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BIOPHARMACEUTICS AND PHARMACOKINETICS OF THE MACROLIDE ANTIBIOTIC - JOSAMYCIN

by

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TO MY PARENTS

ABSTRACT

The investigations detailed herein have been conducted to address various aspects of the biopharmaceutics and pharmacokinetics of josamycin which to-date, have received little or no attention in the literature. Areas of investigation have included the selective determination of josamycin in serum and urine samples, the stability of josamycin in stored biological samples, intrinsic dissolution rates, solubility, acid and alkali stability and bioavailability and pharmacokinetics after dosing with a solution, powder and tablets.

High performance liquid chromatography (HPLC) was used as the main analytical tool throughout these studies and proved to be highly versatile for the determination of josamycin in a number of different media. HPLC analysis afforded simple yet accurate determination of josamycin in samples from dissolution, solubility, tablet content and stability studies. Furthermore, the specificity afforded by HPLC was particularly useful for the separation of josamycin from degradation products formed in acid and alkali media.

Since metabolites of josamycin are microbiologically active, microbiological assays do not determine the concentration solely of josamycin. An analytical method capable of the selective determination of josamycin in serum and urine samples is therefore required for the procurement of reliable bioavailability and pharmacokinetic data. HPLC affords this selectivity and a method for the selective determination of josamycin in serum and urine was successfully developed. The assay was simple yet precise, accurate and sensitive. Furthermore, it was well suited to the determination of josamycin in a large number of biological samples. Its success was largely due to the use of a solid phase extraction step using C_{18} extraction columns, with a highly specific wash sequence followed by a phase separation step after elution from the extraction column. Chromatography was performed on a C_{18} reversed-phase analytical column with UV detection of josamycin and internal standard at 231 nm and at 204 nm respectively using a programmable multi-wavelength detector. Only slight modification of the assay described should enable the selective determination of the metabolites of josamycin. This assay, therefore, lays the groundwork for future investigations into the pharmacokinetics of these metabolites.

The re-usability of extraction columns was assessed in an attempt to reduce the cost of sample analysis. It was found that extraction columns could be used twice for the extraction of serum samples and up to four times for the extraction of urine samples. The difference between the re-usability of extraction columns for serum and urine samples was ascribed to various differences in the composition of the sample

matrix. The stability of josamycin in stored serum and urine samples was also assessed. Josamycin was found to be stable for up to 72 hrs at +4°C and up to 84 days at -15°C in *ex-vivo* and *in-vitro* serum samples. However, josamycin was less stable in urine samples and a significant change in concentration was detected during the storage periods studied.

Intrinsic dissolution rate-pH and solubility-pH profiles were determined over the pH range of the gastro-intestinal tract to assess the possible role of dissolution in the absorption of josamycin. Furthermore, intrinsic dissolution rates at each pH were determined at 50, 100, 200 and 300 rpm, and then at infinite rotation speed (G^{∞}) by an extrapolation procedure. G^{∞} was determined in order to compare dissolution rates in different dissolution media without the influence of a diffusion layer. The intrinsic dissolution rate and solubility were found to be highly pH dependent. G^{∞} decreased >8500 fold from 5.15 $mg.cm^{-2}.s^{-1}$ at pH 1.2 to 5.815 x 10^{-4} $mg.cm^{-2}.s^{-1}$ at pH 7.5. The solubility of josamycin decreased from 212 $mg.ml^{-1}$ at pH 5.45 to 0.18 $mg.ml^{-1}$ at about 9.0. These profiles suggest that josamycin may be subject to dissolution and solubility limited absorption from the intestinal tract, particularly at higher intestinal pH's. This hypothesis is supported by the reduced bioavailability of josamycin from a powder compared to a solution. Determination of intrinsic dissolution rates at infinite rotation speed (when the diffusion layer is theoretically equal to zero) proved to be a useful means by which the dissolution characteristics of pure drug in different dissolution media could be compared.

Dissolution profiles of josamycin powder and two tablet preparations used in bioavailability studies were determined at various pH's over the gastro-intestinal pH-range. The effect of formulation on dissolution from the tablet preparations was assessed by comparison of the powder with tablet dissolution profiles at each pH. Dissolution profiles of Josamycin 200 mg tablets from pH 1.2 to 5.0 showed rapid dissolution and were similar to powder dissolution profiles. However, above pH 5.0, dissolution of the powder was retarded by reduced intrinsic dissolution rate and solubility. Dissolution from the tablet preparation above pH 5.0 was further reduced by formulation factors. Dissolution of these tablets will therefore be rapid in acidic gastric fluids. However, if intact tablets or granules pass into the less acidic environment of the duodenum, dissolution will be significantly reduced and absorption is likely to be dissolution rate limited.

Comparison of josamycin powder with Josacine 500 mg tablet profiles showed that dissolution from these tablets was severely limited by formulation factors over the entire pH range, particularly at higher pH's. Absorption from these tablets is therefore likely to be dissolution rate limited whether dissolution occurs in gastric or intestinal fluids.

Although macrolide antibiotics are known to be susceptible to acid degradation, there is a dearth of information on the stability of josamycin. This prompted a comprehensive investigation into the stability of josamycin from pH 1.0 to 12.0, together with the effect of ionic strength and buffer concentration on the rate of degradation in acid. Further studies were conducted utilising simulated gastric and intestinal fluids to assess the extent of degradation after an oral dose.

The pH-rate profile for josamycin over the pH range 1 - 12 was comprehensively characterised and the equation describing the profile determined. Results indicated that josamycin is subject to specific acid catalysis but catalysis in alkali media appeared to be more complex. The rate constant for catalysis by hydronium ion (k_1) was 54.11 $M^{-1}.hr^{-1}$ whilst the rate constant for catalysis by hydroxide ion (k_3) was 60.35 $M^{-1}.hr^{-1}$. Catalysis due to water was insignificant and the water catalysed rate constant was found to be 3.370×10^{-5} hr^{-1}. The pH of maximum stability was determined as pH 6.5 whilst degradation at pH 1.0 and 12.0 is about five orders of magnitude greater than at pH 6.5. The degradation of josamycin in acid is subject to a significant primary salt effect, however, no secondary salt effect was evident. Consideration of the ionic strength of the reaction medium is therefore essential when undertaking stability studies of josamycin in aqueous media. Concentration *vs.* time profiles for josamycin in acidic media were biphasic which indicated that the degradation reaction did not follow a simple pathway whereby josamycin degrades directly to products. Further investigations demonstrated that josamycin undergoes a reversible isomerisation step, with subsequent degradation of josamycin and possibly its isomer, by cleavage of the mycarose moiety to the desmycarose compounds.

Studies to determine the stability of josamycin in simulated gastric fluids demonstrated that acid degradation could be appreciable after oral administration. However, extensive degradation *in-vivo* will only occur at the most acidic gastric pH's of about pH 1.0 to 2.0. Josamycin was, however, found to be significantly more stable than erythromycin, suggesting that problems with acid degradation *in-vivo* would be less for josamycin than the extensively used macrolide antibiotic, erythromycin.

The administration of josamycin as a solution and powder have hitherto not been reported and the bioavailability of josamycin without the influence of formulation factors has not been established. Bioavailability of josamycin from a solution and powder were therefore conducted. Furthermore, the bioavailability of two tablet preparations was assessed. A multiple dose study was also conducted and parameters obtained from pharmacokinetic modelling of both single dose solution and multiple dose solution profiles compared.

The results showed that josamycin is inherently rapidly absorbed from a buffered solution. After a 1 gram dose, a mean C_{max} of 1.64 μg/ml was reached within a mean t_{max} of 0.39 hrs after a lag time of about 10 mins. Absorption from powder, however, is slower and a mean C_{max} of 0.755 μg/ml was attained with a mean t_{max} = 0.50 hrs. Furthermore, the bioavailability of the powder was only ±50% that of the solution. It was therefore concluded that absorption from the powder was limited by the dissolution rate and solubility. The bioavailability from two different tablet formulations was still lower than from the powder, with absorption hindered not only by the intrinsic dissolution rate and solubility, but also by formulation factors. Furthermore, absorption from the tablets was highly erratic. It was concluded that intact tablets or granules passed into the duodenum, after which dissolution from the tablets or granules was retarded and pH-dependent. Investigations into the *in-vitro* dissolution characteristics of the tablet preparations and comparison with powder dissolution profiles over the entire gastro-intestinal pH range rather than at a limited number of pH's provided invaluable information for the explanation of the poor profiles obtained after tablet administration.

Modelling of single dose solution data showed that the pharmacokinetics of josamycin can be adequately described by a two body-compartment model with rapid and extensive distribution into a peripheral compartment (α = 6.50 hr^{-1}, k_{21} = 1.46 hr^{-1}). The mean volume of distribution was 161.7 l which suggests that josamycin distributes to "deep" tissue and is consistent with the considerable accumulation of josamycin observed in various organs. Josamycin was 51% bioavailable in this study and had a t½ of 1.66 hrs with a total clearance of 4.71 $l.kg^{-1}.hr^{-1}$. The pharmacokinetics of josamycin however, changed significantly after multiple dosing. Both distribution coefficients decreased (α = 2.99 hr^{-1}, k_{21} = 0.41 hr^{-1}) as did the volume of distribution (Vd = 34.3 l). This suggested that saturation of the peripheral compartment occurred. Furthermore, a decrease in clearance (Cl = 0.63 $l.kg^{-1}.hr^{-1}$) with a corresponding increase in elimination half-life (t½ = 3.61) were also observed. This suggested possible saturation of metabolic enzyme pathways. These changes in pharmacokinetic parameters were presumably responsible for the non-linear kinetics observed during the multiple dose studies.

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CHAPTER 1

INTRODUCTION

1.1 ISOLATION, STRUCTURE AND PHYSICO-CHEMICAL PROPERTIES OF JOSAMYCIN

Josamycin is a macrolide antibiotic produced by the actinomycete *Streptomyces narbonensis* var. *josamyceticus*. The yeast was originally isolated from a soil sample collected at Motoyama in Japan by Osono *et al.* in 1967 (1). It is structurally identical to a previously isolated leucomycin, leucomycin A_3, which is produced in minor quantities by the actinomycete *Streptomyces kitasatoensis Hata* (1). The molecular formula of josamycin is $C_{42}H_{69}NO_{15}$ (molecular mass = 827) and is composed of a 16-membered lactone ring and two sugar residues - mycaminose and isovaleryl mycarose. The molecular structure of josamycin is depicted in figure 1.1. Unlike erythromycin which has two sugar residues attached individually to the lactone ring (2), the isovaleryl mycarose moiety of josamycin is attached by an α-glycosidic linkage to the mycaminose moiety which is in turn attached by a ß-glycosidic linkage to position-5 of the lactone ring (3). The initial isolate was obtained from the fermentation broth utilising cation exchange resins and liquid-liquid extraction techniques (1) which yielded colourless needle-like crystals with a melting point of 130 - 133°C. As a powder, josamycin is white to slightly yellow and has an extremely bitter taste. It is easily soluble in methanol, ethanol, acetone, chloroform, ethyl acetate, butyl acetate, dioxane and acidic water; soluble in butanol, ether, carbon tetrachloride, benzene and toluene, and sparingly soluble in petroleum ether, n-hexane and water. It is reported to have a pKa of 6.7 in 50% aqueous ethanol (4) and 7.1 in 40% aqueous methanol (1) and is slightly basic due to the tertiary amino group at position-3 of the mycaminose moiety. The diene system in the lactone ring is responsible for substantial UV absorption at 231 nm (Figure 1.2) with an $E_{1cm}^{1\%}$ of 325 in 0.001N aqueous hydrochloric acid. Josamycin is a highly lipophilic molecule with an octanol-water partitioning coefficient of 70.6 (5), which is about 15 times greater than that of the extensively used macrolide, erythromycin.

Josamycin is a relatively under-utilised macrolide which has been developed to overcome several disadvantageous properties of erythromycin. Josamycin has been marketed as an antibiotic which exhibits significant acid stability compared with erythromycin, does not induce resistance and has excellent tissue distribution. Furthermore, josamycin is reported to be better tolerated than erythromycin and its use is associated with fewer side effects and drug interactions. However, only a limited amount of data on josamycin have appeared in the literature since its

Figure 1.1

Josamycin -	R_1 = H	$R_2 = COCH_2CH(CH_3)_2$
JM-O_1 -	R_1 = OH	$R_2 = COCH_2CH(CH_3)_2$
JM-O_2 -	R_1 = H	$R_2 = COCH_2C(OH)(CH_3)_2$
Delv-JM -	R_1 = H	R_2 = H

Figure 1.2

Figure 1.1 Molecular structure of josamycin and its three major metabolites, hydroxy josamycin (JM-O_1), β-hydroxy josamycin (JM-O_2) and deisovaleryl josamycin (Delv-JM).

Figure 1.2 UV absorbance spectrum of josamycin in methanol illustrating the λ-max at 231 nm.

introduction for clinical use.

1.2 PHARMACOLOGY

1.2.1 Mode of Action

Josamycin, as with the macrolide group of antibiotics, exerts its anti-bacterial activity by the inhibition of protein synthesis through the formation of a complex with the ribosomal 50s subunit (6,7). From studies on erythromycin, macrolides enter bacterial cells by passive diffusion, however, they are accumulated in bacterial cells as a consequence of ribosomal binding (6,8). The concentration of erythromycin in gram-positive bacteria has been found to be up to 90 fold greater than in the extracellular medium (8). However, in gram-negative bacteria, the cell wall prevents the passive diffusion of drug molecules into bacteria and therefore affords protection to the organism against macrolides, rendering the majority of gram-negative bacteria immune to most of these antibiotics. The high lipophilicity of josamycin, however, can overcome this problem in some gram-negative bacteria as has been observed in *Bacteroides fragilis* (9). Resistance to macrolides occurs by modification of the ribosomal structure (10,11) which decreases ribosomal binding (12). However, josamycin is known as a macrolide which is non-resistance inducing and has been shown to have little resistance-inducing activity in *Staplylococcus aureus* (13), which in many cases is highly resistant to erythromycin.

Besides accumulating in bacterial cells, josamycin accumulates in human phagocytic cells (14-16) possibly by virtue of its lipophilicity and/or an active transport mechanism (17). Josamycin is therefore able to exert its antibacterial action on susceptible organisms surviving intracellularly after phagocytosis. Such activity has been observed against *L. pneumophilia* and *H. influenza* whilst a more hydrophilic antibiotic, Penicillin G was unable to penetrate the phagocytic cells. Josamycin therefore has the potential to prevent recurrences of infection caused by the release of viable micro-organisms from phagocytes. Such occurrences can occur with ß-lactam therapy due to protection of bacteria from the antibiotic by the phagocyte (16). Furthermore, josamycin may also stimulate the intracellular killing ability of polymorphonuclearcytes and also exert an opsonising effect on bacteria rendering them more susceptible to phagocytosis (18,19).

1.2.2 Spectrum of Activity

Josamycin is mainly active against gram-positive aerobes. However, it also exhibits activity against selected gram-negative anaerobes. The *in-vitro* activity in comparison with that of erythromycin for a number of common pathogens is tabulated in table 1.1. Josamycin is generally equally or slightly less active that erythromycin against gram-positive aerobic bacteria (20,21). However, one notable exception is its activity against erythromycin resistant *S. aureus* (22). As mentioned earlier, josamycin does not induce resistance in this organism and this is one of its main advantages in clinical use. Josamycin also shows greater activity than erythromycin against various anaerobic bacteria such as the bacteroides species and gram-positive bacilli (20,23-25) and is slightly more or equally effective against *C. jejuni* (26). However, josamycin was found to be ineffective against the anaerobic bacteria Clostridium (33% of species) and Fusobacterium (60% of species) which may limit its use in mixed anaerobic infections (27). Josamycin has also been found to be particularly effective in the treatment of periodontitis and bucco-dental infections caused by a number of micro-organisms including streptococci, facultative anaerobes and anaerobes, probably due to the significant amounts excreted in the saliva (28,29).

1.2.3 Drug Interactions

Josamycin is considered to be the macrolide of choice for co-administration with drugs interacting with this group of compounds. This is discussed further in 1.3.3. Drug-drug interactions with macrolide antibiotics have been extensively reviewed by Ludden (30) with special attention given to the interaction of erythromycin and theophylline which is fairly common and potentially dangerous. Of the clinically used macrolides, troleandomycin exhibits the greatest potential for interaction by inhibiting drug metabolising cytochrome P-450 enzymes systems. This compound has a significant effect on the metabolism of theophylline, methylprednisolone and carbamazepine and can cause ergotism in patients receiving ergot alkaloids and cholestatic jaundice in patients taking oral contraceptives. Erythromycin exhibits a weaker effect than troleandomycin but can also significantly affect the metabolism of theophylline, methylprednisolone, carbamazepine and warfarin as well as increase the bioavailability of digoxin. However, josamycin is the cause of far fewer interactions than either troleandomycin or erythromycin as it is a far less potent inhibitor of metabolism. Selles *et al.* (31) found that josamycin had no effect on the pharmacokinetics of theophylline after chronic dosing. Descotes *et al.* (32) compared the effect of josamycin and troleandomycin on caffeine

Table 1.1 Spectrum of Activity and Minimum Inhibitory Concentrations (90%) for Josamycin and Erythromycin Against some Common Pathogens (Reproduced from Hardy *et al*, Ref. 20).

Organism	MIC (90%) μg/ml Josamycin	MIC (90%) μg/ml Erythromycin
Mycoplasma pneumoniae	0.03	--
Staphylococcus aureus - Methicillin susceptible	8.0	128
S. aureus - Methicillin resistant	>128	>128
S. epidermidis	>128	>128
Streptococcus pyogenes	0.25	0.03
S. pneumoniae	0.12	0.03
S. agalactiae	0.25	0.06
S. viridans	0.25	0.06
Enterococcus species	>128	>128
Corynebacterium species	32	16
Listeria monocytogenes	2	0.5
Branhamella catarrhalis	1	0.25
Neisseria gonorrhoeae	2	0.5
Campylobacter jejuni	1	1
Legionella pneumophilia	1	2
Haemophilus influenzae	16	4
Bordetella pertussis	0.25	0.03
Bacteroides fragilis	8	4
Other *Bacteroides* species	2	4
Clostridium perfringens	1	1
Propionibacterium acnes	0.12	0.03
Peptococcus-Peptostreptococcus species	8	4

disposition in human volunteers. They found that the elimination half-life of caffeine was increased by 14.6% after treatment with josamycin although this was negligible in comparison with the 95% increase elicited by troleandomycin. Josamycin has also been shown to decrease the total clearance of carbamazepine (33,34), probably due to alteration of its biotransformation by decreasing hepato-cellular activity as carbamazepine is extensively metabolised in the liver (98.5%). However, any adverse effect is less with josamycin than erythromycin and troleandomycin. Interestingly, however, Descotes and Evreux (35) observed that josamycin increased the pentobarbital-induced sleeping time in mice whilst this effect was not observed with erythromycin. This effect, which is an indication of cytochrome p-450 inhibition was, however, less than with troleandomycin.

1.2.4 Tolerance and Side Effects

Josamycin is reported to be well tolerated after single and multiple dosing. In a comparative study between josamycin and erythromycin stearate (28), josamycin

was significantly better tolerated. Out of 21 subjects receiving 500 mg of josamycin six or eight hourly, none presented with gastro-intestinal complaints - the most common side effect of macrolides, and no subjects withdrew from the study. However, of seven subjects receiving 500 mg of erythromycin stearate six hourly, subjects presented with severe gastro-intestinal side effects on 21% of the dosage days whilst two were forced to withdraw from the trial. In a separate study investigating the pharmacokinetics after low and high doses, six out of 15 subjects who were given 1.25 gm and/or 2 gm doses of josamycin (36) reported with gastro-intestinal side effects. However, none were sufficiently severe to warrant exclusion from the trial and symptoms subsided within 1 hr after the intake of food. In attempts to determine the cause of these side-effects Itoh *et al.* (37) investigated the effect of 14- and 16-membered macrolides on the gastro-intestinal activity of the canine gut. They found that 14-membered macrolides increased gastro-intestinal motility whilst 16-membered compounds did not and concluded that a structure-physiological activity relationship exists. Qin *et al.* (38) also investigated the effect of erythromycin and josamycin on the gastro-intestinal motility in dogs and similarly found that josamycin did not disturb GI motility whilst the effects of erythromycin were sufficiently severe to induce emesis in the majority of animals in the study.

Erythromycin derivatives, particularly erythromycin estolate, are known to occasionally induce an acute hepatic reaction (39-43). However, there has only been one report of an acute hepatic reaction (36) occurring with josamycin intake which presented as abnormally high bilirubin levels, although other symptoms associated with erythromycin induced toxicity such as fever and pain were absent. This subject was subsequently found to have had abnormally high bilirubin levels before the start of the study in which he was involved and josamycin could not be uniquely implicated. The propensity for hepatic toxicity reactions with josamycin therefore appears to be extremely low.

1.3 PHARMACOKINETICS

The pharmacokinetics of josamycin in humans have been determined by several investigators and a summary of their results is given in table 1.2. The majority of data have been collected using non-specific microbiological techniques unable to distinguish between parent compound and microbiologically active metabolites. For josamycin, this is cause for concern as its metabolites are microbiologically active and appear in considerable concentrations in serum and urine (44). Results from these studies must therefore be considered with a certain degree of reservation.

Table 1.2 Summary of Pharmacokinetic Parameters for Josamycin Appearing in the Literature.

Author	Dosage Regimen	No. of Subjects.	Subject Status and Special Conditions	C_{max} (μg/ml)	t_{max} (hr)	$AUC_{(0-\infty)}$ (μg.hr.ml^{-1})	k_a (hr^{-1})	k_{el} (hr^{-1})	$t_{½}$ (hr)	Vd** (l)	Assay Method	Ref.
1) Bergan *et al.*	1250 mg stat	9	healthy/fasting	2.64	1.0			0.55	1.26		M. luteus	45
	1250 mg stat	9	healthy/with food	1.93	1.5			0.27	2.57			
2) Bergan *et al.*	250 mg stat	6	healthy/fasting	0.20	0.5		3.04	0.40	1.73		M. luteus	36
	750 mg stat	15	"	1.30	0.5			0.53	1.30			
	1250 mg stat	15	"	3.10	0.5			0.43	1.60			
	2000 mg stat	15	"	8.50	0.5-2.0			0.46	1.50			
3) Strausbaugh *et al.*	500 mg q8h x 4/7	7	healthy/fasting	1st. dose - 0.71	1.0			0.47	1.48		S. lutea	28
				day 2* = 1.64 - 0.09	1.0				—			
	500 mg q6h x 4/7	7	"	1st. dose - 0.61	1.0			0.45	1.56			
				day 2* = 2.31 - 0.34	1.0				—			
	1500 mg stat, then	4	"	1st. dose - 5.06	1.0			—	—			
	500 mg q6h x 10/7			day 10* = 2.61 - 1.04	1.0			—	—			
4) Wildfeuer *at al.*	1000 mg q12h x 4/7	12	healthy/fasting	1st. dose - 1.48	1.0	5.0		0.59	1.16		S. lutea	5
				day 4 = 3.34	1.0	11.5		0.30	2.31			
	1000 mg q12h x 8/7	14	"	1st. dose - 1.72	1.0	6.0		0.47	1.46			
				day 8 = 3.16	2.0	12.9		0.56	1.79			
5) Ikeda *et al.*	1000 mg stat	—	healthy/fasting	3.78	0.72	7.9		—	—		M. luteus	46
	1000 mg stat	—	healthy/with food	4.62	1.95	10.8		—	—			
6) Fourtillan *et al.*	1000 mg stat	5	healthy	0.80	0.75	1.62		0.055	12.7	5763	HPLC	47
7) Fraschini *et al.*	1000 mg q12h x 7/7	4 (1BCM)	Bronchopneumopathic/ fasting	1st. dose - 3.03	1	8.38	3.45	0.41	1.72	300	S. lutea	48
				day 7 = 3.40	1	8.16	3.07	0.45	1.54	273		
		6 (2BCM)		1st. dose - 3.75	1	8.50	2.75	0.30	2.34	403		
				day 7 = 4.22	1	9.02	2.74	0.38	1.84	300		
8) Okolicsanyi *et al.*	1000 mg stat/q12h x 4/7	10	healthy/fasting	1st. dose - 1.78	1.45			0.66	1.05		S. lutea	49
				day 4 = 7.43 (n=4)	1.67			—	—			
	1000 mg stat/q12h x 4/7	9	Gilberts Syndrome/ fasting	1st. dose - 3.43	1.22			0.57	1.22			
				day 4 = 7.21 (n=4)	1.00			—	—			
	1000 mg stat/q12h x 4/7	12	cirrhosis/fasting	1st. dose - 4.34	1.17			0.13	5.36			
				day 4 = 9.85 (n=5)	2.40			—	—			

* C_{max} - C_{min}.

** Apparent volume of distribution calculated from oral data.

1.3.1 Absorption

The extensively used macrolide, erythromycin base, exhibits appreciable acid instability (50-53) and undergoes inactivation in gastric fluids which often compromises bioavailability (50). Attempts to overcome this problem include the use of enteric-coated tablets and derivatives insoluble in acid such as erythromycin estolate and stearate (54,55). However, this has led to complex and often unpredictable absorption patterns (56) which can be further affected by the concomitant intake of food (57,58). Josamycin was introduced as a relatively acid stable macrolide thus avoiding the complications in the absorption process encountered with erythromycin. Unfortunately, no intravenous data are available and therefore the absolute bioavailability of josamycin has not been established. Josamycin has only been administered orally and is confined to tablet preparations due to its extremely bitter taste. The tasteless propionate, however, has been formulated into a suspension for paediatric use (59).

The absorption of josamycin has been found to be rapid and complete within 0.5 to 1.5 hrs after administration of a single oral dose of less than 2 gm to fasting volunteers (Table 1.2). However, the time to peak concentration has been reported to be as high as 2 hrs after a single 2 gm dose (36), and has reached 1.67 hrs after multiple dosing in healthy subjects and up to 2.4 hrs in cirrhotic patients (49). After single 1 gm oral doses, mean peak concentrations determined by microbiological methods ranged between 1.48 and 3.78 μg/ml indicating considerable variation in the extent of absorption. However, Fourtillan *et al.* (47) using a specific HPLC method determined peak concentrations to be significantly lower at 0.80 μg/ml.

Bergan *et al.* (36) demonstrated that josamycin exhibits dose-dependent kinetics by administering four different doses of between 250 mg and 2 gm with mean peak concentrations of 0.2 μg/ml and 8.5 μg/ml after the upper and lower doses respectively. Multiple dose studies with 500 mg six or eight hourly (28), or 1 gm twelve hourly (5) to healthy subjects have resulted in significantly increased blood levels with peak concentrations after multiple dosing reaching between 2 and 4 times the peak concentration of the first dose. However, only a slight increase was observed after dosing with 1 gm twelve hourly to bronchopneumopathic patients (48).

Administration of josamycin with food was shown by Bergan *et al.* (45) to retard absorption, increase the t_{max} and decrease the C_{max} in comparison with equivalent doses given to fasting subjects. However, Ikeda *et al.* (46) found that food delayed, rather than retarded, absorption and increased the C_{max} from 3.78 to 4.62 μg/ml. Also, the AUC increased, signifying a slight increase in bioavailability. Similarly, serum concentrations of josamycin propionate were also found to be three times higher when administered with food than on a fasting stomach. Similar anomalous results

have also been obtained from numerous studies on the effect of food on the absorption of erythromycin (60). However, the overall conclusion from studies on erythromycin is that the formulation rather than the drug *per se* is largely responsible for the highly variable results. A similar conclusion cannot, however, be made with josamycin due to the limited amount of data available.

1.3.2 **Distribution**

The distribution of josamycin into various body tissues has been extensively reviewed by Periti *et al.* (61). Josamycin exhibits extensive distribution and Fraschini *et al.* (48), utilising a microbiological assay reported an apparent volume of distribution of 300 L and 403 L for a one body-compartment and two body-compartment model respectively. However, Fourtillan *et al.* (47), utilising a specific HPLC assay reported a value as high as 5763 L. The volume of distribution for erythromycin has been reported to be about 47 L (62). However, since josamycin is approximately 15 times more lipophilic than erythromycin, a significantly greater volume of distribution for josamycin is expected. Values obtained by Fraschini for josamycin therefore compare favourably with the volume of distribution for erythromycin. Whilst one is inclined to believe that the data obtained by Fourtillan using HPLC is a more accurate reflection of the actual josamycin serum concentrations, the extremely high volume of distribution of 5763 L appears to be excessively high.

Josamycin distributes into bronchial aspirates, sputum, aqueous humour, tears, healthy and pathological gums, saliva and suction blister fluids together with long bones and lower jaw bone in concentrations below that of serum but above the minimum inhibitory concentrations of most susceptible pathogens (63). However, josamycin is concentrated in lung tissue (2.8 - 12 times the serum concentration), tonsils (up to 28 times), middle ear exudates (up to 4 times), sinus secretions (3 times), adenoids (3.5 times) and subretinal fluids (2 times) making it particularly useful for the treatment of upper respiratory tract infections. Josamycin is also concentrated in prostate fluids reaching concentrations of 5.0 μg/ml, and as mentioned earlier, into polymorphonuclearcytes.

Josamycin is only 15% protein bound (5) in comparison with the 70-90% for erythromycin (5,64,65) which is predominantly bound to α_1-acidglycoprotein (66,67). Changes in serum composition such as in cirrhotics or displacement from binding sites by other drugs is therefore unlikely to exhibit any significant effect on the unbound fraction, total serum concentration or distribution.

Distribution into the intestinal fluid of rabbits after i.v. administration of josamycin has been documented by Fukuya (68) who found that the concentration in intestinal fluids was 4.3 times that of the serum concentration. Similarly, erythromycin is excreted into the mid-jejunal segments of rabbits against a concentration gradient, probably by an active transport mechanism (69). Josamycin also distributes into maternal milk and reached concentrations of 1.1 times that of the serum six hours after a 500 mg dose (70). This observation has been attributed to the high lipophilicity of josamycin and its affinity for the fat content of milk rather than by an active transport mechanism.

1.3.3 Metabolism

Hydroxylation in the liver at position-15 on the lactone ring to 15-hydroxy josamycin (JM-O_1) and at the ß-position on the isovaleryl moiety to ß'-hydroxy josamycin (JM-O_2) are the major metabolic pathways of josamycin. A third but minor route of metabolism results in cleavage of the isovaleryl moiety to form deisovaleryl josamycin (DeIv-JM) (71,72). This is in contrast to erythromycin which undergoes N-demethylation to form N-des-methyl erythromycin (73) in rabbits and rats. Molecular structures of the metabolites of josamycin are shown in figure 1.1. Metabolism studies in rats have shown that josamycin is easily metabolised and that both josamycin and metabolites are totally excreted mainly into faeces and to a minor extent into urine (71). The ease with which josamycin is metabolised is thought to be largely responsible for its low toxicity.

Further studies using rat livers show that other macrolides, namely troleandomycin (74-76), and to a lesser extent erythromycin (77) induce their own transformation and subsequently form inactive stable metabolite-cytochrome P-450-Fe(II) complexes. In the case of troleandomycin, this can lead to decreased mono-oxygenase activity which is reflected by a decrease in the clearance of antipyrine in humans (76). With erythromycin the overall mono-oxygenase activity remains unchanged (77). Conversely, josamycin does not exhibit any inducing activity nor does it form any inactive complexes (78). It has been suggested that this inducing/inactivating activity is dependent upon the presence of a sterically unhindered, readily accessible N-dimethylamino group, which is required for binding to iron (79). Furthermore, this activity increases with increasing hydrophobicity. Although josamycin is highly hydrophobic, the N-dimethyl amino group is extensively hindered by a bulky mycarose moiety which prevents any such activity. This has been cited as the reason for the lack of interference by josamycin on the metabolism of drugs which are metabolised by the cytochrome P-450 enzyme system (80). It has

therefore been suggested that josamycin is a better option than either troleandomycin or erythromycin for patients requiring therapy with macrolide antibiotics whilst receiving concomitant therapy with drugs such as theophylline and carbamazepine. Furthermore, the usefulness of josamycin in these situations is borne out by the lack of reports describing drug interactions with this antibiotic.

Okolicsanyi *et al.* (49) studied the pharmacokinetics of josamycin in cirrhotics and found that serum levels, AUC and terminal half-lives were all increased compared with normal subjects, probably due to a decreased first-pass metabolism and to impaired biotransformation. Dosage adjustment was therefore recommended in these patients. Increased levels were also observed in patients with biliary occlusion and parenchymatous liver disease (45), and also in Gilberts Syndrome although to a highly variable degree (49).

1.3.4 Excretion

Studies on the excretion of josamycin in mice have shown that over 75% is excreted into the faeces whilst over 23% is excreted into the urine (71). Animal studies have also shown that josamycin is concentrated 100-1000 times in the bile but to-date, no study investigating the excretion of josamycin into bile in humans has appeared in the literature. However, evidence of extensive biliary excretion was obtained from a study conducted by Bergan and Øydvin (45) on subjects with biliary occlusion. In these patients an extremely prolonged absorption phase and elevated elimination half-life were observed. They concluded that normally, intact josamycin is extensively excreted in bile. However, in these patients this route of elimination was not patent which prevented its rapid excretion resulting in the serum concentration *vs.* time profiles reflecting only the absorption process rather than absorption and elimination.

Relatively insignificant amounts are excreted in the urine. Although josamycin and its three main metabolites have been detected in urine by thin-layer chromatography (71), to-date, quantitative urinary excretion data are confined to the total microbiological activity of the excreted compounds. These single dose studies have accounted for up to 1% (81), 0.39% (49) and 1.8 - 8.1% (36) appearing in the urine, depending on the dose administered. Urinary excretion after multiple dosing increases and Strausbergh *et al.* (28) observed an increase from between 4.4% - 14.3% for the first day to between 14.8% and 17.5% after eight days whilst Okolicsanyi *et al.* (49) observed an increase from 0.39% to 2.3% after a four day regimen.

No data concerning the pharmacokinetics of josamycin in patients with renal disease have appeared in the literature. However, since josamycin is only slightly excreted in the urine significant accumulation is unlikely to occur in these patients.

1.3.5 Pharmacokinetic Modelling

Pharmacokinetic modelling of josamycin serum concentration *vs.* time profiles has received little attention with the majority of modelling having been carried out on scanty data (48) from single dose studies. Published reports, do however, suggest that the pharmacokinetics of josamycin follow a two body-compartment model (48,63,47). However, there is a dearth of pharmacokinetic modelling data on multiple dose studies.

In summary, josamycin is a 16-membered macrolide antibiotic which is significantly more lipophilic that erythromycin and is extensively distributed to a variety of tissues in concentrations above the MIC of many pathogens. As with other macrolides, it is particularly useful for the treatment of upper respiratory tract infections. Furthermore, josamycin is an effective alternative for the treatment of erythromycin resistant staphylococci. Josamycin has proved to be well tolerated and causes few gastro-intestinal side effects as well as having no or negligible effect on the pharmacokinetics of other compounds commonly associated with interactions with macrolides. Josamycin is reputed to be appreciably more stable than erythromycin and appears to be well absorbed from the tablet preparations used in pharmacokinetic studies reported in the literature. However, only sparse data on its stability are available and a comprehensive stability study and pH-rate profile is lacking. Although it is well understood that josamycin is extensively distributed into body tissues and is subsequently eliminated predominantly in the bile, the elimination rate of josamycin is a contentious point and requires further investigation utilising specific HPLC techniques capable of the selective detection of josamycin in biological fluids.

CHAPTER 2

QUANTITATIVE DETERMINATION OF JOSAMYCIN

High-performance liquid chromatography (HPLC) has become one of the most widely used analytical techniques for the analysis of compounds of pharmaceutical interest. Numerous analytical methods utilising HPLC are published in scientific journals monthly and HPLC assays are being increasingly adopted by compendia such as the United States Pharmacopoeia as official methods for the analysis of raw materials and dosage forms. The widespread use of HPLC has been encouraged by the development of reliable and reasonably priced equipment, versatility in the choice of detection method and the potential for a high degree of selectivity and sensitivity in a wide diversity of simple and complex matrices. Furthermore, samples can often be rapidly analysed after minimal sample manipulation with easy and accurate quantitation. HPLC has thus become the analytical method of choice for the analysis of many compounds in both biological and non-biological matrices.

A number of HPLC assays for the analysis of josamycin have been published. However, these have been developed specifically for the analysis of josamycin in serum. Two of these required expensive and complex chromatographic systems (47,82) whilst a third was insufficiently sensitive for use in pharmacokinetic studies (83). High-performance liquid chromatographic assays utilising standard equipment and suitable for the analysis of josamycin raw material, josamycin in tablet dosage forms, studies on the biopharmaceutics of josamycin, and the analysis of josamycin in biological samples were required, and therefore developed in our laboratories.

2.1 ANALYTICAL METHODS FOR USE WITH RAW MATERIAL, INTRINSIC DISSOLUTION RATE STUDIES AND DOSAGE FORM ANALYSIS.

2.1.1 HPLC Apparatus

The modular HPLC system consisted of a Beckman 114 constant-flow pump (Beckman Instruments Inc., Fullerton, CA, U.S.A.), a WISP 710B automated sample injector (Waters Assoc., Milford, MA, U.S.A.), an M490 multi-wavelength programmable UV detector (Waters Assoc., Milford, MA, U.S.A.) and an Hitachi 561 dual-pen flat-bed recorder (Hitachi, Tokyo, Japan). Analysis was performed on a 25 cm x 3.9 mm I.D. stainless-steel column, packed in our laboratory with Techsil

micro-particulate bonded (10 μm) octadecylsilane (C_{18}) material (HPLC Technology, Wilmslow, U.K.). The column temperature was maintained at 35°C by a Model LC-22 temperature controller (Bioanalytical Systems, W. Lafayette, IN, U.S.A.).

2.1.2 Chemicals

Chemicals were of analytical grade. The acetonitrile was of distilled-in-glass UV grade (Burdick & Jackson, Muskegon, MI, U.S.A.). Phosphoric acid, citric acid, di-sodium hydrogen phosphate and sodium hydroxide were obtained from Merck (Johannesburg, South Africa). Josamycin base was obtained from Yamanouchi Pharmaceutical Co. (Tokyo, Japan) and oleandomycin phosphate from Pfizer (Pietermaritzburg, South Africa). Water used for extraction and chromatography was initially purified by a reverse-osmosis Milli-RO 15 water purification system (Millipore, Bedford, MA, U.S.A.), followed by further purification by filtration through a Milli-Q system (Millipore, Bedford, MA, U.S.A.) consisting of a Super-C carbon cartridge, two Ion-X ion exchange cartridges, an Organex-Q cartridge and a 0.22 μm Millipak stack filter.

2.1.3 Method Development

2.1.3.1 Principles of Reversed-Phase Chromatography

Reversed-phase column packing material has become the most widely used stationary phase in HPLC due to its versatility and application to the retention and subsequent separation of a wide range of compounds. Reversed-phase materials are prepared by the reaction of n-alkylchlorosilanes with surface silanol groups of a silica stationary phase. This results in conversion of the hydrophilic surface of the silica to a predominantly hydrophobic surface. However, residual unreacted silanols retain an element of hydrophilicity on the stationary support (84), enabling many compounds to be retained by both solvophobic and silanophilic interactions. Such a dual-retention mechanism has been proposed by Bij *et al.* (85). Solvophobic or hydrophobic retention mechanisms involve the preferential partitioning of compounds out of a hydrophilic liquid phase into the hydrophobic environment of the solvated C_{18} moiety of the stationary phase. Silanophilic retention mechanisms involve hydrogen bonding and/or ionic interactions between the compound and ionised silanol groups on the stationary phase (86), with the predominant mechanism depending on the pKa of the compound and silanol groups and pH of the mobile phase (86). The relative importance of each mechanism for solute retention is peculiar to any particular compound and although

C_{18} stationary phases are extremely lipophilic, retention can be predominantly hydrophilic or silanophilic (87).

Elution of compounds exhibiting dual-retention mechanisms from reversed-phase materials can be effected by an organic-aqueous mixture containing either amine modifiers or buffers. The organic constituent serves to elute compounds from the C_{18} moiety of the stationary phase, while the amines and cations of the aqueous constituent displace compounds from silanophilic binding sites by competition for residual silanols (87-89). Interaction between compound and silanols can also be prevented by buffer anions (such as phosphate and acetate) which can act as counter-ions to cationic compounds *e.g.* protonated amines. Basic amine compounds are known to be retained on reversed-phase stationary phases by a dual-retention mechanism (89,90,85) and therefore require such an organic-aqueous mixture for elution. Differences between the retention characteristics of compounds can be exploited to effect separation by adjusting the organic-aqueous ratio and the composition of the aqueous phase (91). This can be used not only to facilitate separation of compounds but also to optimise peak shape once separation has been achieved. This is of particular importance for basic drugs such as josamycin as the efficiency of reverse-phase columns for these drugs is generally low (92). Column efficiency can, however, be maximised by careful selection of the mobile phase constituents.

2.1.3.2 Choice of Column

Josamycin is a weakly basic compound with a pKa of 7.1 and has a tertiary amine situated on the mycaminose moiety (Figure 1.1). This tertiary amine and the lipophilicity of the josamycin molecule ensure retention on reversed-phase material by a dual-retention mechanism. Phenyl, cyano, C_8 and C_{18} columns have all been used successfully for the analysis of numerous macrolides (82,93-100). However, assays published to-date for the analysis of josamycin (83,47,101) have only used C_8 and C_{18} analytical columns. Assay development was therefore initiated utilising a 10 μm C_{18} column, which was subsequently used for the analysis of raw material, dosage forms and samples from intrinsic dissolution rate and bioavailability studies.

Analytical columns (25 cm x 3.9 mm I.D. stainless-steel column) were packed in the laboratory with Techsil reversed-phase micro-particulate bonded (10 μm) octadecylsilane (C_{18}) material utilising a slurry technique and a Shandon column packing apparatus (Shandon Southern Products Ltd., Runcorn, Cheshire, England). Column efficiency of newly packed columns was assessed by the injection of a 5 μl sample of test mixture containing benzamide (15 μg/ml), benzene (125 μg/ml), benzophenone (15 μg/ml) and biphenyl (15 μg/ml) onto the column, with a mobile phase of acetonitrile-water 3:1 at 1 ml/min with UV detection at 254 nm. A typical chromatogram of the test mixture is depicted in figure 2.1. The number of theoretical

plates for the benzophenone peak was then calculated utilising equation 2.1 (102):-

$$N = 5.54 \left(\frac{t_R}{W_{1/2}}\right)^2 \qquad \text{Eq. 2.1}$$

where N = the number of theoretical plates.

t_R = the retention time of the benzophenone peak, and

$W_{1/2}$ = the peak width at half peak height.

Only columns exhibiting theoretical plate counts above 6000 were used. The performance of these columns was good and were used for the analysis of over 2000 samples. However, changes in the retention times of josamycin and oleandomycin were observed with use, often with peak shape and resolution improving.

2.1.3.3 Internal Standard

Oleandomycin (Figure 2.2) belongs to the macrolide group of antibiotics and has a structure similar to that of josamycin in that the molecule consists of a lactone ring with two sugar residues attached. The retention behaviour of oleandomycin on C_{18} reversed-phase material of both analytical and solid-phase extraction columns (used for the extraction of josamycin from biological matrices) has been well established from previous work undertaken in this laboratory (98,100), and was similar to that of josamycin. Oleandomycin was therefore chosen as the internal standard.

2.1.3.4 Choice of Mobile Phase

The use of UV detection at low wavelengths required that all solvents and buffers in the mobile phase exhibit a UV cut-off below 204 nm. Acetonitrile (with a UV cut-off of 190 nm), phosphoric acid and sodium hydroxide were therefore used in the development of a suitable mobile phase.

A series of experiments were conducted to determine the effect of buffer molarity, buffer pH and the organic-aqueous ratio of the mobile phase on the retention of josamycin on C_{18} reversed-phase analytical columns, results of which are depicted in figure 2.3. Increases in buffer molarity, buffer pH and organic content all served to decrease the retention time of josamycin. Once the retention time of josamycin was adjusted to about six minutes, separation of josamycin from internal standard was easily afforded by only slight adjustment of the mobile phase composition.

The final mobile phase was acetonitrile-0.015 M phosphate buffer pH 6.0 (5:2). The buffer was prepared by making 0.96 ml phosphoric acid (85%) up to 1 litre with water and adjusting the pH to 6.0 with sodium hydroxide pellets. The mobile phase was degassed and filtered through a 0.4 μm HVLP Millipore filter prior to use.

Figure 2.1

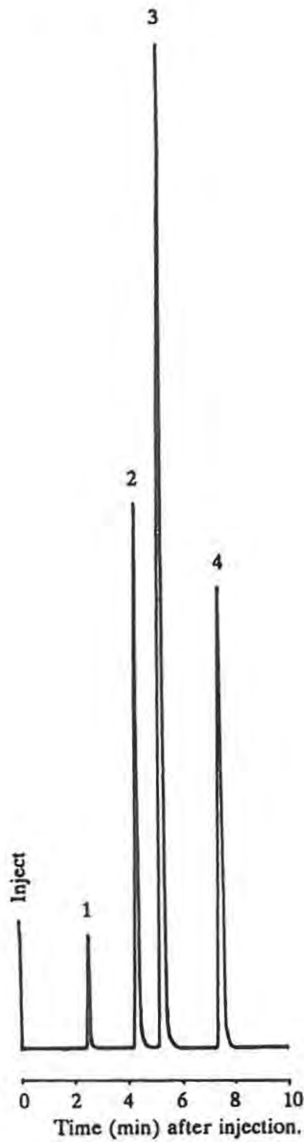


Figure 2.2

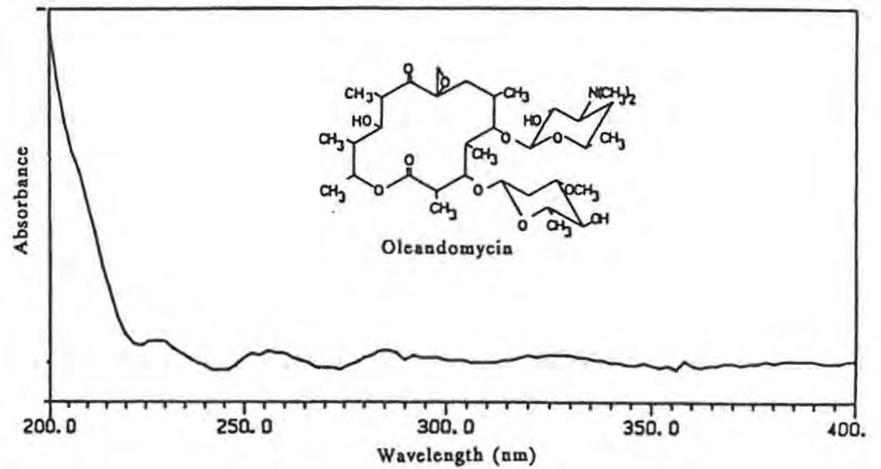


Figure 2.3a

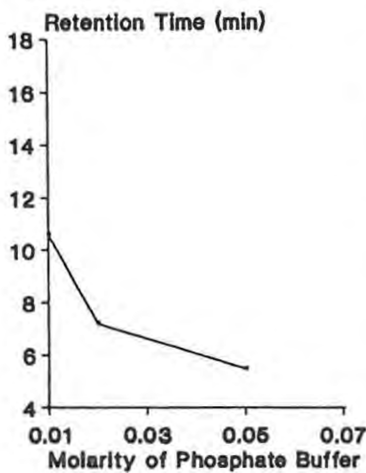


Figure 2.3b

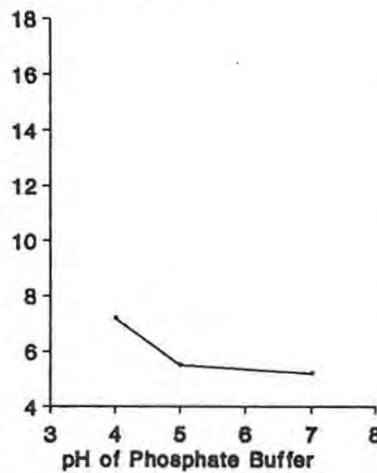


Figure 2.3c

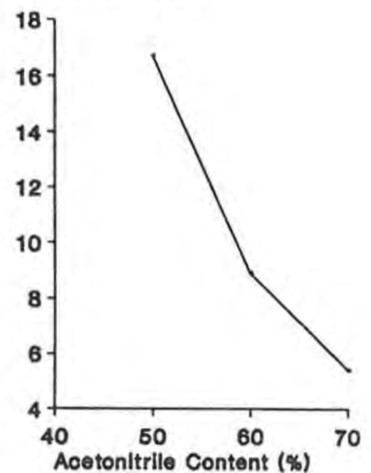


Figure 2.1 Typical chromatogram of a test mixture containing benzamide (1), benzene (2), benzophenone (3) and biphenyl (4) after chromatography on a 10 μm C_{18} analytical column packed in our laboratory.
Figure 2.2 Molecular structure and UV absorbance spectrum of oleandomycin.
Figure 2.3a, b and c Plots showing the effect of (a) buffer molarity at pH 6.0 and acetonitrile/buffer 1:1, (b) buffer pH at 0.025 M and acetonitrile/buffer 1:1 and (c) acetonitrile content of the mobile phase at buffer pH 6.0, 0.015 M on the retention time of josamycin on 10 μm C_{18} stationary phase.

Typical chromatograms of an aqueous sample containing josamycin and oleandomycin at 231 nm and at 204 nm are depicted in figures 2.4a and 2.4b respectively.

2.1.3.5 **UV Detection of Josamycin and Internal Standard**

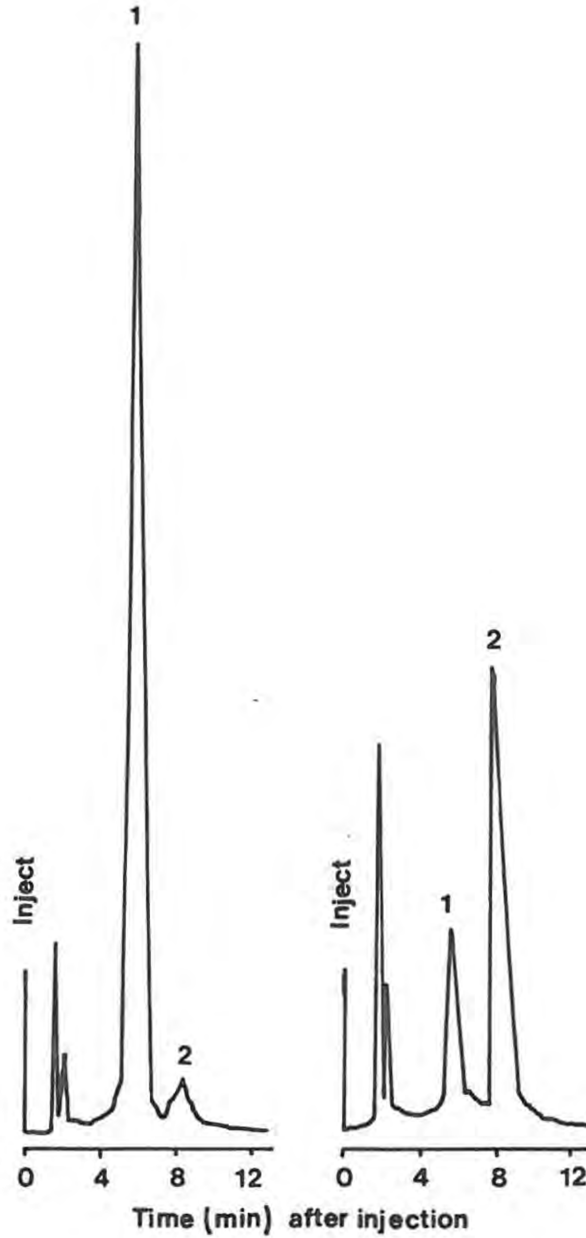
The UV absorbance spectra of josamycin (Figure 1.2) and oleandomycin (Figure 2.2) indicate that both compounds absorb strongly at lower UV wavelengths with a UV λ-max of 231 nm for josamycin, and oleandomycin exhibiting particularly strong absorbance at 200 nm. Oleandomycin, however, shows little or no UV absorbance at 231 nm, the wavelength of maximum absorbance of josamycin. Utilisation of dual-wavelength monitoring, however, enabled the effective use of this internal standard. The eluate was monitored at 231 nm for the detection of josamycin and at 204 nm for the detection of oleandomycin. Monitoring the eluate at 200 nm would be optimum for the detection of oleandomycin. However, excessive baseline noise was obtained at 200 nm. Furthermore, chromatographic interference from small amounts of contaminants is common at low wavelengths. Since detection of the internal standard at the optimum wavelength was not necessary, the eluate was monitored at 204 nm to reduce the baseline noise as well as the chance of chromatographic interference with the internal standard by traces of contaminants. Sufficient internal standard was used in each sample to obtain an adequate response at 204 nm at an attenuation of 0.04 absorbance units full scale (a.u.f.s.) or above.

2.1.4 Chromatographic Conditions

1) Mobile phase flow rate	- 1.2 ml/min
2) Column temperature	- 35°C
3) Column pressure	- 55 bar
4) Retention times	- Josamycin *ca.* 6 min
	- Internal standard *ca.* 8 min
5) Detector settings	- Channel 1:- λ = 231 nm, 0.02 - 0.2 a.u.f.s.
	- Channel 2:- λ = 204 nm, 0.04 - 0.06 a.u.f.s.
	- Time constant = 1.0 s
	- Threshold value = 0%
6) Injection volume	- 2 - 50 μl
7) Chart speed	- 5 mm/min
8) Chart recorder input	- 10 mV full scale

<u>Figure 2.4a</u>
231 nm, 0.1 a.u.f.s.

<u>Figure 2.4b</u>
204 nm 0.1 a.u.f.s.



<u>Figures 2.4a and b</u> Typical chromatograms obtained at a) 231 nm and b) 204 nm, of an aqueous sample of josamycin (1); on-colum load = 2 μg, and oleandomycin (2); on-column load = 20 μg, after analysis on a 10 μm C_{18} analytical column. The mobile phase was acetonitrile-phosphate buffer 0.015 M, pH 6.0, 5:2, 1.2 ml/min.

2.1.5 Intrinsic Dissolution Rate Studies

2.1.5.1 Preparation of Standards

Calibration curves from 0 - 1.0 mg/ml were constructed for the analysis of samples from intrinsic dissolution rate studies at pH 1.2 and 2.2, from 0 - 0.1 mg/ml for the analysis of samples from studies at pH 3.0, 4.0, 5.0, and 6.0, and from 0 - 0.01 mg/ml for the analysis of samples from studies at pH 7.5.

The upper standard for the calibration curve from 0 - 1.0 mg/ml was prepared by accurately weighing 10 mg of josamycin into a 10 ml volumetric flask, dissolving in 2 ml of acetonitrile and making up to volume with water. The upper standard for the calibration curve from 0 - 0.1 mg was prepared by accurately weighing 10 mg of josamycin into a 100 ml volumetric flask, dissolving in 2 ml of acetonitrile and making up to volume with water. A 1 in 10 dilution of this solution was used as the upper standard for the calibration curve from 0 - 0.01 mg/ml. The remaining standards for each calibration curve were prepared by the 1 in 2, 1 in 5 and 1 in 10 dilution of the respective upper standard.

A 50 μl aliquot of each standard was then mixed with 200 μl of internal standard solution (oleandomycin phosphate, 0.5 mg/ml) and vortexed for 30 s. A 50 μl aliquot of the resulting solution was then injected into the chromatographic system described in 2.1.1.

The internal standard solution was prepared by dissolving 10 mg of oleandomycin phosphate in 4 ml of acetonitrile and making up to volume in a 20 ml volumetric flask with 0.1 M phosphate buffer pH 7.5. This buffer was prepared by making 6.4 ml of phosphoric acid (85%) up to 1 litre with water and adjusting the pH to 7.5 with sodium hydroxide pellets. The internal standard solution was prepared with buffer in order to raise the pH of acidic samples to above pH 5.0 which prevented degradation of josamycin in these samples whilst awaiting analysis. Standards were anlaysed in duplicate and calibration curves were constructed by linear regression of plots of peak height ratio *vs.* concentration. These were found to be linear over the concentration ranges studied.

