quickly on an oil-bath to about 250^{o} and maintain at this temperature for about thirty seconds. Cool, dissolve the residue in 1 ml of water, cool in ice water, and add 1 ml of diazoaminobenzene-

sulphonic acid; a cherry-red colour is produced (15).

b) Crystal tests. Micro: lead iodide solution - branching needles (sensitivity: 1 in 400); platinic iodide solution - bunches of curved (sensitivity : 1 in 400) (11).

c) The infrared absorption spectrum of a potassium bromide dispersion of it, previously dried at 105° for 3 hours, exhibits maxima only at the same wavelengths as that of a similar preparation of USP Neostigmine Bromide Reference Standard (12).

d) A solution (1 in 50) responds to the test of bromide (neostigmine bromide) (12).

8.2 Titrimetric Methods

8.2.1 Aqueous Titration

British Pharmacopoeia (1973) (17) reported the following procedure for the assay of neostigmine methyl sulphate:

Dissolve 0.15 g in 20 ml of water, transfer to a semimicro ammonia distillation apparatus, and add 20 ml of a 50 per cent w/v solution of sodium hydroxide. Pass a current of steam through the mixture, collect the distillate in 50 ml of 0.1 N sulphuric acid until the total volume reaches about 200 ml, and titrate the excess of acid with 0.02 N NaOH using methyl red solution as indicator. Repeat the operation without the substance being examined; the difference between the titrations represents the amount of acid required to neutralise the dimethylamine formed from the neostigmine. Each ml of 0.02 N sulphuric acid is equivalent to 0.006688 g of $C_{13}H_{22}N_2O_6S$.

Huang et al (24) described a simple, rapid and accurate method using the application of alternating - current oscillopolarographic titration in pharmaceutical analysis. In

this method neostigmine methyl sulphate in injections was titrated with sodium tetraphenyl borate. To the injection solution (20 ml) containing 10 mg of neostigmine methyl sulphate were added 10 ml of 0.01 N sodium tetraphenyl borate and 10 ml of acetate buffer solution (pH 5.3). The solution was diluted to 50 ml with water, after 5 minutes the precipitate was formed, the contents were filtered. A 25 ml portion of filtrate was treated with 8 drops of 40% sodium hydroxide solution and titrated with 0.01 N TI_2SO_4, the disappearance of the incision of sodium tetraphenyl borate on the curve of d/E/dt vs E being used to indicate the end point. The results were corrected for a blank value. The recovery range from 99.3 - 100.2% and the coefficient of variation was 0.42%.

Tsubouchi et al (25) reported a method of analysis of neostigmine using the application of one-phase end-point change system in two phase titration to amine drug analysis. Neostigmine in aqueous solution (5 or 10 mM) was determined by titration with aqueous 0.01 M tetraphenyl borate with tetrabromo phenolphthalien ethyl ester as an indicator in the organic phase (1,2-dichloroethane), in borate phosphate buffer medium (pH 5.5.-7.5). The end-point was detected by means of color change of the indicator in the organic phase without transfer of indicator between phases. Various common ions (including carbonate, acetate, citrate, and tannate) did not interfere but thiamine, papaverine, dodecyle sulphate and mercury caused interference.

Diamandis and Christopoulos (26) developed a potentiometric titration of neostigmine bromide in pharmaceutical compounds by titrating them with sodium tetraphenyl borate. 25 Ml of aqueous solution (0.2 - 1 mM) of neostigmine bromide and 5 ml of the appropriate buffer solution was added.

The solution was titrated potentiometrically at 0.36 ml per minute with 0.01 M sodium tetraphenyl borate at 22° ± 2° with continuous stirring. The end-point was detected by a tetraphenylborate selective electrode. This method was adopted for determination of neostigmine in powders, injections, drops and syrups.

United States Pharmacopeia XIX (1980) (12) described the following procedure for the assay of neostigmine methyl sulphate:

Place about 100 mg of neostigmine methyl sulphate, accurately weighed, in a 500 ml Kjeldahl flask, dissolve in 150 ml of water, and add 40 ml of sodium hydroxide solution (1 in 10). Connect the flask by means of a distillation trap to a well-cooled condenser that dips into 25 ml of boric acid solution (1 in 25), distil about 150 ml of the contents of the flask, add methyl purple TS to the solution in the receiver, and titrate with 0.02 N sulphuric acid. Perform a blank determination, and make the necessary correction. Each ml of 0.02 N sulphuric acid is equivalent to 6.688 mg of $C_{13}H_{22}N_2O_6S$.

British Pharmacopoeia (1973) (17) reported the following procedure for the assay of neostigmine tablets:

Weigh and powder 2 tablets. Transfer a quantity of the powder, equivalent to 0.15 of neostigmine bromide, to a semi-micro ammonia distillation apparatus, add 20 ml of a 50% w/v solution of sodium hydroxide and 0.5 ml of a 2% solution of octan-2-ol in liquid paraffin and complete the assay described under neostigmine methyl sulphate. (above) begining at the words "Pass a current of steam ....". Each ml of 0.02 N sulphuric acid is equivalent to 0.006064 g of $C_{12}H_{19}BrN_2O_2$.

8.2.2 Titrimetric Methods

Non-aqueous Titration

United State Pharmacopeia XIX (1980) (12) described the following procedure for the assay of neostigmine bromide:

Dissolve about 750 mg of neostigmine bromide accurately weighed, in a mixture of 70 ml of glacial acetic acid and 20 ml of mercuric acetate TS, add 4 drops of crystal violet TS, and titrate with 0.1 N perchloric acid to a blue end-point. Perform a blank determination, and make any necessary correction. Each ml of 0.1 N perchloric acid is equivalent to 30.32 mg of $C_{12}H_{19}BrN_2O_2$ (12).

Bayer and Posgay (27) suggested a perchloric acid titration method for the determination of neostigmine in pharmaceutical preparations. The neostigmine solution was titrated with 0.1 N perchloric acid which has been standardized with anhydrous potassium carbonate with 0.1% gentian violet in acetic acid containing 3% mercuric acetate as indicator.

Surmann et al (28) reported a titration method for the pharmaceutical hydrochlorides and hydrobromides in non-aqueous media. This method involve direct titration of drug with perchloric acid in acetic anhydride medium, with naphthol benzein or Sudan Red B an indicator for hydrochlorides for hydrobromides respectively.

Kracmarova and Kracmar (29) reported the following non-aqueous titration:

Evaporate the sample of the eye drops (5 ml) on a water bath to dryness, dissolve the residue in anhydrous acetic acid (2 x 20 ml), add crystal violet (0.2% in anhydrous acetic acid) (2 drops) as indicator, and titrate with 0.1 N perchloric acid to a blue end-point, add 5% Hg^{II} acetate solution

(5 ml), mix and titrate again to a blue end-point; the difference between the titres corresponds to neostigmine bromide.

8.2.3 Iodimetric Methods

Koka (30) developed an iodimetric determination of proserine (neostigmine methyl sulphate) in drug formulations. The drug sample is dissolved in water and the solution is treated with 2-3 ml of dilute sulphuric acid, 2-3 ml of 10% potassium iodide solution and 5 ml of 0.1 N iodine, diluted to 25 ml with water and filtered; 10 ml of filtrate is titrated with 0.1 N sodium thiosulphate.

Mitchenko and Kirichenko (31) reported the use of iodimetric method for the determination of neostigmine methyl sulphate and pilocarpine HCl in eye-drops. Neostigmine methyl sulphate and pilocarpine HCl are determined by reaction with iodine solution in dark, filtering the mixture and titrating unreacted iodine with sodium thiosulphate solution. When both are present the sum of the two is determined by above method and only pilocarpine is determined alkalimetrically, argentimetrically or mercurimetrically. Neostigmine methyl sulphate is determined by the difference. The relative error in determining the two drugs in eye-drops is 3.56 and 2.85 respectively.

8.3 Biological Method

Buckles and Bullock (32) reported the application of enzyme inhibition to the estimation of small quantities of drugs possessing anticholinestrase activity. The method is based on the inhibition of the pseudocholinesterase activity of horse serum. 5 Ml of sample containing 5 µg of neostigmine is mixed with 1 ml of horse serum, 1 ml of 0.2% cresol red solution and 37 ml of water. The mixture is heated to 40^{o}C and pH adjusted to 7.9. After 15 minutes add 5 ml of 3% acetylcholine perchlorate solution and readjust

the pH to 7.9. Maintain the pH between 7.8 and 8.0 during 15 minutes by dropwise addition of 0.025 N sodium hydroxide and record the volume of alkali used. Correct for non-enzymic hydrolysis by conducting an experiment with 1 ml buffer instead of horse serum and carry out a blank with 5 ml water instead of sample. The percentage inhibition is a linear function of the log concentration of neostigmine methyl sulphate.

8.4 Spectrophotometric Methods

8.4.1 Colorimetric Methods

Belal et al, (33) described a spectrophotometric determination of neostigmine in tablets and ampoules. Tablet preparations containing neostigmine methyl sulphate were powdered and dissolved in 50 ml of water and 1 ml of solution was mixed with 3 ml of citrate-phosphate buffer solution (pH 7.8) and 2 ml of a 0.1% solution of bromothymol blue in 0.01 N sodium hydroxide. The mixture was extracted with chloroform before measurement of its absorbance at 416 nm vs a reagent blank. Alternatively 10 ml of chloroform extract was treated with 10 ml of 0.01 N sodium hydroxide and the aqueous layer was separated and made up to 25 ml with water, its absorbance being measured at 615 nm as a reagent blank. The calibration graph were rectilinear for 0.2-1.6 mg. The recovery by this method was 100.5%.

Belikov et al (34) have used sulphonephthalein dyes as extraction reagents for the extraction of quaternary ammonium compounds. They reported the optimum pH value, λmax, distribution coefficient and sensitivity of reactions for the ion association complexes of neostigmine methyl sulphate. In a general procedure for determining the quaternary ammonium compounds, 0.5 ml of 0.01% solution of compound is added to 5 ml of a buffer solution of appropriate pH, 0.5 ml of 0.1% dye

solution is added, the complex is extracted into 5 ml of chloroform and absorbance of organic phase is measured at 400-434 nm. Relative error is less than 2%.

Tsubouchi (35) reported a spectrophotometric determination of organic cations by solvent extraction with tetrabromophenol-phthalien ethyl ester. 5 Ml of the sample containing less than 0.01 µM of neostigmine was extracted with 2 ml of 0.07% ethanolic tetrabromophenolphthalien ethyl ester (potassium salt) and 5 ml of borate - phosphate buffer (pH 10), dilute to 25 ml with water and shake for 2 minutes with 10 ml of 1,2,dichloroethane. Separate and filter the organic phase and measure its absorbance at 615 nm against reagent blank. The coefficient of variation was 1.11%.

8.4.2 Ultraviolet Spectrophotometric Method

Rita (36) developed a ultraviolet spectrophotometric assay of neostigmine methyl sulphate as the alkaline hydrolysis product, 3-(dimethylamino)phenol. An aliquot of injection preparation equivalent to 5 mg of neostigmine methyl sulphate was acidified and any phenol or p-hydroxy benzoate preservative present was extracted with chloroform. The residual aqueous solution was made alkaline and heated on steam bath for 30 minutes and the 3-(dimethylamino) phenol formed is determined by UV-spectrophotometry.

Kracmarova and Kracmar (29) described a spectrophotometric method for evaluation of neostigmine bromide and neostigmine methyl sulphate with regards to the degradation products. To 5 ml of injection solution, 2 ml of 0.05 N H_2SO_4 was added. The mixture was diluted to 10 ml with water. The absorbance was recorded at 261 nm for neostigmine bromide or at 260 nm for neostigmine methyl sulphate or at 266 nm for both of the compounds.

Mytchenko and Kyrychenko (37) reported a spectrophotometric determination of proserine (neostigmine methyl sulphate) in medicinal formulations. The tablets are grinded in water and shaked. 0.5% solution is filtered and the absorbance of filtrate is directly recorded at 260 nm. This method can also be used for analysis of neostigmine in eye drops (containing also pilocarpine HCl) after dilution. The accuracy of method is within ± 0.66%.

8.4.3 Infrared Spectrophotometric Methods - IR

Mynka et al (38) reported the infrared spectroscopy of drugs of the amide class. The IR spectrum of neostigmine methyl sulphate was determined and interpreted by measurement at 1732 cm^{-1}. Analysis can be done with a maximum error of 1.66%.

Kracmarova and Kracmar (29) reported spectrophotometric analysis of neostigmine in infrared region. Grind the sample with liquid paraffin (12 drops) and record the spectrum in a sodium chloride cell in the range 3-15 μ. Characteristic band for neostigmine bromide and neostigmine methyl sulphate are observed.

8.4.4 Photometric Method

Kracmarova and Kracmar (29) reported a photometric method for the evaluation of neostigmine bromide and neostigmine methyl sulphate as follows:

To the injection solution (1.5 ml) add buffer solution (pH 4.6) (5 ml), bromo-cresol purple solution (2 g in 15 ml of water and 3.2 ml of 0.1 N - potassium hydroxide diluted to 250 ml with water) (5 ml) and chloroform (25 ml). Shake for 1 minute in a separating funnel, and filter the chloroform layer; repeat the extraction with 2 ml of chloroform and dilute the combined extracts with chloroform to 50 ml.

To a 25 ml portion add 0.1 N potassium hydroxide (20 ml), shake for 30 seconds, filter the aqueous layer, dilute to 100 ml with water and measure the extinction at 570 nm.

8.5 Chromatographic Methods

8.5.1 Paper Chromatographic Method

Giebelmann (39) developed a paper chromatographic detection for phenoxy compounds having a phenyl ester functional group using the following solvents - butanol-20% formic acid (4:1), methanol acetone-triethanolamine (100:100:3) and butanol - water (6:1). As little as 2.5-10 µg of neostigmine can be detected with Millon's reagent.

Scott et al, (40) reported an investigation on the metabolism of neostigmine in patients with myasthenia gravis. Neostigmine and m-hydroxyphenyltrimethylammonium bromide are precipitated from urine with aqueous bromine. The precipitate is extracted with 50% methanol. This extract is applied to Amberlite CG-50 resin at pH 6.86. Ten bases are eluted with 0.2 N hydrochloric acid and submitted to chromatography on Whatman NO 541 paper with butanol-ethanol-water-acetic acid (32:8:12:1) as solvent. The R_f value of neostigmine was between 0.5 - 0.3.

Kracmarova and Kracmar (29) described procedures for the determination of neostigmine bromide and neostigmine methyl sulphate in various pharmaceutical preparations, as well as for the chromatographic control of the degradation products; 3 dimethylaminophenol and (3-hydroxyphenyl) trimethylammonium salts, with the use of Whatman No. 1 paper, butanol-conc-aqueous ammonia-water (3:1:4) as solvent, and Dragendorff reagent as the detecting agent.

Clarke (11) described the following:

(1) Paper: Whatman No. 1, sheet 14 x 6 in, buffered by dipping in a 5% solution of sodium hydrogen citrate, blotting, and drying at 25° for 1 hour. It can be stored immediately.

Sample: 2.5 µl of a 1% solution; in 2 N acetic acid if possible, otherwise in 2 N hydrochloric acid, 2 N sodium hydroxide, or ethanol.

Solvent: 4.8 g of citric acid in a mixture of 130 ml of water and 870 ml of n-butanol. This may be used for several weeks if water is added from time to time to keep the specific gravity at 0.843 to 0.844.

Development: Ascending, in tank 8 x 11 x 15½ in; 4 sheets being run at a time. Time of run, 5 hours.

Location under ultraviolet light, absorption; location reagents: Iodoplatinate spray; weak reaction; bromocresol green spray, weak reaction. Rf 0.20.

(2) Paper: Whatman No. 1 or No. 3, sheet 17 x 19 cm, implegnated by dipping in a 10% solution of tributyrin in acetone and drying in air.

Sample: 5 µl of 1 to 5% solutions in ethanol or chloroform.

Solvent: Acetate buffer (pH 4.58).

Equilibration: The beaker containing the solvent is equilibrated in a thermostatically controlled over at 95° for about 15 minutes.

Development: Ascending, the paper is folded into a cylinder and clipped, the cylinder is inserted in the beaker containing the solvent which is not removed from the oven. A plate-glass disk thickly smeared with silicone grease may serve as a cover. Time of run, 15 to 20 minutes.

Location: Under ultraviolet light, absorption, R_f 1.00.

(3) Paper and sample as in 2 above. Solvent: Phosphate buffer (pH 7.4). Equilibration: The peak containing the solvent is equilibrated in a thermostatically controlled oven at 86° for about 15 minutes.

Development: as in 2 above.

Location: Under ultraviolet light, absorption, R_f 0.66.

8.5.2 Thin-Layer Chromatography (TLC)

Anand (41) reported a thin-layer chromatographic and spectrophotometric investigation on the identification of a phenolic impurity in neostigmine. The samples of neostigmine were applied on TLC plates coated with alumina-D and were developed for 30 minutes with chloroform-benzene-methanol (5:4:1) to give a good separation.

Neostigmine was detected with iodine vapours or with Dragendorff's reagent. The ultra violet spectrophotometric technique is also used for quantitative estimation. Neostigmine has a maxima at 261 nm.

Porst and Kny (42) described analysis of neostigmine eye drops using TLC system and absorption spectroscopy. The drug is detected and determined by TLC on cellulose and ultraviolet or visible spectrophotometry is done after formation of colored compound with 3,5-dichloro-p-benzoquinonechlorimine or sodium nitrite or 4-dimethylaminobenzaldehyde.

Clarke (11) described the following:

Plate: Glass plate, 20 x 20 cm, coated with a slurry consisting of 30 g of silica gel G in 60 ml of water to give a layer 0.25 mm like and dried at 110° for 1 hour.

Sample: 1 µl of a 1% solution in 2N acetic acid.

Solvent: Strong ammonia solution: methanol (1.5 : 100). It should be changed after two runs.

Equilibration: The solvent is allowed to stand in the tank for 1 hour.

Development: Ascending, in a tank, 21 x 21 x 10 cm, the end of the tank being covered with filter paper to assist evaporation. Time of run, 30 minutes.

Location reagent: Acidified iodoplatinate spray, positive reaction, R_f 0.02.

8.5.3 Gas Liquid Chromatographic Methods

Chan et al (43) developed a sensitive and selective analytical method for the measurement of neostigmine in human plasma using gas chromatography. Plasma containing neostigmine is washed with ethyl ether and buffered with potassium iodide-glycine solution, then iodide-glycine-drug complexes are extracted into dichloromethane. The extract is evaporated and the residue is dissolved in methanol. 2-5 µl of methanolic solution was injected into gas chromatographic glass column (2 m x 0.25 inches O.D.) packed with Diatomite CQ coated with 3% of OV-1, 3% of OV-17 or 3.8% of SE-30 and silanised; a nitrogen selective detector was used. Pyridostigmine bromide was used as internal standard. The temperature was maintained at 205°C and the flow rate of nitogen was 30 ml per minute. The detection limit was 5 ng/ml and there was no interference with other basic drugs.

Ward et al (44) described a simple GLC assay of neostigmine using diethyl analog of neostigmine as internal standard. Neostigmine and its analog were dissolved in 25 µl of chloroform. 2 µl of chloroform solution was injected into a

gas chromatograph equiped with a flame ionisation detector and 1.2 m x 2 mm I.D. glass column packed with 5% OV-17 on Gas-Chrom Q is used at 210^{o} with helium (40 ml/minute) as a carrier gas and flame ionization detector. For determination of neostigmine in biological fluids, the drug is extracted into chloroform with added 3-diethylcarbamoyloxy-NNN-trimethylanilinium iodide as the internal standard. The relative retention time of the drug and the standard is 1 to 1.35.

De Ruyter et al (45) developed a reversed-phase, ion-pair liquid chromatography of quarternary ammonium compounds for the determination of pyridostigmine, neostigmine, and edrophonium in biological fluids. The method is based on extraction of neostigmine into water-saturated dichloromethane, then back-extraction is done with tetrabutyl ammonium hydrogen sulphate to enhance the recovery to more than 86%. The extracts are submitted to chromatography on a variety of reversed-phased columns fitted with 214 nm detectors. For biological extracts a column (15 cm x 4.6 mm) of Ultrasphere octyl (5 μm) provided with an RP-2 pre-column was used. For neostigmine elution was carried out with a solution containing sodium heptanesulphonate (0.01 M), sodium hydrogen phosphate (0.01 M) and tetramethyl ammonium chloride (2.5 mM) in aqueous 20% acetonitrile. The mobile phase (2 ml per minute) was adjusted to pH 3 with sulphuric acid, edrophonium was used as an internal standard. The calibration graphs were rectilinear for upto 400 ng per ml. The limit of detection was 5 ng per ml. The coefficient of variation was 1.5%.

Davison et al (46) described a method for simultaneous monitoring of plasma level of neostigmine and pyridostigmine in man. The assay involve preliminary ion pair

extraction of the drugs and internal standards (3-diproypl carbamoyloxy) 1-methyl pyridinium bromide) with the use of potassium iodide and of glycine buffer. The extract is analysed by GLC (10% of OV-17 on Chromosorb W-AW (100-120 mesh) with a nitrogen-sensitive detector. The calibration graphs are rectilinear and reproduced over the range 5-100 ng per ml of either drugs in 3 ml samples.

Pohlmann and Cohan (47) developed a simplified detection of quarternary ammonium compounds by gas chromatography. The method has been applied to pyridostigmine, neostigmine and acetyl choline. The quarternary salts in the serum or urine are first converted with potassium triiodide (KI_3) into their iodies which are extracted into chloroform. The extract is evaporated in vacuum and the residue is dissolved in water or hexane. The solution is injected on to a column (1.8 m x 2 mm) of Porapak Q (800-100 mesh), Chromosorb 101 (80-100 mesh) or Chromosorb 105 (80-100 mesh), operated at 145°C-170°C with helium or nitrogen as carrier gas (20 ml per ml). On the column the quarternary ammonium iodides are thermally decomposed, and the methyl iodide released is measured with a ^{63}Ni electron-capture detector. Extraction yields are at least 95% and down to 10 f-mol of quarternary ammonium compound can be detected with good reproducibility.

Chan and Dehghan (48) described a GLC method for isolation and determination of neostigmine, pyridostigmine and their metabolites in human biological fluids. The method involves a preliminary, selective ion-pair extraction of the drugs and their metabolites into dichloromethane and in (dichloromethane-acetone), respectively. The analysis of extract was done by GLC on a column of 3% (drug) or 10% (metabolite) of OV-17 on Chromosorb W AW,

with a nitrogen-sensitive detector. For the determination of 3-hydroxytrimethyl anilinium ion; a major metabolite of neostigmine, 3-hydroxy-N-methylpyridinium ion was used as a internal standard. As little as 3 ng/ml of neostigmine and upto 50 ng/ml of its metabolite can be determined in biological samples.

8.6 Ion-Selective Electrodes Method

Kina et al (49) described a method of neostigmine analysis based on ion-selective electrodes sensitive to some organic compounds used as drugs. Liquid membrane electrodes sensitive to neostigmine have been made by the ion-association extraction method. The exchange components used were crystal violet, dipicrylamine, sodium tetrapentyl borate, 1,10-phenanthroline, 4,7-diphenyl-1,10-phenanthroline. The solvents used for these compounds were 1,2-dichloroethane and nitrobenzene. The choice of solvents affected with selectivities of the membranes but the choice of ion-exchange components did not. The selectivities relative to univalent cations were mostly better than 10^{-4}. The useful concentration range of electrodes was 10 μM to 0.1 M with a response of 59 mV per decade change in the concentration of the drug. The electrodes could be used in analysis of mixed pharmaceutical preparations.

Diamandis and Christopoulos (26) reported a potentiometric titration of neostigmine and other pharmaceutical compound in pharmaceutical formulation with sodium tetrahydroborate. (See Section 8.2.1.). The end point was detected by a tetraphenylborate-selective electrode.

8.7 Polarographic Method

Novotny (50) devised a polarographic method for the determination of neostigmine in pharmaceutical preparations having a concentration of 0.5 mg/ml of the drug. This method is based on the hydrolysis of neostigmine and nitration of resulting phenol, followed by polarography of

resulting derivative. The neostigmine solution (0.2 to 0.6 ml of 0.1%) is evaporated to dryness in a 50 ml beaker on a water bath. 1 Ml of KOH solution (20%) is added and warmed on the bath for 20 minutes and again evaporated to dryness. 0.5 Ml of water and 2 ml of conc. HNO_3 (65%) is added and warmed for 20 minutes and cooled. 10 Ml of KOH solution (20%) is added and the volume made upto 14.5 ml with water. The estimation is carried out by polarography, with a galvanometer sensitive to 10^{-9} amp. To determine the concentration of neostigmine in an unknown solution, solution of the unknown concentration of neostigmine is treated as described above, both with and without a known amount of neostigmine.

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PIRENZEPINE DIHYDROCHLORIDE

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

5,11-Dihydro-11-[(4-methyl-1-piperazinyl) acetyl]-6H-pyrido[2,3-b][1,4] benzodiazepin-6-one dihydrochloride monohydrate.

11-[(4-Methyl-1-piperazinlyl) acetyl]-pyrido [2, 3-b][1,4] benzodiazepin-6(5 H)-one dihydrochloride monohydrate.

1.1.2 Generic Names

L-S 519, Pirenzepine

1.1.3 Trade Names

Bisvanil, Gasteril, Gastrozepin, Leblon, Tabe, Ulcosan.

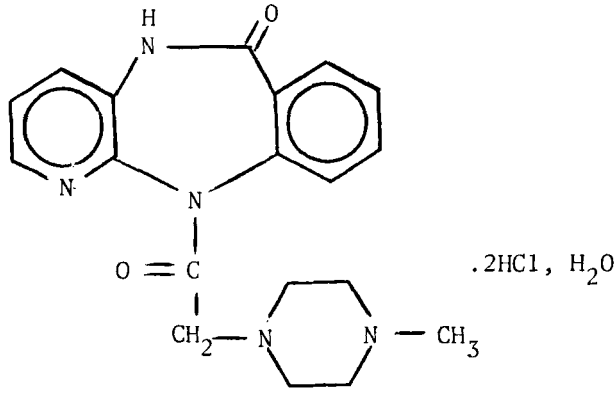
1.2 Formulae

1.2.1 Empirical

$C_{19}H_{23}Cl_2N_5O_2$ (dihydrochloride)

$C_{19}H_{21}N_5O_2$ (base)

1.2.2 Structural



1.2.3 CAS No.

[29868-97-1] as dihydrochloride salt.

[28797-61-7] as free base.

1.3 Molecular Weight

424.33 as dihydrochloride salt.

351.42 as free base.

1.4 Elemental Composition

Dihydrochloride salt: C 53.78%, H 5.46%, N 16.50%, O 7.52%, Cl 16.71% (1).

Free base: C 64.94%, H 6.02%, N 19.93%, O 9.11% (2).

1.5 Appearance

Pirenzepine dihydrochloride is a white crystalline substance (2).

2. Physico-chemical Properties

2.1 Melting Range

Pirenzepine dihydrochloride melts in the range 250-252^{o}C (corrected) with decomposition (1).

2.2 Solubility

Pirenzepine dihydrochloride is readily soluble in water, slightly soluble in methanol and practically insoluble in ether (1).

2.3 Distribution Ratio

Pirenzepine is a tricyclic compound, but its physioco-chemical properties differ considerably from those of other tricyclic compounds which are used as centrally-acting drugs. Pirenzepine is unusual because of its pronounced hydrophilic properties. Comparison of the distribution coefficient of pirenzepine with those of other

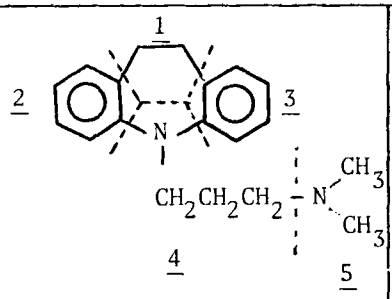
tricyclic drugs at pH 7.4, has been carried out (3). The results depicted in Table 1 show that pirenzepine is 200-13000 less lipid soluble.

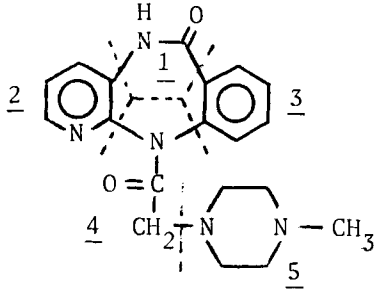
Table 1: Distribution coefficient of pirenzepine and other tricyclic drugs.

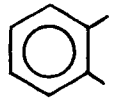
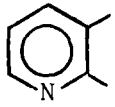
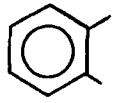
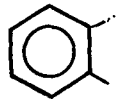
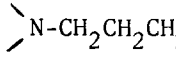
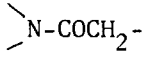
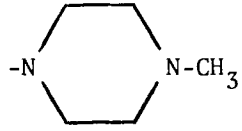
Drug	P_{app} pH 7.4	CNS effects
Chlorpromazine	3160	+
Cyproheptadine	1600	+
Imipramin	250	+
Dibenzepine	51	+
Pirenzepine	0.23	-

Eberlein et al (3) analysed the pirenzepine molecule into five structural elements. Each of the five elements can be allocated a hydrophobic substituent consant (πx) and compared with those assigned for corresponding structural elements in imipramine molecule (Table 2). A positive πx value indicates contribution to the lipophilic character of the molecule and a negative πx value indicates contribution to hydrophilicity of the molecules.

Table 2: Contribution of the different fragments of pirenzepine and imipramine molecules to their distribution coefficients.





Imipramine			Pirenzepine	
Fragment No.	Fragment structure	πx	Fragment structure	πx
1	$-CH_2-CH_2-$	+10.02	-NH-CO-	-1.49
2		+ 1.96		+0.65
3		+ 1.96		+1.96
4		+ 0.50		-0.97
5	$-N(CH_3)_2$	- 0.32		-0.14
Log_{Base} (calculated)		+ 5.12	Log_{Base} (calculated)	+0.010
Log_{Base} (experimental)		+ 4.80	Log_{Base} (experimental pH 7.4)	-0.64

It can be shown that the structural elements 1 and 4, in particular, contribute largely to the hydrophilicity of pirenzepine. Fragments 1 and 4 in pirenzepine consist of amide groupings each with two dipoles which can interact strongly with water molecules. The pyridyl group of fragment 2 in pirenzepine is also expected to contribute to the hydrophilicity of the molecule. Adding up the individual hydrophobic constants for pirenzepine molecular fragments a log P of 0.01 is obtained for the whole molecule, which indicates that pirenzepine as the uncharged free base will be distributed uniformly between the octanol and water. At physiological pH, however, the ratio shifts even more towards water solubility due ionization of the molecule. Under physiological conditions (pH 7.4) the experimentally determined log P is - 0.64 (Table 2), indicating that about 5 times more of the compound is distributed in the aqueous phase than in octanol. Under the same conditions imipramine accumulates much more strongly in the organic phase. As will be shown later, the pronounced hydrophilic properties of pirenzepine explain many of the specific pharmacokinetic characteristics of the drug.

2.4 Spectral Properties

2.4.1 Ultraviolet Spectrum

The ultraviolet absorption spectrum of pirenzepine in methanol/HCl was obtained on a Cary 219 spectrophotometer. The spectrum, shown in Figure 1, is characterized by a maximum at 283 nm and a minimum at 264 nm. The results agree with those reported in the literature (4, 5). It is also reported that pirenzepine in 0.1 N sodium hydroxide solution shows absorption maximum at 295 nm (4).

2.4.2 Infrared Spectrum

The infrared absorption spectrum of pirenzepine obtained from a potassium bromide dispersion was recorded on a Pye

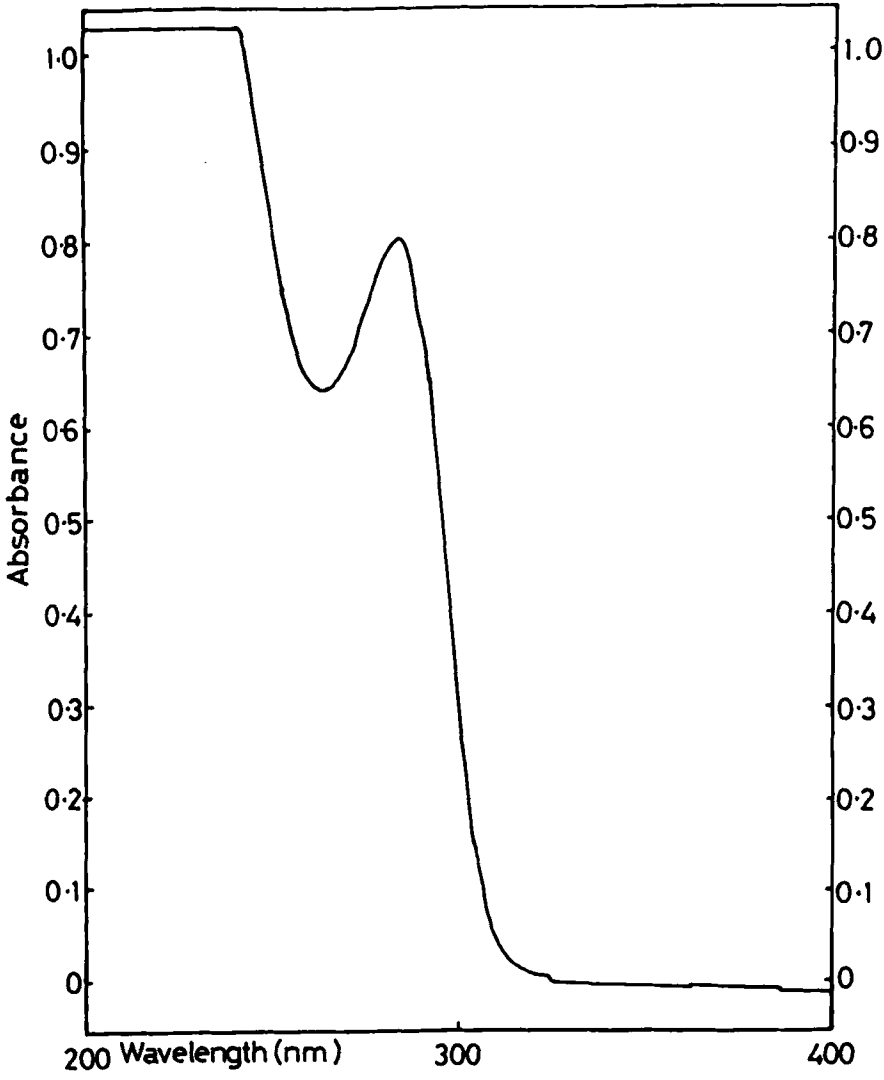


Figure 1 : ULTRAVIOLET SPECTRUM OF PIRENZEPINE DIHYDROCHLORIDE IN METHANOLIC HCl.

Unicam SP 1025 spectrometer and is shown in Figure 2. The characteristic bands of the spectrum with the assignments are listed below:

Frequency (cm^{-1})	Assignment
3520, 3463	Amide N-H stretch
3240	Water molecule
3020, 2990	Aromatic C-H stretch
2700 - 2400	t-Amine salt N-H stretch
1717, 1678	Amide C = O
1605, 1585, 1502,) 1479, 1432)	Aromatic C⋯C, C⋯N stretch.
1370	Aryl C - N stretch
800, 785, 760	Aromatic C - H out-of-plane bending.

2.4.3 ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) Spectrum

Pirenzepine solution in D_2O was used to obtain the proton magnetic spectrum on a Varian-T60 NMR spectrometer with TMS as internal standard. The spectrum is shown in Figure 3, and assignment of the chemical shifts to the different protons is summarized below:

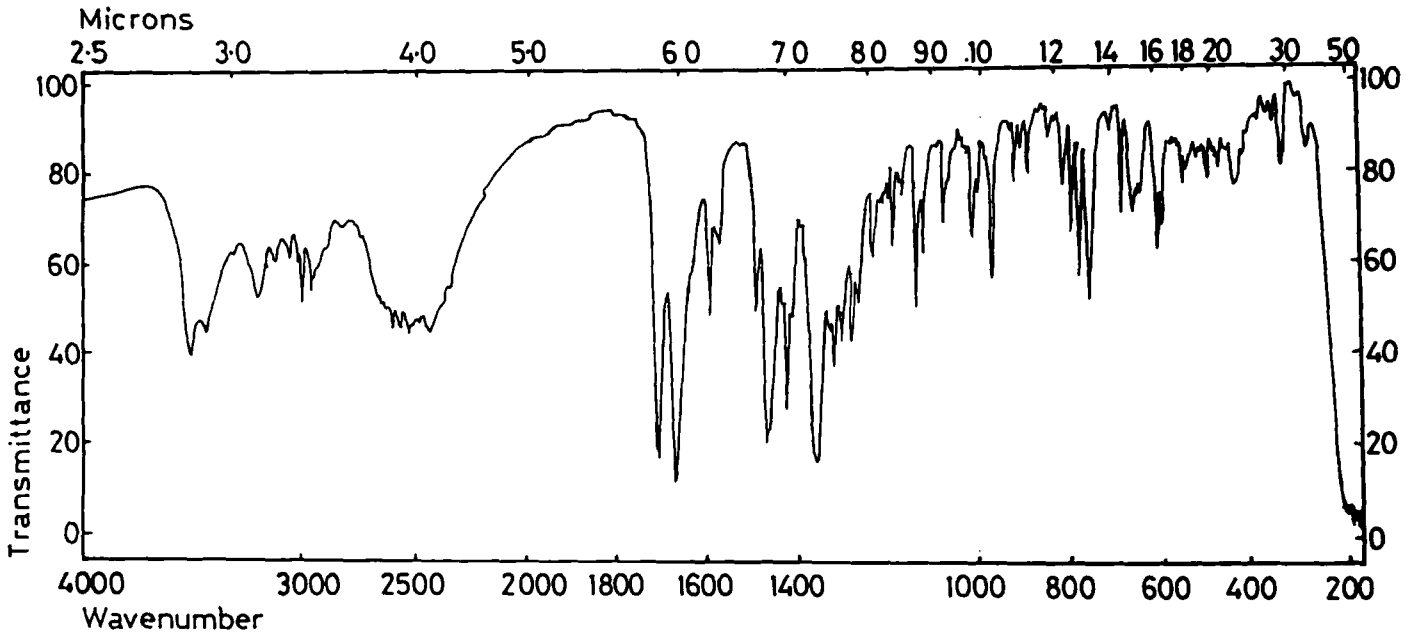


Figure 2 : INFRARED SPECTRUM OF PIRENZEPINE DIHYDROCHLORIDE, KBr DISC.

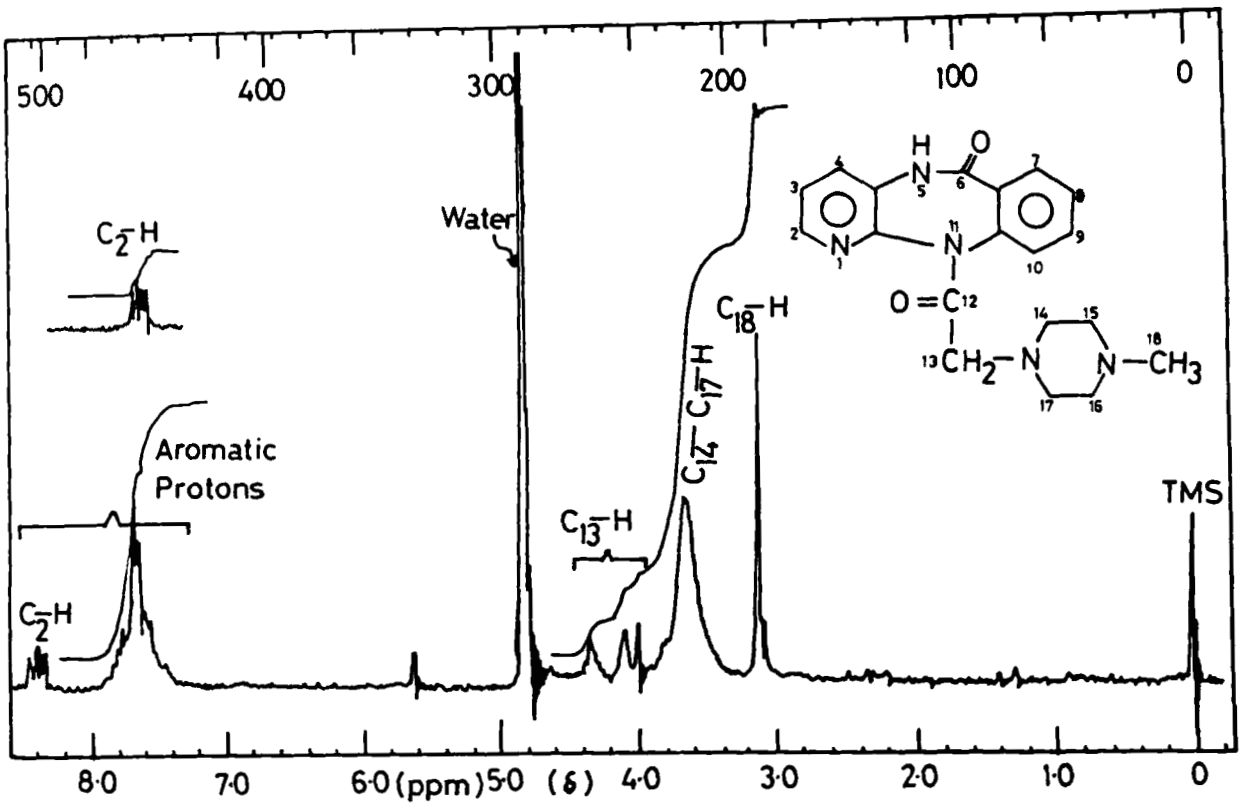
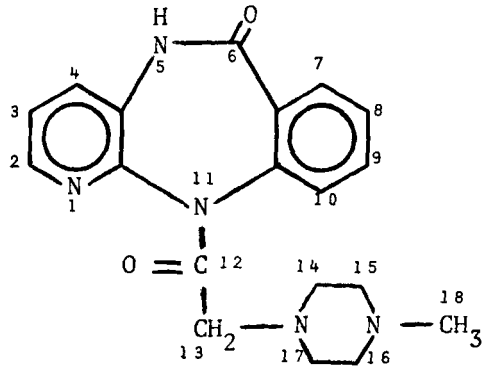


Figure 3 : ^{1}H NMR SPECTRUM OF PIRENZEPINE DIHYDROCHLORIDE IN D_2O WITH TMS AS INTERNAL REFERENCE.



Chemical shift (δ)	Multiplicity	Proton Assignment	No. of protons
3.05	Singlet	-N-$\underline{CH_3}$	3
3.55	Multiplet	-$\underline{CH_2}$-(piperazine)	8
4.15	Multiplet	-$\underline{CH_2}$-C(=O)-	2
7.7	Multiplet	Aromatic-H	6
8.4	Multiplet	Aromatic-H	1

2.4.4 ^{13}C Nuclear Magnetic Resonance (^{13}C NMR) Spectrum

The ^{13}C NMR spectra of pirenzepine in a mixture of D_2O and formic acid using dioxane as internal reference are obtained using a Jeol FX 100 90 MHz spectrometer at ambient temperature. Figure 4 and 5 show the ^{1}H-decoupled and off-resonance spectra, respectively. The carbon chemical shifts, assigned on basis of the off-resonance splitting pattern and the theories of chemical shifts, are presented below:

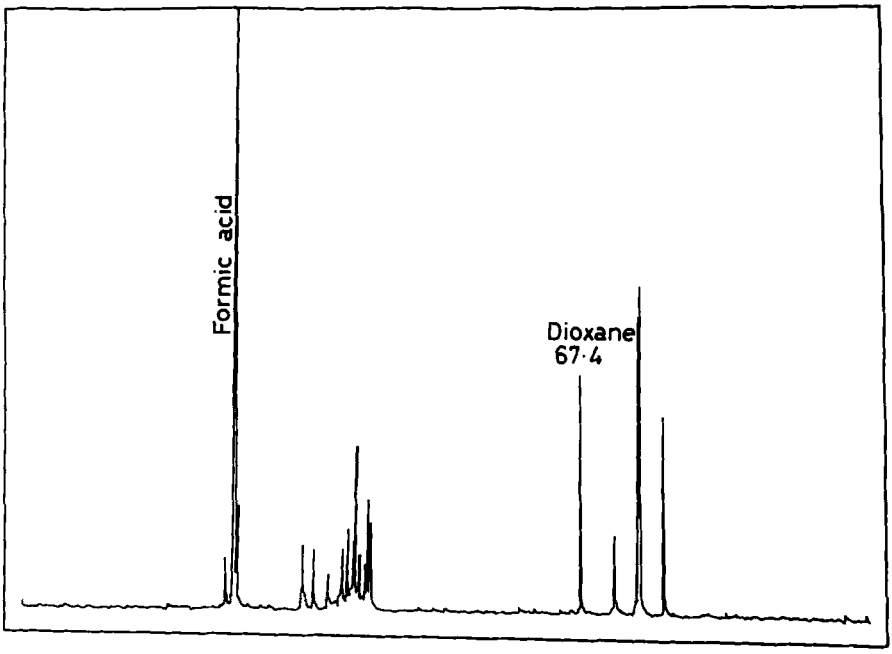


Figure 4 : ^{1}H DECOUPLED ^{13}C NMR SPECTRUM OF PIRENZEPINE DIHYDROCHLORIDE IN D_2O/ FORMIC ACID MIXTURE WITH DIOXANE AS INTERNAL REFERNECE.

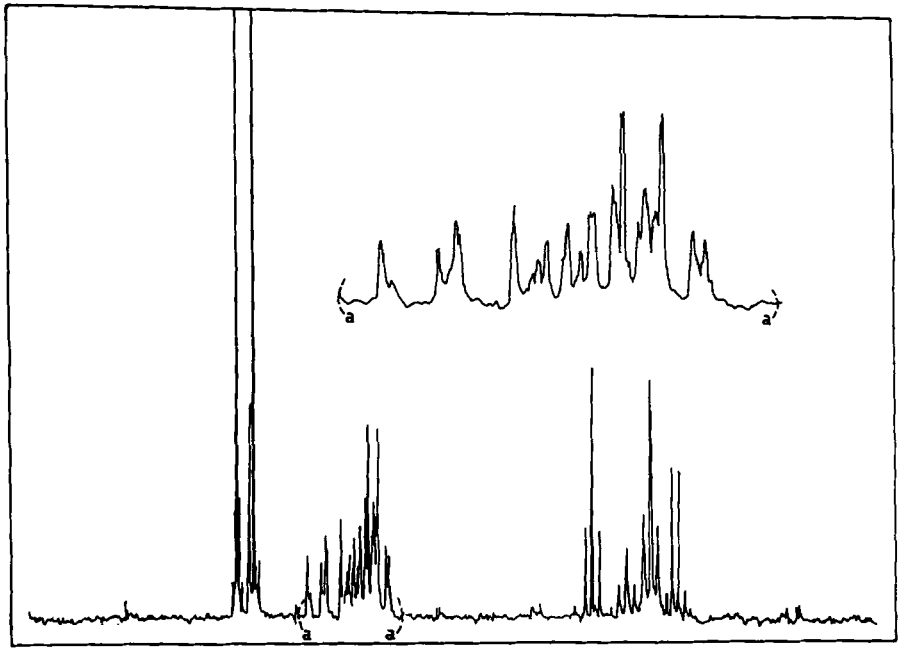
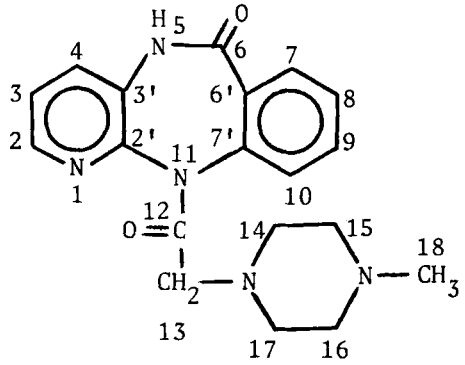


Figure 5 : OFF-RESONANCE ^{13}C NMR SPECTRUM OF PIRENZEPINE DIHYDROCHLORIDE IN D_2O/ FORMIC ACID MIXTURE WITH DIOXANE AS INTERNAL REFERENCE.



Chemical Shift (δ)	Multiplicity	Carbon Assignment
44.83	Quartet	C_{18}
50.72	Triplet	C_{15}, C_{16}
51.87	Triplet	C_{14}, C_{17}
57.77	Triplet	C_{13}
122.41	Doublet	C_{10}
128.22	Doublet	C_{8}
128.88	Doublet	C_{7}
130.57	Doublet	C_{3}
131.57	Singlet	$C_{6'}$
132.15	Doublet	C_{9}
132.74	Doublet	C_{4}
135.24	Singlet	$C_{7'}$
139.43	Doublet	C_{2}
143.61	Singlet	$C_{3'}$
146.77	Singlet	$C_{2'}$
165.21	Singlet	C_{12}
165.31	Singlet	C_{6}

2.4.5 Mass Spectra

The 70 eV electron impact mass spectrum of pirenzepine, presented in Figure 6, was obtained on Varian MAT 311 mass spectrometer using ion source pressure of 10^{-6} Torr, ion source temperature of 180°C and an emission current of 300 μA. The molecular ion is detectable at m/e 351 and the base peak at m/e 113. A proposed fragmentation pattern and the mass/charge ratios of the major fragments is shown in Scheme 1. The direct chemical ionization (DCI) spectrum (Figure 7) was obtained on a Finnigan 4000 mass spectrometer using methane gas a reagent with ion electron energy of 100 eV, ion source pressure of 0.3 Torr, ion source temperature of 150°C and emission current of 300 μA. The spectrum is dominated by a quasi-molecular ion (M + 1). Two peaks appearing at m/e 380 and m/e 392 are attributed to the transfer of carbocations from the carrier gas. The mass spectral assignments of the prominent ions under DCI conditions are shown in Table 3.

Table 3. Mass spectral assignments of pirenzepine using DCI with methane as reagnet gas.

m/e	Species
392	$[M + C_3H_5]^+$
380	$[M + C_2H_5]^+$
352	MH^+
351	M^+

2.5 Crystal Structure

The crystal structure of pirenzepine monohydrate free base was studied by Ruzic-Toros and Kojec-Prodic (6). Pirenzepine monohydrate was to be monoclinic, $P2_{1/c}$ a = 13.160 [7], b = 12.766 [9],

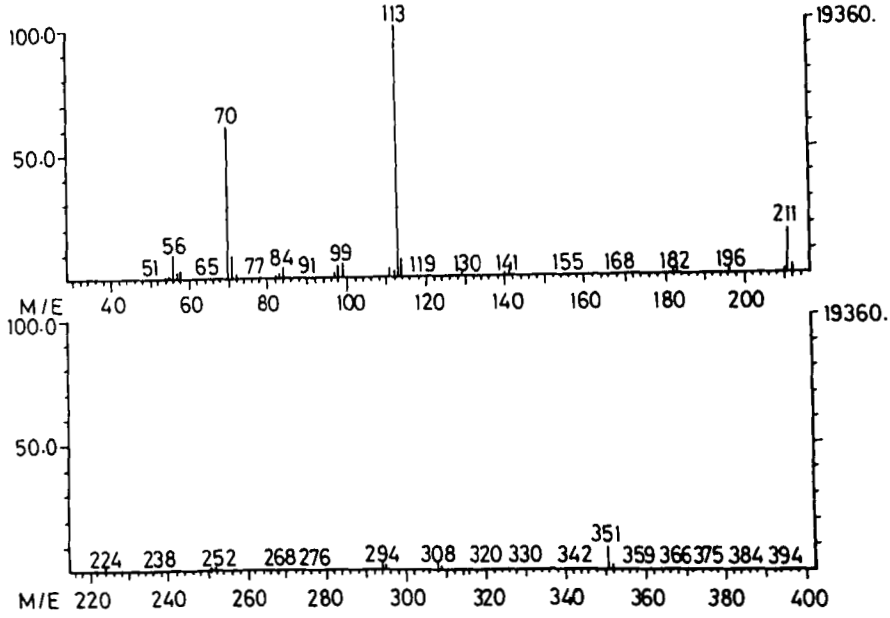


Figure 6 : ELECTRON IMPACT MASS SPECTRUM OF PIRENZEPINE.

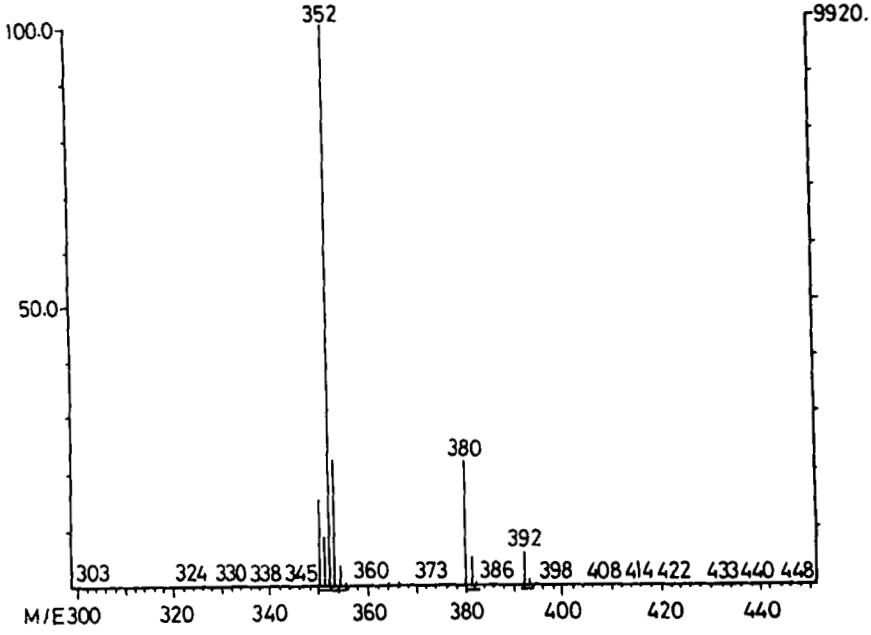
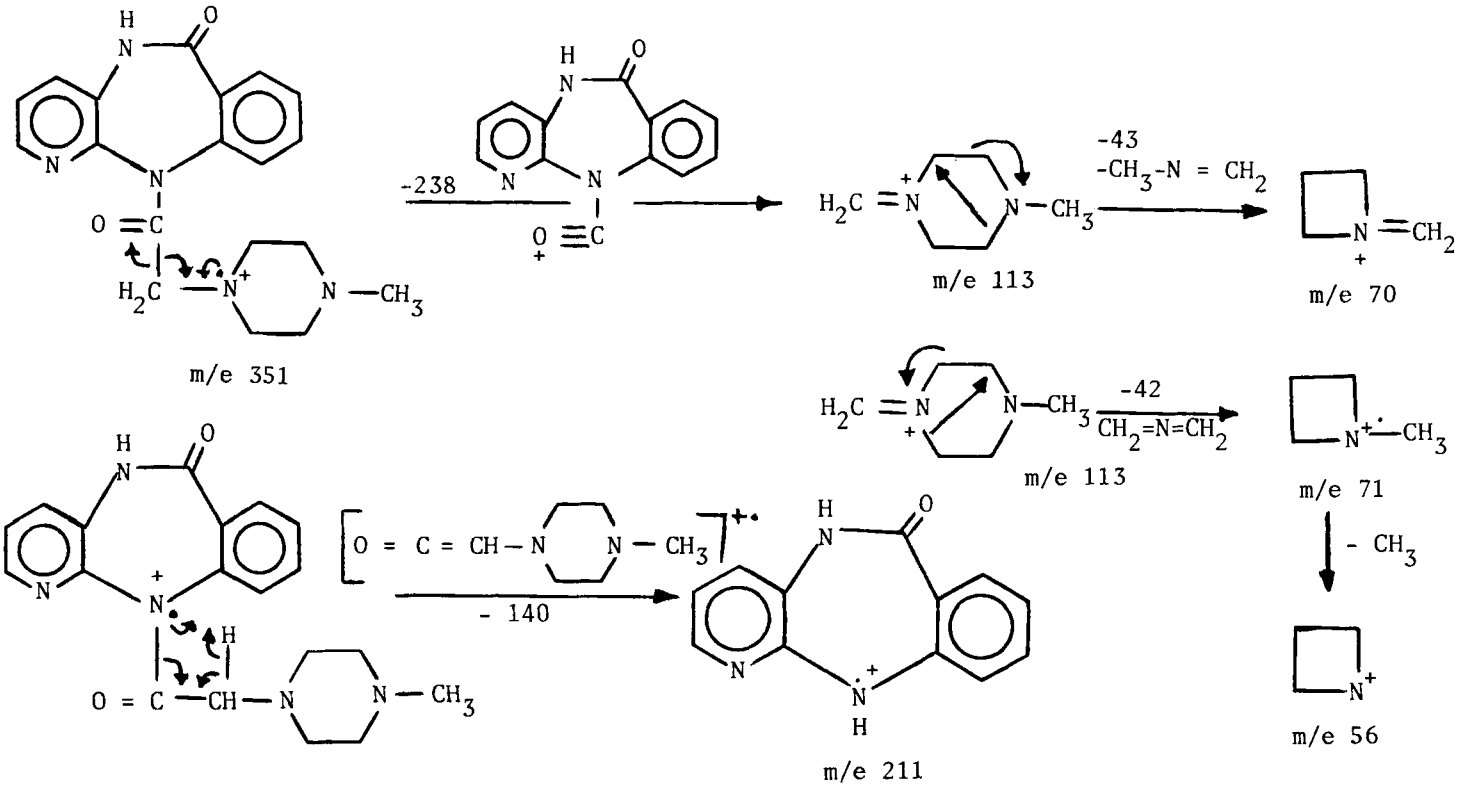


Figure 7 : DIRECT CHEMICAL IONIZATION MASS SPECTRUM OF PIRENZEPINE.



Scheme 1. Proposed fragmentation of pirenzepine.

c = 12.439 [7] A^{o}, β = 113.2 [4]^{o}, U = 1920.8 A^{o3}, Z = 4, D_c = 1.277 Mgm^{-3}, μ(Mo K_α) = 0.128 mm^{-1}. Final R = 0.045 for 2681 observed reflexions.

Bond distances and angles (Table 4) in the fused rings, benzene, diazepine and pyridine, are affected by conjugation. Deviation from the values expected for the given atom type and hybridization could not be explained by the thermal vibrations of the atoms (Table 5). The shortening of C[7] - C[8], 1.372[4]A^{o}, in the benzene ring could be due to intermolecular contacts, involving these atoms, with N[1][3.352[3]; 3.470[3]A^{o}.

The seven-membered diazepine ring is folded at N[5] - C[6] and N[11] to form a boat conformations. The pyridine and benzene rings are planar while the piperazine ring in a chair conformation. A value of 60.9[4]^{o} was obtained for the dihedral angle between the benzene and the pyridine rings.

The water molecule is involved in hydrogen bonds with the amide group of the diazepine ring, N[5] - H[5] .. O[W]{2.800[3]} and O(W)-H(W)2 ... O[6]{2.907[3] A^{o}}. N[4'] of the piperazine ring is hydrogen-bonded to the water molecule by O(W)- H(W)1 ... N[4']{2.821[3]A^{o}}. Molecular packing is also influenced by van der Wall interactions. The relative orientation of the pyridine and piperazine ring, with a dihedral angle of 22.2[4]^{o}, enables a short intramolecular contact N[1] ... N[1'] of 3.087[2]A^{o}. The structural formula and atomic numbering of pirenzepine is given in Figure 8. The conformation of the molecule is shown in Figure 9 and Table 6.

