Analytical Profiles of Drug Substances and Excipients

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PREFACE

The profiling of drug substances as to their physical and analytical characteristics remains as important today as it was when the *Analytical Profiles* series was first initiated. The compilation of concise summaries of physical and chemical data, analytical methods, routes of compound preparation, degradation pathways, and the like, is a vital function to both academia and industry.

It is certainly fair to say that workers in the field require access to current state-of-the-art data, and the *Analytical Profiles* series has always provided information of the highest quality. For this reason, profiles of older compounds are being updated whenever appropriate. The growth of the analytical methodology used to provide the data in these profiles also continues at a phenomenal rate, with many of the significant advances being chronicled in the *Journal of Pharmaceutical and Biomedical Analysis (JPBA)*. The close relationship between these flagship sources of analytical information is evident in that the undersigned is an assistant editor for *JPBA*, and that the North American editor of *JPBA* is a member of the editorial board of the *Analytical Profiles*.

The series' mission was expanded some time ago to include profiles of excipient materials, reflecting that these materials require the full degree of scrutiny historically associated with drug compounds. These highly detailed compilations of excipient properties and analytical methods have been well received by workers in the field, and such profiles will continue to be sought. In the present volume, the series' mission is further expanded to include a profile of a natural product which has been used as a precursor material in the synthesis of new drug candidates. If this information proves to be of interest to the pharmaceutical community, additional chapters of this type will be developed.

As always, the success of the *Analytical Profiles* series will continue to be based on the contributions of the chapter authors and on the quality of their work. A complete list of available drug and excipient candidates is available from the editor. We look forward to hearing from new and established authors and to working with the pharmaceutical community on the *Analytical Profiles of Drug Substances and Excipients*.

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AMOXICILLIN

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Acknowledgements

References

1. INTRODUCTION

Amoxicillin is a broad spectrum penicillin antibiotic that was first marketed by Beecham Pharmaceuticals in 1972. A co-formulation with potassium clavulanate, marketed by Beecham in 1981, extended the antibacterial spectrum to include β-lactamase producing organisms. Amoxicillin is on the World Health Organisation's list of essential drugs.

A profile of amoxicillin was published in this series in 1978 [1]. This updated profile refers to the earlier profile where appropriate but is mainly concerned with the extensive information that has become available since 1978.

Amoxicillin is used as the trihydrate in oral products and as the sodium salt in parenteral products. Many other salts that are not used clinically have been described, mainly in the patent literature.

2. DESCRIPTION

2.1. Nomenclature

2.1.1. CAS numbers

Amoxicillin	26787-78-0
Amoxicillin trihydrate	61336-70-7
Amoxicillin sodium salt	34642-77-8

2.1.2. Chemical names

The Chemical Abstracts index name is:
4-Thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,
6-[[amino(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-,
[2S-[2α,5α,6β(S*)]].

The British Pharmacopoeia prefers the simpler name:
(6R)-6-(α-D-4-Hydroxyphenylglycylamino)penicillanic acid.

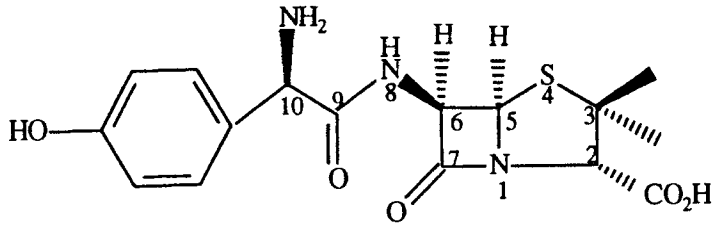
The European Pharmacopoeia, and the US Pharmacopoeia as an alternative to the CA index name, give:
(2S,5R,6R)-6-[(R)-(−)-2-Amino-2-(4-hydroxyphenyl)acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid.

Other names include:
6-[D-(−)-α-Amino-α-(4-hydroxyphenyl)acetamido]penicillanic acid and D-(−)-α-Amino-p-hydroxybenzyl penicillin. In some early literature amoxicillin was referred to as p-hydroxy ampicillin.

2.1.3. Stereochemistry

Amoxicillin has the S,R,R configuration at C2 (equivalent to C3 in conventional penicillin numbering), C5 and C6 respectively that is common to all penicillins. The side-chain configuration at C10 is R. In some of the literature this is referred to as D(−).

2.2. Structure, Formula, Molecular Weight and Elemental Composition



The structure is numbered in accordance with the CA index name. Conventional penicillin numbering, which is used in much of the amoxicillin literature, has S 1, $C(CH_3)_2$ 2, CH(COOH) 3 and N 4.

Formulae and molecular weights:

Amoxicillin	$C_{16}H_{19}N_3O_5S$	365.41
Amoxicillin trihydrate	$C_{16}H_{19}N_3O_5S.3H_2O$	419.45
Amoxicillin sodium salt	$C_{16}H_{18}N_3O_5SNa$	387.39

Elemental composition:

	%C	%H	%N	%O	%S	%Na
Amoxicillin	52.59	5.24	11.50	21.89	8.78	
Amoxicillin trihydrate	45.82	6.00	10.02	30.52	7.64	
Amoxicillin sodium salt	49.61	4.68	10.85	20.65	8.28	5.93

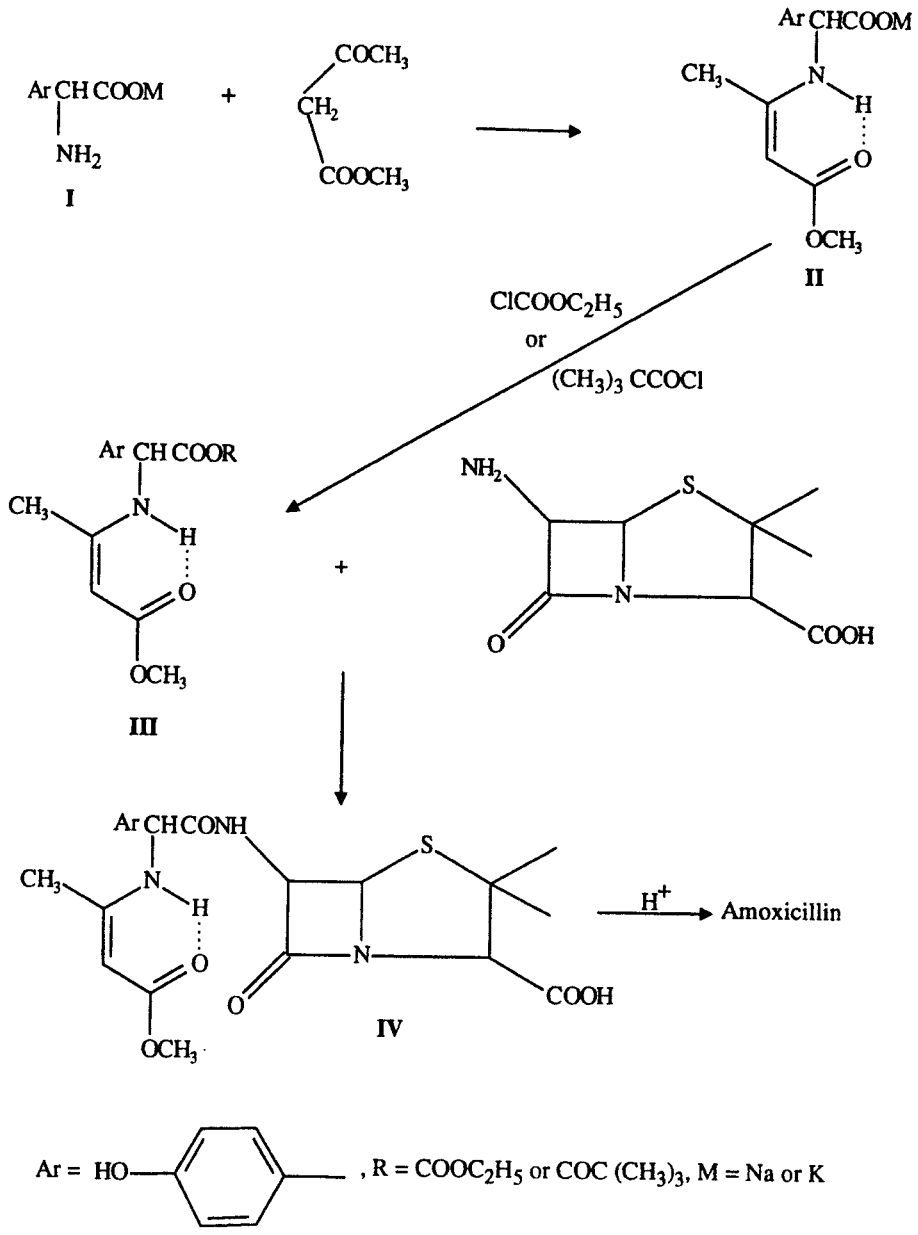
2.3. Appearance

Amoxicillin trihydrate is a white or almost white crystalline powder. Amoxicillin sodium salt is a white or almost white powder, sometimes with a pinkish tinge. As normally produced it is amorphous and hygroscopic. Freshly prepared aqueous solutions may show a transient pink colouration [2]. Both compounds may have the slightly sulphurous odour that is typical of many penicillins.

3. SYNTHESIS

Numerous patents exist describing different syntheses, or variations on existing syntheses, of amoxicillin, but the route generally used today for large scale manufacture [3] has the same chemistry as that described in the 1978 profile [1]. This is shown in Scheme 1. The amino group of D-(−)-p-hydroxyphenylglycine, I, is protected as an enamine by reaction with methylacetoacetate to give the Dane salt, II. This is isolated as a solid by solvent removal, suspended in a solvent such as acetone or dichloromethane and reacted with ethylchloroformate or pivaloyl chloride at −10 to −50°C in the presence of a basic catalyst such as N-methylmorpholine to form the mixed anhydride, III. 6-Aminopenicillanic acid, as a solution of the triethyamine salt in dichloromethane, is added and reacts to give the N-protected amoxicillin, IV. The enamine is hydrolysed by addition of water and hydrochloric acid to pH 1.5 to 2.5 at about 0°C and the dichloromethane layer is removed. Adjustment of the aqueous layer to about pH 5 (the isoelectric point) results in crystallisation of amoxicillin trihydrate. Most major manufacturers use potassium rather than sodium for preparation of the Dane salt and pivaloyl chloride rather than ethylchloroformate for the mixed anhydride [4].

Amoxicillin sodium salt is manufactured either by suspension of the trihydrate in water or a mixture of water and an alcohol such as isopropanol, dissolution by addition of sodium hydroxide solution and isolation by lyophilisation or spray drying, or by a precipitation method in which sodium ethylhexanoate in a solvent such as isopropanol is added to amoxicillin dissolved in an organic solvent by addition of an amine such as triethylamine.


Ar CH COOM
NH2
I
COCH3
CH2
COOCH3
Ar CHCOOM
CH3
N
H
O
OCH3
II
ClCOOC2H5
or
(CH3)3 CCOCl
Ar CH COOR
CH3
N
H
O
OCH3
III
+
NH2
S
N
O
COOH
Ar CHCONH
CH3
N
H
O
OCH3
S
N
O
COOH
IV
H+
Amoxicillin
Ar = HO
, R = COOC2H5 or COC (CH3)3, M = Na or K

SCHEME 1

4. PHYSICAL PROPERTIES

4.1. Solubility

The British and European Pharmacopoeias [2] give descriptive statements on solubility which are equivalent to the following approximate solubilities at 15 to 25 °C.

Solvent	Solubility (mg/ml)	
	Trihydrate	Sodium
Water	1 - 10	>1000
Ethanol	1 - 10	10 - 33
Chloroform	< 0.1	< 0.1
Diethyl ether	< 0.1	< 0.1
Acetone		0.1 - 1.0

More specific figures for the trihydrate, at an unspecified temperature, are about 4, 7.5, 3.4 and 1.3 mg/ml in water, methanol, ethanol and acetone respectively [1].

The solubility of the trihydrate in water varies with pH. A study [5] at 37°C in aqueous potassium chloride (μ 0.5) over the pH range 2 to 8 showed a minimum of about 0.013M in the pH range 4 to 6. The calculated intrinsic solubility at the isoelectric point was 0.013M (5.45 mg/ml).

4.2. Dissociation Constants and pH of Solution

The macro dissociation constants for the three ionisable groups are:

Solvent	Temp.	pK_a			Ref.
	(°C)	COOH	NH_2	OH	
Water	22	2.4	7.4	9.6	6
Aq. KCl, μ 0.5	37	2.67	7.11	9.55	5
Aq. KCl, μ 0.5	35	2.87	7.28	9.65	7
Aq. KCl, μ 1.0	23	2.63	7.55	9.64	8
Aq. KCl, μ 1.0	35	2.61	7.30	9.45	8

The four micro dissociation constants in aqueous KCl, μ 1.0, for the forms in which the carboxy group is ionised are [8]:

Temp. (°C)	pK_a			
	NH_2 (OH)	NH_2 (O^-)	OH (NH_3^+)	OH (NH_2)
23	7.58	8.49	8.70	9.61
35	7.33	8.24	8.51	9.49

The column headed NH_2 (OH) gives the dissociation constant of the NH_2 group for the form in which the carboxy group is ionised and the phenol is not ionised, etc.

The British/European and US Pharmacopoeia limits for pH of a 0.2% solution of amoxicillin trihydrate in water are 3.5 to 5.5 and 3.5 to 6.0 respectively [2,9]. Limits of 8.0 to 10.0 for a 10% solution of the sodium salt are given in the British and European Pharmacopoeias [2]. Typical values for good quality material are close to 4 and 9 for the two forms respectively.

4.3. Partition Coefficient

Apparent partition coefficients into iso-butanol (2-methylpropanol) at 37°C are:

Aqueous phase	P	Ref.
0.1M HCl	0.33	10
0.2M HCl	0.44	10
0.3M HCl	0.56	10
0.5M HCl	0.66	10
pH 7.4	0.13	11
pH 1 - 3	0.79	12
pH 5 - 6	0.13	12
pH 7 - 9.5	0.25	12

P values of 0.87, 0.024 and 0.32 for the cation, zwitterion and anion respectively were calculated from pK_a values and partition measurements into iso-butanol over the pH range 1 to 9.5 [12].

A calculated log P value of 0.87 (equivalent to a P value of 7.4) for partition into n-octanol has been reported [13]. This is clearly out of line with the measurements in iso-butanol listed above and is unlikely to be reliable because it was obtained from a correlation of HPLC retention times with measured log P values for a group of penicillins which did not include any zwitterionic compounds.

4.4. Crystal Forms

The crystal structure of amoxicillin trihydrate has been determined in a single crystal X-ray study [14]. The crystals are orthorhombic, in space group $P2_12_12_1$ with four molecules in the unit cell. The thiazolidine ring conformation has the sulphur atom out of the plane formed by the other four atoms. Comparison with the crystal structure of ampicillin trihydrate showed that the molecular packing and conformation are similar, but an additional element of three dimensional rigidity is provided by hydrogen bonding of the p-OH group to the carboxylate of a neighbouring molecule [14]. This may explain the observation [15] that when amoxicillin trihydrate was dehydrated (e.g. over P_2O_5) it retained some crystallinity, shown by X-ray powder diffractometry, and reverted to the trihydrate, shown by IR and X-ray powder diffractometry, on absorption of water vapour. By contrast, dehydration of ampicillin trihydrate gave an amorphous hygroscopic material which remained amorphous on absorption of water vapour to well above the trihydrate level.

Comparison of the crystal structure of amoxicillin trihydrate with that of ampicillin anhydrate showed [14] that the p-OH group would reduce the benzene ring overlap which contributes to the stability of the anhydrate crystal form. This is consistent with the failure of amoxicillin to crystallise under the conditions used to prepare ampicillin anhydrate [14]. However, a hygroscopic crystalline anhydrate of amoxicillin has been obtained [16], by solid state removal of methanol from a crystalline monomethanolate, but little information is available about this form.

Amoxicillin sodium salt is normally prepared in an amorphous form. An anhydrous crystalline form can be obtained [17] by removal of solvent from various solvates, either in the solid state or by displacement in solution with a solvent of lower dielectric constant.

4.5. X-Ray Powder Diffractogram

The powder diffractogram of amoxicillin trihydrate obtained with copper Kα radiation is shown in Figure 1. Diffraction lines with a relative intensity greater than 10% are listed below, with their d spacings and relative intensities.

Diffraction angle (°2θ)	d (A°)	Relative intensity (%)
12.17	7.27	35
15.13	5.85	100
16.25	5.45	22
17.20	5.15	30
18.04	4.91	91
19.33	4.59	64
19.77	4.49	36
20.20	4.39	27
21.48	4.13	39
22.01	4.04	37
22.46	3.96	23
23.05	3.86	46
23.49	3.79	58
23.82	3.73	21
25.75	3.46	65
26.69	3.34	61
27.10	3.29	23
27.90	3.20	20
28.71	3.11	73
29.47	3.03	54

The diffraction angles are close to those given in the more limited data set in [1], but some of the relative intensities are significantly different. Such differences can arise from differences in the size and orientation of particles in the sample.

Amoxicillin sodium salt, in its usual amorphous form, gives a featureless diffractogram.

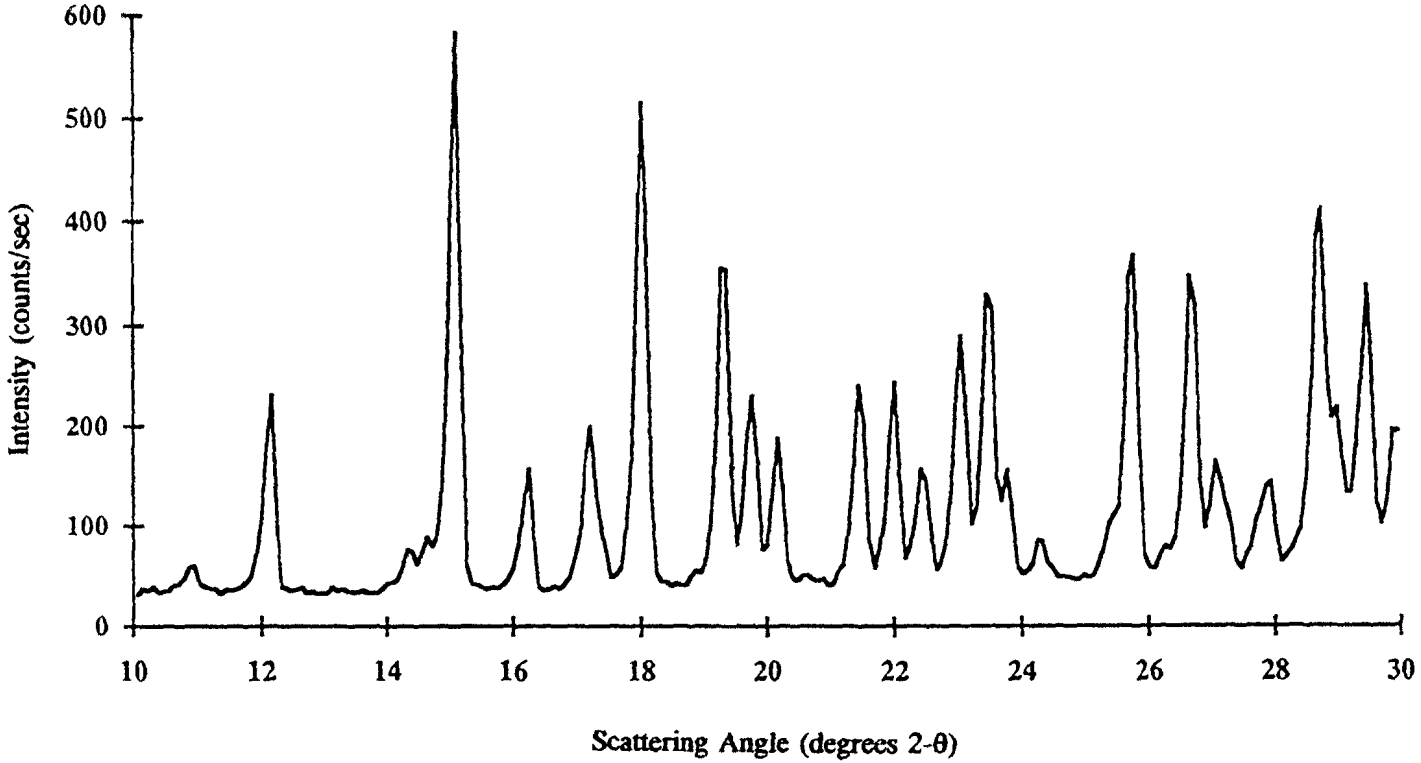


Figure 1. X - Ray Powder Diffractogram of Amoxicillin Trihydrate

4.6. Thermal Analysis

Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) of amoxicillin trihydrate showed loss of the water of crystallisation from about 50 to 150 °C followed by decomposition of the amoxicillin from about 160°C upwards [18]. DSC and TGA of amoxicillin sodium salt in an oxygen atmosphere showed loss of water at about 100°C and thermal decomposition in multiple events from about 200 to 500°C [19]. The techniques are of little practical value for these compounds.

4.7. Optical Rotation and Circular Dichroism

The British and European Pharmacopoeias [2] give specific optical rotation ranges of +290 to +315° and +240 to +290° calculated on the anhydrous basis for the trihydrate (0.2% in water) and the sodium salt (0.25% in 0.4% potassium hydrogen phthalate) respectively.

Optical rotatory dispersion and circular dichroism (CD) spectra in water were given in the 1978 profile [1], and CD results in a pH5.4 buffer [20] and in water [21] have been reported. The main features in the CD spectrum are a strong positive Cotton effect at 230 to 238nm, which is characteristic of all penicillins studied [20,21], a strong negative Cotton effect at 207 to 209nm, the origin of which is uncertain, and a weaker negative at 273 to 278nm due to the phenolic chromophore.

4.8. Ultraviolet Spectrum

The UV spectra of amoxicillin in ethanol, 0.1M KOH and 0.1M HCl were given in the 1978 profile [1]. Wavelengths and molar absorbances are:

Solvent	Wavelength (nm)	Molar Absorbance (m^2/mol)
Ethanol	230	10850
	274	1400
	281(sh)	1160
0.1M HCl	229	9500
	272	1080
	278(sh)	920

UV spectra (continued)

Solvent	Wavelength (nm)	Molar Absorbance (m^2/mol)
0.1M KOH	248	2200
	291	3000
	325(sh)	750

4.9. Infrared Spectrum

IR spectra of amoxicillin trihydrate and amoxicillin sodium salt in a potassium bromide disc are shown in Figures 2 and 3 respectively. The frequencies and assignments of significant bands are listed below.

Amoxicillin trihydrate

Frequency (cm^{-1})	Assignment
3520	Water of crystallisation, OH stretch
3458, 3175	Amide NH and phenol OH stretch
3046	Benzene ring CH stretch
2964	Methyl CH stretch
1775	β-Lactam CO stretch
1686	Amide I, CO stretch
1580	COO^- asymmetric stretch and NH_3^+ asymmetric deformation
1517	Benzene ring C=C stretch
1482	Amide II, NH bend CN stretch combination band and NH_3^+ symmetric deformation
1396, 1378	Gem dimethyl CH deformation and phenol OH combination band
1327	COO^- symmetric stretch
1314	Fused thiazolidine β-lactam skeletal mode
1283	Amide III, NH bend CN stretch in plane combination band
1250	Phenol CO combination band
1143, 1120	Benzene ring CH in plane deformation

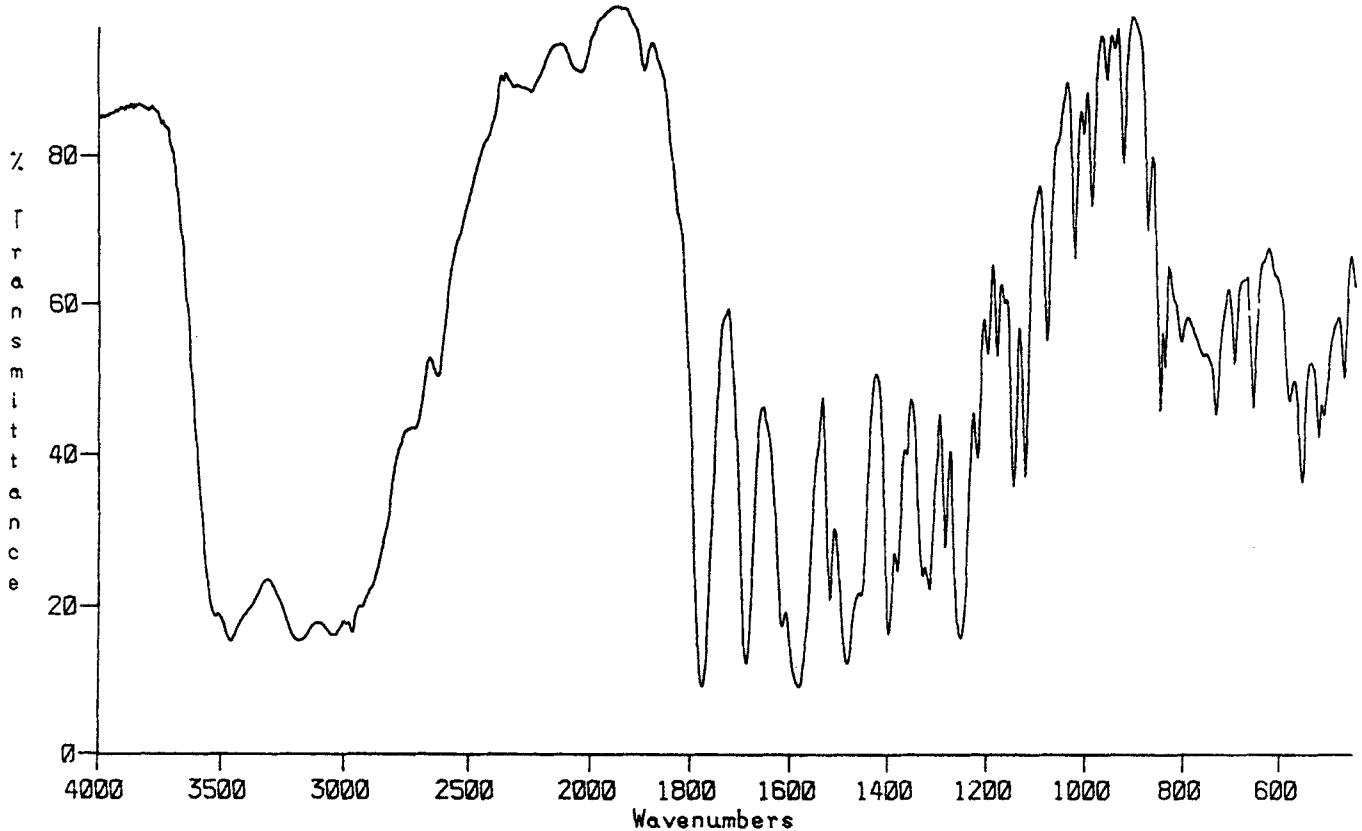


Figure 2. Infrared Spectrum of Amoxicillin Trihydrate

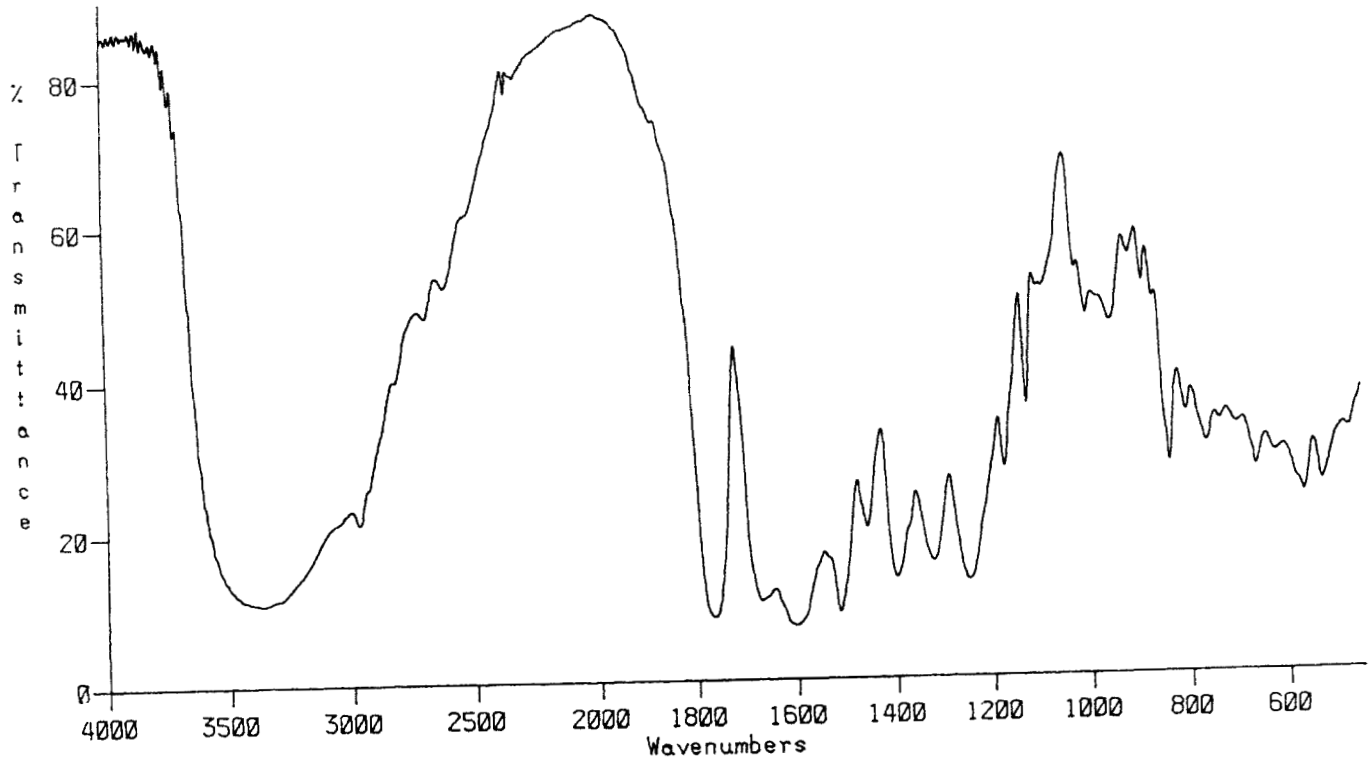


Figure 3. Infrared Spectrum of Amoxicillin Sodium Salt

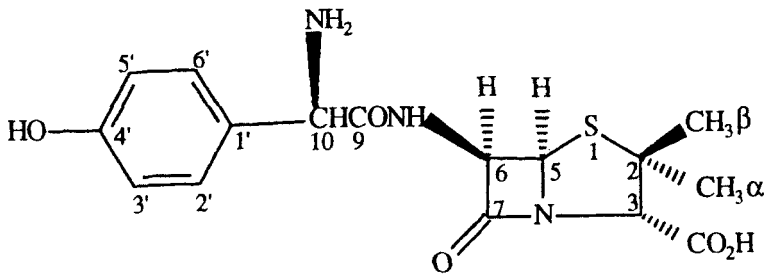
Amoxicillin sodium salt

Frequency (cm^{-1})	Assignment
3366 (v. broad)	Phenol and water OH, amine and amide NH stretches
2969	Methyl CH stretch
1764	β-Lactam CO stretch
1671	Amide I, CO stretch
1601	COO^- asymmetric stretch
1513	Amide II, NH bend CN stretch combination band and benzene ring C=C stretch
1457	Gem dimethyl CH deformation
1398	COO^- symmetric stretch and phenol OH combination band
1321	Fused thiazolidine β-lactam skeletal mode
1248	Phenol CO and Amide III, NH bend CN stretch in plane combination bands
1173,1126	Benzene ring CH in plane deformation

The water contributing to the very broad band at 3366 cm^{-1} is partly present in the sample but also comes from water uptake during grinding with potassium bromide.

4.10. Proton Nuclear Magnetic Resonance Spectrum

The 400 MHz spectrum of amoxicillin trihydrate in D_2O adjusted to pD 8 with NaOD is shown in Figure 4. Chemical shifts relative to external sodium dimethylsilapentane-1-sulphonate and assignments are listed below, using the numbers in the following structure.



Chemical shift (ppm)	Multiplicity	Assignment
1.37	Singlet	α-CH_3
1.42	Singlet	β-CH_3
4.11	Singlet	3-H
4.52	Singlet	10-H
4.68	Singlet	HOD
5.36, 5.39	Quartet, J=3.9Hz	5-H,6-H
6.80; 7.19	Double doublet	3'-H,5'H; 2'-H,6'-H

The α- and β- methyls were assigned from nuclear Overhauser effect experiments relative to 3-H. The HOD signal is due to exchange of the water, carboxy, amine, amide and phenol protons.

The chemical shifts are pH dependent. In D_2O at the natural pD [21], or with addition of a small amount of DCl [1] or trifluoroacetic acid [22], the 5-H and 6-H protons were not separated and the methyl signals were less well separated than at pD 8.

4.11. Carbon - 13 Nuclear Magnetic Resonance Spectrum

The 100 MHz C-13 spectrum of amoxicillin trihydrate in D_2O at pD 8 is shown in Figure 5. The lower trace is the full proton decoupled spectrum and the upper trace is the DEPT 135 spectrum in which signals from the quaternary carbons are suppressed. Chemical shifts relative to external dioxan at 67.4 ppm and assignments are:

Chemical shift (ppm)	Assignment
27.2	α-CH_3
31.1	β-CH_3
58.3	C10
58.7	C6
65.0	C2
67.4	C5
73.9	C3
116.8	C3', C5'
129.4	C2', C6'
131.4	C1'
157.3	C4'
175.2	C7 and COOH
176.3	C9

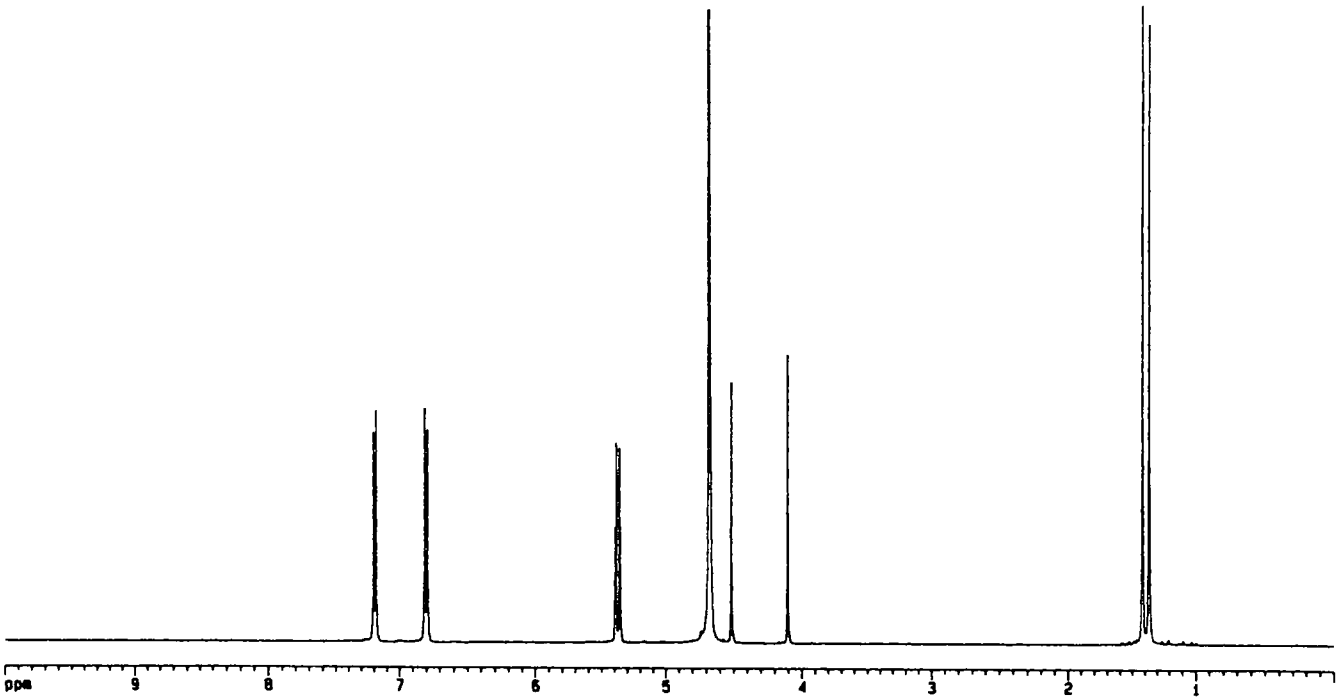


Figure 4. 400 MHz Proton NMR Spectrum of Amoxicillin in D_2O at pD 8

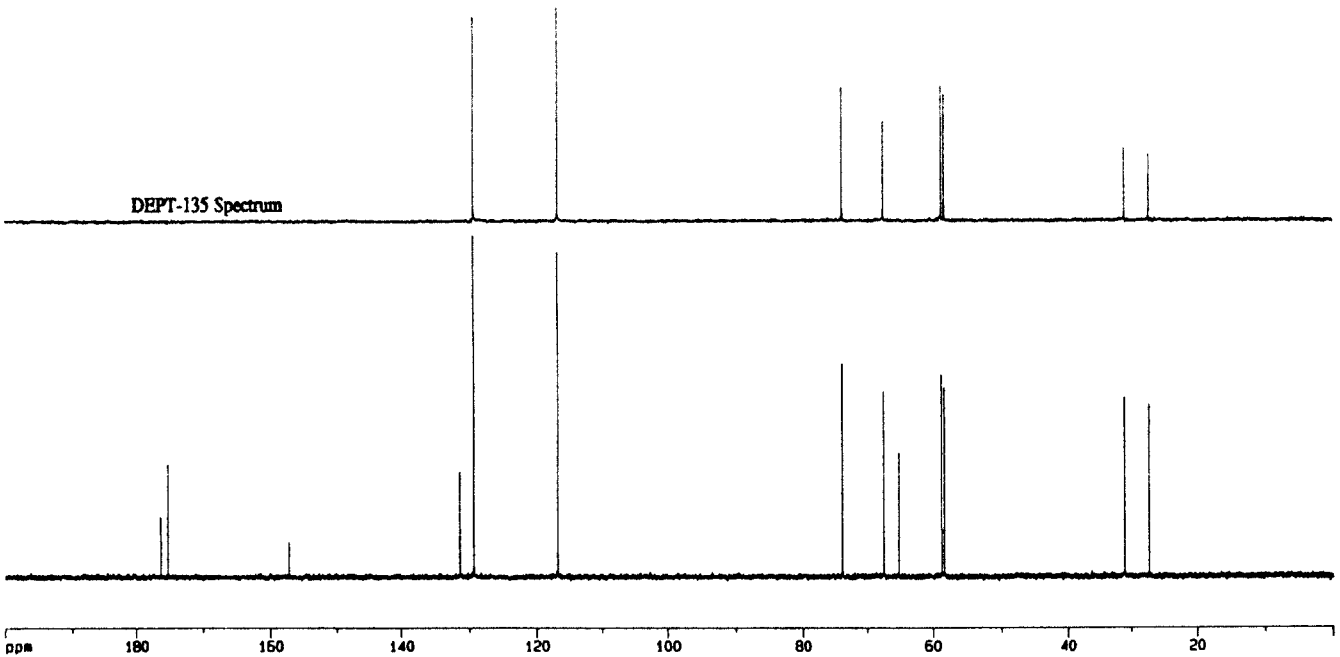


Figure 5. 100 MHz C-13 NMR Spectrum of Amoxicillin in D_2O at pD 8

The assignments are based on 2D COSY and COLOC experiments, as well as the chemical shifts and results of the DEPT spectrum. All the assignments, except C7 and C9 which are interchanged, are consistent with published values for amoxicillin trihydrate dissolved in dimethyl sulphoxide [23], although there are some chemical shift differences due to solvent effects.

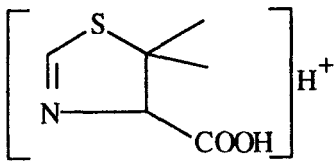
Solid state C-13 NMR spectra of amoxicillin trihydrate and other penicillins have been used to compare thiazolidine ring conformations in the solution and solid states [24]. The results indicated rapid interconversion between the S out of plane and the C3 out of plane conformations in solution, with an equilibrium ratio of 74 to 26 for solutions of amoxicillin sodium salt.

4.12. Mass Spectrum

The positive and negative ion spectra obtained from amoxicillin trihydrate by the fast atom bombardment ionisation technique with a glycerol matrix are shown in Figures 6 and 7 respectively. The glycerol ions have been subtracted. Protonated and deprotonated pseudomolecular ions are seen at 366 and 364 m/z in the positive and negative ion spectra respectively. Significant fragments in the positive ion spectrum arise by loss of the amino group (349) with cleavage across the amide (134), cleavage across the β-lactam (160) and α- to the amide (122). The only significant fragment in the negative ion spectrum, at 223, comes from a complex cleavage of the S-C_2, C_5-N and C_6-C_7 bonds. These fragmentations occur with other penicillins[25]. The structures of the fragment ions are:

349 $[M - NH_2]^+$

223 $[p\text{-}OHC_6H_4CH(NH_2)CONHCH{=}CHS]^-$

160 

134 $[p\text{-}OHC_6H_4CHCO]^+$

122 $[p\text{-}OHC_6H_4CHNH_2]^+$

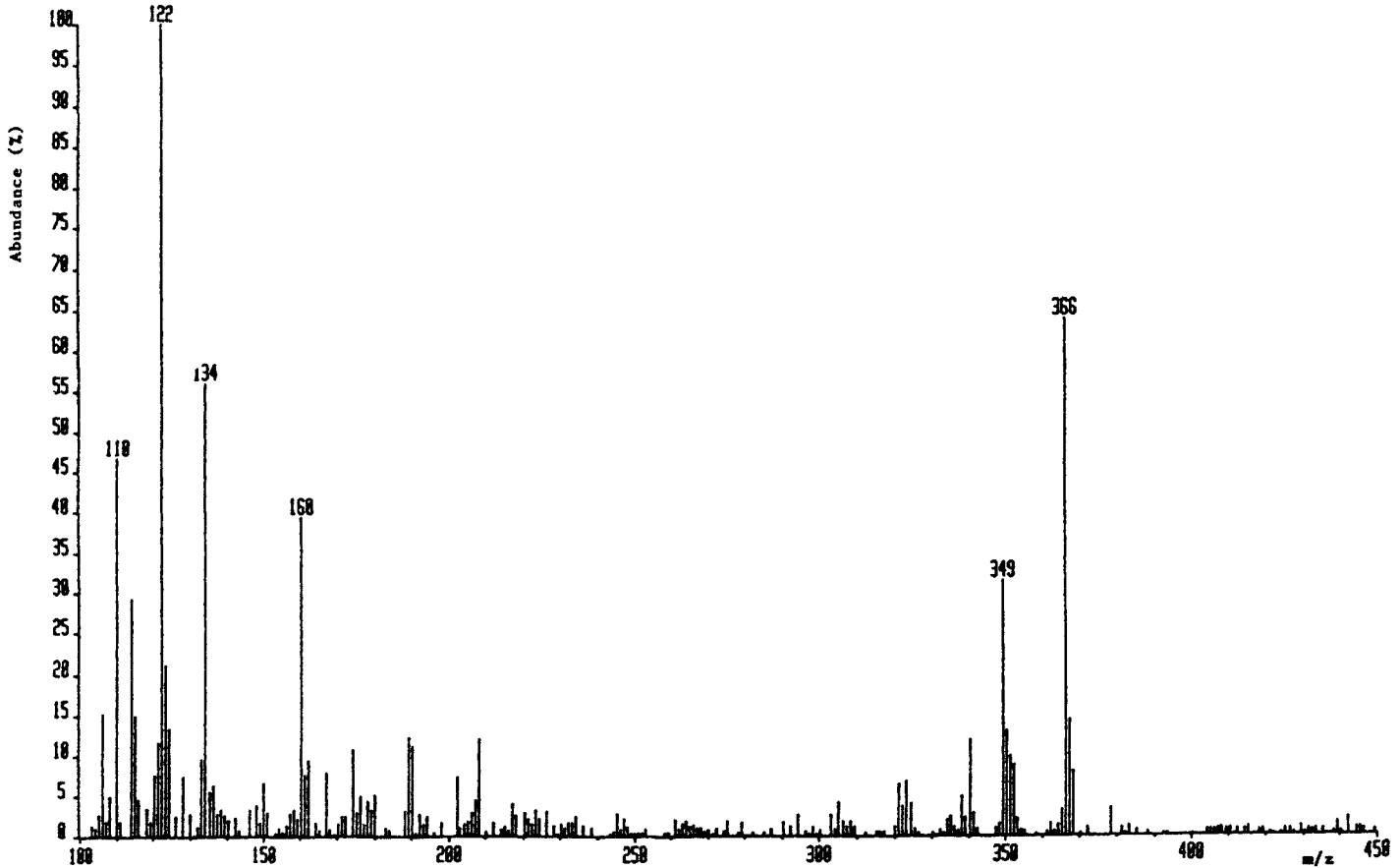


Figure 6. Positive Ion Mass Spectrum of Amoxicillin Trihydrate

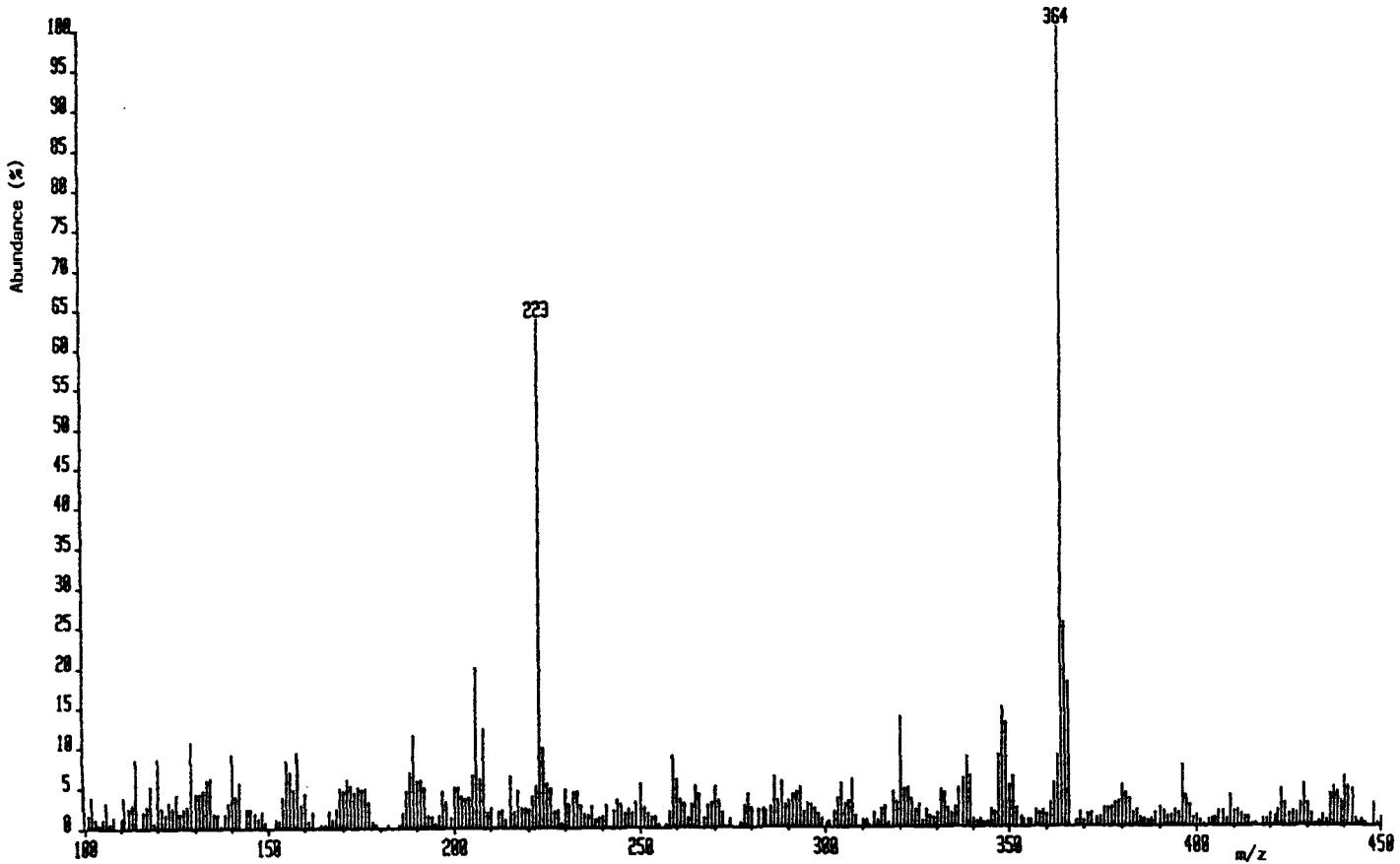


Figure 7. Negative Ion Mass Spectrum of Amoxicillin Trihydrate

Amoxicillin trihydrate did not give a useful mass spectrum by the conventional electron impact ionisation technique, even with a specialised in beam procedure which gave spectra from several other penicillins [26]. Laser desorption [27] and desorption chemical ionisation [28] both gave pseudomolecular ions and the latter technique also gave significant fragmentation.

STABILITY AND DEGRADATION PRODUCTS

5.1. Stability in Aqueous Solution

Kinetic studies of the degradation of amoxicillin in dilute aqueous solution (0.4 to 4 mg/ml) have been carried out over the pH range 1 to 10 at 35°C [5], 1.1 to 10.8 at 35°C [7], 8.2 to 12.6 at 35°C [8], 2 to 7 at 30, 40, 50 and 60°C [29] and 1.5 to 9 at 37°C [30]. At constant pH degradation followed first order or pseudo first order kinetics, with a minimum rate at about pH 6 [5,7,29]. Degradation was subject to general acid base catalysis by citrate and phosphate buffers [5,7], with a 10 fold increase in rate being ascribed to phosphate in one study [30]. Increasing ionic strength was reported to have a positive effect on degradation rate in alkali and a negative effect in acid [31].

Degradation at higher amoxicillin concentrations (25 to 125 mg/ml) at pH 8.6 to 10 and 35°C gave non first order kinetics, indicative of a dimerisation reaction [8]. Interpretation of the results indicated that dimerisation proceeds through attack by the NH_2 group of one molecule on the β-lactam carbonyl of another and is subject to general base catalysis by the NH_2 and phenolic O^- groups in other molecules. The rate of dimerisation of amoxicillin at pH 9 is greater than that of other amino penicillins due, at least partly, to this effect of the phenolic group [32].

The stability of amoxicillin sodium salt in various intravenous infusion fluids has been studied [33-35]. Degradation was faster at the higher amoxicillin concentrations [33] and particularly in fluids containing dextrose, dextran or sorbitol [33-35]. Other studies also showed a deleterious effect of carbohydrates and alcohols on the stability of amoxicillin in solution [36,37].

5.2. Stability in the Solid State

Kinetic studies of the degradation of amoxicillin trihydrate and sodium salt in sealed containers at 37, 50, 80, 90, 100 and 110°C have been reported [38]. Results for the sodium salt were interpreted as indicating a sequential two step degradation. The trihydrate showed first order kinetics at 37 and 50°C but at the higher temperatures its degradation rate was consistent with formation of a solid plus a gas. Rate constants were derived which were extrapolated to 20°C and used to calculate the time for 10% degradation as 1.25 and 3.2 years for the sodium salt and trihydrate respectively. However, these authors made no mention of the possible effect of water content which is well known to be important for the solid state stability of all penicillins.

Results consistent with sequential two step degradation were found for both amoxicillin trihydrate and sodium salt in open containers at 80 to 140°C [39]. The same author found that under controlled humidity conditions degradation was first order at 23 to 90% relative humidity (RH) and 64 to 90°C for trihydrate and at 50 to 90% RH and 40 to 70°C for sodium salt, although at 23% RH sequential reactions occurred with the sodium salt [40]. The logarithm of the first order rate constants at a fixed temperature increased linearly with RH [40] or with the logarithm of the vapour pressure [41], confirming the importance of water for the degradation of these compounds.

5.3. Degradation Products

In common with other penicillins, amoxicillin hydrolyses in aqueous alkali to a penicilloic acid, V. This has been isolated and characterised as the monosodium salt [42], which was subsequently shown [43] to have retained the 5R configuration of the parent penicillin. Epimerisation of the penicilloic acid in aqueous solution has been studied [43,44] and shown [43] to occur at C5, rather than at C6 as stated, without evidence, in [44]. The penicilloic acid can also be obtained by hydrolysis with β-lactamases.

Degradation of amoxicillin in aqueous solution containing phosphate [45], sorbitol and zinc sulphate [46] or diethanolamine and zinc sulphate [47] gave a product which was assumed from its spectroscopic properties, and by analogy with ampicillin degradation, to be the piperazine-2,5-dione, VI. This was subsequently confirmed by characterisation of the isolated product [48,49], which was shown [48] to be formed in significant amount on degradation of concentrated aqueous solutions of amoxicillin sodium

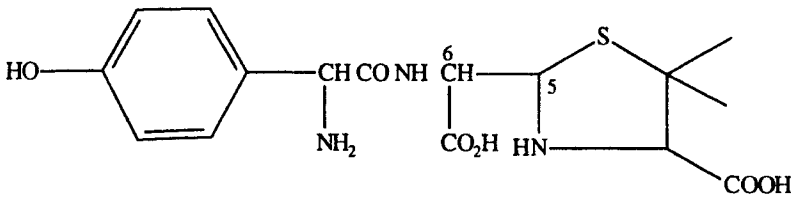
salt. The epimerisation of VI is analogous to that of the penicilloic acid [49]. The facile epimerisation of these compounds, which is characteristic of substituted thiazolidines which are not N-acylated, is significant for HPLC analysis because it produces diastereoisomers which may be resolved as separate components, thus giving a misleading impression of the number of different structural types present (see Section 7.1).

Amoxicillin dimer, present as an impurity in amoxicillin sodium salt, was isolated and characterised as VII [50]. More detailed characterisation, together with data on amoxicillin trimer, was given in [48]. Both compounds were isolated from an aged 20% solution of amoxicillin sodium salt, chromatographic analysis of which also indicated the presence of a tetramer and the penicilloates corresponding to these three oligomers.

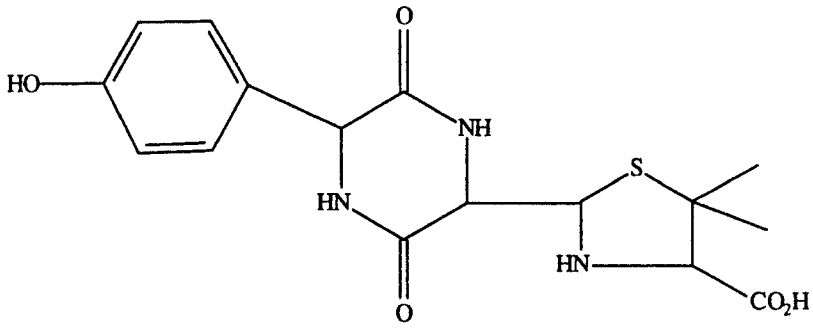
Little information is available about the structure of degradation products formed in neutral or acid solution or in the solid state. The penicilloic acid and small amounts of penicillamine (VIII, R=H) and a pyrazine (assumed to be IX, R=CH_3, but see below) were found [30] under gastrointestinal pH conditions, 1.5 increased stepwise to 8. A small amount of N-formylpenicillamine (VIII, R=CHO) was formed at pH 7 [51].

A fluorescent compound which slowly formed in amoxicillin solutions at pH 1 to 4 was identified [52] as the pyrazine, IX, R=CH_3. However, the identification, which was subsequently confirmed [53], was done on a product formed by degradation in the presence of added formaldehyde. Identity of this material with the product from degradation of amoxicillin alone was established only by thin layer chromatography. However, the methyl group in the analogous pyrazine from ampicillin has been proved to come from the formaldehyde [53], and ampicillin without formaldehyde gives a pyrazine with H in place of the methyl [54]. Consequently the pyrazine reported in [52] as being formed from amoxicillin in the absence of formaldehyde is probably IX, R=H rather than IX, R=CH_3. This is supported by the finding [29] of an HPLC peak in amoxicillin extensively degraded at pH2 to 7 with the same retention time and UV spectrum as an authentic sample of IX, R=H. Other peaks detected in this study [29] were assigned to amoxicillin penicillenic acid (X, R=NH_2) and amoxicillin penamaldic acid (XI). The validity of these assignments, which were based only on UV spectra, is questionable in view of the known instability of the penicillenic (Section 6.3.2) and penamaldic [43] acids of amino penicillins.

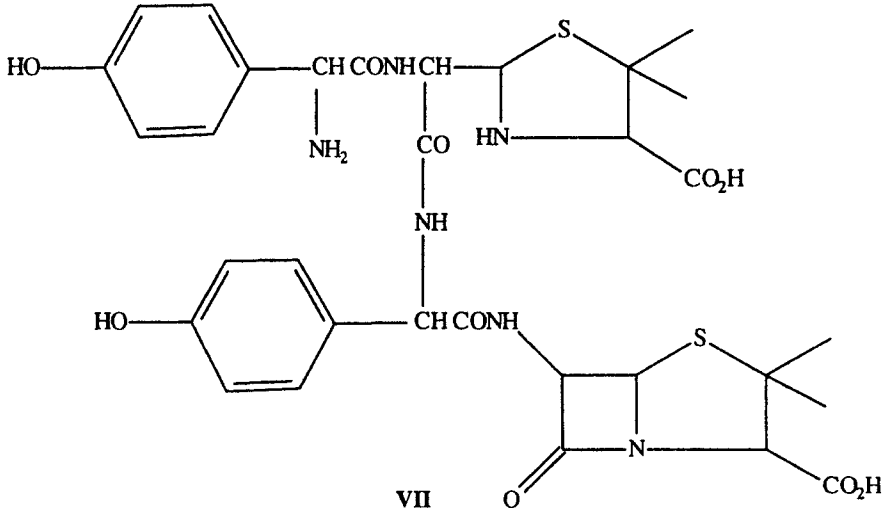
Solid state degradation of amoxicillin trihydrate and sodium salt gave qualitative increases in the piperazine-2,5-dione (VI) and in unidentified peaks which were tentatively ascribed to amoxicillin oligomers and their penicilloates [38]. Amoxicillin penicilloic acid increased in the trihydrate but not in the sodium salt.


HO
CH CO NH
NH2
6
CH
CO2H
5
S
HN
COOH

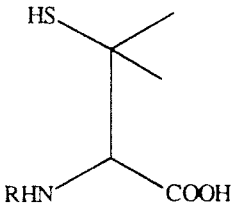
V


HO
O
NH
HN
O
S
HN
CO2H

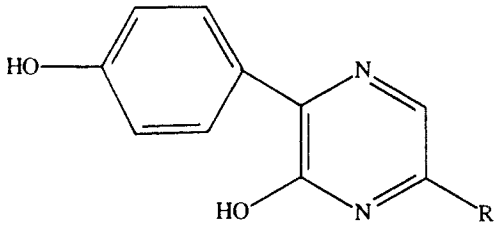
VI


HO
CH CONH CH
NH2
CO
HN
S
CO2H
NH
HO
CH CONH
S
N
O
CO2H

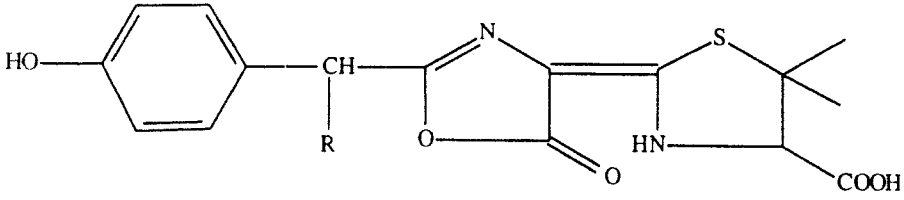
VII


HS
RHN
COOH

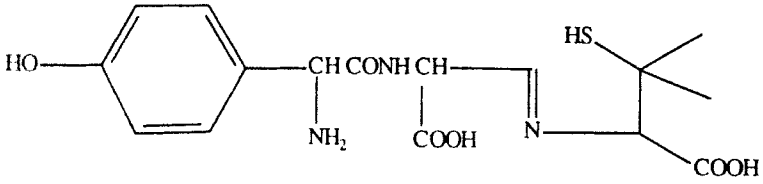
VII


HO
N
HO
N
R

IX


HO
CH
R
N
O
O
S
HN
COOH

X


HO
CH CONH CH
NH2
COOH
N
HS
COOH

XI

Degradation products may be present as impurities in amoxicillin trihydrate and sodium salt. Several samples of trihydrate contained the penicilloic acid and some also contained the piperazine-2,5-dione [55]. Both these compounds were present in samples of the sodium salt, which also contained the dimer [55].

6. METHODS OF ANALYSIS

6.1. Pharmacopoeial Methods

The US Pharmacopoeia gives an HPLC method for assay of the drug substance and for amoxicillin content in various dosage forms [9]. A different HPLC method is specified for amoxicillin content in co-formulation products with potassium clavulanate [56].

The European and British Pharmacopoeias [2] use the mercurimetric titration assay described in Section 6.2.2 for assay of both the trihydrate and sodium salt. The method is also specified for determination of degradation products in the sodium salt monograph.

Pharmacopoeial content requirements, with reference to the anhydrous substances, are:

	Trihydrate	Sodium salt
USP	90.0% min.	
BP/EP	95.0% min.	85.0% min.*

* Degradation products are limited to 9.0% maximum and the total of assay, degradation products, ethylhexanoic acid and sodium chloride to 95.0% minimum with reference to the anhydrous substance.

The BP and the Veterinary BP use the spectrophometric method involving reaction with imidazole described in Section 6.3.2 for amoxicillin content in formulated products [57,58].

Pharmacopoeial identity tests are IR for the trihydrate and TLC for formulated products in the USP (except for co-formulations with clavulanate, where HPLC is used); IR, TLC and a colourimetric test for the drug substances in the BP and EP; various combinations of IR, TLC and colourimetric tests for formulated products in the BP and BP(Vet.).

6.2. Titrimetric Methods

6.2.1. Iodometric

The classical iodometric assay of penicillins [59] is applicable to amoxicillin and was the required procedure in the US Pharmacopoeia [60] prior to introduction of HPLC in 1991. This method is based on the fact that the intact penicillin nucleus does not react with iodine, but reaction does occur after hydrolysis to the penicilloic acid. Any penicilloic acid or other iodine reactive compounds present in the sample as impurities are corrected for by a blank titration of unhydrolysed penicillin. A slight variation of the standard procedure is required for amoxicillin and ampicillin in which a small amount of HCl is added to the blank to release bound iodine which would otherwise cause false results [61]. The reaction between iodine and penicilloic acid does not have an exact stoicheiometry and results are calculated relative to the purity of a reference standard which is assayed simultaneously with the sample.

Two iodine based methods have been published for amoxicillin and other penicillins which claim an exact stoicheiometry so that a reference standard is not required. In one [62] the penicilloic acid was reacted with potassium iodate at pH 5 and the liberated iodine titrated. A sample blank could be carried out as in the conventional iodometric method. In the other [63], the penicillin in 10% HCl was reacted with iodoxybenzoate, potassium iodide added and the released iodine titrated. The same group had previously claimed exact stoicheiometry for a method in which amoxicillin in 10% HCl was titrated with dibromodimethylhydantoin [64]. Both these methods were based on oxidation of products of penicillin degradation in the strong acid conditions. No information was provided about possible interference by potential impurities and the conditions are such that inclusion of a sample blank seems to be impossible.

6.2.2. Potentiometric

Prior to the introduction of HPLC the US Pharmacopoeia included both the iodometric assay and titration of the acidic and basic functions, with a concordance requirement of not more than 6.0% difference between any pair of results [60]. Non aqueous potentiometric titrations in dimethylsulphoxide/methanol and glacial acetic acid, with lithium methoxide and perchloric acid titrants, were used for the acidic and basic

functions respectively. These titrations are not specific for amoxicillin; degradation products and other potential impurities also respond.

The mercurimetric titration method specified in the British and European Pharmacopoeias [2] is based on the fact that the intact penicillin nucleus does not titrate with mercuric ion whereas the thiazolidine ring of penicilloic acids does titrate, with a 1 to 1 mole stoicheiometry [65]. The sample is hydrolysed to penicilloic acid with alkali and titrated at pH 4.6 with mercuric nitrate. A sample blank titration of the unhydrolysed penicillin measures any penicilloic acid or other mercury titratable impurities and the purity is calculated from the titre difference. Preliminary acetylation of the amino group of amoxicillin is needed to prevent its involvement in the titration [65]. This method has good specificity for the penicillin nucleus, does not require a reference standard of the penicillin and assays for both purity and degradation products present as impurities. Different titration conditions have been investigated [66,67] and the method has been applied to amoxicillin in formulated products [68]. A comparison with the hydroxylamine colourimetric method for several penicillins including amoxicillin showed no significant difference in results [69].

Potentiometric titration of amoxicillin and related compounds with cupric sulphate using a single crystal of a cupric salt as indicating electrode has been reported [70].

6.3. Spectrophotometric Methods

6.3.1. Ultraviolet

Methods based on measurement of the second derivative peak at 280.7 nm in pH 5.8 buffer [71] and the fourth derivative peak at 308.5 nm in 0.1M NaOH [72] have been applied to assay of amoxicillin in formulated products.

The identification of thirteen penicillins including amoxicillin by their UV and derivative UV spectra has been achieved [73,74]. Similar spectra were differentiated by use of the Fast Hartley Transform.

6.3.2. Ultraviolet, of a Derivative

Degradation of amoxicillin at pH 5.2 in the presence of cupric ion gives a product absorbing at 320nm. Measurement of this was used as the assay method in the 1973 British Pharmacopoeia [75]. The product was

thought to be the penicillenic acid of amoxicillin, X, R=NH_2 [1], but this is unlikely because this compound is expected to be unstable due to facile reaction of the amino group with the oxazolone ring [76]. This is supported by failure to obtain the penicillenic acid by reaction with imidazole and mercuric chloride under conditions which gave a stable penicillenic acid suitable for assay purposes from penicillins without an α- amino group [77]. Under these conditions amoxicillin gave an unstable product absorbing at 311nm, which was postulated to be the mercaptide of the piperazine-2,5-dione, VI [77].

Acetylation of the amino group followed by reaction with imidazole and mercuric chloride gave the mercaptide of X, R=$NHCOCH_3$ as a stable product with strong absorbance at 325nm [78]. This provided a sensitive assay method (down to 0.5 μg/ml) which was specific for amoxicillin in the presence of its acid and alkali degradation products except for the dimer and higher polymers containing an intact β-lactam ring. Specificity with respect to these polymers could be obtained by preliminary treatment of the sample in 0.1M HCl at 60°C [78]. This method, without the preliminary acid treatment, was introduced into the British Pharmacopoeia in 1980 for assay of amoxicillin and its products [79] and is still used in the current edition for the products [57].

Reaction of penicillins with 1,2,4-triazole and mercuric chloride gave the penicillenic acids in a procedure that was faster and more sensitive than that with imidazole [80]. However, amoxicillin, without acetylation, gave an unstable product and the penicilloic acid interfered, giving a response equivalent to 30% of that from amoxicillin itself.

Conversion to the piperazine-2,5-dione with sorbitol/zinc sulphate [46] or diethanolamine/zinc sulphate [47], followed by measurement at 322nm in 1M NaOH, provided a stability indicating method for amoxicillin. Any piperazine dione present as impurity was corrected for by a sample blank and no interference was found from acid or alkali degradation products or from amoxicillin oligomers.

Reaction with acetylacetone and formaldehyde followed by measurement of the product at 339nm gave a method for amoxicillin in the 5 to 60 μg/ml range [81].

6.3.3. Colourimetric

The colour produced by reaction with formaldehyde and sulphuric acid or with chromotropic acid and sulphuric acid [82,83] has been used as an identity test to differentiate various penicillins and cephalosporins. Neither

procedure differentiates amoxicillin from ampicillin but the formaldehyde reaction is one of the identity tests in the British and European Pharmacopoeia amoxicillin monographs [2].

The well established hydroxylamine method for assay of penicillins [84] is applicable to amoxicillin and was one of three alternative assays in USP XX [85]. This method involves reaction of the β-lactam carbonyl with hydroxylamine at pH 7 to give a hydroxamic acid, reaction of which with ferric ion in strong acid gives a purple colour measured at 480nm. A blank in which the sample is hydrolysed with β-lactamase or alkali can be used to correct for any non-penicillin reactive compounds, but with high quality samples the blank is negligible and the USP method did not include it. Normal penicillin degradation products do not interfere, although amoxicillin dimer (VII) and other polymers with an intact β-lactam ring would be expected to respond.

Colourimetric modifications of iodometric titration methods (see Section 6.2.1) have been reported in which the excess iodine is determined at 520nm after reaction with metol and sulphanilamide [86] or in which iodine liberated by reaction of the penicilloic acid with potassium iodate is measured at 520nm [87]. These methods should retain the high specificity of the titration procedures.

Many other colourimetric methods have been published for the assay of amoxicillin, mainly in formulated products. Few of these papers include any consideration of the specificity of the method relative to amoxicillin degradation products, although in some (e.g. reaction with ninhydrin) it is obvious from the chemistry that they will respond. Methods in which degradation products (other than polymers with an intact β-lactam ring) are unlikely to interfere, based on studies in the paper or on the chemistry of the reaction, are: reaction with 2,3-dichloro-5,6-dicyano-p-benzoquinone [88], complexation with cupric acetate [89], reaction with 2-nitrophenylhydrazine [90] and reaction with 4-aminophenazone [91,92]. Methods in which degradation products are likely to respond are: heating with paramolybdate or ammonium molybdate in sulphuric acid [93,94], heating with ammonium vanadate in concentrated sulphuric acid [95], reaction with ninhydrin [96,97], reaction with p-dimethylaminobenzaldehyde [98], reaction of the phenol ring with sodium nitrite [99], extraction into chloroform with N,N-dimethyl-p-phenylenediamine [100], acid hydrolysis followed by reaction with haematoxylin and chloramine T [101] and reaction with Folin-Ciocalteau reagent [102]. Methods in which the potential for interference by degradation products is difficult to assess are: complexation with chloranilic acid [103] and reaction with dinitrostilbene [104].

6.4. Chromatographic Methods

6.4.1. Thin Layer Chromatography

The main use of TLC in analysis of amoxicillin and its formulated products is as an identity test. A major study [105] using silica gel and silanised silica gel plates with thirty five different mobile phases and iodine vapour detection produced a system, on silanised plates with ammonium acetate/acetone mobile phase, in which amoxicillin was separated from all the other penicillins studied. A slightly modified version of this system was subsequently introduced into the European and British Pharmacopoeia monographs for amoxicillin trihydrate and sodium salt [2]. The British [57,58] and US [9] Pharmacopoeias specify other TLC systems for identification of amoxicillin in formulated products. Simple TLC methods have been developed for identification of several compounds on the WHO essential drugs list, to help combat counterfeiting [106]. For amoxicillin, systems using ethyl acetate/acetic acid/water or acetone/toluene/acetic acid/water with silica gel plates were recommended.

TLC systems that separate amoxicillin from its major degradation products were used to monitor the purity of amoxicillin sodium salt during process development [107] and of isolated degradation products [48].

Penicillins on TLC plates are usually detected by iodine vapour [2,105,106] or a starch iodine spray [57,107], although amino penicillins such as amoxicillin can also be detected with ninhydrin [9,106]. Investigations of alternative detection systems claimed advantages of sensitivity, selectivity or stability of the spot for chloroplatinic acid [108], iodic acid [109] and chloranil [110].

Two unusual approaches to TLC of amoxicillin and other penicillins are the use of silica gel impregnated with tricaprylmethylammonium chloride with a methanol/water mobile phase [111], and the use of micellar solutions as mobile phase [112]. The latter was reported to give better separation of penicillins and their degradation products than organic mobile phases.

6.4.2. High Performance Liquid Chromatography

Table 1 summarises HPLC methods which have been published for analysis of amoxicillin and its impurities and degradation products in the drug substances and formulated products. Most of these methods use the reverse phase mode on C18 columns, with UV detection and a mobile phase containing a small amount of methanol or acetonitrile in phosphate

buffer at pH 4 to 6. More complex conditions, including ion pairing and post column derivatisation, have been used in a few studies, but the simpler conditions used in the first few entries in Table 1 should be adequate for most normal assay purposes.

Table 1. HPLC methods for amoxicillin, its impurities and degradation products

Sample type[a]	Method type and mobile phase[b]	Comments	Ref.
	Assay of amoxicillin content		
DS,FP	RP/C18/UV MeCN/pH5.0 PO_4	USP method	9
FP	RP/C18/UV MeOH/pH4.4 PO_4	USP, with clavulanate	9
DS,FP	RP/C18/UV MeOH/1.25% Ac	Heat degraded samples Correlation with bioassay	113
DS,FP	RP/C18/UV MeOH/pH6.0 PO_4	With clavulanate which is assayed simultaneously	114
DS,FP	RP/C18/UV and EC MeOH/H_2O/Ac	UV preferred to EC for routine assay	115
DS,FP	RP/C18/UV MeCN/pH4.1 PO_4	8 penicillins in presence of degradation products	116
DS,FP	RP/C8/UV MeOH/pH5.0 PO_4	Comparison with chemical methods	117
DS	RP/C18/post col. NaOH/$HgCl_2$/EDTA/UV MeOH/PO_4	Method for 6 penicillins	118
DS	RP/C18/UV MeOH/pH2.5 PO_4	To measure hydrolysis rate, pH2 to 7	29
DS	RP/C18/UV MeOH/pH6.5 PO_4	Stability in simulated gastric juice	30
DS	RP/C18/IP/UV MeCN/Bu_4NOH/PO_4	Stability in intravenous solutions	35

(continued)

Table 1 (continued)

Sample type[a]	Method type and mobile phase[b]	Comments	Ref.
	Assay of amoxicillin, impurities and degradation products		
DS	RP/C8/gradient/UV MeOH/pH7.0 PO_4	Penicilloic, DKP[c] and oligomers	55
DS	RP/C8/gradient/UV MeCN/PO_4	Penicilloic, dimer and polymers	50
DS	RP/C18/UV MeOH/PO_4	Penicilloic measured, other degradation products detected	119
DS	RP/C18/gradient/UV MeOH/PO_4	Solid state stability, qualitative method for degradation products	38
FP	RP/C18/gradient/UV 2 cols. in series MeOH/MeCN/PO_4	Penicilloic, 6-APA and HPG[d]	120
	Miscellaneous studies		
DS	RP/C8/UV MeOH/pH7.0 PO_4	Resolution from the side chain diastereoisomer	121
DS	RP/C18/IP/UV and RP/Ph/UV Various mobile phases	Investigation of retention mechanism	122
DS	RP/C18/UV i-PrOH/pH7.25 PO_4	Effect of temperature on separation of 6 penicillins	123
DS	RP/C18, CN and polymer/UV NP/SiO_2/UV Various mobile phases	Comparison of different column types	124
DS,FP	RP/C18/UV MeCN/MeOH/pH4.7 PO_4	Identity test, separation of 9 penicillins	125

(continued)

Table 1 (continued)

Sample type[a]	Method type and mobile phase[b]	Comments	Ref.
DS	RP/polymer/IP/UV Various mobile phases	Effect of mobile phase on retention of amoxicillin and clavulanate	126
DS	RP/C18/UV Various mobile phases	Effect of crown ether in mobile phase on retention of 8 penicillins	127
DS	RP/C18/IP/UV n-PrOH/pH8.5 borate/cetrimide	Resolution of 7 penicillins	128

a. DS = drug substance; FP = formulated product

b. RP = reverse phase; NP = normal phase; IP = ion pair; col. = column UV = ultraviolet; EC = electrochemical; MeOH = methanol; MeCN = acetonitrile; Ac = acetic acid; i-PrOH and n-PrOH = iso- and normal propanol; PO_4 = Na or K phosphate or phosphoric acid. Where mobile phase pH is not stated the original reference gives a defined composition without specifying pH.

c. DKP = amoxicillin piperazine-2,5-dione

d. 6-APA = 6-aminopenicillanic acid; HPG = p-hydroxyphenylglycine

6.4.3. Gas Chromatography

Amoxicillin is too involatile and thermally unstable to be gas chromatographed without derivatisation. Many attempts to prepare a silyl derivative for GC resulted in mixtures of compounds with different numbers of silyl residues [129]. Successful GC of the methyl ester of the N-benzoyl derivative has been reported [130].

6.5. Miscellaneous Methods

6.5.1. Electrophoresis

The related techniques capillary electrophoresis (CE), isotachophoresis (IT) and micellar electrokinetic capillary chromatography (MECC) have all been applied to amoxicillin. Studies by CE showed that several penicillins

including amoxicillin could be separated [131] and the variation of migration time with pH was established [132]. IT has been used for quantitative analysis of amoxicillin in a specific product [133] and advocated as an excellent QC tool for qualitative and quantitative analysis of β-lactams including amoxicillin [134]. MECC has been said to give better resolution of penicillins in a shorter analysis time than HPLC [135] and to separate penicillins better than CE [136]. More specifically, amoxicillin was separated from various degradation products and potential impurities, and from clavulanate, by MECC [137]. The variation of migration time with pH, and with the use of D_2O rather than H_2O in the buffer, was used to identify the compounds.

6.5.2. Polarography

Amoxicillin has been quantified in drug substance or formulated product by polarography of a pH 6.2 solution [138], an acid hydrolysis product [139] or a bromine oxidation product [140].

6.5.3. Flow injection analysis

Flow injection methods in which amoxicillin was hydrolysed by immobilised β-lactamase, the penicilloic acid reacted with iodine and the blue starch - iodine colour measured [141], and in which the red colour produced by oxidative condensation with 1-nitroso-2-naphthol in the presence of ceric ions was measured [142], have been reported. They are capable of 30 and 40 samples per hour respectively.

6.5.4. Fluorescence

The quenching of the fluorescence of mercurochrome has been used to assay amoxicillin and other penicillins [143]. The method was said to be selective for confirming potency, but the measurement was made at pH 10 so interference by the penicilloic acid, which will be formed at that pH, seems likely.

A stopped flow chemiluminescence method capable of assaying 120 samples per hour was based on inhibition of the reaction between luminol and iodine [144].

There are no reports of the assay of drug substance and formulated products by the fluorescence methods used to assay amoxicillin in biological fluids (see Section 7.2.2).

7. METABOLISM AND PHARMACOKINETICS

7.1. Identification of Metabolites

No antibacterially active metabolites of amoxicillin were detected in human urine by TLC with bioautographic detection using *Bacillus subtilis* on agar gel [145].

Amoxicillin penicilloic acid, V, was detected in human urine by TLC with iodine vapour detection and identified by co-chromatography with the product of alkaline and β-lactamase hydrolysis [146]. The identification was confirmed by other workers using TLC and HPLC [147-149].

A new metabolite detected in human urine by HPLC was identified as amoxicillin penamaldic acid, XI, on the basis of spectroscopic investigations of material, obtained by *in vitro* treatment of amoxicillin penicilloic acid, which was shown to co-elute with the metabolite on HPLC [150]. More detailed UV and NMR studies on material prepared in the same way showed that the assignment of XI was incorrect and that the new metabolite was the 5S diastereoisomer of the penicilloic acid, V, produced by epimerisation of the 5R isomer formed by hydrolysis of amoxicillin [43]. The presence of both isomers in rat and human urine has recently been confirmed by high field proton NMR [151].

Amoxicillin piperazine-2,5-dione, VI, was identified as a metabolite in human urine by co-elution of an HPLC peak with an authentic sample [152], and, in rat and human urine, by NMR [151]. The amount represented about 0.8% of the oral dose of amoxicillin [152].

NMR signals indicating the presence of the amoxicillin dimer, VII, and amoxicillin carbamate (amoxicillin with NHCOOH instead of NH_2) were seen in some samples of rat urine [151]. The latter compound is believed to be formed by reversible reaction of amoxicillin with bicarbonate present at unusually high level in some urine samples.

7.2. Assay in Biological Samples

7.2.1. High Performance Liquid Chromatography

HPLC methods used to assay amoxicillin and its metabolites in biological fluids are summarised in Table 2. The methods are nearly all reverse phase, as for assay of drug substance and formulated products, but the use of ion pairing is more common in the methods for biological fluids

where separation from endogenous components may be aided by this technique. Also, pre- or post- column derivatisation is quite widely used to improve sensitivity and/or separation from endogenous components.

Table 2. HPLC methods for amoxicillin and its metabolites in biological samples

Sample type[a]	Method type[b]	LOQ (LOD) and comments[c]	Ref.
	Assay of amoxicillin only		
S,U	RP/C18/IP/UV Pre col. triazole	0.05 μg/ml, S 0.5 μg/ml, U	153
P	RP/C18/IP/FL On line EC oxidation	0.05 μg/ml	154
P,U	RP/C18/IP/FL Post col. fluorescamine	(0.01 μg/ml, P) (0.025 μg/ml, S) Automated method	155
S,Sp	RP/C18/IP/UV	<2 μg/ml,S <0.7 μg/ml, Sp	156
P	RP/C18/FL[d] Post col. fluorescamine	0.1 μg/ml	157
S	NP/SiO_2/ H_2O-PO_4-CH_3CN/UV	Direct injection of serum	158
S	RP/C18/UV	1 μg/ml Automated method	159
P	RP/C18/IP/UV	0.1 μg/ml	160
P	RP/C8/UV	0.5 μg/ml	161
P	RP/C18/FL Pre col. NaOH/HCl/$HgCl_2$	0.01 μg/ml Complex derivatisation	162
S,U	RP/C18/UV Different mobile phase for S and U	(0.5 μg/ml, S) 100 μg/ml, U	163

(continued)

Table 2 (continued)

Sample type[a]	Method type[b]	LOQ (LOD) and comments[c]	Ref.
U	.RP/C8/UV	0.5 μg/ml	164
P,U	RP/C18/UV Post col. imidazole	(0.025 μg/ml, P) (0.2 μg/ml, U) Automated method	165
P,Sa,U	RP/C8/UV	2.5 μg/ml, P 25 μg/ml, U	166
P, rabbit	RP/C8/UV	0.5 μg/ml	167
P, bovine	RP/C18/UV	(0.1 μg/ml)	168
P,MEE, chinchilla	RP/C8/UV	0.5 μg/ml, both fluids Assay on 75 μl samples	169
S, canine, equine	RP/Ph/IP/UV	(0.05 μg/ml)	170
	Assay of amoxicillin and metabolites		
U	RP/C18/IP/UV Post col. $NaOH/NaClO_4$	(1 μg/ml, amoxicillin, pen-oics and DKP)	152
U	RP/C18/IP/UV	<5 μg/ml, amoxicillin, pen-oic and 'penamaldic'[e]	171, 172
U	RP/C18/FL Post col. fluorescamine	2.5 μg/ml, amoxicillin 5 μg/ml, pen-oic	147
U, rat	RP/C18/UV	Amoxicillin and DKP	151

a. Human origin unless otherwise stated. MEE = middle ear effusion; P = plasma; S = serum; Sa = saliva; Sp = sputum; U = urine

b. RP = reverse phase; NP = normal phase; IP = ion pair; col. = column; UV = ultraviolet; FL = fluorescence; EC = electrochemical

c. LOQ/(LOD) = limit of quantification or (limit of detection); pen-oic = amoxicillin penicilloic acid; DKP = amoxicillin piperazine-2,5-dione

d. Sep Pak cartridge used as the analytical column

e. Structure incorrectly assigned as amoxicillin penamaldic acid, see Section 7.1

7.2.2. Fluorescence methods

Spectrofluorometric methods based on degradation to a fluorescent product which is measured after extraction into an organic solvent have been described that are capable of assaying amoxicillin in plasma or urine down to about 1 μg/ml. The procedures involve vigorous heating under acid conditions with [173,174] or without [175] formaldehyde, vigorous heating in alkali without formaldehyde [176] or alkaline hydrolysis followed by mild heating with mercuric chloride [177]. Penicilloic acid present as a metabolite is corrected for in the latter method by a blank without the alkaline hydrolysis step. The possibility of interference by metabolites appears not to have been considered in the other papers, although the penicilloic acid was subsequently reported [178] to respond in the method given in [174] and in a modified version of this method [147]. The structure of the fluorescent product formed in these methods has not been established using the exact conditions of the assays, but by analogy with the behaviour of ampicillin [53,54] it is probably the pyrazine, IX, with R = CH_3 or H for the methods with or without formaldehyde respectively.

7.2.3. Miscellaneous methods

Microbiological assay by the agar plate diffusion method with a sensitive strain of an organism such as *Sarcina lutea* or *Bacillus subtilis* has been used to assay amoxicillin in biofluids [179,180,181]. However, this lengthy and not very sensitive method has been largely superceded by chromatographic or chemical methods.

An enzyme linked immunosorbent assay (ELISA) has been developed and applied to assay of amoxicillin in lung secretions in comparison with conventional bioassay [182]. The ELISA was capable of measuring amoxicillin down to 10 ng/ml, but it also responded to amoxicillin penicilloic acid and consequently gave results higher than bioassay. A commercially available ELISA system was reported to have a sensitivity of 6 ng/ml for amoxicillin in milk [183].

A method based on polarography of the product formed by alkaline hydrolysis followed by heating with formaldehyde at pH5 had a detection limit of 10 μg/ml for amoxicillin in plasma [184]. Obviously any penicilloic acid in the sample will also respond in this method.

Derivative UV spectrophotometry at 275 to 305 nm can be used to measure amoxicillin in urine down to 2 μg/ml [185], but the possibility of interference by the penicilloic acid and other metabolites appears not to have been investigated.

Amoxicillin and its penicilloic acid and piperazine-2,5-dione metabolites have been quantified in human and rat urine by high field proton NMR [151]. The penicilloic acid has also been assayed by iodometric titration [146,186] and by spectrophotometry after separation by TLC [148].

7.3. Pharmacokinetics

The pharmacokinetics of amoxicillin after oral [187,188] and parenteral [188,189] administration to humans, and after oral administration in combination with potassium clavulanate [190], have been reviewed. These reviews also cover antibacterial activity and therapeutic use.

Amoxicillin is active against a wide range of gram positive and gram negative pathogens, but is inactivated by β-lactamase producing bacteria. However, it is active against such bacteria when co-administered with the β-lactamase inhibitor, potassium clavulanate [190].

Amoxicillin is well absorbed when given orally, with a bioavailability that appears to be much higher than expected in the light of its physico-chemical properties and the pH partition theory [191]. Numerous studies show recovery of intact amoxicillin in urine after oral administration in the range 43 to 80 % after 6 to 8 hours, with most figures in the upper part of this range [147,171,172,187,188,190]. An additional 10 to 25 % of the dose appears in urine as the penicilloic acid [147,148,152,171,172,187] with a ratio of about 2 to 1 of the 5R to 5S isomers [152,171,172]. Amoxicillin is extensively distributed in body tissues and fluids, with adequate levels for antibacterial activity in most of them [187,188,190]. The half life in serum is about one hour and is the same for oral, intramuscular and intravenous administration [187,188,190]. Co-administration with potassium clavulanate does not affect the absorption, distribution and excretion of amoxicillin [190].

Recent studies have investigated the non-linearity of disposition kinetics after intravenous administration using a rat model [192] and the nature of oral absorption [191,193]. The intravenous study indicated that non-linearity arises from renal elimination mechanisms, possibly saturation of active tubular secretion or reabsorption at high doses. Oral studies in the rat [193] showed that the plasma level curve is best fit by a combined zero and first order kinetic function, which also gives a good fit for human plasma level data. This appears to be consistent with a study of absorption kinetics in humans which provided evidence for saturable carrier mediated

absorption, presumed to be via the dipeptide carrier system which has been shown to transport amoxicillin in *in vitro* studies with rodent small intestine [191]. Carrier mediated absorption may explain the unexpectedly high oral bioavailability of amoxicillin referred to above.

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DILTIAZEM HYDROCHLORIDE

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DILTIAZEM HYDROCHLORIDE

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1. INTRODUCTION

1.1. Therapeutic Category (1,2)

Diltiazem hydrochloride is a calcium ion influx inhibitor (slow channel blocker or calcium antagonist). It has generally been indicated for the treatment of angina and, more recently (2), hypertension. Diltiazem hydrochloride is a potent dilator of coronary arteries and has been shown to increase exercise tolerance in man. It is available for dosing as immediate release tablets and as extended or sustained release capsules and is usually well-tolerated. While some adverse reactions have been reported during diltiazem hydrochloride therapy, it is generally considered to be well-tolerated. In most cases, no causal relationship between the events and diltiazem hydrochloride use has yet to be established (1).

1.2. History

Diltiazem hydrochloride belongs to the benzothiazepine class of compounds. It was first synthesized in the laboratories of Tanabe Seiyaku Co., LTD in Japan and was granted its first patent in 1969 (3). The first non-patent literature reference to diltiazem appeared in 1970 (4). Since the introduction of diltiazem hydrochloride in pharmaceutical formulations, it has gained wide acceptance as an anti-anginal and anti-hypertensive agent. A search of Chemical Abstracts (1967-July, 1992) and Pharma-ceutical Abstracts (1974-July, 1992) produced over 200 bibliographic citations dealing with diltiazem or diltiazem hydrochloride.

2. DESCRIPTION

2.1. Chemical Name, Formula and Molecular Weight

The accepted chemical name for diltiazem hydrochloride is :
(2S-cis)-3-(acetyloxy-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxy-phenyl)-1,5-benzothia-zepin-4(5H)-one monohydrochloride.
The CAS Registry number is 33286-22-5.

Diltiazem hydrochloride has also been known as : **(+)-cis-3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one monohydrochloride and (+)-5-[2-(dimethylamino)ethyl]-cis-2,3-dihydro-3-hydroxy-2-(p-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one acetate (ester) monohydrochloride.**
The molecular formula and molecular weight of diltiazem hydrochloride are, respectively, $C_{22}H_{26}N_2O_4S$. HCl and 450.98 g/mole.
It's chemical structure is shown in Figure 1.

2.2. Definition

Diltiazem hydrochloride has principal tradenames of Dilacor® and Cardizem®. It has also been known as CRD-401, RG 83606, Altiazem®, Anginyl®, Angize®, Britiazi®, Bruzen®, Calcicard®, Cardiem®, Dilpral®, Dilzem®, Dilzene®, Herbesser®, Masdil® and Tildiem®. Diltiazem hydrochloride possesses two asymmetric carbons and is supplied as the dextrorotatory cis-isomer. Diltiazem, when referenced, indicates the free base (CAS registry number 42399-41-7).

2.3. Appearance, Odor and Taste.

Diltiazem hydrochloride is a white to off-white crystalline powder. It is odorless and has a bitter taste.

3. SYNTHESIS

A variety of synthetic approaches (both stereospecific and non-stereospecific) have been reported for diltiazem and diltiazem hydrochloride in the scientific literature (4, 5, 6, 7, 8, 9). Patent references for the preparation of the compound also exist in many countries, notably :
USA Patent No. 3,562,257 ; Germany Patent No. 1,805,714 ; Japan Patent No. 5,920,273 ; German Patent No. 3,415,035 and USA Patent No. 4,552,695. One approach (9) to stereospecific synthesis of diltiazem hydrochloride is shown in Figure 2. Diltiazem hydrochloride is prepared from (E)-methyl-4-methoxypropenoate [(1)] via either of the enantiomers of threo-methyl-3-(4-methoxyphenyl)-2,3-dihydroxypropanolate

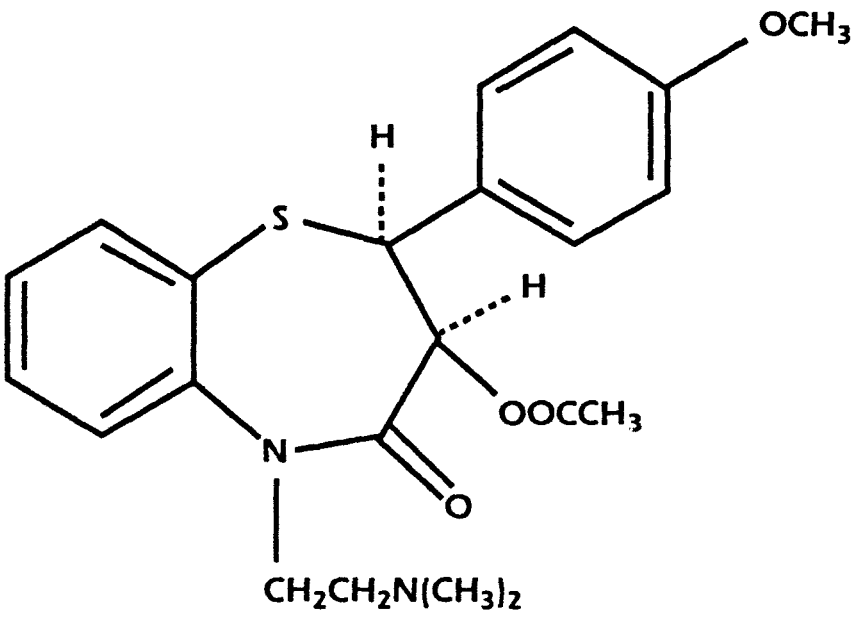


Figure 1. Chemical structure of diltiazem hydrochloride.

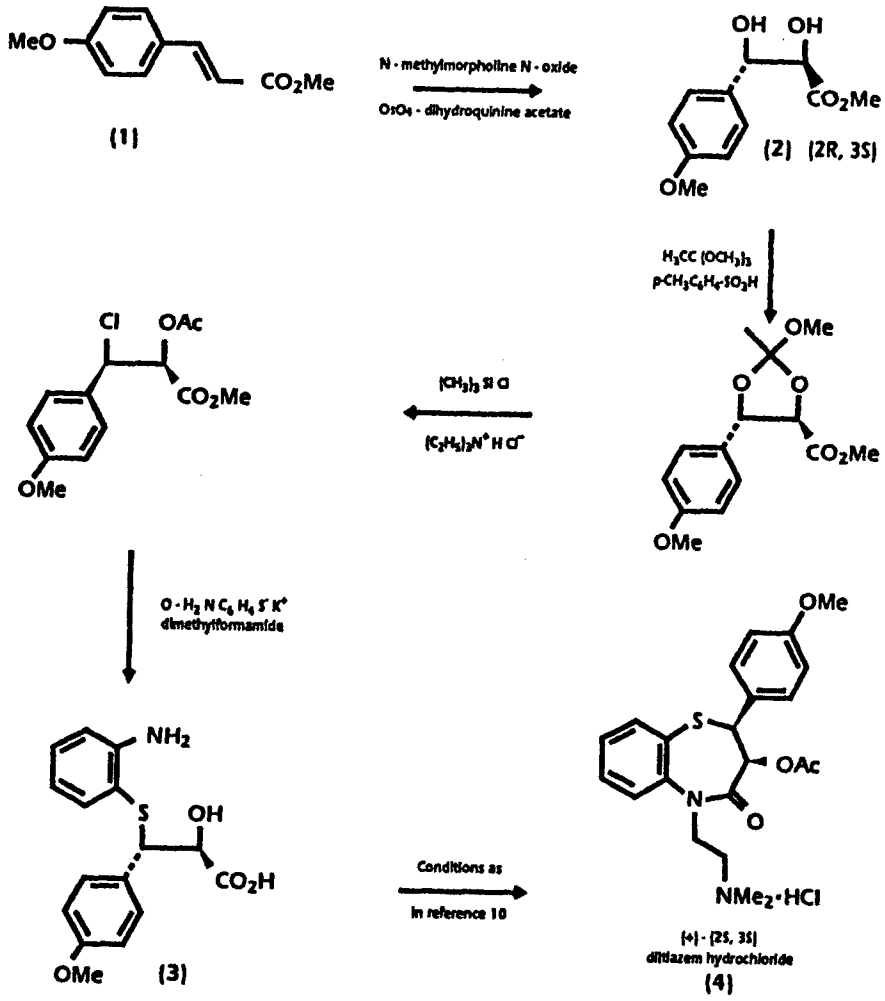


Figure 2. An example of a stereospecific synthesis of diltiazem hydrochloride.

thioether [(3)], an intermediate in the synthesis of diltiazem hydrochloride patented by Tanabe (10). This intermediate is then converted to diltiazem hydrochloride [(4)] according to the Tanabe method (10).

4. PHYSICAL PROPERTIES

4.1. Infrared Spectrum

The infrared spectrum of diltiazem hydrochloride dispersed in potassium bromide was recorded at 4 cm^{-1} resolution on a Nicolet Model 740 FTIR (11). Figure 3 shows the diltiazem HCl vibrational features in the 4000 to 400 cm^{-1} region after spectral subtraction of the absorptions (3430 and 1629 cm^{-1}) due to adsorbed water. Table I lists characteristic frequencies, relative intensities, and vibrational assignments for the primary diltiazem HCl absorption bands.

TABLE I

Infrared Absorption Spectral Assignments for Diltiazem hydrochloride

Frequency (cm^{-1})	Relative Intensity*	Assignment
3056	w	aromatic C-H stretch
3035	w	aromatic C-H stretch
2966	w	aliphatic C-H stretch
2837	w	O-CH_3 C-H stretch
2393	s	amine HCl N-H stretch
1743	s	acetate C=O stretch
1679	vs	lactam C = 0 stretch
839	m	o-substituted aromatic C-H out-of-plane deformation
781	m	p-substituted aromatic C-H out-of-plane deformation

* w = weak, s = strong, m = medium, v = very

The spectrum obtained is consistent with the structure of diltiazem hydrochloride.

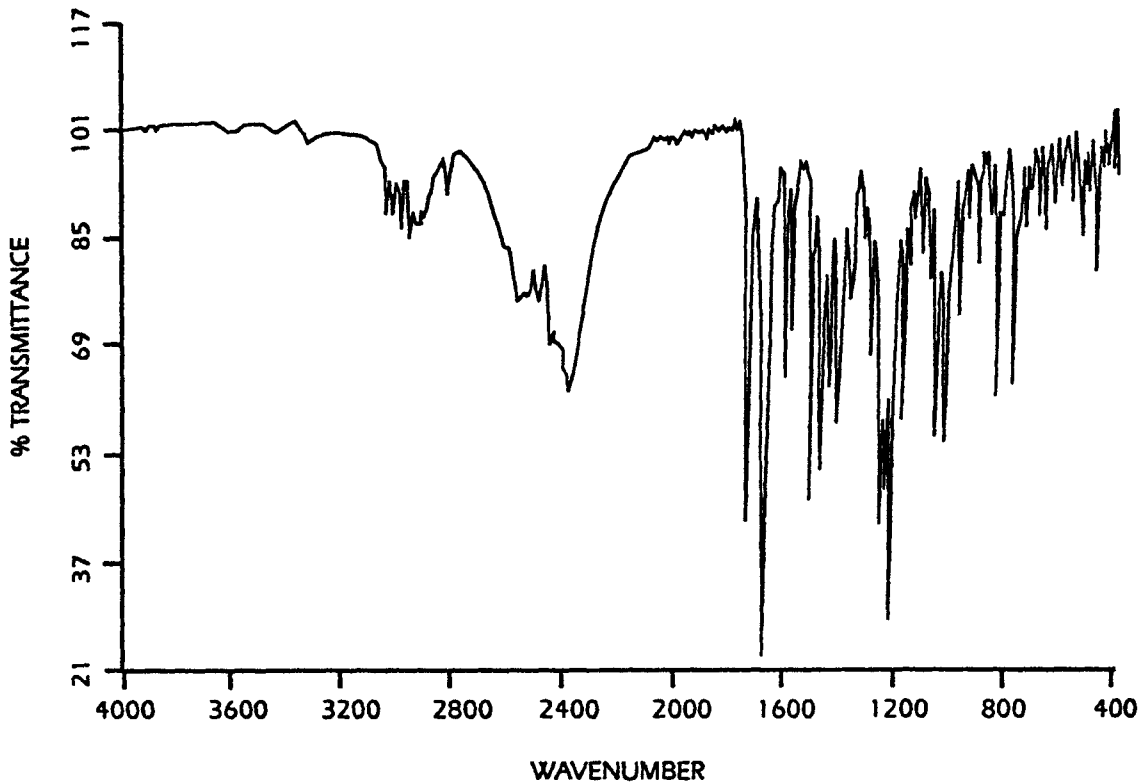


Figure 3. Infrared absorption spectrum of diltiazem hydrochloride in KBr.

4.2. Nuclear Magnetic Resonance Spectrum

4.2.1. Proton NMR

The ^1H NMR spectrum of diltiazem hydrochloride was acquired at 300 MHz on a Bruker AC-F-300 FT instrument (12) (Figure 4). Samples for ^1H NMR spectra were dissolved in $(CD_3)_2SO$. The spectral assignments for ^1H NMR were made based on work by Glaser and Sklarz (13). The small peaks in the ^1H spectra at 1.34, 2.5, and 3.71 ppm are most likely due to the contribution of the 1,5-benzothiazepine-ring-inverted (P, 2S, 3S)-boat (twist-boat) analogue of the major conformation, the (M, 2S, 3S) twist-boat. The ^1H NMR spectra assignments for diltiazem hydrochloride are shown in Table II. Aditional information pertaining to the ^1H NMR spectra under a variety of conditions was reported by Kojic-Prodic et al (14).

4.2.2. Carbon-13 NMR

The ^{13}C NMR spectrum of diltiazem hydrochloride was acquired at 50 MHz on a Varian VXR-200 FT-NMR (12). The spectrum was acquired from a solution of drug in $(CD_3)_2SO$. The spectrum is shown in Figure 5. Spectral assignments (13) are listed in Table III. The small peaks at 19.6, 78.9, 119.5 and 168.2 ppm are most likely due to the contribution of the 1,5-benzothiazepine-ring-inverted (P, 2S, 3S)-boat (twist-boat) analogue of the primary conformation, the (M, 2S, 3S) twist-boat.

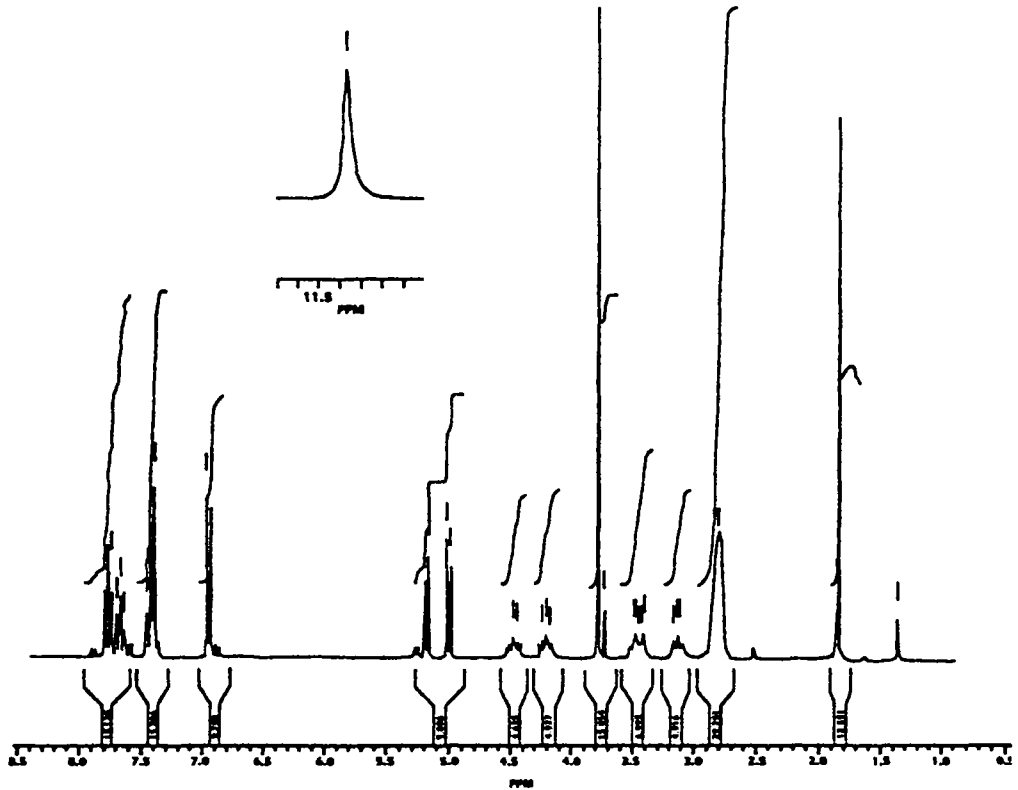
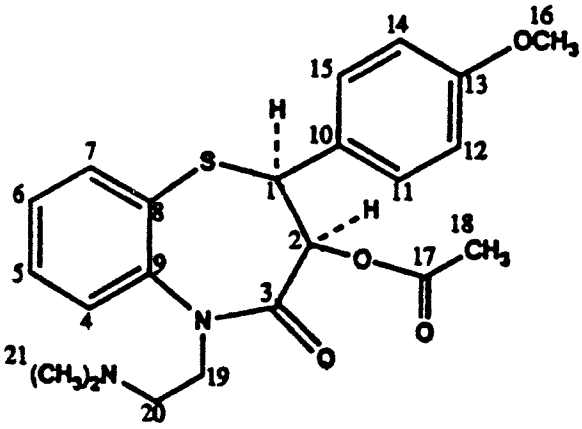


Figure 4. ^{1}H NMR spectrum of diltiazem hydrochloride.

TABLE II

Proton NMR Spectral Assignments for Diltiazem Hydrochloride (13)



Proton designation	Chemical Shift (ppm)
H-1	5.17
H-2	5.00
H-4	7.74
H-5	7.65
H-6	7.43
H-7	7.77
H-11, H-15	7.40
H-12, H-14	6.94
H-16	3.77
H-18	1.83
H-19	4.47, 4.20
H-20	3.47, 3.12
H-21	2.78

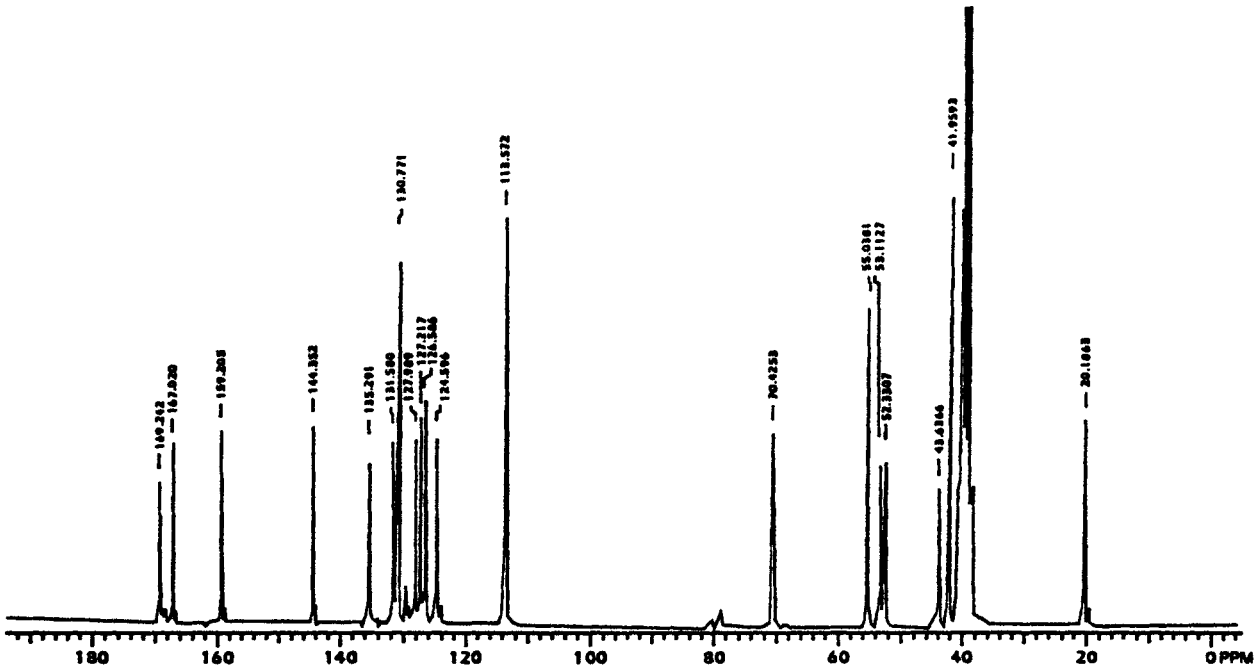
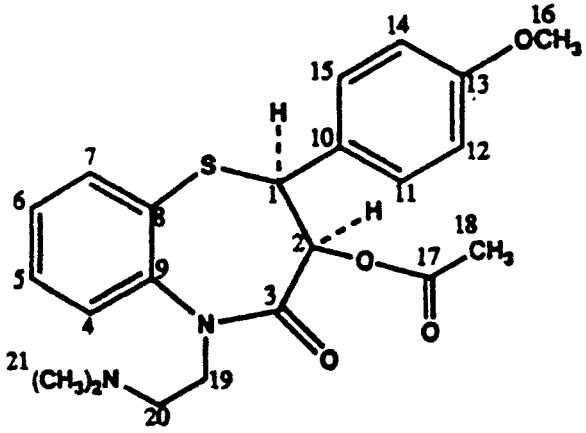


Figure 5. ^{13}C NMR spectrum of diltiazem hydrochloride.

TABLE III

^{13}C NMR Spectral Assignments for Diltiazem Hydrochloride (13)



Carbon designation	Chemical Shift (ppm)
1	53.11
2	70.43
3	167.02
4	124.60
5	131.58
6	127.99
7	135.29
8	127.22
9	144.35
10	126.59
11, 15	130.77
12, 14	113.57
13	159.20
16	55.04
17	169.24
18	20.19
19	52.33
20	43.64
21	41.96

4.3. Ultraviolet Spectrum

The ultraviolet absorption spectrum of diltiazem hydrochloride in 0.1 N HCl (aq.) was obtained using a Beckman DU-6 spectrophotometer. The absorption spectrum, shown in Figure 6, is characterized by maxima at approximately 205 nm and 236 nm. The absorptivity in absorbance units for a 10.1 µg/mL solution in a 1-cm cell is 0.954 at 205 nm and 0.556 at 236 nm.

4.4. Mass Spectrum

The low resolution mass spectrum of diltiazem HCl shown in Figure 7 was obtained using a Finnigan 4500 mass spectrometer operated at 70 eV in the direct probe electron impact mode (15). The molecular ion of the free base is seen at m/z = 414 and the protonated free base ion is observed at m/z = 415. The fragmentation pattern (Figure 8) is consistent with the chemical struture.

4.5. Optical Rotation

Diltiazem hydrochloride possesses two asymmetric carbons and is therefore optically active. The dextrorotatory enantiomer is the more potent biologically active form (16, 17) and is, therefore, the form supplied. The optical rotation of a 1% (w/v) solution of diltiazem hydrochloride in water at 25°C $\left(\left[\alpha_D^{25}\right]\right)$ is between +100° and +116°. An optical rotation of +98.3° ± 1.4° $\left(\left[\alpha_D^{24}\right];c = 1.002\right)$ in methanol has also been reported (3).

4.6. Electroanalytical Behavior

Diltiazem hydrochloride is electrochemically active and exhibits both oxidative and reductive voltammetric responses. Wang et al have reported that, using a static mercury drop electrode, a 1 µM solution of drug in 0.05 M NaOH(aq.) exhibited a single cathodic response at -1.45 V for the solution-phase (non-adsorbed) drug (18). The same authors also reported two anodic peaks at +0.78 V and +0.98 V for a 100 µM solution of drug in 0.05M phosphate buffer (pH = 7.4) using a glassy carbon electrode. A typical cyclic voltammogram of diltiazem

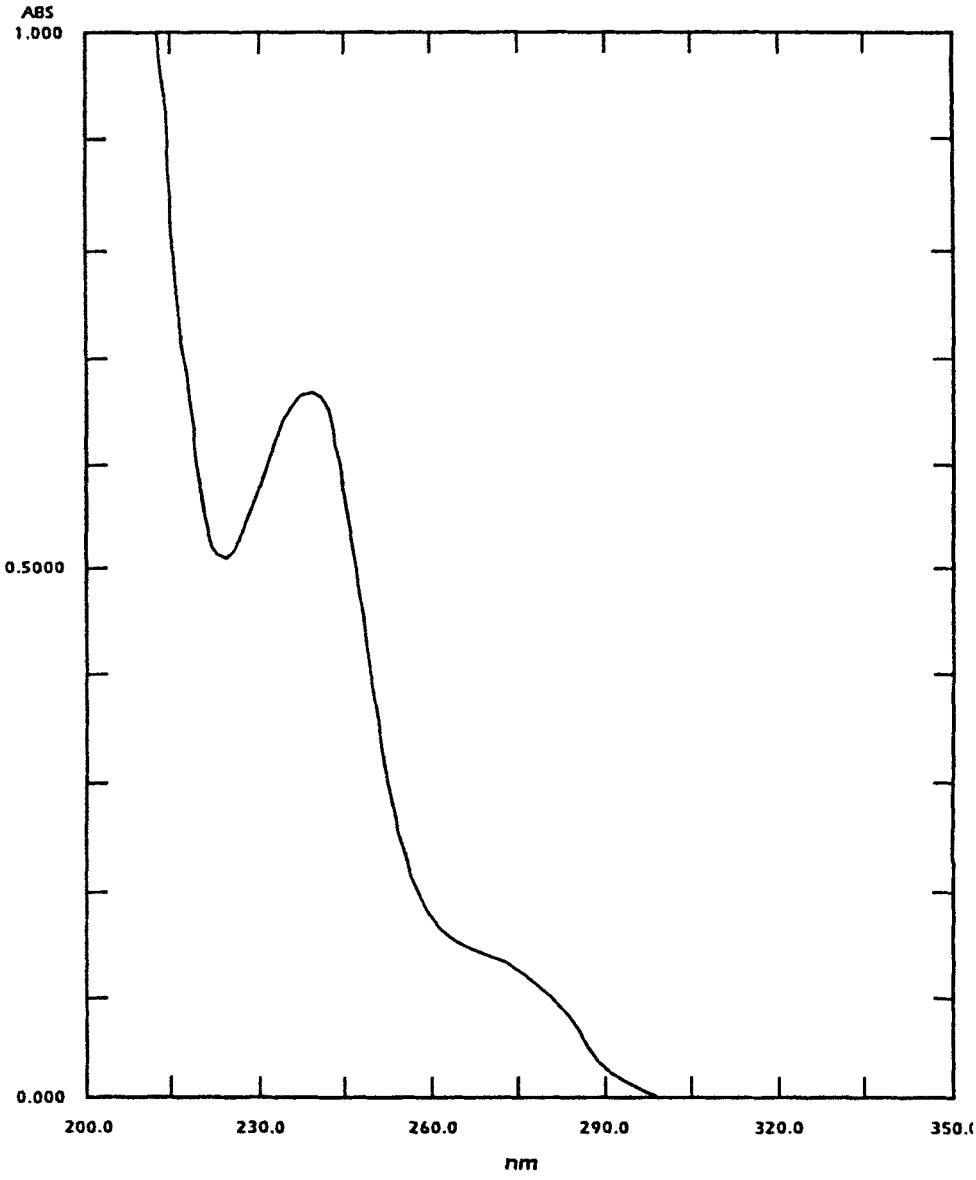


Figure 6. UV absorbance spectrum of diltiazem hydrochloride.

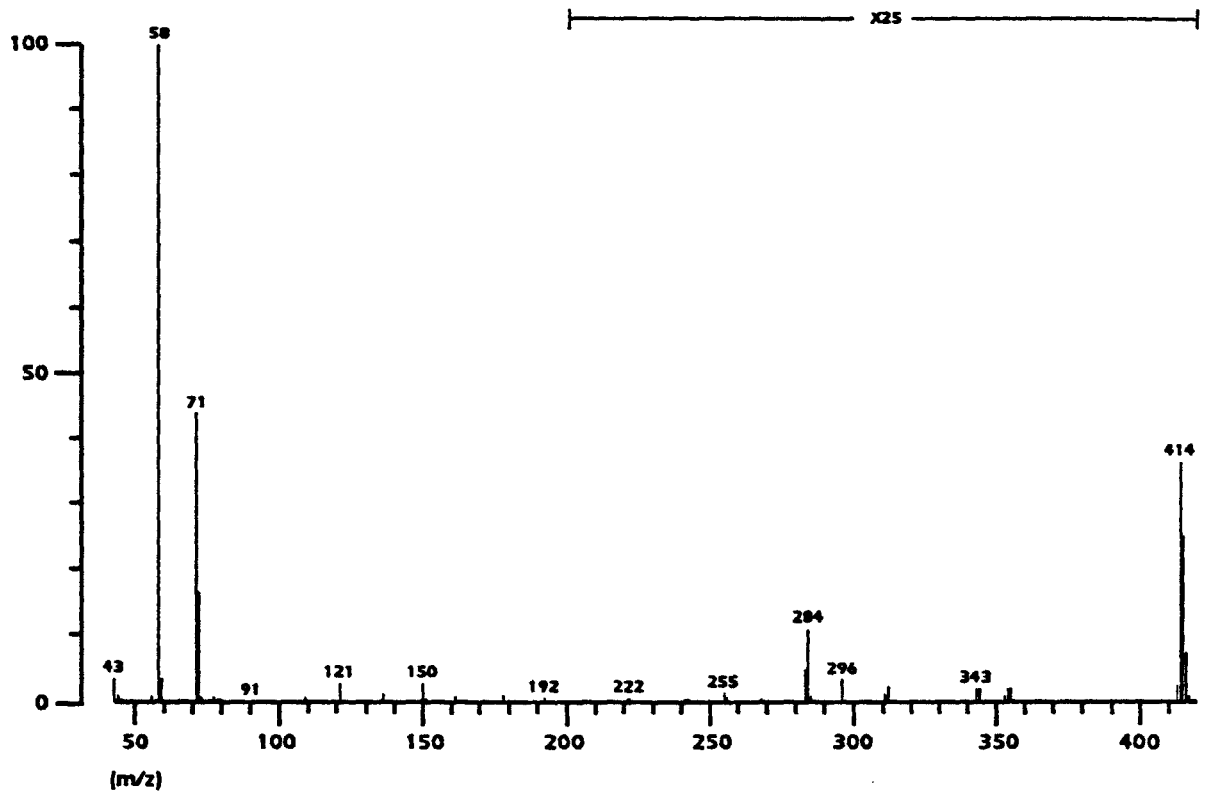


Figure 7. Low resolution mass spectrum of diltiazem hydrochloride.