The attenuation of channel 1 of the detector was set so as to obtain a full scale response for josamycin from the upper standard of each calibration range, and the attenuation of channel 2 set to 0.06 a.u.f.s. for the detection of internal standard.

2.1.5.2 Sample Preparation

A 50 μl aliquot of dissolution medium was removed from the dissolution vessel (4.1.1.3) with a Pipetman fixed volume pipette (Gilson,Villiers-le-Bel, France), added to 200 μl of internal standard solution and mixed on a vortex mixer for 30 s. A 50 μl aliquot of the resulting solution was then injected into the chromatographic system for

analysis. Results of intrinsic dissolution rate studies are detailed in 4.1.2.

2.1.5.3 **Precision and Accuracy**

Calibration curve parameters for each intrinsic dissolution rate study are listed in table 2.1. A high degree of linearity is demonstrated by the R.Sq. values (where R.Sq. is the coefficient of variation) which ranged from 0.9639 to 0.9997 confirming the suitability of this assay to these studies.

2.1.6 Tablet Content Analysis

The tablet content of both Josamycin 200 mg tablets (Yamanouchi Pharmaceutical Co., Tokyo, Japan) and Josacine 500 mg tablets (Pharmuka, Gennevilliers, France) were assessed prior to dissolution testing and bioavailability studies.

2.1.6.1 **Preparation of Standards**

The tablet content of individual tablets was determined by comparing the peak height ratios of test solutions with that of a standard solution equivalent to 100% of the label claim. A linear response for josamycin on-column loads of between 0 and 11 μg was obtained for calibration curves constructed during intrinsic dissolution rate studies at pH 1.2 as detailed in 2.1.5.1. The on-column load of josamycin in tablet content determinations was within this range; *i.e.* approximately 4 μg for the Josamycin 200 mg Tablets and approximately 10 μg for the Josacine 500 mg Tablets. A single calibrator was therefore suitable for the determination of the tablet content of both tablet formulations.

The standards for tablet content analysis of 200 mg and 500 mg tablets were prepared by accurately weighing 200 mg or 500 mg of josamycin respectively, into a 100 ml volumetric flask, dissolving in 50 ml of acetonitrile, and after the addition of 5 ml of internal standard solution, making up to volume with water. A 2 μl aliquot of the resulting solution was then injected into the chromatographic system described in 2.1.1. The standard solution was analysed in triplicate and the mean peak height ratio of the three analyses used as the standard peak height ratio.

The internal standard solution was prepared by dissolving 1 gram of oleandomycin phosphate in 25 ml of acetonitrile in a 50 ml volumetric flask and making up to volume with water.

During this study, the eluate was monitored only at 204 nm as the concentration of josamycin in the samples was sufficient for quantitation at this wavelength. Channel 1 of the detector was therefore set at 204 nm for the detection

of both josamycin and internal standard. An attenuation change was, however, necessary and the detector was programmed to maintain an attenuation of 0.2 a.u.f.s. for the first 7 min after injection for the detection of josamycin, and to effect an attenuation change to 0.02 a.u.f.s. after 7 min for the detection of internal standard.

A typical chromatogram showing the detection of josamycin and internal standard at 204 nm with the programmed attenuation change is depicted in figure 2.5.

2.1.6.2 **Sample Preparation**

Each tablet (n = 6 tablets for each dosage form) was placed in a 100 ml volumetric flask. After the addition of 50 ml of acetonitrile, 25 ml of water and 5 ml of internal standard solution, the flask was sonicated for 10 min to ensure complete disintegration and dissolution of the tablet. The volume was then made up to 100 ml with water and the flask sonicated for a further 5 min. A 1 ml aliquot of the resulting solution was then centrifuged at 12000 rpm for 5 min in a high speed Eppendorf 5414 centrifuge (Eppendorf, Gerätebau, Germany) to remove any suspended particles from the solution. A 2 μl aliquot of the supernatant was then injected into the chromatographic system described in 2.1.1. Each test solution was analysed in triplicate and the tablet content calculated as the mean of the three analyses. The tablet content uniformity was assessed by calculating the standard deviation of the tablet content from the six tablets.

2.1.6.3 **Results and Discussion**

Tablet content and content uniformity results are tabulated in table 2.2. Josamycin 200 mg tablets and Josacine 500 mg tablets were found to contain 109.58% $\pm$1.28% and 105.47% $\pm$0.84% of the label claim, respectively. The content uniformity was highly precise, although a slight excess of the label claim was present in both formulations. The precise content uniformity ensured that all dissolution studies (4.3) were conducted on tablets containing an equal quantity of josamycin, and also that a uniform amount was administered with each dose of either product during bioavailability studies (*vide infra* Chapter 6). Allowance for this excess was taken into account in calculations dependent on the tablet content in dissolution studies.

The relative standard deviation (RSD %) of triplicate injections (Table 2.2) for each tablet was an indication of the precision of the assay. Values for both tablet preparations were all $\leq$ $\pm$4.01 %.

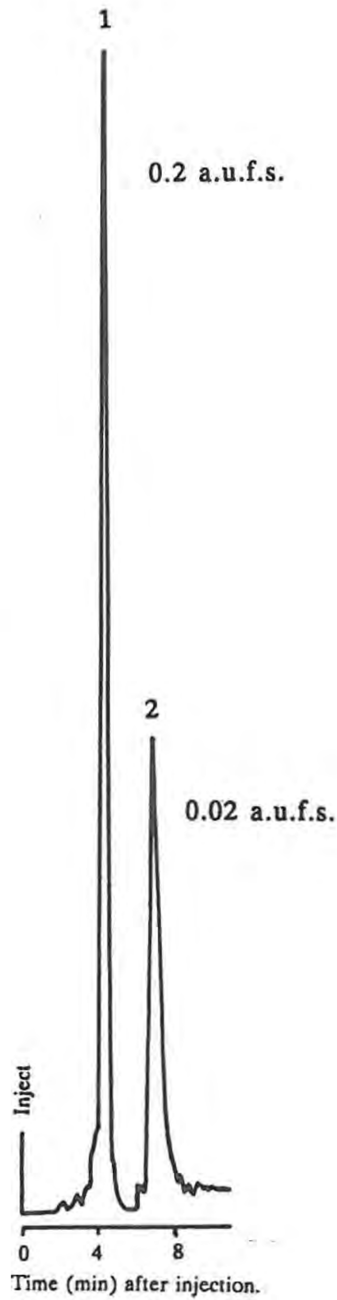


Figure 2.5 Typical chromatogram obtained at 204 nm showing the detection of josamycin (1) at 0.2 a.u.f.s., the programmed attenuation change, and detection of internal standard (2) at 0.02 a.u.f.s. Chromatography was performed on a 10 μm C_{18} analytical column. The mobile phase was acetonitrile-phosphate buffer 0.015 M, pH 6.0, 5:2, 1.2 ml/min

2.1.7 Discussion

The decrease in retention time of josamycin with increasing organic content of the mobile phase indicated the existence of a typical hydrophobic interaction between josamycin and the stationary phase, whereas the decrease in retention time with increasing buffer molarity was indicative of a silanophilic retention mechanism. This buffer effect can be explained by the increased shielding of josamycin from ionic binding sites by buffer ions with increasing buffer molarity. The decrease in retention time with increasing pH, however, was opposite to what was expected for josamycin. As josamycin is slightly basic (with a pKa of 7.1) it becomes less protonated as the pH increases. If the retention mechanism involves hydrophobic bonding, the retention time should therefore increase with increasing pH. This however, was not observed and no suitable explanation could be put forward.

The retention of oleandomycin on C_{18} packing material was also found to be highly sensitive to the organic-aqueous phase ratio and the pH and molarity of the buffer component. Increased organic phase and molarity, and decreased pH all served to reduce the retention time of oleandomycin. However, oleandomycin was more sensitive to buffer molarity and pH and less sensitive to acetonitrile concentration than josamycin. These differences facilitated easy separation of the two compounds on a C_{18} reversed-phase analytical column with the internal standard eluting after josamycin.

A multi-wavelength programmable detector enabled the use of an internal standard which showed little or no UV absorbance at 231 nm, the wavelength of maximum absorbance of the compound of interest. Chromatographically, oleandomycin was highly suitable as an internal standard in this analytical system and its use as such was made possible by the application of dual-wavelength monitoring. The additional programmable facilities of the M490 detector also facilitated the tablet content assay. By employing a programmed attenuation change, the eluate could be monitored at a single wavelength. During these studies, the on-column load of josamycin was sufficient for an adequate response to be obtained at 204 nm (at which wavelength josamycin shows minimal UV absorbance) and at a low attenuation of 0.2 a.u.f.s. However, an attenuation of 0.02 a.u.f.s. was used for the detection of internal standard. Use of an attenuation change reduced the amount of internal standard required in each sample. If the internal standard was monitored at 0.2 a.u.f.s., an excessive amount of oleandomycin would have been required in each test solution in order to obtain an adequate response without increasing the injection volume. Increasing the injection volume was not possible as this led to overloading of the column with josamycin.

Initially, the mobile phase was recycled during the analysis of samples from intrinsic dissolution rate studies to reduce the volume of mobile phase used. However, the retention times of both josamycin and internal standard slowly changed after repeated injections of 50 μl samples consisting of relatively high buffer molarity (samples of 0.1 - 0.2 M in comparison with 0.015 M buffer in the mobile phase). Also, a negative peak immediately before and interfering with the josamycin peak became increasingly apparent after each injection. Recycling of mobile phase was therefore avoided during these studies.

2.2 ANALYTICAL METHODS FOR DEGRADATION, SOLUBILITY, AND TABLET AND POWDER DISSOLUTION STUDIES USING HPLC

Initial investigations into the stability of josamycin in acidic medium utilising the HPLC system detailed in 2.1.1. showed the formation of two early eluting compounds (degradation products P2 and P3) which were well resolved from josamycin, and a third compound (P1) which eluted slightly before but unresolved from josamycin. Figure 2.6 shows a chromatogram of a sample of josamycin following degradation in 0.1 M HCl at 37°C, and taken 10 min after innoculation of the acid medium with josamycin at a concentration of 1 mg/ml. An alternative analytical method was therefore developed to separate josamycin from all three acid degradation products for the analysis of samples from degradation, solubility, and tablet and powder dissolution studies - studies in which degradation of josamycin at low pH's was extensive. The separation of josamycin from alkali degradation products was also investigated in order to optimise an assay for the analysis of samples from alkali degradation studies.

2.2.1 HPLC Apparatus

The modular HPLC system consisted of a Beckman 114 constant-flow pump (Beckman Instruments Inc., Fullerton, CA, U.S.A.), a fixed loop SSI 3XL manual injector (Scientific Systems Inc., State College, PA, U.S.A.) fitted with loop disc No. 3, an M490 multi-wavelength programmable UV detector (Waters Assoc., Milford, MA, U.S.A.), and an Hitachi 561 flat-bed recorder (Hitachi, Tokyo, Japan). The analysis was performed on an Ultrasphere-XL, 3 μm C_{18} analytical column with guard cartridge also containing 3 μm C_{18} packing material (Beckman Instruments Inc., Altex Division, San Ramon, CA, U.S.A.), at ambient temperature.

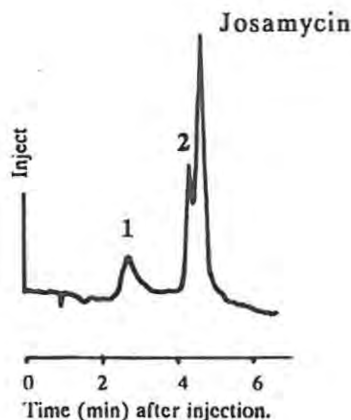


Figure 2.6 Chromatogram showing 2 early eluting acid degradation products P2 and P3 eluting as a single peak (1), and the co-elution of a third degradation product P1 (2) with josamycin after chromatography on a 10 μm C_{18} analytical column. The mobile phase was acetonitrile-phosphate buffer 0.015 M, pH 6.0, 2:1 at 1.2 ml/min.

2.2.2 Method Development

2.2.2.1 Choice of Column

After extensive manipulation of the acetonitrile-phosphate buffer mobile phase, adequate separation of josamycin from degradation product P1 was not obtained on a 10 μm C_{18} column. Three additional commercially produced analytical columns were therefore assessed:-

1) Nova Pak, 10 μm C_{18} (Waters Assoc., Milford, MA, U.S.A.),

2) Waters 10 μm C_8 (Waters Assoc., Milford, MA, U.S.A.), both without guard cartridges, and

3) Beckman Ultrasphere-XL, 3 μm C_{18} analytical column with guard cartridge.

Chromatograms of the most efficient separations obtained for each column are depicted in figures 2.7a,b and c. The mobile phases used are listed in the respective figure legends. Adequate separation was only obtained on the Ultrasphere-XL column and this column was subsequently used for the selective analysis of josamycin in solutions containing both josamycin and degradation products.

2.2.2.2 Mobile Phase

The effect of the buffer pH and the acetonitrile content of the mobile phase on the retention time of josamycin and P1 on the Ultrasphere-XL column was similar for both compounds. Consequently, the two compounds could not be separated by manipulation of these two mobile phase components alone. However, retention of josamycin was more sensitive to buffer molarity than P1 and separation improved as

Figure 2.7a

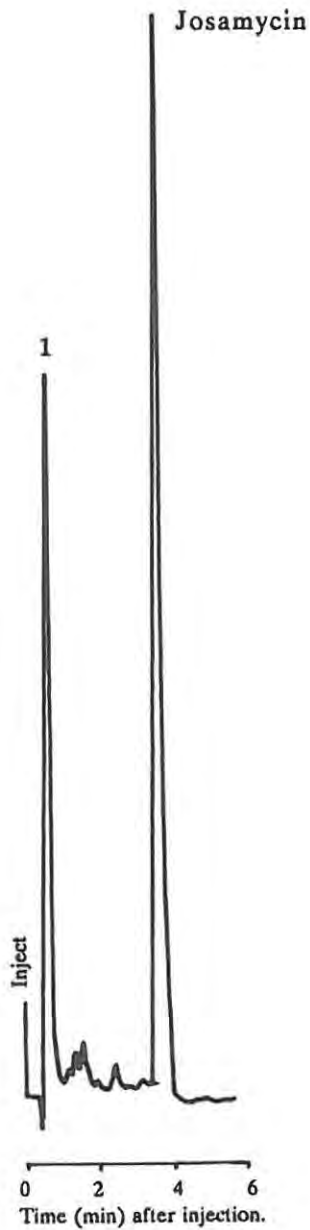


Figure 2.7b

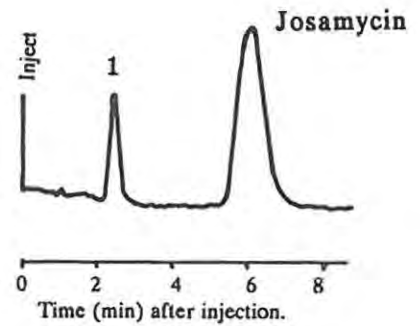
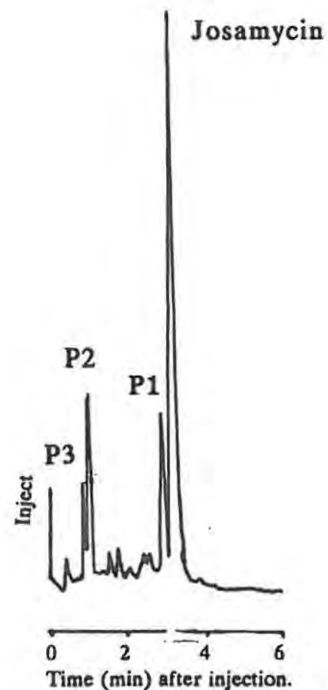


Figure 2.7c



Figures 2.7a, b and c Chromatograms of samples of josamycin following acid degradation in 0.1 N HCl at 37°C for 10 min, after chromatography on:-

a) Waters C_{18} Nova Pak analytical column showing early eluting degradation products P2 and P3 (1) and josamycin with no separation from P1. The mobile phase was acetonitrile-ammonium acetate 0.08 M, 1:1, 1.2 ml/min,

b) Waters 10 μm C_8 analytical column showing early eluting degradation products P2 and P3 (1) and josamycin also without separation from P1. The mobile phase was acetonitrile-phosphate buffer 0.0038 M, pH 3.0, 5:4, 1.2 ml/min, and

c) Beckman Ultrasphere-XL 3μm C_{18} analytical column showing early eluting degradation products P2 and P3, and separation of P1 from josamycin. The mobile phase was acetonitrile-phosphate buffer 0.005 M, pH 4.0, 65:45, 1.6 ml/min.

the buffer molarity decreased below 0.01 M. Adequate separation was finally achieved utilising a 0.005 M phosphate buffer. The retention time of josamycin could then be adjusted without compromising separation by adjusting the buffer pH and acetonitrile content of the mobile phase. The retention time of josamycin was reduced to 3.5 min which facilitated the analysis of up to 200 samples a day.

The final mobile phase was acetonitrile-phosphate buffer 0.005 M, pH 4.0, 65:45, degassed and filtered through a 0.4 μm HVLP Millipore filter prior to use. The buffer was prepared by making 0.32 ml phosphoric acid (85%) up to 1 litre with water and adjusting the pH to 4.0 with sodium hydroxide pellets.

Samples of josamycin following degradation in buffer of pH 12 at 37°C, and taken 10 min after innoculation of the buffer with josamycin to a concentration of 0.1 mg/ml, were analysed on the system described for the separation of josamycin from acid degradation products. Numerous alkali degradation products were detected (Figure 2.8) which eluted both before and after josamycin. However, they were all well separated from josamycin and this system was therefore used without modification for the analysis of samples from degradation studies in alkali media.

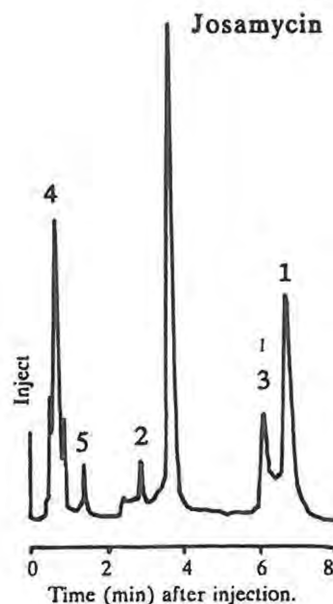


Figure 2.8 Chromatogram of a sample of josamycin degraded at pH 12.0 and at 37°C for 10 mins showing various degradation products (1,2,3,4 and 5) and josamycin, after chromatography on a Beckman Ultrasphere-XL analytical column. The mobile phase was acetonitrile-phosphate buffer 0.005 M, pH 4.0, 65:45, 1.6 ml/min.

2.2.3 Chromatographic Conditions

1) Mobile phase flow rate	- 1.6 ml/min (recycled for 5 days max.)
2) Column temperature	- ambient
3) Column pressure	- 55 bar
4) Retention time	- Josamycin *ca* 3.5 min
5) Detector settings	- λ = 231 nm, 0.01 - 0.03 a.u.f.s.
	- Time constant = 0.5 s
	- Threshold value = 0%
6) Injection volume	- 2 or 5 μl
7) Chart Speed	- 5 mm/min
8) Chart recorder input	- 10 mV full scale

2.2.4 Assessment of Peak Purity

Since the acid and alkali degradation pathways of josamycin have not been fully elucidated, pure samples of degradation products were not available for chromatography. Chromatograms of degradation compounds could not, therefore, be compared with those of intact josamycin and alternative methods for the assessment of peak purity were used. Josamycin peak purity following acid degradation was assessed utilising UV absorbance spectra generated by a diode array detector and ratio plots generated by the M490 programmable UV detector. Peak purity for samples from alkali degradation studies was assessed chromatographically.

2.2.4.1 UV Diode Array Detection

Josamycin at a concentration of 2 mg/ml was degraded in 0.1 M hydrochloric acid at 37°C for 20 min after which a 0.5 μl sample of the solution was injected via the appropriate sample loop into the chromatographic system. Although quantitation of only josamycin was required, the peak purity of the three degradation products was assessed to complement data obtained by thermospray-mass spectrometry for the identification of these compounds (*vide infra* Chapter 5). The mobile phase was therefore adjusted to acetonitrile-phosphate buffer 0.005 M, pH 4.0, 2:3, to separate the two early eluting degradation compounds. This slightly increased the retention times of P1 and josamycin to *ca.* 7 min and 8 min respectively. The eluate was monitored between 200 nm and 400 nm for a period of 8 min after injection using an HP 1040A diode array detector (Hewlett Packard, Palo Alto, CA, U.S.A.) fitted in-line in the chromatographic system in place of the M490 detector. The detector was programmed to take spectra during the up-slope, apex and down-slope portions of

peaks. Peak purity was then assessed by testing the superimposability of the three spectra on one another. The chromatogram obtained at 231 nm and UV spectra obtained during the up-slope, apex and down-slope portions of each peak are depicted in figure 2.9.

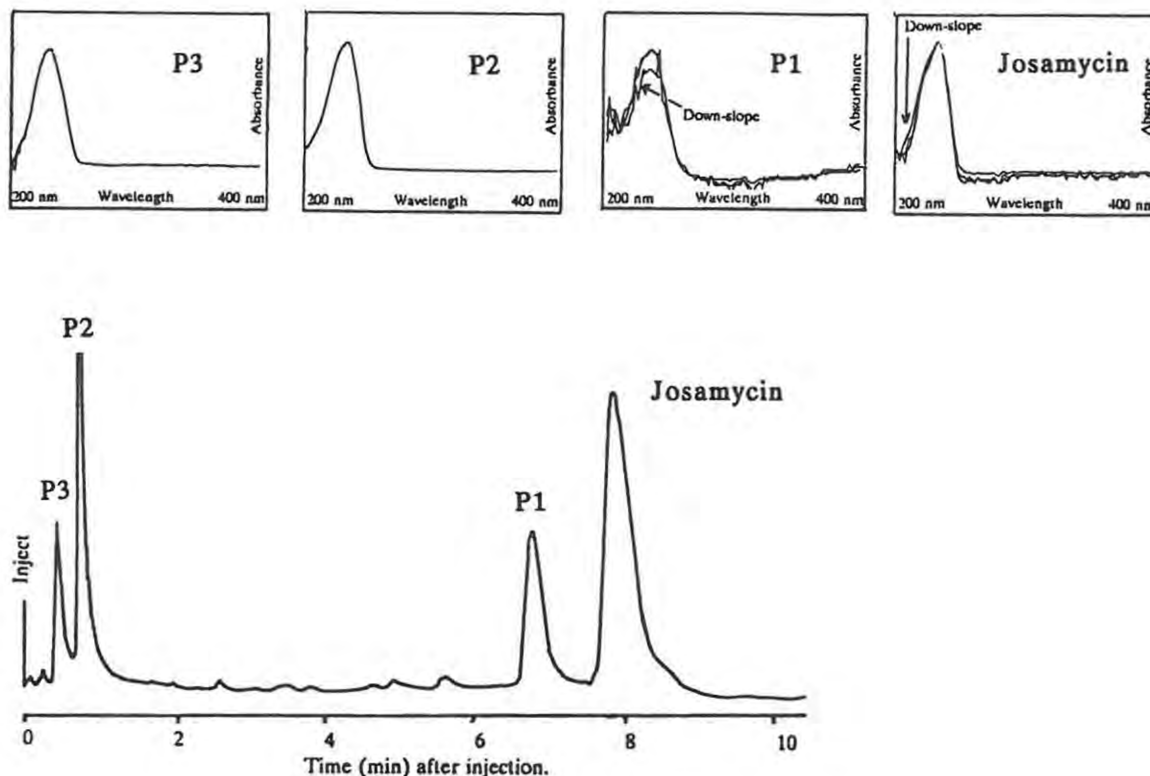


Figure 2.9 Chromatogram of a sample of josamycin following acid degradation, and the corresponding UV absorbance spectra of the up-slope, apex and down-slope portions of peaks of acid degradation products (P1,P2 and P3) and josamycin. Chromatography was performed on a Beckman Ultrasphere-XL analytical column and the mobile phase was acetonitrile-phosphate buffer 0.005 M, pH 4.0, 2:3, 1.6 ml/min.

For P2 and P3, the up-slope, apex and down-slope spectra were all superimposable which indicated a high degree of purity for both peaks. For P1 and josamycin, the up-slope and apex spectra were superimposable, however, the down-slope spectra were disparate from the other two. This indicated possible co-elution of more than one compound during the latter portion of both peaks. The chromatogram exhibited slight shoulders at the base of the down-slope of P1 and josamycin (more pronounced with josamycin) which could account for the slight shifts in the down-slope spectra. However, compounds responsible for these abberations did not elute until after the apex of the josamycin peak and therefore would not interfere with quantitation of josamycin based on peak height measurements.

2.2.4.2 Ratio Plot

Chromatograms at 231 nm and the corresponding ratio plots of absorbance at 231 nm *vs.* 250 nm for a sample of intact josamycin (Figure 2.10a), and for a sample of josamycin degraded in acid as in 2.2.4.1 (Figure 2.10b) were obtained using the M490 multiwavelength programmable detector. The mobile phase optimised as in 2.2.2.2 was utilised, however, the flow rate was reduced to 1.2 ml/min to increase the width of the josamycin peak as a suitable ratio plot could not be obtained from the narrow peak obtained at 1.6 ml/min.

Figure 2.10a

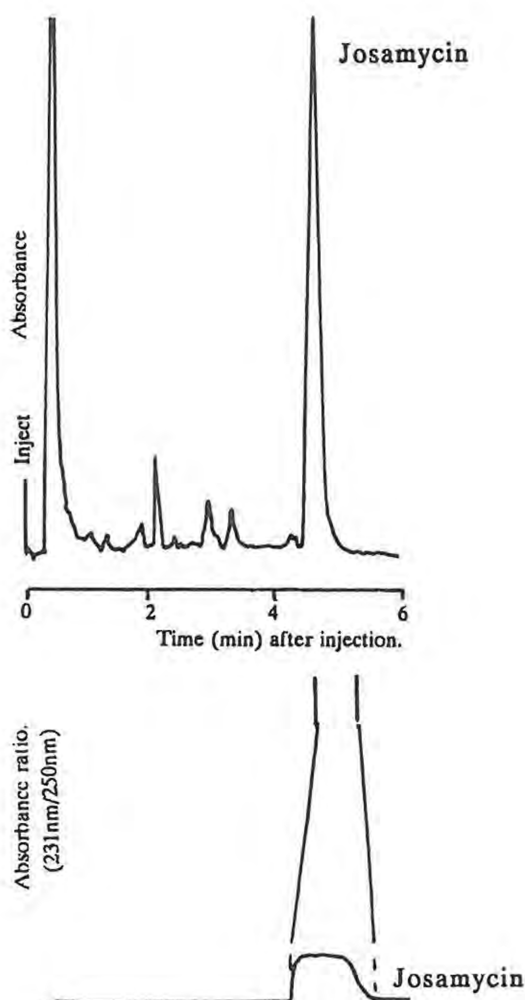


Figure 2.10b

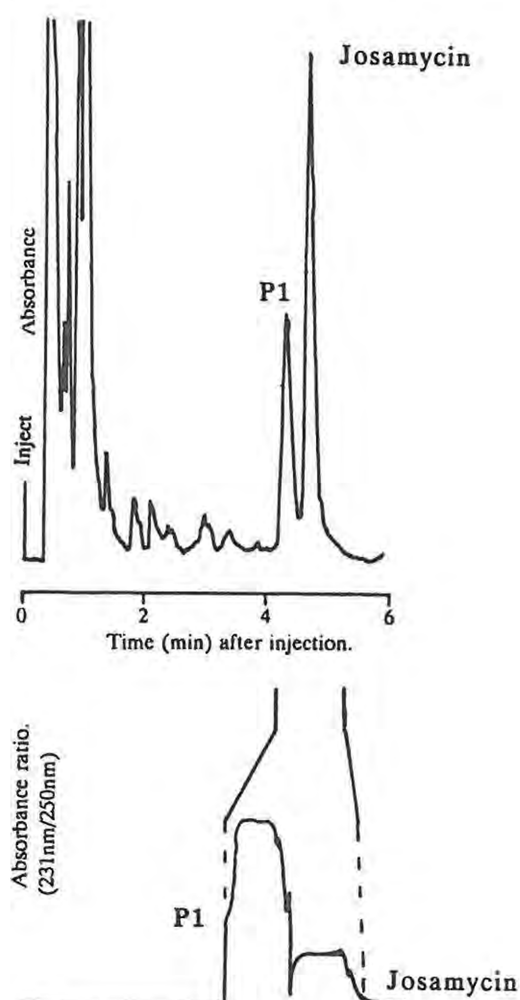


Figure 2.10a and b a) Chromatogram at 231 nm of a sample of intact josamycin dissolved in acetonitrile, and the corresponding ratio plot (absorbance at 231 nm/250 nm) of the josamycin peak, and

b) Chromatogram at 231 nm of a sample of josamycin following acid degradation, and the corresponding ratio plot of P1 and josamycin peaks. Chromatography was performed on a Beckman Ultrasphere-XL analytical column. The mobile phase was acetonitrile-phosphate buffer 0.005 M, pH 4.0, 65:45, 1.2 ml/min.

The irregular ratio plot for P1 indicated the possible co-elution of more than one compound, which corresponds with the dissimilar UV absorbance spectra of the apex and down-slope obtained for this peak in 2.2.4.1. Ratio plots of the josamycin peak in both undegraded and degraded samples were identical, except for a slight abberation in the latter part of the ratio plot of the degraded sample. This abberation was probably caused by the late eluting compound responsible for the shift in the down-slope UV absorbance spectrum as described in 2.2.4.1. This ratio plot indicated that elution of the interfering compound commenced after the apex of the josamycin peak which confirmed that there would be no interference with the quantitation of josamycin based on peak height measurements.

2.2.4.3 Assessment of Josamycin Peak Purity after Alkali Degradation.

Josamycin at a concentration of 0.1 mg/ml was degraded in Teorell and Stenhagen's buffer of pH 12 (103) at 37°C for 20 min, after which a 5.0 μl sample of the solution was injected via the appropriate sample loop into the chromatographic system described in 2.2.1. The eluate was monitored at 231 nm. As stated in 2.2.2.2, numerous degradation products eluted both before and after josamycin (Figure 2.8) but there was no evidence of any compounds co-eluting with josamycin. To substantiate this, the retention time of josamycin was increased in stages to 10 min by altering the pH and molarity of the mobile phase buffer in order to promote separation of josamycin from any co-eluting compounds. No additional compounds were detected and this chromatographic system was therefore used for both acid and alkali degradation studies.

2.2.5 Degradation Studies

2.2.5.1 Preparation of Standards and Controls

The upper standard or stock solution for calibration curves from 0 - 1.0 mg/ml was prepared by accurately weighing 10 mg of josamycin into a 10 ml volumetric flask, dissolving in 2 ml of acetonitrile and making up to volume with water. The remaining standards were prepared by the 1 in 2, 1 in 5, 1 in 10 and 1 in 20 aqueous dilutions of the stock solution. A 50 μl aliquot of each standard was then added to 500 μl of 0.2 M di-sodium hydrogen phosphate solution, and mixed on a vortex mixer prior to the injection of a 2 μl aliquot into the chromatographic system described in 2.2.1.

The upper standard or stock solution for calibration curves from 0 - 0.1 mg/ml was prepared by accurately weighing 10 mg of josamycin into a 100 ml volumetric flask, dissolving in 2 ml of acetonitrile and making up to volume with water. The

remaining standards were prepared as previously described. A 200 μl aliquot of each standard was then added to 200 μl of 0.2 M di-sodium hydrogen phosphate solution and mixed on a vortex mixer prior to the injection of a 2 μl aliquot into the chromatographic system.

Fresh standards were prepared on each day of analysis. A single sample of each standard was analysed and calibration curves constructed by linear regression of plots of peak height *vs*. concentration, which were found to be linear over the concentration ranges studied.

For degradation studies which were conducted over a period of more than one day and in which concentrations were calculated from more than one calibration curve, the day-to-day accuracy of the assay was monitored by the analysis of control solutions to ensure that concentrations determined throughout the study were accurate and precise. The concentration of josamycin in samples from all studies which continued for 2 or more days were within the lower calibration range and controls were prepared accordingly. Control solutions were prepared by accurately weighing 5 mg of josamycin into a 100 ml volumetric flask, dissolving in 2 ml of acetonitrile and making up to volume with water. A 200 μl aliquot was then added to 200 μl of 0.2 M di-sodium hydrogen phosphate solution, and mixed on a vortex mixer prior to the injection of a 2 μl aliquot into the chromatographic system. Controls were freshly made and analysed on each day of analysis throughout each degradation study. Ideally, day-to-day accuracy should be assessed by the analysis of the same control solution on each day of analysis. However, the stability of josamycin in an aqueous medium when stored at -70°C over the study period of 81 days has not been evaluated. Fresh control samples were therefore prepared on each day of analysis and accuracy assessed on a daily basis.

2.2.5.2 Sample Preparation

For samples to be analysed utilising the 0 - 1.0 mg/ml calibration range (5.1.1.1), a 50 μl aliquot was removed from the test medium utilising a Transferpette repeating pipette (Rudolf Brand GMBH and Co., Wertheim-Glashütte, Germany) and added directly to 500 μl of 0.2 M di-sodium hydrogen phosphate solution previously pipetted into a 1.5 ml Eppendorf Micro Test Tube 3810 (Eppendorf-Netheler-Hinz GMBH, Hamburg, Germany). For samples to be analysed utilising the 0 - 0.1 mg/ml calibration range (5.1.1.1), a 200 μl aliquot was removed from the test medium and added directly to 200 μl of 0.2 M di-sodium hydrogen phosphate solution. The diluted sample was then vortexed for 30 s and a 2 μl aliquot of the resulting solution injected into the chromatographic system. The di-sodium hydrogen phosphate solution adjusted the pH of the samples to between pH 5.0 and 7.0 which inhibited degradation of samples awaiting analysis. Samples were diluted to ensure a maximum on-column load

of 0.2 μg after the injection of 2 μl of the diluted sample. Results of degradation studies are detailed in 5.1.2.

2.2.5.3 Precision and Accuracy

Calibration curve parameters and control results for each day of analysis for degradation studies are listed in table 2.3. The R.Sq. values for the calibration curves ranged from 0.9981 to 0.9998, and bearing in mind that these calibration curves were constructed from single injections of each standard using a fixed loop injector and peak height measurements without internal standard, the assay was extremely precise. Furthermore, the percentage difference between spiked control concentrations and concentrations found after analysis of these controls was between 4.77 % and -4.62 % for 20 different control solutions. These results indicate that analysis with a high degree of accuracy was possible with this assay method.

2.2.6 Solubility Studies

2.2.6.1 Preparation of Standards

A stock solution was prepared by accurately weighing 10 mg of josamycin into a 10 ml volumetric flask, dissolving in 5 ml of acetonitrile and making up to volume with water. The upper standard was prepared by the 1 in 10 dilution of the stock solution with an acetonitrile-water 1:1 mixture. The remaining standards were prepared by the 1 in 2, 1 in 5 and 1 in 10 dilution of the upper standard with an acetonitrile-water 1:1 mixture. A 2 μl aliquot of each standard was then injected into the chromatographic system described in 2.2.1. A single sample of each standard was analysed and calibration curves were constructed by linear regression of plots of peak height *vs.* concentration, which were found to be linear over the concentration range studied.

2.2.6.2 Sample Preparation

A 1 ml aliquot of filtered sample (*vide infra* 4.2.1.2) was diluted to a concentration within the linear calibration range with an acetonitrile-water 1:1 mixture. Samples from dissolution media of pH 4.0 and 5.0 were diluted 1 in 500; of pH 6.0, 1 in 250; of pH 6.5, 1 in 100; and of pH 7.5 - 9.0, 1 in 10. A 2 μl aliquot of diluted sample was then injected into the chromatographic system. The solubility was calculated by multiplying the concentration of the diluted sample by the relevant dilution factor. Results of solubility studies are detailed in 4.2.2.

2.2.6.3 **Precision and Accuracy**

The precision and accuracy of the sample dilutions was assessed prior to the commencement of solubility determinations. A solution of josamycin in acetonitrile (5 mg/ml) was diluted in triplicate to 1 in 500 and 1 in 100, and a further solution in acetonitrile (0.5 mg/ml) was diluted in triplicate to 1 in 10. Each solution was then analysed. The precision of the assay was calculated as the RSD % of each set of triplicate injections, and the accuracy of the assay calculated as the difference between the actual spiked concentrations and the concentrations found after analysis. The precision of the assay and found to be $\leq \pm 2.43$ %, and concentrations found after analysis for all samples except for one were within 5 % of the spiked concentrations. The assay procedure was therefore found to be accurate and precise and suitable for solubility studies.

2.2.7 **Powder and Tablet Dissolution Studies - pH 1.2 to 4.0**

2.2.7.1 **Preparation of Standards and Controls**

The upper standard or stock solution was prepared by accurately weighing 11 mg of josamycin into a 20 ml volumetric flask, dissolving in 10 ml of acetonitrile and making up to volume with water. The remaining standards were prepared by the 1 in 2, 1 in 5, 1 in 10, 1 in 20 and 1 in 50 dilution of the stock solution with an acetonitrile-water 1:1 mixture. A 1 ml aliquot of each standard was then mixed with 4 ml of 0.2 M di-sodium hydrogen phosphate solution. After mixing on a vortex mixer for 30 s, a 2 μl aliquot of the resulting solution was injected into the chromatographic system described in 2.2.1. A single sample of each standard was analysed and calibration curves constructed by linear regression of plots of peak height *vs.* concentration, which were found to be linear over the concentration ranges studied.

Two analytical controls of different concentrations were freshly prepared each day of analysis. The upper control was prepared by accurately weighing 8 mg of josamycin into a 20 ml volumetric flask, dissolving in 10 ml of acetonitrile and making up to volume with water. A second control was prepared by the 1 in 5 dilution of this solution with an acetonitrile-water 1:1 mixture. A 1 ml aliquot of each control solution was then mixed with 4 ml of 0.2 M di-sodium hydrogen phosphate solution and prepared for injection as for samples and standards. Analytical control results and calibration curve parameters for dissolution studies on Josamycin 200 mg tablets and josamycin powder (200 mg), and Josacine 500 mg tablets and josamycin powder (500 mg) are listed in tables 2.4 and 2.5 respectively. Accuracy and precision of the assay is discussed in 2.3.2.3.

2.2.7.2 **Sample Preparation**

A 1.2 ml aliquot of the dissolution medium was removed from the dissolution vessel (*vide infra* 4.3.1.3) at various time intervals and centrifuged at 12000 rpm for 30 s in a high speed centrifuge. A 1 ml aliquot of the supernatant was mixed with 4 ml of 0.2 M di-sodium hydrogen phosphate solution. The sample was mixed on a vortex mixer for 30 s and a 2 μl aliquot of the resulting solution injected into the chromatographic system described in 2.2.1. Dilution of samples with 0.2 M di-sodium hydrogen phosphate raised the pH of samples to between pH 5.0 and 7.0 and prevented degradation of samples whilst awaiting analysis. Dilution of samples and standards also ensured that the on-column load was within the linear calibration range.

In order to determine the amount of josamycin degraded or remaining entrapped in the tablet matrix at the end of each dissolution run, solutions prepared as in 4.3.1.5 from mass balance analysis studies were diluted prior to analysis by mixing a 1 ml aliquot of the centrifuged sample with 4 mls of 0.2 M di-sodium hydrogen phosphate. After mixing by vortexing, samples were analysed utilising the calibration curve prepared as in 2.2.7.1 for the respective dissolution run.

2.2.8 **Discussion**

Separation of josamycin from degradation products could only be accomplished on a highly efficient 3 μm Ultrasphere-XL analytical column with a theoretical plate count of 65000 per metre. Separation on this column was highly selective, which was comfirmed with a high degree of certainty by UV diode array detection and UV ratio plot techniques. Furthermore, separation was rapid which afforded short analysis times and enabled expeditious analysis of the large number of samples generated in degradation and dissolution studies.

Use of an automatic injector was desirable and attempts were made to incorporate a WISP 710B into the chromatographic system. Manufacturers specifications state that repeat injections should have a relative standard deviation of <1 % although no injection volume is specified. However, using injection volumes of 2 - 5 μl (without an internal standard), this degree of reproducibility was not always obtained throughout a days analysis and this injector could therefore not be used with confidence during these studies without an internal standard. Attempts were made to optimise the retention of oleandomycin on the Ultrasphere-XL column for use as an internal standard. However, the retention of oleandomycin, josamycin and the acid degradation product P1 on C_{18} reversed-phase material were all highly sensitive to the molarity of buffer in the mobile phase. Consequently, the elution of oleandomycin at a suitable time without compromising the separation of josamycin

from P1 was not achieved. Attempts were also made to use several other macrolides namely tylosin tartrate, rifamycin, spiramycin and midecamycin as an internal standard, but were similarly unsuccessful. A highly precise manual fixed loop injector was therefore used for which the relative standard deviation of the peak heights of 10 consecutive injections was 0.78% for an injection volume of 2 μl and with an overfill of 5 x the loop volume. The excellent precision of this fixed loop injector obviated the need for an internal standard, however, the manual injection of numerous samples was extremely tedious and time consuming.

The linearity of each assay utilising the Ultrasphere-XL column was dependent on the on-column load of josamycin. A linear response was not obtained if more than 0.2 μg of josamycin was injected. The concentration of calibration standards and injection volumes thereof were therefore selected so as to ensure a maximum on-column load of $\leq$ 0.2 μg. All samples were also diluted accordingly.

Solubility studies were initially conducted in dissolution media of pH 1.0 and above. For this reason the analytical method described in 2.2.1 for the separation of josamycin from acid degradation products was used. Ultimately, however, pH 5.45 was the minimum pH at which the solubility of josamycin could be determined (4.2.2) and at which pH no degradation was observed. Nevertheless, the assay developed for low pH work was still utilised.

Samples from dissolution rate studies were initially filtered through a 0.45 μm HVLP filter (Millipore Corp., Bedford, Massachucettes, USA) fitted to a 1 ml syringe. However, filters were rapidly blocked by undissolved tablet components and a sufficient volume of each sample could not be filtered by a single filter. Samples were therefore centrifuged at high speed to remove solid particles from the sample matrix. The clear supernatant was then carefully removed using a fixed volume repeating pipette and diluted for analysis.

The use of disposable guard columns, custom made by the manufacturer for the Ultrasphere-XL system, proved invaluable for the maintenance of column performance. The pressure of the mobile phase increased slowly after repeated injections and guard columns were changed after every 1000 injections to maintain an acceptable column pressure. Guard columns effectively protected the main analytical column from clogging and no deterioration in the performance of the analytical column was observed even after over 4000 injections.

The high flow rate and long periods of analysis used large volumes of mobile phase. However, to decrease the volume of acetonitrile used and the cost thereof, all the mobile phase was pooled in a single reservoir at the end of each analytical run and recycled overnight. Recycling was then stopped immediately prior to the start of the next analytical run but the mobile phase from the previous day re-used. In this way, the volume of mobile phase used was significantly reduced without the possibility of

altering the retention time of josamycin during an analytical run. Two litres of mobile phase could be recycled for up to 5 days of analysis and used for the analysis of up to 1000 samples without affecting chromatography.

The system detailed in this section proved highly versatile for the analysis of josamycin in the variety of media utilised in the studies outlined. Furthermore, calibration curves and control results show the method to be accurate and precise.

2.3 TABLET AND POWDER DISSOLUTION STUDIES USING SPECTROPHOTOMETRIC METHODS

Josamycin is relatively stable at pH 5.0, 6.0, 7.5 and 9.0 (*vide infra* chapter 5). Therefore, the analysis of samples from dissolution studies at these pH's utilising the stability specific HPLC assay developed in 2.2 was unnecessary. As analysis of numerous samples by this HPLC assay was tedious and time consuming, an assay procedure capable of extremely rapid sample analysis was developed utilising a Beckman DU-68 Spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA) fitted with a sipper.

2.3.1 Method Development

Solutions of components used in the preparation of the various dissolution media (citric acid, di-sodium hydrogen phosphate, potassium di-hydrogen phosphate and sodium hydroxide) were scanned over the UV absorbance range of 200 - 400 nm to check for any UV absorbance by these compounds which could interference with the determination of josamycin at 231 nm. As was expected, solutions of all components except citric acid showed negligible UV absorbance in the concentrations present in the dissolution media, citric acid solution showing extremely high absorbance at 231 nm. Consequently, the background absorbance of buffer solutions containing citric acid was excessive and the accurate quantitation of josamycin at 231 nm in these solutions was not possible. However, citric acid shows negligible absorbance at 250 nm whilst josamycin shows appreciably higher absorbance than citric acid at this wavelength (Figure 2.11). Although the absorbance of josamycin at 250 nm is still relatively low, the concentrations of josamycin in the dissolution media were sufficient for an adequate response and accurate quantitation at 250 nm. Analysis of samples at this wavelength therefore allowed accurate quantitation of josamycin without interference from citric acid.

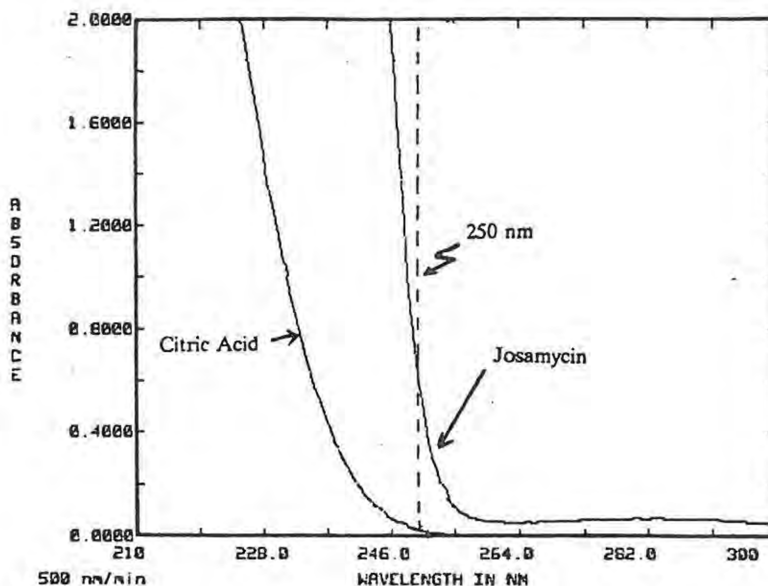


Figure 2.11 UV absorbance spectra of josamycin (0.2 mg/ml in acetonitrile) and a 0.2 M solution of citric acid.

The possibility that tablet components of both 200 mg and 500 mg tablets dissolved in the dissolution media could interfere in the quantitation of josamycin was also investigated. A tablet of each dosage form was sonicated in 100 ml of an acetonitrile-water 1:1 mixture for 10 min. After dissolution of the tablet was complete, the solution was prepared for analysis as in 2.3.3.2 and the amount dissolved determined from a calibration curve prepared as in 2.3.3.1. The amounts dissolved as calculated after spectrophotometric analysis were equal to the tablet contents determined as in 2.1.6. for both dosage forms. The spectrophotometric determination of josamycin was therefore a suitable means of analysis for both powder and tablet dissolution studies at pH 5.0, 6.0, 7.5 and 9.0.

2.3.2 Analysis of Samples from Dissolution Studies at pH 5.0 to 9.0

2.3.2.1 Preparation of Standards and Controls

Standards and controls were freshly prepared as follows for each dissolution study. The upper standard or stock solution was prepared by accurately weighing 11 mg of josamycin into a 20 ml volumetric flask, dissolving in 4 ml of acetonitrile and making up to volume with the relevant dissolution medium. The remaining standards were prepared by the 1 in 2, 1 in 5, 1 in 10, 1 in 20 and 1 in 50 dilution of the upper standard with an acetonitrile-dissolution medium 2:8 mixture also

prepared using the relevant dissolution medium. A 1 ml aliquot of each standard was then added to 4 ml of water and mixed on a vortex mixer for 30 s. The resulting solution was then aspirated via a sipper (3 s aspiration time) into the spectrophotometer for analysis at 250 nm (without utilising the option of using a reference wavelength).

Linear regression analysis of plots of absorbance *vs.* concentration were performed by the Q2LinSet program (program No.7) of the Quant II Linear Soft-Pac^{TM} Module (Beckman-RIIC Ltd., Glenrothes, Scotland), and were found to be linear over the concentration range studied (Tables 2.4 and 2.5).

Upper and lower analytical controls were freshly prepared each day. The upper control was prepared by accurately weighing 8 mg of josamycin into a 20 ml volumetric flask, dissolving in 4 ml of acetonitrile and making up to volume with the relevant dissolution medium. The lower control was prepared by a 1 in 5 dilution of the upper control with an acetonitrile-dissolution medium 2:8 mixture. A 1 ml aliquot of each control solution was then added to 4 ml of water and mixed on a vortex mixer for 30 s. The resulting solution was then aspirated into the spectrophotometer for analysis.

2.3.2.2 **Sample Preparation**

Samples (1.2 ml) withdrawn from the dissolution vessel were centrifuged at 12000 rpm for 30 s in a high speed centrifuge. A 1 ml aliquot of the supernatant was mixed with 4 ml of water and mixed on a vortex mixer for 30 s. The resulting solution was then aspirated into the spectrophotometer and the concentration calculated by the spectrophotometer using the Q2LinSet program. Results of dissolution studies at pH 5.0, 6.0, 7.5 and 9.0 are detailed in 4.3.2.

2.3.2.3 **Precision and Accuracy**

Calibration curve parameters and analytical control results for Josamycin 200 mg tablets and josamycin powder (200 mg), and Josacine 500 mg tablets and josamycin powder (500 mg) for both HPLC and spectrophotometric methods are listed in tables 2.4 and 2.5. Both analytical methods showed a high degree of precision and accuracy. This is illustrated by R.Sq. values of between 0.9979 and 1.0000 for all calibration curves, and percentage differences between spiked concentrations of controls and concentrations found after analysis of < 5 % for all but 5 out of 32 control solutions.

2.3.3 Analysis of Samples from Mass Balance Analysis Studies

2.3.3.1 Preparation of Standards

The upper standard or stock solution was prepared by accurately weighing 10 mg of josamycin into a 20 ml volumetric flask, dissolving in 10 ml of acetonitrile and making up to volume with water. The remaining standards were prepared by the 1 in 2, 1 in 5, 1 in 10, 1 in 20 and 1 in 50 dilution of the upper standard with an acetonitrile-water 1:1 mixture. A 1 ml aliquot of each solution was then added to 4 ml of water and mixed on a vortex mixer for 30 s. The resulting solution was then aspirated into the spectrophotometer for analysis at 250 nm and the calibration curve calculated by the Q2LinSet program using linear regression analysis as in 2.3.2.1.

2.3.3.2 Sample Preparation

A 1 ml aliquot of each sample prepared from tablet dissolution studies as in 4.3.1.5 was added to 4 ml of water and mixed on a vortex mixer for 30 s. The resulting solution was then aspirated into the spectrophotometer and the concentration calculated by the Q2LinSet program. Results of mass balance analysis studies are detailed in 4.3.2.

2.3.4 Discussion

A separate set of standards and controls was prepared using each different dissolution medium in order to minimise the difference between the composition, and therefore the background absorbances of standards, controls and samples. This helped to prevent the occurrence of errors during interpolation of absorbance readings of samples. Problems were, however, encountered in the preparation of standards with dissolution media of pH 6.0, 7.5 and 9.0, and the low solubility and wettability of josamycin at these pH's necessitated the use of 20% acetonitrile as a co-solvent and wetting agent to ensure complete dissolution of josamycin. Acetonitrile was therefore used in the preparation of all standards. However, despite the 20% acetonitrile content of the standards, there was no difference between the absorbances of blank solutions of standards and samples.

The concentration of josamycin approached saturation concentrations during the latter stages of dissolution studies conducted in dissolution media of pH 7.5 and 9.0. Samples from dissolution studies at pH 7.5 and 9.0 were therefore diluted with water to ensure that there could be no precipitation of josamycin from saturated samples awaiting analysis. Standards, and samples from dissolution studies at pH 5.0 and 6.0 were also diluted accordingly. Dilution of samples also allowed for the aspiration of

a large volume of each sample through the spectrophotometer flow cell without removing a correspondingly large volume of dissolution medium from the dissolution vessel. Aspiration of 3 ml of each sample ensured complete washout of the previous sample from the spectrophotometer and prevented cross-contamination of samples.

The concentrations of the standards were entered into the Q2LinSet program as mg/l, with the concentration of the top standard ($\pm$550 mg/l) slightly greater than that obtained after the complete dissolution of one Josacine 500 mg tablet in 1 litre of dissolution fluid. The amount of josamycin dissolved in mg was therefore equivalent to the concentration in mg/l and the amount dissolved calculated directly from the sample absorbance readings by the spectrophotometer. Only minimal data processing was then required for calculation of the % of tablet content dissolved.

Results of control samples indicated that the UV spectrophotometer afforded accurate analysis of josamycin in the various dissolution media used. Furthermore, analysis of samples was rapid and up to three complete sets of dissolution studies, *i.e.* six replicates in 3 different dissolution media could be completed daily when utilising this analytical technique.

Table 2.1 Calibration Curve Parameters for Intrinsic Dissolution Rate Studies.

pH of Dissolution Medium	Calibration Range (mg/ml)	Calibration Curve Parameters		
		m	c	R.Sq.
1.2	0 - 1.0	9.4346	0.1910	0.9962
2.2	0 - 1.0	12.1470	-0.0127	0.9997
2.2	0 - 1.0	14.5310	-0.3242	0.9992
3.0	0 - 0.1	52.1010	0.0099	0.9639
4.0	0 - 0.1	57.9210	0.5388	0.9944
5.0 and 6.0	0 - 0.1	54.8900	0.7042	0.9937
7.5	0 - 0.01	831.3000	0.3010	0.9923

Table 2.2 Results of Tablet Content Uniformity Studies for Josamycin 200 mg Tablets and Josacine 500 mg Tablets.

Josamycin 200 mg Tablets				Josacine 500 mg Tablets		
Tablet No.	% of Label Claim*			Tablet No.	% of Label Claim*	
	Mean (n=3)	± RSD %			Mean (n=3)	± RSD %
1	109.07	3.35		1	104.91	1.65
2	109.56	1.01		2	105.98	0.85
3	109.73	4.01		3	106.08	1.41
4	112.23	3.81		4	103.95	2.02
5	108.61	2.73		5	106.49	0.19
6	108.30	1.74		6	105.41	0.67
Mean % of Label Claim ± S.D.	109.58 ±1.28			Mean % of Label Claim ± S.D.	105.47 ±0.84	

* mean of three repeat injections.

Table 2.3 Calibration Curve Parameters and Analytical Control Results for Degradation Rate Studies.

Day of Analysis	Calibration Curve Parameters			Spiked Conc. of Control mg/ml	Concentration Found after Analysis mg/ml (n=3)		Percentage Difference*
	m	c	R.Sq.		Mean	± RSD %	Mean (n=3)
0	1297.6	2.04	0.9985	0.057	0.058	3.72	3.06
1	1283.3	0.43	0.9996	0.042	0.043	1.71	2.00
2	1273.9	1.54	0.9986	0.068	0.067	1.65	-0.91
3	1301.4	-0.43	0.9997	0.043	0.043	1.45	2.03
4	1309.2	0.51	0.9992	0.049	0.051	1.89	2.71
5	1308.1	0.24	0.9994	0.052	0.054	2.00	4.02
6	1258.8	0.38	0.9990	0.060	0.063	0.59	4.28
7	1310.3	0.27	0.9997	0.037	0.037	1.96	-1.66
8	1347.6	1.05	0.9990	0.058	0.059	0.57	2.32
9	1330.6	-0.30	0.9997	0.059	0.062	8.98	4.77
10	1318.8	0.08	0.9989	0.057	0.057	8.92	-1.03
17	1357.3	-0.13	0.9993	0.051	0.051	1.15	0.78
20	1379.0	-0.97	0.9997	0.054	0.056	1.63	3.49
24	1357.7	-0.47	0.9996	0.055	0.055	2.90	-0.46
28	1294.1	-0.34	0.9998	0.056	0.058	3.91	3.41
32	1302.2	0.50	0.9981	0.048	0.049	3.86	2.40
39	1310.8	0.53	0.9991	0.062	0.059	1.62	-4.62
53	1177.4	-0.74	0.9998	0.060	0.060	2.89	0.44
67	1154.7	-1.04	0.9993	0.065	0.063	1.12	-2.23
81	1154.7	-1.04	0.9993	0.063	0.065	0.63	2.86

* Difference between spiked concentrations and concentrations found after analysis as a percentage of the spiked concentration.

