"SOME APPLICATIONS OF GAS CHROMATOGRAPHY TO THE DETERMINATION OF DRUGS"

by

RAJA RAM WADHWANI

A thesis presented for the degree of

MASTER OF PHILOSOPHY

from the

University of Aston in Birmingham

August 1979

DEPARTMENT OF PHARMACY.

SUMMARY

Some applications of gas chromatography to the determination of drugs.

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The development and application of gas-liquid chromatographic (GLC) methods for the determination of chloramphenicol in pharmaceutical products and lactic acid in cerebrospinal fluid (CSF) is described.

Chloramphenicol was found to convert rapidly and quantitatively into a phenyl boronate ester on treatment with phenyl boronic acid. This derivative whose structure was proven by infra-red and ^{1}H nuclear magnetic resonance spectroscopy and mass spectrometry, was found to possess good chromatographic properties. It was eluted from an OV-17 column within 5 minutes and the incorporation of triphenyl benzene as an internal standard enabled a quantitative response to be achieved. The procedure was found to be applicable to the determination of chloramphenicol in solid, liquid and semi-solid dosage forms after suitable extraction. Details for each procedure are described.

The assay was also suitable for the determination of chloramphenicol in degraded samples. This has been illustrated by a study of the stability of chloramphenicol eye-drops-hydrolytic or photochemical decomposition. The hydrolysis reactions were performed at 89°C in solutions of different pH (3-6) or with different buffer concentrations (0.05N-1.5N). In each case first-order kinetics were observed.

Photochemical degradation was used to assess the efficiency of various containers in protecting chloramphenicol eye-drops from light-initiated decomposition. Again, first-order kinetics were observed and the most efficient container studied was an amber soda glass bottle originally used for packing eye/ear/nose drops. This degradation was also followed by high-performance liquid chromatography (HPLC) and this showed that the initial degradation resulted in the formation of p-nitrobenzaldehyde.

Lactic acid in cerebrospinal fluid (CSF) was estimated by esterification with ethanol. Diethyl malonate was found to be a useful internal standard for quantitative purposes and this method allowed the analysis of lactic acid in 77 samples of CSF from patients with suspected meningitis. It was found that lactate levels were elevated in cases of bacterial meningitis (> 0.3 mg.ml.$^{-1}$) where as non-bacterial meningitis cases had normal (below 0.2 mg.ml.$^{-1}$) levels.

Key Words

Gas liquid chromatography, Assay, Chloramphenicol, Lactic acid, Cerebrospinal fluid.

"We shall not cease from explorating

And the end of all our exploring

Will be to arrive where we started

And know the place for the first time".

T.S.Eliot.

"The greater our knowledge increases

the greater our ignorance unfolds".

J.F.Kennedy.

1962.

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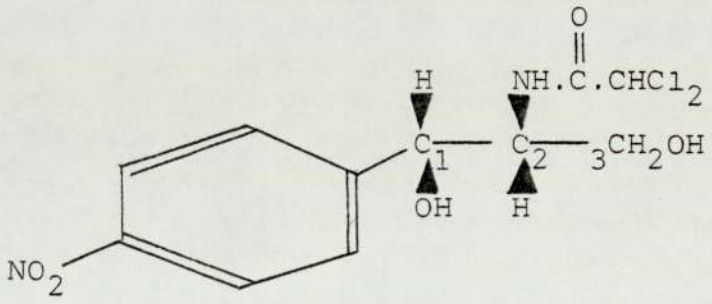
PART I

ASSAY OF CHLORAMPHENICOL.

A. INTRODUCTION.

Chloramphenicol(1) is a chlorine containing antibiotic which was isolated in 1947 from Streptomyces venezuela found in a soil sample in Venezuela.
Chloramphenicol is the active isomer of the four possible[1] stereoisomers and has the structure D-(-)threo-1-(p-nitrophenyl)-2-(2,2-dichloroacetamido)-1,3-propanediol(1). This structure is unusual among natural products in possessing a nitro group(the first such compound identified although azomycin, 2-nitro imidazole[7], is a further member of this group) and a dichloroacetyl substituent.



(1)

(1). Medical uses of chloramphenicol.

Chloramphenicol is a broad spectrum antibiotic with activity against Rickettsia and some viruses. It is highly toxic and the main indications for chloramphenicol therapy are, therefore, typhoid and paratyphoid fever in which no satisfactory alternative is available. A second indication is H.influenzae. It has also been used for treating whooping cough, for various infections by staphylococci which are resistant to other antibiotics and for urinary infections with Proteus.

Chloramphenicol passes cellular barriers more readily than most other antibiotics. It is readily absorbed when given by mouth and diffuses rapidly into tissues, the cerebrospinal fluid, and the foetal circulations. Chloramphenicol was the first broad spectrum antibiotic to be used clinically, however, there is evidence relating toxicity to quantitative exposure to the drug which restricts its use in medicine today. The most serious toxic action of chloramphenicol is its attack on the bone marrow, possibly attributed to its nitrophenyl structure. Chloramphenicol may cause cancer in humans[4]. The suspected target is the haemopoietic system and the main route of exposure is likely to be by injection.

Its use in new born infants and older children with hepatic disease is restricted[5], because of the potential accumulation of toxic quantities, an accumulation resulting from poor or absent hepatic conjugation and occasionally from delayed excretion of the conjugate by the kidney.

Chloramphenicol is currently recommended for initial therapy of life-threatening disease caused by Haemophilus influenzae, because of increasing prevalence of ampicillin-resistant strains[6].

The "gray baby" syndrome is directly related to the higher concentration of the chloramphenicol[7] in plasma.

(II). Biological fate of chloramphenicol.

The dissolution rate of poorly water soluble drug preparations appears to relate to particle size, which is directly related to the total surface in contact with the surrounding liquid, such that

$$dw / dt = KS (C - C^{o}) \quad \text{——————————} \quad (1).$$

Where dw / dt = dissolution rate

K = dissolution rate constant

S = surface area of dissolving solid

C = concentration in diffusing layer

C^{o} = concentration in solution.

The dissolution rate constant, K, is given by

$$K = D / L. \quad \text{——————————} \quad (2).$$

Where D = diffusion co-efficient, and

L = thickness of diffusion layer.

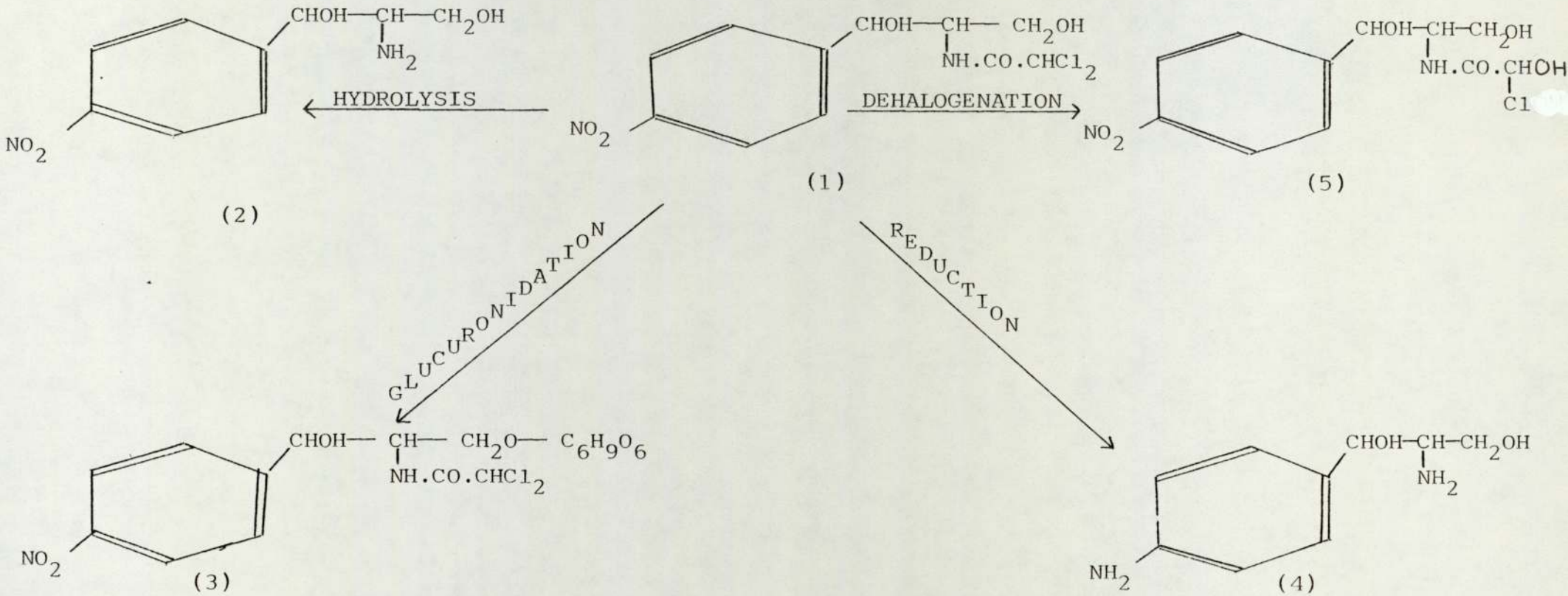
Chloramphenicol is absorbed by simple diffusion mechanism in the intestinal tract but absorption from the stomach appears to be minimal[8].

In the clinical trials with chloramphenicol, Glazko, et al[9], found plasma levels in human to be directly proportional to dosage, while urinary excretion of nitro-compounds indicated essentially complete intestinal absorption. Biochemical changes which occur during metabolism of chloramphenicol were determined by Glazko and his colleagues[9]. Isolation and identification of metabolites found in various body fluids after administration of this antibiotic indicates that its metabolism can occur by

routes shown in scheme I.Comparative information on the metabolism of nitro compounds[9] enabled these studies on the metabolic fate of chloramphenicol to be under taken readily.

Chloramphenicol[9] is rapidly absorbed, inactivated and excreted. In man, about 90% of the drug administered orally is recovered in the urine, in 24 hours, principally in the form of inactive metabolic products, which retain the aryl-nitro group intact (scheme I).

Less than 10% of the dose is excreted as unchanged chloramphenicol, confirming the Ley, *et al*[10], observations. Glazko, *et al*,[11] observed maximum blood levels within 2 hours of dosage by microbiological assay, following oral administration of chloramphenicol to man. The assay indicated that the principal nitro compound in the blood is active chloramphenicol, although, the renal plasma clearance results indicated that chloramphenicol is largely excreted by glomerular filtration, while the inactive metabolic products appear to be excreted mainly by tubular secretion.



(SCHEME I)

Pathways in the metabolic disposition of chloramphenicol (1).

(III). Pharmaceutical aspects of chloramphenicol.

(a). Stability of chloramphenicol.

(i). In solid forms.

Chloramphenicol in the solid state either as the bulk drug or present in solid dosage forms, is a very stable antibiotic[12]. Reasonable precautions taken to prevent excessive exposure to light or moisture are adequate to prevent significant decomposition over an extended period.

(ii). In solution.

The stability of chloramphenicol in aqueous solution is governed by the rate at which hydrolytic processes occur. First reports by the discoverers of chloramphenicol[13] indicated that in aqueous solutions between pH 2 and 9, it was stable for more than 24 hours at room temperature and that it was unaffected by boiling in distilled water for 5 hours.

Later workers [14-21] reported varying degrees of stability in aqueous solution and on heating, but the results were not always comparable because of the variety of solutions and buffers used and the method of assay which include microbiological, chemical, and spectrophotometric.

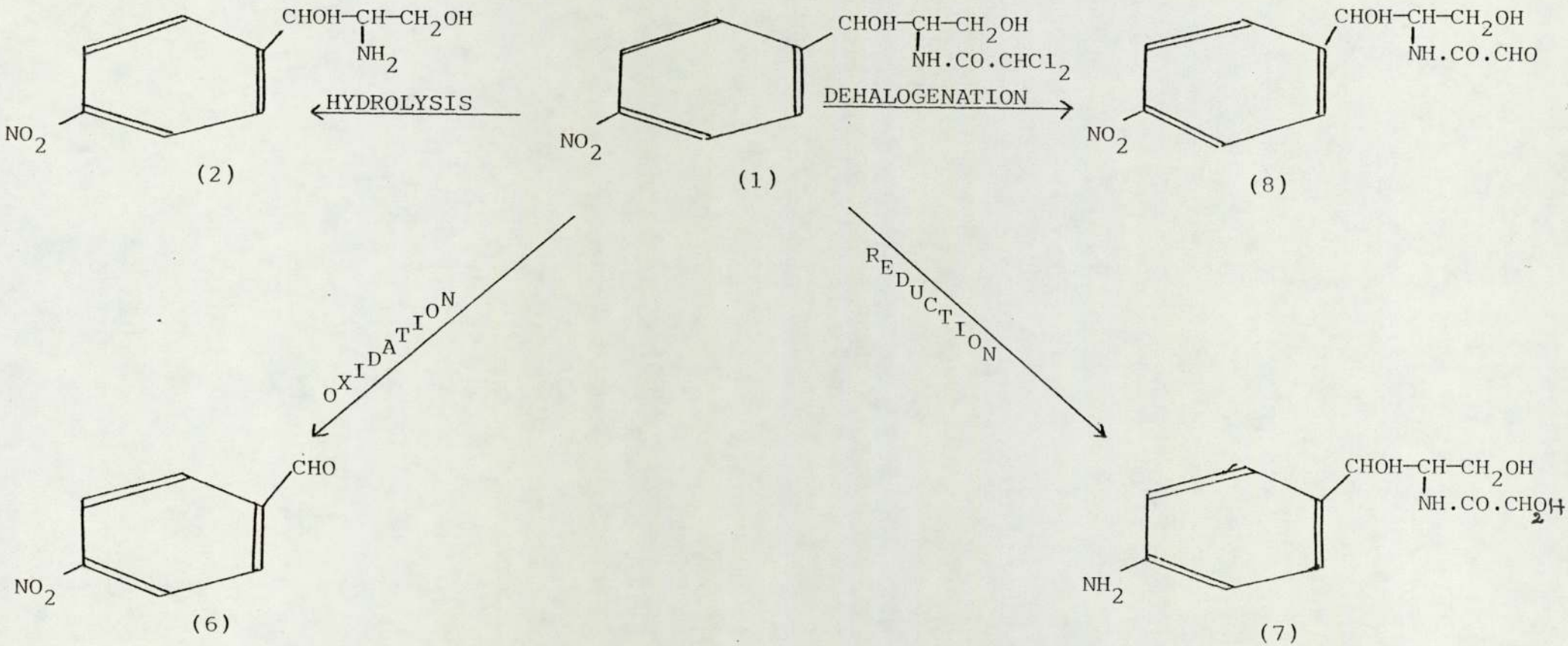
Results from the studies of Brunzell[22] indicate that the antibiotic is more stable in the presence of borate buffer solution than in its absence.

Heward, et al[23], and James and Leach[25], in their separate studies, suggested that the complexation between the antibiotic and borate ion is responsible for the

increased stability and solubility of chloramphenicol in this buffer system. Citrate and phosphate buffers were rejected as they reduced the stability of the solution[19,29].

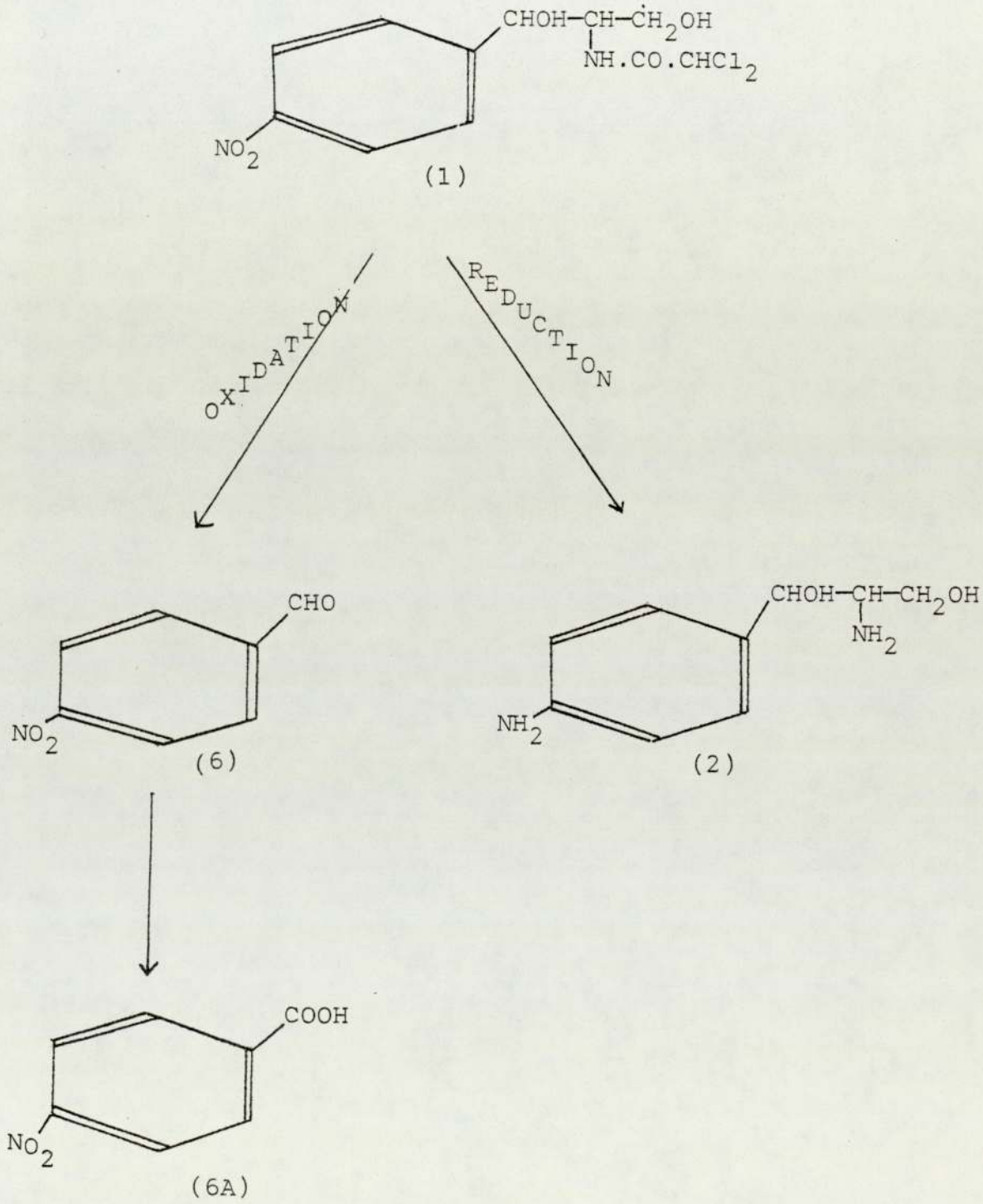
(b). Degradation of chloramphenicol.

The two primary routes of decomposition of chloramphenicol have been determined[26-30], to be (i) amide hydrolysis with the formation of 1-(p-nitrophenyl)-2-amino-1,3-propanediol(2) and dichloroacetic acid(scheme II).
(ii) hydrolysis of covalent chlorine of the dichloroacetic moiety. The hydrolytic cleavage of the amide linkage[27,29] is the major cause of chloramphenicol break down and is the significant route of degradation in solution below pH 7. The rate of loss of antibiotic and the rate of production of amine[26] parallel the break down of chloramphenicol, and are essentially the same. Under most conditions of degradation reported pseudo-first order kinetics are in operation.
Certain bacteria or baterial enzymes are able to decrease the antibiotic potency of chloramphenicol solutions by hydrolysing the amide linkage[31,32] of the drug. Reduction of the nitro group to a primary amine[33](7) or oxidation of the antibiotic to p-nitrobenzaldehyde(6) may also occur (scheme II).
The effect of light is often considered an important factor in drug stability. The major photodegradation products of chloramphenicol were isolated and identified by Shih[30] as p-nitrobenzaldehyde, p-nitrobenzoic acid(6A) and 4,4-azoxy benzoic acid. (scheme III).
The chemistry of the photodegradation products suggests that chloramphenicol in water under the influence of



(SCHEME II)

Decomposition products of chloramphenicol (1).



(SCHEME İII)

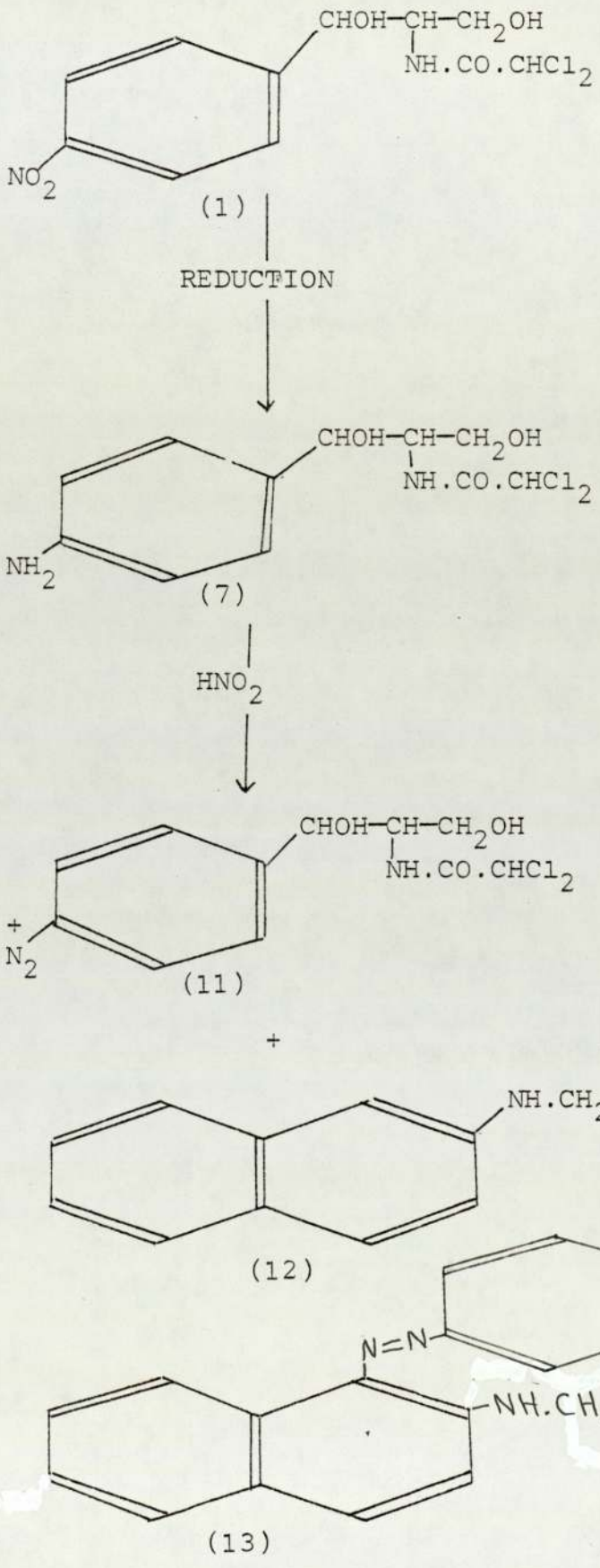
Photodegradation products of chloramphenicol(1).

light undergoes oxidation, reduction and condensation reactions[30] (scheme III).

(IV). Assay methods available for the determination of chloramphenicol.

(a) Chemical.

The initial determination [10,11,34-36] of chloramphenicol was by colorimetry after nitro group reduction and Bratton-Marshall[37] diazo coupling(scheme IV). Although, this method has opened the way for studies on the metabolic fate of chloramphenicol, it is not specific for the active antibiotic. This is because inactive degradation products of chloramphenicol which have retained the nitro-group are also included in the determination. Moreover, the estimation of the amount of chloramphenicol, by the reduction, diazotisation and coupling method, is tedious and subject to many manipulations each of which may be a source of error. Aihara, et al[38], performed a colorimetric analysis by the hydroxamic acid method. This method was not specific because the principal hydrolysis products of chloramphenicol did not yield a significant colour. Hughes and Diamond[39], evaluated the concentration of the chloramphenicol to be the 10 $\mu g.ml.^{-1}$ level in blood and plasma of patients receiving multiple antibiotics, by the method of Kakemi, et al[40]. They extracted the drug from blood or plasma with isoamyl acetate at pH 7,


CHOH—CH—CH2OH
NH.CO.CHCl2
NO2
(1)
REDUCTION
CHOH—CH—CH2OH
NH.CO.CHCl2
NH2
(7)
HNO2
CHOH—CH—CH2OH
NH.CO.CHCl2
+
N2
(11)
+
NH.CH2.CH2.NH2
(12)
CHOH-CH-CH2OH
NH.CO.CHCl2
N=N
NH.CH2.CH2.NH2
(13)

(SCHEME IV)

and then derivatised with iso-nicotinic acid hydrazide and sodium hydroxide. The intensity of yellow colour was found to be proportional to the concentration of chloramphenicol. Although, this procedure enabled the estimation of chloramphenicol in body fluids in the presence of other antibiotics, a lack of precision in the results is possible.

Zuman[41], determined the 2-(2,2-dichloracetamido)--3-hydroxyl-4-nitropropiophenone, a possible toxic contaminant in the synthetic chloramphenicol, by direct polarographic measurement. As this method depends upon the existance of the p-nitrophenyl group, a lack of specificity is again apparent.

Chloramphenicol mono-succinate was separated from chloramphenicol by electrophoresis after ethyl acetate extraction. The nitro containing metabolites remained in the aqueous layer[42]. Chloramphenicol palmitate was synthesized by Swartz[63] as a tasteless derivative of chloramphenicol.

Glazko, et al[11], showed that the intact esters are poorly absorbed from the intestinal tract and must first be hydrolysed by the esterases in the small intestinel before any significant absorption can take place. In general, the chemical methods often co-determine inactive metabolites and suffer from the risk of interference by other drugs.

(b). Microbiological methods.

(i). Cylinder plate method.

This is an agar diffusion procedure using Sarcina lutea ATOC 9341 as the test organism. The response of the assay is produced by the solutions of chloramphenicol in 1% phosphate buffer pH 6 diffusing through an agar layer uniformly inoculated with the test organism.

(ii). Turbidimetric method.

In this method, the concentration of chloramphenicol is determined by measuring the turbidity that is produced by the actively growing test organism in a series of test tubes containing chloramphenicol and inoculated liquid culture medium. By comparing the turbidity of the unknown to that of the reference standard, the potency of drug is found[43].

Generally, microbiological procedures are time-consuming and less accurate than other assay methods. Since decomposition products or metabolites of chloramphenicol do not possess significant antibiotic activity, only intact chloramphenicol is measured providing that no other antibiotics or chemotheraputic agents are present. Because of the sensitivity of the turbidimetric method, it is useful in determining chloramphenicol levels in blood, serum and other clinical specimens.

Bioassays generally require long incubation times, which can delay results, and variable precision and also interference by other antibiotics in the samples are frequent problems.

(c). Chromatographic methods.

Various chromatographic methods, such as paper[44,45] and thin-layer chromatography[46-48], have been established in the past for the identification and determination of chloramphenicol and its metabolites in body fluids and to study the various aspects of chloramphenicol chemistry and pharmaceuticals.

Generally, these methods are time-consuming and less accurate than procedures based upon gas and high-performance liquid chromatography with increased specificity due to the potential for the separation of chloramphenicol from its degradation products.

(i). Gas chromatography.

Gas chromatography, in general, has not found wide-spread application in the analysis of antibiotics. This is principally because these drugs are frequently large, polar and thermolabile molecules which do not chromatograph or derivatise with efficiency.

Neverthless, gas chromatography has been applied to several antibiotic systems and perhaps most success has been obtained with chloramphenicol.

Shaw[49], in 1963, developed a gas chromatographic technique for analysis of chloramphenicol in the presence of structurally related compounds likely to be present in the growth medium or cell free extract of cultures

of S.venezuela.

This technique, which involved chromatography after conversion to trimethyl silyl derivatives, is complicated and time-consuming and also takes about 25 minutes for chloramphenicol to elute, which is a reasonably long time.

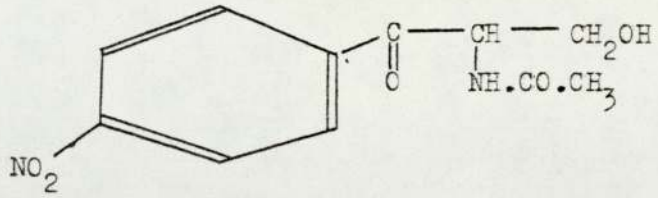
Davies[50] developed a gas chromatographic method for the determination of small amounts of the ortho- and meta- nitro isomers of chloramphenicol as possible impurities in chloramphenicol.

Yamamoto[51], in 1967, determined chloramphenicol in body fluids and pharmaceutical preparations by gas chromatography. Although this method has speed and specificity, the peaks are some what poorly resolved.

(ii). High-performance liquid chromatography.

Vigh and Inczedy[52], in 1974, separated some chloramphenicol intermediates and by-products by high-performance liquid chromatography. Although the procedure is rapid, the need for adjustment of pH of the eluent is a disadvantage.

Again, Vigh and Inczedy[53,54], in 1976, separated six intermediate products or impurities of chloramphenicol(scheme V) by high-performance liquid chromatography. These six compounds eluted within 28 minutes, which is a reasonably long time in terms of HPLC but is a significant improvement on other available methods.



