APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE ANALYSIS, STABILITY AND PHARMACOKINETICS OF ERYTHROMYCIN

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ABSTRACT

Erythromycin is a macrolide antibiotic used mainly in the treatment of infections caused by gram-positive organisms. Erythromycin base is rapidly degraded in acidic media necessitating the use of structurally modified erythromycin derivatives or acid resistant dosage forms in order to decrease gastric inactivation of the drug. The majority of pharmacokinetic studies to-date have utilized relatively non-specific microbiological assay procedures which are unable to differentiate between concentrations of active erythromycin base and the inactive pro-drug derivatives.

A high-performance liquid chromatographic (HPLC) technique is described for the simultaneous determination of erythromycin base and propionate (inactive pro-drug form) in human serum and urine following the oral administration of erythromycin estolate, an acid stable derivative of erythromycin. The method involves a solid-phase extraction step prior to chromatography on a C_{18} reversed-phase column with coulometric electrochemical detection. Sample handling and storage techniques are presented which minimize hydrolysis of the inactive ester moiety between sample collection and analysis, thereby more accurately reflecting the *in vivo* situation than in previously published studies. Results from single dose pharmacokinetic studies indicate that only 10-15% of the total erythromycin concentration *in vivo* is present as the active base component following oral administration of erythromycin estolate. This percentage increases to approximately 25% during multiple dose administration. Novel urinary excretion data are presented which reveal that approximately 40% and 55% of the total erythromycin excreted in urine is excreted as erythromycin base following single and multiple dosages respectively.

Computer fitting of mean serum concentration-time data revealed that an open one compartment model with linear first order absorption and elimination best described the absorption and disposition of erythromycin, although poor computer fits for individual data sets were observed. Some evidence of non-linear elimination is presented utilizing both compartmental and non-compartmental pharmacokinetic techniques. Large intra- and inter-personal variability in erythromycin absorption and disposition was experienced which was evaluated in five subjects who each received one 500 mg erythromycin estolate tablet from the same batch, on three separate occasions.

In addition, an HPLC method is described for the analysis of "total erythromycin" concentrations following erythromycin estolate administration which involves hydrolysis of the ester component prior to chromatography, as well as an HPLC method utilizing amperometric electrochemical detection capable of monitoring the stability of erythromycin base in stored biological fluids. These methods were utilized in various stability studies involving erythromycin base and propionate as well as for the analysis of erythromycin estolate dosage forms.

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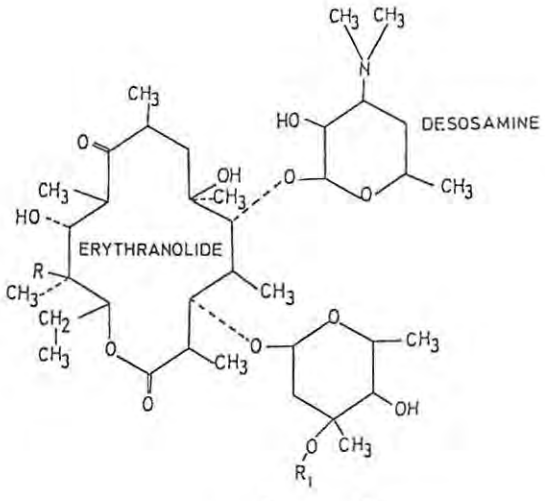
CHAPTER ONE

INTRODUCTION

1.1 CHEMICAL PROPERTIES OF ERYTHROMYCIN

1.1.1 DISCOVERY, CHEMICAL STRUCTURE, AND BIOSYNTHESIS OF ERYTHROMYCIN BASE

Erythromycin is a macrolide antibiotic produced by an actinomycete originally isolated from a soil sample collected in the Phillipine Archipelago. The actinomycete was identified as a strain of *Streptomyces erythreus* and the antibiotic was first isolated by McGuire *et al* (1) in 1952 in the Lilly Research Laboratories U.S.A. The fermentation liquors of *Streptomyces erythreus* contain many related compounds (2,3) the most important being erythromycins A,B and C. These compounds are selectively extracted by various chemical and chromatographic techniques (4,5) to yield erythromycin base of which erythromycin A is the major component. Erythromycin base is a colourless, bitter tasting, weakly basic compound (pKa 8.8) (6) with a poor water solubility at room temperature which decreases with an increase in temperature from 15-55°C (7,8). Table 1.1 lists its solubilities in a range of solvents.



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

Figure 1.1 Chemical structures of erythromycin and its components

SOLVENT	SOLUBILITY mg/ml	
	BASE	ESTOLATE
Water	2.1	0.2
Methanol	>20	>20
Ethanol	>20	>20
Isopropanol	>20	>20
Isoamyl alcohol	9.7	>20
Cyclohexane	0.2	0.1
Petroleum ether	4.7	0.1
Benzene	>20	0.1
Iso octane	0.5	0.1
Carbon tetrachloride	>20	0.1
Ethyl acetate	>20	>20
Isoamyl acetate	>20	1.3
Acetone	>20	>20
Methyl ethyl ketone	>20	>20
Diethyl ether	>20	0.2
Ethylene chloride	>20	>20
Chloroform	>20	>20
Carbon disulfide	5.1	0.1
Pyridine	>20	>20
Formamide	>20	>20
Ethylene glycol	>20	>20
Propylene glycol	-	>20
Dimethyl sulfoxide	-	>20
1,4 Dioxane	>20	>20
0.1N NaOH	-	12.3
0.1N HCl	-	0.2

Table 1.1 Solubilities of erythromycin base and erythromycin estolate (reproduced from references 6 & 142)

Figure 1.1 depicts the chemical structure of erythromycins A,B and C. Erythromycin is a polyhydroxylactone containing two sugars; desosamine, which is a 3-N-dimethyl-aminodeoxy sugar, and L-cladinose which contains no nitrogen and has one methoxy group. The aglycone part of the molecule is a 14-member lactone called erythranolide A. Both cladinose and desosamine are bound to the lactone ring by a β-glycosidic linkage (9). Erythromycin B differs from erythromycin A by the absence of a hydroxyl group at C-12 on the lactone ring, while erythromycin C has the identical lactone ring to erythromycin A but has the sugar L-mycarose replacing L-cladinose at the C-3 position.

The biosynthesis of erythromycin from erythranolide A has been studied by Hung, Marks and Tardrew (10) who proposed two alternative pathways, leading by one route to erythromycin B and C and an intermediate compound and by another route to erythromycin A and another intermediate compound (Fig. 1.2).

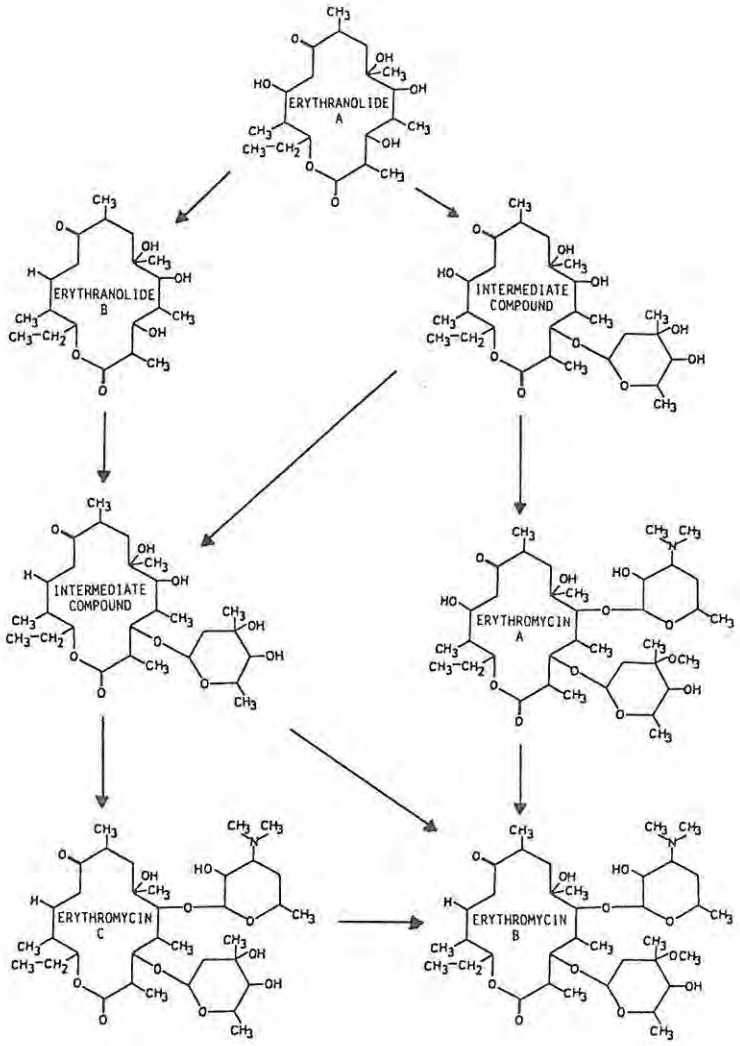


Figure 1.2 Postulated synthetic pathways of erythromycin from erythranolide A: Reproduced from reference (10)

Erythromycin base is thought to have four different crystal forms as a result of varying preparation methods and recrystallization solvents (8). Monohydrate, dihydrate, anhydrate and amorphous structures have been proposed (7,11), however, the so called "dihydrate" has since been shown to be a pseudomorph containing 4.5% entrapped or clathrated water having a melting range of 130-135°C. Similarly, the "monohydrate", which melts at 190°C has been shown to contain 1-2% entrapped water (12). Dissolution studies on five different commercial lots of erythromycin have shown the amorphic form to have the slowest dissolution (but greatest solubility) in pH 7.4 phosphate buffer (8). Similar results have been obtained during *in vivo* absorption studies in humans where a decreased absorption rate for the amorphous form was reported when compared to crystalline products (13). However, erythromycin absorption in humans is a complex process and factors besides dissolution rate alone have been implicated (Section 1.4.2).

1.1.2 STABILITY

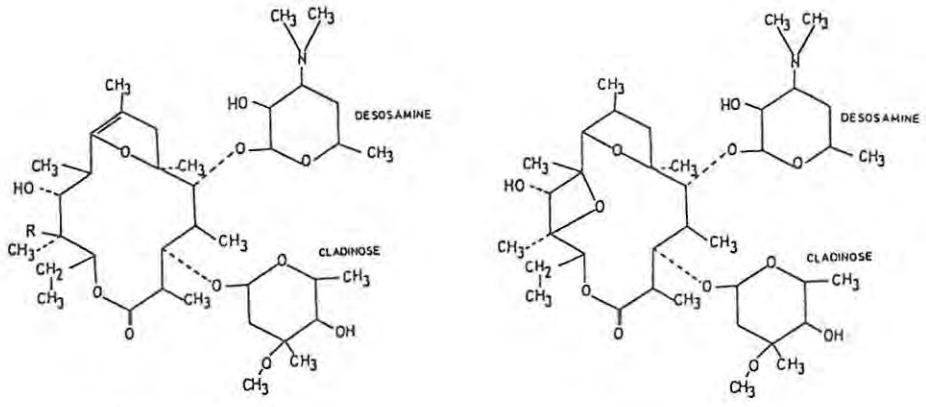
Erythromycin base is extremely unstable in acidic media (14,15). Depending on the conditions of the reaction, two degradative products are formed, the structures of which are depicted in figure 1.3. Mild acid treatment yields erythromycin-6-9-hemiketal while further reaction yields anhydro-erythromycin which is a "spiroketal" of erythromycin. Alternatively, treatment with strong acids results in only the formation of anhydroerythromycin, with no cleavage of the glycosidic linkages being reported (16-19). Reports on the stability of erythromycin have varied in their comprehensiveness which has prompted further exhaustive studies in our laboratory (20). The following important points have been extracted from the available literature.

1) The critical factor in the rate of erythromycin degradation is pH although the concentration and type of buffer is of importance (16,20).

2) The optimum pH for erythromycin stability is between pH 7.0 and 8.0 (16,20-23). The major alkaline degradative product is thought to be dihydroerythromycin (23).

3) Degradation increases with an increase in temperature and follows pseudo first-order kinetics (16,20).

4) Metal ions, particularly Al^{+3}, Fe^{+3} and Cu^{+2} increase the rate of degradation in acidic media (21).



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

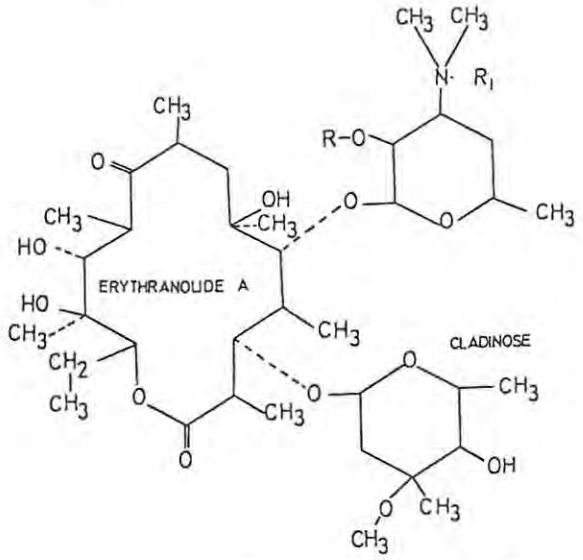
(III)

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

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

1.1.3 CHEMICAL DERIVATIVES OF ERYTHROMYCIN

Oral administration of erythromycin base is complicated by its degradation following dissolution in the acidic gastric contents. Formulation of acid resistant dosage forms (Section 1.4.2) as well as the preparation of acid-stable chemical derivatives have been utilized to overcome this problem. The structural formulae of the most commonly used erythromycin derivatives are depicted in figure 1.4.

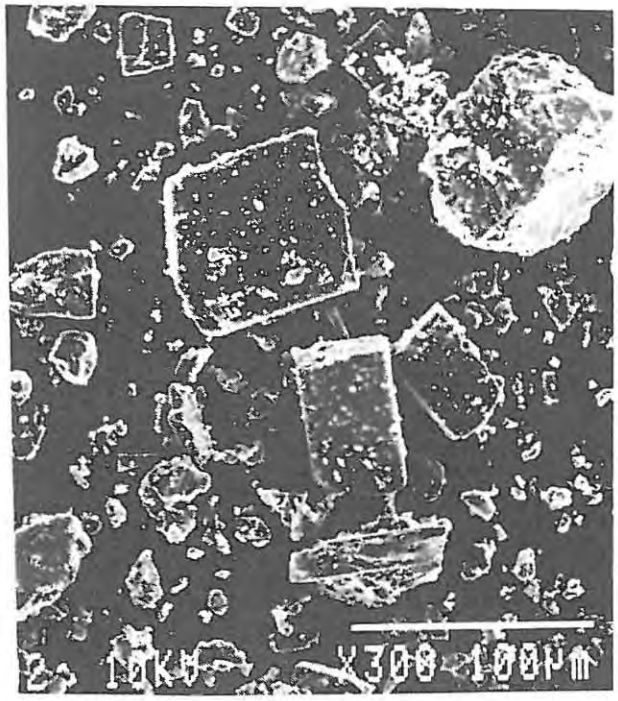


	R	R_1
Erythromycin base	H	
Propionyl erythromycin	CH_2CH_2 CO	
Erythromycin estolate	CH_2CH_2 CO	$C_{12}H_{25}OSO_3$
Erythromycin stearate	H	$C_{17}H_{35}COO$
Erythromycin ethylsuccinate	$CH_2CH_2OOCCH_2CH_2COO$	
Erythromycin lactobionate	H	$C_{11}H_{19}O_9COO$
Erythromycin glucoheptonate	H	$C_6H_{13}O_6COO$

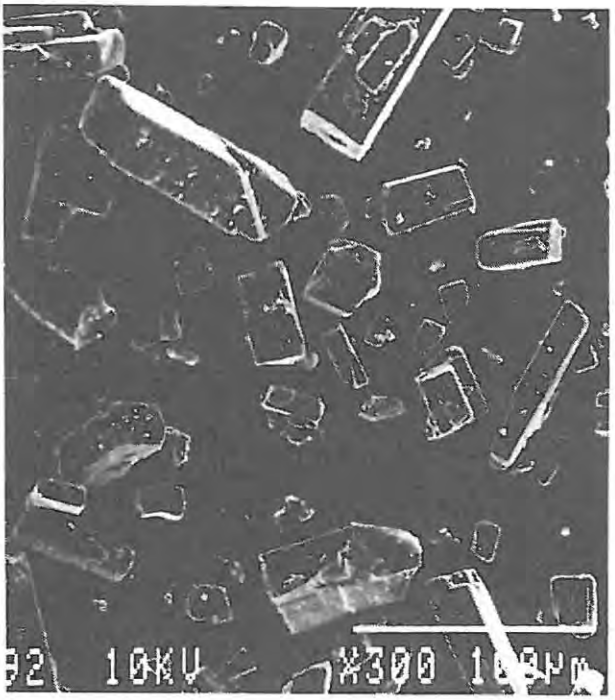
Figure 1.4 Chemical structures of erythromycin and its most common derivatives

Erythromycin stearate was prepared as an acid insoluble and hence stable salt of erythromycin, but has since been shown to be acid-labile (22,24). The propionyl ester is also acid-labile (22) although its lauryl sulphate salt, being insoluble in acidic media (25) (Table 1.1), has enjoyed much success as a tasteless, acid-stable derivative of erythromycin. The ethylsuccinate ester has also been used extensively as an acid-stable derivative although little has been published relating to its acid-stability. These esters of erythromycin are inactive pro-drug forms of the drug which must hydrolyse *in vivo* to the active erythromycin base form prior to activity. This has led to extensive research into the synthesis of

Figure 1.5 Scanning electron micrographs of four erythromycin estolate raw materials



Erythromycin Estolate - USP



Erythromycin Estolate - Batch EL1

Erythromycin Estolate - Batch EL2

Erythromycin Estolate - Batch AI

ACKNOWLEDGENT

The scanning electron micrographs were prepared by the Rhodes University Electron Microscopy Unit utilizing a Jeol J.S.M. model 840 scanning electron microscope. The samples were coated with gold prior to examination.

more rapidly hydrolyzing esters as well as non-esterified acid-stable and active forms of the antibiotic (26-28). The most recent ester reported is erythromycin acistrate, (stearic acid salt of the 2'-acetyl ester) which has undergone some preliminary studies in humans (29).

Two water soluble salts, erythromycin lactobionate and glucoheptonate have been prepared for intravenous (i.v.) use (Fig. 1.4), and have recently been the subject of a comprehensive stability study (30) in a range of i.v. diluents.

Erythromycin estolate also exists in both a crystalline and amorphous form (31). The crystalline material has a melting range of 134-135°C while the amorphous product melts between 128° and 131°C (31). Crystal structure and particle size have both been shown to affect the dissolution of the estolate, further emphasizing the importance of thorough physical characterization of raw materials prior to production (32). Figure 1.5 depicts scanning electron micrographs of the U.S.P. reference standard plus erythromycin estolate raw material obtained from three different sources in which large variations in particle size distribution and particle shape are clearly evident.

Inter-batch differences in the dissolution rates of 10 different erythromycin stearate bulk powders have also been reported along with recommendations regarding the minimum dissolution rate required for acceptable bioavailability (33). Previous attempts at correlating *in vitro* tests with *in vivo* bioavailability were only marginally successful (13,34).

1.2 CLINICAL PHARMACOLOGY

1.2.1 MODE OF ACTION

Erythromycin penetrates the cell wall of sensitive bacteria (35) and attaches to the 50S ribosomal subunit donor site in the presence of ammonium and potassium ions (36). A single molecule of erythromycin attaches to each ribosomal fragment (35). It prevents the incorporation of certain amino acids into the polypeptide linkages, but has no effect on the synthesis of nucleic acid (36).

Although it acts by inhibiting protein synthesis, erythromycin may be either bacteriostatic or bactericidal depending on the sensitivity of the

particular microorganism and the concentration of the drug (37). Its selectivity for micro-organisms is due to the absence of the 50S subunit on the ribosomes of the human host (38). The binding parameters of erythromycin to *Staphylococcus aureus* (39) and *Legionella pneumophilia* (40) have been extensively studied, providing a valuable reference for the screening of new active analogues as well as in assessing interference from co-administered compounds.

Table 1.2 Typical *in vitro* sensitivities of organisms to erythromycin (reproduced from reference 37)

	Typical Minimun Inhibitory Concentrations (µg/ml)	
Organism	Mean	Range
S. aureus	0.4	0.005->100
Streptococcus pyogenes	0.04	0.005-0.8
Streptococcus pneumoniae	0.05	0.006-0.2
Streptococcus viridans	0.06	0.02-0.1
Clostridium perfringens	0.5	0.05-5.0
Listeria monocytogenes	0.16	0.1-0.3
Corynebacterium diphtheriae	0.02	0.006-0.2
Actinomyces israelii	0.5	0.2-0.5
Nocardia asteroides	25.0	0.2->200
Neisseria meningitidis	0.4	0.1-0.8
Neisseria gonorrheae	0.1	0.005-0.4
Haemophilus influenzae	3.1	0.1-6.0
Bordatella pertussis	0.3	0.02-1.56
Bacteroides fragilis	1.5	0.12->128
Brucella species	5.0	0.3-10.0
Mycoplasma pneumoniae	0.005	0.001-0.01
Mycobacterium kansasii	1.0	0.5-2.0
Agent of Legionnaires disease	<0.5	-

1.2.2 ANTIMICROBIAL SPECTRUM

Erythromycin is active against most gram-positive bacteria and some gram-negative organisms, with an overall spectrum of activity similar to that of penicillin G (35). Erythromycin is reported to be more active in alkaline pH which can be of importance in the treatment of urinary tract infections (41,42). The sensitivities of some selected organisms to erythromycin as reported by Nicholas (37) are summarized in table 1.2. A similar, but far more comprehensive table incorporating data on over 70 different organisms taken from 100 original references has recently been published by the staff of the Mayo Clinic, New York, U.S.A. (43,44). Inspection of these and other tables shows that for most susceptible organisms, minimum inhibitory concentrations are in the region of 0.1 to 1.0 µg/ml.

Erythromycin is concentrated intracellularly by polymorphonuclear leukocytes and alveolar macrophages through an active process (45,46). This is thought to be of particular importance in the treatment of chronic infections which are prone to relapse, as well as in the treatment of *Legionella* pneumonia (43).

1.2.3 CLINICAL USES

The penicillins and cephalosporins have supplanted erythromycin for the treatment of serious staphylococcal infections (43), however, the efficacy of this antibiotic against so many gram-positive cocci and against *Haemophilus influenzae* and *Mycoplasma pneumoniae* makes it an excellent antibiotic for the treatment of most upper respiratory tract infections such as sinusitis, laryngitis, tracheitis, acute bronchitis and primary atypical pneumonia (38). For persons allergic to penicillin who require an antibacterial agent with the spectrum of penicillin G or V, erythromycin is the drug of choice, provided that penetration of the blood brain barrier is not required (35). Recently the spectrum of erythromycin usage has been extended to include the treatment of legionnaire's disease for which it is now considered the drug of choice (35,37), as well as some genito-urinary diseases, including gonorrhoea and primary syphilis (47).

Erythromycin is one of the few commonly used antimicrobial agents which is not immunosuppressive and may even potentiate the human immune response (48,49), making it an important drug for the treatment of infections in immunocompromised patients and neonates (50).

The usual oral dose is 1.0 to 4.0 g per day for adults and 25.0 to 50.0 mg/kg per day for children in divided doses, either with or without food depending on the dosage form administered and manufacturers recommendations (Section 1.3.2). In the treatment of primary syphilis the dose is increased to 2.0 to 4.0 g per day for 10-15 days (47).

Intravenous administration is reserved for the treatment of severe infections such as legionnaire's disease with the usual dose being 0.5-1.0 g every 6 hours, which may be given for as long as 4 weeks with no difficulty except for possible episodes of thrombophlebitis at the site of injection (47).

1.2.4. RESISTANCE

Plasmid mediated mechanisms by which the target site and not the antibiotic itself is changed, have been implicated in the development of resistant organisms (37). Metabolites excreted in the urine have also been shown to induce this resistance (51). These theories have been challenged by the recent findings that some strains of *Escherichia coli* produce an erythromycin esterase which hydrolyses the lactone ring to form novel inactive degradative products (52).

Extensive clinical studies have been performed to assess the importance of erythromycin resistance, indicating that the incidence of resistance by strains of *Staphylococcus aureus* has increased in all American hospitals since 1975 particularly in the large medical-school-affiliated institutions (43). Other organisms which may develop resistance include the *Streptococci*, *Pneumococci* and *Corynebacterium diptheria* (43).

1.2.5 ADVERSE REACTIONS

Erythromycin is generally regarded as the least toxic of the commonly used antibiotics (37,44,50). Mild degrees of epigastric distress, nausea, vomiting and diarrhoea are fairly frequently encountered with both oral and i.v. erythromycin, but these symptoms are only rarely serious enough to require cessation of therapy (47,53). The cause of these side-effects may be related to recent findings that erythromycin has a stimulating effect on human gastrointestinal muscle, similar to that of endogenous motilin (54). Erythromycin is known to cause a reversible cholestatic hepatitis in approximately 1 in 1000 patients (35,47) particularly adults, during prolonged administration (37). Originally only erythromycin estolate was implicated in this reaction, but extensive prescription-event monitoring programmes have shown all the erythromycin derivatives to be equally responsible (55).

More recently erythromycin has been used for the treatment of infections due to the *Legionella* species for which i.v. doses of up to 4 g per day are frequently used, often in compromised patients (56). Reversible hearing losses have been reported after both i.v. (56) and oral (57) administration of drug which is in conflict with published pharmacokinetic studies involving patients with renal failure (Section 1.3.6).

1.2.6 DRUG INTERACTIONS

Erythromycin has been reported to affect the elimination of theophylline, carbamazepine, warfarin and other drugs that are principally metabolized by the cytochrome P-450 system in the liver (58,59). The mechanism of this interaction is discussed further in section 1.3.5. The interaction between erythromycin and theophylline has been extensively studied with the result that in order to reduce the possibility of theophylline toxicity, a 25% reduction in theophylline dose is suggested if erythromycin is given continuously for more than six days (60). The effects of concomitant erythromycin administration on the blood levels of carbamazepine and warfarin must constantly be borne in mind (59,61), particularly after the recent death by internal bleeding of a patient stabilized on warfarin who was given a course of erythromycin (62).

1.3 PHARMACOKINETICS OF ERYTHROMYCIN - GENERAL CONCEPTS

1.3.1 OVERVIEW

The study of the absorption, distribution, metabolism and excretion of erythromycin has been extensively researched over the last 35 years. Many of these studies were performed prior to the development of modern pharmacokinetic principles and techniques, but serve as useful background, providing physiologically based interpretations of pharmacokinetically derived observations. Literature relating to the general concepts of erythromycin pharmacokinetics will therefore be discussed in this section while results of the more recent and definitive pharmacokinetic studies will be discussed in section 1.4.

The vast majority of studies have utilized microbiological assay techniques which lack the specificity required for detection of metabolites/ degradation products in biological fluids, but most importantly are unable to differentiate between levels of active erythromycin base and inactive ester pro-drug forms after administration of the estolate and ethyl-succinate derivatives. A discussion of the importance of this differentiation and a summary of the analytical techniques available are available in sections 1.3.7 and 2.1 respectively.

1.3.2 ABSORPTION AND THE EFFECT OF FOOD

The stearic acid salt moiety of erythromycin stearate dissociates in the gastrointestinal contents leaving the parent base to be absorbed as such. Both erythromycin base and stearate are formulated in various modified solid oral dosage forms in order to decrease contact with the acidic gastric fluids. These formulations include enteric coated capsules and tablets, film coated tablets, enteric coated pellets within gelatin capsules and most recently, polymer coated particles compressed into tablets (63). Erythromycin is predominantly absorbed in the small intestine, with absorption continuing in the caecum and large intestine (64). Absorption from the rectal area is sufficient for the successful use of erythromycin base suppositories (65,66).

Erythromycin estolate and ethylsuccinate are acid insoluble and are therefore less prone to degradation by gastric fluids (25). They are formulated as suspensions, capsules and tablets making them ideal for pediatric use. The lauryl sulphate salt moiety of the estolate dissociates in the less acidic environment of the duodenum and is absorbed as the propionyl ester which must then hydrolyse *in vivo* to the active base form prior to activity (67,68). Erythromycin ethylsuccinate, which is absorbed as the inactive ester must similarly undergo hydrolysis prior to activity (68).

The absorption of drugs, particularly those prone to gastric acid degradation and/or intestinal metabolism may be influenced by a variety of factors including the presence or absence of food in the gastrointestinal tract (69-71). The effect of food on the absorption of erythromycin is a contentious point which has been discussed at length in the literature and has been reviewed by Welling (71) in 1976. Table 1.3 is a summary of literature reports which have specifically studied the effect of food on various formulations of erythromycin. This wealth of data only serves to confuse the issue, as opposing results are often obtained in apparently similar experiments. Some important results and trends are summarized in the following discussion.

Table 1.3 Effect of food on the absorption of erythromycin

DRUG	DOSAGE FORM	DOSAGE REGIMEN	FOOD DETAILS	FLUID VOLUME	OBSERVED EFFECT ON ABSORPTION	YEAR PUBLISHED	REF.
BASE	TAB	S	-	-	REDUCED	1953	72
BASE	TAB (EC)	S	-	-	DELAYED	1953	72
BASE	CAPS	S	1 hr pc	-	REDUCED	1953	73
BASE	TABS (EC)	S	1 hr pc	-	DELAYED	1953	73
BASE	TABS (EC)	M	ac	100 ml	REDUCED	1978	143
BASE	TABS (EC)	S	1 hr pc	240 ml	DELAYED	1979	79
BASE	TABS (EC)	S	ac	-	REDUCED	1979	76
BASE	PELLETS	S	pc & ac	50 ml	DELAYED	1979	144
BASE	PELLETS	S	pc & ac	250 ml	NO EFFECT	1981	145
BASE	TABS	S	2 hr pc	-	REDUCED	1981	141
BASE	TABS (EC)	S	2 hr pc	-	NO EFFECT	1981	141
BASE	TABS (EC)	M	pc	-	NO EFFECT	1981	141
BASE	TABS (EC)	S	20 min ac	150 ml	REDUCED	1983	77
BASE	PELLETS	S	pc	50 ml	NO EFFECT	1984	146
BASE	TABS (EC)	M	ac	-	NO EFFECT	1985	147
BASE	PELLETS	M	ac	-	REDUCED	1985	147
STEARATE	FILMTABS	M	ac	-	REDUCED	1959	117
STEARATE	FILMTABS	S	ac	-	REDUCED	1960	118
STEARATE	-	S & M	ac	-	REDUCED	1964	77
STEARATE	FILMTABS	M	ac	100 ml	REDUCED	1978	143
STEARATE	FILMTABS	S	pc	250 ml	REDUCED	1978	74
STEARATE	FILMTABS	S	ac	-	INCREASED	1979	76
STEARATE	FILMTABS	S	pc & ac	50 ml	REDUCED	1979	144
STEARATE	FILMTABS	S & M	pc	250 ml	REDUCED	1979	78
STEARATE	FILMTABS	S	pc & ac	250 ml	REDUCED	1981	145
STEARATE	FILMTABS	S	ac	150 ml	INCREASED	1983	77
STEARATE	TABS (EC)	S	ac	150 ml	REDUCED	1983	77
EES	SUSP	S & M	-	-	INCREASED	1977	70
EES	SUSP	S	pc	240 ml	NO EFFECT	1979	79
EES	FILMTAB	S	pc & ac	120 ml	REDUCED	1980	148
PROP	CAPS	S	ac	-	REDUCED	1959	117
PROP	CAPS	S	ac	-	REDUCED	1960	118
ESTOLATE	SUSP	S	-	-	NO EFFECT	1960	149
ESTOLATE	SUSP	S	-	-	NO EFFECT	1960	150
ESTOLATE	CAPS	S	-	-	REDUCED	1960	118
ESTOLATE	-	S & M	ac	-	NO EFFECT	1964	77
ESTOLATE	CAPS&SUSP	S & M	pc	250 ml	INCREASED	1979	78
ESTOLATE	CAPS	S	pc	240 ml	INCREASED	1979	79

KEY: EES = Erythromycin Ethylsuccinate
PROP = Erythromycin Propionate
TAB (EC) = Enteric Coated Tablets
TAB = Uncoated Tablets
PELLETS = Enteric Coated Pellets within Gelatin Capsule
SUSP = Suspension
S = Single Dose M = Multiple Dose
pc = Dose after Food ac = Dose before Food

The first reports on the effect of food on erythromycin absorption showed the absorption of uncoated erythromycin base formulations to be drastically reduced when administered concurrently with food (72,73). Similar results were obtained with erythromycin stearate film coated tablets (74) and more recently uncoated cores of an enteric coated tablet (75). This phenomenon

did not occur in patients with decreased gastric acid secretions (72,73). These findings suggest that the unprotected drug degrades after dissolving in the increased gastric secretions associated with food intake, and that the drug is thus exposed to these acidic fluids for longer periods due to decreased gastric emptying.

The reverse however, has also been shown in similar studies where increased absorption of erythromycin when taken immediately before food has also been reported for erythromycin stearate film coated tablets (76,77) as well as ethylsuccinate suspensions (70), estolate suspensions (78,79) and estolate capsules (78). This phenomenon has been attributed to the neutralizing and buffering effect of food on the gastric secretions as well as increased dispersion of drug particles due to increased gastric activity (76). It has also been suggested that food may slow down the passage of the drug through the small intestine, thereby increasing its exposure to absorption sites in the upper gastrointestinal tract. A decrease in co-administered water volume from 250 to 25 ml resulted in a decreased absorption from erythromycin stearate tablets in fasting volunteers, which has been attributed to an increase in gastric residence time with the smaller water volume, as well as decreased dissolution (71,74). Trends observed after single dose administration were also reflected in multiple dose studies.

In four unrelated studies, erythromycin stearate film coated tablets exhibited both increased (76,77) and decreased (74,78) absorption in the presence of food. Further examination showed that products from two different manufacturers were used, and that products from the same company performed similarly while those from different companies did not. Large differences in absorption from two different film coated erythromycin base tablets have been reported when administered one hour before a meal, whereas no differences were evident when the same formulations were administered two hours before an identical meal (80).

These and many other findings have led to the current thinking that any observation regarding the influence of food on the absorption of erythromycin in humans, is valid for the specific product studied, not for all similar formulations and certainly not for erythromycin in general (76,77). The manufacturers of erythromycin products should therefore determine the effect of food on their formulations and instruct the health care team accordingly.

1.3.3 DISTRIBUTION

Detailed distribution studies in rats utilizing C_{14} erythromycin were reported as early as 1956 (64). These and other studies suggest that erythromycin is well distributed throughout the body with the exception of the brain, where therapeutic concentrations are only attained if meningeal inflammation is present (58,81). Foetal serum levels are approximately 5-20% of parent serum levels (38) although concentrations in the foetal liver are high (37). Levels of erythromycin in human milk are approximately 50% of serum levels (38) while both useful (82,83) and valueless (84,85) correlations between salivary and plasma concentrations have been reported. Skin blistering techniques have shown adequate erythromycin concentrations in extravascular fluids following oral administration of erythromycin (86). Recent experiments using positron tomography (87) demonstrated effective erythromycin concentrations in the pneumonic lung within ten minutes of an intravenous dose.

Table 1.4 Serum binding of erythromycin

DRUG	SAMPLING	METHOD	% UNBOUND	RATIO UNBOUND BASE/ESTER	REF
BASE	*In vitro*	UF	18.6	4.3	88
PROPIONATE	*In vitro*	UF	4.3		88
BASE	*In vitro*	ED	15.9	4.1	88
PROPIONATE	*In vitro*	ED	3.9		88
BASE	*In vivo*	UF	10.4	8.0	88
PROPIONATE	*In vivo*	UF	1.3		88
BASE	*In vitro*	UF	26.7	3.6	89
PROPIONATE	*In vitro*	UF	7.4		89
BASE	*In vivo*	ED	35.5		91

KEY: UF = Ultrafiltration
ED = Equilibrium Dialysis

1.3.4 PROTEIN BINDING

Erythromycin base and propionate are both highly bound to serum protein (Table 1.4), with erythromycin base being less highly bound than the ester. This is an important consideration when evaluating serum/plasma levels obtained in comparative studies involving erythromycin base and estolate products, since the higher total (bound + unbound) levels associated with the ester formulations may be due to reduced tissue distribution of the ester moiety and not necessarily superior

absorption (88). Considering that free levels of drug in tissue water are in equilibrium with free levels in serum (88) and that bound antibiotics have no activity (89) administration of base or stearate dosage forms may be clinically superior to ester pro-drug forms despite lower reported serum/plasma levels (88).

The principal erythromycin binding protein in human plasma has been identified as α_1-acid glycoprotein, the binding properties of which are well documented (90,91). Extrapolation of pharmacokinetic parameters from normal trial subjects to clinical situations can be complicated by the increased amounts of α_1-acid glycoprotein found in infected patients (92) and the decreased amounts present in patients with liver cirrhosis (93).

1.3.5 METABOLISM

Erythromycin is N-demethylated in the liver of rabbits and rats to form des-N-methylerythromycin (94) which is excreted predominantly in the bile (95,96), with small amounts being found in urine (64). Approximately 45% of the dose administered to rabbits is metabolized by this pathway per hour (94). Other compounds have been detected in urine, bile and faeces which are thought to be formed by combined enzymatic and non-enzymatic reactions resulting from the recycling of compounds to and from the gastrointestinal tract (51,95,97). Metabolism of erythromycin in rats was found to be only marginally induced by chronic administration of phenobarbital (95).

Erythromycin is reported to induce its own metabolism by increasing both the number and activity of the microsomal enzymes involved in its metabolism (98). This effect appears to be negated by the production in both rats and humans of a metabolite which forms an inactive complex with reduced cytochrome p-450 (98,99). This complex has been implicated as the possible cause of the interaction between erythromycin and other drugs metabolized by the cytochrome p-450 system, including theophylline, carbamazepine and warfarin (99). A similar and more clinically significant interaction involving an inactive metabolite has been described for another macrolide antibiotic troleandomycin while midecamycin and josamycin do not form this complex (99).

1.3.6 EXCRETION

The study of the excretion of erythromycin in man is limited to a discussion of the total microbiologically active form excreted due to the lack of suitable specific analytical techniques for the quantitative determination of its metabolites and/or degradation products.

Biliary excretion is thought to be the major excretory pathway for erythromycin and its metabolites (38,58,100). Studies in dogs (101) and humans (102) have reported 5.4% and 4.3% respectively of an intravenous dose to be excreted via this route with an erythromycin bile to serum ratio of 9:1 (100). Patients with alcoholic liver disease have longer elimination half-lives than normal patients, but seldom require adjustment in dosage (103,104). Erythromycin base is excreted via the bile in greater amounts than erythromycin propionate, the reasons for which are unclear (105). Secretion of erythromycin across the wall of the jejunum into the lumen of the small intestine after intravenous administration has been demonstrated in both the rat and rabbit (64,106).

Renal clearance has been estimated as being 13% and 76% of creatinine clearance in humans and dogs respectively (42,95) with tubular reabsorption also being suspected. The percentage of the dose administered which is excreted in the urine is variable and very low, ranging from 1% of an oral dose to 15% of an intravenous dose (Table 1.5). Urinary excretion has been

Table 1.5 Urinary excretion of microbiologically active erythromycin

DRUG	ROUTE	% EXCRETED	YEAR	REF
G-HEPTONATE	I.V.	15.0	1953	123
LACTOBIONATE	I.V.	4.3	1955	102
LACTOBIONATE	I.V.	5.6-7.1	1980	84
LACTOBIONATE	I.V.	1.0-7.5	1986	108
ESTOLATE	ORAL	4.4	1961	110
ESTOLATE	ORAL	4.0	1969	67
STEARATE	ORAL	0.5	1977	126
STEARATE	ORAL	4.4	1982	129
BASE	ORAL	1.4	1977	126
BASE	ORAL	5.0-8.6	1982	129
BASE	ORAL	1.8	1982	128
BASE	ORAL	1.0	1983	151

linked directly to creatinine clearance, however, adjustments in dosage during renal failure are not deemed necessary as the overall clearance of the drug is not significantly reduced in renal failure (107,108). In light of the recently reported incidences of reversible hearing loss during intravenous therapy with high doses (Section 1.2.5) further studies on the effect of renal impairment on erythromycin elimination during chronic intravenous erythromycin administration may be necessary.

1.3.7 ERYTHROMYCIN BASE CONCENTRATIONS FOLLOWING ADMINISTRATION OF ERYTHROMYCIN ESTOLATE

Early pharmacological studies on erythromycin propionate (109) and estolate (110) suggested that the drug was absorbed as the propionyl ester which then hydrolysed partially *in vivo* to give limited concentrations of erythromycin base. Since then, it has been established that the ester form is microbiologically inactive (68), and that following administration of erythromycin estolate, erythromycin base only represents 20-30% of total serum concentrations (67,78,111-115). The numerous comparative bioavailability studies (116-122) reporting the overwhelming superiority of the estolate over the other erythromycin derivatives in which non-specific microbiological assay techniques were used, need to be re-examined using discriminatory assay procedures capable of selectively determining concentrations of both erythromycin base and propionate. Results of similar studies in which discriminatory methods were utilized, suggest that at best, concentrations of erythromycin base after administration of the estolate are equal to those obtained for a similar dose of erythromycin base or stearate (78,113-115).

The discriminatory methods utilized have generally involved either liquid-liquid or paper-chromatographic separations with microbiological end-points which lack the selectivity of modern chromatographic techniques and are tedious to perform. In general, studies on the pharmacokinetics of erythromycin base and propionate in humans following administration of erythromycin estolate have been hampered by the lack of sufficiently sensitive, selective and reproducible analytical techniques in which sufficient account is taken of the possible hydrolysis of the ester between sample withdrawal and analysis.

1.4 PHARMACOKINETICS OF ERYTHROMYCIN - MODELLING OF DATA

1.4.1 INTRAVENOUS STUDIES

Two early studies on the i.v. use of erythromycin glucoheptonate reported serum levels far in excess of those associated with similar oral doses which decayed in a rapid biphasic manner (123,124). The first report on the pharmacokinetics of i.v. erythromycin was published by Welling and Craig (108), who compared disposition kinetics in individuals with normal and impaired renal function. A similar biphasic elimination was reported which was consistent with two-compartment model kinetics with elimination occuring from the central compartment which has since been the model best describing the results of all subsequent i.v. studies (84,85,103,107,125). Table 1.6 lists some average parameter values obtained from these studies.

