HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STUDIES OF THE ACID DEGRADATION, PHARMACOKINETICS AND COMPARATIVE BIOAVAILABILITY OF ERYTHROMYCIN

A thesis submitted to

RHODES UNIVERSITY

in partial fulfilment of the requirements

for the degree of

MASTER OF SCIENCE

(PHARMACY)

by

FIONA GLEW

December 1988

ACKNOWLEDGEMENTS

I am deeply indebted to the following people for their invaluable contributions to this thesis:

My supervisor, Professor I. Kanfer for his guidance and assistance throughout the course of this study.

Dr C. Stubbs for his interest, helpful suggestions and friendship.

Professor H. Parolis and the staff of the School of Pharmaceutical Sciences for their varied forms of assistance and for use of department facilities. Special thanks to Dr A. Kench for her assistance during the clinical trials.

My fellow post-graduate students for their advice, support and friendship.

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

Mr J. Jonas and Dr R. Dowse for help with computer fitting of data.

Mrs D. Wicks for typing this thesis and for her useful suggestions.

For financial assistance during this study, the South African Council for Scientific and Industrial Research, Rhodes University, The Foundation for Pharmaceutical Education and my parents.

The following companies for donation of drug samples; Abbott, Adcock-Ingram, Eli-Lilly, Lennon Limited, and Pfizer.

My parents and James, my brother, for their love, support and encouragement.

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ABSTRACT

Erythromycin is a macrolide antibiotic with a spectrum similar to penicillin and is used mainly in the treatment of infections caused by gram-positive organisms. Since its discovery in 1952, erythromycin has achieved wide-spread clinical use.

Susceptibility of erythromycin base to inactivation by acid results in decreased availability following exposure to acidic gastric fluids. Formulation of acid resistant dosage forms and the preparation of acid stable chemical derivatives have been attempted to improve absorption and subsequent clinical efficacy. Two of the most commonly used erythromycin derivatives are the stearic acid salt (erythromycin stearate) and the lauryl sulphate salt of the propionyl ester (erythromycin estolate).

Although it has been known for many years that erythromycin is susceptible to acid degradation, very few reports on the stability of erythromycin in aqueous solutions appear in the literature. In this study, a high-performance liquid chromatographic system using electrochemical detection was employed for a kinetic study of erythromycin degradation. The effect of varying acid pH on the degradation rate of both erythromycin base and erythromycin stearate, and the effect on the hydrolysis rate of erythromycin estolate is presented. In addition, the effect of temperature on erythromycin degradation was also investigated.

Until recently, the majority of pharmacokinetic and bioavailability studies have utilized relatively non-specific microbiological assay procedures. However, in this study a solid phase extraction, followed by the use of a high-performance liquid chromatographic system using electrochemical coulometric detection was employed for the determination of erythromycin in biological fluids. Human volunteers each received enteric coated erythromycin base pellets in capsule dosage form and also film coated erythromycin stearate tablets on separate occasions.

Results from the clinical trials revealed the enteric coated erythromycin base pellets had a greater bioavailability than the film coated erythromycin stearate tablets. Computer fitting of data revealed no intra-volunteer variability in elimination rate constants, suggesting differences in serum levels following administration of both dosage forms are due to variation in absorption. Results from the clinical trials were also compared with those obtained from a further trial, during which the same volunteers received erythromycin estolate.

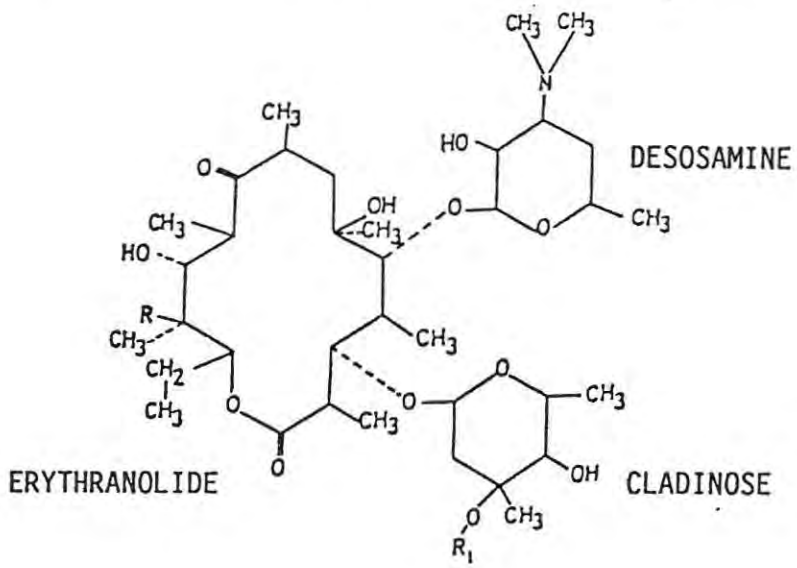
CHAPTER 1

INTRODUCTION

1.1 DISCOVERY

Erythromycin was discovered by McGuire et al in 1952 (1). Erythromycin is an antibiotic produced by the growth of certain strains of Streptomyces erythreus and is a mixture consisting largely of erythromycin A with lesser amounts of erythromycin B and erythromycin C (2). The structure of erythromycin A and its components appear in Fig. 1.1.

Fig 1.1. Structure of erythromycin and its components



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

1.2 PHYSICOCHEMICAL PROPERTIES

Erythromycin is a polyhydroxylactone containing two sugars: L-cladinose which contains no nitrogen but one methoxy group and D-desosamine which is a 3-N-dimethylaminodeoxy sugar (3). The aglycone part of the molecule is a 14 membered lactone called erythranolide A. Both cladinose and desosamine are bound to the lactose ring by a β-glycoside linkage (3).

The biosynthesis of erythromycin from erythranolide A has been studied by Hung, Marks and Tardrew (4). The proposed biosynthetic pathway appears in Fig 1.2.

Erythromycin is a crystalline, white compound which is slightly soluble in water (~2mg/ml) but freely soluble in alcohols, acetone, chloroform, acetonitrile and ethyl acetate (5).

Erythromycin is a weak base (pKa = 8.6) and is unstable in acidic and strongly alkaline solutions, showing its maximum stability between pH 6 and 9.5 (6). The principle acid degradation products are the 6,9-hemiketal of 8,9-anhydroerythromycin (or enol-ether) and 8,9-anhydroerythromycin, the structures of which are depicted in Fig 1.3 (7-10). The dry powder is stable but should be stored below 30°C and protected from light (6). Aqueous, alcoholic solutions buffered at pH 7-8 are stable for about one week under refrigeration at 4°C (6).

Erythromycin A, $C_{37}H_{67}NO_{13}$ has a melting point of 137°C-140°C and $[\alpha]_D^{25}$ = -78°C (c = 2% ethanol), whilst the hydrochloride has a melting point of 170°C - 173°C (3).

Fig 1.2 Postulated synthetic pathways of erythromycin from erythranolide A.

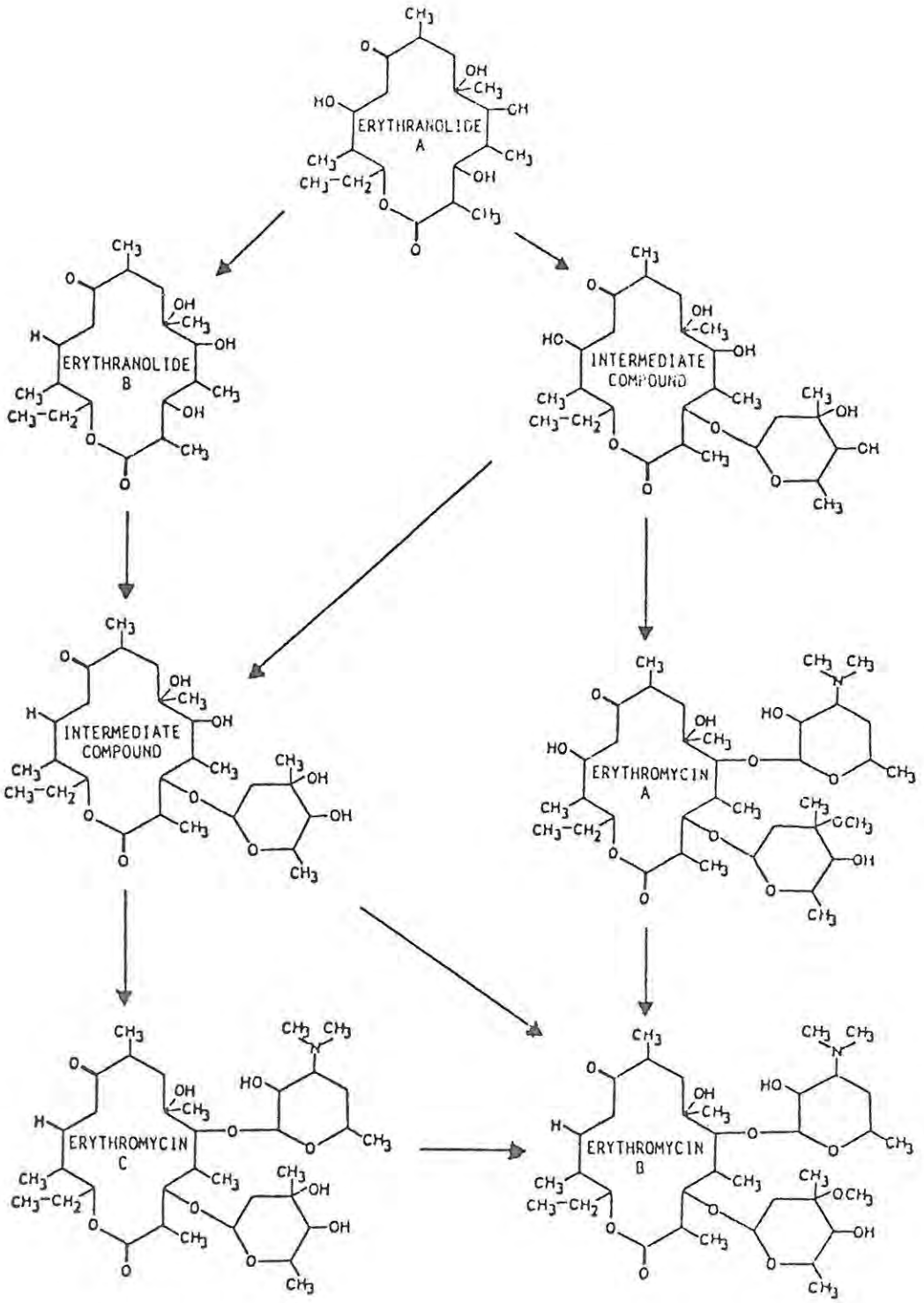
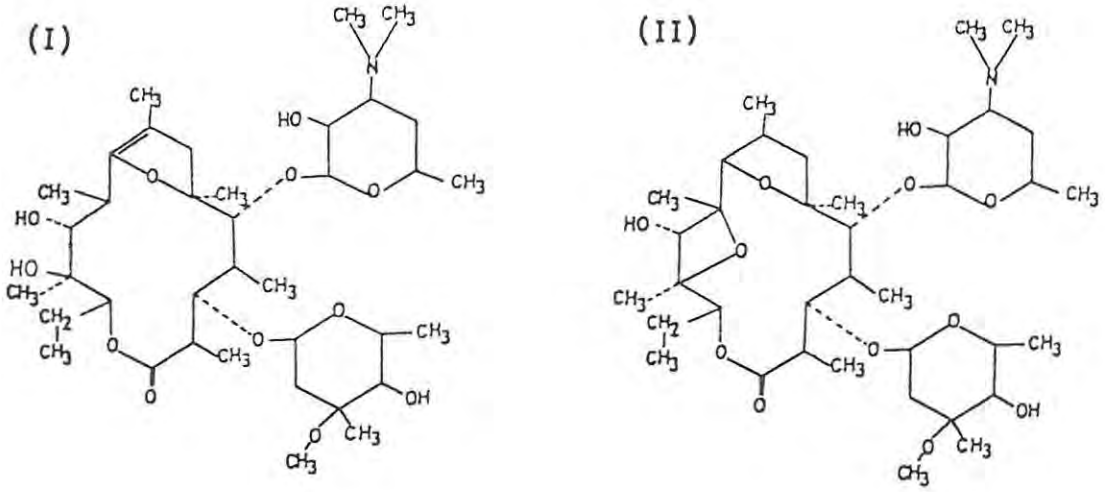


Fig 1.3 Chemical structures of the predominant acid degradation products of erythromycin



(I) 6,9 - hemi-ketal of 8,9 anhydro erythromycin A
(II) 8,9 - anhydro erythromycin A

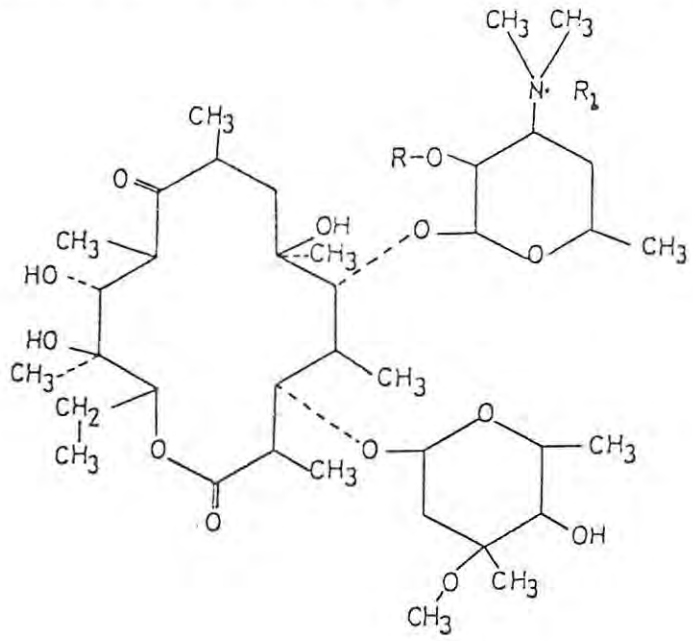
1.3 STABILITY

Susceptibility of erythromycin to inactivation by acid results in decreased absorption following exposure to gastric secretions (11). Formulation of acid resistant dosage forms and the preparation of acid stable chemical derivatives have attempted to improve absorption and subsequent serum levels (12). Modifications in the chemical structure of the molecule includes the formation of a salt (the stearate), an ester (the ethyl succinate or propionate) or the lauryl sulphate salt of the propionyl ester (the estolate). The most recent ester reported is erythromycin acistrate (stearic acid salt of the 2'-acetyl ester) which has undergone some preliminary studies (13). Results from a comparative bioavailability

study reveal erythromycin acistrate produced higher erythromycin serum concentrations than erythromycin stearate.

Given intramuscularly erythromycin is irritant, although certain erythromycin esters have been used as intramuscular preparations. Preparations for intravenous use include erythromycin glucoheptonate and erythromycin lactobionate. The structural formulae of the most commonly used erythromycin derivatives are depicted in Fig 1.4.

Fig 1.4 Structural formula of erythromycin and its derivatives



	R	R_1
Erythromycin base	H	
Erythromycin stearate	H	$C_{17}H_{35}COO$
Erythromycin ethylsuccinate	$CH_2CH_2OOCCH_2CH_2COO$	
Propionyl erythromycin	CH_2CH_2CO	
Erythromycin estolate	CH_2CH_2CO	$C_{12}H_{25}OSO_3$
Erythromycin lactobionate	H	$C_{11}H_{19}O_9COO$
Erythromycin glucoheptonate	H	$C_6H_{13}O_6COO$

1.4 CLINICAL PHARMACOLOGY

1.4.1 Mode of Action

Erythromycin penetrates the cell wall of sensitive bacteria (14) and attaches to the 50S ribosomal subunit in the presence of ammonium or potassium ions (15). Absence of the 50S subunit in the human host leads to selective toxicity for the micro-organism.

A single molecule of erythromycin attaches to each ribosomal fragment. In binding to the 50S subunit erythromycin prevents translocation, the step in polypeptide chain formation which follows elongation, hence inhibiting protein synthesis (15). Erythromycin also prevents the incorporation of certain amino acids into polypeptide linkages (14).

Although erythromycin acts to inhibit protein synthesis, it may be bacteriostatic or bactericidal depending on the concentration of the drug and the sensitivity of the micro-organism (14).

1.4.2 Spectrum

Erythromycin has a spectrum similar to that of penicillin, and is active against most gram positive and some gram negative organisms. Erythromycin is also active against *Treponema pallidum*, members of the rickettsiaceae, as well as *Mycoplasma pneumoniae*, *Legionella pneumophilia*, *Chlamydia* and atypical mycobacteria (14, 16). Typical *in vitro* sensitivities of organisms to erythromycin are summarized in Table 1.1.

Table 1.1 Typical *in vitro* sensitivities of organisms to erythromycin

Organism	Typical Minimum Inhibitory Concentration (ug/ml) 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 veridans	0.06	0.02-0.1
Enterococcus	1.5	0.1->400
Clostridium perfringens	0.5	0.5-5
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	0.2->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$0.05	

1.4.3. Resistance

Certain strains of *Staphylococcus aureus* have been shown to be resistant to erythromycin (16). Some staphylococci resistant to

erythromycin were found to be resistant to all macrolide antibiotics although the degree of resistance varies from strain to strain. Resistance to erythromycin appears to be related to a unique plasmid determined mechanism and is caused by the methylation of specific sites on the 50S subunit which prevents the binding of erythromycin, a process necessary for antibacterial activity (16).

Increased resistance is more common following prolonged treatment with erythromycin than short term treatment and it is therefore thought that erythromycin metabolites may be responsible for induction of resistance (17).

1.4.4. Clinical Uses

The efficiency of erythromycin against so many gram positive cocci and against *Haemophilus influenza* and *Mycoplasma pneumoniae* makes it an excellent respiratory tract antibiotic. Erythromycin is recommended for streptococcal tonsilopharyngitis, and for other pharyngeal infections, for sinusitis, otitis media, laryngitis, tracheitis and acute bronchitis as well as primary atypical pneumonia (18).

Erythromycin is the drug of choice for Rheumatic Fever and is also effective against Legionaires disease, syphilis and gonorrhoea (16).

For persons allergic to penicillin who require an antibacterial agent, erythromycin is the drug of choice provided penetration across the blood brain barrier is not required, such as in bacterial meningitis (15). Erythromycin has also been used in place of penicillin to treat enterococcal endocarditis and urinary tract infections.

Although the dose of erythromycin is dependent on the condition being treated, the usual adult dose is 1-4g per day. Doses should be

divided such that a maximum of 500mg should be ingested as a single dose to minimize gastrointestinal intolerance. For children the usual dose is 25-50mg/kg/day which should be divided so as to be administered 2-4 times daily.

Intravenous administration is reserved to treat severe infections. The usual dose is 0.5 - 1 gram every 6 hours, which may be given for as long as 4 weeks, although thrombophlebitis may become a problem (19).

1.4.5. Adverse Drug Reactions

Erythromycin is regarded as the least toxic of the commonly used antibiotics. Side effects are few and in general not serious (16). Although mild degrees of epigastric distress, diarrhoea, nausea and vomiting are fairly frequently encountered, their severity is generally dose related and these symptoms are only rarely serious enough to require cessation of therapy. Children are more likely to experience nausea and vomiting (19).

Cutaneous eruptions caused by hypersensitivity have been reported, although these cases are rare (16). Another rare complication of erythromycin is auditory impairment which is reversible if the antibiotic is discontinued (19).

The most serious toxicity of erythromycin is cholestatic hepatitis, which may be caused by various forms of erythromycin. Although it was widely accepted that the estolate derivative was primarily responsible for causing cholestatic jaundice (15, 20, 21) recent evidence suggests that this may not be the case (20). In a study using prescription monitoring of 1200 patients receiving erythromycin stearate and estolate, results suggested that the incidence of jaundice caused by the estolate was less than 1 per 1000. In the three cases of jaundice reported all three patients received erythromycin stearate (20).

Symptoms of cholestatic hepatitis begin after 10 - 20 days of treatment. Clinical characteristics include abdominal pain, nausea, and vomiting followed by the development of jaundice, acholic stools, dark urine and hepatomegaly. Patients recover rapidly after erythromycin treatment is discontinued.

Thrombophlebitis occurs relatively frequently with intravenous administration of erythromycin (16), although the lactobionate salt of erythromycin may be less irritating (22). Erythromycin should not be given intramuscularly since it causes severe local pain.

1.4.6. Drug Administration

Erythromycin in therapeutic doses has been shown to chiefly influence the hepatic elimination of several drugs, which might well be prescribed concomitantly with the antibiotic (18). Evidence suggests that erythromycin interacts with theophylline, carbamazepine, warfarin and phenytoin. It has been suggested that erythromycin reduces the capacity of the mixed function oxidase system in the liver.

Extensive studies on the interaction of erythromycin and theophylline have been carried out. Some reports show that concomitant administration of erythromycin and theophylline leads to a significant elevation of serum theophylline levels although other reports show no interactions (23, 24). The mechanism of interference by erythromycin with theophylline disposition is unclear although it may possibly be explained in part, by competitive inhibition of the 1-demethylation pathway by erythromycin which is itself demethylated (24). Clinically it is suggested that a reduction in the theophylline dose may be desirable during concomitant erythromycin treatment (24).

Wong et al (25) reported that although there were no differences in apparent volume of distribution, elimination rate constant or absorption rates, the clearance of a single oral dose of carbamazepine was reduced in the presence of erythromycin. Further studies are indicated since this study was based on a single carbamazepine dosage which does not mimic the clinical situation (25).

After three days of concomitant warfarin and erythromycin therapy, erythromycin was shown to increase the prothrombin time of a warfarin stabilized patient by 40% (26).

A study of clearance of a single dose of phenytoin during erythromycin treatment reported that even though erythromycin failed to significantly decrease mean phenytoin clearance, some individual subjects exhibited pronounced decreases in phenytoin clearance, providing sufficient incentive to closely monitor patients taking both drugs (27).

1.5. PHARMACOKINETICS

1.5.1 Bioavailability Data

Erythromycin stearate which dissociates in the intestinal tract and erythromycin base are absorbed as the free base (28). The lauryl sulphate salt moeity of the estolate, however, dissociates in the intestinal tract and is absorbed as the propionyl ester which must subsequently be hydrolyzed in vivo to the active base form. Similarly the ethylsuccinate must also be hydrolysed in vivo to the bioactive free base (2).

Until recently, the assay of serum erythromycin has been carried out microbiologically (29). Microbiological assays, which are based on comparison of the dose which inhibits growth of a suitable micro-

organism with the dose of the standard preparation which produces the same degree of inhibition, do not distinguish between active parent drug, the unhydrolysed form or metabolites.

It has been shown that erythromycin estolate produces a higher total blood level than other erythromycin derivatives but studies have shown that only a small portion of the estolate is hydrolysed to the active drug in the blood (30). Therefore, although the estolate shows greater efficiency of gastrointestinal absorption, this may not necessarily be indicative of higher therapeutic efficiency.

The rapid *in vitro* hydrolysis of the estolate during microbiological assay procedures has led to an overestimation of biologically active drug in the patient. The estolate also shows a higher degree of protein binding than the free base (31, 32).

Although a vast amount of bioavailability data has been reported in the literature, comparison of bioavailability data between studies is difficult due to differences in study protocols and analytical techniques. Various suggestions regarding protocols and interpretation of results have been reported (12, 33-37). It must be noted, however, that the results of comparative bioavailability studies involving erythromycin and its derivatives are only valid for products evaluated under the same conditions (33, 38).

1.5.2 Absorption

Erythromycin base is very unstable in acid (11). This has led to the formulation of enteric coated dosage forms to protect the base from acid degradation in the stomach. Coating is reported to decrease the acid degradation and also cause a slight delay in absorption (11, 39-41). The absorption of erythromycin from two different enteric

coated preparations, enteric coated tablets and enteric coated pellets has been evaluated. The enteric coated pellets led to a higher, more regular absorption of erythromycin than did coated tablets (42).

The absorption of erythromycin base has been reported to be reduced, delayed or not affected by the presence of food depending on the formulation or dosage regimen used (43).

Smith *et al* reported fasting subjects absorbed well, but if the dose was taken after food, absorption was reduced (44). The absorption of erythromycin base by patients with achlorhydria was not affected by food, although the absorption was slower and peak serum levels occurred later (44).

Erythromycin stearate bioavailability is reported to be sensitive to the presence of food. Reduced bioavailability of erythromycin stearate in the presence of food has been confirmed in single and multiple dose studies (7, 45-47). In a study (46) comparing erythromycin base and erythromycin stearate bioavailability, the mean peak erythromycin concentrations following a single oral 500mg dose of erythromycin base were 1.84 $\pm$ 1.15 µg/ml and 1.91 $\pm$ 1.57 µg/ml when the dose was taken before and after breakfast, respectively. Erythromycin stearate taken before and after breakfast was reported to have mean peak erythromycin concentrations of 2.09 $\pm$ 1.06 µg/ml and 0.37 $\pm$ 0.40 µg/ml respectively, suggesting erythromycin stearate absorption is affected by the presence of food.

The serum levels of erythromycin obtained were not only affected by the presence of food, but were also reduced significantly in individuals when the accompanying water was reduced from 250ml to 20ml (7). Possible explanations are that stomach emptying is faster following the ingestion of a larger volume of water, reducing acid degradation.

Reduced bioavailability of erythromycin stearate given together with food is possibly due to the increased degradation by gastric acid, since the production of acid is increased by food ingestion. Although most reports indicate erythromycin stearate bioavailability is reduced in the presence of food, Clayton and Leslie (48) concluded that erythromycin stearate had a greater bioavailability when administered immediately before food. A further study in non-fasting subjects reported that the peak plasma erythromycin concentration following a 500mg oral dose of erythromycin stearate was reached more rapidly and was twice as high as that observed in fasting subjects. Possibly the food neutralizes and dilutes the acid and in addition, gastric motility may aid dispersion and result in rapid absorption and enhanced bioavailability (35).

In 1964, a single and multiple dose study comparing erythromycin stearate and erythromycin estolate bioavailability was carried out (40). Using a microbiological assay technique, estolate blood levels were reported to be at least twice as high as stearate levels in the fasting state and more than ten times higher in the non-fasting state.

Recently, however, with the development of assays which discriminate between the bioactive and bio-inactive portion in the plasma, the active erythromycin base has been measured. Using discriminatory assay techniques, a single dose of erythromycin estolate gave rise to higher plasma levels of total antibiotic than the equivalent dose of erythromycin stearate, although the estolate produced lower, if somewhat more consistent levels of free base (28, 47, 50).

In a multiple dose study of erythromycin estolate, erythromycin stearate and enteric coated erythromycin base, it was concluded that the area under the curve values derived from the free erythromycin base concentrations following the administration of the

three products was not statistically significant (28).

Erythromycin estolate absorption is reported to be unaffected by the presence of food (47, 51, 52) although it has also been reported that food may enhance absorption (47, 52).

1.5.3. Absorption Kinetics

Numerous studies have been carried out to examine erythromycin pharmacokinetics following intravenous administration as well as single and multiple oral dosage regimens.

A zero order absorption model was found to adequately describe the pharmacokinetics following a single dose (47, 53) and a multiple dosage regimen (54). However, first order absorption has been suggested following multiple dosing (47, 55, 56). A further study concluded that erythromycin is absorbed at a constant rate in some subjects, whereas other subjects followed first order absorption rates (51), or a combination of zero order and first order processes (58).

In many studies, acceptable curve fitting was only possible with the inclusion of an absorption lag time parameter, (11, 47, 53, 57, 59). However, following the administration of a suspension, absorption was not delayed.

Typical pharmacokinetic parameters obtained following oral administration of erythromycin base and erythromycin stearate appear in Table 1.2.

Table 1.2 Typical pharmacokinetic parameters describing erythromycin disposition in relation to oral dose

	Dose (mg Base)			
Serum	250	500	1000	500 stearate
n	20	22	21	22
lag (hr)	1,4(0,3)	1,4(0,3)	1,3(0,4)	1,1(0,4)
ka (hr^{-1})	3,2(1,3)	4,3(2,2)	3,2(1,8)	2,3(0,7)
Ke (hr^{-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)
$T_{1/2}$ (hr)	2,0(2,0)	2,5(1,0)	3,0(1,7)	2,7(2,3)

Standard deviation in parenthesis

Key
- n = number of people in trial
- lag = lag period between administration of dose and commencement of absorption
- Ka = absorption rate constant
- Ke = elimination rate constant
- $AUC_{.1}$ = area under the serum concentration-time curve calculated by the trapezoidal file
- $AUC_{..2}$ = AUC calculated with AUTOAN2/NONLIN program

Reproduced from Josefsson *et al* (80).

1.5.4. Distribution

Erythromycin is widely distributed in the tissues (39). Diffusion into the spinal fluid is poor even in the presence of meningial inflammation (19). Moderate levels of erythromycin appear in tonsil tissue, and pleural fluid and bronchial secretions contain erythromycin in amounts exceeding serum levels (60). Erythromycin is one of the few antibiotics which penetrate the prostatic fluid (61), and animal studies reveal good penetration into the aqueous humour (62). Placental transfer produces low, variable erythromycin levels in foetal blood, although it is concentrated in the foetal liver (63).

1.5.5. Binding

The extent of protein binding is influenced by both drug concentration and albumin concentration. Different values for protein binding have been reported although most reports state that erythromycin is highly protein bound (13, 13, 32, 64). Prandota *et al* (64) reported erythromycin to be 73% protein bound whilst Wiegund and Chun (31) demonstrated erythromycin to be 90% bound to serum proteins.

Erythromycin binds to serum albumin but exhibits a high affinity (at least 50% bound) for α-acid glycoproteins (64). Since this binding is loose and reversible, this does not appear to influence erythromycin tissue levels (18, 40).

1.5.6 Metabolism

A considerable amount of erythromycin is metabolised in the liver (39). Erythromycin is reported to be rapidly demethylated in the liver to yield des-N-methylerythromycin. A study carried out on rats (65), dogs (66) and rabbits (67) demonstrated that a metabolite

excreted in the bile was identical to des-N-methylerythromycin. A further study on the metabolism of erythromycin in rats concluded that des-N-methylerythromycin is the only microbiologically active metabolite, and was excreted in the bile and passed out in the faeces.

A recent study, (68) has concluded that repeated doses of erythromycin may actually induce increased metabolism by increasing both the titre and activity of the enzymes.

1.5.7 Elimination

Erythromycin is excreted in the urine and the bile (39). Although highly variable urine concentrations are achieved, the drug is only minimally excreted by this route. The portion of dose excreted in the urine varies from 0.02% - 15% (63, 69, 70). Jaundiced patients show slightly higher drug excretion in urine than the non-jaundiced patients. Alkalinisation of the urine was reported to have no effect on the urinary concentration of erythromycin or the total daily urinary excretion although it does increase the antibacterial activity of erythromycin present in the urine (71). Erythromycin excretion is not significantly affected by urine alkalination suggesting that active transport processes may be involved. This is in agreement with the findings that renal clearance of erythromycin is only about 13% of creatinine clearance, indicating either an active transport system and/or net tubular re-absorption of erythromycin. However, Lee *et al* (66) investigating the renal clearance of erythromycin in dogs, found that erythromycin clearance was about 70% of creatinine clearance. Since only a small portion of drug is eliminated by the kidneys, the elimination rate is not significantly influenced by altered renal function. There is, therefore, no need to alter the dosage regimens in persons with impaired renal function (72). However, recently reported incidences of reversible hearing loss involving patients with renal failure during chronic intravenous therapy, indicates further study is necessary (72).

Biliary excretion is reported as the main excretion pathway of erythromycin and des-N-methylerythromycin which are excreted in high concentrations following both oral and intravenous dosages (22, 73, 74). The concentration of active drug in the bile often exceeds serum concentrations suggesting an active secretory process (75).

1.5.8 Elimination Kinetics

The pharmacokinetics of distribution and elimination have not been adequately characterized. All intravenous studies reported that the elimination profiles were consistent with a two compartment open model with elimination occurring from the central compartment (43, 58, 72, 76, 77). Although a two compartment open model has been used to characterize the elimination profile following a single oral dose (53), one compartment model elimination following both single and multiple dosing regimens has also been reported (47, 55, 56, 77-79).

An extensive kinetic study using intravenous (58) and oral dosage regimens (57) evaluated elimination profiles using two compartment and one compartment models, respectively. This study concluded that the large subject variability in erythromycin serum concentration profiles after oral administration was related to variability in absorption kinetics and absolute bioavailability rather than variability in disposition kinetics.

Multiple dosage regimens show that the bioavailability of the initial dose was lower than subsequent doses (47, 53, 54, 56) In addition, following a multiple dosage regimen of 250mg oral erythromycin every 6 hours, Colburn *et al* (54) reported the average elimination rate constant on day one was considerably higher than day three. The average halflife increased from 1.8 hours on day one to 2.5 hours on day three, suggesting a degree of saturation of the enzymatic pathways following repeated dosing.

Non-linear kinetics have been suggested following both intravenous (58) and oral (80) regimens. Increasing the i.v. dosage led to a decrease in the elimination rate constant and an increase in the volume of distribution and the fraction eliminated in the urine (58). Following oral administration, Josefsson et al reported a superproportionate increase in the area under the serum concentration-time curve (AUC) with increasing dosages indicating possible dose dependent kinetics. A trend towards an increase in the fraction of erythromycin in the urine was also observed.

Several papers have reported the possibility of enterohepatic recycling (53, 55, 57, 58, 80, 81). This phenomena manifests in the form of irregularities or secondary peaks in the serum concentration-time curve following both oral and intravenous dosing (55, 80, 82, 83). It has been suggested that these irregularities may be linked to food intake (58) although further investigation is necessary.

Typical pharmacokinetic parameters describing erythromycin disposition following the i.v. administration of erythromycin lactobionate (250mg base equivalent) are given in Table 1.3.

1.6 STUDY OBJECTIVE

Although it has been known for many years that erythromycin is susceptible to acid degradation very few reports on the kinetics of the degradation have appeared in the literature. The main objective of this study was to investigate the kinetics of erythromycin base degradation. A comparative study between the chemical kinetics of erythromycin base and erythromycin stearate acid degradation was investigated. A brief investigation into erythromycin estolate hydrolysis was undertaken.

In addition a study using human volunteers was carried out to compare the bioavailability and pharmacokinetics following a single oral dose of erythromycin base and erythromycin stearate.

Table 1.3 Typical pharmacokinetic parameters describing erythromycin disposition following administration of erythromycin lactobionate (250mg base equivalent).

n	24
V_{dss}	44,4(11,4)
$T_{1/2}^{\alpha}$(hr)	0,11(0,07)
$T_{1/2}^{\beta}$(hr)	1,5(0,3)
Cl_s(l/hr)	26,3(7,5)
Cl_r (L/hr)	3,4(2,4)
f_u	0,12(0,07)

Standard deviation in parenthesis

Key
- n = number of people in trial
- V_{dss} = apparent volume of distribution at steady state equilibrium
- $T_{1/2}^{\alpha}$ = distribution half life
- $T_{1/2}^{\beta}$ = elimination half life
- Cl_s = total body clearance
- Cl_r = renal clearance
- f_u = fraction of dose excreted unchanged in urine

Reproduced from Austin et al (58).

CHAPTER 2

DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF ERYTHROMYCIN AND ITS ACID DEGRADATION PRODUCTS

2.1 INTRODUCTION

High performance liquid chromatography (HPLC) is a widely used analytical technique. Due to its relative simplicity, high efficiency, selectivity, speed and precision, HPLC has found popularity for use in the analysis of drugs (84-86).

Like all chromatographic techniques, separation of components in a mixture depends on the relative affinities of the various solute molecules for the stationary and mobile phase (87). The earliest stationary phases of silica were more polar than the mobile phases used, resulting in the retention of polar solutes. More recently, systems employing a non-polar stationary phase and a more polar mobile phase have been developed. This technique referred to as reversed-phase liquid chromatography (RPC), uses a stationary phase prepared by covalent bonding of various hydrocarbons e.g. octyl C_8 or octadecyl C_{18} to a silica support. The mobile phase usually consists of a mixture of water and water miscible polar organic solvents (84, 88).

During preparation of the bonded phase a significant fraction of surface silanols are unreacted with the long chain stationary phase. Reacting the packing material with trimethylchlorosilane, a process called end-capping, to reduce the number of free silanols produces columns of superior batch to batch uniformity, improved column life and reduced peak tailing (89, 90).

The separation of compounds as they move through the column can be seen to be dependent on three factors; retention, column selectivity and column efficiency (91).

Once a column is 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 (89), where K' is the capacity factor and is calculated from Equation 2.1 (see Fig. 2.1)

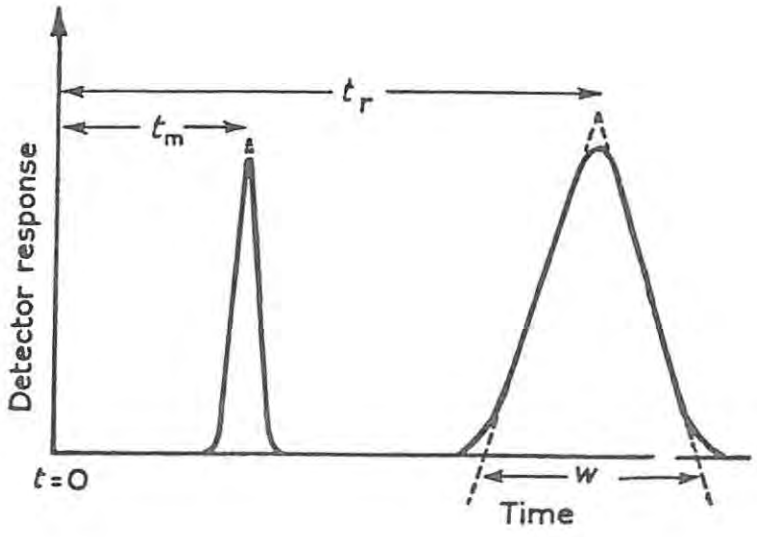
$$K' = \frac{t_r - t_m}{t_m} \qquad \text{Equation 2.1}$$

Where t_m = the time for solvent molecules to traverse the column

t_r = the retention time for the compound of interest.

Large K' values indicate long retention times, which results in diffuse bands that are difficult to detect (84, 91).

Fig 2.1 Schematic diagram of a chromatogram



Column selectivity, α , is the ratio of the capacity factors K_1', and K_2' of two compounds 1 and 2, (Equation 2.2) and is a function of the stationary phase properties and the mobile phase eluting power (84, 89).

$$\alpha = \frac{K_2'}{K_1'} \qquad \text{Equation 2.2}$$

Column efficiency or resolving power of the column can be calculated from the plate number, N. The value is calculated from Equation 2.3 (92).

$$N = 5.54 \frac{t_r^2}{W} \qquad \text{Equation 2.3}$$

Where t_r = peak retention
W = peak width at half the peak height.

Both these values are measured in the same terms of time or distance.