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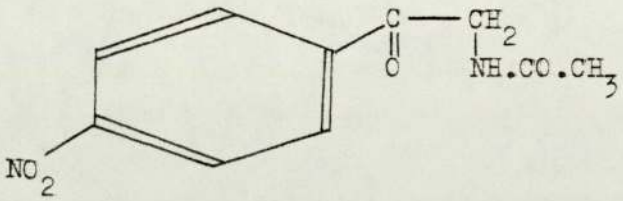

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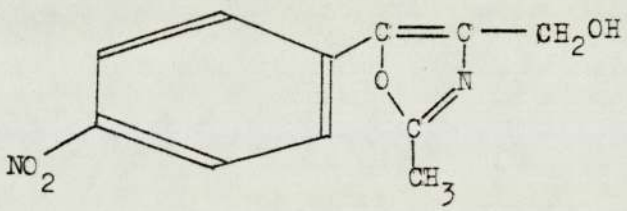

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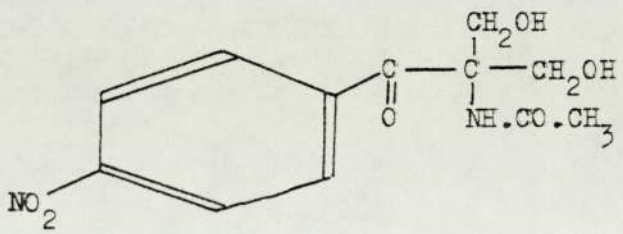
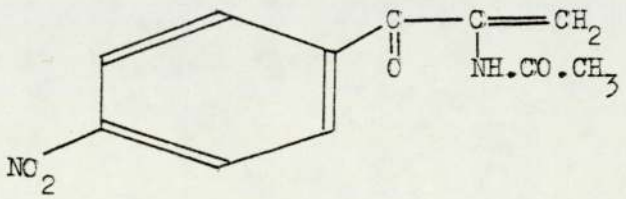
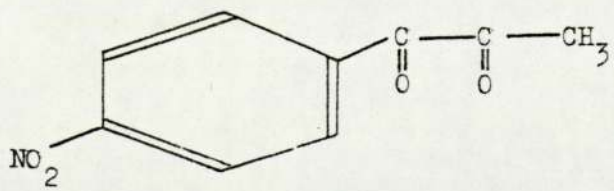


(iv)



(v)



(vi)

(SCHEME V)

Ali[55], in 1978, has separated some hydrolysis products of chloramphenicol by HPLC with a methanol-water-acetic acid solvent system. The appearance of the first product; nitrobenzyl alcohol, is after 5 minutes, a reasonably rapid elution. The peak area estimation technique was used.

Again; Nilsson-Ehle, et al[56], have established a HPLC procedure for quantitation of chloramphenicol in serum and cerebrospinal fluid(CSF). In this assay, chloramphenicol has been extracted in methanol by first standing for 10 minutes at room temperature and then centrifuging the sample for further 10 minutes, which decreases the rapidity of the assay. However, these methods either fail to distinguish between chloramphenicol and its metabolites or require a prior chromatographic separation in terms of the HPLC technique.

Recently, Le Belle, et al[68], have determined chloramphenicol and 2-amino-1-(p-nitrophenyl)-1,3-propanediol in pharmaceutical formulations, by HPLC.

In fact, this method is rapid, precise and accurate for the determination of chloramphenicol and 2-amino-1-(p-nitrophenyl)-1,3-propanediol in pharmaceutical formulations.

(B). RESULTS AND DISCUSSION.

Chloramphenicol, like many other biologically active molecules, has several polar functional groups. Two of these are hydroxyl groups on the 1,3-propanediol chain which are in a close spatial arrangement to one another. These hydroxyl groups, due to their polarity, prevent the efficient use of gas chromatography in the analysis of chloramphenicol. In order to be able to use this technique, it is important to convert the chloramphenicol molecule into a less polar derivative.

The preparation of some aromatic boronate esters of chloramphenicol has been described in conjuction with work on their anti-microbial properties [illegible]. The gas chromatography of these compounds was not described, however, and the demonstrated advantages of boronates in GC analysis of various compounds[57,58] suggested that a useful analytical method for the determination of chloramphenicol could perhaps be based upon these derivatives.

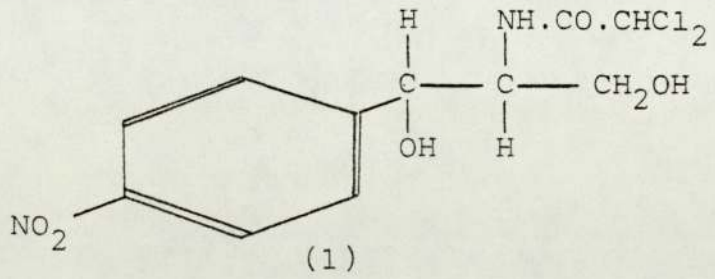
In a recent review, Nicholson[59], suggested that substituted boronic acids will react with a variety of compounds that have two functional groups, each with a reactive hydrogen atom, in close proximity. The more common reagents are methyl cyclohexyl-, phenyl boronic acid, and butyl boronic acid, the most frequently encountered was butyl boronic acid, $C_4H_9.B(OH)_2$, because of its stability and relatively short retention times.

(I). Preparation of the phenyl boronate derivative of chloramphenicol.

Chloramphenicol (0.5 gm.) was dissolved in ethyl acetate (5.0 mls.) and to it (10% excess) of phenyl boronic acid (0.209 gm.) was added. The mixture was placed under reflux for 15 minutes and was then allowed to cool. It was then evaporated to dryness under vacuum. The yellow crystals formed were recrystallised from ethanol (5.0 mls.). The light yellow powder formed was dried in an oven at 40°C under vacuum. The reaction of chloramphenicol (1) with phenyl boronic acid (9) is shown in scheme (VI).

The yield of crystals was(0.706 gm.) 99.58% and the compound melted at 173-174°C.

The purification of the ester derivative (10) was followed by infra-red spectroscopy and thin-layer chromatography. The structure of the compound was confirmed by mass spectrometry and ^1H n.m.r. spectroscopy.

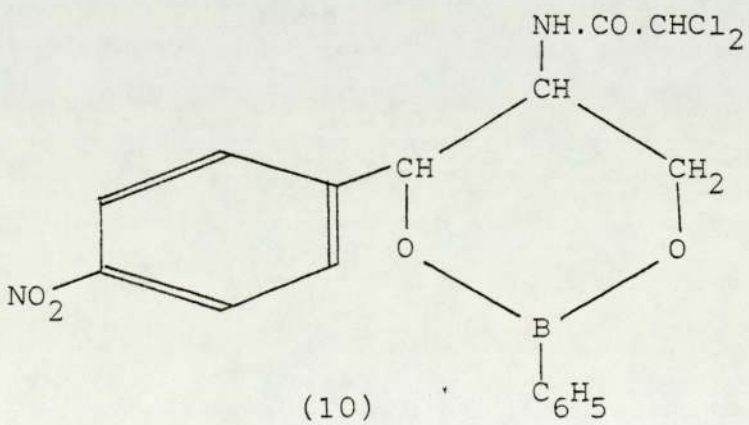


(1)

+

$C_6H_5.B(OH)_2$

(9)



(10)

(Scheme VI)

Formation of phenyl boronate of chloramphenicol ester(10) by esterification of chloramphenicol(1) with phenyl boronic acid(9)

(II). Spectra.

(a). Infra-red.

Fig.1 shows the infra-red spectrum of chloramphenicol(1) and fig.2 shows the infra-red spectrum of the phenyl boronate of chloramphenicol ester(10), both determined by the KBr disc method.

The appearance of a band at 1440cm^{-1}, in the phenyl boronate ester spectrum, may characterise the ν_{B-C} bond which is absent in the chloramphenicol spectrum, and may indicate ester formation. The absorption at 3500-3300 cm^{-1} due to bonded OH in the chloramphenicol spectrum has disappeared in the boronate ester spectrum, which may indicate the participation of these groups in the formation of the boronate ester derivative(10). The appearance of a band in the boronate ester spectrum in the region 3280 cm^{-1} is thought to be due to the amide N-H stretching vibration, which was evidently obliterated by the -OH groups in the chloramphenicol(fig.1). The carbonyl group, which absorbs at 1660 cm^{-1} in the phenyl boronate ester spectrum is also present at 1680 cm^{-1} in the chloramphenicol spectrum. The strong absorption at 1520 cm^{-1} and 1350 cm^{-1}, in both the chloramphenicol and phenyl boronate ester spectra, are probably due to the aromatic nitro, NO_2, symmetrical and asymmetrical stretching vibrations.

Schemes (VII) and (VIII) indicate the probable interpretation of the infra-red spectra by KBr disc method.

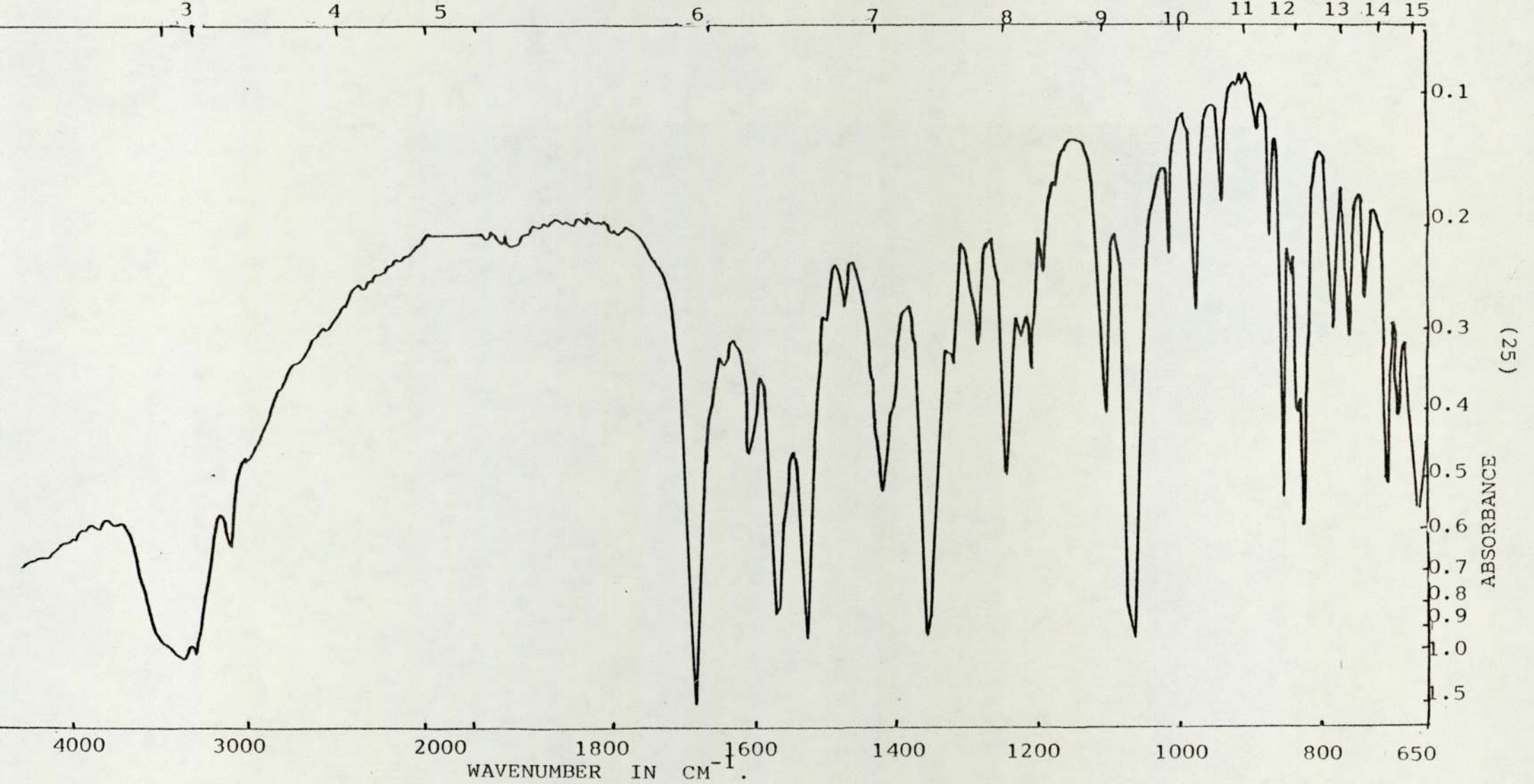


Fig.1. Infra-red spectrum of chloramphenicol by KBr disc.

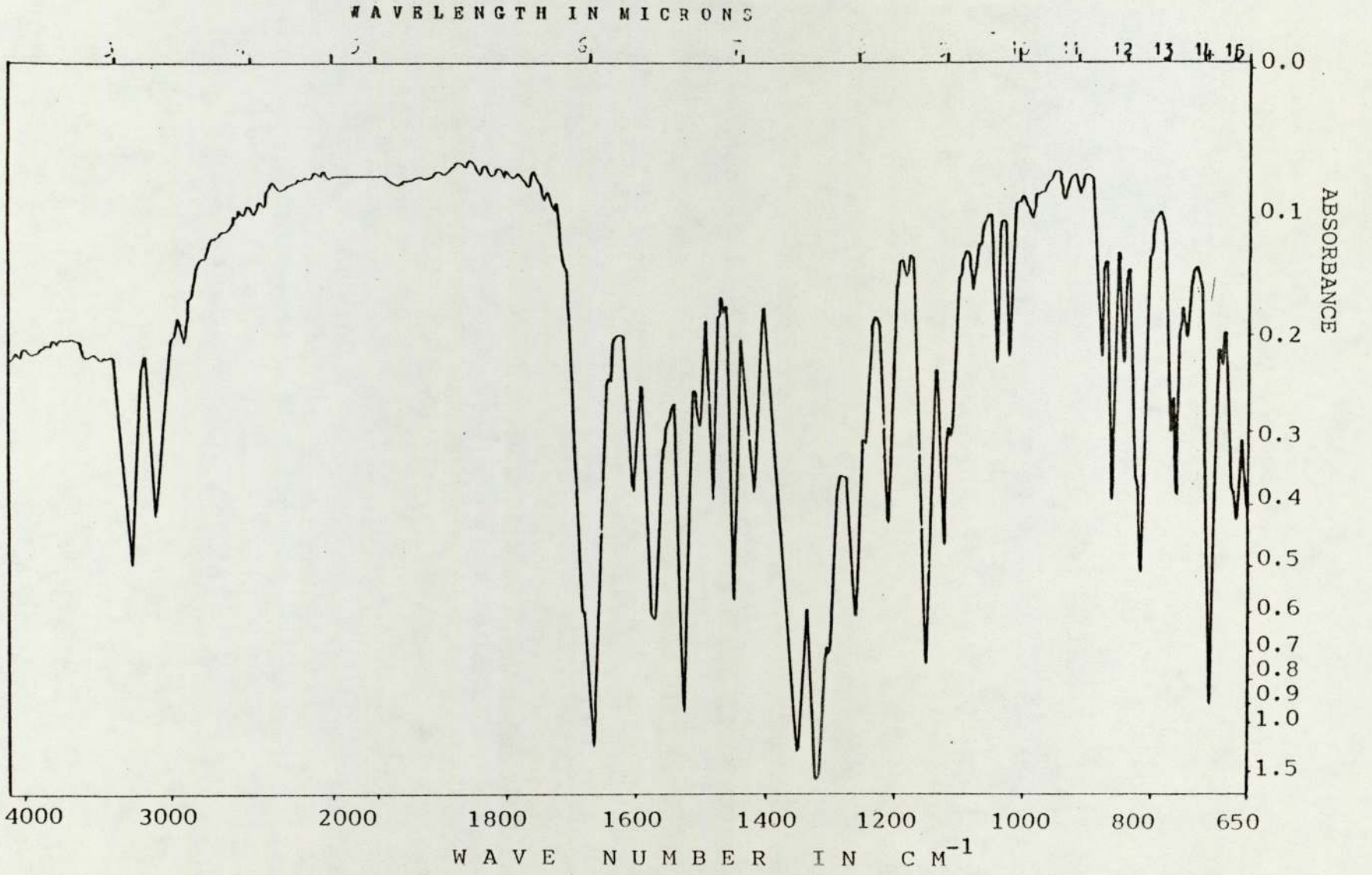
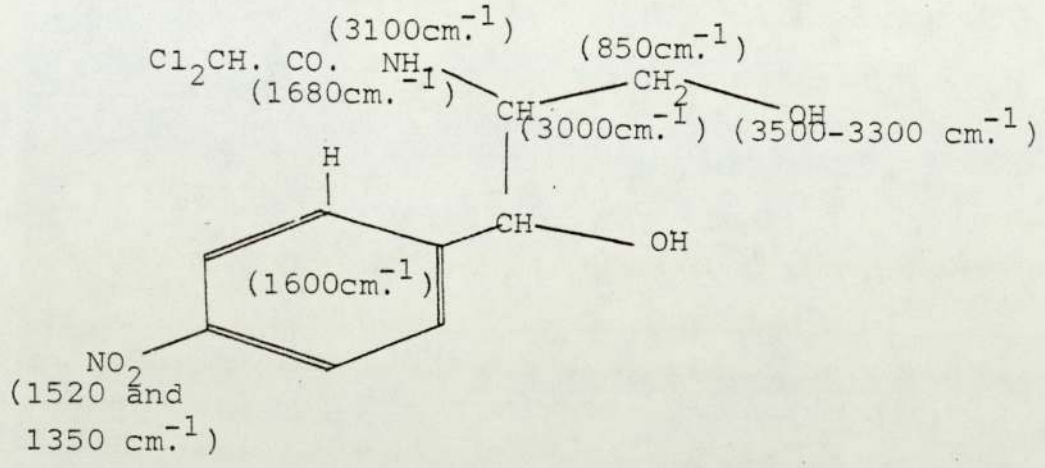
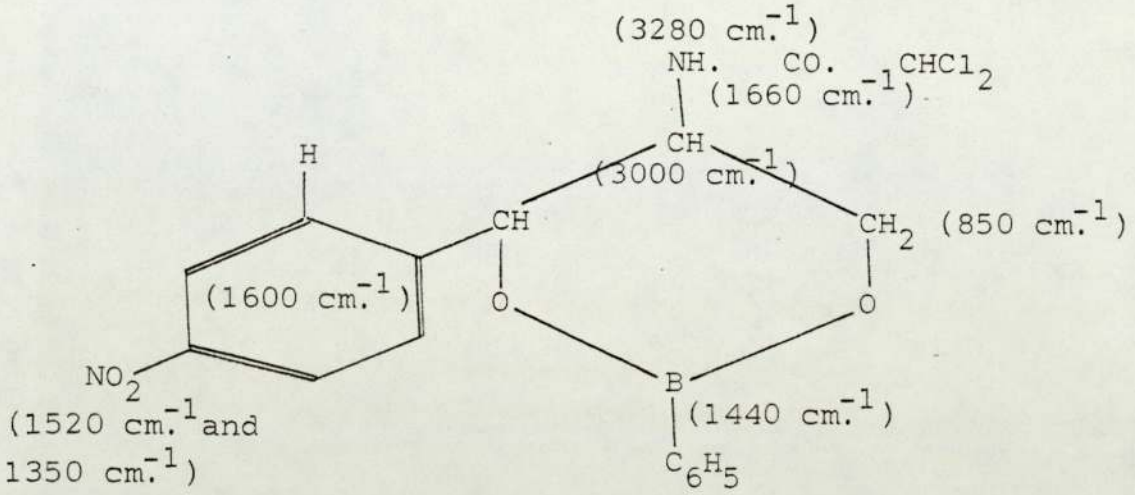


Fig. 2. Infra-red spectrum of phenyl boronate of chloramphenicol by KBr disc.



Scheme (VII) indicates the interpretation of infra-red spectrum of chloramphenicol by KBr disc method.



Scheme (VIII) indicates the interpretation of infra-red spectrum of phenyl boronate of chloramphenicol by KBr disc method.

(b). Nuclear magnetic resonance.

Fig.3 records the ^1H n.m.r. spectrum of the phenyl boronate of chloramphenicol ester(10), and fig.4 records the ^1H n.m.r. spectrum of chloramphenicol it self. A 5ppm expansion of phenyl boronate ester in the aromatic and 5-4σ region is displayed in fig.5.

The peak at 1.95-2.60τ, in the phenyl boronate ester spectrum is thought to be due to phenyl, $-C_6H_5$ group(e), which is not present in the chloramphenicol spectrum, and which confirms the formation of the cyclic ester. (a) protons of the compounds are identified by peaks at 5.43 τ and 5.73 τ in phenyl boronate ester spectrum and at 5.96 τ and 5.79 τ in chloramphenicol spectrum. (c) proton, in chloramphenicol spectrum is identified by peak at 5.43 τ, and in boronate ester spectrum at 5.25 τ. The proton of -OH as (b), in phenyl boronate spectrum, has disappeared which indicates the loss of hydroxyl, -OH, group during esterification. The protons of (f) and (h) portions, in both the chloramphenicol and boronate ester spectra, are identified by peaks at 1.77 τ, 2.33 τ, and 1.84 τ, 2.26 τ, respectively.Also, the strongest peak, in both spectra, at 4.45 τ and 4.35 τ, respectively, is attributed to a proton of a (d) portion of the compounds. Finally, the (g) proton, in chloramphenicol spectrum, at 2.34 τ, is identified at

3.20-3.50 τ in phenyl boronate spectrum which is thought to be shifted due to two chlorine atoms.

Schemes (IX) and (X) show the interpretation of ^{1}H n.m.r. spectra.

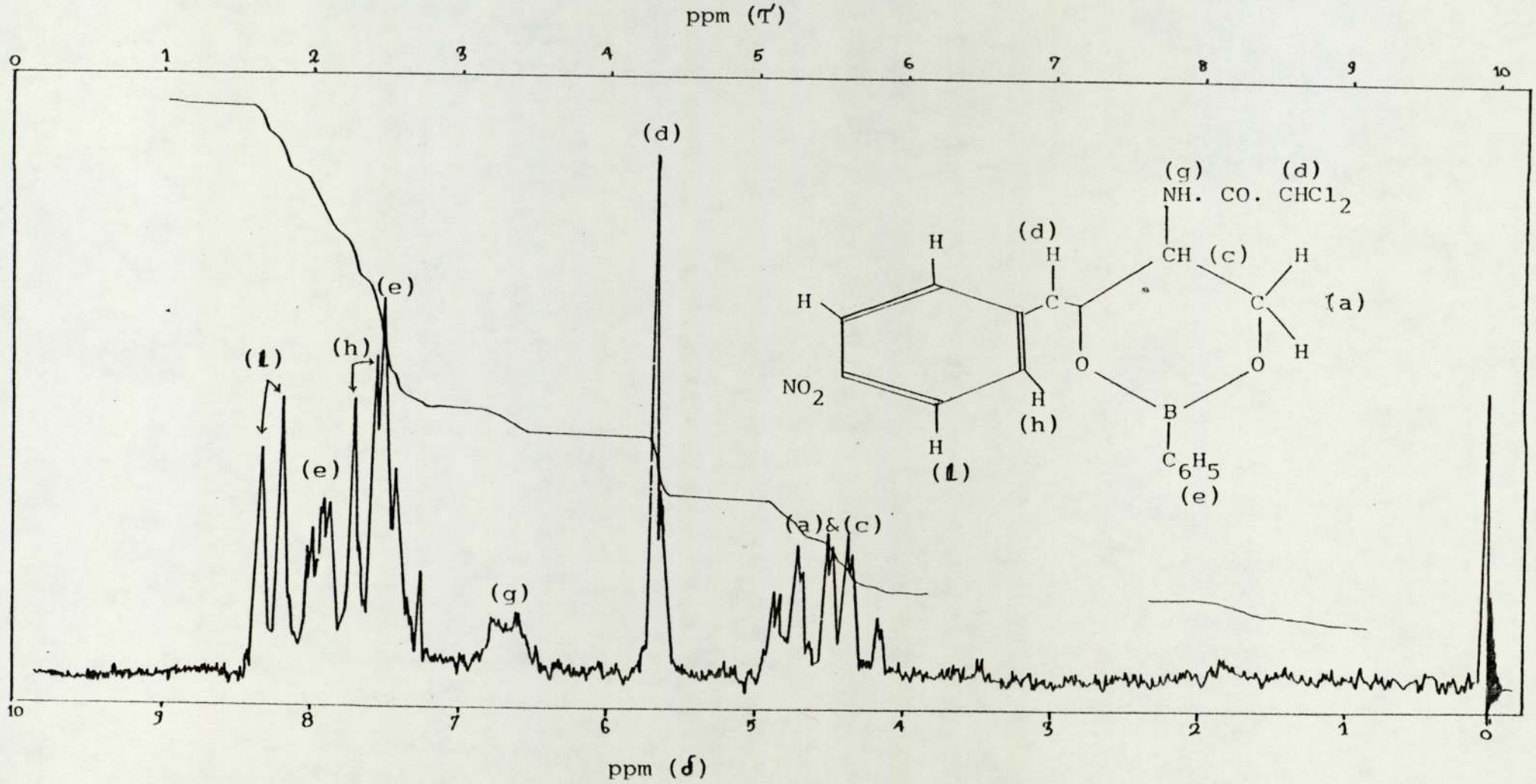


Fig.3. ^{1}H n.m.r. spectrum of phenyl boronate of chloramphenicol in $CDCl_3$.

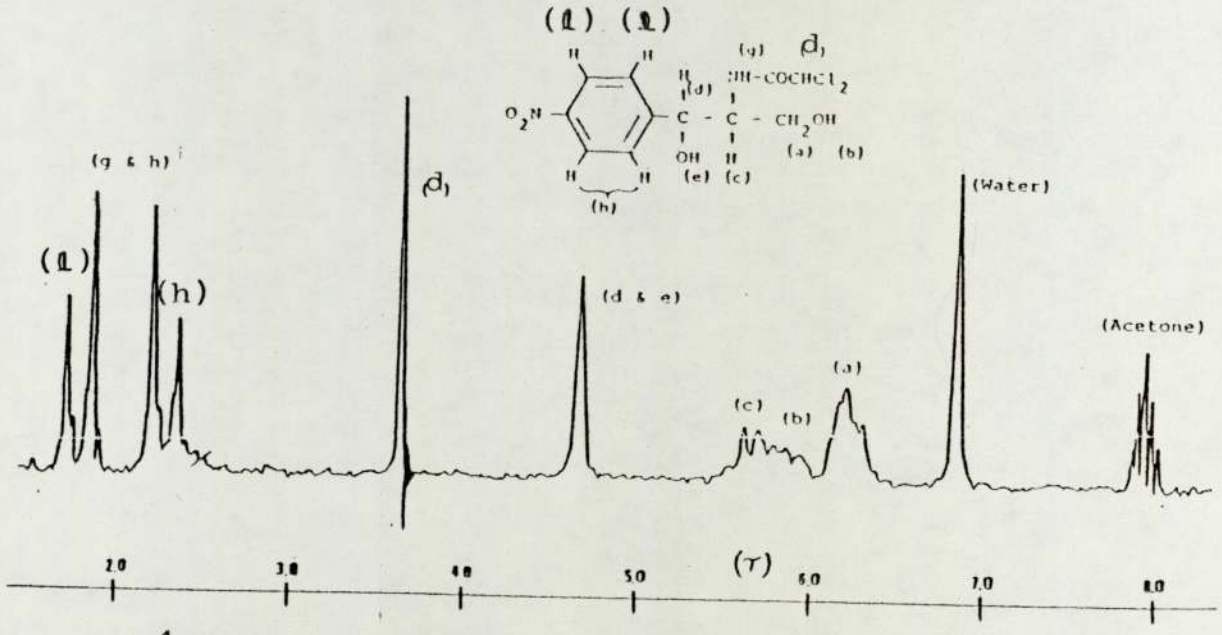


FIG. 4 - NMR SPECTRUM OF CHLORAMPHENICOL, PARKE DAVIS LOT H700171 IN DEUTERATED ACETONE CONTAINING TRIMETHYLSILANE AS INTERNAL STANDARD.

