HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ERYTHROMYCIN IN SERUM AND URINE

by

CHRISTOPHER STUBBS

A Thesis Submitted to
Rhodes University
in Partial Fulfilment of the
Requirements for the Degree of

MASTER OF SCIENCE

January 1985

School of Pharmaceutical Sciences
Rhodes University
Grahamstown

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Professor I. Kanfer for his enthusiasm, guidance and invaluable assistance throughout this study, as well as for making such excellent laboratory facilities available.

Grateful thanks are also due to:

Dr Neil Sparrow for his helpful suggestions and pleasant company in the laboratory.

Professor H. Parolis and the staff of the School of Pharmaceutical Sciences for their varied forms of assistance and for the use of departmental facilities.

Messrs L.H. Purdon and O.I.D. Campbell for their technical assistance.

The South African Council for Scientific and Industrial Research and the H. Bradlow Scholarship for Postgraduate Study, for financial assistance.

The following companies for donation of drug samples; Abbott, Lennons, Lilly, Pfizer and Yamanouchi.

Kathleen Wakashe and Shelley Macrae for their help in preparing this thesis.

My parents, Rich and Sue, for their interest and encouragement.

CONTENTS

CHAPTER 2

CHAPTER 3

DETERMINATION OF ERYTHROMYCIN IN BIOLOGICAL FLUIDS

LIST OF FIGURES

LIST OF TABLES

Abstract

Erythromycin, a macrolide antibiotic used mainly against gram-positive bacteria has been in clinical use since 1952 (1). Previous pharmacokinetic data published on this antibiotic have been derived predominantly from microbiological assay techniques. However, these techniques are relatively imprecise as well as being non-specific and extremely tedious to perform.

A novel high performance liquid chromatographic analysis of erythromycin in human serum and urine using U.V. detection at 200 nm and/or electrochemical detection using both an amperometric and a coulometric electrochemical detector is presented.

The method involves a solid phase extraction procedure followed by a simple phase separation step and chromatography on a reverse phase column. In order to select the optimum U.V. detector for this analysis, five "state of the art" detectors were compared in terms of their signal-to-noise ratios at U.V. wavelengths between 200 and 210 nm. A known metabolite des-N-methylerythromycin is readily detectable using U.V. detection, whilst another metabolite/degradation product anhydroerythromycin is not seen using U.V. detection but is readily observable using an electrochemical detector.

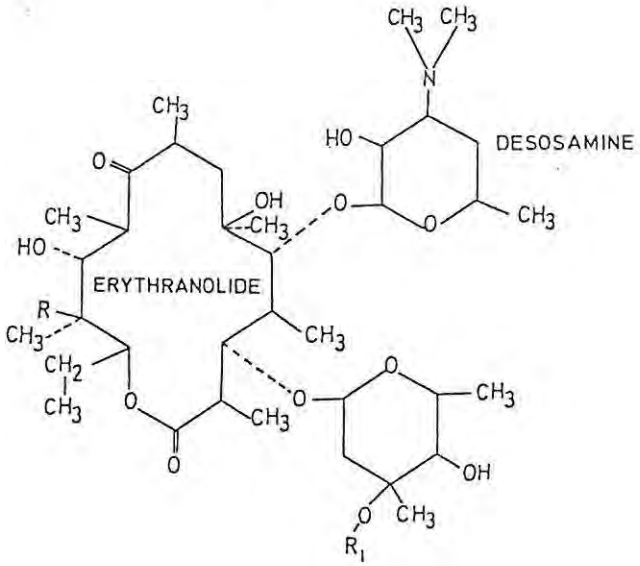
The method has a limit of sensitivity of 0.25 μg/mL and 1.00 μg/mL in serum and urine respectively (U.V. detection) and is sufficiently sensitive to monitor serum and urine concentrations of erythromycin in man after administration of a single 500 mg erythromycin stearate tablet.

CHAPTER ONE

INTRODUCTION

1.1 PHYSICOCHEMICAL PROPERTIES OF ERYTHROMYCIN

Erythromycin was discovered by McGuire *et al.* in 1952 (1) from amongst the metabolic products of a strain of *Streptomyces erythreus* found in a soil sample from the Phillipines (1). The major component of the drug is erythromycin A, with small amounts of erythromycin B and C being present (2). The structures of erythromycin and these two components are depicted in Fig. 1.1. Erythromycin is a polyhydroxylactone containing two sugars, desosamine, which is a 3-N -dimethyl-aminodeoxy sugar, and L-cladinose, which contains no nitrogen and has one methoxy group. The aglycone part of the molecule is a 14-member lactone, called erythranolide. Both cladinose and desosamine are bound to the lactone ring by a β-glycosidic linkage (3).



	R	R_1
Erythromycin A	OH	CH_3
Erythromycin B	H	CH_3
Erythromycin C	OH	H

FIGURE 1.1 Structures of erythromycin and its components

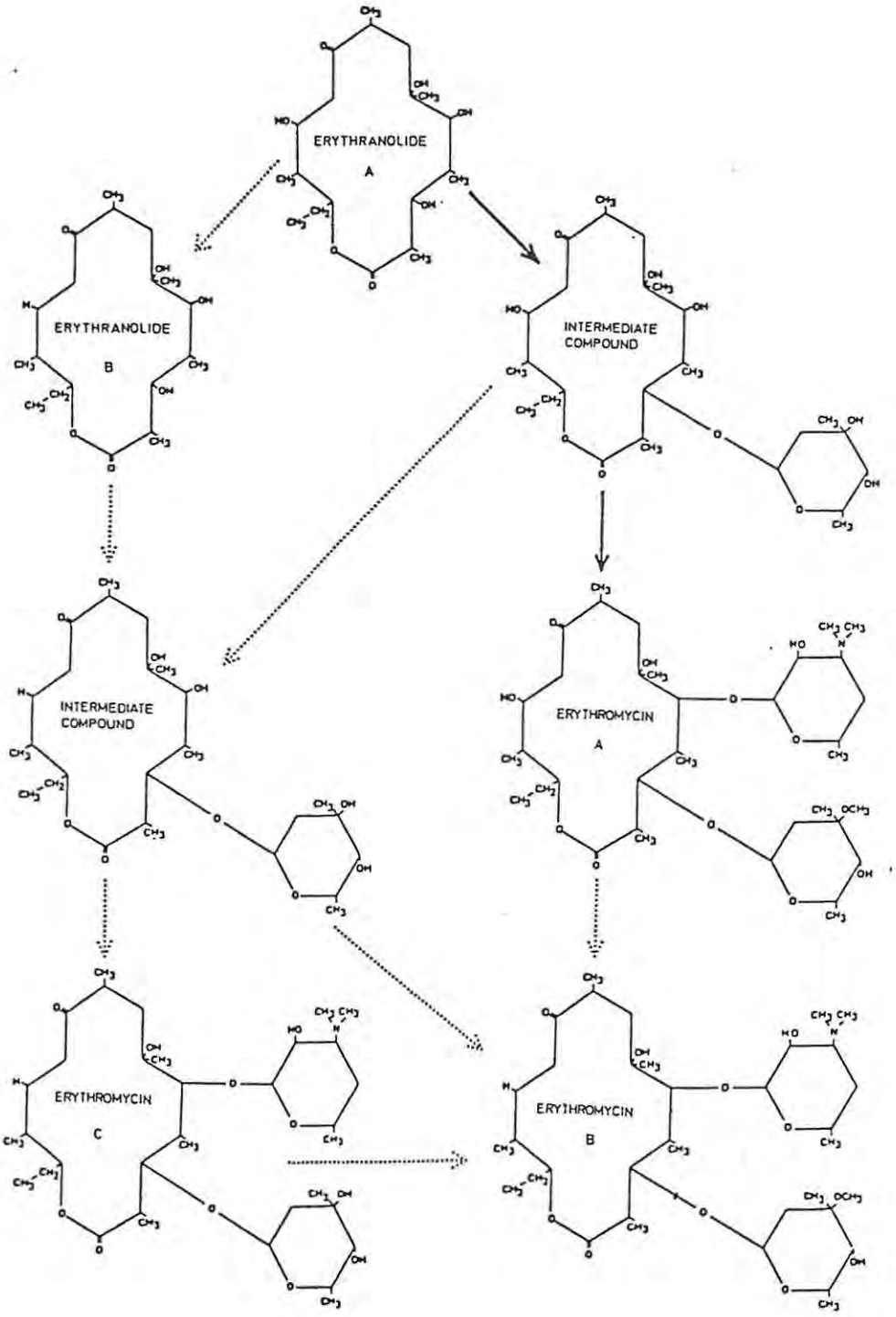
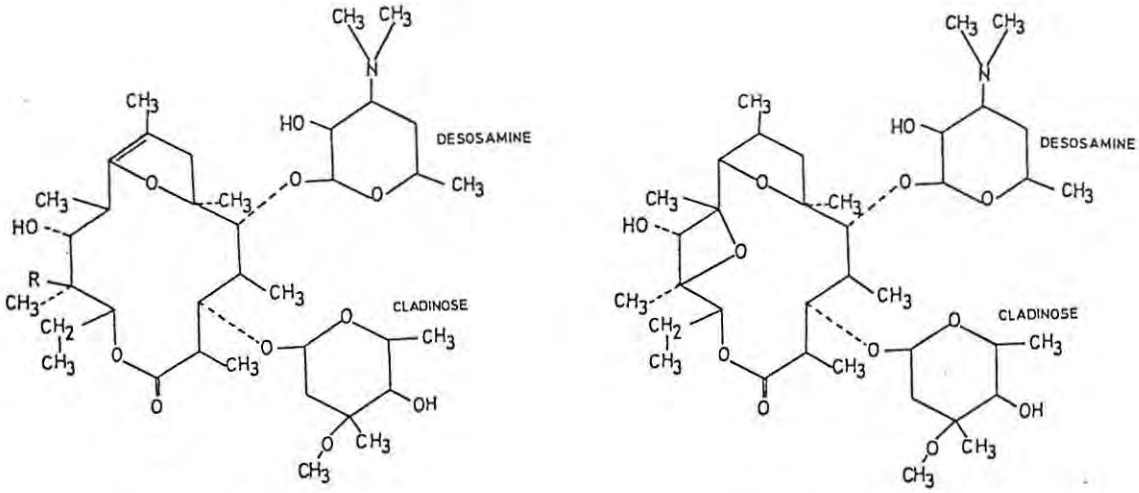


FIGURE 1.2 Postulated synthetic pathways of erythromycin from erythranolide A

Reproduced from Hung, Marks and Tardrew (4).

The formation of erythromycin from erythranolide A involves two alternative pathways during biosynthesis, leading by one route to erythromycin B and C and an intermediate compound and by another route to erythromycin A and another intermediate compound (Fig.1.2) (4). Erythromycin exists in many different forms, including at least two hydrates and an anhydrate (5).

Anhydrous erythromycin has a melting point of 190-193°C and an optical rotation of $[\alpha]_D^{25}$ = -78°C (2% in ethanol); the monohydrate and hydrochloride salt, have melting points of 137-140°C and 170-173°C respectively (3). Erythromycin is a colourless, bitter tasting substance poorly soluble in water (1-2 mg/L), readily soluble in most alcohols, acetone, chloroform and ether (6). It is a weak base (pKa 8.8) which is unstable in acidic or alkaline solutions and shows its maximum stability between pH 6.0 and 9.5 (7). The principal acid degradation products are 8,9 anhydroerythromycin and the 6,9 hemiacetal of 8,9 anhydroerythromycin (Fig.1.3) (8).



(I) R = OH
(II) R = H

(III)

(I) 6,9 - hemi-acetal of 8,9 - anhydroerythromycin A
(II) 6,9 - hemi-acetal of 8,9 - anhydroerythromycin B
(III) 8,9 - anhydroerythromycin A

FIGURE 1.3 Chemical structures of the predominant acid degradation products of erythromycin

Aqueous, alcoholic solutions buffered at pH 7.0-8.0 are stable for one week under refrigeration (4°C) (7) and for three months when frozen (-10°C) (9). The dry powder is stable, but it is suggested that it be stored in the dark below 30°C in airtight containers (6).

1.2 CHEMICAL DERIVATIVES OF ERYTHROMYCIN

As discussed, erythromycin is susceptible to inactivation by acid, resulting in decreased absorption of orally administered erythromycin due to exposure of the drug to gastric secretions (10). Chemical modifications of the basic drug moeity have been performed in order to protect the active moeity from acid degradation. The most commonly used of these erythromycin derivatives are the stearate (an insoluble salt), the ethylsuccinate (an ester) and the estolate (the lauryl sulfate salt of the propionyl ester) (11).

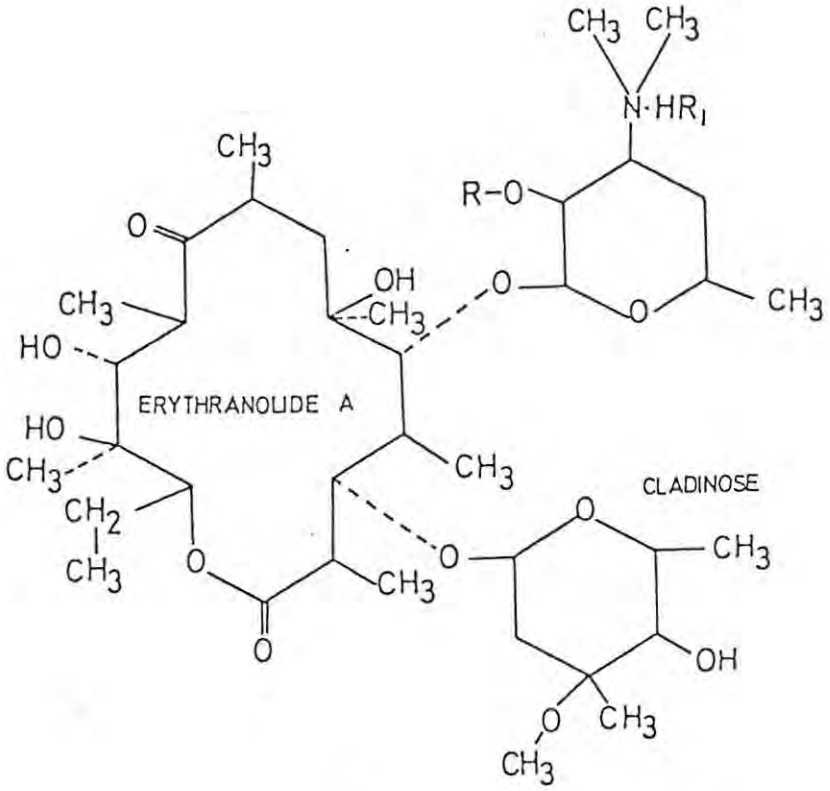
Two water soluble salts, erythromycin lactobionate and glucoheptonate, have been prepared for intravenous (i.v.) erythromycin formulations. The structures of all of the above compounds are depicted in Fig. 1.4.

1.3 CLINICAL PHARMACOLOGY OF ERYTHROMYCIN

1.3.1 Mode of Action

Erythromycin penetrates the cell wall of sensitive bacteria (12) and attaches to the 50S ribosomal subunit donor site in the presence of ammonium or potassium ions (13). A single molecule of erythromycin attaches to each ribosomal fragment (12). It prevents the incorporation of certain amino acids (lysine, phenylalanine) into the polypeptide linkages, but has no effect on the synthesis of nucleic acid (13).

Although it acts by inhibiting protein synthesis, erythromycin may be either bacteriostatic or bactericidal depending on the sensitivity of the particular microorganism and the concentration of the drug (14). Its selectivity for micro-organisms is due to the absence of the 50S subunit on the ribosomes of the human host (15).



	R	R_1
Erythromycin base	H	
Propionyl erythromycin	CH_2CH_2 CO	
Erythromycin estolate	CH_2CH_2 CO	$C_{12}H_{25}OSO_3$
Erythromycin stearate	H	$C_{17}H_{35}COO$
Erythromycin ethylsuccinate	$CH_2CH_2OOCCH_2CH_2COO$	
Erythromycin lactobionate	H	$C_{11}H_{19}O_9COO$
Erythromycin glucoheptonate	H	$C_6H_{13}O_6COO$

FIGURE 1.4 Structural formulae of erythromycin and its derivatives

1.3.2 Antimicrobial Spectrum

Erythromycin is active against most gram-positive bacteria and some gram-negative organisms, with an overall spectrum of activity similar to that of penicillin G (12). Typical *in vitro* sensitivities of organisms to erythromycin as reported by Nicholas (14) are summarized in Table 1.1.

TABLE 1.1. Typical *in vitro* sensitivities of organisms to erythromycin

Organism	Typical Minimum Inhibitory Concentration (micrograms per milliliter) Mean	Range
S. aureus	0.4	0.005->100
Streptococcus pyogenes	0.04	0.005-0.8
Streptococcus pneumoniae	0.05	0.006-0.2
Streptococcus viridans	0.06	0.02-0.1
Clostridium perfringens	0.5	0.05-5
Listeria monocytogenes	0.16	0.1-0.3
Corynebacterium diphtheriae	0.02	0.006-0.2
Actinomyces israelii	0.5	0.2-0.5
Nocardia asteroides	25.0	0.2->200
Neisseria meningitidis	0.4	0.1-0.8
Neisseria gonorrheae	0.1	0.005-0.4
Hemophilus influenzae	3.1	0.1-6
Bordatella pertussis	0.3	0.02-1.56
Bacteroides fragilis	1.5	0.12->128
Brucella species	5	0.3-10
Mycoplasma pneumoniae	0.005	0.001-0.01
Mycobacterium kansasii	1	0.5-2
Agent of Legionnaires' disease	$\leq$0.5	...

Reproduced from Nicholas (14).

In addition, erythromycin is active against some atypical mycobacteria, *Chlamydia* and some rickettsiae (12). The development of resistance by staphylococci, *Haemophilus influenzae*, and some pneumococci has decreased the use of this drug as first-line treatment of infections caused by these bacteria (12).

1.3.3 Resistance

Resistance to erythromycin appears to be related to a unique plasmid-determined mechanism by which the target site and not the antibiotic itself is changed. Strains of *Staphylococcus aureus* resistant to erythromycin have been shown to contain an enzyme which methylates specific sites on the 50S ribosomal subunit inhibiting the binding of erythromycin which is vital for its antibacterial action (14). Metabolites of erythromycin, isolated from human urine, have been shown to be possible inducers of resistance (16).

Staphylococci, resistant to erythromycin, have been reported to be resistant to all other macrolide antibiotics tested. The degree of this cross resistance varies considerably from strain to strain but may extend to all drugs acting on the 50S ribosomal sub-unit (17).

1.3.4 Adverse Reactions

Erythromycin is generally regarded as the least toxic of the commonly used antibiotics (14). Mild degrees of epigastric distress, nausea, vomiting and diarrhoea are fairly frequently encountered with oral erythromycin, but these symptoms are only rarely serious enough to require cessation of therapy (18). Allergic effects occur in about 0.5 per cent of patients (17). Most patients receiving i.v. erythromycin experience burning sensations in the infused vein often with phlebitis, necessitating frequent changes of the infusion site (12).

Cholestatic hepatitis may occur after oral use of erythromycin estolate, and ethylsuccinate (18), the signs and symptoms of which, range from mild to moderate abdominal discomfort with jaundice, to symptoms of acute cholecystitis (12). The reaction usually develops

after 10 to 21 days of continuous therapy and is more prevalent in adults than in children (14). Once medication is discontinued, recovery is prompt, although residual hepatic dysfunction has been reported (15). The generally accepted hypothesis that erythromycin estolate is a more frequent cause of jaundice than any of the other erythromycin derivatives, has recently been challenged. From the results of prescription-event monitoring of over twelve thousand patients receiving erythromycin estolate or erythromycin stearate it was concluded that the incidence of this side effect is unlikely to be more than one in a thousand using either drug (19). In addition, erythromycin stearate was responsible for three cases of erythromycin induced jaundice, whereas erythromycin estolate could not be implicated in any cases. In a controlled clinical study the estolate was found to cause no changes in normal liver function in 32 patients after a ten day period of administration (20). However, in a biochemical study, erythromycin estolate was found to have more cytotoxic potential than erythromycin base or propionate, causing leakage of enzymes from Chang liver cells at far lower concentrations than the other two drugs tested (21).

1.3.5 Drug Interactions

Erythromycin has been reported to affect the hepatic elimination of some drugs, the most important being theophylline and carbamazepine (22). The interaction between erythromycin and theophylline has been extensively studied. Some investigators have reported no interaction between these drugs, although many reports in the literature indicate that in clinical practice an interaction can be anticipated (23,24). This results in increased serum theophylline concentrations and hence possible toxicity. The mechanism of the interference is unclear, but is presumed to be due to competitive inhibition of a demethylation pathway during hepatic metabolism (23).

It has been suggested that in clinical practice a 25% reduction in the theophylline dose may be desirable in patients given more than six days concomitant treatment with erythromycin (23). Decreased serum erythromycin area under the curve values have been reported after the

co-administration of theophylline (25). Various possible explanations for these findings, including decreased absorption, altered protein binding and increased liver blood flow were discussed by the authors.

Increased serum carbamazepine concentrations and associated toxicity have been reported after the co-administration of erythromycin (24). The cause of this interaction is presumed to be similar to that of the erythromycin - theophylline interaction. Recently Wong *et al.* (26) reported that the clearance of oral carbamazepine (single dose) was decreased by the co-administration of erythromycin, whereas the apparent volume of distribution, elimination rate constant and absorption rate constant were unchanged. Further studies in this field using multi-dose regimes are necessary. This interaction should constantly be borne in mind when treating patients on long-term carbamazepine therapy with macrolide antibiotics (22).

Erythromycin has also been shown to increase the prothrombin time of a warfarin stabilized anticoagulant patient by 40% after only three days of concomitant erythromycin therapy (27).

1.3.6 Clinical Uses

The efficacy of this antibiotic against so many gram-positive cocci and against *Haemophilus influenzae* and *Mycoplasma pneumoniae* makes it an excellent antibiotic for the treatment of most upper respiratory tract infections such as sinusitis, laryngitis, tracheitis, acute bronchitis and primary atypical pneumonia (15). For persons allergic to penicillin who require an antibacterial agent with the spectrum of penicillin G or V, erythromycin is the drug of choice, provided that penetration across the blood brain barrier is not required (12).

The spectrum of erythromycin usage has been extended to include the treatment of Legionnaire's disease (14) and some genito-urinary diseases (14), including gonorrhoea and primary syphilis (18).

Recent findings that erythromycin is one of the few commonly used antimicrobial agents which is not immunosuppressive and may even

potentiate host immune responses, may well extend its already extensive clinical usage (28).

The usual oral dose is 1.0 to 4.0 g per day for adults and 25.0 to 50.0 mg/kg per day for children. The dose is administered in divided doses (usually 2-4 times a day) so as to decrease gastro-intestinal intolerance (18). As an alternative to penicillin in the treatment of primary syphilis 2.0 to 4.0 g per day should be administered for 10-15 days (15).

Intravenous administration is reserved for the therapy of severe infections. The usual dose is 0.5-1.0 g every 6 hours which may be given for as long as 4 weeks with no difficulty except for thrombophlebitis at the site of injection (18).

1.4 PHARMACOKINETICS OF ERYTHROMYCIN

1.4.1 Problems Associated with the Interpretation of Data Obtained from Published Pharmacokinetic Studies

The interpretation of data obtained from published studies, concerning both the pharmacokinetics and comparative bioavailability of erythromycin is complicated due to variations in study design, meal composition and timing, concomitant fluid intake and assay methodology, all of which have been shown to affect the pharmacokinetics of erythromycin considerably (11). The microbiological assay methods used in the majority of these studies are only able to detect concentrations of active drug. They are unable to detect the presence of any metabolites/degradation products, but most importantly, cannot distinguish between the amounts of active base and inactive parent ester, present after the administration of the ester derivatives of erythromycin. This has led to much confusion over the advantages of the various erythromycin derivatives available, as well as to some erroneous conclusions.

Numerous criteria for the standardization of pharmacokinetic and comparative bioavailability studies have been proposed (11,29-32). The

most important of these include the need for large numbers of subjects, a discriminatory assay technique and multiple dose studies (31).

The control of study variables and the specificity of the assay method have been used in the following discussion as a means of weighting the significance of the findings of the various studies discussed.

1.4.2 Absorption

1.4.2.1 Erythromycin Base and Stearate

Erythromycin base is absorbed intact, while erythromycin stearate, an insoluble salt of erythromycin, dissociates in the duodenum to erythromycin base, in which form it is absorbed (33). This absorption occurs predominantly in the small intestine, caecum and large intestine (34). Suppositories of erythromycin base are also well absorbed (35,36).

Shortly after the introduction of erythromycin, the absorption of the drug after oral administration of erythromycin base capsules was reported as being adequate but irregular (10). The initial theory of possible gastric acid deactivation of the drug (10), was substantiated by the improved serum concentrations obtained in patients with pernicious anaemia who were known to have no gastric acid secretions (37) and in normal patients after the co-administration of the capsules with aluminium hydroxide gel (10). Specially formulated acid resistant coatings were then applied to erythromycin tablets which resulted in increased absorption of the drug (10,37).

Erythromycin stearate which was originally prepared as an acid stable salt of erythromycin has since been found to be acid labile (38). This drug, which is formulated both as a tablet and a suspension for paediatric use, was also found to have increased absorption in patients with decreased gastric acid secretions (39). It is now predominantly formulated as a film coated tablet which provides sufficient protection for the acid labile drug (40).

Erythromycin base has been formulated as an enteric coated tablet but recently has become available as enteric coated pellets contained within a capsule (41). The effect of co-administration of food and other study variables on the bioavailability of these two drugs in the dosage forms discussed have been extensively studied.

The effect of food on the absorption of drugs in general has been reviewed by Welling (42). In the study of erythromycin absorption, the timing of the dose in relation to food intake is particularly important in that food may delay gastric emptying time and hence expose the acid resistant coating of the formulations to acid for a sufficiently long period to cause their dissolution and hence the acid degradation of the drug (43). The results of these studies are contradictory in many cases and are summarized in the following discussion.

Co-administration of food with the enteric coated erythromycin base formulation resulted in delayed but not decreased absorption (31, 44,45). More consistent and increased serum concentrations were obtained after administration of the formulation containing enteric coated pellets, which were not affected by the co-administration of food (44,46,47,48).

Many workers have found that erythromycin is absorbed better from film coated erythromycin stearate tablets when administered to fasting subjects (43,46,49-53), while others have found the absorption to be increased by the co-administration of food (40,44,54,55,56). A feasible hypothesis for this contradiction has been put forward by Malmborg (44) who stated that any observation on the advantages or disadvantages of concurrent food administration made from a particular make of tablet are valid for that tablet alone and not the substance in general. Bioavailability differences between five different film coated erythromycin stearate tablet formulations have been reported (57), which appears to substantiate this hypothesis.

The volume of water co-administered with erythromycin stearate film coated tablets has been shown to have a dramatic effect on the absorption of the drug (43). The decrease in absorption of

erythromycin in fasting individuals when the accompanying water volume was reduced from 250 to 20 mL is clearly depicted in Fig.1.5. In this particular study (43) the absorption of erythromycin was decreased by food (all subjects received 250 mL water) with the type of meal having no additional effects on this reduction (Fig.1.5).

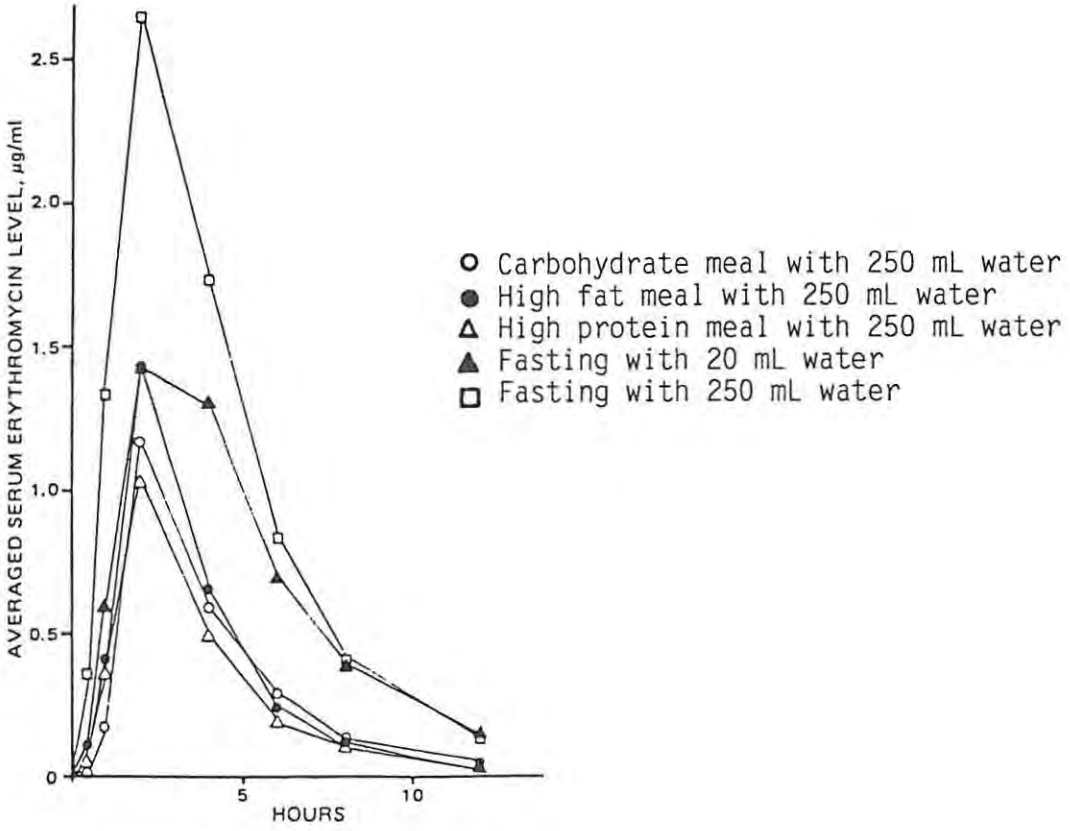


FIGURE 1.5 The effect of concurrent food and water administration on the absorption of erythromycin stearate

Reproduced from Welling *et al.* (43).

Erythromycin base and erythromycin stearate cannot be termed bioequivalent on the results of the majority of the single dose studies discussed. However, after multiple dosage regimes as found in clinical practice, the amount of drug absorbed when measured by the area under the serum concentration time curve (AUC) and maximum serum concentration reached (Cmax) are similar for both drugs (58,59,60). Typical values for these paramaters odtained after a single dose study (58) using different formulations of erythromycin base and stearate are summarized in Table 1.2.

TABLE 1.2 Comparision of maximum erythromycin serum concentrations

		Treatment (500 mg Dose Erythromycin)				
	DOSE	A BASE, ENTERIC COATED TABLET FASTING	B BASE, ENTERIC COATED TABLET NON FASTING	C BASE, ENTERIC COATED PELLETS NON FASTING	D STEARATE, FILM COATED TABLET NON FASTING	SIGNIFICANCE OF DIFFERENCES*
C_{max} (µg/mL)	1.	1.62 (0.70)	0.23 (0.48)	1.52 (0.79)	1.88 (1.19)	A,C,D>B
	4.	2.67 (0.72)	3.49 (1.48)	3.47 (1.23)	3.47 (1.91)	NS
	7.	4.00 (1.43)	3.90 (2.39)	3.97 (2.13)	3.72 (1.90)	NS
AUC_{0-7h} (µg/mL.hr)	1.	4.32 (1.84)	0.31 (0.80)	3.63 (1.96)	4.88 (2.80)	A,C,D>B
	4.	10.03 (3.70)	13.10 (5.32)	12.50 (5.18)	11.15 (6.69)	NS
	7.	15.53 (7.95)	14.75 (10.99)	15.10 (7.09)	11.24 (5.70)	C>D

* Difference significant at $p<0.01$ Standard deviation in parenthesis

Reproduced from Kahela and Järvensivu (58).

1.4.2.2 Erythromycin Estolate

Erythromycin estolate, unlike the base or stearate is acid stable (61). It is absorbed as the propionyl ester of erythromycin which does not have any significant antimicrobial activity until hydrolysed *in vivo* to erythromycin base (62). This hydrolysis occurs slowly and incompletely, with chromatographic studies showing that after an oral dose of erythromycin estolate only 20 per cent of the total drug absorbed is available as the active base (63). This tasteless derivative is formulated as a paediatric suspension or as a capsule.

The non-specific nature of the majority of microbiological analytical methods used for the analysis of this derivative, have been discussed in Section 1.4.1. In 1969 Stephens *et al.* (63) described the first analytical method capable of discriminating between concentrations of inactive propionyl ester and active erythromycin base. The numerous studies (39,49,50,61,64-69), that have reported the overwhelming increase in bioavailability of erythromycin estolate over the base and other derivatives, need to be examined with a new perspective. Some observations made during these studies are not however dependent on the specificity of the assay method, the most important being the influence of concomitant food intake.

Concurrent food intake with this erythromycin derivative has been reported to result in increased (45,53), decreased (49,50) and unaffected absorption (70). Erythromycin estolate has also been shown to have a lower biliary excretion than the base (66).

Bechtol *et al.* (71) have used a discriminatory assay technique (63) to study the bioavailability of the two most commonly formulated erythromycin paediatric suspensions, the estolate and ethylsuccinate. They found serum erythromycin base concentrations to be higher after administration of the estolate than after the ethylsuccinate. These erythromycin base concentrations were similar to those obtained after the administration of equivalent amounts of erythromycin base or stearate. These results have been verified by the work of Yakatan *et al.* (33), DiSanto *et al.* (72) and Welling *et al.* (53) who all

concluded that the estolate formulation was at best equivalent to the base and stearate formulations after multiple dosing, which is in disagreement with the majority of the earlier literature as discussed. Figure 1.6 depicts the considerable difference between the concentrations of the inactive ester and active base after repeated doses of an erythromycin estolate suspension when employing a discriminatory assay technique (53).

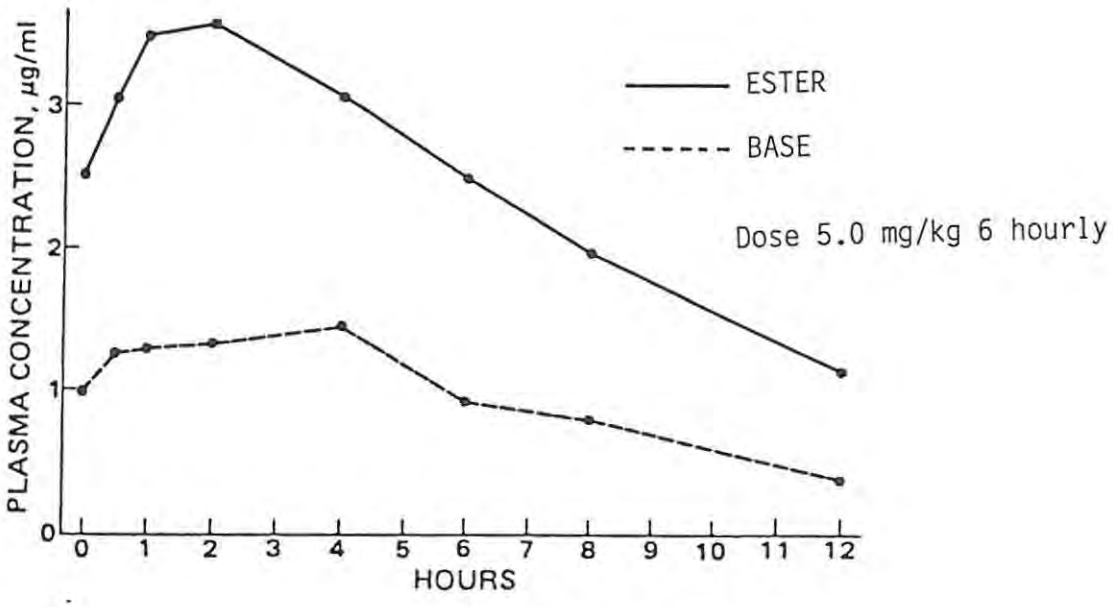


FIGURE 1.6 Plasma concentrations of erythromycin propionate (ESTER) and erythromycin base (BASE) following repeated doses of erythromycin estolate suspension

Reproduced from Welling *et al.* (53).