In comparing column efficiencies a more useful parameter is the height equivalent to a theoretical plate (HETP). The HETP is obtained by dividing N by the length of the column, L. The smaller the HETP, the smaller the zone width and the greater the column efficiency (84).

Column efficiency depends not only on the size and quality of the packing material but also on the column packing technique (89). High quality stationary phase packing material is commercially available. Wet packing, a widely used method of column packing involves the pumping of a slurry of column packing material into the column by a suitable displacement fluid at high pressure. The main variables in slurry packing are the density and viscosity of the solvent

and the flow rate and pressure of the slurry (84). Although various slurry solvents have been used, carbon tetrachloride has been shown to produce high efficiency columns (93, 94).

Following column selection it is necessary to optimize the mobile phase composition until adequate separation is achieved. Selection of mobile phase depends on numerous factors including viscosity, polarity, compressibility, vapour pressure as well as compatibility with the stationary phase and detector (84, 88). Typical mobile phases used in RPC are mixtures of water (or aqueous buffer) and one or more organic solvents (84). The most widely used organic solvents include methanol, acetonitrile and tetrahydrofuran (84). Given a choice between solvents of similar polarity, the less viscous solvent is chosen since it results in lower pressures and hence extends the life of the column (89).

Modification of accessible silanol groups on the stationary phase by the use of buffer salts such as phosphate and citrate leads to reduced peak tailing and improved resolution (89, 95). In addition, pH control with the use of buffers can significantly affect the retention time of easily ionizable species. For silica based bonded materials the useful pH range is 2-8. At pH less than 2, the silica-carbon bond is attacked and at high pH values the silica substrate dissolves (96). Prior saturation of the mobile phase with silica from a silica saturating pre-column may extend the life of the analytical column whilst working at these extreme pH ranges (97). Dissolved gases in the mobile phase can affect detector performance and should therefore be controlled (98). This may be achieved by the use of an in-line vacuum degassing unit or by the constant purging of the mobile phase with helium (89).

A large number of detectors have been described for use in liquid chromatography (99, 100). The most widely used detectors, due to ease of operation, versatility and sensitivity are

spectrophotometric detectors based on the absorption of ultra-violet light (84, 96). Although not universal in application a great many substances do absorb ultraviolet radiation.

Electrochemical methods of detection probably offer the most promising approach to developing a universal detector for HPLC, offering the benefits of both high sensitivity and selectivity (84). Since the initial use of electrochemical detection for monitoring catecholamines in brain tissue (101), the use of electrochemical detection has grown rapidly.

Electrochemical detection results from a current-producing reaction at the surface of the electrode (102). This can either be an oxidation or reduction reaction. The success with which liquid chromatography with electrochemical detection methods can be applied is determined by the electrode potential required to affect oxidation or reduction of the compound of interest.

Most detectors limit mobile phase composition to some degree. However, electrochemical detectors require the mobile phase to be able to carry an ionic current, be electrochemically inert in the desired potential range and dissolve the analyte of interest (102). Direct electrochemical detection is therefore not likely to be useful in normal phase chromotography, since nonpolar organic solvents, due to their inability to support a significant ionic strength are not well suited to electrochemical reactions. Alternatively, reverse phase chromatographic systems using aqueous mobile phase, with or without organic modifiers are ideal for electrochemical detection (102, 103).

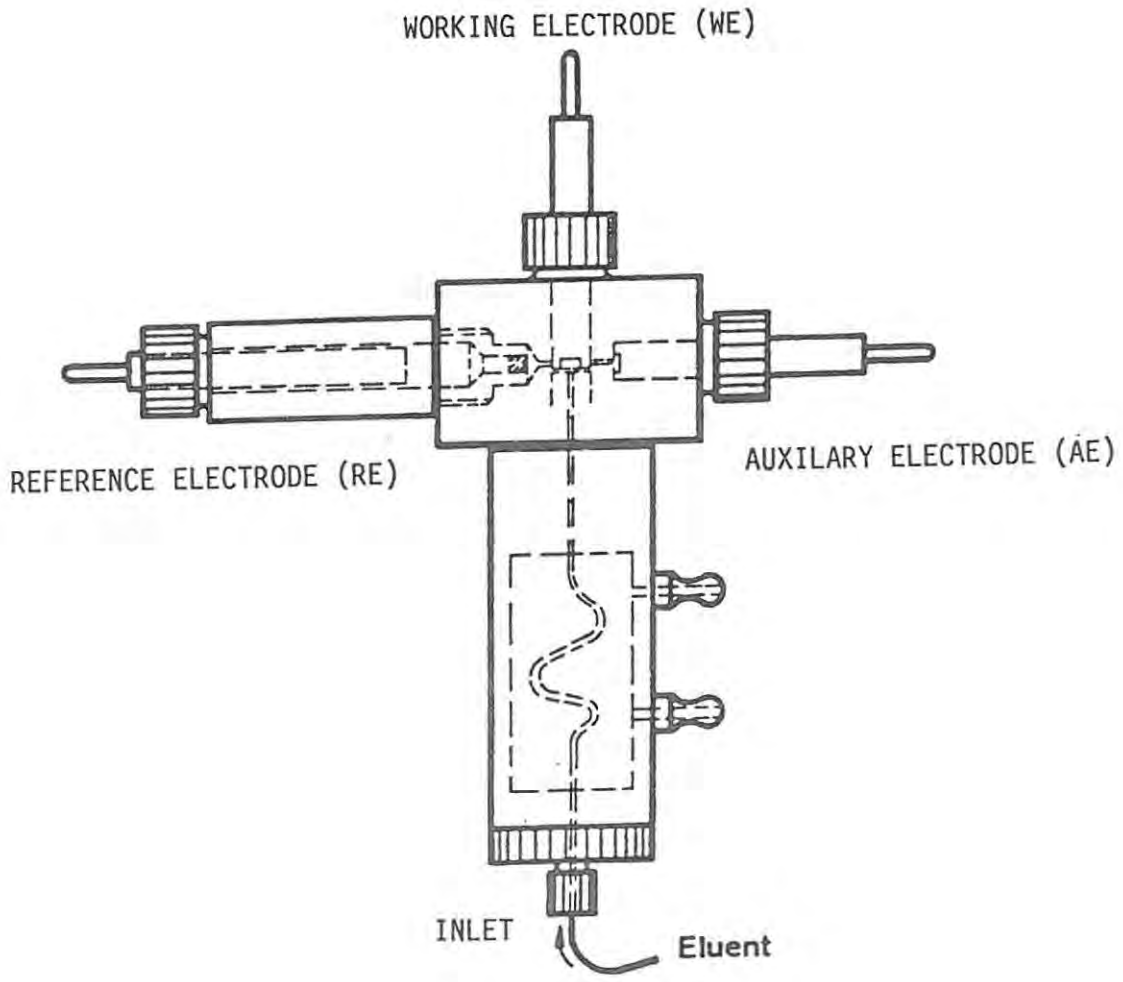
Two different approaches can be used for detection of electroactive species, namely; amperometry and coulometry. Amperometric detectors operate in the limit where the concentration of analyte entering and leaving the cell is the same (conversion efficiency is zero). Coulometric detectors are those

where the concentration of entering analyte is reduced to zero as it exits the cell (conversion efficiency is 100%). For practical purposes amperometric detectors may involve conversion efficiency up to 5% and coulometric detectors above 95% (103).

Although many cell designs for solid working electrode detectors have been devised, there are basically two designs in use, namely; the wall jet and the thin layer configuration (103).

The wall jet principle is based on the impinging of the analyte on the head of the working electrode, where the reaction takes place (104). A detector based on the wall jet principle appears in Fig 2.2.

Fig 2.2 Single electrode, amperometric electrochemical detector flow cell (Metrohm)



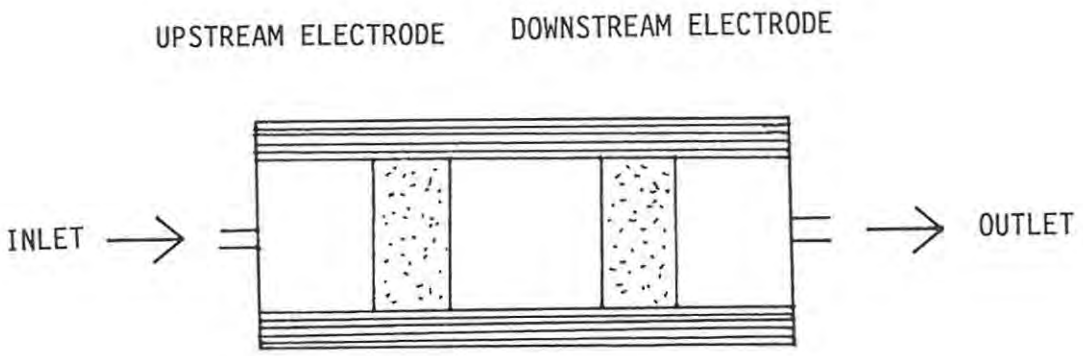
The electric current generated by the reaction at the working electrode (WE), which is proportional to the amount of eluent, is measured by the auxilary electrode (AE), while the reference electrode (RE) together with the electronic potentiostat ensures a constant polarizing voltage. All cells operating in this manner are amperometric.

The type of material used for the RE and AE is not critical but the choice of WE material is important in affecting detection (102). The most widely used WE materials include carbon paste and glassy carbon (105).

Coulometric electrodes 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 (106). The larger area of the working electrode is required in order to obtain complete electrolysis (103). Although only a single electrode is necessary for detection, an innovative approach to electrochemical detection involves the use of two working electrodes operated simultaneously at different potentials (107-109) (Fig 2.3). The upstream electrode is used as a screening electrode. In this operation mode, the upstream electrode is held at an electrode potential slightly lower than the downstream potential. The upstream electrode will therefore completely oxidize and make undetectable other species oxidizable at potentials lower than that of the analyte. The downstream electrode can be set at optimum potential for detecting the analytes (106, 108).

In addition, when operating potentials are high, a guard cell can be incorporated into the HPLC system between the solvent delivery system and injector (106). This cell consists of a single electrode which electrolyses contaminants in the mobile phase prior to chromatography (106). This cell ensures the mobile phase is electrochemically pure since solvents currently available are optically pure but not electrochemically pure.

Fig 2.3 Dual electrode, coulometric electrochemical detector flow cell (Coulochem)



Choice of optimum potential for analyte detection is made from a voltammogram. This is a plot of potential applied versus current generated (106). Two types of voltammograms can be generated, namely, cyclic and hydrodynamic voltammograms. Cyclic voltammograms have the difficulty of requiring analyte to be supplied continuosly for a period of 2-4 minutes while the potential of the electrode is continuosly changed. Although it takes longer to acquire the data, this problem is overcome by producing a hydrodynamic voltammogram. The applied potential is varied and allowed to stabilize. At each applied potential an equivalent amount of analyte is injected into the system. A plot of applied potential versus peak height or area can be constructed. 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 (102, 106).

The potential selected should be lowest potential at which the required sensitivity is achieved. Greater sensitivity can be obtained in this manner, since background currents from the

electrodes and solvents increase with increasing applied potential (102, 109).

2.2 METHODS AVAILABLE

Numerous methods for the separation and quantitation of erythromycin and its degradation products have been reported.

Paper chromatographic (110) and thin layer chromatographic (111-113) methods are available for the separation of erythromycins. However these methods are not suitable for precise quantitative analysis. A gas chromatographic method capable of separating and quantifying erythromycin and degradation products is available. However, this method has not found wide-spread use possibly due to the required lengthy derivatization (24 hours) of samples (2).

Several HPLC methods have been reported for raw material, dosage form and biological fluid analysis of erythromycin (10, 34, 114-121). The first reported HPLC method showed separation of erythromycin A and erythromycin B. However, this report failed to present quantitative data or demonstrate separation of degradation products (144). A novel HPLC method based on fluorometric detection reported by Tsuji (115) capable of separating erythromycin and degradation products has apparently not received wide use, probably due to the instrumentation involved and the complexity of the procedure.

Suitable methods for the quantitative analysis of erythromycin using UV detection have been developed (10. 115-117, 119-121). Using a μBondapak C_{18} reverse phase column with UV detection at 215nm, Tsuji *et al* was able to separate and detect erythromycin and degradation products (117). Similarly, Stubbs (119), used UV detection at 200nm.

Atkins *et al* however, used a Lichrosorb RP-8 5um column with UV detection at 214nm to carry out an acid degradation study (16). The separation of erythromycin and degradation products has also been shown using poly(styrene-divinylbenzene) packing material (122).

More recently however, HPLC with electrochemical detection has been used. Using a C_{18} column and a dual electrochemical detector in the oxidative screen mode, Chen and Chiou (34) reported a simple and sensitive method capable of quantitatively measuring erythromycin and degradation products. The applied cell potential of the screen electrode was set at 0.70V and the sample electrode at 0.90V. A further method on the use of electrochemical detection described the use of a dual electrode coulometric detector in the oxidative mode with the screen potential set at 0.70V and the sample electrode set at 0.80V (123). Alternatively, the amperometric response given by a glassy carbon electrode can also be used to detect erythromycin. However, the detector rapidly loses sensitivity (123). Stubbs (119) reported the use of amperometric and coulometric detectors for the quantitative analysis of erythromycin. Croteau (124) recently described an HPLC method using a Novapack end-capped C_{18} column with amperometric detection for quantitative analysis of erythromycin base and propionate. However, a rapid decrease in detector sensitivity (up to 60% in 24 hours) was reported.

HPLC was chosen for this study because of its inherent advantages over other analytical methods. The application of ultraviolet and electrochemical amperometric and coulometric detection methods was investigated.

2.3 MATERIALS AND APPARATUS

2.3.1. Materials

Drug samples used:

Erythromycin Base USP		(1)	
Erythromycin Base	LOT 85F0251	(2)	
Erythromycin Estolate USP		(1)	
Erythromycin Stearate	LOT-07-616-CD	(3)	
Erythromycin Enol-Ether	LOT 3511-55A	(3)	
Anhydroerythromycin		(3)	see Section 2.4.3
Des-N-methylerythromycin	LOT 164-171-185-2	(4)	
Oleandomycin phosphate	LOT 206/76 7003	(5)	

(1) USP Reference Standards, Rockville, USA
(2) Sigma Chemical Company, St Louis, USA
(3) Abbott Laboratories, North Chicago, USA
(4) Lilly Laboratories, Indianpolis, USA
(5) Pfizer Laboratories, Pietermaritzburg, South Africa

All reagents were of at least analytical grade quality. Hydrochloric acid and sodium hydroxide were obtained from BDH Chemicals, Poole, England, while phosphoric acid was obtained from Carlo Erba, Milan, Italy.

The acetonitrile used in this study was distilled-in-glass grade and purchased from Burdick and Jackson Laboratories, Muskegon, Mich., USA.

HPLC-grade water was prepared by reverse osmosis purification followed by passage through a Milli-Q (Millipore Corp., Bedford, Mass, USA) system.

2.3.2 High-Performance Liquid Chromatographic System

The HPLC system employed was as follows:

Model 6000A liquid chromatographic pump (Waters Associates, Milford, Mass., USA)

Model 710B WISP automated sample injector (Waters Associates, Milford, Mass., USA)

Column - (see Section 2.4.4)

Detector - (see Section 2.4.6)

Model 561 strip-chart recorder (Perkin-Elmer Corp., Norwalk, Conn., USA)

2.3.3 Additional Equipment

The following were employed throughout the study:

Type 2004 MP6 five-figure precision balance (Sartorius GmbH, Gottingen, W. Germany)

Model 8845-30 ultrasonic cleaner (Cole-Parmer Instrument Co., Chicago, Ill., USA)

Model 601 digital ionalyser (Orion Research Incorp, Cambridge, Mass., USA)

Model M-16710-12 'maxi-mix' vortex mixer (Thermolyne Corp., Dubuque, Iowa, USA)

Model HN-SII general purpose centrifuge (International Equipment Co., Needham Heights Mass., USA)

Model MFC BAS microfiltration centrifuge (Bioanalytical Systems Inc., W. Lafayette, USA)

Model SVC-100H Speed Vac Concentrator (Savant) (Savant Instruments Inc., Hicksville, New York, USA)

Model 10302 Vacuum pump (Lammert, Addison, Ill, USA)

Model A-25 Eyela aspirator (Tokyo Rikakikai Co. Ltd. Tokyo, Japan)

Model 3521 reciprocating mechanical shaker (Lab-line Instruments Inc., Melrose Park, Ill., USA)

Fixed and variable volume Transferpettes (Brand, W. Germany)

Magnetic stirrer (GallenKamp, England)

Column packer (HPLC Technology, Runcorn, England)

Heater (Hanke, Dieselstrasse, Berlin, Germany)

Grade A glassware was employed throughout the study. All glassware was cleaned either in chromic acid or Decon 75 concentrate (Atomic Export Import corp. (Pty) Ltd., Johannesburg, South Africa) before being thoroughly rinsed with distilled and HPLC grade water and dried prior to use.

2.4 EXPERIMENTAL

2.4.1 Stock Solution

Stock solutions of all drugs used were prepared by dissolving 25mg of the appropriate drug sample in acetonitrile and making up to volume in a 25ml volumetric flask. Dilutions were made with acetonitrile to produce the concentrations required.

2.4.2 Mobile Phase Preparation

Mobile phase was prepared by mixing the required amount of buffer solution with the required amount of acetonitrile. No correction for volume changes that occured upon mixing were made. The mobile phase was deaerated and filtered through a 0.45µm membrane filter (Type HVLP, Millipore Corp, Bedford, Mass., USA) prior to use.

Phosphate buffer was prepared by pipetting a specific volume of phosphoric acid (depending upon the required molarity) into a 1 litre volumetric flask and making up to volume with HPLC-grade water. Sodium hydroxide pellets were then added to adjust the buffer to the required pH.

2.4.3. Synthesis of Anhydroerythromycin

Anhydroerythromycin obtained from Abbott Laboratories, North Chicago, USA was found to be impure. The synthesis of anhydroerythromycin from erythromycin base (obtained from Sigma Chemical Company, St. Louis, USA) according to the method reported by Wiley *et al* (125) was undertaken. Initially this yielded a product which contained anhydroerythromycin and erythromycin enol-ether. The method was repeated, but the

erythromycin base was allowed to stand in hydrochloric acid for sixty hours to allow complete degradation. A small amount of anhydroerythromycin was obtained, in this manner.

2.4.4 Columns and Mobile Phases

Numerous columns and mobile phases were investigated in order to optimize separation of erythromycin A, erythromycin ethol-ether and anhydroerythromycin (see Table 2.1). Preliminary studies were performed using ultraviolet detection (see Section 2.4.6.1. ii). Using different columns, several mobile phases of phosphate buffer/acetonitrile differing only in aqueous/organic ratios were investigated. The effect of buffer strength and pH on the elution of erythromycin and degradation compounds was examined. In addition, using a mobile phase of 0.05M phosphate buffer (pH 6.3)/acetonitrile (35/65), the effect of varying the flow rate between 1.0ml/min and 2.0ml/min, on retention characteristics was investigated.

(i) C_8 Reverse Phase Column (10µm)

A 25cm x 3.9mm internal diameter (i.d) column was packed with Techsil 10µm octylsilane (C_8) material by the method described in section 2.4.5. Using this column numerous mobile phases varying in aqueous/organic ratio were used. In addition, the effect of varying buffer pH (pH range 3,8-6,32) and buffer molarity were characterised (Table 2.1)

(ii) Licrosorb C_8 Reverse Phase Column (7µm)

A commercially available 25cm x 3.0mm i.d. Lichrosorb column packed with octylsilane (C_8) material was used. Mobile phases of varying aqueous/organic ratios were investigated.

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

This was a 25cm x 3.9mm i.d. column packed with Techsil 10um octadecylsilane (C_{18}) material.

(iv) Novapak End-capped Reverse Phase Column

This commercially available 15cm x 3.9mm i.d. column packed with octadecylsilane (C_{18}) material (Novapak, Waters Associates). A mobile phase of 0.05M phosphate buffer (pH 6.32)/acetonitrile (70/30) was used.

Table 2.1 Columns and mobile phases used

COLUMN	CH_3CN / BUFFER	BUFFER MOLARITY	BUFFER pH
1. C_8 10μm	40/60	0.05	6.32
	65/35	0.05	6.32
	60/45	0.10	6.32
	60/40	0.10	6.32
	60/35	0.10	6.32
	30/70	0.10	4.44
	35/65	0.10	4.44
	40/60	0.10	4.44
	50/50	0.10	4.44
	60/40	0.10	4.44
	40/65	0.10	3.80
	40/60	0.10	3.80
2. Lichrosorb RP-8 7μm	30/70	0.05	6.32
	40/60	0.05	6.32
	50/50	0.05	6.32
3. C_{18} 10μm	40/60	0.05	6.32
	65/35	0.05	6.32
	50/50	0.05	5.00
	65/35	0.05	5.00
	40/60	0.10	5.00
	50/50	0.10	5.00
	65/35	0.10	5.00
4. Novapack C_{18} end-capped	30/70	0.05	6.32

2.4.5 Column Packing

The columns were packed using the slurry packing method in conjunction with an HPLC column packer.

The empty column was thoroughly rinsed with methanol and dried. The column end frits were sonicated in 6N nitric acid for 5 minutes, after which they were thoroughly rinsed. Three grams of column packing material were weighed out and slurried in 40ml of carbon tetrachloride containing 10% methanol and without delay placed in the slurry reservoir. One hundred and fifty millilitres of degassed isopropanol was pumped through the system at a pressure of $3,5 \times 10^3$ kPa. Without interrupting the flow of solvent the column was inverted, and a further 150ml of solvent was pumped through the system. The pump was shut off and the column allowed to stabilize for 30 minutes. The column was removed from the column packer, and checked using a test mixture.

2.4.6 Detectors

2.4.6.1 Ultraviolet Detectors

(i) Hewlett Packard 1040A Diode-Array Detector

The Hewlett Packard 1040A Diode-Array detector in conjunction with an HP85 personal computer, an HP 9121 dual disc drive and an HP 7470A X-Y plotter was used to obtain ultraviolet spectra of erythromycin A, erythromycin enol-ether and anhydroerythromycin. The data storage facility allowed post-run manipulation of chromatograms.

(ii) Pye-Unicam LC-UV Detector

This variable wavelength detector was operated at a wavelength of 200nm at a sensitivity setting of 0.02 absorbance units full scale (AUFS). This detector was used to perform preliminary work on column and mobile phase optimization

since stabilization following mobile phase adjustments occurs more rapidly with UV detection than with electrochemical detection, which may take many hours to stabilize.

2.4.6.2 Electrochemical Detectors

(i) Metrohm Amperometric Electrochemical Detector

A Metrohm Model 656 electrochemical detector was used in conjunction with a Metrohm Model 641 VA current/voltage console. The electrode system, which is depicted in Fig. 2.2, consists of a glassy carbon working electrode (WE), a glassy carbon auxilary electrode (AE), and a silver/silver chloride/KCl reference electrode.

Prior to use the reference electrode must be filled with 3M potassium chloride solution. In addition, the WE and AE must be polished using a polishing cloth and aluminium oxide paste.

(ii) Coulochem Coulometric Electrochemical Detector

The Coulochem Model 5100A was used and consisted of an electronic console, an analytical cell and a guard cell.

The Model 5010 analytical cell included a dual electrode containing two porous graphite in-line working electrodes.

The detector was operated in the single electrode mode using only one of the two electrodes or in the screen mode using both electrodes.

The Model 5020 Guard cell consisted of a single porous graphite working electrode. This was placed between the solvent delivery system and the injector in the HPLC system and operated at a potential of +1.0V. As such the guard cell electrolysed any contaminants in the mobile phase.

Construction of Voltammograms

Hydrodynamic voltammograms were constructed for both detectors. The detectors were set at a relatively low voltage of 0.7V and allowed to stabilize. Twenty microlitres of erythromycin solution (0.05µg/µl) was injected onto the system. The electrode potential was increased, allowed to stabilize and the equivalent amount of erythromycin re-injected into the system.

The peak height measured was converted to nAmps by simple proportion.

2.5 RESULTS AND DISCUSSION

2.5.1 Column and Mobile Phase

The choice of column and optimum mobile phase was dictated by the need to separate erythromycin, erythromycin enol-ether and anhydroerythromycin within a relatively short retention time.

The necessity of having a buffer component in the mobile phase for the elution of erythromycin from a C_{18} and C_8 column has been reported (126, 127). A phosphate buffer was chosen due to its reported column efficiency (88).

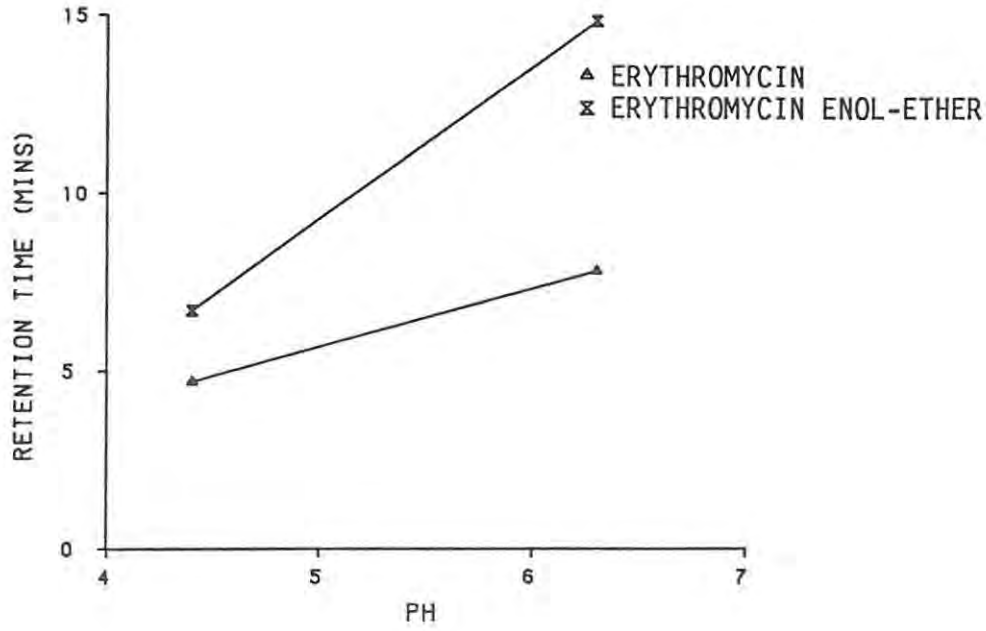
Preliminary studies performed using a C_8 column with ultraviolet detection (200nm) found that the retention time of erythromycin and erythromycin enol-ether and consequently peak shape and separation were affected by numerous factors. Anhydroerythromycin showed poor UV absorption (see Section 2.5.2.1)

The molarity of buffer was found to have a substantial effect on the retention time of the compounds. Increasing the molarity of the

buffer from 0.05M to 0.1M decreased the retention time of erythromycin from 10 minutes to 7.5 minutes, and erythromycin enol-ether from 19.3 minutes to 14.5 minutes.

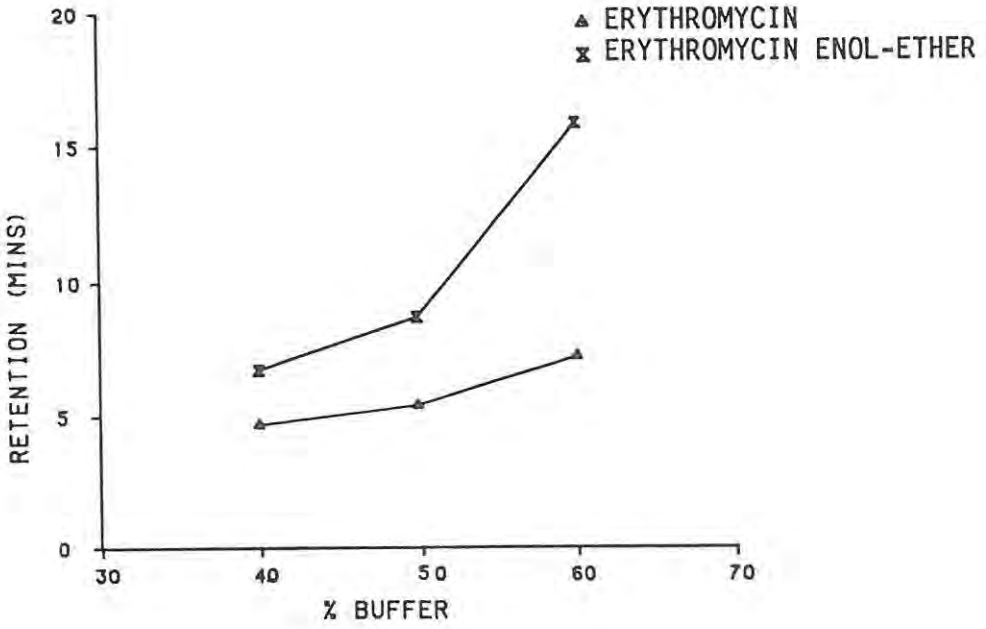
Mobile phase pH was also found to affect retention time. As previously reported (88), the retention time decreased with decreasing pH (Fig 2.4).

Fig 2.4 Effect of buffer pH on retention of erythromycin and erythromycin enol-ether using a C_8 column



The aqueous/organic phase ratio of the mobile phase played an important role in the final choice of mobile phase. Reducing the organic portion ensured the compounds remained on the column longer increasing retention time and hence separation (Fig 2.5).

Fig 2.5 Effect of buffer/acetonitrile ratio on retention of erythromycin and erythromycin enol-ether using a C_8 column

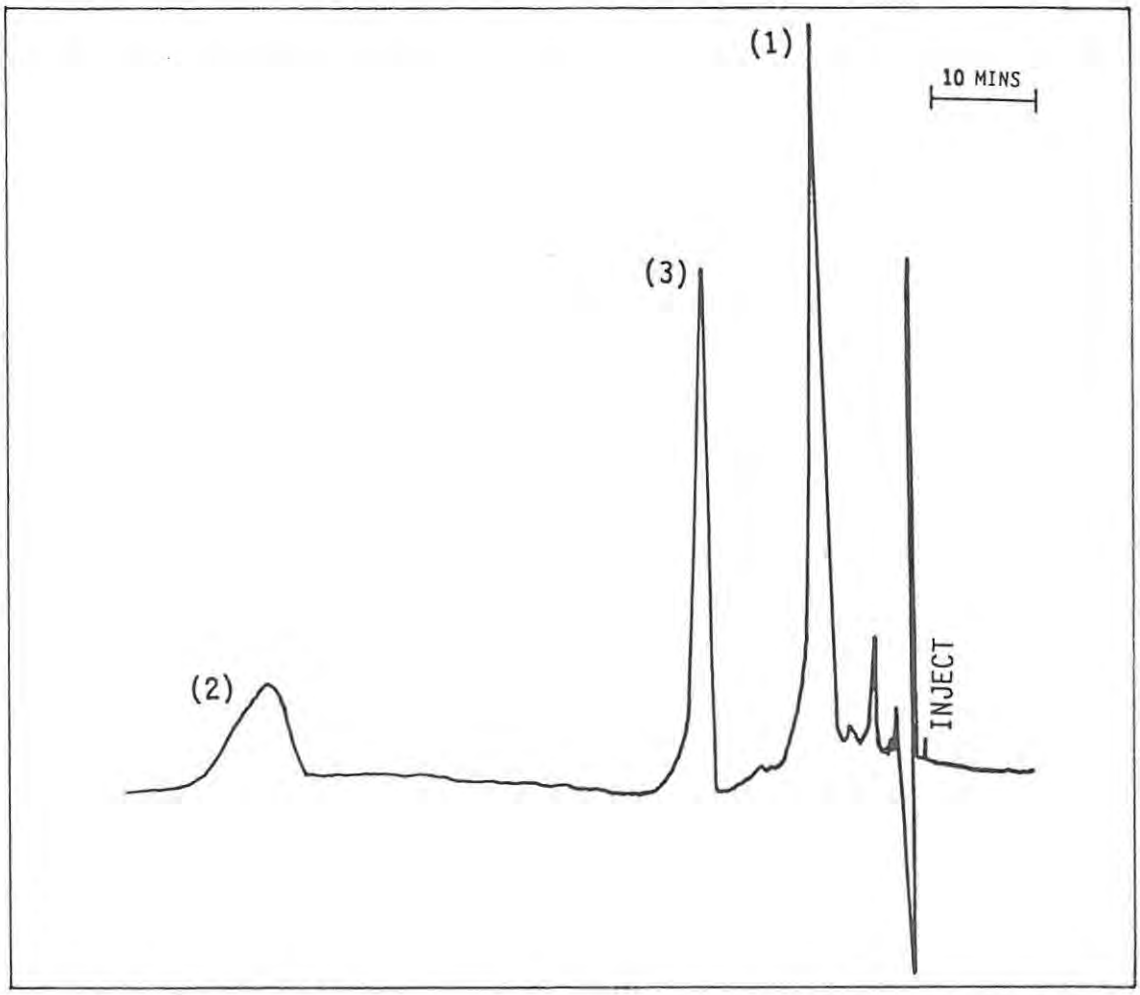


In order to ensure separation of anhydroerythromycin from erythromycin and erythromycin enol-ether, further studies on choice of column and mobile phase were carried out using electrochemical detection.

The Novapak end-capped column, although able to separate erythromycin and its degradation products, was unsuitable due to the extremely long retention time of erythromycin enol-ether (Fig 2.6). This column was excluded from further studies. Similarly, the Lichrosorb C_8 column was excluded from further studies due to poor peak shape.

Fig 2.6 Chromatogram of erythromycin, erythromycin enol-ether and anhydroerythromycin using a Novapack C_{18} column

(1) ERYTHROMYCIN
(2) ERYTHROMYCIN ENOL-ETHER
(3) ANHYDROERYTHROMYCIN



Mobile phase 0.05M phosphate buffer (pH 6.32)/acetonitrile (70/30)

A comparison between the capacity factors of erythromycin, its degradation products and a few derivatives using a mobile phase of 0.05M phosphate buffer (pH 6.3)/acetonitrile (35/65) on a C_8 and a C_{18}

column is reported in Table 2.2. With judicious choice of mobile phase, both the C_8 column and the C_{18} column were seen to resolve erythromycin, anhydroerythromycin and erythromycin enol-ether as seen in Fig 2.7 and Fig 2.8 respectively. As such, both columns were suitable for use in this study.