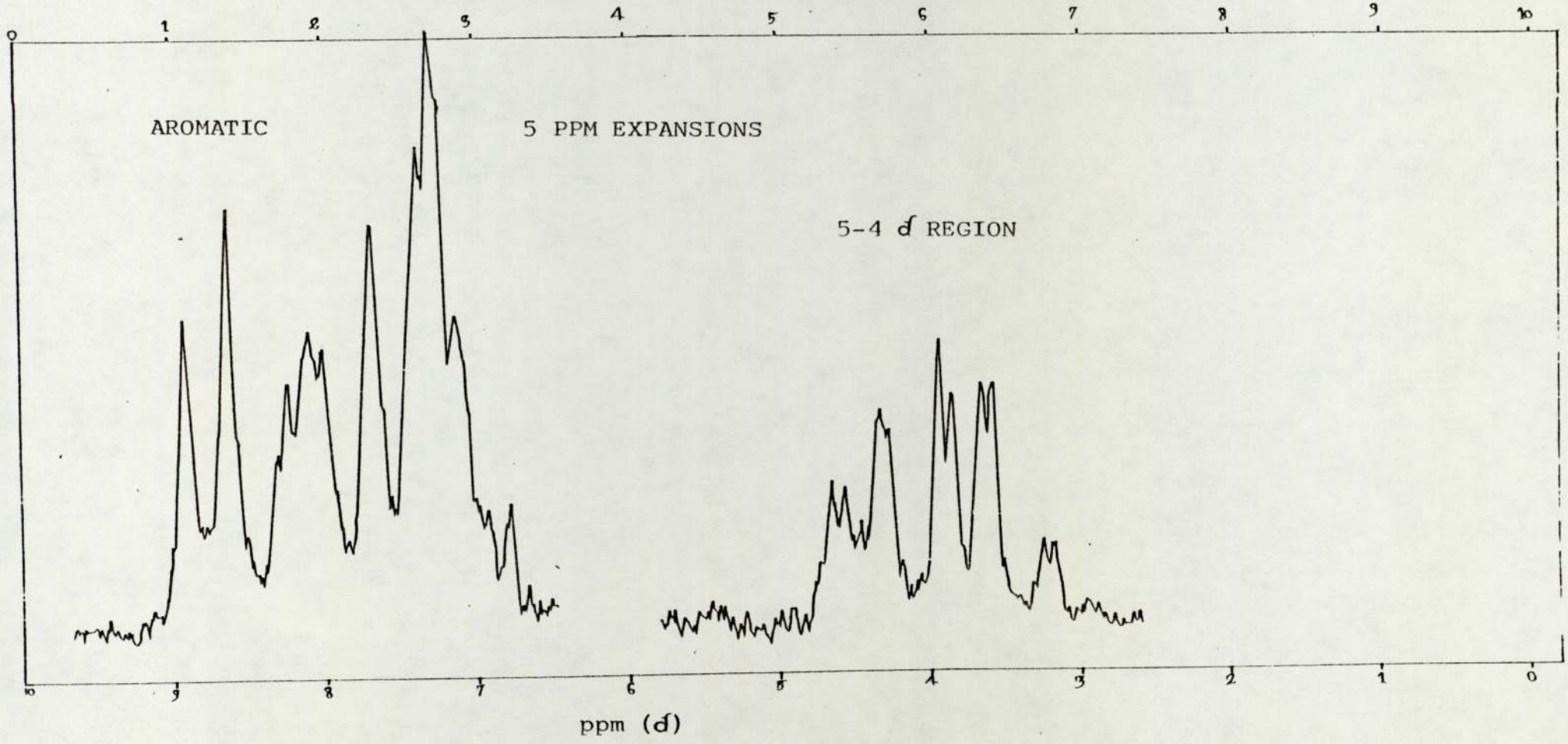
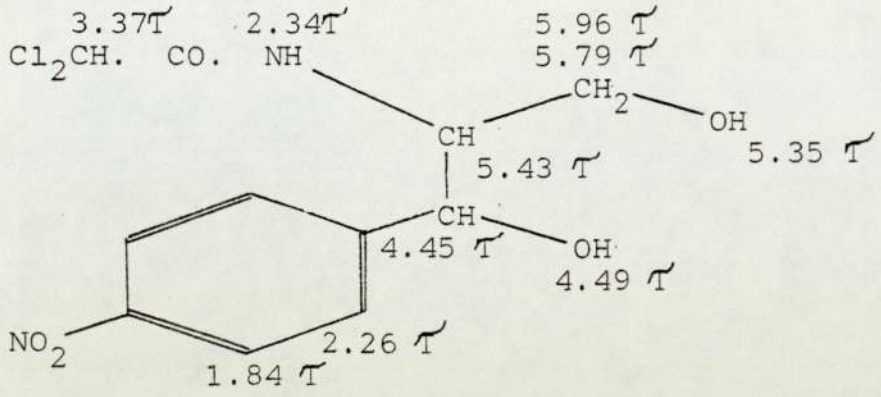
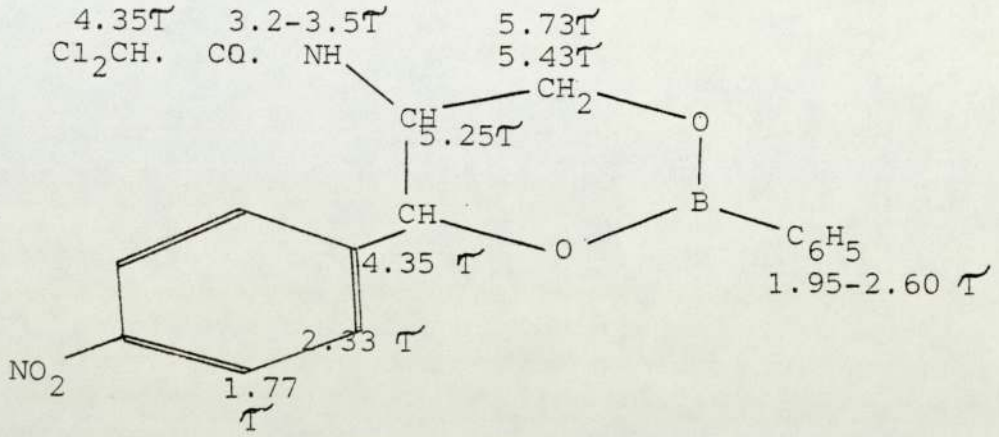


Fig. 5 . 5 ppm expansions of ^{1}H n.m.r. spectrum of phenyl boronate of chloramphenicol.



Scheme (IX) indicates the identified protons in chloramphenicol molecule by ^{1}H n.m.r.

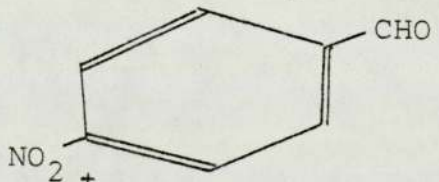


Scheme (X) indicates the interpretation of ^1H n.m.r. spectrum of phenyl boronate of chloramphenicol.

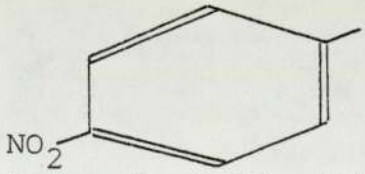
(c). Mass spectra.

Fig.6 indicates the mass spectrum of the cyclic phenyl boronate of chloramphenicol determined at an ionization energy of 70eV. Scheme (XI) records the assignments of the possible fragments in contrast to chloramphenicol, a small molecular ion (Mol.Wt.408) is observed.

The peak at M/e 281 with 47% relative intensity is thought to be due to loss of $(\overset{+}{M}-126+H)$, $NH_2.CO.CHCl_2$ fragment from the molecular ion $\overset{+}{M}$ =408 of the compound, while chlorine being ^{35}Cl isotope. Again, a peak at M/e 257 with 57% relative intensity is considered to be due to loss of $(\overset{+}{M}-151)$,

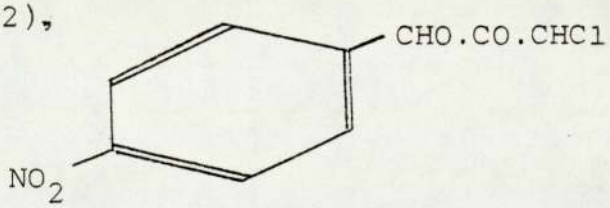


fragment from the molecular ion $\overset{+}{M}$=408. The peak at M/e 222 with 49% relative intensity could attributed by the loss of $(\overset{+}{M}-186)$,



fragment with ^{35}Cl isotope.

The peak at M/e 331 with 18% relative intensity is considered due to the loss of phenyl, $-C_6H_5$ fragment from the compound. The peak at M/e 153 with 78% relative intensity might have come from fragmentation $Cl_2CH.CO.NH.CH.CH_2^+$ by the loss of $(\overset{+}{M}-255)$ from the molecular ion $\overset{+}{M}$ = 408. The peak at M/e 146 with 63% relative intensity is considered due to the loss of $(\overset{+}{M}-262)$,



fragment from the compound. The peak at M/e 297 with 15% relative intensity is attributed due to the loss of ($\overset{+}{M}$-111), $CO.CH.Cl_2$ fragment from the compound with ^{35}Cl isotope. Again, a peak at M/e 324 with 13% relative intensity is considered due to the loss of ($\overset{+}{M}$-83+H), $-CHCl_2$ fragment from the compound. The base peak at M/e 51 is thought to be due to CH_3Cl fragment with ^{35}Cl isotope. The peak at M/e 77 with 52% relative intensity is attributed to phenyl, $-C_6H_5$ fragment by the loss of ($\overset{+}{M}$-331) from the molecular ion $\overset{+}{M}$ = 408. Finally, a peak at M/e 70 with 76% relative intensity is attributed to $CO.NH.CH.CH_2^+$ fragmentation due to the loss of ($\overset{+}{M}$-338) from the molecular ion.

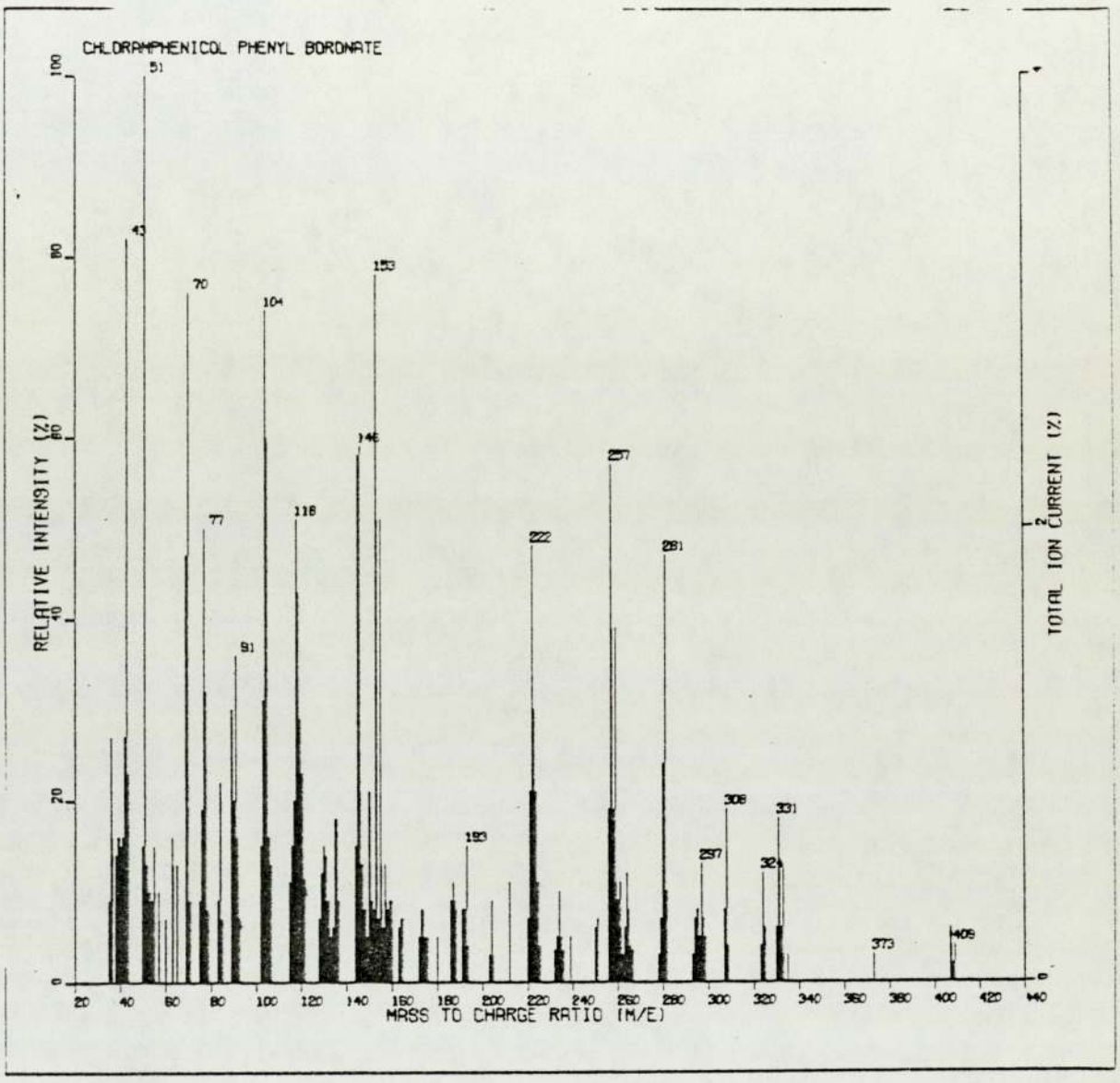
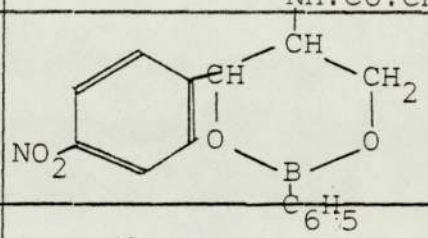
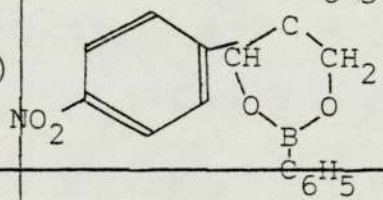
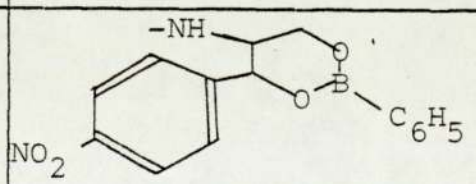
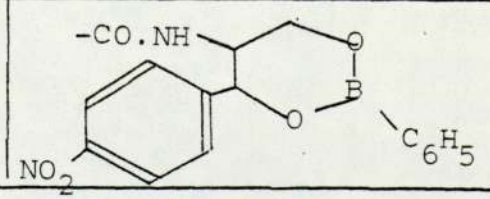


Fig.6. Mass spectrum of cyclic phenyl boronate ester of chloramphenicol at 70 eV ionization energy.

Scheme (XI), indicates the probable fragments of major peaks of phenyl boronate of chloramphenicol by mass spectrometry at 70eV ionising voltage, 3KV accelerating voltage and 300°C.

M/e	RELATIVE INTENSITY.	MOLECULAR ION, ($\overset{+}{M}$ = 408).	FRAGMENTATION.
331	18%	($\overset{+}{M}$-77)	
281	47%	($\overset{+}{M}$-126+H)	
257	57%	(M-151)	$C_6H_5.B{=}O{-}CH_2{-}CH_2{-}NH.CO.CHCl_2$
153	78%	($\overset{+}{M}$-255)	$Cl_2CH.CO.NH.CH.\overset{+}{C}H_2$
222	49%	($\overset{+}{M}$-186)	$C_6H_5.B{=}O{-}CH_2{-}CH_2{-}NH.CO.CHCl$
118	51%	($\overset{+}{M}$-192)	$ClCH.CO.NH.CH{-}\overset{+}{C}H_2$

Scheme (XI) continued.

M/e	RELATIVE INTENSITY.	MOLECULAR ION, ($\overset{+}{M}$ = 408).	FRAGMENTATION.
104	74%	($\overset{+}{M}$-306)	$C_6H_5.B=\overset{+}{O}$
77	50%	($\overset{+}{M}$-331)	$C_6H_5^+$-
70	76%	($\overset{+}{M}$-338)	$CO.NH.CH.CH_2^+$
51	100%	($\overset{+}{M}$-357)	CH_3Cl
43	82%	($\overset{+}{M}$-365)	$^+CH_2-CH_2-CH_3$
146	63%	($\overset{+}{M}$-262)	$C_6H_5-B=O-CH_2-CH_2-NH$
297	15%	($\overset{+}{M}$-111)	
324	13%	($\overset{+}{M}$-83+H)	

(III). Chromatography of chloramphenicol phenyl boronate.

The gas chromatographic properties of chloramphenicol phenyl boronate ester were explored. Initial conditions used a 10.0 $mg.ml.^{-1}$ solution of the boronate ester and a 2% OV-17 on chromosorb W, AW-DMCS, 2 metre glass column was chosen. The boronate ester eluted after 10 minutes with reasonably broad peaks. Then 1.5% OV-17 liquid phase was used in the same column, which did decrease the retention time (8 mins.) and increase the resolution of the boronate ester peak. At this stage, an internal standard, triphenyl benzene, was mixed with the boronate ester solution and the mixture was analysed on the latter column. This column eluted triphenyl benzene after 5 minutes and the boronate ester after 8 minutes, with still less sharp peaks. Again, when the length of column was reduced to 0.5 metre, it decreased the retention time of the boronate ester peak to 4 mins. and triphenyl benzene peak to 2 mins.(which is reasonably rapid elution), as well the peaks were reasonably sharp and well resolved.

The initial oven temperature was 200°C. At this temperature , the peaks were reasonably broad and also retention time was longer.Finally, at 260°C, a reasonable retention time was found with increased sharpness of the peak.

Again, the carrier gas (nitrogen) flow rate was started

from 40 mls.min.$^{-1}$. At this flow rate, the retention time was very long(10 mins.) for the boronate ester peak.Finally, at 60-70 mls.min.$^{-1}$ the flow rate of carrier gas gave best resolution and reasonably rapid retention time (4 mins.) for boronate ester to elute.

The change in flow rates of air and hydrogen gases did not effect too much upon the resolution and retention time of the peak, but at very low flow rates, 30 mls.min.$^{-1}$ for hydrogen and 100 mls.min.$^{-1}$ for air and also at very high flow rates, 150 mls.min.$^{-1}$ for hydrogen and 700 mls.min.$^{-1}$ for air, did decrease the resolution and height of the peak.

The over-all polarity of the chloramphenicol molecule was reduced by reacting with a weak acid, phenyl boronic acid, $C_6H_5.B(OH)_2$, to yield the cyclic phenyl boronate ester and the break down of the structure does not occur at the temperature required for complete volatilisation of the compound, so that it would give small retention time on the column.

Fig.7 is a calibration graph of boronate ester concentration plotted against the peak height ratio(^{drug} / std.) with equation $y = mx + C$, which is linear by passing through the origin.

The sensitivity of 0.01 mg.ml.$^{-1}$ of the compound has been achieved by this technique. This sensitivity may possibly be increased by the use of a electron-capture detector.

Fig.8 shows the chromatogram of the peaks obtained by gas chromatography.

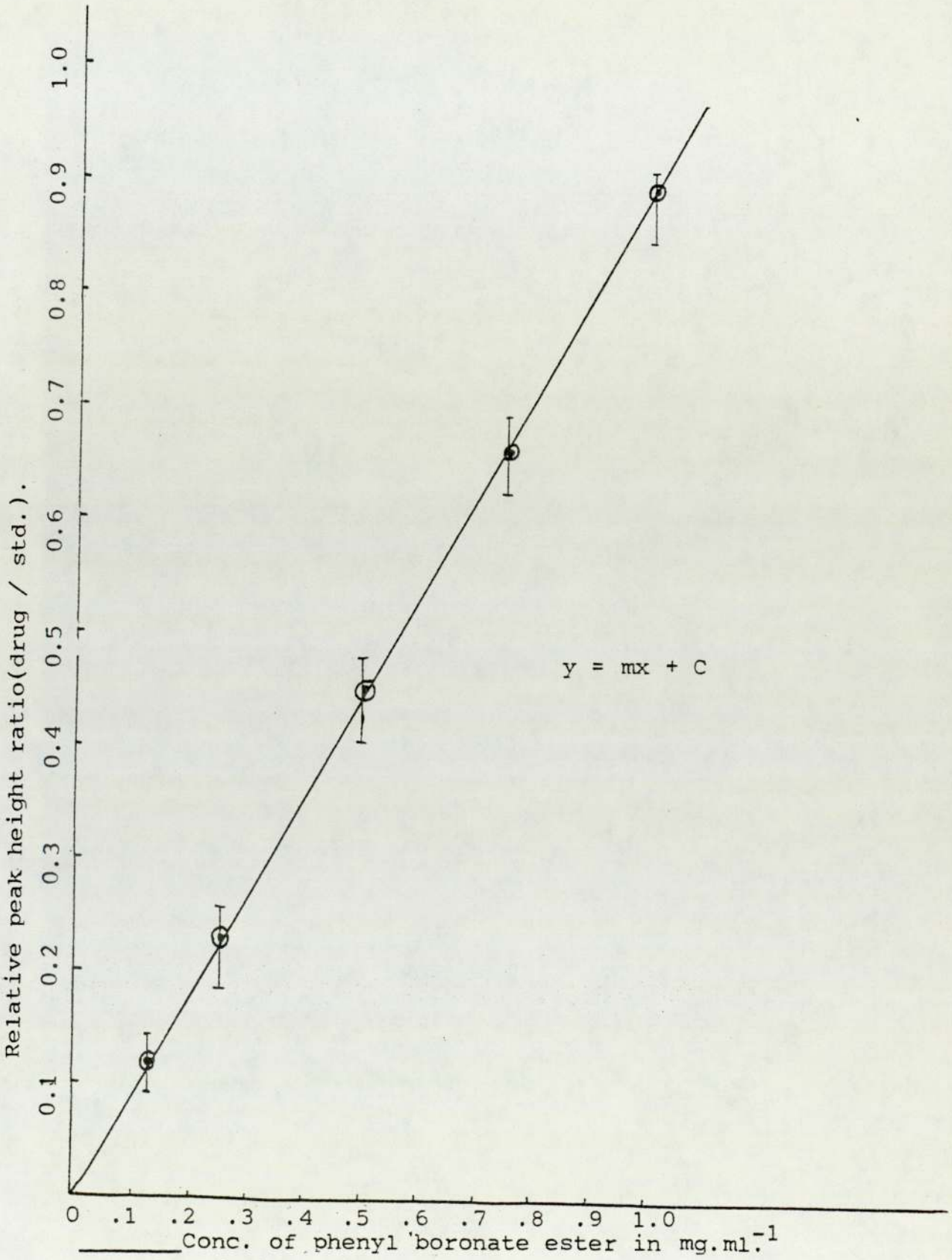


Fig. 7, indicates a calibration graph of phenyl boronate of chloramphenicol determination.

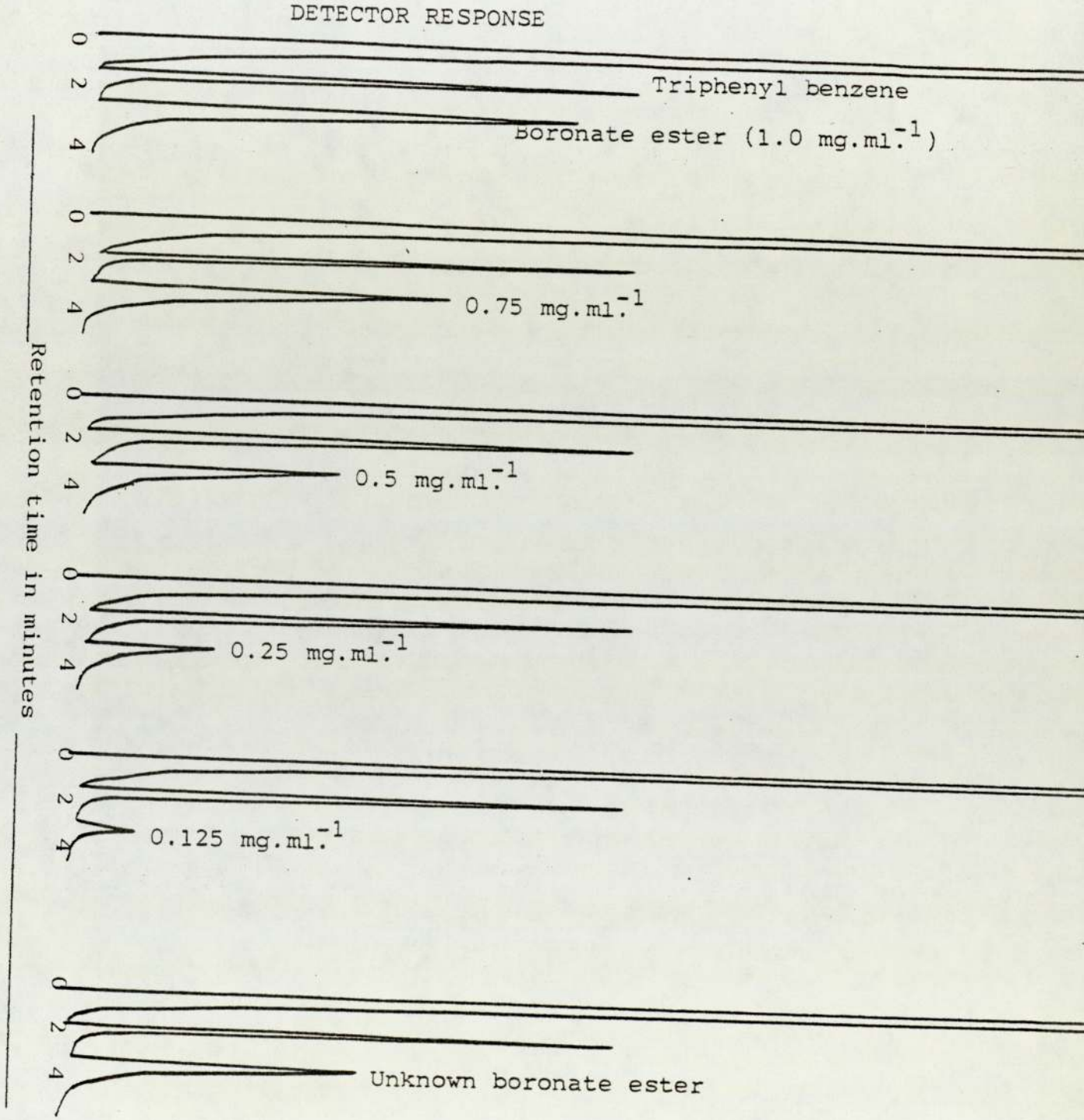


Fig. 8, is a chromatogram of phenyl boronate of chloramphenicol indicating the sensitivity of the GLC assay method.

(IV). Development of in situ assay procedure.

In order to apply the phenyl boronic acid derivitisation procedure to the gas-phase determination of chloramphenicol, it would be convenient[8] to prepare the derivative in situ. The use of the reagent in excess should ensure the complete formation of the cyclic ester and total reaction of the chloramphenicol. Un-reacted phenyl boronic acid is readily eluted during gas chromatography, in the form of its trimeric anhydride[58]. So that the excess of reagent presents no direct problem.

The chromatogram in fig.9 indicates how the chloramphenicol peak increases by the increase of phenyl boronic acid. When 7 $mg.ml.^{-1}$ of phenyl boronic acid is added to 0.5% chloramphenicol solution, the excess of boronic acid is eluted significantly while the formation of phenyl boronate ester is constant and no increase in peak height is observed when 8.0 $mg.ml.^{-1}$ of phenyl boronic acid is added to chloramphenicol solution.

Development time, for the formation of boronate ester, is within 5 minutes and the stability of the derivative is more than 10 weeks, when stored in a refrigerator.

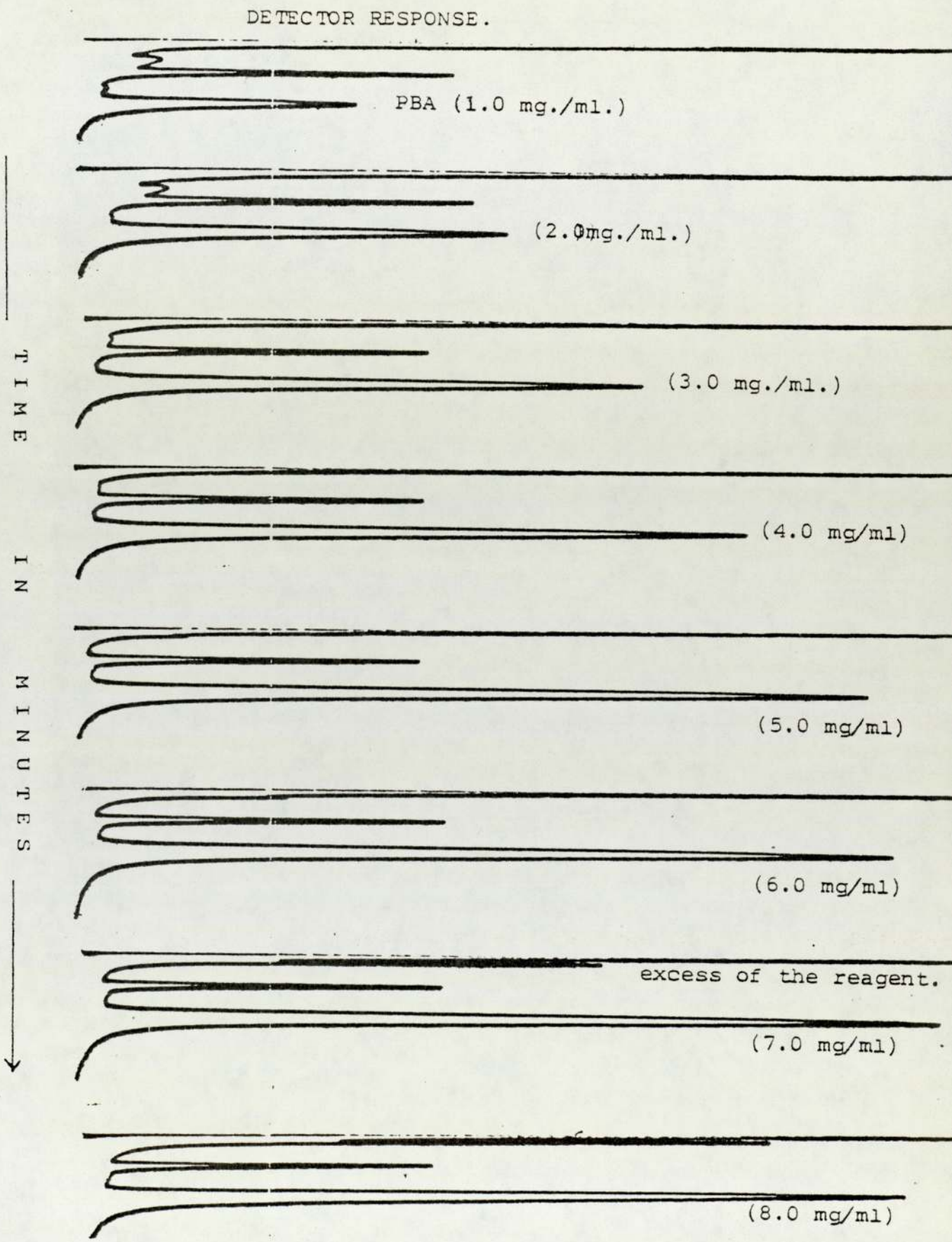


Fig. 9 , indicates the reaction of Chloramphenicol with varying amounts of Phenyl boronic acid.