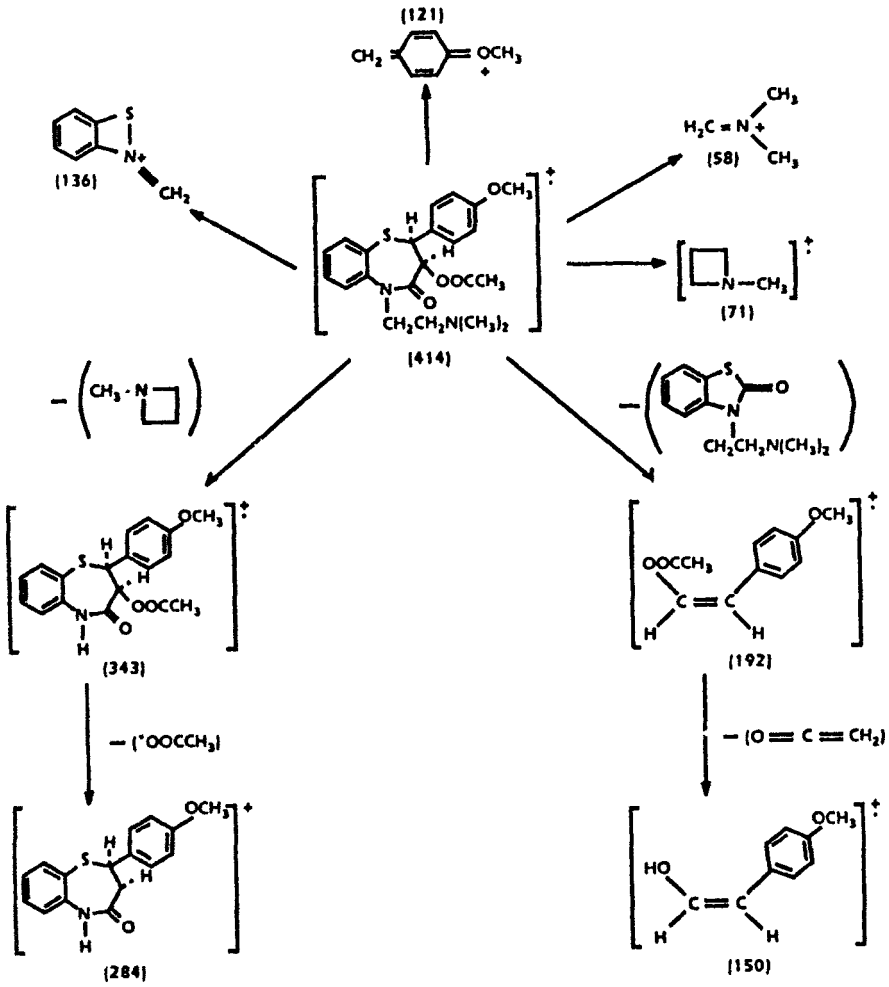


Figure 8. Mass spectral fragmentation pattern of diltiazem hydrochloride.

using a PAR potentiometer and a glassy carbon working electrode. The reference electrode was saturated calomel and the auxilliary electrode was platinum. Solutions of 500 µM, 400 µM and 200 µM diltiazem hydrochloride in 0.05M phosphate buffer were used to generate the voltammograms at a scan rate of 100 mV/sec. All solutions were degased with nitrogen prior to use.
Diltiazem hydrochloride has been shown to interfere with measurements made at Ca^{2+} or K^{+} specific electrodes (20).

4.7. Thermoanalytical Behavior

4.7.1. Melting Point

Diltiazem hydrochloride melts at about 210°C [207.5°C - 212°C (3)] with decomposition at higher temperatures.

4.7.2. Loss on Drying

Diltiazem hydrochloride loses not more than 0.5% of its weight after being dried at 105°C for three hours (21).

4.7.3. Differential Scanning Calorimetric Behavior (22)

The differential scanning calorimetry (DSC) behavior of diltiazem hydrochloride is shown in Figure 10. The thermogram was obtained using a Perkin Elmer Series 7 DSC scanning from 50°C to 300°C at 10°C/min.
A primary endotherm corresponding to melting is observed with a peak onset temperature of ~ 210°C and a peak of temperature of ~ 215°C. Decomposition becomes apparent above 230°C.

4.7.4. Thermogravimetric Analysis Behavior (22)

Thermogravimetric Analysis (TGA) of diltiazem hydrochloride tested under a nitrogen atmosphere indicates no weight loss until a temperature of ~ 230°C is reached. Significant weight loss is observed above 230°C due to decomposition/vaporization. A typical TGA curve is shown in Figure 11. The curve was obtained using a Perkin Elmer Series 7 TGA

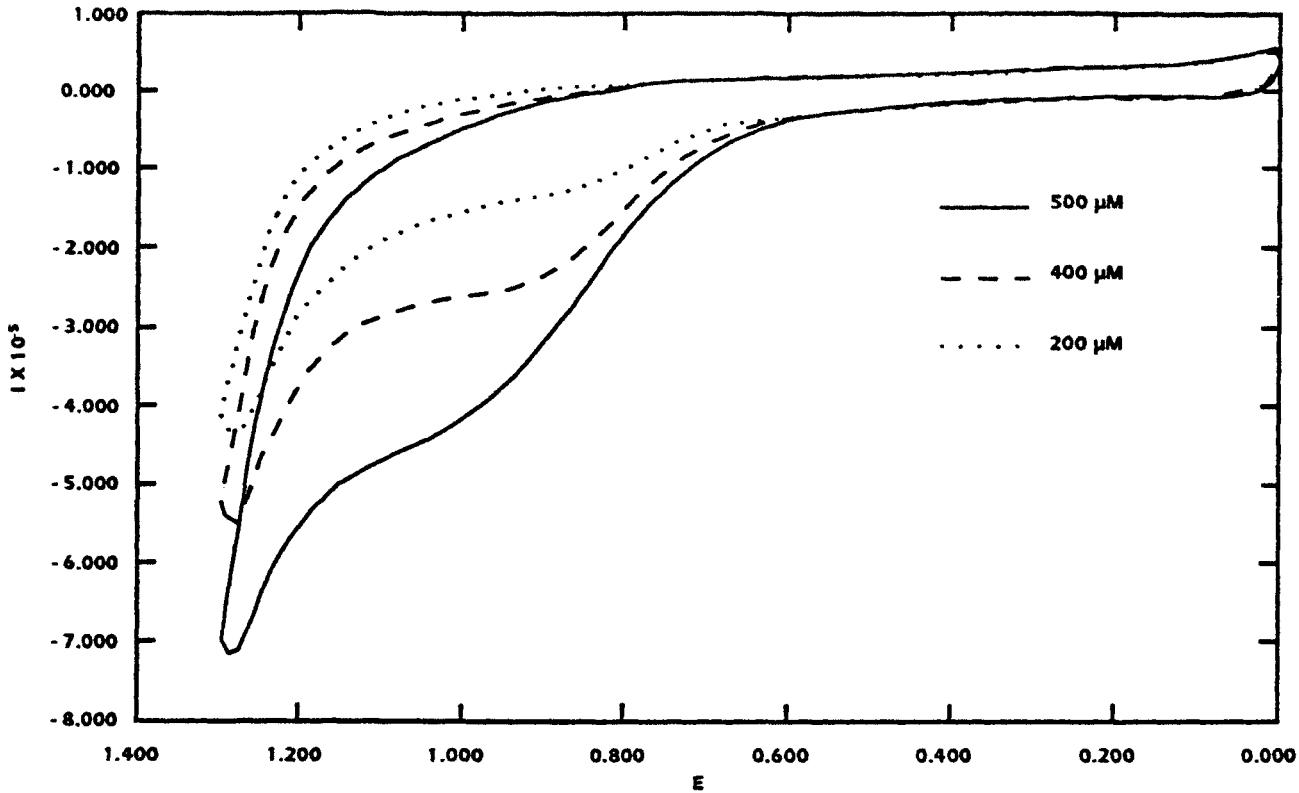


Figure 9. Cyclic voltammogram of diltiazem hydrochloride at a glassy carbon working electrode.

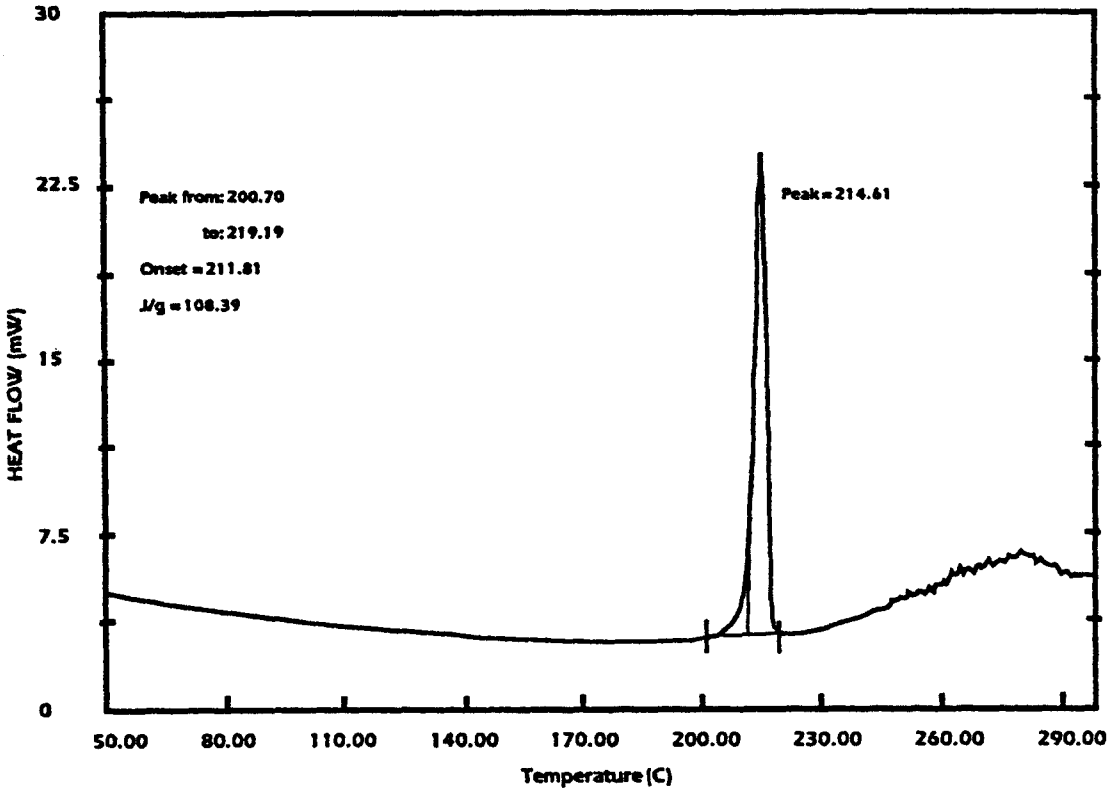


Figure 10. Differential scanning calorimetric behavior of diltiazem hydrochloride.

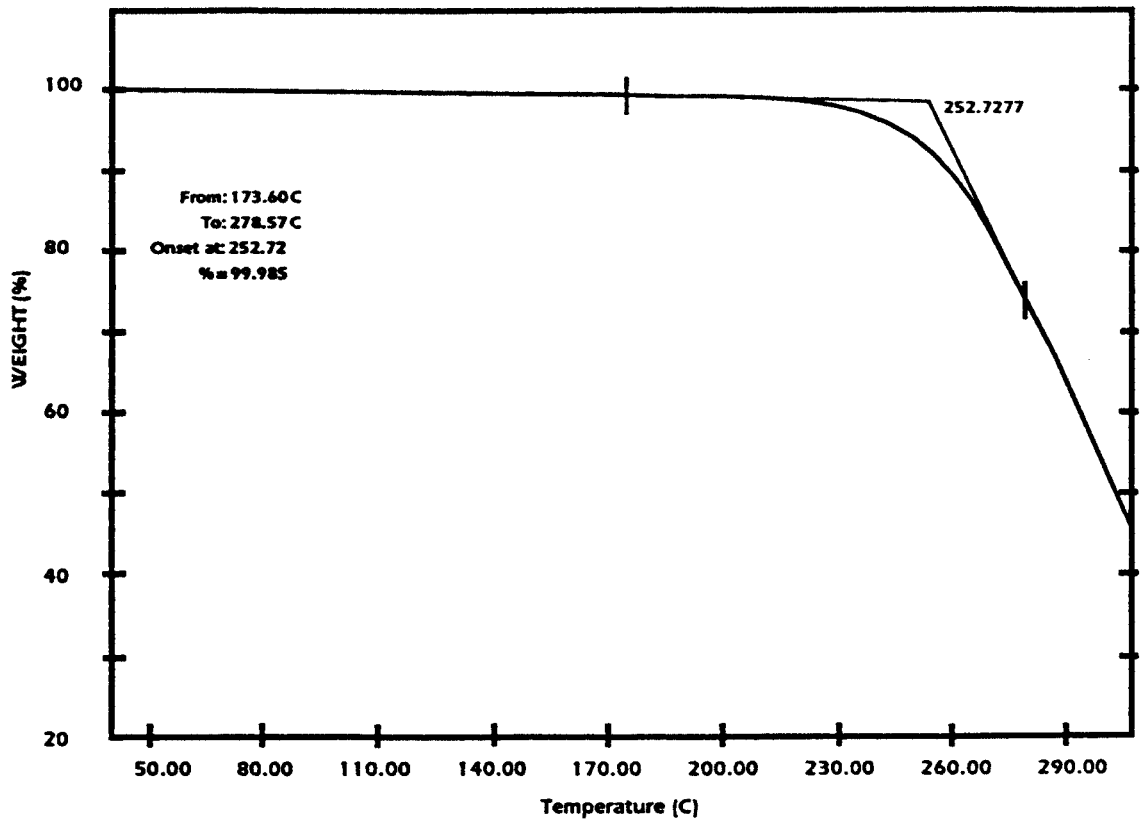


Figure 11. Thermogravimetric analytical behavior of diltiazem hydrochloride (under nitrogen).

scanning from 40°C to 300°C at a scan rate of 10°C/min. A 3.762 mg sample of diltiazem hydrochloride was used.

4.7.5. Residue on Ignition

Typically < 0.1% residue is obtained when a 1 gram sample of diltiazem hydrochloride is ignited in a platinum crucible in the presence of concentrated H_2SO_4 in a muffle furnace at 800°C until no visible residue remains (21).

4.8. Solubilities

The solubility of diltiazem hydrochloride in a variety of solvents is presented in Table IV (3). Note that solubilities are indicated in terms of current USP definitions (23).

TABLE IV

Solubility of Diltiazem hydrochloride at 25°C

Solvent	Solubility
Chloroform	Freely soluble
Formic acid	Freely soluble
Methanol	Freely soluble
Water	Freely soluble
Dehydrated alcohol	Sparingly soluble
Benzene	Practically insoluble
Ether	insoluble

4.9. Crystal Properties and Conformation

Diltiazem hydrochloride exists as a crystalline powder. It has been crystallized as fine needles from ethanol-isopropanol (3).

4.9.1. Polymorphism

No polymorphs of diltiazem hydrochloride have been reported to date.

4.9.2. X-ray Crystallography

X-ray crystallographic measurements of diltiazem hydrochloride including interatomic distances and angles have been thoroughly reported by Kojic-Prodic et al. (14). A powder diffraction pattern of diltiazem hydrochloride was generated using a Enraf-Nonius CAD 4 diffractometer with CuKα radiation. Figure 12 shows the resulting pattern while Table V lists the corresponding numerical data.

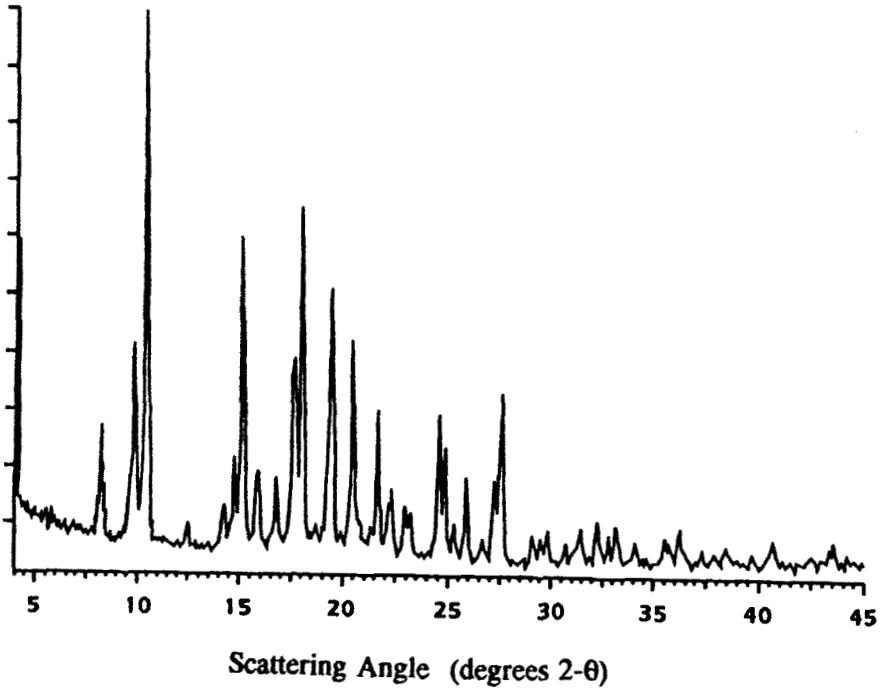


Figure 12. X-ray powder diffraction pattern of diltiazem hydrochloride.

Table IV

Crystallographic Data Associated with the X-ray Powder Diffraction Pattern of Diltaizem Hydrochloride (Obtained Using CuKα Radiation)

Scattering Angle (degrees 2-θ)	D-Spacing (Angstroms)	Relative Intensity (I/I_{max})
4.105	21.5096	39.0
5.850	15.0950	2.1
7.948	11.1150	3.9
8.345	10.5873	21.9
8.589	10.2860	2.2
8.818	10.0198	2.4
9.185	9.6206	2.2
9.435	9.3656	3.2
9.952	8.8804	37.9
10.607	8.3334	100.0
12.543	7.0512	5.0
14.318	6.1807	8.8
14.818	5.9735	18.4
15.239	5.8093	59.0
15.959	5.5489	14.0
16.795	5.2743	12.3
17.757	4.9909	34.7
18.115	4.8930	63.9
18.722	4.7356	2.6
19.532	4.5410	44.9
20.551	4.3182	37.5
20.804	4.2662	3.1
21.724	4.0875	27.5
22.364	3.9721	11.6
23.021	3.8601	8.9
23.261	3.8208	8.2
24.589	3.6174	29.7
24.900	3.5729	21.4
25.331	3.5131	6.7
25.933	3.4329	16.0

Table IV
(continued)

Crystallographic Data Associated with the
X-ray Powder Diffraction Pattern of Diltaizem Hydrochloride
(Obtained Using CuKα Radiation)

Scattering Angle (degrees 2-θ)	D-Spacing (Angstroms)	Relative Intensity (I/I_{max})
26.729	3.3324	4.3
27.331	3.2604	16.7
27.660	3.2223	32.1
28.855	3.0916	2.3
29.187	3.0571	6.3
29.585	3.0169	5.6
29.877	2.9881	5.6
30.752	2.9051	4.0
31.488	2.8388	5.7
32.291	2.7700	7.2
32.859	2.7234	4.8
33.166	2.6989	6.5
35.547	2.5234	5.6
35.774	2.5079	4.3
36.281	2.4740	7.8
36.486	2.4606	2.4
37.317	2.4077	2.5
38.515	2.3355	3.4
38.687	2.3255	2.1
38.825	2.3176	2.3
39.747	2.2659	2.5
40.731	2.2134	5.0
42.547	2.1230	2.4
43.413	2.0827	3.1
43.559	2.0760	2.7
44.209	2.0470	2.4

4.9.3. Structural Conformation

Kojic-Prodic et al have reported that diltiazem hydrochloride exists in a (M, 2S, 3S)-twisted-boat conformation based upon X-ray crystallographic data (14). They also reported, however, that no distinction could be made between boat and twisted boat conformations based upon ultraviolet and circular dichroism spectra obtained from drug in solution.

4.10. pH (18, 19)

The pH of a saturated aqueous solution of diltiazem hydrochloride in water is 3.0. A 1.0% (w/w) solution of drug in water has a pH of 4.2.
A pH of 4.7 $\pm$ 0.3 has been reported for a 1.0% (w/v) solution of diltiazem in water (24).

5. METHODS OF ANALYSIS

5.1. Identification Tests

5.1.1. Ultraviolet Spectrophotometry

Identification testing of diltiazem hydrochloride drug substance and product can be accomplished by UV absorbance analysis. The drug substance samples are diluted in 0.1N aqueous hydrochloric acid and the drug product samples in acetonitrile (the resulting solution is filtered with a 0.45 µm nylon syringe filter). The ultraviolet spectrum of diltiazem is obtained using a 1 cm cell and by scanning from 350 nm to 250 nm at a scan rate of 150 nm/minute. The resulting spectrum is qualitatively compared to the spectrum obtained from a diltiazem hydrochloride reference standard. The drug substance spectrum is characterized by maxima at approximately 205 nm and 236 nm. The drug product is characterized by an absorption maximum at approximately 239 nm.

5.1.2. Infrared Spectroscopy

The infrared spectrum of diltiazem hydrochloride is obtained from a potassium bromide disc and is recorded at 4 cm^{-1} resolution. A disc is made from 1 mg of sample in about

200 mg of potassium bromide. The infrared absorption spectrum of a potassium bromide dispersion of diltiazem hydrochloride exhibits maxima only at the same wavelengths as that of a similar preparation of diltiazem hydrochloride reference standard.

5.1.3. Elemental Analysis

The identity of diltiazem hydrochloride can be determined by the elemental weight determination of carbon, hydrogen, nitrogen, chlorine and sulfur. The elemental analysis of a sample of diltiazem hydrochloride is performed and the results are compared to the following theoretical values :

Element	Percent Theory
C	58.59
H	6.03
N	6.21
Cl	7.86
S	7.11

5.2. Electrochemical Analysis

5.2.1. Ion-Selective Electrodes

The use of ion-selective electrodes as a rapid, inexpensive method for the determination of diltiazem has been explored. Detection limits of 10^{-5} M or lower are attainable. The potentiometric sensor is based on the incorporation of dinonylnaphthalene sulfonic acid and diltiazem into a plasticized membrane resulting in a coated-wire ion-selective electrode (25).

5.2.2. Adsorptive Stripping Voltammetry and Flow Amperometry

Voltammetric procedures can be used for trace measurements of diltiazem. Diltiazem exhibits both reductive and oxidative voltammetic responses. Adsorptive stripping voltammetry can be used due to the reductive capacity of diltiazem. The adsorptive stripping is accomplished with a static mercury drop electrode in conjunction with a stripping analyzer. A detection

limit of 4×10^{-9} M is achievable. The anodic response of diltiazem is used for flow injection analysis at a glassy carbon detector. The flow injection measurements yield a detection limit of 1.5 ng (26).

5.3. Chromatographic Analysis

5.3.1. High Performance Liquid Chromatography

A stability-indicating HPLC procedure may be used for the identity testing of diltiazem hydrochloride. The retention time of the major peak in the sample chromatogram should correspond to that of the diltiazem hydrochloride reference standard (approxi-mately 5.5 minutes). The HPLC procedure is described under Section 9.2.1.1.

Diltiazem hydrochloride and its related compounds can be separated in both bulk drug and finished tablets using a Waters μBondapak C18 column (10 μm particle size, 300 mm x 3.9 mm I.D.) and a mobile phase of buffer : methanol : acetonitrile (50:25:25, v/v) at a flow rate of about 1.6 mL/minute. The buffer is 0.1M aqueous sodium acetate containing 5 mM d-camphorsulfonic acid (99%) adjusted to pH 6.2 with 0.1M aqueous sodium hydroxide. Detection of the compounds is achieved using UV absorbance at 240 nm. The method provides for the resolution of trans-diltiazem and seven known and unidentified related compounds. Diltiazem hydrochloride elutes at approximately 21 minutes under these conditions. The minimum detectable amounts are less than 0.1% for all related compounds except for one of the synthetic intermediates for which there is a limit of about 2% (27).
Optical isomers of diltiazem hydrochloride may be separated by first derivatizing the isomers, followed by HPLC analysis. Diltiazem hydrochloride has two asymmetric carbon atoms. There are two geometric isomers, cis and trans, depending on the position of the substituents at these positions. Each isomer has optical isomers, the d- and l- forms. The enantiomers of diltiazem hydrochloride are separated by first hydrolysis to the desacetyl form followed by the introduction of a chiral species establishing diastereomers. The reaction is performed in the presence of pyridine. The diastereomers are separated using reversed-phase chromatography. The cis and trans isomers

are separated using a μBondapak C 18 column (300 mm x 4 mm I.D.) and a mobile phase of 0.12M aqueous sodium acetate solution containing 1.5 g of d-camphorsulphonic acid (pH 5.5) : acetonitrile : methanol (2:1:1) at a flow rate of 1.6 mL/minute with UV absorbance detection at 254 nm. The separation of diastereomers was achieved using a Nucleosil 5 C 18 column (200 mm x 4 mm I.D.) and a mobile phase of 0.01M aqueous ammonium acetate solution (pH 6.6) : acetonitrile (1:9) at a flow rate of 1.5 mL/minute and with UV absorbance detection at 254 nm (28). Other derivitization methods are available (29).

Another HPLC method allows the determination of the cis(-) impurity in cis(+) diltiazem. The method uses a Chiral-AGP column (100 mm x 4.6 mm) and a mobile phase of 0.01 M aqueous phosphate buffer (pH 7) containing 10% (v/v) of 2-propanol at a flow rate of 0.8 mL/minute. All chromatography is conducted under ambient conditions. Detection is achieved by monitoring UV absorbance at 210 nm. The cis(+) isomer elutes at about 11 minutes and the cis(-) isomer at 20 minutes (30).

5.3.2. Capillary Zone Electrophoresis

A micellar electrokinetic chromatographic method allows the separation of optically isomeric diltiazem hydrochloride using bile salts as chiral surfactants. The chiral separation of diltiazem hydrochloride from several analogs is achieved at ambient temperature using 0.05M sodium taurodeoxycholate in a 0.02M aqueous phosphate-borate buffer solution of pH 7.0. Separation is performed using a fused-silica capillary tube (650 mm x 50 mm I.D.) and a voltage up to +25 kV. Detection is achieved on-column using UV adsorption at 210 nm (31).

5.3.3. Gas Chromatography

Diltiazem can be separated on a fused-silica DB1 capillary column (15 m x 0.32 mm I.D. with a 0.25 μm film thickness) operating the chromatograph in the split mode using helium as the carrier and make-up gas to a nitrogen-phosphorus detector. DB1 is a bonded methyl polysiloxane equivalent to SE-30, OV-1 or SP-2100. The carrier gas velocity is 29 cm/sec. Gas

flow rate to the N-P detector is 4 mL/min for hydrogen, 50 mL/min for air and, to the flame ionization detector, 20 mL/min for hydrogen and 270 mL/min for air. An injection port temperature of 250°C and a temperature program of 8°C/min from 120°C to 280°C with a 5 minute hold at the upper temperature was used. The detector temperature is 300°C (32).

5.4. Spectrophotometric Analysis

5.4.1. Colorimetry

A colorimetric method is available which allows the estimation of diltiazem alone and in solid dosage forms. The method involves the reaction of diltiazem with cobalt thiocyanate in acidic media. The complex is extracted in benzene and monitored at 630 nm (33).

5.4.2. Specific Rotation

The specific rotation, $\left[\alpha_D^{25}\right]$, is determined with freshly prepared 1% solutions of diltiazem HCl in water. The specific rotation for diltiazem HCl bulk drug ranges between +112.0 and +114.0 (27) [between +110 and +116 (21)].

5.4.3. Direct Ultraviolet Spectrophotometry

Diltiazem hydrochloride is characterized by an absorption maximum at approximately 235 nm. This absorption can be used as the basis for the quantitative determination of diltiazem. The assay is performed by comparing the absorbance of the sample dissolved in 0.1N HCl to a standard of a known concentration in 0.1N HCl. The absorbance for the drug product is calculated by subtracting the absorbance of the excipients similarly prepared in 0.1N HCl from the absorbance recorded for the drug product.

6. STABILITY

6.1. Solid State Stability (34)

In the solid state, diltiazem is reported to be highly stable. Storage of the drug substance under conditions of room temperature and 33% or 79% relative humidity for 57 days did not cause any physical or chemical degradation. Diltiazem was stable after three weeks of storage under conditions of 44°C/75% relative humidity. Storage for three weeks at 40°C/75% relative humidity with exposure to UV light did not affect the chemical stability of diltiazem but did cause a slight color change (yellowing).

6.2. Solution Stability

Diltiazem undergoes hydrolysis to desacetyl diltiazem in aqueous buffer solutions (pH 1-7) (35). Diltiazem is most stable at pH 5. The decomposition follows pseudo-first order kinetics. The extrapolated room temperature shelf-life was 42 days or 15.8 days at pH 5 or 2, respectively. Exposure of aqueous buffered solutions of diltiazem at pH 2 or 7 to UV radiation caused more degradation as compared to the same solutions protected from light, demonstrating the light sensivity of the compound in solution (34). The stability of diltiazem hydrochloride in aqueous sugar solutions (fructose, dextrose, sucrose, sorbitol and mannitol) was determined to be superior to that in pH 5 aqueous buffer (36).

7. BIOPHARMACEUTICS AND METABOLISM

7.1. Absorption and Bioavailability

Diltiazem is rapidly absorbed after oral dosing in humans (37). Oral administration of a solution of diltiazem reaches peak plasma levels 38 minutes after dosing. After oral administration, the t-max was determined to be approximately 3 hours (38). Diltiazem has been reported to be 80% protein bound (39). It is lipophilic and has a large volume of distribution (3-8 l/kg) (39). Diltiazem has a bioavailability of 40 to 50% (40) even though it is almost completely absorbed after oral administration. This is due to the fact that it so rapidly undergoes first pass

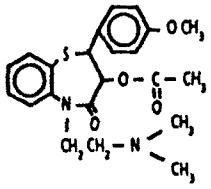
metabolism. During long term treatment, oral clearance decreases and bioavailability increases due to saturation of hepatic first-pass metabolism.

7.2. Metabolism

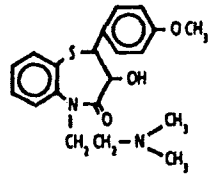
Diltiazem is extensively metabolized, predominantly due to hepatic metabolism (39). Only traces (0.1-4%) (37) of unchanged drug are eliminated in the urine. The three major metabolic pathways are O-desacetylation, N-demethylation and O-demethylation which produce six metabolites (see Figure 13). Desacetyl diltiazem and N-monodemethyl diltiazem undergo further biotransformation to form desacetyl N-monodemethyl diltiazem, desacetyl N,O-didimethyl diltiazem and desacetyl O-demethyl diltiazem (41). Desacetyl diltiazem, N-monodemethyl diltiazem, desacetyl N-monodemethyl diltiazem, desacetyl diltiazem N-oxide are most abundant in the plasma. The most abundant metabolites in urine are N-monodemethyl diltiazem and desacetyl N,O-didimethyl diltiazem (42). N-monodemethyl diltiazem is excreted unconjugated. The other metabolite is excreted in urine as the glucuronide or sulfate conjugate. N-monodemethyl diltiazem and desacetyl diltiazem are active metabolites which are not as potent as the parent compound.

7.3. Pharmacokinetics

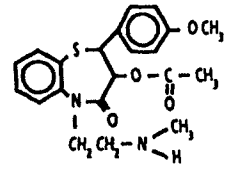
Diltiazem was shown to exhibit dose dependent (30 mg - 120 mg administrated orally, three times a day) nonlinear pharmacokinetics (43) when administered to healthy individuals. The nonlinearity of diltiazem area under the curve (AUC) is a result of dose dependent first pass metabolism and is not reflected in the elimination half-life which is the same regardless of dose. The mean apparent oral clearance (44) and half-life of diltiazem following chronic oral therapy was 20.9 mL/min/kg and 3.5 hours, respectively. After a constant rate infusion, diltiazem was also shown to exhibit (45) nonlinear pharmacokinetics. After IV administration the following pharmacokinetic parameters were determined in healthy male volunteers : the elimination t-1/2 (40) was 11.2 hours and the total clearance was 11.5 mL/min/kg. Diltiazem elimination is affected by liver damage (46) but not by kidney failure.



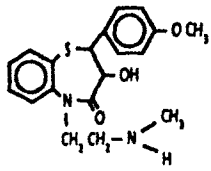
Diltiazem



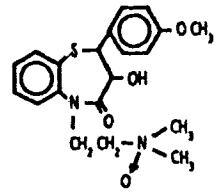
Desacetyl diltiazem



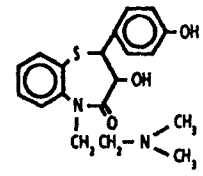
N-monodemethyl diltiazem



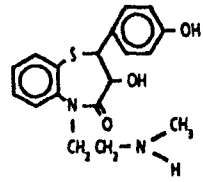
Desacetyl N-monodemethyl diltiazem



Desacetyl diltiazem N-oxide



Desacetyl O-demethyl diltiazem



Desacetyl N, O-didimethyl diltiazem

Figure 13. Diltiazem and its six metabolites found in man.

Pharmacokinetic analysis showed that elimination of diltiazem in the elderly is disturbed, with a prolongation of the t-1/2 and an increase in the AUC as compared to control subjects (47).

8. DETERMINATION IN BIOLOGICAL MATRICES

Diltiazem hydrochloride can be analyzed in blood, plasma and urine using HPLC and GC methods. Numerous HPLC methods have been developed to determine the quantity of diltiazem and its metabolites in biological matrixes. The majority of the HPLC methods developed use a reversed-phase octyldecylsilane column. An HPLC method to determine diltiazem and three metabollites (desacetyl diltiazem, N-monodemethyl diltiazem, and desacetyl N-monodemethyl diltiazem) in plasma using a Nucleosil C18 column is an example of such a method (48). This method uses a 3 µm particle size column and a mobile phase of 37% acetonitrile and 63% 0.1M aqueous ammonium dihydrogen phosphate buffer containing 0.06% triethylamine (pH adjusted to 5.9). The lower limit of detection in serum was 1.5-3.0 ng/mL. An HPLC method to analyze plasma samples has been reported (49) which utilizes solid-phase extraction prior to analysis. The chromatographic conditions used are almost identical to those previously cited. An example of an automated HPLC method for direct injection of diltiazem containing plasma samples can be found in reference 50. The method utilizes a C18 (5 µm particle size Supelcosil LC-18-DB) column and UV absorbance detection, with a mobile phase of 35% 0.05M aqueous phosphate buffer (pH 2.5) containing 0.25% (v/v) triethylamine and 65% acetonitrile. The detection limit is 2 ng/mL. Another reversed-phase HPLC method was developed for the analysis of diltiazem and six of its metabolites in plasma (51). The method uses an Ultrasphere-ODS 5 µm particle size column and a mobile phase consisting of 40% methanol, 36% 0.04M aqueous ammonium acetate and 24% acetonitrile and 0.04% triethylamine adjusted to pH 7.3. UV absorbance detection at 237 nm provides a 10 ng/mL limit of quantification. Diltiazem and 4 of its metabolites can be determined using a reversed-phase HPLC method (41) after extraction from plasma. The method uses a 3 µm particle size Spherisorb ODS 2 column and a mobile phase of 60% acetonitrile and 40% 0.01M aqueous ammonium phosphate buffer containing 0.06% TEA (pH adjusted to 3.75). UV

absorbance detection is at 237 nm. An ion-pairing HPLC (52) method for the determination of diltiazem and desacetyl diltiazem in plasma has also been developed. It uses UV absorbance detection at 237 nm and a 5 µm particle size C18 Spherisorb ODS-II column. Ammonium bromide is employed as the ion-pairing agent. The mobile phase is 40% methanol, 36% 0.04M aqueous ammonium bromide in water and 24% acetonitrile and 0.06 mL of triethylamine to give a pH of approximately 8.5. An alternate reversed-phase method (53) uses a C18 µBondapak column and a mobile phase of 58% 0.06M aqueous acetate buffer, 37% acetonitrile, and 5% methanol, final pH 6.45, to determine diltiazem and desacetyl diltiazem in plasma.

Several reversed-phase methods were also developed which do not use a C18 column. A reversed-phase method using a C8 Spherisorb column has been reported (54) to quantitate diltiazem and two of its metabolites (N-monodemethyl diltiazem and desacetyl diltiazem). A 10 µm particle size PRP-1 column (55), mobile phase of 60% acetonitrile and 0.01M aqueous KH_2PO_4, 40% 0.005M aqueous tetrabutylammonium hydroxide and UV absorbance detection at 254 nm was used to determine diltiazem present in plasma. Several HPLC methods have been developed which use a cyano-bonded column. One such method was developed for the determination of diltiazem and its metabolite desacetyl diltiazem in human plasma (56). The analytes are extracted from plasma made basic with 0.5M aqueous dibasic sodium phosphate (pH 7.4) using 1% 2-propanol in hexane. The method uses a cyanopropylsilane column with a mobile phase of 45% acetonitrile and 55% 0.05M aqueous acetate buffer (pH 4.0). The minimum detectable limit was 2 ng/mL in plasma. A similar HPLC method was developed by Johnson and Pieper (57) for the determination of diltiazem and three of its metabolites. Also, an HPLC method was developed (58) for the analysis of diltiazem and desacetyl diltiazem in plasma using UV detection at 237 nm, a Zorbax CN 6 µm particle size column and a mobile phase of 45% methanol, 55% 0.05M aqueous ammonium dihydrogen phosphate and 0.25% triethylamine adjusted to pH 5.

Several gas chromatographic methods were developed to assay diltiazem and its metabolites. A method to determine

serum diltiazem levels was developed which uses a glass column packed with 1% OV-17 on Chromasorb W (59) with electron capture detection. An alternative GC method (60) using a glass column packed with 3% OV 7 on GasChrom Q, 80-100 mesh, and electron capture detection was used to quantitate diltiazem and desacetyl diltiazem in plasma after derivitization using N-methyl-N-(trimethylsilyl)-trifluoroacetamide. The limits of quantitation were 2 ng/mL and 3 ng/mL, respectively. A capillary gas chromatographic method utilizing a cross-linked fused-silica column, electron-capture detection and the derivitizing agent, BSTFA (bis(trimethylsilyl)-trifluoroacetamide), was developed for the analysis of diltiazem and desacetyl diltiazem in plasma or serum (61). The minimum measurable concentrations were 3 and 1 ng/mL, respectively.

9. DETERMINATION IN PHARMACEUTICALS (62)

9.1. Dissolution Testing

The dissolution testing of diltiazem is accomplished using USP Apparatus II (paddles) with a stirring rate of 100 ± 4 ppm. The dissolution medium is 900 mL of water heated to 37.0 ± 0.5°C. Samples are introduced into the dissolution baths and are collected at appropriate time intervals through a filter tip (Disteck, 20 µm pore size). Sample solutions are passed through a UV flow-cell and the absorbance is measured against a standard using an UV absorbance spectrophotometer at 236 nm. The dissolution limits are defined for each sampling interval.

9.2. Potency

9.2.1. Assay

9.2.1.1. High Performance Liquid Chromatography

A stability-indicating high performance liquid chromatographic method is available for the formulated product of diltiazem hydrochloride. Diltiazem hydrochloride is separated from its major potential degradate, desacetyl diltiazem using an isocratic HPLC method. The method utilizes a Phenomenex IB

SIL CN column (5 µm particle size, 150 mm x 4.6 mm I.D.) and a mobile phase of 0.05M aqueous potassium phosphate (monobasic) solution containing 0.05% (v/v) triethylamine (pH 4.0) : acetonitrile (75:25). The separation is achieved at ambient temperature using a flow rate of 1.0 mL/minute. UV absorbance detection is accomplished at a wavelength of 236 nm. The standard and sample solutions are prepared in a diluent of acetonitrile and 20 µL are injected onto the column. The retention time of diltiazem is approximately 13 minutes while that of the potential degradation product, desacetyl diltiazem, is 9 minutes. The analysis is completed within 17 minutes and allows quantitation of both components. A typical HPLC chromatogram is shown in Figure 14.

Twenty dosage units are ground to a fine powder and a portion of the powder equivalent to one dosage unit is dissolved in acetonitrile. The sample is sonicated for approximately 15 minutes. After the appropriate dilution of the cooled solution, the sample is centrifuged and the supernatant liquid is injected into the HPLC.

Tablets must contain not less than 90.0 percent and not more than 110.0 percent of the labeled amount of diltiazem hydrochloride.

Numerous other reversed-phase methods exist for diltiazem hydrochloride tablets. One such method uses a Waters µBondapak C18 HPLC column (10 µm particle size, 300 mm x 3.9 mm I.D.). The separation is achieved at ambient temperature with a mobile phase of 40% (v/v) acetonitrile in 0.05M aqueous potassium dihydrogen orthophosphate at a flow rate of 2 mL/minute. Detection is achieved using UV absorbance at 254 nm. Ethanol is the extraction solvent resulting in a final concentration of the sample of 0.30 mg/mL and 20 µL is injected onto the column (63). Another similar chromatographic method is available for diltiazem hydrochloride in tablets (64).

Another example of an HPLC method using clonazepam as the internal standard is available for the study of the stability of diltiazem tablets in the presence of its degradation products. The method utilizes a Micropak MCH-5 reversed-phase column

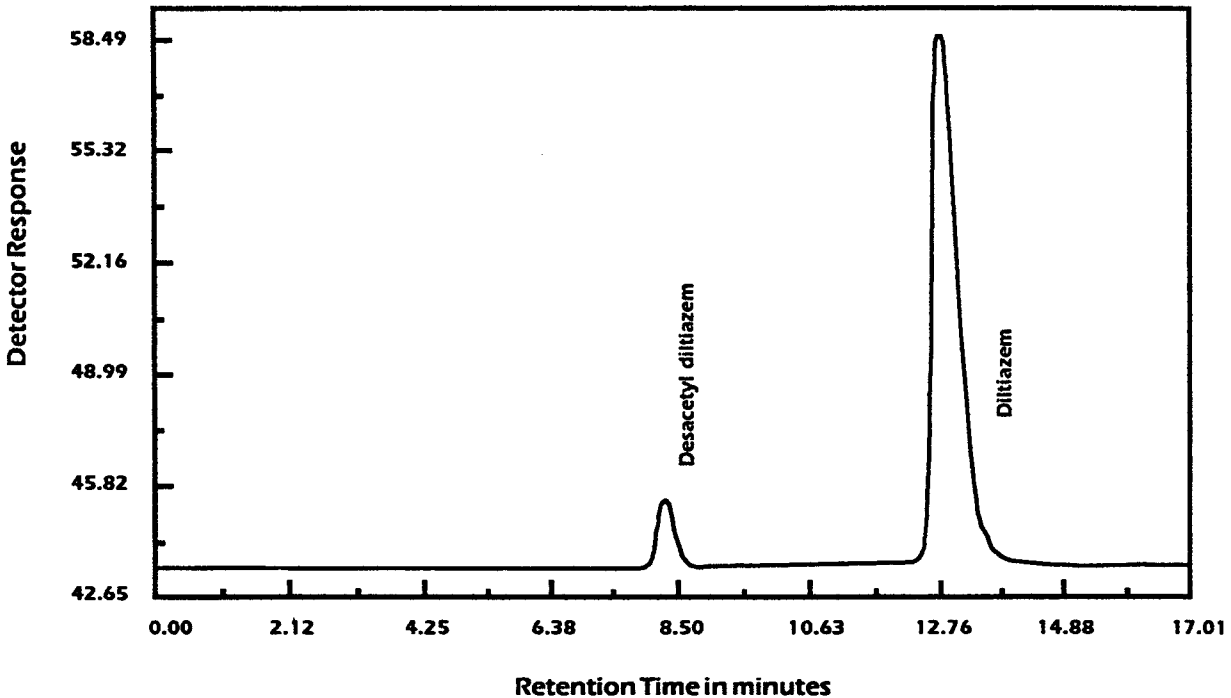


Figure 14. Typical HPLC chromatogram of diltiazem hydrochloride drug product containing the principal degradation product, desacetyl diltiazem.

(5 μm particle size) at ambient temperature with a mobile phase of acetonitrile-water (48:52) at pH 3 containing 0.01M sodium-n-octanesulfonate as the ion pairing agent. The flow rate of the mobile phase is 1 mL/minute and the effluent is monitored at 239 nm using UV absorbance detection. The retention times of the internal standard and diltiazem are 3 and 9 minutes, respectively. The detection limit for diltiazem using this method is 0.2 μg/mL (65).

9.2.1.2. Capillary Zone Electrophoresis

Micellar electrokinetic chromato-graphy can be applied to the determination of diltiazem. The separation of diltiazem using capillary zone electrophoresis is not successful under neutral and alkaline conditions because diltiazem migrates with the same velocity as the electroosmotic flow. The use of bile salts as the micellar phase will allow the migration of diltiazem with a high number of theoretical plates and within a short time. Diltiazem hydrochloride can be separated from its transformation products at ambient temperature using a fused-silica capillary tube (650 mm x 50 mm I.D.), an applied voltage of 20 kV and a buffer of 0.02M aqueous phosphate-borate (pH 8.0) containing 0.01M aqueous sodium cholate. Detection is accomplished on-column using UV absorption at 210 nm. Using this method, the detection limit is about 0.1% (66).

9.2.1.3. Gas Chromatography

A gas chromatographic assay allows the determination of diltiazem hydrochloride in tablet formulations. The separation uses a 3% OV-210 on a Chromosorb W 80/100 mesh column with an oven temperature of 260°C (isothermally). A carrier gas flow rate of 50 mL/minute is used. The method allows the quantitation of diltiazem in the concentration range of 0.05 mg/mL to 0.2 mg/mL (67).

9.2.2. Dosage Uniformity

Tablets must meet the U.S.P. requirement for content uniformity testing (62). The content uniformity assay is performed by assaying ten tablets individually. The samples are

dissolved with the aid of sonication in acetonitrile and then appropriately diluted. The sample is centrifuged prior to testing. The chromatographic procedure for the potency assay (Section 9.2.1.1.) is then used.

9.2.3. Stability Testing

The stability testing requirements for diltiazem hydrochloride include an HPLC assay and dissolution test. The HPLC assay is described in the section for the potency assay (9.2.1.1.) and the dissolution test method is described in Section 9.1.

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DIOSGENIN

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1. DESCRIPTION
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1.1.1. Chemical Names
1.1.2. Synonym
1.2. Formula
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1.2.2. Structural
1.3. Molecular Weight
1.4. Elemental Composition
1.5. Appearance
1.6. Occurrence
1.7. Use
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2.2. Specific rotation
2.3. Solubility
2.4. Thermal Analysis
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2.5.2. Absorbance reflectance spectrum
2.5.3. Near Infra Red (NIR) spectrum
2.5.4. Infra Red (IR) spectrum
2.5.5. Mass spectrum
2.5.6. Nuclear Magnetic Resonance (NMR) spectrum
2.5.6.1. ^{1}H–NMR spectrum
2.5.6.2. ^{13}C–NMR spectrum
3. ISOLATION
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5.2. TLC methode of analysis (densitometric)
5.3. HPLC
5.4. GLC
5.5. Spectrophotometric
5.6. Separation diosgenin and tigogenin
6. BIOLOGICAL ACTIVITY OF DIOSGENIN
7. REFERENCES

1. DESCRIPTION

1.1. Nomenclature

1.1.1. Chemical Names

(25R)–Spirost–5–en–3β–ol ; 25R–5–Spirosten–3β–ol ; 3β–Hydroxy–5–Spirostene

1.1.2. Synonym

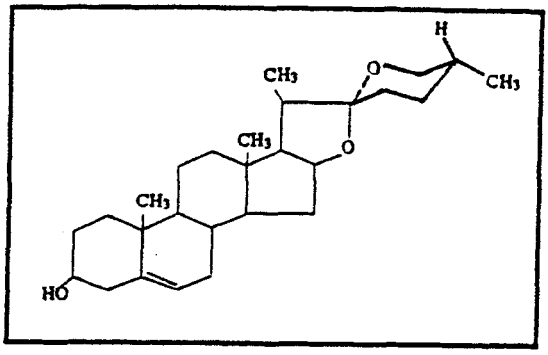
Nitogenin

1.2. Formula

1.2.1. Empirical

$C_{27}H_{42}O_3$

1.2.2. Structural



1.2.3. CAS Registry No.

512-04-9

1.3. Molecular weight

414.61

1.4. Elemental composition

C : 78.21 %, H : 10.21 %, O : 11.58 %

1.5. Appearance

A fine white odourles and tasteless crystalline powder

1.6. Occurrence

Diosgenin was accumulated in various plants mostly in Family Dioscoreae, Zingeberaceae, and Liliaceae as glycoside. It was reported by Mahato et al. [1], that 63 kinds of saponins have diosgenin as its aglycon. Diosgenin was produced commercially mostly from rhizomes of *Dioscorea spp.* (5-8%) and *Costus spp.* (1-3%) [2].

1.7. Use

Diosgenin was used as raw material for producing steroid hormone in pharmaceutical industry.

2. PHYSICAL PROPERTIES

2.1. Melting point

204 - 207 ^{o}C [3]

2.2. Specific rotation

$[\alpha_D]$ at 25^{o}C (c =1.4 in $CHCl_3$) = -129^{o} [3]

2.3. Solubility

The solubility data of diosgenin are listed in Table 1.

Table 1. Diosgenin solubility in various solvents

Solvents	Solubility (mg/ml, 30°C)
Methanol	20
Ethanol absolute	> 100
Chloroform	> 100
Ethyl acetate	> 100
n–Hexane	9
Cyclohexane	30
Water	1

2.4. Thermal Analysis

The DTA (differential thermal analysis) thermograms of diosgenin were obtained on a Shimadzu DT-30 Thermal Analyser. The heating rate was 10°C/min. The thermograms are presented in Figure 1.

The DTA curve **a** revealed a endothermic residual moisture peak and a single sharp endhotermic melting peak of diosgenin (onset 206°C). Thermogram **b** was recorded from 25°C to 122°C, after cooling the sample was heated again from 25°C to 223°C (curve **c**) and showed only a single endhotermic melting point of diosgenin.

2.5. Spectral properties

2.5.1. Ultra Violet (UV) spectrum

The ultraviolet spectrum of diosgenin in methanol was shown in Figure 2. It exhibits a maximum at 205 nm, with absorbance = 0.986 for 100 ppm solution. The first order derivate spectrum with $d\lambda$ =2nm was also shown. The spectra was recorded on a Shimadzu UV 265 spectrophotometer.

2.5.2. Absorbance reflectance spectrum

The visible absorbance reflectance spectrum of diosgenin on Kieselgel GF254 TLC precoated (E.Merck), eluted with $CHCl_3$/ethyl acetate (3/1) and visualized by anisaldehyde–sulphuric acid spray reagent (100°C, 10 min) was shown in Figure 3. It showed a maximum at 430 nm. The spectrum was recorded on a Shimadzu CS-930 TLC Scanner.

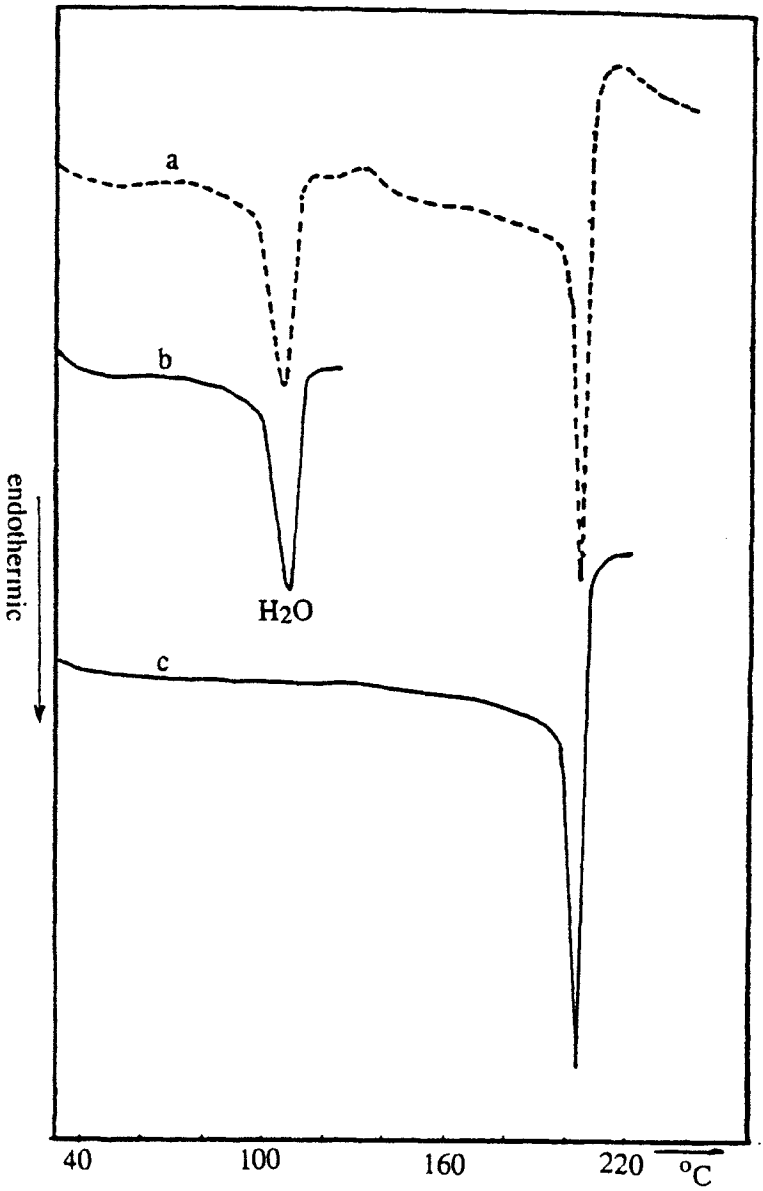


Figure 1 : The DTA Thermogram of Diosgenin (Sigma)

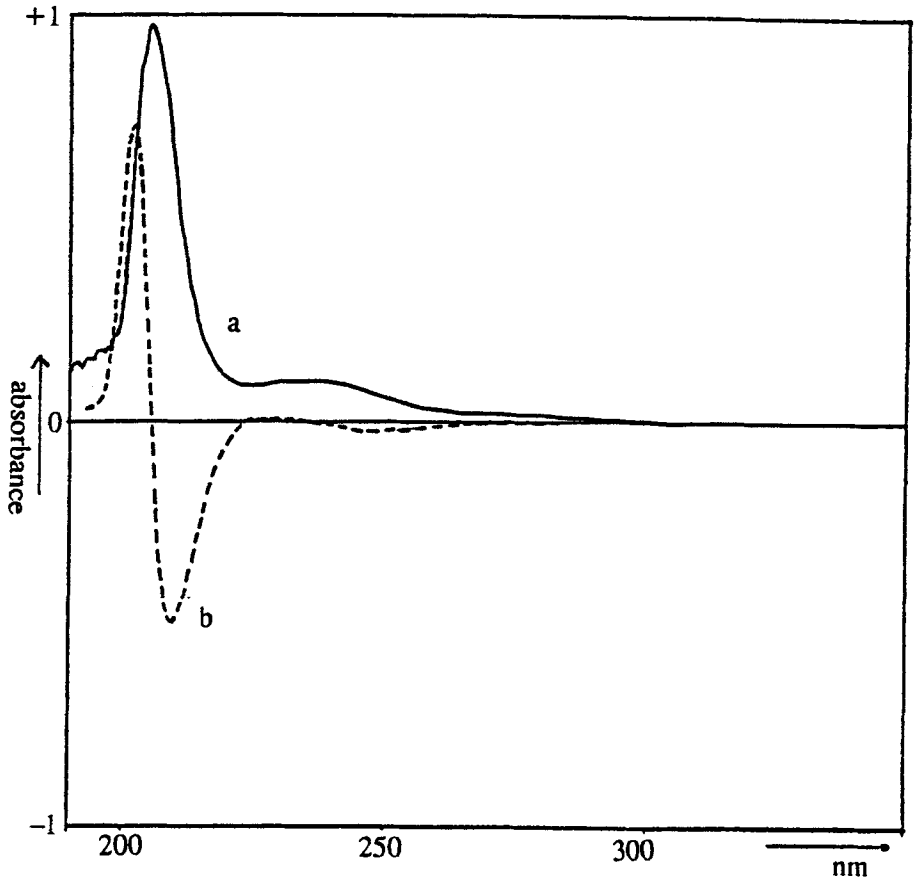


Figure 2 : Ultra Violet Spectrum of diosgenin (Sigma) in methanol (a). The first order derivative spectrum with di λ = 2 nm wits also presented (b)

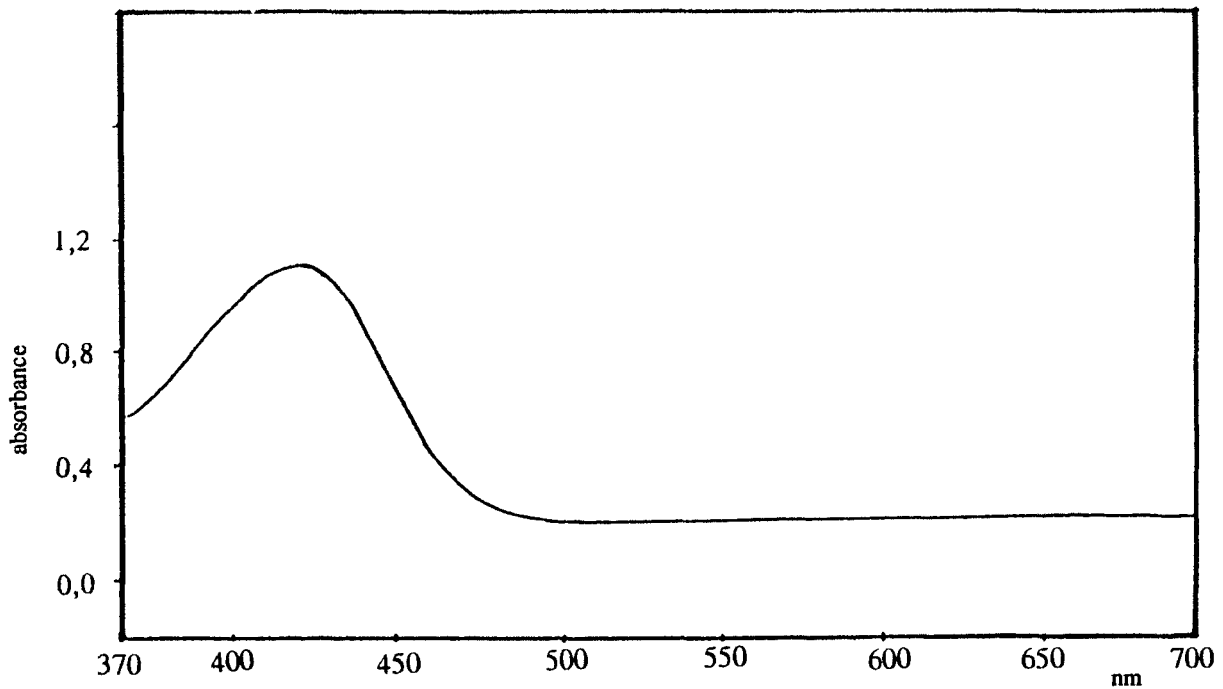


Figure 3 : Absorbance Reflectance Spectrum of diosgenin (Sigma)

2.5.3. NIR (Near Infrared) spectrum

The NIR (near infrared) spectrum of 1 % diosgenin in CCl_4 was recorded by using a Shimadzu 365 spectrophotometer was shown in Figure 4. The λ_{max} data are shown in table 2.

Table 2. NIR characteristics of diosgenin

λ max (nm)	Assignment
1415	OH stretching
2231	CH stretching
2357	
2435	
2458	

2.5.4. IR (Infra Red) spectrum

The IR (infra red) spectrum of diosgenin as KBr disc (2mg/200mg) was recorded on a Jasco 5300 FT– IR spectrophotometer to which the IR spectrum was shown in Figure 5. The characteristic bands for diosgenin are shown below.

Table 3. IR Characteristics of diosgenin

Frequencies (cm-1)	Assignment
3460	stretching vibration of OH
2906 – 2848	stretching vibration of CH steroid skeleton
1660	vibration of C = C bond
1452	deformation vibration of methyl
1377	and methylene

Beside the characteristic assigments which described in the table 3, the spiroketal ring of diosgenin was shown with characteristic bands as follows 837, 870, 899, 921, 952, 963 and 981 cm^{-1} and 25R configuration was shown by more intensive band at 899 than 921 cm^{-1} [4].

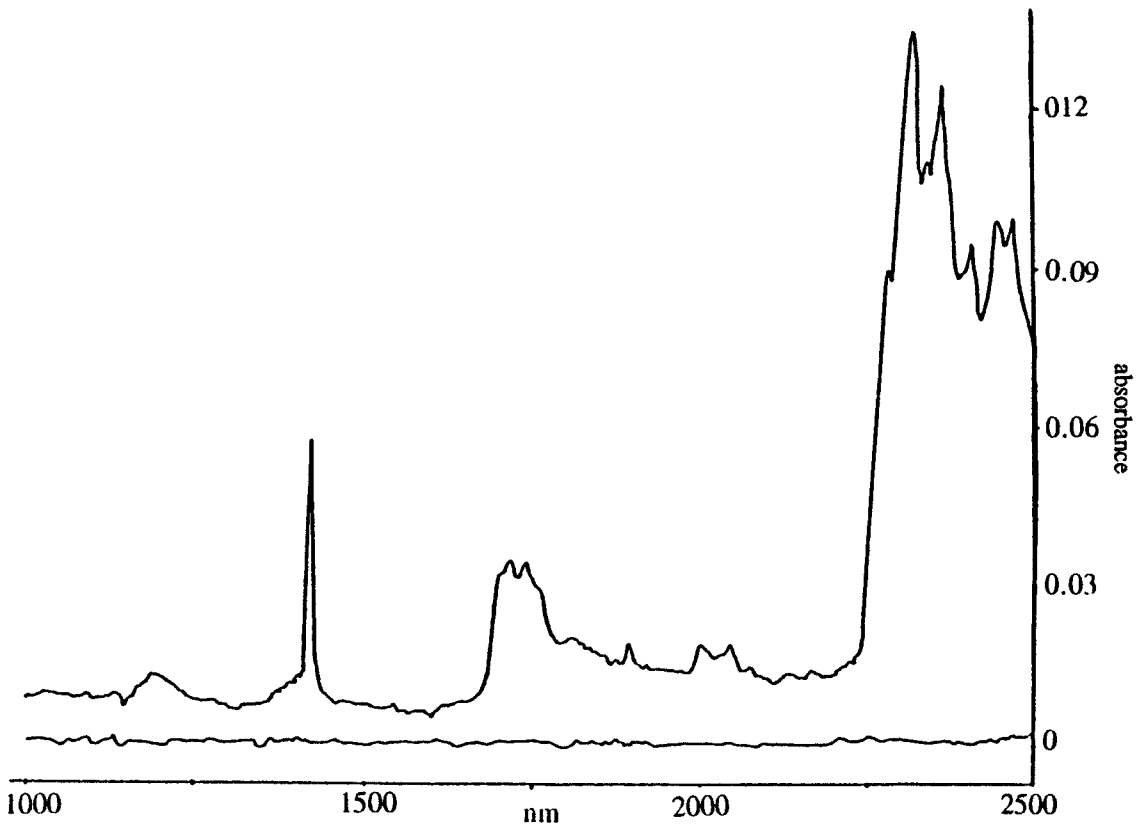


Figure 4 : Near Infra Red Spectrum of 1% diosgenin (Sigma) in CCl_4

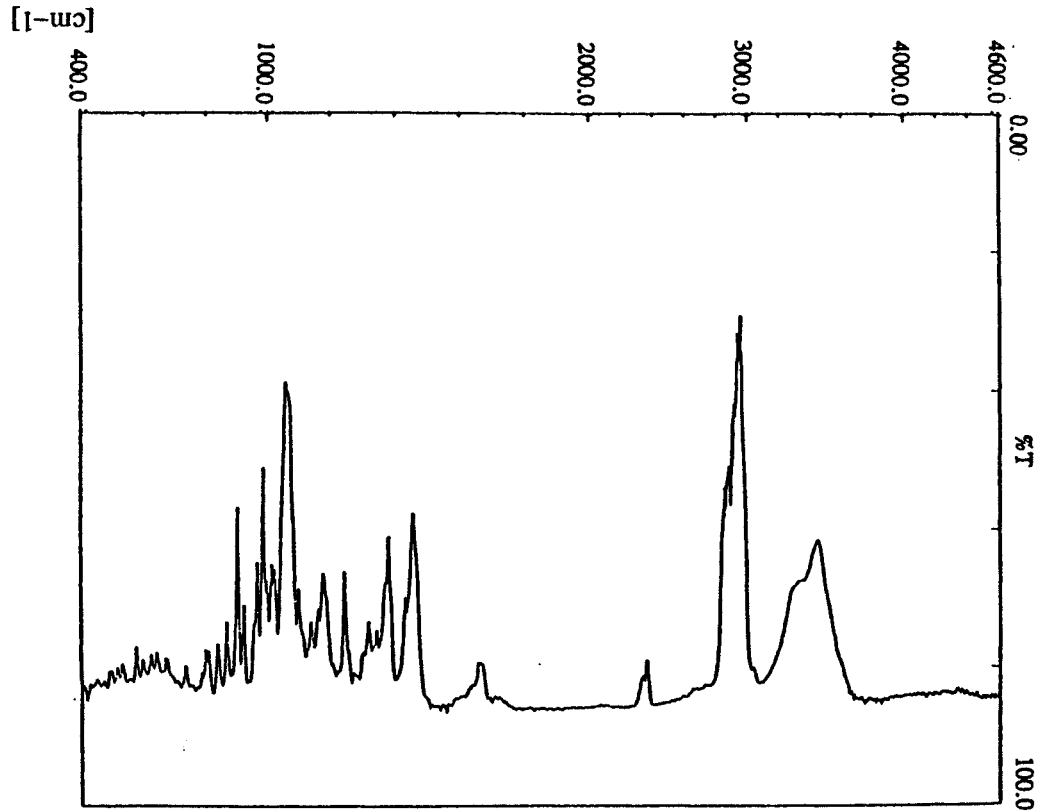


Figure 5 : Infra Red Spectrum of diosgenin (Sigma)

2.5.5. Mass Spectrum

The MS (mass spectrum) of diosgenin presented in Figure 6A and Figure 6B were obtained by electron impact (EI) and chemical ionization (CI) using methane as reagent on a Jeol, JMS–DX–303 Mass spectrophotometer. The ionizing electron beam energy was at 70 eV. The main fragments of diosgenin are given in the table 4.

Table 4. The main fragments of diosgenin

species *	m/z (rel. int.)	
	EI	CI
$M^+ + 29$		443 (5)
$M^+ + 1$		415 (73)
M^+	414 (17)	
		397 (83)
a	342 (10)	
b	300 (25)	
(b–H_2O)	282 (55)	
c	285 (15)	
(c–H_2O)	267 (10)	
d	217 (15)	
e	139 (100)	139 (8)
f	115 (15)	115 (18)

* See scheme 1.

The fragment of m/z 139 and 115 were characteristic for spiroketal ring [5,6] of diosgenin.

2.5.6. Nuclear Magnetic Resonance Spectrum

2.5.6.1. ^{1}H–Nuclear Magnetic Resonance Spectrum

The ^{1}H-NMR spectrum of diosgenin in $CDCl_3$ recorded on a Jeol FT–NMR spectrophotometer (400 MHz) and on a Hitachi FT–NMR R-1900 spectrophotometer (90 Mhz) using TMS as the internal standard. The spectra are shown in Figure 7 and 8. The characteristics proton chemical shifts, multiplicities and assignment are given in the table 5 are identical with previously reported spectra [6,7].

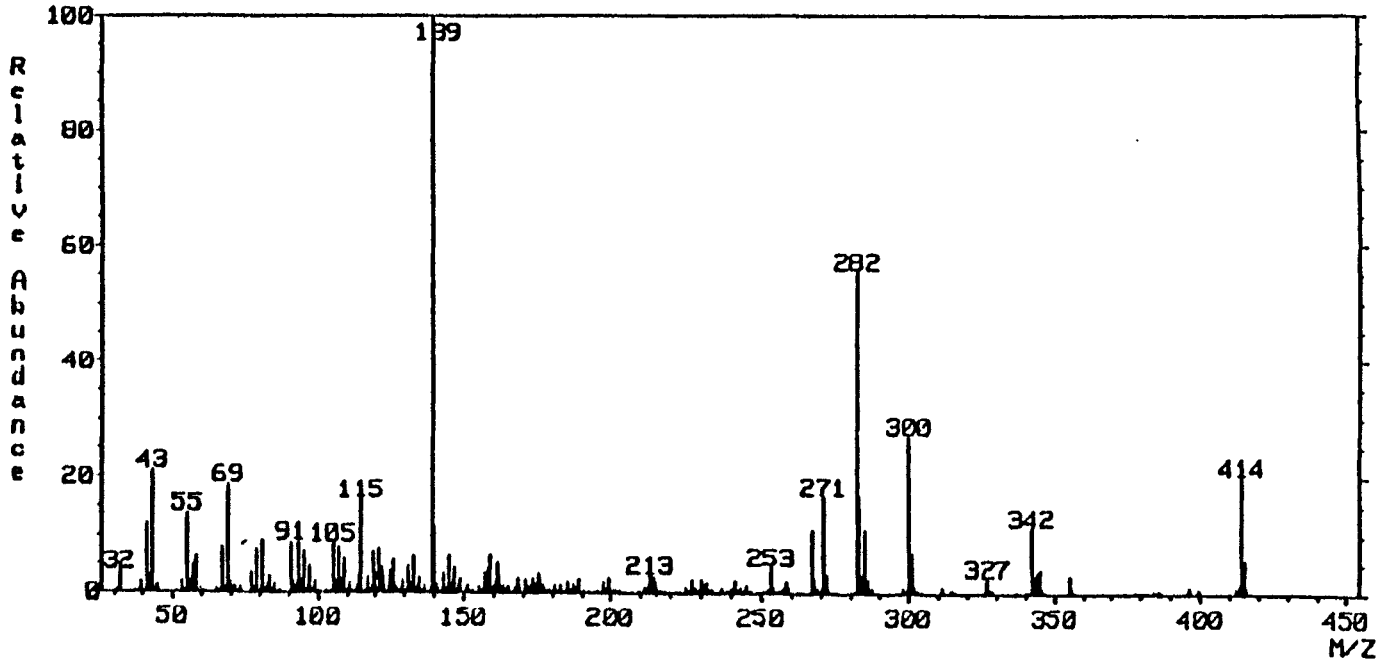


Figure 6A : El Mass Spectrum of diosgenin (Sigma)

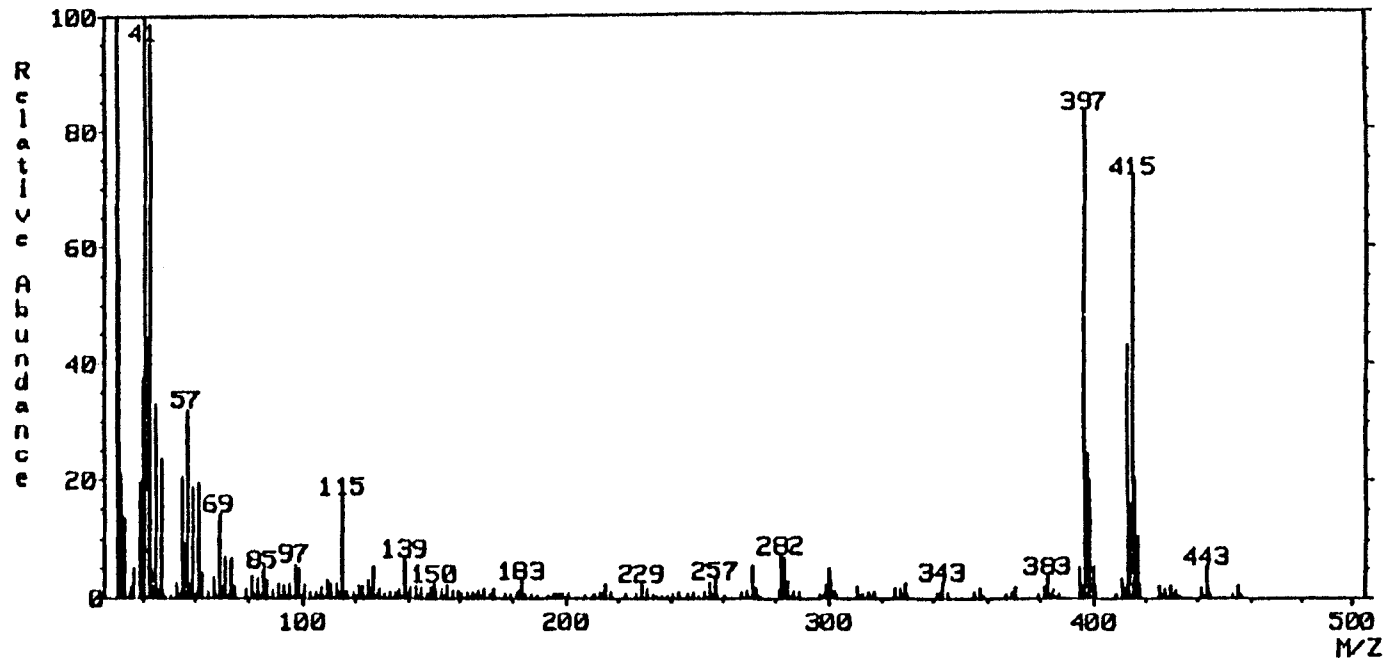
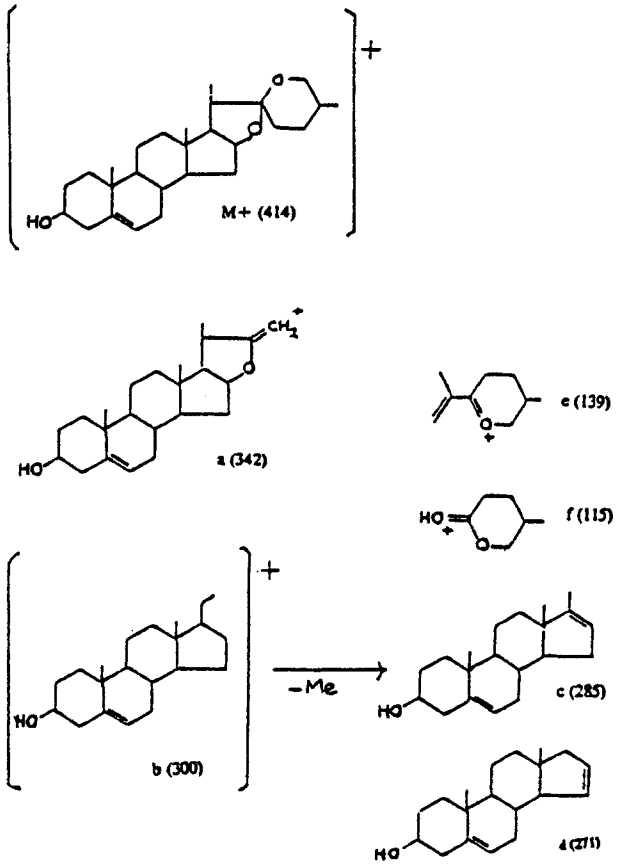


Figure 6B : CI Mass Spectrum of diosgenin (Sigma)

Scheme 1 : EI – MS Fragmentation of Diosgenin



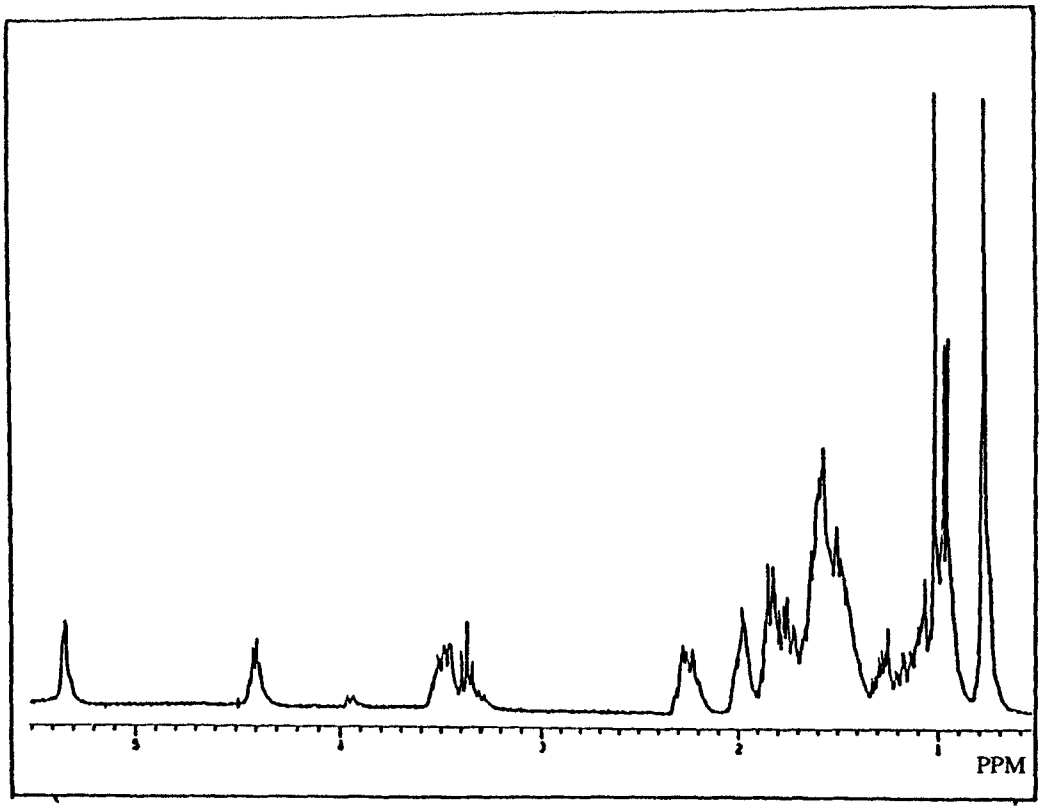


Figure 7 : ^{1}H – NMR (400 MHz) Spectrum of diosgenin (Sigma) in $CDCl_3$

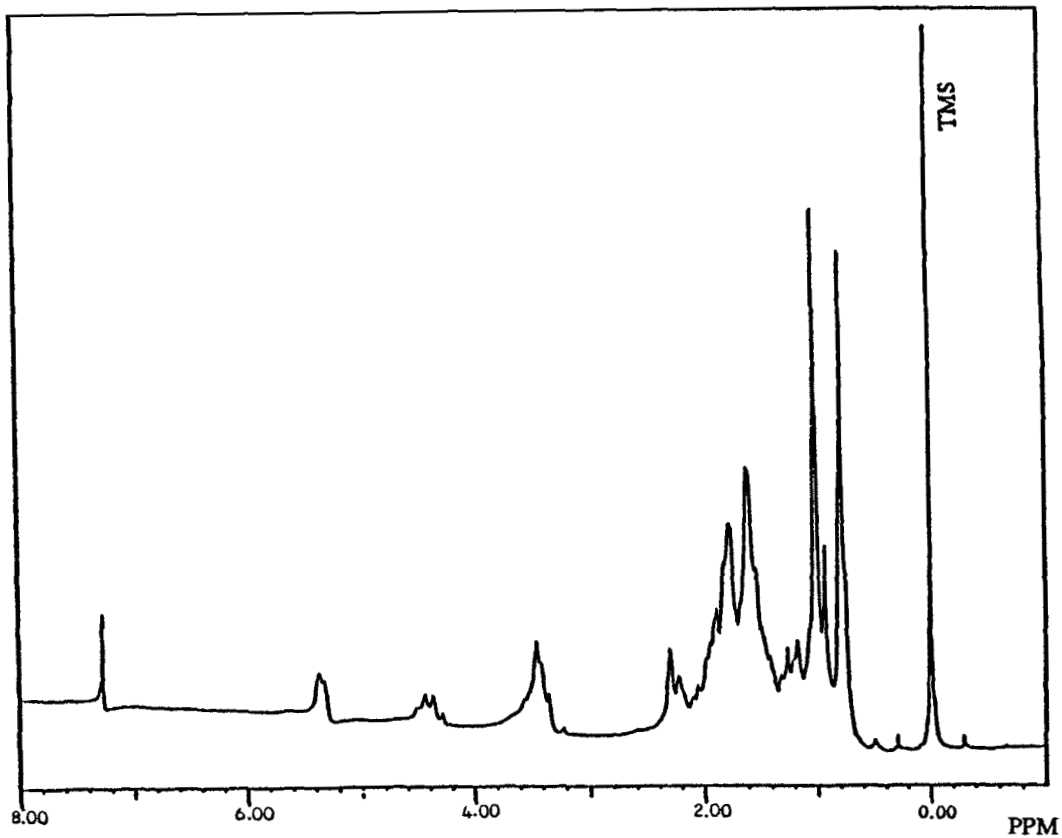


Figure 8 : ^{1}H – NMR (90 MHz) Spectrum of diosgenin (Sigma) in $CDCl_3$

Table 5. ^{1}H-NMR characteristics of diosgenin

Chemical shift (δ ppm)		Multiplicity** (J Hz)	Assignment
400 Mhz	90 Mhz		
0.77	0.79	s	Me–27
0.78	0.79	s	Me–18
0.97	0.98	d(6.4)	Me–21
1.02	1.03	s	Me–19
1.99	*	m	H–11eq
3.36		t (10.5 ; –10.5)	H–26ax.
3.49		dd (4.1 ; –10.5)	H–26eq.
3.53		m	H3ax
4.40	4.40*	q(6.4;6.4;6.4)	H–16
5.35	5.31	br.d	H–6

* : not resolved
** : For 400 Mhz ^{1}H–NMR Spectrum

2.5.6.2. ^{13}C-Nuclear Magnetic Resonance Spectrum

The ^{13}C–NMR spectrum of diosgenin in $CDCl_3$ (Figure 9) was recorded in a Hitachi R–1900 FT NMR (22.1 Mhz) using TMS as internal standard. The data are presented in the table 6.

The characteristic ^{13}C NMR signals for 25R configuration of diosgenin are C23 (31.3 ± 0.3 ppm), C24 (28.8 ± 0..3 ppm), C26 (66.9 ± 0.2 ppm) and C22 (108.7 – 110.0 ppm), and for Δ^5 are C5 (141.0 ± 0.8 ppm) and C6 (121.0 ± 0.4 ppm) [9].

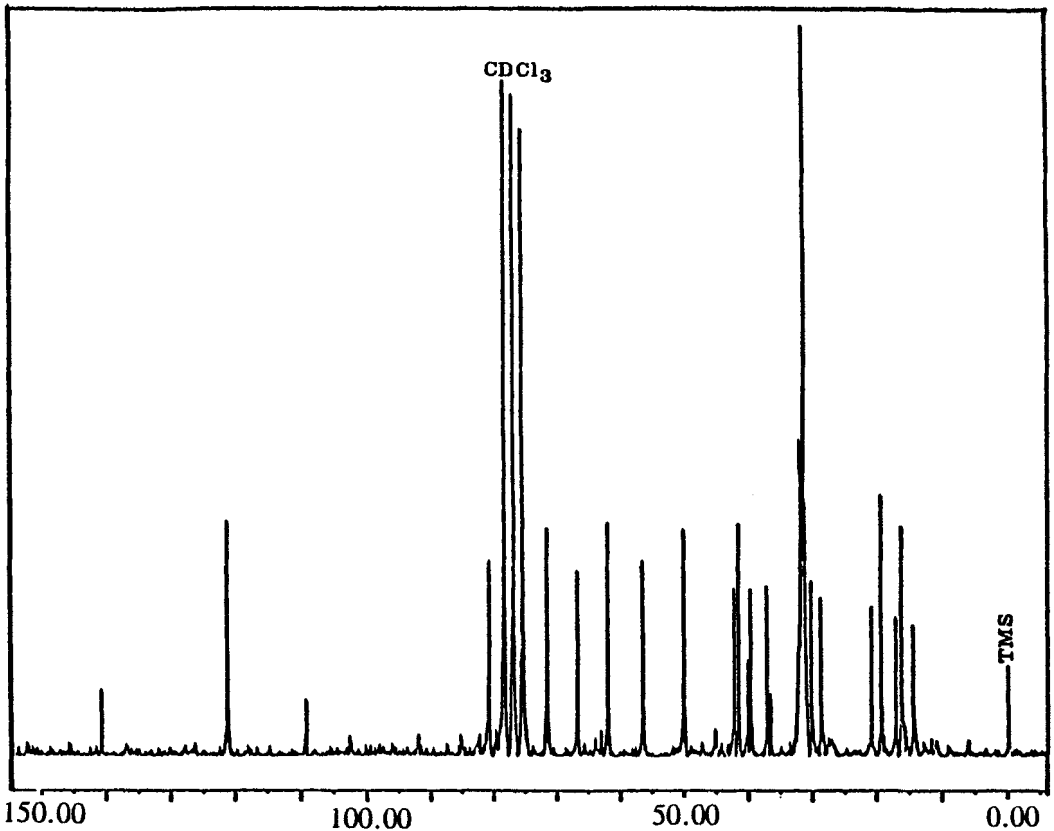


Figure 9 : ^{13}C – NMR Spectrum of diosgenin (Sigma) in $CDCl_3$

Table 6. ^{13}C-NMR characteristics of diosgenin

Carbon No.	Chemical shift (δ, ppm; in $CDCl_3$)	Carbon No.	Chemical shift (δ, ppm; in $CDCl_3$)
1	37,3	14	56.5
2	31.6	15	31.8
3	71.6	16	80.7
4	42.3	17	62.1
5	140.6	18	16.3
6	121.3	19	19.4
7	32.0	20	41.6
8	31.4	21	14.5
9	50.1	22	109.2
10	36.6	23	31.4
11	20.9	24	28.8
12	39.8	25	30.3
13	40.3	26	66.8
		27	17.1

3. ISOLATION

In our laboratory we isolated diosgenin from the rhizome of *Costus spiralis* Rosc. with the following procedure : 750 gram of fresh weight rhizomes was boiled with 1l ethanol 96 % for 3 hours, 2 times. After concentrated the extract to a quarter volume then hydrolyzed with HCl 2N for 2 hours (100°C). After cooling the hydrolysate was neutralized with saturated solution of sodium bicarbonate then filtered. The residue was extracted with petroleum ether (40–60°) in a Soxhlet apparatus for 18 hours. After evaporating the petroleum ether extract, the crude diosgenin was purified on a column chromatography (Kieselgel 40; E. Merck) using n-hexane/ethyl acetate (8/2) as eluent.

4. CHEMICAL CONVERSION OF DIOSGENIN TO STEROID HORMONES

In order to obtain steroid hormone like materials, ring E and F of diosgenin attached to C16 and C17 must first be removed. Ring E and F of diosgenin can be easily be degraded to

16-dehydropregnenolone acetate (16-DPA), which is an exellent material for the preparation of most type of steroid hormone (Marker degradation) [10,11,12]. In scheme 2 the conversion of diosgenin to some steroid hormone was presented.

5. METHOD OF QUANTITATIVE ANALYSIS

5.1. Methods of diosgenin extraction from sample

Method of Blunden et al [13]

After maceration of the fresh *Dioscorea spp.* tubers, hydrolysis was refluxing with 2N HCl for 2 hours,extraction of diosgenin from dry acid insoluble residue with petroleum ether (40-60). After evaporating to dryness the extract was dissolved in $CHCl_3$ before analysis.

Method of Carle [14]

100 mg powdered dry plant material (or 500 mg of tissue cultures biomass) was refluxing with 40 ml 2N HCl/MeOH 2 hours (75°C). After cooling the extract was neutralized with 6 ml NH_3 25% , and evaporated to dryness. The residue was taken with 10 ml ice water and filtered (Seitz-filter). The residue on filter was washed with 25 ml NH_3 10 % then 25 ml cool water. The residue on filter was heated overnight (60° C) to dryness. The dry residue on Seitz -Filter was refluxing with 50 ml $CHCl_3$ (70°C), then evaporated to dryness. The dry $CHCl_3$ extract was dissolved in $CHCl_3$ before analysis.

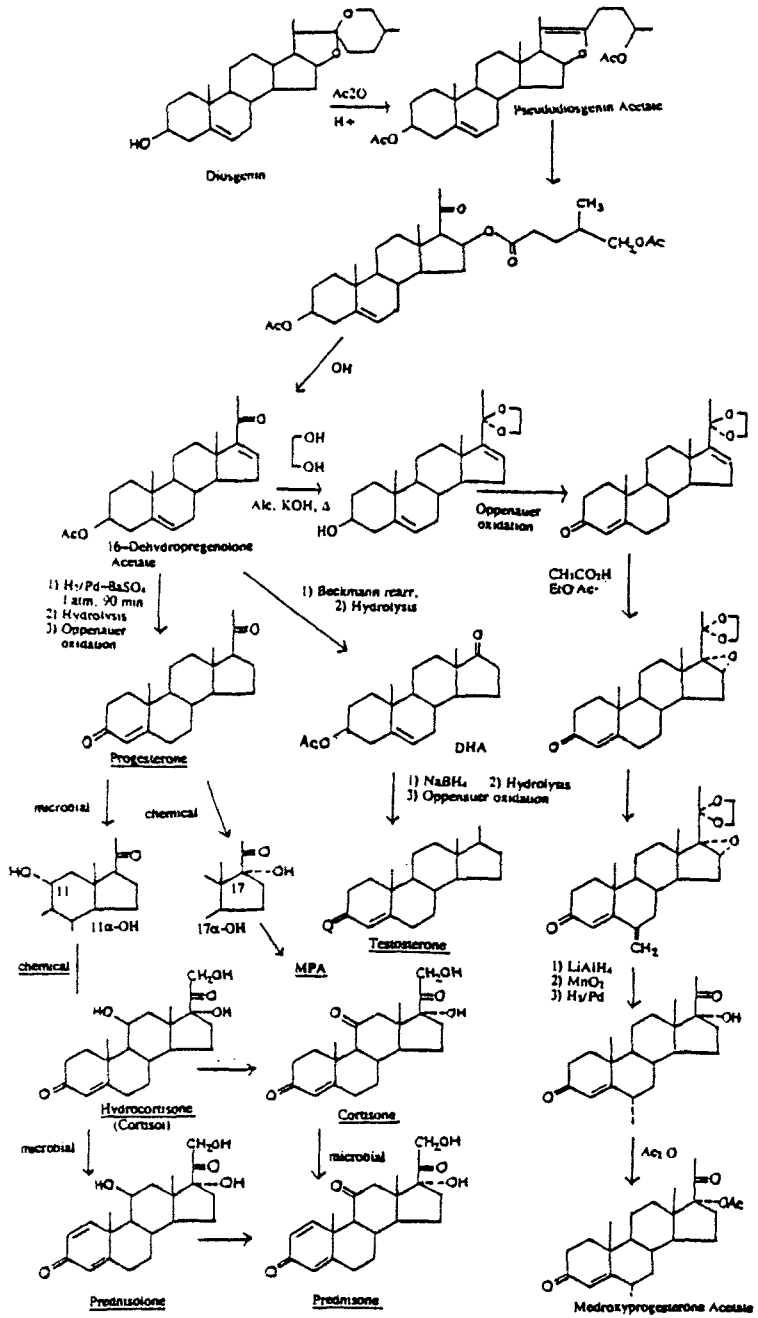
Method of Tal and Golberg [15]

100 mg lyophilized plant cells materials were refluxing with 2N HCl for 2 hours, filtered, washed with water and lyophilized. The dried cells were extracted with $CHCl_3$ for 3 hour at 60°C. The $CHCl_3$ was filtered (0.46 M) and evaporated to dryness. The extract was dissolved in small volume of $CHCl_3$ before injection to HPLC.

Method of Indrayanto et al. [16]

To separate free sterols and other non polar soluble component of the plant biomass from sapogenin steroid extraction was done in the following method. 1 gram (accurately weight) of dry biomass was extracted 3 times using a vortex-mixer with 5 ml $CHCl_3$ for each. The residue was hydrolyzed with 2 N HCl (100°C, 2 hours), cooled, neutralized with 10N NaOH, extracted 3 times with 5 ml $CHCl_3$. The chloroform phase was collected and evaporated in N_2 to dryness. The hydrolysate extract was dissolved in 1 ml $CHCL_3$ (accurately) before analysis (for TLC) or in methanol (for HPLC).

Scheme 2 : Conversion of Diosgenin to several Steroid Hormone (11)



According to Görög and Szàsz [12] to extract diosgenin from seeds, the sample must be defatted with petroleum ether (40-60).

5.2. TLC method of analysis (densitometric)

The $CHCl_3$ extract was applied on Kieselgel 60 precoated plates (E. Merck). n-Hexane : ethyl acetate (3:1) was used as eluents. The diosgenin spots (Rf. 0.36) are visualized by anisaldehyde- sulphuric acid spray reagent. Quantitation was done by measuring the maximum absorbance reflectance at 427 nm (Shimadzu CS-930 TLC Scanner). By this method linearity of diosgenin was achieved at concentration 0.01 g to 12.00 g/spot ($r = 0.9928$, $n = 10$), Limit of detection = 0.004 g/spot; Limit of quantitation = 0.012 g/spot. Accuracy with standard addition was 98.86 0.65 % (mean SD, n=5) [16]. Blunden et al. [12] using n- Hexane : Ethyl acetate (4:1) as eluents and antimony trichloride- sulphuric acid as sprayed reagents. According to the author experiences it is impossible to separate diosgenin and tigogenin with conventional TLC methods, so the absence or presence of tigogenin must be confirmed with other methods of analysis.

5.3. HPLC

Analysis was performed by using a stainless steel (4 mm x 25 cm) Micropack MCH 10 column (RP C18 Variant) with a mixture of solvent containing acetonitril 83 %, methanol 10 % and chloroform 10 %. A refractive index detector was used [15]. Lambert et al. [17] reported HPLC analysis of diosgenin with a Lichrospher 100 CH- 8/II RP (25 x 0.4 cm) and acetonitril:water (75:25) as mobile phase, detection UV 210 nm. In our laboratory we used a 25x0.4 cm Partisil ODS-3 column (Whatman) and Methanol:Water (95:5) as mobile phase and monitored by UV-Detector (205 nm). With this method linearity was achieved at concentration 0.049 g to 09.96 g/peak; limit of detection was 0.040 g/peak; limit of quantitation was 0.136 g/peak. Using this method diosgenin, solasodine, solanidine, cholesterol and stigmasterol/sitosterol could be separated. A typical HPLC chromatogram of an extract of *Costus spp.* rhizomes ,which analysed in our laboratory , was presented in figure 10.

5.4. GLC

Carle [14] reported analysis of diosgenin by using a glass column (1/8 inch x 6 ft) with 3% SE-30 on Gaschrom Q 100-120 mesh, oven

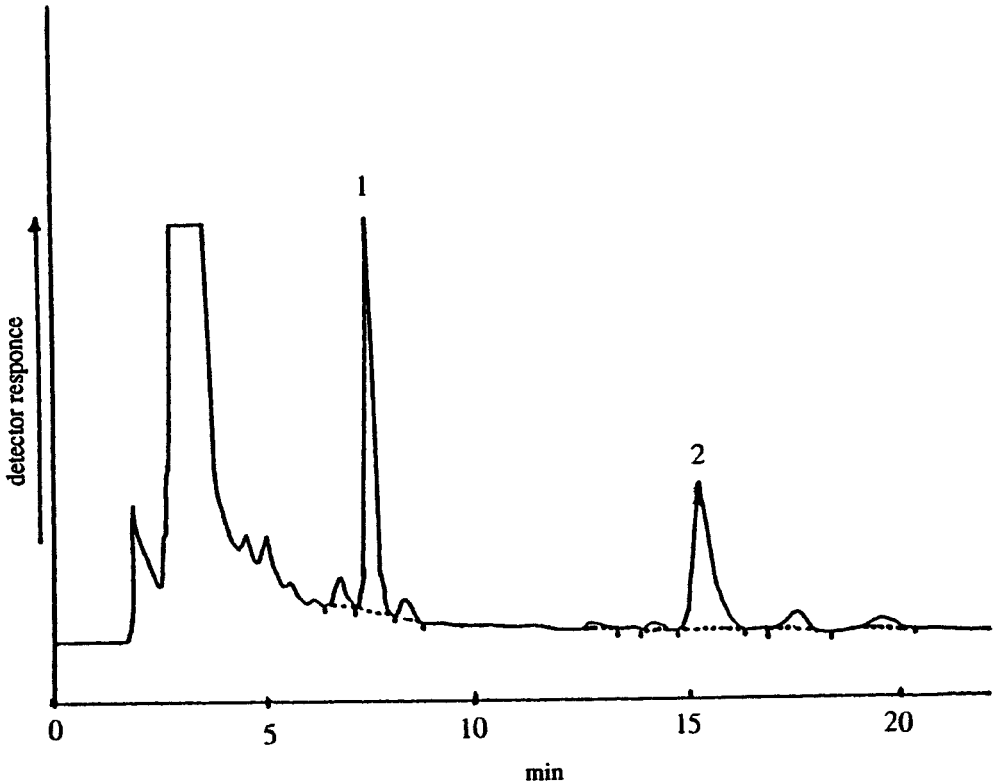


Figure 10 : HPLC Chromatogram of an extract of
Costus spp. Rhizomes, Condition : see text
1 Diosgenin : 2 Stigmasterol

temperature, FID and injector were 300°C. Lambert et al [17] used a OV-17 capillary column (15 m x 0.1 mm i.d.) with oven temperature was 245°C.

5.5. Spectrophotometric

Diosgenin and other sapogenin are UV inactive compounds so must be reacted before determinated spectrophotometrically. Diosgenin can be reacted with 70 % perchloric acid at room temperature. The yellow colour remain constant for at least 3° minutes (λ_{max} 410 nm). This method is spesific for Δ^5-sapogenins [12].

5.6. Separation diosgenin and tigogenin

By using a fused silica column, 50 m x 0.22 mm i.d., CP-SII 5 CB, film thickness 0.12 m and oven temperature 270°C, carrier gas hydrogen (linear velocity 49 cm/sec), Van Gelder [18] reported the separation of various alkaloid and sapogenin steroids (including diosgenin and tigogenin). By using derivatization with TFAA before injection in GLC, Carle [14] could separated diosgenin and tigogenin by using a glass column (1/8 inch x 10 ft.) with 3 % QF-1 as stationary phase, and oven temperature 220°C. Diosgenin and tigogenin could be separated also by HPLC method of analysis by using Sperisorb SSW and Ultrasphere ODS with n-hexane/ethanol and water/methanol solvents systems [19].

6. Biological activity of diosgenin

Thewles et al. [20] reported that biliary cholesterol output in rat was stimulated over 3-fold by feeding diosgenin for 5 days, whereas billiary outputs of phospholipid and bile salts were not changed. Diosgenin treatment also produced a significant decrease in biliary alkaline phosphodiesterase. The saponins from *Costus speciosus* has a stimulating effect and anti inlammatory activity on uterus and produced proliferative changes in vagina and uterus, showing similiar effect to that produced by stilbestrol [21].

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ECONAZOLE NITRATE

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1. Description
   1.1. Nomenclature, Formulae, Molecular Weight and Structure
   1.2. Drug properties
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   2.3. Mass Spectrum
   2.4. Infrared Spectrum
   2.5. Ultraviolet Spectrum
   2.6. Thermal properties
   2.7. Crystal Properties
   2.8. Solubility
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4. Stability
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   5.1. Drug Metabolism
   5.2. Pharmacokinetics
6. Methods of Analysis
   6.1. Identification
   6.2. Spectrophotometric Analysis
   6.3. Titrimetric Analysis
   6.4. Chromatographic Analysis
   6.5. Microbiological Analysis
7. Acknowledgements
8. References

1. DESCRIPTION

1.1. Nomenclature, Formulae, Molecular Weight and Structure

Econazole nitrate was first synthesized in 1969 by GODEFROI et al[1]. The compound has been marketed under the following trade names; 'Ecostatin'; 'Epi-Pevaryl'; 'Gyno-Pevaryl'; 'Ipenee'; 'Palavale'; 'Pevaryl'; 'Spectazole' and 'Econacort' (with hydrocortisone).

The chemical name of econazole nitrate is 1-(2-(4-chlorophenylmethoxy)-2-(2,4-dichlorophenyl) ethyl)-1H-imidazole. The molecule contains an asymmetric carbon and is thus capable of existing in two stereoisomeric forms. However, stereochemical procedures are not employed in its synthesis and it is used only as a racemic mixture.

The molecular formula of econazole nitrate is C_{18} H_{15} Cl_3 N_3O and the molecular weight is 444.7. The structural formula is given in figure 1.

1.1.1. Nomenclature

1.1.1.a. Chemical name - econazole is referred to under the following names:
1-[2-[(4-chlorophenyl)methoxy]-2-(2,4 dichlorophenyl)ethyl]-1H-imidazole
or
1-[2,4-dichloro-β-[(p-chlorobenzyloxy)phenylethyl] imadazole.

1.1.1.b Generic name - Econazole nitrate.

1.1.1.c Proprietary name - Ecostatin, Econacort (with hydrocortisone), Gynopevaryl, Pevaryl, Spectrazole, Mycopevaryl.

1.1.2. Formulae

1.1.2.a Empirical - C_{18} H_{16} Cl_3 N_3 O_4

1.1.2.b Structural - see figure 1

1.1.2.c Wiswesser line notation
- T5N CNJ A01R DG& B2R BG DG &WNQ

1.1.2.d CAS Registry number - 68797-31-9

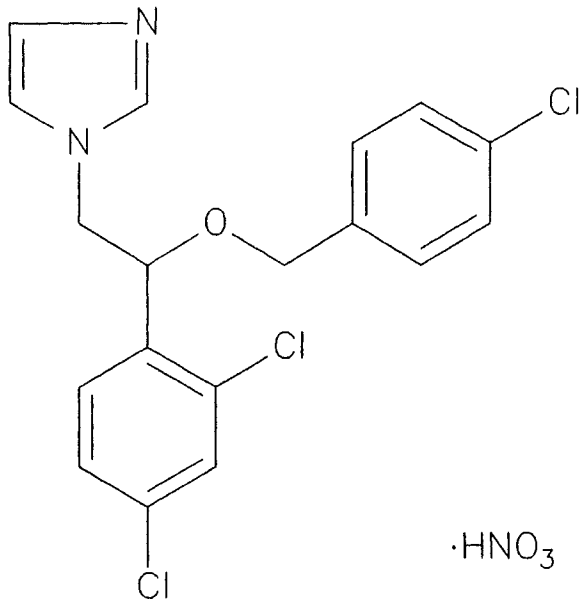

FIGURE 1: Structure of econazole nitrate

1.1.3. *Molecular mass*
444.7 (HNO_3 content 14.16%)

1.1.4. Elemental composition

C	=	48.62%
H	=	3.63%
N	=	9.45%
O	=	14.39%
Cl	=	23.92%

1.2. Pharmacological Properties

Econazole is an imidazole antifungal agent which is similar in structure to another imidazole derivative. It is administered topically as the nitrate for the treatment of dermatomyeoses and vaginal candidosis. In standard *in-vitro* sensitivity tests econazole has been shown to be active against a wide variety of dermatophytes, yeasts, *actinomycetes*, moulds and other fungi, as well as some Gram-positive bacteria (but not Gram-negative bacteria)[2]. Econazole, like other imidazole antifungals appears to act primarily by disruption of cell membrane systems. The compound has also been investigated for systemic usage - oral and intravenous route. Intravenous infusions of 200 to 600 mg daily for up to two months, have been well tolerated in a few patients.

1.3. Appearance, Colour, Odour and Taste

Econazole nitrate is a white (or almost white) crystalline powder which is almost odourless.

1.4. Hazard Data

Econazole nitrate has no known hazards asociated with its use and is considered to be of a low order of toxicity (LD_{50} oral in mice 463 mg/kg, LD_{50} oral in rats 668 mg/kg)[3]. In use it may cause local irritation and sensitivity reactions may occur (erythema, pruritis). Normal precautions should be taken when handling it, i.e avoid ingestion, inhalation of the dust and contact between the substance and skin. If skin contact occurs, wash the affected part immediately and thoroughly with soap and water. If the substance is introduced into the eye, flood the eye with copious quantities of water. In cases of ingestion, give large quantities of water as a mouthwash although this should not be swallowed. If the substance is swallowed or inhaled, drink large quantities of water or milk.

2. PHYSICAL PROPERTIES

2.1. Elemental Analysis

Elemental analysis of duplicate samples of econazole nitrate was performed using a Perkin-Elmer 2400 CHN analyser. Approximately 3-mg quantities of material were combusted and measured against an acetanilide standard. The chlorine determination was based on oxygen flask combustion followed by titration against mercuric perchlorate which had been standardized against sodium chloride and chlorobenzoic acid. The oxygen content of the samples was calculated by difference.

The data are given in table 1.

TABLE 1: Elemental analysis data for econazole nitrate

Element	% w/w Theory	% w/w Found
C	48.62	48.79
H	3.63	3.59
N	9.45	9.31
Cl	23.92	23.62
O	14.39	14.69

2.2. Nuclear Magnetic Resonance Spectra

2.2.1. Proton magnetic resonance

The proton NMR spectrum (Figure 3) of a 3 %w/v solution of econazole nitrate is deuterated dimethyl sulphoxide at ambient temperature (approximately 22°C), was obtained using a Bruker AC300 spectrometer operating at a nominal frequency of 300 MHz. The chemical shifts given in Table 2 and the proton assignments are as indicated in Figure 2.

Solvent peaks can be observed at 2.5 ppm and 3.4 ppm (broad). A second broad, low peak due to the nitrate counter-ion proton is seen downfield at 14.33 ppm. The protons associated with the methoxy- and ethyl-carbons are deshielded by their adjacent groups (aromatic and heterocyclic rings and oxygen). Consequently they appear shifted downfield, between 4.3 and 5.2 ppm.

The aromatic protons and those of the ethylenic carbons of the imidazole ring appear between 7.1 and 7.8 ppm. The proton associated with C2 of the imidazole ring is shifted downfield by its adjacent nitrogens and appears at 9.05 ppm.

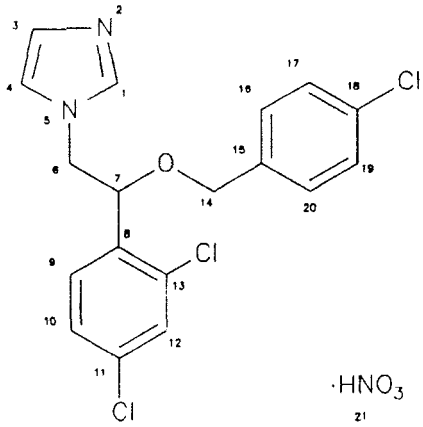

FIGURE 2: Structural assignments for econazole nitrate

2.2.2. Carbon magnetic resonance

The carbon-13 proton decoupled NMR spectrum (Figure 4) of a 3 %w/v solution of econazole nitrate in deuterated dimethyl sulphoxide at ambient temperature (approximately 22°C), was obtained by using a Bruker AC300 spectrometer operating at a nominal frequency of 300 MHz. The chemical shifts are given in table 3 in p.p.m.

The spectrum indicates the presence of five quaternary carbons between 132 and 137 ppm. One of these obscures the imidazole ethylenic carbon (C1) at 136.21 ppm whilst the other two ethylenic carbons (C3 & C4) appear further upfield at 119.78 and 122.94 ppm. The remaining benzenoid carbons resonate between 128 and 130 ppm. These include two pairs of equivalent carbons (C17/19 & C16/20). The alkyl-bridge carbons (C6/7/14) appear further upfield at 75.05, 51.81 and 65.59 ppm respectively. The complex series of resonances around 40 ppm arise from the solvent.

2. MASS SPECTRUM

The mass spectrum (Figure 5) of econazole nitrate was obtained by direct insertion of a sample into a JEOL HX-110 double-focussing mass spectrometer using desorption electon-ionization techniques. The source was heated to 135°C. Desorption was achieved using a 2 A/minute heating ramp. The mass spectrometer was operated at an accelerating voltage of 10 kV. A JEOL complement data system was used to acquire and process the data. The full scan low-resolution spectrum was acquired at 3000-resolution (M/deltaM) by scanning the magnetic field over a range of 30 - 600 m/z.

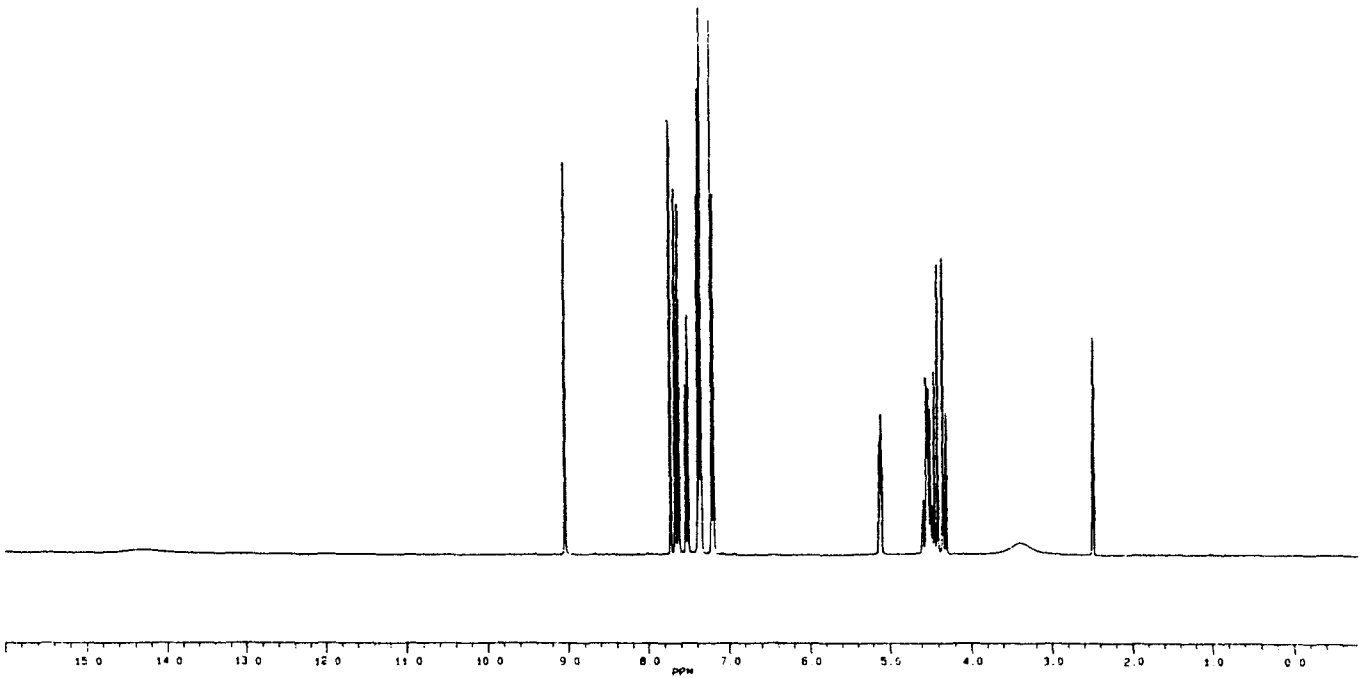

FIGURE 3: Proton NMR spectrum for econazole nitrate

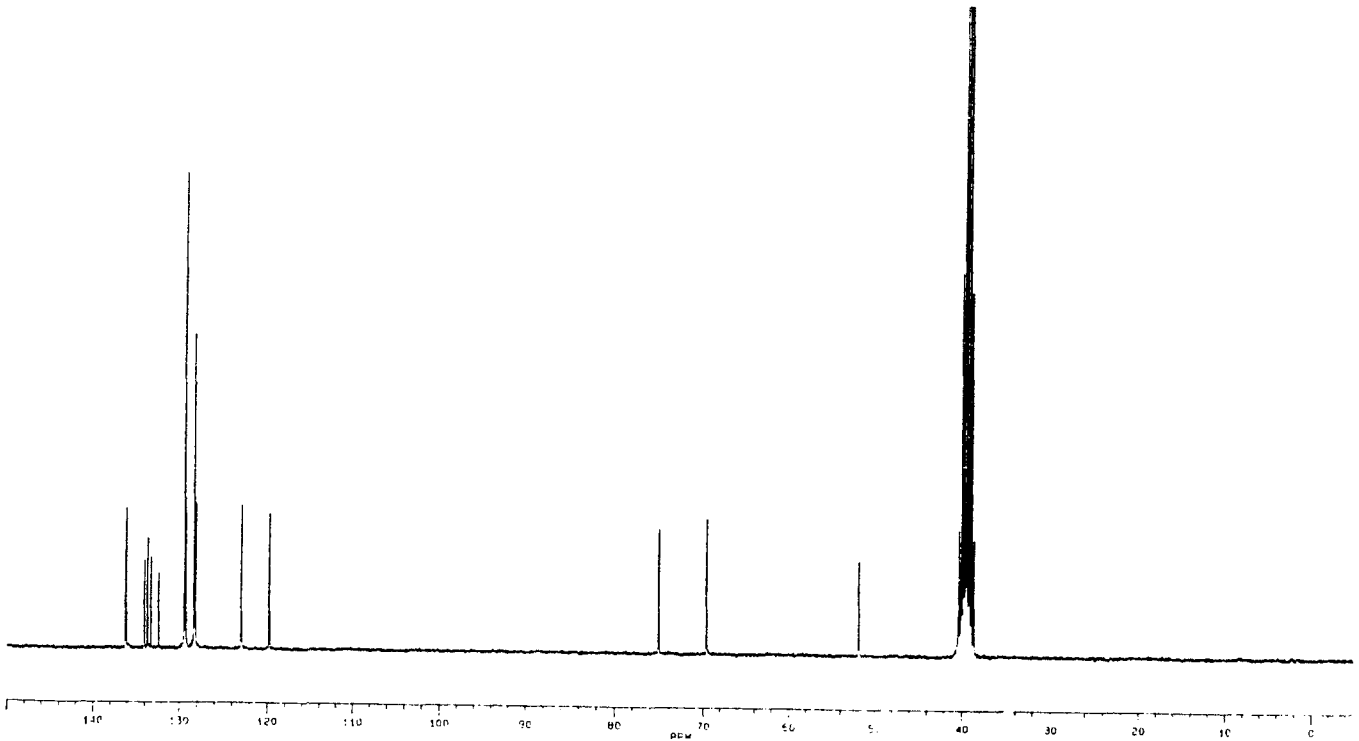

FIGURE 4: Carbon-13 NMR spectrum for econazole nitrate

TABLE 2: Proton chemical shift assignments for econazole nitrate

Protons at Carbon	Chemical Shift (ppm)	Intensity	Multiplicity#
1	9.05	1	T
3,4	7.63 - 7.73	2	T
6	4.48 - 4.60	2	M
7	5.12	1	M
9	7.38	1	D
10	7.52	1	DD
12	7.73	1	D
14	4.31 - 4.45	2	M
16,17,19,20	7.15 - 7.35	4	M
21	14.33	1	S (broad)

S = singlet, D = doublet, DD = double doublet, T = triplet, M = multiplet

TABLE 3: Carbon chemical shift assignments for econazole nitrate

Carbon Number	Chemical Shift (ppm)#
1	136.21
3,4	119.78 - 122.94
6	51.81
7	75.05
8	Q
9	129.39
10	128.16
11	Q
12	129.29
13	Q
14	69.59
15	Q
16,17,19,20	128.32 - 129.39
18	Q

Q = quaternary carbons at 132.37, 133.28, 133.68,134.06 and 136.22 ppm.