Table 2.2 Capacity factors of erythromycin and various degradation products and derivatives

COMPOUND	C_8 COLUMN K'	C_{18} COLUMN K'
Des-N-methylerythromycin	3.00	2.67
Erythromycin	3.75	3.83
Anhydroerythromycin	4.75	4.83
Erythralosamine	4.77	5.17
Erythromycin enol-ether	8.00	9.33

Mobile phase : 0.5M phosphate buffer (pH 6.3)/CH_3CN (35/65)

Fig 2.7 Chromatogram of erythromycin, erythromycin enol-ether and anhydroerythromycin using a Techsil C_8 10um column
Mobile phase 0.1M phosphate buffer (pH 4.4)/acetonitrile (65/35)

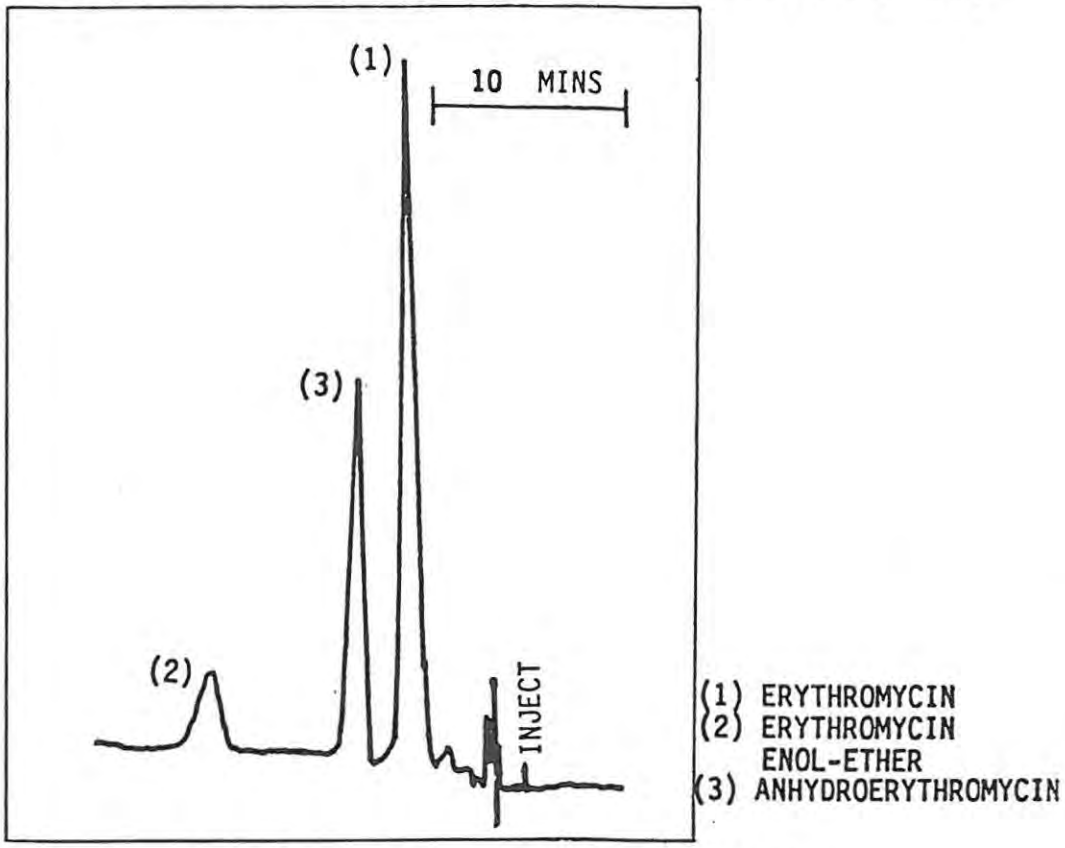
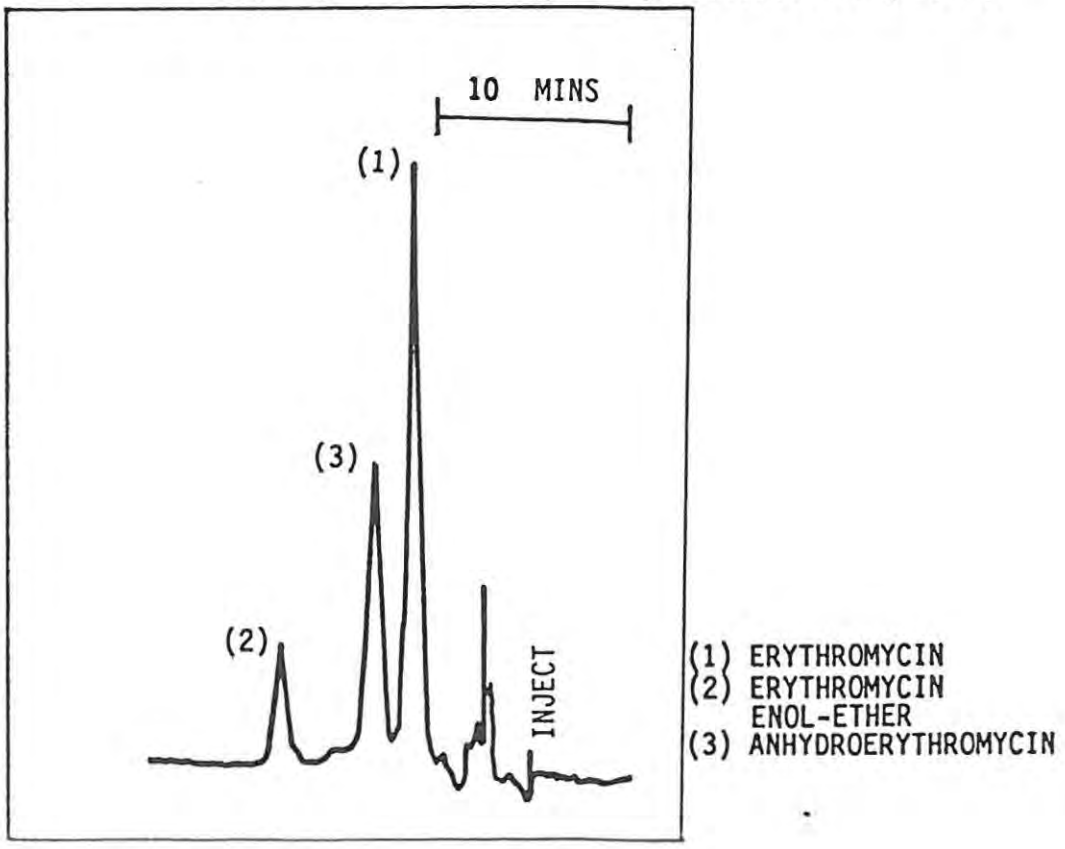
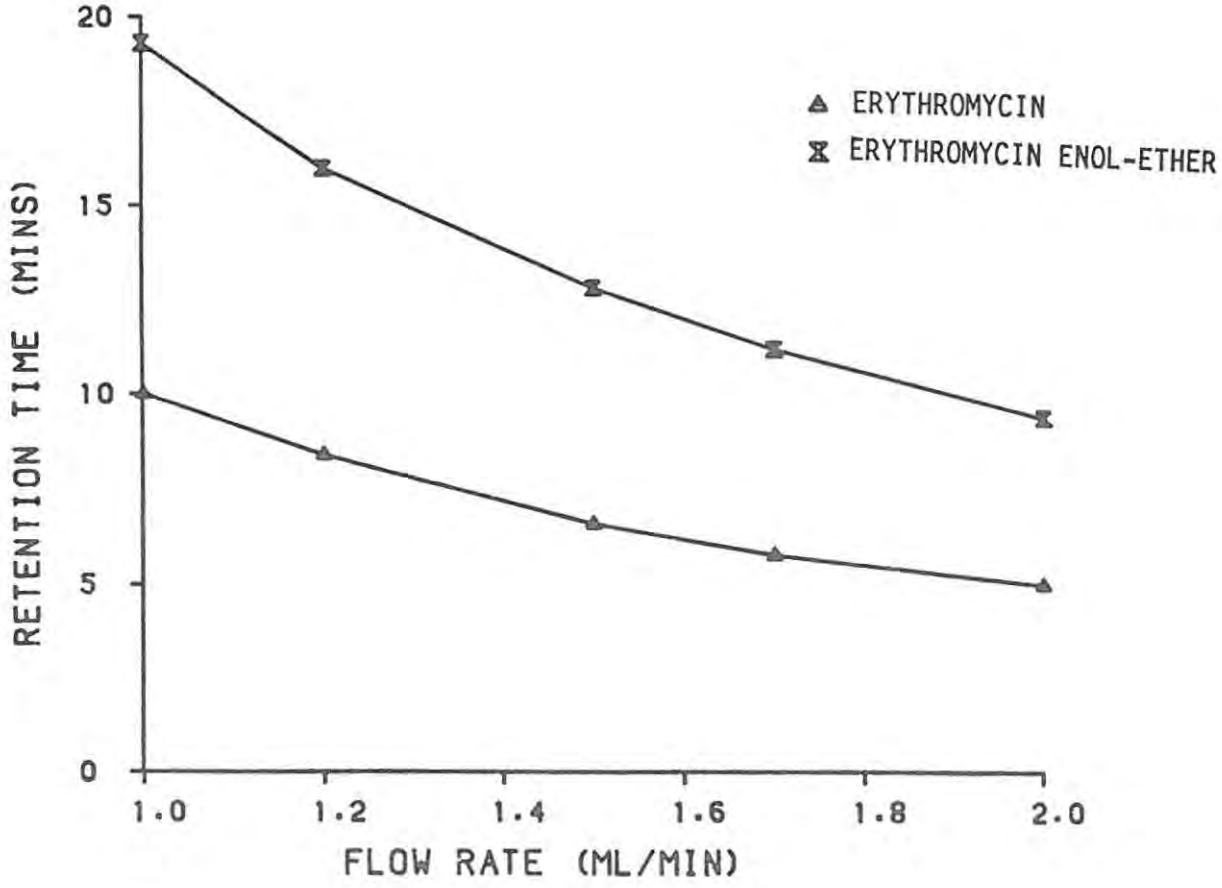


Fig 2.8 Chromatogram of erythromycin, erythromycin enol-ether and anhydroerythromycin using a Techsil C_{18} 10um column
Mobile phase 0.1M phosphate buffer (pH 5.0)/acetonitrile (60/40)



Increasing the flow rate of the mobile phase from 1.0ml/min to 2.0ml/min greatly reduced analysis time (Fig 2.9). However, due to prohibitive back pressures, which would reduce column life, an optimum flow rate of 1.3ml/min was selected.

Fig 2.9 Effect of mobile phase flow rate on retention of erythromycin and erythromycin enol-ether



2.5.2 Detectors

2.5.2.1 Ultraviolet Detectors

The ultraviolet spectrum of erythromycin is shown in Fig 2.10. In the wavelength range examined (194nm-294nm) it can be seen that erythromycin only significantly absorbs UV radiation below 215nm with maximum UV absorption at 194nm. Anhydroerythromycin has a UV spectrum similar to erythromycin, showing a UV maximum at 194nm (Fig 2.11). However the ultraviolet spectrum of erythromycin enol-ether revealed a UV maximum at 210nm (Fig 2.12).

Anhydroerythromycin showed very poor UV absorption characteristics (119) and a UV spectrum was only obtained following an on-column load of 100µg.

Fig 2.10 Ultraviolet spectrum of erythromycin

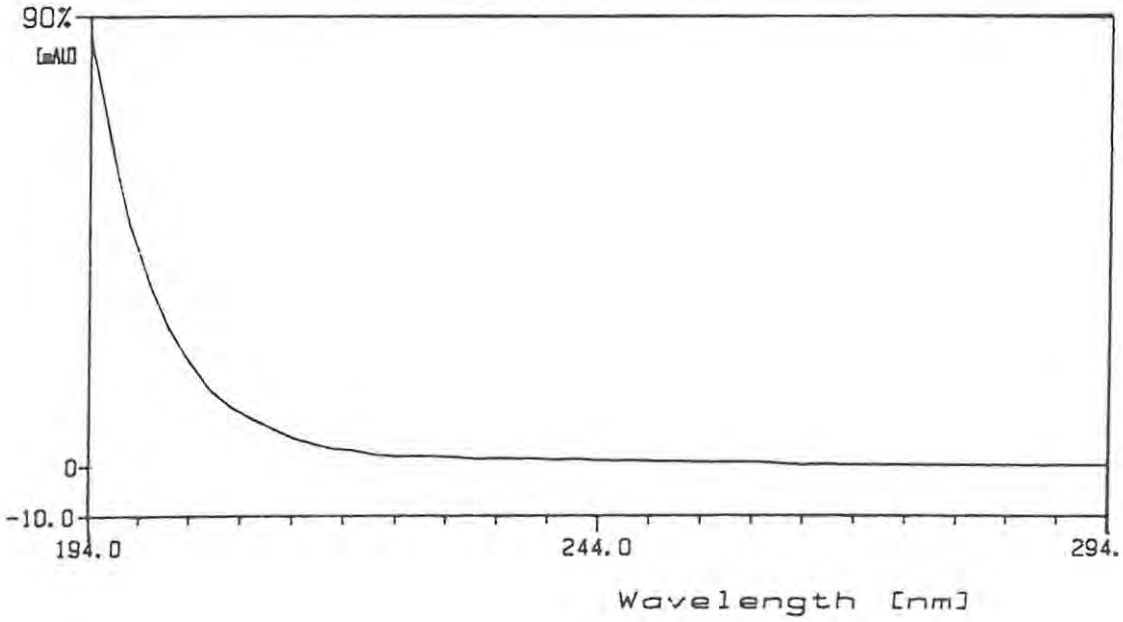


Fig 2.11 Ultraviolet spectrum of anhydroerythromycin

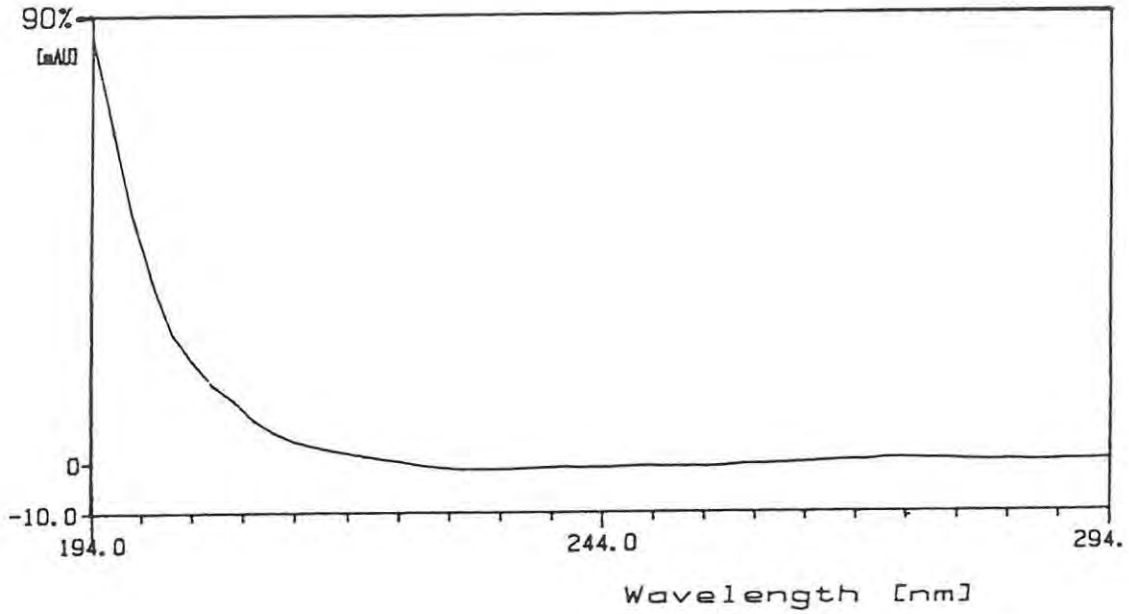
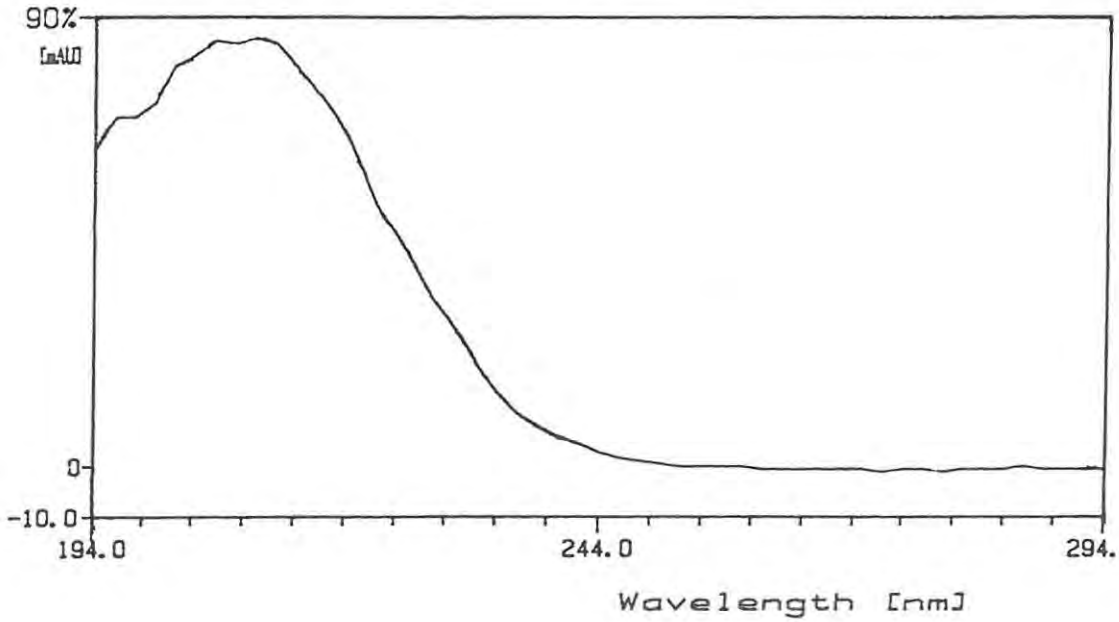


Fig 2.12 Ultraviolet spectrum of erythromycin enol-ether



2.5.2.2 Electrochemical Detectors

Erythromycin has previously been analysed by electrochemical detection in the oxidative mode using a voltage of 0.80V (34, 119, 123). In this study only the oxidative mode was used. The voltammograms prepared are shown in Fig 2.13. It can be seen that erythromycin has a lower half wave potential (0.80V) at the porous graphite electrode than at the glassy carbon electrode (1.15V).

For the Metrohm detector, the best compromise between detector response and baseline noise was an electrode potential of 1.10V.

For the Coulochem detector operating in the single electrode mode a voltage of 0.90V was used. However, when both electrodes were used the upstream screening electrode was operated at 0.70V and the downstream electrode at 0.90V.

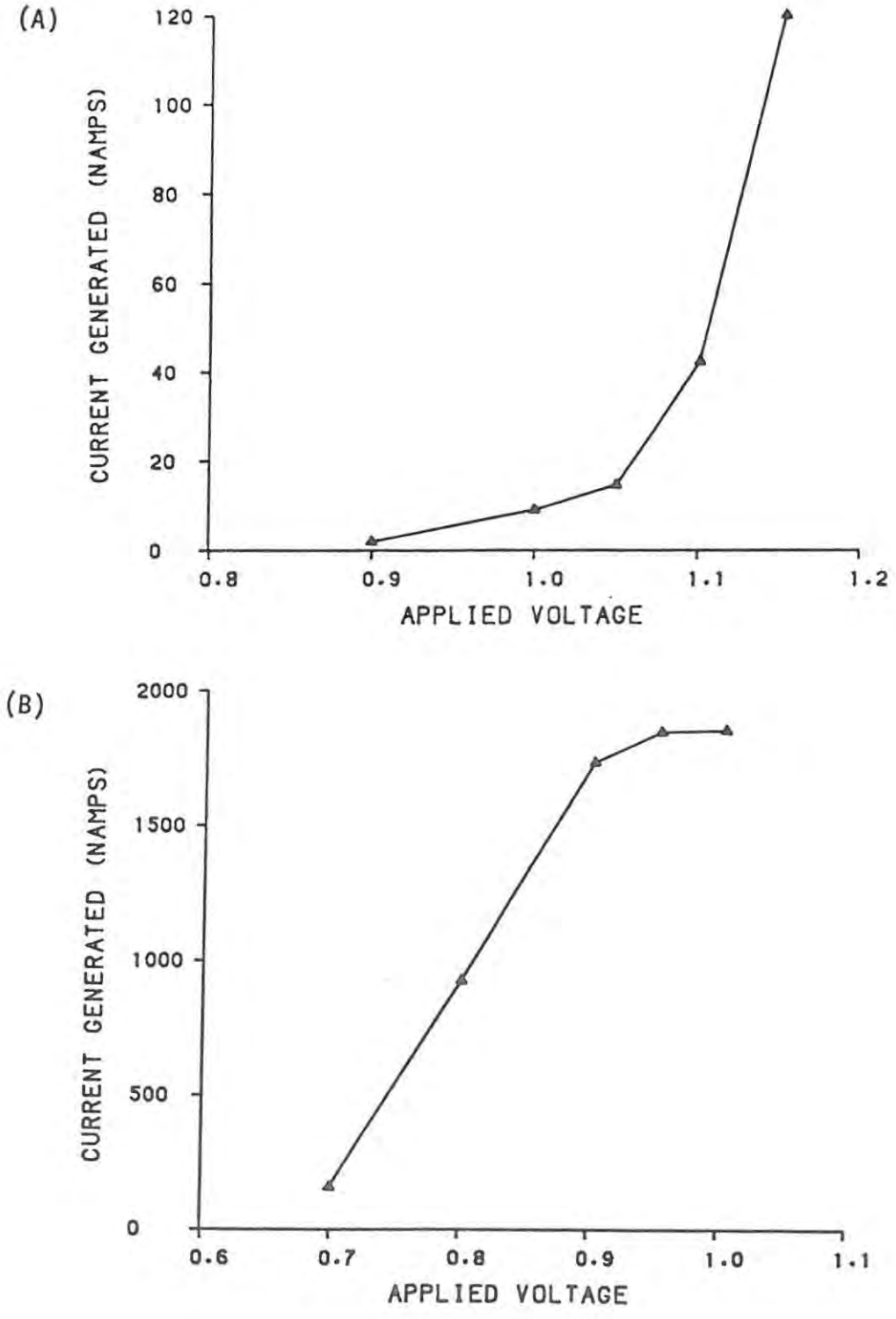
Use of electrochemical detection showed greatly increased sensitivity to anhydroerythromycin.

2.5.2.3 Choice of Detector

A sensitive reproducible means of detection capable of measuring erythromycin, erythromycin enol-ether and anhydroerythromycin was required. Although absolute sensitivity of the compounds was not a major concern for the *in vitro* studies, in order to quantify all three compounds simultaneously it was necessary that they have sensitivities of similar magnitude. As such, although ultraviolet detection (200nm) of erythromycin and erythromycin enol-ether was adequate, due to the low UV absorptivity of anhydroerythromycin, ultraviolet detection was unsuitable.

FIG 2.13 Hydrodynamic voltammograms of erythromycin

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

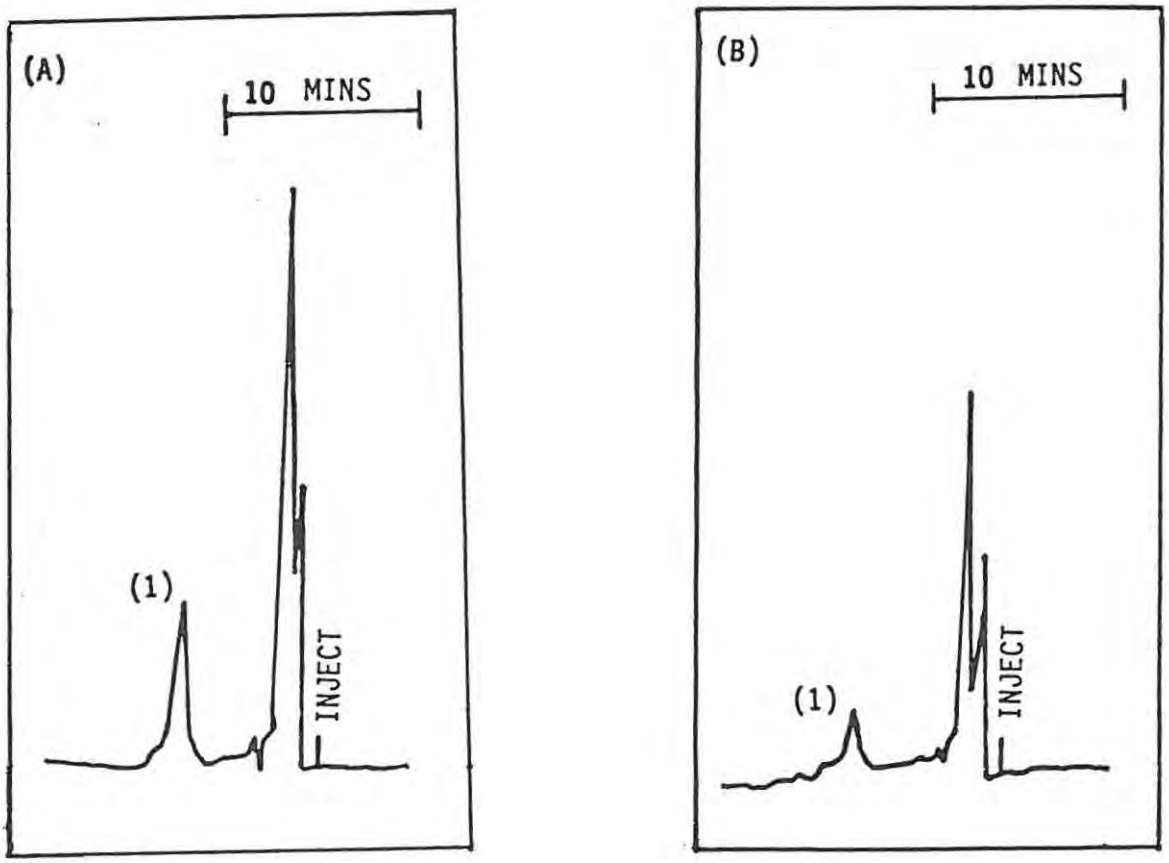


This lack of sensitivity of anhydroerythromycin was overcome by the use of electrochemical detection. The amperometric detector, although able to detect all three compounds, rapidly lost sensitivity, necessitating the frequent repolishing of electrodes. An example of the rapid loss of sensitivity is shown in Fig. 2.14. This rapid loss of sensitivity by a glassy carbon electrode has been reported in the literature (123, 124). The amperometric detector was therefore not used in subsequent studies.

Fig. 2.14 Chromatogram of erythromycin

(A) Time = 0 hours
(B) Time = 18 hours later

Load 1µg



(1) ERYTHROMYCIN

The coulometric detector operated in the oxidative mode was therefore adopted for all further studies since it provided a suitable means of simultaneously detecting all three compounds. Although the coulometric electrode did not exhibit the rapid loss of sensitivity seen in the amperometric detector, a gradual decrease in sensitivity did occur after constant use. Cleaning the electrodes with 6N HNO_3 returned cells to the original condition. However, sensitivity of older cells showed limited improvement following cleaning.

Sudden decreases in sensitivity were observed when new batches of mobile phase were introduced. However, by recycling the mobile phase a general increase in sensitivity to a plateau was observed, presumably due to elimination of electroactive contaminants by the guard cell.

The coulometric detector was also found to provide the necessary sensitivity for the analysis of erythromycin in serum and urine at concentrations found in humans after the administration of oral dosage forms. (*vide infra* Chapter 4).

2.5.3 Solvent degassing

Although the mobile phase was degassed prior to use, an in-line vacuum degassing unit was used to ensure a low dissolved gas concentration in the mobile phase.

2.6. CONCLUSIONS

An HPLC method using either a Techsil C_{18} 10μm stationary phase with a mobile phase of 0.1M phosphate buffer (pH 5.0)/acetonitrile (60/40) or

a Techsil C_8 10μm stationary phase with a mobile phase 0.1M phosphate buffer (pH 4.4)/acetonitrile (65/35) was suitable for separating erythromycin, erythromycin enol-ether and anhydro-erythromycin.

The Coulochem coulometric detector operated in the single electrode mode at a polarizing voltage of 0.90V provided a suitable means of detection.

CHAPTER 3

ACID DEGRADATION STUDIES OF ERYTHROMYCIN

3.1 INTRODUCTION

Chemical kinetics is concerned with the rates of reactions and the mechanisms by which reactions occur (128). The first goal in a kinetic investigation is the determination of the rate equation (129). The rate equation defines the relationship between the reaction rate and the reactant concentrations.

Zero order reactions are reactions in which the rate is unaffected by changes in reactant concentration and therefore the rate of change of concentration of the reactant with time is constant, K^o. (Equation 3.1)

$$\frac{-dC_a}{dt} = K^o \qquad \text{Equation 3.1}$$

Integration of this equation yields Equation 3.2.

$$Ca = Ca^o - K^o t \qquad \text{Equation 3.2}$$

where C_a = concentration of reactant at any time, t

C_a^o = concentration of reactant at time zero

t = time

From Equation 3.2 it can be seen that a plot of C_a against time, t, will be linear and the slope is equal to the zero order rate constant, K^o. In general however, the rate of a chemical reaction is dependent on the concentration of the reactants. For the simple first order generalized reaction A --> B, the rate equation is defined in Equation 3.3.

$$\frac{-dC_a}{dt} = Kt \qquad \text{Equation 3.3}$$

Integration of this equation yields the exponential Equation 3.4.

$$C_a = C_a^0 e^{-Kt} \qquad \text{Equation 3.4}$$

From Equation 3.4 it can be seen that a plot of ln C_a against, time, t, will be linear and the slope is equal to the first order rate constant, K.

The half-life, $t_{1/2}$ is defined as the time for the concentration of reactant to reach half the initial concentration. In a first order reaction the half-life is independent of concentration and can be calculated using Equation 3.5.

$$t_{1/2} = \frac{0.693}{K} \qquad \text{Equation 3.5}$$

For the generalized reaction A + C --> D, if the concentration of C at time = 0, is much less than the concentration of A, the reaction will behave as if it were a first order reaction; it is said to be a pseudo-first order reaction.

Therefore in order to investigate the rate of degradation of a reactant using the integral method, the concentration variation of the reactant with time must be measured.

However, if a reaction is extremely slow in terms of the time available to study it, or if its kinetic behaviour becomes complicated in its later stages, it may be preferable to use the 'initial rates' method. It is then only necessary to monitor the initial 10% of the degradation reaction (130). Throughout this period of observation, the change in concentration of reactant is then assumed to be negligible (C_a approximately equal to C_a^0) and the initial velocity of the reaction is a constant. K^0 is obtained from the slope of the plot of C_a against time. The first order rate constant is subsequently calculated using Equation 3.6 (see Appendix I).

$$K = \frac{K^o}{C_a{}^o}$$ Equation 3.6

It has been suggested however, that instead of monitoring drug reactant concentration with time, monitoring degradation product with time may provide an alternative and better approach to kinetic studies. Using this method for the generalized reaction A --> B, the concentration of product, B, is measured for as long as it is necessary to evaluate $\left(\frac{dC_b}{dt}\right)_{t=0}$, where $\left(\frac{dC_b}{dt}\right)_{t=0}$ is the rate of change of product formed over the initial part of the reaction. The first order rate constant, K, is calculated using Equation 3.7.

$$K = \frac{\left(\frac{dC_b}{dt}\right)_{t=0}}{C_a{}^o}$$ Equation 3.7

A few reports on the use of the initial rates method have appeared in the literature (10, 130-134). Taylor *et al* (134) reported good agreement between rate constants obtained by measuring parent drug decrease and degradation product increase following a study on the degradation of 3,5 diiodoaspirin. A further study also showed the use of initial rates method to be a reliable means of calculating rate constants for the simple case of a drug decomposing irreversibly to a single product (133). However, when this method was applied to a more complex tetracycline degradation only limited success was achieved (132). A kinetic study on the degradation of erythromycin by Atkins *et al* (10) obtained rate constants using the initial rate method, monitoring both

erythromycin disappearance and erythromycin-enol-ether appearance. Results obtained using both methods are summarized in Table 3.1. Unfortunately, no attempt was made to calculate rate constants using the integral method which would have enabled a comparison between the integral and the initial rates methods.

Table 3.1 Rate constants for erythromycin degradation using the 'inital rates' method

Buffer	pH	$10^{-5}K(min^{-1})^a$	
		Erythromycin disappearance	Erythromycin A 6,9-hemiketal appearance
0.1 M phosphate	6.5	9.11	6.25
	7.0	2.37	2.42
0.1 M phosphate	7.0	6.59	2.74
0.2 M succinate	6.0	81.80	36.90

[a] Obtained by the initial rates method

Reproduced from Atkins *et al* (10).

The rate constant for the reaction can be empirically related to absolute temperature by the Arrhenius equation (Equation 3.8).

$$K = Ae^{\frac{-E_A}{RT}} \qquad \text{Equation 3.8}$$

where A = pre-exponential factor
E_a = activation energy
R = gas constant
T = absolute temperature

From the logarithmic form of the Arrhenius equation (Equation 3.9) it can be seen that the activation energy can be calculated from the slope of a plot of log K against the reciprocal of the absolute temperature.

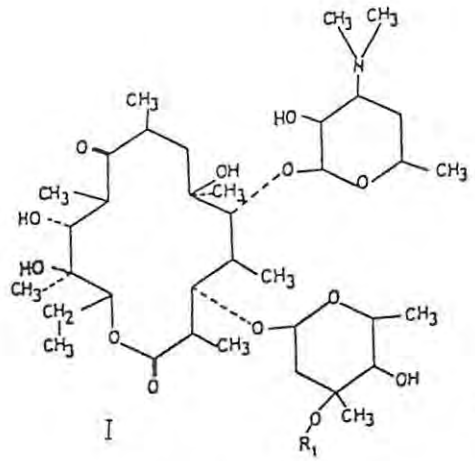
$$\log K = \log A - \frac{E_A}{2.303R} \cdot \frac{1}{T} \qquad \text{Equation 3.9}$$

The effects of varying acid pH on the degradation rates of both erythromycin base and erythromycin stearate, in addition to the effect of pH on the hydrolysis rate of estolate are presented. An investigation on the temperature dependence of erythromycin base degradation was also undertaken.

3.2 PREVIOUS REPORTS ON ERYTHROMYCIN DEGRADATION

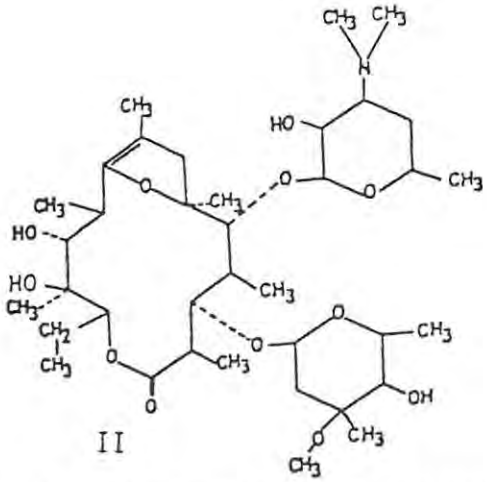
Although there are very few reports in the literature on the stability of erythromycin in aqueous solutions, it has been known for many years that erythromycin is susceptible to acid degradation. The acidic degradation is reported to yield two products depending on the conditions of the reaction (7, 9, 10). The proposed pathway of degradation appears in Fig. 3.1 (9, 10).

Fig 3.1 Proposed acid degradation pathway of erythromycin



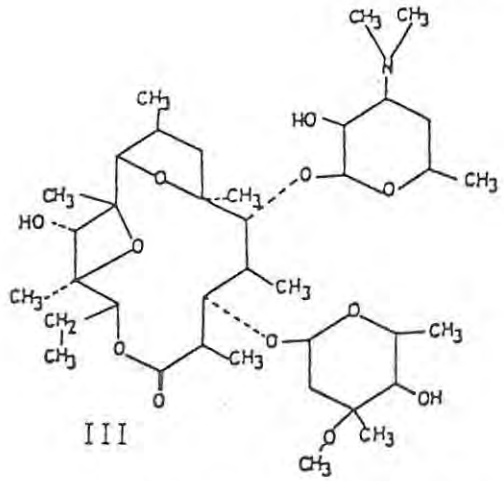
I

ERYTHROMYCIN A



II

ERYTHROMYCIN A 6-9-HEMIKETAL



III

ANHYDROERYTHROMYCIN A

In 1957, Wiley et al (125) reported that very mild acid treatment, pH 2.5 for a few minutes or pH 4.0 for several hours resulted in the formation of anhydroerythromycin (III) (Fig. 3.1). However Kurath et al (9) reported that treatment of erythromycin with glacial acetic acid at room temperature for two hours yields erythromycin A 6-9- hemiketal (erythromycin enol-ether) (II) (Fig. 3.1), whilst further treatment in acid (dilute methanolic hydrochloric acid) for one hour yields the anhydroerythromycin. Alternatively erythromycin A treated in strong acid (hydrochloric acid, pH 2.0, 30 minutes) only yields the anhydroerythromycin.

Although the formation of erythromycin enol-ether and anhydroerythromycin occurs at acidic pH values, at pH 8.8 and above dihydroerythromycin is the major degradation product (116).

An early report investigating the effects of both pH and temperature on the rates of degradation of erythromycin concluded the pH of maximum stability was approximately pH 8.0 and this decreased with increasing temperature. A further study on the stability of erythromycin found the pH of optimum stability to be between pH 7.0 and pH 7.5 (135). Although this latter study was extensive, no details of assay method were given. Atkins et al (10), investigating a variety of buffer species in the pH range 3.06 - 7.46, and temperature and buffer concentration on the rates of erythromycin degradation concluded that pH is the critical factor in determining erythromycin stability in acidic aqueous solutions.

Unlike erythromycin base, erythromycin stearate is thought to be acid stable, primarily due to its limited acidic solubility. However conflicting reports on the acid stability of erythromycin stearate have appeared in the literature. Garrod et al (39) claimed erythromycin stearate to be acid stable whilst Wade (136) stated erythromycin stearate is acid labile. Using microbiological

assay techniques Stephens et al (8) and more recently Boggiano and Gleeson (51) also reported that in vitro exposure of erythromycin stearate to acid results in rapid degradation.

Like erythromycin stearate, erythromycin estolate is also reported to be acid stable primarily due to its acid insolubility (36, 51). The estolate which is primarily absorbed from the gastro-intestinal tract as the propionyl ester is hydrolysed in serum to the active base. As such, a few reports on the rate of hydrolysis in serum have appeared in the literature (137). However there is little reported on the aqueous hydrolysis of the estolate.

3.3 MATERIALS AND APPARATUS

3.3.1 Materials

As in Section 2.3.1

3.3.2 High-Performance Liquid Chromatographic System

The HPLC system used in this study has been previously described (section 2.3.2). A C_{18} 10um column and a dual electrode electrochemical detector was used.

3.3.3 Additional Equipment

As in Section 2.3.3

3.4. EXPERIMENTAL

3.4.1 Stock Solutions

Stock solutions of erythromycin A were prepared by dissolving 50mg of drug sample in acetonitrile and making up to volume in a 10ml volumetric flask. Stock solutions of erythromycin enol-ether and

anhydroerythromycin were prepared by dissolving 25mg of appropriate drug sample in acetonitrile and making up to volume in a 25ml volumetric flask. Dilutions were made with acetonitrile to produce the required concentrations.

3.4.2 Chromatographic Conditions

HPLC	As in Section 2.3.2
Mobile phase	0.1M phosphate buffer (pH 5.0)/acetonitrile (60/40)
Flow rate	1.3 ml/min
Detection	Coulometric +0.90V
Recorder input	10mV

3.4.3 Test Solutions

The rate of degradation in several solutions was investigated. These test solutions were prepared as follows:

A) Phosphate Buffer Test Solution

Phosphate buffer test solutions were prepared by pipetting 6.7ml of phosphoric acid into a 1 litre volumetric flask and making up to volume with HPLC-grade water. The solution was adjusted to the required pH using analytical grade sodium hydroxide pellets.

B) Hydrochloric Acid Test Solution

Hydrochloric acid test solutions were prepared by diluting concentrated hydrochloric acid with HPLC-grade water.

3.5 ERYTHROMYCIN BASE

3.5.1 Experimental

3.5.1.1 Sample Preparation

Solutions of erythromycin, erythromycin enol-ether and anhydro-erythromycin were prepared as in Section 3.4.1.

3.5.1.2 Calibration Curves

Stock solutions of erythromycin, erythromycin enol-ether and anhydroerythromycin were diluted to yield four different concentrations. Each concentration was assayed in triplicate. The linearity of peak height with concentration was established for the three compounds. Calibration curves were constructed on the days samples were assayed.

3.5.1.3 Sampling Procedure

Method A

The study was initiated by adding 20ml of test solution maintained in a water bath at the required temperature to the pre-weighed drug sample. The solution was then vortexed for 30 seconds. Samples were withdrawn at appropriate times and analysed for erythromycin, erythromycin enol-ether and anhydroerythromycin by HPLC.

Method B

The study was initiated by adding 250µl of erythromycin stock solution (5.0mg/ml) to 5ml of test solution maintained in a waterbath at the required temperature. The test solution was

vortexed for 30 seconds. Samples were withdrawn at appropriate times and analysed for intact erythromycin by HPLC.

Where degradation of erythromycin was rapid, time between sampling was short. Consequently, there was insufficient time to assay the sample before the next sampling time. To overcome this problem it was necessary to use a separate reaction mixture for each individual exposure time in the reaction solution. In order to ensure that the use of different starting samples did not affect the results, five samples were assayed for erythromycin immediately after the addition of 20ml of test solution to the erythromycin sample (method A) and five samples were assayed immediately after the addition of 250µl stock solution to 5ml of test solution (method B).

3.5.1.4 Precision

Within-run precision was assessed by degrading five erythromycin samples (method B) in hydrochloric acid, pH 3.2 for 10 minutes. The samples were assayed for intact erythromycin by HPLC.

3.5.1.5 Degradation in Phosphate Buffer Test Solution

A Erythromycin Powder

All studies were performed in phosphate buffer test solution prepared as in Section 3.4.3. The method was carried out as reported in Section 3.5.1.3 (Method A) using 4.7mg erythromycin base with all samples maintained at 37°C. The degradation was carried in duplicate using the following test solutions.

TEST SOLUTION	pH
1	3.2
2	4.3
3	5.5
4	6.5
5	7.0

B Erythromycin Solution

All studies were performed in phosphate buffer test solution prepared as in Section 3.4.3. The method was carried out as reported in Section 3.5.1.3 (method B) with all samples maintained at 37°C. The degradation was carried out in duplicate using the following test solutions.

TEST SOLUTIONS	pH
1	3.2
2	4.3
3	5.5
4	6.5
5	7.0

3.5.1.6 Degradation in Hydrochloric Acid Test Solution

All studies were performed in hydrochloric acid test solution. The method was carried out as in Section 3.5.1.3. (method B) in duplicate, with all samples maintained at 37°C, using the following test solutions.

TEST SOLUTIONS	pH
1	2.2
2	2.9
3	3.2

3.5.1.7 Effect of Temperature

All studies were performed in 0.1M phosphate buffer, pH 3.0. The method was carried out as in Section 3.5.1.3 (method B) with samples maintained at constant temperature in a water bath. The degradation was carried out in duplicate.