Table 2.4 Calibration Curve Parameters and Analytical Control Results for Dissolution Studies of Josamycin 200 mg Tablets and Josamycin Powder (200 mg).

pH of Dissolution Media	Calibration Curve Parameters			Spiked Concentration of Control (mg/ml)	Concentration Found after Analysis (mg/ml)	Percentage Difference ***
	m	c	R.Sq.			
1.2 *	0.2885	1.4044	0.9997	327.00	335.00	1.02
				65.40	63.57	-2.80
2.2 *	0.3052	1.1370	0.9998	379.00	369.75	-2.44
				75.80	74.90	-1.19
3.0 *	0.3061	-0.1856	1.0000	393.00	395.94	0.75
				78.60	82.29	4.69
4.0 *	0.3373	0.2036	1.0000	405.00	408.59	0.89
				81.00	85.39	5.41
5.0 **	2275.9	-5.5680	1.0000	410.00	413.20	0.78
				82.00	83.19	1.45
6.0 **	2256.0	1.2645	0.9999	430.00	432.16	0.50
				86.00	100.53	16.89
7.5 **	2191.0	-2.5570	0.9979	403.50	400.59	-0.72
				80.70	80.70	0.00
9.0 **	2278.0	-4.0280	0.9994	421.00	417.43	-0.85
				84.00	84.82	0.98
						x = 1.46
						SD = 4.51

* HPLC.
** DU-68 Spectrophotometer.
*** difference between spiked concentration and concentration found after analysis as a percentage of the spiked concentration.

Table 2.5 Calibration Curve Parameters and Analytical Control Results for Dissolution Studies of Josacine 500 mg Tablets and Josamycin Powder (500 mg).

pH of Dissolution Media	Calibration Curve Parameters			Spiked Concentration of Controls (mg/ml)	Concentration Found after Analysis (mg/ml)	Percentage Difference ***
	m	c	R.Sq.			
1.2 *	0.4602	-2.5920	0.9999	465.00	425.43	-8.51
				46.50	44.74	-3.78
2.2 *	0.4320	-2.0908	0.9999	415.50	430.78	3.68
				83.10	88.18	6.11
3.0 *	0.4775	-1.5298	0.9999	413.00	390.61	-5.42
				82.60	82.78	0.22
4.0 *	0.4355	-1.9511	0.9998	435.00	450.58	3.58
				87.00	89.44	2.81
5.0 **	2251.6	-2.3040	1.0000	437.00	439.01	0.46
				218.50	222.86	2.00
6.0 **	2341.5	-1.9080	0.9998	502.00	503.85	0.37
				100.40	101.12	0.72
7.5 **	2287.0	-1.2450	0.9998	479.00	485.89	1.44
				95.80	99.38	3.74
9.0 **	2332.9	-4.7500	0.9997	449.00	466.36	3.87
				89.80	95.9	6.82
						x = 1.13
						SD = 3.95

* HPLC.
** DU-68 Spectrophotometer.
*** difference between spiked concentration and concentration found after analysis as a percentage of the spiked concentration.

CHAPTER 3

DETERMINATION OF JOSAMYCIN IN BIOLOGICAL FLUIDS

The analysis of anti-bacterial agents in biological fluids has traditionally been accomplished by microbiological methods. However, these methods are generally non-selective and unable to differentiate between parent drug and microbiologically active metabolites. Furthermore, they can be tedious to perform. However, the advent of HPLC has enabled the rapid and selective analysis of parent drug and metabolites in biological fluids. The selectivity together with the high degree of precision, accuracy and sensitivity afforded by HPLC makes it an ideal tool for the determination of anti-microbial agents in biological fluids for pharmacokinetic studies. This is particularly relevant with josamycin as the metabolites are microbiologically active. Most data published to date on the pharmacokinetics of josamycin have been obtained using microbiological assay techniques (36,45,48,104). However, a few HPLC assays for the determination of josamycin in biological fluids have been published. Fourtillan *et al.* (47) adapted the HPLC assay developed by Tsuji (94) for the determination of erythromycin in plasma to determine josamycin in plasma. This method involves on-line post-column derivatisation, extraction and fluorescence detection and requires complex and expensive equipment. Ducci and Scalori (83) described an HPLC assay for josamycin with a detection limit of 0.3 μg/ml, which is insufficiently sensitive for pharmacokinetic studies. Räder *et al.* (82) described a sensitive HPLC assay but a dual-pump system and expensive column-switching apparatus are required. A selective HPLC assay with sufficient sensitivity, precision and accuracy utilising only standard equipment was developed and is described herein.

3.1 HPLC APPARATUS, CHEMICALS AND MOBILE PHASE

The modular HPLC system used for the determination of josamycin in both serum and urine was as described in 2.1.1. Chemicals used were as described in 2.1.2. The analytical column, mobile phase and UV detection of josamycin and internal standard (oleandomycin) was as described in 2.1.3. This mobile phase afforded separation of josamycin from its two major metabolites, which eluted before josamycin (*vide infra* 3.8.1), and from the internal standard which eluted after josamycin. The early elution of metabolites was of particular importance for the success of the assay as josamycin is extensively metabolised and significant concentrations of metabolites were detected in serum (and urine) samples.

3.2 CHROMATOGRAPHIC CONDITIONS

1) Mobile phase flow rate	- 1.2 ml/min
2) Column temperature	- 35°C
3) Column pressure	- 55 bar
4) Retention times	- Josamycin *ca.* 6 min
	- Internal standard *ca.* 8 min
5) Detector settings	- Channel 1:- λ = 231 nm, 0.02 a.u.f.s. for serum and 0.06 a.u.f.s. for urine.
	- Channel 2:- λ = 204 nm, 0.06 a.u.f.s. for serum and urine.
	- Time constant = 1.0 s
	- Threshold value = 0%
6) Injection volumne	- 2 - 10 μl
7) Chart speed	- 5 mm/min
8) Chart recorder input	- 10 mV full scale

3.3 EXTRACTION OF JOSAMYCIN FROM SERUM

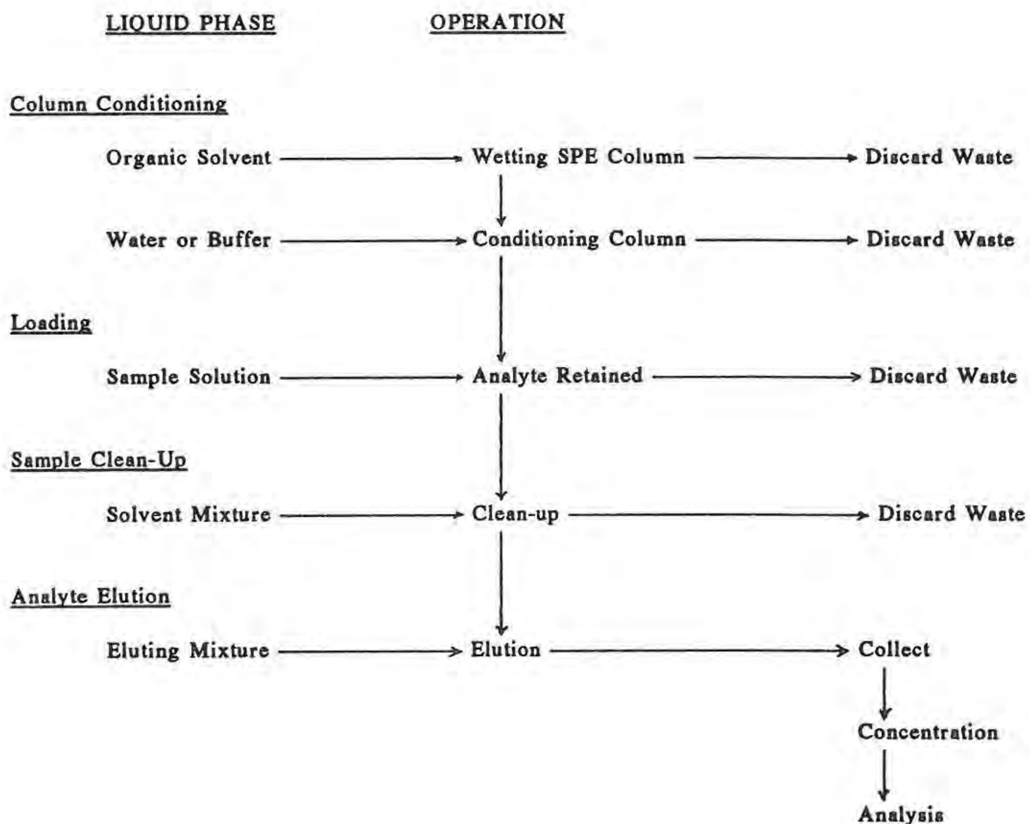
3.3.1 Method Deveplopment

The use of solid-phase extraction (SPE) techniques has become increasingly popular in isolating compounds of interest from complex biological matrices prior to analysis by HPLC (105,106). SPE has many advantages over conventional procedures (107,108). In particular, this technique is highly efficient and selective and a large number of samples can be handled at any one time. These advantages make it ideal for use in bioavailability and pharmacokinetic studies.

SPE involves the retention of compounds onto a stationary phase from the sample matrix followed by either selective elution of the compound of interest from the extraction column whilst leaving contaminants retained on the column, or removal of retained contaminants by a wash solution followed by elution of the compounds of interest. The principles of retention and elution of compounds in reversed-phase SPE are the same as those for chromatography on reversed-phase analytical columns, and have been discussed in 2.1.3.1. SPE techniques exploit differences between the retention characteristics of analyte and contaminants. The type of stationary phase required and the wash and elution mixtures used therefore depend on the

chromatographic or retention characteristics of the compound to be analyzed as well as those of the retained contaminants. A range of SPE materials from the most polar (silica) through to the most non-polar (reversed-phase C_{18}) materials is now available (107), and with the correct extraction column and extraction procedure SPE can be highly efficient and produce samples free from biological contaminants.

The applicability of reversed-phase stationary phases to the separation and extraction of basic drugs has been reported previously (86). More specifically, Räder *et al.* (82) used an in-line C_{18} sample clean-up column for the analysis of josamycin. Various other macrolide antibiotics have also been successfully extracted from biological matrices utilising C_{18} SPE columns (98,100). Method development was therefore initiated using C_{18} SPE columns for the extraction of josamycin from serum and urine. A general scheme for sample preparation using C18 SPE columns is shown below.



A scheme similar to this was followed in the development of an SPE procedure for josamycin from serum and urine.

An important characteristic displayed by macrolides (100) when adsorbed on reversed-phase C_{18} material is that they are efficiently eluted by an acetonitrile-buffer mixture but not by an acetonitrile-water mixture, and josamycin is no exception. This is demonstrated by the increased retention of josamycin on C_{18} analytical columns with decreasing buffer molarity in the mobile phase as shown in figure 2.3, and by the inability of pure acetonitrile to elute josamycin from a C_{18} extraction column but its

efficient elution with an acetonitrile-buffer mixture (Table 3.1). This characteristic was exploited in developing a procedure for the extraction of josamycin from serum and urine.

Initial work on the extraction of josamycin from serum was carried out using pooled human serum dialysed against normal saline to remove any unwanted compounds which may have been ingested and absorbed by the donor prior to phlebotomy. An assay procedure was successfully validated for the determination of josamycin in dialysed serum. However, serious problems were encountered when serum samples obtained from bioavailability studies (non-dialysed) were extracted using this extraction procedure. The assay procedure and associated problems are discussed in detail in 3.4. All subsequent developmental work, as detailed in this section, was therefore conducted using non-dialysed serum. Whole blood was obtained from healthy male donors who had refrained from taking any medication for the previous two weeks and caffeine for the previous 24 hours. Blood was withdrawn and allowed to clot for 30 min after which time the tubes were centrifuged for 20 min at 1600 x g, the serum harvested and stored at -15°C until required.

3.3.1.1 Extraction Column and Sample Preparation

Preparation of C_{18} extraction columns prior to the application of aqueous biological samples involves wetting of the hydrophobic stationary phase followed by conditioning. Wetting serves to solvate and open up the hydrocarbon chains which increases the surface area available for interaction with the analyte. This was achieved by passing 5 ml of acetonitrile through the extraction column. Conditioning of the column optimises conditions for retention of the analyte by removing excess acetonitrile from the stationary phase leaving the solvated C_{18} chains in an aqueous environment favourable for hydrophobic retention of lipophilic molecules. This was achieved by passing 5 ml of water through the extraction column. Failure to carry out this step resulted in loss of josamycin during the loading procedure, possibly due to the *in-situ* formation of an acetonitrile-buffer/amine mixture with endogenous serum salts or amines which could reduce or prevent retention of the analyte.

Prior to loading samples onto the extraction column, serum proteins were precipitated to prevent clogging of the columns and to release protein-bound drug. This was achieved by the addition of an equal volume of acetonitrile to the serum sample (1 ml) followed by mixing on a vortex mixer. The internal standard (*vide infra* 3.3.1.5) was then added and after further mixing on a vortex mixer, the sample was centrifuged at 1600 x g for 5 min. The eluotropic strength of the protein-free supernatant containing acetonitrile, serum salts and other endogenous components was sufficient to prevent retention of josamycin and internal standard on the SPE column. Hence, the eluotropic strength was reduced by diluting the supernatant with

5 ml of water in a clean test-tube. Josamycin and internal standard were then loaded onto the extraction column by passing the dilute sample over the stationary phase under gravity with the aid of a custom made glass reservoir.

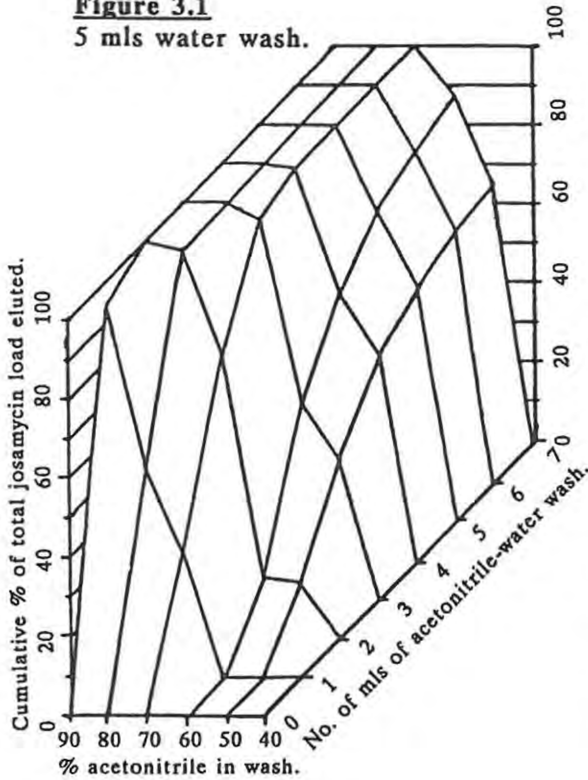
The internal standard solution was added after protein precipitation since an aliquot of the aqueous internal standard solution added to the serum sample prior to protein precipitation would result in a corresponding increase in the volume of acetonitrile required for complete precipitation of serum proteins. Dilution of the supernatant in a larger volume of water would then be necessay. This, however, was avoided by adding the internal standard directly after the acetonitrile.

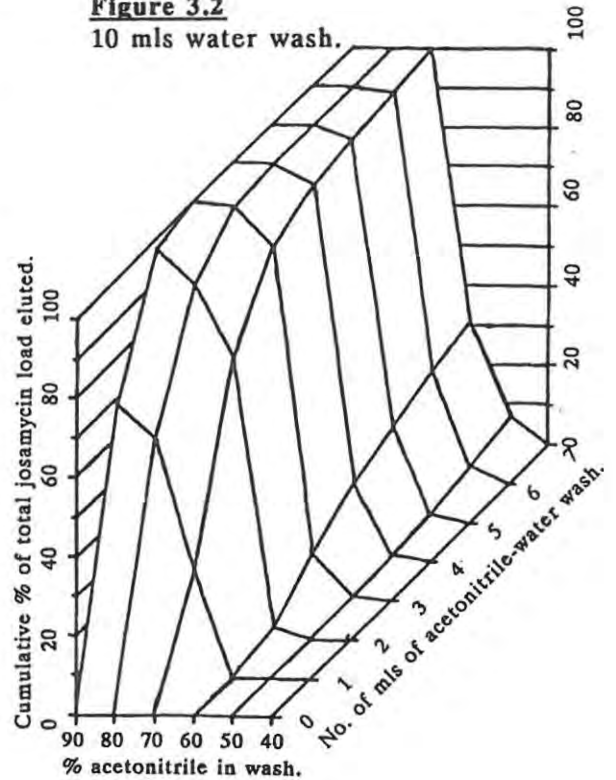
3.3.1.2 Determination of the Wash Sequence

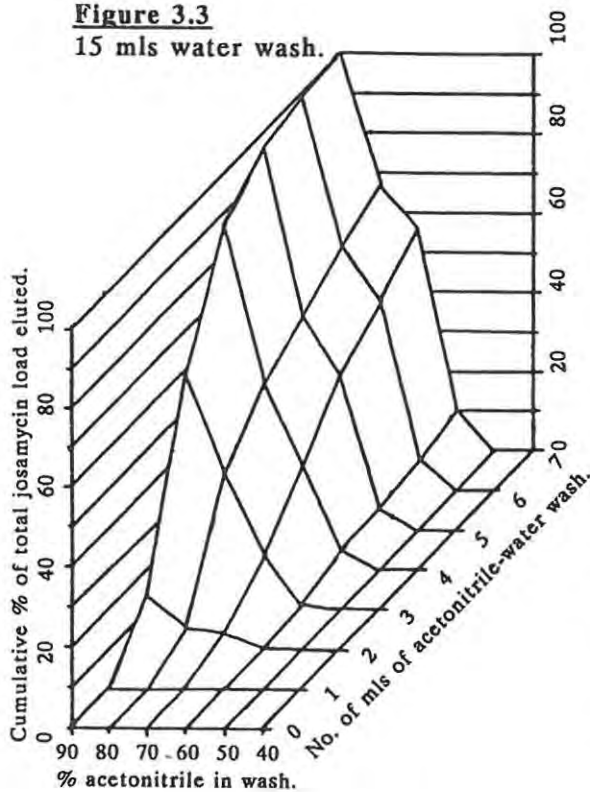
As an assay exhibiting low detection limits and high precision and accuracy is a pre-requisite for pharmacokinetic studies, a high percentage recovery and chromatograms devoid of interfering peaks are desirable. These required the selective removal of endogenous serum contaminants from the extraction column prior to elution of josamycin and internal standard with minimal loss of both analytes. A two-phase wash sequence immediately after loading consisting of a water wash followed by an acetonitrile-water wash was necessary in order to obtain adequate sample clean-up without compromising percentage recovery. Throughout the development of the extraction procedure, the extraction of oleandomycin as the internal standard did not present a problem and development was therefore focused on josamycin.

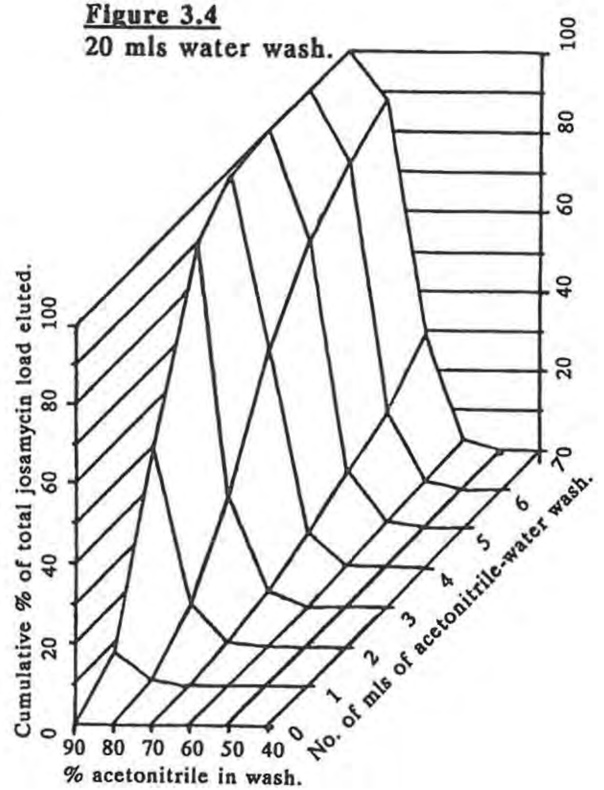
The cleanliness of extracts was dependent mainly on the eluotropic strength and volume of the acetonitrile-water wash which improved with increasing acetonitrile content and volume. However, loss of josamycin from the extraction column during the extraction procedure occurred almost entirely during the acetonitrile-water wash, the extent of which was also closely related to the wash composition and volume. Loss increased with increasing acetonitrile content and volume of the wash. However, the loss of josamycin during this wash was also highly dependent on the volume of the previous water wash. Extensive investigations into the amount of josamycin lost during acetonitrile-water washes of various compositions after 5, 10, 15 and 20 mls of water wash were conducted, and the cleanliness of the resulting extracts monitored in order to determine the most suitable wash sequence.

The experimental design of these investigations is detailed in 3.4.5.1 and results are depicted in 3-dimensional diagrams in figures 3.1 - 3.4. After either a 5, 10, 15 or 20 ml water wash, an acetonitrile-water wash composed of 40% acetonitrile did not elute any josamycin from the extraction column. However, even after 7 mls of this wash all extracts were insufficiently clean; this wash was therefore unsuitable. For washes composed of 50% or more of acetonitrile, josamycin was eluted by the acetonitrile-water wash, although, loss was reduced as the volume of the preceding









Figures 3.1 - 3.4 Cumulative elution profiles of josamycin from extraction columns during acetonitrile-water washes of various compositions after a water wash of 5, 10, 15 or 20 mls respectively, for non-dialysed serum extracts.

water wash increased. For a wash composed of 50% acetonitrile, about 65% of the josamycin load was eluted by 7 mls of the acetonitrile-water wash after a 5 ml water wash, but was reduced to about 8% after a 10 ml water wash and 0% after 15 and 20 mls of water wash. After 7 mls of an acetonitrile-water wash composed of 60% acetonitrile, about 88% of the josamycin load was lost after a 5 ml water wash, but was reduced to 30% after a 10 ml water wash, 10% after a 15 ml water wash and about 3% after a 20 ml water wash. For acetonitrile-water washes composed of 70% or more of acetonitrile, the elution of josamycin also decreased with an increasing volume of water wash. However, significant loss was still observed after a 20 ml water wash even with small volumes of acetonitrile-water. The cleanliness of extracts improved dramatically as the acetonitrile content of the wash increased above 40%. Use of the smallest volume of an acetonitrile-water wash of the lowest possible eluotropic strength which afforded adequate sample clean-up with the minimum loss of josamycin was preferable as this provided maximum confidence with which josamycin could be extracted from serum. A 20 ml water wash followed by 4 mls of an acetonitrile-water (1:1) mixture was found to be most suitable and afforded both excellent sample clean-up with minimal loss of josamycin.

In conclusion, the removal of serum salts and highly polar compounds from the extraction column using a large volume of water prior to the acetonitrile-water wash is vital to prevent the *in-situ* formation of an acetonitrile-buffer/amine mixture during the acetonitrile-water wash. Such a mixture was able to elute josamycin from the extraction column and result in a significantly reduced recovery. Results of this investigation are discussed further in 3.4.5.2 and 3.4.5.3.

3.3.1.3 Determination of Elution Mixture

A small quantity of sample contaminants remained on the extraction column after a wash sequence of 20 ml of water followed by 4 ml of a 1:1 acetonitrile-water mixture. The production of clean extracts therefore required the selective elution of josamycin from the extraction column. The volume of elution mixture required for complete elution of josamycin and internal standard was dependent on the acetonitrile-buffer ratio of the elution mixture as shown in table 3.2. As the amount of acetonitrile in the elution mixture increased, a smaller volume was required. However, when all josamycin was eluted with the minimum volume of different elution mixtures, samples eluted with mixtures of lower acetonitrile content tended to be cleaner. Since samples were to be evaporated to dryness, the smallest possible elution volume was desirable. The volume and composition of the elution mixture was therefore a compromise between elution volume and sample cleanliness. Elution with 3 x 500 μl aliquots of a 3:2 acetonitrile-phosphate buffer 0.05 M, pH 5.8, mixture was found to be the most suitable and resulted in complete elution of josamycin and

internal standard with chromatographs free from peaks due to interfering contaminants.

3.3.1.4 Sample Concentration and Reconstitution

A final elution volume of 1500 μl required an injection volume of over 300 μl for the detection of josamycin at the lower limits of the calibration range. Injection of this volume of sample onto the analytical column produced an unacceptably long solvent front and interference in the quantitation of the analyte. Therefore, evaporation of the sample to dryness and reconstitution in a smaller volume was necessary. The eluate was evaporated to dryness under vacuum at 40°C in a rotary vacuum centrifuge (Savant, Hicksville, NY, U.S.A.) after which a plug of buffer and analyte remained. Both water and acetonitrile were required for complete dissolution of the residue. However, the volumes used were critical to the success of the assay (98). If the volumes of acetonitrile and water used for reconstitution resulted in a single homogenous solution, serious chromatographic interference was observed when an aliquot of this solution was injected into the chromatographic system. This was probably due to excessive buffer concentration in the sample. However, reconstitution with 20 μl of water and 50 μl of acetonitrile resulted in salting-out of the organic component and a subsequent micro phase-separation with partitioning of josamycin and internal standard predominantly into the upper organic layer. Injection of aliquots of the upper acetonitrile layer into the chromatograph produced chromatograms with no interfering peaks and afforded easy quantitation of josamycin. This phase-separation served to remove buffer from the sample for analysis and acted as both a concentration and purification step.

The extraction ratio of josamycin into the acetonitrile layer was investigated by evaporating to dryness 1.5 ml of elution mixture spiked with 5 μg of josamycin, reconstituting the residue with 20 μl of water and 50 μl of acetonitrile and determining the amount of josamycin present in the acetonitrile layer. The fraction of the added amount of josamycin extracted into the acetonitrile layer was then calculated. Two additional elution mixtures of different acetonitrile-buffer ratios were investigated to determine the effect of the amount of buffer residue on the extraction ratio. Results are tabulated in table 3.3 and show that the extraction ratio was not significantly influenced by the amount of buffer present. This was therefore not a factor to consider in determining the optimum elution mixture.

3.3.1.5 Internal Standard

Chromatographic conditions for the separation of josamycin and oleandomycin with oleandomycin eluting after josamycin had previously been optimised and oleandomycin found to be a suitable internal standard (2.1.3.3/4 and 5).

The internal standard solution, containing 0.5 mg/ml of oleandomycin phosphate was prepared by dissolving 10 mg of oleandomycin phosphate in 2 ml of acetonitrile in a 20 ml volumetric flask and making up to volume with water.

3.3.1.6 Final Serum Extraction Method

A 1 ml volume of serum was mixed in a vortex mixer with 1 ml of acetonitrile for 5 s. Internal standard (0.2 ml) was added and mixed for an additional 5 s. Precipitated proteins were separated by centrifugation for 5 min at 1600 x g and the supernatant was transferred to a 10 ml test tube containing 5 ml of water. The diluted supernatant was then loaded onto a 1 ml disposable C_{18} extraction column which had been pre-treated by wetting with 5 ml of acetonitrile followed by 5 ml of water, with the aid of a 20 ml custom-made glass reservoir. On completion of the loading process, the column was washed with 20 ml water followed by 4 ml of acetonitrile-water (1:1). Loading and washing was effected by allowing the respective solutions to flow through the extraction column under gravity. This ensured that the column was not sucked to dryness at any stage before the elution step. After completion of the loading and wash sequence, the column was dried by suction (under a vacuum of 5-15 mmHg), using a Baker No. 10 extraction system (J.T. Baker, Phillipsburg, NJ, U.S.A.). Drug and internal standard were eluted into a 2 ml tapered collection tube (Kimble, Vineland, NJ, U.S.A.) with 3 x 0.5 ml aliquots of acetonitrile-phosphate buffer 0.05 M, pH 5.8 (3:2), the column being dried under vacuum between each aliquot. The sample was evaporated to dryness under vacuum at 40°C in a rotary vacuum centrifuge (Savant, Hicksville, NY, U.S.A.). Reconstitution was effected by the addition of 20 µl of water, mixing for 1 min, addition of 50 µl of acetonitrile, mixing for an additional 1 min, and centrifugation for 30 s at 1600 x g. An aliquot (30-40 µl) of the clean supernatant was transferred to a WISP limited-volume insert (Waters Assoc., Milford, MA, U.S.A.) with a microsyringe. Aliquots (2-10 µl) of this sample were injected into the chromatographic system.

Chromatograms of extracts of serum samples collected before and after the administration of 1 g of josamycin powder to a human volunteer are shown in figures 3.5 and 3.6.

3.3.2 Validation of the Assay

3.3.2.1 Linearity

Linearity over the concentration range of 0 - 2 µg/ml was established by the extraction of standards prepared by the serial dilution of a serum stock solution. An aqueous josamycin stock solution was prepared by accurately weighing 10 mg of

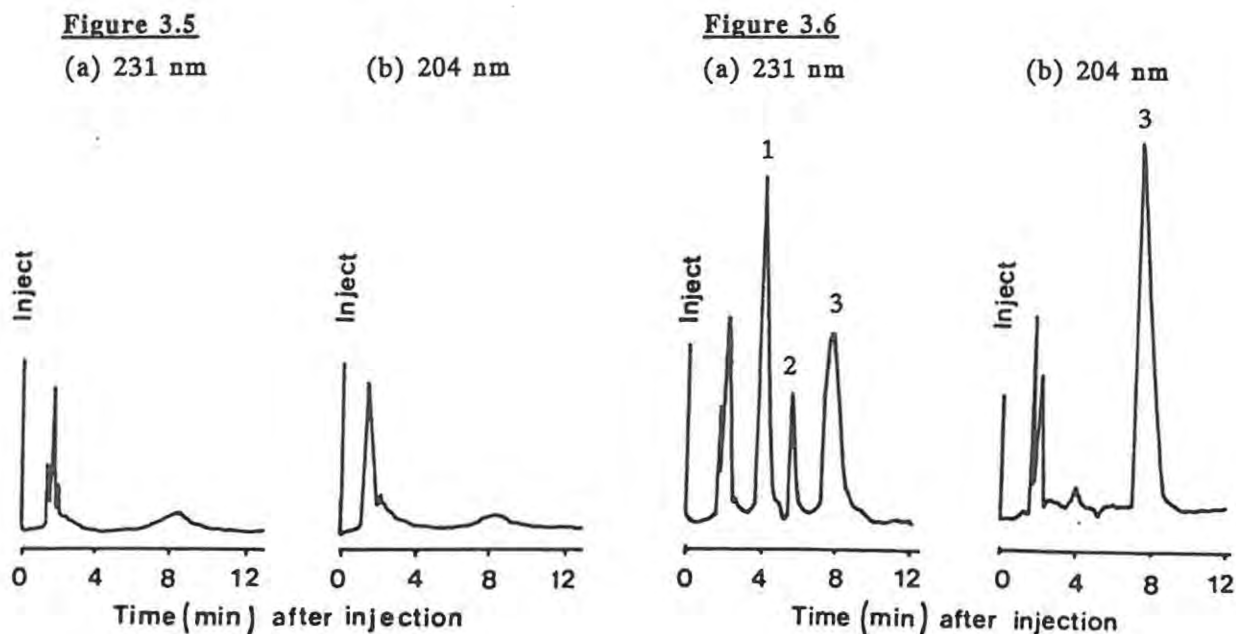


Figure 3.5 Chromatograms of a blank serum extract monitored at 231 nm (a) and 204 nm (b).
Figure 3.6 Chromatograms of a serum extract showing metabolite (1), josamycin 0.29 μg/ml (2) and internal standard (3), monitored at 231 nm (a) and 204 nm (b). Samples were withdrawn from a volunteer immediately before and 30 min after the oral administration of 1 g of josamycin powder to a healthy male volunteer.

josamycin into a 10 ml volumetric flask, dissolving in 2 ml of acetonitrile and making up to volume with water (solution A). A 1 ml aliquot of solution A was then diluted to 10 ml with water (solution B). The upper standard (2 μg/ml) was prepared by pipetting a 1 ml aliquot of solution B into a 50 ml volumetric flask and making up to volume with serum. The remaining standards containing 1.5, 1.0, 0.6, 0.3 and 0.1 μg/ml of josamycin were prepared by the serial dilution of the upper standard.

3.3.2.2 Precision and Accuracy

The precision of the assay was determined by calculating the relative standard deviations of the peak height ratios of replicate samples of standards, and the relative standard deviations of the concentrations of replicate accuracy samples. Accuracy was assessed at concentrations of 1.818 μg/ml and 0.303 μg/ml after the extraction of replicate samples of serum solutions prepared by an independent analyst, by comparing the means of concentrations found with the actual spiked concentrations. Sample concentrations were calculated by interpolation of peak height ratios from the calibration curve.

3.3.2.3 Percentage Recovery

The percentage recovery from spiked serum samples was assessed by comparing peak height ratios of samples to which internal standard was added after completion of the extraction process with the peak height ratios of aqueous reference samples containing josamycin and internal standard in amounts equivalent to 100% recovery. Replicate samples of two serum solutions containing different concentrations of josamycin were extracted, evaporated to dryness and reconstituted as described in 3.3.1.6 but without internal standard. After reconstitution of each sample, as much of the upper acetonitrile layer as possible was transferred to a 2 ml collection tube containing 0.2 ml of internal standard solution (0.5 mg/ml). Samples were then again evaporated to dryness which resulted in a residue free from buffer. The residue was reconstituted with 20 μl of water and 50 μl of acetonitrile leaving the extracted josamycin plus internal standard dissolved in a specific volume of solvent. An aliquot of this sample was injected into the chromatograph. Reference samples were prepared by the addition of an amount of josamycin equivalent to 100% recovery to collection tubes containing internal standard. After evaporating to dryness, the reference sample residues were reconstituted as were residues of the test samples. The ratios of peak height of josamycin to that of internal standard for the test samples were then compared with those of the reference samples.

Removal of the acetonitrile layer followed by evaporation to dryness and subsequent reconstitution in a precise volume was necessary as the volume of the acetonitrile layer was unknown. This was due to the partial miscibility of the two solvents after salting out and consequently the amount of josamycin present in the acetonitrile layer could not be calculated unless the procedure described above was followed.

3.3.3 Results and Discussion

3.3.3.1 Linearity

A calibration curve was constructed by linear regression of a plot of peak height ratio of josamycin to that of internal standard *vs.* josamycin concentration (Table 3.4), which was found to be linear over the calibration range studied (Figure 3.7). The linear regression equation was $y = 1.0855x - 0.0237$ with an R.Sq. value of *0.9983*.

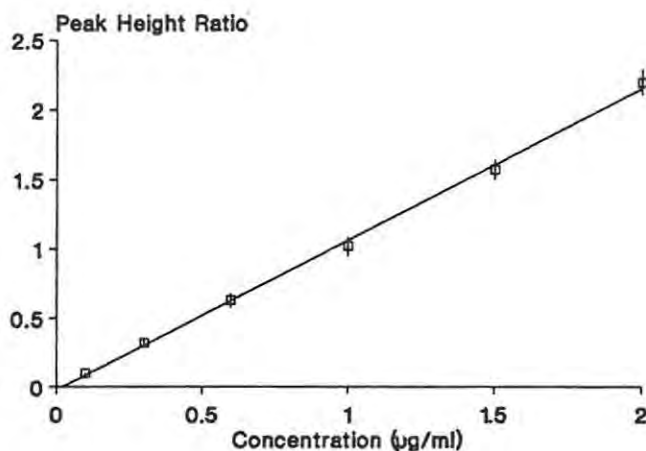


Figure 3.7 Calibration curve constructed by linear regression of mean peak height ratios *vs.* concentration after the extraction of replicate samples of non-dialysed serum standards. Linear regression equation:- $y = 1.0855x - 0.0237$, R.Sq = 0.9983.

3.3.3.2 Precision and Accuracy

Results from precision and accuracy studies are tabulated in table 3.4. Relative standard deviations for replicate samples of all calibration standards and accuracy studies were below 10%. The difference in the mean concentrations found in samples prepared by an independent analyst and the actual spiked concentrations were 2.31% and 10.98% at the upper and lower concentrations respectively.

3.3.3.3 Percentage Recovery

Results from percentage recovery studies are tabulated in table 3.5. The mean overall percentage recoveries at the upper and lower ends of the calibration range were 77% and 71% respectively. Results of studies to determine the extraction ratio of josamycin into the acetonitrile layer of the final reconstitution step show that 87% of josamycin eluted from the extraction column will partition into the acetonitrile layer after reconstitution (Table 3.3). By calculation 11% and 18% of the initial load was therefore lost from the column prior to the elution step, and a further 12% and 11% of the initial load lost during the phase separation step at the upper and lower concentrations respectively.

3.3.3.4 Detection Limit

The detection limit of josamycin in serum was 0.025 mg/l, based on a signal-to-noise ratio of 3 and determined with a 10-μl injection volume and a sensitivity setting of 0.01 a.u.f.s. This corresponds to an on-column load of < 5 ng and 20 %

of the final amount extracted. Injection of a higher percentage load produced chromatograms in which interference from contaminants was occasionally observed.

3.3.4 Sample Contamination from Plastic Extraction Apparatus

Manufacturers of SPE columns supply vacuum manifolds to assist in the extraction and elution of multiple samples. Extraction columns are fitted onto the manifold by a luer fitting either moulded into the plastic top as in early Bond-Elut manifolds (Analytichem Internaltional, Harbour City, CA, U.S.A.) or into a luer fitting stainless steel needle which passes through the plastic top of the manifold as in the J.T.Baker & Co. manifold (J.T. Baker, Phillipsburg, NJ, U.S.A.). Manifolds with luer fittings moulded into the plastic top allow the eluent to come onto contact with the plastic directly after leaving the extraction column. During development of this assay, aliquots of elution mixture filtering through SPE columns fitted into the moulded plastic luer fittings of the Bond-Elut manifold, were contaminated by unknown compounds. These contaminants eluted from the analytical column at about the same time as josamycin and interfered with the quantitation of josamycin. Investigation into the source of this peak revealed that various compounds were eluted from the manifold by the elution mixture. Figure 3.8 shows a chromatogram of a sample of elution mixture collected after passing directly through the Bond-Elut manifold without having passed through an extraction column. The Bond-Elut manifold was therefore modified to prevent contamination of samples in this manner by fitting stainless steel luer needles (which protruded through the plastic top) in place of the moulded plastic luer fittings. SPE columns could then be fitted directly into these needles, thereby avoiding contact between the elution mixture and plastic manifold.

Interference by compounds eluted from the plastic of extraction columns has also been observed by Junk *et al.* (243). An extensive study revealed that alkanes, alkenes, plasticisers and antioxidants contained in SPE column blanks could have been responsible for extraneous peaks observed during the analysis of pesticides by GC. Contaminating samples unknowingly in this manner could be a major source of frustration during assay development and it is therefore important to ensure that plastic components used during any extraction procedure are not a source of sample contamination.

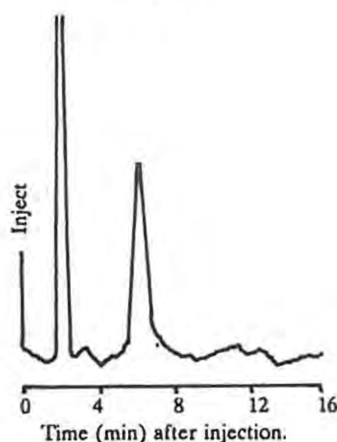


Figure 3.8 Chromatogram obtained after injection of a 5 μl aliquot of the upper acetonitrile layer obtained after reconstituting the dried residue of a 1.5 ml sample of elution mixture (acetonitrile-phosphate buffer 0.05 M, pH 5.8, 6:4) which had passed directly through the Bond-Elut manifold without stainless-steel needles. The interfering peak can be seen eluting after 6 mins.

3.3.5 Discussion

A rapid, sensitive and selective (*vide infra* 3.8) assay with the accuracy and precision required for pharmacokinetic studies has been developed for the analysis of josamycin in human serum. SPE proved to be highly suitable for the extraction of josamycin from serum which was facilitated by judicious exploitation of the dual-retention mechanism displayed by macrolide antibiotics. With the aid of custom built racks and reservoirs, and allowing the diluted sample and washes to percolate through the extraction column under gravity, over 80 samples can be extracted with ease during a normal working day and assayed overnight using an automated injection system.

The presence of endogenous salts and amines in serum samples was a constant problem throughout the wash procedure. As shown, a large volume water wash after loading is essential for the removal of these contaminants from the extraction column prior to the acetonitrile-water wash. It is also of paramount importance that this wash does not become contaminated with these endogenous compounds. Contamination can occur from the diluted serum sample if pasteur pipettes used during loading to remove bubbles from the extraction column are also used during the 20 ml water wash or acetonitrile-water wash procedures, or from diluted sample remaining on the side of the reservoir after loading. Clean pasteur pipettes must therefore be used at each step in the extraction procedure and reservoirs must be rinsed with HPLC grade water prior to the commencement of the 20 ml water wash. Contamination of the water or acetonitrile-water wash can lead to excessive loss of josamycin during the acetonitrile-water wash and serious problems with accuracy and precision. The necessity of preventing contamination was also highlighted during the extraction of erythromycin

utilising a similar solid-phase extraction procedure developed in our laboratory by Stubbs (98,109). Internal standard (oleandomycin) appeared to be randomly lost from SPE columns during the extraction process. Investigation into this revealed that if pasteur pipettes contaminated by endogenous serum salts during the loading process were used during the wash sequence, oleandomycin was lost from the extraction column during the acetonitrile/water wash. During the extraction of josamycin, however, oleandomycin appeared to be relatively unaffected by contamination in this manner but josamycin was highly sensitive to the presence of these contaminants.

Oleandomycin was retained on and eluted from C_{18} extraction columns in a similar manner to josamycin. Oleandomycin was easily eluted from the SPE column during the final elution step, however, loss during the extraction procedure was only slight and few problems were encountered with extraction of this internal standard. Oleandomycin was therefore ideally suited for use as an internal standard in the SPE of josamycin. As previously mentioned in 2.1.3.5, monitoring the eluate at 200 nm would be optimum for the detection of oleandomycin. However, many endogenous compounds in biological samples show strong UV absorbance in this region and it was difficult to obtain chromatographs free from interfering peaks at 200 nm and at low attenuations (0.01-0.02 a.u.f.s.). Interference was mainly due to late eluting peaks which could be eliminated if acetonitrile-water washes with a high acetonitrile content were used. However, these washes severely reduced the recovery and precision of josamycin extraction and their use was therefore not appropriate. To overcome this problem a large amount of internal standard (100 μg oleandomycin) was used in each extraction. This allowed the eluate to be monitored at 204 nm at an attenuation of 0.06 a.u.f.s. which effectively eliminated interference with the oleandomycin peak.

3.4 EXTRACTION OF JOSAMYCIN FROM DIALYSED SERUM AND ASSOCIATED PROBLEMS

The development of procedures for the extraction of compounds from serum is routinely carried out using pooled human serum which has been dialysed against normal saline. Pooled serum obtained from blood banks may contain unwanted drugs which have been administered and absorbed prior to phlebotomy particularly if discarded serum from polycythaemic patients, which generally contains various drugs being used by the patient, is used. Dialysis renders the serum free from exogenous compounds and thus eliminates the risk of possible chromatographic interference from these contaminants. A method for the extraction of josamycin from serum was therefore initially developed using pooled human serum dialysed in our laboratory with

standard 16mm dia. Visking Dialysis Tubing (Serva, Feinchemica GMBH, Heidelberg, Germany) against normal saline. However, serious problems were encountered when samples withdrawn from a human volunteer after the administration of an oral dose of josamycin were extracted. The extraction procedure, validation and problems encountered are discussed below.

3.4.1 Extraction Procedure

The initial extraction procedure validated for the extraction of josamycin from dialysed serum was similar to that for non-dialysed serum (3.3.1.6) but with the following differences:-

a) a water wash of only 5 mls in place of 20 mls was used, and

b) 7 mls of a 6:4 acetonitrile-water mixture in place of 4 mls of a 1:1 acetonitrile-water mixture for the final wash was used.

3.4.2 Validation Parameters

Linearity over the concentration ranges of 0 - 1 μg/ml and 0 - 5 μg/ml was established by the extraction of standards prepared by the serial dilution of a serum stock solution. An aqueous josamycin stock solution was prepared by accurately weighing 10 mg of josamycin into a 10 ml volumetric flask, dissolving in 2 ml of acetonitrile and making up to volume with water (solution A). A 1 ml aliquot of solution A was then diluted to 10 ml with water (solution B). The upper standard (5 μg/ml) was prepared by pipetting a 1 ml aliquot of solution B into a 20 ml volumetric flask and making up to volume with serum. Standards containing 2.5, 1.0 and 0.5 μg/ml of josamycin were prepared by the 1 in 2, 1 in 5 and 1 in 10 dilution of the upper standard, and standards containing 0.25, 0.10 and 0.025 μg/ml of josamycin were prepared by the 1 in 2, 1 in 5 and 1 in 10 dilution of the 0.50 μg/ml standard. The 0 - 5 μg/ml calibration curve was constructed using replicate samples of the upper 5 standards with a detector attenuation of 0.04 a.u.f.s. at 231 nm for the detection of josamycin. The 0 - 1.0 μg/ml calibration curve was constructed using replicate samples of the lower 5 standards with a detector attenuation of 0.01 a.u.f.s. Calibration curves over two concentration ranges were constructed so that serum concentrations as low as 0.05 μg/ml and as high as 5.0 μg/ml *i.e.* a 100 fold difference, could be determined if necessary. Ultimately, however, a calibration range from 0 - 2 μg/ml was used for the determination of josamycin in samples from bioavailability trials.

The precision of the assay was determined by calculating the relative standard deviations of the peak height ratios of replicate samples of standards, and the percentage recovery was determined as in 3.3.2.3 at 5.0, 0.5 and 0.1 μg/ml.

3.4.3 Validation Results

Calibration, precision and recovery data for the determination of josamycin in dialysed serum are tabulated in table 3.6. A calibration line obtained by plotting the ratio of josamycin peak height to that of internal standard *vs.* josamycin concentration was found to be linear over both calibration ranges studied. The linear regression equation of the upper calibration range was $y = 0.7863x - 0.0826$ with an R.Sq. value of *0.9986*, and of the lower calibration range was $y = 2.6670x - 0.0409$ with an R.Sq. value of *0.9998*. The upper and lower calibration curves obtained are depicted in figures 3.9 and 3.10 respectively.

Figure 3.9

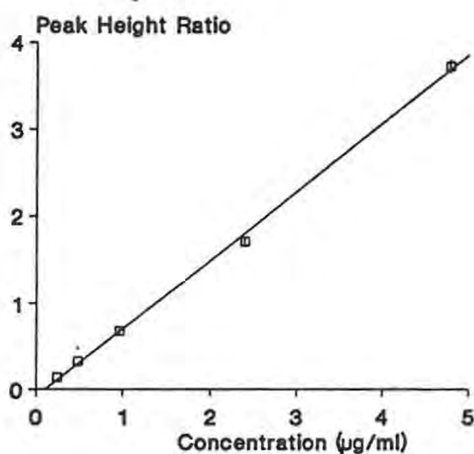
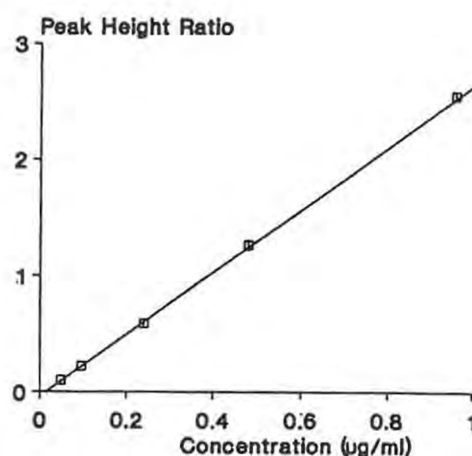


Figure 3.10



Figures 3.9 and 3.10 Calibration curves for the upper and lower calibration ranges constructed by linear regression of mean peak height ratios *vs.* concentration after the extraction of replicate samples of dialysed serum standards. Linear regression equations:- upper calibration range, $y = 0.7863x - 0.0826$, R.Sq. = 0.9986; lower calibration range, $y = 2.6670x - 0.0409$, R.Sq. = 0.9998.

Relative standard deviations of replicate samples were $\leq \pm 4.16$ % for the five upper standards and $\leq \pm 5.99$ % for the five lower standards. This analytical method was therefore highly precise for the determination of josamycin in dialysed serum over a wide range of concentrations.

The mean percentage recoveries at the upper, middle and lower concentrations investigated were 78%, 70% and 60% respectively.

3.4.4 Pilot Trial

After validation of the assay, a pilot trial was conducted to obtain preliminary pharmacokinetic data. An oral dose of five Josamycin 200 mg tablets was administered together with 200 mls of water to a healthy male volunteer, who had fasted overnight. A light breakfast and lunch were given 2 and 5 hours after administration of the dose. Blood samples were withdrawn from an arm vein via an indwelling catheter immediately prior to dosing and then at 0.33, 0.66, 1.0 ,1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 hours after administration of the dose. Samples were allowed to clot and the serum harvested after centrifuging the clotted blood at 1600 x g for 20 min. Urine samples were collected immediately prior to dosing and then at 2.0, 4.0, 8.0, 12.0, 16.0 and 24.0 hours after administration of the dose. The volumes voided after each collection time were noted for the calculation of a cumulative urinary excretion profile. Serum and urine samples were stored at -15°C and analysed the following day.

After the extraction of serum samples as in 3.4.1, josamycin was not detected in any of the samples withdrawn from the volunteer during the trial period. However, josamycin was detected in the urine (using the same extraction procedure as for serum, *vide infra* 3.5) and concentrations ranged between 0.57 and 5.65 μg/ml. The cumulative urinary excretion curve obtained is depicted in figure 3.11.

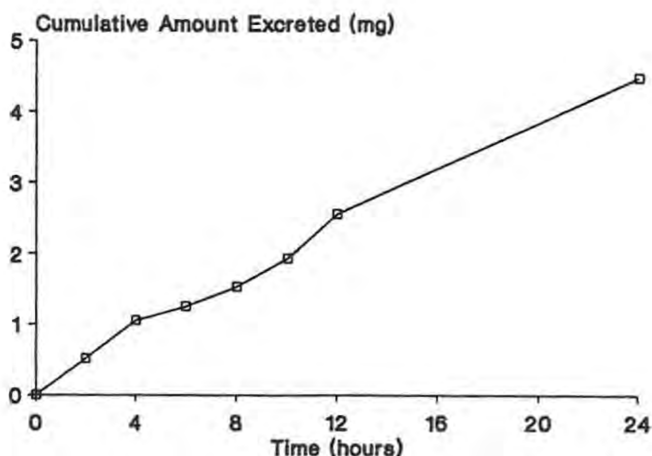


Figure 3.11 Cumulative urinary excretion profile of josamycin, obtained after the oral administration of five Josamycin 200 mg Tablets to a healthy male volunteer.

The failure to detect even trace levels of josamycin in volunteer serum samples indicated that either josamycin was not absorbed or josamycin in the sample was lost during the extraction procedure. A peak serum concentration of between 0.8 and 2.0 μg/ml was expected (36,45). However, since excretion of josamycin in urine was observed, some absorption of the drug must have occurred and should therefore have been detected in the serum. Re-extraction of serum samples failed to yield any positive results. As the extraction of standards prepared with dialysed serum was successful, it was suspected that serum samples from the volunteer were not behaving in the same manner as the standards during the extraction procedure. In order to determine if differences in the extractability of josamycin from dialysed and non-dialysed serum did exist, aliquots of non-dialysed blank serum were spiked with appropriate amounts of drug and extracted simultaneously with standard solutions prepared from dialysed serum. Josamycin was consistently lost during the extraction of only non-dialysed serum samples. The dialysis of serum was therefore assumed to be responsible for the discrepancy observed and further method development was subsequently carried out using non-dialysed serum. The extraction procedure was subsequently modified and validated using non-dialysed serum as detailed in 3.3.

3.4.5 Investigation into the Loss of Josamycin during Extraction of Non-Dialysed Serum Samples

3.4.5.1 Experimental

In order to overcome the problem outlined in 3.4.4 it was essential to determine during which stage of the extraction procedure josamycin was lost. Non-dialysed serum was spiked with josamycin to a concentration of 5 μg/ml and a 1 ml aliquot extracted using the assay procedure validated for the extraction of josamycin from dialysed serum. During the load and wash sequences, the waste liquors were collected in 1 ml aliquots as they flowed from the extraction column. Loss of josamycin from the SPE column during these steps was assessed by assaying the samples collected, in the following manner. After the addition of 0.6 mls of phosphate buffer 0.05 M, pH 5.8 to each sample collected (added to effect a phase separation on reconstitution), samples were evaporated to dryness. The residue was then reconstituted with 20 μl of water and 50 μl of acetonitrile after which a 20 μl aliquot of the upper acetonitrile layer was injected into the chromatographic system. The amount of josamycin found in each 1 ml aliquot of waste was then calculated as a percentage of the initial amount present in the serum sample. This was done by comparing the peak height of josamycin for each sample of waste with the cumulative sum of the peak heights of josamycin of all waste samples together with the peak

height of josamycin in the final acetonitrile-buffer elution sample. Serum samples were spiked to a high enough concentration so that a low detector sensitivity setting of 0.1 a.u.f.s. could be used. This, together with slight sample clean-up during the phase separation step made it possible to detect josamycin in the aliquots of waste solutions collected although they contained large amounts of serum contaminants.

The possibility that josamycin was not eluted during the final elution step was investigated by repeating the elution step and analysing the sample for josamycin.

3.4.5.2 **Results**

No josamycin was present in any of the aliquots collected during the load or water wash steps. Therefore, all josamycin was retained by the SPE column during loading and no josamycin was lost during the water wash. However, 0%, 25%, 25%, 20%, 10% and 7% of the initial josamycin load was present in the 1st, 2nd, 3rd, 4th, 5th, 6th and 7th aliquot collected during the acetonitrile-water wash. The cumulative amount of josamycin lost during the acetonitrile-water wash was therefore 0%, 25%, 50%, 70%, 80% and 87% after 1, 2, 3, 4, 5, 6 and 7 mls of wash respectively. Only 13% of the load was eluted during the final elution step of the extraction process. After a 5 ml water wash, 7 mls of a 6:4 acetonitrile-water wash (which did not elute josamycin from the SPE column after extraction from dialysed serum) eluted almost all josamycin from the SPE column after extracticn from non-dialysed serum. A plot of the cumulative amount eluted during the 6:4 acetonitrile-water wash *vs.* the volume of wash after a 5 ml water wash is depicted in figure 3.1. Previous work (98,100) showed that a water wash directly after loading of a serum sample onto an SPE column was vital to prevent loss of macrolides from the column during any subsequent acetonitrile-water wash. An inadequate water wash was suspected as a possible cause of the problem. The relationship between the volume of water wash, the volume and composition of the acetonitrile-water wash and the amount of josamycin eluted during the acetonitrile-water wash after extraction from non-dialysed serum samples, was therefore extensively investigated. The procedure detailed in 3.4.5.1 was repeated using 5, 10, 15 and 20 ml water washes, each followed by 7 mls of an acetonitrile-water wash of either 90%, 80%, 70%, 60%, 50%, or 40% acetonitrile. Three-dimensional plots of the cumulative amount of josamycin eluted from extraction columns during acetonitrile-water washes *vs.* volume *vs.* composition of the acetonitrile-water wash, after either 5, 10, 15 or 20 mls of water wash are depicted in figures 3.1 - 3.4. These results show that the amount of josamycin eluted by any of the acetonitrile-water washes decreased as the volume of the preceding water wash increased. For example, the 6:4 acetonitrile-water wash which was used in the extraction procedure validated for dialysed serum eluted almost 90% of josamycin from the extraction column following a 5 ml water wash (Figure 3.1) compared with

about 2% after a 20 ml water wash (Figure 3.4). The volume of the water wash was thus critical to the successful extraction of josamycin from non-dialysed serum. Josamycin could therefore be efficiently extracted from non-dialysed serum using a 20 ml water wash followed by 7 mls of a 6:4 acetonitrile-water wash. However, as discussed in 3.3.1.2, only 4 mls of a 1:1 acetonitrile-water wash was required to produce clean samples after a 20 ml water wash. A water wash of 20 mls followed by 4 mls of a 1:1 acetonitrile water wash was subsequently used in the final extraction procedure.

