STUDY OF BIOLOGICALLY ACTIVE COMPOUNDS BY HPLC.

A THESIS

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BY

CHITRALEKHA B. JADHAV

DIVISION OF ORGANIC CHEMISTRY
NATIONAL CHEMICAL LABORATORY
PUNE - 411 008 (INDIA)

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CERTIFICATE

Certified that the work incorporated in the thesis "STUDY OF BIOLOGICALLY ACTIVE COMPOUNDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY" submitted by Miss C. B. Jadhav, was carried out by the candidate under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

V H Deshpande

(V. H. DESHPANDE)
(Research Guide)

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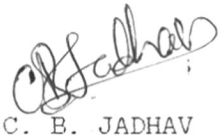


Ms. C. B. JADHAV

NCL, Pune

February 1991.

ABBREVIATION	
TLC	Thin layer chromatography
GC	Gas chromatography
LC	Liquid chromatography
HPLC	High performance liquid chromatography
$CDCl_3$	Deuterated chloroform
M.P.	Melting point
s	Singlet
d	Doublet
t	Triplet
q	Quartet
m	Multiplet

ABSTRACT

This research work is about the High Performance Liquid Chromatography (HPLC) of the drugs, Carbamazepine (Tegretol,[R] Ciba-Geigy) and ambroxol, and their respective synthetic intermediates and preparation of some analogues of ambroxol. The thesis is divided into three chapters.

Chapter I:

This chapter presents a brief introductory overview of liquid chromatography with special reference to high performance liquid chromatography.

Chapter II:

A simultaneous, isocratic, near-baseline HPLC separation has been reported for carbamazepine and its synthetic intermediates for the first time. This was achieved using bonded octadecylsilane column and simple and inexpensive mobile phase [methanol-buffer (pH 6.9)].

Chapter III:

Section A: In this section, some synthetic analogues of ambroxol have been prepared starting from 2-amino-3,5-dibromo-benzaldehyde and amine derivatives.

Section B: This section describes a simultaneous, isocratic, baseline HPLC separation of ambroxol and its synthetic intermediates for the first time on a bonded octadecylsilane column using acetonitrile-buffer (pH 6.9) 55:45 as the mobile phase.

I N D E X

CHAPTER-I

BRIEF OVERVIEW TO HPLC

Chromatography, in its many forms has become a widely practiced, versatile, separation and analytical technique. The word 'Chromatography', derived from the Greek 'Chroma' meaning colour and 'graphein' meaning to 'to write', was coined by Tswett[1] when he first separated plant pigments in 1903.

The basic principle involved in the separation of components of a mixture by this technique is the selective distribution between two phases, namely the stationary and mobile phases.

We have used high-performance liquid chromatography (HPLC) for the separations in the present work. Therefore, a brief overview of liquid chromatography with special reference to HPLC is presented here.

Liquid chromatography has become the most powerful separation tool in chemistry. It has gained prominence over gas chromatography(GC), because an overwhelming majority of organic compounds are not sufficiently volatile or thermally stable, and hence can not be analyzed by GC without prior modification.

HPLC applications span such wide and diverse areas as biotechnology, biochemistry, diagnostic medicine, drugs and pharmaceuticals, environmental chemistry, explosives and propellants, foods and beverages, fossil fuels, pesticides etc.

Liquid chromatography modes

1) Partition or Bonded phase chromatography :

In this mode, chemically bonded stationary phases prepared from silica by reacting the surface silanol groups with an organochlorosilane or alkoxysilane are used. These stationary phases have Si-O-Si-R (R = C_8, C_{18} or R = CN, R = NH_2) linkages which confer hydrolytic stability. This partition chromatography can be carried out in normal phase or reversed-phase mode depending on the polarity of the mobile phase.

2) Liquid - Solid (Adsorption) chromatography (LSC):

This involves solid particles with active sites as stationary phases, such as silica, alumina, activated carbon and chemically bonded polar reversed-phase packings e.g. $-NH_2$, $-CH_2OH$ or -CN. Solvent molecules in the mobile phase compete with the solute molecules for sites on the adsorbent. An equilibrium exists between the adsorbent, solute, and the solvent as shown below

If an adsorbent possesses a polar surface e.g. silica or alumina, non-polar groups will not be retained while components with polar groups will be strongly adsorbed. In the case of non-polar adsorbent such as charcoal, polar molecules will be less strongly retained (Fig. 1.1).

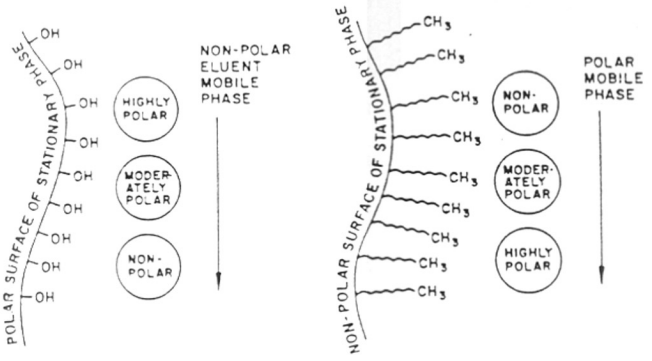


Fig.1.1. Graphical illustration of normal and reversed phase liquid chromatography.

3) Ion pair chromatography (IPC):

IPC is used for the separation of ionic or ionizable compounds such as quaternary ammonium salts, sulfonates, amino acids and amino phenols. IPC may operate either by the partition or the ion-exchange mode. In the partition mode ionic molecules having insufficient lipophilic characters form an ion-pair with a suitable counter ion present in the mobile phase. The resulting increase in the lipophilic character of the sample, makes the sample develop affinity for the stationary phase. In the ion-exchange mode, the polar counter ion is sorbed by the hydrocarbonaceous stationary phase, creating on ion exchange site. A polar sample molecule is electrostatically attracted to this site. IPC can be applied to the separation of samples containing both ionic as well as non-ionic components by a suitable choice of pH.

4) Ion-Exchange chromatography (IEC):

Ionic compounds are often best separated by ion-exchange chromatography with the help of ion-exchangers as the stationary phase.

Charged stationary phases with the counter ions are available to exchange with solute ions of the same charge in the mobile phase.

The ion exchange can be represented as follows

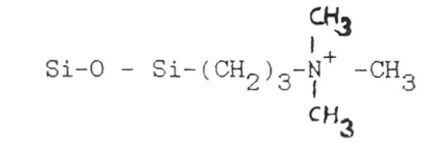
$R^+ Y^- + X^- \rightleftharpoons R^+ X^- + Y^-$ Anion exchange

$R^- Y^+ + X^+ \rightleftharpoons R^- X^+ + Y^+$ Cation exchange

Anion exchangers contain basic groups, e.g. $-NR_3$ (strong) or NH_2 (weak).

Cation exchangers contain acidic groups, e.g. SO_3 (strong) or COO^- (weak).

These ion-exchange resins are covalently bonded, via their functional groups, to silica matrix as shown below.



IEC requires a careful control of ionic strength and pH of mobile phase.

5. Size Exclusion Chromatography (SEC or GPC):

It is also known as gel-permeation chromatography. This technique separates substances according to their molecular size and shape. Molecules are eluted in order of decreasing molecular size. Compounds with similar molecular size are not resolved.

The distinguishing and salient features of HPLC in comparison to classical LC are summarized as follows:

	Classical LC	HPLC
1.	Packing	
	Particle size: 60-120 mesh	Typically 3, 5 or 10 μm
	Size distribution (125-250 μm) wide	Narrow (e.g. 95% particle population between 3.6 to 5.8 μm for a nominally 5 um designated packing).

2.	Sample application	
	Dependent and time consuming	Precise (syringe or injection valves)
3.	Effluent flow	
	By gravity	Precisely controlled by metering pumps.
4.	Detection	
	Off-line (usually by thin layer chromatography)	On-line
5.	Time	
	Analysis requires several hours.	Typically 10-15 min.

A typical liquid chromatograph consists of the following modules (Fig. 1.2):

1) A solvent delivery system.

2) A valve injector to introduce sample onto the column head.

3) A column packed with microparticles (5 or 10 μm) of a suitable packing.

4) A detector to detect eluted components. (Ultraviolet and refractive index detectors are the most common).

5) A recorder to record the chromatogram.

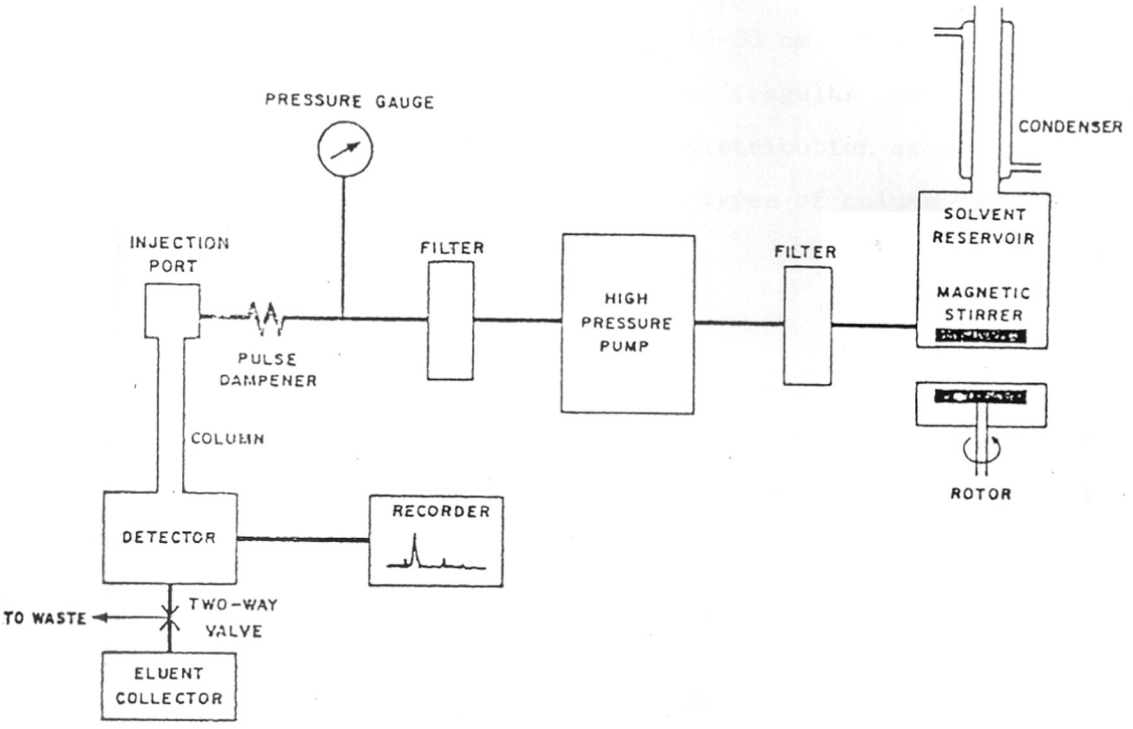


Fig.1.2. Schematic diagram of a typical HPLC.

Column and column packings

The column is the heart of any high-performance liquid chromatograph while the detector system represents the eyes.

The HPLC columns generally used in practice have an internal diameter in the range 2-5 mm and are 10-30 cm long. Packings with 5 or 10 μ average particle diameter (regular or irregular shape) and fairly narrow particle size distribution are commonly used to pack HPLC columns. The various types of column packings used in HPLC are shown in Fig. 1.3.

Detectors:

The detectors used in HPLC are of two very broad types.

(1) Specific property detectors: These measure a specific property of the solute, which is either not possessed or possessed to a much smaller degree by the mobile phase,e.g. ultraviolet absorption (UV). UV detector is used for substances which absorb UV radiation, e.g. olefins, aromatics and compounds containing functional groups like C=O, C=S, N=O etc. Fig. No.1.4 shows a schematic diagram of a dual beam UV absorbance detector with the taper cell.

UV detector is relatively less sensitive to temperature and flow changes unlike the R.I. detector.

(2) Bulk property detector: It measures difference of a bulk property of the solute and mobile phase, e.g. Refractive Index (RI). Fig. 1.5 shows schematic diagram of ultraviolet and refractive index are most common.

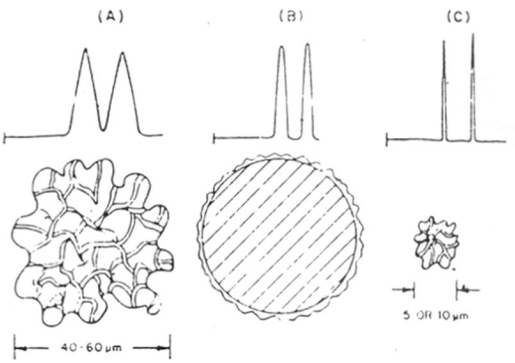


Fig.1.3 Various types of column packings used in liquid chromatography: (a) body porous, (b) pellicular, (c) body porous microparticle. The chromatogram above the particles visualize the peak broadening.

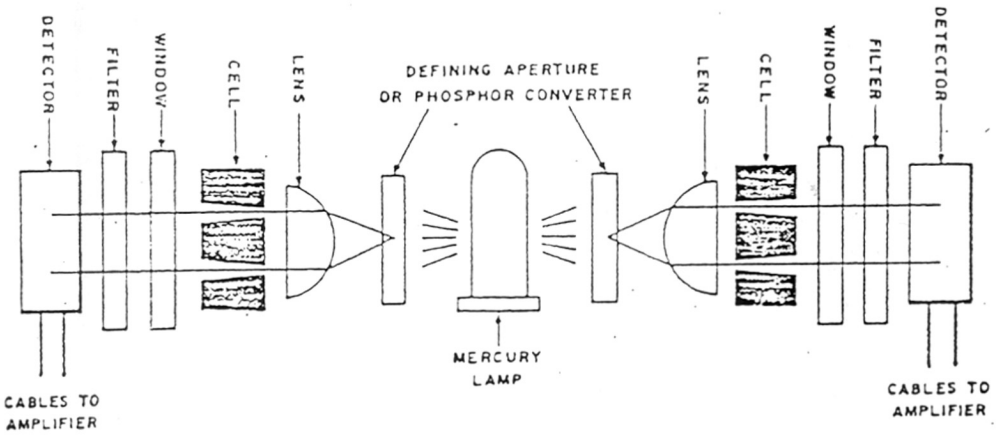


Fig.1.4. Ultraviolet absorbance detector.