3.5.1.8 'Initial Rates' Method

All studies were performed in 0.1M phosphate buffer, pH 4.3, pH 5.5 and pH 7.0. The method was carried out as in section 3.5.1.3 (method A) with samples maintained at 37°C in a water bath. The studies were carried out in duplicate. Samples were analysed in order to compare rate constants calculated by the integral method as well as the initial rates method using erythromycin decrease and erythromycin enol-ether and anhydroerythromycin increase.

The first order rate constants were calculated from the apparent zero order decrease in erythromycin concentration using the relationship (129) shown in Equation 3.10

$$K = \frac{\left[\frac{dC_a}{dt}\right]_{t=0}^{*}}{C_a^{o}} \qquad \text{Equation 3.10}$$

Similarly, rate constants were also calculated using the increase in both erythromycin enol-ether and anhydroerythromycin using Equation 3.11 and Equation 3.12 respectively.

$$K = \frac{\left[\frac{dC_b}{dt}\right]_{t=0}^{*}}{C_a^{o}} \qquad \text{Equation 3.11}$$

$$K = \frac{\left[\frac{dC_d}{dt}\right]_{t=0}^{*}}{C_a^{o}} \qquad \text{Equation 3.12}$$

* KEY

a ERYTHROMYCIN
b ERYTHROMYCIN ENOL-ETHER
d ANHYDROERYTHROMYCIN

3.5.2 Results

3.5.2.1 Linearity and Calibration Curves

Linearity was established for erythromycin, erythromycin enol-ether and anhydroerythromycin over the range of concentrations studied. Details of the linearity data and the calibration curves for the three compounds are shown in Table 3.2 and Fig. 3.2 respectively.

Table 3.2 Linearity data for erythromycin, erythromycin enol-ether and anhydroerythromycin

Compound	Slope	Y-intercept	Correlation Coefficient
Erythromycin	495.600	-0.0246	0.9997
Erythromycin enol-ether	1265.300	-0.522	0.9990
Anhydroerythromycin	424.630	-0.095	0.9986

3.5.2.2 Sampling Procedure

A relative standard deviation for the assay at time = 0 was found to be 4.6% (n=5) and 2.0% (n=5) for method A and method B respectively.

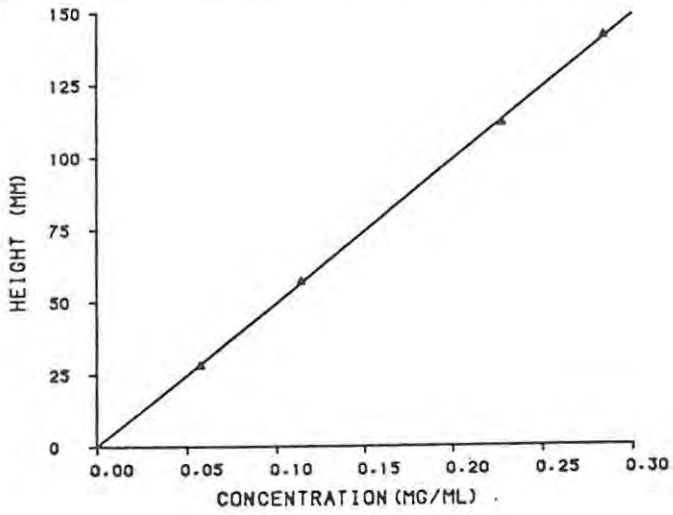
Fig 3.2 Calibration curves for erythromycin, erythromycin enol-ether and anhydroerythromycin

20µl injection volume

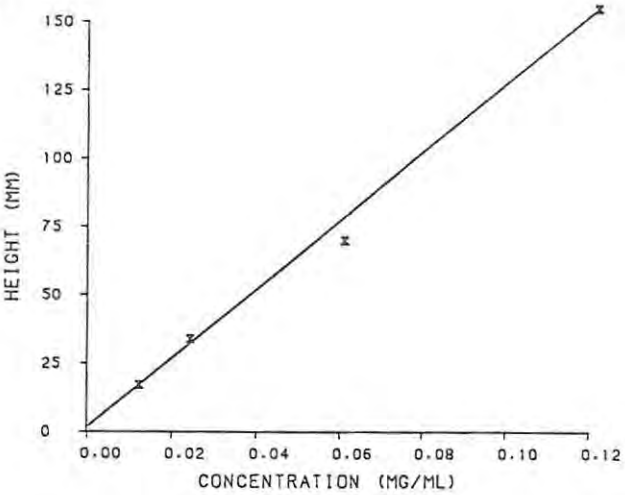
Three samples per data point

(1) ERYTHROMYCIN
(2) ERYTHROMYCIN ENOL-ETHER
(3) ANHYDROERYTHROMYCIN

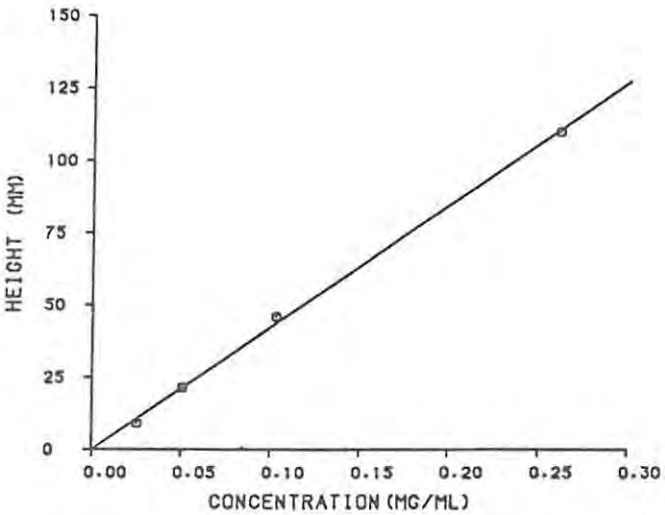
(1)



(2)



(3)



Although there is a slight variation in drug concentration at time zero, the variation is insignificant. As such, where degradation was rapid, a separate reaction mixture for each exposure time in the reaction was used. In addition, therefore, to the normal limitations in experimental assay reproducibility, each result contained a slight deviation due to the variation of drug concentration at the start of the time period.

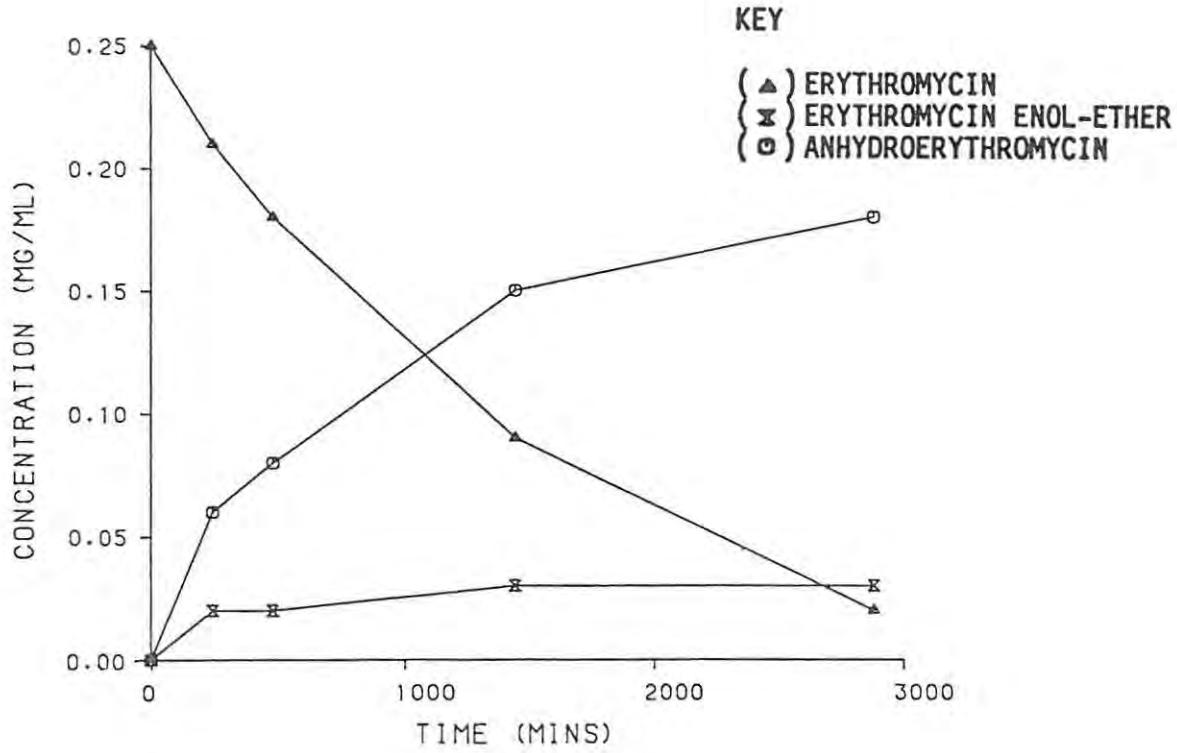
3.5.2.3 Precision

Using five samples a relative standard deviation for assay precision of 3.4% was obtained.

3.5.2.4 Test Solutions

At all pH values investigated the concentration of erythromycin decreases exponentially with time, whilst erythromycin enol-ether shows a rapid increase to approximately 10% of the expected value. This result is in agreement with that reported by Atkins *et al* (10). The subsequent degradation of erythromycin enol-ether to anhydroerythromycin occurs and the concentration of erythromycin enol-ether slowly decreases. An example of the relationship between the concentration of each of the three species in the reaction as a function of time appears in Fig. 3.3. Below pH 5.5 erythromycin enol-ether and anhydroerythromycin (Fig. 3.4) are formed. However, between pH 5.5 and pH 8.0, in addition to the formation of erythromycin enol-ether and anhydroerythromycin, a third compound forms (Fig. 3.5). At pH 8, erythromycin degrades only to the unidentified peak (Fig. 3.6) Tsuji and Goetz (116) reported that above pH 8.8 the major degradation product was dihydroerythromycin. Comparing the retention time of this compound with published data on dihydroerythromycin suggests that the unidentified peak may well be dihydroerythromycin.

Fig 3.3 Plot of concentration versus time for erythromycin, erythromycin enol-ether and anhydroerythromycin



3.5.2.5 Phosphate Buffer Test Solution

A Erythromycin Powder

The individual concentration-time data for erythromycin, erythromycin enol-ether and anhydroerythromycin are listed in Appendix 2 and the curves are depicted in Fig. 3.7.

Fig 3.4 Chromatograms of erythromycin degradation below pH 5.5

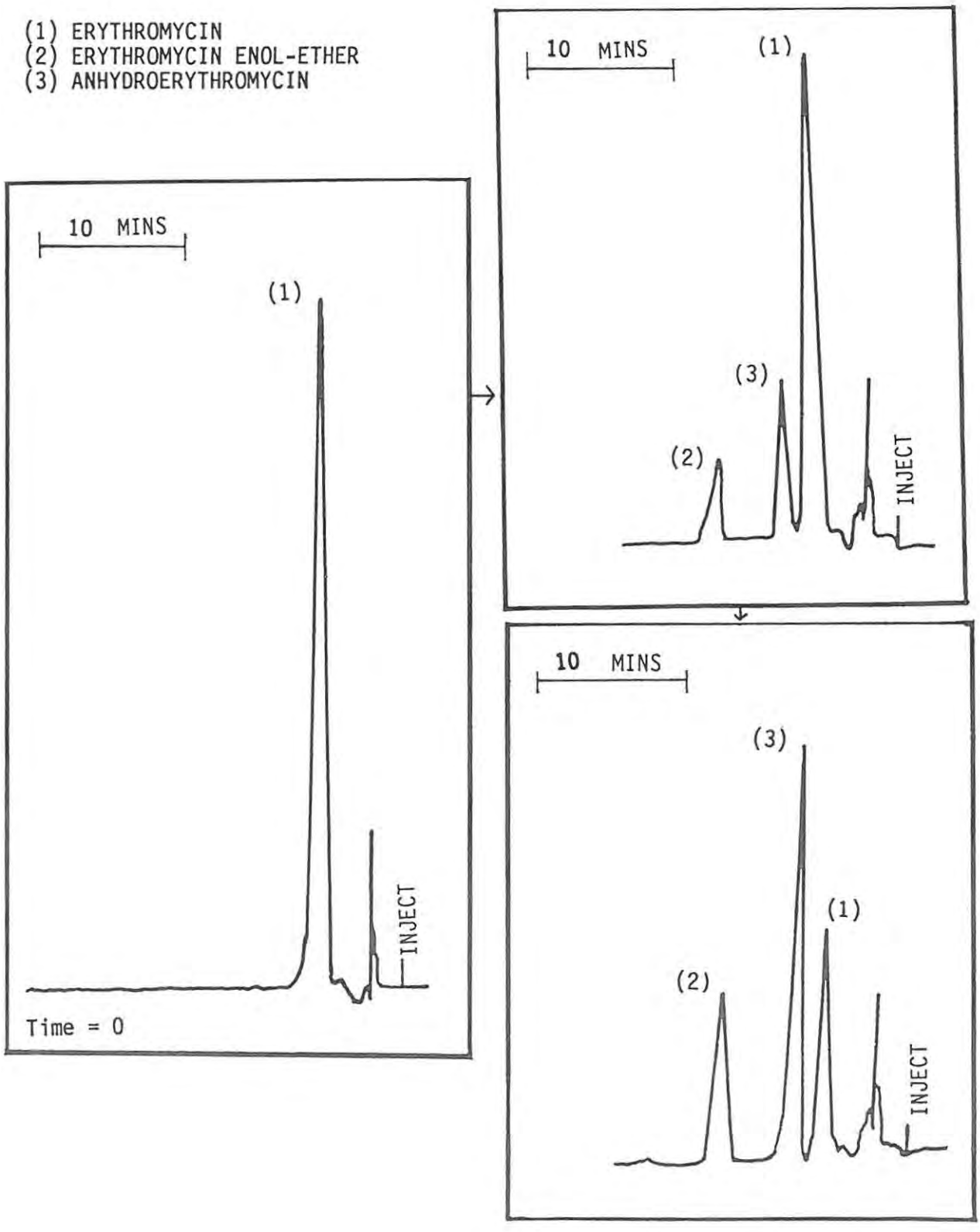


Fig 3.5 Chromatograms of erythromycin degradation at pH 6.5

(1) ERYTHROMYCIN
(2) ERYTHROMYCIN ENOL-ETHER
(3) ANHYDROERYTHROMYCIN
(4) POSSIBLY DIHYDROERYTHROMYCIN

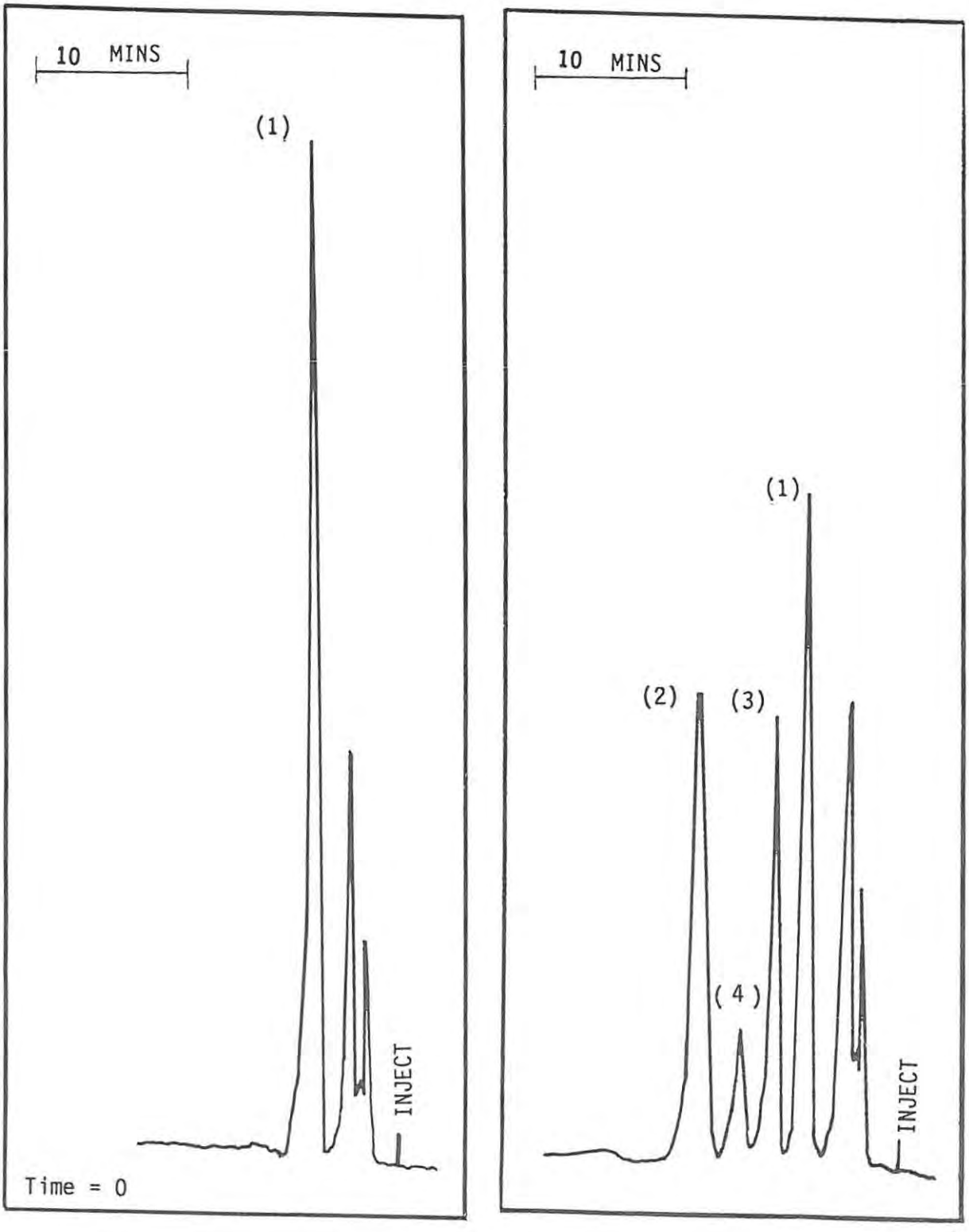
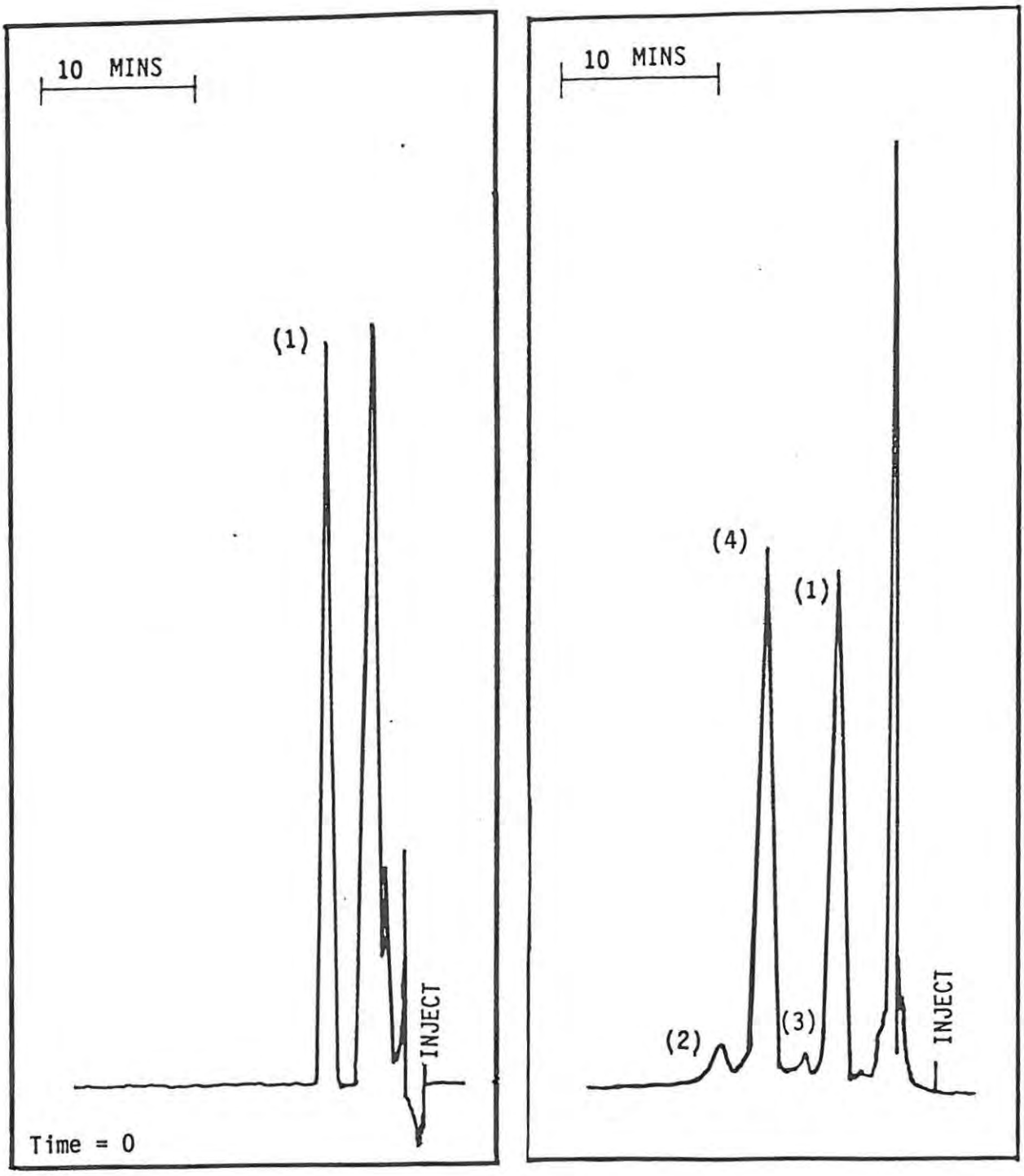


Fig 3.6 Chromatograms of erythromycin degradation at pH 8.0

(1) ERYTHROMYCIN
(2) ERYTHROMYCIN ENOL-ETHER
(3) ANHYDROERYTHROMYCIN
(4) POSSIBLY DIHYDROERYTHROMYCIN



Logarithmic plots of erythromycin concentration against time (Fig. 3.8) yielded straight lines suggesting that the degradation of erythromycin is a pseudo-first order process. The observed first order rate constants were calculated from the slopes of the straight lines obtained.

B. Erythromycin Solution

The individual concentration time data are listed in Appendix 3 and the curves are depicted in Fig. 3.9. Semilogarithmic plots of erythromycin concentration against time are depicted in Fig. 3.10. The observed first order rate constants were calculated from the slopes of the straight lines obtained and are compared in Table 3.3 with those obtained using erythromycin powder.

Table 3.3 Rate constants for erythromycin degradation in 0.1M phosphate buffer

pH	Rate constant			
	$K(min^{-1} \times 10^{-3})^a$	R	$K(min^{-1} \times 10^{-3})^b$	R
3.2	33.600	0.999	74.500	0.993
4.3	4.530	0.996	6.700	0.986
5.5	0.735	0.999	0.518	0.999
6.5	0.084	0.981	0.067	0.996
7.0	0.023	0.936	0.038	0.995

a rate constant obtained using Method A.
b rate constant obtained using Method B.
R correlation coefficient

Fig 3.7 Plots of concentration versus time for erythromycin, erythromycin enol-ether and anhydroerythromycin in 0.1M phosphate buffer

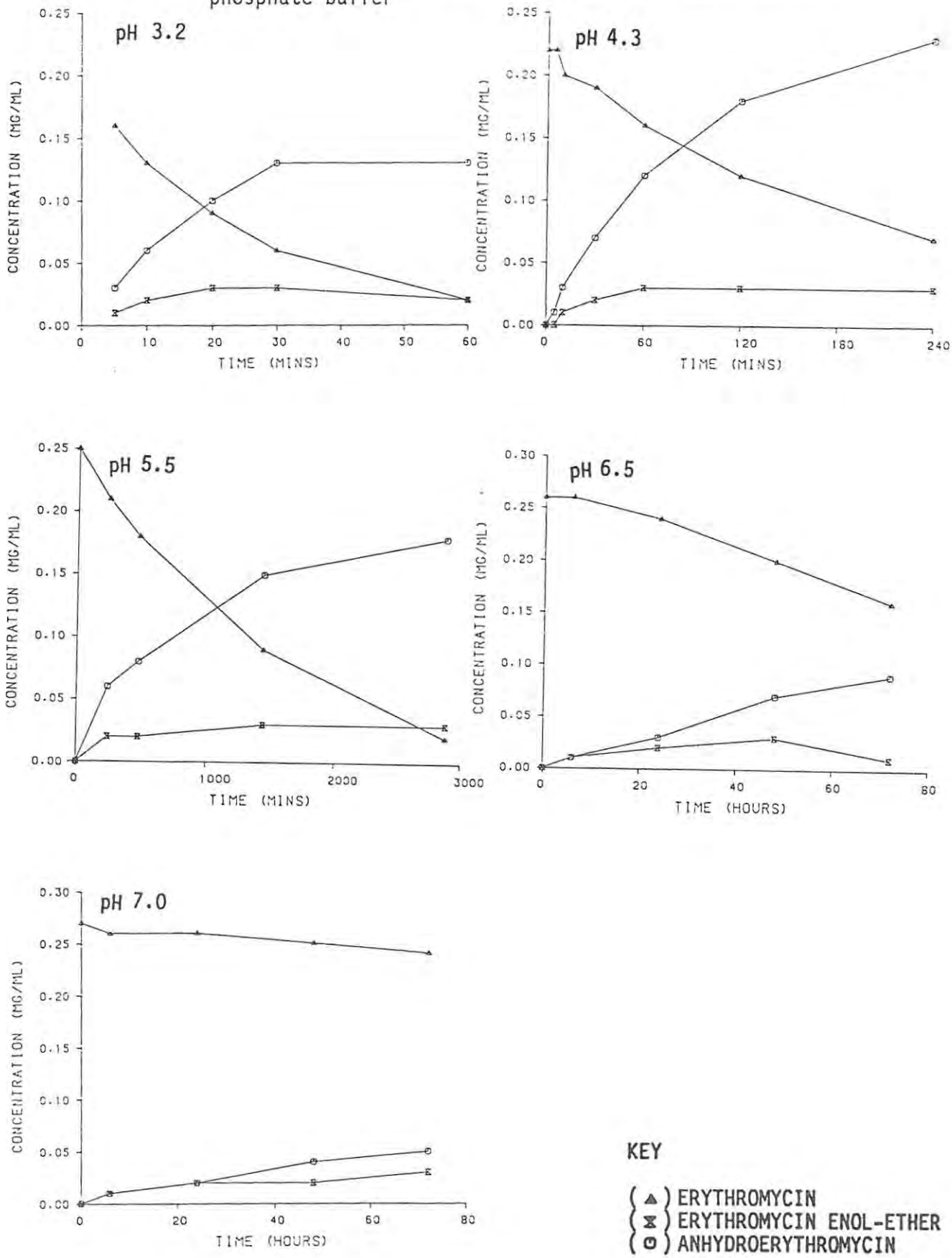


Fig 3.8 Semilogarithmic plots of erythromycin concentration versus time in 0.1M phosphate buffer

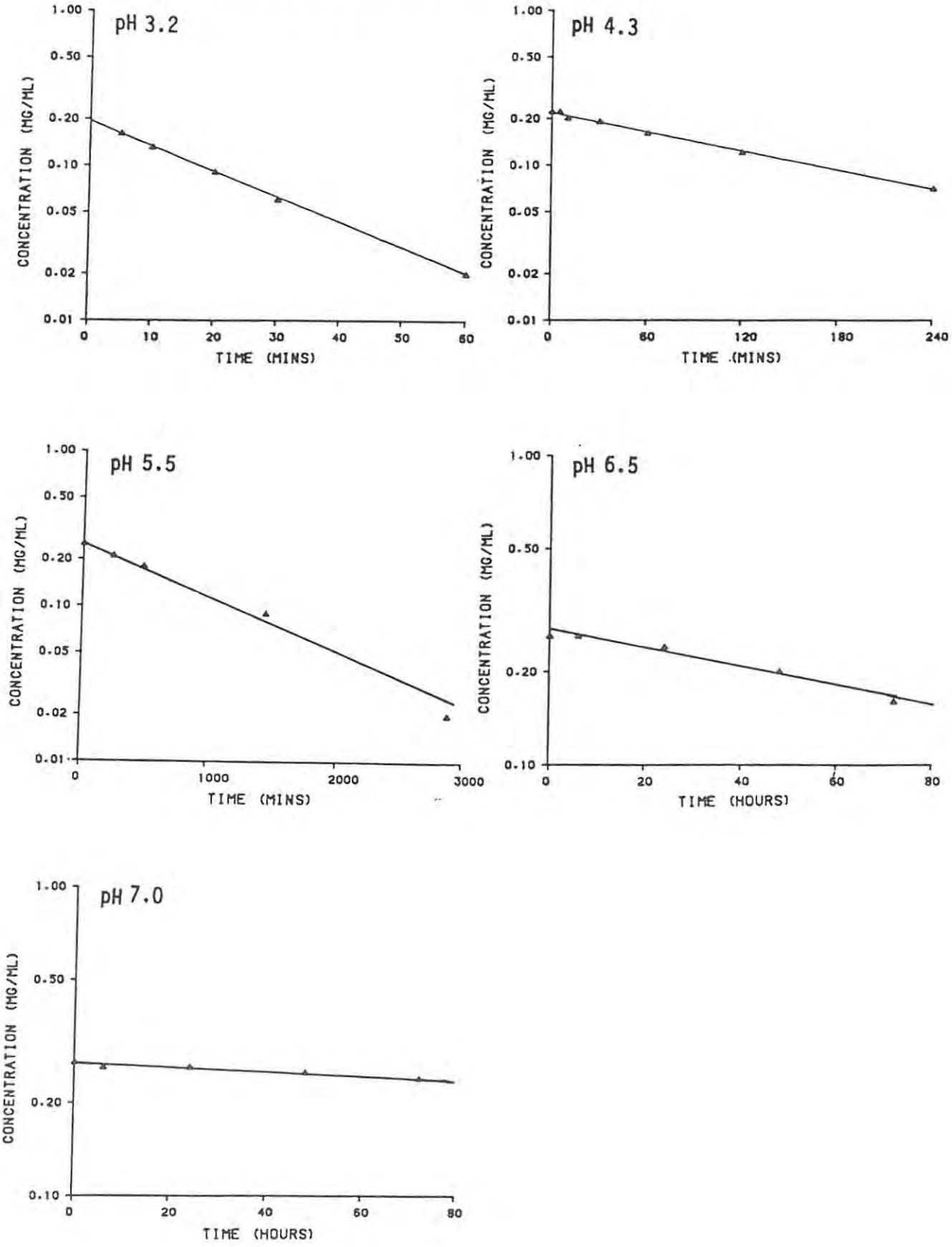


Fig 3.9 Plots of erythromycin concentration versus time in 0.1M phosphate buffer

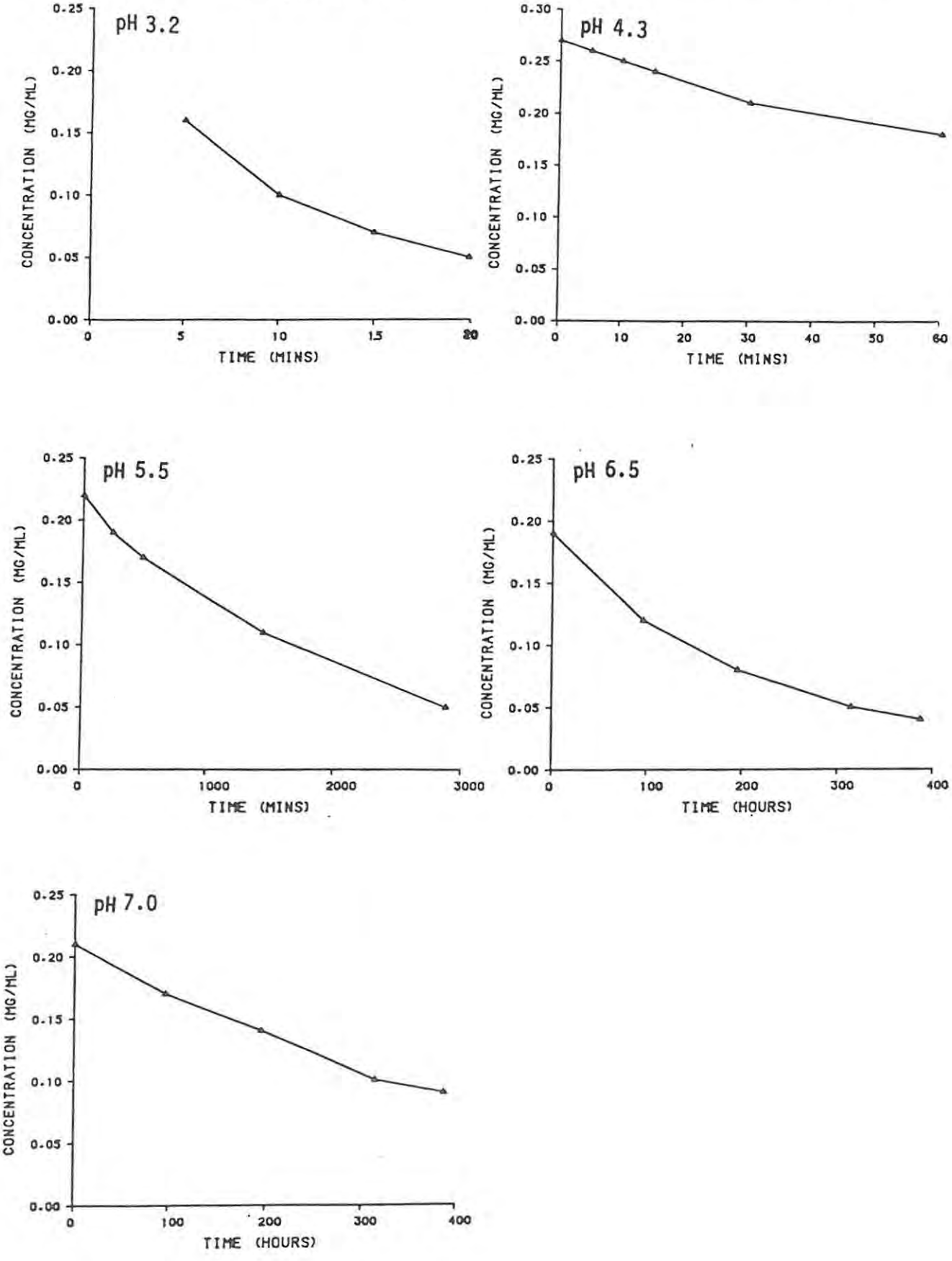
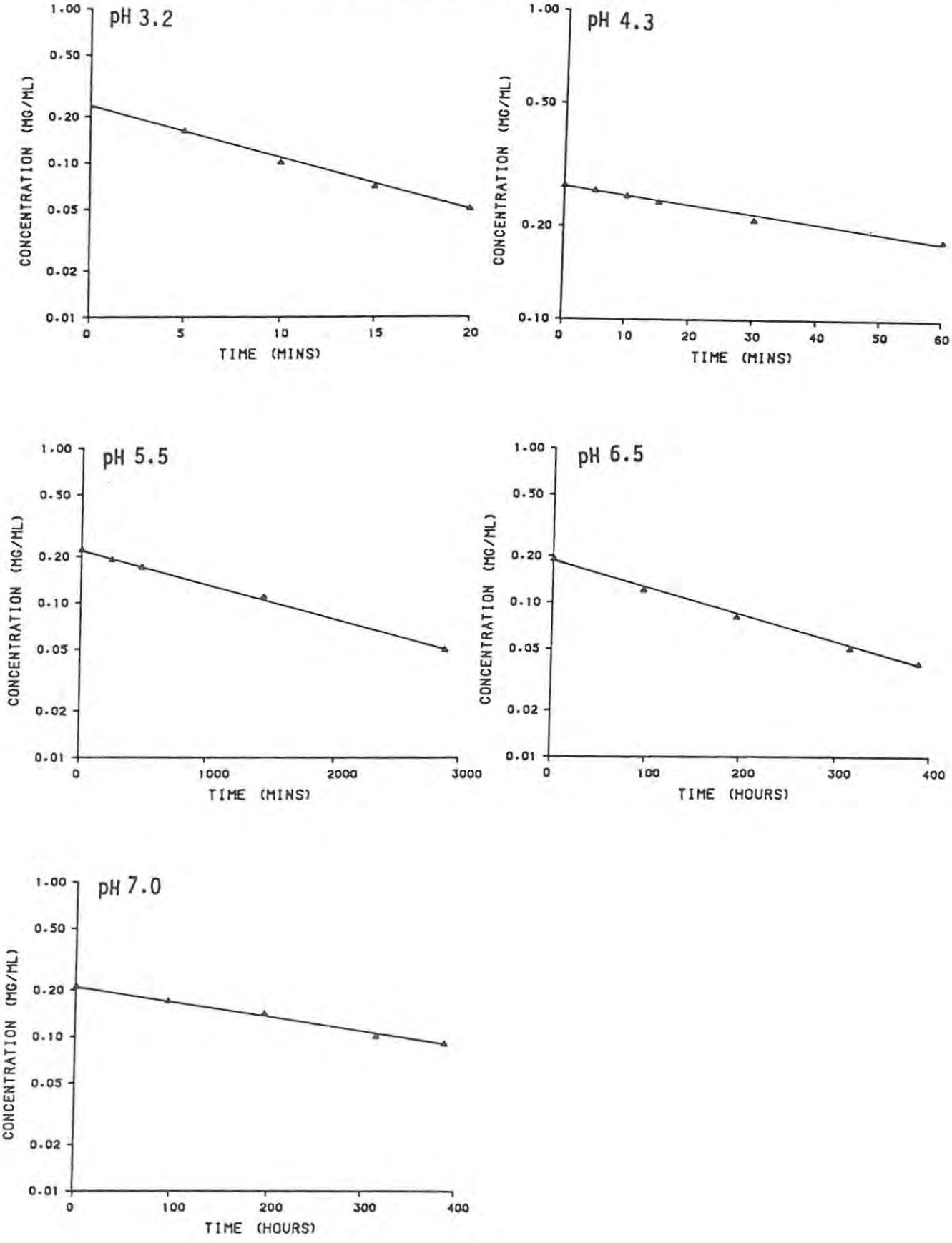


Fig 3.10 Semilogarithmic plots of erythromycin concentration versus time in 0.1M phosphate buffer.



As expected there is very little difference between the rate constants obtained using both methods. However, at pH 3.2 the dissolved erythromycin (method B) degrades faster than the erythromycin powder (method A). At this low pH the rate of degradation is so fast, dissolution of the powder may have become the rate limiting factor leading to a slower degradation rate.