Trummlitz et al. (7), using X-ray analysis, determined the crystal structures of pirenzepine dihydrochloride and its monoprotonated form, pirenzepine monohydrochloride (Figure 10). Molecular mechanics (MMPI) and semiempirical quantum chemical (MNDO) calculations showed that the calculated minimum energy conformations of the tricycle and of the exocyclic amide group are

Table 4. Bond distances (A) and angles (^{o})

N(1)-C(2)	1.348 (3)	C(14)-C(15)	1.388 (3)
N(1)-C(12)	1.315 (3)	C(16)-O(16)	1.229 (3)
C(2)-C(3)	1.364 (4)	C(16)-C(17)	1.513 (4)
C(2)-H(2)	0.99 (2)	C(17)-N(1')	1.452 (3)
C(3)-C(4)	1.375 (3)	C(17)-H(17)1	1.01 (2)
C(3)-H(3)	0.97 (2)	C(17)-H(17)2	1.05 (2)
C(4)-C(13)	1.395 (3)	N(1')-C(2')	1.460 (3)
C(4)-H(4)	0.97 (2)	N(1')-C(6')	1.454 (3)
N(5)-C(6)	1.362 (3)	C(2')-C(3')	1.505 (4)
N(5)-C(13)	1.403 (3)	C(2')-H(2')1	1.04 (2)
N(5)-H(5)	0.86 (2)	C(2')-H(2')2	1.04 (2)
C(6)-O(6)	1.231 (3)	C(3')-N(4')	1.465 (3)
C(6)-C(14)	1.484 (4)	C(3')-H(3')1	1.05 (2)
C(7)-C(14)	1.397 (4)	C(3')-H(3')2	1.01 (3)
C(7)-H(7)	0.97 (2)	N(4')-C(5')	1.462 (4)
C(7)-C(8)	1.372 (4)	N(4')-C(7')	1.468 (5)
C(8)-C(9)	1.377 (4)	C(5')-C(6')	1.502 (4)
C(8)-H(8)	1.00 (3)	C(5')-H(5')1	1.03 (2)
C(9)-C(10)	1.383 (4)	C(5')-H(5')2	1.01 (2)
C(9)-H(9)	0.92 (2)	C(6')-H(6')1	1.04 (2)
C(10)-C(15)	1.381 (3)	C(6')-H(6')2	1.03 (3)
C(10)-H(10)	0.94 (2)	C(7')-H(7')	1.09 (3)
N(11)-C(12)	1.438 (3)	C(7')-H(7')2	0.97 (4)
N(11)-C(15)	1.433 (3)	C(7')-H(7')3	1.05 (3)
N(11)-C(16)	1.362 (2)	O(W)-H(W)1	0.93 (3)
C(12)-C(13)	1.387 (3)	O(W)-H(W)2	0.78 (5)

C(2)-N(1)-C(12)	116.7 (2)	C(12)-N(11)-C(16)	124.9 (2)
C(1)-C(2)-C(3)	122.9 (2)	C(15)-N(11)-C(16)	120.6 (2)
N(1)-C(2)-H(2)	115 (1)	N(1)-C(12)-N(11)	117.0 (2)
C(3)-C(2)-H(2)	121 (1)	N(1)-C(12)-C(13)	125.0 (2)
C(2)-C(3)-C(4)	119.6 (2)	N(11)-C(12)-C(13)	118.0 (2)
C(2)-C(3)-H(3)	120 (1)	C(4)-C(13)-N(5)	119.8 (2)
C(4)-C(3)-H(3)	120 (1)	C(4)-C(13)-C(12)	116.8 (2)
C(3)-C(4)-C(13)	118.7 (2)	N(5)-C(13)-C(12)	123.1 (2)
C(3)-C(4)-H(4)	123 (1)	C(6)-C(14)-C(7)	118.1 (2)
C(13)-C(4)-H(4)	118 (1)	C(6)-C(14)-C(15)	123.2 (2)
C(6)-N(5)-C(13)	129.8 (2)	C(7)-C(14)-C(15)	118.6 (2)
C(6)-N(5)-H(5)	114 (1)	C(10)-C(15)-N(11)	120.5 (2)
C(13)-N(5)-H(5)	115 (1)	C(10)-C(15)-C(14)	120.9 (2)
N(5)-C(6)-O(6)	119.3 (2)	N(11)-C(15)-C(14)	118.5 (2)
N(5)-C(6)-C(14)	119.3 (2)	N(11)-C(16)-O(16)	120.8 (2)
O(6)-C(6)-C(14)	121.2 (2)	N(11)-C(16)-C(17)	117.9 (2)
C(8)-C(7)-C(14)	120.5 (2)	O(16)-C(16)-C(17)	121.3 (2)
C(8)-C(7)-H(7)	121 (2)	C(16)-C(17)-N(1')	113.2 (2)

Table 4. Contd.....

C(14)-C(7)-H(7)	118 (2)	C(17)-N(1')-C(2')	111.0 (2)
C(7)-C(8)-C(9)	120.2 (3)	C(17)-N(1')-C(6')	111.7 (2)
C(7)-C(8)-H(8)	120 (2)	C(2')-N(1')-C(6')	110.6 (2)
C(9)-C(8)-H(8)	120 (2)	N(1')-C(2')-C(3')	109.9 (2)
C(8)-C(9)-C(10)	120.4 (2)	C(2')-C(3')-N(4')	110.8 (2)
C(8)-C(9)-H(9)	123 (1)	C(3')-N(4')-C(5')	109.3 (2)
C(10)-C(9)-H(9)	117 (1)	C(3')-N(4')-C(7')	110.9 (2)
C(9)-C(10)-C(15)	119.4 (2)	C(5')-N(4')-C(7')	110.9 (2)
C(9)-C(10)-H(10)	121 (1)	N(4')-C(5')-C(6')	110.1 (2)
C(15)-C(10)-H(10)	119 (1)	N(1')-C(6')-C(5')	110.6 (2)
C(12)-N(11)-C(15)	114.3 (1)	H(W)1-O(W)-H(W)2	110 (4)

Table 5. Final atomic coordinates (x 10^4 for C, N and O; x 10^3 for H) and isotropic thermal parameters (x 10^2)

U_{eq} is derived from the anisotropic thermal parameters by $U_{eq} = \frac{1}{3} \Sigma_i \Sigma_j U_{ij} a^* a^*_j a^*_j a_i . a_j$

	x	y	z	U_{eq} or U (A^2)
N(1)	6263 (1)	1423 (1)	6087 (1)	4.2 (2)
C(2)	6199 (1)	1306 (1)	4985 (1)	4.6 (2)
C(3)	6380 (1)	372 (2)	4557 (1)	4.7 (2)
C(4)	6615 (1)	-503 (1)	5255 (1)	4.3 (2)
N(5)	7023 (1)	-1266 (1)	7162 (1)	4.3 (2)
C(6)	6726 (1)	-1540 (1)	8057 (1)	7.2 (2)
O(6)	7131 (1)	-2330 (1)	8634 (1)	6.4 (2)
C(7)	5060 (1)	-1465 (1)	8510 (1)	4.8 (3)
C(8)	4228 (2)	-932 (2)	8677 (1)	5.1 (3)
C(9)	4186 (1)	143 (2)	8615 (1)	5.1 (3)
C(10)	4978 (1)	698 (1)	8379 (1)	4.5 (2)
N(11)	6635 (1)	716 (1)	7947 (1)	3.7 (2)
C(12)	6537 (1)	588 (1)	6761 (1)	3.4 (2)
C(13)	6695 (1)	-404 (1)	6403 (1)	3.5 (2)
C(14)	5866 (1)	-922 (1)	8266 (1)	3.9 (2)
C(15)	5818 (1)	162 (1)	8216 (1)	3.7 (2)
C(16)	7373 (1)	1361 (1)	8752 (1)	4.2 (2)
O(16)	7400 (1)	1426 (1)	9729 (1)	6.2 (2)
C(17)	8146 (1)	1997 (1)	8376 (2)	4.6 (3)
N(1')	8712 (1)	1375 (1)	7805 (1)	3.8 (2)

Table 5. Contd....

	x	y	z	U_{eq} or U (A^2)
C(2')	9214 (1)	2042 (1)	7195 (2)	4.9 (3)
C(3')	9766 (2)	1377 (2)	6583 (2)	5.8 (3)
N(4')	10604 (1)	696 (1)	7417 (1)	5.0 (2)
C(5')	10082 (2)	28 (1)	8008 (2)	5.4 (3)
C(6')	9536 (2)	688 (2)	8627 (2)	4.7 (2)
C(7')	11162 (3)	63 (3)	6827 (3)	8.4 (4)
O(W)	2165 (2)	2153 (1)	8845 (2)	7.9 (2)
H(2)	603 (2)	195 (2)	451 (2)	6.1 (7)
H(3)	632 (2)	32 (2)	375 (2)	5.2 (6)
H(4)	676 (2)	-118 (2)	499 (2)	3.9 (6)
H(5)	737 (2)	-175 (2)	696 (2)	4.3 (6)
H(7)	509 (2)	-222 (2)	854 (2)	5.7 (7)
H(8)	366 (2)	-133 (2)	886 (2)	7.1 (8)
H(9)	362 (2)	53 (2)	871 (2)	5.2 (6)
H(10)	496 (2)	143 (2)	834 (2)	4.2 (6)
H(17)1	868 (2)	237 (2)	909 (2)	6.3 (7)
H(17)2	768 (2)	259 (2)	780 (2)	5.7 (7)
H(2')1	857 (2)	248 (2)	661 (2)	6.0 (7)
H(2')2	978 (2)	255 (2)	777 (2)	6.6 (8)
H(3')1	918 (2)	91 (2)	594 (2)	6.6 (9)
H(3')2	1017 (2)	184 (2)	621 (2)	8.3 (9)
H(5')1	951 (2)	-46 (2)	741 (2)	6.6 (7)
H(5')2)	1068 (2)	-44 (2)	857 (2)	7.0 (8)
H(6')1	1012 (2)	112 (2)	929 (2)	5.1 (6)
H(6')2	917 (2)	21 (2)	904 (2)	7.5 (8)
H(7')1	1175 (3)	-45 (3)	747 (3)	11 (1)
H(7')2	1152 (3)	55 (3)	648 (3)	9 (1)
H(7')3	1057 (3)	-38 (3)	615 (3)	10 (1)
H(W)1	162 (3)	167 (3)	843 (3)	10 (1)
H(2)2	223 (4)	217 (4)	950 (4)	14 (2)

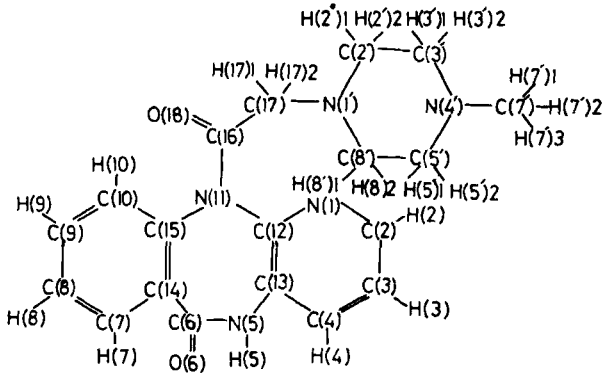


Figure 8: STRUCTURAL FORMULA OF PIRENZEPINE WITH ATOMIC NUMBERING (6).

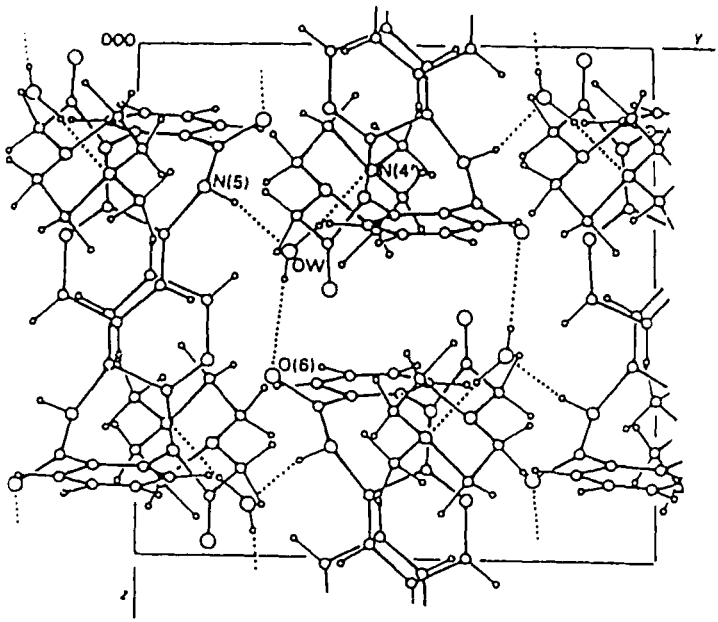


Figure 9: A VIEW OF THE CRYSTAL STRUCTURE SHOWING THE PACKING, HYDROGEN BONDS AND CONFOR-MATION OF THE BENZODIAZEPINE AND PIPERAZINE RINGS (6).

Table 6. Torsion angles (°)

C(12)-N(1)-C(2)-C(3)	1.4 (3)	C(14)-C(15)-N(11)-C(12)	69.5 (2)
N(1)-C(2)-C(3)-C(4)	1.5 (4)	N(11)-C(12)-C(13)-N(5)	-4.7 (3)
C(2)-C(3)-C(4)-C(13)	-2.0 (3)	C(12)-C(13)-N(5)-C(6)	38.3 (3)
C(3)-C(4)-C(13)-C(12)	-0.4 (3)	C(13)-N(5)-C(6)-C(14)	5.3 (3)
C(3)-C(4)-C(13)-N(5)	-174.7 (2)	C(15)-N(11)-C(12)-C(13)	-64.5 (2)
C(4)-C(13)-C(12)-N(1)	3.6 (3)	C(13)-N(5)-C(6)-O(6)	-178.8 (2)
C(4)-C(13)-C(12)-N(11)	-178.9 (2)	C(12)-N(11)-C(16)-O(16)	179.8 (2)
C(13)-C(12)-N(1)-C(2)	-4.1 (3)	C(12)-N(11)-C(16)-C(17)	0.2 (3)
C(7)-C(8)-C(9)-C(10)	0.3 (3)	N(11)-C(16)-C(17)-N(1')	-50.0 (2)
C(8)-C(9)-C(10)-C(15)	-0.5 (3)	C(16)-C(17)-N(1')-C(2')	165.2 (1)
C(9)-C(10)-C(15)-C(14)	1.1 (3)	C(17)-N(1')-C(2')-C(3')	-178.4 (1)
C(9)-C(10)-C(15)-N(11)	178.7 (2)	N(1')-C(2')-C(3')-N(4')	-57.9 (2)
C(10)-C(15)-C(14)-C(7)	-1.4 (3)	C(2')-C(3')-N(4')-C(5')	58.7 (3)
C(10)-C(15)-C(14)-C(6)	177.8 (2)	C(2')-C(3')-N(4')-C(7')	-178.7 (2)
C(15)-C(14)-C(7)-C(8)	1.1 (3)	C(3')-N(4')-C(5')-C(6')	-58.6 (2)
C(14)-C(7)-C(8)-C(9)	-0.6 (3)	N(4')-C(5')-C(6')-N(1')	58.6 (2)
N(5)-C(6)-C(14)-C(15)	-42.4 (3)	C(5')-C(6')-N(1')-C(2')	-57.7 (2)
C(6)-C(14)-C(15)-N(11)	0.2 (3)		

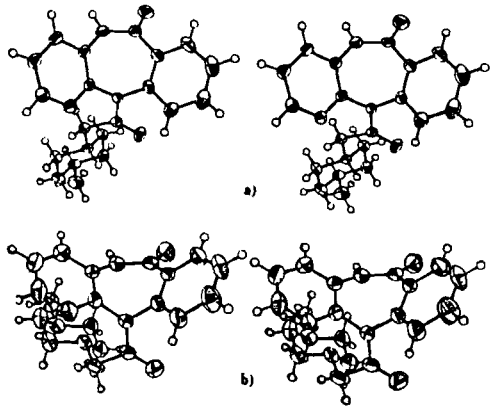


Figure 10: STEREO-PAIRS OF DRAWINGS FOR THE MOLECULAR STRUCTURES OF PIRENZEPINE DIHYDROCHLORIDE (a) AND PIRENZEPINE MONOHYDROCHLORIDE (b) OBTAINED FROM X-RAY ANALYSIS (7).

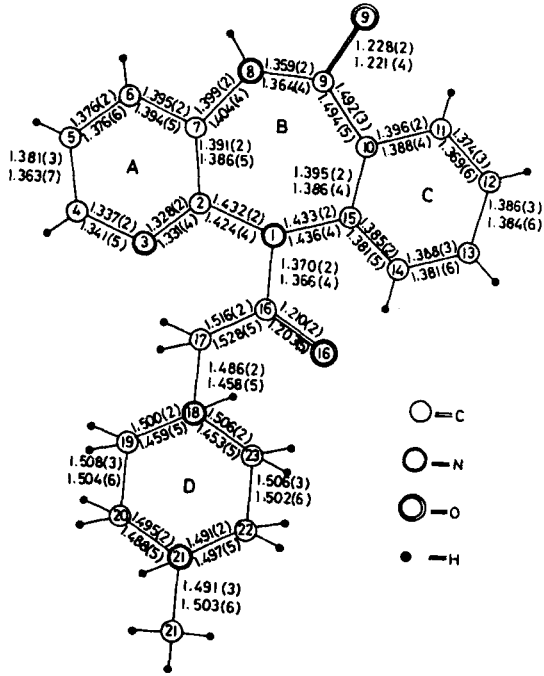


Figure 11: ATOM NUMBERING SCHEME AND CRYSTALLOGRAPHIC BOND ANGLES (Å, STANDARD DEVIATIONS IN PARENTHESES) FOR PIRENZEPINE DIHYDROCHLORIDE (FIRST VALUE) AND PIRENZEPINE MONOHYDROCHLORIDE (SECOND VALUE) (7).

in agreement with the crystal structures. The tricyclic ring system consists of the planar rings A and C and nonplanar ring B (Figure 11). For both compounds the seven-membered ring B has a distarted boat shape. This ring is folded along a folding line passing through N1 and the middle of the bond N8 - C9. This similarity in the geometry of the tricyclic systems of both compounds does not hold for the side chains which have totally different arrangements with respect to the tricyclic system in both compounds (Figures 10 and 12). The steric situation is quite similar along the bond N1 - C16, in that the carbonyl groups are almost in eclipsed positions to N1 - C15. Significant differences are observed, however, along the C16 - C17 bond. For the dihyrochloride N18 is trans to N1(τ N18-C17-C16-N1 = 171.3^{o}) whereas in the monohydrochloride N18 and N1 are in gauche positions (τ N18-C17-C16-N1 = 64.1^{o}). In this arrangement the piperazine ring will be situated below the tricyclic ring system in the crystal structure of pirenzepine monohydrochloride. The crystal structures of both salts showed strong N-H ... Cl^- hydrogen bonds for the protonated nitrogens of the piperazine rings. A relatively weak O ... Cl^- hydrogen bonds also exist for the distorted water molecule in the crystal lattice in the dihydrochloride salts. The amide nitrogen N8 of the monohydrochloride salt shows a further N-H ... Cl^- hydrogen bond which is not observed in the dihydrochloride structure.

3. Synthesis

Pirenzepine, together with other 11-substituted 11H-pyrido[2,3-b] [1,5] benzodiazepin-5(6H)-ones, had been prepared by Dr. Karl Thomae (8). The compounds are prepared by treatment of an 11H-pyrido[2,3-b][1,5] benzodiazepin-5(6H)-one with a dihalogen compound, XCH_2COX' in which X and X' may be alike or different and designate an atom of Cl, Br and I. The 11-haloacetyl intermediate is then treated with the appropriate amine. Thus, a boiling solution of 21 g 11H-pyrido[2,3-b][1,5]benzodiazepin-5(6H)-one) in 300 ml

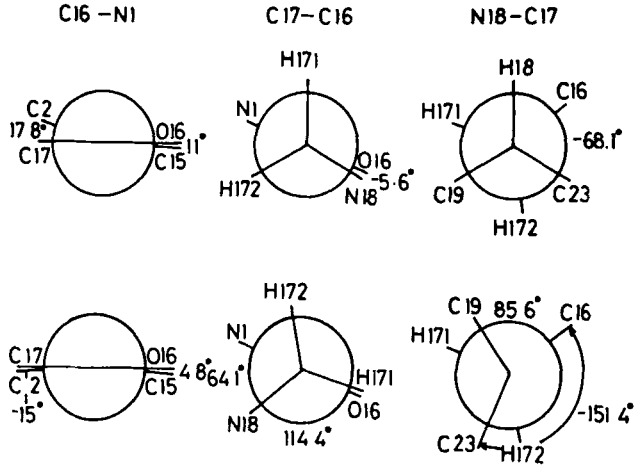
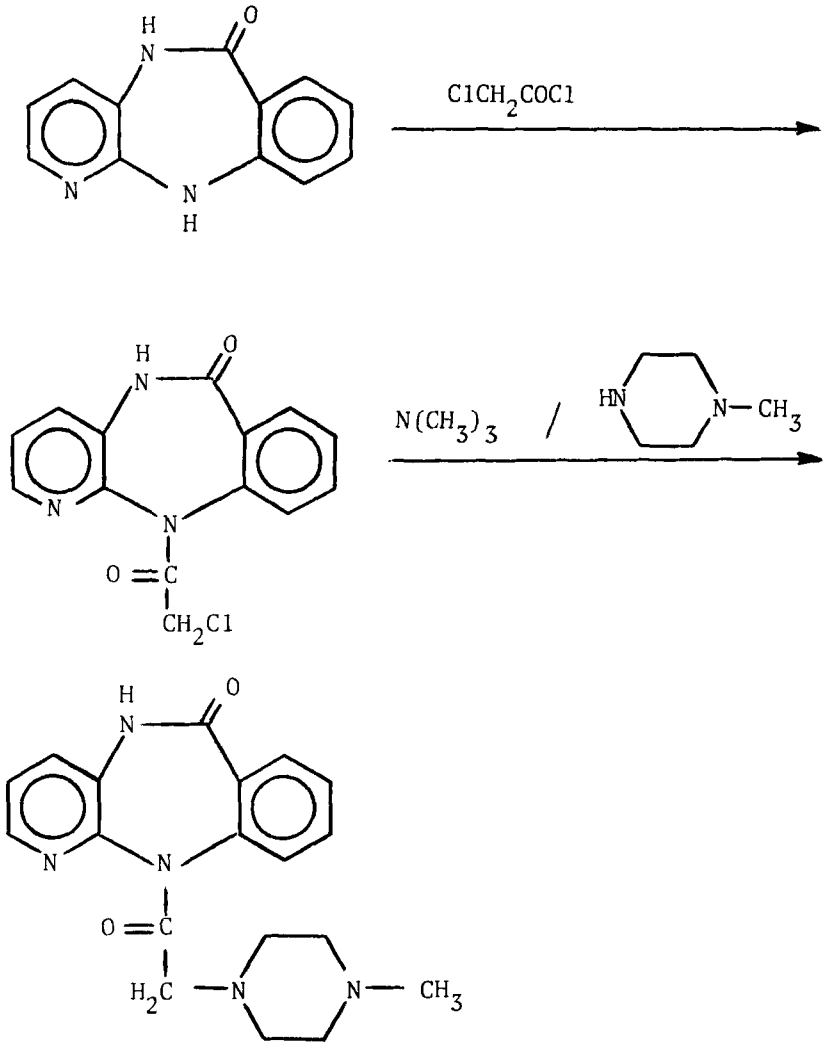


Figure 12: NEWMAN PROJECTIONS DOWN THE BONDS $C_{16}-N_1$, $C_{17}-C_{16}$ & $N_{18}-C_{17}$ FOR PIRENZEPINE DIHYDROCHLORIDE (ABOVE) AND THE MONOHYDROCHLORIDE (BELOW) (7).

absolute dioxane treated dropwise simultaneously in 30 min with 15.8 g $ClCH_2COCl$ in 40 ml absolute dioxane and 14.4 g Et_3N in 30 ml absolute dioxane, the mixture refluxed for 8 hr and the hot filtrate evaporated in vacuo gave 11-chloroacetyl-11H-pyrido[2,3-b][1,5] benzodiazepin-5(6H)-one (Scheme 2). The chloroacetyl derivative and N-methylpiperazine in absolute alcohol refluxed for 18 hr filtered and the hot filtrate evaporated in vacuo to give pirenzepine.



Scheme 2. Synthesis of pirenzepine

4. Stability

Stability of pirenzepine depends on both moisture contents and pH and the effect of the moisture contents could be minimized by adjusting to optimum pH (9). Pirenzepine dihydrochloride in tablet was shown stable to exposure to light at 50 - 55°. No change in color, smell, taste or general appearance or presence of degradation products was observed (10).

5. Methods of Analysis

5.1 Spectrophotometric Methods

The determination of pirenzepine dihydrochloride spectrophotometrically using ΔA method have recently been reported (4). The mean percentage recovery for tablets, each labelled to contain 25 mg, was 100.2 ± 1.15. The drug shows maximum absorption at 280 nm in hydrochloric acid and at 295 nm in 0.1 N sodium hydroxide due to keto-enol tautomerism. ΔA at 300 nm was found to be linearly related to the drug concentration over a range of 10-80 μg/ml with a relative standard deviation of 0.4%.

A rapid and direct method for the determination of pirenzepine dihydrochloride in the presence of degradation products using first derivative spectrophotometry has also been reported (5). The method was applied using synthetic mixtures of the intact drug and degradation products. The procedure is suitable for monitoring the drug stability.

5.2 Chromatographic Methods

5.2.1 Thin-Layer Chromatographic (TLC) Methods

Thin layer chromatography have been used for the analysis of pirenzepine (10). Precoated TLC plates with 0.2 mm silica gel F_{254} were used.

5.2.2 High-Pressure Liquid Chromatography (HPLC)

Daldrup et al (11) have used reversed-phase liquid chromatography as a test for screening pharmaceuticals, drugs and insecticides.

High performance reverse - phase liquid chromatography retention data for pirenzepine and other compounds (560 compounds) are given. The relative retention times were calculated as the ratios of retntion times of compound and reference compound, 5-(p-methylphenyl)-5-phenyl hydantoin. The UV detector wavelength was 220 nm, where most of the compounds gave response. Two solvent programs and a prepacked column C-18 SIL-X-10 were used for the analysis.

Babhair (12) reported a simple and sensitive method for the determination of pirenzepine, in dosage forms and in biological fluids, using high-performance liquid chromatography with U.V. detector. A tablet of 25 mg drug was ground, suspended in 10 ml of water, shaken and then filtered. A known volume of the filtrate is adjusted to appropriate concentration. Twenty µl of this solution were injected. Plasma or urine samples were made alkaline with ammonia before extraction with chloroform which was evaporated and the residue was dissolved in the mobile phase, 20 µl of this solution were injected. The determination limit for quantitation was about 1 µg/ml of pirenzepine. Complete separation of the drug was achieved in about 5.4 minutes under the present chromatographic conditions. A 30 X 3.9 cm i.d. commercially available stainless steel C-18 column was used. Mobile phase consisted of acetonitrile, methanol and 5% acetic acid (70:40:15).

High-performance liquid chromatographic determination of pirenzepine dihydrochloride in its pharmaceutical formulation have also

been reported (13). Normal phase liquid chromatography has been performed on a Micropack Si-10 column using ammonium hydroxide (28-30% NH_3) in methanol (0.75 : 99.25% v/v) mobile phase and a flow rate of 2 ml/min. Clobazam has been used as internal standard with retention times of 1.9 and 2.8 minutes for clobazam and pirenzepine dihydrochloride, respectively at 254 nm. Tablets each labelled to contain 25 mg pirenzepine dihydrochloride gave mean percentage result of 99.98 ± 0.4. The method have also been reported to be a stability indicating method.

5.3 Radioimmunoassay

A radioimmunoassay system developed for pirenzepine was used to specify the requirements for using such system in pharmacokinetics and to illustrate the general strategy in establishing specificity, sensitivity and reliability (14). The type of error in the final data was considered. It is a widespread assumption that weak cross-reactions in metabolites result in a relative error and, therefore, have no relevance to the detection of the parent drug. In contrast to this assuption, it was shown that such weak cross-reactivity in biological samples gives rise to an absolute error which is independent of the concentration and analogous to that caused by a blank value in chemical analysis.

Tanswell and Zahn (15) applied monoclonal assays for studying the pharmacokinetics of pirenzepine.

6. Pharmacokinetics

The marked physicochemical properties of pirenzepine result in the drug being highly hydrophilic at physiological pH. This property determines the fate of pirenzepine in the organism due to the limited ability of the drug to penetrate lipid membranes. This limited penetration is reflected in its absorption, distribution, biotransformation and excretion.

6.1 Absorption

Orally administered pirenzepine was almost completely absorbed by rabbits and dogs but only about 11% absorbed by rats and the blood level maxima reached after 3 hr (16).

After oral administration of aqueous solutions or tablets 20-30% of the dose is absorbed (17). In an international pharmacokinetic studies 20% of the oral dose administered to the panel of 87 volunteers was absorbed (18). Uniform plasma levels were observed in this large panel from 10 different countries after a single oral dose of 50 mg, with minimal interpatient variation. Peak serum concentration were approximately 50 ng/ml occurring within 2 hrs. After oral dose of 20 mg/kg, the drug appeared in the blood at a concentration of 0.17 µg/ml only 15 min after administration. Afterwards, however, increase in the concentration was shown reaching the maximum of 0.42 µg/ml approximately 3 hr after administration (19). The results are comparable to those of Hammer et al (16) who reported that the maximum blood concentration was attained 3 hr after administration at the level of 0.55 µg/ml.

After oral administration of 25 mg of the drug, the maximum blood level occurred in 2 - 3 hr and was about 24 ng/ml. Computer simulation was used to derive a single dosage scheme for pirenzepine consisting of an initial dose of 50 mg and maintenance dose of 25 mg at 12 hr - intervals. This schedule maintained a maximum plasma level of $\geq$ 24.1 ng/ml in normal subjects (17).

The maximum plasma level appeared 2 hr after the administration of 12.5 - 150 mg pirenzepine orally to volunteers (20). After multiple administration (25 - 50 mg a day) the plasma level was increased for the first 3 days of administration and remained constant thereafter.

Simulated plasma levels of pirenzepine after an oral therapeutic dosage regimen (50 mg, twice daily) on the basis of single administration data, showed steady state within 2 days of treatment (18). Pirenzepine administered i.m. was completely absorbed in rabbits and dogs. Rats that poorly absorb oral pirenzepine showed complete absorption of i.m. dose of the drug.

Using monoclonal assays (15), plasma pirenzepine levels and urinary excretion were measured over time

in 10 healthy male subjects after 10 mg i.v. or i.m. dose, in randomized crossover study. The data were fitted to a 3-compartment open model. Absorption after i.m. injection was rapid and complete (mean bioavailability 100.3%).

After i.v. bolus injection high plasma level peaks are reached which last only for a short time period since due to the distribution of pirenzepine into body tissues the plasma level declines rapidly with an initial half-life of about 5 min. (17, 21). The peak concentration after i.v. administration (22) was much higher than the therapeutic level after oral administration. In the first minutes after i.v. injection of 10 mg pirenzepine (0.15 mg/kg), the plasma concentrations were almost 10-fold higher than the steady state level reached after daily doses of oral 100 mg. In less than one hour, however, the plasma level of the i.v. dose is comparable to the steady state concentrations after oral administration (18).

The absorption of s.c. dose of pirenzepine is very swift and comparable to that of i.v. (4) and unlike that of i.m. reported by Hammer et al (16).

6.2 Distribution

Informations provided by whole-animal autoradiography of rat 10 minutes after intravenous injection of 2 mg/kg of radioactive pirenzepine (15, 21), showed that the drug is widely distributed in the body. The radioactive drug is found in all internal organs and skeletal muscles. No radioactivity was detected in the brain of the rat or the fetuses of gestating mouse suggesting that the blood-brain barrier represents a genuine barrier to pirenzepine and that the drug does not cross the placental barrier. Continuous intravenous infusion of pirenzepine over a period of 2 days at a rate of 2 mg/hr/8-9 kg baboon (10 times the human infusion dose), showed that the highest levels are in the organs responsible for the elimination of the drug i.e. liver and kidneys. The other internal organs, the skin and skeletal muscles, however, showed a higher pirenzepine concentration per g of tissue than the plasma.

Extremely low drug concentration was found in the brain.

The distribution of pirenzepine in the tissues after one-time oral administration was investigated by Kobayashi et al (19) after isolation of tissues and whole-body autoradiography technique. After 3 hr, the time of maximum blood concentration, high concentration of pirenzepine was observed in tissues like liver, kidney, pancreas, lung, pituitary gland, salivary gland, adrenal gland etc. These results, therefore, agree with those of Hammer and Koss (21) in that radioactive pirenzepine was found distributed in all tissues except the central nervous system. The radioactivity distributed in this way decreased as time passed. Twenty-four hours after administration, the concentration was slightly hgih only in the gastrointestinal tract and liver. Radioactivity in the testis showed a tendency to decrease gradually during 72 hr. Kaubisch et al (23) observed strong radioactivity distributed in the gastrointestinal tract, kidney and salivary glands in a whole-body autoradiography performed after i.v. administration of ^{14}C-pirenzepine in a dose of 2 mg/kg. The presence of radioactivity in the gastrointestinal tract was considered to result from the excretion of the radioactivity into the inside of the intestinal tract via the blood vessels and intestinal wall. This is supported by the finding of Kobayashi et al (19) who observed clean pictures of radioactivity distribution at the wall or mucus of the stomach and intestines in autoradiography 3 hr and 9 hr after administration and considered that the radioactivity was secreted from the gastrointestinal wall or was deposited there.

After multiple oral dose, Kobayashi et al (19), observed a slight tendency towards accumulation in the liver, kidney and testis whereas radio-activity decreased slowly in one-day administration. However, the radioactive concentration in each tissue did not increase in proportion to the administration times but decreased as time passed and left only traces one week after the termination of administration. In multiple administration

almost all of the radioactivity was found excreted into urine and feces 24 hr after each administration. It is, therefore, considered that the accumulation of the drug in the body is small. Jaup and Blomstrand (24) measured serum and cerebrospinal concentrations of pirenzepine in healthy volunteers after therapeutic dosage over 2-5 days. The cerebrospinal fluid-to-serum ratio was 0.095 to 1, indicating that pirenzepine passed the blood-brain barrier, but only to a small extent. Pirenzepine concentrations in the cerebrospinal fluid was about 10% those of serum.

That the penetration of pirenzepine through the blood-brain barrier is very limited was also demonstrated by other studies (18, 25-27).

6.3 Metabolism

Pirenzepine undergoes only slight metabolism irrespective of the route of administration and there is no significant first-pass effect. Only small amounts of metabolites have been detected (16-18, 21), of which the desmethyl derivative accounted for significantly less than 10% of the dose. The desmethyl compound has no pharmacological effect. The tricyclic moiety, despiperazinly pirenzepine was also detected as a metabolite in negligible amounts (17). Unlike cimetidine, pirenzepine does not inhibit the hepatic mixed function oxidase system (28).

6.4 Excretion

In man as well as in other animals, pirenzepine is excreted unchanged in urine and feces (17, 18, 21). Due to the special physicochemical properties of pirenzepine only about 10% of the dose is bound to plasma proteins (17, 20) and hence it is almost completely filtered into the kidneys. The volume of distribution of pirenzepine is identical to the extracellular space which indicates that pirenzepine rapidly equilibrates between plasma and the extracellular fluids of the peripheral tissues. The total plasma clearance of pirenzepine is 255 ml/min. Because of its limited

lipid solubility, the drug cannot be reabsorbed from the renal tubules so that the renal clearance of 129 ml/min corresponds to the glumerular filtration rate. Hammer et al (16) reported that approximately equal amounts of pirenzepine were excreted via the bile and urine. The hepatic clearance, mostly biliary excretion is about 125 ml/min (17).

Following oral administration of a therapeutic dose of labeled drug, 82% of the substance excreted in urine is unchanged, as is 92% of that in the feces. The drug is eliminated from the organism fully and almost equally in urine and feces, elimination being almost complete after 4 days (17). Kobayashi et al (19) reported that in single as well as in multiple administration, almost all of the dose was excreted in urine and feces. In i.v. administration, 44% and 50% of the administered dose were excreted into urine and feces, respectively 24 hr after administration and 46% and 53% respectively, 4 days after administration. Similar results were obtained with subcutaneous administration. Hammer et al (16) reported that 36% and 56% of the administered dose were excreted in urine and feces, respectively, following i.v. administration. 42% of a 10 mg dose was also reported (15) eliminated renally with total clearance of 253 ml/min and renal clearance of 107 ml/min. The mean transit time was 9.3 hr. A urinary excretion rate of 7-12% was reported by Ohashi et al (20) within 4 days after multiple administration of pirenzepine (25-50 mg, 3 times a day for 4 days). Hammer et al (16) reported approximately 4% of the administered dose was excreted in urine after oral administration. According to Kobayashi et al (19) about 8% was excreted in urine after single oral administration and about 4.5% in multiple administration. The difference in the value was attributed to the difference in absorption rates between fasted and nonfasted animals.

Elimination half-life of 10 hr was reported (21) following i.v. dose of 8 mg pirenzepine to humans. Oral administration of 25 mg tablets to volunteers resulted in elimination half-life of

11 hr (21). Ohashi et al (20) reported an elimination half-life of 13 hr. The long elimination half-life was attributed to slow redistribution of pirenzepine from the tissues rather than to slow excretion (21).

Intravenous administration of ^{14}C-pirenzepine to dams in a dose of 2 mg/kg caused the appearance of radioactivity in milk almost equivalent to that in blood (19).

Pretreatment of healthy volunteers with therapeutic doses of pirenzepine over 5 days showed no significant effect on the absorption, distribution or elimination of antipyrine (28).

7. Pharmacology

7.1 Antisecretory Effects

Secretory studies with pirenzepine clearly demonstrate that pirenzepine suppresses both basal and stimulated acid and pepsin secretion (30-52). Intravenous pirenzepine reduces basal acid secretion by 90% and pentagastrin-stimulated secretion by approximately 50% (30). Fritsch et al (31) reported that oral 50 mg of pirenzepine, in patients with duodenal ulcer, inhibited the basal acid secretion by 39.9% and peptone-induced acid output by 21.3%. The percentage inhibition nearly doubled if the same dose is given after a treatment with 50 mg pirenzepine twice daily for 3 to 7 days. No acute effect on serum gastrin levels can be demonstrated by a single dose of pirenzepine. After pretreatment only small increase of basal gastrin levels is observed, serum gastrin does not change during stimulation. Oral pirenzepine (25 mg) reduces acid output by 50% and pentagastrin-stimulated secretion by 30-40%. Doubling the dose increases the degree of inhibition of stimulated secretion by ~ 10% (30). Jaup et al (32) reported that pirenzepine 50 mg PO b.i.d. reduced basal gastric acid secretion by 54% at two hours following the last dose. Pentagastrin-stimulated secretion of hydrochloric acid (1 mcg/kg/hr) by continuous i.v. infusion was reduced by 31%. Secretory studies by

Jaup et al (33) clearly demonstrated that pirenzepine suppresses basal, pentagastrin-stimulated and insulin-stimulated acid secretion in a dose-related manner. In a double-blind, placebo controlled, randomized studies on patients with dyspepsia, Bianchi Porro et al (34) reported a 48% decrease of gastric secretion in the first hour and 30% in the second hour after 50 mg oral dose of pirenzepine. Pirenzepine caused a reduction to 20% of the concentration of the acid produced by vagal stimulation (35). The drug inhibited the vagally transmitted acid secretion by about 45% even when the vagally stimulated acid secretion amounts to more than 50% of the pentagastrin-stimulated secretion. These effects of pirenzepine are similar to those of atropine but different from the effects of cimetidine. Eugenides et al (36) reported that histamine-stimulated acid output per two hours in healthy volunteers was reduced with 25 mg pirenzepine b.d. by 12.5% and with 50 mg t.d.s. by 24%. Stockbrugger et al (37) studied the effect of three different doses of pirenzepine on acid secretion stimulated by modified shamfeeding (MSF) on healthy volunteers. Oral pirenzepine 25 mg b.i.d., 50 mg b.i.d. or 50 mg t.i.d. reduced basal output (0-30 min) by 48%, 59% and 66% respectively, in stimulated acid output (0 - 120 min) by 45%, 58% and 48% respectively. When the acid secretion was calculated as volume corrected for duodenal-gastric reflux and pyrolic losses, the corresponding figures were 33.7%, 36.3% and 42.6%. Mignon et al (38) reported a dose-related reduction of meal-stimulated acid response with increasing doses of i.m. pirenzepine. Results with 0.5 mg/kg of pirenzepine showed 53% reduction in acid secretion. Pirenzepine 10 mg given to duodenal ulcer patients 45 min and again 10 min before the start of MSF blocked 48% of the response (39).

Pirenzepine (25 µg/kg) administered i.m. to dogs 10 min before infusion of bombesin, significantly inhibited the hypersecretion induced by the peptide in the main stomach and in a dose of 100 µg/kg pirenzepine vertually abbolished the acid response to bombesin (40). In Heidenhain pouch both doses fully prevented acid secretion. Pirenzepine injected during the secretory plateau

induced by bombesin, produced a mean peak inhibition of acid output from the main stomach of 76.5% and 80% with doses of 25 and 100 g/kg respectively. Pirenzepine (25 and 100 µg/kg i.v., 10 min before a meal test) fully prevented acid secretion for Hiedenhain pouch. The drug showed no effect on bombesin - or meal-stimulated gastrin release. Acid and pepsinogen secretion induced by bethanechol in isolated perfused mouse stomach are inhibited by pirenzepine (41).

7.2 Efficacy

Pirenzepine is effective in the treatment of gastric and duodenal ulcers. It is also effective in non-ulcer dyspepsia and in preventing ulcer recurrence. Pirenzepine in combination therapy with cimetidine can be used successfully for the treatment of cimetidine-resistant conditions such as duodenal ulcer and Zollinger-Ellison syndrome. The literature reports a number of studies on the efficacy of pirenzepine in the treatment of gastric and duodenal ulcers (43, 53-130). The published world literature (from 1977 to 1981) on the efficacy of pirenzepine had been the subject of a review and commentary by Bianchi Porro and Petrillo (53). The review examined the data published in Europe which deal with pirenzepine in the treatment of gastric and duodenal ulcers, taking into account only those results obtained from controlled endoscopic trials. In 12 prospective randomized double-blind placebo-controlled studies pirenzepine was administered to 475 duodenal ulcer patients with incidence of endoscopically proven healing of 74% when the patients received daily dosage of 100 or 150 mg pirenzepine, but only 55% if the dosage of the drug was 75 mg or less. Similar healing rates were observed (72% when daily dose of pirenzepine was 100 or 150 mg, 55% when the dose was 75 mg or less) in 4 randomized double-blind placebo -controlled studies in which 84 patients with gastric ulcer were admitted.

In another review, Texter and Reilly (83) evaluated the clinical evidence concerning the healing effect of pirenzepine in gastric and duodenal ulcers. The reviewers subjected the

results of the previous double-blind, therapeutic studies on ulcer healing to mathematical treatments. The therapeutic studies reviewed included a large number of patients in Europe and Japan. The dose-response curve for duodenal ulcer incorporating 16 data points from 8 trials in 530 patients, 343 of whom received pirenzepine, resulted in a correlation coefficient $r = 0.887$ with $P < 0.005$. Although the analsis is not strictly mathematically correct, it suggests that pirenzepine heals duodenal ulcer compared to placebo with a positive relationship between dose and effect. The predicted healing rate at 150 mg was 79%. The analysis also shows that endoscopic healing from gastric and duodenal ulcers is linear with respect to time upto 6 weeks ($r = 0.75$, $P < 0.001$) and suggests that healing rates improve with time on therapy. This observation on time course is strengthened by the results of a large Japanese multicentre trial on pirenzepine. Therapeutic studies on gastric ulcer, showed endoscopic healing rate of 66.7%, using 75 mg/day pirenzepine for 8 weeks, which increased to 72% with 150 mg dose for 8 weeks.

Brunner (94) reported that pirenzepine is equally or at least not essentially less effective than cimetidine in the treatment of duodenal ulcer. The effect of pirenzepine and cimetidine on healing, symptoms and relapse rate of duodenal ulcer was studied by Eichenberger et al (95). No significant differences in ulcer healing between the efficacy of the drugs when 1 gm/day of cimetidine and 75 mg/day pirenzepine were used. The relapse rate after treatment with pirenzepine was lower than after treatment with cimetidine.

Do et al (96) reported the efficacy of pirenzepine, 100 mg daily, in the treatment of duodenal ulcers in a placebo-controlled study. Pirenzepine produced healing in 14 out of 18 patients (78%) following 6 weeks of treatment as compared to 7 of 20 patients (35%) receiving placebo. Relief of daytime and nighttime pain was greater in pirenzepine-treated patients, as well as lower consumption of antacid.

Cheli et al (97) reported that pirenzepine in 100 mg orally daily for 28 days resulted in clinical cure in 34 of 44 duodenal ulcer patients. Sixteen patients completing treatment, were given pirenzepine 50 mg daily for 6 months, resulted in ulcer relapse in 18.7%. In 16 other patients, who interrupted treatment completely without pirenzepine, clinical recurrence of duodenal ulcer occured in 68.7%, with endoscopic recurrence in 62%, in the following 6 months.

Pirenzepine was shown by Dal Monte et al (98) to be effective in the maintenance treatment of duodenal ulcers to prevent ulcer recurrence when given in oral doses of 100 mg daily for one year. Pirenzepine has also been effective in doses of 50 mg daily for one year (100, 104, 107). However, other investigators have reported no significant difference between pirenzepine 25 mg orally b.i.d. and placebo in the prevention of duodenal ulcer relapse in a one-year study (102). Hoffenberg (103) reported pirenzepine in oral dose of 50 mg b.i.d. for 4 weeks, initially, followed by 50 mg b.i.d. for 4 weeks, was no more effective than placebo in the treatment of duodenal ulcer (48% versus 40% complete healing). These data suggest that longer courses of therapy (6-8 weeks) may be indicated.

Pirenzepine in oral daily doses of 100 - 150 mg has been effective in producing healing of 74% of patients with duodenal ulcers in controlled clinical trials (range 52 - 90%) (53, 55, 57, 60, 74, 76, 104, 105), whereas lower doses of 75 mg daily or less have produced lower response rates ($\tilde{-}$ 55%) (53, 54, 106). These results are supported by more recent clinical studies with pirenzepine in oral daily doses of 100 - 150 mg and this appears to be the optimal dose (107 - 113).

Available clinical trials on gastric ulcer suggest that oral doses of 100-150 mg daily can produce healing in approximately 72% of patients with gastric ulcer, similar to duodenal ulcer, doses of 75 mg daily or less produce lower healing rates (53, 89, 114, 115).

Oral 50 mg daily dose of pirenzepine was reported effective in the treatment of non-ulcer dyspepsia in a double-blind comparative trial in 59 patients for a period of 4 weeks (116). A daily dose of 100 mg was also shown to be effective (117). Short-term pirenzepine therapy (100 mg daily for 4 weeks) was reported effective in improving the endoscopic appearance of acute conjestive and erosive gastritis and non-ulcer-associated severe duodenitis (118). The drug was similarly effective as cimetidine 1 gm daily for 4 weeks.

Comparative efficacy of pirenzepine 100 mg PO daily and cimetidine 1 gm PO daily in 6-week treatment of duodenal ulcer in outpatients has been indicated by several controlled clinical trials (67, 68, 81, 107, 108). Porro et al (109) have also reported that pirenzepine 150 mg PO daily was comparable to cimetidine 1 gm daily in the treatment of active duodenal ulcer in 90 patients treated for a period of 4 weeks (72% versus 75% respectively). In most clinical studies, the efficacy of both drugs was approximately 70%. Both drugs have been equally effective in preventing duodenal ulcer recurrence following healing with conventional treatment with either drug (99, 101, 120, 121). Combination therapy with cimetidine has been effective in Zollinger-Ellison syndrome resistant to cimetidine alone (121).

A single 400 mg oral dose of cimetidine demonstrated approximately twice the antisecretory activity of 50 mg pirenzepine single oral dose in both basal and stimulated gastric acid secretion following pentagastrin stimulation. With pirenzepine in doses of 50 mg, inhibition of pentagastrin-stimulated acid secretion was similar to that of an oral 200 mg cimetidine, this cimetidine dose, however, had a greater inhibitory effect on gastric basal acid secretion (122). The authors indicate that high doses of pirenzepine should be avoided due to antimuscarinic effects such as dry mouth and palpitations and that cimetidine is indicated as agent of choice for reduction of gastric acid secretion. Palpitations occurred in several patients in this study with doses of 50 mg. In a single-blind multicenter study Brunner et al (108) evaluated the efficacy of pirenzepine 100 mg daily

(50 mg before breakfast and dinner) compared with that of cimetidine 1 gm daily (200 mg t.i.d. with meals and 400 mg h.s.) in the treatment of endoscopically-proven duodenal ulcer in 254 patients. Endoscopy was repeated after 4 weeks of treatment with both regimens; the endoscopist was blinded as to treatment regimens. Healing rates of 64% and 73% occurred with pirenzepine and cimetidine, respectively, which was not statistically significant. Pain relief was achieved rapidly with both drugs and the incidence of side effects was similar.

Pirenzepine in an oral dose of 50 mg b.i.d. was reported similarly effective as cimetidine 400 mg b.i.d. oral dose (low-dose therapy) in the treatment of duodenal ulcers in multicenter trial (125). Following 4 weeks of therapy, healing occured in 27 of 37 pirenzepine-treated patients (73%) and 29 of 38 cimetidine-treated patients (76%). Side effects were similar in each group, except for more frequently reported antimuscarinic effects in pirenzepine-treated patients. No adverse effects on intragastric milieu were reported. Intragastric microbial concentrations were increased significantly with both drugs but remained within normal limits; nitrile concentration prior to or following treatment did not exceed the normal range. Oral 300 mg daily dose ranitidine was reported (104) equally effective as oral 100 mg daily dose of pirenzepine in the treatment of duodenal ulcer in one controlled study (response rates of 87% in each group). Pirenzepine was equally effective as atropine as gastric antisecretory agent, but unlike atropine, pirenzepine had no effects on CNS, increase in heart rate, mydriasis, urinary bladder contraction or motor function of gastroenteric tract (124).

Londong et al (125) reported that concomitant pirenzepine and cimetidine therapy result in significantly greater suppression of stimulated acid secretion than either drug alone. It is suggested that combination therapy may exhibit synergistic effects on parietal cell function and could be advantageous in patients with gastrinoma,

ulcers and hypersecretion resistant to single drug therapy, and in prophylaxis of stress ulcer bleeding in critically-ill patients.

Weberg et al (126) has demonstrated that pirenzepine enhances and prolongs the effects of antacids on intragastric acidity. Addition of pirenzepine to antacid therapy resulted in a more sustained pH elevation than that observed with twice the amount of antacid administered alone.

Combination therapy with H_2-receptor antagonists has proven more effective than H_2-antagonist therapy alone (118, 127). The combination of pirenzepine 50 mg h.s. and ranitidine 150 mg b.i.d. given for a period of 6-12 weeks was effective in all of 7 patients with active recurrent duodenal ulcer. Continuous maintenance therapy with ranitidine 150 mg daily and pirenzepine 50 mg daily resulted in prevention of ulcer relapse in 5 patients who, in the previous year, continually relapsed while receiving single-agent (cimetidine or ranitidine) therapy. These results suggest the efficacy of combined H_2-antagonist and pirenzepine therapy in highly recurrent or resistant duodenal ulcers (127). Mignon et al (121) reported the effectiveness of combination therapy of pirenzepine and cimetidine in 3 of 4 patients with Zollinger-Ellison syndrome who were unresponsive to cimetidine alone.

Unlike cimetidine, pirenzepine appears to lack inhibitory effects on the hepatic mixed function oxidase system and it should be safe to administer concomitantly with drugs that are metabolized by this system (128).

The available informations suggest that pirenzepine possesses selective antimuscarinic effects and minimal CNS effects. Studies presented so far do not show that the drug is more effective than cimetidine or ranitidine and no significant differences in toxicity have been observed. Pirenzepine in combination therapy with H_2-receptor antagonists, however, suggest that pirenzepine may be very useful in the treatment of resistant duodenal ulcer and Zollinger-Ellison syndrome.

7.3 Adverse Reactions

The major side effects of pirenzepine are due to its anticholinergic activity which indicates that the selectivity of the drug is not absolute and the anticholinergic effects can occur which appear to be dose-dependents.

Dry mouth is the most common side effect of pirenzepine. Salivary secretion effects have been noted in many therapeutic trials. In the pharmacological studies in man, pirenzepine in a dose of 50 mg b.i.d. inhibited salivation compared to placebo (131) but not as much as ℓ-hyoscyamine in a dose of 0.6 mg b.i.d. which was approximately equipotent in suppression of gastric acid. Ten of 18 patients in the trials reported dry mouth with pirenzepine and 17 of 18 on ℓ-hyoscyamine. Dry mouth is reported to occur in 13.5% of patients receiving pirenzepine in doses of 150 mg daily. The incidence is reduced to 4% with doses of 100 mg daily and to 2.5% with doses of 75 mg daily or less (54). Dry mouth does not appear to be severe enough to warrant withdrawal of treatment in most cases (106).

Pirenzepine in high doses inhibits the contractile (132) activity of the stomach and colon (132). Bianchi Porro et al (34), however, reported pirenzepine (50 mg orally) showed no effects on gastric emptying. Similar results were reported by Stacher et al (133) when healthy men received either 50 mg pirenzepine twice daily or placebo. Despite the considerable effects of pirenzepine on antral motility, its delaying effect on gastric emptying was insignificant. Constipation has occurred with pirenzepine in approximately 2.6% of patients receiving doses of 150 mg oral daily dose (54). Diarrhea has also occurred with pirenzepine therapy, however, the incidence appears to be less than that of constipation (23, 106, 134). Jaup (135) measured esophageal peristaltic activity after pirenzepine, 10 mg i.v. or 50 mg b.i.d. (orally) in comparison with atropine 0.5 mg i.v. or ℓ-hyoscyamine 0.6 mg b.i.d. The latter two agents reduced esophageal peristaltic pressure compared to placebo while pirenzepine showed

marked effects only shortly after i.v. dosing, when plasma levels were high. Oral pirenzepine slightly decreased (- 15%) peristaltic amplitude compared to placebo while ℓ-hyoscyamine decreased this parameter over 50%. Other studies (136, 137) have shown decreased amplitude of esophageal contractions as well as inhibition of lower esophageal spincter pressure with parenteral administration of pirenzepine. Colonic motility is reduced by 0.3 mg/kg i.m. (138) and gastric smooth muscle effects are slight at 0.2 mg/kg i.m. (139) or 50 mg b.i.d. (131). Despite pirenzepine effects in altering esophageal and colonic motility, the available data does not suggest the drug will increase the risk of peptic gastro-esophageal reflux (140).

The effects of intravenous and oral pirenzepine on various gastrointestinal hormones and metabolic parameters are similar to those observed for antimuscarinic agents except for the lack of depression of pancreatic bicarbonate secretion (141, 142) and possibly lack of gastrin (143-145). Pirenzepine produced no changes in gastric inhibitory polypeptide, insulin, glucagon, neurotensin, vasoactive intestinal polypeptide, somatostatin, secretin, free fatty acids, lactate, pyruvate, β-hydroxybutyrate, acetoacetate, prolactin and growth hormone. Pancreatic secretion volume, amylase, trypsin, chymotrypsin, lipase, pancreatic polypeptide, enteroglucagon, pepsin and gastric acid secretions are decreased. One study (146) noted a slight prolongation of gastrin post-prandially, while others (143, 145) observed decreases in contrast to atropine and another trial (144) showed no change. Masala et al (147) reported that pirenzepine in single doses of 75 mg reduced prolactin levels in females but produced only minimal effects on prolactin in males. Single doses of 75 mg pirenzepine were reported by Alagna et al (148) to produce no direct effect upon the adrenal glands. The responsiveness of the adrenal glands to exogenous ACTH was unchanged. However, a reduction in basal cortisol levels occurred with pirenzepine, suggesting a reduction in ACTH secretion. The drug has not produced adrenal insufficiency.

Visual disturbances have been reported in 42% of subjects (149). Brunner (94) however, reported that only small number of patients treated with pirenzepine complained about impairment of vision. Blurred vision and double vision have occurred during pirenzepine treatment in approximately 6.3% of patients receiving oral daily doses of 150 mg (75, 83). Hoffenberg (103) described amblyopia in 24% of patients receiving dose of 100 mg daily.

Urinary retention has been reported in 0.3% of patients receiving pirenzepine in doses of 100 mg daily (83). Kuhn et al (150) reported that pirenzepine (8 mg i.m.) decreased the pulse rate and systolic and diastolic blood pressure. Tachycardia has been observed during pirenzepine treatment (103). In most studies, however, no effects on heart rate were observed (83). Pirenzepine in dose of 50 mg daily caused palpitations (122). CNS toxicity has been minimal in most studies with pirenzepine (83, 151). This can be explained on the bases of pirenzepine selectivity and also to hydrophilic characteristics. Dizziness, mental confusion, asthenia and headache have been reported in a few patients (152, 153). Studies in healthy volanteers showed that no psychotropic effects occurred that are commonly encountered with tricyclic antidepressants, indicating that pirenzepine lack appreciable CNS toxicity (154). Fink and Erwin (149) also reported lack of CNS effects by pirenzepine with doses upto 150 mg.

7.4 Tissue Selectivity

Pirenzepine blocks the acetylcholine receptors of the parietal cells of the stomach and is thereby a marked inhibitor of gastric acid secretion both in animals and men. Pharmacological and clinical studies have shown this action of pirenzepine to be selective, since in doses which significantly inhibit gastric secretion, side effects typical of anticholinergic agents do not occur or minimal (30, 33, 45) and much higher doses are needed to inhibit salivation (132) and contraction of stomach and colon (138) or to induce tachycardia (52, 132).

Engelhorn (155) has evaluated the selectivity of pirenzepine and atropine and his experiments indicated substantial differences between the influence of either antimuscarinic agent on gastric ulcer and gastric secretion on one hand and on smooth muscle organs on the other. Although the anti-ulcer and antisecretory effects of pirenzepine approach those of atropine, significant differences are demonstrated in isolated muscle preparations after acetylcholine stimulation as well as after transmural stimulation. Pirenzepine was 16.5 times weaker than atropine in reducing salivation in rabbit and is 100 times less effective than atropine in doubling papillary diameter. Atropine caused a dose-dependent inhibition of the gastrointestinal transport while pirenzepine, even at higher doses, did not cause a decrease but rather an increase. Atropine caused a dose-dependent increase in heart rate which, however, could only be obtained with pirenzepine in doses 10 to 100 times greater than with atropine. Jaup et al (131, 156) compared the effects of pirenzepine and ℓ-hyoscyamine when taken in equipotent doses in inhibiting gastric secretion. Both drugs affected the salivary secretion but the inhibition by pirenzepine was significantly less than that by ℓ-hyoscyamine. Gastric emptying was significantly delayed by ℓ-hyoscyamine compared to pirenzepine. Swallowing induced eosphageal peristalsis was significantly inhibited in 51%. Pupil size and near point distance were significantly affected by ℓ-hyoscyamine but not by pirenzepine. The minimal effects of pirenzepine on the CNS have been attributed to the drug selectivity and hydrophilicity.

7.5 Mechanism of Action

It is generally accepted that pirenzepine is an antimuscarinic drug. Among other examples demonstrating this, is the competitive inhibition by the drug of the carbachol effect on the spontaneously beating guinea pig atrium (157), the selective antagonism of carbachol-stimulated acid secretion in the perfused rat stomach while no such antagonism was noticed when histamine or

pentagastrin was used as a stimulus (48), as well as the selective antagonism, by pirenzepine, of the muscarinic response to bethanechol on conscious cats with gastric fistula (157) and the finding that pirenzepine, in greater amount that atropine, blocked only the muscarinic effects of acetylcholine without altering the nicotinic component (151). Similar to other antimuscarinic agents, pirenzepine affects only the volume and acid output, and not gastric pH (83). Baldi et al (158) have demonstrated that pirenzepine (0.15 mg/kg, i.m.) inhibits the motor response in the human colon to a cholinergic stimulus with prostigmine.

Studies on receptor binding, pharmacological investigation on isolated tissues and studies in man have shown that pirenzepine distinguishes between different subclasses of muscarinic receptors (83, 150, 159-170). Using pirenzepine as novel tool that reveals heterogeneity of muscarinic receptors, evidences have been provided (164-166) of the existence of three distinctive types of muscarinic receptors classified as types A, B and C. With pirenzepine the log affinity constant for the A-type is about 7.7, for the B-type about 6.6 (10-fold weaker) and 4-fold further weaker for the C-type. In the same system the log affinity for atropine is about 8.5 to 9, indicating that the pharmacological response of pirenzepine as compared to that of atropine will be about 10-fold weaker at the A-type, 100-fold weaker at the B-type and 400-fold weaker at the C-type. The pharmacological data available on the potency of pirenzepine as well as data from binding studies suggest that in the calf sympathetic ganglia there seems to be predominantly A-receptors and in the rat sympathetic ganglia more of B-receptors. The myocardium have predominantly C-receptors, the conduction tissues in the heart contain B-type receptors while the ileum seems to have both type B and C receptors and the CNS both type A and B receptors. In most of the cases there is good quantitative agreement between the binding analysis and the pharmacological results (132, 155). Evidence for the heterogeneity of muscarinic receptors is also presented (171-173) using the selective ganglionic receptor agonist

McN-A-343 [4-(m-chlorophenylcarbamoyloxy)-2-butenyltrimethylammonium chloride]. The compound selectively stimulates the muscarinic receptors on ganglionic receptors with minimal influence on the muscarinic receptors in the heart and smooth muscle. Those receptors stimulated selectively by McN-A-343 have been named as M1-receptors and those stimulated by conventional muscarinic drugs are called M2-receptors. Further investigations are needed on pirenzepine and McN-A-343 to determine which nomenclature to follow. It appears that M_1 and M_2 receptors correspond to the A-type and C-type receptors respectively. Pirenzepine may also have a cytoprotective effect in addition to its antisecretory effects (174, 175). Other mechanism for pirenzepine ulcer-heating effects were also reported (176).