Chromatographic theory :

Although a detail discussion of chromatographic theory can be found in HPLC text books,[2-4] it is pertinent to give an account of some of the basic elements of chromatographic theory useful for a practicing chromatographer.

Resolution (R_s): It is a quantitative description of the degree of separation obtained between two peaks.

Synder[5] gives an account of how much resolution is needed for qualitative and quantitative work.

$$R_s = \frac{t_2 - t_1}{1/2\ (W_1 + W_2)}$$

where t_1 and t_2 = Retention times of peaks 1 and 2 (min), W_1 and W_2 = Peak widths of peak 1 and 2 at the base (min).

For high resolution there must be large difference in retention time and peaks must be narrower as shown in Fig. 1.6.

Capacity factor (k')

$$k' = \frac{t_1 - t_0}{t_o} \quad \frac{v_1 - v_0}{v_o} \qquad \text{Eqn. 1}$$

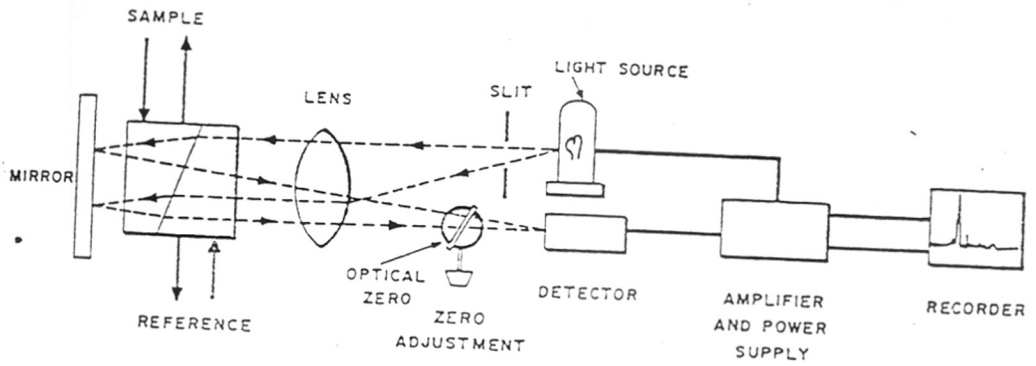


Fig.1.5 Schematic diagram of a refractometer.

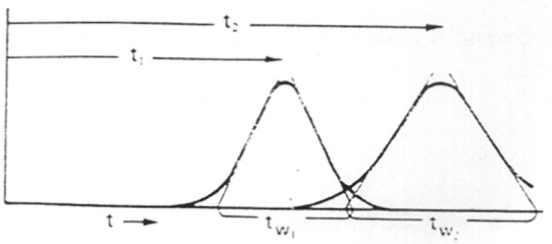


Fig.1.6. Resolution in LC.

V_o and t_o are elution volume and elution time, respectively, of an unretained component.

The term t_o can be defined as:

$$t_o = \frac{L}{u} \qquad \text{Eqn. 2}$$

where L is the length of column (in cm) and u = linear velocity of the mobile phase (in cm/sec).

Rearranging Eqn. 1, we get:

$$t_1 = t_o\ (1+k') \text{ ---------- Eqn. 3}$$

Substituting for t_o in Eqn. 3 from Eqn. 2:

$$t_1 = \frac{L}{u\ (1 + k')}$$

Selectivity factor (α):

$$\alpha = \frac{k'_2}{k'_1}$$

This is the most important term in optimizing the resolution. A small increase in α will improve the resolution considerably.

Efficiency (N):

It is a quantitative measure of how a peak broadens with time.

$$N = \frac{16\ (\ t_R\ )^2}{W}$$

where t_R is the uncorrected retention time and W is the peak width at the base line measured in units of time.

Height equivalent of theoretical plates (HETP):

$$HETP = \frac{L}{N}$$

where HETP is a measure of column efficiency per unit length, L is the length of the column and N is the efficiency of the column.

There are four processes leading to molecular spreading:

1. Eddy diffusion
2. Mobile phase mass transfer
3. Stagnant mobile phase mass transfer
4. Stationary phase mass transfer.

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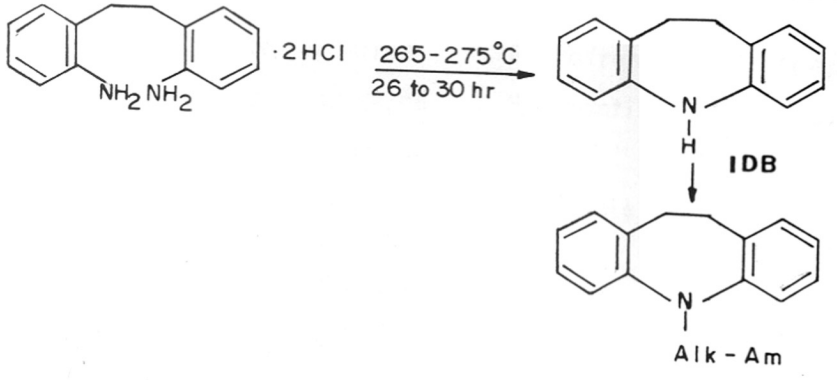
CHAPTER-II

SIMULTANEOUS REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CARBAMAZEPINE AND ITS SYNTHETIC INTERMEDIATES

Introduction:

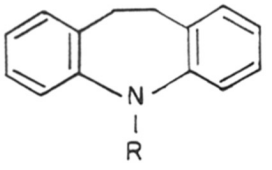
Carbamazepine[1] (Tegretol, Ciba-Geigy), 5H-dibenz[b,f]azepine -5-carboxamide, is widely used in the treatment of convulsive disorders and trigeminal neuralgia.

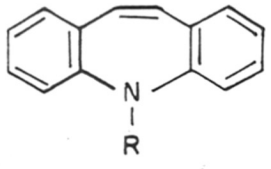
The 5H-dibenz[b,f]azepine[2] nucleus was first reported in 1899 by Thiele and Holzinger. Thermal cyclization of o,o´-diaminobibenzyl hydrochloride gave rise to 10,11-dihydrodibenz-[b,f]azepine (IDB) also known as iminobibenzyl. In a 1951 patent, Haeflinger and Schindler[3] reported a series of aminoalkyl iminobibenzyls.



Although this class of compounds was initially patented for anti-allergic activity, it was subsequently found to possess significant antidepressant activity as well. The initial patent and several that soon followed, prompted an extensive investigation of related compounds. Some of the major dibenz[b,f]azepines that have grown out of these studies are shown in Table 2.1.

TABLE - 2·1

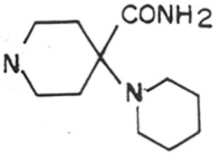




(i) R = $-(CH_2)_3\ NMe_2$

(ii) R = $-(CH_2)_3\ NHMe$

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

(iv) R = $-(CH_2)_3-$ 

(v) R = $-(CH_2)_3-$  $-(CH_2)_2-OH$

(vi) R = $-CONH_2$

Compound	Name	Trade Name	Activity
(i)	Imipramine	Tofranil	Antidepressant
(ii)	Desipramine	Pertofrane	Antidepressant
(iii)	Trimepramine	Surmontil	Anidepressant
(iv)	Carpipramine		Psychotropic
(v)	Opipramol	Ensidone	Anidepressant and Antipsychotic
(vi)	Carbamazepine	Tegretol	Analgesic and Anticonvalsant

The chemistry of 5H-dibenz[b,f]azepine has been comprehensively reviewed by Renfroe et al.[4] in 1984. Several synthetic methods have been developed for the preparation of carbamazepine[5-18] (SCHEME-1).

Although a process for the preparation of carbamazepine (as outlined in SCHEME-2) was developed in this laboratory, it was necessary to develop a simple and reliable method to monitor the various reaction steps.

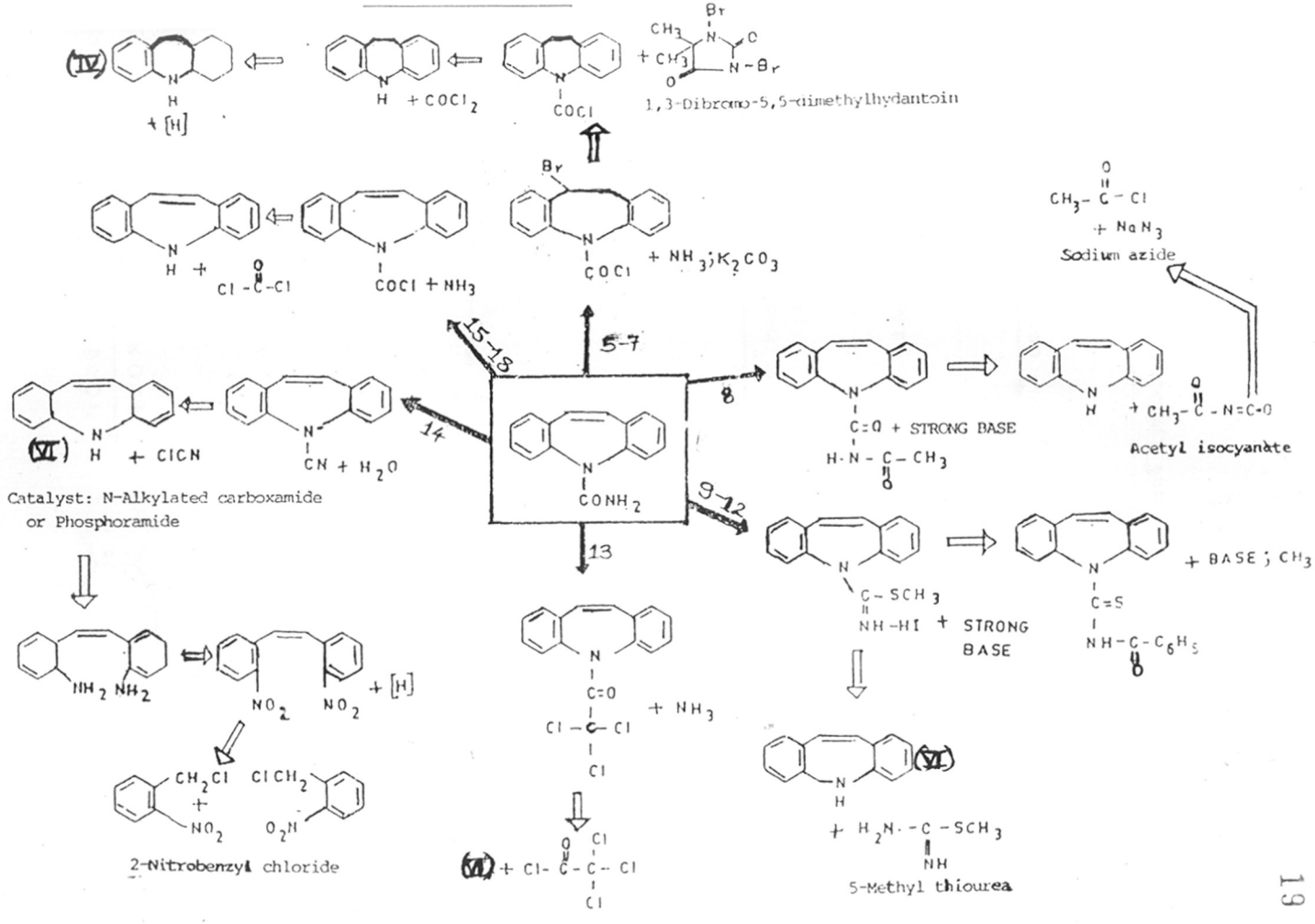
Thin layer chromatography (TLC) has been used to monitor related substances in carbamazepine drug raw material and tablet formulations.[19,20] However, it was found that TLC was inadequate for some of the steps of our synthetic scheme.

The major disadvantage of gas chromatographic (GC) method is the thermal lability of carbamazepine at temperatures required to volatilize it, which may give rise to iminostilbene, 9-methyl acridine and other decomposition products[21,22].

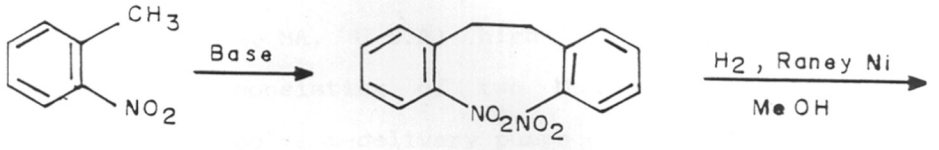
High performance liquid chromatography (HPLC) of carbamazepine using a silica,[23] an octadecylsilane[24,25] and a diol[26] has been reported. However, simultaneous separation of carbamazepine and its intermediates mentioned in our Scheme 2 (I-VII) has not been reported so far.

A simultaneous separation of carbamazepine and its synthetic intermediates on a bonded octadecyl silane using a simple and inexpensive mobile phase was undertaken.