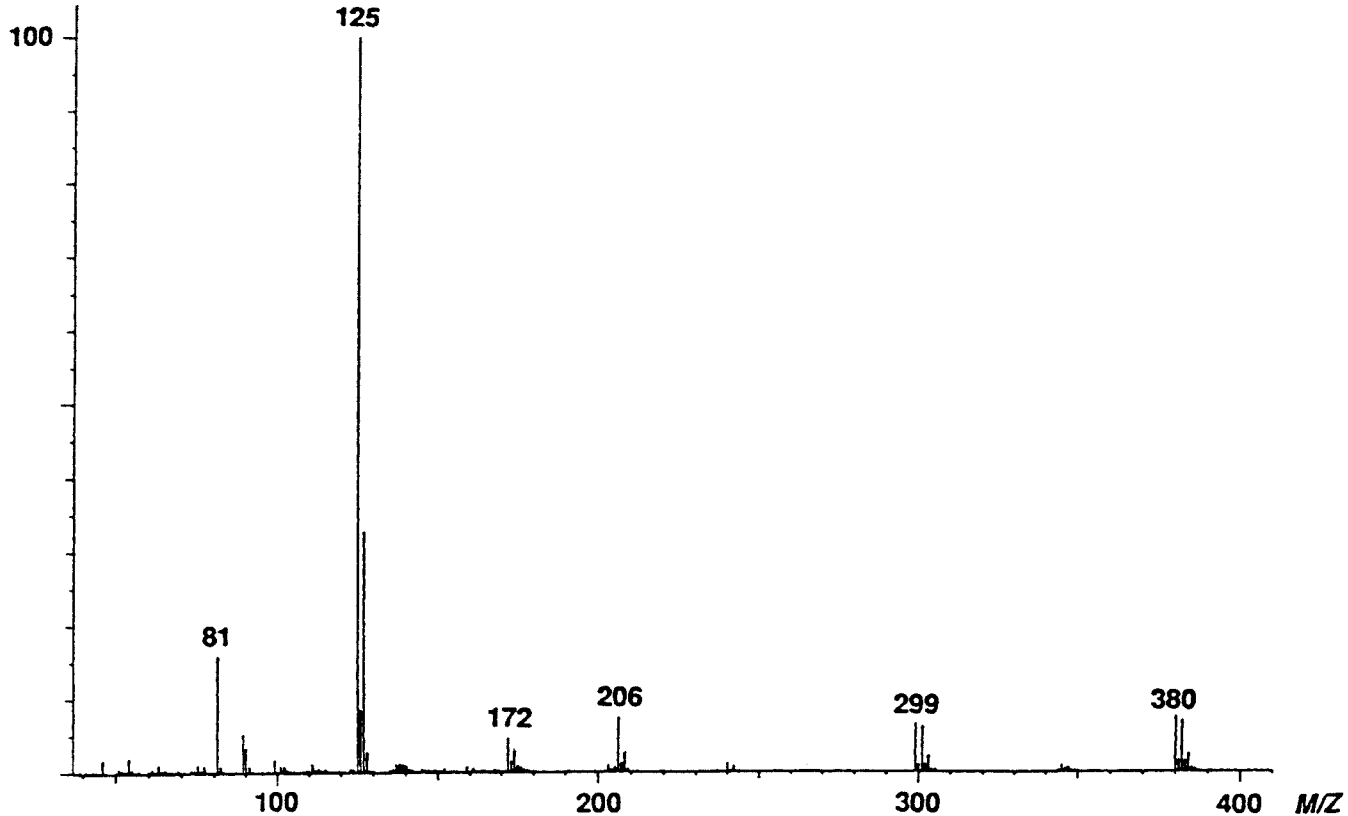

Figure 5. Mass spectrum of econazole nitrate

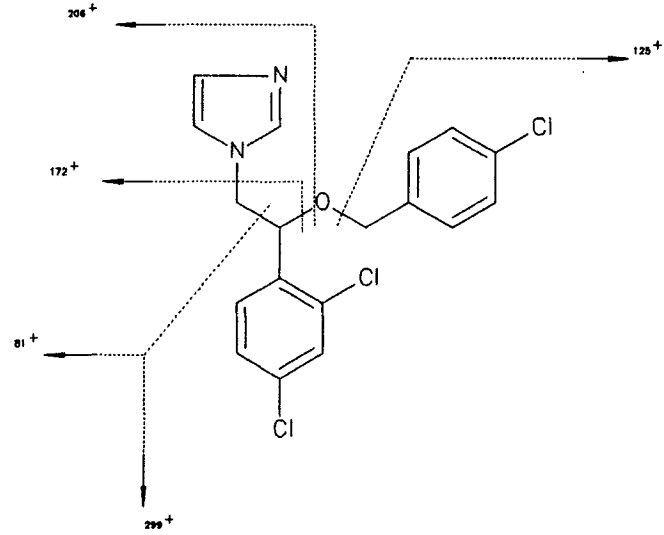

FIGURE 6: Fragmentation pattern for econazole nitrate

Accurate mass measurements were performed on the molecular ion and significant fragment ions at a resolving power of 10000. High-resolution measurements were made using accelerating voltage scanning. Accurate mass data is listed in table 4 and fragment ions are depicted in figure 6. The data indicates some formation of the molecular ion (M^+) although there is significant fragmentation to yield a p-chlorobenzylium base peak (m/z 125). The spectrum shows multiple peaks around some of the major fragments due to the presence of chlorine atoms in their strucures which give rise to isotopic peaks.

TABLE 4: Accurate mass measurements of selected fragment ions in the electron-ionization mass spectrum of econazole nitrate.

Theoretical Mass	Measured Mass	Fragment Strucure	Loss of
380.0250	380.0237	$C_{18}H_{15}ON_2Cl_3$	-#
298.9797	298.9798	$C_{14}H_{10}OCl_3$	$C_4H_5N_2$
206.0611	206.0611	$C_{11}H_9N_2Cl$	C_7H_6OCl, Cl
171.9846	171.9850	$C_8H_6Cl_2$	$C_3H_3N_2$, $C_7H_6OCl_2$
125.0158	125.0162	C_7H_6Cl	$C_{11}H_9N_2OCl_2$

Molecular-ion of base-form

2.4. Infra-red Spectrum

The infra-red spectrum of econazole was obtained as a dispersion in potassium bromide on a Perkin Elmer FT1600 FTIR (Figure 7) The infra-red spectrum of econazole nitrate is also published in the British Pharmacopoeia[4].

2.5. Ultraviolet Spectrum

The ultraviolet spectrum of econazole nitrate was obtained using a Hewlett Packard HP8452A diode-array spectrophotometer configured with a 1-nm spectral bandwidth. The spectrum of a 0.002% w/v solution in methanol was measured over the range 190 - 300nm (figure 8). This reveals a significant K-band absorbance pattern between 200 and 230 nm, comprising a sharp, strongly absorbing peak at 202 nm and a weaker, broad peak around 225 nm.

A 0.05% w/v solution in methanol was used to measure the extinction coefficients of characteristic but weaker B-band absorption between 250 and 290 nm (table 5 and figure 8 - inset). This region includes analytically useful absorbance maxima at 265, 271 and 280 nm.

TABLE 5: Selected ultraviolet spectral extinction coefficients for econazole nitrate 0.05% w/v in methanol

Wavelength (nm)	Extinction coefficient	
	Specific (A 1%,1cm)	Molar
265	9.4	418
271	9.7	430
280	4.9	218

2.6 Thermal Properties

2.6.1. Melting point

The following melting point ranges have been reported.

Melting Range (°C)	Reference
164 - 165	1
162	5
162 - 166	6
161 - 166	7

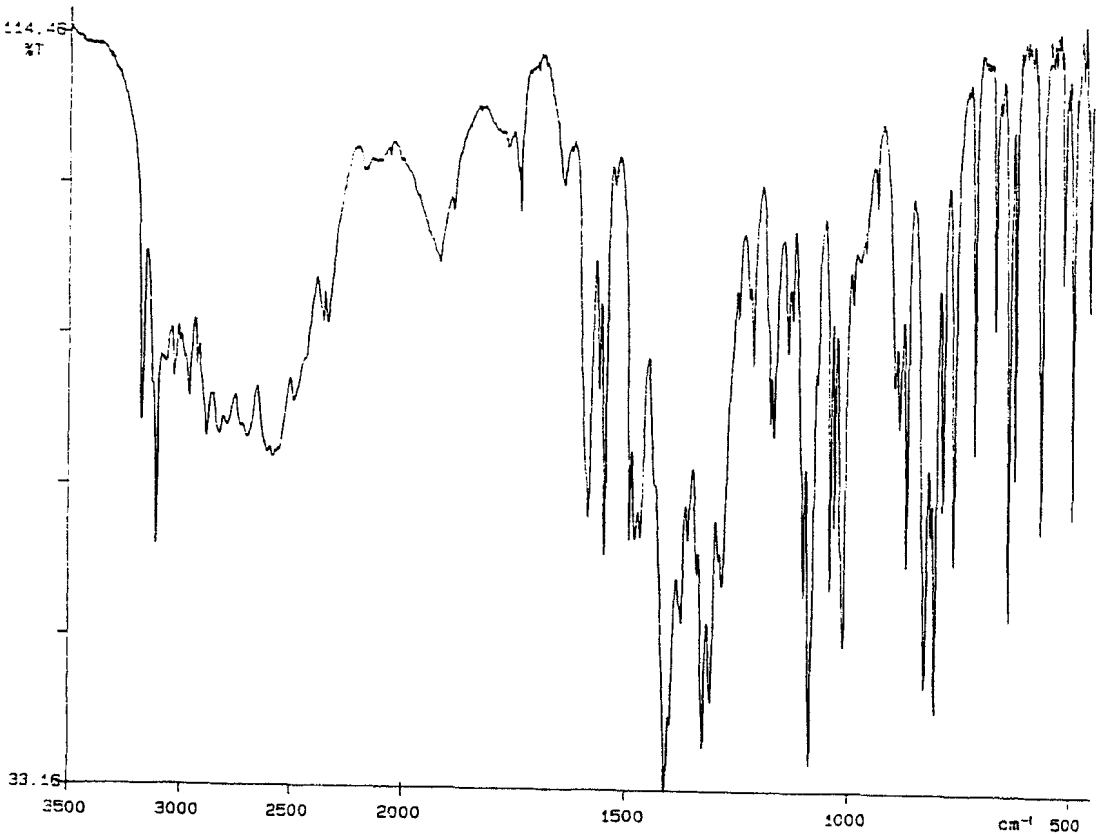

FIGURE 7: Infra-red spectrum for econazole nitrate

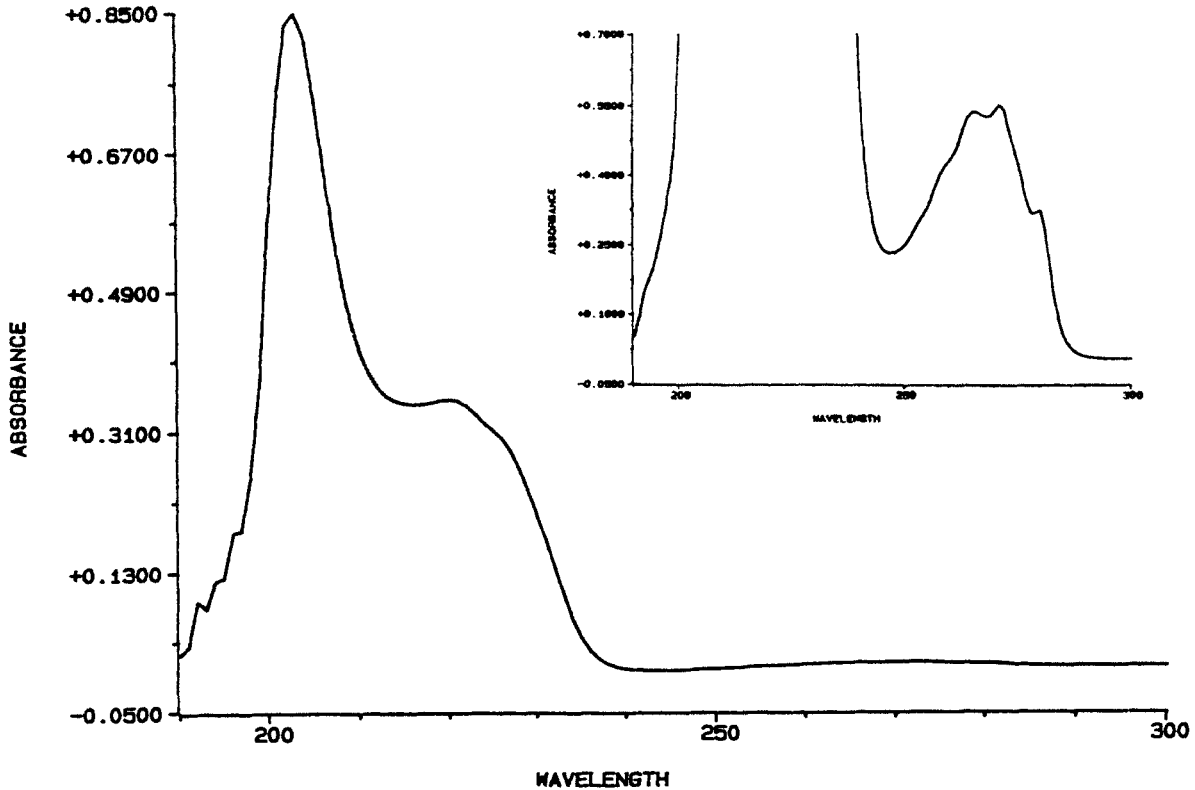

Figure 8. Ultraviolet absorption spectrum for 0.002% w/v econazole nitrate in methanol. The spectrum obtained at 0.05% w/v is shown in the insert.

2.6.2 Differential scanning calorimetry

A DSC trace of a 4.7 mg sample of econazole nitrate is presented in figure 9. The trace was obtained using a DuPont 2000 thermal analyzer operated over the range 90 - 225°C at a ramp rate of 1°C per minute. Econazole nitrate shows a sharp endotherm commencing at approximately 156°C and which reaches a maximum at 161.98°C. This is in good agreement with the melting point data reported in 2.6.1. The endotherm is followed by a large, irregular exotherm which makes quantitation of the endotherm difficult. Nevertheless, the latent heat of fusion ($delta_F$) can be estimated at approximately 28.6 KJ/mol.

2.6.3. Thermogravimetric analysis

A TGA trace of a 10.3 mg sample of econazole nitrate if presented in figure 10. The trace was obtained on a DuPont 2000 thermal analyzer operated over the range 50 - 200°C at a ramp rate of 2°C per minute. Econazole nitrate shows a gradual loss of mass which commences at approximately 168°C and which proceeds at a rate of approximately 1.3% mass per °C until it slows down at around 190°C. This loss of mass is due to vaporization of the melt.

2.7. SOLUBILITY

Solubility data for econazole nitrate in water, acetone and ethanol (96%) are given in table 6.

TABLE 6: Solubility data for econazole nitrate in water, acetone and ethanol (96%)[8].

Solvent	Solubility# g/100ml
Water	<0.1
Ethanol (96%)	2.0
Acetone	1.5

at 20°C

2.7 CRYSTAL PROPERTIES

The X-ray powder diffraction pattern of econazole nitrate was obtained on a Philips model APD3720 powder diffraction system, equipped with a vertical goniometer in the °theta/°2theta geometry. The X-ray generator (Philips model XRG31000) was operated at 45 kV and 40 mA using the copper Ktheta-line at 1.544056e as the radiation source.

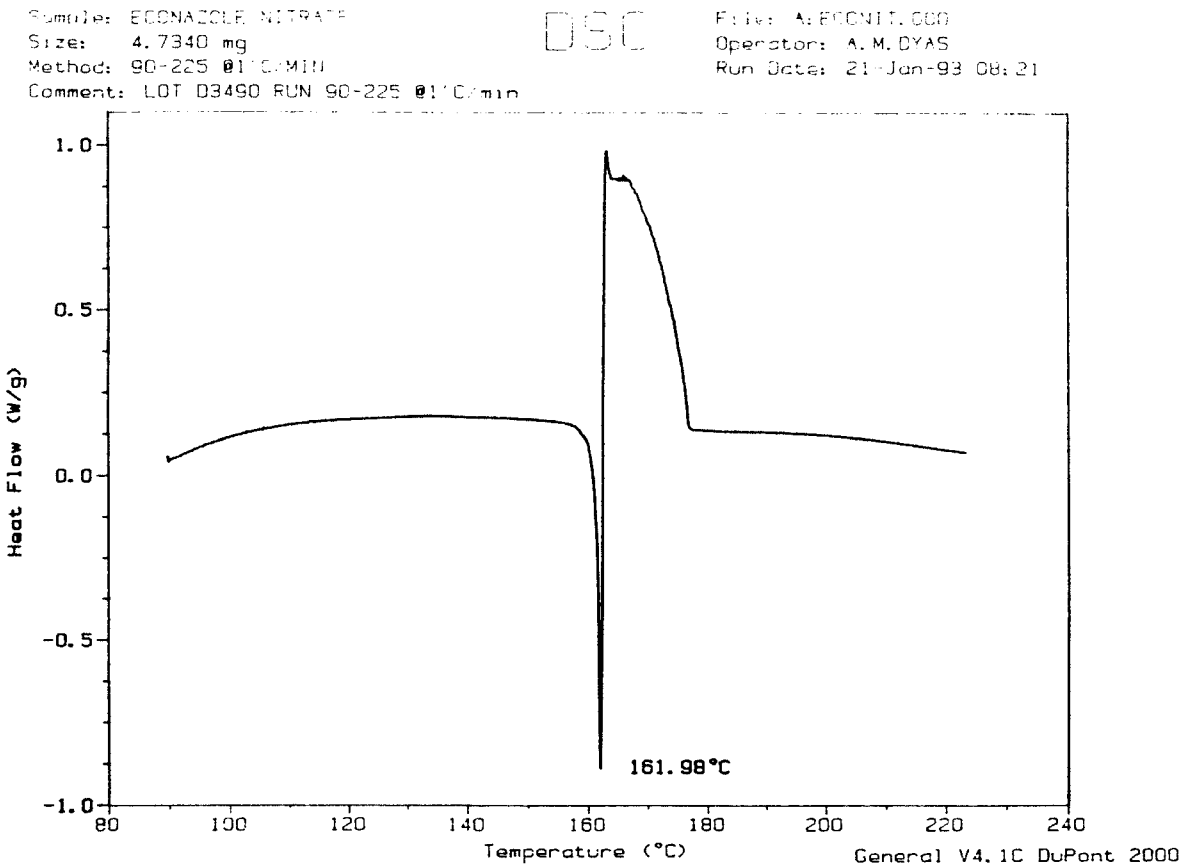

FIGURE 9: DSC trace for econazole nitrate

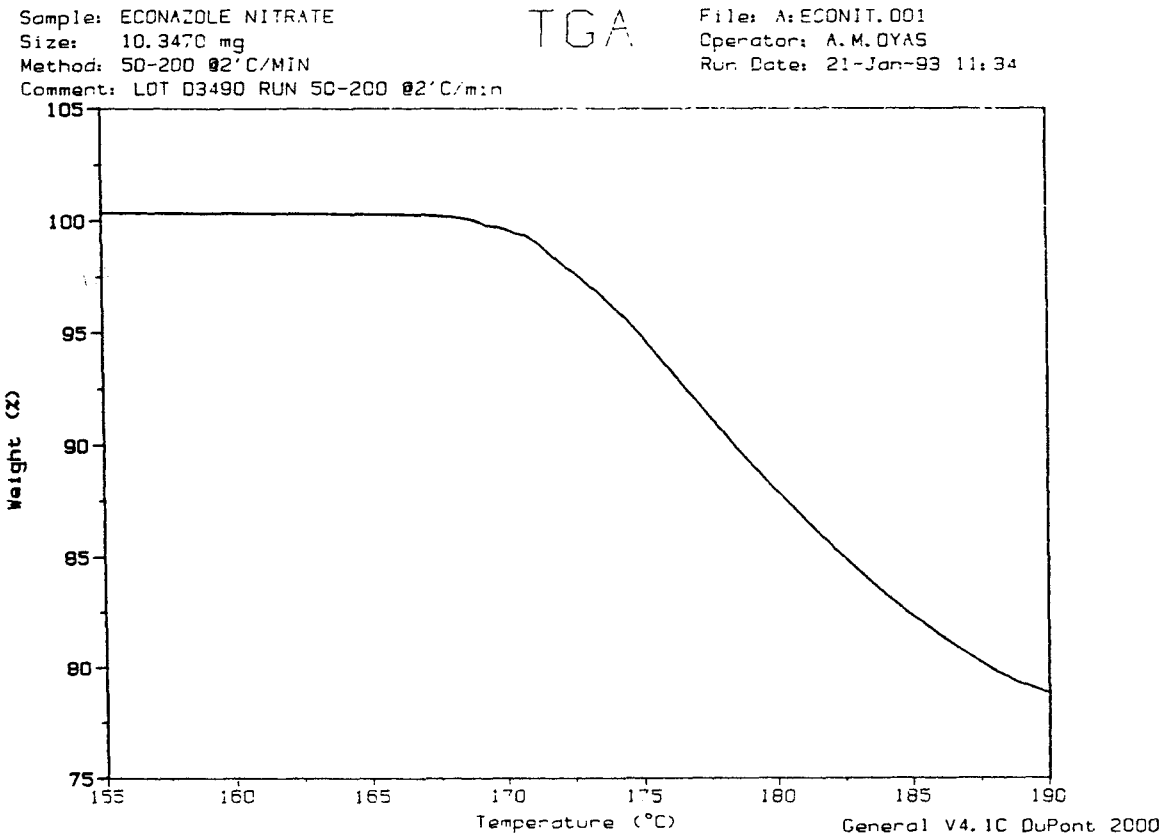

FIGURE 10: TGA trace for econazole nitrate

Each sample was scanned between 2 and 32 °2theta in step sizes of 0.04 °2theta. The material was crystalline, as evidenced by the numerous α-peaks present in the powder patern. The peak positions (°2theta) and relative peak intensities are summarized in table 7. There are significant scatters (°2theta) at 16.34 and 20.38° with subsidiary scatters between 22.5 and 24.5° (four peaks), between 26.0 and 27.0° (two peaks) and at 29.03°.

TABLE 7: X-ray diffraction data for econazole nitrate

PEAK NUMBER	SCATTERING ANGLE (°2theta)	D-SPACING (angstroms)	RELATIVE INTENSITY (%I/I_{max})
1	5.04	17.51	5
2	10.15	8.71	13
3	11.66	7.58	4
4	12.68	6.97	6
5	13.80	6.41	12
6	14.06	6.29	7
7	15.26	5.80	6
8	16.34	5.42	91
9	16.74	5.29	8
10	18.22	4.87	10
11	19.22	4.61	19
12	19.82	4.48	17
13	20.38	4.35	100
14	21.03	4.22	4
15	22.29	3.99	9
16	22.76	3.90	37
17	23.33	3.81	33
18	23.98	3.71	28
19	24.47	3.64	22
20	24.75	3.59	11
21	25.34	3.51	7
22	25.60	3.48	14
23	25.87	3.44	13
24	26.25	3.39	52
25	26.49	3.36	45
26	28.05	3.18	10
27	28.34	3.15	11
28	29.03	3.07	49
29	29.73	3.00	10
30	30.71	2.91	5
31	31.18	2.87	14
32	31.41	2.85	12
33	31.69	2.82	11

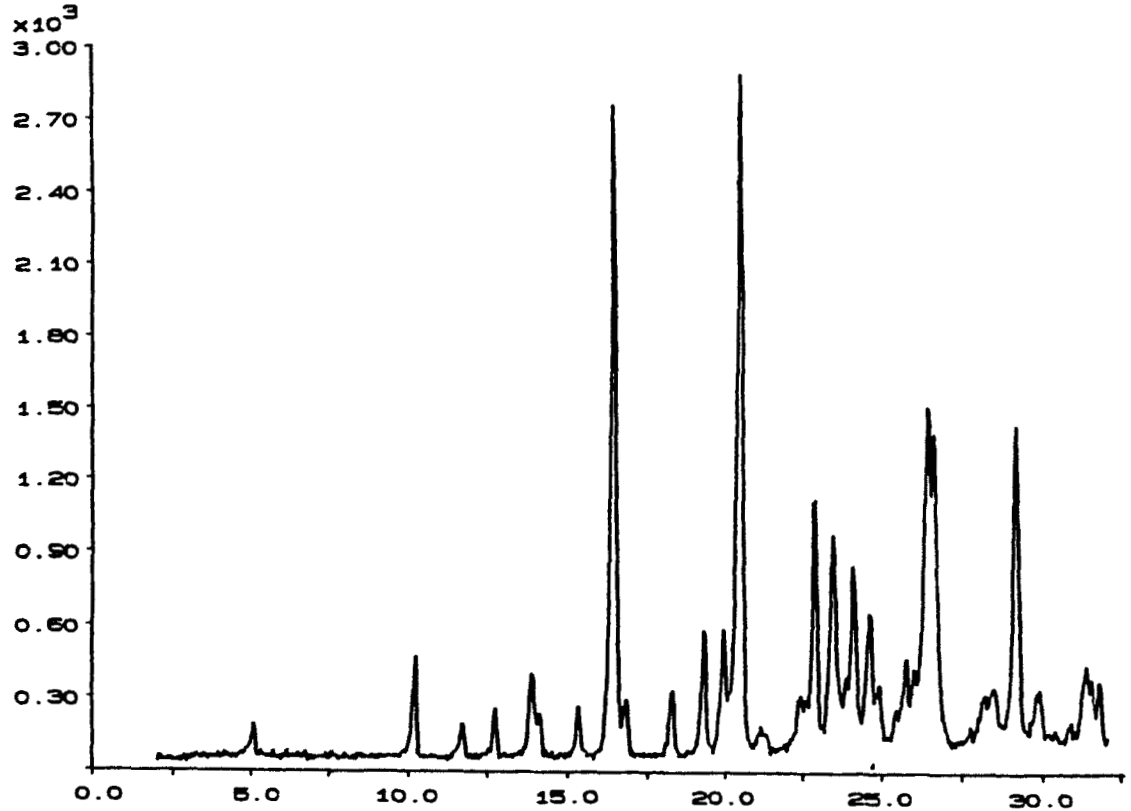

FIGURE 11: X-ray diffraction pattern for econazole nitrate

3. SYNTHESIS

Econazole nitrate may be synthesised by the reaction pathway shown in Figure 12 . This reaction scheme, taken from Godefroi et al[1], has optional pathways at each stage. Imidazole is coupled with brominated 2,4-dichloroaceto -phenone and the resultant ketone is oxidized with sodium borohydride to the corresponding alcohol. This product is coupled with 2,4-dichlorotoluene by means of sodium hydride in hexamethylphosphoramide (an aprotic solvent) before being extracted with nitric acid to give econazole nitrate.

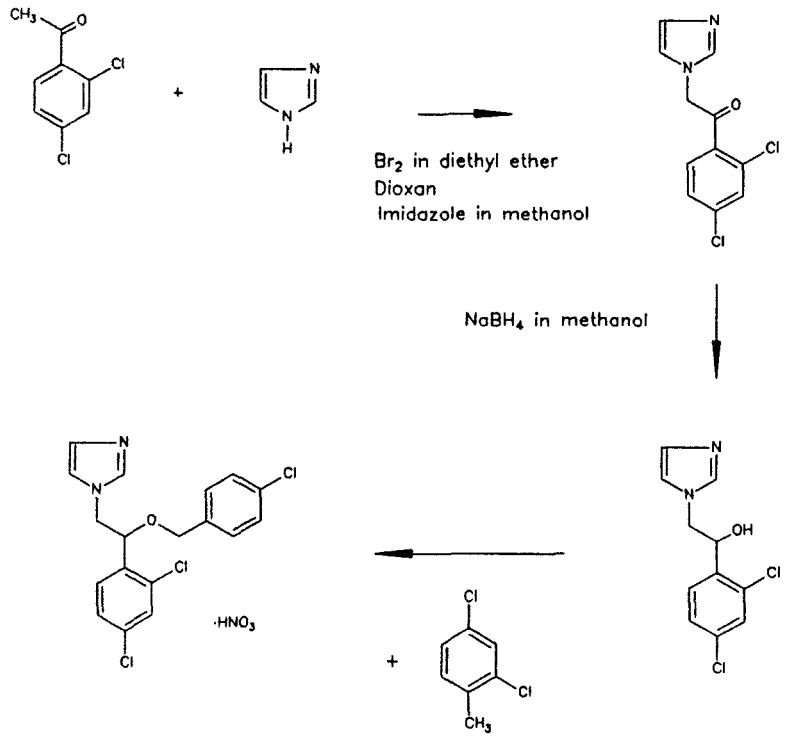

FIGURE 12: Synthetic pathway for econazole nitrate (from Godefroi et al[1])

4. STABILITY

Little is available in the literature on this subject. In a paper describing a validated stability indicating assay for econazole nitrate in cream and lotion formulations[9], the potential degradation products, 4-chlorobenzylic alcohol and 1-(2,4-dichloro-β-hydroxyphenethyl) imidazole are described. Both degradants would be produced by hydrolysis of the ether in linkage in parent molecule. Both the bulk drug substance and formulated product (econazole nitrate 1%w/w in a cream base) are known to be stable at room temperature (undefined) for up to five years[10].

5. METABOLISM, PHARMACOKINETICS, BIOAVAILABILITY

5.1. Metabolism

The metabolism of econazole has been studied in man[12] following oral administration whilst the metabolism of ^{3}H-labeled econazole nitrate has been studied in pregnant and non-pregnant rats and in monkeys[11]. The major plasma metabolites M1-M3 were found after single oral dosing 10mg/kg and 50mg/kg in pregnant and non-pregnant rats, 10mg/kg in monkeys and after a 500mg single dose in man. No unchanged econazole nitrate was found in rats dosed at 10mg/kg. Increasing the dose in this species to 50mg/kg resulted in measurable levels of the parent compound. This suggests saturatable first pass metabolism. Following single oral doses of econazole base (500mg) to human subjects, unchanged drug was detected in plasma for 24 hours.

Overall the main mechanisms involved in metabolism are oxidation of the heterocyclic ring, O-deschlorobenzylation and conjugation of subsequent alcohol. A Double maxima observed in plasma concentration/time curves following oral dosing is indicative of enterohepatic recycling. Plasma and urinary metabolites are given in Figure 13.

5.2. Pharmacokinetics

Rats given oral econazole at 10mg/kg and at 50mg/kg demonstrated two distinct maxima at 0.5 - 2 hours and after 8 hours in their plasma concentration - time curves[11]. The maximum concentration was found to be 2.5 μg/ml following the 10mg/kg dose and 4.6mg/ml following the 50mg/kg dose. The plasma half-lines measured in three test groups of rats were 14, 15 and 22 hours.

A single maximum was observed in monkeys (mean peak plasma concentration of 2.5 μg/ml of base at 4 hours)

following a single oral dose of 10mg/kg. The plasma concentration time curve declined in a bioxponential manner with mean terminal base lines of 4.5 and 69 hours.

5.3. Bioavailability

The absolute bioavailability of econazole nitrate has been estimated as approximately 75% following oral administration[13].Absorption of econazole nitrate is not significant when econazole nitrate is applied to the skin or vagina[14].

6. METHODS OF ANALYSIS

6.1. Identification

The BP identification tests for econazole nitrate are based on three procedures[15] :

(a) infra-red spectroscopy - samples must compare with a published reference spectrum[4]
(b) ultraviolet spectroscopy - a 0.02% w/v solution of econazole nitrate in methanol-0.1M hydrochloric acid solution (9:1) must exhibit absorption maxima at 265, 271 and 280 nm.
(c) Colour test - addition of potassium chloride and diphenylamine solutions to a 0.2% w/v suspension of econazole nitrate in ice-cooled water followed by dropwise addition of sulphuric acid should produce an intense blue colour.

Two TLC procedures have been used for the identification of econazole nitrate is also used; In the first[16], The sample spot must have the same Rf value as that of the standard material. The second is based on that given for the identification of nitrogenous drugs[17] . 10 microlitres of a 0.1% w/v solution of the test substance in methanol is spotted onto the plate. After development with methanol-strong ammonia solution, the plate is visualised under UV light. A spot due to the econazole nitrate can be observed at R_f = 0.80. The structurally-related drugs clotrimazole and miconazole give similar R_f values of 0.77 and 0.80 respectively when tested in this fashion.

6.2. Spectrophotometric Analysis

Ultraviolet spectrophotometric assay of Econazole nitrate 1% solution has been used[16]; 5 ml of sample diluted

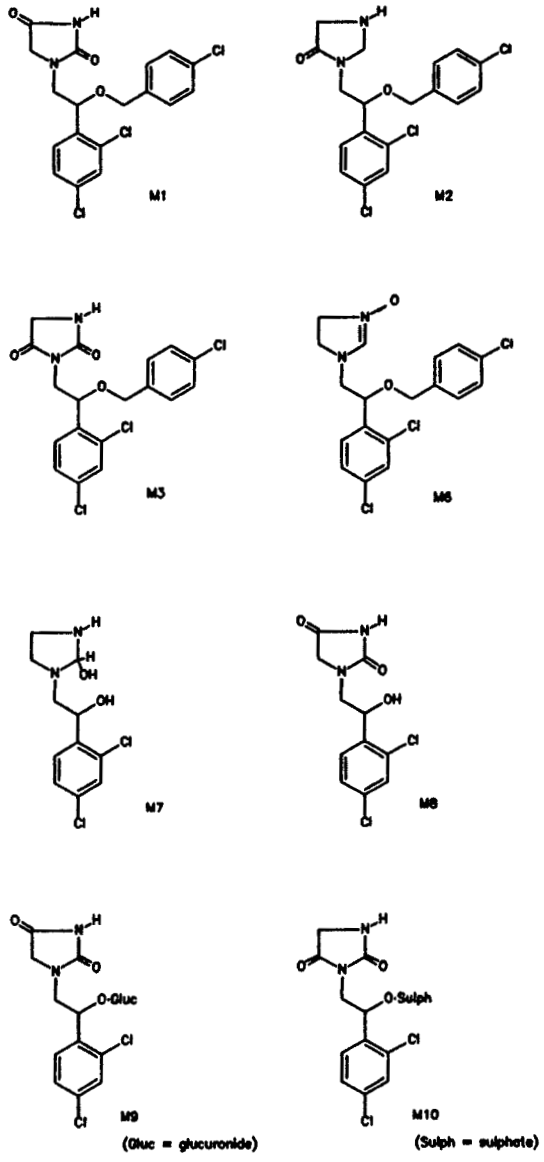

FIGURE 13: Plasma (M1 - M3) and urinary (M6 - M10) metabolites of econazole nitrate.

to 100 ml with methanol is compared to a 0.05% W/V solution of standard substance dissolved in methanol. The absorbance is measured at the maximum around 271 nm.

6.3. Titrimetric Analysis

The EP/BP/USP assays of econazole nitrate[15] bulk drug substance is performed by non-aqueous titration. The sample, 0.4g in anhydrous glacial acetic acid is titrated against 0.1M perchloric acid VS to a potentiometric end-point. Each ml of 0.1M perchloric acid VS is equivalent to 44.47 mg of econazole nitrate.

The above titration has also been performed to a visual end-point using either crystal violet or α-naphtholbenzine as indicators.

6.4 Chromatographic Analysis

6.4.1. Gas liquid chromatography

A gas chromatographic procedure is cited in the BP for the assay econazole nitrate in a cream base[18]. Econazole nitrate is extracted into methanol - 0.6m sulphuric acid, whilst other lipid soluble excipients are extracted into carbon tetrachloride. The aqueous extracts are made alkaline with 2 M ammonia and extracted into Chloroform. An internal standard, 1,2,3,4-tetraphenylcyclopenta-1,3-diene is added to the chloroform extracts and then evaporated to a low volume. Extracts were chromatographed on a 1.5 m x 2.0 mm OV-17 (or equivalent column) at 270°C using nitrogen as carrier gas and flame ionization detection.

6.4.2. High performance liquid chromatography

Econazole nitrate has been determined in tablets, creams, lotions and powders by HPLC[19] . Samples were extracted in methanol prior to analysis. Chromatography was performed on a Micro-Pak C18 column (30 cm x 4.0 mm) using a mobile phase consisting of methanol - aqueous 0.05 M ammonium dihydrogen phosphate (85:15) at a flow rate of 2.0 ml/min. Clotrimazole was used as internal standard. Recovery of active substance from cream, lotion and powder formulations was 99.3, 100.6 and 98.5% respectively.

A stability-indicating assay of econazole nitrate in cream and lotion formulations has been reported[9]. Samples of cream or lotion are dispersed in tetrahydrofuran and extracted into methanolic and aqueous methanolic solutions. They are then filtered prior to analysis. Chromatography was performed on a Waters uBondapak C18 Column (30 cm x 3.9mm) and UV detection at 220 nm was used. The mobile phase consisted of 780 ml of methanol, 20 ml of tetrahydrofuran and 200 ml of ammonium carbonate solution (0.1% w/v), at a flow rate of 2.0 ml/min. The method was shown to be capable

of separating econazole nitrate and its two possible degradation products viz; 4-chlorobenzylic alcohol and 1-(2,4-dichloro-β-hydroxyphenylethyl)imidazole. Quantitation is by peak area ratio against an internal standard (miconazole).

A similar stability indicating HPLC procedure has also been reported[20] , for the determination of econazole nitrate in a cream base containing hydrocortisone. Econazole nitrate is extracted into acetonitrile, the extract is diluted using phosphate buffer, filtered and separated by HPLC using a Hypersil C18 5um column (20 cm x 4.6 mm) eluted with acetonitrile-phosphate buffer (75:25) at 2 ml/min (phosphate buffer consisted of 0.25% KH_2PO_4 and 0.25% K_2HPO_4). Detection was by UV absorbance at 232 nm.

HPLC has also been used to determine econazole in plasma[21] where the samples were made alkaline with potassium hydroxide and extracted in diethyl ether. The extracts were evaporated to dryness and the residue dissolved in methanol. Chromatography was performed on a Partisil 10 ODS column using a mobile phase consisting of methanol-aqueous potassium dihydrogen phosphate (0.01 M, 70:30, v/v) adjusted to pH 4.5 at a flow rate of 2.0 ml/min. Detection was by UV absorbance at 220 nm and quantitation was by peak height ratio against an internal standard (miconazole). The recovery of econazole from plasma was 84% ± 9.2, SD., n = 25).

Procedures have been investigated for the separation and quantitation of the stereoisomers of econazole by HPLC[22]. Indirect resolution was achieved following derivatization of econazole to yield its O,O-dibenzoyltartaric acid monoesters. These derivatives are diastereomeric and can be resolved on Lichrosorb Si60 (7 um) with an eluent comprising dichloromethane-methanol-acetonitrile-glacial acetic acid (70/23/5/2 by volume). Direct resolution of the enantiomers was achieved using both $acid_1$-α-glycoprotein and ovomucoid phases, with the latter giving the better separation.

6.4.3. Thin Layer Chromatography

In addition to the two thin layer chromatographic (TLC) identification procedures cited in section 6.1., a TLC procedure is described in the USP XXII monograph on Econazole Nitrate under the heading 'Chromatographic Purity'. This first appears in the 2nd Supplement to USP XXII[23] where the solvent system consists of chloroform, methanol and formic acid (7:2:1). In the 7th Supplement to USP XXII[6] the solvent system is changed to 1,4-dioxan, toluene and 13.5M ammonium hydroxide (60:40:1). In both cases 0.25 mm silica gel plates are used and visualisation is by exposure of the plates to iodine vapour. The current EP[24] and BP 1993[7] monographs also use a similar solvent system to that employed in reference 6, albeit with silica gel plates containing a fluorescent indicator.

Although these methods are used to separate the parent compound and related substances, these identity of the related substances are not given.

6.5. Microbiological Analysis

Godefroi et al., analysed the in ratio microbiological activity of econazole nitrate[1], againstthe mould *Aspergillus fumigatus* (AF), the yeast *Candida albicans* (CA), the dermatophytes *Microsporum canis* (MC), *Trichophyton rubrum* (TR) and *Trichophyton mentagrophytes* (TM), and the gram positive bacteria *Erysipelothrix insidiosa* (EI) and *Staphylococcus haemolyticus* (SH). In the methodology employed[1] the compound was assayed against griseofulvin and nystatin. Results are presented in table 8 as the lowest level (in μg/ml) causing total inhibition of growth of the test organism.

TABLE 8: Lowest levels (μg/ml) of antimicrobial agent causing total inhibition of growth of test organism

Antimicrobial Agent	Test Organism CA	MC	TM	TR	AF	EI	SH
Econazole Nitrate	100	0.1	0.01	0.1	<1	0.01	<1
Griseofulvin	X	10	10	10	X	X	X
Nystatin	33	333	333	333	333	-	-

Where x = denotes partial growth at 100μg/ml.
CA = *Candida albicans*
MC = *Microsporum canis*
TM = *Trichophyton mentagrophytes*
TR = *Trichophyton rubrum*
AF = *Aspergillus fumigatus*
EI = *Erysipelothrix insidiosa*
SH = *Staphylococcus haemolyticus*

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HYOSCYAMINE

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HYOSCYAMINE

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Acknowledgement

References

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

α-(Hydroxymethyl) benzeneacetic acid 8-methyl-8-azabicyclo [3.2.1] oct-3-yl ester.
Benzeneacetic acid, α-(hydroxymethyl)-8-methyl-8- azabicyclo [3.2.1] oct-3-yl ester, [3(*S*)-endo].
1αH, 5αH-Tropan-3α-ol (-) tropate (ester).
3α- Tropanyl *S*-(-)-tropate.
l-Tropic acid ester with tropine.
l-Tropine tropate
Tropine (-) tropate

For the sulfate salt:
Benzeneacetic acid, α-hydroxymethyl-8-methyl-8-azabicyclo [3.2.1] oct-3-yl ester, [3(S)-endo], sulfate (2:1), dihydrate.
1αH, 5αH-Tropan-3α-ol (-)-tropate (ester) sulfate (2:1) (salt) dihydrate.
(1R,3r,5S)- Tropan-3-yl (*S*)-tropate sulphate dihydrate.

1.1.2 Generic Names

Hyoscyamine, l-Hyoscyamine, Daturine, Duboisine.

1.1.3 Trade Names

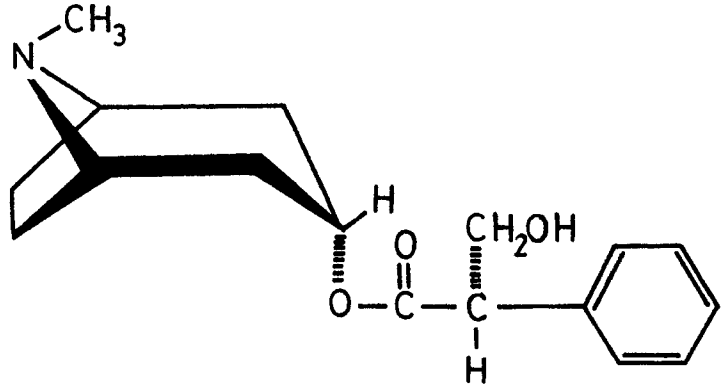
Cystospaz; Levsin (The base) Egacene; Egazil; Duretter; Peptard; (Hyoscyamine sulfate dihydrate).

1.2 Formulae

1.2.1 Empirical

$C_{17}H_{23}NO_3$ (Hyoscyamine)
$C_{34}H_{48}N_2O_{10}S, 2H_2O$ (Hyoscyamine sulfate dihydrate)
$(C_{17}H_{23}NO_3)_2, H_2SO_4, 2H_2O$

1.2.2 Structural



1.2.3 CAS Registry Number

[101-31-5] Hyoscyamine
[6835-16-1] Hyoscyamine sulfate dihydrate

1.3 Molecular Weight

289.374 (hyoscyamine).
712.85 (hyoscyamine sulfate dihydrate).

1.4 Elemental Composition

C 70.56%, H 8.01%, N 4.84%, O 16.59%.

2. Physical Properties

2.1 Appearance, Color and Odor

Hyoscyamine occurs as silky tetragonal needles (from alcohol), colorless and odorless.

Hyoscyamine sulfate occurs as needles (from alcohol) (1), or as a white crystalline powder or colorless needles (2).

2.2 Melting Range

108.5° (1)
Between 106° and 109° (3) Hyoscyamine Melts between 108°-111° (4)
Hyoscyamine sulfate dihydrate melts at:
206° (1)
About 203° (with decomposition) (2)
Not less than 200° (3).

2.3 Solulbility Data

One gram of hyoscyamine dissolves in: 281 ml water; 1 ml chloroform; 69 ml ether; 150 ml benzene, freely soluble in alcohol and dilute acids (1).
One gram of hyoscyamine sulfate dihydrate dissolves in 0.5 ml water; in about 5 ml alcohol, very slightly soluble in chloroform and ether (1).

2.4 Acidity

The acidity of hoscyamine sulfate dihydrate has been reported as follows:
pH of (1 in 100) is 5.3 (1).
pH of (2% w/v solution) is between 4.5 to 6.2 (2).

2.5 Specific Optical Rotation

Hyoscyamine
$[\alpha]D^{20}$ - 21.0° (alcohol) (1).
$[\alpha]D^{15}$ - 22.0° (50% ethanol) (5).
Between - 20° and - 23°, determined in dilute alcohol (1 in 2) containing 100 mg of hyoscyamine in each 10 mL (3).
Hyoscyamine sulfate dihydrate
$[\alpha]D^{15}$ - 29.0° (c=2) (1).
Not less than - 24.0°, determined in a solution containing 500 mg in each 10 mL (3).
In a 5% w/v solution, - 24.0° to - 29.0° (2).

2.6 Loss on Drying

Hyoscyamine: Drying in a vacuum over silica gel to constant weight; it loses not more than 0.2% of its weight (3).
Hyoscyamine sulfate dihydrate: Drying it in a vacuum at 105° for 16 hours; it loses between 2.0% and 5.5% of its weight (3).

2.7 Dissociation Constant

pKa for hyoscyamine at 21° is 9.7 (6).

2.8 Crystal Structure

The crystal structure of (-)-hyoscyamine hydrobromide, $C_{17}H_{23}NO_3$, HBr has been investigated (7):
Space groups $P2_1$ with cell dimensions a = 12.021±0.004, b = 7.404±0.002, c = 10.790±0.004, B = 114.18±0.03°. The methyl group attached to the nitrogen atom is in the *equatorial* position with respect to the six-membered ring, this is in contrast to scopolamine hydrobromide where the N-methyl group is in the *axial* position (8). The distances of the nitrogen atom from the oxygen atoms of the molecule are : N-O (1) 3.74 A°, N-O (2) 5.29 A° and N-O (3) 7.71 A°. Figure 1, represents projection of 4 element cells along *b-axis*. In crystals the hydrophyllic and hydrophobic groups arranged perpendicularly (to the *b-axis* down the paper plane) (7).
The relationship between the molecular structure and the pharmacological mode of operation has also been discussed with reference to known properties of the acetylcholine receptor. Bonding to the receptor is achieved through the tertiary amino group with hydrogen bond of 3.7 and 7.7 A° to the anionic site of the receptor.

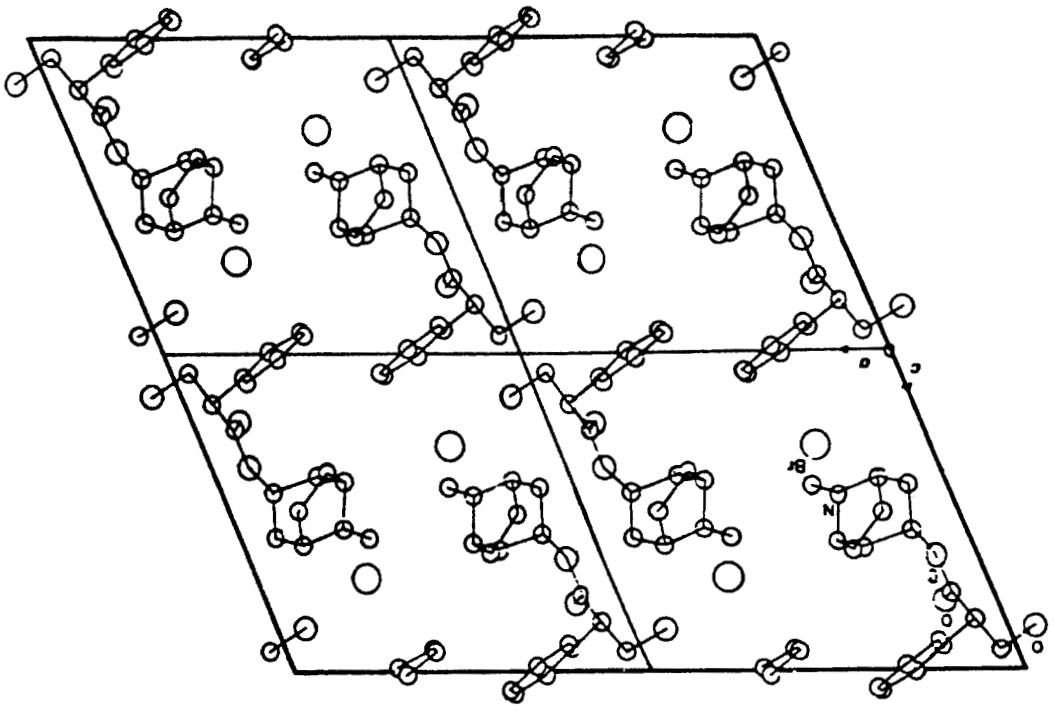


Figure 1: **Projection of 4 Element Cells along *b-axis* for Hyoscyamine.**

2.9 Spectral Properties

2.9.1 Ultraviolet (UV) Spectrum

The UV absorbance spectrum of hyoscyamine in methanol was scanned from 200 to 400 nm using Varian DMS 90 Spectrometer. This is shown in Figure 2. Hyoscyamine showed the following absorptivity values (Table 1).

Table 1 : UV Absorptivity Values

max. nm	ϵ	A(1%, 1cm)
247	149.88	5.18
252	164.39	5.70
258	177.08	6.12
264	147.60	5.10

Clarke (9) reported the following UV data:
Hyoscyamine in ethanol, maxima at *247.5 nm* (E 1%, 1 cm 4.5), *252 nm* (E 1%, 1 cm 5), *255 nm* (E 1%, 1 cm 5.4), and *264 nm* (E 1%, 1 cm 5).
In aqueous acid at *252 nm* (A1 = 6.2); *258 nm* (A1 = 8.2); *264 nm* (A1 = 6.2) (6).

2.9.2 Infrared (IR) Spectrum

The IR absorption spectrum of hyoscyamine as KBr-pellet (2%) was recorded on a Perkin Elmer 1310 IR Spectrophotometer. The spectrum is presented in Figure 3.
Assignment of the functional groups have been correlated with the following frequencies (Table 2).

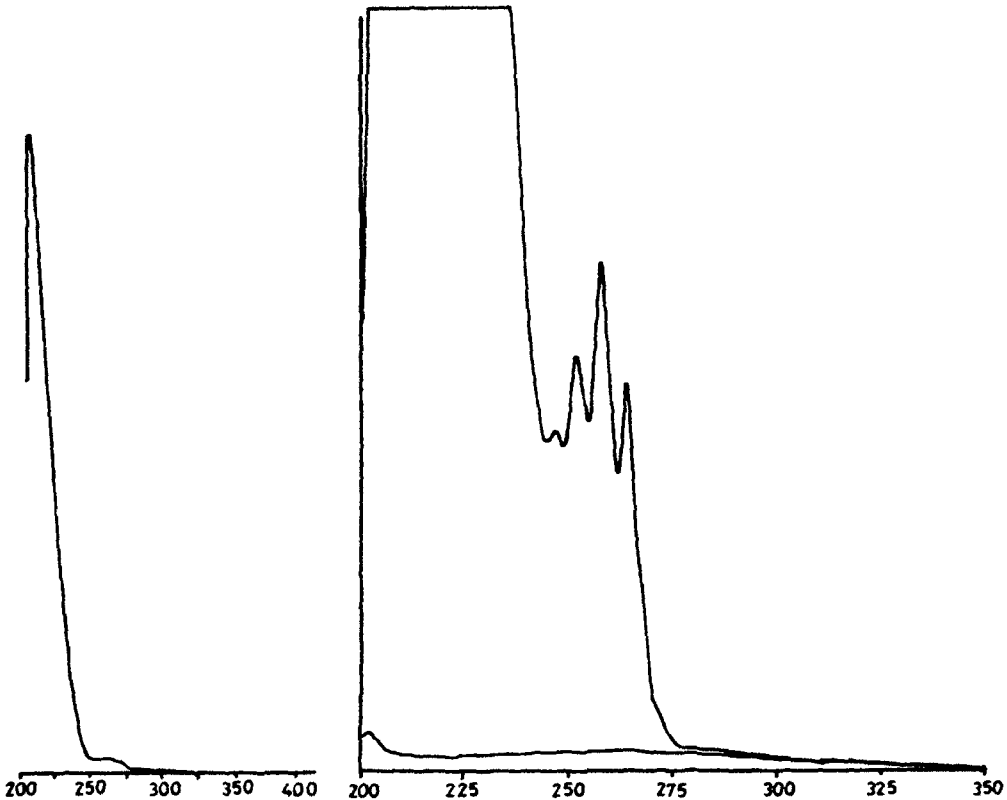


Figure 2: UV Spectrum of Hyoscyamine.

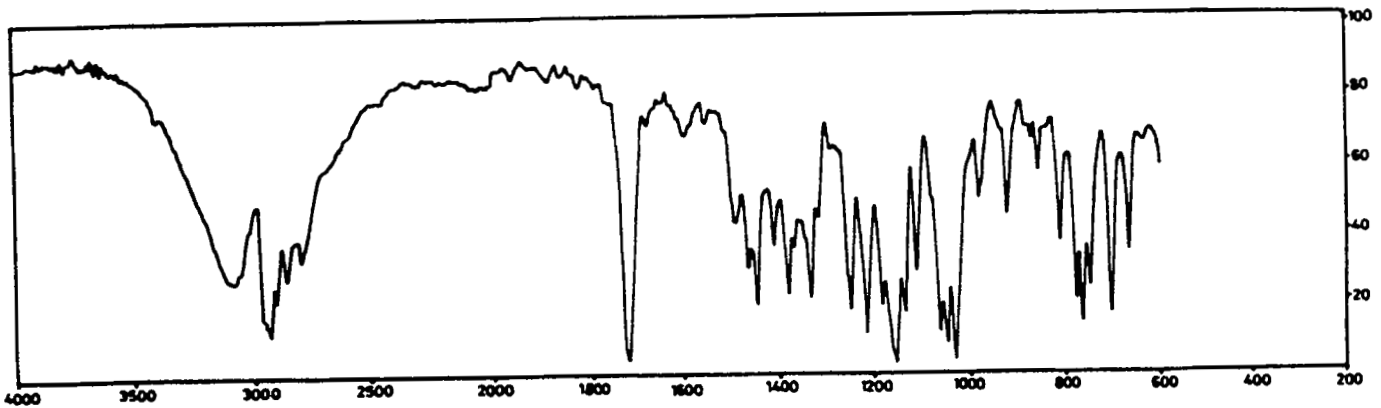


Figure 3: **IR Spectrum of Hyoscyamine.**

Table 2 : IR Characteristics of Hyoscyamine

Wave number (cm^{-1})	Functional Group
3095	OH (stretch)
2940	CH (stretch)
2805	$HN^{+}-CH_3$
1715	O-C- (ester)
1595	C=C (aromatic)
1215, 1155, 1030	C-O-C (ether)
760, 745, 695	Out of plane bending of monosubstituted aromatic

The IR of hyoscyamine exhibited the following unassigned absorption bands:
2860, 1485, 1465, 1445, 1410, 1380, 1330, 1245, 1170, 1135, 1110, 1060, 1045, 978, 918, 850, 805, 772, 640 cm^{-1}.
Clarke (9) reported the following principal peaks:
1712, 1154, 1032 cm^{-1} for hyoscyamine base in KBr disc. Principal peaks for hyoscyamine hydrobromide (KBr disc), 1738, 1160, 1025, 1145, 1225 and 1050 cm^{-1} (6).

2.9.3 ^{1}H-NMR Spectra

The proton magnetic resonance spectrum of hyoscyamine is shown in Figure 4. It was obtained for a solution in $CDCl_3$ on a Varian XL 200 NMR Spectrophotometer, using TMS as an internal reference.
The spectral interpretation is listed in Table 3. This was assigned applying chemical shifts, intensities and COSY which is presented in Figure 5. COSY was also obtained on Varian XL 200 in $CDCl_3$ using standard varian software.

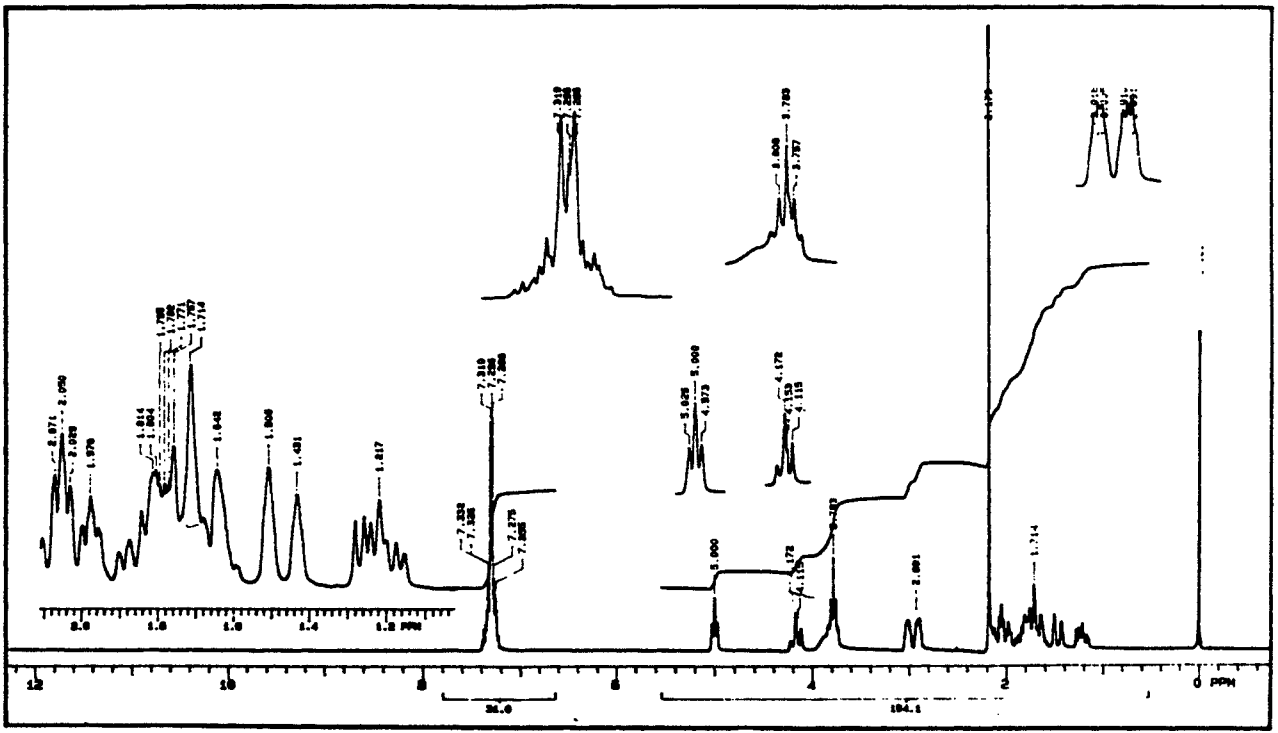


Figure 4: ^{1}H-NMR Spectrum of Hyoscyamine.

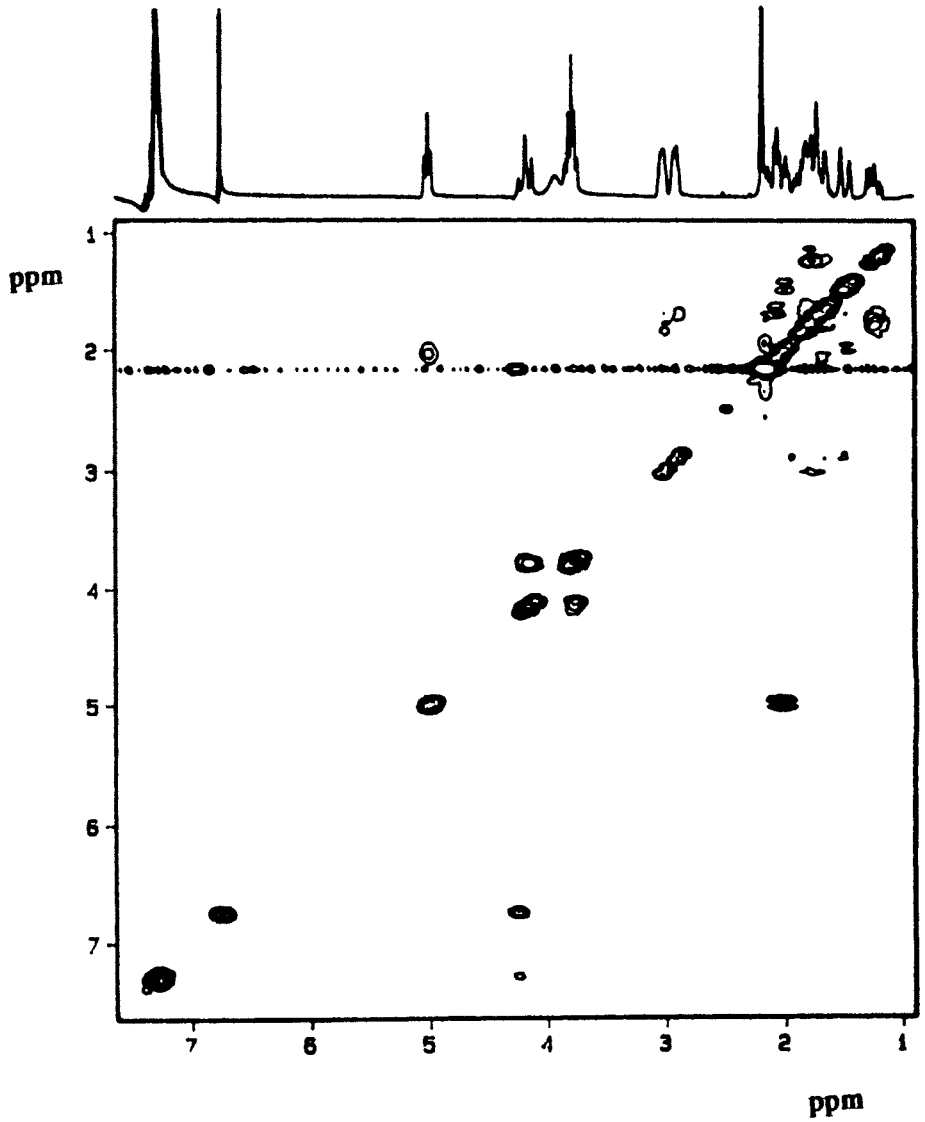


Figure 5: Proton Correlation Spectroscopy (COSY) for Hyoscyamine.

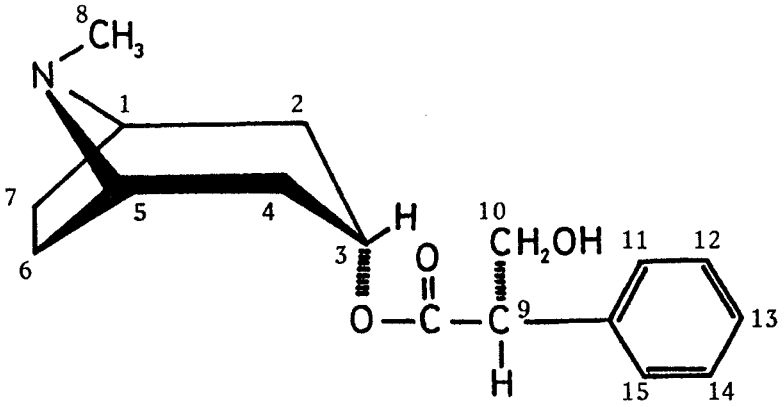


Table 3 : ^{1}H-NMR Characteristics for Hyoscyamine

Proton Assignment	Chemical Shifts δ (pmm)
5 H aromatics (at C_{11}, 12, 13, 14 and 15)	7.310, 7.296, 7.286 (m)
H at C_3	5.00 (t)
H at C_9	4.172
CH_2 OH at C_{10}	4.115 (exchangable)
2 H at C_{10}	3.783 (m)
H at C_1 & H at C_5	2.891 (d)
3H at C_8 ($N-CH_3$)	2.179 (s)
2H each at C_2, C_4, C_6, C_7	1.27 - 2.07 (m)

s=singlet; d= doublet; t = triplet, m = multiplet. Other ^{1}H-NMR data for hyoscyamine (atropine) were also reported (10-12).

2.9.4 Carbon-13 NMR Spectra

The ^{13}C-NMR decoupled spectrum of hyoscyamine in $CDCl_3$ was obtained on a Varian XL-200 NMR Spectrophotometer, using TMS as an internal reference. The spectrum is shown in Figure 6. The spectral interpretation is presented in Table 4. This was assigned by applying chemical shifts, intensities, APT and DEPT which is presented in Figure 7. APT and DEPT were obtained on the same instrument using standard Varian software.

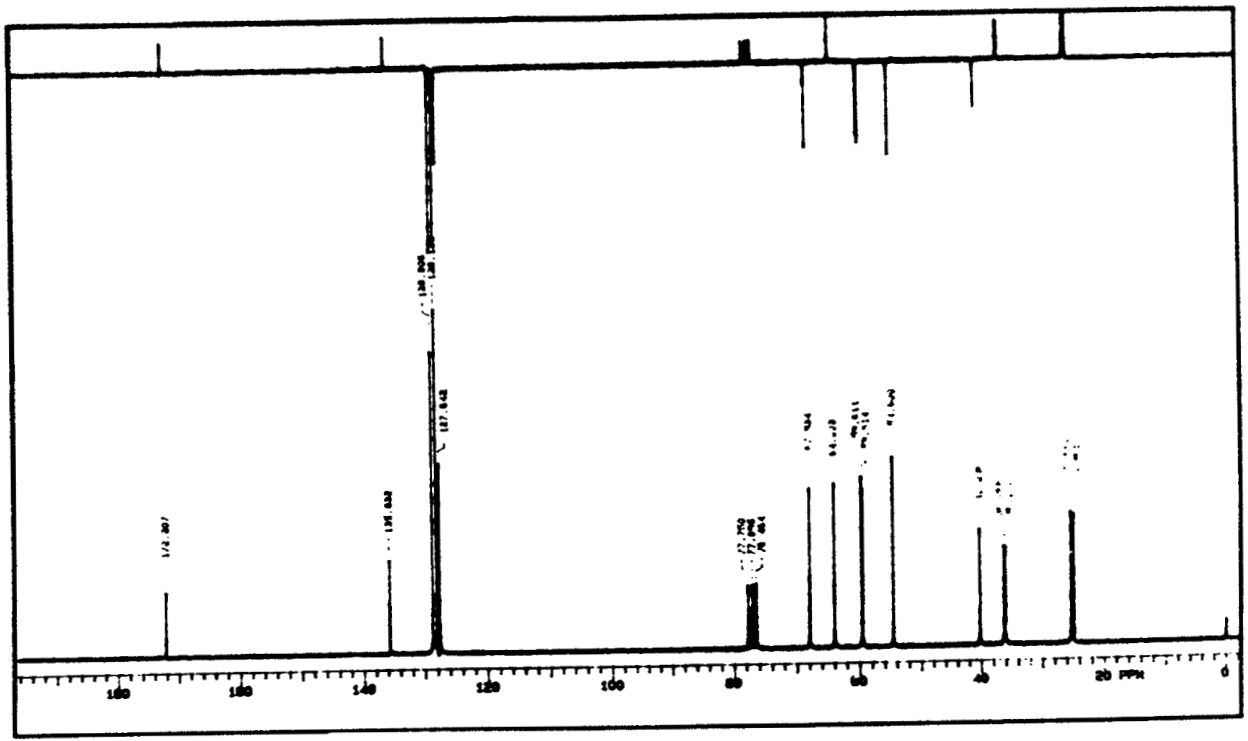


Figure 6: Carbon-13 NMR Spectrum of Hyoscyamine.

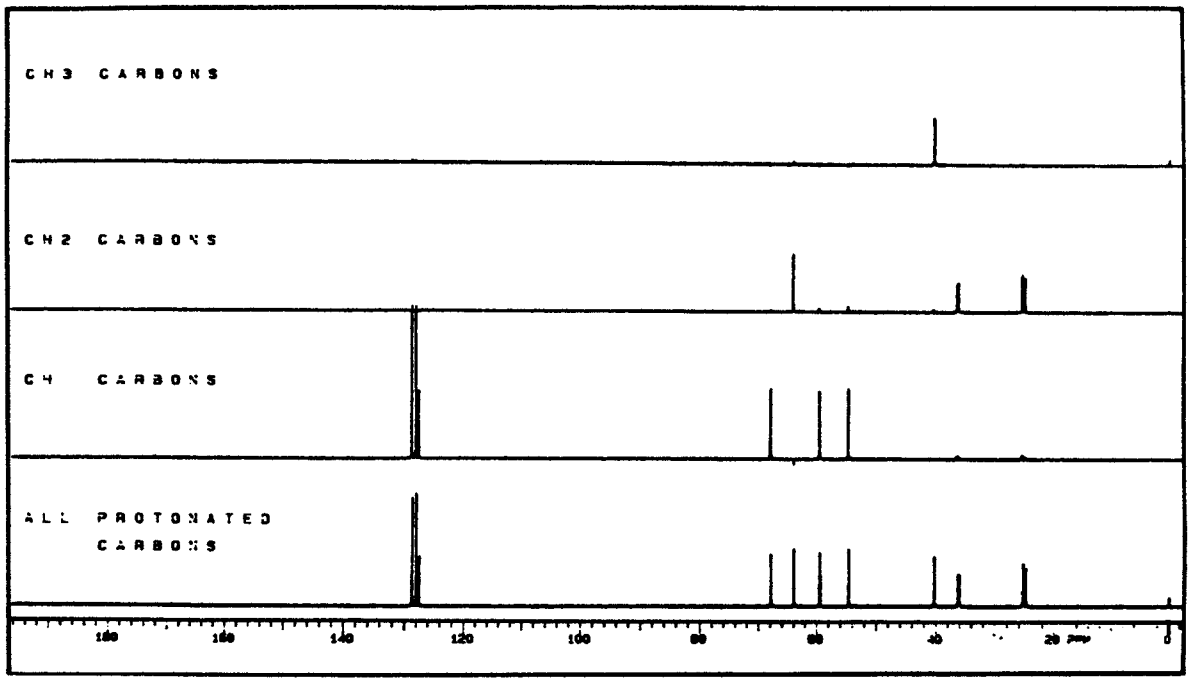


Figure 7: **Distortionless Enhancement by Polarization Transfer (DEPT) for Hyoscyamine.**

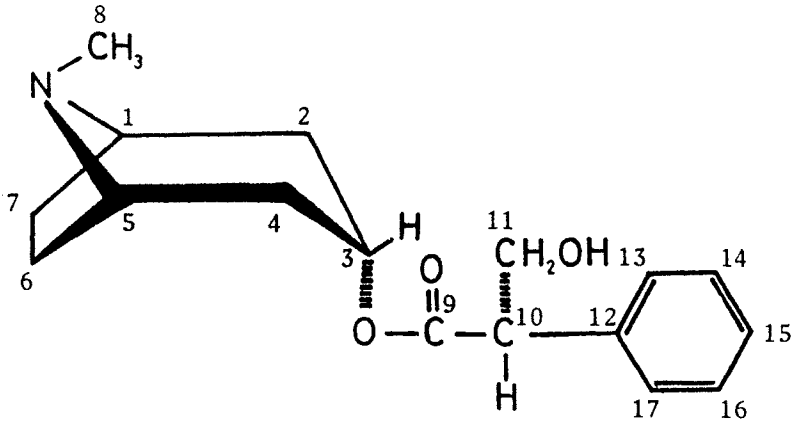


Table 4: Carbon-13 Chemical Shifts of Hyoscyamine.

Carbon Assignment	Chemical Shift pmm (δ)	Multiplicity
C_9	172.22	Singlet
C_{12}	135.84	Singlet
C_{13}, C_{17}	128.79	Doublet
C_{14}, C_{16}	128.10	Doublet
C_{15}	127.63	Doublet
C_3	67.95	Doublet
C_{11}	64.03	Triplet
C_1	59.62	Doublet
C_5	59.53	Doublet
C_{10}	54.65	Doublet
C_8	40.31	Quartet
C_2	36.34	Triplet
C_4	36.13	Triplet
C_7	25.42	Triplet
C_6	24.95	Triplet

Other carbon-13 data for hyoscyamine (atropine) have also been reported (10, 13, 14).

2.9.5 Mass Spectrum (MS)

The electron impact ionization (EI) mass spectrum of hyoscyamine is presented in Figure 8. The spectrum was obtained

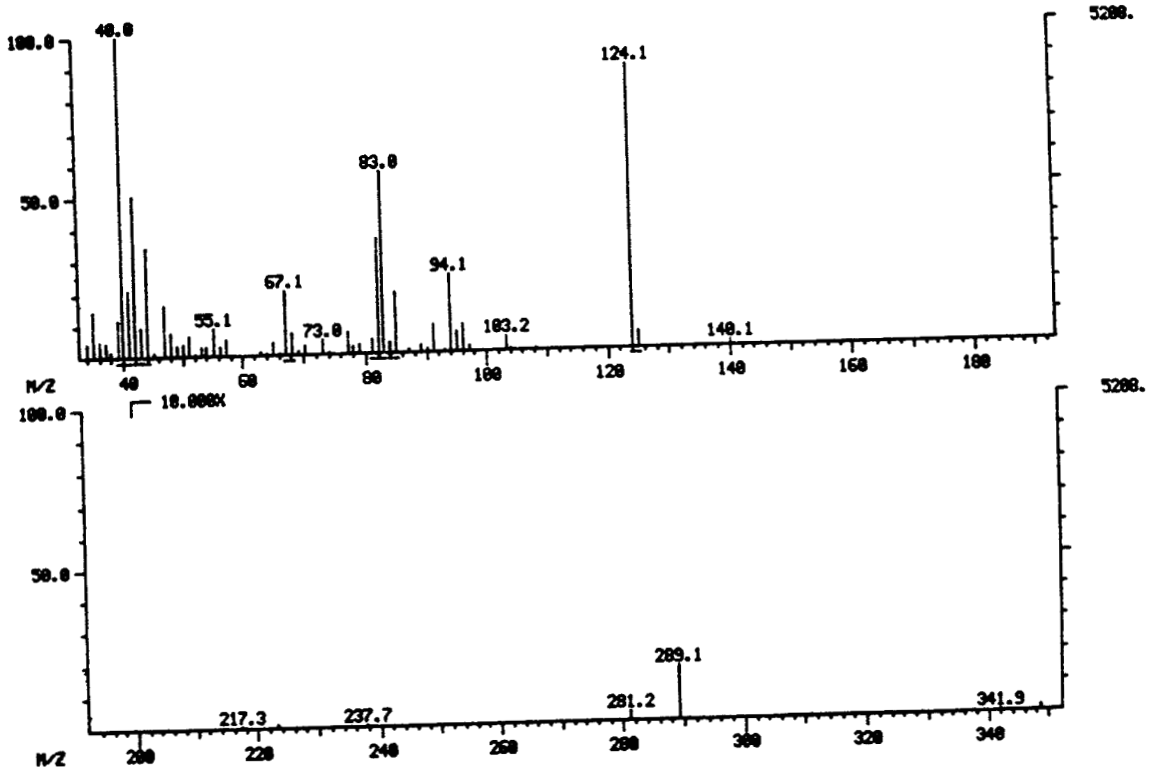
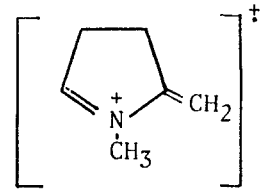
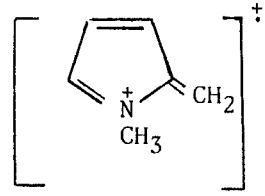
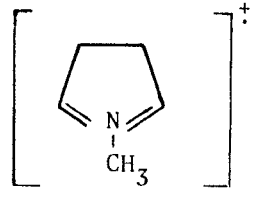
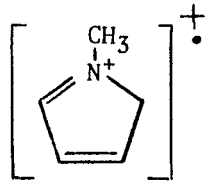


Figure 8: Mass Spectra of Hyoscyamine.

using a Fannigan MAT 5100 series GC/MS spectrometer operating with an ionization potential of 70 cV.
The spectrum exhibited a molecular ion peak at a mass/charge (m/z) ratio of 289 with relative intensity of 18.6% and a base peak at m/z ration of 124 with relative intensity of 90%.
The most prominent ions, their relative intensities as well as some proposed ion fragments are shown in Table 5.

Table 5 : Mass Fragments of Hyoscyamine

m/z	Relative Intensity %	Ions
289	17.0	M^+
281	5.0	-
124	90.0	-
103	6.4	-
96	10.1	
95	7.2	96-H
94	26.0	
83	57.4	

82	36.4	
67	20.0	-
55	9.8	$[CH_2{=}\overset{+}{N}{=}CH{-}CH_2]^{+\bullet}$
47	18.0	-
44	33.3	-
43	9.0	44-H
42	50.6	$[CH_2{=}N^{+}{=}CH_2]^{+\bullet}$
41	20.5	42-H
40	100.0	41-H

Other mass spectral data for hyoscyamine (atropine) have been reported (15-19).

2.9.6 Differential Scanning Calorimetry (DSC)

The DSC thermogram for hyoscyamine is shown in Figure 9. This was obtained at a heating rate of 10 °C/minute under a flow of nitrogen (150 min^{-1}), using Dupont TA 9900 computer thermal analyzer applying purity program V1.1A.
The thermogram is characterized by a single melting endotherm with an extrapolated onset temperature for melting of 103.4 °C. The purity of the sample was found to be 99.34%.

3. Isolation of Hyoscyamine

Hyoscyamine and/or atropine occur in several plants of the family *Solanaceae*, such as species of *Atropa, Datura, Hyoscyamus, Duboisia, Mandragora* and *Scopolia* (20). In some of these plants e.g. belladonna and scopolia, hyoscyamine is the dominant alkaloid throughout the life cycle of the plant. In *Datura stramonium*, hyoscyamine is the principal alkaloid at the time of flower-

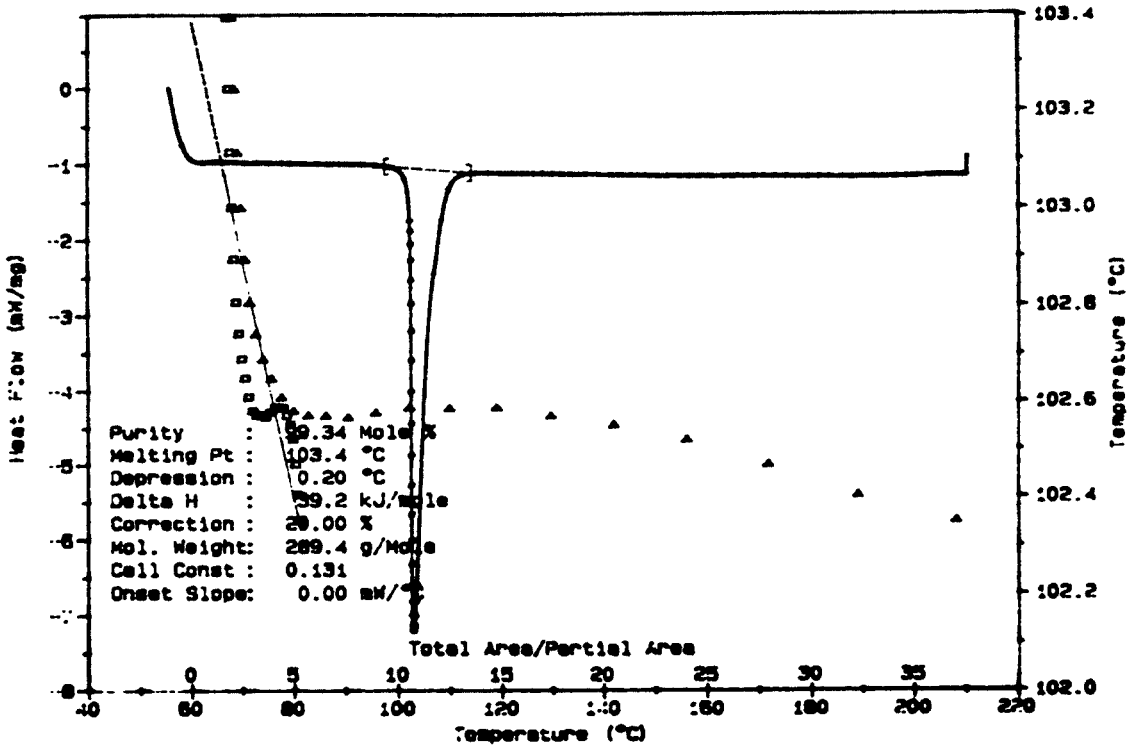


Figure 9: DSC Curve of Hyoscyamine.

ing and after (20). *Hyoscyamus muticus* and *Duboisia myoporoides* being the best sources to obtain hyoscyamine.

One of the methods for the isolation of hyoscyamine is described as follows (21):

The powdered solanaceous plant is thoroughly moistened with an aqueous solution of sodium carbonate and extracted with ether or benzene. The alkaloids are extracted from the solvent with dilute acetic acid, the acid solution is shaken with ether as long as it takes up coloring matter. The acid layer is then rendered alkaline with sodium carbonate, the precipitated alkaloids are collected, washed and dried, then dissolved in ether or acetone.

The resulting organic solution is dehydrated with anhydrous sodium sulfate and filtered. After concentration and cooling of the solution, atropine and hyoscyamine crystalize from the solution as masses and collected by filteration.

Hyoscyamine and atropine so obtained are converted to their oxalate salts and separated from each other by fractional crystallization from acetone and ether (hyoscyamine oxalate is the more soluble one).

3.1 Preparation of Hyoscyamine

Since hyoscyamine is the levorotatory enantiomorph of atropine, it can therefore, be obtained by the resolution of atropine (22).

4. Synthesis of Hyoscyamine

4.1 Partial Synthesis

Since hyoscyamine is an ester of the aminoalcohol *tropine* and *(-)-tropic acid*, it can therefore be obtained by heating tropine with (-)-tropic acid in the presence of hydrogen chloride as the same manner as atropine (23).

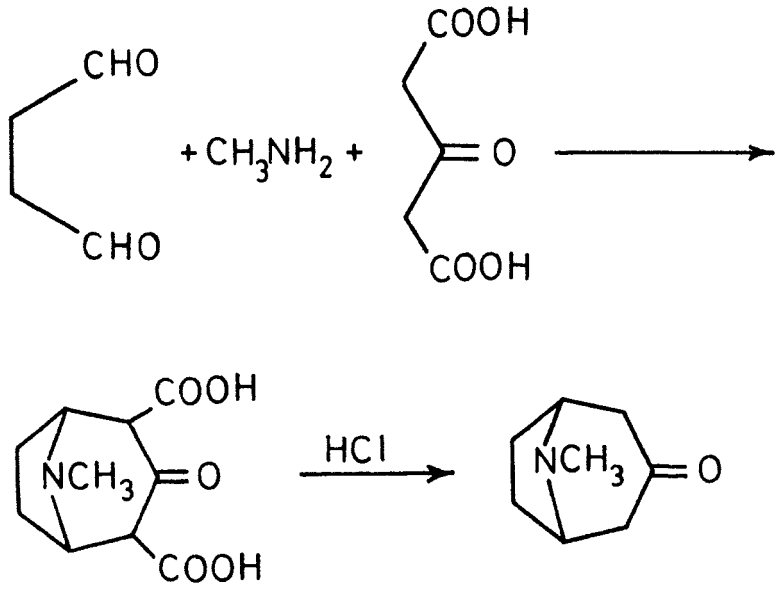
The esterification can be also performed by treating tropine hydrochloride with (-)-acetyltropyl chloride in nitrobenzene (24).

4.2 Total Synthesis

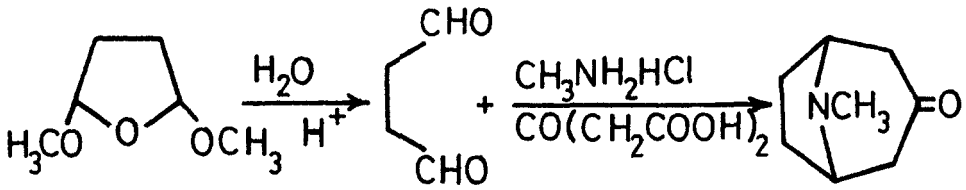
Total synthesis of hyoscyamine requires two different schemes, the first is the synthesis of tropine and the other is the synthesis of (-)-tropic acid.

4.2.1 Total Synthesis of Tropine

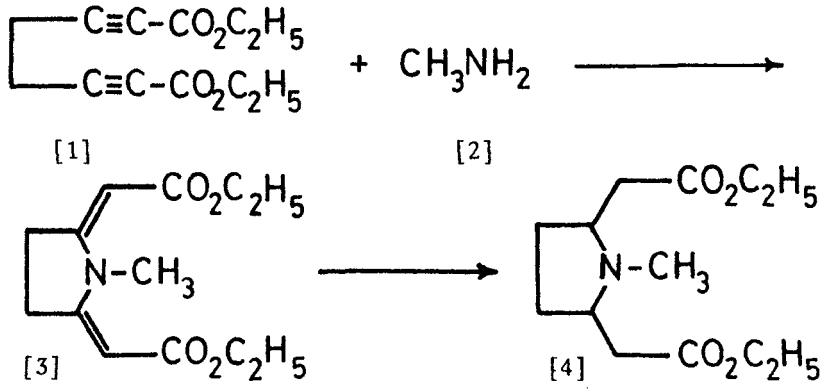
It was Willstatter (25) who first synthesized tropine from suberone (cyclopentanone). For this synthesis refer to analytical profile of atropine (26).
But the most reliable biomimetic type of synthesis of tropine was achieved in 1917 by Robinson (27). In this method, succindialdehyde, methylamine and acetone dicarboxylic acid were mixed at pH 5.6 for 30 minutes to give tropinone in one step which was then reduced to tropine by an established process (25). A much better yield (almost 40%) was obtained by using calcium acetondicarboxylate; the calcium salt so produced is converted into tropinone by warming with hydrochloric acid (28).

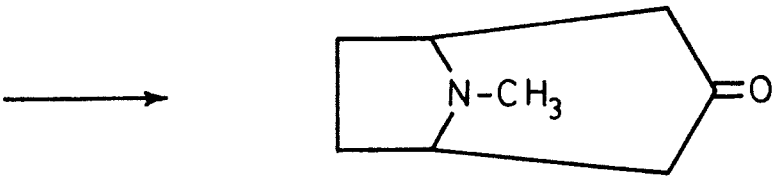


Schopf and Lehmann (29) have also obtained a yield of 70-85% by modifying Robinson's synthesis. A dilute buffered solution of succindialdehyde, methylamine hydrochloride and acetone dicarboxylate were mixed at 25° and at pH 5.0.
Elming et al. (30) have synthesized tropinone (in 81% yield) by using methylamine hydrochloride, acetone dicarboxylic acid and generating succindialdehyde *in situ* by the action of acid on 2,5-dimethoxytetrahydrofuran which is commercially available.



An alternative synthesis of tropinone has been reported (31):
In this method, diyne diester [1] was condensed with methylamine [2] to give the pyrrolidine diester [3]. Catalytic hydrogenation of [3] produced [4] which on Dieckmann cyclization (32), hydrolysis and decarboxylation gave tropinone [5].





[5]

Tropinone can better be hydrogenated into tropine in the presence of Raney nickel (33, 34).

4.2.2 Total Synthesis of (-)-Tropic Acid

Several schemes for the total synthesis of tropic acid are known [For these schemes refer to the analytical profiles of atropine (26) and of scopolamine (35)].
It was Landenburg and Rugheimer who first synthesized tropic acid from acetophenone (36).
McKenzie and Wood (37) also have synthesized tropic acid from acetophenone and described a method for the resolution of isomers.
Muller (38) employed ethylphenyl acetate to prepare tropic ester and this upon hydrolysis yielded tropic acid.
Chambon used ethyl α-bromophenyl acetate as the starting substance to synthesize tropic acid (39).
Blicke et al. later (40), have synthesized tropic acid by boiling phenylacetic acid with isopropylmagnesium chloride in ethereal solution, and then treating the Grignard product so formed with formaldehyde.
Other methods of synthesis of tropic acid have been placed in patents (41,42).(-)-Tropic acid can be obtained by resolution of (±)-tropic acid (37).

5. Biosynthesis of Hyoscyamine

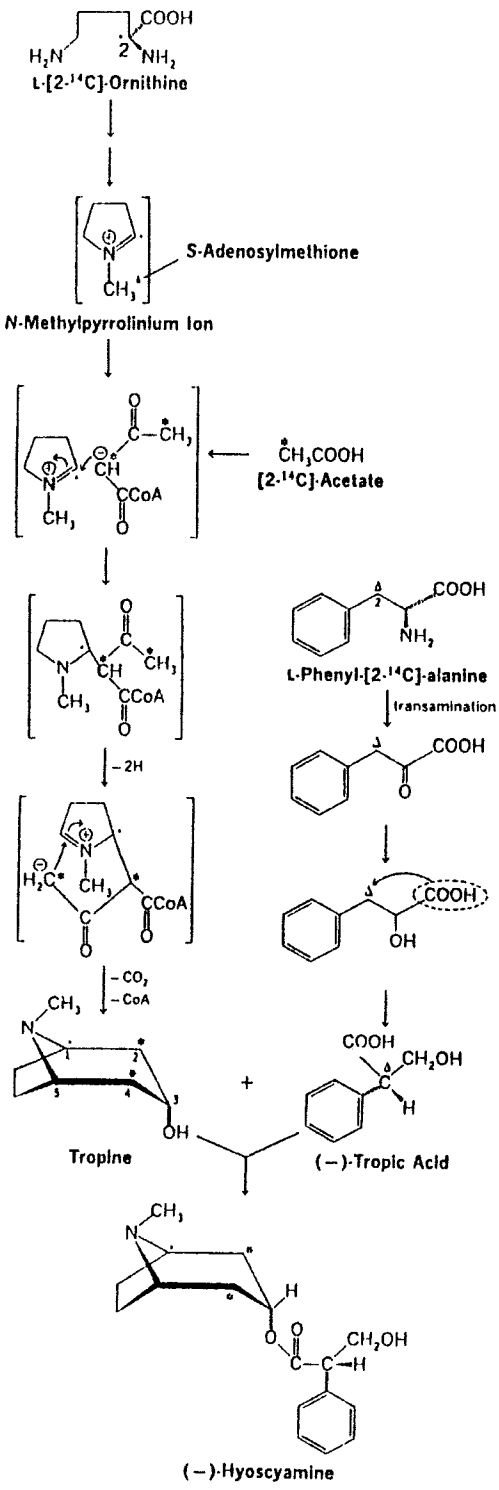
Early work with isotopes has established that ornithine or one of the related aminoacids (glutamic acid or proline) (43) as well as acetate (44,45) are precursors of the tropine moiety (as [2-C^{14}]-ornithine; [5-C^{14}]-proline and [1-C^{14}]-acetate were all incorporated into hyoscyamine (isolated from fed plants). It is believed that the incorporation of glutamic acid or proline is considered to occur via ornithine (43), and the acetate is incorporated via acetoacetic acid, as feeding with [1,3-C^{14}]-acetoacetate resulted in the isolation of labelled hyoscyamine (43).

It has been shown that putrescine was incorporated into hyoscyamine (46) however, it was predicted that certain enzymes are capable of converting putrescine into N-methyl putrescine which is an established precursor of tropine (43). It has also been reported that (+)-(*2R*)-hygrine serves as a precursor of the tropane alkaloids of *Datura innoxia* (47), this is true as (*2R*)-hygrine is formed by an attack of the acetoacetate on the pyrrolinium salt (43) as it is evident from the biosynthetic scheme of hyoscyamine.

Recently, it has been shown that the biosynthetic pathway between ornithine and hyoscyamine must be different in *Datura and Hyoscyamus albus* (48), i.e. the conversion of ornithine to the 1-methyl-Δ^1-pyrrolinium salt (a precursor of hyoscyamine) can proceed by two pathways, one of which (*in Datura*) cannot involve free putrescine (48).

Phenylalanine is the established precursor of tropic acid. Tracer studies have shown that the side chain of the aminoacid undergoes intramolecular rearrangement during the conversion to tropic acid (49), thus upon feeding L-phenyl[2-C^{14}]-alanine, radioactive (-)-tropic acid is resulted. Biological esterification of (-)-tropic acid with tropine produces hyoscyamine.

The biosynthetic pathway of hyoscyamine is presented in the following scheme [after (50)].


L-[2-14C]-Ornithine
S-Adenosylmethione
N-Methylpyrrolinium Ion
[2-14C]-Acetate
L-Phenyl-[2-14C]-alanine
Transamination
-2H
-CO2
-CoA
Tropine
(-)-Tropic Acid
(-)-Hyoscyamine

6. Pharmacokinetics

6.1 Drug Absorption

Hyoscyamine is well and completely absorbed from the gastrointestinal (GI) tract following oral doses of the drug. Food does not appear to affect its absorption (51,52).

A commercially available extended-release capsule (*Levsinex*R; *Timecaps*R) contains small beads of hyoscyamine sulfate which are surrounded by a porous membrane that permits fluids to enter and dissolve the drug. The manufacturer states that 0.375 mg of drug is delivered from a capsule at an approximate rate of 0.125 mg/4 hours. In a crossover study comparing extended-release capsules and conventional tablets, bioavailability (as determined by area under the plasma concentration-time curve) during the first 4 hours after administration of a single 0.375 mg dose as extended-release capsules, was about 43% that of a single 0.125 mg dose as conventional tablets when the data were corrected for difference in dose (51).

6.2 Onset of Action

After oral administration, the onset of action of hyoscyamine is approximately 20 to 30 minutes (51,52).

Following parenteral administration of hyoscyamine sulfate, the drug has an onset of action of 2 to 3 minutes; peak pharmacologic action occurs within 15 to 30 minutes and persists for up to 4 hours.

When hyoscyamine sulfate conventional tablets are chewed or administered sublingually or when the drug is given orally as an elixir or solution, the drug has an onset of action of 5 to 20 minutes. Pharmacologic action peaks within 30 to 60 minutes and persists for about 4 hours. Following oral administration of hyoscyamine sulfate ex-

tended- release capsules, the drug has an onset of action of about 20 to 30 minutes; pharmacologic action peaks within 40 to 90 minutes and persists for about 12 hours (51).

6.3 Drug Distribution

Hyoscyamine is well distributed throughout the body. The drug crosses the blood-brain barrier. It also crosses the placenta and found in placental tissues. Small quantities of the drug distribute into breast milk. Hyoscyamine is about 50% bound to plasma proteins (51,52).

6.4 Metabolism

Hyoscyamine is metabolized in the liver to (-)-tropic acid, tropine and hyoscyamine glucuronide.

6.5 Elimination

The majority of a dose of hyoscyamine is excreted unchanged in the urine within 12 hours (53). In studies using atropine (dl-hyoscyamine), approximately 30-50% of a dose was excreted in urine unchanged (51). The drug is also eliminated in urine as metabolites (52). Elimination of hyoscyamine is prolonged in individuals with renal dysfunction.
In a crossover study comparing single dose administration of extended-release capsules of hyoscyamine and administration of 3 doses of conventional tablets at 4-hours intervals, urinary excretion during the first 24 hours for extended-release capsules was about 80% that for conventional tablets (51).
Hyoscyamine is excreted into breast milk in trace amount (52).

6.6 Half-Life

The serum half-life of hyoscyamine is about 3.5 hours (51,52).

The apparent plasma half-life of hyoscyamine that is released from the sustained-release formulation is approximately 7 hours (52).

7. Drug Stability and Storage

Hyoscyamine is readily racemized to atropine when warmed in an ethanolic alkaline solution or by the action of heat (27,54). It racemizes slowly in ethanol (4).
The conversion of (-)-hyoscyamine to (±)-atropine results in loosing almost 50% of its antimuscarinic activity (as hyoscyamine has approximately twice the antimuscarinic potency of atropine).
Hyoscyamine, its salts (the sulfate and the hydrobromide) and its pharmaceutical formulations are sensitive to light (51).
They should be stored at controlled room temperature 15 to 30° (59 to 86°F) (52,53).
Preserved in tight, light-resistant containers (3).
Hyoscyamine sulfate injection, preserved in single-dose or in multiple-dose containers, preferably of Type I glass (3).

Hyoscyamine in the dry state, stored as above, showed no decomposition after five years, when examined by different physical and chemical methods including spectroscopic evidence (55).
Hyoscyamine and the sulfate salt are incompatible with alkalis, mercury salts and tannic acid, while hyoscyamine hydrobromide is incompatible with alkalis, silver salts and tannic acid (54).

8. Methods of Analysis

8.1 Identification

The following identification tests are mentioned in the USP XXI (3) under hyoscyamine.
A: Transfer 30 mg of hyoscyamine and 36 mg of USP hyoscyamine sulfate RS to individual 60 mL separators with the aid of 5 mL portions of water. To each separator add 1.5 ml of 1N sodium hydroxide and 10 mL of chloroform.

Shake for 1 minute, allow the layers to separate, and filter the chloroform extracts through separate filters of about 2 g of anhydrous granular sodium sulfate supported on pledgets of glass wool. Extract each aqueous layer with two additional 10 mL portions of chloroform, filtering and combining with the respective main extracts. Evaporate the chloroform solutions under reduced pressure to dryness, and dissolve each residue in 10 mL of carbon disulfide. The infrared absorption spectrum, determined in a 1-mm cell, of the solution obtained from the test specimen exhibits maxima only at the same wavelengths as that of the solution obtained from the Reference Standard (RS).
B: Dissolve 60 mg of hyoscyamine in 1 mL of 0.2N hydrochloric acid, and add gold chloride TS, dropwise shaking, until a definite precipitate separates. Add a small amount of 3N hydrochloric acid, dissolve the precipitate with the aid of heat, and then allow to cool; lustrous golden yellow scales are formed (*distinction from atropine and scopolamine*).
Melting range: between 106° and 109°.
Specific rotation: between -20° and -23°, calculated on the dried basis, determined in a solution in dilute alcohol (1 in 2) containing 100 mg of hyoscyamine in each 10 mL.

The following identification tests are mentioned in the USP XXI (3) under hyoscyamine sulfate.
A: The infrared absorption spectrum of a potassium bromide dispersion of it, previously dried, exhibits maxima only at the same wavelengths as that of a similar preparation of USP Hyoscyamine Sulfate RS.
B: To about 1 mL of a solution (1 in 20) add gold chloride TS, dropwise with shaking, until a definite precipitate separates. Add a small amount of 3N hydrochloric acid, dissolve the precipitate with the aid of heat, and

allow the solution to cool; lustrous golden yellow scales are formed (*distinction from atropine and scopolamine*).
C: A solution of (1 in 20) responds to the tests for sulfate.
Melting temperature: not less than 200°.
Specific rotation: not less than -24°, calculated on the dried basis, determined in a solution containing 500 mg of hyoscyamine sulfate in each 10 mL.

The following identification tests are reported in the BP (2) under hyoscyamine sulphate.
A. The infra-red absorption spectrum, concordant with the spectrum of standard hyoscyamine sulphate.
B. Specific optical rotation: In a 5% w/v solution, -24° to -29°.
C. To 1 mg add 0.2 ml of fuming nitric acid and evaporate to dryness on a water-bath. Dissolve the residue in 2 ml of acetone and add 0.2 ml of a 3% w/v solution of potassium hydroxide in methanol. A violet colour is produced.
D. Heat 0.5 ml of a 5% w/v solution with 2 ml of 2M acetic acid. To the hot solution, add 4 ml of a 1% w/v solution of 2,4,6-trinitrophenol and allow to cool, shaking occasionally. Collect the crystals so formed, washed with two 3-ml quantities of iced water and dry at 100° to 105°. The melting point of the dried crystals is 164° to 168°.
E. Yields the reactions characteristic of sulfate.

Other identification tests are as follows:
One of the most sensitive tests for atropine, hyoscyamine and scopolamine is their mydriatic reaction in the pupil of the eyes of young dogs; cats and rabbits (Induction of mydriasis).
A neutral alcohol-free aqueous solution of the sulfate salt is dropped into the conjunctival sac of the eye. Mydriasis can be observed in the test eye.

It has been reported (56) that 1 part in 40,000 of atropine sulfate will cause a distinct dilation of the pupil of the eye in 1 hour.
Gerrard Reaction: Add 1 ml of 2% solution of $HgCl_2$ in 5% aqueous methanol, to about 5 mg hyoscyamine and warm the mixture for a while. A deep red color is produced.
Gold Chloride Test: To a few ml of 1% aqueous solution of hyoscyamine acidified with hydrochloric acid, add a few drops of gold chloride solution; a lemon oily precipitate is formed which crystallizes after a while. The precipitate is recrystallized from boiling water acidified with diluted hydrochloric acid, and dried. The aurichloride of hyoscyamine so formed appears as golden yellow hexagonal plates, has a melting point of 165°.

8.2 Microcrystal Tests

Picric acid gives with hyoscyamine solution (as sulfate salt), rosettes of needles or plates (sensitivity 1 in 400) (9).
Potassium triiodide produces with hyoscyamine (or salts) small plates and prisms often in crosses and arrowheads (sensitivity 1 in 1000) (9).
Microcrystal tests can be performed to identify tropane alkaloids from animal tissues (57).
These alkaloids are extracted from animal tissues with the universal buffer solution or with acid solution at pH 4 to 5 (using oxalic or tartaric acid). The alkaloids are then identified micro crystallographically by reaction with Rieneck's salt and by Vitali's reaction (57).

8.3 Titrimetric Determinations

8.3.1 Aqueous Titrations

Belladonna, hyoscyamus and their galenical preparations contain 0.4 to 1% tropane alkaloids, mainly as hyoscyamine.
The BP (2) recommends an aqueous titration method for the assay of these preparations.
The method of assay depends on the extraction and purification of the total alkaloids by solvent extraction technique. The residue of total alkaloids is then dissolved in several ml of chloroform, 20 ml of 0.01M sulfuric acid VS is added and the chloroform is removed by evaporation on a water-bath. The excess acid is titrated with 0.02M sodium hydroxide VS using methyl red solution as indicator.
Each ml of 0.01M sulfuric acid VS is equivalent to 0.005788 g of total alkaloids calculated as hyoscyamine.

Determination of small amounts of nitrogenous bases (including tropine alkaloids) in aqueous solutions such as eye-drops and injections has been described (58). The method depends on precipitation of the alkaloids with tetraphenylboron at pH 3.7 and the excess of the reagent is then determined by back-titration with a standard solution of quaternary ammonium salt to visual end-point.
Melting points of the organic tetraphenylboron salts may be used in the identification of many of these compounds (58,59).

8.3.2 Non-aqueous Titration

The USP XXI (3) describes a non-aqueous titration method for the assay of hyoscyamine and its salts (the hydrobromide and the sulfate):

Dissolve about 500 mg of hyoscyamine, accurately weighed, in 50 mL of glacial acetic acid, add 1 drop of crystal violet TS, and titrate with 0.1N perchloric acid VS to a green end-point. Perform a blank determination, and make any necessary correction.
Each mL of 0.1N perchloric acid is equivalent to 28.94 mg of $C_{17}H_{23}NO_3$.

Dissolve about 700 mg of hyoscyamine hydrobromide, accurately weighed, in a mixture of 50 mL of glacial acetic acid and 10 mL of mercuric acetate TS. Add 1 drop of crystal violet TS, and titrate with 0.1N perchloric acid VS to a blue-green end-point. Perform a blank determination and make any necessary correction.
Each mL of 0.1N perchloric acid is equivalent to 37.03 mg of $C_{17}H_{23}NO_3 . HBr$.

Dissolve about 1 g of hyoscyamine sulfate, accurately weighed, in 50 mL of glacial acetic acid and titrate with 0.1N perchloric acid VS, determining the end-point potentiometrically. Perform a blank determination, and make any necessary correction.
Each mL of 0.1N perchloric acid is equivalent to 67.68 mg of $(C_{17}H_{23}NO_3)_2 . H_2SO_4$.

The BP (2) recommends the following method for the assay of hyoscyamine sulfate:
Dissolve 0.5 g in 25 ml of anhydrous glacial acetic acid. Carry out the non-aqueous titration method using 0.1M perchloric acid and determining the end-point potentiometrically.
Each ml of 0.1M perchloric acid VS is equivalent to 0.06770 g of $(C_{17}H_{23}NO_3)_2 . H_2SO_4$.

Many other non-aqueous titration methods for the assay of hyoscyamine and related tropine alkaloids have been reported, some of these are in references (60-64).

8.3.3 Potentiometric Titrations

Potentiometric titration may be carried out by using a glass electrode and a standard calomel cell as reference electrode (2):
Titrate with the titrant to the color change of the indicator that corresponds to the maximum value of dE/dV (where E is the electromotive and V is the volume of titrant).

Hydrochlorides of several alkaloids and related substances including hyoscyamine were titrated in dimethyl sulfoxide medium with 0.1 M $AgNO_3$. Ends points were determined by conductimetric, potentiometric and polarimetric techniques and the results were satisfactory by these three techniques (65).

8.4 Gravimetric Methods

Atropine and hyoscyamine mixture or in galenicals can be determined gravimetrically by precipitation with potassium tetraphenyl borate (66). Recoveries varying from 81.9 to 99.6% were obtained according to the volume of solution analysed. Reasonable results were obtained by reducing the volume of solution to 25 ml and with 10 to 25 mg alkaloids (atropine or hyoscyamine). The ratio atropine/hyoscyamine was determined by dissolving the alkaloidal tetraphenyl borate in 25% hydrochloric acid and measuring the specific rotation which is - 43° for hyoscyamine in this solvent (66).

Another gravimetric method utilizing potassium iodobismuthate for the determination of small quantities of atropine and other alkaloids has been described (67).

8.5 Polarographic Determinations

Several alkaloids including atropine/hyoscyamine were determined qualitatively and quantitatively by a polarographic method (68):
Concentration of 0.001% (or lower) to 1% of alkaloids can be performed. Alkaloids as salts (nitrates, hydrochlorides, hydrobromides, sulfates and acetates) were qualitatively separated at the cathode of an electrolytic cell. Of the several kinds of electrodes tested, aluminum as anode (wrapped in parchment) and a steel one as cathode were the most satisfactory.
The alkaloids collected at cathode were washed, dried and their melting points were determined for identification. Additional of NaCl to the electrolyzed solution hastened the separation of alkaloids. Some alkaloids were quantitatively determined (68).

Oscillopolarographic studies of several alkaloids with tropane and isoquinoline linkages have been reported (69):
Various related alkaloids can be distinguished even in mixtures, by the oscillograms. Thus it was possible to distinguish atropine, hyoscyamine, scopolamine, homatropine HBr, cocaine, hydrastine, berberine as well as other alkaloids.

The oscillopolarographic behaviour of atropine/hyoscyamine and other alkaloids has been reported (70):
A dropping mercury electrode served as polarizable electrode, and a graphite one as a reference. The depolarizing potentials were referred to the potentials of Tl ions. Semiquantitative determinations from the depth of the characteristic Cuts-in on the curve was possible in alkaline solutions. Oscillopolarographic curve of atropine was obtained.

Other polarographic techniques were also reported (71-73).

8.6 Spectrophotometric Methods

8.6.1 Colorimetric Determinations

Morin (74) suggested the use of Vitali's reaction for the determinations of small amounts of atropine.
Allport and Wilson (75) have also adopted Vitalis reaction for the rapid determination of the alkaloids in *belladonna* and *stramonium*.
The method was found not applicable to *Hyoscyamus niger* or its galenical preparations (59).
Allport and Jones (76) confirmed that atropine and hyoscyamine react quantitatively and the method is also applicable to scopolamine.

A summary of this determination can be described as follows (59):
A quantity of alkaloidal solution or tablets containing between 1.6 to 2.4 mg of atropine is rendered alkaline and extracted with chloroform. The alkaloid is re-extracted from the chloroform with 6% acetic acid and ethanol. An exact aliquot of the resulting extract is transferred into an evaporating dish and evaporated just to dryness on a water bath, fuming nitric acid (0.2 ml) is immediately added to the residue and again evaporated to dryness. The resulting residue is dissolved in acetone (about 3 ml) and made up to volume (10 ml). A 3.0% potassium hydroxide in methanol (0.1 ml) is added and the mixture allowed to stand for 5 minutes. A purple color is developed and the intensity of this color is then measured in a photoelectric absorptiometer. The concentration is calculated from a calibration curve with quantities of 0.025 mg to 0.15 mg of pure hyoscyamine treated similarly.
Ashley (77) stated that, this method gives accurate and reproducible results only when the reagents, reaction times and water content of the acetone are controlled (not more than 0.2% of water should be present in the acetone). He

also suggested the use of pyridine (analytical grade) instead of acetone for the determination of scopolamine (produces higher color intensity).

A newer approach to the Vitali type of reaction has been reported by Freeman (78):

A measured quantity of alkaloidal solution (containing about 0.05 to 0.15 mg alkaloid) is evaporated to dryness on a water-bath, the residue is nitrated with fuming nitric acid (0.2 to 0.3 ml) and evaporated again to dryness. The residue is transferred to a 10-ml graduated flask with the aid of small quantities of dimethylformamide, 25% w/w aqueous solution of tetramethylammonium hydroxide (0.3 ml) is added to the flask which diluted to volume with dimethylformamide. The resulting mixture is allowed to stand for 5 minutes and the extinction of the developed color is measured at *540 nm* in 1-cm cells against dimethylformamide. The alkaloidal content is ascertained from a calibration curve which is linear (59,78).

A colorimetric method for the determination of small amounts of tropic acid, mandelic acid and their esters (atropine, hyoscyamine, scopolamine and homatropine) has been reported (79):

The alkaloid was nitrated for 15 minutes at 50° with a solution of 20% KNO_3 in concentrated H_2SO_4. On making the nitrated product alkaline with hot 18-20% NaOH solution, a color developed in 30 minutes. This color was estimated by using an S_{42}, S_{47} or S_{50} filter in the Pulfrich photometer.

The sensitivity was 50 and 60 µg of hyoscyamine (atropine) per ml. The probable error was ± 3.0%.

Colorimetric estimation of atropine and related alkaloids in pharmaceutical preparations has been reported by two procedures (80).

Procedure (a): An aliquot chloroformic extract prepared by the USP method (containing 0.25 to 1.0 mg of alkaloids) was evaporated to dryness on a water

bath. Fuming nitric acid was added and heated till fumes ceased. The residue was then dried at 105° for 15 minutes and allowed to cool. The residue was dissolved in acetone and diluted to 25 ml. An aliquot (5 ml) was mixed with isopropylamine (2 ml) and 0.1% methanolic KOH (0.1 ml). The extinction of the produced color was measured at *540 nm* after one minute.

Procedure (b): The residue was nitrated as in procedure (a) and dissolved in 50% ethanol (10 ml). The ethanolic solution was heated on a water bath with 10% HCl (2.5 ml) and zinc dust (0.1 g) for 10 minutes, cooled and filtered. The zinc residue was washed with water and the washings were added to the filtrate. 1% $NaNO_2$ (1 ml) was added, mixed and allowed to stand for 10 minutes. 2.5% solution of ammonium sulfamate (1 ml) was added, the mixture was shaked and allowed to stand for 10 minutes. 2.5% solution of ammonium sulfamate (1 ml) was added, the mixture was shaked and allowed to stand for 10 minutes. 1% N-1-naphthylethyl-enediamine dihydrochloride solution (1 ml) was added and diluted to 25 ml with water. The extinction of the produced color was measured after 30 minutes at *550 nm*. The concentration was calculated by reference to a standard curve. Recovery experiments in both procedures indicated an accuracy of ±1%.

A photometric method for the quantitative determination of tropane alkaloids has been described (81).

The determination of atropine/hyoscyamine or scopolamine is based on the reaction of the alkaloid with p-dimethylaminobenzaldehyde reagent in concentrated H_2SO_4. The intensity of the color so produced being measured in a photoelectric absorptiometer using a green filter.

The above method can be applied for the micro-determination of hyoscyamine (or atropine) which required special

treatment, and measuring the extinction at *500 nm* using a suitable spectrophotometer (82).

A colorimetric method for the determination of tropane alkaloids or tropane quaternary salts has been reported (83): In this method, the alkaloids are precipitated with molybdophosphoric acid, the complex precipitate can be dissolved and reduced to molybdium blue which can be colorimetrically measured. The following procedure was described:
To 1 ml sample (containing 0.2 to 1 mg alkaloid) add 10% H_2SO_4 (1 drop), 5% NH_4Cl solution (0.2 ml) and 0.5% molybdophosphoric acid (dropwise 1 ml). After 10 minutes, filter the mixture, repeat the filtration, wash the precipitate with 0.15% H_2SO_4 (5x1 ml) and with water (5x1 ml), dissolve the precipitate in acetone (2 ml), add ethanol (2 ml) and 2% ascorbic acid solution (1 ml), set aside for 15 minutes, add acetone (1 ml), cool to 20° and dilute with water to 10 ml. Measure the extinction at *430 nm* (or use an appropriate filter) against water in 2-ml cells. Beer's law is obeyed with stated concentrations. The error is < 3.0%.

The colorimetric reaction of Tropaeolin 00 with alkaloids under acidic conditions is used for microquantitation of alkaloids (Haussler 84):
The aqueous solution of an alkaloid (5 ml) (containing 100 µg) is mixed with a similar quantity of an acetic buffer (pH 4.6) and 3 ml of a saturated aqueous Tropaeolin 00. The resulting mixture is then extracted with chloroform (4x5 ml). The combined extracts are acidified with 2 ml of an acid reagent (1 ml concentrated H_2SO_4 and 99 ml methanol) and diluted to 25 ml with chloroform. The alkaloid is then determined spectrophotometrically at *545 nm* and calculated from a standard curve.

The above method was applied for the determination of small amounts of hyoscyamine and scopolamine in crude drugs (85):
These alkaloids were first separated by paper chromatography with the solvent n-butanol-glacial acetic acid (10:1), saturated with water. After development, the spots were eluted, treated and determined as above.

Two acid-dye methods have been reported for the colorimetric determination of tropane alkaloids (86,87).
In the first procedure, hyoscyamine (or atropine) can be selectively determined in the presence of scopolamine by using bromcresol purple, which forms a chloroform-extractable complex with hyoscyamine at pH 6.6. This complex is separated and measured *at 420 nm.*
Scopolamine and the hydrolytic product tropine do not interfere.
In the second procedure, both hyoscyamine and scopolamine form chloroform-extractable complexes using bromthymol blue at pH 5.6, and measuring the extinction *at 420 nm.* In this technique, both alkaloids can be determined without preliminary separation, as low as 0.05 mg of each alkaloid could be estimated, with average percentage recoveries of 98.6 and 101.5 for hyoscyamine and scopolamine respectively.
The first procedure can be adopted to estimate hyoscyamine content in belladonna tincture using as little as 0.1-0.2 ml of the tincture. Bromcresol purple is used at pH 6.6 and the complex formed is treated with 0.1 N NaOH and measured at *580 nm.* Neither scopolamine nor tropine interferes (86).

The above procedure has been modified to determine tropane alkaloidal salts as follows (88).
Alkaloidal salt (1 mg) is dissolved in water (100 ml). To an aliquot of this (1 ml), mM bromocresol purple (3 ml) and buffer solution of pH 4.0 (2 ml) are added. The so produced color is ex-

tracted with chloroform (4x5 ml). The combined extracts are diluted to 10 ml with chloroform.
The absorbance of this at the absorption maxima *(405 to 410 nm)* is measured. The alkaloid content is then calculated from a calibration graph. Hydrolysis products of alkaloids do not interfere unless present in threefold amounts.
The relative error of the method does not exceed ± 1%.

Other colorimetric methods have also been described (89-93).

8.6.2 Ultraviolet Determinations

An unit-dose assay of tropine alkaloids and their synthetic analoges has been reported (94).
Samples of powdered tablets, injection solutions, eye drops were extracted with 1,2-dichloroethane.
A volume of the extract containing 0.05 to 0.15 mg of alkaloid was mixed with mM iodine in dichloroethane (1 ml). This mixture was diluted to 10 ml with dichloroethane. The extinction of the charge transfer complex at the maximum *at 280 nm or 295 nm* was measured against a reagent blank. The procedure has been successfully applied to atropine, hyoscyamine, scopolamine, homatropine and their N-butyl analoges (94).

Some alkaloids including atropine (hyoscyamine) can be determined spectrophotometrically (95). This method can also be applied to pharmaceutical preparations.
To a solution of the alkaloid (1 mg) in phosphate buffer solution of pH 7.0 (20 ml), 1% sodium picrate was added (3 ml). The resulting mixture was extracted with chloroform, this was shaked with phosphate buffer solution of pH 11.2 to 11.5 (40 ml). The aqueous phase was collected, diluted with the same buffer to 100 ml, the extinction of resulting solution was measured at *355 nm*.