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STREPTOMYCIN

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1. HISTORY

Streptomycin is a water soluble organic base biosynthesised by appropriate strain of the actinomycetes, *Streptomyces griseus*. Prior to the development of Penicillin Waksman and his collaborators began to study the production of antibiotic substances in the department of microbiology of New Jersey Agricultural Experiment Station, Rutgers University in 1939 (1). In the succeeding years they examined some thousands of actinomycetes, hundreds of fungi and many bacteria and extracted from their culture media a number of antibacterial substances. Particular attention was devoted to obtaining substances which acted prominently on gram negative bacteria. As a result of this work, the organism producing streptomycin was isolated from heavily manured soil and from a chicken's throat (2,3). The first publication on streptomycin was made by Schatz, Bugie and Waksman (3) in 1944. A complete bibliography of the work on streptomycin from 1944-1952 was recorded by Waksman in "The Literature on Streptomycin" (4). The cultural descriptions and the nutritional requirement of *Streptomyces griseus* are also discussed by Waksman (5,6). The early promising clinical reports of 1944 based on crude Streptomycin prepared in the Merck Research Laboratories showed the importance of the crude drug and started an intensive development program for its large-scale production. The general line of development of the work on streptomycin have closely followed those of the work on penicillin. Rapid progress in the study of streptomycin was greatly helped by the experience gained with penicillin and by the fact that there already existed organizations for large-scale manufacture and exploitation of the latter.

2. DESCRIPTION

2.1 Nomenclature

2.11 Chemical Names

a) 0-2-Deoxy-2-(methylamino)-α-L-glycopyranosyl-(1 → 2)-0-5-deoxy-3-C-formyl-α-L-lyxofuranosyl-(1 → 4)-N,N'-bis (aminoiminomethyl)-D-streptamine (7).

b) 4-0-(2-0-(2-Deoxy-2-methylamino-α-L-glycopyranosyl)-5-deoxy-3-C-formyl-α-L-lyxofuranosyl)-N N'-diamidino-D-streptamine (8).

c) 2,4-Diguanidino-3,5,6-trihydroxycyclohexyl-5-deoxy-2-O-(2-deoxy-2-methylamino-α -L-glucopyranosyl)-3-formyl-β-L lyxopentanofuranoside (9).

d) N-Methyl-L-glucosamidinostreptosidostreptidine (10).

e) 2-(N-methyl-α-L-glucopyranosaminido)-3-C-formyl-5-desoxy-L-aldopentofuranose (11).

2.12 Generic Name

Streptomycin A. (12)

2.13 Trade Names

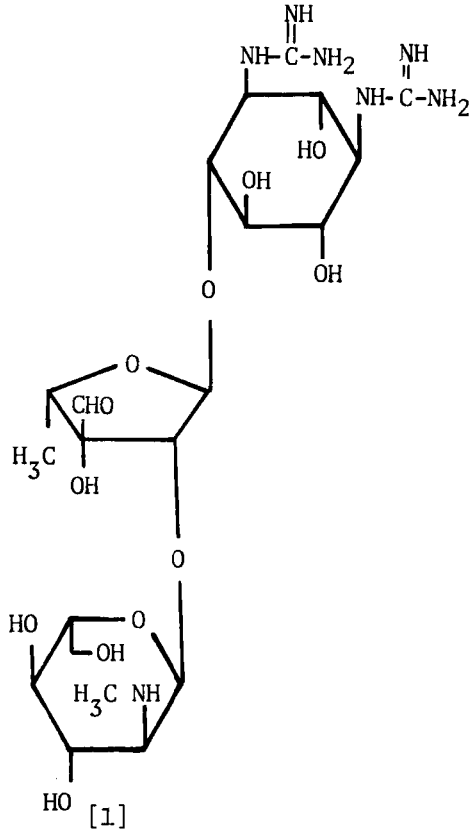
a) Estreptomicina (8)
b) Streptomycinum (13)
c) Streptomyseen (13)
d) Streptomycini (14)
e) Streptovex (15)
f) Stryzolin (15)
g) Crystamycin (15)
h) Dimycin (15)
i) Oroject NS (15)
j) Streptopen (15)

2.2 Formulae

2.21 Empirical

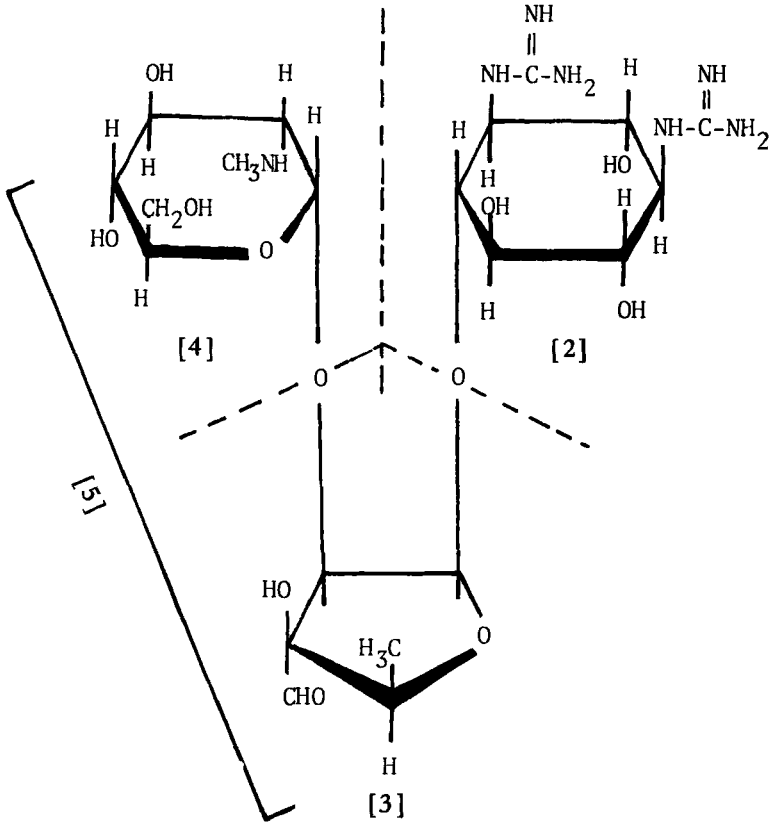
a) Streptomycin = $C_{21}H_{39}N_7O_{12}$
b) Streptomycin sulphate = $(C_{21}H_{39}N_7O_{12})_2 3H_2SO_4$
c) Streptomycin Hydrochloride = $C_{21}H_{39}N_7O_{12}.3HCl$.
d) Streptomycin calcium chloride = $(C_{21}H_{39}N_7O_{12}.\ 3HCl)_2\ CaCl_2$

2.22 Structural



[1]

Streptomycin is an optically active, triacidic base, $C_{21}H_{39}N_7O_{12}$; possessing an aldehydic carbonyl group. The high proportion of oxygen molecule is characteristic of a carbohydrate. Streptomycin [1] is made up of three components : Streptidine [2], Streptose [3], and N-methyl-L-glucosamine [4], linked together by glycosidic bonds (16).



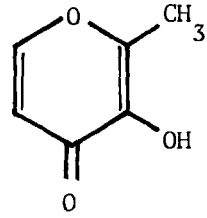
Streptomycin

[1]

2.23 Structural elucidation

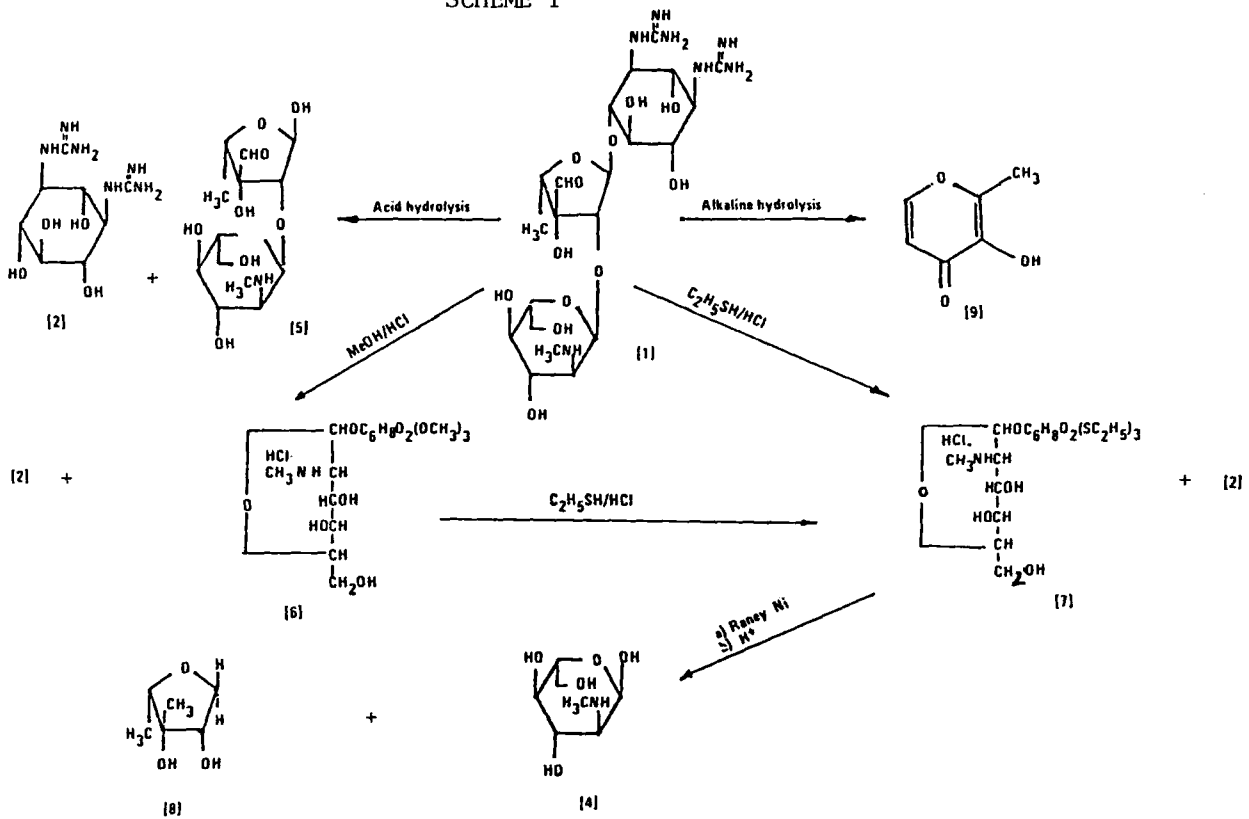
The difficult task of elucidating the structure of streptomycin was carried out principally by four groups of workers led by K. Folkers, O. Wintersteiner, H.E. Carter and M.L. Wolfrom. Their work has excellently reviewed for several times (11, 17-21). The structural elucidation of streptomycin is based on studies of its various degradation products.

1) On acid hydrolysis, the weaker glycosidic bond in streptomycin between streptidine and streptose splits to give streptidine [2] and streptobiosamine [5](16,22-26).
2) Mild methanolysis of streptomycin [1] with methanolic hydrogen chloride gives streptidine [2] and methyl streptobiosaminide dimethyl acetal hydrochloride [6]. Treating [6] with ethyl mercaptan and hydrogen chloride gives ethyl thiostreptobiosaminide diethyl mercaptal hydrochloride [7] which is also obtained by the action of ethyl mercaptan and hydrogen chloride on streptomycin. By Raney-nickel and subsequent acid hydrolysis of [7], it gives N-methyl-L-glucosamine [4] and bisdeoxy-streptose [8](27-28).
3) Hydrolysis of streptomycin with N1 sodium hydroxide for three minutes at 100^{o} or eighteen hours at 40^{o} yields a weakly acid substance, which has been characterized as maltol [9] (29).



The degradation reactions of streptomycin is well summarized in Scheme I.

SCHEME I


Acid hydrolysis
Alkaline hydrolysis
MeOH/HCl
C_2H_5SH/HCl
C_2H_5SH/HCl
a) Raney Ni
b) H^+
[1]
[2]
[4]
[5]
[6]
[7]
[8]
[9]

Streptidine

Streptidine [2], $C_8H_{18}N_6O_4$, is a meso Form of 1,3-diguanido-2,4,5,6-tetrahydrocyclohexane (24,25, 30, 31). The structure of streptidine [2] was confirmed by synthesis from D-glucosamine (32,33).

Streptobioasamine

The structure of Nitrogen containing disaccharide, streptobiosamine [5] was determined by a study of hydrolysis products of the methyl ether and the ethyl thio-ether formed by the action of hydrogen chloride in methanol and ethyl mercaptan respectively (27, 29, 34, 35). The hydrolysis of methyl-streptobiosaminide dimethyl acetal gave N-methylglucosamine [4](27,28). The structure of N-methyl-L-glucosamine has been established by synthesis from L-arabinose (36). Because of its instability, it is very difficult to isolate streptose [3], the second component of streptobiosamine [5]. The molecular formula $C_6H_{10}O_5$ was calculated from the known formulae of streptomycin [1] , Streptidine [2] and N-methyl-L-glucosamine [4] and its structure was deduced by identifying oxidation and reduction products. It was finally shown to be 3-C-formyl-5-deoxy-L-lyxose, a pentose containing the reactive aldehyde group associated with streptomycin molecule (29,37-40). The structure of streptomycin [1] was finally confirmed by total synthesis (Scheme II).

2.24 CAS Registry Number

1) Streptomycin	(57-92-1)
2) Streptomycin sulphate	(3810-74-0)
3) Streptomycin Hydrochloride	(6160-32-3)

2.25 Wiswesser Line Notation

Streptomycin sulphate :

T60TJ B1Q CQ -DQ EM1 FO- DT50TJ B CV H CQ EO -
AL6T J BQ CQ DMYZ UM EQ EMYZU M & WSQQ 3. (41)

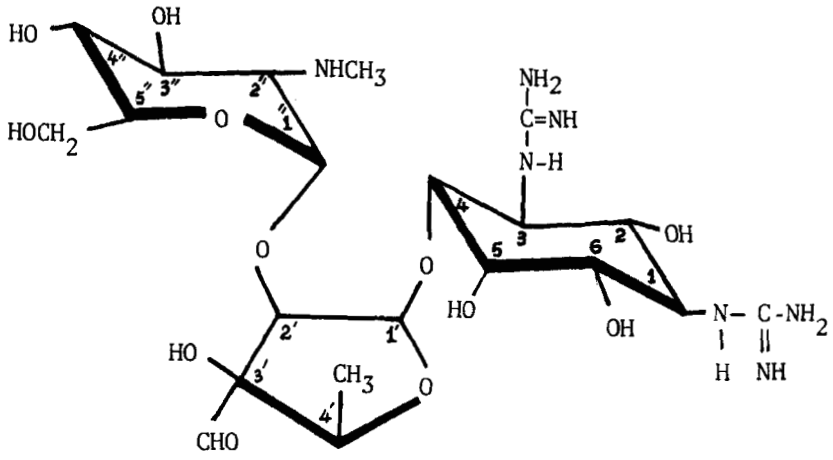
2.26 Stereochemistry and Absolute Configuration

The chemistry of the component fragments of streptomycin was thoroughly studied and their structures were established (23-40). The absolute configuration of streptomycin has been elucidated by chemical (applying Reeve's Copper complexing method to various degradation products of streptomycin, dihydrostreptomycin and streptobiosamine) (42,43) and optical correlations (44). The absolute stereochemistry of streptose was established to be (R) at C-4 and (S) at C-2 (37,40,45). The streptidine was shown to possess all *trans* configuration (46). Although streptidine is a meso form, the asymmetric attachment of streptobiosamine to it causes each of the ring carbon of streptidine in streptomycin to be asymmetric. The asymmetry of streptidine ring in streptomycin is 1(R), 2(R), 3(S), 4(R), 5(R), 6(S). The streptobiosamine moiety of streptomycin has been shown to be attached to position 4 of streptidine in either R or S absolute configuration (46-48). The three units of the streptomycin are attached together by glycosidal bonds which were shown to be formed by condensations between :

(i) the C-2 hydroxyl of streptose and C-1 hydroxyl of N-methyl-L-glucosamine (28,38,39,49-51).

(ii) C-1 hydroxyl of streptose and a hydroxl group of streptidine that is adjacent to only one guanidino group (i.e., position 4) (22,38,39,52-54).

All glycosidic linkages have an α-S configuration which was confirmed by NMR and crystallographic studies (44, 55-57). By using the configurational assignment, the structure of streptomycin in complete stereochemical details may be written as indicated below :



2.3 Molecular Weight

a) Streptomycin =	581.6
b) Streptomycin sulphate =	1457.4
c) Streptomycin hydrochloride =	691.0
d) Streptomycin calcium chloride =	1492.9 (8)

2.4 Appearance, Color, Odor and Taste

Streptomycin occurs as white to slightly pink or pale brownish powder or granules; odor: odorless or with a slight odor; taste : slightly bitter. Streptomycin is hygroscopic and may deliquensce on exposure to air but not affected by air or light (13).

2.5 pH Range

A 25 per cent w/v solution of streptomycin has a pH between 5 and 7.

3. PHYSICAL PROPERTIES

3.1 Melting Range

Indefinite (12).

3.2 Solubility

1) Streptomycin : It is very soluble in water; almost insoluble in alcohol (95%), chloroform, ether and petroleum ether (13).

2) Streptomycin sulphate : It is very soluble in water; practically insoluble in alcohol acetone, chloroform, ether and petroleum ether (8).

3) Streptomycin hydrochloride : It is very soluble in water, practically insoluble in alcohol, chloroform and ether (8).

4) Streptomycin calcium chloride : It is very soluble in water, practically insoluble in alcohol, chloroform and ether (8).

3.3 Loss on Drying

Streptomycin loses when dried over phosphorus pentoxide at 60°C and at a pressure not exceeding 5 mm. of mercury, for 3 hours, not more than 5 per cent (13).

3.4 Optical Rotation

Streptomycin is optically active with levo rotation. The following optical rotations were reported for the various salts of streptomycin (7,12) :

	$[\alpha]_D^{25}$	Solvent
1. Streptomycin hydrochloride.	-84^o	Water (C =1.0)
2. Streptomycin calcium chloride.	-76^o	Water (C =1.0)

The specific rotation for streptomycin sulphate was determined in our laboratory on a Perkin Elmer Polarimeter, Model 241 MC and found to be $[\alpha]_D^{25}$ - 75^o in water (C = 1.0).

3.5 Crystal Structure

Neidle, et al (57) have reported the X-ray crystallographic analysis of streptomycin oxime selenate. Streptomycin oxime selenate, $C_{21}H_{40}N_8O_{12}$ $[1\frac{1}{2}H_2SeO_4]$, $4H_2O$ crystallizes from aqueous methanol as monoclinic needles, space group C2, a = 17.10, b = 14.36, c = 16.13 Å, B = 108°, D_m = 1.54 g.cm^{-3}, D_c = 1.55 g. cm^{-3} for four formula units per cell. The [001] projection of one streptomycin oxime ion in selenate crystal is shown in Fig. 1.

3.6 Spectral Properties

3.61 Ultraviolet spectrum

The UV spectrum of streptomycin sulphate in water was scanned from 200 to 400 nm on DMS 100 Varian AG Spectrophotometer (Fig. 2). Three maxima were observed at 241.6, 270 and 328.2 nm. Streptomycin in 0.2N sulphuric acid, showed a maxima at 280 nm with $E^{1\%}_{1cm}$ = 0.2 (9).

3.62 Infrared spectrum

The infrared spectrum of streptomycin sulphate as KBr disc recorded on a Pye Unicam infrared spectrophotometer is shown in Fig. 3.

The structural assignments have been correlated with the following frequencies (Table 1):

Table 1 : IR Characteristics of Streptomycin Sulphate.

Frequency cm^{-1}	Assignment
3100-3500	NH and OH stretch (vb).
1630-1710	-C(H)=O and -C=NH stretch (vb).
1480	(vb).
1100-1200	C-O-C-stretch (vb).

vb = Very broad.

Other reported IR data (41) are 3330, 1670, 1470, 1390, 1110 and 1040 cm^{-1}.

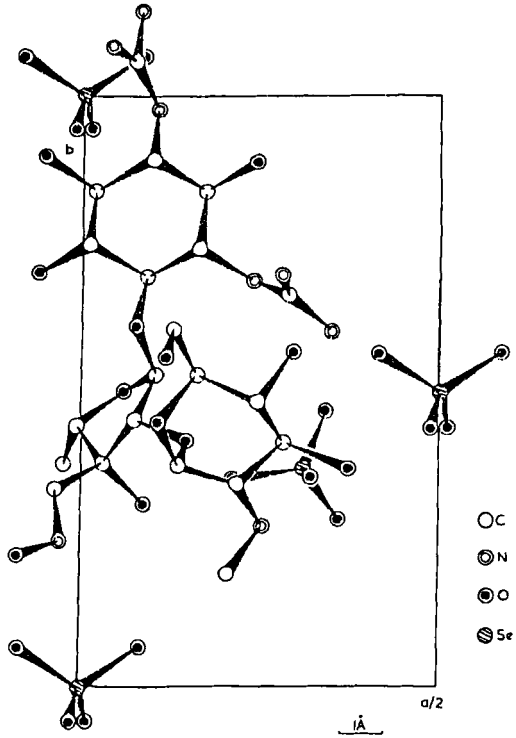


Fig. 1 : [001] Projection of one Streptomycin oxime ion in the Selenate Crystal.

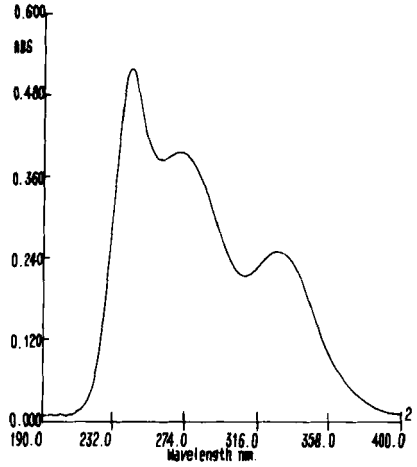


Fig. 2 : UV Spectrum of Streptomycin Sulphate in Water.

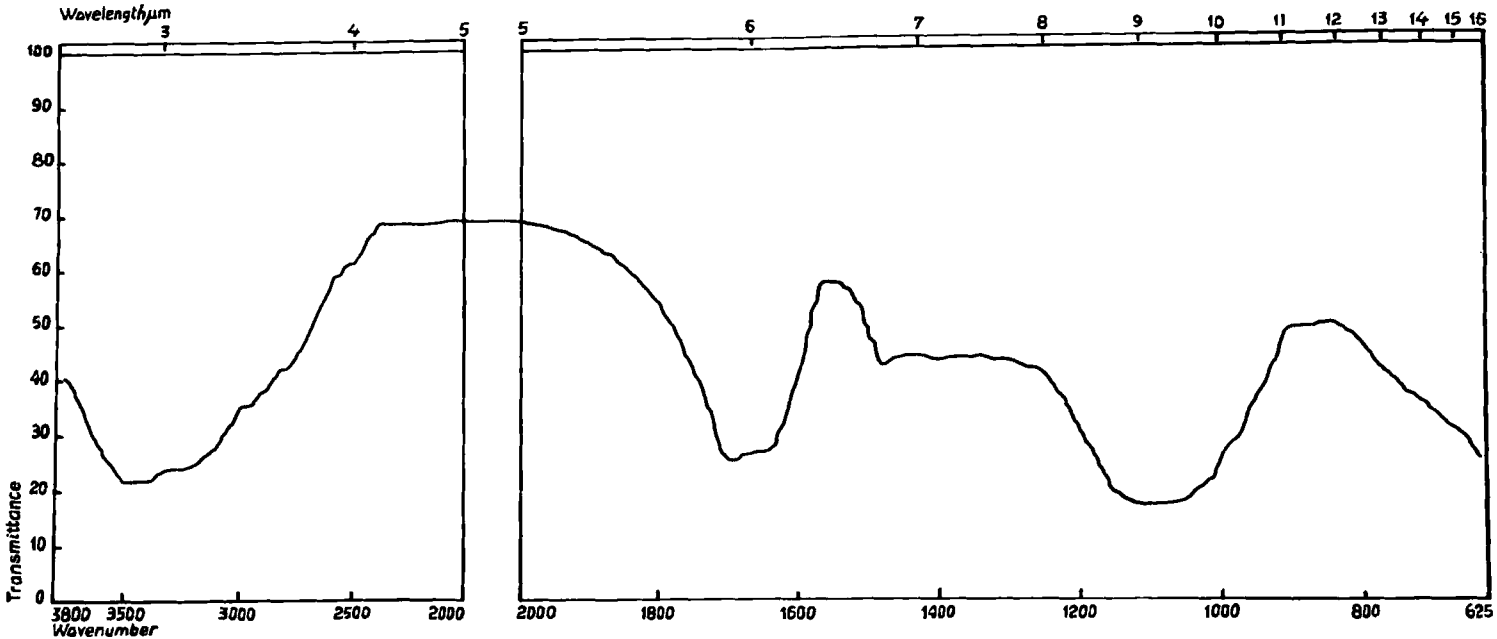


Fig. 3 : IR Spectrum of Streptomycin Sulphate as KBr disc.

3.63 Nuclear Magnetic Resonance Spectra

3.631 ^{1}H-NMR Spectra

The ^{1}H-NMR spectra of streptomycin in D_2O (Fig. 4) were recorded on a T60A MHz NMR spectrometer with TMS as internal reference. The proton chemical shifts are shown in Table 2.

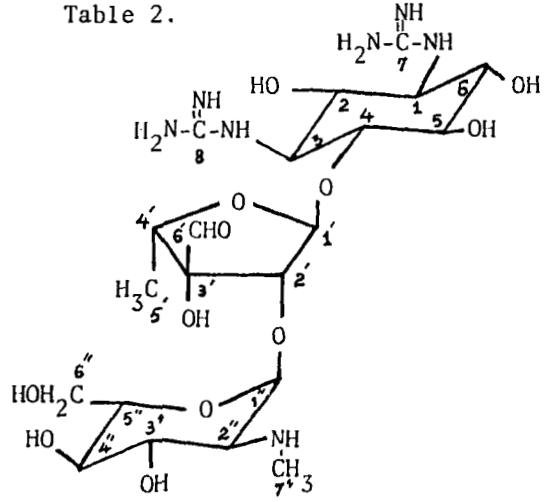


Table 2 : PMR Characteristics of Streptomycin Sulphate

Group.	Chemical shift. D_2O
H-1 N-methylglucosamine	5.53
H-1 Streptose	5.30
3 -H Formyl streptose	5.06
	3.83
	3.56
2 -N Me of N-methyl glucosamine.	2.86
4 - Sec-Me of Streptose.	1.23

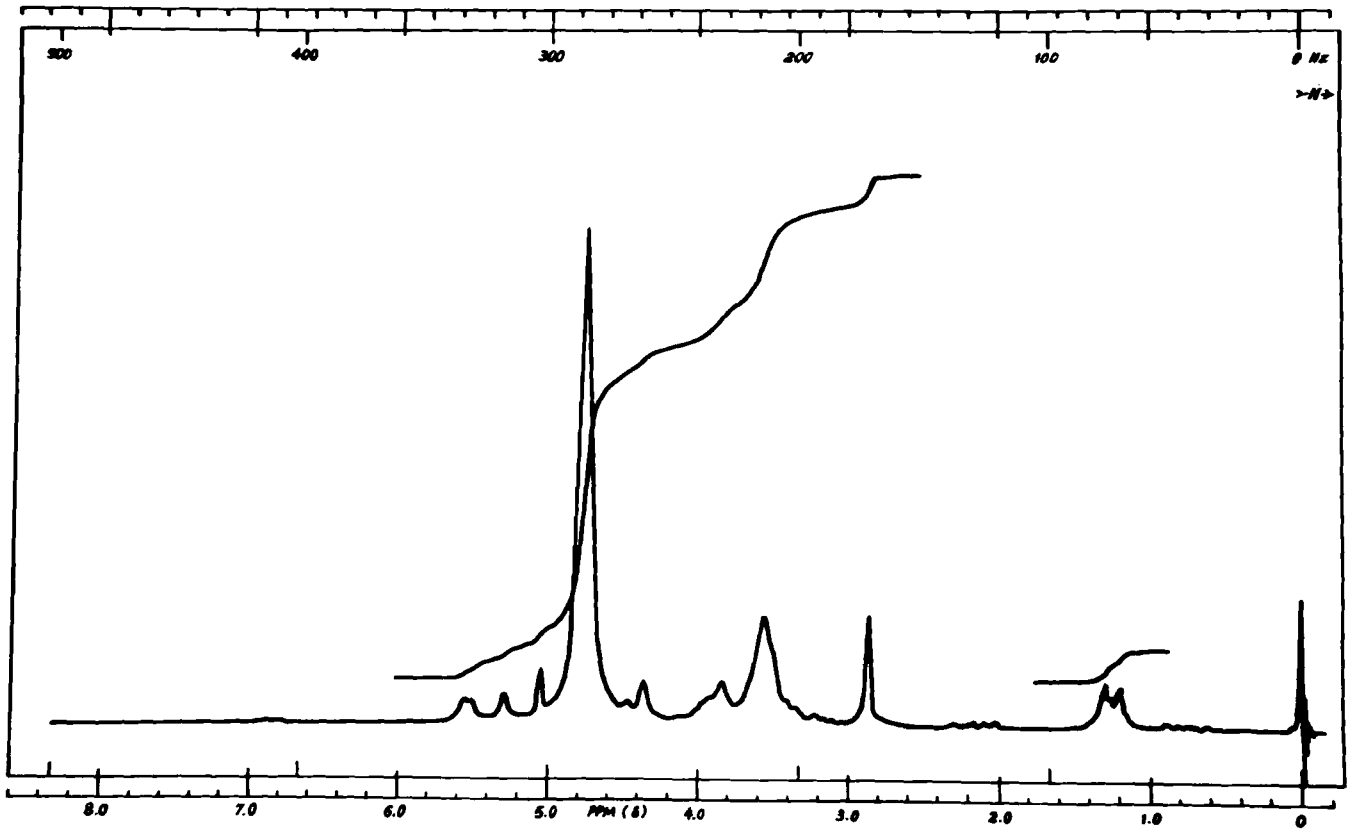


Fig. 4 : ^{1}H-NMR Spectrum of Streptomycin Sulphate in D_2O and TMS.

3.632 ^{13}C-NMR Spectra

The natural abundance C-13 NMR noise-decoupled and single frequency off-resonance decoupled (SFORD) spectra (Fig. 5 and Fig.6) in deuterium oxide were obtained on a Joel FX 90 - 90-MHz instrument. The carbon chemical shifts were assigned on the basis of the theory of chemical shift and SFORD splitting pattern (Table 3).

Table 3 : Carbon Chemical Shifts of Streptomycin Sulphate.

Carbon No.	Chemical Shift δ ppm	Multiplicity
1	57.06	d
2	87.13	d
3	60.82	d
4	108.61 or 97.11	d
5	63.11	d
6	61.52	d
7	160.33	s
8	160.80	s
1'	108.61 or 97.11	d
2'	71.92	d
3'	84.89	s
4'	80.67	d
5'	34.81	q
6'	186.31	d
1''	108.61 or 97.11	d
2''	64.50	d
3''	73.33	d
4''	74.21	d
5''	80.14	d
6''	75.97	t
7''	15.20	q

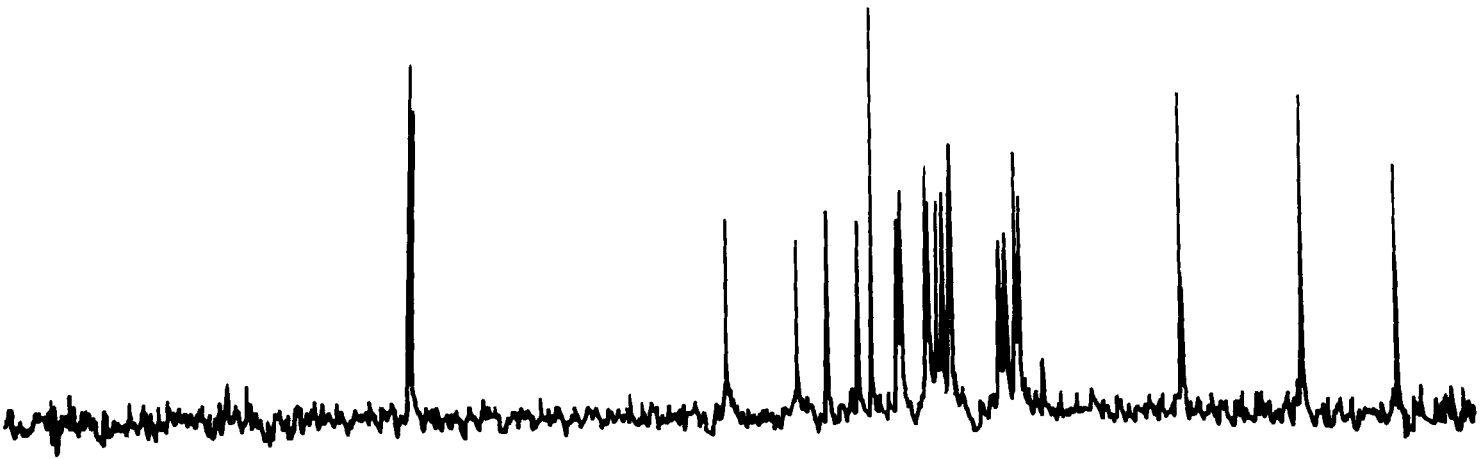


Fig. 5 : ^{13}C-NMR Noise-decoupled Spectrum of Streptomycin Sulphate in D_2O and TMS.

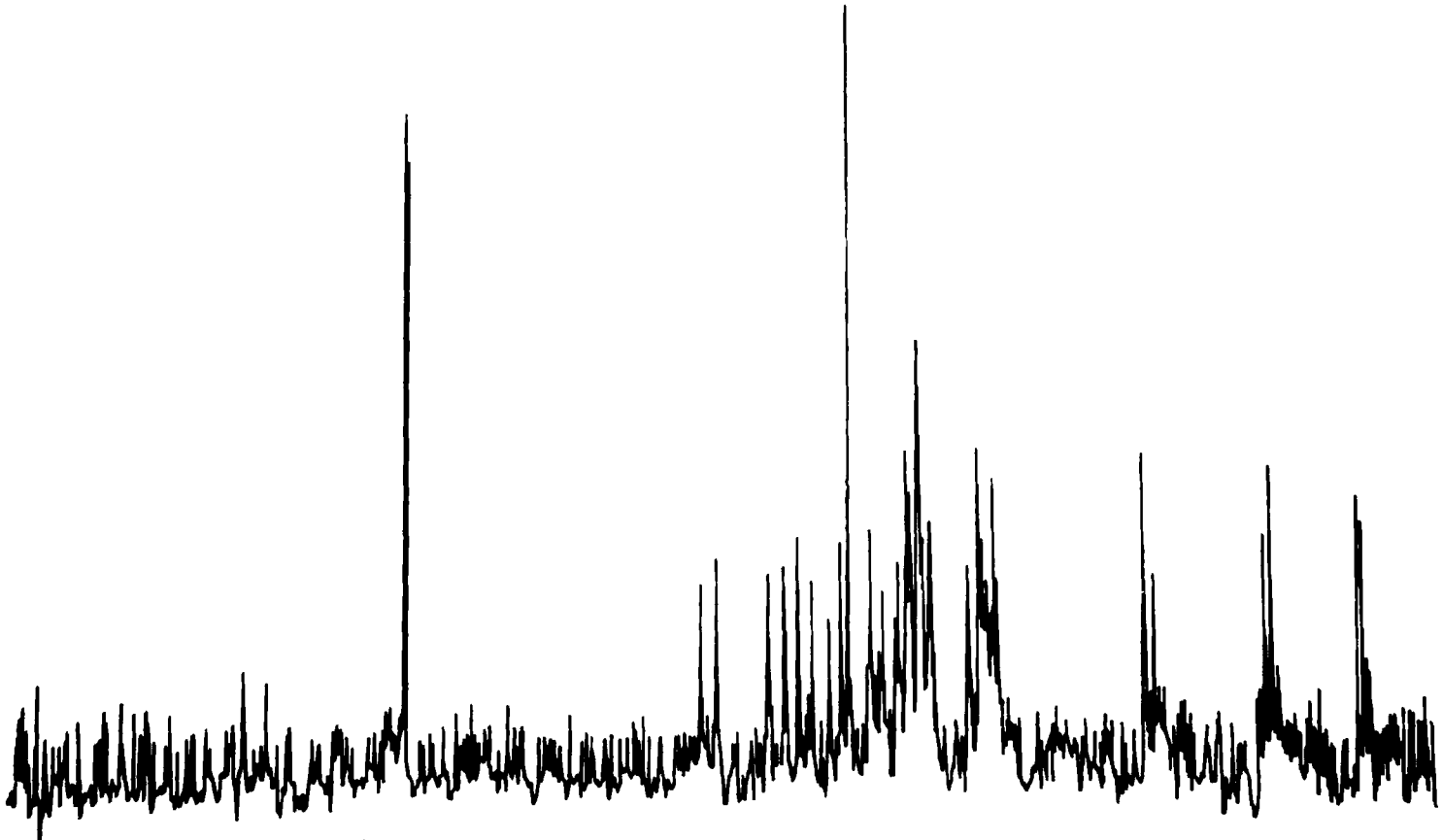
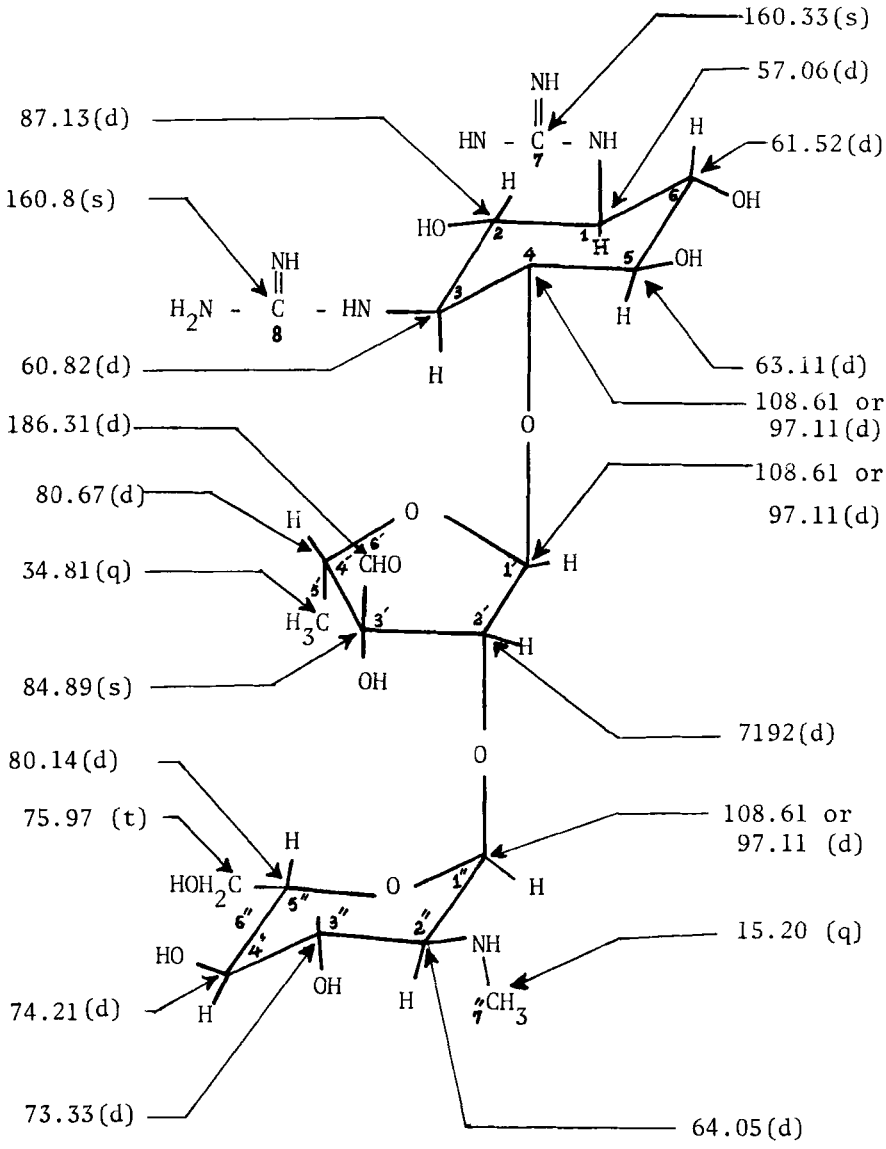


Fig. 6 : ^{13}C- NMR Off-Resonance Spectrum of Streptomycin Sulphate in D_2O and TMS.


160.33(s)
57.06(d)
87.13(d)
61.52(d)
160.8(s)
60.82(d)
63.11(d)
108.61 or
97.11(d)
186.31(d)
108.61 or
97.11(d)
80.67(d)
34.81(q)
84.89(s)
7192(d)
80.14(d)
75.97 (t)
108.61 or
97.11 (d)
15.20 (q)
74.21(d)
73.33(d)
64.05(d)

4. PRODUCTION OF STREPTOMYCIN

Streptomycin is produced by the micro-organism *streptomyces griseus*. It is also produced by other strains of organisms like *Actinomyces globisporus* (58), pink variant of *streptomyces griseus* (59), *streptomyces galbus* (60). However, for large scale production, appropriate strain of *streptomyces griseus* is used.

Streptomyces griseus can biosynthesize streptomycin in a medium containing glucose, sodium citrate and inorganic salts, but much higher yields are obtained by using the media containing also an organic source of nitrogen such as meat extract, yeast autolysate, Soybean flour, corn steep liquor or fermentation solubles (61-88). One or more of these are used for industrial biosynthesis. Tremendous amount of work has been done on the production of streptomycin (64, 89-103).

The large-scale production has been accomplished by either surface culture or deep culture process (64,65). The deep culture process is more often used for large-scale production of streptomycin. A brief outline of the production of streptomycin is well summarised below (104):

a) Production of Spore Suspension

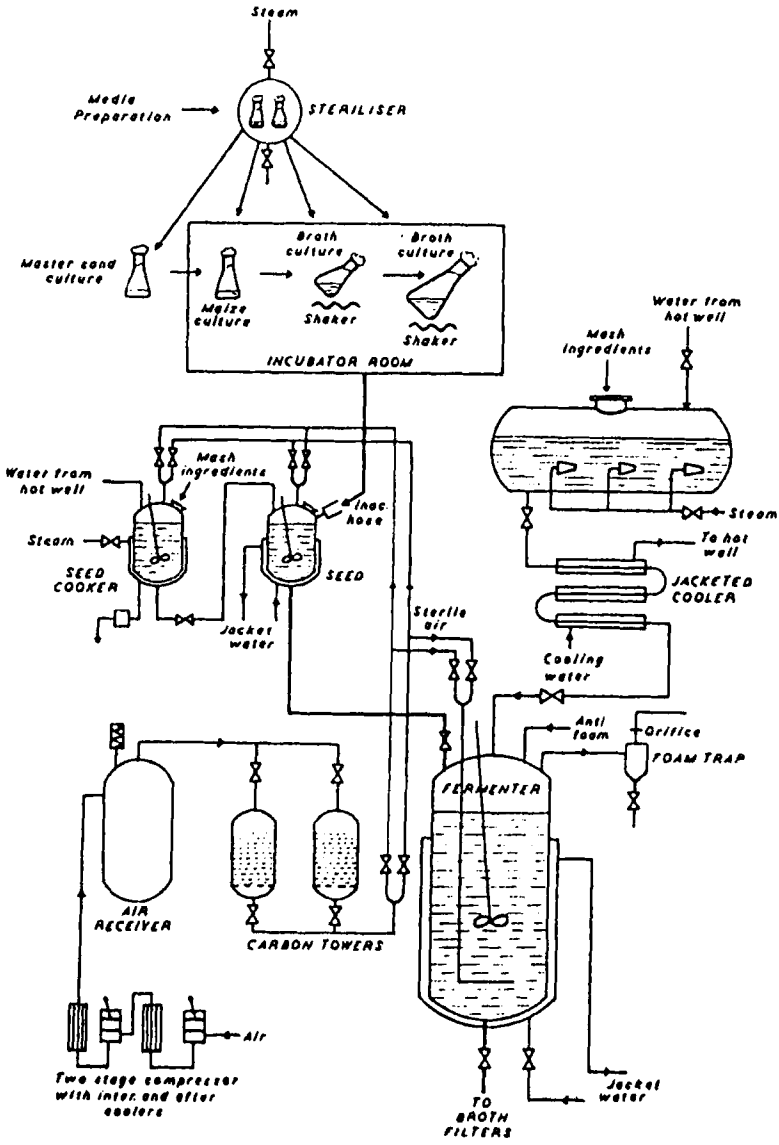
The spores of *streptomyces griseus* required for inoculation is obtained from rapidly growing culture on solid medium (64, 105). The spores are either suspended in sterile water or in skimmed milk which is inoculated with the medium in the shake flasks.

b) Preparation of Medium

All the solid constituents of the culture medium are placed in the fermenter through the charge hole and water is added to make up the volume. The medium is sterilized by the direct introduction of steam. Sterilization is carried out at 15 lb pressure for 25 minutes. After shutting off the steam, water is passed through the jacket and an air pressure of 10 lb is maintained in the fermenter. The sitrrer is working during sterilization, cooling and fermentation.

c) Preparation of the inoculum

The sterilized medium in the seed tank (115 L. capacity, provided with stirrer and inoculum measuring tank) is



Flow Sheet 1 : The production of Streptomycin.

inoculated with the content of shake flask. After the inoculation, the mycelium is grown for 36 hours with an aeration rate of 100 L per minute. After 36 hours, a heavy growth of mycelium is obtained. About twenty liters of this mycelium suspension is blown into the measuring tank and finally to the fermenters through pipe-line.

d) Fermentation

The fermenters are made up of stainless steel, having the capacity of 15000 gallons and provided with sitrrer, anti-foam nozzle, air sparger and air outlet. After the inoculum from the seed tank has been added to the fermenter, aeration is started at once. Then the fermentation is carried out at an optimum temperature of 25^{o}-30^{o}C. Foaming during fermentation is controlled by the addition of antifoaming agents. The final concentration of streptomycin (50,000 units per litre) will be reached in 72 hours. The production of streptomycin is given in flow sheet I.

e) Isolation and Purification

The streptomycin from the culture fluid is separated by adsorption on the charcoal and by subsequent elution with methanolic hydrochloric acid or other suitable solvents (106-117).

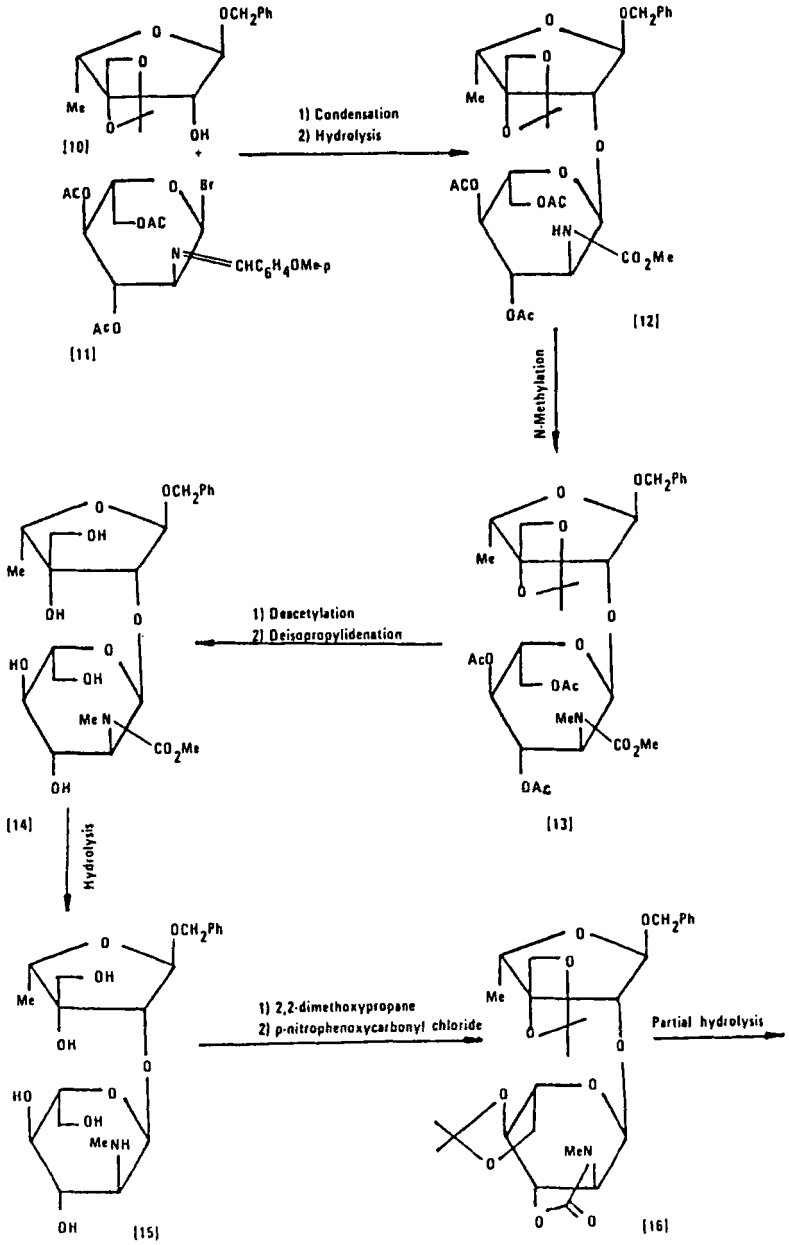
Further purification is carried out by chromatography on alumina (118-122) or ion exchange resins (123-164) and by precipitation with various base precipitants (165-179). Solvent extraction techniques (180-188), electrodialysis (189) and electroösmotic (190) procedures are also employed.

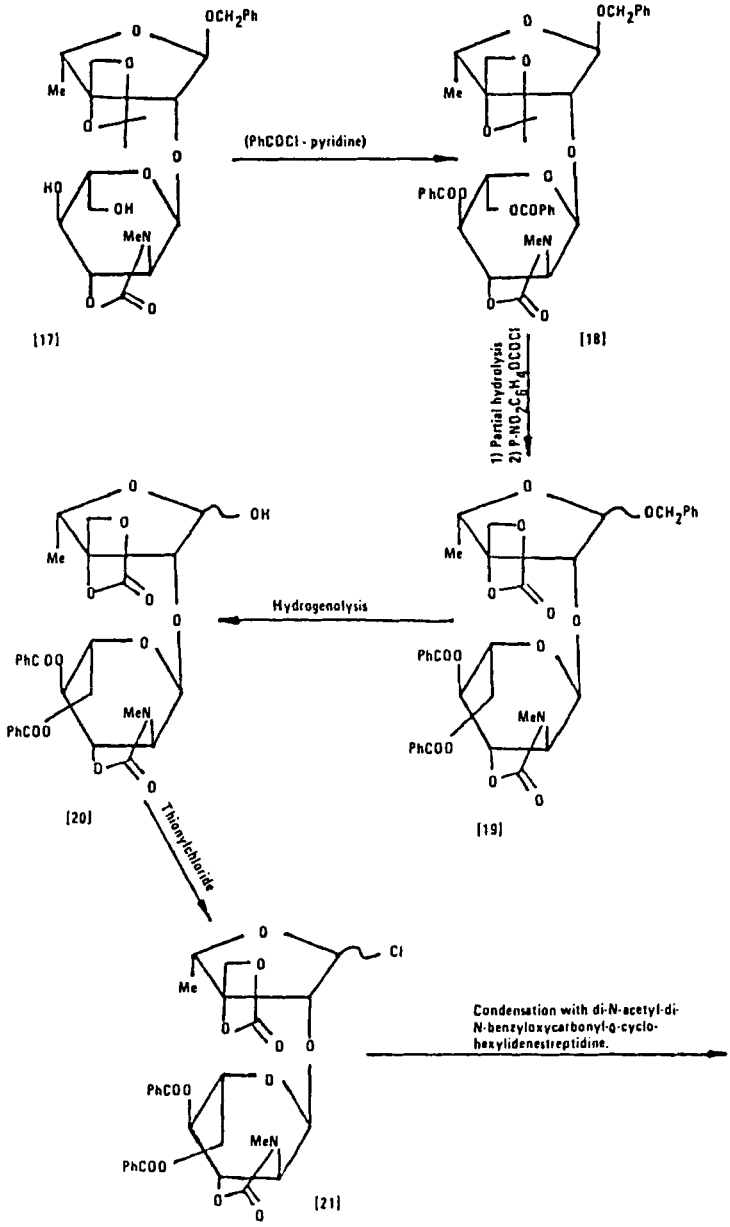
5. SYNTHESIS OF STREPTOMYCIN

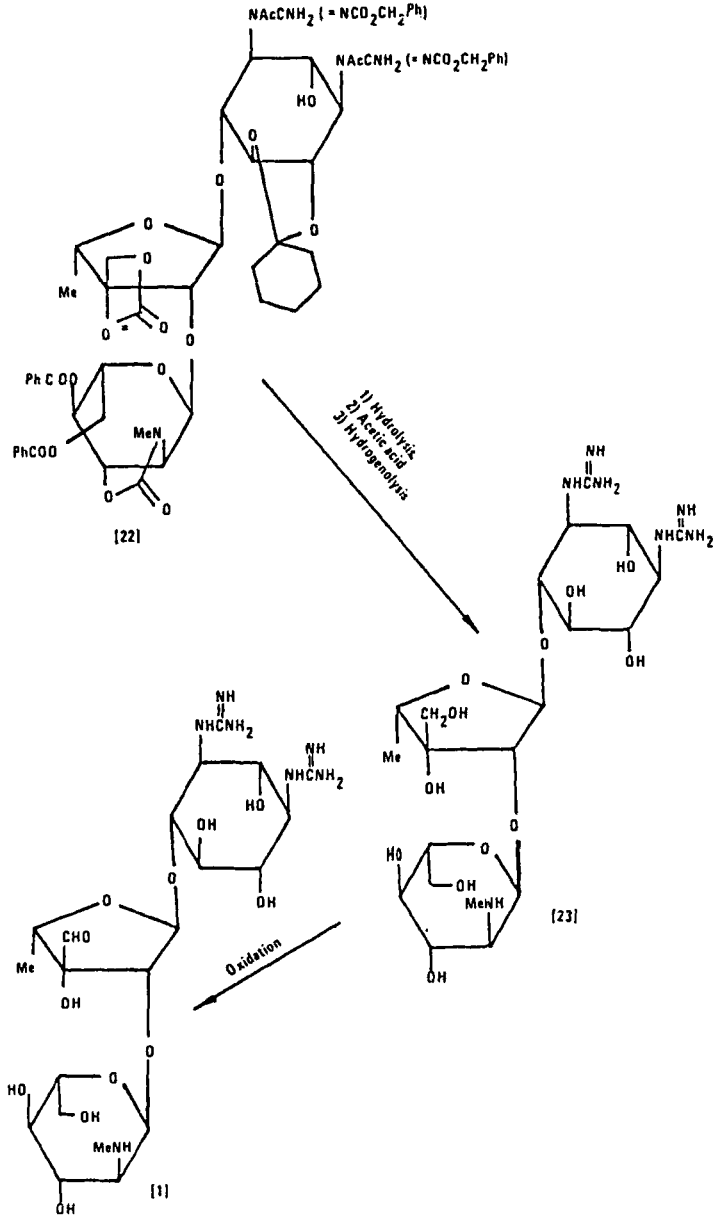
Although streptomycin was the first aminoglycoside antibiotic to be discovered, it was not synthesised until some 30 years later. The total synthesis of streptomycin was achieved by Sumio Umezawa et al, in 1973 (191,192). The reactions involved are in shown in Scheme II & summarized below:

Condensation of blocked derivative of dihydro-streptose (benzyl α-L-dihydro-streptose)[10] with the glucosamine derivative (L-glucopyranosyl bromide)[11] in benzene in the presence of mercuric cyanide, followed by hydrolysis of the Schiff base with 50% acetic acid and

SCHEME II


1) Condensation
2) Hydrolysis
[10]
[11]
[12]
N-Methylation
[13]
1) Deacetylation
2) Deisopropylidenation
[14]
Hydrolysis
[15]
1) 2,2-dimethoxypropane
2) p-nitrophenoxycarbonyl chloride
Partial hydrolysis
[16]


OCH2Ph
Me
HO
OH
MeN
(PhCOCl - pyridine)
PhCOO
OCOPh
[17]
[18]
1) Partial hydrolysis
2) P-NO2-C6H4OCOCl
OH
Hydrogenolysis
PhCOO
[20]
[19]
Thionylchloride
Cl
Condensation with di-N-acetyl-di-N-benzyloxycarbonyl-q-cyclo-hexylidenestreptidine.
[21]


NAcCNH2 (= NCO2CH2Ph)
NAcCNH2 (= NCO2CH2Ph)
HO
Me
PhCOO
PhCOO
MeN
[22]
1) Hydrolysis
2) Acetic acid
3) Hydrogenolysis
NH
NHCNH2
NH
NHCNH2
HO
OH
OH
CH2OH
Me
OH
HO
OH
MeNH
[23]
Oxidation
CHO
Me
OH
HO
OH
MeNH
OH
[1]

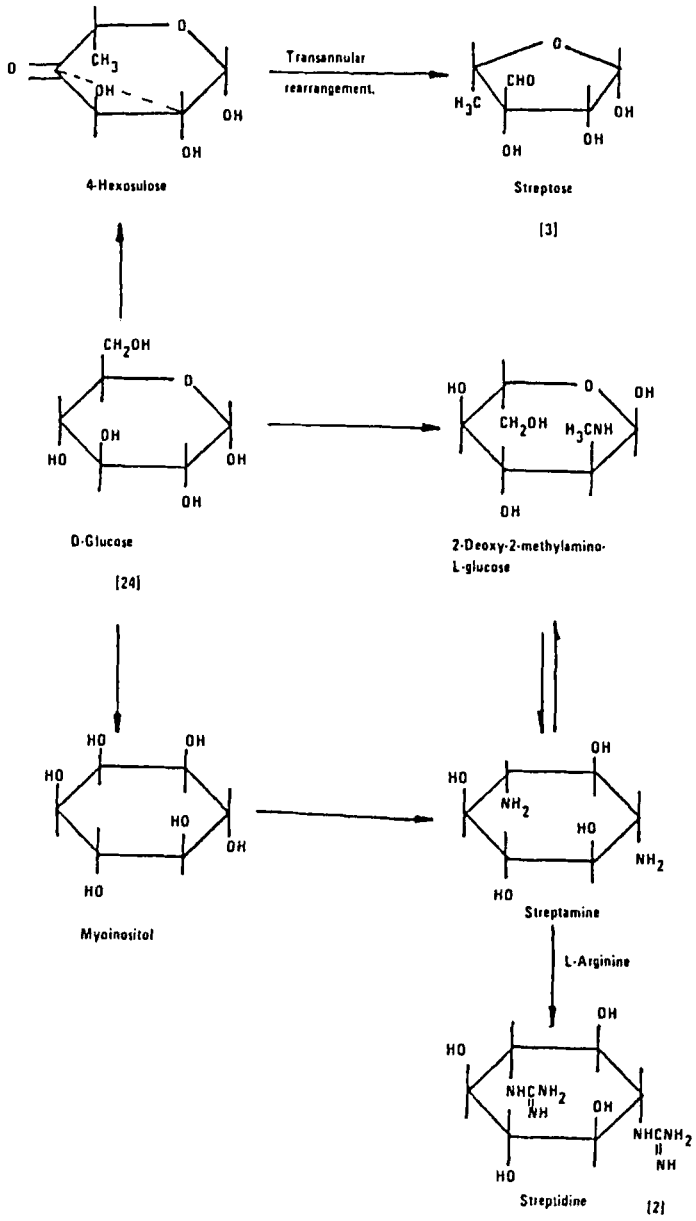
reaction with methyl chloroformate (Na_2Co_3, aq. acetone) gave the protected streptobiosaminide[12]. N-Methylation of [12] gave [13] Deacetylation followed by deisopropylidenation (IN HCl, Aqueous MeOH) gave [14] which was identical with benzyl α-S-dihydrostreptobiosaminide. Hydrolysis of [14] with 10% $Ba(OH)_2$ afforded benzyl α-L-dihydro-Streptobiosaminide [15]. Treatment of [15] with 2,2-dimethoxypropane (p-toluenesulfonic acid) gave a diisopropylidene derivative and further blocking by use of p-nitrophenoxycarbonylchloride (NaOH, aqueous acetone) gave [16]. Partial hydrolysis of [16] (25% AcOH in MeOH) gave the diol [17]. This was converted into the dibenzoyl derivative [18]. Partial hydrolysis of [18](75% Acetic acid) removed the isopropylidene group giving a diol which was transformed ($p\text{-}NO_2C_6H_4OCOCl$, pyridine) into the carbonate [19]. Hydrogenolysis of the glycoside linkages (Palladium Black-dioxane) gave free sugar [20]. The reaction of [20] with thionyl chloride at room temperature offered the α-glycosyl chloride [21]. Direct treatment of [15] with p-nitrophenoxycarbonylchloride or phosgene followed by benzolyation also gave [19]. But the yield is very poor.