SCHEME - I

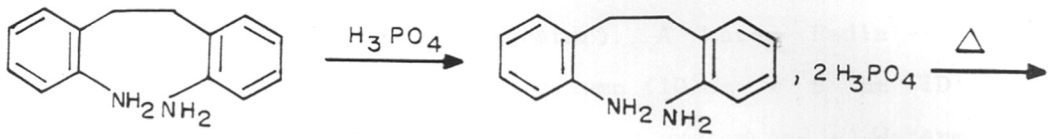

(IV)
+ [H]
+ COCl2
COCl
1,3-Dibromo-5,5-dimethylhydantoin
+ NH3;K2CO3
+ NaN3
Sodium azide
15-18
5-7
8
14
9-12
13
(VI)
+ ClCN
CN + H2O
Catalyst: N-Alkylated carboxamide
or Phosphoramide
CONH2
C=O + STRONG BASE
Acetyl isocyanate
+ BASE ; CH3
STRONG BASE
+ [H]
2-Nitrobenzyl chloride
+ NH3
+ Cl-C-Cl
5-Methyl thiourea

SCHEME - 2

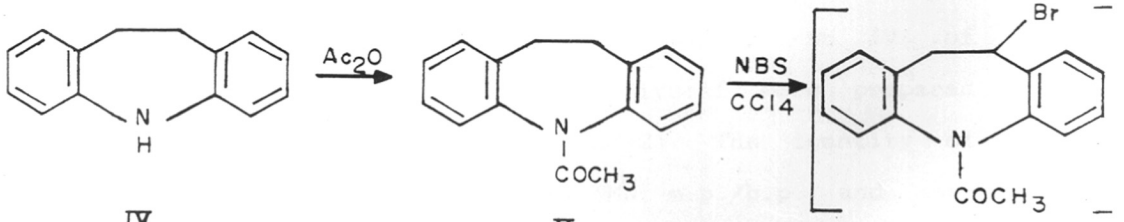


I
O - Nitrotoluene (ONT)

II
O,O'- Dinitrodibenzyl (DNDB)

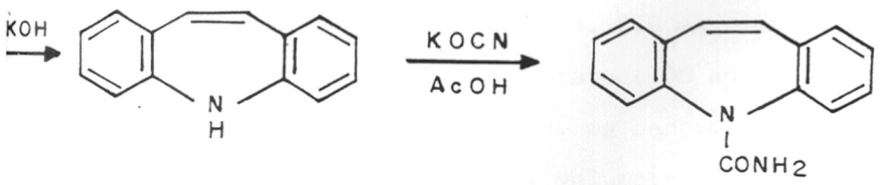


III
O,O'- Diaminodibenzyl
(DADB)



IV
Iminodibenzyl
IDB

V
Iminodibenzyl acetate
(IDB acetate)



VI
Iminostilbene(ISB)

VII
Carbamazepine (CBZ)

EXPERIMENTAL

Liquid Chromatograph:

The method was developed on a Millipore-Waters Chromatography Division (Milford, MA, U.S.A) high-performance liquid chromatographic system consisting of two Model 510 series dual-head reciprocating solvent-delivery pumps controlled by a Model UGK universal injector and a Model 481 Lambda-Max LC spectrophotometer operating at 280 nm. The analogue output of the detector was recorded and processed with a Waters Model 730 data module (printer, plotter and integrator). A Water Radial-PAK Bondapak phenyl (10 μm) polythene column (10 cm x 8 mm ID) mounted inside a Z-module radial compression system and a Waters Novapak C_{18} (4 μm) stainless steel column (15 cm x 3.9 mm ID) were used.

Authentic samples: The synthetic intermediates (**I** to **IV**) of carbamazepine and the carbamazepine (**VII**) itself were prepared and purified in our laboratory (SCHEME-2). The identity of individual compounds was confirmed by NMR, m.p./b.p. and mass spectrometry.

Standard solutions: The authentc samples, 2.5 mg o-nitrotoluene (ONT) (**I**), 8.5 mg o,o´-dinitrodibenzyl (DNDB) (**II**), 5.5 mg, o,o´-diaminodibenzyl (DADB) (**III**), 2.0 mg iminodibenzyl (IDB) (IV), 25.0 mg iminodibenzyl acetate (IDB acetate) (**V**), 3.0 mg imino stilbene (ISB) (**VI**) and 2.5 mg carbamazepine (CBZ) (**VII**) were weighed separately in 10 ml volumetric flasks, dissolved and diluted with the mobile phase.

Artificial mixture solution of carbamazepine and its synthetic intermediates were prepared by mixing an equal volume of each of the above standard solutions (I-VII). About 70 µl volume of this artificial mixture was injected into the liquid chromatograph.

Reagents and chemicals: Analytical reagent grade triethylamine, orthophosphoric acid, CH_3COONa, KH_2PO_4 and Na_2HPO_4 were used to prepare the buffer solutions. Methanol was purified to HPLC quality in our laboratory. A Milli Q system (Millipore, Bedford, MA, U. S.A) was used to purify water.

pH 3.1 buffer: A stock buffer solution of triethylammonium phosphate (ca. 0.7 M) was prepared by mixing 10.0 ml triethylamine with about 80 ml water and the pH was adjusted to 2.2 with 85% (w/w) orthophosphoric acid (ca. 8 ml). The solution was cooled to room temperature and diluted to the volume in a 100 ml volumetric flask. A 1 ml volume of this stock solution was diluted to 100 ml with water to obtain the working buffer solution (7 mM; pH 3.1).

pH 5.0 buffer: 4.1 g anhydrous sodium acetate was dissolved and diluted to the volume in a 1000 ml volumetric flask with water. The pH of this solution was adjusted to 5 using a few drops of glacial acetic acid.

pH 6.9 buffer: 3.40 g anhydrous KH_2PO_4 and 3.76 g $Na_2HPO_4,2H_2O$ were dissolved and diluted to the volume in a 1000 ml volumetric flask with water.

Mobile phase: Methanol-buffer [with required pH (60:40)] was used as the mobile phase at a flow-rate of 2.0 ml/min for Radial PAK μ Bondapak phenyl (Z-module) and (65:35), 0.7 ml/min for Novapak C_{18} column.

Column dead time (to): This was determined by injecting 50 μl of methanol-buffer (with required pH) (65:35) into the mobile phase stream. The peak-through combination caused by the change in refractive index was used as a marker and the point of baseline cross was taken as to.

RESULTS AND DISCUSSION

Our aim was to develop a good simultaneous HPLC separation of carbamazepine (**VII**) and its six synthetic intermediates (I-VI) in a reasonable time (ca. 20 min) and using a simple and inexpensive mobile phase.

All the above compounds (I-VII) are weakly basic and have an aromatic ring system. However, these compounds differ in the polarity of functional groups. This prompted us to try a Radial-PAK u Bondapak phenyl column (Z module) first. Initially, the attempts were made to separate the intermediates using methanol in combination with plain water and buffers (pH 3.1, 5.0 and 6.9). Under these conditions, methanol-buffer at pH 3.1 (60:40) system produced the best separation (Fig. 2.1) compared to that of the other pH 5.0 (Fig. 2.2) and 6.9 (Fig. 2.3) systems. However, the separation of **III-VII** and **IV-V** peak pairs was still inadequate. As could be expected from the weakly basic nature of these compounds, pH played only a small role in their separation.

It was then decided to use a more efficient octadecyl column (Novapak C_{18}) to improve the separation and tried similar binary mobile phase combinations as mentioned above. This resulted in a substantial improvement in overall separation as compared to the previous attempt. The best separation for all the peak pairs was achieved using methanol-buffer at pH 6.9 (65:35), (Fig. 2.4). The separation patterns obtained at pH 3.1, 5.0 and plain water are shown in Figs. 2.5, 2.6, 2.7 respectively.

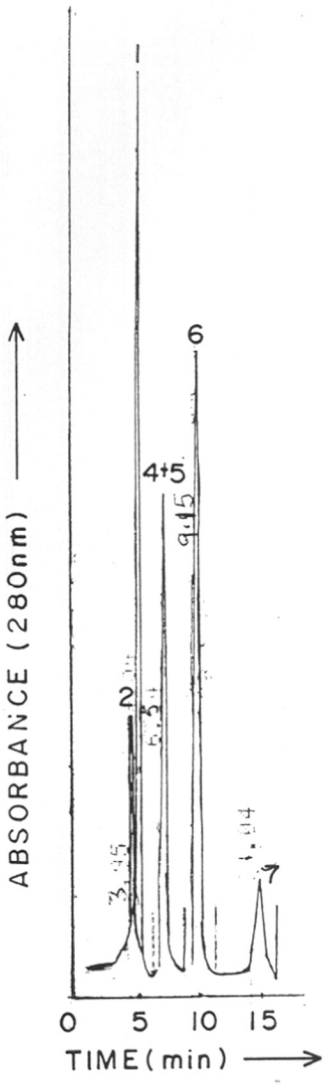


FIG. 2.1

HPLC Separation of an artificial mixture of Carbamazepine and its synthetic intermediates:

Column	: Radial PAK ϰ Bondapak phenyl (Z-module) (Waters) 10 cm x 8 mm i.d.); dp = 10 µm
Mobile phase	: Methanol-buffer [pH 3.1] (60:40)
Flow rate	: 2.0 ml/min.
Chart speed	: 0.2 cm/min.
UV Detection	: 280 nm

Peaks: 1. Carbamazepine (CBZ) (**VII**); 2. o,o'-Diaminodibenzyl (DADB) (**III**); 4. Iminodibenzyl acetate (IDB acetate) (**V**); 5. Iminostilbene (ISB) (**VI**); 6. Iminodibenzyl (IDB) (**IV**); 7. o,o'-Dinitrobenzyl (DNBB) (**II**).

NOTE: O-Nitrotoluene (ONT (**I**) was not included.

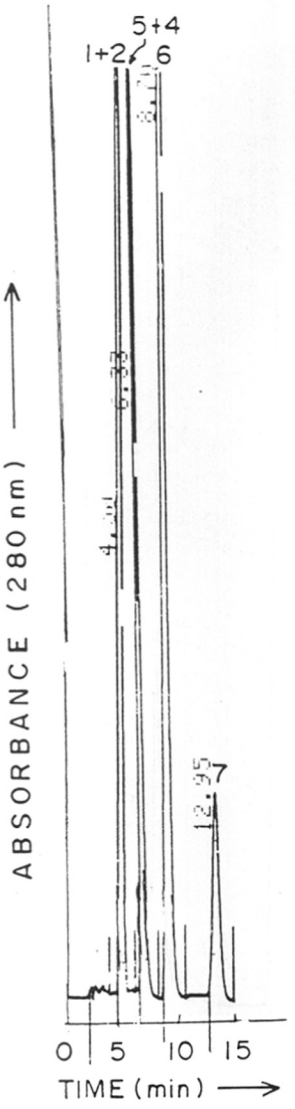


FIG. 2.2

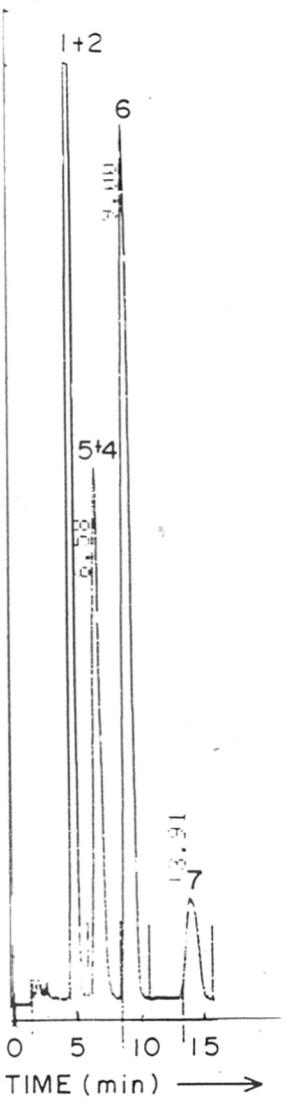


FIG. 2.3

HPLC Separation of an artificial mixture of Carbamazepine and its synthetic intermediates:

Column	: Radial PAK Ⅱ Bondapak phenyl (Z-module) (Waters) 10 cm x 8 mm i.d.); dp = 10 µm
Mobile phase	: Methanol-buffer [**pH 5.0**] (60:40)- **FIG. 2.2** Methanol-buffer [**pH 6.9**] (60:40)- **FIG. 2.3**
Flow rate	: 2.0 ml/min.
Chart speed	: 0.2 cm/min.
UV Detection	: 280 nm

Peaks: 1. Carbamazepine (CBZ) (**VII**); 2. o,o'-Diaminodibenzyl (DADB) (**III**); 4. Iminodibenzyl acetate (IDB acetate) (**V**); 5. Iminostilbene (ISB) (**VI**); 6. Iminodibenzyl (IDB) (**IV**); 7. o,o'-Dinitrobenzyl (DNBB) (**II**).

NOTE: O-Nitrotoluene (ONT) (**I**) was not included.