Table 1.6 Pharmacokinetics of erythromycin - intravenous studies

DOSE (mg)	MODEL	$t_{½}$ (h)	V (l)	Cl_p (l/h)	Cl_r (l/h)	REF.
500	2BCM	2.0	57.0-90.0	16.5-27.2	1.26	108
125	2BCM	1.3	33.0±25.1	28.1±5.9	1.5±0.4	84
250	2BCM	1.3	40.4±25.1	28.9±14.4	1.3±0.8	84
500	2BCM	2.4	70.3±11.4	25.3±4.3	2.7±0.5	84
900	2BCM	2.4	76.7±26.6	26.3±11.8	4.4±3.6	84
500	2BCM	0.5	-	-	-	85
500	2BCM	1.6	0.8 l/kg	0.5 l/h/kg	-	103
500	2BCM	2.0	-	-	-	120
500	2BCM	2.75	-	8.7-24.2	0.1-1.5	107

KEY: $t_{½}$ = Elimination half-life V = Volume of distribution
Cl_p = Plasma Clearance Cl_r = Renal Clearance

The extensive tissue penetration reported for erythromycin (58,64,81,86,87) is reflected in the fairly large values obtained for the volume of distribution at steady state (108). The low values for renal clearance are expected as only a small percentage of the drug is excreted unchanged in the urine (Section 1.3.6). Some evidence of dose dependent i.v. kinetics has been reported (84) with clearance being unchanged but volume of distribution and terminal half-life increasing with dose. Inter-subject variations in disposition kinetics after i.v. administration to human volunteers has been shown to be less than the variability associated with oral erythromycin therapy, suggesting that drug disposition is less variable than drug absorption (84). In summary, erythromycin disposition kinetics after i.v. administration have been well described by a two compartment body model with some evidence of dose dependency and a terminal half-life ranging from 1.3 to 2.8 hours.

1.4.2 ERYTHROMYCIN BASE AND STEARATE - SINGLE DOSE

The study of the pharmacokinetics of oral erythromycin is complicated by the variety of formulations and protocols utilized. The results of numerous reports in which the single dose pharmacokinetics of either erythromycin base or stearate have been studied are summarized in table 1.7. Some of the more important findings and trends observed in these studies are highlighted in the following discussion.

The pharmacokinetics of erythromycin base and stearate from 0-12 hours post administration is best described by an open one compartment body model (74,78,83,126-129) with elimination half-lives ranging from 1.4 to 3.0 hours. Kroboth *et al* (104) have reported similar results for 0-12 hour data, but have shown that continued sampling to 24 hours introduces a biphasic component of the elimination profile best described by a two compartment model with a terminal elimination half-life of 6.0 to 7.0 hours.

Table 1.7 Pharmacokinetics of erythromycin base and stearate - single dose studies

PRODUCT	DOSE (mg)	MODEL	$t_{½}$ (h^{-1})	k_a (h)	T max (h)	T lag (h)	FOOD	TIME	REF
S FCT	500	1CBM	0.6	1.9	3.3	0.8	p.c	0-12	74
S FCT	500	1CBM	0.6	2.0	2.7	0.1	fasting	0-12	74
S FCT	250	1CBM	3.0	1.5	0.9	0.9	fasting	0-6	126
B ECP	250	1CBM	2.2	1.4	2.7	0.9	fasting	0-6	126
S FCT	500	1CBM	zero-order	2.3	4.0	-	p.c	0-12	78
S FCT	500	1CBM	zero-order	3.5	2.4	-	fasting	0-12	78
S FCT	250	1CBM	mixed	-	2.0	1.3	fasting	0-6	127
B ECP	250	1CBM	mixed	-	5.0	3.0	p.c	0-6	127
S FCT	500	1CBM	-	3.0	-	-	fasting	0-12	83
B ECP	250	1CBM	2.6	1.4	2.3	1.4	p.c	0-12	128
B ECP	500	1CBM	3.8	1.9	2.5	1.4	fasting	0-12	128
B ECP	250	1CBM	3.2	2.0	2.0	1.4	fasting	0-11	129
B ECP	500	1CBM	4.3	2.5	2.0	1.4	fasting	0-11	129
B ECP	1000	1CBM	3.2	3.0	2.0	1.3	fasting	0-11	129
S FCT	500	1CBM	2.3	2.7	2.0	1.1	fasting	0-11	129
B ECT	500	2CBM	zero-order	6.6	6.3	3.1	fasting	0-24	104

KEY: B ECT = Base enteric coated tablets
B ECP = Base enteric coated pellets
S FCT = Stearate Film coated tablets
Mixed = Zero and first-order processes described data equally well
ka = First-order absorption rate constant
t½ = Elimination half-life
FOOD = Conditions of study
TIME = Period over which blood samples were collected

Erythromycin absorption has been interpreted as zero-order, first-order and variable first-order processes (Table 1.7) both with and without lag times. Longer lag times and increased Tmax (time to reach maximum serum concentration) values have been associated with the enteric coated pellet formulations which is expected since dissolution of active drug only occurs in the alkaline pH of the small intestine. Elimination half-lives obtained in studies assuming zero-order absorption are on average longer than those from other studies. Increased elimination half-lives have also been reported for supine volunteers when compared with active persons (130).

Comparison of AUC (area under the serum concentration versus time curve) and Cmax (maximum serum concentration) values between studies is not very informative due to differences in study protocols and analytical techniques. Superproportionate AUC values following the administration of 3 increasing oral dosages of erythromycin base have been reported (129), indicating possible dose-dependent pharmacokinetics. As was found following i.v. administration (84) elimination half-lives increased with increased dosages, suggesting a degree of capacity-limiting saturation of the microsomal enzymes involved in erythromycin metabolism. This saturation may be linked to earlier discussions on erythromycin metabolism (section 1.3.5) where erythromycin was reported to induce its own metabolism, as well as to induce the production of an inactive metabolite which binds to cytochrome p-450 inhibiting metabolism of itself and other similarly metabolized drugs. Serum concentration peaks during the elimination phase have been observed in some volunteers after both oral and intravenous administration, dramatically affecting the calculation of both AUC and elimination half-life. Entero-hepatic recycling (as reported for josamycin (131)) and reabsorption of drug following active secretion into the gastrointestinal tract are the most likely causes (84,106,129).

1.4.3 ERYTHROMYCIN BASE AND STEARATE - MULTIPLE DOSE STUDIES

The pharmacokinetics of erythromycin following repeated administration of either erythromycin base or stearate is characterized by serum levels and AUC values at steady-state, far in excess of those calculated from single dose data, irrespective of the model used (78,126-128,132). The absolute bioavailability of a stearate dosage form, obtained by comparison with intravenous data, has been reported to increase from 30% after dose 1 to 90% after dose 9, while values for erythromycin base have remained constant

at 40% in both situations (127). A saturation of metabolic processes as previously discussed, is generally accepted as the most feasible mechanism, particularly since elimination half-lives have been shown to increase (132) while absorption rates have remained constant or decreased (78) following multiple dose administration.

Table 1.8 Pharmacokinetics of erythromycin base and stearate - multiple dose studies

PRODUCT	DOSE (mg)	MODEL	k_a (h^{-1})	$t_{½}$ (h)	TIME	REF
B ECT	250 q6h	1CBM	zero-order	-	6 hr	132
B ECP	250 q6h	1CBM	1.7	1.9	6 hr	126
S FCT	250 q6h	1CBM	1.9	1.8	6 hr	126
S FCT	250 q6h	1CBM	0.5	2.0	12 hr	78
S FCT	250 q6h	1CBM	0.5	2.1	12 hr	78
S FCT	250 q6h	1CBM	mixed	-	6 hr	127
B ECP	250 q6h	1CBM	mixed	-	6 hr	127
B ECP	250 q6h	1CBM	2.6	2.1	9 hr	128
B ECP	500 q12h	1CBM	2.2	1.9	9 hr	128

KEY: B ECT = Base Enteric Coated Tablets
B ECP = Base Enteric Coated Pellets
S FCT = Stearate Film Coated Tablets
Mixed = Zero and first-order absorption
k_a = First-order absorption rate constant
$t_{½}$ = Elimination half-life
TIME = Period over which blood samples were collected

As is evident from table 1.8 the interpretation of the absorption process after multiple dosages of erythromycin is as diverse as after single dose administration, with apparently similar studies reporting conflicting results. Colburn *et al* (132) have suggested that dose-to-dose variations in the rate and extent of absorption and day-to-day variability in the kinetics of elimination must be considered when analysing data obtained from multiple dose protocols. Circadian effects on the metabolism of erythromycin have also been reported (133) but are unlikely to explain the variations found over three days of multiple dose administration.

1.4.4 ERYTHROMYCIN ESTERS - SINGLE AND MULTIPLE DOSE

The pharmacokinetics of erythromycin estolate and ethylsuccinate appear to be similar to that of erythromycin base after both single and multiple doses with only the elimination half-lives being slightly increased for the esters (78,83,134,135). Welling *et al* (78) have reported no differences in the pharmacokinetics of both total erythromycin (base + ester) and the propionyl ester alone, following administration of single and multiple

doses of the estolate. These authors found a one compartment model with zero and first-order absorption best described single and multiple dose data respectively, while Patamasucon *et al* (134) found a first-order absorption process adequate for both dosage regimens in infants. Koeleman *et al* (135) have shown some advantages of non compartmental methods over compartmental methods when performing comparative bioavailabilty studies involving erythromycin estolate.

1.5 COMPARATIVE BIOAVAILABILITY STUDIES

The concept of comparative bioavailability is complex, and has been plagued by a lack of definitive criteria and many misunderstood myths (136,137). In the light of the problems associated with the study of erythromycin pharmacokinetics following oral administration it is obvious that interpretation of comparative bioavailability studies is equally if not more complex. This subject has been regularly discussed in the literature and various suggestions regarding study design and interpretation of results have been made (35,80,138-141).

Table 1.9 Comparative bioavailability studies involving various erythromycin derivatives

COMPOUNDS STUDIED	REFERENCES				
BASE-BASE	141(S+M)	139(S+M)	97(S+M)	147(S)	152(S+M)
	153(S)	154(S+M)			
BASE-STEARATE	141(S+M)	139(S+M)	97(S+M)	76(S)	154(S+M)
	77(S)	66(S)	146(S)	145(S)	113(S+M)
BASE-ESTOLATE	119(S)	121(S)			
BASE-ESTOLATE*	113(S+M)	114(S+M)			
BASE-E/SUCCINATE	139(S+M)				
BASE-E/SUCCINATE*	155(S+M)				
STEARATE-STEARATE	156(S)	77(S)	143(S+M)		
STEARATE-ESTOLATE	122(S)	116(S)	157(S+M)	118(S)	83(S)
	117(S)				
STEARATE-ESTOLATE*	78(S+M)				
STEARATE-E/SUCCINATE	139(S+M)	130(S)	158(S+M)		
ESTOLATE-ESTOLATE	135(S)				
ESTOLATE-E/SUCCINATE	120(S)	112(S+M)	134(S)		
ESTOLATE-E/SUCCINATE*	115(S+M)				

KEY: (S) = Single dose study
(S+M) = Single and multiple dose study
* = Discriminatory analytical technique

It must constantly be borne in mind that the results of comparative bioavailability studies involving erythromycin derivatives, are only valid for the specific products evaluated under the experimental conditions described (80,139). It is impossible to evaluate findings of these studies unless a detailed description of the study protocol and analytical method employed accompany the results. Table 1.9 summarizes the various comparative bioavailability studies performed on erythromycin derivatives. It is not possible to comment on the findings of each study, but in addition to the previously mentioned reservations, the following three points are worth noting:

1. Multiple dose protocols are preferred to single dose studies.

2. Comparisons involving the ester derivatives of erythromycin should be performed utilizing analytical techniques which are capable of determining individual concentrations of both the ester and base components in biological fluids.

3. The estolate derivative, which for many years was thought to exhibit superior absorption over erythromycin base and stearate, has been shown to yield lower concentrations of active erythromycin base when discriminatory assay procedures have been utilized.

CHAPTER TWO

THE SIMULTANEOUS DETERMINATION OF ERYTHROMYCIN BASE AND PROPIONATE IN HUMAN SERUM AND URINE

2.1 INTRODUCTION

The need for discriminatory assay techniques, capable of selectively determining concentrations of erythromycin base and propionate in biological fluids following the oral administration of erythromycin estolate has been discussed (Section 1.3.7). This is of particular importance when comparing erythromycin estolate with other erythromycin derivatives as well as in pharmacokinetic studies. It is worth noting that some standard pharmacological texts used by drug prescribers (47,159) do not differentiate between the various derivatives of erythromycin, thereby assuming no significant differences in their basic pharmacokinetics.

Both the USP (160) and BP (161) official assays for the analysis of erythromycin base and estolate employ microbiological techniques. Various similar procedures are also used (162-164) all of which rely on the total antimicrobial activity of the sample versus a susceptible bacterium usually *Staphylococcus aureus*, *Sarcina lutea* or *Bacillus pumilus*. While being sufficiently sensitive for most applications, these methods lack specificity, are relatively imprecise and are extremely tedious to perform (165). The analysis of erythromycin estolate is further complicated by the microbiological inactivity of the drug and its rapid and continuous hydrolysis in collected biological samples (68,166). Following the administration of erythromycin estolate, a large but unknown percentage of the ester in serum/plasma samples is hydrolysed to the active base during the assay procedure. This hydrolysis continues during storage even at -20°C (166). The significance of the above when comparing assay methodologies and in designing new ones, is that concentrations obtained utilizing analytical methods which do not include a separation step are not representative of the concentrations of erythromycin base in the samples at the time of withdrawal and that continued hydrolysis during storage results in overestimation of erythromycin base concentrations. More importantly, if two identical samples are subjected to even slight differences in sample collection, handling and/or storage, differences will be obtained due to variation in the extent of hydrolysis. For comparative studies involving erythromycin estolate products, these problems may be overcome by the

incorporation of a hydrolysis step, in which all the propionyl ester is converted to erythromycin base prior to detection, thereby determining "total erythromycin" concentrations (*vide infra* chapter 3).

In 1969 Stephens *et al* (67) developed the first discriminatory assay for erythromycin, a two-step paper chromatographic-bioautographic technique, capable of determining concentrations of erythromycin base and propionate in body fluids. While no special precautions regarding sample collection procedures were mentioned, valuable information regarding the proportions of circulating erythromycin base and propionate was obtained. Erythromycin base was found to contribute approximately one quarter of the total erythromycin concentrations in both serum and urine. This early procedure and a thin-layer chromatographic (TLC) adaptation (167), appear to effect the separation adequately but suffer from the general disadvantages associated with microbiological methods (165) as well as being tedious to perform with low sample throughputs. Tserng and Wagner (166) developed the first chemical assay based on a liquid-liquid separation of the two components at pH 6.0 preceeding spectrofluorimetric detection of the derivatized species. Using this method, they showed that the hydrolysis of the ester to the base after sample withdrawal was significant and that it continued during storage of samples even at low temperatures. They attempted to minimize this error by spiking their standards on the same day as their *in vivo* experiments and then storing them under identical conditions. The liquid-liquid separation described, has been modified by various workers to incorporate microbiological end-points (78,113,115) and modified sample handling techniques (78,113-115), the most popular being immediate freezing (-70°C) of serum/plasma after separation from whole blood. Welling *et al* (78) determined the concentrations of erythromycin base by subtraction of ester concentrations from "total erythromycin" concentrations obtained after hydrolysis, thus making the determination of erythromycin base highly dependent on complete and reproducible hydrolysis of the ester fraction. Yakatan *et al* (113) performed TLC-bioautographic runs on all samples to confirm the success of the liquid-liquid separation procedure. The separation methods employed by these workers are extremely time consuming and are not conducive to automation, while the detection techniques employed lack the selectivity of modern chromatographic methods.

Tsuji (168) described a high-performance liquid chromatographic (HPLC) technique for the simultaneous analysis of erythromycin base and erythromycin ethylsuccinate in human serum which involved an ethereal

extraction prior to chromatography and a complex post-column extraction and derivatization procedure preceeding fluorimetric detection. The complex apparatus required for this analysis and the relatively poor chromatography reported, made this particular HPLC method unattractive for possible adaptation to erythromycin propionate analysis, although HPLC was chosen as the general analytical tool for these studies. More recently, an HPLC method has been described by Croteau *et al* (169) for the simultaneous quantitative analysis of erythromycin base and propionate as well as erythromycin base and ethylsuccinate in biological fluids. This method involves amperometric electrochemical detection following separation of the compounds of interest on the identical column to that used in section 3.2.2 of this study. The major disadvantages of this method are the extremely long retention time reported for erythromycin propionate (34.5 min) and the rapid decrease in detector sensitivity (up to 60% in 24 hours). In addition, insufficient account appears to have been taken of the reported hydrolysis (166) of erythromycin propionate during sample handling and storage procedures.

High-performance liquid chromatography is applicable to an enormous variety of compounds due to advances in instrumentation, column packings and methods of detection (170). Selective separations are accomplished with great speed, sensitivity and precision, making it an ideal technique for the analysis of drugs in biological fluids. An HPLC technique employing ultraviolet (UV) detection at 200 nm has previously been reported by the author (171) for the analysis of erythromycin base in human serum and urine. Deployment of UV wavelengths below 215 nm is normally associated with a loss of selectivity and an increase in baseline noise, especially at high detector amplifications. The sensitivity required for the detection of erythromycin base over a 12 hour period following the administration of erythromycin estolate, however, made UV detection impractical. Two papers recently published on the analysis of erythromycin base in biological fluids (172,173) have shown advantages of electrochemical detection over spectrophotometric methods.

Electrochemical detection in HPLC has advanced from being a relatively specific tool in catecholamine analysis to a relatively common detection technique with a broad application, especially in the analysis of easily oxidizable drugs in biological fluids (174-176). Various detection modes are now available to induce reactions in unreactive species but the conventional oxidative mode is still the most commonly used due to the

excessive interference and other operational problems associated with the pure reductive and combination modes (174,176,177). Coulometric electrodes are most commonly made of porous graphite or carbon composites with inert plastics through which the eluent flows (178) whilst amperometric electrodes are usually glassy carbon or carbon paste surfaces. Coulometric electrodes, by definition, react with 100% of the passing analyte while this percentage is typically 1-10% for amperometric electrodes (174). The increased reaction of the coulometric electrodes does not always result in the most favourable signal-to-noise ratio due to associated increases in background currents (174). These electrodes, however, do have distinct advantages when operated as screening electrodes in dual electrode electrochemical cells. The two previously mentioned methods (172,173) for the analysis of erythromycin base in biological fluids by HPLC with electrochemical detection both employed the same dual electrode coulometric detector operated in the oxidative screen mode. In this mode, the upstream electrode is set at a potential just lower than that required for detection of the drug, serving as a screen by oxidizing most co-eluting contaminants prior to detection at the downstream analytical electrode. Erythromycin is only oxidized at electrode potentials above 0.8 V (172,173,179) necessitating the use of high operating potentials which are associated with sensitivity and stability problems due to high detector noise (174). The relatively high detector sensitivity required for the analysis of erythromycin base in biological fluids further compounds the problem, requiring highly selective pre-chromatographic extraction procedures in order to remove co-eluting contaminants and to facilitate sample concentration.

Sample clean-up and concentration has traditionally been performed by adaptations of standard chemical procedures involving liquid-liquid extractions of compounds after adjustment of pH (180). Solid-phase extraction procedures (both on and off-line) have recently become increasingly popular (181) and would appear to be increasing in popularity due to their ease of use, rapid sample throughputs and most importantly, their ability to be fully automated by robotic and other specialized systems. Literature regarding solid-phase extraction procedures has been predominantly directed towards the broadening of its application and the subject of automation, with very little being reported on the unique problems it has presented to analytical chemists. Variabilities in recovery, but not assay reproducibility have been reported for an ibuprofen assay when using different batches of extraction columns (181) and

permanent adsorption losses have also been discussed (180). Precipitated fibrin from thawed plasma samples is known to interfere with the flow of directly loaded samples (181) while more recently, variations have been observed between identical samples extracted before and after freezing which have been attributed to increases in the binding strength between the drug and serum protein on freezing, thereby making less of the drug available for interaction with the solid-phase extraction matrix (182).

2.2 EXPERIMENTAL

2.2.1 REAGENTS

All reagents were of at least analytical grade. The acetonitrile was distilled-in-glass UV grade (Burdick & Jackson) and the water was purified through a Milli-Q system (Waters Associates) containing two ion exchange cartridges (Ion-Ex), an organic selective cartridge (Organ-X) and a carbon filter (Super-C) immediately following deionization by reverse-osmosis.

Drug samples used:

Erythromycin Base USP[1]	Lot No MD H-1
Erythromycin Estolate USP[1]	Lot No MD F
Erythromycin Ethylsuccinate[2]	Lot 51-039-CD
Erythromycin Stearate[2]	Lot 07-616-CD
Erythromycin B [2]	Lot 84-991-AX
Anhydroerythromycin[2]	Lot 86-188-AX
Erythromycin Enol Ether[2]	Lot 3511-55A
Erythralosamine[2]	Lot 26-978-AX
Propionyl Erythromycin[3]	Lot 758G28279
Des-N-methylerythromycin[3]	Lot 164-171-185-2
Oleandomycin phosphate[4]	Lot 206/767003
Triacetyl oleandomycin[4]	Lot 07305-76EA
Josamycin[5]	Lot W-285

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

2.2.2 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM

The analysis was performed with a modular high-performance liquid chromatograph consisting of the following components:

a) Model M6000A constant-flow pump (Waters Associates)
b) Model ERC-3510 solvent degasser (Erma Optical Works)
c) Model 710B WISP automatic sample injector (Waters Associates)
d) Column: 25 cm x 3.9 mm i.d steel column , custom packed in our laboratory to contain 10 µm C_{18} material (Techsil, HPLC Technology)
e) Guard Column: guard-pak (Waters Associates) unit, fitted with a cartridge containing 40-60 µm glass beads (Supelco Inc.)
f) Coulochem 5100A electrochemical detector with a model 5010 analytical cell and a 5020 guard cell (Environmental Sciences Associates)
g) Model LC-22 temperature controller (Bioanalytical Systems Inc.)
h) Model 56 strip chart recorder (Perkin Elmer)

2.2.3 ADDITIONAL EQUIPMENT

a) Model 601 digital ionalyser (Orion Research)
b) Speed-Vac rotary vacuum centrifuge (Savant Instruments)
c) Whirlimixer (Fisons)
d) Model 2004MP analytical balance (Sartorius)
e) Model HN-SII centrifuge (Damon/IEC Division)
f) Model 5414 high speed micro-centrifuge (Eppendorf Industries)
g) Column Packer (HPLC Technology)
h) Fixed volume transfer pipettes (Rudolph Brand & Co.)
i) Model 710-SNR micro-syringe (Rheodyne Inc.)
j) Model 1040A diode-array ultraviolet detector (Hewlett Packard)
k) Microlab-P programmable automatic pipette (Hamilton-Bonaduz AG)
l) Apple II Europlus Computer

2.2.4 TREATMENT OF GLASSWARE

All glassware involved in sample extraction procedures was rinsed in water before use, and then soaked in a chromic acid mixture for 24 hours (70 g/l potassium dichromate in concentrated sulphuric acid). After numerous water rinses, the glassware was finally rinsed in deionized water and dried in an oven. Glassware for general use was treated with a detergent solution (Decon 75: Atomic Export Company, SA.).

2.3 METHODOLOGY

2.3.1 MOBILE PHASE PREPARATION

Phosphate buffer (0.05 M) (buffer A) was prepared by adding 3.2 ml phosphoric acid to 1 litre of water. Sodium hydroxide was then used to adjust the solution to pH 6.3. The mobile phase was prepared by mixing 650 ml acetonitrile with 350 ml buffer (A). The solvent mixture (pH 7.0) was degassed and filtered through a 0.45 µm filter (Millipore, Type HVLP). The mobile phase which was constantly degassed using an in-line degassing unit, was recycled until prolongation of retention times was observed.

2.3.2 CHROMATOGRAPHIC CONDITIONS (SYSTEM A)

The mobile phase was used at a flow-rate of 1.3 ml/min for the analysis of both serum and urine samples, with a resulting pressure of 1500 p.s.i. The electrochemical detector was operated in the oxidative screen mode with the upstream electrode set at +0.70 V, the downstream electrode at +0.90 V and the guard cell at +1.00 V. The column temperature was maintained at 35 °C.

2.3.3 COLUMN PACKING PROCEDURE AND COLUMN CHOICE

Four different cclumns were employed with numerous mobile phase combinations in order to obtain the desired separation between erythromycin base, propionate and the internal standard.

a) 25cm x 3.9mm i.d steel column packed with 5 µm C_{18} material
b) 25cm x 3.9mm i.d steel column packed with 10 µm C_{18} material
c) 15cm x 3.9mm i.d. steel column containing 5 µm highly end-capped C_{18} material (Novapak, Waters Associates).
d) 7.5cm x 4.6mm i.d. steel high speed column containing 3 µm C_{18} material (Ultrasphere-ODS, Beckman Instruments)

Columns (25 cm x 3.9 mm i.d.) were packed as follows: Three and a half grams of packing material (Techsil C_{18}, 5 or 10 µm) were slurried in 30 ml carbon tetrachloride and sonicated until dispersed. After loading the carbon tetrachloride slurry into the column reservoir or "bomb", 120 ml methanol was pumped through the column at a pressure of 5 000 p.s.i. After

this period the column was inverted without interuption of flow and a further 120 ml methanol was pumped through the column. After removal from the column packer, the column was equilibrated with a 75:25 acetonitrile/water mixture and tested using a standard laboratory test mixture containing benzamide, benzophenone and benzene (179).

2.3.4 CALIBRATION STANDARDS

Stock solutions of erythromycin base (0.25 mg/ml) and erythromycin propionate (0.50 mg/ml) were prepared in acetonitrile and were stored at 4 °C for no longer than one week. Three dilutions were prepared by adding 1.0, 2.0 or 4.0 ml of either stock solution to a 20.0 ml volumetric flask which was made up to volume with acetonitrile. Immediately preceeding any study, aliquots of between 20 and 100 µl of these dilutions were added to 1.0 ml blank serum or 0.5 ml blank urine using a programmable automatic pipette producing calibration curves over the ranges 0.25 - 4.00 µg/ml (base), 0.50 - 8.00 µg/ml (ester) in serum and 1.00 - 20.00 µg/ml (base & ester) in urine. Total erythromycin concentrations, expressed as erythromycin base equivalents, were obtained from the sum of erythromycin base concentrations and the base equivalent of propionyl concentrations (ester concentration corrected by molecular mass conversion constant of 0.696). The standards were vortexed briefly, snap frozen in liquid air and stored at -15 °C in insulated holders. Serum used in the preparation of standards was originally obtained from a local hospital and dialysed versus normal saline for three days prior to use. During the final phases of the experiments fresh serum was obtained from drug-free student volunteers. Aqueous oleandomycin phosphate solutions (20.00 µg/ml serum, 40.00 µg/ml urine) were prepared for use as the internal standard.

2.3.5 ELECTROCHEMICAL VOLTAMMOGRAMS AND ULTRAVIOLET SPECTRA

Hydrodynamic voltammograms were constructed as follows utilizing chromatographic system (A): The detector was set at a low electrode potential (+0.5 V) and the chromatographic system left to stabilize. Identical injections of a mixture of erythromycin base and propionate were chromatographed at increasing potentials until either the baseline noise became excessive or the drug response reached a plateau or began to decrease. The peak heights of each injection, normalized to one sensitivity setting were then converted to a value in microamps and plotted against the

working electrode potential. Voltammograms were performed on both electrodes of the dual electrode coulometric cell whenever the chromatographic conditions were altered and as a routine trouble-shooting guide.

A diode-array UV detector was used during the early stages of method development to obtain UV spectra of the compounds of interest.

2.3.6 EXTRACTION OF BIOLOGICAL SAMPLES

Although the basic outline of the extraction procedure remained constant throughout development, numerous variations in both the composition and volume of the wash solutions were required to overcome various developmental problems. The final method as used in the biological studies was as follows:

One hundred microlitres of internal standard solution and 1.0 ml acetonitrile were added to the freshly thawed serum samples followed by vortex mixing and centrifugation at 1600xg for 10 min in order to precipitate serum protein. The supernatant was transferred to a culture tube containing 5.0 ml water and mixed well. To the 0.5 ml urine samples 100 µl internal standard solution and 5.0 ml water were added followed by thorough vortex mixing prior to loading onto the extraction columns. The 1 ml C_{18} extraction columns (Bondelut, Analytichem Int.) were pre-washed prior to loading with 5.0 ml acetonitrile, followed by 5.0 ml water under atmospheric pressure. The diluted sample was then added to the extraction column with the aid of a 25-ml custom made glass reservoir and a pasteur pipette. After the load solution had passed through the extraction column, it was washed with 20.0 ml water (serum) or 10.0 ml water (urine) followed by 3.0 ml acetonitrile-water (1:1). The compounds of interest were eluted into a 2-ml tapered collection tube with two successive 500 µl aliquots of buffer (A) - acetonitrile (2:3). The sample was then taken to dryness under vacuum in a rotary vacuum centrifuge and the residue in the collection tube was reconstituted in 20 µl of water and vortex-mixed for 1 min. On addition of 100 µl acetonitrile, two layers formed as previously described (171). This mixture was vortex-mixed for 1 min and then centrifuged for 1 min at 1600xg to ensure complete separation of the two layers. A portion of the top acetonitrile layer (60-80 µl) was then transferred to a WISP limited-volume insert using a micro-syringe. Aliquots (5-20 µl) of this sample were injected onto the column.

2.3.7 CONTROL OF ESTER HYDROLYSIS DURING ASSAY PROCEDURE

The stability of the propionyl ester during the various phases of the extraction procedure was assessed in order to detect any problem areas and to assist in method development. Erythromycin propionate standards were prepared in acetonitrile and buffer (A) and stored for 12 hours at either room temperature (22-25°C), in the automatic injector (28-30°C), in the refrigerator (4° C) or in the deep-freeze (-15°C). Samples were analysed by HPLC at regular intervals over this time period. Similar experiments were performed on standards that had undergone a phase separation step as previously described (Section 2.3.6). Samples from a pilot trial were re-injected after 12 hours storage in the automatic injector and the concentrations of both base and ester compared with results from the original analysis.

Hydrolysis during the drying stage in the rotary vacuum centrifuge was carefully monitored in 6 samples at two different concentrations, particularly since the centrifuge is heated to 37°C. The overall hydrolysis of aqueous standards was assessed by comparing base/ester ratios of extracted samples with identical unextracted standards.

2.3.8 CONTROL OF ESTER HYDROLYSIS DURING SAMPLE COLLECTION AND STORAGE

Five millilitre blood samples were collected from the forearm of human volunteers via an indwelling catheter. Approximately 3 ml was immediately transferred to a teflon centrifuge tube and spun at 16000xg for 2 min in a high-speed micro-centrifuge. A 1ml aliquot of serum was then accurately transferred to a glass tube which was immediately immersed into liquid air. The frozen sample was then transferred to an insulated holder and stored at -15°C for a maximum of 6 hours prior to analysis. Urine samples were collected during prescribed intervals, the volume recorded and a 0.5 ml aliquot immersed in liquid air prior to storage for no longer than 24 hours at -15°C.

The extent of the hydrolysis during sample withdrawal and storage under various conditions, with and without snap freezing in liquid nitrogen, was evaluated and compared.

2.3.9 LINEARITY, PRECISION AND ACCURACY

Samples over the following concentration ranges were extracted in triplicate: Serum erythromycin base 0.25 - 4.00 µg/ml
Serum erythromycin propionate 0.5 - 8.00 µg/ml
Urine erythromycin base and propionate 1.00 - 20.00 µg/ml

Calibration curves were obtained by plotting the ratio of the peak height of these compounds to that of the internal standard using a linear regression computer programme written for the Apple desk top computer (183). The operator is required to input the concentrations and respective peak heights of the standards and then the identification and peak heights of all analysed samples. Interpolation is performed automatically and results are printed in a clear tabular format.

The precision and accuracy of the method were assessed in the same experiment. A calibration curve was constructed as above using duplicate standards at three different concentrations. Replicate samples (n=6) at three different concentrations (serum or urine) were prepared using different stock solutions and were extracted as previously described. The mean concentrations obtained for the replicates were a measure of the accuracy of the method whilst the relative standard deviations of the six samples at any one concentration provided measure of precision.

2.3.10 EXTRACTION EFFICIENCY

The overall percentage recovery of the extraction procedure was determined as follows: Spiked serum and urine samples were analysed by extracting replicate (n=5) samples at five different concentrations without the addition of internal standard. After reconstitution, as much as possible of the acetonitrile (upper) layer was removed and transferred to a second collection tube and re-dried. Amounts of drug corresponding to a 100% recovery were spiked into similar tubes and taken to dryness. Both the extracted samples and standards were reconstituted in a 20 µl aliquot of acetonitrile containing oleandomycin as an external standard for comparison of the respective peak height ratios.

2.3.11 PILOT TRIALS

Pilot trials were originally performed on six volunteers who were participating in a double cross-over comparative bioavailability study involving two erythromycin estolate tablet dosage forms (500mg as erythromycin base equivalent). Results from these pilot studies showed deficiencies in the method which led to extensive further investigation (*vide infra* section 2.4.4). The final pilot trial was performed on a single drug-free volunteer utilizing the following protocol: The subject received one tablet of erythromycin estolate (500 mg base equivalent) after an overnight fast, accompanied by 250 ml water. Blood samples were drawn at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10 and 12 hours after 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. All samples were handled and stored as previously described. Serum samples were collected in duplicate for repeat analysis after 24 hours at -15°C.

2.4 RESULTS AND DISCUSSION

2.4.1 CHROMATOGRAPHY

Erythromycin exhibits interesting behaviour on C_{18} silica based columns, being highly dependent on buffer ion concentration for its elution (179). Propionyl erythromycin is more lipophilic than erythromycin base and hence has a longer retention on reversed-phase columns under identical chromatographic conditions. Attempts at utilizing a highly end-capped C_{18} column (Novapak) resulted in extremely long retention of erythromycin propionate at the optimum conditions for erythromycin base analysis (179). These results are similar to those reported by Cachet *et al* (184) who reported improved chromatography for erythromycin base and a wide variety of related substances on aged C_{18} columns in which the polarity of the packing material had increased, and are consistent with the long retention time for erythromycin propionate (34.5 min) reported by Croteau *et al* (169) utilizing the same highly end-capped column (Novapak).

Adequate resolution between erythromycin base, propionate and internal standard could not be obtained using the 3µm high speed C_{18} column despite numerous variations in mobile phase composition, temperature and flow-rate. The two columns packed in our laboratories with a non end-capped C_{18} material (Techsil) performed similarly, resulting in adequate and fairly

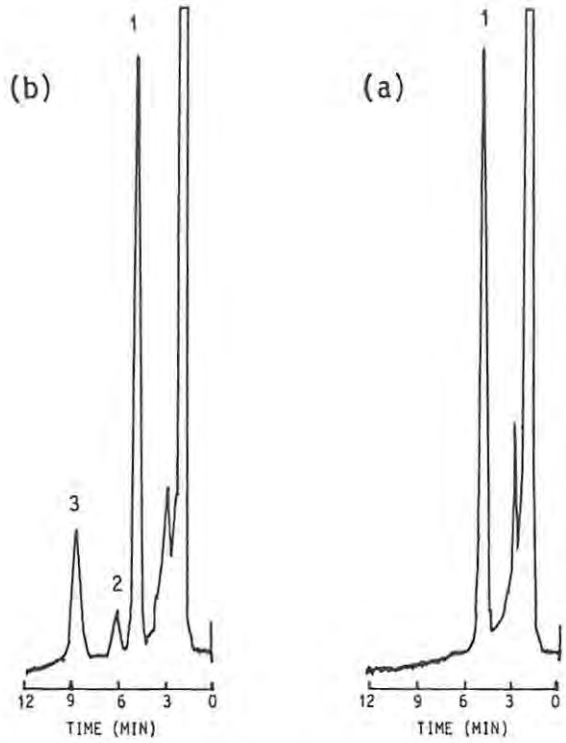


Figure 2.1 (a) Chromatogram of a blank serum extract
(b) Chromatogram of an extract of serum 5 hours after the oral administration of a tablet containing 500 mg erythromycin estolate (chromatographic system A)

rapid separation of the three compounds of interest. The column containing 10 µm material was finally chosen as the 5 µm column generated very high back-pressures (2500 psi) without any chromatographic advantages. Although the mobile phase composition was varied throughout the study due to changes in column performance, a mobile phase consisting of 65% acetonitrile and 35% buffer (A) was used in all further studies (chromatographic system A). A chromatogram of a serum extract containing erythromycin base, propionate and internal standard is depicted in figure 2.1 (b) and a similar chromatogram of a urine sample is depicted in figure 2.2 (b). The resolution between peaks is good and despite the relatively long retention of erythromycin propionate, the peak shape is not distorted. The retention times for erythromycin base and propionate plus a variety of related macrolide compounds employing chromatographic system (A) are reported in table 2.1. Urine samples collected during the latter stages of single dose trials and throughout multiple dose studies, contained two additional peaks which interfered with both the base and ester peaks (Fig. 2.2 (c)). These two compounds were identified by retention times alone as being anhydroerythromycin and erythromycin enol ether, two known acid degradation products of erythromycin. Attempts at identifying these peaks utilizing the diode-array UV detector were unsuccessful due to their poor UV absorption characteristics (179).

Table 2.1 Retention times for erythromycin and related macrolides (chromatographic system A)

COMPOUND	RETENTION (min)
Oleandomycin phosphate	4.6
Des-N-methylerythromycin	5.0
Josamycin	5.5
Erythromycin base	5.9
Anhydroerythromycin	6.9
Erythromycin ethylsuccinate	7.4
Erythralosamine	7.7
Erythromycin propionate	8.6
Triacetyl oleandomycin	8.9
Erythromycin enol ether	11.2
Propionyl josamycin	16.0

The glass beads guard column proved to be very successful, which unlike commercially available C_{18} cartridges did not affect chromatography on the analytical column. During routine analysis the guard column was back-flushed with methanol during mobile phase changeover periods (usually once a week) and then returned to the system. The same glass beads cartridge and analytical column were used throughout this study, further evidence of the advantages of a clean sample extraction procedure and the use of guard columns.

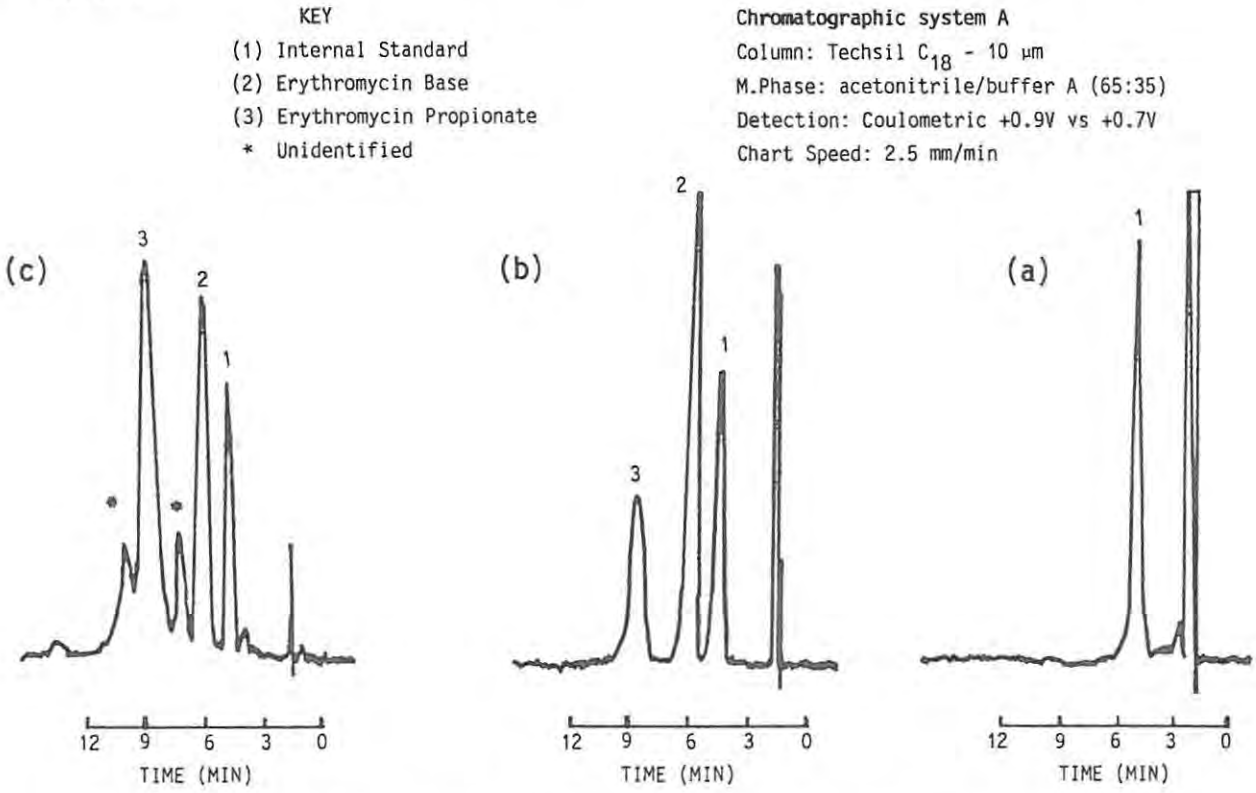


Figure 2.2 (a) Chromatogram of a blank urine extract
(b) Chromatogram of an extracted urine sample - 2 hours after administration of an erythromycin estolate tablet
(c) Chromatogram of an extracted urine sample - 12 hours after administration of an erythromycin estolate tablet
(chromatographic system A)

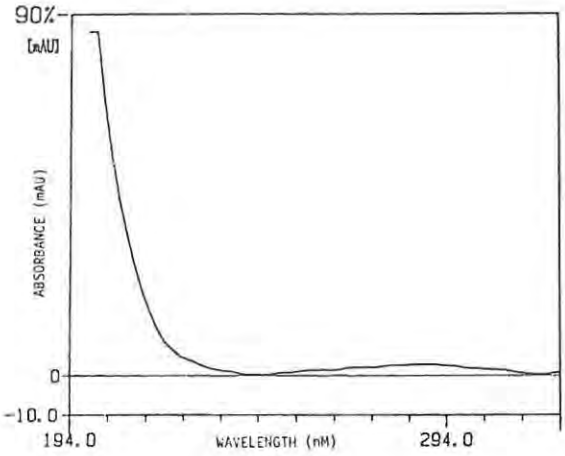


Figure 2.3 Ultraviolet spectrum of erythromycin base in mobile phase (chromatographic system A)

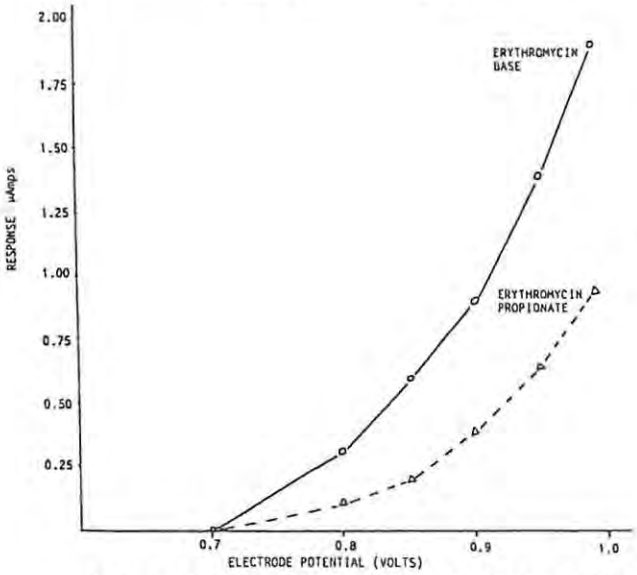


Figure 2.4 Hydrodynamic voltammograms of erythromycin base and erythromycin propionate (chromatographic system A)

2.4.2 DETECTION AND LIMITS OF SENSITIVITY

Both erythromycin base and propionyl erythromycin have poor UV absorption characteristics, with the only significant absorption being in the low UV wavelength range (Fig. 2.3). Increased sensitivity and some degree of increased selectivity was obtained by using a coulometric electrochemical detector operated in the oxidative screen mode as previously reported for erythromycin base (172,173,179). Similar levels of sensitivity could not be obtained using amperometric electrochemical detection due to the low buffer content of the mobile phase employed (*vide infra* chapter 6).