Finally condensation of [21] with di-N-acetyl-di-N-benzyloxycarbonyl-O-cyclohexylidenestreptidine gave the 4-O-glycoside [22]. Hydrolysis of [22] with 0.05 N $Ba(OH_2)$ and dioxane successively removed the carbamate, carbonate, benzoyl and acetyl groups. Removal of the remaining protecting groups with 50% acetic acid followed by hydrogenolysis with Palladium black gave dihydrostreptomycin [23]. Subsequent mild oxidation of [23] afforded streptomycin[1].

6. BIOSYNTHESIS OF STREPTOMYCIN

Biosynthesis of streptomycin has been quite extensively studied. Streptomycin is formed from glucose as a primary precursor (193). Biosynthesis of the components of streptomycin, streptidine, streptose and N-methyl-glucosamine are well summarised by Horner (193) & Snell (194). General indication of the metabolic relationships of glucose [24] to the various components of streptomycin is shown in Scheme III.

SCHEME III


Transannular
rearrangement.
4-Hexosulose
Streptose
[3]
D-Glucose
[24]
2-Deoxy-2-methylamino-
L-glucose
Myoinositol
Streptamine
L-Arginine
Streptidine
[2]

Experiments with labeled compounds demonstrated that the specifically labeled glucose [24] is converted into streptomycin [1] in which each of the sub-units is labeled at the corresponding carbon (Scheme IV). This was established by several groups (195-203). Some of the intermediates in the biosynthetic pathway of streptomycin have been isolated,but some of the key intermediates are not yet detected. Recent studies established the presence of streptidine-6-phosphate and dihydro-streptose-dTDP, in the streptomycin biosynthetic pathway (204). Some intermediates between glucose and dihydrostreptose are known as a result of the work of Grisebach (205) who demonstrated that [^{14}C-]-glucose-dTDP was converted to 4-Keto-4,6-dideoxyglucose-dTDP which in turn was converted to dihydrostreptose-dTDP.

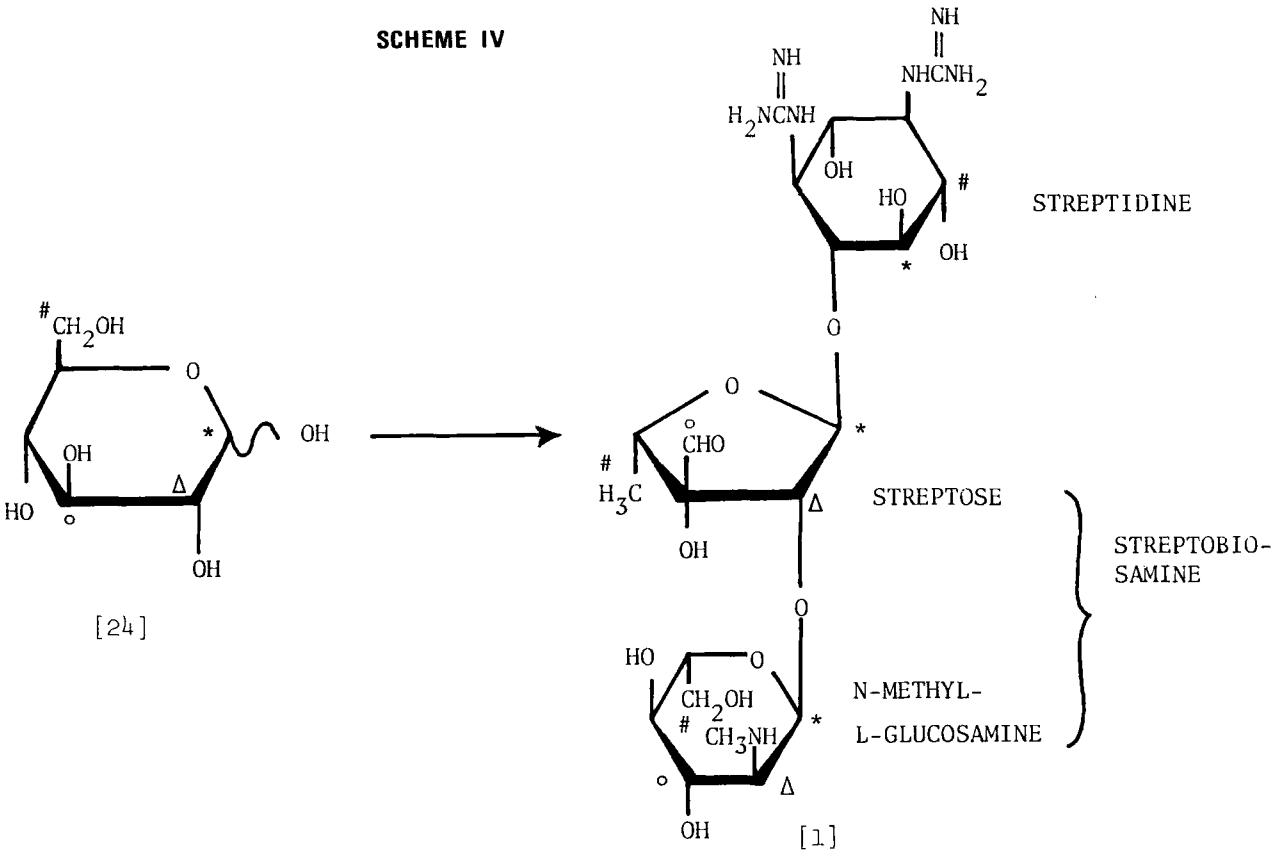
Nothing is known yet regarding the intermediate between glucose and N-methylglucosamine. Recently Kniep and Grisebach (206) established the role of dihydrostreptosyl-streptidine 6-phosphate [25] in the biosynthesis of streptomycin. They concluded that the nucleotide-bound N-methyl-4-glucosamine moiety in *S. griseus* was catalized and transferred to dihydrostreptosyl streptidine 6-phosphate [25] which in turns was converted to dihydrostreptomycin 6-phosphate [26]. Further oxidation of [26] gave streptomycin-6-phosphate[27](207) which on hydrolysis yield streptomycin [1] (208). The reaction is summarized in Scheme V.

7. PHARMACOLOGY AND THERAPEUTIC CATEGORY

Streptomycin is an aminoglycoside antibiotic which is active against a wide range of bacteria, both Gram positive and Gram negative (209). It is a valuable therapeutic agent for all forms of tuberculosis (210-252). Streptomycin is effective against Tularemia (253,254). It is highly specific and one of the most effective agent for the treatment of all forms of plauge (255). In combination with Penicillin G, it is considered to be the drug of choice in infections with *Streptococcus viridans* and *Enterococcus* (256). It is also effective against *staphylococcus albus* (257).

Streptomycin is a drug of second choice in infections due to susceptible strains of anaerobic *streptococci*, bacteroides, *Aerobacter aerogenes*, *A. fecalis*, *Shigella*, *Brucella* and *Vibrio comma* and in combination with tetracycline, in *Porteus* infections and in brucellosis (256,258-264). Since streptomycin is effective against *H. influenzae*, *E. coli*, *Klebsiella pneumoniae* and *Salmonella*, in combination with

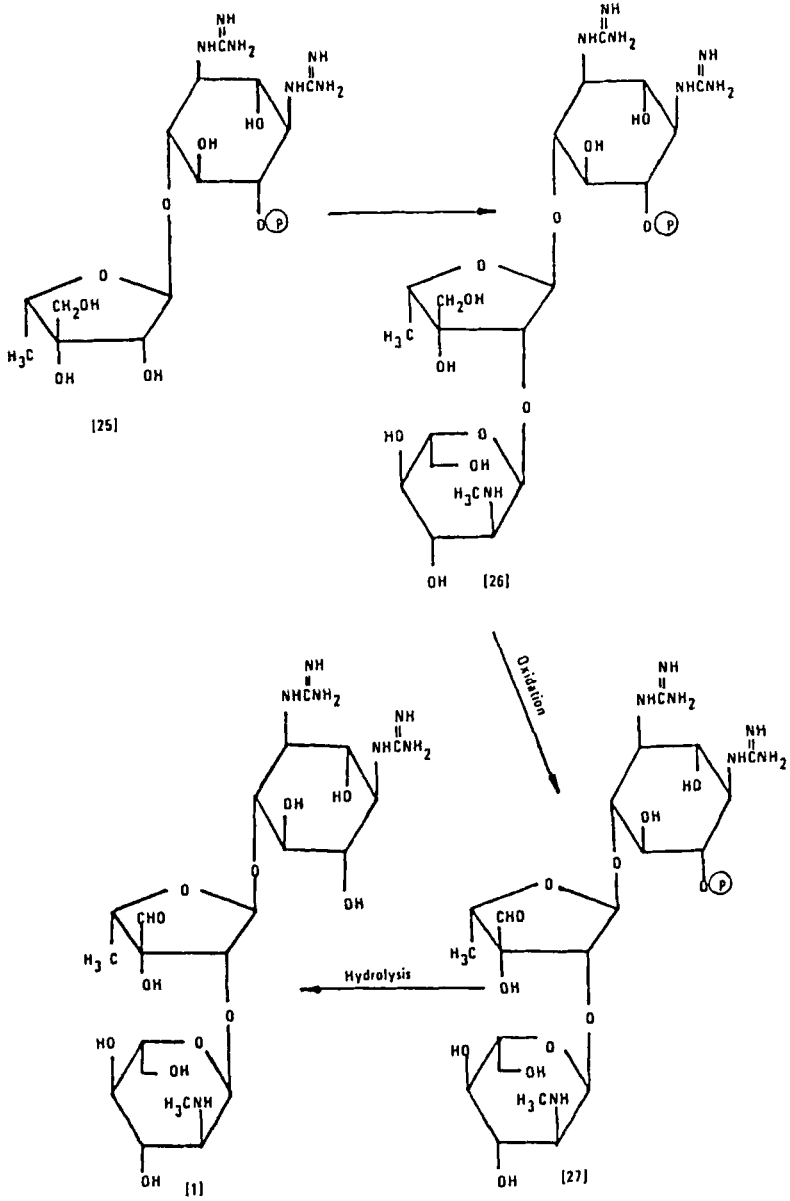
SCHEME IV



[24]

[1]

SCHEME V



other antibiotics it is useful in the treatment of respiratory tract infections, bacterial meningitis and urinary tract infections (256,265-273). Streptomycin is also effective for typhoid fever (274-277), whooping cough (278), diphtheria (279) and in murine leprosy (280-283).

8. PHARMACOKINETICS

8.1 Absorption

Streptomycin is absorbed poorly and irregularly from the gastrointestinal tract (255,284). Therefore, the oral route is not suitable for administration of streptomycin for systemic action. However, the oral route has been recommended in the treatment of intestinal disorders due to streptomycin sensitive strains of bacteria or to reduce the bacterial flora of the intestine prior to operation on intestinal tract. Streptomycin is rapidly absorbed when administered by subcutaneous, intramuscular or intravenous routes (255,256,285). Streptomycin can also be absorbed through rectum when administered as cocoa butter or carbowax mixture (286,287). Parentral administration must be employed to ensure therapeutically adequate plasma and tissue levels of streptomycin. After an intramuscular dose of 1 g of streptomycin, peak serum concentrations of 25 to 50 mg/ml are attained in 0.5-2 hours, falling to 5 to 10 mg/ml at 12 hours (9, 15). The effective minimum serum concentration is about 7 mg/ml (15).

8.2 Distribution

No metabolite of streptomycin has so far been detected. About one third the circulating streptomycin is bound to serum protein. Streptomycin is widely distributed to all organs except brain.

The drug penetrates rapidly into the peritoneal, pericardial, pleural and synovial fluids. Very little enters into cerebrospinal fluid except in meningitis (256,285,288,289).

8.3 Excretion

About 50-80% of streptomycin injected is excreted in urine unchanged in 24 hours (15,285,290,291). Most of the oral dose is eliminated in the faeces (15,256). About 0.5% is excreted in the breast milk in 24 hours and 1% is excreted in bile (15,286).

8.4 Half-life

Serum half-life of streptomycin is about 2.5 hours in young adults. This will be increased in the new born and in adults above 40 years. In renal function impairment, the half-life may be increased to 2-5 days (15).

9. TOXICITY

Allergic reactions are relatively very common with the administration of streptomycin. The usual allergic symptoms are eosinophilia, fever and skin rashes. The drug also causes headache, nausea, arthralgia, blurring of vision, vertigo, renal irritation and neurotoxicity. The most serious toxic effect is damage to the eighth cranial nerve, causing deafness which is permanent. Damage to renal tubules, bone marrow and liver rarely occurs at the usual therapeutic tissue level. Application of streptomycin directly to the peritoneum or its intravenous injection in large doses may cause death by paralysis of the respiration (285,286,288, 292-303).

10. DOSAGE

About 0.5 to 1 gram by intramuscular injection daily or at long intervals. As an internal antiseptic, 500 milligrams by mouth every eight hours (304).

11. PHARMACEUTICAL FORMS

1) Streptomycin sulphate U.S.P. (305).
2) Streptomycin sulphate injection B.P. (304).
3) Streptomycin calcium chloride B.P. (8).
4) Streptomycin hydrochloride B.P. (8).

12. MECHANISM OF ACTION

The antibacterial action of streptomycin is a consequence of its combination with the 30S ribosomes of susceptible organisms, thereby distorting the translation of the genetic code. As a result of this condition the wrong amino acids are incorporated into the nascent portion of the protein molecule, the protein synthesis is disrupted and the cell dies (256, 306, 307).

13. METHODS OF ANALYSIS

13.1 Elemental Composition

C = 43.7%	N = 16.86%
H = 6.76%	O = 33.01% (7).

13.2 Identification

13.21 Pharmacopeial Tests

The following tests have been described in British Pharmacopoeia for the identification of streptomycin (304).

a) Dissolve about 0.2 g of streptomycin in a mixture of 2 ml of methyl alcohol and 0.1 ml of sulphuric acid, filter if necessary and allow to stand at about 25°; crystals of streptidine sulphate separate in the course of 2 or 3 days. Dissolve the crystals in 10 ml of hot water containing 0.1 g of trinitrophenol and cool. The melting point of the precipitate after recrystallisation from hot water is about 283°C.

b) Boil a small quantity of streptomycin with 1N sodium hydroxide for a few minutes and add a slight excess of hydrochloric acid and a few drops of ferric chloride test solution; a brilliant violet color is produced.

13.22 Color Tests

The following color tests have been described for the identification of streptomycin :

a) Glucosamine Test

i) A 10 mg of streptomycin is dissolved in 1 ml of water, 2 ml of 1N hydrochloric acid is added and the solution is heated on a steam bath for 10 minutes. Then 2 ml of 2N sodium hydroxide and 1 ml of 20% aqueous acetylacetone are added. The solution is again heated for 5 minutes and cooled. About 1 ml of ethanol and 25 ml of concentrated hydrochloric acid

are then added. A cherry red color is produced (308).

ii) To about 2 ml of an aqueous solution of streptomycin, 1 ml of a 2% solution of acetylacetone in water and 1 ml of 1N sodium hydroxide are added. The mixture is heated for 10 minutes in a boiling water bath and then cooled. A pink color is developed on addition of 2 ml of a solution of Ehrlich's aldehyde (309).

b) Guanido Test

i) A 10 mg of streptomycin is dissolved in 1 ml of water and 1 ml of oxidized nitroprusside reagent is added (the reagent is prepared by mixing equal volumes of 10% sodium nitroprusside, 10% potassium ferricyanide and 10% sodium hydroxide, in that order; after the color becomes bright green-yellow, 1 ml is diluted to 100 ml with water), a red color develops (310).

ii) Aqueous solution of streptomycin when treated with diacetyl in the presence of oxygen gives a pink color which is intensified by the addition of 1-naphthol (311).

iii) When streptomycin is treated with 1-naphthol and sodium hypobromite, a red color is produced (312,313).

iv) When streptomycin is treated with sodium hypobromite in strong alkaline solution, a volatile bromo amine is produced which turns acidified starch iodide solutions to dark blue color (314).

c) Other color Tests

i) An alcoholic solution of streptomycin gives a pink color with sulphuric acid (315).

ii) An alcoholic solution of streptomycin produces a yellowish-red color with phosphoric acid (315).

iii) With Sakaguchi solution (100 mg of boric acid in 10 ml of concentrated sulphuric acid) streptomycin gives pale-green after 3-5 minutes (315).

13.23 Microbiological Tests

a) Test with Streptococcus lactis

Streptomycin when treated with milk inoculated with *Streptococcus lactis*, prevents the coagulation of the milk in the usual way (316).

b) Triphenyltetrazolium Chloride (TTC) Test

When streptomycin is treated with a culture of *Streptococcus thermophillus* on a sterile milk medium containing triphenyltetrazolium chloride, prevents the usual color change from leucoform to red (317,318).

c) Water-blue Test

A dilute solution of streptomycin prevents the formation of the blue color when treated with a culture of *Klebsiella pneumoniae* containing water blue dye (319).

13.3 Titrimetric Methods

13.31 Aqueous

Delaby and Stephan (320) have reported an iodimetric method for the determination of streptomycin. The method is as follows :

Dissolve about 0.7 to 1 g of streptomycin in 15 ml of water, add 15 ml of Nessler's reagent and 1 ml of 10% potassium iodide. Then add 20 ml of water, neutralize with 2N hydrochloric acid and add 1 ml in excess. Add 15 ml of 0.1N iodine solution. Shake and titrate the excess of iodine with 0.05N sodium thiosulphate solution.

An iodometric method involving the reaction of streptomycin with sodium hyphobromite has been also reported (321). The method is as follows :

> The sample containing streptomycin sulphate is added to 5-10 ml basic sodium hyphobromite solution and air is swept successively through the sample and 2 U-tubes each containing 5 ml of 0.1N sulphuric acid and 0.1-0.2 g potassium iodide. After sweeping air (10-15 bubbles/sec) through the system for about 15 minutes, the liberated iodine is titrated with 0.005N sodium thiosulphate. Analysis of 4-8 mg portions of 90.2% pure streptomycin sulphate showed values of 89.2, 88.7 and 88.4%.

Huttenrauch and Keiner (322) have described a titrimetric method for the determination of streptomycin sulphate. The method is as follows :

> The sample containing about 50 to 150 mg of streptomycin sulphate is dissolved in 25 ml of water; 2 ml of 1N sodium hydroxide is added and the mixture is heated at 100° for 5 minutes. After cooling, it is acidified with 1N sulphuric acid, then diluted to 100 ml with water and about 0.1 ml of 2% ferric chloride solution is added. The solution is set aside for 7 minutes and then titrated with 0.01N ceric sulphate until the characteristic red colour is discharged. This method can also be employed for the determination of streptomycin in culture media (323).

Modification of Huttenrauch and Keiner method by replacing ceric sulphate solution with potassium permanganate solution, was reported by Chandra et al (324). This modified method gives results with sharper end point.

13.32 Non-aqueous

Penau et al (325) have described a nonaquous method of determination of antibiotics including

streptomycin. The specified amount of Streptomycin sulphate is dissolved in perchloric acid and 2 ml of ethyleneglycol. Then about 0.05M benzidine solution in acetic acid is added. After 10 minutes, 2 drops of indicator (1% thymolphthalein in ethanol or 0.5% thiazole yellow in ethanol) is added and the excess of perchloric acid is titrated with acid potassium phthalate solution. 1ml of 0.1N $HClO_4$ acid is equivalent to 581.6/30 mg of streptomycin base.

Gautier and Pellerin (326) have reported a non-aqueous method of estimation of sulphate of the organic bases like streptomycin, dihydrostreptomycin, and others by direct titration of perchloric acid in glacial acetic acid. The procedure is as follows :

To a 0.1N solution of the sample in glacial acetic acid, add sufficient benzidine (as 0.05M solution in glacial acetic acid) to precipitate 95 % of the sulphate; allow the precipitate to settle and titrate the supernatant liquid with 0.1 N perchloric acid in glacial acetic acid.

13.4 Gasometric

Gasometric determination of streptomycin was reported by Yamagishi and Yokoo (327) and Viala (328). Pure streptomycin in solution or in complex preparations or in association with penicillins, are determined by measuring the volume of nitrogen evolved by streptidine fraction of the molecule under the action of sodium hypochlorite or sodium hypobromite and comparing it with that freed by reference solutions of guanidine hydrochloride or guanidine acetate.

13.5 Electrometric

13.51 Polarographic

Streptomycin is reducible at the dropping mercury electrode. A polarographic analysis has been developed (329). The concentration - diffusion current curve is linear for concentrations from 0.1 to 1.0 mg/ml. The solution of streptomycin in 3% tetramethyl ammonium

hydroxide is polarographed at 13.6°; the $E_{\frac{1}{2}}$ is 1.45 volts vs a mercury electrode. The rate of decomposition of streptomycin in alkaline solution was polarographically studied by Bricker and Vail (330).

Goodey et al (331) has described a polarographic method for the assay of streptomycin in fermenter broth and associated recovery stages. A Tinsley Mark 19 pen recording polarograph is used, with mercury capillaries of drop times between 2 and 3 seconds at 50 cm height. Lithium hydroxide is used as base electrolyte.

Doan and Riedel (332) have reported a polarographic method for the estimation of streptomycin sulphate. Solutions containing 100-400 γ /ml were used for the determination. The pH of the solution was adjusted to 12.4 by adding 2 ml of 1N sodium hydroxide. The dissolved oxygen was removed by bubbling nitrogen through the sample for 5 minutes after that the surface was flushed with nitrogen. A mercury drop rate of 4-5 seconds was maintained. For all concentrations the half wave potential ($E_{\frac{1}{2}}$) was 1.28-1.29 volts. Plotting the diffusion current against concentration resulted in a straight line.

Cunha (333) has published a similar polarographic method for determination of streptomycin sulphate. The limit of identification of streptomycin sulphate was 0.58-72.87 γ/ml.

Caplis et al (334) have reported a method for determination of antibiotics including streptomycin by alternating-current polarography.

A survey of the applications of polarographic methods in the production control of antibiotics including streptomycin was described by Weissbuch and Unterman (335,336).

13.52 Amperometric

Streptomycin forms water insoluble salt with various anionic dyes. This can be used for amperometric estimation of streptomycin (337).

The method is based on the precipitation of streptomycin with a trivalent dye which is synthesised by coupling diazotized para rosaniline with 1-naphthol-4-sulphonic acid. About 1-3 mg of streptomycin is dissolved in 5 to 10 ml of 0.02M triethylamine citrate buffer, pH 2.8 and is titrated with standard solution of the dye. The potential is maintained at -0.08 volts.

Konopik (338) has described the application of metal salt solutions to amperometric determination of organic compounds including streptomycin.

13.53 Potentiometric

Machek (339) has carried out a potentiometric titration for the determination of streptomycin in mixed preparations. The method is as follows :

> Mix the sample with 15 ml of water and 20 ml of butyl acetate and adjust to pH 2.1 by addition of 2N hydrochloric acid and shake for five minutes. Extract a 1 ml aliquot of the filtered upper layer with 10 ml of phosphate buffer solution (pH 7.2) and determine the penicillin in the extract iodimetrically or microbiologically. Mix the filtered lower layer with 20 ml of butyl acetate, adjust to pH 9.5 by addition of 2N sodium hydroxide, add Barium chloride (2ml) and shake for 1 minute; centrifuge, separate and dilute the aqueous phase to 50 ml, adjust the pH of a 20 ml aliquot to 5.8 by addition of 2N hydrochloric acid and titrate the streptomycin potentiometrically with 0.1N NaOH to pH 9.6.

A potentiometric microbiological assay for the antibiotic substances including streptomycin was proposed (340). The method for the determination of tetracycline hydrochloride with suspension of *Escherichia coli* and a potentiometric CO_2 sensor (HNC Model 10-22-00 or an Orion Model 95-02) has been extended to the determination of streptomycin. Sample solutions containing 2-4 $\mu g\ ml^{-1}$ of streptomycin

was used. The calibration graph was rectilinear with range 0.33 to 16.67 μg ml^{-1}.

13.6 Spectroscopic Methods

13.61 Colorimetric Methods

13.611 The maltol Method

When streptomycin is heated in dilute alkaline solution it forms maltol (29). Maltol reacts with ferric ions to give a purple color (304) and with the Folin-Ciocalteu phenol reagent it produces a blue color (341). This fact may be used for the assay of streptomycin.

A colorimetric assay involving the maltol formation, for the estimation of streptomycin is described in the European Pharmacopoeia (342). The procedure is as follows :

Dissolve 0.1g of streptomycin in water and dilute to 100.0 ml with the same solvent. To 5.0 ml of this solution add 5.0 ml of 0.2N sodium hydroxide and heat for exactly 10 minutes in a water bath. Cool in ice for exactly 5 minutes, add 3 ml of a 1.5 per cent w/v solution of ferric ammonium sulphate in 0.5N sulphuric acid and sufficient water to produce 25 ml and mix. Exactly 20 minutes after the addition of the ferric ammonium sulphate, measure the extinction of a 2 cm layer at about 525 nm, using as the compensation liquid a solution prepared in the same manner, omitting the substance to be examined. The extinction is not less than 90.0 per cent of that obtained by carrying out the operations simultaneously, using streptomycin sulphate CRS instead of the substance to be examined. Calculate the percentage with reference to the substance to be examined and the streptomycin sulphate CRS, both dried for 4 hours

at 60^{o} over phosphorus pentoxide at a pressure not exceeding 5 Torr.

Pharmacopoeia of United States, 1955 (343) described a similar maltol colorimetric assay for determination of streptomycin in streptoduocin for Injection, U.S.P.

Boxer et al (344) have reported a simple and rapid colorimetric assay for streptomycin in clinical preparations, urine and broth. The method is based on the formation of maltol from streptomycin, separation of the maltol from the interfering substances by extraction with chloroform and subsequent determination by either phenol reagent or acid ferric ammonium sulphate.

Eisenman and Bricker (345) have described a colorimetric method for the estimation of streptomycin in which the maltol is separated from the reaction mixture by steam distillation. The method is successfully applied for the determination of streptomycin in fermentation broths, concentrates and clinical samples. If more than 500 units of streptomycin are present the colorimetric reaction with ferric ion is employed and the color is read at 550 nm. If less thean 500 units are present, the phenol reagent is used and the color is read at 775 nm. The standard curves prepared from pure streptomycin are linear.

Bagdasarian and Michalska (346) have reported the maltol method for determination of streptomycin in urine without extracting the streptomycin prior to the assay, provided that dosage was moderate. This method is recommended for routine use, but cannot be applied to determining streptomycin in concentration below 100 γ/ml.

Doery and Mason (347) and Kartseva et al (348) separated streptomycin from other fermentation products by ion exchange chromatography on cation exchange resin before convertion into maltol. Final determination of streptomycin was done by Maltol method.

Bruns et al (349), have reported a colorimetric method for the determination of streptomycin from the broth. The antibiotics are collected from the solution at pH 8.3-8.5 on silica gel and extracted in sulphuric acid. The content is determined by maltol method.

Desai et al (350) have described a direct method for estimation of streptomycin in fermenter broth sample. The broth sample was deproteinized by the addition of 4 ml of trichloroacetic acid, filtered centrifuged and diluted. After the addition of 2 ml of 2N sodium hydroxide to 5 ml of the broth, the mixture was heated in a boiling water bath for three minutes and cooled. The absorbance was read after addition of ferric ammonium sulphate. A blank was run concurrently, consisting of the same sample without heat.

Ferrari et al (351) have adapted the maltol method for automated analysis of streptomycin in fermentation media using dialysis to separate maltol from the interfering substances. The maltol thus separated was automatically treated with stream of ferric chloride in 2.2N hydrochloric acid and the extinction of the reaction product at 530 nm was automatically recorded. Errors were claimed to be less than with conventional manual methods.

Further development of the auto-analyzer method for evaluation of streptomycin were described by Shaw and Fortune (352). They claimed that high precision could

be maintained even with considerable simplification to allow for high throughput of samples.

Iodimetric-absorptiometric method was adopted by Sauciuc and Ionescu (353) for micro-determination of streptomycin in fermentation liquid. The method is as follows :

The sample containing 25-150 µg of streptomycin is heated with 0.1N sodium hydroxide for 4 minutes. The solution is then adjusted to pH 7.5 by adding 0.055N phosphoric acid then treated with freshly diluted 0.001N iodine in potassium iodide solution. After 5 to 10 minutes 5 ml of reagent A (300 ml water, 200 ml acetic acid,6g potassium bromide and 1ml hydrochloric acid) is added followed by a strong jet of 1 ml of reagent B (100 ml water, 0.72g Sulphanilamide 0.02 g N-naphthyl-ethylenediamine dihydrochloride and 5g hydroxylammonium chloride). After 10 minutes the extinction of the solution is measured against the blank solution which is similarly treated.

Krystyna (354) has reported the maltol colorimetric method for the determination of streptomycin residues in milk. Antibiotic - fortified milk samples were cooled, defatted by centrifugation, deproteinized with zinc sulphate in the presence of barium hydroxide, concentrated and again defatted with light petroleum. Then the sample was treated with sodium hydroxide and heated on the water bath. The maltol formed was extracted into chloroform from the acidic medium and back-extracted into water at alkaline pH. The aqueous extract was mixed with Folin Ciocalteu phenol reagent, after 10 minutes the absorbance was measured at 660 nm and referred to a calibration graph.

The maltol method has been extensively used in studies of stability (355) and in the analysis of streptomycin in pharmaceutical preparations (356), feeds (357) and urine (358). Minor modifications have been suggested by Borowiecka (359), Kartseva and Bruns (360) and Wahbi et al (361).

13.612 Guanido reaction Method

Both streptomycin and dihydrostreptomycin form orange-red color with oxidized nitroprusside reagent (310,362). Based on this principle Valedinskaya (363) has described a colorimetric method for estimation of streptomycin. The procedure is as follows :

10 ml of oxidized nitroprusside reagent is added to 10 ml of aqueous sample solution containing approximately 0.1 mg of streptomycin or dihydrostreptomycin. After 3 minutes the absorbance is determined at 490 nm against a reagent blank prepared by substituting 10 ml of water for the sample aliquot. A working curve is prepared with the appropriate compound following the same procedure. The color is stable for about ½ hour.

Vieira and Pereira (364) have adapted the nitroprusside method for the determination of streptomycin in urine. The urine sample is treated with acetone and centrifuged after 30 minutes. Then the precipitate is stirred with water and filtered or centrifuged. To an aliquot of the clear solution, water is added to bring to 2.5 ml, then treated with nitroprusside reagent and the absorbance is measured.

Halliday (311) described the application of the modified Voges-Proskauer reaction to streptomycin, in which a pink color is generated by the reaction of the guanidine moiety with diacetyl and

1-napthol. Quantitative analytical procedure was developed by Szafir and Bennett (365), Ashton et al (366) and Banerjee (367). The method is as follows :

Transfer 2 ml of the sample solution to a test tube, add 15 ml of water and then 1 ml of 0.4 per cent diacetyl solution, 1 ml of 20% potassium hydroxide solution and 1 ml of 10% solution of 1-naphthol in anhydrous ethanol, in that order. Mix the contents of the tube by inversion after each addition. Forty minutes later determine the extinction at 525 nm in a 1 cm cell against water. Prepare a standard graph from suitable dilutions of a standard streptomycin solution, using the same procedure for color development as described for the sample. Determine the potency of the diluted sample solution from the standard graph. A standard graph should be prepared for each determination to allow for differences in room temperature, reagents, and so on.

Schulz and Posada (368) have developed a colorimetric method involving guanidino group reaction for determination of streptomycin. The aqueous sample solution is treated with an ethanolic reagent containing diacetyl and naphthalene-2,7-diol; ethanolic sodium hydroxide is added and the mixture is set aside at room temperature for 45 minutes. The extinction is then measured at 555 nm and the streptomycin content is calculated with reference to a standard solution containing 50 µg of streptomycin sulphate per ml.

The Sakaguchi reaction (312,313) has been adapted by Buck et al (369) to the analysis of streptomycin and dihydrostreptomycin, using spectrophotometric determination of the red color formed by the reaction of 1-naphthol and sodium hypobromite with the antibiotics.

Natarajan and Tayal (370) have reported a rapid method of colorimetric estimation of streptomycin, based on the Sakaguchi reaction. The procedure is as follows :

Mix an aqueous solution of the sample with 1 ml of 10% sodium hydroxide, and 2 ml of 0.02% ethanolic 1-naphthol. After 15 minutes, add 1 ml of sodium hypobromite reagent and 10 ml of carbontetrachloride. Shake well, and add 5 ml of absolute ethanol, shake again and measure the extinction of the aqueous phase at 530 nm in a 1 cm cuvette with a reagent blank. The results were comparable with those from microbiological method.

The application of the Sakaguchi reaction for the colorimetric determination of streptomycin was also reported by Fontes (371).

Modification of the above method by using 8-hydroxyquinoline was described by Tibaldi (372).

Szilagyi and Szabo (373) have reported a similar colorimetric method for the analysis of Sakaguchi-positive antibiotics, using N-bromosuccinimide. The method is as follows :

5 ml Of the solution of the sample is treated with 2 ml of 1-naphthol reagent. After being shaken and set aside for 3 minutes, the mixture is rapidly poured into 0.5 ml of N-bromosuccinimide solution, the mixture is shaken vigorously and 1 ml of 40% urea solution is added in 15 second. A vivid red colour develops, the extinction of which is read in 5 minutes against blanks, with a blue filter, results being referred to standard curves. The relative error of the method is ± 4%.

13.613 Glucosamine reaction Method

The glucosamine test for the identification of streptomycin using acetyl acetone and Ehrlich's reagent, can be used quantitatively (309). Based on this principle, Kamata et al (374) have described a colorimetric assay for streptomycin. The procedure of the method is as follows :

To 4 ml of streptomycin solution, add 1 ml of 2.5N sulphuric acid. Boil for 1 hour, cool, neutralize and dilute to 10 ml. To about 2 ml of this solution add 5.5 ml of reagent A (2.225 ml acetylacetone, 113.75 ml 2N sodium carbonate 34.1 ml 1N hydrochloric acid and water), stopper, boil for 20 minutes, cool, add 10 ml. of ethanol, finally add reagent B (0.8 of Ehrlich's aldehyde, 30 ml of concentrated hydrochloric acid and absolute ethanol). Measure the indentsity of the color at 532 nm. The method is reliable only for samples of high purity.

The acetyl acetone-Ehrlich's aldehyde method has been adapted by Masquelier (375) for the determination of streptomycin in urine. The urine sample containing streptomycin is treated with lead acetate,the excess of lead is removed by sodium sulphate and filtered. To about 1 ml of the filtrate 1 ml of acetyl acetone reagent and 2 drops of sodium hydroxide are added. The solution is heated in a water bath for 10 minutes and cooled. 2 ml of the Ehrlich's aldehyde reagent is then added and the red color developed is compared with a standard solution of streptomycin and with a blank of urine sample.

13.614 Other Methods

The aldehyde group of streptomycin was exploited by Marshall et al (358) who developed a quantitative procedure based on the formation of a colored semicarbazone with 4-[4-(p-chlorophenazo)-1-naphthyl] semicarbazide. The excess of the reagent is extracted with chloroform and hydrochloric acid is added. The color developed is measured at 580 nm.

Savitskaya and Kartseva (376) used 2,4-dinitrophenyl hydrazine which reacts with streptomycin to form a yellow hydrazone. The excess reagent is extracted with butyl acetate and the concentration of the streptomycin is determined from calibration curve prepared with pure streptomycin.

Mattick (377) has reported a colorimetric method for the determination of antibiotics

including streptomycin in milk which is based on the inhibition of the reduction of nitrate to nitrous oxide by *Micrococcus pyrogenes*. The nitrous oxide produced is determined colorimetrically after diazotisation with sulphanilic acid and coupling with 1-naphthylamine.

Barilari and Katz (378) have reported a method using copper tartrate and phosphomolybdic acid reagents for colorimetric determination of streptomycin. The freshly prepared solution of streptomycin sulphate is heated for 8 minutes with 2 ml of water and 2 ml of copper tartrate solution. After cooling the phosphomolybdic acid is added and the blue color developed is read photocolorimetrically.

A photocolorimetric method for the determination of streptomycin with reineckate salt was described by Barilari et al (379). Streptomycin is heated with water at 40^{o} and equal volume of ammonium reineckate solution is added. The precipitate thus formed is separated, washed with ethanol and dissolved in acetone water mixture. Then acetone is evaporated. The solution is then treated with sodium hydroxide, diluted with water and neutralized with nitric acid. 5 ml of this solution is treated with 5 ml of ferric nitrate and the concentration of streptomycin is determined colorimetrically. The application of this method in the pharmaceutical analysis was reported by Basu and Dutta (380).

Colorimetric determination of aldehyde and ketones with oxalic acid dihydrazide was adapted by Bartos and Burtin (381) for determination of streptomycin. About 1 ml of the sample is treated with 2 ml of phosphate-citrate-borate buffer and 2 ml of oxalic acid dihydrazide reagent. The mixture is allowed to stand for 4 hours. The blue or violet color resulting is read colorimetrically.

Using picric acid reagent, Agrawal and Dutta (382) have developed a rapid colorimetric assay method for streptomycin in mixture with dihydrostreptomycin. The test sample and

the blank are treated with 3 ml of 0.1% picric acid,2 ml of 5% sodium hydroxide; heated for 3 minutes, cooled diluted to 10 ml and the extinction is measured at 500 nm. Dihydrostreptomycin does not give this color reaction, but can be determined after being converted into streptomycin.

Yavors'kli (383) has reported the application of dinitrobenzene and their derivatives as reagent in colorimetric analysis of organic medicinal substances including streptomycin.

A new spectrophotometric method for the assay of streptomycin, by salt formation with bromothymol blue was reported by Nour El-Deen et al (384).

Barilari et al (385) have reported a new photocolorimetric method for the determination of streptomycin. Streptomycin solution is treated with potassium iodate solution and sodium arsenite solution. After the disappearance of the yellow color, 0.03 ml of phenol and 4 ml of sulphuric acid are added, boiled, cooled and the absorbance is read at 490 nm.

A spectrophotometric method for the estimation of streptomycin by salt formation with tropaeolin 000, was reported by Nour El-Deen et al (384) and Beltagy et al (386). The method is as follows :

To about 3 ml of sample in water add about 1 ml of cold, dilute sulphuric acid, and 3 ml of cold aqueous solution Tropaeolin 000. Mix and cool in ice for one hour, centrifuge, decant and wash the precipitate with 10 ml of cold dilute sulphuric acid. Dissolve the residue in 2 ml of sodium hydroxide, adjust to 100 ml with water. Dilute a mixture of 5 ml of the solution and 1 ml of dilute sulphuric acid to 50 ml and measure the extinction at 485 nm.

A new sensitive colorimetric determination of streptomycin by thiobarbituric acid method was proposed by Erno (387). The method is

specific and sensitive. The procedure is as follows :

The solution containing streptomycin is mixed with 0.1M potassium iodate in 0.05 ml of 0.5M sulphuric acid, after 15 minutes, a 2% solution of sodium arsenite in 5 ml hydrochloric acid is added. When the brown color is disappeared, 1 ml of thiobarbiturate reagent is added. The mixture is heated for 10 minutes and then the extinction of the solution is measured at 415 nm against a blank.

The modification of thiobarbituric acid method was reported by Erno et al. (388) and Fukumoto Hisako (389).

Alykov (390) has described an absorptiometric method for the determination of amino glycoside antibiotics in biological fluids. Streptomycin and other amino glycosides form a complex with stilbazochrome P in acetate or citrate buffer medium. The blood sample is treated with 1 ml of water and 0.5 ml of trichloroacetic acid solution and centrifuged. To the supernatant liquid 1 ml of stilbazochrome P reagent in citrate buffer solution, 1 ml of 1 mM $PrCl_3$ and 6 ml of ethanol added. After 20 minutes the mixture is centrifuged and the absorbance of the supernatant liquid is measured at 560 nm.

A new and selective spectrophotometric determination of streptomycin using O-hydroxyhydroquinonephthalein [2',3',6',7'-tetrahydroxyspiro (isobenzofuran;1(3H),9'-(9H) xanthen)-3-one)] and manganese, was reported (391). The method involves formation of a ternary complex in a medium containing 40% of methanol and 1% of polyvinylpyrrolidinone. Measurements are made at 570 nm at pH 10 to 10.3.

A photometric method for the determination of streptomycin and other aminoglycoside antibiotics was proposed by Alykov (392). The method involves the measurement of the decrease in the absorbance at 575 nm, due to Acid black S caused by precipitation of a complex of C.I Acid Black 24 with the antibiotics.

Eriochrome Black T was used as a precipitant in the photometric determination of streptomycin and other antibiotics in biological fluids (393).

Ninhydrin spectrophotometric method has been adapted for the determination of aminoglycoside antibiotics in pharmaceutical preparations (394). About 10 µl portions of the solutions of streptomycin in water were applied to pre-coated Silica gel plate; the chromatogram was developed in a solution of 1g of ninhydrin in 50 ml of 95% ethanol-anhydrous acetic acid (5:1). After drying the colored bands were transferred to test tubes and shaken with 5 ml of propan-2-ol containing 5 drops of ammonium hydroxide and the tubes were centrifuged. The absorbance spectrum of supernatant solution was recorded in the range 400 to 800 nm against water.

Divakar et al (395) have reported a spectrophotometric method for the determination of streptomycin, tetracycline and chloramphenicol using brucine and sodium periodate. About 10 ml of streptomycin solution was boiled under reflux for 45 minutes with 10 ml of 2M hydrochloric acid and the excess of the acid was removed in vacuo. The residue was dissolved in 20 ml of warm water and diluted to 50 ml. About 0.5 to 3.0 ml of the solution was treated with 3 ml of 5mM-brucine, 1.5 ml of 5m.M.sodium metaperiodate and 2 ml of 2.3M.-sulphuric acid. The solution is then diluted, boiled for 15 minutes, cooled and the absorbance was measured at 430-450 nm.

13.62 Ultraviolet Methods

When streptomycin is heated with 0.1N sodium hydroxide, it produce maltol (29), which exhibits an absorption maximum at 322 nm in alkaline solution. Therefore the streptomycin can be assayed by measuring the absorbance at 322 nm before and after hydrolysis in sodium hydroxide solution (396).

Katz (397) has reported an ultraviolet spectroscopic method for the determination of

streptomycin in feeds. The streptomycin in feeds was extracted with dilute sulphuric acid, separated, neutralized, further separated, concentrated and purified by ion exchange. The streptose moiety was converted into maltol and quantitatively determined by measuring its ultraviolet absorbance.

13.63 Fluorimetric Methods

Boxer and Jelinek (398) have reported a fluorimetric method for the determination of streptomycin in blood and spinal fluid. The basis of this method is the formation of hydrazone of streptomycin with fluorescent 9-hydrazinoacridine hydrochloride. The excess of the reagent together with hydrazones of acidic, neutral and basic compounds are separated from the strongly basic streptomycin hydrazone by extraction from the solution with benzyl alcohol. The aqueous base containing streptomycin hydrazone is centrifuged and the intensity of the fluorescence of the clear solution is measured. The method has been extended to determine streptomycin in urine and tissues (399).

Streptomycin and dihydrostreptomycin in alkaline solution, produce a strong fluorescence with sodium 1,2-naphthaquinone-4-sulphonate (400). Based on this principle, Faure and Blanquet (401) have developed an analytical procedure for the determination of streptomycin. Aqueous solution of streptomycin was treated with sodium hydroxide and sodium 1,2-naphthaquinone-4-sulphonate solution. Then the solution was set aside in the dark for 1 hour at 20^{o} and then the fluorescence was measured. The detailed studies of fluorescence spectra were made in subsequent publications (402,403). Application of the method for the determination of streptomycin in blood serum or plasma was also explored (404-406).

A continuous-flow Auto-Analyzer fluorimetric procedure for the determination of lactic acid in milk was applied to the detection of antibiotics (407). The procedure was based on the

inhibition by antibiotics of the ability of starter cultures to produce lactic acid.

Alykov (408) has described a new fluorimetric method for the determination of aminoglycoside antibiotics. The method involves extraction of fluorescein complexan-Pr^{3+} aminoglycoside species from an aqueous medium of pH 5.8 to 6.2 into isoamyl alcohol-benzene (1:1) reextraction of fluorescein complexan associated with the aminoglycoside into aqueous 0.1M sodium fluoride and Fluorimetric determination of fluorescein complexan in this aqueous solution at 533 nm. The method is suitable for the determination of streptomycin and the related antibiotics in blood, milk and urine. Proteins are removed from the blood samples by precipitation with trichloroacetic acid.

13. 7 Chromatographic Methods

13.71 Column Chromatography

Column chromatographic (CC) method has been used extensively for the separation and purification of streptomycin. Several reports have been published regarding the application of charcoal (106-117), acid washed alumina (118-122) and ion exchange resins (123-164) for the separation and purification of streptomycin.

Okumura et al (409) have used columns of a magnesium alumino-silicate for purification of basic antibiotics. Resolutions such as streptomycin and kanamycin from oleandomycin were described. The method appears to have applicability in the isolation of the antibiotics from biological fluids such as culture media, blood and urine.

St. John et al (410) used column of cation exchange resin. Amberlite IRC-50 to separate streptomycin and streptomycin B from fermentation broths, antibiotic being determined colorimetrically (maltol method), with good agreement with microbiological procedure. Application of the above method for determination of

streptomycin in animal feed (397) and in formulations (411) were reported. Amberlite CG 120 ion exchange resin was utilized by Conca and Pazdera (412) for separation of streptidine in streptomycin.

Comparative study of carboxylic cation exchangers in respects of the sorption and desorption of streptomycin was reported (413).

Other reports have described in details the use of permutit (414), phenoxy-acetic acid cation exchanger and phenolic resins (415), the resolution of components of streptomycin complex (416), the separation of streptomycin from fermentation broths using continuous process (417) and evaluation of various exchangers for separating colored impurities (418). The chromatographic behaviour of streptomycin on Sephadex G-10 has been described by Storl (419).

13.72 Paper Chromatography

Horne and Pollard (420) have described a method for identification of streptomycin by using paper-strip chromatogram. The position of the antibiotic were detected by means of the Sakaguchi reagent. 3% ammonium chloride was found to be necessary for good separation.

Winsten and Eigen (421) were successful in resolving mixtures of streptomycin and closely related substances. Butanol-piperidine-toluene sulphonic acid mixture was used as a developing agent. The position of the antibiotics were revealed by bioautography on agar.

Solomons and Regna (422), Stodolla et al (423) Kowezyk (424) have applied similar method for the separation of streptomycin. Peterson and Reineke (425) described a similar method for the determination of streptomycin in salt-free preparations and applications of the method to studies of biosynthesis were published by Hunter et al (199). Effects of salts on the movement and zone shape of streptomycin in paper chromatography was discussed by Sokolski and Lummis (426).

A detailed study by Heding (427,428) produced a system capable of clearly resolving streptomycin, and its related compounds with a development time of 8 hours. The developer used was prepared by mixing equal volume of amyl alcohol containing 1% of bis-(2-ethylhexyl) phosphate and 0.5% sodium chloride solution in borate buffer. The spots were detected either by using a spray of 1-naphthol-diacetyl or by bioautography on a nutrient agar plate seeded with spores of *Bacillus subtilis*.

Other reports of resolution have been published by Makisumi (429), Kondo et al (430) and Pant and Agrawal (431). The former used butanol, acetic acid, pyridine. water (4:1:1:2) and butanol: 6N hydrochloric acid (7:3) as developing reagent and Sakaguchi's reagent and Jaffe's reagent for detection. The R_f values of streptomycin in the above solvent systems were 0.17 and 0.08 respectively.

Albu-Budai and Unterman (432) reported a quantitative procedure based on paper chromatographic resolution, elution and spectrophotometric determination after color development with 1-naphthol-diacetyl. MN 261 paper strips previously impregnated with phosphate buffer of pH 7 were used. The developing solvent were pentachlorophenol-butanol-sodium hydroxide-water.

Numerous spray reagents have been used in the detection of streptomycin based largely on colorimetry. This includes potassium permanganate (433), iodine (434), sodium periodate with picric acid (435), the aniline, lactic acid, picric acid mixture (436) and bromine-starch-potassium iodide, reagent (9).

Couling and Goodey (437) have described a new method of streptomycin chromatography and its use in the examination of the reaction between streptomycin and ammonia. Paper previously impregnated with phosphate buffer at pH 7 and sodium hydroxide-pentachlorophenol-butanol as developing solvent were used. To detect the compounds, dip the chromatogram in periodate acetone and then in benzidine - acetone to

give yellow or white spot in a blue background; or dip in a mixture of diacetyl and 1-naphthol to give red spot on a white background.

13.73 Thin-Layer Chromatography

Thin layer chromatographic technique was extensively used for the separation and identification of streptomycin and its related compounds by Kondo (430) and Borowiecka (438). The former used plates coated with activated carbon whereas the latter used plates coated either with silica gel G or Kieselguhr G. VonSchantz et al (439) studied the application of metal plates for thin layer detection of pharmaceutical compounds.

Several reports have appeared on the application of thin layer chromatography in identifying aminoglycoside antibiotics. The solvent system and the R_f values are reported in table 4.

Voigt and Bared (443) and Katayama and Ikeda (444) have reported two-dimensional TLC method for separation and identification of streptomycin and its related compounds.

Sato and Ikeda (445) were able to resolve streptomycin and its dihydro-and dihydrodeoxy derivatives by ascending TLC procedure. Similar claims were made by Katayama and Ikeda (446).

Thin layer-bioautographic method has been employed for the separation and identification of streptomycin (447,448). The spots obtained from streptomycin by TLC procedure have been eluted with phosphate buffer and examined microbiologically with *Bacillus subtilis* spores. A similar procedure has been adapted by Zuidweg et al (449) and Hamann et al (450) to resolve streptomycin and other antibiotics. The former used the plates coated with sephadex and the latter used the cellulose layer.

Application 4-Dimethylaminobenzaldehyde-antimony trichloride as a detecting agent for antibiotic in TLC was reported by Mathis and

Table 4 : TLC of Streptomycin

Adsorbent	Solvent System.	Spray Reagent	R_f value	Reference
1. Silica gel G/ Aluminium oxide.	Propanol— Ethyl acetate-water- Ammonium hydroxide (50:10:30:10).	Ninhydrin.	0.62-07	440
2. Silica gel G	Butanol-water-methanol-tolune -p-sulphonic acid (40:20:10:1)	Sodium Nitroprusside, potassium permanganate and sodium hydroxide mixture.	0.4	441
3. Silica gel G	1) Acetic Acid : butanol (7:2) 2) Benzene-acetone acetic acid 2:2:1.	10% aqueous solution Copper sulphate with 2% ammonia. ,,	0.32 0.32	442

Schmitt (451). Paunez (452) described a TLC method utalizing the layer of ion-exchange resin.

More extensive examination of streptomycin group was reported by Heding (453) and More recently Borowiecka (454). Forty seven solvent systems were investigated in deciding on optimum conditions for resolution of streptomycin and related compounds. Visualization was made by a spray reagent of sodium nitroprusside-potassium permanganate.

13.74 High Performance Liquid Chromatography

Bagon (455) has developed an assay of antibiotics in pharmaceutical preparations using reversed-phase high-pressure liquid chromatography. Adapting this method, antibiotics like streptomycin were determined by HPLC on a column packed with Spherisorb S5-ODS. Aqueous methanol at a flow rates of upto 1 ml/min. was used as an eluent. The elutes were monitored by spectrophotometric detection.

Whall (456) has reported a HPLC method for the determination of streptomycin and dihydrostreptomycin sulphate. The method is as follows:

Samples were dissolved in water and diluted to 50 ml., 3 ml of each solution is then diluted to 50 ml with mobile phase containing 0.02 M sodium hexanesulphonate - 0.025 M sodium phosphate in aqueous acetonitrile, adjusted to pH 6. A 25 µl portion was subjected to HPLC on a column packed with µ Bondapak C_{18}. The column was fitted with a reversed-phase guard column and elution was carried out at 1 ml/min. at 25°. Solutes being detected at 195 nm.

HPLC assay of pyrazinoic acid in human plasma in the presence of antituberculosis drugs using an automatic sampler was proposed by Ratti et al (457). This method could also be used to determine streptomycin. The chromatographic conditions are : Mobile phase : 0.01M ammonium dihydrogen phosphate - acetonitrile (3:7).

Column condition : Column of 25 cm length 4.6 mm width packed with Lichrosorb-NH_2. Flow rate at 2 ml/min. The elutes are detected at 254 nm.

Inchauspe and Samain (458) have described a ion-pair reversed-phase high performance liquid chromatographic method for separating aminoglycoside antibiotics using perfluorinated carboxylic acids. The aminoglycoside antibiotics were separated at ambient temperature on a column (25 cm x 4.6 mm) packed with ultrasphere C_{18} (5 µm) with methanol - 50 mM-pentafluoropropionic acid (21:29), methanol - 50 mM-heptafluorobutyric acid (3:2), methanol - 50 mM - camphorsulphonic acid (29:21) or 0.2 M trifluoroacetic acid as mobile phase and refractive index detection.

13.75 Electrophoresis

Foster and Ashton (459) described a method for separation of streptomycin and related compounds by paper electrophoresis. The test solutions were applied to the paper which is then wetted with a buffer solution of pH 5. After passage of current for 16 hours the paper was removed and dried. The spots were revealed by one of following reagents : 1) diacetyle; 2) naphtharesorcinol spray; 3) Elson Morgans reagent. Alternatively, the potency was determined microbiologically by laid down the papers on agar plates seeded with suitable organisms.

Takahashi and Amano (460) employed the paper electrophoresis method for the identification of various antibiotics.

Philippe et al (461) have reported a similar method of electrophoresis for the separation of streptomycin and dihydrostreptomycin. Whatman 3MM paper in borate buffer at pH 9 were used. The electropherogram was run for 3 hours at 500 V and the spots were developed with Monastero's alkaline nitroprusside-ferric cyanide reagent.

Other reports of resolution of streptomycin and other antibiotics have been published by Paris

and Theallet (462) Lightbrown and de Rossi (463). The application of electrophoresis for separation and identification of the antibiotics in Pharmaceutical mixtures was reported by Apreotesei and Teodosiu (464).

Katayama and Ikeda (465) achieved a similar resolution using electrophoresis layers of silica gel. They also described a two dimensional technique using a combination of electrophoresis and chromatography on silica gel (466).

Gorge and Monteoliva (467) used two dimensional techniques on paper (chromatography in one direction and electrophoresis in the second) for identification of many guanidino compounds including streptomycin. Application of electrphoresis to the identification of several aminoglycoside antibiotics has been reported by Garber and Dobrecky (468) and Ochab (469).

Langner et al (470) have described a method of determination of antibiotics including streptomycin in mixtures at ultra-micro levels by rapid low-voltage electrophoresis. The antibiotics could be separated on microscopic slides coated with agarose gel. After electrophoresis at 50 V per cm. for 5 to 30 minutes, the antibiotics were located by Bioautography on agar seeded with *Bacillus subtilis*.

13.76 Counter-Current Distribution

Titus and Fried (471) have reported the use of counter-current distribution technique for the analysis of streptomycin preparation. The Craig technique of counter-current distribution was employed to show the presence of a related substance in crude streptomycin by distribution between butanol and water.

Craig countercurrent distribution technique has been extensively employed for the separation of streptomycin from mannosidostreptomycin by Plaut and McCormack (472) and O'Keeffe et al (473). The former obtained a better resolution by using the solvent systems of an aqueous phase containing 0.5% sodium bicarbonate and 1% sodium chloride and solvent phase

(Pentasol-amyl alcohol) containing 5% stearic acid and the latter employed a system composed of lauric acid in amyl alcohol and aqueous phosphate-borate buffer.

Meyer (474) has discussed in details the different methods of counter-current distribution techniques employed for the analysis of various compounds including streptomycin.

13.8 Bio-Assay Methods

The principle of the biological assay is based on the comparison of the inhibition of the growth of bacteria produced by known concentration of the standard preparation with that produced by measured concentration of the sample being tested.

13.81 Agar-Diffusion Method on Solid Media

Many agar-diffusion methods with the suitable inoculum of the following test organisms have been reported :

Escherichi coli (475-477), *Bacillus subtilis* (478-483), *Bacillus circulans* (484), *Bacillus licheniforms* (485), *Bacillus cereus* var. *mycoides* (486-487), *Staphylococcus aureus* (488-490), *Streptococcus lactis* (491) *Klebsiella pneumoniae* (492) *Lactobacillus bulgaricus* (493) acid resistant microbacterium V-5(494). The method has been extensively used in determination of streptomycin in biological body fluid (495-499), urine (500). food and animal feed (501-505) and pharmaceutical preparations (506).

Modifications of agar diffusion method have been reported. Paper disk method has been adapted for the determination of streptomycin by Loo et al (507), Koelzer and Giesen (508), Reilly and Sobers (509), and Kanazawa & Kuramata (510). Resazurin disc has been employed by Shahani and Badami (511).

Waksman and Reilly (512) applied Agar-streak method for assaying streptomycin and other antibiotic substances: The potency of the antibiotics preparations can be determined by

incorporating graded dilutions in standard nutrient agar media in petri plates and streaking the agar surface with selected test bacteria. After overnight incubation, the potency is read and expressed as the reciprocal of the highest dilution inhibiting the different test organisms.

McGuire et al (513) has suggested a new linear diffusion method for microbiological assay of streptomycin. Slide-cell method (514) and modified drop-plate method were also reported. A comparative study of cup, paper-disk, pulp disk and superposition assay mthods was reported by Ishida et al (516).

Dewart et al (517) and Light Brown et al (518) have described a diffusion assay for antibiotics by an automated procedure. The method is as follows :

Disposable plastic Petri dishes loaded with agar prepared for assay are carried automatically to a device that punches holes in the agar, then to a dispensing unit that dispenses measured volumes (e.g., 50 or 70 µl) of test or standard solution into the holes; after incubation, the area of zones of inhibition are measured.

Continuous automatic microbiological assay of antibiotics was reported by Shaw and Duncombe (519). The method was based on the measurement of respiratory carbon dioxide.

Improvements of Agar diffusion method for assaying streptomycin were also reported (520,521).

British Pharmacopoeia 1958 (304) and Egyptian Pharmacopoeia 1953 (13) describe a microbiological Assay method for determination of streptomycin.

Assay Procedure :

Fill in uniform thickness petri-dishes, or rectangular trays, to a depth of 3 to 4 mm. with agar culture medium which has been previously inoculated with a suitable quantity of a suspension of spores of a suitable strain of *Bacillus subtilis*.

Allow the inoculated plates to dry at room temperature and keep at 5°C., until used. Place on the surface of the inoculated medium, small sterile cylinders of uniform size approximately 10 mm high or having an internal diameter of approximately 5 mm., made of glass, porcelain or aluminium, and keep at about 150°C. Five, six, eight cylinders, or any other numbers which can be conveniently adjusted to the size of the agar plate, may be placed on each. Bore in the medium, in place of cylinders, holes, 5 to 8 mm in diameter, by means of a sterile borer.

Prepare in sterile phosphate buffer solution, pH 8.0, solutions of the standard preparation of streptomycin, of known concentrations, as for example 0.5 to 2 units per ml and solutions of the sample to be tested presumed to be of the same order of concentration. Fill into the cylinders or holes, the solutions of the standard preparation and of the sample to be tested in alternate order by means of a pipette which delivers a standard amount of solution sufficient almost to fill the holes when they are used.

Place the plates in a refrigerator for about 2 hours, and then incubate at about 30° to 37°C for about 16 hours. Measure, with the greatest possible accuracy, the diameters of the inhibition zones produced by the varied concentrations of the standard preparation of Streptomycin and of the sample to be tested, and from the results the potency of the latter is calculated.

Limit of Error: The limits of error (P=0.99) that can be obtained with this method are 79 and 121 per cent., where 10 cylinders or holes are used; 85°and 115°, where 20 cylinders or holes are used; 89.5° and 110.5° where 40 cylinders or holes are used; and 92.5° and 107.5° where 80 cylinders or holes are used for the standard preparation of streptomycin and for the sample to be tested, respectively.

13.82 Liquid Culture Method

13.821 Turbidimetric Method

Oswald and Knudsen (522) have reported a turbidimetric method for assay of streptomycin. Two series of tubes, one with graded amounts of standard streptomycin and other with the unknown are inoculated with the same amount of a standard suspension of the test organism, incubated at 37° for 4 hours. The percentage of transmission read in a photoelectric colorimeter. Potency of the sample under test can be read directly from the standard curve.

Applications of the turbidimetric method for the analysis of streptomycin in urine, serum and cerebrospinal fluid were reported by Gibb (523) and Whitlock et al(524).