(v). Development of an in situ assay.

When 150 mg., 100 mg., 50 mg., 25 mg., and 12.5 mg. of chloramphenicol(1) was dissolved in phenyl boronic acid(9) in ethyl acetate (1.0 ml.; 6.0 $mg.ml.^{-1}$) and mixed well and left for 5 minutes at room temperature, then triphenyl benzene in ethyl acetate (0.2 ml.; 3.0 $mg.ml.^{-1}$) as internal standard was added to each solution and the contents were thoroughly mixed. Finally, 1 µl from each solution was taken into 5 µl syringe and injected into the GC column.

The detector response was linear as shown in fig.10, which indicates the calibration graph of concentration of chloramphenicol plotted against the relative peak height ratio (drug / std.).

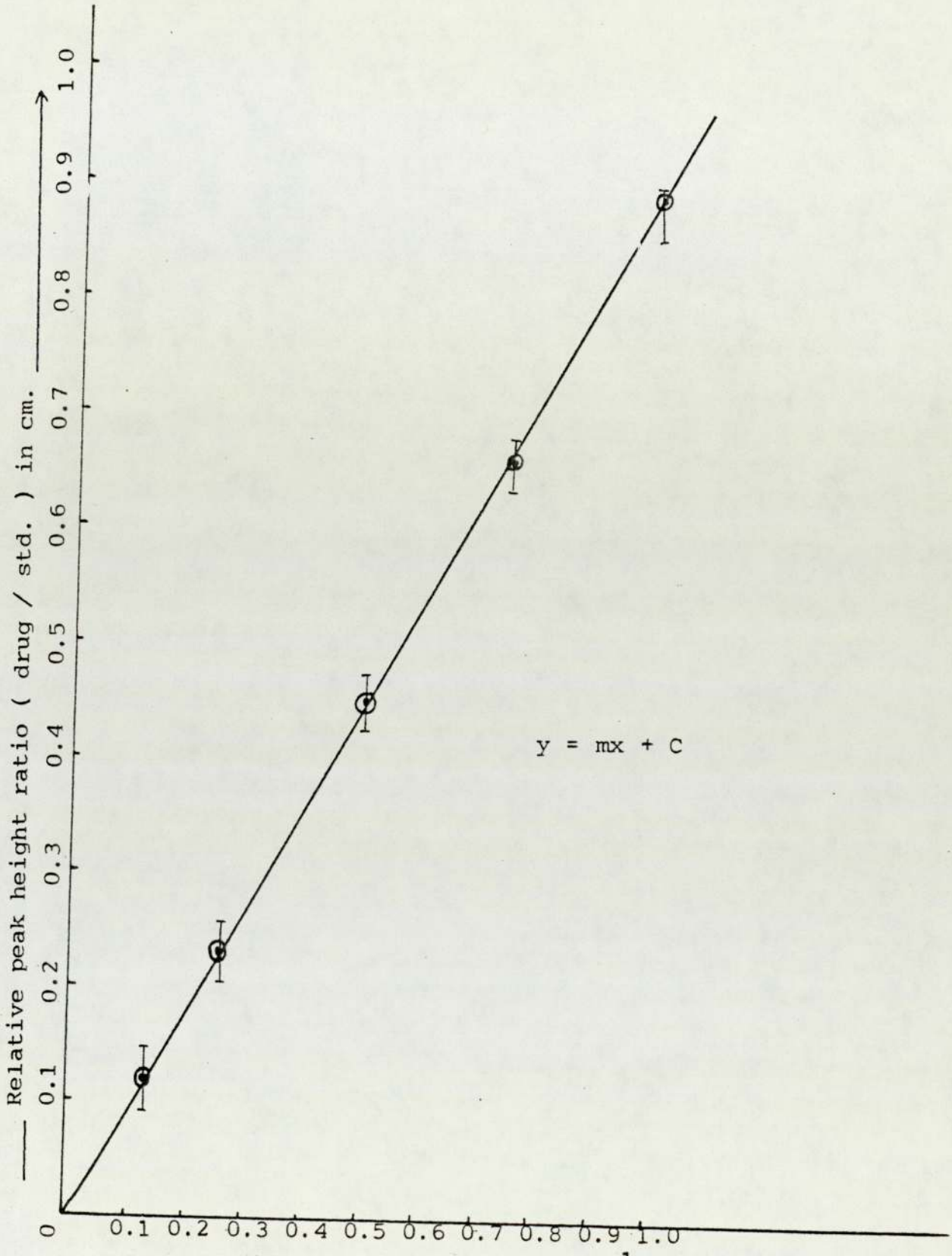


Fig.10. Calibration graph of chloramphenicol.

(VI). Detection limit of chloramphenicol.

The detection limit of 0.01 $mg.ml.^{-1}$, for chloramphenicol, has been achieved using a Pye unicam 104 gas chromatogram equipped with dual flame ionization detector using 20×10^{1} amplification while other conditions remain the same.

(VII) Assay of chloramphenicol in pharmaceuticals.

The assay procedure based upon the derivitisation of chloramphenicol to yield the cyclic phenyl boronate ester is very satisfactory for the analysis of bulk samples of chloramphenicol. To extend this to the assay of pharmaceuticals, however, there are certain problems to be over-come. These are concerned with the extraction of the drug efficiently from the formulations so that quantitative results may be obtained to achieve adequate sensitivity for the concentrations encountered, and to efficiently derivatise the drug so that reliable estimates of the chloramphenicol level in the formulations may be obtained.

The assay method has been extended to determine the amount of antibiotic present in several formulations, chloramphenicol capsules, eye-drops, ear-drops, eye-ointment, cream, and solutions A, B, and C.

(a). Chloramphenicol assay.

Bulk samples of chloramphenicol (0.05-0.30 gm.) were dissolved in ethanol (4.0 ml.) and the solutions were diluted to 10.0 mls. with distilled water. An aliquot (1.0 ml.) was diluted to 5.0 mls. with distilled water and 1.0 ml. of the resulting solutions($1.0-6.0 mg.ml.^{-1}$) were extracted with ethyl acetate(1.0 ml.). To the separated ethyl acetate layers(0.5 ml.) were added phenyl boronic acid in ethyl acetate($1.0 ml.; 1.0 mg.ml.^{-1}$) and triphenyl benzene in ethyl acetate($0.2 ml.; 0.5 mg.ml.^{-1}$)

as internal standard. The mixtures were allowed to stand for 5 minutes at room temperature and then 1 µl was injected into the gas chromatographic column.
A calibration curve of response ratio V/S concentration of the drug was constructed.

(b). Capsule assay.

Chloramphenicol capsules contain 250 mg. of the drug. This drug is not readily soluble in water. It was possible to dissolve the contents of a capsule in water by warming the solution, but the dangers of degradation of the drug suggested that this was not an ideal procedure.

The equivalent of the contents of one capsule was dissolved in ethanol(20.0 mls.) and this solution was diluted to 100 mls. with distilled water(2.5 $mg.ml.^{-1}$). Extraction of this solution(1.0 ml.) with ethyl acetate and derivitisation as in (a) was performed.

Table 1 contains the statistics of the quantitative determination of chloramphenicol in pharmaceuticals, which indicates that 2.434 $mg.ml.^{-1}$(97%) of the drug is recovered in a capsule.

(c). Eye drops assay.

Chloramphenicol eye-drops(BPC) contain chloramphenicol 500 mg., boric acid 1.5 gm., borax 300 mg., and phenyl mercuric acetate or nitrate 2 mg., and volume upto 100 mls. with distilled water.

The eye-drops solution(1.0 ml.;nominally 0.5% w/v in a borate buffer) was diluted to 5.0 ml. with distilled water(1.0 $mg.ml.^{-1}$). Extraction of 1.0 ml. of this solution with ethyl acetate (1.0 ml.), followed by derivitisation as in (a) was performed.

Fig.11 shows the GC traces of the formulations including eye-drops and table 1 contains the statistics of the estimation of chloramphenicol as 0.975 $mg.ml.^{-1}$ (97.5%) recovered in the eye-drops.

(d). Ear drops assay.

The ear-drops solution (0.1 ml.; nominally 10% w/v in propylene glycol, $CH_3.CH(OH).CH_2(OH)$), were diluted to 5.0 mls. with distilled water(2.0 $mg.ml.^{-1}$). Extraction of 1.0 ml. into ethyl acetate(1.0 ml.) and derivitisation as in (a) was performed.

Fig.11 shows the GC traces of the formulations including ear-drops and table 1 contains the statistics of the estimation of chloramphenicol as 1.958 $mg.ml.^{-1}$ (98%) recovered in the ear-drops.

(e). Eye ointment assay.

The eye-ointment (0.5 gm.; nominally 1% w/w in a liquid paraffin-wool fat base) was dissolved in petroleum ether(3.0 ml.; 40-60^{o}C fraction) and aceto nitrile, CH_3CN, (2.0 ml.)was added. After agitation the phases were separated by centrifugation and the lower layer of aceto nitrile(0.5 ml.) was derivatised by the

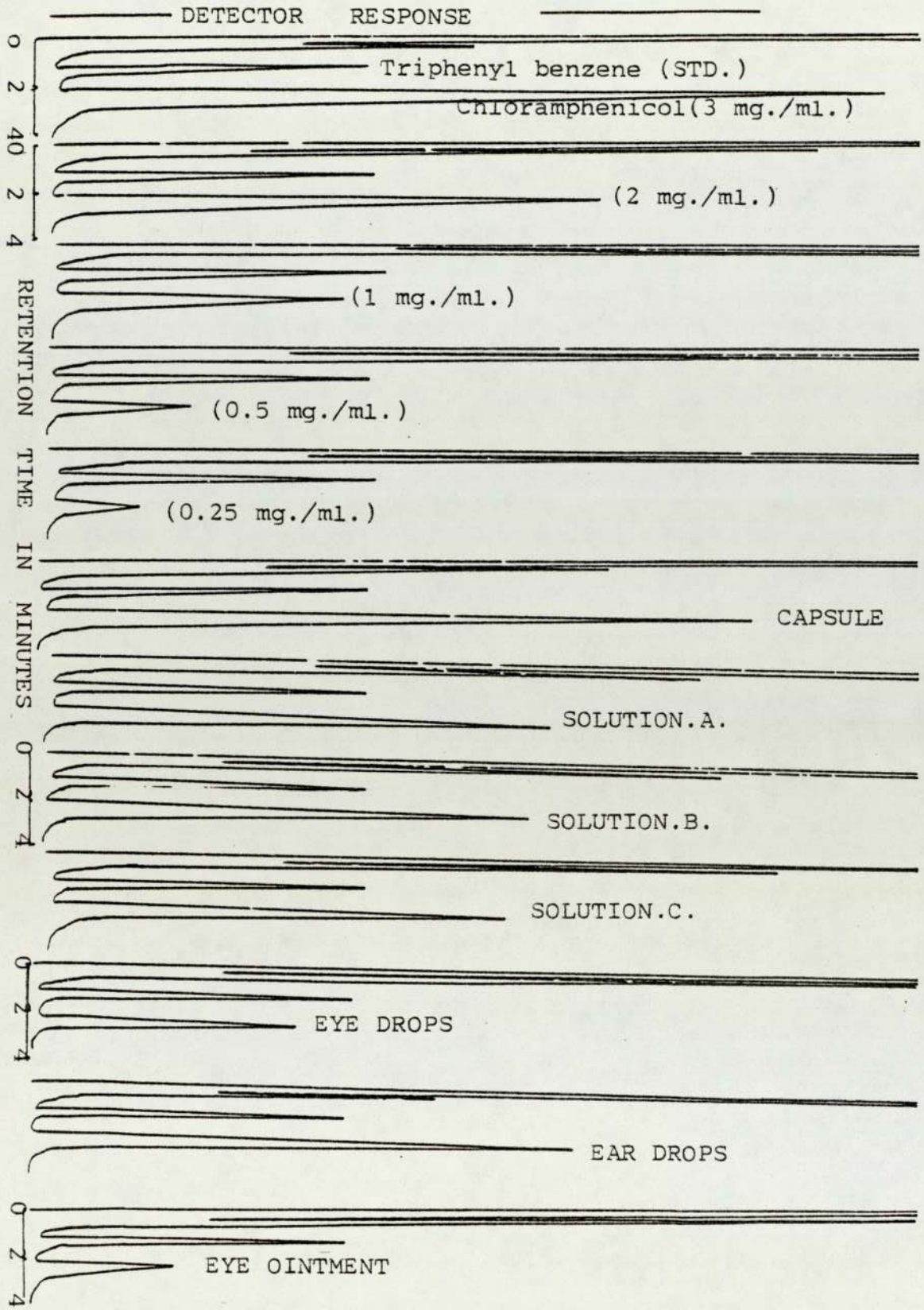


Fig. 11 , indicates the chromatogram of chloramphenicol in pharmaceuticals.

Table 1, indicates computer results for the estimation of chloramphenicol in pharmaceutical preparations.

****

4:4:79 PEAK HEIGHT RATIO CHLORAMPHENICOL IN FORMULATIONS.

THERE ARE 5 X-VALUES AND 5 REPLICATES OF EACH Y-VALUE

	X-VALUES	Y-VALUES				
1	3.000	2.63	2.62	2.63	2.62	2.63
2	2.000	1.75	1.75	1.75	1.75	1.75
3	1.000	0.90	0.90	0.90	0.90	0.90
4	0.500	0.45	0.45	0.45	0.45	0.45
5	0.250	0.26	0.27	0.26	0.26	0.26

B (SLOPE) = 8.6181231E-01 A(INTERCEPT)= 3.5345385E-02

SOURCE OF VARIANCE	DEGREES OF FREEDOM	VARIANCE	VARIANCE RATIO
REGRESSION	1	19.3107	4036843.1784
DEVIATION	3	0.0007	155.1900
BETWEEN X-S	4	4.8282	1009327.1871
RESIDUAL	20	0.0000	
TOTAL	24	0.8047	

THE F STATISTIC (1,3) = 26012.2702 (100.00%)

THE VARIANCE OF Y ABOUT THE REGRESSION LINE IS 0.0001

COVARIANCE = 0.9336 CORRELATION COEFFICIENT = 0.9999

THIS ACCOUNTS FOR 99.9880% OF THE VARIATION OBSERVED

Table 2 indicates the comparison of chloramphenicol originally present in the drug with estimated amount of chloramphenicol by GLC assay method.

DRUG	CP ORIGINALLY PRESENT IN DRUG, $mg.ml.^{-1}$	CP ESTIMATED BY GLC ASSAY, $mg.ml.^{-1}$	ERROR $mg.ml.^{-1}$	ACCURACY	95% CONFIDENCE LIMIT.
CAPSULES	2.5	2.434	0.07	97.4%	2.421 TO 2.447
SOLUTION "A"	1.80	1.794	0.006	99.7%	1.781 TO 1.806
SOLUTION "B"	1.75	1.745	0.005	99.71%	1.733 TO 1.757
SOLUTION "C"	1.65	1.64	0.01	99.4%	1.628 TO 1.652
EYE-DROPS	1.0	0.975	0.025	97.5%	0.967 TO 0.981
EAR-DROPS	2.0	1.958	0.042	98.0%	1.945 TO 1.970
EYE OINTMENT	0.5	0.48	0.20	96.1%	0.465 TO 0.490

Table 3. Data for 3 replicate runs of peak height ratio for chloramphenicol determination in pharmaceutical preparations by GLC assay method.

	1	2	3	MEAN	S.DEV.	CV(%)
a	17.55	17.58	17.56			
a/std.	2.627	2.624	2.629	2.627	0.0027	0.1036
b	11.75	11.70	11.80			
b/std.	1.754	1.751	1.748	1.751	0.0027	0.1518
c	6.15	6.10	6.08			
c/std.	0.904	0.902	0.901	0.902	0.002	0.2169
d	3.02	3.08	3.00			
d/std.	0.452	0.454	0.451	0.452	0.001	0.2268
e	1.75	1.78	1.77			
e/std.	0.262	0.266	0.264	0.264	0.0021	0.8123

Where a, b, c, d, and e are the peak heights of chloramphenicol, in cm.

addition of phenyl boronic acid in ethyl acetate (1.0ml.; 1.0 mg.ml.$^{-1}$). Then triphenyl benzene in ethyl acetate (0.2 ml.; 0.5 mg.ml.$^{-1}$) as internal standard was added to the solution. It was then mixed well and 1 µl was directly injected into gas chromatographic column. Fig. 11 shows the GC traces of the estimation of chloramphenicol in formulations including eye-ointment, and table 1 contains the statistics of the estimation of chloramphenicol as 0.48 mg.ml.$^{-1}$(96.1%) recovered in the eye-ointment.

The problem of dissolving the eye-ointment into a suitable solvent and extracting the drug into the efficient reagent was considered seriously as the ointment is not soluble in water.

The eye-ointment was found to dissolve in petroleum ether(40-60^{o}C) and hexane solvents. But the former was more efficient. As ethyl acetate is miscible with petroleum ether and also in hexane, the extraction of the drug into ethyl acetate was not possible. Although,it was tried to extract the drug into ethyl acetate together with dimethyl sulfoxide, $CH_3.\underset{\underset{O}{|}}{S}.CH_3$,and also with aceto nitrile, $CH_3.CN$, but the extraction by this method decreased the efficiency of extraction of the drug by 13% compared to that only into aceto nitrile, or dimethyl sulfoxide.

The reagents aceto nitrile and dimethyl sulfoxide were found suitable for the extraction of chloramphenicol from the eye-ointment, although aceto nitrile extracted about 3% more than dimethyl sulfoxide.

(f). Cream assay.

Chloramphenicol cream contains 1.0 gm. chloramphenicol 50 gm. propylene glycol, and 49 gm. macrogol 4000. As macrogol 4000 is an emulsifying agent, the problem of a very thick emulsion after adding water, was created. To a certain extent, this problem of emulsion formation was over-come by adding 0.5 gm. of the potassium chloride into the mixture of chloramphenicol cream (0.15 gm.) dissolved into ethanol(1.0 ml.) and volume upto 5.0 mls. with distilled water and chloramphenicol extracted into ethyl acetate(1.0 ml.). But the sensitivity of this procedure on GC was very poor and it could not improved by other means.

(g). Solutions A, B, and C.

A and B are the solutions in which 1.80 $mg.ml.^{-1}$ and 1.75 $mg.ml.^{-1}$ of the chloramphenicol, respectively, was dissolved in ethanol, and solution C contained 1.65 $mg.ml.^{-1}$ of chloramphenicol together with sulphacetamide dissolved in ethanol.

Each solution (1.0 ml.) was diluted to 5.0 mls. into distilled water. Extraction of this solution with ethyl acetate (1.0), followed by derivitisation as in (a) was performed.

Table 1 indicates the statistic data of computer results, which estimates the amount of the drug present in the solutions, and fig. 11 shows the GC traces of the estimation of antibiotic in formulations including solutions A, B, and C.

(VIII)Estimation of chloramphenicol in pharmaceuticals.

Fig. 12 shows the calibration plot for the estimation of chloramphenicol in pharmaceutical preparations, e.g., capsule, eye-drops, ear-drops, eye-ointment, and solutions A, B, and C, which gives a linear response. Fig. 11 is the chromatogram for the estimation of chloramphenicol in pharmaceutical preparations, which indicates that none of excipients have interfered with the estimation of the drug.

Table 1 records typical computer results for this plot estimating the amount of chloramphenicol in the pharmaceutical preparations with some parameters and table 2 shows the accuracy, precision, and extraction efficiency of this assay method. Table 3 shows the data for 3 replicate runs of peak height ratio (drug / std.) for chloramphenicol determination in pharmaceutical formulations by GLC technique.

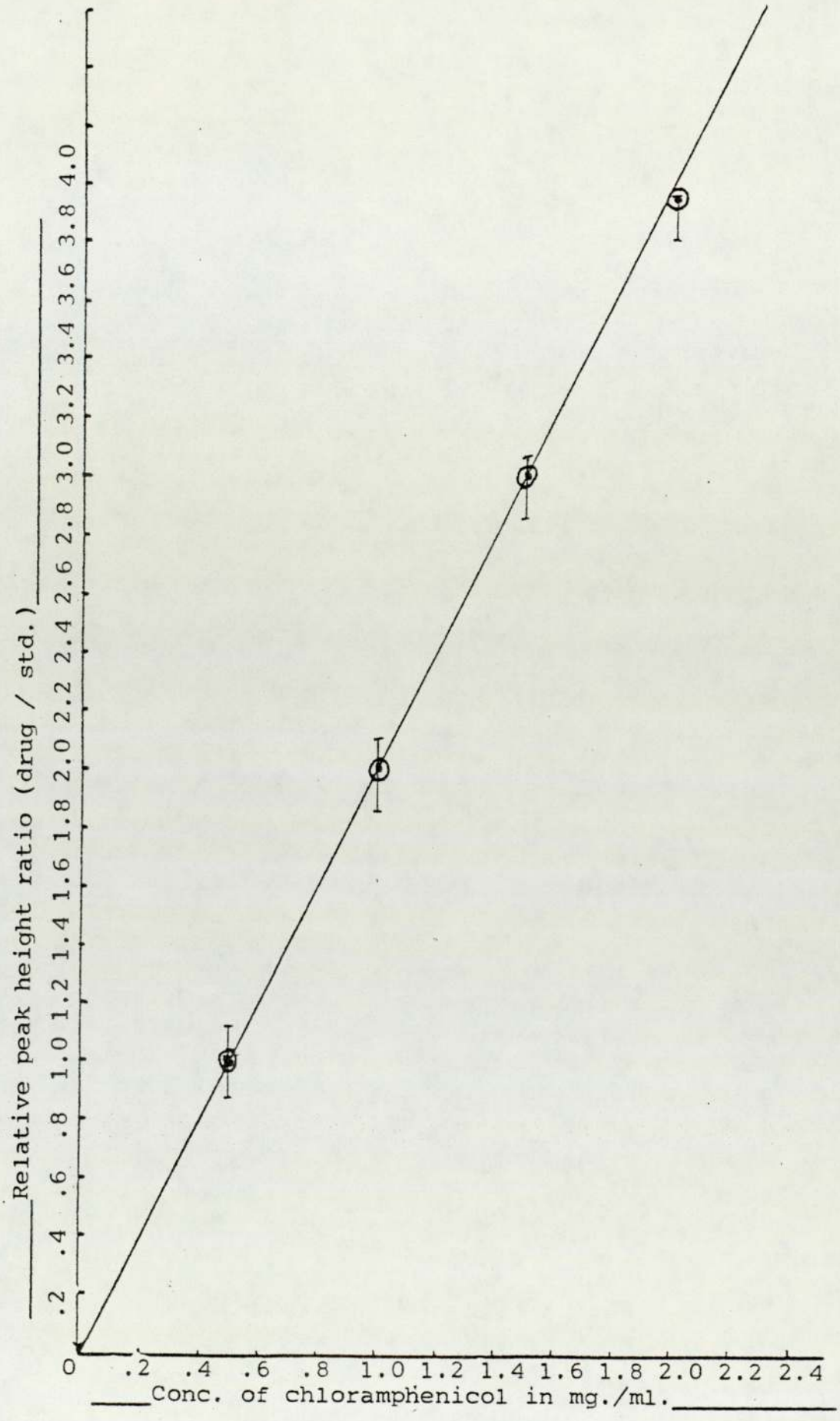


Fig. 12, indicates the calibration graph for the estimation of chloramphenicol in pharmaceuticals.

(IX). Hydrolytic degradation of chloramphenicol.

This GC assay technique may also be used to monitor the kinetic degradation of chloramphenicol. It is useful because degraded samples may then be assayed or kinetic profiles of chloramphenicol degradation may be obtained. Acetate buffers of pH in the range of 3-6 with buffers strength from 0.05N to 1.5N were used and degradations were monitored at 89°C (± 1°C).

The extent of any degradative reaction can be readily established by analysing solutions of chloramphenicol at various stages of degradation for the residual drug. Any differences between initial concentration of chloramphenicol and that of residual chloramphenicol after degradation can be attributed to the formation of amine[26].

This comparison has been carried out for aqueous solutions of chloramphenicol in acetate buffer at pH 3.95, 4.1, 4.5, 4.9, and 5.6 and at buffer strengths 0.05N, 0.1N, 0.25N, 0.5N, 1.0N and 1.5N utilizing GLC technique for quantification of the amount of chloramphenicol present in the degraded solutions.

The rate of antibiotic disappearance from buffered aqueous solutions appears to be first order with respect to antibiotic concentration (fig. 13). This relationship is apparently valid over widely differing hydrogen-ion-concentrations (fig. 14).

The linear logarithmic plots have been found to be

extremely reproducible under all encountered experimental conditions and in no case have they deviated significantly from the over-all first order relationship. The rate equation for a first order reaction is expressed as:

$$\ln (A)_o / (A) = Kt \quad \text{(1)}$$

$$\text{or } (A) = (A)_o e^{-Kt} \quad \text{(2)}$$

Where $(A)_o$ is concentration at the start of the reaction.

(A) is concentration after degradation (t=n).

and K is rate constant of the reaction.

Fig. 13 indicates the over-all degradation rate of chloramphenicol in acetate buffered aqueous solution at 89°C in a water bath to be first order with respect to the antibiotic over a range of buffer pH 4-5.6. Table 4 shows the computer results for the kinetics of chloramphenicol degradation in acetate buffers of different pH values.

Fig. 14 indicates the dependency of degradation of chloramphenicol at the strength of the buffer, which demonstrate that hydrogen-ion catalysis and base catalysis play a role in the degradation of chloramphenicol at pH 4.8, and table 5 shows the parameters for the kinetics of degradation of chloramphenicol.

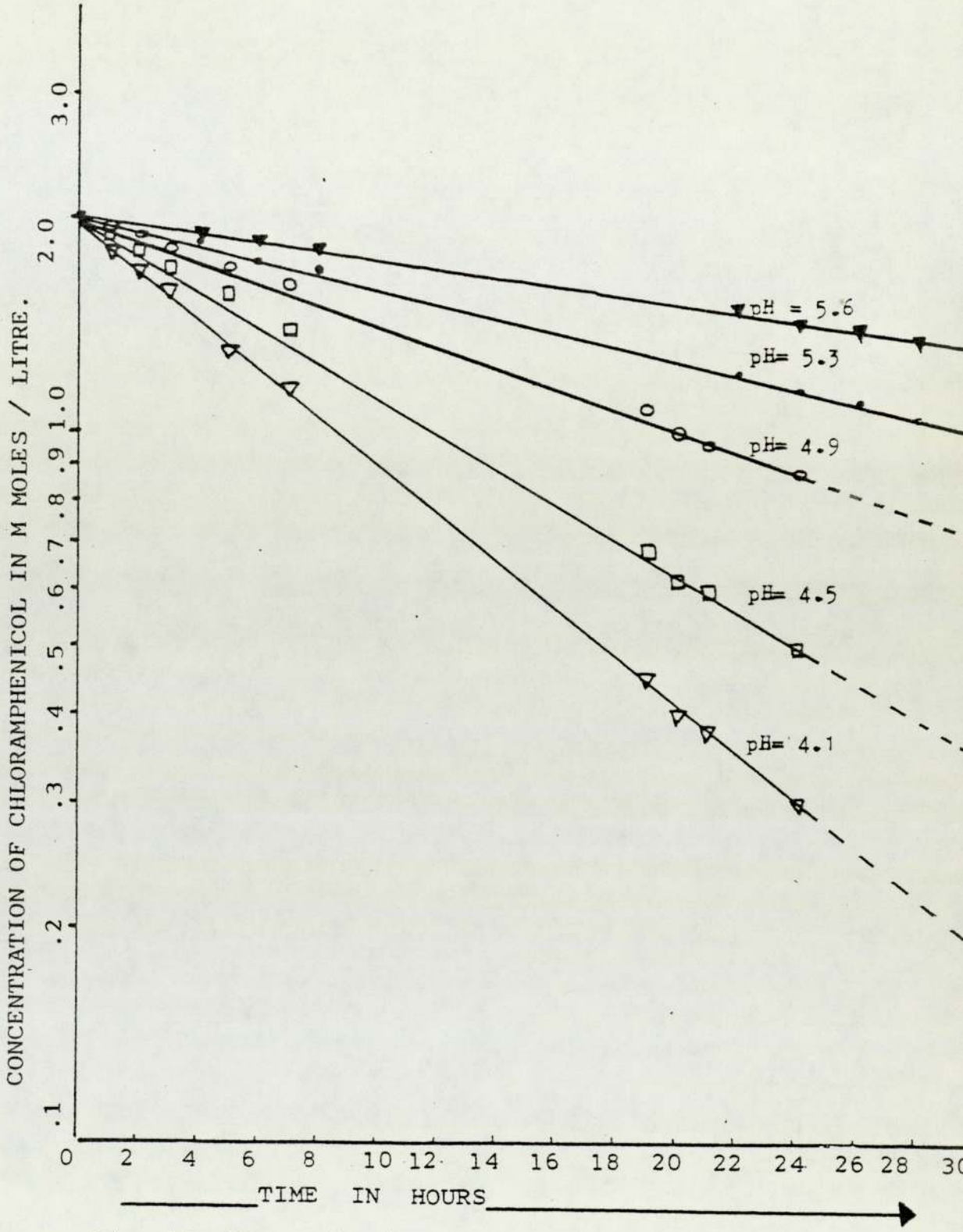


Fig. 13. Plots showing the Over-all character of Chloramphenicol degradation in acetate buffer at 89°C to be first order with respect to antibiotic concentration.