Hydrodynamic voltammograms of erythromycin base and propionate are depicted in figure 2.4. From these voltammograms it can be seen that erythromycin base is more readily detectable than erythromycin propionate at all electrode potentials, which is fortunate since lower levels of the base are obtained in biological fluids. It can also be seen that relatively high electrode potentials are required for adequate detection of the drugs, with a compromise having to be reached between signal strength and baseline noise, which increases with increasing potentials. Optimum results were obtained with an upstream screen electrode potential of 0.7 V (at which

very small amounts of the compounds are oxidized) and a downstream analytical electrode potential of 0.9 V where substantial reaction occurs. Electrochemical detectors are renowned for their variability in response over extended analyses (176). They are also extremely sensitive to pump malfunctions, exhibiting baseline noise and drift due to the slightest piston seal leak or check-valve problem, which are not detected on spectrophotometric detectors even when operated at low wavelengths and high amplifications. The mobile phase was constantly recycled with approximately one litre being used per week. The overall system sensitivity was found to increase during the first few days following a mobile phase change and then to reach a plateau, presumably due to the "scrubbing" action of the guard cell which removes most electroactive mobile phase contaminants oxidizing at less than 1.0 V. Sudden and dramatic sensitivity decreases were observed when new batches of mobile phase were introduced (Fig. 2.5). Re-injection of calibrators over a 24 hour run-time showed some slight variations in detector response which were compensated for in the construction of the calibration curve. Detector sensitivity was also shown to change due to potential shifts, with voltammograms generally shifting to higher potentials after constant use. Cleaning of electrodes with 30 ml 6N HNO_3 generally returned cells to their original condition although older cells appeared to reach a point where further cleaning seldom improved performance.

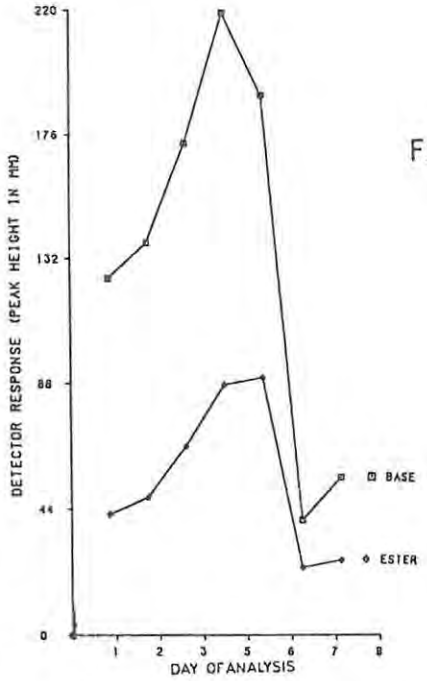


Figure 2.5 Day-to-day sensitivity variations in electrochemical detector response (chromatographic system A)

Determination of the sensitivity limits of HPLC-electrochemical procedures is complicated by variability in detector response. In this study, day-to-day variations in detector response were monitored by the daily injection

of a mixture of erythromycin base and propionate (in acetonitrile). Adjustments to detector gain settings or injection volume were then made prior to automated analysis. As previously reported, calibration standards were analysed throughout chromatographic runs in order to detect dramatic shifts in detector response. In general, no changes were found in calibration curves prepared from standards analysed at the beginning and end of 12-18 hour runs. Small changes in detector sensitivity were experienced over the same time periods, which were compensated by the employment of the internal standard. During the least sensitive analysis periods, serum concentrations of 0.10 µg/ml of erythromycin base and 0.25 µg/ml erythromycin ester were well within detection limits utilizing 10-20 µl injection volumes. However, during more sensitive periods similar results were attained employing injection volumes of only 5-10 µl. Urine analysis was complicated by the large concentration ranges encountered (0.50 - 30.00 µg/ml) for both compounds, but the method proved both sufficiently sensitive and flexible to cope with these problems.

2.4.3 ESTER HYDROLYSIS DURING SAMPLE MANIPULATION

Initial studies on the hydrolysis of the propionyl ester during the various steps in the extraction procedure were performed by comparing the ratio of the erythromycin base peak height to that of the ester. This is a very sensitive measure, as a small amount of hydrolysis results in a large change in this ratio, due to both the numerator increasing and the denominator decreasing. The experiments reported and discussed in this section were designed primarily to identify and limit hydrolysis of the ester during sample manipulation. A specific study on the rate of hydrolysis of the ester in serum and aqueous samples under various conditions was performed and is reported in chapter 4.

The employment of an automatic sample injector, required reconstituted samples to resist hydrolysis for up to 18 hours prior to injection during lengthy chromatographic runs. Samples reconstituted in water showed substantial degradation on storage. After 1 hour in the sample injector and 2 hours at room temperature the base/ester ratio had increased by more than 10% which continued at a similar rate for 4 hours. Identical samples prepared in acetonitrile showed no signs of hydrolysis throughout the 12 hour study period. Stored samples which had undergone a phase separation showed a 5% increase in the base/ester ratio after six hours, probably due

to some buffer solution being carried-over into the separated upper acetonitrile layer. Extracted biological samples showed a 5% increase in the base/ester ratio after 12 hours in the automatic sampler suggesting a possible problem, but calculation of the respective drug concentrations of similarly stored standards injected at the beginning and end of 18 hour chromatographic runs showed no significant increases in base concentrations or corresponding decreases in ester concentrations. The hydrolysis of the propionyl ester during storage in the sample injector was therefore most fortuitously minimized by the employment of the phase separation step.

The sample evaporation step was originally seen as a major obstacle in the assay development, since samples spend approximately one hour in a heated (37°C) chamber in a solution containing 40% phosphate buffer, conditions which are highly conducive to ester hydrolysis. However, due to the latent heat of evaporation of the solvents under vacuum, samples remain relatively cool in the rotary vacuum centrifuge with some actually freezing if heat transfer with the tube holders is insufficient. A series of aqueous standards, injected prior to and after extraction and reconstitution showed increases of less than 5% in base/ester peak height ratios, suggesting that sample hydrolysis during the entire extraction procedure was minimal.

Significant hydrolysis of erythromycin propionate has been reported during blood collection procedures (166) which was shown to decrease on addition of sodium fluoride, suggesting enzymatic influences in addition to chemical mechanisms. It was felt that the routine addition of sodium fluoride and the exact control of the intervals between sample withdrawal, centrifugation, serum harvesting and sample storage would be too complex in a clinical trial situation. Using a high speed microcentrifuge it was possible to separate the serum fraction from blood samples within two minutes, a technique which proved easy to use and minimized the possibility of hydrolysis considerably. The addition of sodium fluoride had no effect on hydrolysis during this period, which was expected as the time interval between blood withdrawal and serum harvesting was considerably shorter than in the published study (166).

Hydrolysis of the ester during storage at -15°C is a major problem as it is seldom possible to analyse samples immediately following withdrawal. Studies on the hydrolysis of the ester in stored serum samples spiked with erythromycin propionate showed definite degradation of samples after 12 hours at -15°C. It was possible that a significant proportion of the

hydrolysis during this 12 hour storage period could have occurred during the freezing process, therefore the experiment was repeated with samples being snap frozen in liquid-air prior to storage. Base/ester peak height ratios in six samples (5.0 µg/ml propionate) increased slightly from an initial value of 0.10 to 0.20 after 12 hours at -15°C with snap freezing, as opposed to a ratio of 0.50 obtained without prior freezing. This showed the advantages of initial freezing which was confirmed in less stringent but possibly more meaningful studies in which the concentrations of ester in spiked samples (1.0 & 5.0 µg/ml (n=4)) were monitored for one week at -15°C after initial snap freezing. The concentrations of ester were shown to decrease 6% overnight (12 hours) and 30% after 4 days. Similar studies using serum concentrations of 0.5 and 2.5 µg/ml showed less than 5% differences in serum sample ester concentrations when monitored two hourly for eight hours. It was therefore felt that adequate control of ester hydrolysis during blood sample collection and storage could be obtained by the combination of immediate centrifugation of blood samples followed by snap freezing of serum, particularly if analysis could be performed within eight hours of sample storage at -15°C.

Plasma samples are normally collected when rapid separation of blood cells is required, since this eliminates the clotting time required prior to centrifugation. Similar concentrations of erythromycin base and propionate were obtained in duplicate samples collected as either serum or plasma (Table 2.2). Collection of plasma samples involved an extra manipulation

Table 2.2 Comparison of serum and plasma duplicates

Sample Identification	Matrix	Concentration Base (µg/ml)	Concentration Ester (µg/ml)
P1 4hr Single dose	Plasma	0.12	0.98
	Serum	0.17	1.00
P2 4hr Single dose	Plasma	0.27	1.76
	Serum	0.33	1.77
P3 4hr Single dose	Plasma	0.16	1.32
	Serum	0.17	1.89
P4 6hr Multiple dose	Plasma	1.42	4.37
	Serum	1.40	3.58
P5 6hr Multiple dose	Plasma	1.38	3.95
	Serum	1.43	3.48
P6 6hr Multiple dose	Plasma	1.43	3.87
	Serum	1.40	3.59

since blood had to be transferred from the syringe to the heparinized tube and then only transferred to the centrifuge tube after gentle mixing.

The collection and storage of urine samples did not receive the same priority as serum samples. Samples were snap frozen as soon as possible after being voided and stored at -15°C for a maximum of 24 hours. Detailed hydrolysis experiments were not performed, but data obtained from studies performed in serum and buffer solutions, suggest that hydrolysis would not exceed 10% during the 24 hour storage period.

2.4.4 EXTRACTION AND PILOT TRIALS

The solid-phase extraction procedure utilized in this study was based on the observation that erythromycin does not elute from a C_{18} column using mixtures of acetonitrile and water, unless a buffer component is included in the eluate (179). The original extraction procedure for the simultaneous determination of erythromycin base and propionate in serum was developed using dialysed serum and was almost identical to the extraction procedure described previously for erythromycin base (171). Good precision, linearity and adequate recovery was obtained for both drugs during system development using a 5 ml water wash of the loaded sample, followed by a 5 ml acetonitrile/water wash (1:1) prior to elution. However, serum profiles obtained from all six patients in the first pilot trial had very low propionyl erythromycin concentrations which were seldom greater than those obtained for erythromycin base (Fig.2.6).

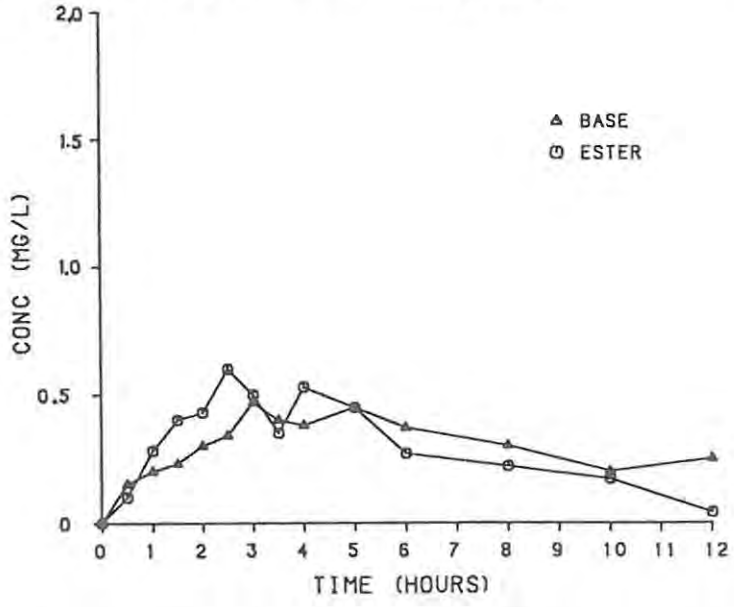
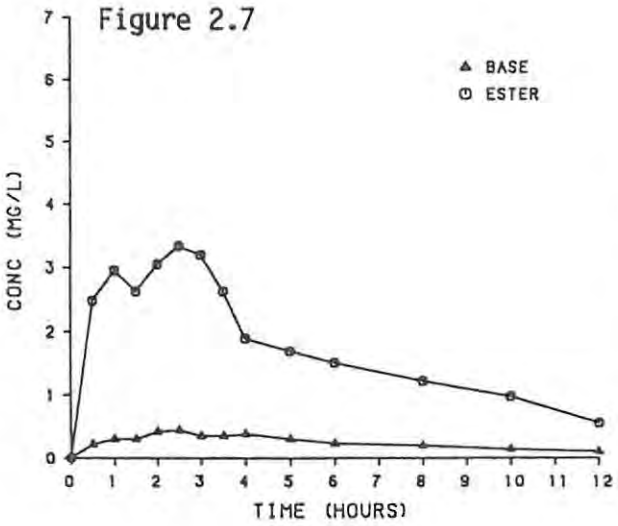


Figure 2.6 Serum concentration-time profile of erythromycin base and propionate - first pilot trial

Total erythromycin concentrations at each sampling time, obtained from the sum of the base concentration and the base equivalent of the propionate concentration were far lower than total concentrations obtained after hydrolysis of the ester component (Chapter 3). This suggested that a significant proportion of the ester in the trial samples, but not in the calibrators was being lost during any one of the procedures employed in the analysis. Similar problems had been experienced by a colleague utilizing a similar extraction procedure for the analysis of josamycin (a macrolide antibiotic) in biological fluids where differences in drug recovery between samples prepared in dialysed and fresh non-dialysed serum occurred (185). Large amounts of josamycin were found in the acetonitrile/water wash from fresh serum samples but not from dialysed serum samples. These losses were decreased by the employment of larger volume water washes prior to the acetonitrile/water wash or by decreasing the eluting power of the acetonitrile/water wash. Similar investigatory procedures on erythromycin propionate in dialysed and non-dialysed serum showed identical results and trends, while erythromycin base and the internal standard were not effected. The volume and eluting strength of the acetonitrile/water wash is vital to the assay as it is this wash that removes the greatest amount of co-eluting compounds and thereby determines the cleanliness of the extract. A 3 ml acetonitrile/water wash was found to be the minimum required for serum extracts while a 20 ml water wash improved recovery in the non-dialysed samples compared to dialysed samples. A possible explanation for this unique phenomenon is that during the dialysis procedure, some buffer salts may be removed from the serum and are therefore not loaded onto the extraction column. During the acetonitrile/water wash these salts, if present, are eluted to form a buffer containing acetonitrile/water mixture which is known to elute macrolide compounds. Increased water washes may remove these salts from the extraction column prior to the acetonitrile/water wash, thereby eliminating the problem.

The final extraction procedure employed (Section 2.3.6) yielded clean sample extracts, allowing the unhindered determination of the compounds of interest (Figs. 2.1 & 2.2). Injection of samples reconstituted in water alone i.e. without a phase separation step, was unsuccessful due to the large number of interfering peaks associated with co-eluting contaminants. The micro liquid-liquid extraction performed during the reconstitution and phase separation step provided a most important and convenient increase in the selectivity of the extraction procedure.



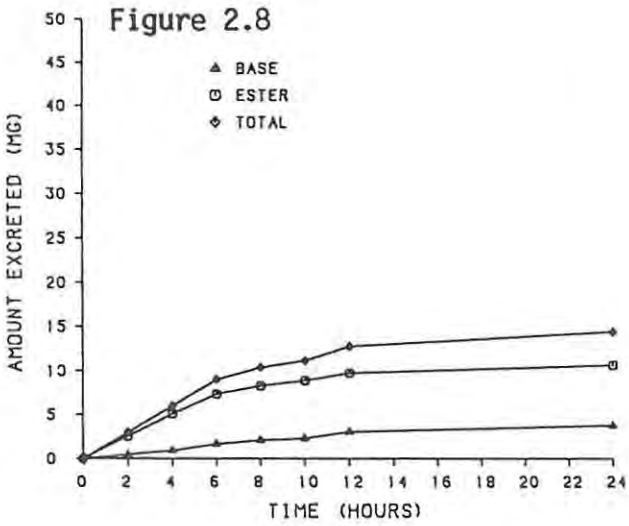
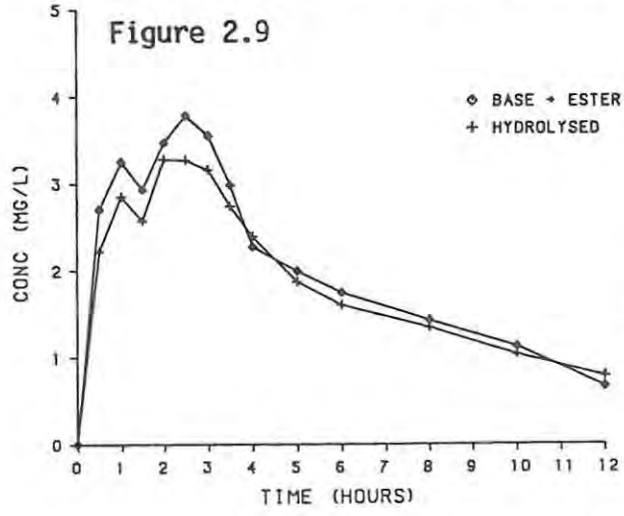


Figure 2.7 Serum concentration-time profile of erythromycin base and propionate - second pilot trial

Figure 2.8 Cumulative urinary excretion profile for erythromycin base and propionate - second pilot trial

Results of the second pilot study showed that the problems initially encountered had been solved and that the levels of erythromycin propionate obtained were similar to those reported in the literature. Figures 2.7 and 2.8 depict serum concentration versus time and cumulative urinary excretion curves respectively, obtained in the final pilot trial. These findings are discussed in detail as part of a more comprehensive experiment (Chapter 6) but show the utility of the method to simultaneously determine concentrations of erythromycin base and propionate in human serum and urine after the oral administration of erythromycin estolate. Total erythromycin concentrations obtained using this discriminatory assay compared well with



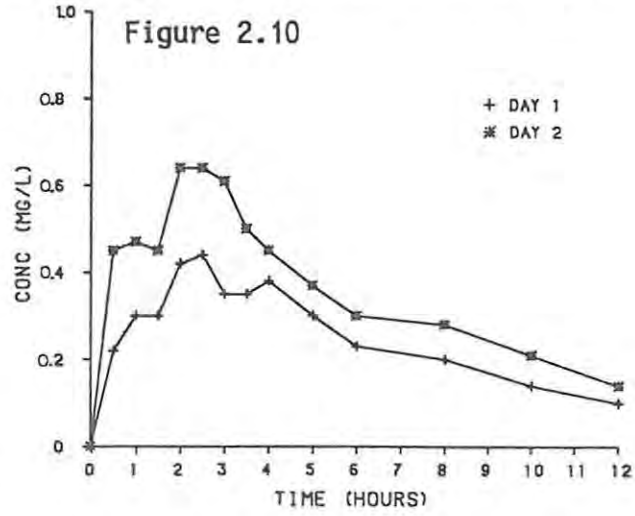


Figure 2.9 Comparison of the discriminatory analytical technique with the method involving prior hydrolysis of serum samples

Figure 2.10 Erythromycin base concentrations in duplicate serum samples (stored at -15°C) analysed 24 hours apart

similar concentrations obtained by analysis of hydrolysed serum samples (Fig. 2.9) indicating that unlike in the previous pilot trial, no major drug losses had occured.

Duplicate serum samples collected during this trial and analysed after 24 hours at -15°C showed increased amounts of erythromycin base over identical samples analysed within 6 hours of sample withdrawal (Fig. 2.10). These results substantiate the findings of previously discussed simulated studies (Section 2.4.3), and further emphasize the importance of specialized sample collection and storage techniques in this analysis.

Table 2.3 Calibration data - erythromycin base and propionate in serum and urine

	Serum	Urine
Concentration Range - Base	0.25 - 4.00 µg/ml	1.00 - 20.00 µg/ml
Concentration Range - Ester	0.50 - 8.00 µg/ml	1.00 - 20.00 µg/ml
Slope - Base	0.3738	0.1862
Slope - Ester	0.1572	0.0649
Intercept - Base	0.0020	0.0212
Intercept - Ester	0.0074	0.0047
Correlation coefficient-Base	0.9999	0.9997
Correlation coefficient-Ester	0.9998	0.9993

The solid-phase extraction procedure was ideally suited to the requirements of this analysis, since many samples could be extracted simultaneously without constant operator attention. Using a system of custom support racks, serum samples from three volunteers a day were analysed in two batches of forty, 6 and 12 hours after drug administration.

2.4.5 LINEARITY, PRECISION AND ACCURACY

Linearity was established for both compounds in serum and urine over the concentration ranges studied. The calibration data are summarized in table 2.3. Due to variations in detector response, new calibration curves were extracted daily which were consistently linear for both drugs with low values for the y-axis intercepts. These calibrators were extracted in two batches, the second being approximately 12 hours after preparation and storage (Section2.3.4), indicating that hydrolysis of the ester during this short storage period was negligible.

Table 2.4 Accuracy and precision - erythromycin base and propionate in serum and urine

	Base added+ (µg/ml)	Base found (µg/ml)	R.S.D.* (%)	Ester added+ (µg/ml)	Ester found (µg/ml)	R.S.D.* (%)
SERUM	0.25	0.29	4.5	0.25	0.27	6.7
	1.00	1.04	1.2	2.00	2.01	3.2
	2.00	2.01	3.4	4.00	4.03	3.8
URINE	1.05	0.98	4.6	0.99	0.93	7.9
	10.48	10.69	3.5	9.88	10.27	7.5
	26.20	26.14	4.9	24.70	24.85	5.3

\+ 6 samples were analysed at each concentration.

* Relative standard deviation

Table 2.4 lists results of the accuracy and precision studies. The low relative standard deviations for both drugs at all concentration levels indicates good system reproducibility while the accuracy of the method was confirmed by the high degree of agreement obtained between spiked and experimentally obtained concentrations.

Preparation and serial dilution of a stock calibrator sample is the generally accepted method of calibrator sample preparation, but was not feasible in this assay due to the possibility of ester hydrolysis during the mixing times required to obtain homogeneous standards. The linear calibration curves and good precision obtained using individual spiking of serum and urine blanks with small volumes of concentrated drug solutions suggested that this method was a most satisfactory substitute.

2.4.6 EXTRACTION EFFICIENCY

Results of recovery studies are summarized in table 2.5. Variations in recovery were observed at the five different concentration levels studied, although no concentration related trends could be detected. The recovery was generally low, especially for erythromycin propionate in serum, but since precision data showed these losses to be highly consistent and the necessary levels of sensitivity could be easily obtained, this was not seen as a major problem. Throughout the development of this assay, a compromise had to be constantly sought between sample recovery and the chromatographic cleanliness of extracts, since attempts at increasing recovery by decreasing the strength or volume of the acetonitrile/water

wash resulted in un-usable sample extracts as previously discussed (Section 2.4.4). Sample losses during the solid-phase extraction alone, were estimated as being 12% for the base and 30% for the ester (0.5 & 5.00 µg/ml (n=4)). Recovery was not increased by further elution of the extraction columns.

The recovery during the phase separation extraction procedure (approximately 80% for both compounds) was not improved by the addition of extra buffer nor by saturation of the aqueous layer with NaCl. Adjustment of the sample pH below 5.0 resulted in degradation of the compounds whilst no changes in recovery were seen at higher pH's. Varying the volume of acetonitrile indicated that addition of 100 µl acetonitrile to the 20 µl aqueous phase proved more successful than addition of a 25 µl aliquot. This proved quite advantageous as the larger final sample volume allowed multiple injections of calibrators.

Table 2.5 Recovery data - erythromycin base and propionate in serum and urine

	Concentration (µg/ml)	n	Base (%)	Ester (%)
SERUM	0.5	6	79.1	59.1
	1.0	6	64.2	68.9
	2.0	6	69.2	50.1
	3.0	6	65.0	52.0
	4.0	6	70.0	49.7
	Mean		69.5±8.5	55.6±15.0
URINE	3.0	5	73.3	55.6
	10.0	5	86.2	63.2
	20.0	5	86.1	68.9
	Mean		81.8±7.4	62.6±6.7

It must also constantly be borne in mind that results from extraction efficiency studies are obtained from adaptations of the original method and may introduce artifactual losses. For example, recovery values obtained in this study were highly dependent on the removal of the entire acetonitrile layer during the adapted phase separation step which was almost impossible due to the small sample volumes employed, making the values obtained, only approximations of true sample losses.

In summary, the HPLC method presented here has the necessary precision, sensitivity and accuracy to allow the simultaneous determination of erythromycin base and its propionyl ester in serum and urine with post-sampling hydrolysis being minimized. It has proved extremely useful for the pharmacokinetic characterization of both compounds after the oral administration of erythromycin estolate to human volunteers.

CHAPTER THREE

DETERMINATION OF TOTAL ERYTHROMYCIN CONCENTRATIONS IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOLLOWING THE ORAL ADMINISTRATION OF ERYTHROMYCIN ESTOLATE

3.1 INTRODUCTION

"Total erythromycin" concentrations in biological samples following the oral administration of erythromycin estolate may either be determined by the summation of erythromycin base and propionate concentrations (Section 2.3.4) or by determination of the erythromycin base concentration following complete hydrolysis of the ester moiety. Both the USP (160) and BP (161) official methods for the analysis of erythromycin estolate raw material and dosage forms, involve hydrolysis of the ester moiety in a buffer solution at 37 °C prior to microbiological analysis. Standard microbiological assay procedures (162-164) have been employed in the majority of published studies regarding the pharmacokinetics of erythromycin following the administration of erythromycin estolate. During the lengthy incubation period associated with these microbiological assay techniques, substantial amounts of the microbiologically inactive propionyl ester undergo hydrolysis to erythromycin base which is detected by its inhibition of the test organism. The extent of this hydrolysis has not been determined, resulting in erythromycin base concentrations obtained utilizing these methods being overestimations of true values. A hydrolysis step similar to that described for *in vitro* samples (160,161) is required prior to microbiological analysis to ensure complete conversion of the inactive ester. Welling *et al* (78) extracted the compounds of interest into an organic solvent prior to dilution and hydrolysis of the dried samples as previously described (160,161) whilst Yakatan *et al* (113) reported complete hydrolysis of erythromycin propionate in human serum after incubation at 60°C for 2 hours.

Prior to the development of the discriminatory assay procedure for erythromycin base and propionate in biological fluids described in the previous chapter, our laboratory was requested to perform numerous comparative bioavailabilty studies involving erythromycin estolate dosage forms. It was therefore decided to adapt a previously described HPLC method for the analysis of erythromycin base in biological fluids (171) to the

analysis of "total erythromycin" concentrations following administration of estolate dosage forms by incorporating a hydrolysis step prior to chromatography.

It is essential that data obtained from the analysis of drugs in biological fluids be subjected to thorough within-run validation processes (186,187). The basic requirements of such validation studies are that the accuracy of the calibration curve be monitored daily by a set of *in vitro* standards and that replicate samples obtained from patients during the study (*in vivo* standards) be re-analysed throughout the duration of the analysis (188). These procedures should be able to detect day-to-day variations in system response as well as degradation of samples on storage.

3.2 EXPERIMENTAL

3.2.1 REAGENTS AND EQUIPMENT

In addition to the reagents and equipment described in sections 2.2.1 and 2.2.3 respectively, a re-circulating waterbath (Colora, West Germany) was employed in all hydrolysis procedures.

3.2.2 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM

The HPLC equipment utilized was identical to that described in section 2.2.2 with the exception that a highly end-capped C_{18} column (Novapak-Waters Associates) was employed.

3.3 METHODOLOGY

3.3.1 CHROMATOGRAPHIC CONDITIONS (SYSTEM B)

The mobile phase consisted of 30% acetonitrile and 70% buffer (B). It was pumped at a flow-rate of 1 ml/min with a resulting pressure of 1500 psi at a column temperature of 35°C. The upstream screen electrode of the electrochemical detector was set at 0.7V and the downstream analytical electrode at 0.9V. The chart speed was set at 5mm/min.

3.3.2 HYDROLYSIS CONDITIONS

One millilitre of acetonitrile was added to 1.0 ml serum followed by vortex mixing and centrifugation at 1600 xg for 5 min in order to de-proteinize the biological material. The supernatant was transferred to a culture tube containing 4.0 ml water and tightly sealed with a teflon-lined screw-cap after gentle vortex mixing. The samples were then incubated at 37°C for 12 hours in a re-circulating water bath. After incubation, the samples were allowed to equilibrate at room temperature prior to addition of 250 µl of the aqueous oleandomycin phosphate (12.0 µg/ml) internal standard solution.

3.3.3 EXTRACTION OF SERUM SAMPLES

Samples were extracted utilizing a similar solid-phase extraction procedure to that described in section 2.3.6. with the following changes:

a) After the load solution had passed through the extraction column, it was washed with 5.0 ml water and 5.0 ml acetonitrile/water (1:1)

b) The elution solvent was a mixture of equal volumes of acetonitrile and buffer (A)
c) Samples were reconstituted with 20 µl water and 25 µl acetonitrile.
d) Aliqouts of between 1 and 5 µl were injected onto the column.

3.3.4 CALIBRATION STANDARDS

Calibration standards containing erythromycin base (0.5-8.0 µg/ml) were prepared by serial dilution of a stock serum sample spiked with erythromycin base. Sufficient quantities of each calibrator were stored at -15°C to enable daily analysis of duplicate samples for at least 10 days. Calibration curves were constructed from plots of the peak height ratio of erythromycin base and the internal standard as previously described (Section 2.3.4).

3.3.5 *IN VITRO* STANDARDS

Four sets of *in vitro* standards were prepared as in section 3.3.4 by an independent analyst to contain concentrations of erythromycin base within the range of the calibration standards. Replicate samples of each standard were analysed daily.

3.3.6 *IN VIVO* STANDARDS

Thirty millilitres of blood was withdrawn from four of the trial subjects at pre-determined sampling times. Serum from each patient was divided into six aliquots and stored at -15°C. These *in vivo* standards were coded by an independent analyst and introduced into the sample pool for analysis on the first and final days of analysis, as well as on three randomly selected interim days. The same analyst was responsible for the collation of all *in vitro* and *in vivo* validation data, and the preparation of a separate quality control report.

3.4 RESULTS AND DISCUSSION

3.4.1 CHROMATOGRAPHY AND EXTRACTION OF SERUM SAMPLES

Erythromycin base and the internal standard were well resolved with retention times of 6.2 and 4.2 min respectively (Fig. 3.1). The coulometric electrochemical detector operated in the oxidative screen mode was both more sensitive and selective than the UV detector utilized in earlier studies (171). Serum extracts were almost totally devoid of interfering compounds and (Fig. 3.1) provided the required sensitivity.

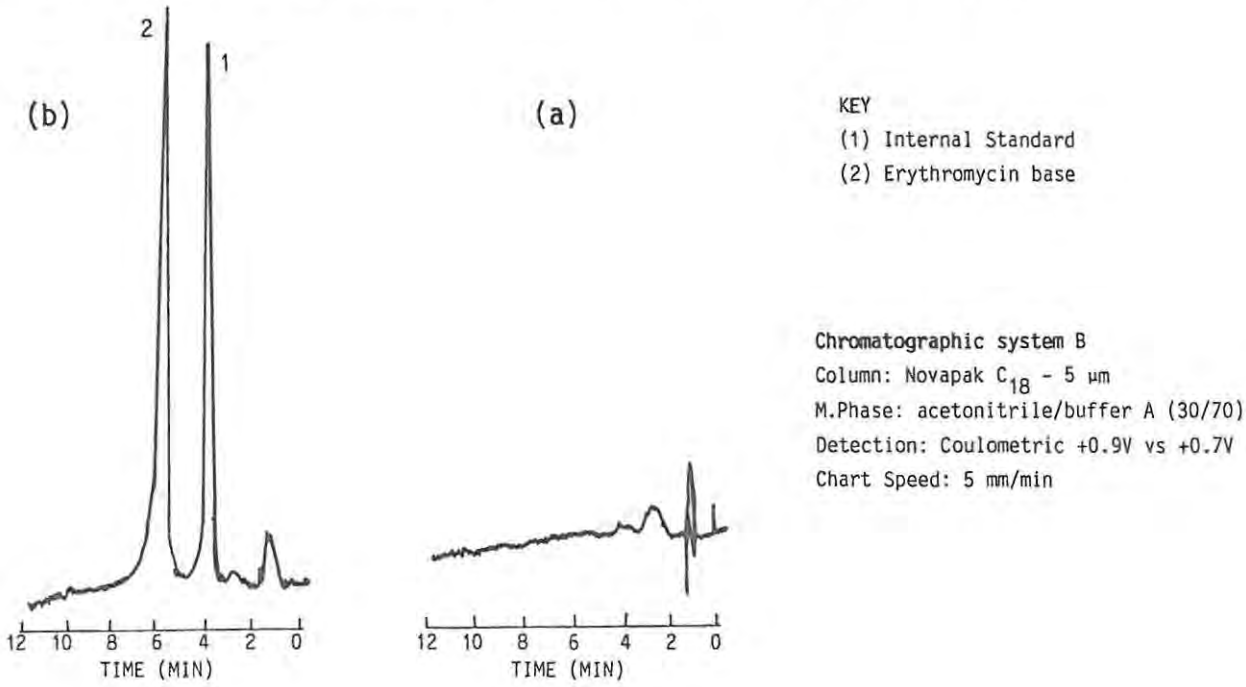


Figure 3.1 (a) Chromatogram of a blank serum extract
(b) Chromatogram of an extract of a hydrolysed serum sample after the oral administration of erythromycin estolate (chromatographic system B)

Slight variations in detector response were experienced during lengthy periods of analysis which were minimized by the recycling of the mobile phase as previously described (Section 2.3.1).

In the early stages of this analysis a commercially available extraction station was employed (Vac-Elut, Analytichem International) which allowed simultaneous extraction of ten samples. A system of custom made support racks and sample reservoirs was later introduced which enabled the simultaneous extraction of 60 samples as described in section 2.4.4. During the scale-up process, problems were encountered with selected samples in which the internal standard was either completely lost or drastically reduced. Various investigations into the cause of this problem, including a detailed study on the flow-rate through each extraction column, proved unsuccessful. It was subsequently noticed that the problem was caused by contamination of the pasteur pipette used to assist in the loading of the acetonitrile/water wash, with small amounts of the original load solution. The presence of minute amounts of endogenous buffer molecules in the acetonitrile/ water wash was presumably sufficient to elute the internal standard. This problem was easily overcome by rinsing the pasteur pipettes prior to addition of the acetonitrile/water wash.

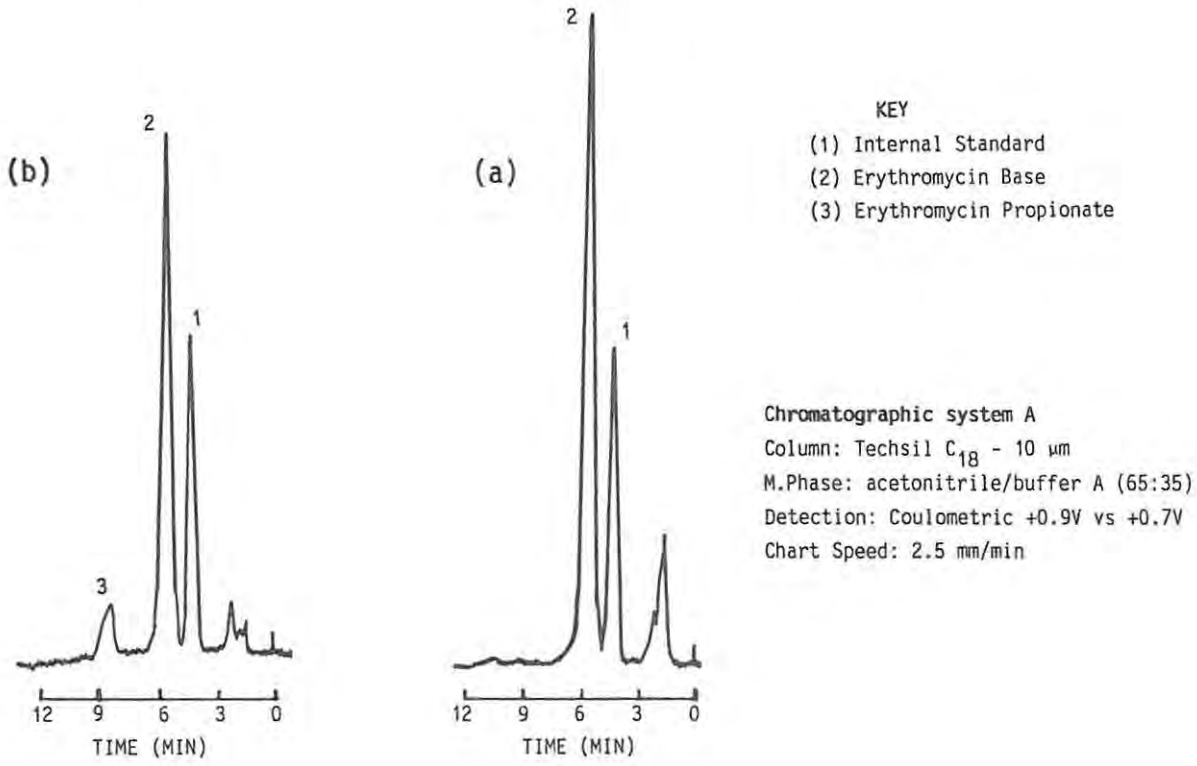


Figure 3.2 Chromatograms of serum samples containing erythromycin propionate following hydrolysis at 37°C for 12 hours
(a) Serum precipitated and diluted with water
(b) Untreated serum (chromatographic system A)

3.4.2 HYDROLYSIS OF SERUM

It was not possible to monitor the degradation of erythromycin propionate utilizing chromatographic system (B) due to the long retention (>25 min) of the ester and its correspondingly poor peak shape. During initial hydrolysis studies, however, chromatographic system (A) was utilized to qualitatively monitor the presence of the ester despite the fact that it was only in the early stages of development. These studies showed that significant amounts of the propionyl ester were still present in both spiked and *in vivo* serum samples which had been incubated for 12 hours at 37°C or for three hours at 55°C (Fig. 3.2). These data showed that incomplete hydrolysis occured during the incubation period of the standard microbiological assay which was in conflict with the results of Yakatan *et al* (113). No erythromycin propionate was present in precipitated and diluted serum samples (Section 3.3.2) which had undergone hydrolysis at 37 °C for 12 hours (Fig. 3.2).

These initial findings were later confirmed in a quantitative study utilizing chromatographic system (B) in which the amount of erythromycin base per sample was compared with calculated values following hydrolysis of serum samples spiked with erythromycin propionate. Serum samples which had been precipitated and diluted (n=6) as previously described, yielded 93.0 % of calculated erythromycin base concentrations following incubation at 37°C for 12 hours. This small discrepancy between observed and calculated concentrations may well be due to the fact that the raw material was not 100% pure, since complete conversion of the ester under identical conditions had been demonstrated (Fig. 3.2). Untreated serum samples yielded only 72.5% of expected erythromycin base concentrations.

3.4.3 VALIDATION OF THE ANALYTICAL METHOD

The results of the within-run quality control procedures recorded during the analysis of serum samples from a comparative bioavailability trial involving two erythromycin capsule formulations are presented as an example of the applicability of the analytical method to the analysis of multiple samples.

Table 3.1 Calibration data - total erythromycin

Day	Date	Regression Equation	Correlation Coefficient
1	23/9/86	y = 0.2042 (x) - 0.0070	0.999
2	24/9/86	y = 0.2165 (x) - 0.0055	0.999
3	25/9/86	y = 0.2348 (x) - 0.0100	0.999
4	26/9/86	y = 0.2286 (x) - 0.0120	0.999
5	30/9/86	y = 0.2024 (x) + 0.0111	0.999
6	1/10/86	y = 0.2054 (x) + 0.0060	0.999
9	13/10/86	y = 0.1987 (x) + 0.0172	0.999
10	14/10/86	y = 0.2133 (x) + 0.0150	0.999

Calibration concentrations

A = 8.90 µg/ml B = 4.05 µg/ml C = 2.02 µg/ml D = 0.81 µg/ml E = 0.41 µg/ml

Table 3.2 *In vitro* quality control - total erythromycin

Actual Concentration of Drug (µg/ml)

Sample 1	Sample 2	Sample 3	Sample 4
0.70	1.40	3.50	7.00

Measured Concentration (µg/ml)

DAY	Sample 1	Sample 2	Sample 3	Sample 4
1	0.72	1.41	3.71	7.40
2	0.72	1.41	3.67	7.27
3	0.72	1.40	3.47	7.08
4	0.75	1.45	3.72	7.45
5	0.74	1.43	3.60	7.31
6	0.70	1.38	3.57	7.13
10	-	1.47	3.82	7.62
No. of analysis	6	7	7	7
Mean	0.73	1.42	3.65	7.32
S.D.	0.02	0.03	0.11	0.19
R.S.D.%	2.4	2.2	3.1	2.6

Table 3.3 *In vivo* quality control - total erythromycin

	Total Erythromycin Concentration (µg/ml)			
Sample Identification	P1S2	P3S6	P4S8	P8S7
As Analysed	1.28	1.23	2.84	3.94
Date				
23/9/86	1.01	0.98	2.83	4.25
30/9/86	1.08	1.18	3.20	-
1/10/86	1.04	1.04	2.75	4.10
Mean	1.10	1.11	2.90	4.09
S.D.	0.12	0.12	0.20	0.15
R.S.D. %	11.0	10.5	6.9	3.8

Table 3.1 lists the calibration data for each day of the analysis. The data appear linear with low y-intercept values and correlation coefficients consistently greater than 0.999. Day-to-day changes in the slope of the calibration curve were encountered which were attributed to varying detector response.