3.4.5.3 Conclusion

It is well known that amines can effectively block silanol binding sites on C_{18} reversed-phase stationary phases and compete for these binding sites with compounds retained by a dual-retention mechanism. Dialysed and non-dialysed serum samples were assayed in a clinical laboratory for electrolytes and amine containing compounds (urea, urates and creatinine). The results are tabulated in table 3.7. Although the electrolyte balance was virtually unchanged, urea, urates and creatinine were almost totally removed from serum by dialysis. It was therefore concluded that endogenous serum amines acted as silanol blocking agents and that they prevented the retention of josamycin by silanophilic interaction during the extraction of non-dialysed serum samples, but not during the extraction of dialysed samples. Cox and Pullen (110) observed a decrease in recovery of a 21-aminosteroid from 90% for aqueous samples to only 50% for serum samples and also ascribed this to the presence of a non-protein amine modifier in serum. Josamycin was therefore probably retained from non-dialysed serum samples predominantly by solvophobic interaction and subsequently eluted by an acetonitrile-water mixture. Alternatively, endogenous amines still present on the column after an inadequate water wash produced a mixture at the onset of the acetonitrile-water wash capable of eluting josamycin retained by both solvophobic and silanophilic interaction. However, a combination of both possibilities is the most likely scenario. Removal of these components from the extraction column prior to the commencement of the acetonitrile-water wash was therefore essential for the successful extraction of josamycin from non-dialysed serum samples. A 5 ml water wash was sufficient to remove traces of endogenous amines from SPE columns which remained in the serum after dialysis, however, a much larger volume was required to do this effectively for non-dialysed serum. A 20 ml water wash was found to be optimum and was therefore included in the extraction procedure. This enabled the successful development of a precise and accurate assay with good sample recovery, however, the precision of the assay for dialysed serum was generally greater than was obtained for non-dialysed serum.

The extraction of oleandomycin from non-dialysed serum using the procedure for dialysed serum was less variable than josamycin. The retention of oleandomycin on SPE columns was therefore less susceptible to the presence of endogenous serum compounds than josamycin, and adjustments made to the dialysed serum extraction procedure for the efficient extraction of josamycin from non-dialysed serum maintained the integrity of oleandomycin as a reliable internal standard.

Re-extraction of pilot-trial samples using the method described in 3.3.1.6 did not yield any measurable serum concentrations of josamycin. However, josamycin concentrations were successfully determined in samples from a second pilot trial in which josamycin was administered as a powder. This discrepancy is discussed and possible explanations are put forward in Chapter 6.

Problems in method development sited in this section show that any SPE procedure developed for bioavailability or pharmacokinetic studies must be developed and validated using media whose composition is no different from samples obtained from volunteers. Failure to do this can lead to the validation of an assay which is in fact unsuitable for the task for which it is intended. In the case of josamycin, the limitation of the assay developed using dialysed serum was obvious, however, any less noticable limitation could have far-reaching consequences if not detected by an astute analyst.

3.5 URINE EXTRACTION

3.5.1 Extraction Procedure

Urine samples were extracted using the method validated for the extraction of josamycin from non-dialysed serum (3.3.1.6). Clean extracts were obtained without modification of the extraction procedure. Chromatograms of extracts of urine samples collected before and after the administration of 1 g of josamycin to a human volunteer are shown in figures 3.12 and 3.13.

3.5.2 Validation of the Assay

3.5.2.1 Linearity

Linearity over the concentration range of 0 - 5 μg/ml was established by the extraction of replicate samples of standards prepared by the serial dilution of a urine

Figure 3.12
(a) 231 nm (b) 204 nm

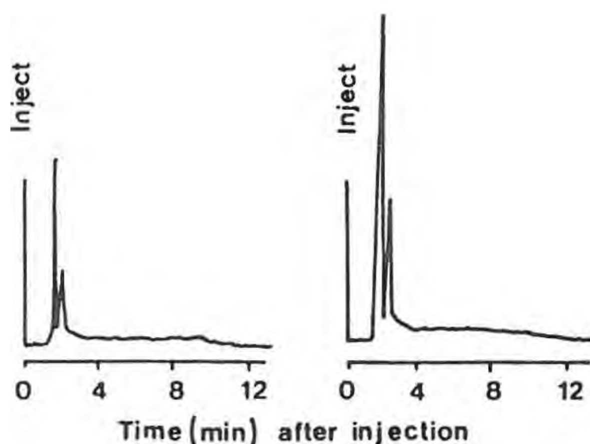


Figure 3.13
(a) 231 nm (b) 204 nm

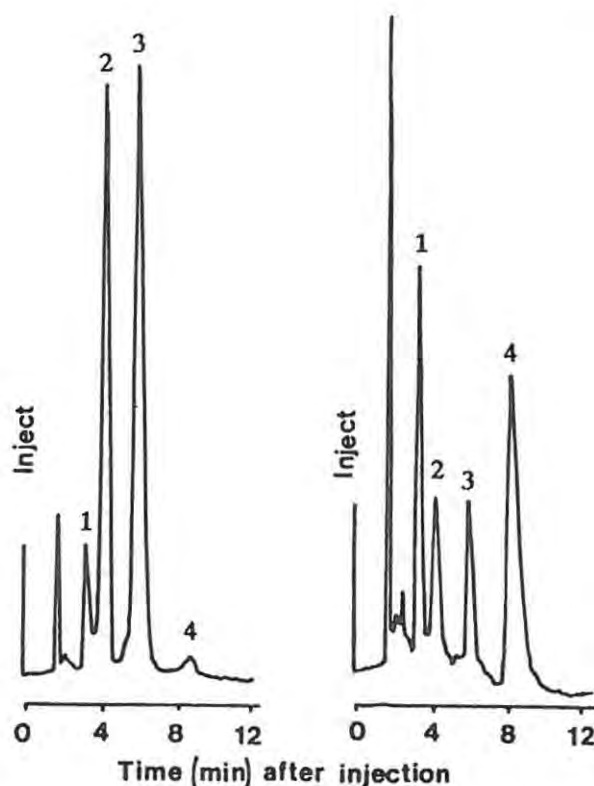


Figure 3.12 Chromatograms of a blank urine extract monitored at 231 nm (a) and at 204 nm (b).
Figure 3.13 Chromatograms of a urine extract showing metabolites (1 and 2), josamycin 5.28 μg/ml (3) and internal standard (4), monitored at 231 nm (a) and 204 nm (b). Urine was collected immediately before and four hours after the oral administration of 1 g of josamycin powder to a healthy male volunteer.

stock solution. An aqueous josamycin stock solution was prepared by accurately weighing 10 mg of josamycin into a 10 ml volumetric flask, dissolving in 2 ml of acetonitrile and making up to volume with water (solution A). A 1 ml aliquot of solution A was then pipetted into a 10 ml volumetric flask and made up to volume with water (solution B). The upper standard was prepared by pipetting 1 ml of solution B into a 20 ml volumetric flask and making up to volume with urine. The remaining standards were prepared by the 1 in 2, 1 in 5, 1 in 10 and 1 in 20 dilution of the upper standard with urine.

3.5.2.2 Precision and Accuracy

The precision of the assay was determined by calculating the relative standard deviations of the concentrations of replicate samples of both standards and accuracy samples. Accuracy was assessed at concentrations of 4.540 μg/ml and 1.488 μg/ml after the extraction of replicate samples of solutions which were prepared by an independent analyst, by comparing the means of concentrations found with the actual

spiked concentrations. Sample concentrations were calculated by interpolation of peak height ratios from a calibration curve prepared by the investigator.

3.5.2.3 Percentage Recovery

The percentage recovery from urine samples was determined as detailed in 3.3.2.3 for serum samples. Replicate samples of two different urine stock solutions spiked to concentrations of 5.00 and 0.25 μg/ml were extracted.

3.5.3 Results and Discussion

3.5.3.1 Linearity

A calibration curve was constructed by linear regression of a plot of the ratio of josamycin peak height to that of internal standard *vs.* josamycin concentration, and was found to be linear over the calibration range studied (Figure 3.14). The linear regression equation was $y = 0.3142x - 0.0073$ with an R.Sq. value of 1.0000. Josamycin is only slightly excreted in the urine (± 5%) and a calibration range with an upper concentration of 5 μg/ml is suitable for quantitation of josamycin in the majority of urine samples. Samples with concentrations above the upper calibration limit should be diluted with blank urine prior to analysis.

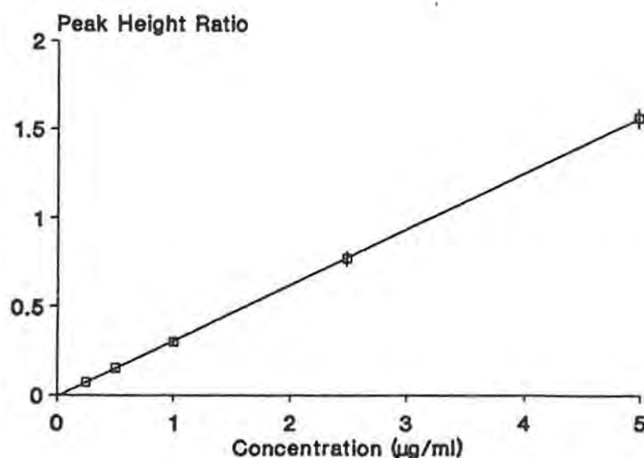


Figure 3.14 Calibration curve constructed by linear regression of mean peak height ratios *vs.* concentration after the extraction of replicate samples of urine standards. Linear regression equation:- $y = 0.3142x - 0.0073$, R.Sq. = 1.0000.

3.5.3.2 Precision and Accuracy

Results from precision and accuracy studies are tabulated in table 3.4. Relative standard deviations for replicate samples of all calibration standards and accuracy samples were $\leq \pm 6.2\%$. The difference in the mean concentrations found in samples prepared by an independent analyst and the actual spiked concentrations were 10.22% and 3.70% at the upper and lower concentrations respectively.

3.5.3.3 Percentage Recovery

Results from percentage recovery studies are tabulated in table 3.5. The mean overall percentage recoveries at the upper and lower ends of the calibration range were 76% and 80% respectively. By calculation as in 3.3.3.3, 13% and 8% of the initial load was therefore lost from the column prior to the elution step, and a further 11% and 12% of the initial load lost during the phase separation step at the upper and lower concentrations respectively.

The extraction procedure and chromatographic system developed initially for the determination of josamycin in serum proved equally suitable for the determination of josamycin in urine without modification. Furthermore, the determination of josamycin in urine was generally more precise than in serum and chromatograms were generally devoid of unknown peaks. A lower concentration of compounds in urine than in serum which may be capable of acting as silanol blocking agents and subsequently interfering with the extraction process could be the reason for the increased precision. Also, endogenous constituents present in urine are generally more polar than those found in serum and are more easily eluted from the SPE columns during water and acetonitrile-water washes, hence producing chromatograms completely free from interfering peaks at both 204 nm and 231 nm.

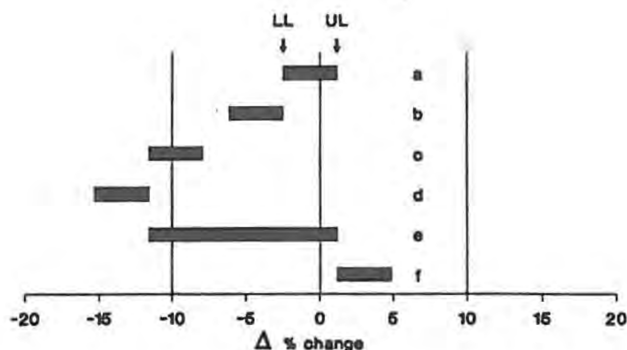
3.6 STABILITY OF JOSAMYCIN IN STORED SAMPLES

Assessment of the stability of compounds in samples awaiting analysis is essential for the procurement of reliable pharmacokinetic data. There is, however, a dearth of information in the literature on stability study design and statistical treatment of data for biological samples. Data are often presented merely as a comparison of concentrations found in freshly prepared samples with concentrations found in samples stored under various conditions for various periods of time. There is also frequently no statistical evaluation of the data, calculation of maximum storage times (111,112) or precise definition of what constitutes a significant change in

concentration. Some authors (113-115) have used a simple t-test to test for any significant difference between stored and freshly prepared samples but do not suggest limits beyond which samples can no longer be considered suitable for analysis. Furthermore these tests do not take the precision of the assay into consideration.

Timm *et al.* (116) have presented an approach which takes into account the precision of the assay and furthermore, they suggested criteria for the interpretation of stability data. The true percentage change in concentration between freshly prepared and stored samples (Δ) can only be found if concentrations are determined without analytical error, which is thus improbable. Timm, therefore, constructed 90% confidence intervals within which the true percentage change (Δ) lies with 90% certainty, and from which one can determine with 90% certainty that a pharmacokinetically relevant change has or has not taken place. These confidence intervals are calculated using the actual measured percentage response differences between stored and freshly prepared samples (D) for a number of replicate samples and are therefore dependent on the precision of the assay. Degradation greater than 10% was considered pharmacokinetically relevant. Furthermore, Timm stated that a statistically significant change is not important unless it is pharmacokinetically relevant and suggests precise criteria to decide when a statistically significant change can be considered pharmacokinetically relevant. Decisions on the stability of samples are based on the position of these 90% confidence intervals in relation to 0 and -10% true percentage change limits (116). The interpretation of all permutations of the positions of confidence intervals in relation to these limits is given in figure 3.15. This approach makes interpretation of stability data both simple and meaningful.

Stability studies are usually carried out using biological fluids spiked after the absence of chromatographic interference from unknown compounds has been confirmed. Results from these studies are then taken as an indication of the stability of the drug in *ex-vivo* samples *i.e.* samples obtained from volunteers during bioavailability studies. However, the presence of metabolites and their stability as well as differences in enzyme activity between freshly withdrawn and pooled serum could have a marked effect on the stability of the drug in biological samples, and *ex-vivo* samples obtained from volunteers may not behave in a similar manner to spiked samples. Data obtained from stored spiked samples may therefore not be relevant to stored *ex-vivo* samples. In order to store trial samples and *in-vitro* standards and controls with equal confidence, stability studies on both *ex-vivo* and *in-vitro* serum and urine samples were conducted and results assessed following guidelines suggested by Timm.



LL = lower limit; UL = upper limit of the confidence interval.
(a) change of response, not significant and not relevant.
(b) decrease of response, significant but not relevant.
(c) decrease of response, significant and possibly relevant.
(d) decrease of response, significant and relevant.
(e) decrease of response, not significant but possibly relevant.
(f) increase of response, significant.

Figure 3.15 Interpretation of confidence intervals derived from sample stability data as described by Timm *et al.* (Ref. 116)

3.6.1 Experimental

3.6.1.1 Sample Preparation

Ex-vivo serum and urine samples were obtained from a healthy volunteer after the oral administration of 1 gm of josamycin as an aqueous suspension. The volunteer had abstained from taking any medication during the previous two weeks and caffeine and alcohol for the previous 24 hours. Prior to administration of the dose, blank serum and urine were collected and later analysed to ensure that both fluids were free from contaminating peaks. Serum with josamycin concentrations at the upper and lower ends of the calibration range was obtained from the volunteer by withdrawing 100 ml of blood via an arm vein using 10 ml Vac-U-Test tubes (Radem Laboratory Equipment, Johannesburg, R.S.A.) at 20 min and again at 150 min after dosing. The whole blood was allowed to clot for 30 min after which time the tubes were centrifuged at 1000 x g for 20 min. The serum within each set of 10 tubes was then harvested and pooled. Urine was voided every two hours post dose and collected at the end of the 0 - 2 and 4 - 6 hour periods (upper and lower concentrations respectively). Five replicate aliquots of each *ex-vivo* sample (two serum and two urine) were then pipetted into individual test-tubes and analysed immediately for 0 time concentrations. Four individual 6 ml aliquots of each solution were then pipetted into test-tubes for storage under the appropriate conditions.

In-vitro serum and urine samples were prepared from pooled blank non-dialysed serum and urine which had been confirmed free from interfering contaminants. An aqueous stock solution was prepared by accurately weighing 10 mg of josamycin into a 100 ml volumetric flask, dissolving in 2 ml of acetonitrile and making up to volume with water. A serum solution containing about 2 μg/ml of josamycin was prepared by pipetting 1 ml of the stock solution into a 50 ml volumetric flask and making up to volume with serum. A second solution containing about 0.24 μg/ml of josamycin was prepared by pipetting 6 ml of this solution into a 50 ml volumetric flask and making up to volume with serum. A urine solution containing about 10 μg/ml of josamycin was prepared by pipetting 5 ml of the aqueous stock solution into a 50 ml volumetric flask and making up to volume with urine. A second solution containing about 2 μg/ml of josamycin was prepared by pipetting 10 ml of this solution into a 50 ml volumetric flask and making up to volume with urine. Five replicate 1-ml samples of each *in-vitro* solution (two serum and two urine) were pipetted into individual test-tubes and analysed immediately for 0 time concentrations. Four individual 6 ml aliquots of each solution were then pipetted into test-tubes for storage under the appropriate conditions.

3.6.1.2 **Storage Conditions and Analysis of Samples**

Samples were stored under conditions as tabulated in table 3.8. Storage conditions were designed to assess the stability of serum and urine samples during both long term storage at -15 °C and short term storage in a refrigerator at +4 °C.

Replicate serum and urine samples (n=5) were analysed as in 3.3.1.6 and 3.5.1 respectively immediately after preparation (zero time) and then after each of the stated storage intervals. Concentrations were interpolated from a calibration curve constructed on each day of analysis from freshly prepared standards.

3.6.2 **Results and Discussion**

Confidence intervals were calculated using the formulae published by Timm *et al.* (116). However, instead of calculating confidence intervals using the measured percentage response difference between stored and freshly prepared standards, confidence intervals were calculated using the measured percentage concentration difference between zero time concentrations and concentrations of stored samples determined from a freshly prepared calibration curve. Confidence intervals for the percentage change in concentration after storage from the initial concentration were then represented diagramatically.

3.6.2.1 Serum

Results from stability studies of josamycin in *ex-vivo* and *in-vitro* serum samples are depicted in figures 3.16 and 3.17 respectively. For *ex-vivo* and *in-vitro* samples at both concentrations, either a slight increase, a not significant and not

Figure 3.16

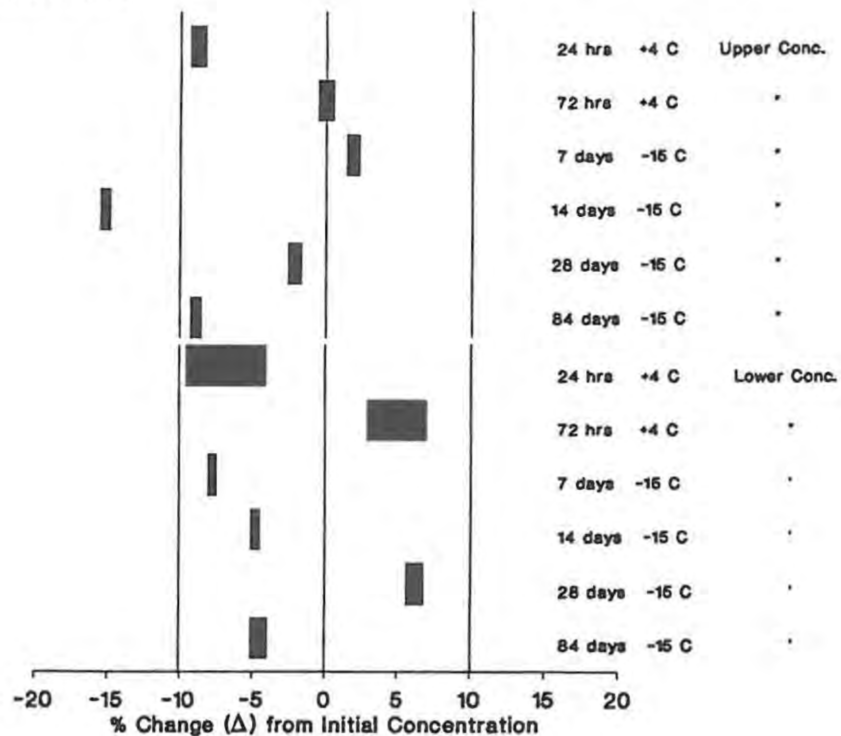


Figure 3.17

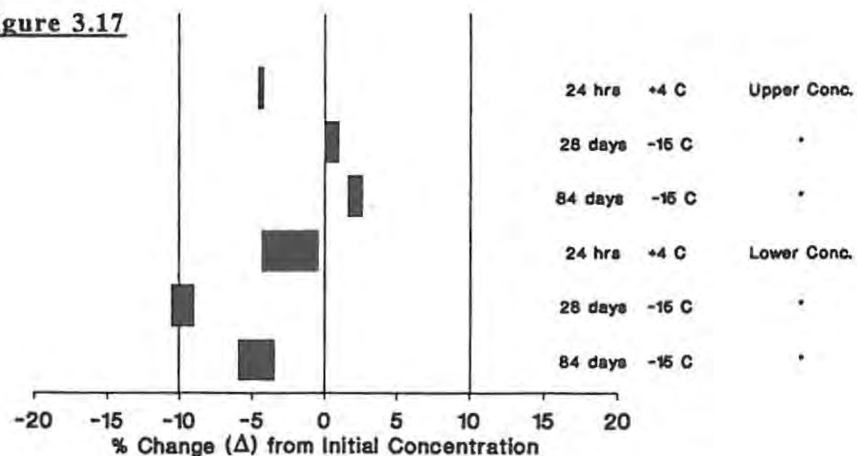


Figure 3.16 Stability of josamycin at two different concentrations in *ex-vivo* serum samples stored at +4°C and -15°C. Time zero concentrations = 0.56 μg/ml and 0.25 μg/ml.

Figure 3.17 Stability of josamycin in *in-vitro* serum samples spiked to 1.88 μg/ml and 0.23 μg/ml and stored at +4°c and -15°c.

relevant change, or a significant but not relevant decrease in concentration was detected after storage under all but two of the conditions investigated. *Ex-vivo* samples stored at -15°C for 14 days showed a significant and relevant change in concentration, and *in-vitro* samples stored at -15°C for 28 days showed a significant and possibly relevant change in concentration. However, subsequent analysis after extended storage confirmed that no relevant change had occurred. This may have been due to slightly inaccurate analysis of samples on that particular day. Josamycin was found to be equally stable in *ex-vivo* and *in-vitro* samples, and no pharmacokinetically relevant degradation occurred in either medium after storage at 4°C for 24 and 72 hours respectively, or after storage at -15°C for 84 days. Serum samples from bioavailability studies can therefore stored in a refrigerator for 72 hours after phlebotomy whilst awaiting analysis. However, if analysis within this period is not possible, samples can be stored at -15°C for up to 84 days with confidence. *In-vitro* standards and control samples can be stored for 24 hours in a refrigerator whilst awaiting anlaysis, and can be prepared up to 84 days in advance of their analysis if stored at -15°C.

3.6.2.2 **Urine**

Results from stability studies of josamycin in *ex-vivo* and *in-vitro* urine samples are depicted in figures 3.18 and 3.19 respectively. *Ex-vivo* urine samples collected during the two different time intervals were found to contain josamycin in equal concentrations. Results for only one set of samples are therefore depicted. The stability of josamycin in urine was in sharp contrast with that in serum and a relevant decrease in concentration was detected in *ex-vivo* samples after storage at -15°C for 28, and in *in-vitro* samples after storage at -15°C for 84 days. No relevant decrease was detected in *ex-vivo* or *in-vitro* samples stored at +4°C over the storage periods investigated. Consideration of the stability of josamycin in urine samples is therefore important if samples are to be analysed after a period of storage. *In-vitro* standards and controls can be prepared up to 28 days in advance of their analysis and can be stored at +4°C for 24 hours prior to analysis. *Ex-vivo* samples can be stored for 72 hours at +4°C and should be analysed in less that 28 days after storage at -15°C if storage is necessary. However, a more detailed study is required to determine the precise time for which urine samples can be stored at -15°C.

Figure 3.18

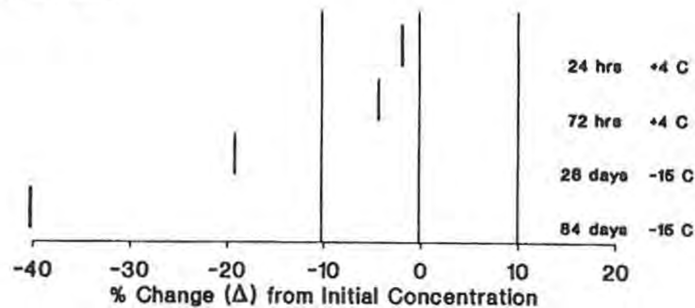


Figure 3.19

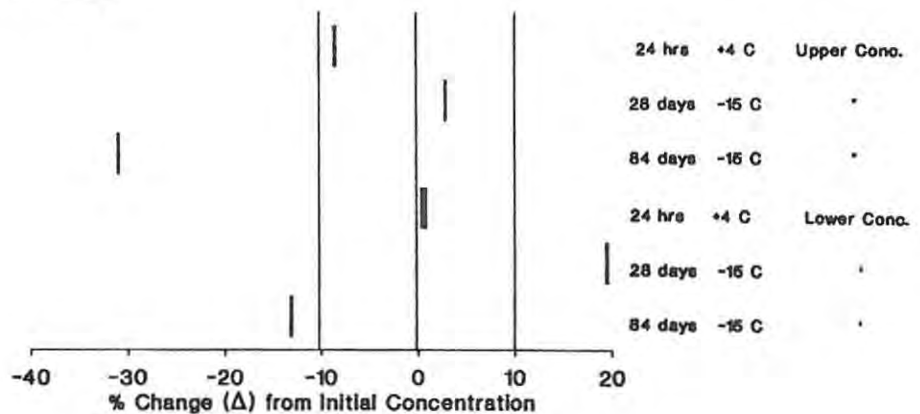


Figure 3.18 Stability of josamycin in *ex-vivo* urine samples stored at +4°C and -15°C. Time zero concentration = 9.62 μg/ml.
Figure 3.19 Stability of josamycin in *in-vitro* urine samples spiked to 9.47 μg/ml and 1.88 μg/ml and stored at +4°C and -15°C.

3.7 RE-USABILITY OF EXTRACTION COLUMNS

SPE columns are generally considered disposable and for single use only. However, the large number of assays required for pharmacokinetic studies and the increasing cost of SPE columns made it worthwhile to consider their re-use in our laboratory. Although solid-phase extraction techniques are becoming increasingly popular for the extraction of compounds from biological matrices, the use and re-use of SPE columns is not without pitfalls. Some assays have shown inconsistent sample recovery between different batches of extraction columns (117,118) while others appear to be unaffected (119). Differences in recoveries between SPE columns containing C_{18} packing material from different manufacturers have also been observed.

Ruane and Wilson (88) noted this during the extraction of a beta-blocking agent from serum, however, this was not observed during the extraction of erythromycin base from serum using extraction columns from the same two manufacturers (120). These differences may be due to variations in the volume of packing material in the SPE columns (119) or to variations in the extent of C_{18} coverage, end-capping and silanol deactivation of the silica support between batches and manufacturers (118). Changes in the packing material can therefore have a profound effect on the retention of a particular analyte. Endogenous serum components have been shown to adversely affect the retention of analytes on C_{18} reversed-phase material (110 and section 3.4.5.3), possibly by binding to and modifying the nature of the solid-phase surface. It is therefore reasonable to expect that these compounds may build up on SPE columns after each successive re-use and subsequently have an increasingly deliterious effect on the extraction procedure. The successful re-use of SPE columns for the extraction of cyclosporin from serum has previously been reported (121). However, as the retention characteristics of each compound are highly specific, the re-use of extraction columns must be validated for each extraction procedure before implementation of routine re-use. A feasibility study on the re-use of SPE columns for the extraction of josamycin from serum and urine was subsequently conducted.

3.7.1 Experimental

3.7.1.1 Preparation and Analysis of Samples

Two solutions containing different concentrations of josamycin were prepared with non-dialysed serum and urine as detailed for *in-vitro* samples in 3.6.1.1.

Extraction columns were re-used for four successive extractions using one set of 10 columns for serum and a second set of 10 for urine. Five replicate samples at each concentration were extracted each time for both serum and urine, and columns and samples within each set of ten were randomised before each extraction. Serum and urine samples were analysed as in 3.3.1.6 and 3.5.1 respectively. Concentrations were calculated from a calibration line constructed from standards extracted together with the first set of extractions.

3.7.1.2 Statistical Treatment of Results

The approach and equations presented by Timm *et al.* (116) for the interpretation of stability data was adopted for the analysis of results obtained in this study. Concentrations obtained after the 2nd, 3rd and 4th extractions were compared to those obtained in the first extraction, and 90% confidence intervals for the true observed difference in concentration between the first and each subsequent set of

extractions constructed. Decisions on the relevance of any changes observed were made according to the guidelines suggested by Timm.

3.7.2 Results and Discussion

Results of SPE column re-useability studies for serum and urine are depicted in figures 3.20 and 3.21 respectively. At the lower serum concentration, a not significant but possibly relevant change for the first re-use, and a significant and relevant change for the second and third re-use was observed. At the higher serum concentration, a significant but not relevant change for the first re-use, and a

Figure 3.20

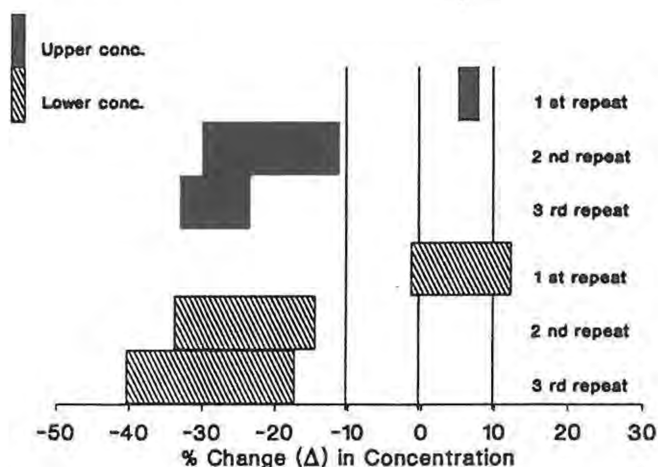


Figure 3.21

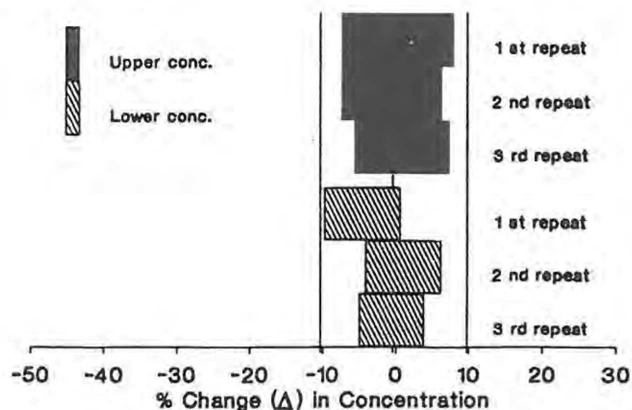


Figure 3.20 Re-usability of C_{18} SPE columns for the extraction of josamycin from serum. Stock solutions of non-dialysed serum were spiked to concentrations of 1.82 μg/ml and 0.30 μg/ml.
Figure 3.21 Re-usabiltiy of C_{18} SPE columns for the extraction of josamycin from urine. Stock solutions of urine were spiked to concentrations of 9.09 μg/ml and 1.82 μg/ml.

significant and relevant change for the second and third re-use was observed. At both the lower and upper urine concentrations, not significant and not relevant changes were observed for all three repeat extractions.

These data indicate that SPE columns could be confidently used twice for the extraction of josamycin from serum and up to four times for the extraction of josamycin from urine. Re-use of SPE columns for the extraction of josamycin from urine had no effect on the precision and accuracy of the assay even after a third re-use. Columns could well be re-used a greater number of times, however further validation is necessary before such extensive re-use can be utilised.

An interesting observation is that loss of josamycin from the extraction columns during the extraction of serum samples increased successively after the first re-use, however, no such loss during the extraction of urine samples was observed. As mentioned earlier, re-use of SPE columns may be limited by the build up of endogenous compounds acting as silanol blocking agents, and the difference in their re-usability between serum and urine could be due to the presence of these compounds in serum but not in urine. This is in agreement with the greater precision of urine determinations using the procedure developed for the extraction of josamycin from serum.

In conclusion, SPE columns could be successfully re-used for the extraction of josamycin from serum and urine. However, the nature of the sample matrix significantly affected their re-usability and validation of their re-use for each specific sample matrix was essential.

3.8 SELECTIVITY OF THE ASSAY

Josamycin is extensively metabolised in the liver, predominantly to 15-hydroxy-josamycin ($JM-O_1$) and to a lesser extent to ß'-hydroxy-josamycin ($JM-O_2$) (Figure 1.1). Fourtillan *et al.* (47) was able to quantitate $JM-O_1$ in serum and found that concentrations were considerable and over twice those of josamycin in samples obtained from a human volunteer after the administration of 1 g of josamycin. As metabolic changes to josamycin do not interfere with the diene chromophore of the lactone ring responsible for UV absorbance at 231 nm, their UV absorbance spectra are very similar to that of the parent compound (71). Chromatographic separation of josamycin from its metabolites is therefore vital in order to accurately quantitate unchanged drug by HPLC with UV detection.

3.8.1 Confirmation of Assay Selectivity

Chromatograms of serum samples collected after the administration of 1 g of josamycin to healthy male volunteers showed the appearance of an unknown peak at *ca.* 4 mins (Figure 3.6), whilst chromatograms of urine samples collected from the same volunteers show the appearance of two extra peaks at *ca.* 3 mins and 4 mins (Figure 3.13). Both peaks were absent from chromatographs of samples taken before administration of the dose and when present, eluted before josamycin as would be expected from more polar metabolites. Identification of these peaks by comparing retention times of metabolite standards with those of the unknown peaks was not possible as pure samples of metabolites were not available. However, UV spectra of the unknown peaks were obtained with the use of an HP 1040 diode array detector connected in-line with the chromatographic system. Three dimensional plots of wavelength *vs.* retention time *vs.* absorbance indicate the separation of two and three compounds in serum and urine extracts respectively (Figures 3.22 and 3.23).

Spectra obtained at the apex of the unknown peaks eluting at 3 mins and 4 mins were almost superimposable on that of josamycin (Figure 3.24). The two compounds eluting at these times were thus probably identified as JM-O_2 and JM-O_1 respectively. This indicates that josamycin was adequately separated from its major and minor metabolites and that the assay procedures described in this chapter are suitable for the selective quantitative analysis of josamycin in serum and urine.

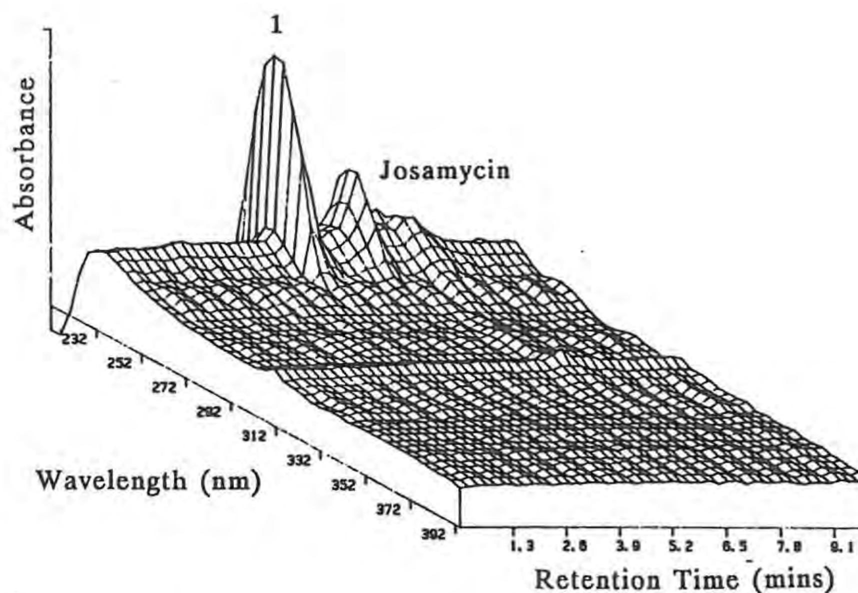


Figure 3.22 Three-dimensional plot of absorbance *vs.* wavelength *vs.* retention time for a serum extract obtained after the administration of 1 g of josamycin to a healthy male volunteer, showing separation of JM-O_1 (1) eluting after 4 min, from josamycin.

Figure 3.23

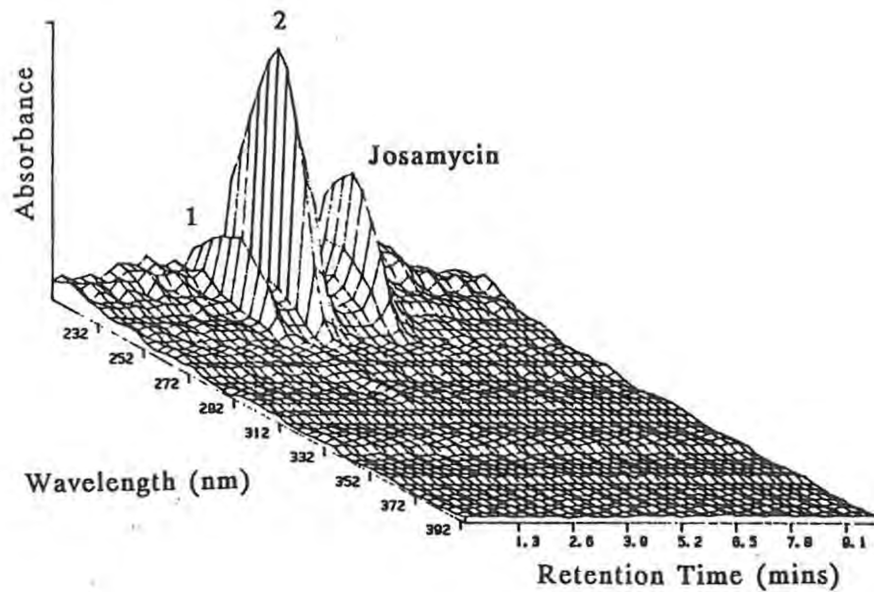


Figure 3.24

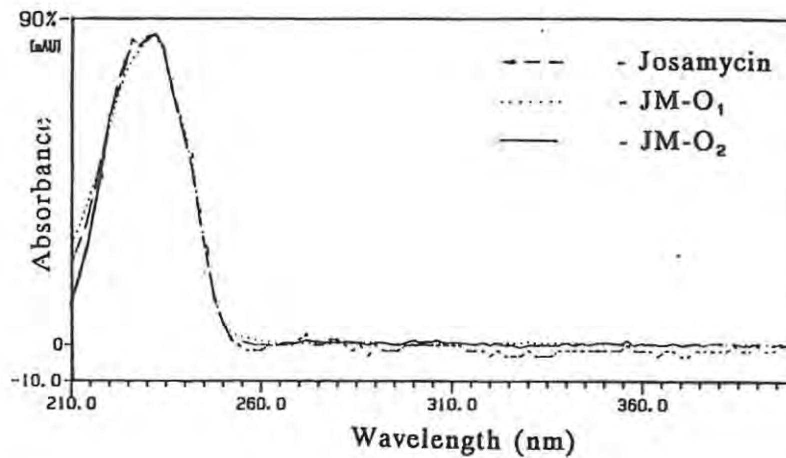


Figure 3.23 Three-dimensional plot of absorbance *vs.* wavelength *vs.* retention time for a urine extract obtained after the administration of 1 g of josamycin to a healthy male volunteer showing separation of JM-O_2 (1), eluting after 3 min, and JM-O_1 (2), eluting after 4 min, from josamycin.
Figure 3.24 UV absorbance spectra obtained at the apex of peaks eluting at 3, 4 and 6 mins after injection of an extract of a urine sample, using an HP1040 diode array detector.

Table 3.1 Elution of Josamycin from C_{18} SPE Columns by Various Volumes of Acetonitrile followed by Elution by an Acetonitrile-Buffer Mixture (On-column load = 2 μg).

	Volume of Acetonitrile used for Elution (mls)					
	0.10	0.25	0.50	1.00	1.50	2.00
Mean % of Load Recovered (n=2 columns) in Acetonitrile	0	0	0	0	0	0
Mean % of Load Recovered (n=2 columns) in Elution Mixture*	108	95	100	85	89	98

* Elution mixture = 3 x 500 μl acetonitrile-phosphate buffer 0.05 M, pH 5.8, 3:2.

Table 3.2 Elution Profile of Josamycin from C_{18} SPE Columns by Consecutive Aliquots of Elution Mixture for Various Acetonitrile-Buffer Mixtures.

Composition of Elution Mixture *		Aliquot of Elution Mixture 1 st	2 nd	3 rd	4 th
	Volume of Aliquot (ml) Cumulative Volume (ml)	1.0 1.0	0.5 1.5	0.5 2.0	0.5 2.5
50:50	% of Load Eluted Cumulative % Eluted	15 15	47 62	30 92	8 100
55:45	% of Load Eluted Cumulative % Eluted	51 51	41 92	8 100	0 100
60:40	% of Load Eluted Cumulative % Eluted	88 88	12 100	0 100	0 100
65:35	% of Load Eluted Cumulative % Eluted	96 96	4 100	0 100	0 100

* acetonitrile-phosphate buffer, 0.05 M, pH 5.8.

Table 3.3 Partitioning of Josamycin into the Acetonitrile Layer formed after the Reconstitution of Residues which Remained after 1.5 ml Aliquots of Various Elution Mixtures were Evaporated to Dryess.

Elution Mixture Composition Acetonitrile-Buffer *	7:3	3:2	1:1
% of Total Josamycin in Acetonitrile Layer **	83	87	89

* phosphate buffer 0.05 M, pH 5.8.
** samples reconstituted with 20 μl water and 50 μl acetonitrile.

Table 3.4 Calibration, Precision and Accuracy Data for the Extraction of Josamycin from Non-Dialysed Serum and Urine.

	Calibration Standards		Accuracy Standards		
	Concentration (µg/ml)	Peak Height Ratio * Mean ±R.S.D. %	Spiked Concentration (µg/ml)	Concentration Found Mean ±R.S.D. %	Percentage Difference**
Serum ***	0.10	0.098 4.10 (n=5)	0.303	0.270 3.27 (n=5)	-10.98
	0.30	0.320 9.09 (n=3)	1.818	1.776 5.64 (n=5)	-2.31
	0.60	0.630 8.33 (n=3)			
	1.00	1.020 7.02 (n=5)			
	1.50	1.570 4.53 (n=3)			
	2.00	2.190 4.10 (n=5)			
Urine ****	0.25 (n=6)	0.072 6.20 (n=6)	1.488	1.433 3.14 (n=3)	-3.70
	0.50 (n=6)	0.151 4.50 (n=6)	4.540	4.960 3.47 (n=3)	10.22
	1.00 (n=6)	0.300 5.00 (n=6)			
	2.49 (n=6)	0.770 5.75 (n=6)			
	4.99 (n=6)	1.561 3.48 (n=6)			

* Peak height ratio of josamycin/internal standard.
** Difference between concentration found and spiked concentration as a percentage of the spiked concentration.
*** Linear regression equation :- y = 1.0855x - 0.0237, R.Sq. = 0.9983
**** Linear regression equation :- y = 0.3142x - 0.0073, R.Sq. = 1.0000

Table 3.5 Percentage Recovery Data after the Extraction of Josamycin from Non-Dialysed Serum and Urine.

	Spiked Sample Concentration (µg/ml)	Reference Samples PHR* ±R.S.D. %	Extracted Samples PHR* ±R.S.D. %	Percentage Recovery
Serum	2.00	1.565 2.42 (n=5)	1.208 4.73 (n=3)	77 %
	0.25	1.170 5.64 (n=5)	0.850 6.85 (n=3)	71 %
Urine	5.00	3.345 0.76 (n=4)	2.513 5.25 (n=6)	76 %
	0.25	0.243 5.48 (n=4)	0.195 8.69 (n=6)	80 %

* Peak height ratio of Josamycin/Internal Standard.

Table 3.6 Calibration, Precision and Percentage Recovery Data for the Extraction of Josamycin from Dialysed Serum.

	Calibration and Precision Data		Percentage Recovery			
	Concentration of Standard (µg/ml)	Peak Height Ratio * Mean ±R.S.D.%	Spiked Sample Concentration (µg/ml)	Reference Samples Peak Height Ratio * Mean ±R.S.D. %	Extracted Samples Peak Height Ratio * Mean ±R.S.D. %	Recovery
Upper Range **	0.240	0.135 2.13 (n=6)	0.100	0.259 3.63 (n=5)	0.154 8.20 (n=5)	60 %
	0.480	0.316 4.16 (n=6)	0.500	0.210 4.25 (n=5)	0.146 8.24 (n=5)	70 %
	0.960	0.674 2.19 (n=6)	5.000	3.390 4.60 (n=5)	1.860 4.64 (n=5)	78 %
	2.390	1.701 2.91 (n=6)				
	4.780	3.720 2.06 (n=6)				
Lower Range ***	0.048	0.095 5.99 (n=5)				
	0.096	0.215 1.36 (n=5)				
	0.240	0.583 2.52 (n=5)				
	0.480	1.261 3.81 (n=5)				
	0.960	2.543 0.85 (n=5)				

* Peak height ratio of josamycin/internal standard.
** Linear regression equation :- y = 0.7863x - 0.0826, R.Sq. = 0.9986, detector attenuation = 0.04 a.u.f.s. at 231 nm.
*** Linear regression equation :- y = 2.6670x - 0.0409, R.Sq. = 0.9998, detector attenuation = 0.01 a.u.f.s. at 231 nm.

Table 3.7 Clinical Laboratory Report for Serum Before and After Dialysis.

	Before Dialysis	After Dialysis
Sodium	138.0 mmol/l	152.0 mmol/l
Potassium	5.1 mmol/l	0.0 mmol/l
Chloride	101.0 mmol/l	114.0 mmol/l
Urea	4.4 mmol/l	0.0 mmol/l
Creatinine	93.0 μmol/l	0.0 μmol/l
Phosphorus	1.5 mmol/l	0.03 mmol/l
Urate	0.39 mmol/l	0.02 mmol/l
Serum Albumin	43.0 g/l	39.0 g/l
Alkaline Phosphatase	45.0 U/l	34.0 U/l
Aspartate Transaminase	23.0 U/l	20.0 U/l
Alanine Transaminase	12.0 U/l	7.0 U/l
Glutamyl Transferase	22.0 U/l	18.0 U/l

Table 3.8 Storage Conditions for Stability Studies to Determine the Stability of Josamycin in *Ex-Vivo* and *In-Vitro* Serum and Urine Samples.

	Storage Times			
Storage Temperature	Serum		Urine	
	Ex-Vivo	*In-Vitro*	*Ex-Vivo*	*In-Vitro*
ambient	0 hrs	0 hrs	0 hrs	0 hrs
+4°C	24 hrs	24 hrs	24 hrs	24 hrs
+4°C	72 hrs	72 hrs	72 hrs	----
-15°C	7 days	7 days	----	----
-15°C	14 days	14 days	----	----
-15°C	28 days	28 days	28 days	28 days
-15°C	84 days	84 days	84 days	84 days

CHAPTER 4

DISSOLUTION AND SOLUBILITY STUDIES

4.1 INTRINSIC DISSOLUTION RATE STUDIES

Dissolution may be described as the process of dissolving, where molecules in the solid phase leave the solid surface to form a solution in the dissolution medium. Solubility, however, refers to the extent to which dissolution can occur before saturation is reached.

Gastro-intestinal absorption of drugs requires that the drug molecules are in solution and dissolution is therefore a pre-requisite for drug absorption when a drug is administered in the solid state. It follows then that the rate at which drug molecules dissolve can determine the rate of absorption if the dissolution rate is slow in comparison with the absorption rate. The importance and influence of dissolution rate on the bioavailability of drugs is now well recognised and has been reviewed by Wagner (122) and investigated by others (123-125).

The first investigator to describe the dissolution process mathematically was Fick (126), who derived the expression:

$$\frac{dW}{dt} = -DA\frac{dC}{dx} \qquad \text{Eq. 4.1}$$

which describes the rate (dW/dt) at which a solute diffuses from an area (A) perpendicular to the plane of a concentration gradient (dC/dx) with mixing by molecular agitation only (W = amount dissolved, t = time, A = area, C = concentration, x = distance perpendicular to the plane of A, and D is the diffusion coefficient). Noyes and Whitney (127) later derived the expression:

$$\frac{dC}{dt} = k(C_s - C) \qquad \text{Eq. 4.2}$$

to describe the rate of dissolution from a constant surface area utilising rotating cylinders of benzoic acid and lead chloride, and considered the bulk of the solution to be of uniform concentration, where dC/dt = rate of change of concentration, C is the concentration of the bulk solution at time t, C_s is the solubility and k is a constant. They assumed that a layer of saturated solution formed adjacent to the solid surface and that the rate of dissolution was controlled by the rate at which molecules diffused from this layer into the bulk solution. Furthermore, they showed that the rate of dissolution was directly proportional to the difference in concentration between a

saturated solution and the bulk solution. The equation was subsequently modified to the form (128):

$$\frac{dC}{dt} = kS(C_s - C) \qquad \text{Eq. 4.3}$$

to include the surface area S from which dissolution proceeds. Nernst (129) and Brünner (130) later applied Fick's theory of dissolution to show that k in equation 4.3 was a function of the diffusion coefficient (D) of the solute as defined by Fick, the area (A) from which dissolution proceeds, the solution volume (V) and the diffusion layer thickness (h) such that k = DA/Vh. They proposed that the saturated layer advocated by Noyes and Whitney actually consisted of a stagnant layer, across which a concentration gradient was set up (Figure 4.1). Solvated molecules in equilibrium with the solid surface diffuse across the concentration gradient to a distance (h) from the solid surface, beyond which there is uniform mixing. This relationship resulted in the well known modern form of the Noyes-Whitney equation where:

$$\frac{dW}{dt} = \frac{D}{h}S(C_s - C) \qquad \text{Eq. 4.4}$$

and presupposes that the rate of dissolution is a diffusion controlled process.

Equation 4.4 shows that as the concentration (C) in the bulk solution increases, the rate of dissolution dW/dt decreases. However, under sink conditions C is negligible in comparison to C_s and Nelson (131) showed that equation 4.4 could be reduced to:

$$\frac{dW}{dt} = KSC_s \qquad \text{Eq. 4.5}$$

which after integration yields:

$$W = KSC_s t \qquad \text{Eq. 4.6}$$

The slope (KC_s) of a plot of W/S *vs.* t will therefore yield the initial dissolution rate (G) where W/S = Gt (W = mg, S = cm^2, t = s and G = $mg.cm^{-2}.s^{-1}$) *i.e.* the rate of dissolution uninfluenced by dissolved solute - also referred to as the intrinsic dissolution rate. G can be described as the initial amount of solid dissolving from a uniform surface composed of pure drug of 1 unit area, in 1 unit time into fresh solvent, and is a measure of the rate at which the solute can dissolve under sink conditions in the specified dissolution medium.

The intrinsic dissolution rates of numerous compounds have been determined utilising a number of experimental conditions (Table 4.1). The majority of

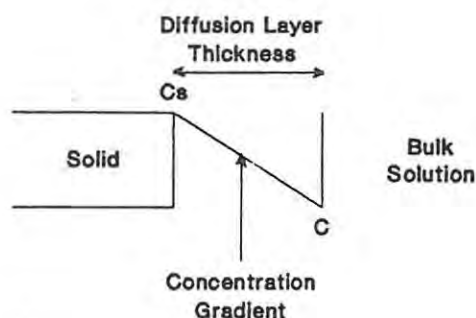


Figure 4.1 Schematic representation of the diffusion or stagnant layer model proposed by Nernst and Brünner, where Cs is the concentration of the saturated solution adjacent to the solid surface and C is the concentration of the bulk solution.

Table 4.1 Experimental Conditions Employed by Various Investigators for Intrinsic Dissolution Rate Determinations.

Author	Date	Equation Used	Rotation Speed (rpm)	Disc Mounting	Ref.
Nelson E.	1957	N & W [1]	500	H C [5]	123
Nelson E.	1958	N & W [1]	---	V Suspended [6]	124
Nelson E.	1958	N & W [1]	500	H C [5]	237
Levy G. and Sahli B.	1962	N & W [1]	550	H C [5]	125
Levy G. and Tanski W.	1964	N & W [1]	3.0-400	H C [5]	238
Wood J.H. *et al.*	1965	N & W [1]	430	H C [5]	132
Kaplan S.A.	1974	N & W [1]		H C [5]	144
Nelson K.G. and Shah A.C.	1975	N & W [1]	200-400	H C [5], V C [7]	133
Shah A.C. and Nelson K.G.	1975	Levich [2]	V F R [4]	Flow Through [8]	239
Tsuji A. *et al.*	1978	Levich [2]	228	H C [5]	240
Nicklasson M. *et al.*	1981	N & W [1]	∞	H C [5]	135
Nicklasson M. and Brodin A.	1982	N & W [1]	∞	H C [5], V E [9]	137
Nicklasson *et al.*	1983	M T [3]	∞	V E [9]	138
Nicklasson *et al.*	1985	Levich [2]	∞	H E [10]	141
Nicklasson M. and Magnusson A-B.	1985	Levich [2]	∞	H C [5], H E [10]	142
Graffner *et al.*	1985	N & W [1]	∞	V E [9]	241
Nicklasson M. *et al.*	1988	N & W [1]	∞	H C [5], H E [10]	143
Farraj N.F. *et al.*	1989	N & W [1]	102	H Stationary [11]	242
Shah J.C. *et.al*	1989	N & W [1]	100	H C [5]	154

[1] Noyes and Whitney equation ($dW/dt = Dh.S.(C_s - C)$).
[2] Levich equation ($G = 0.61.D^{2/3}.\nu^{-1/6}.C_s.\omega^{1/2}$).
[3] Mass transfer.
[4] Variable flow rate.
[5] Horizontal centric mounting.
[6] Vertically suspended.
[7] Vertical centric mounting.
[8] Flow through cell, stationary disc.
[9] Vertical excentric mounting.
[10] Horizontal excentric mounting.
[11] Horizontal stationary mounting.

investigators have used a centrically mounted disc composed of pure drug compressed into a flat surfaced pellet and mounted so as to present a constant surface area to the dissolution medium, *e.g.* Woods apparatus (132), whilst others have used an excentrically mounted disc. Discs have been rotated at a variety of rotation speeds

and G calculated utilising equation 4.6 at a single rotation speed. However, G is influenced by the degree of agitation of the bulk solution (133), or the rotation speed of a rotating disc, as described by the Nernst-Brünner film theory (129,130). This theory states that the thickness of the film or diffusion layer of a rotating disc is inversely proportional to the rotation speed under laminar flow conditions, and diffusion across the diffusion layer will become more rapid as the thickness of this layer decreases. This theory was investigated further by Levich (134) who derived the well known Levich equation (Equation 4.7)

$$G = 0.62.\ D^{\frac{2}{3}}.\ \nu^{-\frac{1}{6}}.\ C_s.\ \omega^{\frac{1}{2}} \qquad \text{Eq. 4.7}$$

where (G) = intrinsic dissolution rate ($mg.cm^{-2}.s^{-1}$), (D) = diffusion coefficient ($cm^2.s^{-1}$), (ν) = kinematic viscosity ($cm^2.s^{-1}$), C_s = solubility in the relevant dissolution medium ($mg.cm^{-3}$) and (ω) = angular velocity ($rad.s^{-1}$). This equation shows that the intrinsic dissolution rate is directly proportional to the square root of the angular velocity or rotation speed. Levich also derived equation 4.8,

$$h = 1.612.\ D^{\frac{1}{3}}.\ \nu^{\frac{1}{6}}.\ \omega^{-\frac{1}{2}} \qquad \text{Eq. 4.8}$$

which shows that the diffusion layer thickness (h cm) is inversely proportional to the square root of the angular velocity or rotation speed. The wide diversity of experimental conditions used in the determination of intrinsic dissolution rates has made comparison of results from different authors difficult. Furthermore, comparison of intrinsic dissolution rate data obtained for compounds in various media at a single rotation speed is complicated by the uncharacterized effect of rotation speed on the diffusion layer in each instance. More meaningful comparison of intrinsic dissolution rates could be made if they were not influenced by a diffusion layer. Nicklasson and co-workers (135-143) have endeavoured to remove some of the ambiguity in determining and interpreting intrinsic dissolution rate data and have proposed a method to determine the "true" intrinsic dissolution rate - the intrinsic dissolution rate without the influence of a diffusion layer. This they accomplished by determining G at various rotation speeds using centrically or excentrically mounted discs and employed an extrapolation procedure to determine G at infinite rotation speed (G^∞), when the diffusion layer thickness is theoretically equal to zero. Both vertically and horizontally mounted discs were employed (136,137). They found that vertically mounted discs were most efficient in removing the diffusion layer although flow over the disc was found to be quite turbulent. Nicklasson derived equation 4.9 for the calculation of G^∞ utilising this system. From equation 4.9 a plot of 1/G is a linear function of $1/\omega$, and extrapolation to $1/\omega = 0$ will yield $1/G^\infty$ ($1/G^\infty = 1/k_1$ for $\omega \rightarrow$

∞, *i.e.* G approaches k_1 where k_1 is the "true" intrinsic dissolution rate and is the rate at which molecules leave the solid surface when unhindered by molecules in solution), where ω is the angular velocity (rotation speed) in rad.s^{-1} and k_2 is a constant.

$$\frac{1}{G} = \frac{1}{k_1} + \frac{k_2}{\omega} \qquad \text{Eq. 4.9}$$

The effect of the intrinsic dissolution rate of a compound on its bioavailability is well recognised. Kaplan (144) suggested that drugs exhibiting aqueous intrinsic dissolution rates lower than 0.016 mg.cm^{-2}.s^{-1} when utilising Woods apparatus at 50 rpm may exhibit dissolution rate-limited absorption, whilst drugs with aqueous intrinsic dissolution rates lower than 0.0016 mg.cm^{-2}.s^{-1} are highly likely to do so. Whilst these limits have been shown to be useful, prediction of the absorption characteristics of a compound based on the intrinsic dissolution rate in a single dissolution medium is likely to be inaccurate as the intrinsic dissolution rate can be markedly influenced by the pH of the dissolution medium. More accurate predictions and more meaningful explanations of *in-vivo* absorption characteristics could be made if an intrinsic dissolution rate - pH profile of the compound over the gastro-intestinal pH range was obtained. Such data would also be useful for the prediction and explanation of *in-vitro* release characteristics of compounds from dosage forms in simulated gastro-intestinal fluids. Intrinsic dissolution rates of josamycin were therefore determined in dissolution media covering the gastro-intestinal pH range of pH 1.2 to pH 7.5 in order to assess the role of intrinsic dissolution rate on absorption.