1.4.2.3 Erythromycin Ethylsuccinate

Erythromycin ethylsuccinate is a tasteless (6) acid stable derivative of erythromycin (55). It is absorbed as the intact ester, which has been reported to be biologically inactive and is subsequently hydrolysed to the active base *in vivo* (62). The reported increased bioavailability of this derivative in early studies was probably due to the lack of specificity of the microbiological assay method used. Since the development of a discriminating assay, it has been found that this ester results in slightly lower concentrations of active base than after the administration of erythromycin base, stearate or estolate (45,71, 73), but is still regarded as being clinically equivalent (11,14, 24,74).

1.4.2.4 Results of Specific Pharmacokinetic Studies on Erythromycin Absorption

The following points have been summarized from the numerous pharmacokinetic studies which have attempted to characterize the absorption of erythromycin in terms of established pharmacokinetic concepts.

A zero-order rate constant was found to most adequately fit the absorption data in three single dose (32,53,75) and one multiple-dose study (76). Of the remaining multiple dose studies, two obtained best results when using a first-order absorption rate constant (53,77), while another found a combination of zero and first-order constants, or a series of first-order rate constants to fit the data most satisfactorily (78).

The rate of absorption was found to change from dose to dose, being consistently lower after the third dose on any one day than after the second (76). It was also found to decrease after multiple dosing, with the rate being consistently lower at "steady state" than after the first dose (53).

The absolute bioavailability of erythromycin stearate has been reported as being 53% for capsules, 48% for film coated tablets and 55% for a suspension in a single dose experiment (51) and 65% for a film coated tablet in a multiple dose trial (78). These bioavailability percentages are particularly low in that the first pass extraction for erythromycin has been reported to be less than 10% (78).

Various workers have found it necessary to incorporate a lag-time function into curve-fitting programs after the administration of solid dosage forms of erythromycin (41,53,60,75-78) but not after the administration of suspensions (53). The formulations of enteric coated beads appear to have the longest lag-times (48).

1.4.3 Distribution

Erythromycin is well distributed throughout the body. Diffusion across the meninges is poor although therapeutic concentrations may be attained in the spinal fluid if meningeal inflammation is present (17). Placental transfer produces low serum concentrations in foetal blood although concentrations in the foetal liver are high (14,17).

Moderately high concentrations of erythromycin appear in middle ear exudate and tonsil tissue (65) and good penetration into aqueous humor and milk has been shown in animal studies (79). Erythromycin is one of the few antibiotics which penetrates well into prostatic fluid (79). A small percentage of the drug is found in the urine, but sufficient concentrations for the inhibition of most pathogens are obtained (80). Recent studies using positron tomography (81) showed that an effective concentration of erythromycin was reached in the pneumonic lung within ten minutes after i.v. administration.

Erythromycin is highly bound to serum proteins. The percentage of unbound drug after the administration of an erythromycin stearate formulation has been estimated to be 10 and 27 per cent by Wiegand and Chun (82) and Gordon *et al.* (83) respectively. The same workers found the percentage of unbound propionyl erythromycin to be 1.5 and 7.0 per cent respectively, after the administration of an oral erythromycin

estolate product. All of the commonly used analytical methods for the analysis of erythromycin measure the concentrations of both bound and unbound drug. Patients, having the same serum concentrations of erythromycin base after administration of erythromycin base and estolate formulations would therefore have approximately three to four times more drug available after the former therapy.

Erythromycin, not surprisingly, has been shown to not bind exclusively to serum albumin. At least half of the bound drug has been associated with binding to α - acid glycoprotein (84). A significant correlation was found between increased erythromycin serum concentrations and increased amounts of α- acid glycoprotein present in infected patients (84). This has important implications in the extrapolation of pharmacokinetic data obtained from normal trial subjects to the use of the drug in clinical situations.

1.4.4 Metabolism

Erythromycin is N-demethylated in the liver of rabbits and rats to form des-N-methylerythromycin (85) which is excreted predominantly in the bile (79,86), with small amounts being found in urine (87). Approximately 45% of the dose administered to rabbits is metabolized by this pathway per hour (85). In the rat, about half of the drug eliminated in the urine, bile and faeces was found to be unchanged (87). In a recent report (88) it was established that erythromycin induces its own metabolism after repeated doses, by both increasing the number and activity of the microsomal enzymes involved in its metabolism. In humans, it has been shown that concentrations of erythromycin in bile exceed those of serum considerably (89).

1.4.5 Excretion

The amount of unchanged erythromycin eliminated in urine is small, averaging from 2-10% after oral dosage (60,77,90) to 15% after i.v. administration (80). Renal clearance has been estimated as being 13% and 76% of creatinine clearance in humans and dogs respectively (91). Renal tubular reabsorption in humans is suspected (91,92).

Erythromycin and des-N-methylerythromycin are excreted through the bile, which contains high concentrations of these two substances after both oral and i.v. dosing. Biliary excretion has been proposed as being the major excretion pathway of erythromycin and its metabolites (15,17,89). Secretion of erythromycin across the wall of the jejunum into the lumen of the small intestine has been demonstrated in both the rat and rabbit after i.v. administration (86,93).

The pharmacokinetics of the distribution and elimination of erythromycin have not yet been convincingly characterized. In all of the i.v. studies reported (43,53,75,79,80,94) an open two-compartment model was found to describe the serum concentration - time curve most satisfactorily.

In a comprehensive study, involving both i.v. (94) and oral dosages (78) of erythromycin to the same subjects, the i.v. and oral serum concentration-time profiles were best described by two-compartment and one-compartment models respectively. Similar results were obtained by Welling *et al.* (53). The large inter-subject variations observed after oral administration (78) were not found after i.v. dosage (94), indicating that these effects were presumably due to varied absorption and not differences in distribution or elimination.

Of the other oral pharmacokinetic studies reported, a one-compartment model was found to best describe the data after single and multiple doses by some workers (43,77,95) while a two-compartment model was found to be most applicable by Kroboth *et al.* (75) after a single dose study.

The most significant difference between single and multiple oral dose erythromycin studies was that the AUC, and hence bioavailability of the first dose, is consistently less than that of the same dose of drug after a multiple dosing schedule. Using data from previous i.v. studies (94), Mather *et al.* (78) showed that the absolute bioavailability of an oral erythromycin stearate tablet was 30% after the first dose and 65% after the ninth dose. Similar results have been obtained by other workers (51,53,60,76). Colburn *et al.* (76) reported

an increase in the elimination half-life ($t_{½\beta}$) of erythromycin from 1.8 hr on day 1 to 2.6 hr on day 3 which may suggest saturation of an enzymatic degradation pathway after day 1, leading to decreased elimination of the drug. From the findings of Danan *et al.* (88), who reported that erythromycin induces its own metabolism, an increase in metabolism would be expected (Section 1.4.4). Welling *et al.* (53) found the same increase in bioavailability after multiple dosing as discussed, with a concomitant decrease in the rate of absorption, indicating that this phenomenon was unlikely to be due to increased absorption. In a controlled chronopharmacokinetic study, DiSanto *et al.* (96) found the noon dose (180° from local midnight) to result in the highest AUC value on any one day. This brief evidence of circadian effects on the pharmacokinetics of erythromycin does not, however explain the variations found over three days of multiple dosing.

Impaired renal function has been reported not to affect the clearance of erythromycin (80), which would be expected, as only a very small percentage of the drug is excreted via the renal route (60,77,90).

Evidence of non-linear erythromycin pharmacokinetics after i.v. administration has been reported (94). In this study a dose related increase in elimination half-life, volume of distribution and fraction of drug excreted in the urine were noticed. In a single dose oral study, Josefsson *et al.* (60) reported a non-linear increase in AUC values for increasing doses of erythromycin, as well as a trend towards an increased elimination half-life and fraction of dose excreted unchanged in the urine.

This evidence certainly suggests some capacity limited degradation of erythromycin, presumably in the liver (60), resulting in decreased clearance and an increase in the unchanged drug available for urinary excretion. Changes in protein binding could explain the change in volume of distribution reported by Austin *et al.* (94). The extensive biliary excretion of erythromycin (89) and the active secretion of the drug into the small intestine (93) may well result in the increased concentrations of erythromycin found after multiple doses, as well as the apparent non-linear excretion. Similar observations made for

TABLE 1.3 Typical pharmacokinetic parameters describing erythromycin disposition in relation to oral dose

	(DOSE mg BASE)			
	250	500	1000	500 (stearate)
SERUM				
n	20	22	21	22
lag (h)	1.4 (0.3)	1.4 (0.8)	1.3 (0.4)	1.1 (0.4)
ka (h-1)	3.2 (1.3)	4.3 (2.2)	3.2 (1.8)	2.3 (0.7)
ke (h-1)	0.8 (0.3)	0.5 (0.1)	0.4 (0.1)	0.6 (0.2)
AUC^1 (mg/L/hr)	4.5 (1.7)	11.2 (4.3)	27.2 (10.6)	7.5 (3.4)
AUC^2 (mg/L/hr)	5.4 (3.4)	13.2 (4.1)	28.6 (11.2)	8.0 (3.6)
T½ (hr)	2.0 (2.0)	2.5 (1.0)	3.0 (1.7)	2.7 (2.3)
URINE				
n	10	11	10	10
% EXCRETED	5.0 (3.00)	7.0 (2.00)	9.0 (5.00)	4.0 (3.00)
Cl_R(L/h)	3.0 (1.2)	3.5 (1.6)	2.9 (1.4)	2.7 (1.1)

Standard deviation in parenthesis

KEY

n = number of people in trial
lag = lag period between administration of dose and commencement of absorption
k_a = absorption rate constant
k_e = elimination rate constant
AUC^1 = area under the serum concentration-time curve calculated by the trapezoidal rule
AUC^2 = AUC calculated with the AUTOAN2/NONLIN program.
% EXCRETED = Percentage of biologically active erythromycin excreted in the urine during 9 hours
Cl_R = Renal clearance

Reproduced from Josefsson *et al.* (60)

another macrolide antibiotic, josamycin, have been explained by entero-hepatic recycling (97). Biphasic serum concentration curves were evident in a few subjects in both an i.v. (94) and an oral study (60) indicating the possibility of significant entero-hepatic recycling. This phenomenon may be more prevalent than reported, as in many other studies individual variations such as these could have been ascribed to random variations and lost in the averaging of the data (94). Other explanations of these "humps and bumps" on serum concentration-time profiles have been discussed by Benet (98).

Typical values for standard pharmacokinetic parameters obtained after oral administration of two erythromycin formulations are depicted in Table 1.3.

1.5 STUDY OBJECTIVES

In view of the problems associated with microbiological methods currently used and the lack of suitable techniques to quantitatively measure erythromycin concentrations in biological fluids, the main objective of this study was to develop a specific, sensitive, reproducible and rapid method for the quantitative determination of erythromycin and possibly some of its known metabolites and/or degradation products in serum and urine by HPLC with ultraviolet (U.V.) detection employing a conventional variable wavelength U.V. detector. It was felt that such a method would provide a powerful tool for the study of the biopharmaceutics and pharmacokinetics of erythromycin.

Whilst not a primary objective of this study and in view of the recent reports published using HPLC with electrochemical detection (9,99),the latter system was also investigated.

CHAPTER TWO

DEVELOPMENT OF AN HPLC METHOD FOR THE ANALYSIS OF ERYTHROMYCIN

2.1 INTRODUCTION

Like all chromatographic techniques, HPLC may be defined as a differential migration process where sample components are selectively retained by a stationary phase (100). What sets HPLC apart from other techniques is its ability to accomplish this separation with great speed, sensitivity, and precision, and its applicability to an enormous variety of compounds (101). Recent advances in instrumentation, column engineering and theory have extended its field of application to virtually all branches of science and technology (102).

The earliest stationary phases e.g. silica, were more polar than the mobile phases, which consisted of mixtures of non-polar organic solvents, resulting in an increased adsorption and hence retention for polar solutes. More recently, systems have been developed, where the relative polarities of the two phases are reversed and these are termed reverse phase HPLC. These systems employ non-polar stationary phases which are prepared by the covalent bonding of various hydrocarbon chains e.g octyl (C_8) or octadecyl (C_{18}) to a silica support, and using mobile phases consisting of polar organic solvents mixed with water. In this form of chromatography the mechanism for solute interaction is a combination of partition chromatography, where molecules actually penetrate the bonded hydrocarbon layer, and adsorption chromatography where the non-polar molecules are attracted to the non-polar stationary phase. The solute behaviour is opposite to that in normal phase chromatography and yields advantages in the separation of certain classes of compounds, so much so that it is currently used for the majority of pharmaceutical applications (103).

In the preparation of reverse phase packing materials, residual silanol groups on the silica packing support are known to interfere with the process of reverse phase chromatography. A second silanization reaction where the residual hydrogens of the

silanol groups are replaced with trimethylsilane functions is called "end-capping" and produces columns of superior batch-to-batch uniformity, improved column life and reduced peak tailing (103,104).

The object of chromatography is to separate components, within a reasonable, time into separate zones as they migrate through the column. This separation, or resolution, depends on three main factors; retention, column efficiency and column selectivity.

In order to attain maximum resolution between two compounds, the compounds must be retained on the column for an adequate period of time. This is mainly due to the fact that the difference in elution time between two peaks becomes smaller as retention decreases, regardless of the column's efficiency or selectivity (101).

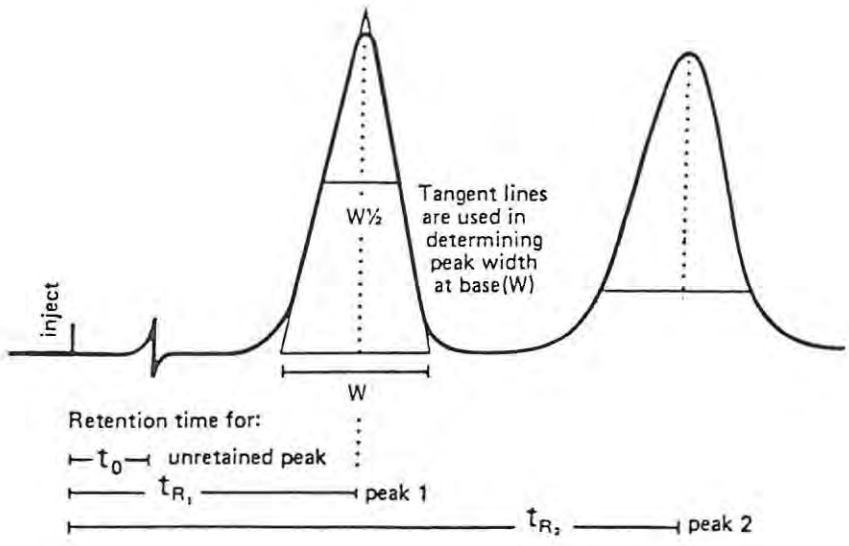


FIGURE 2.1 Calculation of column performance parameters

In order to help in choosing the correct retention for maximum resolution between compounds it is necessary to define the parameter, the column capacity factor, k'. This is defined as follows for each peak (Fig. 2.1):

$$k_1' = \frac{t_{R_1} - t_0}{t_0} \qquad \text{(Equation 1)}$$

Once a column has been chosen, it is necessary to adjust the eluent strength so that the retention times for the peaks of interest are in the range of k' values between 1 and 10.

Compounds which elute very late (k' >10), result in broad peaks which affect the detection limits. This is due to the slow movement of the zone of separation for these compounds through the column and hence from the column through the detector. The zone of separation is the same width for all peaks, irrespective of their retention. However, for early eluting peaks, it leaves the column travelling faster, resulting in sharper and hence more easily quantifiable peaks (101).

There are two fundamental ways of improving resolution as depicted in Fig. 2.2. The first is by decreasing peak width (zone width) while holding the zone centre separation constant and the second by increasing the zone centre separation while holding the peak width constant. The former method is termed the efficiency of the column and the latter the selectivity.

Column efficiency, which is a measure of zone spreading, is affected by three concurrent phenomena; longitudinal diffusion, multiple flow paths and resistance to mass transfer (101).

Longitudinal diffusion along the column axis is of little consequence in HPLC as the analysis is completed in a short time when compared to diffusion rates within the solvent. This is in direct contrast to gas chromatography, where diffusion rates within the carrier gases are much faster, resulting in significant decreases in column efficiency (101). Multiple flow paths lead to flow velocity

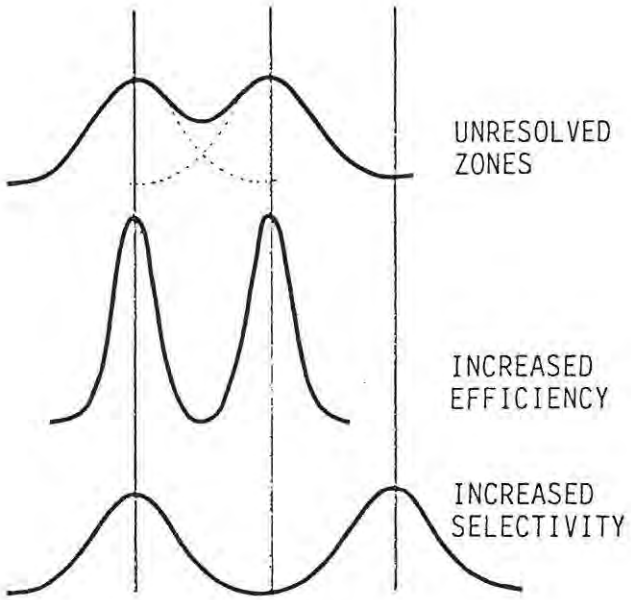


FIGURE 2.2 Improving peak resolution by increasing efficiency or selectivity

inequalities and eddy diffusion, causing some molecules to travel faster than others resulting in increased zone widths. This is minimized by homogeneous packing surfaces and uniformly packed particles (105).

Resistance to mass transfer or sorption-desorption kinetics is the major source of zone spreading, and may be reduced in numerous ways including the use of small particle-size packing substrates with monolayers of active surface, increased temperature and changes in flow rate (103).
When comparing column efficiencies, the height equivalent to a

theoretical plate (HETP) is a useful parameter. The smaller the HETP value the smaller the zone width and consequently the greater the efficiency of the column. This parameter is most easily obtained from the calculation of the number of theoretical plates (N) of a column as follows (Fig 2.1):

$$N = 16\left(\frac{t_{R_1}}{W}\right)^2 \qquad \text{(Equation 2)}$$

The HETP is then obtained by dividing N by the length of the column (L). Modern columns have HETP values of 0.01 to 0.1 mm giving a 25 cm column with an HETP of 0.2 mm about 12 500 plates. Increasing N and decreasing HETP leads to more efficient columns but it must be remembered that resolution, which is the ultimate goal of chromatography, is only proportional to the square root of the plate count (N).

The selectivity of a column, or the separation factor, is simply the ratio of the capacity factors (Equation 1) of the peaks.

$$\text{(Column Selectivity)}\ \alpha = \frac{k'_2}{k'_1} \qquad \text{(Equation 3)}$$

Column selectivity is a function of the properties of the stationary phase and eluting power of the mobile phase. The ability to change column selectivity by small changes in mobile phase composition is the most powerful tool in the optimization of resolution in HPLC (106).

The choice of the solvent for use in reverse phase chromatography is complex and depends on numerous factors including surface tension, dielectric constant, viscosity and eleutropic strength which are mainly linked to the polarity of the solvent (103). As a general rule, the column efficiency decreases with an increase in solvent viscosity and polarity. Water, the weakest eluent, is both the most viscous and polar, while methanol and acetonitrile which are strong eluents are less viscous and relatively non-polar. A mixture of these solvents normally provides a suitable mobile phase for the desired separation (101,103).

Column performance can be further increased by the addition of buffer salts such as citrate or phosphate, to the aqueous component of the mobile phase. Easily ionizable compounds interact with the polar residual silanol groups present on the packing substrate surface leading to increased retention and decreased peak shape. Buffer salts, besides keeping the pH of the mobile phase constant throughout the column are known to decrease this effect by masking the residual silanol groups via the reaction of the oxygen atom of the buffer molecule (107).

In addition, the judicious choice of mobile phase pH can have a significant effect on the retention of weak acids and bases by suppression of ionization. Weak acids are made more hydrophobic, resulting in an increased retention by lowering of pH while the reverse is true for weak bases (101,106). Columns with silica substrates however, fail rapidly if a mobile phase of pH <2.00 or >7.00 is used due to the increased solubility of silica in these pH ranges especially at increased temperatures (108). The life of the analytical column may be extended when working at these extreme pH ranges by the prior saturation of the mobile phase with silica from a silica saturating pre-column, or to a lesser degree, by the use of a conventional guard column (109).

The particle size of the packing material and the flow rate used have an important effect on the efficiency of a given column. There are four major points which must be noted (110,111).

1) The pressure resulting from a set flow rate of the same mobile phase increases when the particle size of the packing is decreased.
2) There is an optimum flow rate at which the HETP for a column is minimum. Working at this flow rate yields the best resolution but does not necessarily provide the shortest analysis time.

3) Column efficiency is inversely proportional to the size of the column packing material. Shorter columns packed with small particles can provide similar separations to conventional columns, or columns of the same length can provide increased efficiencies when packed with smaller particles.
4) The flow rate at which the column efficiency is maximum is inversely proportional to the particle diameter.

As a consequence of the last two relationships the use of small particles can lead to decreased analysis time by the use of both shorter columns and increased flow rates (110).

This has been the major cause of the trend towards using smaller particle size packing substrates <10 μm compared to the larger 50 μm pellicular packings originally used (110,112). These large particle size pellicular packings are still used in guard columns due to their ability to be inexpensively dry packed. These guard columns which are placed prior to analytical columns to protect them from contamination, have been known to affect peak shape considerably when using highly efficient analytical columns (112). This has been circumvented by the production of guard columns containing small disposable cartridges, slurry-packed with the same packing material as the analytical column.

A further application of this decrease in the particle size of the packing substrate has led to the development of high speed liquid chromatography (111). In this form of HPLC, extremely short columns (5.0 - 10.0 cm) are filled with packing substrates with an average diameter of only 3-5 μm. Additional advantages of these columns can best be seen from a plot of HETP vs flow rate. Figure 2.3 shows such plots (Van Deemter curves) for three columns packed with C_{18} stationary phase particles of 3, 5 and 10 μm diameter. From this plot it can be seen that for the column packed with 10 μm substrate, as the flow rate is increased above the optimum of 0.5 to 1.0 mL/min the HETP value increases, decreasing column efficiency. The curve is much flatter for the column packed with 3 μm packing material allowing the use of higher flow rates

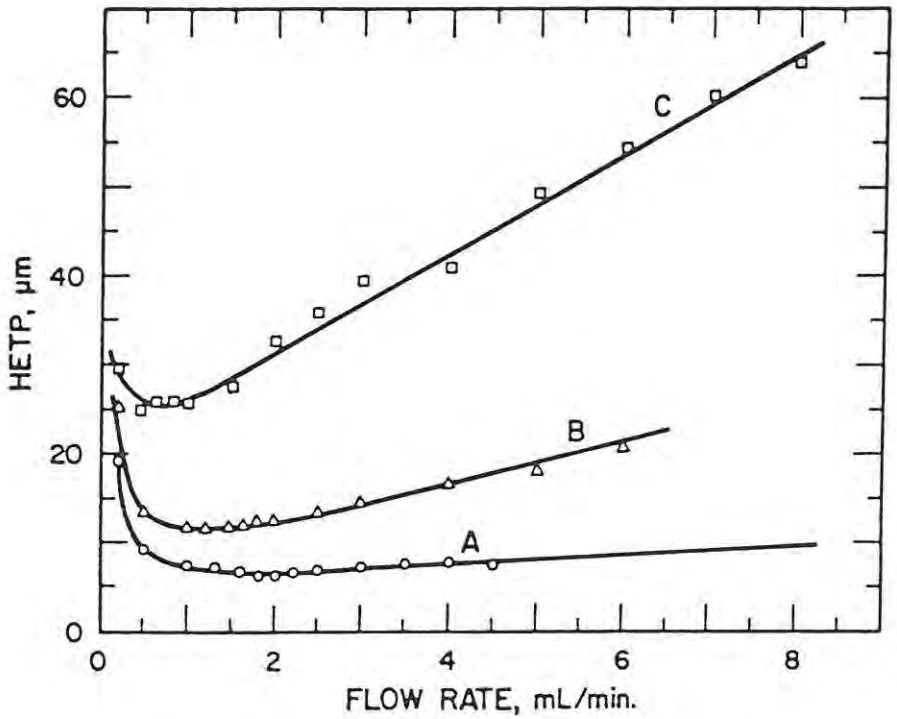


FIGURE 2.3 Relationship of the HETP to the mobile phase flow rate for three different columns containing C_{18} packing substrates with different particle diameters:
A) 3 µm B) 5 µm C) 10 µm

Reproduced from Di Cesare, Dong and Ettre (111).

of 2.5 - 3.0 mL/min without a decrease in column efficiency. The associated increase in inlet pressure generated limits the use of even higher flow rates (111).

The practical advantages of high speed liquid chromatography include a significantly improved chromatographic performance per unit time, leading to shorter analysis times and decreased solvent consumption. There are, however, distinct disadvantages which have affected its widespread acceptance. The main disadvantage

is the need for specialized equipment to decrease the effects of extra-column band width broadening which can negate the extra efficiency of the column, leading to the design of detectors containing micro-flow cells (1-3 μL) and extremely fast response times (111). Special injectors with by-pass loops are also necessary to decrease the formation of column top-end voids caused by the pressure fluctuations of conventional injectors which are a distinct problem at the high pressures encountered.

The packing of columns containing microparticulate packing substrates requires the use of high pressure slurry techniques to ensure maximal homogeneity and subsequent column efficiency (112). The main variables in slurry packing are the density and viscosity of the solvent and the composition, flow rate and pressure of the slurry (105). The use of carbon tetrachloride as the slurry solvent has been reported to result in columns of superior efficiency when compared to traditional slurry solvents such as methanol (113), presumably due to decreased particle size distribution during packing (114).

There are various detectors available for use in HPLC which have recently been reviewed by White (115,116). The ultraviolet (U.V.) detector was one of the earliest HPLC detectors and is still the most popular due to its ease of operation, versatility and sensitivity (115). Any solute with a U.V. absorption can be monitored using this mode of detection. Recent technological advances in computers and electronics have led to a new generation of U.V. detectors having the ability to scan peaks on-line over a pre-determined wavelength range, giving U.V. spectra that may be compared to documented U.V. spectra for positive peak identification. Data storage facilities have led to the post-run manipulation of chromatograms resulting in the most convenient choice of sensitivity and peak integration parameters. The advent of the diode-array detector has further extended the powers of the U.V. detector by having the facility to compare U.V. spectra at three different parts of a peak in order to validate peak purity and to detect any possible co-eluting contaminants.

There are no universally accepted criteria for the comparison of U.V. detectors but the concept of the signal-to-noise ratio (S/N) is well established (117). Any disturbance of a detector output that is unrelated to an eluted solute can be defined as detector noise. Short term noise which manifests itself as a "fuzzy" baseline is the most important and is mainly due to pump pulsations, dissolved gases in the mobile phase and unstable detector electronics (115,118).

The limit of detection or sensitivity is defined as the amount of solute that will produce a signal, equivalent to twice (or three times) the short-term noise level (115,117). By injecting equivalent amounts of a compound using identical chromatographic conditions, two detectors may be compared in this manner by calculating their S/N ratios.

It is not until the detector signal is fully amplified that the diverse nature of this noise is revealed (119). When working at high sensitivities and low wavelengths (below 220 nm) the use of carefully purified solvents, correctly chosen for their U.V. cut off values, non-absorbing buffer salts, freshly prepared HPLC grade water and meticulous cleaning of glassware are essential (103,119,120,121). Baseline noise caused by dissolved gasses in the mobile phase is due to the interaction between oxygen and the solvent and is best controlled by constant degassing. This may be achieved by the constant purging of the mobile phase with helium (103), or by the use of an in-line vacuum degassing unit. Methods such as periodic ultrasonication or helium purging are less popular, as they cause sudden changes in mobile phase gas content resulting in detector baseline instability (118).

The need for strict adherence to these precautions and others in high sensitivity bioanalyis is unfortunately not generally seen as being of great importance and is best described by Jones (119); "There is much ignorance of such problems amongst colleagues who carry out HPLC on similar samples at high analyte concentration in various chemical and pharmaceutical development laboratories where the knob of the detector amplifier is rarely turned past the halfway mark."

2.2 METHODS AVAILABLE FOR THE ANALYSIS OF ERYTHROMYCIN IN RAW MATERIAL AND IN PHARMACEUTICAL DOSAGE FORMS

2.2.1 Requirements of a Suitable Method

The need for an analytical method to quantitatively determine erythromycin in biological fluids after the administration of the various erythromycin derivatives available, has been discussed in Section 1.4.1. Analytical techniques for the assay of erythromycin and its derivatives in raw material and in pharmaceutical dosage forms must be able to indicate both the purity and stability of the compound, before and after manufacture and storage. This is particularly important in that different batches of erythromycin raw material have been reported to differ in the amount of the various erythromycin components present (2,122). In addition, solutions of erythromycin base are only stable for one week (4°C), even when buffered at pH 7.00 - 8.00 (7), making the detection of degradation products of the utmost importance.

2.2.2 Microbiological Analysis

Both the USP (123) and BP (124) official assays for the analysis of erythromycin use microbiological techniques. The capability of the sample to inhibit growth of a susceptible organism is measured and the results compared with those obtained with standard solutions of known concentrations of erythromycin. *Staphylococcus aureus* is employed for the turbidometric procedure (125) and *Sarcina lutea* or *Bacillus pumilus* for the plate assay (123,124,126,127). The inability of these methods to differentiate between erythromycin base and any other components or degradation products present, has led to the development of more selective methods for the analysis of erythromycin in raw material and/or dosage forms. While being sufficiently sensitive, these microbiological assay methods have been reported as being relatively imprecise (128) and are extremely tedious to perform.

2.2.3 Ultraviolet Spectrophotometric Analysis

The ultraviolet spectrophotometric assay for erythromycin first described by Kuzel *et al.* in 1954 (129), has since been automated by the same workers (130) and is applicable to dosage form and fermentation broth analyses. The method is based on mild alkaline hydrolysis of erythromycin with 0.25 N sodium hydroxide at 65°C which produces a chromophore having an absorbance peak at 236 nm. In order to determine materials other than erythromycin which absorb at this wavelength, a sample blank is prepared by mild acid treatment of the sample with 0.50 N sulphuric acid. This converts the erythromycin present to anhydroerythromycin which does not absorb U.V. radiation at the wavelength used. While being able to perform up to 300 determinations per day, this method is non-specific and hence not stability-indicating.

2.2.4 Colorimetric Analysis

Kuzel and Coffey (131) developed a sensitive and precise method based on the ion-pair-dye complex of bromocresol purple (5,5,-dibromo-o-cresol-sulphonphthalein) and the desosamine moeity of erythromycin in pH 1.2 buffer. This method, like that of Sanghavi and Chandramohan (132) who used p-dimethyl amino benzaldehyde as the coupling agent, lacks specificity for erythromycin since all tertiary amines are measured. These methods are capable of detecting erythromycin in dosage forms and fermentation broths in a concentration range of 10.00-100.00 µg/mL.

2.2.5 Chromatographic Analysis

2.2.5.1 Column Chromatography

A technique using a silica gel glass column, for the separation of erythromycin on a preparative scale has been reported by Banaszek *et al.* (133).

2.2.5.2 Thin Layer Chromatography

A number of thin layer chromatographic systems (tlc) have been described for the separation of erythromycin, from its derivatives and components (7,133-138). The method of Radecka *et al.* (135) using densitometric detection is the only method available for the quantitative analysis of erythromycin by tlc. Thin layer and paper chromatographic methods combined with bio-autography have been described for the analysis of erythromycin and its derivatives in biological fluids (Section 3.1). These methods however, can only detect microbiologically active erythromycin components and are thus unsuitable for detailed analysis of erythromycin in raw materials and/or dosage forms since they are not stability-indicating.

2.2.5.3 Gas Chromatography

A gas chromatographic method for the determination of erythromycin and its derivatives following silylation with a combination of three trimethylsilyl derivatives in pyridine has been reported by Tsuji and Robertson (2). This method is able to quantify the proportions of erythromycin A, B, C, anhydroerythromycin, erythralosamine and the propionate esters of erythromycin in dosage forms following chromatography on an OV-225 or PPE-20 column at 275°C with flame ionization detection. Ten micrograms of silanized erythromycin were injected onto the column although this could be decreased to 100 ng with the use of a lower attenuation setting. Results of samples analysed by this technique were found to compare well with those determined by microbiological assay methods. This procedure, however, has not found wide-spread use possibly due to the lengthy silylation time (24 hrs at 75°C) and the instability of the g.c. column (7).

2.2.5.4 High Performance Liquid Chromatography (HPLC)

The first reported HPLC technique for the determination of erythromycin was by Omura *et al.* in 1973 (139). These workers used a reverse phase Jascopak column for the separation of macrolide antibiotics in general. They reported retention times of 3.0 and 2.4 min for erythromycin A and

B respectively using U.V. detection at 232.5 nm.

White *et al.* (140) also reported the separation of erythromycin on a reverse phase column (Lichrosorb C_{18} 10 μm) when investigating the general applicability of HPLC with small particle size columns to antibiotic analyses. A refractive index detector was used which was able to detect an on-column load of 10.00 μg.

A normal phase method using a Corasil 2 (silica gel) column with chloroform as mobile phase has been used to distinguish between erythromycin and anhydroerythromycin (141).

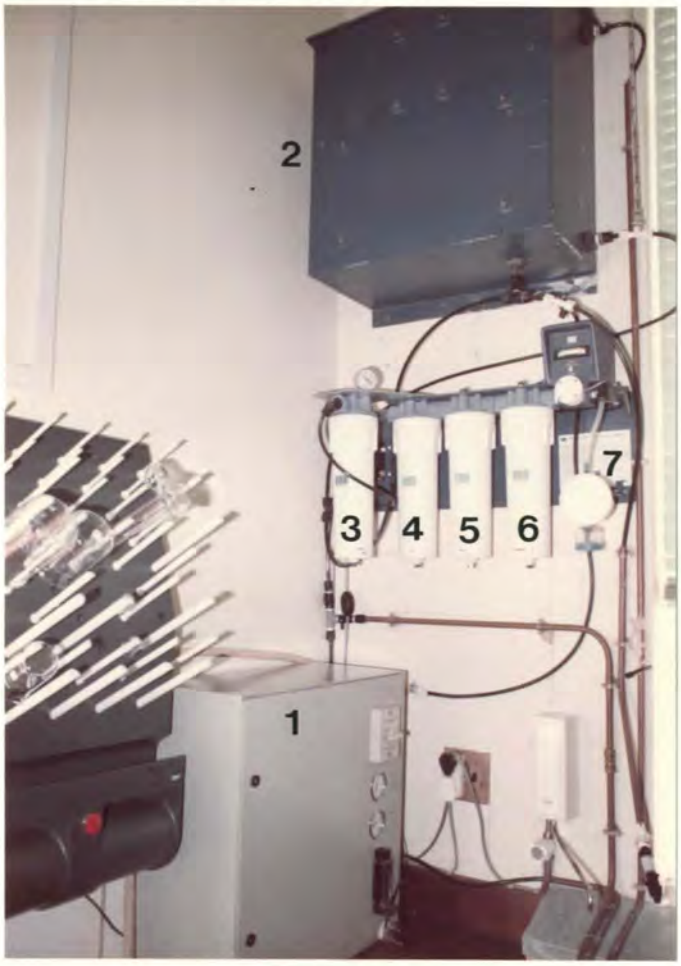
These methods (140,141), while being able to detect erythromycin and possibly one major degradation product, are not suitable for the detailed quantitative analysis of erythromycin in raw material or in pharmaceutical dosage forms. Suitable methods for this purpose have been developed by Tsuji *et al.* (142-145). The first method reported (142) was able to separate erythromycin from its components using a μBondapak C_{18} reverse phase column with U.V. detection at 215 nm. This method was later adapted for use in monitoring erythromycin in fermentation broths (144). The analytical column was changed to a Lichrosorb C_{18} 5 μm column preceded by a C_{18} guard column for the analysis of erythromycin ethylsuccinate and its components in dosage forms (143). Both the analytical and guard columns were maintained at 70°C resulting in good separation of all components with excellent peak shape.

A further method utilizing an increased column temperature (70°C) for the separation of erythromycin base and its components was reported as an improvement over the original method (145). An internal standard, calusterone or megestrol acetate was utilized in the analysis of erythromycin base tablets which compared favourably with results obtained by the microbiological assay method (123).