Results of the *in vitro* validation procedure are summarized in table 3.2. The excellent correlation observed beween the calculated and observed erythromycin concentrations of the *in vitro* standards indicated a high degree of accuracy, whilst the low relative standard deviations obtained on repeat analyses of these standards reflected good overall precision, but most importantly, showed that results were consistent over the entire analysis period. Similar results were obtained for the *in vivo* replicate samples (Table 3.3), although a slight decrease in precision was observed. The results of both the *in vitro* and *in vivo* validation studies enabled results obtained over the entire analysis period to be treated with confidence and provided a valuable in-house control of analytical procedures, particularly since the entire validation process was monitored by an independent analyst.

3.4.4 COMPARISON OF RESULTS

Results of a pilot trial have been discussed (Section 2.4.4) in which good correlation was observed between "total erythromycin" concentrations obtained by addition of erythromycin base and ester concentrations (System A) and "total erythromycin" concentrations obtained following hydrolysis of the ester moiety as described in this chapter (system B). Similar comparisons of data recorded during more extensive pharmacokinetic studies (Appendices 2-5) show "total erythromycin" concentrations obtained utilizing the discriminatory assay to be consistently greater than those obtained by prior hydrolysis of the ester moiety. These differences are in the order of 5-10% and are most likely due to differences in the purity of the various raw materials used.

In conclusion, the HPLC method described is highly suited to the routine analysis of erythromycin base in human serum following hydrolysis of the propionyl ester moiety. The hydrolysis conditions ensure total conversion of the ester which does not occur in un-treated serum samples incubated at 37°C for 12 hours, suggesting that incomplete hydrolysis probably occurs during the standard microbiological assay procedure for erythromycin.

CHAPTER FOUR

ASPECTS OF ERYTHROMYCIN STABILITY

4.1 INTRODUCTION

The study of the stability of a drug is generally accomplished by measuring the concentration variation of intact drug over a specific time interval (189). For this purpose, any analytical method capable of selectively determining concentrations of intact drug in the presence of its degradation products may be termed a stability indicating analytical technique. Drug stability may also be studied using the initial rate method which is based on the quantitative analysis of the degradation product or products (190). A major advantage of this method of stability testing is that due to inherent assay precision errors, it is possible to determine small increases in the degradative products far more readily than corresponding decreases in the original drug concentration (189). For these purposes it is obvious that a stability indicating assay must be able to quantitatively determine concentrations of all the degradative products formed during the reaction. In the studies performed in this section, drug stability was assessed by monitoring the concentration of intact drug with time, in addition, the detection of degradative products was used as a further indicator of drug instability.

Erythromycin base has excellent stability in the dry form, as well as in solid dosage forms, while buffered solutions (pH 7.0 - 8.0) show evidence of degradation after 4 days at room temperature and after one week in the refrigerator (6). The acid instability of erythromycin derivatives, and the degradative products formed, have been discussed in section 1.1.2. Numerous chromatographic methods have been previously described which are capable of separating these degradative products *in vitro*, including TLC (191,192), gas liquid chromatographic (193,194) and high-performance liquid chromatographic techniques (168,171-173,195,196). For the purposes of our intended study on the stability of erythromycin base in stored biological samples, an analytical technique was required which was capable of the selective quantitative analysis of erythromycin base and its major acid degradation product (anhydroerythromycin) in biological fluids. The author had previously reported an HPLC method utilizing low wavelength UV monitoring for the analysis of erythromycin base in biological fluids (171)

but had also shown that anhydroerythromycin, which was readily detected by electrochemical methods could not be determined by UV analysis (179).

The HPLC methods described by Chen and Chiou (172) and Duthu (173) for the analysis of erythromycin base in biological fluids both involved the deployment of a sophisticated coulometric electrochemical detector operated in the oxidative screen mode as earlier described in chapter 2. The success of these methods was totally dependent on the screening capabilities of the detector, since only single-step liquid-liquid extractions were performed which resulted in relatively poor sample extracts. The only electrochemical detector available for the first part of this study (Part A) was a simple single-electrode amperometric detector which obviously had no screening facilities and required selective pre-chromatographic extraction procedures for optimum results. Attempts at utilizing previously reported extraction procedures (172,173) were unsuccessful due to excessive sample interference. However, good results were obtained utilizing the highly selective extraction procedure developed for the UV assay (171). Despite decreased selectivity, amperometric detectors are often associated with increased sensitivity due to decreased background interference and are generally easier to maintain. A major disadvantage of the afore- mentioned coulometric detector is that the porous electrodes are prone to blockages and associated increases in back-pressure which result in permanent cell damage, often necessitating replacement of this relatively expensive component.

Two distinctly different aspects of erythromycin stability were addressed in this chapter. Part (A) concerns the stability of erythromycin base in stored biological fluids whilst part (B) is a study of the hydrolysis of erythromycin propionate in spiked human serum and in a variety of aqueous solutions.

No information has been reported relating to the stability of erythromycin base in stored biological samples. This stability was of particular importance to our laboratory since the performance of contractual biopharmaceutical studies usually involved a delay between sample collection and analysis due to a variety of reasons including transport and availability of equipment and personnel.

The hydrolysis of erythromycin propionate to erythromycin base has previously been discussed with respect to the problems associated with the simultaneous analysis of both components in biological fluids (Section 2.1) and in the analysis of total erythromycin concentrations following hydrolysis of serum samples (Section 3.1). Previous attempts at determining the rate of this hydrolysis in whole blood (67,166) and plasma (167,169) have varied in their thoroughness and, with the exception of Croteau *et al* (169) have utilized relatively non-specific analytical techniques. Additional studies were performed utilizing chromatographic system (A) in an attempt to further characterize the rate of this reaction and to confirm previous findings that hydrolysis of untreated serum for 12 hours at 37°C does not result in complete conversion of the ester fraction (Section 3.4.2).

Erythromycin estolate raw material is available from various suppliers and differences in crystal structure, particle size and dissolution characteristics have been reported (Section 1.1.3). However, no studies have been performed in which the *in vivo* and *in vitro* rates of hydrolysis of these esters have been compared, which may be of particular importance, since the microbiological activity of the ester derivatives is dependent on this hydrolysis.

PART A: THE STABILITY OF ERYTHROMYCIN BASE IN STORED BIOLOGICAL FLUIDS

4.2 EXPERIMENTAL

4.2.1 REAGENTS

All reagents and equipment utilized were identical to those described in section 2.2.1 and 2.2.3.

4.2.2 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM

The same liquid chromatograph described in section 3.2.2 was utilized with the exception that the coulometric detector was replaced with a Metrohm model 656 amperometric electrochemical detector fitted with a glassy carbon working electrode used in conjunction with a model 641 VA current/voltage measuring device (Metrohm Ltd., Switzerland).

4.3 METHODOLOGY

4.3.1 CHROMATOGRAPHIC CONDITIONS (SYSTEM C)

The working electrode potential of the amperometric detector was set at 1.15V using a silver/silver chloride reference electrode. All other chromatographic conditions were the same as in section 3.3.1.

4.3.2 CALIBRATION STANDARDS AND EXTRACTION OF BIOLOGICAL SAMPLES

Calibration standards were prepared by serial dilution of a stock solution of either serum or urine spiked with erythromycin base. Serum samples were prepared to contain 0.50, 1.00, 2.00, 3.00 and 5.00 µg/ml erythromycin base while urine calibrators contained 1.25, 2.50, 5.00, 15.00 and 25.00 µg/ml. Anhydroerythromycin calibrators were similarly prepared to contain 0.30, 3.00 and 6.00 µg/ml in serum and 3.00, 6.00 and 12.00 µg/ml in urine.

All samples were extracted utilizing the solid-phase extraction procedure described in section 3.3.3 with oleandomycin phosphate as the internal standard.

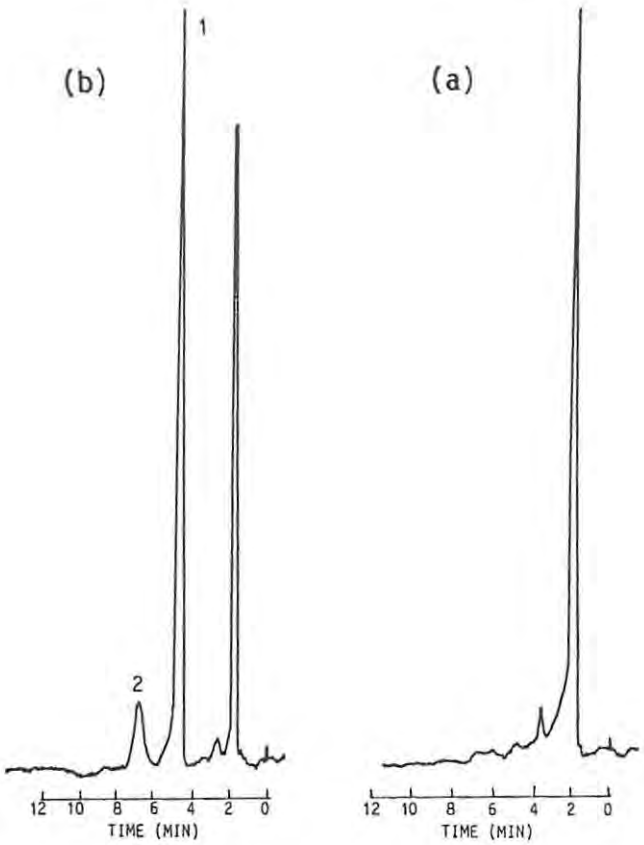
4.3.3 PREPARATION OF STORED SAMPLES

Blank human dialysed serum (pH 7.2) was spiked with erythromycin base to yield final concentrations of 1.00 and 3.00 µg/ml whilst blank urine (pH 5.6) was spiked to contain 3.00 and 15.00 µg/ml. The samples were separated into 10.0 ml aliquots and stored at 4°C and -15°C. Triplicate samples from each batch were analysed after 1, 2, 4, 8 and 12 weeks.

4.4 RESULTS AND DISCUSSION

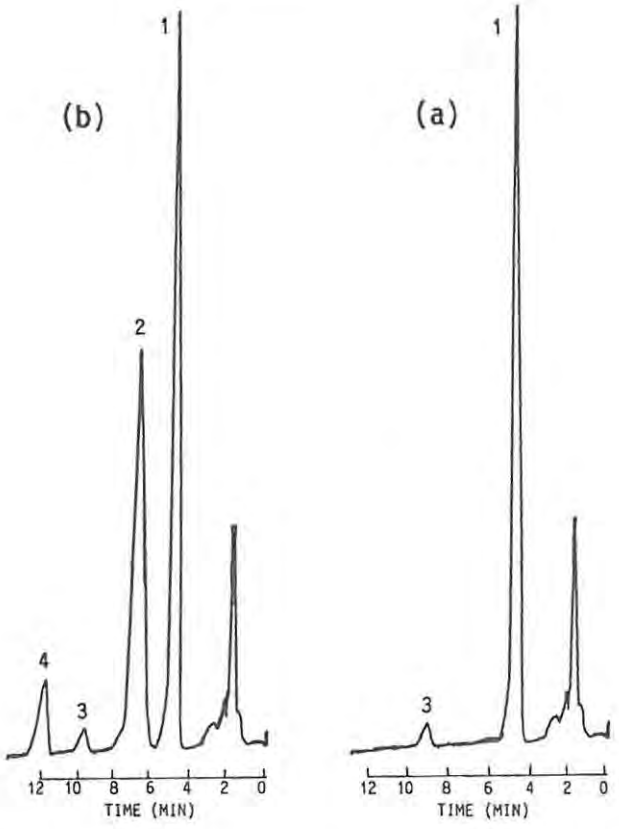
4.4.1 CHROMATOGRAPHY

Under the conditions of this assay, a working electrode potential of 1.15 V provided the best compromise between detector response and background noise, enabling the detection of low concentrations of both erythromycin base and anhydroerythromycin. Despite the normally large interferences associated with the use of high working electrode potentials, the solid-phase extraction procedure provided sufficiently clean extracts for the quantitative analysis of erythromycin in serum and urine over the required



Chromatographic system C
Column: Novapak C_{18} - 5 µm
M.Phase: acetonitrile/buffer A (30/70)
Detection: Amperometric +1.15V
Chart Speed: 5mm/min

Figure 4.1 (a) Chromatogram of a blank serum extract
(b) Chromatogram of an extract of a serum sample containing erythromycin base (1.0 µg/ml) stored for 8 weeks at 4°C (chromatographic system C)



KEY
(1) Internal Standard
(2) Erythromycin base
(3) Unidentified
(4) Anhydroerythromycin

Figure 4.2 (a) Chromatogram of a blank urine extract
(b) Chromatogram of an extract of a urine sample containing erythromycin base (15.0 µg/ml) stored for 8 weeks at -15°C (chromatographic system C)

concentration range. Figure 4.1 (A) depicts a blank serum extract while figure 4.1 (B) shows a typical chromatogram obtained for erythromycin base and the internal standard following extraction of a serum sample containing 1.00 µg/ml erythromycin base stored at 4°C for 8 weeks. Chromatograms of a blank urine extract and a urine sample (15.00 µg/ml) stored at -15°C for 8 weeks are depicted in figures 4.2 (A) and (B) respectively. The blank extract contains a resolved unidentified peak while the stored erythromycin sample contains a peak which corresponds with the retention time of anhydroerythromycin (11.4 min) in addition to the drug and internal standard peaks.

As previously discussed (Section 2.4.2), it is difficult to define the sensitivity limits of electrochemical systems, however, based on a minimum signal-to-noise ratio of 3, the limit of sensitivity throughout most of this study was approximately 0.1 µg/ml erythromycin base in serum. Previous attempts at utilizing the amperometric detector for the analysis of erythromycin base and propionate (System A) were unsuccessful due to a lack of sensitivity using the mobile phase required for the separation of these two compounds on the 10 µm Techsil C_{18} column. The good sensitivity obtained utilizing the Novapak column (System C) with a mobile phase containing a larger buffer component (70% for System C versus 35% for system A) suggests that the amperometric detector is more dependent on its ionic environment than the coulometric detector.

4.4.2 VALIDATION OF THE AMPEROMETRIC METHOD

Calibration plots determined with 5 different concentrations of erythromycin base were linear over the ranges studied. The calibration line in serum prepared as in section 4.3.1.2 had a slope of 0.227 and a y-intercept of 0.004 with a correlation coefficient of 0.999 whereas the urine line had a slope of 0.099 and a y-intercept of -0.0373 with a correlation coefficient of 0.999. Three point standard curves for anhydroerythromycin in serum and urine showed good linearity with slopes of 0.245 and 0.128 and y-intercepts of -0.019 and 0.055 respectively. Correlation coefficients for serum and urine were both 0.999.

Within-run precision was assessed by extracting six spiked serum and urine samples each at the upper and lower limits of the concentration ranges studied and three spiked samples at the intermediate concentrations used to generate the calibration line. Intra-assay relative standard deviations

ranged from 1-6% for both serum and urine (Table 4.1). Similar experiments were performed for anhydroerythromycin using the samples analysed to generate the regression lines in both serum and urine with relative standard deviations in the range of 1-5% (Table 4.2).

Table 4.1 Precision data - erythromycin base in serum and urine

SERUM			URINE		
Erythromycin Conc.µg/ml	No. of Samples	R.S.D. %	Erythromycin Conc.µg/ml	No. of Samples	R.S.D. %
0.50	6	6.0	1.25	6	3.5
1.00	3	5.1	2.50	3	5.0
2.00	3	4.2	5.00	3	5.7
3.00	3	4.6	15.00	3	2.7
5.00	6	1.6	25.00	6	2.8

Table 4.2 Precision data-anhydroerythromycin in serum and urine

SERUM			URINE		
Anhydro-erythromycin Conc.µg/ml	No. of Samples	R.S.D. %	Anhydro-erythromycin Conc.µg/ml	No. of Samples	R.S.D. %
0.30	3	1.7	3.00	3	4.5
3.00	3	4.4	6.00	3	5.3
6.00	3	2.2	12.00	3	4.8

4.4.3 STABILITY OF SAMPLES ON STORAGE

The results of the stability study described in section 4.3.3 are summarized in table 4.3. Serum samples appeared to be stable when stored at both 4°C and -15°C for up to 12 weeks with no evidence of anhydroerythromycin formation. However, erythromycin was found to degrade in urine samples after one week at 4 °C with significant amounts of anhydroerythromycin being detected, while at -15°C samples showed some evidence of degradation after 2 weeks with significant degradation occuring after 4 weeks (Table 4.3). It was not possible to explain the finding that urine samples (15.0 µg/ml) stored at -15°C for 8 weeks contained more intact erythromycin base with correspondingly less anhydroerythromycin than similar samples stored for 8 weeks at the same temperature. Degradation of erythromycin base was more rapid in urine than in serum which was most probably due to the lower pH of the urine or may be related to the fact that a significant proportion of the drug would be bound to serum protein and hence less susceptible to chemical degradation. Long term storage of

urine samples containing erythromycin base may be facilitated by adjustment of sample pH towards neutrality and/or by stabilization with toluene (197).

In summary, the chromatographic system described was highly suited to the requirements of the stability study and helped to provide valuable information regarding the long term stability of erythromycin in stored biological samples. The utilization of the simple single electrode amperometric detector eliminates the need for more costly and maintenance intensive coulometric detectors and makes erythromycin analysis in biological fluids readily accessible.

Table 4.3 Long term storage of erythromycin base in spiked serum and urine

		Concentration of Erythromycin base (µg/ml)				
		1 week	2 weeks	4 weeks	8 weeks	12 weeks
Serum 4°C	1.00	1.25	1.04	0.99	1.08	0.97
	3.00	3.90	3.32	3.43	3.23	2.86
Serum -15°C	1.00	1.15	1.05	1.13	1.20	1.04
	3.00	3.46	3.06	3.00	3.56	3.10
Urine 4°C	3.00	1.99+	1.50++	0.99++	1.00++	0.73++
	15.00	12.41+	11.53++	9.20++	4.90++	3.41++
Urine -15°C	3.00	2.61	3.16+	2.15++	2.10++	1.90++
	15.00	14.02	16.60+	11.75++	15.05+	6.51++

KEY: + Some evidence of Anhydroerythromycin
++ Large amounts of Anhydroerythromycin

PART B: HYDROLYSIS OF ERYTHROMYCIN PROPIONATE AND ESTOLATE

4.5 METHODOLOGY

4.5.1 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Chromatographic system (A) was utilized for the simultaneous determination of erythromycin base and propionate in both serum and *in vitro* samples.

4.5.2 HYDROLYSIS OF ERYTHROMYCIN PROPIONATE IN SERUM

Serum samples were extracted as described in section 2.3.6 prior to chromatography. Seventy millilitres of blank human serum was spiked with sufficient erythromycin propionate to yield a final concentration of 3.5 µg/ml and gently inverted for 5 min. Six 1 ml samples were removed and immediately snap frozen in liquid air and stored at -15°C, whilst the

remaining serum was divided into 1 ml aliquots prior to hydrolysis at either room temperature (17-19°C), 37°C or 55°C in suitably equilibrated water-baths. Triplicate samples were withdrawn from the 37°C water-bath after 1, 2, 4, 6, 8, 10 and 12 hours, from the 55°C water-bath after 1, 2, 3 and 4 hours and from the room temperature bath after 1, 2, 4, 8 and 12 hours. Immediately following withdrawal from the water-baths, the samples were snap frozen and stored at -15°C for a maximum of six hours. A calibration curve was prepared as in section 2.3.4 over the concentration range 0.50 to 4.00 µg/ml for both erythromycin base and propionate. The mean erythromycin propionate concentration obtained for the six zero-time samples served as a 100% intact ester reference value. From these data, plots of the percentage intact ester versus time were constructed and the pseudo-first order rate constants for the hydrolysis obtained from the slope of the respective semi-log plots using a linear regression programme on an Apple II Europlus desk-top computer.

4.5.3 *IN VITRO* HYDROLYSIS OF ERYTHROMYCIN ESTERS

4.5.3.1 GENERAL METHODOLOGY

Approximately 10 mg of drug was added to a 10 ml volumetric flask and made up to volume with acetonitrile. After brief vortex mixing, 1 ml of this standard solution was transferred to a 20 ml volumetric flask containing the relevant solvent. The contents of the volumetric flask were transferred to a 50 ml boiler tube which was immersed in a water-bath set at the required temperature. Fifty microlitre aliqouts were removed at the specified time intervals and immediately transferred to a WISP limited volume insert prior to injection of a 10 µl aliquot. All experiments were performed in duplicate.

Buffer B (pH 7.2) was prepared as previously described (Section 2.3.1).

4.5.3.2 COMPARISON OF ESTERS

Erythromycin estolate powder obtained from four different manufacturers and one erythromycin propionate sample, were subjected to hydrolysis at 37°C in both buffer (B) and deionized water. Erythromycin estolate samples were obtained from the USP Authorities, Adcock-Ingram S.A., Eli-Lilly S.A and Lennon Ltd. S.A. The erythromycin propionate sample was obtained from Eli-Lilly Laboratories U.S.A.

4.5.3.3 EFFECT OF TEMPERATURE

Solutions of U.S.P erythromycin estolate were prepared in buffer (B) and were subjected to hydrolysis at room temperature (17-19°C), 37°C, 45°C and 55°C. Ester hydrolysis at room temperature was monitored hourly for 15 hours whereas heated samples were analysed at intervals of between 15 and 20 minutes.

4.5.3.4 EFFECT OF ADDITIVES

Hydrolysis of the USP estolate sample at 37°C was repeated in solutions of buffer (B) containing 5%, 10% and 20% acetonitrile. Hydrolysis of the propionyl ester sample was performed in water at 37°C both with and without the addition of 50% acetonitrile or 0.25% sodium lauryl sulphate, a commonly used wetting agent.

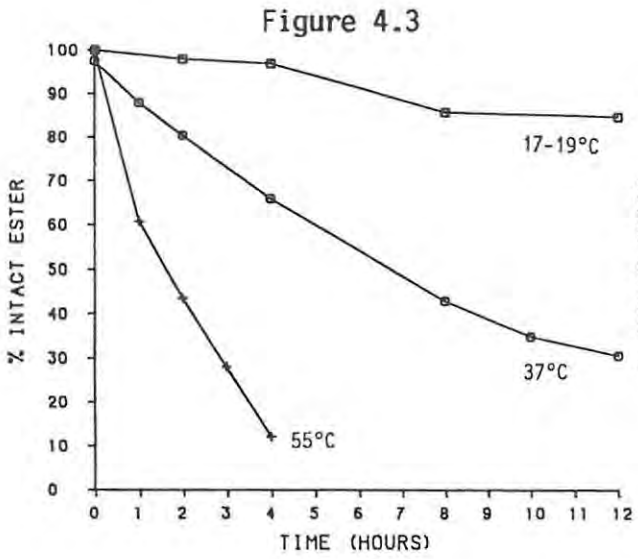
4.6 RESULTS AND DISCUSSION

4.6.1 HYDROLYSIS OF ERYTHROMYCIN PROPIONATE IN SERUM

The hydrolysis of erythromycin propionate in spiked human serum was rapid at both 37°C and 55°C (Fig. 4.3) with degradation half-lives of 7.05 and 1.38 hours respectively, while at room temperature, the hydrolysis appeared slower than in earlier reported studies (Section 2.4.3). Semi-log plots of the data summarized in figure 4.3 yielded straight lines suggesting that the hydrolysis of erythromycin propionate in serum was a pseudo first-order process. Similar results were reported by Tserng and Wagner for the hydrolysis of erythromycin propionate in whole blood (166).

Temperature		k (h^{-1})	$t_{½}$ (h)	Correlation
37°C	Serum	0.098	7.05	0.964
	Buffer	0.020	0.57	0.999
55°C	Serum	0.502	1.38	0.971
	Buffer	0.091	0.13	0.999
Room (17-19°C)	Serum	0.016	44.14	0.893
	Buffer	0.004	2.83	0.999

Table 4.4 Hydrolysis of erythromycin propionate in serum and buffer (B) - effect of temperature



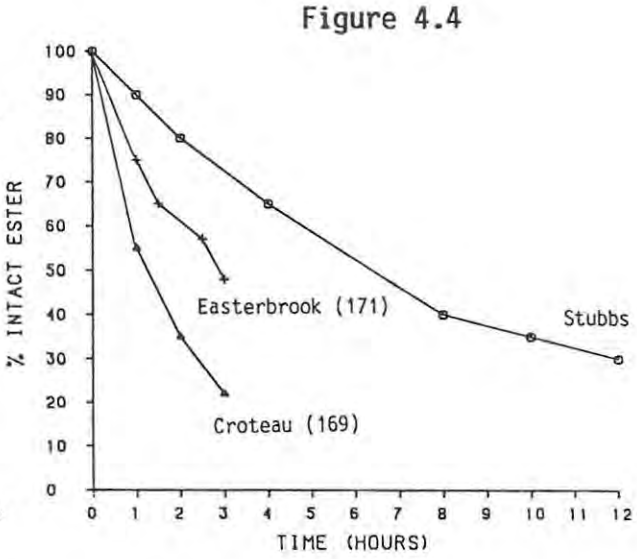
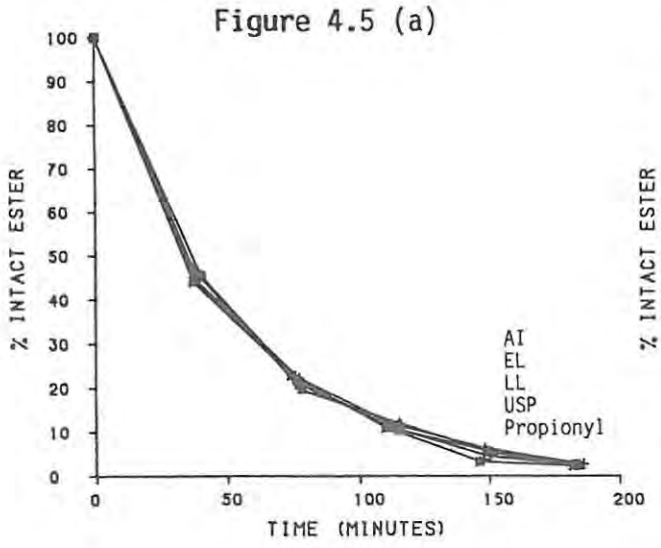


Figure 4.3 Hydrolysis of erythromycin propionate in serum - effect of temperature

Figure 4.4 Hydrolysis of erythromycin esters in serum at 37°C - comparison with published studies



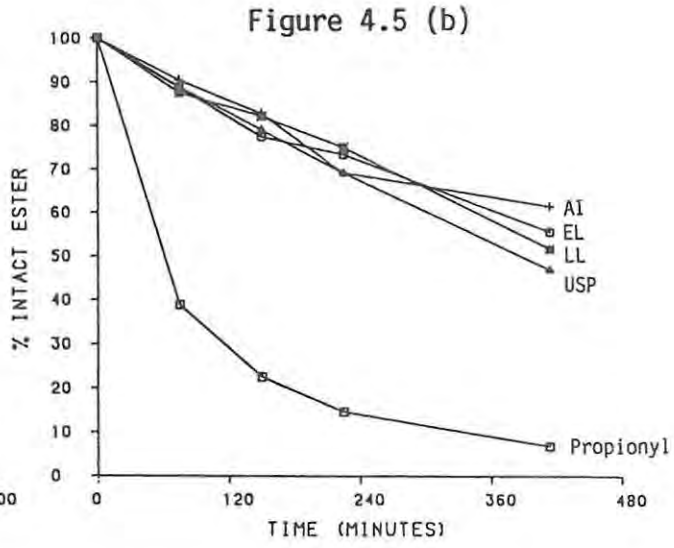


Figure 4.5 *In vitro* hydrolysis of erythromycin esters at 37°C
(a) Hydrolysis in buffer (B)
(b) Hydrolysis in water

Table 4.4 clearly indicates that as previously reported, hydrolysis of the propionyl ester was slower in serum than in buffer solutions, presumably due to unreactivity of the bound drug fraction (67,167).

Figure 4.3 shows that total conversion of erythromycin propionate to erythromycin base does not occur in human serum after 12 hours at 37°C or after 2 hours at 55°C, confirming initial observations reported in section 3.4.2. Correlation between the rate of hydrolysis of erythromycin propionate in serum calculated in this study, with previously documented studies (167,169) involving erythromycin estolate hydrolysis in human plasma was poor (Table 4.6 & Fig. 4.4). It is unclear why these workers (167,169) used erythromycin estolate in their studies, since erythromycin propionate and not erythromycin estolate, is the circulating chemical moiety *in vivo*. Other possible reasons for these discrepancies include differences between serum and plasma samples and varying degrees of hydrolysis during sample handling and analysis.

4.6.2 *IN VITRO* HYDROLYSIS OF ERYTHROMYCIN PROPIONATE AND ESTOLATE

The four different erythromycin estolate samples and the one erythromycin propionate sample, were shown to have almost identical rates of hydrolysis in buffer (B) at 37°C (Fig. 4.5 (A) & Table 4.5).

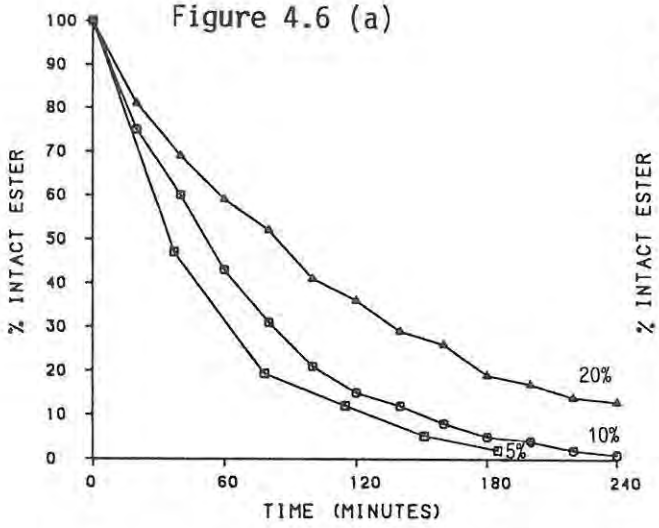
Table 4.5 Hydrolysis of erythromycin esters in buffer (B) and water at 37°C

Ester	Buffer			Water		
	k (min^{-1})	$t_{½}$ (min)	Correlation	k (min^{-1})	$t_{½}$ (min)	Correlation
USP	0.020	34.5	0.995	0.002	378.7	0.995
Propionyl	0.019	35.3	0.999	0.006	113.6	0.939
A.Ingram (AI)	0.019	36.5	0.998	0.001	577.5	0.952
Ilosone (EL)	0.021	33.4	0.999	0.001	489.7	0.992
Lennon (LL)	0.022	31.8	0.998	0.002	450.0	0.978

k = Pseudo-first order rate constant, obtained from the slope of the log-linear plot.

$t_{½}$ = Hydrolysis half-life.

Correlation = Correlation of data with linear regression line obtained from log-linear plot.



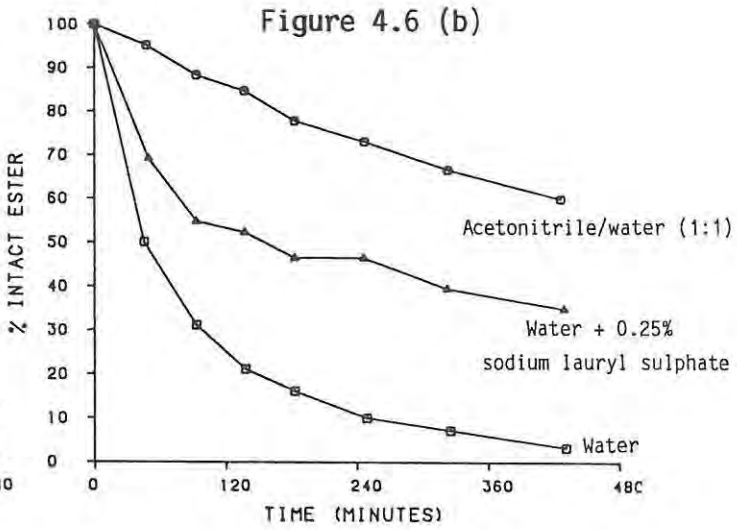
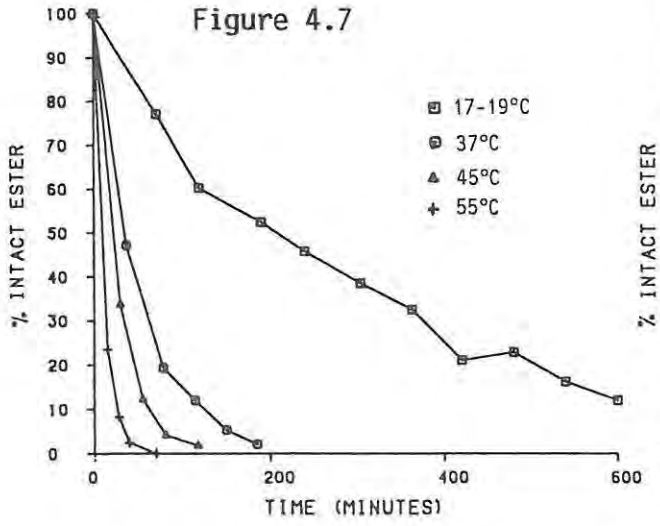


Figure 4.6 (a) Hydrolysis of erythromycin propionate in buffer (B) - effect of acetonitrile

(b) Hydrolysis of erythromycin propionate in water - effect of acetonitrile and sodium lauryl sulphate



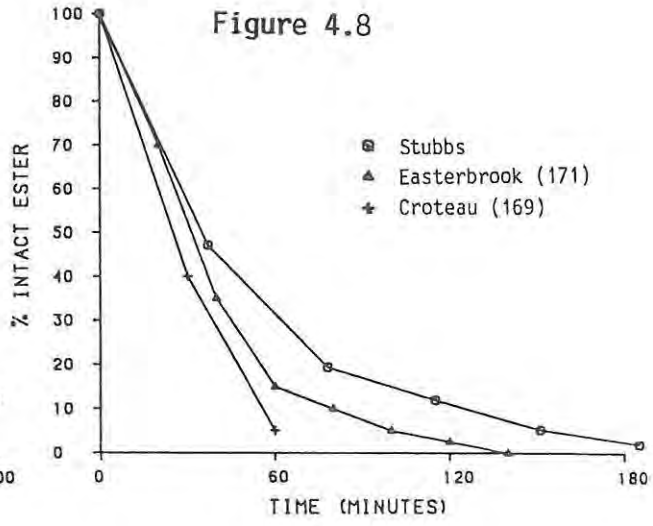


Figure 4.7 Hydrolysis of erythromycin estolate in buffer (B) - effect of temperature

Figure 4.8 Hydrolysis of erythromycin estolate in buffer (B) - comparison with published studies

An interesting extension of this work would be to perform similar studies in spiked human serum or serum obtained from human volunteers having been administered different erythromycin estolate formulations. Similar experiments in water resulted in the four estolate products having decreased but similar hydrolysis rates which were all significantly slower than the erythromycin propionate sample (Fig. 4.5 (B) & Table 4.5). Hydrolysis of the propionate sample in water was retarded by the addition of 0.25% sodium lauryl sulphate (Fig. 4.6 (B)) which may explain the slower dissolution rate of the estolate samples, since this same compound is released into the reactant mixture on dissolution of the estolate products (Section 1.1.3). Addition of varying amounts of acetonitrile decreased the hydrolysis of erythromycin propionate in buffer solution (Fig. 4.6 (A)) suggesting that an acetonitrile/water mixture containing sodium lauryl sulphate may suppress hydrolysis sufficiently to enable dissolution studies to be performed on erythromycin estolate products without the complications caused by ester hydrolysis during the dissolution procedure itself, or during storage of samples prior to analysis.

Table 4.6 Hydrolysis of erythromycin esters in serum and buffer - comparison with published results

		k (min^{-1})	$t_{1/2}$ (min)	Correlation
Buffer	Stubbs	0.021	33.7	0.999
	Easterbrook (167)	0.031	22.1	0.994
	Stephens (169)	0.049	13.9	0.955
Serum Plasma	Stubbs	0.098	423.0	0.9664
	Easterbrook (167)	0.008	83.4	-
	Croteau (169)	0.004	180.9	-

As expected, the rate of ester hydrolysis in buffer solution increased with an increase in temperature (Figure 4.7). Hydrolysis of erythromycin estolate USP in buffer at 37°C was slower than previously reported (Table 4.6), presumably due to differences in solvent composition and other experimental variables.

CHAPTER FIVE

ANALYSIS OF ERYTHROMYCIN ESTOLATE DOSAGE FORMS

5.1 INTRODUCTION

In order to estimate the within-batch variation of tablets used in pharmacokinetic trials as well as to analyse formulations prior to comparative bioavailability studies, it was necessary to develop a reproducible and accurate analytical method for the analysis of erythromycin estolate in various dosage forms. Both the USP (160) and BP (161) official assays involve hydrolysis of the ester moiety with subsequent microbiological determination of the resultant erythromycin base. These methods determine the total erythromycin content of the dosage form as the erythromycin base equivalent, but cannot differentiate between amounts of erythromycin base or estolate. Further qualitative investigations utilizing paper chromatography (161) or infrared spectroscopy (160) are required in order to differentiate between these compounds. As discussed in the previous chapter, selective analytical procedures are essential in the study of product stability.

Several paper and thin-layer chromatographic methods are available for the separation of erythromycin base and estolate (6,198-201), none of which are suitable for routine quantitative analysis. A gas chromatographic method has been reported for the selective quantitative analysis of erythromycin estolate in pharmaceutical dosage forms, however, a 24 hour derivatization reaction is required prior to analysis, making routine analysis extremely tedious and time consuming (194). The HPLC method of Tsuji and Kane (196) is the most suitable reported technique for the analysis of erythromycin and its derivatives *in vitro*. The analysis of erythromycin base in solid dosage forms was described utilizing a C_{18} reversed-phase column with UV detection at 215 nm which correlated well with results obtained utilizing microbiological assay techniques. The major problem with this method is that in order to attain suitable resolution and peak shape, the column was heated to 70°C which resulted in rapid column deterioration. A retention time of 12 min was quoted for erythromycin estolate utilizing a modified mobile phase to that utilized for erythromycin base analysis, but no mention was made of the analysis of erythromycin estolate in dosage forms.

The HPLC system described in chapter 2 (System A) fulfilled our basic requirements and was therefore adapted to the specific needs of the analysis. A UV detector was incorporated into the system immediately after the electrochemical detector since UV detectors appear to be more commonly used in routine analysis, presumably due to the large day-to-day variations associated with electrochemical detectors.

5.2 EXPERIMENTAL

5.2.1 REAGENTS AND DOSAGE FORMS

All reagents and equipment were the same as previously described (Sections 2.2.1 and 2.2.3). Erythromycin estolate raw material was obtained from the same manufacturers as recorded in section 4.5.3.2 while erythromycin estolate dosage forms were obtained from the following sources:

Eli-Lilly SA	Ilosone 500 mg tablets	EL 1
	Ilosone 250 mg capsules	EL 2
	Ilosone 125 mg/5ml suspension	EL 3
Adcock-Ingram SA	500 mg tablets	EL 1
	250 mg capsules	EL 2
Lennon SA	500 mg tablets	LL 1
	250 mg capsules	LL 2
	250 mg capsules	LL 3
	125 mg/5ml suspension	LL 4
	125 mg/5ml suspension	LL 5
	125 mg/5ml suspension	LL 6
	250 mg/5ml suspension	LL 7
	250 mg/5ml suspension	LL 8
	250 mg/5ml suspension	LL 9
Geo Schwulst SA	125 mg/5ml suspension	GS 1

5.2.2 HIGH-PERFORMANCE LIQUID CHROMATOGRAPH

The high-performance liquid chromatograph described in section 2.2.2 was utilized throughout this study. In addition, a Kratos Instruments Model 769 variable wavelength UV detector was placed in-line immediately after the electrochemical detector.

5.3 METHODOLOGY

5.3.1 CHROMATOGRAPHIC CONDITIONS

The high-performance liquid chromatograph was operated under the same chromatographic conditions as described in section 2.3.2 (System A). The UV detector was set at a wavelength of 200 nm, a variable time constant of 1 second and at sensitivity settings of between 0.01 and 0.05 absorbance units.

5.3.2 ANALYSIS OF TABLETS

Intact tablets were placed in a 200 ml volumetric flask to which 100 ml deionized water was added. After shaking for 15 min on a mechanical shaker, 50 ml acetonitrile was added, followed by further shaking for 10 min. This process was repeated following addition of a second 50 ml acetonitrile aliquot after which the sample was made up to volume with water. The flask was then sonicated for 5 min to facilitate dissolution and allowed to equilibrate at room temperature. One millilitre was accurately removed using a glass pipette and transferred to a 10.0 ml volumetric flask containing 1.0 ml of the internal standard solution (oleandomycin phosphate (2.0 mg/ml) in acetonitrile/water 1:1) and made up to volume with acetonitrile. Duplicate samples were obtained by repeating this step. After gentle mixing, approximately 3.0 ml was transferred to a teflon centrifuge tube and spun at 12 000 rpm in a high speed micro-centrifuge for 6 min in order to precipitate any insoluble adjuvants. Sufficient sample was transferred to a WISP limited volume insert prior to injection of 1-3 µl aliquots.

Six tablets from each manufacturer were analysed in duplicate by comparison of their peak height ratios with those obtained from a set of calibration standards prepared as in section 5.3.5. These calculations yielded the amount of erythromycin estolate per tablet, which was then converted to the erythromycin base equivalent by a molecular mass correction constant of 0.6949.

5.3.3 ANALYSIS OF CAPSULES

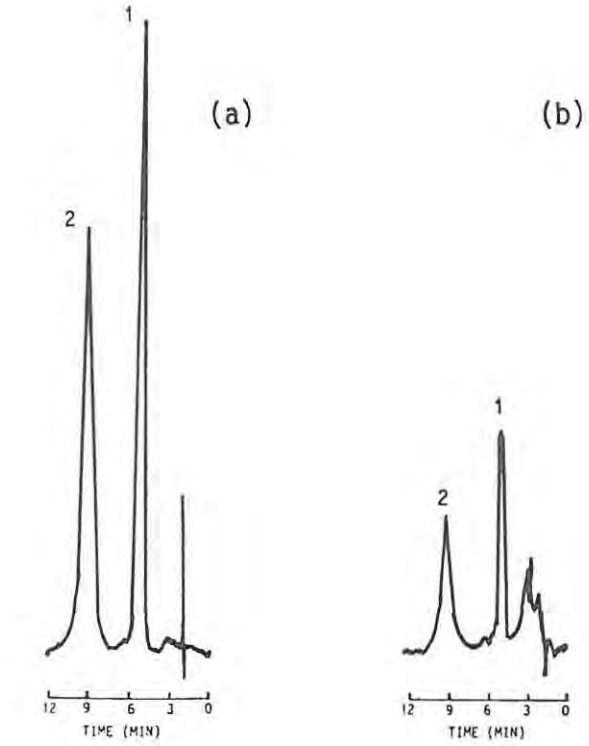
The mass of the capsule contents was obtained by subtraction of the mass of the capsule shell from that of the filled original capsule. Approximately 5.0 mg was accurately determined and transferred to a 10.0 ml volumetric flask to which 1 ml internal standard solution (Section 5.3.2) and 5 ml acetonitrile was added. After vortex mixing for 1 min, the sample was made up to volume with acetonitrile, sonicated for 5 min and allowed to equilibrate at room temperature. Samples were centrifuged , injected and the results calculated as described in section 5.3.2. Six samples from each product were analysed.