13.822 Other Methods

Bonifas and Chesni (525) have developed the dilution method for the determination of streptomycin : *Klebsiella pneumoniae* was allowed to grow at pH 9-9.5 in the synthetic medium of Monod to which sugar and indicator cresol red were added. The reaction shifted towards the acid side upon splitting the sugar. Decreasing amounts of streptomycin were added to the medium. The inhibiting amount of streptomycin was determined by noting the dilution in the tube immediately preceding that in which these was the first change of color of the indicator.

Dilution-method was extended to determine streptomycin in urine and body fluids (526).

A microbiological assay of antibiotics based on inhibition of ammonia production was reported by Kobos (527). The test solution was incubated for 2 hours at 37° with a suspension of *E. coli* in 6% peptone medium., the pH of the mixture was

adjusted with sodium hydroxide and the concentration of ammonia was determined with use of an Orion 95-10 ammonia sensor.

13.9 Biochemical Methods

A radioimmuno assay (528,529) was developed for determination of streptomycin in plasma and urine. The method involves enzyme-catalysed introduction of a labelled group into the molecule and separation of the labelled product by paper chromatography before counting.

Schwenzer and Anhalt (530) have reported an automated fluorescence polarization immunoassay for streptomycin in blood serum. In the assay described fluorescein-labelled streptomycin was used as the tracer and antiserum against streptomycin was raised in rabbits. On mixing of tracer, sample and antibody, the polarization of tracer fluorescence was measured in an Abbott TDX fluorimeter.

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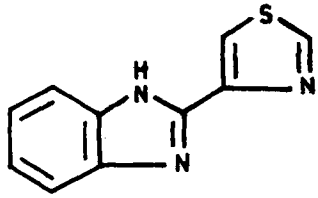
THIABENDAZOLE

Vijay K. Kapoor

1. Description

1.1 Name, Formula and Molecular Weight

Thiabendazole, an anthelmintic and antifungal agent, was developed by scientists of Merck laboratories[1] in 1961. Thiabendazole is 2-(4-thiazolyl)-1H-benzimidazole. The CAS Registry number is 148-79-8. The most common proprietory name is Mintezol.



$C_{10}H_7N_3S$ Molecular Weight: 201.25

1.2 Appearance, Color, Odor

White to cream-colored, odorless or almost odorless powder.

1.3 Salts

Thiabendazole is a weak base. The hydrochloride (sublimed at 265°) and sulfate (melted at 262-6°) have been prepared.[2] Water-soluble lactate of thiabendazole has also been prepared along with the citrate, fumarate and salicylate which had limited solubility in water.[3]

2. Physical Properties

2.1 Infrared Spectrum

The infrared spectrum[4] (KBr disc) of thiabendazole is presented in Figure 1. The principal peaks are at 1408, 906 and 740 cm^{-1}.

2.2 Ultraviolet Spectrum

The light absorption, in the range 230 to 350 nm, of a 2-cm layer of a 0.0004 per cent w/v solution in 0.1N hydrochloric acid exhibits two maxima, at 243 nm and at 302 nm; absorbance at 243 nm, about 0.47 and at 302 nm, about 0.98.[5]

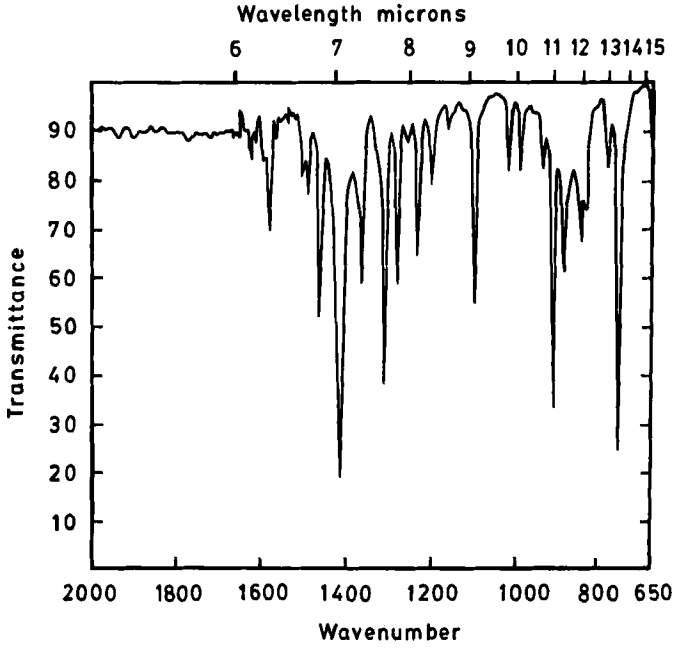


Figure 1. Infrared Spectrum of Thiabendazole

2.3 Optical Rotation

Thiabendazole exhibits no optical activity.

2.4 Melting Range

The melting range[6] of thiabendazole is between 296° and 303°.

2.5 Solubility

The solubility[7] of thiabendazole at ordinary room temperature is as follows:

Solvent	Solubility, mg/ml
Water	practically insoluble
Alcohol	6.66
Chloroform	3.33
Ether	0.5
Acetone	slightly soluble
Dilute mineral acids	soluble

2.6 Crystal Properties

Crystal structure of thiabendazole is described.[8] Crystals of thiabendazole are orthorhombic, space group P b c a, with a = 17.052(7), b = 10.998(4), and c = 10.030(8) Å. There are eight formula units per cell; observed and calculated densities are 1.414 and 1.421 g cm^{-3}. Intensity data were collected on an automatic diffractometer; the structural parameters were refined by full-matrix least-squares to an R index of 0.066 for 1805 reflections. The two ring systems are approximately planar, but are twisted by 10° with respect to each other. The C-C bond connecting the two ring systems is 1.442(10) Å long. Molecules are linked together by N(1)-H(1)----N(14) hydrogen bonds to form chains parallel to the c axis.

X-ray powder diffraction data for thiabendazole is given in Table 1.[9] The data were obtained by diffractometer and Debye-Scherrer camera techniques, and are tabulated in terms of the lattice spacings and the relative intensities of the line. Patterns using three different X-ray wavelengths with the camera method are given.

3. Synthesis

Brown et al.[1] from Merck laboratories first reported the synthesis (Scheme I) of thiabendazole by the reaction of 4-thiazolecarboxamide with O-phenylenediamine in polyphosphoric acid at 250° for three hours. In a patent[10] the synthesis of thiabendazole is reported through another route (Scheme II). O-Nitroaniline was condensed with lactic acid or the acid halide to give N-lactoyl-2-nitroacetanilide which on oxidation followed by bromination gave N-(bromopyruvoyl)-2-nitroanilide. The latter on refluxing overnight with thioformamide hydrate followed by zinc dust reduction gave thiabendazole.

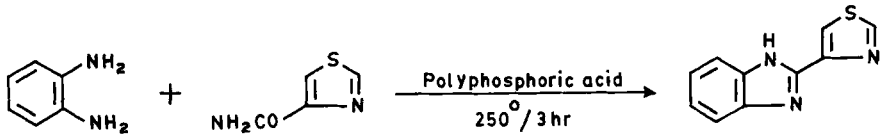
Alternate methods of synthesis have also been described.[11,12] Synthesis of ^{14}C- or ^{35}S-labeled thiabendazole is also described.[13]

Table 1

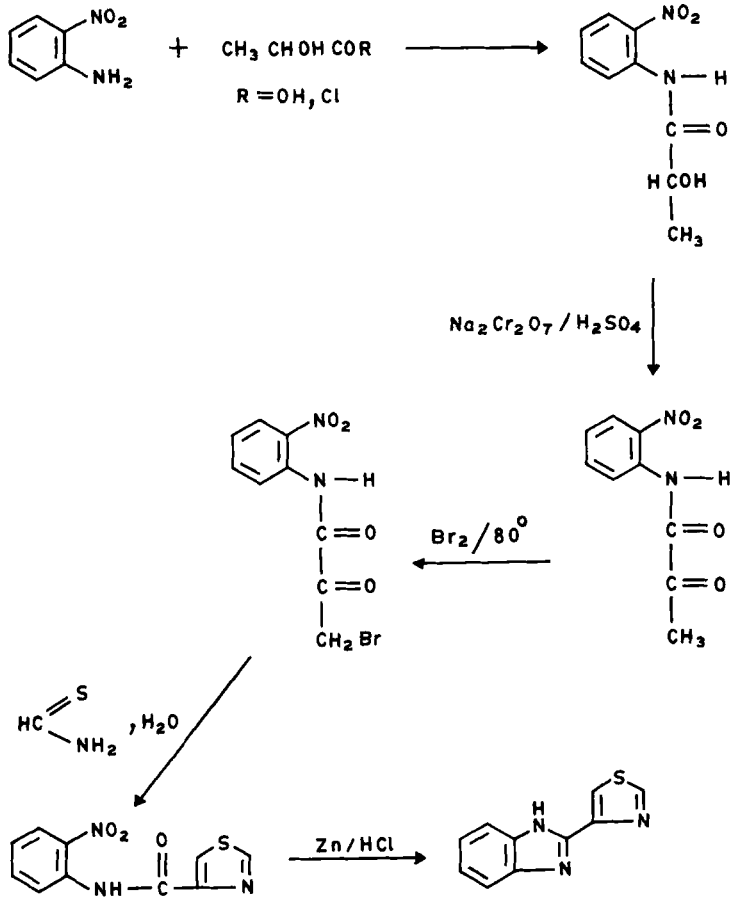
X-Ray Diffraction Data for Thiabendazole

Diffractometer		Camera					
CuKα		CuKα		CoKα		CrKα	
d(Å)	$1/1_1$	d(Å)	$1/1_1$	d(Å)	$1/1_1$	d(Å)	$1/1_1$
6.81	8	6.76	17	6.76	17	6.76	13
5.64	4	-	-	-	-	-	-
5.56	7	5.57	18	5.57	14	5.57	11
5.07*	43*	5.03*	68*	5.04*	75*	5.03*	63*
4.23*	100*	4.21*	100*	4.21*	100*	4.21*	100*
-	-	4.02	3	4.01	5	-	-
3.72	14	3.71	26	3.71	27	3.70	20
3.39*	43*	3.38*	51*	3.38*	51*	3.38*	43*
3.27	2	3.26	5	3.26	7	3.25	7
-	-	3.11	3	3.10	4	-	-
2.814	10	2.803	19	2.806	21	2.808	14
2.695	2	2.681	6	2.685	7	-	-
-	-	2.629	3	2.630	7	-	-
2.525	3	2.517	12	2.515	12	2.519	8
-	-	2.419	1	2.418	17	-	-
2.327	3	2.321	9	2.316	21	2.311	7
-	-	2.253	4	2.252	5		
-	-	2.137	5	2.132	4		
2.062	2	2.055	7	2.056	7		
2.025	1	2.022	4	2.000	5		

*Most intense lines



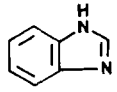
Scheme I. Synthesis of thiabendazole



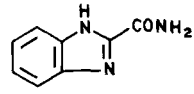
Scheme II. Alternate synthetic pathway to thiabendazole

4. Stability and Degradation

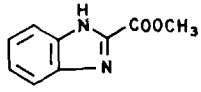
In general thiabendazole is a stable compound. The USP XXI NF XVI directs that it should be packed and stored in well-closed containers.[6] Thiabendazole, however, has been reported[14-17] to be slightly uv (sunlight) sensitive. Photolytic degradation products of thiabendazole have been identified as benzimidazole, benzimidazole-2-carboxamide, triazole-4-carboxamide, thiazol-4-ylamidine and methyl thiazol-4-carboxylate.[14,16] A recent study involving photooxidation of thiabendazole in methanol in presence and absence of a photosensitizer, methyleneblue also identified dimethyl oxalate, thiazole-4-(N-carbomethoxy)-carboxamide, methyl benzimidazole-2-carboxylate, benzimidazole-2-carboxamide and benzimidazole as the main products of photolysis.[19] Figure 2 gives the structures of the main degradative products.



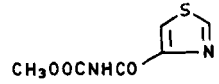
Benzimidazole



Benzimidazole-2-carboxamide



Methyl benzimidazole-2-carboxylate



Thiazole-4-(*N*-carbomethoxy)-carboxamide

Figure 2. Main products of photolysis of thiabendazole

A recent review discusses the different breakdown products due to environmental factors of various fungicides including thiabendazole.[20]

5. Metabolism and Pharmacokinetics

Thiabendazole is metabolized chiefly to 5-hydroxythiabendazole which is excreted in the urine conjugated either as glucuronide or as the sulfate (Figure 3).[13] Thiabendazole is rapidly absorbed from the gastrointestinal tract and peak plasma levels of 13 to 18 μg/ml are found within an hour of ingestion. The drug and its metabolites are excreted rapidly, 87% in the urine and 5% in

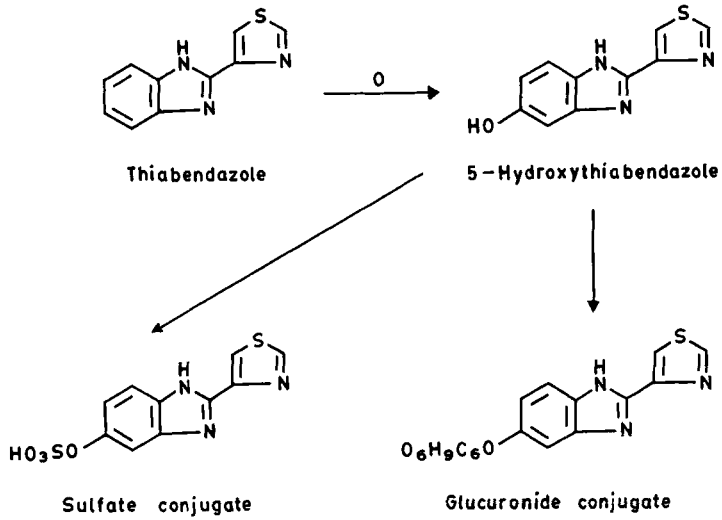


Figure 3. Metabolism of thiabendazole

the faeces within 48 hours.[21] Small amounts of both unchanged thiabendazole and free 5-hydroxy-thiabendazole may be found in both the plasma and urine. Gastrointestinal absorption and secretion of thiabendazole has been studied.[22] Wilson et al.[23] have reported that 5-hydroxylation of thiabendazole in rat liver microsomal preparation is dependent on cytochrome P-450 and has a Km of 3.92×10^{-4}M and V_{max} of 0.484 μmole of 5-hydroxythiabendazole produced/hr/mg of protein. 5-Hydroxythiabendazole gave a type II binding spectrum with cytochrome P-450. Desmethylimipramine and ethoxyquin are reported to inhibit the hepatic microsomal hydroxylation of thiabendazole.[24] A single oral dose of desmethylimipramine (80 mg/kg) administered to rats inhibited the hydroxylation of thiabendazole by 45% in vitro at 5 hr after dosage but did not decrease cytochrome P-450. A single oral dose of ethoxyquin (200 mg/kg) to rats inhibited the hydroxylation by 65% in vitro at 1 hr after dosage, inhibition was less at 5 hr. Oral ethoxyquin (400 mg/kg) or desmethylimipramine (80 mg/kg) administered 30 minutes before oral thiabendazole (100 and 200 mg/kg, respectively) delayed absorption of thiabendazole and resulted in its decreased plasma concentrations. Bauer

et al.[25] have studied the pharmacokinetics of thiabendazole and its metabolites in an anephric patient undergoing hemodialysis and hemoperfusion. The half-life, volume of distribution, and clearance of thiabendazole were 1.17 hr, 2.76 l/kg, and 27.2 ml/min/kg, respectively. While thiabendazole and the 5-hydroxy metabolite did not accumulate during multiple dosing, the glucuronide and sulfate conjugates accumulated extensively despite hemodialysis and hemoperfusion.

Pharmacokinetics of thiabendazole has been studied in cattle,[26,27] sheep,[28] and avian[29] species. The findings are consistent with the literature data that metabolism and excretion of thiabendazole are more extensive in cattle than in sheep.

6. Methods of Analysis

6.1 Elemental Analysis

The elemental analysis of thiabendazole is as follows:

Element	% Calcd.	% Found[11]
C	59.68	59.74
H	3.51	3.62
N	20.88	20.57
S	15.93	15.99

6.2 Identification Color Test

The following color reaction can be used to identify a sample of thiabendazole.[5,6] About 5 mg of the sample are dissolved in 5 ml of 0.1N hydrochloric acid and 3 mg of p-phenylenediamine dihydrochloride are added followed by shaking to dissolve the contents. About 0.1 g of zinc dust is then mixed and the mixture allowed to stand for 2 minutes. 5 ml of a solution prepared by dissolving 20 g of ferric ammonium sulphate in 75 ml of water, and 10 ml of 1N sulphuric acid are then added. When diluted with water to 100 ml a blue or blue-violet color is developed.

6.3 Titrimetric Analysis

The titration with perchloric acid is the method of choice to assay thiabendazole.[5,6]

The sample is dissolved in glacial acetic acid. After specific quantities of acetic anhydride, mercuric acetate and two drops of crystal violet solution are added, the titration with 0.1N perchloric acid to a blue-green end point is carried out. A blank determination is performed and any necessary correction is made. Each ml of 0.1N perchloric acid is equivalent to 20.13 mg of $C_{10}H_7N_3S$.

A titration method utilizing the complex formed by thiabendazole with silver ions is also described.[30]

6.4 Bioassay

Several microbiological assay methods using sensitive fungi for the quantitative determination of thiabendazole have been reported.[31-38] These methods have been employed for determining the residue of the fungicide on plant materials or in soil. A simple and rapid bioassay for the direct determination of thiabendazole residue in soil involves placing pellets composed of mixtures of soil (200-500 mg) and agar on an agar medium pre-inoculated with the test organism *Penicillium digitatum*.[37] After cold pre-incubation followed by incubation at 27°, the size of the inhibition zone is measured (Figure 4). The lowest detectable concentration of thiabendazole in a sandy soil was found to be 1.0 μg/g. In another method[35] extracts from citrus fruit and bananas were applied as spots to silica gel in Petri dishes which were then uniformly inoculated with *Penicillium cyclopium* spores. Fungicide concentration was determined from comparison of inhibition zones with those of a standard 48-hr incubation. Detection threshold was 0.5 μg thiabendazole.

Other microorganisms employed in the bioassay studies are *Penicillium exansum*,[32] *Graphium ulmi*[36] and *Sporobolomyces roseus*.[38]

6.5 Polarographic Analysis

A polarographic method for determining thiabendazole is described.[39] The electrochemical behavior of thiabendazole was investigated by sampled d.c. polarography and differential pulse polarography. Thiabendazole showed one wave peak in the polarogram when a short drop time (0.4 s) was used. Detection was most sensitive at pH 8.

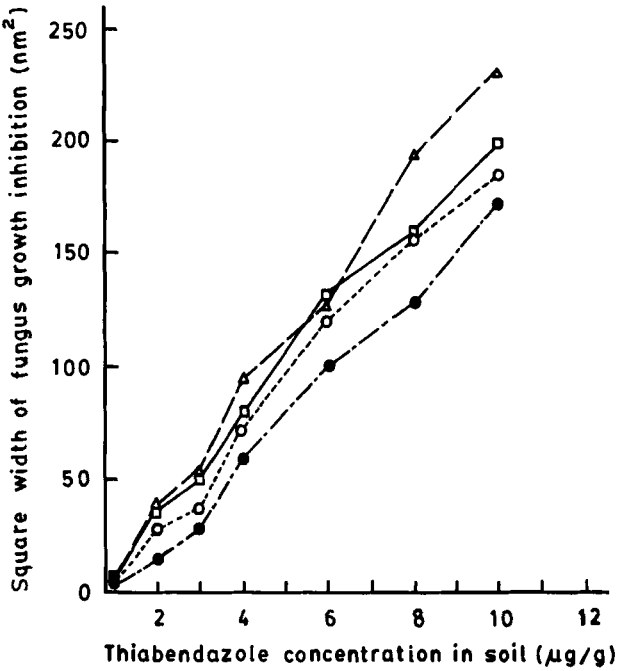


Figure 4. Effect of thiabendazole concentration in soil on growth inhibition of Penicillium digitatum using the soil-agar pellet technique. Growth inhibition is expressed as the square width of the inhibition zone. Pellet composition (ml agar/mg soil): Δ- - -Δ 0.5/500, □——□ 0.4/400, O- - -O 0.35/300, ●- - -● 0.3/200.

The current measured was proportional to the concentration. The detection limit was 0.5 ppm. Differential pulse polarography has been used for quantitative determination of thiabendazole in citrus fruit peels.

6.6 Colorimetric Analysis

Thiabendazole can be analyzed colorimetrically in feeds.[40-43] The method involves: (a) complete extraction of thiabendazole from feed with 0.2N HCl in 50% methanol; (b) separation from other substances by extraction with chloroform from alkaline solution; (c) further purification by reextraction into dilute HCl; (d) reduction with

zinc dust in the presence of p-phenylenediamine to form a complex; (e) subsequent oxidation with a ferric solution to form a thiazine dye; and (f) extraction of the dye into butanol and measurement at 605 nm against reference standard thiabendazole. The method gives most accurate and reproducible results. The method described above was automated by White.[44] In the automated system the solution was reduced with 0.3% zinc dust in glycerol, and the products were coupled with 0.17% p-phenylenediamine in 2N H_2SO_4 and 15% Fe $NH_4(SO_4)_2$ in 0.1N H_2SO_4. The blue complex was measured at 610 nm. The relative standard deviation was 1.2%, and the recovery was 95-100% from feed containing 1% thiabendazole. A modified method was used to determine the residues of thiabendazole in citrus fruits.[45]

A method for estimating thiabendazole in the milligram and submilligram ranges (0.1-2.0 mg/ml) has been described which involves measuring of absorbance of a copper-thiabendazole-tetramethylguanidine complex at 350-400 nm.[46] A modified method in which absorbance of the complex was measured at 311 nm permitted estimation of 2 µg/ml thiabendazole. An improved procedure involves shaking an alkaline aqueous suspension containing thiabendazole with solution of cupric acetate and 1-dimethylamino-2-propanol in chloroform to produce a green color in the chloroform phase.[47] The analysis is completed by spectrophotometric measurement. Thiabendazole in concentration range of 0.1-1.0 mg/ml can be estimated by this method.

A simple colorimetric method involving the reaction of thiabendazole in chloroform with alkaline buffered bromocresol green is also described.[48]

6.7 Spectrophotometric Analysis

6.71 Ultraviolet

Thiabendazole is estimated in various fruits as a residual fungicide by ultraviolet spectrophotometry.[49-60] The method in general involves extraction with ethyl acetate-ammonium hydroxide, purification by acid-base procedure, and measuring the absorbance of the acid solution at 302-3 nm. In another purification procedure the

fruit peels or pulps are extracted with chloroform, the extract is acidified and chloroform evaporated. The acidic concentrate is cleaned up with chloroform, and thiabendazole extracted at pH 9.5. Using this method thiabendazole was determined spectrophotometrically at 302-3 nm at concentrations of 0.1 ppm in 100 g peel or pulp, or 0.03 ppm in whole citrus fruit. Aspect of protein interference in thiabendazole determination is discussed.[61]

6.72 Infrared

Thiabendazole can also be analyzed by infrared spectrophotometry.[62] The method involves dissolving 0.12 g of thiabendazole sample in 5 ml of N,N-dimethylformamide and adding 1 g of anhydrous sodium sulphate. A portion of the supernatant is used for infrared spectrophotometry, scanning between 2.5 and 16.7 μm. Absorbance at 11.09 μm is used for quantitation. The statistical analysis of multiple determination by this method gave a relative error of $\ll$ 2% and a coefficient of variation $\ll$ 1%.

6.8 Spectrofluorometric Analysis

Thiabendazole is analyzed by spectrofluorometric method also.[54,63-68] The fungicide can be detected in crops by acetone extraction followed by partitioning in acetone-water and ethyl acetate and clean-up on magnesium oxide-celite-aluminium oxide column. The compound is then quantitatively measured by direct fluorometry. The excitation and emission wavelengths are 305 and 345 nm, respectively for thiabendazole. The method is reported to be sensitive to 0.02 ppm thiabendazole.[63] An automated fluorometric method for the determination of thiabendazole is also described which gives the minimum level of detection of the fungicide as $\sim$0.05 μg/ml.[64] A sensitive fluorometric method for determination of thiazole containing compound is recently described.[68]

6.9 Chromatographic Analysis

6.91 Paper Chromatography

The following system has been described.[4]

Paper:	Whatman No.1, sheet 14 x 6 in, buffered by dipping in a 5% solution of sodium dihydrogen citrate, blotting, and drying at 25° for 1 hour.
Solvent:	4.8 g of citric acid in a mixture of 130 ml of water and 870 ml of n-butanol.
Equilibration:	None
Development:	Ascending. Time of run, 5 hours.
Location:	a. Examination under ultraviolet light b. Bromocresol green spray c. Potassium permanganate spray
R_f:	0.06

An alternate system employs acetone-water (1:6) in ascending mode which gives Rf value of thiabendazole as 0.49.[69] Location is done by allowing the paper to react with iodine vapor for 1 minute.

6.92 Thin-Layer Chromatography

A variety of thin-layer chromatographic systems using silica gel or silica gel GF_{254} plates for identification and evaluation of thiabendazole have been reported. These are tabulated below:

Solvent System	Reference
Benzene-acetic acid-acetone-water (10:4:1:0.4)	70
Benzene-acetic acid-acetone-water (70:20:10:2)	71,72
Ethyl acetate-ethyl methyl ketone-formic acid-water (50:30:10:10)	65,67
Ethyl acetate-benzene (50:50)	73
Diethyl ether-glacial acetic acid-methanol (100:5:2)	74
Acetone	74

Solvent System	Reference
Light petroleum (60-80°)-acetone (30:10)	74
Chloroform-acetone (80:20)	75

Using aluminium oxide F_{254} neutral plate [74] diethyl ether-methanol is the system employed.

Cole et al.[76] has given the Rf values of thiabendazole in different solvent systems. These are given below:

Solvent System	Rf Value x 100
Ethyl acetate	55
Ethyl acetate saturated with ammonia	60
Ethyl acetate-toluene-ammonia (60:40:2)	41
Ethyl acetate-toluene-ammonia (40:60:2)	31
Ethyl acetate-toluene-ammonia (20:80:2)	21
Toluene-ethanol-ammonia (80:20:2)	43
Toluene-ethyl acetate-ethanol-ammonia (60:10:30:2)	58
Toluene-ethyl acetate-ethanol-ammonia (70:15:15:2)	52
Toluene-ethyl acetate-ethanol-ammonia (60:10:10:2)	38

Detection of spot of thiabendazole on the plate has been carried out by different methods such as visualization under ultraviolet light,[74,76,77] by spraying with potassium iodobismuthate solution followed by exposure to bromine vapors[74] or by exposure to iodine vapors.[76] A biological method for detection and evaluation of thiabendazole has also been described.[73] It is done by spraying the plates with spores of any of the thirtytwo fungal species followed by incubation. Quantitative relations were established between spot surface area and the amount of fungicide. An enzyme inhibition

technique is also reported.[75]

Using thin-layer chromatography quantitative determination of thiabendazole has been carried out spectrophotometrically[70,71,72,78,79] or fluorometrically[65,67,80,81]. Otteneder and Hezel[65] have reported a method by which thiabendazole can be determined directly on the chromatogram by reflectance-absorption photometry with measurement at 302 nm or fluorometrically at 355 nm, with excitation at 313 nm. Compared with the elution and subsequent photometry, direct evaluation on the chromatogram has the advantage that it saves time and requires less substance per spot.

6.93 Gas Chromatography

Gas chromatographic methods have been used to determine residue of thiabendazole.[50,53,67,82-94] An earlier method to determine the fungicide in whole citrus fruit and in citrus and banana pulp is described by Mestres *et al.*[50] Samples were made alkaline with ammonia solution and macerated with ethyl acetate and filtered. The filtrate was cleaned up by extraction into 0.1N HCl, and making the aqueous phase alkaline and reextracting back into ethyl acetate. The concentrated extract was chromatographed on a 10% DC-200 stationary phase using FPD set in the mode for determining sulfur. The limit of detection was of the order of 0.1 μg. Hey[53] used SE-30 as stationary phase with a nitrogen-sensitive detection. Tanaka and Fujimoto[83] determined thiabendazole as its methyl derivative with flame ionization detection and a column of 10% DC-200 on Gas-Chrom Q at 240°. It permits determination in concentrations down to 0.1 ppm.

Thiabendazole was determined by Nose *et al.*[84] as N-pentafluorobenzoyl derivative by electron-capture gas chromatography following reaction of thiabendazole with pentafluorobenzoyl chloride. The minimum amount of thiabendazole detectable by this method was 0.01 ppm. Principal operating conditions were: 1.5 m x 3 mm I.D. glass column packed with 5% OV-101 on Gas Chrom G (HP) (80-100 mesh), injection port and detector temperature 270°, column temperature 230°. Nitrogen was used as a carrier gas at a flow-rate of

40 ml/min. Combined gas chromatography-mass spectrometry was operated with column temperature 230°, separator temperature 260°, ion source temperature 290° and flash heater temperature 270°. The carrier gas was helium at a flow-rate of 30 ml/min. Recently, Bardalaye and Wheeler[85] employed this technique to determine thiabendazole in yams with operating conditions as: 1.83 m x 4 mm I.D. glass column packed with 5% OV-17 on 80-100 mesh Gas-Chrom Q, argon-methane carrier gas (95 + 5) at a flow-rate of 60 ml/min. Temperatures of oven, detector and injectors used were 240, 300 and 200°, respectively. Tjan and Jansen[86] have used pentafluorobenzoyl bromide for derivatization of thiabendazole.

A combined gas chromatography - mass spectrometry confirmatory assay for thiabendazole and 5-hydroxythiabendazole at 0.1 ppm in animal tissue isolates has been developed.[95] On-column methylation converts these compounds to their N-methyl and N,O-dimethyl derivatives, respectively. Identification and quantitation are achieved by selective ion monitoring of M-1, M and M+1 ions from N-methylthiabendazole and the M and M-15 ions from N,O-dimethyl derivative of 5-hydroxy-thiabendazole.

6.94 High Performance Liquid Chromatography

In recent years high performance liquid chromatography (HPLC) has become one of the major analytical tools to determine the residual thiabendazole alone[96-100] or simultaneously with other additives[101-111] in fruits. Isshiki et al.[96] have described a simple HPLC method for determining thiabendazole in fruits. Thiabendazole is extracted with methanol and the extract is washed with n-hexane saturated with methanol. The HPLC determination is carried out using LiChrosorb RP-8 as stationary phase and methanol-2.8% ammonia water (60:40) as mobile phase; 2-methylindole is added as an internal standard. The detection is carried out fluorometrically and the limit of detection is 0.1 µg thiabendazole/g. Recoveries are 92%. Collinge and Noirfalise[99] have determined thiabendazole in curds (artificial marmalades) and orange and lemon marmalades

by using aqueous solution containing 0.8% of ammonium nitrate and 0.7% of ammonia (buffered solution)-methanol (1:1) as mobile phase for curds, and buffered solution-methanol (3:2) for marmalades. The flow-rate in column was 1.0 ml/min, the column temperature was 22-26° and the detection wavelength was 305 nm. Yamada et al.[100] have described a detailed procedure for the separation of thiabendazole in fruits in which the uv-absorbing contaminants are removed by fractional separation with an aqueous-ethyl acetate system. The HPLC conditions for thiabendazole determination reported are: column, Unisil C-18 10 μ 4.6 mm x 300 mm; eluant, methanol-0.16 M KH_2PO_4 water (50:50, v/v %); flow rate, 1.0 ml/min; pressure, 60 kg/cm^2; detector uv (298 nm); range 0.01 AUF; and sample size 5 μl. The detection limit for this method was found to be 0.08 ppm, and it was shown that 1 ppm of thiabendazole in the fruits could be estimated within ± 10%. An ion-pair HPLC has been described by Belinky.[98]

A simultaneous HPLC determination of thiabendazole, O-phenylphenol and diphenyl residues in citrus fruits without prior clean up has been described by Kitada et al.[106]

A rapid, sensitive and precise HPLC method using fluorescence detection has been developed by Watts et al.[112] for the simultaneous determination of thiabendazole and unconjugated 5-hydroxythiabendazole in human serum. Sample pretreatment consists only of protein precipitation with acetonitrile containing the internal standard, 2-methylindole. Detection limits were found to be 0.1 μg/ml serum for thiabendazole and 0.4 μg/ml serum for 5-hydroxythiabendazole. A microenzymatic method for the conversion of the glucuronide and sulfate esters of 5-hydroxythiabendazole was also developed using β-glucuronidase and sulfatase, respectively. Thus, quantitation of the separate metabolites was possible. A special adaptation of the chromatographic procedure was utilized for the determination of 5-hydroxythiabendazole metabolites in the sera of uremic patients, which can contain large amounts of interfering fluorescent substances. The method should be of particular use for monitoring thiabendazole therapy in

patients to eliminate the potentially toxic metabolites.

HPLC methods for determining thiabendazole in waste waters[113,114] and in textile products[115-117] are also described.

6.10 Miscellaneous

A radioactive indicator method for the determination of thiabendazole residues has been described.[118]

7. Limits of Tolerance

Tolerances in ppm for the residues of the fungicide thiabendazole in or on various raw agricultural commodities established under the Federal Food, Drug, and Cosmetic Act fixed by the United States Environmental Protection Agency are: 0.02 for sweet potatoes;[119] 0.1 for dried beans,[120] eggs,[121] meat;[121] and soyabeans;[122] 0.4 for banana pulp[119] and milk;[123] 1 for hubbard squash;[124] 3 for bananas[119] and rough rice;[125] 5 for strawberries;[126] 6 for sugar beets;[121] 8 for rice hulls;[127] 10 for apples,[128] carrots,[129] citrus fruits,[130] grapes,[123] pears,[128] potatoes[131], rice straw,[125] sugar beet tops[132] and wheat grains;[131] 15 for cantaloupes;[126] and 40 for mushroom.[133]

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TIMOLOL MALEATE

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1. Introduction

1.1 Therapeutic Category (1)

Timolol maleate is a beta adrenergic blocker which is non-selective between $beta_1$ and $beta_2$ adrenergic receptors. It does not have significant intrinsic sympathomimetic, direct myocardial depressant or local anesthetic (membrane-stabilizing) activity. Timolol maleate is effective in lowering intraocular pressure and is widely used in patients with open-angle glaucoma and aphakic glaucoma. Timolol maleate is also indicated both for the treatment of hypertension (alone or in combination with other anti-hypertensive agents, especially thiazide-type diuretics) and to reduce cardiovascular mortality and the risk of reinfarction in patients who have survived the acute phase of myocardial infarction and who are clinically stable. Timolol maleate, available for oral dosing as tablets and for injection and ophthalmic dosing as distinct sterile aqueous solutions, is usually well tolerated with most adverse effects being mild and transient. However, a number of adverse effects have been reported with other beta-adrenergic blocking agents and should be considered potential adverse effects of timolol maleate. A number of adverse reactions considered to be causally related to timolol maleate have been reported. For example, severe respiratory reactions and cardiac reactions, including death due to bronchospasm in patients with asthma and rarely death in association with cardiac failure, have been reported (1).

1.2 History

Timolol maleate, belonging to the thiadiazole class of compounds, was first synthesized in the Merck-Frosst Laboratories in Montreal, Canada. The first non-patent literature reference to timolol maleate appeared in 1972 (2). Timolol maleate, since its introduction in pharmaceutical formulations, has gained a wide acceptance as an anti-hypertensive and anti-glaucoma agent. A search of Chemical Abstracts (1966 - 1986) and Pharmaceutical Abstracts (1974 - 1986) produced over 210 unique bibliographic citations for works dealing with timolol maleate.

2. Description

2.1 Chemical Name, Formula, Molecular Weight

The accepted chemical name for timolol maleate (MK-950) is: (S)-1-[(1,1-dimethylethyl)-amino]-3-[[4-(4-morpholinyl)-1,2,5-thiadiazol-3-yl]oxy]-2-propanol, (Z)-butenedioate (1:1) salt. The CAS registry number is 26921-17-5.

Other names which have been used for timolol maleate include the maleate salts of (s)-(-)-3-(3-tert-butyl-amino-2-hydroxypropoxy)-4-morpholino-1,2,5-thiazide, (-)-3-morpholino-4-(3-tert-butylamino-2-hydroxypropoxy)-1,2,5-thiadiazole and (-)-1-(tert-butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol.

2.2 Definition

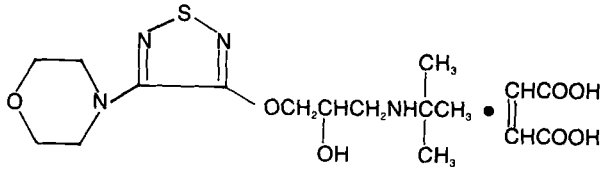
Timolol maleate possesses an asymmetric carbon atom in its chemical structure and is provided as the levoisomer. It has tradenames of BLOCADREN® and TIMOPTIC®. Timolol, when referred to, indicates the free base (CAS Registry Number = 26839-75-8).

2.3 Appearance, Color, Odor

Timolol maleate is a white, odorless, crystalline powder.

3. Synthesis

Timolol maleate has been prepared through a series of synthetic steps beginning with D-mannitol and acetone (3). The synthetic route is presented in Figure 1. The starting material, I, is reacted with acetone and the product (II) is washed with benzene and crystallized from hexane. This substituted mannitol (II) is then oxidized in tetrahydrofuran with lead tetraacetate to the substituted glyceraldehyde (III), hydrogenated with t-butylamine (IV) and treated with benzaldehyde to form a substituted phenyloxazolidine (V). In a separate reaction sequence,



$C_{13}H_{24}N_4O_3S \cdot C_4H_4O_4$ Molecular Weight 432.49 g/mole

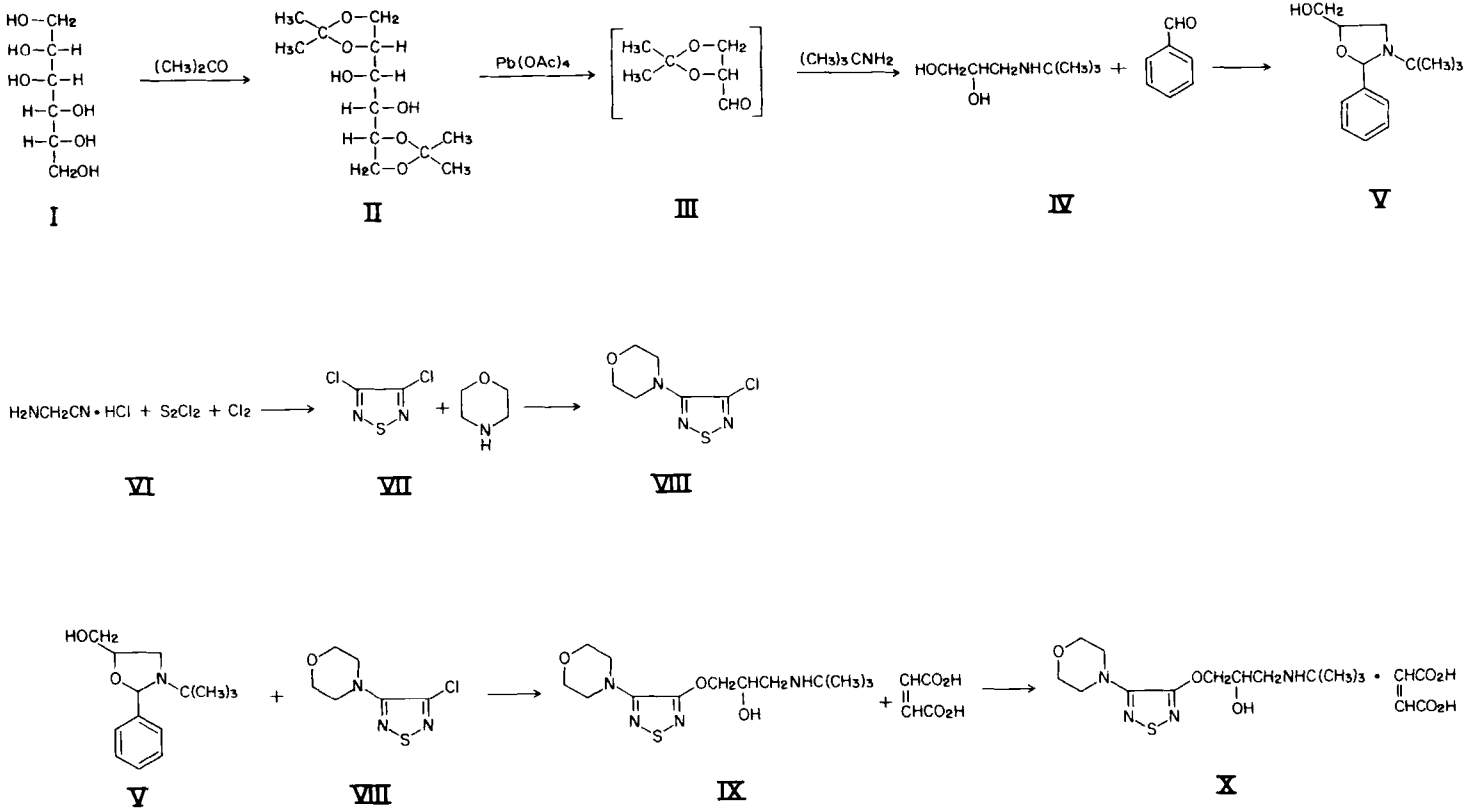


Figure 1. Synthetic route to timolol maleate.

aminoacetonitrile hydrochloride (VI) is reacted in the presence of chlorine with sulfur monochloride to form a chlorinated thiadiazole (VII), which, in turn, is reacted with morpholine to form VIII. Compounds VI and VIII are reacted to timolol free base (IX) which is then converted to timolol maleate by refluxing with maleic acid in acetone (X).

4. Physical Properties

4.1 Infrared Spectrum

The infrared spectrum of timolol maleate taken in a KBr pellet is shown in Figure 2 (4). A Digilab Model FTS-15C Fourier transform infrared spectrophotometer was used to acquire the spectrum. Frequency assignments for some of the characteristic bands are listed in Table I.

Table I

Infrared Spectral Assignments for Timolol Maleate

Frequency (cm^{-1})	Assignment	
3250	O-H	stretch
3000	N-H	stretch
1700	C=N	stretch
1200	C-O(C-OH)	stretch
1100	C-O-C	stretch

The infrared spectrum of timolol maleate taken in a mineral oil mull is shown in Figure 3 (4). Both of these spectra are consistent with the structure of timolol maleate.

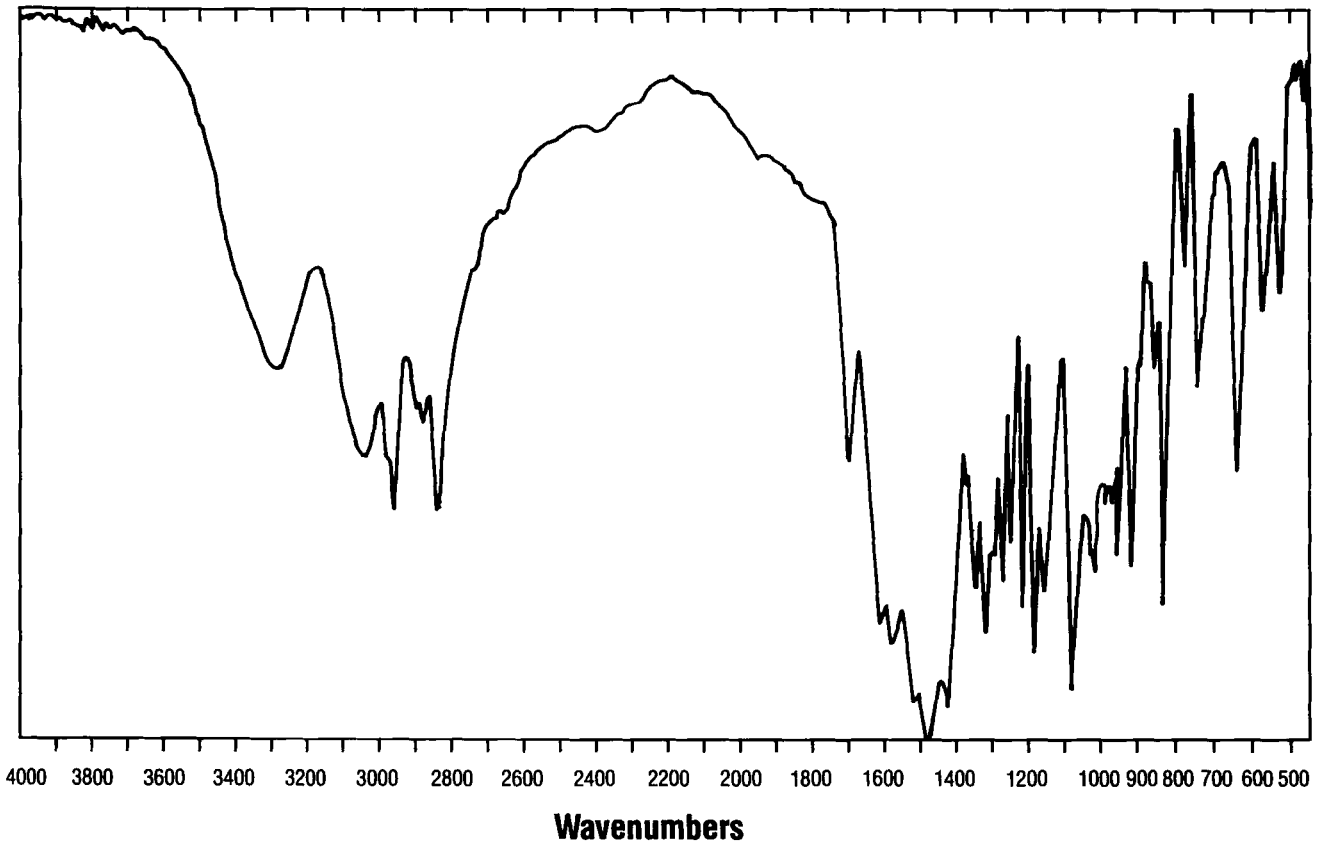


Figure 2. Infrared spectrum of timolol maleate taken in a KBr pellet.

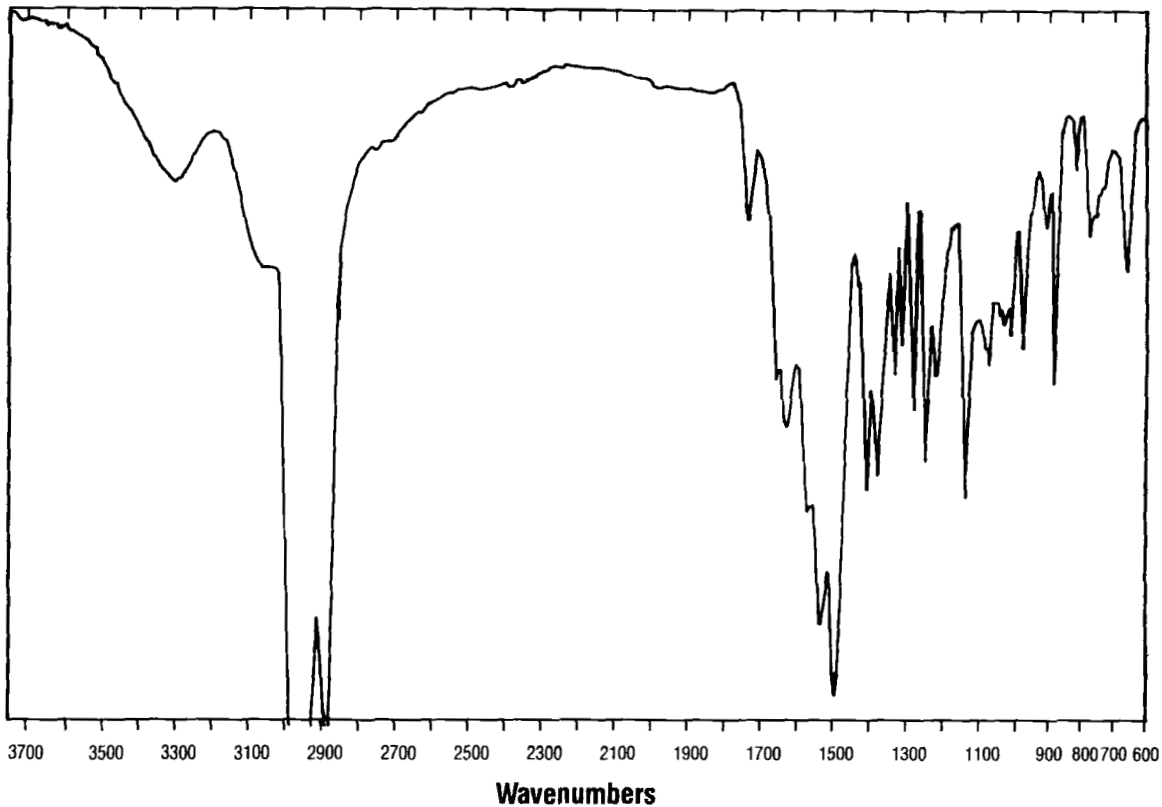


Figure 3. Infrared spectrum of timolol maleate taken in a mineral oil mull. Mineral oil contributes to the spectrum at ca. 1350-1450, 1500-1750 and 2875-3000 wavenumbers.

4.2 Nuclear Magnetic Resonance Spectrum

4.2.1 Proton NMR Spectrum

The proton magnetic resonance spectrum of timolol maleate was obtained using a Nicolet NT 360 spectrometer. The spectrum was acquired from an approximately 0.02 M solution using d_6 - DMSO as the solvent. The spectrum is shown in Figure 4 and the spectral assignments are listed in Table II (4,5).

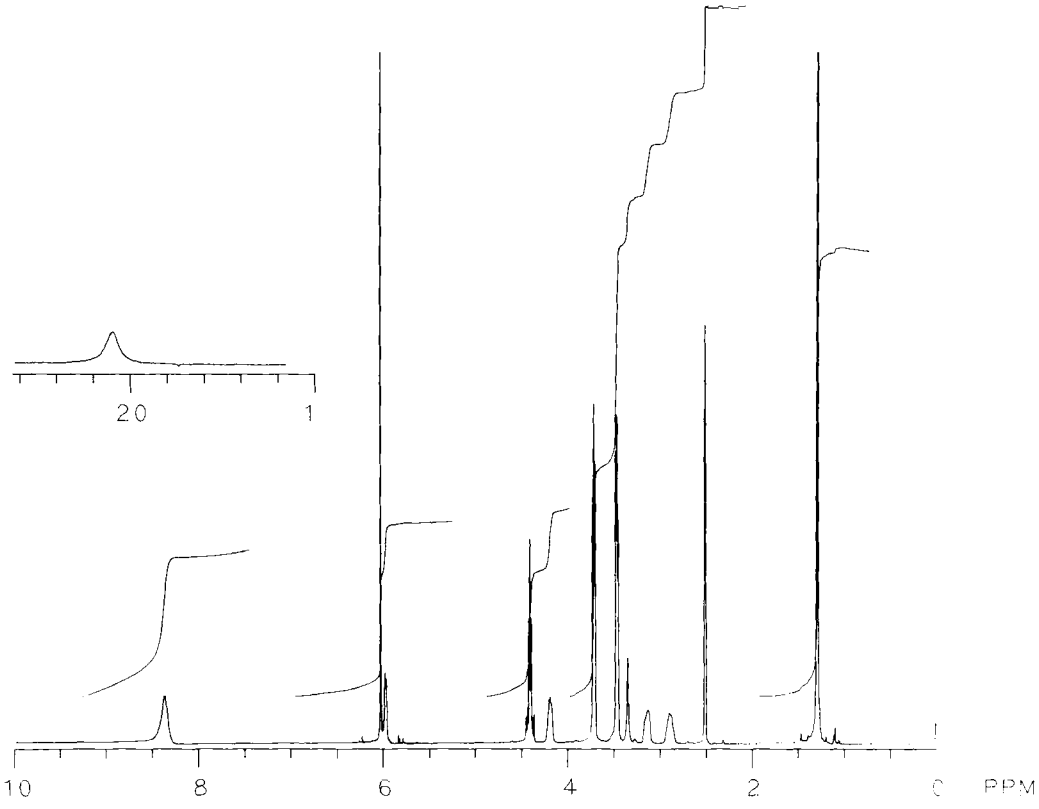
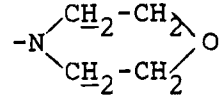
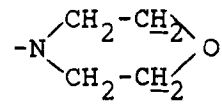


Figure 4. Proton NMR spectrum of timolol maleate.

Table II

Proton NMR Spectral Assignments for Timolol Maleate

δ (ppm)	Relative No. Protons	Assignment
1.3	9	$-C(C\underline{H}_3)_3$
2.5	-	d_6 - DMSO solvent effect
2.9 – 3.1	2	$-CH-C\underline{H}_2-N$
3.35	-	residual H_2O/HOD
3.47	4	
3.72	4	
4.2	1	$-C\underline{H}-$ $\|$ O
4.4	2	$O-C\underline{H}_2-$
5.97	1	$-O-\underline{H}$
6.03	2	$\underline{H}C=C\underline{H}$
8.38	2	$-\overset{\underline{H}}{\underset{\underline{H}}{N}}-C(CH_3)_3$
20.1	2	$-C=C(COO\underline{H})-CO_2^-$

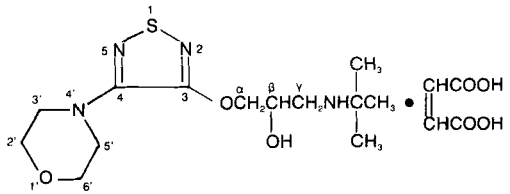
4.2.2 C^{13} NMR Spectrum

The C^{13} NMR spectrum shown in Figure 5 was obtained on a Varian CFT-20 spectrometer using a 0.5 M timolol maleate solution in d_6 - DMSO (5). The C^{13} spectral assignments are listed in Table III.

Table III

C^{13} NMR Spectral Assignments for Timolol Maleate

δ (ppm)	Assignment
167.4	CO_2H/CO_2^-
153.3	C_3
149.8	C_4
135.9	$H\underline{C}=\underline{C}H$
72.0	$-O\underline{C}H_2-$ (Cα)
65.6	$O-(\underline{C}H_2-)_2$ (a)
65.0	$-\underline{C}HOH-$ (Cβ)
56.5	-NH-C-
47.5	$-N-(CH_2-)_2$ (b)
43.7	$-NH\underline{C}H_2-$ (Cγ)
24.9	$C(\underline{C}H_3)_3$



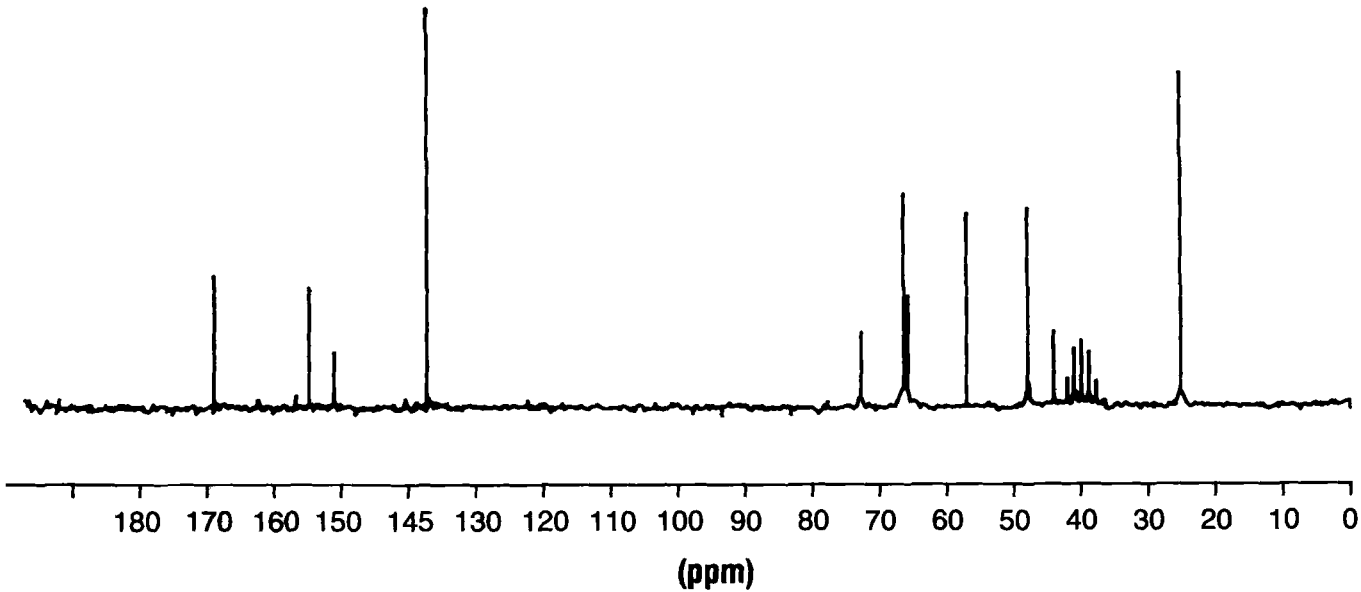


Figure 5. C^{13} NMR spectrum of timolol maleate.

a) The C_2' and C_6' carbons are equivalent; therefore, only a single line is observed for these two carbons.

b) The C_3' and C_5' carbons are equivalent; therefore, only a single line is observed for these two carbons.

Both the proton and C^{13} NMR spectra are consistent with the timolol maleate structure.

4.3 Ultraviolet Spectrum

The ultraviolet absorption spectrum of timolol maleate in 0.1 N aqueous hydrochloric acid is characterized by maxima at approximately 210 nm and 294 nm.

The absortivity in absorbance units of a 1% (w/w) drug solution in a 1 cm cell ($A^{1\%}_{1cm}$) is 200 at 294 nm. The UV spectrum shown in Figure 6 was obtained using a Hewlett-Packard Model HP8451A diode array spectrophotometer.

4.4 Mass Spectrum

A mass spectrum of timolol is shown in Figure 7. This spectrum was obtained using an LKB model 9000 mass spectrometer operated at 70 eV in the direct probe-electron impact mode. The fragmentation pattern observed is consistent with the chemical structure of timolol.

The molecular ion is noted at m/e = 316. A strong M-minus-methyl peak is observed at m/e = 301. The loss of $C_4H_{10}N$ leads to m/e = 244. Loss of C_4H_9 produces a fragment at m/e = 259 which lacks the intensity to be seen in a high resolution spectrum. The loss of $C_4H_9N(H)\text{-}CH_2$ gives rise to m/e = 230 with the peak at m/e = 86 representing this lost group. Principle fragmentations of the morpholine ring are seen

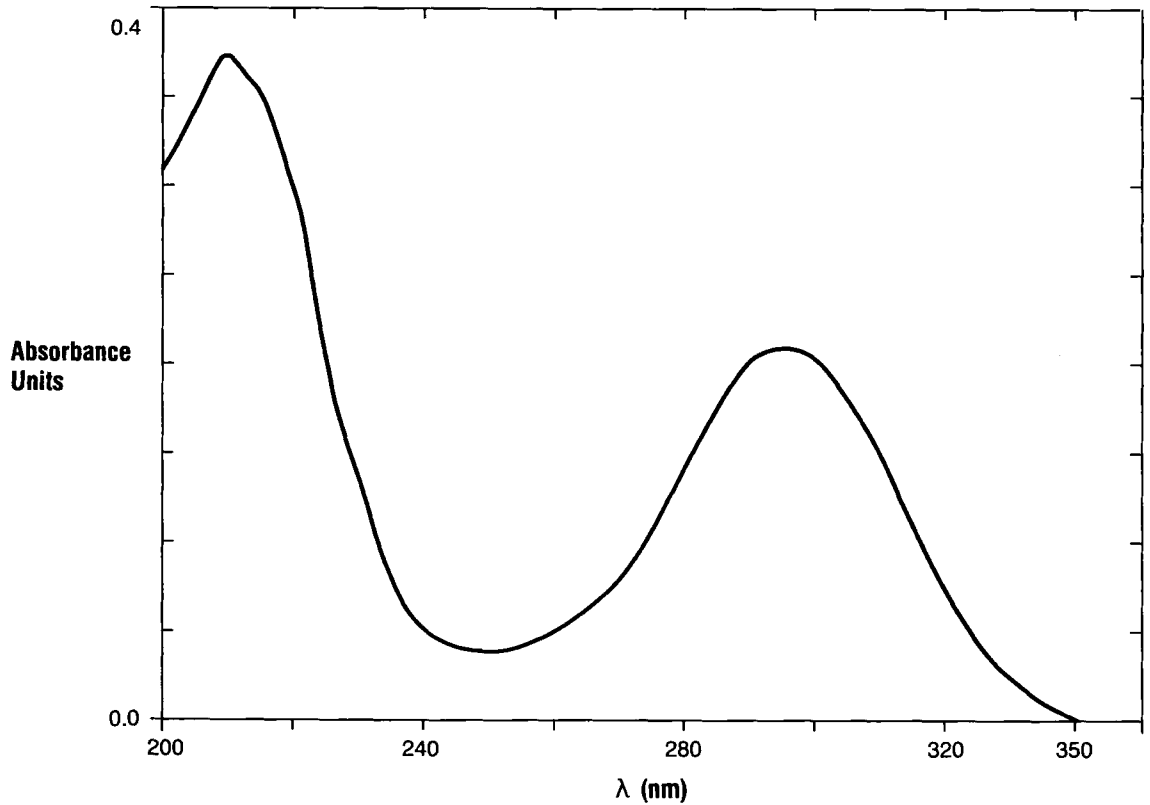


Figure 6. Ultraviolet spectrum of a solution of timolol maleate in 0.1 N HCl (aq).