4.1.1 Experimental

4.1.1.1 Dissolution Media

The USP lists only two fluids for simulation of gastric and intestinal fluids - one of pH 1.2 (145) and one of pH 7.5 (146). The pH of gastro-intestinal fluids can vary between these two pH values and to ensure adequate characterization of the intrinsic dissolution rate - pH profile of josamycin over the gastro-intestinal pH range, five additional dissolution media of intermediate pH were used. USP Simulated Gastric Fluid without pepsin (pH 1.2), McIlvaine's buffer consisting of 0.1M citric acid adjusted to pH 2.2, 3.0, 4.0, 5.0 and 6.0 with 0.2 M di-sodium hydrogen phosphate, and USP Simulated Intestinal Fluid without pancreatin (pH 7.5) were utilised as dissolution media. McIlvaine's buffer was chosen as it afforded adequate buffer capacity throughout this pH range and was easily prepared.

4.1.1.2 Disc Preparation

Discs of pure drug with a diameter of 11.5 mm and surface area of 0.95 cm^2 were prepared by compression of 200 mg of josamycin powder in a Carver Press utilising a tablet punch and die, modified to produce a flat surfaced disc. Discs for experiments designed to determine the effect of compression force on the intrinsic dissolution rate were compressed at 0.5, 1.0, 1.5, 2.0 and 3.0 tonnes pressure for 30 s. All other discs were compressed at 2.0 tonnes pressure for 30 s.

Discs for experiments conducted on centrically mounted discs were left in the die and the die itself attached to a stirring shaft and lowered into the dissolution medium (Figure 4.2).

Discs for experiments conducted on excentrically mounted discs were carefully removed from the die and coated on the underside and circumference with a water insoluble lacquer (Tipp-Ex[R] fluid) to ensure that a single flat surface only was exposed to the dissolution medium (135). The discs were then mounted vertically on a circular perspex holder 40 mm in diameter, with the surface of the disc parallel to the axis of rotation (Figure 4.3). A small portion of the circumference of the holder was flattened and the disc affixed to that area.

Figure 4.2

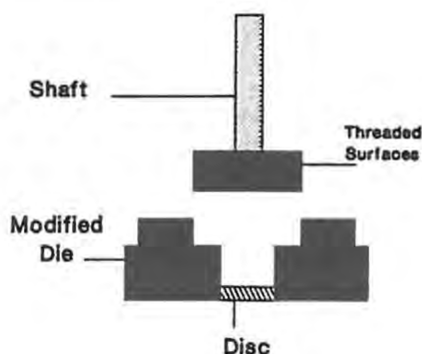


Figure 4.3

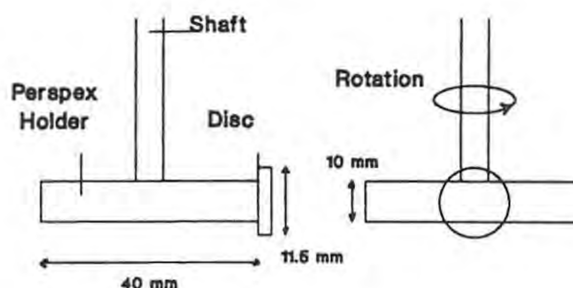


Figure 4.2 Cross-section of modified tablet die showing shaft attachment and centrically mounted disc.
Figure 4.3 Perspex holder with vertical excentrically mounted disc.

4.1.1.3 Determination of Intrinsic Dissolution Rates

After preparation and mounting of discs, the die or perspex holder was screwed onto the stirrer shaft of a Pharmatest[R] basket dissolution apparatus (Type PTW S, Hainburg, Germany) and lowered whilst rotating into 100 ml of dissolution medium such that the under surface of the die or perspex holder was 20 mm below the surface of the dissolution medium. The dissolution medium was maintained at 37°C. Sampling of the dissolution medium was carried out at relevant times over sampling

periods of 2 min at pH 1.2, 2.2, 3.0 and 4.0, of 10 min at pH 5.0 and 6.0 and of 30 min at pH 7.5. Samples were withdrawn with a 50 μl repeating pipette without replacement, and prepared for analysis as in 2.1.5. Care was taken to withdraw samples from the same point each time. Although josamycin degrades rapidly in highly acidic media (*vide infra* 5.1.2.1), no significant degradation was detected during the short sampling period of 2 min utilised at low pH's. Degradation of samples whilst awaiting analysis was prevented by adding samples to an internal standard solution prepared with a 0.2 M di-sodium hydrogen phosphate solution (see 2.2.5.2).

Experiments to determine the effect of compression force on the intrinsic dissolution rate were carried out in duplicate at each compression force in dissolution medium of pH 2.2 utilising excentrically mounted discs rotated at 50 rpm.

In order to determine whether G^{∞} should be determined using centrically or excentrically mounted discs, intrinsic dissolution rates from both mountings determined in duplicate at 100, 200 and 300 rpm in dissolution medium of pH 1.2 were compared. Thereafter, intrinsic dissolution rates were determined in duplicate using excentrically mounted discs rotated at 50, 100, 200 and 300 rpm (or angular velocities of 5.24, 10.47, 20.96 and 31.45 $rad.s^{-1}$ respectively) in dissolution media of pH 1.2, 2.2, 3.0, 4.0, 5.0, 6.0 and 7.5.

4.1.1.4 **Calculation of Intrinsic Dissolution Rates**

Intrinsic dissolution rates were calculated by linear regression analysis of plots of amount dissolved per unit area ($mg.cm^{-2}$) *vs.* time (s) utilising equation 4.6. An example of these plots is depicted in figure 4.4, which shows the amount dissolved per unit area *vs.* time for excentrically mounted discs rotated at 50, 100, 200 and 300 rpm in dissolution medium of pH 1.2.

Intrinsic dissolution rates at infinite rotation speed were determined utilising equation 4.9, where $1/k_1$ is the y-intercept ($1/\omega = 0$) of the linear regression line of a plot of $1/G$ *vs.* $1/\omega$. At infinite rotation speed, $1/\omega = 0$, therefore $k_1 = G^{\infty}$.

4.1.2 Results and Discussion

4.1.2.1 **Influence of Compression Force on Intrinsic Dissolution Rate**

Results depicted in figure 4.5 indicate that the dissolution rate decreased slightly from discs compressed at 0.5 to 1.5 tonnes pressure, and was only marginally influenced by compression force beyond 1.5 tonnes. Surface cracks were, however, observed in discs compressed at 3.0 tonnes. A compression force of 2.0 tonnes was therefore selected for all subsequent intrinsic dissolution rate determinations.

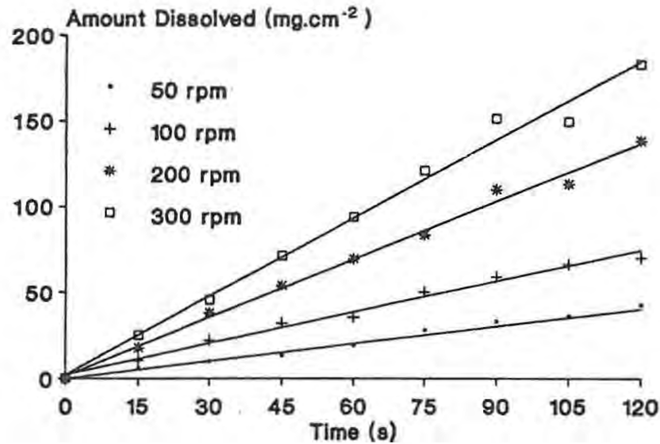


Figure 4.4 Typical plots of W/S ($mg.cm^{-2}$) *vs.* t (s) showing the linear dissolution of josamycin with time from a constant surface area (excentrically mounted discs in dissolution medium of pH 1.2).

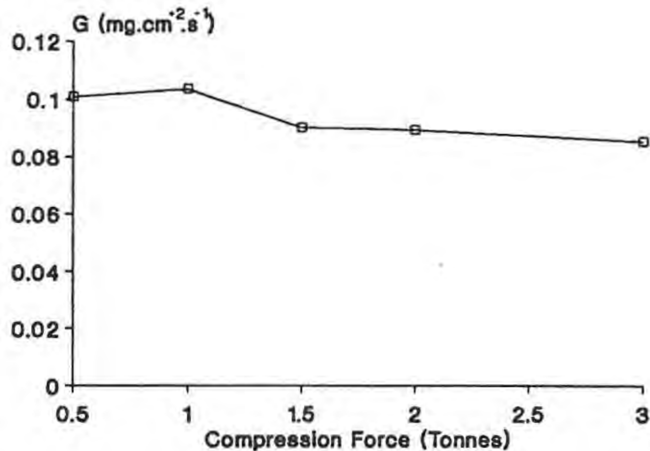


Figure 4.5 Effect of disc compression force on intrinsic dissolution rate (excentrically mounted disc, 50 rpm in dissolution medium of pH 2.2).

4.1.2.2 Comparison of Intrinsic Dissolution Rates from Centrically and Excentrically Mounted Discs

G values at each rotation speed for centrically and excentrically mounted discs in dissolution medium of pH 1.2 are tabulated in table 4.2. Data for plots of 1/G *vs.* $1/\omega$ for both mountings, and results of linear regression analysis of these plots for the determination of G^{∞} are tabulated in tables 4.3 and 4.4 respectively. Plots of 1/G *vs.* $1/\omega$ for both mountings at pH 1.2 are depicted in figure 4.6. The mean intrinsic dissolution rates (n=2) obtained with excentrically mounted discs were 4.89, 6.00,

5.98 and 12.42 times those obtained with centrically mounted discs at 100 rpm, 200 rpm, 300 rpm and at infinite rotation speed respectively. Theoretically, G^{∞} should be solely a measure of the rate at which a compound leaves the solid surface and independent of any experimental factors or variables such as the flow of dissolution media over the disc surface or diffusion layer thickness. It follows then that apparatus design should not affect the intrinsic dissolution rate at infinite rotation speed. However, extrapolation of data to infinite rotation speed for centrically and excentrically mounted discs did not yield a single G^{∞} value as may have been expected and G^{∞} for josamycin was dependent on the design of the apparatus. Furthermore, the slope of the linear regression line of a plot of $1/G$ *vs.* $1/\omega$ for centrically mounted discs was 3.91 times greater than that for excentrically mounted discs. Rotation speed therefore had a greater effect on the dissolution rate from excentrically than centrically mounted discs. This was also observed by Nicklasson during experiments on acetylsalicylic acid (136) and can be explained by the more efficient replacement of fresh dissolution medium adjacent to the disc surface of an excentrically mounted disc by better hydrodynamic flow and greater turbulence at the disc surface (137). These results indicate that the dissolution of josamycin is a diffusion controlled process and that the diffusion layer was eliminated more efficiently with excentrically mounted discs (137).

The objective of this study was to determine intrinsic dissolution rates without the influence of the diffusion layer so that data obtained in different dissolution media could be meaningfully compared. Excentrically mounted discs were therefore used for the characterization of the intrinsic dissolution rate - pH profile.

4.1.2.3 Influence of Rotation Speed on Intrinsic Dissolution Rate

Plots of $1/G$ *vs.* $1/\omega$ for excentrically mounted discs at each pH are depicted in figures 4.7 - 4.11. Data for these plots are tabulated in table 4.3 and results of linear regression analyses for the determination of G^{∞} are tabulated in table 4.4. Figures 4.7 - 4.11 show that the intrinsic dissolution rate was highly dependent on pH and rotation speed. However, G was influenced more strongly by rotation speed at low pH than at higher pH's. This is illustrated in a 3-dimensional plot of G^x/G^{50} (where G^x = G at either 50, 100, 200 or 300 rpm, and G^{50} = G at 50 rpm) *vs.* rotation speed *vs.* pH (Figure 4.12), which shows the increase in the intrinsic dissolution rate with rotation speed relative to G at 50 rpm, in each dissolution medium. For example, at pH 1.2, G at 300 rpm was 4.28 times greater than at 50 rpm whilst at pH 7.5, G at 300 rpm was only 2.63 times greater than at 50 rpm. This is also illustrated by the convergence towards higher values of the linear regression lines of plots of log G *vs.* pH at 50, 100, 200 and 300 rpm (Figure 4.13).

Figure 4.6

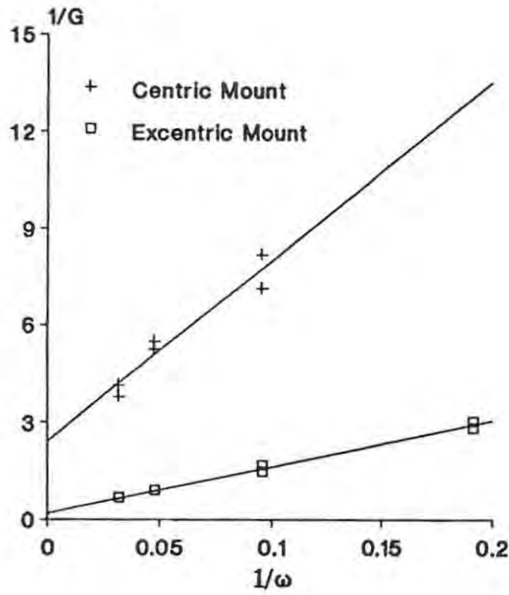


Figure 4.7

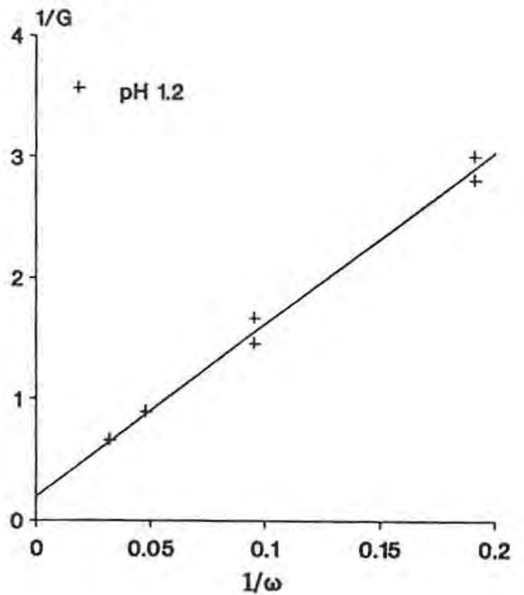


Figure 4.8

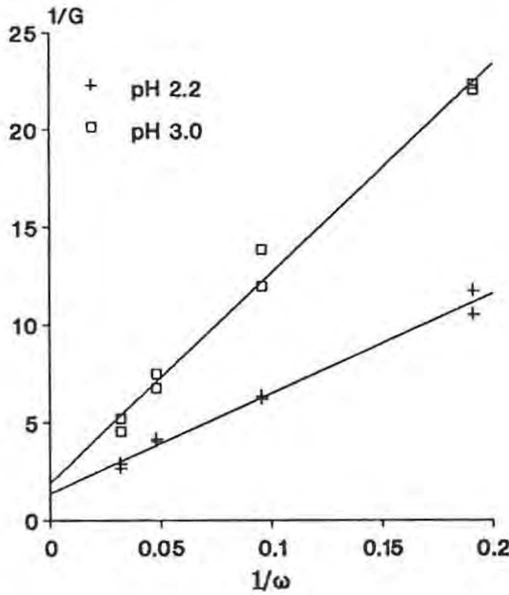


Figure 4.9

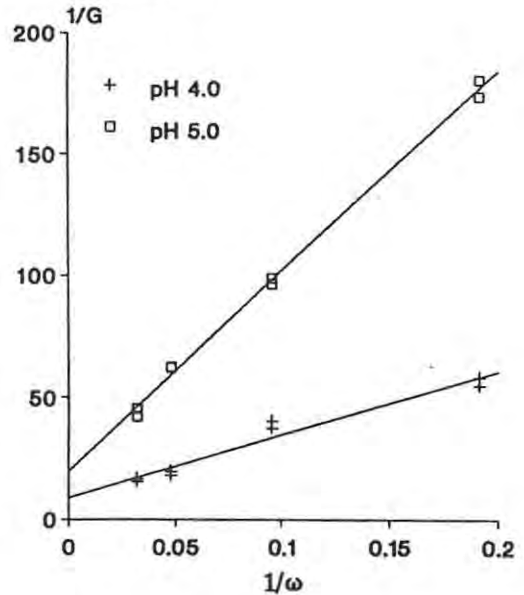


Figure 4.10

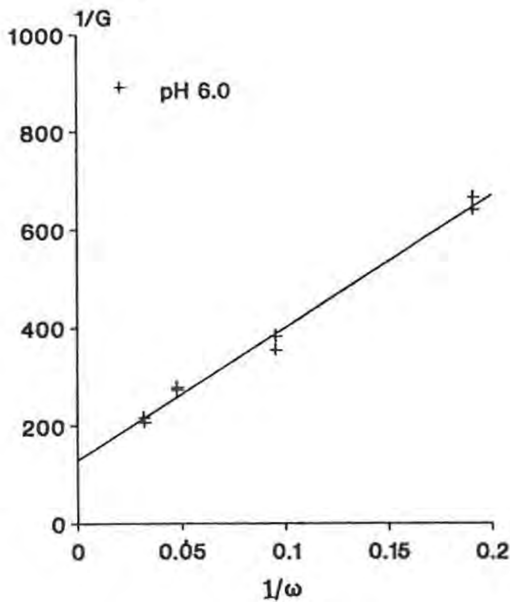


Figure 4.11

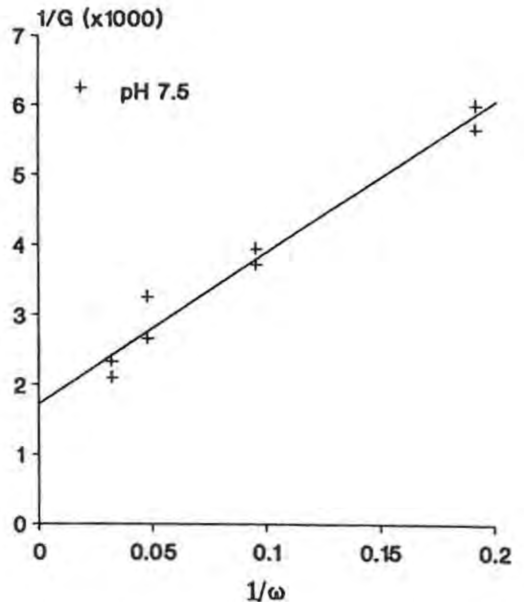


Figure 4.6 Plot of 1/G *vs.* $1/\omega$ for centrically and excentricaly mounted discs in dissolution medium of pH 1.2 showing the dependence of G on the design of the disc mounting.
Figures 4.7 - 4.11 Plots of 1/G *vs.* $1/\omega$ for excentrically mounted discs in each dissolution medium showing the linear regression line for calculation of G^{∞}.

Figure 4.12

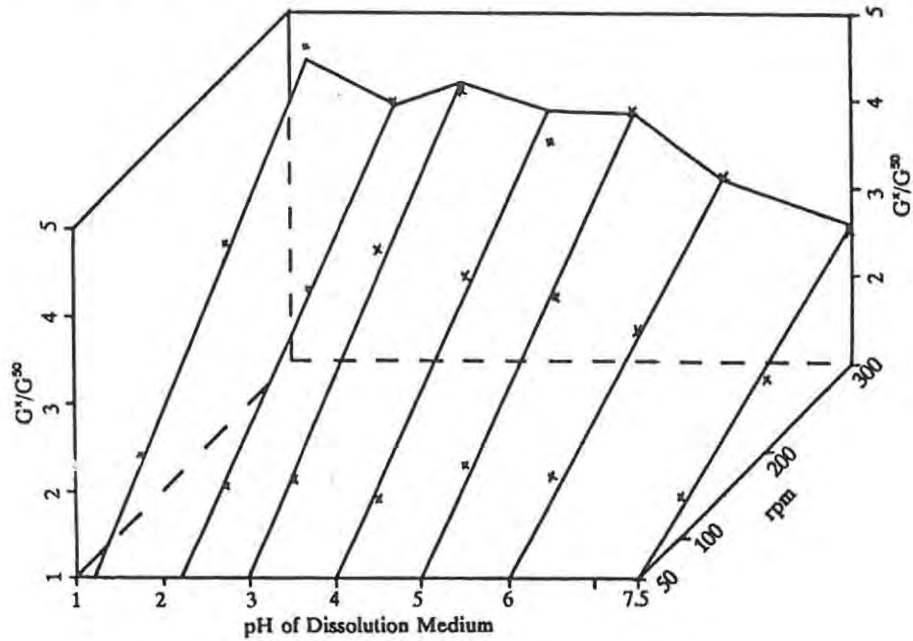


Figure 4.13

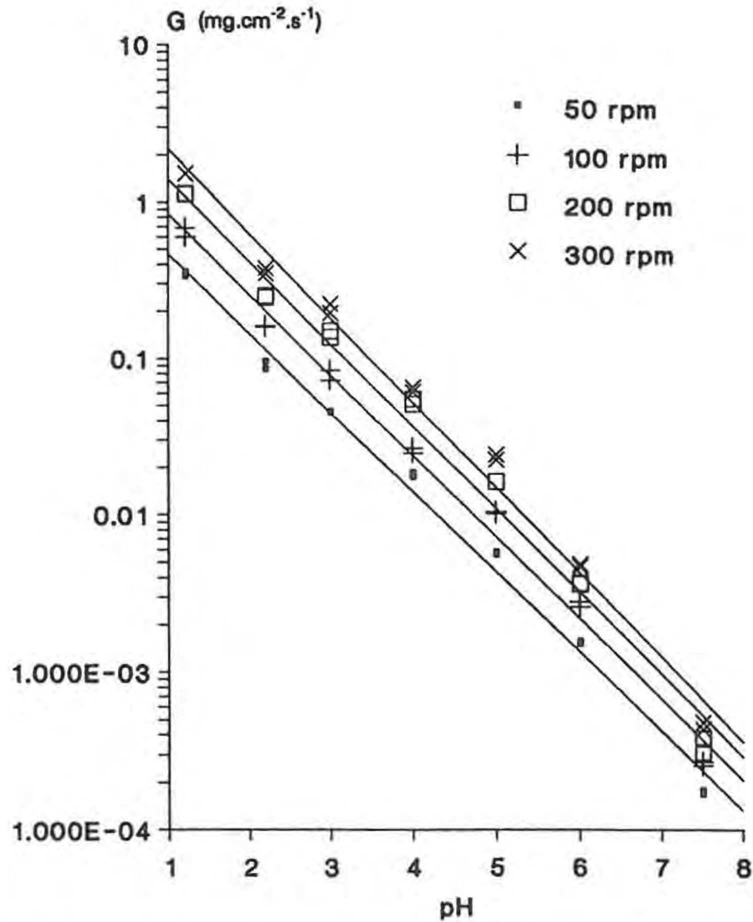


Figure 4.12 3-dimensional plot of G^x/G^{50} *vs.* rotation speed *vs.* pH (where G^x = G at either 50, 100, 200 and 300 rpm) showing the effect of rotation speed on G at each pH.
Figure 4.13 Log G *vs.* pH profiles at 50, 100, 200 and 300 rpm showing the pH dependency of G at each rotation speed, and the convergence of the linear regression lines of the profiles due to the varying influence of rotation speed on G at each pH.

Consequently, G values at different pH's obtained at either 50, 100, 200 or 300 rpm could not be compared on an equal basis as the influence of rotation speed on the diffusion layer, and therefore on G, was different at each pH. For example, at 50 rpm, G was 2002 times greater at pH 1.2 than at pH 7.5, but at 300 rpm, G was 3341 times greater at pH 1.2 than at pH 7.5. However, at infinite rotation speed the diffusion layer no longer contributes to the dissolution process and the determination of G^{∞} values at each pH allowed the comparison of intrinsic dissolution rates which were theoretically not influenced by the diffusion layer. Comparing G^{∞} values was therefore a simple means by which intrinsic dissolution rates determined in different dissolution media could be compared without the complication of the variable influence of rotation speed.

4.1.2.4 Influence of pH on Intrinsic Dissolution Rate

Plots of log G *vs.* pH at 50, 100, 200 and 300 rpm are depicted in figure 4.13 and a plot of log G^{∞} *vs.* pH is depicted in figure 4.14. Linear relationships between log G and pH at each rotation speed, and log G^{∞} and pH were obtained. Results of linear regression analysis of these plots are tabulated in table 4.5. These plots show that the intrinsic dissolution rate was highly dependent on the pH of the dissolution medium over the entire pH range studied. G^{∞} was 5.16, 0.73, 0.53, 0.11, 5.09×10^{-2}, 7.73×10^{-3} and 5.81×10^{-4} $mg.cm^{-2}.s^{-1}$ at pH 1.2, 2.2, 3.0, 4.0, 5.0, 6.0 and 7.5 respectively and G^{∞} varied by a factor of >8500 over the pH range studied.

In dissolution media of pH 1.2 - 4.0 a sampling period of 2 min was sufficient to determine the dissolution rate. Distortion of the disc surface was apparent after dissolution of approximately 30 mg of josamycin and was particularly pronounced at lower pH's but absent at higher pH's (Figures 4.15 and 4.16). This distortion, also observed by Nicklasson (137), could disrupt the flow of dissolution medium over the disc surface and increase the disc surface area during the dissolution run. Furthermore, eddy currents could also be generated at the edge of the disc due to the recession of the disc surface from the edge of the water-insoluble lacquer coating. Deviations from linearity of plots of W/S ($mg.cm^{-1}$) *vs.* t (s) for dissolution runs where these conditions were encountered could therefore be expected. However, linear regression lines with correlation coefficients of 0.9904 and 0.9947 were obtained for plots of duplicate experiments conducted in dissolution medium of pH 1.2 with a disc rotation speed of 300 rpm - the experimental conditions producing the maximum amount of distortion. As no significant deviation from linearity was detected, it was unnecessary to consider these factors during the calculation of intrinsic dissolution rates. At lower pH's shorter sampling period would have been preferable in order to obtain data prior to distortion of the disc surface. However, sample manipulation demanded a minimum sampling interval of 15 s, and a sampling

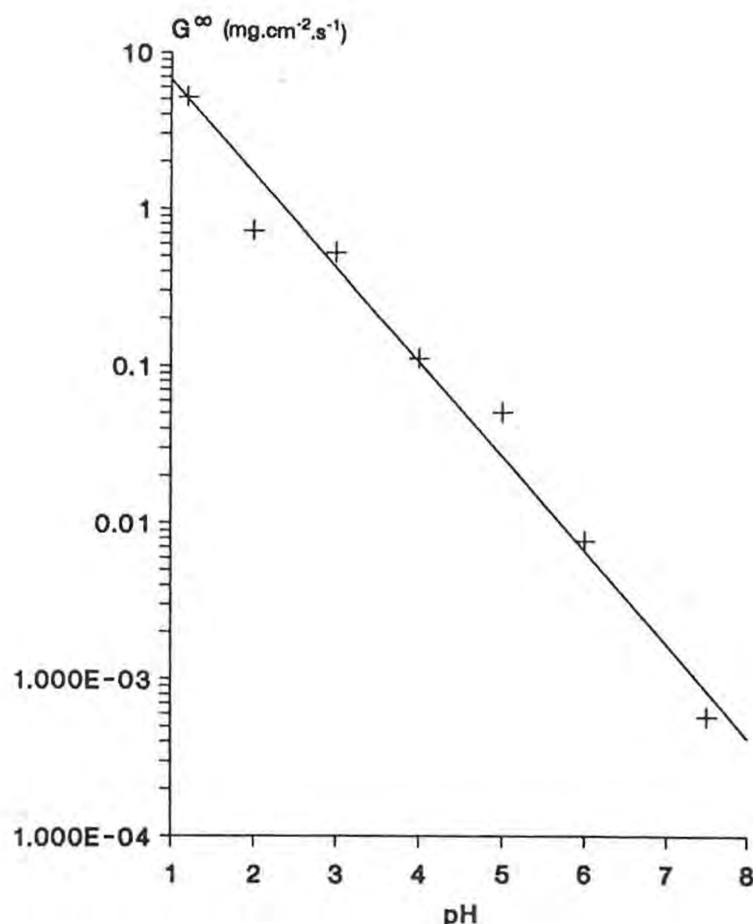


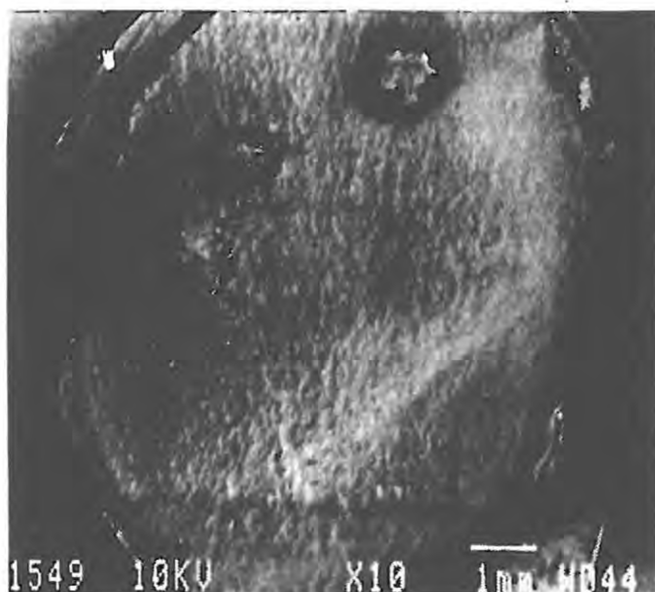
Figure 4.14 Log G∞ - pH profile of josamycin over the pH range of pH 1.2 - 7.5 at 37°C (replicate determinations n=3).

period of 2 min was therefore required for adequate characterisation of dissolution profiles. Use of a spectrophotometer with a flow cell attachment providing continuous monitoring of the dissolution medium may have been a more suitable analytical technique under these circumstances. However, this type of apparatus was not available for use.

At pH 5.0 and 6.0, a sampling period of 10 min was utilised. At pH 7.5 a sampling period of 30 min was required for adequate characterization of the dissolution rate, after which, at 300 rpm, less than 1 mg of josamycin was dissolved. Concentrations of josamycin in higher pH dissolution media were therefore extremely low and HPLC was the ideal analytical technique for analysis of these samples.

As mentioned earlier, Kaplan (144) suggested that the absorption of drugs can be dissolution rate limited if the aqueous intrinsic dissolution from centrically mounted discs rotated at 50 rpm rate is below 0.016 $mg.cm^{-2}.s^{-1}$, and highly likely if it is below

Figure 4.15 **Figure 4.16**



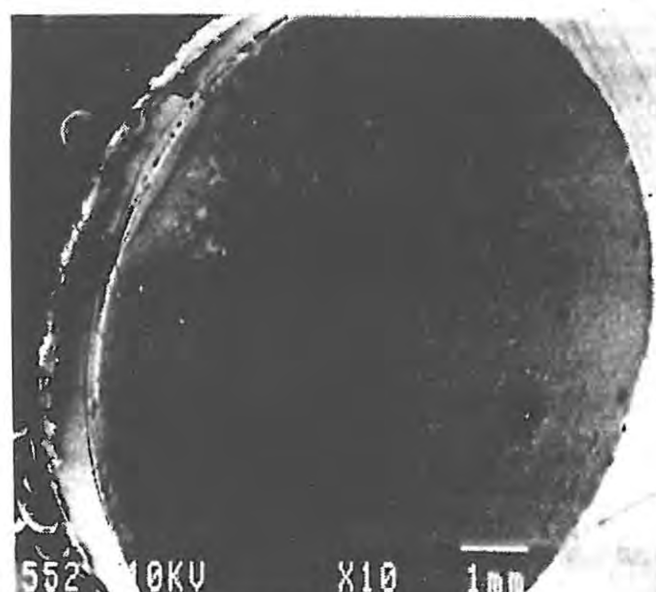


Figure 4.15 Electron micrograph of disc surface after excentric rotation of the disc at 300 rpm in dissolution medium of pH 1.2 showing the surface abberations developed after 30 s.
Figure 4.16 Electron micrograph of disc surface after excentric rotation of the disc at 300 rpm in dissolution medium of pH 7.5 showing the lack of surface abberations after 30 min.

0.0016 $mg.cm^{-2}.s^{-1}$. As G at any rotation speed is considerably higher for excentrically than centrically mounted discs, direct comparison between these limits and intrinsic dissolution rates determined in this study cannot be made. However, comparison will provide a very conservative estimate of the possible role of intrinsic dissolution rate on the absorption of josamycin.

Calculation of intrinsic dissolution rates from the linear regression equation of the plot of log G^{∞} *vs.* pH (Table 4.5) shows that G^{∞} for josamycin was 5.10 $mg.cm^{-2}.s^{-1}$ at pH 1.2 and decreased rapidly with increasing pH to 0.016 $mg.cm^{-2}.s^{-1}$ at pH 5.4, and to below 0.0016 $mg.cm^{-2}.s^{-1}$ at pH 7.0. Consequently, absorption of josamycin could be dissolution rate limited from intestinal fluid of pH 5.4 - 7.0 and highly likely to be dissolution rate limited from intestinal fluid above pH 7.0. Also, G^{∞} varied by a factor of 31 over the normal intestinal pH range of pH 5.0 - 7.5. Small changes in intestinal fluid pH could therefore result in significant changes in the dissolution rate, and subsequently have a profound effect on the absorption rate of the drug. The pH of the intestinal tract may therefore be critical in determining the bioavailability of josamycin after oral administration. Furthermore, due to the intra- and inter- individual variation in intestinal pH (147),

intra- and inter- individual variation in the absorption rate of josamycin could be expected.

4.2 SOLUBILITY

When a drug dissolves, an equilibrium between ionised and unionised species exists. The ratio of the concentrations of these two species is determined by the dissociation constant (pKa) of the drug and the pH of the dissolution medium, and for a weak base, is given by the following form of the Henderson-Hasselbalch equation (Equation 4.10).

$$pH = \log \frac{[B]}{[BH^{+}]} + pKa \qquad \text{Eq.4.10}$$

where [B] is the concentration of the unionised form, $[BH^+]$ is the concentration of the ionised form and pKa is the dissociation constant. However, the concentrations which these two species can attain at any pH is dependent on the solubility of the drug in that dissolution medium. For most drugs, mainly the dissolved unionised form is absorbed from the gastro-intestinal tract. Both the solubility and pKa are therefore important factors in determining the amount of undissociated drug available for absorption. Solubility and pKa can therefore strongly influence the absorption characteristics and bioavailability of a compound (148) and it has been reported in the literature that compounds with a solubility of less than 1% over the gastro-intestinal pH range may exhibit solubility dependent absorption (149).

The importance of the intrinsic dissolution rate on the bioavailability of a compound has previously been discussed. Moreover, solubility (C_s) and intrinsic dissolution rate are related as can be seen from equation 4.6. Log-log plots of G *vs.* C_s for various compounds have been found to be linear by a number of researchers (133,135,136,142), and generally, the less soluble a compound the slower the intrinsic dissolution rate. Solubility, intrinsic dissolution rate and pKa are therefore factors of equal importance in understanding the absorption characteristics of a compound. Although the pKa of josamycin has previously been reported (1,4), no data on the solubility of this drug have yet appeared in the literature. The solubility of josamycin over the gastro-intestinal pH range was therefore determined in this study.

4.2.1 Experimental

4.2.1.1 Dissolution Media

Solubility determinations were carried out in de-ionised water (HPLC grade) and in McIlvaine's buffer of pH 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, prepared as in 4.1.1.1, and in a buffer solution of pH 9.0, prepared by adjusting McIlvaine's Buffer of pH 8.8 (the maximum pH of this buffer system) to pH 9.0 with sodium hydroxide pellets.

4.2.1.2 Determination of Solubility

Excess josamycin (Table 4.6) was weighed into screw-capped test-tubes (Kimax, Kimble, Vineland, NJ, U.S.A.) followed by the addition of 2 ml of buffer (n=3 at each pH). The test-tubes were vigorously shaken and sonicated for 20 s to ensure wetting of the powder, after which the solutions were maintained at 37°C in a constant temperature water-bath (Colora, Ultra Thermostat, Lorch, Germany) whilst shaken by a mechanical shaker (Griffin Flask Shaker, Griffin and George Ltd., UK). Samples were withdrawn after 8 hours with a 1 ml syringe (Terumo Corporation, Tokyo, Japan) and filtered through a 0.45 μm filter (Millipore, Bedford, MA, U.S.A.). The pH of each filtered solution was then measured and the sample analysed immediately, after suitable dilution as in 2.2.6.2. Syringes and filters were pre-heated to 37°C in an incubator prior to use to maintain the temperature of the sample at 37°C until filtration was complete. This was done to prevent any further dissolution of josamycin after sampling, which would have occurred if the sample cooled during the filtration procedure as josamycin is more soluble at room temperature (21°C) than at 37°C. Preliminary studies showed that in all dissolution media there was no change in concentration after 4 hours of equilibration indicating that solution saturation was achieved within this time. Nevertheless, samples were aspirated for analysis after 8 hours. Josamycin was stable between pH 5.0 and 9.0 and no degradation products were detected after the required equilibration time.

4.2.2 Results and Discussion

Solubility data of josamycin in McIlvaine's Buffer and de-ionised water at 37°C are tabulated in table 4.6 and depicted graphically in figure 4.17.

The solubility of josamycin in buffer solution was 212 $mg.ml^{-1}$ at pH 5.45 and decreased rapidly with increasing pH to ~0.21 $mg.ml^{-1}$ at ~pH 8.0. Above pH 8.0 there was only a slight decrease in solubility to ~0.18 $mg.ml^{-1}$ at ~pH 8.9. Josamycin has a pKa of 7.1 and this basic property can be attributed to the tertiary

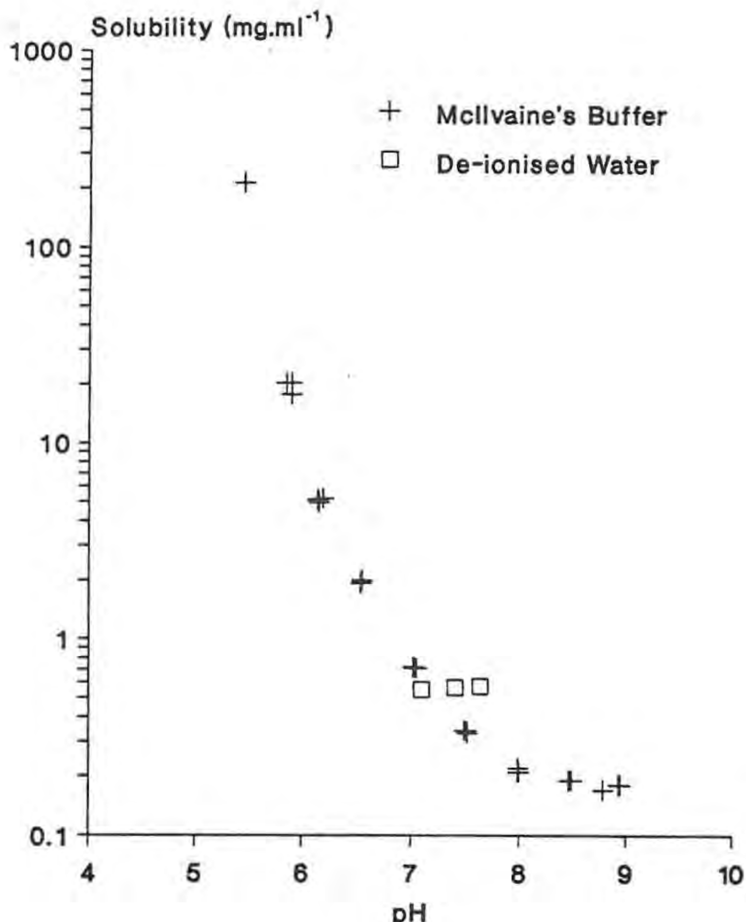


Figure 4.17 pH-solubility profile of josamycin in McIlvaine's buffer at 37°C (triplicate determinations).

amine attached to position 3 of the mycaminose moiety (Figure 1.1). The nitrogen, however, is strongly shielded by adjacent molecular units which inhibit the activity of the lone pair of electrons on the nitrogen and make josamycin only weakly basic. This was evident in the pH-solubility profile which is typical of a weak base.

Triplicate saturated solutions of josamycin in de-ionised water were of pH 7.40, 7.09 and 7.63 with josamycin concentrations of 0.56, 0.55 and 0.57 mg.ml^{-1} respectively. The pH and solubility determinations for replicate samples in saturated buffer solutions were less variable than those of saturated solutions of josamycin in de-ionised water. If a compound is sparingly soluble, variable solubility can be caused by interference from highly soluble impurities (150). However, in this instance, the pH electrode may have been insufficiently sensitive at the low ionic strength of the de-ionised water solutions, which may account for the variation in the pH measurements obtained.

Josamycin was more soluble in de-ionised water than in McIlvaine's Buffer of equal pH (Figure 4.17), *i.e.* at pH ~7.5, josamycin had a solubility of ~0.56 $mg.ml^{-1}$ in de-ionised water and a solubility of only ~0.34 $mg.ml^{-1}$ in McIlvaine's Buffer of pH 7.5. This could be due to a "salting out" effect whereby the solubility of a slightly soluble organic compound is reduced by the presence of dissolved salts (151).

Attempts were made to determine the solubility of josamycin between pH 1.0 and pH 5.0. However, addition of josamycin to all buffers below pH 5.0 resulted in the formation of a clear highly concentrated solution from which a sticky mass suddenly precipitated before excess josamycin could be added. After precipitation, a saturated supernatant with an equilibrium pH of 5.45 and a concentration of 212 $mg.ml^{-1}$ was obtained irrespective of the initial pH of the buffer. This was observed even when 1.0 M HCl was used as the dissolution medium. Similar dissolution behaviour was also observed by Hajdu *et al.* during preformulation studies on pelrinone hydrochloride (152). This phenomenon may be explained by the formation and subsequent precipitation of a polymorph less soluble than the original compound once dissolution of the original had occurred (153). Investigations into the properties of this precipitate were not conducted. However, the possible occurrence of this phenomenon in the acidic environment of the stomach together with any subsequent effect on the bioavailability of josamycin should not be overlooked.

The pH-solubility profile of josamycin can be expected to have far-reaching implications on its *in-vivo* behaviour. Josamycin is highly soluble in gastric fluid but as drug molecules will be highly ionised at this pH, absorption from the stomach should be extremely poor (148). On the other hand, josamycin, with a favourable lipid solubilitiy and a pKa of less than 10 should be rapidly absorbed from the more alkaline medium of the intestine (148). However, Kaplan (144), and Smythe and Hottendorf (149) indicated that solubility limited absorption may be observed if the aqueous solubility of a compound is less than 1% or 10 mg/ml. For example, Shah *et al.* (154) found the solubility of etoposide to be less than 0.02% in aqueous media of between pH 2.0 and 8.0 and also concluded that poor aqueous solubility could be responsible for its low and erratic bioavailability, which was between 20% and 90% after oral administration (155). The solubility of josamycin at intestinal pH's ranged from 21% at pH 5.45 to 0.019% at about pH 8.5 *i.e.* the solubility varied considerably and by a factor of 1105 over this pH range. The solubility of josamycin in de-ionised water is well below 1% and found to be 0.56 $mg.ml^{-1}$ or 0.056%. Furthermore, the solubility of josamycin in buffer is about 1% at about pH 6.0 and decreases sharply with increasing pH. Absorption of josamycin could therefore be solubility limited from an intestinal enviroment of pH 6.0 and higher, and would be increasingly hindered by poor solubility from increasingly alkaline intestinal fluid. This suggests that

josamycin may be subject to solubility limited absorption which is reinforced by results obtained from the intrinsic dissolution rate studies.

4.3 *IN-VITRO* DISSOLUTION TESTING

Drugs are often formulated into tablets for ease and convenience of manufacture, handling and administration. However, to be effective, the rate and extent of dissolution of drug from the tablet must ensure that absorption is facilitated such that therapeutic concentrations in the body can be attained. Ideally, dissolution testing should be conducted *in-vivo*. However, routine testing in this manner is not possible and *in-vitro* dissolution testing is thus utilised. It is well known that poor bioavailability can often be explained by poor *in-vitro* dissolution characteristics of a formulation. It has been reported that the poor bioavailability of 80% of formulations exhibiting bio-inequivalence that were tested by the FDA, could be ascribed to poor dissolution characteristics (156). As the *in-vitro* dissolution characteristics of a formulation may provide valuable information on the behaviour of the formulation *in-vivo*, *in-vitro* dissolution testing has become an integral part of the development of effective formulations, and is an important aspect of investigations designed to unravel the biopharmaceutics of drug products (157,158).

Since the early 1960's, numerous investigators have published data on the relationship between *in-vitro* dissolution and *in-vivo* bioavailability. Abdou (159) has published a comprehensive review on this subject. He has cited numerous cases in which dissolution and bioavailability are well correlated. However, in many instances dissolution testing failed to predict or explain poor *in-vivo* performance of the formulation. In most of these studies dissolution was investigated in only one or two dissolution media - usually 0.1 M HCl or simulated gastric fluid (145) to represent dissolution in the stomach, or a buffer of pH 7.5 or simulated intestinal fluid (146) to represent dissolution in the intestine. However, the pH of the stomach and intestinal tract can vary considerably (147,160). Correlations were therefore conducted on a limited amount of data representing dissolution at only the extremes of pH values encountered throughout the gastro-intestinal tract. Better *in-vitro/in-vivo* correlations may have been obtained with dissolution profiles determined in dissolution media of intermediate pH's. Some investigators have, however, tried to simulate the gastro-intestinal tract more closely. Ogata *et.al* (161) and Kaniwa *et.al* (162) used a system in which the pH of the dissolution medium was changed from pH 1.2 to pH 6.5 and then to pH 7.5 by the addition of buffer salts after suitable time intervals during the dissolution run. Utilising this system, they obtained good *in-vitro/in-vivo*

correlations between dissolution rate and bioavailability for nalidixic acid and flufenamic acid. Ogata *et.al* (163) also conducted an extensive investigation into the dissolution of five chloramphenicol preparations in dissolution media covering the pH range of 1.2 to 9.0. The value of this was illustrated when he found that the dissolution of one formulation was highly pH dependent and that bioavailability data for all five preparations were well correlated with dissolution data at pH 4.0. The dissolution characteristics of controlled release formulations are commonly determined in buffers of various pH's to simulate the passage of the dosage form through the gastro-intestinal tract. The usefulness of this procedure in identifying bioavailability problems has been amply demonstrated. For instance, investigations conducted by Skelly (164) on the poor bioavailability of a generic quinidine gluconate preparation, demonstrated that dissolution was highly pH-dependent between pH 1.0 and 7.4 and that bioavailability problems were due to poor dissolution of the product between about pH 2.0 and 5.8. Although no *in-vitro/in-vivo* correlations were made, general trends in the dissolution characteristics of the product were easily visualised and their relevance to the *in-vivo* data was obvious.

Dissolution of tablets formulated for immediate release should, theoretically, be completed in the acidic environment of the stomach. However, non-disintegrated tablets or tablet granules can pass through the pyloric sphincter into the duodenum (165-167), where dissolution in a more alkaline environment will be necessay for absorption to occur. Dissolution profiles in media covering the entire gastro-intestinal pH range can therefore also provide a detailed picture of the dissolution characteristics likely to be exhibited by an immediate release dosage form after oral administration, and meaningful conclusions may then be drawn when comparing *in-vitro* dissolution profiles with *in-vivo* bioavailability data.

The release of drug from a tablet matrix is initiated by disintegration of the tablet into granules followed by disintegration of the granules into smaller aggregates and primary particles. The dissolution rate of the drug particles is then determined by the physico-chemical properties of the drug, such as intrinsic dissolution rate, solubility and wettability, and the degree of agitation of the dissolution medium - whether *in-vitro* or *in-vivo*. Some drug particles, however, may remain entrapped in the tablet matrix. This may result in retardation of the dissolution rate since the porosity of the matrix and the rate of diffusion of dissolved drug through the hydrated matrix into the bulk solution can become additional rate limiting factors (168). Dissolution of drug from a tablet is therefore a complex procedure and determined by both the characteristics of the formulation and the physico-chemical properties of the drug, with the overall rate of dissolution (and subsequent absorption) often being controlled by the slowest step in the dissolution process (169). The rate of dissolution

of a powder, on the other hand, is unhindered by the processes of disintegration, de-aggregation and diffusion. A comparison of the dissolution profiles of powder with those of tablets containing an equal quantity of drug, under the same conditions, can usually indicate the contribution made by formulation factors to the overall dissolution profile of the tablet. Tablet formulations exhibiting dissolution profiles close to those of the powder are unlikely to have bioavailabiltiy problems (157) provided that the drug powder exhibits good bioavailability. If however, dissolution of drug from the tablet is significantly slower than from the powder, formulation factors may explain any bioavailability problems observed with the tablet.

A comprehensive study to determine the effect of pH and formulation on the dissolution characteristics of two tablet formulations used in bioavailability and pharmacokinetic studies (*vide infra* Chapter 6) was conducted. Dissolution profiles of Josamycin 200 mg tablets (Yamanouchi Pharmaceutical Co., Tokyo, Japan) and Josacine 500 mg tablets (Pharmuka, Gennevilliers, France) which contained 200 mg and 500 mg of josamycin base per tablet respectively, were determined in dissolution media of pH 1.2 - 9.0 covering the entire gastro-intestinal pH range. Dissolution profiles of 200 mg and 500 mg of powder were also determined for comparison with the tablet dissolution profiles. Determination of multiple dissolution profiles over this pH range was considered to be relevant to formulations containing josamycin as the intrinsic dissolution rate and solubility of josamycin are particularly sensitive to pH.

Numerous *in-vitro* dissolution testing methods have appeared in the literature over the last few decades, each with its associated advantages and disadvantages. These are extensively described in several review articles (170-172). However, compendia in both the U.S.A. - the U.S.P. (173), and U.K. - the B.P. (174) list only two official methods - the Rotating Basket Method and the Paddle Method. Consequently, the majority of dissolution testing for both official and non-official purposes is performed utilising one of these two methods. These methods do, however, have some inherent problems such as difficulty in maintaining sink conditions throughout the dissolution run for sparingly soluble drugs, and difficulty in simulating the passage of dosage forms along the gastro-intestinal tract which is of particular relevance to controlled release dosage forms. Some additional methods and equipment which attempt to address these problems *e.g.* the Langenbucher Flow-Through Method (175) and the Bio-Dis Apparatus (G.B. Caleva Ltd., Ascot, England) are, however, currently being considered by the compendial authorities and may significantly improve the assessment of a dosage forms performance *in-vivo*.

The Rotating Basket method was originally described by Pernarowski *et al.* (176) who modified the Beaker Method of Levy (177) by the addition of a rotating basket assembly in which to confine the dosage form. The Paddle Method was

originally described by Poole (178) and consisted of a round-bottomed flask and stirrer. It has since undergone various refinements into its present form. Conflicting evidence has been presented as to the ability of each method to detect differences between formulations (179-181) or to provide data with better *in-vitro/in-vivo* correlations.

The Basket Method is reported to be less sensitive to variations in the alignment and centering of the stirring assembly than the Paddle Method, can keep floating formulations submerged, and can also confine non-disintegrated portions of a dosage form to a restricted area ensuring exposure of the dosage form to uniform agitation until disintegration has been completed (158). The main disadvantages of the Basket Method are that dissolved gas in the dissolution fluid can form bubbles on the basket mesh which interfere with the flow of dissolution fluid past the tablet and retain disintegrated particles in the basket. Also, granules of a disintegrated tablet which drop through the basket can remain unstirred at the bottom of the flask. However, the intensity of agitation is generally greater with the Paddle Method and this problem can be avoided if this method is utilised (180). One disadvantage of the Paddle Method is that a tablet may come to rest on the side of the flask and the position that the tablet settles can have a pronounced effect on the dissolution profile, especially at lower agitation rates. However, this problem is rarely observed (182). The suitability of each method to a particular dissolution study is therefore determined by the experimental particulars and requirements of each study.

4.3.1 Experimental

4.3.1.1 Dissolution Apparatus

Since a dissolution monograph does not currently exist in either the U.S.P. or B.P. for tablets containing josamycin, the choice of apparatus was determined by the nature of the formulations tested. Both tablet formulations consisted of immediate release formulations and thus containment of the dosage form within a confined area by the use of a basket was deemed unnecessary. Furthermore, use of the Basket Method for dissolution testing of powders was impractical. The Paddle Method was therefore chosen as the most suitable method and the U.S.P. Paddle Method used throughout all dissolution investigations.

The water bath of the dissolution apparatus (PHARMATEST Type PTW S, Hainburg, West Germany) was allowed to equilibrate for a minimum of two hours prior to the commencement of a dissolution study. One litre of dissolution fluid was placed in each flask and allowed to equilibrate to 37°C for about 1 hour whilst being stirred at 100 rpm. Flasks were sealed during this period to ensure that no

evaporation of the dissolution fluid took place. The temperature of the dissolution medium was monitored until the start of the dissolution run to ensure that the correct equilibrium temperature was attained. This was done with the aid of an electronic thermometer manufactured in the laboratory and capable of detecting temperature changes of less than 0.1°C. The temperature of the water bath was monitored with the *in-situ* temperature monitoring system. Prior to introduction of the test sample, bubbles which had formed within the flask were removed by rapid stirring. The paddle rotation rate was maintained at 100 rpm throughout each study.