From Table 3.3 and Fig. 3.11 it is evident that erythromycin shows increasing stability with increasing pH reaching a maximum stability at approximately pH 7.0. Although extensive alkaline degradation studies were not carried out, studies carried out at pH 8.0 indicate erythromycin base is susceptible to alkaline degradation when exposed over a lengthy period. Rate constants obtained show good agreement with those published in the literature (Table 3.4).

Fig 3.11 pH-rate profile of erythromycin in 0.1M phosphate buffer

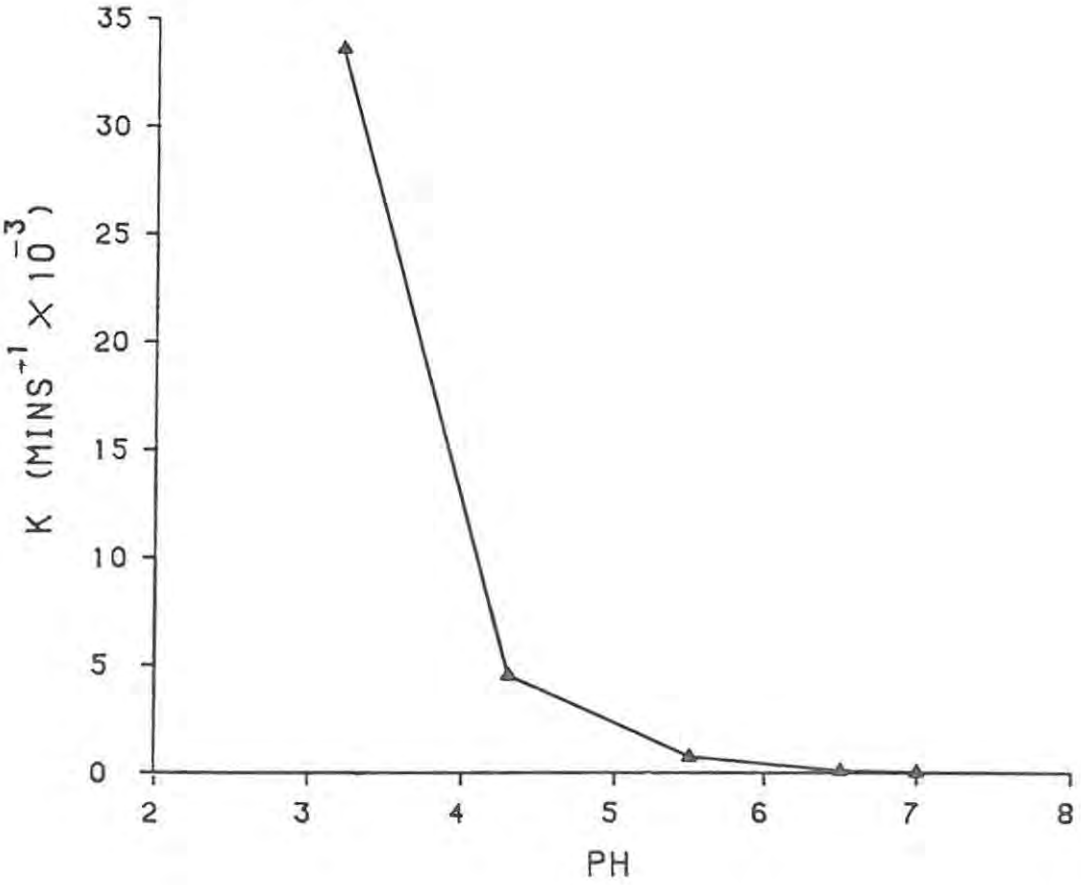


Table 3.4 Comparison of reported rate constants

Phosphate Buffer molarity	pH	Temperature (°C)	Rate constant (mins x 10^{-5})	
0.1	7.0	31.5	2.8	Amer
0.1	6.5	37	5.7	Atkins
0.1	7.0	37	2.1	
0.1	6.5	37	6.7	Glew*
	7.0	37	3.8	

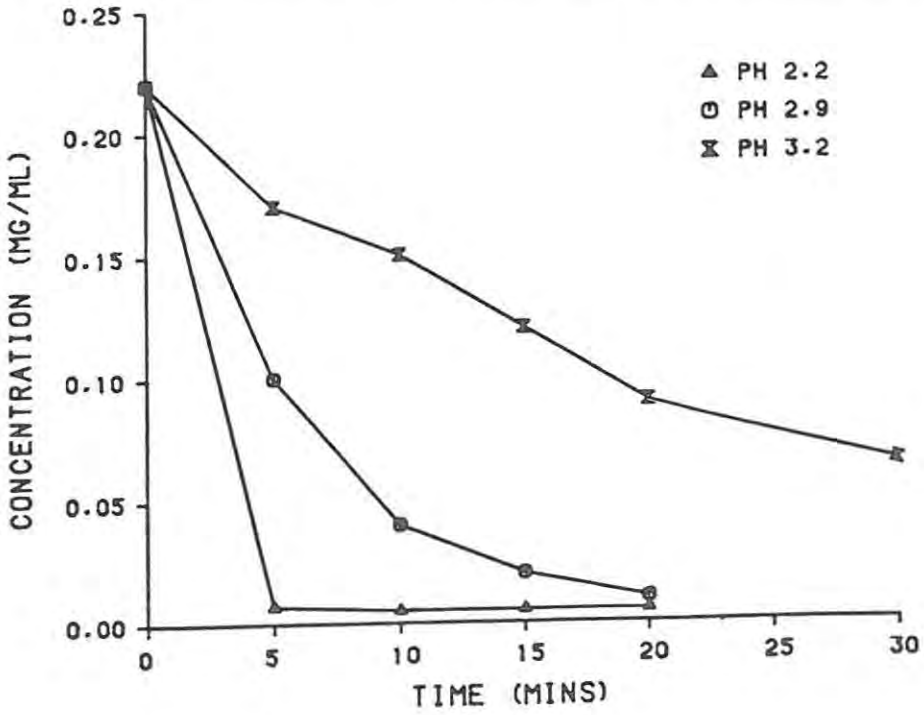
* Method B

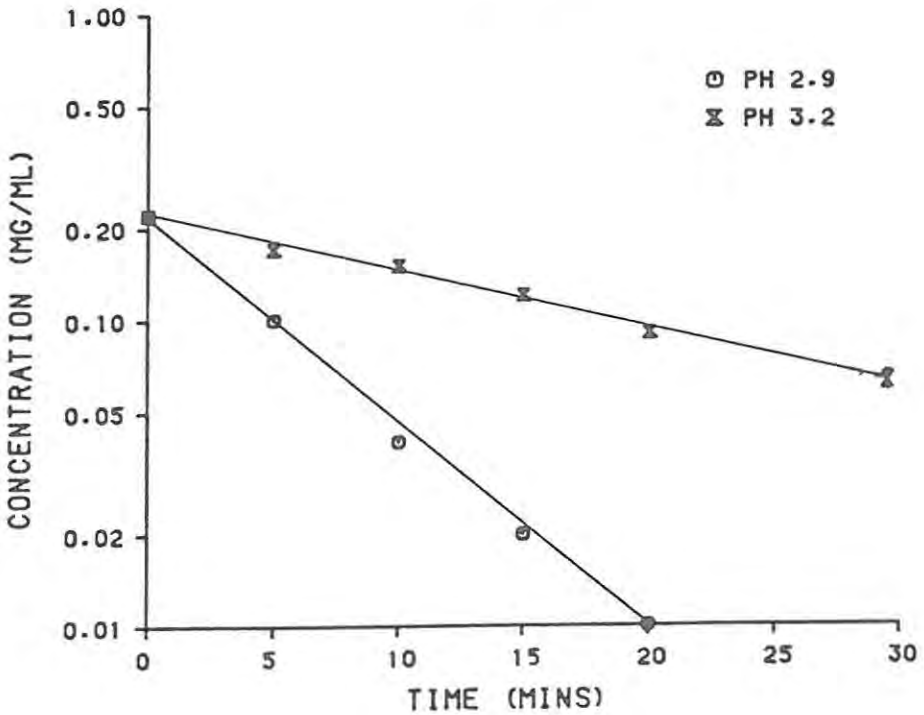
3.5.2.6 Hydrochloric Acid Test Solution

The individual concentration time data are listed in Appendix 4 and the curves depicted in Fig. 3.12. At all three pH values the erythromycin was rapidly degraded. At pH 2.2 the degradation was so rapid the rate

constant could not be calculated. However, the rate constants obtained at pH 2.9 and pH 3.2 are 155.68 x 10^{-3} and 38.46 x 10^{-3} min^{-1} respectively.

Fig 3.12 Plots of erythromycin concentration versus time, and log concentration versus time in hydrochloric acid





3.5.2.7 Effect of Temperature

The individual concentration-time data are listed in Appendix 5 and the curves are depicted in Fig. 3.13. The observed first order rate constants were calculated from the slopes of the straight lines obtained from the plots of log concentration against time (Fig. 3.14) and are shown in Table 3.5. A linear Arrhenius plot was obtained and is depicted in Fig 3.15.

Fig 3.13 Plots of erythromycin concentration versus time, in 0.1M phosphate buffer, pH 3.5, at 23°C, 28°C and 37°C

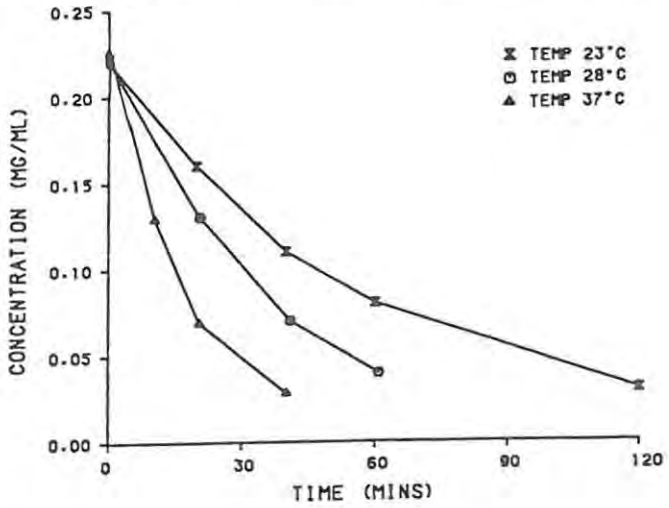


Fig 3.14 Semilogarithmic plots of erythromycin concentration versus time in 0.1M phosphate buffer, pH 3.5, at 23°C, 28°C and 37°C

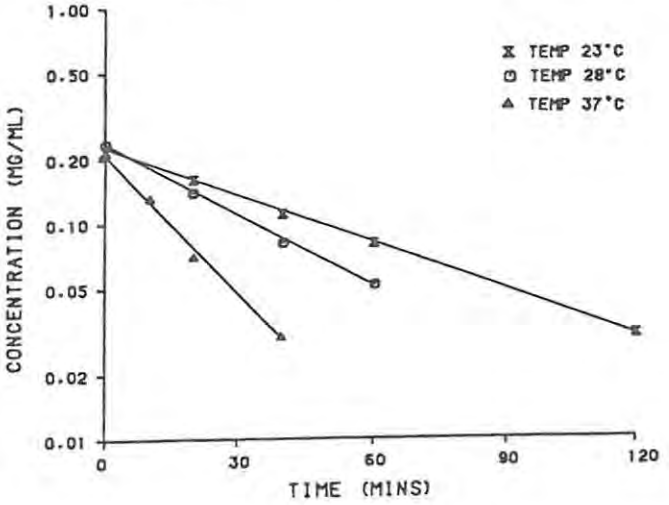
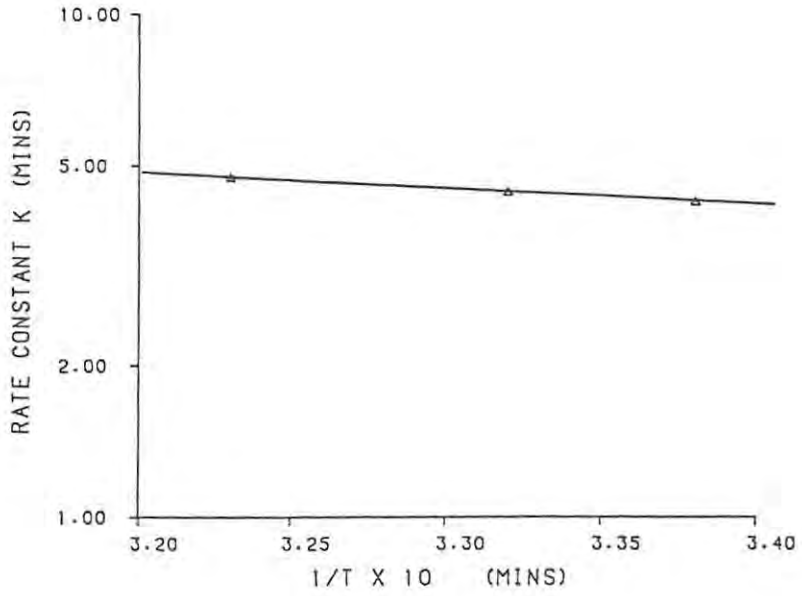


Table 3.5 Rate constants for erythromycin degradation in 0.1M phosphate buffer, pH 3.5, at 23°C, 28°C and 37°C.

Temperature	Rate constant	
	$K(min^{-1} \times 10^{-3})$	R
23	16.5	0.999
28	25.6	0.999
37	50.7	0.992

R correlation coefficient

Fig 3.15 Arrhenius plot of erythromycin in 0.1M phosphate buffer, pH 3.5



3.5.2.8 'Initial Rates' Method

In Table 3.6, the rate constants obtained using the initial rates method are compared with those obtained using the integral method. Comparable results were obtained using the integral method and the 'initial rates' method using erythromycin decrease. However slight differences were seen when the rate constants for degradation were calculated using the increase in erythromycin enol-ether and anhydroerythromycin. This is presumably due to errors in detecting the low concentrations of erythromycin enol-ether and anhydroerythromycin.

Table 3.6 Comparison of rate constants calculated using the integral and 'initial rates' methods

pH	Rate constant ($min^{-1} \times 10^{-3}$)			
	Integral method	Initial rates method		
		I	II	III
4.3	4.530	4.72	2.91	13.6
5.5	0.735	0.664	0.664	0.996
7.0	0.023	0.023	0.047	0.047

I Rate constant calculated using erythromycin decrease
II Rate constant calculated using erythromycin enol-ether increase
III Rate constant calculated using anhydroerythromycin increase

3.6 ERYTHROMYCIN STEARATE

3.6.1 Experimental

3.6.1.1 Sample Preparation

As in Section 3.4.1

3.6.1.2 Calibration Curves

As in Section 3.5.1.2

3.6.1.3 Degradation in Phosphate Buffer Test Solution

All studies were performed in aqueous phosphate buffer prepared as in Section 3.4.2. The method was carried out as previously reported in Section 3.5.1.3 (method A) using 6.6mg erythromycin stearate. All samples were maintained at 37°C. The degradation was carried out in duplicate using the following test solutions.

Test solution	pH
1	3.2
2	4.3
3	5.5

3.6.2 Results

3.6.2.1 Linearity and Calibration

As in Section 3.5.2.1.

3.6.2.2 Phosphate Buffer Test Solution

The individual concentration-time data for erythromycin, erythromycin enol-ether and anhydroerythromycin are listed in Appendix 6 and the

curves are depicted in Fig. 3.16. The erythromycin stearate dissolved (the sum of erythromycin, erythromycin enol-ether and anhydroerythromycin was equivalent to the amount of erythromycin base at time = 0) and the subsequent erythromycin concentration showed an exponential decrease with time. Logarithmic plots of erythromycin concentration against time yielded straight lines (Fig. 3.17) suggesting a pseudo-first order process. Rate constants obtained are shown in Table 3.7.

Fig 3.16 Plots of concentration versus time for erythromycin, erythromycin enol-ether and anhydroerythromycin in 0.1M phosphate buffer

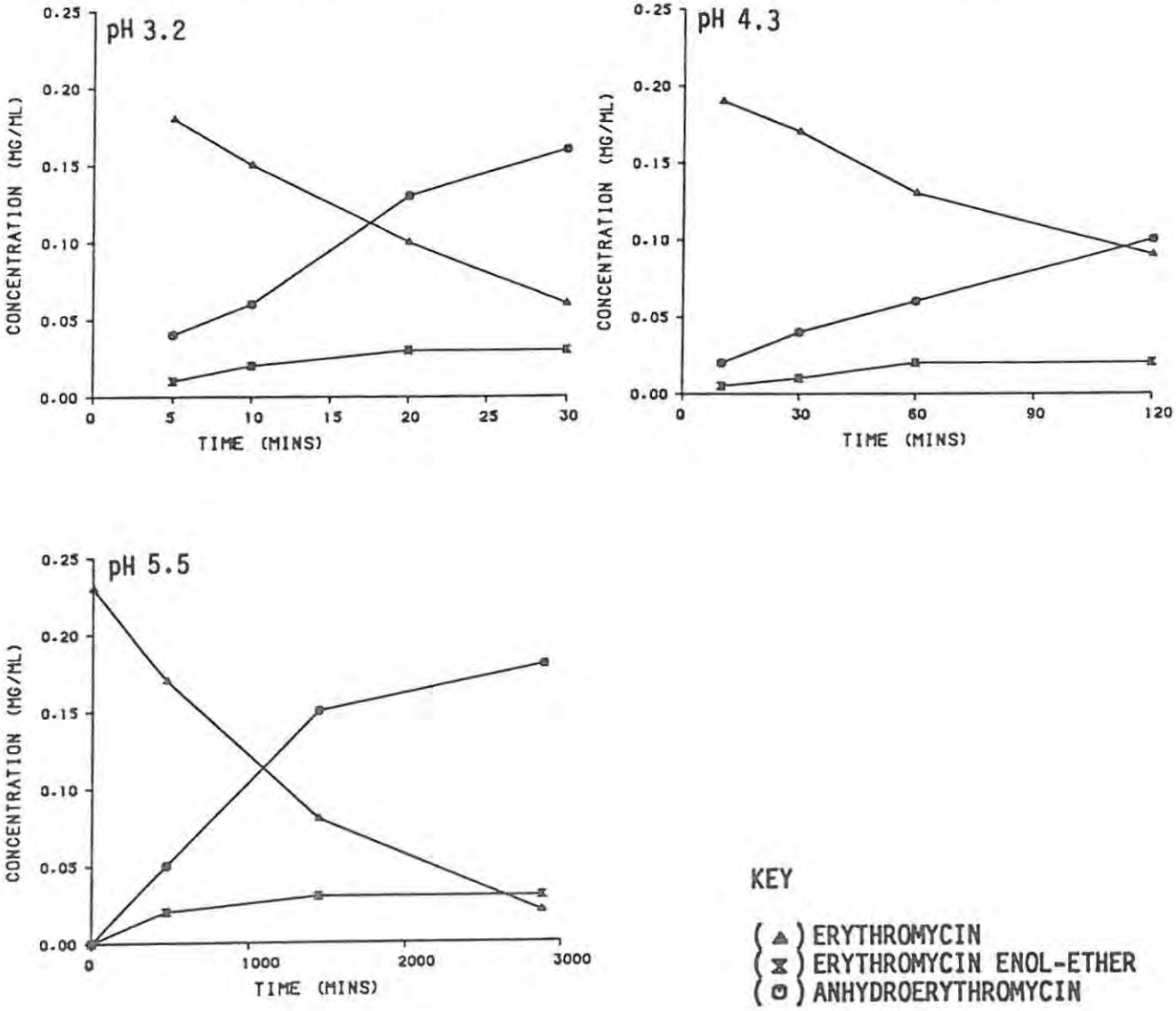


Fig 3.17 Semilogarithmic plots of erythromycin concentration versus time in 0.1M phosphate buffer

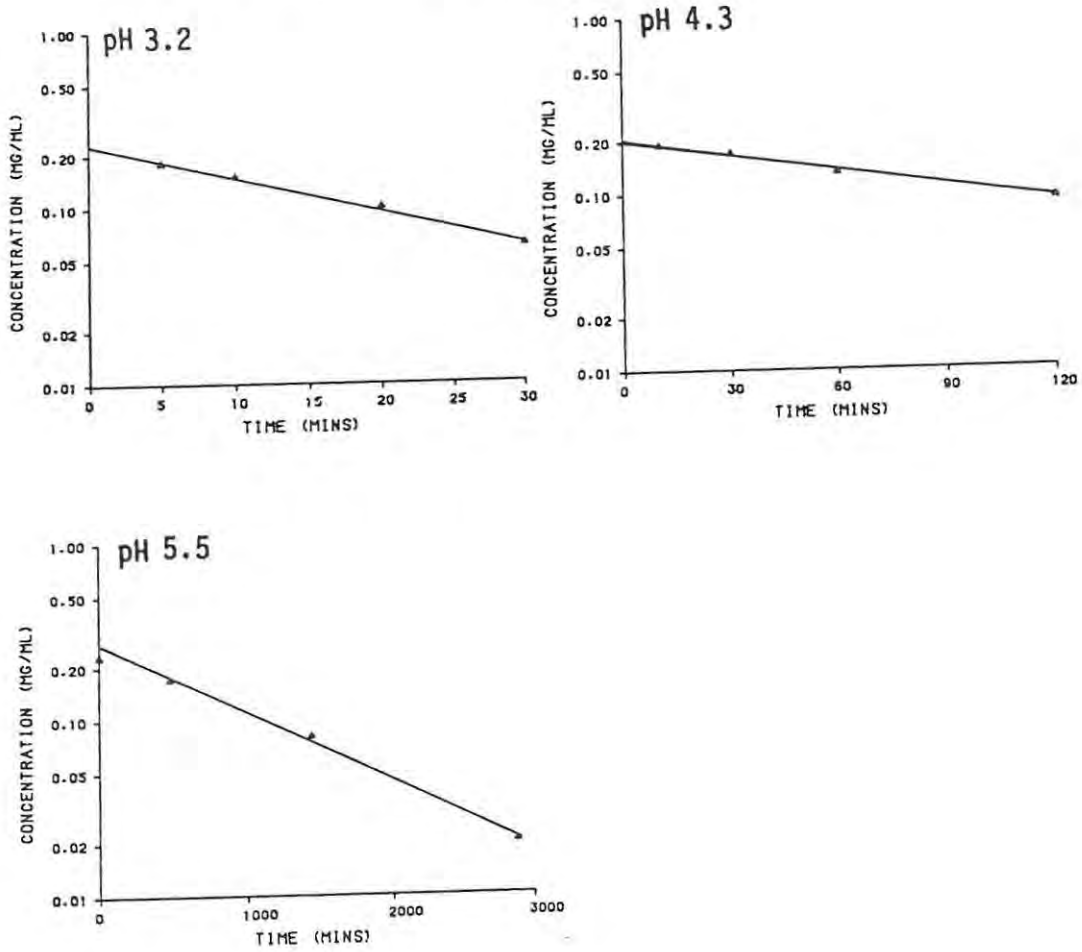


Table 3.7 Rate constants for erythromycin stearate degradation in 0.1M phosphate buffer

pH	Rate constant	
	$K(min^{-1} \times 10^{-3})$	R
3.2	18.90	0.995
4.3	4.15	0.988
5.5	0.37	0.993

R correlation coefficient

Although rate constants obtained for erythromycin stearate are slower than those obtained for erythromycin base powder, the rate of degradation did not appear to be significantly decreased by the use of the stearate salt. These findings are in agreement with Boggiano and Gleeson (51) that erythromycin stearate is in fact very sensitive to acid degradation *in vitro*.

3.7 ERYTHROMYCIN ESTOLATE

3.7.1 Experimental

3.7.1.1 Sample Preparation

Stock solutions of erythromycin estolate were prepared by dissolving 25mg of drug sample in acetonitrile and making up to volume in a 25.0ml volumetric flask with acetonitrile. Dilutions were made with acetonitrile to produce the required concentrations. Other stock solutions were prepared as in Section 3.4.1.

3.7.1.2 Calibration Curves

A stock solution of erythromycin estolate was diluted to yield four different concentrations. Each concentration was assayed in triplicate. The linearity of peak height with concentration was established.

3.7.1.3 Phosphate Buffer Test Solution

All studies were performed in aqueous phosphate buffer as in Section 3.5.1.3 (method A). Twenty millilitres of test solution maintained at

37°C in a water bath was added to 6.8mg erythromycin estolate. The degradation was carried out using the following test solutions.

Test solution	pH
1	3.2
2	4.3
3	5.5
4	6.5
5	7.0

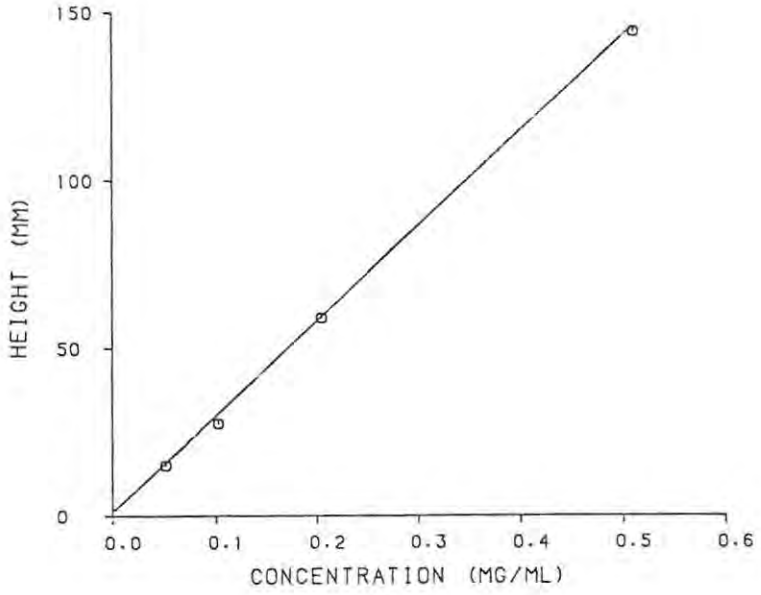
3.7.2 Results

3.7.2.1 Linearity and Calibration

Linearity was established for erythromycin estolate. Details of the linearity data and the calibration curve are shown in Fig. 3.18.

Fig 3.18 Calibration curve for erythromycin estolate

Three samples per data point



3.7.2.2 Phosphate Buffer Test Solution

At pH 6.5 and 7.0 the erythromycin estolate was rapidly hydrolysed to the free base (Fig. 3.19). The concentration-time data for erythromycin estolate are listed in Appendix 7 and the curves are depicted in Fig. 3.20.

Fig 3.19 Chromatogram of erythromycin estolate hydrolysis

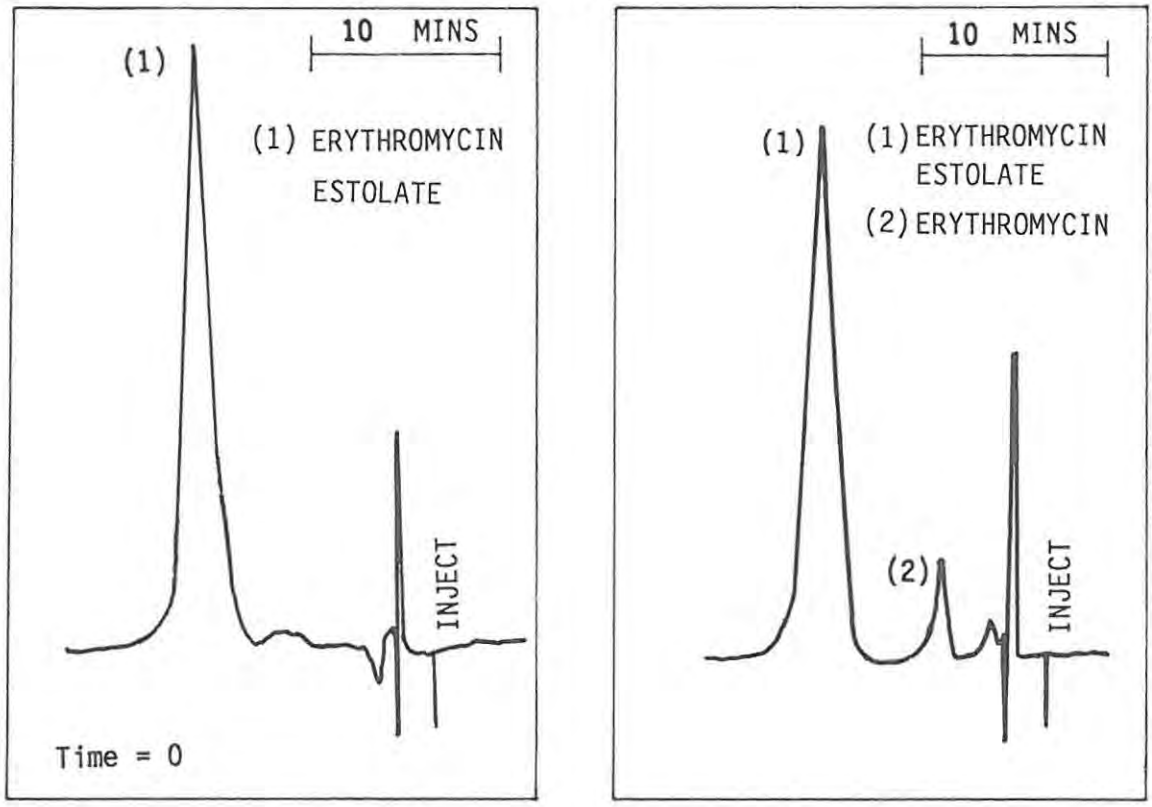
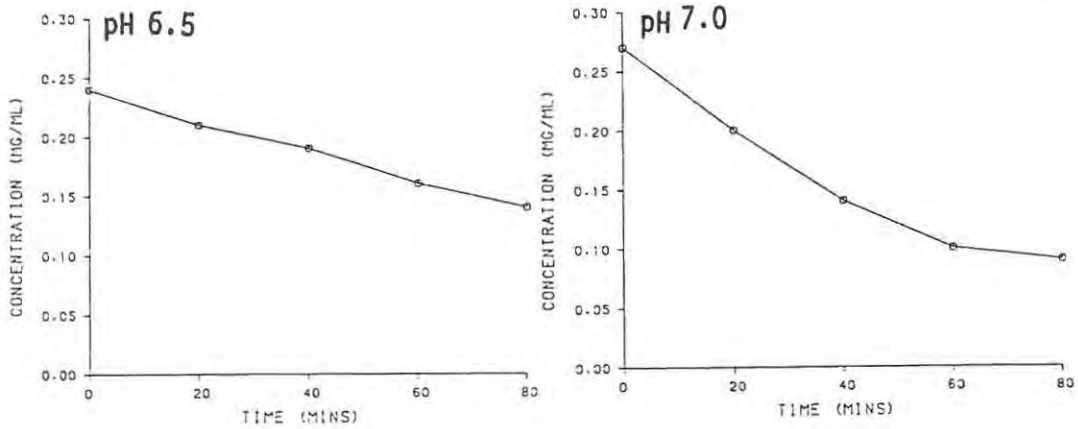


Fig 3.20 Plots of erythromycin estolate concentration versus time, in 0.1M phosphate buffer



Logarithmic plots of erythromycin concentration against time (Fig. 3.21) yielded straight lines suggesting the hydrolysis of erythromycin estolate is a pseudo-first order process. The observed first order rate constants are shown in Table 3.8.

Fig 3.21 Semilogarithmic plots of erythromycin estolate concentration versus time, in 0.1M phosphate buffer

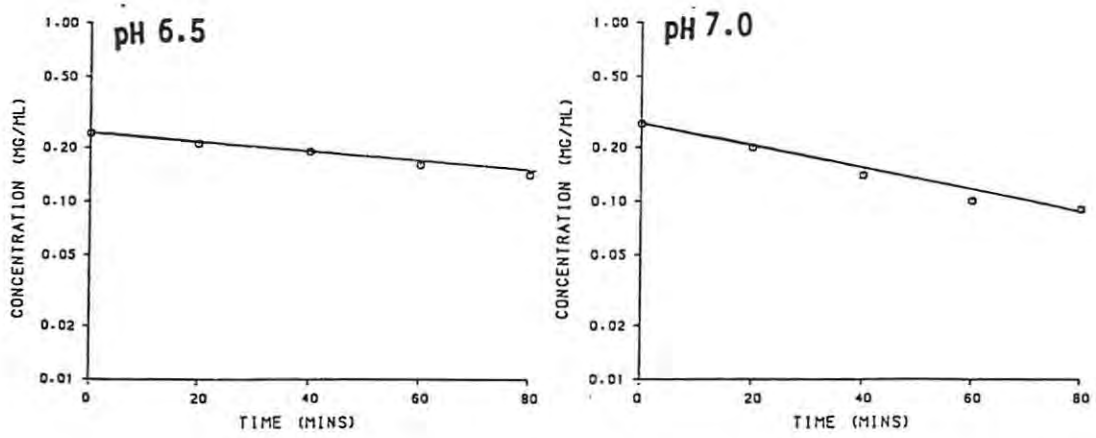


Table 3.8 Rate constants for erythromycin estolate hydrolysis in 0.1M phosphate buffer

pH	Rate constant	
	K(min^{-1} x 10^{-3})	R
6.5	2.85	0.994
7.0	6.30	0.979

R correlation coefficient

However, as the pH decreased, the rate of hydrolysis decreased, presumably due to the low acid solubility of the estolate limiting the exposure of drug to the solution environment. At pH 5.5 and below, any erythromycin base resulting from dissolution of the estolate and subsequent hydrolysis of the propionate to produce the free drug moeity, was rapidly degraded to erythromycin enol-ether and anhydroerythromycin. Due to difficulty in resolving erythromycin estolate and erythromycin enol-ether no attempt was made to measure hydrolysis rates at pH 5.5 and below.

CHAPTER 4

CLINICAL TRIALS AND THE DETERMINATION OF ERYTHROMYCIN IN BIOLOGICAL FLUIDS

4.1 INTRODUCTION

In vivo trials, using healthy volunteers were conducted to compare the bioavailability of erythromycin following the administration of a single oral dose equivalent to 500mg erythromycin base as either erythromycin stearate (film coated tablet) or erythromycin base (enteric coated pellets in a capsule). These trials also enabled further elucidation of the pharmacokinetic parameters of erythromycin.

In order to undertake *in vivo* trials a suitable method for the analysis of erythromycin in human serum and urine was required. Until recently, bioavailability data published on erythromycin have been derived predominantly from microbiological assay techniques, although several HPLC methods using spectrophotometric (5, 114-119, 138, 139) and more recently, electrochemical (34, 119-121, 123, 124) methods of detection have also been published.

However, for this study a recently published HPLC assay developed by Stubbs *et al* (121), which involved a solid-phase extraction procedure followed by a simple phase separation step was used. Detection was carried out using a dual-electrode electrochemical detector in the oxidative screen mode. Prior to use, this method was validated and a pilot trial using one volunteer was conducted to check the effectiveness of the analytical method. In addition, a comparison between mean serum data and pooled serum data, obtained from the clinical trials, was undertaken.

4.2 ANALYSIS OF ERYTHROMYCIN IN SERUM

4.2.1 Experimental

4.2.1.1. Sample Preparation

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

An internal standard stock solution was prepared by dissolving 20mg oleandomycin phosphate in 10ml acetonitrile. The internal standard solution (20µg/ml) was then prepared by diluting 1.0ml of this stock solution to 100.0ml with acetonitrile.

4.2.1.2 Chromatographic Conditions

HPLC System	:	As in Section 2.3.2
Column	:	Techsil C_{18} 10µm
Mobile Phase	:	0.05M Phosphate buffer (pH6.3)/ acetonitrile (35/65)
Flow rate	:	1.3ml/min
Temperature	:	35°C
Detection	:	Coulometric screen +0.70V analytical +0.90V
Chart speed	:	2.5mm/min
Recorder input	:	10mV

4.2.1.3 Extraction Procedure

One millilitre of serum was vortexed for 30 seconds mixed with 100µl of the internal standard solution. The sample was then deproteinated by the addition of 1.0ml acetonitrile, followed by vortexing for 1 minute and centrifugation for 5 minutes at

1600xg. The supernatant was transferred into a culture tube containing 5ml water for loading onto a disposable 1.0ml-C_{18} extraction column which had been previously washed with 5.0ml acetonitrile and 5.0ml water. The extraction column was subsequently washed with 20.0ml water and 5.0ml water/acetonitrile (50/50). Elution from the column into tapered collection tubes was carried out using two successive 500.0ul aliquots of 0.05M phosphate buffer (pH 6.3)/acetonitrile (40/60) under vacuum. The sample was then dried in a rotary vacuum centrifuge. The residue in the collection tube was reconstituted in 20.0ul water and vortexed for 1 minute. The sample was vortexed for a further 1 minute following the addition of 100.0ul acetonitrile, centrifuged for 1 minute at 1600xg and 20ul aliquots of the top layer were injected onto the column.

4.2.1.4 Calibration Curves

The serum stock solution was diluted to yield four different concentrations, the stock solution itself providing the fifth concentration. Concentrations ranged from 0.25ug/ml to 5.0ug/ml and each concentration was assayed in triplicate. The calibration curve was constructed by plotting the ratios of peak height of erythromycin to that of the internal standard versus the respective erythromycin concentrations. A straight line fit of the data was made by least squares linear regression analysis.

4.2.1.5 Precision

Within-run precision was assessed by extracting six spiked serum samples each at the upper and lower limits of the concentration range studied (0.25ug/ml - 5.00ug/ml).

4.2.1.6 Extraction Efficiency

Three different concentrations of spiked serum samples were assayed in triplicate. All samples were extracted as described in

section 4.2.1.3, except that the internal standard was incorporated in the final reconstitution and was thus not carried through the extraction procedure.

Standard solutions of erythromycin corresponding to those extracted above were made up in 1.0ml of 0.05M phosphate buffer (pH 6.3)/acetonitrile (40/60) and taken to dryness in a rotary vacuum centrifuge. These samples were reconstituted as for the serum samples, with the internal standard included in the 100.0ul acetonitrile aliquot.

To determine the percentage recovery, the ratio obtained from the serum extracts were compared to those resulting from the equivalent concentration of standard solutions.

4.2.2 Results and Discussion

4.2.2.1 Linearity and Calibration Curve

Linearity was established for the range of concentrations studied (0.25ug/ml - 5.00ug/ml). Details of the linearity data and the calibration curves for erythromycin in serum are shown in Fig 4.1 Chromatograms of a blank serum extract and a serum extract containing erythromycin and internal standard are depicted in Fig 4.2. The retention time of erythromycin was 7.6 minutes and that of the internal standard 5.6 minutes.

4.2.2.2 Precision

Relative standard deviations of 2.3% (n=6) and 4.3% (n=6) were obtained for the within-run precision studies for the upper and lower concentrations respectively.