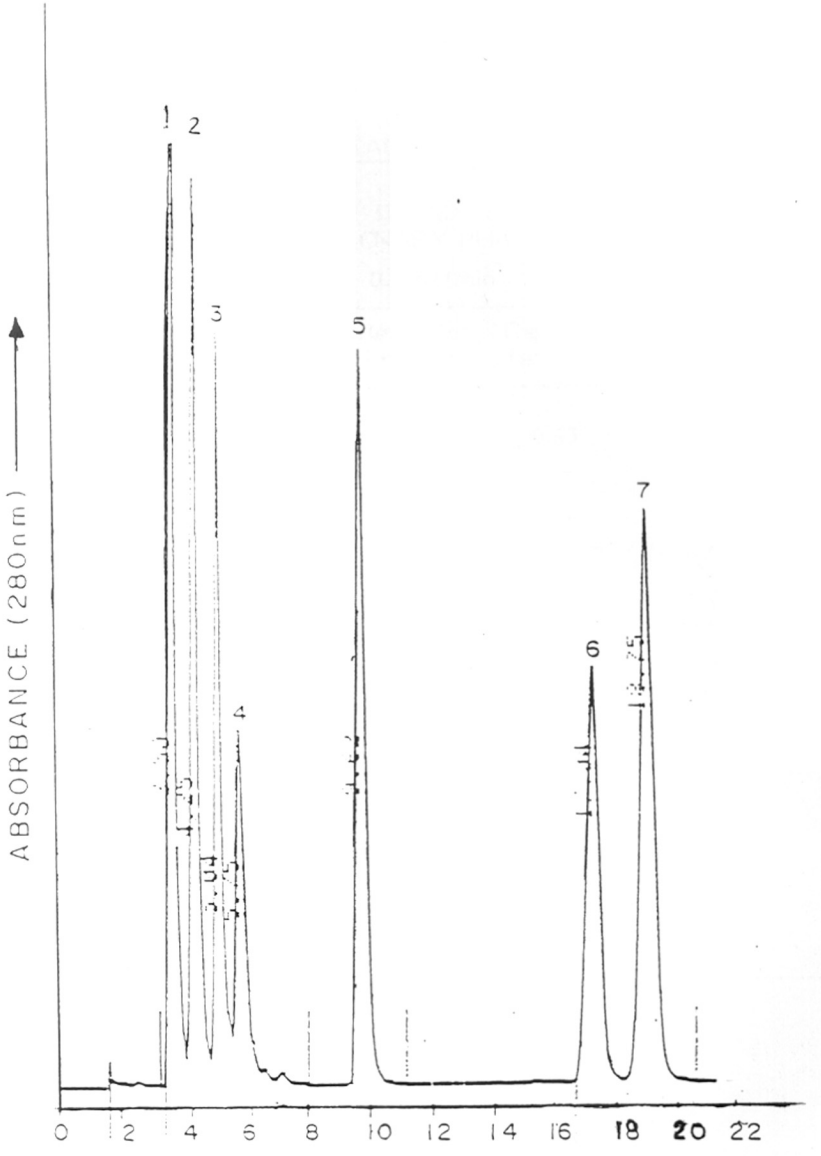


FIG. 2.4

HPLC separation of an artificial mixture of carbamazepine and its synthetic intermediates.

Column: NOVA-PAK C_{18} (Waters) (15 cm x 3.9 mm i.d.); dp = 4 µm.
Mobile phase: Methanol-buffer [pH 6.9] (65:35).
Flow rate: 0.7 ml/min.
Chart speed: .75 cm/min.
UV detection: 280 nm
Peaks: 1. Carbamazepine (CBZ) (VII); 2. o,o'-Diaminodibenzyl (DADB) (III); 3. o-Nitrotoluene (ONT) (I); 4. Iminodibenzylacetate (IDB acetate) (V); 5. Iminostilbene (ISB) (VI); 6. Iminodibenzyl (IDB) (IV); 7. o,o'-Dinitrodibenzyl (DNDB) (II).

TABLE 2.2

HPLC OF CARBAMAZEPINE AND ITS SIX SYNTHETIC INTERMEDIATES ON A NOVA-PAK C_{18} (WATERS) STATIONARY PHASE AT pH 6.9. (FIG. 2.4)

Void volume time, to = 2.15 min at 0.7 ml/min

Compound	max nm	Retention time(min)	Capacity factor, k'	Selectivity factor,
Carbamazepine (CBZ) (VII)	285	3.50	0.63	1.49
O,O'-Diaminodibenzyl (DADB) (III)	-	4.16	0.94	1.42
O-Nitrotoluene (ONT) (I)	-	5.00	1.33	1.26
Iminodibenzyl acetate (IDB acetate) (V)	-	5.75	1.67	2.090
Iminostilbene (ISB) (VI)	262 310 360	9.66	3.49	1.99
O,O'-Dinitrodibenzyl (DNDB) (II)		17.04	6.93	1.10
Iminodibenzyl (IDB) (IV)	205 262	18.58	7.60	

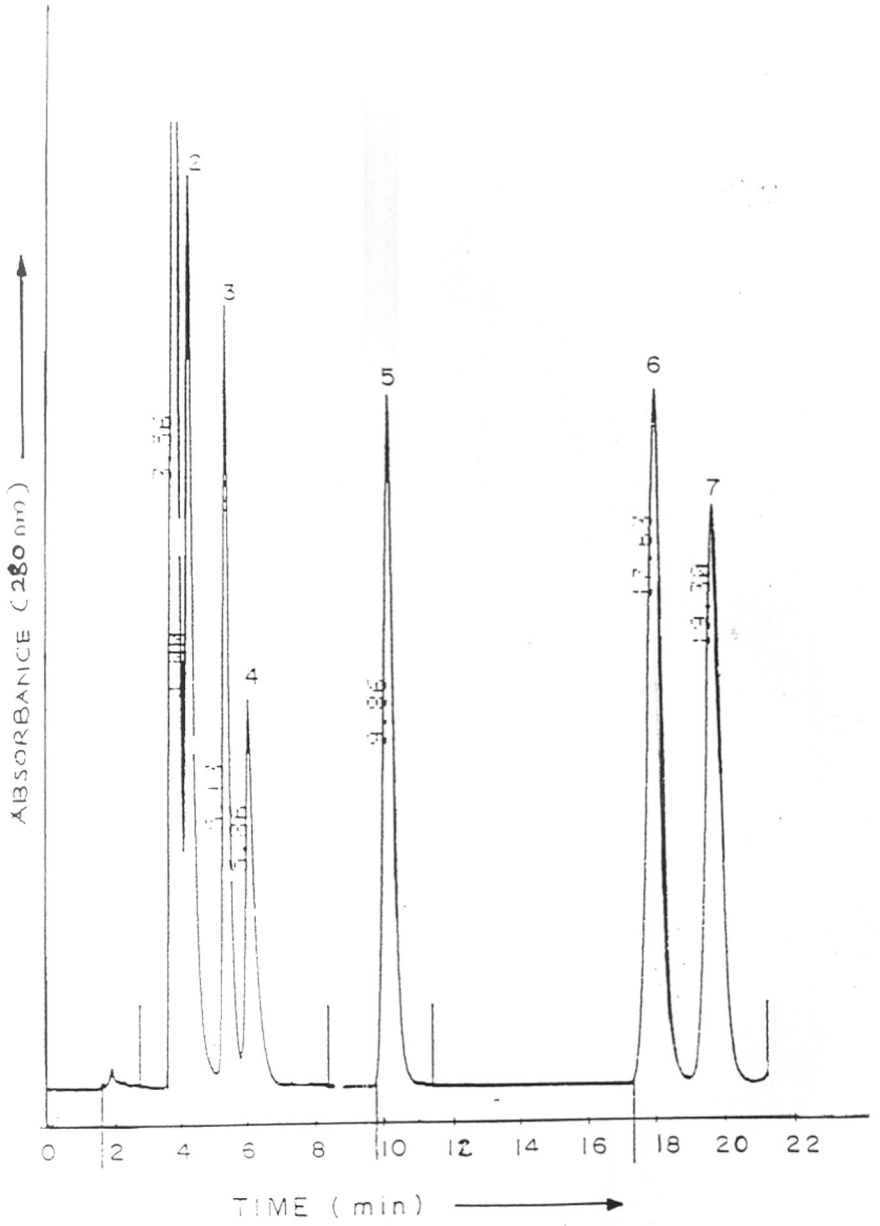


FIG. 2.5

HPLC separation of an artificial mixture of carbamazepine and its synthetic intermediates.

Column: NOVA-PAK C_{18} (Waters) (15 cm x 3.9 mm i.d.); dp = 4 µm.
Mobile phase: Methanol-buffer [pH 3.1] (65:35).
Flow rate: 0.7 ml/min.
Chart speed: .75 cm/min.
UV detection: 280 nm
Peaks: 1. Carbamazepine (CBZ) (**VII**); 2. o,o'-Diaminodibenzyl (DADB) (**III**), 3. o-Nitrotoluene (ONT) (**I**); 4. Iminodibenzylacetate (IDB acetate) (**V**); 5. Iminostilbene (ISB) (**VI**); 6. Iminodibenzyl (IDB) (**IV**); 7. o,o'-Dinitrodibenzyl (DNDB) (**II**).

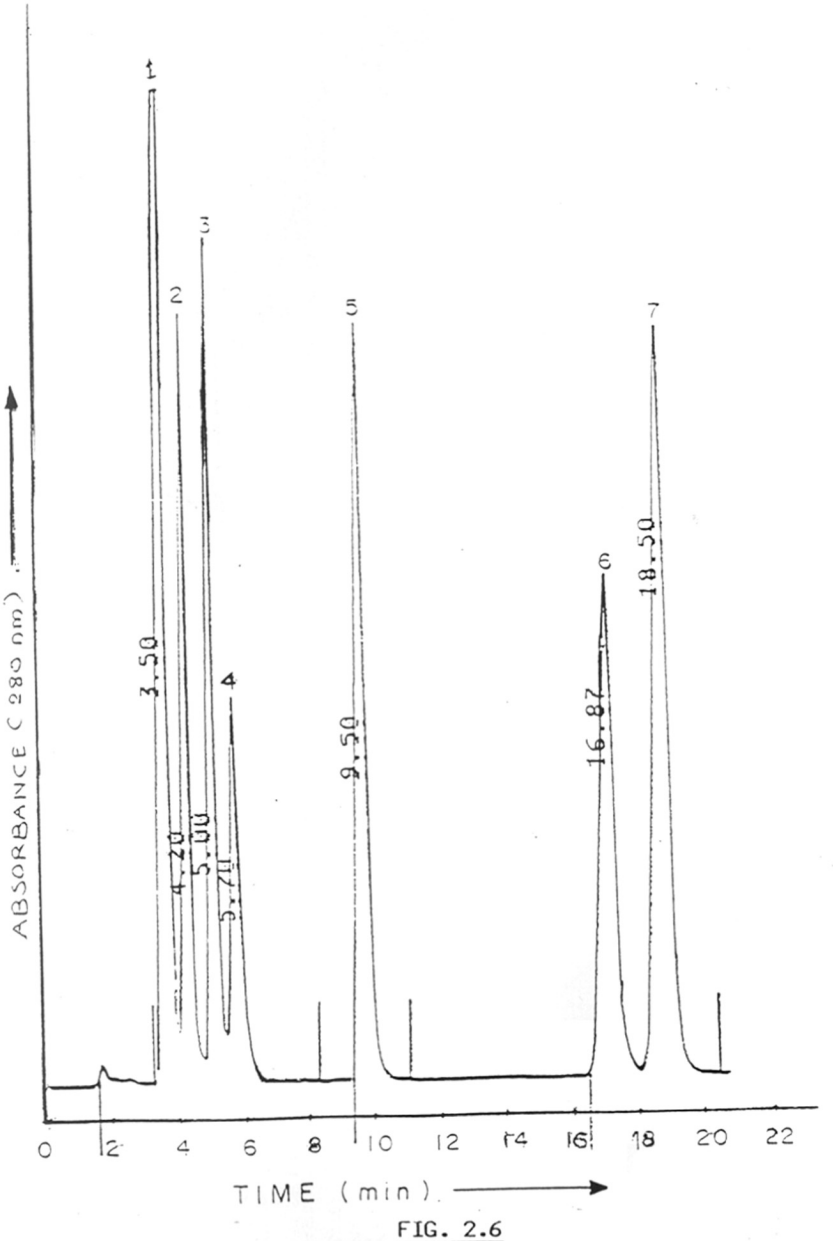


FIG. 2.6

HPLC separation of an artificial mixture of carbamazepine and its synthetic intermediates.

Column: NOVA-PAK C_{18} (Waters) (15 cm x 3.9 mm i.d.); dp = 4 µm.
Mobile phase: Methanol-buffer [pH 5.0] (65:35).
Flow rate: 0.7 ml/min.
Chart speed: .75 cm/min.
UV detection: 280 nm
Peaks: 1. Carbamazepine (CBZ) (VII); 2. o,o'-Diaminodibenzyl (DADB) (III); 3. o-Nitrotoluene (ONT) (I); 4. Iminodibenzylacetate (IDB acetate) (V); 5. Iminostilbene (ISB) (VI); 6. Iminodibenzyl (IDB) (IV); 7. o,o'-Dinitrodibenzyl (DNDB) (II).