4.3.1.2 Dissolution Fluid

Dissolution testing of each product and powder was conducted in dissolution media of pH 1.2 - 9.0, covering the entire gastro-intestinal pH range.

Dissolution media consisted of U.S.P. simulated gastric fluid (pH 1.2) (145) and U.S.P. simulated intestinal fluid (pH 7.5) (146), both without enzymes; McIlvaine's buffer (183) at pH 2.2, 3.0, 4.0, 5.0, and 6.0; and McIlvaine's buffer (pH 8.0) adjusted to pH 9.0 with sodium hydroxide pellets. McIlvaines buffer was prepared by the addition of a 0.2 M solution of di-sodium hydrogen phosphate to a 0.1 M solution of citric acid in sufficient quantities to obtain a solution of the desired pH. All reagents used were of analytical grade and all water used was of HPLC grade. These dissolution media were the same as those used for solubility determinations.

4.3.1.3 Dissolution Testing of Powder and Tablets

Josamycin powder (200 mg or 500 mg) was weighed into a 20 ml screw cap Kimax tube (Kimble, Toledo, OH, USA). The powder was wetted to prevent its flotation on the surface of the dissolution fluid by the addition of 5 mls of water followed by vortexing for 1 min. The volume of water used was capable of dissolving only 1.68 mg of powder which was considered negligible. The josamycin suspension was then introduced into the dissolution fluid at a point mid-way between the stirrer shaft and the side of the dissolution vessel. The Kimax tube was then rinsed with 2 x 5 ml aliquots of dissolution fluid, which had been removed from the flask prior to the commencement of the dissolution run. The sampling intervals in the early stages of the powder dissolution runs were extremely short to ensure adequate characterisation of the dissolution profile. Stirring was therefore started before the introduction of the powder to ensure homogenous mixing before the first sampling time.

Dissolution of Josamycin 200 mg tablets and josamycin powder (200 mg) was monitored for 60 min at pH 1.2, 2.2, 3.0, 4.0, 5.0 and 6.0 and for 240 min at pH 7.5 and 9.0. Dissolution of Josacine 500 mg tablets and josamycin powder (500 mg) was

monitored for 60 min at pH 1.2, 2.2, 3.0, 4.0 and 5.0, and for 240 min at pH 6.0, 7.5 and 9.0. Samples (1.2 ml) of the dissolution fluid were removed at the relevant times with a 1 ml tuberculin syringe (Terumo Coorporation, Tokyo, Japan). Sampling from a consistent position within the dissolution vessel was ensured by insertion of the syringe into the dissolution media to its maximum extent via a sampling port in the dissolution vessel cover. The importance of sampling from a single point throughout the dissolution run has been cited in the literature (184) and the U.S.P. lists criteria for its precise location. Sampling in the abovementioned manner ensured that these sampling criteria were met. Furthermore, turbulence caused by the sampling probe can affect the dissolution profiles (185,186). The sampling syringes were, therefore, held in the dissolution fluid for as short a time as possible. Dissolution fluid was not replaced after sampling and the total amount removed during each run did not exceed 25 mls.

The procedure detailed above was followed for the dissolution testing of tablets. However, sampling times were adjusted to ensure that both the rapid dissolution from Josamycin tablets and the slower dissolution from Josacine tablets were adequately characterised. The dosage forms were introduced into the dissolution vessel via the sampling port.

The solubility of josamycin was sufficient to ensure that sink conditions were maintained during dissolution runs at pH 6.0 and below. However, sink concentrations were exceeded during dissolution of 200 mg and 500 mg of josamycin at pH 7.5 and 9.0. The maintenance of sink conditions during dissolution at these pH's was not practical as the circulation of up to 12.5 l of fluid through each dissolution vessel was nesessary to achieve this. All dissolution runs were therefore carried out in 1 l of dissolution medium.

4.3.1.4 Analysis of Samples

Samples from dissolution runs at pH 1.2, 2.2, 3.0 and 4.0 were analysed by HPLC as described in 2.2.7, and those from dissolution runs of pH 5.0, 6.0, 7.5 and 9.0 were analysed spectrophotometrically as described in 2.3.2.

Analytical controls were prepared as in 2.2.7.1 and 2.3.2.1 and analysed concurrently with samples as a quality control measure. Control results are tabulated in table 2.4 and 2.5.

4.3.1.5 Mass Balance Analysis

Mass balance analysis was carried out to determine the amount of josamycin remaining undissolved at the end of each dissolution run and to determine the extent to which degradation occurred during the dissolution run.

At pH 1.2 to 6.0, where josamycin is highly soluble, any josamycin remaining

undissolved must be entrapped in the tablet matrix. However, above pH 6.0 where josamycin has only limited solubiltiy, any undissolved josamycin could be either entrapped in the tablet matrix or could exist as undissolved particles in the dissolution medium. Data obtained from this procedure were therefore useful in assessing the extent of release of josamycin from the tablet matrix.

The extent of degradation was taken as the fraction of josamycin added to the dissolution vessel which could not be accounted for as either dissolved or undissolved at the end of the dissolution run.

To determine the amount remaining undissolved, the dissolution medium was filtered under vacuum through a 0.45 μm HVLP Millipore filter after the dissolution run was complete. The solid residue was quantitatively transferred to a 100 ml volumetric flask containing 50 ml acetonitrile and made up to volume with water. The mixture was then sonicated for 10 mins to ensure complete disintegration of any remaining granules and dissolution of all undissolved josamycin, after which about 1 ml of the solution was centrifuged at 12000 rpm for 5 mins in a high speed centrifuge. A 1 ml aliquot was then diluted to 10 ml with an acetonitrile/water mixture (1:1). Samples of this solution were then prepared for subsequent analysis either by HPLC (2.2.7) or spectrophotometrically (2.3.3).

4.3.2 <u>Results and Discussion</u>

4.3.2.1 Dissolution Profiles of Josamycin Powder (200 mg) and Josamycin 200 mg Tablets.

Dissolution profiles of josamycin powder (200 mg) and Josamycin 200 mg tablets from 0 to 60 min are depicted in 3-dimensional plots in figures 4.18 and 4.19 respectively. Dissolution profiles from 0 to 240 min of powder and tablets at pH 7.5 and 9.0 are depicted in figures 4.20 and 4.21 respectively. Lag times (taken as the sample time in which josamycin was first detected in the dissolution medium), disintegration times (measured during each dissolution run), times taken to reach 50% and 100% dissolution (fd = 0.5 and fd = 1.0 respectively, where fd is the fraction dissolved) and the maximum concentrations reached, for both powder and tablet dissolution profiles are tabulated in table 4.7. Results of mass balance analysis are tabulated in table 4.8.

Dissolution profiles of josamycin powder (200 mg) show both rapid and complete dissolution at pH 1.2 through to pH 5.0. fd = 1.0 was attained within 7 min and the rate and extent of dissolution was pH-independent over this pH range. At pH 1.2 and 2.2, the concentration of josamycin decreased after dissolution was complete. This was due to acid degradation of josamycin which was confirmed by the

Figure 4.18

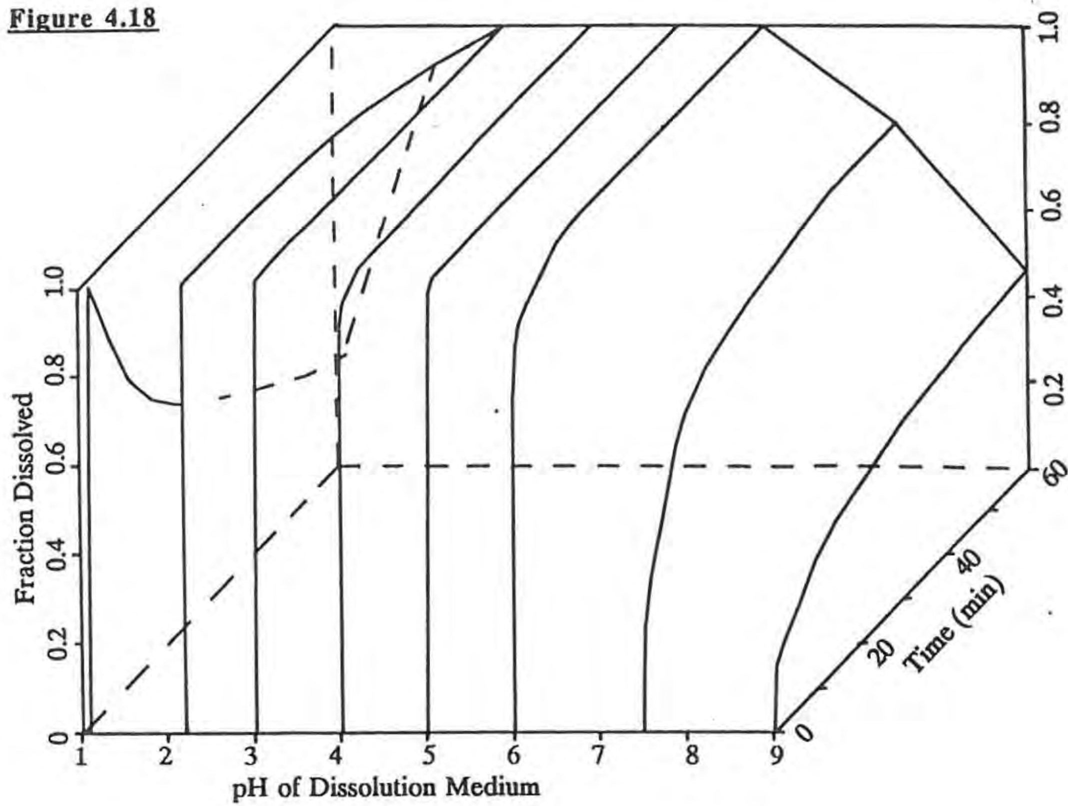


Figure 4.19

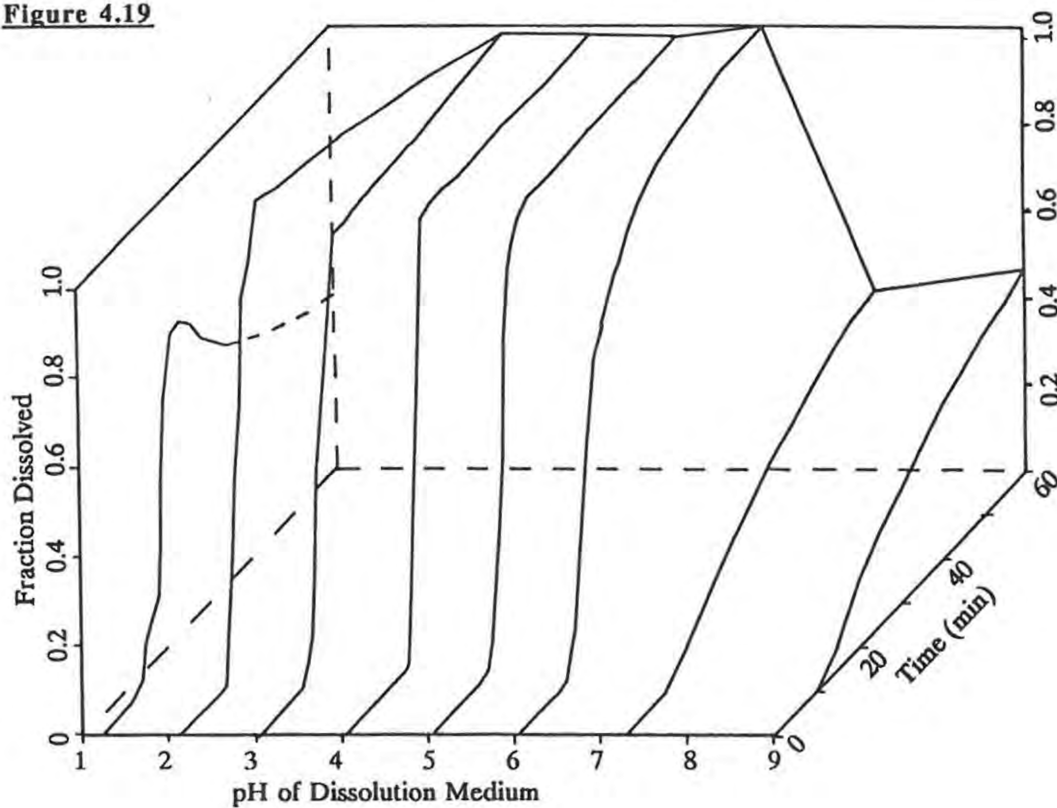


Figure 4.18 3-dimensional plot of the fraction of powder dissolved *vs.* time *vs.* pH for josamycin powder (200 mg) showing the effect of pH on the rate and extent of dissolution.

Figure 4.19 3-dimensional plot of the fraction of tablet content dissolved *vs.* time *vs.* pH for Josamycin 200 mg tablets, showing the effect of pH and formulation on the rate and extent of dissolution.

Figure 4.20

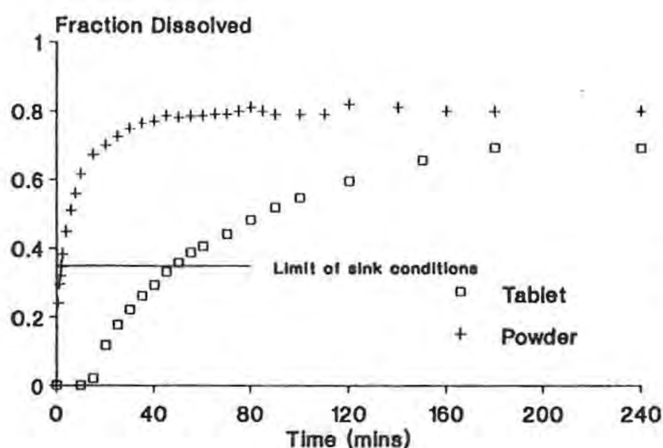
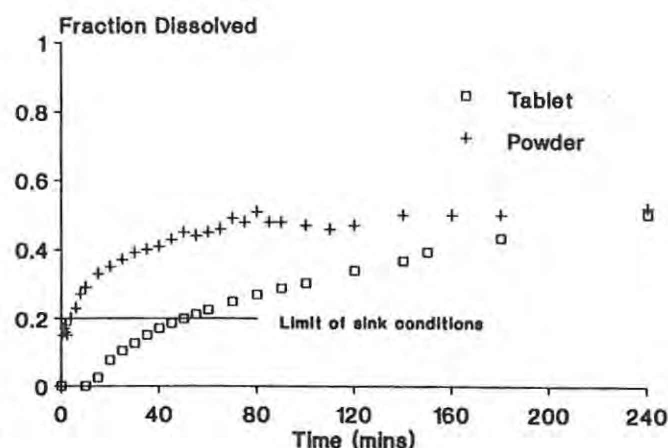


Figure 4.21



Figures 4.20 and 4.21 Dissolution profiles of josamycin powder (200 mg), +; and Josamycin 200 mg tablets, □; from 0 to 240 min at pH 7.5 and 9.0 respectively, showing the prolonged dissolution times and the retardation of dissolution of josamycin from the tablets by formulation factors both before and after sink conditions were exceeded.

appearance of acid degradation products in chromatograms of these samples after analysis utilising a stability indicating HPLC assay. Above pH 5.0, the shape of the dissolution profiles changed considerably with pH. At pH 6.0, dissolution for most of the profile was rapid but slowed slightly after *fd* = 0.8 and the time taken to reach *fd* = 1.0 increased to 20 min. At pH 7.5 and 9.0, dissolution was rapid until sink concentrations were exceeded (Figures 4.20 and 4.21), after which dissolution was clearly pH-dependent with the rate and extent of dissolution decreasing with increasing pH. At pH 7.5, *fd* = 0.5 was attained after 6.0 min, however, a maximum *fd* of only 0.8 was reached after 50 min. At pH 9.0, *fd* = 0.5 was attained after 70 min and a maximum *fd* of only 0.51 attained after 240 min.

Josamycin 200 mg tablets were sugar coated and dissolution was delayed until the coating had been penetrated by the dissolution medium. Lag times of between 10 and 15 min were observed and were independent of pH. Disintegration of all tablets was complete within 5 min after the lag period and was also independent of pH.

In simulated gastric fluid (pH 1.2) dissolution was complete 17 min after the start of the dissolution run, and after a lag period of 11 min. Dissolution after the lag period was therefore very rapid. However, a maximum *fd* of only 0.74 was attained and after 17 min the concentration of josamycin in the dissolution medium decreased due to acid degradation. Mass balance analysis (Table 4.8) confirmed that no josamycin remained undissolved at the end of the dissolution runs and that 61% of the tablet content had degraded during this period.

At pH 2.2, 3.0, 4.0 and 5.0, the dissolution profiles were very similar with rapid dissolution after the lag period, and dissolution complete (*fd* = 1.0) between

14 and 24 min after the start of the dissolution run. Slight degradation was evident at pH 2.2 and from mass balance analysis 12% of the tablet content had degraded by the end of the dissolution run. As with the powder, dissolution of the tablets was pH-independent over the pH range 1.2 to 5.0. However, above pH 5.0, dissolution was clearly pH-dependent; the rate and extent of dissolution decreasing dramatically with increasing pH. At pH 6.0, the dissolution profile up until $fd = 0.5$ was very similar to those at lower pH's, however, after this point the dissolution rate slowed considerably and dissolution was complete after 58 min. At pH 7.5 and 9.0, dissolution was very slow throughout the entire dissolution run irrespective of whether concentrations were above or below sink concentrations (Figure 4.20 and 4.21). $fd = 0.5$ was attained after 83 min at pH 7.5 and not at all at pH 9.0. Dissolution reached a maximum fd of 0.69 at pH 7.5, and of 0.50 at pH 9.0, after 240 min.

Complete dissolution of both the powder and tablet was expected at pH 7.5 at which pH the solubility of josamycin is 0.34 mg/ml. However, the maximum fd was only 0.80 for the powder and 0.69 for the tablets. This incomplete dissolution may have been due to poor wettability of the josamycin particles at this pH. Also, in the case of the tablets mass balance analysis showed that 31% of the tablet content remained undissolved, some or all of which may have remained entrapped in the tablet matrix. A saturated solution should have been attained at pH 9.0 as the solubility of josamycin at this pH is only 0.18 mg/ml. However, the maximum concentration reached was 0.103 mg/ml ($fd = 0.52$) for the powder, and 0.100 mg/ml ($fd = 0.50$) for the tablets. Again, this may have been due to poor wettability of the josamycin particles, or in the case of the tablets, up to 50% of the tablet content may have remained entrapped in the tablet matrix.

The trends observed for both tablets and powder can, in part, be explained by the high sensitivity of the intrinsic dissolution rate and solubility of josamycin to pH over the pH range studied. As shown earlier, the intrinsic dissolution rate (G^{∞}) decreased by a factor of >8500 from pH 1.2 to 7.5 and the solubility decreased by a factor of >1150 between pH 5.45 and 8.90. From pH 1.2 to 5.0, the intrinsic dissolution rate and solubility were high enough to ensure rapid dissolution and no difference in the dissolution profiles was detected. However, above pH 5.0 the intrinsic dissolution rate and solubility were low enough to significantly limit the dissolution rate and their effect on the dissolution profiles became evident.

Dissolution from the tablets, however, was further complicated by formulation factors. Comparison of tablet with powder dissolution profiles shows that apart from the lag time, the profiles were equivalent and pH-independent between pH 1.2 and 5.0. Furthermore, release of josamycin from the tablet matrix over this pH range was complete and formulation factors appeared to have had a negligible effect on the rate and extent of dissolution. Above pH 5.0, however, the effect of pH on dissolution

was significantly more pronounced with the tablets than with the powder. Formulation factors severely limited the rate of dissolution from the tablets above pH 5.0, and release of josamycin from the granule matrix may have been incomplete at pH 7.5 and 9.0.

4.3.2.2 Disslution Profiles of Josamycin Powder (500 mg) and Josacine 500 mg Tablets.

Dissolution profiles of josamycin powder (500 mg) and Josacine 500 mg tablets from 0 to 60 min are depicted in 3-dimensional plots in figures 4.22 and 4.23 respectively. Dissolution profiles from 0 to 240 min of powder and tablets at pH 6.0, 7.5 and 9.0 are depicted in figures 4.24 - 4.26 respectively. Lag times, disintegration times, times taken to reach 50% and 100% dissolution and the maximum concentrations reached for both powder and tablet dissolution profiles are tablulated in table 4.7. Results of mass balance analysis are tabulated in table 4.8.
Dissolution profiles of josamycin powder (500 mg) were very similar to those of josamycin powder (200 mg) over the entire pH range studied. Dissolution was rapid and complete at pH 1.2 through to pH 6.0 and *fd* = 1.0 was attained in less than 7 min throughout this pH range. Extensive degradation was observed at pH 1.2 whilst degradation at pH 2.2 was only slight. Dissolution from pH 1.2 to 6.0 was pH-independent, however, above pH 6.0 dissolution was pH-dependent and increasingly limited by solubility and the intrinsic dissolution rate with increasing pH. At pH 7.5 and 9.0, dissolution was initially rapid but incomplete by the end of the dissolution run. At pH 7.5, *fd* = 0.5 was attained after 2 min but a maximum *fd* of 0.65 attained after 240 min. At pH 9.0, a maximum *fd* of 0.25 was attained after 240 min.

Josacine 500 mg tablets were film coated, however, the coating was rapidly dissolved and dissolution commenced almost immediately after introduction into dissolution media of pH 1.2 through to pH 7.5. Only at pH 9.0 was a lag period evident which delayed dissolution for 7 min. Disintegration at all pH's, however, was prolonged and complete between 39 min (at pH 7.5) and 60 min (at pH 1.2) after the start of the dissolution run.

At pH 1.2 to 5.0, dissolution from the tablets was slow but complete within 68 min and was pH-independent. In simulated gastric fluid a maximum *fd* of 0.46 was attained despite the high solubiltiy of josamycin at this pH. The shape of this dissolution profile was due to slow release of josamycin from the tablet matrix with concurrent degradation. Mass balance analysis confirmed that no josamycin remained entrapped in the tablet matrix and that the remaining 54% of the tablet content had degraded during the dissolution run. Dissolution profiles at pH 2.2, 3.0, 4.0 and 5.0 were very similar and apparently linear during the disintegration period with *fd* = 0.5

Figure 4.22

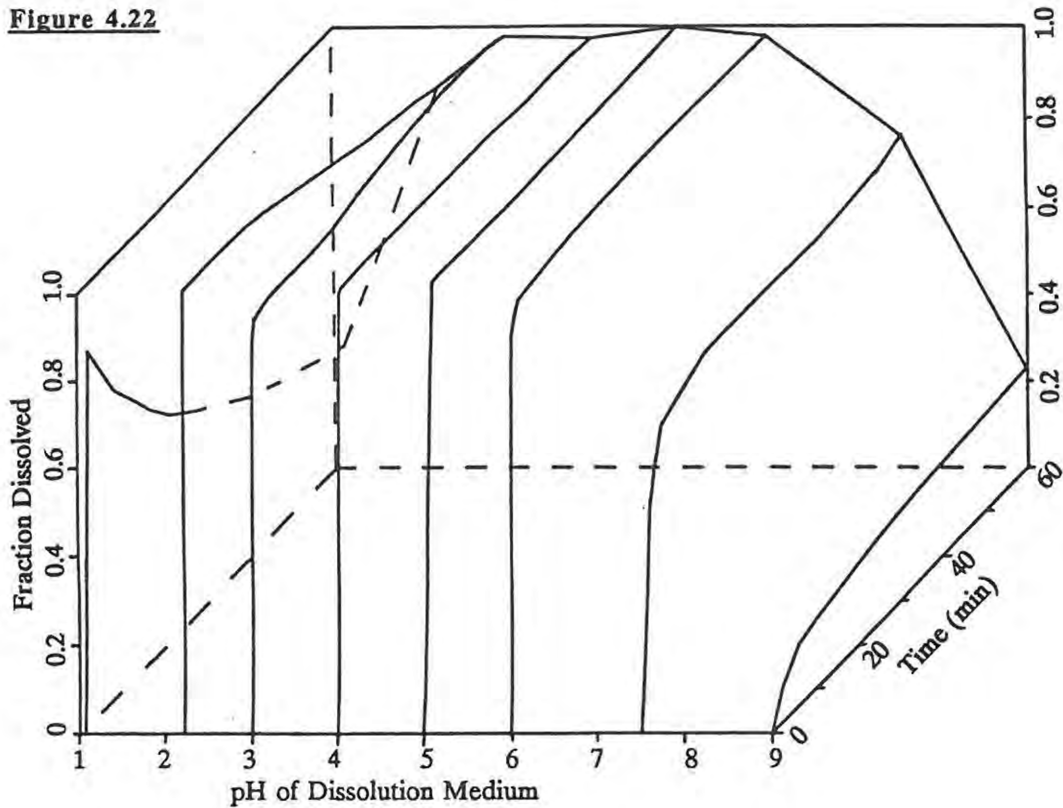


Figure 4.23

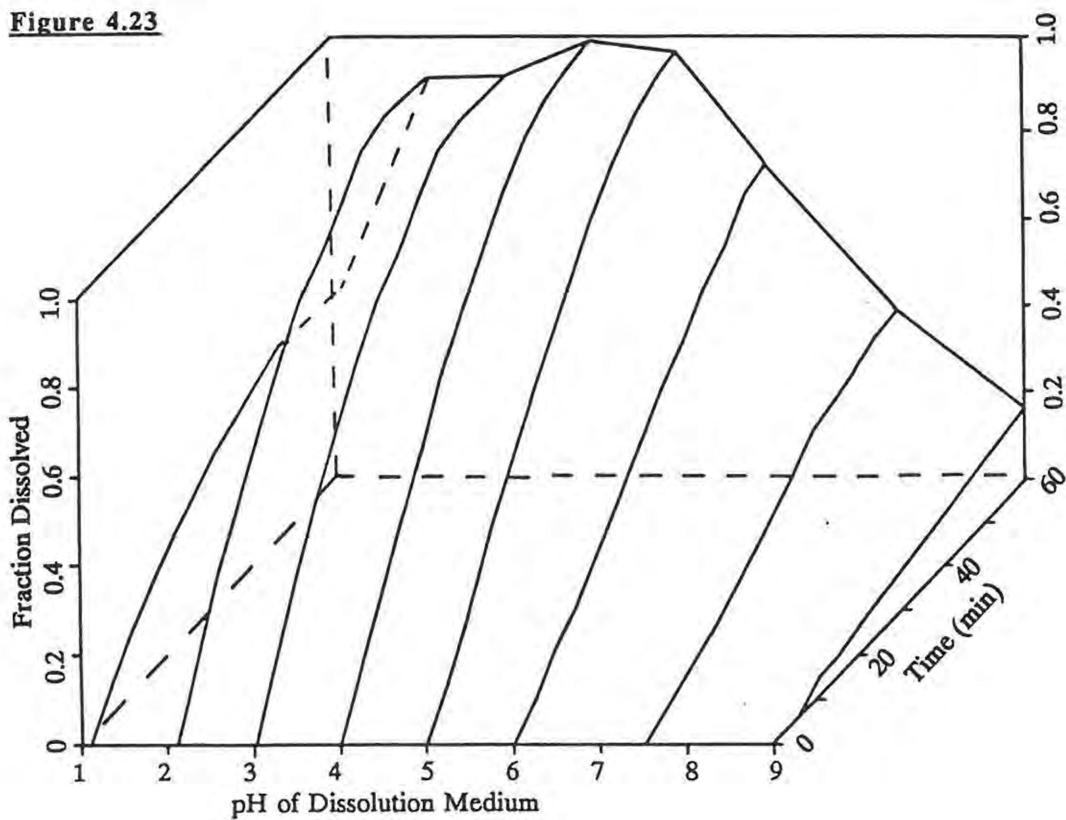


Figure 4.22 3-dimensional plot of the fraction of powder dissolved *vs.* time *vs.* pH for josamycin powder (500 mg) showing the effect of pH on the rate and extent of dissolution.
Figure 4.23 3-dimensional plot of the fraction of tablet content dissolved *vs.* time *vs.* pH for Josacine 500 mg tablets, showing the effect of pH and formulation on the rate and extent of dissolution.

Figure 4.24

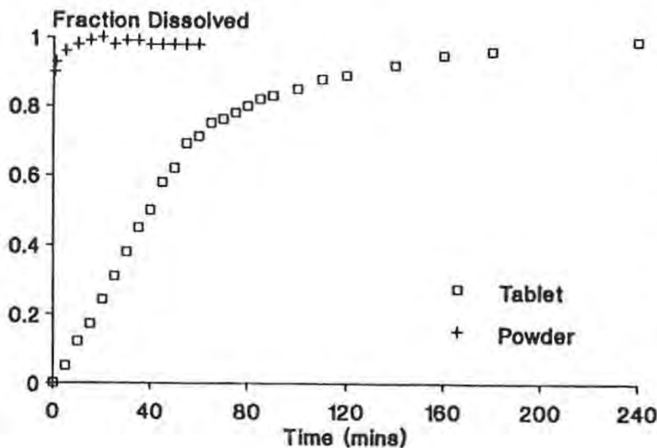


Figure 4.25

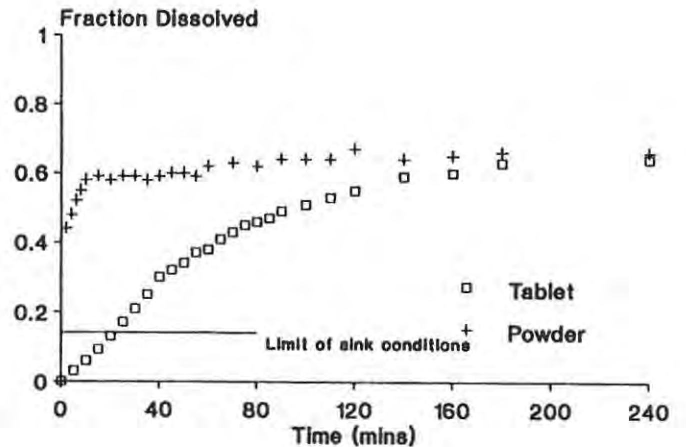
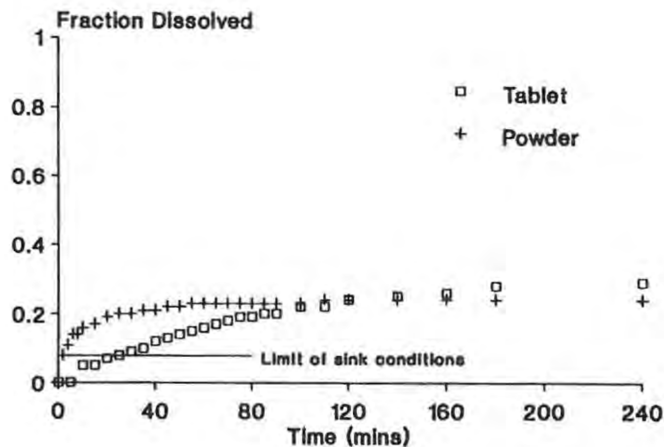


Figure 4.26



Figures 4.24 - 4.26 Dissolution profiles of josamycin powder (500 mg), +; and Josacine 500 mg tablets, □,; from 0 to 240 min at pH 6.0, 7.5 and 9.0 respectively, showing the prolonged dissolution times and the retardation of dissolution of josamycin from the tablets by formulation factors both before and after sink conditions were exceeded.

attained between 20 and 23 min and maximum dissolution attained between 51 and 68 min after the start of the dissolution run. Above pH 5.0, however, dissolution was pH-dependent with the rate and extent of dissolution decreasing with increasing pH. At pH 6.0, fd = 0.5 was attained after 40 min and fd = 1.0 attained after 240 min (Figure 4.24). At pH 7.5, fd = 0.5 was attained after 93 min, however, a maximum fd of only 0.64 was attained after 240 min (Figure 4.25) and at pH 9.0, a maximum fd of only 0.29 was attained after 240 min (Figure 4.26).

As discussed in 4.3.2.1., the pH-dependence of the dissolution profiles can be explained in part by the pH-dependence of the intrinsic dissolution rate and solubility of josamycin, with their limiting effect on dissolution becoming apparent above pH 5.0. This was particularly evident when sink conditions were exceeded. Sink

conditions were maintained throughout the dissolution runs at pH 1.2 to 6.0, but were exceeded at pH 7.5 when $fd = 0.14$ *i.e.* almost immediately for the powder but after 20 min for the tablets, and at pH 9.0 when $fd = 0.08$ *i.e.* again almost immediately for the powder but after 35 min for the tablets. Saturated solutions should have been attained at pH 7.5 and 9.0, however, these concentrations were not reached, also possibly due to poor wettability of the josamycin powder.

Comparison of tablet with powder dissolution profiles show that dissolution from the tablets was markedly slower than from the powder in all dissolution media. Formulation factors therefore severely restricted the dissolution of josamycin from the tablets throughout the entire pH range studied. At pH 1.2 - 5.0, maximum dissolution of the tablets was only attained shortly after the end of a prolonged disintegration period and the dissolution rate appeared to be limited by the disintegration rate at these pH's. At pH 6.0, 7.5 and 9.0, however, dissolution continued well after disintegration was complete and disintegration of the tablets into granules was not considered to significantly influence the dissolution rate. However, incomplete release of josamycin from the granule matrix most likely contributed to the poor dissolution profiles obtained. Mass balance analysis shows that up to 40% and 72% of the tablet content was undissolved at the end of the dissolution runs at pH 7.5 and 9.0 respectively. The presence of numerous non-disintegrated granules in the solid residue remaining after the filtration of dissolution medium for mass balance analysis indicated that part or all of the undissolved josamycin may have remained entrapped in the granule matrix after dissolution at these pH's.

4.4 CONCLUSION

Investigations have shown that the intrinsic dissolution rate and solubility of josamycin is highly dependent on pH, to the extent that bioavailability could be altered significantly by small changes in the pH of the intestinal content at the sight of absorption. The possibility of this was also apparent from the dissolution profiles of josamycin powder (200 mg and 500 mg) when the extent of dissolution was severly limited in dissolution media at intestinal pH's. These dissolution studies also show that the rate and extent of dissolution from Josamycin 200 mg tablets were severely limited by formulation factors in comparison with the already poor dissolution profiles of josamycin powder at pH's above 5.0, and that formulation factors severely limited the rate and extent of dissolution from Josacine 500 mg tablets compared to the powder over the entire pH range studied. Incomplete release of josamycin from tablet matrices and slow disintegration rates were identified as possible causes with their effect on the

dissolution rate being more distinct at higher pH's. Dissolution profiles also show that josamycin would be susceptible to acid degradation in gastric fluids of pH 1.2 and therefore the gastric residence time of dissolved josamycin would significantly influence bioavailability.

Intrinsic dissolution rate and solubility data together with powder dissolution profiles show that after oral administration of the powder, dissolution would be rapid in the acidic contents of the stomach. However, as josamycin is highly ionised in acidic media absorption is unlikely to take place from the stomach (148) but will occur from the intestinal tract where the environment is more alkaline. The rate of absorption will therefore depend on the gastric emptying rate together with the pH of the intestinal tract.

Dissolution profiles of Josamycin 200 mg tablets suggest that if disintegration occurs in the stomach, the bioavailability and absorption characteristics of josamycin from these tablets should be the same as from the powder. However, if complete tablets or intact granules pass into the duodenum (Josamycin 200 mg tablet are relatively small oval shaped tablets, 4 mm diameter x 6 mm long, and could pass throught the pyloric sphincter in as little as 10 min after administration (187)), dissolution would be slow and incomplete as demonstrated by tablet dissolution profiles in dissolution media above pH 5.0. The bioavailability of these tablets can therefore be expected to be significantly less than the powder.

Dissolution profiles of Josacine 500 mg tablets suggest that dissolution in the stomach would be slow and determined by the disintegration rate, after which the rate of absorption would depend on the gastric emptying rate, and subsequently the pH of the intestinal tract. Complete dissolution of the tablet contents would, however, occur although slow dissolution could compromise bioavailability. If non-disintegrated tablets or intact granules passed into the duodenum, the rate and extent of dissolution would be severely limited and extremely poor bioavailabiltiy could be expected.

The pH of the gastric and intestinal fluids together with the gastric retention behaviour of complete or disintegrated tablets could therefore have a profound effect on the bioavailability of josamycin from both these tablet preparations.

Table 4.2 Results of Linear Regression Analysis of Plots of Amount Dissolved (W/S mg.cm^{-2}) *vs.* Time (s) at Each Rotation Speed for Excentrically Mounted Discs at pH 1.2, 2.2, 3.0, 4.0, 5.0, 6.0 and 7.5, and for Centrically Mounted Discs at pH 1.2.

pH	rpm	ω (rad.s^{-1})	G (mg.cm^{-2}.s^{-1}) (slope)	Intercept	Corr. Coeff.
1.2 *	50	5.24	0.3556	-0.7189	0.9955
	50	5.24	0.3327	0.09244	0.9851
	100	10.47	0.6860	5.1511	0.9879
	100	10.47	0.5997	2.3122	0.9939
	200	20.94	1.1247	1.6642	0.9969
	200	20.94	1.1148	5.1813	0.9974
	300	31.42	1.5115	5.8220	0.9964
	300	31.42	1.5158	2.2369	0.9947
2.2 *	50	5.24	0.08517	0.7222	0.9930
	50	5.24	0.09497	0.5853	0.9889
	100	10.47	0.1618	2.7984	0.9755
	100	10.47	0.1584	1.7273	0.9922
	200	20.94	0.2413	2.9282	0.9881
	200	20.94	0.2490	2.5862	0.9918
	300	31.42	0.3452	5.1296	0.9837
	300	31.42	0.3749	3.6520	0.9893
3.0 *	50	5.24	0.04544	0.07778	0.9820
	50	5.24	0.04479	0.2027	0.9839
	100	10.47	0.07244	0.2444	0.9957
	100	10.47	0.08378	-0.1344	0.9966
	200	20.94	0.1486	-0.4711	0.9956
	200	20.94	0.1342	-0.2111	0.9890
	300	31.42	0.1932	-0.4000	0.9974
	300	31.42	0.2212	-1.0116	0.9902
4.0 *	50	5.24	0.01827	-0.01489	0.9649
	50	5.24	0.01717	-0.1267	0.9782
	100	10.47	0.02664	-0.07533	0.9940
	100	10.47	0.02473	0.2304	0.9948
	200	20.94	0.05464	0.2791	0.9978
	200	20.94	0.05038	0.05289	0.9987
	300	31.42	0.06474	0.07422	0.9961
	300	31.42	0.06026	0.07689	0.9970
5.0 *	50	5.24	5.5451×10^{-3}	0.1814	0.9952
	50	5.24	5.7661×10^{-3}	0.1231	0.9982
	100	10.47	0.01040	0.2210	0.9976
	100	10.47	0.01014	0.2757	0.9968
	200	20.94	0.01610	0.2265	0.9990
	200	20.94	0.01615	0.2586	0.9982
	300	31.42	0.02386	0.2109	0.9996
	300	31.42	0.02212	0.2033	0.9998
6.0 *	50	5.24	1.5613×10^{-3}	0.07476	0.9938
	50	5.24	1.5026×10^{-3}	-1.0741×10^{-3}	0.9949
	100	10.47	2.8181×10^{-3}	0.03698	0.9993
	100	10.47	2.6111×10^{-3}	0.1068	0.9958
	200	20.94	3.6614×10^{-3}	-8.9229×10^{-3}	0.9988
	200	20.94	3.5948×10^{-3}	0.03240	0.9968
	300	31.42	4.6254×10^{-3}	0.1295	0.9975
	300	31.42	4.8225×10^{-3}	0.01608	0.9995
7.5 *	50	5.24	1.6671×10^{-4}	6.3299×10^{-3}	0.9941
	50	5.24	1.7656×10^{-4}	8.5422×10^{-3}	0.9958
	100	10.47	2.5345×10^{-4}	0.01772	0.9950
	100	10.47	2.6867×10^{-4}	-2.4936×10^{-3}	0.9938
	200	20.94	3.0793×10^{-4}	0.03737	0.9846
	200	20.94	3.7570×10^{-4}	0.02194	0.9905
	300	31.42	4.2937×10^{-4}	0.01132	0.9904
	300	31.42	4.7754×10^{-4}	5.8696×10^{-3}	0.9902
1.2 **	100	10.47	0.1223	1.0120	0.9833
	100	10.47	0.1405	0.7603	0.9956
	200	20.94	0.1910	1.2290	0.9864
	200	20.94	0.1825	3.2390	0.9886
	300	31.42	0.2420	4.4770	0.9982
	300	31.42	0.2640	5.0056	0.9956

* excentrically mounted discs.
** centrically mounted discs.

Table 4.3 Data for Plots of 1/G *vs.* 1/ω for Excentrically Mounted Discs at pH 1.2, 2.2, 3.0, 4.0, 5.0, 6.0 and 7.5 and for Centrically Mounted Discs at pH 1.2.

	1/G			
pH of Dissolution Medium	50 rpm ω = 5.24 rad.s^{-1} 1/ω = 0.1910	100 rpm ω = 10.47 rad.s^{-1} 1/ω = 0.0955	200 rpm ω = 20.96 rad.s^{-1} 1/ω = 0.0477	300 rpm ω = 31.45 rad.s^{-1} 1/ω = 0.0318
pH 1.2 *	2.812 3.006	1.458 1.668	0.889 0.897	0.662 0.660
pH 2.2 *	11.737 10.526	6.180 6.313	4.144 4.016	2.897 2.667
pH 3.0 *	22.007 22.331	13.805 11.936	6.729 7.452	5.176 4.521
pH 4.0 *	54.735 58.241	37.538 40.437	18.302 19.849	15.446 16.595
pH 5.0 *	180.34 173.43	96.154 98.619	62.112 61.920	41.911 45.208
pH 6.0 *	640.61 665.34	345.86 383.00	273.15 278.16	216.22 207.34
pH 7.5 *	5998.8 5662.5	3944.8 3721.6	3247.8 2661.7	2328.8 2094.2
pH 1.2 **	--- ---	8.1766 7.1174	5.2356 5.4795	4.1322 3.7836

* excentrically mounted discs.
** centrically mounted discs.

Table 4.4 Linear Regression Analysis of Plots of 1/G *vs.* 1/ω for Excentrically Mounted Discs at pH 1.2, 2.2, 3.0, 4.0, 5.0, 6.0 and 7.5 and for Centrically Mounted Discs at pH 1.2.

pH	slope	y - intercept	Corr. Coeff.	G$^{\infty}$ (mg.cm^{-2}.s^{-1})	Log G$^{\infty}$
1.2*	14.2244	0.1938	0.9962	5.1500	0.7126
2.2*	51.1826	1.3779	0.9938	0.7257	-0.1392
3.0*	107.600	1.8976	0.9947	0.5270	-0.2782
4.0*	258.740	8.9674	0.9791	0.1115	-0.9527
5.0*	822.980	19.659	0.9985	0.05087	-1.2936
6.0*	2709.55	129.40	0.9952	0.007728	-2.1119
7.5*	21708.9	1719.7	0.9854	0.0005815	-3.2355
1.2**	55.5980	2.4109	0.9357	0.4148	-0.3822

* excentrically mounted discs.
** centrically mounted discs.

Table 4.5 Results of Linear Regression Analysis of Plots of log G *vs.* pH at 50, 100, 200, 300 rpm and at Infinite Rotation Speed.

Disc Rotation Speed (rpm)	Slope	Intercept	Correlation Coefficient
50	-0.5067	0.1728	0.9923
100	-0.5155	0.4389	0.9913
200	-0.5268	0.6740	0.9859
300	-0.5409	0.8812	0.9888
Infinite	-0.6007	1.4288	0.9904

Table 4.6 Solubility of Josamycin in McIlvaine's Buffer and De-Ionised Water at 37°C.

Initial pH of Buffer	Mass (mg) of Josamycin Added to Buffer (2 mls)	pH of Saturated Solution	Solubility C_s mg.ml^{-1}	Molar Solubility C_s M.ml^{-1}
4.0	600.00	5.45	212.00	0.25
	600.00	5.45	212.00	0.25
	600.00	5.45	212.00	0.25
5.0	73.17	5.85	20.25	0.025
	72.74	5.90	19.75	0.024
	73.73	5.90	20.25	0.025
6.0	30.57	6.19	5.20	6.29×10^{-3}
	30.48	6.14	5.13	6.20×10^{-3}
	30.49	6.15	4.98	6.02×10^{-3}
6.5	20.82	6.55	1.97	2.38×10^{-3}
	20.15	6.54	1.92	2.32×10^{-3}
	20.13	6.55	1.99	2.41×10^{-3}
7.0	6.77	7.02	0.72	8.71×10^{-4}
	6.57	7.03	0.71	8.59×10^{-4}
	7.26	7.05	0.71	8.59×10^{-4}
7.5	6.53	7.49	0.34	4.11×10^{-4}
	6.16	7.52	0.33	3.99×10^{-4}
	6.47	7.51	0.34	4.11×10^{-4}
8.0	6.76	7.99	0.22	2.66×10^{-4}
	6.93	7.99	0.21	2.54×10^{-4}
	7.13	8.00	0.21	2.54×10^{-4}
8.5	3.24	8.47	0.19	2.30×10^{-4}
	4.31	8.49	0.19	2.30×10^{-4}
	3.70	8.47	0.19	2.30×10^{-4}
9.0	3.33	8.94	0.18	2.18×10^{-4}
	4.19	8.79	0.17	2.06×10^{-4}
	3.82	8.95	0.18	2.18×10^{-4}
De-ionised Water	5.11	7.40	0.56	6.77×10^{-4}
	6.88	7.09	0.55	6.65×10^{-4}
	7.40	7.63	0.57	6.89×10^{-4}

Table 4.7 Dissolution Data for Josamycin Powder (200 mg), Josamycin 200 mg Tablets, Josamycin Powder (500 mg) and Josacine 500 mg Tablets .

		Josamycin Powder (200 mg)			Josamycin 200 mg Tablets						
pH of Dissolution Medium	Run Time (min)	Time to fd * =0.5 (min)	Time to fd * =1.0 (min)	Maximum Conc. Reached (mg/ml) **	Lag Time (min)	Disintegration Time (min)	Time to fd * =0.5 (min)	Time to fd * =1.0 (min)	Maximum Conc. Reached (mg/ml) **	Solubility (mg/ml)	Limit of Sink Conditions (mg/ml) ***
		x ±S.D.	x ±S.D.	x ±S.D.	x ±S.D.	x ±S.D.	x ±S.D.	x ±S.D.	x ±S.D.		
1.2 n = 6	60	—	0.58 0.18	0.195 0.009	11 3	16 2	15 2	na ****	0.162 0.010	> 212.00	> 31.80
2.2 "	60	—	0.83 0.24	0.225 0.007	12 1	16 3	15 2	17.8 3.3	0.202 0.002	> 212.00	> 31.80
3.0 "	60	—	2.75 2.25	0.219 0.004	12 1	17 3	15 1	19.0 2.9	0.196 0.004	> 212.00	> 31.80
4.0 "	60	—	5.00 0.00	0.200 0.002	15 1	19 2	18 1	23.8 1.9	0.196 0.003	> 212.00	31.80
5.0 "	60	—	7.00 1.91	0.211 0.007	15 2	18 2	18 2	14.2 13.7	0.195 0.002	21.00	4.20
6.0 "	60	—	20.0 5.00	0.217 0.005	12 2	15 2	18 1	58.3 3.7	0.202 0.002	5.00	0.75
7.5 "	240	6.00 1.15	na ****	0.160 0.007	13 3	17 2	83 5	na ****	0.138 0.022	0.34	0.07
9.0 "	240	173.0 29.8	na ****	0.103 0.021	12 2	16 2	na ****	na ****	0.100 0.002	0.18	0.04

		Josamycin Powder (500 mg)			Josacine 500 mg Tablets						
pH of Dissolution Medium	Run Time (min)	Time to fd * =0.5 (min)	Time to fd * =1.0 (min)	Maximum Conc. Reached (mg/ml) **	Lag Time (min)	Disintegration Time (min)	Time to fd * =0.5 (min)	Time to fd * =1.0 (min)	Maximum Conc. Reached (mg/ml) **	Solubility (mg/ml)	Limit of Sink Conditions (mg/ml) ***
		x ±S.D.	x ±S.D.	x ±S.D.	x	x ±S.D.	x ±S.D.	x ±S.D.	x ±S.D.		
1.2 n = 6	60	—	na ****	0.433 0.011	< 5	60 9	na ****	na ****	0.230 0.010	> 212.00	> 31.80
2.2 "	60	—	0.5 0.0	0.528 0.002	< 5	45 6	20 0	51 5	0.460 0.010	> 212.00	> 31.80
3.0 "	60	—	5.1 2.7	0.498 0.006	< 5	46 7	22 2	57 6	0.470 0.010	> 212.00	> 31.80
4.0 "	60	—	0.0 0.0	0.531 0.005	< 5	42 6	23 3	56 5	0.510 0.010	> 212.00	31.80
5.0 "	60	—	2.5 0.0	0.509 0.009	< 5	52 4	23 3	68 3	0.520 0.020	21.00	4.20
6.0 "	240	—	6.9 5.2	0.512 0.021	< 5	44 3	40 3	240 0	0.500 0.010	5.00	0.75
7.5 "	240	2.0 0.0	na ****	0.330 0.013	< 5	39 3	93 6	na ****	0.320 0.010	0.34	0.07
9.0 "	240	na ****	na ****	0.123 0.003	< 5	43 5	na ****	na ****	0.150 0.010	0.18	0.04

* fd - fraction of tablet content dissolved.
** maximum concentration attainable = 0.2 mg/ml or 0.5 mg/ml when fd = 1.0 for 200 or 500 mg tablets or powder respectively.
*** sink conditions calculated as 20% of solubility.
**** na - not attained.

Table 4.8 Mass Balance Analysis Results for Dissolution Studies on Josamycin 200 mg Tablets and Josacine 500 mg Tablets.

		Josamycin 200 mg Tablets			
pH of Dissolution Media	Run Time (min)	Fraction Dissolved at End of Run (fd_e)*	Fraction Undissolved at End of Run (fu_e)**	Fraction Unchanged at End of Run ***	Fraction Degraded at End of Run ****
		x ±S.D.	x ±S.D.	x ±S.D.	x ±S.D.
1.2 n = 6	60	0.39 0.03	0.00 0.00	0.39 0.03	0.61 0.03
2.2 "	60	0.88 0.01	0.00 0.00	0.88 0.01	0.12 0.01
3.0 "	60	0.98 0.02	0.00 0.00	0.98 0.02	0.02 0.02
4.0 "	60	0.98 0.01	0.00 0.00	0.98 0.01	0.02 0.01
5.0 "	60	0.97 0.01	0.00 0.00	0.97 0.01	0.03 0.01
6.0 "	60	1.01 0.01	0.00 0.00	1.01 0.01	0.00 0.01
7.5 "	240	0.69 0.02	0.32 0.03	1.01 0.02	0.00 0.01
9.0 "	240	0.50 0.01	0.51 0.01	1.01 0.02	0.00 0.01

		Josacine 500 mg Tablets			
pH of Dissolution Media	Run Time (min)	Fraction Dissolved at End of Run (fd_e) *	Fraction Undissolved at End of Run (fu_e) **	Fraction Unchanged at End of Run ***	Fraction Degraded at End of Run ****
		x ±S.D.	x ±S.D.	x ±S.D.	x ±S.D.
1.2 n = 6	60	0.46 0.02	0.00 0.00	0.46 0.02	0.54 0.02
2.2 "	60	0.91 0.01	0.00 0.00	0.91 0.01	0.09 0.01
3.0 "	60	0.92 0.02	0.00 0.00	0.92 0.02	0.08 0.02
4.0 "	60	0.97 0.03	0.00 0.00	0.97 0.03	0.03 0.03
5.0 "	60	1.04 0.03	0.00 0.00	1.04 0.03	0.00 0.03
6.0 "	240	0.99 0.01	0.00 0.00	0.99 0.01	0.00 0.01
7.5 "	240	0.64 0.02	0.40 0.01	1.04 0.02	0.00 0.02
9.0 "	240	0.29 0.01	0.72 0.07	1.01 0.05	0.00 0.05

* fd_e = fraction of tablet content dissolved at end of run.
** fu_e = fraction of tablet content undissolved at end of run.
*** $fd_e + fu_e$ = fraction unchanged at end of run.
**** fraction degraded = tablet content - fraction unchanged.

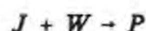
CHAPTER 5

STABILITY OF JOSAMYCIN IN AQUEOUS SOLUTION

If a drug is to be administered orally in a formulation where dissolution in the stomach is facilitated, stability over the entire gastro-intestinal pH range is essential for good bioavailability. For example, erythromycin, a commonly used macrolide antibiotic, exhibits appreciable instability in acid when it exists as the free base. Erratic bioavailability profiles after oral administration have thus been ascribed to this instability (188,189). The instability of erythromycin at gastric pH's was first reported by Wiley in 1957 (52) and later by Kurath in 1971 (53) who investigated the degradation products formed under strongly acid conditions. More recently, the rates of reaction and degradation pathways have been studied by Atkins *et al.* (190) and Vinckier *et al.* (51). Both authors found the acid degradation of erythromycin to be complex and have presented evidence for various possible pathways. Boggiano and Gleeson (50) assessed the stability of erythromycin from a clinical viewpoint and found that up to 90% of an oral dose of the base could be inactivated in the stomach within five minutes. Overcoming this problem has been of major concern and has led to the emergence of enteric-coated erythromycin base formulations, and also to the development of more stable derivatives such as erythromycin estolate (191), which is more stable in acid by virtue of its insolubility at low pH, and azithromycin (192). Erythromycin stearate was also developed as an insoluble acid stable derivative (50,193), however, it has been shown to exhibit acid instability similar to erythromycin base (50). In a recently published paper, Stella *et al.* (194) found that another macrolide antibiotic Rhizoxin also exhibited poor stability characteristics. Despite the fact that josamycin has been marketed for a number of years, only sparse information is available on its stability and no comprehensive stability study has been published. It is, however, reported to be *remarkably stable compared to erythromycin* over the pH range of 2 - 4 (195). This absence of stability data, the known susceptibility of most macrolides to acid degradation, and the erratic bioavailability profiles obtained after the oral administration of two commercially available tablet preparations containing josamycin (*vide infra* chapter 6), prompted a comprehensive investigation into the pH-stability of josamycin.

The main objective of most pharmaceutical stability studies is to determine the order of the reaction, rate constant for the reaction, the factors which influence the rate constant and the pathway by which degradation occurs. This can be achieved by monitoring the change in concentration of the parent compound with time together with the formation of any degradation products under varoius reaction conditions. For

example, consider a reaction involving a drug molecule J and a second molecule W which are present in the reaction medium in similar concentrations, and which react to give some product P, *i.e.*



$$J + W \rightarrow P$$

The rate of reaction is proportional to the product of the concentrations of the two reacting species (196) *i.e.*

$$rate \propto [J][W] \qquad \text{Eq. 5.1}$$

The rate of change in the concentration of J can be written as -d[J]/dt and the differential rate equation for the reaction can therefore be written as

$$rate = -\frac{d[J]}{dt} = k_2[J][W] \qquad \text{Eq. 5.2}$$

Such a reaction is said to be an overall second order reaction as the rate is dependent on the concentration of both the reacting species, and k_2 is known as the second order rate constant. If [J] and [W] are equal, equation 5.2 can be written as (197)

$$\frac{d[J]}{dt} = -k_2[J]^2 \qquad \text{Eq. 5.3}$$

and when integrated between the limits of t = 0 and t = t when [J] at t = 0 is $[J]_0$, *i.e.*

$$\int_{[J]_0}^{[J]} \frac{d[J]}{[J]^2} = -\int_0^t k_2.dt \qquad \text{Eq. 5.4}$$

yields

$$[J]_t^{-1} = [J]_0^{-1} + k_2 t \qquad \text{Eq. 5.5}$$

If reciprocal concentration *vs.* time data are plotted according to equation 5.5 and a straight line is obtained, the reaction can be said to be second order with the slope of the line equal to the second order rate constant k_2 with units of $conc.^{-1}.time^{-1}$. However, the majority of bi-molecular degradation reactions of drugs involve hydrolysis by water and if the drug is in solution, then the concentration of water is greatly in excess of the concentration of drug. For a reaction which proceeds according to $J + W \rightarrow P$, where W is a water molecule, the change in concentration of W will be negligible in comparison to that of J. The concentration of water can

therefore be assumed to be constant for the duration of the reaction and equation 5.2 can be rewritten as (196)

$$-\frac{d[J]}{dt} = k_1[J] \qquad \text{Eq. 5.6}$$

where [W] is incorporated into the constant k_2 such that $k_1 = k_2[W]$. The rate of change in [J] is therefore now dependent only on [J] and the reaction is said to be an "apparent or pseudo first-order reaction". Integration of equation 5.6 from t=0 to t=t where [J] at t=0 is $[J]_0$, (196) *i.e.*

$$\int_{[J]_0}^{[J]} \frac{d[J]}{[J]} = -\int_0^t k_1 dt \qquad \text{Eq. 5.7}$$

yields

$$\ln [J] = \ln [J]_0 - k_1 t \qquad \text{Eq. 5.8}$$

If log concentration *vs.* time data are plotted according to equation 5.8 and a straight line is obtained, the reaction can be said to be pseudo first-order with the slope of the line equal to the pseudo first-order rate constant k_1 with units of $time^{-1}$, and with the y-intercept equal to $[J]_0$.