2.3 EXPERIMENTAL

2.3.1 Reagents

Reagents were of at least analytical grade and the acetonitrile (Burdick and Jackson) was distilled-in-glass grade. The HPLC water used in the mobile phase was purified through a Milli-Q System (Waters Associates) (Fig. 2.4).



1) Deomed reverse osmosis system - for initial purification of water
2) Storage tank
3) Super-C cartidge - for removal of dissolved organic material
4 & 5) Ion-Ex cartridges - mixed bed deionizing resins for removal of dissolved inorganics
6) Organex-Q cartidge - for removal of last traces of organics such as some amino compounds generated by the anionic exchange resins
7) Milli-stack unit - for removal of all microorganisms and particles larger than 0.22 µm

FIGURE 2.4 Milli-Q-Water Purification System

Drug samples used:

Erythromycin Base USP [1]	Lot No MD H-1
Erythromycin Estolate USP [1]	Lot No MD F
Erythromycin Ethylsuccinate [2]	Lot 51-039-CD
Erythromycin Stearate [2]	Lot 07-616-CD
Erythromycin B [2]	Lot 84-991-AX
Anhydroerythromycin [2]	Lot 86-188-AX
Erythromycin Enol Ether [2]	Lot 3511-55A
Erythralosamine [2]	Lot 26-978-AX
Des-N-methylerythromycin [3]	Lot 164-171-185-2
Oleandomycin phosphate [4]	Lot 206/767003
Triacetyl oleandomycin [4]	Lot 07305-76EA
Josamycin [5]	Lot W-285

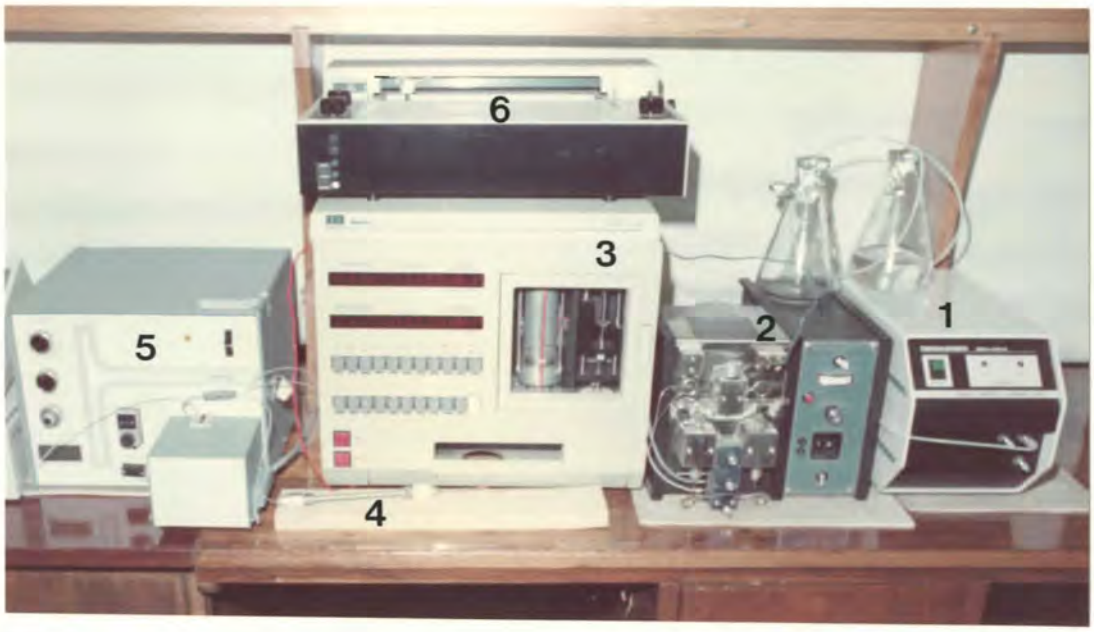
1) USP Reference Standards, Rockville, USA.
2) Abbott Laboratories, North Chicago, USA.
3) Lilly Laboratories, Indianapolis, USA.
4) Pfizer Laboratories, Pietermaritzburg, South Africa.
5) Yamanouchi International Ltd., Tokyo, Japan.

Solutions of these samples were prepared in A-grade glassware by the addition of HPLC grade water, U.V. grade acetonitrile or a mixture of both.

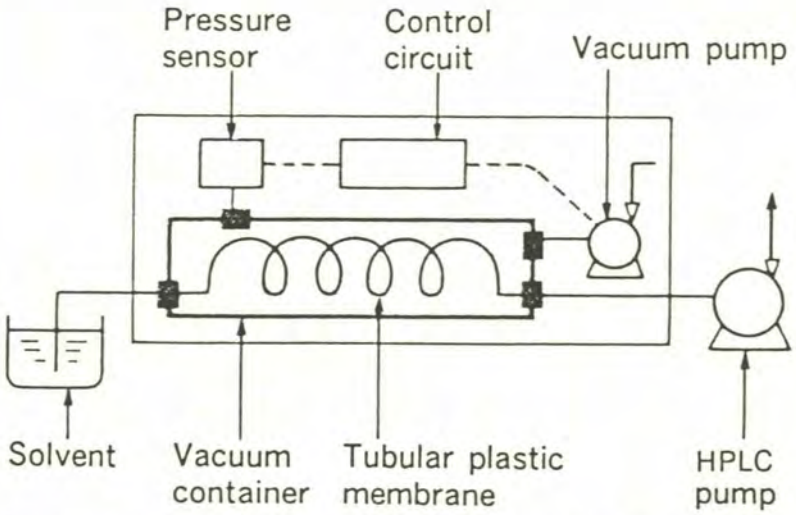
2.3.2 High Performance Liquid Chromatographic System

The high performance liquid chromatographic system used throughout this analysis is depicted in Fig. 2.5.

FIGURE 2.5 High Performance Liquid Chromatographic System.



1) Model ERC-350 Solvent degasser (ERMA Optical Works) Fig. 2.6
2) Model M 6000A Solvent delivery system (Waters Associates)
3) Model 710B WISP Automated sample injector (Waters Associates)
4) Column - see section 2.3.5
5) Model 769 Variable wavelength U.V. detector (Kratos Analytical Instruments)
6) Model 561 Strip chart recorder (Perkin-Elmer)



The solvent to be degassed passes through the tubular plastic membrane, with its surroundings maintained in a vacuum. Only gas is allowed to cross the membrane while the solvent is passing through it

FIGURE 2.6 Solvent Degasser: Principle of Operation

2.3.3 Additional Equipment

1) Model 601 digital ionalyser (Orion Research)
2) Speed-Vac concentrator (Savant Instruments)
3) Whirlimixer (Fisons)
4) Type 2474 five figure precision balance (Sartorious)
5) Ultrasonic cleaner (Branson)
6) Microfiltration centrifuge (Bioanalytical systems)
7) Type BD filter membrane (Millipore Corp)
8) Model LC-22 temperature controller (Bioanalytical systems)
9) Model 730 Data module (Waters Associates)
10) Magnetic stirrer (Gallenkamp)
11) Column Packer (HPLC Technology)

2.3.4 Ultraviolet Detectors

1) Kratos Instruments Model 769 Variable Wavelength Detector

This detector which was fitted with an 8 µl flow cell was operated predominantly at 200 nm at the maximum sensitivity setting of 0.01 absorbance units full scale (AUFS). The variable time constant was varied between 1.0 and 2.0 sec. Set at these conditions, this detector was used as the standard for the comparison of the other detectors tested. For this purpose, it was placed in-line after the detector being evaluated, which was set at 200 nm with a time constant equal to that of the Kratos detector and at the maximum sensitivity setting which allowed an acceptable baseline. A sample of erythromycin, or internal standard, was injected into the chromatographic system in order to compare the peak height to baseline noise (S/N ratios) of the two detectors. Factors such as signal stability (drift) and peak shape were also considered.

2) Waters Associates Model 490 Variable Wavelength Detector

The model used was a prototype, fitted with a standard taper-cell design flow cell. Various additional features such as multiple

wavelength programming and the ability to scan peaks over a specified wavelength range are available on the commercial product. It was operated at wavelengths between 200 and 210 nm with a time constant of between 1.0 and 5.0 sec and at sensitivity settings of between 0.001 and 0.05 AUFS depending on the wavelength used.

3) Beckman Instruments Model 165 Variable Wavelength Detector

This detector had similar extra features to that of the Waters 490 detector with the ability to substantiate peak identification by the scanning of a peak over a set wavelength range. It was fitted with a flow cell having a 13 µl illuminated volume and was operated at a wavelength of 200 nm and at a sensitivity of either 0.01 or 0.02 AUFS. The response time was controlled by the setting of the baseline slope parameter to a value of 300. This introduced an active filter which set the variable time constant to 2 sec for maximum baseline stability, reverting to 0.01 sec on detection of a peak.

4) Philips PU 4021 Diode-Array Detector

This was the first of two diode-array detectors used, which in general have many additional features for the validation of peak purity and identity. This detector had an 8 µl flow cell and was operated at 200 nm and 0.01 AUFS. A few of the additional features including the scanning of peaks were also tested.

5) Hewlett Packard 1040A Diode-Array Detector

This detector which had a standard 3.5 µL flow cell was controlled and assisted by the following components; An HP 85 personal computer, an HP 9121 dual disc drive and an HP 7470A X-Y plotter. It had the usual facilities of a diode-array detector with the additional advantage of a data storage facility which allowed the post-run manipulation of chromatograms as well as the ability to monitor and permanently store the results of chromatograms monitored at eight different wavelengths. The detector was usually set to monitor four different wavelengths; 195, 200, 205 and 210 nm with the sensitivity adjusted during post-run

manipulations to give the best S/N ratio. It was also used for the scanning of peaks and the generation of 3- dimensional plots of absorption vs time vs wavelength.

2.3.5 Mobile Phase Preparation

The 0.05 M phosphate buffer used in these studies was prepared by adding 3.2 mL phosphoric acid to 1.0 L of freshly prepared HPLC grade water. Sodium hydroxide pellets were then added to the acid/water mixture with constant stirring on a magnetic stirrer until the desired pH of the buffer had been attained. All other buffers used were prepared by dilution of this buffer solution.

Mobile phase composition is reported as the relevant volumes of organic and aqueous components that were added to make 100.0 mL of mobile phase without correcting for molar volume changes, i.e. 500.0 mL of a mobile phase, 0.05 M phosphate buffer/acetonitrile (70/30), pH 6.50 was prepared by mixing 350.0 mL of 0.05 M phosphate buffer with 150.0 mL acetonitrile. Where the final mobile phase was adjusted to a pH value after addition of the two phases, this has been reported as the corrected pH. The solvent mixture was degassed and filtered through a 0.6 μm filter prior to use.

2.3.6 Columns and Mobile Phases

(i) C_{18} Reverse Phase Column (10 μm)

This, the first column used, was a 25 cm x 3.9 mm i.d. steel column packed with Techsil 10 μm octadecylsilane (C_{18}) material (batch number T-06-10, HPLC Technology) by the method described in section 2.3.8. Preliminary studies were performed on this column. These included the affect of both buffer strength and mobile phase pH on the retention of erythromycin. The choice of the optimum mobile phase and operating conditions for this column are summarized as system A in Table 2.1.

(ii) C_{18} Reverse Phase Column (5 μm)

A 25 cm x 3.9 mm i.d. column was packed with Techsil 5 μm C_{18} material. The optimum operating conditions for this column are summarized as system B in Table 2.1. The effect of increased temperature on the resolution between erythromycin and the internal standard (oleandomycin phosphate) was studied over a range from 25°C to 48°C.

(iii) C_{18} Reverse Phase High Speed Column (3 μm)

This commercially available high speed column was only 7.5 cm long with an internal diameter of 4.6 mm. It was packed with 3 μm C_{18} material (Ultrasphere - ODS, Beckman Instruments). Numerous mobile phases, differing in organic/aqueous phase ratios, buffer strength and mobile phase pH operated at flow rates of between 1.1 and 2.5 mL/min were tested in order to obtain a suitable separation between erythromycin and the internal standard. The best combination of these variables is reported as system C in Table 2.1. When the formation of a top-end void became evident it was repaired by application of a paste of Techsil 5 μm C_{18} in methanol.

(iv) Novapak C_{18} End -Capped Reverse Phase Column (5 μm)

This commercially available 15 cm x 3.9 mm i.d. column was packed with end-capped 5 μm C_{18} material (Novapak, Waters Associates). Two slightly different sets of operating conditions for this column are reported as systems D and E in Table 2.1. The use of a guard column and of a silica saturating pre-column were investigated in addition to the effects of changes in flow rate and column temperature. During these studies the use of an in-line vacuum degassing unit as well as in-line ultrasonic degassing of the mobile phase were compared.

TABLE 2.1 High performance liquid chromatographic systems used

HPLC SYSTEM	COLUMN	MOBILE PHASE (PHOSPHATE BUFFER)	FLOW RATE	TEMPERATURE	PRESSURE
A	C_{18} REVERSE PHASE 10 µm (TECHSIL)	0.05 M BUFFER/ ACETONITRILE (50/50), pH 7.00	1.0 mL/min	Ambient	1100 psi
B	C_{18} REVERSE PHASE 5µm (TECHSIL)	0.05 M BUFFER/ ACETONITRILE (50/50), pH 7.00	1.0 mL/min	45°C	2000 psi
C	C_{18} REVERSE PHASE HIGH SPEED 3 µm (ULTRASPHERE)	0.005 M BUFFER/ ACETONITRILE (50/50), pH 7.00	2.0 mL/min	Ambient	3000 psi
D	C_{18} REVERSE PHASE END-CAPPED 5 µm (NOVAPAK)	0.05 M BUFFER/ ACETONITRILE (70/34), pH 7.00	1.0 mL/min	Ambient	1500 psi
E	C_{18} REVERSE PHASE END-CAPPED 5 µm (NOVAPAK)	0.05 M BUFFER/ ACETONITRILE (70/30), pH 7.00	1.0 mL/min	35°C	1500 psi

2.3.7 Pre-Columns and Guard Columns

(i) Silica Saturating Pre-Column

This column when used, was placed in-line between the solvent delivery system and injector. It consisted of a 25 cm x 3.9 mm i.d. steel casing dry packed with 37-53 µm silica material (Solvecon pre-column gel, Whatman).

(ii) Rheodyne Guard Column Unit

This guard column, which was adapted from a loop column system used with the model 7125 Rheodyne injector, was placed in-line immediately prior to the analytical column. It consisted of a guard column holder fitted with a 3 cm cartridge, slurry - packed with porous 10 µm C_{18} material.

(iii) Guard-Pak Guard Column Unit (Waters Associates)

This unit, which consisted of a holder for disposable guard column discs containing densely packed beads of 10 µm particles was placed in-line immediately prior to the analytical column. Commercially available discs containing C_{18} and cyano (CN) material were used, as well as a disc packed in the laboratory with 40 µm glass beads.

2.3.8 Column Packing Procedure

Columns were packed as follows using an HPLC column packer. Packing material from a used, 25 cm x 3.9 mm i.d. steel casing (µBondapak, Waters Associates) was removed and the column thoroughly rinsed with acetone. The column end-frits and sieves were soaked in concentrated nitric acid for 5 min after which they were thoroughly rinsed with water and sonicated for 20 min in methanol. Three and a half grams of packing material (Techsil C_{18}, 5 or 10 µm) was slurried in 30.0 mL U.V.- grade carbon tetrachloride and sonicated until dissolved. After loading the carbon tetrachloride mixture into the column packing reservoir, 120.0 mL of degassed and filtered U.V. grade methanol was pumped through the column at a pressure of 5 000 psi. After this period the column was inverted without interuption of flow and a further 120.0 mL methanol was pumped through the column. The methanol reservoir was constantly degassed by sonication. After removal from the column packer, the column was washed with 50.0 mL acetonitrile/water (75/25) and tested using this mobile phase and a standard laboratory test mixture prepared as follows:

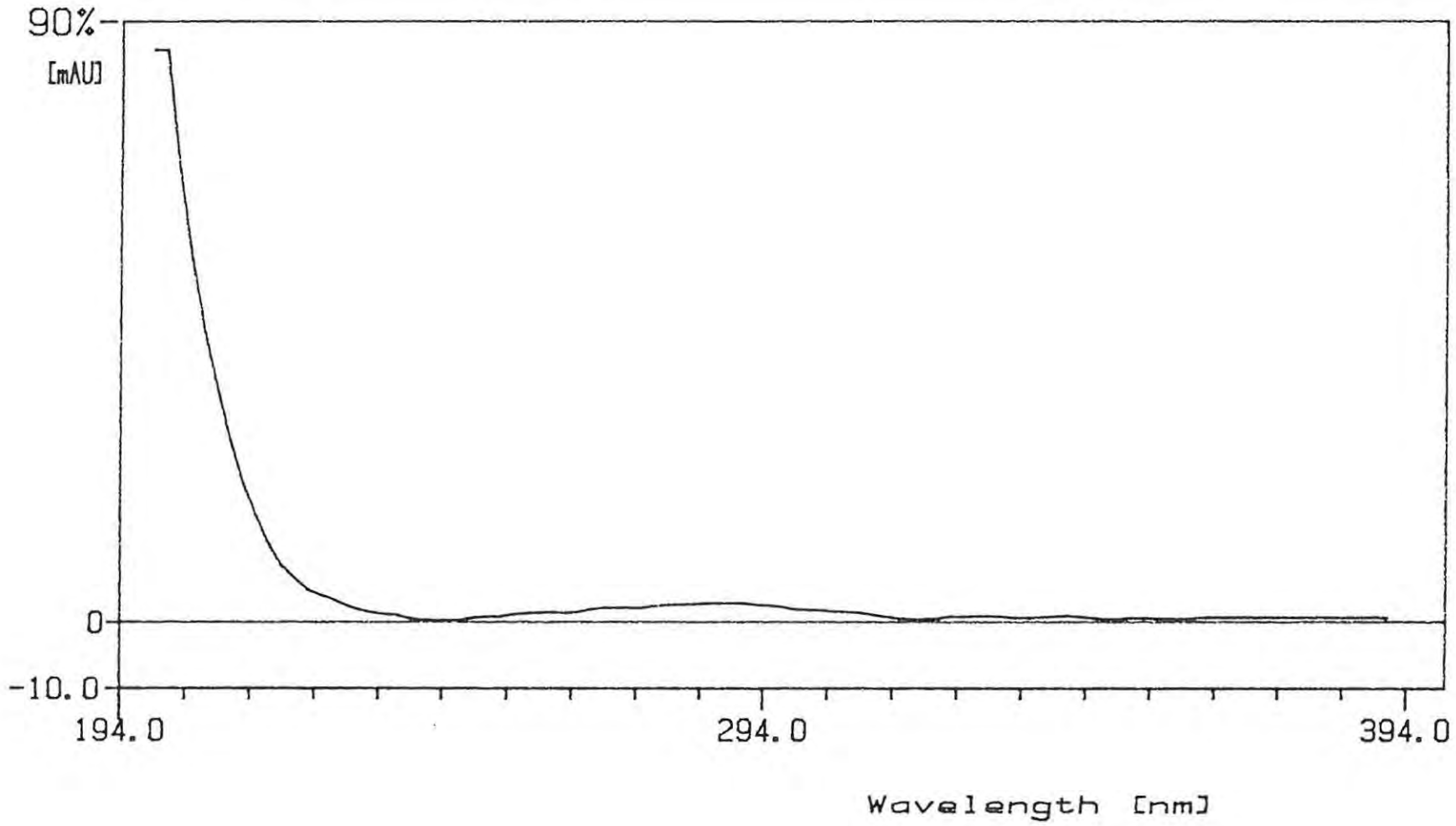


FIGURE 2.7 Ultraviolet spectrum of erythromycin in mobile phase 0.05 M phosphate buffer/acetonitrile (70/30), pH 7.00

Ten milligrams of both benzamide and benzephenone, plus 0.5 mL benzene were made up to 20.0 mL with acetonitrile. One millilitre of this solution was diluted to 50.0 mL with acetonitrile/water (75/25).

Two microlitres of this solution were injected onto the column at a flow rate of 1.0 mL/min, with U.V. detection at 254 nm, a sensitivity setting of 0.04 AUFS and a chart speed of 1 cm/min.

2.4 RESULTS AND DISCUSSION

2.4.1 Detector Wavelength

Erythromycin absorbs in the low ultraviolet wavelength range but its inherent molar absorptivity is poor (146). The need to detect low concentrations of erythromycin in biological fluids (0.25-5.00 μg/mL) necessitated the choice of a wavelength where the absorbance was relatively high. From Fig. 2.7 it can be seen that erythromycin only significantly absorbs U.V. radiation below 215 nm with a wavelength of 195 nm giving the highest absorptivity. A wavelength of 215 nm has been shown by Tsuji and Goetz (142) to provide sufficient sensitivity when monitoring high concentrations of erythromycin such as those found when analysing dosage forms of the drug. The need for maximum sensitivity led to the choice of 200 nm as the wavelength of detection, as wavelengths below 200 nm yielded unacceptable baseline noise and drift, while those above did not provide sufficient sensitivity. Figure 2.8 depicts the decrease in detector sensitivity resulting from an increase in detector wavelength from 195 to 210 nm.

2.4.2 Detector Choice (See Table 2.2)

The Kratos detector was found to have the most favourable S/N ratio at 200 nm when set at its maximum sensitivity of 0.01 AUFS. As a result, it was used as the standard for the comparison of the various detectors as previously described and was used for the majority of analyses throughout this study. The Waters prototype M490 detector

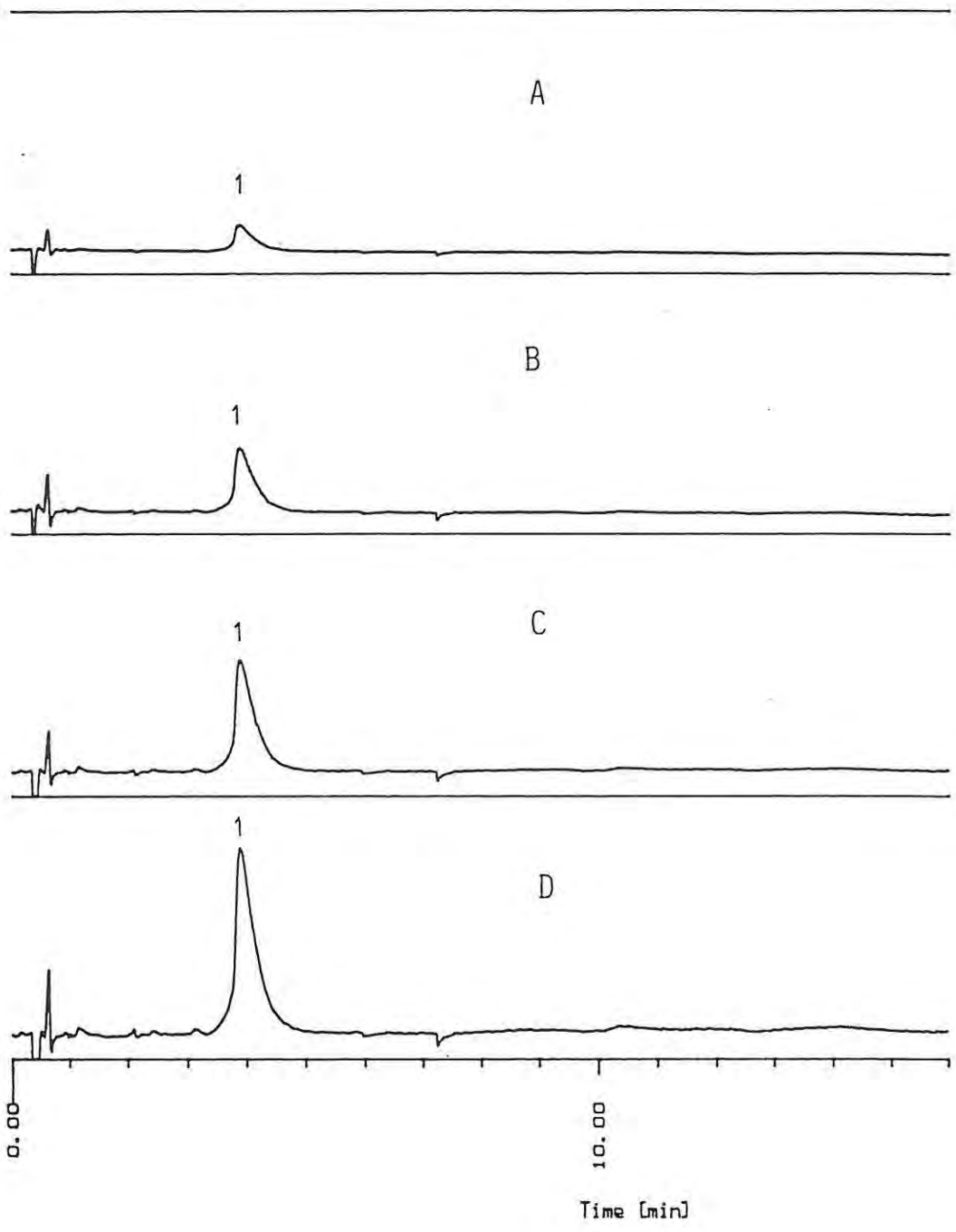


FIGURE 2.8 Chromatogram of erythromycin (1) monitored simultaneously at four different wavelengths
A) 210 nm
B) 205 nm
C) 200 nm
D) 195 nm

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/34), pH 7.00

TABLE 2.2 Comparison of U.V. detectors

DETECTORS	WAVELENGTH (nm)	SENSITIVITY SETTING (AUFS)	* S/N RATIO	+ COMPARATIVE S/N RATIO
KRATOS SF769	200	0.01	44.0	4.0
WATERS 490	200	0.03	11.0	
KRATOS SF769	200	0.01	18.4	1.0
WATERS 490	210	0.002	19.4	
KRATOS SF769	200	0.01	38.0	3.0
BECKMAN 165	200	0.02	14.3	
KRATOS SF769	200	0.01	57.6	3.0
PHILIPS PU4021	200	0.01	18.2	
KRATOS SF769	200	0.01	67.2	3.0
HEWLETT-PACKARD 1040A	200	0.01	21.3	

* The Signal-to-noise ratios (S/N) were calculated as in section 2.3.4.

\+ The comparative S/N ratio is the ratio of the S/N value of the Kratos detector to that of the detector under evaluation.

introduced the concept of the compromise between the wavelength of maximum absorption and the wavelength at which maximum amplification of signal is possible. This detector, when set at the same conditions as the Kratos detector (i.e 200 nm, 0,01 AUFS, filter 2.0 sec), resulted in a baseline which was unusable due to excessive noise. Adjustment of the sensitivity setting to 0,03 AUFS resulted in a baseline similar to that of the Kratos detector with an expected decrease in the S/N ratio. Increasing the detector wavelength above

200 nm resulted in a dramatic decrease in baseline noise. This enabled the utilization of a detector sensitivity of 0,002 AUFS at a wavelength of 210 nm resulting in a comparable S/N ratio to that of the Kratos detector. The approximate four fold decrease in erythromycin absorption from 200 to 210 nm (Fig. 2.8) was thus compensated by a five fold increase in detector sensitivity.

The Beckman 165 detector was found to have a far inferior S/N ratio than that of the Kratos detector when operated at 200 nm. This was mainly due to the increased baseline noise which only allowed a maximum sensitivity setting of 0.02 AUFS to be used.

The Philips PU4021 detector was also found to have an inferior S/N ratio to that of the Kratos detector when operated at 200 nm and at a maximum sensitivity of 0.01 AUFS. The lack of a data storage capability inhibited the use of the peak scanning facilities to their full potential as these manipulations had to be performed during a chromatographic run prior to the injection of the next sample which increased the overall analysis time and solvent consumption considerably.

The data storage facility of the Hewlett - Packard 1040A detector was found to be extremely useful. Extensive-post run manipulation of the sensitivity parameters at various wavelengths however, still resulted in the optimum S/N ratio being inferior to that of the Kratos detector. This detector was mainly used for special applications, such as the monitoring of a chromatogram at four different wavelengths (Fig. 2.8) and the generation of three dimensional plots of absorbance vs time vs wavelength. These plots, provide a powerful means of determining both peak purity and the wavelength of choice for a mixture of compounds. The ability to detect des-N-methylerythromycin and the inability to detect anhydroerythromycin in the U.V. wavelength range from 195 to 300 nm is depicted by such a plot (Fig. 2.9).

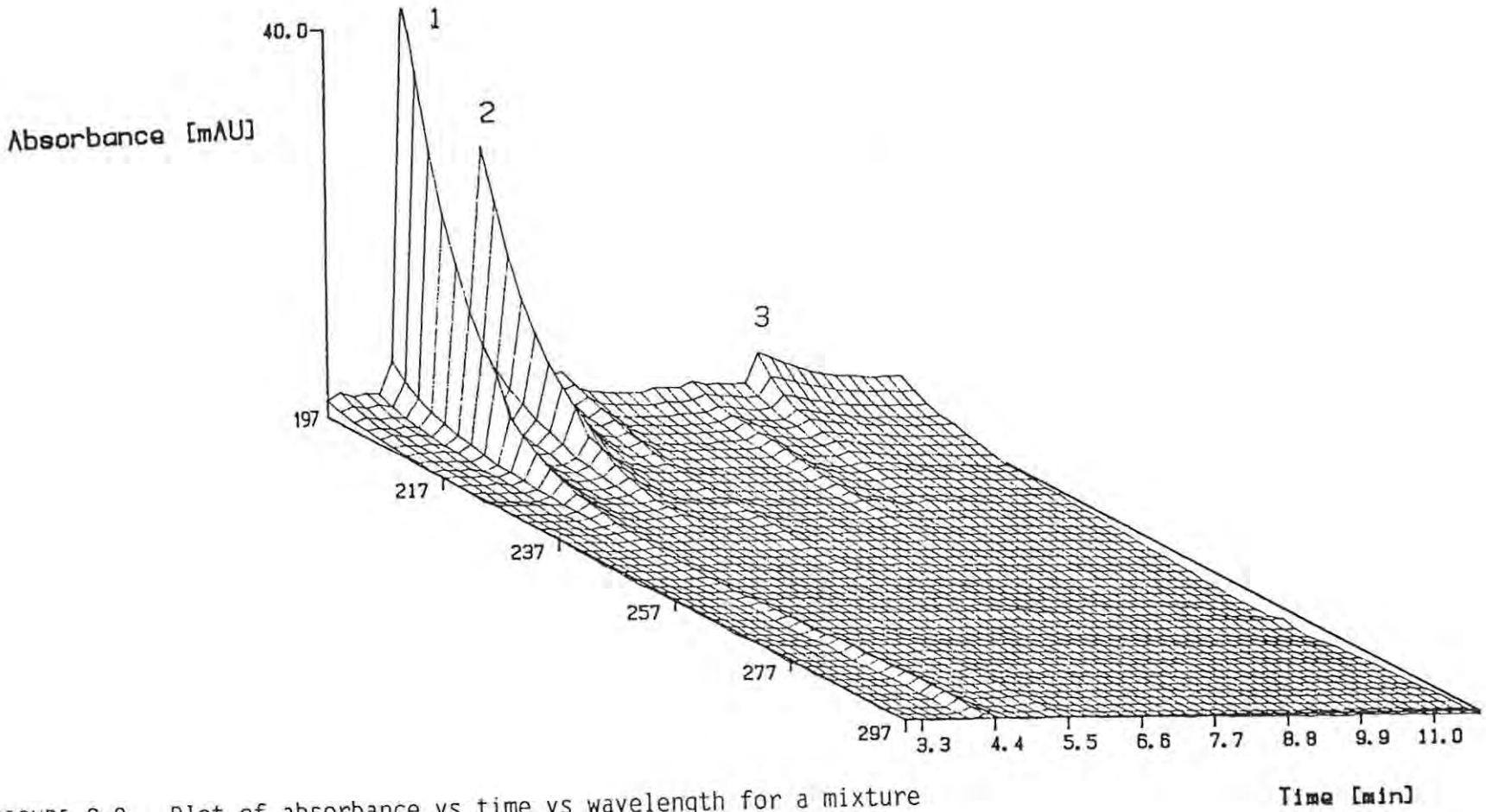


FIGURE 2.9 Plot of absorbance vs time vs wavelength for a mixture of des-N-methylerythromycin (1), erythromycin (2) and anhydroerythromycin (3), using the Hewlett-Packard diode-array U.V. detector

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/34), pH 7.00

2.4.3 Choice of Internal Standard

Oleandomycin phosphate was chosen as the internal standard from two other macrolide antibiotics, triacetyloleandomycin and josamycin. It was readily detectable at 200 nm and eluted prior to erythromycin on all chromatographic systems used. This necessitated the development of extremely efficient sample clean up procedures in order to separate both erythromycin and oleandomycin from interfering serum and urine components. Furthermore, oleandomycin co-eluted (Table 2.3) with des-N-methylerythromycin, a known metabolite of erythromycin (85). Josamycin could not be used due to its long retention and relatively weak U.V. absorption at 200 nm, while triacetyloleandomycin was not detectable at all, using the chromatographic conditions optimized for erythromycin analysis (Table 2.3).

2.4.4 Mobile Phase Selection

The choice of the optimum mobile phase for the different columns used was governed by the need to separate erythromycin and the internal standard, the peak shape and the necessity of having a retention time for the internal standard of approximately 4 min in order to allow for the elution of endogenous components when applying the method to the analysis of biological fluids.

The utilization of a detector wavelength of 200 nm limits the choice of possible mobile phase constituents. HPLC grade methanol, which has a U.V. cut-off of 205 nm (120) may be used in small percentages, but HPLC grade acetonitrile was chosen as it is superior both in transparency (U.V. cut off 195 nm) (103,120) and in eluting strength from reverse phase columns (Eleutropic value on octadecylsilane, 3.1 vs methanol 1.0) (120).

The necessity of having a buffer component in the mobile phase for the elution of erythromycin from a C_{18} column was established in preliminary studies (147,148). Erythromycin did not elute at all from these columns when a mobile phase of methanol/water or acetonitrile/water was used, while a mixture of either of these

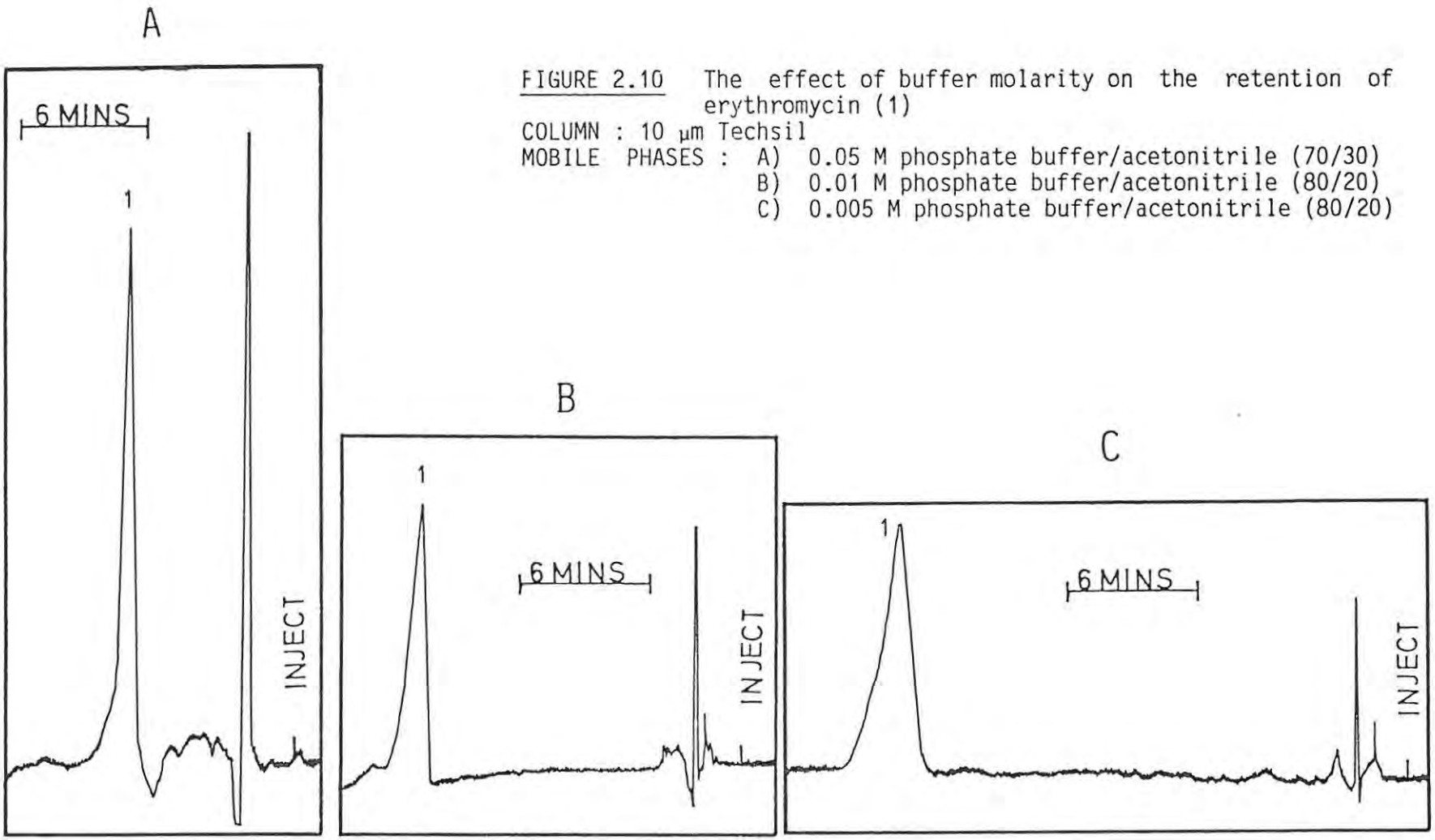


FIGURE 2.10 The effect of buffer molarity on the retention of erythromycin (1)
COLUMN : 10 µm Techsil
MOBILE PHASES : A) 0.05 M phosphate buffer/acetonitrile (70/30)
B) 0.01 M phosphate buffer/acetonitrile (80/20)
C) 0.005 M phosphate buffer/acetonitrile (80/20)

solvents with a buffer component easily eluted the drug. A phosphate buffer was chosen due to its reported column efficiency and U.V. transparency at 200 nm (103).