5.3.4 ANALYSIS OF SUSPENSIONS

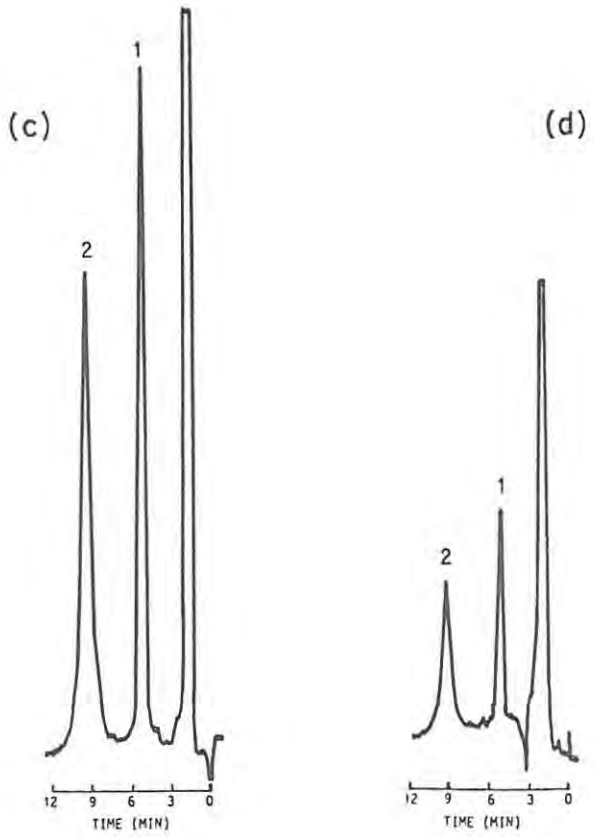
In order to overcome the problems associated with the transfer of small volumes of supensions, the density of each suspension was calculated in triplicate by determining the mass of a 20.0 ml sample. Approximately 100 µl of the 125 mg/5ml suspensions or 50 µl of the 250 mg/5ml suspensions were transferred to a 10.0 ml volumetric flask and the mass of the transferred sample accurately determined. One millilitre of internal standard solution (Section 5.3.2) and 4.0 ml water were added, the sample vortex mixed (1 min) and made up to volume with acetonitrile and treated as described for the analysis of capsules (Section 5.3.3). The volume of the original sample was calculated utilizing the pre-determined density value and the results expressed as milligrams erythromycin base equivalent per 5 ml.

The product GS 1 required reconstitution prior to analysis which was performed according to the label instructions. All products were well shaken prior to analysis in triplicate.

Figure 5.1 Chromatograms of erythromycin estolate dosage forms (chromatographic system A)
(a) Tablet EL1 - electrochemical detection
(b) Tablet EL1 - UV detection
(c) Suspension GS1 - electrochemical detection
(d) Suspension GS1 - UV detection



KEY
(1) Internal Standard
(2) Erythromycin Estolate



Chromatographic system A
Column: Techsil C_{18} - 10 µm
M.Phase: acetonitrile/buffer A (65:35)
Detection: Coulometric +0.9V vs +0.7V
U.V 200 nm
Chart Speed: 2.5 mm/min

5.3.5 VALIDATION OF ASSAY PROCEDURES

Linearity was assessed by preparation of four erythromycin estolate USP standards in acetonitrile to contain approximately 80-120 % of the label claim of the products being analysed. One millilitre of internal standard solution was added, the samples made up to volume with acetonitrile and chromatographed as previously described. Calibration curves were obtained by plotting the peak height ratios of erythromycin estolate and internal standard versus the respective calibrator concentrations.

Within-run precision was assessed by the repeat analysis (n=6) of selected dosage forms. Sample recovery was determined by replacing erythromycin estolate tablets with erythromycin estolate raw material samples (415.9 mg, 521.0 mg and 637.7 mg) and proceeding as described for the analysis of tablets (Section 5.3.2). Experimentally determined values were compared with calculated values and expressed as a percentage recovery. Sample stability in the automatic injector was assessed by re-injection of analysed samples after overnight storage.

5.4 RESULTS AND DISCUSSION

5.4.1 CHROMATOGRAPHY AND SAMPLE STABILITY

Adequate separation between erythromycin estolate, base and internal standard was obtained with no interference from dosage form adjuvants being observed. Representative chromatograms of erythromycin estolate tablets, capsules and suspensions are depicted in figure 5.1. Adequate sensitivity was obtained by UV analysis at 200 nm allowing the same sample dilutions to be utilized for both the UV and electrochemical detectors. Utilization of a detector wavelength of 215 nm as previously described (196) resulted in a dramatic decrease in sensitivity requiring larger sample loads, thereby increasing the possibility of column overloading and interference from sample contaminants. The electrochemical detector was more sensitive than the UV detector and could be operated at lower amplifications than required in previous analyses (Chapter 2) which resulted in a more stable response with associated decreases in day-to-day sensitivity variations. Tables 5.1-5.4 summarize results obtained utilizing both detection methods which clearly indicate the applicability of either technique. The major advantage of the electrochemical detector is its increased sensitivity and

Table 5.1 Dosage form analysis - stability of samples in the automatic sample injector (24 hours)

Product		Electrochemical		Ultraviolet	
		Original Analysis	Repeat Analysis	Original Analysis	Repeat Analysis
EL1	Mean Contents (mg)	547.6	552.7	525.6	519.3
AI1	Mean Contents (mg)	508.5	501.1	469.8	457.6
LL1	Mean Contents (mg)	482.9	470.0	438.0	413.2

Table 5.2 Dosage form analysis - precision data

Product	n	Electrochemical		Ultraviolet	
		Mean Contents(mg)	R.S.D. %	Mean Contents(mg)	R.S.D. %
Tablets					
EL1	6	547.6	1.8	525.6	1.4
LL1	6	508.5	0.7	469.8	1.9
AI1	6	482.9	2.3	438.0	1.8
Capsules					
EL2	6	287.3	1.3	299.6	2.2
LL2	6	273.0	0.8	278.2	1.8
AL2	6	219.8	1.5	214.3	2.5
Suspensions					
EL3	6	132.3	1.1	131.9	4.8
GS1	6	90.5	6.7	90.5	8.1
LL7	6	132.4	1.4	131.3	2.5

Mean Contents = Erythromycin base equivalent per tablet, capsule or 5ml of suspension

Table 5.3 Dosage form analysis-recovery data (tablet analysis)

Sample Number	Estolate added(mg)	Electrochemical		Ultraviolet	
		Estolate found(mg)	Recovery %	Estolate found(mg)	Recovery %
1	415.9	407.6	98.2	402.8	97.0
2	415.9	396.0	95.4	402.8	97.0
3	415.9	407.6	98.2	422.2	102.0
4	521.8	512.5	98.2	481.3	92.2
5	521.8	512.5	98.2	501.0	96.0
6	521.8	512.5	98.2	501.0	96.0
7	637.7	675.5	106.0	628.7	98.6
8	637.7	628.9	98.6	628.7	98.6
9	637.7	652.2	102.3	638.5	100.1
	Overall recovery :		99.3 ± 3.1		97.5 ± 2.8

its application to analyses involving detection of acid degradation products while the UV detector is more suited to lengthy automated analyses especially when performed by less skilled technical staff.

Samples were stable in the automatic injector over a 24 hour period (Table 5.1).

5.4.2 VALIDATION

Calibration curves prepared from standards containing 2.11, 2.60, 3.21 and 4.07 mg/10ml were linear with a slope of 0.17, a y-intercept of 0.00 and a correlation coefficient of 0.994.

Precision data for the analysis of erythromycin estolate tablets, capsules and suspensions utilizing both UV and electrochemical detection are summarized in table 5.2. For the tablet and capsule analysis precision was excellent with relative standard deviations being less than 2.5 %. The advantage of incorporating an internal standard is clearly evident, since sample volume variations during centrifugation and storage in the sample injector are of no consequence. The technique utilized in the tablet assay allowed for tablet disintegration and de-aggregation in water prior to dissolution of the poorly water-soluble drug in acetonitrile. This procedure proved highly successful and reduced the possibility of drug entrapment within intact granules which may occur if organic solvents are added directly to crushed tablet matrices.

Table 5.4 Chromatographic responses of erythromycin estolate raw materials (chromatographic system A)

	Supplier	Estolate peak height (mm)	Mass powder (mg)	Relative response (mm/mg)
Batch 1	USP	95.5	10.5	9.1
	AI	94.5	11.3	8.4
	LL	92.0	11.0	8.4
	EL	88.0	9.8	9.0
Batch 2	USP	48.0	7.4	6.5
	AI	62.0	10.6	5.9
	LL	67.5	10.4	6.5
	EL	70.5	10.6	6.6

Mass powder = Mass of erythromycin estolate powder dissolved in 10ml acetonitrile.

Relative standard deviations obtained for the two "syrupy" suspensions (EL3,LL7) were less than 5% indicating good precision, however, poor precision was obtained with sample GS 1, presumably due to sampling problems (Section 5.4.3).

As expected, the recovery study indicated minimal sample losses (Table 5.3) since no complex sample manipulations were performed. The accuracy of these assay procedures is dependent on the chromatographic response per unit mass of each erythromycin estolate raw material being identical to that of the USP standard used in the calibration curve. Comparison of these responses (Table 5.4) indicated some slight variations between samples, with the Adcock-Ingram sample exhibiting the lowest response in both sets of standards.

5.4.3 DOSAGE FORM ANALYSIS

Results obtained from the analysis of erythromycin estolate tablets, capsules and suspensions utilizing both UV and electrochemical detection are summarized in table 5.5.

The content uniformity of all three tablet products appeared to be excellent, with relative standard deviations obtained from the analysis of six tablets per batch being less than 3.5 % in all cases. Mean values obtained for the amount of drug per tablet, expressed as a percentage of the label claim, show the three different products to cover the entire permissible range of 90-120% (160).

The content uniformity of capsules is highly dependent on the method of capsule filling, and is reflected in the uniformity of the capsule mass. Products EL2 and LL2 had capsule mass variations in the order of 1 - 3 % with similar values for variations in drug contents, however, two capsules from the product AI2 were over-filled making the removal of the contents extremely difficult which resulted in 10% variations in both capsule mass and drug content. The mean drug content of capsules EL1 and LL2 were almost identical (110%) which was within the pharmacopoeial limits (160) for erythromycin estolate capsules (90-115%).

The product AI2 had a mean content of 94% if the results of all six capsules were incorporated. However, if the results of the two over-filled capsules were included, the mean drug content was only 86% of label claim. The wide variations in capsule contents reported for product AI2 would make it an unsuitable product for pharmacokinetic studies and would certainly complicate the performance of comparative bioavailability studies unless capsules were pre-weighed prior to administration.

It is possible that the lower drug content values obtained for the tablet AI1 and the capsule AI2 may be related to the discrepancy noted in the previous section regarding the decreased chromatographic response of the AI raw material compared to the USP standard.

Table 5.5 (a) Analysis of tablets and capsules
(b) Analysis of suspensions

5.5 (A) - TABLETS AND CAPSULES

Product	n	Electrochemical			Ultraviolet		
		Mean contents (mg)	% Label claim	R.S.D. %	Mean contents (mg)	% Label claim	R.S.D. %
Tablets							
EL1	6	547.6	109.5	3.1	540.8	108.2	4.8
LL1	6	506.1	101.2	2.5	499.8	100.0	3.4
AI1	6	464.6	92.9	1.6	457.8	91.6	1.4
Capsules							
EL2	6	279.4	111.8	2.3	281.5	112.6	3.0
LL2	6	278.3	111.3	3.3	280.5	112.2	2.7
AI2	6	234.2	93.6	10.5	230.7	92.3	10.3

5.5 (B) SUSPENSIONS

		Electrochemical			
		Mean contents (mg/5ml)	% Label claim	R.S.D. %	Base/Ester ratio x100
EL3	1	132.3	105.8	3.3	131.9
GS1	1	90.5	72.4	1.1	90.5
LL4	1	132.4	106.0	4.2	131.3
LL5	1	123.9	99.1	6.4	118.5
LL6	1	123.0	98.4	6.1	114.5
LL7	1	266.8	106.7	5.9	249.5
LL8	1	232.2	93.3	6.2	227.2
LL9	1	258.5	103.4	6.0	245.0

KEY: n = Number of each product tested (suspensions analysed in triplicate)
Mean contents = Erythromycin base equivalent.
Base/ester = Ratio of base peak height to ester peak height x100, used as a measure of ester hydrolysis.

Sampling from suspensions is difficult, either due to their "syrupy" nature or as was the case with product GS1, their rapid settling rate and granular precipitates. In order to facilitate the transfer of small volumes of these suspensions, disposable pipette tips were modified by removing 5mm from their ends, thereby increasing their bore considerably. Of the 8 different erythromycin estolate suspensions analysed, only one was not within the USP content limits of 90-115% of label claim. This product, GS1, is formulated as dry granules for reconstitution, but the resultant suspension settles rapidly, making removal of a representative sample extremely difficult which may have been the cause of the low assay value. Similar dose irregularity problems could be experienced in clinical practice as well as in comparative bioavailability studies involving this formulation.

Comparison of the peak height ratios of erythromycin base and estolate for each of the suspensions (Table 5.5), shows product GS1 to have far less erythromycin base than the other suspensions which was to be expected since it is formulated as dry granules thereby decreasing the possibility of ester hydrolysis on storage. The products LL6 and LL9 were manufactured 3 years prior to analysis yet showed no signs of excessive estolate hydrolysis or formation of any other degradative compounds.

In summary, the HPLC method described, utilizing either UV or electrochemical detection was ideally suited for the analysis of erythromycin estolate dosage forms. The sample manipulations employed resulted in excellent within-run precision with minimal sample hydrolysis.

CHAPTER SIX

COMPARISON OF SERUM AND URINE CONCENTRATIONS OF ERYTHROMYCIN BASE AND PROPIONATE IN HUMANS FOLLOWING THE ORAL ADMINISTRATION OF ERYTHROMYCIN ESTOLATE

6.1 INTRODUCTION

Early literature reports (67,109,110) indicated that samples of biological fluids obtained from human volunteers who had been administered oral erythromycin estolate contained both erythromycin propionate and erythromycin base (Section 1.3.7). Various analytical techniques have been developed in order to selectively determine concentrations of both components in biological fluids. Interpretation of data obtained utilizing these analytical methods is complicated by the varying extent of ester hydrolysis which occurs between sample withdrawal and analysis (Section 2.1).

Two different methods have previously been used to assess the relative amounts of erythromycin base and propionate *in vivo* following administration of erythromycin estolate (Table 6.1). The first method involves comparison of erythromycin base and propionate concentrations at each sampling interval (78,112-114) whilst the second is based on a comparison of area under the serum concentration-time curve (AUC) values calculated for each drug (111,115). Although ratios of erythromycin base and propionate concentrations are reported to be similar over the entire sampling period and therefore give some indication of the amount of each component present, comparison of AUC values is more meaningful since this parameter is commonly used in the comparative assessment of drug absorption in biopharmaceutical studies (137). In all published studies to-date (Table 6.1), the amount of erythromycin base *in vivo* relative to the amount of erythromycin propionate has been reported to increase during multiple dose administration. No detailed studies on the urinary excretion of either component have been reported, although Stephens *et al* (67) briefly mentioned that similar results were observed in serum and urine following multiple dose administration of erythromycin estolate to a human volunteer.

Table 6.1 Erythromycin base and propionate concentrations following the oral administration of erythromycin estolate - summary of literature

Reference	Assay Technique	Protocol	Method of Calculation	% Base
67	TLC-Bioautography	SD	Drug Concentrations	20-35
112	TLC-Bioautography	SD	Drug Concentrations	20
112	TLC-Bioautography	MD	Drug Concentrations	40
78	Liquid-Liquid Microbiological	MD	Drug Concentrations	28
113	Liquid-Liquid Microbiological	MD	Drug Concentrations	20
114	Liquid-Liquid	SD	Drug Concentrations	16
114	Fluorimetric	MD	Drug Concentrations	27
115	Liquid-Liquid Microbiological	MD	Comparison of AUC's	17-19
169	HPLC	MD	Comparison of AUC's	27

KEY: SD = Single dose study
MD = Multiple Dose Study
% BASE = The percentage of erythromycin *in vivo* present as erythromycin base.

The aim of this study was to simultaneously determine the concentrations of erythromycin base and propionate in human serum and urine following both single and multiple dose administration of erythromycin estolate utilizing a newly developed HPLC assay procedure in which ester hydrolysis prior to, and during analysis was decreased to a minimum (Chapter 2). Data obtained from these studies were evaluated in terms of the amount of each moiety present *in vivo* and the amount of each component excreted unchanged in urine. The non-compartmental pharmacokinetic parameters Tmax (time to reach maximum serum concentration), Cmax (Maximum serum concentration), $t_{1/2}$ (elimination half-life) and the $AUC_{0-\infty}$ were calculated in order to evaluate the pharmacokinetics of both components in humans (*vide infra* Section 7.1).

6.2 CLINICAL TRIALS

6.2.1 VOLUNTEERS

All four studies were performed using the same non-smoking male volunteers judged to be healthy on the basis of medical history, physical examination and clinical pathology tests. These tests included:

Haematology- full blood count
Blood Chemistry- glucose, total bilirubin, alkaline phosphatase, aspartate transaminase, alanine transaminase, creatinine and urea.
Urinalysis - specific gravity, pH, glucose, protein, bilirubin and microscopic examination.
Hepatitis B antigen.

The following exclusion criteria were employed:

1) Regular use of medication or abuse of alcoholic beverages
2) History or presence of gastro-intestinal, liver or renal disease
3) History of hypersensitivity to erythromycin and related compounds
4) Body mass more than 10% above or below suggested normal values for corresponding height and age.

Volunteers signed a combined protocol/consent form (Appendix 1) prior to each study and were paid for their participation. Demographic data of the trial subjects are summarized in table 6.2.

Table 6.2 Demographic data of trial subjects

NAME	CODE	AGE (years)	MASS (kg)	HEIGHT (cm)	SEX
A.Wellman	P1	21	79	180	M
O.Emberger	P2	20	88	185	M
M.Cawood	P3	21	85	184	M
D.Bramsen	P4	20	74	182	M
K.Fletcher	P5	23	88	186	M
L.Hillary	P6	23	77	196	M

6.2.2 TREATMENTS

Single dose 1 (S1): Only 5 patients were used in this study (P1-P5). Each patient received one Ilosone 500 mg tablet (Batch L045790 Eli-Lilly S.A.) containing the equivalent of 500 mg erythromycin base as erythromycin estolate. The same tablet (EL 1) was used in all subsequent studies.
Single dose 2 (S2): Patients P1-P6 received a single dose of tablet EL 1.
Single dose 3 (S3): Patients P1-P6 received a single dose of tablet EL 1.
Multiple dose (MD): Patients P1-P6 received one tablet (EL 1) every six hours for five doses. Serum and urine were collected after the fifth dose.
Each study was separated by a minimum of two weeks.

6.2.3 SUBJECT RESTRICTIONS AND PROTOCOLS

Subjects refrained from taking any medication, including over-the-counter preparations for two weeks prior to each study. Ingestion of alcoholic and caffeine containing beverages and foods was not permitted 48 hours before and during each trial period. Strenuous physical activity was not allowed for the duration of the trials. Food and water intake were strictly controlled throughout the studies and were only administered at the times indicated on the protocol sheets (Figs. 6.1 & 6.2). All patients were confined to bed in a semi-reclining position for the first five hours of the study except during the voiding of urine samples. Adverse reactions were recorded by the investigator and reported to the attending medical practitioner.

Figure 6.1 Protocol for single dose studies (S1-S3)

All studies commenced at 8.0 am. following an overnight fast.

Time	Sample		Additional Information
	S	U	Volunteers supine
0			Dose administered with 200 ml water
0.5	S		
1	S		
1.5	S		200 ml water
2	S	U	
2.5	S		Breakfast - 3 slices of toast, lightly spread with margarine and jam plus 200 ml orange juice
3	S		
3.5	S		
4	S	U	200 ml water
5	S		Lunch -1 piece boiled chicken, rice and vegetables, mixed fruit, 200 ml orange juice
6	S	U	
8	S	U	
10	S	U	Supper - volunteers choice
12	S	U	
24	S	U	

After lunch the volunteers continued with daily tasks and took water as required.

KEY S = Serum Sample
U = Urine Sample

Figure 6.2 Protocol for multiple dose study (MD)

Studies commenced at 8.0 am. following an overnight fast.

Time	Sample		Additional Information
0	S	U	Report to School of Pharmaceutical Sciences. Dose 1 administered with 200 ml water.
2			Breakfast - as in Figure 6.1
4			Lunch - as in Figure 6.1
6			Dose 2 with 200 ml water
10			Supper - volunteers choice
12			Dose 3 with 200 ml water
14			Report to central venue
16			Light snack served
18	S		Dose 4 with 200 ml water
24	S	U	Dose 5 with 200 ml water Protocol identical to Figure 6.1
48	S	U	

Blood and urine samples were collected according to the schedules summarized in figures 6.1 and 6.2. Samples were manipulated and stored as described in section 2.3.8.

6.2.4 ANALYSIS OF SAMPLES

Serum and urine samples from the single dose studies (S2 & S3) and the multiple dose study, were analysed for erythromycin base and propionate by HPLC as described in chapter 2 and duplicate serum samples were re-analysed for "total erythromycin" concentrations following hydrolysis as described in chapter 3. Serum samples collected during single dose study 1 were only analysed for "total erythromycin" concentrations following hydrolysis and could therefore not be included in this section.

6.3 RESULTS

6.3.1 SERUM PROFILES

The serum concentration versus time profiles (0-12 hours) of erythromycin base and propionate for all six patients in single dose studies S2 and S3 as well as the multiple dose study (0-24 hours) are depicted in figures 6.3-6.5 and the mean profiles for each study in figure 6.6. Serum concentrations from all three trials are presented in Appendices (2-5).

Figure 6.3 Serum concentration-time profiles of erythromycin base and erythromycin propionate - single dose 2 (S2)

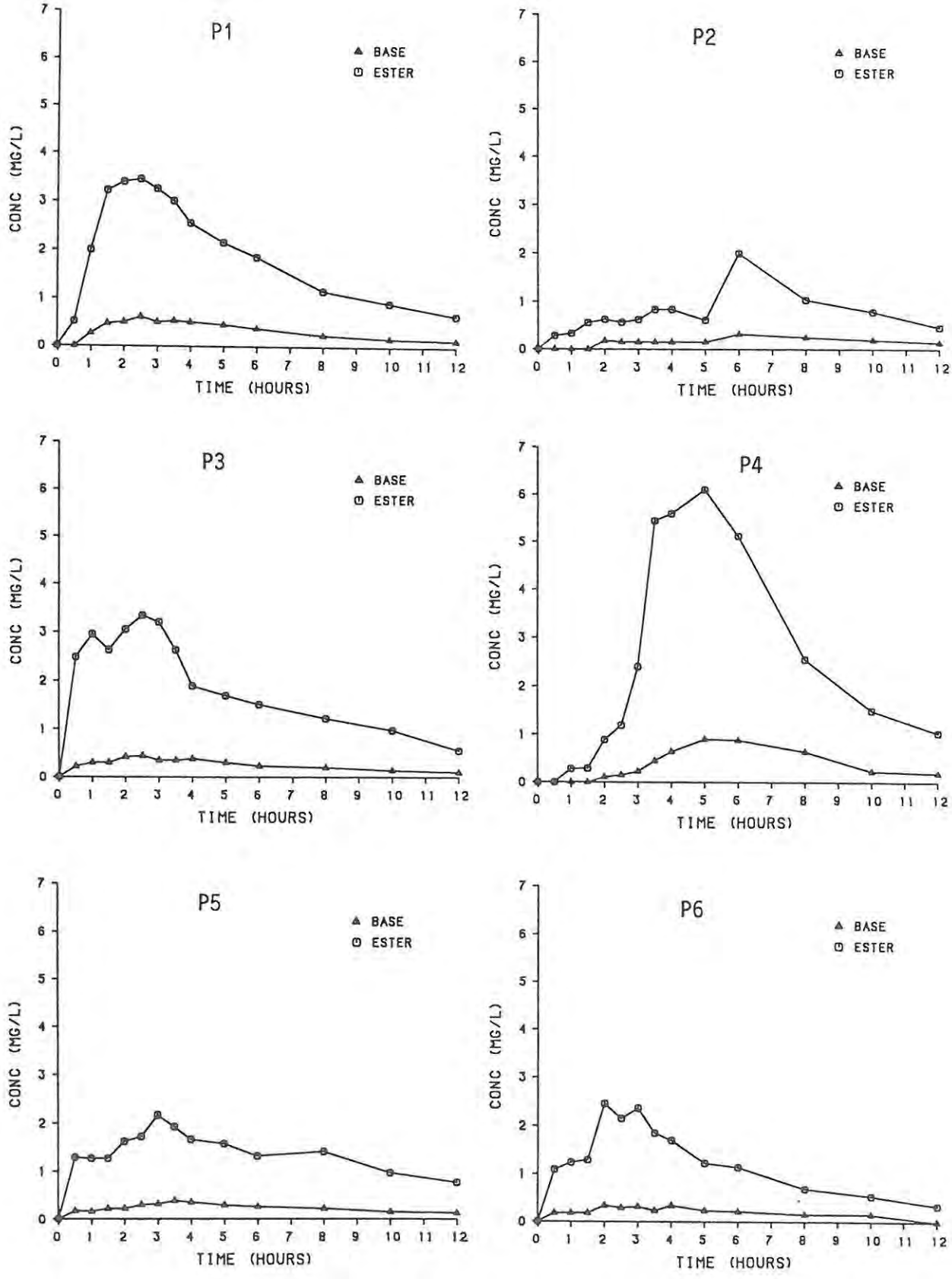


Figure 6.4 Serum concentration-time profiles of erythromycin base and erythromycin propionate - single dose 3 (S3)

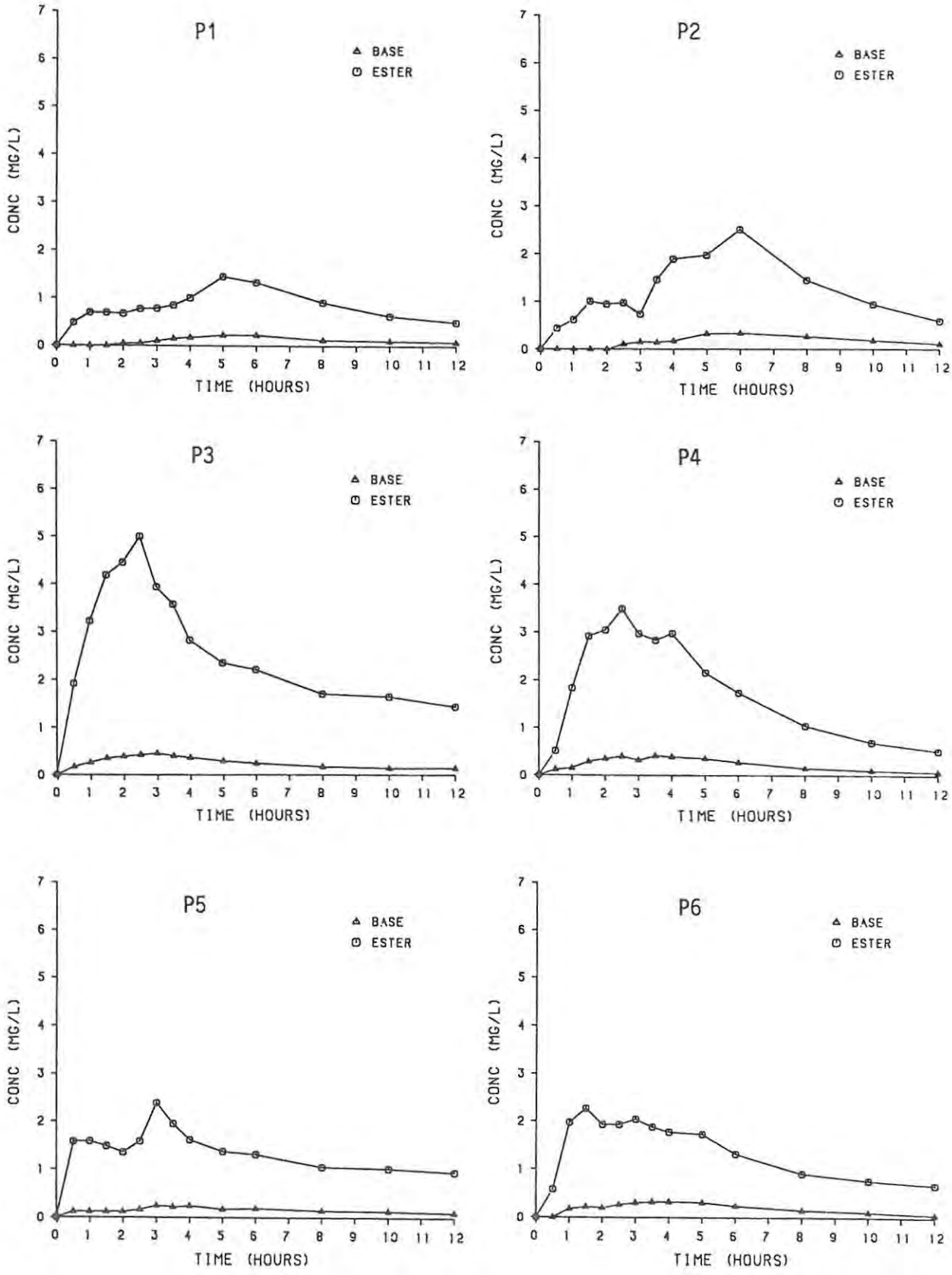


Figure 6.5 Serum concentration-time profiles of erythromycin base and erythromycin propionate - multiple dose (MD)

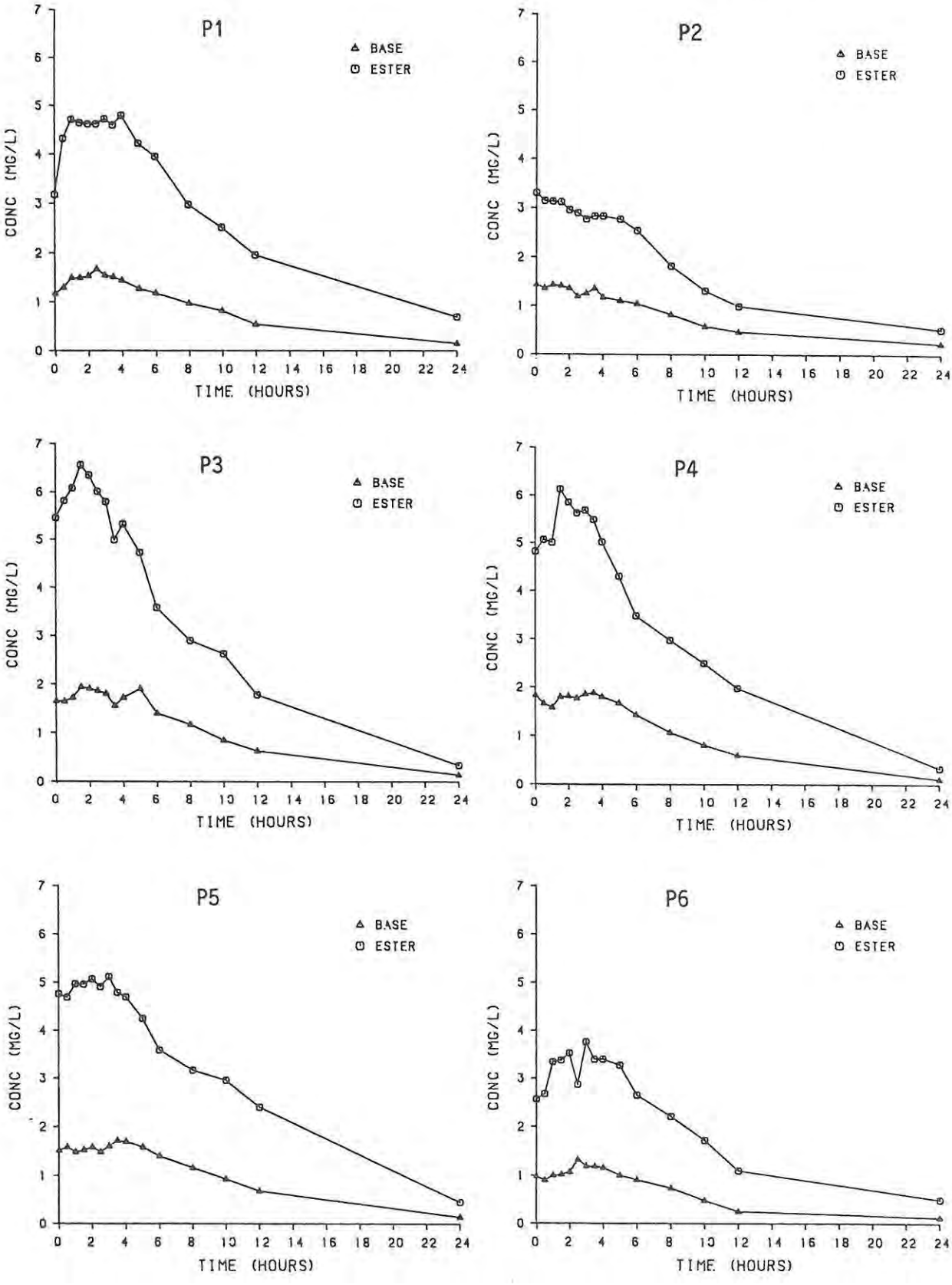


Figure 6.6 Mean serum concentration-time and cumulative urinary excretion profiles for erythromycin base and propionate - studies S2, S3 and MD

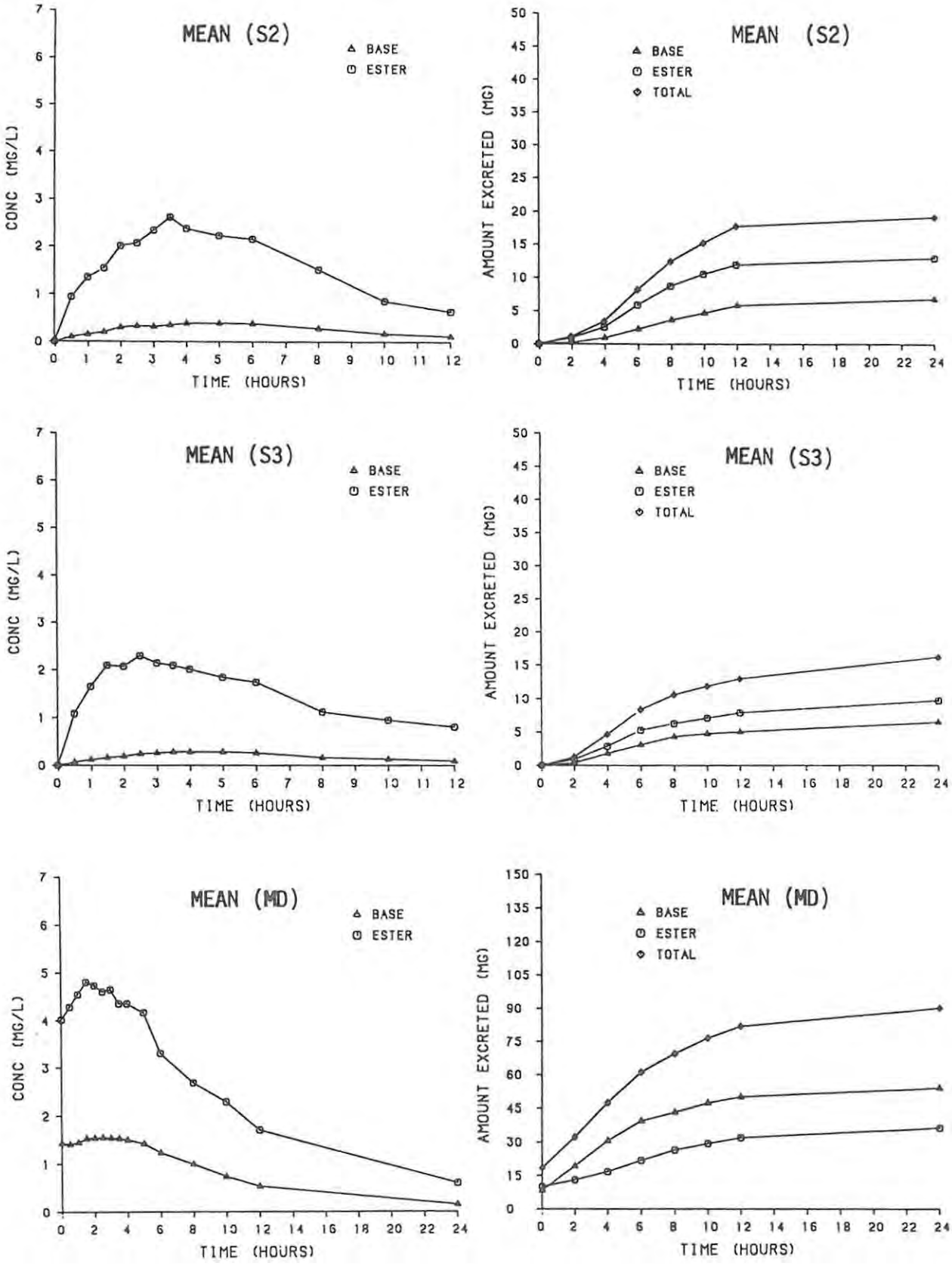


Figure 6.7 Cumulative urinary excretion profiles for erythromycin base and propionate - single dose 2 (S2)

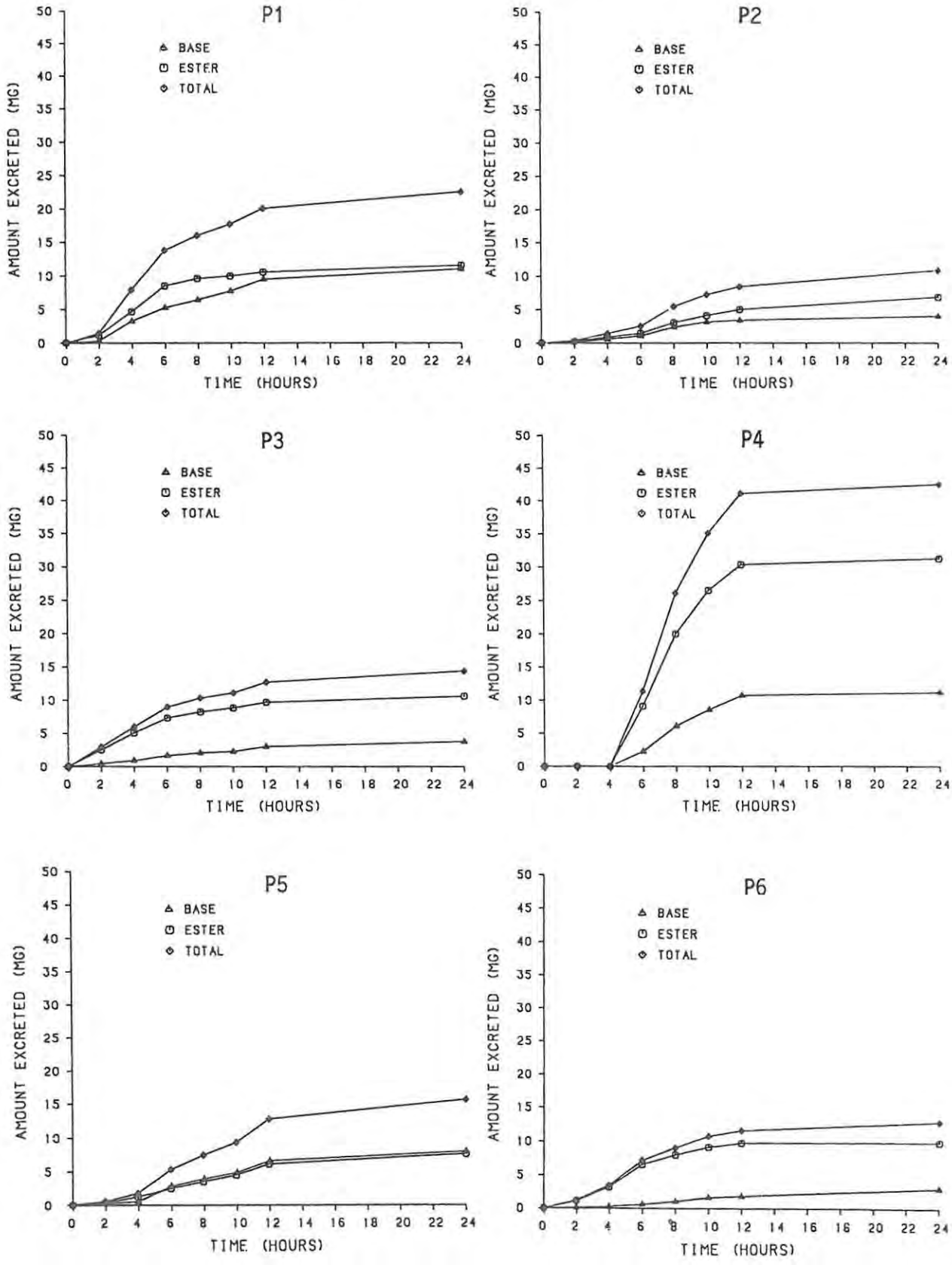


Figure 6.8 Cumulative urinary excretion profiles for erythromycin base and propionate - single dose 3 (S3)