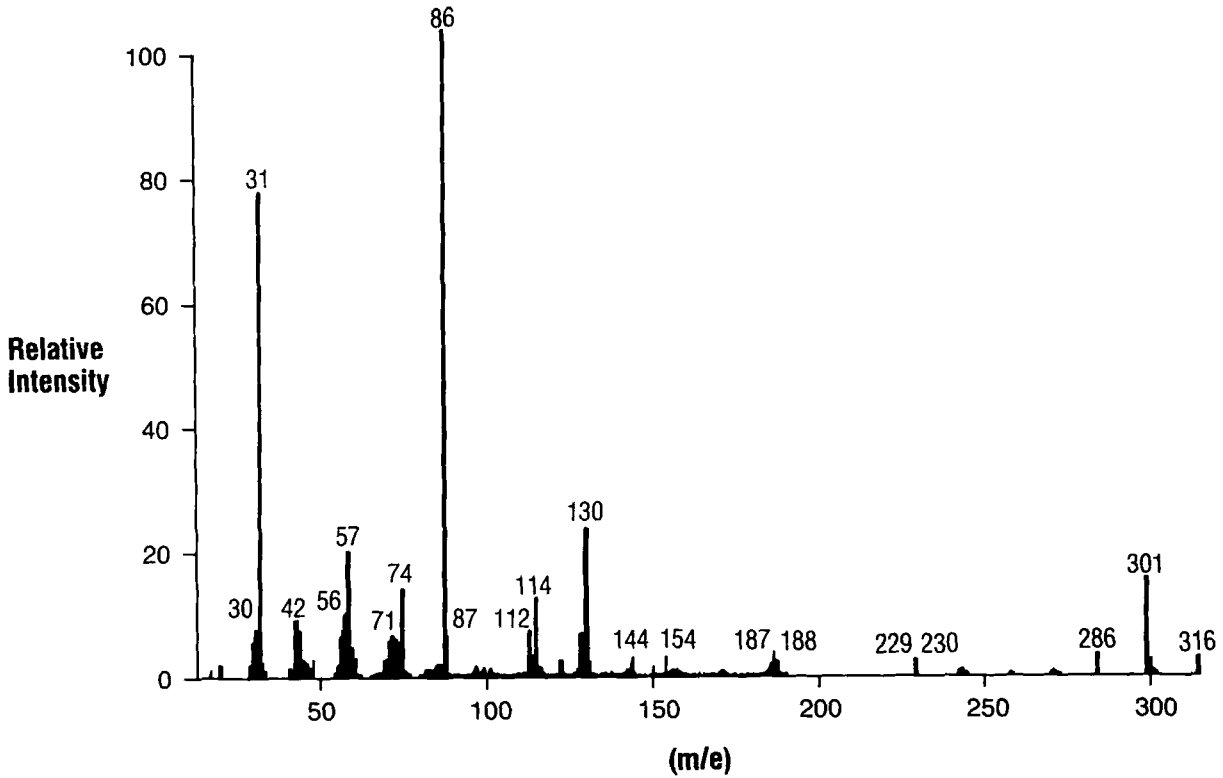


Figure 7. Low resolution mass spectrum of timolol maleate taken in the electron impact mode.

at m/e = 286 and m/e = 272. A MacClafferty rearrangement leads to m/e = 187 and two ions at m/e = 130 (see Figure 8). Fragmentation of the heterocyclic nucleus produces ions at m/e = 144 and possible M-144 at m/e = 172. The peak at m/e = 243 is due to the loss of C_4H_9O from the molecular ion. This fragment also appears as a characteristic doubly charged ion at m/e = 121.5 (due to the same decomposition). The origin of this peak is apparent from the metastable peak at m/e = 196 which corresponds to the transition from m/e = 301 to m/e = 243.

4.5 Optical Rotation

Timolol maleate, having one chiral carbon, is optically active. The levorotatory enantiomer is the biologically active form. The optical rotation of a 5% (w/v) solution of timolol maleate in 1.0 N aqueous HCl at 25 °C and 405 nm [A^{25}_{405}] is -12.2° (A^{25}_{D} = -4.2 °) (6).

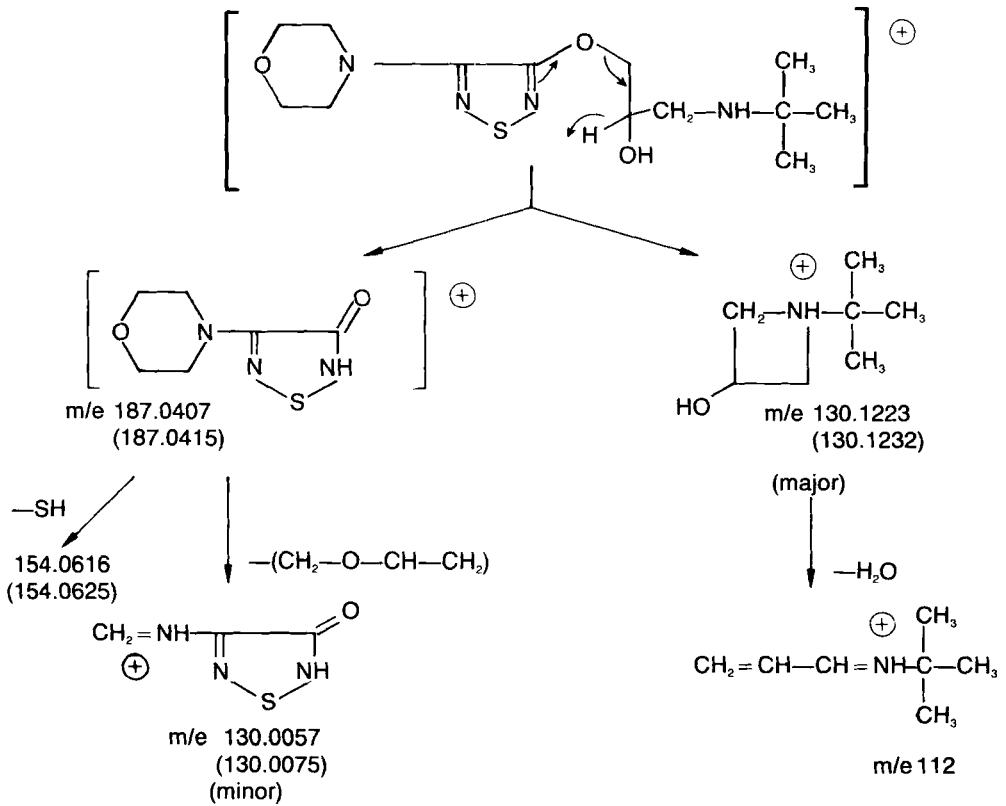


Figure 8. Schematic representation of the mass spectral fragmentation of timolol maleate.

4.6 Electroanalytical Behavior

Timolol maleate is electrochemically active and it undergoes a single irreversible electro-oxidation. The $E_{1/2}$ of an approximately 40 µg/mL solution of timolol maleate in 0.01 M aqueous KCl is +730 mV (vs Ag/AgCl). Figure 9 shows the voltammogram of this solution obtained using a Princeton Applied Research Model 174A Polarographic Analyzer with a glassy carbon working electrode, a silver/silver chloride reference electrode and a platinum auxillary electrode.

4.7 Thermoanalytical Behavior

4.7.1 Melting Point

The melting point of timolol maleate varies between 201.5 °C and 202.5 °C as determined by the USP class Ia capillary method (6).

4.7.2 Differential Thermal Analysis Behavior

The differential thermal analysis (DSC) behavior of timolol maleate under nitrogen is shown in Figure 10. This curve was obtained using a Perkin-Elmer Series 7 DSC scanning from 40 °C to 250 °C at 10 °C/minute. A primary endotherm corresponding to melting is observed with an actual peak temperature of 205.8 °C. An additional endotherm with a peak temperature of ca. 215 °C is noted corresponding to compound decomposition.

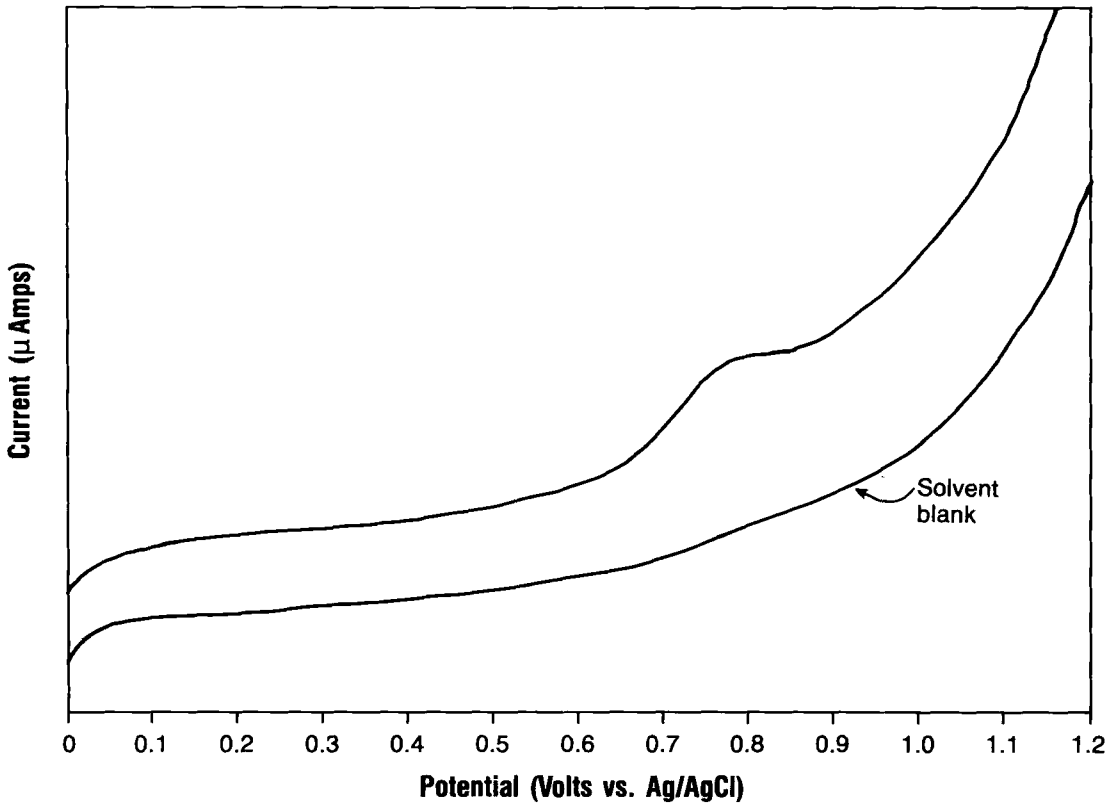


Figure 9. Scanning voltammogram of timolol maleate in 0.1 M KCl (aq).

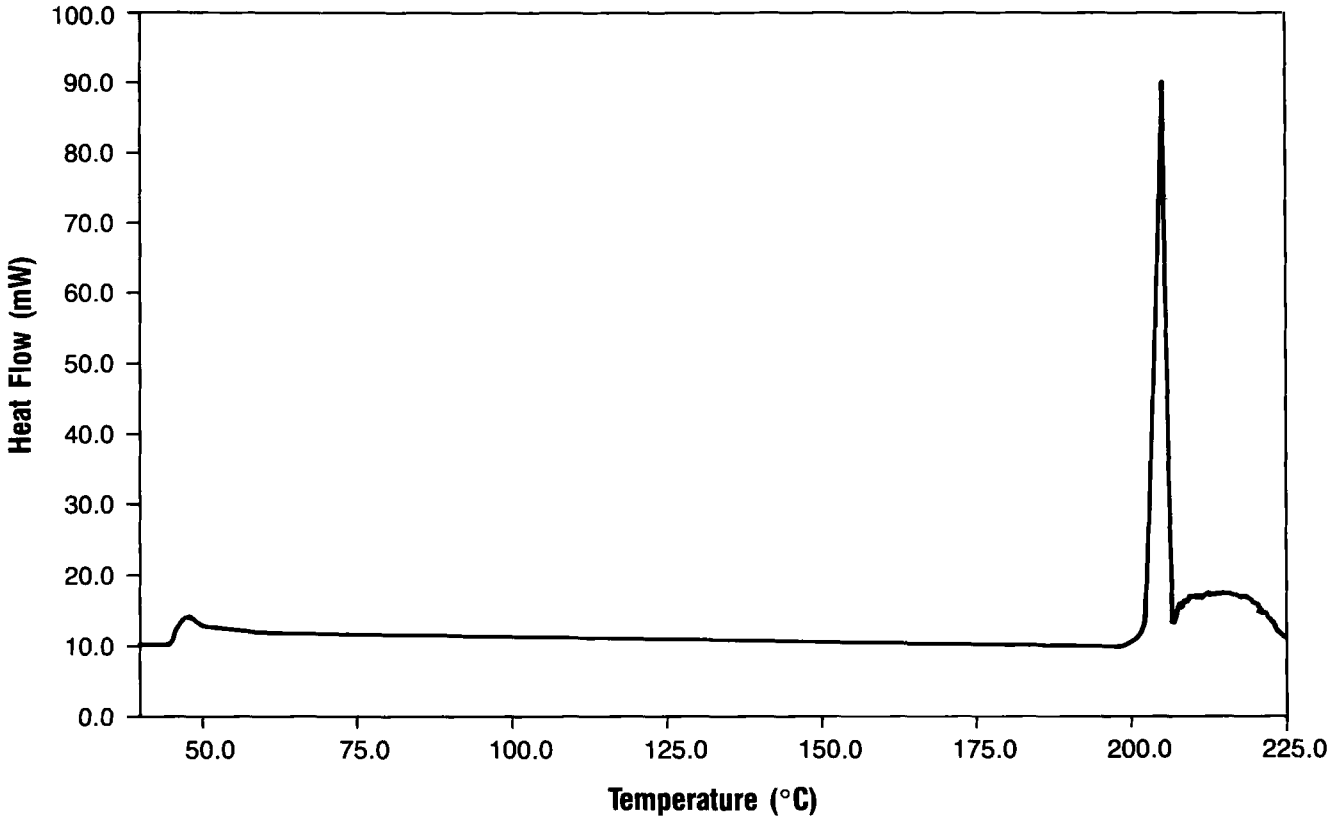


Figure 10. Differential scanning calorimetry behavior of timolol maleate.

4.7.3 Thermogravimetric Analysis Behavior

Thermogravimetric analysis of timolol maleate indicates no weight loss until 205.8 °C. From 205.8 °C to ca. 350 °C there is an approximately 80% weight loss due to decomposition/vaporization. A typical TGA curve for timolol maleate is shown in Figure 11. The curve was obtained using a Perkin-Elmer Series 7 TGA scanning from 30 °C to 400 °C at 10 °C/minute. A sample of 3.523 mg of timolol maleate was used.

4.8 Solubilities

The solubilities of timolol maleate in a variety of solvents at room temperature (≈25 °C) is presented in Table IV (6). Note that these solubilities are stated in terms of the current U.S.P. definitions (7).

Table IV

Solubility of Timolol Maleate at Room Temperature

Solvent	Solubility
Water	Soluble
Methanol	Soluble
Ethanol	Soluble
Chloroform	Sparingly Soluble
Propylene Glycol	Sparingly Soluble
Ether	Practically Insoluble
Cyclohexane	Practically Insoluble
Isooctane	Practically Insoluble

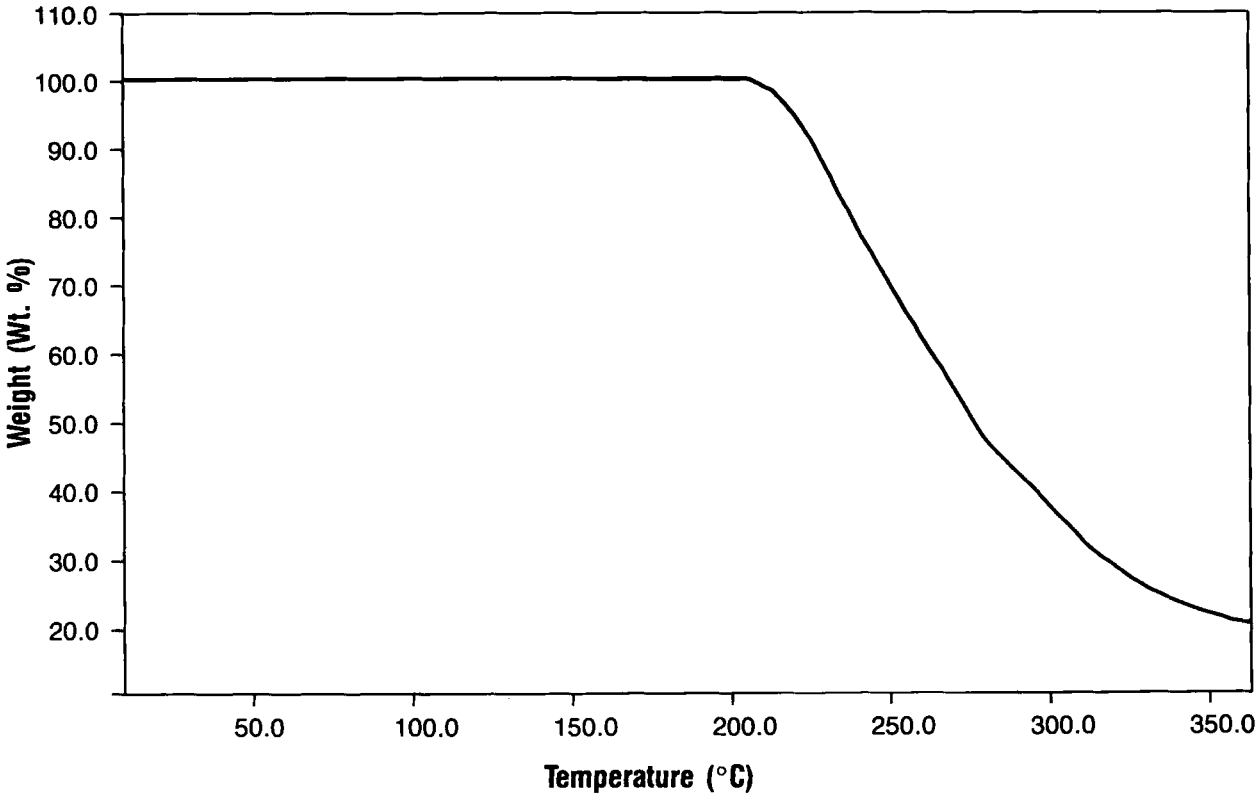


Figure 11. Thermogravimetric analysis behavior of timolol maleate.

4.9 Crystal Properties (8)

Timolol maleate exists as a crystalline powder. No polymorphs of timolol maleate have been reported. Figure 12 shows the x-ray powder diffraction pattern of timolol maleate obtained with a Phillips Electronics model APD3720 powder diffractometer using CuKα radiation. Table V lists the interplanar distances and the relative intensities of the major lines in the x-ray powder diffraction pattern of timolol maleate.

Table V
X-Ray Powder Diffraction of Timolol Maleate

Peak Angle (2θ°)	D Spacing (Å)	(I/Imax)(%)
7.1	12.49	13
9.9	8.95	13
11.4	7.76	6
14.2	6.23	100
14.8	5.96	27
15.6	5.68	12
16.3	5.44	30
18.1	4.90	43
19.0	4.67	62
19.5	4.56	24
19.8	4.47	18
20.5	4.33	52
20.6	4.31	54
20.9	4.24	67
21.4	4.14	75
22.4	3.97	35
23.0	3.86	9
23.8	3.74	50
24.8	3.58	45
26.0	3.42	7
26.5	3.36	16
27.1	3.29	60
28.3	3.16	32
29.1	3.07	13
29.5	3.03	5
31.6	2.82	26
34.0	2.63	12
38.8	2.32	19
39.3	2.29	14

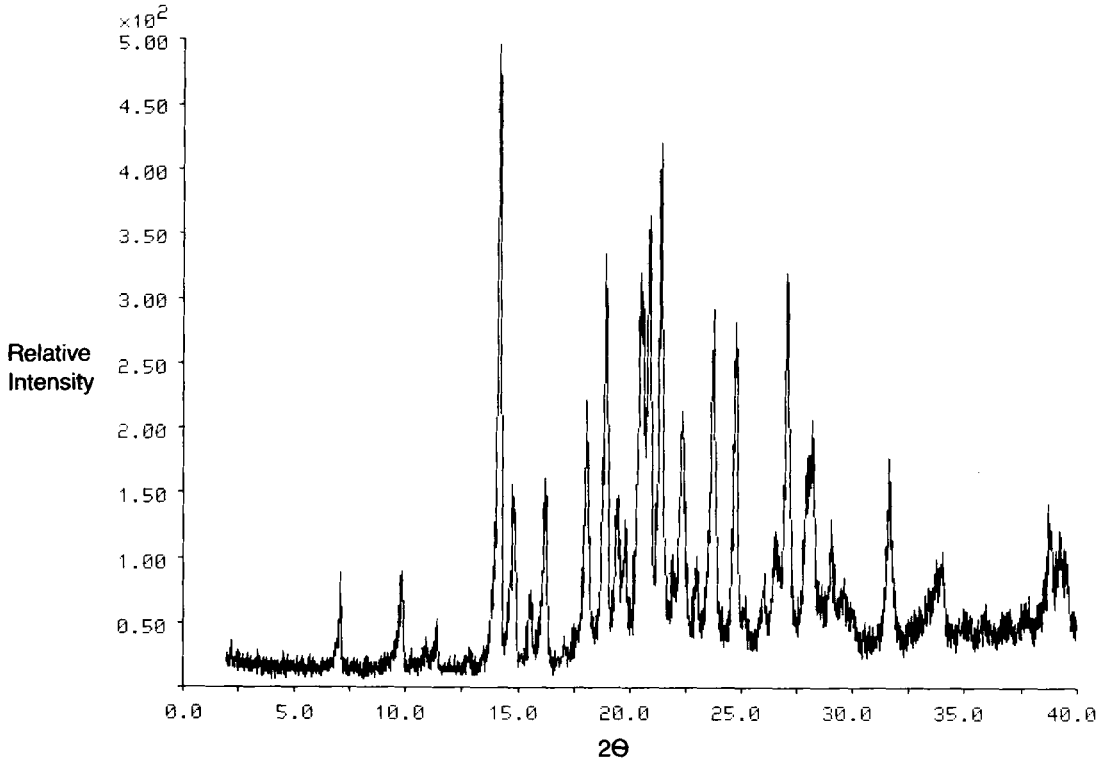


Figure 12. X-ray powder diffraction pattern of timolol maleate.

4.10 Hygroscopicity (9)

The maleate salt of timolol is a stable anhydrous compound. No hydrates of timolol maleate have been reported. The free base of timolol, however, exists both as a stable crystalline hemihydrate and anhydrous compound. Anhydrous timolol maleate is non-hygroscopic when stored under ambient temperature conditions (≈25 °C) and relative humidity conditions ranging from 33% to 76%.

4.11 Dissociation Constant (9)

The pKa of timolol base obtained by potentiometric titration in water at 25 °C is approximately 9.2. The native pH of a saturated aqueous solution of timolol maleate is ≈ 4.

5. Methods of Analysis

5.1 Identification Tests

5.1.1 Ultraviolet Spectrophotometry (10)

Ultraviolet spectrophotometry is used to identify timolol maleate. A solution in 0.1 N HCl (aq) scanned from 200 nm to 400 nm qualitatively exhibits the same absorbance characteristics at identical wavelengths as does a similarly prepared and concomitantly measured solution of a timolol maleate standard. Quantitatively, equimolar sample and standard solutions will exhibit absorbances at ≈ 294 nm (λmax) which differ by no more than 3% ($A^{1\%}_{1cm}$ ≈ 200).

5.1.2 Infrared Spectroscopy

Infrared spectroscopy may also

be used to identify timolol maleate. The infrared spectrum prepared as a mineral oil dispersion compares qualitatively (with maxima only at the same frequencies) to the spectrum of a similarly prepared timolol maleate standard.

5.1.3 Elemental Analysis

Timolol maleate may be identified through the use of elemental analysis. The results of an elemental weight percent determination of carbon, nitrogen, sulfur, hydrogen and oxygen are compared to the respective theoretical values of 49.35%, 17.71%, 10.15%, 7.65% and 15.17%.

5.2 Titrimetric Analysis (10)

Timolol maleate may be determined by perchloric acid titration to a visual or potentiometric endpoint. An accurately weighed sample (≈ 800 mg) is dissolved in ≈ 90 mL glacial acetic acid and titrated with 0.1 N perchloric acid in dioxane to a green endpoint. Alternatively, the endpoint may be determined potentiometrically as that point at which the greatest change in potential per unit volume change of titrant is noted. For potentiometric titrations, the electrode system should consist of an indicating electrode prepared by emptying a sleeve type calomel electrode, drying it and filling with 0.1 N $LiClO_4$ in acetic anhydride. A platinum ring reference electrode is to be used.

5.3 Spectrophotometric Analysis

5.3.1 Direct Ultraviolet Spectrophotometry

Timolol maleate exhibits an ultraviolet absorption band near 294 nm attributed to the thiadiazole core structure. This absorption is the basis for the quantitative determination of the drug. Assay of the compound is performed by comparing the net absorbance at 294 nm of a sample dissolved in 0.1 N HCl (aq) with the net absorbance of a standard in 0.1 N HCl (aq) of known concentration. The net absorbance is calculated by subtracting the drug free matrix contribution to absorbance at the wavelength of determination from the absorbance of the drug solution at the same wavelength.

$$C_{sam} = \{[(A_{sam} - A_b)C_{std}]/(A_{std} - A_b)\}$$

where:

C_{sam} = Concentration of the sample solution
C_{std} = Concentration of the standard solution
A_{sam} = Absorbance at 294 nm of the sample solution
A_{std} = Absorbance at 294 nm of the standard solution
A_b = Absorbance at 294 nm of the drug free solution matrix

5.3.2 Ultraviolet Spectrophotometry via Flow Injection Analysis

Ultraviolet absorbance is also used as the detection mode for timolol maleate determinations by flow injection analysis (11). In the case of the flow injection technique assay, timolol maleate sample and

standard solutions are periodically injected into a flowing stream. The resulting changes in UV absorbance at 294 nm of the stream are measured relative to the analyte-free stream. Calculation of the concentration of timolol is made in an identical fashion as direct UV spectrophotometry.

5.3.3 Specific Rotation

The specific rotation of timolol maleate is determined in 0.1 N HCl (aq). Using a solution of ≈ 50 mg/mL, the angle of rotation at 405 nm, 25 °C, in a 10 cm cell is determined with a suitable photoelectric polarimeter. Specific rotation, calculated on the dried basis, is between -11.7° and -12.5°.

5.4 Chromatographic Analysis

5.4.1 Thin Layer Chromatography (10)

Normal-phase thin layer chromatography on silica gel has been employed for timolol maleate. A developing solvent system consisting of chloroform/methanol/(28% ammonia water) [80/20/1 (v/v)] is used to develop a spot resulting from 10 µL of an approximately 5 mg/mL solution of the drug in methanol. A reference spot for impurity purposes applied as 10 µL of ≈ 20 µg/mL solution of timolol maleate in methanol is also employed. R_f for timolol maleate in this system is ≈ 0.7. Detection is made under visible light after the developed and air-dried plate has been exposed

to iodine vapor for 2 hours. Sensitivity is estimated to be within an order of magnitude of the amount of timolol maleate in the reference spot solution (≈ 200 ng drug).

5.4.2 High Performance Liquid Chromatography (12)

High performance liquid chromatography may be employed to analyze timolol maleate. A chromatographic system consisting of a column (300 mm x 4.6 mm i.d.) packing with μ-phenyl stationary phase (10 μm particle size) has been used with a mobile phase consisting of 1 part methanol and 2 parts 0.005 M aqueous hexane sulfonic acid (pH adjusted to) with acetic acid). A column temperature of 30 °C and a flow rate of 2.00 mL/min are used. Detection is by UV absorbance at 280 nm. Typically, 20 μL injections of a timolol maleate solution are made. Under these conditions, timolol is separated from its known potential process impurities.

6. Stability - Degradation

6.1 Solid State Stability (13)

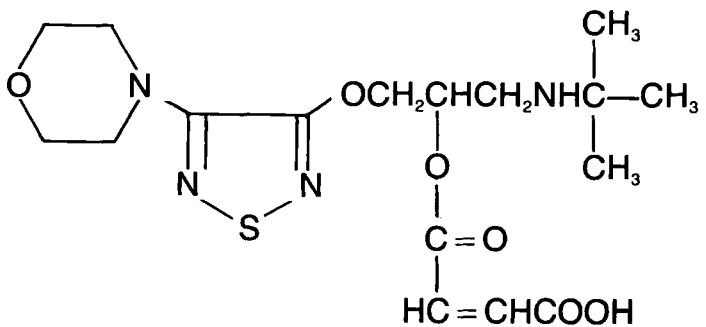
Timolol maleate is an extremely stable compound at room temperature in the solid state. In fact, prolonged exposure to extremes of temperature and humidity caused minimal degradation of the solid compound. For example, timolol maleate stored for ≈ 2 months at 105 °C in an air atmosphere (ambient relative humidity) showed no difference in potency by TLC chromatographic

profile when compared to a sample of unstressed solid. Only heating timolol maleate to above its melting point and keeping it molten for 10 minutes produced discoloration and an approximate 5% loss in potency. (TLC of this sample did detect small quantities of several unknown decomposition products.) Timolol maleate, when heated at 95 °C for ≈3 weeks in an air atmosphere (100% relative humidity), also exhibited a loss in potency of ≈5% (by UV assay). Two degradates were isolated from this sample and were identified as the cis/trans isomer pair of timolol O-fumarate ester and timolol O-maleate ester (Figure 13).

Timolol maleate exhibited surface discoloration when exposed in an open dish to intense UV light (≈ 20 times normal sunlight). This sample did not, however, show any loss of potency by UV assay nor were any decomposition products detected by TLC.

6.2 Solution Stability (13)

Timolol maleate is extremely stable in aqueous solutions (protected from light) with no decomposition being detected in injectable formulations stored at room temperature for ≈ 5 years. Timolol maleate can be degraded in solution if exposed to elevated temperatures or intense ultraviolet radiation. The solution decomposition of the drug is pH dependent with maximum stability occurring near pH = 4. Solutions of timolol maleate prepared in each of 0.1 N HCl (aq) (pH = 1) and phosphate buffer (pH = 7.2) and then stored in sealed glass vials for ≈ 2 months at 105 °C yielded considerable amounts of degradation products. A solution prepared in distilled water and stored identically



cis and trans

Figure 13. Potential solid state degradates of timolol maleate exposed to 105 °C/100% relative humidity.

was found to be virtually the same as unstressed control solution. Studies of timolol aqueous solutions (pH ≈5) under autoclave conditions indicated that timolol maleate degrades at the rate of approximately 1%/hour at 120 °C. Three decomposition products were isolated from solutions which were autoclave degraded. Figure 14 depicts the proposed degradation mechanism and indicates the three modes of thermally induced solution degradation; (1) rearrangement to isotimolol; (2) ether cleavage to form 4-hydroxy-3-morpholino-1,2,5-thiadiazole; and (3) oxidation followed by ether cleavage to form 4-hydroxy-3-morpholino-1,2,5-thiadiazole 1-oxide.

Aqueous solutions of timolol maleate (pH ≈ 5) have also been shown to be susceptible to degradation when exposed to intense ultraviolet radiation. Exposure for 2 hours to UV light equivalent to ≈ 20 times normal sunlight produced approximately 2% loss in potency. Evidence of the three thermal degradation products mentioned above as well as several unidentified degradation products was found in this solution. Because of the potential for light-induced degradation, it is recommended that aqueous timolol maleate solutions be stored protected from light.

7. Biopharmaceutics and Metabolism

7.1 Absorption and Bioavailability

Timolol maleate is rapidly and completely absorbed after oral administration (14). Maximum blood plasma concentrations ranging from 10 ng/mL to 100 ng/mL are attained within 1 to 2.4 hours after either acute or

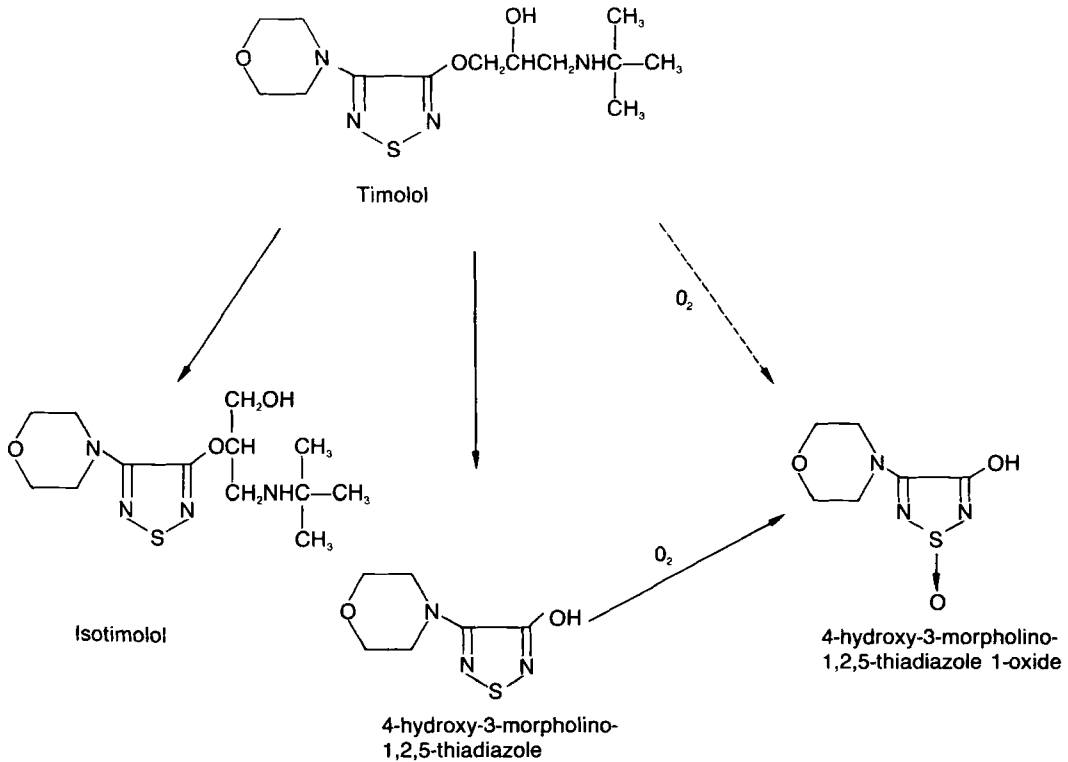


Figure 14. Proposed aqueous solution degradation mechanism for timolol maleate in solutions exposed to autoclave conditions.

chronic administration of 2.5 mg to 20 mg of timolol maleate twice daily (15-16). The bioavailability of oral timolol is reported to be 61% to 75% of a reference intravenous dose (17-18). Bioavailability of less than 100% is attributed to first-pass metabolic extraction by the liver after oral administration rather than to incomplete gastrointestinal absorption (14). The effect of food on the rate and extent of oral absorption of timolol maleate is not significant (19).

Timolol maleate administered directly to the eye as an ophthalmic drop appears to be rapidly absorbed, producing a decrease in intraocular pressure within three hours (20). Systemic absorption of timolol also occurs after ocular administration, producing peak blood plasma levels of no more than 2 to 5 ng/mL at 0.5 hours to 1.5 hours after instillation of 2 drops of 0.5% timolol maleate ophthalmic solution in each eye (21). Some small but statistically significant cardiovascular effects have been reported after ophthalmic administration (20,22). Supporting studies in rabbits indicate that timolol reaches peak levels in most tissues of the eye at ten minutes after ocular administration with blood serum concentrations peaking at 30 to 60 minutes (23).

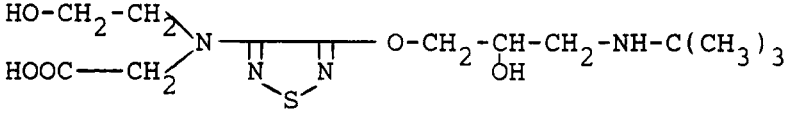
Timolol free base is rapidly absorbed through intact skin (24).

7.2 Metabolism

After oral administration of a 4 mg dose of ^{14}C-timolol maleate to man, 72% of the ^{14}C label is excreted within 84 hours with 66% in the urine and 6% in the feces. Only 20% of the timolol

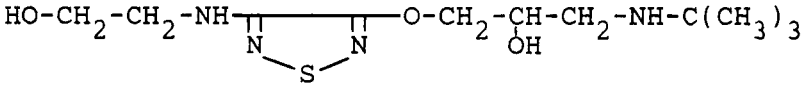
dose is recovered unchanged from the urine. Over 80% of the blood plasma radioactivity represents timolol metabolites (14).

Oxidation and hydrolytic cleavage of the morpholine ring of an oral dose of ^{14}C- timolol maleate produces two major urinary metabolites in man (25).



Metabolite I

N-{{4-[3-(1,1-dimethylethyl)amino]-2-hydroxypropoxy}-1,2,5- thiadiazol-3-yl}-N-(2-hydroxyethyl) glycine.

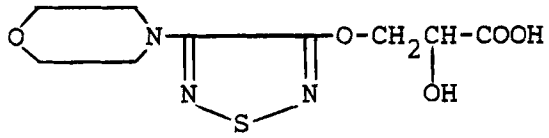


Metabolite II

1-(1,1-dimethylethylamino)-3-{[4-(2-hydroxyethylamino)-1,2,5-thiadiazol-3-yl]oxy}-2-propanol.

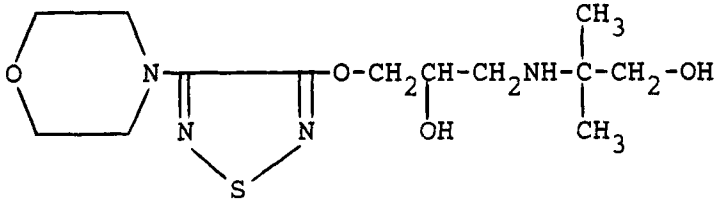
Metabolite I represents approximately 30% of the total urinary radioactivity while Metabolite II accounts for 10% (14,25).

Oxidation of the basic oxypropanolamine side chain of orally administered timolol maleate results in two other minor urinary metabolites in man. Metabolites III and IV represent 6% and 3%, respectively, of administered radioactivity (26).



Metabolite III

2-hydroxy-3-[{4-(4-morpholinyl)-1,2,5-thiadiazol-3-yl}oxy]propanoic acid



Metabolite IV

1-{[(1,1-dimethyl-2-hydroxy)-ethyl]amino}-3-{[4-(4-morpholinyl)-1,2,5-thiadiazol-3-yl]oxy}-2-propanol.

Biological testing of synthetic samples of Metabolites I, II and III shows only Metabolite II to have significant β-adrenergic blocking activity. However, activity was only one-seventh that of timolol maleate in anesthetized dogs (26).

These four metabolites are also produced by other species. Metabolite III represents the major metabolic pathway for timolol maleate in the dog. Other metabolites in rodents result from hydroxylation and oxidation of either the morpholino ring or the oxypropanolamine side chain (25).

7.3 Pharmacokinetics

Oral administration of timolol maleate in doses ranging from 5 mg to 20 mg

results in peak plasma concentrations of timolol maleate within one to two hours, followed by an exponential decline of plasma concentrations with time (15). The mean apparent half-life of elimination of unmetabolized drug from plasma is approximately 2.5 hours (15,17), although plasma half-lives as long as five hours have been reported in normal volunteers (27). Half-life is independent of the administered dose. Timolol maleate appears to follow simple linear kinetics over the dosage range studied; area under the plasma concentration versus time curve is proportional to the administered dose (15). The mean renal clearance of unchanged timolol maleate in normal volunteers was reported as 69.6 mL/minute (27). Pharmacokinetic parameters do not change upon one week chronic administration of oral timolol maleate (15).

Following intravenous administration, plasma levels decrease biexponentially with time (28). Reported half-lives for timolol maleate elimination from plasma, 3.3 to 4.1 hours, are comparable to the oral half-life.

The pharmacodynamics of oral timolol maleate have been well characterized (15,16). Maximal suppression of exercise-induced tachycardia, a measure of β-blockade, occurs when timolol maleate plasma levels reach 27 to 30 ng/mL. Fifty percent of maximum inhibition of exercise-induced tachycardia is produced by timolol maleate plasma concentrations of 3 to 4 ng/mL.

8. Determination in Biological Matrices

Timolol maleate may be determined in blood plasma and urine by isolation and

derivatization followed by gas-liquid chromatography with electron capture detection (29). Timolol and the internal standard, desmethyltimolol, are extracted from either 1.0 mL of alkalinized plasma or 0.1 mL of alkalinized urine into 4% isoamyl alcohol in heptane. The compounds are then back-extracted into 0.1 N hydrochloric acid, the acidic phase alkalinized, and the compounds extracted into methylene chloride. After evaporation of the methylene chloride, timolol maleate and the internal standard are derivatized by addition of 0.1 mL of heptaflurobutyrylimidazole-ethyl acetate reagent and immersion in a boiling water bath for one hour. The diheptafluorobutyryl derivatives are recovered in n-heptane, which is evaporated at 60 °C under a stream of nitrogen to concentrate the sample. Typically, 1 to 5 µl of sample is chromatographed on a 1.83 m X 0.64 cm column packed with 1% OV-17 on 80-100 mesh Gas Chrom Q. The chromatograph is operated isothermally with injection port, oven and detector temperatures of 250 °C, 185 °C and 300 °C, respectively. Helium, the carrier gas, and 10% methane in argon, the purge gas, are maintained at 75 mL per minute. The ^{63}Ni electron-capture detector was operated in the pulse mode of voltage with a pulse interval of 50 microseconds. Retention times of derivatized timolol maleate and internal standard are 7.6 and 6.0 minutes, respectively. Limits of detection are 2 ng/mL in plasma and 20 ng/mL in urine.

A capillary column gas-liquid chromatographic-mass spectrometric assay with selected-ion monitoring for timolol maleate and ^{13}C-timolol maleate in blood plasma is reported to have a sensitivity of 0.5 ng/mL (30). This method provides the capability to examine administration of drug and its stable isotope-labeled counterpart simultaneously for comparison of two different routes of administration. The

isolation procedure prior to assay consists of passing 0.5 to 1.5 mL of plasma, containing ^{2}H-timolol maleate as an internal standard, through a cartridge containing an octadecyl-silica bonded reversed-phase packing. The cartridge is flushed and the compounds are then eluted with acetonitrile-0.05 N hydrochloric acid, which is subsequently alkalinized. Back extraction from cyclohexane-toluene into an aqueous phase followed by extraction into methylene chloride precedes evaporation and derivatization with bis(trimethylsilyl)-trifluoroacetamidepyridine. One to two µL are injected onto a 16 m X 0.25 mm capillary column coated with SE-30. The splitless injection port temperature is 260 °C and oven temperature is 225 °C. Carrier gas (helium) velocity is 30 cm/second.

The mass spectrometer is operated in the electron-impact ionization mode at an ionizing potential of 70 eV, a filament emission current of 0.8 mamp and electron multiplier setting of 1900 V. Selected ion monitoring at m/e 86, 89 and 95, base peaks corresponding to the side chains of timolol maleate, ^{13}C-timolol maleate and ^{2}H-timolol maleate, respectively, allows construction of a standard curve from the ratios $\text{Intensity}_{86}/\text{Intensity}_{95}$ and $\text{Intensity}_{89}/\text{Intensity}_{95}$.

Other gas-liquid chromatographic methods employing mass fragmentographic detection (31) or nitrogen-selective flame ionization detection (18) have been reported.

High performance liquid chromatography with electrochemical detection may also be employed for analysis of timolol maleate in human blood plasma and breast milk (32). A 25 cm X 4.6 mm i.d. column packed with PXS 5/25 Partisil ODS 3 (5 µm) is used with a mobile phase of methanol :0.2 M sodium dihydrogen phosphate :88% orthophosphoric acid :water (500:200:3:297) pumped at a flow

rate of 1.0 mL per minute. The applied potential for oxidative electrochemical detection is +1.2 V for maximum sensitivity. A glassy carbon working electrode is employed with a silver/silver chloride reference electrode and a platinum auxillary electrode. Timolol maleate and the internal standard, propranolol for plasma or serum, pindolol for breast milk, are extracted from biological matrices with diethyl ether. Compounds are extracted from the organic phase into dilute orthophosphoric acid. For plasma or serum, a hexane wash of the organic phase is sufficient to prepare the acidic aqueous phase for injection of 100 µL into the chromatographic column. For breast milk, the acidic aqueous phase must be alkalinized and the compounds back-extracted into ether. Timolol maleate and internal standard are once again extracted into dilute orthophosphoric acid and the hexane wash and assay are performed as for plasma. Calibration curves are linear from the 2 ng/mL limit of detection up to 100 ng/mL.

9. Determination in Pharmaceuticals

9.1 Dissolution Testing

The dissolution of timolol maleate tablets is evaluated based on methodology and apparatus described in USP XXI(33). The acidic dissolution medium (500 mL of 0.1 N hydrochloric acid) is maintained at 37 $\pm$ 0.5 °C and basket speed is 100 rpm. After introduction of the tablet, filtered aliquots of the dissolution medium are withdrawn for assay for timolol maleate content against appropriate standards. The official assay is a high performance liquid chromatographic method developed for a system with an ultraviolet detector. The wavelength for detection is 295 nm. A mobile phase consisting of pH 2.8 phosphate

buffer: methanol (3:2) is pumped at a rate of 1.8 mL/minute through a 30 cm X 3.9 mm column packed with octadecyl-bonded silica. About 15 µl of dissolution sample is injected onto the column. Not less than 80% of the labeled amount of timolol maleate is dissolved in 20 minutes. Alternative assay methods, as outlined in the previous sections (direct ultraviolet spectrophotometry, ultraviolet spectrophotometry via flow injection analysis and high performance liquid chromatography), can be used if an official compendial method is not required.

9.2 Potency Testing

9.2.1 Assay (33)

Official compendial methods are available for both timolol maleate tablets and ophthalmic solution. Tablets are prepared for assay by weighing and finely powdering not less than twenty tablets. An accurately weighed portion of powder is dispersed in 0.05 M monobasic sodium phosphate and sonicated for five minutes. Acetonitrile is added followed by an additional five minutes of sonication. Water is then added and the dispersion is shaken for ten minutes. After dilution to volume with water, the sample may be centrifuged to remove dispersed solids from the timolol maleate solution. The supernatant liquid may be assayed with appropriate standards with the chromatographic system outlined above (Dissolution, Section 9.1) or by an alternative assay if an official compendial method is

not required. Tablets must contain not less than 90.0 percent and not more than 110.0 percent of the labeled amount of timolol maleate.

The official compendial assay for timolol maleate opthalmic solution requires that an aliquot of the solution be diluted to volume with water to produce a 0.5 mg per mL timolol maleate solution. Five mL each of this solution and reference standard solutions are transferred to separators and made basic with pH 9.7 carbonate buffer. Timolol is extracted into toluene from the alkaline aqueous phase. The aqueous phase in each separator is then transferred to a second set of separators for an additional toluene extraction. The aqueous phase in the second set of separators is then discarded. Additional pH 9.7 buffer is added to the toluene remaining in the first set of separators and, after extraction, the aqueous phase is transferred to the second set of separators. After shaking, the aqueous phase is discarded and the toluene from both sets of separators is combined. Timolol in the combined toluene solutions is extracted into four portions of 0.1 N sulfuric acid. The sulfuric acid extracts are diluted to volume with additional acid solution. The extracts are assayed against a 0.1 N sulfuric acid blank at 294 nm with an ultraviolet spectrophotometer. The timolol maleate ophthalmic solution must

contain not less than 90.0 percent nor more than 110.0 percent of label claim.

9.2.2 Dosage Uniformity (33)

Tablets must also meet official compendial standards for uniformity of dosage units. Ten tablets, assayed individually must exhibit 85.0 to 115.0 percent of the label claim and have a relative standard deviation less than or equal to 6.0 percent. If one unit is outside the label claim range or the relative standard deviation is greater than 6.0 percent, or both, an additional twenty tablets must be tested. Of the thirty tested, not more than one may be outside the 85.0 to 115.0 percent range, and that must be within the 75.0 to 125.0 percent of label claim range (34).

Direct ultraviolet spectrophotometry, ultraviolet spectrophotometry via flow injection analysis and reversed-phase high performance liquid chromatography with ultraviolet detection, all as previously described, are acceptable techniques for uniformity determinations. Typically sample preparation involves the disintegration and dissolution of single tablets in solvents identical to those used for assay. A set of extractions and other sample preparation steps identical to those described in the potency assay (9.2.1) are then employed.

9.2.3 Stability Testing (33)

Timolol maleate is tested for stability using the high performance liquid chromatographic method described in the USP XXI. Based upon data generated in accelerated and room temperature stability studies, timolol maleate tablets carry a five year expiration dating.

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TRAZODONE HYDROCHLORIDE

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1. Introduction

1.1. History

Trazodone hydrochloride was originally synthesized in 1966 by Palazzo and Silvestrini (1) in the chemical laboratories of the firm Francesco Angelini. The molecule consists of a triazolopyridine ring and a phenyl piperazinylalkyl moiety. It is the first triazolopyri -dine derivative to be used clinically, and therefore, represents a new chemical class of antidepressant drugs. Its development was a result of an organized approach rather than fortuitous discovery and was based on the hypothesis that the mechanisms responsible for physical and mental pain are the same (2,3).

The history, pharmacology and clinical data on trazodone hydrochloride is well documented in a number of review articles and monographs (3-8). In addition, an analytical profile has been described (9).

1.2. Therapeutic Category

Trazodone hydrochloride is an antidepressant drug indicated in the symptomatic treatment of moderate to severe depression. Its major advantages include a low incidence of anticholinergic and cardiovascular side effects along with minimal stimulatory effects upon dopamine and norepinephrine receptors and is, therefore, considered particularly useful in the geriatric population (5).

2. Description

2.1. Nomenclature

2.1.1. Chemical Name

2-{3-[4-(3-chlorophenyl)-1-piperazinyl] propyl}-1,2,4-triazolo-[4,3-a]pyridin-3(2H)-one monohydrochloride

2.1.2. Generic Name

Trazodone hydrochloride

2.1.3. Proprietary Names

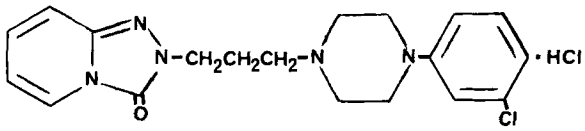
Desyrel, Manegan, Molipaxin, Pragmazone, Thombran, Trittico

2.2. Formulae

2.2.1. Empirical

$C_{19}H_{22}ClN_5O$ HCl

2.2.2. Structural



2.2.3. Registry Numbers

Chemical abstracts;
Trazodone:19794-93-5
Trazodone hydrochloride:25332-39-2

2.3. Molecular Weight

408.33

2.4. Elemental Composition

C, 55.89%; H, 5.68%; Cl 17.36%; N, 17.15%; O, 3.92%

2.5. Appearance, Color and Odor

White, odorless crystals (plates) with a bitter taste.

2.6. Patent Information

U.S. Pat. 3,381,009 (1968 to Angelini Francesco)(1). Brit. Pat. 1,117,068 (11).

3. Physico-Chemical Properties

3.1. Melting Range

The melting point for trazodone free base is 96°C (9). The hydrochloride salt melts with decomposition in the range 222-228°C (9,10,11). Under vacuum decomposition does not occur and a melting range of 231-232.5°C is reported (9).

3.2. Solubility

Trazodone hydrochloride is soluble in chloroform, sparingly soluble in water, ethanol and methanol (10) and insoluble in common organic solvents (9).

3.3. Stability and Storage

Trazodone hydrochloride tablets should be stored at room temperature in tight, light resistant containers and protected from temperatures in excess of 40°C (13).

3.4. Dissociation Constant

The reported pKa for trazodone, in 50% ethanol, is 6.14 (9). This value was obtained potentiometrically using a glass-calomel electrode.

3.5. Crystal Structure

The crystal and molecular structure for trazodone hydrochloride has been determined (14). X-ray diffraction data were obtained using an Oak Ridge diffractometer employing molybdenum Kα radiation with a niobium filter.

Trazodone hydrochloride, $C_{19}H_{22}ClN_5O \cdot HCl$, crystals are monoclinic with space group $P2_1/c$ and with cell dimensions a=16.866[3], b=10.66[2], c=11.230[2], A, =93.04(1)° and Z=4 with the final R factor of 0.042 for 3017 reflections. The measured and calculated densities are 1.344 and 1.345 mg m^{-3} respectively for Z=4.

Figure 1 depicts the 3-dimensional, X-ray derived, structure of trazodone hydrochloride (58). Bond lengths and angles are tabulated in Tables 1 and 2.

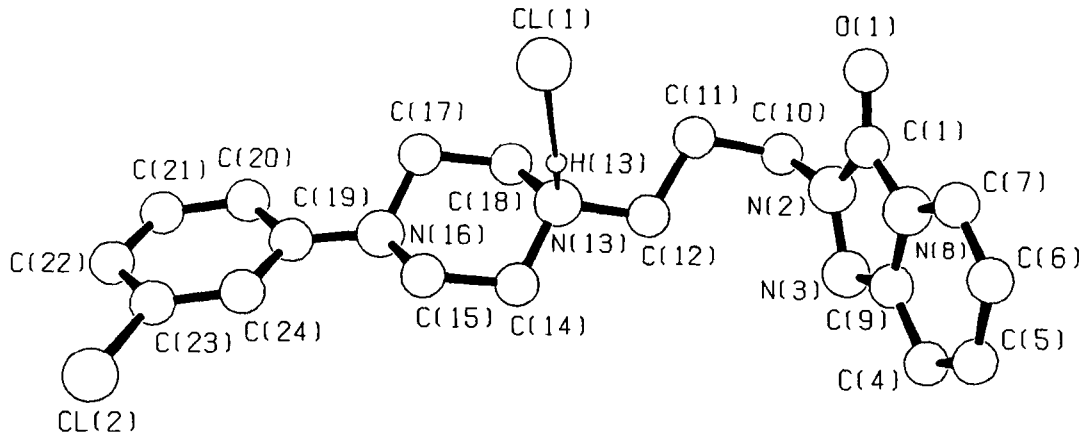


Figure 1. Three-Dimensional, X-Ray Derived, Structure of Trazodone Hydrochloride.

Table 1. Intramolecular Bond Lengths (Å) of Trazodone Hydrochloride

Distances	Å	Distances	Å	Distances	Å
C(1)-O(1)	1.222	N(2)-C(10)	1.451	C(17)-C(18)	1.506
C(1)-N(2)	1.349	C(10)-C(11)	1.518	C(18)-N(13)	1.495
N(2)-N(3)	1.386	C(11)-C(12)	1.512	N(16)-C(19)	1.407
N(3)-C(9)	1.308	C(12)-N(13)	1.498	C(19)-C(20)	1.394
C(9)-N(8)	1.384	N(13)-H(13)	0.872	C(20)-C(21)	1.378
C(9)-C(4)	1.419	N(13)-Cl(1)	3.052	C(21)-C(22)	1.380
C(4)-C(5)	1.344	H(13)-Cl(1)	2.180	C(22)-C(23)	1.363
C(5)-C(6)	1.421	N(13)-C(14)	1.491	C(23)-C(24)	1.383
C(6)-C(7)	1.337	C(14)-C(15)	1.497	C(24)-C(19)	1.391
C(7)-N(8)	1.384	C(15)-N(16)	1.455	C(22)-Cl(2)	1.747
N(8)-C(1)	1.394	N(16)-C(17)	1.463		

Table 2. Bond Angles (°) of Trazodone Hydrochloride

Angles	(°)	Angles	(°)	Angles	(°)
O(1)-C(1)-N(1)	130	C(7)-N(8)-C(9)	123	C(18)-N(13)-C(14)	108
O(1)-C(1)-N(8)	128	N(8)-C(9)-C(4)	118	C(17)-N(16)-C(19)	118
C(1)-N(2)-C(10)	125	C(1)-N(8)-C(7)	129	C(15)-N(16)-C(19)	117
C(1)-N(2)-N(3)	114	N(2)-C(10)-C(11)	113	N(16)-C(19)-C(20)	121
C(1)-N(8)-C(9)	108	C(10)-C(11)-C(12)	112	N(16)-C(19)-C(24)	121
N(2)-C(1)-N(8)	103	C(11)-C(12)-N(13)	113	C(19)-C(20)-C(21)	120
C(10)-N(2)-N(3)	121	C(12)-N(13)-C(14)	109	C(20)-C(21)-C(22)	122
N(2)-N(3)-C(9)	104	C(12)-N(13)-H(13)	114	C(21)-C(22)-C(23)	117
N(3)-C(9)-C(4)	130	N(13)-H(13)-Cl(1)	172	C(22)-C(23)-C(24)	118
N(3)-C(9)-N(8)	112	N(13)-C(14)-C(15)	112	C(23)-C(24)-C(19)	120
C(9)-C(4)-C(5)	119	C(14)-C(15)-N(16)	111	C(24)-C(19)-C(20)	118
C(4)-C(5)-C(6)	122	C(15)-N(16)-C(17)	111	Cl(2)-C(23)-C(22)	119
C(5)-C(6)-C(7)	121	N(16)-C(17)-C(18)	111	Cl(2)-C(23)-C(24)	118
C(6)-C(7)-N(8)	118	C(17)-C(18)-N(13)	111		

This study revealed a number of interesting features about trazodone which may be useful in proposing structural requirements for neuroleptic activity. The piperazine ring is in the normal chair conformation with N(13) extending upward and N(16) down. The bond lengths and angles about N(13) resemble that of a protonated amine and those associated with N(16) demonstrate sp^2 character due to the attached chlorophenyl ring. Torsional angles about N(2)-C(10)(93.2°) C(11)-C(12) (178.2°) suggest that the propylene side chain is fully extended. In addition, the triazolopyridine nucleus (including C(10)) and the chlorophenyl moiety are planar groups.

3.6. Differential Scanning Calorimetry

A differential scanning calorimetry (DSC) curve for trazodone hydrochloride has been reported (9). Data obtained using a Perkin Elmer DSC 1B instrument was used as a purity index for trazodone.

3.7. Spectral Properties

3.7.1. Ultraviolet Spectrum

The ultraviolet spectrum of trazodone hydrochloride was scanned from 200 to 360 nm with a Gilford Response spectrophotometer at a concentration of 30 and 75 μ moles/L in methanol and 30 μmoles/L water (Figure 2). Each spectrum is characterized by two well defined and two less prominent peaks. The spectra in methanol show maxima at 213, 256, 278 and 312 nm while that in water displays maxima at 211, 246, 275 and 312 nm. The latter are in agreement in previously reported data (9,10).

The reported A (1%, 1 cm) and molar absorptivity values for trazodone hydrochloride in water are summarized in Table 3 (9).