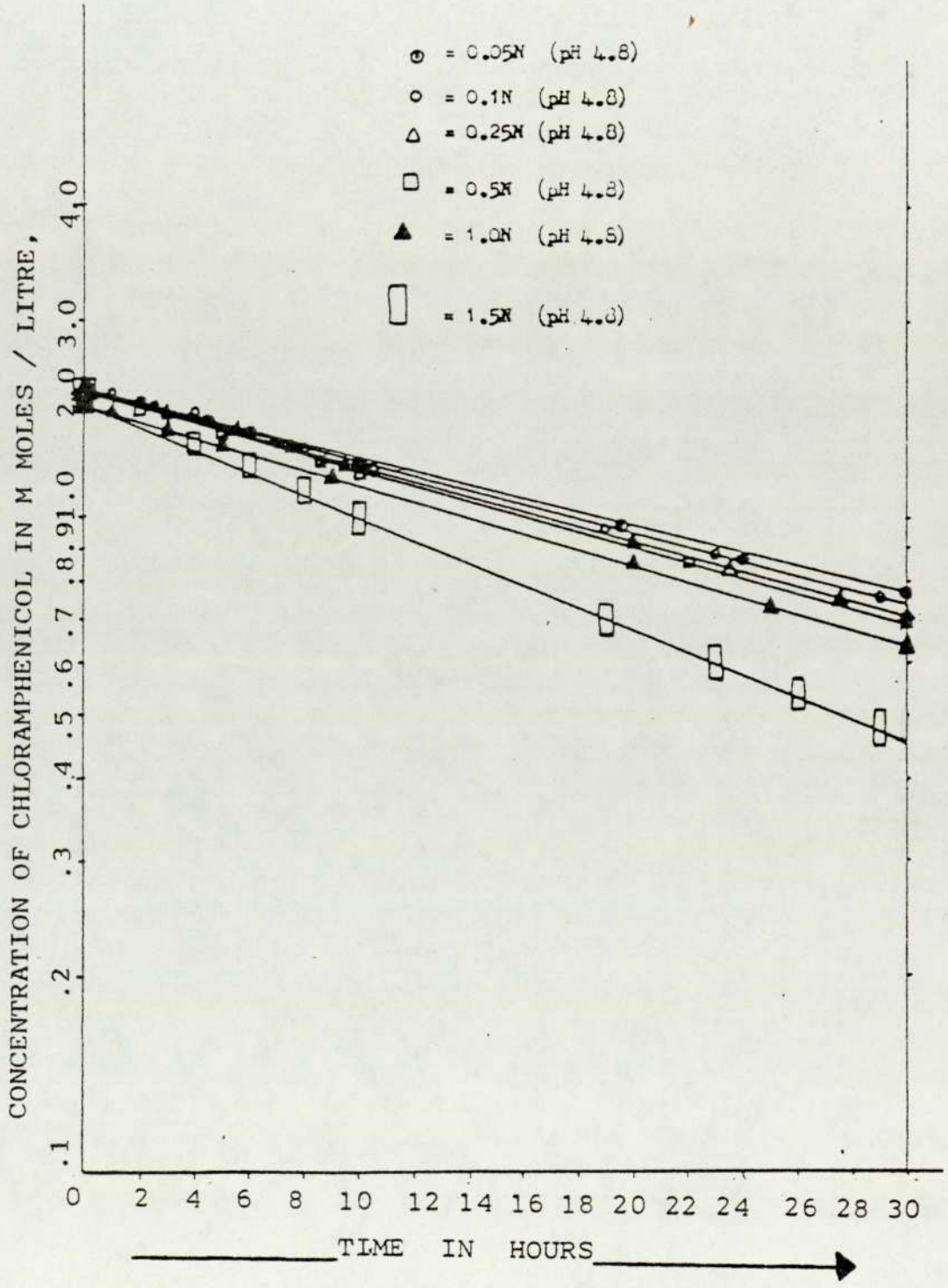


Fig 14 , indicates the dependency of degradation of Chloramphenicol at varying buffer strength of pH 4.8 at 89^0C.

Table 4, showing the computer results for kinetics of chloramphenicol in acetate buffer(0.1N) at 89°C.

BUFFER pH	$R_{**}2$	RATE CONSTANT, K.	HALF-LIFE IN HOURS, $T_{1/2}$ = ln2 / K.
4.1	99.9%	0.104 hr^{-1}	6.66
4.5	99.8%	0.079 hr^{-1}	8.77
4.9	99.6%	0.057 hr^{-1}	12.16
5.3	99.9%	0.035 hr^{-1}	19.80
5.6	99.7%	0.0235hr^{-1}	29.49

Table 5, shows some parameters for the kinetics of degradation of chloramphenicol in acetate buffer(pH 4.8) at 89°C.

BUFFER STRENGTH.	R_{**}^2	RATE CONSTANT, K, hr^{-1}	HALF-LIFE IN HOURS. $T_{\frac{1}{2}} = \ln 2 / K$.
o.o5N	99.83%	0.0242	28.63
o.1N	99.80%	0.0255	27.15
o.25N	99.96%	0.0262	26.46
o.5N	99.90%	0.0264	26.37
1.oN	99.97%	0.0278	24.92

(X). Photodegradation of chloramphenicol eye-drops (BPC) under UV light.

An aqueous solution of chloramphenicol in borate buffer at pH 6.95 was degraded upon exposure to UV light. After about 7 hours of irradiation, samples were analysed by the GLC assay method and also their UV absorbances were determined, which indicates that the degradation products were not present initially in the chloramphenicol solution ($\lambda_{max.}$ 275) but were produced as a result of a photochemical reaction ($\lambda_{max.}$ 267), as can be seen in fig. 15 .

250 mls. of the chloramphenicol eye-drops (BPC) in borate buffer was prepared in a volumetric flask and exposed to UV light . As photolysis progressed, the sample solution slowly turned yellow, and then orange yellow precipitants were observed to form after 20 mins. of irradiation.

The distance between light source and the samples was 5 cm.After appropriate time, 1.0 ml. fraction of the sample solution was with-drawn and chilled to stop the further reaction ,which was, then, extracted with ethyl acetate(1.0 ml.). To the separated ethyl acetate layer(0.5 ml.) was added phenyl boronic acid in ethyl acetate(1.0 ml.;6.0 $mg.ml.^{-1}$) and triphenyl benzene in ethyl acetate(0.2 ml.; 3.0 $mg.ml.^{-1}$) as internal standard. The mixture was allowed to stand for 5 mins. at room temperature and then 1 μl was injected into GC column.

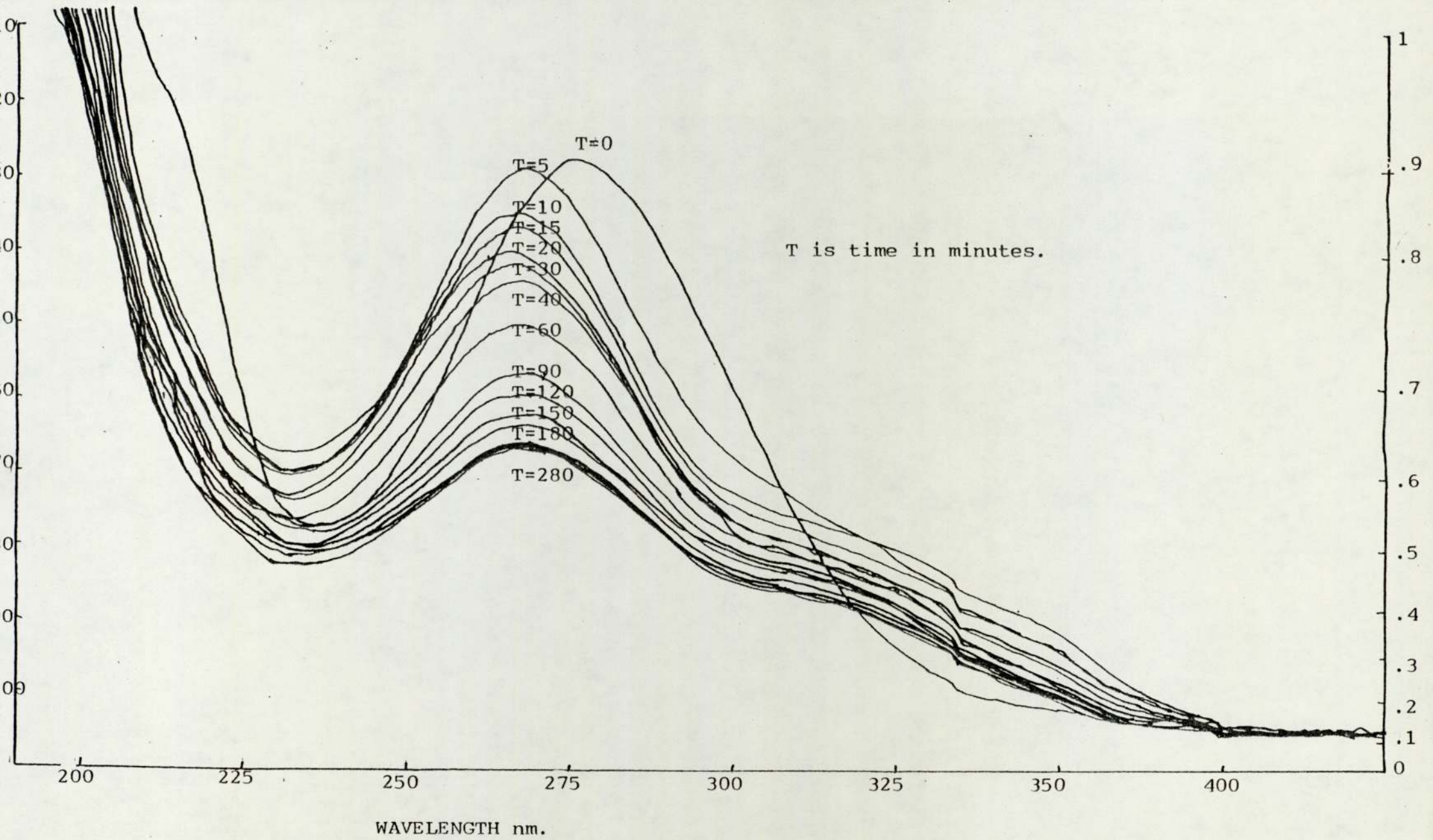


Fig. 15. UV spectrum of chloramphenicol and its photodegraded products.

(i). In different containers.

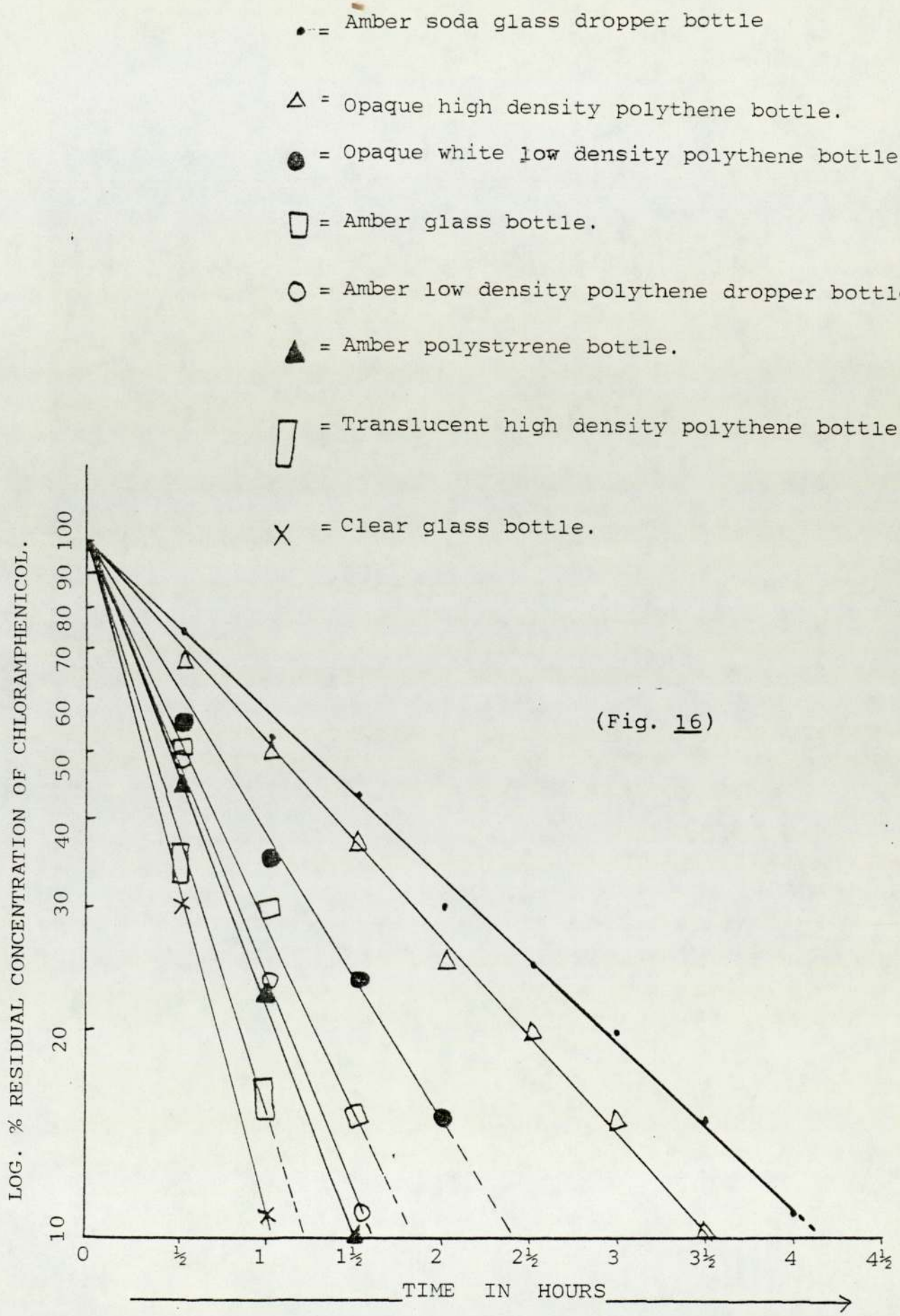
Table 6 shows the computer results for kinetics of chloramphenicol eye-drops (BPC) in different containers under UV light, placed 5 cm. away from the sample solutions. These results indicate that the slowest rate of reaction ($0.0092\ hr.^{-1}$) and highest half-life in hours of the antibiotic is achieved when the sample solution is irradiated in amber soda glass dropper bottle.This means amber glass soda dropper container offers more resistance to the UV light than other containers examined in this investigation , as can be seen in fig. 16.

Table 7 shows the computer results for kinetics of chloramphenicol eye-drops(BPC) under UV light passing through different filters(1 x 3 mm i.d.) from various containers when sample solution is placed in a quartz glass tube covered by aluminum foil.

Again, from these results, it clearly demonstrate that the slowest rate of reaction ($0.0004\ hr^{-1}$) and highest half-life of chloramphenicol (1677.55 hr.) is achieved when the UV light passes through amber soda glass dropper bottle filter while sample solution is placed in the quartz glass tube covered by aluminum foil.

From fig.17, it is evident that the 2% photodegradation, which is maximum, has reached after 45 hours of irradiation the chloramphenicol eye-drops(BPC) solution through amber soda glass dropper bottle filter. Hence, it

suggests that for the storage of chloramphenicol eye-drops(BPC) in multidose, an amber soda glass dropper bottle would be the best one for longer life of chloramphenicol.


= Amber soda glass dropper bottle
= Opaque high density polythene bottle.
= Opaque white low density polythene bottle.
= Amber glass bottle.
= Amber low density polythene dropper bottle.
= Amber polystyrene bottle.
= Translucent high density polythene bottle.
= Clear glass bottle.
LOG. % RESIDUAL CONCENTRATION OF CHLORAMPHENICOL.
10
20
30
40
50
60
70
80
90
100
0
½
1
1½
2
2½
3
3½
4
4½
TIME IN HOURS

(Fig. 16)

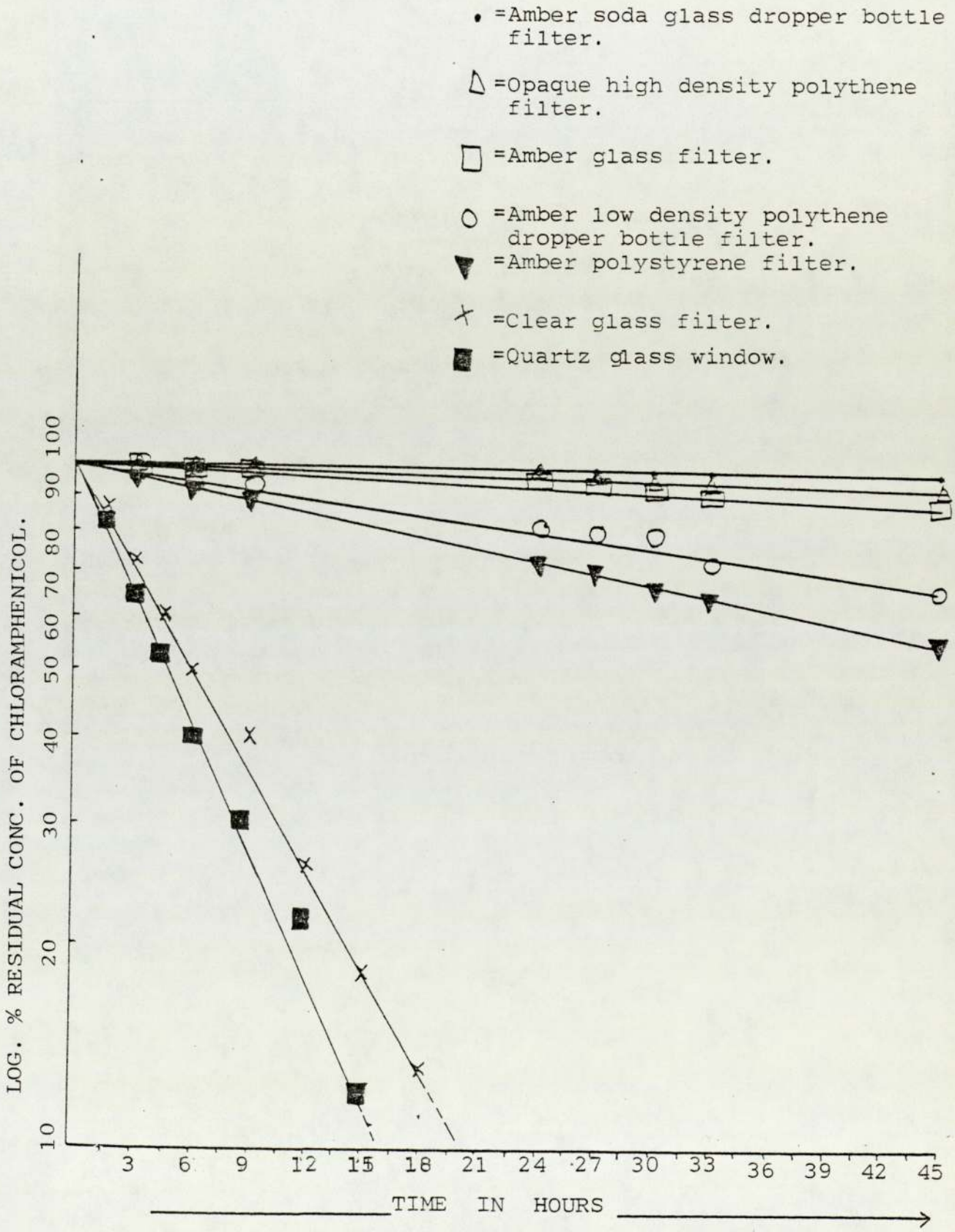


Fig. 17, Photodegradation of chloramphenicol eye-drops through different filters from the containers.

Table 6, indicates the computer results for kinetics of chloramphenicol eye-drops(BPC) in different containers under UV-light.

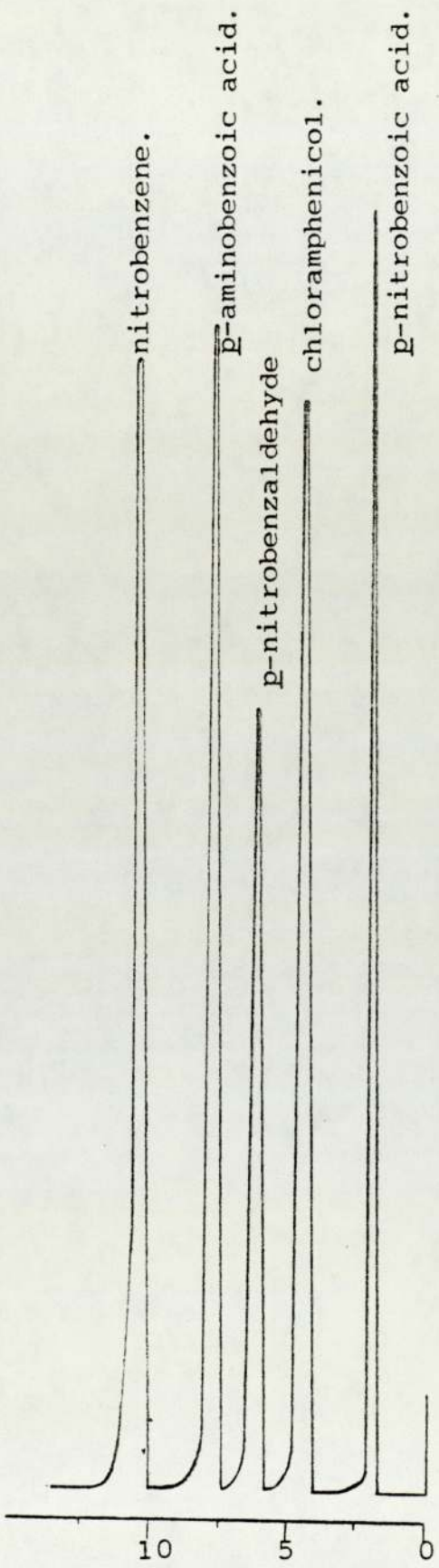
CONTAINER	$R_{**}2$ (%)	RATE CONSTANT K, hr^{-1}.	HALF-LIFE IN HOURS $T_{\frac{1}{2}} = \ln 2/K$.
Amber soda glass dropper bottle.	99.67	0.00921	75.269
Opaque high density polythene bottle.	99.33	0.01097	63.186
Opaque low density polythene bottle.	99.77	0.01582	43.816
Amber glass bottle.	99.82	0.02033	34.095
Amber low density polythene dropper bottle.	99.99	0.02129	32.5575
Amber polystyrene bottle.	99.85	0.02333	29.711
Translucent high density polythene bottle.	99.08	0.03120	22.216
Clear glass bottle.	99.64	0.03719	18.638

Table 7, indicates the computer results for kinetics of chloramphenicol eye-drops(BPC) under UV-light passing through different filters.

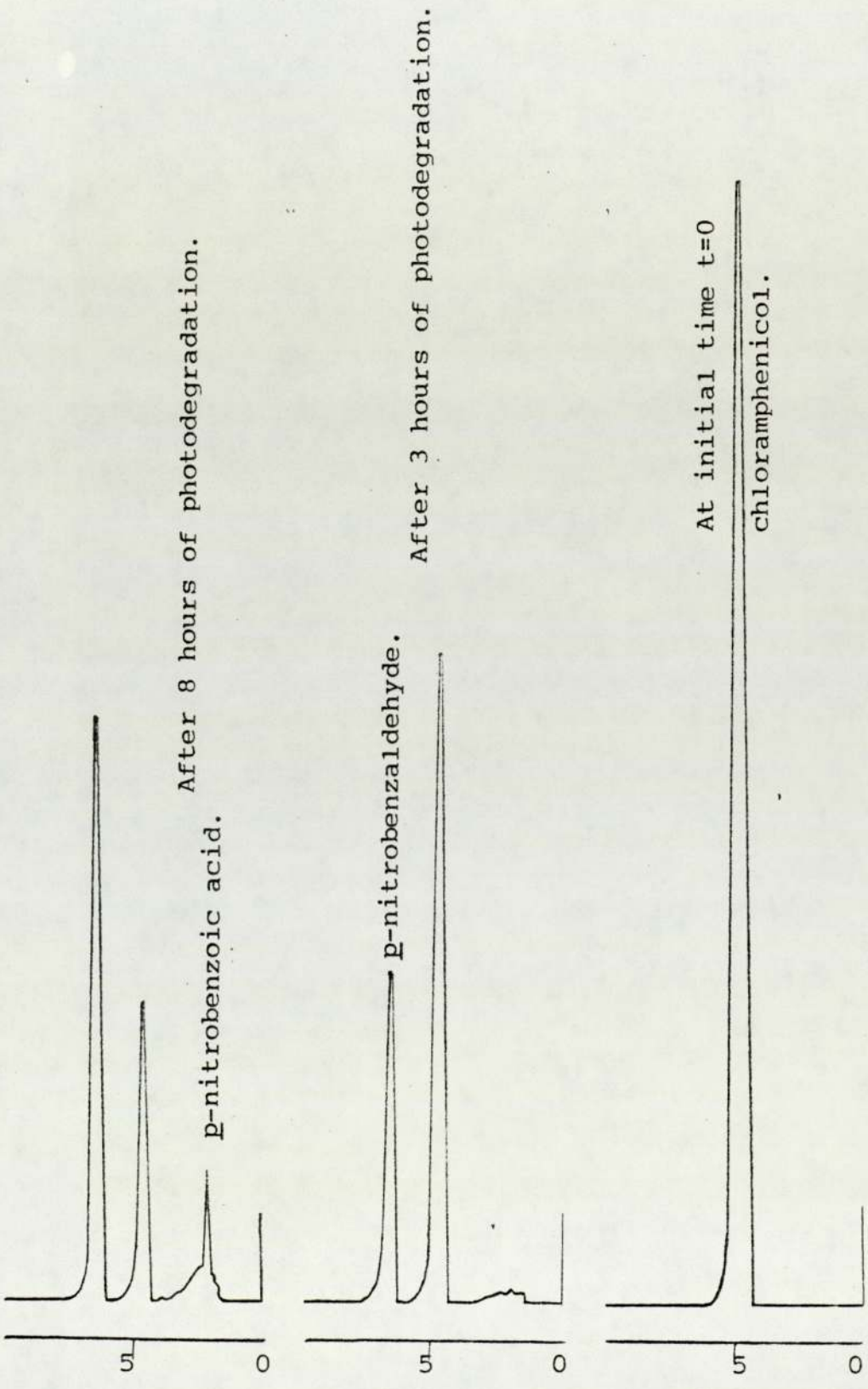
FILTER	$R_{**}2$ (%).	RATE CONSTANT K, $hr.^{-1}$	HALF-LIFE IN HOURS, $T_{1/2}$ = ln2 /K.
Amber soda glass dropper bottle filter.	86.88	0.0004127	1677.549
Opaque high density polythene filter.	97.16	0.001449	478.364
Amber glass filter.	97.52	0.002590	267.625
Amber low density polythene dropper bottle filter.	97.99	0.009459	73.279
Amber polystyrene bottle filter.	99.26	0.01310	52.917
Clear glass filter.	99.57	0.1140	6.08
Quartz glass window.	98.25	0.1306	5.31

(XI). High-performance liquid chromatography of photodegraded products of chloramphenicol.

These results indicate that the GLC procedure is also suitable for the determination of the kinetics of photodegradation of chloramphenicol. To monitor the progress of the reaction further, high-performance liquid chromatography (HPLC) was used. Fig. 18 shows the chromatogram of a mixture of some products which may result from the photodegradation reaction of chloramphenicol. Fig. 19 shows the products identified after an extended (96 hr.) reaction period, in which the primary photodegradation of chloramphenicol is shown to be the fission of the chloramphenicol molecule to yield p-nitrobenzaldehyde. Other products, such as p-nitrobenzoic acid do not appear in significant amounts until a large proportion of the chloramphenicol has disappeared.


nitrobenzene.
p-aminobenzoic acid.
p-nitrobenzaldehyde
chloramphenicol.
p-nitrobenzoic acid.
10
5
0
TIME IN MINUTES.

(Fig. 18)



TIME IN MINUTES.
(Fig. 19)

(C). EXPERIMENTAL.

(I). Apparatus and conditions.

Chromatography was performed with flame-ionization on a Pye 104 gas chromatograph equipped with 0.5 m x 3 mm I.D. glass column packed with 1.5% OV-17 on Supasorb M, AW-DMCS, 100-120 mesh. The column was maintained at 260°C and the injection port was held at 300°C. The nitrogen carrier gas flow rate was 65 ml.min.$^{-1}$, the air pressure was 0.5 Kg.cm.$^{-2}$ and that of the hydrogen was 1.3 Kg.cm.$^{-2}$. Mass spectra were determined using a GC-interfaced VG Micromass 12B mass spectrometer operated with a trap current of 100 μA, an accelerating voltage of 3KV and an ionisation energy of 22 eV. ^{1}H n.m.r. spectra were measured in deuterio chloroform solution with tetramethyl silane as internal standard on a Varian A-60A spectrometer, and infra-red spectra were determined using KBr disc method on a SP 200 spectro-photometer.

(II). Thin layer chromatography.

The purity of the compound was examined by TLC using a silica gel GF 254 plates with Toluene-Methanol-Ethyl acetate (60:20:15) as the mobile phase.

The two dimensional TLC of chloramphenicol phenyl boronate showed a single spot under UV light at Rf value 0.75.

(III). Preparation of phenyl boronate of chloramphenicol.