Fig 4.1 Calibration curve for erythromycin in serum
Three samples per data point

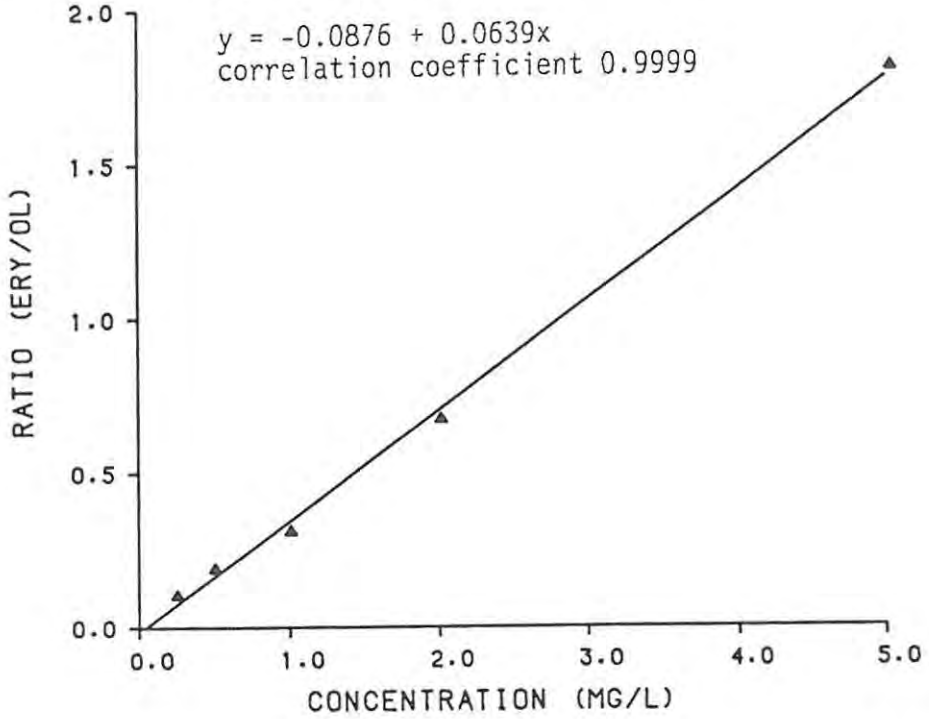
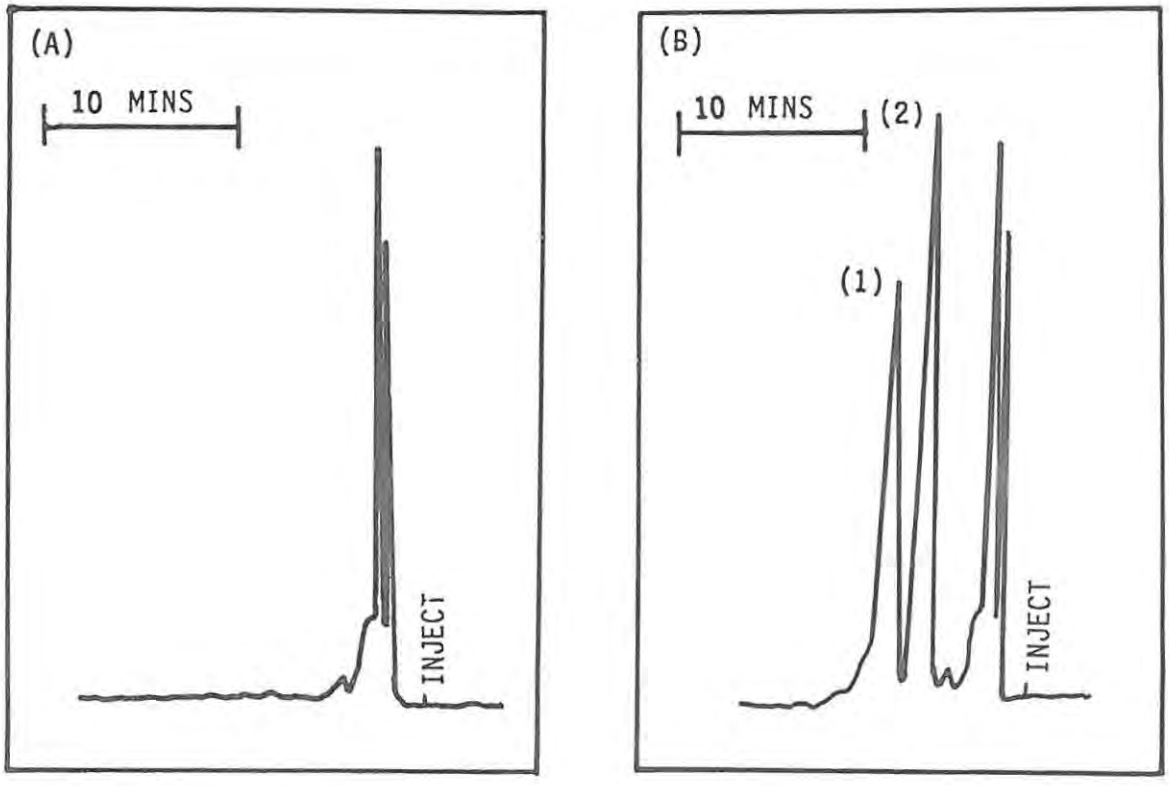


FIG 4.2 Chromatograms of a blank serum extract (A) and a serum extract following the oral administration of erythromycin (B)

(1) ERYTHROMYCIN
(2) INTERNAL STANDARD



4.2.2.3 Extraction Efficiency

Table 4.1 shows that analytical recoveries of erythromycin from serum. A mean recovery of 84.7% was obtained.

Table 4.1 Recovery data for erythromycin in serum

SERUM CONCENTRATION (µg/ml)	PERCENTAGE RECOVERY SAMPLE NUMBER 1	2	3	MEAN	(SD)	RSD
0.50	85.9	89.8	92.3	89.3	(2.6)	2.9%
1.00	86.3	79.2	86.0	83.8	(3.3)	3.9%
2.00	78.2	83.0	82.3	81.2	(2.1)	2.6%
Mean Percentage Recovery (n=9)				84.7	(4.4)	5.1%

4.2.2.4 Sensitivity and Detection

Under the conditions of this assay and based upon a signal to noise ratio of 3, the detection limit for erythromycin in serum was 0.10µg/ml. However, the detection limit could be increased by the use of a larger sample injection volume.

4.3 ANALYSIS OF ERYTHROMYCIN IN URINE

4.3.1. Experimental

4.3.1.1 Sample Preparation

Twenty five milligrams of erythromycin was dissolved in 10.0ml acetonitrile and 1.0ml of this solution was made up to 50.0ml with blank urine.

The internal standard stock solution was prepared by dissolving 20mg oleandomycin phosphate in 10ml acetonitrile. The internal standard solution was then prepared by diluting 0.5ml of this stock solution to 20.0ml with acetonitrile.

4.3.1.2 Extraction Procedure

One millilitre of urine was mixed with 100µl of internal standard solution and vortexed. The sample was transferred to a culture tube containing 5.0ml water. The samples were then extracted as for the serum samples (Section 4.2.1.3).

4.3.1.3 Calibration Curves

A calibration curve was constructed, as described for the analysis of erythromycin in serum (Section 4.2.1.4), over the concentration range of 5.0µg/ml to 50.0µg/ml.

4.3.1.4 Precision

Within-run precision was assessed by extracting six spiked urine samples each at upper and lower limits of the concentration range studied.

4.3.1.5 Extraction Efficiency

The analytical recovery of erythromycin in urine was assessed as for serum samples (Section 4.2.1.6).

4.3.2 Results and Discussion

4.3.2.1 Linearity and Calibration Curve

Linearity was established for the range of concentrations studied (5.0ug/ml - 50.0ug/ml). Details of the linearity data and the calibration curve for erythromycin in urine are shown in Fig 4.3. Chromatograms of a blank urine and a urine extract containing erythromycin and internal standard are depicted in Fig 4.4.

Fig 4.3 Calibration curve of erythromycin in urine

Three samples per data point

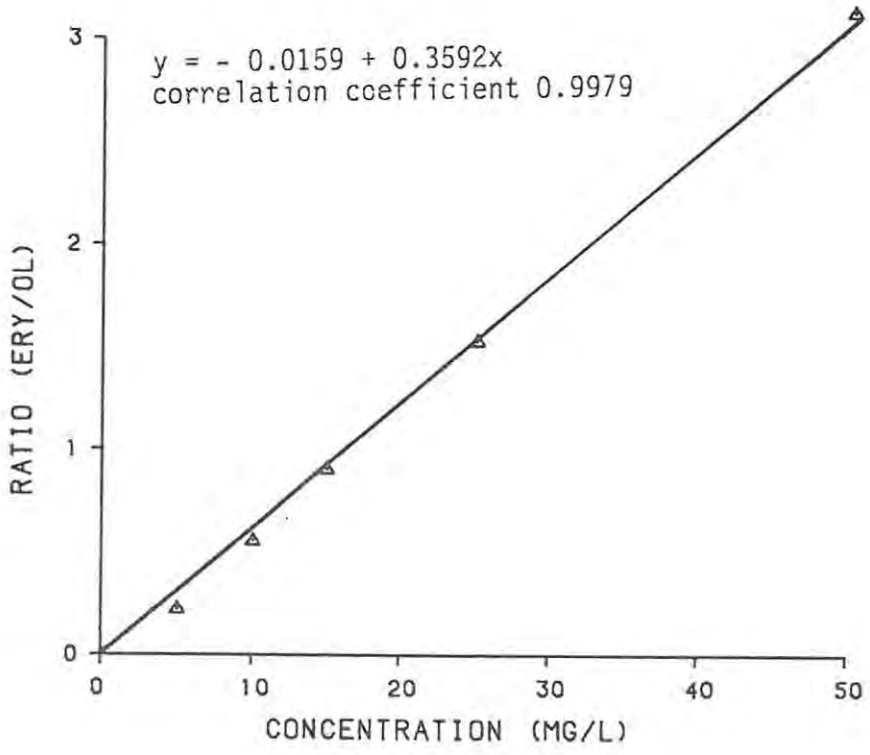
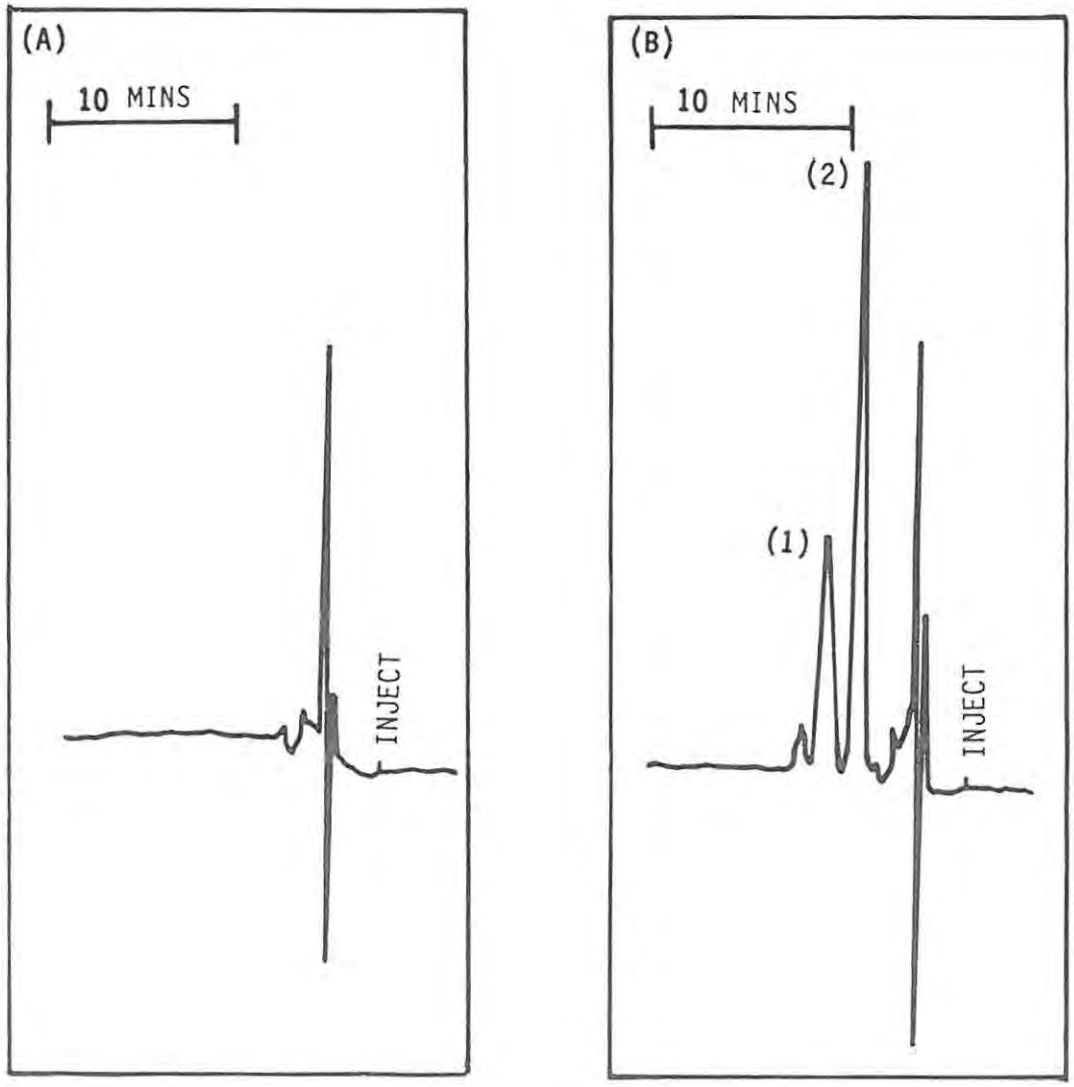


FIG 4.4 Chromatograms of a blank urine extract (A) and a urine extract following the oral administration of erythromycin (B).

(1) ERYTHROMYCIN
(2) INTERNAL STANDARD



4.3.2.2 Precision

Relative standard deviation of 2.3% (n=6) and 1.7% (n=6) were obtained for the within-run precision study for the upper and lower concentrations respectively.

4.3.2.3 Extraction Efficiency

Table 4.2 shows that analytical recoveries of erythromycin in urine. A mean recovery of 82.6%, was obtained.

Table 4.2 Recovery data for erythromycin in urine

URINE CONCENTRATION (µg/ml)	PERCENTAGE RECOVERY SAMPLE NUMBER 1	2	3	MEAN	(SD)	RSD
5.0	85.5	87.7	79.4	84.2	(3.5)	4.2%
25.0	79.2	84.4	79.2	80.9	(2.5)	3.0%
Mean Perentage Recovery (n = 6)				82.6	(3.4)	4.2%

4.4 PILOT TRIAL

4.4.1 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 after the oral administration of a commercially available erythromycin stearate formulation (equivalent to 500mg erythromycin base). The volunteer was a healthy male who was a non-smoker with no previous history of kidney or liver disease, and who infrequently partook of alcoholic beverages. The volunteer received an honorarium for participating in the trial and adhered to the same standard procedures as those described in Section 4.5.1.3.

The volunteer received two film coated tablets each containing erythromycin stearate (equivalent to 250mg erythromycin base) accompanied with 250ml water following an overnight fast.

Blood samples were withdrawn at 0, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 2.50, 3.00, 4.00,, 5.00, 6.00, 10.00, 12.00 and 24.00 hours after the ingestion of the medication. Urine samples were collected during the time intervals 0-2, 2-4, 4-6, 6-8, 8-10, 10- 12 and 12-24 hours. Samples were frozen at -20^{o}C until analysis.

Serum and urine samples were analysed according to the methods described in Section 4.2.1.3 and Section 4.3.1.2 respectively.

4.4.2 Results and Discussion

The HPLC method was suitable for the determination of erythromycin in serum and urine following a single oral 500mg erythromycin dose. The serum concentration-time profile and cumulative urinary excretion profile from this study are depicted in Fig 4.5 and Fig 4.6 respectively.

The patient achieved a C_{max} of 1.71µg/ml at t_{max} of 2.00 hours. The analysis of urine samples collected in the pilot study indicated only a small percentage (0.9%) of the administered dose was excreted as unchanged erythromycin after 24 hours.

Fig 4.5 Serum erythromycin concentration-time profile after the ingestion of 500mg erythromycin administered as two film coated tablets containing erythromycin stearate

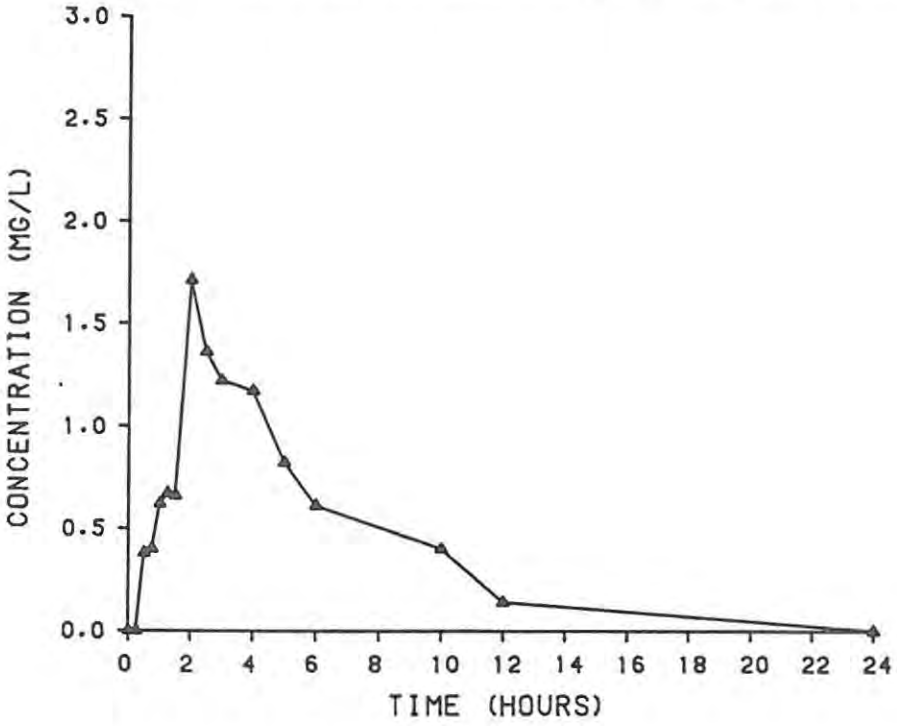
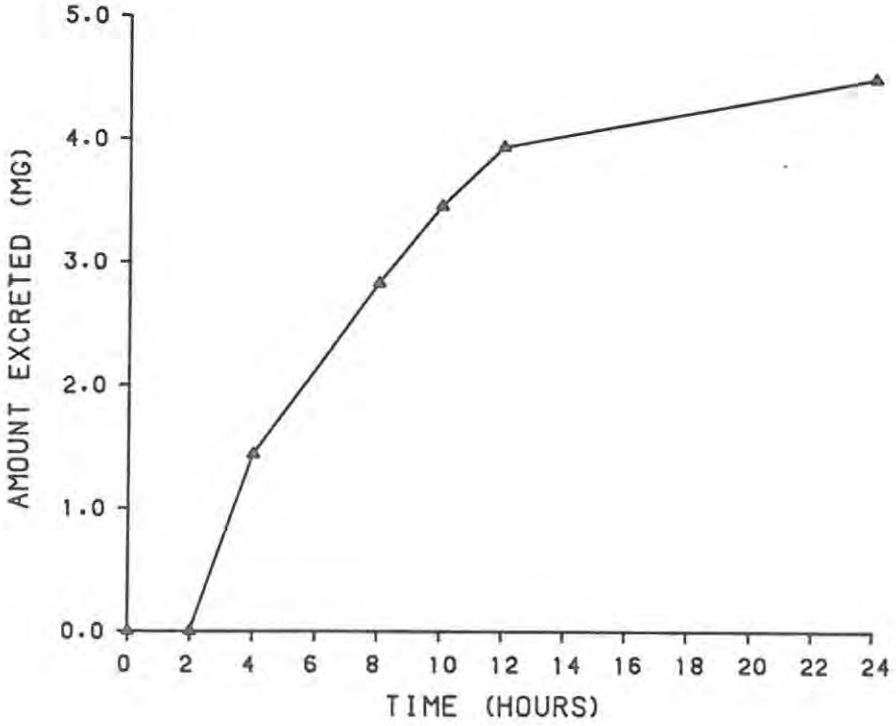


Fig 4.6 Cumulative urinary excretion profile after the ingestion of 500mg erythromycin administered as two film coated tablets containing erythromycin stearate



4.5 CLINICAL TRIALS

4.5.1 Experimental

4.5.1.1. Four Patient Study

Four healthy non-smoking volunteers participated in the trials. The demographic data of the volunteers are summarized in Table 4.3. Volunteers were chosen on the basis of physical health and the following haematology, blood chemistry and urinalysis tests:

Haematology : Haemoglobin, haematocrit, WBC, RBC, platelet count, differential count.

Blood chemistry : Serum creatinine, serum urea, blood glucose, serum bilirubin, serum alkaline phosphatase, SGPT and SGOT.

Urinalysis : Specific gravity, bile, pH, protein, glucose, RBC, WBC, epithelial cells, granular casts.

Table 4.3 Demographic data of the volunteers

NAME	CODE	AGE (YEARS)	MASS (Kg)	HEIGHT (cm)	SEX
M. Cawood	P1	21	84	184	M
K. Fletcher	P2	23	90	186	M
W. Brunyee	P3	24	84	183	M
L. Hilliary	P4	23	85	196	M
MEAN (SD)		22.8 (1.1)	85.8 (2.5)	187.3 (5.2)	

Volunteers signed a combined protocol/consent form (Appendix 8) prior to each trial. Volunteers were paid an honorarium for participating in the trial and adhered to the standard procedures described in section 4.5.1.3.

4.5.1.2 Treatments

A. Test dose 1 (TD1) consisted of a single oral dose of two capsules each containing 250mg of enteric coated erythromycin base pellets. (Erymax Lot Number: 107157)
B. Test dose 2 (TD2) consisted of a single oral dose of two film coated erythromycin stearate tablets containing the equivalent of 250mg erythromycin base. (Erythrocin Lot Number: 96007M4)

The volunteers received the treatments as shown in Table 4.4. Trial 2 was carried out two weeks after Trial 1.

Table 4.4 Treatments received by volunteers

VOLUNTEER	TRIAL 1	TRIAL 2
P1	TD1	TD2
P2	TD2	TD1
P3	TD1	TD2
P4	TD2	TD1

4.5.1.3 Standard Procedures

All volunteers had to conform to the following restrictions:

(a) No drugs, including over-the-counter preparations, were allowed for at least a week before the trial and for the duration of the trial.

(b) No alcohol was to be consumed for at least 24 hours before the trial and for the duration of the trial.
(c) No food or drink was to be ingested for 10 hours before the start of the trial.
(d) All volunteers were confined to a semi-reclining position for the first five hours of the study except during the voiding of urine samples.

A standardized breakfast consisting of three slices of toast, with margarine and jam, and 250ml orange juice was served two and half hours after the start of the trial. A standard lunch consisting of chicken, rice and vegetables followed by fresh-fruit salad was given to all the volunteers three hours later.

4.5.1.4 Sampling Schedule

The sampling schedule used for both Trial 1 and Trial 2 is given in Table 4.5.

4.5.1.5 Collection and Storage of Blood Samples

An indwelling 0.8mm butterfly catheter (21G, Teruma Corporation, Tokyo, Japan) was inserted into a suitable vein in the forearm of each volunteer and strapped into position with adhesive tape so as to facilitate mobility of the arm. Each 10ml blood sample was withdrawn from the butterfly through a sterile hypodermic needle (0.8mm, Promex (Pty) Ltd., Bergvlei, South Africa) by syringe aspiration. The butterfly was then flushed with 1.5ml of sterile saline solution containing heparin (50 iu./ml). Immediately prior to sampling, the butterfly was cleared of the heparin solution by the withdrawl of 2ml of blood which was discarded. Ten millilitres of blood was then collected using a new syringe and needle. The blood sample was transferred into a labelled vacutainer tube which was stoppered and

allowed to stand for 30 minutes. The tube was centrifuged at 3000 r.p.m. and the serum transferred to a labelled vacutainer tube. The serum samples were stored at -20°C, for a maximum of two days, until analysis.

Table 4.5 BLOOD SAMPLING SCHEDULE

TIME	
0	Dose administered with 250ml water
0.5	
1.0	
1.5	
2.0	
2.5	Breakfast - slices of toast with margerine and jam, 250ml orange juice
3.0	
4.0	
5.0	Lunch - 1 piece boiled chicken, rice and vegetables, fruit salad, 200 orange juice
6.0	
8.0	
10.0	Supper - volunteers choice
12.0	
24.0	

After lunch the volunteers took water as required

4.5.1.6. Collection and Storage of urine samples

The collection of urine samples was controlled by the volunteers themselves, and was, therefore, not subject to the same degree of control as the collection of blood samples.

The urine output was collected in a large measuring cylinder. The volume was measured and a 10ml sample was transferred to a labelled vacutainer tube, the remainder of the urine was discarded. Urine samples were stored at -20°C until analysis.

4.5.1.7 Analysis of Serum Samples

Serum samples were brought to room temperature and then mixed on a vortex mixer before being analysed. The samples were prepared according to the method described in section 4.2.1.3. Between 10µl and 20µl of the final reconstituted sample was injected on to the column.

4.5.1.8 Analysis of Urine Samples

Urine samples were brought to room temperature, mixed using a vortex mixer and analysed, according to the method described in Section 4.3.1.2.

4.5.1.9 Pooled Serum

Using the serum samples collected following TD1 administration, pooled serum samples, at each sampling time were obtained by mixing 250ul serum from each volunteer. These 1ml serum samples were vortexed and prepared for injection according to the method described in Section 4.2.1.3.

4.5.2 Results and Discussion

Four healthy volunteers, mean age 22.8 ± 1.1 years, weighing 85.8 ± 2.5kg and of 187.3 ± 5.2cm average height, participated in the clinical study. The same volunteers participated in both trials ensuring that all four volunteers took both preparations.

Erythromycin base and erythromycin stearate appeared to be well tolerated by the volunteers for the duration of the trial. Gastric discomfort did however occur in some of the volunteers following dosing, although this effect was mild and transient.

The use of an indwelling catheter for the collection of blood samples was found to be convenient. However, in one subject (P2) the butterfly became blocked. The delay caused by the removal and subsequent replacement of the butterfly resulted in omission of the blood sample scheduled to be drawn from P2 at 2 hours 30 minutes following TD2 administration.

The individual serum concentration-time and urinary excretion data following administration of TD1 and TD2 are listed in Appendices 9 and 10 and the curves are depicted in Figs. 4.7 - 4.10.

Mean serum concentration-time profiles and mean urinary excretion profiles are shown in Fig. 4.11 and Fig. 4.12 respectively.

These results show peak blood concentrations following TD1 administration ranged between 1.69 μg/ml and 2.96 μg/ml, whilst those obtained following TD2 administration ranged between 0.77 μg/ml and 1.89 μg/ml. All volunteers achieved a greater C_{max} following TD1 administration than TD2 administration. However, due to the omitted sample, it is unknown whether P2 achieved a higher concentration following TD2 administration than the concentration reported at 3.0 hours. The average time to reach C_{max} is 3.1 ± 0.54

hours and 2.87 ± 1.1 hours following administration of TD1 and TD2 respectively. However, following TD2 adminstration P1 exhibited a rapid absorption showing a C_{max} at 1 hour 30 minutes. All the volunteers exhibited an absorption lag-time of one hour or more following both TD1 and TD2 administration. The lag-time following TD2 administration however, appeared to be more erratic, with two volunteers (P3 and P4) only showing detectable amounts of drug after two hours.

It is well known that erythromycin base absorption is impaired by exposure to acidic gastric fluids. Both the preparation of erythromycin stearate and the formulation of enteric coated erythromycin base dosage forms have attempted to reduce the gastric degradation and hence improve absorption and clinical efficacy.

Results from the clinical trials carried out reveal, on average, the erythromycin base formulation produces higher serum concentrations than the erythromycin stearate formulation. The time to reach maximum serum concentration was however, similar following the administration of both preparations.

Reports in the literature (8, 51) and acid stability studies carried out in our laboratory suggest erythromycin stearate is acid labile. As such the lower erythromycin serum levels following administration of the erythromycin stearate formulation may be related to acid degradation of the erythromycin in the stomach. If the film coated erythromycin stearate tablet remained in the acid stomach for a period of time, the film coat will dissolve and expose the erythromycin stearate. The acid-labile erythromycin stearate would therefore be subject to acid degradation in the stomach leading to reduced biovailability. Alternatively, if the capsule of the erythromycin base formulation releases the pellets in the stomach, the enteric coating will still protect the erythromycin base until the pellets pass through the pyloric sphincter, prior to absorption.

Fig 4.7 Serum concentration-time profiles following administration of test dose 1 (TD1)

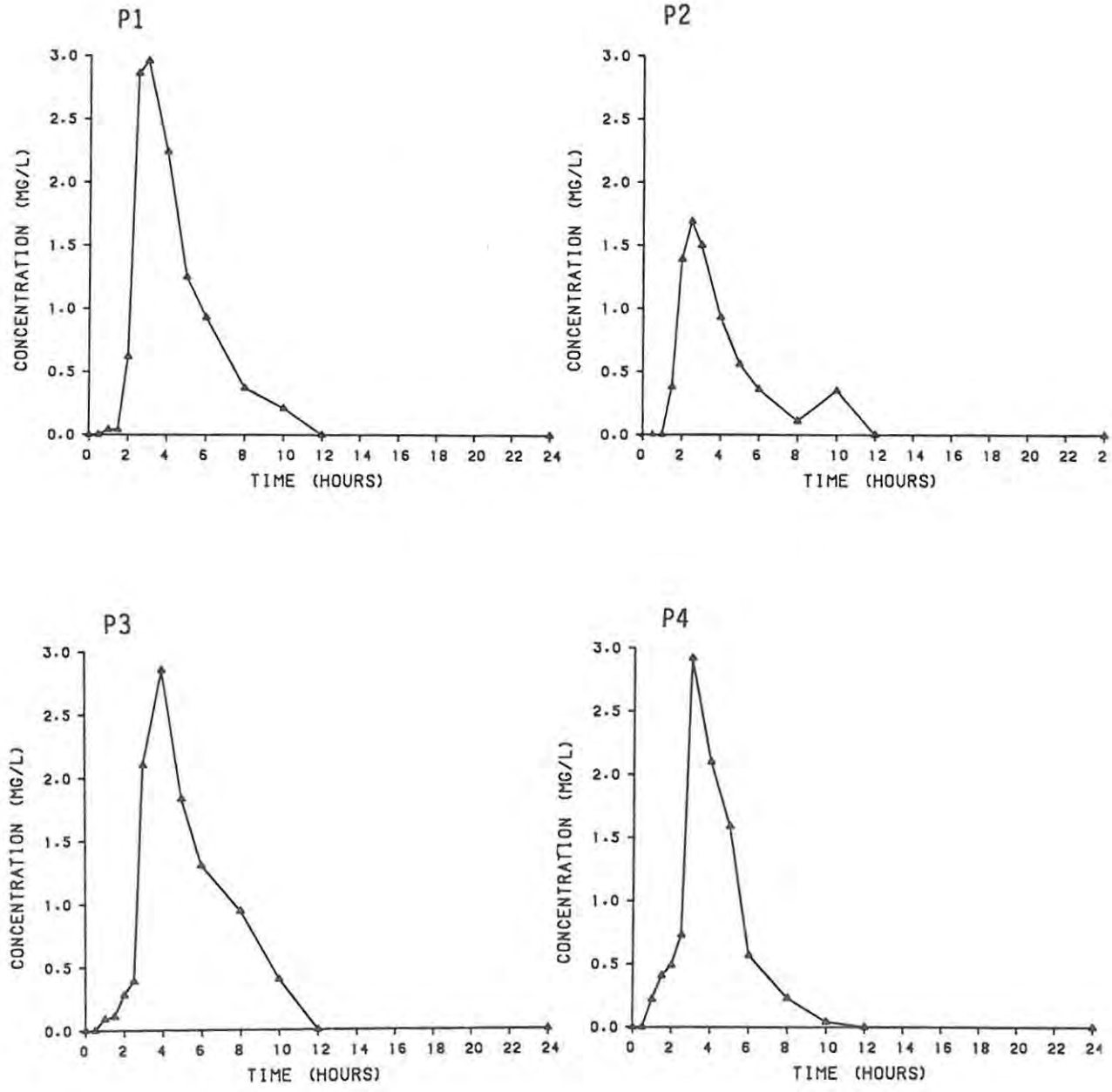


Fig 4.8 Serum concentration-time profiles following administration of test dose 2 (TD2)

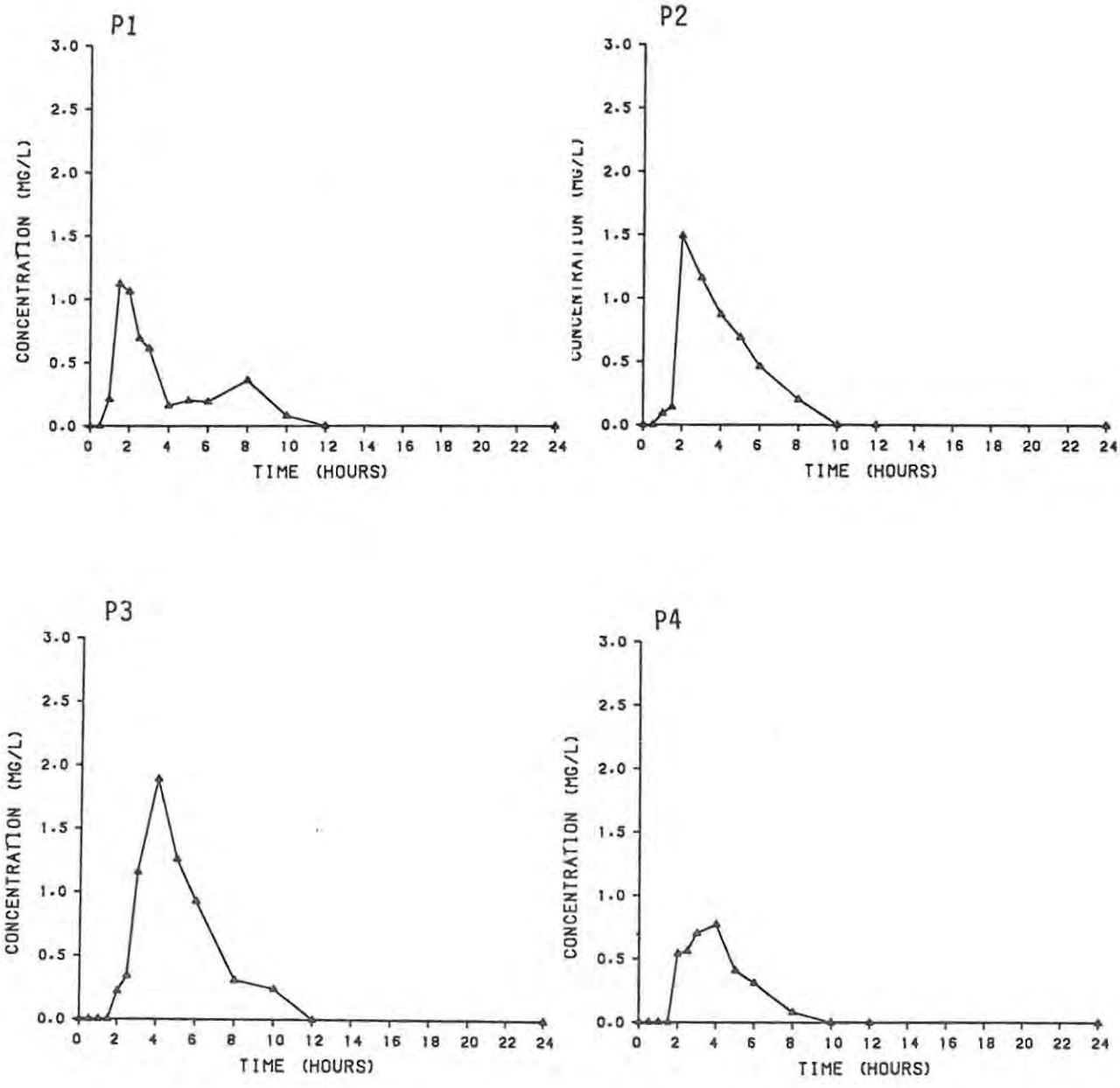


Fig 4.9 Cumulative urinary excretion profiles following administration of test dose 1 (TD1)

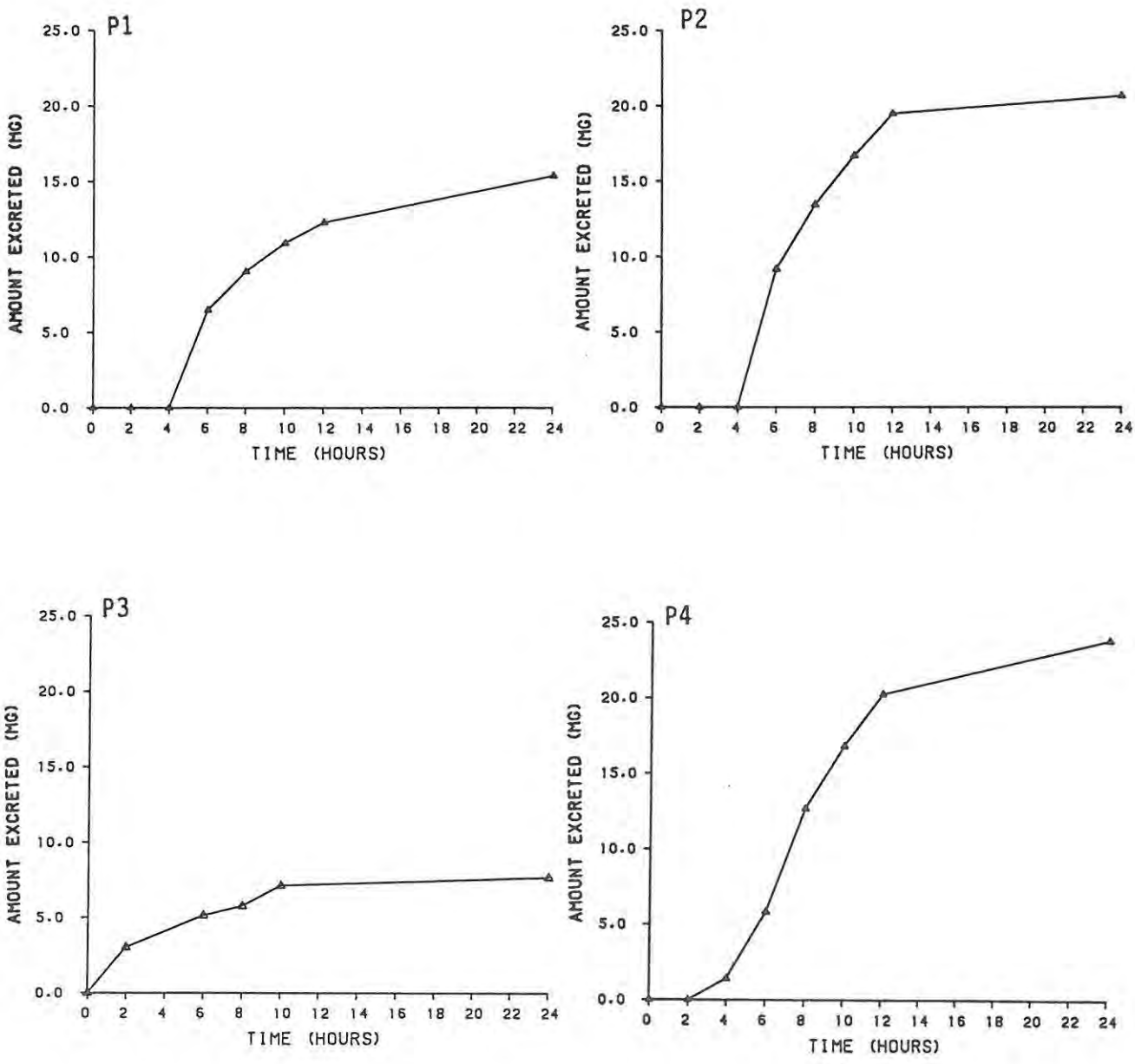


Fig 4.10 Cumulative urinary excretion profile following administration of test dose 2 (TD2)