Table 3. Ultraviolet Characteristics of Trazodone Hydrochloride

max (nm)	A(1%,1cm)	ε
211	1225	50,100
246	287	11,730
274	94	3,840
312	94	3,840

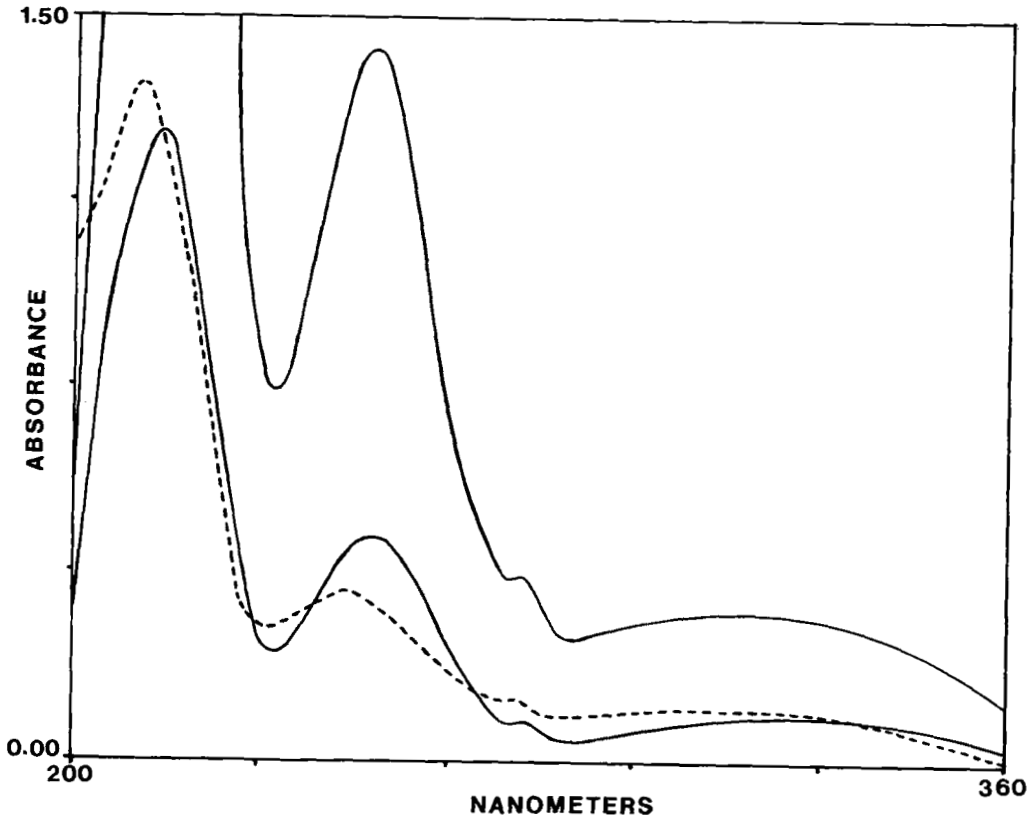


Figure 2. Ultraviolet Spectra of Trazodone Hydrochloride (——, methanol; ----, water).

3.7.2. Fluorescence Spectrophotometry

Trazodone hydrochloride, like most 2-alkyl-substituted-1,2,4-triazolo-[4,3-a] pyridin-3 (2H)-ones, shows an intense fluorescence when irradiated with ultraviolet light. The excitation and emission wavelengths for trazodone are 317 and 455 nm respectively (9).

3.7.3. Infrared Spectrum

The infrared spectrum of trazodone hydrochloride is shown in Figure 3. The spectrum was obtained with a Beckman AccuLab 4 infrared spectrophotometer from a compressed KBr disc. Structural assignments of some of the characteristic absorption bands are presented in Table 4.

Table 4. Infrared Characteristics of Trazodone Hydrochloride

Frequency (cm^{-1})	Intensity	Assignment
2975-2840	W	Aliphatic CH
2535,2460	M	+N-H stretching
1705	S	C=0 stretching
1640	M	triazoline C=N stretch
1595	S	aromatic C=C stretch

M=Medium; S=Strong; W=Weak

Other characteristic bands appear at 1540, 1491, 1447, 1350, 1273, 943 and 745 cm^{-1}. Principle peaks, wavenumbers and structural assignments are in agreement with previously published results (9,10).

3.7.4. Nuclear Magnetic Resonance (NMR) Spectra

3.7.4.1. Proton Magnetic Resonance (PMR) Spectra

The PMR spectra of trazodone base and trazodone hydrochloride were recorded on a Bruker AM 300 NMR spectrometer employing a frequency of 300.13 MHz. Spectral assignments were confirmed by homodecoupling, COSY and heteronuclear correlation experiments.

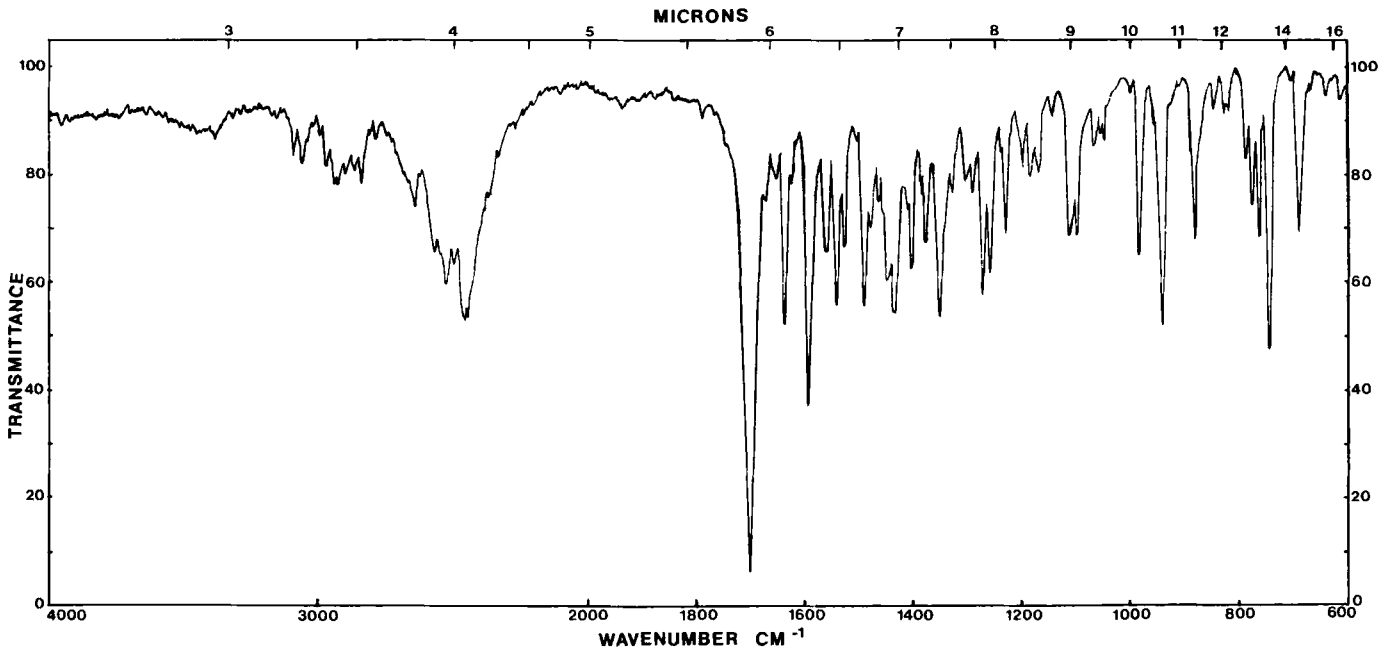
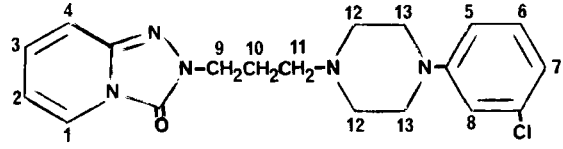


Figure 3. Infrared Spectrum of Trazodone Hydrochloride-KBr Disc.

The spectrum of trazodone base obtained in $CDCl_3$ is presented in Figure 4. Scale expansion in the region 6.4 to 7.8 ppm is illustrated in Figure 5. The chemical shifts, multiplicities and assignments are compiled in Table 5. These data are consistent with the results reported using a 60 MHz instrument (9).

Table 5. Proton Magnetic Resonance Assignments for Trazodone Base



Chemical Shift δ (ppm)	Multiplicities	Number of Protons	Assignment
7.75	d	1	1
7.11	m	3	3,4,6
6.84	m	1	8
6.76	m	2	5,7
6.46	m	1	2
4.09	t	2	9
3.13	t	4	13
2.56	t	4	12
2.49	t	2	11
2.05	m	1	10

d=doublet; t=triplet; m=multiplet

The PMR spectrum of trazodone hydrochloride in DMSO-d_6 is shown in Figure 6. Scale expansion in the region 6.5 to 8.0 ppm is depicted in Figure 7. Chemical shifts and assignments are given in Table 6. It can be seen that protonation of the piperzine ring nitrogen nearest the propyl chain affects the chemical shift and splitting pattern of adjacent protons in the piperazine ring.

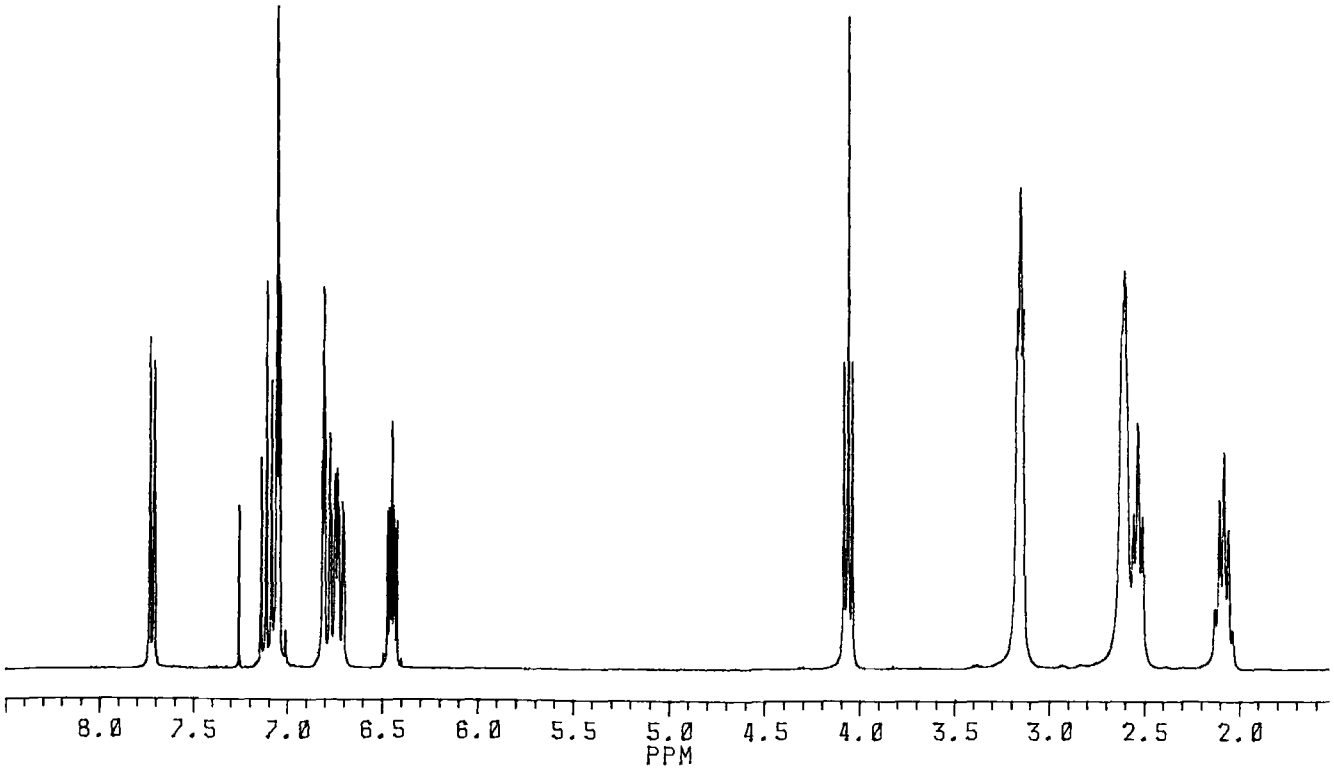


Figure 4. Proton Magnetic Resonance Spectrum of Trazodone Base in $CDCl_3$.

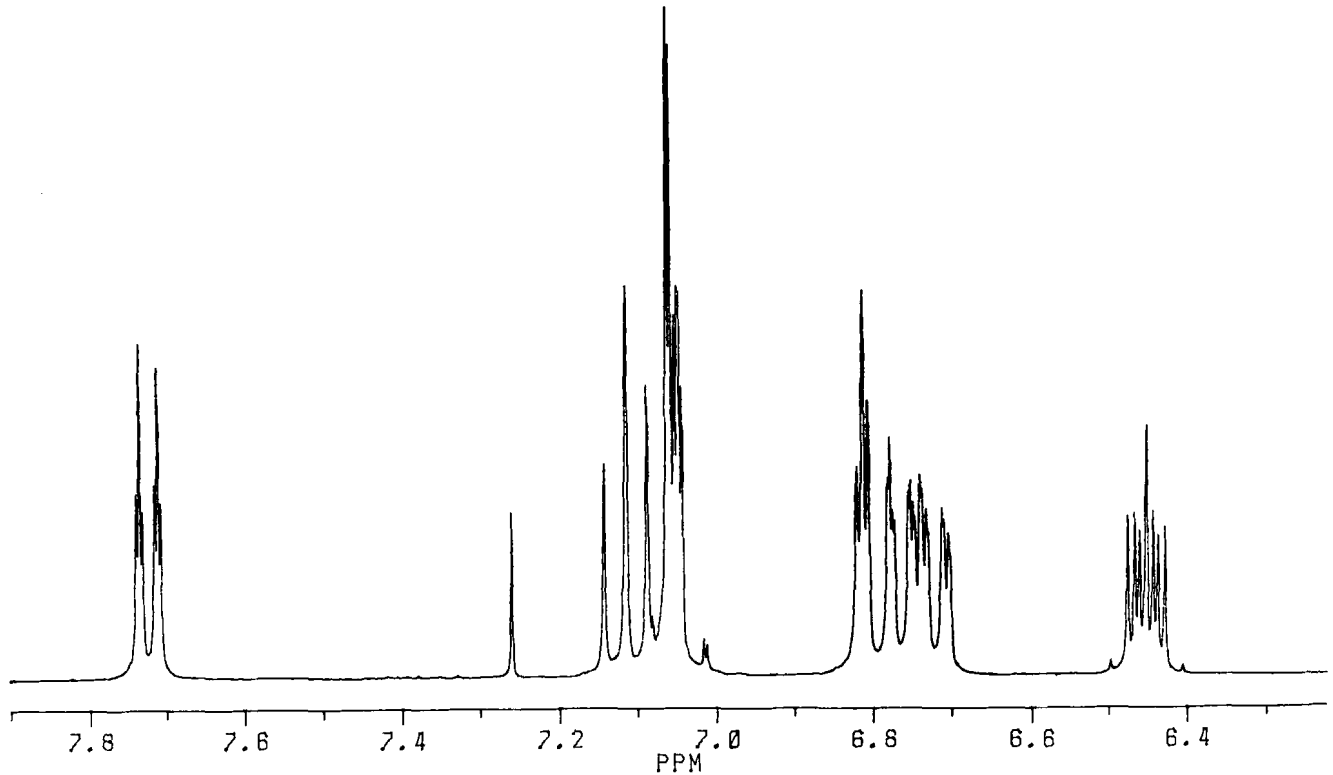


Figure 5. Proton Magnetic Resonance Spectrum of Trazodone Base in $CDCl_3$ with Scale Expansion (6.4-7.8 ppm).

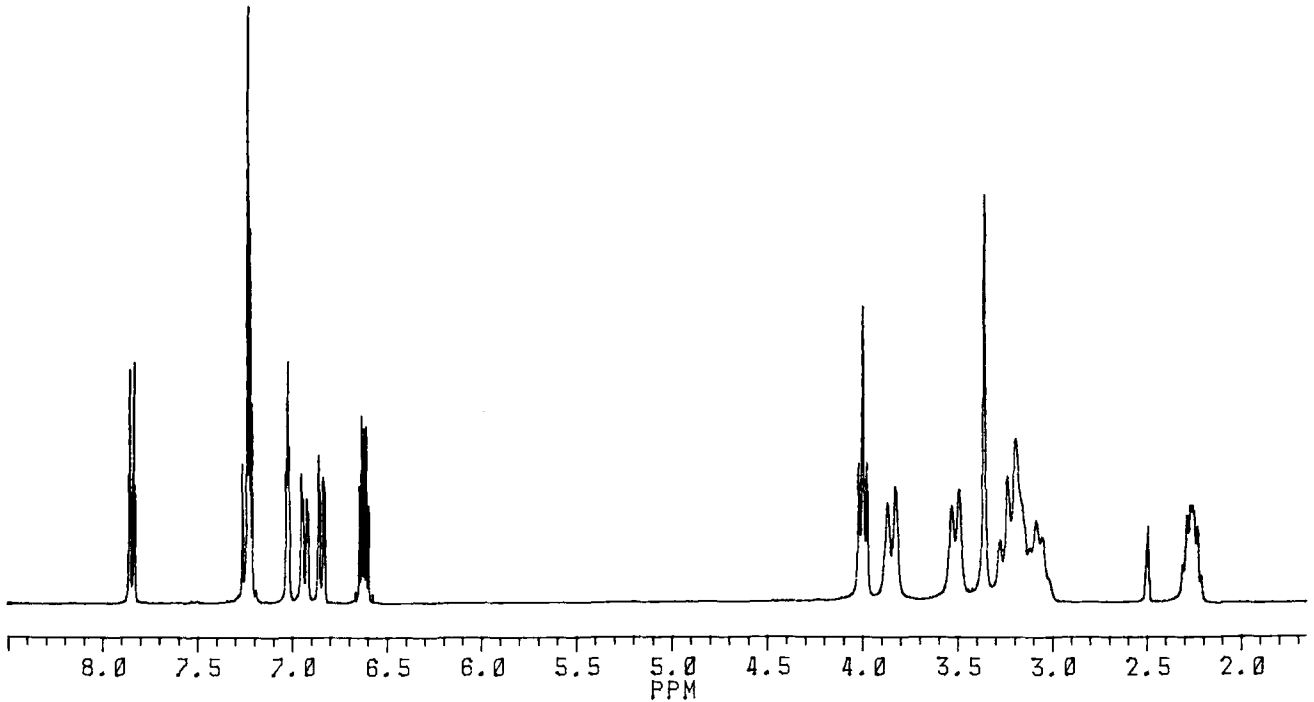


Figure 6. Proton Magnetic Resonance Spectrum of Trazodone Hydrochloride in DMSO-d_6.

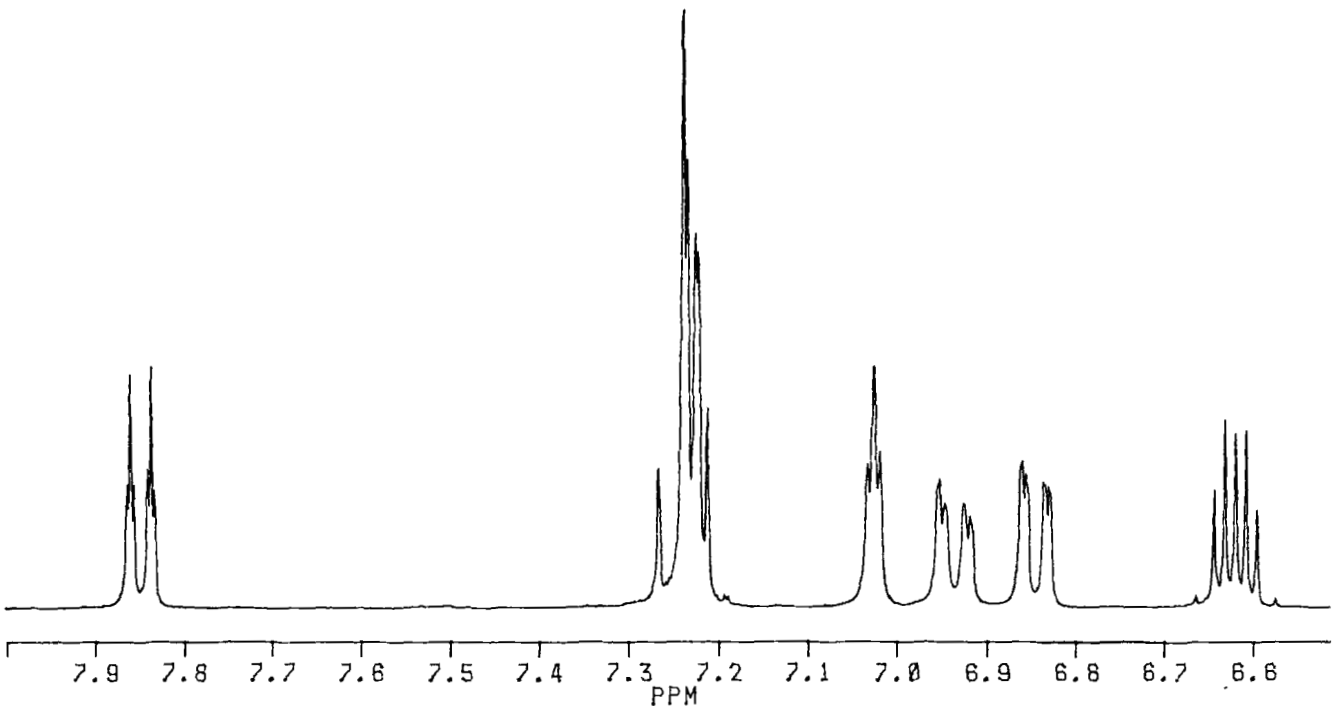
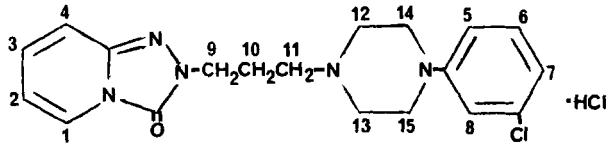


Figure 7. Proton Magnetic Resonance Spectrum of Trazodone Hydrochloride in DMSO-d_6 with Scale Expansion (6.5-8.0 ppm).

Table 6. Proton Magnetic Resonance Assignments for Trazodone Hydrochloride



Chemical Shift δ (ppm)	Multiplicities	Number of Protons	Assignment
7.87	m	1	1
7.25	m	3	3,4,6
7.04	m	1	8
6.95	dd	1	5
6.86	dd	1	7
6.64	m	1	2
4.01	t	2	9
3.86	d	2	12,13
3.53	d	2	12,13
3.15	m	6	11,14,15
2.25	m	2	10

d=doublet; dd=doublet,doublet; t=triplet
m=multiplet

3.7.4.2. Carbon-13 Nuclear Magnetic Resonance Spectrum (^{13}C-NMR)

The ^{13}C-NMR spectrum of trazodone hydrochloride base obtained in DMSO-d_6 is given in Figure 8. The spectrum was obtained at ambient temperature on a Bruker AM 300 NMR spectrometer at 75.47 MHz with broad band proton decoupling. Chemical shifts, and assignments are outlined in Table 7. Spectral assignments were confirmed by C-13/H-1 heteronuclear correlation experiments and published chemical shift data.

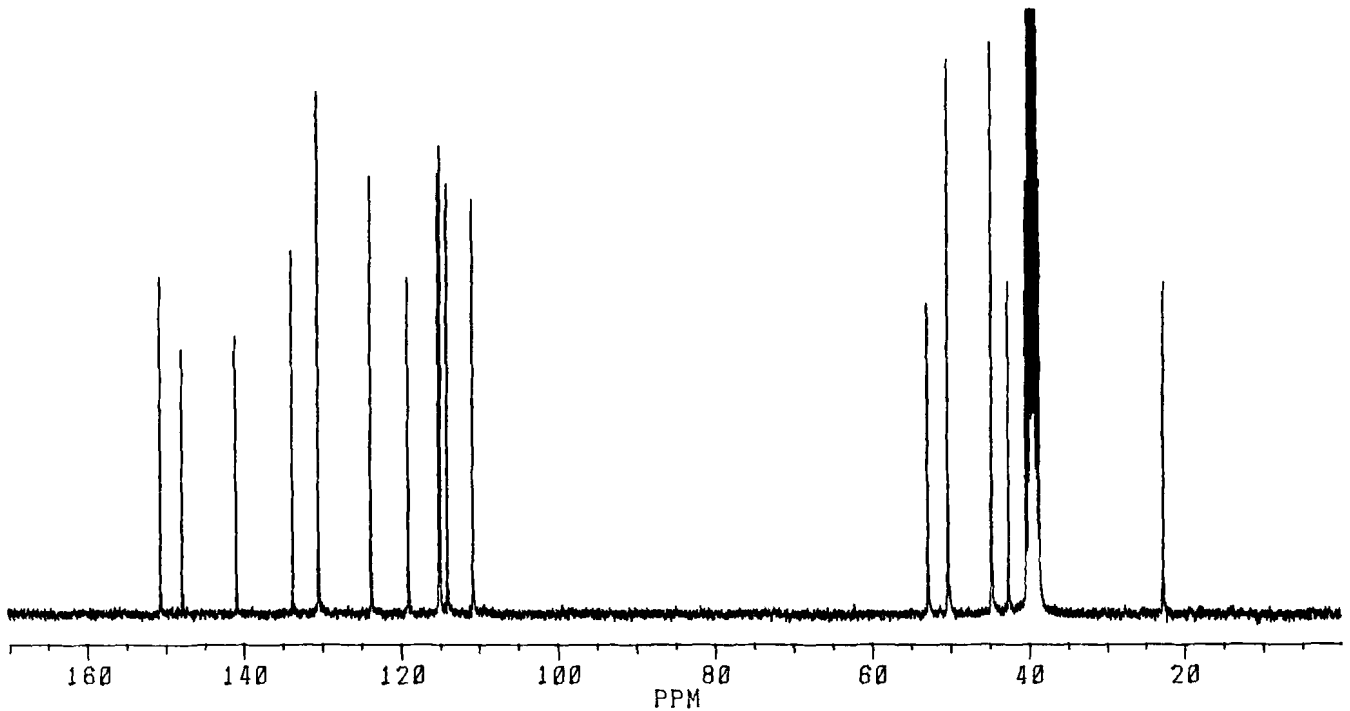
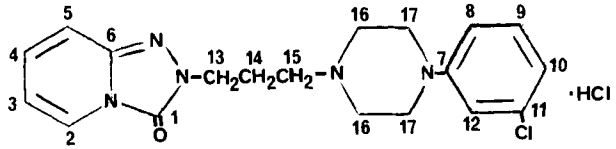


Figure 8. Carbon-13 Nuclear Magnetic Resonance Spectrum of Trazodone Hydrochloride in DMSO-d_6.

Table 7. Carbon-13 Nuclear Magnetic Resonance Assignments for Trazodone Hydrochloride

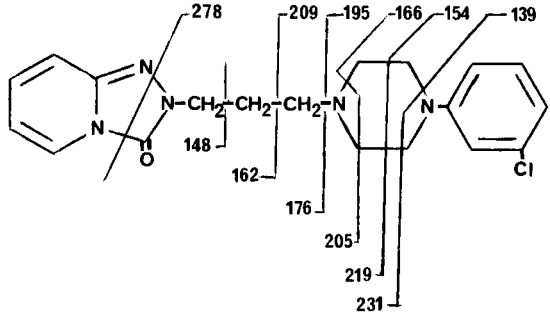


Chemical Shift δ (ppm)	Assignment	Chemical Shift δ (ppm)	Assignment
150.69	6	114.92	4,5 or 9
147.88	1	113.99	8
141.06	7	110.76	3
133.84	11	52.79	13
130.51	4,5 or 9	50.23	17
130.47	4,5 or 9	44.66	16
123.82	2	42.54	15
119.03	10	22.78	14
115.12	12		

3.7.5. Mass Spectrometry

3.7.5.1. Electron Impact (EI)

An electron impact mass spectrum of trazodone hydrochloride is presented in Figure 9. This was obtained by direct solid insertion probe at an electron energy of 70 eV and a source temperature of 180°C using a VG Analytical MM 16F single focussing mass spectrometer linked to a VG 2035 data system. The spectrum shows a molecular ion peak M^+ at a mass/charge (m/z) ratio of 371 with a relative intensity of 13.2% and a base peak at 205. Prominent diagnostic ions are observed at m/z 70, 136, 166, 176, 209, 231 and 278. The fragmentation pattern leading to the various ions is outlined below.



Mass spectra of trazodone and other related compounds have been reported elsewhere (9,15,16,17,18).

3.7.5.2. Chemical Ionization (CI)

The chemical ionization spectrum of trazodone hydrochloride is shown in Figure 10. This spectrum was also determined on a VG Analytical MM 16F mass spectrometer, however, using ammonia as a reagent gas. The spectrum shows a pseudo molecular ion at a mass to charge ratio of 372.

4. Synthesis

A number of synthetic pathways have been described for the synthesis of trazodone hydrochloride (1,11,19,20). All of the reported routes employ 1,2,4-triazo-[4,3-a]-pyridin-3(2H)-one (I) or 2-substituted derivatives as starting material. The more important methods are outlined in Scheme 1.

The original synthesis reported by Palazzo and Silvestrini (1) consisted of the condensation of 1-(3-chlorophenyl)-4-(3-chloropropyl)-piperazine with the sodium salt of I. Other related patented methods were simultaneously developed in the same laboratories (11).

More recently, modications of existing pathways were reported (19). One method involves the condensation of the usual triazopyridine (I) with bromochloropropane to yield the 2-(3-chloropropyl)-derivative (II). Subsequent treatment with N-(3-chlorophenyl)piperazine gives trazodone. Alternatively, I is alkylated with bromochloropropane and treated with diethanolamine to form the di-alcohol (III). This product is then chlorinated with thionyl chloride and subsequently reacted with 3-chloroaniline to yield trazodone.

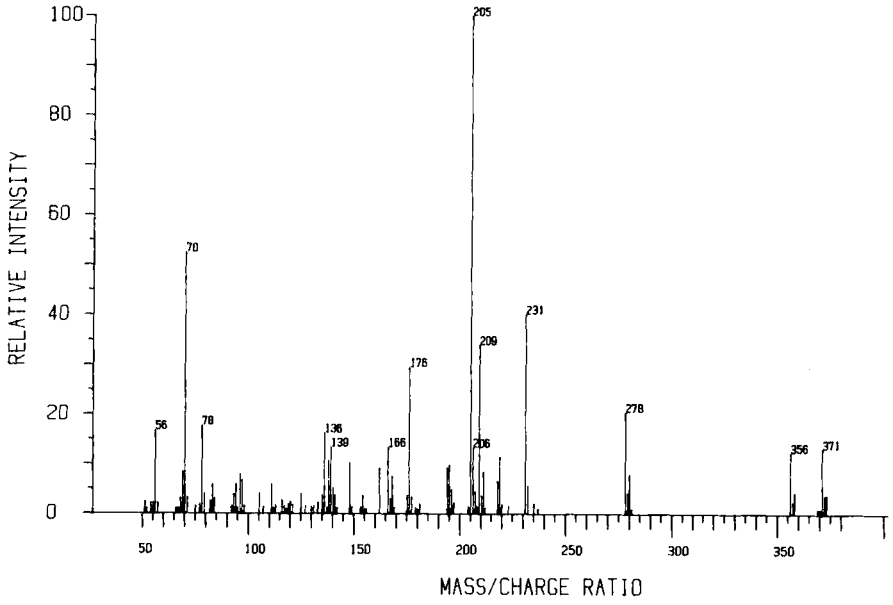


Figure 9. Mass Spectrum of Trazodone Hydrochloride Electron Impact.

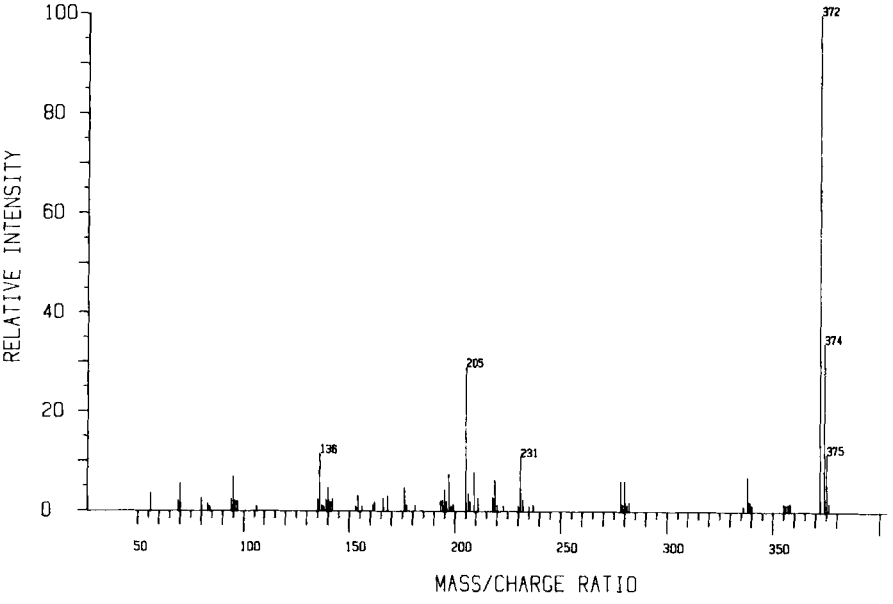
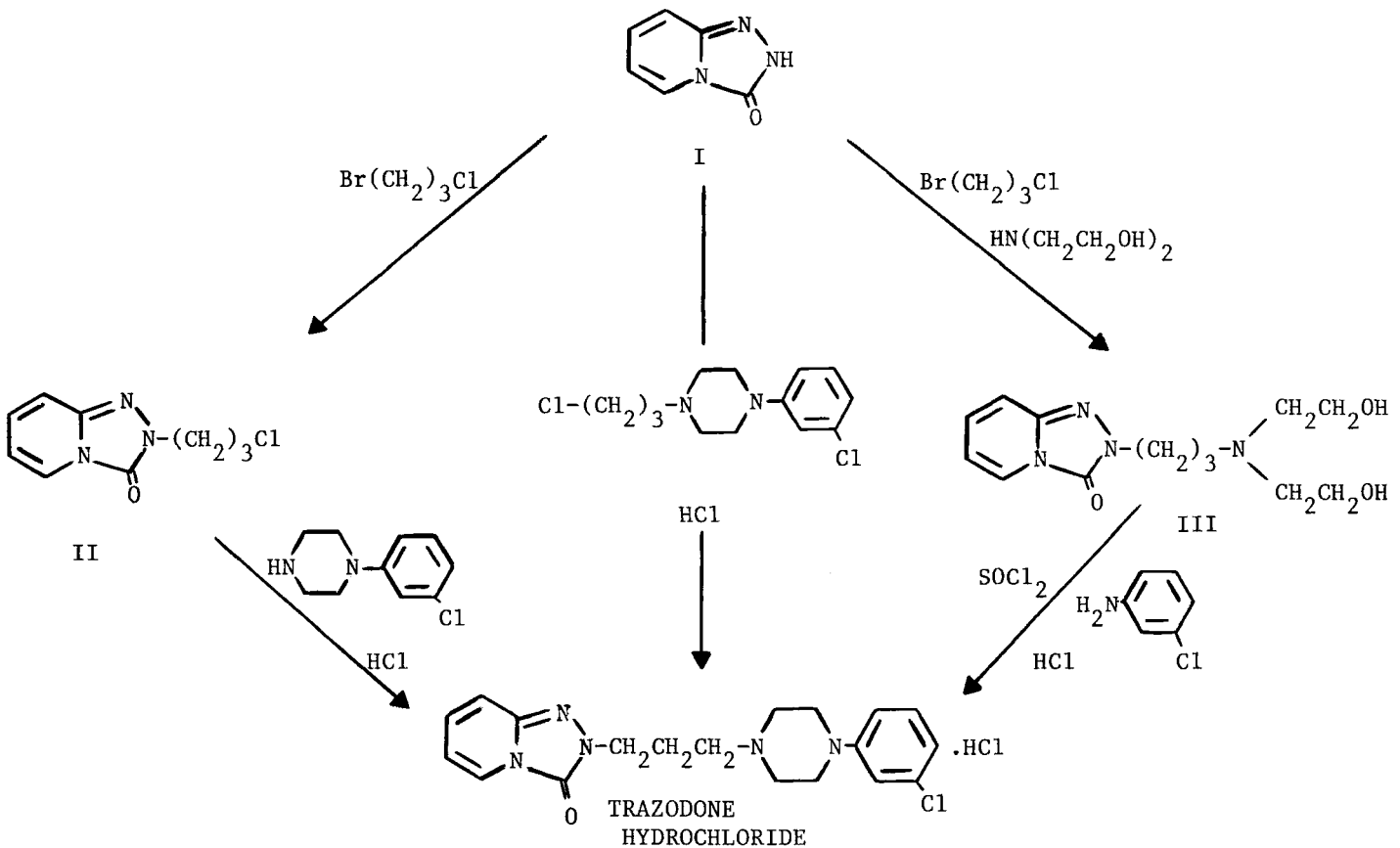


Figure 10. Mass Spectrum of Trazodone Hydrochloride-Chemical Ionization.



Scheme 1. Synthesis of trazodone hydrochloride

The synthesis of ^{14}C-labelled trazodone hydrochloride has also been published (20). This procedure also employs the classical condensation reaction described in the original patent.

5. Pharmacokinetics

5.1. Absorption

Following oral administration to man, trazodone is rapidly absorbed. The time to reach maximum plasma concentrations typically varies from 0.5 to 4.0 hours in fasting subjects (21-26). Peak plasma concentrations following oral administration of a single 50 mg dose range from 0.5 to 1.0 µg/mL (21,24-26). Following oral ingestion of a single 100 mg trazodone hydrochloride dose average maximum plasma concentrations of 1.61 µg/mL and 1.66 µg/mL were reported for a capsule and liquid formulation respectively (22).

Oral and intramuscular bioavailability of trazodone is almost complete as shown by the virtually identical plasma concentration-time profiles obtained following oral intramuscular and intravenous administration to healthy volunteers (23). Ingestion of food delays the absorption of trazodone from the gastrointestinal tract but does not seem to affect its bioavailability (23,27). Studies in rats and rabbits have shown that food delays the oral absorption by decreasing the transfer rate of drug from the stomach to the duodenum (23).

5.2. Distribution

Trazodone is a weak base with a pKa of 6.14 (9), and therefore, exists mostly in the unionized form at physiological pH. The drug distributes in a relatively large volume: V_d values reported in the literature or calculated from published data range approximately from 1-2 L/kg for healthy volunteers (21,22,24,25,28). Trazodone is highly bound to plasma proteins. *In vitro* protein binding studies with human plasma have shown that trazodone is 96, 98 and 97% bound at concentrations of 1.0, 0.1 and 0.01 µg/mL respectively (29).

Studies in laboratory animals show that trazodone readily crosses the blood brain barrier but is minimally transferred across the placenta (30,31). Little information is available in man concerning its distribution in organs and body fluids. Excretion of trazodone in human breast milk has been studied and found to be very small (32). The average milk/plasma ratio was 0.14, therefore, exposure to babies via breast milk would be minimal.

5.3. Elimination

In general, plasma concentrations of trazodone in man decline in a biphasic manner with a mean half-life of approximately 4 hours during the period 3-10 hours following dosing and a terminal elimination half-life of 6-12 hours (21,22,25,28,32). Total body clearance (assuming an oral bioavailability of 100%) ranges from 150 mL/min. to 400 mL/min. (21,24,32). Patients treated with trazodone on a twice daily dosage schedule show significantly higher plasma levels in the morning as compared to the levels at night (33). A possible diurnal variation in clearance may be responsible.

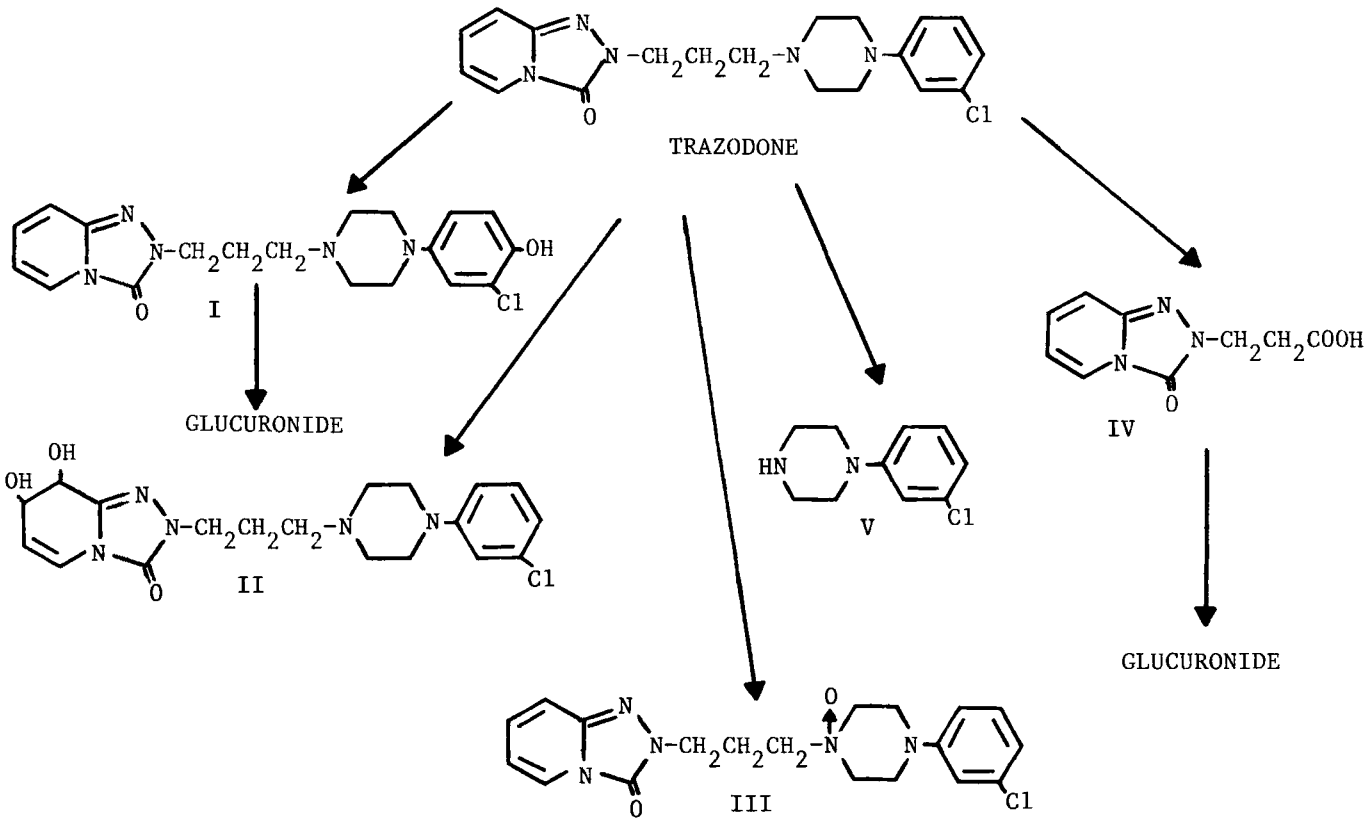
Trazodone is extensively metabolized via hydroxylation, N-oxidation and hydrolysis (Scheme 2) (34). Hydroxylation occurs in the benzene ring (4-hydroxyphenyl metabolite, I) and in the triazolopyridine ring (dihydrodiol, II) while N-oxidation occurs on a piperazine nitrogen (N-oxide, III). A hydrolytic reaction results in the formation of 3-oxo-1,2,4-triazolo[4,3-a]-pyridin-2-yl propionic acid (IV) and 1-m-chlorophenylpiperazine (V), a pharmacologically active metabolite (35,36). Small concentrations of 1-m-chlorophenylpiperazine, approximately 1% of trazodone plasma concentrations, have been reported in man (37). Whether these small concentrations contribute to the overall effects of trazodone remains unclear. Approximately 70-75% of an oral dose of the drug is excreted in urine within 72 hours of administration, most as metabolites. Only less than 1% of the dose is recovered in urine as unchanged drug (26,34). The remainder of an oral dose is excreted in feces, involving biliary excretion, mainly as trazodone metabolites (23,27).

Results from animal studies indicate that trazodone does not induce its own metabolites (38).

6. Methods of Analysis

6.1. Identification

Trazodone hydrochloride reacts with certain reagents to produce characteristic colors which may be useful for its identification. When treated with Liebermanns Test reagent at 100°C (sodium nitrite-sulphuric acid) trazodone gives a transient violet color whereas with Mandelin's reagent (ammonium vanadate-sulphuric acid) a grey color develops which later changes to violet (10).



Scheme 2. Biotransformation pathways of trazodone in man

An infrared spectroscopic method is described which is useful for the rapid emergency identification of certain psychtropic drugs including trazodone (39). The method employs chloroform extraction of patient's urine at different pH's and utilizes the infrared finger print region and spectra of authentic standards to confirm identity. Infrared spectra are determined as compressed potassium bromide discs from dried chloroform extracts.

6.2. Chromatographic Analysis

6.2.1. Thin-Layer Chromatography (TLC)

A number of thin-layer chromatographic systems have been developed for the determination of trazodone. These systems have been employed for the identification (9,10,40,41,42) and quantitation (43,44,45) of the drug both as pure drug substance as well as in biological fluids and tissues.

A variety of detection methods have been used and most systems use silica gel plates as absorbent. A summary of some of the reported TLC methods is given below.

System I (9)

Absorbent: Merck Kieselgel-0.25 mm
Mobile Phase:
i) cyclohexane-benzene-diethylamine (5:4:1)
ii) chloroform-methanol-isopropanol-ether-NH_3 (60:25:25:2)
iii) n-butanol:water:NH_3 (20:10:2)
iv) abs. ethanol:NH_3 (25:5)
Detection: ultra-violet light (366 nm), ninhydrin, rhodanine, ammonium sulphocyanide and cobaltous chloride or potassium dichromate, sulphuric acid with glacial acetic acid.

System II (10)

Absorbent: Silica gel G, 0.25 mm treated with potassium hydroxide in methanol.
Mobile Phase:
i) methanol:NH_3 (100:1.5)
ii) cyclohexane:toluene:diethylamine (75:15:10)
Detection: ultra-violet light, Dragendorff's reagent or acidified iodoplatinate solution.

System III (40)

Absorbent: Silica gel, F254, 0.25 mm
Mobile Phase:
i) methanol:NH_3 (100:1.5)
ii) cyclohexane:toluene:diethylamine (75:15:10)
iii) chloroform:methanol (9:1)
iv) acetone
Detection: Dragendorff's reagent

System IV (42)

Absorbent: Silica gel 60, F254, 0.25 mm
Mobile Phase:
i) ethylacetate:methanol:30% NH_3 (85:10:5)
ii) cyclohexane:toluene:diethylamine (65:25:10)
iii) ethylacetate:chloroform (50:50)
iv) acetone
Detection: 10% sulphuric acid followed by Dragendorff's reagent and acidified iodoplatinate solution

System V (45)

Absorbent: Silica gel, 0.25 mm
Mobile Phase:
i) methanol:ammonia (100:1.5)
ii) ethylacetate:methanol:ammonia (85:10:5)
Detection: 5% sulphuric acid, iodoplatinate solution followed by Dragendorff's reagent

6.2.2. Gas-Liquid Chromatography (GLC)

A number of GLC procedures have been published for the determination of trazodone. The technique has been used for routine detection of the drug (9,10,17), biopharmaceutics and pharmacokinetic studies (16,18,28,47), as well as in therapeutic and overdose stiuations.(45,46) Chromatography of trazodone is readily accomplished without derivatization using a variety of stationary phases in packed or capillary columns. Detectors employed include flame ionization (FID), nitrogen-phosphorous (NPD) and mass spectrometry (MS). The conditions for the reported GLC methods are as follows:

System I (9)

Type of sample: drug substance
Column: 6 ft x 1/4" I.D. glass column packed with 1.5% OV-17 on Chromosorb W HP 80-100
Carrier gas: Nitrogen, 13 lb/inch^2
Temperature: column 268°C, injector 320°C
Retention time: approximately 3.5 min.
Detector: not stated, presumably FID

System II (10)

Type of sample: general
Column: 2 m x 4 mm I.D. glass packed with 2.5% SE-30 on chromosorb G 80-100
Carrier gas: nitrogen, 45 mL/min.
Temperature: column, retention index - 10
Retention index: 3250
Detector: FID or NPD

System III (16)

Type of sample: plasma (rat)
Column: 80 cm x 4 mm I.D. glass column packed with 1% OV-1 on Chromosorb Q 100-120
Carrier gas: nitrogen, 35 mL/min.
Temperature: column, 240° C; injector, 270°C
Retention time: 2 min.
Detector: FID and MS

System IV (45)

Type of sample: biological fluids and tissue
Column: 4 m x 0.25 mm I.D. DB-1 fused-silica capillary (0.25 µm film)
Carrier gas: hydrogen, 40 cm/sec.
Temperature: column, 265°C
Retention time: 2.87 min.
Detector: FID (or NPD)
In addition:

i) **Column:** 1.8 m column with 3% SP 2250 on Supelcoport 100-120
Temperature: 10°C/min. from 140-300°C, hold 5 min.
Retention time: 3.89 min.
Detector: NPD (MS)

ii) **Column:** 1.8 m column with 3% SP 2100 on Supelcoport 100-120
Temperature: 13°C/min from 160-320°C, hold 7 min.
Retention time: 3.10 min.
Detector: NPD (MS)

System V (28)

Type of sample: plasma
Column: 1.23 m x 2 mm I.D. glass column packed with 3% OV-101 on Chromosorb W HP 80-100
Carrier gas: helium, 30 mL/min
Temperature: column, 260°C, injector, 310°C; detector, 275°C
Retention time: 5.1 min.
Detector: NPD

System VI (47)

Type of sample: plasma, brain tissue (rat)
Column: 1 m x 3 mm I.D. glass column packed with 3% OV-1 on Gas Chrom Q 80-100
Carrier gas: nitrogen, 30 mL/min.
Temperature: 270°C
Retention time: 4.2 min.
Detector: NPD (MS)

System VII (17)

Type of sample: urine (rat)
Column: 60 cm x 1 mm I.D. nickel capillary column packed with 20% UCC-W (Hewlett Packard) on Chromosorb W AW DMCS 80-100
Carrier gas: helium, 7 mL/min.
Temperature: column, 20°C/min. from 100-300°C; injector, 270°C
Detector: MS

System VIII (18)

Type of sample: plasma
Column: 4 m x 0.25 mm I.D. DB-1 fused-silica capillary (0.25 μm film)
Carrier gas: helium, 200 cm/sec.
Temperature: column, 170°C for 1 min., 15°C/min. to 255°C; injector, 250°C; separator oven 255°C
Retention time: 3 min.
Detector: MS

6.2.3. High Performance Liquid Chromatography (HPLC)

Several HPLC methods have been developed for the determination of trazodone; most of which have been employed in analysis of biological fluids and tissues for unchanged drug and metabolites. The majority of the procedures utilize reverse-phase chromatography with mobile phases comprised of an aqueous buffer with organic solvent modifiers (21,32,43,48,50-52,54). Others include

reversed-phase with ion-pairing agents (49,56,57) and normal-phase systems (53,55). Detector systems include ultra-violet (UV) at various wavelengths, fluorescence and electrochemical oxidation. A summary of the reported HPLC systems is presented below.

System I (48)

Type of sample: serum or plasma
Column: 150 x 4.6 mm Supelcosil LC-8 DB
Mobile phase: phosphate buffer 0.01M (pH 4.6): methanol: acetonitrile (47:37:16)
Temperature: ambient
Flow rate: 2.1 mL/min.
Retention time: 3.2 min.
Detection: UV at 206 nm

System II (32)

Type of sample: breast milk
Column: 150 x 3.9 mm Nova-Pak C18 (4 µm)
Mobile phase: methanol:phosphate buffer 0.082 M (pH 6.5): acetonitrile (39:47:14)
Temperature: ambient
Flow rate: 1.5 mL/min.
Retention time: 5 min.
Detection: UV at 211 mm

System III (49)

Type of sample: plasma and tissue
Column: 250 x 4.6 mm trimethyl silyl (5 µm)
Mobile phase: acetonitrile:phosphate buffer (pH 3.0) (27:73) with 0.02 M heptane sulfonic acid and 0.04 M triethylamine
Temperature: ambient
Flow rate: 1.5 mL/min.
Retention time: 12.8 min.
Detection: UV at 214 nm

System IV (50)

Type of sample: plasma
Column: 300 x 4.6 mm Bondapak C18 (10 µM)
Mobile phase: phosphate buffer 0.05 M (pH 4.7): acetonitrile (72:8)
Temperature: 60°C
Flow rate: 2.5 mL/min.
Retention time: 8 min.
Detection: UV at 214 nm

System V (51)

Type of sample: serum
Column: 250 x 4.6 mm LiChrosorb 10RP8
Mobile phase: acetonitrile: phosphate buffer, 0.6%; pH 3.0 (1:1)
Temperature: ambient
Flow rate: 2 mL/min.
Retention time: 3.8 min.
Detection: UV at 240 nm

System VI (52)

Type of sample: biological fluids
Column: 250 x 4.6 mm Ultrasphere ODS (octadecylsilyl) 5 μm)
Mobile phase:
i) phosphate buffer, 0.1 M (pH 7.0): acetonitrile (10:3.8); 3.0 mL/min; 65°C; retention time (10:3.8); 8 min.
ii) phosphate buffer 0.1 M (pH 3.0): acetonitrile (10:3); 1.5 mL/min.; 60°C; retention time, 1.25 min.
Detection: UV at 242 nm

System VII (43)

Type of sample: serum and urine
Column: ODS (C18)
Mobile phase: methanol: 0.011 M phosphate buffer (pH 7.5): acetonitrile (60:38:2)
Detection: UV at 250 nm

System VIII (21)

Type of sample: plasma
Column: 250 x 4.6 mm Spherisorb S5 ODS
Mobile phase: acetonitrile: 0.05 M sulphuric acid (18:1)
Temperature: ambient
Flow rate: 2 mL/min.
Retention time: 6.8 min.
Detection: UV at 254 nm

System IX (53)

Type of sample: drug substance
Column: 125 mm Spherisorb S5W Silica
Mobile phase: methanolic ammonium perchlorate, 10 mM, pH 6.7
Temperature: ambient
Flow rate: 2 mL/min.
Relative retention time: 0.31
Detection: UV at 254 nm, electrochemical, + 1.2 V

System X (54)

Type of sample: plasma
Columns: 250 x 4.6 mm MC-18 with 20% carbon load (5 µm), 300 x 4.6 mm Bond-apak C18 (10 µm)
Mobile phase: phosphate buffer, 0.2 M (pH 4.7): tetrahydrofuran: acetonitrile (90:5:5)
Temperature: 45°C
Flow rate: 1.5 mL/min.
Retention time: 39 min.
Detection: UV at 254 nm

System XI (55)

Type of sample: biological fluids
Column: 125 x 5 mm Spherisorb S5W Silica (5 µm)
Mobile phase: methanol with 0.02% perchloric acid
Temperature: ambient
Flow rate: 2.0 mL/min.
Retention time: 3.5 min.
Detection: Fluorescence, λ ex=200nm

System XII (56)

Type of sample: plasma
Column: 150 x 4.6 mm Ultrasphere octyl (5 µm)
Mobile phase: acetonitrile: water (50:50) with 0.05% of each of tetramethylammmonium hydroxide and perchloric acid (70%)
Temperature: ambient
Flow rate: 1.0/min.
Retention time: 7.7 min.
Detection: Fluorescence, λex=320 nm; λem=440 nm

System XIII (57)

Type of sample: plasma
Column: 250 x 4.6 mm LC-1 (trimethysilyl) (5 µm)
Mobile phase: phosphate buffer (0.05 M, pH 3.0): acetonitrile (90:10) with 0.005 M of each of sodium heptane sulfonate and n-nonylamine
Temperature: ambient
Flow rate: 2.2 mL/min.
Retention time: 13 min.
Detection: electrochemical, +1.15 V

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YOHIMBINE

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From the historical point of view yohimbine is the most important of the complex indole alkaloid types. It was known since ancient times, in the curde drug bark or the impure form, as an aphrodisiac by the West African natives. It occurs in various tropical trees such Pausinystalia yohimbe Pierre and related speices, Aspidosperma quebrachoblanco Schlecht and as a minor alkaloid in some Rawaolfia spp. The first isolation of the drug was reported by Spiegel in 1896 (1) and Witkop (2) in 1943 suggested its correct constitution.

1. Description

1.1 Nomenclature

1.1.1 Chemical Name

17α-Hydroxyyohimban-16α-carboxylic acid methyl ester (3, 4).

1.1.2 Generic Names

Quebrachine, Corynine, Aphrodine (3,4,5,6).

1.1.3 Wiswesser Line Notation

a) Yohimbine base: T F6 D5 C666 E
M ON&TTTTJ T
Q UVO1 ..... (4).

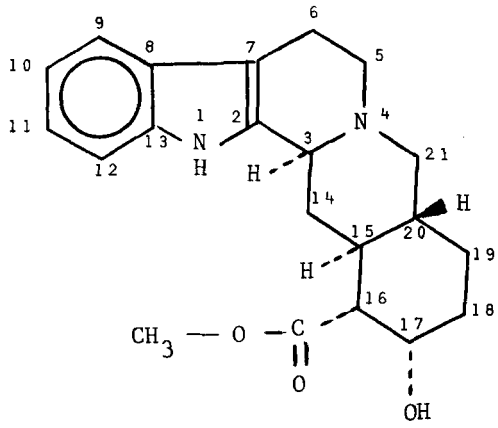
b) Yohimbine hydrochloride: T F6 D5 C666 E
M ON&TTTTJ T
Q UVO1 &GH ..(4).

1.2 Formulae

1.2.1 Empirical

$C_{21}H_{26}O_3N_2$

1.2.2 Structural



1.3 Molecular Weight

354.45

1.4 Elemental Composition

C 71.16%, H 7.39%, N 7.90%, O 13.54%.

2. Physical Properties

2.1 Color, Odour and Taste

Colorless needles when crystallized from ethanol, colorless or white in bulk. Taste is bitter, odourless (4,6,7).

2.2 Melting Point

240° - 241°.

2.3 Sublimation

Under vacuum the base sublimes at 159°/0.01 mm (7).

2.4 Solubility

Almost insoluble in water, freely soluble in ethanol, chloroform and sparingly soluble in diethyl ether (4,6).

2.5 Salts and Their Melting Point

The salts are well crystalline and the hydrochloride is colorless plates m.p. 300° - 302°C , the nitrate m.p. 269-270°C, the tartarate hexahydrate m.p. 213°C, followed by solidification and remelting at 278°C. The thiocyanate is of rectangular crystals (m.p. 233° - 234°C) and the colorless methiodide plates from acetone have the m.p. 249 - 250°C. The melting point for the O-acetate is 133°C and the O,N-diacetyl derivative melting point is 183°C (7, 8).

2.6 Specific Rotations of Base and Salts

Various specific rotations have been recorded for the base. $[\alpha]_D^{20}$ + 107.9° in pyridine; + 56 in EtOH (9); + 62.2° in EtOH (7); the hydrochloride $[\alpha]_D^{22}$ + 103.3 (H_2O) (7).

3. Spectral Properties

3.1 Ultraviolet Spectrum

The U.V. spectra of yohimbine in methanol and in 0.1 N H_2SO_4 were scanned from 200 to 400 nm using Varian DMS 90 spectrophotometer and the following maxima and (E 1%, 1 cm) values were found:

In methanol: 3 maxima at 289, 282 and 225 nm were recorded. The (E 1%, 1 cm) found were : 210 at 289 nm, 247 at 282 nm and 1079 at 225 nm. In the acidic solution a shift to lower wavelengths with some hyperchromic effect was observed (E 1%, 1 cm) at 280 nm was 225, at 272 nm was 233 and at 221 nm was 1110. Previous reports in 0.1 N H_2SO_4 (6) were as follows:

(E 1%, 1 cm) at 278 nm 204; at 272 nm 208 and 220 nm 1064. Figure 1 illustrates the UV spectrum behaviour for both the methanolic and the acidic solutions of yohimbine.

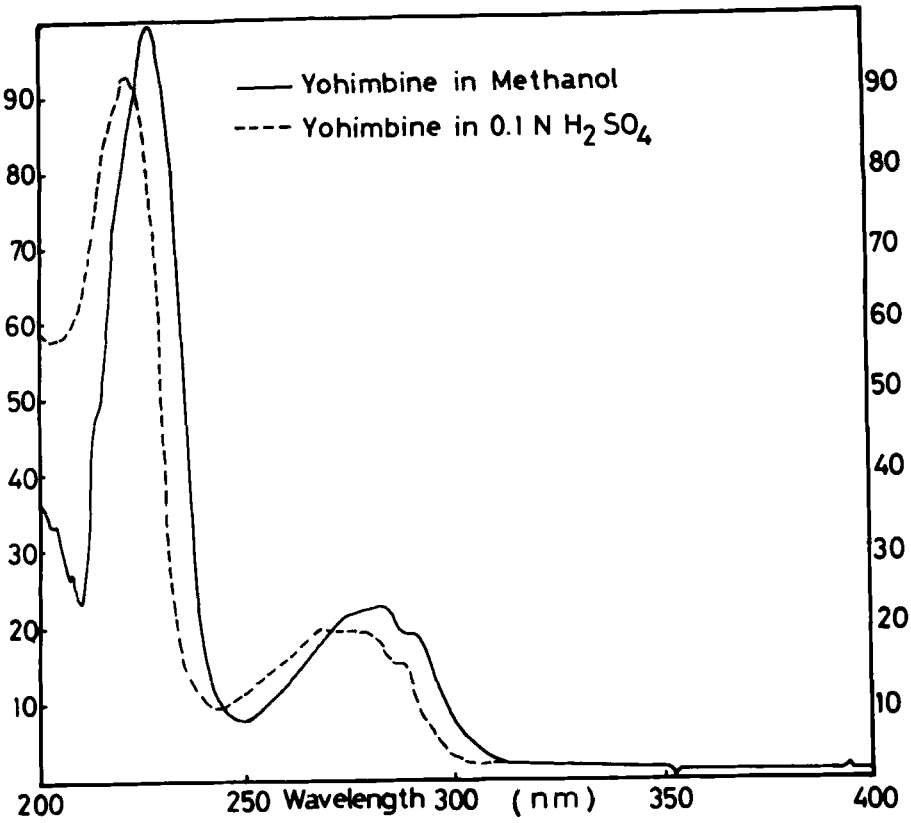


Figure 1: Ultraviolet spectrum of yohimbine in methanol and in 0.1 N sulphuric acid.