In solution, numerous factors such as pH, buffer concentration and ionic strength can also markedly affect the rate of reaction. Any change in these factors during a reaction leads to complex rate equations. However, by keeping these solution variables constant throughout a reaction the study can be simplified and the pseudo first-order rate constants can be calculated. The effect of each solution variable on the pseudo first-order rate constant can be determined by following the course of reaction under pseudo first-order conditions at different concentrations of the species under investigation.

Effect of pH:- For most reactions involving hydrolysis, the rate of reaction is determined by the catalytic effect of the solvated proton or hydronium ion (H^+) on hydrolysis, referred to as specific acid catalysis; the catalytic effect of the solvated hydroxide ion (OH^-) on hydrolysis, referred to as specific base catalysis; and to the catalytic effect of water itself on hydrolysis. The general equation describing the rate of reaction with respect to pH can then be written as (198)

$$rate = k_1[H^+]^n[J] + k_2[J] + k_3[OH^-]^m[J] \qquad \text{Eq. 5.9}$$

where k_1 ($conc^{-1}.time^{-1}$) is the second order rate constant describing catalysis due to

the hydrogen ion, k_2 (time^{-1}) is the first order rate constant describing catalysis due to water, and k_3 (conc^{-1}.time^{-1}) is the second order rate constant describing catalysis due to the hydroxide ion. Values of n and m are the orders of reaction with respect to H^+ and OH^- respectively and are usually equal to +1 and -1 if a compound undergoes specific acid and specific base catalysed hydrolysis. If however, the pH is maintained at a constant level during a reaction and it proceeds under pseudo first-order conditions, the rate of reaction can be written as

$$rate = K[J] \qquad \text{Eq. 5.10}$$

where K is the overall pseudo first-order rate constant for the reaction. Combining equations 5.9 and 5.10, an equation describing K with respect to $[H^+]$ and $[OH^-]$ and therefore pH is obtained as in equation 5.11.

$$K = k_1[H^+]^n + k_2 + k_3[OH^-]^m \qquad \text{Eq. 5.11}$$

Plots of log K *vs.* pH yield the pH-rate profile for the compound and if the reaction adheres strictly to equation 5.9, the profile will be either V-shaped, if the reaction is purely acid and base catalysed, or U-shaped if catalysis by water is significant. The shape and the point of maximum stability is dependent on the relative importance of each type of catalysis. The catalytic rate constants can be determined directly from the pH-rate profile as follows. At regions of low pH were k_2 and k_3 can be considered negligible in comparison with k_1,

$$K = k_1[H^+]^n \qquad \text{Eq. 5.12}$$

and therefore

$$\log K = \log k_1 - n.pH \qquad \text{Eq. 5.13}$$

A plot of log K *vs.* pH will yield a straight line with a negative gradient, indicating dependence of the rate of reaction on the concentration of the hydronium ion, with the gradient equal to -n and a y-intercept equal to log k_1. Conversely, at regions of high pH, k_1 and k_2 can be considered negligible in comparison with k_3, and a plot of log K *vs.* pH will also yield a straight line but with a positive gradient of +m (198) indicating dependence of the rate of reaction on the concentration of the hydroxide ion. Under these conditions

$$K = k_3[OH^-]^m \qquad \text{Eq. 5.14}$$

and taking logs of equation 5.14,

$$\log K = \log k_3 - m.pOH \qquad \text{Eq. 5.15}$$

k_3 can be obtained from the y-intercept of a plot of log K *vs.* pOH. Once k_1, k_3, n and m have been calculated, k_2 can be calculated utilising equation 5.11 together with a value of K determined experimentally at any pH. Once all constants are calculated, the pH-rate profile for the reaction is fully characterised.

Effect of ionic strength:- Besides pH, the ionic strength (μ) of the reaction medium can also influence the rate of reaction if charged species are involved in any part of the reaction process (198,199). For example, if a drug is positively charged and its hydrolysis is catalysed by the hydronium ion, then an increase in ionic strength will cause an increase in the reaction rate. Conversely, if a drug is positively charged and its hydrolysis is catalysed by the hydroxide ion, then an increase in ionic strength will cause a decrease in the reaction rate. This is known as the "primary or kinetic salt effect" and is described by the Brønstead-Bjerrum equation:-

$$\log K = \alpha + 2.Q.Z_A.Z_B.\sqrt{\mu} \qquad \text{Eq. 5.16}$$

which is derived from transition-state theory and the Debye-Hückel limiting law (200), where K is the reaction rate constant, α and 2Q are constants and Z_A and Z_B are the charges of the reacting species. The Debye-Hückel limiting law, and therefore equation 5.16, is valid only for solutions of low ionic strength (μ = <0.01). For solutions of higher ionic strength the modified Debye-Hückel equation can be used which results in equation 5.17

$$\log K = \alpha + 2.Q.Z_A.Z_B.\frac{\sqrt{\mu}}{1 + \sqrt{\mu}} \qquad \text{Eq. 5.17}$$

A plot of log k *vs.* $\sqrt{\mu}/(1+\sqrt{\mu})$ should therefore be linear if equation 5.17 holds over the range of μ studied. This equation has successfully described the effect of ionic strength on the rate of reaction for ionic strengths as high as 1.2 (201,202). Ionic strength is therefore an important variable to consider during stability studies and all buffer solutions used should be adjusted to a constant ionic strength with an inert electrolyte *e.g.* NaCl or KCl.

Buffers used in maintaining the pH of a reaction medium constant can also affect the reaction rate if any of the buffer species act as a catalyst. Any catalytic effect dependent on the buffer concentration is known as the "secondary salt effect", and can be investigated by conducting reactions in solutions of varying buffer concentrations but of a constant ionic strength. Catalysis of this nature can also be

affected by ionic strength if the secondary salt effect is due predominantly to either the ionised or un-ionised form of the buffer and the ionic strength is high enough to alter the ionisation constant of the buffer (198,199).

5.1 KINETIC STUDIES

5.1.1 Experimental

5.1.1.1 pH Effects

The pH-dependent hydrolysis of josamycin in aqueous media was investigated from two different approaches:-

Study A:- Determination of the pH-rate profile from pH 1.0 to 12.0 using solutions of constant ionic strength.

Solutions used for this investigation were prepared as follows:- Hydrochloric acid was diluted to provide solutions of pH 1.0, 1.2 and 1.5. At pH 2.2, 3.0, 4.0, 4.5, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, McIlvaine's buffer prepared as in 4.1.1.1 was used. At pH 8.5, 9.0, 10.0, 11.0 and 12.0, Teorell and Stenhagen's buffer (103) was used. Each solution was adjusted to μ = 1.0 with an appropriate quantity of sodium chloride. The high molarity of the McIlvaine buffers ($\pm$0.1 M at pH 2.2 to $\pm$0.2 M at pH 8.0) ensured that the pH could be maintained constant over long periods of time. This was essential for the determination of degradation rates between pH 4.0 and 8.0. However, the high molarity required that all solutions be adjusted to an unusually high ionic strength of 1.0 in order to accommodate all the buffers used.

Study B:- Investigation of the degradation of josamycin in simulated gastric and intestinal fluids.

For these investigations, hydrochloric acid at pH 1.0 and 1.5 unadjusted for ionic strength, USP simulated gastric fluid without enzymes (pH 1.2) and USP simulated intestinal fluid without enzymes (pH 7.5) were used.

For both studies, a stock solution was prepared by weighing 200 mg of josamycin into a 10 ml volumetric flask and dissolving and making up to volume with acetonitrile. Each reaction was initiated by inoculating 5 mls of buffer, previously pipetted into a 15 ml Kimax screw-capped test-tube and heated to 37°C, with 250 μl of stock solution for buffers of pH 1 - 4 (resulting in a josamycin concentration of

1.0 mg/ml or 1.2092×10^{-3} M), or 25 µl of stock solution for buffers of pH 4.5 - 12.0 (resulting in a josamycin concentration of 0.1 mg/ml or 1.2092×10^{-4} M). The inoculated buffer solutions were then mixed by vortexing for 10 s to ensure uniform mixing and immediately returned to a constant temperature water-bath maintained at 37°C. Light was excluded for the duration of the reaction, apart from short intervals at each sampling time. At each sampling time, the solution was mixed by vortexing after which a 200 µl aliquot was removed. To prevent further degradation, the sample was immediately added to a 200 µl aliquot of 0.2 M di-sodium hydrogen phosphate solution previously pipetted into an Eppendorf 1.5 ml teflon reaction tube and mixed by vortexing for 10 s. Samples were then analysed as detailed in 2.2.5. All reactions were done in triplicate.

5.1.1.2 Ionic Strength and Buffer Concentration Studies

The effect of ionic strength on the degradation rate of josamycin in acidic media was determined using solutions of hydrochloric acid of pH 1.0 adjusted to ionic strengths of 0.1, 0.2, 0.5 and 1.0 with sodium chloride.

The effect of buffer concentration on the degradation rate of josamycin in acidic media was determined using undiluted McIlvaine's buffer of pH 2.2 together with three additional solutions prepared by the 1 in 5, 1 in 4 and 1 in 2 dilution of this buffer. All solutions were adjusted to an ionic strength of 1.0. Reactions were initiated in triplicate and samples taken and prepared for analysis as described in 5.1.1.1.

5.1.1.3 Methods to Determine the Order of Reaction

The order of a reaction may be determined from first-order (ln concentration *vs.* time) or second-order (reciprocal concentration *vs.* time) plots according to equations 5.8 and 5.5 respectively. As stated earlier, a first-order reaction will yield a straight line for a ln concentration *vs.* time plot whilst a second order reaction will yield a straight line for a reciprocal concentration *vs.* time plot. The order of reaction can also be determined utilising equation 5.18 (197),

$$\log(-R)_x = \log k + n \log[J_0]_x \qquad \text{Eq. 5.18}$$

where R is the apparent zero-order rate of reaction (with units of conc.time^{-1}) determined by linear regression of a cartesian plot of concentration *vs.* time data for the initial 10% of the reaction, and $[J_0]$ is the initial concentration of josamycin. By determining R at various initial concentrations, the order of reaction can be determined from a plot of $\log(-R)_x$ *vs.* $\log[J_0]_x$ which yields a straight line with a slope equal to the order of reaction. Values for R were determined with $[J_0]$ at 0.268,

0.201, 0.134 and 0.067 mg/ml at pH 2.2 and an ionic strength of 1.0 to confirm the order of reaction in acidic media utilising equation 5.18.

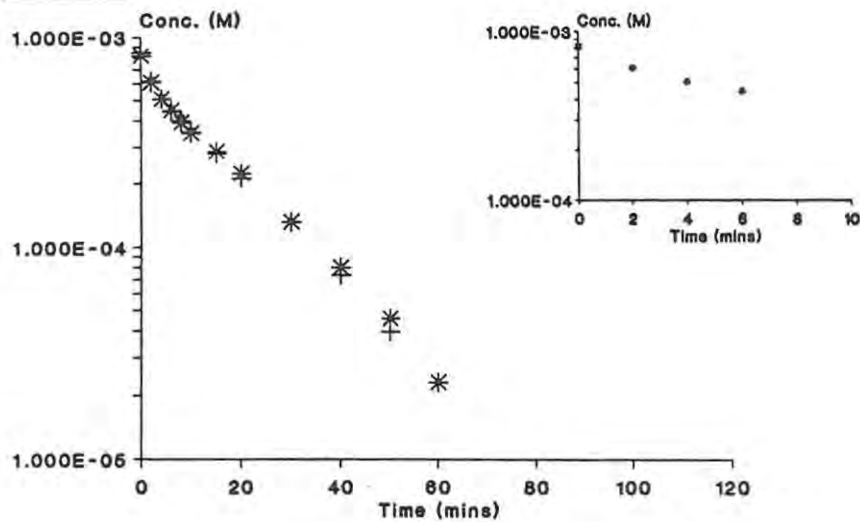
5.1.2 Results and Discussion

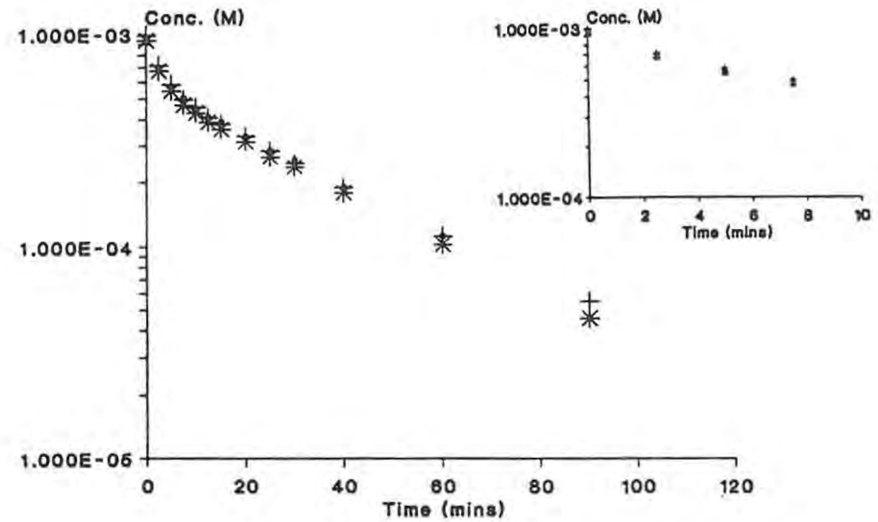
5.1.2.1 Degradation Rates and pH-Rate Profile

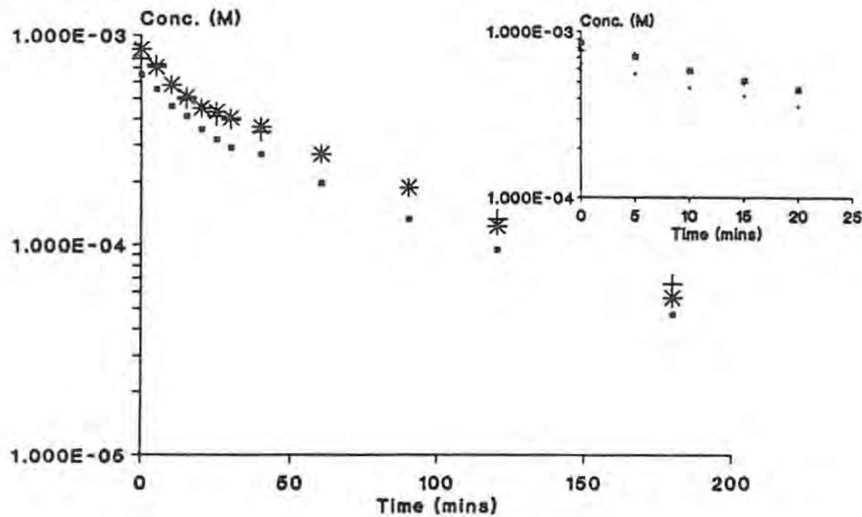
Semilogarithmic plots of the molar concentrations of josamycin remaining *vs.* time at each pH with μ equal to 1.0 are shown in figures 5.1 to 5.19. From pH 1.0 to 6.5, three degradation products were evident in the HPLC chromatograms (Figure 2.7c) and a single reaction pathway appeared to be followed over this pH range. From pH 1.0 to 5.0 (Figures 5.1 - 5.8), first-order plots were biphasic, each with an initial linear phase followed by a terminal linear phase. As the same reaction pathway appeared to be followed from pH 1.0 to pH 6.5, biphasic plots were also expected at pH 5.5, 6.0 and 6.5 had the reactions been followed to completion. However, as degradation at these pH's was extremely slow only a small portion of the reaction was monitored and only the initial linear phase characterised. Above pH 6.5 the reaction was more complex and up to five degradation products were detected (Figure 2.8). For the initial portion of the reaction at pH 7.0 and for the entire reaction (*i.e.* to completion) at pH 7.5 to 9.0 (Figures 5.12 - 5.16) first-order plots were linear, whilst at pH 10.0 to 12.0 (Figures 5.17 - 5.19) slightly biphasic first-order plots were obtained. For all biphasic plots, apparent first-order rate constants were determined for the initial linear portion (K_{init}) and for the terminal linear portion (K_{term}) by linear regression of the data points within each linear region. For the monophasic plots, a single apparent first-order rate constant (K_{init}) was determined by linear regression of all points. First-order plots of the data points used for determining K_{init} at pH 1.0 to 5.0 and pH 10.0 to 12.0 are depicted in the inserts of figures 5.1 to 5.8 and 5.17 to 5.19 respectively. Linear regression was performed by the computer program LINREG (197). The rate constants K_{init} and K_{term} obtained are listed in table 5.1 and the pH-rate profile depicted graphically in figure 5.20.

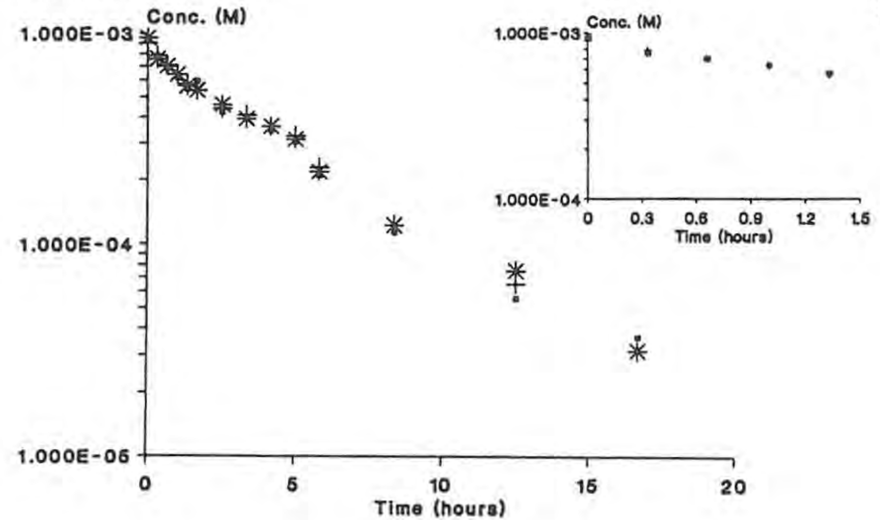
The pH-rate profile for K_{init} was V-shaped showing a marked dependence of the degradation rate on pH. Josamycin undergoes rapid degradation at low pH with a maximum K_{init} of 7.2716 hr^{-1} at pH 1.0 (μ = 1.0). As the pH increased above 1.0, K_{init} rapidly decreased over five orders of magnitude to a minimum of 1.4609×10^{-4} hr^{-1} at pH 6.5. Thereafter, K_{init} rapidly increased with increasing pH again over five orders of magnitude to a maximum of 1.4842 hr^{-1} at pH 12.0. The K_{term} *vs.* pH profile (Figure 5.20) was parallel to that of K_{init}.

The V-shaped pH-rate profile is typical of a compound that undergoes specific acid and specific base catalysed reactions. For K_{init}, the linear portion of the profile

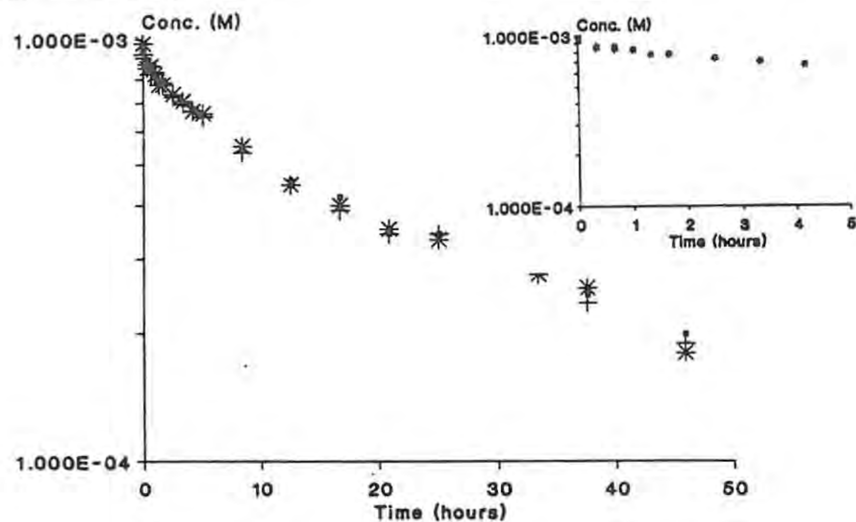


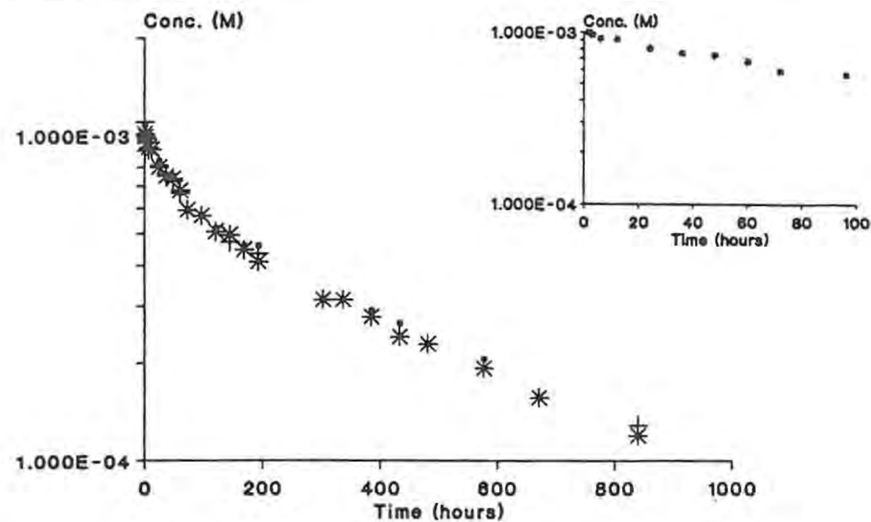


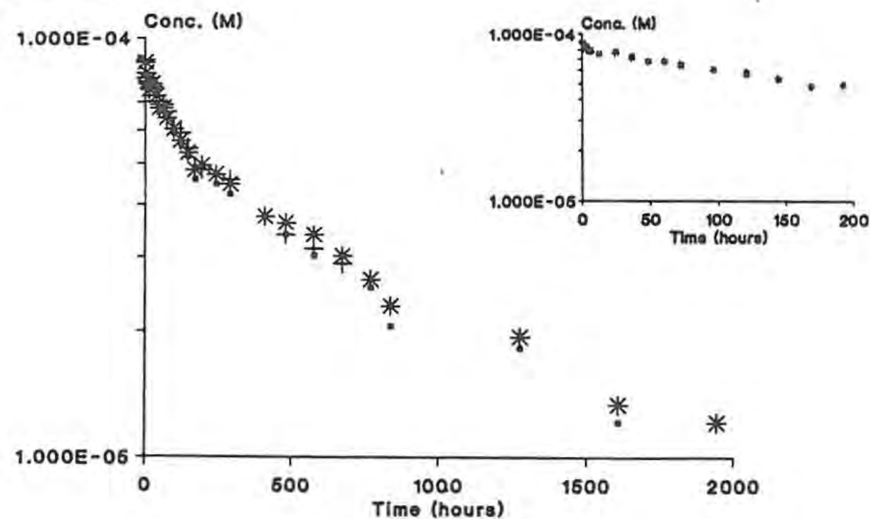


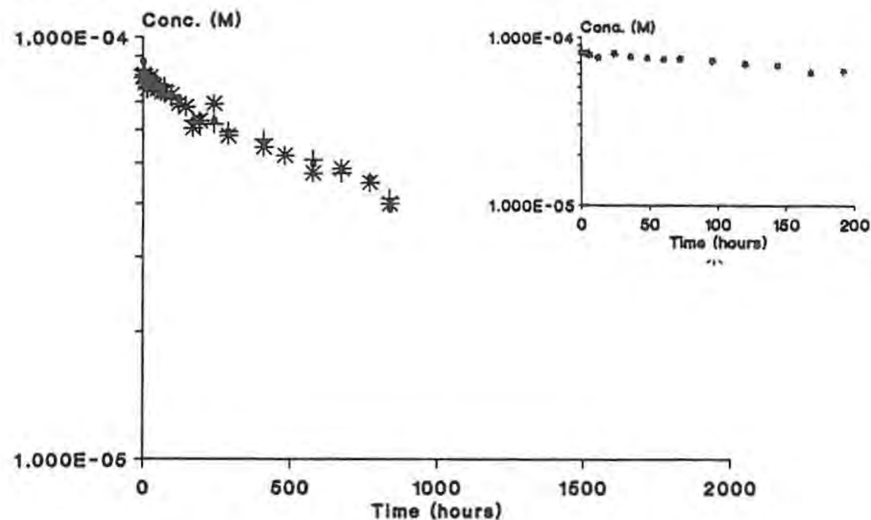


Figures 5.1 - 5.4 First-order concentration *vs.* time plots for josamycin in aqueous media at pH 1.0, 1.2, 1.5 and 2.2 respectively, at 37°C with μ = 1.0. Inserts are expanded plots of the initial linear portion of the respective profiles used for the determination of K_{init} by linear regression (▪, + and ⁎ represent replicate experiments).

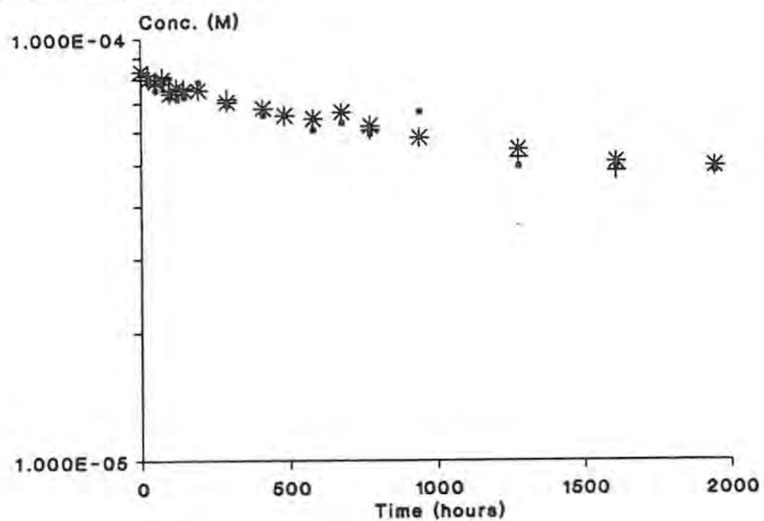


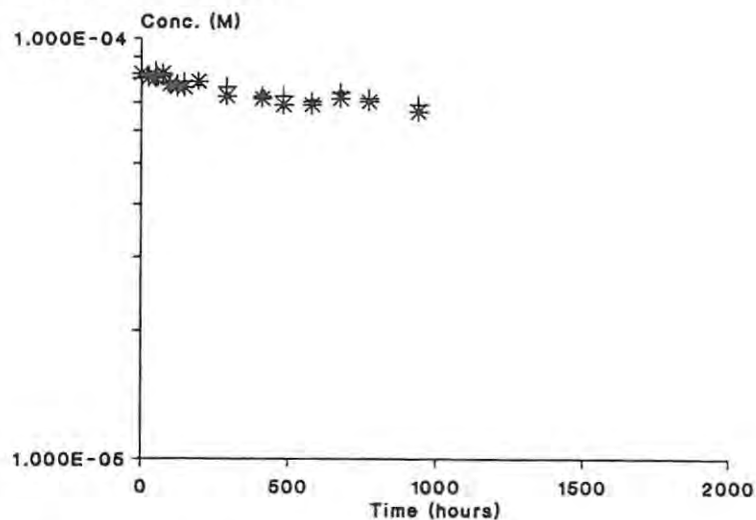


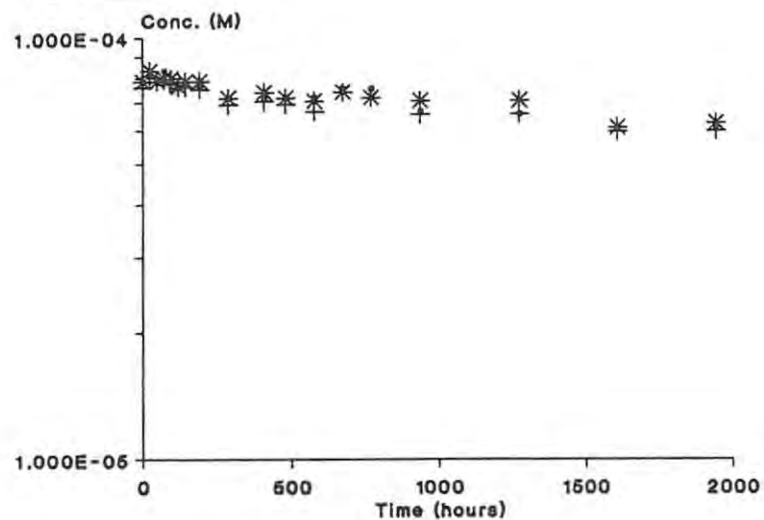


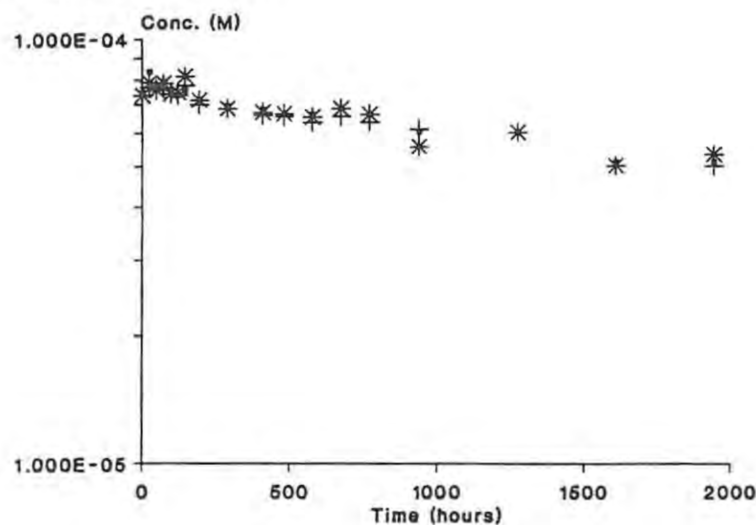


Figures 5.5 - 5.8 First-order concentration *vs.* time plots for josamycin in aqueous media at pH 3.0, 4.0, 4.5 and 5.0 respectively, at 37°C with μ = 1.0. Inserts are expanded plots of the initial linear portion of the respective profiles used for the determination of K_{init} by linear regression (▪, + and ✱ represent replicate experiments).

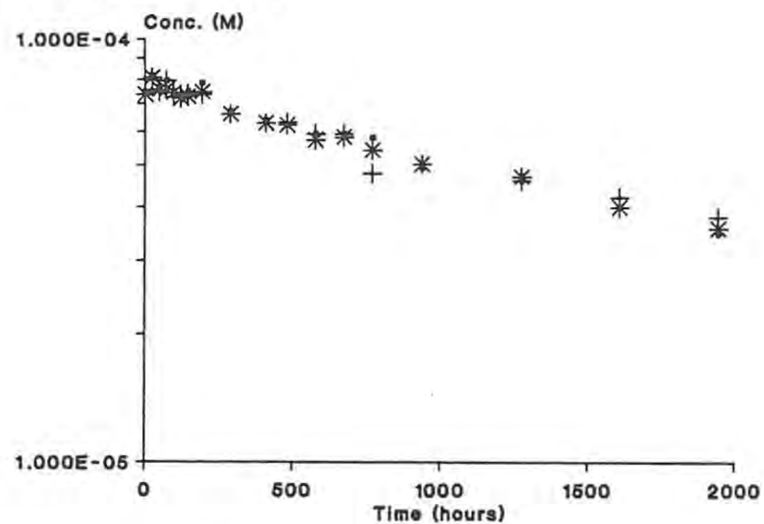


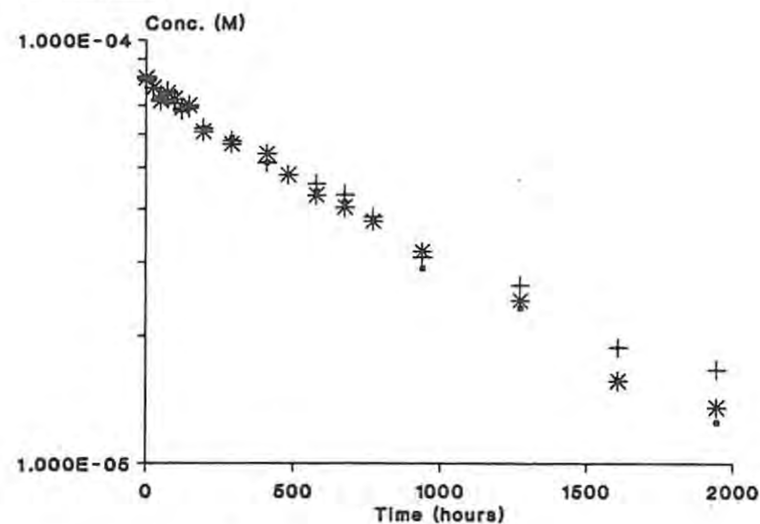


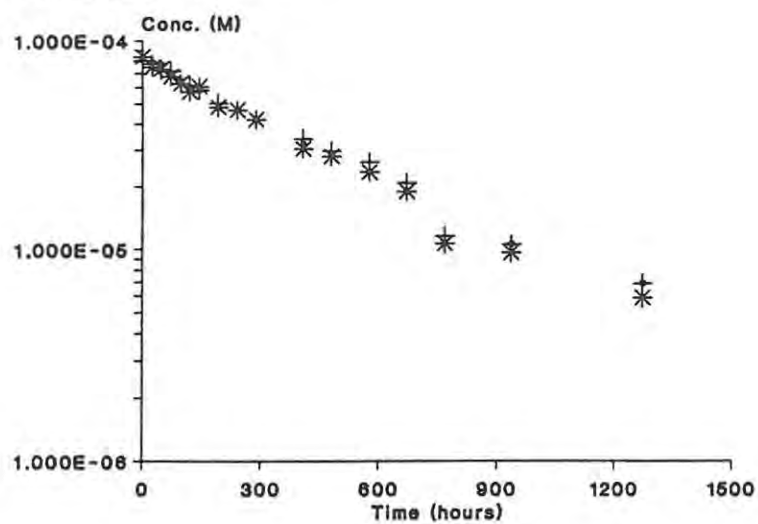


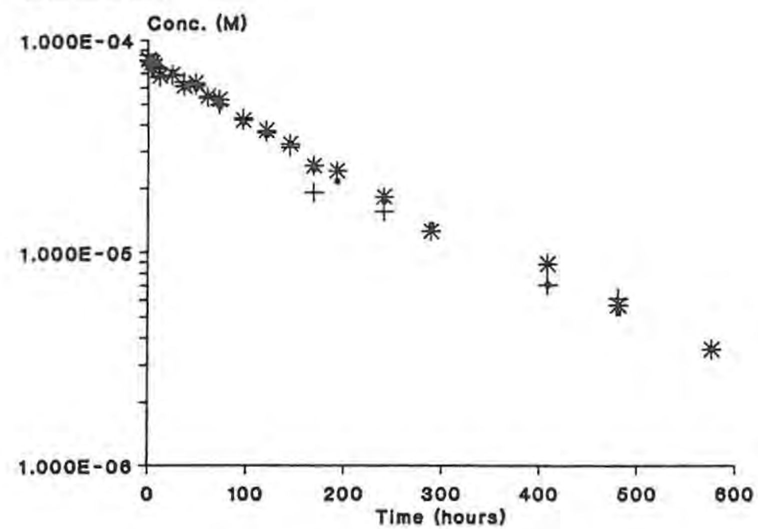


Figures 5.9 - 5.12 First-order concentration *vs.* time plots for josamycin in aqueous media at pH 5.5, 6.0, 6.5 and 7.0 respectively, at 37°C with μ = 1.0 (▪, + and * represent replicate experiments).

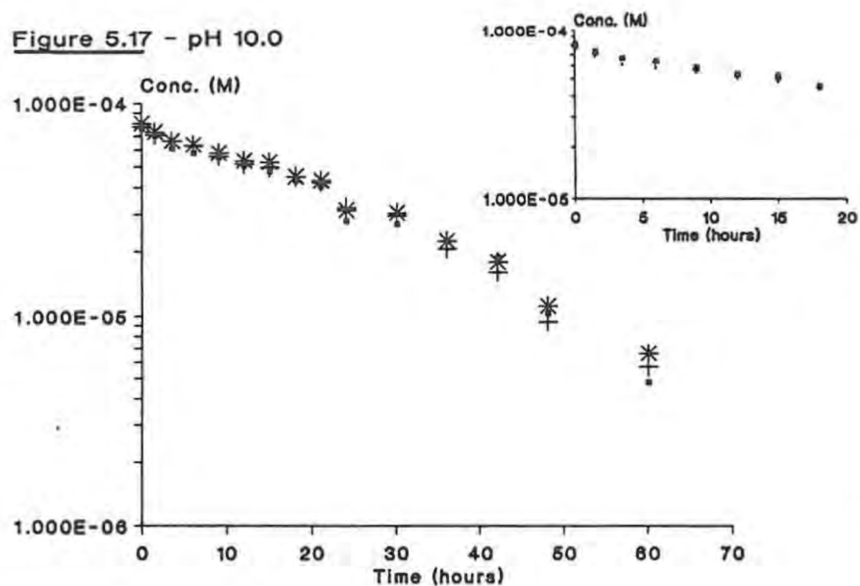


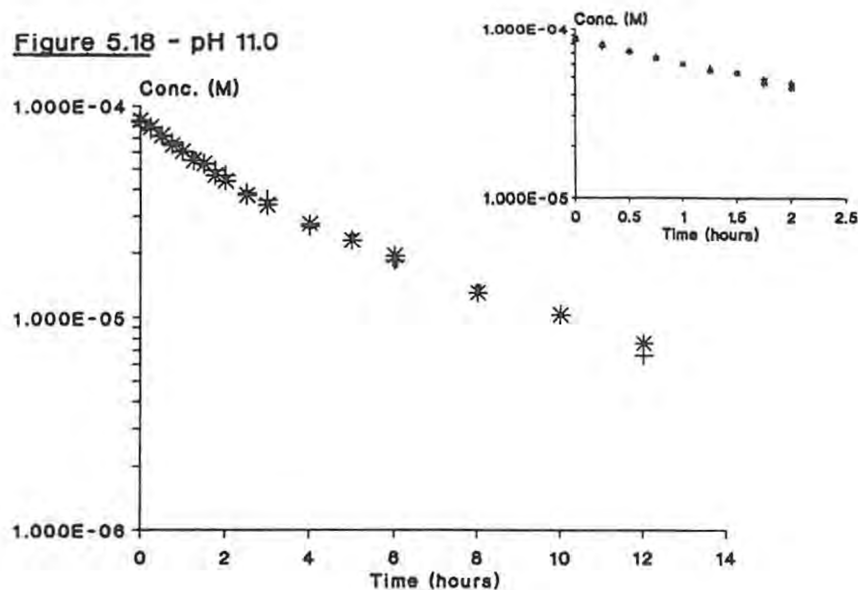


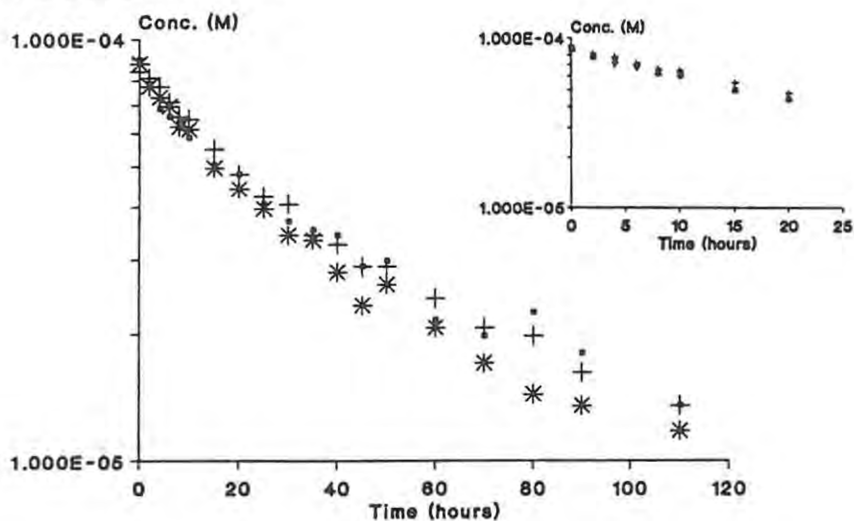




Figures 5.13 - 5.16 First-order concentration *vs.* time plots for josamycin in aqueous media at pH 7.5, 8.0, 8.5 and 9.0 respectively, at 37°C with μ = 1.0 (▪, + and * represent replicate experiments).







Figures 5.17 - 5.19 First-order concentration *vs.* time plots for josamycin in aqueous media at pH 10.0, 11.0 and 12.0 respectively, at 37°C with μ = 1.0. Inserts are expanded plots of the initial linear portion of the respective profiles used for the determination of K_{init} by linear regression (▪, + and ✻ represent replicate experiments).

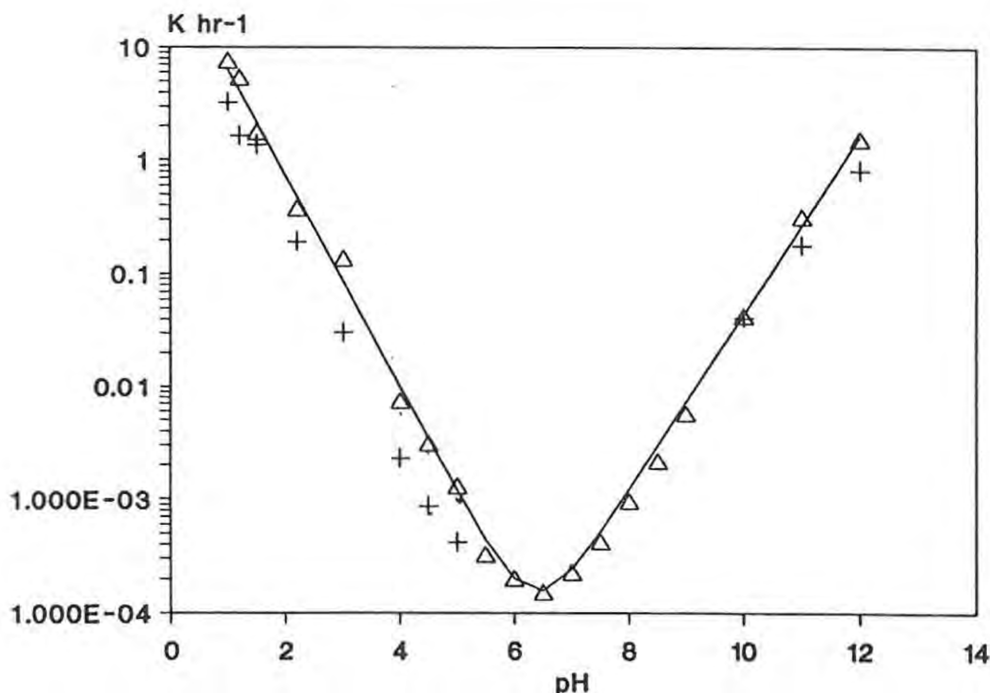


Figure 5.20 pH-rate profile for Josamycin at 37°C with μ = 1.0. (Δ) = K_{init}, (+) = K_{term}, showing experimental data and the line of best fit for K_{init} according to equation 5.11.

from pH 1.0 to 5.5 was accurately described by equation 5.13 for which a correlation coefficient of 0.9975 was obtained. The rate constant for catalysis by the hydronium ion (k_1) was found to be 54.11 $M^{-1}.hr^{-1}$ and the order of the reaction (n) with respect to the hydronium ion was close to unity with a value of 0.9334 indicating specific acid catalysis. For K_{init}, the linear portion of the profile from pH 7.0 to 12.0 was accurately described by equation 5.15 for which a correlation coefficient of 0.9986 was obtained. The rate constant for catalysis by the hydroxide ion (k_3) was 60.35 $M^{-1}.hr^{-1}$. However, the order of the reaction (m) with respect to the hydroxide ion was substantially less than unity with a value of 0.7848 indicating that hydrolysis in alkali media is not purely catalysed by OH^- ions. Usually, for a simple pseudo first-order reaction n and m are equal to +1 and -1 respectively, however, the low value obtained for m could be a reflection of a complex alkali degradation pathway for josamycin. The water catalysed rate constant k_2 was calculated using equation 5.11 with values for k_1, k_3, n and m as cited above at a pH of 6.5. A value of 3.370 x 10^{-5} hr^{-1} was obtained. This very low rate constant is consistent with the marked V-shape of the pH-rate profile which indicates that catalysis by water is of little significance compared with catalysis by either H^+ or OH^- ions. The linear nature of the log K_{term} *vs.* pH profile in acidic media shows that reactions determining K_{term} over this pH range were also subject to specific acid catalysis. For K_{term}, the rate

constant for catalysis by the hydronium ion (k_1) was found to be 39.41 $M^{-1}.hr^{-1}$ with the order of reaction (n) equal to 0.9062 and a correlation coefficient of 0.9882. The value of k_1 for K_{term} was lower than that for K_{init} which is consistent with the degradation rate being slower over the terminal linear portion than over the initial linear portion. For K_{term}, k_3 and m were not calculated as biphasic curves were only obtained at the very high pH values of pH 10 and above, providing only a limited number of data points. The factors responsible for the biphasic first-order plots are discussed in 5.2.

5.1.2.2 Effect of Ionic Strength and Buffer Concentration

Plots of the observed rate constants K_{init} and K_{term} *vs.* ionic strength in accordance with equation 5.17 are shown in figure 5.21. These plots were highly linear and demonstrated that both K_{init} and K_{term} were significantly dependent on ionic strength. The maintenance of constant ionic strength throughout any reaction to determine the stability of josamycin in aqueous media is therefore essential.

Plots of K_{init} and K_{term} *vs.* buffer concentration are shown in figure 5.22. K_{init} decreased slightly with increasing buffer concentration whilst K_{term} was unaffected. However, as the rate constants did not change significantly, determination of rate constants for the pH rate profile was carried out at a single buffer concentration and no extrapolation to zero buffer concentration was made.

Figure 5.21

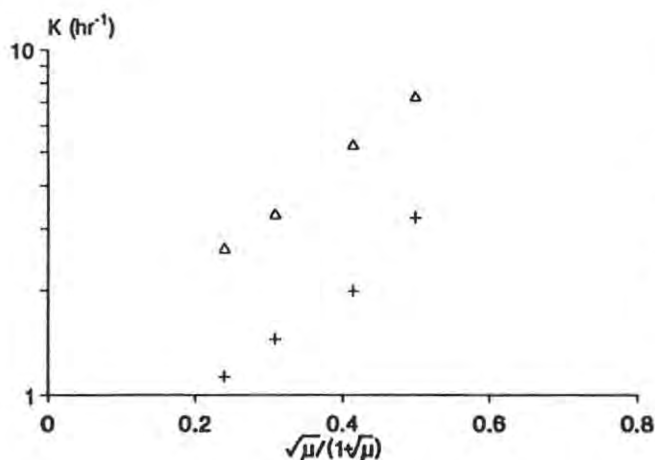


Figure 5.22

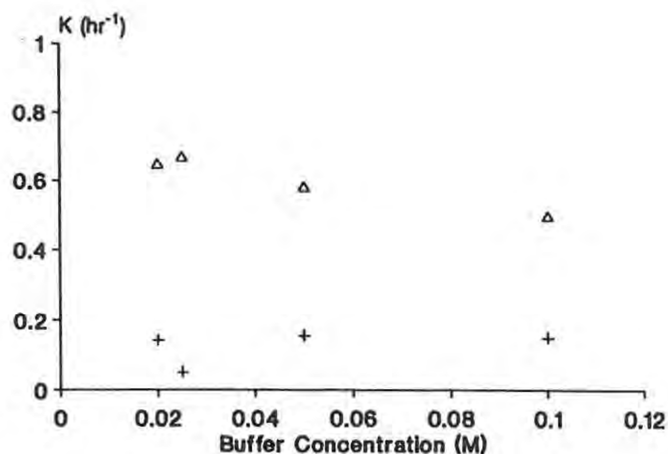


Figure 5.21 Plots of log K_{init} (Δ) and log K_{term} (+) *vs.* $\sqrt{\mu}/(1+\sqrt{\mu})$ according to equation 5.17.
Figure 5.22 Plot of K_{init} (Δ) and K_{term} (+) *vs.* buffer concentration (M) at pH 2.2 with μ = 1.0.

5.1.2.3 Determination of Order of Reaction

To determine the order of reaction, ln concentration (first-order) and reciprocal concentration (second-order) *vs.* time plots at each pH were inspected. Generally, all first-order plots at acidic pH's were biphasic and consisted of an initial and a terminal *linear* portion (the biphasic nature of these plots may be accounted for by the proposed reaction pathways *vide infra* - 5.2.3.1). The reaction can therefore be said to follow first-order kinetics during each linear phase. However, at low pH's where degradation is rapid, the initial linear phase is less well defined. The order of reaction during the initial phase was therefore confirmed using equation 5.18 as detailed in 5.1.1.3 at pH 2.2 with $\mu = 1.0$. Apparent zero-order initial rates of reaction at each concentration were determined by linear regression of cartesian plots of degradation data over the initial 10% of the reaction. Linear regression of a plot of these rates in accordance with equation 5.18 gave a straight line of $y = 1.0837x - 1.2620$ with a correlation coefficient of 0.9924 (Figure 5.23). The slope of the line was close to unity indicating that the degradation of josamycin in acidic media follows first-order kinetics.

At pH 7.0 and above, first-order plots were also generally linear indicating that the reaction in alkali media was first-order. The possibility that second-order kinetics were followed was discounted since data obtained at pH 1.0 and 9.0 and plotted according to the second-order rate equation (Equation 5.5) did not yield straight line plots, as is shown in figures 5.24 and 5.25.

5.1.2.4 Degradation of Josamycin in Simulated Gastric and Intestinal Fluids

The percentages of josamycin remaining intact with time in simulated gastric fluids of pH 1.0 (0.1 N HCl), pH 1.2 (USP simulated gastric fluid) and pH 1.5 (0.03 N HCl) prepared with hydrochloric acid; in McIlvaines buffer at pH 2.2 and 3.0, and in USP simulated intestinal fluid are tabulated in table 5.2. As expected, significant amounts of josamycin were rapidly lost at highly acidic pH's and after 10 mins, 65% of the initial amount remained at pH 1.0, 70% at pH 1.2 and 83% at pH 1.5. After 60 mins the amounts remaining at the respective pH's were only 26%, 34% and 55%. However, as the pH increased above 1.5, loss of intact josamycin was markedly slower and after 60 mins, 60% remained at pH 2.2 and 88% at pH 3.0. In simulated intestinal fluid, no discernable degradation was detected within 60 mins. The half-life for the gastric emptying of solutions has been observed to range between 10 and 50 mins (166,203). The residence time of dissolved drug in gastric fluids can therefore be both variable and considerable. As the stability of josamycin is highly pH sensitive, the gastric pH and gastric residence time of dissolved josamycin will therefore have a profound effect on bioavailability of josamycin. For instance, with a mean gastric residence time as low as 10 mins in gastric fluid of pH 1.0, 35% of

Figure 5.23

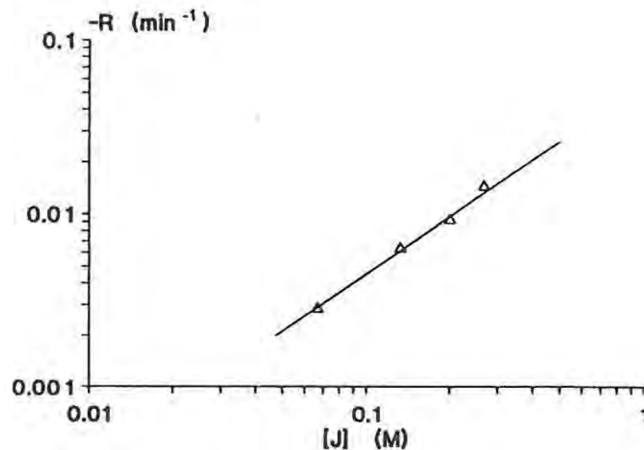


Figure 5.24

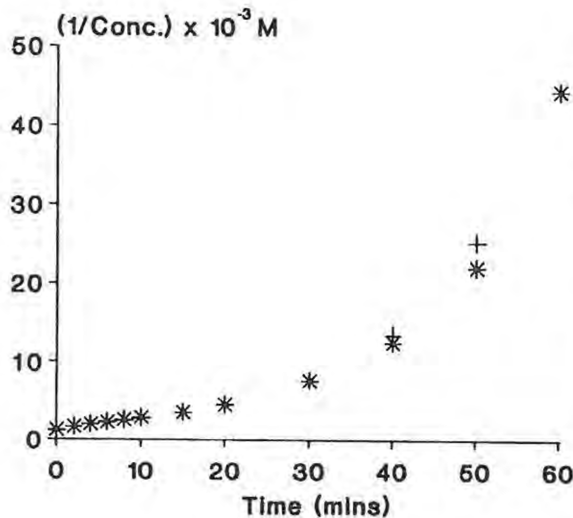


Figure 5.25

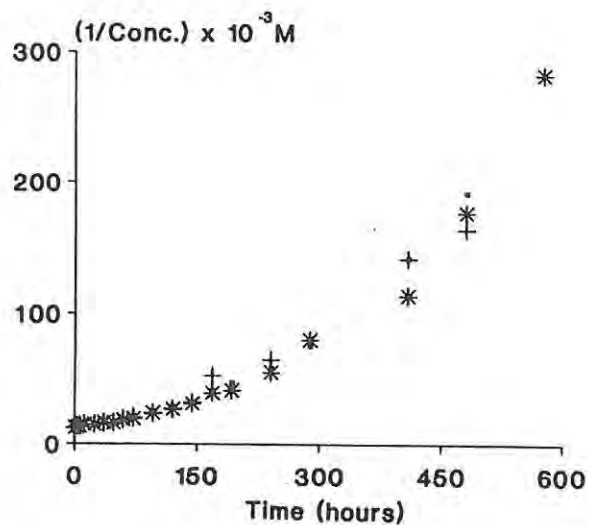


Figure 5.23 Plot of log (-R) *vs.* log $[J]_0$ according to equation 5.18 to determine the order of reaction (slope = 1.0837).
Figure 5.24 and 5.25 Second order reciprocal concentration *vs.* time plot for josamycin at pH 1.0 and 9.0 respectively, at 37°C with μ = 1.0 (▪, + and ✱ are replicate experiments).

the administered dose could be lost due to degradation in the stomach. A mean gastric residence time of 60 mins would result in the loss of 74% of the administered dose. At intestinal pH's, however, josamycin is highly stable. Due to the instability of josamycin at gastric pH's, the gastric pH and gastric emptying time will be important factors determining the overall bioavailability of josamycin from any immediate release oral dosage form.