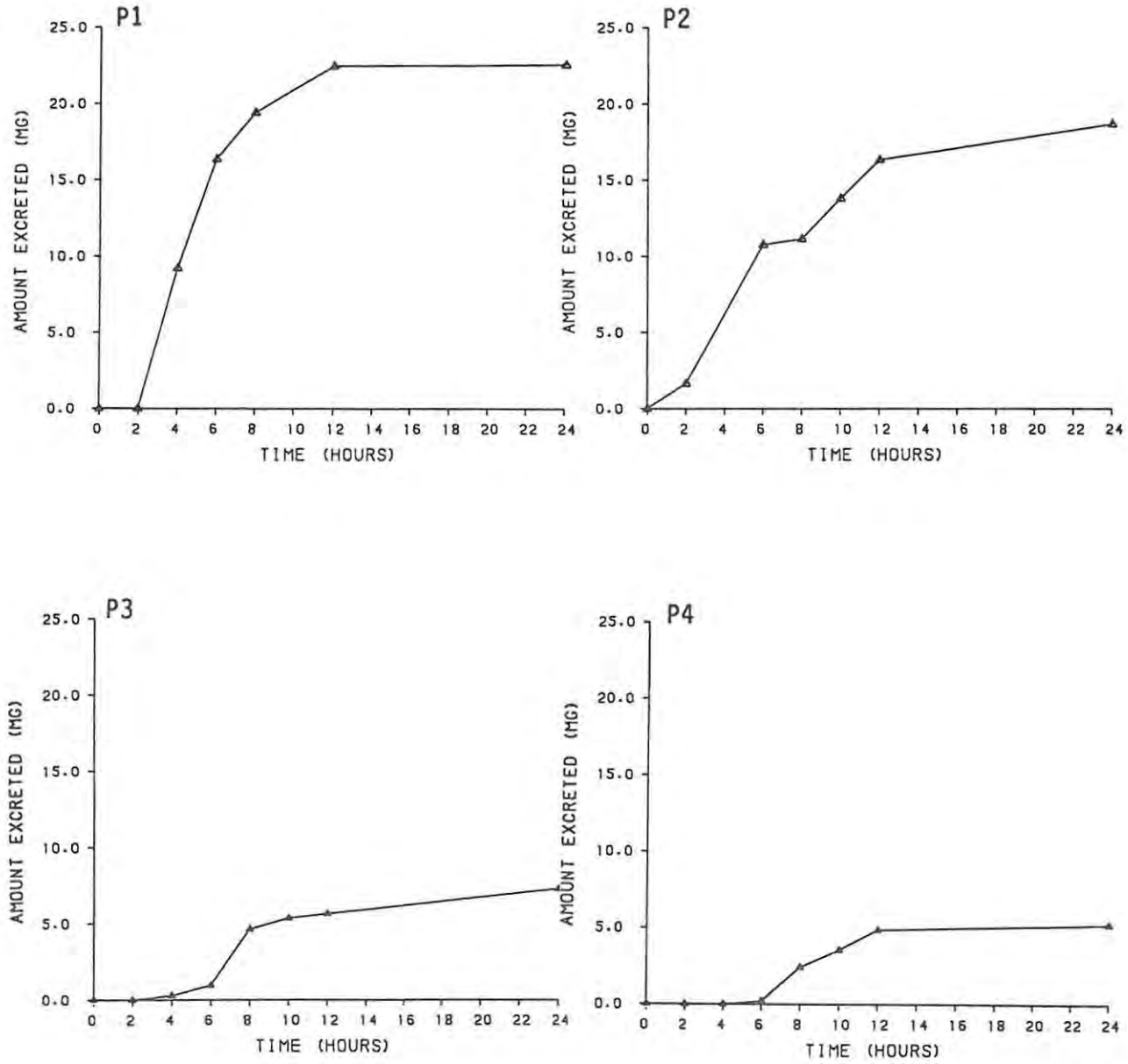
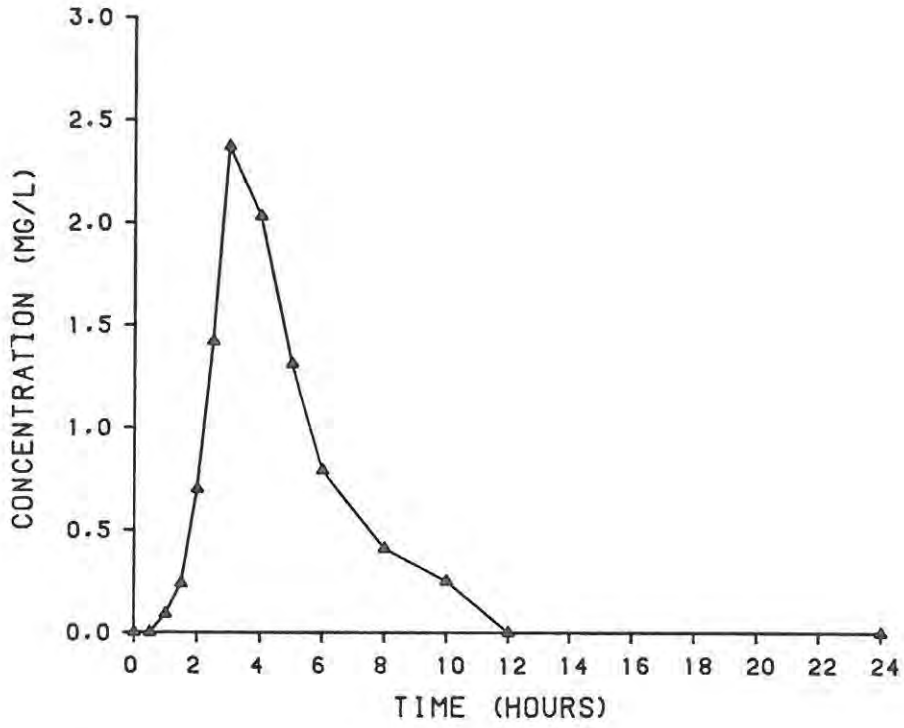


Fig 4.11 Mean serum concentration-time profiles following administration of test dose 1 and test dose 2



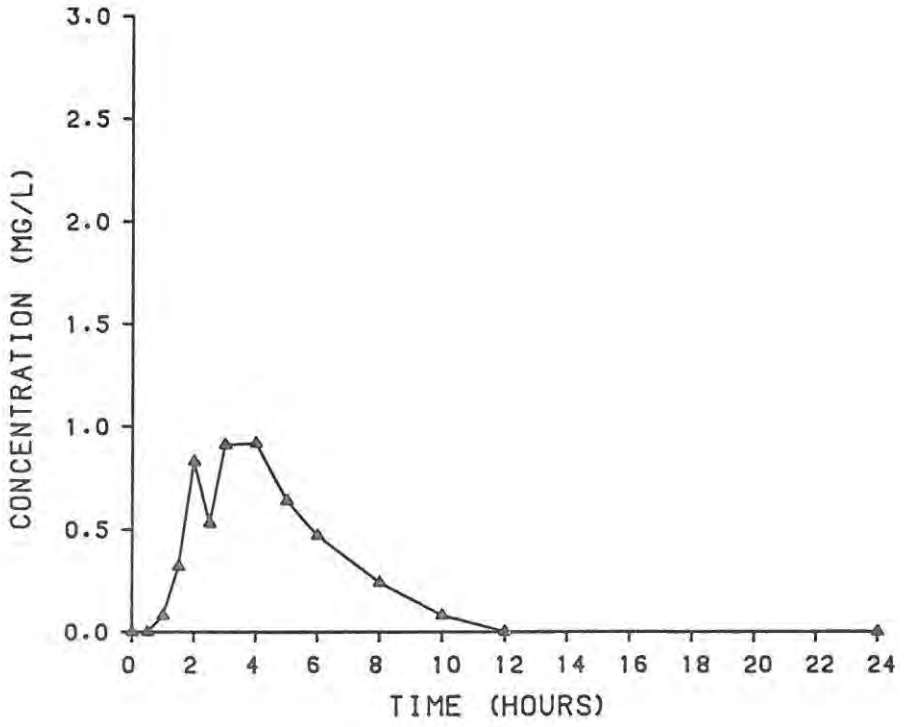
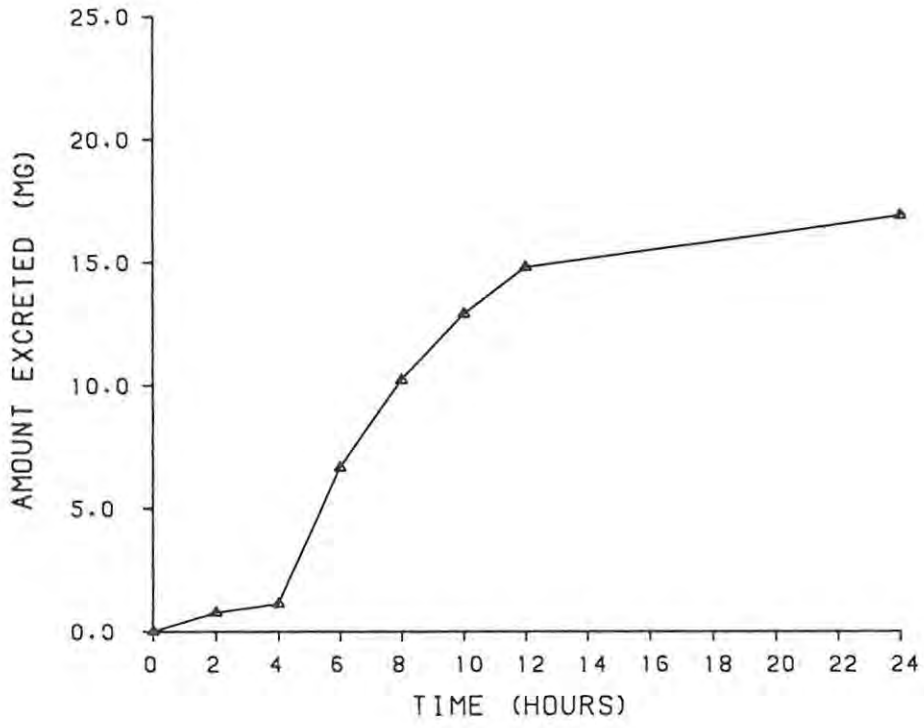
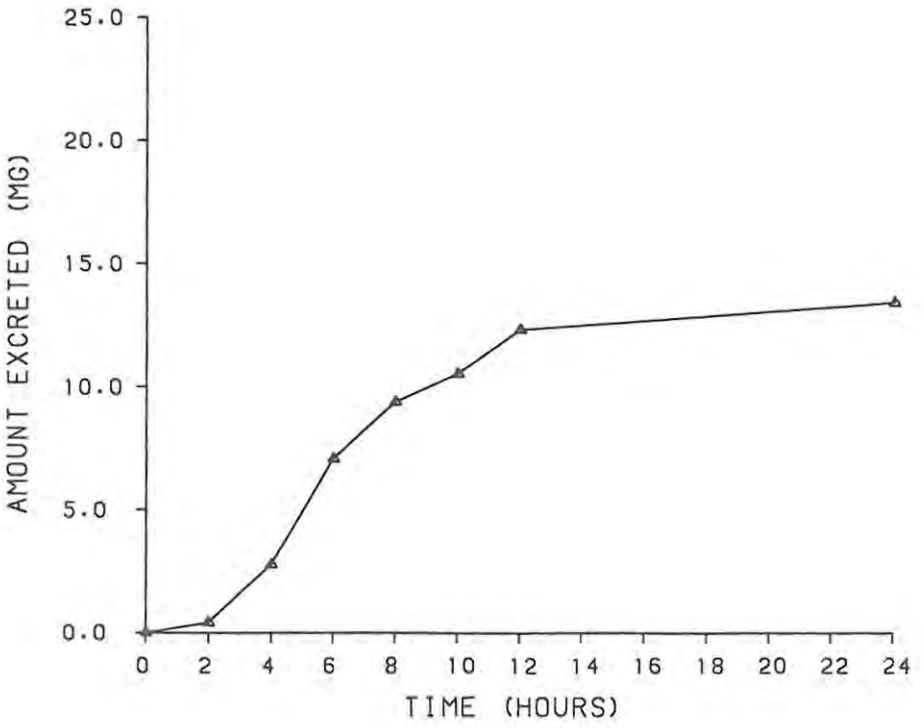


Fig 4.12 Mean urinary excretion profiles following administration of test dose 1 and test dose 2





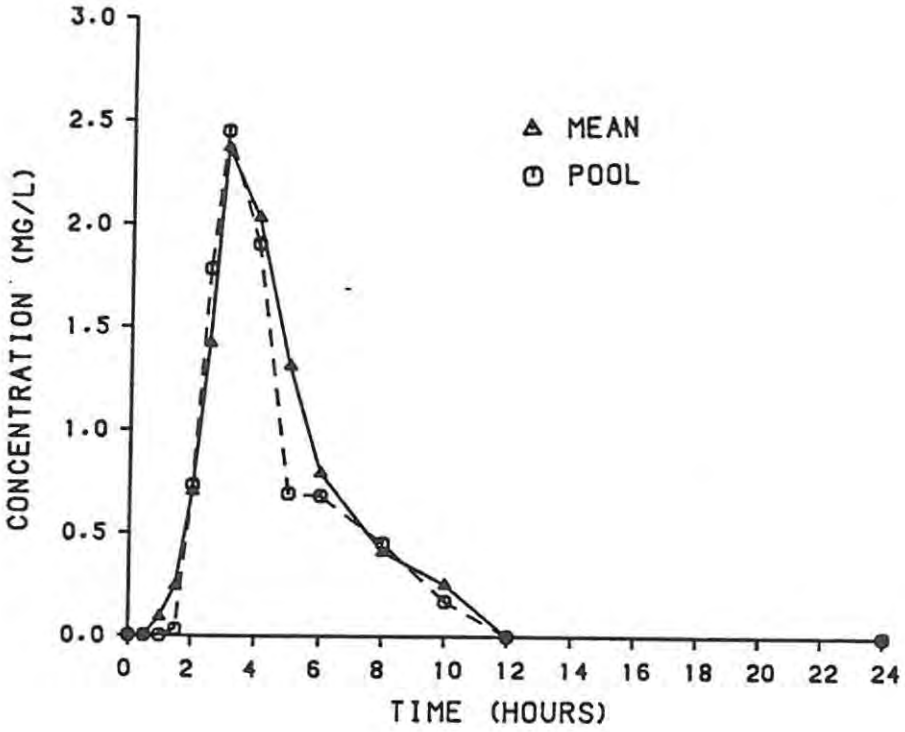
In addition, pellets pass through the pyloric sphincter easier than tablets which may remain in the stomach and therefore be exposed to the gastric fluids for a longer period of time. The erythromycin stearate tablets remaining in the stomach may have resulted in increased erythromycin stearate degradation, reducing serum concentration, and may also be responsible for the erratic lag time following administration of erythromycin stearate.

The results from the clinical trials are in agreement with several reports in the literature (55, 56, 140), that higher erythromycin serum concentrations and more regular absorption is obtained following administration of a capsule containing enteric coated erythromycin base pellets than from a film coated erythromycin stearate formulation.

Cumulative urinary excretion profiles indicate that a small but variable percentage of the administered dose was excreted as unchanged erythromycin after 24 hours. The mean percentage of original drug excreted unchanged in the urine is 3.38% and 2.69% following TD1 and TD2 administration respectively. No evidence of linearity was observed between the total amount of drug excreted and the area under the curve (vide infra Chapter 5). Urine samples collected during the later stages of the trials contained varying amounts of degradation products/metabolites indicating that not all the drug is excreted as intact base.

The individual serum concentration-time data from the pooled serum samples are given in Appendix 11. From Fig. 4.13 it can be seen that the pooled serum samples resulted in similar concentrations to those obtained by analysing the samples individually and calculating the mean concentrations. Use of pooled samples would greatly reduce analytical costs and time. However, results obtained would be limited and would not reveal any large inter-individual variations.

Fig 4.13 Serum concentration-time profiles following administration of test dose 1 - mean and pooled data



In summary, the HPLC method was effective for the determination of erythromycin base in serum and urine. Data obtained following the clinical trials revealed higher serum concentrations of erythromycin base were obtained following TD1 administration than following TD2 administration. The small and variable amounts of base eliminated in the urine and the poor correlation between serum and urine concentration suggests that erythromycin urinary excretion data are of little value in pharmacokinetic studies.

CHAPTER 5

PHARMACOKINETICS AND COMPARATIVE BIOAVAILABILITY OF ERYTHROMYCIN

5.1 INTRODUCTION

The purpose of pharmacokinetics is to study the time course of drug and metabolite concentrations in biological fluids, tissue and excreta, and to construct suitable models to interpret such data (141). Traditionally, pharmacokinetic analysis has involved the use of a 'compartmentalized' system, with the behaviour of a drug in a biological system described in terms of either a one, two or multicompartmental model (142). This method, based upon linear processes, uses a series of differential equations to describe the transfer of drug between compartments. Numerous reports on compartmental analysis, as well as computer programs available for optimization of pharmacokinetic parameters have appeared in the literature (142-146).

More recently, however, there has been a move away from compartmental analysis and curve fitting towards noncompartmental and model independent approaches (147-149). These methods may be applied to virtually all linear systems since no assumption of any specific compartment model is made.

Bioavailability is defined as the rate and the extent to which a drug becomes available in the systemic circulation (142). Peak serum concentration (C_{max}) and time to peak (t_{max}), which can be obtained directly from the serum concentration-time profile give a rough indication of the rate of absorption (150), while the extent of absorption can be obtained from the area under the curve (AUC). Several methods however, have been developed to quantify the rate of absorption in terms of a constant, K_a (147-155). An estimate of the absorption rate constant, K_a, can be obtained by 'feathering' the curve

(142) and subsequent computer-fitting of the serum concentration versus time profile, or from the Wagner-Nelson method (153), or various other such techniques (143, 149). An alternative method, dependent only on the time course of the drug was described in 1978 by Yamoaka (147) and Cutler (151). This noncompartmental method has been extensively reviewed by Riegelman and Collier (152). This method of statistical moments assumes that irrespective of the route of drug administration, the zero and first moments are calculated as follows:

$$AUC = \int_0^{\infty} C.dt \qquad \text{Equation 5.1}$$

$$MRT = \frac{\int_0^{\infty} t.C.dt}{\int_0^{\infty} C.dt} = \frac{AUMC}{AUC} \qquad \text{Equation 5.2}$$

where AUC, the zero moment, is the area under the curve, AUMC, is the area under the moment curve, and MRT, the first moment, is the mean residence time. The use of statistical moments has found widespread application in comparative bioavailability studies.

As previously discussed in Section 1.5 various pharmacokinetic models for erythromycin have been reported in the literature. However, following administration of either erythromycin base or erythromycin stearate, pharmacokinetics of erythromycin are best described by an open one compartment body model, with absorption described as either zero order, first order or as a variable first order process (7,47, 55, 56, 58, 80, 120).

Conflicting reports on the bioavailability of erythromycin following administration of erythromycin base and erythromycin stearate have appeared in the literature. Several reports (37, 46, 56, 80, 140) indicate that enteric coated pellets produce higher C_{max} and AUC values than film coated stearate tablets. However, Clayton et al (48) and Malmborg (35) reported erythromycin stearate tablets to have a greater bioavailability than enteric coated pellets.

In this study, both model-independent and compartmental models were used for the pharmacokinetic analysis of the erythromycin serum concentration-time data.

5.2 METHODS OF CLINICAL TRIAL DATA ANALYSIS

5.2.1 Model-Independent Analysis

The parameters C_{max} and t_{max} were obtained directly from the individual serum concentration-time data. The elimination rate constant, K_e, was calculated from the terminal slope of the semilogarithmic plot of serum concentration versus time, whilst the AUC values from time zero to infinity were calculated by the log-linear trapezoidal method. Extrapolation to infinity involved the addition of the residual area, given by dividing the final measured serum concentration by K_e.

The mean residence time (MRT) was calculated using Equation 5.2. The clinical trial data were then analysed by the manual feathering of the semilogarithmic plots of serum concentration versus time to calculate K_a. In addition K_a was calculated from the construction of percentage drug absorbed versus time plots as described by Wagner (153).

5.2.2 One Compartment Model - First Order Absorption (Model I)

Absorption of drug is described by a constant input function which is independant of the amount of drug present at the site of absorption. Drug elimination is assumed to be first order.

The following equation (120) was used to characterize this model:

$$\frac{dC}{dt} = \frac{K_a D e^{-K_a(t-t_{lag})}}{V} - K_e C_1 \qquad \text{Equation 5.3}$$

$\frac{dC}{dt}$ = rate of change of drug concentration in the central compartment
K_a = first order absorption rate constant
D = dose of the drug
V = volume of central compartment
K_e = elimination rate constant
C_1 = concentration in the central compartment

5.2.3 Two Compartment Model - First Order Absorption (Model II)

The two compartment model attempts to characterize drug concentrations if the drug does not rapidly distribute throughout the body. This model allows for a distribution phase during which the drug distributes between the central compartment and a peripheral compartment. The model utilized in this study assumes drug elimination occurs only from the central compartment. The differential equation describing the rate of change of drug in the peripheral and central compartments are as follows (120):

$$\frac{dC}{dt} = \frac{K_a D e^{-k_a(t-t_{lag})}}{V} + K_{21}A_2 - \frac{K_{12}C_1}{V} - K_e C_1 \qquad \text{Equation 5.4}$$

K_{12} = first order rate constant for transfer of drug from the central compartment to the peripheral compartment
K_{21} = first order rate constant for transfer of drug from peripheral compartment to central compartment
A_2 = amount in the peripheral compartment
K_e = first order rate constant for elimination of drug from central compartment.

5.2.5 Computer Analysis

An integrated program PEELFIT (145) operated on an Apple IIe microcomputer was used to computer fit the experimental data to Model I and Model II. PEELFIT does not reqire any initial estimates or limits of parameters in order to run the program, and as such estimates of the parameters are calculated without any constraint. All the data received equal weighting.

The computer fitting of experimental data to Model I was subsequently carried out using the nonlinear regression program FLEXPLEX (156) operated on a VAX STATION 2000 computer. Prior to running of the programme, initial estimates and limits of all parameters were required. Initial estimates for parameters were obtained from 'feathering' of the curves and from the Wagner - Nelson method. During modelling, the elimination rate constants were constrained at the K_e value obtained from 'feathering' the curves, since estimates of K_e and K_a were found to converge to the same value if neither parameter was constrained. This convergence has been explained by Tse and Welling (157), and in order to overcome this, it has been suggested that during computer fitting the K_e value be constrained at the value obtained for K_e from 'feathering' (47). As with the PEELFIT program all data received equal weighting.

Initially very poor computer fits were obtained, presumably related to the long lag - time which was exhibited in all the patients. In order to overcome this problem and acheive a better computer fit and consequently to give a more accurate estimate of the parameters, it was necessary to adapt the model. As such, when the time following the administration of the dosage form was less than the lag - time calculated from the program, the predicted concentration was maintained at 0.0 ug/ml. Using this computer program, the curve fitting continues

until the percentage difference between the last and present sum of squares is less than or equal to 0.00001%.

5.3 RESULTS AND DISCUSSION

5.3.1 Model Independent Analysis

The values of C_{max}, t_{max}, AUC, K_e and $t_{1/2}$ obtained from the serum data following TDI and TD2 administration are presented in Table 5.1. As reported in Section 4.5.2. peak blood concentrations following TD1 (erythromycin base formulation) administration ranged between 1.69 µg/ml and 2.96 µg/ml, whilst those obtained following TD2 (erythromycin stearate formulation) administration ranged between 0.77 µg/ml and 1.89 µg/ml. All volunteers achieved a higher AUC value following TD1 administration except P2 who showed a slightly greater AUC value following TD2 administration.

The elimination rate constant, K_e, and $t_{1/2}$, were obtained from the terminal slope of the semilogarithmic plots of serum concentration, versus time. Secondary peaks or 'bumps' in the elimination phase of some of the volunteers, possibly due to enterohepatic recycling, made it difficult to calculate the elimination rate constant. 'Bumps' were therefore omitted in the calculation of K_e, since it was felt that inclusion of these concentrations in the slope would lead to errors in the estimation of K_e. Data points omitted in the calculation of K_e include, in P2 following TD1 administration, time = 10.0 hours, and in P1 following TD2 adminstration, time = 4.0 and 8.0 hours. The K_e values for these two volunteers were subsequently calculated using fewer available data points.

The semilogarithmic plots of the individual concentration-time data shown in Fig. 5.1 and Fig. 5.2, reveal the decline in erythromycin concentration to be, in general, monoexponential. The calculated elimination rate constants for all volunteers following TD1 administration did not differ to any great extent except for subject

Table 5.1 Model independent pharmacokinetic parameters

TEST DOSE	VOLUNTEER	PARAMETERS				
		t_{max} (h)	C_{max} (μg/ml)	AUC (μg/ml.h)	k_e (h^{-1})	$t^{1/2}$ (h)
TD1	P1	3.0	2.96	10.07	0.39	1.79
	P2	2.5	1.69	4.91	0.29	2.39
	P3	4.0	2.85	11.95	0.30	2.31
	P4	3.0	2.92	7.74	0.52	1.33
	MEAN (SD)	3.1 (0.54)	2.61 (0.52)	8.66 (2.63)	0.38 (0.09)	1.95 (0.43)
TD2	P1	1.5	1.12	2.98	0.26	2.67
	P2	2.0	1.49	5.25	0.33	2.10
	P3	4.0	1.89	6.60	0.37	1.87
	P4	4.0	0.77	2.34	0.55	1.26
	MEAN (SD)	2.9 (1.1)	1.31 (0.42)	4.29 (1.72)	0.38 (0.10)	1.97 (0.5)

Fig 5.1 Semilogarithmic plots of serum concentration versus time following administration of test dose 1

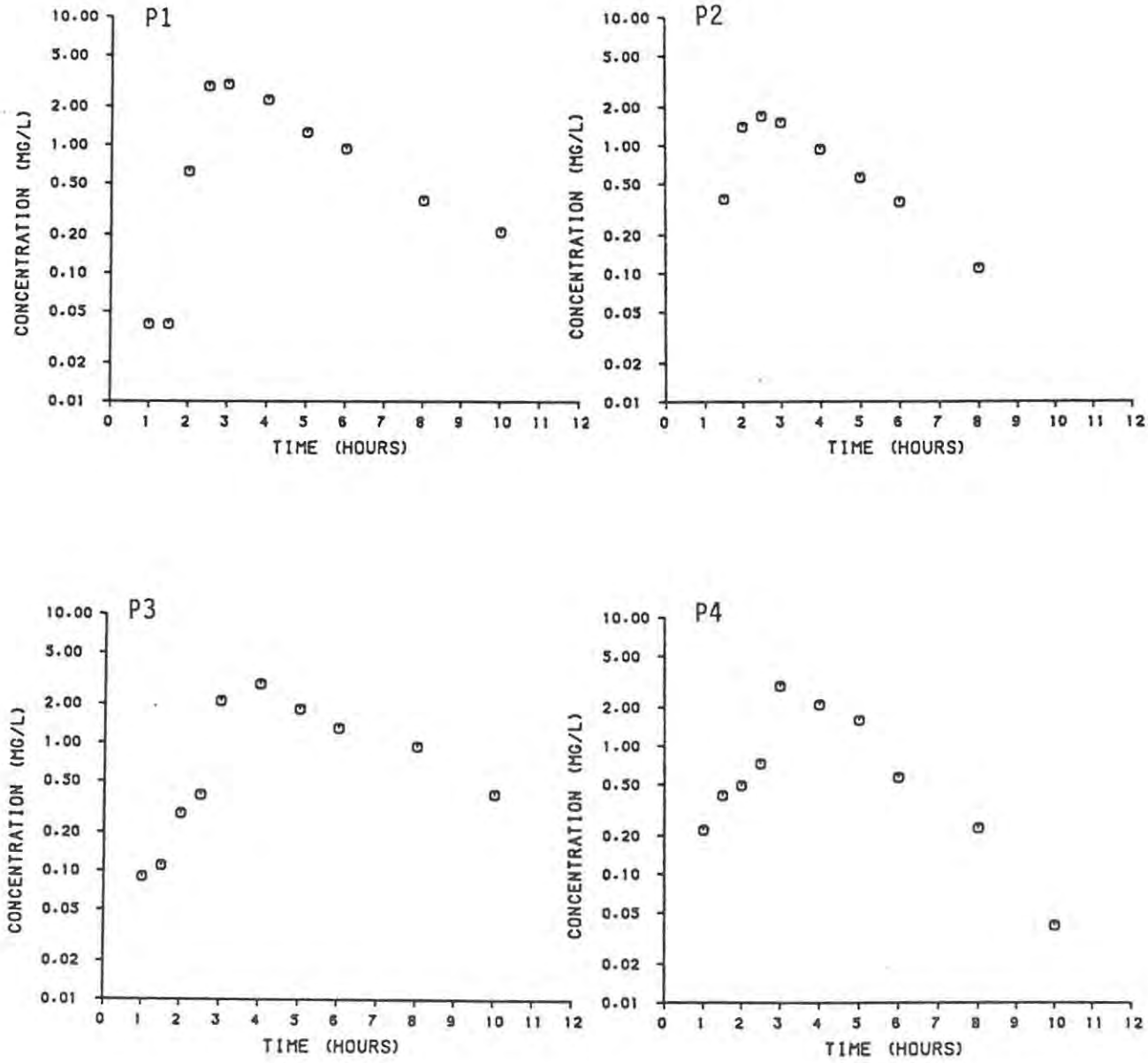
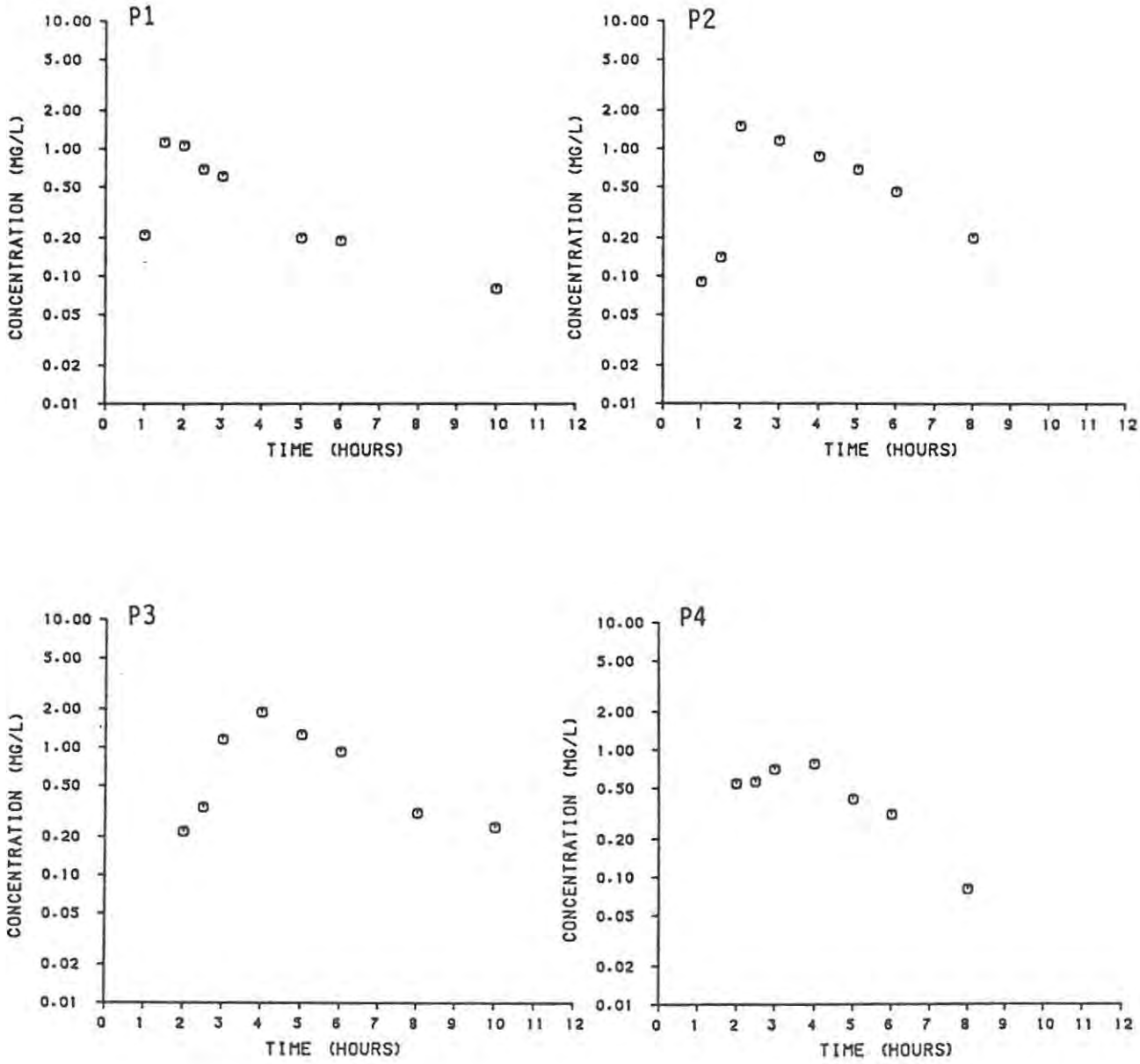


Fig 5.2 Semilogarithmic plots of serum concentration versus time following administration of test dose 2



P4, who displayed a larger K_e and consequently a shorter $t_{1/2}$. This pattern was repeated following TD2 administration suggesting that due to intra-individual variation, P4 eliminates erythromycin faster than the other volunteers. The faster elimination rate of P4 is however unlikely to be the sole factor contributing to the low C_{max} and AUC following TD2 administration, since following TD1 administration, the K_e value was the same, although significantly higher C_{max} and AUC were obtained. Excluding the K_e value of P4, the above results reveal both low intra- and inter-personal variability in K_e. These results are in agreement with Austin et al (58), Jossefsson et al (55) and more recently Tjandramaga et al (140) who also reported low interpersonal variation in K_e values. These reports all suggest that variations in serum concentration and AUC values are due to variability in the absorption process.

Results from the statistical moment analysis are shown in Table 5.2. Although the use of statistical moments has found widespread application in comparative bioavailability studies, results of little significance were obtained using this method, with similar values being obtained for TD1 and TD2. The slightly shorter transit time found for P4 following administration of both dosage forms is possibly explained in terms of the greater elimination rate value for that volunteer.

Table 5.2 Relative bioavailability of test dose 1 with respect to test dose 2

	MRT (h)	
VOLUNTEER	TD1	TD2
P1	4.78	5.26
P2	5.06	4.82
P3	6.25	5.78
P4	4.34	4.74
MEAN (SD)	5.10 (0.71)	5.51 (0.41)

5.3.2 Computer Analysis

Observed and calculated serum concentrations obtained by computer fitting of data to Model I are reported in Appendix 12.

Model I appeared to fit the data adequately, provided the predicted concentration was constrained at 0.0ug/ml, when the time following dosage form administration was less than the lag-time predicted by the model. Constraining the model to incorporate the lag-time ensured the estimates of K_a were a better reflection of the absorption rate constant than if the model was not constrained, and the rate constant was subsequently calculated as a combination of the lag-time and the absorption rate. Results of computer fitting using Model I are graphically represented in Fig. 5.3 and Fig. 5.4. The above results are in agreement with reports in the literature that suggest erythromycin shows first order absorption (7,55,56,80).

Using PEELFIT, however, neither Model I nor Model II appeared to fit the data adequately. Unlike FLEXPLEX, PEELFIT does not require any initial estimates or limits on parameters. In addition, the elimination rate constant cannot be constrained and the model could not be adapted to accomodate the long lag-time, consequently serum concentration-time profiles correlated extremely poorly with experimental data. Results of computer fitting using Model I obtained from PEELFIT are shown graphically in Fig. 5.5 and Fig. 5.6.

From Table 5.3 results of the K_a and K_e values obtained from Model I are shown. These values are compared with those obtained using PEELFIT, as well as 'feathering' the curve and the Wagner-Nelson method.