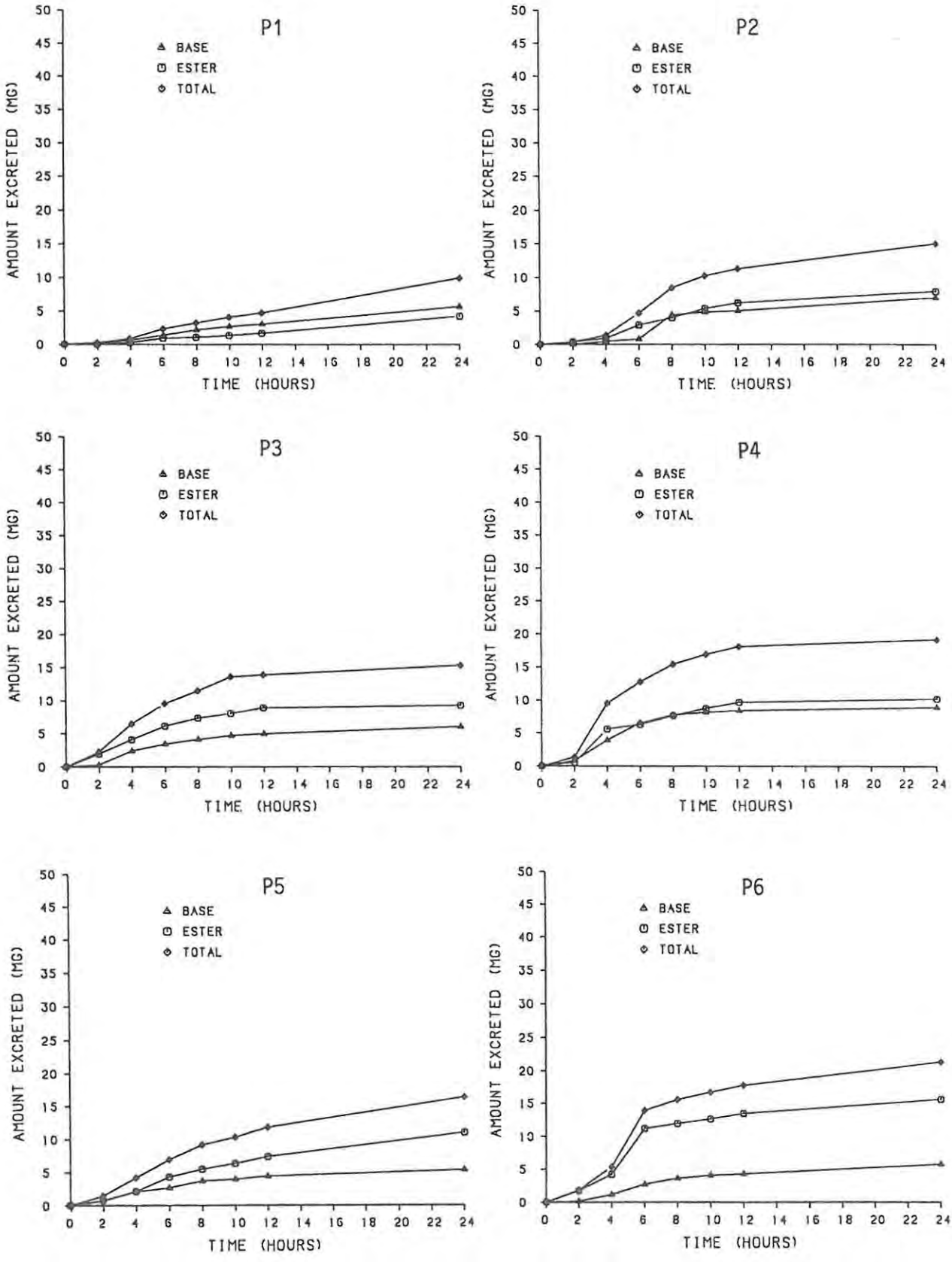
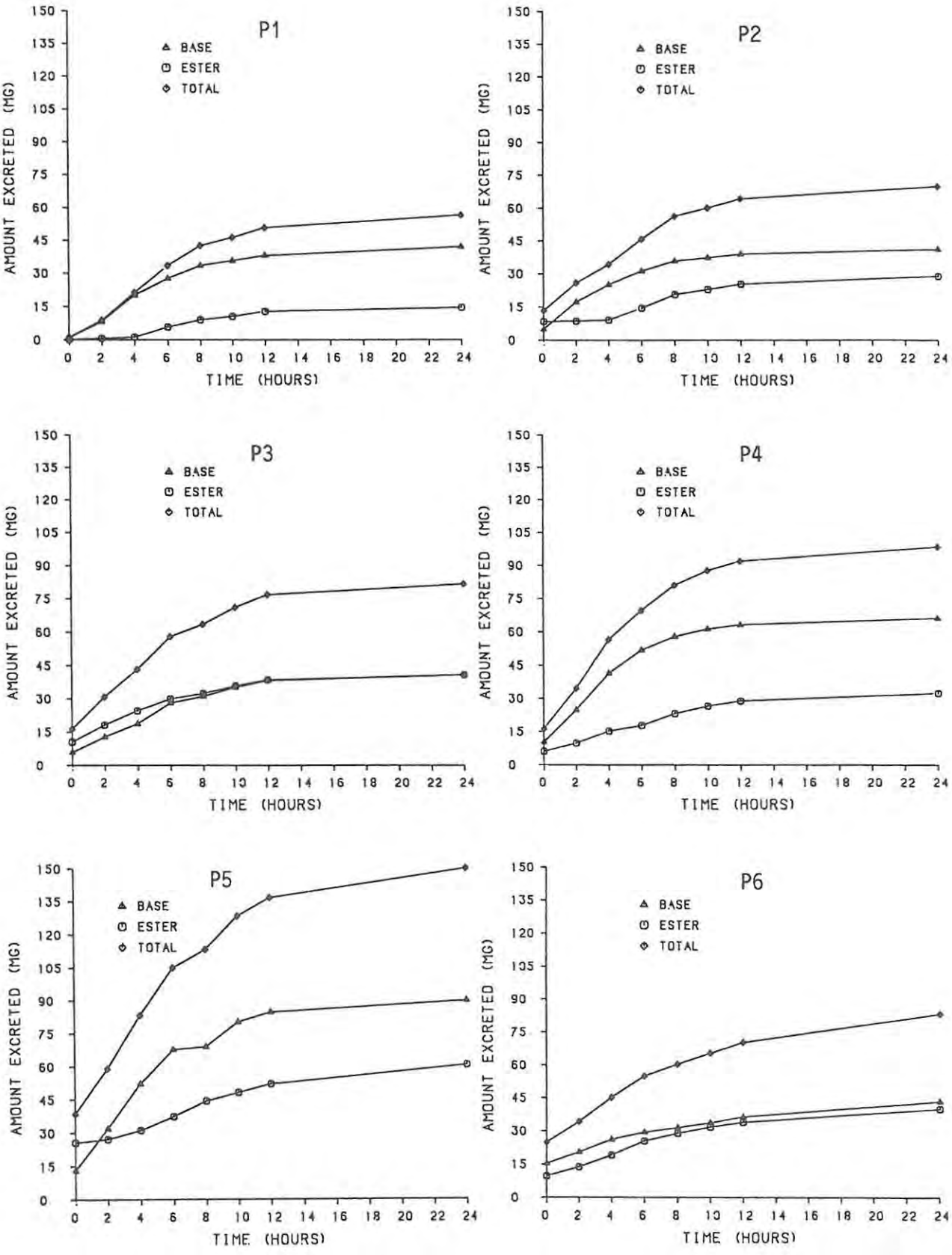


Figure 6.9 Cumulative urinary excretion profiles for erythromycin base and propionate - multiple dose (MD)



6.3.2 CUMULATIVE URINARY EXCRETION PROFILES

Cumulative urinary excretion profiles of erythromycin base, erythromycin propionate and the sum of the two components are depicted in figures 6.7-6.9 and the mean profiles for each study in figure 6.6. Urine concentrations and cumulative data are presented in Appendices (2-5).

6.3.3 COMPARISON OF ERYTHROMYCIN BASE AND PROPIONATE

Table 6.3 lists the $AUC_{0-\infty}$ of erythromycin base (linear trapezoidal rule) as a percentage of the $AUC_{0-\infty}$ for the total erythromycin concentration (Base + ester) for each patient during the three phases of the study. The mean percentages of erythromycin base in serum samples from each volunteer are summarized in table 6.4. Both these parameters have been used as a measure of the relative amounts of base and ester *in vivo* following the oral administration of erythromycin estolate (Section 6.1).

Table 6.3 Relative amounts of erythromycin base and propionate *in vivo* - comparison of AUC values

Patient	% Erythromycin Base of Total Erythromycin *in vivo*. Single dose S2	Single dose S3	Multiple dose 0-6 hr
1	15.4	11.9	23.4
2	16.9	12.0	29.9
3	12.4	8.7	24.4
4	13.4	11.7	25.4
5	14.5	10.5	25.1
6	14.4	11.7	25.0
Mean	14.3	11.2	25.2

Table 6.4 Relative amounts of erythromycin base and propionate *in vivo* - comparison of serum concentrations

Patient	% Erythromycin Base of Total Erythromycin Concentration*. Single dose S2	Single dose S3	Multiple dose 0-6 hr
1	15.5 ± 2.6	12.2 ± 2.6	24.1 ± 1.7
2	18.7 ± 3.1	11.6 ± 4.1	29.9 ± 1.8
3	12.8 ± 2.8	9.5 ± 1.7	25.0 ± 2.6
4	12.5 ± 3.7	10.9 ± 2.1	25.3 ± 1.8
5	14.3 ± 1.9	9.9 ± 2.1	24.6 ± 1.8
6	15.1 ± 3.4	12.3 ± 3.1	25.0 ± 2.5
Mean	15.0 ± 3.9	11.0 ± 2.8	25.0 ± 2.8

* Mean value of 14 data points per subject.

The percentage of the total amount of unchanged erythromycin base and propionyl ester excreted in urine as erythromycin base was calculated for each patient (Table 6.5) as was the fraction of the dose excreted as either erythromycin base or propionate (Table 6.6).

Table 6.5 Percentage of total erythromycin (base+propionate) excreted in urine as erythromycin base

	% Erythromycin Base		
Patient	Single Dose S2	Single Dose S3	Multiple Dose
1	48.9	57.0	74.4
2	36.8	47.0	58.7
3	26.2	39.5	50.0
4	26.4	46.7	67.1
5	51.2	32.8	59.7
6	23.4	26.9	52.0
Mean	35.5	40.0	59.8

Table 6.6 Percentage of dose excreted in urine as either erythromycin base or propionate

	% Dose Excreted	
Patient	Single Dose S2	Single Dose S3
1	4.5	2.0
2	2.2	3.0
3	2.9	3.1
4	9.1	3.8
5	3.1	3.3
6	2.6	4.9
Mean	3.9	3.2

6.3.4 PHARMACOKINETIC PARAMETERS

Table 6.7 summarizes values obtained for the non-compartmental pharmacokinetic parameters Cmax, Tmax, $t_{½}$ and $AUC_{0-\infty}$ during all three phases of this study (*vide infra* Section 7.2.3.1).

Table 6.7 Model independent pharmacokinetic parameters for erythromycin base and propionate

Cmax (μg/ml)	BASE			ESTER		
Patient	S2	S3	MD	S2	S3	MD
1	0.62	0.22	1.67	3.45	1.45	4.72
2	0.32	0.33	1.43	2.00	2.51	3.30
3	0.44	0.45	1.94	3.34	4.99	6.56
4	0.90	0.41	1.88	6.11	3.49	6.13
5	0.38	0.25	1.71	2.16	2.38	5.12
6	0.34	0.33	1.32	2.45	2.04	3.76
Mean	0.39	0.28	1.55	2.62	2.29	4.73
Tmax (h)						
1	2.5	5.5	2.5	2.5	5.0	3.0
2	6.0	6.0	1.0	6.0	6.0	0.5
3	2.5	3.0	1.5	2.5	2.5	1.5
4	5.0	3.5	3.5	5.0	2.5	1.5
5	3.5	3.0	3.5	3.0	3.0	3.0
6	4.0	3.5	2.5	2.0	3.0	3.0
Mean	4.5	4.0	2.5	3.5	2.5	2.0
AUC (μg/ml.h)						
1	4.55	2.12	8.24	24.88	13.98	26.70
2	3.10	2.75	7.56	12.19	17.87	17.78
3	3.53	3.51	10.51	24.96	51.86*	32.67
4	5.62	2.90	10.37	36.15	22.58	30.43
5	3.68	3.06	9.40	24.51	32.59	28.01
6	2.27	2.58	6.42	14.94	24.64	19.24
Mean	3.72	2.85	8.79	21.90	27.59	26.04
$t_{½}$(h)						
1	3.93	6.93	6.30	4.08	4.95	6.30
2	4.95	3.85	4.95	3.01	2.89	4.33
3	-	4.07	4.95	4.95	9.90	5.78
4	2.48	3.01	4.62	2.67	3.30	7.45
5	5.33	8.66	5.78	4.62	13.86	11.55
6	1.82	3.30	2.56	3.65	9.90	4.62
Mean	3.47	4.95	4.95	3.30	8.67	6.30

* Excluded from calculation of Mean
AUC = $AUC_{0-\infty}$

6.4 DISCUSSION

The serum concentration versus time profiles depicted in figures 6.3-6.6 clearly indicate that as previously reported, concentrations of erythromycin propionate exceed those of erythromycin base over the entire 12 hour trial period. In the two single dose studies, most 24 hour serum samples contained detectable amounts of erythromycin propionate but no erythromycin base. Erythromycin base and propionate appeared to reach maximum serum concentrations at similar times in most individuals (Table 6.7), however, there were both large inter- and intra-personal variations between patients and treatments, indicating that absorption of the drug is a complex process. Maximum serum concentrations for both compounds were attained considerably earlier following the fifth dose in the multiple dose study than in the two single dose experiments.

Inspection of serum versus time profiles and the $AUC_{0-\infty}$ data summarized in table 6.3 clearly showed that the amount of erythromycin base relative to the propionyl ester increased considerably following multiple administration of erythromycin estolate. From these $AUC_{0-\infty}$ values it was calculated that following single dose administration of erythromycin estolate approximately 11% to 15% of the total erythromycin present *in vivo* over a 12 hour period consisted of erythromycin base, whilst over a single dosage interval of 6 hours following multiple dose administration this percentage increased to approximately 25 % (Table 6.3). These results compare favourably with those of Croteau *et al* (111) who reported AUC values for erythromycin base and propionate of 6.5 and 17.7 µg/ml.h respectively following eight hourly administration of erythromycin estolate (10 doses) from which it may be calculated that approximately 27 % of the total erythromycin present was erythromycin base. Previous studies in which the relative concentrations of erythromycin base and ester in serum samples were used as an indicator of the ratio of each drug in circulation (Table 6.1), revealed that approximately 16-20% of total erythromycin concentrations were due to erythromycin base following single dose administration and 20-40% after multiple administration. Similar data obtained in this study (Table 6.4) suggest that only 10-15% of total erythromycin concentrations are due to erythromycin base following single dose administration and 20-25% following multiple administration. These differences are most probably due to the additional precautions employed in this study in order to decrease ester hydrolysis between sample withdrawal and analysis.

The observation that increased amounts of erythromycin base are present *in vivo* following multiple dose administration was further substantiated by comparison of Cmax values obtained for both compounds after single and multiple dose administration (Table 6.7). Maximum serum concentrations of erythromycin propionate increased approximately two fold during multiple administration whereas erythromycin base Cmax values increased four-to-five fold. Possible explanations for this phenomenon include: increased absorption of erythromycin base or decreased absorption of the propionyl ester, increased elimination of the ester or decreased elimination of the base or time-related variability in the *in vivo* hydrolysis of the ester. Calculation of $t_{½}$ and $AUC_{0-\infty}$ values was complicated in some individuals who exhibited bi-phasic elimination in one study and monoexponential elimination in the others. Despite these problems (discussed in chapter 7), there was no evidence of any major changes in the elimination half-lives of

either erythromycin base or propionate between the single and multiple dose studies (Table 6.7), which suggested that varying elimination of either compound was an unlikely cause of the differences encountered between these two dosage regimens. As previously reported (Tables 1.8 & 6.7) elimination half-lives for erythromycin base following administration of erythromycin estolate were longer than corresponding values obtained following administration of oral erythromycin base or i.v. erythromycin lactobionate (20,108).

Cumulative urinary excretion profiles (Figs. 6.7-6.9 & 6.6) clearly indicate a reversal in the proportions of erythromycin base and propionate excreted unchanged following single and multiple dose administration. The percentage of erythromycin base in the total erythromycin excreted increased from 35-40% following single dose administration to approximately 60% during the multiple dosage regimen (Table 6.5) which is presumably due to the increased amount of erythromycin base available for excretion after multiple dose administration. Decreased urinary excretion of erythromycin base or increased elimination of erythromycin propionate can therefore also not explain the increased amounts of erythromycin base *in vivo* after multiple dose administration suggesting that variations in the *in vivo* rate of ester hydrolysis may be a major cause of this phenomenon. As mentioned earlier (Section 2.4.1) urine samples collected during the latter stages of the single dose studies and throughout the multiple dose studies contained varying amounts of at least two metabolites/degradation products indicating that not all the drug excreted in urine is in the intact base or ester form. The mean percentage of the original dose excreted in urine as either erythromycin base or propionate varied from 3.9% to 3.2% in single dose studies S2 and S3 respectively.

Four patients exhibited absorption lag-times of one hour or more (P2 & P4 in S2, P1 & P2 in S3) which were reflected in their respective cumulative urinary excretion profiles. Some evidence of a linear relationship was observed between the total amount of erythromycin excreted and corresponding $AUC_{0-\infty}$ values for single dose study S2 if patient P3 was excluded (correlation of linear regression line was 0.987), however, no evidence of linearity was observed for single dose study S3.

In summary, the HPLC method utilized in these studies proved highly effective for the simultaneous determination of erythromycin base and propionate in human serum and urine. Data obtained in these studies may be

treated with confidence since hydrolysis of the ester moiety prior to analysis was strictly controlled and therefore reflects the situation *in vivo* as accurately as possible. Serum concentrations of erythromycin base were considerably lower than those of the inactive propionyl ester throughout the entire study interval, further indication of the need for selective erythromycin base determination when performing comparative studies involving other erythromycin derivatives and/or dosage forms. Comparative studies involving erythromycin estolate should also only be performed after multiple drug administration since the proportion of erythromycin base and propionate *in vivo* varies between dosage regimens. Future studies are required in which serum concentration-time profiles for both erythromycin base and propionate are constructed after each dose in a multiple dose study in order to assess at what stage an equilibrium is reached between the respective amounts of erythromycin base and propionate *in vivo*.

Although valuable information was obtained regarding the relative amounts of erythromycin base and propionate excreted in urine following single and multiple dose administration, the small amount of either compound eliminated via this route and the poor correlation between serum and urine concentrations suggested that urinary excretion data is of little value in pharmacokinetic studies. Future HPLC-mass spectrographic studies need to be directed towards the identification of the metabolites detected in urine. Large inter- and intra-personal variations in the model-independent pharmacokinetic parameters were observed, however, the relative amounts of erythromycin base and propionate *in vivo* appeared to be fairly constant in the single dose studies.

CHAPTER SEVEN
PHARMACOKINETIC ANALYSIS OF DATA

7.1 INTRODUCTION

Pharmacokinetics is the study of the time course of drug absorption, distribution, metabolism and excretion and is concerned with the relationship of these processes to the intensity and time course of pharmacologic effects of drugs and chemicals (202). The classic approach in establishing these relationships has been the employment of pharmacokinetic models which serve to reproducibly characterize the behaviour or "fate" of a drug in biological systems when administered via a particular route in a particular dosage form (203). Sheiner (204) has described two major purposes of pharmacokinetic modelling: Investigation of correlation which is performed to establish the kind and degree of relationship between dependent and independent observations and investigation of causation which involves examination of the underlying mechanisms responsible for the association.

Usually the behaviour of a drug in a biological system can be described in terms of pharmacokinetic models incorporating one, two or multiple compartments with a series of differential equations to describe the transfer of drug between compartments. Numerous texts are available which describe the concepts and mathematical equations involved in pharmacokinetic modelling as well as the computer programs available for optimization of pharmacokinetic parameters (137,202-206). In this study, "total erythromycin" data obtained following the oral administration of erythromycin estolate tablets to human volunteers in three separate single dose experiments were analysed according to open one and two-compartment models with linear first order absorption and elimination as well as a one compartment model with zero-order absorption. Although Welling *et al* (78) described the pharmacokinetics of erythromycin propionate following the oral administration of erythromycin estolate, similar analyses of erythromycin base data were not performed.

As previously discussed, reports on the pharmacokinetics of erythromycin have varied in the choice of the pharmacokinetic model following oral administration, whereas all reports involving i.v. administration of the drug have found an open two-compartment model to best describe the disposition of the drug (Section 1.4). The lack of agreement on the

pharmacokinetics of orally administered erythromycin is presumably due to the complex processes involved in the absorption of enteric coated products, or as is the case with erythromycin estolate, the absorption of the acid insoluble ester derivative. In order to fully understand the absorption process of a drug, it is necessary to acquire information regarding the time-course of this process. This is of particular importance in comparative bioavailability studies in which both the rate and extent of absorption of a drug are compared following administration of different dosage forms. Although the extent of absorption is usually the more important factor in antibiotic therapy, the rate of absorption is also important since it can significantly influence the time-course of drug concentrations especially during multiple dosage regimens (202). Although there are numerous methods available for the calculation of absorption rate constants (137,202,203,207), due to the low percentage of erythromycin excreted unchanged in urine and the lack of i.v. data, only curve-fitting techniques and the method described by Wagner and Nelson (137) were employed in this study.

In recent years there has been a shift away from computer based curve fitting of experimental data towards non-compartmental or model-independent pharmacokinetic techniques (202). These techniques have traditionally been used in the estimation of the bioavailability of drugs and since they involve relatively simple calculations do not require complex computer-aided operation. Since no assumption of any specific compartment model is made these methods may thus be applied to virtually all linear systems.

The use of statistical moment theory in pharmacokinetics was first described by Yamouka *et al* (208) and Cutler (209), and has further increased the scope of non-compartmental methods. These methods are based on the fact that irrespective of the route of drug administration, the first two (zero and first) moments are calculated as follows (202):

$$AUC = \int_0^\infty C\,dt \qquad (7.1)$$

$$MRT = \frac{\int_0^\infty tC\,dt}{\int_0^\infty C\,dt} = \frac{AUMC}{AUC} \qquad (7.2)$$

where, AUC and MRT (mean residence time) are termed the zero and first moments, respectively of the drug concentration-time curve. Riegelman and Collier (210) have applied these statistical moment theories to the oral

absorption of drugs and have estimated mean *in vivo* dissolution times by comparison of MRT values obtained following oral administration of solid dosage forms and solutions of the same compounds. Mean absorption times (MAT) of orally administered drugs have also been calculated by comparison of the MRT values obtained following oral and i.v. administration (209).

Calculation of these parameters is highly dependent on accurate estimations of AUC values, for which purpose the trapezoidal rule is usually employed. The area between the final data point and infinity is usually estimated by dividing the last serum concentration value by the terminal elimination rate constant. The accuracy of this method depends largely on sufficient serum concentration-time points over the trial period, and is reported to both overestimate and underestimate the area depending on the shape of the curve (211). The log trapezoidal rule which involves logarithmic transformation of data prior to calculation of the area by a modification of the linear trapezoidal rule has been found to be more appropriate when applied to data which decline exponentially such as is found during the linear elimination of drugs. Large errors are however encountered if the log trapezoidal rule is used during the steeply ascending portion of serum concentration-time curves associated with the absorption process and is therefore used in combination with the linear trapezoidal rule to yield optimal estimates of area (212).

Large intra- and inter-personal variability in the pharmacokinetics of numerous drugs have been observed, particularly following extravascular administration of compounds whose pharmacokinetics are complicated by such factors as varied dissolution in gastrointestinal fluids, gastric acid degradation, gut wall metabolism, high first-pass extraction ratios, site-specific absorption etc. (137,153,203,205,213,214). Several authors have reported large inter-personal variations in erythromycin pharmacokinetics following oral administration of various erythromycin derivatives and dosage forms (126,152,153,215), whereas Austin *et al* (84) reported low inter-personal variations following i.v. administration, suggesting that the absorption process is the principle cause of these variations. In a study on the intra- and inter-personal variability in erythromycin absorption following oral administration of two different erythromycin base dosage forms, Graffner *et al* (153) employed discrepancy values (d%) to compare the differences in intra-personal variabilities observed between the two products. These values are calculated from AUC values obtained following the administration of each drug and attempt to describe the

overall extent of these variations. It is important that the extent and possible causes of this variability in drug absorption, disposition, metabolism and excretion be evaluated and minimized, since the power of standard statistical procedures utilized in most biopharmaceutical studies decreases dramatically with an increase in the error term associated with each observation (216).

7.2 METHODOLOGY

7.2.1 SELECTION OF DATA

Pharmacokinetic analyses were performed utilizing "total erythromycin" data obtained for each patient (plus the mean data) during each of the three single dose studies described in section 6.2 (Appendices 2,3,6). In addition, the mean data for erythromycin base and propionate obtained in studies S2 and S3 (Appendix 5) were also analysed.

7.2.2 PHARMACOKINETICS

7.2.2.1 MODEL INDEPENDENT ANALYSIS

The parameters Cmax and Tmax were obtained from individual serum concentration-time data. The terminal rate constant (K) was calculated from the slope of the terminal phase of a semi-log plot of serum concentration versus time and was used in the calculation of the elimination half-life ($t_{1/2}$).

Area under the curve (AUC) and was calculated using the linear trapezoidal rule during the absorption phase and the log-linear trapezoidal rule during the elimination phase. Similarly, MRT was calculated using equation 7.2. Calculation of K in studies S2 and S3 was complicated by the fact that serum concentrations at the 24 hour time interval were often greater than values obtained by extrapolation of 12 hour serum concentrations. In order to overcome this problem values for K and therefore $t_{1/2}$, AUC and MRT were calculated using both sets of data.

7.2.2.2 ONE COMPARTMENT MODEL - FIRST ORDER ABSORBTION (MODEL 1)

The one compartment model is the simplest of pharmacokinetic models. It considers the body as being represented by a single compartment into which

drugs are rapidly distributed. Loss of drug is assumed to be first order, with the apparent first order elimination rate constant being the sum of the rate constants of all excretory pathways. For a drug that enters the body and is eliminated by an apparent first order process and which distributes according to a one compartment model, the following equation may be used to characterize these processes (207):

$$\frac{dC}{dt} = k_a \frac{D}{V} e^{-k_a(t-t_o)} - k C_1 \qquad (7.3)$$

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 the central compartment
k = elimination rate constant (only for model 1)
C_1 = concentration in the central compartment

7.2.2.3 TWO COMPARTMENT MODEL - FIRST ORDER ABSORPTION (MODEL 2)

Most drugs do not instantaneously distribute between the blood and other body fluids and tissues as is assumed in the one compartment model. The two compartment model attempts to characterize this phenomenon by allowing for a distribution phase during which the drug may slowly distribute between the central compartment from which samples are withdrawn, and a peripheral compartment. The model utilized in this study is the simplest of all two compartment models in which elimination of the drug is assumed to only occur from the central compartment. The differential equations describing the rate of change of drug in the peripheral and central compartments are as follows:

$$\frac{dA_2}{dt} = k_{12}C_1V_1 - k_{21}A_2 \qquad (7.4)$$

$$\frac{dC_1}{dt} = k_a \frac{D}{V_1} e^{-k_a(t-t_o)} + k_{21}\frac{A_2}{V_1} - k_{12}C_1 - k_{10}C_1 \qquad (7.5)$$

dA_2/dt = rate of change in amount of drug in the peripheral compartment
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_{10} = first order rate constant for elimination of drug from central compartment

7.2.2.4 ONE COMPARTMENT MODEL - ZERO-ORDER ABSORPTION (MODEL 3)

The same assumptions regarding distribution and elimination of drug are made as for model 1. However, absorption of drug is described by a constant input function which is independent of the amount of drug present at the site of absorption (217):

$$C = \frac{k_0(e^{k_{10}T} - 1)e^{-k_{10}t}}{Vk_{10}} \qquad (7.6)$$

k_0 = apparent zero-order absorption rate constant
T = a constant corresponding to the absorption time, after absorption ceases (during absorption, T is a variable and equal to t).

7.2.2.5 COMPUTER ANALYSIS

The nonlinear regression program NONLIN (218) operated on a CDC Cyber 170 series 825 computer was used for all computer fitting of experimental data to the previously described pharmacokinetic models. The DFUNC subroutine of NONLIN was modified for each model using differential forms of equations 7.3-7.6 (207). Initial estimates as well as upper and lower limits for each parameter were either obtained from manual curve-stripping of data (203) or from previously published results. All data points received equal weighting although various weighting functions were utilized in early set up procedures. Since no accurate assessment of the bioavailability factor (F) was available, no attempt was made to incorporate such a term in the DFUNC routines. However, the limits of the volume term were increased so as to compensate for dose-to-dose variations in the absolute bioavailability of the drug. As described by Welling *et al* (78) values of k were constrained when fitted to model 1. Goodness of fit was assessed from the correlation coefficient calculated for each set of observed and predicted data, and by visual inspection of the resulting serum concentration-time curves. Akaike's information criteria (AIC) was used to select the model best describing the experimental data (219). The minimum AIC value being regarded as the most appropriate (217).

$$AIC = n \ln Re + 2p \qquad (7.7)$$

n = number of observations
Re = Weighted sum of squared deviations of data set
p = number of parameters

7.2.2.6 ASSESSMENT OF RATE OF ABSORPTION

In addition to the computer fitting techniques previously described, estimations of k_a were obtained by manual curve-stripping of log serum concentration-time plots (203) as well as by construction of percentage drug absorbed versus time plots as described by Wagner (137).

7.2.3 ASSESSMENT OF THE VARIABILITY IN ERYTHROMYCIN ABSORPTION AND DISPOSITION

7.2.3.1 COMPARISON OF PHARMACOKINETIC PARAMETERS

Values of non-compartmental pharmacokinetic parameters obtained from "total erythromycin" data (Section 7.2.2.1) were tabulated and the relative standard deviations calculated for each parameter, both between the 5 participants in each trial, as well as between the values obtained for each patient during the three administration phases. These data reflected inter- and intra-personal variations respectively.

7.2.3.2 CALCULATION OF DISCREPANCY VALUES

Discrepancy values (d%) for the 5 patients who completed studies S1-S3 were calculated utilizing an adaptation of the method described by Graffner *et al* (153). Results obtained for patient P1 (total erythromycin) in studies S1-S3 have been used as an example of the method (Fig. 7.7):

1) An average serum concentration versus time profile was obtained by averaging the serum concentrations obtained in all three studies at each time interval.
2) The serum concentration versus time profile obtained in each study was plotted together with the average profile.
3) The total area of the shaded portions of figure 7.7 (a)(b) and (c) which represent the differences in area between the average profile and that of the study under evaluation, was obtained using the linear trapezoidal rule. In figure 7.7 (b) and (c), this was simply obtained from the difference in AUC_{0-12} values , whereas in figure 7.7 (a) the differences in area from 0-5 hours and from 5-12 hours were added.
4) The d% value for each administration was obtained by expressing the total area difference of the two profiles as a percentage of the AUC_{0-12} of the average profile.

Figure 7.1 Semi-log plots of "total erythromycin" serum concentration versus time curves (S1,S2,S3).

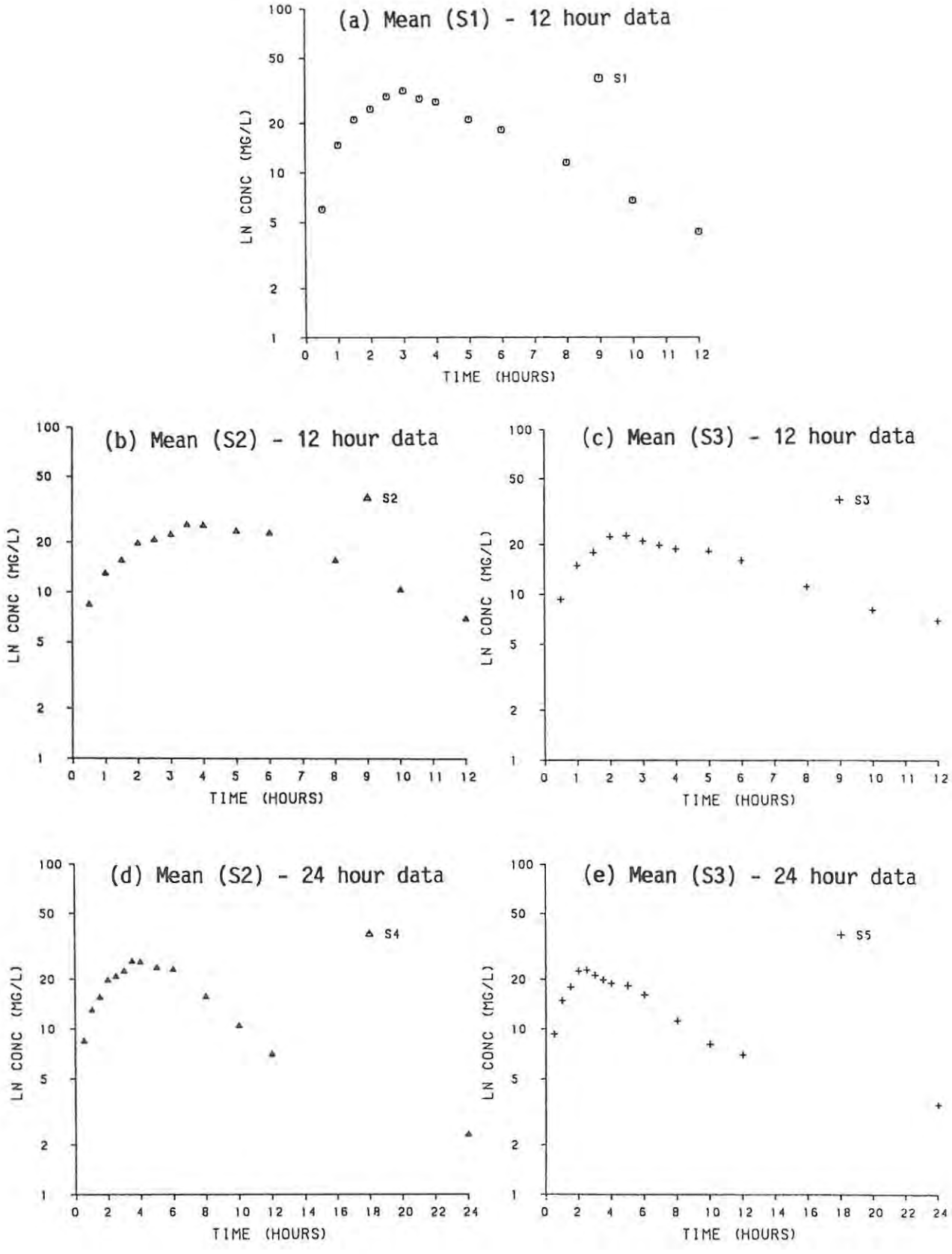


Table 7.1 Model independent pharmacokinetic parameters obtained from total erythromycin data - single dose studies

Parameter	Study	P1	P2	P3	P4	P5	P6	Mean
C max	S1	2.37	3.30	4.54	3.56	3.21	-	3.14
(μg/ml)	S2	3.40	1.53	3.28	6.76	2.07	2.25	2.55
	S3	1.70	2.42	3.86	4.10	2.08	2.46	2.27
T max	S1	4.0	3.0	2.5	3.5	3.0	-	3.0
(h)	S2	2.5	6.0	2.0	5.0	3.0	2.0	3.5
	S3	5.0	6.0	2.5	2.5	3.0	2.5	2.5
K (12h)	S1	0.21	0.29	0.26	0.23	0.20	-	0.23
(h^{-1})	S2	0.18	0.22	0.12	0.27	0.16	0.18	0.20
	S3	0.17	0.23	0.08	0.21	0.02	0.06	0.12
K (24h)	S2	0.11	0.03	0.10	0.37	0.09	0.08	0.10
(h^{-1})	S3	0.06	0.06	0.09	0.07	0.06	0.05	0.06
$t_{½}$ (12h)	S1	3.30	2.39	2.70	3.01	3.41	-	3.05
(h)	S2	3.96	3.15	5.78	2.60	4.33	3.85	3.47
	S3	4.08	3.01	8.67	3.30	28.99	11.55	5.78
$t_{½}$ (24h)	S2	6.30	23.1	7.14	1.87	7.70	9.24	6.93
(h)	S3	11.55	11.55	7.70	9.90	11.55	13.86	11.55
AUC_{0-12}	S1	14.25	16.87	24.15	22.71	14.99	-	18.55
(μg/ml.h)	S2	21.77	8.97	21.59	38.12	15.74	12.20	19.70
	S3	10.96	15.12	24.42	20.33	13.54	14.36	16.60
AUC_{0-24}	S2	27.29	13.82	27.53	41.16	21.32	14.92	24.85
(μg/ml.h)	S3	15.91	20.67	32.16	24.83	20.28	19.90	22.68
MRT (12h)	S1	7.71	5.05	5.36	5.83	6.17	-	5.97
(h)	S2	6.76	8.29	8.38	7.33	8.19	6.25	7.27
	S3	8.65	7.78	11.51	5.85	40.53	15.13	9.13
MRT (24h)	S2	9.23	36.76	9.94	6.94	12.16	10.40	9.93
(h)	S3	17.89	16.33	10.67	10.72	16.61	17.36	15.12
$AUC_{0-\infty}$(12h)	S1	16.86	17.85	25.72	24.99	16.88	-	20.50
(μg/ml.h)	S2	25.49	10.94	28.14	42.70	20.01	13.98	23.26
	S3	14.04	17.81	37.27	22.72	47.68	24.81	22.39
$AUC_{0-\infty}$(24h)	S2	29.45	32.20	30.37	41.19	24.62	16.88	27.06
(μg/ml.h)	S3	24.91	31.00	35.49	28.37	26.45	26.63	28.58

5) The mean d% value for each patient was obtained from the d% values calculated for each administration.

7.3 RESULTS AND DISCUSSION

7.3.1 MODEL INDEPENDENT ANALYSIS

Interpretation of the pharmacokinetics of erythromycin was complicated by large inter- and intra-personal variability (Section 7.3.4) and the apparent non-linearity of erythromycin elimination utilizing the 24 hour data point. Semi-log plots of erythromycin serum concentrations versus time for all patients in study S1, for which only 12 hour data were available, revealed linear elimination profiles as was reflected in the mean plot of these data (Fig. 7.1 (a)). Similar results were obtained for the mean profile of study S2 utilizing 12 hour data (Fig. 7.1 (b)) whereas for study S3 the terminal portion of the log-linear profile was slightly concave due to the serum concentration at 12 hour being slightly higher than expected (Fig. 7.1 (c)). This trend is clearly evident in figures 7.1 (d) and (e) which represent the mean log-linear profiles of studies S2 and S3 utilizing data collected 24 hours post-administration. The elimination phase in these cases was definitely biphasic and could not be described by a single pseudo-first order elimination rate constant. A similar observation has been reported for erythromycin base by Kroboth *et al* (104) (Section 1.4.2). Further studies are required in which serum concentrations are monitored at more frequent time intervals between 10 and 24 hours since this evidence of non-linear elimination is based on the results of only one data point per subject. The high-performance liquid chromatographic analytical methods described in this study are ideally suited for this purpose since they have been shown to be both sufficiently sensitive and precise for the determination of low concentrations of erythromycin in serum.

Values for K calculated utilizing 12 hour data (K(12)) and 24 hour data (K(24)) differed significantly, as did subsequent calculations of $t_{1/2}$ (12) and $t_{1/2}$(24). These differences were particularly apparent in subject P4 in study S2 and patients P3 and P5 in study S3 (Table 7.1). Similar trends were observed following multiple dose administration (Table 7.2). The values of $t_{1/2}$(12) obtained for studies S1 and S2 were similar to the mean $t_{1/2}$ value (3.7 h) reported by Welling *et al* (78) for "total erythromycin" following administration of erythromycin estolate under similar

experimental conditions (12 hour data). The values for $t_{½}(12)$ in study S3 and for $t_{½}$ (24) in studies S2 and S3 were all greater than published values (78).

Large errors were encountered during the calculation of $AUC_{0-\infty}$ values due to the lack of an accurate estimation of K which is required for the calculation of the area after the final data point. Values for AUC_{0-12} and AUC_{0-24} (Table 7.1) were therefore used in conjunction with $AUMC_{0-12}$ and $AUMC_{0-24}$ values in the calculation of MRT values (Equation 7.2). These parameters reflect large intra- and inter-personal variability in erythromycin pharmacokinetics and clearly show the differences obtained when 12 and 24 hour data are utilized, making comparisons with published reports of limited value. It was not possible to test for linearity by comparison of $AUC_{0-\tau}$ values obtained following multiple dose administration (at steady-state) with those following single dose administration (202) since accurate estimations of $AUC_{0-\infty}$ were not available. Values of AUC_{0-12} and AUC_{0-24} were however considerably lower than $AUC_{0-\tau}$ values (Tables 7.1 and 7.2).

Table 7.2 Model independent pharmacokinetic parameters obtained from total erythromycin data - multiple dose study

Parameter	Patient						
	P1	P2	P3	P4	P5	P6	Mean
C max (µg.ml)	5.96	4.47	7.21	7.82	6.67	5.03	5.89
T max (h)	2.0	0.0	1.5	3.5	3.5	3.0	1.5
$AUC_{0-\tau}$ (µg/ml.h)	32.88	23.95	38.67	40.49	35.78	25.12	32.83
K (12h) (h^{-1})	0.11	0.12	0.12	0.09	0.11	0.13	0.12
K (24h) (h^{-1})	0.11	0.07	0.13	0.10	0.12	0.08	0.10
$t_{½}$ (12h) (h)	6.30	5.80	5.78	8.06	6.30	5.33	5.58
$t_{½}$ (24h) (h)	6.3	11.00	5.33	7.29	5.78	8.67	6.93

Figure 7.2 Comparison of observed and predicted (Models 1&2) mean "total erythromycin" serum concentrations (S1,S2,S3).