3.2 Infrared Spectrum

The infrared spectrum of yohimbine hydrochloride is presented in Figure 2. The spectrum is obtained from KBr disc on a Perkin-Elmer Infrared Spectrophotometer model 580 B. It shows the following:

Frequency (cm^{-1})	Assignments
3540	OH - stretch (free)
3190	NH - stretch
2900, 2960	CH - stretch
1730	C = O - stretch
1460, 1472, 1448	C = C - aromatic

Other characteristic absorption bands are :

1172, 1150, 1025, 1000, 970, 750 and 702 cm^{-1}.

IR data of yohimbine hydrochloride have also been reported (3, 5, 6).

Clarke (6) reported the principlal peaks at 1705, 741, 1160 or 1197 or 1436 cm^{-1}.

3.3 Nuclear Mangetic Reonance Spectra (NMR)

3.3.1 Proton Nuclear Magnetic Resonance (PMR) Spectra

The PMR spectrum of yohimbine hydrochloride in DMSO-d_6 and in DMSO-d_6 plus D_2O were shown in Figures 3a and 3b respectively. The spectra were recorded on a Varian T60 A NMR spectrometer using tetramethylsilane

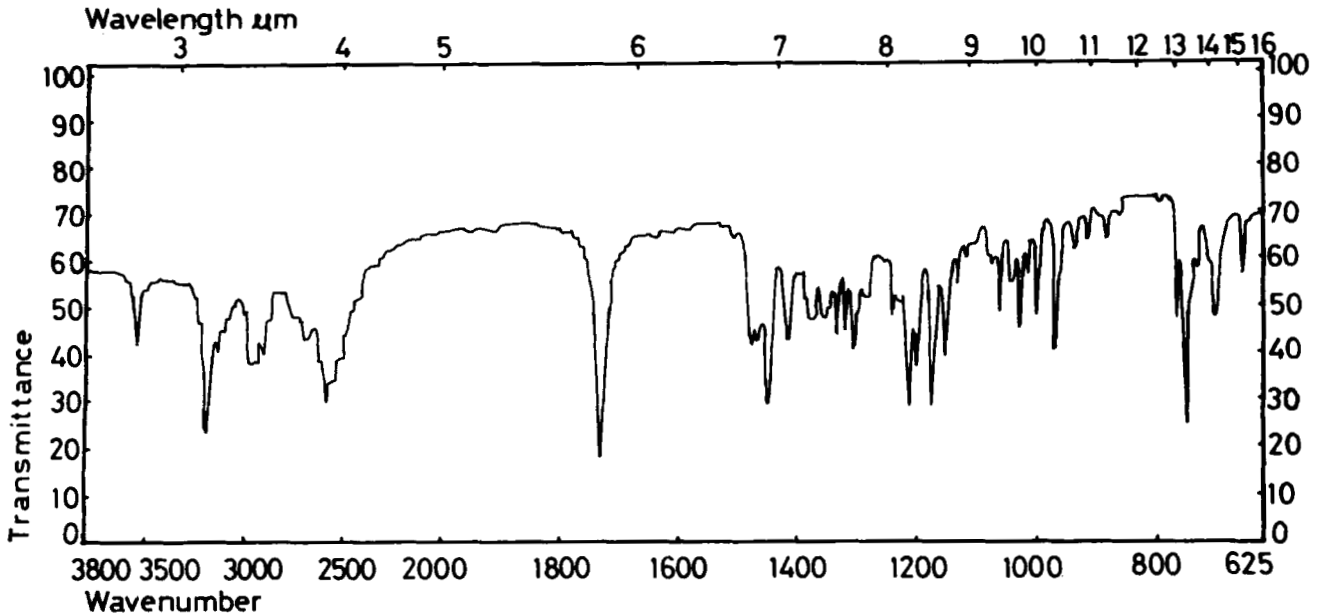


Figure 2: Infrared spectrum of yohimbine, KBr disc.

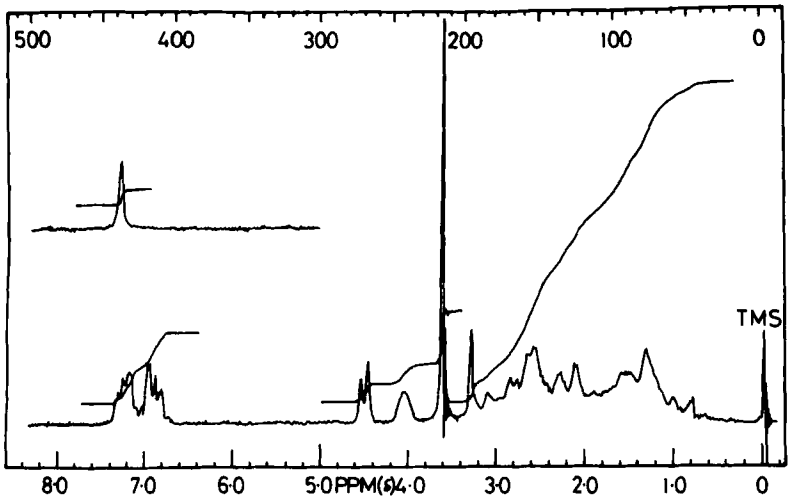


Figure 3a: Proton magnetic resonance spectrum of yohimbine hydrochloride in DMSO-d_6 using TMS as reference standard.

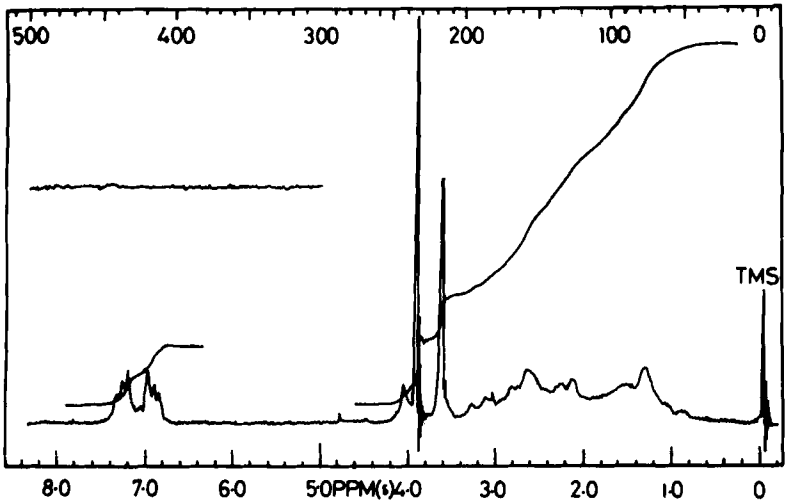


Figure 3b: Proton magnetic resonance spectrum of yohimbine hydrochloride in DMSO-d_6 plus D_2O using TMS as reference standard.

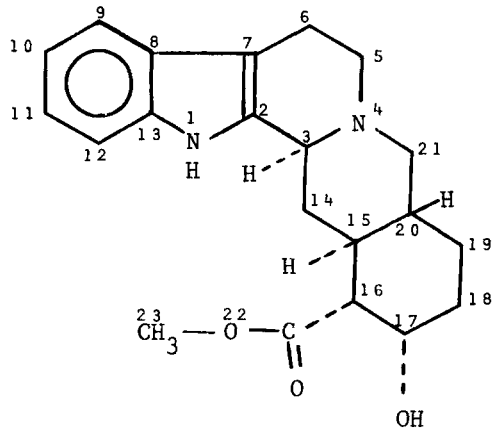
(TMS) as reference standard. The structrual assignments as follows:

Chemical shift (PPM)	Assignments
Multiplets at 0.7-4.60	CH_2 and CH protons
Singlet at 3.60	CH_3 protons
Multiplet at 6.7-7.4	Aromatic protons
Singlet at 4.05	NH (exchangable with D_2O).
Singlet at 10.65	OH (exchangable with D_2O).

Mills et al (3) have reported the proton NMR spectrum of yohimbine.

3.4 Carbon-13 Nuclear Magnetic Resonance Spectrum (C-13 NMR)

The proton-decoupled and off-resonance carbon-13 NMR spectrum of yohimbine have been determined on Jeol FX 100 MHz spectrometer at an ambient temperature and are presented in Figures 4 and 5 respectively. The sample was dissolved in deuterated chloroform and DMSO-d_6 using TMS as reference standard. The carbon assignments are based on chemical shift values and off-resonance spectrum and are listed below:



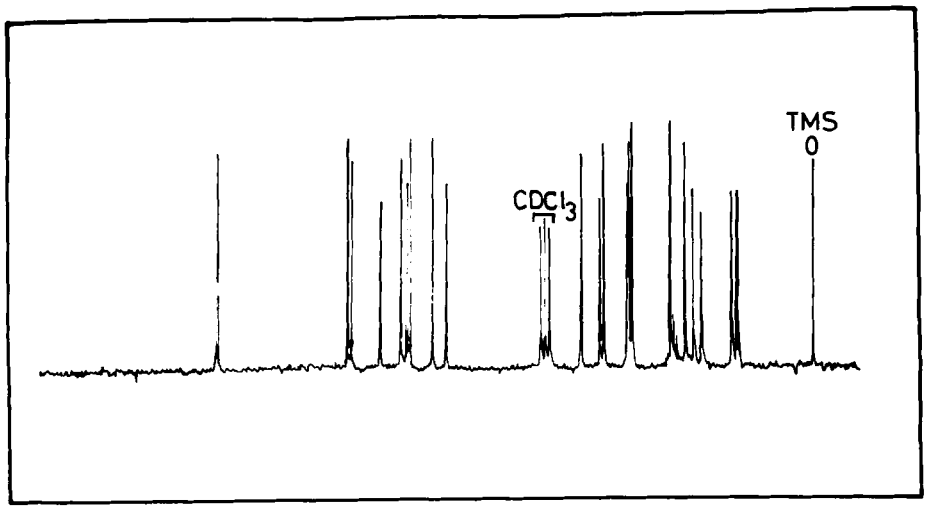


Figure 4: Proton-decoupled carbon-13 NMR spectrum of yohimbine in $CDCl_3$ and DMSO-d_6 using TMS as reference standard.

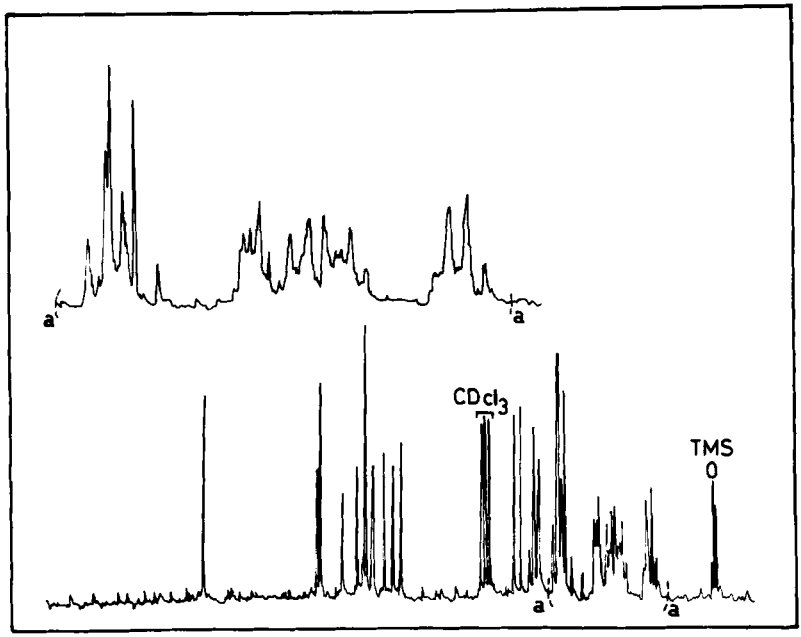


Figure 5: Off-resonance carbon-13 NMR spectrum of yohimbine in $CDCl_3$ and DMSO-d_6 using TMS a reference standard.

Chemical shift (δ)	Multiplicity	Assignment
135.1	Singlet	C_2
70.2	Doublet	C_3
52.6	Triplet	C_5
21.7	Triplet	C_6
106.9	Singlet	C_7
127.0	Singlet	C_8
117.6	Doublet	C_9
118.5	Doublet	C_{10}
120.5	Doublet	C_{11}
111.0	Doublet	C_{12}
136.1	Singlet	C_{13}
34.7	Triplet	C_{14}
36.5	Doublet	C_{15}
52.4	Doublet	C_{16}
66.8	Doublet	C_{17}
31.9	Triplet	C_{18}
23.2	Triplet	C_{19}
39.8	Doublet	C_{20}
61.3	Triplet	C_{21}
174.8	Singlet	C_{22}
51.6	Quartet	C_{23}

3.5 Mass Spectra

The electron impact (EI) mass spectrum at 70 eV recorded on Varian Mat 311 mass spectrometer and the chemical ionisation (CI) mass spectrum obtained with Finnigan 4000 mass spectrometer are shown in Figures 6 and 7 respectively. The EI spectrum (Fig. 6) shows a base peak at m/e 353 and another peak at m/e 354 (M^+) of almost equal abundance (98%), other major fragments are at m/e 184, m/e 169 and at m/e 156. The CI spectrum (Fig. 7) shows a base peak at m/e 355 ($M^+ + 1$). Other fragments are at m/e 337 and at m/e 323.

Other mass spectrum data has also been reported (3).

4. Natural Sources and Isolation

Yohimbine is the major alkaloid of the bark of Pausinystalia yohimba (Syn. Corynanthe yohimbe) and also present in the related species P. macroceras, P. paniculata and P. trillesii (f. Rubiaceae) (7,4,8). Several Rauwolfia species were reported to contain yohimbine (e.g. R. serpentina)(4,10). R. caffra (11), R. tetraphylla (12) and R. vomitoria(13), (f. Apocynaceae).

The isolation of yohimbine from the bark of species containing it as the major alkaloid can be done by the method of Le Hir et al (8), where yohimbine in a mixture with the other isomers (α-yohimbine) was boiled with d-tartaric acid, the yohimbine tartarate crystallized immediately while α-yohimbine tartarate remained in solution and crystallized later. The extraction of the alkaloids from their natural sources is generally carried out by the conventional method. The drug powder is treated with Na_2CO_3, dried and extracted with a suitable organic solvent e.g. benzene or chloroform (14,15). Le Hir et al (8) also used column chromatographic separation. Court and his students (16-20), working on Rauwolfia species, used preparative thin-layer chromatography as a routine technique for the isolation and characterisation of indole alkaloids. Although preparative HPLC was not used in the routine isolation of such compounds but the technique might be of routine use in the future.

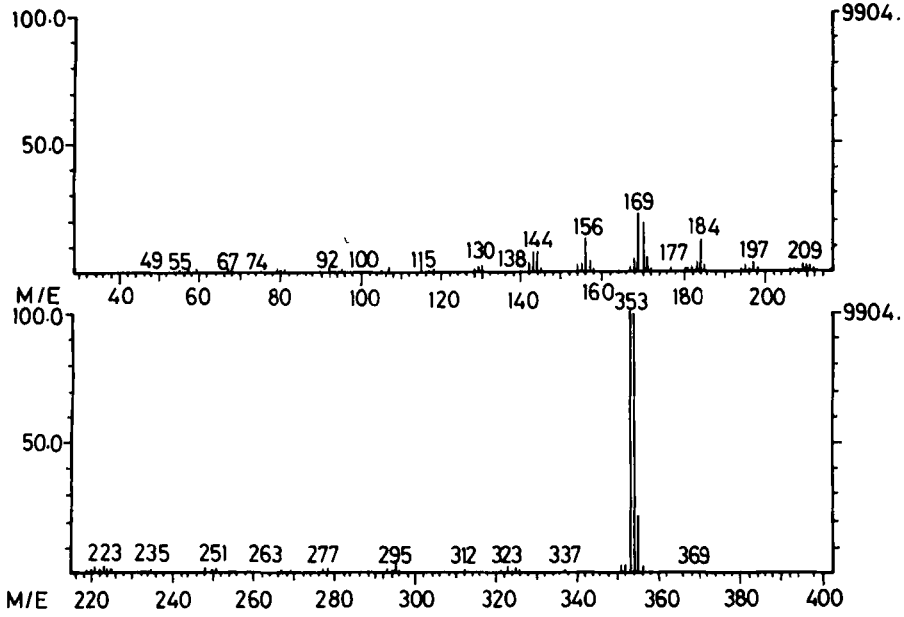


Figure 6: Electron impact(EI) mass spectrum of yohimbine.

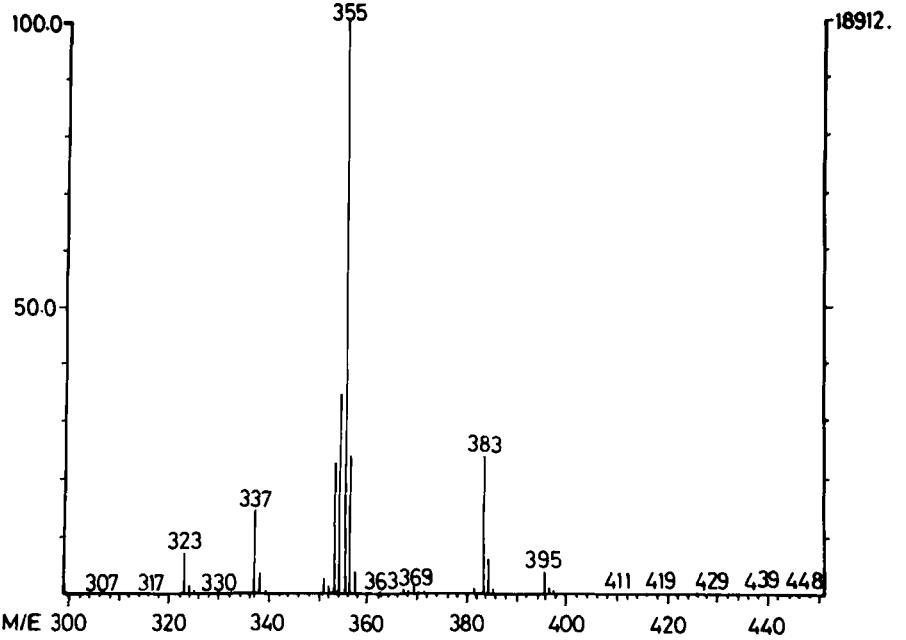


Figure 7: Chemical ionization (CI) mass spectrum of yohimbine.

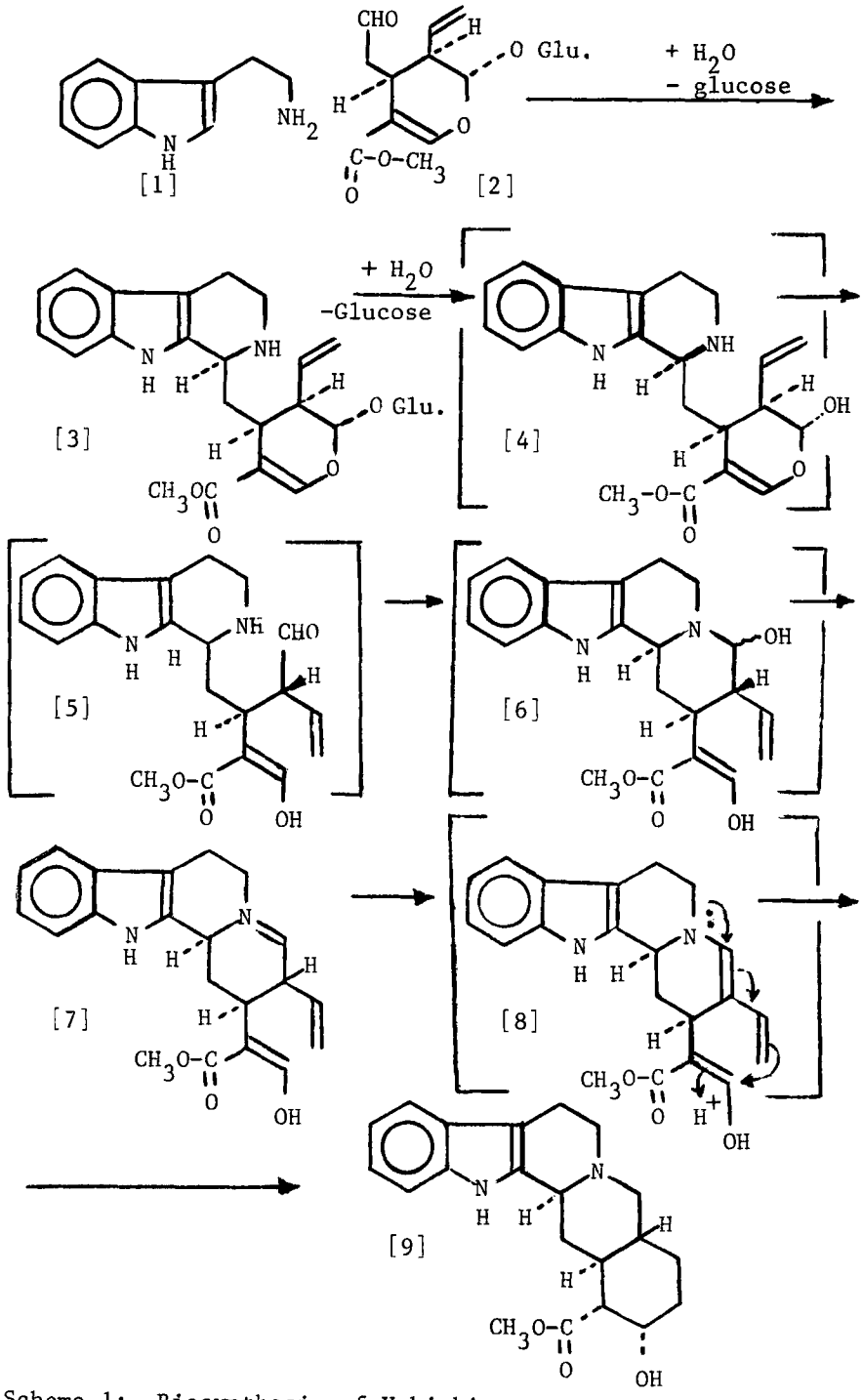
5. Biosynthesis of Yohimbine

The major features of the probable biosynthetic pathway to yohimbine were summarized by Atta-ur-Rahman and Basha (21). Scheme 1 illustrates how yohimbine is biosynthesized from tryptamine and secologanin. The condensation of tryptamine [1] with secologanin [2] is catalysed by the enzyme strictosidine synthetase which controls the C-ring closure mechanism, and results in the formation of strictosidine [3]. A second glycosidase enzyme converts strictosidine via a series of reactive intermediates [4]-[6] to 4,21-dehydrocorynantheine aldehyde [7] by D-ring closure. This aldehyde can then isomerize to [8] which cyclizes to yohimbine.

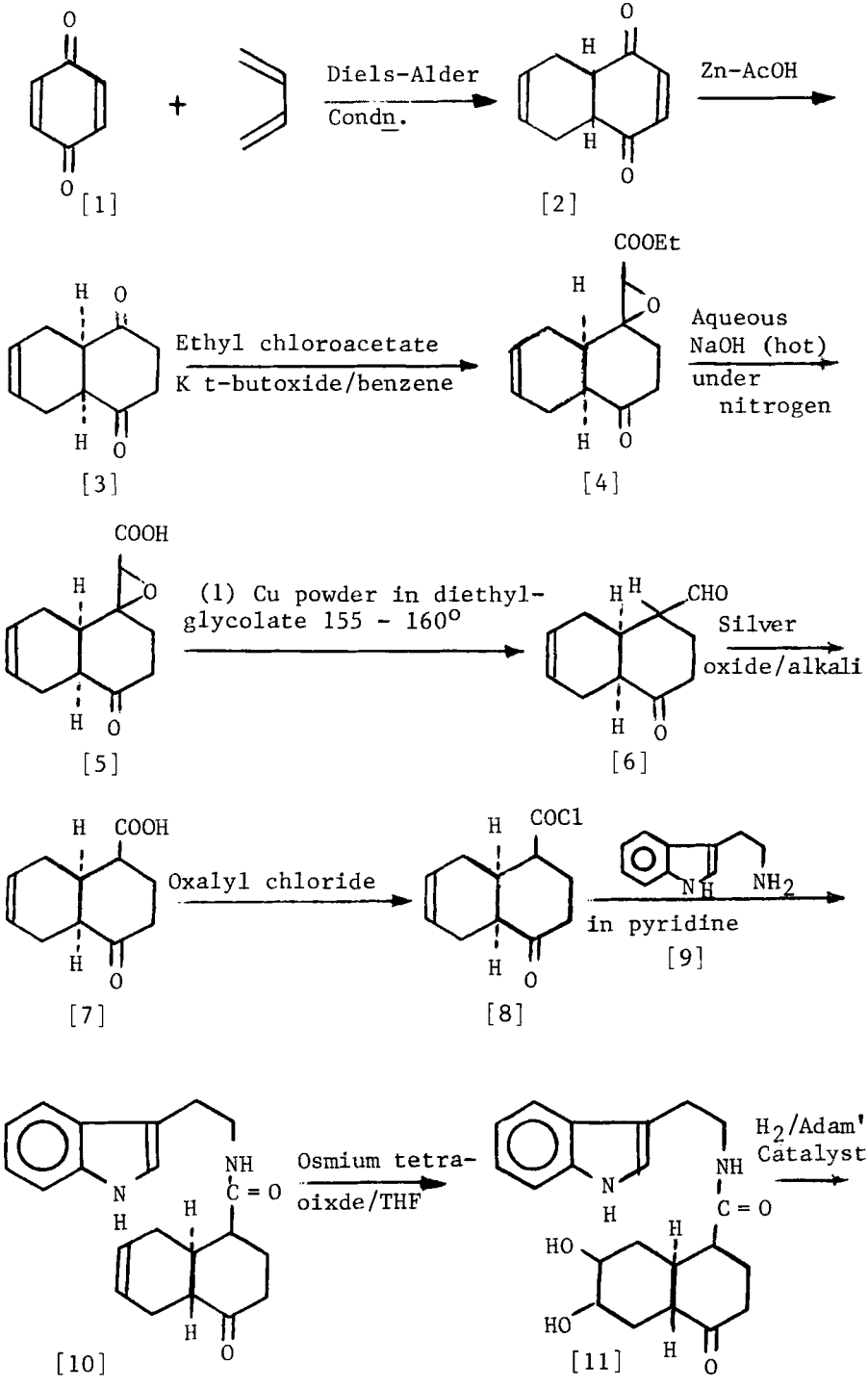
6. Total Synthesis of Yohimbine

The following total synthesis of yohimbine was described by Van Tamelen *et al* (22).

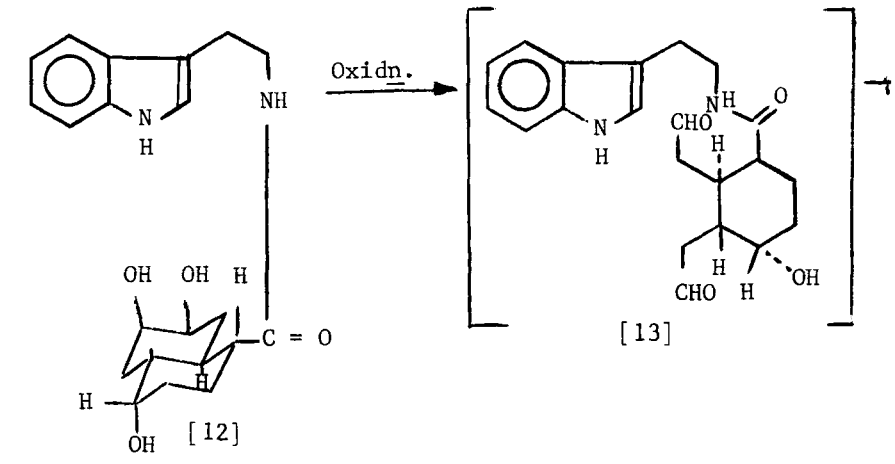
p-Benzophenone [1] was condensed with 1,4-butadiene to give the adduct 1,4,5,8,9,10-*cis*-hexahydronaphthalene-1,4-dione [2]. Selective reduction by zinc metal and acetic acid gives the octahydronaphthalenedione [3] which was converted to glycidic acid ester [4], subsequent hydrolysis to the glycidic acid [5] and then final decarboxylation to the ketoaldehyde [6]. The ketoaldehyde [6] was oxidized to the corresponding keto-acid [7] using silver oxide in the presence of alkali. The keto acid [7] was then converted to its acid chloride [8] and the latter was reacted with tryptamine [9] in the presence of pyridine yielding tryptamide [10]. The tryptamide [10] is then converted to the *cis*-diol [11] using solution of osmium tetraoxide in tetrahydrofuran added to a solution of amide [10] in pyridine-THF and cooled at dry-ice-acetone temperature. Hydrogenation of the keto-diol [11] over Adams' catalyst in ethanol gave a good yield of the desired triol [12]. Intermediate [12] was converted to the dialdehyde [13] by cleavage of the triol amide [12] in aqueous acetone and then was cyclized *in situ* using phosphoric acid and heating at 70°, where intermediate [14] was obtained. The lactol [14] was then transformed into the lactol-lactam methyl ether [15] by means of p-toluenesulfonic acid catalyzed methanolysis. Lithium aluminium hydride reduction of [15] in THF afforded the lactol ether base [16]. Hydrolysis of [16] by means of aqueous hydrochloric

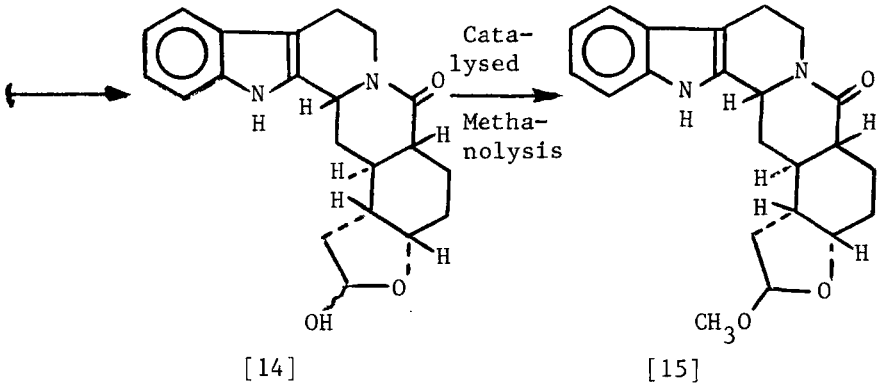


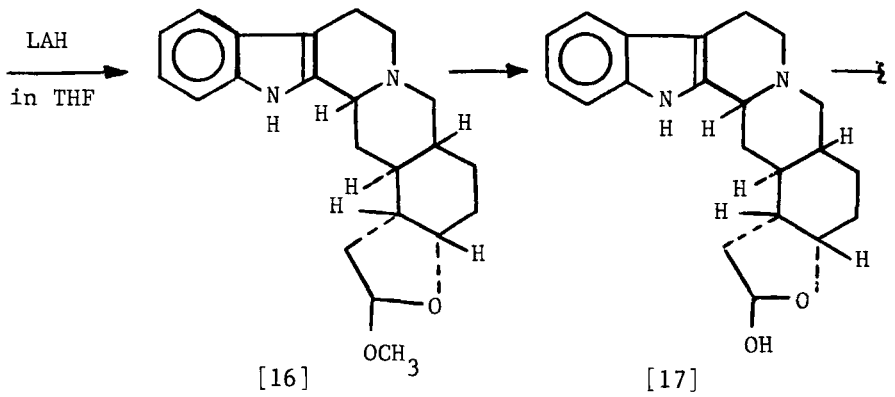
Scheme 1: Biosynthesis of Yohimbine.



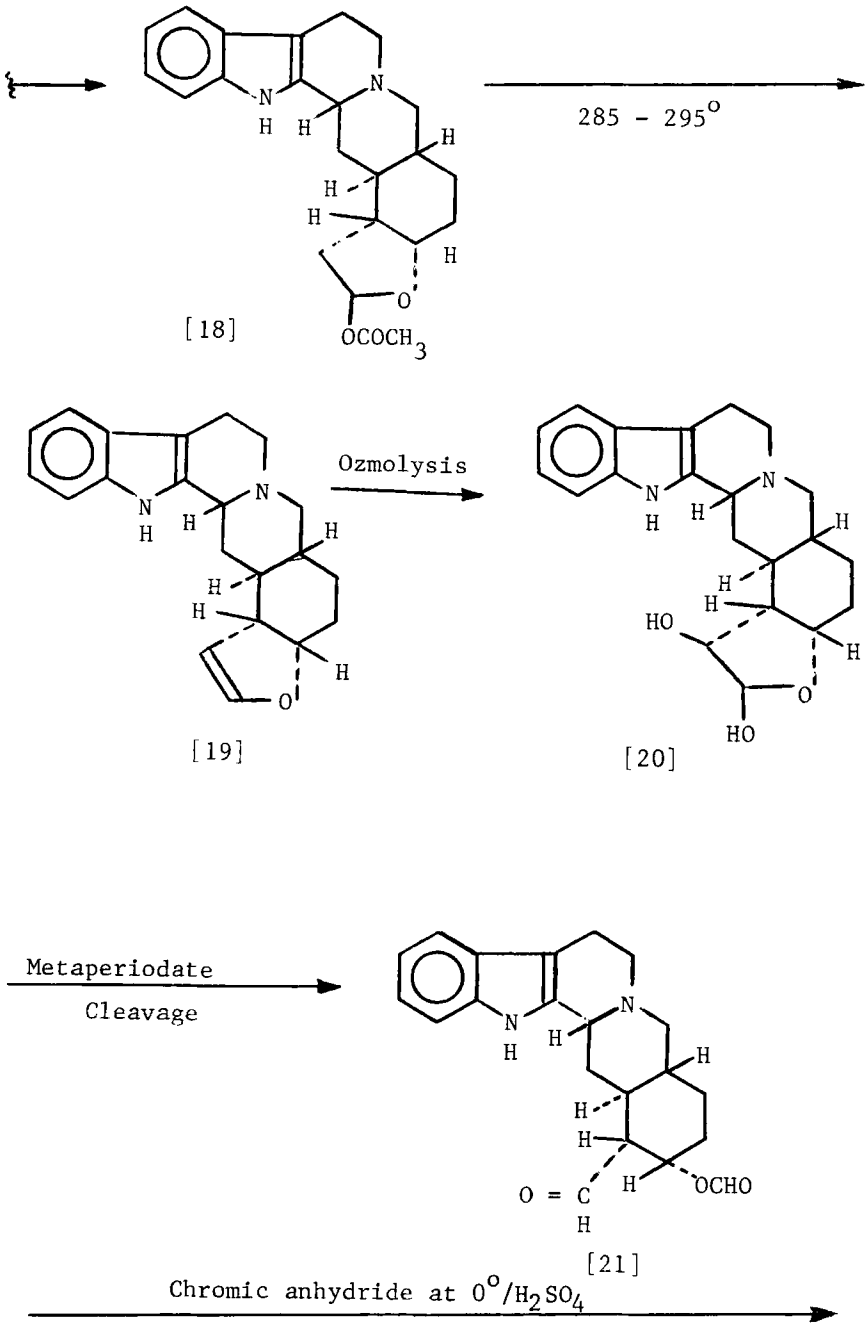
Scheme 2: Total Synthesis of Yohimbine.



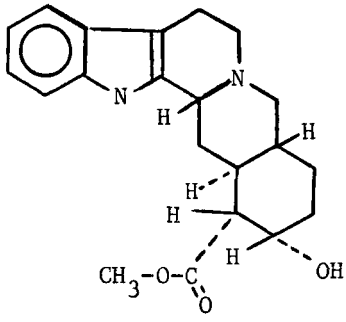




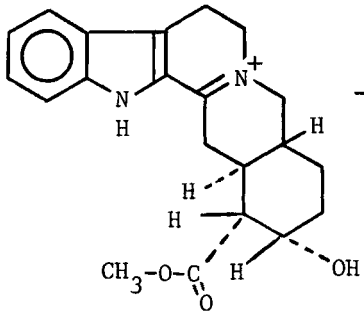
Scheme 2: Continued.

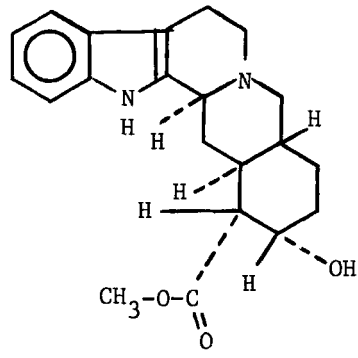


Scheme 2: Continued.



[22]





[23]

Scheme 2. Continued.

acid gave the lactol base [17]. The latter was then converted to the lactol acetate [18] (as acetate salt) by treatment with acetic anhydride-pyridine. The acetate salt [18] was subjected to pyrolysis conditions, where a limited amount of acetate salt under 0.01 mm pressure in a sublimer was exposed to a metal bath previously heated to 285-295°, where the enol ether [19] have been formed, as the acetate salt. The olifinic bond in the cyclic enol ether system was oxidized using osmium tetraoxide-periodate. The ozmylation was carried in the enol ether at -78° in THF-pyridine, where the diol [20] was obtained. Metaperiodate cleavage of 1,2-glycol [20] gave the O-formate of pseudoyohimbaldehyde [21]. The crude periodate was dissolved in methanol-acetone and treated at 0° with chromic anhydride (2.0-3.5 equiv.) in sulfuric acid for 30 minutes. After removal of the formyl group, pseudoyohimbine [22] was obtained (low yield) using alumina chromatography. The resolution of the synthetic base was accomplished by means of l-camphor sulphonic acid where pseudoyohimbine epimierizes to yohimbine [23]. Scheme 2 illustrates the sequence of synthesis described above.

7. Pharmacology and Medicinal Uses

Yohimbine produces a competitive α-adrenergic blockade of limited duration and it is relatively selective for the presynaptic α_2-receptor. At high doses it also blocks postsynaptic α-adrenergic receptors. It also blocks 5-HT receptors. It has a little effect on smooth muscle and readily penetrates the central nervous system and produces a complex pattern of responses in doses lower than the required to produce peripheral α-adrenergic blockade. These include vasopressin release and hence manifests an antidiuretic effect. General CNS excitation including elevated blood pressure, increased heart rate, increased motor activity, irritability and tremors also occured. Parentral administration may induce sweating, nausea and vomiting (23 - 33). Lecrubier *et al* (29) investigated the possible use of yohimbine to improve the condition of orthostatic hypotension induced by clomipramine. The controversial aphrodisiac property of yohimbine was recently investigated by many authors. Morales *et al* (30) reported the ability of 6 mg of yohimbine, given 3 times a day, to improve parathesia of the lower limbs in 4 patients out of 6 diabetic patients. Steers *et al* (31)

studied the pharmacological effects of yohimbine in in-vitro preparations of human and rabbit penis and in vivo on rabbits and concluded that, in the penis, yohimbine exhibits α-adrenergic blocking properties and does not affect catecholamine level in this tissue. Condra et al (32) studied the effectiveness of yohimbine in the treatment of impotence and Nakatau et al (33) have studied the pharmacokinetics of yohimbine in man. However, the possible effectiveness of yohimbine as an aphrodisiac, at least in rats, has now received support from a study carried out by Clark et al (34). The teams of Condra and Nakatau (32,33) found that in patients with clear organic impotence, treatment with yohimbine hydrochloride exhibited a positive response rate of approximately 30% and postulated that the failure to improve the conditions of the other two thirds of patients may be due to interindividual differences in the pharmacokinetics of the drug which appears to be cleared in individuals by metabolism.

8. Methods of Analysis

8.1 Qualitative Analysis

8.1.1 Color Tests

Yohimbine when reacted upon by ammonium molybdate gives a blue color that changes slowly to green (sensitivity 0.025 µg); with ammonium vanadate test it gives a blue changing to green and fading away (sensitivity is 0.1 µg). With Vitali's test the colour sequence is yellow/yellow/violet (6). Other non-specific tests are the xanthydrol reagent and 4-dimethylamino benzaldehyde in methanolic HCl.

8.1.2 Microcrystal Tests

Wachsmuth (35) determined the precipitating properties of flavinic acid (in ethanol-diethyl ether solution) on several alkaloidal and other nitrogen containing substances and reported that abundant precipitates were obtained with yohimbine (sensitivity 1:100). Habib and Aziz (36) found that yohimbine gives irregular large rosettes of curved hairs with 0.5% aqueous ammonium reineckate

which can be identified microscopically. With potassium cyanide solution, yohimbine gives rosettes of rods and with sodium carbonate solution it gives rosettes of rods or dense rosettes (sensitivity in both reagents 1:1500) (4). Characteristic crystals with different reagents obtained in our laboratory, are illustrated in Figures 8-17.

8.2 Quantitative Analysis

8.2.1 Gravimetric Methods

Raymond (11) reviewed methods used at his time in gravimetric analysis. He extracted powdered bark of yohimbehe with ether : chloroform (4:1) mixture using 10 ml of solvent for each gram of bark material Feldhoff (15) modified the techniques used before him by liberating the bases in the bark of Yohimbe with Na_2CO_3 treatment and extracting the drug in a soxhlet with di-or-trichloroethylene mixed in the receiver with aqueous oxalic acid solution. The organic solvent was evaporated, the aqueous acid solution was treated with NH_4OH solution to liberate the alkaloids which were then extracted with ether. The alkaloids were precipitated with an alcoholic solution of HCl in a beaker and the aqueous alcoholic part was washed twice with ether and methyl acetate. The secondary alkaloids remained in solution after crystallisation of yohimbine HCl. After being left 24 hours in a refrigerator, the crystalline yohimbine-HCl was filtered on a weighed filter paper, washed and dried at 102°C to constant weight.

8.2.2 Titrimetric Methods

a) Precipitation Titration

Vytras and Riha (37) carried out precipitation titrimetry for 9 alkaloids including yohimbine using sodium tetraphenylborate reagent and a Crytur valinomycin ion-selective electrode.

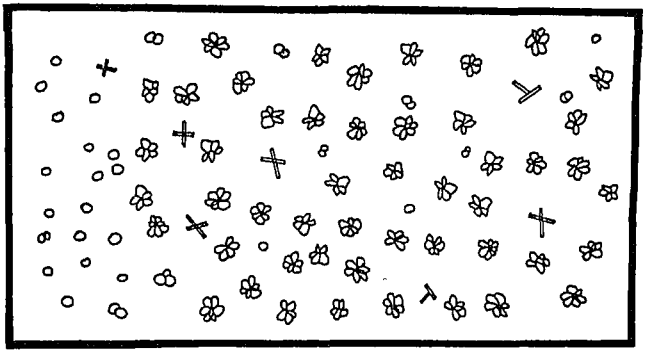


Figure 8 : Rosettes of circular plates of yohimbine with picric acide reagent (after 3 minutes).

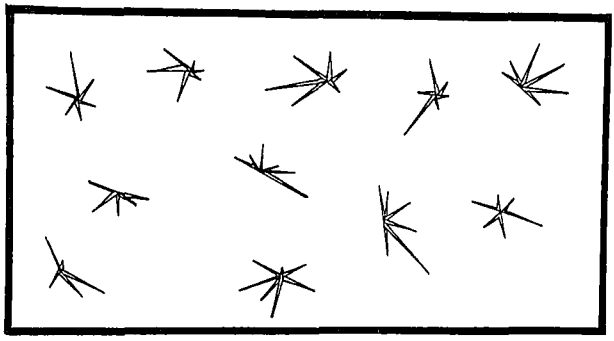


Figure 9 : Radiating pointing plates of yohimbine with mercuric chloride reagent (after 14 minutes).

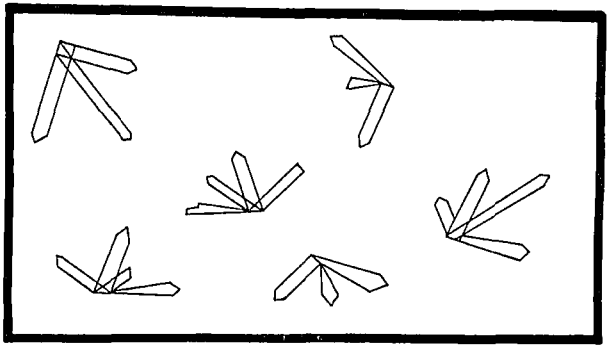


Figure 10 : Orange coloured radiating relatively wide crystal plates of yohimbine with potassium chromate reagent (after 9 minutes).

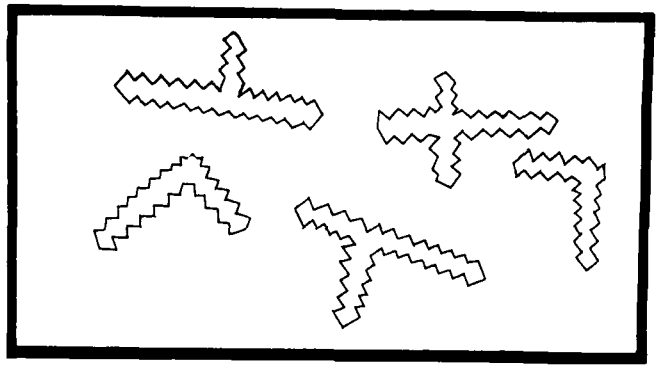


Figure 11 : Tagged plates of crystals of yohimbine with Dragendorff's reagent (after 5 minutes).

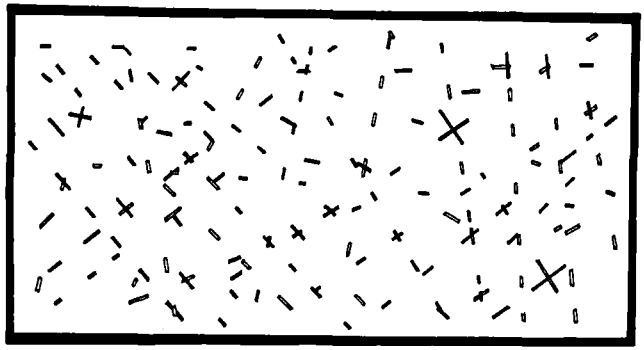


Figure 12 : White crystalline minute rods of yohimbine after reaction with lead iodide reagent (immediately produced).

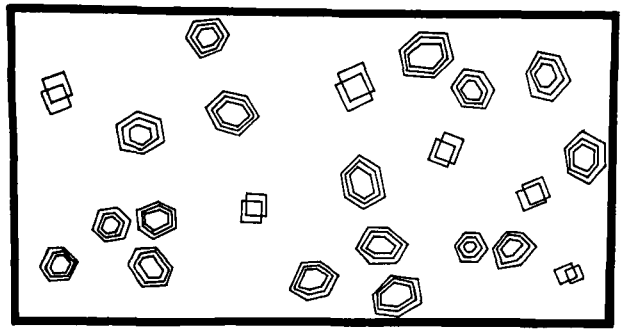


Figure 13 : Crystal of tetragonal or hexagonal structures after addition of Marme's reagent (after 5 minutes).

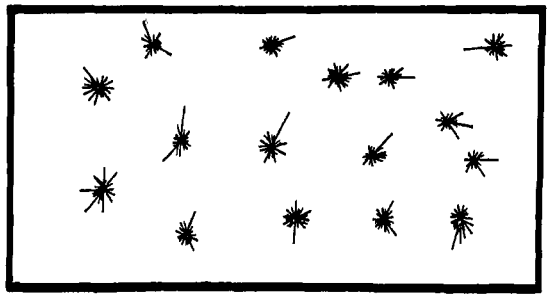


Figure 14 : Radiating rod clusters of yohimbine and Na_2CO_3 solution (after 10 minutes).

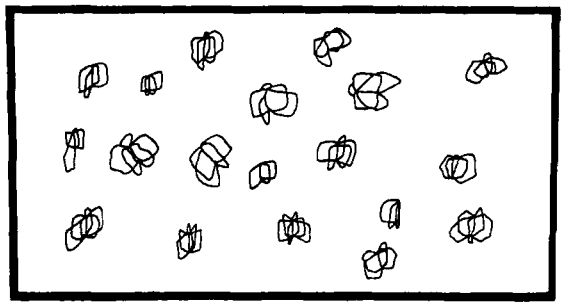


Figure 15 : Crystal plates, characteristic, to reaction of yohimbine with $NH_4S\,CN$ aqueous solution (after 5 – 7 minutes).

Figure 16 : Immediate sandy crystals with Wagner's reagent.

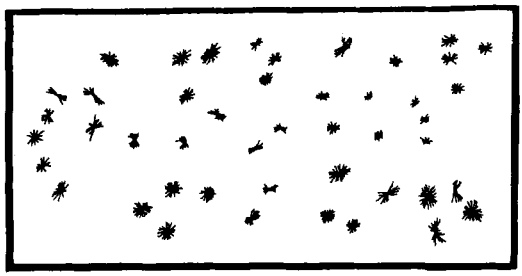


Figure 17 : Fine clusters of rods with KCN solution (after 15 minutes).

b) Micro-heterometric Titration

In their trial to analyze large organic nitrogen - containing compounds, Bobtelsky and Brazily (38) studied the behaviour of micro amounts of yohimbine together with nitron, quinone, strychnine, papaverine, nicotine, atropine, morphine or phenanthroline when titrated heterometrically with tungstosilicic, tungstophosphoric or molybdophosphoric acid at pH 1 or 7. They found that the influence of the acidity of the solution was different if the organic molecule contained 1 or 2 heterocyclic N atoms.

8.2.3 Spectrophotometric Methods

a) Colorimetry

Colorimetric assays of the weakly basic tertiary alkaloid were first done on color reactions characteristic of the indole group (8, 29). In presence of other indole containing compounds, such as those naturally present in the natural source, such a method is of little value. However, Kolsck (39) used the colour reaction of yohimbine indole nucleus with p-dimethylaminobenzaldehyde and measured the colour produced photometrically with filter S 39 or S 49. The method is summarized as follows:

Yohimbine-HCl was dissolved in water, if desired with addition of little ethanol. In a 10 ml volumetric flask to 1 ml of the solution containing 0.05 - 3 mg of the alkaloid salt, 2 ml of p-dimethylylamino-benzaldehyde were added and the mixture was left to cool for 10 minutes and 0.05 ml of a 5% H_2O_2 (or 1% $NaNO_2$) solution was added, shaken, and the color was allowed to develop. To this, distilled water was added to make 10 ml of mixture and density was measured photometrically using a filter S 39 or S 49. The mechanism of this reaction was studied by Pindur (40).

Jung assayed reserpine and yohimbine (41,42) photometrically with xanthydrol reagent in presence of other nitrogen containing compounds. The indole nucleus of the two compounds forms a colored compound with xanthydrol and the extinction is measured at 515 mu. In this method a 50-150 µg of yohimbine were dissolved in 5 ml of a freshly prepared solution of 0.4% xanthydrol in glacial acetic acid containing 1% v/v HCl heated on a boiling water bath for 15 minutes, cooled and the extinction was measured at 515 mu. The color is stable for 5 hr. The procedure can be used for tablets, injections or mixtures after extracting the alkaloid by a suitable method. The method was also used by Budavari et al (43).

The yohimbine-methyl orange complex color as a quantitative method was utilized by Mohamed and Elsayed (44) for assaying the base in tablet formulations. A solution of the powdered tablets ($\approx$ 50 mg of yohimbine hydrochloride) in 50 ml of H_2O was prepared. A measured volume containing about 300 mg of tablet base was diluted to 50 ml. A 2 ml portion of this solution was transferred to a separating-funnel containing 2 ml of buffer (pH 4.0) and 1 ml of 0.1% methyl orange solution in 20% ethanol. The yohimbine-methyl orange complex was extracted successively with chloroform, diluted to 25 ml with chloroform and the extinction was measured at 421 nm. For 5 determinations the recovery was 97.6 - 102.5%.

8.2.4 Spectrofluorimetric Methods

In their spectrophotofluorimetric study of a vast number of organic compounds of pharmaceutical interest, Underfried et al (45) using a Xe arc - source emitting a continuum from 200-800 mu in their spectrofluorometer, reported that yohimbine at pH 7 had its maximum activation at 270 mu and its maximum emission of fluorescence at 360 mu.

Chiang and Chen (46) assayed yohimbine after increasing its fluorescence in solutions by subjecting its heated solution in presence of H_2O_2 to ultraviolet light. Auterhoff and Schroeder (47) carried a similar assay and evaluated yohimbine in Yohimbe bark. They extracted 1 g of the bark with ethyl ether after alkalinisation with 10% aqueous ammonia. The ethereal extract was re-extracted with 0.1 N H_2SO_4 solution and the acid solution was basified with NH_4OH, extracted with chloroform, concentrated and diluted to 10 ml with chloroform. The chloroformic extract was chromatographed on a MN 300 Polygram cellulose plate impregnated with formamide : dimethylformamide : acetone (4:3:12) and developed with heptane : ethylmethyl ketone : 0.25% aqueous ammonia (4:2:1) for 15 cm and the zone of R_f 0.2, corresponding to yohimbine, was extracted with $CHCl_3$: CH_3OH (100 ml). The extract was filtered, the solvent removed under reduced pressure at 35°C and the residue dissolved in 10 ml of 30% acetic acid. To 1 ml of this solution, 1 ml of 3% H_2O_2 and 4 ml of 30% acetic acid were added. A similar solution of standard yohimbine was prepared. Both solutions were heated for 2 hr at 80°C, cooled and the volume was adjusted to 10 ml with 30% acetic acid. After excitation at 365 nm the fluorescence was measured at 496 nm.

Clarke (6) reported the quantitative estimation of yohimbine as follows:

The drug when in a mixture consisting of reserpine, ajmaline and yohimbine may be separated by thin-layer chromatography and determined by spectrophotometry or spectrofluorometry as described previously for indole alkaloids by some authors (48,49).

8.2.5 Chromatographic Methods

8.2.5.1 Paper Chromatography (P.L.C.)

Zaffaroni's system (50) was tried by Machovicova (51) on paper chromatography and was found to be suitable for the separation of reserpine, reserpic acid and yohimbine. Quantitation of yohimbine using P.L.C. by Auterhoff and Schroeder (47) is described in section 8.2.4 above

Clarke (6) reported the following paper chromatography systems for the separation of yohimbine:

a) Paper: Whatman No. 1, sheet 16 x 4 inch, buffered by dripping in a 5% solution of sodium dihydrogen citrate, blotting, and drying at 25° for 1 hour. (It can be stored indefinitely).

Sample: 2.5 µl of a 1% solution; in 2 N acetic acid if possible, otherwise in 2 N hydrochloric, 2 N sodium hydroxide, or ethanol.

Solvent: 4.8 of citric acid in a mixture of 130 ml of water and 870 ml of n-butanol. This may be used for several weeks if water added from time to time to keep the specific gravity at 0.843 to 0.844.

Development: Ascending, in a tank 8 x 11 x 15½ inch, 4 sheets being run at a time. Time of run, 5 hour.

Location: Under ultraviolet light, pale blue fluorescence; location reagent : iodoplatinate spray, week reaction; bromocresol green spray; weak reaction.

b) Paper: Whatman No. 1 or No. 3, sheet 17 x 19 cm, impregnated by dipping in a 10% solution of tributyrin in acetone and drying in air.

Sample: 5 µl of 1 to 5% solution is ethanol or chloroform.

Solvent: Acetate buffer (pH 4.58).

Equilibration: The beaker containing the solvent is equilibrated in a thermostatically controlled oven at 95° for about 15 minutes.

Development: Ascending. The paper is folded into a cylinder and clipped, the cylinder is inserted in the beaker containing the solvent which is not removed from the oven. A plate-glass disk thickly smeared with silicone grease may serve as a cover. Time of run, 15 to 20 minutes.

Location: Under ultraviolet light, blue fluorescence. R_f value 0.64.

c) Paper and Sample: As above in (b).

Solvent: Phosphate buffer (pH 7.4).

Equilibration: The beaker containing the solvent is equilibrated in a thermostatically controlled oven at 86° for about 15 minutes.

Development: As above in (b).

Location: As above in (b). R_f value 0.04.

8.2.5.2 Thin-Layer Chromatography (T.L.C.)

Silica gel G. plates preapred with 0.1 M NaOH or 0.1 M $KHSO_4$ developed by 5 solvent systems were used by Fike (52) in the study of the behaviour of 140 basic drugs including yohimbine and the R_f values of those drugs were recorded and successful separation was achieved but none of the indole alkaloids related to yohimbine were included in the study. However, Court and

and Habib (16) and Court and Timmins (17), in their studies on Rauwolfia alkaloids and their behaviour on T.L.C. have given valuable informations on the colour reactions and R_f values of such related alkaloids on silica gel layers employing 10 solvent systems and emphasis can be concentrated on the related alkaloids:

Yohimbine, α-yohimbine, serpentine, sarpagine and ajmalicine which come in the strongly basic fraction of Rauwolfia root extract.

Quantitation photodensitometrically on silica gel layers of alkaloids of plant sources was carried out by Massa et al (53) who found that fluorscence reflectance readings of unsprayed spots of fluorescent alkaloids were favourable in activated silica gel plates. They used a double-beam photodensitometer with recorder and integrator functioning in the visible and ultraviolet lights. Nine solvents systems were used and suitable concentrations of standards were run and the study was done on 25 alkaloids of which 14 alkaloids sprayed with Dragendorff-Schuette reagent, were detected at 400 nm and 11 others were determined by inhibition of fluorescence at 528 nm and 13 alkaloids estimated by their natural fluorescence or induced by a suitable reagent such as $KMnO_4$, Iodine or dichloroacetic acid and heat, and excited at 358 nm. However, Court and Habib (16) quantitated 10 Rauwolfia alkaloids by combined preparative T.L.C. and colorimetry and found that their method was best suitable for β-carboline alkaloids.

Clarke (6) described the following TLC system for the isolation of yohimbine:

Plate: Glass plates 20 x 20 cm, coated with a slurry consisting of 30 g of silica gel G in 60 ml of water to give a layer 0.25 mm thick and dried at 110° for 1 hour.

Sample: 1 µl of a 1% solution in 2 N acetic acid.

Solvent: Strong ammonia solution : methanol (1.5 : 100). It should be changed after two runs.

Equilibration: The solvent is allowed to stand in the tank for 1 hour.

Development: Ascending, in a tank 21 x 21 x 10 cm, the end of the tank being covered with filter paper to assist evaporation. Time of run: 30 minutes.

Location reagent: Acidified idoplatinate spray, positive reaction. R_f value 0.55.

8.2.5.3 Gas-Liquid Chromatography (G.L.C.)

Dusci and Hackett (54) described a direct procedure for analysis of yohimbine and other neutral drugs in tissues. They used Hewlett-Packard gas chromatograph model 5700A equipped with a flame ionization detector. The column was a 4 ft x 4 mm i.d. glass column pakced with 3% OV-17 on Gas Chrom Q, 80-100 mesh. The instrument settings were as follows:

Injection port temperature, 250°C; detector temperature, 300°C; nitrogen carrier gas flow rate, 60 ml/min. Oven temperature programmed as 150°C held for 2 minutes, increased at 8°C/min to 290°C and the final temperature was held for 4 minutes. Using cholesterol as a standard, the relative retention time (RRT) of yohimbine was 0.89.

Clarke (6) reported the following GC system for the separation of yohimbine.

Column: 5% SE-30 on 60-80 mesh Chromosorb W AW, 5 ft x 1/8 inch internal diameter stainless steel column.

Column temperature: 230°C.

Carrier Gas: Nitrogen.

Gas Flow: 30.7 ml per minute.

Detector: Flame ionization detector, hydrogen 22 ml per minute.

Retention time: 0.38 relative to codeine.

8.2.5.4 High-Speed Liquid Chromatography (HPLC)

The advent of HPLC in the field of separation and quantitation of almost all types of chemical mixtures made the tasks of the analytical chemists far easier than two decades ago. HPLC on alkaloids using various types of chromatographic separation modes was carried out by various authors. Yohimbine separation on adosrption HPLC was successful and Verpoorte and Svendsen (55) were able to separate it from reserpine on Mercksorb S1 70 (5 μm) column using 6 systems of eluent and a Packard Model 8200 liquid chromatograph equipped with a 254 nm and 280 nm UV detector was used. The column was a stainless steel tube (30 cm x 2 mm i.d.). The column temperature was maintained at 20°C and pressures of 50-250 kg/cm^2 were employed to control flow rates. However, the relatively high[E 1%, 1 cm]of yohimbine in U.V. light at 226 nm makes the detection of minute quantities possible if the instrument U.V. wavelength can be varied to the optimum. Rodgers (56) was able to analyse several classes of tranquilizer drugs including Rauwolfia alkaloids by this technique using adsorption columns of silica gel and cation exchangers.

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