Chloramphenicol (0.5 gm.) was dissolved in ethyl acetate (5.0 mls.) and phenyl boronic acid (0.21 gm.)was added.

The mixture was heated under reflux for 30 minutes. Evaporation under reduced pressure to low bulk and crystallisation of the residue from aqueous ethanol yielded the cyclic boronate ester 0.706 gm.(99.6% yield) with melting point 173-174°C.

(IV). Calibration curve.

Calibration curves were constructed by adding varying amounts, 1.0 $mg.ml.^{-1}$, 0.75 $mg.ml.^{-1}$, 0.5 $mg.ml.^{-1}$, 0.25 $mg.ml.^{-1}$, and 0.125 $mg.ml.^{-1}$ of phenyl boronate ester dissolved in ethyl acetate with triphenyl benzene in ethyl acetate (0.1 ml.; 3.0 $mg.ml.^{-1}$) as internal standard. The peak height ratios of the phenyl boronate ester to internal standard was plotted against the amount of ester added (fig.7.). The mean normalised peak height ratios were used to calculate the amount of chloramphenicol in unknown sample.Table 8 indicates the computer results for 3 replicate runs of peak height ratio for phenyl boronate of chloramphenicol ester.

(V). Pharmaceutical preparations.

(a). Chloramphenicol in situ.

Chloramphenicol standard (12.5-150 mg.) were dissolved in ethanol (4.0 mls.) and the solutions were diluted to 10 mls. with distilled water. An aliquot (1.0 ml.) from each was diluted to 5.0 mls. with distilled water, and 1.0 ml. of the resulting solutions (0.25-3.0 $mg.ml.^{-1}$) ware extracted with ethyl acetate (1.0 ml.). To the

Table 8. Data for 3 replicate runs of peak height ratio for phenyl boronate of chloramphenicol.

	1	2	3	MEAN	S.DEV.	CV(%)
a	7.90	7.88	7.90			
a/std.	0.888	0.890	0.888	0.8887	0.0014	0.1572
b	6.00	5.85	5.90			
b/std.	0.776	0.663	0.664	0.6644	0.0014	0.2091
c	4.05	4.00	4.02			
c/std.	0.450	0.454	0.453	0.452	0.0013	0.2933
d	2.10	2.10	2.10			
d/std.	0.236	0.236	0.236	0.236	0.0029	1.218
e	1.10	1.02	1.00			
e/std.	0.122	0.122	0.116	0.119	0.0029	2.455

Where a, b, c, d, and e are the peak heights in cm. for antibiotic concentrations.

separated ethyl acetate layers(0.5 ml.) was added phenyl boronic acid in ethyl acetate (1.0 ml.; 1.0 $mg.ml.^{-1}$) and triphenyl benzene in ethyl acetate (0.2 ml.; 0.5 $mg.ml.^{-1}$) as internal standard. The mixtures were allowed to stand for 5 mins. at room temperature and then 1 µl was injected into the gas chromatographic column. A calibration curve of response ratio V/S concentration of drug was constructed (Fig. 12.).

(b). Chloramphenicol capsule.

The equivalent of the contents of one capsule(nominally 250 mg.of chloramphenicol) was dissolved in ethanol (20 mls) and this solution was diluted to 100 mls. with distilled water (2.5 $mg.ml.^{-1}$). Extraction of this solution (1.0 ml.) with ethyl acetate and derivitisation as in (a) was performed.

(c). Chloramphenicol eye-drops.

The eye-drops solution (1.0 ml.; nominally 0.5% w/v in a borate buffer) was diluted to 5.0 mls. with distilled water (1.0 $mg.ml.^{-1}$). Extraction of this solution (1.0 ml.) with ethyl acetate (1.0 ml.), fallowed by derivitisation as in (a) was performed.

(d). Chloramphenicol ear-drops.

The ear-drops solution (0.1 ml.; nominally 10% w/v in propylene glycol) was diluted to 5.0 mls. with distilled water (2.0 $mg.ml.^{-1}$). Extraction of this solution (1.0 ml.) into ethyl acetate (1.0 ml.) and derivitisation as in (a) was performed.

(e). Chloramphenicol eye-ointment.

The eye-ointment (0.5 gm.; nominally 1% w/w in a liquid paraffin-wool fat base) was dissolved in petroleum ether (3.0 ml.; 40-60°C fraction) and acetonitrile (2.0 ml.) was added. After agitation the phases were separated by centrifugation and the lower layer acetonitrile phase (0.5 ml.) was derivatised by the addition of phenyl boronic acid in ethyl acetate (1.0 ml.; 1.0 $mg.ml.^{-1}$) with triphenyl benzene in ethyl acetate (0.2 ml.; 0.5 $mg.ml.^{-1}$) as internal standard. The mixture was allowed to stand for 5.0 mins. at room temperature and then 1 µl was injected into gas chromatographic column.

(f). Chloramphenicol solutions A, B, and C.

Solutions A and B contained 1.80 and 1.75 $mg.ml.^{-1}$ of chloramphenicol dissolved in ethanol, and solution C contained 1.65 $mg.ml.^{-1}$ of chloramphenicol with 1% sulphacetamide in ethanol.

The solutions A, B, and C (1.0 ml.)from each were diluted to 5.0 mls. with distilled water. Extraction of 1.0 ml. from each of these solutions with ethyl acetate (1.0 ml.), followed by derivitisation as in (a) was performed.

(VI). Degradation of chloramphenicol.

All reagents used in this investigation were of analytical grade.

The buffers refered were prepared by dissolving reagent grade chemicals in distilled water, adjusting and measuring pH with a Pye model 78-pH meter.

Chloramphenicol, 500 mg., was accurately weighed into 100 mls. volumetric flask with the aid of a small glass funnel. Any of the compound adhering to the sides of the funnel was washed into the flask with approximately 90 mls. of the appropriate buffer solution. The flasks were then placed into a constant temperature water bath at 89°C ±0.5°C and the contents were allowed to attain the temperature of the bath. At that time the contents of the flask were made upto the mark with proper buffer solution and the flasks were securely stoppered. The first sample was with drawn from each of the reaction vessels only after visible inspection showed that all of the chloramphenicol in each vessel had dissolved and the timing of the reaction was then started.

1.0 ml. portions of the samples were with drawn in to volumetric pipettes, after appropriate intervals, into an approximately 5.0 mls. capacity glass vial, and the further reaction was stopped by chilling the samples immediately after with drawn from the flasks, and then aliquots (1.0 ml.) were extracted with ethyl acetate(1.0 ml.).

To the separated ethyl acetate layers (0.5 ml.) was added phenyl boronic acid in ethyl acetate (1.0 ml.; 6.0 mg.ml.$^{-1}$) and triphenyl benzene in ethyl acetate (0.2 ml.;3.0mg.ml.$^{-1}$) as internal standard. The mixtures were allowed to stand for 5.0 mins. at room temperature and then 1 µl was injected into the gas chromatographic column.

(VII). Photodegradation of chloramphenicol eye-drops(BPC) under UV light.

(a). In different containers.

Chloramphenicol eye-drops (BPC) were made by dissolving boric acid (1.5 gm.), borax (0.3 gm.) and phenyl mercuric acetate (2.0 mg.) in 90 mls. of distilled water by heating at 40°C. Then chloramphenicol (0.5 gm.) was added and temperature was adjusted at 40°C till all the antibiotic had dissolved and the volume of the flask was made upto 100 mls. with distilled water. After mixing very well, the first sample (1.0 ml.) was with drawn at time t=0. Then the solution was distributed among the containers. These were placed at a distance of 5 cm. from the UV light.

The 1.0 ml. portions of the samples were with drawn into volumetric pipettes, after appropriate intervals, into a glass vial, and the further reaction was stopped by chilling the samples immediately after sampling.

Ethyl acetate (1.0 ml.) was added to each sample and the mixtures were agitated thoroughly.

To the separated ethyl acetate layer (0.5 ml.) was added phenyl boronic acid in ethyl acetate (1.0 ml.; 6.0 mg.ml^{-1}.) and triphenyl benzene in ethyl acetate (0.2 ml.; 3.0 mg.ml^{-1}.) as internal standard. The mixtures were allowed to stand for 5 mins. at room temperature and then 1 µl was injected into the gas chromatographic column.

(b). Using filters from various containers.

Chloramphenicol eye-drops (BPC) in borate buffer were prepared as in (a).

Experiments were conducted by placing the chloramphenicol eye-drops solutions (50 mls.) into a quartz glass tube and the tube was covered by baconfoil and a window of 3 X 1 inch was made into each tube. Then, the filters from various containers were placed infront of the window at a distance of 4 cm. away from the UV light source and 1 cm. away from the sample tubes.

Samples (1.0 ml.) were with drawn after the appropriate time, extraction of these solutions (1.0 ml.) with ethyl acetate and derivitisation as in (a) was performed.

(a). CONTAINERS USED.

1. Amber soda glass dropper bottle (for nose/ ear drops).
2. Opaque high density polythene(polyethylene) bottle.
3. Opaque white low density polythene dropper bottle.
4. Amber low density polythene dropper bottle.
5. Translucent high density polythene bottle.
6. Amber polystyrene bottle.
7. Amber glass bottle.
8. Clear glass bottle.

(b). FILTERS USED FROM THE FOLLOWING CONTAINERS.

1. Amber soda glass dropper bottle filter.
2. Opaque high density polythene bottle filter.
3. Amber low density polythene dropper bottle filter.
4. Amber glass bottle filter.
5. Amber polystyrene bottle filter.
6. Clear glass bottle filter.
7. Quartz glass window.

(VIII) High-performance liquid chromatography of the chloramphenicol photodegraded samples.

A liquid chromatograph consisting of a ALTEX model 100A pulseless pump with Pye UV detector operating at 275 nm and a 7120 Rheodyne injection valve was used. The range of the detector was set at 0.64 a.u.f.s. A 25 cm. X 4.6 mm i.d. reversed-phase column at 1.5 ml.min.$^{-1}$ flow rate was used. Injections (20 µl) were made of all solutions for analysis.

The mobile phase consisted of methanol-water-phosphoric acid, 50:50:1 , of HPLC grade.

(D). SUMMARY AND CONCLUSION.

A gas liquid chromatographic procedure for the specific determination of chloramphenicol in pharmaceutical preparations such as chloramphenicol capsules, eye-drops, ear-drops and eye-ointment has been developed.

This method is also applicable to the determination of chloramphenicol in products showing hydrolytic or photochemical degradation. A high-performance liquid chromatographic procedure for the determination of chloramphenicol in photodegradation products, has also been developed.

The speed, accuracy, simplicity, specificity, and the reproducibility of results obtained using this technique can claim that this method has superiority over many other assay methods.

Extension of this gas liquid chromatographic procedure into the nanogram range is required for separating and estimating chloramphenicol in biological fluids. The use of an electron capture detection system using the native electron-capturing ability of the chloramphenicol molecule or possibly specific detection using a boron selective detector could be an advantage to increase the sensitivity of the method.

The over-all degradation rate of chloramphenicol in aqueous solutions has been shown to be first order with respect to antibiotic over a range of acetate buffers(3-6) and its

strength (0.05N-1.5N).

The reproducibility of the experimental results along with the adherence to first order dependency indicate that the reaction involving amide cleavage might be responsible for the entire observed loss of the antibiotic.

These results demonstrate that the specific hydrogen-ion catalysis play a role in the degradation of chloramphenicol at the pH values and ionic strengths studied.

The chloramphenicol eye-drops (BPC) assay results indicate that for storage of solution in multidose, an amber soda glass dropper bottle would be the best to ensure an extended life for the antibiotic.

Also HPLC results indicate that the major photodegradation products of chloramphenicol were p-nitrobenzaldehyde and p-nitrobenzoic acid, although p-nitrobenzoic acid did not appear in significant amount until a large proportion of the chloramphenicol has disappeared.

PART II

ASSAY OF LACTIC ACID.

A. INTRODUCTION.

Rapid and accurate examination of cerebrospinal fluid(CSF) may be an important emergency procedure carried out in the microbiological laboratory. The classical CSF findings in purulent meningitis have been well documented[84] but often it is impossible to say, before the results of CSF culture are available, whether the etiological agent is bacterial, viral, tuberculous, or fungal. This is some times due to partially effective treatment which can alter the CSF findings and obscure the results of CSF Gram stain and culture[85].

CSF Gram stain may be negative in less than or equal to 25% of cases of bacterial meningitis[63,85,86]. Viral meningitis can present with a predominance of polymorphonuclear leukocytes(PMNL) in the CSF, mumps meningitis with a low CSF glucose[87] and early tuberculous meningitis such as Listeria can be present with lymphocytosis and high protein[88].

It has been known for 50 years that CSF lactate increases in some cases of bacterial meningitis[89] and recent workers have published reports of the value of CSF lactate assay in a differential diagnosis of bacterial tuberculous or viral meningitis[121-124]. It was suggested over 40 years ago that lactic acid measurements gave a more reliable index of the progression of infection than sugar content[90] and recently it has been shown that elevated

levels of lactic acid came down within 72 hours of appropriate antibacterial therapy[91,92].

Kopetsky and Fishberg[93], postulated that the source of increased CSF lactic acid observed in purulent meningitis might be "anaemia of the brain" induced by increased intracranial pressure with reduced oxygenation in the region of brain.

Since Levinson[94] has attributed the evidently fixed decrease in alkalinity of the CSF in meningococcus meningitis to a production of lactic acid and the temporary decrease in tuberculous meningitis to carbon dioxide, a study has been made of the lactic acid concentration of the CSF in the meningitis.

Clinically, elevations of CSF lactic acid levels occur in hypocapnia[95] and in meningitis[88,96-98].

Mc Illwain[99], Umbreit[100], and Davson[101], in their separate studies , demonstrated that lactic acid rises in excess of pyruvic acid. According to Wittgenstein[102] an elevation of lactic acid in the CSF occurs as a result of muscular excercise, and enters from the brain rather than from the circulating blood[103-107].

(I). Assay methods available.

Many methods have been introduced to aid in the examination of CSF in cases of meningitis. Some have gained limited acceptance but each of them has drawbacks.

Nishimura[108], in 1924, adopted Clausen's method[109] for the study of the lactic acid of spinal fluid. This method is based upon the extraction of lactic acid from glucose-free filtrate with ether and the lactic acid, then is oxidised to acetaldehyde with potassium permanganate. This acetaldehyde is distilled into sodium bisulphite solution and finally this bisulphite is titrated with iodine. This method is very complicated and time consuming.

Again, in 1926, Glaser[110], adopted the Clausen's sulphuric acid method[109] for the analysis of lactic acid in CSF and blood. In this method, the CSF proteins are precipitated with copper sulphate by Sluiter's method[111]. Also, this method is very complicated and time consuming.

Alexander, et al[112], determined the lactate concentration of CSF by the method of Mitchell[113]. Initially, CSF protein was precipitated with copper sulphate and calcium hydroxide. This method, like others, is time consuming and less accurate.

Posner, et al[114], in 1967, performed an experiment on the determination of CSF lactate. In this method, CSF samples were deproteinised in 6% perchloric acid. The lactate value is determined by Barker and Summerson method[115].

This method is also less accurate and time consuming. Later, in 1968, Prockop[88], determined the CSF lactate by modifying Kiebs Ringer bicarbonate solution[100]. Richard, et al[116], in 1974, determined CSF lactic acid level in patients. In this method the CSF samples were chilled in perchloric acid (8%). Lactic acid measurements were made within 72 hours of the collection, using the enzymatic spectrophotometric method[117]. Also this method is less accurate and time consuming.

In a recent review, Frank and Gary[118], have reported a coupled enzymatic method to measure blood lactic acid. This method is based on the enzymatic oxidation of blood lactate to pyruvate in the presence of coenzyme nicotinamide adenine dinucleotide (NAD) and enzyme lactate dehydrogenase (LDH). The reaction product , NADH, is then oxidised in the presence of horseradish peroxidase, as in scheme I.

(i). $NADH + \frac{1}{2} O_2 + H^+ \xrightarrow[Mn^{2+}]{HPO} NAD + H_2O$

(ii). $CH_3.CHOH.COO^- + NAD \xrightarrow{LDH} CH_3.CO.COO^- + H^+$

(Scheme I)

The maximum rate of oxygen depletion, which is directly proportional to the amount of lactate ion present in sample is amperometrically monitored by a membrance oxygen electrode.

Although, this method is simple and rapid, but the use of buffer and adjustment of pH for the rate of coupled reaction, may produce inaccuracy in results.

Controni,et al[119], have developed a GLC lactate determination procedure for rapid diagnosis of meningitis in CSF lactic acid. The method of extraction reported here, is similar to that of Controni,et al, but the lactic acid is converted into ethyl lactate rather than methyl lactate. This ethyl lactate has eluted at slightly longer retention time which allows a greater resolution than is available with the literature procedure (Fig. 1).

This poor resolution could have been caused by the stainless steel column used by Controni,et al, in methyl lactate technique, or could have been caused by thermal conductivity detector used by Controni,et al, for the flame ionisation detector is more sensitive than the thermal conductivity detector[125].

The use of an internal standard, in the technique reported here, has increased the precision of the analysis and the accuracy of the quantification procedure for ethyl lactate determination in comparison to the determinations of Controni,et al.

The assay method for the determination of lactic acid in human CSF, reported here, has advantages over other assay methods of being rapid, sensitive, accurate, and specific.

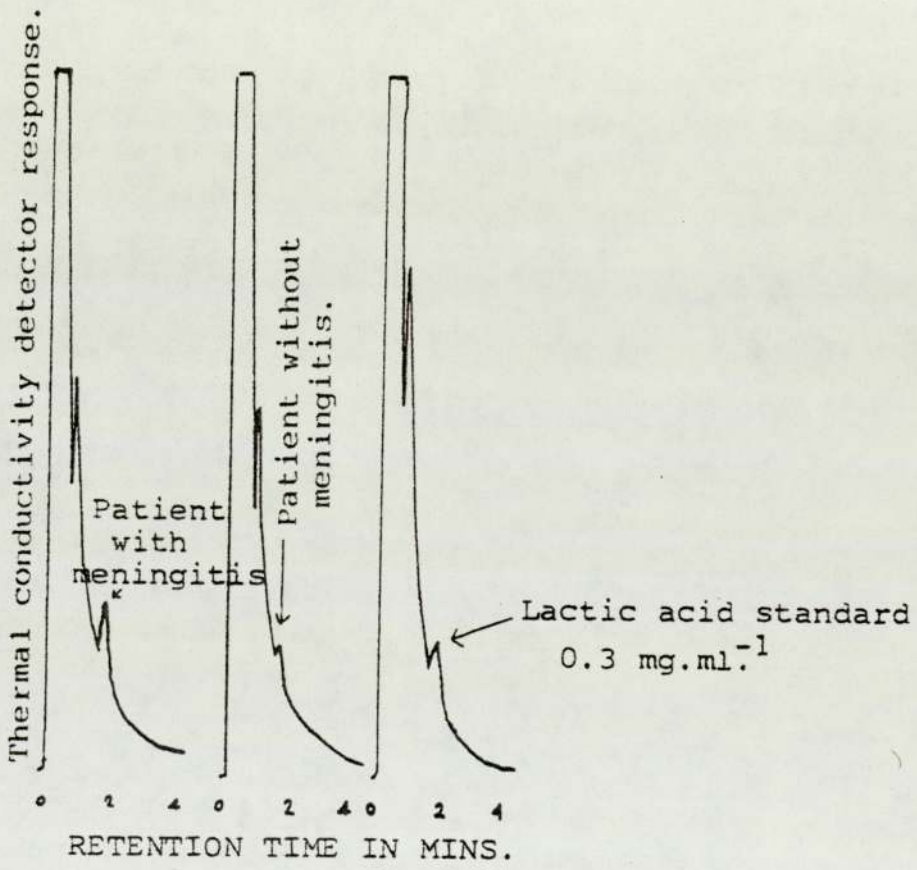


Fig. 1 . Lactic acid determination in CSF samples by Controni,et al, GLC assay method.

The lactic acid is eluted within 2.0 minutes of injecting into the gas chromatographic column, and the sensitivity upto 0.04 $mg.ml.^{-1}$ has been achieved by this assay method.

(B). RESULTS AND DISCUSSION.

(I). Estimation of CSF lactate by gas chromatography.

The assay of lactic acid in CSF is based upon the method of Controni, et al[92,119]. The modifications of Controni, et al, method enabled the procedure reported here, to achieve base line separation of the lactate peak which facilitates quantification of the analysis. Chromatography was found to be satisfactory using ethyl lactate, rather than methyl lactate.

The sensitivity of the analysis, under conditions reported here, easily enabled the lower lactate level (0.06 $mg.ml.^{-1}$) to be measured. The incorporation of diethyl malonate as internal standard was found to increase the precision of the analysis and on a routine basis precision of better than $\pm$ 5% was readily achieved. Typical tracings are recorded in figs. 2, 3, and 4. In several samples of the CSF extra peaks, with retention times intermediate between those of the lactate and malonate peaks, were found. Of these pyruvate has been positively identified by mass spectrometry. The lactate and malonate peaks were identified in CSF samples by both retention time and mass spectral comparisons. It was necessary to store some of the CSF samples prior to analysis. The monitoring of the same samples over a period of three months showed that no significant loss occurs when samples are stored at $-40^{o}C$. Fig. 5 is the chromatogram of CSF samples monitored before

storing and fig. 6 is the chromatogram of same samples after adding standard lactic acid and monitored over a period of three months.

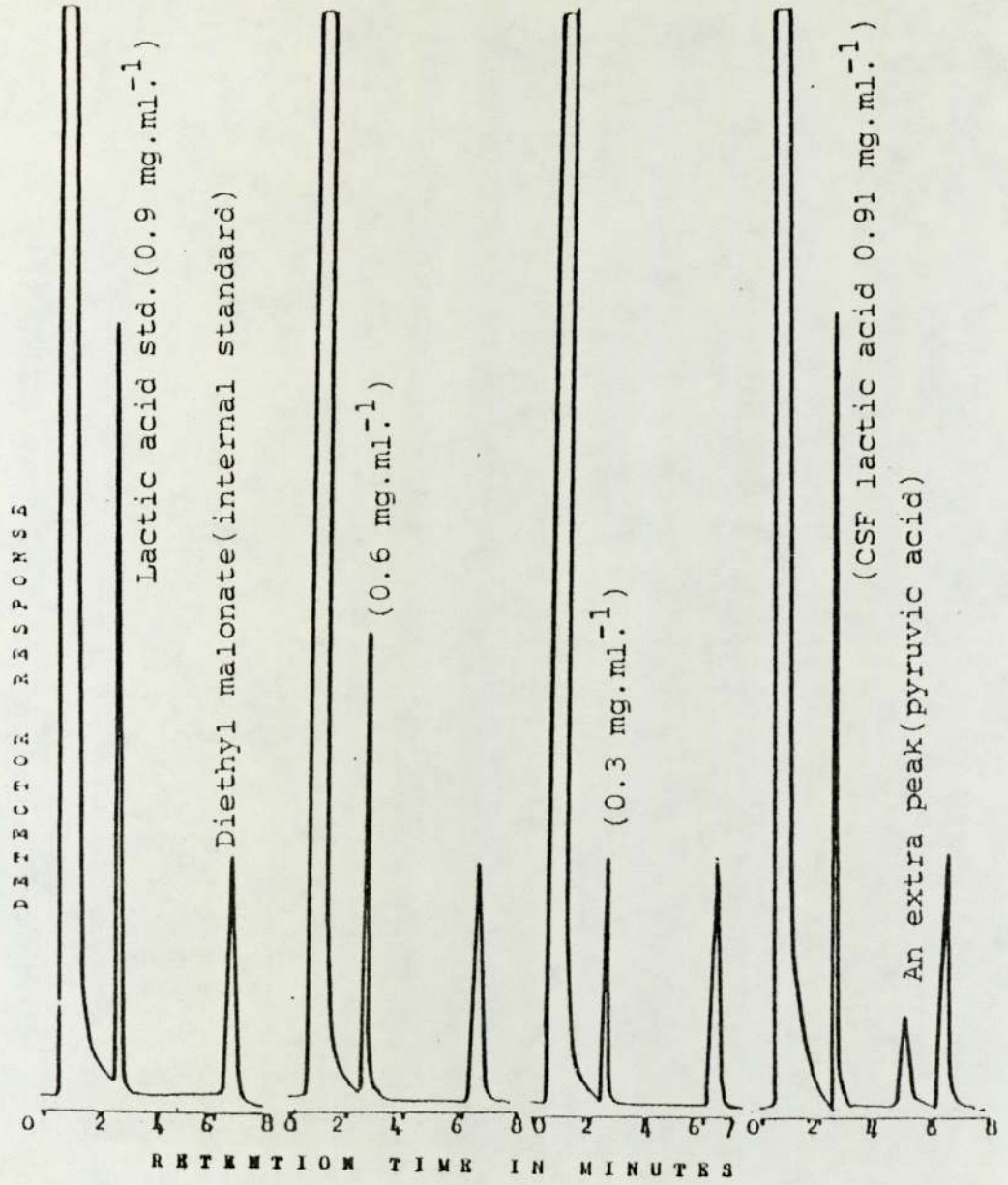


Fig. 2, indicates the highest value of lactic acid (0.91mg.ml.$^{-1}$) found in CSF no.56.

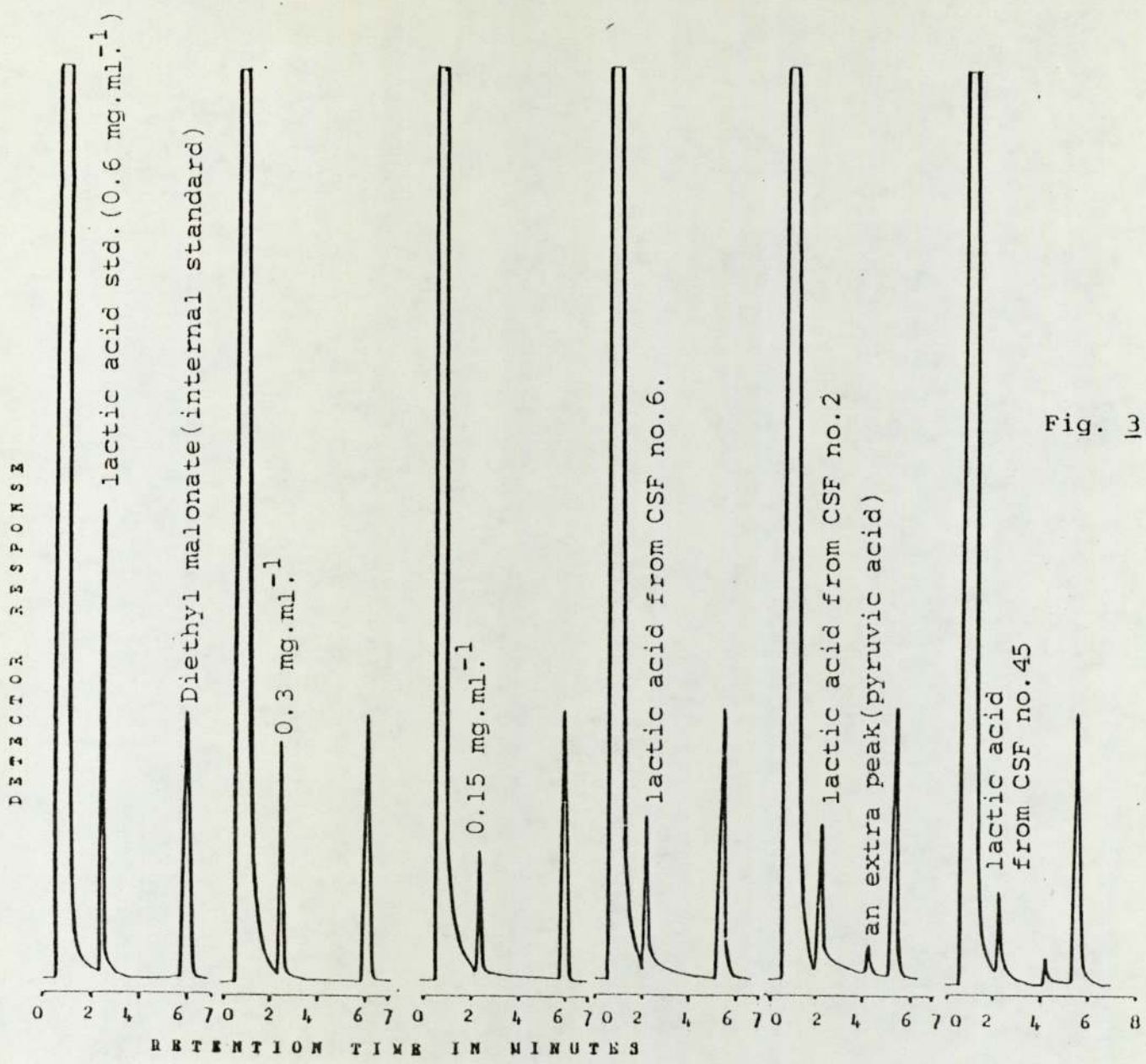


Fig. 3, indicates varying amounts of lactic acid found in CSF samples.