The molarity of the buffer was found to have a dramatic effect on the retention time and subsequent peak shape of erythromycin (Fig.2.10). It was not possible to use a mobile phase of acetonitrile/0,05 M phosphate buffer (80/20) due to the insolubility and resulting precipitation of the buffer salts on mixing.

Mobile phase pH was also found to have a substantial effect on the retention of erythromycin. As previously reported (142), the retention of erythromycin increased with an increase in mobile phase pH. The use of a mobile phase above the normally accepted pH limit for C_{18} columns of 7.00 was investigated by the introduction of a silica saturating pre-column. This column, however, resulted in an unstable baseline when monitored at 200 nm, suggesting that the utilization of low U.V. wavelengths prevents the succesful application of this approach, possibly due to the U.V. absorption by the dissolved silica particles in the saturated mobile phase.

As expected, the organic/aqueous phase ratio of the mobile phase had a considerable effect on both the selectivity and efficiency of the various columns used, making it an important parameter in the optimization of chromatographic conditions (Table 2.1).

2.4.5 Column Choice

The 25 cm column packed with 10 µm C_{18} material (Techsil) was found to be adequate for the initial studies of the effects of mobile phase composition on the retention of erythromycin. It, however, was unable to resolve erythromycin from the internal standard, which led to the choice of a column of the same length packed with 5 µm C_{18} material (Techsil). This column provided adequate resolution between these two compounds as is depicted in Fig. 2.11 and was subsequently used for a large proportion of the initial application of the HPLC method to the analysis of biological fluids. The high speed column proved to be less

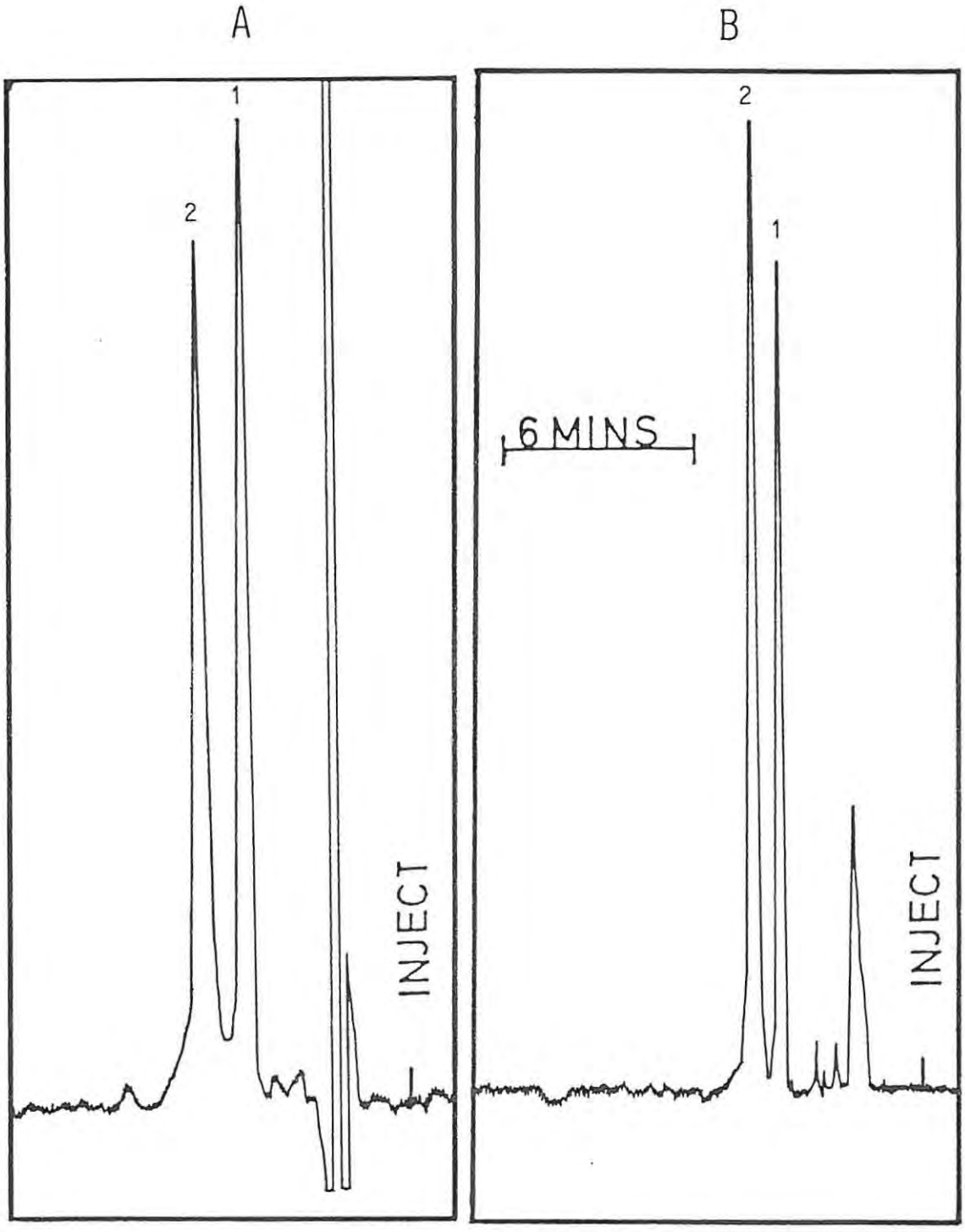


FIGURE 2.11 Chromatograms of oleandomycin (1) and erythromycin (2)

COLUMNS : A) 10 µm Techsil
B) 5 µm Techsil
MOBILE PHASE : 0.05M phosphate buffer/acetonitrile (50/50), pH 7.00

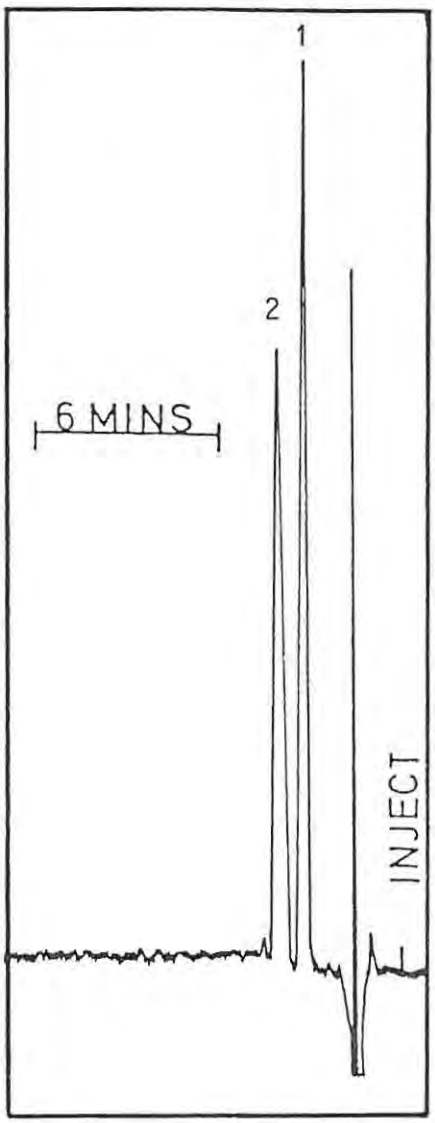


FIGURE 2.12 Chromatogram of oleandomycin (1) and eythromycin (2)

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/34), pH 7.00

efficient than the 5 μm C_{18} column probably due to the extra-column bandwidth broadening effects caused mainly by the relatively large detector cell volume of 8 μL (Kratos detector). The Novapak column provided the best peak shape and resolution of erythromycin and the internal standard, with the k' values being 4.0 and 3.1 respectively (Fig. 2.12) using chromatographic system D (Table 2.1).

By small adjustments in mobile phase composition, this method could be adapted to biological fluid analysis with the internal standard being separated from early eluting biological fluid components. The retention of erythromycin and some of its derivatives and components are reported in Table 2.3 using the Novapak column (system D) (Table 2.1).

TABLE 2.3 Retention times of erythromycin and various other macrolides

COMPOUND	RETENTION TIME (min)	k'
OLEANDOMYCIN PHOSPHATE	2.8	3.1
DES-N-METHYLERYTHROMYCIN	3.0	3.3
ERYTHROMYCIN (USP)	3.6	4.0
ERYTHROMYCIN STEARATE	3.6	4.0
ERYTHROMYCIN B	7.0	7.7
JOSAMYCIN	11.0	12.2
ANHYDROERYTHROMYCIN	9.4	10.4
ERYTHRALOSAMINE	11.0	12.2
ERYTHROMYCIN ENOL ETHER	20.0	22.0
ERYTHROMYCIN ESTOLATE	44.0	48.8
ERYTHROMYCIN ETHYLSUCCINATE	50.0	55.6
TRIACETYLOLEANDOMYCIN	UNKNOWN	

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/34), pH 7.00

From Table 2.3 it can be seen that this chromatographic system was ideally suited for the analysis of erythromycin and its components. The extremely long retention times of the two esters of erythromycin, erythromycin ethylsuccinate and estolate did not allow the direct application of this method to the analysis of these esters. Any attempt at decreasing the retention times of these compounds would have resulted in a shorter retention time for erythromycin making the application of this method to biological fluid analysis impossible. The retention times for these esters on the non-end-capped C_{18} columns were considerably shorter. An assay procedure for the analysis of these compounds could be developed using the high speed or, 5 μm C_{18} columns used in this study with small changes in the mobile phase composition.

2.4.6 Evaluation of Column Packing Techniques

Reverse phase columns packed by the method described, have been shown to be consistently comparable to similar commercially available columns at significantly reduced costs. The use of carbon tetrachloride as the slurry solvent and the time taken between the loading of the slurry and commencement of pressurization are vital to this technique. The constant degassing of the follow up-solvent (methanol) was an added precaution, as methanol is known to rapidly re-absorb oxygen after degassing (118).

2.4.7 Effect of Flow Rate

The optimum flow rate, as recommended by the manufacturers, for the Novapak column is 0.5 mL/min, which provided adequate separation between erythromycin and the internal standard at ambient temperature. An increase of flow rate in 0.1 mL increments provided comparable separation and improved peak shape leading to the choice of 1.0 mL/min as the optimum flow rate for this column. Changes in flow rate had a more dramatic effect on the efficiency of the high speed column. Flow rates below 2.0 mL/min resulted in very poor peak shape and resolution between erythromycin and the internal standard (Fig. 2.13). A flow rate of 2.0 mL/min was chosen as the optimum flow rate for the high speed column as the backpressure of 3000 psi prohibited the use of

higher flow rates. In addition, from the typical Van Deemter curve for a column of these dimensions (Fig. 2.3) an increase in flow rate above 2.0 mL/min would not be expected to increase column efficiency, but only to result in a decrease in analysis time.

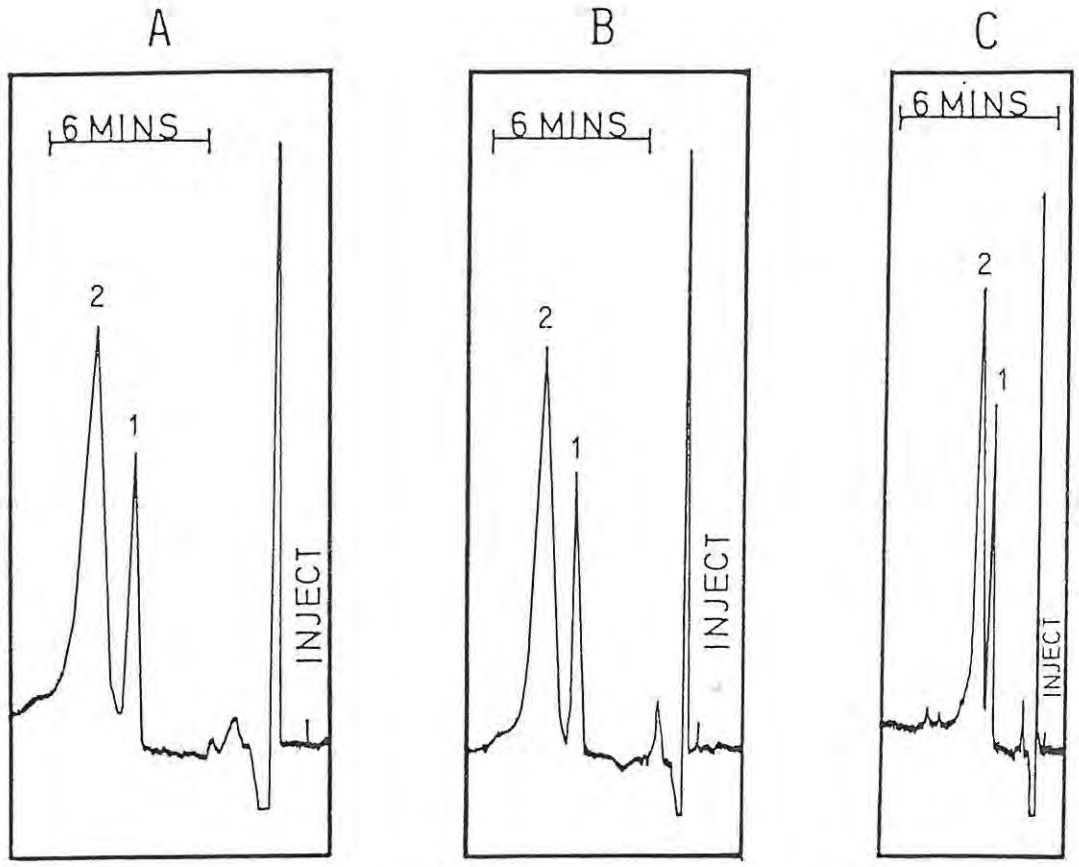


FIGURE 2.13 The effect of flow rate changes on the separation of oleandomycin (1) and erythromycin (2)
A) 1.3 mL/min
B) 1.6 mL/min
C) 2.0 mL/min

COLUMN : Ultrasphere High Speed
MOBILE PHASE : 0.005 M phosphate buffer/acetonitrile (50/50), pH 7.00

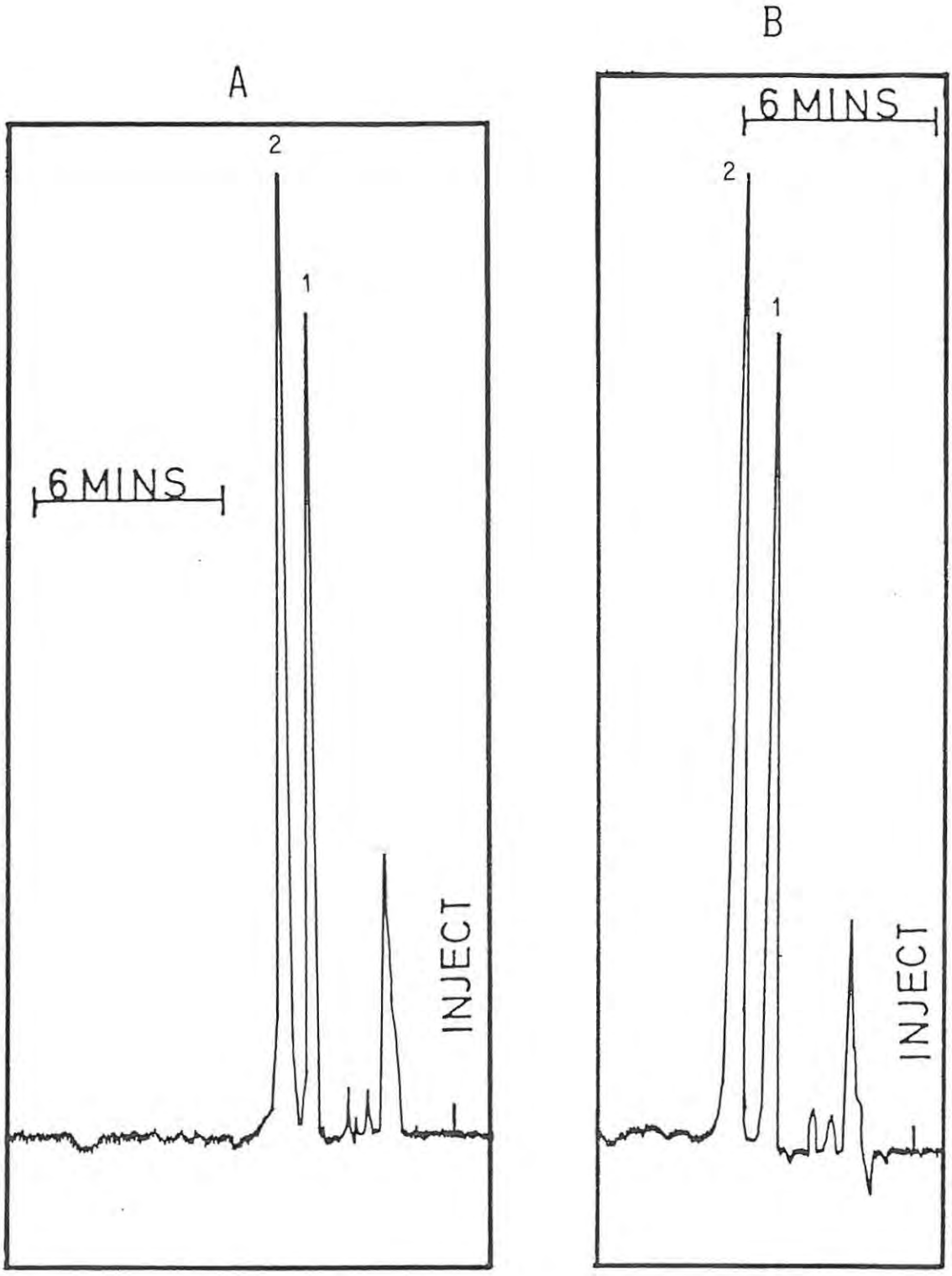


FIGURE 2.14 The effect of column temperature on the separation of oleandomycin (1) and erythromycin (2) A) 25°C
B) 35°C

COLUMN : 5 µm Techsil
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (50/50), pH 7.00

2.4.8 The Effect of Column Temperature

The improvement in column efficiency, as represented by the peak shape and resolution of erythromycin from other macrolide antibiotics has been previously reported (143,149). The effect of an elevation of column temperature from ambient temperature (20-25°C) to 45°C on the resolution of erythromycin and the internal standard when using the 5 μm C_{18} column is depicted in Fig 2.14. The Novapak column provided adequate resolution between erythromycin and the internal standard at room temperature. However, in the application of this method to the analysis of biological fluids, a column temperature of 35°C helped in assuring the separation of the internal standard from the early eluting biological fluid components.

2.4.9 Guard Column Choice

The use of the Rheodyne guard column fitted with a C_{18} cartridge resulted in an increase in retention times with associated decreases in peak shape and sensitivity for erythromycin and the internal standard.

Erythromycin did not elute at all when using the Guard-Pak unit fitted with a C_{18} cartridge, prior to the Novapak analytical column (Fig. 2.15). This was most probably due to the activity of the residual silanol groups on the pellicular guard column packing substrate when using a mobile phase optimized for the highly end - capped packing substrate of the analytical column. The use of cyano (CN) or glass bead cartridges had a minimal effect on the chromatography. The mean peak height ratios of erythromycin and internal standard for three urine samples at five different concentrations were unchanged after chromatography both with and without a glass bead guard column in-line (Table 2.4).

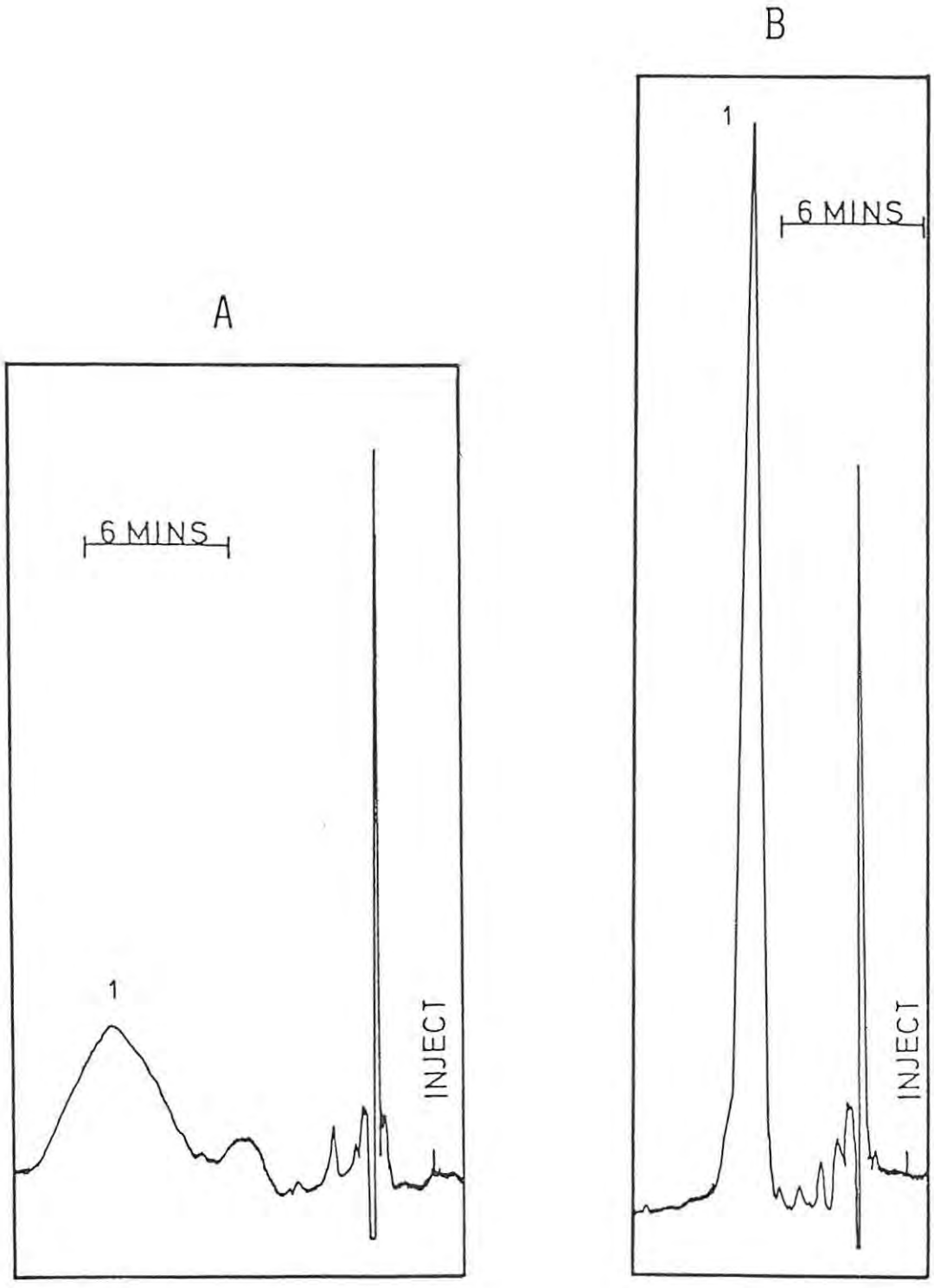


FIGURE 2.15 Chromatogram of erythromycin (1) using a Guard-Pak guard column unit A) C_{18} Cartridge
B) CN Cartridge

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/30), pH 7.00

TABLE 2.4 The effect of a glass bead guard column on the peak height ratios of extracted urine samples

CONCENTRATION OF URINE SAMPLE µg/mL	MEAN PEAK HEIGHT RATIO (n=3) ERYTHROMYCIN/INTERNAL STANDARD	
	Without Guard Column	With Guard Column
10.00	0.93	0.93
15.00	1.35	1.30
20.00	1.70	1.70
25.00	2.20	2.13
50.00 (0.5 mL Sample)	1.92	1.90

2.4.10 Solvent Degassing

Although the mobile phase was degassed before use, it was found that during lengthy analysis, the baseline remained more stable to drift and short term baseline noise when an in-line vacuum solvent degasser was used. Within-run degassing by periodic ultrasonication was initially used but was found to cause sudden baseline drifts. This has previously been reported by Bakalyar *et al.* (118).

2.5 CONCLUSIONS

The high performance liquid chromatographic methods using the 5 μm Techsil and Novapak columns described in this chapter compare favorably with the established HPLC method of Tsuji and Kane (145) for the analysis of erythromycin in pharmaceutical dosage forms.

The Novapak column, which yielded excellent peak shape at ambient temperatures, would be expected to have a considerably longer analytical life than the column used by Tsuji and Kane (145) which was operated at 70°C. Although the retention time for erythromycin was shorter on the Novapak column (3.6 min vs 9.0 min), this column could not be used for the analysis of the esters of erythromycin due to the extremely long retention times recorded for these compounds (Table 2.3). The method developed using the 5 μm Techsil column could be adapted for this purpose.

The optimization of mobile phase components and operating conditions to allow the utilization of a U.V. detection wavelength of 200 nm was not of any particular importance in the analysis of erythromycin *in vitro*, as sensitivity is seldom a major concern in these studies. It did, however, provide the necessary sensitivity for the development of an HPLC technique using U.V. detection for the analysis of erythromycin in serum and urine at the concentrations found in humans after the administration of oral dosage forms.

The comprehensive testing of various U.V. detectors at the conditions of their envisaged deployment, proved to be invaluable in the final selection of the Kratos detector for this analysis.

CHAPTER THREE

DETERMINATION OF ERYTHROMYCIN IN BIOLOGICAL FLUIDS

3.1 INTRODUCTION

Microbiological assay methods, as previously described, have been used for the majority of studies involving the analysis of erythromycin in biological fluids. These methods, while being sufficiently sensitive, lack the specificity necessary for detailed pharmacokinetic studies.

In 1969 Stephens *et al.* (63) reported a paper chromatographic technique for the separation of erythromycin from the inactive propionate ester after the administration of erythromycin estolate. This method utilizes a microbiological end-point (bio-autography) and hence suffers from similar specificity problems to that of conventional microbiological methods. This method was later adapted to thin layer chromatography - bioautography by Easterbrook and Hersey (150).

A chemical assay for the analysis of erythromycin and erythromycin propionate was reported by Tserng and Wagner (151). In this method, serum samples were adjusted to pH 6.00 for the ethereal extraction of the ester and to pH 9.00 for extraction of erythromycin base. The extracts were then measured fluorimetrically after complexation with a fluorescent dye (Tinopal G.S.).

Tsuji (152) described an HPLC technique based on the same detection method as Tserng and Wagner (151) for the analysis of erythromycin, erythromycin ethylsuccinate and some of their components/degradation products in serum. A 25 cm C_{18} column was used at a temperature of 70°C, following an extensive liquid - liquid extraction. A relatively complex post-column derivatization and extraction procedure preceded fluorimetric detection, necessitating the use of expensive and complicated apparatus. In addition, erythromycin was not well resolved from its metabolites/degradation products (presumed to be erythralosamine and anhydroerythromycin) present in human serum after oral administration of an erythromycin ethylsuccinate suspension. This

method has not been used in any published studies to date.

A recent paper by Chen and Chiou (9) described the HPLC analysis of erythromycin in biological fluids using electrochemical detection following separation on a C_{18} column. A novel dual electrode electrochemical detector operated in the oxidative screen mode was used. This procedure was reported as being extremely sensitive, allowing the use of small sample aliquots (0.1 to 1.0 mL). Serum samples were extracted using the same ether extraction as reported earlier (151) for serum and a simple precipitation step for urine. However, in order to reduce background currents and enhance detector stability, it was necessary to maintain the HPLC system in operation continuously even when not in use. Furthermore, the choice of erythromycin B as internal standard may lead to anomalies during pharmacokinetic studies since varying amounts of this compound are usually present in batches of raw material (2,122). Using this chromatographic method, two metabolites/degradation products (anhydroerythromycin and an unidentified compound), were detected in serum and urine of dogs after oral administration of erythromycin.

A further recent report by Duthu (99), using the same detector system as Chen and Chiou (9), described the analysis of erythromycin in serum only, using a similar extraction procedure but with different chromatographic conditions. A diphenyl reverse phase column was used, which was reported to cause less peak tailing than C_8 or C_{18} columns. A drug-related peak was observed in serum of human subjects after oral administration of a suspension of erythromycin but was absent in serum of subjects receiving an enteric coated erythromycin product. This compound was tentatively identified as the internal spiroketal of erythromycin. These two methods (9,99) are extremely sensitive and will no doubt find widespread use in the analysis of erythromycin in serum and urine. However, problems associated with the use of electrochemical detectors in general, may limit their widespread acceptance. The comparison of HPLC methods using electrochemical and U.V. detection for the analysis of erythromycin and its components is extensively discussed in Section 4.3.6.

The analysis of compounds in biological fluids by chemical methods usually necessitates a sample preparation stage (153). This is particularly important for non-chromatographic techniques in which selectivity is predominantly attained by the meticulous elimination of possible contaminants by extensive sample work-up procedures (153).

The increased selectivity afforded by HPLC has limited the extent of this sample preparation stage in the majority of analyses. A simple protein precipitation step performed by the addition of a strong acid, or an alcohol, followed by centrifugation, normally suffices when the concentration of the compound is sufficiently high and the detection method sufficiently selective (154). Where extra sample clean-up prior to chromatography has proved necessary, liquid - liquid extraction systems have predominantly been used (154). These extraction procedures rely on the selective separation of the unionized compound into a water immiscible solvent by the adjustment of the sample pH. The sample is then taken to dryness and reconstituted in a suitable solvent for chromatographic analysis, providing both a sample clean-up and concentration procedure (153).

Various other extraction procedures such as thin layer chromatography, ion-pair formation and solid phase extractions have been used for this purpose (153,154).

In solid phase extractions, the compound of interest is selectively adsorbed onto the extraction column matrix which is then washed to remove interfering compounds. The compound of interest is then eluted from the extraction column into a collecting tube by a further solvent mixture. Many different solid phases have been used, such as charcoal, silica, alumina, ion exchange resins and X-AD-2 (a hydrophobic polystyrene resin) (153). Recently, extraction columns packed with the same solid phases as those used in conventional HPLC have become available, which have extended the use of this technique.

The main advantages of solid phase extraction systems over liquid - liquid extraction methods are as follows.

1) Cleaner extracts are obtained, due to increased selectivity (153,155).

2) Sample losses due to emulsion formation do not occur (154).

3) Smaller volumes of solvents are used resulting in decreased analysis costs (156).

4) Less hazardous solvents are used (156).

6) Compounds which are unstable at the extreme pH ranges needed for their extraction into organic solvents may be extracted using solid phase techniques (117).

The principle disadvantage of these methods is the possibility of permanent adsorption losses of compounds with high affinity for the stationary phase, resulting in decreased sample recoveries (153).

When comparing the ability of different extraction procedures to remove interfering endogenous serum and urine components prior to chromatography, it is useful to have some indication of the amount of the original biological sample and hence potential interference, that has been loaded onto the column. A term, the "percentage sample loaded" has been used for this purpose throughout this study and is defined as follows:

The percentage sample loaded, is the percentage of the final reconstitution volume which is loaded onto the column. The original sample volume (i.e. volume of serum or urine to be analysed), must be constant for all extractions compared using this parameter.

3.2 DEVELOPMENT OF AN EXTRACTION METHOD FOR ERYTHROMYCIN FROM SERUM AND URINE

3.2.1 Experimental

3.2.1.1 Sample Preparation

Aqueous solutions of erythromycin (0.50 µg/mL) and oleandomycin (3.00-12.00 µg/mL) were prepared as in Section 2.3.1. During these developmental stages, spiked serum and urine samples of different concentrations were prepared by the addition of appropriate amounts of the aqueous solutions of erythromycin to drug free serum or freshly collected urine. The concentration ranges of both serum and urine varied between 1.00 and 10.00 µg/mL depending on the stage of the method development. Transfer pipettes were used for the dispensing of 0.5 and 1.0 mL aliquots while a programmable automatic pipette (Hamilton Microlab-P) was used for the addition of aliquots between 20.0 and 100.0 µL.

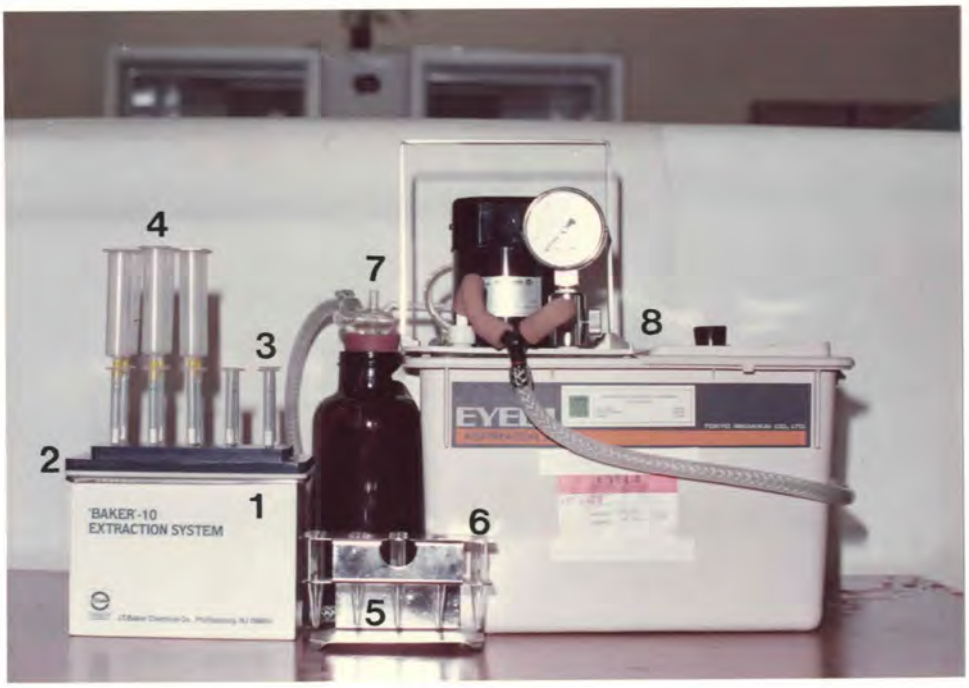
3.2.1.2 Chromatographic Conditions

HPLC System	As in Section 2.3.2
Column and mobile phase	Systems A, B, D, E (Table 2.1)
Flow rate	1.0 mL/min
Pressure and temperature	Table 2.1
Detection wavelength	U.V. 200 nm (Kratos detector)
Sensitivity	0,01 AUFS
Recorder input	10 mV

3.2.1.3 Solid Phase Extraction System

A "Baker - 10" Extraction system (J.T. Baker Chemical Co.) was used which consisted of the following components.

FIGURE 3.1 "Baker 10" solid phase extraction system



1) "Baker 10" vacuum manifold
2) Removable lid
3) Disposable extraction columns containing 40 µm C_{18} material (1 mL capacity)
4) Sample reservoirs (15 mL)
5) Collection rack
6) Glass collection tubes (2 mL)
7) Vacuum trap
8) Model A2-S Aspirator (Rikakikai Co. Ltd.)