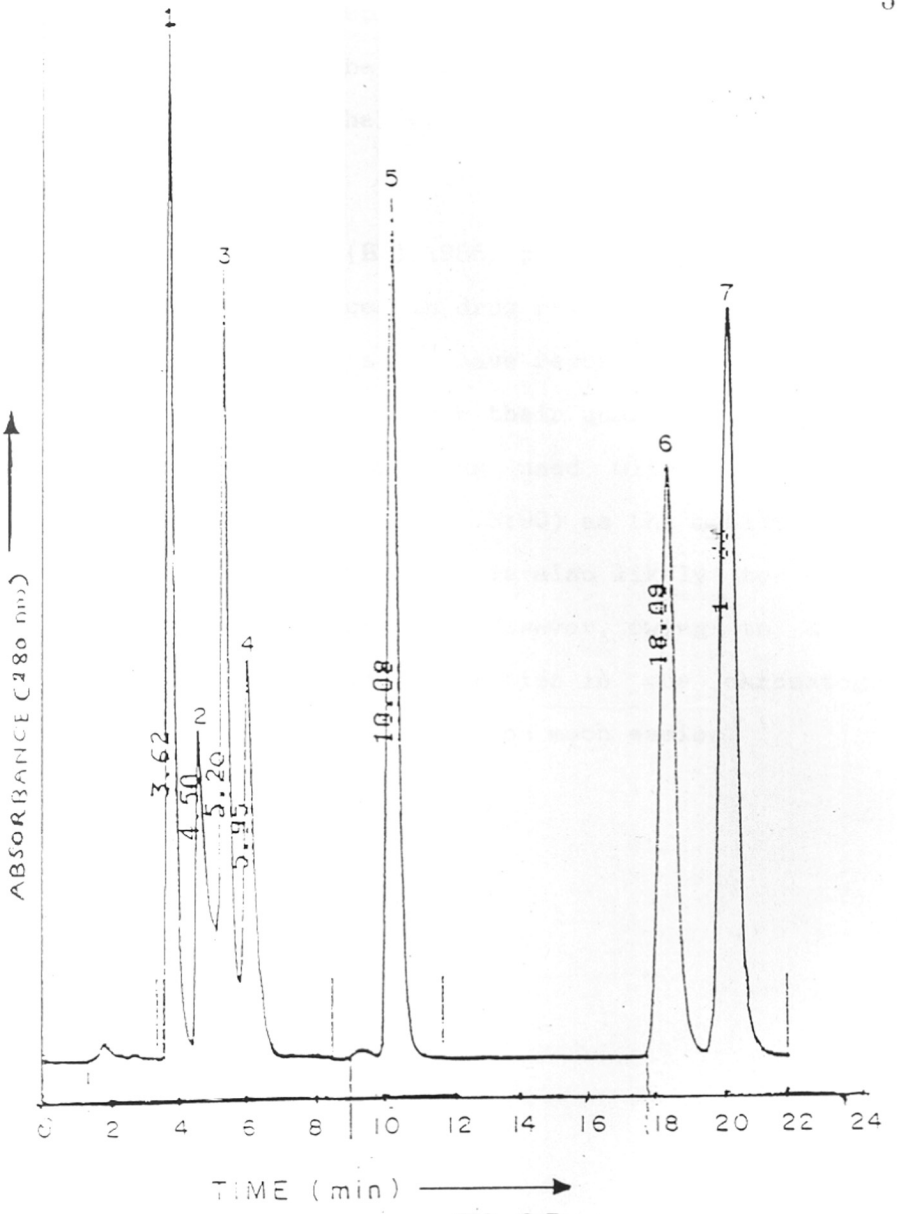


FIG. 2.7

HPLC separation of an artificial mixture of carbamazepine and its synthetic intermediates.

Column: NOVA-PAK C_{18} (Waters) (15 cm x 3.9 mm i.d.); dp = 4 µm.
Mobile phase: Methanol-plain water (65:35).
Flow rate: 0.7 ml/min.
Chart speed: .75 cm/min.
UV detection: 280 nm
Peaks: 1. Carbamazepine (CBZ) (VII); 2. o,o'-Diaminodibenzyl (DADB) (III) 3. o-Nitrotoluene (ONT) (I); 4. Iminodibenzylacetate (IDB acetate) (V); 5. Iminostilbene (ISB) (VI); 6. Iminodibenzyl (IDB) (IV); 7. o,o'-Dinitrodibenzyl (DNDB) (II).

The HPLC data for this separation at pH 6.9 is presented in TABLE 2.2. As methanol is the cheapest organic modifier, there was no incentive in trying other organic modifiers such as acetonitrile and tetrahydrofuran.

British Pharmacopoeia (BP) 1988, prescribes TLC for the carbamazepine related substances in drug raw material and tablet formulations. Recently Cyr et al.[27] have reported that poor peak shapes of IDB (IV) and ISB (VI) makes their quantitation unreliable when a bonded diol column was used with acetonitrile-methanol 0.05% aqueous acetic acid (5:5:90) as the mobile phase. Carbamazepine prepared by our route is also likely to contain these two as carry-over impurities. However, owing to a much better peak shape for both the impurities in our chromatogram (Fig. 2.4) should make their quantitation much easier.

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CHAPTER III

SECTION A: PREPARATION OF AMBROXOL ANALOGUES:

Introduction:

The research and development in the area of drugs and drug intermediates is very important as newer drugs always replace the old ones. Realising the importance of organic chemistry, which can play a vital role in the synthesis of basic drugs, challenging problems related to the area of drugs and drug intermediates have been taken up at NCL. The main emphasis in this area is to find innovative routes in organic synthetic methodology for a better living and also offer chemical challenges to the scientific community.

NCL`s activity in this area is mostly restricted to the synthesis of known drugs. The synthesis may be accomplished by following totally new approaches in respect of raw materials, intermediates and reaction conditions. The manufacture of drugs and drug intermediates which are being used by public and private pharmaceutical companies and technologies are based on the methodologies developed in the basic research programme.

NCL interacts with industries by undertaking projects on their behalf on sponsorship basis. In this connection, a Delhi based pharmaceutical company sponsored a project on a process

development of ambroxol hydrochloride. Ambroxol is used as an expectorant drug. It is a metabolite of an another expectorant drug, bromhexine[1] which is also known as bisolvon. Although bromhexine has been used as an expectorant in India and other countries, ambroxol has not been introduced in India so far. It is mainly used in European countries.

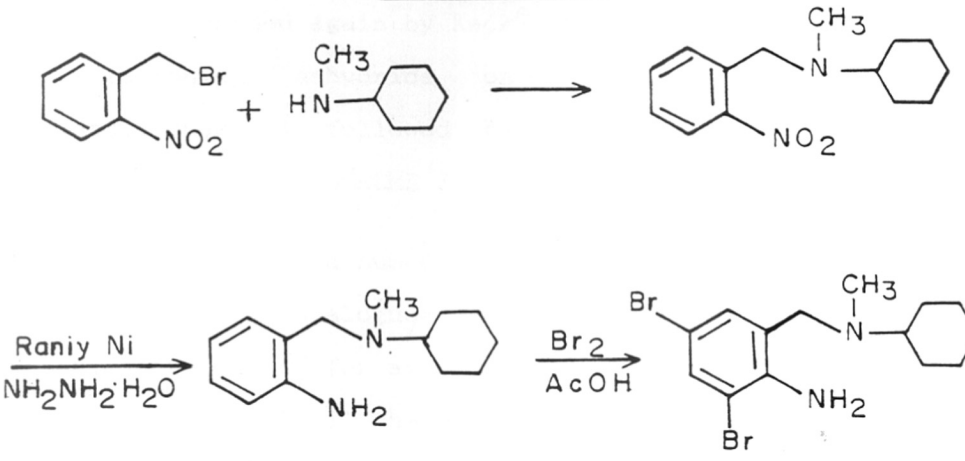
Bromhexine is 2-amino-3,5-dibromo-N-cyclohexyl-N-methyl benzene methanamine or N-cyclohexyl-N-methyl-2-(2-amino-3,5-dibromo)benzyl ammonium. It was first synthesised in 1963 by Keck.[2] The synthesis starts with displacement of halogen on 2-nitrobenzyl bromide by N-methyl cyclohexylamine, followed by Raney Nickel and hydrazine reduction of the nitro group. Bromination in acetic acid then affords bromhexine (SCHEME 1).

Bromhexine was subjected to pharmacological[3] and extensive clinical trials and the following observations were made:

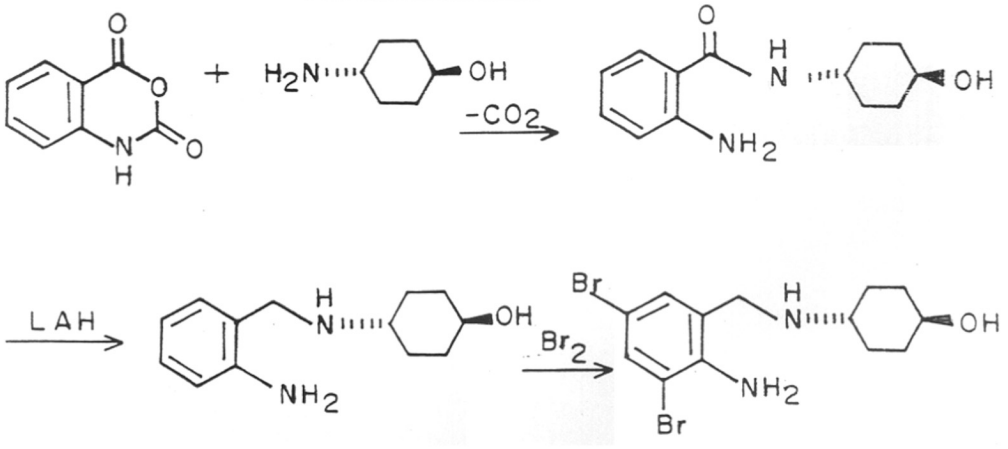
1. It proved to have marked expectorant action; however, the potency was less than that of codeine.
2. Side effects on blood pressure and respiration occurred only with high dosages.
3. Chronic and acute toxicity proved to be very less.
4. Optimal dosage proved to be 1 tablet (12-16 mg) 3 or 4 times daily.
5. The preparation has a pronounced expectorant effect. The majority of patients reported relief in breathing.
6. Undesired side effects were not observed.

It is known that newer drugs always replace the old ones. Ambroxol[4] was also found to be better than bromhexine. As men-

SCHEME - 1



SCHEME - 2

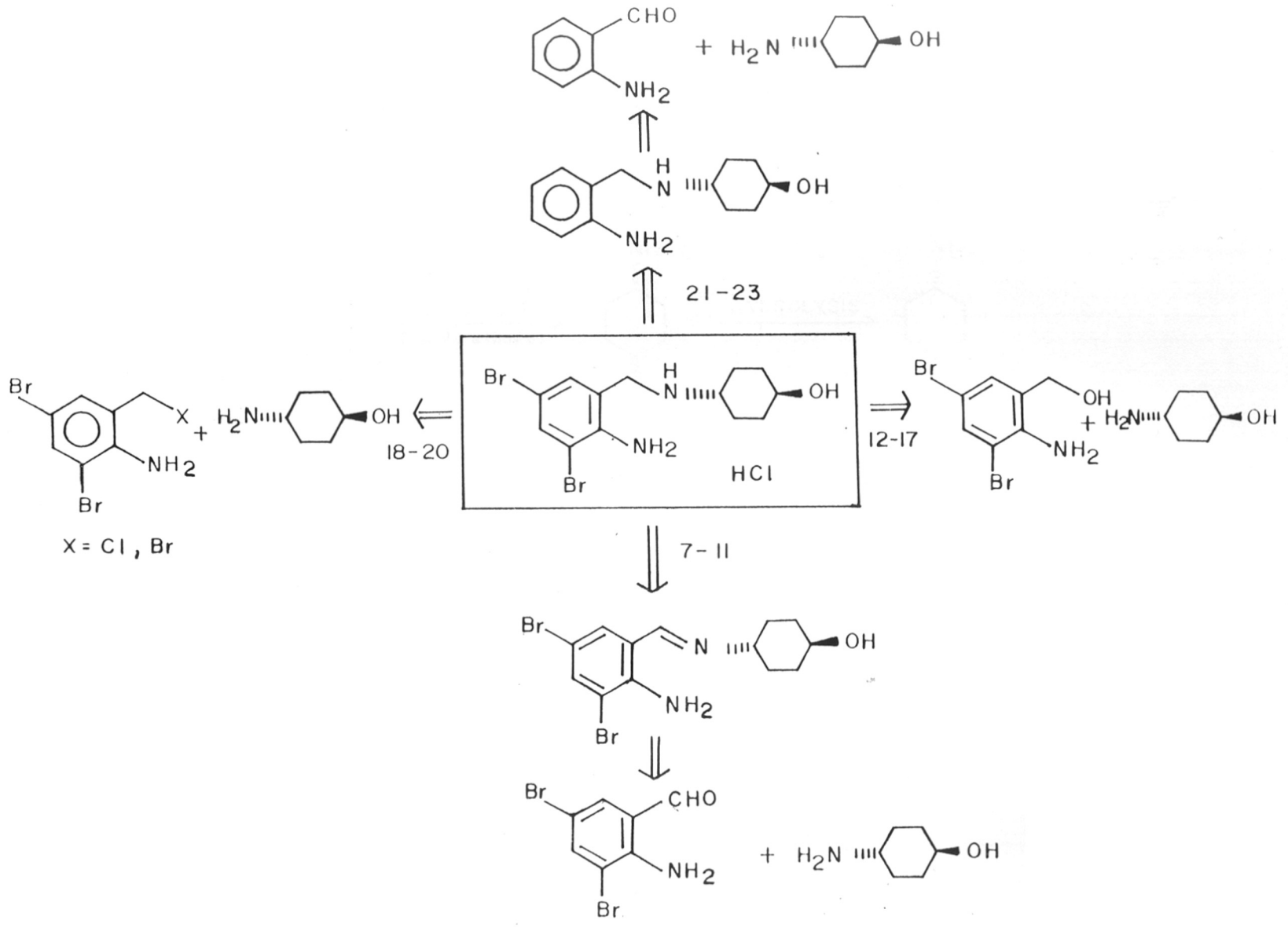


tioned earlier, ambroxol[5] is a metabolite of bromhexine. It was first synthesised again by Keck[6] in 1967.

Isatonic anhydride on condensation with trans-4-aminocyclohexanol followed by LAH reduction and bromination afforded ambroxol (SCHEME 2).

Subsequently, a number of synthetic routes for ambroxol and a new synthetic analogues have been reported. A classified synthetic methods[7-23] for ambroxol are shown in SCHEME-3. It is not possible to give all the synthetic analogues of ambroxol here. However, it should be mentioned here that Thomae et al.[24] have reported in the patent the preparation of 385 analogues of ambroxol.