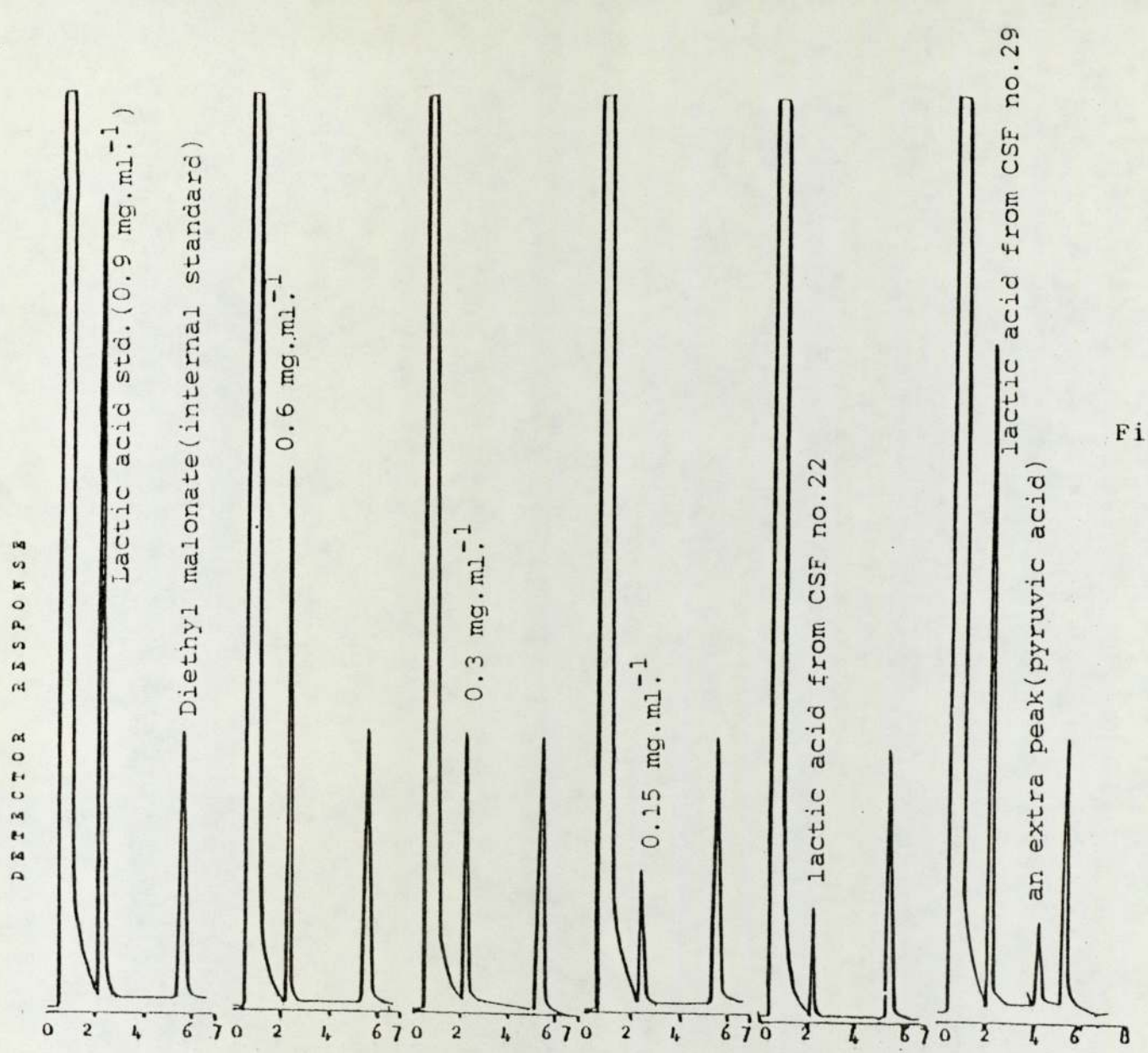


Fig. 4, indicates comparision between bacterial and non-bacterial cases.

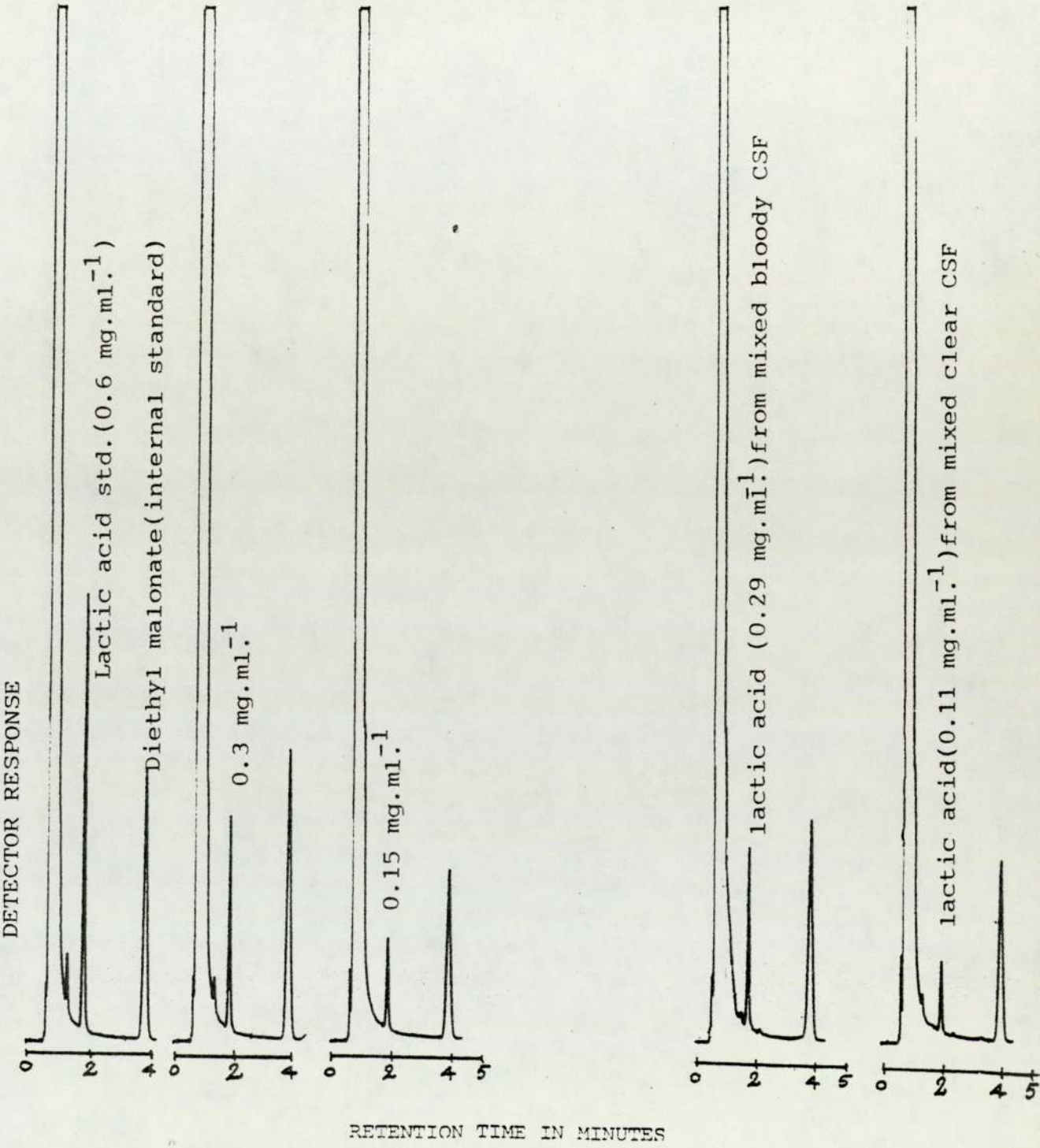


Fig. 5, indicates the chromatogram of mixed bloody CSF and mixed clear CSF monitored before storing at -40°C.

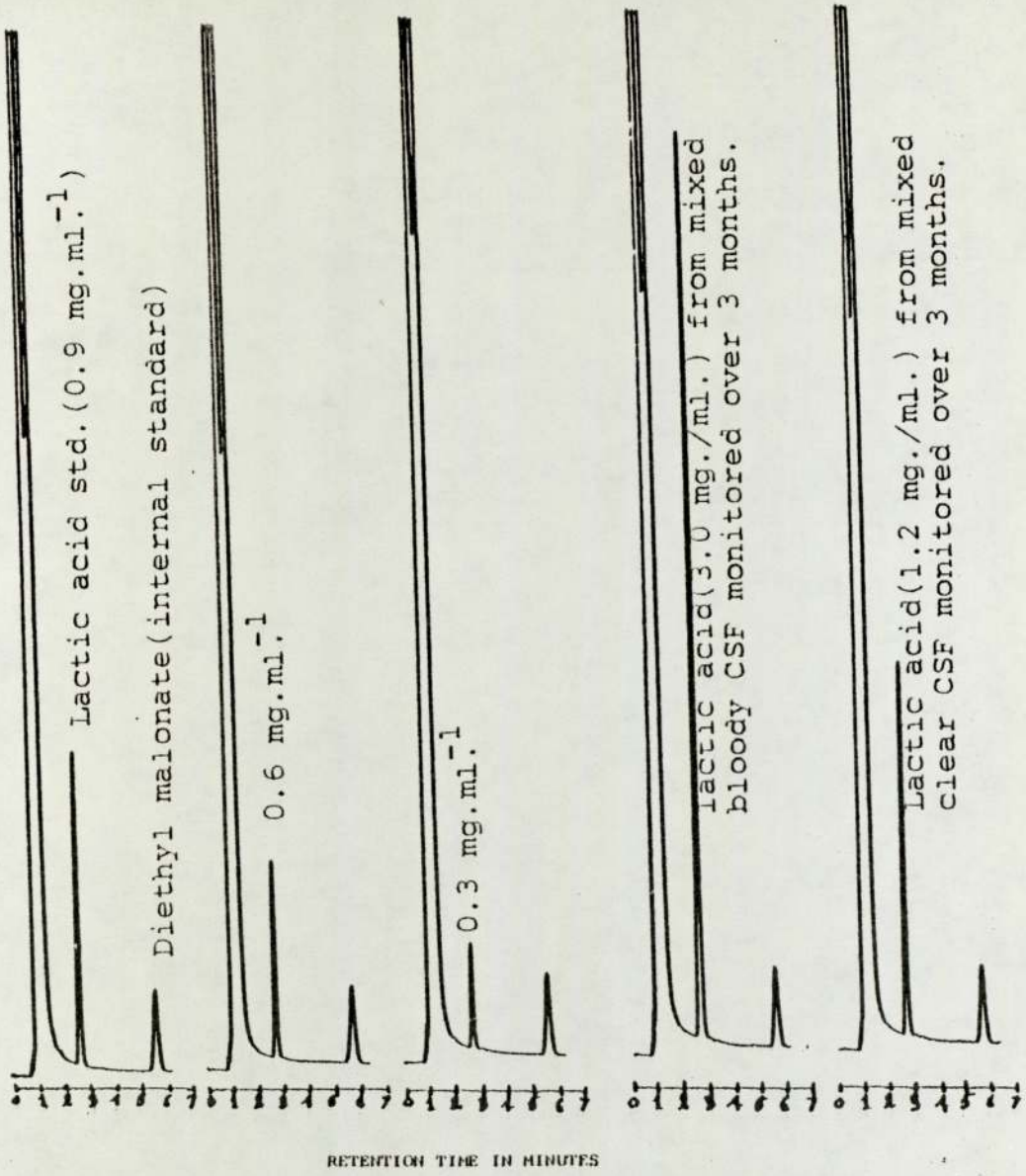


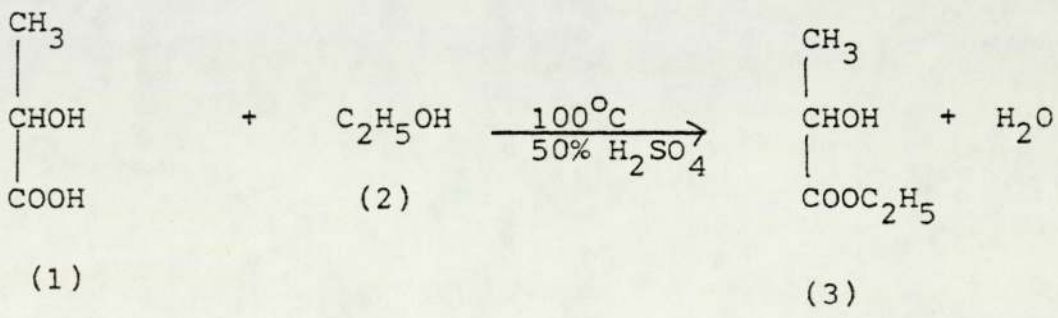
Fig. 6, indicates the chromatogram of mixed bloody CSF and mixed clear CSF monitored over a period of 3 months.

(II). Preparation of ethyl lactate ester derivative and calibration curve.

All reagents used in this analysis were of analytical grade.

Samples of cerebrospinal fluid (CSF) were assayed for lactic acid content by treating aliquots of CSF (1.0 ml.) with sulphuric acid (0.2 ml.; 50%) in a 3.0 ml. capacity reactivial. Absolute ethanol (1.0 ml.) was added, the container was sealed and the mixture was then heated to 100°C and maintained at this temperature for 5 minutes. On cooling, chloroform (0.5 ml.) contained diethyl malonate (0.125 $mg.ml^{-1}$) as internal standard was added and the whole was shaken to equilibrate the phases. After separation of the phases (and centrifugation where necessary to disperse emulsified droplets) an aliquot of the lower chloroform layer (1 µl) was injected into the gas chromatographic column.

REACTION.



The ratio of the peak heights of the ethyl lactate peak (2.0 mins.) and the diethyl malonate peak (4.8 mins.) was used to calculate the lactate level of the CSF by interpolation onto a calibration line. The calibration line was obtained by preparing solutions of lactic acid containing 0.1-0.9 $mg.ml.^{-1}$. Aliquots of each solution (1.0 ml.) were derivatised and analysed in parallel with the CSF samples to yield a linear calibration(fig.7.). In those cases where less than 1.0 ml. of CSF was available proportionate reduction in the scale of derivatisation and extraction down to 0.1 ml. of CSF (0.02 ml.50%-sulphuric acid, 0.1 ml. ethanol and 0.05 ml. chloroform) was successful with little loss in precision.
Photograph 'A' shows the reactivials used for reacting lactic acid with ethanol, and photograph 'B' is the gas chromatograph used for the analysis of lactic acid in CSF samples.
This assay of CSF ethyl lactate is a relatively simple, cheap, precise, accurate and quick procedure for any laboratory equipped with a gas liquid chromatograph and can be performed as an emergency procedure.

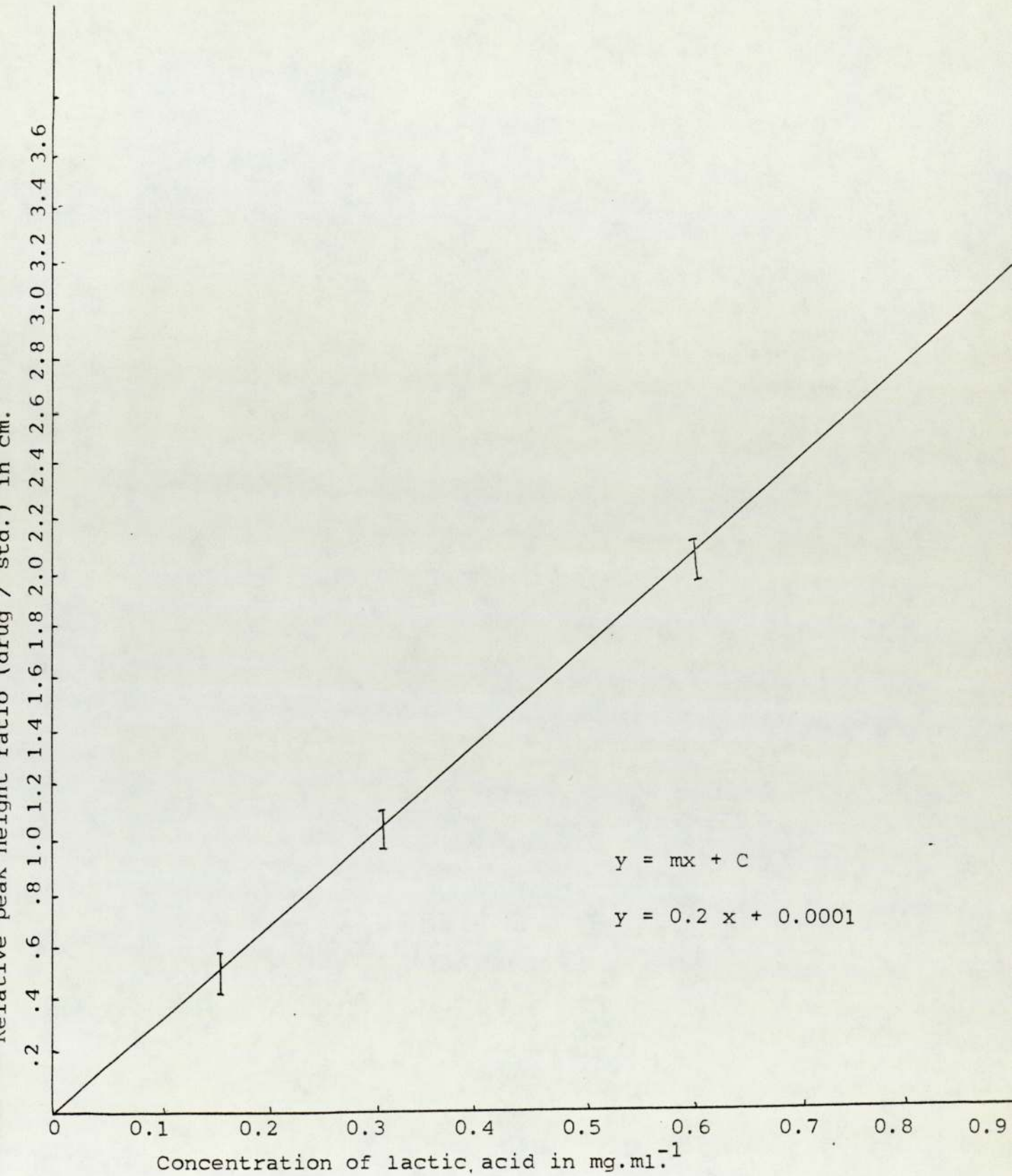
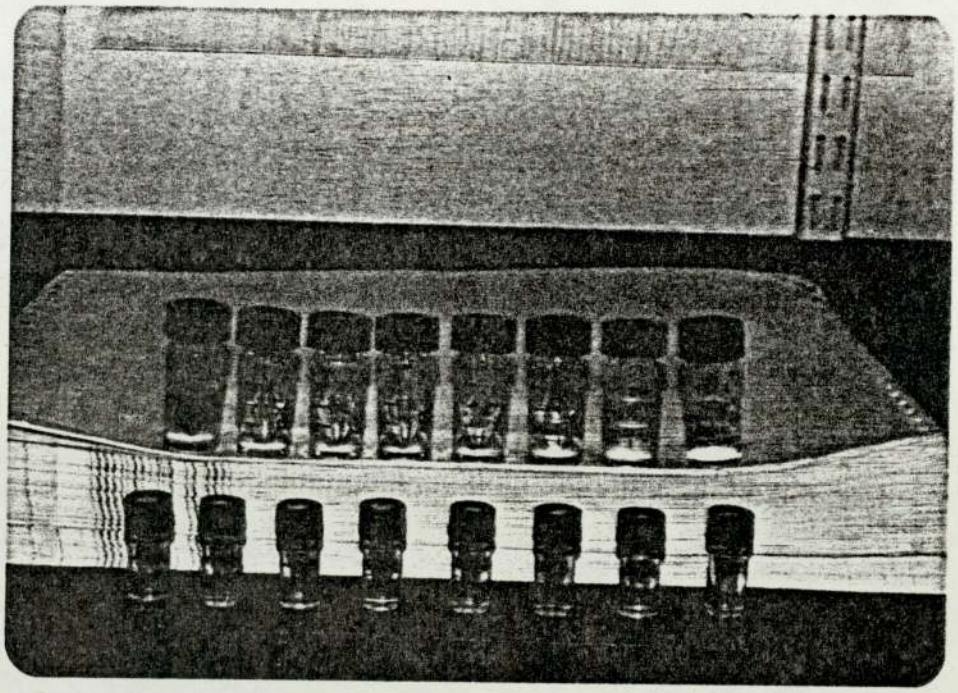
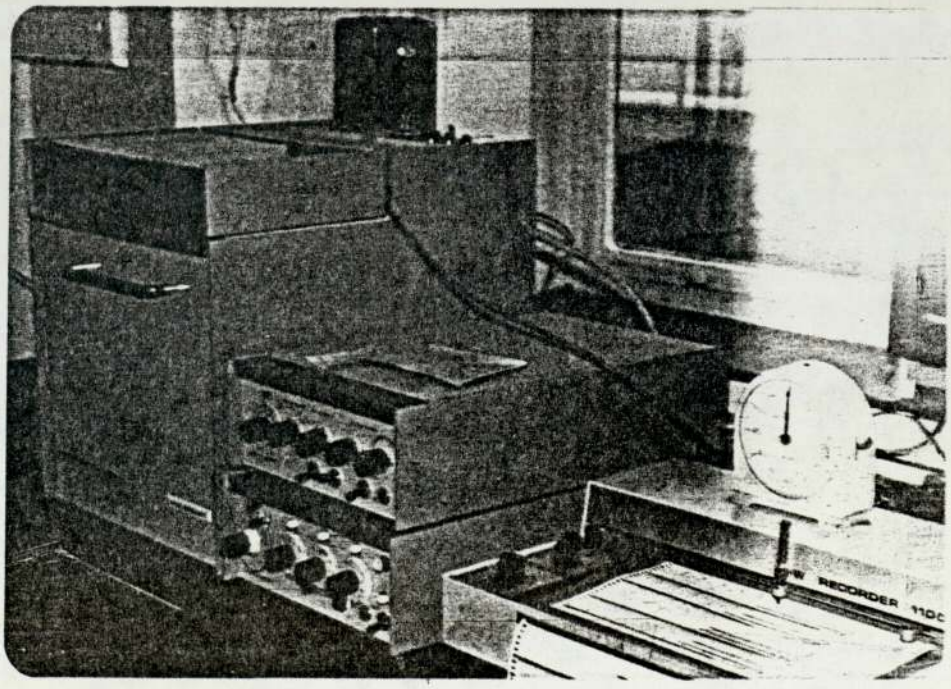


Fig. 7, indicates the calibration graph of determination of lactic acic in CSF samples.



Photograph 'A' , reactivials used for reaction.



Photograph 'B', gas liquid chromatograph used
for quantitative analysis of lactic acid.

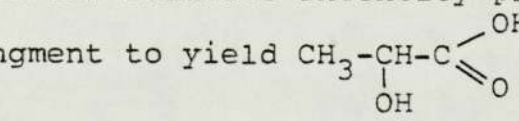
(III). Identification and quantification procedure.
The peaks obtained from CSF samples were identified as lactic acid(1), by GC technique, if they had retention times equal to the lactate standard. Also, lactic acid in CSF samples was confirmed by GC-MS technique comparing the retention times of the peaks as well as mass spectra. The amount of lactic acid in CSF samples were quantitated by adding equal amount of diethyl malonate as internal standard. The mean ratio of peak heights of drug to the peak heights of internal standard were plotted against the concentrations of the standard lactic acid. Fig.7 indicates the calibration curve of the above plot which gives linear response to the detector by passing through the origin with equation $y = 0.2 x + 0.0001$.
This method is sensitive (0.06 $mg.ml.^{-1}$), accurate, rapid and precise ($\pm$ 5%).

(IV). Mass spectral identification.

Figs. 8, 9, and 10 indicate the mass spectra of ethyl lactate, diethyl malonate, and ethyl pyruvate(possibly) respectively taken by GC-MS at 22 eV ionising voltage, 3KV accelerating voltage, and 170°C temperature.

Tables 1, 2, and 3 indicate the identified fragmentations of the major peaks of ethyl lactate, diethyl malonate, and ethyl pyruvate respectively.

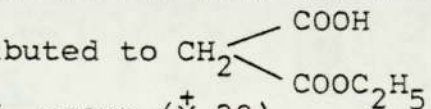
In fig. 8, the peak at M/e 103 with 11% relative intensity is possibly attributed to $HO^{+}=CH-COOC_2H_5$ fragment, by the loss of $-CH_3$ group ($\overset{+}{M}-15$) from the molecular ion. The peak at M/e 90 with 16% relative intensity probably results from a rearrangment to yield



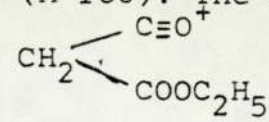
fragmentation, by the loss of $-CH_2=CH_2$,($\overset{+}{M}-28$) from the molecular ion.

Again, the peak at M/e 73 with 89% relative intensity is thought to be attributed by $CH_3-CH(OH)-C\equiv O^{+}$ fragment, by the loss of $-C_2H_5O$ group ($\overset{+}{M}-45$) from the molecular ion. The base peak at M/e 45 might have come from $CH_3-CH=OH^{+}$ fragment, by the loss of $\overset{+}{M}-73$ from the molecular ion.

In fig. 9 , the peak at M/e 132 with 75% relative intensity is probably attributed to



fragment, by the loss of $-CH_2=CH_2$ group ($\overset{+}{M}-28$) from the molecular ion ($\overset{+}{M}=160$). The base peak at M/e 115 could be attributed by



fragment , by the loss

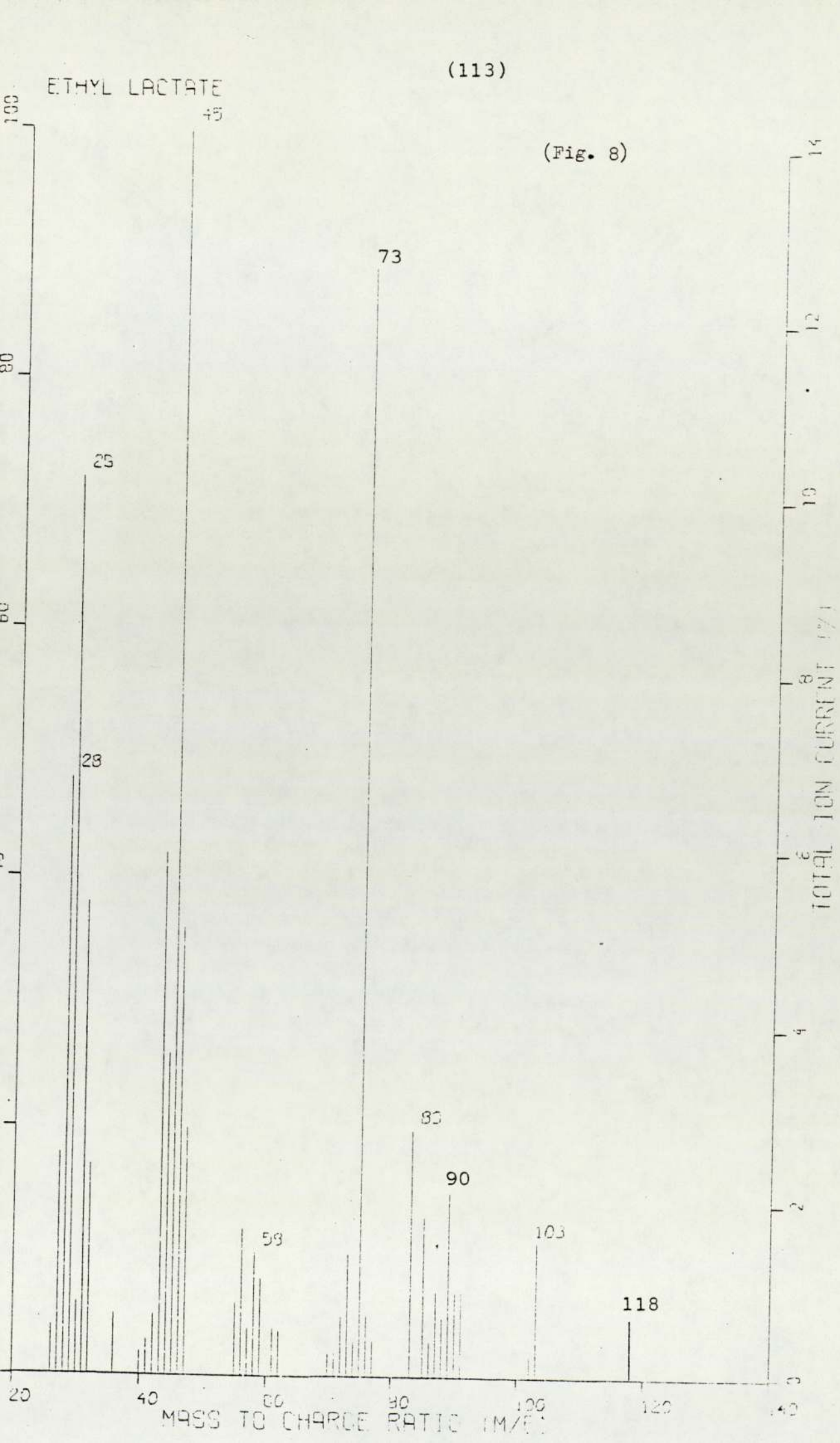
of $-C_2H_5O$ group ($\overset{+}{M}$-45) from the molecular ion. Again, a peak at M/e 104 with 13% relative intensity might have come from CH_2(COOH)(COOH) fragment, by the loss of $\overset{+}{M}$-56 from the molecular ion $\overset{+}{M}$=160. The peak at M/e 87 with 50% relative intensity is supposed to be given by $^{+}CH_2\text{-}COOC_2H_5$ fragment, by the loss of $\overset{+}{M}$-73 from the molecular ion. Finally, the peak at M/e 59 with 27% relative intensity is probably due to $^{+}CH_2\text{-}COOH$ fragment, by the loss of $\overset{+}{M}$-101 from the molecular ion $\overset{+}{M}$=160.