The BP (2) describes the following UV procedure to detect apo-atropine in hyoscyamine sulfate.
0.1 g of hyoscyamine sulfate is dissolved in sufficient 0.01 M hydrochloric acid to produce 100 ml. The A (1%, 1 cm) of the resulting solution *at 245 nm* is not more than 4.0 (about 0.5%).

A spectrophotometric assay method for some narcotics and other alkaloids in galenical preparations has been reported (96).
The alkaloidal salt (particularly in ampoules) was dissolved in aqueous solution and diluted to volume. The extinction of this, was determined at the wavelength for maximum absorption *(257 to 286 nm)* and compared with that progressively diluted samples of stock solution.

The use of charge-transfer complexation in the spectrophotometric determination of hyoscyamine has been reported (97). A complex was formed between hyoscyamine and iodine in carbon tetrachloride medium as follows.
A solution of hyoscyamine base in CCl_4 and 1 ml of 1 m M iodine in CCl_4 were mixed and diluted to 25 ml with the solvent and set aside for 30 minutes. Two spectrophotometric methods were applied. In one the absorbance *at 390 nm* was measured in the conventional way against a reagent blank. In the other the treated sample solution was placed in the reference beam, the transmittance was adjusted to 100% and the apparent absorbance of the reagent blank was measured *at 520 nm.*
The two calibration graphs (of both assays) were rectilinear for 0.5 to 2.5 mg of hyoscyamine. Both methods gave quantitative recoveries.

Hyoscyamine and atropine as solutions in 0.1 N HCl can be determined by UV spectroscopy (98). The absorbance of such solution is measured in 3-cm cells

at 238 to 320 nm. (For more detail of this technique, see under quantitative TLC).

8.6.3 Fluorimetric Analysis

A fluorimetric method for the determination of atropine and other related alkaloids has been described (99). The method is based on the formation of fluorescent complex between atropine and eosine: To a solution of atropine in chloroform (9 ml) 0.1% eosine solution is added (1 ml). The mixture is shaken thoroughly and the fluorescence intensity *at 556 nm* (excitation *at 365 nm*) is measured after 10 minutes. Beer's law is obeyed with 1 to 5 µg of atropine per ml; the coefficient of variation is 2.6%.

Hyoscyamine can be determined by fluorimetric technique as follows (100). A solution of hyoscyamine or an eluate of it from a LiChrosorb DIOL HPLC column is treated with 9,10-dimethoxy anthracene-2-sulfonate solution. The resulting derivative is determined fluorimetrically *at 446 nm* with excitation *at 383 nm*.
This technique can be applied as post column derivatization using fluorimetric ion-pair HPLC. When hyoscyamine was determined by HPLC applying the above method, the following data were obtained (100):
The regression : Linear in the range 40 to 600 ng of hyoscyamine per injection.
The standard deviation : 1.2% at 200 ng.
The limit of detection : 500 pg.

8.6.4 Infrared Determinations

The application of IR spectrophotometry to the quantitative determinations of tropine alkaloids has been reported (101). The pressed KBr pellet technique was applied in these determinations. Recoveries from standard

mixtures showed a mean value of 104% for atropine and hyoscyamine and 98.2% for scopolamine.

8.6.5 Circular Dichroism (CD) Determination

CD spectra in aqueous buffer solution can be used to distinguish between stereoisomers including hyoscyamine and atropine (102). Hyoscyamine can be determined from its CD spectrum *at 222 nm* in the presence of atropine, and the sum of the two alkaloids can be determined from their absorbance *at 257 nm.*

8.7 Chromatographic Methods

8.7.1 Paper Chromatography

Clarke (9) described the following two chromatographic systems for the identification of hyoscyamine.

I) Whatman No.1, sheet (14 x 6 inches), buffered by dipping in 5% solution of sodium dihydrogen citrate, blotting and drying at 25° for one hour. It can be stored indefinitely. A solvent composed of 4.8 g of citric acid in a mixture of 130 ml of water and 870 ml of n-butanol was used (103).
Rf value of hyoscyamine is 0.37.
Location reagent : Iodoplatinate spray.

II) 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.
Solvent system: acetate buffer (pH 4.58).
Equilibration: The solvent in a beaker was equilibrated in a thermostatically controlled oven at 95° for about 15 minutes (104).
Rf value of hyoscyamine is 0.94.
Location reagent: as for I.

Paper chromatography has been used for quantitative determinations of the tropane alkaloids or for their separation from crude drugs prior to determination.

The following system was reported for the microdetermination of solanaceous alkaloids (105). Ethereal extract of a crude drug was applied to strips of filter paper impregnated with potassium chloride solution. The chromatograms were developed in the solvent n-butanol-aqueous HCl by the ascending technique for 20 hours. After development, the chromatograms were dried, sprayed with modified Dragendorff's reagent and areas of the spots were measured. The relative concentrations of the individual alkaloids were calculated by reference to calibration curves. Rf values : hyoscyamine 0.85; scopolamine 0.63 and atropine 0.34.

Tropane alkaloids in crude drugs can be qualitatively identified, separated by paper chromatography and quantitatively assayed by colorimetric determination (106). Whatman No.3 filter paper was used and the chromatograms were developed with the solvent water saturated n-butanol-glacial acetic acid (25:1) for 18 hours. Corresponding areas on the chromatograms were identified, cut off, eluted with ethanol and determined colorimetrically.
Rf values for hyoscyamine and atropine 0.53, for scopolamine 0.35.

Other paper chromatographic systems have been reported (107-110).

8.7.2 Paper Electrophoresis

Measurements of the electrophoretic mobility of 68 different alkaloids including hyoscyamine were reported (111). This was performed in an LKB apparatus on Whatman No.1 paper (18 x 46 cm) at 8v/cm for 3 hours in the presence of universal buffers at various pH values.

The relative displacements (rd) for l-hyoscyamine at various pH values were reported as follows:

pH 2.3, 4.3, 6.4, 8.2, 10.5 and 11.4
rd 66, 66, 103, 88, 69 and 18

The alkaloids were identified in filtered UV or by spraying with various alkaloidal reagents.

8.7.3 Thin Layer Chromatography (TLC)

Many TLC systems have been reported for the identification of hyoscyamine and other tropane alkaloids. Several of these systems are presented in table 6.

The USP (3) recommends that foreign alkaloids and other impurities in hyoscyamine can be detected by TLC technique using 0.5 mm layers of silica gel and chloroform, acetone, diethylamine (5:4:1) as the solvent system.
TLC has also been used for quantitative determinations of the tropane alkaloids. Belladonna alkaloids can be determined by quantitative TLC (Samples of *Belladonna* are extracted according to the method of Pharm. Helv. V (Swiss Pharmacopeia V). The alkaloids so extracted are then separated on kiesel gel G plates with the solvent acetone-aqueous ammonia (19:1). After development, the chromatograms are dried, sprayed with Dragendorff's reagent and the areas of the spots are measured.
The results obtained by this procedure agree with those obtained by the modified Pharm. Helv. V methods (116).

Hyoscyamine and scopolamine can be determined by direct photodensitometry on thin layer chromatograms (117).
After extraction of alkaloids from powdered hyoscyamus (4 g) and purification, an aliquot is applied to 0.5 mm layers of silica gel G which developed for 1 hour with the solvent ahydrous methanol-aqueous ammonia (200:1). After development, the plates are dried at room temperature, sprayed with modified

Table 6 : TLC of Hyoscyamine

	Chromatogram	Solvent System	R_f	Ref.
1.	Silica gel G (0.25 mm layers) acidified iodoplatinate spray reagent	Methanol-strong ammonia (100:1.5)	0.18	(9,112)
2.	Silica gel G 250 µm thick, dipped in 0.1M KOH in methanol	Chloroform-methanol (90:10)	0.18	(6)
3.	Silica gel G layers	Chloroform-acetone-diethylamine (5:4:1)	0.38	(113)
4.	" "	Chloroform-diethylamine (9:1)	0.40	(113)
5.	" "	Cyclohexane-chloroform-diethylamine (5:4:1)	0.16	(113)
6.	Silica gel GF_{254}	Acetone-water-25% ammonia soln (90:7:3)	0.24	(114)
7.	Aluminium oxide G. layers	Cyclohexane/$CHCl_3$ (7:3) + 0.05% diethylamine 3 drops/100 ml	0.10	(113)
8.	Basic silica gel G.	Methanol	0.17	(113)
9.	Silica gel GF_{254} layers	Chloroform-acetone-ammonical ethanol (5:4:1)	0.45	(98)
10.	Alkaline silica gel G layer dipped in 0.5N KOH	70% Ethanol-25% ammonia (99:1)	0.22	(115)

Dragendorff's reagent. The spots are scanned at *490 nm* with a recording and integrating densitometer.
The integrator count is a rectilinear function of the weight of alkaloid over the range of 4 to 65 µg for hyoscyamine and 7 to 60 µg for scopolamine. The results agreed well with those obtained by the spectrophotometric method of Freeman (78).

Tropane alkaloids in galenical preparations *(syrups, tablets, suppositories)* can be determined colorimetrically after separation on TLC plates (118).
The alkaloids are extracted with ammonical ethyl-ether-chloroform mixture. The organic layer of the extract is evaporated and dissolved in ethanol. This is applied into thin layers of silica gel GF_{254} which developed in the solvent ethanol-water-triethanolamine (5:5:2). After development, the zones are scraped from the plates and dissolved in dilute nitric acid. The acid solution is evaporated and the resulting residue is dissolved in anhydrous ethanol-acetone (3:97), 3% methanolic KOH is added and the extinction of hyoscyamine at *575 nm* is measured within 1 minute. The concentration of the substance is determined by reference to a calibration graph.

Determination of l-hyoscyamine plus atropine from belladonna by TL chromatographic technique has been described (119).
Extraction of alkaloids was effected with warm 0.1 N H_2SO_4. After filtration, the filterate was adjusted to pH 9-10 with aqueous NH_4Cl-NH_3 buffer. Purification was done through Extrelut column and eluted with $CHCl_3$. The concentrated eluate was applied to TLC plates coated with silica gel F_{254} which developed in the solvent system acetone-water-25% ammonia solution (90:7:3). After development, the plates were immersed in Dragendorff's reagent and scanned by densitometry *at 520 nm.*

A combined TLC and UV determination method for atropine and hyoscyamine was reported (98). The test solution (150-250 µl) containing 1% of the alkaloid (atropine or hyoscyamine) was applied as 8 cm long bands to 0.25 mm thick layers of silica gel GF_{254} plates. The chromatograms were developed for 12 cm with a mixture of $CHCl_3$-acetone-ammonical ethanol (5:4:1) at pH 12.4. The bands at Rf 0.44 (vissible under 254 nm radiation) were scraped off, placed on G_5 filters and extracted with 0.1 N HCl (2 ml) followed by filtration. The extraction was repeated 4 times and the combined extracts were made up to volume with HCl (10 ml). The absorbance of this was recorded in 3. cm cell *at 238 to 320 nm* and the amount of each alkaloid was then calculated.

A comparison between TLC-densitometry and HPLC for the determination of hyoscyamine and scopolamine in Datura leaves, fruits and seeds has been studied (120).
The alkaloids were extracted into ethanol and purified on a column chromatography of diatomaceous earth moistened with 2.5 M H_2SO_4. Non-alkaloidal materials were first eluted with ethylether, alkaloids were then eluted with $CHCl_3$ saturated with aqueous ammonia. After evaporation of eluate, a solution of the residue in chloroform was submitted to TLC plates of silica gel 60 F_{-254} which developed in the solvent trichloroethane-diethylamine (9:1). After drying at 105° for 2 hours, the plates were sprayed with a solution of 4-dimethylaminobenzaldehyde in ethanol-4 M H_2SO_4 (1:1) and again dried at 105° for 60 minutes. Hyoscyamine and scopolamine spots were determined with the use of high-speed scanner *at 495 nm*. The calibration graph was rectilinear for 0.2 to 2.0 mg ml^{-1} of alkaloid, and the detection limit was 50 ng. The method was rapid and comparable with

HPLC method on a column of Zorbax TMS or Lichrosorb RP-2 (at 40°) with detection *at 259 nm.*

Other TLC systems have also been reported (121-125).

8.7.4 Gas Liquid Chromatography (GLC)

Many GLC systems have been reported for the identification and quantitation of tropane alkaloids either in crude drugs or in pharmaceutical formulations.
System I: This system is recommended to determine hyoscyamine in tablets by GLC method (126).
Extraction: Tablets containing 0.3 mg of hyoscyamine (I) were powdered, made alkaline with aqueous ammonia and ground with enough diatomaceous earth to produce a flowing powder which was packed into chromatographic tube. Hyoscyamine was eluted with chloroform. The eluate was washed with 0.01 N H_2SO_4 and the aqueous acid phase was adjusted to pH 11 with conc. ammonia solution and extracted with chloroform. The extract was reduced to small volume.
An aliquot of this together with homatropine HBr (II) and ethyl morphine (III) (as internal standards) in chloroform was analyzed by GLC.
Column: A glass (1 m x 4 mm) packed with 3% OV-17 on Chromosorb W AW-DMCS (80-100 mesh) and operated at 225°.
Carrier Gas: Nitrogen (90 ml min^{-1}).
Detection: Flame ionization detector.
Calculation: Retention times were measured and the hyoscyamine content was calculated from the peak area relative to that of II or of III. For 0.37 mg ml^{-1} of hyoscyamine (I), the accuracy was 95.8% with II as internal standard and 96.3% with III as internal standard (n=13). The coefficient of variation was ± 2.6%.

System II: The following GLC method was reported for selective determination of belladonna alkaloids (hyoscyamine, atropine and scopolamine) in phar-

maceutical preparations in the presence of chlorpheniramine and phenyl propanolamine (127).

Procedure: Sample capsule contents (20 µg alkaloid) were mixed with 0.01 N H_2SO_4, 0.05% homatropinium bromide solution in 0.01 N H_2SO_4 was added (2 ml) as internal standard, the resulting mixture was diluted with the acid solution (50 ml) and centrifuged at 2000 r.p.m. (for 15 minutes).

The supernatant liquid (25 ml) was washed first with chloroform, then after adjusting to pH 7.9-8.1 with phosphate buffer, was again washed with cyclohexane. After washings, the pH of the aqueous phase was adjusted to 9.0 and extracted with dichloromethane and this was evaporated to dryness under nitrogen. The residue was dissolved in dichloromethane (0.9 ml), an aliquot of this (10µl) was submitted into GLC.

Column: A glass column (4 ft x 4 mm) containing 3% OV 17 on Gas-Chrom Q (80-100 mesh) operated at 210°.

Carrier Gas: Helium (50 ml min^{-1}).

Detection: Flame ionization.

Calculation: Peak heights were measured and the alkaloid content were calculated. Atropine and hyoscyamine have the same retention time.

The mean recoveries and standard deviations (n=6) were 100.2 ± 0.8% for hyoscyamine plus atropine and 10.5 ± 3.7% for scopolamine.

System III: The following system was reported for quantitative determination of scopolamine and atropine plus hyoscyamine in unit doses of tablets and elixirs (128).

Extraction: A unit dose form containing 6 µg scopolamine 100 µg atropine plus hyoscyamine was extracted. The extract and washing were evaporated to 0.1 ml and 1 to 2 µl were submitted to the system.

Column condition: A glass column (60cm x 3mm) containing 3% OV-17 on Gas Chrom Q (80-100 mesh), maintained at 210°.

Carrier gas: Helium (60 ml/min^{-1}).

Detection: Flame ionization.
Calculation: The peak height ratio was measured and the concentration of each alkaloid was calculated from a calibration graph.
The co-efficient of variations (10-determinations) were : for scopolamine ± 4.8%, for atropine plus hyoscyamine ± 2.5%.

System IV: This system has been described as a simplified quantitative analysis of atropine and other alkaloids in scopolia extract. The method is also applied to gastrointestinal drugs of these alkaloids (129). The alkaloids are separated as their trimethylsilyl derivatives. Diphenhydramine is used as internal standard.
Column condition: A column (1 m x 3 mm) packed with 0.75% of Dexsil 300 GC on Gas-chrom Q, column temperature was maintained at 180°.
Carrier gas: Nitrogen (40 ml min^{-1}).
Detection: Flame ionization.
Calculation: The calibration graphs (peak-height ratio vs alkaloid content) was rectilinear for 25 to 75 ng of atropine and 2.5 to 7.5 ng of scopolamine.

System V: This GLC system is the USP method to assay hyoscyamine tablets, hyoscyamine sulfate elixir and other formulations (3).
Extraction: Weigh and finely powdered not less than 20 hyoscyamine tablets. Transfer an accurately weighed portion of the powder, equivalent to about 0.43 mg of hyoscyamine, to a separator containing 5 mL of pH 9.0 buffer, and add by pipet, 2.0 mL of internal standard solution (25 mg of homatropine HBr in 50 mL water), adjust with 1N sodium hydroxide to a pH of 9.0. Extract with 2 x 10 mL portions of methylene chloride, filter the organic extracts through 1 g of anhydrous sodium sulfate supported by a small cotton plug in a funnel into 50-mL beaker and evaporate

under nitrogen to dryness. Dissolve the residue in 2.0 mL of methylene chloride and apply into the following GLC system.
Column: A chromatographic glass column (1.8m x 2mm) packed with 3% liquid phase G2 on support S1 AB (cured as directed in the USP). The column is maintained at 225°.
Carrier Gas: Nitrogen at a flow rate of 25 ml min^{-1}.
Procedure: Inject 1-µL portions of the assay preparation and the standard preparation (according to the USP) successively into the gas chromatograph. Measure the areas under the peaks for hyoscyamine and homatropine in each chromatogram. Calculate the ratio, Au, of the area of the hyoscyamine peak to the area of the internal standard peak in the chromatogram from the assay preparation, and similarly calculate the ratio A_s in the chromatogram from the standard preparation.
Calculate the quantity in mg of $C_{17}H_{23}NO_3$ in the portion of tablets taken by the formula (289.37/676.82) (W/10) (Au/As), in which 289.37 and 676.82 are the molecular weights of hyoscyamine and anhydrous hyoscyamine sulfate respectively and W is the weight in mg of USP hyoscyamine sulfate RS taken for the standard preparation.

Many other GLC have been reported for the analysis of hyoscyamine as well as other tropane alkaloids. Some of these systems are presented in table 7.

8.7.5 High Performance Liquid Chromatography (HPLC)

Many HPLC have been reported for the identification and quantitation of tropine alkaloids including hyoscyamine.

System I: The following HPLC system has been recommended for the detection and determination of hysocyamine, atropine and scopolamine in tinctures of solanaceous drugs (137).

Table 7 : The GLC of Hyoscyamine

Column condition	Carrier gas (Gas flow)	Detection	Recommended for: Retention time (tR) or Index (RI)	Ref.
1- A stainless steel (5 ft x 1/8 inch) packed with 5% SE-30 on (60-80 mesh) Chromosorb W AW, Column temperature 230°	Nitrogen (30.7 ml min^{-1})	Flame ionization Hydrogen (22 ml min^{-1})	The identification of tropane alkaloids t_R of hyoscyamine 0.59 (relative to codeine).	(9,130)
2- A glass (2m x 4mm) packed with 2.5% SE-30 on (80-100 mesh) Chromosorb G (acid washed and dimethyl-dichlorosilane treated) Temperature 100-300°.	Nitrogen (45 ml min^{-1})		Most thermally stable drugs including several alkaloids. RI for hyoscyamine is 2192. (Reference = n-alkanes with an even number of carbon atoms).	(6,131)
3- A glass (6 ft x 0.075 inch) packed with 2.5% SE-30S on acid washed silanized Chromosorb G (80-100 mesh). Temperature programmed from 150° to 275° at 6° per minute. Inlet port maintained at 315°	Helium (100 ml min^{-1})	Flame ionization	Determination of tropane alkaloids in powdered belladonna or stramonium. Peak areas are measured. The precision for each alkaloid is ±2.5% The method is quantitative for amounts in the range 2 µg to 53 mg.	(132)

(Table continues)

(continued)

4- Glass column (6 ft x 0.075 inch) containing 2.5% of SE-30/S on acid washed Chromosorb G DMCS (80-100 mesh). Temperature maintained at 200° for 6 minutes, then at 290° at 6° per minute.	Helium (100 ml min^{-1})	Flame ionization	Quantitative determination of hyoscyamine and scopolamine. Peak areas are measured.	(133)
5- A glass column (150 cm x 4 mm) packed with 1.5% of SE-30 on Chromosorb W (80-100 mesh) and operated at 194°	Helium (190 ml min^{-1})	Flame ionization	Quantitative analysis of hyoscyamine in *Duboisia myoporoides* and in *D. leichhardtii*. Peak areas are measured.	(134)

Other GLC techniques have also been reported (91,135,136).

Extraction: The alkaloids were extracted from the tincture sample with ethylether-chloroform. The extract was evaported to dryness and the resulting residue was dissolved in methanol for analysis by HPLC.
Column: A column (30 cm x 3.9 mm) packed with μ-Bondapak C_{18} (10 μm).
l-Hyoscyamine and scopolamine HBr were used as the external standards.
Mible phase: 3% Aqueous acetic acid-methanol (7:3) for detection and (3:1) for determination (1 ml min^{-1}).
Detection: UV at 254 nm.
The calibration graphs were rectilinear for < 2 mg ml^{-1} and the coefficient of variations (n=6) were 0.5 to 1.7%.

System II: The following is a reversed phase ion-pair HPLC system described for the separation and determination of tropane alkaloids in crude drugs (138).
Extraction: A sample (0.5 g) was extracted with chloroform (10 ml) which contained 25% aqueous ammonia (0.15 ml). A portion of the organic extract (2 ml) was evaported to dryness and the residue dissolved in 0.02% benzylamine hydrochloride in methanol (1 ml) as an internal standard.
The resulting solution was diluted to 5 ml with the mobile phase for analysis by HPLC.
Column: A column (15 cm x 4 mm) packed with TSK gel 120 A ODS (5 μm).
Mobile phase: 66.7 mM sodium phosphate (pH 3.5)-methanol (12:13), containing 17.5 mM sodium dodecyl sulfate (35°, 1 ml min^{-1}).
Detection: UV at 210 nm.
Notes: *Calibration graphs were rectilinear from 20 to 100 μg ml^{-1}.*
Detection limit : 7.5 to 10 ng.
Recoveries were 98.1 to 106.4% with coefficient of variation of 1.8 to 4.3%.

System III: This is also an ion-pair HPLC system for the analysis of hyoscyamine and scopolamine in solanaceous crude drugs (139).

Extraction: Hyoscyamine and scopolamine were determined by HPLC after direct extraction of the crude drugs with the mobile phase by heating under reflux or after fractionation of the alkaloids by extraction at alkaline pH with ethylether and back extraction with chloroform in the presence of H_2SO_4.
Column: A (15 cm x 4 mm) column of TSK gel 120 A ODS (5 µm).
Mobile phase: 0.067 M phosphate buffer (pH 2.5)-acetonitrile (13:7) containing 17.5 mM sodium dodecyl sulfate (1.5 ml min^{-1}) at 35°.
Detection: UV at 210 nm.
Notes: *The calibration curves were rectilinear for 1.5 to 70 µg ml^{-1} (of both alkaloids), detection limit was 10 ng.*

System IV: The following HPLC system was recommended for analysis and quantitation of belladonna alkaloids and can be applied to elixirs, tablets and capsules of these alkaloids containing phenobarbitone (140).
Extraction: The sample was treated with H_2SO_4 and mixed with CH_2Cl_2, after shaking, the organic layer was discarded. Carbonate buffer solution (pH 9.4) was added to the aqueous phase and the mixture was extracted with CH_2Cl_2. The organic phase was filtered, evaporated, theophylline (as internal standard) was added and the solvent evaporated to dryness. The residue was treated with MeOH-HCl and evaporated to dryness, the residue was re-dissolved in methanol and the methanolic solution was again evaporated to dryness. The final residue was dissolved in water and applied to the following HPLC system.
Column: A stainless steel (25cm x 4mm) of Spherisorb ODS (5µm).
Mobile phase: Water-methanol (21:10, pH 2.0) containing tetramethylammonium phosphate (0.8 ml min^{-1}).
Detection: UV at 220 nm.

The calibration graphs were rectilinear in the range of 0-0.5 mg ml^{-1} (hyoscyamine plus atropine), 0-0.32 mg ml^{-1} (scopolamine).
The coefficient of variation was 1.4% for scopolamine and 0.5% for hyoscyamine (n=6).
The detection limit was 0.02µg for each alkaloid.
Recovery of the compounds was 93.0 to 106.1%.

System V: This system is a combination of ion-pair and column switching HPLC to determine tropane alkaloids, mainly hyoscyamine, in complex preparations of gastrointestinal drugs using three pumps in the system (141).
Technique: The tropane alkaloid fraction immediately eluted by the primary mobile phase without sodium dodecyl sulfate (SDS) from a pretreatment column. The fraction was then transferred to an analytical column and separated by the ion-pair mobile phase. A tertiary pump was used to supply SDS to the trapped fraction in the loop after eluation the pretreatment column.
Columns: A pretreatment column (50mm x 4mm) and an analytijcal column (250mm x 4mm), both were packed with TSK Gel 120 A (5µm). The column temperature was maintained at 35°.
Mobile phases: The primary mobile phase for the pretreatment column was a mixture of 1/15 M sodium phosphate solution (adjusted to pH 3.5 with 1/15 M phosphoric acid) and methanol (48:52). The secondary mobile phase for the analytical column was the primary mobile phase containing 17.5 mM SDS. The tertiary mobile phase was the primary mobile phase containing 175 mM SDS. The flow rates of the primary, secondary and tertiary mobile phases were 0.9, 1.0 and 0.1 ml min^{-1} respectively.
Detection: UV detector at *210 nm. The calibration graphs were linear in the range 0.3 to 300 µg/ml for hyoscyamine (atropine). Hyoscyamine levels in model*

Table 8 : The HPLC of Hyoscyamine

	Column	Mobile Phase	Detection	Recommended for	Ref.
1.	A stainless steel (12.5cm x 4.9 mm) packed with silica (Spherisorb S_5W) (5μm)	Solution containing 1.175 g (0.01M) of ammonium perchlorate in 1000 ml methanol and adjusted to pH 6.7 by 1ml of 0.1M NaOH in methanol	UV/electrochemical	Identification of several drugs including hyoscyamine. *K'* value for hyoscyamine is 3.7	(6)
2.	A silica gel Si 100 (10cm x 4.6mm)	Chloroform saturated with picric acid (0.35 ml min^{-1})	UV	Identification and quantitation of hyoscyamine which was separated in < 15 minutes. Coefficient of variation 1.2%	(142)
3.	Two reversed-phase columns, each (122cm x 2.3mm) one was packed with Corasil C_{18} and the other with Corasil phenyl	-Absolute methanol- 1% $NH_4H_2PO_4$ (3:2,1:1,2:3) -Absolute methanol- 1% $NH_4H_2PO_4$-1% $(NH_4)_2HPO_4$ (3:1:1,2:1:1,4:3:3) -Absolute methanol-0.5% $(NH_4)_2CO_3$(3:2,1:1,2:3) (1.4 ml min^{-1})	UV at *254 nm*	Determination of eight antispasmodic mixtures including hyoscyamine sulfate. Each determination can be achieved in approximately 15 minutes with an accuracy of 1-2%.	(143)
4.	A column (10cm x3mm) packed with silica gel of pore size 100 nm coated with 0.06 M picric acid in citric acid buffer solution of pH 6.0	Chloroform saturated with the buffer. (The picrate ion-pair of alkaloids were injected as solutions in $CHCl_3$)	Spectroscopically monitored	Identification and separation of hyoscyamine, scopolamine and ergotamine	(144)

5. Reversed phase column (10cm x3 mm) chemically bonded (10μm) silica gel of the diol and CN type Li Chrosorb	25% methanol in water adjusted to pH 3.5 with 0.1N NaH_2PO_4	Fluorigenic ion-pair extraction detector	Analyiss of basic drugs including hyoscyamine as well as pesticides	(145)
6. A stainless steel (1m x 4.6mm) dry packed with Sil-X adsorbent	A mixture of ammonium hydroxide (28% NH_3 by weight in water) and tetrahydrofuran (1:100 v/v).	Differential RI (refractive index) having a range of 1.30 to 1.45 RI units and highly sensitive UV *at 254 nm*	Separation and quantitative determination of of tropane alkaloids including hyoscyamine. The average deviation of concentration range 5-50 μg was within ± 1% for all alkaloids	(146)
7. A column (25cm x 4.6 mm) of Li Chrosorb DIOL	Aqueous phosphate buffer	Fluorimetric ion-pair technique	Determination of amine drug including hyoscyamine The limit of detection for hyoscyamine was 200 pg.	(100)

Other HPLC have also been reported (147-150).

preparations of gastrointestinal drugs determined by this column switching system were with the recovery of about 97%.

Many other HPLC systems have been reported for identification and quantitation of hyoscyamine and other tropane alkaloids. Several of these are presented in the following table 8.

8.7.6 Gas Liquid-Mass Spectroscopy

Belladonna alkaloids including hyoscyamine have been analyzed by capillary GC and GC-mass selective detection (MSD) (151).
The retention data and characteristic ions of the alkaloids and their trimethylsilane (TMS) derivatives are given. Derivatization with different silylation reagents was compared. N-methyl-N- (trimethylsilyl) trifluoroacetamide was a better reagent than *bis* (trimethylsilyl) acetamide or *bis* (trimethylsilyl) trifluoroacetamide).

8.8 Immunoassays

8.8.1 Radio-immunoassays (RIA)

A radio-immunoassay method for the determination of atropine and hyoscyamine in blood plasma or blood serum has been reported (152).
An antiserum was raised by immunization of rabbits with an immunogen prepared by *coupling hyoscyamine to human serum albumin* and using [^{3}H]-atropine as tracer. Atropine and hyoscyamine reacted to equal extents with the antibodies. Some structurally related drugs e.g. homatropine or scopolamine as well as atropine hydrolysis products (tropine and tropic acid) did not interfere in the assay.
It was possible to detect down to 9 n M atropine or hyoscyamine in 0.01 ml of serum or plasma.

The recovery of atropine (hyoscyamine) added to various concentrations to pooled normal human plasma was near 100%.

A rapid and sensetive RIA procedure has been developed for the quantitation of atropine and hyoscyamine from unpurified ethanolic extracts of *Atropa belladonna* (153).
The antiserum used was highly specific for d-hyoscyamine (the unnatural enantiomer). A racemization step (converting l-hyoscyamine into atropine) had to be included in the assay procedure. There was practically no interference in the assay from other compounds present in the extracts.

8.8.2 Enzyme immunoassays

Competitive solid-phase enzyme immunoassay for the evaluation of tropane alkaloids in plant materials using *anti-dl-tropic acid antibodies* has been reported (154).

8.9 Radioligand Assay

Anticholinergic drugs including hyoscyamine indicating that their incubation with muscarinic receptor at 0° before and after addition of the radiolabeled ligand [^{3}H]-dexetimide can provide lower detection limits (by a factor of 2.5 to 9) with coefficient of variations 3 to 9% (155).

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INDAPAMIDE

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1. INTRODUCTION

1.1 Therapeutic Category

Indapamide is a mild diuretic and antihypertensive agent containing both a polar sulfamoyl chlorobenzamide moiety and a lipid-soluble methylindoline moiety (1). It is the lipid soluble moiety which distinguishes the activity of indapamide from other diuretics (2). Indapamide has been shown to be a potent long acting antihypertensive agent when used alone or along with other therapeutic agents (2). Indapamide has been successfully combined with beta-blockers, methyldopa and other antihypertensive reagents (3).

Once-daily administration of 2.5 mg of indapamide has shown the drug to be a safe and effective agent to use in lowering blood pressure of hypertensive patients with normal renal function as well as patients with various degrees of renal impairment and those undergoing long-term maintenance hemodialysis (4). Results from clinical trials indicate that indapamide effectively reduces arterial blood pressure in approximately two-thirds of patients with mild to moderate hypertension, is well tolerated and does not induce biochemical abnormalities that constitute cardiovascular risk factors (3). Indapamide has also been shown to produce a clinically significant decrease in edema (5) as well as a reduction in total peripheral resistance (6).

1.2 History

The sulfonamides were discovered in Germany in the middle of the twentieth century subsequent to investigation of diazo dyes (7,8). There have been a number of structural modifications of sulfonamides producing numerous medicinal agents including antibiotics, thiazide diuretics and carbonic anhydrase inhibitors (8). One modification of the sulfonamides involving the addition of indoline was responsible for the production of indapamide. The first patent and USA patent citations may be found in References 9 and 10, respectively. One of the first non-patent literature references to indapamide appeared in 1974 (11). A search of the literature from 1966-1992 yielded over 330

citations for works dealing with analysis and pharmacologic effects of indapamide.

2. DESCRIPTION

2.1 Chemical name, Formula, Molecular Weight

The accepted chemical name for indapamide is 3-(aminosulfonyl)-4-chloro-N-(2,3-dihydro-2-methyl-1H-indol-1-yl)benzamide. The CAS registry number is 26807-65-8. The chemical structure is shown in Figure 1. The molecular formula is $C_{16}H_{16}ClN_3O_3S$ and its molecular weight is 365.84 g/mole.

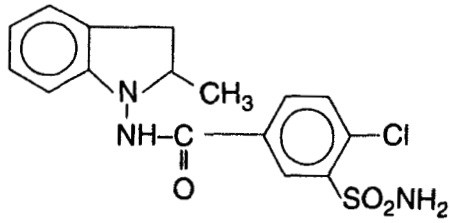

Figure 1. Chemical structure of indapamide.

2.2 Dosage Form Names

Dosage form names include Bajaten, Damide, Fludex, Indaflex, Indamol, Ipamix, Lozol, Natrilix, Noranat, Pressural, Tandix and Veroxil.

2.3 Appearance, Color

Indapamide is a white to yellow-white crystalline powder consisting of tetragonal crystals.

3. SYNTHESIS

Indapamide is synthesized by a six step process (12). The synthetic scheme is shown in Figure 2. In step I, 2-methylindole is reduced to 2-methylindoline and consequently converted into N-amino-2-methylindoline hydrochloride in step II via the N-nitroso intermediate. The product of Step II is then saved for reaction in Step VI. Step III entails the production of 3-chlorosulfonyl-4-chlorobenzoic acid from the reaction of chlorosulfonic acid with p-chlorobenzoic acid. Next, 3-chlorosulfonyl-4-chlorobenzoic acid is taken to 3-sulfamoyl-4-chlorobenzoic acid (Step IV) and to 3-sulfamoyl-4-chlorobenzoyl chloride (Step V). Step VI entails the reaction of the products from Step II and Step V to give the final product. The synthesis of other diuretics related to indapamide has also been described (13,14,15).

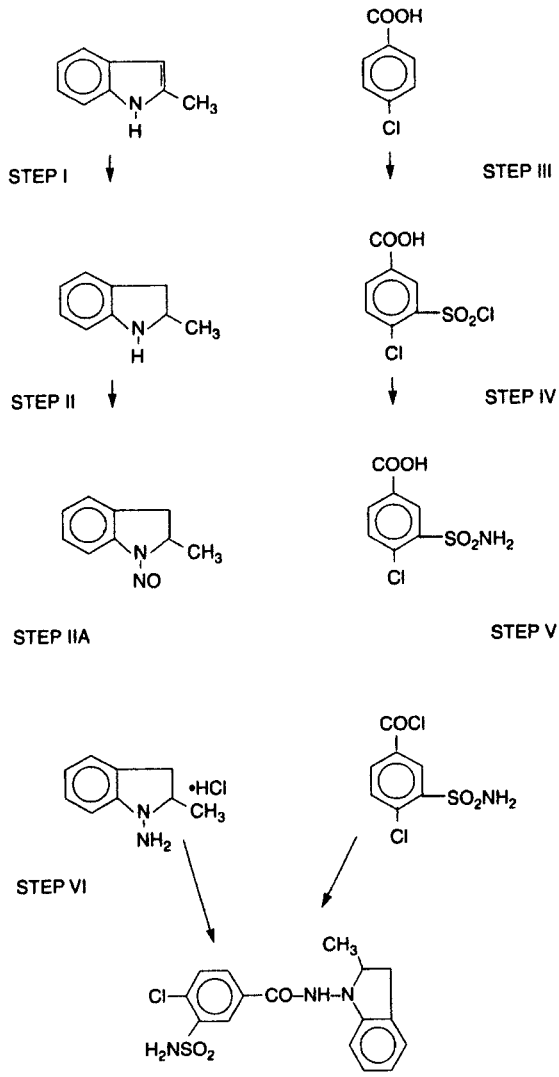

Figure 2. General scheme for the synthesis of indapamide.

4. PHYSICAL PROPERTIES

4.1 Infrared Spectrum

The infrared spectrum of indapamide in a potassium bromide dispersion is shown in Figure 3 (16). A Nicolet model 740 FTIR spectrometer was used to acquire the spectrum. Frequency assignments for some characteristic bands are listed in Table I (17).

TABLE I

Vibrational Assignments for Selected Indapamide Infrared Absorptions

Frequency (cm^{-1})	Relative Intensity*	Assignment
3654	m	O-H stretch
3504	m	O-H stretch
3315	w	amide N-H stretch
3224	s	amide N-H stretch
1660	vs	amide C=O stretch
1599	s	aromatic C=C
1341	vs	antisymmetric SO_2
1301	m	C-N stretch
1257	m	aromatic C-H in-plane
1176	vs	antisymmetric SO_2
753	s	aromatic C-H out-of-plane

*m=medium; s=strong; v=very; w=weak.

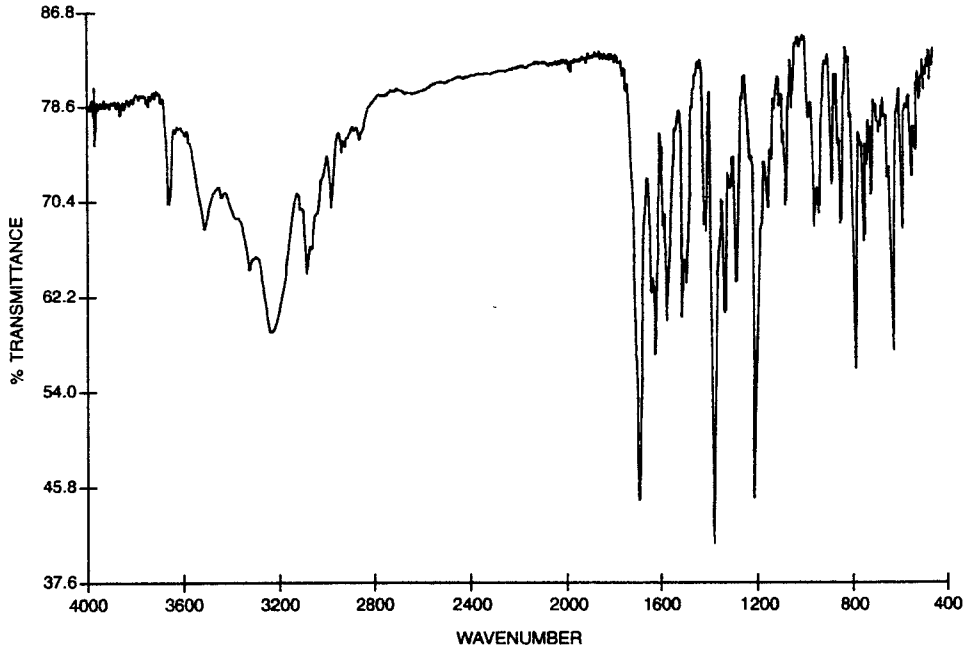

Figure 3. Infrared spectrum of indapamide taken in a KBr disk.

4.2 Nuclear Magnetic Resonance Spectrum

All spectra were obtained using a Varian VXR 200 Fourier transformer NMR Spectrometer (18). The NMR spectra presented are consistent with the chemical structure of indapamide.

4.2.1 Proton NMR

The proton magnetic resonance spectrum of indapamide was obtained from an approximately 50 mg/mL solution using deuterated methanol as the solvent. The spectrum is shown in Figure 4 and spectral assignments are listed in Table II. Figure 4 also shows the structural lettering applied to indapamide to facilitate proton NMR spectral assignment. A listing of instrument parameters used in obtaining this spectrum is provided in Table III.

TABLE II

Proton NMR Spectral Assignments of Indapamide

Proton	Chemical Shift (ppm)
a	exchangeable
b	8.58, 8.59
c	8.01-8.05
d	7.66, 7.71
e	6.57-7.11
f	3.83
g	2.64 - 3.20
h	1.36, 1.40

TABLE III

Parameter Table for the ^1H NMR Spectrum of Indapamide

Observe:

Nucleus	1.250	
Spec. Width	4000.0	Hz
Acq. Time	2.000	sec
Pulse Width	7.0	μsec
Freq.	200	MHz
Offset	800	Hz
Delay	1.000	sec
Transients	64	

Decouple:

Nucleus	1.250	
Mode	NNN	
Modulation:Mode	C	
Offset	-43.9	Hz
Power	20	db
Freq.	200	Hz

Plot/Processing:

FN	16	K		
Width	2040.8	Hz/ppm	Start	-40.0 Hz/ppm

Experiment:

Pulse Sequence	STD1H
Solvent	CD_3OD

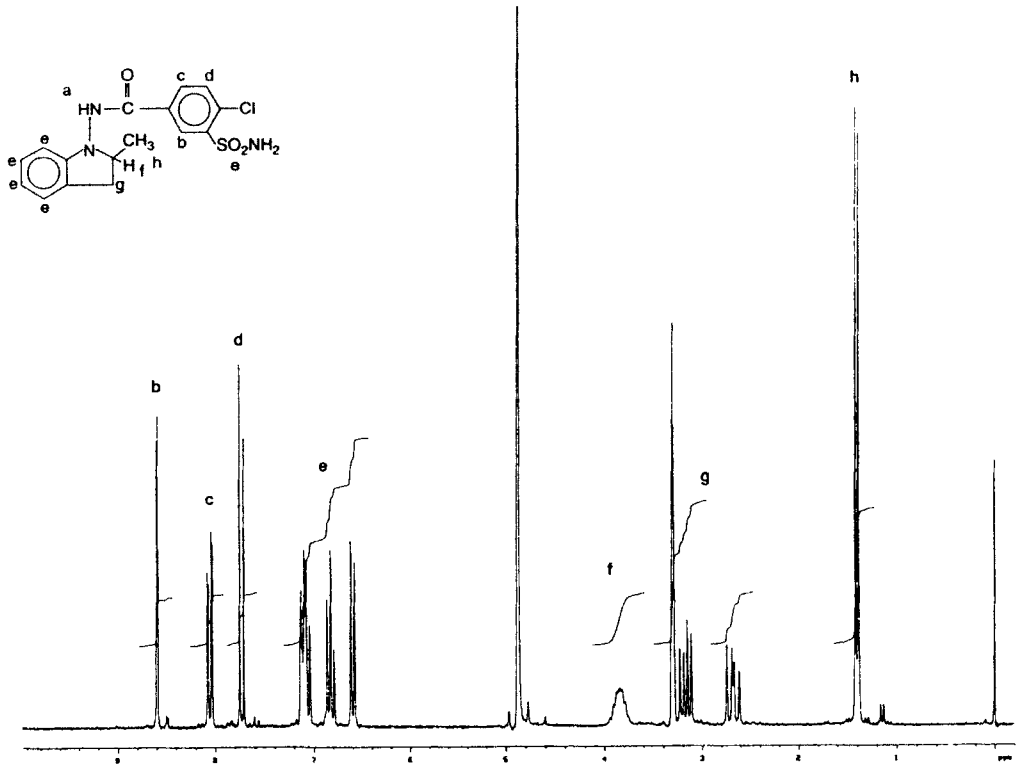

Figure 4. Proton NMR spectrum of indapamide.

4.2.2 Carbon Magnetic Resonance Spectrum

The carbon-13 NMR spectrum shown in Figure 5 was obtained using a 200 mg/mL solution of indapamide in deuterated methanol. Figure 5 also shows the structural numbering of indapamide to facilitate carbon NMR spectral assignments. The carbon-13 NMR spectrometer parameters and tentative spectral assignments are listed in Tables IV and V, respectively.

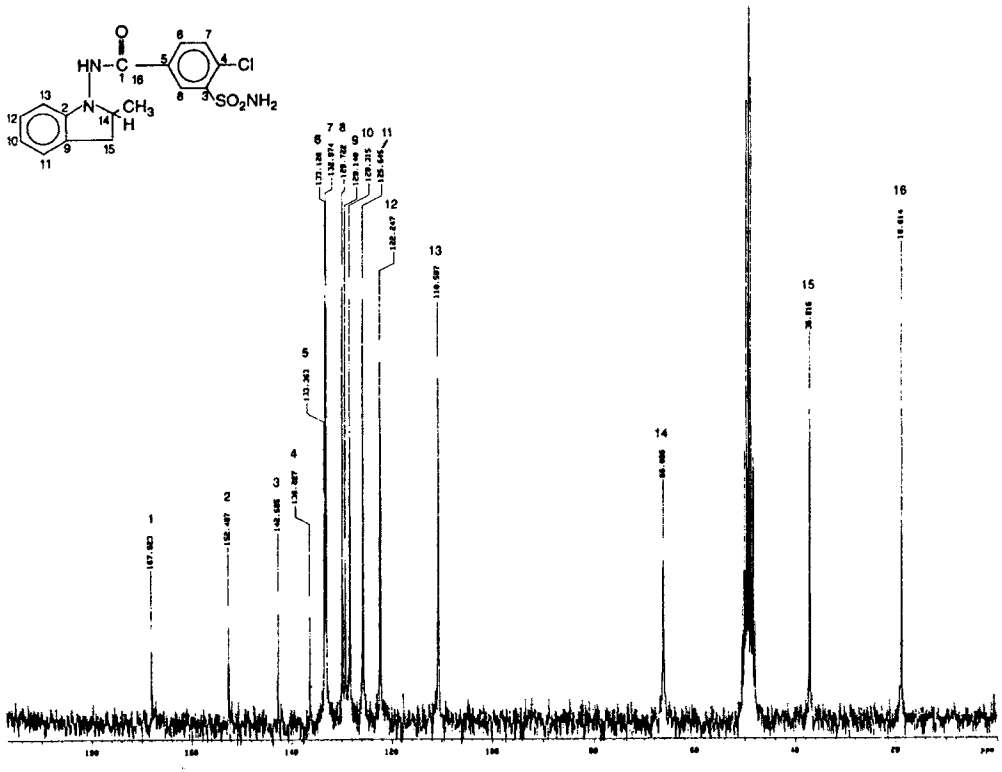

Figure 5. Carbon 13 NMR spectrum of indapamide.

TABLE IV

Parameter Table for ^{13}C Spectrum of Indapamide

Observe:

Nucleus	13.250	
Spec. Width	10000.0	Hz
Acq. Time	0.400	sec
Pulse Width	6.0	μsec
Freq.	50	MHz
Offset	0	Hz
Delay	2.000	sec
Transients	1536	

Decouple:

Nucleus	1.250	
Mode	YYY	
Modulation:Mode	S	
Pulse Width	13.0	μsec
Offset	-43.9	Hz
Power	5	db
Freq.	9900	Hz
Power Mode	64.0	

Plot/Processing:

FN	8	K		
LB	2.000	Hz		
Width	10000.0	Hz/ppm	Start -91.6	Hz/ppm

Experiment:

Pulse Sequence	STD13C
Solvent	CD_3OD

TABLE V

^{13}C NMR Spectral Assignments of Indapamide

Carbon No.	Chemical Shift (ppm)
1	167.94
2	152.48
3	142.59
4	136.23
5	133.36
6	133.14
7	132.96
8	129.73
9	129.14
10	128.32
11	125.64
12	122.24
13	110.59
14	66.09
15	36.83
16	18.61

4.3 Ultraviolet-Visible Spectrum

The ultraviolet-visible spectrum for indapamide was obtained with a 1-cm cell using a solution with a concentration of 8 micrograms/mL with methanol as a solvent (12). The spectrum (Figure 6) exhibits characteristic absorption bands with maxima at 242 nm (A 1% = 630), 278 nm (A 1% = 98) and 286 nm (A 1% = 100). Aqueous solutions of indapamide (17) display an increase in absorptivity at approximately 242 nm with an increase in pH.

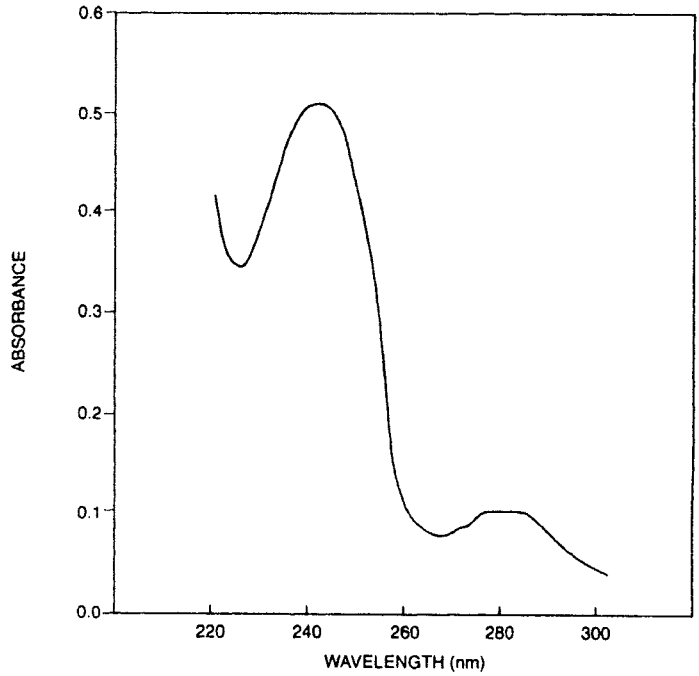

Figure 6. Ultraviolet absorption spectrum of indapamide in methanol.

4.4 Mass Spectra

The electron impact (EI) and fast atom bombardment (FAB) mass spectra of indapamide were obtained on a VG70-SE mass spectrometer with the resolution tuned to approximately 1000 (19). Using 3-nitrobenzyl alcohol as a matrix, the FAB spectrum shows the protonated ions of m/z 366 and 368 and molecular ions of 365 and 367, an isotope pattern consistent with the presence of one chlorine atom (Figure 7). The 70 eV EI spectrum shows the major fragment ions of m/z 218, 147 and 131, consistent with the structure of indapamide (Figure 8). The structures of the major fragment ions (17) are given in Figure 9.

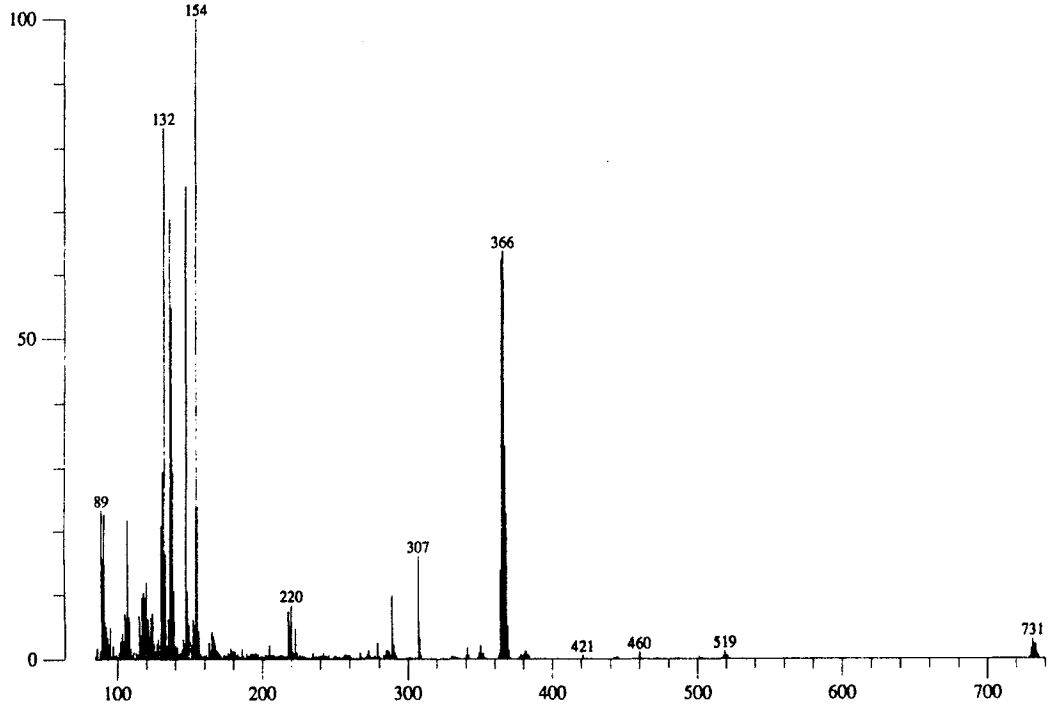

Figure 7. Fast atom bombardment mass spectrum of indapamide.

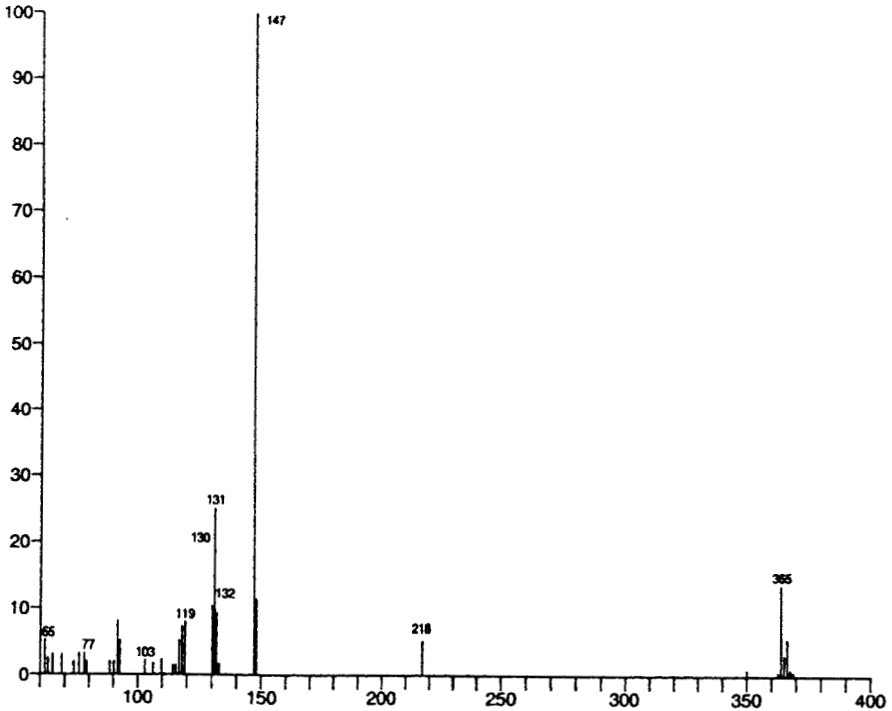

Figure 8. Mass spectrum of indapamide in the electron impact mode.

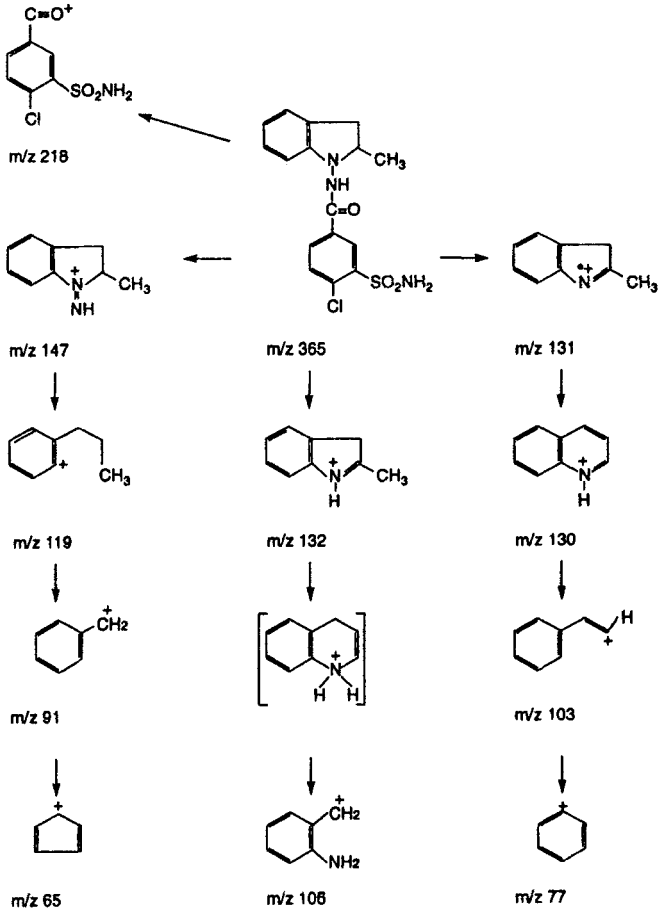

Figure 9. Principal mass spectral fragmentations of indapamide.

4.5 Thermoanalytical Behavior

4.5.1 Melting Point

The melting point of indapamide varies between 160-162°C as determined by the USP class Ia capillary method.

4.5.2 Loss on Drying

Weight loss is typically less than 3% for an accurately weighed sample of indapamide heated to constant weight at 105°C.

4.5.3 Differential Thermal Analysis Behavior

The differential thermal analysis (DSC) behavior of indapamide is shown in Figure 10 (20). The thermogram was obtained using a Perkin Elmer Series 7 DSC scanning from 40°C to 220°C at 10°C/minute. A primary endotherm corresponding to melting is observed at a peak onset temperature of 165°C.

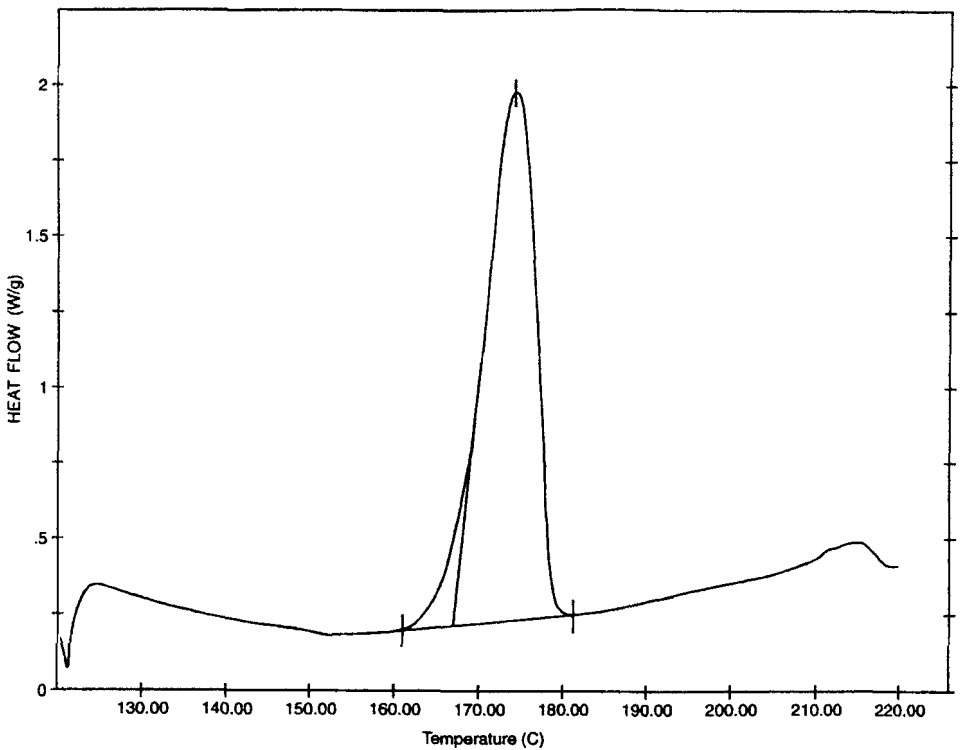

Figure 10. Differential thermoanalytical behavior of indapamide.

4.5.4 Thermogravimetric Analysis Behavior

Thermogravimetric analysis (TGA) of indapamide indicates weight loss of 1.6% from 40 to 100°C. A typical TGA curve is shown in Figure 11. The curve was obtained using a Perkin Elmer Series 7 TGA scanning from 40°C to 370°C at 10°C/minute (20). Significant weight loss is observed above 250°C due to decomposition/vaporization.

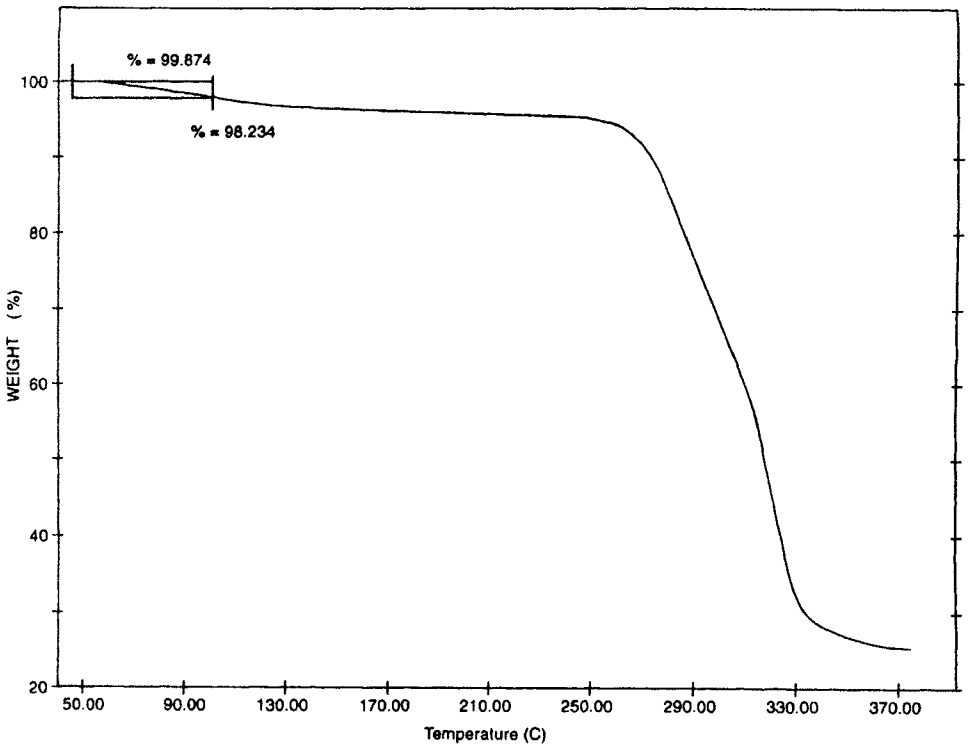

Figure 11. Thermogravimetric behavior of indapamide.

4.5.5 Residue on Ignition

Typically <0.1% residue is obtained when a sample (1 gram) of indapamide is ignited in a platinum crucible in the presence of concentrated sulfuric acid in a muffle furnace at 800°C.

4.6 Solubilities

Indapamide is soluble in methanol, acetic acid and ethyl acetate; it is very slightly soluble in chloroform.

The equilibrium solubilities of indapamide in aqueous buffer solutions over the pH range of 1 through 10 are shown in Table VI and Figure 12 (12). From the data it can be seen that the aqueous solubility of the compound is 0.1 mg/mL or less at pH values of 8 or below. The rapid increase in solubility beginning at about pH 8 is the result of the formation of the more soluble ionized species.

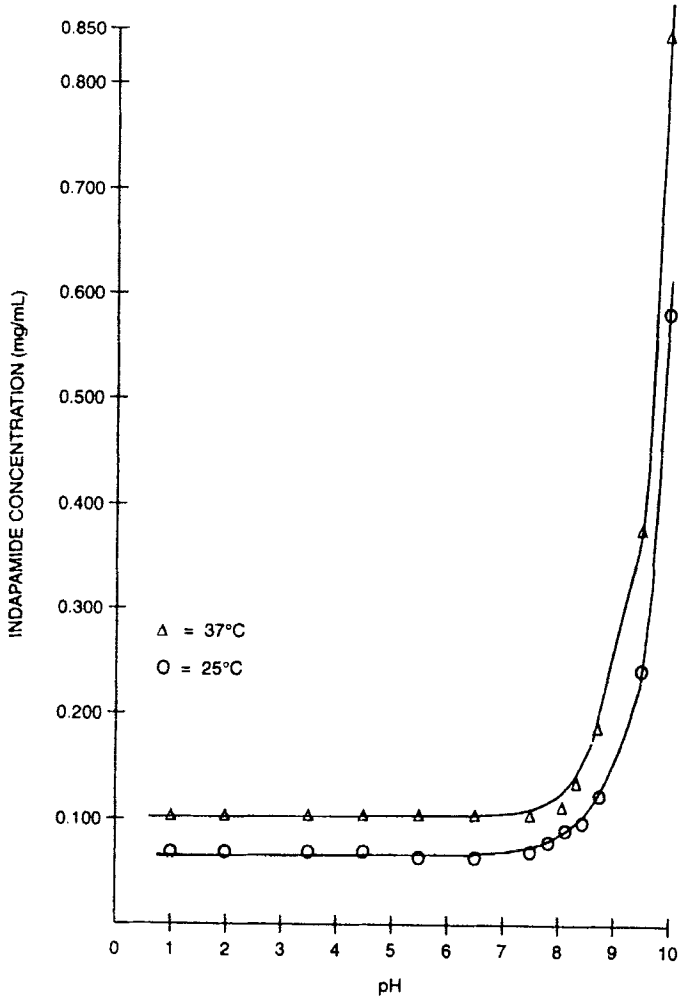

Figure 12. Aqueous pH solubility plot of indapamide.

TABLE VI

pH Solubility Profile of Indapamide at Various Temperatures

pH	Solubility at 25°C (mg/mL)	Solubility at 37°C (mg/mL)
1.0	0.063	0.095
2.0	0.061	0.094
3.5	0.061	0.093
4.5	0.059	0.091
5.5	0.061	0.094
6.5	0.059	0.097
7.5	0.063	0.098
7.8	0.069	---
8.0	0.076	0.103
8.4	0.088	0.130
8.7	0.111	0.178
9.3	0.231	0.370
9.9	0.573	0.860

4.7 Crystal Properties and X-Ray Powder Diffraction

No evidence of polymorphism has been seen from microscopic and thermal analysis of various lots of indapamide (12). Studies have indicated that the compound is capable of forming non-stoichiometric clathrate-type solvates with such solvents as dichloromethane and methanol. Such clathrates will form a glassy (amorphous) state following solidification from the molten state. Material in the amorphous state exhibits an enhanced initial solubility, a behavior typical of an amorphous form.

A Philips diffractometer with graphite-monochromated CuKα radiation was used (37). The wavelength was 1.5406 Ångstroms. Table VII displays the X-ray diffraction data for indapamide. Figure 13 shows the X-ray diffraction pattern of indapamide.

4.8 Dissociation Constant

In the case of poorly water-soluble substances, determination of pKa by acid-base titration is not always possible. In the case of indapamide, the pKa value has been estimated based upon pH-solubility data (21). Indapamide behaves as a weak monoprotic acid, exhibiting a pKa of 8.8 ± 0.2 at 25°C. Potentiometric titration of indapamide in dimethylformamide with tetrabutylammonium hydroxide shows two inflection points. The first inflection point has been assigned to the ionization of the sulfonamide group. The second inflection point has been assigned to ionization of the amide function.

4.9 Distribution Constants

The distribution constant for indapamide was determined in chloroform/water and octanol/water systems at 25°C for various pH values of water (17). The results are indicated in Table VIII.

TABLE VII

X-Ray Powder Diffraction Pattern of Indapamide

2θ	dÅ	I/I_o
7.400	11.9364	5.6
10.334	8.5530	1.3
10.891	8.1170	13.0
11.076	7.9816	2.5
11.306	7.8195	2.4
11.775	7.5091	3.0
12.196	7.2509	2.9
12.521	7.0636	2.3
12.785	6.9186	2.5
13.061	6.7729	2.6
13.303	6.6501	2.4
13.783	6.4197	13.2
14.071	6.2888	66.8
14.870	5.9525	100.0
15.248	5.8059	1.4
18.454	4.8037	3.8
18.754	4.7276	9.9
19.241	4.6091	2.6
19.651	4.5137	7.4
19.893	4.4596	2.7
20.272	4.3770	1.0
20.760	4.2751	1.8
21.076	4.2117	1.6
21.249	4.1778	1.8
21.827	4.0685	20.6
22.375	3.9701	4.2
22.964	3.8695	6.2
23.627	3.7625	8.2
24.209	3.6734	1.5
24.631	3.6113	10.2
25.079	3.5479	4.3
25.256	3.5234	4.9
25.661	3.4687	16.4
25.879	3.4399	13.6
26.286	3.3876	3.9

TABLE VII (cont.)

X-Ray Powder Diffraction Pattern of Indapamide

2θ	dÅ	I/I_o
26.720	3.3335	3.4
27.722	3.2153	4.3
28.045	3.1790	4.6
28.387	3.1415	1.1
29.981	2.9780	7.4
30.310	2.9464	1.3
30.521	2.9265	1.0
31.240	2.8608	2.7
31.845	2.8078	2.8
32.147	2.7821	9.2
32.637	2.7414	2.9
32.817	2.7268	2.7
33.208	2.6956	1.4
37.213	2.4142	2.0
37.654	2.3869	1.2
39.050	2.3047	1.3
39.235	2.2943	1.3
42.178	2.1407	1.3
42.544	2.1232	2.1
43.684	2.0704	1.2
44.146	2.0498	1.1

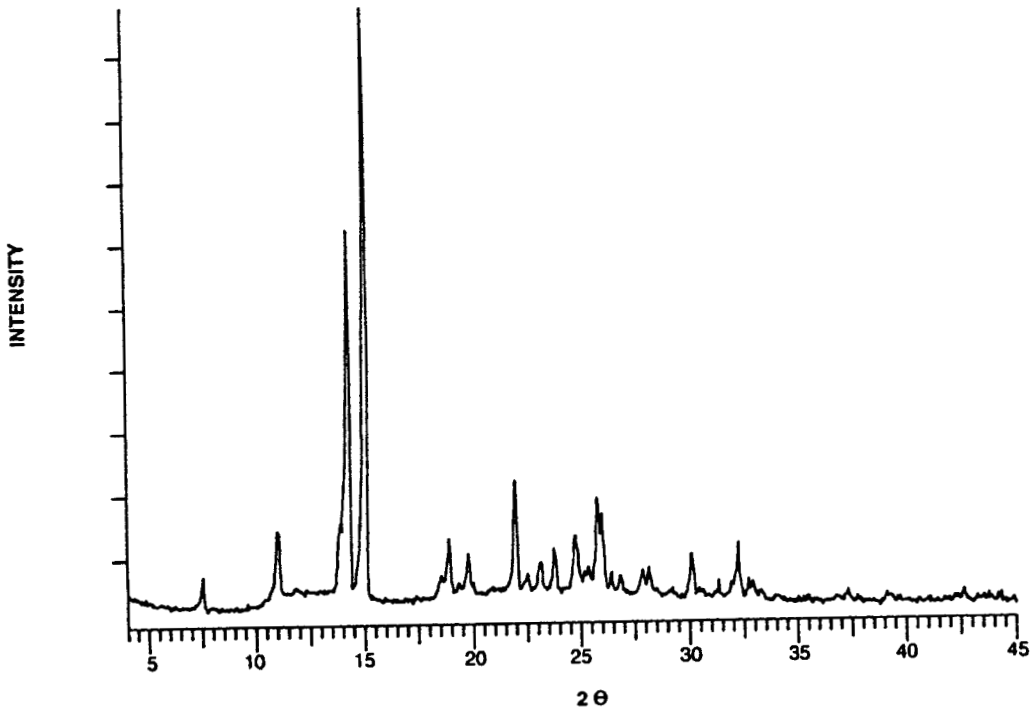

Figure 13. X-Ray Powder Diffraction Pattern of Indapamide

TABLE VIII

Partition Coefficients of Indapamide
in Chloroform-Water and Octanol-Water at 25°C

pH	Chloroform - Water	pH	Octanol - Water
2.05	12.51	1.86	25.3
3.10	12.60	3.05	26.1
4.28	12.60	3.69	24.7
5.22	12.73	4.45	25.5
6.33	12.73	5.38	24.4
7.20	12.42	6.25	25.4
7.83	11.85	6.97	24.7
8.20	11.56	7.73	22.9
8.54	10.50	8.24	20.1
8.93	7.87	8.88	14.8
9.15	6.90	9.33	10.9
9.40	5.31	9.83	7.6
9.61	3.96	10.51	2.5
9.79	2.85	10.92	1.0
10.36	0.95	11.49	0.3
11.10	0.20	11.71	0.2
11.56	0.07	11.87	0.1
12.05	0.04	---	---

5. METHODS OF ANALYSIS

5.1 Identification Tests

5.1.1 Ultraviolet Spectrophotometry

Identification testing of indapamide drug substance can be accomplished by UV absorbance spectrophotometric analysis. A 35-40 mg sample of indapamide is weighed into a 50-mL volumetric flask. The drug substance is dissolved and brought to volume with methanol. The solution is then diluted 1 to 10 with methanol and a portion placed in a 1-cm cell. The spectrum is recorded from 260 to 325 nm using methanol as a blank. There should be maxima at about 286 nm and 278 nm and minima at about 282 nm and 271 nm. The resultant spectrum also exhibits characteristics at the same wavelengths as a similarly prepared and concomitantly measured indapamide reference standard.

5.1.2 Infrared Spectrophotometry

Infrared spectroscopy can be used to identify indapamide. The infrared absorption spectrum of indapamide is obtained as a potassium bromide disc. A disc is made from 1 mg of sample in about 200 mg of potassium bromide. The infrared absorption spectrum of the potassium bromide dispersion of indapamide exhibits maxima only at the same wavelengths as that of a similar preparation of indapamide reference standard.

5.1.3 Elemental Analysis

The identity of indapamide can also be determined by an elemental weight percent determination of carbon, hydrogen and nitrogen. The elemental analysis of an indapamide sample is performed and the results are compared to the following theoretical values (12).

Element	Theory (%)
C	52.53
H	4.41
N	11.49

5.2 Titrimetric Analysis

Indapamide may be determined by a quantitative non-aqueous titration in acetone. An indapamide solution is prepared by weighing 180 mg of previously dried drug substance in 30.0 mL of acetone. The solution is titrated potentiometrically with standardized 0.1 N tetrabutylammonium hydroxide in toluene using a combination glass/calomel electrode (12).

5.3 Chromatographic Analysis

5.3.1 Thin-layer Chromatography

Two normal-phase, thin-layer chromatographic methods are available which separate indapamide from its potential impurities. The methods use silica gel GF plates (250 micrometer particle size) with visualization using shortwave UV light at 254 nm (21). System I uses a developing solvent consisting of toluene:ethyl acetate (70:30) and system II uses a developing solvent of chloroform:methanol:glacial acetic acid (95:5:1). Standard and sample solutions are prepared in methanol to produce a 3% (w/v) solution of indapamide. The chambers are allowed to equilibrate with mobile phase for one hour. Ten microliters of standard and sample solution are applied to the plates and then developed for 15 cm. System I produces an Rf value for indapamide of 0.25 while system II produces an Rf value of 0.39.

5.3.2 High Performance Liquid Chromatography

A stability-indicating high performance liquid chromatographic method (22) is available for indapamide. The drug substance is separated from its related substances using a Octadecyl silane column (4 mm ID x 300 mm, 10 μm particle size) and a mobile phase consisting of water, acetonitrile, methanol and acetic acid (650:175:175:1, v/v) at a flow rate of 2.0 mL/minute. Detection of the analytes is achieved at ambient temperature using UV absorbance at 254 nm. The standard and sample solutions are prepared in a diluent consisting of mobile phase and 5 μL are injected onto the column. All related substances are eluted within 30 minutes and may also be quantitated. The retention time of indapamide is approximately 15 minutes.

6. STABILITY

6.1 Solid State Stability

Indapamide is a stable compound in the solid state. After storage for two years at room temperature and one and one-half years at 35°C, no change in indapamide assay was found (23). Additionally, no change in physical appearance was observed.

7. BIOPHARMACEUTICS AND METABOLISM

7.1 Absorption and Bioavailability

Indapamide is rapidly absorbed by humans after oral administration. Absorption of the drug was shown to be complete after ^{14}C-indapamide was given intravenously to beagle dogs which were used in an oral study. The absolute availability following both oral and intravenous administration was 94% (24). In man and other species (rat and dog), 10 mg of indapamide is rapidly absorbed with maximal plasma levels of 140 ng/mL occurring within 0.5-1 hour after administration (25).

The bioavailability of indapamide is not significantly reduced when taken with food or antacids. Steady-state blood concentrations of indapamide appear to be independent of body weight. The volume of distribution for indapamide has been estimated from blood concentrations to be 25 to 27 L. The volume of distribution estimated from plasma concentrations was 110 L (26).

The blood concentration of indapamide has been shown to increase linearly with increasing doses following both single and multiple doses. Other pharmacokinetic parameters are not dose-related (27).

Indapamide is preferentially and reversibly taken up by erythrocytes in the peripheral blood (24, 25, 26). The red cell:plasma ratio is approximately 4:1. The drug is localized at the vascular wall (24). Approximately 71 to 79% of indapamide is reversibly bound to plasma proteins. Greater than 40% of the dose is located in the blood

compartment as indapamide one hour after dosing (24,25,28).

7.2 Metabolism

Indapamide plasma concentrations decline biphasically with a short, rapid half-life of 2 hours followed by a more prolonged half-life of about 15-18 hours (25,29). Indapamide is extensively metabolized to at least 15 different metabolites. Only about 5% is excreted as unchanged drug in the urine (25,29). Several workers have suggested that hydroxylation of the indoline ring with conjugation may be a major route of metabolism (25). The glucuronide and sulphate conjugates of indapamide have been detected in the urine of patients (26).

7.3 Elimination

Elimination of indapamide is predominately in the urine, 60%, and approximately 20% in the feces (24,26,28). Indapamide is filtered by the kidney but has a renal clearance of only 5 mL/min (28). The mean peak urinary excretion rate following a single dose of 40 mg of indapamide is approximately 3 μg/min (26). Peak urinary excretion after oral administration is 3 hours for unchanged indapamide (26). Comparison of the average urinary excretion rate profiles for 4 beagle dogs after intravenous and oral doses (1 mg/kg) reveals no significant differences in the amount or rate of urinary excretion of unchanged indapamide (29).

The fecal elimination is caused by the enterohepatic biliary recirculation of indapamide metabolites which in some subjects can be continued for 96 hours (25). However, elimination is essentially complete after 144 hours. The slow elimination in man may be due to enterohepatic recirculation and the long gastrointestinal tract transit time (24). Metabolic products in the feces are at least a molecular weight of 500 indicating probable glucuronide and sulphate conjugates (26).

The elimination half-life of indapamide has been estimated at 17 hours after administration of 2.5 mg of indapamide for 15 days (26).

7.4 Pharmacokinetics in Altered Renal Function

One study indicated that indapamide in a single daily dose of 2.5 mg is safe and effective for hypertensive patients with various degrees of renal impairment. Indapamide did not significantly affect the function of patients with renal impairment and did not accumulate in the blood. In addition, the calculated half-life for indapamide in hypertensive hemodialysis patients is found to be very similar to the half-life observed in hypertensive patients with normal and compromised renal function (30). No adverse effect of indapamide on renal function is evident in normal volunteers, hypertensive patients or geriatric hypertensive patients after monitoring glomerular filtration rate or effective renal plasma flow (24).

8. DETERMINATION IN BIOLOGICAL MATRICES

Indapamide can be analyzed in blood, plasma and urine by HPLC and fluorescence methods. A specific and sensitive assay method for the analysis of indapamide in urine, blood and plasma was developed (31). The method uses a 250 x 4.6 mm i.d. Zorbax ODS (5 μm particle size) column and a mobile phase of 0.1 M sodium acetate buffer (pH=3.6)/acetonitrile at a ratio of 65/35 (v/v). This method uses an internal standard of sulfanilanilide. Calibration curves obtained by plotting the ratio of the peak height of indapamide to that of sulfanilanilide versus the concentration of indapamide were linear over the concentration ranges of 25-200 ng/mL for plasma and 50-400 ng/mL for blood and urine using UV detection at 241 nm. Another publication reports a high performance liquid chromatographic assay method for monitoring indapamide and its major metabolite in urine (32).

A semiautomated fluorescence procedure for the determination of indapamide in plasma and whole blood has also been reported (33). The procedure requires pre-extraction of the biological sample followed by continuous flow analysis of the solution. The assay is sensitive to indapamide levels of 25 ng/mL in plasma and blood with a linear response from 25 to 200 ng/mL.

9. DETERMINATION IN PHARMACEUTICALS

9.1 Dissolution Testing

Dissolution testing of indapamide is accomplished using the rotating basket method (USP Method I) with 900 mL of simulated gastric fluid T.S. without enzymes heated to 37°C as the dissolution medium and a basket rotation of 100 rpm. Samples are placed in the baskets and the baskets are then lowered into the dissolution medium with samples withdrawn at appropriate intervals. The samples are filtered through 0.45 μm Millipore filters. The dissolution medium is assayed for indapamide against appropriate standards using an UV absorbance assay (23).

9.2 Potency

9.2.1 Assay

The assay of finished product tablets is accomplished with an HPLC method. The method employs a Octadecyl silane 100 x 4.6 mm i.d. column (3 μm particle size) with a mobile phase consisting of 1.08 g octane sulfonic acid, sodium salt in 700 mL of water, 10 mL of glacial acetic acid and 300 mL of acetonitrile. The flow rate is 1.0 mL/minute with UV absorbance detection at 242 nm.

Twenty tablets are ground to obtain a homogeneous powder and an accurately weighed portion of the powder is dispersed in the solvent consisting of water/acetonitrile (25/75). The sample is sonicated for 20 minutes and cooled to room temperature. After dilution to volume with water/acetonitrile diluent, the sample is mixed and centrifuged to remove any dispersed solids. The solution is then appropriately diluted depending upon tablet strength and the resultant solution is injected into the HPLC system. Tablets must contain not less than 90.0 percent and not more than 110.0 percent of the labeled amount of indapamide (34).

9.2.2 Dosage Uniformity

Tablets must meet the U.S.P. (35) requirement for dosage uniformity testing. The requirement for uniformity of the

dosage form is met if the individual assay of each of 10 dosage units lies between 85.0% and 115.0% of the label claim and the relative standard deviation of all 10 assays is less than or equal to 6.0%. Twenty additional dosage units must be tested if one unit is outside of this range and no unit is outside of the range of 75.0% to 125.0% of the label claim or if the relative standard deviation is greater than 6.0% or both. The 30 dosage units tested meet the requirements if not more than one unit is outside the 85.0% to 115.0% range, none are outside of 75.0 to 125.0% range and the relative standard deviation does not exceed 7.8%.

The content uniformity assay is performed by assaying ten tablets individually. Each tablet is ground to a fine powder and transferred quantitatively to a 50-mL volumetric flask. About 40-mL of water/acetonitrile (25/75 v/v) diluent are added to the flask and the solution is sonicated for 20 minutes. The solution is then treated as described in the potency assay procedure.

9.2.3 Stability Testing

Stability testing of indapamide tablets is accomplished by an HPLC assay and dissolution testing. The conditions for the HPLC assay are described in the section for potency assay (9.2.1). The dissolution test methodology is described in Section 9.1.

9.3 Disintegration Test

A disintegration test with discs is performed for indapamide tablets. The apparatus is described in USP XXII under section [701] Disintegration (36). The test is accomplished by placing a tablet in each of the six tubes of the basket. The amount of time needed for the tablets to disintegrate completely in 0.1 N hydrochloric acid at 37°C is recorded. The disintegration test needs to be repeated for 12 additional tablets if one or two tablets fail to disintegrate completely. Of the 18 tablets, not less than 16 of the tablets must disintegrate completely.

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LINCOMYCIN HYDROCHLORIDE

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1. DESCRIPTION :

1.1. Nomenclature :

1.1.1. Chemical Names :

1.1.1.1 Methyl 6-amino- 6,8 - dideoxy - N - [(2S, 4R)- 1 - methyl - 4 - propylprolyl] - 1 - thio - α - D - erythro - D - galacto - octopyranoside hydrochloride.

1.1.1.2 Methyl 6,8 - dideoxy - 6 - [[(1 - methyl - 4 - propyl- 2 -pyrrolidinyl) carbonyl] amino] - 1 - thio-D-erythro- α -D- galacto - octopyranoside hydrochloride.

1.1.2. Generic Name :

Lincomycin hydrochloride

1.1.3. Registry Number :

Chemical abstract CAS number for :

Lincomycin hydrochloride (anhydrous) 859 - 18 - 7

Lincomycin hydrochloride (monohydrate) 7179 - 49 - 9

1.1.4. Forms Available :

Lincomycin hydrochloride monohydrate.

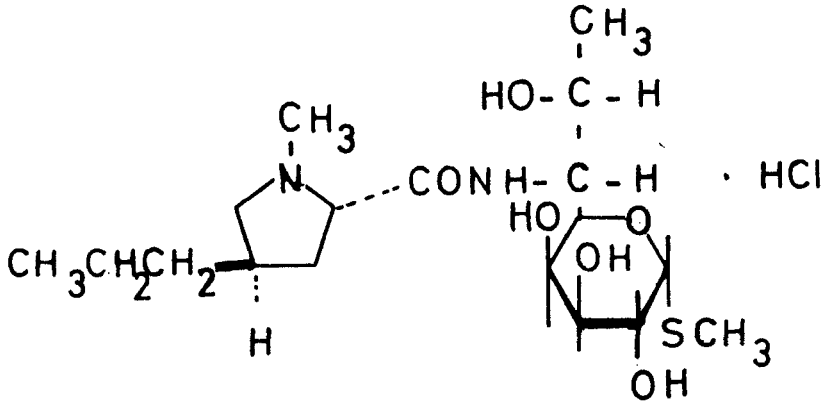
1.2. Formula :

1.2.1. Emperical Formulae :

$C_{18}H_{34}N_2O_6S.HCl.H_2O$

1.2.2. Molecular Weight : 461.01

1.2.3. Structural Formula :



Structural studies showed that lincomycin consists of a carbohydrate portion, methyl 6 - amino - 6,8 - dideoxyl - 1 - thio - D - erythro - α - D - galacto - octopyranoside, bound by an amide linkage to 1 - methyl - trans - 4 - propyl - L - pyrrolidine - 2 - carboxylic acid.

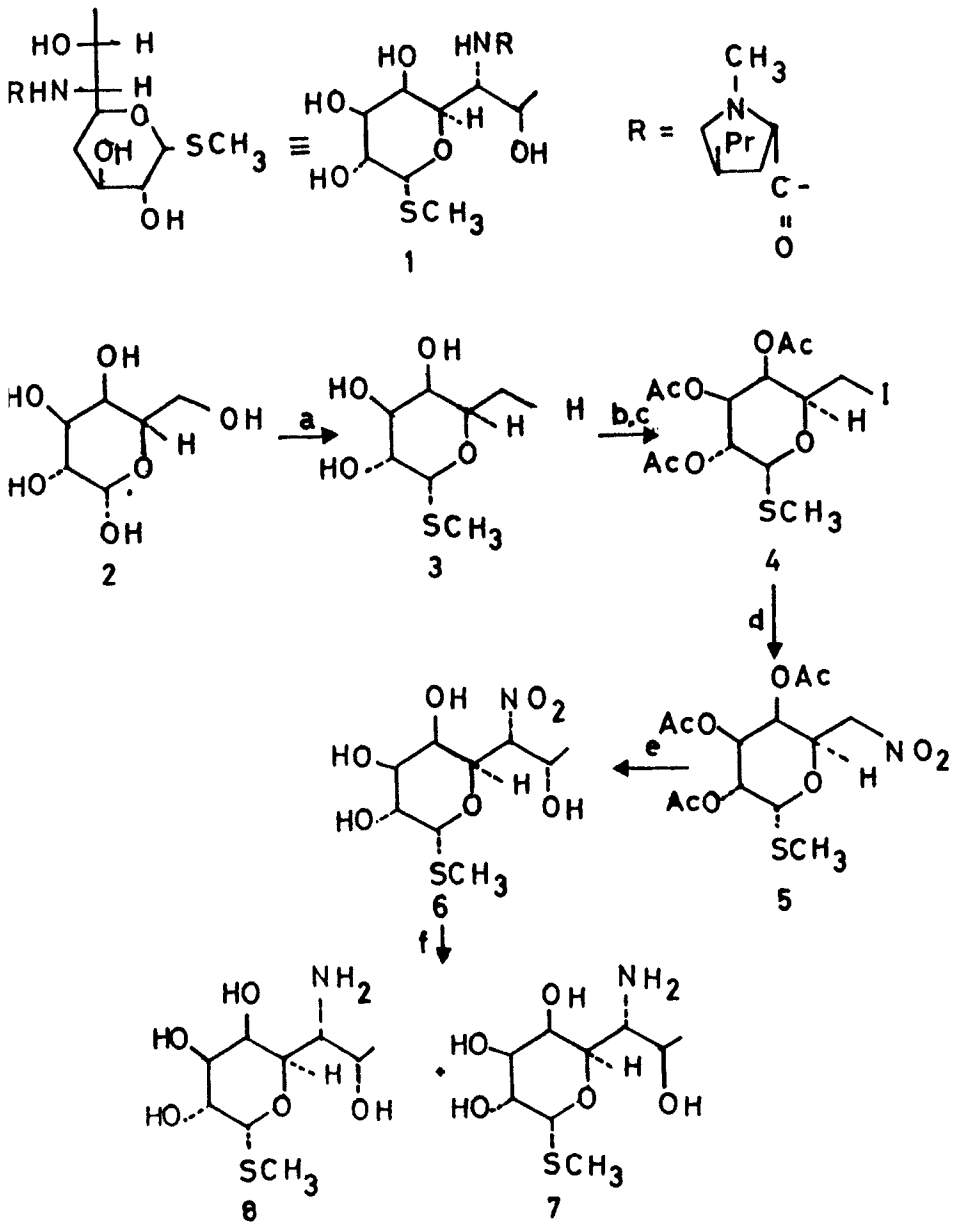
1.3. Appearance, Color, Odor and Taste :

Lincomycin hydrochloride monohydrate is a white or almost white, crystalline powder. It is odorless or has a faint mercaptan - like odor and has a bitter taste.

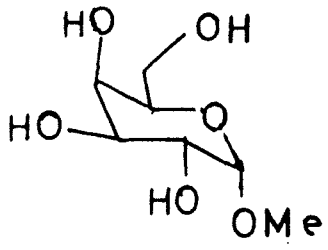
2. SYNTHESIS :

Different methods were developed to synthesize lincomycin (1). Most of these methods directed towards the synthesis of 6 - amino - 6,8 - dideoxy - D - erythro - D - galac - tose commonly named lincosamine - the sugar component of the antibiotic lincomycin. The D - galactose - derived aldehyde is the most frequently used synthon. Other direction were concentrated on the synthesis of lincomycin from non - carbohydrate precursors through the application of furan compound (2) or hetero Diels - Alder Approach (3). Scheme (i) shows the partial synthesis of lincomycin (4). The starting methyl thiogalactoside 3, formed when D - galactose 2 was treated with methanethiol, was converted to 6 - iodo sugar 4 via selective tosylation of the primary hydroxy group. Condensation of nitro derivative 5 with acetaldehyde yielded a 7 - epimeric mixture of nitro alcohol 6. Reduction of 6 gave lincomycin 7 and its 7 - epimer 8. Coupling with trans - 1 - methyl - 4 - propyl - L - proline led to the isolation of lincomycin 1 and its 7 - epimer, respectively.

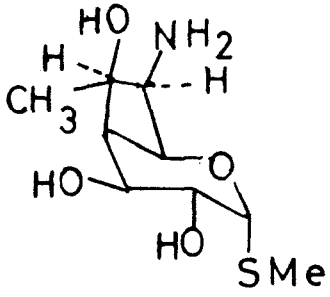
Stereocontrolled lincomycin was synthesized from methyl - D - galactopyranoside, I, of methyl thio - lincosa - minide, II, using the direct synthesis precursor to lincomycin (5). The off - pyranose stereocenters C - 6 and C - 7 were controlled by an intramolecular nitrogen delivery reaction using epoxy alcohol.



Scheme (I) a) MeSH , HCl ; b) TsCl , Py ; Ac_2O , Py ; c) NaI , acetone , d) $NaNO_2$, DMF , e) MeCHO , MeONa , MeOH , f) LAH , THF .

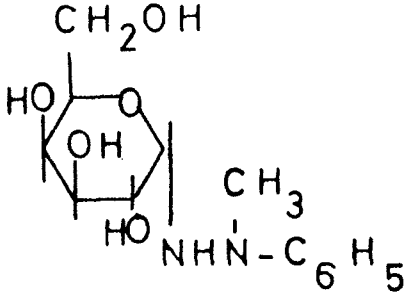


I

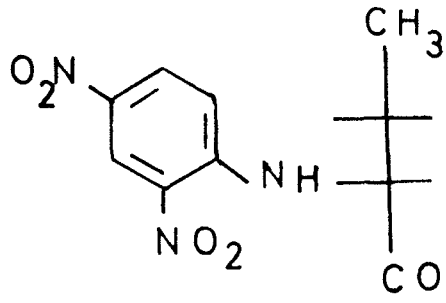


II

The stereochemistry of the carbohydrate moiety was acheived by cleavage the amide bond with refluxing hydrazine followed by a series of selected chemical reactions (6). The structure and stereochemistry of six asymmetric centers of the carbohydrate moiety was established by isolation of D - galactose - α - methyl - phenylhydrazone , III , and N - 2,4 - dinitrophenyl - D - allothreonine , IV , following oxidative cleavages of the appropriate derivatives. The configuration of the thiomethyl group was assigned from considerations of optical rotations.



III



IV

3. POLYMORPHIC FORMS :

Lincomycin hydrochloride has two polymorphic forms (A and B). This was detected by their x-ray diffraction which showed the following interplanar spacing for the two crystalline forms (7).

Interplanar spacings of the two polymorphs

Polymorph A	Polymorph B	Polymorph A	Polymorph B
17.48[a]	14.02[a]	4.77	4.13
16.51	10.52	4.57	4.04
13.80	9.40	4.39	3.88
12.99[a]	8.53	4.33	3.67
10.77	6.96	4.22	3.50
8.58[a]	6.19[a]	4.01	3.45
8.04	5.90	3.86	3.28
7.28	5.55	3.77	3.02
6.80	5.34	3.54	2.90
6.37	5.15	3.40	2.76
6.10	5.01	3.26	2.57
5.68	4.62[a]	3.18	——
5.34	4.48	2.85	——
5.02	4.26	2.71	——

(a) Major peaks

The two forms showed two different infra - red spectra (8) (Figure I). Form A is a hemihydrate and consisted of light , fluffy , needle - like crystals (9) . The second form of crystals , B , existed as small , dense cubic crystals , m.p. 145 - 147 °C (10) . This form existed as a monohydrate . Polymorphic form B is the commercially available material. The two forms result from differing conditions of crystallization . Form A was obtained when acetone was added quickly without stirring to an aqueous solution of lincomycin hydrochloride. The mixture was allowed to stand 3 - 5 minutes while maintained at 5 - 10 °C , during which time crystallization took place . However , form B separated when the acetone was added slowly (at a rate of 20 - 25 ml. per minute) and the mixture maintained at 30 - 35 °C with stirring for four hours .

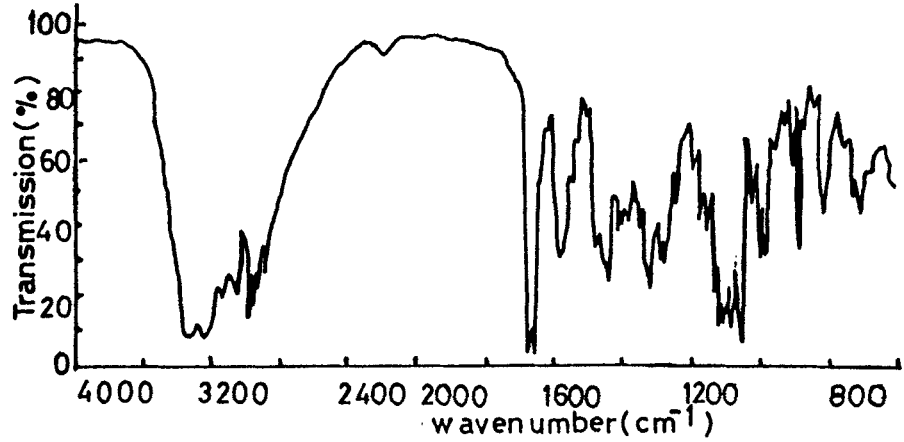
4. PHYSICO - CHEMICAL PROPERTIES :

4.1. Melting Range :

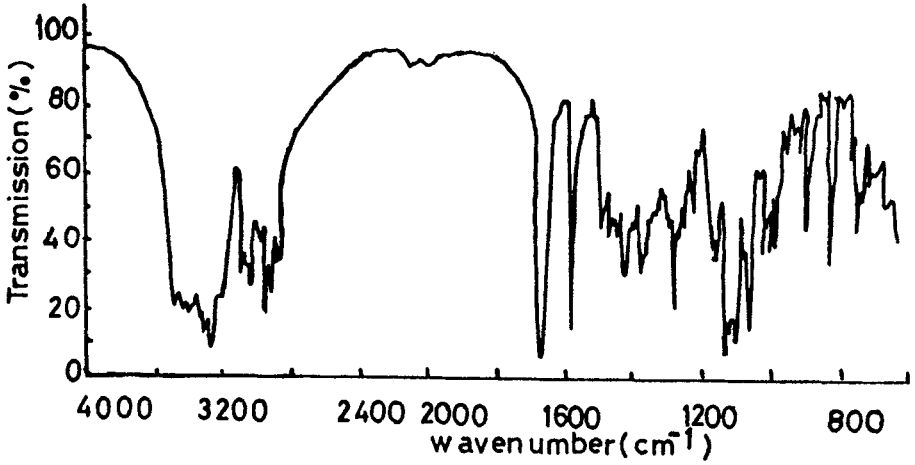
Lincomycin hydrochloride hemihydrate = 146 - 148 °C.
Lincomycin hydrochloride monohydrate = 156 - 158 °C.

4.2. Specific Optical Rotation (7) :

Optical rotatory dispersion measurements showed lincomycin hydrochloride to be dextrorotatory with no maximum and $[\alpha]_D^{25}$ = + 137 °C. (C = 1 , water) .



Lincomycin hydrochloride hemihydrate (Polymorph A)



Lincomycin hydrochloride monohydrate (Polymorph B)

Figure (i) : I.R. Spectra of Lincomycin hydrochloride polymorphs (8) in KBr.

4.3. Dissociation Constant, pKa (7) :

The pKa of lincomycin (free base) has been reported as 7.6 .

4.4. Crystallization :

Partitation chromatography and countercurrent distribution procedures were used for purification and crystallization of lincomycin hydrochloride (7). Anhydrous lincomycin hydrochloride is hygroscopic, consequently different crystal forms have been observed due to the degree of hydration. Crystallization of lincomycin hydrochloride from concentrated and supersaturated aqueous solution gave 70 - 90 µm size crystals (11).

4.5. Differential Scanning Calorimetry (DSC) (12) :

Thermograms of lincomycin hydrochloride polymorphic forms are shown in Figure (II). These thermograms were obtained using Mettler TA 3000 DSC - 20 unit. The heating rate was 10 °C min^{-1} and the sample size ranged between 5 - 10 mg. Lincomycin hydrochloride monohydrate has one endothermic peak, showing no signs of decomposition at the melting point. However, lincomycin hydrochloride hemihydrate undergoes loss of water of hydration at 90.7 °C. Above 200 °C decomposition occurs for both polymorphs with loss of methyl mercaptan.

Lincomycin hydrochloride (monohydrate)	Δ H ENDO (mJ) 960.44	Δ H, J/G 98.415	Peak Temp. 155.5 °C
Lincomycin hydrochloride (hemihydrate)	178.66 310.72	24.111 41.933	90.7 °C 150.0 °C

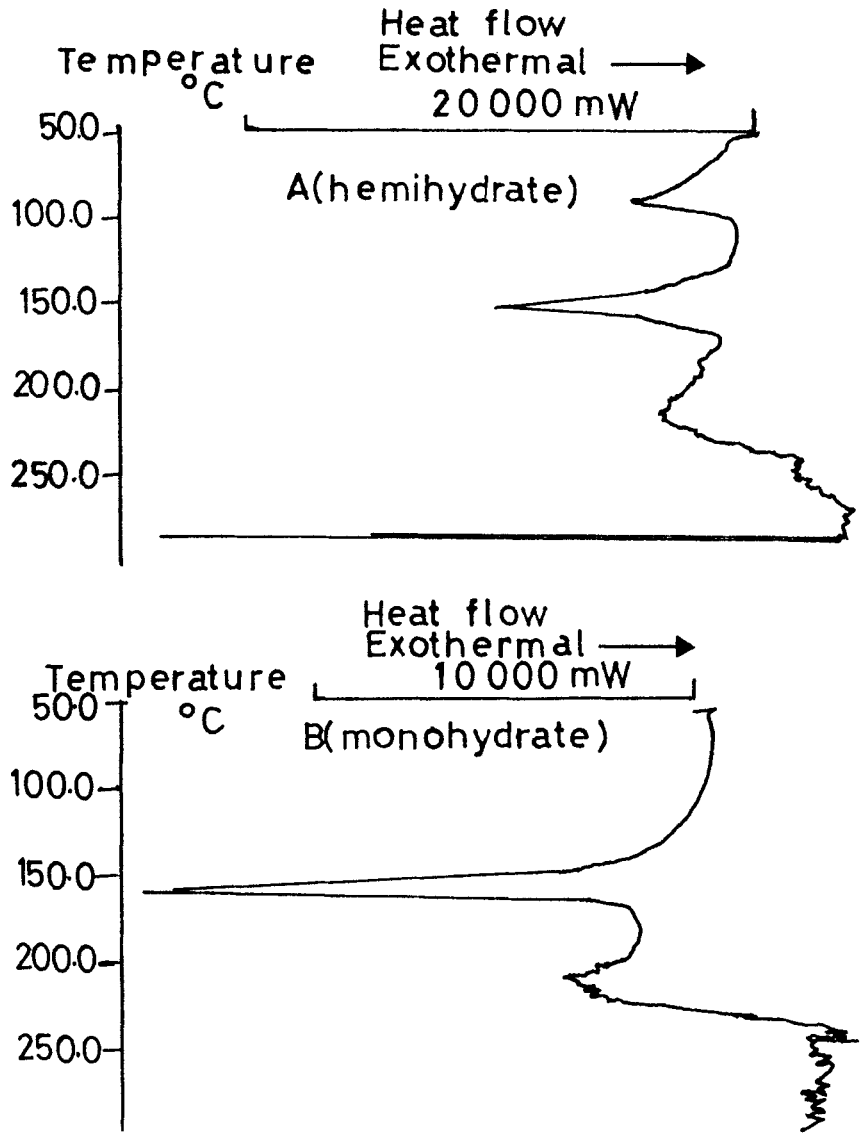


Figure (II) . DSC curves of lincomycin hydrochloride forms (A and B) .

4.6. Reference Preparation (13) :

A suitable sample of lincomycin hydrochloride monohydrate was obtained by the generosity of the Upjohn Company (USA) with the following date :

$[\alpha]_D^{25}$	+ 143 °C
pH (1 % aqueous solution)	4.8
Water (Karl Fischer)	3.85 %
Equivalent weight	460
Potency	865 ug/ml on an undried basis
Lincomycin B content	1.98 %

The purity of the sample in acetone - water mixture (93:7) and in ethyl acetate - ethanol mixture (1 : 1) was found to be 99.8 $\pm$ 0.2 % and 99.7 $\pm$ 0.6 % , respectively. This sample was used for the standard preparation of FDA and has assigned potency of 865 ug/ml undried. After being distributed into ampoules, the moisture content of the material was determined by Karl Fischer method and found to be 2.08 % w/w. The potency of the material corrected to 881 ug/mg due to the change in water content from 3.85 w/w to 2.08 w/w. The material in the ampoules increased in weight by approximately 1.0% w/w in 10 minutes when exposed to the atmosphere at a relative humitidy of 55 %.

4.7. Solubility :

Lincomycin hydrochloride is soluble in 2 parts of water, in 40 parts of ethanol (96 %), in 20 parts of dimethylformamide, very slightly soluble in acetone and practically insoluble in chloroform and in ether.

4.8. Stability :

4.8.1. Stability in Dry Form :

It was found to be stable as dry form for 6 months at 70 °C (7). In dry selection nutrient agar media , the stability of lincomycin was high (14). Stable to light and air , but undergoes loss of water of hydration between 50 °C and 150 °C (15).

4.8.2. Stability in Solution :

Lincomycin undergoes hydrolysis of the thioglycoside group (catalyzed by acid) and hydrolysis of the amide function (probably both acid and base catalyzed). In very acidic medium, lincomycin degrades at the same rate of clindamycin . Above pH 1.5 , lincomycin is more stable than clindamycin (16). Ultrasonic nebulization of lincomycin should be avoided during the preparation of solution due to the formation of a bad smell which may be attributed to the liberation of methanethiol (17). Lincomycin hydrochloride retained its initial antimicrobial activity for a minimum of one month at 5 °C in both normal saline and 5 % aqueous dextrose solution (18).

At pH 1 to 10 lincomycin - 2 - phosphate hydrolyzed to lincomycin at a rate greater than it undergoes thioglycoside or amide hydrolysis . pH - rate studies show that lincomycin - 2 - phosphate is most stable at pH 6 - 10 (19).

The stability of lincomycin hydrochloride injection of pH 6 - 6.5 containing benzyl alcohol (1 %) as a preservative and a local anesthetic was studied at different temperatures. The results show that these injections were stable and loss $\leqslant$ 15 % activity in 3 years if kept at 25 - 30 °C. The half - life of lincomycin hydrochloride in solutions heated at 50 °C was 22 days (20) .

5. SPECIAL PROPERTIES

5.1. Ultraviolet Spectrum :

Lincomycin showed no characteristic absorption in the region 230 - 400 nm .

5.2. Infra - red Spectrum :

A typical potassium chloride disc spectrum of lincomycin hydrochloride monohydrate is shown in Figure (I), form B . The spectrum shows two strong bands at 1655 cm^{-1} and 1564 cm^{-1} attributed to the amide group in addition to a broad band at 3700 - 3100 cm^{-1} (λOH and λNH). Other bands were observed at 1262 cm^{-1}, 1104 cm^{-1}, 1075 cm^{-1} and 1040 cm^{-1}.

5.3. Mass Spectrum :

The mass spectrum of lincomycin showed five characteristic and diagnostic ions (molecular ion and four other ions) which can be considered to arise from molecular ions with charge localization at either the amino nitrogen or the pyran oxygen (Figure III , Table I scheme ii) (21). These ions provided considerable insight into the structure elucidation of lincomycin . The base peak (c) involves the hygric acid portion (trans - 4 - propyl - L - hygric acid). The major ion arising from fragmentation of the sugar portion of the molecule was ion (b). These two peaks represented the possible degradation products .

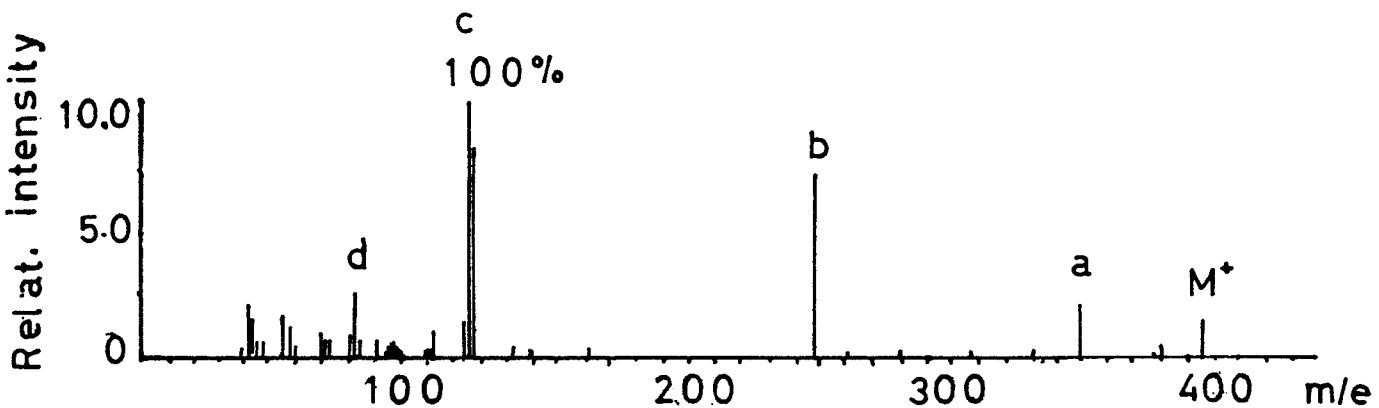
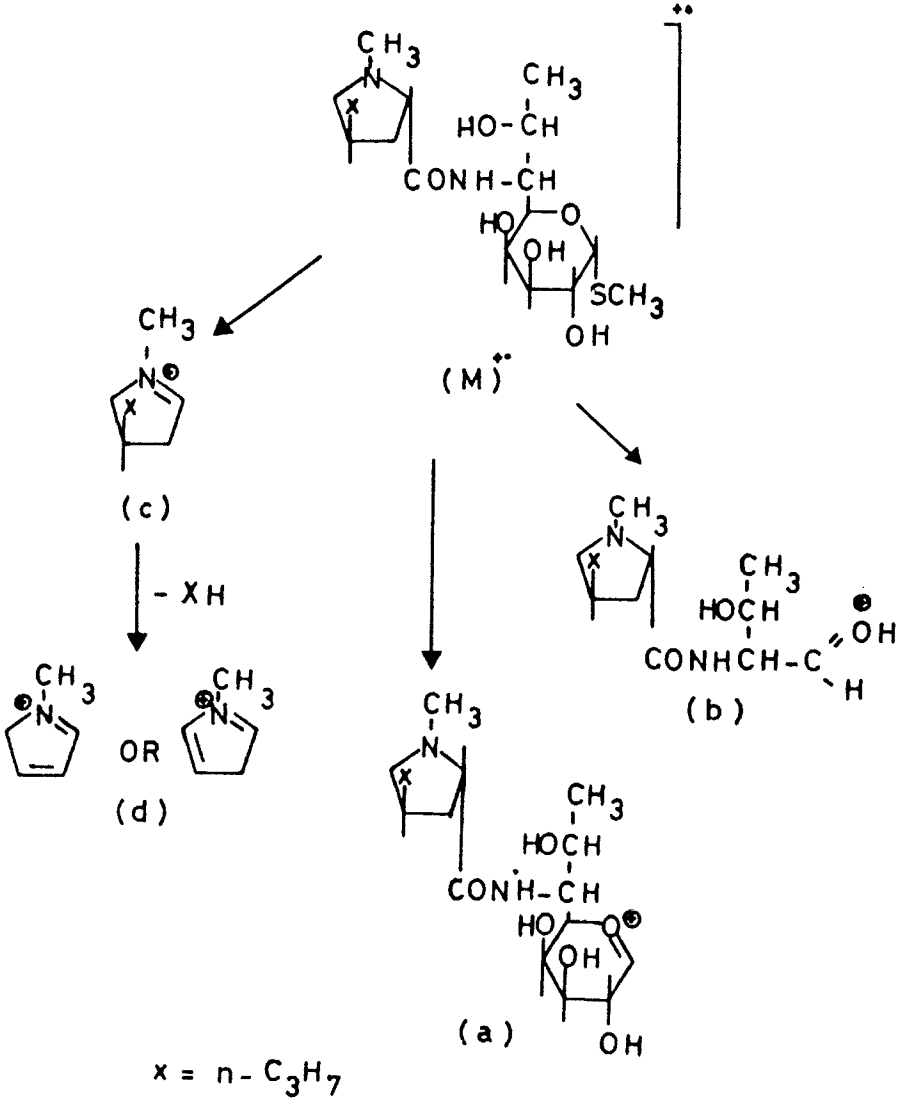


Figure (III) Mass Spectrum of lincomycin


CH3
CH3
HO-CH
CONH-CH
HO
O
OH
SCH3
OH
(M)+·
(c)
- XH
(d)
OR
(b)
CONHCH-C
H
(a)
x = n-C3H7

Scheme (II)

Table I

Partial Mass Spectrum of Lincomycin

Fragment ion	m/e (% relative intensity)
[M]	406 (1.6)
[a]	359 (1.8)
[b]	257 (7.1)
[c]	126 (100.0)
[d]	82 (2.9)

5.4. Nuclear Magnetic Resonance Spectrum :

The structure of lincomycin and its degradation products were studied by 'H-NMR (22). It was found that the spectrum was complex, containing many super - imposed multiplets which were difficult to factor. This spectrum was analyzed after hydrolyzing the molecule at the amide linkage into an amino acid (L 4 - trans - n - propyl hygric acid) and an amino thiosugar (methyl thiolincosaminide). The combination of the NMR results of the two parts with their chemical information afforded the complete structure for the antibiotic (Cf. Figure IV a - c). NMR values obtained for the sugar moiety of lincomycin are reported in Table (II). The NMR Spectrum of the amino acid portion was found to be a propyl - 1 - substituted hygric acid by comparison with known L - hygric acid. The location and configuration of the alkyl group could not be determined from the spectrum because the 2 - and 5 - hydrogen multiplets could not be factored.

The relative lincomycin quantities in clindamycin can be determined by 'H-NMR spectroscopy (23).

^{13}C - NMR Spectrum and Spin - Lattice relaxation times were used to study the structure of lincomycin (24). The results of analysis are reported in Table (III) and Figure (V). The signal assignments were based on spectral comparison of the structurely related compounds and on other known techniques.

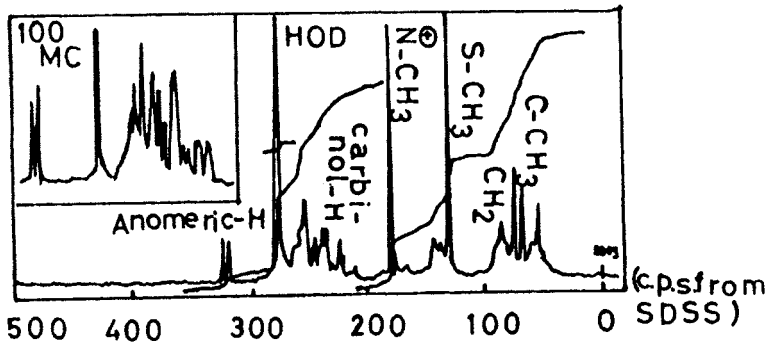


Figure (IVa) . Nmr spectrum of lincomycin hydrochloride (22).

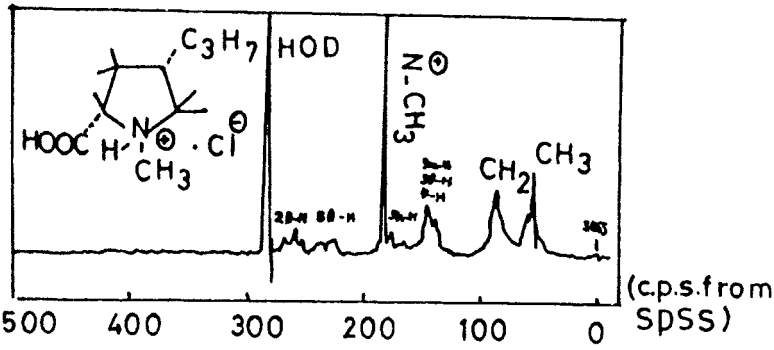


Figure (IVb) . Nmr spectrum of amino acid portion L-4-trans-n-propylhygric acid hydrochloride in D_2O (22).

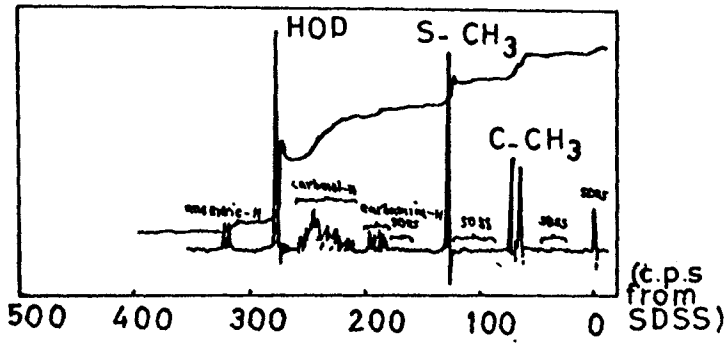


Figure (IVc) . Nmr spectrum of methyl thiolincos - aminide in D_2O (22) .

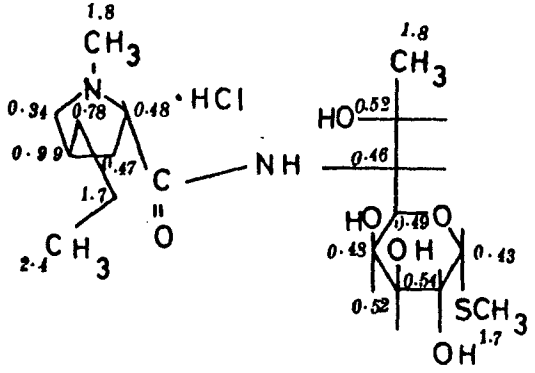
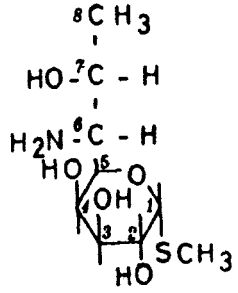


Figure (V) . Carbon - 13 spin - lattice relaxation times (seconds) for Lincomycin.HCl .