SCHEME - 3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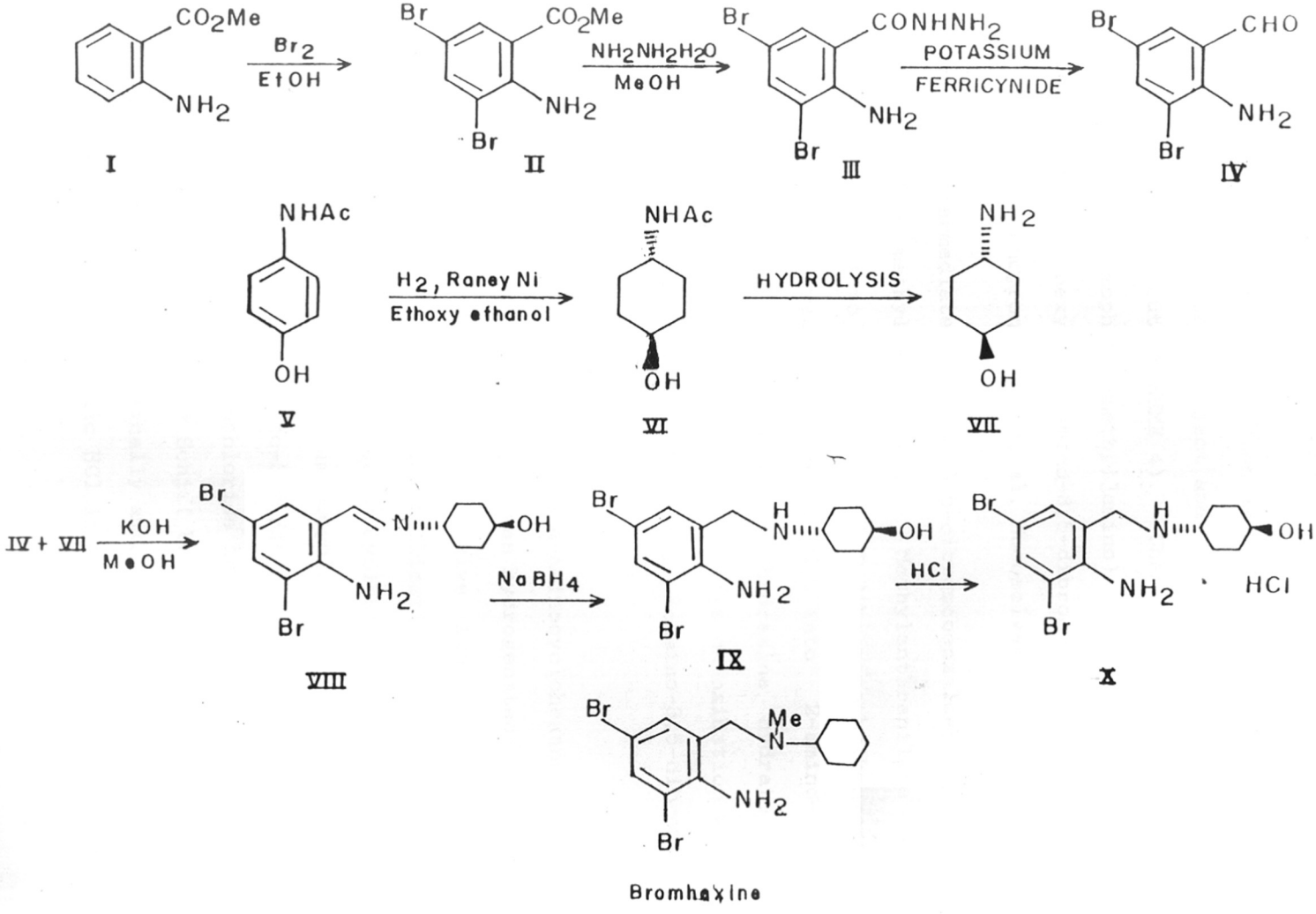
NH2
+ H2N
OH
H
N
OH
NH2
21-23
Br
X
+
H2N
OH
NH2
Br
X = Cl , Br
18-20
Br
H
N
OH
NH2
Br
HCl
12-17
Br
OH
+ H2N
OH
NH2
Br
7-11
Br
N
OH
NH2
Br
Br
CHO
NH2
Br
+ H2N
OH

SCHEME – 4


CO2Me
NH2
Br2
EtOH
Br
CO2Me
NH2
Br
NH2NH2H2O
MeOH
CONHNH2
POTASSIUM
FERRICYNIDE
CHO
I
II
III
IV
NHAc
OH
H2, Raney Ni
Ethoxy ethanol
HYDROLYSIS
NH2
V
VI
VII
IV + VII
KOH
MeOH
N
OH
NaBH4
H
HCl
HCl
VIII
IX
X
Me
Bromhexine

PRESENT WORK

The process development work on ambroxol hydrochloride was undertaken as a sponsored project and the process has been successfully carried out (SCHEME 4). Chemically ambroxol is 4-{[(2-amino-3,5-dibromophenyl-methyl]amino} cyclohexanol or N-(trans- p-hydroxycyclohexyl) (2-amino-3,5-dibromobenzyl)amine and trade names are Brochopront, Duramucal, Mucosolvan, etc.

The key intermediate, 2-amino-3,5-dibromobenzaldehyde (IV) was prepared[25] from methylanthranilate. Methylanthranilate was brominated in ethanol to give 2-amino-3,5-dibromomethylbenzoate in 90% yield. It was then converted into 2-amino-3,5-dibromobenzoylhydrazine on treatment with hydrazine hydrate in methanol in 86% yield. The hydrazine derivative on oxidation with potassium ferricynide afforded the 2-amino-3,5-dibromo-benzaldehyde (IV) in 89% yield.

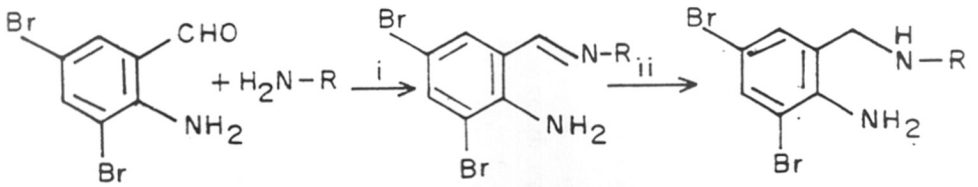
The other intermediate, trans-4-aminocyclohexanol was prepared from paracetamol. Paracetamol was hydrogenated in ethoxy ethanol in presence of Raney Nickel to give trans-4-acetamido-cyclohexanol in 45% yield on crystallisation from acetone. Hydrolysis of the trans-4-acetaminocyclohexanol with 15% hydrochloric acid yielded trans-4-aminocyclohexanol-hydrochloride (78%). Condensation of 2-amino-3,5-dibromobenzaldehyde with trans-4-amino-cyclohexanol-hydrochloride in presence of potassium hydroxide in methanol gave the Schiff base which on reduction with $NaBH_4$ afforded ambroxol. Finally ambroxol was converted into its hydrochloride with methanolic HCl in 62% yield.

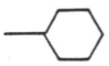
Pharmacology (1967), toxicity (1978) and metabolism and clinical studies (1987) for ambroxol were carried out.[4]

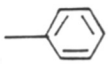
The HPLC separation of the synthetic intermediates of ambroxol was carried out in the present work. In addition to this, some synthetic analogues of ambroxol were prepared starting from 2-amino-3,5-dibromobenzaldehyde and various amines.

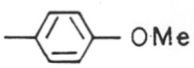
2-Amino-3,5-dibromobenzaldehyde was condensed with various amines such as cyclohexylamine, aniline, p-anisidine, 4-methyl-3-nitroaniline and β and α naphthylamine. The 2-amino-3,5-dibromobenzaldehyde was treated with amines in ethanol in presence of potassium hydroxide at room temperature and the corresponding imine derivatives thus obtained were reduced with sodium borohydride in ethanol to give the desired derivative (see TABLES 3.1 and 3.2), SCHEME 5.

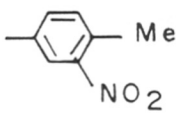
SCHEME - 5

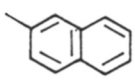


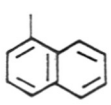
1, R =  1a

2, R =  2a

3, R =  3a

4, R =  4a

5, R =  5a

6, R =  6a

Reagents i) KOH, EtOH, r·t

ii) EtOH, $NaBH_4$

TABLE 3.1

2-Amino-3,5-dibromo-benzaldehyde	Amines	Quantity of amine	Quantity of KOH	EtOH	Product No.	Yield
4.2 g (0.015 mole)	Cyclohexylamine	1.548 g. (1.8 ml) (0.0156 mole)	0.840 g. (0.015 mole)	25 ml	1	3.9 g. 72%
2.8 g (0.01 mole)	Aniline	1.022 g. (0.0101 mole) (1 ml)	0.615 g. (0.0101 mole)	20 ml	2	2.4 g. 70%
2.0 g (0.007 mole)	p-Anisidine	0.861 g. (0.007 mole)	0.400 g. (0.007 mole)	20 ml	3	1.7 g. 72%
1.4 g (0.005 mole)	4Me-3NO_2 aniline	0.762 g. (0.005 mole)	0.280 g. (0.005 mole)	15 ml	4	1.4 g. 67%
2.0 g. (0.007 mole)	β-nap. amine	1 g. (0.007 mole)	0.392 g. (0.007 mole)	20 ml	5	3.1 g. 55%
2.8 g. (0.01 mole)	α-nap. amine	1.43 g. (0.01 mole)	0.560 g. (0.01 mole)	20 ml	6	2.8 g. 71%

TABLE 3.2

Imine	Quantity of imines	$NaBH_4$	EtOH	Product No.	Yield
Cyclohexyl imine	3.9 g. (0.0108 mole)	0.758 g. (0.020 mole)	25 ml.	1a	3.6 g. 91%[a], Crude 3.39 84%, distilled
Aniline imine	1.0 g. (0.0023 mole)	0.214 g. (0.0056 mole)	20 ml	2a	600 mg, 75%[b]
P-Anisidine imine	1.5 g. (0.0039 mole)	0.296 g. (0.0078 mole)	20 ml	3a	900 mg, 60%[b]
4-Me-3-NO_2 aniline imine	1.0 g. (0.0024 mole)	0.184 g. (0.0048 mole)	20 ml	4a	500 mg, 50%[b]
β-Nap. imine	1.7 g. (0.0042 mole)	0.319 g. (0.0084 mole)	20 ml	5a	1.423 g, 84%[a] 1.2 g. 70%[b]
α-Nap. imine	2.0 g. (0.0049 mole)	0.376 g. (0.0098 mole)	20 ml	6a	1.5 g., 75%[a] 1.0 g., 59%[b]

a = Crude product; b = Purified product

EXPERIMENTAL PROCEDURE

All b.ps and m.ps are uncorrected. IR spectra were recorded as films for liquids or in Nujol for solids on Perkin-Elmer spectrophotometer model 683. ^{1}H-NMR spectra on Bruker WH-90 FT spectrophotometer using TMS internal standard and mass spectra on Finnigan MAT 1020 automated GC/MS spectrophotometer at 80 eV. The column chromatography was carried out over silica gel (60-120 mesh). 2-Amino-3,5-dibromobenzaldehyde prepared in this laboratory from methylanthranilate and commercial grade amines were used.

Preparation of Imines : General Procedure

A mixture of 2-amino-3,5-dibromobenzaldehyde (0.01 mole), an amine (R) (0.01 mole) and ethanol was taken in a 50 ml round bottom flask. To this was added, potassium hydroxide (0.01 mole). The reaction was stirred for 24 hrs. at room temperature. The solvent was stripped off and the residue was diluted with water. The solid obtained was filtered, washed with water and used as such for next reaction. The analytical samples were purified by column chromatography and/or by crystallisation.

2-Amino-3,5-dibromobenzyledene cyclohexylamine (1):

Crystallized from chloroform-pet. ether, m.p. 72-73°C.

IR (Nujol): 3450, 3340, 1660, 1600 cm^{-1}.

^{1}H-NMR ($CDCl_3$): 1.1-1.92 (m, 10H, 5 x CH_2); 3.13 (m, 1H, CH), 6.94 (bs, 2H, NH_2/D_2O exchangeable), 7.0 (d, J = 2Hz, 1H, Ar-H), 7.34 (d, J=2Hz, 1H, Ar-H), 8.57 (s, 1H, Ar-HC=N).

MS (m/z) M^+ 360

Found: C, 42.8; H, 4.5; N, 8.0.

$C_{13}H_{16}Br_2N_2$ requires C, 43.3; H, 4.4; N, 7.8%.

2-Amino-3,5-dibromobenzyledene aniline (2):

Crystallized from pet. ether, m.p. 73-74°C.

IR (Nujol, $CHCl_3$): 3460, 1670, 1615, 1600, 1590 cm^{-1}.

^{1}H-NMR ($CDCl_3$): 7.18 (m, 5H, Ar-H), 7.36 (d, J=2Hz, 1H, Ar-H), 7.51 (d, J=2Hz, 1H, Ar-H), 8.35 (s, 1H, Ar-H C=N).MS: m/z (M^+ 354).

Found: C, 44.5; H, 3.2; N, 8.3.

$C_{13}H_{10}Br_2N_2$ requires: C, 44.0; H, 2.8; N, 7.9%.

2-Amino-3,5-dibromobenzyledene-4-methoxyaniline (3):

Crystallized from pet. ether, m.p. 107°C.

IR (Nujol): 3435, 1665, 1610, 1600 cm^{-1}

^{1}H-NMR ($CDCl_3$): 3.77 (s, 3H, OCH_3, 6.86 (d, J=9Hz, 2H, 7.12 (d, J=9Hz, 2H, Ar-H), 7.33 (d, J=2Hz, 1H, Ar-H), 7.51 (d, J=2Hz, 1H, Ar-H), 8.34 (s, 1H, Ar-HC=N).