3.2.1.4 Additional Equipment

1) Culture tubes 15mL (Kimble)

2) WISP limited volume inserts (Waters Associates)

3) Model 701SN Analytical syringe (Hamilton-Bonaduz)

4) N-Evap analytical evaporator (Organomation Associates)

5) Transfer pipettes (Brand)

6) Microlab-P automatic pipette (Hamilton-Bonaduz)

7) An Apple II micro-computer was used for the linear least squares regression analysis

3.2.1.5 General Extraction Method

Two main procedures were developed, the first for the initial developmental studies using aqueous sample loads and the second for the extraction of spiked serum and urine samples.

In the first method, the extraction column was washed successively with methanol (2.0 mL), water (2.0 mL) and acetonitrile/water (50/50 - 2.0 mL) with a vacuum of between 0 and 10 mm Hg being maintained. The sample (0.1 to 1.0 mL) was then loaded onto the extraction column followed by a wash of acetonitrile/water (70/30 - 2.0 mL), after which the extraction column was allowed to go to dryness. The vacuum was then broken, in order to place the collection tubes in position, and then re-applied to assist in the elution of the compounds of interest from the extraction columns with three 500 µL aliquots of acetonitrile/0.05 M phosphate buffer (50/50), pH 7.00. The concentration of erythromycin in the eluate (1.5 mL) was usually sufficient and did not require a further concentration step prior to chromatography. Where necessary, the eluate was taken to dryness under a stream of nitrogen at 40°C and reconstituted to 500 µL with water.

The relative efficiencies of the various eluting solvent systems and sequences used were compared by quantification of the peak heights obtained from a calibration curve. The recoveries were calculated by comparison of the amount of erythromycin eluted, to the original amount of erythromycin loaded onto the extraction column.

For the analysis of serum and urine, the initial wash cycle was changed to acetonitrile (3.0 mL) followed by water (3.0 mL). One millilitre of serum or urine was precipitated with 1.0 mL acetonitrile and centrifuged at 1 600xg, after which the supernatant was transferred to a culture tube containing 4.0 mL water. This mixture was then loaded onto the pre-treated extraction column with the aid of a sample reservoir.

The extraction column was washed with water (5.0 mL) followed by acetonitrile/water (50/50 - 5.0 mL), after which it was allowed to go to dryness under vacuum. The drug was then eluted from the extraction column with two successive 500 µL volumes of acetonitrile/0.05 M phosphate buffer (50/50), pH 7.00. The eluate was taken to dryness in a rotary vacuum centrifuge (Savant) and reconstituted as follows:

Twenty microlitres of water were added to each dried extract followed by vortex mixing for 1 min. On addition of 25.0 µL acetonitrile, two layers formed, which were separated after vortex mixing by centrifugation at 1 600xg for 2 min. Approximately 15.0 - 20.0 µL of the upper (acetonitrile) layer were transferred using an analytical syringe to a WISP limited volume insert for injection into the chromatographic system.

This extraction technique was later adapted for the extraction of 2.0 mL serum samples by the addition of 2.0 mL acetonitrile followed by centrifugation and dilution of the supernatant with 8.0 mL water. The internal standard (oleandomycin phosphate) was added either in the acetonitrile used for sample precipitation, or as a 250 or 500 µL aliquot immediately prior to sample precipitation.

3.2.2 Results and Discussion

3.2.2.1 Choice of Extraction Technique

A highly selective extraction procedure was necessary for the analysis of erythromycin in biological fluids using the HPLC method discussed in the previous chapter, due to the low and relatively nonspecific U.V. wavelength employed (105,157) the high detector sensitivity and the extensive sample concentration needed to obtain the desired sensitivity limits (0.25 μg/mL serum, 5.00 μg/mL urine).

A solid phase extraction technique was chosen for this purpose as it was felt that such a system would provide the most simple yet efficient extraction procedure. Octadecylsilane extraction columns were chosen as the retention of erythromycin on this solid support was well understood after the development of the HPLC technique previously discussed.

3.2.2.2 Extraction of Aqueous Samples

During the development of an HPLC method for the analysis of erythromycin, it was noticed that erythromycin did not elute from a C_{18} column if the mobile phase did not contain a suitable buffer component (Section 2.4.4). Similar results were found when aqueous samples of erythromycin were loaded onto C_{18} extraction columns. A mixture of acetonitrile/0.05 M phosphate buffer (50/50) was the only solvent combination which eluted the drug from the extraction column (Fig. 3.2). Other solvents tested included acetonitrile, methanol, water, phosphate buffer, acetonitrile/water and methanol/water.

The initial wash cycle of methanol (2.0 mL), water (2.0 mL) and acetonitrile/water (50/50 - 2.0 mL) was found to be necessary in order to both condition and clean the extraction column prior to extraction. A pure methanol wash was necessary before loading the extraction column with aqueous samples. The polar organic solvent is reported to be necessary in order to "activate" the non-polar solid support prior to contact with aqueous samples (156). Elution of the drug was found to be more efficient when the same volume of eluting solvent was applied in

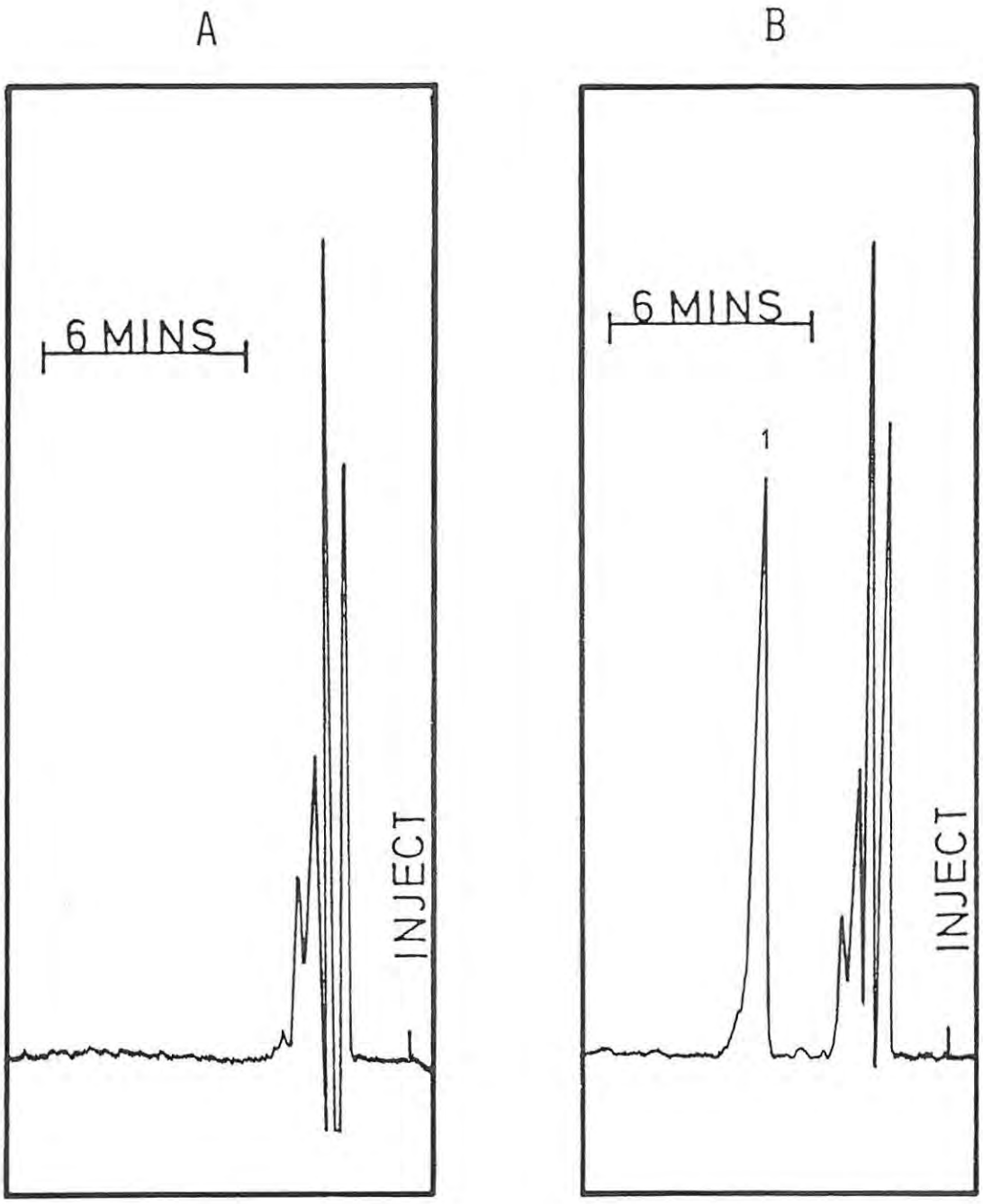


FIGURE 3.2 Chromatogram of an extraction of an aqueous sample of erythromycin (1) in which the drug was eluted with
A) acetonitrile/water (50/50)
B) acetonitrile/0.05 M phosphate buffer (50/50)
COLUMN : 10 µm Techsil
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (50/50), pH 7.00

smaller aliquots, with the extraction column being allowed to go to dryness between each elution. When using an acetonitrile/0.05 M phosphate buffer (50/50), pH 7.00 eluting solvent, a single 500 µL volume eluted 72% of the drug whereas a serial wash of 300 and 200 µL eluted 82% of the drug. Similar results were obtained by Good and Andrews (158) for the extraction of benzodiazepines in serum using the same extraction columns. A total volume of 1.0 ml of this eluting solvent was chosen as being the ideal volume of eluting solvent, as it eluted 95% of the drug. A change in composition of the eluting solvent to acetonitrile/0.05 M phosphate buffer (70/30), pH 7.00 had no effect on the amount of erythromycin eluted from the extraction column, with 500 µL of this solution eluting 80% of the drug.

Oleandomycin, the internal standard, performed similarly to erythromycin on the C_{18} extraction columns. It did not elute from the extraction column with an acetonitrile/water wash while it was eluted with a mixture of acetonitrile and 0.05 M phosphate buffer.

3.2.2.3 Extraction of Serum and Urine Samples

The major problem in the adaptation of the basic method described for aqueous samples to the analysis of serum samples, was the precipitation of serum proteins when loaded onto the pre-washed extraction columns. This led to clogging of the extraction columns even when using small serum aliquots (250 µL). The problem was alleviated by the pre-precipitation of 1.0 mL serum samples with 2.0 mL acetonitrile/water (50/50) prior to loading. However, when these samples were washed with a mixture of acetonitrile/water, the necessity of which is described later, erythromycin eluted from the extraction column (Fig. 3.3). This was possibly due to the eluting power of the mixture of the acetonitrile and endogenous ions present in the serum sample. The precipitated sample was then diluted with 4.0 mL of water after centrifugation and immediately prior to loading onto the extraction column as it was known that a pure water wash did not elute the drug from these samples (Fig.3.3). Similarly the subsequent acetonitrile/water (50/50) wash did not elute the drug from samples loaded in this manner (Fig. 3.3).

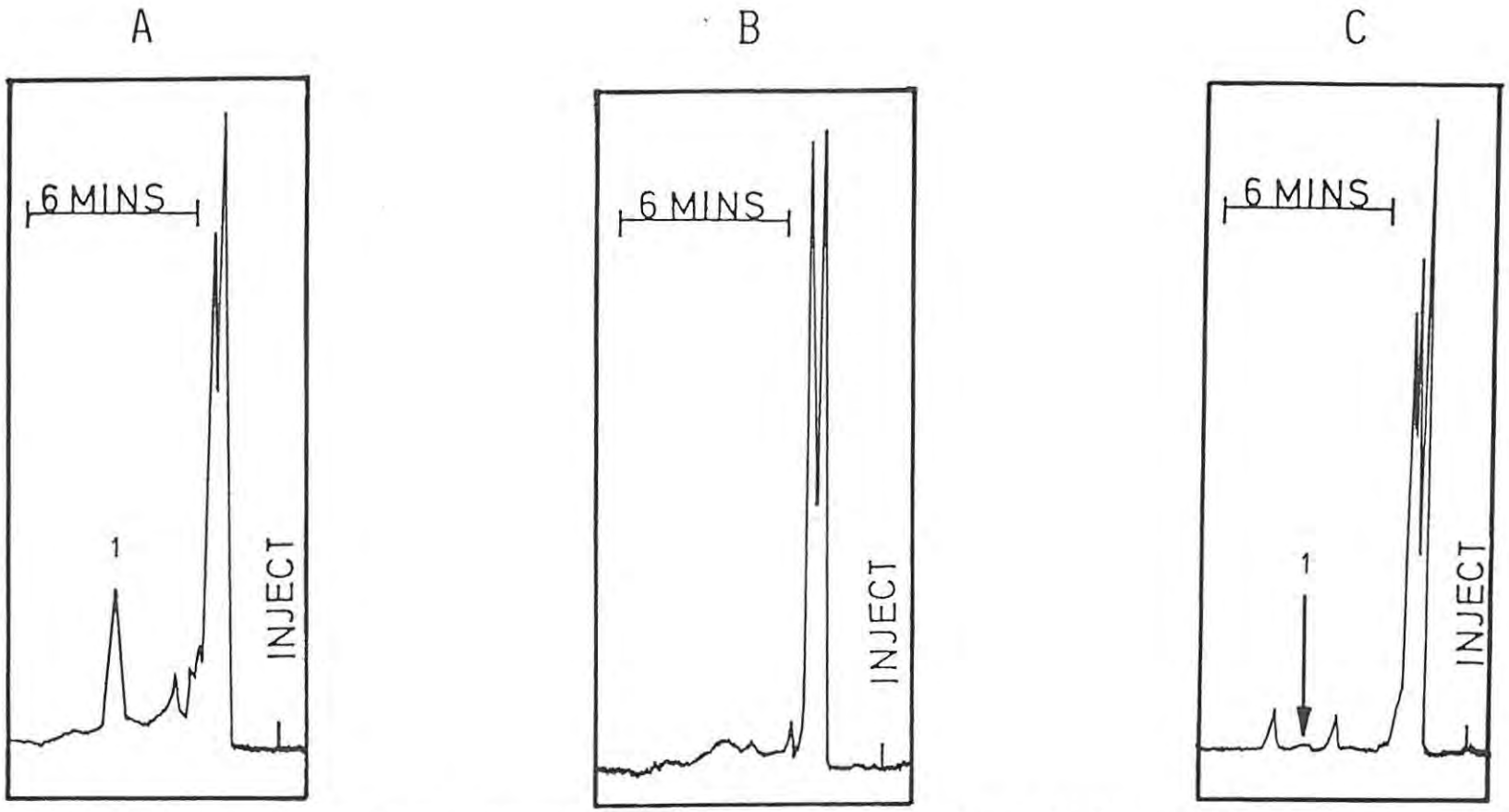


FIGURE 3.3 Chromatogram of an extraction of a pre-precipitated serum sample containing erythromycin (1) after the extraction column had (A) been washed with acetonitrile/water(50/50) and (B) been washed with pure water. In chromatogram (C) the same pre-precipitated sample had been diluted with 4.0 mL water prior to loading and the extraction column washed with acetonitrile/water (50/50).

COLUMN : 10 µm Techsil
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (50/50), pH 7.00

Although a water wash did not elute the drug from the extraction column, it did not result in a clean final extract which was sufficiently free from interference from endogenous serum components. For this purpose it was found that an acetonitrile/water wash containing at least 50% acetonitrile was essential, as chromatograms of samples washed with a 10% acetonitrile/water wash were not clean enough for quantification of the erythromycin peak (Fig. 3.4). The effect of urine components on the chromatograms of extracts is depicted in Fig.3.5. In this experiment, an aqueous sample of erythromycin was extracted along with identical samples to which 0.25, 0.5 and 1.0 mL aliquots of urine were added. The resulting chromatograms show increased interference which may be directly related to the amount of urine added. Five millilitre acetonitrile/water (50/50) washes were found to clean the samples sufficiently and were used in all subsequent analysis.

3.2.2.4 Concentration of the Elution Volume

The initial extraction procedure used, in which samples were reconstituted to 500 µL with mobile phase, yielded quantifiable chromatograms for the analysis of serum and urine samples of erythromycin in the concentration range 50.00-100.00 µg/L. The range of concentrations found after administration of dosage forms of erythromycin is however, considerably lower (0.25-5.00 µg/mL for serum and 5.00-50.00 µg/mL for urine - Table 1.2). The dried extracts (1.0 mL Serum) were then reconstituted in 100 µL mobile phase in order to provide a potential five fold increase in sensitivity. On injection of various volumes of these samples, it was found that the interference from serum or urine components became a serious problem when the injection volume was greater than 10.0-20.0 µL (Fig.3.6). This represented a percentage sample load for 1.0 mL samples of 10-20%.

It was also established that the chromatographic interference was not only due to endogenous compounds but was also due to the buffer component which had been concentrated. One millilitre of a mixture of erythromycin and oleandomycin in acetonitrile was taken to dryness and reconstituted in 50.0 µL acetonitrile of which 10.0 µL were then

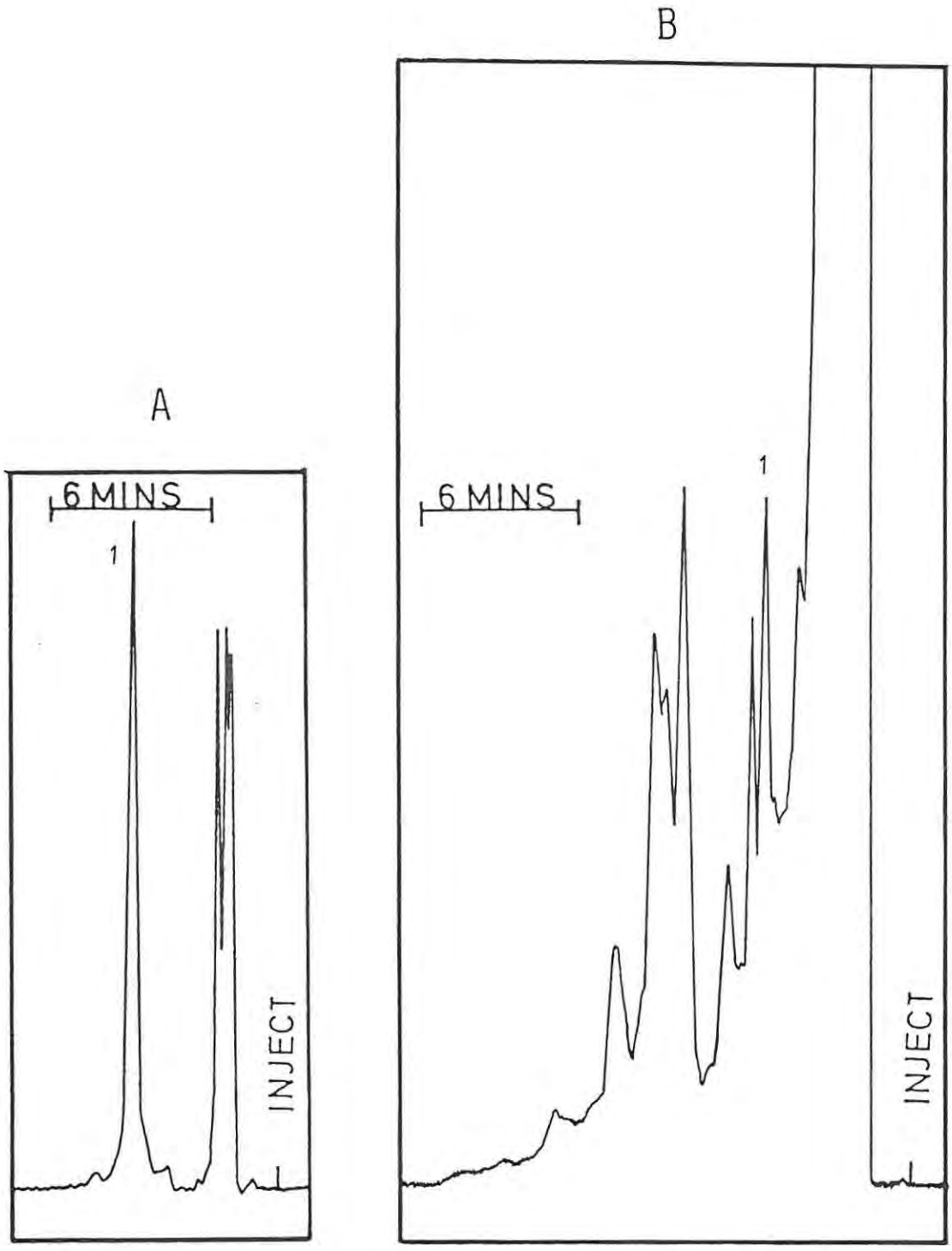


FIGURE 3.4 Chromatogram of a 1.0 mL serum extract containing erythromycin (1) after elution with 2.0 mL 0.05 M phosphate buffer/acetonitrile (50/50). The extraction column had been washed with 2.0 mL
A) acetonitrile/water (50/50)
B) acetonitrile/water (10/90)

COLUMN : 5 µm Techsil
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (50/50), pH 7.00

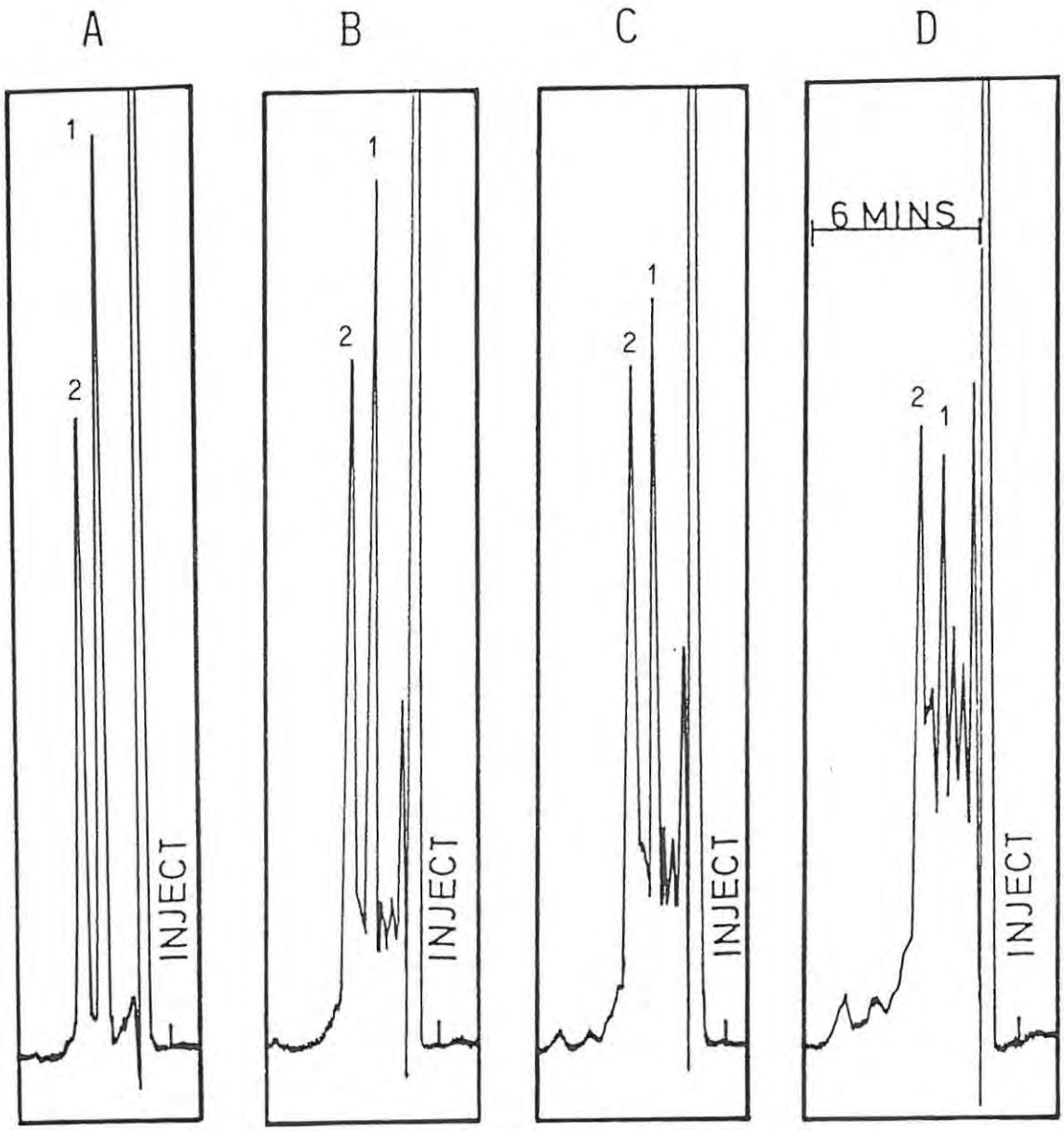


FIGURE 3.5 Chromatogram of an aqueous extract of oleandomycin (1) and erythromycin (2) to which

A) No urine was added
B) 0.25 mL urine was added
C) 0.50 mL urine was added
D) 1.00 mL urine was added

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/34), pH 7.00

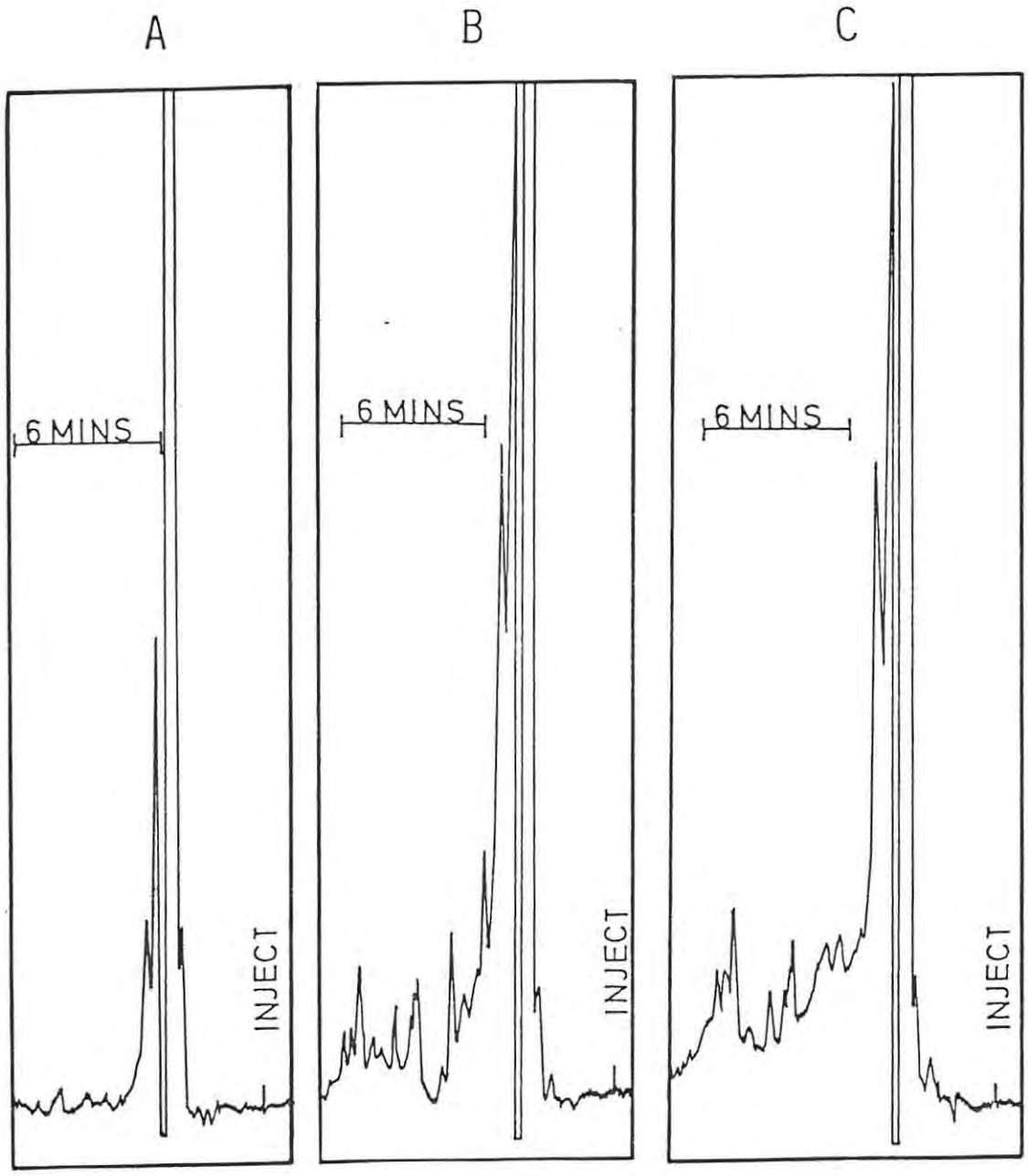


FIGURE 3.6 Chromatogram of a blank serum extract (1.0 mL) without the phase separation step. The percentage samples loaded were A) 10%
B) 20%
C) 30%

COLUMN : 5 µm Techsil
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (50/50), pH 7.00

injected onto the column. Figure 3.7 compares the chromatogram of such a sample, to that of an identical sample to which 300 µL of 0.05 M phosphate buffer were added and treated as above. The increase in the number of interfering peaks caused by the buffer component is clearly evident. The amount of buffer added, represented the amount of buffer present in 1.0 mL of a acetonitrile/buffer elution solvent (70/30).

Numerous attempts were made to separate erythromycin from the aqueous phase containing the buffer components, into an organic phase. Salting out of the acetonitrile and buffer layers immediately after elution with both NaCl and Na_2SO_4 was troubled by the carry over of the salts into the organic solvent, which inhibited the reconstitution of the dried extracts into small solvent volumes. The separation of erythromycin into ether, carbon tetrachloride, chloroform, cyclohexane, isooctane and pentane, with the pH adjusted with sodium carbonate (151), proved unsuccessful. An attempt to differentially freeze the acetonitrile (freezing point -43°C) and aqueous components of the eluate also failed.

3.2.2.5 Reconstitution of Samples

The composition of the reconstitution solvent proved to be the key to increasing the percentage sample load and hence sensitivity of the assay. Two distinct layers formed on addition of 50.0 µL acetonitrile/ 0.05 M phosphate buffer (50/50) to dried extracts due to the "salting out" (117) of the organic layer by the high concentration of buffer salts present in the aqueous layer. Injection of these separate layers into chromatographic system E (Table 2.1), after extraction of a 2.0 mL spiked serum sample (0.25 µg/mL) showed that erythromycin was present in the upper (acetonitrile) layer and was well resolved from the endogenous serum components, while the aqueous layer contained many co-eluting compounds which made the identification of the drug impossible (Fig.3.8).

Initial attempts at consistently separating the two layers of this mixture with a pasteur pipette were not successful as the relatively large pipette tip caused some disturbance and hence mixing of the two

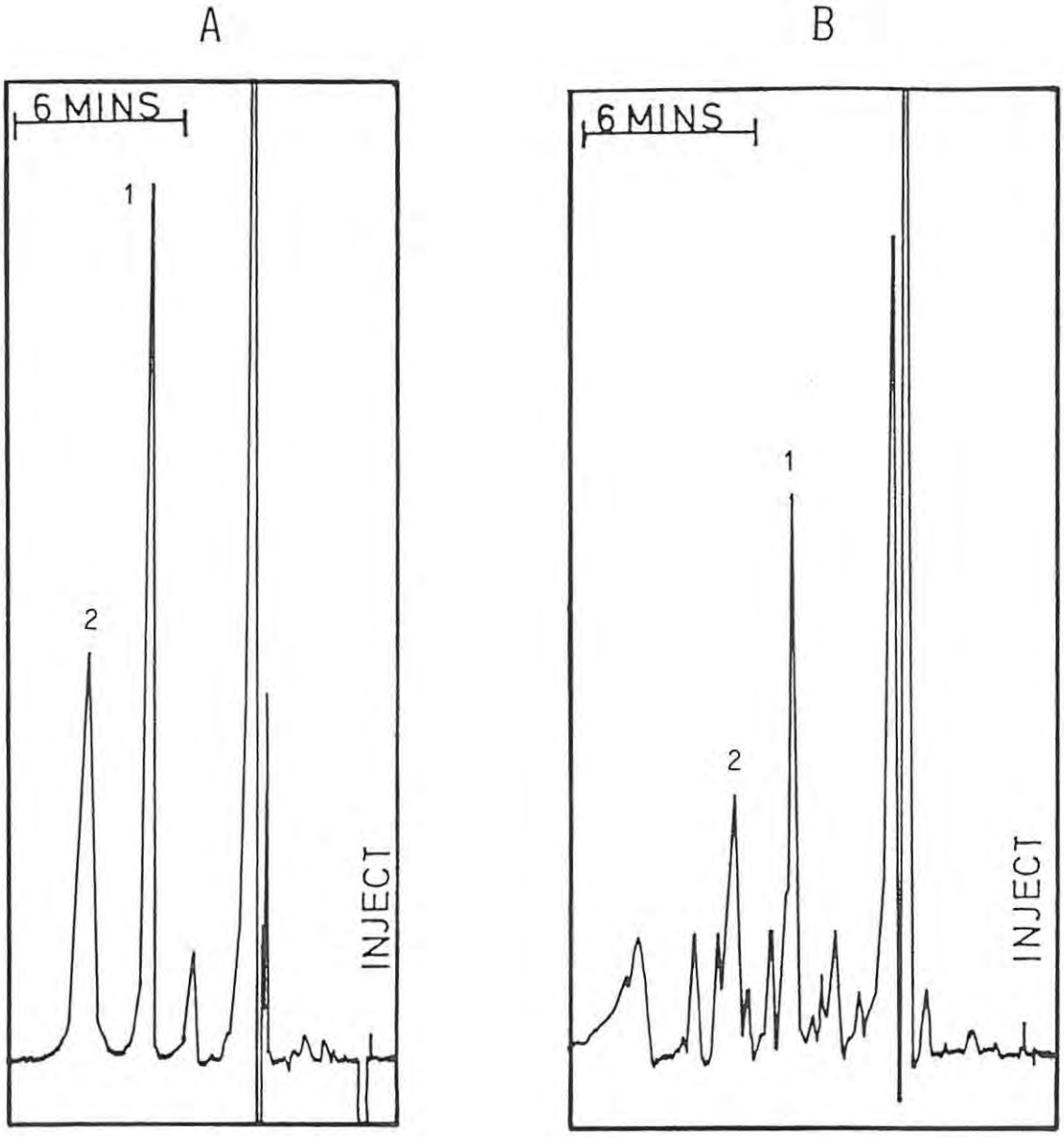


FIGURE 3.7 Chromatogram of a mixture of oleandomycin (1) and erythromycin (2) in (A) acetonitrile and (B) acetonitrile/buffer (70/30) after being taken to dryness and reconstituted in acetonitrile (50.0 µL)

COLUMN : 5µm Techsil

MOBILE PHASE : 0.05 M phosphate buffer (50/50), pH 7.00

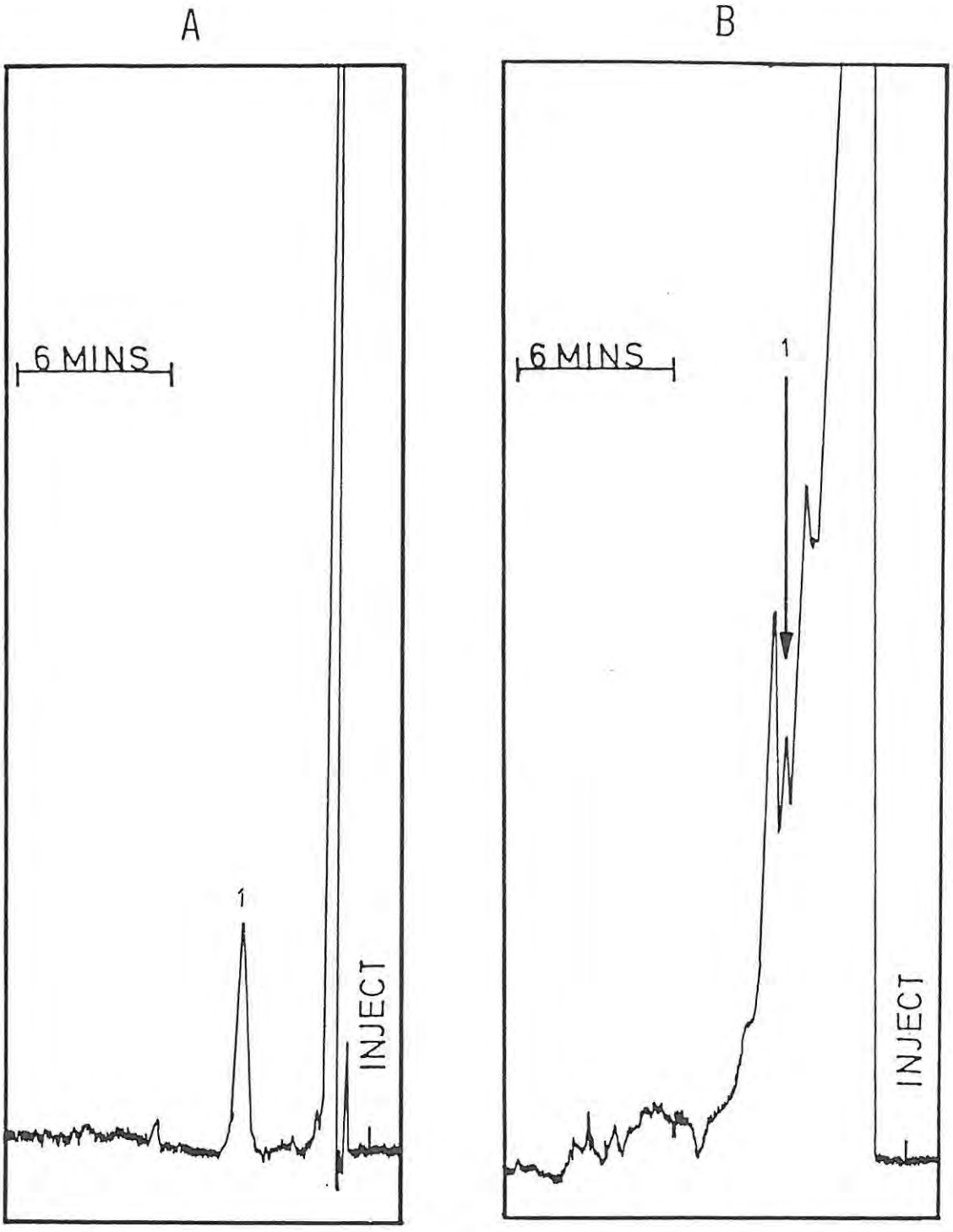


FIGURE 3.8 Chromatogram of (A) the upper layer (acetonitrile) and (B) lower layer (aqueous) after reconstitution of a 2.0 mL serum extract containing erythromycin (1) with water (20.0 µL) and acetonitrile (25.0 µL)

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/30), pH 7.00
INJECTION VOLUME : 5 µL

layers. Cleaner extracts were obtained when an analytical syringe with a narrow needle was used.