Fig. 10 is the mass spectra of an extra peak observed in the GC traces of CSF samples, which is thought to be of ethyl pyruvate, $CH_3\text{-}CO\text{-}COOC_2H_5$, which might have formed by the reaction of pyruvic acid present in the CSF samples with ethanol, and is identified in MS spectra as fallow:

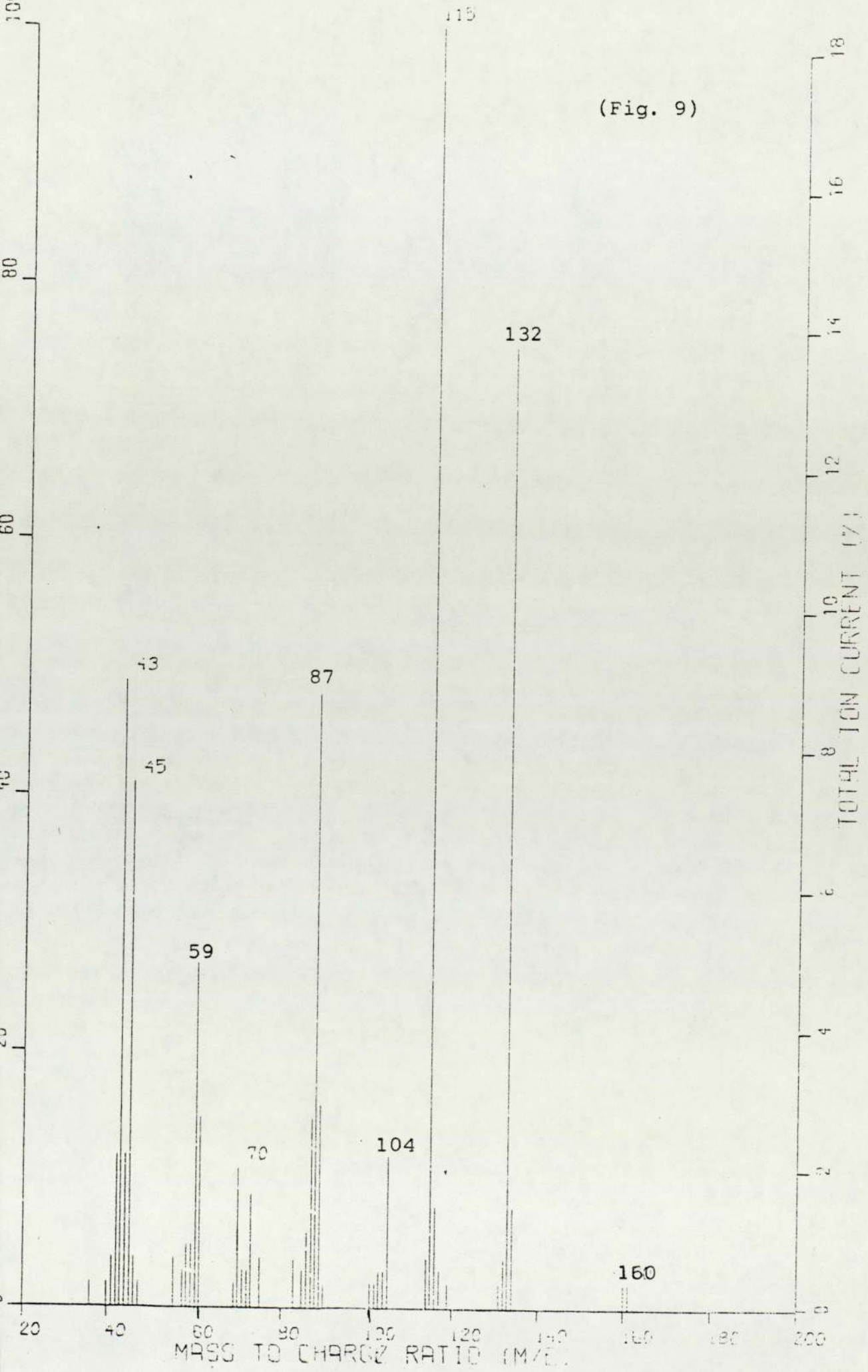
The peak at M/e 101 with 8% relative intensity is probably attributed to $^{+}CO.COOC_2H_5$ fragment, by the loss of $-CH_3$ group from the molecular ion $\overset{+}{M}$=116. The peak at M/e 88 with 39% relative intensity is thought to be by $CH_3.CO.COOH$ fragment, by the loss of $-CH_2{=}CH_2$ group from the molecular ion $\overset{+}{M}$=116. The peak at M/e 73 with 9% relative intensity is possibly attributed by $^{+}COOC_2H_5$ fragment, by the loss of $-CH_3CO$ group from the molecular ion. Again, the peak at M/e 71 with 29% relative intensity is supposed to be attributed by $CH_3.CO.CO^{+}$ fragment, by the loss of C_2H_5O group ($\overset{+}{M}$-45) from the molecular ion $\overset{+}{M}$=116. The peak at

M/e 59 with 33% relative intensity is probably given by $^{+}CH_2$-COOH fragment, by the loss of $\dot{M}$-57 from the molecular ion. Finally, the base peak at M/e 43 is possibly attributed by CH_3CO^{+} fragment, by the loss of $\dot{M}$-73 from the molecular ion $\dot{M}$=116.

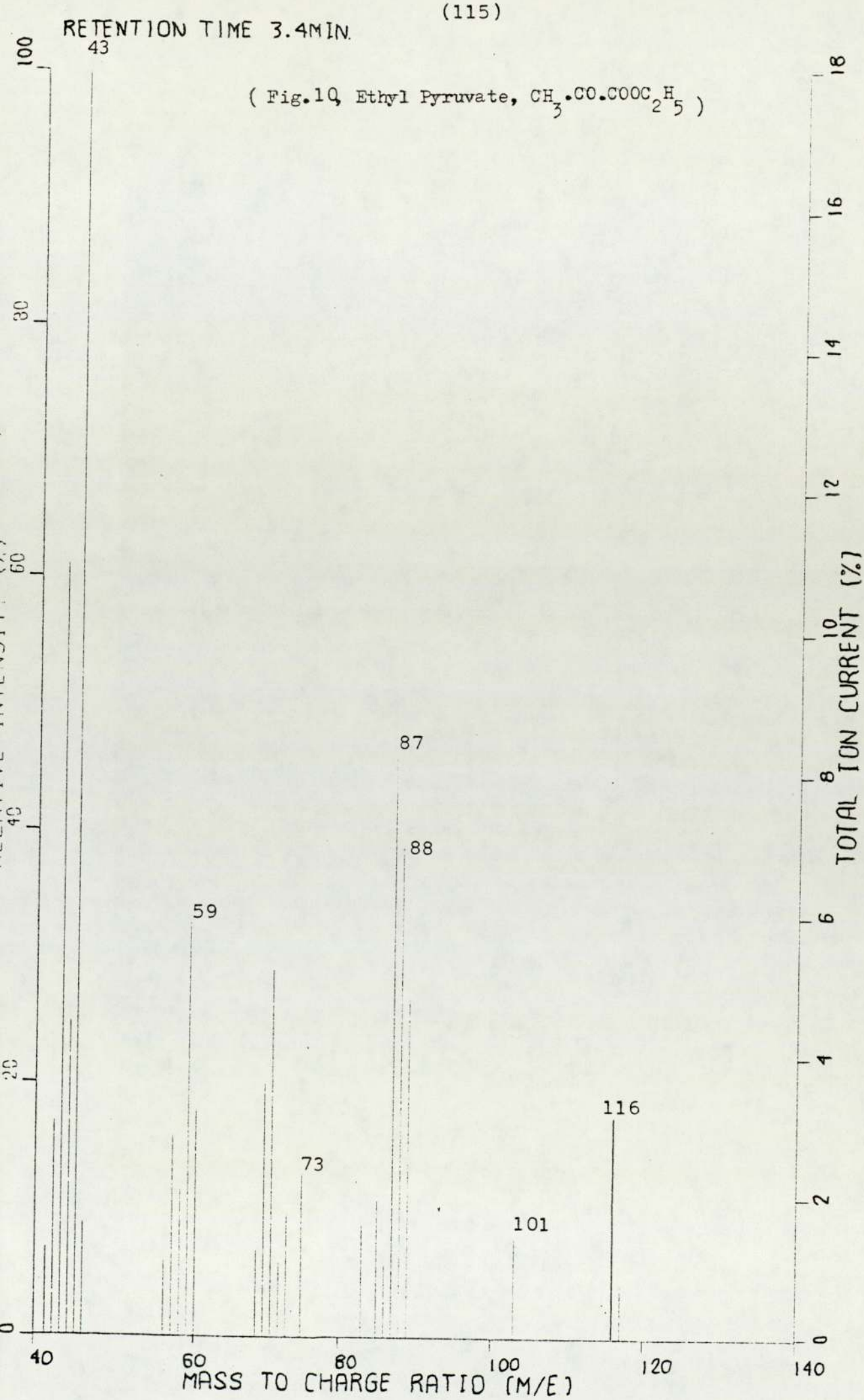
(Fig. 8)


ETHYL LACTATE
45
73
29
28
83
90
59
103
118
20
40
60
80
100
120
140
MASS TO CHARGE RATIO (M/E)
TOTAL ION CURRENT (%)
2
4
8
10
12
14

DIETHYL MALONATE



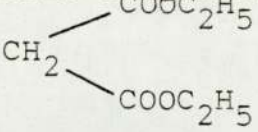
(Fig. 9)



(Fig.10, Ethyl Pyruvate, $CH_3.CO.COOC_2H_5$)

Table .1. indicates the possible fragments of ethyl lactate, $CH_3.CH(OH).COOC_2H_5$, by GC-MS.

M / e	RELATIVE INTENSITY	MOLECULAR WEIGHT, $\overset{+}{M}$ = 118	FRAGMENTS.
103	11%	$\overset{+}{M}$-15	$H\overset{+}{O}=CH-\ COOC_2H_5$
90	16%	$\overset{+}{M}$-28	$CH_3-CH(OH)-C(=O)-OH^+$
73	89%	$\overset{+}{M}$-45	$CH_3-CH(OH)-C\equiv O^+$
45	100%	$\overset{+}{M}$-73	$CH_3-CH=\overset{+}{O}H$

Table.2. indicates the probable fragments of diethyl malonate, 

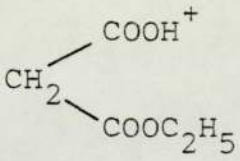
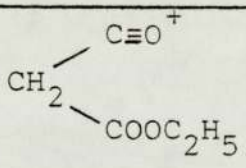
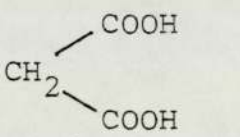
M/e	RELATIVE INTENSITY.	MOLECULAR WEIGHT, $\overset{+}{M}$ = 160	FRAGMENTS.
132	75%	$\overset{+}{M}$-28	
115	100%	$\overset{+}{M}$-45	
104	13%	$\overset{+}{M}$-56	
87	50%	$\overset{+}{M}$-73	$^{+}CH_2-COOC_2H_5$
59	27%	$\overset{+}{M}$-101	$^{+}CH_2-COOH$

Table.3. shows the possible fragments of ethyl pyruvate, $CH_3.CO.COOC_2H_5$.

M/e	RELATIVE INTENSITY.	MOLECULAR WEIGHT, $M^+ = 116$	FRAGMENTS.
101	8%	M^+-15	$^+CO.COOC_2H_5$
88	39%	M^+-28	$CH_3.CO.COOH$
73	9%	M^+-43	$^+COOC_2H_5$
71	29%	M^+-45	$CH_3CO.CO^+$
59	33%	M^+-57	$^+CH_2-COOH$
43	100%	M^+-73	CH_3CO^+

(V). CSF lactate value determination.

Fig. 2 is the chromatogram of CSF sample no. 56, indicates the highest level of lactic acid (0.91 $mg.ml.^{-1}$) found in a CSF sample with an extra peak which is believed to be by pyruvic acid. Table 4 records the computer results for this analysis.

Fig. 3 is a gas chromatogram of CSF samples no. 2, 6, and 45 indicating the varying amounts of lactic acid in CSF samples as well as extra peaks(pyruvic acid), and table 5 gives the computer results of this analysis indicating that these samples could be non-bacterial cases.

Again, fig. 4 is a gas chromatogram of CSF samples no. 22 and 29 which indicate sample no. 22 might be non-bacterial case and sample no. 29 (second highest value of lactic acid(0.69 $mg.ml.^{-1}$) found with an extra peak observed) obviously a bacterial case. Table 6 records the computer results of this analysis.

Table 7 records the data for 3 replicate runs of peak height ratio for lactic acid determination in CSF samples by GLC assay method.

Table 4. computer results.

5:4:78 GC CALIBRATION (PEAK HEIGHT RATIO) FOR ASSAY OF LACTIC ACID

There are 3 X-values and 6 replicates of each Y-value

	X-values	Y-values				
1	0.900	3.19	3.16	3.17	3.12	3.14	3.18
2	0.600	2.10	2.05	2.05	2.08	2.08	2.05
3	0.300	1.02	1.05	1.04	1.03	1.07	1.06

B (SLOPE) = 3.5218333E 00 A(INTERCEPT)= -2.1538889E-02

SOURCE OF VARIANCE	DEGREES OF FREEDOM	VARIANCE	VARIANCE RATIO
REGRESSION	1	13.3956	27189.5073
DEVIATION	1	0.0051	10.4153
BETWEEN X-S	2	6.7004	13599.9613
RESIDUAL	15	0.0005	
TOTAL	17	0.7887	

THE F STATISTIC (1,1) = 2610.5440 (98.57%)

THE VARIANCE OF Y ABOUT THE REGRESSION LINE IS 0.0008

COVARIANCE = 0.2237 CORRELATION COEFFICIENT = 0.9995

THIS ACCOUNTS FOR 99.907% OF THE VARIATION OBSERVED

GIVE AVERAGE OF Y-VALUE FOR CALCULATION OF UNKNOWN CONC.

YOUR INTERPOLATED CONCENTRATION IS 0.1404 $mg.ml.^{-1}$

THE 95% CONFIDENCE LIMITS ARE 0.126 TO 0.155

****

GIVE AVERAGE OF Y-VALUE FOR CALCULATION OF UNKNOWN CONC.

YOUR INTERPOLATED CONCENTRATION IS 0.906 $mg.ml.^{-1}$

THE 95% CONFIDENCE LIMITS ARE 0.893 TO 0.919

Table 5. computer results

****

24:1:78 GC CALIBRATION (PEAK HEIGHT RATIO) FOR ASSAY OF LACTIC ACID.

THERE ARE 4 X-VALUES AND 6 REPLICATES OF EACH Y-VALUE

	X-VALUES	Y-VALUES					
1	0.900	1.94	1.93	1.98	1.94	1.92	1.74
2	0.600	1.23	1.24	1.24	1.22	1.21	1.22
3	0.300	0.63	0.63	0.63	0.64	0.64	0.63
4	0.150	0.31	0.31	0.31	0.31	0.31	0.31

B (SLOPE) = 2.1149718E 00 A(INTERCEPT)= -1.1257062E-02

SOURCE OF VARIANCE	DEGREES OF FREEDOM	VARIANCE	VARIANCE RATIO
REGRESSION	1	8.9071	4827.3975
DEVIATION	2	0.0039	2.0997
BETWEEN X-S	3	2.9716	1610.5323
RESIDUAL	20	0.0018	
TOTAL	23	0.3892	

THE F STATISTIC (1,2) = 2299.0602 (99.97%)

THE VARIANCE OF Y ABOUT THE REGRESSION LINE IS 0.002

COVARIANCE = 0.1831 CORRELATION COEFFICIENT= 0.9975

THIS ACCOUNTS FOR 99.50% OF THE VARIATION OBSERVED

GIVE AVERAGE OF Y-VALUE FOR CALCULATION OF UNKNOWN CONC.

YOUR INTERPOLATED CONCENTRATION IS = 0.0857 $mg.ml.^{-1}$

THE 95% CONFIDENCE LIMITS ARE 0.054 TO 0.117

****

Table 6. computer results

15:3:78 GC CALIBRATION FOR ASSAY OF LACTIC ACID

THERE ARE 4 X-VALUES AND 6 REPLICATES OF EACH Y-VALUE

	X-VALUES	Y-VALUES					
1	o.900	3.30	3.26	3.35	3.26	3.17,	3.13
2	0.600	2.12	2.10	2.07	2.20	1.99	2.04
3	0.300	1.02	1.02	1.02	1.01	1.02	0.99
4	0.150	0.52	0.51	0.51	0.50	0.50	0.50

B (SLOPE) = 3.6421431E 00 A (INTERCEPT) = -7.0419774E-02

SOURCE OF VARIANCE	DEGREES OF FREEDOM	VARIANCE	VARIANCE RATIO
REGRESSION	1	26.4143	10965.9840
DEVIATION	2	0.0194	8.0358
BETWEEN X-S	3	8.8177	3657.6852
RESIDUAL	20	0.0024	
TOTAL	23	1.1522	

THE F STATISTIC (1,2) = 1363.5256 (99.95%)

THE VARIANCE OF Y ABOUT THE REGRESSION LINE IS 0.0040

COVARIANCE = 0.3153 CORRELATION COEFFICIENT = 0.9984

THIS ACCOUNTS FOR 99.67%)F THE VARIATION OBSERVED

GIVE AVERAGE OF Y-VALUE FOR CALCULATION OF UNKNOWN CONC.

YOUR INTERPOLATED CONCENTRATION IS 0.125 $mg.ml.^{-1}$

THE 95% CONFIDENCE LIMITS ARE 0.100 TO 0.150

****

GIVE AVERAGE OF Y-VALUE FOR CALCULATION OF UNKNOWN CONC.

YOUR INTERPOLATED CONCENTRATION IS 0.692 $mg.ml.^{-1}$

THE 95% CONFIDENCE LIMITS ARE 0.668 TO 0.716

Table 7. Data for 3 replicates runs of peak height ratio for lactic acid determination in CSF samples by GLC assay.

	1	2	3	mean	S.Dev.	CV(%)
a	13.05	14.35	14.35			
a/std.	2.90	2.87	2.87	2.88	0.0457	0.159
b	9.95	10.05	9.65			
b/std.	1.951	1.951	1.950	1.951	0.0082	0.418
c	4.50	4.30	4.10			
c/std.	0.957	0.955	0.954	0.955	0.0048	0.498
d	2.17	2.10	2.05			
d/std.	0.477	0.477	0.477	0.477	0.0023	0.490

Where a, b, c, and d are the peak heights of lactic acid standard.

(C). EXPERIMENTAL.

(I). Instrumentation.

(a). GC.

Gas chromatograph	:	Pye 104 (model 4)
Detector	:	Dual flame ionisation.
Recorder	:	W+W 1100.
Column	:	1.5 m X 4 mm i.d. glass
Packing material	:	Carbowax 20M (8%) + KOH (2%) on Chromosorb W, AW-DMCS, 100-120 mesh.
Oven temperature	:	$150^{o}C$
Injection port temperature	:	$180^{o}C$
Amplification	:	20×10^{2}
Chart speed	:	0.5 cm. $min.^{-1}$
Nitrogen(carrier) gas F/R	:	70 $ml.min.^{-1}$
Air pressure	:	o.5 $Kg.cm.^{-2}$
Hydrogen pressure	:	1.3 $Kg.cm.^{-2}$

(b). MS.

Mass spectrometer	:	Micro mass 12B.
Ionising voltage	:	22eV.
Accelerating voltage	:	3 KV.
M.S. source temperature	:	$280^{o}C$
Trap current	:	100 μA

(II). Development of ethyl lactate derivative.

During a six months period, 77 CSF samples obtained by lumbar puncture on 60 patients (tables 8-14) at various stages of treatment were spun down and the supernatant deep frozen at -40°C.

Samples of cerebrospinal fluid (CSF) were assayed for lactic acid content by treating aliquots of CSF (1.0 ml.) with sulphuric acid (0.2 ml.; 50%) in a 3 ml. reactivial. Absolute ethanol (1.0 ml.) was added, the container was sealed and the mixture was then heated to 100°C($\pm$ 0.5°C) and maintained at this temperature for 5 minutes. On cooling chloroform (0.5 ml.) containing diethyl malonate (0.125 mg.ml.$^{-1}$) as internal standard was added and the whole was shaken to equilibrate the phases. After separation of the phases (and centrifugation where necessary to disperse emulsified droplets) an aliquot of the lower chloroform layer (1 µl) was injected into the GC column. In those cases where less than 1.0 ml. of CSF was available proportionate reduction in the scale of derivatisation and extraction down to 0.1 ml. of CSF (0.02 ml. 50% sulphuric acid, 0.1 ml. ethanol and 0.05 ml. chloroform) was successful with little loss in precision.

(III). Stability of lactate derivative in CSF & blood.

(a). 5 bloody CSF samples were mixed together.

(b). 8 clear CSF samples were mixed together.

These (a) and (b) mixtures were derivatised as in (II) and were analysed by GLC technique as containing 0.29 $mg.ml^{-1}$ and 0.11 $mg.ml^{-1}$ of lactic acid, respectively (fig. 5.).

Then bloody mixed CSF samples were acidified with 0.03 ml. of N/10 lactic acid standard and clear mixed CSF samples were acidified with 0.5 ml. of N/10 lactic acid.

Again, these mixtures were derivatised as in (II).

They contained 3.0 $mg.ml^{-1}$ and 1.2 $mg.ml^{-1}$ of lactic acid in mixed bloody samples and mixed clear samples, respectively (fig. 6.).

These samples were stored at $-40^{o}C$ over a period of three months and monitoring of these samples after and during that period showed no significant loss in concentration of lactic acid.

Note: All CSF samples were with drawn and mixed in a air tight glove box and all syringe cone tips used in the CSF samples were diped in 5% phenol solution, in order to destroy the bacteria.

Table 8, indicates the lactic acid value determined of CSF samples by GLC assay method.

No.	Name of Patient.	CSF sample date	Lactic acid determined.
1	M.R.	3.8.1977	0.125mg/ml
2	D.S.	3.8.1977	0.17mg/ml*
3	Z.E.	5.8.1977	0.13mg/ml
4	R.S.	5.8.1977	0.12mg/ml
5	P.K.	15.8.1977	0.40mg/ml
6	E.C.	16.8.1977	0.19mg/ml
7	V.K.	17.8.1977	0.16mg/ml
8	R.S.	17.8.1977	0.13mg/ml
9	P.B.	20.8.1977	0.15mg/ml
10	Y.D.	23.8.1977	0.14mg/ml
11	R.S.	25.8.1977	0.15mg/ml

* indicates an extra peak observed.

Table 9, indicates the lactic acid value determined of CSF samples by GLC assay method.

No.	Name of patient.	CSF sample date	Lactic acid determined.
12	T.P.	25.8.1977	0.19mg/ml
13	D.M.	1.9.1977	0.09mg/ml
14	N.M.	3.9.1977	0.19mg/ml
15	R.G.	3.9.1977	0.15mg/ml
16	B.P.	5.9.1977	0.13mg/ml
17	I.T.	5.9.1977	0.12mg/ml
18	Y.B.	17.9.1977	0.12mg/ml
19	C.P.	20.9.1977	0.06mg/ml
20	E.B.	26.9.1977	0.13mg/ml
21	B.R.	27.9.1977	0.13mg/ml
22	M.S.	27.9.1977	0.13mg/ml

* indicates an extra peak observed.

Table 10, indicates the lactic acid value determined of CSF samples by GLC assay method.

No.	Name of patient.	CSF sample date.	Lactic acid determined.
23	D.B.	3.10.1977	0.24mg/ml*
24	R.S.	11.10.1977	0.05mg/ml
25	D.B.	12.10.1977	0.14mg/ml
26	A.G.	12.10.1977	0.11mg/ml
27	E.D.	18.10.1977	0.10mg/ml
28	G.H.	18.10.1977	0.10mg/ml
29	D.B.	30.10.1977	0.69mg/ml*
30	J.H.	7.11.1977	0.21mg/ml
31	J.H.	7.11.1977	0.22mg/ml
32	M.A.	10.11.1977	0.12mg/ml
33	F.T.	10.11.1977	0.12mg/ml

* indicates an extra peak observed.

Table 11, indicates the lactic acid value determined of CSF samples by GLC assay method.

No.	Name of patient.	CSF sample date.	Lactic acid determined.
34	C.C.	11.11.1977	0.19mg/ml*
35	R.M.	15.11.1977	0.06mg/ml
36	W.W.	15.11.1977	0.58mg/ml*
37	J.O.	16.11.1977	0.12mg/ml
38	J.H.	17.11.1977	0.22mg/ml
39	D.T.	12.11.1977	0.10mg/ml
40	J.A.	12.11.1977	0.12mg/ml
41	J.H.	17.11.1977	0.10mg/ml
42	T.M.	21.11.1977	0.17mg/ml
43	J.S.	23.11.1977	0.16mg/ml
44	J.S.	23.11.1977	0.15mg/ml

* indicates an extra peak observed.

Table 12, indicates the lactic acid value determined of CSF samples by GLC assay method.

No.	Name of patient.	CSF sample date	Lactic acid determined.
45	M.K.	28.11.1977	0.10mg/ml*
46	J.H.	28.11.1977	0.11mg/ml
47	C.G.	1.12.1977	0.08mg/ml
48	F.S.	2.12.1977	0.13mg/ml
49	D.M.	2.12.1977	0.17mg/ml
50	A.B.	2.12.1977	0.09mg/ml
51	T.K.	6.12.1977	0.17mg/ml
52	C.D.	7.12.1977	0.14mg/ml
53	A.M.	7.12.1977	0.11mg/ml
54	M.O.	7.12.1977	0.11mg/ml
55	A.S.	9.12.1977	0.08mg/ml

* indicates an extra peak observed.

Table 13, indicates the lactic acid value determined of CSF samples by GLC assay method.

No.	Name of patient.	CSF sample date	Lactic acid determined.
56	R.F.	11.12.1977	0.91mg/ml*
57	D.K.	12.12.1977	0.11mg/ml
58	A.A.	15.12.1977	0.31mg/ml
59	A.A.	22.12.1977	0.48mg/ml
60	T.P.	15.12.1977	0.10mg/ml
61	T.P.	15.12.1977	0. 105mg/ml
62	C.D.	17.12.1977	0.10mg/ml
63	J.O.	16.12.1977	0.12mg/ml
64	P.K.	20.12.1977	0.11mg/ml
65	M.J.	24.12.1977	0.11mg/ml
66	S.B.	8.9.1977	0.17mg/ml

* indicates an extra peak observed.

Table 14, indicates the lactic acid value determined of CSF samples by GLC assay method.

No.	Name of patient.	CSF sample date.	Lactic acid determined.
67	B.K.	18.10.1977	0.04mg/ml
68	P.M.	3.12.1977	0.08mg/ml
69	D.D	16.8.1977	0.25mg/ml
70	N.R.	6.10.1977	0.11mg/ml
71	K.U.	4.4.1978	0.26mg/ml
72	W.B.	7.4.1978	0.29mg/ml
73	D.S.	11.4.1978	0.21mg/ml
74	R.G.	11.4.1978	0.15mg/ml
75	W.W		0.13mg/ml
76	F.T.		0.10mg/ml
77	J.A.		0.18mg/ml

(D). SUMMARY AND CONCLUSION.

The GLC assay, for the determination of lactic acid in cerebrospinal fluid (CSF) of humans, with respect to bacterial or non-bacterial characterstics, has been established and found superior to other assay methods. When one compares the rapidity of the technique with others, it was found this assay method to be among the fastest. This technique compared rather favourably with limulus, CLE, and NBT techniques which take more than half an hour to perform.

The time required to prepare the CSF lactate derivative for technique reported here, is less than 15 minutes, and the retention time for complete analysis of the peaks is less than 5 minutes. The method reported here, for the quantitative determination of lactic acid by GLC has the advantages over other published assay methods[45,57,68,69,120] of being simple, accurate, sensitive, rapid, specific, and cheap, and can be performed as an emergency procedure for any laboratory equipped with a gas liquid chromatograph.

The measurement of lactate in CSF is undoubtedly useful but must always be considered as merely one parameter when assessing each patient.

As much as 0.91 $mg.ml.^{-1}$ of lactic acid was observed in this analysis which clearly indicates a bacterial case, and as low as 0.05 $mg.ml.^{-1}$ of lactic acid has been quantified by this technique, which obviously indicates the non-bacterial meningitis. The stability of the

samples has been assessed over three months period in order to make sure that the lactic acid levels are constant.

The monitoring of the same samples over a period of three months showed that no significant loss occurs when CSF samples are stored at -40^{o}C.

FURTHER WORK.

Due to the toxicity of Chloramphenicol, its use in new born infants and older children with "hepatic disease" is restricted. Also, as it may cause cancer in humans, chloramphenicol injections are prohibited. It is recommended for initial therapy of life-threatning disease, because of ampicillin-resistant strains.

But recent changes in medical practice and antibiotic suseptibility of pathogenic bacteria suggests that chloramphenicol may be used with increasing frequency in infants and children in future while the chemist might require an exact evaluation of the molar concentration of an antibiotic in solution, in the field of kinetic studies and the like, the clinician seeks information as to the distribution and behaviour of the antibiotic in the body fluids.

The concentration of chloramphenicol is reported to be higher in CSF than those of other antibiotics. Trials sooner or later necessitate estimation of the drug in blood, urine, and CSF as well as its presence in pus and exudates. Monitoring of milk concentrations are of particular importance in deciding whether a mother undergoing antibiotic therapy should breast feed her child. Thus, this GLC assay method of chloramphenicol could be usefully developed further to monitor plasma concentration of the drug in patients who are under going chloramphenicol therapy.

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