MS: m/z (M^+ 384).

Analysis: Found C, 44.3; H, 3.4; N, 7.6.

$C_{14}H_{11}Br_2N_2O$ requires C, 43.8; H, 2.9; N, 7.3%.

2-Amino-3,5-dibromobenzyledene-3-nitro-4-methylaniline (4):

Crystallized from ethyl acetate + pet. ether, m.p. 155°.

IR (Nujol): 3460, 3235, 1610, 1600 cm^{-1}.

^{1}H-NMR ($CDCl_3$): 2.57 (s, 3H, CH_3), 7.21-7.77 (m, 5H, Ar-H), 8.40 (s, 1H, Ar-HC=N).

MS: m/z (M^+ 413).

Found: C, 41.1; H, 3.3; N, 10.3

$C_{14}H_{11}Br_2N_3O_2$ requires C, 40.6; H, 2.7; N, 10.2%.

2-Amino-3,5-dibromobenzyledene-2-naphthylamine (5):

Crystallized from pet. ether, m.p. 123°.

IR (Nujol): 3460, 3370, 1665, 1630, 1605 cm^{-1}.

^{1}H-NMR ($CDCl_3$): 7.21-7.87 (m, 9H, Ar-H) 8.51 (s, 1H, Ar-HC=N).IR (Nujol): 3435, 3420, 1612, 1600, 1570 cm^{-1}.

MS: m/z (M^+ 404)

Found: C, 50.9; H, 3.2; N, 6.8.

$C_{17}H_{12}Br_2N_2$ requires C, 50.4; H, 3.0; N, 6.9%.

2-Amino-3,5-dibromobenzyledene-1-naphthylamine (6):

Crystallized from pet ether, m.p. 122-123°C.

IR (Nujol): 3435, 3420, 1612, 1600, 1570 cm^{-1}.

^{1}H-NMR ($CDCl_3$): 6.95-8.2 (m, 9H, Ar-H), 8.44 (s, 1H, Ar-HC=N).

MS: m/z (M^+ 404).

Analysis: Found: C, 50.0; H, 3.2; N, 7.4.

$C_{17}H_{12}Br_2N_2$ requires C, 50.5; H, 3.0; N, 6.9%.

GENERAL PROCEDURE:

Preparation of Amines:

The crude Schiffs base (0.001 mole) was taken in ethanol (25 ml) and an excess of $NaBH_4$ was slowly added to this over a period of 1 hr. The reaction mixture was stirred for 12 hrs at room temperature. The solvent was stripped off and the residue diluted with water. The solid obtained was filtered, washed with water. The solid prodducts were purified by column chromatography and/or

crystallization. In the case of **1a**, after the reaction, the solvent was removed on rotavapour, diluted with water and extracted with ethyl acetate (2 x 50 ml). The organic layer washed with water, brine and then dried (Na_2SO_4). On removal of the solvent, the product was distilled under reduced pressure.

N-(Cyclohexyl)-(2-amino-3,5-dibromobenzyl)amine (1a):

Distilled under reduced pressure, b.p. 200°C at 6 mm.

IR (Nujol): 3435, 3370, 1625, 1610, 1580 cm^{-1}.

^{1}H-NMR ($CDCl_3$): 1.1-2.0 (m, 10H, 5 x CH_2), 2.42 (m, 1H, CH), 3.71 (s, 2H, CH_2), 5.35 (bs, 2H, NH_2, D_2O exchangeable), 7.0 (d, J=2Hz, 1H, ASr-H), 7.47 (d, J = 2Hz, 1H, Ar-H). MS: m/z (M^+ 362).

Found: C, 43.3; H, 5.4; N, 7.4.

$C_{13}H_{18}Br_2N_2$ requires C, 43.1; H, 5.8; N, 7.7%.

N-(Phenyl)-2-amino-3,5-dibromobenzyl)amine (2a):

Crystallized from petroleum ether, m.p. 63°C.

IR (Nujol): 3400, 3290, 3260, 1620, 1605, 1560 cm^{-1}.

^{1}H-NMR $CDCl_3$): 3.6 (bs, 1H, NH, D_2O exchangeable), 4.11 (s, 2H, CH_2), 4.60 (bs, 2H, NH_2, D_2O exchangeable), 6.59-7.45 (m, 7H, Ar-H).

MS: m/z (M^+ 356).

Found: C, 43.6; H, 3.6; N, 8.3

$C_{13}H_{12}Br_2N_2$ requires: C, 43.5; H, 3.4; N, 7.9%.

N-(4-Methoxyphenyl)-(2-amino-3,5-dibromobenzyl)amine (3a):

Crystallized from ethyl acetate + pet. ether, m.p. 79°C.

IR (Nujol): 3390, 3260, 1620, 1600, 1570 cm^{-1}.

^{1}H-NMR ($CDCl_3$): 3.71 (s, 3H, OCH_3): 4.11 (s, 2H, CH_2), 6.57 (d, J=8Hz, 2H, Ar-H), 7.33 (d, J=8Hz, 2H, Ar-H); 7.11 (d, J=2Hz, 1H, Ar-H), 7.42 (d, J=2Hz, 1H, Ar-H).

MS: m/z (M^+ 386).

Found: C, 44.0; H, 3.8; N, 7.6.

$C_{14}H_{14}Br_2N_2O$ requires: C, 43.5; H, 3.6; N, 7.3%.

N-(3-Nitro-4-methylphenyl)-(2-amino-3,5-dibromobenzyl)amine (4a):

Crystallized from chloroform + pet. ether, m.p. 118°C.

IR (Nujol): 3400, 3270, 1625, 1600, 1570 cm^{-1}.

^{1}H-NMR ($CDCl_3$): 2.51 (s, 3H, CH_3), 4.2 (s, 2H, CH_2), 4.31 (s, 1H, NH, D_2O exchangeable), 4.52 (bs, 2H, NH_2, D_2O exchangeable), 6.73-7.51 (m, 5H, Ar-H).

MS: m/z (M^+ 415).

Found: C, 41.0; H, 3.4; N, 9.6.

$C_{14}H_{13}Br_2N_3O_2$ requires: C, 40.5; H, 3.1; , 10.1%.

N-(2-Naphthyl)-(2-amino-3,5-dibromobenzyl)amine (5a):

Crystallized from chloroform + pet. ether, m.p. 99-100°.

IR (Nujol): 3385, 3280, 1640, 1610, 1590 cm^{-1}.

^{1}H-NMR ($CDCl_3$): 4.29 (s, 2H, CH_2), 6.9-7.7 (m, 9H, Ar-H).

MS: m/z (M^+ 406).

Found: C, 50.7; H, 3.8, N, 7.1.

$C_{17}H_{14}Br_2N_2$ requires C: 50.3; H, 3.5; N, 6.9%.

N-(1-Naphthyl)-(2-amino-3,5-dibromobenzyl)amine (6a):

Crystallized from chloroform : pet. ether, m.p. 110°C.

IR (Nujol): 3450, 3260, 1615, 1600, 1585 cm^{-1};

^{1}H-NMR ($CDCl_3$): 4.35 (s, 2H, CH_2), 6.75-7.73 (m, 9H, Ar-H).

MS: m/z (M^+ 406).

Found: C, 50.1; H, 3.6; N, 7.2.

$C_{17}H_{14}Br_2N_2$ requires: C, 50.3; H, 3.5; N, 6.9%.

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SECTION B:

Simultaneous separation of Ambroxol and its four synthetic intermediates by Reversed Phase High Performance Liquid Chromatography:

Gas Chromatography (GC),[1] high-performance liquid chromatography (HPLC)[2-4] of ambroxol in biological materials has been reported in literature. However, to the best of our knowledge, simultaneous high-performance liquid chromatographic (HPLC) separation of ambroxol as bulk drug substance and its synthetic intermediates has not been reported so far.

In the present work a simultaneous separation of ambroxol (IX) and its four synthetic intermediates (I-IV) by reversed phase HPLC on a bonded C_{18} stationary phase have been studied.

EXPERIMENTAL

Liquid Chromatograph:

The method was developed on a Millipore-Waters Chromatographic Division (Milford, M.A., U.S.A.) HPLC system consisting of two model 510 series dual-head reciprocating solvent delivery pumps controlled by a model 680 Automated Gradient Controller, a model U6K universal injector and a Model 481 Lamda-Max spectrophotometric detector operating at 247 nm. The analogue output of the detector was recorded and processed with a model 730 data module (printer, plotter and integrator). A Waters Novapak C_{18} (4 μm) stainless steel column (15 cm x 3.9 mm ID) was used.

Authentic Samples:

Authentic samples of ambroxol (IX) and its intermediates (I-IV) were prepared and purified in our laboratory. The identity of individual compounds was confirmed by physical methods (melting point, IR, NMR and mass spectrometry).

Standard Solutions:

The following compounds, 5.0 mg methyl anthranilate (I), 6.5 mg 2-amino-3,5-dibromomethyl benzoate (II), 4.5 mg 2-amino-3,5-dibromobenzoyl hydrazine (III), 4.0 mg 2-amino-3,5-dibromobenzaldehyde (IV) and 6.0 mg ambroxol (IX) were weighed separately in 10 ml volumetric flasks and dissolved and diluted with mobile phase.

Artificial mixture solution of ambroxol (IX) and its intermediates (I-IV):

This was prepared by mixing an equal volume of each of the above standard solutions. About 50 µl of this artificial mixture solution was injected into the liquid chromatograph.

Reagents and Chemicals:

Analytical reagent grade triethylamine, orthophosphoric acid, CH_3COONa, KH_2PO_4 and Na_2HPO_4 were used to prepare buffer solutions. Methanol was purified to HPLC quality in our laboratory. A Milli Q system (Millipore, Bedford, M.A., U.S.A.), was used to purify water.

pH 3.1 Buffer:

A stock buffer solution of triethylammonium phosphate (ca. 0.7 M) was prepared by mixing 10.0 ml triethylamine with about 80

ml water and the pH was adjusted to 2.2 with 85% (w/w) orthophosphoric acid (ca. 8 ml)_. The solution was cooled to room temperature and diluted to the volume in a 100 ml volumetric flask. A 1 ml volume of this stock solution was diluted to 100 ml with water to obtain the working buffer solution (7 mM, pH 3.1).

pH 5.0 Buffer:

4.1 g. anhydrous sodium acetate was dissolved and diluted to the volume in a 1000 ml volumetric flask with water. The pH of this solution was adjusted to 5 using a few drops of glacial acetic acid.

pH 6.9 Buffer:

3.40 g anhydrous KH_2PO_4 and 3.76 g Na_2HPO_4, $2H_2O$ weredissolved and diluted to the volume in a 1000 ml volumetric flask with water.

Mobile Phase:

(i) Methanol-buffer (with appropriate pH) (70:30) was used as the mobile phase at a flow rate of 0.7 m/min.

(ii) Acetonitrile-buffer (with appropriate pH) (55:45) was used as the mobile phase at a flow rate of 0.7 ml/min.

Column dead time (to):

This was determined by injecting 50 ul of methanol-buffer (65:35) into the mobile phase stream. The peak-trough combination caused by the change in refractive index was used as a marker and the point of baseline cross over was taken as to.

RESULTS AND DISCUSSION:

It was felt necessary to have a HPLC method to monitor the progress of the reaction sequence described in the SCHEME 4 which would also enable to ascertain the quality of ambroxol produced.

Initially, attempts were made to separate ambroxol (IX) and its intermediates (I-IV) on a Novapak C_{18} column using an inexpensive methanol - aqueous buffer (pH 3.1, 5.0 and 6.9) (70:30) as the mobile phase system. Under these conditions the best separation was achieved using the pH 3.1 buffer (TABLE 3.1 and Fig. 3.1). Compared to that of pH 5.0 and pH 6.9 (Fig. 3.2) under these pH (5.0 and 6.9), there was no separation involving ambroxol (IX) and (III).

In order to improve the separation further, acetonitrile-aqueous buffer (pH 3.1, 5.0 and 6.9) (55:45) as the mobile phase system was tried. However, only in acetonitrile-aqueous buffer (pH 6.95) produced a separation (TABLE 3.1, Fig.3.3) which was even better than that obtained using the above mentioned methanol-pH 3.1 buffer system, particularly with respect to two peak pairs involving ambroxol (IX). The separation pattern obtained in acetonitrile-buffer (pH 5.0) is shown in Fig. 3.4.