Table II

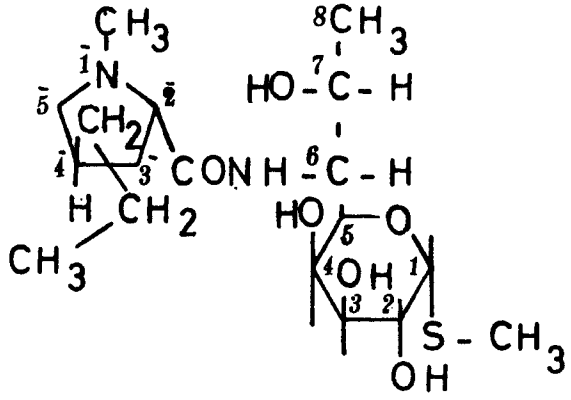
Chemical Shifts and Coupling of the Sugar Moiety of lincomycin in D_2O with Sodium 2,2 - dimethyl - 2 - silapentane - 5 - sulfonate as Internal Reference (22).



No.	Chemical Shift		Coupling Constant (cps)	Description
	Position	Frequency (cps)		
1.	C_8	68.0 (d)	6.7	Carbinol methyl group coupled to one neighboring hydrogen (C_7).
2.	C_1	127 (s)	----	S - methyl group
3.	C_6	188.5 (2d)	10.5 , 3.0	Carbamine hydrogen coupling to two neighbors.
4.	C_1 - C_4	212 - 256 (m)	----	Carbinol hydrogen of the sugar.
5.	HOD	276 (s)	----	Represents six exchangeable hydrogens on oxygen and nitrogen
6.	C_5	320 (d)	5.5	Anomeric hydrogen of the sugar.

Table III

^{13}C Chemical Shifts (24)



Position	Chemical Shift (cps)
C - 1	89.2
C - 2	68.8
C - 3	71.4
C - 4	69.5
C - 5	70.0
C - 6	54.9
C - 7	67.4
C - 8	17.2
S - CH_3	14.2
C - 2′	69.5
C - 3′	36.4
C - 4′	37.4
C - 5′	68.4
α - CH_2	35.0
β - CH_2	21.5
δ - CH_3	14.3
N - CH_3	41.8
C = O	170.1

5.5. Near - Infra-red Spectrum (25) :

Near infra - red reflectance analysis via Mahalanobis distance determination was found to be a useful technique for determining the concentration of lincomycin in an agricultural premix. The results obtained were comparable to the corresponding values collected with the use of the reference sample.

6. CHROMATOGRAPHIC METHODS

6.1. Thin - Layer Chromatography :

Different methods were developed to detect lincomycin by TLC . These methods can be summarized as follows :

6.1.1. Silica gel GF 254 thin layer plate has been used with developing solvent of methanol - chloroform (1 : 3) to detect lincomycin (26). Lincomycin has an Rf of 0.65 detected by charring, iodine vapors, or permanganate spray. The latter consists of 10 gm of potassium carbonate, 8 gm of sodium periodate, and 1 gm of potassium permanganate in 500 ml of water. The mixture was left standing for 16 hours and filtered.

6.1.2. Silica gel G and kieselguhr (1 : 2) thin layer plate has been applied to detect lincomycin using a mixture of methanol - ethyl acetate - water - 25 % ammonium hydroxide - pyridine - 3.85 % ammonium acetate (100 : 20 : 60 : 20 : 10 : 300) as a mobile phase (27). Lincomycin has an Rf of 0.8 .

6.1.3. TLC Silica gel plate carrying lincomycin has been developed in 80/20 chloroform / methanol mixture and sprayed with 5 % Vanillin in methanol and 20 % w/v sulfuric acid in methanol (28). The plate was gently heated to observe lincomycin yellow spots. The Rf of lincomycin is 0.5. To quantitate samples, the plates were heated until red brown spots developed which were stable and amenable to TLC direct analysis. Tenths of micrograms of lincomycin were detectable by this method (28).

6.2. Paper Chromatography (7) :

Lincomycin hydrochloride monohydrate can be detected by paper chromatography. The method employed was paper chromatography followed by bioautography on agar trays seeded with Sarcinea Lutea. The following solvent systems were used :

I. 1-Butanol : water (84 : 16 v/v), 17 hrs.
II. 1-Butanol : water (84 : 16 v/v), plus 0.25 % p - toluene sulfonic acid (w/v), 16 hrs.
III. 1-Butanol : acetic acid : water (2 : 1 : 1 w/v), 16 hrs.
IV. 1-Butanol : water (84 : 16 v/v) plus 2 % piperidine v/v .
V. 1-Butanol : water (4 : 96 v/v), 5 hrs.
VI. 1-Butanol : water (4 : 96 v/v) plus 0.25 % p - toluene sulfonic acid (w/v) , 5 hrs.

Figure (VI) shows the bioautogram of lincomycin on Sarcinea Lutea in which the breaking appeared under system V indicated the purified preparations.

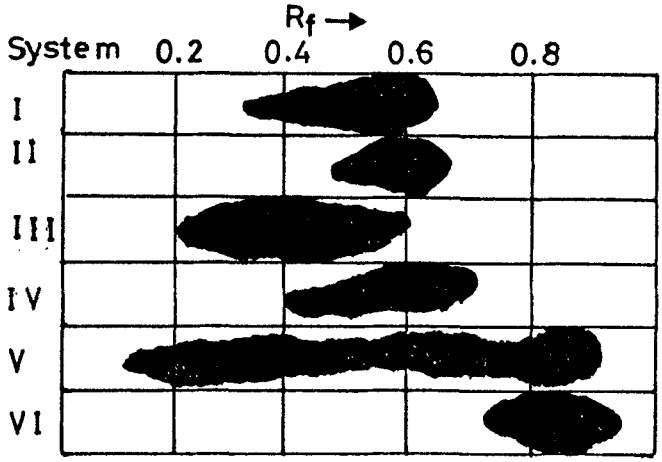


Figure (VI) Paper chromatography of lincomycin . The antibiotic was detected by bioautography on Sarcina Lutea seeded agar (7) .

6.3. High Pressure Liquid Chromatography :

Lincomycin hydrochloride and Clindamycin hydrochloride were chromatographed by an ion - pair HPLC under the following conditions (Figure VII) (29) :

Column :

30 cm X 4 mm ID prepacked with C_{18} μ Bonapack.

Mobile phase :

1 gm sodium diethyl sulfosuccinate, 1.0 ml formic acid, 125 ml water, and completed to 500 ml with anhydrous methanol.

Detection :

Refractive index detector (9.6 X 10^{-3} Rf units full scale).

Ion - pair HPLC column was employed to separate a synthetic mixture containing lincomycin, lincomycin B, clindamycin B, 7 - epicilindamycin and clindamycin (Figure VIII) (30). RI detection was utilized with the mobile phase consisting of a 60/40 ratio methanol - water, 2 ml acetic acid per litre (0.035 μ), and 0.005 M, DL - 10 - sodium camphor sulfonate adjusted to pH 6.0 using a 30 cm X 3.9 mm ID prepacked with $C_{18}\mu$ Bondpack column at a flow rate of 1.0 ml/min. Detection by UV can be employed at 214 nm for an additional sensitivity utilizing a mobile phase composed of a 60 : 40 ratio methanol / water, 0.01 M phosphate buffer, and 0.005 M sodium pentane sulfonate.

Lincomycin was determined quantitatively by HPLC at 214 nm using methanol - acetonitrile - 0.05 M phosphate buffer (16 : 16 : 68) as a mobile phase (31).

Lincomycin in bulk or in tablets was determined by HPLC after diluted with ammonium phosphate buffer (pH 3) using dodecyl sodium sulfate - water - acetonitrile (0.29 : 68 : 32) as a mobile phase. The recovery and coefficients of variations for tablets were 100.3 and 0.99 %, respectively, (32).

Lincomycin in plasma (human or povine) and urine determined by HPLC at 214 nm using ion - pair - HPLC column with an acetonitrile - trifluoroacetic acid (0.1 - 0.2 % v/v in the mixture) as an eluent (33). More than 1800 samples were assayed by this method. Soil - phase extraction (SPE) procedure was used in this analysis followed by an evaporation step.

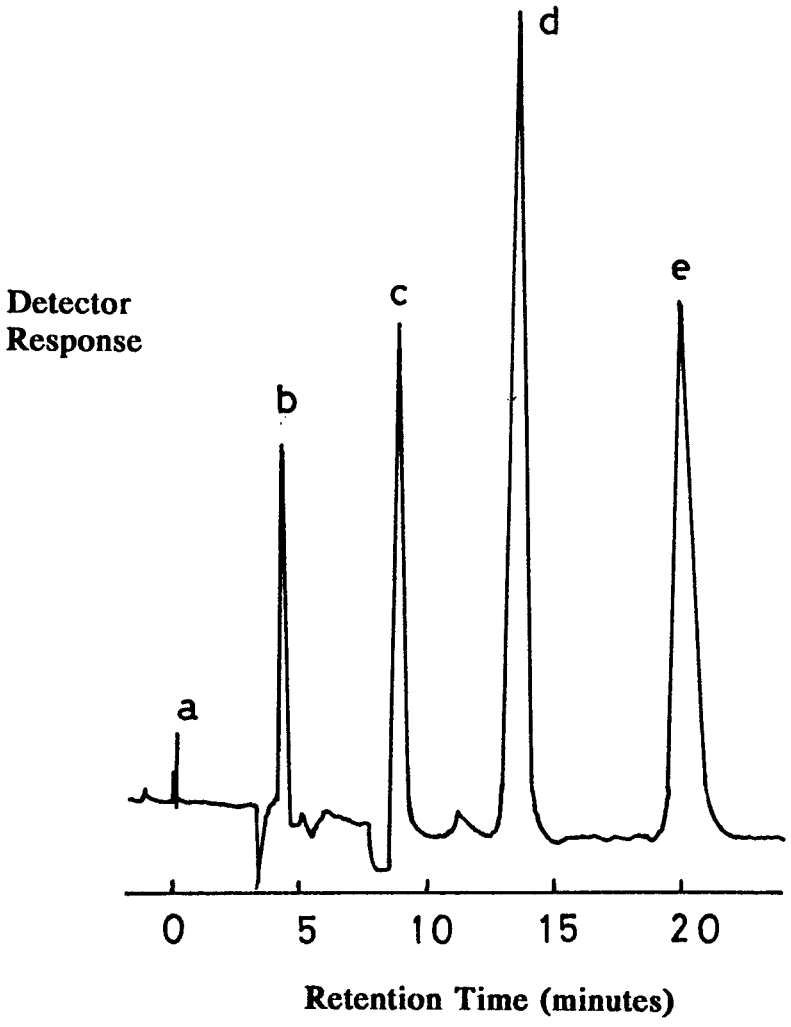


Figure (VII). HPLC Chromatogram of : (a) injection, (b) solvent, (c) lincomycin, (d) clindamycin, (e) testosterone propionate as internal standard (29).

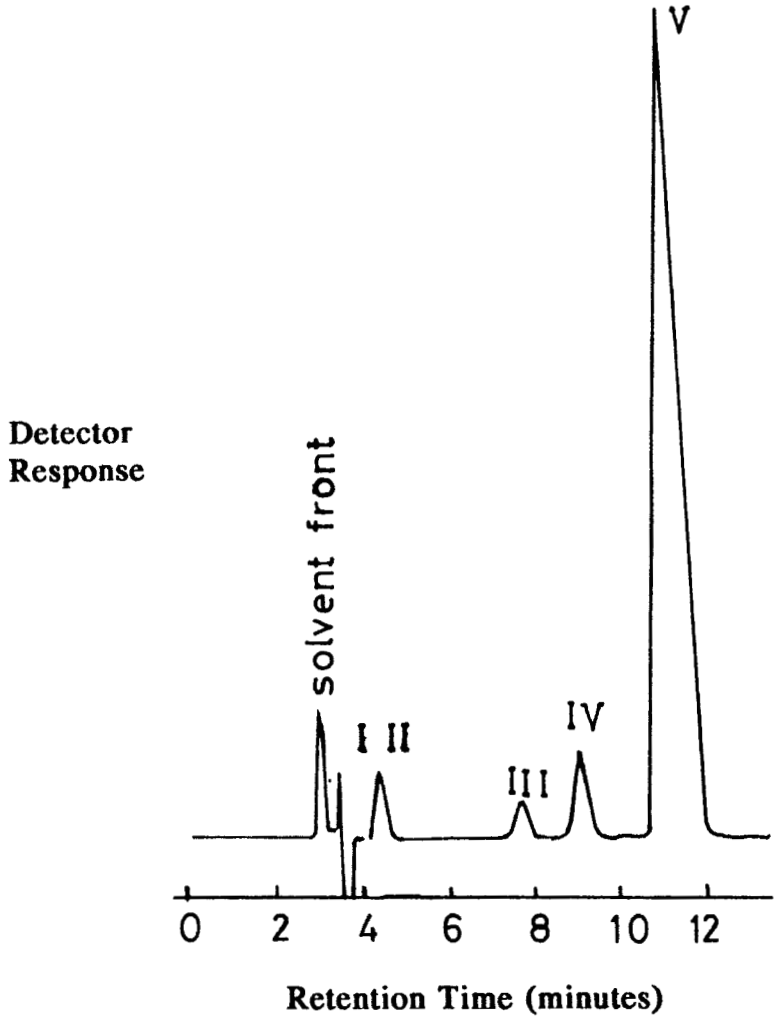


Figure (VIII). Chromatogram of a synthetic mixture of lincomycin (I), lincomycin B (II), clindamycin B (III), 7 - epiclindamycin (IV, and clindamycin (V), (30).

6.4. Gas Chromatography :

A vapor - phase chromatographic method was reported for the determination of lincomycin in preparation certifiable under the Federal Food and Cosmetic Act (34).

A gas - liquid chromatographic method was used for the determination of both lincomycin and lincomycin B. In this method lincomycin was silylated in pyridine followed by direct chromatographic analysis (35). The method has some difficulties due to the reactivity of the silylating reagent towards the labile protons. This means that lincomycin must be separated from the compounds containing hydroxyl, primary and secondary amines and sulfhydryl. These compounds occured in the dosage forms as solvent and additives. Also, because of the excessive silylating reagent, the electrode of the gas chromatograph rapidly become contaminated and the analysis was reduced. Accordingly, this method was modified to minimize these difficulties by extracting the trimethylsilyl ether derivative with cyclohexane before the use of gas chromatography (36). The modified method can be applied to determine the lincomycin content in the capsules, syrups, injectables and powder. In this method, trimethylsilyl ether derivative of lincomycin was prepared by adding 0.2 ml of the silylating reagent to the dried sample obtained from the drug (~4 mg lincomycin) in 1 ml of pyridine. The reaction mixture was allowed to stand for 30 minutes. To this mixture, 1.0 ml of the internal standard solution (saturated solution of tetraphenyl cyclopentadienone in cyclohexane) and 2 ml of water were added. The mixture was shaked vigorously. The phases were separated by gravity or centrifugation. A 5 µl of the cyclohexane phase were injected into the gas chromatograph and the area of each peak was measured by planimetry or by disc integration. Figure (IX) shows a chromatogram obtained under the following conditions :

Glass column : U - shaped 6 ft by 3 mm I.D packed with 5 % SE - 30 ; operating conditions : column temperature 237 °C ; detector temperature 280 °C, injector temperature 280 °C ; carrier gas ; nitrogen at 20 p.s.i (150 ml/min).

McMurray et al (37) developed an assay for determination of lincomycin in animal feeds using gas - chromatography - mass spectrometry.

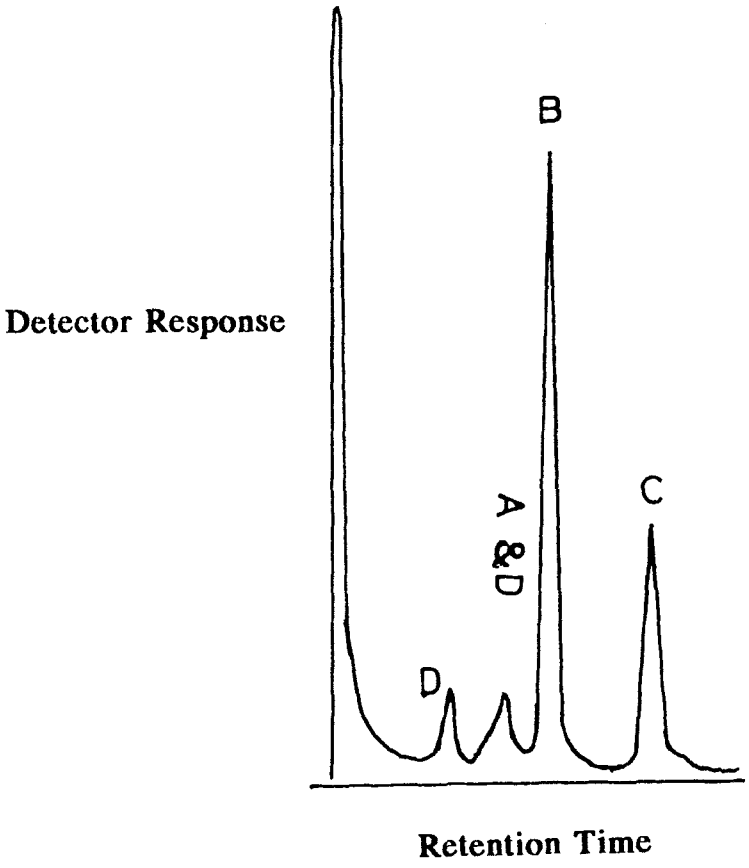


Figure (IX) Chromatogram of silylated compounds capsules by the improved method. Key: A = lincomyc B = lincomycin; C = internal standard; D = lactose (36).

6.5. Pyrolysis - Gas Chromatography :

Pyrolysis - gas chromatography in two temperature ranges (200°C - 400°C) and (600°C - 1300°C) has been used to characterize the structure of antibiotic substances (38). The low temperature pyrogram (375 °C) of lincomycin showed many fast moving peaks and a broad peak at a retention time of 24.6 minutes. Samples of this material were collected and analyzed by mass spectroscopy. It was shown that the broad peak was identified as 1- methyl - 3 - propylpyrrole (Figure X). The 4 - ethyl analogue gave an identical chromatogram except the shift in the retention time of the broad peak to 15.8 minutes.

6.6. Chemical - Ionization Mass Spectrometry (39) :

The election - ionization mass spectrum of lincomycin using isobutane or ammonia as gas reagent produced the protonated molecular - ion $[MH^+]$ as most aboundant peak (base peak) in addition to few fragments of low levels. This technique can be used to determine the purity of lincomycin and to monitor the reactions performed by chemical or biochemical methods.

7. OPTICAL ROTATION MEASUREMENT (40) :

Lincomycin hydrochloride in injections was determined by measuring its optical rotation. In this test, lincomycin hydrochloride in injection was diluted with water and the optical rotation of the resulting solution was determined by polarimetry.

8. METHODS OF ANALYSIS :

8.1. Non - aqueous Titration (41) :

Lincomycin hydrochloride monohydrate (100 mg) was dissolved in a mixture of 5 % mercuric acetate (3 ml) and glacial acetic acid (30 ml). The resulted solution was titrated with 0.1 N $HClO_4$ in which the end - point was detected potentiometrically. Each ml of $HClO_4$ is equivalent to 23.05 mg of lincomycin hydrochloride monohydrate.

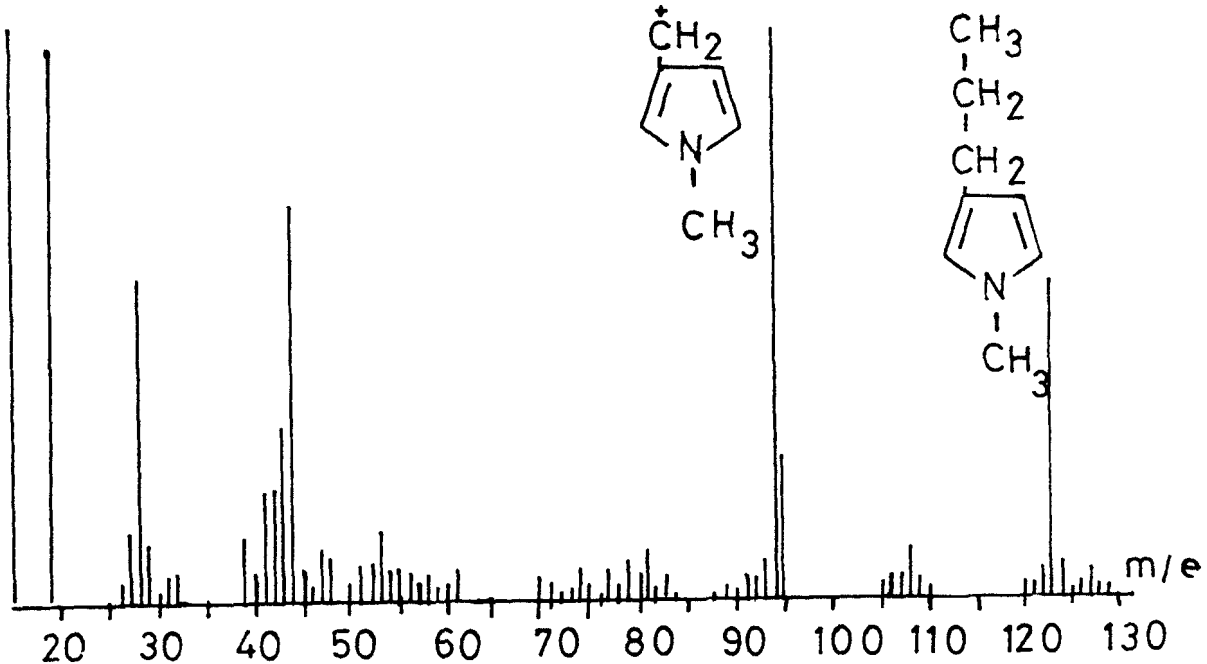


Figure (X) Mass spectrum of the 24.6 minute peak from the lincomycin pyrolysis (38).

8.2. Microbiological :

A microbiological assay was described for lincomycin in animal feed using an agar diffusion assay and an agar slant of Sarcina Lutea ATCC No. 9341 as the test organism for the analysis of lincomycin (42).

A standard curve was prepared by using different concentrations of lincomycin (0.15 , 0.3 , 0.6 , 1.2 and 2.4 µg/ml) as well as a reference concentration of 0.6 ug/ml in phosphate buffer (pH 8). An accurate weight of lincomycin hydrochloride was transfered to 250 ml centrifuge bottle. 0.1 N hydrochloric acid - methanol mixture (1 : 4 , 75 ml) was added and the mixture was shaked for 20 minutes. The mixture was centrifuged and the supernate layer was decanted into 250 ml separator. 75 ml of hexane was added and the mixture was shaked moderately. The lower aqueous layer was drained into 100 ml beaker , then 2.5 - 5 ml aliquot was pipetted into 50 ml mixing cylinder. The volume was adjusted with phosphate buffer solution , (pH 8) , to 40 ml and 1 drop of 4 N sodium hydroxide was added. The content of the mixture was shaked vigorously and made up to 50 ml with buffer of pH 8 . The final concentration of lincomycin base should be 0.5 - 0.8 µg/ml. The mixture was left at 32 °C for 24 hours and the potency of lincomycin was determined from the standard curve.

Food and Drug Administration (FDA) replaced the above mentioned method , " Microbiological agar diffusion assay " , with a microbiological turbidimetric assay because the modified method are more accurate and reliable than agar diffusion assay (43).

The AOAC® official action method for lincomycin has been modified to include the broiler 2 g/ton level and the swine feed levels as high as 2600 gm/ton (44). The procedures involved three different samples preparatory methods with progressive simplification as the concentration of the antibiotic is increased. A 7 - 8 ml agar monolayer was used with slightly lower standard concentration which produce a more sensitive response by the organism and allows for a reduction in sample size for 2 g/ton feeds. Samples prepared by all three techniques were analyzed by bio - plate system.

8.3. Colorimetric Methods :

8.3.1. A colorimetric method was developed by Baudet (45) for determination of lincomycin hydrochloride. The method consists of hydrolysis of the antibiotic and the intensity of the color formed as a result of the condensation reaction occured between the liberated methanethiol and 2,3 - dichloroquinone chlorimide was measured. The intensity of the color formed was found to be proportional to the drug concentration.

8.3.2. Lincomycin hydrochloride can form a color complex with lead salt and gave a distinct peak at λ max 385 nm under visible spectrum. It is possible by this method to determine lincomycin in dry preparation, aqueous solutions and n - butanol extracts (46) as follows :

8.3.2.1. Determination of lincomycin in dry preparations and aqueous solution :

0.1 - 1 ml of lincomycin hydrochloride solution and 0.5 ml of a 0.02 M solution of lead chloride ($PbCl_2$) were introduced into a test tube and the volume was brought up to 3 ml with 1 N hydrochloric acid. The reference solution was prepared by the addition of 2.5 ml of 1 N hydrochloric acid to 0.5 ml of 0.02 M $PbCl_2$ solution. After mixing the content of the tubes, the solution was subjected to photometry for 15 minutes at λ max 385 nm. The resulting absorption satisfies the Bouguer - Lambert - Bar law over the range of lincomycin hydrochloride concentrations from 10 to 1000 AU/ml.

8.3.2.2. Determination of lincomycin hydrochloride in n - butanol extracts :

In order to determine lincomycin hydrochloride in n - butanol extracts, the butanol solution must be diluted to four times of its original volume with ethanol, after which 0.1 - 1 ml of the resulting solution is subjected to the above mentioned steps.

8.3.3. Lincomycin hydrochloride formed a molybdenum blue heteropolycomplex with Folin's reagent. This method was used to monitor lincomycin in the workplace air of antibiotic manufacturing plant at wavelength 600 nm (47).

8.3.4. Lincomycin can form a ternary complex with eosin and palledium (II). This behavior was used for the determination of lincomycin in solutions at 595 nm (48).

8.3.5. A rapid colorimetric assay for the determination of lincomycin injections was developed by Huang and Cao (49).

8.4. Iodometric Titration (50) :

This method was used for the determination of the liberated methanethiol resulted from the destruction of lincomycin in 16 - 23 % sulfuric acid.

8.5. Isotachophoretic Assay (51) :

Lincomycin hydrochloride and aminoglycoside hydrochloride were determined in pharmaceuticals by Isotachophoresis. In this method, a mixture of 0.02 M potassium acetate and 0.3 % hydroxypropylmethyl cellulose (15,000) was used as an aqueous electrolyte system, whereas a mixture of 20 um 1,4 - aminobutyric acid in acetic acid (pH 4.72) or 0.02 M glycylglycine or B - alanine was used as the terminator. The analysis was performed at a constant current of 200 μA at 5 °C. It was found that the minimum amount that could be quantitated by this method was 1.6 nmol and the relative standard deviation was 2 %.

8.6. Mass Fragmentograph (52) :

The amount of lincomycin in blood can be analyzed quantitatively by mass fragmentograph after extraction with Amberlite XAD - 2 resin followed by the formation of trimethyl silyl derivative.

9. BIOSYNTHESIS :

Earlier biosynthesis studies on the lincomycin published by scientists at " The Upjohn Co. " in the 1960's and early 1970's provided the first biosynthetic of a C_2- or C_3 proline unit formed from L - tyrosine. Other scientists have postulated a common biosynthetic pathway arising from tyrosine leading to a common branch - point compound which can be converted to either a C_2 - proline unit (lincomycin B) or a C_3 - proline unit (lincomycin A) (56 , 57). Brahme et al (58) provided the formation of methyl thiolincosaminide from glycose. This was achieved by studying the biosynthesis of the propyl L - hygric acid (PHA) and the methyl thiolincoaminide (MTL) moieties using both carbon - 13 labeled substractes in combination with carbon - 13 NMR and mass spectral analysis. The pathway and the intermediates were postulated from the biosynthetic information obtained from ^{13}C - ^{13}C spin - coupling and those carbon remain on MTL or PHA which are enriched from carbon - 13 .

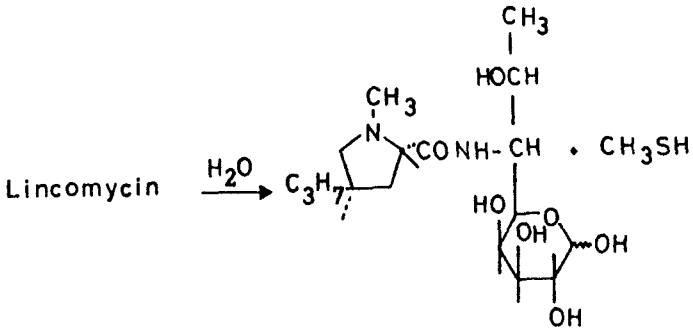
10. MICROBIAL KINETICS :

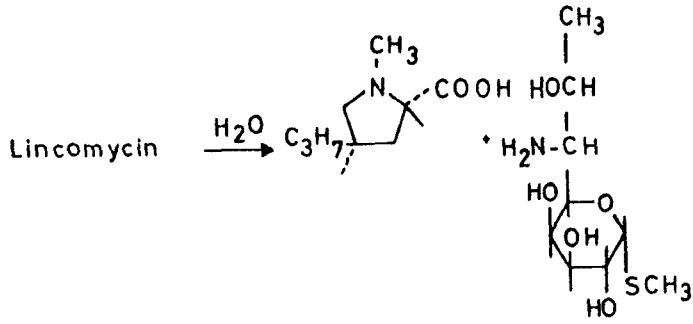
Microbial kinetic studies were applied to the action of lincomycin (59) and clindamycin (60) against Staphylococcus aureus , a representative Gram - positive Coccus . The effects of lincomycin and clindamycin combinations on Staphylococcus aureus were also studied (61). It was found that the effective ratio of a clindamycin - lincomycin combination against Staphylococcus aureus was ranged between 5 : 1 and 9 : 1 . The effective ratio against E. Coli was fixed at 6 : 1 over a wide concentration range. This difference was attributed to the differences in bio - availability and/or binding characteristics of the drug for bioreceptors. Mixture containing equipotent fractions of lincomycin and clindamycin show equivalence of effects on Staphylococcus aureus.

11. DRUG KINETICS (16) :

11.1. Reactions and Rate Equation :

Lincomycin undergoes thioglycoside hydrolysis and amide hydrolysis.



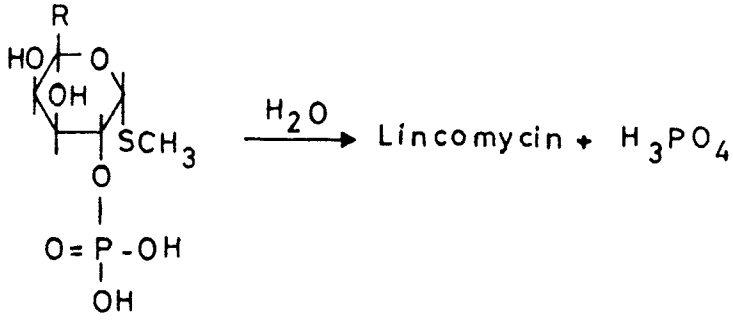


Both reactions are acid-catalyzed. Forist et al (62) studied the rate of loss of lincomycin in 0.1 N HCl (pH 1.10) at 70 °C , finding a half - life of 39 h (K_{obs} = 4.85 X $10^{-6}S^{-1}$). The reaction was first order in lincomycin. Since these hydrolysis reactions are probably similar to the corresponding reactions of clindamycin , the rate equation for overall degradation in the acid region probably can be written as follows :

$$\text{Rate} = K\ [LH^+]\ [H^+]$$

where LH represents protonated lincomycin.

The stabilities of some esters of lincomycin have been studied. The 2-, 3-, and 4- monohexanoate of lincomycin undergo interconversion in alkaline medium, achieving an equilibrium mixture of the three isomers; the isomers then hydrolyzed to lincomycin (63). Lincomycin - 2 - phosphate hydrolyzed to lincomycin and phosphate (19) as follows:



In very acidic medium, it is probable that thioglycoside hydrolysis occurs first, followed by phosphate ester hydrolysis. The hydrolysis reactions of p - substituted o - benzilidene acetals at the 3,4 - positions of lincomycin were studied at different pH's (64). The results show that pH profiles for all the acetals are straight lines in the pH range 1.0 - 3.5 at 37 °C and are in agreement with the following rate law:

$$\text{Rate} = K\,[\text{acetal}]\,[H^+].$$

The reaction is first order in lincomycin acetal.

11.2. pH - Rate Profile:

At pH 1.1 lincomycin has a half - life of 39 h (62). The full pH - rate profile for lincomycin degradation has not been reported. However, on the basis of the known reactions and published data (19, 62 and 64), it can be expected that lincomycin is more stable than clindamycin at all pH values (above about 1.5). In the acidic region, thioglycoside hydrolysis and amide hydrolysis will take place; in the alkaline region, amide hydrolysis is the predominant reaction. The pH - rate profile for the degradation of lincomycin - 2 - phosphate from about pH 0 to 10, at 90 °C, has been reported (19).

The resulted curve obtained show that the hydrolysis of the phosphate group at pH values above 7 is very slow, a plateau from about pH 1 to 6, and a sharp rise below pH 1, resumbly due to acid - catalyzed thioglycoside hydrolysis. Comparing the rates of degradation of lincomycin - 2 - phosphate, at pH 1 to 10; the most labile portion of the lincomycin - 2 - phosphate molecule is the phosphate group, that is, lincomycin - 2 - phosphate hydrolyzes to lincomycin in this pH range before any other portions of the molecule undergo substantial change (19).

12. FORMULATION AND COMBINATIONS (16):

12.1. Degradation Reactions:

Stabililty studies of lincomycin formulations don't appear to have been reported. However, the most probable degradation pathways are thioglycoside hydrolysis and amide hydrolysis (cf. 11.1).

It has been reported that lincomycin cycloamate dihydrate is more thermally stable in the solid state than lincomycin hydrochloride monohydrate (65).

A pediatric formulation of lincomycin - 2 - phosphate at pH 7.5 containing sucrose, sorbitol, glycerin, alcohol, saccharin, preservatives, flavor and color yielded hydrolysis rate constants in reasonable agreement with those predicted from the simple aqueous buffer kinetic data (19).

12.2. Stabilization Methods (16):

The stability of lincomycin formulations solution can be improved by controlling the pH and temperature. In comparison with clindamycin, it can reveal that lincomycin is generally the more stable compound above pH 1.5; below this pH, both compounds have equal susceptibility to hydrolysis.

13. TOXICITY (66) :

Lincomycin has received extensive toxicologic investigation. In initial acute toxicity studies, a high level of tolerance for the antibiotic was shown by four species of laboratory animals. The LD value upon intraperitoneal administration in the mouse was 1000 mg/kg. In the rat, the oral LD value was > 4000 mg/kg. Oral administration of 300 mg/kg for 5 days was well tolerated by the cynomolgus monkey and dog. Blood serum assays indicated that the antibiotic was well absorbed from the gastrointestinal tract and was extensively distributed in body tissues and fluids. Dogs remained clinically healthy when given intramuscular and intravenous doses of 150 mg/kg twice daily for several days. A sublethal intravenous dose of 943 mg/kg produced transient prostration in one dog; oral doses of 4000 mg/kg for 5 days produced no untoward effects.

Long term oral toxicity studies of one month and six months duration in the beagle were evaluated by clinical observations, hematologic data, blood chemistry determinations, organ function values, urinalyses, gross and microscopic examinations of tissues. In neither study was evidence of drug effect observed in groups of dogs that received daily doses of 300 mg/kg or less. Other dogs dosed at 500 and 700 mg/kg for a 3 weeks period also gave no indication of intoxication due to lincomycin. Oral testing was also conducted in white rats at the same levels of administration as for the dogs (30, 100, 300 mg/kg) for 1, 3, and 12 months. In the one month study, the average comparative gains in body weight of the dosed groups were superior to those of the control rats with the exception of the 300 mg/kg group females (14 % less).

In all three studies, terminal hemograms and organ weight data were regarded as normal; no drug - related tissue changes were observed. Subacute parenteral studies were conducted as the oral studies in both species. Excellent subcutaneous tolerance in the rat and intramuscular tolerance in the dog to 30 daily injections of 60 mg/kg of lincomycin was shown.

Six investigation were made on the effect of lincomycin on reproduction in the rat and dog and on the newborn of these species. In one study similar to that recommended by FDA officials (67), breeding rats received daily subcutaneous doses of 75 mg/kg throughout 2 consecutive matins. In another study, pregnant beagle bitches were injected with 50 mg/kg daily during gestation, and their offspring were studied for teratogenic evidence. No drug relationship could be associated with the results of either of these studies. Assay for lincomycin of fetuses and suckinge of dosed rat dams of a third study revealed that placental and milk transfer of the antibiotic doses occur but that the small amounts received by these routes caused no adverse effects. The subcutaneous LD value in newborn rats was 783 mg/kg; the newborn : adult LD ratio was established as approximately 1 : 5. In parallel multiple - dose studies, subcutaneous injections were made for 5 weeks in newborn rats (30 mg/kg) and for 2 weeks in partial litters of newborn beagle pups (30, 60, 90 mg/kg, respectively). Neither series of injections appeared to affect the normal development of these baby animals.

Lincomycin was found to have comparatively low musculoirritant property in a standard test in rabbits. After one week, lions injected with 100 mg in 1 ml had essestially normal appearance. Erythromycin was found to be significantly more irritating; a dose of 50 mg/ml produced a muscle lesion comparable to that caused by a sixfold greater dose of lincomycin.

No drug - related impairments of cochlear or vestibular functions of the eighth cranial nerve were dissolved in groups of cats injected daily with 30 or 60 mg/kg doses of lincomycin for 2 months.

During an antigenicity study, subcutaneous injections of various levels of lincomycin resulted in the eventual loss of several guinea pigs. The underlying cause of death was believed to be associated with alternations of the gram - positive flora of the intestine in this species, similarly as has been demonstrated for other antibiotics that are active against gram - positive bacteria.

14. MODE OF ACTION (68 , 69) :

Lincomycin , an antibiotics produced by Streptomyces lincolnensis, has been used widely in the treatment of infections caused by gram - positive organisms. Lincomycin inhibited microbial protein synthesis by interacting strongly and specifically with the 50 S ribosomal subunit at mutually related sites . The results show that such interaction was not influenced by any of the chemical modifications of lincomycin structure . One molecule of antibiotic bound per 50 S subunit. It inhibited peptide bond formation , a mixture of protein synthesis which was catalyzed by a peptidyl transferase centre on the 50 S subunit. It interfered with substrate binding at the P- or A- site on the catalytic centre. It probably bound to the ribosome in positions at or close to the peptide transferase centre.

15. PHARMACOLOGY :

Lincomycin increased the TEM - 2 ß - lactamase activity of Escherichia Coli (E. Coli) K - 12 cells carrying plasmid RP4 at a concentration which slightly inhibited cell growth (70). In a control culture , ß - lactamase activity reached its maximal level in late log phase, where as when lincomycin was present, ß - lactamase activity continued to increase into the stationary phase. Lincomycin (100 µg/ml) inhibited both cell growth and protein synthesis by about 35 % but the activity of stimulated ß - lactamase was 2.5 - fold per ml of culture and about 4 - fold per cell after 20 hours of growth. Inactivation of ß - lactamase appeared to be faster when lincomycin was present . This was determined by measuring the decrease in ß - lactamase activity when phenethyl alcohol was present to prevent maturation of the enzyme.

The inhibitation effect of lincomycin on the growth of E.Coli carrying plasmid RP4 was studied using the pulse - labeled cultures with [^{3}H] leucine chased with non - radioactive leucine and immunoppted with anti - ß – antiserum (71). The results show that subinhibitory concentrations of lincomycin slightly inhibited the growth of E. Coli and caused a 2 - fold increase in TEM - 2 ß - lactamase . The synthesis rate of ß- lactamase was two times higher in inhibited cultures than in control culture. No significant decrease of labeled enzyme occured during the 30 minutes chase, indicating no degradation of ß - lactamase.

The rate of maturation of pre - ß - lactamase was determined by measuring the decrease in the amount of pre- ß - lactamase after one minute labeling interval. There was no significant difference between the control and lincomycin treated cultures, indicating that posttranslational translocation is not involved in the stimulation.

The effect of antimicrobial agents on serum folate level was studied using the standard autoclaving technique of Lactobacillus casei microbial assay. The blood for serum folate assay was obtained from adult human subjects before and 2 hours after a 24 hours test dose of the antibiotic (72). The results show that lincomycin produced a falsely low serum folate level.

Gryaznova et al (73) studied the effect of lincomycin on metabolism of Actinomyces reseolus producing lincomycin. The results show that lincomycin inhibited protein synthesis by 50 % at 1000 µg/l. When mycelio were cultured for 24 - 69 hours, their sensitivity to lincomycin decreased.

The effect of lincomycin resistance mutations in Escherichia Coli on the alteration of ribosomal proteins and functions was analyzed by Hummel et al (74). The results show that lincomycin did not seem to alter any ribosomal function.

Enzymatic hydrolysis rates for different positional and structural esters of lincomycin were determined in dog serum and simulated USP intestinal fluid (75). In general, the hydrolysis rates were faster in simulated intestinal fluid than in dog serum, indicating a higher esterase activity in simulated intestinal fluid. The 2 - propionate ester of lincomycin was hydrolyzed slower than the longer chain 2 - hexanoate ester, with the greatest difference in rates occuring in simulated intestinal fluid. Sterically hindered esters were hydrolyzed at extremely slow rates.

16. STRUCTURAL - ACTIVITY RELATIONSHIP :

Since the discovery of lincomycin, different structurally modified analogs have been prepared with a view to understanding structural - activity relationships. Replacement of the 7 (R) - hydroxyl group of lincomycin by a 7 - chloro substituent in the 7 (R) configuration increased the in vitro antibacterial activity by a factor of 4. The latter compound, 7 - deoxy-7 (s)-chloro lincomycin hydrochloride, was also more potent than the parent antibiotic on in vivo assay (76 - 77). An increase in the size of the alkyl group at C - $^{-}$4 in the pyrrolidine nucleous also led to enhance antibacterial activity. Lengthening the S - alkyl side chain at the C - 1 position of the amino sugar resulted in increased in vitro activity against Streptococcus aureus, but the effect was reversed in vivo (76).

Modification at the pyrrolidine nitrogen atom resulted in a change of antibacterial spectrum. Thus, N - demethyl - N - ethyl lincomycin hydrochloride was slightly less active than lincomycin hydrochloride against gram - positive organisms when tested in vitro, but about eight times more active against gram - negative organisms. Removing the N - CH_3 group caused reduction in the bioactivity of lincomycin against Sarcina Lutea to 1/20 (77).

The in vitro antibacterial activity of members of leucomycin complex increased with increasing partition coefficient and removal of testification of the three hydroxyl of the macrolide ring. The in vitro activity of the NH lincomycin was positively correlated with log partition coefficeint (Optimum > 2.7) . Electronic and steric factors also influenced the activity of leucomycin and lincomycin antibiotics (78).

17. ACTIVATION ENERGY (16) :

On the basis of the chemical similarities between lincomycin and clindamycin, in very acidic medium, the two compounds should behave similarly. At pH 1.1, the Arrhenius activation energy of clindamycin hydrolysis is 38.0 ± 1.2 kcal/mol (62); under the same conditions, lincomycin is to have the same activation energy. At higher pH values, the reactions of the two compounds will differ. The activation energy for the hydrolysis of the phosphate group in lincomycin - 2 - phosphate in the pH range 6.2 to 8.7 is 32.1 ± 1.2 kcal/mol (19). In the pH range 3.5 to 10, lincomycin - 2 - phosphate is stable (< 10 % hydrolysis) for 2 years at 25°C.

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LOMEFLOXACIN

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1 HISTORY AND THERAPEUTIC CATEGORY

2 DESCRIPTION

- 2.1 Nomenclature
  - 2.1.1 Chemical Name
  - 2.1.2 Generic Name (USAN)
  - 2.1.3 Chemical Abstract Services (CAS)
- 2.2 Structure, Formula, Molecular Weight
- 2.3 Salt forms, Racemates
- 2.4 Appearance

3 SYNTHESIS

4 PHYSICAL PROPERTIES

- 4.1 Infrared Spectrum
- 4.2 Nuclear Magnetic Resonance
  - 4.2.1 Proton Nuclear Magnetic Resonance
  - 4.2.2 Carbon Nuclear Magnetic Resonance
  - 4.2.3 Fluorine Nuclear Magnetic Resonance
- 4.3 Ultraviolet Spectra
- 4.4 Fluorescence Spectra
- 4.5 Mass Spectrum
- 4.6 Melting Range
- 4.7 Thermogravimetric Analysis
- 4.8 Differential Scanning Calorimetry
- 4.9 X-Ray Analysis
  - 4.9.1 X-Ray Crystallography
  - 4.9.2 X-Ray Powder Diffraction
- 4.10 Dissociation Constants
- 4.11 Solubility
- 4.12 Partition Coefficient

5 METHODS OF ANALYSIS

- 5.1 Potentiometric Assay
- 5.2 Spectrophotometric Assay
- 5.3 Microbiological Assay

5.4 High Performance Liquid Chromatography
5.4.1 Assay for *in vitro* studies
5.4.2 Assay for *in vivo* studies

6 STABILITY

7 BIOPHARMACEUTICS

7.1 Mechanism of Antibacterial Action and Resistance
7.2 Pharmacokinetics
7.4 Toxicity

8 REFERENCES

9 ACKNOWLEDGMENTS

1. HISTORY AND THERAPEUTIC CATEGORY

Lomefloxacin is member of the fluoroquinolone class of antimicrobial drugs. It is active against a wide range of gram-positive and gram-negative organisms (1).

The quinolone class of drugs were discovered in the 1960s when Lesher *et al.* isolated nalidixic acid as a by-product of chloroquine synthesis (2). More than a thousand quinolones and analogs have since been synthesized and evaluated in an attempt to reduce toxicity and increase antimicrobial potency. The attachment of a fluorine to C-6 and a piperazine or methylpiperazine to C-7 has led to more active agents such as norfloxacin, ciprofloxacin, ofloxacin and lomefloxacin (3).

Quinolone analogs commercially available in the United States are lomefloxacin (Maxaquin), norfloxacin (Noroxin), ciprofloxacin (Cipro), enoxacin (Penetrex) and ofloxacin (Floxin). Agents such as enoxacin, pefloxacin and ofloxacin are also available in Europe. Other agents such as fleroxacin are expected to be available soon (4).

2. DESCRIPTION

2.1 Nomenclature

2.1.1 Chemical Name

(a) 1-Ethyl-6,8-difluoro-1,4-dihydro-7-(3-methyl-1-piperazinyl)-4-oxoquinoline-3-carboxylic acid;

(b) 6,8-Difluoro-1-ethyl-7-(3-methyl-1-piperazinyl) 4-oxo-1,4-dihydroquinoline-3-carboxylic acid;

(c) 1-Ethyl-6,8-difluoro-1,4-dihydro-4-oxo-7-(3-methyl-1-piperazinyl)-3-quinolinecarboxylic acid

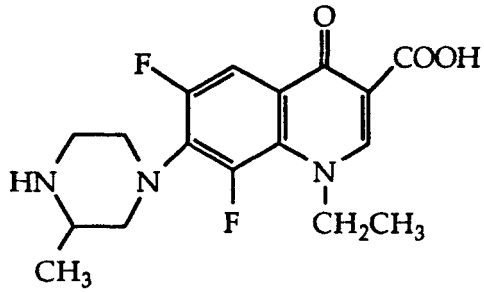
2.1.2 Generic Name (USAN)

Lomefloxacin

2.1.3 Chemical Abstract Services (CAS)
98079-51-7

2.2 Structure, Formula and Molecular Weight

Structure:



Molecular Formula: $C_{17}H_{19}O_3N_3F_2$

Molecular Weight: 351.35

2.3 Salts
Lomefloxacin hydrochloride
Lomefloxacin mesylate

2.4 Appearance, Taste and Odor
A white odorless crystalline powder with a bitter taste (5).

3. SYNTHESIS

Route 1

Lomefloxacin was synthesized by Faba *et al.* (6) by the acidic hydrolysis of 1-ethyl-6,8-difluoro-1,4-dihydro-7-(3-methyl-1-piperazinyl)-4-oxoquinoline-3-carbonitrile with sulfuric acid in a hydro-alcoholic medium. Methanol, ethanol or isopropanol may be used as the reaction solvent and the reaction temperature (75°C-125°C) depends on the composition of the medium. Organic acids or other

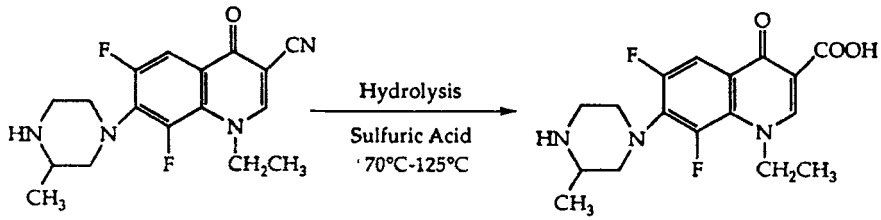


Figure 1. **Synthesis of Lomefloxacin from 1-ethyl-6,8-difluoro-1,4-dihydro-7-(3-methyl-1-piperazinyl)-4-oxoquinoline-3-carbonitrile.**

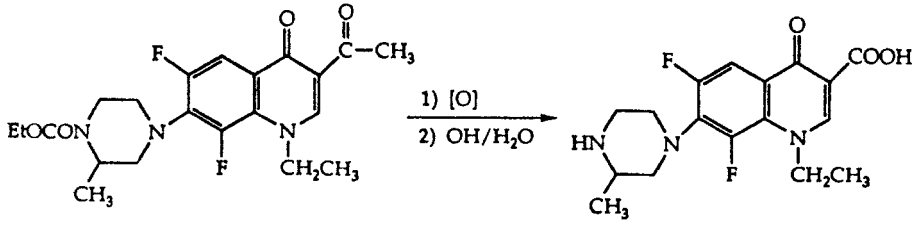


Figure 3. **Synthesis of Lomefloxacin from 3-acetyl-6,8-difluoro-1-methyl-7-(4-ethyxycarbonyl-3-methyl-1-piperazinyl)-4-oxo-1,4-dihydroquinoline.**

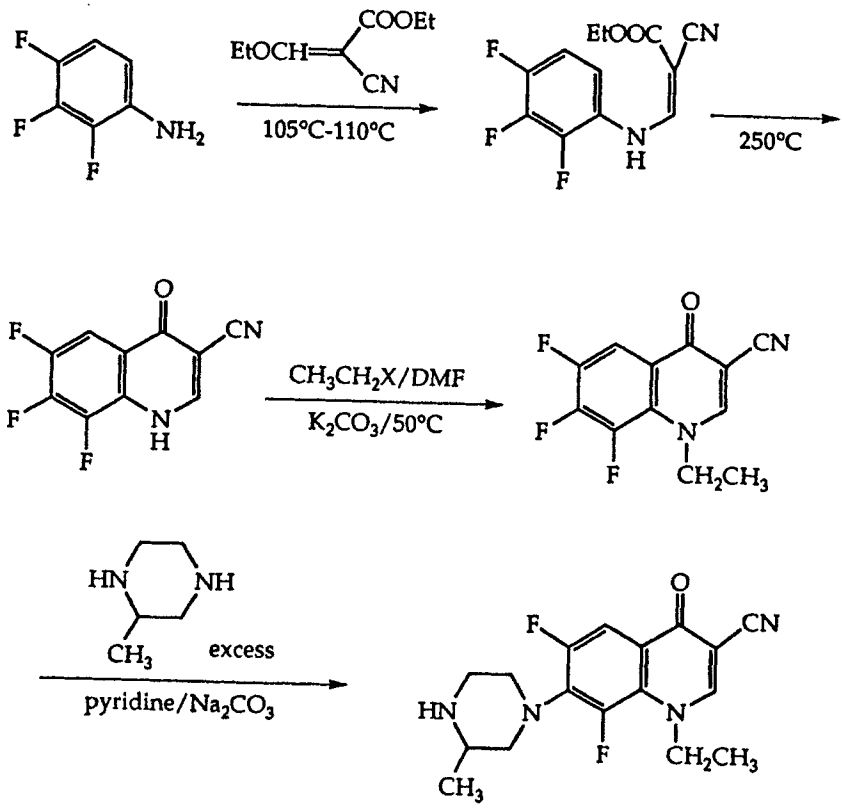


Figure 2. **Synthesis of 1-ethyl-6,8-difluoro-1,4-dihydro-7-(3-methyl-1-piperazinyl)-4-oxoquinoline-3-carbonitrile.**

inorganic acids may be used for the hydrolysis. Isolation and purification was done using conventional methods. The reaction yield was 71 %. The results of the elemental analysis were:

% Theoretical: C: 58.06, H: 5.40, N: 11.95, F: 10.81
% Experimental: C: 58.16, H: 5.67, N: 11.29, F: 10.61

The reaction scheme is depicted in Figure 1.

The synthesis of the carbonitrile intermediate has been described by Marquillas Olondriz *et al.* (7) as a 4-step procedure via a quinoline carbonitrile derivative which is then N-ethylated and condensed with 2-methylpiperazine (Figure 2). The reaction yielded 89 % of the final product.

Route 2

Marquillas Olondriz *et al.* (8) described a synthetic route consisting of oxidation of 3-acetyl-6,8-difluoro-1-methyl-7-(4-ethoxycarbonyl-3-methyl-1-piperazinyl)-4-oxo-1,4-dihydroquinoline to yield the 3-carboxylic analog, followed by deprotection of the piperazinyl amino group by aqueous alkaline hydrolysis. The oxidation may be carried out with sodium hypochlorite or sodium hypobromite. Sodium hydroxide or potassium hydroxide may be used for the alkaline hydrolysis. The reaction yield was 72 %. The results of the elemental analysis were:

% Theoretical: C: 58.11, H: 5.45, N: 11.96
% Experimental: C: 58.17, H: 5.32, N: 12.07

The reaction scheme is depicted in Figure 3.

4. PHYSICAL PROPERTIES

4.1 Infrared Spectrum

The infrared absorption spectrum of lomefloxacin obtained as a Nujol mull on a Bio-Rad FTS-7 spectrophotometer is given in Figure 4. Proposed assignments for the major absorption bands are given inTable1.

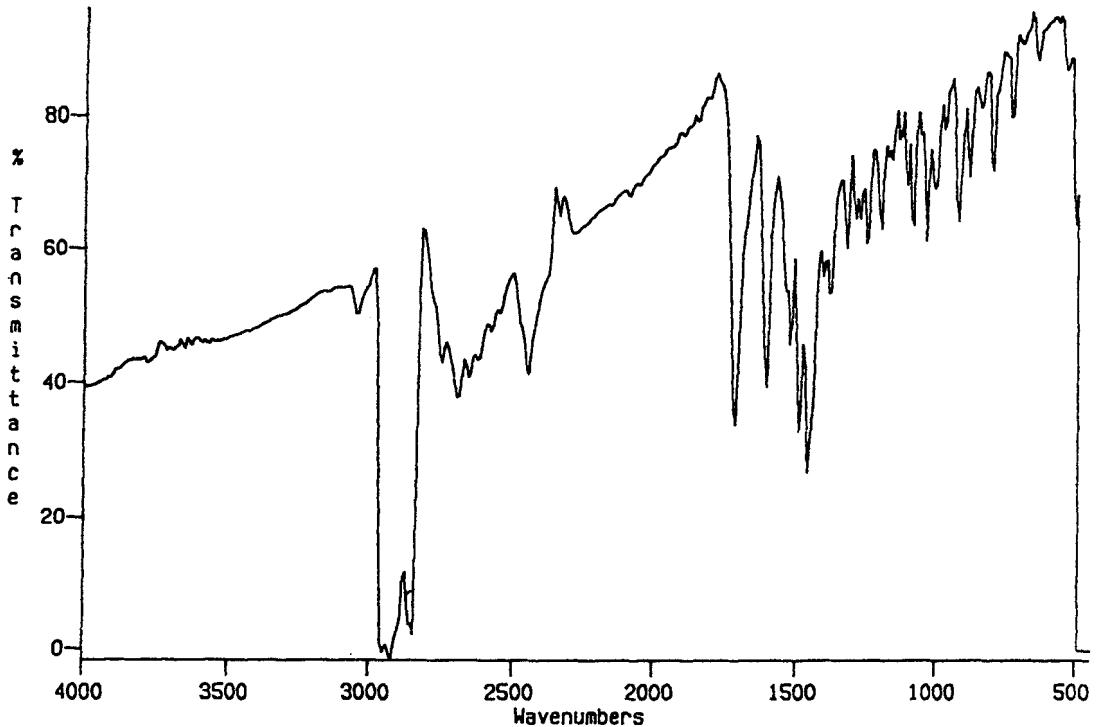


Figure 4. Infrared spectrum of Lomefloxacin hydrochloride.

Table 1
Proposed infrared absorption assignments of lomefloxacin

Wavenumber (cm^{-1})	Assignment
3000-2850	Nujol
2700 (broad)	$-NH_2^+$ stretch, symmetrical and asymmetrical
2464	$-NH_2^+$ overtones and combinations
1726	C=O stretch of carboxylic acid
1620	C=O stretch of pyridone carbonyl
1466	$-CH_2$ scissors vibration and $-CH_3$ antisymmetric deformation
1380	$-CH_3$ symmetric deformation
1208	absorption of aryl fluoride(s)
807	C-H bending of tri-substituted double bond

4.2 Nuclear Magnetic Resonance

4.2.1 Proton Nuclear Magnetic Resonance

The proton nuclear magnetic resonance spectrum of lomefloxacin mesylate obtained in D_2O at 25° C is given in Figure 5 (9). The spectrum was obtained on a Bruker AM-500 NMR Spectrometer operating at 500.13 MHz and was referenced to external TSP [3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid]. The chemical shifts and spectral assignments are provided in Table 2 (9, 10). The effect of increasing concentrations of Al^{3+} on the

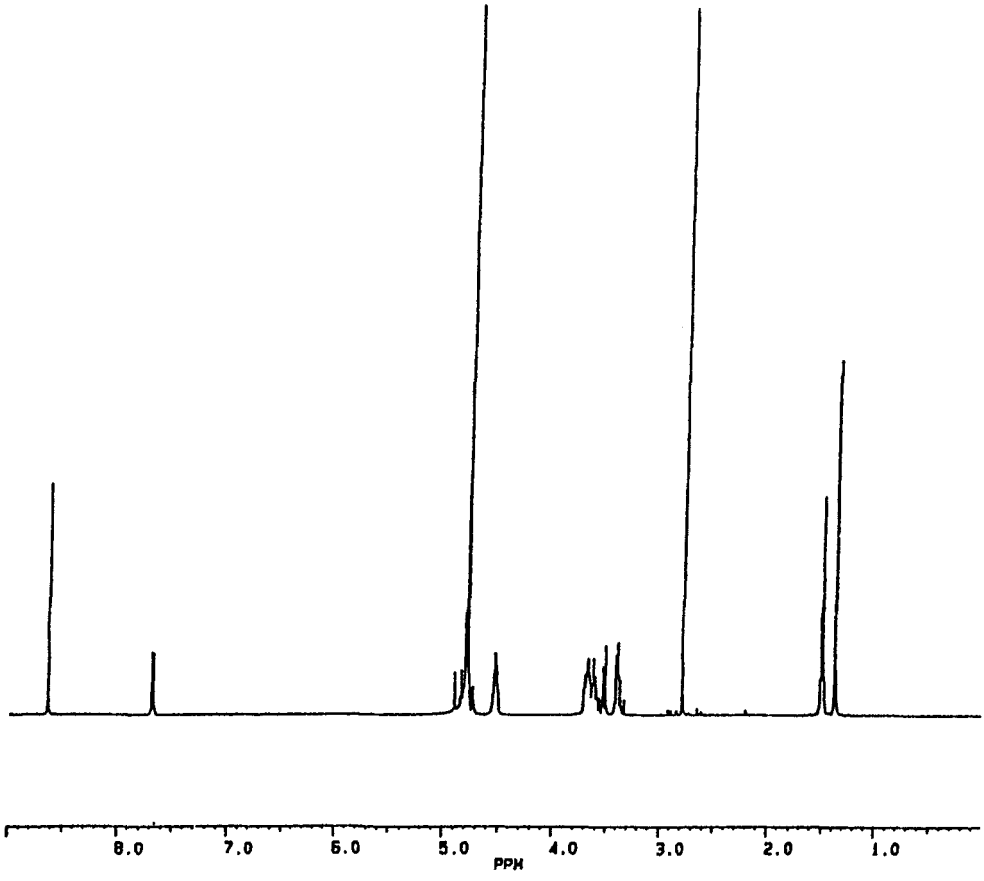


Figure 5. Proton NMR spectrum of Lomefloxacin mesylate.

proton spectrum of lomefloxacin is given in Figure 6 (9, 10).

4.2.2 Carbon Nuclear Magnetic Resonance

The carbon nuclear magnetic resonance spectrum of lomefloxacin mesylate obtained in D_2O at 25°C is given in Figure 7 (9). The spectrum was obtained on a Bruker AM-500 NMR Spectrometer operating at 125.76 MHz and was referenced to external TSP [3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid]. The ^{13}C spectrum was obtained with proton broad-band decoupling and the carbon assignments for lomefloxacin were made using a combination of 1-D and 2-D NMR techniques. The two-dimensional, heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra were taken with standard Bruker pulse programs. The HMQC and HMBC spectra are given in Figures 8 and 9 (9). The chemical shifts and spectral assignments are provided in Table 2 (9, 10). The effect of Al^{3+} on the carbon spectrum of lomefloxacin is shown in Figure 10 (9).

4.2.3 Fluorine Nuclear Magnetic Resonance

The fluorine nuclear magnetic resonance spectrum of lomefloxacin obtained in D_2O at 25°C is given in Figure 11. The spectrum was obtained on a Bruker AM-500 NMR Spectrometer operating at 470.56 MHz and was referenced to external $CFCl_3$. The chemical shifts and spectral assignments are provided in Table 2 (9, 10). The effect of Al^{3+} on the fluorine spectrum of lomefloxacin is given in Figure 12 (9).

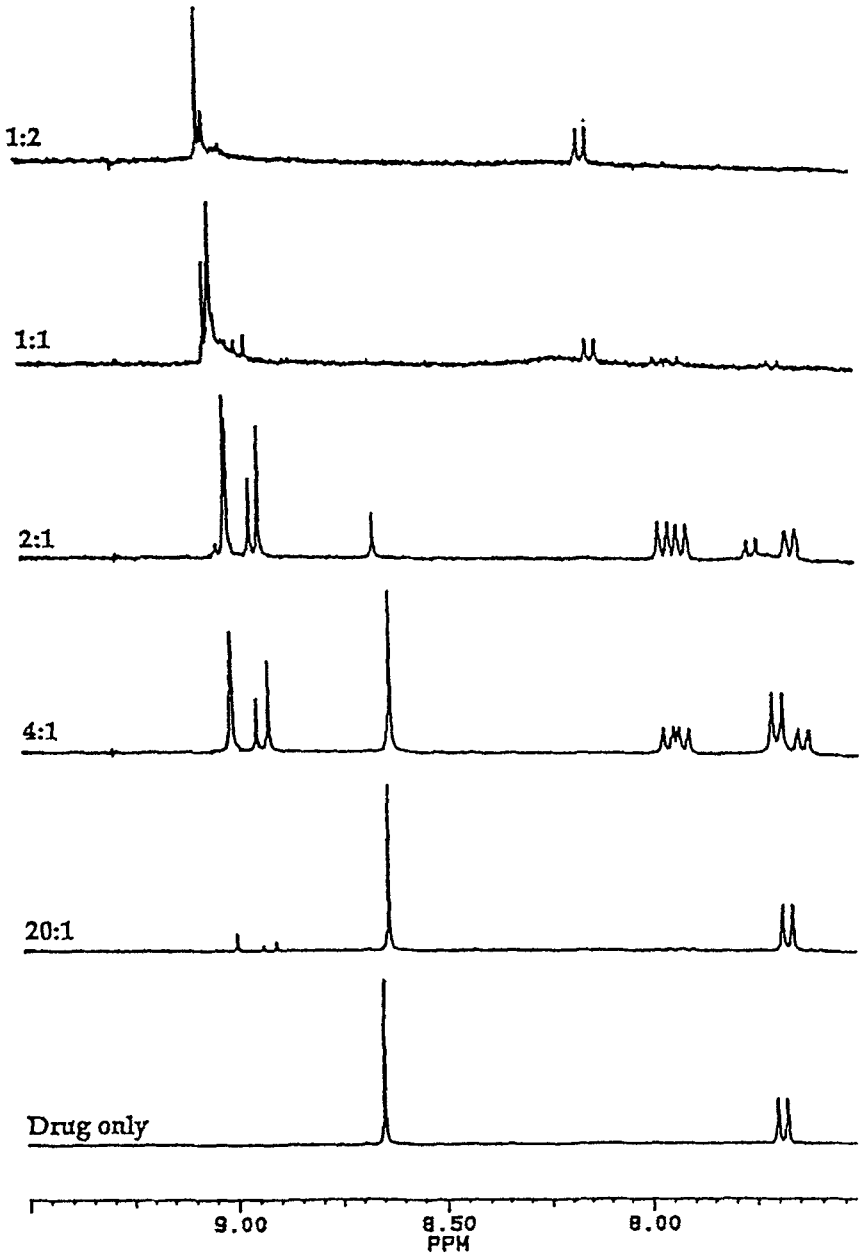


Figure 6. Proton NMR spectrum of the Al(III) complex of Lomefloxacin, expanded to illustrate the aromatic region.

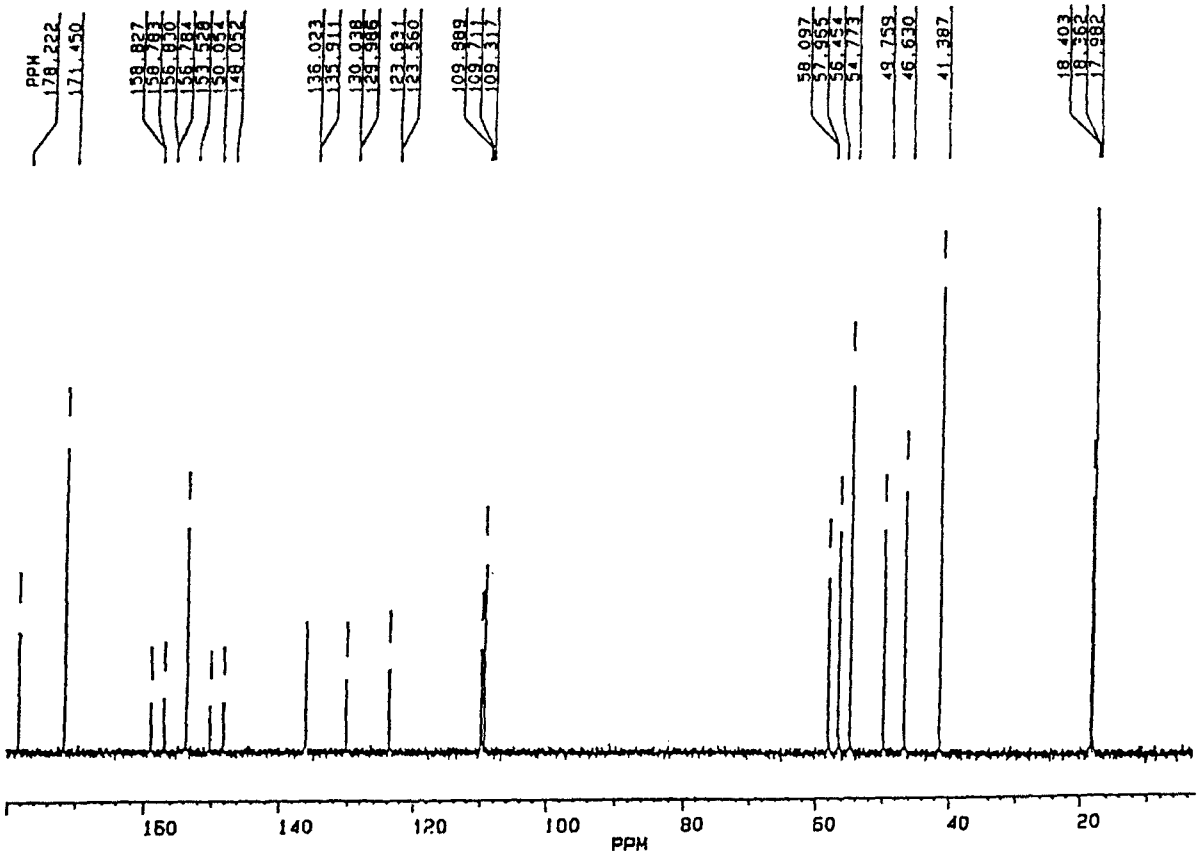


Figure 7. Carbon NMR spectrum of Lomefloxacin mesylate.

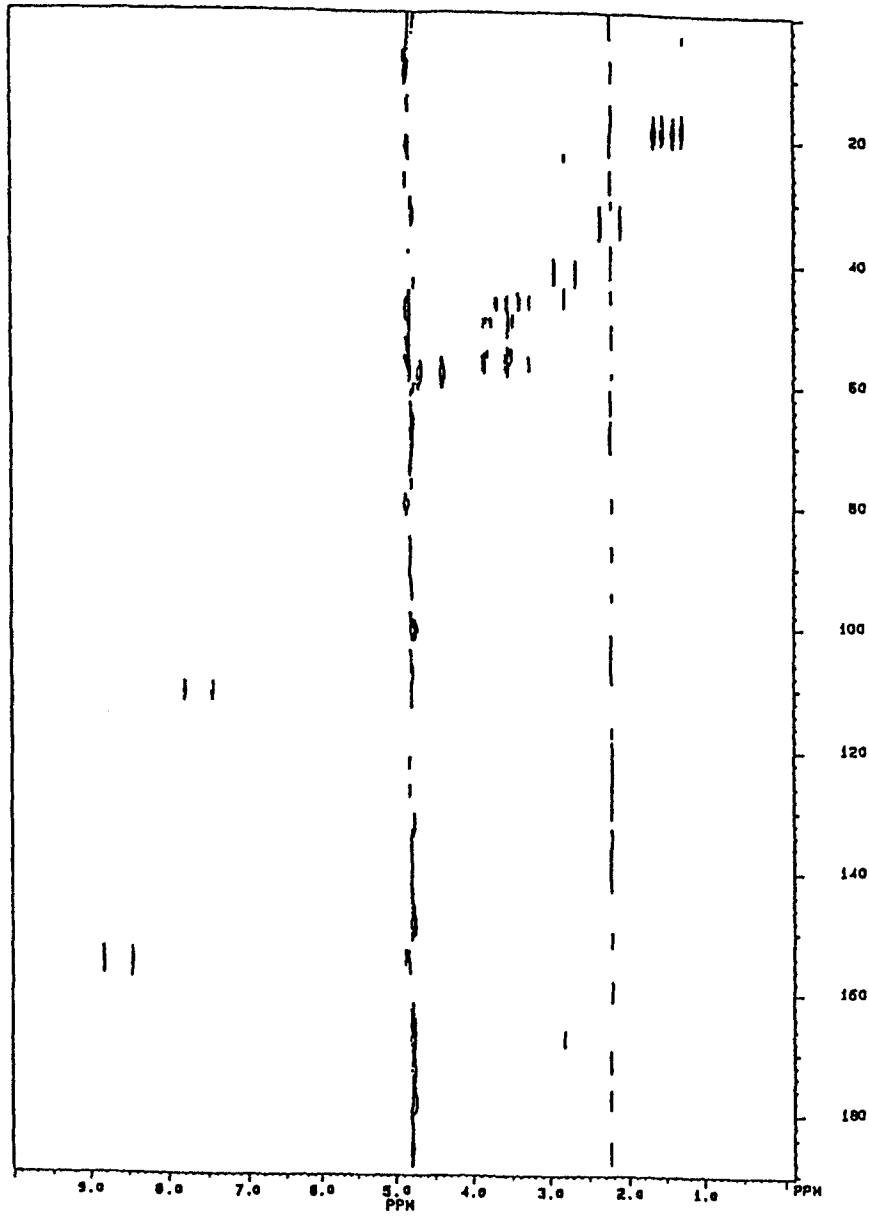


Figure 8. Heteronuclear multiple quantum correlation (HMQC) spectrum of Lomefloxacin mesylate.

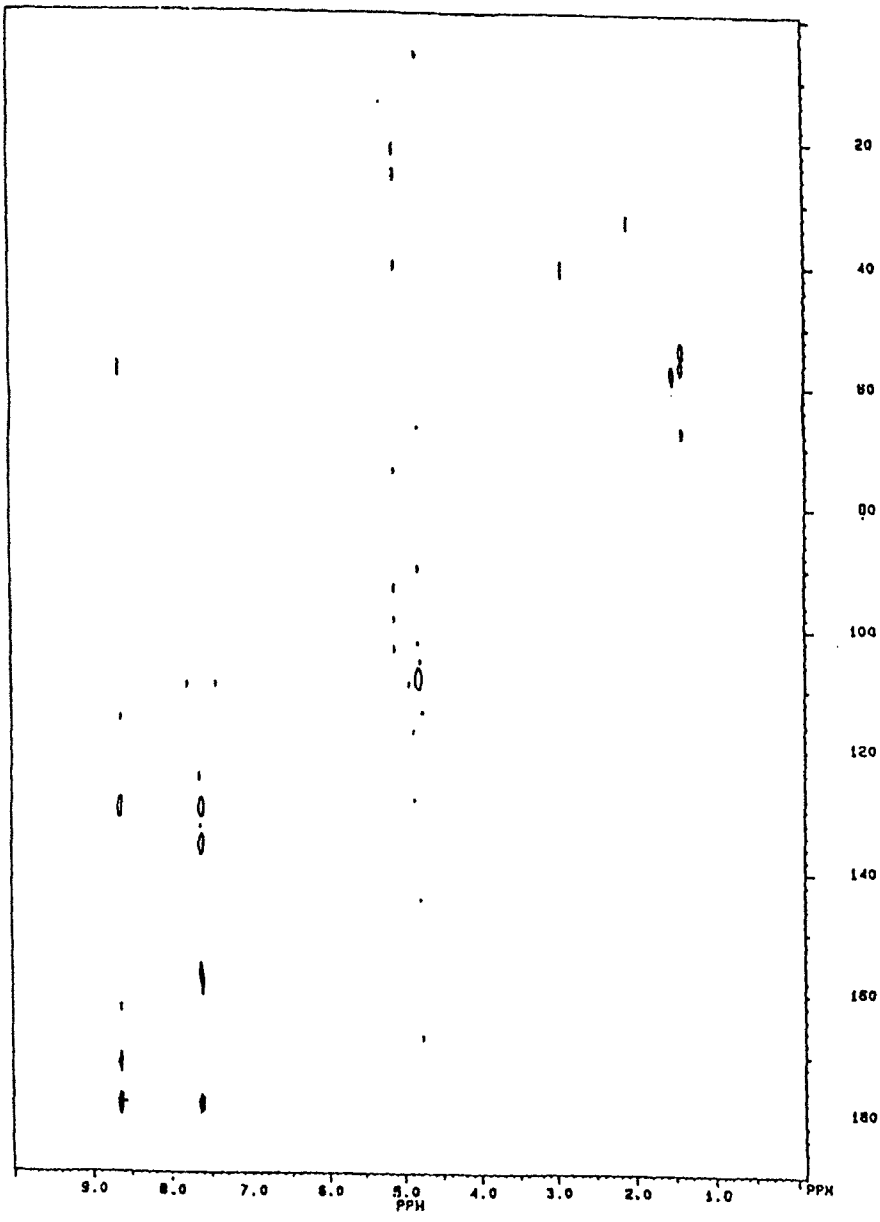


Figure 9. Heteronuclear multiple bond correlation (HMBC) spectrum of Lomefloxacin mesylate.

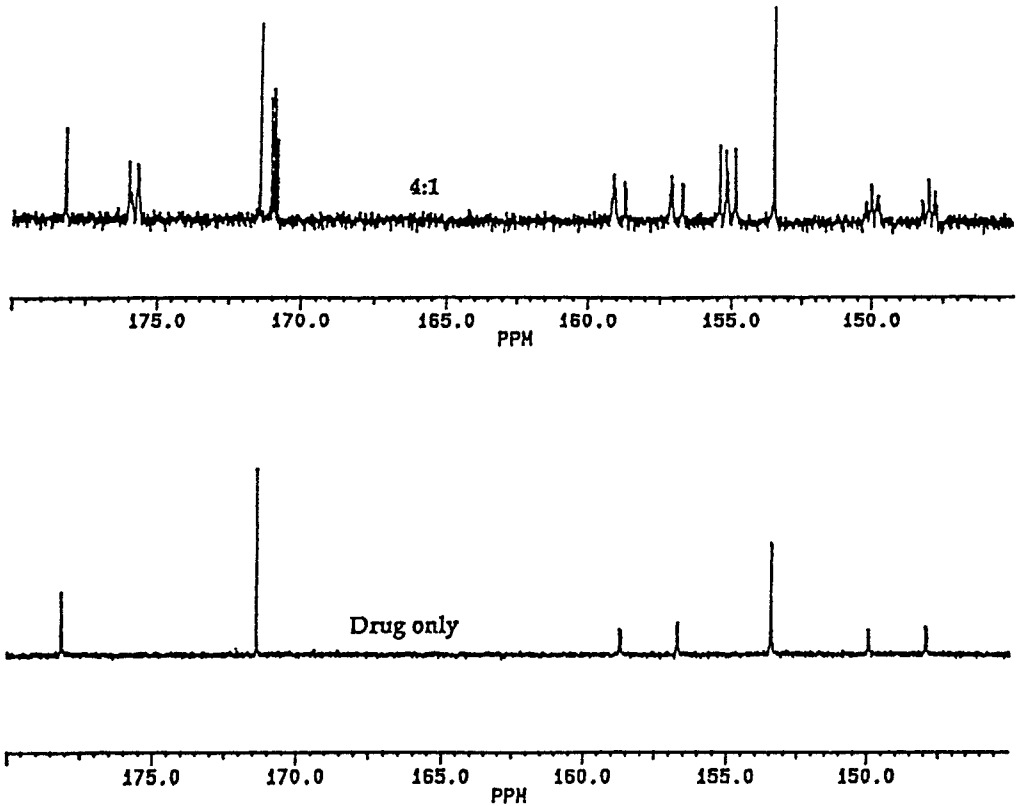


Figure 10. Carbon NMR spectrum of the Al(III) complex of Lomefloxacin, expanded to illustrate the aromatic region.

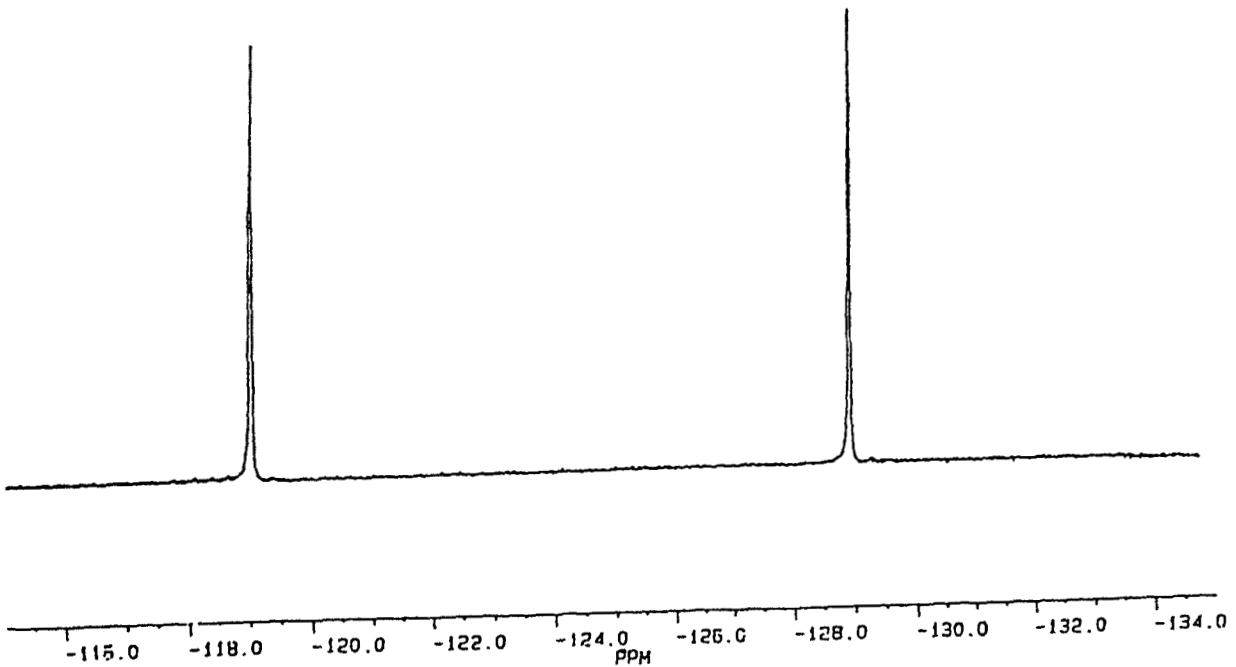


Figure 11. Fluorine NMR spectrum of Lomefloxacin mesylate.

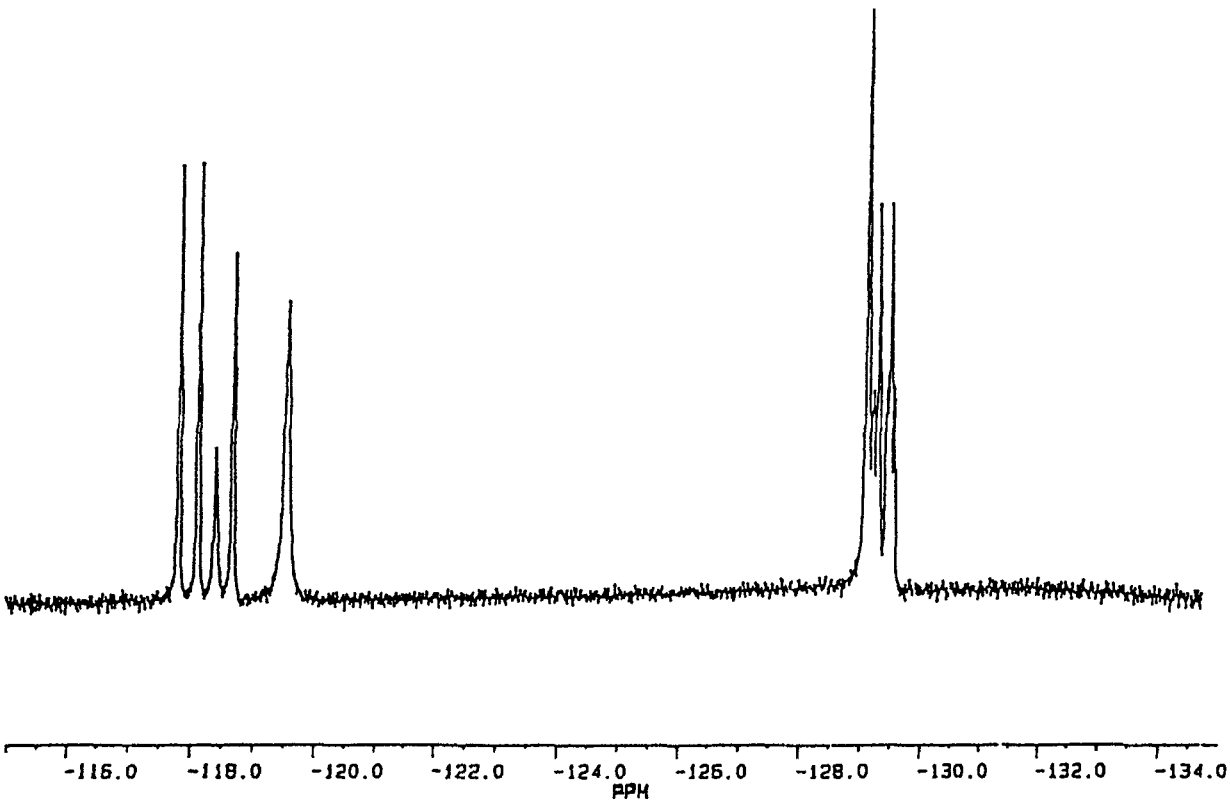
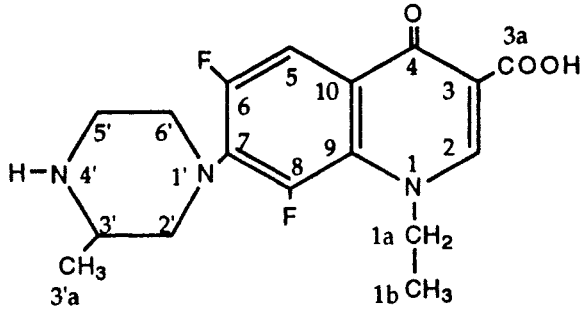


Figure 12. Fluorine NMR spectrum of the Al(III) complex of Lomefloxacin.



Structure of lomefloxacin illustrating the numbering system of the carbons

Table 2

Chemical shifts (ppm) for lomefloxacin mesylate at 25° C and pH 5.3 in D_2O.

Carbon Atom	^1H	^{13}C	^{19}F
4		178.2	
3a		171.5	
6		157.8 (d) J=252 Hz	-119.0 (t) J=12 H
2	8.7	153.8	
8		149.0 (d) J=252 Hz	-128.8 (d) J=12 H
10		136.0 J=14 Hz	
9		130.0 J=7 Hz	
7		123.6 J=9 Hz	
5	7.7 (d) J=12 Hz	109.8 J=22 Hz	
3		109.3	
1a	4.6 (q) J=7 Hz	58.0	
2',6' & 3',5'	3.5 (m)	56.5, 54.8, 49.8, 46.6	
1b	1.5 (t) J=7 Hz	18.4	
3'a	1.4 (d) J=6 Hz	18.0	

4.3 Ultraviolet Absorption Spectra

The ultraviolet absorption spectra for lomefloxacin were obtained on a Hewlett Packard 8451A Diode Array Spectrophotometer as a function of pH. The lomefloxacin solutions were prepared in 0.15 M acetate buffer, 0.05 M phosphate buffer, and 0.15 M borate buffer (μ = 0.15 M with NaCl) at pH 5, 7, and 9, respectively. These spectra are given in Figure 13. The molar absorptivities (a) and absorption maxima as a function of pH are summarized in Table 3.

Table 3

Ultraviolet absorption maxima and molar absorptivities (a) as a function of pH

pH	λmax (nm)	a x 10^{-3}
	226	15.5
5	288	36.7
	320	13.5
7	282	31.5
	326	13.3
9	282	29.3
	328	13.2

4.4 Fluorescence Spectra

The fluorescence spectra for lomefloxacin were obtained on a SLM-Aminco 4800 Spectrofluorometer. The excitation spectra were scanned as the emission wavelength was held constant at 420 nm, while the emission spectra were scanned holding the excitation wavelength constant at 350 nm. The spectra were obtained as a function of pH at a constant concentration

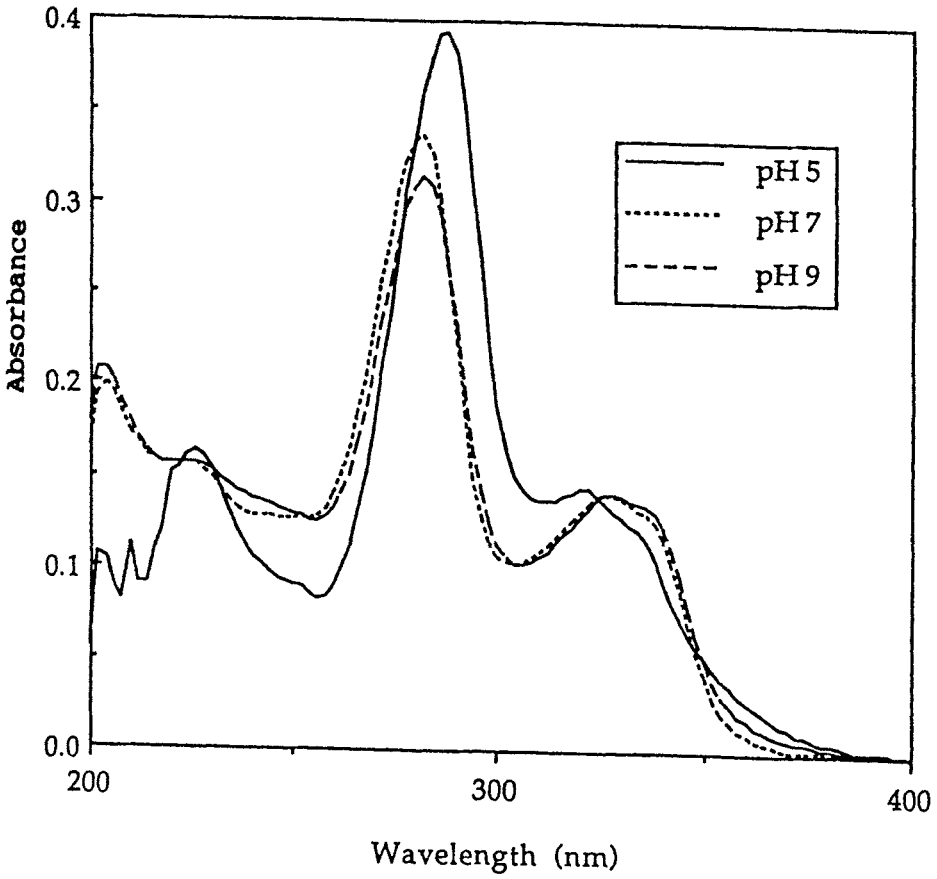


Figure 13. **Ultraviolet absorption spectra of Lomefloxacin as a function of pH.**

of 0.1 mg mL^{-1}. The lomefloxacin solutions were prepared in 0.15 M acetate buffer, 0.05 M phosphate buffer, and 0.15 M borate buffer (μ = 0.15 M with NaCl) at pH 5, 7, and 9, respectively. The fluorescence spectra at pH 5, 7, and 9 are shown in Figures 14-16. Table 4 summarizes the excitation and emission maxima for lomefloxacin as a function of pH.

Table 4

Fluorescence excitation and emission maxima as a function of pH

pH	excitation wavelength (nm)	emission wavelength (nm)
5	255, 355	450
7	255, 310, 350	405
9	255, 310, 350	405

4.5 Mass Spectrum

The electron ionization (EI) and chemical ionization (CI) mass spectra of lomefloxacin were obtained on a Nermag R10-10 quadrupole GC/MS system with SPECTRAL 30 data system. Samples were desorbed from a heated wire in the ion volume using a heating current of 50 mA/s programmed to 500 mA. The source temperature was 250°C. The emission current was 200 mA and 70 eV electron energy was used. The EI spectrum of lomefloxacin is shown in Figure 17. An interpretation of the spectrum is given in Table 5. CI spectra were obtained with ammonia as the reagent gas at a source pressure previously optimized using perfluorotributylamine derived ions. The CI spectrum gave an M+1 signal of 352. Determination of the exact mass of the fragments was performed by high resolution mass spectroscopy as a positive EI voltage scan on a VG

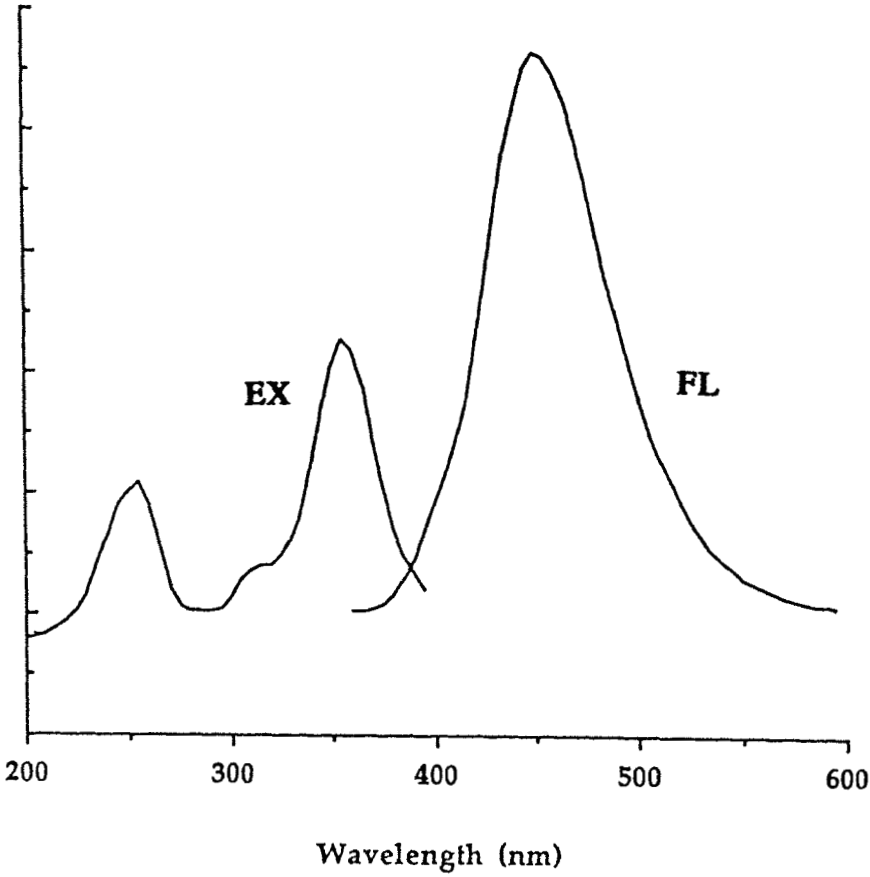


Figure 14. Excitation (EX) and fluorescence (FL) spectra of Lomefloxacin at pH 5.

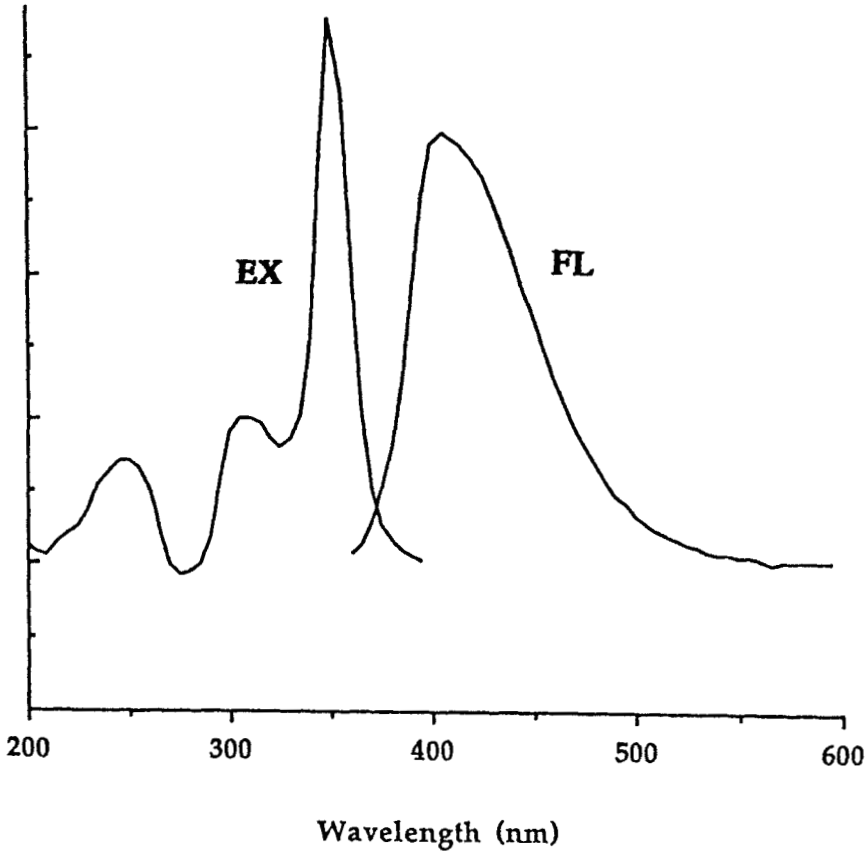


Figure 15. Excitation (EX) and fluorescence (FL) spectra of Lomefloxacin at pH 7.

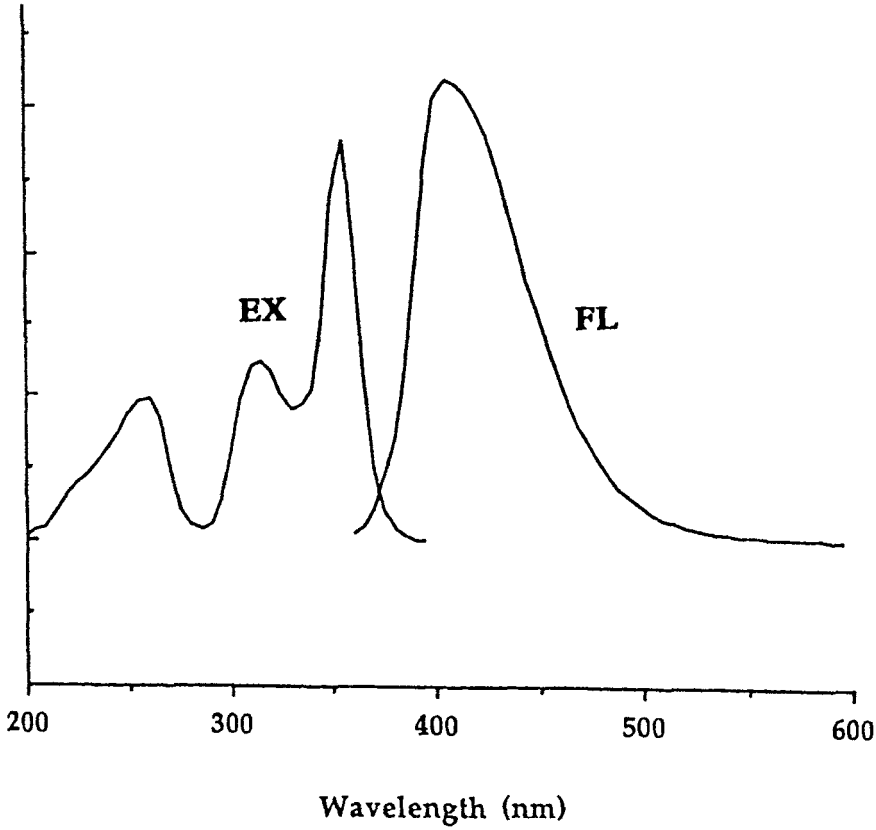


Figure 16. **Excitation (EX) and fluorescence (FL) spectra of Lomefloxacin at pH 9.**

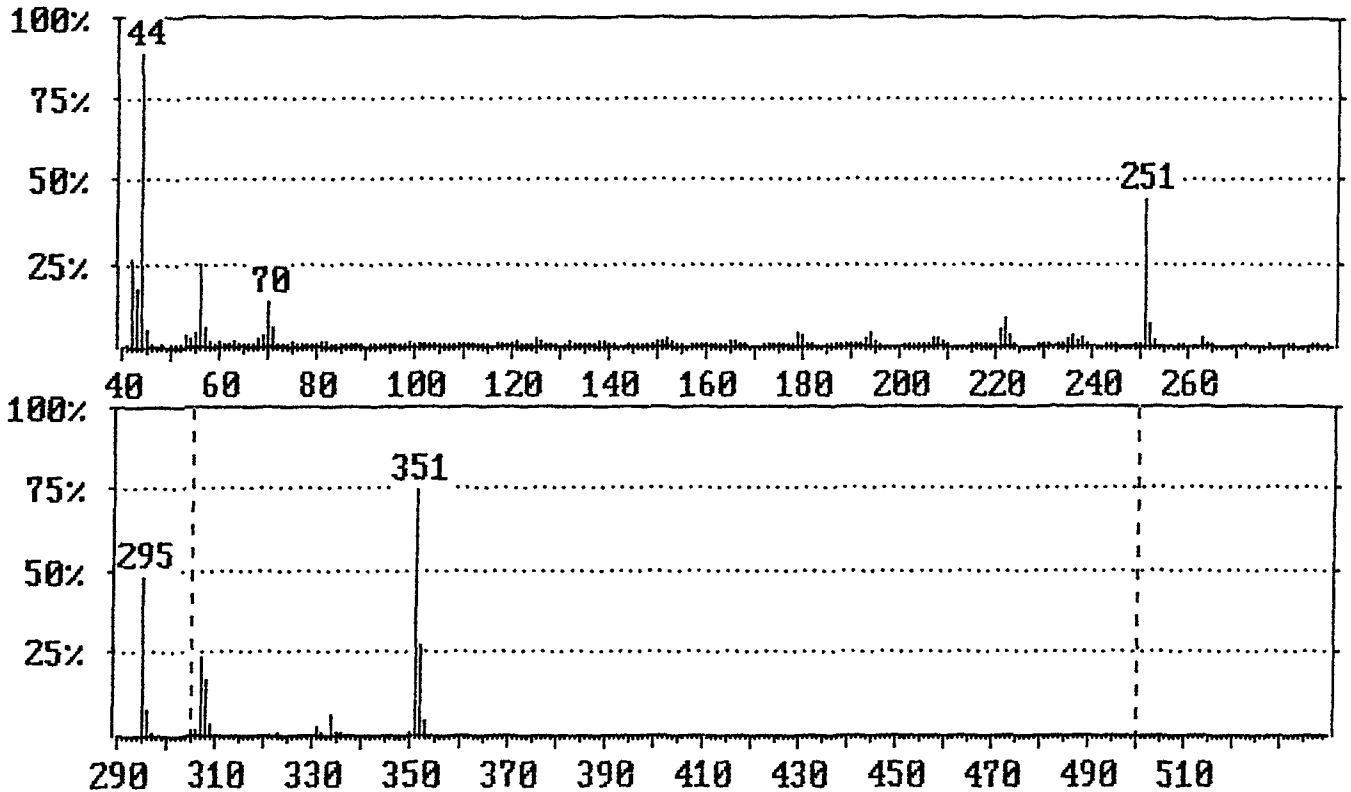
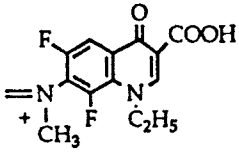
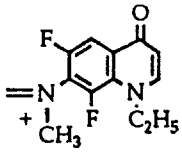
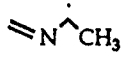


Figure 17. Electron-impact mass spectrum of Lomefloxacin.

Table 5

Mass spectral assignments for lomefloxacin

m/z	Assignment	Experimental Exact Mass	Calculated Exact Mass
351	$C_{17}H_{19}N_3O_3F_2$ M^+ (free base)	351.1384	351.1394
295	$C_{14}H_{13}N_2O_3F_2$ 	295.0896	295.0895
251	$C_{13}H_{13}N_2OF_2$ 	251.1017	251.0996
70	C_4H_8N	70.0626	70.0656
56	C_3H_6N 	56.0494	56.0500
44	CO_2	43.9898	43.9898

AutoSpecEQ Spectrometer. Results are shown in Table 5.

4.6 Melting Range

The melting point of lomefloxacin was determined visually (11). The melting range for lomefloxacin was 235-245° C for the solid collected immediately after titrating to pH 7 (the zwitterionic species). The range for the mesylate salt was 314-319° C. The Merck Index reports the melting point for lomefloxacin to be 239-240.5° C and the hydrochloride salt to be 290-300° C. The salt form decomposes at that temperature (12).

4.7 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis of lomefloxacin hydrochloride was performed on a Shimadzu TGA-50 instrument. The compound was heated from room temperature to 350°C at a rate of 5°C/min. The temperature was held constant at 120°C for 20 minutes and 350°C for 5 min. The thermogram is given in Figure 18. A gradual weight loss from room temperature to 300°C is shown, followed by a rapid weight loss above 300°C. The total weight loss was approximately 46%.

4.8 Differential Scanning Calorimetry (DSC)

The differential scanning calorimetry thermogram of lomefloxacin hydrochloride obtained on a Shimadzu DSC-50 instrument at a scan rate of 5°C/min is given in Figure 19. The thermogram exhibits a sharp melt peak with an onset temperature of 294°C and an extrapolated melting point of about 304°C followed by decomposition.

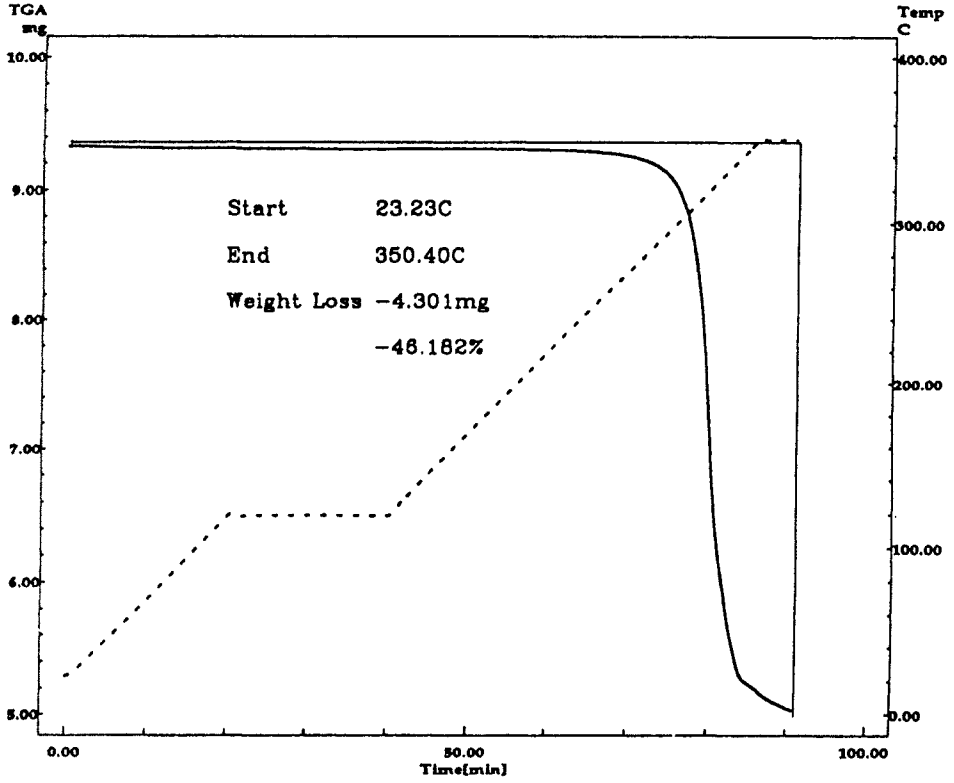


Figure 18. Thermogravimetric analysis of Lomefloxacin hydrochloride.

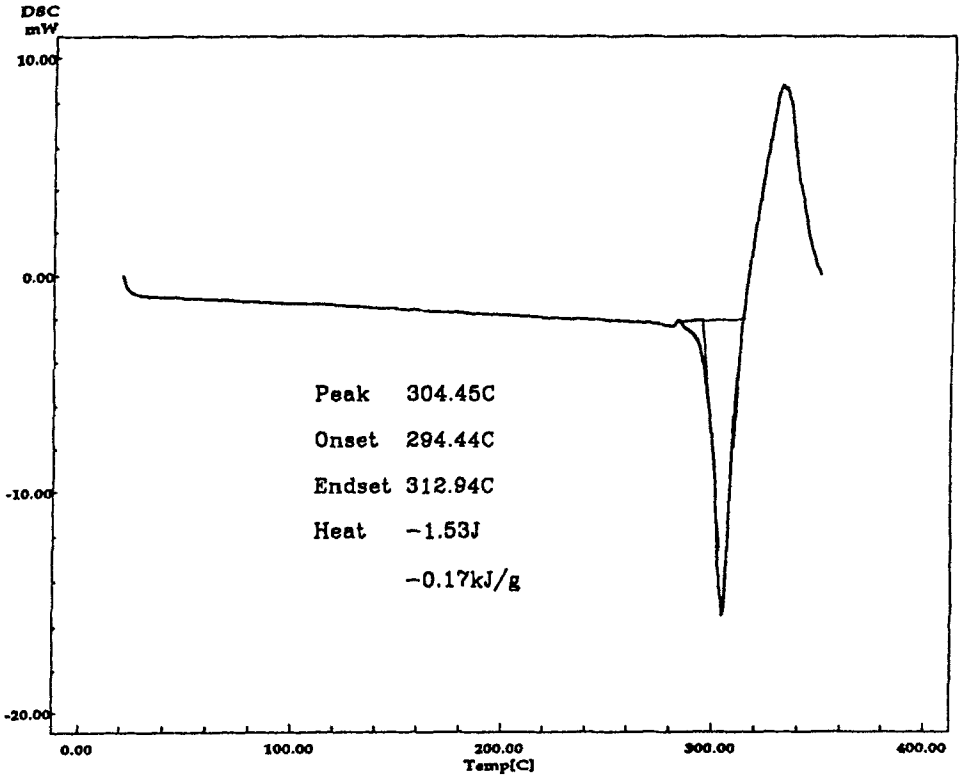


Figure 19. Differential scanning calorimetry thermogram of Lomefloxacin hydrochloride.

4.9 X-Ray Analysis

4.9.1 X-Ray Crystallography

The X-ray crystallography of lomefloxacin has been reported by Riley, *et al.* (10). A colorless crystal of $C_{17}H_{19}F_2N_3O_3 \cdot 0.59H_2O$ having approximate dimensions of 0.100 x 0.300 x 0.300 mm, obtained by recrystallization from a saturated aqueous solution, was mounted in a glass capillary. All measurements were made on a Rigaku AFC5R diffractometer with graphite monochromated Cu Kα radiation and a 12 kW rotating anode generator. Cell constants and an orientation matrix for data collection, obtained from a least-squares refinement using the setting angles of 23 carefully centered reflections in the range $69.43 < 2\theta < 69.96°$ corresponded to a monoclinic cell with dimensions: a = 22.826(3), b = 14.184(1), c = 11.464(1) Å, β = 93.81(2)°, V = 3703.4(6) $Å^3$. For Z = 8 and F.W. = 360.79, the calculated density was 1.294 g cm^{-1}. Based on the systematic absences, h + k = 2n +1 in *hkl* and l = 2n + 1 in *h0l*, the space group was determined as C2/c. The data were collected at a temperature of 298 K. The structure was refined to a final R = 0.064 for 2391 observed reflections ($R^2 > 0.01\ \sigma(R^2)$) (9, 10).

4.9.2 X-Ray Powder Diffraction

The X-ray powder diffraction pattern obtained for lomefloxacin hydrochloride on a Philips model APD 3720 system, equipped with a vertical goniometer in the θ/2θ geometry and a theta compensating slit, is shown in Figure 20. The copper Ka line at 1.544056 Å was used as the radiation source, and the sample was scanned between 2 and 32 degrees 2-θ, in the step sizes of

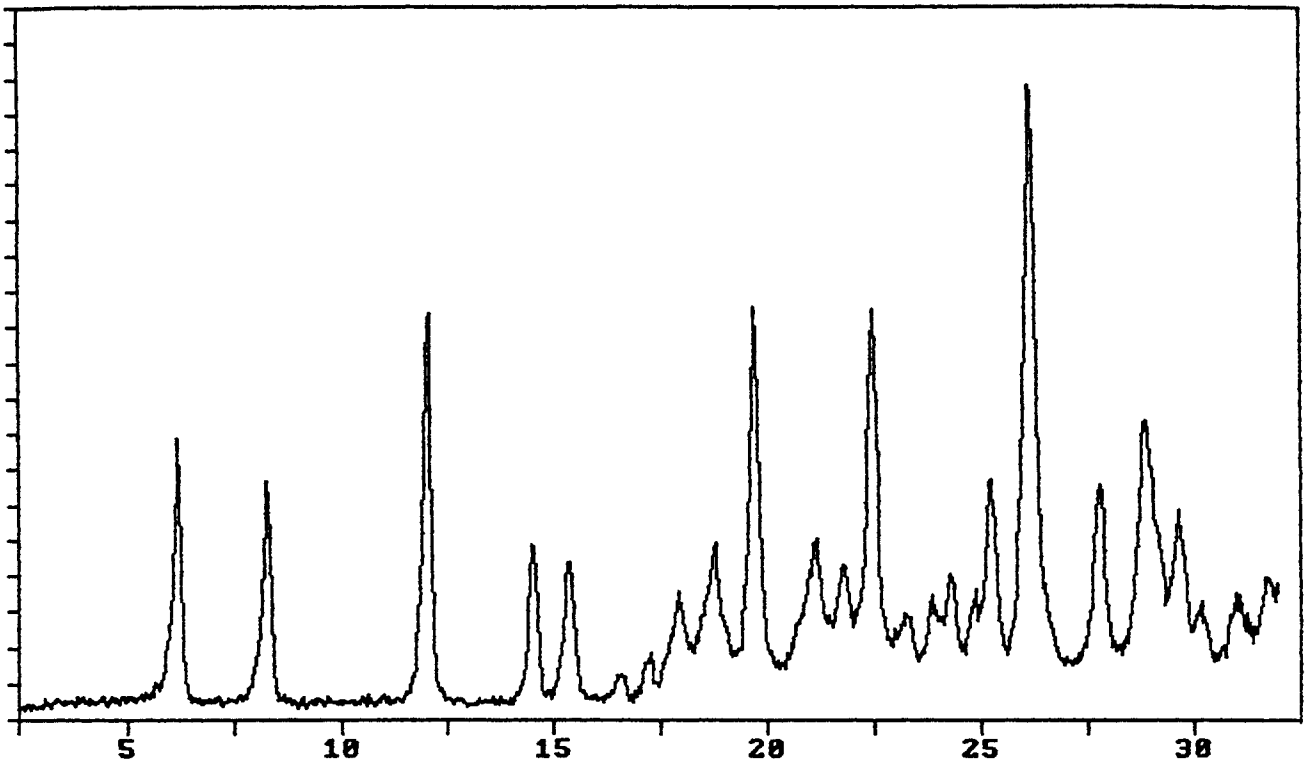


Figure 20. X-ray powder diffraction pattern of Lomefloxacin hydrochloride.

Table 6

Crystallographic Data Associated with the X-Ray Powder Diffraction Pattern of Lomefloxacin HCl

Scattering Angle (degrees 2-q)	d-Spacing (Å)	Relative Intensity (I/I_{max})
6.205	14.2676	100.0
8.310	10.6576	59.6
12.020	7.3752	76.6
14.520	6.1105	25.1
15.350	5.7819	21.6
16.570	5.3589	3.9
17.195	5.1655	5.3
17.895	4.9649	14.5
18.750	4.7404	20.3
19.680	4.5185	47.7
21.115	4.2145	18.2
21.745	4.0938	14.9
22.445	3.9677	41.6
23.270	3.8289	8.7
23.835	3.7394	10.5
24.300	3.6689	11.4
24.785	3.5982	8.8
25.205	3.5392	21.0
26.140	3.4147	56.7
27.765	3.2184	18.6
28.800	3.1051	23.0
29.605	3.0224	15.5
30.175	2.9666	7.2
31.045	2.8855	8.3
31.705	2.8269	9.3

0.04 degrees 2-θ. The data associated with the X-ray diffraction pattern are presented in Table 6.

4.10 Dissociation Constants

Lomefloxacin has two ionizable functional groups: the carboxylic acid function at position 3 and on the piperazinyl ring at position 7. The macroscopic ionization of lomefloxacin is shown in Figure 21. The apparent pKa values for lomefloxacin were determined spectrophotometrically to be 5.82 and 9.30 (11, 13), and potentiometrically to be 5.49 and 8.78 (14), at 25° C.