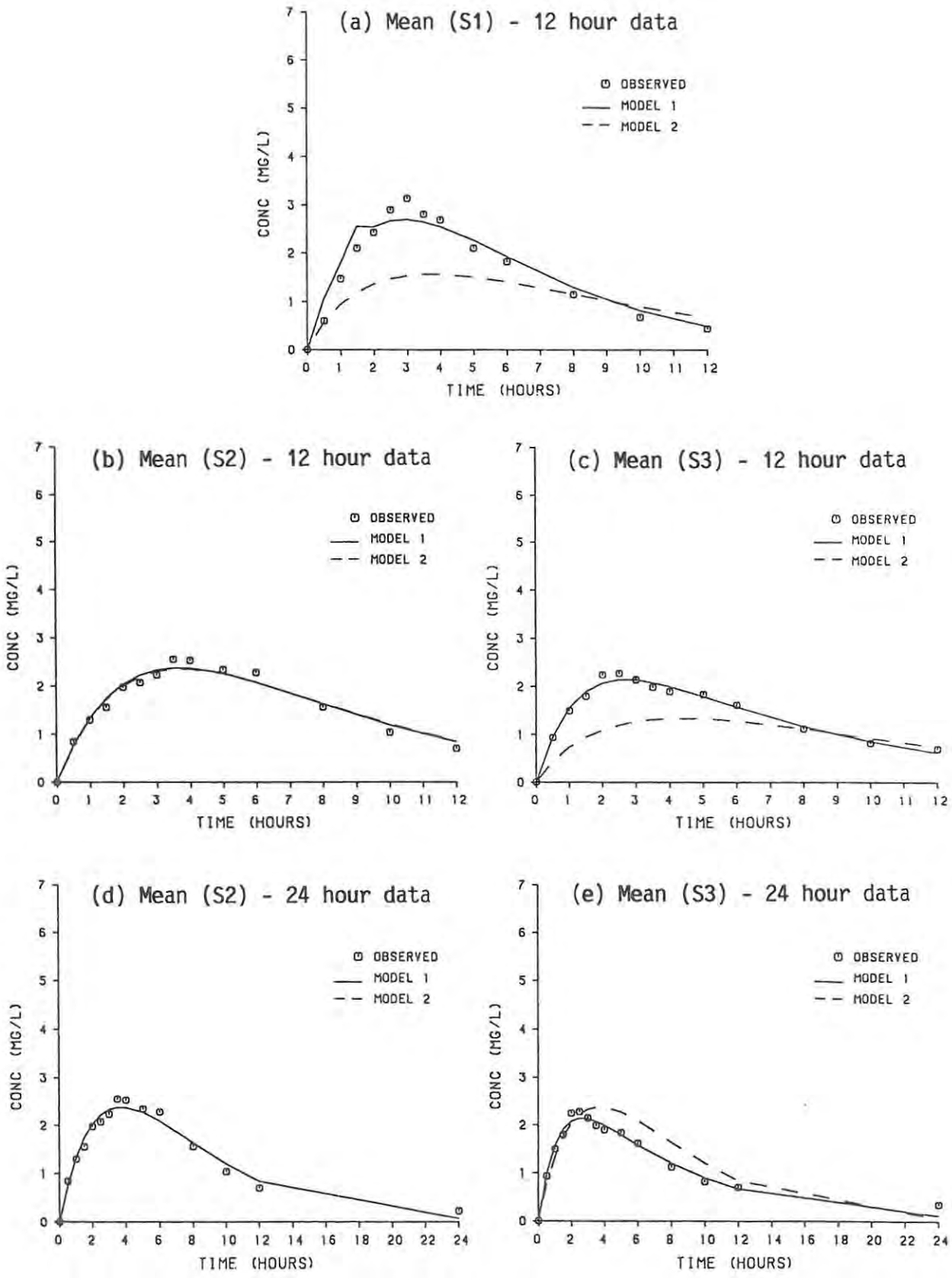


Figure 7.3 Comparison of observed and predicted (Models 1&2) individual "total erythromycin" serum concentrations (S2).

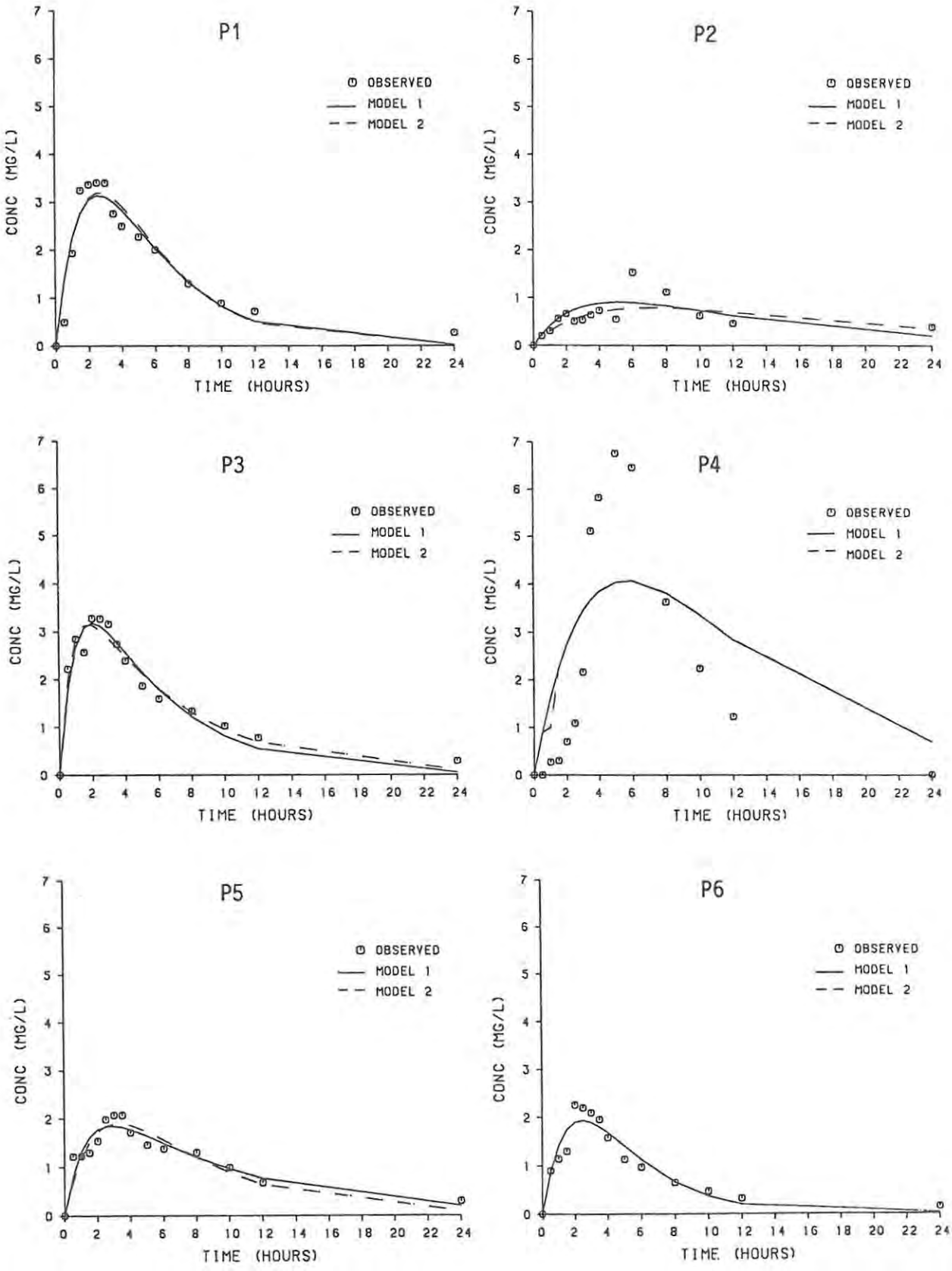


Figure 7.4 Comparison of observed and predicted (Model 3) mean "total erythromycin" serum concentrations (S1,S2,S3).

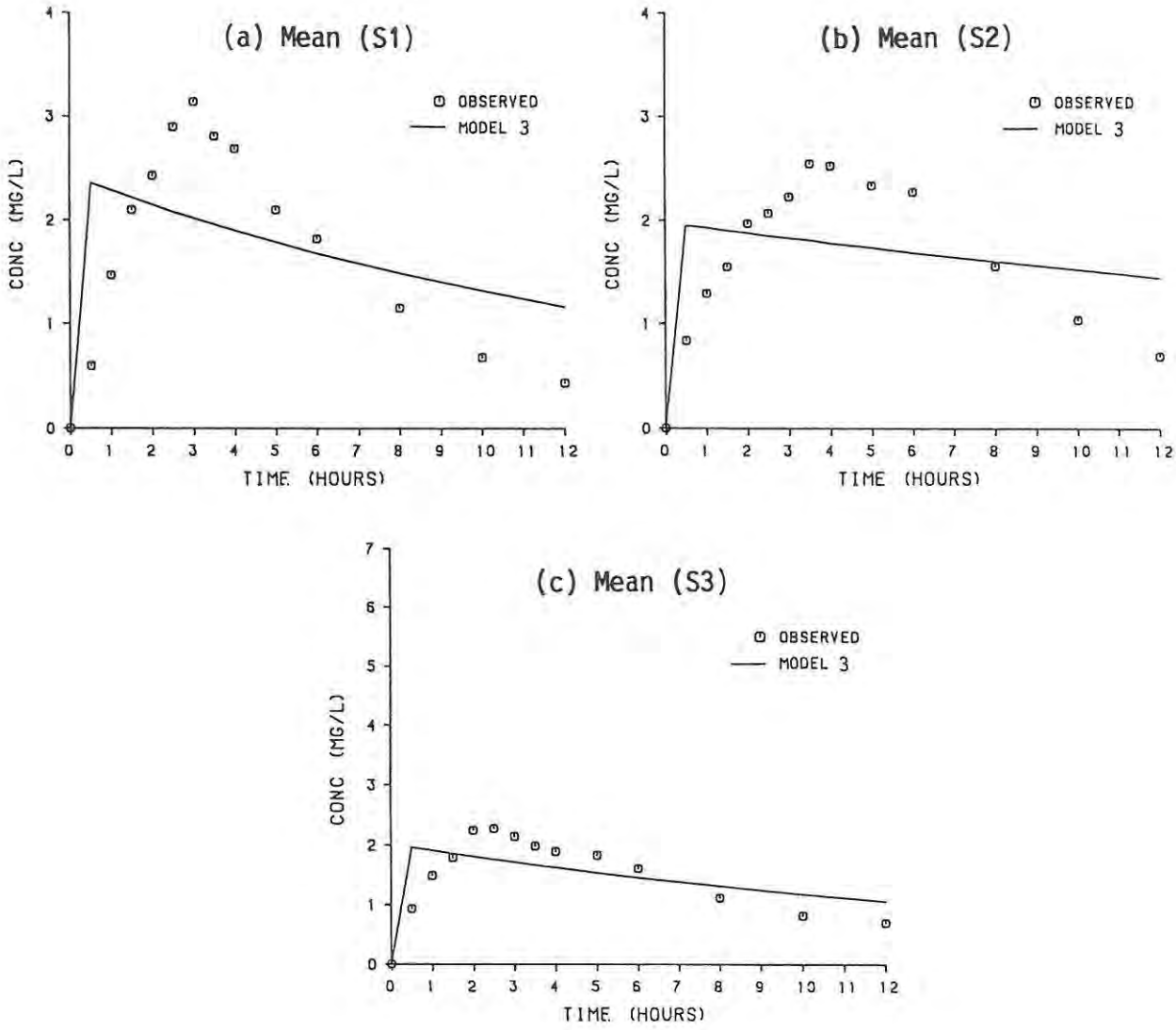


Table 7.3 Comparison of pharmacokinetic models - Akaike's information criteria (AIC)

Study	Model 1		Model 2	
	Correlation coefficient	AIC	Correlation coefficient	AIC
Mean S1	0.980	1.40	0.312	53.17
Mean S2	0.987	-15.20	0.970	- 1.49
Mean S3	0.992	-27.10	0.170	44.58
Mean S2	0.987	-15.41	0.987	-10.79
Mean S3	0.989	-21.49	0.987	-10.79

7.3.2 COMPARTMENTAL ANALYSIS - TOTAL ERYTHROMYCIN CONCENTRATIONS

Observed and calculated "total erythromycin" serum concentrations obtained by computer fitting of data to models 1 and 2 are reported in appendices 6-11. Inspection of these tables and of corresponding individual serum concentration-time curves reveals that in many cases the appropriateness of the respective models varied when 12 and 24 hour data were fitted. This phenomenon is illustrated in figure 7.2 in which the results of computer fitting of mean 12 and 24 hour data from studies S1-S3 using models 1 and 2 are graphically presented. For study S1 (only 12 hour data were available), model 1 appeared to be more appropriate than model 2 (Fig.7.2(a)) although the computer derived data did not correlate with experimental data in the region of the maximum serum concentration. In study S2 both models 1 and 2 appeared equally appropriate when both 12 and 24 hour data were utilized (Fig.7.2(b)&(d)), whereas in study S3 model 1 proved superior to model 2 in both cases (Fig.7.2(c)&(e)). As was found for study S1, model 1 did not describe the mean data of studies S2 and S3 perfectly, but replicated the general trend of the experimentally determined concentration-time profiles. Comparison of AIC values for these mean data sets (Table 7.3) revealed that model 1 was superior to model 2 in all instances. Although the mean data of studies S1-S3 appeared to be reasonably well represented by model 1, modelled data obtained for the individual patients showed varied degrees of correlation with experimentally obtained data, and in some cases were extremely poorly simulated. Individual serum concentration-time plots of experimental and model generated data for patients P1-P6 in study S2 are depicted in figure 7.3. Although both models 1 and 2 yield reasonable approximates of experimentally obtained data for patients P1,P3, and P5, extremely poor correlations were obtained for the other three patients, particularly for patient P6. Serum concentration-time profiles generated using model 3 correlated extremely poorly with experimental data (Fig. 7.4) which was in contrast to the findings of Welling *et al* (78)(Section1.4.4). More complex models incorporating multiple rate constants (207,220-222) and multiple compartments would presumably be required for more accurate simulations of these data.

Estimates of k_a and k for study S1 were found to converge to the same value when fitted to model 1 without either parameter being constrained (Appendix 12). This trend has been explained in terms of data variance due to a lack of data points (223) and it has been suggested that k values

Table 7.4 Comparison of k_a values obtained from NONLIN

Total Erythromycin Concentrations - 12 hour data.

	Model 1 k not constrained		Mode 1 k set at value (1)*		Model 1 k set at value (2)*		Values obtained by curve stripping		Wagner-Nelson Method
+	k_a	k	k_a	k	k_a	k	k_a	k	k_a
Mean S1	0.34	0.34	0.47	0.24	0.49	0.23	1.00	0.23	0.61
Mean S2	0.27	0.27	0.34	0.21	0.35	0.20	0.48	0.19	0.36
Mean S3	0.16	0.72	0.86	0.13	0.86	0.13	1.14	0.13	0.86
Individual Sublects (S3)									
P1	0.52	0.27	0.75	0.18	0.78	0.17	1.83	0.17	1.67
P3	0.99	0.20	1.50	0.12	1.57	0.14	2.06	0.13	1.92
P4	0.17	0.17	0.12	0.25	0.12	0.26	-	0.26	0.57
P5	0.12	0.76	0.51	0.18	0.76	0.12	0.96	0.13	0.64
P6	0.40	0.41	0.63	0.18	0.80	0.19	0.98	0.19	0.48

KEY * Value (1) = Value for k obtained by linear regression analysis of terminal slope of log linear serum concentration time curve.

* Value (2) = Value for k obtained by curve stripping techniques.

+ = All in h^{-1}

which are more easily obtainable by manual curve stripping techniques, be constrained during computer fitting (78). Table 7.4 lists k_a values obtained using model 1 for selected mean and individual data sets with values of k not constrained, set at the value obtained by manual curve stripping and at the value obtained by linear regression of the terminal phase of a semi-log plot of serum concentration versus time. In addition, values for k_a obtained by curve stripping and the Wagner-Nelson method are tabulated. Good correlation was observed between k_a values calculated by the Wagner-Nelson method and those obtained from model 1 with k fixed at terminal slope values, for the mean data of studies S2 and S3, but not for the individual patient data or the mean profile of study S1. Values of k_a calculated by curve stripping techniques did not correlate with values obtained by other methods. Gibaldi and Perrier (202) have shown that curve stripping techniques yield unsatisfactory results unless the k_a is at least three times greater than the value of the elimination rate constant.

Computer fitting techniques have also been shown to yield substantial errors for parameter estimates if, as is the case for erythromycin, data from i.v. studies suggests a two compartmental system whereas data following oral administration may be fitted to a one compartment model (202). In addition, k_a values calculated by the methods used in this study, are estimations of the first order loss of drug from the intestinal tract and not of the first order appearance of drug *in vivo* (202). Any loss of drug due to gastric acid degradation or gut wall metabolism as well as variations in gastric emptying or gastrointestinal motility effects the validity of the calculated k_a values considerably (224). Despite these problems, k_a values obtained using these methods are considered good enough approximations for the comparison of relative absorption rates in comparative studies (225) although the large intra-personal variations in k_a encountered in these studies suggest that this parameter would be of limited use in comparative studies unless large numbers of patients were employed.

The values for volume of distribution (V) obtained from computer analysis of data utilizing model 1 (Appendix 12) are not accurate estimations of this parameter, since i.v. data are required for this purpose (202,205) (unless an accurate estimation of F which remains relatively constant for all subjects during all administrations is available). In order to compensate for changes in F, the term D/V in equation 7.5, which should actually be FD/V, constantly accommodates by varying the value of V, since D is defined as a constant in the DFUNC routine. It is important that when setting the initial estimates in the data file of NONLIN that the limits of this parameter are sufficiently large to allow for these fluctuations.

Table 7.5 The effect of weighting functions on NONLIN

Mean S1 Total Erythromycin 12 hr data.

	k_a (h^{-1})	V (ℓ)	k (h^{-1})	t_{lag} (h)	r^2	Weighted Σ squares	Cor	AIC
Equal	0.34	68.90	0.34	0.03	0.947	0.720	0.980	1.40
$1/y$	0.34	72.12	0.34	0.03	0.951	0.668	0.980	0.35
$1/y^2$	0.33	80.33	0.33	0.03	0.966	0.459	0.981	-4.90
$1/\sqrt{y}$	0.34	70.23	0.34	0.03	0.948	0.711	0.980	1.22

Figure 7.5 Comparison of observed and predicted (Models 1&2) mean erythromycin base and propionate serum concentrations (S2,S3).

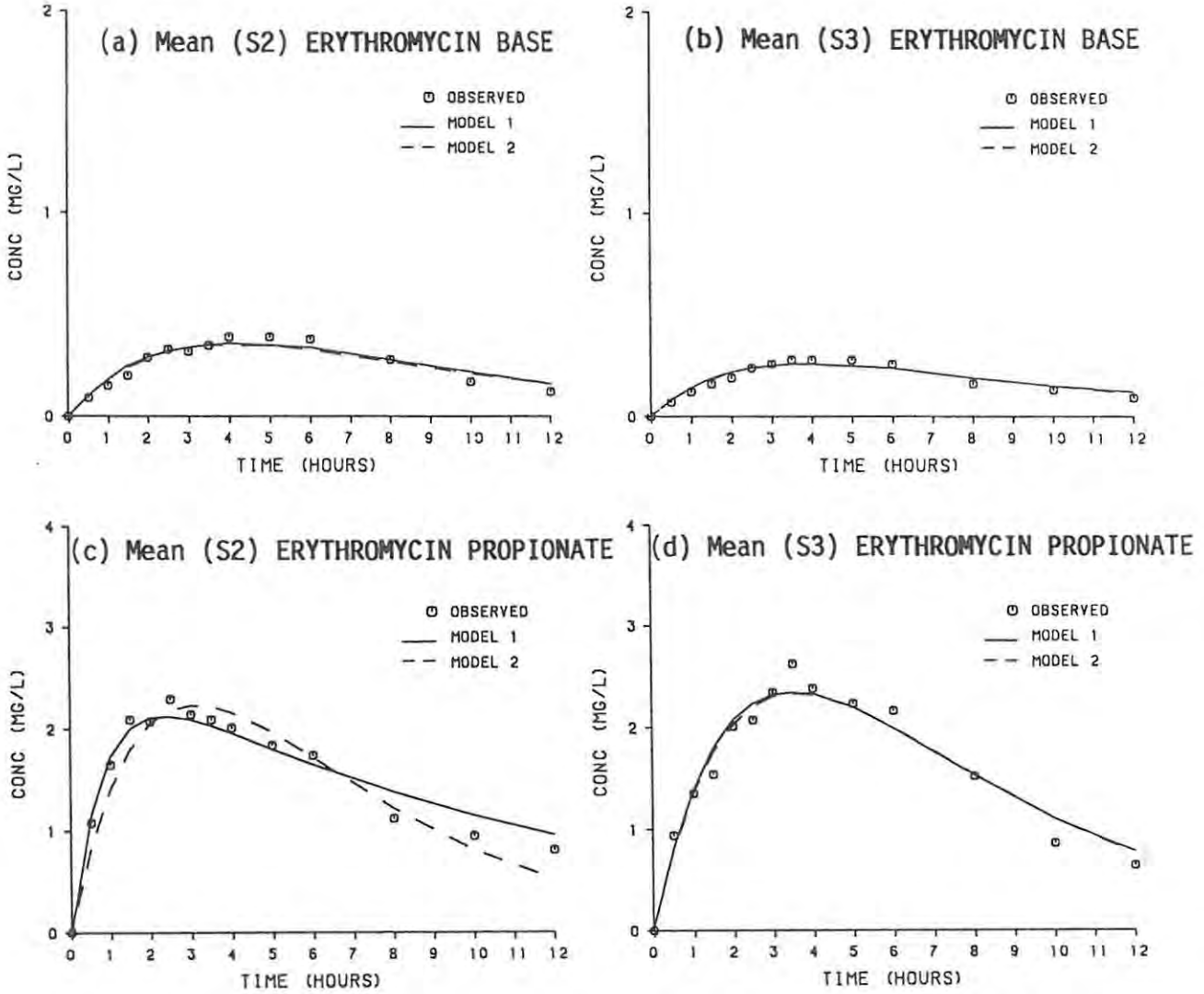


Table 7.6 Pharmacokinetic parameters (model 1) for erythromycin base and propionate

Study	k_a (h^{-1})	V (ℓ)	k (h^{-1})
Base - (S2)	0.27	58.25	0.21
Base - (S3)	0.40	108.66	0.15
Ester - (S2)	0.37	102.84	0.21
Ester - (S3)	1.19	197.36	0.09

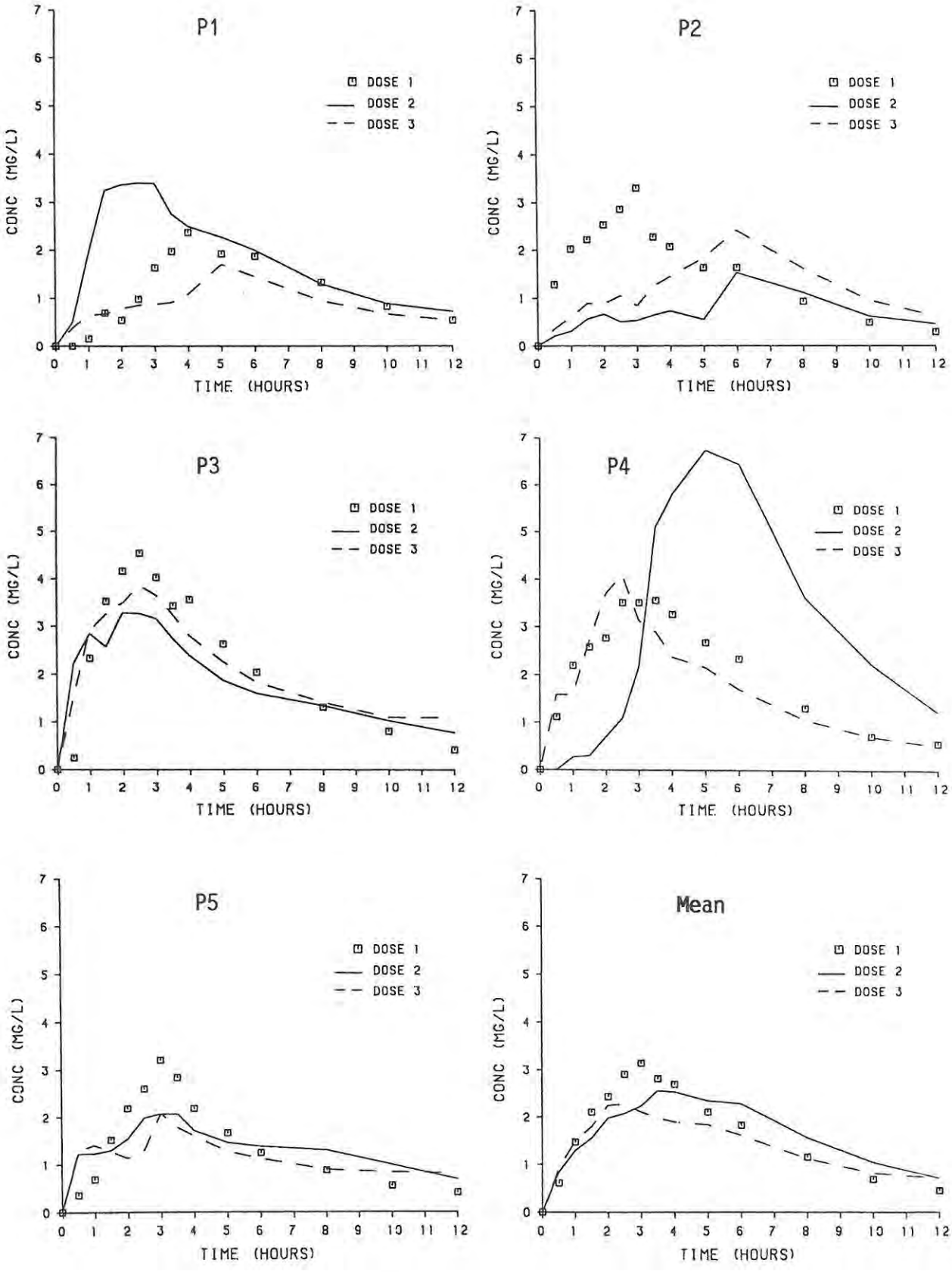
Attempts were made to vary the weighting of data during operation of NONLIN using model 1. The weighting function $1/Y^2$ yielded the best computer fits for the data set tested (Table 7.5). However, equal weighting of data was still used throughout the study since calculated serum concentration values were unchanged and because the implication of these functions was unclear (226). Peck *et al* (227) discussed the limitation of the weighting functions available using NONLIN and have suggested a revised system of functions based on extended least squares analysis which may be investigated for future studies.

In summary, "total erythromycin" concentrations following single dose administration were best described by an open one-compartment model with linear first order absorption and elimination. Values for k_a varied dramatically depending on the method of calculation as well as between patients and administrations making comparisons with published data of little use and suggesting that erythromycin is not a good candidate for detailed compartmental pharmacokinetic analysis.

7.3.3 COMPARTMENTAL ANALYSIS - ERYTHROMYCIN BASE AND PROPIONATE

Mean experimental and computer derived (models 1&2) serum concentration versus time profiles for erythromycin base and propionate obtained for studies S2 and S3 are depicted in figure 7.5 and the observed and calculated serum concentrations are summarized in appendix 11. From visual inspection of figure 7.5 and from the calculation of AIC values it was found that model 1 best described the absorption and disposition of both compounds although erythromycin base concentrations were more accurately simulated than those of the ester. Values for k were held constant during computer fitting using model 1 as previously described, therefore, no additional values for this parameter were obtained (Table 7.6). The calculated values for k_a (Table 7.6) were obviously effected by the same previously discussed limitations but did provide some means of comparing the rate of erythromycin base absorption following erythromycin estolate administration with that following erythromycin base administration. The k_a and k values calculated for erythromycin base are actually composite terms of the rates of absorption and elimination respectively, and the *in vivo* rate of hydrolysis of the propionyl ester. Additional unpublished data (20) obtained following oral administration of erythromycin base and stearate to three of the volunteers used in this study under identical experimental protocols showed that both the rate of absorption and elimination of

Figure 7.6 Total erythromycin serum concentration versus time plots for single dose studies S1-S3.



erythromycin base are slower following erythromycin estolate administration. The decreased k may be explained by the additional input from the hydrolysis of the propionyl ester during the elimination phase. The fact that the k_a for erythromycin base following the administration of erythromycin estolate is lower than that following administration of erythromycin base suggests that the majority of erythromycin base *in vivo* following estolate administration does not result from erythromycin base absorption (formed by hydrolysis of the ester in the gastrointestinal fluids) but from the *in vivo* hydrolysis of the propionyl ester.

7.3.4 INTRA- AND INTER-PERSONAL VARIABILITY IN ERYTHROMYCIN ABSORPTION AND DISPOSITION

Total erythromycin serum concentrations following 3 separate administrations of the same erythromycin estolate tablet are presented in figure 7.6. From these curves it is evident that both intra- and inter-personal variations in erythromycin absorption and disposition occurred, but it is not possible to evaluate or quantify these differences. Comparison of the relative standard deviations calculated for some non-compartmental parameters (Table 7.7) revealed that intra-personal variations in these parameters were of a similar magnitude to inter-personal variations but the data were too scattered to make any definitive judgements as to the significance of these differences. Graffner *et al* (153), who introduced discrepancy values for comparison of intra-personal variability (Fig. 7.7) also proposed tentative limits for the degree of these discrepancies.

Table 7.7 Intra- and inter-personal variability in model independent parameters - single dose studies

Parameter	Inter-Personal Variation (R.S.D. %)			Intra-Personal Variation (R.S.D. %)				
	S1	S2	S3	P1	P2	P3	P4	P5
C max (h)	23.0	59.7	38.2	34.4	41.4	16.2	35.6	26.7
T max (h)	17.8	45.2	42.2	32.8	34.7	12.4	34.3	4.7
AUC_{0-12} (µg/ml.h)	24.5	52.3	30.2	35.3	30.4	6.7	35.7	7.6
$t_{½}$(12h) (h)	14.3	30.8	55.5	11.1	14.2	52.2	11.8	>100.0
MRT (12h) (h)	17.2	9.1	27.9	12.3	24.8	36.5	13.6	18.2

Figure 7.7 Calculation of discrepancy values - Patient 1

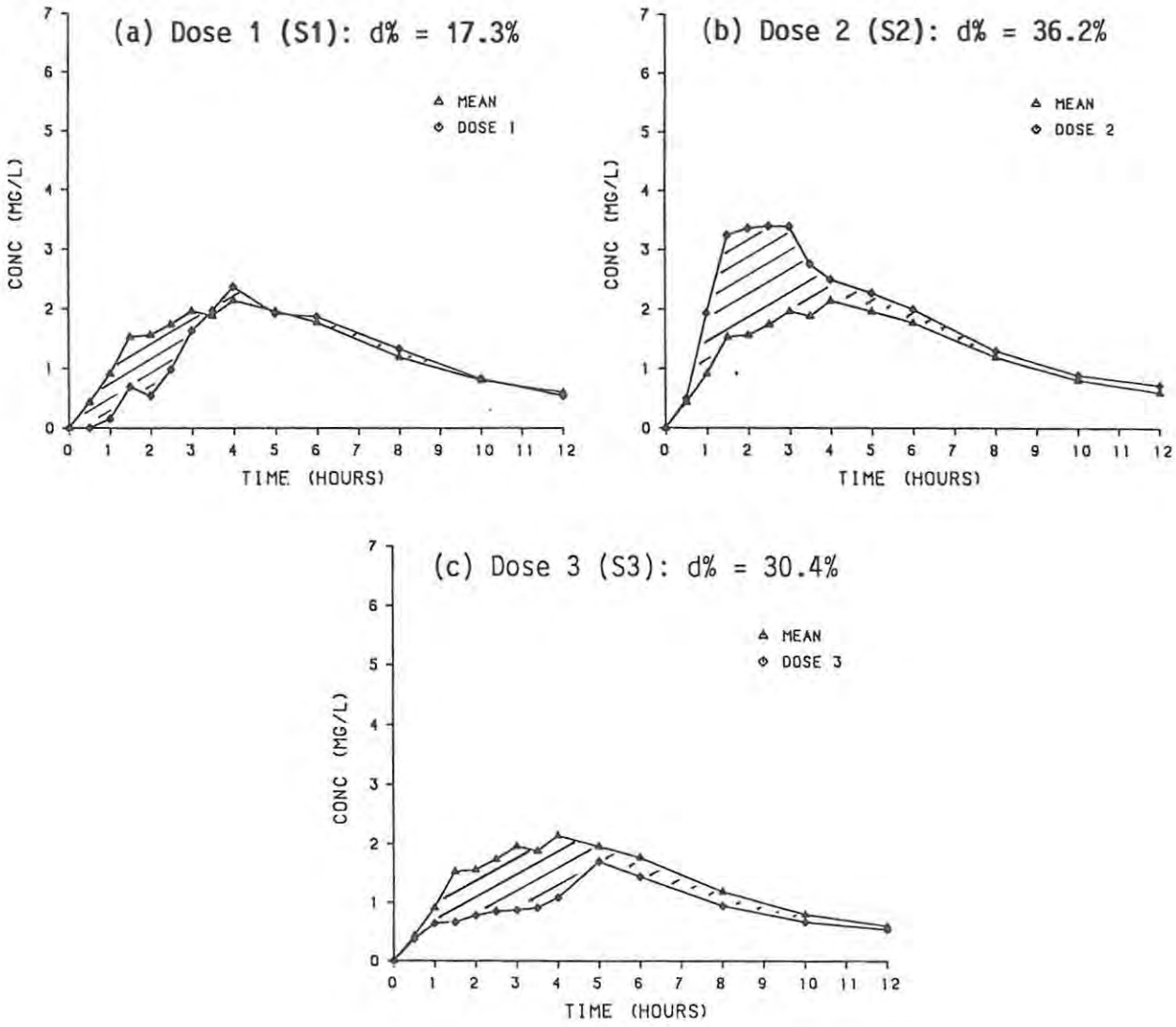


Table 7.8 Discrepancy values - total erythromycin data

	Patients			(d % values)		
Treatments	P1	P2	P3	P4	P5	Mean
S1	17.3	48.9	16.9	29.0	22.2	14.1
S2	36.2	34.4	7.2	53.2	9.7	18.6
S3	30.4	31.8	3.8	43.3	12.6	11.0
Mean	28.0	38.4	9.3	41.8	14.8	14.6

KEY d % = 0-20 % - small
= 21-35 % - moderate
= >35 % - large

Values for d% from 0-20%, 21-35% and >35% were proposed as being small, moderate and large discrepancies, respectively. Application of these limits to the results summarized in table 7.8 reveal that 2 patients had small discrepancies (P3,P5), 1 moderate (P1) and 2 large (P2,P4). The mean curves for each administration fell into the small discrepancy class in all cases, while the mean of all individual discrepancies (26.5%) was classified as moderate. This value was similar to the value previously obtained (153) for an erythromycin base dosage form containing enteric coated pellets (23.5%) but was smaller than that reported for an enteric coated erythromycin base tablet (73.2%).

In summary, calculation of discrepancy values proved to be a valuable method for the comparison of intra-personal variability in erythromycin absorption and disposition as well as providing a means for comparing these variations with previously published studies. Considering that variations in tablet uniformity were minimal and the extent to which differences in trial protocols were controlled, the intra-personal variations in erythromycin absorption and disposition reported in this study should constantly be borne in mind during the design of comparative bioavailability studies involving erythromycin estolate dosage forms.

CONCLUSIONS

Various aspects of the stability and pharmacokinetics of erythromycin have not been previously addressed due to the lack of suitable analytical techniques. A major problem has been the interpretation of results from biopharmaceutical studies in which the acid-stable (e.g. erythromycin estolate) pro-drug forms of erythromycin have been involved, since selective determination of the active erythromycin base and inactive ester forms is complicated by continued hydrolysis of the ester moiety during sample manipulation and storage.

The method described in this study which involves solid-phase extraction of biological samples prior to chromatography on a reversed-phase column with coulometric electrochemical detection provided the required sensitivity, precision and accuracy for the study of the pharmacokinetics of both erythromycin base and propionate following the oral administration of erythromycin estolate. The sample collection and storage procedures have been shown to minimize ester hydrolysis prior to chromatography, thereby allowing an accurate assessment of the situation *in vivo*. Results of pharmacokinetic studies utilizing these analytical techniques have shown that only 10-15% of the total erythromycin *in vivo* following single dose administration of erythromycin estolate was due to erythromycin base. Previous estimates of this value have varied between 16 and 35%. Published findings that the amount of erythromycin base relative to erythromycin propionate increases considerably during multiple dose administration were confirmed in this study.

Studies in urine showed that only 3-4% of a single dose of erythromycin estolate was excreted in urine as either erythromycin base or propionate. The percentage of erythromycin base in the total amount of erythromycin excreted in urine varied from 35-40% after single dose administration to 60% during multiple dose administration. Two other unidentified compounds were excreted in urine, particularly during multiple dose administration. It is hoped that relatively minor modifications to the chromatographic system employed in this study will allow mass spectrographic identification of these compunds.

Analysis of erythromycin estolate dosage forms was performed by HPLC with both UV and coulometric electrochemical detection. Similar results were obtained with either of these two modes of detection, both of which proved

equally successful. Samples of erythromycin suspensions stored for up to three years at room temperature contained only minimal amounts of erythromycin base, indicating excellent stability of these dosage forms.

The stability of erythromycin base in stored serum and urine was assessed by HPLC with amperometric electrochemical detection. This analytical procedure proved highly successful and was also able to detect the presence of the principle acid degradation product of erythromycin, anhydro-erythromycin. Serum samples containing erythromycin base were shown to be stable for up to 12 weeks at both 4°C and -15°C whereas urine samples were shown to degrade after 1 week at 4°C and 2 weeks at -15°C. This information has proved most useful in the performance of large scale biopharmaceutical trials since it is seldom possible to analyse samples immediately following the clinical phase of these experiments.

The analytical method described for the determination of "total erythromycin" concentrations in human serum by prior hydrolysis of serum samples proved invaluable during comparative bioavailability studies since sample collection and storage procedures could be greatly simplified. In addition, the method served as a valuable control of the discriminatory assay procedure during the pharmacokinetic trials described in this study. The solid-phase extraction procedure employed with both these methods was highly suited to the semi-automated analysis of large numbers of samples since up to 60 samples could be simultaneously processed without constant operator attention. Total erythromycin concentrations obtained using the discriminatory assay procedure were consistently higher (10%) than those obtained by prior hydrolysis of serum samples. Hydrolysis studies showed that the hydrolysis conditions employed in this study ensured total conversion of the propionyl ester prior to chromatography. Similar studies suggested that this was not true for the conventional microbiological techniques nor for the conditions proposed by Yakatan *et al* (113).

Pharmacokinetic analysis of both "total erythromycin" and erythromycin base and propionate data revealed that a one compartment model with linear first-order absorption and elimination best described the absorption and disposition of erythromycin. Individual data sets were not as well simulated by this model as were the mean data sets. Some evidence of non-linear elimination of erythromycin was evident over the 10-24 hour time period, however, further studies are required in order to substantiate these findings. This phenomenon affected the calculation of both model

dependent and model independent pharmacokinetic parameters and made the comparison of parameter values obtained in this study with previously published studies extremely difficult.

Large intra- and inter-personal variations in erythromycin absorption and disposition were apparent, which were evaluated by the repeat administration of identical erythromycin estolate tablets to the same volunteers on three separate occasions under identical experimental conditions. These variations were classified as moderate, utilizing a previously reported method (153) of discrepancy values and should constantly be borne in mind during the design of pharmacokinetic studies involving erythromycin estolate dosage forms.

Appendix 1 Consent forms - Single and multiple dose studies

CONSENT TO ACT AS A RESEARCH STUDENT-SINGLE DOSE TRIAL

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 check the effectiveness of a recently developed analytical method for the determination of erythromycin propionate and erythromycin base in human serum after the oral administration of a tablet containing 500 mg of erythromycin estolate.

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 p.m. on the day preceeding the trial. I will arrive at the School of Pharmaceutical Sciences at 7.30 a.m. on the day of the trial and a blood sample will be drawn. I will then be given the tablet containing erythromycin estolate with a 200 ml glass of water followed immediately by a light breakfast. Blood samples will be drawn at one half, one, one and a half, two, two and a half, three, four,six, eight, ten and twelve hours after taking the dose. I will not take any drugs for one week preceeding the trial and I will follow all dietry instructions during the study.

3. The incidence of untoward effects associated with the use of erythromycin estolate is low but reversible jaundice and liver disease have been associated with this erythromycin derivative. Any history of liver disease would then proclude the subject from this study. Other possible side effects include gastric irritation as well as hypersensitivity reactions including 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 Mr. C. Stubbs and Professor I. Kanfer. I understand that they will answer any questions I may have concerning this investigation.

6. I will be paid for participating 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 jeapardy to my standing whatsoever.

CONSENT TO ACT AS A RESEARCH SUBJECT - MULTIPLE DOSE TRIAL

1. Erythromycin is a macrolide antibiotic used in the treatment of many common infections particularily those caused by gram positive organisms. The purpose of this study is to study the pharmacokinetics at steady state of both erythromycin propionate and erythromycin base, after the administration of 5 doses of erythromycin estolate (500 mg) given at 6 hourly intervals.

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 two day trial. I will arrive at the School of Pharmaceutical Sciences at 7.45 am on the first day of the trial and a blood sample will be drawn. I will then be given the first tablet containing erythromycin estolate with a 200 ml glass of water. A light breakfast will be served at 10.00 am and and a light lunch at 12.00 noon. I will be given the second dose at 2.00 pm. (with 200 ml water) after which I will fast for at least 2 hr. I will eat supper at exactly 6.00 pm and receive the third dose at 8.00 pm. I will then report to 1 Bowles Street (before 10.00 pm) where I will be served a light snack at 12 midnight and will receive the fourth dose at 2.00 am. The following morning I will be taken to the School of Pharmaceutical Sciences at 7.45 am and a blood sample will be taken afterwhich I will receive the fifth dose. Blood samples will be drawn at ½, 1, 1½, 2, 2½, 3, 3½, 4, 5, 6, 8, 10, 12 and 24 hrs. Urine will be collected at 0 hrs, 0-2, 2-4, 4-6, 6-8, 8-10, 10-12 and 12-24 hr intervals. I will receive breakfast at 10.30 am and lunch at 1.15 pm as well as 200 ml water every 2 hrs. I will not take any drugs for one week preceeding 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. The risk of these side-effects does increase with the administration of multiple doses of the drug.

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 Professor I Kanfer. I understand that he will answer any questions I may have concerning this investigation.

6. I will be paid for participating 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 jeapardy to my standing whatsoever.