This method of sample reconstitution and solvent separation enabled the injection of between 5.0 and 10.0 µL of the 25.0 µL acetonitrile volume after extraction serum samples (i.e. a 20 to 40% sample load) with sufficient separation between erythromycin, the internal standard and the interfering peaks, to allow successful quantification of these compounds. The distribution of erythromycin and oleandomycin between the two phases was studied by the addition of appropriate amounts of the drugs to 1.0 mL acetonitrile/0.05 M phosphate buffer (50/50). The samples were then taken to dryness and reconstituted as described. Samples from each layer where analysed by HPLC and the amount of drug in each phase compared. Table 3.1 shows the percentages of the two drugs present in the aqueous phase compared to the acetonitrile layer.

TABLE 3.1 The distribution of erythromycin and oleandomycin between the two layers formed on reconstitution of extracts.

	ERYTHROMYCIN	OLEANDOMYCIN
SAMPLE CONCENTRATION µg/mL (n=3)	PERCENTAGE DISTRIBUTION AQUEOUS/ACETONITRILE LAYER	PERCENTAGE DISTRIBUTION AQUEOUS/ACETONITRILE LAYER
0.50	14.0	16.0
1.00	16.6	13.5
2.50	17.0	18.0
5.00	18.0	14.0
10.00	11.0	16.0
20.00	10.3	15.0
Mean Value	14.5	15.4

The mean percentage of erythromycin remaining in the aqueous phase was 14.5%. Due to the larger volume of the acetonitrile layer (25.0 vs 20.0 µL) and the possible contamination of the aqueous layer with some of the erythromycin rich acetonitrile layer, the estimation of the amount of erythromycin present in the aqueous layer was presumed to be slightly exaggerated. It was therefore assumed that a 10 to 15% range would be a more accurate estimation of the amount of erythromycin remaining in the aqueous layer.

3.2.2.6 Re-Use of Extraction Columns

The extraction columns were found to be re-usable. Six spiked urine samples at two different concentrations were extracted as in section 3.4.1.3., after which a further six samples were extracted using the same extraction columns. There were no significant differences in peak height ratios or relative standard deviations between the two extractions at either concentration. These results are summarized in Table 3.2. The chromatograms from both series of extractions were similar, indicating that there was no accumulation of contaminants.

TABLE 3.2 Within-run precision after re-use of extraction columns

URINE CONCENTRATION µg/mL	MEAN PEAK HEIGHT RATIOS (n=3) ERYTHROMYCIN/INTERNAL STANDARD		RELATIVE STANDARD DEVIATION	
	1st EXTRACTION	2nd EXTRACTION	1st EXTRACTION	2nd EXTRACTION
10.00	0.69	0.73	3.3%	1.5%
20.00	1.39	1.40	3.0%	2.7%

3.3 ANALYSIS OF ERYTHROMYCIN IN SERUM

3.3.1 Experimental

3.3.1.1 Sample Preparation

An erythromycin serum stock solution (5.00 µg/mL) was prepared as follows:

Twenty five milligrams of erythromycin were dissolved in 50.0 mL water and 0.5 mL of this solution was made up to 50.0 mL with blank serum. Appropriate dilutions of this stock solution were then made.

The internal standard stock solution was prepared by dissolving 24.0 mg oleandomycin phosphate in 1.0 mL acetonitrile which was then made up to 100.0 mL with water. The internal standard solution (12.00 µg/mL) was then prepared by diluting 5.0 mL of this stock solution to 100.0 mL with water.

3.3.1.2 Chromatographic Conditions

HPLC System	As in Section 2.3.2
Column	Novapak
Mobile phase	0.05 M phosphate buffer/acetonitrile (70/30), pH 7.00 (System E, Table 2.1)
Flow rate	1.0 mL/min
Pressure and temperature	Table 2.1
Detection wavelength	U.V. 200 nm (Kratos detector)
Sensitivity	0.005 AUFS (Kratos detector 0.01 AUFS coupled to the Spectrum filter on a x2 amplification setting and a 0.01 sec filter setting)
Recorder input	10 mV

3.3.1.3 Extraction Procedure

Two millilitres of serum were mixed with 250 µl of the internal standard solution. Addition of 1.0 mL acetonitrile followed by vortex mixing for 1 min and centrifugation for 5 min at 1 600xg resulted in deproteinization of the serum sample. The supernatant was transferred to a culture tube containing 8.0 mL water for direct loading onto a 1.0 mL disposable C_{18} extraction column, which had been pre-washed under 10-15 mm Hg vacuum, by successively passing 3.0 mL acetonitrile and 3.0 mL water through each. The diluted sample was then added to the extraction column with the aid of a sample reservoir. The extraction column was washed with 5.0 mL water and then 5.0 mL acetonitrile/water (50/50) with vacuum maintained until dry. Erythromycin and the internal standard were eluted into tapered collection tubes with two successive 500 µL aliquots of acetonitrile/0.05 M phosphate buffer (50/50). The sample was then taken to dryness in a rotary vacuum centrifuge. The residue in the collection tube was reconstituted in 20.0 µL water and vortexed for 1 min. After addition of 25.0 µL acetonitrile the mixture was vortexed and then centrifuged for 1 min at 1 600xg. Five microlitre aliquots of the top layer were injected onto the column.

3.3.1.4 Calibration Curve

The serum stock solution was diluted to yield four different concentrations, the stock solution itself providing the fifth. Concentrations ranged from 0.25 to 5.00 µg/mL and each concentration was assayed in triplicate. The calibration curve was constructed by plotting the ratio of the peak height of erythromycin to that of the internal standard, versus the respective erythromycin concentration. A straight-line fit of the data was made by least-squares linear regression analysis.

3.3.1.5 Precision

Within-run precision was assessed by extracting six spiked serum samples each at the upper and lower limits of the concentration ranges studied (0.50 and 2.00 µg/mL).

3.3.1.6 Extraction Efficiency

For this study, in which the analytical recovery of erythromycin from serum was assessed, spiked serum samples were assayed in triplicate at three different concentrations. All samples were extracted as previously described except that the internal standard was incorporated into the 25.0 µl aliquot used in the final reconstitution and phase separation step and was thus not carried through the extraction procedure.

Standard solutions of erythromycin corresponding to those extracted above, were made up in 1.0 mL of acetonitrile/0.05 M phosphate buffer (50/50) and were taken to dryness in a rotary vacuum centrifuge. These samples were then reconstituted as for the serum samples with the internal standard being added in the 25.0 µL acetonitrile aliquot. To determine the percentage recovery, the ratios obtained from the serum extracts were compared to those resulting from the relevant standard solutions of equivalent concentrations.

3.3.2 Results and Discussion

3.3.2.1 Linearity and Calibration Curve

Linearity was established for the range of concentrations studied (0.25-5.00 µg/mL). The curve had a slope of 0.0295, a y-intercept of 0.2938 with a correlation coefficient of 0.9997. The calibration curve for erythromycin in serum is shown in Fig.3.9. Chromatograms of a blank serum extract and a serum extract containing erythromycin and internal standard are depicted in Fig. 3.10. The retention time for erythromycin was 6.2 min and that of the internal standard 4.2 min.

FIGURE 3.9 Calibration curve of erythromycin in serum

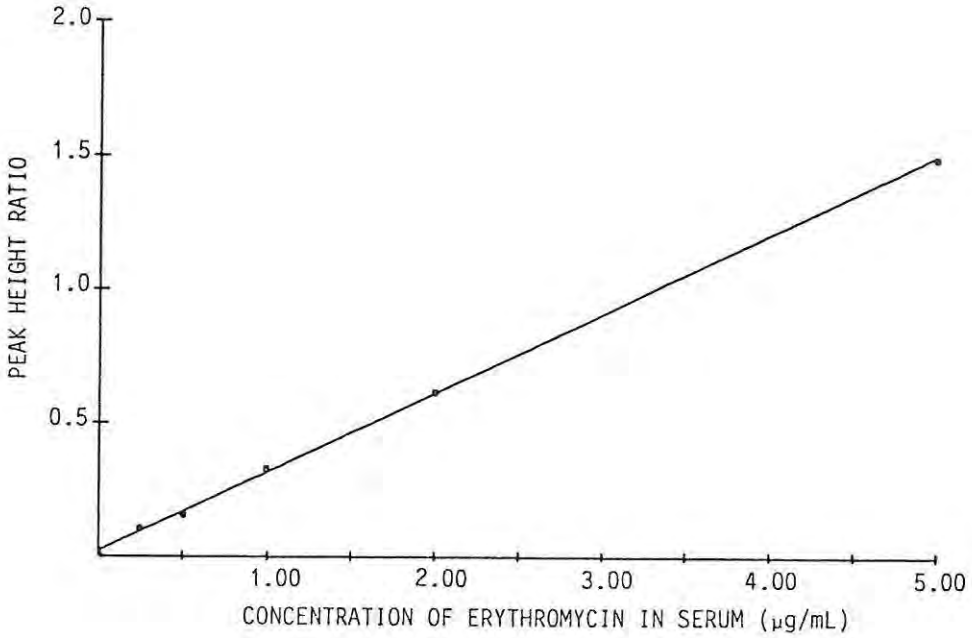


TABLE 3.3 Within-run precision study on serum assay

SPIKED CONC	CONCENTRATION MEASURED (µg/mL)							
	SAMPLE NUMBER							
(µg/mL)	1	2	3	4	5	6	MEAN (SD)	R.S.D.
0.50	0.49	0.48	0.52	0.53	0.52	0.50	0.51 (0.02)	3.92%
2.00	2.02	1.94	1.98	2.00	2.01	2.01	1.99 (0.03)	1.50%

TABLE 3.4 Analytical recoveries of erythromycin in serum.

SERUM CONC	% RECOVERY				
(µg/mL)	1	2	3	MEAN (SD)	R.S.D.
0.50	85.0	89.6	79.2	84.6 (5.2)	6.2%
1.00	82.8	76.7	82.8	80.8 (3.5)	4.4%
2.00	85.3	79.9	80.0	81.7 (3.1)	3.8%
MEAN PERCENTAGE RECOVERY (n=9)				82.4 (3.9)	4.7%

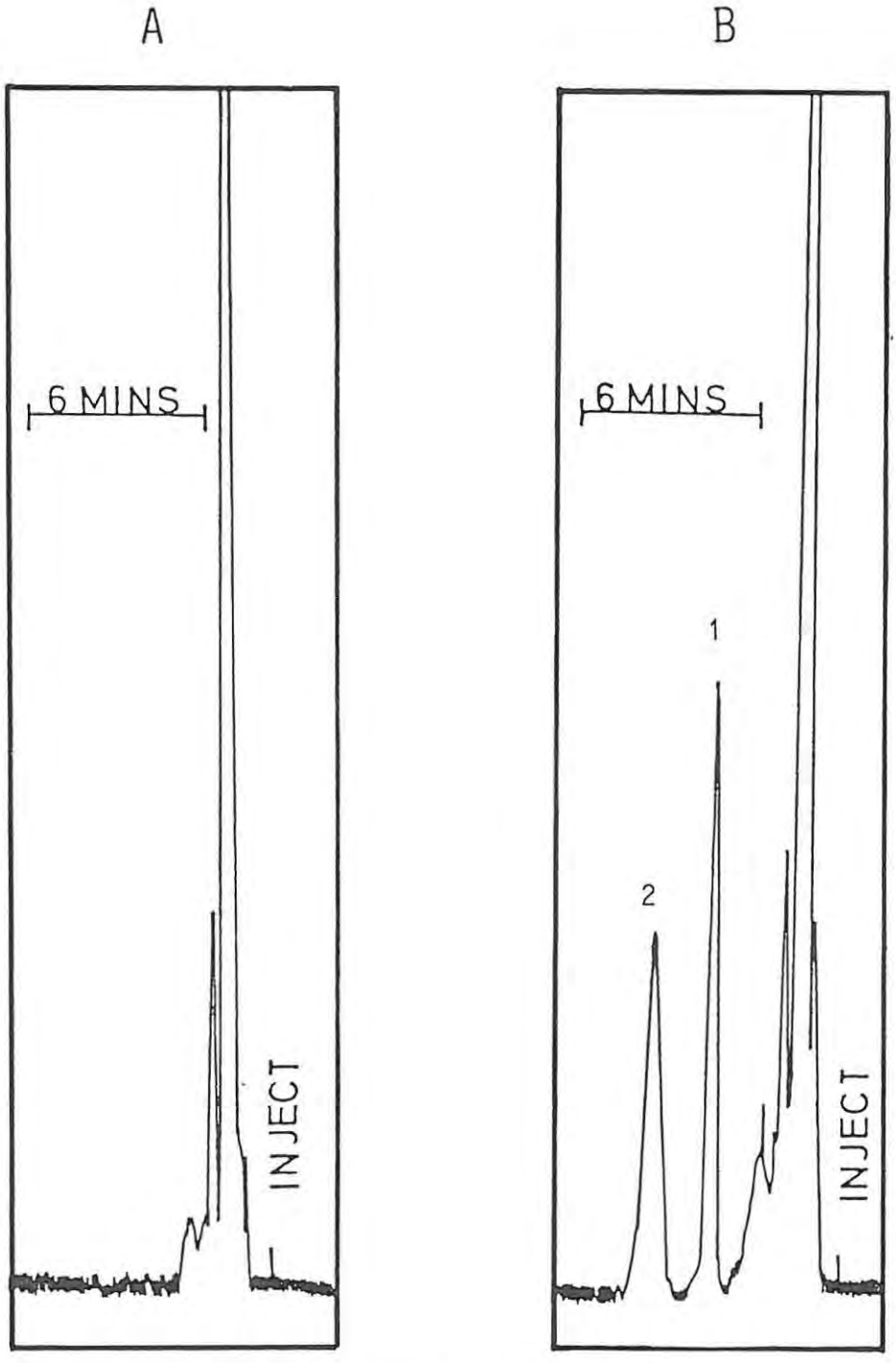


FIGURE 3.10 Chromatogram of (A) a blank serum extract (2.0 mL) and (B) a serum extract (2.0 mL) containing oleandomycin (1) and erythromycin (2)

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/30), pH 7.00
INJECTION VOLUME : 5 µL
ERYTHROMYCIN CONCENTRATION : 1.50µg/mL

3.3.2.2 Precision

Results of the within-run precision study are depicted in Table 3.3. Relative standard deviations (RSD) at both the upper and lower concentration limits fall within the accepted limits for drug determinations. The method, as indicated by the above results was found to be reproducible.

3.3.2.3 Extraction Efficiency

Table 3.4 shows the analytical recoveries of erythromycin in serum. The mean recovery value of 82.4% obtained for serum, does not take into account the 10-15% of the drug remaining in the aqueous phase (Section 3.2.2.5.). The overall recovery values may thus be adjusted by 10-15%. This 10-15% loss due to the final phase separation step was considered unimportant since the resulting extract enabled a percentage sample load of 30-40% from 2.0 mL serum samples which was free from sample interference. The limit of sensitivity was thus increased by approximately 6 to 8 times compared to extracts omitting this step, from which a maximum percentage sample load of only 10-15% was possible for 1.0 mL serum samples (Section 3.2.2.3.) due to extensive interference from other sample components.

3.3.2.4 Sensitivity and Detection Limits

Under the conditions of this assay and based on a minimum signal to-noise ratio of 3, the detection limit for erythromycin in serum was 0.25 μg/mL. The detection limit was increased where necessary by the use of a larger injection volume, which was associated with a slight increase in the interference from serum components.

3.3.2.5 Stability of Samples on Storage

Serum samples (1.00 μg/mL) were found to be stable for 1 month at 4°C (95.0%, n=3). However, similar serum samples (1.00 μg/mL) stored for 1 month at -10°C suggested that some degradation may have occurred (85.0%, n=3). The absence of any degradation products, suggested that

the low concentrations obtained may have been slightly in error. Erythromycin has been reported as being stable for three months in an acetonitrile solution when stored at -10°C (9) but only for 1 week at 4°C (7). Samples containing higher concentrations of erythromycin were used in further studies on the stability of erythromycin in urine samples, in order to facilitate the detection of any possible degradation products.

3.4 ANALYSIS OF ERYTHROMYCIN IN URINE

3.4.1 Experimental

3.4.1.1 Sample Preparation

An erythromycin urine stock solution (50.00 µg/mL) was prepared as follows:

Twenty five milligrams of erythromycin were dissolved in 50.0 mL water and 5.0 mL of this solution was made up to 50.0 mL with blank urine. Appropriate dilutions of this stock solution were then made. The same internal standard solution was used as previously described for the analysis of serum samples (Section 3.3.1.1).

3.4.1.2 Chromatographic Conditions

These were identical to those in section 3.3.1.2 except that the spectrum-filter was not used i.e. Kratos detector 0.01 AUFS.

3.4.1.3 Extraction Procedure

One millilitre of urine was mixed with 500 µL of the internal standard solution and 1.0 mL of acetonitrile. After centrifugation for 5 min at 1 600xg the supernatant was transferred to a culture tube containing 8.0 mL water. The samples were then extracted as for serum samples (Section 3.3.1.3). Between 1.0 and 5.0 µL were injected onto the column.

3.4.1.4 Calibration Curve

A calibration curve was constructed as described for the analysis of erythromycin in serum (Section 3.3.1.4). Concentrations ranged from 5.00 to 50.00 µg/mL.

3.4.1.5 Precision

Within-run precision was assessed by extracting six spiked urine samples each at the upper and lower limits of the concentration ranges studied (5.00 and 50.00 µg/mL).

3.4.1.6 Extraction Efficiency

The analytical recovery of erythromycin in urine was assessed as for serum samples (Section 3.3.1.6), except that four concentrations of urine samples were analysed.

3.4.2 Results and Discussion

3.4.2.1 Linearity and Calibration Curve

Linearity was established for the range of concentrations studied (5.00 to 50.00 µg/mL). The curve had a slope of 0.0127, a y-intercept of 0.0686 with a correlation coefficient of 0.9997. The calibration curve for erythromycin in urine is shown in Fig. 3.11.

Initial studies using 0.5 mL urine for samples containing greater than 30.00 µg/mL erythromycin showed that the linearity at these higher concentrations was improved when 1.0 mL of urine was used throughout. A calibration curve using 1.0 mL samples in the concentration range 7.50 to 30.00 µg/mL had a correlation coefficient of 0.9997, whereas the same calibration curve using 0.5 mL of the 30.00 µg/mL sample had a correlation coefficient of 0.9962. It was therefore decided to use 1.0 mL samples throughout the study.

Chromatograms of a blank urine extract and a urine extract containing erythromycin and internal standard are depicted in Fig. 3.12. The retention time for erythromycin was 6.2 min and that of the internal standard 4.2 min.

3.4.2.2 Precision

Initial precision studies, performed with the internal standard being added in the acetonitrile used to precipitate the urine proteins, resulted in poor results. Table 3.5 shows the results of duplicate injections, of five identical spiked urine sample extracts, prepared with the internal standard being added as above. The within-run precision was poor, with a relative standard deviation of 16.8%. Duplicate injections of the same samples yielded almost identical results, indicating that the chromatography was not the cause of the poor precision.

TABLE 3.5 Inter-sample and within-run precision on five urine samples (10.00 µg/mL) with the internal standard being added in 0.5 mL acetonitrile

	PEAK HEIGHT RATIOS ERYTHROMYCIN/INTERNAL STANDARD			
SAMPLE	1ST INJECTION	2ND INJECTION	AVERAGE	
1	0.80	0.80	0.80	MEAN RATIO = 0.89 (n=5)
2	0.95	1.01	0.98	SD = 0.15
3	1.10	1.10	1.10	RSD = 16.8%
4	0.78	0.82	0.80	
5	0.74	0.75	0.75	

This was subsequently established as being due to variations in the volume of internal standard added by the following experiment. Sufficient internal standard was added to 3.0 mL of urine, after which the samples were then separated into three equal aliquots and extracted as described. The relative standard deviation for these samples was only 1.2%. This effect may be due to either evaporation of the volatile acetonitrile during sample preparation, or due to a varying wetting phenomenon between the plastic tip and the solvent.

The internal standard was then added as an aqueous solution (0.5 mL) prior to addition of the acetonitrile. This resulted in a dramatic increase in precision, with the relative standard deviations being 4.0% at 5.00 µg/mL and 1.50% at 50.00 µg/mL (Table 3.6), which was well within the accepted limits for drug determinations.

3.4.2.3 Extraction Efficiency

Table 3.7 shows the analytical recoveries of erythromycin in urine. The mean recovery of 79.5%, may be adjusted by 10-15% as discussed previously. This value corresponds well with the value obtained for the recovery of serum samples (82.4%).

3.4.2.4 Sensitivity and Detection Limits

Using 1.0 mL urine samples, the minimum detectable limit for erythromycin in urine under the above conditions was 1.00 µg/mL. In view of the accessibility of large volumes of urine samples, this limit could be increased by the use of larger sample aliquots.

3.4.2.5 Stability of Samples on Storage

Spiked urine samples were found to be unstable when stored at 4°C for one week, but were found to be relatively stable after two weeks when stored at -10°C (Table 3.8-A-B). Extracted urine samples which had been reconstituted for injection, were found to have degraded after three days, even when stored at 4°C (Table 3.8-C). Samples extracted and taken to dryness, but not reconstituted, were relatively stable for one

FIGURE 3.11 Calibration curve of erythromycin in urine

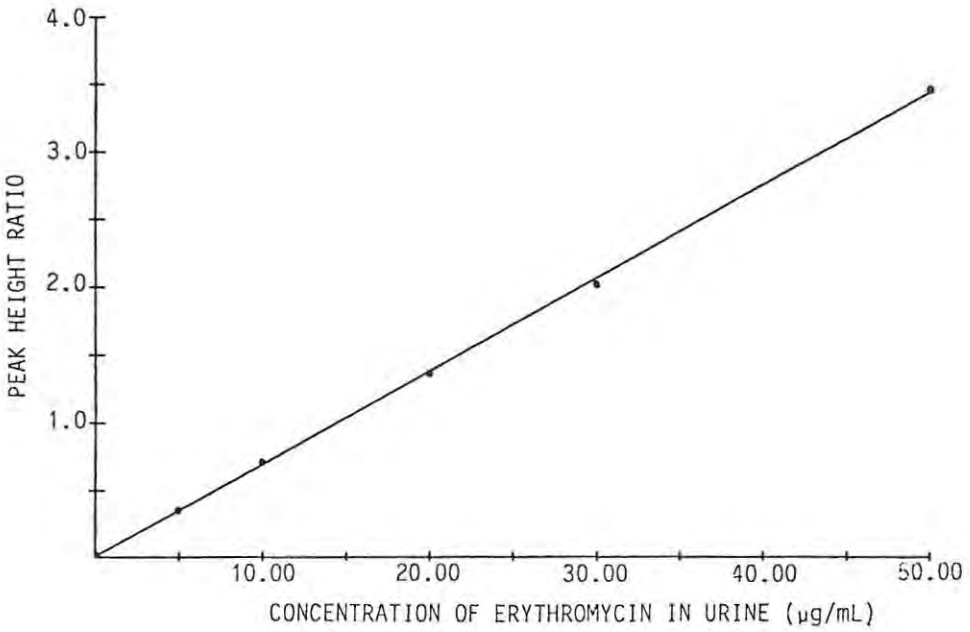


TABLE 3.6 Within-run precision study on urine assay.

SPIKED CONC	CONCENTRATION MEASURED (µg/mL) SAMPLE NUMBER							
(µg/mL)	1	2	3	4	5	6	MEAN (SD)	R.S.D.
5.00	5.01	5.16	5.25	5.22	4.71	4.95	5.05 (0.20)	4.0%
50.00	47.50	48.30	48.91	48.70	49.50	48.00	48.48 (0.70)	1.5%

TABLE 3.7 Analytical recoveries of erythromycin from urine.

URINE CONC	% RECOVERY SAMPLE NUMBER				
(µg/mL)	1	2	3	Mean (SD)	R.S.D.
5.00	85.0	80.0	85.0	83.3 (2.9)	3.5%
10.00	77.5	82.5	82.5	80.8 (2.9)	3.6%
20.00	75.0	75.0	75.0	75.0	0%
50.00	76.0	80.0	80.0	78.7 (2.3)	2.9%
Mean Percentage recovery (n=12)				79.5 (3.7)	4.7%

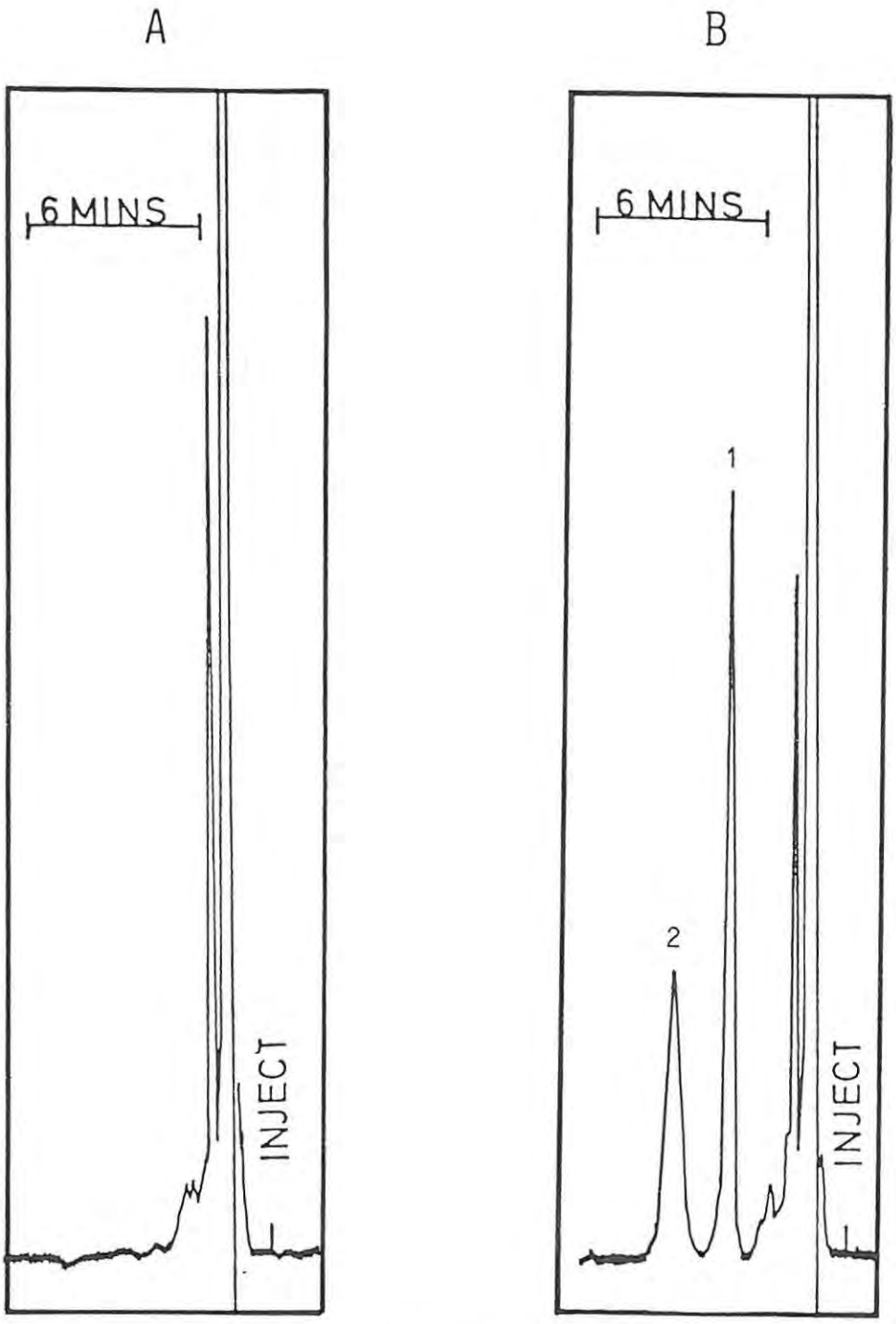


FIGURE 3.12 Chromatogram of (A) a blank urine extract (1.0 mL) and (B) a urine extract (1.0 mL) containing oleandomycin (1) and erythromycin (2)

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/30) pH 7.00
INJECTION VOLUME : 3 µL
ERYTHROMYCIN CONCENTRATION : 5.00µg/mL

week at 4°C, whilst signs of degradation were evident after the second week (Table 3.8-D).

Extracted urine samples stored dry on the extraction columns prior to elution, also showed some evidence of degradation when analysed after storage for one week at 4°C (Table 3.8-E).

TABLE 3.8 Stability of urine samples

SAMPLE	STORAGE CONDITIONS	TIME OF STORAGE	ERYTHROMYCIN CONCENTRATION BEFORE STORAGE µg/mL	MEAN ERYTHROMYCIN CONCENTRATION AFTER STORAGE µg/mL (n=3)
A URINE SAMPLES	4°C	1 WEEK	5.0 20.0	3.5 14.5
B URINE SAMPLE	-10°C	1 WEEK	5.0 50.0	4.9 24.9
		2 WEEKS	5.0 25.0 50.0	5.0 22.5 45.0
C URINE EXTRACTS, RECONSTITUTED FOR INJECTION	4°C	3 DAYS	5.0 10.0 50.0	3.8 7.7 38.2
D URINE EXTRACTS, PRIOR TO RECONSTITUTION	4°C	1 WEEK	5.0 50.0	4.7 48.5
		2 WEEKS	5.0 50.0	4.0 42.0
E URINE EXTRACTS, STORED ON EXTRACTION COLUMN	4°C	1 WEEK	5.0 50.0	4.2 42.0
		2 WEEKS	5.0 50.0	4.0 30.0

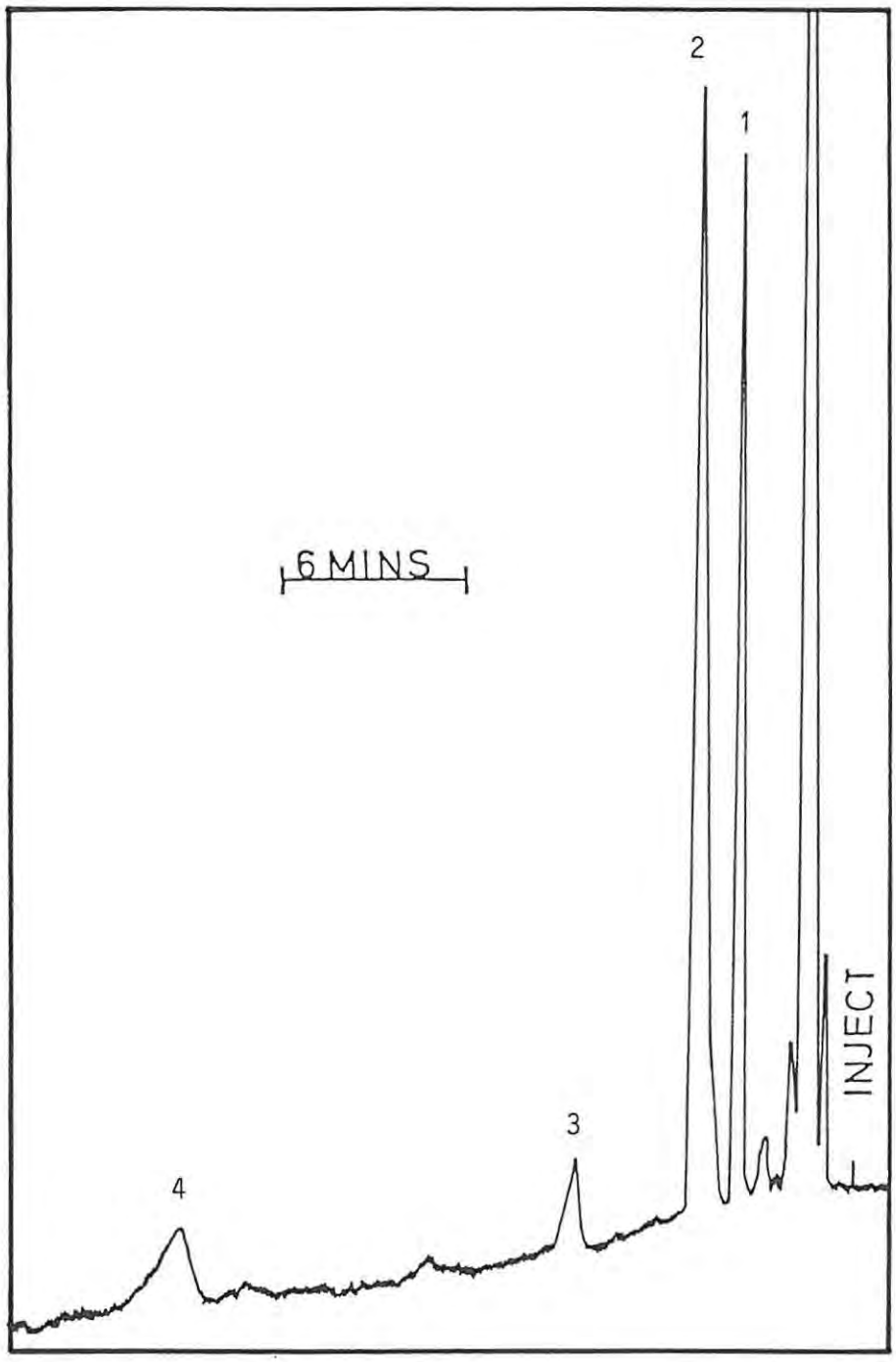


FIGURE 3.13 Chromatogram of a urine sample (1.0 mL) containing erythromycin (20.00 µg/mL) stored at 4°C for one week.
1) oleandomycin
2) erythromycin
3) degradation product - possibly anhydroerythromycin
4) degradation product

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/30) pH 7.00
INJECTION VOLUME : 3 µL

All the samples which were presumed to have degraded on storage, had small peaks of a similar retention to anhydroerythromycin (Fig.3.13). Due to the inability of the U.V. detection method to adequately detect this acid degradation product, the amount of this compound present could not be quantified. This would however be possible by performing these studies using electrochemical detection, as discussed in Chapter 4, which would lead to a better understanding of the stability of erythromycin in biological fluids.