4.11 Solubility

The aqueous solubility of lomefloxacin was studied (11) as a function of pH and temperature with constant ionic strength of 0.15 M with NaCl. The intrinsic solubility at 25° C was 2.93×10^{-3} M or 1.03 mg mL^{-1}. The aqueous solubility of lomefloxacin mesylate at 25°C and 37°C, pH 5, 7, and 9 is given in Table 6. It was found that the presence of metal ions (Al^{3+}, Ca^{2+}, Mg^{2+} and Fe^{3+}) increased the aqueous solubility of lomefloxacin. In addition, the aqueous solubility of lomefloxacin increased to a constant value with increasing Bi^{3+}, followed by a marked decrease in solubility at higher Bi^{3+} concentrations. (15). Lomefloxacin is known to bind to all of the above metal ions. (16)

Table 7

Aqueous solubility of lomefloxacin as a function of pH

pH	Aqueous Solubility at 25°C (mg mL^{-1}) ± *	Aqueous Solubility at 37°C (mg mL^{-1}) ± *
5	256 ± 7.21	296 ± 37.7
7	1.40 ± 0.03	1.64 ± 0.06
9	2.46 ± 0.18	3.75 ± 0.24

* standard deviation, n=3

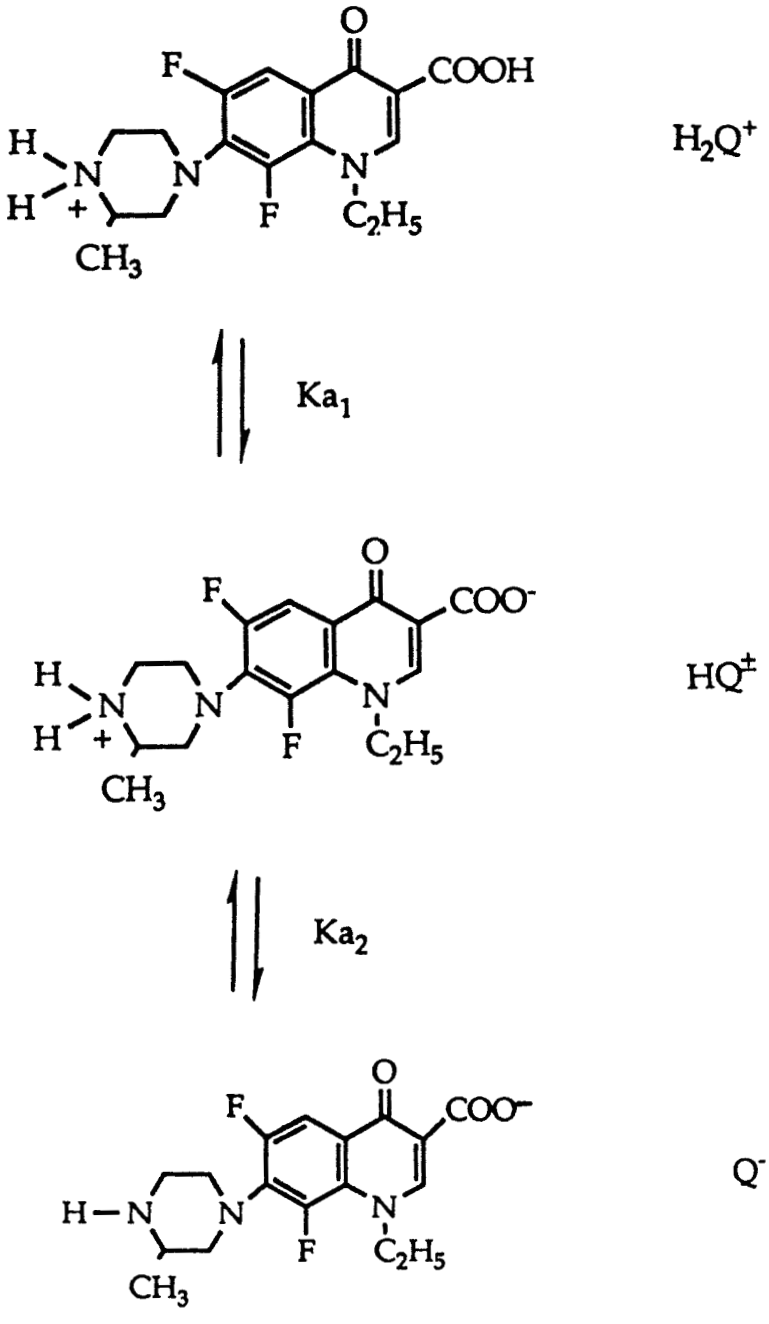


Figure 21. Macroscopic ionization of Lomefloxacin.

4.12 Partition Coefficient

The distribution of lomefloxacin between 1-octanol and aqueous buffers was studied at 25°C, pH 5, 7 and 9 (17). The apparent partition coefficients of lomefloxacin as determined by the analysis of both the organic and aqueous phases using spectrophotometry were 0.118 ± 0.001, 0.139 ± 0.004 and 0.112 ± 0.002, respectively. The thermodynamic partition coefficient was determined to be 0.146 ± 0.005. Takacs-Novac, *et al.* (18) measured the octanol-water partition coefficient spectrophotometrically by analysis of the aqueous phase only and obtained a value of 0.150. It was found that the presence of metal ions (Al^{3+}, Ca^{2+}, Mg^{2+}, Bi^{3+} and Fe^{3+}) decreased the partition coefficient of lomefloxacin. (15, 19).

5. METHODS OF ANALYSIS

5.1 Potentiometric Assay

Takacs-Novak *et al.* (14) used a potentiometric assay in combination with UV spectroscopy and NMR to study the acid-base properties and protonation equilibria for lomefloxacin and other quinolone antibacterials. Okabayashi *et al.* used potentiometric measurements to determine the stability constants of metal ions with several quinolones including lomefloxacin (20).

5.2 Spectrophotometric Assay

Ross and Riley (11) determined the macroscopic dissociation constants for lomefloxacin using UV spectrophotometric measurements at 266 nm. Takacs-Novak *et al.* used UV spectrophotometry to determine octanol/water partition coefficients for lomefloxacin and related quinolones and related them to their protonation equilibria (18).

5.3 Microbiological Assay

Leigh *et al.* used a microbiological assay to study the antibacterial properties of lomefloxacin (21). Disc susceptibility tests were carried out by measuring zones of bacterial growth inhibition following application of 5 and 10 µg lomefloxacin discs on sensitivity test agar. Lomefloxacin was found to have marked activity against Gram-negative bacteria with minimum inhibitory concentrations (MICs) of 0.25 mg/L or less. Gram-positive bacteria were more resistant, particularly streptococci, where MICs varied between 1 and 8 mg/L. Leigh *et al.* also used this assay to study the effect of the drug on the faecal flora of human volunteers (22). minimum bactericidal concentrations (MBCs) were also determined. The results were similar to those described *in vitro* .

5.4 High Performance Liquid Chromatography

5.4.1 Assays for *in vitro* studies

Ross and Riley (11) reported an HPLC method for determination of solubilities of lomefloxacin and other fluoroquinolones. An MOS Hypersil (C_8) reversed-phase column (5 µm, 15 cm x 4.6 mm) was used with UV detection at 280 nm. The mobile phase was tetrahydrofuran-acetonitrile-H_3PO_4 (10 mM)-triethylamine (10 : 30 : 60 : 0.03 by volume) with a flow rate of 1.5 mL/min.

Pascual *et al.* (23) combined HPLC with fluorescence detection to measure the uptake of lomefloxacin and related quinolones by human neutrophils. A Bondapak C18 column was used with a mobile phase of 0.025 M H_3PO_4 adjusted to pH 3.0 with tetrabutyl-ammonium hydroxide/ acetonitrile (75 : 25) delivered at a flow rate of 1.5 mL/min. The excitation and emission wavelengths for the fluorescence detector were

340 nm and 425 nm respectively. Simanjuntak *et al.* (24) have described a reversed phase HPLC assay with fluorometric detection for the study of transport of lomefloxacin and ofloxacin in rat erythrocytes *in vitro*. The mobile phase used was acetonitrile-0.05 M citric acid-1 M ammonium acetate (16:83:1 v/v/v) at a flow rate of 1 mL/min. The excitation and emission wavelengths for the fluorescence detector were 340 nm and 460 nm respectively.

5.4.2 Assays for *in vivo* studies

Some representative HPLC assays used for analysis of lomefloxacin from biological samples have been summarized in Table 8 (25-28).

6. STABILITY

Leigh *et al* (21) tested the stability of lomefloxacin solutions in serum, in darkness, shaded light and bright sunlight at 23°C and 4°C and of lomefloxacin impregnated discs at 37°C, 23°C, 4°C and -20°C. Lomefloxacin concentrations were determined at preset time intervals, by the microbiological assay using the sensitivity agar method.

Lomefloxacin solutions (50 mg/L) in serum showed considerable rapid degradation in light at 23°C and 4°C, with a 52 % loss in activity in shaded light in 1 h and an 80 % loss in activity in bright sunlight in 30 min. Degradation was more rapid for solutions with lower concentrations of 10 and 1 mg/L. Lomefloxacin discs were stable at all temperatures in dark and shaded light. However in sunlight at 23°C, a 79 % loss in activity was seen in 6 h. These results have an important bearing on laboratory storage, handling and testing of lomefloxacin samples.

Matsumoto *et al.* (29) observed the susceptibility of aqueous lomefloxacin solutions when exposed to long-wavelength ultraviolet (UVA) radiation from 0.3 to 3 J/cm^2. Changes in

Table 8

HPLC methods used for analysis of lomefloxacin from biological samples

Application	Column	Mobile Phase	Detection	Reference
Plasma (50 ng/ml) Urine (100 ng/ml)	μBondaPak C18 Radial Pak Cartridge 0.8 x 10 cm	Acetonitrile/14.7 mM acetate, 9.5 mM citrate, 7.2 mM triethylamine, adjusted to pH 4.8 (230:800), 2 ml/min	Fluorescence, excitation, 280 nm emission, 430 nm	25
Plasma (50 ng/ml) and other clinical specimens	Lichrosorb RP-18 250 mm x 4 mm, 10μm	Methanol/Acetonitrile/0.4 M citric acid (3:1:10)	Ultraviolet, 275 nm	26
Serum (500 ng/ml)	μBondaPak C18 Radial Pak Cartridge 0.8 x 10 cm	18 mM KH_2PO_4 with 0.13 mM heptane sulfonic acid/methanol/ conc. phosphoric acid (700:300:1)	Fluorescence, excitation, 288 nm emission, 475 nm	27
Plasma (10 ng/ml)	Nucleosil C18 4.6 mm x 26 cm	Acetonitrile/0.05 M citric acid/1 M ammonium acetate (22:77:1)	Fluorescence, excitation, 280 nm emission, 455 nm	28

UV absorption spectra were seen accompanied by a decrease in antibacterial activity. Other fluoroquinolones were also studied in a similar fashion and the general observations indicate that the substituent in the 8 position played a major role in the photostability of this class of compounds. An 8-methoxy analog was found to be stable to UVA exposure, while the unsubstituted analog (8-H) and the 8-F analogs (e.g.. lomefloxacin) were unstable to such exposure.

7. BIOPHARMACEUTICS

7.1 Mechanism of Antibacterial Action and Resistance

Lomefloxacin and quinolone agents in general are known to exert their antibacterial action by antagonism of the enzyme DNA gyrase also known as bacterial topoisomerase II. DNA gyrase has also been shown to play an important role in the mechanism of bacterial resistance to these agents (30).

Reactions catalyzed by DNA gyrase within living bacteria include introduction of negative supertwists and the decatenation of interlocked circles and the consequent facilitation of DNA replication and repair. Quinolone agents act on this enzyme and cause inhibition of DNA synthesis, antagonism of RNA and protein synthesis and ultimately cell death.

Bacteria have been shown to present either acquired or intrinsic acquired resistance to the quinolone agents. Acquired resistance occurs due to genetic mutations affecting the structure of the A subunit of DNA gyrase as well as membrane permeation of the agents. The mechanism of intrinsic resistance has not yet been defined. It is expected that resistant DNA gyrase or altered drug permeation or both will be involved (30).

7.2 Pharmacokinetics

Pharmacokinetic behaviour of lomefloxacin following oral administration has been well studied (31-33). Doses up to 800 mg have been studied and found to be well absorbed and tolerated and provide appreciable plasma and urine concentrations for 24 h. Single oral 400 mg doses of lomefloxacin produce maximum plasma concentrations of approximately 4 μg/mL which are significantly higher than those produced by equivalent doses of norfloxacin and ciprofloxacin. Total body clearance has been determined to be about 259 mL/min. Also, its elimination half-life determined to be about 8 h is longer than that of other quinolone agents and may permit once-daily dosing in clinical situations. Hooper *et al.* (34) determined the effect of food on absorption and pharmacokinetics of lomefloxacin. Oral administration of a 400 mg dose after high-carbohydrate or high-fat meals resulted in a longer time for peak plasma concentrations but did not change the peak plasma concentration or the total amount of drug absorbed. Shimada *et al.* (35) observed that bioavailability of lomefloxacin decreased by about 40 % when it was administered concomitantly with aluminum- and magnesium-containing antacids. This was explained by formation of chelate complexes of lomefloxacin with Al^{+3} and Mg^{+2} and the reduced absorption of these complexes when compared to the free drug.

Salivary and fecal samples indicate that oral administration of 400 mg lomefloxacin once daily for seven days, results in oral and intestinal aerobic gram-negative microorganisms, while aerobic gram-positive and anerobic microorganisms were unaffected. The oral and intestinal microflora returned to normal levels two weeks after administration was discontinued (36).

Okezaki *et al.* (37) studied the protein binding of lomefloxacin *in vitro* and found it to be 70-80 % unbound in rat, dog and human sera. No significant

species differences in lomefloxacin were seen. Pharmacokinetics of lomefloxacin were studied in patients with impaired renal function (38, 39). Up to five-fold higher elimination half lives were observed indicating the necessity of dosage adjustments in such patients.

Fluoroquinolones are used in the treatment of purulent bronchitis and pneumonia and are hence likely to be administered concomitantly with theophylline. Drugs such as enoxacin and ciprofloxacin have been shown to reduce theophylline clearance by inhibiting metabolism. Lomefloxacin on the other hand has been shown to have no clinically significant effect on theophylline metabolism (40, 41).

7.3 Toxicity

Tesh *et al.* have shown that lomefloxacin when administered in pregnant female rats from day 7 to day 17 of gestation and from day 17 of gestation to day 21 of lactation, did not produce any significant toxic or teratogenic effects (42, 43).
Fluoroquinolones such as ofloxacin, enoxacin, lomefloxacin and ciprofloxacin have been shown to cause cutaneous phototoxicity and photosensitivity in mice induced by oral administration followed by ultraviolet-A irradiation. These reactions have further been shown to be mediated via reactive oxygen species such as hydroxyl radicals and singlet oxygens and not by generation of toxic photoproducts (44).

The effect of lomefloxacin on cultured rabbit corneal epithelial cells was evaluated because of its potential use for ophthalmic application. The results along with pharmacokinetic data indicate that therapeutically effective concentrations may be achieved without any cytotoxic effects (45).

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METHOCARBAMOL

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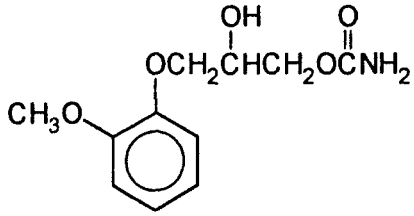
1. Description

1.1 Nomenclature, Formula and Molecular Weight

(±)-Methocarbamol, described in the early literature as AHR-85, is known as 3-(2-methoxyphenoxy)-1,2-propanediol 1-carbamate; 3-(*o*-methoxyphenoxy)-2-hydroxypropyl 1-carbamate; 2-hydroxy-3-(*o*-methoxyphenoxy)propyl 1-carbamate, or guaifenesin carbamate. The term 'methocarbamol' refers to the racemate unless otherwise specified.

Chemical Abstracts Registry No.: [532-03-6]



$C_{11}H_{15}NO_5$ M.W. 241.24

1.2 Appearance, Color, and Odor

Colorless (white) crystals or powder; odorless, or with a slight characteristic odor.

1.3 History

Methocarbamol was developed in 1956 in the laboratories of A.H. Robins. Studies were directed towards the development of propanediol derivatives which possessed muscle relaxant properties superior to those of mephenesin, which had low potency and a short duration of action[1,2,3].

2. Synthesis of Methocarbamol (Figure 1)

A typical synthetic route is illustrated in Figure 1. Reaction of 3-(2-methoxyphenoxy)-1,2-propanediol (guaiacol glyceryl ether) with phosgene[1,4,5], diethylcarbonate[6-9], or ethyl chloro-carbonate (ethyl chloroformate)[10,11], followed by ammonolysis (ammonia or ammonium hydroxide) affords the desired carbamate. Carbon dioxide (at 6500 hPa) as the carbonyl source has also been employed[12]. The guaiacol glyceryl ether is readily prepared from guaiacol (2-methoxyphenol) and epichlorhydrin[11,12]. Synthesis of the S-enantiomer from R-guaiacol glyceryl ether has been reported[9].

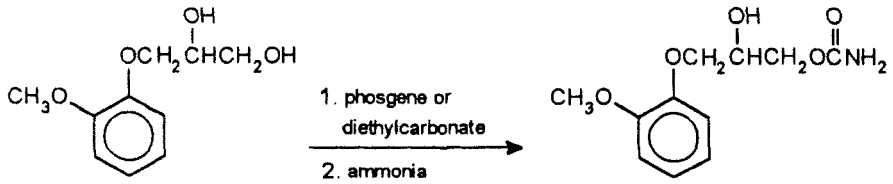


FIGURE 1. Synthesis of Methocarbamol

3. Physical Properties

3.1 Infrared Spectrum

The infrared spectrum of methocarbamol (KBr disc) was recorded on a Nicolet 7199 Fourier Transform spectrometer and is presented in Figure 2. The principal absorption bands include (cm^{-1}):

3442	1439	1116
3330	1401	1091
1681	1390	1057
1610	1369	1031
1509	1256	1016
1466	1223	750
1458	1127	733

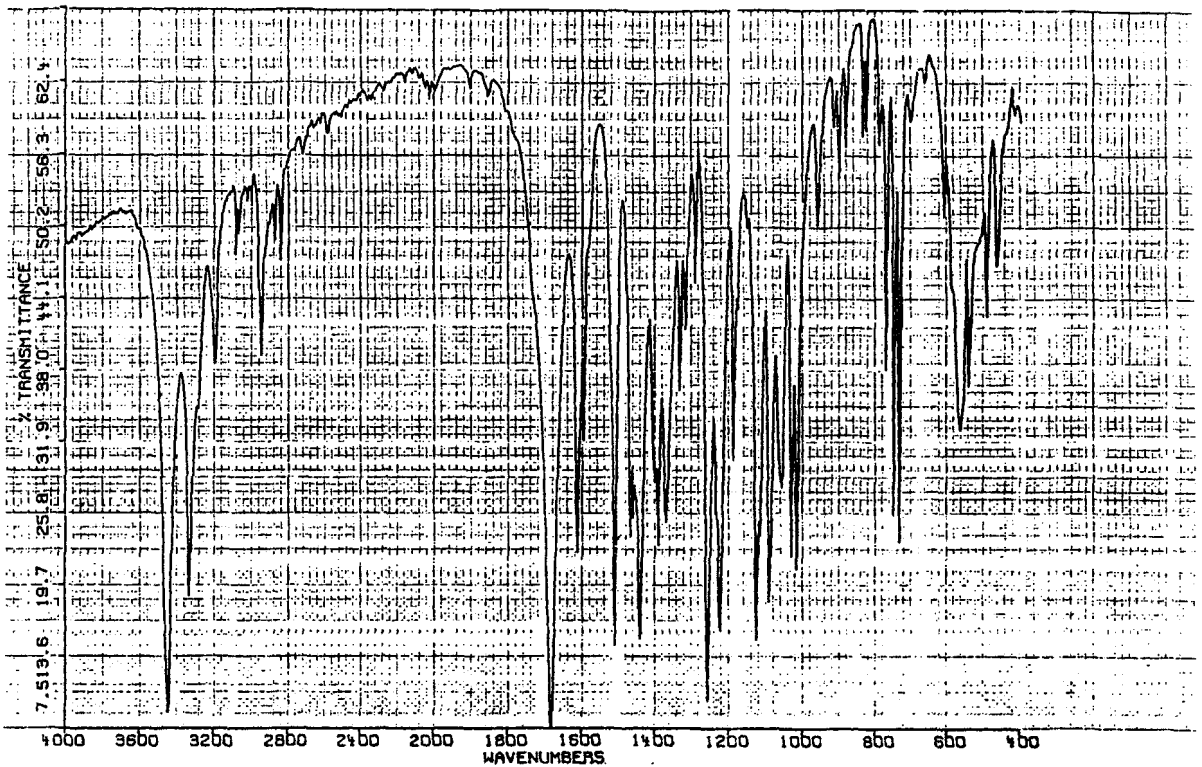


FIGURE 2. IR Spectrum of Methocarbamol. KBr Pellet.

Some suggested assignments include: peaks at 3442 and 3330 cm^{-1} (N-H stretching; secondary OH also expected to overlap these absorptions), 1681 (C=O stretching), 1610 (N-H in-plane bending or C=C str), 1509 (C=C str), 1466 (CH_3 bending), 1458 (CH_2 bending), C-N str is expected from 1020-1340; C-O-C asym and sym str of aryl ethers are usually seen between 1230-1270 and 1020-1075 cm^{-1} respectively, C-O stretching in saturated secondary alcohols often appears as a band around 1100 cm^{-1}; 733 or 750 (aromatic C-H out-of-plane bending - ortho substitution). The complete IR spectrum (KBr) has been previously published[13] as has the region from 650-2000 cm^{-1} [14].

IR spectroscopy has been utilized to measure methocarbamol and ethoxybenzamide in pharmaceutical preparations[15] and diffuse reflectance FT-IR for direct analysis of methocarbamol tablets[16].

3.2 NMR Spectra

Proton and carbon-13 spectra of methocarbamol, in $CDCl_3$, were obtained on a Bruker AM-300 FT NMR spectrometer. The respective spectra are illustrated in Figures 3 and 4 and assignments presented in Tables 1 and 2.

TABLE 1

300 MHz Proton NMR Spectrum of Methocarbamol in $CDCl_3$

Chemical Shift (ppm from TMS)	Number of Protons	Assignment
6.90 m	4	aromatic H
4.76 s	2	NH_2
4.22 m	3	CH, CH_2OCO
4.06 d,d (J = 10, 4)	1	OCH_2
4.00 d,d (J = 10, 7)	1	OCH_2
3.84 s	3	OCH_3
3.32 d (J = 4)	1	OH

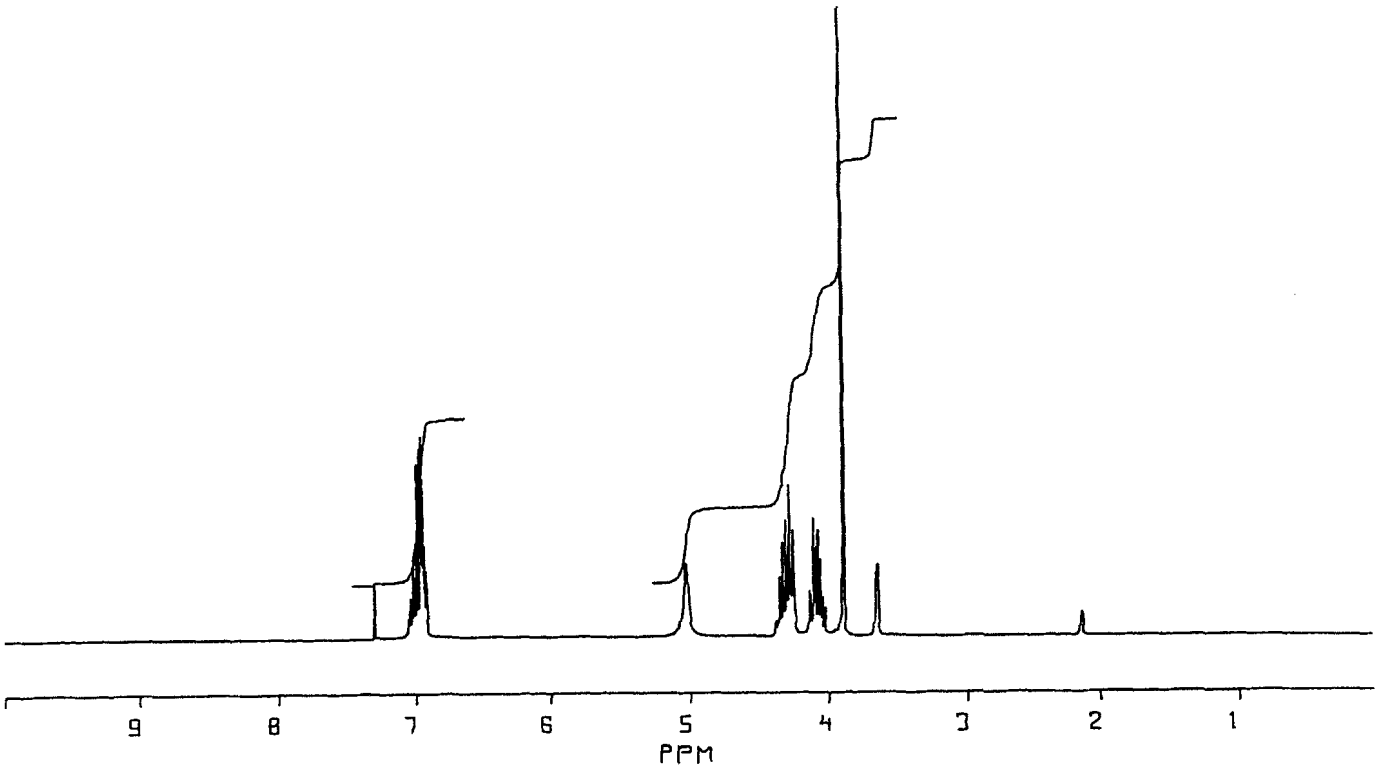


FIGURE 3. Proton NMR Spectrum of Methocarbamol in $CDCl_3$.

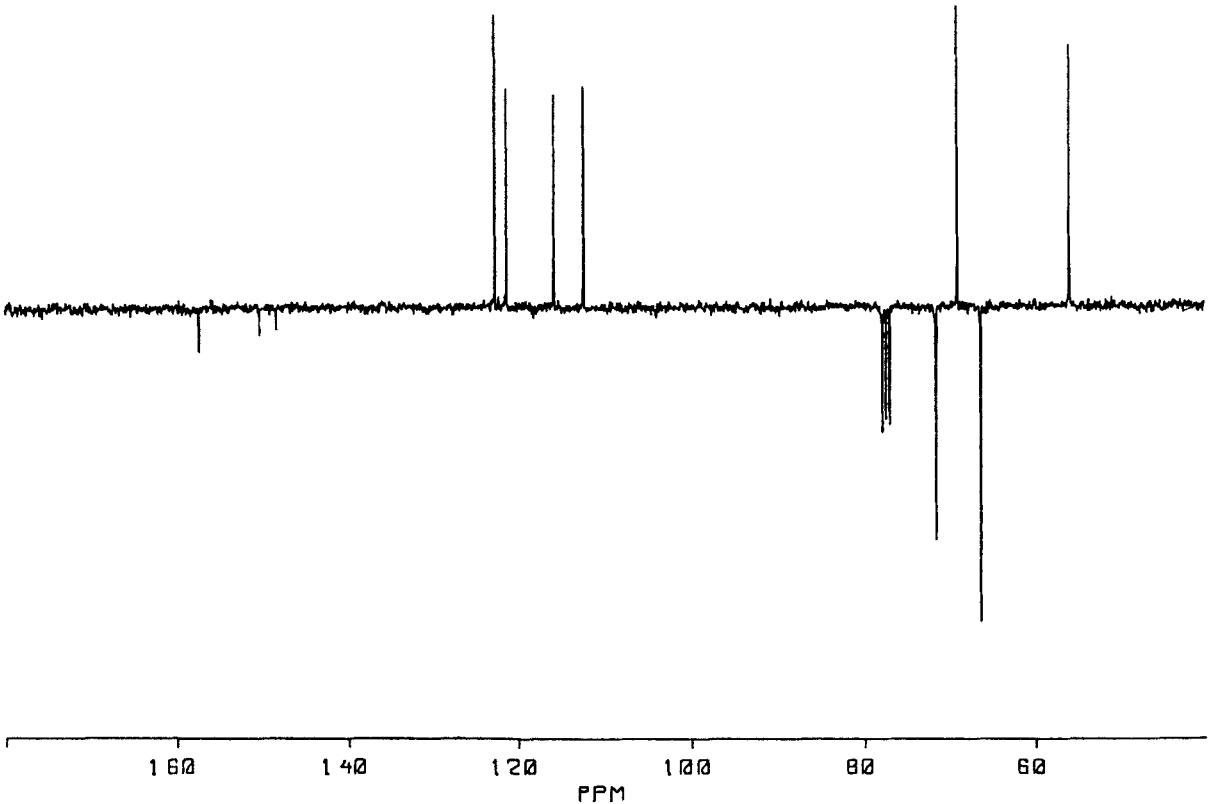
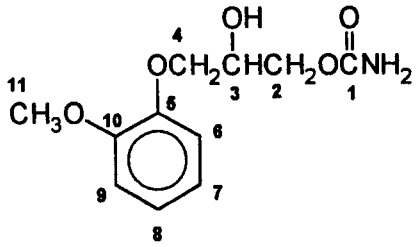


FIGURE 4. ^{13}Carbon NMR Spectrum of Methocarbamol in $CDCl_3$.

TABLE 2

75 MHz Carbon-13 NMR Spectrum of Methocarbamol in $CDCl_3$ [a]



Chemical Shift [b]	Assignment [c]
55.80	11
66.01	2
68.70	3
71.17	4
77	$CDCl_3$
112.08	9
115.58	6
121.06	7 or 8
122.44	7 or 8
147.97	5
149.97	10
157.07	1

[a] In Figure 4 the CH_3 and CH groups are shown as signals possessing an anti-phase with respect to the $CDCl_3$ signal, while quaternary carbons, CH_2 and carbonyls are in phase. A computer program (C-13 NMR Module for ChemWindow), available from Softshell International, Grand Junction, CO, was used to assist assignment of aromatic carbons.

[b] ppm from TMS

[c] carbon numbering as shown in structure

3.3 Mass Spectrum

The positive ion electron impact mass spectrum was recorded on a Kratos MS 50 double focusing magnetic sector mass spectrometer. Operating conditions: mass range 50.0156-731.9606, sampling rate 25, signal level threshold 1, minimum peak width 7, scan rate (sec/dec) 10.0, # of scans averaged 9. High resolution MS: M^+ calculated, 241.0950; found, 241.0951. Mass spectral data and suggested structures for fragment ions are shown in Figure 5. The mass spectrum and fragmentation pathways have been previously published[17,18].

Field desorption MS provided abundant molecular ions for several carbamate drugs, including methocarbamol; comparison with electron impact MS was also made[19].

Methocarbamol is mentioned, without specific details, in a report describing tandem MS as a rapid screening method for drugs in racing animals[20]. The mass spectral properties of methocarbamol, its permethylation product [3-(2-methoxyphenoxy)-1,2-dimethoxypropane], desmethylmethocarbamol, and various glucuronides have been described[18]. GC-MS (1.83 m, 1% SE 30 column) data from a permethylated rat bile sample were also provided[18]. Fragments (% relative abundance in brackets) obtained following GC-MS on a 25 m x 0.2 mm i.d., 5% methyl-silicone capillary column, have been reported: m/z 198 (12), 131 (12), 125 (9), 124 (100), 122 (8), 109 (78), 101 (13), 95 (10), 81 (17), 77 (28)[21].

Supercritical fluid chromatography-MS on a 5μm silica column (20 cm x 2.1 mm i.d.) has been described using a CO_2 mobile phase modified with 15% methanol (Rt = <3 min). The mass spectrum (above 150 a.m.u.) exhibited intense M+1 (m/z 242) and M - 42 (m/z 199) fragments and an m/z 151 (≈15%) ion[22].

Significant Ions	Measured Mass	%Relative Abundance
$C_{11}H_{15}NO_5$ (M^+)	241.0951	9.05
$C_{10}H_{14}O_4$	198.0892	10.69

Significant Ions	Measured Mass	%Relative Abundance
$C_9H_{11}O_3$	167.0709	5.24
$C_8H_9O_2$	137.0605	2.54
$C_7H_8O_2$	124.0527	67.74
$C_7H_7O_2$	123.0446	5.74
$C_7H_6O_2$	122.0369	6.84
$C_4H_8NO_3$	118.0505	100.00
$C_6H_5O_2$	109.0290	42.86
C_6H_7O	95.0498	5.31
C_6H_5	77.0391	11.98
$C_3H_7O_2$	75.0444	7.80
C_5H_5	65.0401	4.70
CH_4NO_2	62.0261	10.47
C_3H_5O	57.0364	16.47

3.4 Optical Rotation and Absolute Configuration

The optical rotation of R-methocarbamol (sodium D line, 1 dm cells, methanol as solvent) was obtained with a Perkin Elmer Model 241 polarimeter. With a 40 mg/mL solution the rotation was found to be +0.027; $[\alpha]^{26}_D = +0.68$.

Based on the optical rotation value obtained under the conditions described above, the absolute configuration is R-(+)-methocarbamol.

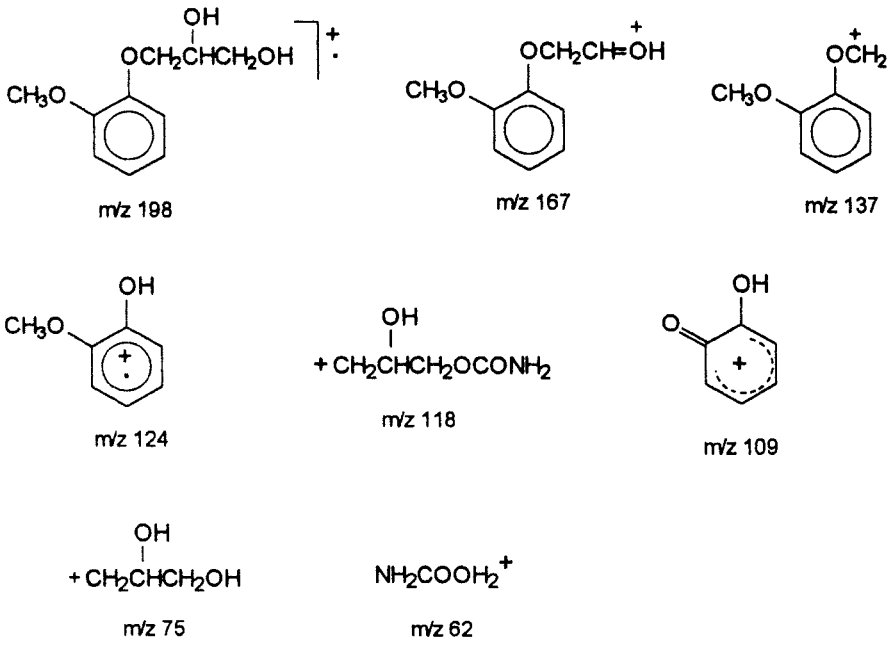


FIGURE 5. Mass Spectral Data

3.5 Ultraviolet Spectrum

The spectrum in water (conc. 0.0010 g/100 mL) was recorded on a Phillips (PU8740) spectrometer and is shown in Figure 6. The absorptivities are:

λmax (nm)	E (1%, 1 cm)
222	298
274	94

The ultraviolet spectrum of methocarbamol in methanol has been reported[14]; λmax 275 nm (E1%, 1cm = 101). UV spectra have been recorded[23] in a variety of solvents [strongest absorption maxima (nm) are underlined]: 0.1N HCl (222, 272, 279s); 0.1N NaOH (222, 272); ethanol (224, 274, 280s). The molar absorptivities (brackets) in ethanol solutions have also

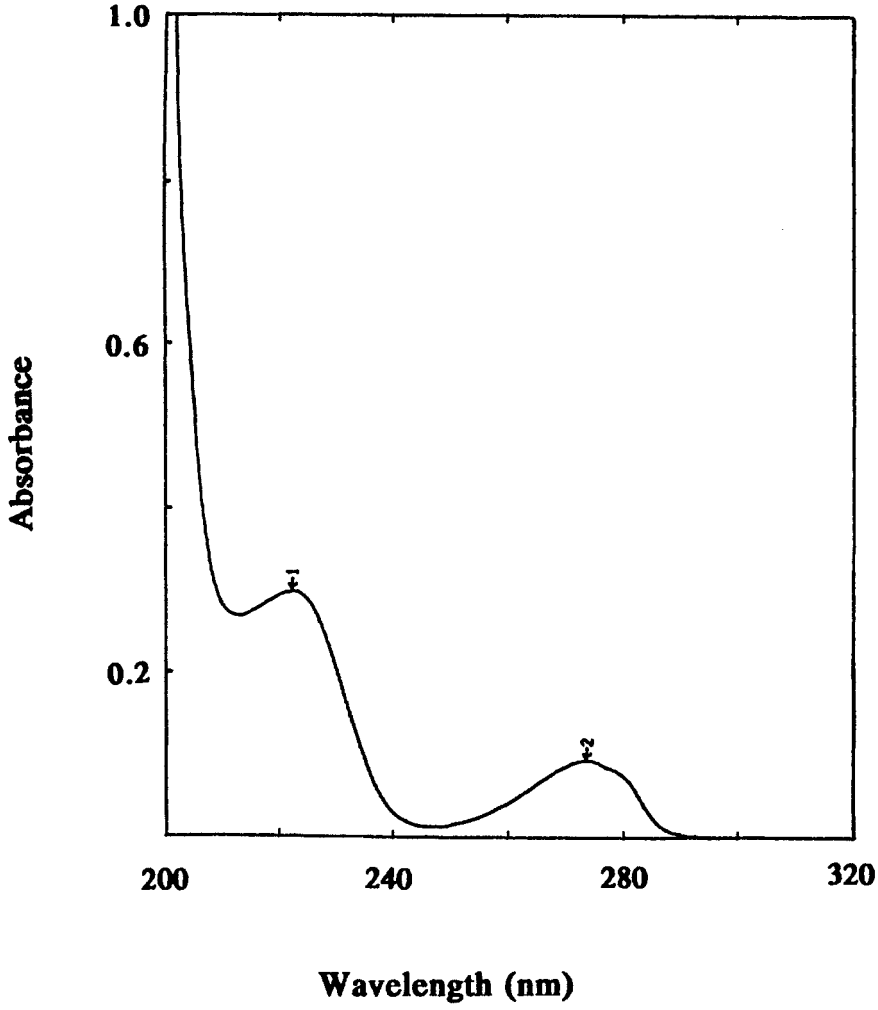


FIGURE 6. Ultraviolet Spectrum of Methocarbamol.

been reported[23]: 224 (7600), 274 (2500). Spectra recorded in ethanol, 0.1M H_2SO_4, or 0.1M NaOH gave absorption maxima at 272 nm[21]. Other studies have also described the effect of solvent (0.1N HCl, 0.1N NaOH, MeOH, EtOH, water) on UV absorption[24].

3.6 Differential Scanning Calorimetry

The DSC thermogram of (±)-methocarbamol is shown in Figure 7. The analysis was conducted on a TA Instruments DSC 2910, under a nitrogen atmosphere, at a scan rate of 10°C/min over the range of 50°C to 150°C. The melt onset temperature was found to be 94.06°C and the maximum temperature 98.06° C. The thermogram did not exhibit any exotherms or endotherms other than that associated with the melt. The estimate of the heat of fusion (ΔH) was 163.9 joules/gm.

3.7 Melting Points

(±)-methocarbamol	92-93°C (from H_2O) [4]
	92-94°C (C_6H_6) [1]
	95-97°C (C_6H_6/light petroleum) [5]
	96°C (EtOAc) [6]
	93.4-95.5°C (*i*-PrOH) [7]
	95-96°C (Et_2O) [11]
	94°C (MeOH) [25]
(+)-methocarbamol	108°C
(-)-methocarbamol	108°C

3.8 Ionization Constant

Methocarbamol is essentially a neutral compound that can be extracted from aqueous acidic or alkaline media using organic solvents[26].

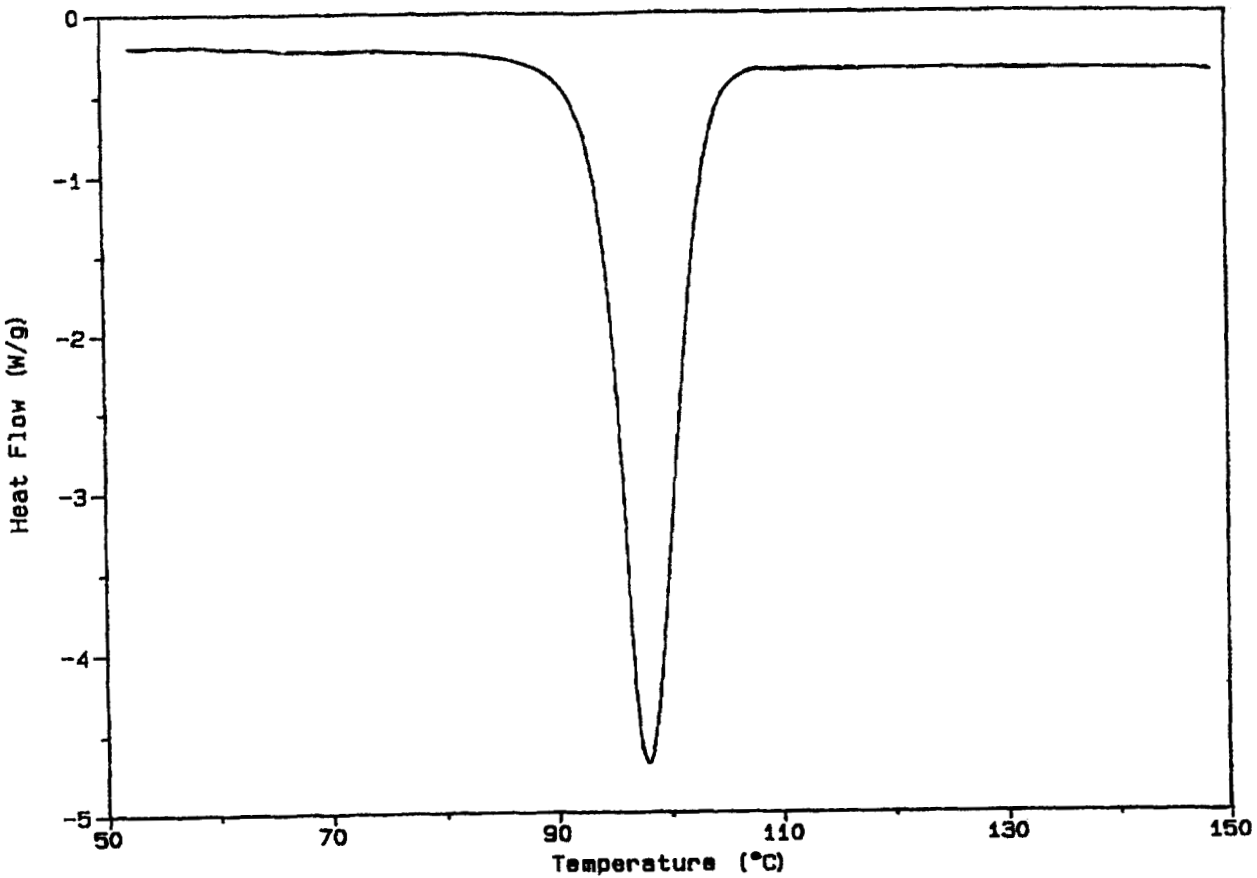


FIGURE 7. DSC Thermogram of (±)-Methocarbamol.

3.9 Lipophilic Index

The lipophilic index (log K'_0) of methocarbamol was 1.54 as determined by correlation of HPLC retention times with those of formamide. Chromatographic conditions: Nucleosil 5 C18 (4.6 mm i.d. x 150 mm), mobile phase - water (30-90%):methanol (70-10%), UV detection (220 nm), column temp 40°C[27].

3.10 Distribution Coefficient

The logP value has been calculated using CLOGP (version 3.54) and found to be -0.06[28].

3.11 Solubility

Methocarbamol is reported to be soluble in 1 in 40 of water[14,29] (2.5 g in 100 mL at 20°C)[30]. Soluble in propylene glycol, soluble in ethanol with heating, sparingly soluble in chloroform, practically insoluble in *n*-hexane[14,29].

3.12 Stability

An aqueous solution (pH 7) of methocarbamol was reported to be stable for 18 months at room temperature[31]. It is, however, thermally labile and will decompose in the injection port of a gas chromatograph[22].

3.13 Color Tests[14]

Liebermann test - violet; Mandelin test - green; Marquis test - blue-violet; Fröhde test (ammonium molybdate test) - greenish blue; Vitali test - yellow/yellow/yellow. The reagent for Liebermann's test is prepared by adding 5 g of sodium nitrite to 50 mL of sulfuric acid. The violet color results from the presence of O-alkyl substituents on the benzene ring. The Mandelin's test reagent is prepared by dissolution of 0.5 g of ammonium vanadate in 1.5 mL of water and the resulting solution diluted to 100 mL with sulfuric acid. The Marquis reagent consists of a mixture of 1 volume formaldehyde solution and 9 volumes of

sulfuric acid. The Mandelin and Marquis tests are generally used for the detection of alkaloids and other nitrogenous bases.

4. Methods of Analysis

4.1 Elemental

The calculated elemental analysis for methocarbamol [$C_{11}H_{15}NO_5$]:

C	54.77%
H	6.27%
N	5.81%
O	33.16%

4.2 Polarography

Polarographic and oscillopolarographic studies on methocarbamol and related compounds have been described[32].

4.3 Colorimetry

For colorimetric analysis, plasma and urine samples are prepared by alkalinization and chloroform extraction. Methocarbamol couples with diazotized dinitroaniline to give an orange-red compound[33]. Alternatively, colorimetric determination with chromotropic acid is possible after alkaline hydrolysis (10 mins. in boiling water) of the carbamate and periodate oxidation to formaldehyde[34].

4.4 Spectrophotometry

Ultraviolet spectrophotometry can be used for quantitative purposes[35]. The UV analysis of phenobarbital and methocarbamol in mixtures has been described[36] as has the determination of methocarbamol (at 227.6 nm) and paracetamol in tablets using second derivative UV spectrophotometry[37]. The influence of grinding on the physical separation of tablet components (including methocarbamol) and the resulting effect

on spectrophotometric reproducibility of assay data has been reported[38]. A recent spectrophotometric assay (560 nm) involves sequential treatment of an ethanol solution of methocarbamol with dilute (1:1) H_2SO_4 and CH_2O-H_2SO_4 reagent, or assay (500 nm) following acidification (H_2SO_4) of an ethanol solution and treatment with $Ce(SO_4)_2$[39].

4.5 Thin Layer Chromatography

Most reports are drug screening methods and methocarbamol is one of many compounds included. Unless otherwise specified, R_f values are reported relative to that of a reference standard and are not included herein. Examples include:

Kieselgel F254 plates, developing solvent - CH_2Cl_2:acetone, (4:1)[40].

Silica gel plates, developing solvents (R_f in brackets) - the lower layer obtained by shaking acetic acid:CCl_4:$CHCl_3$:H_2O, 100:60:90:50 (0.22) or Kieselguhr G (impregnated with formamide) plates, developing solvent - C_6H_6:$CHCl_3$, 30:120 saturated with formamide (0.31). Color reaction with various detection spray reagents (furfural-HCl, furfural-H_2SO_4, vanillin-H_2SO_4) also reported[41].

Silica gel plates, developing solvents (R_f in brackets) - $CHCl_3$:acetone, 4:1 (0.09); acetic acid: toluene:ether:MeOH, 18:120:20:1 (0.09); EtOAc:MeOH:ammonia, 85:10:5 (0.40); EtOAc (0.33); dioxane:toluene:ammonia 20:75:5, (0.02); $CHCl_3$:MeOH, 9:1 (0.36). Response to several visualization sprays (furfural reagent, acidified permanganate, and acidified iodoplatinate) also reported[42].

Normal phase (Toxi-Grams Blank A), developing solvents (R_f in brackets) - EtOAc:MeOH:H_2O, 95.2:3.2:1.6 (0.67) and reversed phase plates (Toxi-Grams C8), CH_3CN:H_2O:*i*-PrOH:EtOAc, 48:48:2:2 (0.86)[43].

Silica gel $60F_{254}$ plates, developing solvents - $CHCl_3$:MeOH, 9:1; MeOH:conc. ammonia, 100:1.5 and $CHCl_3$:acetone, 9:1. Spots were visualized by UV (254 nm) and by spray reagents (I_2 in MeOH, modified Ludy-Tenger reagent, and I_2 in MeOH and copper chloride)[44].

Kieselgel $60F_{254}$ (0.2 mm) plates, developing solvents (R_f in brackets) - ethyl acetate:MeOH:ammonia:water, 43:3:0.5:1.5 (0.57), or methanol:ammonia, 50:0.5 (0.72). Spots visualized with furfuraldehyde plus H_2SO_4 or 1% Fast Black K salt plus NaOH[45].

Silica gel $60GF_{254}$ (0.25 mm) plates, developing solvents (R_f in brackets) - $CHCl_3$:MeOH, 9:1 (0.46); toluene: dioxane:acetic acid, 90:25:5 (0.18); $CHCl_3$:ethyl acetate:MeOH: propionic acid, 65:15:15:7 (0.80); ethyl acetate:MeOH:ammonia, 85:10:5 (0.75); $CHCl_3$:MeOH:propionic acid, 72:18:10 (0.93); CH_2Cl_2:dioxane 2:1 saturated with water (0.60); $CHCl_3$:MeOH, 4:1 (0.84). Spots visualized as follows: sequential treatment with 1% m/V mercury (I) nitrate solution, Mandelin reagent, and Dragendorff reagent; sequential treatment with Ehrlich reagent and Mandelin reagent; Bratton-Marshall reagent[21].

Silica gel G (250 μ) plates treated with 0.1M KOH in methanol, developing solvents (R_f in brackets) - methanol:strong ammonia solution, 100:1.5 (0.70); silica gel G (250 μ) plates, $CHCl_3$:acetone, 4:1 (0.07); ethyl acetate:methanol:strong ammonia solution, 85:10:5 (0.49); ethyl acetate (0.23)[14].

Silica gel $60GF_{254}$ (250 μ) plates, developing solvents - acetone:benzene:$CHCl_3$, 25:40:40. Visualization was accomplished by sequential treatment with 10% furfural in ethanol plus HCl. Quantification is possible by sequential treatment of plates with acetone:glacial acetic acid (3:1), 1% *p*-dimethylaminobenzaldehyde in benzene, and antimony trichloride:$CHCl_3$:acetic anhydride (1:4:1), followed by heating and measurement of absorbance at 550 nm[46].

The TLC separation of drugs (including methocarbamol) from serum lipids has been described[47] as well as a sequential spray procedure ($FeCl_3$-HCl and aqueous KI solution) capable of detecting nitrogen-containing functional groups[48].

4.6 Gas and Liquid Chromatographic Assays

4.6.1 Stereospecific

Sample preparation and chromatographic conditions are summarized in Table 3. To our knowledge there has been one published report describing enantioseparation in a

clinical application[49]. Methocarbamol has been resolved, without derivatization, on an S-*t*-leucine/R-1-(α-naphthyl)ethylamine covalent urea column using a mobile phase consisting of hexane:1,2-dichloroethane: ethanol-trifluoroacetic acid (60:35:5, ethanol-TFA proportion 20:1), with UV detection; R_t of enantiomers, 10.63 and 11.31 min (α = 1.09)[50].

4.6.2 Non-stereospecific

Sample preparation and chromatographic conditions are summarized in Table 3.

There are several other reports in which methocarbamol was included in qualitative drug screening programs. Although specific analytical details (e.g. accuracy, precision, sensitivity, specificity) are generally not provided, the experimental (instrument) parameters may be applicable to quantitative situations.

Some examples using GC include: dimethyl silicone capillary column (0.53 mm i.d. x 10m), oven temp 130°C-230°C (5°C/min), flame ionization detection, relative R_t (based on a reference standard) reported[51]; 3% OV-17 glass column (3 mm i.d. x 4 ft), oven temp 180°C, flame ionization detection, relative R_t reported[52]; 3% OV-101 or OV-17 glass columns (0.25 in i.d. x 6 ft), flame ionization detection, relative R_t reported[44]; columns (2 m x 0.3 cm i.d.) packed with 2.5% SE-30 (R_t 0.9 min) or 3% OV-17 (R_t 2.6 min), oven temp 100°C-280°C (10°C/min), flame ionization detection, could not be detected with the NPD[21]; a GC analysis (6 ft, 3% OV-1 column) of a permethylated rat bile sample has been described[18].

Examples using HPLC include: Spherisorb S5W silica column (4.9 mm i.d. x 125 mm), eluent - methanolic ammonium perchlorate (10 mM) plus 1 ml/L methanolic NaOH (0.1M), UV detection (254 nm), no electrochemical response, relative R_t reported[53]; μBondapak C18 column, eluent - methanol:phosphate buffer (2:3), ratio of UV absorbance (254 and 280 nm) measured, relative R_t reported[54]; μBondapak RP-18 column (20 x 0.6 cm i.d.), eluent - methanol:water (7:3), UV detection (267 nm), R_t 5.3 min[21]; Zorbax C8 column, gradient elution using 0.1% H_3PO_4 and 0.1% H_3PO_4/10% water in

TABLE 3: CHROMATOGRAPHIC METHODS

EXTRACTION/DERIVATIZATION	COLUMN/MOBILE PHASE APPROXIMATE RETN TIME	DETECTION SENSITIVITY	REF
alkalinized plasma extracted with ethyl acetate	C18 μBondapak Radial-pak (10x.8 cm) acetonitrile:water (25:75); 3.7 min	UV (274 nm) 0.5 μg/ml	73
plasma extracted with 1-butanol:1-chlorobutane at neutral pH	not reported	fluorescence 22 mg/L	78
acetonitrile added to blood, sample centrifuged, supernatant injected	C18 μBondapak (30 x 0.4 cm) acetonitrile:phosphate buffer (14.5:85.5); 4 min	UV (210 nm) 10 mg/L	86
plasma alkalinized with sodium borate solution and extracted with dichloromethane	Ultrasphere (7.5 x 0.46 cm) acetonitrile:water (20:80); 3.8 min	UV (274 nm) 0.1 μg/ml	76
blood or urine extracted with toluene at acidic pH; toluene reextracted with methanolic trimethyl anilinium hydroxide	3% OV-1 (1.8m x 6.25 mm) GC column; 6 min	flame ionization 50 mg/L	35
alkalinized plasma or urine extracted with ethyl acetate; derivatization with S-(+)-naphthylethyl isocyanate	Partisil 5 silica (25 x 0.46 cm) hexane:isopropanol (95:5) 33 and 41 min for diastereomers	UV (280) 0.5 μg/ml for each enantiomer $\alpha = 1.24$; R = 2.1	49

acetonitrile, R_t 12.6 min, or PRP-1 column, gradient elution using 1% NH_4OH and 1% NH_4OH in acetonitrile, R_t 11.9 min, detection by photodiode array UV[55]; reverse-phase HPLC with UV detection has been used to screen post-mortem blood[56].

HPLC has been used to determine methocarbamol in tablet or injection formulations[57] as well as in analgesic mixtures[58]. Chromatographic conditions: Zorbax ODS column (5-6 μm particle size, 4.4 mm i.d. x 25 cm), eluent - water:methanol (75:25) UV detection (274 nm)[57]; μBondapak CN (10 μm particle size, 4.4 mm i.d. x 30 cm) or μPorasil (10 μm particle size, 4.4 mm i.d. x 30 cm) columns, eluent - various proportions of $CHCl_3$ and CCl_4, hexane, butyl ether; CH_2Cl_2 or CH_2Cl_2:CCl_4; or THF mixed with toluene, hexane, or butyl ether, refractive index detection[58]. The performance characteristics of HPLC methods for methocarbamol analyses (tablets and injections) have been evaluated using the computer program "FDACHEMIST"[59].

Methocarbamol in tablets[60] and other dosage forms[61] has also been assayed by GC. Chromatographic conditions: 3% OV-17 glass column using flame ionization detection[60]; OV-210 column with flame ionization detection[61].

5. Pharmacodynamics and Pharmacokinetics

Methocarbamol has been described as a centrally active muscle relaxant with the site of action identified as the spinal interneucial neuron[2,62]. A supraspinal locus of activity has also been suggested based on the protection provided against pentylenetetrazole- and electroshock-induced convulsions, and prolongation of the hexobarbital sleeping time[2]. However, direct evidence for a specific receptor has not been delineated and a complete understanding of the mechanism of action is not yet apparent[63]. The inhibition of carbonic anhydrases I and II by methocarbamol is not sufficiently strong to have implications in its mechanism of action[64]. A significant anxiolytic action for methocarbamol has been proposed recently[65]. Several early clinical studies (1958 and 1959) assessed the efficacy of methocarbamol in a variety of conditions including acute trauma and chronic neurological states[66], post-operative pain in gynecological patients[67], neuromuscular disease[68,69], and

orthopedic situations[70].

Published pharmacokinetic information is comparatively scarce. Early studies in animal models (rat and dog), based on radioactive tracer and colorimetric techniques, showed that methocarbamol was readily absorbed from the intestine and widely distributed in body fluids and tissues, particularly liver and kidney[71]. The reported bioavailability in animal models (dog, rat, horse) and in humans ranges from 36% to 90%[71-75]. Reduced absorption and first pass effects were proposed to explain the lowest values. Evaluations in the horse[76] and rat[73] revealed dose-dependent elimination on the basis of HPLC determination of plasma levels. The plasma half-life is reported to be between 0.5 and 1.6 hr in animal models[72,73,76,77] whereas in healthy human subjects the range is 1 - 2 hr[74,78]. It appears that 98% of the dose is eliminated in the urine, with <1% as the unchanged drug, and the remainder as free and conjugated metabolites. Most of the excretion seems to occur in the first 8 hr[14,71,75,77]. The major pathways of metabolism are O-demethylation and *p*-hydroxylation followed by conjugation with glucuronic acid or sulfate[14,71,75,77]. Major species differences in the metabolism and disposition of methocarbamol have not been reported[71,77].

Methocarbamol is available by either prescription or as an over-the-counter product, often in combination with analgesics (acetylsalicylic acid, acetaminophen, codeine). It appears to be a relatively safe drug, one which is well-tolerated and widely used. Nevertheless, cases of fatal intoxication have been reported[35,79,80] and a potential for abuse has also been evaluated in individuals with a history of recreational substance abuse[81,82].

Methocarbamol has an asymmetric carbon and is marketed as a racemic drug at comparatively high doses (up to 12 g/day in severe conditions)[83]. Published information on the pharmacodynamics and pharmacokinetics of methocarbamol enantiomers is limited. There were no significant differences in muscle relaxant activities when the enantiomers were evaluated using a traction test[84]. Significant stereoselectivity has been observed in the rat after intravenous and intraperitoneal administration of the racemate, with S-methocarbamol being generally predominant in plasma. There was no evidence of dose-

dependent kinetics in the dose range of 30-120 mg/kg[85]. In humans, stereoselectivity seems less pronounced compared to the rat. Nevertheless, S-methocarbamol is predominant in human plasma after oral administration of a 2 g dose of the racemate. Two known poor metabolizers of debrisoquine were observed to have elevated plasma levels of methocarbamol enantiomers relative to the levels observed in extensive metabolizers of debrisoquine[85], suggesting that methocarbamol could be a substrate for the P450IID6 isozyme.

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NATAMYCIN

(SUPPLEMENT)

Harry Brik

Gist-Brocades NV

Delft

The Netherlands

NATAMYCIN

(supplement)

Harry Brik

1. Description
   1.1. Name, Formula, Molecular Weight
   1.2. Chemical Structure and Configuration
   1.3. Nomenclature
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8. Acknowledgements

9. References

Introduction

This supplement to the main profile of natamycin (Volume 10, pp. 513-561, 1981) [1] contains updated information and new data from the literature. The literature has been surveyed from 1981 until April 1993. The numbering of the sections is the same as in the main profile.

1. DESCRIPTION

1.1. Name, Formula, Molecular Weight

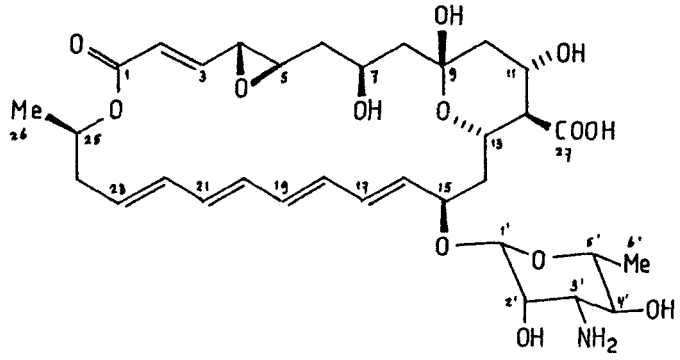
Generic name: natamycin

Synonym: pimaricin

Trade names: Natacyn; Natafucin; Pimafucin; Delvocid

Chemical name: 22-(3-amino-3,6-dideoxy-ß-D-manno-pyranosyl)oxy-1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28-trioxatricyclo [22.3.1.$0^{5,7}$]octacosa-8,14,16,18,20-pentaene-25-carboxylic acid

Structural formula *



* In contradiction to the IUPAC chemical name the C-atoms in the structural formula are numbered in the classical way to facilitate comparison with literature data which refer to parts of the molecule.

Empirical formula	$C_{33}H_{47}NO_{13}$
Molecular weight	665.75
Code designation	CL 12,625; Antibiotic A-5283
CAS registry nr	7681-93-8
Wiswesser line notation	T F3-24-6 A AO GO KVO IU OU QU SU UUTJ BQ DQ M1 C&VQ D&Q WO-BT6OTJ CQ DZ EQ F1

1.2. Chemical Structure and Configuration

Natamycin belongs to the large group of polyene antifungal antibiotics. Characteristic for this group is a macrocyclic lactone-ring with a number of conjugated carbon-carbon double bonds.

By means of sophisticated 2D NMR experiments, using D-mycosamine as an internal chiral reference, and by the preparation of some hydrogenated derivatives, Lancelin and Beau [2] succeeded in the elucidation of the complete stereostructure of natamycin.
The configuration was established as 4R, 5R, 7S, 9R, 11S,12R, 13R, 15R, 25R, 1'R. The structural formula, shown in section 1.1, now reflects the complete stereostructure of natamycin.

The structural relation of natamycin to a number of other tetraene antibiotics is shown in Figure 1.

1.3. Nomenclature

The natamycin-producing Streptomyces strain was found in a soil sample from the neighbourhood of Pietermaritzburg, a town in the province of Natal, South Africa. Therefore the strain in question was called *S. natalensis*.

The name natamycin is commemorative of this strain, the old name pimaricin (still used occasionally but not accepted by the WHO since antibiotics which are produced by a Streptomyces strain should have the suffix "mycin") is called after **Pietermaritzburg**.

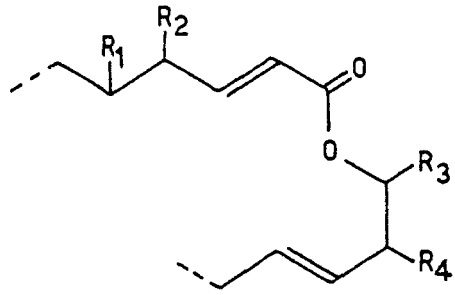


Figure 1

Structural relation of natamycin to other tetraenes which differ only in R1 to R4

Tetraene	R1	R2	R3	R4
natamycin	-O-		Me	H
lucensomycin	-O-		n-Bu	H
arenomycin B	OH	H	n-Bu	H
tetramycin A	OH	H	Me	Et
tetramycin B	OH	OH	Me	Et
tetrin A	OH	H	Me	Me
tetrin B	OH	OH	Me	Me

1.4. Appearance

Natamycin is a white to cream-coloured, almost tasteless and odourless, crystalline powder.

1.5. Standards and Regulatory Status

Natamycin is included in the United States Pharmacopeia [3], in the Code of Federal Regulations as drug substance [4] as well as food additive [5], and in the Minimum Requirements for Antibiotic Products of Japan [6].
The microbiological activity of natamycin is expressed in μg anhydrate per mg. The FDA master standard has an assigned potency of 922 μg of anhydrous natamycin per milligram. This standard is identical to Gist-brocades natamycin trihydrate reference standard lot 711-EN-78-1.
The second USP reference standard (lot G, catalog nr 45750) is identical to Gist-brocades natamycin trihydrate reference standard lot 714-EN-93-1.

Both standards were prepared by repeated crystallization of a selected lot of natamycin.

2. CHEMICAL PROPERTIES

Like several other polyenes, natamycin forms an inclusion complex with gamma-cyclodextrin [7]. The complex shows biological activity[1] and is much more soluble in water than the parent antibiotic.
The structure of the complex is probably analogous to the complex of gamma-cyclodextrin and amphotericin B [9]. In this case the hydrophobic part of the natamycin molecule (about half of the lactone ring) would fit in the central cavity of the cyclodextrin torus.
No complex is formed with alpha- or beta-cyclodextrin as the polyene molecule does not fit in their smaller cavities.

If natamycin is dissolved in methanol (0.1 mg/ml) containing a small amount of hydrochloric acid (0.001 M), within 2 hours almost complete conversion to the methyl ester takes place. Upon addition of water the ester is slowly hydrolyzed to natamycin [10].

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[1] It is more likely that the activity arises from the free polyene, formed after dissociation of the complex [8].

Upon heating with alkali most polyene antibiotics give a retroaldol reaction. Triggered by a ketonic carbonyl, characteristic aldehydes and/or ketones are formed. In the sixties and seventies this reaction was a valuable aid in the elucidation of the structure of polyene antibiotics. Natamycin, and all other polyenes mentioned in Figure 1, forms a pentaene aldehyde (from C13-C25) , acetone (from C8-C10), and ethanal. Sofar the origin of the ethanal never has been elucidated clearly. While investigating tetrin A, Pandey et al. [11] used a variant of the above retroaldol reaction: after acidification of the alkaline reaction mixture an additional amount of ethanal was formed. Pandey presumed that this additional amount was obtained from malonaldehydic acid, an intermediate formed upon heating with alkali (from C11-C12-C27).
However, this hypothesis is very unlikely as (1) authentic malonaldehydic acid[1] is completely converted to ethanal upon treatment with hot alkali (not a trace of a carbonyl is formed after acidification), and (2) the additional amount of ethanal is only obtained from mycosamine, natamycin or its degradation products with a mycosamine-group [12].
At the first stage all the latter compounds also give ethanal in an alkaline medium.
In summary, from the results of the retroaldol reaction of natamycin, a number of its degradation products (with or without a mycosamine-group), of borohydrid-reduced natamycin and of mycosamine itself, in particular the presence or absence of ethanal in the volatile fractions, it is evident that the ethanal originates both from the lactone-ring (C11-C12) and from the aminosugar (most likely C5'-C6') [12].

3. PHYSICAL PROPERTIES

3.1.3. Polymorphism

Thirty-five years after the discovery of natamycin a second crystalline form was observed. The material has been isolated in a minute quantity from a rejected lot of natamycin in the form of a fine birefringent powder [12].

---

[1] This very unstable compound was prepared from ethyl-3,3-diethoxypropionate by hydrolysis with Amberlyst-15.

It was not possible to study the crystal habit with an optical microscope due to the very small dimensions of the crystals (< 1 μm). Although the material was undeniable identified as natamycin, the remarkable low solubility, compared to the regular product, suggested it to be another crystalline form. Indeed the X-ray diffraction pattern was completely different. Further information was obtained by thermal analysis (see Section 3.10.2). The results of the thermal analysis strongly suggests that the new modification (called the β-polymorf) is the more stable form of the two and is also in the possession of 3 moles of crystal water [13].

3.4. Proton NMR spectrum

For comparison with amphotericin B, Brown and Sidebottom [14] recorded the proton NMR spectrum of natamycin in d_6-dimethyl sulphoxide and of N-acetylnatamycin in pyridine. Chemical shifts of assigned protons are given.

3.10.12. Thermal analysis

Both polymorphic forms of natamycin (Section 3.1.3) were examined by means of differential scanning calorimetry (DSC) and by thermogravimetric analysis (TGA) [13]. Figures 2 and 3 show the DSC curves of the two polymorphs, recorded on the DSC 30 of the Mettler TA 4000 System. The obtained data are shown in Table 1. Also shown are the weight losses at the specified temperatures, obtained from a TGA curve, run on a Mettler TG 50 thermobalance.

The weight losses around the first and the second endotherm correspond roughly with 2 moles respectively 1 mole of crystal water (theoretically 5.0 respectively 2.5%). The weight loss at the first endotherm includes some unbound water ($< 1\%$). The position as well as the surface of the endotherms strongly suggests that the β-polymorph is the more stable form.

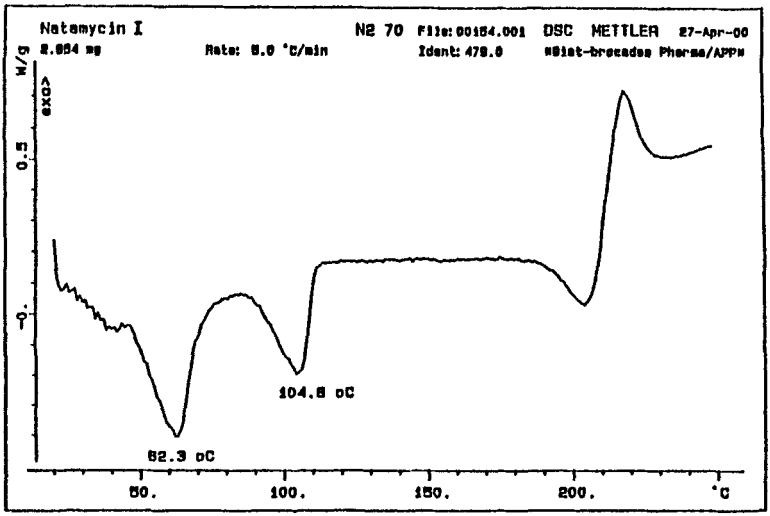

Figure 2. DSC curve of natamycin α-polymorph

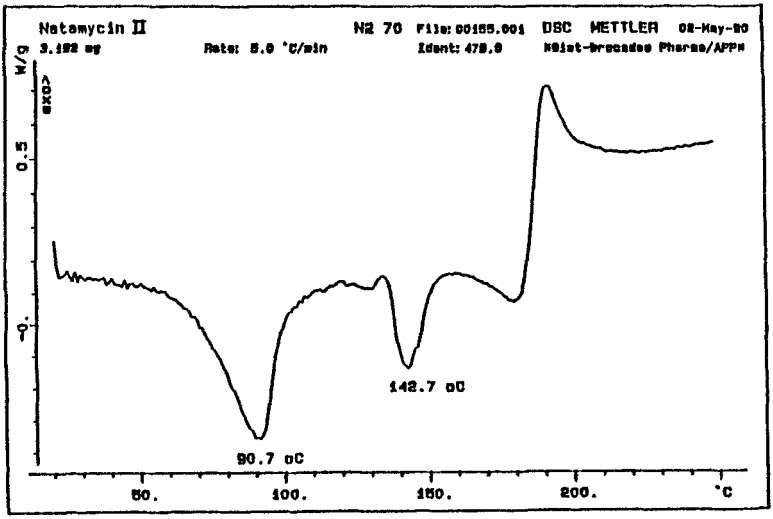

Figure 3. DSC curve of natamycin β-polymorph

Table 1. Thermal analysis of natamycin [13]

polymorf	1st endotherm (minus 2 H_2O)			2nd endotherm (minus 1 H_2O)			exotherm (degradation)	
	T_{top} (°C)	ΔH (J/g)	$-\Delta m$ (%)	T_{top} (°C)	ΔH (J/g)	$-\Delta m$ (%)	T (°C)	$-\Delta m$ (%)
alpha	62.3	71.3	5.5-5.9	104.8	50.4	1.9-2.1	≥184	16.4-16.6
beta	90.7	129.1	5.5-5.8	142.7	35.1	2.1-2.1	≥160	19.7-20.2

The operating conditions were as follows:

	DSC	TGA
sample weight	about 3 mg	about 8 mg
purge gas	nitrogen	nitrogen
heating range	20-250 °C	25-250 °C
heating rate	5 °C/min	5 °C/min
cup	Al, piersed lid	alumina, open

3.12. Solubility

For the solubility of pure natamycin in 87% w/w glycerol at 20 °C a value of 0.18 mg per ml was found (procedure see ref.1, p.540, second note).

4. PRODUCTION

4.4. Synthesis

Brooks and Palmer [15] synthesized two chiral precursors, representing the two halves of the aglycone of natamycin. Both fragments were prepared from dimethyl-3-hydroxy-glutarate.
Oppong et al. [16] described the synthesis of a hemiacetal chiron, a compound which could serve as a starting point for the synthesis of natamycin.

Another approach was followed by Duplantier and Masamune [17] who succeeded in synthesizing the methyl ester of the aglycone of natamycin.
A synthon, used earlier for the synthesis of amphotericin B aglycone, served as starting material.

5. STABILITY

Alsop et al. [18] studied the stability of natamycin eye-drops (a 5% aqueous suspension) after autoclaving at 116 °C.
The suspension was divided over a number of ampoules which were sealed under air or nitrogen. The ampoules were heated at 116 °C, removed at various time intervals and assayed spectrophotometrically by the base-line method (principle see Figure 4-A). From the obtained data, shown in Table 2, it was concluded that during a sterilization period of 30 minutes at 116 °C only a slight, acceptable degradation occurs.
The results were confirmed by a semi-quantitative bioassay.
The stability of natamycin as such and several of its pharmaceutical preparations has been investigated by Ruzicková et al. [19]. Samples were kept in a climate chamber at a temperature of 25 °C and 40 °C (24 hours cycle) and a relative humidity of 75%. Natamycin substance was packed in a closed bottle, the preparations were kept in their commercial packagings. The samples were analyzed spectrophotometrically by measuring the absorbance of their solutions at the maximum at 303 nm. After 70 days at 40 °C the content of the natamycin substance was decreased by 4%. After 3 months at 40 °C the content of Pimafucin oral tablets was decreased by 1%, that of Pimafucin vaginal tablets by 2.6%.

Table 2. Stability of a 5% natamycin suspension at 116 °C [18]

Time of heating (hours)	% natamycin left (air)	% natamycin left (nitrogen)
0	100	100
0.25	103	
0.5	97	
0.67	95	91
1.0	89	
1.5	92	
3	94	85
6	87	80
12	74	66
24	35	30
48	0	0
rate constant	2.66%/hr	2.80%/hr

Gutteridge et al. [20] investigated the autoxidation of four polyene antibiotics, amphotericin B, candicidin, natamycin and nystatin. The polyenes were treated in such a way that free radical damage occurred. This was done by autoxidation of 0.1% solutions of the polyenes in dimethyl sulphoxide:
(a) for 1 week under normal laboratory lighting, (b) for 6 hours under normal laboratory lighting after addition of 0.4 mM ferrous ion, and (c) for 6 hours in open disks, exposed to long-wavelength UV light. The solutions have been monitored for biological activity, changes in absorbance (main UV maxima) and their reactivity with thiobarbituric acid (TBA).
A positive reaction with TBA -formation of a fluorescent compound upon heating in 50% acetic acid- suggests the presence of peroxidic compounds in the polyenes. The TBA reactivity was most increased by the presence of ferrous ions. Little, if any, change in the reactivity occurred in solutions kept under normal lighting. The decrease in biological activity -about the same with all polyenes investigated- was the most in the UV-treated samples. Loss of UV absorption was observed after all treatments. Of the four polyenes, natamycin showed the least increase in TBA reactivity.

7.2. Spectrophotometric Analysis

All of the following procedures were used for the determination of natamycin in cheese. Riedl [21] used the second derivative of the main peaks of the UV spectrum to minimize the interference of the matrix. In pure methanolic solutions 20 ng of natamycin per ml could be detected. In cheese extracts the detection limit amounted up to 150 ng per ml or 2.5 mg per kg cheese or 30 μg per dm^2 cheese surface.
A base-line method was used by three authors [22,23,24]. In all cases a correlation between the ultraviolet method and HPLC was observed, in those cases where the concentration was considerably higher than the detection limit. The detection limit was 0.27 mg per kg [22,23] or 0.3 to 1.7 mg per kg [24]. The latter range was found in an interlaboratory study (see also Section 7.5.3). The base-line method used differed from the original method for determination of natamycin in pharmaceuticals [1, page 547]. In the latter the base-line absorbance was calculated from the absorbances at the minima at 295 and 311 nm and the maximum at 303 nm (Figure 4-A).

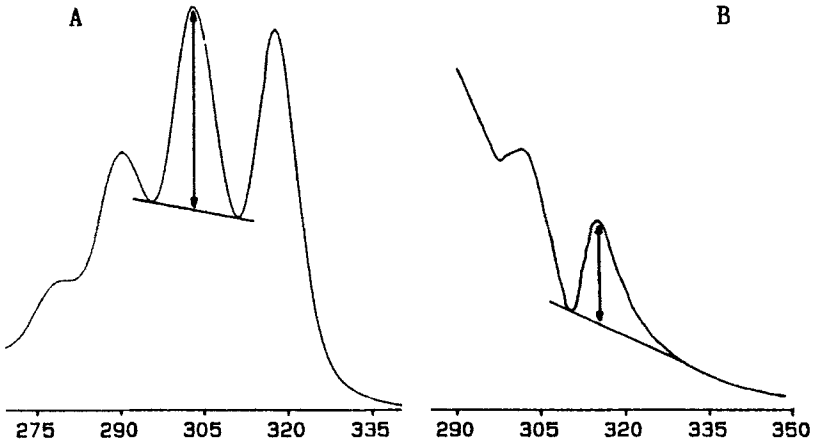

Figure 4.

Determination of natamycin by the base-line method.
A. Pure natamycin (in methanol + 1% acetic acid).
B. Natamycin in cheese extract (in methanol - water 2:1)

For cheese extracts this method is not suitable due to the interference of the matrix. However, it works well if a part of the spectrum at higher wavelengths is used for the calculation. This alternative base-line method uses the straight line between the absorbance at 311 and 329 nm as the base line. The net absorbance may be taken from the UV spectrum as the difference between the absorbance at the maximum at 317 nm and the base-line (Figure 4-B). It may also be calculated from:

$$E = E_{317} - {}^{2}/_{3} \cdot E_{322} - {}^{1}/_{3} \cdot E_{329} \quad [24]$$

7.5.2. Thin Layer Chromatography (TLC)

Thomas et al. [25] compared the composition of a number of samples of nystatin and the related polyfungin by means of TLC. Other polyenes, among which natamycin, were used as reference. Precoated silica gel plates were used with three different mobile phases. The best results has been obtained by chloroform-methanol-water (20:22:10, lower phase). Spots were visualized by spraying with 10% (v/v) sulphuric acid in ethanol and subsequent heating at 100 °C. Natamycin was separated from lucensomycin, amphotericin A and the main components of nystatin and polyfungin. R_f-values has not been given.

Lehmann et al. [26] determined natamycin in cheese rind by TLC of the methanolic extract. Silica gel was used as the stationary phase and glacial acetic acid-butylacetate-butanol-water (2:3:8:5) as the mobile phase. The spots were detected by spraying with 0.1% potassium permanganate solution, after 15 minutes followed by spraying a 0.01% bromphenol blue solution. The detection limit was 1 μg.

By reaction of natamycin (or its decomposition products containing the mycosamine group) with NBD-chloride (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole) a fluorescent derivative was obtained. Upon chromatography on silica gel the spots were quantified by means of a spectrofluorometer. The detection limit on the plate was 8 ng [27]. This procedure has been applied for the determination of residual natamycin in food.

Shenin et al. [28] separated a great number of polyenes by means of TLC. The R_f-values of natamycin and the constitution of the mobile phases are given in Table 3. The silica gel plates were activated by heating for 2 hours at 110 °C.
Spots were visualized by UV-light and by autobiography. In most systems the separation of natamycin from the closely related lucensomycin was poor.

Table 3. TLC of natamycin [28]

Mobile phase	Impregnant	R_f-value
1	none	0.78
1	sodiumborate	0.41
2	none	0.35
3	ammoniumsulphate	0.49
3	sodiumborate	0.46

Mobile phases:
1. n.butanol-acetone-water (4:5:1)
2. n.propanol-triethylamine-water (2:1:1)
3. methanol-25% ammonia-water (20:1:4)

7.5.3. High Performance Liquid Chromatography (HPLC)

Table 4 summarizes a number of HPLC methods. All methods have been used for the determination of traces of natamycin in cheese. The repeatability and reproducibility of one of these methods [24] were subjects of an interlaboratory study, carried out by 37 laboratories in 13 countries [29].

7.10. Enzyme Immuno Assay

Maertlbauer et al. [34] developed a highly specific and sensitive enzyme immuno assay for the quantitative determination of natamycin in cheese rind. Rabbits were immunized with a natamycin-HSA conjugate to produce a specific antiserum. A labelled ligand was produced by coupling natamycin to horseradish peroxidase. The range of this ELISA test was between 0.2 and 2 ng per ml of sample solution. This allowed the determination of natamycin in cheese rind down to concentrations of 5 ng/dm^2 or 0.1 mg/kg. The recovery in a range of 1 to 80 mg/kg was 76 to 84%. The cross-reactivity with amphotericin B and nystatin was $<0.001\%$.

The detection limit of less than 0.2 ng of natamycin per ml for pure solutions is more than 100 times lower than the most sensitive procedure described [21] (see also Section 7.2).

Table 4. Summary of HPLC methods for the determination of natamycin

Sample	Anal. column	Mobile phase	Flow rate	Detection	Detection limit	Ref.
ethanolic extract[1] of cheese rind	Lichrosorb RP-8 10 μm 250x4.6 mm	MeOH-phosphate buffer pH 4.45 (65:35)	1.5 ml/min	303 nm	20 ng/dm^2 cheese surface	30
methanolic extract[1] of cheese rind	Lichrosorb RP-8 250x4.6 mm	MeOH-phosphate buffer pH 6.85 (65:35)		303 nm	10 ng	31
methanolic extract[1] of cheese	Lichrosorb RP-8 5 μm [2]) 150x4.6 mm	MeOH-water-acetic acid (60:40:5)	1 ml/min	303 nm	1 ng or 50 μg per kg cheese	32
methanolic extract[1] of cheese	Lichrosorb RP-8 5 μm [2]) 150x4.6 mm	MeOH-water-acetic acid (60:40:5)		303 nm	0.5 mg per kg cheese	24
methanolic extract of cheese	Lichrosorb RP-8	MeOH-water acetic acid (60:40:5)			0.14 mg per kg cheese	22

[1] Frozen or chilled to remove most of the co-extracted impurities.

[2] Guard column: Perisorb RP-8, 30-40 μm, 100x2.1 mm.

Table 4. (continued)

Sample	Anal. column	Mobile phase	Flow rate	Detection	Detection limit	Ref.
methanolic extract of cheese	Zorbax ODS	MeOH-water-acetic acid (60:40:10)	1 ml/min	303 nm	0.14 mg per kg cheese	23
methanolic extract[1] of cheese or cheese rind	Novapak C-18	5 mM acetate buffer pH 4.4 grad. elution with MeCN		UV	0.5 mg per kg or 5 $\mu g/dm^2$ cheese surface	33
methanolic extract of cheese rind	Lichrospher RP-8, 5 μm 125x4 mm	50 mM phosph. buffer pH 5.0 - MeCN [3])	2 ml/min	240-370 nm diod.array detector	not given (at least 1 μg per g cheese)	34
extract of cheese with mob. phase	TSK ODS-80TM	phosph. buff. pH 6.8 - MeCN (72:28)		305 nm	0.2 μg per g cheese	35
methanolic extract of cheese or tablets	Nucleosil C-18, 5 μm	MeOH - PrOH - 1 M LiCl - water (45:7:2:46)		polarogr. at -1.1 V	0.1 μg	36

[1] Frozen or chilled to remove most of the co-extracted impurities.

[3] Linear gradient from 80:20 to 50:50 in 10 minutes.

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SALICYLIC ACID

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1 Introduction

Salicylic acid occurs (1) as white, fine needle-like crystals or as a fluffy, white, crystalline powder. Synthetic salicylic acid is white and odorless. The acid prepared from natural salicylate may have a slightly yellow or pink tint and a faint wintergreen-like odor. The acid has sweetish, afterward acrid, taste. It is stable in the air.

2 Description

2.1 Nomenclature

2.1.1 Chemical Names

a) Benzoic acid, 2-hydroxy (1-8).

b) O-Hydroxybenzoic acid (1).

2.1.2 Generic Name

Salicylic acid.

2.1.3 Proprietory Names

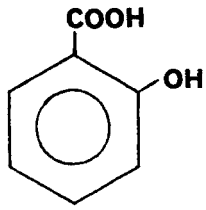
Aserbine, Coroplast, Dithrolan, D volfilm, Egocappol, Fomac, Fosten, Gehwol, Guttaplast. Gyan, Hydrisalic, Ionilplus, Keralyt, Klaron, Komed. Malatex, Monphytol, Occlusal, Pernox, Phytodermine, Pragmatar, Psorin, Pyralvax, Salac, Salactol, Salicyl, Saligel, Sasha, Sebcur, Sebulex, Sebucare, Soluver, Stichasan, Squvamasol, Verrugon, Viron, Wart.

2.2 Formulae

2.2.1 Empirical

$C_7H_6O_3$

2.2.2 Structural



2.2.3 CAS (Chemical Abstract Service Registry Number)

69-72-7

2.2.4 Density (7)

1.443_4^{20}

2.2.5 Refrective Index (2,7)

1.565

2.2.6 Wiswesser Line Notation (7)

QVR BQ

2.3 Molecular Weight (2,4,5,6,8)

138.12

2.4 Elemental Composition

C 60.87%, H 4.38%, O 34.75%

2.5 Appearance, Color, Odor and Taste

Colourless acicular crystals or a white crystalline powder with a sweetish acrid taste. The synthetic form is white and odourless but if prepared from natural methyl

salicylate it may have a slightly yellow or pink tint, and a faint, mint-like odour (3).

3 Physical Properties

3.1 Melting Point (2)

157-159°.

3.2 Boiling Point (2)

About 211° (20 mm Hg).

3.3 pH (2)

pH of saturated solution is 2.4.

3.4 Solubility (2)

One gram dissolves in 460 ml H_2O, 15 ml boiling H_2O, 2.7 ml alcohol, 3 ml acetone, 42 ml chloroform, 3 ml ether, 135 ml benzene, 52 ml oil turpentine, about 60 ml glycerol, about 80 ml fats or oils, solubility increased by sodium phosphate, borax, alkali acetate, or citrate.

3.5 Loss on Drying (4)

Dry it over silica gel for 3 hours, it loses not more than 0.5% of its weight.

3.6 Residue on Ignition (2)

Not more than 0.05%.

3.7 LD_{50} (2)

In mice 500 mg/kg.

3.8 Crystal Growth

Crystal growth of an industrially manufactured salicylic acid (9) powder (60 μm stated particle size) was demonstrated. A microscopic examination about eight months after its preparation showed a maximum particle size of 200-500 μm. About one year later, examination showed further crystal growth, particle size now was 500-800 μm. A further examination after eight months showed no further particle size growth. Particle size growth of salicylic acid occurred even in the dry powder state and not only in ointments.

3.9 Spectral Properties

3.9.1 Ultraviolet Spectra (UV)

UV spectra (10) of salicylic in ethanol (4 mg%) was scanned from 200-400 nm (Fig. 1) using LKB 4054 UV/Vis spectrophotometer, salicylic acid exhibited the following UV data (Table 1).

Table 1. UV data of salicylic acid in ethanol

nm λ_{max}	Absorbance	Molar Absorptivity (ϵ)	A_1^1
210	2.461	8342.5414	604.055
234	1.583	5466.1602	395.811
303	1.040	3591.1602	260.04

3.9.2 Infrared Spectra (IR)

The IR spectrum (10) of salicylic acid as KBS disc was recorded on a Perkin Elmer 1310 infrared spectrometer. (Fig. 2) shows the infrared spectrum of

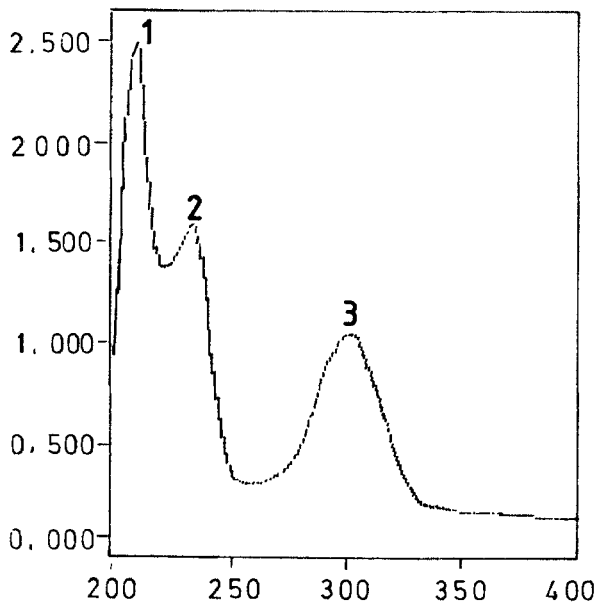


Fig (1) Ultraviolet spectra of Salicylic Acid in Ethanol (4mg %)

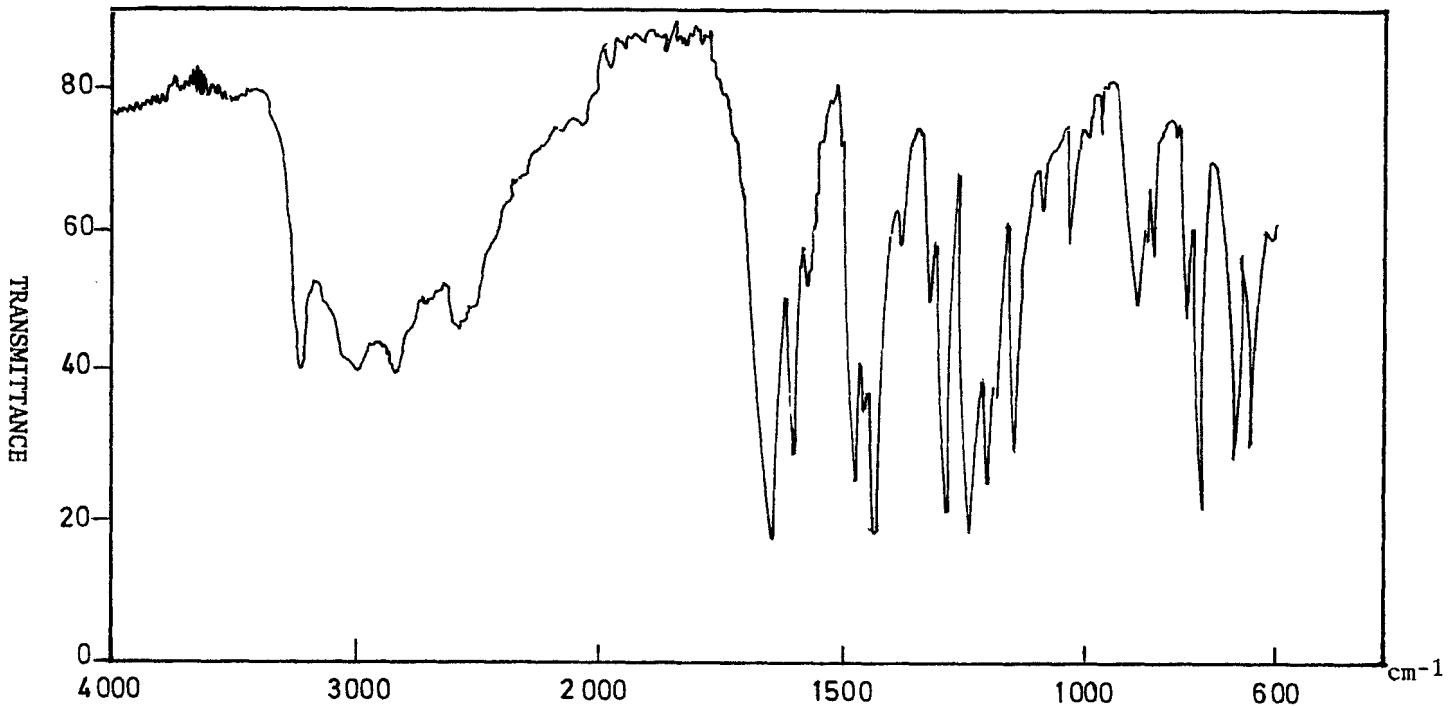


Fig (2). Infrared spectra of Salicylic Acid as KBr disc.

salicylic acid. The structural assignments of salicylic acid have been correlated with band frequencies and are given in table 2.

Table 2. IR characteristics of salicylic acid

Wavenumber cm^{-1}	Assignments
3220	-OH
3050	Aromatic C - H stretch
1650	- C = O (carboxylic)
1440	C = C stretch
690 760	Aromatic C-H bending.

3.9.3 Nuclear Magnetic Resonance Spectra

3.9.3.1 Proton Spectra (PMR)

The PMR spectra (10) of salicylic acid in DMSO-d_6 (Fig. 3,3a) was recorded on a Varian XL-200 MHz NMR spectrometer using TMS as an internal reference. The following structural assignments have been made (Table 3).

3.9.3.2 ^{13}C-NMR Spectra

^{13}C-NMR spectra (10) of salicylic acid in DMSO-d_6 (Figs. 4-6) was recorded on a Varian XL-200 NMR spectrometer. The multiplicity of the resoanance was obtained from APT (Attached Proton Test) program. The ^{13}C-NMR spectrum displayed all the seven carbons resonances. The carbon chemical shifts assignments are presented in Table 4.

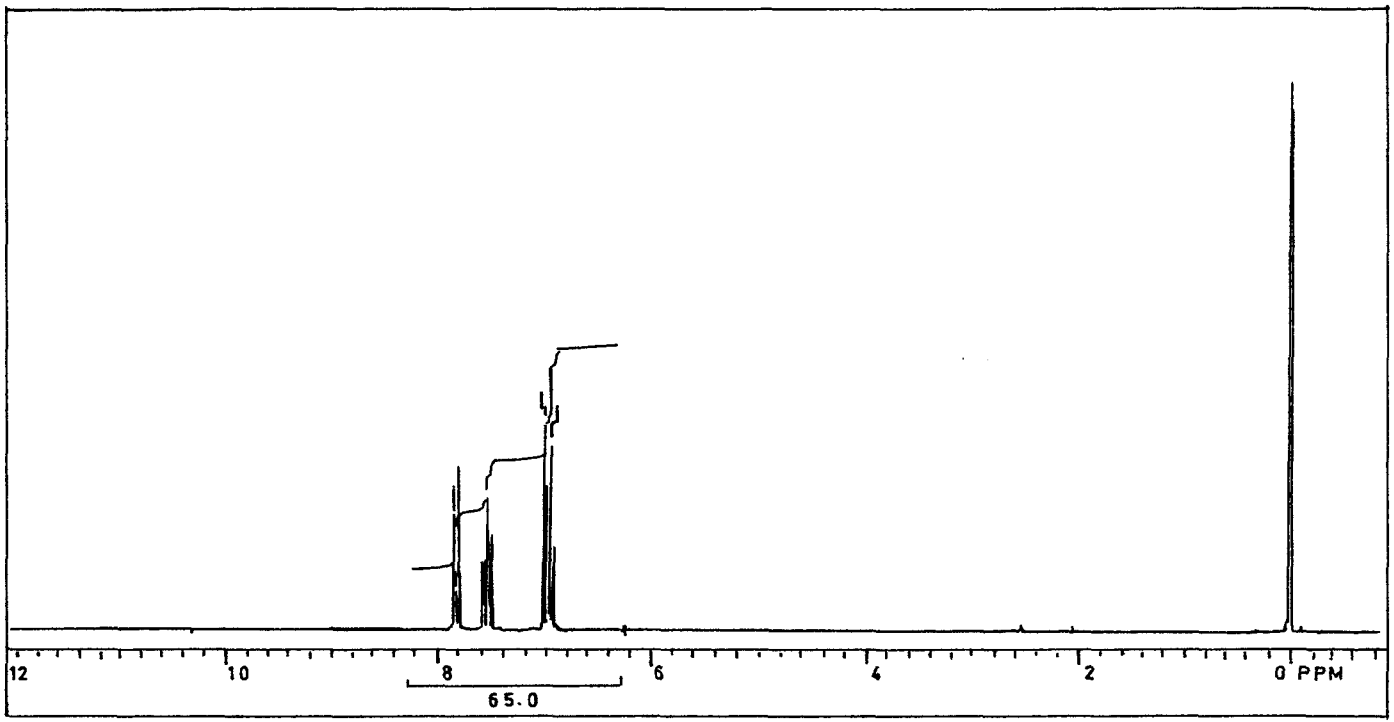


Fig. 3: PMR spectra of salicylic acid in DMSO-d_6.

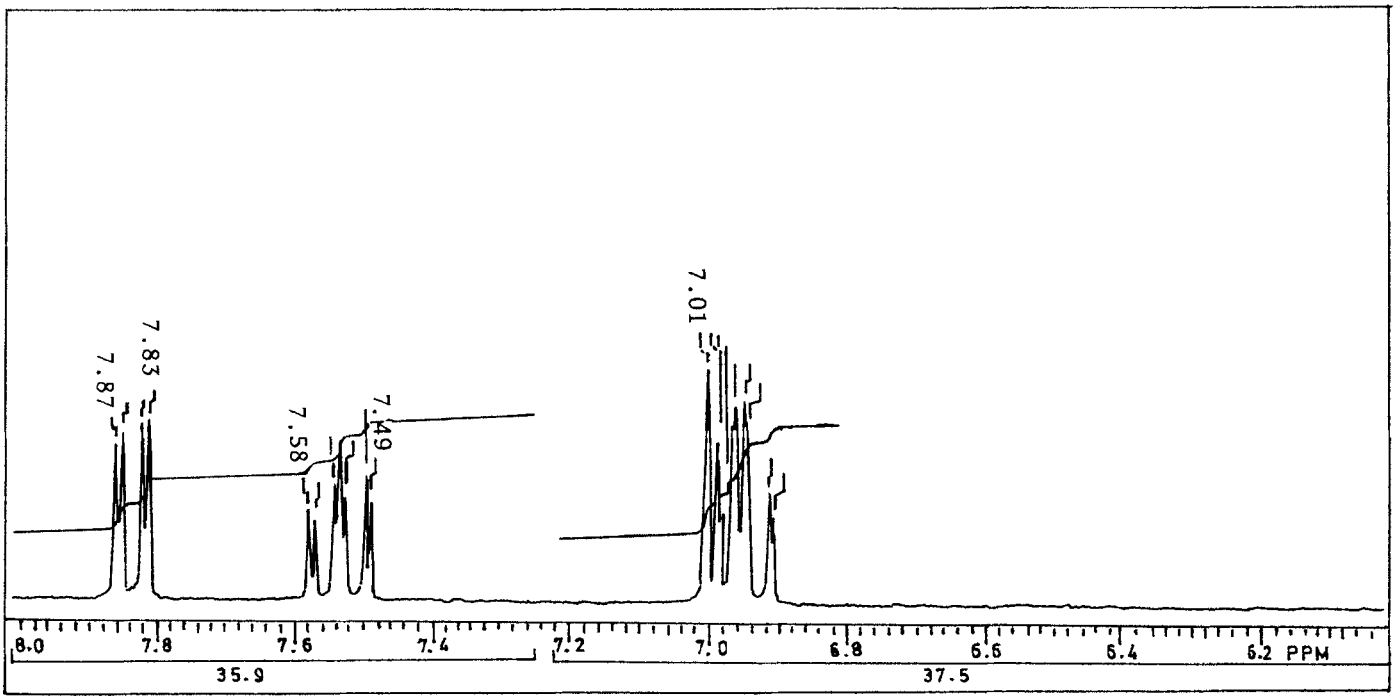


Fig. (3a): PMR spectra of salicylic acid in DMSO-d_6 (expansion of peaks).

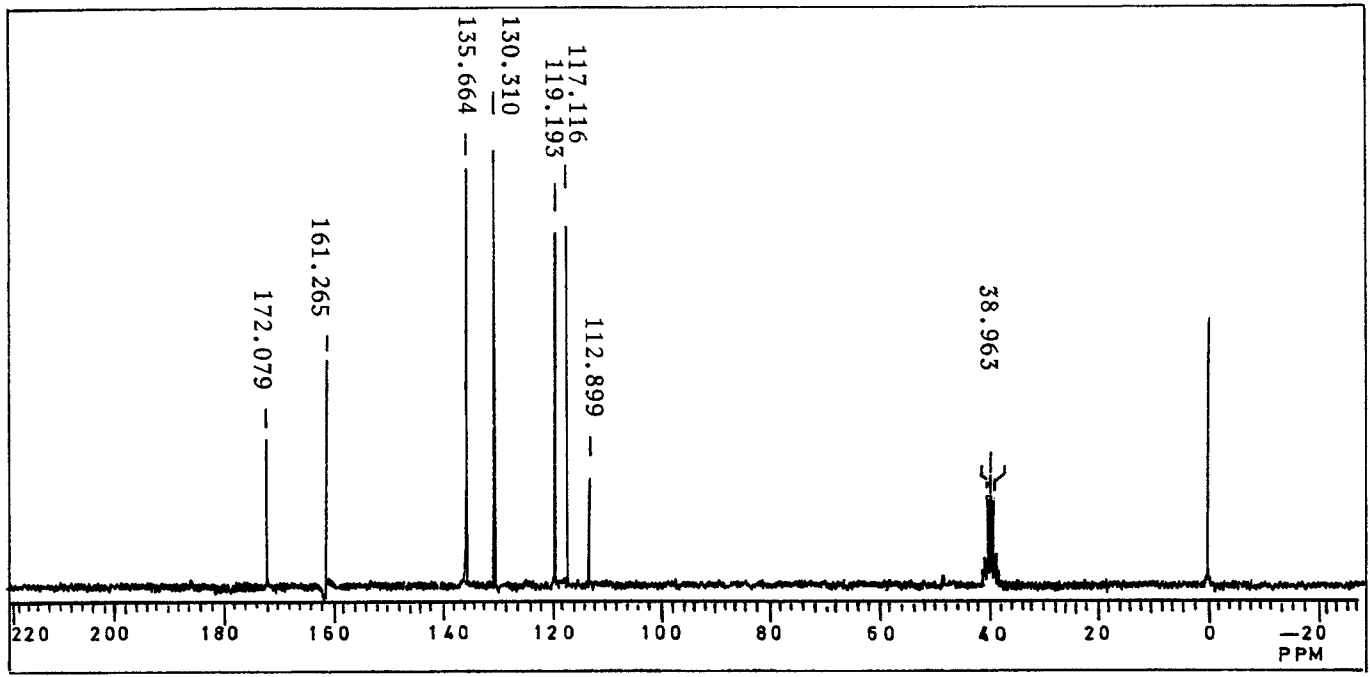


Fig (4) ^{13}C-NMR spectra of Salicylic Acid.

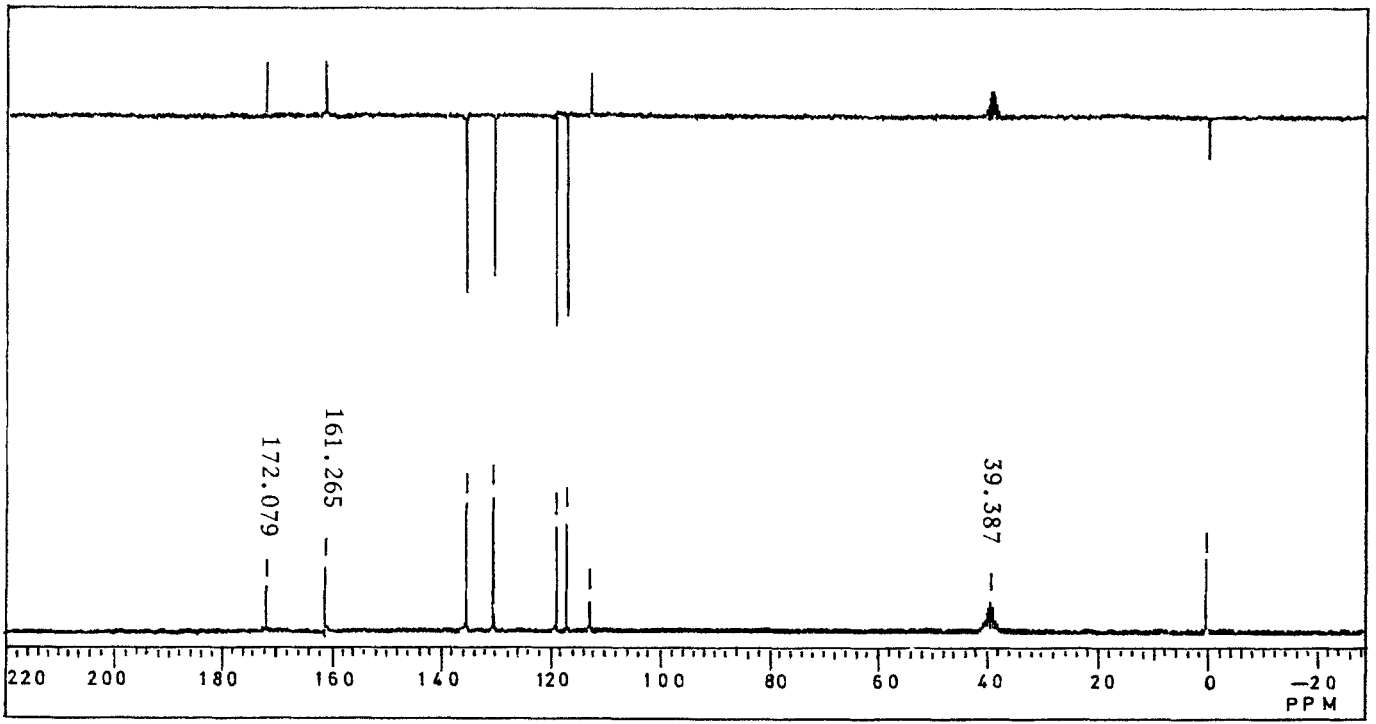


Fig (5) ^{13}C-NMR spectra of Salicylic Acid in DMSO-d_6 (APT).

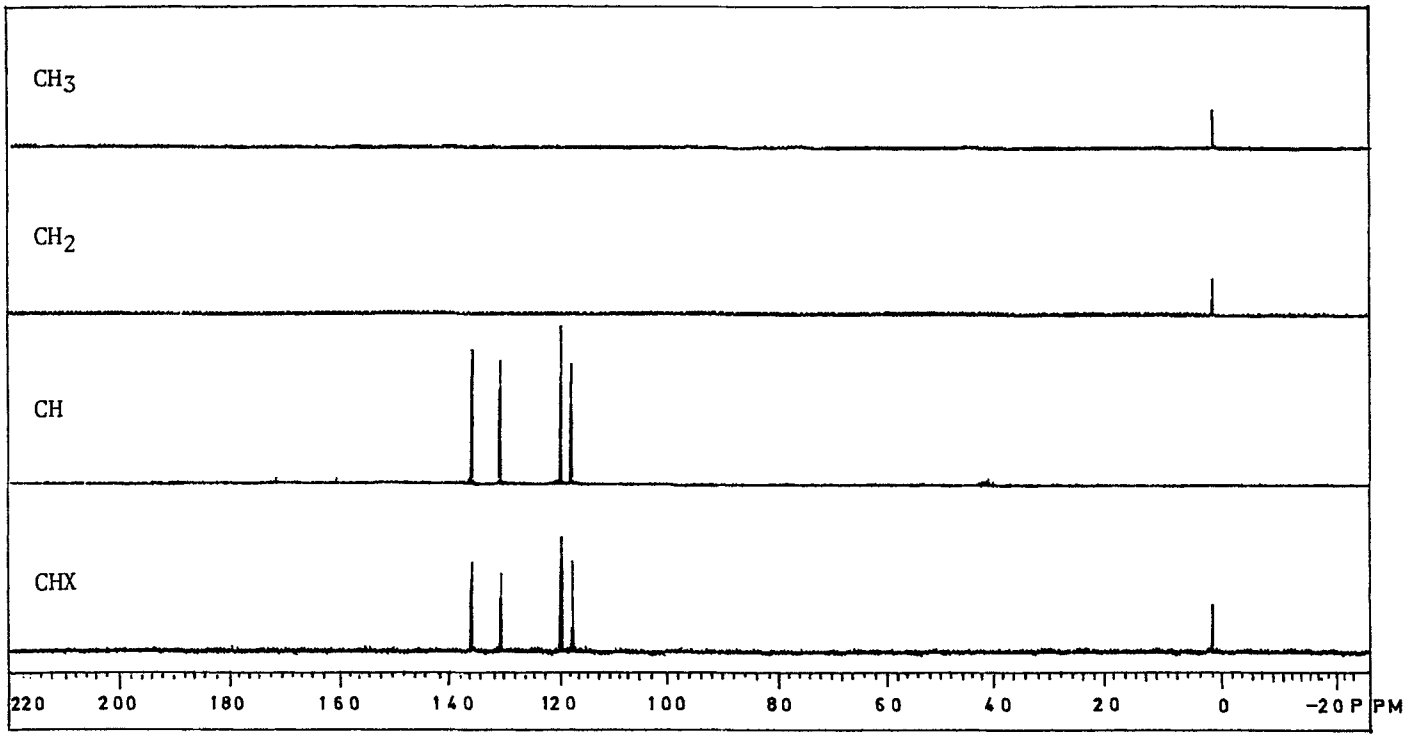
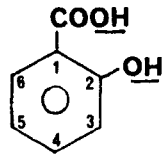


Fig (6) ^{13}C-NMR spectra of Salicylic Acid in DMSO-d_6 (DEPT).

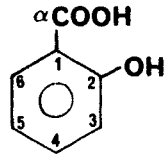
Table 3. PMR assignments of salicylic acid.



Chemical shift δ	Multiplicity	Assignment
2.56	broad (s)	O - H
6.91-7.01	m	3-CH and 5-CH
7.49-7.58	Overlapping doublets	4-CH
7.83-7.87	d	6-CH

s = singlet, m = multiplet, d = doublet

Table (4) Carbon-13 chemical shifts of salicylic acid

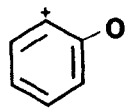
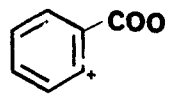


Chemical shift (δ)	Assignment
112.90	C_1
117.12	C_3
119.19	C_5
130.31	C_6
135.66	C_4
161.26	C_2
172.08	C_α

3.9.4 Mass Spectrum

The mass spectra (10) of salicylic acid was obtained by electron impact ionization (Fig. 7) and was recored on Finigen MAT 90 mass spectrometer. The spectrum was scanned from 50-250 a.m.a. Electron energy was 70 ev. Emission current 1 mA and ion source pressure 10^{-6} torr. The base peak is with a relative intensity 100%. Table 5 shows the most prominent fragments and their relative intensities.

Table (5) Mass spectrum of salicylic acid

m/z	Relative intensity %	Ion
45	18	$^{+}$COOH
64	50	—
92	100	
120	80	
138	38	M^{+}

3.10 X-Ray Powder Diffraction

The x-ray diffraction pattern of salicylic acid was determined [(Fig. 7(a)] using Philips full automated x-ray diffraction spectrogoniometer equipped with PW 1730/10 generator. Radiation was provided by copper target (cu anode 2000 W) high intensity x-ray tube operated at 40 KV and 35 MA. The monochromator was a curved single crystal one (PW 1752/00). Divergance slit and the receiving slit were 1 and 0.1° respectively. The scanning speed of the goniometer used as 0.02-2 θ per second.

The instrument is combined with Philips PM 8210 printing recorder with both analogue recorder and digital printer. The goniometer was aligned using silicon sample before use. Values of scattering angle (2 θ), interplanner distance (dÅ) and % relative intensity (I/Io x 100) are listed in Table 5a.

Table (5a): The x-ray powder diffraction lines of salicylic acid.

2 θ	d (Å)	I/Io %	2 θ	d (Å)	I/Io %
11.080	7.9850	100	39.835	2.2629	2.442
15.263	5.8050	2.442	43.870	2.0637	0.919
15.819	5.6022	1.332	45.126	2.0091	0.824
17.082	5.1908	5.469	46.683	1.9457	2.125
17.360	5.1081	46.507	47.195	1.9258	0.919
18.796	4.7209	0.253	50.178	1.8180	1.015
19.771	4.4903	0.666	50.963	1.7919	0.888
25.128	3.5438	2.379	51.337	1.7797	1.998
25.380	3.5093	5.868	54.273	1.6901	0.666
28.113	3.1740	8.692	54.831	1.6743	0.761
28.843	3.0953	1.998	56.098	1.6394	0.380
30.008	2.9778	0.697	56.431	1.6305	0.666
30.676	2.9145	1.935	57.282	1.6083	2.125
31.816	2.8125	1.110	58.151	1.5863	0.571
32.885	2.7235	1.173	58.786	1.5707	3.362
33.447	2.6791	1.522	59.498	1.5536	0.380
33.798	2.6520	1.110	60.044	1.5408	0.507
35.040	2.5608	1.015	60.392	1.5327	0.539
35.667	2.5172	1.237	62.190	1.4927	0.380
36.580	2.4564	0.824	62.782	1.4800	1.332
37.312	2.4100	0.666	63.307	1.4690	0.285
38.038	2.3656	0.824	63.692	1.4612	0.348

2 θ = scattering angle, d (Å) = interplanner distance, I/Io % = relative intensity based on highest as 100.

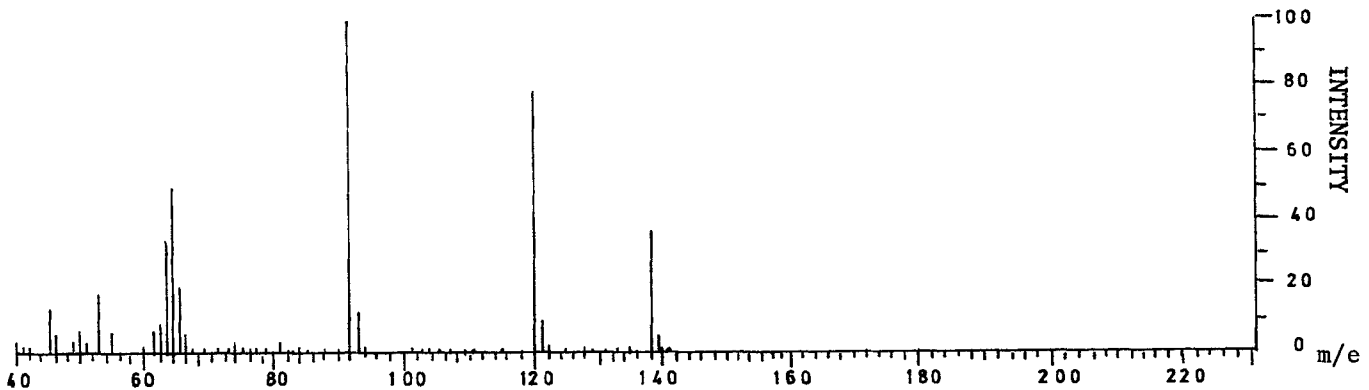


Fig (7) Mass-spectra of Salicylic Acid.

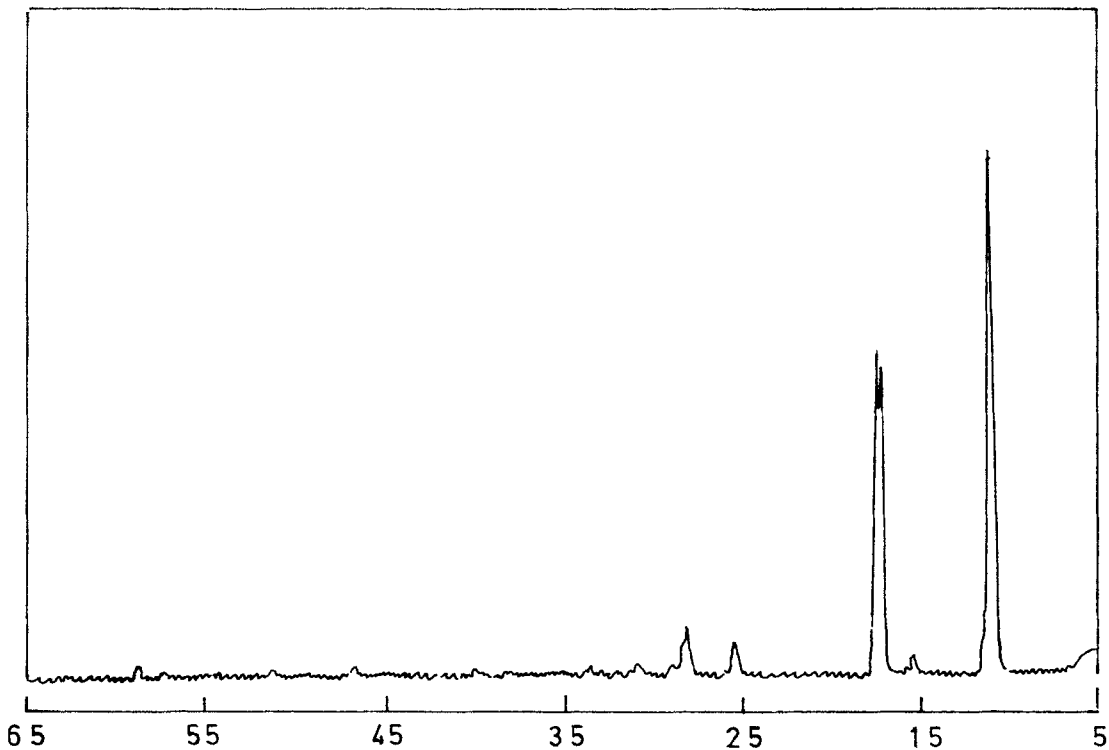


Fig. (7a): X-ray powder diffraction lines of salicylic acid.