NAME: L. Hillary DATE: 5/5/87

SIGNATURE: 

Appendix 2 Serum and urine data (S2)

	P1				P2				P3			
TIME(HRS)	BASE	ESTER	TOTAL[1]	TOTAL[2]	BASE	ESTER	TOTAL[1]	TOTAL[2]	BASE	ESTER	TOTAL[1]	TOTAL[2]
0	-	-	-	-	-	-	-	-	-	-	-	-
0.5	-	0.52	0.52	0.49	-	0.20	0.28	0.20	0.22	2.48	2.70	2.22
1	0.27	2.00	2.27	1.93	-	0.33	0.33	0.30	0.30	2.95	3.25	2.85
1.5	0.48	3.22	3.70	3.24	-	0.56	0.56	0.56	0.30	2.63	2.93	2.57
2	0.51	3.40	3.91	3.36	0.18	0.63	0.81	0.66	0.42	3.05	3.47	3.28
2.5	0.62	3.45	4.07	3.40	0.15	0.57	0.72	0.50	0.44	3.34	3.78	3.27
3	0.51	3.26	3.77	3.39	0.15	0.63	0.78	0.53	0.35	3.20	3.55	3.16
3.5	0.54	3.01	3.55	2.76	0.15	0.84	0.99	0.64	0.35	2.63	2.98	2.74
4	0.51	2.56	3.07	2.95	0.15	0.84	0.99	0.73	0.38	1.89	2.27	2.39
5	0.46	2.16	2.62	2.27	0.15	0.62	0.77	0.55	0.30	1.69	1.99	1.87
6	0.38	1.86	2.24	2.00	0.32	2.00	2.32	1.53	0.23	1.51	1.74	1.60
8	0.24	1.17	1.41	1.30	0.25	1.04	1.29	1.12	0.20	1.22	1.42	1.34
10	0.17	0.92	1.09	0.89	0.19	0.79	0.98	0.62	0.14	0.98	1.12	1.03
12	0.14	0.67	0.81	0.72	0.14	0.47	0.61	0.46	0.10	0.56	0.66	0.78
24	-	0.21	0.21	0.27	0.20	0.35	0.35	0.39	-	0.26	0.26	0.29
INTERVAL (HRS)	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH
0	-	-	-	5.44	-	-	-	5.28	-	-	-	5.70
0-2	272.0	1121.0	1393.0	6.36	116.0	223.3	339.3	6.79	446.7	2502.8	2949.5	5.94
2-4	3244.0	4621.0	7865.0	6.72	564.0	867.3	1431.3	6.57	903.7	5079.8	5983.5	5.82
4-6	5251.4	8477.9	13729.3	6.43	1075.0	1458.6	2533.6	6.77	1647.0	7316.4	8963.4	5.84
6-8	6416.2	9565.4	15981.6	6.70	2410.3	3041.3	5451.6	6.79	2075.8	8262.9	10338.7	5.99
8-10	7751.8	9960.2	17712.0	7.23	3138.3	4097.3	7235.6	6.03	2273.8	8854.0	11127.8	5.90
10-12	9502.3	10561.2	20063.5	7.10	3406.9	5011.9	8418.8	5.16	3025.8	9731.0	12756.8	5.66
12-24	11052.5	11568.6	22612.1	5.94	3999.7	6860.5	10860.2	5.29	3777.8	10640.5	14418.3	5.65

	P4				P5				P6			
TIME(HRS)	BASE	ESTER	TOTAL[1]	TOTAL[2]	BASE	ESTER	TOTAL[1]	TOTAL[2]	BASE	ESTER	TOTAL[1]	TOTAL[2]
0	-	-	-	-	-	-	-	-	-	-	-	-
0.5	-	-	-	-	0.17	1.29	1.46	1.22	0.18	1.09	1.27	0.90
1	-	0.28	0.28	0.27	0.16	1.27	1.43	1.23	0.18	1.24	1.42	1.14
1.5	-	0.29	0.29	0.30	0.22	1.27	1.49	1.30	0.18	1.29	1.47	1.30
2	0.11	0.89	1.00	0.70	0.22	1.62	1.84	1.54	0.34	2.45	2.79	2.25
2.5	0.15	1.19	1.34	1.09	0.30	1.72	2.02	1.98	0.29	2.15	2.44	2.19
3	0.23	2.40	2.63	2.16	0.32	2.16	2.48	2.07	0.32	2.36	2.68	2.09
3.5	0.45	5.45	5.90	5.11	0.38	1.92	2.30	2.07	0.23	1.85	2.08	1.95
4	0.64	5.60	6.25	5.82	0.35	1.66	2.01	1.71	0.34	1.70	2.04	1.58
5	0.90	6.11	7.01	6.76	0.29	1.57	1.86	1.46	0.23	1.23	1.46	1.13
6	0.88	5.13	6.01	6.46	0.26	1.32	1.58	1.38	0.21	1.14	1.35	0.97
8	0.64	2.56	3.20	3.63	0.22	1.41	1.63	1.30	0.15	0.69	0.84	0.65
10	0.23	1.50	1.17	2.24	0.15	0.97	1.12	0.99	0.15	0.54	0.69	0.47
12	0.19	1.04	1.23	1.23	0.13	0.77	0.90	0.68	-	0.34	0.34	0.32
24	-	-	-	-	-	0.22	0.22	0.30	-	-	-	0.15
INTERVAL (HRS)	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH
0	-	-	-	7.32	-	-	-	6.80	-	-	-	5.65
0-2	-	-	-	6.75	244.8	316.8	561.6	7.32	88.4	1090.7	1179.1	5.27
2-4	-	-	-	6.70	516.4	1228.6	1745.0	7.42	268.6	3173.6	3442.2	5.55
4-6	2276.4	9110.7	11387.1	6.07	2833.9	2459.5	5293.5	6.99	600.4	6574.2	7174.6	5.24
6-8	6089.4	20036.7	26126.1	6.10	3936.5	3478.7	7415.2	6.67	1058.0	8001.6	9059.6	5.46
8-10	8565.6	26560.8	35126.4	5.49	4866.7	4468.2	9334.9	6.61	1638.4	9143.8	10782.2	6.10
10-12	10753.5	30402.3	41155.8	5.86	6635.3	6127.9	12763.2	6.80	1843.6	9783.7	11627.3	5.47
12-24	11236.5	31355.7	42592.2	5.42	8002.3	7621.0	15623.3	6.50	3024.9	9927.5	12952.4	6.57

BASE = Erythromycin base concentrate (µg/ml).
ESTER = Erythromycin propionate concentration (µg/ml).
TOTAL[1] = Total erythromycin concentrations obtained by addition of erythromycin base and propionate concentrations (Chapter 2).
TOTAL[2] = Total erythromycin concentrations obtained by hydrolysis of samples prior to chromatography (Chapter 3).
CUM BASE = Cumulative amount of erythromycin base excreted in urine (µg).
CUM ESTER = Cumulative amount of erythromycin propionate excreted in urine (µg).
CUM TOTAL = Cumulative amount of erythromycin (base + propionate) excreted in urine (µg).

Appendix 3 Serum and urine data (S3)

	P1				P2				P3			
TIME(HRS)	BASE	ESTER	TOTAL 1	TOTAL 2	BASE	ESTER	TOTAL 1	TOTAL 2	BASE	ESTER	TOTAL 1	TOTAL 2
0	-	-	-	-	-	-	-	-	-	-	-	-
0.5	-	0.48	0.48	0.37	-	0.43	0.43	0.35	0.17	1.91	2.08	1.51
1	-	0.69	0.69	0.64	-	0.61	0.61	0.60	0.26	3.22	3.48	2.90
1.5	-	0.69	0.69	0.66	-	1.00	1.00	0.89	0.35	4.18	4.63	3.25
2	0.06	0.67	0.73	-	-	0.94	0.94	0.88	0.39	4.45	4.84	3.49
2.5	0.06	0.77	0.83	0.85	0.10	0.97	1.07	1.05	0.42	4.99	5.41	3.86
3	0.10	0.78	0.88	0.87	0.15	0.73	0.88	0.84	0.45	3.93	4.38	3.65
3.5	0.15	0.85	1.00	0.91	0.14	1.46	1.60	1.25	0.40	3.57	3.97	3.24
4	0.17	1.00	1.17	1.09	0.17	1.89	2.06	1.25	0.36	2.82	3.18	2.80
5	0.22	1.45	1.67	1.70	0.32	1.97	2.29	1.83	0.29	2.35	2.64	2.26
6	0.22	1.33	1.55	1.45	0.33	2.51	2.84	2.42	0.24	2.21	2.54	1.83
8	0.12	0.91	1.03	0.95	0.26	1.45	1.71	1.62	0.17	1.37	1.54	1.41
10	0.10	0.63	0.72	0.67	0.18	0.94	1.12	0.96	0.14	1.64	1.78	-
12	0.08	0.68	0.74	0.54	0.11	0.59	0.70	0.62	0.14	1.44	1.58	1.09
24	0.00	0.53	0.61	0.67	-	0.38	0.38	0.32	-	0.34	0.34	0.34
INTERVAL (HRS)	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH
0	-	-	-	5.86	-	-	-	5.33	-	-	-	5.92
0-2	213.0	6.0	219.0	7.67	-	369.4	369.4	6.08	298.2	1919.4	2217.6	7.31
2-4	627.4	226.1	853.5	7.32	462.0	908.4	1370.4	6.73	2388.2	4071.0	6459.2	7.07
4-6	1406.5	920.4	2326.9	6.67	1803.9	2895.9	4699.8	6.65	3423.2	6120.3	9543.5	7.06
6-8	2167.0	1060.2	3227.2	7.61	4469.6	4011.1	8480.7	7.49	4127.9	7347.0	11474.9	6.41
8-10	2712.0	1359.0	4071.0	7.10	4872.4	5392.4	10264.8	5.56	4696.7	8057.4	13601.6	5.47
10-12	3043.5	1674.4	4717.9	7.10	5070.4	6240.7	11311.1	5.39	4984.0	8904.9	13888.9	5.36
12-24	5187.0	4228.7	9915.7	6.27	7066.5	7972.8	15039.3	6.00	6060.0	9267.1	15327.1	6.06

	P4				P5				P6			
TIME(HRS)	BASE	ESTER	TOTAL 1	TOTAL 2	BASE	ESTER	TOTAL 1	TOTAL 2	BASE	ESTER	TOTAL 1	TOTAL 2
0	-	-	-	-	-	-	-	-	-	-	-	-
0.5	0.12	1.51	1.63	1.58	0.12	1.57	1.69	1.29	-	0.58	0.58	0.45
1	0.15	1.83	1.98	1.58	0.12	1.58	1.70	1.41	0.18	1.97	2.15	1.81
1.5	0.29	2.92	3.21	2.72	0.12	1.48	1.60	1.28	0.22	2.26	2.48	1.95
2	0.35	3.05	3.40	3.70	0.12	1.35	1.47	1.13	0.20	1.93	2.13	2.02
2.5	0.40	3.49	3.89	4.10	0.16	1.58	1.74	1.31	0.27	1.93	2.20	2.46
3	0.32	2.97	3.29	3.14	0.25	2.38	2.63	2.08	0.31	2.04	2.35	2.04
3.5	0.41	2.84	3.25	2.91	0.22	1.95	2.17	1.78	0.33	1.88	2.21	1.76
4	0.39	2.98	3.37	2.38	0.24	1.61	1.85	1.60	0.33	1.77	2.10	1.55
5	0.35	2.16	2.51	2.16	0.17	1.37	1.54	1.27	0.31	1.73	2.04	1.75
6	0.27	1.74	2.01	1.70	0.19	1.31	1.50	1.12	0.24	1.32	1.56	1.13
8	0.15	1.05	1.20	1.06	0.13	1.05	1.18	0.89	0.15	0.91	1.06	0.77
10	0.10	0.70	0.80	0.71	0.14	1.02	1.16	0.83	0.11	0.77	0.88	0.90
12	0.07	0.52	0.59	0.52	0.10	0.95	1.05	0.81	0.06	0.68	0.76	0.61
24	-	0.57	0.57	0.25	-	0.42	0.42	0.37	-	0.45	0.45	0.34
INTERVAL (HRS)	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH
0	-	-	-	5.92	-	-	-	5.97	-	-	-	5.67
0-2	817.6	554.8	1372.4	7.31	691.2	723.2	1414.4	7.15	146.4	1689.0	1835.4	5.85
2-4	3933.1	5567.5	9500.6	7.07	2046.6	2100.2	4146.8	6.78	1160.6	4190.4	5351.0	6.20
4-6	6509.1	6268.9	12778.0	7.06	2652.6	4272.2	6924.8	5.94	2716.1	11138.3	13854.4	5.72
6-8	7784.4	7643.7	15428.1	6.41	3683.0	5468.2	9151.2	6.65	3628.7	11905.1	15533.8	6.87
8-10	8162.7	8757.2	16919.9	5.47	3960.4	6330.8	10291.2	5.78	4075.9	12570.7	16646.6	6.28
10-12	8415.9	9679.2	18095.1	5.36	4404.1	7369.0	11773.1	6.52	4295.9	13399.0	17694.9	5.44
12-24	8970.9	10256.7	19227.6	6.06	5346.3	10969.3	16315.6	5.31	5740.1	15540.2	21280.3	5.90

BASE = Erythromycin base concentrate (µg/ml).
ESTER = Erythromycin propionate concentration (µg/ml).
TOTAL 1 = Total erythromycin concentrations obtained by addition of erythromycin base and propionate concentrations (Chapter 2).
TOTAL 2 = Total erythromycin concentrations obtained by hydrolysis of samples prior to chromatography (Chapter 3).
CUM BASE = Cumulative amount of erythromycin base excreted in urine (µg).
CUM ESTER = Cumulative amount of erythromycin propionate excreted in urine (µg).
CUM TOTAL = Cumulative amount of erythromycin (base + propionate) excreted in urine (µg).

Appendix 4 Serum and urine data (MD)

	P1				P2				P3			
TIME(HRS)	BASE	ESTER	TOTAL^1	TOTAL^2	BASE	ESTER	TOTAL^1	TOTAL^2	BASE	ESTER	TOTAL^1	TOTAL^2
0	1.17	3.17	4.34	4.02	1.43	3.30	4.73	4.47	1.65	5.45	7.10	6.79
0.5	1.29	4.32	5.61	5.24	1.36	3.14	4.50	4.13	1.64	5.81	7.45	6.69
1	1.49	4.71	6.20	5.90	1.43	3.13	4.56	4.24	1.72	6.08	7.80	7.15
1.5	1.49	4.64	6.13	5.81	1.41	3.12	4.53	4.34	1.94	6.56	8.50	7.21
2	1.53	4.62	6.15	5.96	1.36	2.96	4.32	4.25	1.90	6.35	8.25	6.95
2.5	1.67	4.62	6.29	5.71	1.20	2.90	4.77	3.96	1.86	6.01	7.87	7.21
3	1.54	4.72	6.26	5.67	1.26	2.78	4.04	4.06	1.80	5.79	7.59	6.88
3.5	1.51	4.60	6.11	5.45	1.36	2.84	4.20	3.93	1.55	4.99	6.45	6.64
4	1.44	4.80	6.24	5.81	1.18	2.84	4.02	4.03	1.72	5.32	7.04	6.27
5	1.27	4.22	5.49	5.33	1.11	2.78	3.89	3.67	1.90	4.73	6.63	5.36
6	1.18	3.95	5.13	4.63	1.05	2.55	3.60	3.36	1.40	3.58	4.98	5.08
8	0.97	2.98	3.95	3.83	0.83	1.83	2.66	2.66	1.17	2.90	4.07	4.07
10	0.82	2.52	3.34	3.05	0.58	1.32	1.90	1.95	0.85	2.63	3.48	3.05
12	0.84	1.96	2.50	2.32	0.47	1.00	1.47	1.60	0.63	1.78	2.41	2.38
24	0.15	0.71	0.86	0.68	0.23	0.73	0.96	0.76	0.14	0.34	0.48	0.48
INTERVAL (HRS)	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH
0	993.6	99.0	1092.6	6.95	4950.8	8311.1	13261.9	6.22	5800.0	10396.0	16196.0	5.81
0-2	8156.6	569.3	8725.9	7.23	17225.7	8551.9	25777.6	7.37	12640.0	18106.0	30746.0	6.45
2-4	20168.6	1107.1	21275.7	7.22	25134.8	9050.9	34185.7	6.82	18660.0	24518.0	43178.0	6.22
4-6	27691.2	5689.1	33380.3	6.75	31275.1	14482.8	45757.9	6.31	28056.0	29918.0	57974.0	6.59
6-8	33455.7	8959.9	42415.6	6.71	35745.0	20614.9	56359.9	5.54	31064.5	32409.5	63474.0	6.19
8-10	35712.5	10505.5	46218.0	6.54	37305.1	22878.1	60183.2	5.35	35222.5	35863.5	71086.0	5.99
10-12	38016.5	12742.3	50758.8	5.89	38954.2	25289.3	64243.5	5.20	38354.5	38554.5	76909.0	6.04
12-24	42071.9	14505.2	56577.1	6.50	41117.9	28872.6	69990.5	5.30	40846.5	40878.5	81725.0	5.79

	P4				P5				P6			
TIME(HRS)	BASE	ESTER	TOTAL^1	TOTAL^2	BASE	ESTER	TOTAL^1	TOTAL^2	BASE	ESTER	TOTAL^1	TOTAL^2
0	1.83	4.82	6.65	6.32	1.51	4.76	6.27	5.74	0.98	2.56	3.54	3.61
0.5	1.66	5.06	6.72	6.31	1.58	4.69	6.27	5.74	0.90	2.67	3.57	3.57
1	1.58	5.01	6.59	7.40	1.48	4.97	6.45	6.37	1.00	3.34	4.34	4.04
1.5	1.80	6.13	7.93	7.08	1.52	4.96	6.49	6.29	1.02	3.37	4.39	4.52
2	1.81	5.85	7.66	7.12	1.58	5.07	6.65	5.64	1.07	3.52	4.59	4.09
2.5	1.77	5.62	7.39	6.71	1.48	4.91	6.39	5.66	1.32	2.87	4.19	4.87
3	1.86	5.68	7.54	7.20	1.60	5.12	6.72	6.40	1.19	3.76	4.95	5.03
3.5	1.88	5.48	7.36	7.82	1.71	4.79	6.50	6.67	1.19	3.39	4.58	4.80
4	1.80	5.02	6.82	7.25	1.69	4.70	6.39	6.41	1.16	3.39	4.55	4.31
5	1.67	5.73	7.40	6.06	1.58	4.25	5.83	5.67	1.00	3.27	4.27	3.82
6	1.43	3.48	4.91	5.18	1.40	3.59	4.99	4.97	0.91	2.65	3.56	3.41
8	1.07	2.98	4.05	3.47	1.16	3.17	4.33	4.07	0.74	2.21	2.95	2.67
10	0.81	2.50	3.31	3.00	0.93	2.96	3.89	2.95	0.48	1.72	2.19	2.07
12	0.60	1.98	2.58	2.46	0.68	2.40	3.08	2.74	0.25	1.10	1.35	1.55
24	0.11	0.34	0.45	0.77	0.14	0.46	0.60	0.62	0.12	0.77	0.89	0.62
INTERVAL (HRS)	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH
0	10174.5	5991.3	16165.8	6.64	12901.5	25589.5	38491.0	5.40	15313.6	9571.0	24884.6	6.16
0-2	24774.5	9703.3	34477.8	7.31	31876.5	27097.0	58973.5	7.78	20444.6	13743.0	34187.6	6.54
2-4	41384.5	15065.8	56450.3	6.95	52096.5	31132.0	83228.5	7.25	26162.4	19021.5	45183.9	6.46
4-6	51922.5	17661.8	69584.3	7.05	67696.5	37324.0	105020.5	6.95	29485.2	25418.7	54903.9	5.39
6-8	57954.5	23053.8	81008.3	5.74	68967.8	44297.4	113265.2	6.68	31512.5	28891.9	60404.4	5.37
8-10	61314.5	26448.8	87763.3	5.35	80237.8	48062.5	128300.3	6.89	33653.1	31748.1	65401.2	5.50
10-12	63270.5	28800.8	92071.3	5.18	84712.8	51975.0	136687.8	6.11	36401.3	33848.9	70250.2	6.41
12-24	66267.5	32435.0	98702.5	5.45	89919.0	60677.0	150596.0	5.40	43332.6	40011.7	83344.3	6.20

BASE = Erythromycin base concentrate (µg/ml).
ESTER$_1$ = Erythromycin propionate concentration (µg/ml).
TOTAL^1 = Total erythromycin concentrations obtained by addition of erythromycin base and propionate concentrations (Chapter 2).
TOTAL^2 = Total erythromycin concentrations obtained by hydrolysis of samples prior to chromatography (Chapter 3).
CUM BASE = Cumulative amount of erythromycin base excreted in urine (µg).
CUM ESTER = Cumulative amount of erythromycin propionate excreted in urine (µg).
CUM TOTAL = Cumulative amount of erythromycin (base + propionate) excreted in urine(µg).

Appendix 5 Mean serum and urine data (S2,S3,MD)

	SINGLE DOSE 2				SINGLE DOSE 3				MULTIPLE DOSE			
TIME(HRS)	BASE	ESTER	TOTAL[1]	TOTAL[2]	BASE	ESTER	TOTAL[1]	TOTAL[2]	BASE	ESTER	TOTAL[1]	TOTAL[2]
0	-	-	-	-	-	-	-	-	1.43	4.01	5.44	5.16
0.5	0.09	0.94	1.03	0.84	0.07	1.08	1.15	0.93	1.41	4.28	5.69	5.28
1	0.15	1.35	1.50	1.29	0.12	1.65	1.77	1.49	1.45	4.54	5.99	5.85
1.5	0.20	1.54	1.74	1.55	0.16	2.09	2.25	1.79	1.53	4.80	6.33	5.89
2	0.29	2.01	2.30	1.97	0.19	2.07	2.36	2.24	1.54	4.73	6.27	5.67
2.5	0.33	2.07	2.40	2.07	0.24	2.29	2.54	2.27	1.55	4.60	6.15	5.69
3	0.32	2.34	2.64	2.23	0.26	2.14	2.40	2.10	1.54	4.64	6.18	5.87
3.5	0.35	2.62	2.97	2.55	0.28	2.09	2.37	1.98	1.53	4.35	5.85	5.89
4	0.39	2.38	2.77	2.53	0.28	2.01	2.29	1.89	1.50	4.35	5.85	5.68
5	0.39	2.23	2.62	2.34	0.28	1.84	2.12	1.83	1.42	4.16	5.58	4.99
6	0.38	2.16	2.54	2.28	0.26	1.74	2.00	1.61	1.23	3.30	4.53	4.44
8	0.28	1.52	1.70	1.56	0.16	1.12	1.28	1.12	0.99	2.68	3.67	3.46
10	0.17	0.86	1.03	1.04	0.13	0.95	1.08	0.81	0.73	2.28	3.01	2.79
12	0.12	0.64	0.76	0.70	0.09	0.81	0.90	0.70	0.53	1.70	2.23	2.18
24	0.04	0.17	0.21	0.23	-	0.45	0.45	0.35	0.15	0.60	0.75	0.66
INTERVAL (HRS)	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH	CUM BASE	CUM ESTER	CUM TOTAL	pH
0	-	-	-	6.03	-	-	-	5.78	8355.6	9993.0	18348.6	6.20
0-2	194.7	875.8	1070.5	6.41	361.0	877.0	1238.0	6.90	19186.3	12961.8	32148.1	7.11
2-4	916.1	2495.1	3411.2	6.46	1769.7	2843.9	4613.6	6.86	30601.1	16649.2	47250.3	6.82
4-6	2280.7	5899.6	8180.3	6.22	3085.2	5269.3	8354.2	6.52	39354.4	21749.0	61103.4	6.51
6-8	3664.4	8731.1	12395.5	6.29	4310.1	6239.2	10549.3	6.91	43116.6	26371.2	69487.8	6.04
8-10	4705.8	10514.1	15219.9	6.35	4746.7	7077.9	11824.6	5.94	47240.9	29251.1	76492.0	5.92
10-12	5861.2	11936.3	17797.5	6.15	5035.6	7877.9	12913.5	5.86	49951.6	31868.5	81820.1	5.81
12-24	6849.0	12995.6	19284.6	5.87	6478.5	9705.8	16184.3	5.93	53925.9	36230.0	90155.9	5.77

BASE = Erythromycin base concentrate (µg/ml).
ESTER$_1$ = Erythromycin propionate concentration (µg/ml).
TOTAL^1 = Total erythromycin concentrations obtained by addition of erythromycin base and propionate concentrations (Chapter 2).
TOTAL^2 = Total erythromycin concentrations obtained by hydrolysis of samples prior to chromatography (Chapter 3).
CUM BASE = Cumulative amount of erythromycin base excreted in urine (µg).
CUM ESTER = Cumulative amount of erythromycin propionate excreted in urine (µg).
CUM TOTAL = Cumulative amount of erythromycin (base + propionate) excreted in urine (µg).

Appendix 6 Observed versus predicted total erythromycin serum concentrations (S1 - 12 hour data)

	P1			P2			P3			P4			P5			MEAN		
TIME (HRS)	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0	0.40	0.34	1.28	1.24	0.53	0.25	1.67	1.11	1.11	1.32	0.85	0.35	0.91	0.44	0.60	1.06	0.57
1.0	0.15	0.74	0.59	2.02	2.02	0.89	2.33	2.73	1.77	2.19	2.22	1.37	0.68	1.53	0.74	1.47	1.78	0.94
1.5	0.69	0.99	0.78	2.22	2.46	1.13	3.52	3.37	2.16	2.58	2.79	1.71	1.52	1.93	0.95	2.10	2.56	1.19
2.0	0.54	1.19	0.93	2.53	2.66	1.29	4.16	3.69	2.37	2.77	3.13	1.91	2.18	2.17	1.10	2.43	2.54	1.36
2.5	0.98	1.35	1.05	2.86	2.70	1.40	4.54	3.79	2.47	3.51	3.29	2.02	2.60	2.28	1.21	2.90	2.67	1.47
3.0	1.63	1.46	1.13	3.30	2.63	1.47	4.03	3.74	2.48	3.51	3.31	2.06	3.21	2.31	1.27	3.14	2.70	1.53
3.5	1.97	1.55	1.20	2.27	2.49	1.50	3.43	3.58	2.43	3.56	3.25	2.06	2.84	2.27	1.31	2.81	2.65	1.56
4.0	2.37	1.60	1.24	2.07	2.31	1.51	3.10	3.36	2.34	3.27	3.12	2.02	2.18	2.18	1.34	2.69	2.55	1.56
5	1.92	1.63	1.29	1.63	1.90	1.48	2.63	2.83	2.11	2.68	2.75	1.88	1.66	1.94	1.33	2.10	2.27	1.51
6	1.87	1.59	1.29	1.63	1.51	1.39	2.04	2.30	1.83	2.34	2.33	1.68	1.24	1.65	1.28	1.82	1.93	1.41
8	1.33	1.41	1.21	0.93	0.87	1.17	1.31	1.39	1.30	1.31	1.55	1.27	0.87	1.11	1.11	1.15	1.3	1.16
10	0.83	1.18	1.07	0.49	0.47	0.92	0.81	0.79	0.87	0.72	0.96	0.90	0.54	0.70	0.92	0.68	0.82	0.90
12	0.54	0.94	0.92	0.29	0.25	0.70	0.42	0.43	0.57	0.57	0.58	0.62	0.40	0.43	0.73	0.44	0.49	0.67

Obs = Observed Serum Concentration µg/ml
Pred^1 = Predicated Serum Concentration µg/ml Model 1
Pred^2 = Predicated Serum Concentration µg/ml Model 2

Appendix 7 Observed versus predicted total erythromycin serum concentrations (S2 - 12 hour data)

	P1			P2			P3			P4			P5			P6			MEAN		
TIME (HRS)	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0.49	1.36	1.34	0.20	0.22	0.17	2.22	1.77	1.48	0	0.87	0.87	1.22	0.80	0.62	0.90	0.87	0.80	0.84	0.76	0.74
1.0	1.93	2.24	2.22	0.30	0.40	0.32	2.85	2.68	2.34	0.27	1.60	1.60	1.23	1.31	1.07	1.14	1.42	1.32	1.29	1.32	1.30
1.5	3.24	2.77	2.75	0.56	0.55	0.44	2.57	3.08	2.80	0.30	2.19	2.20	1.30	1.60	1.40	1.30	1.74	1.63	1.55	1.74	1.71
2.0	3.36	3.04	3.04	0.66	0.65	0.55	3.28	3.18	2.99	0.70	2.69	2.69	1.54	1.77	1.62	2.25	1.89	1.80	1.97	2.03	1.99
2.5	3.40	3.14	3.16	0.50	0.74	0.63	3.27	3.12	3.02	1.09	3.09	3.09	1.98	1.84	1.76	2.19	1.93	1.86	2.07	2.22	2.18
3.0	3.39	3.11	3.15	0.53	0.80	0.70	3.16	2.97	2.94	2.16	3.41	3.41	2.07	1.86	1.84	2.09	1.89	1.84	2.23	2.33	2.30
3.5	2.76	3.00	3.05	0.64	0.84	0.76	2.74	2.78	2.80	5.11	3.65	3.65	2.07	1.83	1.86	1.95	1.80	1.77	2.55	2.37	2.35
4.0	2.50	2.85	2.90	0.73	0.87	0.80	2.39	2.57	2.62	5.82	3.83	3.83	1.71	1.78	1.85	1.58	1.68	1.67	2.53	2.37	2.35
5	2.27	2.45	2.51	0.55	0.89	0.85	1.87	2.15	2.23	6.76	4.04	4.05	1.46	1.64	1.75	1.13	1.41	1.43	2.34	2.27	2.26
6	2.00	2.03	2.09	1.53	0.88	0.88	1.60	1.78	1.85	6.46	4.09	4.10	1.38	1.49	1.59	0.97	1.13	1.17	2.28	2.08	2.08
8	1.30	1.32	1.35	1.12	0.81	0.85	1.34	1.21	1.24	3.63	3.88	3.89	1.30	1.18	1.22	0.65	0.67	0.72	1.56	1.63	1.64
10	0.89	0.81	0.83	0.62	0.71	0.78	1.03	0.81	0.81	2.24	3.46	3.47	0.99	0.94	0.87	0.47	0.37	0.42	1.04	1.19	1.21
12	0.72	0.49	0.49	0.46	0.60	0.68	0.78	0.54	0.53	1.23	2.95	2.97	0.68	0.78	0.60	0.32	0.20	0.24	0.70	0.84	0.86

Obs = Observed Serum Concentration µg/ml
$Pred_1$ = Predicated Serum Concentration µg/ml Model 1
$Pred_2$ = Predicated Serum Concentration µg/ml Model 2

Appendix 8 Observed versus predicted total erythromycin serum concentrations (S2 - 24 hour data)

	P1			P2			P3			P4			P5			P6			MEAN		
TIME (HRS)	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0.49	1.37	1.35	0.20	0.23	0.16	2.22	1.77	2.00	0	0.89	0.89	1.22	0.82	0,70	0.90	0.87	0.87	0.84	0.76	0.76
1.0	1.93	2.25	2.24	0.30	0.41	0.29	2.85	2.67	2.85	0.27	1.62	1.00	1.23	1.32	1.18	1.14	1.42	1.42	1.29	1.32	1.32
1.5	3.24	2.77	2.78	0.56	0.55	0.40	2.57	3.07	3.13	0.30	2.23	2.23	1.30	1.61	1.51	1.30	1.74	1.74	1.55	1.74	1.74
2.0	3.36	3.04	3.08	0.66	0.66	0.48	3.28	3.18	3.14	0.70	2.73	2.73	1.54	1.77	1.72	2.25	1.89	1.89	1.97	2.02	2.02
2.5	3.40	3.13	3.19	0.50	0.75	0.56	3.27	3.12	3.02	1.09	3.12	3.13	1.98	1.83	1.84	2.19	1.94	1.93	2.07	2.21	2.21
3.0	3.39	3.11	3.18	0.53	0.81	0.61	3.16	2.97	2.85	2.16	3.43	3.44	2.07	1.84	1.89	2.09	1.89	1.89	2.23	2.33	2.32
3.5	2.76	3.00	3.08	0.64	0.85	0.66	2.74	2.78	2.67	5.11	3.67	3.68	2.07	1.82	1.89	1.95	1.80	1.81	2.55	2.37	2.37
4.0	2.50	2.84	2.92	0.73	0.88	0.70	2.39	2.57	2.48	5.82	3.85	3.85	1.71	1.77	1.86	1.58	1.69	1.69	2.53	2.37	2.37
5	2.27	2.45	2.53	0.55	0.91	0.75	1.87	2.16	2.12	6.76	4.04	4.04	1.46	1.64	1.73	1.13	1.41	1.41	2.34	2.27	2.27
6	2.00	2.03	2.09	1.53	0.90	0.78	1.60	1.79	1.81	6.46	4.07	4.08	1.38	1.49	1.56	0.97	1.13	1.13	2.28	2.09	2.09
8	1.30	1.33	1.35	1.12	0.83	0.79	1.34	1.22	1.31	3.63	3.82	3.83	1.30	1.20	1.20	0.65	0.67	0.67	1.56	1.64	1.63
10	0.89	0.83	0.83	0.62	0.73	0.75	1.03	0.82	0.95	2.24	3.37	3.38	0.99	0.97	0.89	0.47	0.37	0.37	1.04	1.20	1.20
12	0.72	0.51	0.48	0.46	0.62	0.69	0.78	0.55	0.69	1.23	2.85	2.86	0.68	0.77	0.64	0.32	0,20	0.20	0.70	0.84	0.84
24	0.27	0.02	0.02	-	0.20	0.35	0.29	0.05	0.10	0.01	0.07	0.71	0.30	0.20	0.09	0.15	0.03	0.03	0.23	0.07	0.07

Obs = Observed Serum Concentration µg/ml
$Pred_1$ = Predicated Serum Concentration µg/ml Model 1
$Pred_2$ = Predicated Serum Concentration µg/ml Model 2

Appendix 9 Observed versus predicted total erythromycin serum concentrations (S3 - 12 hour data)

	P1			P2			P3			P4			P5			P6			MEAN		
TIME (HRS)	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0.37	0.30	0.18	0.35	0.35	0.25	1.51	1.82	0.74	1.58	1.48	0.61	1.29	0.80	-	0.45	1.00	0.46	0.93	0.95	0.43
1.0	0.64	0.54	0.31	0.60	0.64	0.43	2.90	2.82	1.24	1.58	2.41	1.03	1.41	1.25	-	1.81	1.59	0.81	1.49	1.55	0.73
1.5	0.66	0.73	0.40	0.89	0.87	0.56	3.25	3.31	1.58	2.72	2.94	1.33	1.28	1.48	-	1.95	1.91	1.09	1.79	1.89	0.94
2.0	0.78	0.87	0.48	0.88	1.06	0.67	3.49	3.49	1.81	3.70	3.19	1.54	1.13	1.59	-	2.02	2.04	1.31	2.24	2.07	1.09
2.5	0.85	0.98	0.54	1.05	1.21	0.75	3.86	3.48	1.96	4.10	3.24	1.69	1.31	1.62	-	2.46	2.07	1.49	2.27	2.14	1.19
3.0	0.87	1.06	0.58	0.84	1.33	0.81	3.65	3.36	2.05	3.14	3.16	1.78	2.08	1.60	-	2.04	2.02	1.63	2.14	2.14	1.27
3.5	0.91	1.12	0.62	1.25	1.41	0.86	3.24	3.19	2.09	2.91	3.00	1.84	1.78	1.56	-	1.76	1.93	1.74	1.98	2.08	1.31
4.0	1.09	1.15	0.65	1.45	1.48	0.90	2.80	2.98	2.10	2.38	2.79	1.87	1.60	1.50	-	1.55	1.82	1.82	1.89	2.00	1.33
5	1.70	1.17	0.69	1.83	1.54	0.94	2.26	2.55	2.03	2.16	2.31	1.85	1.27	1.38	-	1.75	1.56	1.92	1.83	1.79	1.33
6	1.45	1.15	0.71	2.42	1.54	0.96	1.83	2.14	1.91	1.70	1.83	1.77	1.12	1.25	-	1.13	1.31	1.95	1.61	1.57	1.28
8	0.95	1.02	0.70	1.62	1.43	0.93	1.41	1.49	1.57	1.06	1.07	1.52	0.89	1.02	-	0.77	0.89	1.87	1.12	1.17	1.12
10	0.67	0.85	0.66	0.96	1.25	0.85	1.25	1.02	1.21	0.71	0.58	1.22	0.83	0.82	-	0.68	0.61	1.70	0.82	0.85	0.92
12	0.54	0.68	0.60	0.62	1.04	0.75	1.09	0.70	0.09	0.52	0.31	0.95	0.81	0.67	-	0.61	0.40	1.48	0.70	0.62	0.74

Obs = Observed Serum Concentration µg/ml
$Pred_1$ = Predicated Serum Concentration µg/ml Model 1
$Pred^2$ = Predicated Serum Concentration µg/ml Model 2

Appendix 10 Observed versus predicted total erythromycin serum concentrations (S3 - 24 hour data)

	P1			P2			P3			P4			P5			P6			MEAN		
TIME (HRS)	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2	OBS	PRED^1	PRED^2
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0.37	-	0.30	0.35	-	0.34	1.51	1.82	1.80	1.58	1.48	1.48	1.29	-	-	0.45	1.00	1.00	0.93	0.97	0.76
1.0	0.64	-	0.53	0.60	-	0.63	2.90	2.82	2.84	1.58	2.41	2.41	1.41	-	-	1.81	1.59	1.61	1.49	1.56	1.32
1.5	0.66	-	0.72	0.89	-	0.86	3.25	3.31	3.37	2.72	2.94	2.94	1.28	-	-	1.95	1.90	1.93	1.79	1.90	1.73
2.0	0.78	-	0.86	0.88	-	1.05	3.49	3.49	3.56	3.70	3.19	3.19	1.13	-	-	2.02	2.04	2.07	2.24	2.07	2.02
2.5	0.85	-	0.97	1.05	-	1.20	3.86	3.49	3.55	4.10	3.24	3.24	1.31	-	-	2.46	2.06	2.08	2.27	2.13	2.21
3.0	0.87	-	1.05	0.84	-	1.32	3.65	3.37	3.40	3.14	3.16	3.13	2.08	-	-	2.04	2.01	2.02	2.14	2.12	2.32
3.5	0.91	-	1.11	1.25	-	1.41	3.24	3.20	3.19	2.91	3.00	3.00	1.78	-	-	1.76	1.92	1.91	1.98	2.07	2.37
4.0	1.09	-	1.15	1.45	-	1.47	2.80	2.99	2.95	2.38	2.79	2.79	1.60	-	-	1.55	1.81	1.79	1.89	1.99	2.37
5	1.70	-	1.17	1.83	-	1.54	2.26	2.56	2.46	2.16	2.31	2.31	1.27	-	-	1.75	1.56	1.51	1.83	1.79	2.27
6	1.45	-	1.15	2.42	-	1.54	1.83	2.16	2.02	1.70	1.83	1.83	1.12	-	-	1.13	1.32	1.26	1.61	1.57	2.09
8	0.95	-	1.03	1.62	-	1.44	1.41	1.50	1.41	1.06	1.07	1.07	0.89	-	-	0.77	0.91	0.89	1.12	1.20	1.63
10	0.67	-	0.87	0.96	-	1.26	1.25	1.03	1.05	0.71	0.58	0.59	0.83	-	-	0.68	0.63	0.67	0.82	0.90	1.20
12	0.54	-	0.70	0.62	-	1.06	1.09	0.71	0.85	0.52	0.31	0.31	0.81	-	-	0.61	0.43	0.55	0.70	0.67	0.84
24	0.30	-	0.13	0.32	-	0.25	0.34	0.07	0.42	0.25	0.04	0.04	0.37	-	-	0.34	0.04	0.30	0.35	0.12	0.06

Obs = Observed Serum Concentration µg/ml
$Pred_1$ = Predicated Serum Concentration µg/ml Model 1
$Pred^2$ = Predicated Serum Concentration µg/ml Model 2

Appendix 11 Observed versus predicted mean erythromycin base and propionate concentrations (S2,S3)

	MEAN DATA (n=6) Single Dose 2						MEAN DATA (n=6) Single Dose 3					
	ERYTHROMYCIN BASE			ERYTHROMYCIN PROPIONATE			ERYTHROMYCIN BASE			ERYTHROMYCIN PROPIONATE		
TIME (HRS)	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]	OBS	PRED[1]	PRED[2]
0	0	0	0	0	0	0	0	0	0	0	0	0
0.5	0.09	0.10	0.10	0.94	0.80	0.77	0.07	0.08	0.08	1.08	1.15	0.84
1.0	0.15	0.18	0.18	1.35	1.37	1.34	0.12	0.14	0.14	1.65	1.73	1.41
1.5	0.20	0.25	0.24	1.54	1.79	1.75	0.16	0.19	0.18	2.09	2.00	1.80
2.0	0.29	0.29	0.28	2.01	2.07	2.02	0.19	0.22	0.22	2.07	2.11	2.05
2.5	0.33	0.32	0.32	2.07	2.23	2.20	0.24	0.24	0.24	2.29	2.12	2.18
3.0	0.32	0.34	0.34	2.34	2.32	2.30	0.26	0.25	0.25	2.14	2.09	2.23
3.5	0.35	0.35	0.35	2.62	2.34	2.33	0.28	0.26	0.26	2.09	2.03	2.22
4.0	0.39	0.36	0.35	2.38	2.33	2.32	0.28	0.26	0.26	2.01	1.96	2.16
5	0.39	0.35	0.35	2.23	2.20	2.20	0.28	0.25	0.25	1.84	1.80	1.97
6	0.38	0.34	0.33	2.16	1.99	2.00	0.26	0.24	0.24	1.74	1.65	1.72
8	0.28	0.28	0.27	1.52	1.53	1.54	0.16	0.19	0.19	1.12	1.38	1.22
10	0.17	0.22	0.21	0.86	1.10	1.10	0.13	0.15	0.15	0.95	1.15	0.81
12	0.12	0.16	0.16	0.64	0.78	0.76	0.09	0.12	0.11	0.81	0.96	0.52

Obs = Observed Serum Concentration µg/ml
$Pred_1$ = Predicated Serum Concentration µg/ml Model 1
$Pred^2$ = Predicated Serum Concentration µg/ml Model 2

Appendix 12 Pharmacokinetic parameters derived by computer fitting (Model 1) of total erythromycin serum concentrations (S1,S2,S3 - 12 hour data)

		k_a	V	k	t_{lag}	r^2	Weighted squares	Cor
	P1	0.21	117.39	0.21	0.24	0.730	2.145	0.903
	P2	0.42	68.76	0.42	0.02	0.942	0.752	0.970
Dose 1	P3	0.40	49.27	0.39	0.02	0.896	3.26	0.953
	P4	0.35	65.74	0.35	0.02	0.971	0.549	0.988
	P5	0.34	79.81	0.34	0.02	0.798	2.76	0.911
	Mean	0.34	68.89	0.34	0.03	0.947	0.72	0.980
	P1	0.52	79.51	0.27	0.02	0.910	1.59	0.956
	P2							
Dose 2	P3	0.99	107.25	0.20	0.02	0.953	0.823	0.978
	P4	0.17	48.32	0.17	0.50	0.523	39.22	0.788
	P5	0.12	30.40	0.76	0.03	0.873	0.515	0.936
	P6	0.40	95.87	0.41	0.03	0.903	0.650	0.950
	Mean	0.27	78.03	0.27	0.03	0.972	0.22	0.987
	P1	0.16	119.84	0.26	0.03	0.758	0.556	0.872
	P2	0.18	120.00	0.18	0.03	0.711	1.47	0.850
Dose 1	P3	0.19	20.03	0.89	0.03	0.957	0.75	0.978
	P4	0.41	57.29	0.41	0.03	0.895	2.02	0.946
	P5	0.10	24.98	0.99	0.30	0.699	0.958	0.844
	P6	0.20	40.20	0.76	0.03	0.896	0.739	0.947
	Mean	0.16	34.11	0.72	0.03	0.984	0.094	0.992

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