Table 5.3 Comparison of ka and ke values

TEST DOSE	VOLUNTEER	MODEL I K_e constrained		Model I Peelfit		Values obtained by curve stripping		Wagner-Nelson method
		K_a	K_e	K_a	K_e	K_a	K_e	K_a
TD1	P1	3.36	0.38	0.38	0.32	0.62	0.38	2.27
	P2	3.10	0.30	0.55	0.30	2.42	0.29	2.86
	P3	1.13	0.32	0.28	0.24	1.89	0.30	1.09
	P4	0.97	0.53	0.36	0.32	0.66	0.53	1.73
	MEAN	2.14	0.38	0.39	0.30	1.40	0.38	1.49
TD2	P1	3.09	0.28	0.84	0.39	3.40	0.33	-
	P2	4.41	0.34	0.42	0.36	0.63	0.31	2.23
	P3	1.30	0.38	0.29	0.24	1.02	0.38	1.84
	P4	0.79	0.56	0.38	0.33	0.92	0.51	0.64
	MEAN	2.39	0.39	0.64	0.33	1.49	0.38	1.24

Fig 5.3 Comparison of observed and predicted erythromycin serum concentrations following administration of test dose 1

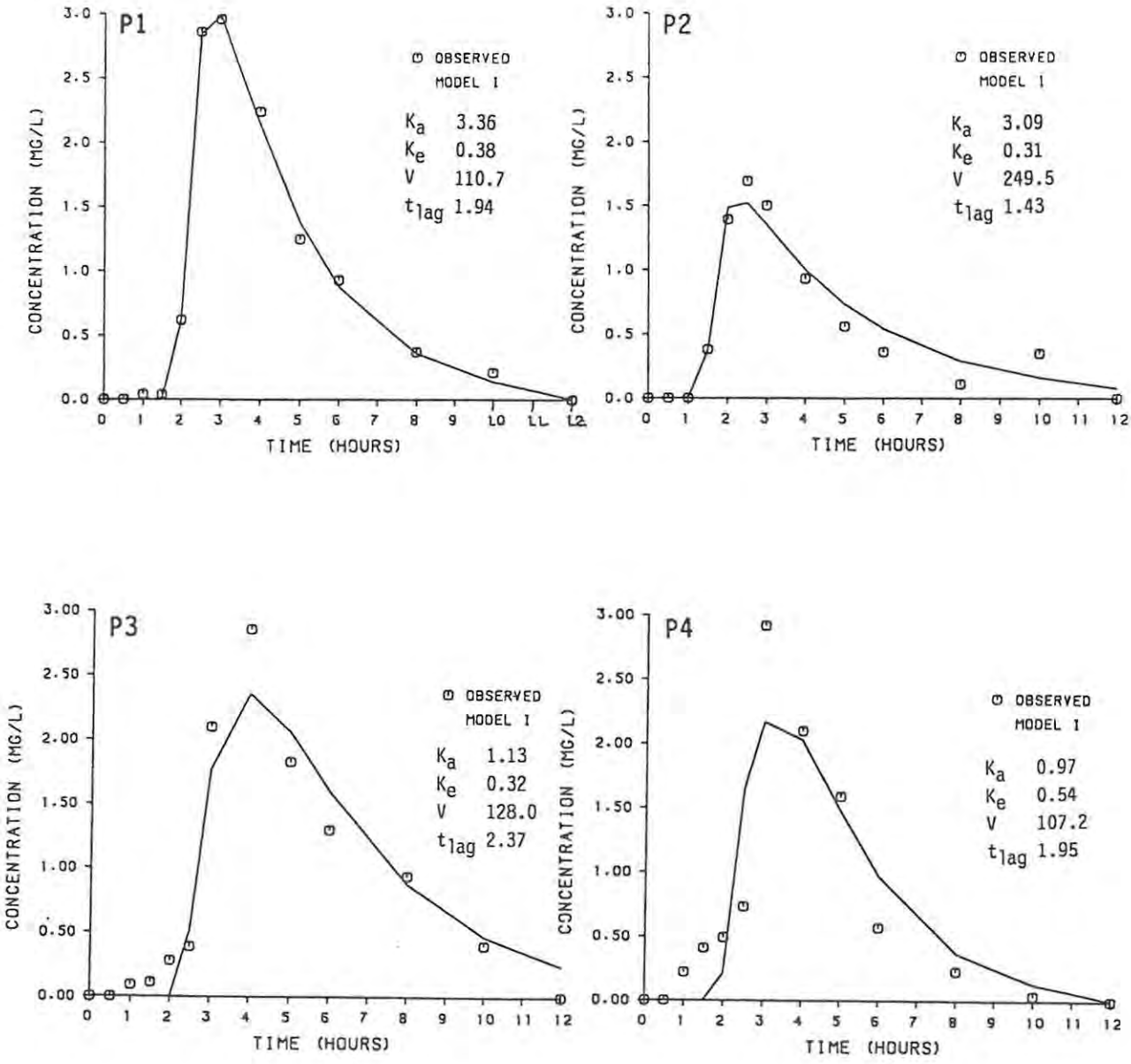


Fig 5.4 Comparison of observed and predicted erythromycin serum concentrations following administration of test dose 2

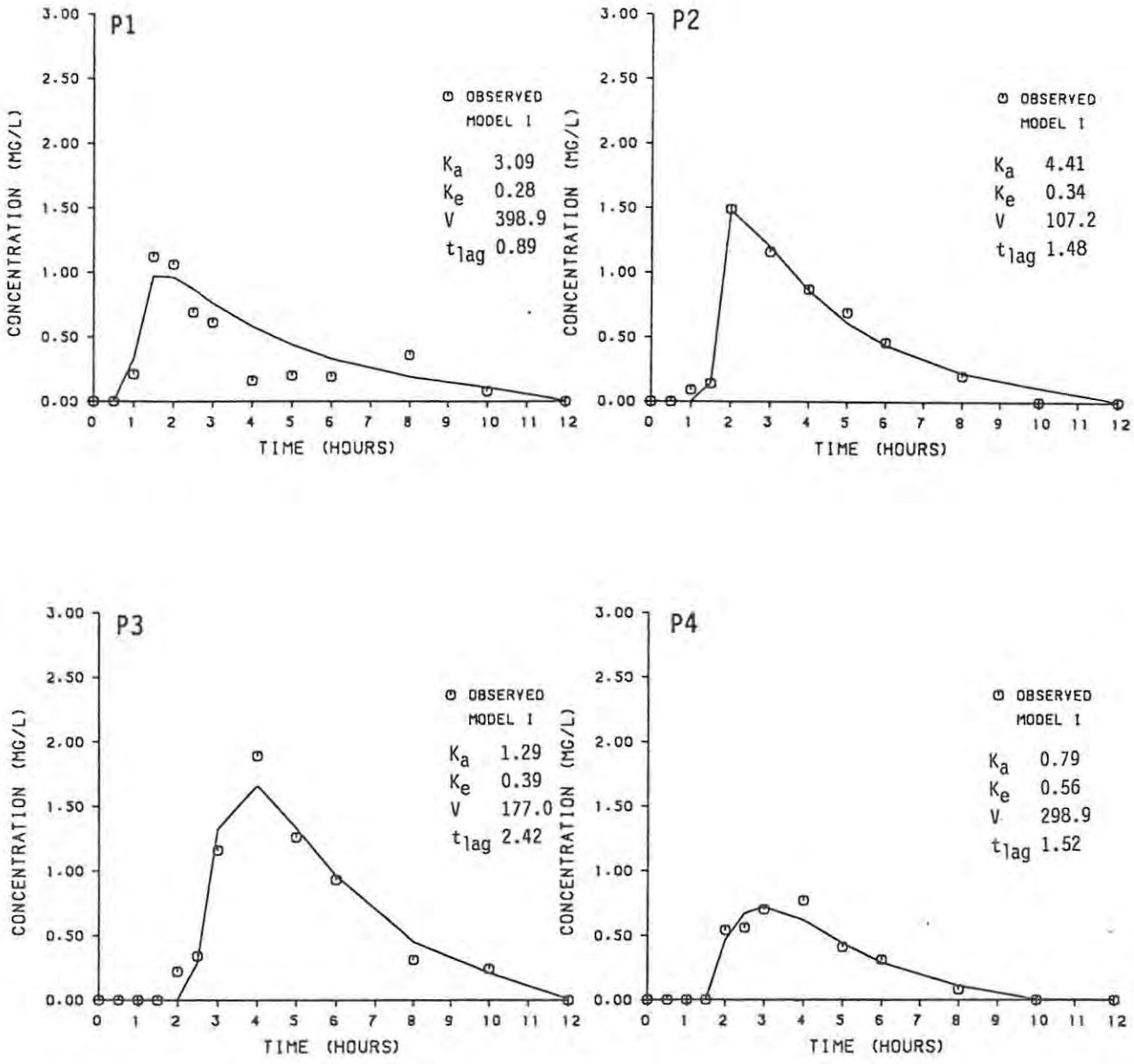


Fig 5.5 Comparison of observed and predicted (PEELFIT) erythromycin serum concentrations following administration of test dose 1

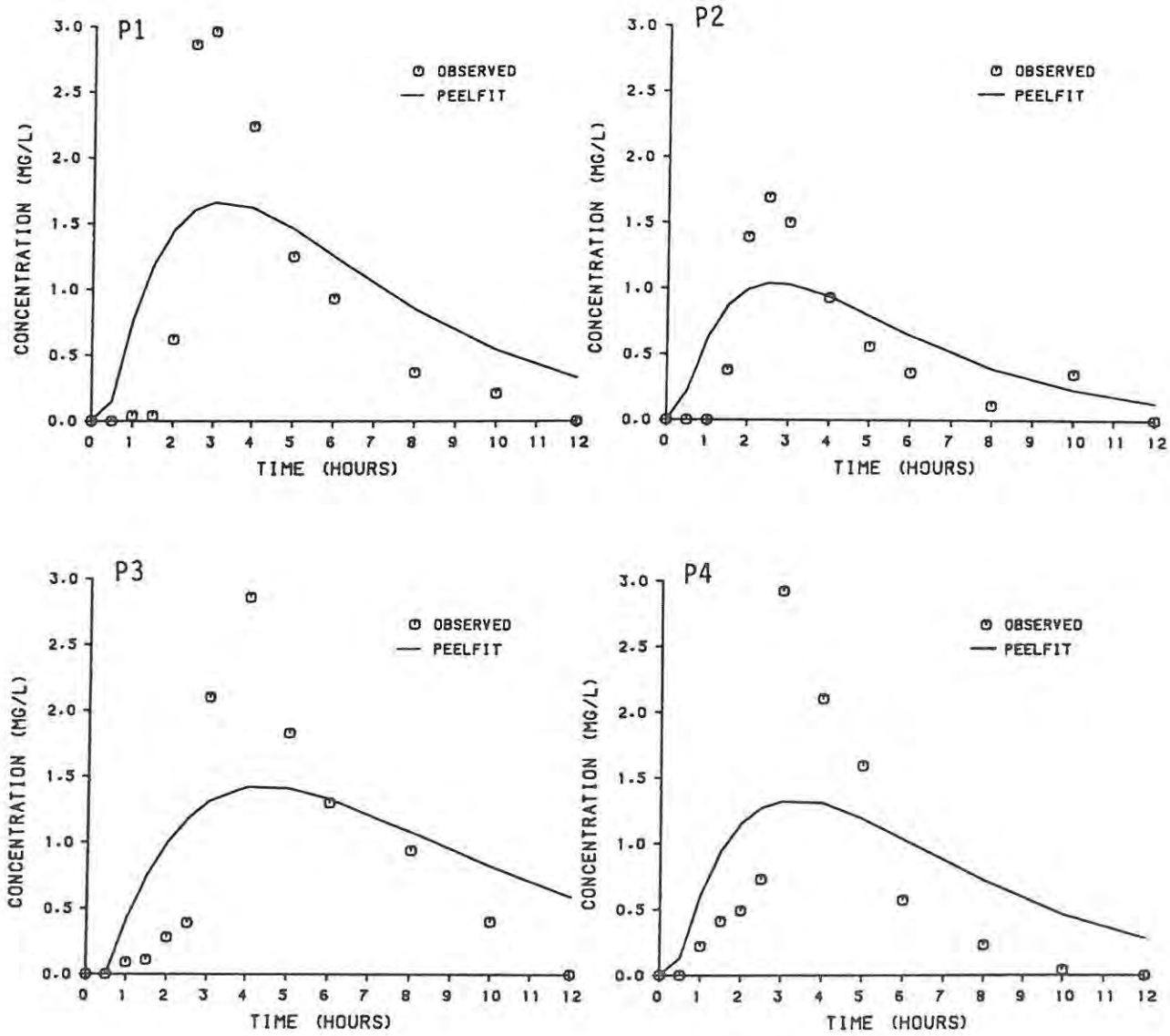
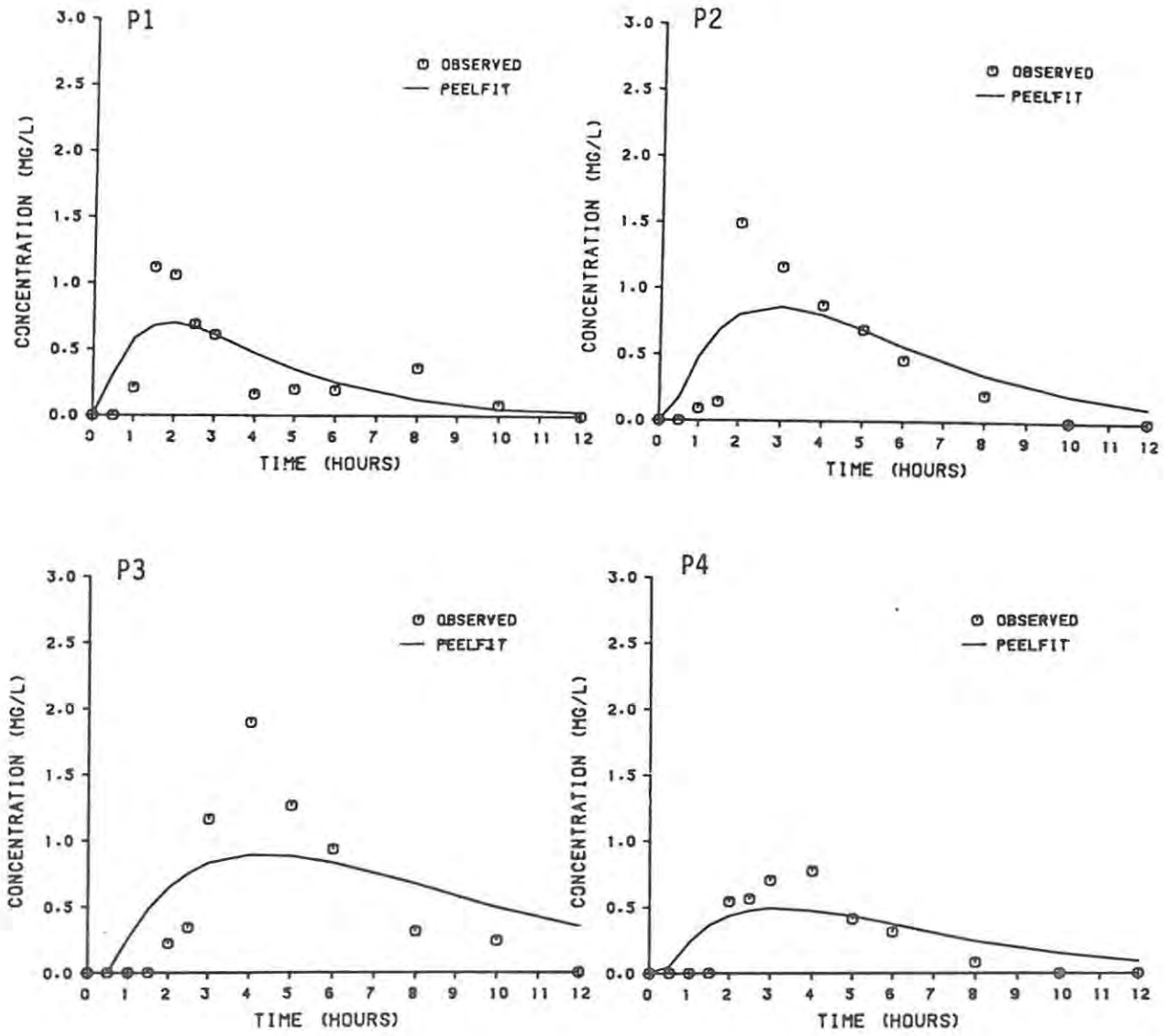


Fig 5.6 Comparison of observed and predicted (PEELFIT) erythromycin serum concentrations following administration of test dose 2



As can be seen from Table 5.3 the absorption rate constants show great intra-personal variability and differ dramatically depending on the method of calculation, a factor which must be borne in mind when comparing data with other literature reports. However, the estimated K_a values obtained can be used to give some indication of the relative absorption rates during comparative bioavailabiity studies, provided the K_a values are obtained using the same method. Due to the rapid absorption and erratic profile in P1 following the administration of TD2, no estimate for K_a was obtained using the Wagner - Nelson method for this volunteer. Results from PEELFIT reveal that mean K_a values of 0.39 h^{-1} and 0.64 h^{-1} were obtained following TD1 and TD2 administration respectively. However, if the high K_a value of P1 following TD2 administration was omitted, the mean K_a value decreased to 0.36 h^{-1}. Computer fitting the data using FLEXPLEX reveal mean K_a values of 2.14 h^{-1} and 2.39 h^{-1} following TD1 and TD2 administration respectively whilst from the Wagner - Nelson method mean K_a values of 1.49 h^{-1} and 1.24 h^{-1} were obtained.

In general, however, the mean K_a values obtained using the same method, following TD1 and TD2 administration did not appear to be significantly different, irrespective of the method of calculation.

Erythromycin concentrations following single dose administration can thus be adequately described by an open one compartmental model with first - order absorption and linear elimination. Following the administration of both erythromycin base and erythromycin stearate, the K_e values obtained for each volunteer did not differ significantly, suggesting little intra-volunteer variability. The limited intra-volunteer variability in elimination rate constants suggests that differences in serum levels and AUC values, following administration of both test doses may be due to variation in absorption, probably related to degradation of the drug in the stomach.

5.3.3 Comparative Bioavailability

The individual AUC values for each test dose were compared in order to ascertain the relative bioavailability of TD1 to TD2. The relative bioavailability F_{rel}, was calculated from the ratio of the AUC values and is presented in Table 5.4. The mean data reveal that TD1 has a greater bioavailability, in terms of the F_{rel} than TD2. In two of the volunteers,the observed AUC following the administration of the erythromycin base formulation is three fold greater than following the administration of the erythromycin stearate formulation, although in P2, the bioavailability of both dosage forms was similar.

Table 5.4 Relative bioavailability of test dose 1 with respect to test dose 2

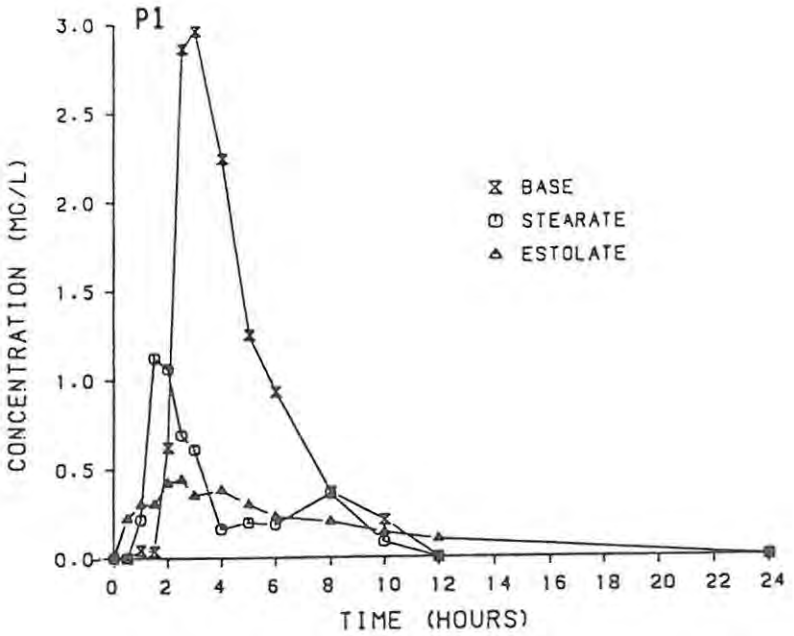
	VOLUNTEERS				
	P1	P2	P3	P4	MEAN (SD)
F_{rel}	3.38	0.94	1.81	3.31	2.36 (1.03)

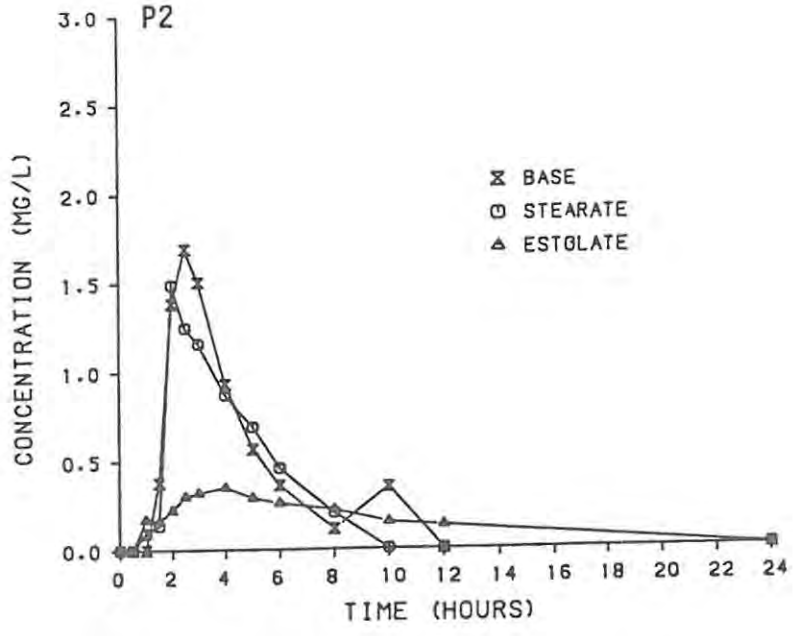
In summary, the clinical trials carried out in this study revealed, for these volunteers, that the enteric coated erythromycin base formulation appears to have a greater bioavailability than the film coated stearate tablets.

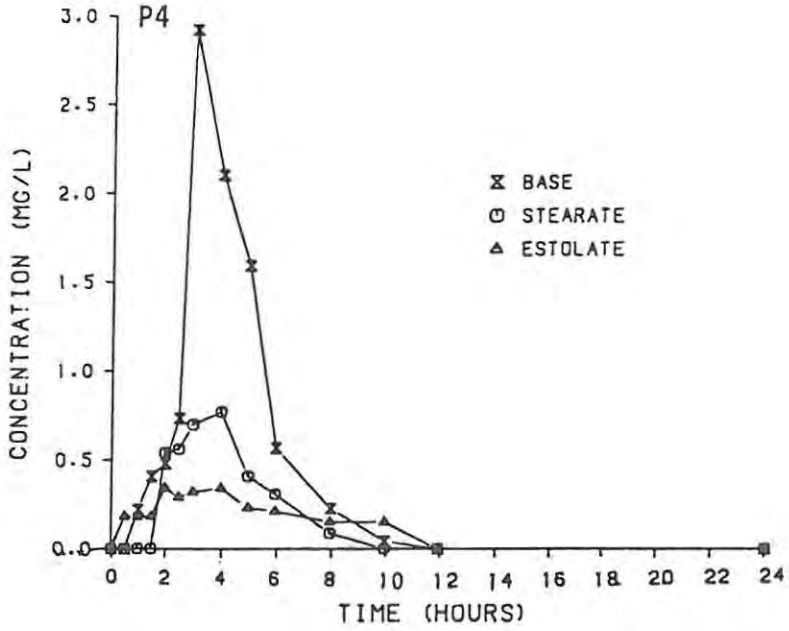
Three volunteers (P1, P2 and P4) who participated in this study also took part in a clinical trial, carried out in our laboratory, during which the volunteers each received erythromycin estolate (Ilosone) equivalent to 500mg erythromycin base (120). Identical protocols with regard to food and exercise, as used in the clinical trials in this study, were employed in the erythromycin estolate trial. Using a discriminatory assay method capable of assaying both erythromycin base and propionate in the serum, the samples collected were analysed for free erythromycin base (120).

Results from the erythromycin estolate trial, as well as results from the clinical trials reported in this study are shown graphically in Fig. 5.7.

Fig 5.7 Erythromycin serum concentration-time profiles after the administration of a single dose of 500mg erythromycin as enteric coated pellets, stearate tablets and estolate tablets.







From Fig. 5.7 and Table 5.5 it can be seen that the erythromycin base formulation resulted in the highest concentration of active erythromycin base (C_{max}) in all three volunteers. The mean AUC value for the erythromycin base formulation was greater than the AUC following administration of both erythromycin stearate and erythromycin estolate. There appeared to be no differences between the three formulations with respect to the time to reach maximum serum concentrations (C_{max}). However, the elimination rate of erythromycin following administration of the estolate product appears to be slower (highest $t_{1/2}$) than following administration of the erythromycin base and erythromycin stearate formulations. This longer elimination rate following erythromycin estolate administration, is presumably due to the rate limiting *in vivo* hydrolysis of the propionyl ester, which must occur in order to form the active erythromycin base.

Table 5.5 Model independent pharmacokinetic parameters following administration of a single dose of 500mg erythromycin as enteric coated pellets, stearate tablets or estolate tablets

	VOLUNTEERS				
	P1	P2	P4	MEAN	(SD)
C_{max} (µg/ml)					
Base	2.96	1.69	2.92	2.52	(0.59)
Stearate	0.12	1.49	0.77	1.13	(0.29)
Estolate	0.44	0.38	0.34	0.39	(0.04)
AUC (µg/ml.h)					
Base	10.07	4.91	7.74	7.57	(2.11)
Stearate	2.98	5.25	2.34	3.52	(1.25)
Estolate	3.53	3.68	2.29	3.16	(0.63)
tmax (h)					
Base	3.0	2.5	3.0	2.83	(0.24)
Stearate	1.5	2.0	4.0	2.50	(1.08)
Estolate	2.5	3.5	4.0	3.33	(0.63)
$t_{1/2}$ (h)					
Base	1.79	2.39	1.33	1.84	(0.43)
Stearate	2.67	2.10	1.26	2.01	(0.56)
Estolate	4.07	5.33	1.82	3.74	(1.45)

Formulation of enteric coated erythromycin base preparation and the production of erythromycin stearate and erythromycin estolate have attempted to reduce gastric degradation, and subsequently to improve absorption and clinical efficacy. Results from the clinical trials however, indicate in these volunteers, that the bioavailability following the administration of enteric coated erythromycin base is greater than following the administration of either erythromycin stearate or erythromycin estolate. Erythromycin stearate, although reported to be acid stable, was found to be acid labile and, as such, susceptible to gastric degradation. While the amount of erythromycin base available following erythromycin estolate administration, is contingent not only upon absorption, but also on the in vivo hydrolysis to release the active erythromycin base. Results from this study suggest that erythromycin base, although acid labile, if suitably formulated to allow protection from the gastric acid, may result in better therapeutic efficiency following oral administration,than either erythromycin stearate or erythromycin estolate.

Appendix 1 Derivatization of initial rates equation

Equations for zero order and first order degradations are given by Equation A1 and Equation A2 respectively.

$$C_a = C_a^o - K^o t \qquad \text{Equation A1}$$

$$C_a = C_a^o e^{-Kt} \qquad \text{Equation A2}$$

Equation A2 may be expanded to yield Equation A3:

$$C_a = C_a^o \left[1 - \frac{Kt}{1!} + \frac{(Kt)^2}{2!} - \frac{(Kt)^3}{3!} \right] \qquad \text{Equation A3}$$

If Kt is sufficiently small (rate constant very small) Equation A3 can be reduced to Equation A4.

$$C_a = C_a^o \quad [1 - Kt] = C_a^o - C_a^o Kt \qquad \text{Equation A4}$$

Under these circumstances (Kt sufficiently small) zero order and first order are practically indistinguishable. From Equation A1 and Equation A4 it can be seen that

$$C_a = C_a^o - K^o t = C_a^o - C_a^o Kt$$

As such $K^o = C_a^o K$

the first order rate constant can subsequently be calculated using Equation A5.

$$K = \frac{K^o}{C_a^o} \qquad \text{Equation A5}$$

Appendix 2 Acid degradation data - phosphate buffer test solution (method A)

pH		Concentration (mg/ml)		
		Erythromycin	Erythromycin Enol Ether	Anhydro-erythromycin
3.2	Time (mins)			
	5	0.16	0.01	0.03
	10	0.13	0.02	0.06
	20	0.09	0.03	0.10
	30	0.06	0.03	0.13
	60	0.02	0.02	0.13
4.3	Time (mins)			
	0	0.22	0.00	0.00
	5	0.22	<0.01	0.01
	10	0.20	0.01	0.03
	30	0.19	0.02	0.07
	60	0.16	0.03	0.12
	120	0.12	0.03	0.18
	240	0.07	0.03	0.23
5.5	Time (mins)			
	0	0.25	0.00	0.00
	240	0.21	0.02	0.06
	480	0.18	0.02	0.08
	1440	0.09	0.03	0.15
	2880	0.02	0.03	0.18
6.5	Time (hours)			
	0	0.26	0.00	0.00
	6	0.26	0.01	0.01
	24	0.24	0.02	0.03
	48	0.20	0.03	0.07
	72	0.14	0.01	0.09
7.0	Time (hours)			
	0	0.27	0.00	0.00
	6	0.26	0.01	0.01
	24	0.26	0.02	0.02
	48	0.25	0.02	0.04
	72	0.24	0.03	0.05

Appendix 3 Acid degradation data - phosphate buffer test solution (method B)

pH		Erythromycin concentration (mg/ml)
3.2	Time (mins)	
	5	0.16
	10	0.10
	15	0.07
	20	0.05
4.3	Time (mins)	
	0	0.27
	5	0.26
	10	0.25
	15	0.24
	30	0.21
	60	0.18
5.5	Time (mins)	
	0	0.22
	240	0.19
	480	0.17
	1440	0.11
	2880	0.05
6.5	Time (hours)	
	0	0.19
	96	0.12
	195	0.08
	314	0.05
	397	0.04
7.0	Time (hours)	
	0	0.21
	96	0.17
	195	0.14
	314	0.10
	387	0.09

Appendix 4 Acid degradation data - hydrochloric acid test solution

pH	Time (mins)	Erythromycin concentration (mg/ml)
2.2	0	0.22
	5	<0.01
	10	<0.01
	15	<0.01
	20	<0.01
2.9	0	0.22
	5	0.10
	10	0.04
	15	0.02
	20	0.01
3.2	0	0.22
	5	0.17
	10	0.15
	15	0.12
	20	0.09
	30	0.07

Appendix 5 Acid degradation data - effect of temperature

Temperature (°C)	Time (mins)	Erythromycin concentration (mg/ml)
23	0	0.22
	20	0.16
	40	0.11
	60	0.18
	120	0.03
28	0	0.23
	20	0.14
	40	0.08
	60	0.05
37	0	0.23
	10	0.13
	20	0.07
	40	0.03

Appendix 6 Acid degradation data - phosphate buffer test solution (erythroymcin stearate)

pH		Concentration (mg/ml)		
		Erythromycin	Erythromycin Enol Ether	Anhydro-erythromycin
3.2	Time (mins)			
	5	0.18	0.01	0.04
	10	0.15	0.02	0.06
	20	0.10	0.03	0.13
	30	0.06	0.03	0.16
4.3	Time (mins)			
	0	-	0.00	0.00
	10	0.19	<0.01	0.02
	30	0.17	0.01	0.04
	60	0.13	0.02	0.06
	120	0.09	0.02	0.10
5.5	Time (mins)			
	0	0.23	0.00	0.00
	480	0.17	0.02	0.05
	1440	0.08	0.03	0.15
	2880	0.02	0.03	0.18

Appendix 7 Acid degradation data - phosphate buffer test solution (erythromycin estolate)

pH		Erythromycin estolate concentration (mg/ml)
6.5	Time (mins)	
	0	0.27
	20	0.20
	40	0.14
	60	0.10
	80	0.09
7.0	Time (mins)	
	0	0.24
	20	0.21
	40	0.19
	60	0.16
	80	0.14

Appendix 8 Consent form

CONSENT TO ACT AS A RESEARCH SUBJECT

1. Erythromycin is a macrolide antibiotic used in the treatment of many common infections particularly those caused by gram positive organisms. The purpose of this study is to study the pharmacokinetics after the administration of two tablets containing 250 mg of erythromycin.

2. To participate in this trial, I must be in good health on the basis of an interview and physical examination. If I agree to be a subject, the following will occur. I will begin fasting at 10.00 pm on the day preceeding the trial. I will arrive at the School of Pharmaceutical Sciences at 7.45 am on the day of the trial and a blood sample will be drawn. I will be given two tablets each containing erythromycin stearate with a 250 ml glass of water. Blood samples will be drawn at 30 minutes, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12, and 24 hours. Urine will be collected at 0 hrs, 0-2, 2-4, 4-6, 6-8, 8-10, 10-12, 12-24 hour intervals. I will not take any drugs for one week preceding the trial and I will follow all dietry instructions during the study.

3. The incidence of untoward effects associated with the use of erythromycin preparations is low. In very rare cases mild gastric irritation as well as hypersensitivity reactions may occur which include fever, eosinophilia and skin eruptions.

4. These procedures will have no direct benefit to me but information may be obtained concerning erythromycin concentrations in the human body.

5. This information was given to me by Miss F. Glew. I understand that she will answer any questions I may have concerning this investigation.

6. I will be paid for participation in this study.

7. Participation in this study is voluntary. I have the right to refuse to participate or may withdraw at any time without jeopardy to my standing whatsoever.

NAME: ..... SIGNATURE: .....

DATE: .....

Appendix 9 Serum data - test dose 1 and test dose 2

TEST DOSE 1

	CONCENTRATION OF ERYTHROMYCIN IN SERUM (ug/ml)					
TIME HOURS	P1	P2	P3	P4	MEAN	(SD)
0	0.00	0.00	0.00	0.00	0.00	
0.05	0.00	0.00	0.00	0.00	0.00	
1.0	0.04	0.00	0.09	0.22	0.09	(0.08)
1.5	0.04	0.38	0.11	0.41	0.24	(0.16)
2.0	0.62	1.39	0.28	0.49	0.70	(0.42)
2.5	2.86	1.69	0.39	0.73	1.42	(0.96)
3.0	2.96	1.50	2.10	2.92	2.37	(0.61)
4.0	2.24	0.93	2.85	2.10	2.03	(0.69)
5.0	1.25	0.56	1.83	1.59	1.31	(0.48)
6.0	0.93	0.36	1.30	0.57	0.79	(0.36)
8.0	0.37	0.11	0.94	0.23	0.41	(0.32)
10.0	0.21	0.35	0.40	0.04	0.25	(0.14)
12.0	0.00	0.00	0.00	0.00	0.00	
24.0	0.00	0.00	0.00	0.00	0.00	

TEST DOSE 2

TIME HOURS	CONCENTRATION OF ERYTHROMYCIN IN SERUM (ug/ml)					
	P1	P2	P3	P4	MEAN	(SD)
0.	0.00	0.00	0.00	0.00	0.00	
0.5	0.00	0.00	0.00	0.00	0.00	
1.0	0.21	0.09	0.00	0.00	0.08	(0.09)
1.5	1.12	0.14	0.00	0.00	0.32	(0.47)
2.0	1.06	0.49	0.22	0.54	0.83	(0.48)
2.5	0.69	-	0.34	0.56	0.53	(0.14)
3.0	0.61	1.16	1.16	0.70	0.91	(0.25)
4.0	0.16	0.87	1.89	0.77	0.92	(0.62)
5.0	0.20	0.69	1.26	0.41	0.64	(0.40)
6.0	0.19	0.46	0.93	0.31	0.47	(0.28)
8.0	0.36	0.20	0.31	0.08	0.24	(0.11)
10.0	0.08	0.00	0.24	0.00	0.08	(0.09)
12.0	0.00	0.00	0.00	0.00	0.00	
24.0	0.00	0.00	0.00	0.00	0.00	

Appendix 10 Urine data - test does 1 and test dose 2

TEST DOSE 1

TIME (HOURS)	CUMULATIVE AMOUNT OF ERYTHROMYCIN BASE EXCRETED IN URINE (mg)					
	P1	P2	P3	P4	MEAN	(SD)
0 - 2	0	0	3.02	0	0.76	(1.3)
2 - 4	0	0	-	1.41	1.11	(1.2)
4 - 6	6.49	9.17	5.15	5.84	6.66	(1.5)
6 - 8	9.04	13.43	5.77	12.69	10.23	(3.1)
8 - 10	10.92	16.68	7.15	16.84	12.90	(4.1)
10 - 12	12.29	19.46	-	20.29	14.80	(5.4)
12 - 24	15.38	20.65	7.67	23.89	16.89	(6.1)

TEST DOSE 2

TIME (HOURS)	CUMULATIVE AMOUNT OF ERYTHROMYCIN BASE EXCRETED IN URINE (mg)					
	P1	P2	P3	P4	MEAN	(SD)
0 - 2	0	1.64	0	0	0.41	(0.7)
2 - 4	9.21	-	0.27	0	2.77	(3.7)
4 - 6	16.38	10.76	0.95	0.21	7.08	(6.8)
6 - 8	19.43	11.14	4.63	2.43	9.41	(6.6)
8 - 10	-	13.81	5.36	3.56	10.54	(6.4)
10 - 12	22.48	16.33	5.66	4.87	12.33	(7.4)
12 - 24	22.59	18.66	7.28	5.24	13.44	(7.4)

Appendix 11 Pooled serum data

TIME (HOURS)	CONCENTRATION OF ERYTHROMYCIN IN SERUM (ug/ml)
	TD1
0.	0.00
0.5	0.00
1.0	0.00
1.5	0.03
2.0	0.73
2.5	1.78
3.0	2.45
4.0	1.90
5.0	0.69
6.0	0.68
8.0	0.45
10.0	0.17
12.0	0.00
24.0	0.00

Appendix 12 Observed versus predicted erythromycin concentration following test dose 1 and test dose 2 administration

test does 1

	P1		P2		P3		P4	
TIME (H)	OBS	PRED	OBS	PRED	OBS	PRED	OBS	PRED
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.0	0.04	0.00	0.00	0.00	0.09	0.00	0.22	0.00
1.5	0.04	0.00	0.38	0.37	0.11	0.00	0.41	0.00
2.0	0.62	0.62	1.39	1.48	0.28	0.00	0.49	0.22
2.5	2.86	2.84	1.69	1.51	0.39	0.52	0.73	1.65
3.0	2.96	2.99	1.50	1.35	2.10	1.77	2.92	2.17
4.0	2.24	2.14	0.93	1.00	2.85	2.36	2.10	2.03
5.0	1.25	1.38	0.56	0.74	1.83	2.06	1.59	1.50
6.0	0.93	0.88	0.36	0.54	1.30	1.61	0.57	0.97
8.0	0.37	0.36	0.11	0.29	0.94	0.89	0.23	0.37
10.0	0.21	0.14	0.35	0.16	0.40	0.47	0.04	0.13
12.0	0.00	0.01	0.00	0.17	0.00	0.25	0.00	0.01

OBS = Observed
PRED = Model I - K_e constrained

test dose 2

	P1		P2		P3		P4	
TIME (H)	OBS	PRED	OBS	PRED	OBS	PRED	OBS	PRED
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1.0	0.21	0.33	0.09	0.00	0.00	0.00	0.00	0.00
1.5	1.12	0.95	0.14	0.14	0.00	0.00	0.00	0.00
2.0	1.06	0.96	1.49	1.48	0.22	0.00	0.54	0.46
2.5	0.69	0.87	-	-	0.34	0.28	0.56	0.69
3.0	0.61	0.76	1.16	1.20	1.16	1.32	0.70	0.72
4.0	0.16	0.58	0.87	0.86	1.89	1.66	0.77	0.62
5.0	0.20	0.44	0.69	0.61	1.26	1.33	0.41	0.44
6.0	0.19	0.33	0.46	0.43	0.93	0.96	0.31	0.29
8.0	0.36	0.19	0.20	0.22	0.31	0.45	0.08	0.11
10.0	0.08	0.11	0.00	0.11	0.24	0.21	0.00	0.01
12.0	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00

OBS = Observed
PRED = Model I - K_e constrained

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