4 Synthesis

Industrial Process (11)

Reaction

$$C_6H_5OH + NaOH \longrightarrow C_6H_5ONa + H_2O$$

$$C_6H_5ONa + CO_2 \longrightarrow HOC_6H_4COONa$$

$$2HOC_6H_4COONa + H_2SO_4 \longrightarrow 2HOC_6H_4COOH + Na_2SO_4$$

Material Requirements

Based on the one ton of salicylic acid,

Phenol	1600 lb	CO_2	1000 lb
Caustic soda	690 lb	H_2SO_4	900 lb

Process

Phenol is run into an equimolar amount of hot aqueous caustic soda in mixers. The resulting solution is heated to about 130°C and evaporated to dryness in stirred autoclaves under vacuum. This operation is sometimes carried on in heated ball mills to insure dryness as well as grinding of the resulting sodium phenate. After drying, the temperature is reduced to about 100°C, and dry carbon dioxide gas is introduced under about 6 atm (90 to 100 psi) pressure. After the desired amount of carbon dioxide is absorbed, the charge is generally heated at 150 to 170°C for several hours.

The crude autoclave product, after cooling, is dissolved in about an equal amount of water and filtered. It may be precipitated with acid and dried or purified in one of two ways before the precipitation. Contamination with iron must be avoided in these operations.

One method consists of decolorizing the crude sodium salicylate solution with activated carbon containing zinc dust. After filtering, the clear, almost

colorless solution is acidified with an excess of sulfuric or hydrochloric acid. The precipitated salicylic acid is centrifuged and dried in rotary dryers to give a high grade of salicylic acid.

The crude sodium salicylate solution may also be purified by crystallization in the form of its hexahydrate ($C_7H_5O_3Na.6H_2O$). At a temperature below 20°C, the hexahydrate crystallizes out after seeding, leaving many impurities behind. The crystals are separated from the mother liquor by centrifuging and washed with small amounts of cold water to remove adherent mother liquor. This material may be recrystallized to yield USP sodium salicylate, or it may be acidified and precipitated as described previously, to give a very good grade of acid.

USP salicylic acid is generally obtained by subliming the technical acid.

5 Pharmacokinetics

5.1 Metabolism

Acetyl salicylic acid is rapidly hydrolysed to salicylic acid (SA) by ubiquitous esterases. Salicylic acid is removed from the body by five parallel and competing pathways: renal excretion; conjugation with glycine to form two glucuronides, SAG and SPG, and hydroxylation to yield salicyluric acid (SU); and conjugation of the carboxyl or hydroxyl group to form gentisic acid (GA). The biologic half-life of acetylsalicylic acid in man is only 15 to 20 min following an intravenous or rapidly absorbed oral preparation, whereas the apparent half-life of salicylic acid varies from 2.4 to 19 hr, because of two easily saturable SA biotransformation pathways. Acetylsalicylic acid must be prescribed with care since the therapeutic concentration range of salicylic acid is quite narrow and a small change in dose may have a pronounced effect on steady-state blood drug levels (12).

Numerous past and present reports of drug levels in biologic fluids following acetylsalicylic acid administration, purporting to describe acetylsalicylic acid absorption and bioavailability, have employed analytical techniques that measure "total salicylate." These nonspecific procedures determine an assorted composite or total sum of acetylsalicylic acid, SA, SU, SAG, SPG, and GA. Consequently, there is little specific information on a particular salicylate, such as the presence of intact acetylsalicylic acid blood levels following administration of a selected product. Nevertheless, it is known that although acetylsalicylic acid is absorbed from the stomach after oral administratio, the major site is in the upper portion of the small intestine. When a rapidly absorbed oral acetylsalicylic acid preparation is compared with an intravenous dose, it is found that about 30% of the oral dose is hydrolyzed to SA before systemic distribution. The acetylsalicylic acid in compressed tablets is generally all absorbed (as acetylsalicylic acid or SA), but the absorption of enteric-coated tablets may be delayed, slow, and/or incomplete. The observed absorption of rectal acetylsalicylic acid has been slow and the amount absorbed dependent on the time between insertion and defecation. On the basis of current knowledge, it is important that specific analytical methods be used to describe acetylsalicylic acid absorption, distribution, and elimination, thereby delineating more clearly the relationship between vascular or tissue levels and response. (Fig. 8) shows the metabolic fate of salicylic acid (12).

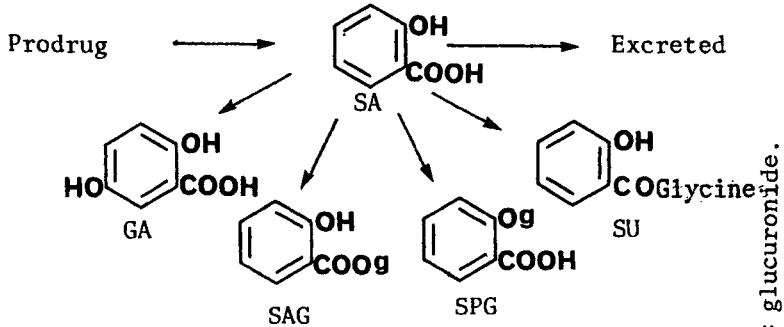


Fig. 8. Metabolic Fate of Salicylic Acid (SA).

The biotransformation of salicylic acid to salicyluric acid in Rhesus monkey has also been reported by Wan *et al.* (13). The dose of salicylic acid given intravenously to the monkey is equivalent to a 625-mg, dose for a 70-kg. man. The terminal half life for the animal studied was 50 min. (Fig. 8), which is shorter than the 3-4-hr. average half-life in man. The clearance value for salicylic acid estimated from the area under the curve was 10 ml/min or 1.8 ml/min/kg, in marked contrast to the data obtained for benzoic acid in rabbits where clearance was found to be between 44 and 115 ml/min or 12.6-28.8 ml/min/kg. In a single intravenous study in the monkey, benzoic acid was found to have a plasma clearance of 140 ml/min or 38.1 ml/min/kg. Plasma clearance of salicylic acid in man, at a dose level of about 500 mg administered intravenously, was found to be in the range of 0.57 ml/min/kg body weight according to the data of Riegelman *et al.* (14). The fraction of the dose excreted as salicyluric acid in 16 hr. was found to be 72%.

5.2 Toxicity

Not only salicylic acid but all the salicylates cause severe toxicity (salicylism). The most toxic salicylate is oil of wintergreen (methyl salicylate); death has been reported from ingestion of < 1 tea spoon in a young child (15).

The early symptoms of salicylism are CNS stimulation, with vomiting, hypernea, hyperactivity, hyperthermia, and even convulsions. This quickly turns to depression, with lethargy, respiratory failure, and collapse (15).

Hyperpnea causes a loss of CO_2 through expired air and therefore a decrease in plasma carbonic acid. This tends to produce an increase in plasma pH (respiratory alkalosis), and the kidneys respond by excreting large quantities of base in the form of bicarbonate. Sodium, potassium, and large amounts of organic acids are also lost along with the bicarbonate (15).

Adults and older children tend to correct the disturbance at this point. Young children, however, quickly develop a fall in the blood pH (metabolic acidosis), due to salicylate stimulation of metabolism. The toxic effects of salicylate and the loss of buffer base interfere with metabolic processes, and ketosis develops. Because the respiratory alkalosis and metabolic acidosis occur simultaneously, a child may present with a mixed disturbance and a relatively normal pH, or with frank acidosis. The PCO_2 will be lower than expected. Children < 4 yr develop metabolic acidosis more rapidly, without concurrent respiratory alkalosis. Dehydrationj is a serious problem because of insensible water loss and increased renal water loss (from an increased urine solute load). Severe losses of sodium and potassium are not uncommon (15).

5.3 Laboratory Findings and Diagnosis

A useful qualitative screening test for salicylic acid is performed by adding a few drops of glacial acetic acid or 0.1N hydrochloric acid to 1 ml of urine, followed by 3 drops of 10% ferric chloride solution. A burgundy red color appears and persists if salicylic acid is present (color may turn reddish brown in the presence of phenothiazines). A serum salicylate level can be obtained in most laboratories. Commerically available test strips may be used with urine as well as serum or plasma to determine the presence of salicylic acid. These tests react only with salicylic acid and therefore do not work on stomach contents or pills, but any salicylate is hydrolyzed in the body to salicylic acid and would be present as such in blood or urine (15).

Other laboratory tests to assist in assessment and treatment include blood pH, serum CO_2, or PCO_2 (any 2); serum sodium; serum potassium, BUN; blood glucose; and urine pH and sp gr. These determinations and the serum salicylate level should be followed serially during therapy (15).

The manifestations of salicylate toxicity are related to the peak level rather than the level of a given moment. For single-dose ingestions of salicylate, an estimate of the relative severity of the illness can be determined by use of a nomogram, provided the approximate time of ingestion and a single serum salicylate level are known (15).

5.4 Uses and Adverse Effects

Salicylic acid (1) is not employed internally as an analgesic due to its local irritating effect on the gastrointestinal tract. It is employed externally on the skin, where it exerts a slight antiseptic action and a marked keratolytic action. The latter property makes salicylic acid a beneficial agent in the local treatment of warts, corns, fungous infections, and certain forms of eczematoid dermatitis. Tissues cells swell, softer, and ultimately desquamate. Salicylic acid is applied as a 2 to 20% concentration in collodion, lotions, or ointments, and as a 10 to 40% concentration in plasters. Salicylic acid plaster is used for the destructive effect of salicylic acid on hardened, keratinized tissue. The so-called corn plaster are typical.

Salicylic acid (2) is also used as preservative of food products, but its use for this purpose is forbidden in some countries, many methyl salicylates, acetylsalicylic acid and other salicylates, dyes, as reagents in analtyical chemistry.

Salicylic acid (3) has kertolytic properties and is applied topically in the treatment of hyperkeratic and scaling skin conditions; such as dandruff, ichthyosis, and psoriasis. Initially a concentration of about 2% is used, increased to about 5% if necessary. It is often used in conjunction with many other agents, such as benzoic acid, coal tar, resorcinol, and sulpher. Salicylic acid is also used in the form of a paint in a collodion basis (10 to 14%) or as a plaster (20 to 250%) to destroy warts or corns. It also possesses fungicidal properties and is used topically in the treatment of such fungal skin infections as tinea.

Salicylic acid (3) is a mild irritant and application of salicylic acid preparations to the skin may cause dermatitis. Symptoms of acute systemic salicylate poisoning have been reported after the application of asalicylic acid to large areas of the body.

6 Methods of Analysis

6.1 Elemental Analysis

C 60.87%, H 4.38%, O 34.75%

6.2 Identification Methods

1. Ferric chloride produces a violet colour even in dilute solutions of salicylic acids or salicylates (1).

2. The addition of dilute mineral acids to moderately concentrated solution of salicylates produces a white crystalline precipitate of the salicylic acid which when washed with cold water and dried, has the melting point of salicylic acid (1).

3. Dissolve 0.2 g in 10 ml of 96% ethanol, add 20 ml of water, and titrate with 0.1M sodium hydroxide Vs using phenol red solution as indicator. Each ml of 0.1M sodium hydroxide Vs is equivalent ot 0.01381 g of $C_7H_6O_3$ (5).

6.3 Titrimetric

1. The method (16) describes the titrimetric micro-determination of salicylic acid, aspirin and p-hydroxybenzoic acid by amplification reactions. The cited acids are brominated with Br to form tribromophenyl hypobromite, which after removal of unconsumed Br wtih formic acid, is treated with iodide to give tribromophenol and free iodine. The liberated iodine is extracted with $CHCl_3$ and reduced to iodide, which is determined by oxidation with Br and iodimetric titration of the iodate produced with $Na_2S_2O_3$ solution. This six fold amplification

method was used to determine 0.05 to 2 mg of the acids.

2. Another titrimetric method was done by Liu (17). In this method the perylenequinone pigments bambusicolin A and B were isolated from the fungus Shiraia bambusicola (P. Heen). Bambusicolin A is slightly soluble in ethanol, and soluble in alkalaline solution, acetone and $CHCl_3$. It changes colour from red to emerald green over the pH range from 8.6-9.4 and is suitable end point indicator for titration of citric, salicylic and other acids with pK values of about 6.

3. Salicylic acid, Na salicylate and aspirin can be determined by titration (18) with standard $KBrO_3$ solution. The incision on the oscillopolarogram is used for end point detection.

4. The samples of the formulated creams (19) containing 0.1-0.2 mequiv of alkaloid halides. Salicylic acid, hexochlorophane or methyl salicylate dispersed in cetamacrogol, cetrimide or lanette wax, are dissolved in 35 ml of anhydrous acetic acid (with addition of 5 ml of 3% Hg acetate in the example of alkoids) or dimethylformamide, and are titrated with 0.1M $HClO_4$ or 0.1M tetrabutylammonium hydroxide, respectively at 0.5 ml min^{-1} with use of glass-calomel system of a Metrohm E536 potentiograph. The potentiograms for the titrations of various drugs are shown. Recoveries were from 98-102% for 2 or 3% additions of the drugs to the creams. The interferences of sod. lauryl sulphate in titrations with tetrabytylammonium chloride is avoided by adding 2 ml aq. $Ba(NO_3)_2$.

6.4 Spectrophotometric

1. Spectrophotometric determination of salicylic acid in aspirin was done by Wahbi *et al.* (20). A portion of powdered tablets or bulk aspirin powdered was extracted with ethanolic 1% monochloroacetic acid. After filtration, the first derivative spectrum of the solution, was

recorded. The trough at 316 nm was used for the determination of salicylic acid.

2. Derivative and derivative-difference spectrophotometric methods based on the zero crossing technique are described for the analysis of mixture of drugs along with salicylic acid which is determined by second-derivative spectrophotometry at 314 nm in methanol medium (21).

3. In this method salicylic acid is determined in its compound preparations. A sample of 1 g of ointment or 1 ml of liquid preparation, containing 6% of salicylic acid and 12% of benzoic acid is dissolved in boiling water. The cooled solution is diluted to 500 ml with H_2O and filtered, and a 100 ml portion of the titrate is diluted to 100 ml with H_2O. Salicylic acid is determined in the resulting solution by differential spectrophotometry at 297 nm (22).

4. Another spectrophotometric analysis for salicylic acid, the sample diluted in 95% ethanol and absorbance of the solution is measured at 303 nm by single-wavelength spectrophotometry (23).

5. Another spectrophotometric determination of salicylic acid in pharmaceutical formulations using copper acetate as a color developer. Different vol. of salicylic acid solution (10 mg/ml) were mixed with an equal vol. of copper acetate solution and made up to 10 ml with Na acetate: acetic acid buffer (pH 5.6 to 6.0). The absorbance of the stable, yellowish-green complex was measured within 30 h at 730 nm (24).

6. Another quantitative analysis of three-acid powder by dual wavelength linear-regression spectrophotometry in pharmaceutical preparations has been reported (25).

7. A semi-automated u.v. method for determination of aspirin in enteric coated tablets is described, involving dissolution of the sample by stirring in simulated gastric and intestinal fluid. The acidified stream of fluid is extracted with $CHCl_3$, and the absorbance of the extract is

monitored at 280 nm. The *in vitro* rate of dissolution of aspirin from the tablets is measured automatically. Aspirin in suppositories is determined by dissolving them in $CHCl_3$, diluting an aliquot with 1% acetic acid in $CHCl_3$ and measurement of the absorbance of the solution at 280 nm (26).

8. Determination of salicylic acid in aspirin by second derivative U.V. spectrophotometry. The powdered aspirin was extracted with anhydrous ethyl ether, filtered extract was evaporated and the residue was dissolved in dioxan. This solution was analysed by second derivative spectrophotometry at 380 nm (27).

9. Another method for the determination of salicylic acid in aspirin powder by second derivative ultra-violet spectrometry was done by Kitamura *et al.* (28). A differentiator with electronic derivative circuit and incorporating three circuits with differencial time constants of 27, 82 and 22 was connected to a double beam spectrophotometer; derivative spectra were recorded with a slit width of 1 mm and scanning speed f 120 nm min^{-1}. The second derivative spectrum of salicylic acid showed a trough at 292 nm and a satellite peak at 316 nm, in the presence of large amounts of aspirin, the trough disappeared, but the peak was unaffected. The relationship between the height of this peak and the concentration of salicylic acid was rectilinear for 1 to 10 $\mu g\ ml^{-1}$.

10. Spectrophotometric determination of salicylic acid was done by Qu (29). 0.5 ml of sample solution containing up to 70 μg of salicylic acid is mixed with NaCl (0.2 g) and 1 nM-pentachloronitrosyliridate (1 ml) and set aside in the dark for one hour. Then (0.2 ml) of 2% ascorbic acid solution is added, the mixture is diluted to 40 ml with 4M-HCl, and the absorbance is measured at 446 nm vs a reagent blank. Beer's law is obeyed from 0.5 to 13 $\mu g\ ml^{-1}$ of salicylic acid. No interference is caused by the presence of boric or benzoic acid.

11. Wang *et al.* (30) have also reported a spectroscopic method for the determination of salicylic acid along with chloramphenicol. Both were determined in ethanolic solution at 230 and 277.8 nm, respecitvely. Calibration graphs were rectilinear for 5 to 25 μg/L of salicylic acid and chloramphenicol, respectively.

6.5 Colorimetric

A rapid method (31) for the determination of salicylate in biological fluids is presented, based on a reagent containing ferric nitrate, mercuric chloride and hydrochloric acid, which precipitates the proteins and simultaneously reacts with salicylic acid to give a purple color.

Reagents

Color reagent. With the aid of heat, 40 g of A.R. mercuric chloride are dissolved in 850 ml of water. The solution is cooled and 120 ml of N-HCl and 40 g of ferric nitrate, $Fe(NO_3)_3$, $9H_2O$, are added. When all the ferric nitrate has dissolved, the volume of the solution is made to 1 L with water. This solution is stable indefinitely.

Stock salicylate solution. 580 mg of sodium salicylate, $C_7H_5O_3Na$, are dissolved in sufficient water to make 250 ml of solution. A few drops of $CHCl_3$ are added as a preservative. This solution contains 200 mg of salicylic acid/100 ml.

Standard salicylate solution. 20 ml of the stock solution are added to sufficient water to make 100 ml of solution. A few drops of $CHCl_3$ are added as a preservative. This solution contains 40 mg of salicylic acid/100 ml. Both stock and standard solutions keep for at least 6 months if stored in a refrigerator.

Procedure

For cerebrospinal fluid (c.s.f.) plasma or whole blood. Wintrobe's anticoagulant (32) is used for plasma

and whole blood. 1 ml of fluid is placed in a cylindrical centrifuge tube and 5 ml of color reagent are added; the tube is shaken during the addition. The contents of the tube are shaken for a few seconds to ensure that the protein precipitate is finely dispersed. The tube is centrifuged at 2000 g for 2 min and the supernatant fluid, which should be optically clear, is transferred to a test tube. A photoelectric colorimeter is set at full-scale deflexion (optical density, 0) with a blank prepared by mixing 1 ml of water with 5 ml of color reagent. If a spectrophotometer is used the wavelength should be set at 540 mμ. The optical density of the purple color is constant for at least 60 min. The salicylate content of the sample is read from a graph prepared by treating 1 ml quantitites of sodium salicylate solutions containing the equivalent of 0.1, 0.2, 0.3, 0.4 and 0.5 mg of salicylic acid, with 5 ml of color reagent and plotting the optical densities of the resultant colored solutions. The quantities shown above correspond to blood salicylic acid levels of 10-50 mg/100 ml and the colors obey Beer's law over this range. By this method the optical densities obtained on analysing solutions containing the equivalent of 12-40 mg of salicylic acid/100 ml, lie between 0.2 and 0.7, the range in which the relative error is least (33). The optical density of the unknown is greater than 0.7, the analysis is repeated, using a smaller sample diluted to 1 ml with water.

For urine. The urine is diluted with water so that it contains between 10 and 40 mg of salicylic acid/100 ml. The diluted urine is analysed as for serum. After obtaining the optical density of the unknown, a blank reading is obtained by setting the instrument with water and reading the optical density of a solution prepared by mixing 1 ml of diluted urine with 5 ml of color reagent and 0.1 ml of syrupy phosphoric acid (sp.gr. 1.75), using the same cells and filter as before. Urine solutions often do not require centrifuging; if centrifuging is necessary both unknown and urine blank are centrifuged.

Urine salicylic acid mg/100 ml = (mg salicylic

acid/100 ml in diluted unknown - mg salicylic acid/100 ml in diluted urine blank) x dilution factor.

Some other colorimetric assays involving diazotization with p-nitroaniline and nitrous acid (34), use of folin and Cioalteu phenol reagent (35), complexation with cupric ion in nitrous acid (36-38) or estimation as the nitro derivative (39) have been reported in the literature.

6.6 Radiometry

The method (40) describes the separation of organic poisons in chemicotoxicological analysis using radiometry and also radiometric methods in studies of losses of organic poisons during separation from biological material. The samples were cadaver liver homogenates with ^{14}C-labeled salicylic acid added at 20 to 100 $\mu g\ g^{-1}$. The homogenates were deproteinised by addition of $(NH_4)_2SO_4$, acidification with H_2SO_4 (to pH 4) and boiling for 3 minutes. The filtered extracts were acidified to pH 1 with 5N-H_2SO_4 and extracted with ethyl ether. After evaporation of the ether the residue was dissolved in acetone. The total loss of salicylic acid during the separation was 33.59% (deproteinisation 19.47%, extraction 10.68% and drying and evaporation 3.44%).

6.7 Fluoremetric

1. This method (41) involves the determination of salicylic acid in human serum and aspirin formulations by second-derivative synchronous fluorescence sepectrometry. Serum containing salicylic acid was sonicated with 2 ml of 1% acetic acid: $CHCl_3$ and was then centrifuged for 10 min at 1500 g. In a 1 ml portion of organic layer was diluted to 2 ml with 1% acetic acid: $CHCl_3$ solution and the solution was analysed by second derivative synchronous fluorescence spectrometry, by simultaneous scanning the excitation and emission monochromators with a constant wavelength difference δ, of 130 nm (excitation wavelength at 380 nm) for salicylic acid.

2. Street, K.W. *et. al.* (42) determined salicylic acid fluorimetrically. The relationship derived for primary inner filtering were compared with observed inner filtering results obtained in the fluorimetric analysis of salicylic acid in methanol.

3. In this method salicylic acid was determined in platelet free blood after hydrolysis and extraction into 1,2-dichloroethane and fluorimetry at 406 nm (43).

4. Salicylic acid has also been determined by spectrofluorometry in plasma. A 5-ml sample of whole blood was placed immediately into a centrifuge tube containing 50 μl heparin solution and 50 μl potassium fluoride solution, the tube being kept in an ice bath. The blood was gently mixed and kept in the ice bath for 2 min (final blood temperature = + 2°). The blood was then centrifuged, and the plasma pipetted off and stored in dry ice until required for analysis. 1 ml aliquot of the ethereal extract of the acidified plasma was re-extracted with 5 ml phosphate buffer (pH 7.0) in a centrifuge tube. The sample was centrifuged, excess ether sucked off, and nitrogen blown through the phosphate buffer for 30 sec to remove the dissolved ether (which modifies the spectrophotofluorometric readings). The solutions were then read on the spectrophotofluorometer, activation wavelength, 315 mμ (uncorrected); emission wavelength, 420 mμ (uncorrected). Fluorescent measurements of standard solutions of salicylic acid in phosphate buffer (0.1-1.0 mcg/ml) were made at the same time (44).

6.8 Infrared Spectroscopy

Method describes the quantitative determination of salicylic acid in aspirin tablets by infra-red spectrophotometry. 200 mg samples of aspirin was dissolved in $CHCl_3$ and centrifuged. The supernatant solution was then analysed for salicylic acid by I.R. spectrophotometry at 1160 cm^{-1} with the use of a standard-additions method (45).

6.9 Chromatographic Methods

6.9.1 Paper Chromatography

1. In this method (46) powdered aspirin (0.1 g) were shaken with anhyd. ethnaol - anhyd. acetic acid (657.10) and a portion of the upper clear liquid was analysed by chromatography on paper pre-treated with 0.2M-phosphate (pH 8), with light petroleum (boiling-range 30° to 60°) ethyl acetate. $CHCl_3$ anhyd. acetic acid (30:10:6:5) as mobile phase. Under 254 nm radiation the spot of free salicylic acid should be a lighter blue that of the standard; its concentration, should be less than 0.4% to be acceptable.

2. Another method for the chromatographic separation of salicylic acid on paper impregnated with ferric hydroxide was done by Rawat *et al.* (47). Strip (14 cm x 3 cm) of Whatman No. 3 paper were dipped in 0.1M-$Fe(NO_3)_3$ for 15 to 20 seconds dried at room temperature, dipped in 0.1M NaOH for 40 to 45 seconds and again dried, the strips were then twice washed with H_2O to remove excess of reagent, and dried. The ethanolic test solution (1 or 2 drops) were applied to such a strip and a chromatogram was developed (for 11 cm) with 1M, 0.1M- or 0.01M-$NaNO_3$, H_2O, ethanol, H_2O-ethanol (1:1) or 1M-NaCl. Salicylic acid was detected by the yellow color of the spot.

3. Phosphometric analysis of several salicylic acid derivatives was done by using filter paper. 1 μl Portion of 1M-NaOH and 1M-Nal were applied to filter paper (No. 51; Toyo Rosh: Co) a portion of the alcoholic sample solution was applied to the same spot and the filter paper was dried at 50° for about 30 minutes under an I.R. lamp. The filter paper was then mounted in a holder, and the phosphorescence was measured at 605 nm (excitation at 300 nm). The calibration graph was rectilinear for 3-630 ng of salicylic acid or aspirin and for 1.5 ng to 700 ng of phenyl salicylate (48).

6.9.2 Gas Chromatography

Osman *et al.* (49) developed a method for the determination of salicylic acid in urine. When a silicon-selective H atmosphere f.i.d is doped with vapours of metal-containing compounds (e.g. ferrocene) in excess of the amount required for positive responses to Si-containing compounds an ionisation quenching effect occurs, which produces negative peaks. This negative response is greatest with 35 ppm of ferrocene, and detector sensitivity is best, when the electrode is located 30 mm from the flame. However, this position gives poor selectivity by increasing the electrode hight to 110 mm, selectivity is improved but selectivity is impaired by a factor of 6, although still remaining 2 to 3 times better than that achieved in the positive response mode. Alcohols, hydrocarbons, ethers, fluorocarbons and N-containing compounds produce only positive peaks, whereas compounds containing Cl, Br, S, P and Fe produce potentially interferring peaks.

Plasma salicyic acid and aspirin has been estimated (50) as their respective trimethylsilyl derivatives by GLC. The method involved the use of fluoride and cooling to minimize the hydrolysis of aspirin in whole blood. Following solvent extraction, internal standards were added to each plasma extract. The extracts were evaporated to dryness and the residue were trimethylsilylated with hexamethyldisilazane in acetone. The analysis was done on a gas chromatograph equipped with dual flame-ionization detector. The signal from the detectors was fed into a strip-chart recorder, with a 1.0 mv full-scale response. Dual coiled columns, 1.8 m in length and 4 mm (id), containing 3% OV-25 coated onto 60-80 mesh chromosorb G, were used. The operating temperatures were 240°, 160° and 240° for injectors, oven and detectors, respectively. The gas flow rates were 55 ml/min, 40 ml/min and 600 ml/min for helium hydrogen and air respectively.

6.9.3 High Pressure Liquid Chromatography (HPLC)

Heideman *et. al.* (51) developed a rapid HPLC method for the estimation of salicylic acid in aspirin tablets. Method involves the blending of tablets with acidic ethanol to extract the aspirin and salicylic acid rapidly. The resulting preparation is then immediately injected on to a 4.6 mm x 3 cm 5 micron reverse-phase column. Aspirin and free salicylic acid are determined simultaneously. The run time is less than 2 minutes.

Salicylic acid as an impurity was investigated in 12 different brands of aspirin formulations readily available in Lagos (Nigeria). The HPLC method adopted for the investigation involved a mobile phase of methanol/water (20/80, v/v) adjusted to pH 2.5 with phosphoric acid and was run on a 50 mm column monitored at 240 nm. The limit of detection for salicylic acid was 5 ng. Only three of the formulations showed the presence of salicylic acid impurity and all these contained salicylic acid in excess of the USP 1980, limit of 0.3% salicylic acid per tablet (52).

Fogel *et. al.* (53) also developed a reversed phase HPLC method for the simultaneous assay of aspirin and salicylic acid in film-coated aspirin tablets. As little as 0.1% salicylic acid (relative to aspirin) can be quantitatively determined. Using a 5-microns octadecylsilane column with water-acetonitrile-phosphoric acid (76-24: 0.5) as the mobile phase enabled the chromatographic separation to be completed in 4 minutes. Due to the slow rate of decomposition of aspirin to salicylic acid in the extraction solvent, acetonitrile-methanol-phosphoric acid (92:8:0.5), the analysis of many samples was routinely performed by means of automated HPLC equipment.

Tang *et al.* (54) have also reported HPLC determination of serum salicylic acid and aspirin concentrations. Serum (0.1 ml) was treated 1 μl of carbamazepine (internal standard) solution (0.65 mg/ml), 0.1 ml of 0.1M-phosphate buffer of pH 7.4, 10 μl of 21.25% H_3PO_4 and 1.2 ml of ethyl ether prior to centrifugation at 4000 rpm

for 5 minutes; 0.8 ml of ethereal layer was evaporated and the residue was redissolved in 100 μl of methanol. Salicylic acid and aspirin in 20 μl of the solution were simultaneously determined by HPLC on a column (25 cm x 4.6 mm) of ultrasphere-ODS (5 μm) with aq. 52% methanol containing 1% acetic acid as mobile phase (1 ml/min) and detection at 228 nm.

Summary of some of the HPLC conditions for the determinations of salicylic acid is also presented in Table 6 (55-72).

6.9.4 Thin Layer Chromatography (TLC)

Summary of some of the TLC conditions used for the analysis of salicylic acid is presented in Table 7 (73-78).

6.10 Thermal Analysis (DSC)

A differential scanning calorimetry curve of salicylic acid was obtained (Fig. 9) on a Perkin Elmer DSC 20 differential calorimeter. Nitrogen was used as purge gas. Scan was performed at the rate of 10°C/min from 100-200°C. The DSC curve revealed an endothermic melting peak (Max. 158.2°C).

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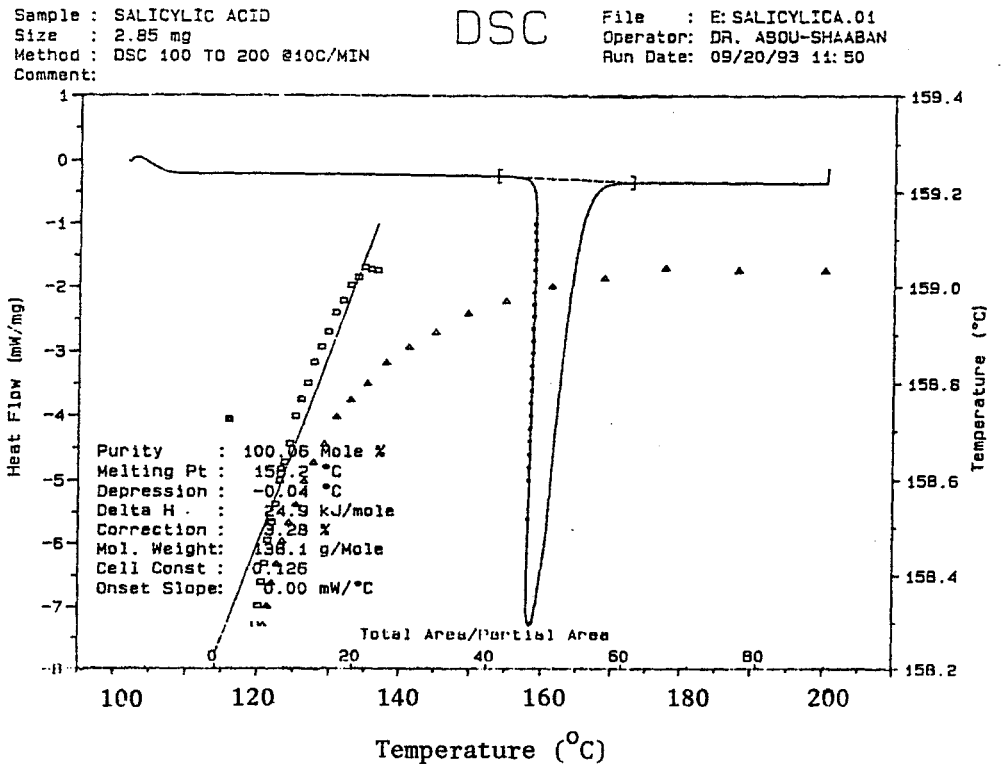

Fig. 9: Thermal curve of salicylic acid.

Table (6): Summary of HPLC conditions for the determination of salicylic acid

Column	Mobile phase	Flow rate	Detection	Sample	Ref.
μBondapak phenyl column	30% of methanol in 5 mM-tetrabutyl amm. phosphate buffer (pH 7.5)	-	-	Semi-solid formula-tions	55
(25 cm x 4 mm) of LiChrosorb RP-18 (5 or 10 μm)	0.1% of acetic acid (plus sod. acetate) in methanol (30 or 50% or a gradient of 20 to 60% of methanol in 0.1% acetic acid H_2O-acetonitrile conc. acetic acid	-	-	Meat	56
15 cm column of Ulre ODS (5trasphe μm)	H_2O acetonitrile: conc. acetic acid (17:2:1)	0.7 ml/min	UV	Blood	57
(25 cm x 4.5 mm) of Spherisorb ODS (5 μm)	H_2O-acetonitrile-methanol (13:5:2) adjusted pH 3 with H_3PO_4	-	-	Serum	58

Conti..... (Table 6).

Column	Mobile phase	Flow rate	Detection	Sample	Ref.
Precolumn (4 mm x 4 mm) of LiChrosorb 100 RP-18 on to a column (25 cm x 4 mm) of Lichrospher RP-8 (5 μm)	Gradient elution of (1:9 to 7:3 over 25 min) of 1.25 mM-tetrabutyl amm. dihydrogen phosphate in acetonitrile in 1.25 mM-tetrabutyl amm. dihydrogen phosphate in aq. 5% acetonitrile.	-	230 or 260 nm	-	59
Hypersil 5 ODS (5 μm) (12.5 cm x 4 mm)	Methanol: 0.01M phosphate buffer pH 2 (11:9)	2 ml/min	305 and 320 nm	-	60
(15 cm x 4 mm) of LiChrosorb R (5 μm)	40% Methanol, pH 3	1.5 ml/min	280 nm	Plasma or urine	61
Radial-PAK C_{18} Cartridge (10 cm x 5 mm)	5 mM tetrabutyl ammonium hydrogen sulphate in 0.1M oxalic acid (pH 4) - acetonitrile (3:1)	5 ml/min	310 nm	Serum	62

Conti ..... (Table 6).

Column	Mobile phase	Flow rate	Detection	Sample	Ref.
(30 cm x 3.9 mm) of μBondapak C_{18} or one (25 cm x 4.6 mm) of LiChrosorb 60RP-18 equipped with guard column	Aq. methanol or H_2O-methanol-anhyd. acetic acid	-	UV	Creams	63
Column of μBondapak C_{18}	Buffer solution with methanol contents from 5-60% (v/v)	-	280 nm	-	64
Column of (30 cm x 3.9 mm) packed with μ Bondapak C_{18}	Buffer of pH (2.7-7.3) mixed with 50% methanol	-	-	-	65
Column (25 cm x 4.6 mm) of Spherisorb ODS (5 μm)	H_2O-0.2M Phosphate buffer pH (2.5) - acetonitrile (7:8:5)	-	235 nm	Plasma	66
μBondapak C_{18}	Methanol-0.01M KH_2PO_4 buffer pH 3.5 (2:3)	-	254 nm	-	67
Plastic C_{18} column (10 cm x 5 mm)	28% methanol in acetate buffer (pH 3.6)	1 ml/min	280 nm	Serum	68

Conti ..... (Table 6).

Column	Mobile phase	Flow rate	Detection	Sample	Ref.
	ODS-2 colum	-	-	Urine	69
(C10 cm x 4.6 mm) of LiChrosorb RP-18 (5 μm) with good column (5 cm x 3.2 mm) of LiChrosorb RP-18	Citrate buffer pH 5.4 methanol (9:1)	0.8 ml/min	295 nm	Plasma	70
μBondapak C_{18} column	6% Acetonitrile in 4 mM phosphate buffer (pH 5.7)	-	237 nm	Plasma or urine	71
μBondapak C_{18}	Methanol-5% acetic acid (3:2)	-	248 nm	Serum	72

Table (7). Summary of conditions used for the TLC of salicylic acid

Plate	Developing solvent	Detection	Ref.
Silica gel (20 cm x 20 cm)	Methanol and 10mM pot. phosphate buffer (pH 2,4,7, 9 or 11)	-	73
Glass tubes (13 cm x 2.3 mm, o.d.) coated with silica gel	-	UV	74
Silica gel G	Toluene-acetic acid-ether methanol (120:18:60:1)	$FeCl_3$ spray reagent	75
Silica gel G	Cyclohexane-acetic acid (9:1)	1st spryaed with aq. 3% H_2O_2, heated and sprayed aq. 2% $FeCl_3$.	76
MN 300 cellulose	-	-	77
Silica gel 60 F_{254} plates	Light petroleum-ethylacetate-anhydrous acetic acid (85:18:3)	440 nm	26

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SULFACETAMIDE

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1. INTRODUCTION

Sulfacetamide is the *N*′-acetyl derivative of sulfanilamide. The sodium salt of sulfacetamide, because of its effectiveness and low toxicity, continues to be the most widely prescribed sulfonamide in the form of eye-drops and ointment for ophthalmic infections. It was introduced in Europe as "Albucid" in 1938 for various eye and other topical infections. Since its use in the treatment of corneal ulcers (1), sulfacetamide is still popular in ophthalmology. The sodium salt is highly soluble at the physiologic pH of 7.4, and is especially suited, as a 10-30% solution, for repeated topical application in the local management of ophthalmic infections (2-4). It is used mainly in the treatment of acute conjunctivitis and in the prophylaxis of ocular infections after injuries or burns (5). Several reviews on various aspects of sulfacetamide have been published (6-10).

2. DESCRIPTION

2.1 Nomenclature

2.1.1 Chemical names

p-Aminobenzenesulfoacetamide (11).
N-Acetylsulfanilamide (11).
N-Sulfanilylacetamide (3,4,11,12).
N-[(4-Aminophenyl) sulfonyl] acetamide (11,12).

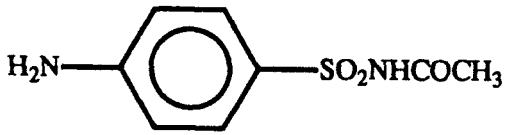
2.1.2 Generic names

Acetosulfaminum; Sulfacetamide; Sulfacetamide Sodium (3).

2.1.3 Proprietary names (Sulfacetamide Sodium)

Acetopt; Albucid; Antebor; Beocid-Isoptal; Bleph-10; Buco-Albucid; Cetamide; Cortucid (with hydrocortisone acetate); Isopto Cetamide (with methyl cellulose); Locula; Ocusol (with zinc sulfate); Op-Sulfa; Optamide; Optosulfex; Prontamed; Sebizon; Sodium Sulamyd; Sulf-10; Sulf-30; Sulfacyl; Sulphacalyre; Sulfapred (with prednisolone sodium phosphate); Supracid; Vasosulf (3-5, 11).

2.2 Formula and Molecular Weight



$C_8H_{10}N_2O_3S$ = 214.24

The CAS registry No. is 144-80-9.

Official monographs for sulfacetamide are given in Argentinian (1966), British (1988), Austrian (1981), Czechoslovak (1987), French (1982), Indian (1985), Netherlands (1983), Polish (1965) and United States (1990) Pharmacopeias (3).

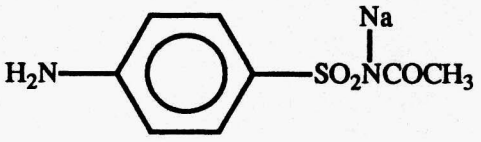
2.3 Appearance, Color, Odor and Taste

A white or yellowish white, odorless, crystalline powder with an acid, slightly saline taste (3).

2.4 Packaging and Storage

It should be preserved in well-closed, light-resistant containers (12).

2.5 Sulfacetamide Sodium



$C_8H_9N_2NaO_3S,H_2O$ = 254.24

The CAS registry No. is 6209-17-2.

Sulfacetamide sodium is the monohydrate of the sodium salt of sulfacetamide. It is highly soluble in water and is used for the preparation of eye-drops and eye ointments. 1.2 g of sulfacetamide sodium is approximately equivalent to 1 g of sulfacetamide (5).

3. SYNTHESIS

Sulfacetamide was first prepared by Dohrn and Diedrich (14) in 1938 and independently by Crossley *et al.* (15) in 1939. Sulfanilamide (I) is acetylated by acetic anhydride to give N^1,N^4- diacetyl derivative (II). The N^4-acetyl group is removed by alkaline hydrolysis to yield sulfacetamide (III) which is crystallized by acidifying with hydrochloric acid.

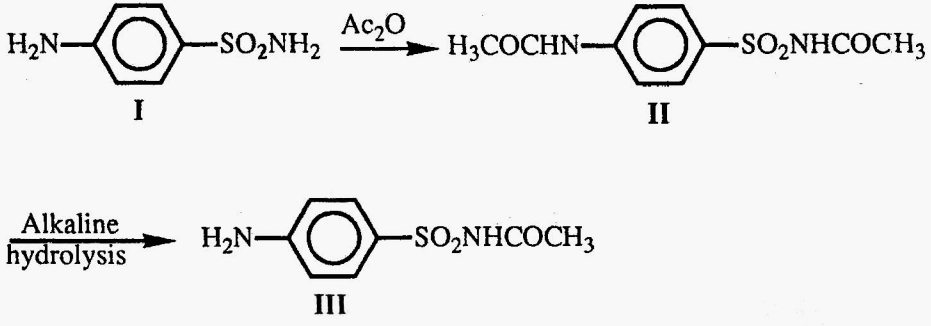


Figure 1. Synthesis of Sulfacetamide.

4. PHYSICAL PROPERTIES

4.1 Melting Point

It melts at 181° to 184°C (4).

4.2 Solubility

It is soluble in 150 parts of water, 15 parts of alcohol and 7 parts of acetone; very slightly soluble in chloroform; slightly soluble in ether; soluble in mineral acids and solutions of alkali hydroxides and carbonates. The sodium salt is soluble in 1.5 parts of water; slightly soluble in ethanol (96%); practically insoluble in chloroform and ether (3,4).

4.3 Clarity and Color of Solution

A solution of about 200 mg in 5 ml of 1 N sodium hydroxide is yellow to faintly yellow having not more than a trace of turbidity (12).

4.4 Acidity

A solution (1 in 150) is acid to litmus. The pH of a 5% w/v solution of sulfacetamide sodium is 8.0 to 9.5 (12).

4.5 Dissociation Constant

Sulfacetamide sodium pK_{a1} (amino group) 1.8; pK_{a2} (sulfonamide group) 5.4 (25°) (4,5).

4.6 Water Content

Not more than 0.5% w/w; sodium salt, not more than 8.1% w/w (12).

4.7 Residue on Ignition

Not more than 0.1% (12).

4.8 Selenium Content

Not more than 0.003%, a 200 mg test specimen being used (12).

4.9 Sulfate Content

Not more than 0.04% (12).

4.10 Heavy Metal Content

Not more than 0.002% (12).

4.11 Ultraviolet (UV) Spectrum

The ultraviolet absorption characteristics of sulfacetamide and its sodium salt are used for the identification of these compounds (4,13). The sulfonamide spectra are sensitive to pH. In alkaline solution the primary amino group is retained as auxochrome, but in acid solution quaternisation occurs to a coordinatively saturated auxochrome (16).

The ultraviolet spectra of sulfacetamide in 0.1 M hydrochloric acid and in 0.1 M sodium hydroxide solutions were recorded on a Shimadzu 240 UV-Visible spectrophotometer and are shown in Figure 1. The hypsochromic shift of the maximum at 270 nm to 256 nm in alkaline solution results from the ionisation of sulfonamide fraction (pKa 5.4) of the molecule. The UV spectral data for sulfacetamide and sulfacetamide sodium are reported in Table I.

Table I

UV Spectral Data for Sulfacetamide and Sulfacetamide Sodium

Compound	Solvent	λ_{max}, nm	A(1%, 1 cm)	Molar Absorptivity	Ref.
Sulfacetamide	Water	260			17
		258		17700	18
	0.1 M HCl	220			19
	0.1 M HCl	215			20
		269			
	0.1 M HCl	217		18900	*
		270		8900	
	0.1 M NaOH	255			21
	0.1 M NaOH	256	765		20
	0.1 M NaOH	256		17300	*
	EtOH	272	878		20
Sulfacetamide	Water	260			17
sodium	pH 7.0 (PO_4 buffer)	255	660-720		13
	0.1 M HCl	271	207		5
	Aq. acid	271	260		4
	0.1 M NaOH	257			22
	0.1 M NaOH	256	626		5
	Aq. alkali	256	750		4

*Values determined by the authors.

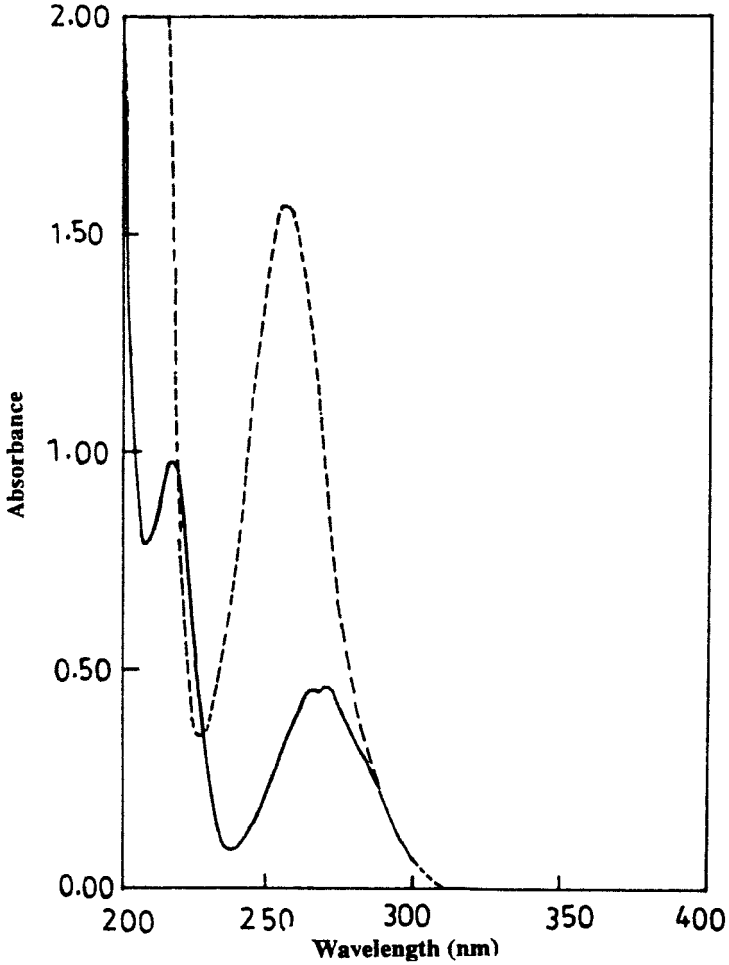


Figure 1. Ultraviolet spectra of sulfacetamide in 0.1 M HCl (——) and 0.1 M NaOH (•••) solutions.

4.12 Infrared (IR) Spectrum

The infrared absorption spectrum of sulfacetamide sodium has been determined in KBr disc (4). The principal peaks appear at 825, 1090, 1145, 1264, 1552, 1600 cm^{-1}. The infrared stretching frequencies of the amino group have been used to calculate the force constant, the band angle and the "S" character of the nitrogen orbitals of the N-H band (23,24). Infrared measurements of sulfonamides have been performed to study the imide-amide tautomerism (25) and to see if there is any change in the electronegativity of the SO_2 group (26,27). Sulfacetamide in eye-drops and ointments has been identified by attenuated total reflectance (ATR) infrared spectra (28).

The infrared spectrum of sulfacetamide as KBr disc was obtained with a Jasco IRA-1 Infrared spectrophotometer and is shown in Figure 2. The assignments for characteristic bands are given in Table II.

Table II

IR Spectral Assignments for Sulfacetamide

Frequency, (cm^{-1})	Assignment
3450	-NH asymmetric stretching
3365	-NH symmetric stretching
1680	$-COCH_3$ stretching
1630	$-NH_2$ deformation
1590	C = C stretching, skeletal vibrations of aromatic ring
1500	
1460	
1410	
1320	$-SO_2$ asymmetric stretching
1155	$-SO_2$ symmetric stretching

4.13 Nuclear Magnetic Resonance (NMR) Spectrum

The ^1H-NMR and ^{13}C-NMR spectra of sulfacetamide in D_2O were determined at 400 MHz and 75.4 MHz respectively on a Bruker AM-300 NMR spectrometer using TMS (tetramethylsilane) as reference standard and are shown in Figure 3-4. The spectral assignments are listed in Table III.

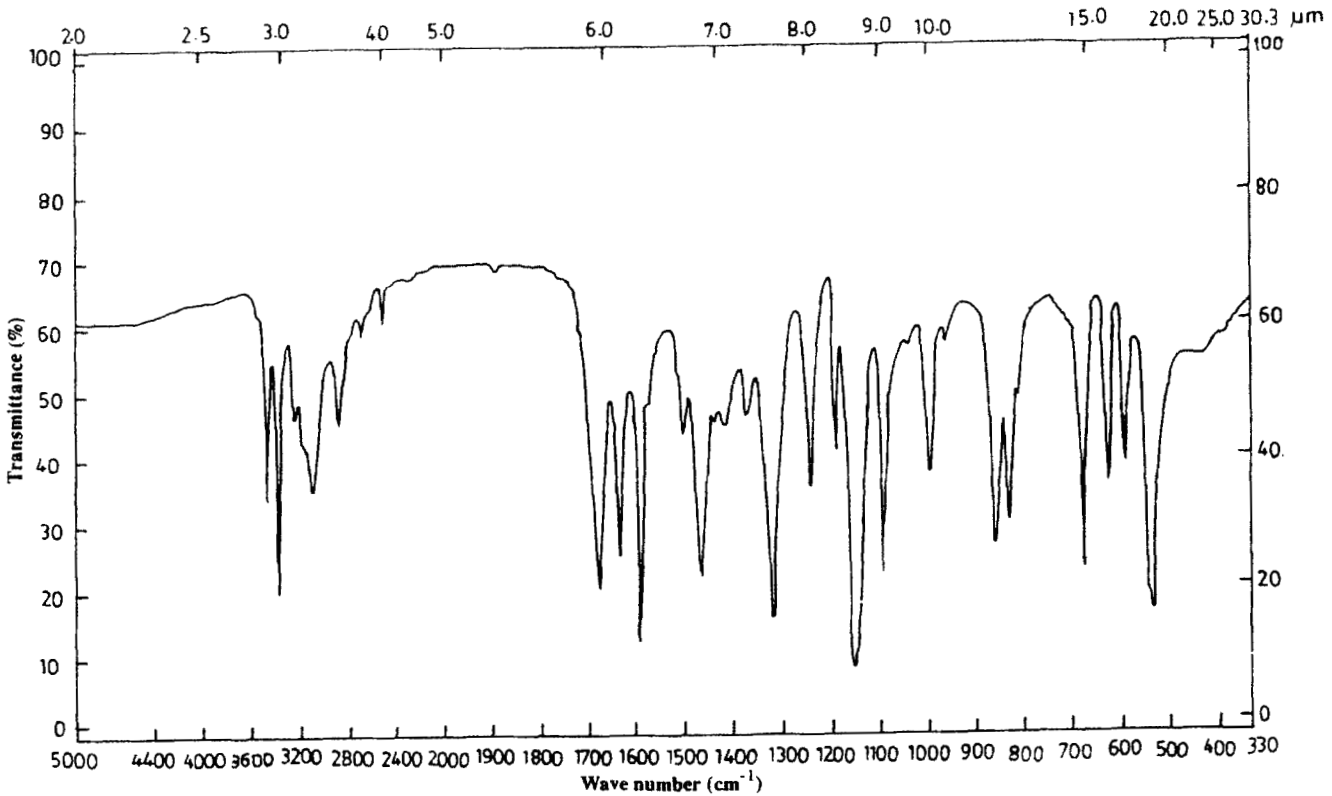


Figure 2. Infrared spectrum of sulfacetamide (KBr disc)

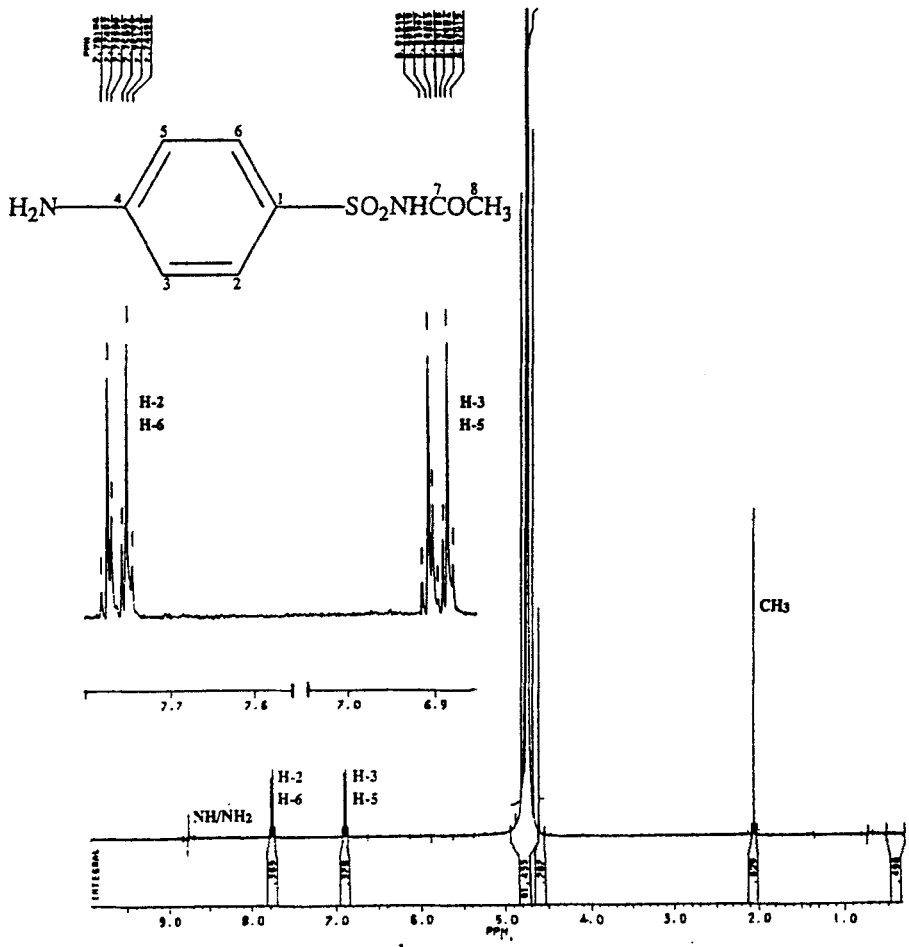


Figure 3. 400 MHz ^{1}H-NMR spectrum of sulfacetamide (D_2O).

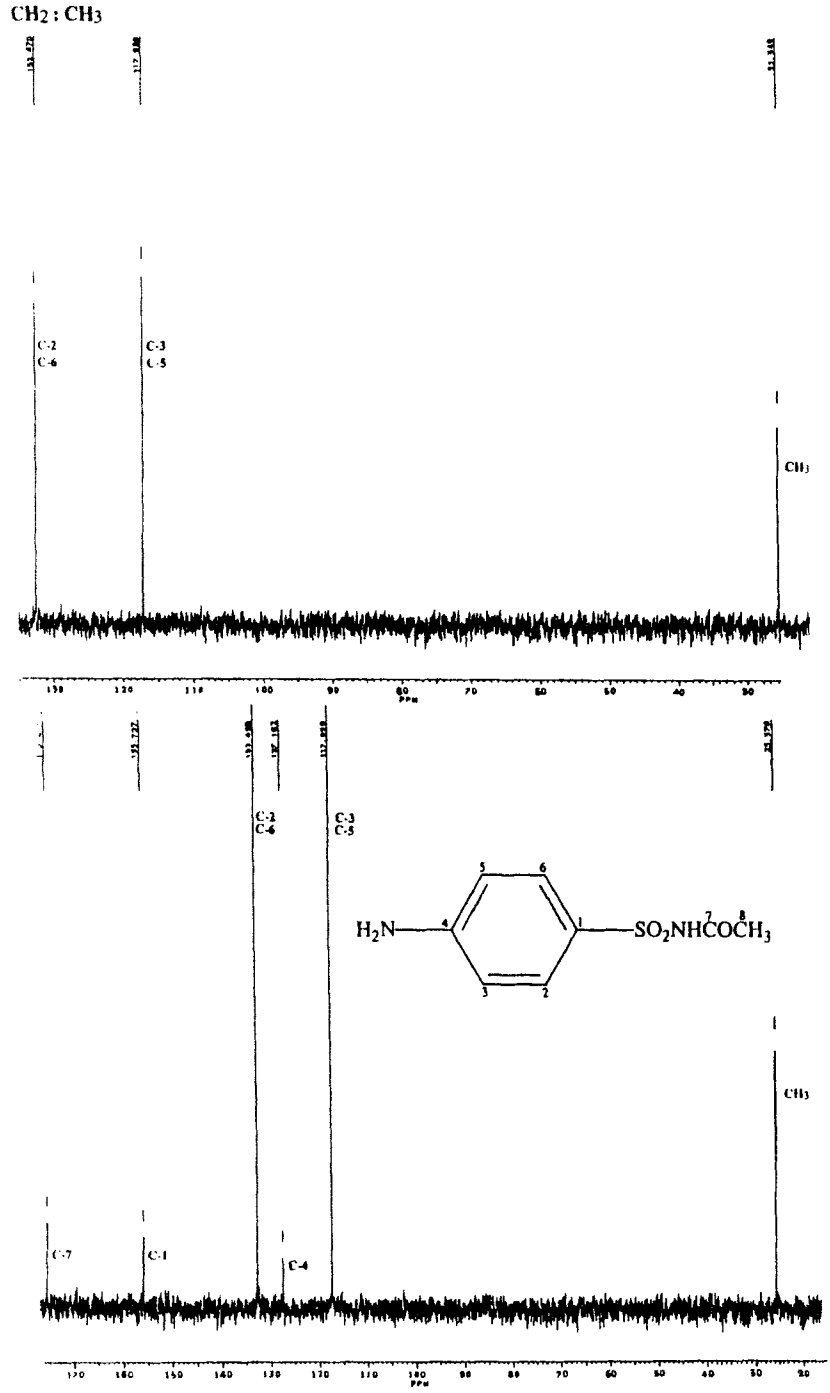


Figure 4. 75 MHz ^{13}C-NMR decoupled spectrum of sulfactamide

Table III

^{1}H-and ^{13}C-NMR Chemical Shifts and Coupling Constants for Sulfacetamide

^{1}H-NMR Chemical shift (ppm)	Proton	Coupling constant (J in Hz)	^{13}C-NMR Chemical shift (ppm)	Carbon
			155.72	1
7.76(1H,d)	2,6	8.9	132.47	2,6
6.89(1H,d)	3,5	8.9	117.08	3,5
			127.19	4
			175.47	7
2.06(3H,s)	8		25.37	8
8.76	NH/NH_2			

4.14 Mass Spectrum

The electron impact ionization spectrum of sulfacetamide was obtained at 70 eV using a solid probe insertion and is shown in Figure 5. The spectrum was run on a Finnigan Mat 112S double focusing mass spectrometer connected to a PDP 11/34 (DEC) computer system. It shows a molecular ion peak M^+ at m/z 214. Since the molecule contains one sulfur atom, M+2 peak appears at m/z 216. The proposed fragmentation pattern and prominent ions are given in Table IV.

4.15 Dissolution

A comparative *in vitro* study of the release of sulfacetamide sodium from eye ointments by dissolution and agar diffusion methods has been made and the effect of viscosity and particle size on the release examined. The aqueous based ointment gives a higher release of the drug than the greasy-based products. A linear relationship exists between agar diffusion release and the quantity of sulfacetamide released after sixty minutes as determined by the dissolution method (29).

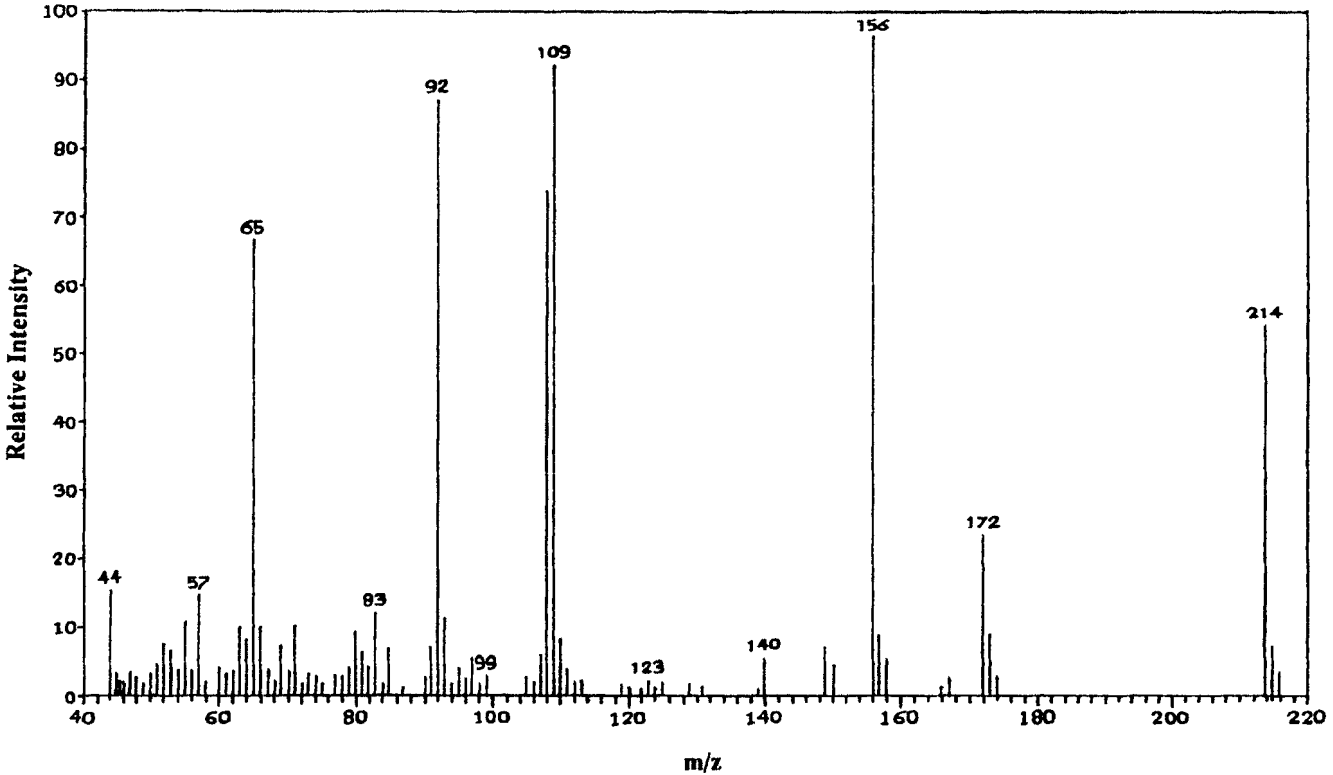
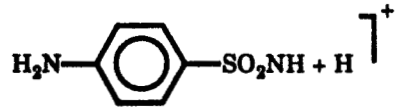
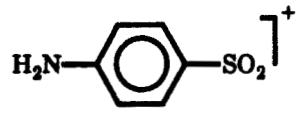
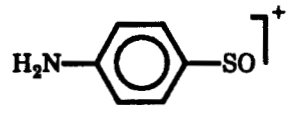
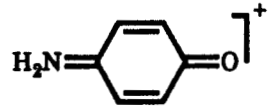
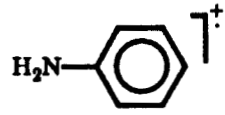
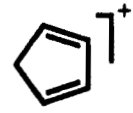


Figure 5. Electron impact mass spectrum of sulfacetamide

Table IV

Proposed Fragmentation Pattern of Sulfacetamide

m/z	Relative Intensity, %	Ion
214	54.3	M^+ ($C_8H_{10}N_2O_3S$)
172	23.7	
156	100.0	
140	5.7	
108	73.5	
92	87.0	
65	66.5	

5. QUALITATIVE TESTS

5.1 Identification (12,13)

5.1.1 The Infrared absorption spectrum of a KBr dispersion of it, previously dried, exhibits maxima only at the same wavelengths as that of a similar preparation of USP Sulfacetamide RS.

5.1.2 The light absorption of sulfacetamide sodium in the range 230 to 350 nm of a 0.001% w/v solution in citro-phosphate buffer pH 7.0 exhibits a maximum only at 255 nm. The A (1% 1cm) at 255 nm is 660 to 720.

5.1.3 Place about 500 mg of sulfacetamide in a test tube, heat gently until it boils, and cool: an oily liquid, which has the characteristic odor of acetamide, condenses on the walls of the test tube (distinction from the sublimates of sulfadiazine, sulfamerazine, sulfamethazine, and sulfapyrazine, which are solids at room temperature).

5.1.4 Dissolve 1 g of sulfacetamide sodium in 10 ml of water, add 6 ml of 2 M acetic acid and filter. Wash the precipitate with cold water and dry at 100° to 105°C for 4 hours. The melting point of the precipitate is 181° to 185°C.

5.1.5 Dissolve 0.1 g of the precipitate obtained in test 5.1.4 in 5 ml of ethanol (96%), add 0.2 ml of sulfuric acid and heat. Ethyl acetate, recognisable by its odor, is produced.

5.1.6 Dissolve 0.1 g of the precipitate obtained in test 5.1.4 in 1 ml of water with the aid of heat. The solution yields the reactions characteristic of primary amines.

5.1.7 A 5% w/v solution of sulfacetamide sodium yields the reactions characteristic of sodium salts.

5.2 Color Tests (4)

5.2.1 Place 1 drop of a solution of sulfacetamide on a filter paper, add 1 drop of coniferyl alcohol (0.1g/10 ml ethanol), and expose the paper to hydrochloric acid fumes. An orange color indicates the presence of an aromatic primary amine in which the amino group is attached directly to a bezene ring.

5.2.2 Dissolve the sample in a minimum volume of 0.1 M sodium hydroxide and add a 1% solution of copper sulfate, drop by drop, until the color change is complete. A blue color indicates the presence of sulfacetamide.

5.2.3 Dissolve the sample in 1 ml of ethanol, add 1 drop of a 1% solution of cobalt nitrate in ethanol, followed by 10 μl of pyrrolidine, and agitate the mixture. Sulfacetamide gives a blue-violet color (Koppanyi-Zwikker test).

5.3 Test for Sulphanilamide (13)

Carry out thin-layer chromatography using silica gel HF 254 as the coating substance, spread in a layer about 0.25 mm thick, and a solvent system butan-1-ol-absolute ethanol-water-13.5 M ammonia (50:25:25:10, v/v). Apply separately to the chromatoplate 5 μl of each of four solutions in water containing (1) 10.0% w/v of the substance being examined, (2) 0.05% w/v of sulfanilamide, (3) 0.025% w/v of sulfanilamide and (4) 0.050% w/v of sulfanilamide in solution (1) and develop the chromatogram. After removal of the plate, allow it to dry in air and spray with a 2% w/v solution, prepared without heating, of 4-dimethylaminobenzaldehyde in a 55% v/v solution of hydrochloric acid. Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2) and not more than one such spot is more intense than the spot in the chromatogram obtained with solution (3). The test is not valid unless the chromatogram obtained with solution (4) shows two clearly separated principal spots.

6. METHODS OF ANALYSIS

6.1 Titrimetric Methods

6.1.1 Sodium nitrite titration

The USP method (12) for the assay of sulfacetamide is based on nitrite titration followed by electrometric end-point determination using platinum electrodes. A 500 mg quantity of the drug is transferred to an open vessel. 20 ml of hydrochloric acid and 50 ml of water are added, the solution stirred until the content dissolved, cooled to about 15°, and slowly titrated with 0.1 M sodium nitrite. Each ml of 0.1 M sodium nitrite is equivalent to 21.42 mg of $C_8H_{10}N_2O_3S$. In the BP method (13), sulfacetamide sodium is assayed by amperometric titration. A 0.5 g quantity of the substance is dissolved in a mixture of 50 ml of water and 20 ml of 2 M hydrochloric acid and 3 g of potassium bromide are added. The solution is cooled in ice and sulfacetamide content determined amperometrically by titration with sodium nitrite. Each ml of 0.1 M sodium nitrite is equivalent to 0.02362 g of $C_8H_9N_2NaO_3S$.

The use of sodium-4-(benzylphino)-azobenzene-4′-sulfonate as internal indicator in the diazometric determination of sulfonamides has been investigated (30). The color changes from deep rose to colorless or full yellow. A nitrite titration method in the presence of a standard solution of 4,4′-sulfonyldianiline and diphenylamine as indicators has been developed (31). An intense red color appears during titration as a result of simultaneous diazotization and coupling process and changes to yellow with a sharp end-point. The method gives an error of 0.4%.

6.1.2 Argentometric titration

Argentometric titration method has been applied to the determination of sulfonamide mixtures. The sulfonamides are quantitatively precipitated by the addition of excess standard silver nitrate solution, the precipitated silver salts removed by filtration, and the excess silver nitrate titrated with standard ammonium thiocyanate using ferric alum as indicator (32).

6.1.3 Bromination

Sulfonamides in mixtures have been determined by titration with 0.02 M chloramine-T after bromination. The ortho dibromination is prevented by acetylation of the aromatic amino group but the reaction of side-chain substituents remains unaffected. The relative standard deviations are about 1%. Interferences even in small amounts, from barbituric acid, isoniazid, ascorbic acid, methionine, thymol and penicillins are observed (33).

Bromine water has been used as a reagent for the determination of sulfa drugs in bulk and in dosage forms by an indirect titrimetric method based on the reaction of sulfonamides with an excess of saturated bromine water to form the corresponding *N*- bromo derivatives. These derivatives on reaction with iodide release equivalent amounts of iodine which could be determined titrimetrically with thiosulfate by using starch as indicator. The recoveries range from 97.8 to 102.1% and the relative standard deviation is 0.1-2.2% (34).

6.1.4 Nonaqueous titration

Sulfonamides possess the SO_2NH-group whose acidity is considerably enhanced in basic solvents such as butylamine or ethylenediamine. Mixtures of sulfonamides can be determined by taking advantage of the differences in their acid strengths in various solvents. Sulfacetamide and other sulfonamides having an acidic hydrogen may be determined by titration with sodium or potassium methoxide in

dimethylformamide using thymol blue or azo-violet as indicator (35,36). A nonaqueous titrimetric method has been described without the exclusion of air, moisture or carbon dioxide. The dried sulfonamide is dissolved in anhydrous dimethylformamide and titrated with tetramethylammonium hydroxide in anhydrous benzene-ethanol (1:1) using thymolphthalein or *o*-cresolphthalein as indicator (37). Sulfacetamide may be determined by nonaqueous titration in dimethylsulfoxide-isopropanol (1:1) with thymol blue or other indicators (38).

Sulfacetamide has been determined in glacial acetic acid and isopropanol by titration with perchloric acid (39,40). The end-point in glacial acetic acid is improved by adding acetic anhydride to the medium and can be detected by potentiometry or with gention violet, solvent blue 19, or quinidine red indicators. Sulfacetamide can be determined in ophthalmic preparations containing phenylephrine hydrochloride or phenyltoloxamine dihydrogen citrate by this method. Recoveries range from 100.1 to 101.2% (41).

6.2 Spectroscopic Methods

6.2.1 Ultraviolet spectrophotometry

Sulfacetamide, *N*'-benzoyl sulfanilamide and sulfathiazole have been determined in pharmaceutical preparations by measuring absorbance of the mixture in 0.1 N hydrochloric acid at 220, 235 and 280 nm respectively (42). Madsen *et al.* (43,44) have performed computer analysis of the multicomponent UV spectra of sulfonamides. The errors in concentration determined from the spectra between 240 and 272 nm are lower when the spectra are analysed by a linear-squares method considering the data over the whole wavelength range compared with the determination using the data at a single wave length. The method has been applied to the assay of sulfacetamide sodium eye-drops.

A differential spectrophotometric method for the determination of sulfacetamide in the presence of sulfanilamide has been reported (45). Sulfacetamide in eye drops containing ethyl *p*-hydroxybenzoate may be determined in pH 5.8 phosphate buffer at 257 and 223 nm. The UV absorption of ethyl *p*-hydroxybenzoate does not change within pH 1-6. The method is simple and rapid, with the coefficient of variation and recovery of 0.63 and 99.58% respectively (46).

A stability-indicating spectrophotometric method for the assay of sulfacetamide in thermally degraded solutions containing sulfanilamide

has been reported (47). The method is based on the difference in absorption maxima of sulfanilamide and sulfacetamide at pH 4.0 and their simultaneous determination by a two-component assay at 258 and 268 nm. The error involved in the method is within 4%. A spectrophotometric method has been applied to the study of the thermal and photodegradation of sulfacetamide solutions. The thermal and photoproducts are determined by absorbance measurements at 370, 420 and 520 nm (48). A multicomponent spectrophotometric method has been developed for the determination of sulfacetamide and its photoproducts, sulfanilamide and azobenzene-4,4′-disulfonamide at pH 4.0. Absorbance measurements are made at 258, 268 and 320 nm and the concentrations of the compounds are determined by solving the matrix equations. The reproducibility of the method lies within ±5% (49).

6.2.2 Colorimetry

Various colorimetric methods have been employed for the determination of sulfonamides. In general, these are used for the determination of small amounts of sulfonamides in mixtures or in degraded solutions. The most common of these methods is the Bratton and Marshall method (50), which consists of diazotizing the sulfonamide with sodium nitrite in dilute acid, decomposing the excess nitrite with sulfamic acid and coupling the diazo compound with *N*-1-naphthylethylenediamine. The intensity of the pink color is measured at 545 nm. A critical study of the Bratton and Marshall method shows a relative standard deviation of 2% (51). The method has been applied to the determination of sulfacetamide after chromatographic separation on thin-layer plates (52). Kuhnan (53) and Werner (54) have proposed a method for determining sulfonamides by the color produced with dimethylaminobenzaldehyde in acid solution. The color intensity is somewhat affected by the pH of the solution (55). Furthermore the absorptivity is relatively low, making the method more subject to interference from colored impurities. The method has been used to study the rate of hydrolysis of sulfacetamide in ophthalmic solutions, after separation of sulfanilamide by TLC. The precision of the method is low and the coefficient of variation for a single determination is about 7% (56).

Sulfonamides in pharmaceutical formulations have been determined by diazotization and coupling with *m*-aminophenol, α-naphthylamine and *N*-1-naphthylethylenediamine dihydrochloride in sulfuric acid-trichloroacetic acid, and measuring the absorbance at 435, 520 and 545 nm, respectively. Absorbance is linear in the concentration

range 1-11 μg/ml for *m*-aminophenol, 1-8 μg/ml for α-naphthylamine, and 1-7 μg/ml for the naphthylethylenediamine. Recoveries are 98-101% for all coupling agents (57). Sulfonamides on hydrolysis yield aromatic primary amines which quantitatively react with *N*-substituted *p*-aminophenols at pH 3. The absorbance with the various reagents is : metol 520-530 nm, *p*-(*N*-benzylamino) phenol 530-540 nm, *p*-(*N*-salicylamino)phenol 530-550 nm, and *p*-(*N*-dimethylamino) phenol 540-570 nm. Recoveries from pharmaceutical formulations are 98.6-100.2% and the sensitivity is 2-8 μg/ml (58).

A colorimetric method for microdetermination of sulfonamides based on diazotization of the drug with sodium nitrite and hydrochloric acid has been reported (59). The diazonium salt is then coupled with 8-hydroxyquinoline in alkaline medium and the absorbance of the developed color measured at its maximum wavelength. A similar method involves diazotization and coupling of the sulfonamide with indole in alkaline solution to form an intense yellow azo dye which exhibits maximum absorption at 449 nm. Beer's law is obeyed over the concentration range 1-32 μg/ml with a relative standard deviation of less than 2% (60). The reaction of sulfonamides with chloramine-T in sulfuric acid gives a yellow product which is suitable for the determination of sulfonamides in different formulations. It has an accuracy similar to that of the Bratton and Marshall method (61).

Sulfacetamide in ophthalmic solutions can be determined by its reaction with phenothiazine and *N*-bromosuccinimide at pH 6 to yield a blue product which absorbs at 605 nm. The recoveries are 96.7-100.7% (62). Sulfacetamide has been determined by its reaction with *o*-chloranil at pH 9 to yield a purple product with absorption maximum at 525 nm. Beer's law is obeyed over the concentration range 10 to 70 μg/ml of sulfacetamide sodium (63).

A modified method for the determination of sulfonamides based on the Bratton-Marshall reaction with derivative spectrophotometry has been proposed (64). Various sulfonamides give excellent precision by using first- to fourth-order derivative spectra. The method has been applied to the determination of sulfonamides in pharmaceutical formulations and urine without pretreatment of the sample.

6.2.3 Nuclear magnetic resonance spectrometry

Nuclear magnetic resonance spectrometry has been employed for the determination of various sulfonamides using a 5% NaOD solution in

D_2O. Spectral characteristics of sulfacetamide and sulfonamides with heterocyclic side chains are reported (65).

6.3 Electrochemical Methods

6.3.1 Potentiometry

Sulfacetamide sodium and other weak bases which do not show a distinct end point on titration in aqueous solution can be determined by use of the half-neutralization point. Three methods are employed for locating the midpoint from the potential (mV) versus volume added curve of a pH titration. The measured error is about 1% (66).

Sulfacetamide and other sulfonamides in pharmaceutical dosage forms have been determined with a silver sulfide electrode over the concentration range 10^{-4}-10^{-1} M. The sulfonamide is dissolved in dimethylformamide, 0.01 N silver nitrate is added, the solution filtered, and the resulting precipitate washed and the excess silver nitrate determined by potentiometric titration with 0.01 N sodium chloride (67,68).

Sulfa drug sensitive electrodes have been used for the potentiometric titration of sulfonamides using standard sulfuric acid solution as titrant with distinct potential jump at the equivalence point and a maximum error of 0.7%. No significant interference is caused by the common inorganic anions (69). Ion-pair complexes of sulfa drugs and quaternary ammonium agents such as cetyltrioctylammonium have been employed as membrane electrodes for the potentiometric determination of sulfonamides (70).

6.3.2 Polarography

Sulfacetamide as technical grade material or in tablets can be determined by a.c. oscillopolarographic titration. The drug is dissolved in 6 M hydrochloric acid containing KBr, titrated with standard $KBrO_3$ solution and determined by bromimetry a.c. oscillopolarography. The detection limit is more than 10^{-4} M or 2.5 mg. (71).

A highly sensitive differential pulse polarographic method has been developed for the determination of trace levels of sulfa drugs after diazotization and coupling with *N*-1-naphthylethylenediamine dihydrochloride. For the coupled diazo product of sulfacetamide, at pH 1.6 and with 0.01 M KH_2PO_4 as base electrolyte, the peak current at -0.070V versus SCE is increased linearly in the concentration range

$4.4x10^{-8}$ to $2.0x10^{-6}$ M. The coefficient of variation is 4.4% (72).

6.3.3 Voltammetry

Sulfonamides have been studied for cathodic and anodic activity at rotating disk electrodes of gold and platinum in 0.1 M H_2SO_4 and 0.1 M Na_2CO_3 solutions. All the sulfonamides display anodic activity. The free (protonated) amino group of the sulfonamides is oxidized by a one-electron reaction to the protonated radical cation imine (73).

6.4 Chromatographic Methods

6.4.1 Thin-layer chromatography (TLC)

Sulfacetamide and related sulfa drugs can be extracted from pharmaceutical formulations and separated by TLC using silica gel or alumina as adsorbent. Since many sulfonamides including sulfacetamide are amphoteric, both acidic and basic solvents have found considerable use in their TLC. Detection of sulfacetamide can be performed non-specifically on fluorescent layers in UV light. More sensitive detection of the free *p*-amino group is, however, carried out with Ehrlich's reagent or with Bratton-Marshall reagent. A 2% solution of vanillin in acetic acid has also been employed (74) . Another spray reagent is a mixture of equal volumes of a 0.2 M solution of 1-phenyl-3-methyl-2-pyrozoline-5-one in pyridine and of aqueous potassium cyanide, applied after exposing the plate to chlorine (75). The use of mercuric chloride-diphenylcarbazone reagent, Van Urk reagent and acidified potassium permanganate solution has been reported (76). The details of TLC systems and R_f values of sulfacetamide are given in Table V.

The characterization of sulfonamide mixtures by TLC method on silica gel plates using metal ions as reagents has been attempted by Walash and Agarwal (87, 88). Copper acetate has been found to produce spots of varying colors, whereas ceric sulfate gives yellow or purple spots with all sulfonamides. A TLC method using Silufol UV 254 plates and a solvent system 1,2-dichloroethane-isopropanol-25%NH_4OH-methenol (130:65:10:65) has been used for the separation of hydrolysis products of sulfacetamide. The degradation products are identified by UV light of 254 nm combined with a Bratton-Marshall reaction. The method can be used for the stability study of sulfacetamide in dosage forms (89).

Quantitative TLC has been applied to the determination of sulfonamides in mixtures and in degraded solutions. Bican-Fister and

Table V

Thin-Layer Chromatography of Sulfacetamide

Adsorbent	Solvent System	Spraying Agent	Color	R_f	Ref.
Silica gel G	Butanol:methanol: acetone:diethylamine (90:10:10:10)	*p*-dimethylamino-benzaldehyde	yellow	0.38	77
Silica gel G	Chloroform:ethanol: heptane:water (30:30:30:1.5%)	*N*-1-naphthyl-ethylenediamine	reddish purple	0.42	78
Silica gel G	Chloroform:butanol: pet.ether (60^{o}-80^{o}) (1:1:1)	*p*-dimethylamino-benzaldehyde	yellow	0.31	79
Silica gel G	Strong ammonia solution:methanol (1.5:100)	alkaline β-naphthol	yellow	0.70	80
Silica gel G	Chloroform:methanol: water (80:20:1.25)	*N*-1-aphthyl-ethylenediamine	reddish purple	0.47	81
Silica gel G	Chloroform:methanol (80:55)	*p*-dimethylamino-benzaldehyde	yellow	0.17	82
Silica gel G	Chloroform:heptane: ethanol (1:1:1)	*p*-dimethylamino-benzaldehyde	yellow	0.21	82
Silica gel G	Cyclohexane:acetone: acetic acid (40:50:10)	*p*-dimethylamino-benzaldehyde	yellow	0.49	83
Silica gel G	Hexanol	acidified potassium permanganate solution	yellow brown	0.53	84
Silica gel G dipped in 0.1 M KOH in methanol	Methanol:strong ammonia solution (100:1.5)	ninhydrin	pink	0.70	75
Silica gel G	Chloroform:acetone (4:1)	Van Urk reagent	yellow	0.17	75

(Table continues)

Table V, *continued*

Adsorbent	Solvent System	Spraying Agent	Color	R_f	Ref.
Silica gel G	Acetone:methanol: strong ammonia solution (85:10:5)	Van Urk reagent	yellow	0.05	75
Silica gel G	Ethyl acetate	Van Urk reagent	yellow	0.42	75
Alumina	Methanol:water (96:8)	*p*-dimethylamino-benzaldehyde	yellow	0.48	85
Alumina	Butanol:water (90:9)	*p*-dimethylamino-benzaldehyde	yellow	0.28	85
Alumina	Benzene:ethanol (9:1)	iodine vapours	brown	0.05	86
Alumina	Ether:methanol (9:1)	iodine vapours	brown	0.26	86
Alumina	Ether:ethanol (9:1)	iodine vapours	brown	0.20	86
Alumina	Chloroform:methanol (80:15)	iodine vapours	brown	0.24	86
Alumina	Chloroform:amyl-alcohol (80:15)	iodine vapours	brown	0.17	86
Alumina	Chloroform:acetone (1:1)	iodine vapours	brown	0.17	86
Alumina	Chloroform:acetic acid (95:5)	iodine vapours	brown	0.40	86
Alumina	Chloroform:acetic acid (90:10)	iodine vapours	brown	0.56	86
Alumina	Acetone: ammonia solution, 25% (80:15)	mercuric chloride-diphenylcarbazone	blue	0.37	86
Alumina	Chloroform:methanol (70:30)	marcuric chloride-diphenylcarbazone	blue	0.04	86

Kajganovic (90) have used Bratton-Marshall reagent for quantitation after separation and elution of sulfonamide mixtures containing sulfacetamide. The solvent system chloroform-methanol-25% ammonia solution (90:15:2.4) gives good separation. The results show a relative standard deviation of about 4%. Sarsunova *et al.* (86) have used chloroform-ethanol (100:8) as solvent system for the separation of sulfacetamide, sulfathiazole and sulfadimidine in tablets. The sulfonamides are assayed by extraction with acetone-ethanol, separation on alumina, and determination at 270 and 290 nm. The relative standard deviations of 2.5%, 4.1% and 3.2% have been obtained for the three components with six replicates.

The hydrolysis of sulfacetamide solution has been studied colorimetrically after separation of the degradation products on silica gel with acetone-methanol-diethylamine (90:10:10) as solvent system (56). The method has a low precision with a coefficient of variation of about 7% . Sulfanilamide, in degraded sulfacetamide formulations, has been separated by TLC followed by spectrophotometric determination of the eluted component (91).

Recovery patterns for individual sulfonamides in mixtures have been studied by TLC. The sulfonamides are separated on silica gel H layers impregnated with sodium hydroxide using chloroform-methanol (88:12) as solvent system. The spots are eluted with acidic alcohol or 0.1 N NaOH and determined by UV spectrophotometry at 270 or 255 nm. The average recoveries of sulfonamides including sulfacetamide range from 87.9 to 95.7% and are about the same with both extracting solvents (21).

Automated densitometry after TLC separation has been used for the determination of sulfacetamide, sulfamethazine and sulfathiazole in a compound preparation. The sulfonamide mixture is chromatographed on polyamide plates with dioxane-propanol-ethyl acetate (4:3:2), chloroform-methanol (16:3), chloroform-ethyl acetate-ethanol (2:2:1), and benzene- isobutanol-pyridine (4:3:0.5). Combinations of the first and third and second and third solvents are used for two-dimensional TLC of the compounds followed by determination by densitometry (92).

6.4.2 Gas liquid chromatography (GLC)

A GLC method for the separation and identification of sulfonamides has been developed by Roeder and Stuthe (93). It is based on the conversion of sulfonamides into the *N'*-methyl derivatives and

analysis by gas chromatography. The method has been applied to the determination of sulfonamides and their N^4-acetyl metabolites in blood and urine. The sulfonamides are extracted from the blood and urine and then methylated with diazomethane. The methyl derivatives are determined using a column of 3% OV-101 on Gas-Chrom Q with a relative standard deviation of 5% for the free sulfonamide and 7% for the acetyl conjugates (94).

6.4.3 High pressure liquid chromatography (HPLC)

Sulfacetamide in biological fluids has been determined using silica column (Spherisorb, 5 μm, 25 cm x 4 mm internal diameter) and cyclohexane-ethanol-acetic acid (85.7:11.4:2.9) as eluent. The k′ value (column capacity ratio) of sulfacetamide for this system is 7.7 (95).

Sulfacetamide and prednisolone acetate in a compound sulfacetamide ophthalmic ointment have been determined by HPLC. The peak of sulfacetamide sodium is well separated from those of the other constituents when using methyl cyanide-1% acetic acid solution (15:85) as the mobile phase on μ-Corasil C_{18} column. The two compounds are determined by calculation of the peak area ratios using thiamine hydrochloride and methyl paraben as the internal standards. The maximum errors are 0.39% and 1.2% respectively (96).

HPLC on μBondapak Ph column with UV detection at 280 nm has been used for the determination of sulfacetamide, sulfabenzamide and sulfathiazole in a triple sulfa cream. The mobile phase consists of a 7:3 mixture of 0.01 M ammonium hydrogen phosphate and methanol (pH 7.2). The proposed method has been shown to give better results for sulfacetamide assay compared with those of the USP method (97). Sulfacetamide, sulfadiazine, sulfamerazine and sulfamethazine in mixtures and in pharmaceuticals have been determined by HPLC using a nonpolar column with methanol-0.02 M potassium dihydrogen phosphate in water as the mobile phase and a variable wavelength UV detector. The method is precise with relative standard deviations of 2.1, 0.6, 1.9 and 1.6% respectively for the four compounds. The preservatives do not interfere with the method (98).

The usefulness of amperometric detection in the analysis of sulfonamides by HPLC has been investigated (99). The UV response of sulfacetamide at 254 nm and that at its absorption maximum are compared with the electrochemical signal obtained. The median limit of detection of the amperometric detector is 1.0 *n*g on-column and the

median gain in sensitivity, compared with UV detection, is 22.5.

The stability of sulfacetamide in 1 M HCl at 40-70°C has been studied by HPLC methods. Three different stationary phases have been employed for the determination of sulfacetamide and its hydrolysis product, sulfanilamide, in degraded solutions (100). HPLC has been used to study the acid hydrolysis of sulfacetamide at elevated temperatures and for the identification of its degradation products (101).

7. STABILITY AND DEGRADATION

7.1 Oxidation

The oxidation of sulfacetamide sodium by peroxydisulfate ion to azobenzene derivatives follows second-order kinetics. The reaction is very sensitive to pH and the rate increases with increase in pH in the region 1.2-7.76. The rate decreases with decrease in dielectric constant of the medium, and a plot between log k_2 and D^{-1} is linear. The reacion is inhibited by the presence of diphenylpicryl hydrazyl (102,103).

7.2 Thermolysis

Aqueous solutions of sulfacetamide sodium are affected by temperature. A freshly prepared solution in nitrogen filled ampoules, when sterilized by autoclaving at 115°C, produces no color whereas the air filled ampoules become appreciably darker (104). Sulfacetamide solutions autoclaved at 115°C have been shown to deposit the crystals of sulfanilamide (105).

The hydrolysis of sulfanilamide solutions at sterilization temperatures has been studied. The rate of hydrolysis appears to be independent of pH in the range 7-9. About 1% loss results from heating at 120°C for 20 minutes or 115°C for 30 minutes, while steaming at 100°C for 30 minutes gives less than 0.5% degradation. The reaction is first-order with respect to sulfanilamide (56). Heating of sulfacetamide solutions at 100°C increases the rate of hydrolysis and the likelihood of sulfanilamide crystallization. The crystallization is minimised by buffering the solutions at pH 9.0 to 9.5. The concentration of sodium metabisulfite does not appear to have any effect on the extent of crystallization (106).

The stability of sulfacetamide solutions at different pH and in the presence of antioxidants has been investigated (107). A 30% solution

with 0.5% sodium thiosulfate, on sterilization at 100°C, remains stable for one year. It has been reported that heat sterilization of sulfacetamide solutions produces immediate degradation (108).

The kinetics of stability of sulfacetamide solutions at pH 7.5 to 9.3 by heating at 90° to 120°C has been studied and the activation energy of the reaction has been determined (109). The optimum pH for the stability of sulfacetamide solutions at 90° to 120°C is 8-8.5 (110). The first-order rate constants for the degradation of sulfacetamide solutions at 120°C in the presence or absence of sodium metabisulfite have been determined. Sulfanilamide, in heat degraded solutions, has been shown to be insignificant in the development of color due to heat stress (48).

The effect of heat, pH and buffers on the hydrolysis of aqueous sulfacetamide solutions has been investigated. The solutions are satisfactorily autoclaved provided there is no subsequent refrigeration. The hydrolytic degradation of sulfacetamide is essentially pH-independent between pH 5 and 11 but is subject to catalysis by buffer constituents. Below pH 4 specific hydronium ion catalysis occurs. The value of Arrhenius activation energy for the hydrolysis of sulfacetamide at pH 7.4 is 22.9 ± 1.1 kcal /mol. Extrapolation of the Arrhenius plot at 25°C yields a shelf life (t_{90}) of 4.2 years (111).The degradation of sulfacetamide solutions in the pH range 1-13 at 70,80 and 90°C has been studied using a stability-indicating method and the thermodynamic parameters have been determined (47). The kinetic treatment of the degradation reactions of sulfacetamide has been presented by Connors *et al.* (10).

The pH is the most important factor in the stability of sulfacetamide sodium solutions. Among the 20% ophthalmic solutions of the drug, the greatest stability (to two years) is shown by the solutions with a pH of 8-8.5 (112). The addition of 200mg/100 ml of sulfacetamide to 30% ophthalmic solutions of sulfacetamide sodium decreases the pH from 9.3-9.6 to 7.3-8.4 and increases the shelf life of the solution after sterilization at 100°C for 30 minutes to more than one year (113).

The stability of sulfacetamide in 1 M hydrochloric acid at 40-70°C has been studied. Sulfacetamide and its hydrolysis product sulfanilamide have been determined by HPLC methods and the kinetics of hydrolysis has been evaluated (100).

7.3 Photolysis

It is well known that the solutions of sulfacetamide sodium darken on standing particularly when exposed to light. Sulfacetamide sodium eye-drops turn brown on storage in colorless glass containers (106). The color develops in few hours on bubbling oxygen through the solution, while the addition of hydrogen peroxide causes the formation of brown color within one hour followed by the deposition of brown crystals (114). The aerobic discoloration of sulfacetamide sodium at pH 7-9 has been studied using a fluorescent tube. The rate of discoloration is affected by the pH, the unionised molecule being more susceptible to oxidation than the ionised form (105, 115). The azo and azoxy derivatives of sulfanilamide have been detected in the solutions of sulfacetamide sodium stored for one year. The solutions become deep red-brown indicating the formation of colored oxidation products (116). An increase in discoloration of sulfacetamide solutions on the addition of sulfanilamide has been observed (106). The color development in sulfacetamide solutions is due to the formation of oxidation products absorbing at 450 nm (48). The photolysis of sulfacetamide sodium follows zero-order kinetics. The degradation proceeds through sulfanilamide to give azobenzene-4,4′-disulfonamide and azoxybenzene-4,4′-disulfonamide. A reduction in pH lowers the rate of degradation. Decreasing the dielectric constant of the medium by the addition of propylene glycol up to 80% favours degradation of sulfacetamide sodium (22, 117).

Aqueous solutions of sulfacetamide and its sodium salt have been subjected to photolysis with 254 nm radiation. There have been found 18 degradation products in the sulfacetamide solution, and 16 products in the solution of its sodium salt by paper chromatography (118). Sulfacetamide solutions on UV irradiation undergo hydrolysis and oxidation to form a number of products identified as sulfanilamide, azobenzene-4,4′-disulfonamide,axoxybenzene-4,4′-disulfonamide, sulfanilic acid, azobenzene,4,4′-disulfonic acid and a blue product. Several unknown products have also been detected by thin-layer chromatography and a scheme for the degradation pathways has been proposed (119). The spectral changes in the UV irradiated solutions of sulfacetamide exhibit a gradual decrease in absorption at 268 nm and a simultaneous increase in absorption in the 280-380 nm region and are in accordance with the formation of the colored azo and azoxy derivatives (49).

The kinetics of photolysis of sulfacetamide solutions at pH 1-13 and in the presence of antioxidants at pH 7 has been studied. The apparent first-order rate constants for the photolysis of sulfacetamide in buffered and unbuffered solutions show relatively higher values in buffered solutions probably due to the catalytic effect of buffer ions (120). The pH-rate profile indicates that around pH 5, the rate of photolysis is at the maximum. At pH values below 5, $\dot{N}H_3$ group is considerably less efficient as an auxochrome and therefore less susceptible to photolysis. In the alkaline region, the photolysis of the anion appears to be almost independent of pH over the range 7-11 (49). The quantum yields of the photolysis of sulfacetamide at pH 1-13 have been determined . The values range from 0.012-0.210 and 0.039-0.120 in buffered and unbuffered solutions respectively (121).

The flash photolysis of sulfacetamide leads to the formation of the excited triplet state, the cation radical and the solvated electron. The ionization of sulfacetamide occurs by a biphotonic process (122). The photolysis of sulfanilamide and sulfacetamide has been studied using the spin traps 2-methyl-2-nitrosopropane and 5,5-dimethyl-1- pyrroline-1-oxide. The radicals trapped during the photolysis of sulfanilamide in aqueous solution are H. and $H_2NSO_2C_6H_4NH.$ (α-fission), $H_2NSO_2.$ and $H_2NC_6H_4.$ (γ-fission), $H_2NC_6H_4SO_2.$ and $H_2N.$ (δ-fission). The $H_2NSO_2C_6H_4.$ and $SO_3^{-}.$ radicals detected in the reaction are not formed directly by homolytic bond fission. Homolytic bond fission is also observed during the irradiation of sulfacetamide (α,δ), which generates $RHNSO2C_6H_4.$ and $SO_3^{-}.$ radicals by a different process. The free radicals generated by these agents may play an important role in their phototoxic and photoallergic effects (123).

7.4 Radiolysis

The effect of γ–irradiation on sulfacetamide sodium in aqueous solutions and in the solid state has been studied. Pulse radiolysis and steady state experiments demonstrate that hydrated electrons ($\bar{e}_{aq}$) and hydroxyl radicals (OH.) are mainly responsible for the degradation between 10^{-4} and 1.2 M solute concentrations. The reaction with $\bar{e}_{aq}$ yields sulfanilic acid and an unidentified product (124).

8. STABILIZATION

Discoloration of sulfacetamide by oxidation can be prevented by removal of oxygen from aqueous solutions and by sealing in colored ampoules. The 20-30% ophthalmic solutions of sulfacetamide sodium

show maximum stability at pH 7.3-8.5 (112,113). Several antioxidants have been used to stabilize sulfacetamide solutions. Sodium thiosulfate is a good stabilizer for photodegradation (22). Sodium metabisulfite has some stabilizing effect in preventing color formation, but it acts as a catalyst for the hydrolytic reaction. In the absence of oxygen, 1% metabisulfite causes a 3.6 fold increase in the rate of hydrolysis. In the presence of oxygen, no rate increase is observed since the metabisulfite is itself oxidized by the oxygen (48, 105). Sodium editate is a very effective inhibitor of photo-oxidation and probably complexes with trace metal ions in the solution which catalyse the oxidation of sulfacetamide.

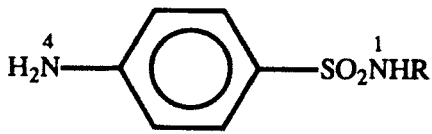
Sodium metabisulfite and thiourea have been shown to be the best antioxidants for photostabilization of sulfacetamide solutions. Sodium editate has also a stabilizing effect on sulfacetamide solutions. The activity of antioxidants in preventing the photodegradation of sulfacetamide solutions appears to depend on their redox potentials. The lower is the redox potential of antioxidant the higher is the stabilization effect (125).

9. METABOLISM AND PHARMACOKINETICS

9.1 Metabolism

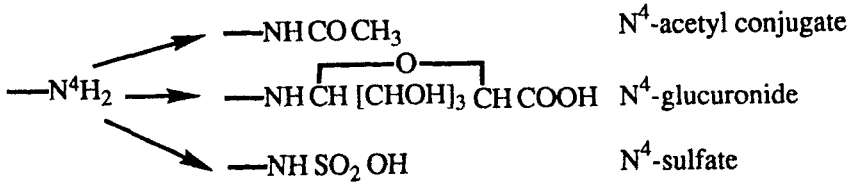
After oral administration sulfacetamide is readily absorbed and rapidly excreted in the urine, mainly as unchanged drug. However, it is usually only used topically in the treatment of eye infections; absorption into the blood may occur after application to the eye if the conjunctiva is inflamed (4).

The most important metabolic reactions of the sulfonamides involve the N^1 and N^4-amino groups shown in the following general structure.



R = H (sulfanilamide)
- $COCH_3$ (sulfacetamide)
or a heterocyclic group

Metabolism of the N^4-amino group is usually the more important and this group can undergo three reactions, namely N^4-acetylation, N^4-glucuronide formation, and N^4-sulfate synthesis (126).



Of these reactions acetylation is the most extensive but the extent to which it occurs varies with the sulfonamide, species and individual. The acetylation involves the enzymatic transfer of the acetyl group from acetyl coenzyme A by acetyl-transferase, and although, usually considered to occur in hepatic tissue, has been shown to take place in the reticulo-endothelial cells (127).

9.2 Pharmacokinetics

Very high aqueous concentrations of sulfacetamide sodium (30%, pH 7.4) are nonirritating to the eye and are effective against susceptible microorganisms. The drug penetrates into ocular fluids and tissues in high concentrations (128). It is bound to plasma protein to the extent of 15 to 18% (4). The plasma half life is reported as 7 to 12.8 hours (129).

Exposure of rats to a low frequency magnetic field alters the pharmacokinetics of sulfacetamide. The drug levels in plasma are lowered and its concentrations in renal and splenic tissues are increased (130).

The concentrations of N^4-acetylsulfanilamide in plasma exceed that of sulfanilamide 4 hours after i.v. administration of 14 mg sulfanilamide/kg to calves. Urinary excretion of sulfanilamide is 10-16% of the dose and that of N^4-acetylsulfanilamide more than 69%. Traces of N^1-acetyl and the N^3,N^4-diacetyl metabolites are present in urine. The binding of N^1-acetyl metabolite to milk components is 88% (131).

Pharmacokinetic studies of sulfacetamide, sulfadimidine and sulfanilamide have been conducted in West African Dwarf goats injected

i.v. at 50 mg/kg. The semilogarithmic graph of the plasma concentration-time profile of these sulfonamides exhibits a biphasic decline, suggestive of 2-compartment open model kinetics. The mean elimination half-life of sulfacetamide and sulfanilamide is 1.88 and 4.39 hour, respectively. The approximate degree of *in vitro* and *in vivo* binding of sulfacetamide (12-16%) and sulfanilamide (22-24%) to plasma protein is independent of drug concentration (132).

10. TOXICITY AND SIDE EFFECTS

Sulfacetamide is a sulfonamide of low toxicity. When given orally to dogs the LD_{50} is 8g /kg (133). Sensitivity reactions to sulfacetamide are rare, but the drug should not be used in patients with known hypersensitivity to sulfonamides (128). Skin sensitivity arising after the use of sulfacetamide preparations has been reported (134). A case of fatal response lupus erythematosis associated with the use of an ophthalmic preparation containing sulfacetamide sodium has been recorded (135). A patient using sulphamethoxazole developed the Stevens-Johnson syndrome shortly after starting treatment with sulfacetamide sodium 30% eye-drops (136).

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TALC

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1. Description

1.1 Name, Formula, and Molecular Weight

Talc is a magnesium silicate commonly referred to as "soapstone". It is obtained from natural sources and may contain small amounts of aluminum silicate.

The Chemical Abstracts identification number is CAS-14807-96-6. In the United States, it is identified in the Color Index as 77019, and is denoted as C.I. Pigment White 26 [1].

The chemical formula is $Mg_3Si_4O_{10}(OH)_2$ [2], which corresponds to a formula weight of 379.3 daltons. The elemental composition is MgO 31.7%, SiO_2 63.5%, and H_2O 4.8% [3].

1.2 Appearance

Crude naturally occurring talc can range in color from white to green and brown [3]. After being ground to powder form, pure talcs range in color from white to grayish white. Morphology of the powder is reported as foliated (plates), fibrous, and steatite [4].

1.3 General Chemical Properties

Talc is a soft, impalpable, crystalline powder which is odorless and tasteless. Its specific gravity is reported as 2.6-2.8 [4]. It is rated as one of the softest materials available, with a hardness of 1-1.5 on the Mohs Mineralogical Scale [5]. It is characterized by hydrophobic-organophilic surface properties and a slippery feel. The amount of softness and slip depend on the source of the talc material.

The material is thermally stable up to 900°C and inert in most chemical reagents. Talc is practically insoluble in water, dilute mineral acids, and dilute solutions of alkali halides [6] and alkaline hydroxides [4]. It is soluble in hot concentrated phosphoric acid [3].

1.4 Uses and Applications

In pharmaceutics, talc is used as a glidant for improved powder flow [7-9] and as a lubricant in tablet formulations [10-13]. Because of its insolubility, it is employed as a filling and dispensing medium [4]. Talc is also used extensively in the cosmetic industry in face, body, and foot powders, as well as in aerosol formulations [14]. Cosmetic and pharmaceutical talcs differ only in particle size [4].

2. Method of Preparation

Talc occurs naturally and is mined, therefore the mineral composition may vary depending on the source [15]. The most frequent impurities are alumina, calcium oxide, and iron oxide [4]. The mined material is pulverized, and the talc is separated from impurities using a floatation process [16]. The fine talc is boiled in dilute hydrochloric acid to remove iron and other soluble impurities. It is then washed with water and dried.

The formation of talc from dolomite and quartz has also been reported [17].

3. Physical Properties

A summary of the physical properties of talc is available in the Handbook of Excipients [5]. This reference also summarizes commercial availability and pharmacopeial specifications.

Three representative samples of USP grade talc were characterized in the present work. Samples were obtained from Charles B. Chrystal Co., Penta Manufacturing, and Whittaker, Clark and Daniels, Inc.

3.1 Crystallographic Properties

Although the unit cell of talc was initially reported to be monoclinic [18], further studies have concluded that the unit cell is triclinic [19-21]. The material is composed of a brucite (MgO) sheet between silicon-oxygen layers, as shown in Figure 1. Each layer is electrically neutral and adjacent layers are held together by weak van der Waals forces. The slippery property of talc is the result of these layers sliding over one another.

The x-ray powder pattern of talc has been reported [22] and peak positions and intensities for a standard are given in Table I. The powder patterns for the three

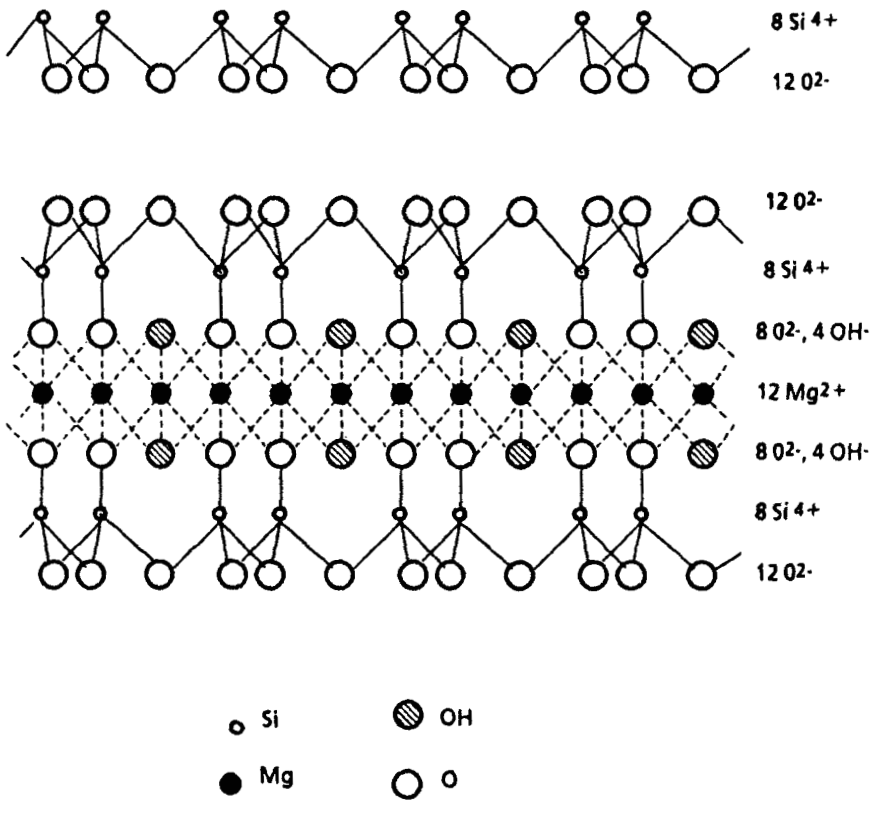

Figure 1. Structure of Talc.

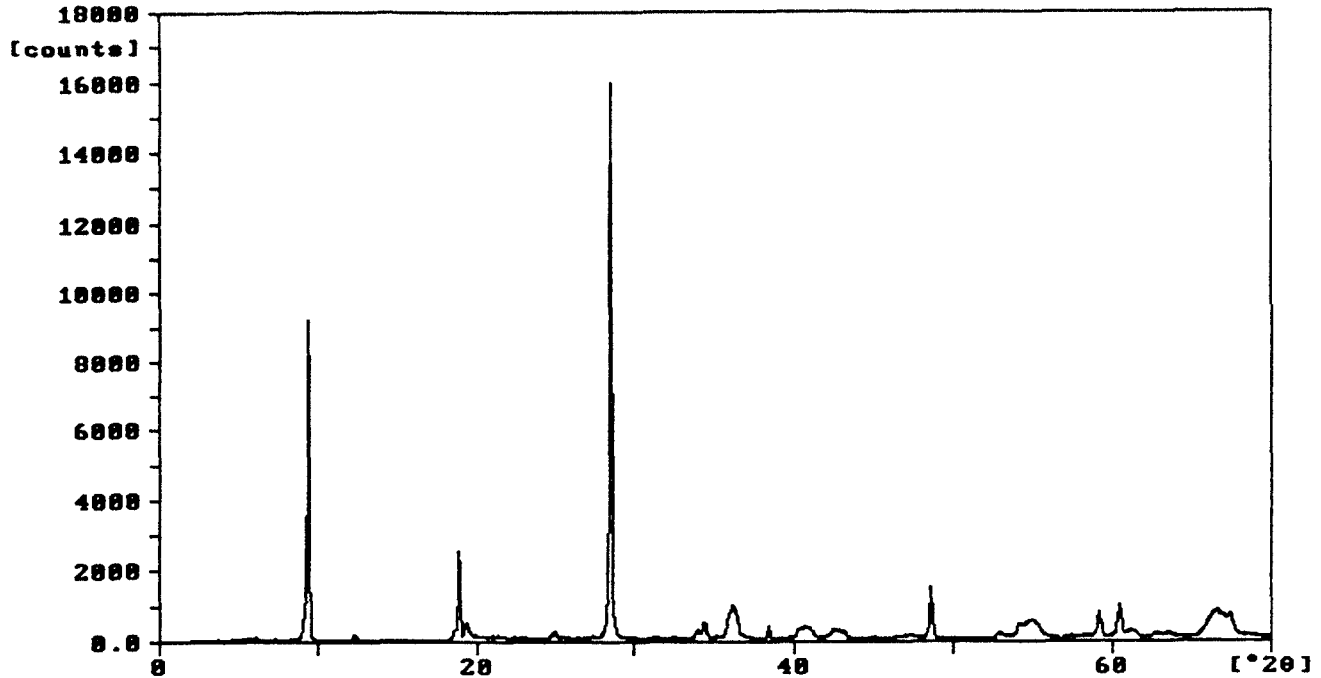

Figure 2. X-ray powder diffraction pattern of talc, Chrystal lot 4087.

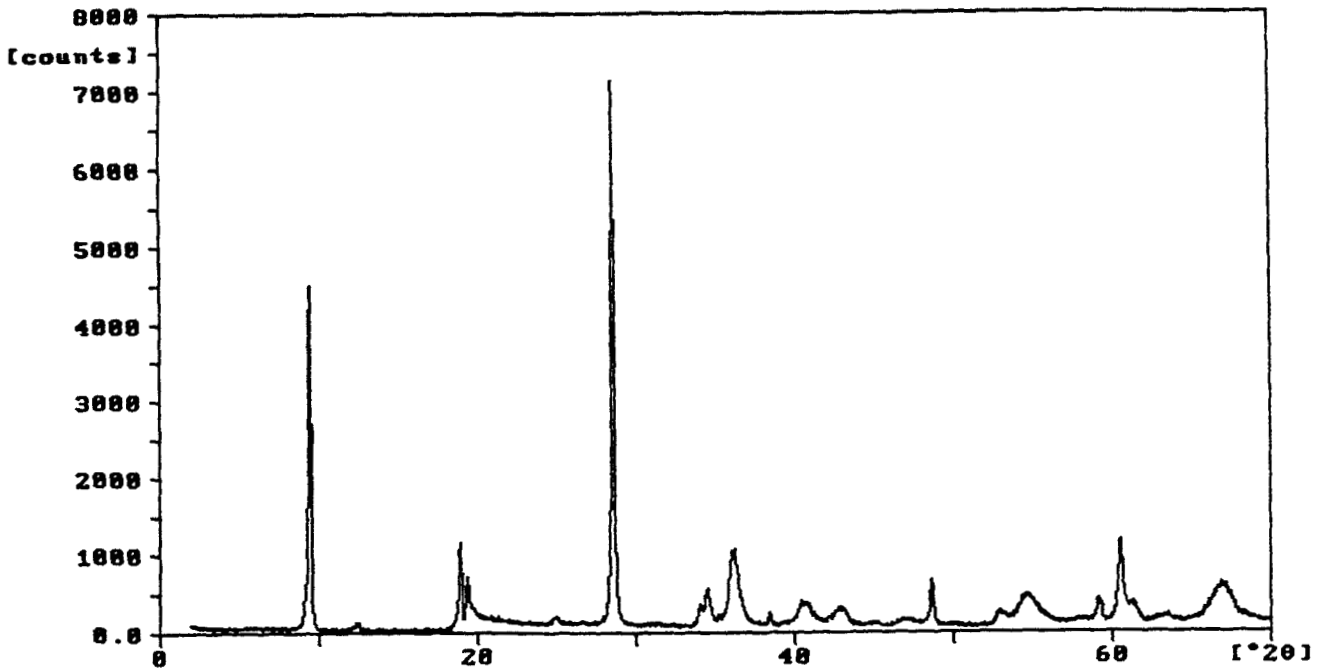

Figure 3. X-ray powder diffraction pattern of talc, Penta lot 23252.

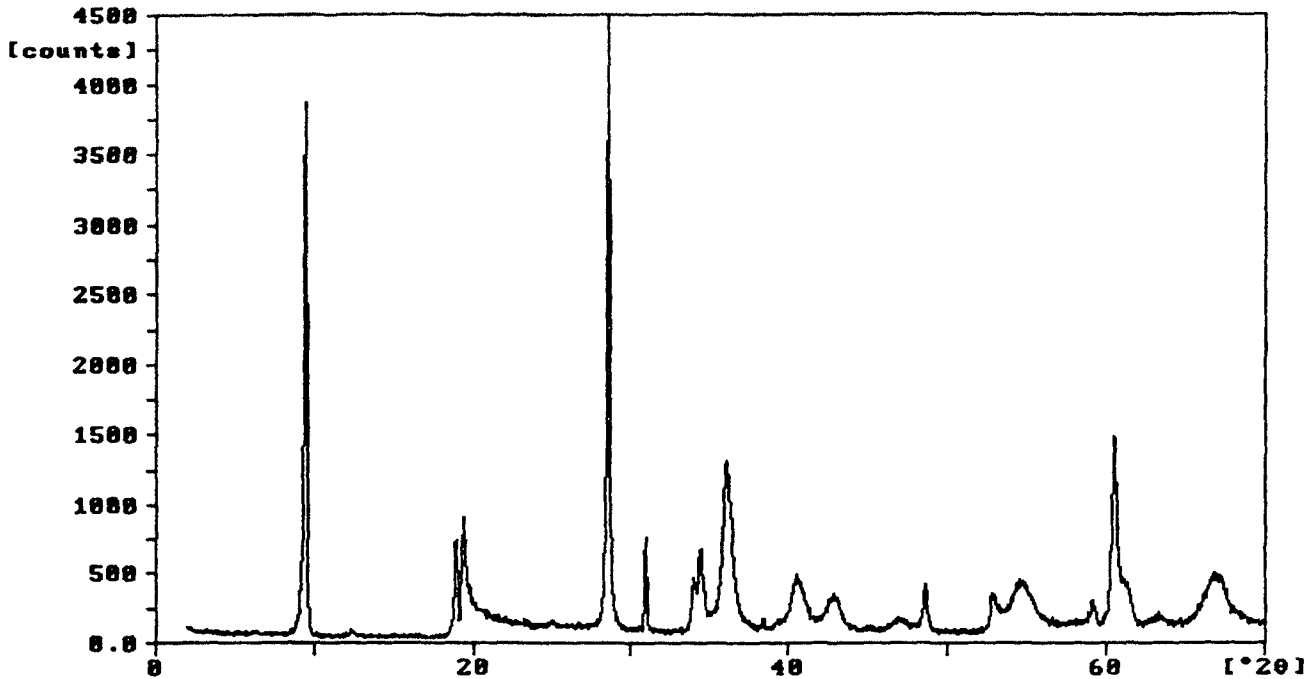

Figure 4. X-ray powder diffraction pattern of talc, Whittaker lot 2576.

Table I.