The anilino group in these compounds as well as the $-CONHNH_2$ group in (III) are extremely weakly basic (e.g. ambroxol (pka^1 = -0.3). Thus, the retention times of the intermediates bearing only the anilino functionality (II and IV) are very slightly affected by the pH changes. The secondary amino group (pka^1, = 8

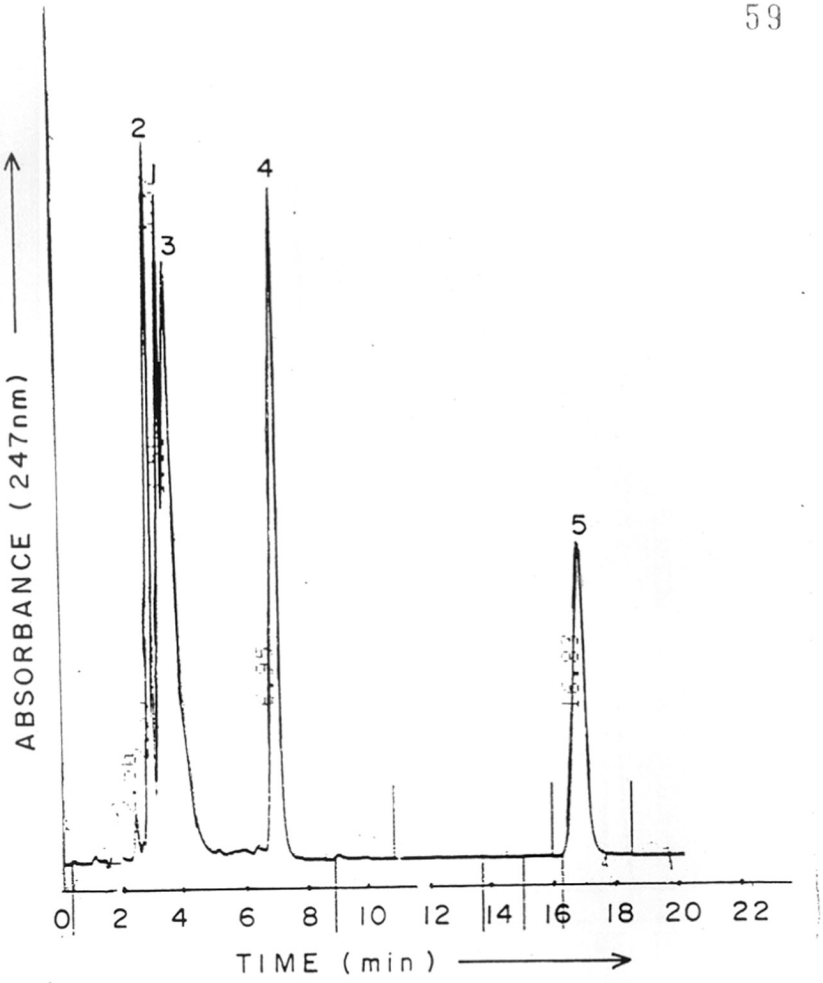

FIG. 3.1

HPLC Separation of an artificial mixture of Ambroxol and its synthetic intermediates:

Column	: NOVA-PAK C_{18} (Waters) (15 cm x 3.9 mm i.d.); dp = 4 µm
Mobile phase	: Methanol-buffer [pH 3.1] (70:30)
Flow rate	: 0.7 ml/min.
Chart speed	: 0.5 cm/min.
UV Detection	: 247 nm

Peaks: 1. 2-Amino-3,5-dibromobenzoylhydrazine (**III**); 2. Methyl anthranilate (**I**); 3. Ambroxol (**IX**); 4. 2-Amino-3,5-dibromobenzaldehyde (**IV**); 5. 2-Amino-3,5-dibromomethylbenzoate (**II**).

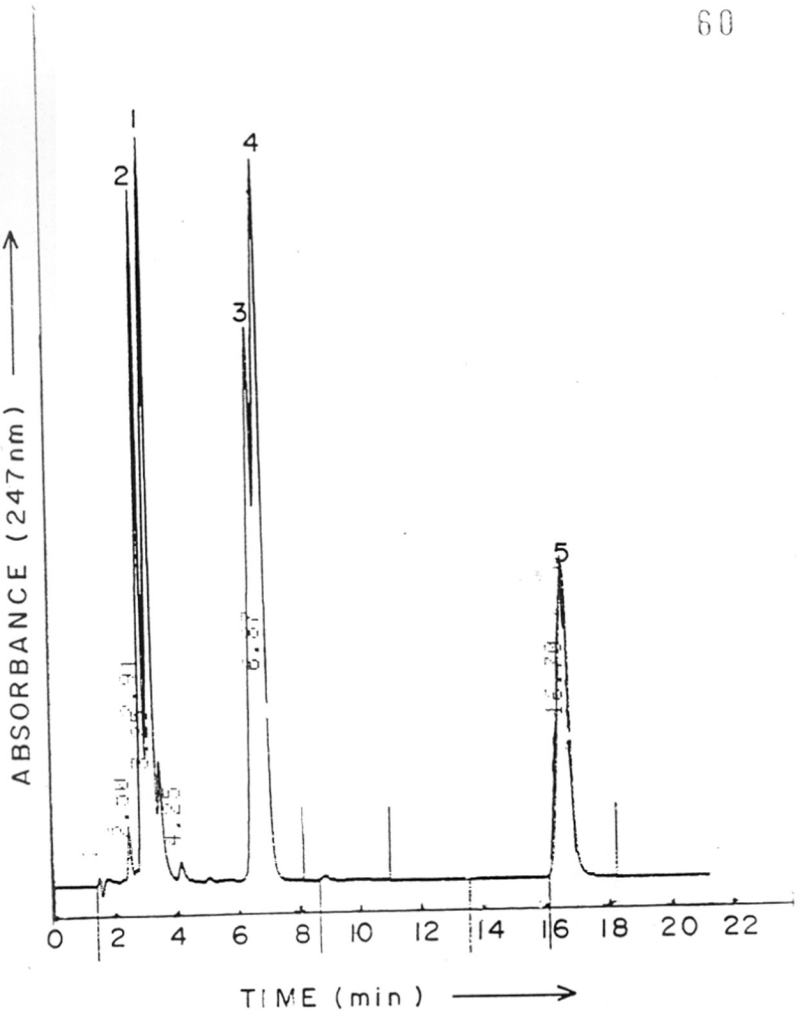

FIG. 3.2

HPLC Separation of an artificial mixture of Ambroxol and its synthetic intermediates:

Column	: NOVA-PAK C_{18} (Waters) (15 cm x 3.9 mm i.d.); dp = 4 µm
Mobile phase	: Methanol-buffer [**pH 6.95**] (70:30)
Flow rate	: 0.7 ml/min.
Chart speed	: 0.5 cm/min.
UV Detection	: 247 nm

Peaks: 1. 2-Amino-3,5-dibromobenzoylhydrazine (**III**); 2. Methyl anthranilate (**I**); 3. Ambroxol (**IX**); 4. 2-Amino-3,5-dibromobenzaldehyde (**IV**); 5. 2-Amino-3,5-dibromomethyl benzoate (**II**).

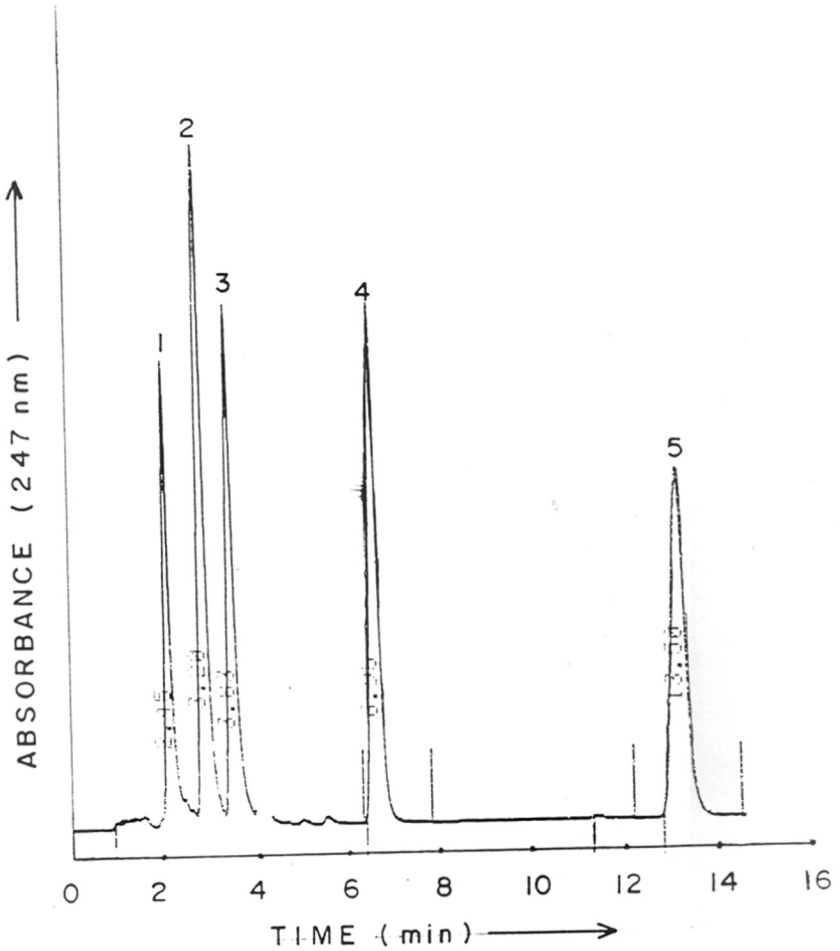

FIG. 3.3

HPLC Separation of an artificial mixture of Ambroxol and its synthetic intermediates:

Column : NOVA-PAK C_{18} (Waters) (15 cm x 3.9 mm i.d.); dp = 4 µm

Mobile phase : **Acetonitrile**-buffer [**pH 6.95**] (55:45)

Flow rate : 0.7 ml/min.

Chart speed : 0.75 cm/min.

UV Detection : 247 nm

Peaks: 1. 2-Amino-3,5-dibromobenzoylhydrazine (**III**); 2. Methyl anthranilate (**I**); 3. Ambroxol (**IX**); 4. 2-Amino-3,5-dibromobenzaldehyde (**IV**); 5. 2-Amino-3,5-dibromomethyl benzoate (**II**).

TABLE 3.1

HPLC OF AMBROXOL AND ITS FOUR SYNTHETIC INTERMEDIATES ON A Nova-Pak C_{18} (WATERS) COLUMN

Void volume time, to = 2.15 min at 0.7 ml/min.

Compound	Methanol buffer (pH 3.1)(70:30) (FIG.3.1)			Acetonitrile-buffer (pH 6.9) (55:45) (FIG.3.3)		
	Retn. time (min)	Capacity factor,	Selectivity factor	Retn. time (min)	Capacity factor	Selectivity factor
Methyl anthranilate (I)	2.83	0.32		3.18	0.48	
			1.53			4.68
2-Amino-3,5-dibromo-benzoylhydrazine (III)	3.20	0.49		2.37	0.10	
			1.32			1.57
Ambroxol (IX)	3.54	0.65		3.77	0.75	
			2.89			3.05
2-Amino-3,5-dibromo-benzaldehyde (IV)	6.16	1.87		7.09	2.30	
			3.65			2.35
2--Amino-3,5-dibromomethyl benzoate (II)	16.83	6.83		13.77	5.40	

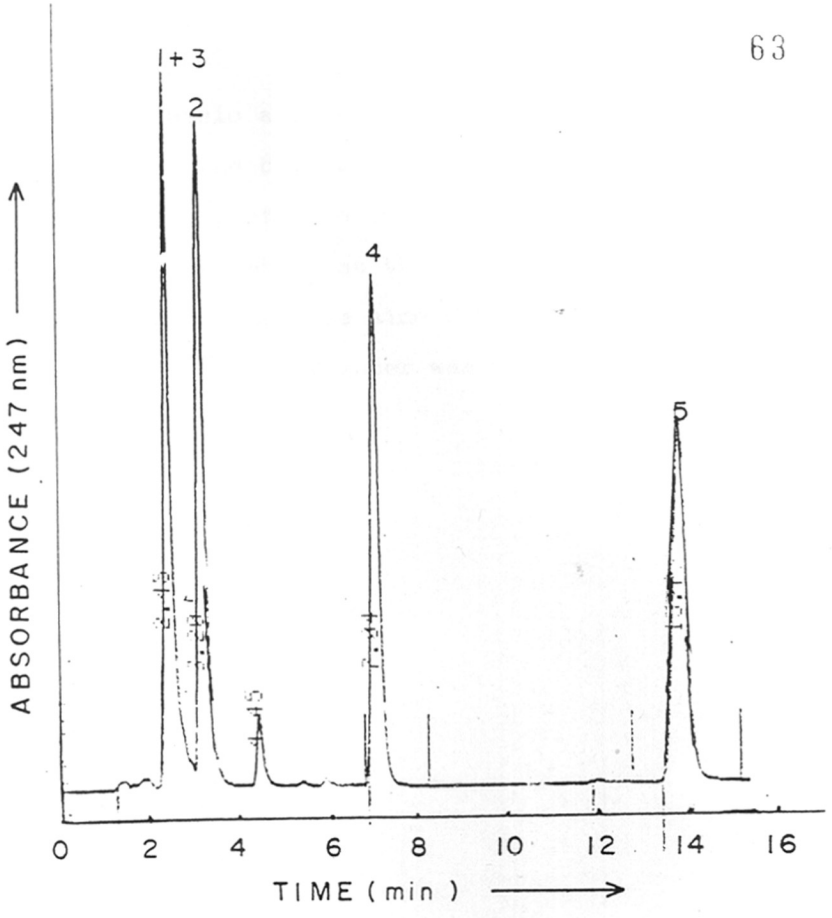

FIG. 3.4

HPLC Separation of an artificial mixture of Ambroxol and its synthetic intermediates:

Column	: NOVA-PAK C_{18} (Waters) (15 cm x 3.9 mm i.d.); dp = 4 µm
Mobile phase	: **Acetonitrile**-buffer [**pH 5.0**] (55:45)
Flow rate	: 0.7 ml/min.
Chart speed	: 0.75 cm/min.
UV Detection	: 247 nm

Peaks: 1. 2-Amino-3,5-dibromobenzoylhydrazine (**III**); 2. Methyl anthranilate (**I**); 3. Ambroxol (**IX**); 4. 2-Amino-3,5-dibromobenzaldehyde (**IV**); 5. 2-Amino-3,5-dibromomethyl benzoate (**II**).

in ambroxol is moderately basic and therefore, its retention time appreciably increases when the buffer pH is changed from 5.0 to 6.9. The retention time is expected to increase still further if the pH is raised beyond 8. However, at this high pH the rate of dissolution of the silica backbone is also expected to increase considerably. Therefore, this pH parameter was not explored.

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