3.5 CLINICAL STUDY

3.5.1 Experimental

A pilot trial using one volunteer was conducted to check the effectiveness of the analytical method to measure serum and urine concentrations of erythromycin base and any possible metabolites or degradation products after the oral administration of a commercially available erythromycin stearate formulation (ERYTHROCIN 500 - Abbott Laboratories).

The patient received one film coated tablet, containing 500 mg erythromycin stearate after an overnight fast, accompanied by 250 mL water immediately followed by a light breakfast. Blood samples were drawn at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10 and 12 hr after ingestion of the medication. The serum was separated by centrifugation and frozen until analyzed. Urine samples were collected during the time intervals 0-2, 2-4, 4-6, 6-8, 8-12 and 12-24 hrs and representative samples were frozen until analyzed.

3.5.2 Results and Discussion

The HPLC method was extremely suitable for the determination of erythromycin in serum and urine following a single 500 mg oral dose. The serum concentration-time profile and cumulative urinary excretion profile from this study are depicted in Figs. 3.14 and 3.15 respectively. No metabolites or degradation products were detected

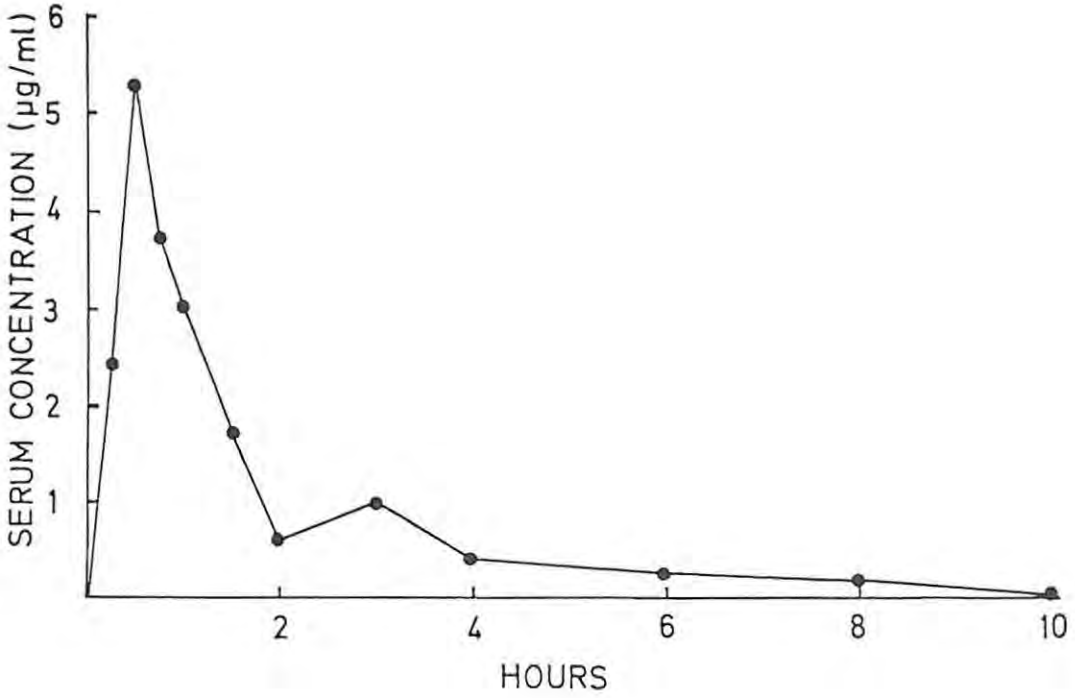


FIGURE 3.14 Serum erythromycin concentration-time profile of a human volunteer after administration of a single 500 mg erythromycin stearate tablet.

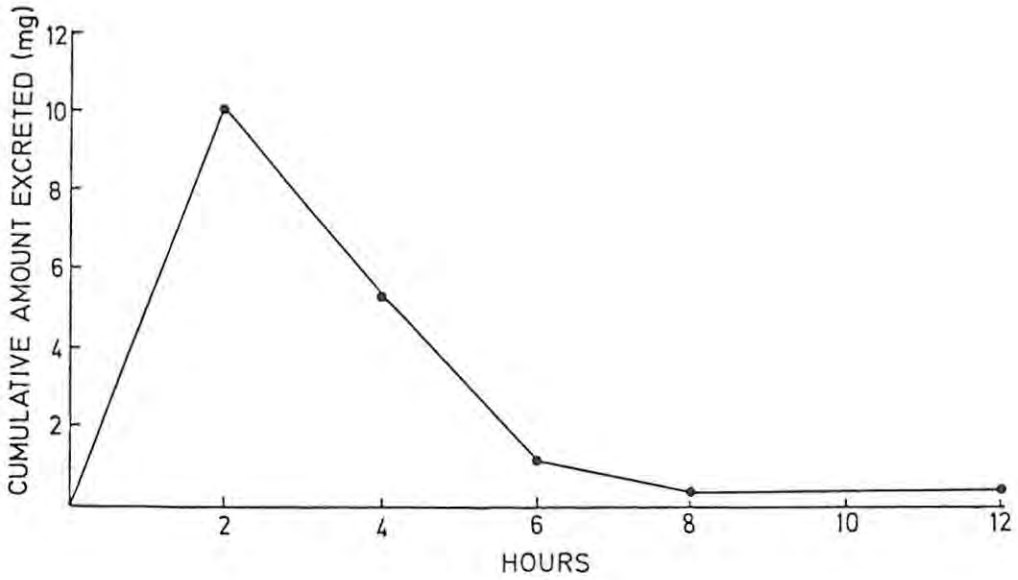


FIGURE 3.15 Cumulative urinary erythromycin concentration-time profile of a human volunteer after administration of a single 500 mg erythromycin stearate tablet.

using U.V. detection, however, subsequent analysis of the urine samples from this study (stored at -10°C) indicated the presence of high concentrations of anhydroerythromycin. It was not known whether this product had formed through the degradation of erythromycin on storage, or whether it had indeed been present on collection of the sample. Further studies in our laboratory have indicated the presence of anhydroerythromycin in urine samples on collection, after oral administration of the same dosage form as above. No attempt has been made to quantify the amount of this product excreted via this route.

The presence of anhydroerythromycin and an unknown product in serum and urine of dogs after oral administration of erythromycin has been reported using HPLC with electrochemical detection (9). A compound of similar retention characteristics to the unknown product reported in the previous study discussed (9), was reported in serum of humans after oral administration of a suspension of erythromycin (99) (Section 3.1).

In addition, des-N-methylerythromycin has been detected in the biliary fluid, intestinal contents and urine of rats (87), and erythralosamine in human serum (152). No studies have been reported to date, in which the ratios of these metabolites/degradation products in biological fluids has been studied.

CHAPTER FOUR

HPLC OF ERYTHROMYCIN WITH ELECTROCHEMICAL DETECTION

4.1 INTRODUCTION

The first practical electrochemical detector for liquid chromatography was developed for the purpose of monitoring catecholamines in brain tissue (159). Following this initial publication, high performance liquid chromatography with electrochemical detection (HPLC - EC) has become very popular for the determination of trace amounts of easily oxidizable or reducable substances, mainly due to three distinct advantages: sensitivity, selectivity and economy (160).

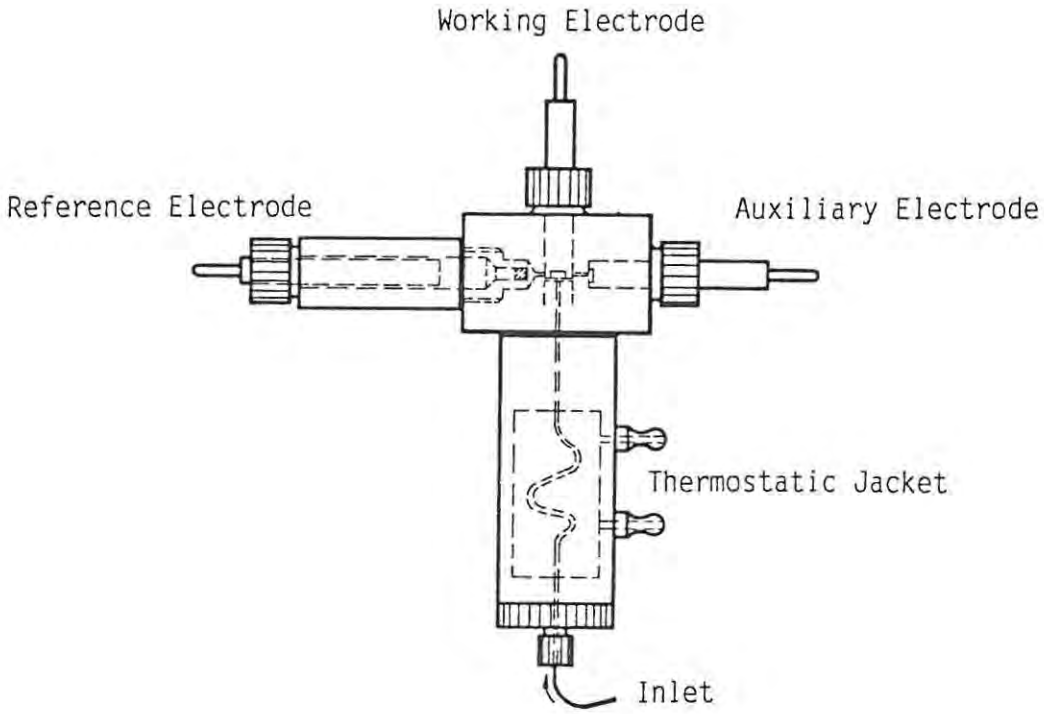
Electrochemical detection results from a current-producing reaction at an electrode surface (160). This can either be an oxidation or a reduction reaction. The general oxidation reaction for a compound XH_2 can be depicted as follows.

$$XH_2 \rightleftharpoons X + 2H^+ + 2e^- \qquad \text{(Equation 4)}$$

This reaction takes place in a low volume flow-through cell which is connected to the column outlet (Fig.4.1). The working electrode (WE) is the electrode at which the reaction takes place and is set at a constant voltage, called the electrode potential, which is compared to a stable reference electrode (RE). The current resulting from the reaction at the WE, which is proportional to the amount of the eluting compound present (160) is measured by the auxiliary electrode (AE). The sensitivity (detector current) of the instrument is governed by the amplification of this signal.

All cells operating in this fashion, with the WE held at a constant potential, are said to be amperometric. The WE typically causing the oxidation of 1-10% of the passing analyte. When this percentage reaches 100%, the electrode is said to be coulometric (160). The reaction at the WE is dependent on the electrochemical properties of the compounds of interest at the specific electrode surface utilized, in

FIGURE 4.1 Single electrode, amperometric electrochemical detector flow cell (Metrohm)



the mobile phase required for their separation (159).

As a general rule, electrochemical detection is not possible in normal phase chromatography due to the totally non-aqueous solvent systems used, whilst in reverse phase and ion-exchange chromatography the mobile phases which consist of small proportions of polar organic solvents mixed with aqueous phases containing dissolved salts, provide a suitable medium for electrochemical reactions (161).

In HPLC - EC the oxidative mode of detection is most often used (160). The presence of oxygen in the mobile phase and the sample, has proved to be the major deterrent for utilization of HPLC-EC in the reductive mode (162). In this study, only the oxidative mode was used. However, the general discussion of electrochemical detection is equally applicable to the reductive mode.

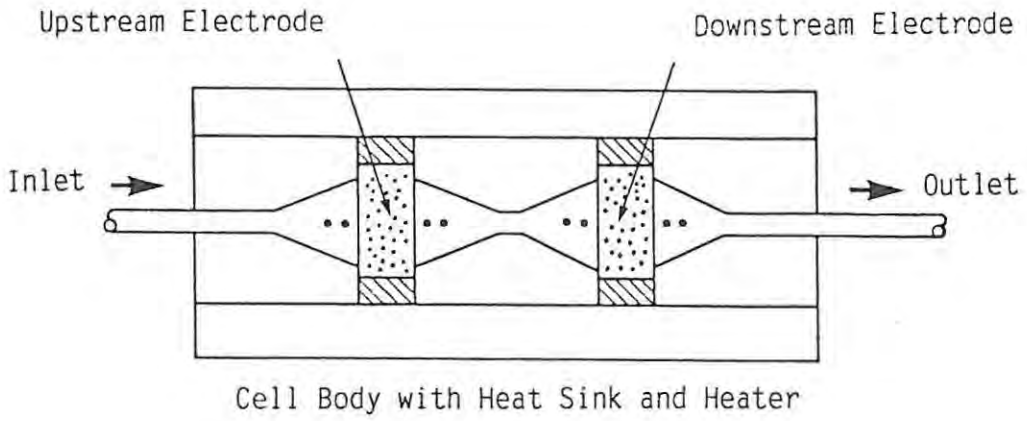
The choice of the optimum electrode potential for a compound is made from a voltammogram. This is a plot of current generated versus electrode potential applied under the conditions of analysis (163). Cyclic voltammetry, in which a constant supply of analyte is scanned over a range of electrode potentials and the resulting current recorded may be used, but in HPLC-EC, hydrodynamic voltammograms are more easily prepared (163). In the preparation of these voltammograms, equivalent amounts of analyte are successively injected into the chromatographic system at varying electrode potentials. The resulting response, measured by peak height or area, is then plotted versus the electrode potential applied. Hydrodynamic voltammograms of erythromycin and two metabolites/degradation products at glassy carbon and porous graphite electrodes are depicted in Fig.4.3. The electrode potential which results in half of the maximum oxidation, is called the half-wave potential and is used to compare the potentials at which different compounds are oxidized (163).

The optimum electrode potential is generally chosen as the lowest potential necessary to provide the desired sensitivity. This results in low background currents and hence a reduction in detector noise with a concomitant increase in selectivity and decrease in the rate of electrode contamination which is most evident at high operating potentials (160,164). Carbon electrodes are generally preferred over metallic electrodes such as gold or platinum due to their versatility, chemical inertness and large potential ranges of operation (typically -1.0 to +1.0 V) (165). In amperometric cells, glassy carbon electrodes, as used in the amperometric cell described in Fig.4.1, are most commonly employed whilst for special applications, dual glassy carbon electrode cells in various orientations have also been designed (166). In a cell which is operated on the wall-jet principle, the WE is not merely placed in the flow path of the cell but is continuously impinged upon by a stream of eluent, resulting in maximum contact between the electrode surface and reacting compounds (167).

Coulometric electrodes (Fig.4.2) are most commonly made of porous graphite or carbon composites with inert plastics through which the eluent flows, although glassy carbon and carbon paste beds have been

used (163). The large surface area available allows the reaction of 100% of the passing analyte, resulting in the maximum possible signal. This signal, however, does not result in the most favourable signal-to-noise ratios due to associated increases in background currents (160). The main advantages of coulometric electrodes are their ability to maintain suitable operation with up to 95% of their surface area contaminated, good signal stability and excellent screening efficiencies in dual electrode cells (168). The screening abilities of these electrodes are demonstrated by the operation of a dual porous graphite electrode coulometric cell, in the oxidative screen mode (Fig. 4.2). In this operation mode, the upstream electrode is set at an electrode potential just lower than the rising part of the voltammogram prepared for the compound of interest. All easily oxidized compounds and sample constituents are oxidized at this electrode and are thus not detected at the downstream electrode which is set at the optimum electrode potential for the detection of the compounds of interest. If an amperometric electrode is used as the upstream electrode the maximum screening efficiency can only be 10%.

FIGURE 4.2 Dual electrode, coulometric electrochemical detector flow cell (Coulochem)



When operating potentials are high (> + 0.8 V) a guard cell can be incorporated into the HPLC system between the solvent delivery system and the injector (115). This cell consists of a single coulometric electrode which pre-oxidizes any electroactive impurities in the mobile phase prior to chromatography, resulting in decreased baseline drift due to constant background currents (163). At these high operating potentials, electrochemical detectors are extremely sensitive to pump pulsations and contamination due to graphite impregnated teflon material used in many automatic samplers, pumps and manual injection systems (169). These effects can be greatly reduced by passivation of all equipment prior to use, which normally involves a strong acid wash, or the use of ethylenediamine tetracetic acid to remove electroactive compounds from the wetted surfaces of all chromatographic components (163). Compounds which must be analysed at these high potentials suffer a loss in detection limits due to high detector noise (160). For maximum sensitivity and decreased background currents only the highest grade chromatography solvents should be used, but even then, their electrochemical purity can not be guaranteed, as they are prepared predominantly for use with optical detectors (163) particularly at low U.V. wavelengths.

The variation in detector response, either due to reference voltage shifts or electrode contamination (161), is still a major problem in HPLC-EC, although recent advances in electrode design (170) and electrode stabilization procedures, have limited these effects considerably (99).

4.2 EXPERIMENTAL

4.2.1 Sample Preparation

Serum samples containing 1.00 µg/mL and urine containing 5.00 µg/mL erythromycin were prepared as in section 3.3.1.1. An aqueous mixture of erythromycin, anhydroerythromycin and des-N-methylerythromycin was prepared as follows:

Five milligrams of each of these compounds were dissolved in 10.0 mL water and 1.0 mL aliquots of each solution were added to an injection vial. A three microlitre injection of this mixture represented an on-column load of 0.50 µg of each compound.

4.2.2 Chromatographic Conditions

HPLC System	As in Section 2.3.2
Column	Novapak
Mobile phase	0.05 M phosphate buffer/acetonitrile (70/30), pH 7.00
Flow rate	1.0 mL/min
Pressure	1500 psi
Temperature	35°C
Detection	U.V. 200 nm (Kratos Detector) Electrochemical
Sensitivity	0,01 AUFS (U.V.)
Recorder input	10 mV

4.2.3 Instrumentation

4.2.3.1 Amperometric Electrochemical Detector

A Metrohm 656 electrochemical detector was used in combination with a Metrohm Model 641 VA current/voltage measuring device. The standard wall-jet cell consisted of a glassy carbon working electrode, a glassy carbon auxiliary electrode and a silver/silver chloride reference electrode. This detector cell, which is depicted in Fig.4.1, was placed in line after the U.V. detector.

4.2.3.2 Coulometric Electrochemical Detector

An Environmental Sciences Associates Model 5100A Coulochem dual electrode electrochemical detector fitted with a standard model 5011 analytical cell was used. This cell consisted of two high surface area

porous graphite coulometric electrodes and was operated either as a conventional single electrode cell or in the screen mode using both electrodes. The detector was placed in line after the U.V. detector, and the cell is depicted in Fig.4.2. During the initial studies, a model 5020 guard cell operated at a potential of + 1.0 V was used.

4.2.4 Construction of Voltammograms

Hydrodynamic voltammograms for both electrochemical detectors were constructed as follows:

The detector was set at a low electrode potential (+ 0.5 V) and the chromatographic system left to stabilize under normal operating conditions. Three microlitres of a mixture of erythromycin, anhydroerythromycin and des-N-methylerythromycin were injected onto the column. The peak heights of each compound were converted to a value in microamps by simple proportion. This procedure was continuously repeated after the electrode potential had been increased by 0.05 V increments each time, with sufficient time being allowed for electrode stabilization.

4.2.5 Extraction Procedures

4.2.5.1 Solid Phase Extraction of Serum and Urine

One millilitre samples of urine and 2.0 mL samples of serum were extracted as previously described (Section 3.3.1.3 and 3.4.1.3). Five microlitre aliquots were injected onto the column.

4.2.5.2 Liquid - Liquid Extraction of Serum

The extraction method of Chen and Chiou (9) was used, which was similar to that reported by Duthu (99), for the extraction of erythromycin from serum for HPLC-EC analysis.

One millilitre of serum and 100 µL of saturated sodium carbonate were vortexed for 15 sec. Two millilitres of ether were added, the tube

stoppered, vortexed for 1 min and centrifuged for 5 min at 1 600xg. The ether layer was then transferred to a 2 mL tapered collection tube and evaporated to dryness under vacuum in a rotary vacuum centrifuge. The residue was reconstituted with 50.0 µL of mobile phase and centrifuged. Approximately thirty microlitres were transferred to a WISP limited volume insert of which 10.0-20.0 µL were injected onto the column.

4.2.5.3 Liquid - Liquid extraction of Urine

The extraction method of Chen and Chiou (9) for the extraction of erythromycin from urine for HPLC-EC was used.

Two hundred microlitres of acetonitrile were added to 100 µL urine. The tube was then vortexed for 30 sec and centrifuged at 1 600xg for 5 min. One hundred microlitres were transferred to a WISP limited volume insert of which 10.0-20.0 µL were injected onto the column.

4.3 RESULTS AND DISCUSSION

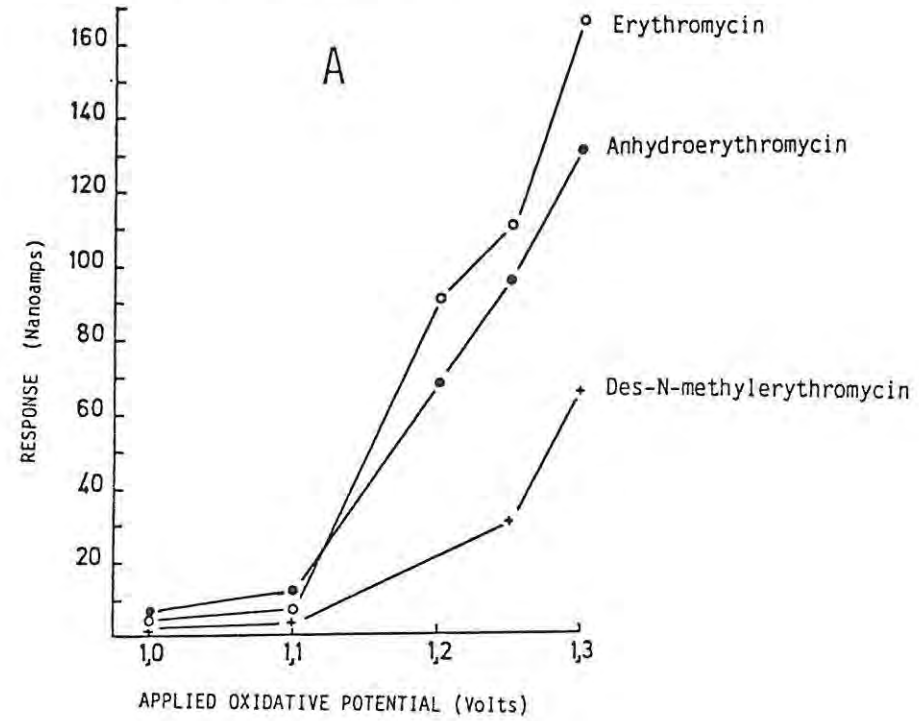
4.3.1 Selection of Electrode Potentials

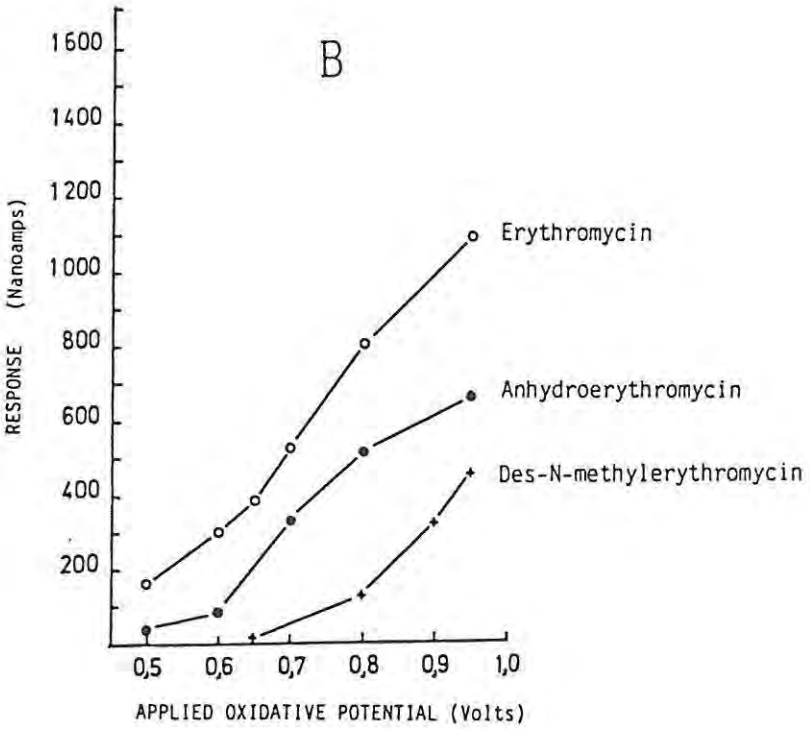
Erythromycin has been analyzed by HPLC-EC at electrode potentials above +0.8 V (9,99). Under the conditions of this study, similar electrode potentials were necessary (Fig. 4.3). From the voltammograms prepared it can be seen that erythromycin has a higher half-wave potential (+1.15 V) at the glassy carbon electrode than at a porous graphite electrode (+0.7 V).

For the Metrohm detector, an electrode potential of +1.15 V was found to yield the best compromise between detector response and baseline noise. For the Coulochem detector operated in the single electrode mode +0.90 V was chosen as the optimum electrode potential whilst in the oxidative screen mode, electrode potentials of +0.70 V for the upstream electrode and +0.90 V for the downstream electrode were selected. These potentials were the best compromise between efficient screening and detector sensitivity.

FIGURE 4.3 Hydrodynamic voltammograms of erythromycin, des-N-methylerythromycin and anhydroerythromycin using:

(A) Glassy carbon electrode (Metrohm)
(B) Porous graphite electrode (Coulochem)





From Fig.4.3 it can be seen that both des-N-methylerythromycin and anhydroerythromycin have higher half-wave potentials than erythromycin. At the electrode potentials chosen for the analysis of erythromycin, des-N-methylerythromycin was not easily detectable whereas anhydroerythromycin was.

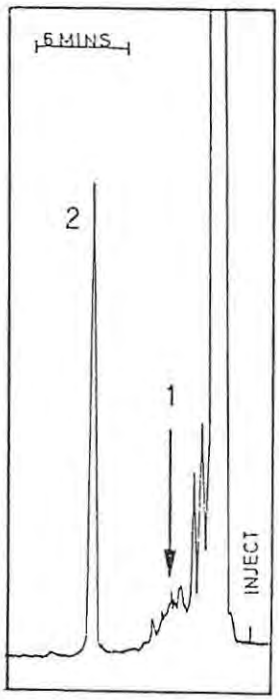
The inability to detect des-N-methylerythromycin by HPLC-EC has previously been reported by Duthu (99). Chen and Chiou (9), however, stated that this metabolite could be detected under their operating conditions (oxidative screen mode at +0.70 V for the upstream electrode and +0.90 V for the downstream electrode) although no chromatograms were shown, nor was the sensitivity of detection given.

An electrochemical pretreatment procedure for glassy carbon electrodes has been reported by Ravichandran and Baldwin (171). This procedure involves sequential application of brief positive and negative pulses to the polished electrode which lowers the required electrode potential compared to that needed at conventional untreated glassy carbon electrodes. This procedure may be of use in this study as increased selectivity is obtained due to the lower electrode potentials used, with the added advantage of increased sensitivity.

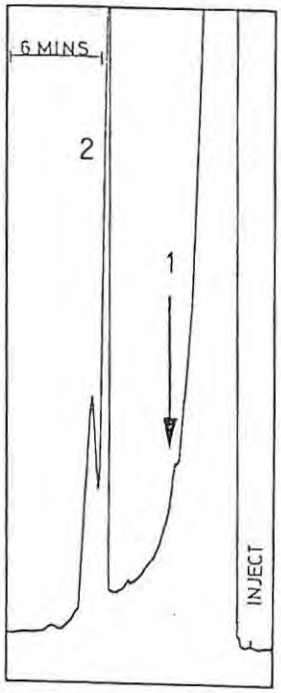
4.3.2 Sensitivity of Electrochemical Detection: Comparison to U.V. Detection at 200 nm

When operated at the electrode potentials discussed in section 4.3.1, the Metrohm amperometric detector was approximately five times more sensitive than the U.V. detector when monitoring erythromycin (Fig.4.5). The detector current (sensitivity) when set at 100 nA provided the best compromise between sensitivity and baseline noise or drift.

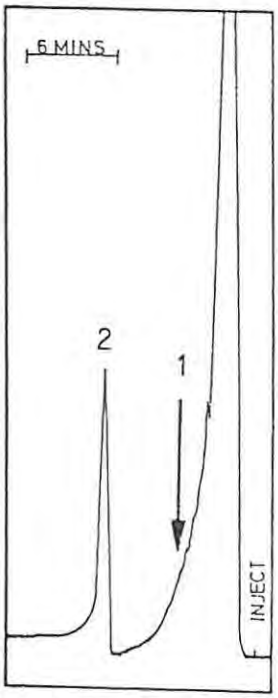
The Coulochem detector operated in the single electrode mode at +0.90 V was found to generate high background currents allowing a maximum sensitivity setting of 1 x 30. This resulted in a smooth baseline, but decreased the sensitivity of the detector considerably to about twice that of the U.V. detector (Fig. 4.5).



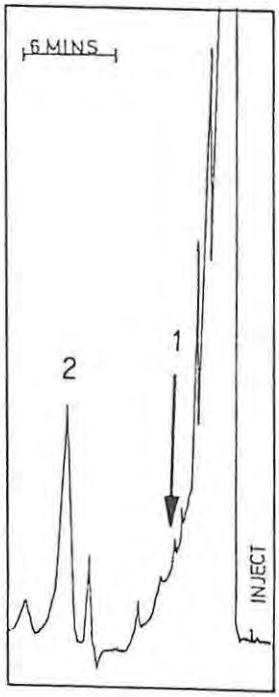
U.V. DETECTOR 200 nm 0,01 AUFS



COULOCHEM ELECTROCHEMICAL DETECTOR
OXIDATIVE SCREEN MODE
SENSITIVITY SETTING 10 x 10



COULOCHEM ELECTROCHEMICAL DETECTOR
SINGLE ELECTRODE
SENSITIVITY SETTING 10 x 3



METROHM ELECTROCHEMICAL DETECTOR
SINGLE ELECTRODE
DETECTOR CURRENT 100 nA

FIGURE 4.4 Blank serum extracts (2.0 mL) extracted using the method of Chen and Chiou (9), monitored by four different detection systems. (1) Expected retention of erythromycin
(2) Unidentified peak

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/30), pH 7.00

The Coulochem detector operated in the oxidative screen mode as discussed in Section 4.3.1, generated far lower background currents, enabling the sensitivity setting to be increased to 10 x 10 or even 15 x 10. This resulted in an approximate ten fold increase in sensitivity over the U.V. detector (Fig. 4.7).

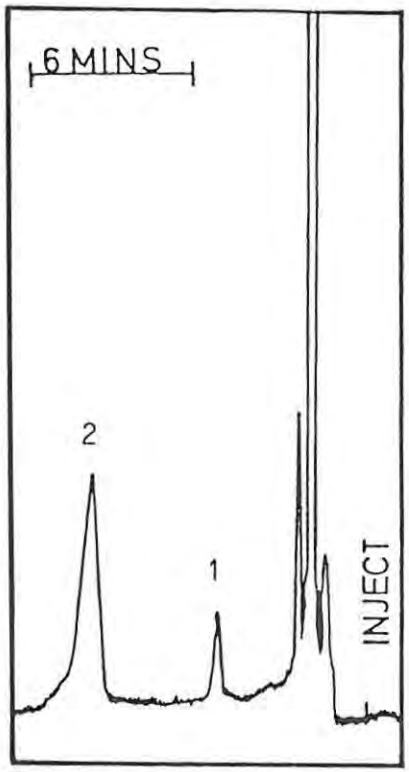
4.3.3 Liquid - Liquid extraction of Serum and Urine

Chromatograms of blank serum samples (2.0 mL) extracted using the liquid-liquid procedure described (9) and monitored by electrochemical and U.V. detection are depicted in Fig 4.4. From these chromatograms it can be seen, that the high levels of interfering compounds would inhibit the quantification of erythromycin, irrespective of the detection method. Chen and Chiou (9) overcame this problem by increasing the retention of erythromycin to 9.6 min in order to separate the drug from interfering compounds. Similar results were obtained for the analysis of urine samples extracted by this procedure.

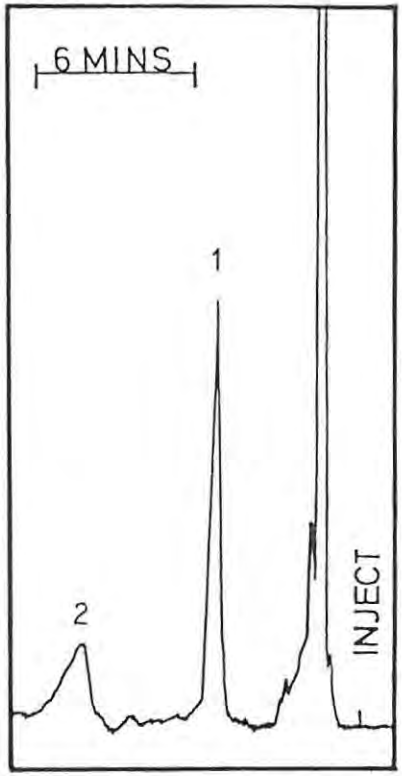
4.3.4 Solid Phase extraction of Serum and Urine

Injection of serum or urine samples extracted by the solid phase technique resulted in chromatograms similar to those obtained when using U.V. detection at 200 nm but with increased sensitivity, as discussed in Section 4.3.2. Chromatograms of solid phase serum extracts containing 1.00 µg/mL erythromycin monitored by U.V., amperometric electrochemical detection at a single glassy carbon electrode and coulometric detection at a single porous graphite electrode, are depicted in Fig. 4.5. The lack of interference from co-eluting serum components is shown in all three chromatograms, allowing the unhindered quantification of erythromycin. The solid phase extraction method has been shown to not only be suitable for U.V. detection at low wavelengths, but also for electrochemical detection at high electrode potentials using even single glassy carbon electrode amperometric cells.

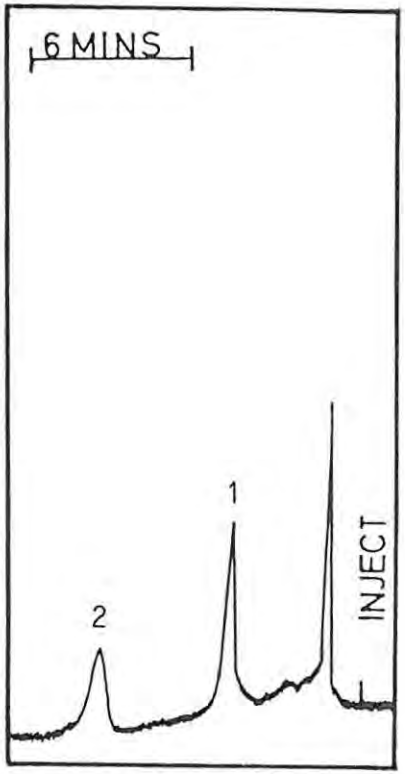
The solid phase extraction system used with the Coulochem electrochemical detector resulted in chromatograms relatively free from



U.V. DETECTOR 200 nm 0,01 AUFS



METROHM ELECTROCHEMICAL DETECTOR
SINGLE ELECTRODE
DETECTOR CURRENT 100 nA



COULOCHEM ELECTROCHEMICAL DETECTOR
SINGLE ELECTRODE
SENSITIVITY SETTING 10 x 3

FIGURE 4.5 Serum samples containing erythromycin (1.00µg/mL) extracted using the solid phase method described, monitored by U.V. and single electrode electrochemical detection systems
(1) Erythromycin
(2) Unidentified peak

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/30), pH 7.00

co-eluting serum and urine components with an increased sensitivity for erythromycin over the U.V. detection method.

For the analysis of urine samples, in which erythromycin concentrations are seldom lower than 1.00µg/mL the Coulochem detector was operated at low sensitivity settings (15 x 1 or 30 x 1). Under these conditions, comparable sensitivities for erythromycin were obtained for both the U.V. and electrochemical detectors. Chromatograms from the electrochemical detector contained far less interference from co-eluting urine components than those from the U.V. detector (Fig.4.6).