Peak Positions and Relative Intensities for Talc Samples

Peak	ICDD Standard[a]		Chrystal		Penta		Whittaker	
	Angle	I/I_o	Angle	I/I_o	Angle	I/I_o	Angle	I/I_o
1	9.49	48	9.4	58	9.4	63	9.4	87
2	18.99	20	18.9	16	18.9	15	18.9	14
3	19.50	60	19.3	3	19.4	9	19.4	19
4	28.59	90	28.6	100	28.6	100	28.6	100
5	-	-	-	-	-	-	31.0	15
6	-	-	34.0	2	34.0	4	34.0	8
7	34.61	20	34.4	3	34.4	7	34.5	14
8	36.19	30	36.2	6	36.2	14	36.1	28
9	-	-	38.4	2	38.4	2	38.5	2
10	-	-	-	-	-	-	39.4	2
11	40.42	8	40.7	2	40.4	4	40.7	10
12	42.57	6	42.5	2	-	-	-	-
13	-	-	-	-	42.9	4	43.0	5
14	43.67	3	-	-	-	-	-	-
15	-	-	-	-	-	-	46.9	2
16	48.57	8	48.6	9	48.6	8	48.6	8
17	53.14	6	-	-	52.8	2	52.9	5
18	-	-	54.3	2	54.2	3	54.8	6
19	-	-	55.0	3	-	-	-	-
20	59.05	3	59.2	5	59.2	4	59.2	4
21	60.72	30	60.5	6	60.5	14	60.5	30
22	-	-	-	-	61.3	3	61.4	5
23	-	-	66.5	5	-	-	66.9	8
24	67.31	3	67.4	4	67.0	6	-	-

[a] ICDD - International Centre for Diffraction Data [22]

lots of talc are presented in Figures 2-4, and the peaks are summarized in Table I. It is evident that extra peaks are observed for the talc lots when compared to the accepted powder pattern (only impurity peaks greater than 1% in intensity were included). These extra peaks are attributed to the various impurities in the sample from the region in which it was mined.

3.2 Infrared Spectroscopy

The Fourier transform mid-infrared (FTIR) spectra of the talc materials from the various vendors were measured by the neat, diffuse reflectance technique. Spectra of the three materials, acquired at a spectral resolution of 1 cm^{-1}, are presented in Figure 5. Identical spectra, including peak frequencies, peak width at half height, and peak shape, were measured for the three sources of talc. The major absorption bands and assignments are detailed in Table II [23].

3.3 Nuclear Magnetic Resonance Spectroscopy

The structural distortions of phyllosilicate minerals have been investigated by solid-state ^{29}Si nuclear magnetic resonance spectroscopy (NMR) [24]. In trioctahedral layer silicates such as talc, the ^{29}Si chemical-shift value is related to the amount of ^{IV}Al substitution and/or distortion of the tetrahedral sheet structure.

The solid-state ^{29}Si NMR spectrum of talc displays a single resonance at -98.5 ppm [24]. Upon ^{IV}Al substitution, the ^{29}Si chemical shift value is shielded (more positive ppm value), whereas further distortion of the tetrahedral sheet is reflected in a decreased chemical shift value (deshielded, increased negative ppm value). The authors of this report suggest that solid-state ^{29}Si NMR may be used to determine the compositional nature of phyllosilicate minerals. In each of these studies, the spectra were obtained at a frequency of 71.5 MHz on a Fourier transform spectrometer interfaced to a 8.45-T magnet. Tetramethylsilane (TMS) was used as an external chemical-shift standard.

3.4 Thermal Properties

Differential thermal analysis (DTA) data for talc exhibit an endotherm at approximately 950-975°C. This thermal event has been attributed to decomposition of the talc to $MgSiO_3$, SiO_2, and water [25, 26]. DTA has also been used to determine mineral impurities in talc batches [27].

It has also been reported that endothermal transitions in DTA curves and weight

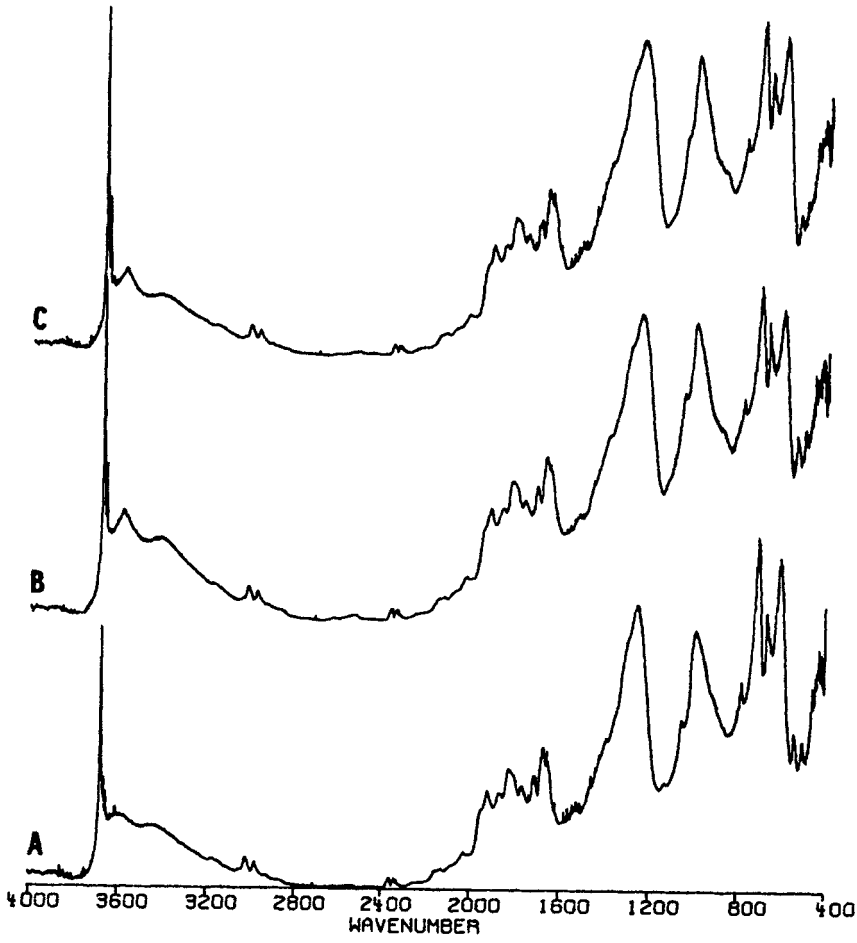

Figure 5. Diffuse reflectance mid-IR spectra for three sources of talc: (A) Chrystal, lot 4087, (B) Penta, lot 23252, and (C) Whittaker, lot 2576.

Table II

IR Peak Table and Assignments

IR Frequency (cm^{-1})	Assignment[a]
3675.7	-OH stretch
3659.7	-OH stretch
1248.4	Si-O stretch
1001.0	Si-O stretch
703.7	O-Si-O bend
666.1	O-Si-O bend
603.5	O-Si-O bend

[a] Assignments based upon pubiished correlation tables and data presented in reference 23 and references contained within.

loss in thermogravimetric (TG curves) above 300°C represent the decomposition of mixed carbonate impurities [27-29].

The thermogravimetric analysis of the three vendor lots of talc resulted in a total volatile content less than 1% for all samples at 300°C.

3.5 Particle Morphology

The particle morphology of the talc materials from the various vendors was investigated with scanning electron microscopy. All the talc materials were found to be flaky and plate-like in appearance as shown in Figures 6-8. When viewed at 3000x, the lamellar orientation of the plates is evident, as well as the presence of smaller crystals growing on the surface.

3.6 Particle Size

The particle size distributions of the three talc lots were obtained using optical microscopy and image analysis. The particle size distributions are summarized in Table III and presented graphically in Figure 9. The majority of particles for all three lots were found to be less than 15 μm in length. The average particle sizes (shown in Table IV) were also similar for the samples.

3.7 Surface Area

The surface area of the talc lots was obtained using a five-point BET analysis after outgassing the samples at room temperature using a vacuum. The results are summarized in Table IV. The surface area of the materials ranged from approximately 5 m^2/g to 9 m^2/g.

Talc samples which were deaerated at 100°C and 10^{-4} mm of Hg for 1 hr reported surface areas in the range of 4-8 m^2/g [30]. However, talc samples outgassed at 200°C for 5 hours resulted in a surface area of 20 m^2/g [31]. Outgassing conditions should be carefully chosen when collecting surface area data for these materials.

3.8 Density

The true density of the talc samples was measured using helium pycnometry, and the data is presented in Table IV. The true densities were similar for the three lots and the minor differences can be attributed to the impurities in the lots.

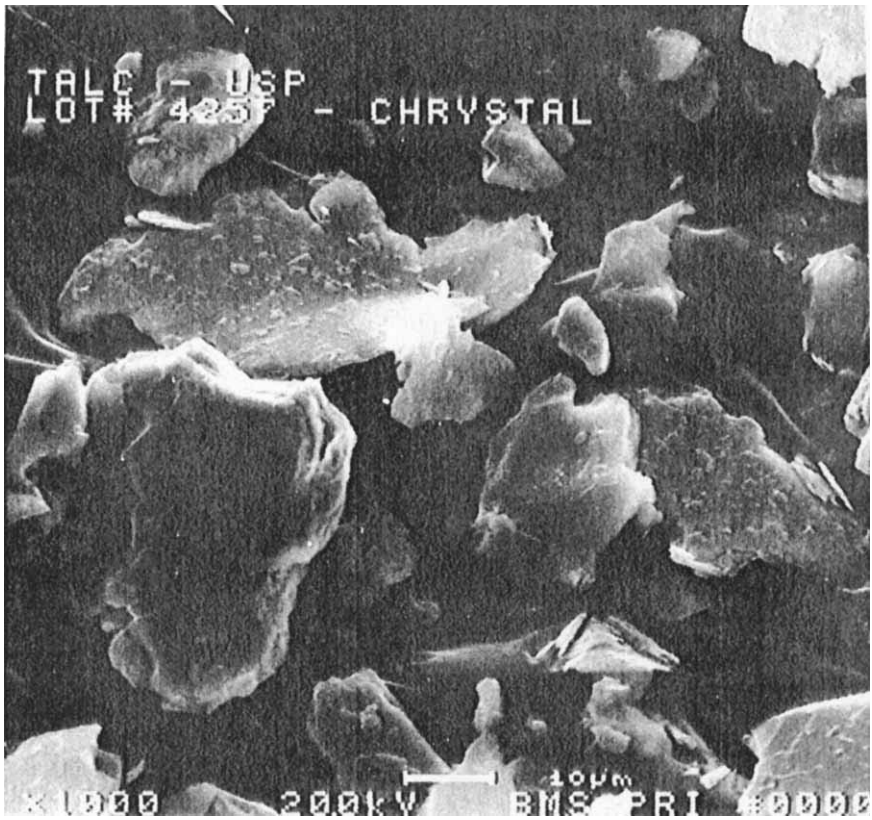

Figure 6a. Scanning electron micrograph of talc, Chrystal lot 4257, at a magnification of 1000x

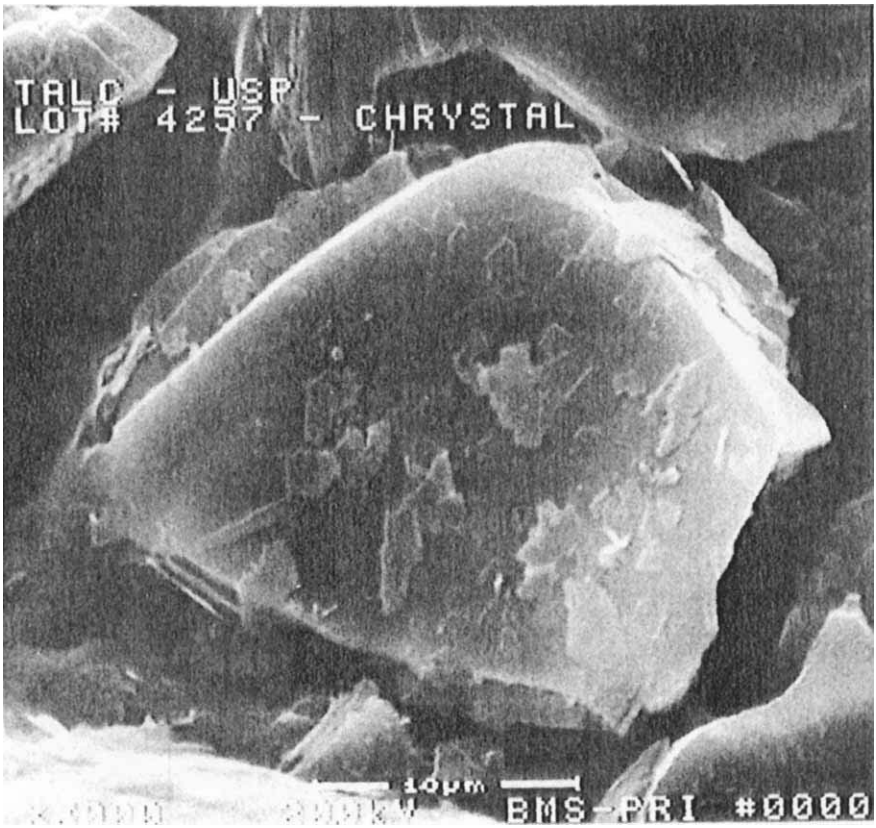

Figure 6b. Scanning electron micrograph of talc, Chrystal lot 4257, at a magnification of 3000x

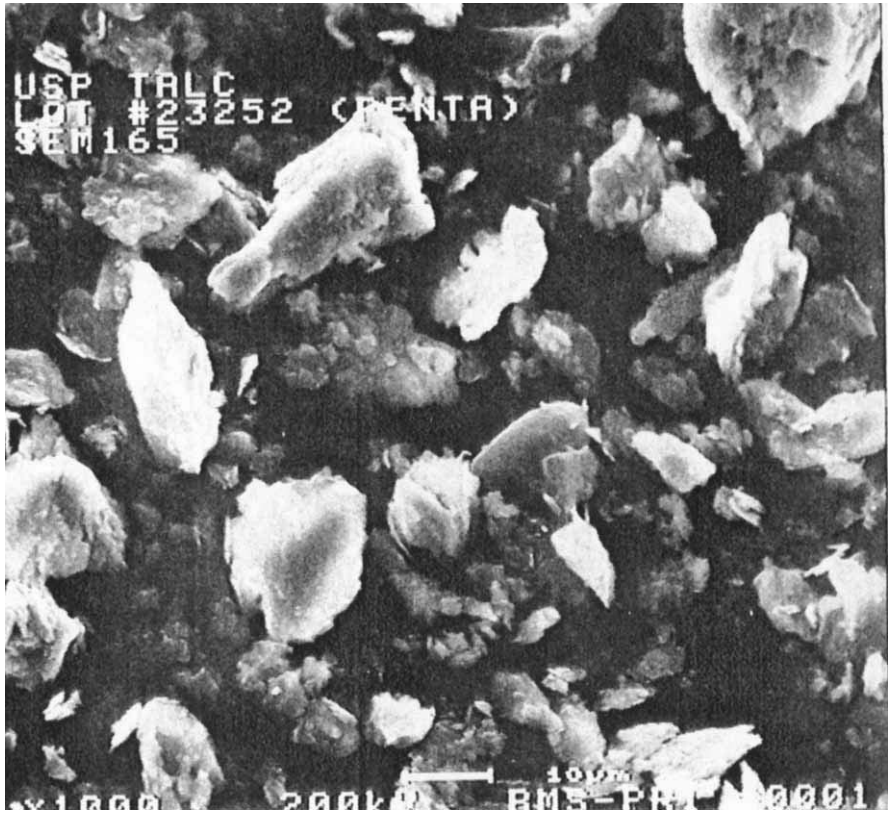

Figure 7a. Scanning electron micrograph of talc, Penta lot 23252, at a magnification of 1000x

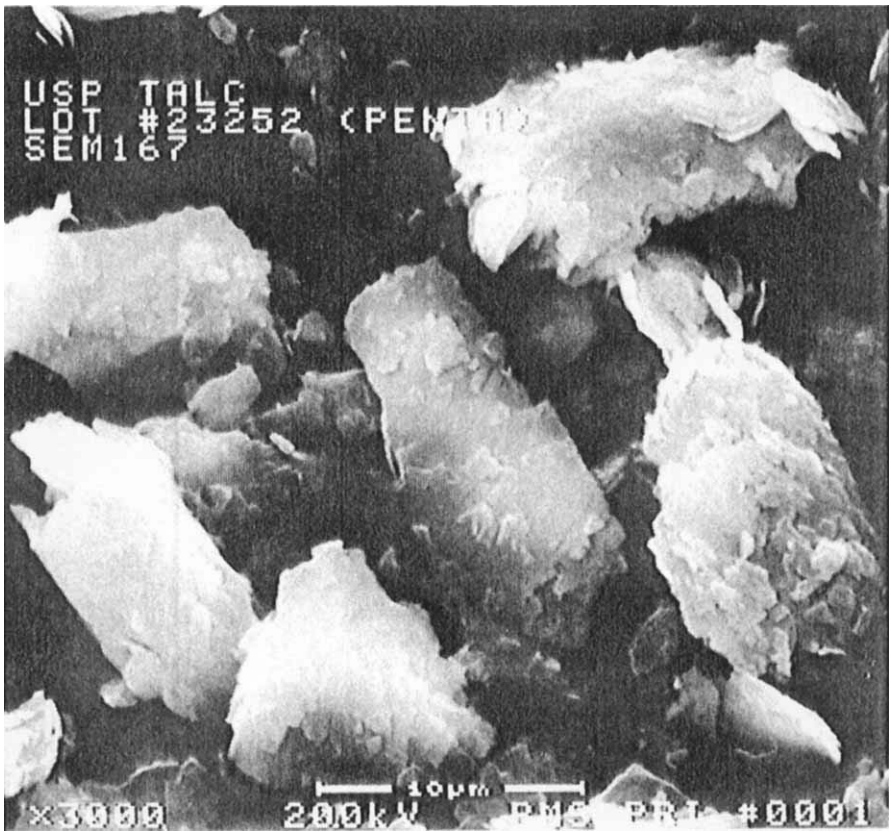

Figure 7b. Scanning electron micrograph of talc, Penta lot 23252, at a magnification of 3000x.

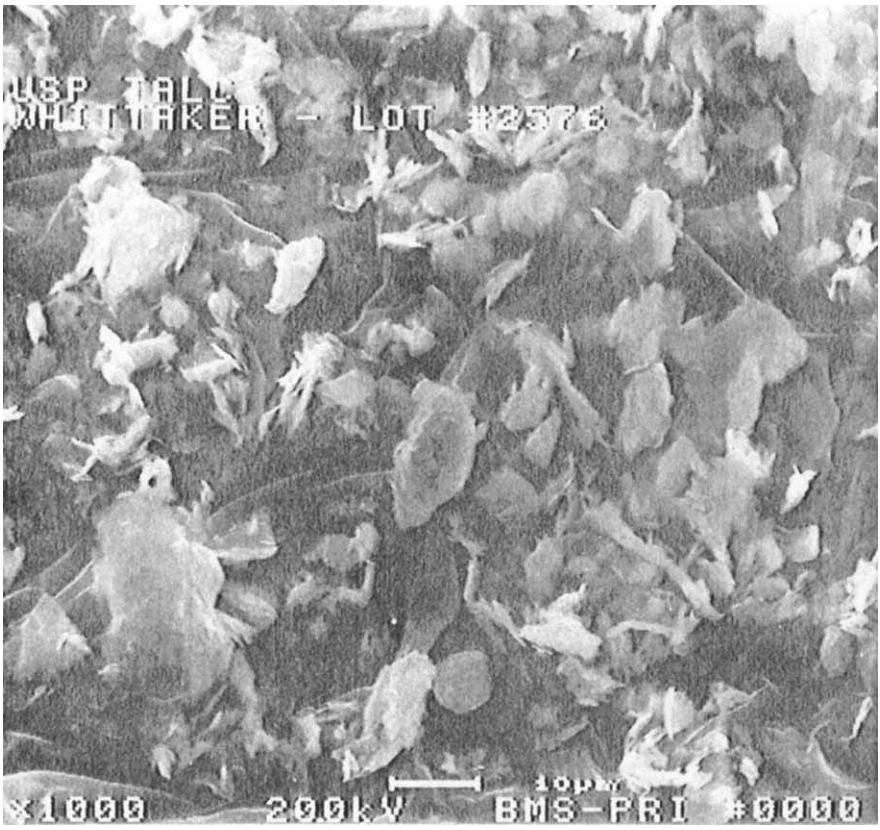

Figure 8a. Scanning electron micrograph of talc, Whittaker lot 2576, at a magnification of 1000x.

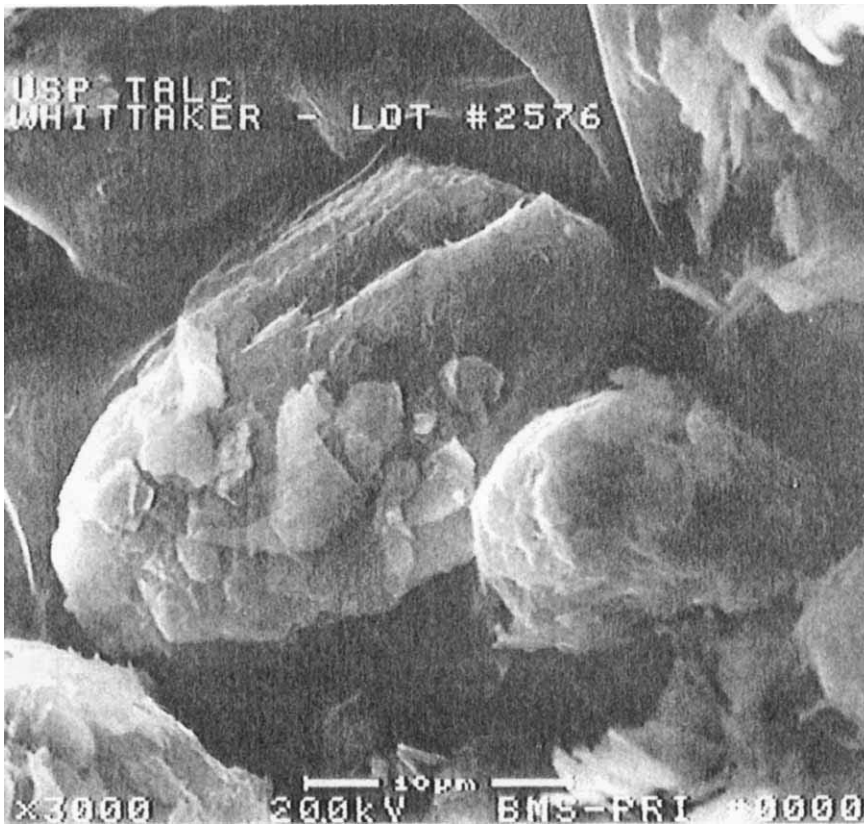

Figure 8b. Scanning electron micrograph of talc, Whittaker lot 2576, at a magnification of 3000x.

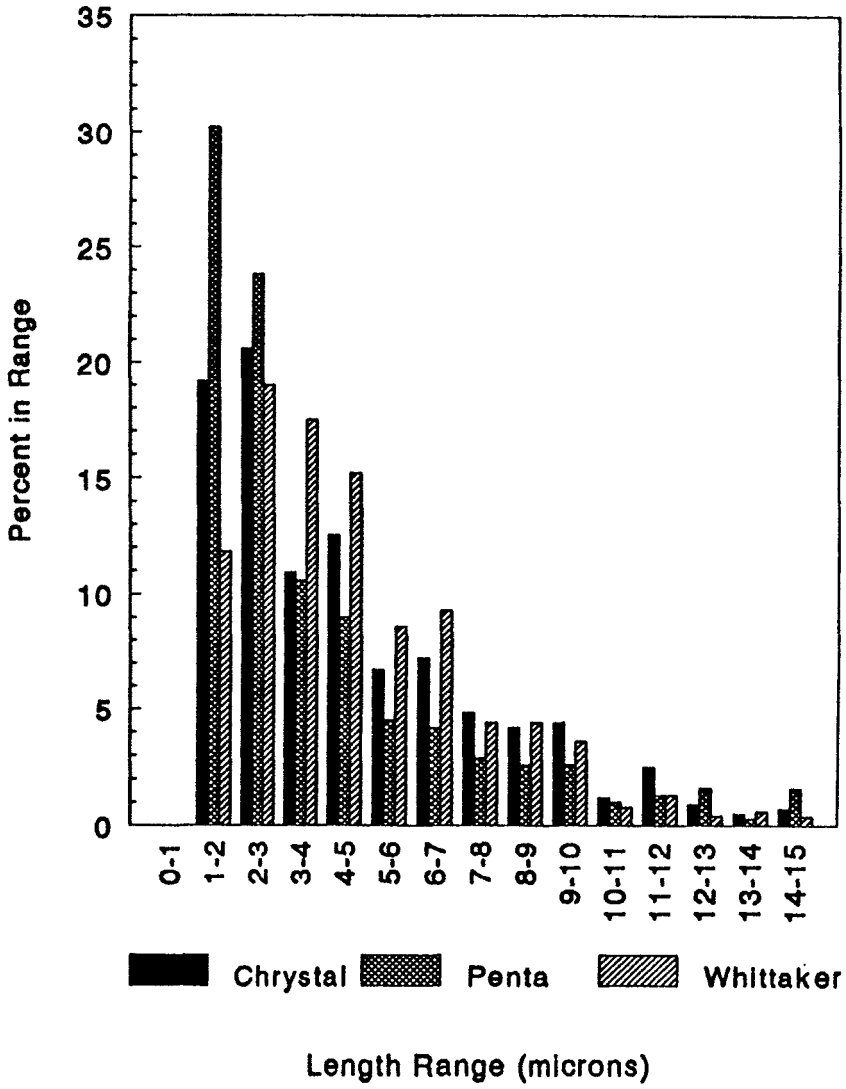

Figure 9. Particle size distribution of talc, as obtained from different vendors.

Table III

Particle Size Distributions for Three Lots of Talc, Obtained Using Optical Microscopy

Length Range (μm)	Percent in Range		
	Chrystal Lot 4087	Penta Lot 23252	Whittaker Lot 2576
0-1	0.0	0.0	0.0
1-2	19.2	30.2	11.8
2-3	20.6	23.8	19.0
3-4	10.9	10.6	17.5
4-5	12.5	9.0	15.2
5-6	6.7	4.5	8.6
6-7	7.2	4.2	9.3
7-8	4.8	2.9	4.4
8-9	4.2	2.6	4.4
9-10	4.4	2.6	3.6
10-11	1.2	1.0	0.8
11-12	2.5	1.3	1.3
12-13	0.9	1.6	0.4
13-14	0.5	0.3	0.6
14-15	0.7	1.6	0.4

Table IV

Micromeretic Properties of Talc Lots from Different Vendors

Property	Chrystal Lot 4087	Penta Lot 23252	Whittaker Lot 2576
Average Particle Size: L x W (μm)	3.3 x 5.3	3.0 x 4.7	3.1 x 5.2
Surface Area (m^2/g)	4.7	7.3	9.2
True Density (g/mL)	2.9	2.6	3.0
Bulk Density (g/mL)	0.38	0.38	0.31
Tap Density (g/mL)	0.48	0.47	0.35
Compressibility (%)	22	19	12

The bulk densities for the Chrystal and Penta lots were 0.38 g/mL, whereas the Whittaker lot was slightly lower at approximately 0.31 g/mL (Table IV). The tap densities of the Chrystal and Penta lots were similar with values of 0.48 and 0.47, respectively. However, the Whittaker material exhibited a tap density of 0.35 g/ml. The compressibility of the Chrystal and Penta lots (22 and 19, respectively) was found to be higher than the compressibility of the Whittaker lot (compressibility value of 12).

3.9 Powder Characteristics

Powder characteristics were measured following the procedure of Carr [32]. Mass flow rates were also measured for comparison. The data is summarized in Table V. The mass flow rates of all three materials is less than 0.2 g/sec, constituting a poor flowing powder. This characteristic is attributable to the small particle size and plate-like structure of the material. The dispersibility value, related to the dustiness and floodability of the powder, was similar for all three vendors. The cohesion, a measure of the apparent cohesive force existing on the surfaces of the particles, was found to differ for the lots, with the Whittaker lot being higher than the other two vendors. The relative flowability of the talc samples were calculated as the flow index and these values are similar for the three lots. The floodability index, or the measure of an unstable or gushing flow of a solid, was also calculated and was found to be similar for the three lots. From this data, it appears that the three lots would have similar bulk handling properties.

3.10 Hygroscopicity

The moisture uptake profile of the talc samples was investigated using an automated moisture sorption system with a microbalance. The materials were exposed to relative humidities ranging from approximately 10% to 95% RH in 10% intervals, as shown in Figure 10. The weight percent absorbed and the equilibrium moisture content (EMC) [33] for the three samples are summarized in Table VI. The talc samples were not hygroscopic and sorbed less than 0.7% weight at the highest relative humidity.

Table V

Powder Characteristics of Talc from Different Vendors

Property	Chrystal Lot 4087	Penta Lot 23252	Whittaker Lot 2576
Mass Flow Rate (g/sec)	0.06	0.2	0.1
Dispersibility	6	4	7
Cohesion	7	7	20
Flow Index	25	25	25
Flood Index	63	68	59

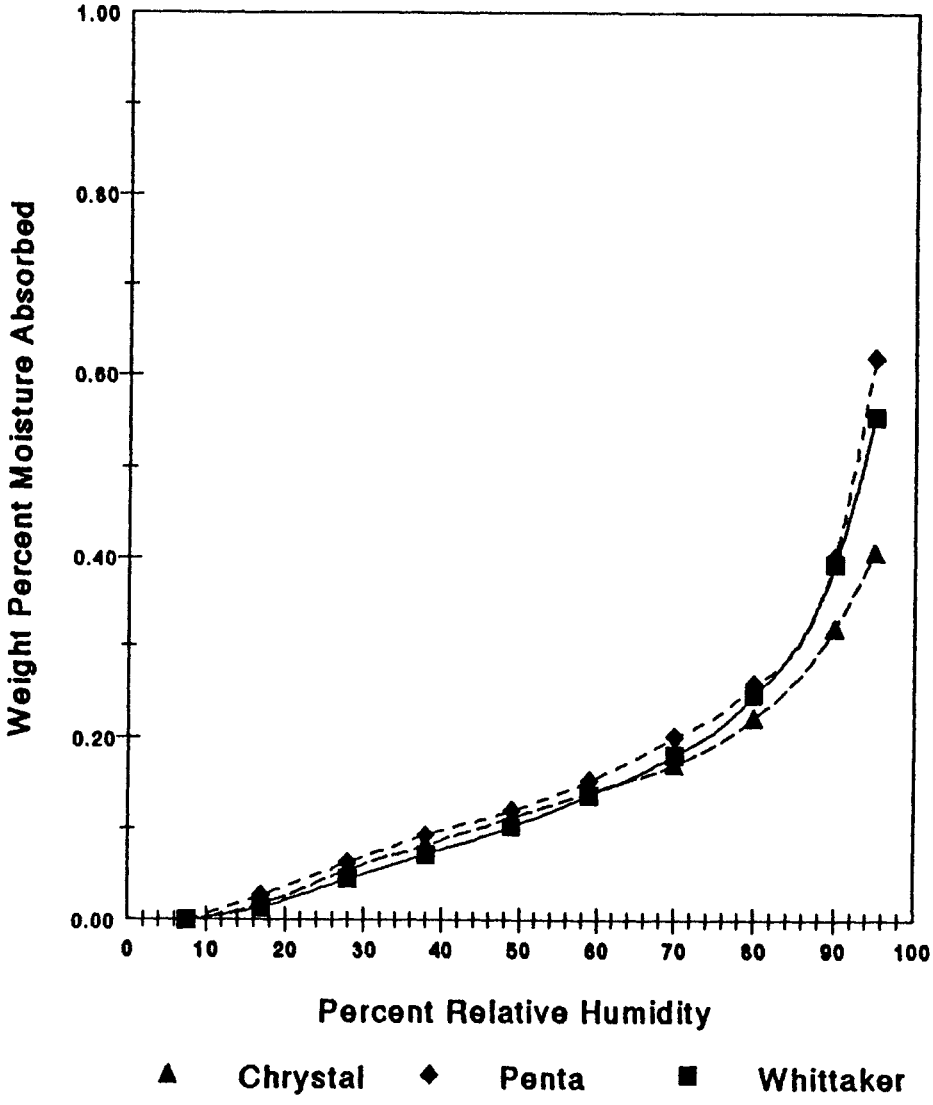

Figure 10. Moisture uptake curves of talc, as obtained from different vendors.

Table VI

Moisture Uptake Data for Talc from Different Vendors

Approximate Relative Humidity (%)	Chrystal Lot 4087		Penta Lot 23252		Whittaker Lot 2576	
	%WT	EMC	% WT	EMC	% Wt	EMC
10	0.00	0.54	0.0	0.60	0.00	0.73
20	0.02	0.62	0.03	0.63	0.01	0.81
30	0.06	0.64	0.06	0.64	0.05	0.83
40	0.08	0.63	0.09	0.63	0.07	0.83
50	0.11	0.63	0.12	0.63	0.10	0.83
60	0.14	0.63	0.15	0.63	0.14	0.83
70	0.17	0.63	0.20	0.65	0.18	0.84
80	0.22	0.65	0.26	0.66	0.25	0.86
90	0.32	0.79	0.40	0.74	0.39	0.94
95	0.41	0.69	0.62	0.81	0.55	0.96

EMC = Equilibrium moisture content

4. Methods of Analysis

4.1 Compendial Tests

The United States Pharmacopeia [34] includes the following assays for talc:

Identification: Talc is added to a melt of anhydrous sodium carbonate and anhydrous potassium carbonate and heated until fused. Hot water and hydrochloric acid are then added to the cooled mixture, and heated to dryness. The remaining solid is boiled in water, and the insoluble silica residue is filtered off. Ammonium chloride and ammonium hydroxide are dissolved in the filtrate. The solution is filtered if necessary and dibasic sodium phosphate TS is added to the filtrate. A white crystalline precipitate of magnesium ammonium phosphate separates.

Microbial limit: Total bacteria count does not exceed 500 per gram.

Loss on ignition: Following the general test method <733>, the material is ignited to 1000°C to constant weight. The material can not lose more than 6.5% of its own weight.

Acid-soluble substances: The sample is digested in hydrochloric acid, brought to original volume with water, mixed, and filtered. Sulfuric acid is added to the filtrate, evaporated to dryness, and ignited to constant weight. The weight of the residue can not exceed 2% of the original sample weight.

Reaction and soluble substances: The sample is boiled in water and filtered. One half of the filtrate is evaporated to dryness, and dried further at 105°C. The weight of the residue cannot exceed 0.1% of the original sample weight.

Water-soluble iron: The other half of the filtrate from the previous test is acidified with hydrochloric acid. Potassium ferrocyanide TS is added to the solution. The liquid should not acquire a blue color.

Arsenic: The test solution is prepared by adding hydrochloric acid to the solid in a flask. The mixture is refluxed, cooled, and transferred to a beaker. The solids are allowed to settle. The supernatant is decanted through filter paper into a volumetric. The solid is rinsed with hot water and the liquid is decanted into the volumetric. The test solution is brought to volume with water. The arsenic content is determined using

general test <211>. The limit is 3 ppm.

Heavy metals: To determine the heavy metal content, the test solution prepared above is used in general test <231>. The limit is 0.004%.

Lead: To determine the lead content, the test solution prepared above is used in general test <251>. The sample cannot contain more than 5 μg of lead.

4.2 Atomic Absorption Spectroscopy

Atomic absorption spectroscopy has been used to determine the amount of impurities in talc samples based on the chemical composition [35]. The detection of calcium, iron, and aluminum gave an indication of the mineral and chemical purity of the talc, whereas, analyses for chromium, manganese, nickel, and copper were of toxicological interest. The sample preparation involved an acid extraction with dilute hydrochloric acid to remove magnesium and calcium carbonates. Total dissolution of the sample was achieved with nitric/hydrofluoric acid mixture, followed by nitric/perchloric acid mixtures. Calcium was determined in the nitrous oxide/acetylene flame and the other elements were detected in the air/acetylene flame.

The atomic absorption technique has also been used to investigate the homogeneity variations within and between batches of talc bulk material [36]. Samples were prepared by boiling talc in hydrochloric acid, cooling, and filtering the mixture. An air acetylene flame was used for the determination of iron, copper, and calcium. Homogeneity variations of the different impurity elements were shown to be significant between bags but not within them.

4.3 Zeta Potential Measurements

Zeta potential was used to study the dispersiblity of talc and other minerals in water [30]. It was found that higher zeta potential values corresponded to better dispersion. Talc exhibited a value greater than 70 mV and good dispersion in distilled water.

Another study which measured zeta potentials in a variety of talc samples was investigating the floatability of this material [37]. No correlation was found because the zeta potentials of plate-like talc were not indicative of surface charges that are uniformly distributed across the mineral surface.

4.4 Spectroscopic Techniques

Near-IR photoacoustic spectra have been reported for talc and other minerals [38]. Samples were passed through a 240-mesh (64μm) sieve, and were ground first if necessary. The powder was oven-dried overnight at 105°C prior to analysis. Bands were observed at 1.45 (O-H band), 2.14, 2.18, 2.27, 2.36, 2.43, and 2.50 μm. The UV-visible photoacoustic spectrum was featureless.

X-ray photoelectron spectroscopy has been used to investigate the silicon-oxygen bonding in talc and other silicate materials [39]. Samples were coated with gold and referenced to the gold $4f_{7/2}$ peak at 84.0 eV. Aluminum Kα radiation was used for the photoionization. A silicon 2s binding energy of 154.4 eV was reported for talc. The binding energy for the oxygen 1s electron was 532.6 eV for both the silicate oxygen and the water oxygen. The silicon and oxygen binding energies were correlated to the calculated charge of the element and the Si-O percent ionic character for silicate minerals.

High resolution silicon Kβ x-ray spectra have also been reported for talc and other minerals [40]. The energy of the Si-Kβ peak was reported at 1832.3 eV with a full width half maximum (FWHM) of 3.3 for talc. The Si-Kβ' peak energy was measured at 1818.6 eV. Comparison of the data from a variety of minerals concluded that the participation of the Si 3p orbitals in bonding is influenced by the local structure around the silicon atom and the molecular orbital picture of the bonding was established.

4.5 Chrysotile Determination

The determination of the impurity chrysotile in talc was developed for routine assays [41]. The test is based on the adsorption of bromocresol blue by chrysotile. Since talc will not absorb the dye, the final measurement is a spectrophotometric determination of the dye left in solution after adsorption.

5. Stability

5.1 Stability

Talc reacts with selected hot, concentrated mineral acid solutions. It is stable with respect to light, oxidation, and changes in pH of suspensions. It is thermally stable up to 900°C, where it loses water and undergoes a solid state transformation.

5.2 Incompatibilities

Talc is chemically unreactive in general. For aspirin formulations, it was found that talc impurities, such as calcium carbonate and calcium silicate, were responsible for the aspirin degradation observed [42]. Impurities such as iron oxide and aluminum silicate did not affect the stability of the aspirin.

Mixtures of mandelic acid and talc were ball-milled and analyzed by IR spectroscopy to investigate the optical isomer effect on the mechanochemical interaction [43]. The IR spectra showed that a complex was formed between talc and the hydroxyl group and the COOH group of mandelic acid. It was also found that the covalent character in the carboxyl group was greater for (RS)-mandelic acid than for the R-isomer and the S-isomer.

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TOLNAFTATE

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1. History and Therapeutic Properties

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8 Acknowledgements

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1. History and Therapeutic properties

Noguchi et al. (1) first reported a series of naphthiomates with antifungal activity. Their studies further revealed that O-2-Naphthyl m, *N*-dimethylthiocarbanilate (Tolnaftate) was the most promising compound of this series. As a topical antifungal agent, tolnaftate is used for the treatment of various dermatophytes and several forms of microsporon that can cause infectious conditions like *Tinea pedis* (athlete's foot), *Tinea cruris* (jock itch) and *Tinea capitis* (body ringworm). Tolnaftate is not effective against *Candida albicans* or gram negative and gram positive bacteria. When used alone, this agent is not effective in the treatment of fungal infections of the hair or nails. The mechanism of action of tolnaftate is still unknown but it is believed to be due to the inhibition of ergosterol biosynthesis (2). Tolnaftate also inhibits the growth and biosynthesis of aflatoxin formation in *Aspergillus parasiticus* (3).

2. Description

2.1 Nomenclature

2.1.1 Chemical Name

2-Naphthyl-*N*-methyl-*N*-(3-Tolyl)thionocarbamate; O-2-Naphthyl m, *N*-dimethylthiocarbanilate; [Methyl (3-methylphenyl)carbamathoic acid.

2.1.2 Generic Name

Tolnaftate

2.1.3 Trade Name

Aftate, Focusan, Foot work, Pediderm, Sporiderm, Sporiline, Tinactin, Tinaderm, Timoped, Tonoftal.

2.1.4 CAS Registry Number

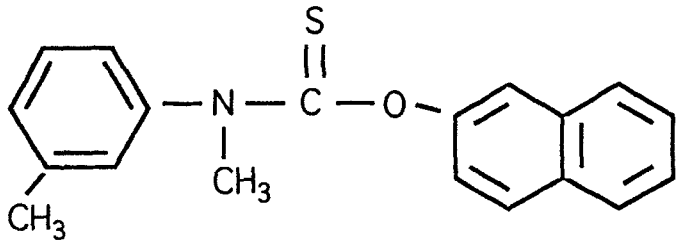
2398-96-1

2.2 Formula and Molecular Weight

$C_{19}H_{17}NOS$ mol wt 307.41

2.3 Elemental Composition

C 74.23%, H 5.58%, N 4.56%, O 5.20%, S 10.43% (4).



Tolnaftate

2.4 Appearance, Color and Odor

Tolnaftate is a white to creamy white, crystalline, odorless fine powder.

2.4 Pharmaceutical Dosage Forms

Tolnaftate is available in the following dosage forms: cream (1% w/w); powder (1% w/w); topical solution (1% w/v); topical aerosol powder (1% w/w); topical aerosol solution (1% w/v); topical spray solution (1% w/v).

3. Synthesis

Tolnaftate is synthesized (scheme 1) by the condensation of *N*-Methyl-m-tolylamine with 2-Naphthyl chlorothionoformate in the presence of sodium bicarbonate or other dehydrochlorinating agent in acetone solution and purified by recrystallization from ethanol (5).

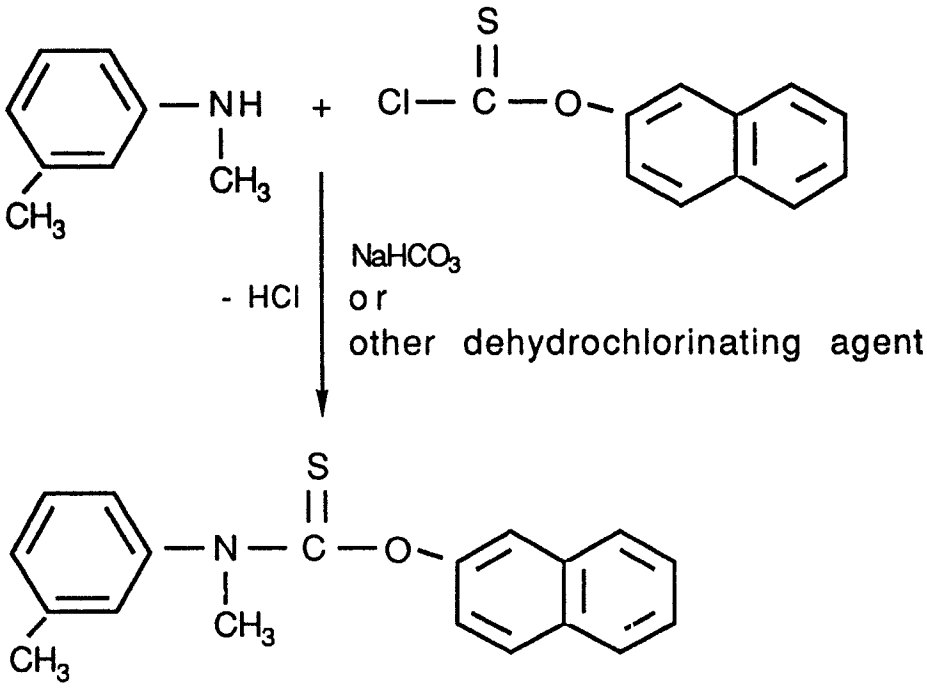
4. Physical Properties

4.1. Infrared Spectrum

The infrared spectrum of tolnaftate is shown in Figure 1. The spectrum was obtained in potassium bromide disk (0.5% w/w) using a FTIR (model 1600, Perkin-Elmer) spectrophotometer. Assignments of the characteristic bands in the spectrum are listed in Table 1 (6).

4.2 ^{1}H - Nuclear Magnetic Resonance Spectrum

The 300-MHz proton nuclear magnetic resonance spectrum of tolnaftate was obtained on a Bruker AM 300 NMR spectrometer and is shown in Figure 2. The spectrum was recorded



Tolnaftate

Scheme 1. Synthesis of Tolnaftate

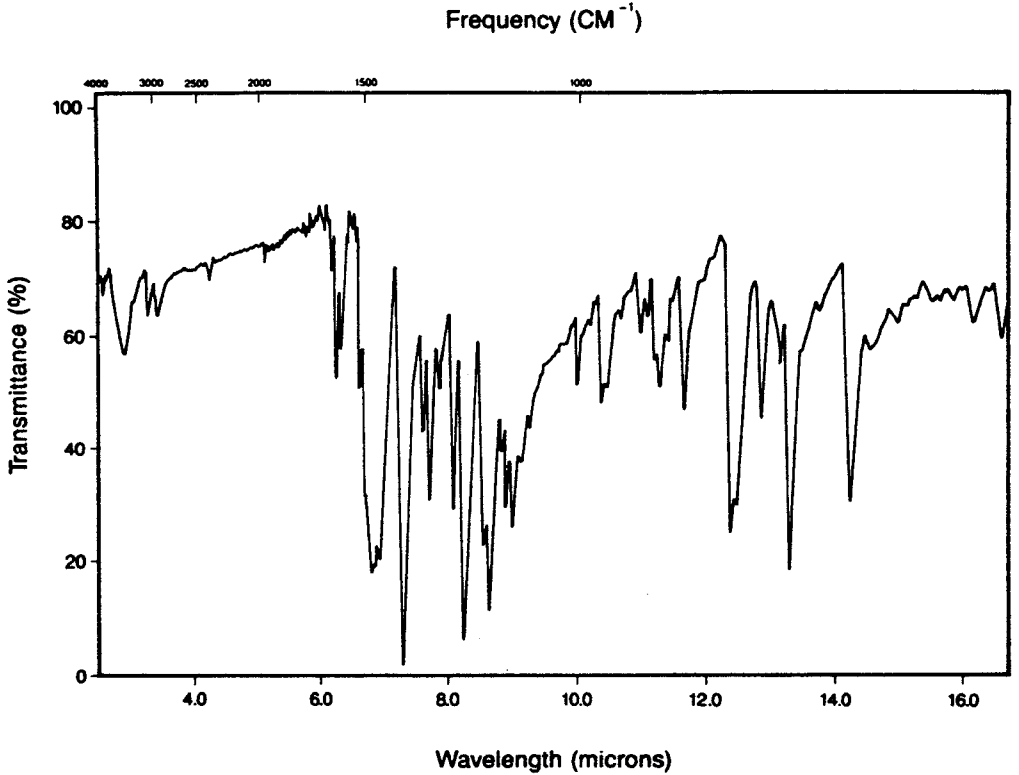


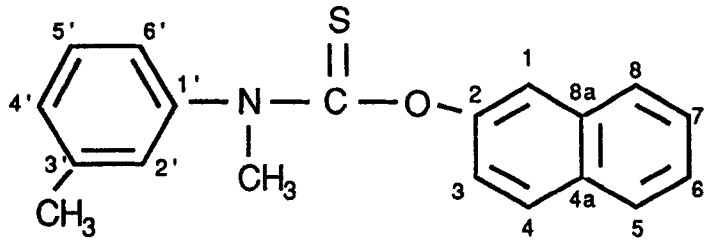
Figure 1. Infrared Spectrum of Tolnaftate.

Table 1
Infrared Spectral Assignments for Tolnaftate.

Wavenumber, cm^{-1}	Assignment
3087, 3057	Aromatic C-H stretching (m)
2923	Aliphatic C-H stretching (m)
1600	Aromatic C=C stretching (m)
1466, 1236	C=S stretching (linked to nitrogen)(s)
1369, 1297, 1210	C-N stretching (s)
1153, 1112	C-O strecthing (s)

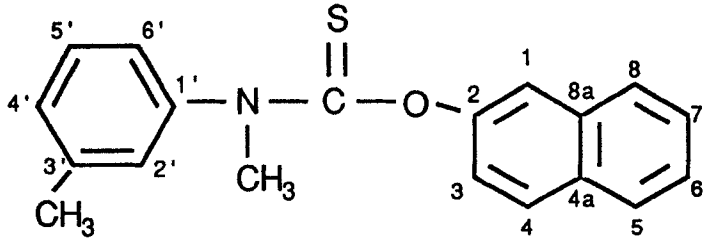
(m) = Medium
(s) = Strong

at an ambient temperature (≈ 25°C). Deuterated chloroform ($CDCl_3$) was used as the solvent with tetramethylsilane as a reference standard. The chemical shifts, multiplicities and assignments are given in Table 2 (6).



4.3 ^{13}C - Nuclear Magnetic Resonance Spectrum

The 200-MHz ^{13}C -NMR spectrum of tolnaftate obtained in deuterated chloroform ($CDCl_3$) is given in Figure 3 (6). The spectrum was recorded on a Bruker AM 200 NMR spectrometer at an ambient temperature (≈ 25°C). Chemical shifts and structural assignments are outlined in Table 3.



4.4 Ultraviolet Spectrum

The ultraviolet spectrum of tolnaftate solution

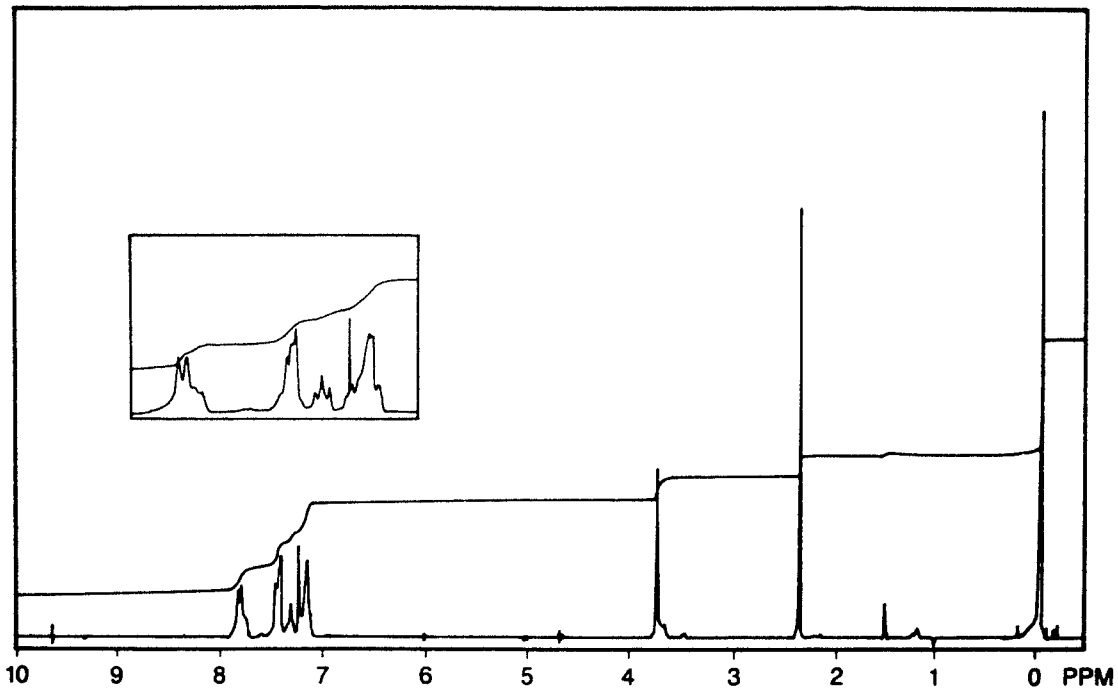


Figure 2. ^{1}H NMR Spectrum of Tolnaftate in $CDCl_3$.

Table 2
^{1}H NMR Spectral Assignments for Tolnaftate.

Chemical Shift (ppm)	Multiplicities	Number of protons	Assignment
2.40	s	3	3'-CH_3
3.75	s (b)	3	N-CH_3
7.81	m	3	C_1, C_3, $C_{6'}$
7.77-7.45	m	3	Ar protons*
7.42-7.28	m	2	Ar protons*
7.22-7.13	m	3	Ar protons*

s = singlet
m = multiplet
(b) = broad
*Aromatic protons

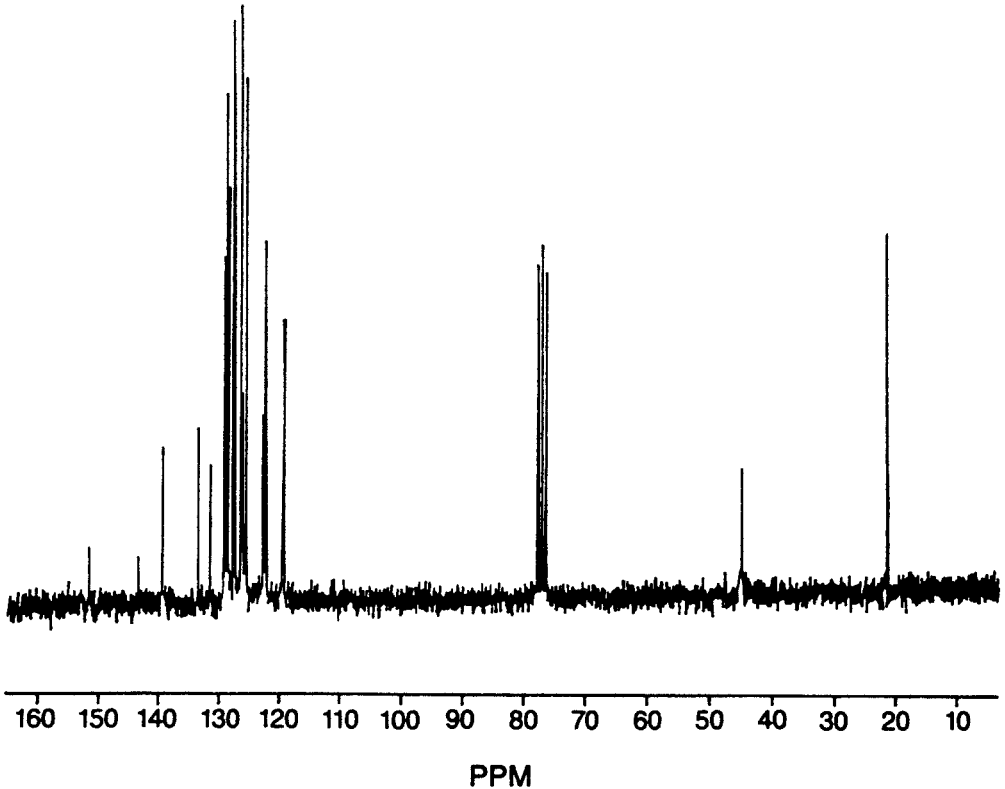


Figure 3. ^{13}C NMR Spectrum of Tolnaftate in $CDCl_3$.

Table 3
^{13}C NMR Spectral Assignments for Tolnaftate.

Chemical Shift (ppm)	Assignment
21.29	3'-methyl
44.77	N-methyl
119.26-129.15	Eleven Aromatic Carbons
131.42	3'
133.56, 139.44	4a, 8a
143.42	1'
151.53	2
155.00	C=S

in methanol (10 μg/mL) was obtained using a UV-VIS spectrophotometer (model UV160U, Shimadzu). Two absorption maxima were detected at 258 and 222 nm as shown in Figure 4 (6).

4.5 Mass Spectrum

The mass spectrum of Tolnaftate is shown in Figure 5. The spectrum was obtained by electron impact at an electron energy of 70 eV and a source temperature of 180°C using a Finnigan INCOS-50B, Quadrupole mass spectrometer linked to a Hewlett-Packard gas chromatograph. The GC column used was a 15 m SPB-5 (Supelco). The spectrum shows a molecular ion at a mass/charge (m/z) ratio of 307 with a relative intensity of 13.3% and a base peak at 148. The most prominent ions with their relative intensities and also the corresponding proposed fragment ions are shown in Table 4 (6).

4.6 Thermal Behavior

The differential scanning calorimetric (DSC) Curve of tolnaftate is shown in Figure 6. The sample was heated in a nonhermetically crimped aluminum pan at a rate of 10°C/min on a differential scanning calorimeter (model 910, Du Pont). A single endothermic peak corresponding to the melting of the compound was observed at 113°C. The enthalpy of fusion was found to be 62.7 J/g (6).

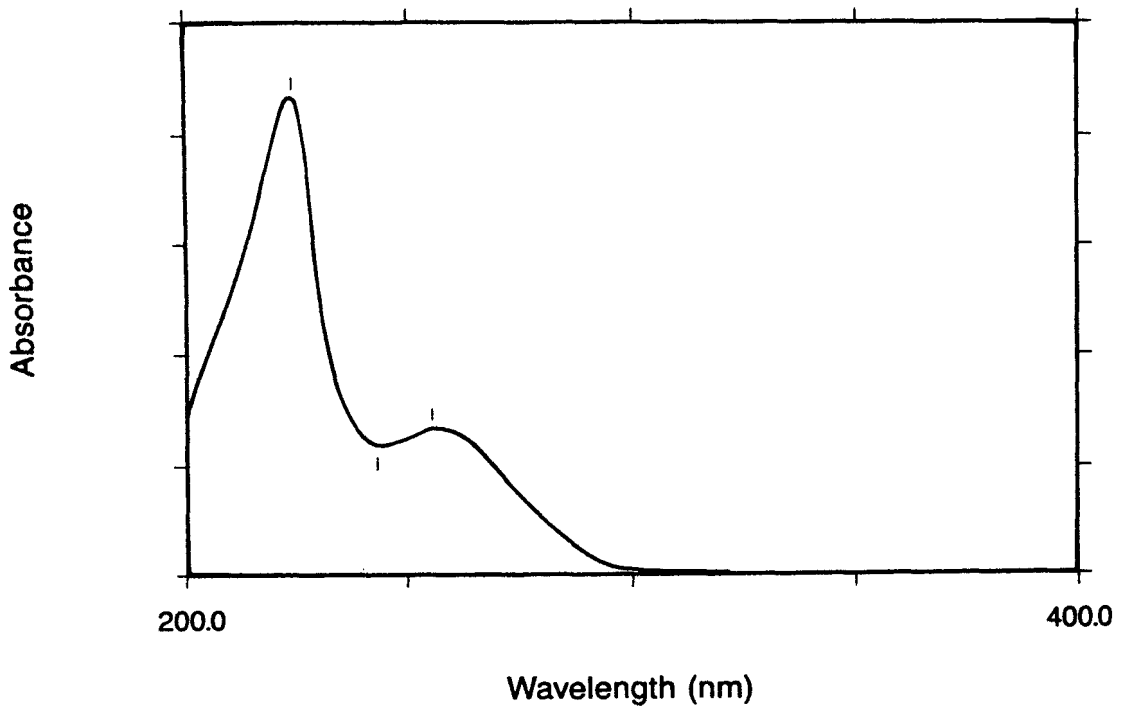


Figure 4. Ultraviolet Spectrum of Tolnaftate in Methanol.

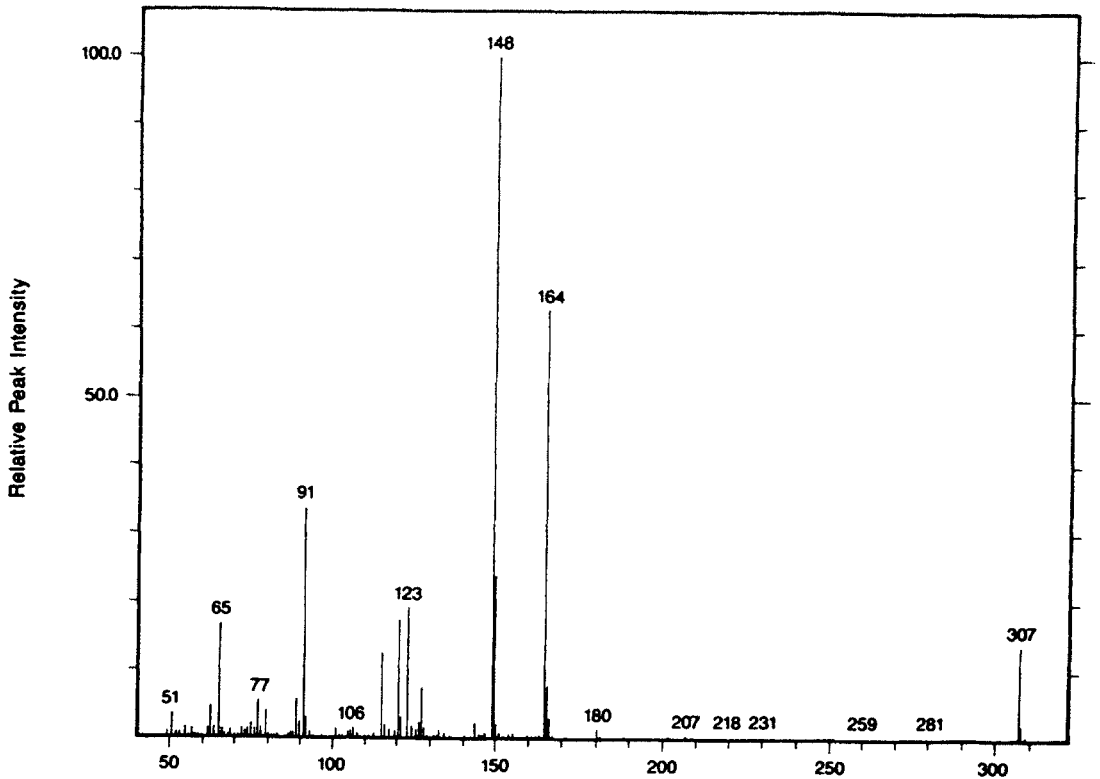


Figure 5. Mass Spectrum of Tolnaftate-Electron Impact.

Table 4
Mass Spectrum Data for Tolnaftate - Electron Impact.

m/z ratio	relative intensity (%)	Fragment ion
307	13.3	$C_{19}H_{17}NOS$
164	63.3	$C_9H_{10}NS$
148	100	$C_9H_{10}NO$
123	18.9	C_7H_7S
91	32.3	C_7H_7 (tropylium ion)
65	16.4	C_5H_5

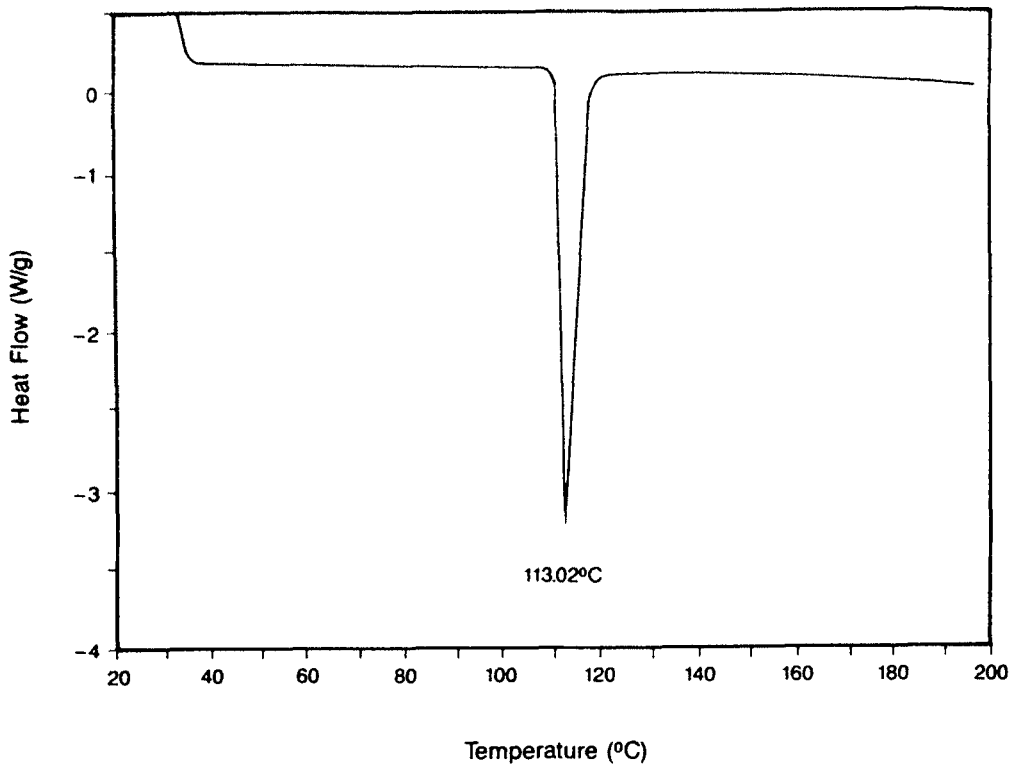


Figure 6. Differential Scanning Calorimetric (DSC) Curve of Tolnaftate.

4.7 Melting Point

Tolnaftate melts between 110-113°C (4).

4.8 Solubility

The solubility of tolnaftate in various solvents at room temperature has been reported (4,5). It is practically insoluble in water, soluble in 1 in 4000 of ethanol, 1 in 7 of acetone, 1 in 9 of carbon tetrachloride, 1 in 55 of ether and 1 in 1.5 of chloroform.

4.9 X-Ray Powder Diffraction Pattern

The powder pattern data for tolnaftate was obtained in a wide angle X-Ray diffractometer (model D500, Siemens) and is shown in Figure 7. The calculated d-spacings for the diffraction pattern are given in Table 5.

4.10 Partition Behavior

Attempts to determine the octanol/water partition coefficient of tolnaftate (at 24°C) were unsuccessful. Tolnaftate could not be detected in the aqueous phase.

5. Methods of Analysis

5.1 Identification (7,8)

A. Infrared absorption spectra of a potassium iodide dispersion of tolnaftate should show the absorption maxima at the same wavelengths as

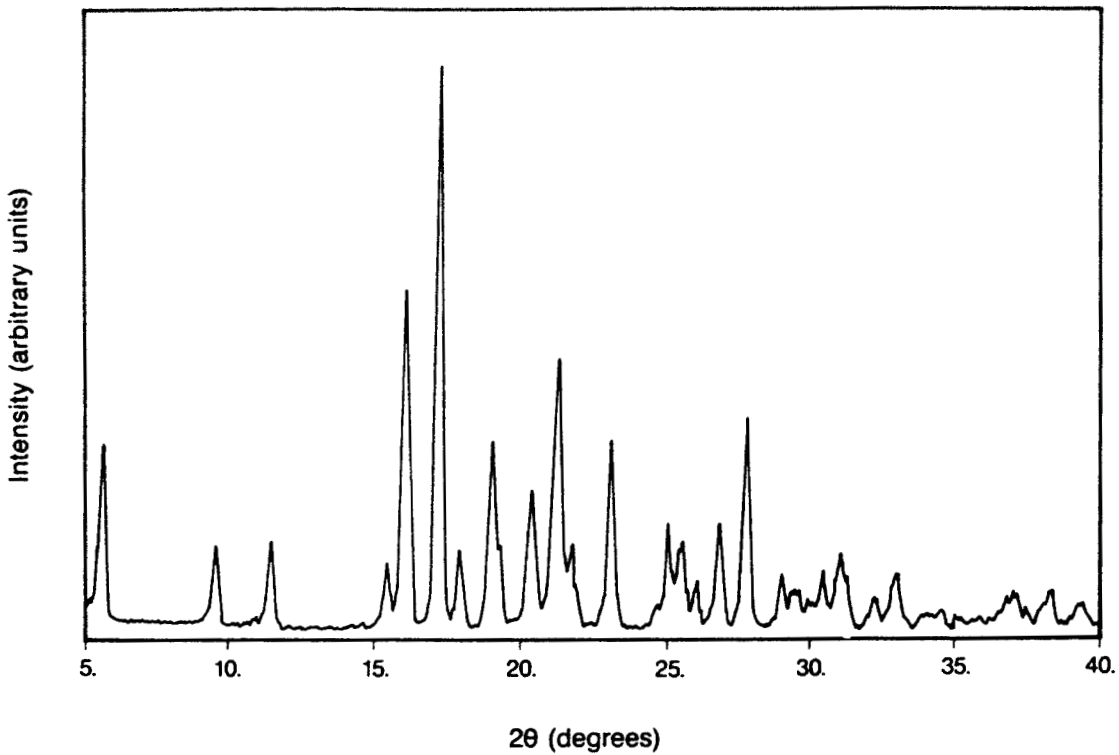


Figure 7. X-Ray Diffraction Pattern of Tolnaftate.

Table 5
Powder X-ray Diffraction Data for Tolnaftate

Peak No	2Θ	d-Space	Relative Intensity (%)
1	5.70	15.49	36.11
2	9.60	9.21	17.14
3	11.5	7.69	18.82
4	16.10	5.50	67.58
5	17.25	5.14	100
6	17.95	4.94	15.35
7	19.05	4.85	36.64
8	19.35	4.58	15.73
9	20.45	4.54	25.80
10	21.30	4.16	52.53
11	21.70	4.09	17.29
12	23.10	3.85	39.20
13	25.05	3.55	22.08
14	25.55	3.48	19.09
15	26.95	3.32	21.80
16	27.80	3.21	42.04

that of a USP Reference Standard.

B. Ultraviolet absorption spectrum of a 1 in 100,000 solution of tolnaftate in methanol should show the same uv spectrum at the same wavelengths as that of a similar solution of USP reference standard.

C. Color Reactions (9)

1. Mandelin's Test: A drop of the Mandelin's reagent when added to a tolnaftate sample on a white tile produces brown color. The Mandelin's reagent consists of 0.5 g of ammonium vanadate in 1.5 mL of water and diluted to 100 mL with sulfuric acid, and the solution is filtered through glass wool.

2. Marqui's Test: A drop of the Marqui's reagent when added to a tolnaftate sample on a white tile produces blue green color. The Marqui's reagent consists of 1 volume of formaldehyde solution with 9 volumes of sulfuric acid.

5.2 Spectrophotometric Method

The United States Pharmacopeia XXII (8) lists a UV Spectrophotometric method for the assay of tolnaftate. The absorbance of tolnaftate solution in methanol (10 μg/mL) is measured at the wave length of maximum absorbance at about 258 nm. Quantitation is based on the net

absorbance at the same wave length of a solution of USP Tolnaftate Reference Standard of known concentration.

5.3 Chromatographic Methods

5.3.1 Thin Layer Chromatography

Tolnaftate is dissolved in a mixture of benzene and ethyl alcohol (1:1 v/v). A 100 μL portion of this solution (10 μg/mL) is applied to a silica Gel G thin layer chromatographic plate. The chromatogram is developed, using toluene as the mobile phase. The spot is visualized using UV light (254 nm) and/or a 1 in 100 solution of iodine in carbon tetrachloride sprayed on the plate and air dried (7).

5.3.2 High Performance Liquid Chromatography (HPLC)

The USP assay method for tolnaftate in powder and aerosol powder product requires extraction of the drug with methanol prior to HPLC analysis. Progesterone is used as the internal standard. The mobile phase consists of acetonitrile:water (2:1 v/v) at a flow rate of 1 mL/min. This method uses a C-18 column, and the column effluent is monitored at 258 nm (8). The LC method developed by Thompson and Carlson (10) for the quantification of tolnaftate in

pharmaceutical formulations, uses acetonitrile:water (80:20 v/v) acidified with 0.5 mL of phosphoric acid per liter (apparent pH = 2.4) as the mobile phase. The flow rate of the mobile phase is 1 mL/min. This method uses a C-18 column and the column effluent is monitored at 257 nm. The LC method developed by Dash (11) uses a C-18 column. The mobile phase consists of methanol:25mM potassium dihydrogen phosphate solution (apparent pH = 6.5) at a flow rate of 1.5 mL/min. Progesterone is used as an internal standard and column effluent is monitored at 258 nm.

5.4 Biological Method

A microbiological assay for the analysis of tolnaftate was developed by Weinstein et al. (12). This method uses an agar diffusion method using *Aspergillus niger* (ATCC 10535) as a test organism. The organism was grown on sabouraud agar at 28°C for 7 days. The standard curve was linear over a concentration range of 1-6 μg/mL

6. Stability and Degradation

Stability of 1% (w/v) solution of tolnaftate in propylene glycol was studied at different temperatures and reported to be stable under such conditions (13). Plastic vials containing 1% (w/v) of the drug were exposed to boiling water (100°C)

for 15 minutes, liquid nitrogen (-160°C) for 24 h and frozen at -5°C for 72 h. In all these instances, no color change and no change in the antifungal activity in the solution was observed (13). When tolnaftate in propylene glycol (1% w/v) was sterilized under 15 lbs of pressure at 120°C for 15 minutes in a glass vial, no change in color and the antifungal activity of the solution were noticed (13). The degradation of tolnaftate in 0.1N NaOH solution at 50°C for 24 h was found to be negligible (11). Tolnaftate solution in acetonitrile:water (80:20 v/v) at an apparent pH of 2.4 was found to be stable at room temperature over a period of 60 days (10).

7.0 Pharmacokinetics, Metabolism and Toxicity

Tolnaftate is ineffective when administered by oral and parenteral routes (13). Therefore, it is administered only by topical route, and no reports on its pharmacokinetics and systemic metabolism have been documented. The LD_{50} of this drug in different animals is shown in Table 6 (4). In dogs, no toxicity was observed when a massive dose (14 g/kg) was administered orally, and in rabbits, no sensitization or irritation reaction was observed when 1% (w/v) tolnaftate solution was applied topically for 20-90 days (14). Results of this study also revealed that tolnaftate is not absorbed percutaneously. The toxicity of tolnaftate to man on topical application or by an oral administration is virtually negligible (13). Subtotal body inunction (application to 90% of the body surfaces) of 1% w/v tolnaftate solution for 7 days did not show any acute systemic toxicity (16).

Table 6
LD_{50} of Tolnaftate in Rat and Mice.

Animal species	Route of Administration	LD_{50} (g/kg)
Mice	oral	> 10
Mice	subcutaneous	> 6
Rat	oral	> 6
Rat	subcutaneous	> 4

8.0 Acknowledgements

The author thanks Dr. R. Suryanarayanan, University of Minnesota and Mr. William Ihm, Creighton University for the powder x-ray diffraction and mass spectrum studies respectively. The useful suggestions of Dr. Shanker L. Saha, Creighton University are greatfully acknowledged.

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TRIAMTERENE

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INDIA

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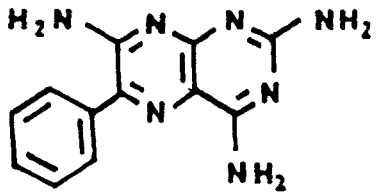
1. Description

1.1 Name, Formula and Molecular Weight

Triamterene, also known as SKF-8542 and triamterenum, is a diuretic. Chemically, it is 2,4,7-triamino-6-phenylpteridine; also named as 6-phenyl-2,4,7-pteridinetriamine. The CAS registry number for triamterene is 396-01-0.



$C_{12}H_{11}N_7$ Molecular Weight: 253.27

The common proprietary names are Dyrenium, Dytac, Jatropur, Teriam, Triamteril and Urocaudal.

1.2 Appearance, Color and Odor

Triamterene is a yellow, crystalline powder; odorless.

2. Physical Properties

2.1. Solubility

Triamterene is very slightly soluble in water (1 in 1000), in ethanol (1 in 3000) and in chloroform (1 in 4000).[1,2] It is practically insoluble in ether. it is soluble (1 in 30) in formic acid.[2] The solubilities of triamterene in various other solvent systems (determined at 20°C) have been reported[3] as: 4.2 mg/mL in dimethylformamide-water (1:1 v/v), 3.25 mg/mL in butanol-water (1:1 v/v), 1.28 mg/mL in methanol-water (1:0.5 v/v), 1.25 mg/mL in dry methanol, 0.183 mg/mL in water, 28.5±10.3 mg/mL in Sörensen buffer between pH 4.0 and pH 6.0, and 0.091 mg/mL in ethyl acetate-water (1:1 v/v). Triamterene is

insoluble in benzene, toluene, absolute butanol, dimethylformamide, ethyl acetate and absolute ethanol and methanol.[3] Various solubility studies on triamterene have been carried out[4-10]. Profiles of apparent solubility at 37°C of triamterne as a function of pH were determined in the presence of HCl, HNO_3, H_2SO_4, H_3PO_4 and acetic acid.[4] The effect of pH on the buffer solubility of triamterene and its metabolite have been studied.[8] Triamterene is solubilized[6] for intravenous administration by mixing lactic acid or glutamic acid in a ratio of 1:2 or 1:4. Mixture of glycols and lactic acid or lactate has also been used to solubilize triamterene or its combination with hydrochlorothiazide.[9] In a recently conducted study solid dispersions of triamterene in polyethylene glycols or Gelucire 44/14 were investigated for liquid filling of hard gelatin capsules.[10] The phase equilibria of the drug and carriers were determined by differential thermal analysis and hot stage microscopy. Particle size analysis was carried out using double image microscopy, while phase solubility techniques and dissolution methods were used to study solubility, dissolution and ageing. Triamterene forms monotectics with polyethylene glycols or Gelucire 44/14. The aqueous solubility of the drug is increased by polyethylene glycols and Gelucire 44/14, with the latter producing greater increase. Other studies have also shown an increase in the dissolution rate for triamterene in solid dispersions with PEG-600 as compared to the pure drug.[11,12] The increase in drug solubility has been attributed to the wettability and microdispersion of the drug in the vehicle.[12]

Aldehyde adducts of triamterene have been prepared to increase triamterene solubility and therefore its bioavailability.[13]

2.2 Dissociation Constant

pka 6.2[2]

2.3 Optical Activity

Triamterene exhibits no optical

activity.

2.4 Crystal Properties

Crystal properties of triamterene have been studied[3,14-16] Microscopic examination of triamterene crystals obtained from various solvents in the absence and presence of water or dimethylformamide (DMF) showed different habits.[3] Crystals obtained from DMF-water solutions are colorless parallelepiped crystals with dimensions of 0.20x0.10x0.07-0.10 mm in general, whereas those obtained from butanol, methanol, ethanol, and ethyl acetate were identical in shape and revealed needle growth. Crystals obtained from ethanol in the presence of water (1:1 v/v) were columnar shaped and truncated, and appeared similar to those obtained in butanol-DMF and DMF-water mixtures. Triamterene crystals obtained from solutions of butanol-water appeared as spiny plates, those obtained after recrystallization from ethyl acetate solutions in the presence of water revealed needle growth. Crystals from the benzene-ethanol (butanol) mixtures showed needle growth, similar to those from solutions of ethyl acetate in the absence of water. One can conclude from this crystal analysis that in the case of triamterene, the solvent systems induce the crystal habits rather than the operating conditions.

The crystalline structure of triamterene has been reported[3], revealing a N,N-dimethylformamide molecule and a water molecule within the crystal lattice. Triamterene crystallizes in a face-centered orthorhombic space group, Fdd2 (#43), when grown from aqueous solutions of dimethylformamide, with unit cell dimensions of a=24.291(4), b=28.191(6), c=9.813(2) A^{o}, V=6747.1A^{o}, z=16, ρ_{calc}= 1.38g.cm^{-3} and ρobs= 1.378(5)g.cm^{-3} (CuK_{α} = 1.54178 A^{o}). The atomic parameters for this crystal form are given in Table I and II, and the intramolecular and intermolecular distances, as well as important conformational angles are listed in Table III, IV and V.[3] The molecular conformation of triamterene with numbering pattern of different atoms has been shown in Figure 1. Location of the DMF and

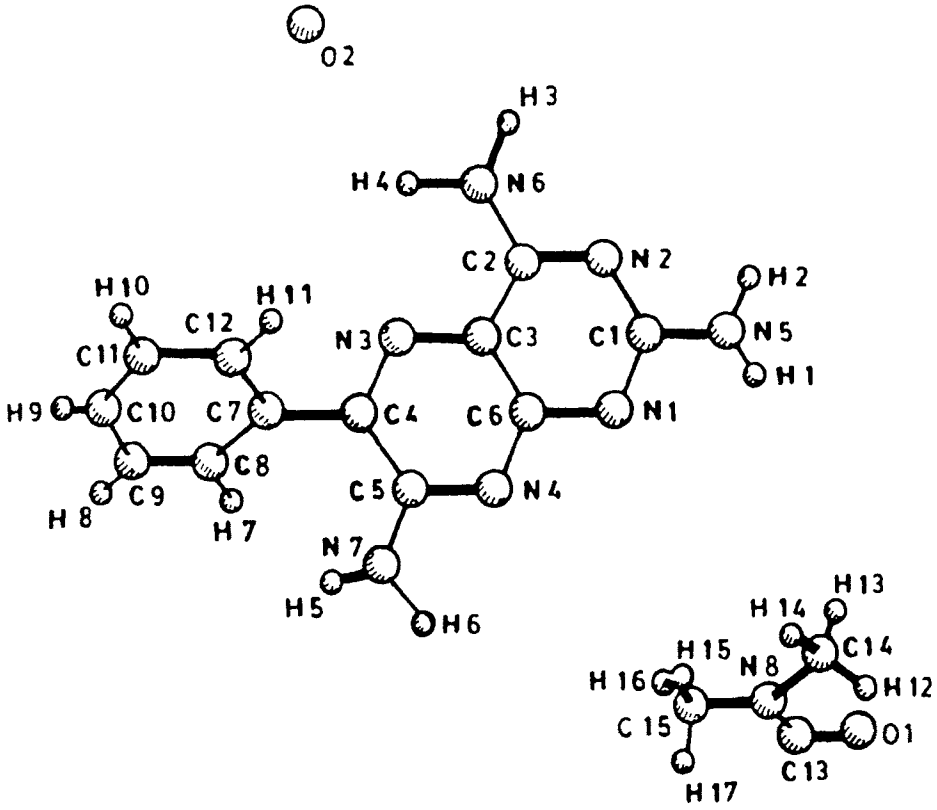


Figure 1 - Molecular conformation of triamterene.

water molecules is also shown. The structure is held together by five hydrogen bonds; namely between atoms O(1) and N(7), with a distance of 2.86 A^{o}, O(1) and N(5), with a distance of 2.96 A^{o}, O(2) and N(1), at two different symmetry sites of 2.99 A^{o}, and between N(2) and N(7), with a distance of 2.91 A^{o}.

Table I: Atomic Coordinates and Equivalent Isotropic Temperature Factors[a]

Atom	X	Y	Z	B_{eq}
O1	0.9255	0.1320	0.0	7.4
N8	0.8414	0.1426	0.091	5.6
C13	0.8756	0.1303	-0.006	8.0
C14	0.8610	0.1603	0.214	15.0
C15	0.7869	0.1417	0.066	14.0
H12	0.8851	0.1369	0.2469	18.7
H13	0.8824	0.1879	0.1879	18.7
H14	0.9327	0.1673	0.2667	18.7
H15	0.7685	0.1512	0.1423	14.6
H16	0.7801	0.1621	-0.0100	14.6
H17	0.7780	0.1099	0.0405	14.6
N1	0.6778	0.2726	0.5370	2.9
N2	0.6710	0.3536	0.6250	2.7
N3	0.5340	0.3107	0.5570	3.2
N4	0.5979	0.2315	0.4890	2.9
N5	0.7536	0.3143	0.600	4.1
N6	0.5892	0.3914	0.650	3.0
N7	0.5203	0.1892	0.453	4.6
C1	0.6987	0.3130	0.587	3.2
C2	0.6178	0.3517	0.614	2.6
C3	0.5896	0.3103	0.566	2.2
C4	0.5094	0.2704	0.516	3.0
C5	0.5421	0.2294	0.484	2.5
C6	0.6203	0.2724	0.529	2.0
C7	0.4499	0.2721	0.506	2.4
C8	0.4233	0.2559	0.389	4.1
C9	0.3667	0.2576	0.378	4.9
C10	0.3366	0.2745	0.482	5.0
C11	0.3603	0.2904	0.498	5.4
C12	0.4193	0.2896	0.607	4.7
H1	0.7688	0.2871	0.5946	4.9
H2	0.7678	0.3415	0.6474	4.9
H3	0.6078	0.4227	0.6962	3.7
H4	0.5442	0.3922	0.6344	3.7
H5	0.4874	0.1810	0.4496	5.5
H6	0.5428	0.1564	0.4523	5.5
H7	0.4445	0.2431	0.3151	5.6
H8	0.3486	0.2475	0.2987	6.1
H9	0.2977	0.2751	0.4761	5.7
H10	0.3387	0.3045	0.6683	7.3
H11	0.4367	0.3002	0.6899	5.6
O2	0.500	0.5000	0.549	10.0

[a] Units = $Å^3$; $B_{eq} = 8/3\ \pi^2 \Sigma_i \Sigma_j U_{ij} a_i^* a_j^* \cdot a_i a_j$

Table II: $\underline{U}$ Values for C(15), H(17), N(8), and O(2)

Atom	$\underline{U}11$	$\underline{U}22$	$\underline{U}33$	$\underline{U}12$	$\underline{U}13$	$\underline{U}23$
O1	0.061	0.082	0.14	-0.026	0.027	-0.022
O2	0.11	0.21	0.05	0.01	0.0	0.0
N1	0.016	0.037	0.059	0.004	0.006	-0.005
N2	0.021	0.029	0.054	0.001	0.007	-0.012
N3	0.033	0.056	0.033	0.016	0.005	0.005
N4	0.025	0.029	0.054	-0.001	0.001	-0.008
N5	0.028	0.018	0.11	0.010	0.002	-0.022
N6	0.039	0.025	0.051	0.009	-0.003	-0.018
N7	0.027	0.015	0.13	-0.008	-0.003	-0.032
N8	0.033	0.08	0.10	0.011	0.022	0.02
C1	0.017	0.05	0.054	-0.016	-0.001	-0.000
C2	0.030	0.04	0.031	0.001	0.001	-0.009
C3	0.016	0.003	0.066	-0.001	-0.008	-0.008
C4	0.040	0.05	0.028	-0.014	-0.004	-0.015
C5	0.032	0.016	0.045	0.018	0.013	-0.006
C6	0.026	0.014	0.037	-0.003	-0.006	0.006
C7	0.032	0.019	0.041	0.010	0.003	-0.018
C8	0.039	0.08	0.037	0.008	-0.000	-0.029
C9	0.032	0.08	0.07	-0.006	-0.014	-0.04
C10	0.020	0.08	0.09	0.002	-0.007	-0.04
C11	0.024	0.09	0.09	0.003	0.018	-0.05
C12	0.048	0.08	0.049	0.011	-0.015	-0.04
C13	0.069	0.10	0.15	0.01	0.07	0.03
C14	0.30	0.11	0.17	0.06	-0.08	-0.04
C15	0.10	0.19	0.26	-0.01	-0.00	-0.14
H1	0.0619	-	-	-	-	-
H2	0.0619	-	-	-	-	-
H3	0.0463	-	-	-	-	-
H4	0.0463	-	-	-	-	-
H5	0.0695	-	-	-	-	-
H6	0.0695	-	-	-	-	-
H7	0.0711	-	-	-	-	-
H8	0.0766	-	-	-	-	-
H9	0.0718	-	-	-	-	-
H10	0.0925	-	-	-	-	-
H11	0.0714	-	-	-	-	-
H12	0.2372	-	-	-	-	-
H13	0.2372	-	-	-	-	-
H14	0.2372	-	-	-	-	-
H15	0.1845	-	-	-	-	-
H16	0.1845	-	-	-	-	-
H17	0.1845	-	-	-	-	-

Table III: Intramolecular Distances and Bond Angles Involving the Hydrogen Atoms

Atom1	Atom2	Distance	Atom1	Atom2	Distance
N5	H1	0.851	C11	H10	0.953
N5	H2	0.960	C12	H11	0.965
N6	H3	1.091	C14	H14	0.890
N6	H4	1.107	C14	H12	0.937
N7	H5	0.837	C14	H13	0.971
N7	H6	1.076	C15	H15	0.912
C8	H7	0.963	C15	H17	0.956
C9	H8	0.939	C15	H16	0.957
C10	H9	0.950			

Atom1	Atom2	Atom3	Angle[a]	Atom1	Atom2	Atom3	Angle[a]
C1	N1	C6	114(1)	N3	C4	C7	116(1)
C2	N2	C1	115(1)	C5	C4	C7	124(1)
C4	N3	C3	117(1)	N7	C5	N4	117(1)
C6	N4	C5	117(1)	N7	C5	C4	122.0(9)
C13	N8	C15	119(2)	N4	C5	C4	121(1)
C13	N8	C14	120(2)	N4	C6	C3	122.4(8)
C15	N8	C14	120(2)	N4	C6	N1	115(1)
N1	C1	N5	116(1)	C3	C6	N1	122(1)
N1	C1	N2	128.2(7)	C12	C7	C8	118.0(9)
N5	C1	N2	116(1)	C12	C7	C4	121(1)
N2	C2	N6	117(1)	C8	C7	C4	121(1)
N2	C2	C3	123(1)	C9	C8	C7	121(1)
N6	C2	C3	120.4(9)	C10	C9	C8	120(1)
C6	C3	N3	123(1)	C9	C10	C11	121.5(9)
C6	C3	C2	117.7(8)	C10	C11	C12	118(1)
N3	C3	C2	120(1)	C7	C12	C11	121(1)
N3	C4	C5	119.9(8)	O1	C13	N8	126(2)

[a]Numbers in parentheses are estimated standard deviations in the least significant digits.

Table IV: Intramolecular Distances Involving the Nonhydrogen Atoms

Atom1	Atom2	Distance[a]	Atom1	Atom2	Distance[a]
O1	C13	1.22(2)	N8	C15	1.35(2)
N1	C1	1.34(1)	N8	C14	1.39(3)
N1	C6	1.41(1)	C2	C3	1.43(1)
N2	C2	1.30(1)	C3	C6	1.36(1)
N2	C1	1.38(1)	C4	C5	1.44(1)
N3	C4	1.35(1)	C4	C7	1.46(1)
N3	C3	1.36(1)	C7	C12	1.34(2)
N4	C6	1.33(1)	C7	C8	1.39(1)
N4	C5	1.36(1)	C8	C9	1.39(1)
N5	C1	1.35(1)	C9	C10	1.34(2)
N6	C2	1.37(1)	C10	C11	1.36(2)
N7	C5	1.29(1)	C11	C12	1.44(1)
N8	C13	1.31(2)			

[a]Numbers in parentheses are estimated standard deviations in the least significant digits.

Table V: Intramolecular Distances Involving the Nonhydrogen Atoms

Atom1	Atom2	Distance[a]	Atom1	Atom2	Distance[a]
O1	N7	2.86(1)	O2	C1	3.56(2)
O1	N5	2.96(1)	O2	C1	3.56(2)
O1	N6	3.52(1)	N1	N5	3.03(1)
O2	N1	2.99(2)	N2	N7	2.91(1)
O2	N1	2.99(2)	N4	N6	3.09(1)
O2	N5	3.47(2)	N5	N6	3.13(1)
O2	N5	3.47(2)	N6	C13	3.54(2)
O2	C11	3.53(2)	N6	C11	3.56(2)
O2	C11	3.53(2)	C6	C9	3.54(2)

[a]Numbers in parentheses are estimated standard deviations in the least significant digits.

2.5 Melting Range

Triamterene is reported[17] to have melting point of 316°, when crystallized from butanol as yellow plates. It is reported[18] to melt at 327° when crystallized from dimethylformamide. A detailed physicochemical study[3] of triamterene has shown that crystals grown from water-free organic solutions (e.g., methanol, ethanol, n-butanol, and DMF) have melting points of 321-324°. Crystalline material grown from organic solvents in the presence of water have melting points in the range of 310-318° (under decomposition), similar to that reported earlier.[17] Upon drying of these crystals under reduced pressure at 65° in the presence of calcium chloride and (or) silica, the melting points were determined to be 327-329°. Thus, the differences in melting points as reported by earlier workers,[17,18] are probably due to the recrystallization methods applied, including possible drying of the crystalline triamterene samples.

2.6 Thermal Analysis

The differential calorimetric curves (DSC) of the various crystalline forms of triamterene grown from organic solutions containing water and from absolute organic solutions, and the DSC curves of triamterene crystals dried under reduced pressure have been described.[3] The differential thermal analysis-thermogravimetry analysis (DTA-TG) thermograms are also given.

2.7 Infrared Spectrum

The infrared spectrum (KBr disc) (Figure 2) of triamterene exhibits principal peaks at wavenumbers 1574, 1610, 1584, 1536, 761 and 822.[2] The infrared spectra of the triamterene crystals obtained from different solvents have been described.[3]

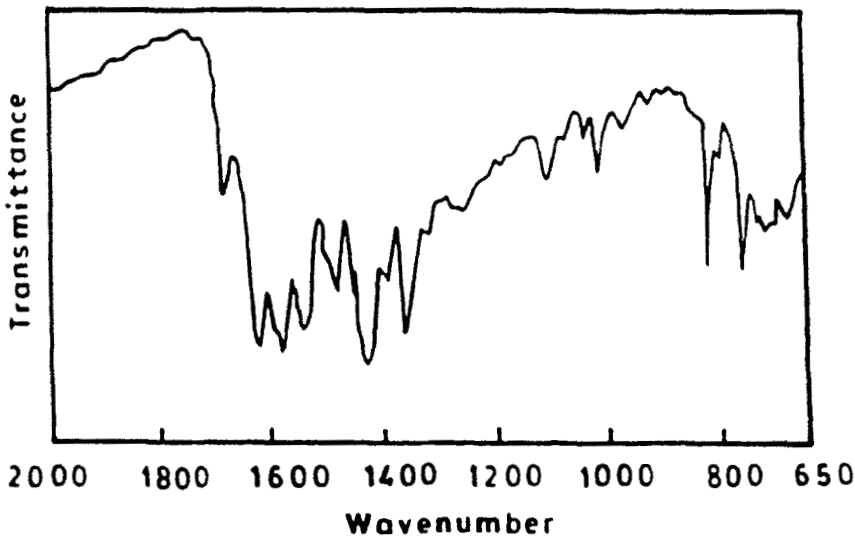


Figure 2 - Infrared spectrum of triamterene.

2.8 Ultraviolet Spectrum

The light absorption, in the range of 255 to 380 nm of a 0.001% w/v solution of triamterene in a mixture of 1 volume of 1M hydrochloric acid and 9 volumes of ethanol (96%) exhibits two maxima, at 262 nm and 360 nm, and a shoulder at 285 nm.[1]

2.9 Raman Spectra

Raman spectra of triamterene crystals obtained from different solvents have been described.[3]

2.10 Mass Spectrum

The mass spectrum of triamterene exhibits principal peaks at m/z 253, 252, 43, 104, 254, 235,77 and 89.[1]

3. Synthesis

Triamterene has been prepared[17,18] by condensation of 2,4,6-triamino-5-nitrosopyrimidine (**1**) with phenylacetonitrile (**2**)

in hot ethoxyethanol in the presence of one equivalent of sodium (Figure 3). Several patent processes based on this method have been reported.[19-26]

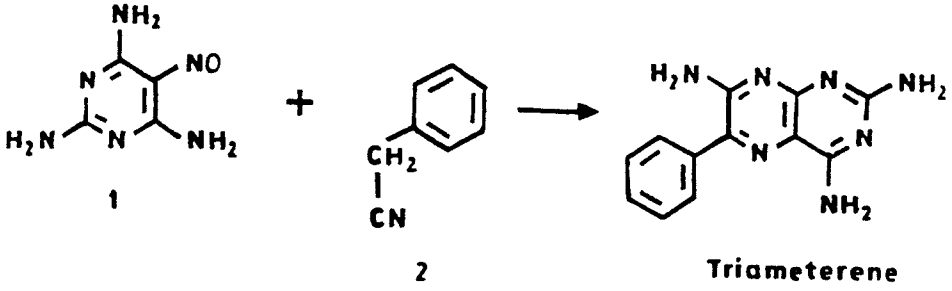


Figure 3- Synthesis of triamterene.

An alternate route of synthesis (Figure 4) has also been described.[27] This involves treatment of 2,4,5,6-tetraaminopyrimidine (3) hydrochloride in ethanolic acetic acid with benzaldehyde (4) and hydrogen cyanide to give 2,4,6-triamino-5-(∝-cyanobenzylamino)pyrimidine (5). The nitrile (5) when heated briefly under reflux with methanolic sodium methoxide and the product worked-up yielded triamterene.

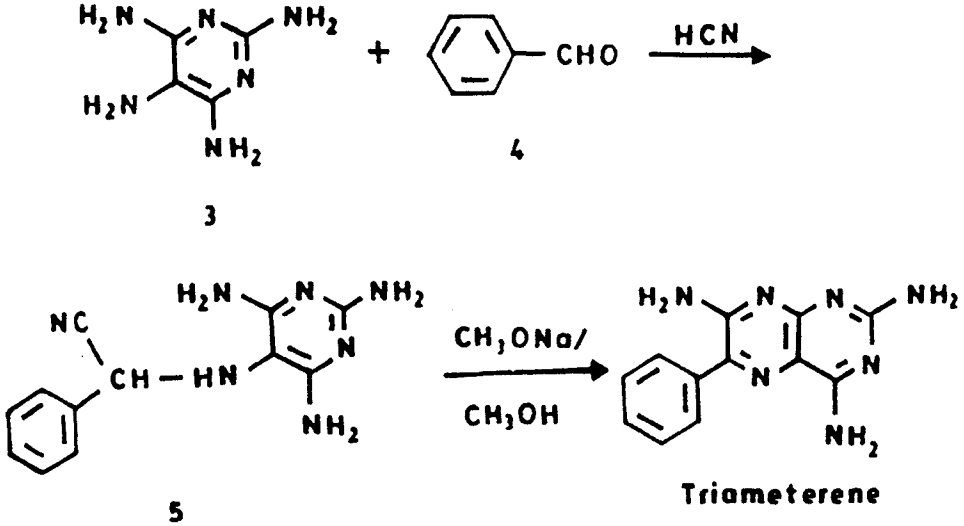


Figure 4 - Alternate synthetic route to triamterene.

A procedure for purifying crude triamterene via salt formation with acids has been reported.[28]

Triamterene labelled with tritium or carbon-14 has also been prepared.[29] Syntheses of 2,4,7-triamino-6-(p-hydroxyphenyl)pteridine[30] and sodium hydroxytriamterene sulfate,[31] the metabolites of triamterene, have been reported.

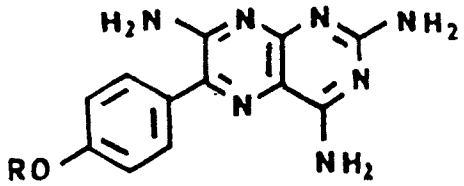
Some isomers[32,33] and derivatives[34,35] of triamterene have been prepared for biological evaluation. Recently, synthesis, natriuretic, antikaliuretic and antimagnesiuretic properties of an acid triamterene derivative have been described.[36]

4. Stability and Degradation

Triamterene is required to be stored in well- closed containers and protected from light.[1] A study in which flash photolysis and steady-state photosensitized oxidation experiments were used, has indicated that there is an interaction or cross-sensitization between triamterene and hydrochlorothiazide when irradiated with UV light in the same solution.[37] The size of the effect observed, however, was not sufficient to conclude that a combination diuretic formulation of triamterene and hydrochlorothiazide would be markedly less stable to the effect of UV light compared to the individual drugs, nor to suggest that it could lead to a greatly increased incidence of adverse phototoxic responses for the combination diuretic formulation. In a study conducted earlier decomposition rate of triamterene in the presence of 2-amino-4-hydroxypteridine and lumazine (2,4-dihydroxy-pteridine) in buffered aqueous solution irradiated by light with $\lambda \leqslant 320$ nm has been determined.[38] These pterines have been shown <u>in vitro</u> to act as photosensitizers producing oxidative decomposition of amino acids and purine bases under certain conditions. In a recent study on solid-state stability testing, the activation energy and degradation rate of triamterene at 25° calculated by conventional methods and by isothermal calorimetry have been compared and discussed.[39]

5. Metabolism

Metabolism of triamterene has been studied by several investigators.[8,40-46] Lehmann[40] was the first to isolate and identify the major metabolites of triamterene as 2,4,7-triamino-p-hydroxyphenylpteridine (p-hydroxytriamterne) (1) and its sulfate ester (p-hydroxytriamterene sulfate) (2) (Figure 5). This suggests a stepwise



(1) R = H

(2) R = SO_3H

Figure 5- Principal metabolites of triamterene.

para-hydroxylation and sulfuric acid esterification of triamterene. Sörgel et al.,[45] using healthy volunteers, showed that 29% of an oral dose of triamterene is excreted as the sulfate ester, 5.5% of the parent drug is excreted unchanged and <1.5% of the dose is excreted as p-hydroxytriamterene. In addition they showed that bioavailability of the oral triamterene is quite variable, with 11-60% of the dose found in the urine after 72 hours. Coadministration of hydrochlorothiazide did not affect the metabolic fate of triamterene, nor did it affect renal clearance of the parent compound or its metabolites. In another study[46] twenty-nine human subjects were treated with 1 mg/kg of triamterene orally. Twenty-four hour urinary excretion of triamterene, p-hydroxytriamterene and the sulfate conjugate averaged 15.3%, 6.3% and 78.4%, respectively. The excretion of triamterene plus the two metabolites averaged 43.1% of the ingested dose.

p-Hydroxytriamterene, the phase I

metabolite of triamterene is reported[47] to increase Na^+ excretion without affecting urinary volume or K^+ excretion, when administered orally at 15 and 30 mg/kg to rats on Na^+-deficient diets. The urine of these animals contained p-hydroxytriamterene sulfate, the phase II metabolite of triamterene, but did not contain p-hydroxytriamterene.

Triamterene and its metabolites have been identified as components of kidney stones passed by patients receiving that drug.[48,49]

6. Pharmacokinetics

Pharmacokinetics of triamterene has been extensively investigated.[43,50-68] Studies have been done on guinea pigs and baboons,[52] rats,[53,54,57] rabbits[55] and on human subjects.[43,51,52,56,58-68] Triamterene is variably but fairly rapidly absorbed from the gastrointestinal tract. It is extensively metabolised and is mainly excreted in the urine in the form of metabolites with some unchanged triamterene. The plasma and urinary levels of triamterene and the two metabolites have been measured using specific method of analysis.[58] Urinary excretion was completed after 48 hours which permitted a rough estimate of triamterene's half-life as 6-12 hours. After single oral doses of 150 to 300 mg to seven subjects, peak plasma triamterene concentrations of 0.03 to 0.15 μg/ml (mean about 0.1) were attained in 1 to 2 hours; peak plasma concentrations of p-hydroxytriamterene sulfate averaged about 1 μg/mL at 2 to 4 hours; both unchanged drug and the metabolite exhibited a second peak concentration several hours later, possibly due to enterohepatic circulation. The area under the curve were 672.5 and 1311.3 μg/mL x h after 150 mg and 300 mg triamterene orally, respectively, and correspondingly, 4.2 and 3.7% of the doses were excreted as unchanged drug. The principal metabolite of triamterene was found to be p-hydroxytriamterene sulfate conjugate. The area under the curve of this metabolite amounted to 6.672 and 11.941 μg/mL x h after the triamterene doses of 150 and 300 mg, respectively. The urinary excretion of the metabolite varied

between 25.0 and 17.5% of the dose after either dose.[58] Following a single oral dose of 100 mg to six normal male volunteers, the plasma concentration time course of the sulfate conjugate paralleled that of triamterene in all subjects, but concentrations of the metabolite were more than 10 times higher than unchanged triamterene concentrations at identical sampling times.[61] Interestingly, the renal clearance of the sulfate conjugate was less than that of triamterene. These characteristic features of triamterene disposition were fitted to a compartment model incorporating a first-pass metabolic process. Unbound fractions of triamterene and metabolite in plasma were 0.39 and 0.10 (mean of six subjects), respectively. The low unbound fraction of the metabolite in plasma most probably accounts for the low renal clearance of the sulfate conjugate as compared with triamterene.

Triamterene clearance was markedly decreased (92% reduction) in seven patients with alcoholic cirrhosis and ascites.[64] This resulted in prolongation of its natriuretic effect, which lasted for up to 48 hours in cirrhotic patients compared with 8 hours in healthy controls. The pharmacokinetics of triamterene has also been investigated in geriatric patients comparing the data with the corresponding values of young healthy volunteers; peak concentration of triamterene was found to be markedly higher in the geriatric patients.[67]

A chronic-dosing pharmacokinetic study has been carried out in six healthy subjects to examine the potential for cimetidine to reduce the renal and hepatic clearance of triamterene.[66] Blood and urine samples were collected frequently for 24 hours after dosing with triamterene alone (100 mg/day) for 4 days and concomitant cimetidine (400 mg twice daily) for an additional 4 days. Cimetidine reduced the clearance of triamterene by hydroxylation by 32%, renal clearance of triamterene by 28%, with no change in its protein binding. The renal clearance of the active sulfate conjugate was not altered by cimetidine. There was a reduced recovery of triamterene and its metabolites in urine after cimetidine,

suggesting decreased absorption. These results were consistent with cimetidine inhibiting cytochrome P450 enzymes in the liver and also competing with triamterene for renal tubular secretion. Despite the pharmacokinetic interaction, cimetidine caused minimal alteration to the natriuretic and antikaliuretic effects of triamterene.

Pharmacokinetic studies of triamterene in combination with other drugs have also been done. In human subjects pharmacokinetics of triamterene with xipamide,[68] with hydrochlorothiazide,[69] with propranolol and hydrochlorothiazide combination,[70] and with oxprenolol and hydrochlorothiazide combination,[71] have been studied. Pharmacokinetic and pharmacodynamic studies of the combination of furosemide retard and triamterene have been done in detail in healthy volunteers.[72-75] In another study triamterene is reported to reduce the extrarenal elimination of digoxin, but induced no changes in digoxin-elicited inotropy.[76]

Pharmacokinetics[77] and pharmacodynamics[78] of acidic and basic triamterene analogs have also been investigaed.

7. Bioavailability

Bioavailability of triamterene has been studied in healthy human subjects.[59,62,65,79] The bioavailability of triamterene was found to be 52%, corresponding to absorption of 83%, in a study done in six volunteers after intravenous administration of the drug.[62] Another study reported that triamterene bioavailability from capsules was poorer and more variable than that from suspension.[65] Effect of various vehicles and vehicle volumes on oral bioavailability of triamterene has been investigated.[80] Addition of a combination of a surfactant and carbonate or bicarbonate as diluents to a triamterene gelatin capsule is reported to result in better bioavailability of the drug.[81] When both calcium carbonate and cetylpyridinium chloride were present in a triamterene capsule the dissolution rates were greatly increased over triamterene capsules containing neither diluent or either

diluent alone. It has been reported that food intake decreases the bioavailability of triamterene.[82] Certain aldehyde adducts of triamterene are reported to increase the bioavailability of the drug.[13]

Bioavailability studies on triamterene in combination with hydrochlorothiazide have been carried out.[83-88] Studies have also been done on the bioavailability of triamterene and bemetizide,[89] and triamterene and furosemide retard[90] combinations.

8. Protein Binding

Triamterene is about 45 to 70% bound to plasma proteins.[2,91] Influence of disease on binding of triamterene to plasma proteins has been studied.[92] Plasma from uremic patients had a decreased binding of triamterene. Hypoproteinemia and hypoalbuminemia decreased the binding fraction. Drug binding was impaired by cirrhosis also.[92]

9. Toxicity

Triamterene has been reported to cause photosensitivity reactions, increase in uric acid concentration, and blood dyscrasias.[91] Nephrolithiasis may occur in susceptible patients. Megaloblastic anemia has been reported in patients with depleted folic acid stores such as those with hepatic cirrhosis. In a study conducted on rats, daily treatment of the animals with doses of 1.5, 3 and 4.5 mg/100g over the period of three weeks caused severe degenerative changes of renal cortical and medullary tubules resembling osmotic nephrosis.[93] Reversible acute renal failure from combined triamterene and indomethacin in healthy subjects is reported.[94] It is recommended that this potentially nephrotoxic association be avoided.

In a mutagenic and testicular toxicity study[95] in mice triamterene had no adverse effects on mating or fertility and did not induce dominant lethal mutations in germ cells of male mice when

given for five days at 5-100 mg/kg/day. Oral exposure to mice under identical conditions had no effect on testicular weight, DNA content, or activity of the de novo pathway for thymidine synthesis from deoxy[6-^{3}H]uridine. A prospective toxicity evaluation study done on forty chemicals using computer-optimized molecular parametric analysis of chemical toxicity (COMPACT) procedure predicted toxicity/carcinogenicity in ten chemicals which included triamterene also.[96]

10. Methods of Analysis

10.1 Elemental Composition

The elemental composition[18] of triamterene is as follows:

Element	Per cent
C	56.91
H	4.38
N	38.72

10.2 Identification Color Tests

A 0.1% w/v solution of triamterene in anhydrous formic acid, when examined under ultraviolet light (365 nm), exhibits an intense blue fluorescence.[1] Solutions in other acids also exhibit a blue fluorescence. When 2 or 3 drops of Liebermann's reagent are added to the sample on a white tile a red-orange color is produced.[2] When a drop of a mixture of 1 volume of formaldehyde solution and 9 volumes of sulfuric acid is added to the sample on a white tile a yellow color is produced.[2]

10.3 Titrimetric Analysis

Triamterene is assayed by non-aqueous titration.[97] The method used is as follows. Transfer about 0.5 g of triamterene, accurately weighed, to a 400 mL beaker, and dissolve in 250 mL of a solvent previously prepared by mixing, in the order named and with cooling prior to use, 1 volume of formic acid, 1 volume of acetic anhydride, and 2 volumes of

glacial acetic acid. Titrate with 0.1N perchloric acid, determining the end point potentiometrically. Perform blank determination, and make any necessary correction. Each mL of 0.1N perchloric acid is equivalent to 25.33 mg of $C_{12}H_{11}N_7$.

10.4 Polarography

In a polarographic study of pteridines the half-wave reduction potentials of triamterene, designated as $E^1_{1/2}$ and $E^2_{1/2}$, were found to be 0.625 and 1.075, respectively.[98] Triamterene may be determined potentiometrically in a dilute acetic acid solution using a mercury electrode.[99] The method is reported to have a sensitivity of 1 μg per mL of biological fluids.

10.5 Voltammetry

A voltammetric method for determination of pyrimidine derivatives has been developed based on adsorption of nitrogen-containing heterocycles on mercury electrodes.[100] The detection limit of the method was 10^{-2}M with the adsorption rate of 10^7L/mol. It is reported[101] that the detection limit of some organic compounds, such as triamterene, by cathodic stripping differential pulsed voltammetry with adsorption preconcentration was improved by an order of magnitude by using digital data processing for background current compensation. Detection limits of $5x10^{-12}$M were observed.

10.6 Spectrophotometric Analysis

10.6.1 Fluorometric

Triamterene can be estimated by fluorometric method using excitation wavelength 370 nm and registration wavelength 440 nm.[102] The method can be used for its estimation in blood,[103] in serum,[104] and in pharmaceutical preparations.[105-107]

10.6.2 Ultraviolet

Dual wavelength ultraviolet spectrophotometric methods have been described for the determination of triamterene in combination with hydrochlorothiazide,[108] and with benzthiazide[109] in tablets. Difference spectrophotometry, a technique in which no prior separation of the drug from other materials is required and which provides for automatic cancellation of interferences while retaining the inherent convenience of direct reading, has been described.[110]

10.6.3 Infrared

Infrared spectroscopy has been employed in the study of different crystal forms of triamterene obtained from different solvents.[3]

10.7 Mass Spectrometry

Selected ion monitoring (SIM) and fast atom bombardment (FAB) techniques of mass spectrometry have been used for detection and quantification of different crystalline forms of triamterene.[3] The use of electron impact mass spectrometry in the analysis of diuretic agents has been described.[111]

10.8 Chromatographic Analysis

10.8.1 Paper Chromatography

Paper chromatography has been employed in the separation of ^{14}C-labelled triamterene and its metabolites.[42]

10.8.2 Thin-Layer Chromatography

The following thin-layer chromatography (TLC) systems have been used for the identification of triamterene.

Solvent System	Plate	Reference
Methanol-Strong ammonia solution (100:1.5)	Silica gel G,250 μm thick, dipped in, or sprayed with,0.1M KOH in methanol, and dried	2
Cyclohexane-Toluene-Diethylamine(75:15:10)	Same plate as above	2
Chloroform-Methanol (90:10)	Same plate as above	2
Ethyl acetate-Methanol-25% Ammonia (80:10:10)	Silica gel G, containing fluorescence indicator	112
Ethyl methyl ketone-Methanol-Ammonia (80:10:10)	Silufol	113
n-Butanol-Methanol-Chloroform-25% Ammonia (50:15:15:15)	Silica gel	62

The location of spot can be done under ultraviolet light when a blue fluorescence is produced.[2] Triamterene and its principal metabolites have been determined simultaneously in biological materials by TLC followed by fluorometric evaluation of the plates.[44,62]

The techniques of normal and reversed-phase thin-layer chromatography,[114,115] and high performance thin-layer chromatography (HPTLC)[116] have also been described.

10.8.3 Gas Chromatography

Gas chromatography[117] and gas-chromatography-mass spectrometry[118,199] have

been described.

10.8.4 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) has been most extensively used in the analyis of triamterene and its metabolites in plasma,[64,120-126] and urine[120,122,124-128]. In one of the techniques for determination of triamterene in human plasma and urine the drug was extracted from the sample with ethyl acetate and chromatographed in reversed phase on μBondapak C_{18} column using 45% methanol in aqueous 0.1% w/v potassium dihydrogen phosphate (pH 3.8) as mobile phase at a flow rate of 2 mL/min.[122] Coefficients of variations of peak area ratios were 11,3 and 2% for 1,20 and 40 ng triamterene/mL plasma and 3,2 and 2% for 100, 300 and 800 ng triamterene/mL urine. Recoveries were 69.9-90.3 and 79.4-88.3% for plasma and urine measurements, respectively. A rapid and highly sensitive HPLC assay for triamterene, hydroxytriamterene and hydroxytriamterene sulfate has been reported.[125] Plasma samples were processed by protein precipitation, while urine was used untreated. Three different solvent systems were used to analyse (a) triamterene in plasma (3% acetonitrile, pH 4.0; internal standard furosemide; sensitivity limit: 1 ng/mL); (b) hydroxy-triamterne and hydroxytriamterene sulfate in plasma (12% acetonitrile, pH 5.5; internal standard cefamendole; sensitivity limits: 20 and 2 ng/mL, respectively); and (c) triamterene, hydroxy-triamterene and hydroxytriamterene sulfate in urine (13% acetonitrile, pH 5.3; internal standard hydroflumethiazide; sensitivity limits: 0.04 μg/mL, 0.5 μg/mL and 0.1 μg/mL, respectively). Fluorescence detection of compounds was performed at 365 nm excitation and 440 nm emission wavelengths.[125] Recovery of triamterene and its metabolites from plasma was complete, and the calibration curves were linear. Various other conditions of HPLC employed for analysis of triamterene and its metabolites in biological fluids are given below.

Column	Mobile Phase	Ref.
LiChrosorb Si 60 (5 μm)	Dichloromethane-Hexane-Methanol -70% Perchloric acid (57:35:8:1); 2 ml/min flow rate	120
μBondapak C_{18}	Acetonitrile-Water-Acetic acid (60:39.5:0.5); 1 mL/min flow rate	123
Octadecyl-silane C_{18}	Acetonitrile-Phosphate buffer	124
Nova-Pak C_{18}	Buffered acetonitrile-Methanol	126

Recently, a HPLC method using a 0.05 mol dm^{-3} sodium dodecyl sulfate-3% propanol mobile phase and a C_{18} column has been described.[129]

Methods for analysis of triamterene in combination with other drugs using HPLC technique have also been reported.[68,85,130-133] Using a mobile phase of methanol-acetonitrile-tetrahydrofuran-water (1:1:1:7) and Zorbax TM-CN column separation of triamterene, bemetizide and bupranolol has been achieved in approximately six minutes with isocratic elution.[130] Tablets containing triamterene and hydrochlorothiazide have been analysed by extracting with hot methoxyethanol and chromatographing on a column of LiChrosorb RP-2 with tetrahydrofuran-isopropyl alcohol-water (31:5.5:63.5) adjusted to pH 3.8 with phosphoric acid, at 2 mL/min and detection at 266 nm.[131] Impurities like 2,7-diamino-4-hydroxy-6-phenylpteridine and 2,4-diamino-7-hydroxy-6-phenylpteridine from triamterene, and 5-chloro-2,4-disulfamoylaniline from hydrochlorothiazide could also be detected.[131] The application of thermospray and plasmaspray high-performance liquid chromatography-mass spectrometry to the analysis of diuretics and probenecid has been investigated.[133]

11. References

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