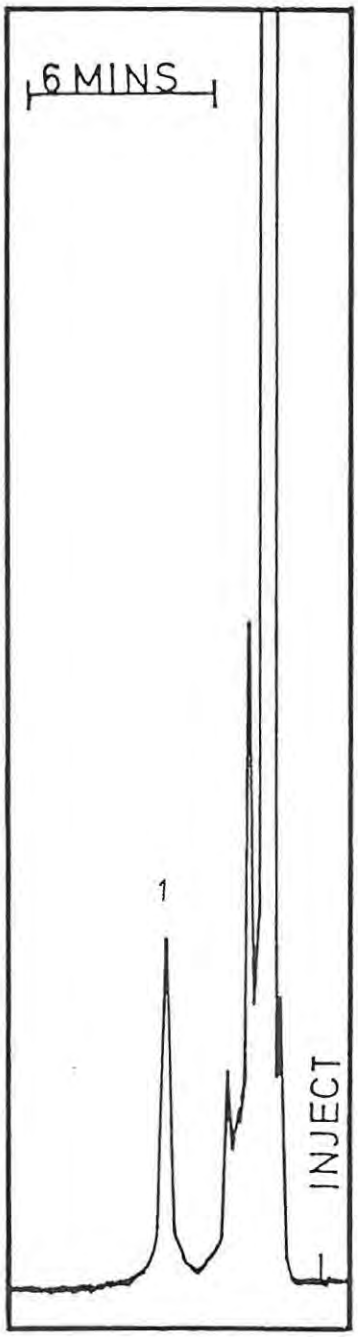
For the analysis of erythromycin in serum using the solid phase extraction system the increased sensitivity of the Coulochem electrochemical detector proved most useful. During these analyses the sensitivity of this detector was increased to the maximum setting which still resulted in a smooth baseline (10 x 10 or 15 x 10). An approximate 10 fold increase in sensitivity for erythromycin over the U.V. method was obtained under these conditions without any dramatic increase in the interference from co-eluting serum components (Fig.4.7).

4.3.5 Detection of Metabolites/Degradation Products

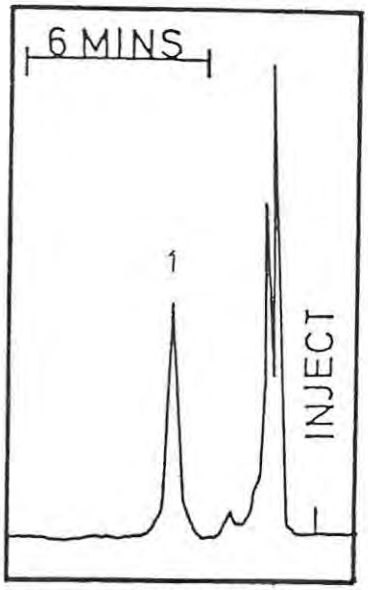
As previously discussed, anhydroerythromycin was readily detectable by electrochemical detection at the electrode potentials used for the analysis of erythromycin, whilst des-N-methylerythromycin was not. The reverse was true for these compounds when using U.V. detection. This is clearly depicted in Fig.4.8.

4.3.6 Comparison of Electrochemical and U.V. Detection

In the HPLC analysis of erythromycin, electrochemical detection was definitely more sensitive than U.V. detection. The liquid - liquid extractions used by other workers in this field (9,99) could not be successfully adapted to the chromatographic conditions of this study, irrespective of the detection method used. The solid phase extraction procedure developed for the U.V. assay, however, has been



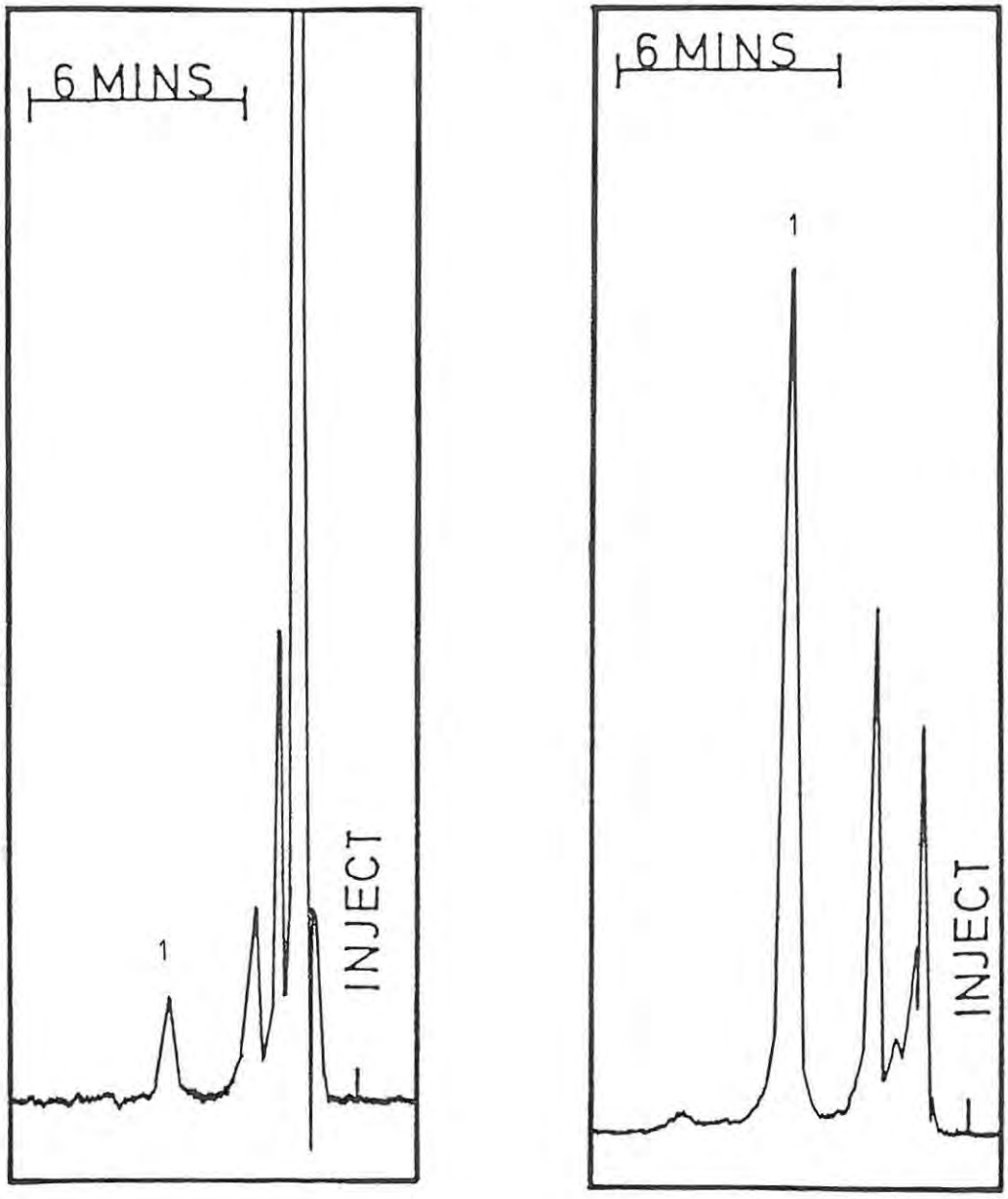
U.V. DETECTOR 200nm
SENSITIVITY 0.01 AUFS



COULOCHEM ELECTROCHEMICAL DETECTOR
OXIDATIVE SCREEN MODE
SENSITIVITY SETTING 1x30

FIGURE 4.6 Urine samples containing erythromycin (1) (5.00 µg/mL) extracted using the solid phase method described, monitored by U.V. and dual electrode electrochemical detection in the oxidative screen mode, at conditions of comparable sensitivity

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/30), pH 7.00



U.V. DETECTOR 200nm
SENSITIVITY 0.01 AUFS

COULOCHEM ELECTROCHEMICAL DETECTOR
OXIDATIVE SCREEN MODE
SENSITIVITY SETTING 15x10

FIGURE 4.7 Serum samples containing erythromycin (1) (1.00 µg/mL) extracted using the solid phase method described, monitored by U.V. and dual electrode electrochemical detection in the oxidative screen mode, at conditions of maximum sensitivity

COLUMN : Novapak
MOBILE PHASE : 0.05 M phosphate buffer/acetonitrile (70/30), pH 7.00

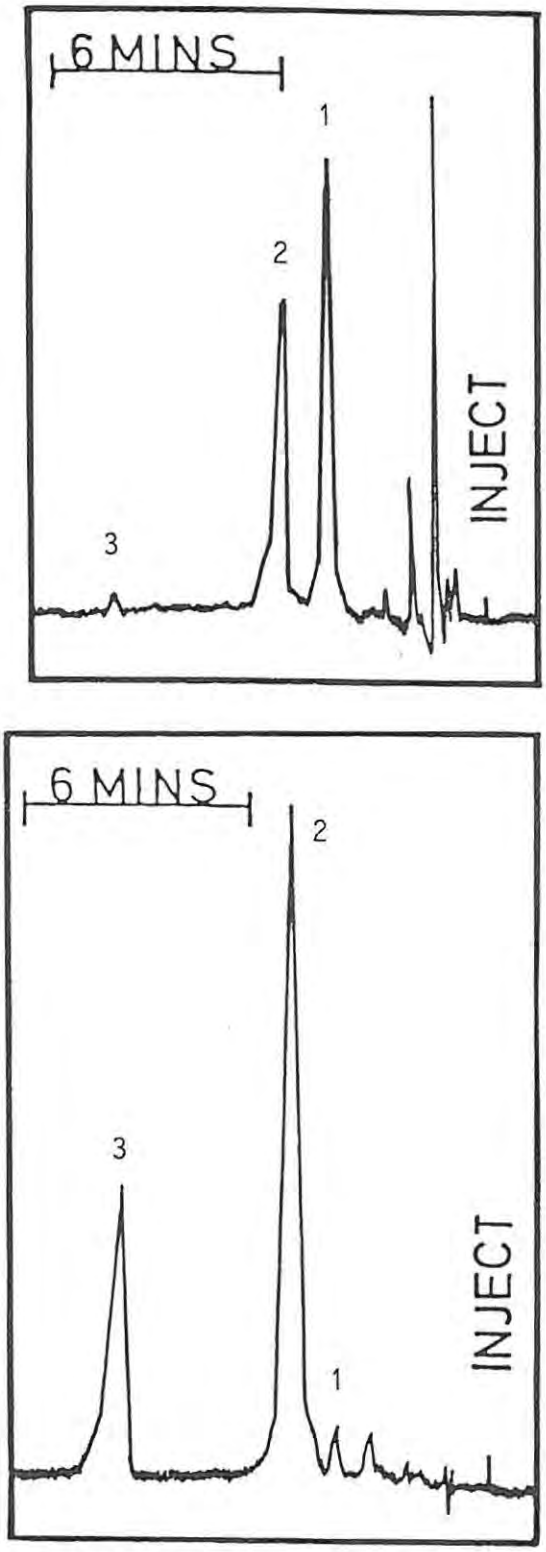


FIGURE 4.8 Chromatogram of des-N-methylerythromycin (1), erythromycin (2) and anhydroerythromycin (3) monitored by:
(A) U.V. detection at 200 nm, 0.01 AUFS
(B) Electrochemical detection in the oxidative screen mode. Sensitivity setting 1x60

shown to be entirely suitable when coupled with electrochemical detection. The use of simple single electrode electrochemical detectors is now possible, unlike previously reported (9,99), due to the effectiveness of this novel extraction procedure.

The utility of the U.V. detector to detect des-N-methylerythromycin and not anhydroerythromycin with the reverse being true for electrochemical detection, shows the potential advantages of using both detection methods in combination, for future pharmacokinetic and metabolic studies.

Despite adherence to prescribed procedures and precautions necessary for optimal use of electrochemical detectors (9,99,163), changes in day-to-day response were clearly evident, which is in distinct contrast to the stable and reproducible response associated with U.V. detectors used in HPLC. The stability of the electrochemical signal over a lengthy period of analyses and the linearity of response over a wide concentration range using the conditions of this assay, have not yet been shown.

SUMMARY AND PROPOSED AREAS OF FUTURE RESEARCH

The HPLC method described using U.V. detection has been shown to be specific, precise and sufficiently sensitive to monitor the concentrations of erythromycin in serum and urine after the administration of oral dosage forms.

The solid phase extraction system developed for the analysis of erythromycin in serum and urine using low wavelength U.V. detection has also been shown to be applicable when using a simple and inexpensive electrochemical detector. The combination of the extraction procedure together with a sophisticated dual-electrode electrochemical detector operated in the oxidative screen mode resulted in cleaner sample extracts than those obtained by other workers (9,99) using this form of detection and also resulted in increased sensitivities over the U.V. detection method described..

The utility of the HPLC-UV method to detect des-N-methylerythromycin should provide useful information relating to both the rate of formation and rate of excretion of this metabolite, particularly if this method could be adapted to the analysis of biliary fluids.

The reported detection of anhydroerythromycin in serum and urine of dogs after oral administration of erythromycin (9) and the results of preliminary studies in our laboratory in which quantifiable concentrations of this degradation product were found in urine after the administration of an oral erythromycin stearate formulation to humans (using HPLC-EC), should yield valuable data relating to the absorption/transformation of erythromycin in man.

A further analytical method may be developed for the analysis of the esters of erythromycin in serum and urine which would enable the comparison to be made between the concentrations of active erythromycin base and inactive ester during pharmacokinetic and comparative bio-availability studies.

As previously discussed in section 1.4 numerous studies have been performed to elucidate the pharmacokinetics of erythromycin in man after both i.v. and oral administration. There is still much disagreement over many aspects of these studies. However, the basic pharmacokinetic parameters reported are largely similar. All these studies have been performed using microbiological assay techniques which are only able to quantify the concentrations of microbiologically active erythromycin present and are therefore unable to distinguish between concentrations of erythromycin and any inactive or relatively inactive metabolites and/or degradation products present in serum and urine. The present HPLC method described which is capable of selective quantification of these compounds, should provide a powerful analytical tool for the study of various aspects of the absorption and disposition characteristics of erythromycin in man.

Various aspects of previous studies may now be re-examined using this analytical procedure, which should lead to a more comprehensive characterization of the pharmacokinetics of this antibiotic. These areas of future research include:

1) Re-investigation of the pharmacokinetics of erythromycin after i.v. dosage and the reported evidence of non-linear elimination of the drug.
2) Re-investigation of the pharmacokinetics of erythromycin after single and multiple dose oral studies with particular attention to differences in the pharmacokinetics of the various derivatives of erythromycin.
3) Studies on the comparative bio-availability of erythromycin and its derivatives by comparison of serum and urine concentrations of both active drug and inactive ester as well as any metabolites/ degradation products present.
4) Re-investigation of the reported differences between the bio-availability of erythromycin after single and multiple doses, with particular attention being paid to the concentrations of any metabolites/degradation products present after the two dosing schedules.

5) Studies on intra-subject variations in erythromycin pharmacokinetics after oral administration of single and multiple doses.
6) Studies of the effects of possible entero-hepatic recycling and active secretion of the drug into the jejunum.
7) Re-investigation of the work of Chelvan *et al.*(89) on the biliary excretion of erythromycin, with the added advantage of being able to quantify the amount of des-N-methylerythromycin excreted via this route.
8) Studies on the effect of concurrent food and water administration the bio-availability of erythromycin and its derivatives using the concentrations of anhydroerythromycin in serum and/or urine, as an indicator of the extent of gastric degradation of the drug.
9) Studies on the metabolism of erythromycin in tissue cultures of liver and gut-mucosal cells, as well as the metabolism due to commensal gut flora.
10) Further studies on the effects of erythromycin on theophylline kinetics and *vice versa*, with particular attention to any possible changes in the ratios of metabolites in serum and/or urine, occuring after co-administration of the two drugs.

The HPLC method described is ideally suited for further studies on the stability of erythromycin in serum and urine as well as *in vitro* studies on the rates of degradation of erythromycin and its derivatives in both acidic and basic media. Studies on the stability of erythromycin during the incubation period associated with microbiological assay methods are also envisaged.

BIBLIOGRAPHY

1. McGuire J.M., Burch J.L., Anderson R.C., Baaz H.E., Flynn H.E., Powell H.M. and Smith J.W., Antibiot. Chemother., 2, 281 (1952).
2. Tsuji K. and Robertson J.H., Anal. Chem., 43, 818 (1971).
3. "Antibiotics", edited by Hash J.H., Methods in Enzymology Vol. XL111, Academic Press, 1975, p.34-52.
4. Hung P.P., Marks C.L. and Tardrew P.L., Appl. Microbiol., 13, 216 (1965).
5. Allen P.V., Rahn P.D., Sarapu A.C., and Vanderwielen A.J., J. Pharm. Sci., 67, 1087 (1978).
6. Martindale, "The Extra Pharmacopoeia", Pharm. Press, London, 28th edition, 1982, p. 1158-1162.
7. Koch W.L., in "Analytical Profiles of Drug Substances", edited by Florey K., Academic Press Inc., New York, Vol. 8, 1979, p.159.
8. Kurath P., Jones P.H., Egan R.S. and Perun T.J., Experientia, 27, 362 (1971).
9. Chen M. and Chiou W.L., J. Chromatogr., 278, 91 (1983).
10. Josselyn L.E. and Sylvester J.C., Antibiot. Chemother., 3, 63 (1953).
11. Fraser D.G., Am. J. Hosp. Pharm., 37, 1199 (1980).
12. Meade R.H., Am. J. Hosp. Pharm., 36, 1185 (1979).
13. Oleinick N.L. and Corcoran J.W., J. Biol. Chem., 244, 727 (1969).
14. Nicholas P., N. Y. State J. Med., 77 , 2088 (1977).
15. Straughan J.L., S. Afr. med.J., 53, 527 (1978).
16. Majer J., Antimicrob. Agents Chemother., 19, 628 (1981).
17. Garrod L.P., Lambert H.P., O'Grady F and Waterworth P.M., "Antibiotic and Chemotherapy", Churchill Livingstone, London, 5th edition, 1981, Chap. 8, p.183-190.
18. "The Pharmacological Basis of Therapeutics", edited by Goodman Gilman A., Goodman S. and Gilman A., The MacMillan Co., N.Y., 6th edition 1980, Chap. 54, p.1222-1225.
19. Inman W.H.W. and Rawson N.S.B., Br. Med. J., 286, 1954 (1983).
20. Grönroos J.A., Saarimaa H.A. and Kalliomäki J.L., Curr. Ther. Res., 9, 589 (1967).

21. Zimmerman H.J., Kendler J. and Libber S., Proc. Soc. Exp. Biol. Med., 144, 759 (1973).
22. D'Arcy P.F., Pharm. Int., 5, 30 (1984).
23. Jonkman J.H.G. and Upton R.A., Clin.Pharmacokinet., 9, 309 (1984).
24. Straughan J.L. and Anderson R., S. Afr. med. J., 64, 197 (1983).
25. Iliopoulou A., Aldhous M.E., Johnston A. and Turner P., Br. J. Clin. Pharmac., 14, 495 (1982).
26. Wong Y.Y., Ludden T.M. and Bell R.D., Clin. Pharmacol. Ther., 33, 460 (1983).
27. Husserl F.E., Arch. Intern. Med., 143, 1831 (1983).
28. Banck G. and Forsgren A., Antimicrob. Agents Chemother., 16, 554 (1979).
29. Nightingale C.H., J. Am. Pharm. Assoc., 16, 203 (1976).
30. Chun A.H.C. and Seitz J.A., J. Am. Pharm. Assoc., NS14, 407 (1974).
31. DiSanto A.R. and Chodos D.J., Antimicrob. Agents Chemother., 20, 190 (1981).
32. Parsons R.L., Paddock M. and Hossack A., Infection, 5, Suppl.1, 23 (1977).
33. Yakatan G.J., Poynor W.J., Breeding S.A., Lankford C.E., Dighe S.V., Martin A.N. and Doluisio J.T., J. Clin. Pharmacol., 20, 625 (1980).
34. Chun A.H.C. and Seitz J.A., Infection, 5, Suppl. 1, 14 (1977).
35. Pozzi E., Luisetti M. and Coppi G., Curr. Ther. Res., 33, 681 (1983).
36. Pozzi E., Ferrara A., Sardi A., Berti M.A. and Coppi G., Curr. Ther. Res., 31, 530 (1982).
37. Smith J.W., Dyke R.W. and Griffith R.S., J.A.M.A., 151, 805 (1953).
38. Boggiano B.G. and Gleeson M., J. Pharm. Sci., 65, 497 (1976).
39. Bell S.M., Med. J. Aust., 2, 1280 (1971).
40. Posti J. and Salonen M., Int. J. Pharmaceutics., 17, 225 (1983).
41. McDonald P.J., Mather L.E. and Story M.J., J. Clin. Pharmacol., 17, 601 (1977).
42. Welling P.G., Postgrad. Med., 62, 73 (1977).
43. Welling P.G., Huang H., Hewitt P.F., Lyons L.L., J. Pharm. Sci., 67, 764 (1978).

44. Malmborg A.S., J. Antimicrob. Chemother., 5, 591 (1979).
45. Bechtol L.D., Timothy Bessent C. and Perkal M.B., Curr. Ther. Res., 25, 618 (1979).
46. Rutland J., Berend N. and Marlin G.E., Br. J. Clin. Pharmac., 8, 343 (1979).
47. Graffner C., Josefsson K. and Stockman O., Abstracts from the 2nd International Conference on Drug Absorption, Edinburgh, 1983, p.59.
48. Digranes A., Josefsson K. and Schreiner A., Curr. Ther. Res., 35, 313 (1984).
49. Hirsch H.A. and Finland M., Am. J. Med. Sci., 237, 693 (1959).
50. Clapper W.E., Mostyn M. and Meade G.H., A.M.and C.T., 7, 91 (1960).
51. Triggs E.J. and Ashley J.J., Med. J. Aust., 2, 121 (1978).
52. Mäntylä R., Ailio A., Allonen H. and Kanto J., Ann. Clin. Res., 10, 258 (1978).
53. Welling P.G., Elliott R.L., Pitterle M.E., Corrick-West H.P., Lyons L.L., J. Pharm. Sci., 68, 150, (1979).
54. Berend N., Rutland J. and Marlin G.E., Curr. Med. Res. Opin., 6, 118 (1979).
55. Malmborg A.S., Curr. Ther. Res., 27, 733 (1980).
56. Clayton D. and Leslie A., J. Int. Med. Res., 9, 470 (1981).
57. Yakatan G.J., Poynor W.J., Harris R.G., Martin A., Leonard R.G., Briggs A.H. and Doluisio J.T., J. Pharmacokinet. Biopharm., 7, 355 (1979).
58. Kahela P. and Järvensivu P., Abstracts from the 2nd International Conference on Drug Absorption, Edinburgh, 1983, p.69.
59. Triggs E. and Neaverson M.A., Med. J. Aust., 2, 344 (1973).
60. Josefsson K., Bergan T. and Magni L., Br. J. clin. Pharmac., 13, 685 (1982).
61. Bell S.M. and Lake B., Med. J. Aust., 1, 1152 (1967).
62. Tardrew P.L., Mao J.C. and Kenney D., Appl. Microbiol., 18, 159 (1969).
63. Stephens V.C., Pugh C.T., Davis N.E., Hoehn M.M., Ralston S., Sparks M.C. and Thompkins., J. Antibiot., 22, 551 (1969).
64. Griffith R.S. and Black H.R., Clin. Med., 76, 16 (1969).

65. Ginsburg C.M., McCracken G.H. and Culbertson M.C., J. Pediatrics, 89, 1011 (1976).
66. Hammond J.B. and Griffith R.S., Clin. Pharmac. Ther., 2, 308 (1961).
67. Griffith R.S. and Black H.R., Antibiot. Chemother. 12, 398 (1961).
68. Hall G.A., Roberts C.E., Perry D.M. and Kirby W.M.M., A.M. and C.T., 7, 231 (1960).
69. Mänisto P., Tuomisto J. and Räsänen R., Arzneim.-Forsch. 25, 1828 (1975).
70. Welling P.G., J. Pharmacokinet. Biopharm., 5, 321, (1977).
71. Bechtol L.D., Stephens V.C., Pugh C.T., Perkal M.B. and Coletta P.A., Curr. Ther. Res., 20, 610 (1976).
72. DiSanto A.R., Tserng K.Y., Chodos D.J., DeSante K.A., Albert K.S. and Wagner J.G., J. Clin. Pharmacol., 20, 437 (1980).
73. Bechtol L.D., DeSante K.A., Foglesong M.A., Spradlin C.T. and Winely C.L., Curr. Ther. Res., 29, 52 (1981).
74. Thompson P.J., Burgess K.R. and Marlin G.E., Antimicrob. Agents Chemother., 18, 829 (1980).
75. Kroboth P.D., Brown A., Yon J.A.I., Kroboth F.J. and Juhl R.P., Antimicrob. Agents Chemother., 21, 135 (1982).
76. Colburn W.A., DiSanto A.R. and Gibaldi M., J. Clin. Pharmacol., 17, 592 (1977).
77. Josefsson K., Steinbakk M., Bergan T., Midtvedt T. and Magni L., Chemother., 28, 176 (1982).
78. Mather L.E., Austin K.L., Philpot C.R. and McDonald P.J., Br.J. clin. Pharmac., 12, 131 (1981).
79. Wilson J.T. and van Boxtel C.J., Antibiot. Chemother., 25, 181 (1978).
80. Welling P.G. and Craig W.A., J. Pharm. Sci., 67, 1057 (1978).
81. Wollmer P., Pride N.B., Rhodes C.G., Sanders A., Pike V.W., Palmer A.J., Silvester D.J. and Liss R.H., Lancet, 2 , 1361 (1982).
82. Wiegand R.G., Chun A.H.C., J.Pharm. Sci., 61, 425 (1972).
83. Gordon R.C., Regamey C. and Kirby W.M.M., J. Pharm. Sci., 62, 1074 (1973).

84. Aldhous M.E., Drew P.J.T., Chaput De Saintonge A., Iliopoulou A. and Turner P., Proceedings of the B.P.S., 8th-10th September, 1982, p147.
85. Mao J.C.H., and Tardrew P.L., Biochem. Pharmac., 14, 1049 (1965).
86. Lee C., Anderson R.C. and Chen K.K., J. Pharmac. Exp. Ther., 117, 265 (1956).
87. Lee C., Anderson R.C. and Chen K.K., J. Pharmacol. Exp. Ther., 117, 274 (1956).
88. Danan G., Descatoire V. and Pessayre D., J. Pharmacol. Exp. Ther., 218, 509 (1981).
89. Chelvan P., Hamilton-Miller J.M.T. and Brumfitt W., Br. J. Clin. Pharmac., 8, 233 (1979).
90. Philipson A., Sabath L.D. and Charles D., Clin. Pharmacol. Therap., 19, 68 (1975).
91. Sabath L.D., Gerstein D.A., Loder P.B. and Finland M., J.Lab. Clin. Med., 72, 916 (1968).
92. Lee C., Anderson R.C. and Chen K.K., Proc. Soc. Exp. Biol. Med., 88, 584 (1955).
93. Holland D.R. and Quay J.F., J.Pharm. Sci., 65, 417 (1976).
94. Austin K.L., Mather L.E., Philpot C.R. and McDonald P.J., Br. J. clin. Pharmac., 10, 273 (1980).
95. Houin G., Tillement J.P., Lhoste I., Rapin M., Soussy C.J. and Duval J., J.Int. Med. Res., 8, 9 (1980).
96. DiSanto A., Chodos D. and Halberg F., Chronobiologica, 2, Suppl.1, 17 (1975).
97. Bergan T. and Øydvin B., Pharmacology, 7, 36 (1972).
98. Benet L.Z., in "Pharmacokinetics During Drug Development: Data Analysis and Evaluation Techniques", edited by Bozler G. and Van Rossum J.M., Gustav Fischer Verlag, Stuttgart, 1981, p.114.
99. Duthu G.S., J. Liq. Chromatogr., 7, 1023 (1984).
100. Hadden N., in "Basic Liquid Chromatography", Varian Aerograph, USA, 1971, Chap.1, p. 1-1.
101. Bakalyar S.R., in "Liquid Chromatography in Clinical Analysis", edited by Kabra P.M. and Marton L.J., Humana Press Inc., New Jersey, 1981, Chap.1, p. 3-19.

102. Horváth C., in "High-Performance Liquid Chromatography: Advances and Perspectives", edited by Horváth C., Academic Press, New York, Vol. 2, 1980, Conspectus, p. IX.
103. Melander W.R. and Horváth C., in "High-Performance Liquid Chromatography: Advances and Perspectives", edited by Horváth C., Academic Press, New York, Vol. 2, 1980, Chap. 3, p. 114-303.
104. Product Information, Waters Associates, Milford, Mass., 1983.
105. Hamilton R.J. and Sewell R.A., " Introduction to High Performance Liquid Chromatography", Chapman and Hall, U.S.A., 2nd Edition, 1982.
106. Karger B.L., Le Page J.N. and Tanaka N., in "High-Performance Liquid Chromatography: Advances and Perspectives", edited by Horváth C., Academic Press, New York, Vol. 1, 1980, Chap. 3, p.113-200.
107. Otto M. and Wegscheider W., J. Liq. Chromatogr., 6, 685 (1983).
108. Horváth C., Melander M. and Molnar I., Anal. Chem., 49, 142 1977.
109. Atwood J.G., Schmidt G.J. and Slavin W., J. Chromatogr., 171, 109 (1979).
110. Guiochon G., in "High-Performance Liquid Chromatography: Advances and Perspectives", edited by Horváth C., Academic Press, New York, Vol 2, 1980, Chap. 1, p. 1-54.
111. Di Cesare J.L., Dong M.W. and Ettre L.S., "Introduction to High Speed Liquid Chromatography", Perkin-Elmer Corp., Analytical Instruments, Norwalk, U.S.A., 1981.
112. Majors R.E., in "Liquid Chromatography in Clinical Analysis", edited by Kabra P.M. and Marton L.J., Humana Press Inc., New Jersey, 1981, Chap. 3, p. 51-92.
113. Siemion C.C., J.Liq. Chromatogr., 6, 765 (1983).
114. Webber T.J.N. and McKerrell E.H., J. Chromatogr., 122, 243 (1976).
115. White P.C., Anal., 109, 677 (1984).
116. White P.C., Anal., 109, 973 (1984).
117. Smith R.V. and Stewart J.T., "Textbook of Biopharmaceutic Analysis", Lea and Febiger, Philadelphia, 1981.
118. Bakalyar S.R., Bradley M.P.T. and Honganen R., J. Chromatogr., 158, 277 (1978).

119. Jones C.R., in "Drug Metabolite Isolation and Determination", edited by Reid E. and Leppard J., Plenum Press, New York, 1983, Art. A-3, p. 33-40.
120. "Solvent Guide" Burdick and Jackson Laboratories Inc., Muskegon, Michigan, U.S.A., (1980).
121. Gabler R., Hegde R. and Hughes D., J. Liq. Chromatogr., 6, 2565 (1983).
122. Chatterjee N.R. and Badve V.M., Hind. Antibiot.Bull., 25, 131 (1983).
123. National Formulary, 20th edition (NF XX), American Pharmaceutical Association, Washington, DC, 1980, p. 289.
124. "British Pharmacopoeia", Her Majesty's Stationery Office, London, 1980, Vol I, p. 176.
125. Dennen D.W., in "Analytical Microbiology", edited by Kavanagh F., Academic Press, New York, Vol. 1, 1963, p.209.
126. Grove D.C. and Randall W.A., "Assay Methods of Antibiotics: A Laboratory Manual", Medical Encyclopedia Inc., New York, 1955, p. 96.
127. Bell S.C., Hamman J.W. and Grundy W.C., Appl. Microbiol., 17, 88 (1969).
128. Nilsson-Ehle I., J. Liq. Chromatogr., 6, Suppl. 2, 251 (1983).
129. Kuzel N.R., Woodside J.M., Comer J.P. and Kennedy E.E., Antibiot. Chemother., 4, 1234 (1954).
130. Kuzel N.R. and Coffey H.F., J. Pharm. Sci., 56, 522 (1967).
131. Kuzel N.R. and Coffey H.F., in "Automation in Analytical Chemistry", Medical Inc., White Plains, N.Y., 1967, p. 235-239.
132. Sanghavi N.M. and Chandramohan H.S., Can. J. Pharm. Sci., 10, 59 (1975).
133. Banaszek A., Krowicki K. and Zamojski A., J. Chromatogr., 32, 581 (1968).
134. Jones P.H., J. Chromatogr., 14, 127 (1964).
135. Radecka C., Wilson W.L. and Hughes D.W., J.Pharm Sci., 61, 430 (1972).
136. Graham K.C., Wilson W.L. and Vilim A., J. Chromatogr., 125, 447 (1976).
137. Vilim A., LeBelle M.J., Wilson W.L., Graham K.C., J. Chromatogr., 133, 239 (1977).

138. Richard G., Radecka C., Hughes D.W. and Wilson W.L., J. Chromatogr., 67, 69 (1972).
139. Omura S., Suzuki Y., Nakagawa A. and Hata T., J. Antibiot., 26, 794 (1973).
140. White E.R., Carrol M.A. and Zarembo J.E., J. Antibiot., 30, 811 (1977).
141. Montoya E.F., Chromatographic Application, Spectra-Physics, Santa Clara, Ca., 1975.
142. Tsuji K. and Goetz J.F., J. Chromatogr., 147, 359 (1978).
143. Tsuji K. and Goetz J.F., J. Chromatogr., 157, 185 (1978).
144. Tsuji K. and Goetz J.F., J.Antibiot., 31, 302 (1978).
145. Tsuji K. and Kane M.P., J. Pharm. Sci., 71, 1160 (1982).
146. The Merck Index, Merck and Co., Rahway, N.J., 9th edition, 1976, p. 482.
147. Kanfer I., Personal Communication.
148. Sugden K., Cox G.B. and Loscombe C.R., J. Chromatogr., 149, 377 (1978).
149. Kennedy J.H., J. Chromatogr., 281, 288 (1983).
150. Easterbrook S.M. and Hersey J.A., J. Chromatogr., 121, 390 (1976).
151. Tserng K. and Wagner J.G., Anal. Chem., 48, 348 (1976).
152. Tsuji K., J. Chromatogr., 158, 337 (1978).
153. Reid A., Anal., 101, 1 (1976).
154. Stewart M.J., in "Drug Metabolite Isolation and Determination", edited by Reid E. and Leppard J., Plenum Press, New York, 1983, Art. A-4, p. 41-46.
155. Skellern G.G., in "Drug Metabolite Isolation and Determination", edited by Reid E. and Leppard J., Plenum Press, New York, 1983, Art. A-1, P. 3-12.
156. Product Information, J.T.Baker Chemical Co., Phillipsburg, U.S.A.
157. Reid E., in "Drug Metabolite Isolation and Determination", edited by Reid E. and Leppard J., Plenum Press, New York, 1983, Art. E, p. 255-270.
158. Good T.J. and Andrews J.S., J. Chromatogr. Sci., 19, 562 (1981).
159. Kissinger P.T., Refshauge C.J., Dreiling C.J. and Adams R.N., Anal. Lett., 6, 465 (1973).
160. Kissinger P.T., Anal. Chem., 49, 447A (1977).

161. Kissinger P.T., in "Liquid Chromatographic Detectors", edited by Vickrey M., Chromatographic Sciences Series, Vol. 23, 1983, Marcell Decker Inc., New York., Chap. 4, p. 128.
162. Caudill W.L., Doctoral Thesis, Michigan University, U.S.A. (1984), University Microfilms International, No.84-06784.
163. Coulochem Instruction Manual, 5th Edition, Environmental Sciences Associates Inc., Bedford, Mass. January 1984.
164. Roston D. and Kissinger P.T., Anal. Chem., 54, 429 (1982).
165. Lankelma J. and Poppe H., J. Chromatogr., 125, 375 (1976).
166. Goto M., Sakurai E. and Ishii D., J. Liq. Chromatogr., 6, 1907 (1983).
167. "Electrochemical detection in HPLC", Metrohm Ltd., Herisau, Switzerland, April 1981.
168. Bratin K., Blank C.L., Krull I.S. Lunte C.E. and Shoup R.E., Int. Lab., 14, 24 (1984).
169. Downer R.G.H., Bailey B.A. and Martin R.J., Chromatography Review (Spectra-Physics Inc.), 11, 5 (1984).
170. Tallman D.E. and Weisshaar D.E., J. Liq. Chromatogr., 6, 2157 (1983).
171. Ravichandran K. and Baldwin R.P., J. Liq. Chromatogr., 7, 2031 (1984).