5.2 ADDITIONAL INVESTIGATIONS

As mentioned earlier, the stability of josamycin has received scant attention in the literature. It is known that treatment of josamycin with acid forms desmycarose josamycin by cleavage of the mycarose moiety (4,79), and isoleucomycin A_3 by allylic rearrangement of the hydroxyl group from C-9 of the lactone ring to C-13 (204). However, the stability-indicating assay used during the aforementioned investigations showed the formation of three additional compounds from josamycin in acid media (Figure 2.7c), whilst five were detected in alkali media (Figure 2.8). Besides the lack of a pH-rate profile for josamycin, no study on the acid degradation pathway has been published. Preliminary investigations into the reaction pathway and identification of the acid degradation products observed, utilising HPLC and thermo-spray mass-spectroscopy were therefore conducted. Furthermore, these investigations were conducted to determine the reason for the biphasic first-order plots encountered in acidic media. Since degradation at physiological pH's above 7.0 was not significant, only the acid degradation of josamycin was studied further.

5.2.1 Chromatographic Evidence for the Reaction Pathway

The peak height *vs.* time profiles of josamycin and the products formed at pH 1.2 are shown in figure 5.26, and at pH 12.0 are shown in figure 5.27.

Figure 5.26

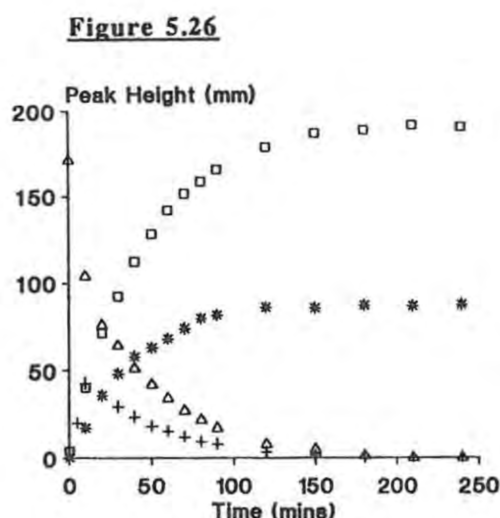


Figure 5.27

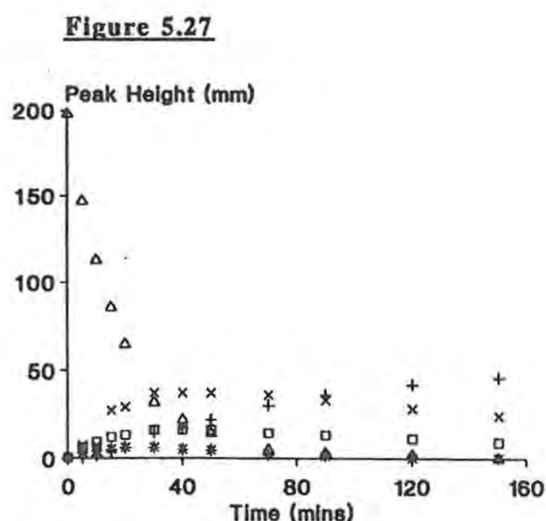


Figure 5.26 Peak height *vs.* time profiles of josamycin and acid degradation products at pH 1.2 (see figure 2.7c for chromatogram), Δ = josamycin, + = P1, * = P2 □ = P3.
Figure 5.27 Peak height *vs.* time profiles of josamycin and alkali degradation products (1,2,3 and 4) at pH 12.0 (see figure 2.8 for chromatogram, Δ = josamycin, + = 1, * = 2, □ = 3, × = 4).

Chromatographic data previously indicated that josamycin and acid degradation product P1 showed very similar retention properties (2.2.2.2). It is therefore postulated that P1 may be very similar in structure to josamycin. Acid degradation products P2 and P3 also showed very similar retention properties, but eluted appreciably earlier than josamycin and P1. The molecular structures of P2 and P3 were thus most likely similar to each other, but different, however, from josamycin. The concentration *vs.* time profile of P1 was consistent with that of an intermediate product as it showed an increase in concentration from zero to a maximum followed by a decline to zero concentration at the completion of the reaction whilst the concentration *vs.* time profiles of P2 and P3 were typical for end products. The immediate conclusion to be drawn from figure 5.26 is that the degradation of josamycin proceeds as a consecutive reaction through an intermediate P1 to the end products P2 and P3. However, no lag period in the formation of either P2 or P3 is evident. This suggests that P2 and P3 could be formed directly from josamycin rather than through an intermediate. However, this is by no means conclusive as a lag period in the formation of the end products could be easily masked if the conversion of the intermediate to the end product is much faster than the formation of the intermediate. Semilogarithmic plots for simple reactions involving a single rate constant and proceeding under pseudo first-order conditions should be linear. However, deviations from linearity such as those observed in this study can be obtained if the reaction is of a complex nature. Such deviations can be due to the formation of an intermediate (198), or alternatively, to the presence of reversible/equilibrium reactions in the reaction pathway. This has been observed by Yuen and Sokoloski (205) during their investigation into the degradation of tetracycline, Muhammad *et al.* (206) during their investigation into the degradation of vinpocetine and also by Saab *et al.* (207) for the isomerisation and subsequent decomposition of cephalosporin esters. P1 therefore appears to be formed directly from josamycin but may be involved in a competitive reversible reaction or in a consecutive reaction. The end products P2 and P3 could be formed either directly from josamycin and/or via P1. The observations cited above prompted further investigations into the presence of reversible reactions between josamycin and P1. Furthermore, the ratio of the peak heights of P2 and P3 remained constant throughout the reaction which prompted investigations into the presence of a reversible reaction between these two compounds.

5.2.1.1 Evidence for Reversible Reactions between Josamycin and P1

A few micrograms of P1 were isolated as follows:- Josamycin, at a concentration of 1 mg/ml, was degraded in 0.1 M HCL for 10 mins (when the concentration of P1 was at a maximum) after which the reaction was stopped by the

addition of an equal volume of 0.2 M di-sodium hydrogen phosphate. Aliquots (5 μl) of the resulting solution were injected into the system described in 2.2 and the eluate containing P1 selectively collected. After 40 injections the collected eluate was evaporated to dryness in a Savant vacuum centrifuge and the residue reconstituted with 0.5 ml of water. An aliquot of the resulting solution was then immediately chromatographed where P1 and a small amount of josamycin was detected. On acidification of this solution, the peak height of josamycin increased at the expense of P1 indicating a reversible reaction between josamycin and P1. At the completion of the reaction no josamycin or P1, but only P2 and P3 were detected.

5.2.1.2 **Evidence for a Reversible Reaction between P2 and P3**

P2 and P3 were similarly isolated as described for P1 using the HPLC system described in 2.2, but using a highly polar mobile phase of acetonitrile/phosphate buffer (0.015 M, pH 6.0) 3:17 to improve separation of these two peaks. The retention times of P2 and P3 were 4 and 2 mins respectively. On acidification of a solution of P2, the peak height of P3 increased at the expense of P2 from zero to a constant height such that the ratio of the peak heights of P2/P3 was 0.45. Similarly, on acidification of a solution of P3, the peak height of P2 increased at the expense of P3 from zero also to a constant height with a peak height ratio of 0.45. A reversible reaction between P2 and P3 therefore existed. Furthermore, the formation of no other compounds was detected confirming that P2 and P3 were end products in the acid degradation of josamycin.

5.2.2 Thermo-Spray Mass Spectroscopy

Thermo-Spray Mass-Spectroscopy (TS-MS) is a relatively new analytical technique which enables the coupling of two hitherto incompatible analytical techniques - that of HPLC, which produces relatively large volumes of liquid eluate, and that of mass-spectroscopy which requires that samples are introduced into the apparatus such that the high vacuums necessary for its operation can be maintained during the entire analytical procedure. This incompatibility has been overcome by the use of an interface between the HPLC system and the mass-spectrometer, called the thermospray probe, which allows the HPLC eluent to be pumped directly into the mass-spectrometer. The probe consists of a heating element which rapidly heats and vaporises the HPLC eluate. The vaporised eluate is then introduced directly into the mass-spectrometer after which analytes are detected in the normal manner and waste vapours are removed from the vacuum chamber by highly efficient vacuum pumps. The mobile phase usually consists of organic/aqueous mixtures with or without

buffers. One drawback of this system, however, is that the buffers used must be volatile (usually ammonium acetate and acetic acid) so as to avoid blockage of the thermospray probe when the mobile phase is vaporised, and to allow their evacuation from the mass-spectrometer chamber. Unfortunately, this can be a serious limitation on the efficiency of the HPLC system in separating compounds. Ionisation of analytes occurs by chemical ionisation during the vaporisation process. Solvents and buffers are responsible for the formation of highly charged droplets which impart a positive charge to the solvated analyte molecules within the droplet (208). This results in the formation of the protonated or ammoniated molecular adduct ions (209,210). As this form of ionisation is "soft", it is generally accompanied by very little fragmentation of the parent molecule. TS-MS is therefore primarily suited to obtaining simple mass-spectra and molecular weights of the non-fragmented molecule rather than more complex fragmentation patterns. Ionisation, however, does not necessarily have to be totally reliant on the vaporisation process but can be enhanced by directing a beam of high energy electrons produced by a heated filament into the vapour stream as it exits the thermospray probe. The filament may be switched on when increased sensitivity is required and/or when poorly ionisable molecules such as non-polar molecules are to be ionised (208).

Samples can be injected into the TS-MS system using two different injection techniques, Firstly, samples can be injected into the mobile phase stream without an in-line analytical column fitted. Known as the "direct injection" technique, it has the advantage that no band spreading, as happens during chromatography, occurs and maximum sensitivity can therefore be attained. This technique, however, is best suited to the analysis of samples containing only a single compound as separation of multiple components does not occur and if more than one compound is present all compounds are detected simultaneously. Alternatively, the individual components of a complex mixture can first be separated by chromatography on an analytical column followed by immediate mass-spectrometry of each compound as they elute. Although the sensitivity of this method is often far lower than with the direct injection technique, isolation and purification of compounds prior to mass-spectrometry is not required. This technique was therefore ideal for obtaining mass-spectral data of the unidentified acid degradation products of josamycin without the need for lengthy separation and purification procedures.

5.2.2.1 Experimental

Separation of josamycin from P1 could not be accomplished using ammonium acetate buffers necessary for TS-MS. However, P2 and P3 were separated using the HPLC system described in 2.2 but with a mobile phase consisting of a 3:8 mixture of acetonitrile/ammonium acetate buffer (ammonium acetate, 0.05 M, adjusted to pH 4.0

with acetic acid). The mobile phase flow rate was 1 ml/min with retention times of *ca.* 15.0 and 7.0 mins for P2 and P3 respectively. This mobile phase and flow rate was used for all TS-MS investigations with or without an in-line analytical column fitted. The thermospray used was an HP 5988A Thermospray. The stem temperature of the thermospray probe, determined by a probe survey (208), was set at 104°C and the vacuum maintained at 1.8 x 10^{-6} torr. The mass-spectrometer was operated in the filament-on mode with the filament voltage set to obtain maximum ionisation.

The mass-spectrum of josamycin was obtained by injecting a 20 μl aliquot of a sample containing josamycin (1 mg/ml) dissolved in acetonitrile/0.1 M ammonium acetate 2:8 into the mass-spectrometer by the direct injection technique. A sample of P1 was prepared by HPLC as in 5.2.1.1 followed by extraction of the 0.5 ml aqueous sample into an equal volume of ether to remove any non-volatile buffer salts. After evaporating to dryness the residue was reconstituted in 0.5 ml of acetonitrile/0.1 M ammonium acetate 2:8. The mass-spectrum of P1 was obtained by injecting a 20 μl aliquot of this sample into the mass-spectrometer also using the direct injection technique. For P2 and P3, a 50 μl aliquot of a solution containing both compounds was injected into the HPLC system fitted with an in-line analytical column and mass-spectra of each compound obtained after chromatographic separation. The sample was prepared by degrading josamycin at a concentration of 1 mg/ml in 0.1 M HCl and allowing the reaction to go to completion. The acidic solution was then diluted with an equal volume of a 0.1 M ammonium acetate solution prior to injection to conserve the analytical column.

5.2.2.2 **TS-MS Results and Discussion**

TS-MS operating conditions were optimised for each sample and are listed with mass-spectral data obtained for josamycin, P1, P2 and P3 in table 5.3. The spectra and TIC's (Total Ion Count) obtained for each compound are shown in figures 5.28 to 5.31.

The TS-MS of josamycin exhibits a peak at *m/z* 829 which can be attributed to the protonated molecular adduct ion - $[J+1]^+$. An abundance of 6.2872 x 10^4 was obtained after the direct injection of 20 μg of josamycin. A single major fragment ion was observed at *m/z* 769 which can be attributed to the protonated form of the fragment remaining after the loss of acetic acid *i.e.* $[J(-)CH_3COOH+1]^+$. The loss of acetic acid is a common phenomenon during chemical ionisation (211) and this could result from the loss of the acetate group together with a hydrogen attached to position-3 of the lactone ring (Figure 1.1).

The TS-MS of the sample of P1 shows significant peaks at *m/z* 829 and *m/z* 769 which can be attributed to the same molecular adduct ions as for josamycin. Significant peaks were also obtained at *m/z* 749 and *m/z* 717. However, no obvious

Figure 5.28

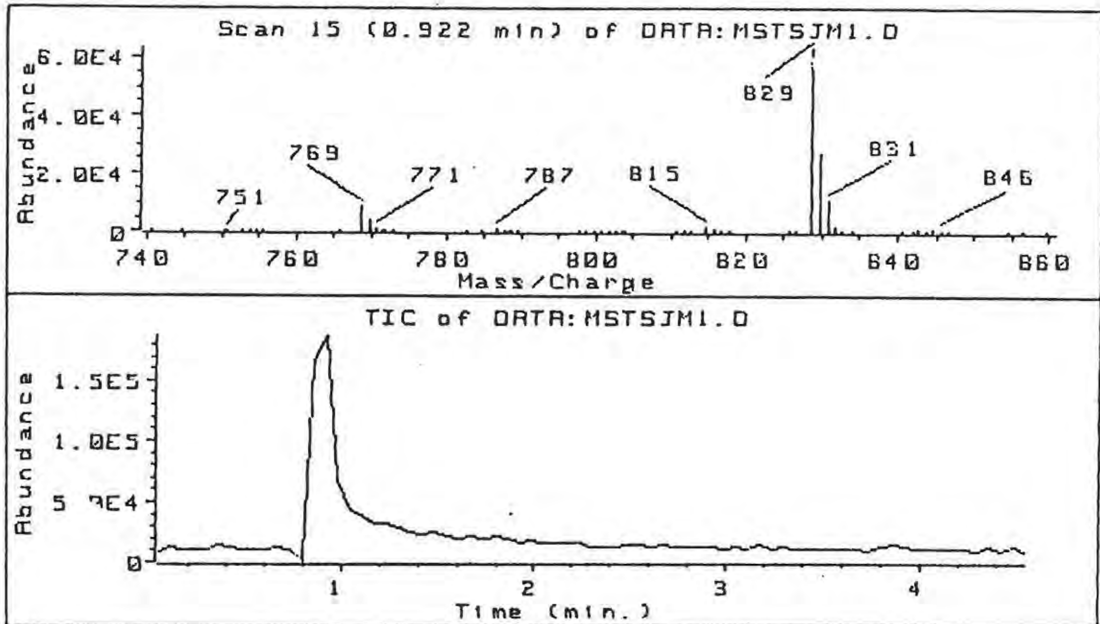


Figure 5.29

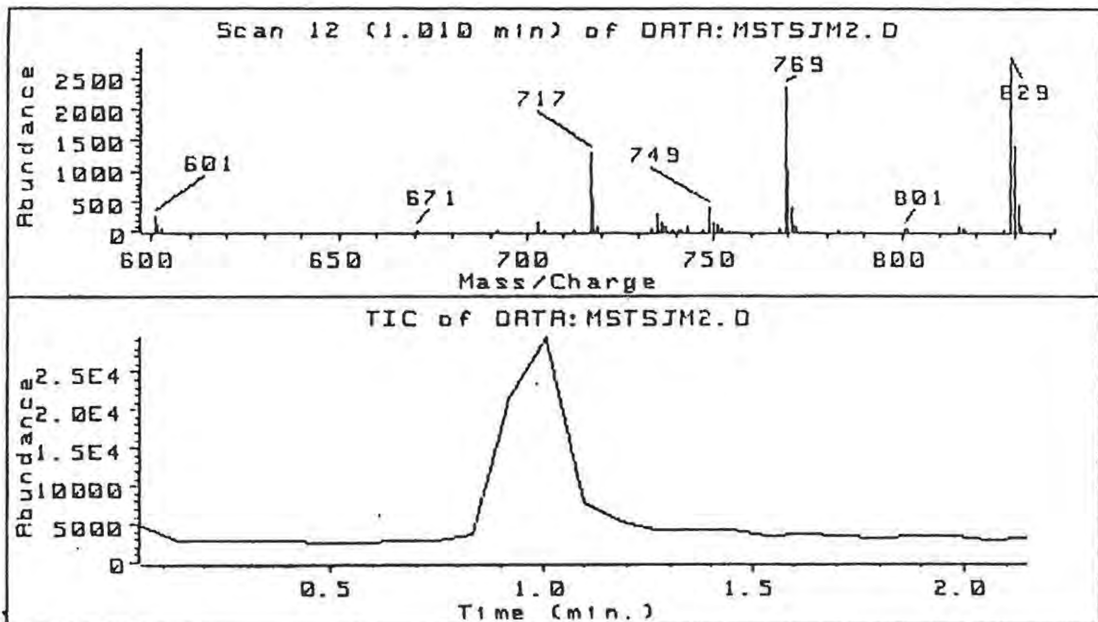


Figure 5.28 TS-MS and corresponding total ion count (TIC) for josamycin obtained after the injection of 20 μg of josamycin into the TS-MS by the direct injection technique.
Figure 5.29 TS-MS and corresponding TIC's obtained after the injection of a sample of P1 by the direct injection technique.

Figure 5.30

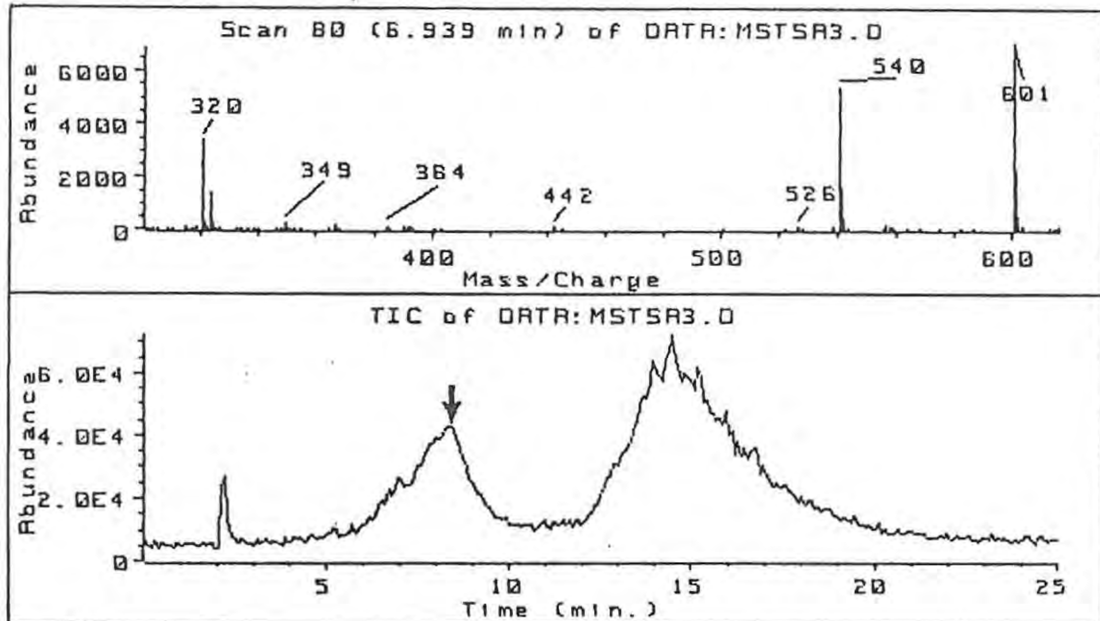


Figure 5.31

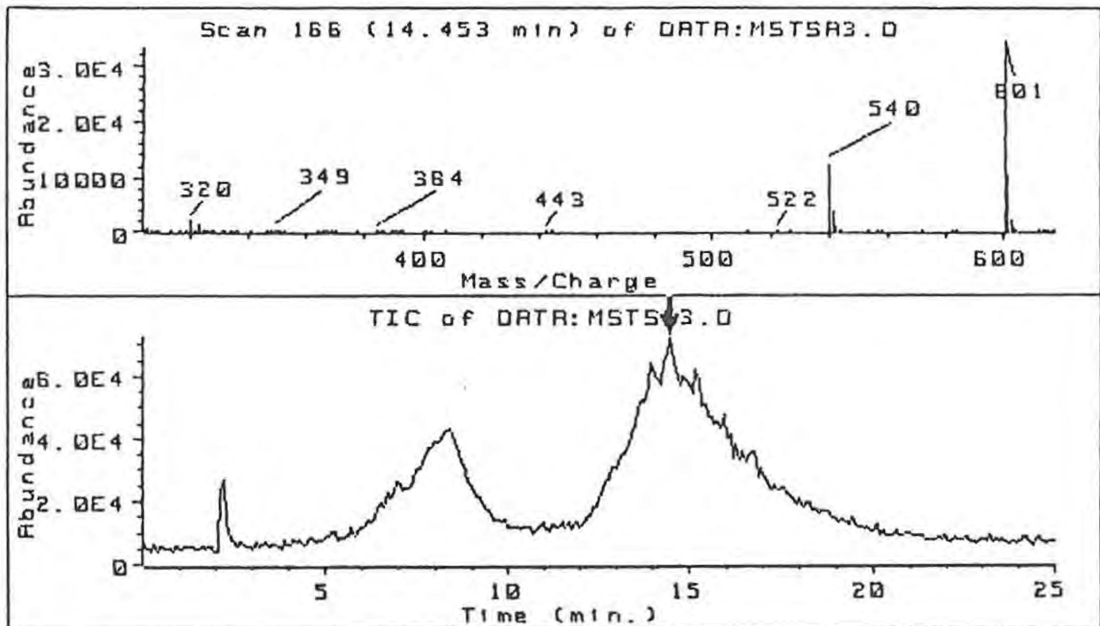


Figure 5.30 and 5.31 TS-MS and corresponding TIC of P2 and P3 obtained at 6.9 min and 14.4 min respectively, after the injection of a sample containing both compounds with separation by HPLC.

molecular structures could be assigned to these and they were therefore probably fragment ions or contaminants originating during the collection of P1. As mentioned in 5.2.1.1, it was not possible to obtain a sample of P1 completely free from josamycin due to the formation of josamycin from P1 during collection. As a reversible reaction between josamycin and P1 was previously established it was postulated that a simple hydration or dehydration reaction may have been taking place and that a minor peak for josamycin plus a major peak for P1 would be detected in the sample of P1 isolated, with *m/z* values differing only by a value equal to the molecular mass of water. However, only the molecular ion attributable to $[J+1]^+$ at *m/z* 829 and the fragment ion attributable to $[J(-)CH_3COOH+1]^+$ were detected. Josamycin and P1 are therefore of equal molecular mass and possibly isomers. A reversible reaction between the two compounds is therefore quite possible which confirms the observations from HPLC to this effect, however, a dehydration/hydration reaction was not taking place. The reversible reaction is possibly due to the formation of a hemi-acetal by reaction of the aldehyde attached to position-6 of the lactone ring with the OH-groups attached to either position-9 of the lactone ring or position-2 of the mycaminose moiety (212), or due to the reversible rearrangement of the hydroxyl group between C9 and C13 of the lactone ring (Figure 1.1). Either of these reactions would produce compounds of equal molecular mass but with different chromatographic properties, as was observed.

The TS-MS taken at *ca.* 7.0 and 14.5 mins after injection of a sample containing both P2 and P3 onto the analytical column were identical with significant peaks at *m/z* 601 and *m/z* 540. The peak at *m/z* 601 can be attributed to the protonated molecular adduct ion of the portion of the macrolide molecule remaining after cleavage of the glycosidic linkage between the mycaminose and mycarose moieties with a hydroxyl group at position-4 of the mycaminose moiety *i.e.* desmycarose josamycin (molecular weight = 599). The peak at *m/z* 540 can be attributed to a protonated fragment of this parent molecule, arising from the loss of acetic acid from position-3 of the lactone ring. Again, P2 and P3 could also be isomers formed by reaction of the same functional groups as in josamycin and P1, and probably isomers of desmycarose josamycin. The chromatographic behaviour of josamycin, P1, P2 and P3 support the presence of two pairs of isomers. However, P2 and P3 were more easily separated than josamycin and P1, since the hydroxyl group which undergoes rearrangement may influence chromatography more markedly for the two lower molecular weight compounds than for the two larger molecules.

5.2.3 Non-Linear Curve Fitting of Concentration *vs.* Time Profiles

5.2.3.1 Possible Reaction Pathways and Mathematical Models

Scheme 1 is proposed on the basis of the following observations made from HPLC data on the acid degradation of josamycin (where J is josamycin):-

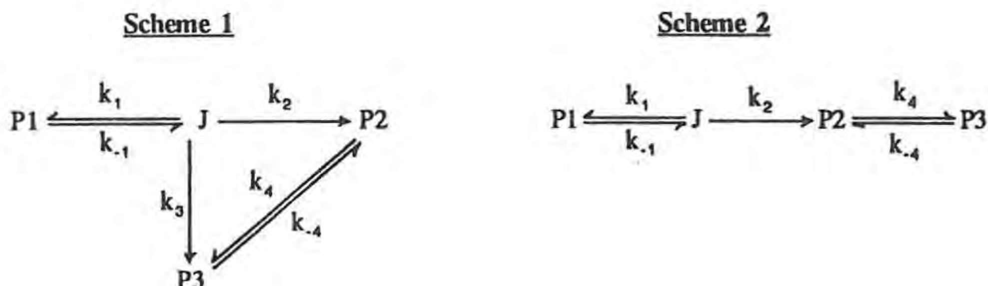
a) the degradation of josamycin goes to completion where P2 and P3 are end products,

b) josamycin and P1 are similar compounds between which a reversible reaction occurs,

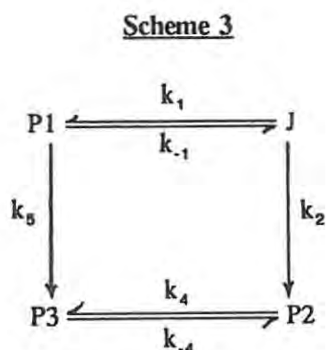
c) P2 and P3 are similar compounds between which a reversible reaction occurs, and

d) P1 is probably not an intermediate compound as the absence of any apparent lag period in the formation of P2 or P3 suggests that both P2 and P3 are formed directly from josamycin.

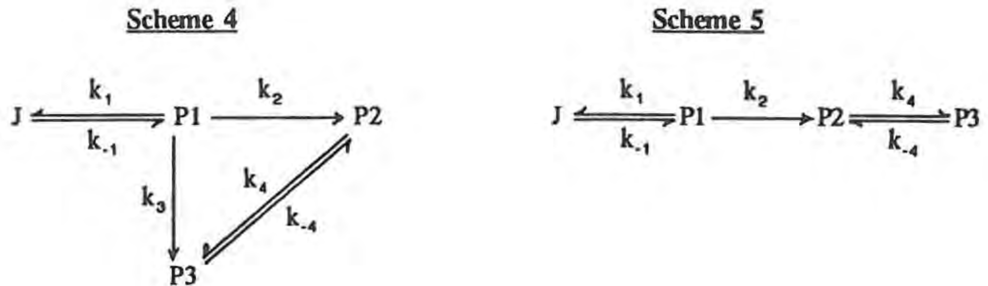
However, TS-MS studies provide strong evidence that P2 and P3 are isomers. In this case only one of the end products (*e.g.* P2) will be formed directly from josamycin. Furthermore, it appears that equilibrium between P2 and P3 is rapidly attained as a lag period in the formation of P3 was not evident. Scheme 2 is therefore proposed, and scheme 1 can be excluded as a viable reaction pathway.



If, however, josamycin and P1 are isomers and the reaction involves the establishment of an equilibrium between josamycin and P1, with hydrolysis of the glycosidic linkage between the mycaminose and mycarose moieties of josamycin and P1 to the respective products P2 and P3, then scheme 3 could be a possible reaction pathway (205).



On the basis that P1 is an intermediate between josamycin and both end products, HPLC data suggests scheme 4. However, since P1 and josamycin are most likely to be isomers, as are P2 and P3, scheme 5 is proposed and scheme 4 can be excluded.



The constants k_1, k_{-1}, k_2, k_3, k_4, k_{-4} and k_5 are the first-order rate constants for each individual reaction.

For scheme 2 then, the differential rate equations pertaining to [J] can then be written as

$$-\frac{d[J]}{dt} = k_1[J] + k_2[J] - k_{-1}[P1] \qquad \text{Eq. 5.19}$$

where

$$\frac{d[P1]}{dt} = k_1[J] - k_{-1}[P1] \qquad \text{Eq. 5.20}$$

After integration (197) using Laplace transforms a mathematical model for scheme 2 is obtained - model 1 (Equation 5.21), describing the [J] with respect to time.

MODEL 1

$$[J]_t = \frac{[J]_0}{\beta_2-\beta_1}. [(k_{-1}-\beta_1). \exp(-\beta_1 t) - (k_{-1}-\beta_2). \exp(-\beta_2 t)] \qquad \text{Eq. 5.21}$$

where

$$\beta_1 = \frac{k_1+k_{-1}+k_2 + \sqrt{(k_1+k_{-1}+k_2)^2 - 4k_{-1}k_2}}{2} \qquad \text{Eq. 5.22}$$

and

$$\beta_2 = \frac{k_1+k_{-1}+k_2 - \sqrt{(k_1+k_{-1}+k_2)^2 - 4k_{-1}k_2}}{2} \qquad \text{Eq. 5.23}$$

For scheme 3, the differential rate equations pertaining to [J] can be written as

$$-\frac{d[J]}{dt} = k_1[J] + k_2[J] - k_{-1}[P1] \qquad \text{Eq. 5.24}$$

where

$$\frac{d[P1]}{dt} = k_1[J] - k_{-1}[P1] - k_5[P1] \qquad \text{Eq. 5.25}$$

and

$$\frac{d[P2]}{dt} = k_2[J] \qquad \text{Eq. 5.26}$$

and

$$\frac{d[P3]}{dt} = k_5[P1] \qquad \text{Eq. 5.27}$$

After integration using Laplace transforms (197), model 2 (Equation 5.28) is obtained.

MODEL 2

$$[J]_t = \frac{[J]_0}{\beta_2-\beta_1}.\ [(k_{-1}+k_5-\beta_1).\ \exp(-\beta_1 t) - (k_{-1}+k_5-\beta_2).\ \exp(-\beta_2 t)] \qquad \text{Eq. 5.28}$$

where

$$\beta_1 = \frac{k_1+k_{-1}+k_2+k_5 + \sqrt{(k_1+k_{-1}+k_2+k_5)^2 - 4(k_1k_5+k_{-1}k_2+k_2k_5)}}{2} \qquad \text{Eq. 5.29}$$

and

$$\beta_2 = \frac{k_1+k_{-1}+k_2+k_5 - \sqrt{(k_1+k_{-1}+k_2+k_5)^2 - 4(k_1k_5+k_{-1}k_2+k_2k_5)}}{2} \qquad \text{Eq. 5.30}$$

For scheme 5 then, the differential rate equations pertaining to [J] can be written as

$$-\frac{d[J]}{dt} = k_1[J] - k_{-1}[PI] \quad \text{Eq. 5.31}$$

where

$$\frac{d[PI]}{dt} = k_1[J] - k_{-1}[PI] - k_2[PI] \quad \text{Eq. 5.32}$$

After integration using Laplace transforms (197), model 3 (Equation 5.33) is obtained.

MODEL 3

$$[J]_t = \frac{[J]_0}{\beta_2-\beta_1}.\ [(k_{-1}+k_2-\beta_1).\ \exp(-\beta_1 t) - (k_{-1}+k_2-\beta_2).\ \exp(-\beta_2 t)] \quad \text{Eq. 5.33}$$

where

$$\beta_1 = \frac{k_1+k_{-1}+k_2 + \sqrt{(k_{-1}+k_2)^2 - 4k_1k_2}}{2} \quad \text{Eq. 5.34}$$

and

$$\beta_2 = \frac{k_1+k_{-1}+k_2 - \sqrt{(k_{-1}+k_2)^2 - 4k_1k_2}}{2} \quad \text{Eq. 5.35}$$

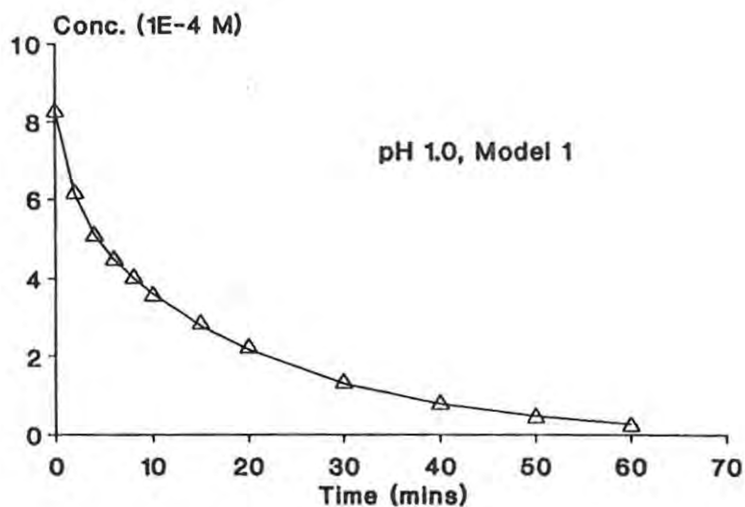
For the purposes of obtaining integrated rate equations describing the [J] with time, k_4 and k_{-4} can be omitted from the differential rate equations as these rate constants are concerned with reactions occurring subsequent to the irreversible first-order reactions and they thus have no effect on the rate of disappearance of J.

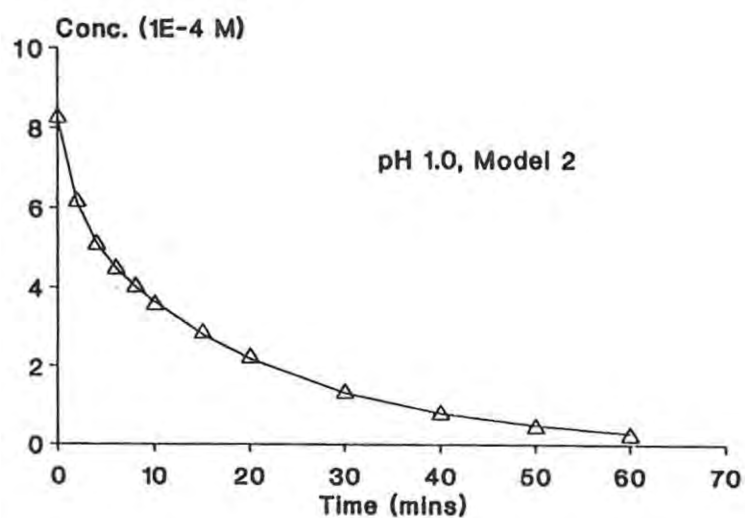
In order to determine if the schemes 2, 3 and 5 could describe the acid degradation of josamycin, models 1, 2 and 3 were fitted to concentration *vs.* time data obtained from stability studies at pH 1.0 to 5.0. The equations were fitted by non-linear regression using the computer program MINSQ (Micromath Scientific Software, Salt Lake City, Utah, U.S.A.).

5.2.3.2 **Results and Discussion**

For each data set, the initial estimate for both k_1 and k_{-1} was taken as the value of the apparent rate constant K_{init} determined as in 5.1.2.1, and the value of the apparent rate constant K_{term} used as the initial estimate for k_2 and k_5. The lower and upper limits for each k were set at 0.0001 and +infinity, and the lower and upper limits for $[J]_0$ set at 50% and 100% of the concentration of J determined by HPLC at time zero. The curve fitting process was initiated and after convergence was attained, the process was repeated using the estimates for each parameter obtained during the previous fitting as the new initial estimates. In most cases there was no difference between parameter values obtained at convergence for the first and second fits. Plots showing mean experimental data (n = 3) and the corresponding fitted data for Models 1, 2 and 3 at each pH (μ = 1.0) are shown in figures 5.32a, b and c to figures 5.39a, b and c respectively. The sum of squares and parameter values obtained for each model are listed in tables 5.4 to 5.6.

Good fits were obtained for Models 1 and 2, however, Model 3 was fitted less well to the experimental data. This is immediately apparent by visual inspection of the plots and also by comparison of the minimum sum of squares obtained for each scheme. The lowest values for the sum of squares was obtained for Model 1 although those for Model 2 were only slightly higher. Sum of squares values for Model 3 however, were about one order of magnitude greater than for either Model 1 or 2. Furthermore, k_2 appeared to be a parameter of little significance in Model 3. A value could not be obtained as k_2 tended to the lower limit irrespective of the limit, without causing any change in k_1, k_{-1} or the sum of squared deviations. Schemes 2 and 3 are therefore viable pathways whilst scheme 5 can be excluded. There is thus considerable evidence that P1 is involved in a competitive reversible reaction with josamycin. Furthermore, P2 is formed directly from josamycin. P3 may be formed via P2 (scheme 2), or alternatively as scheme 3 suggests, via P1 and P2. However, the significantly lower values of k_5 in comparison with k_2 suggest that the formation of P2 via P1 as in scheme 3 is an alternative route of minor importance. The values of the apparent rate constant for the terminal slope, K_{term}, were close to those of k_2 for both Model 1 and 2. The reaction J $\rightarrow$ P2 therefore appears to determine the rate of degradation (K_{term}) after equilibrium between josamycin and P1 has been established - *i.e.* this reaction becomes the rate limiting step once equilibrium between josamycin and P1 has been rapidly attained, which is consistent with these models, and explains the biphasic first-order plots obtained. The apparent rate constant for the initial linear portion of the reaction, K_{init}, was less well predicted by the models. K_{init} should be equal to (k_1+k_2) but experimental values obtained consistently lay between k_1 and (k_1+k_2). However, this was not of sufficient significance to consider Models 1 and 2 invalid. From curve fitting of only the josamycin concentration *vs.* time data,





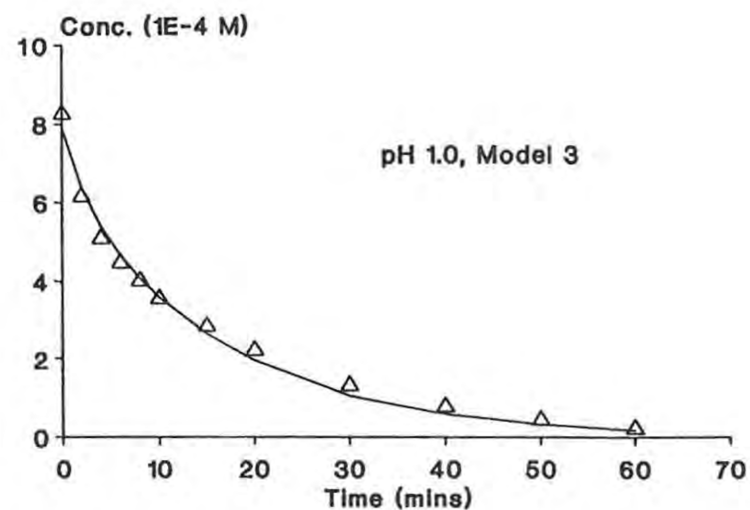
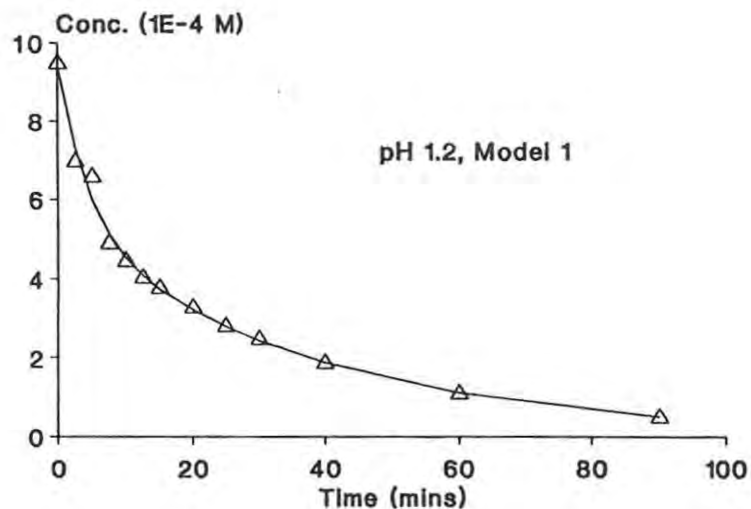
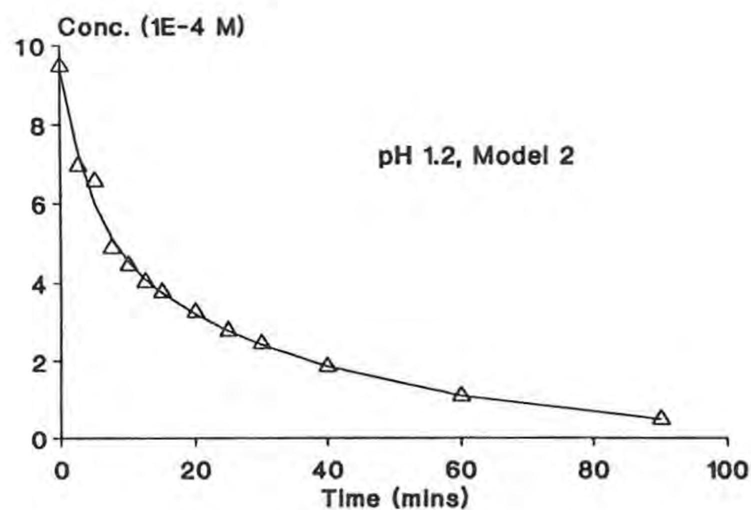


Figure 5.32a, b and c Experimental and fitted data for Model 1 (a), Model 2 (b) and Model 3 (c) at pH 1.0 (37°C with μ = 1.0).





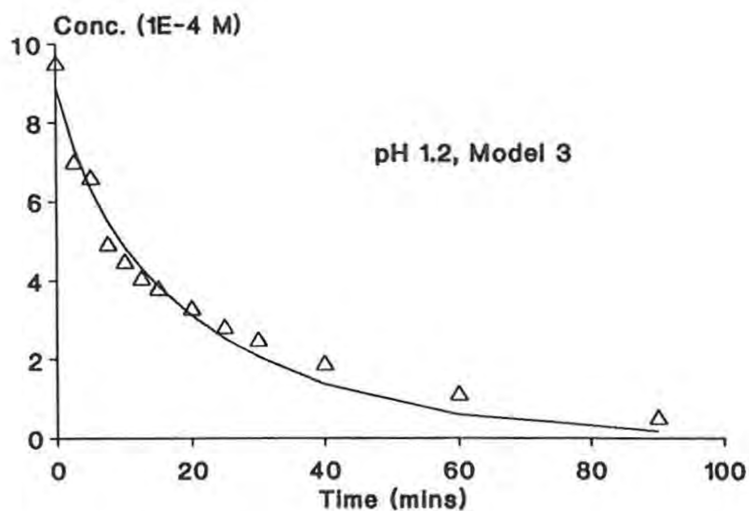
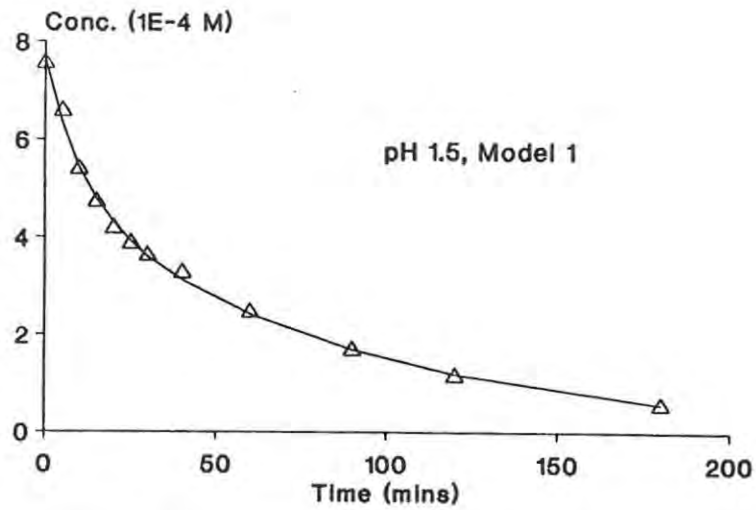
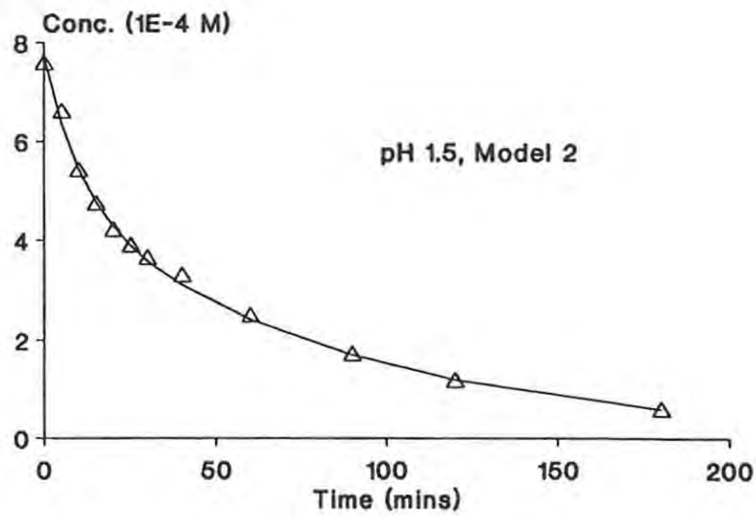


Figure 5.33a, b and c Experimental and fitted data for Model 1 (a), Model 2 (b) and Model 3 (c) at pH 1.2 (37°C with μ = 1.0).





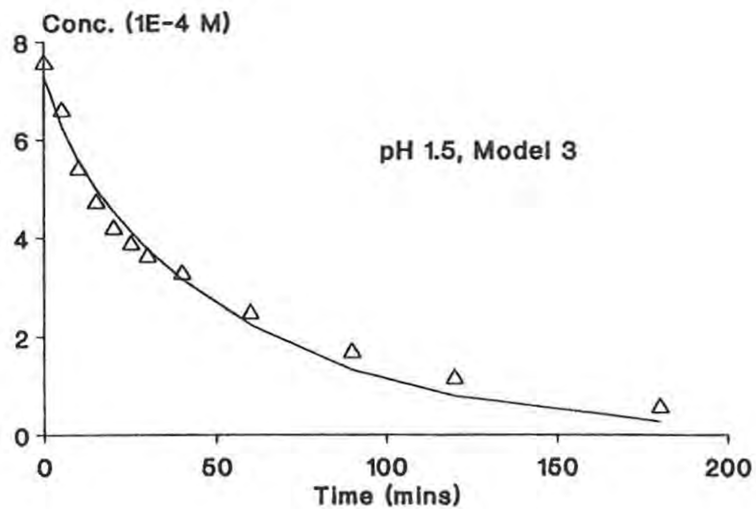
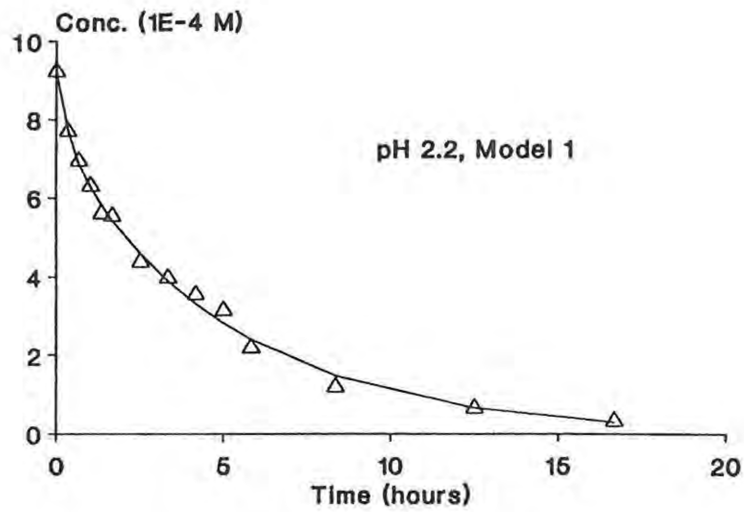
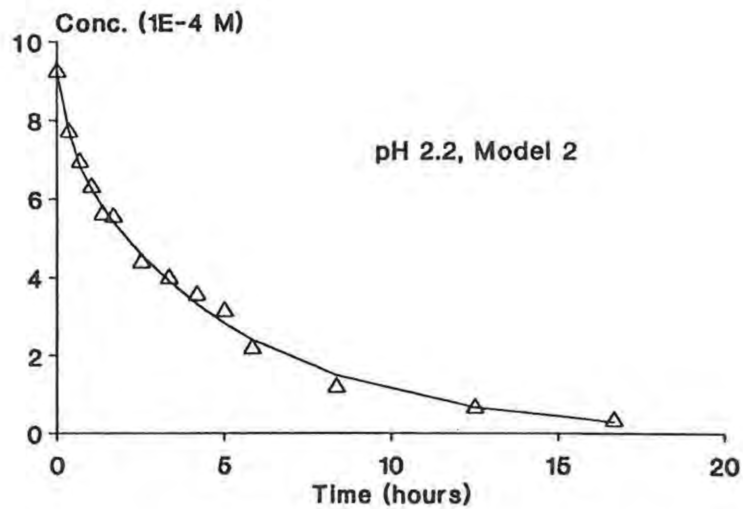


Figure 5.34a, b and c Experimental and fitted data for Model 1 (a), Model 2 (b) and Model 3 (c) at pH 1.5 (37°C with μ = 1.0).





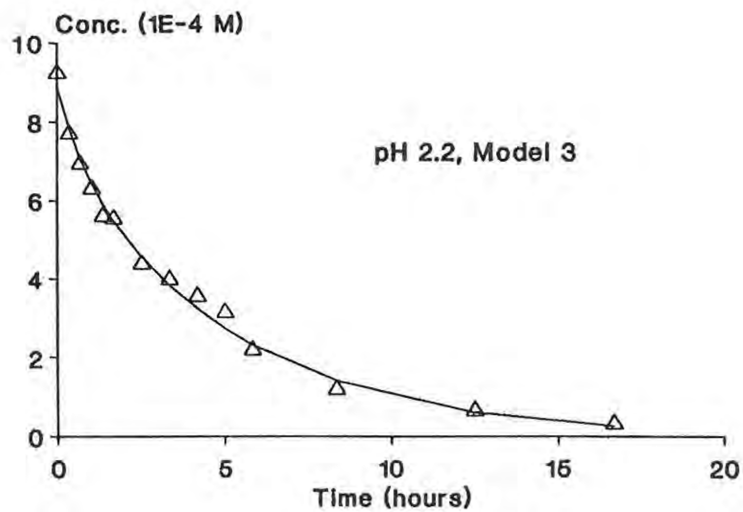
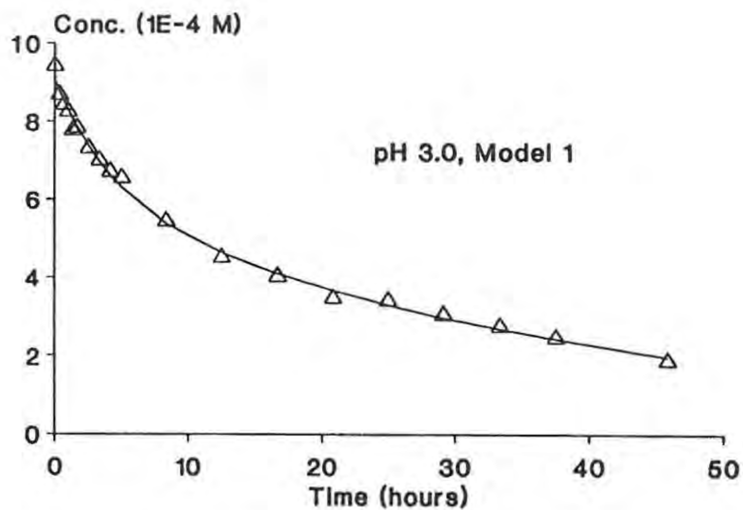
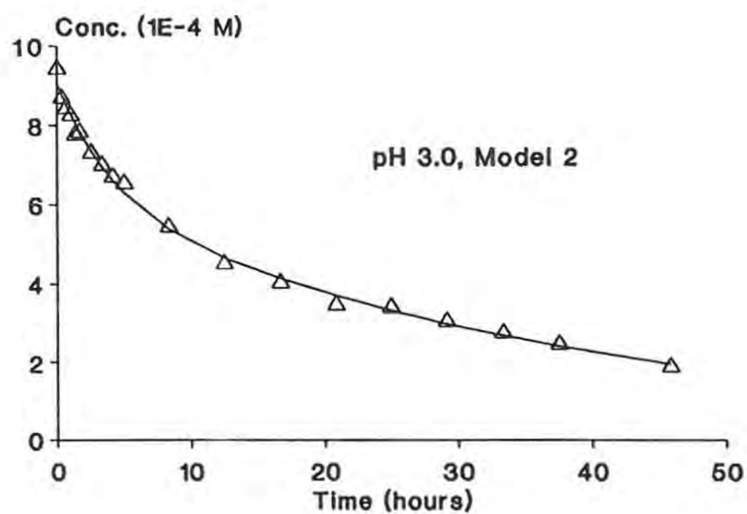


Figure 5.35a, b and c Experimental and fitted data for Model 1 (a), Model 2 (b) and Model 3 (c) at pH 2.2 (37°C with μ = 1.0).





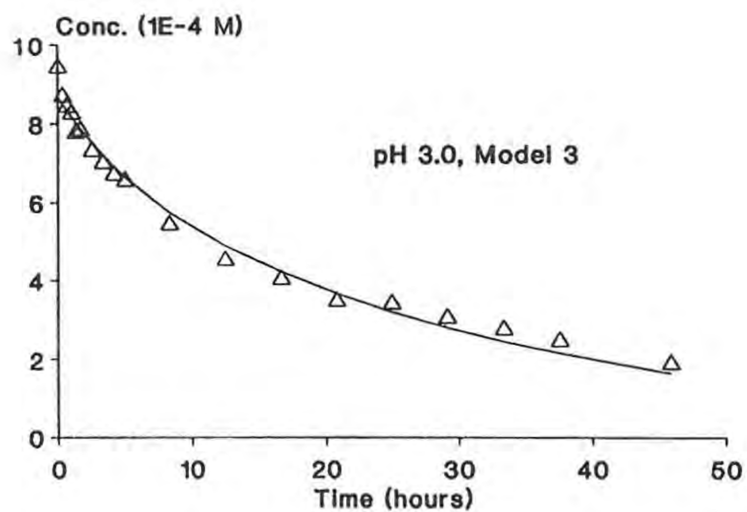
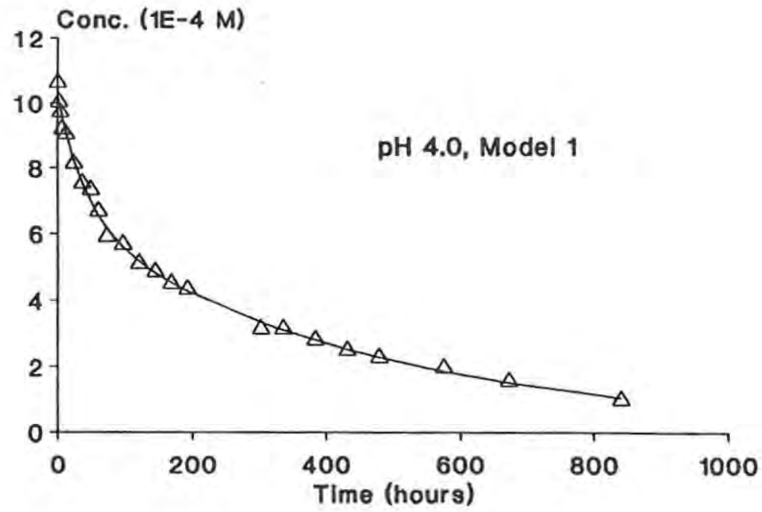
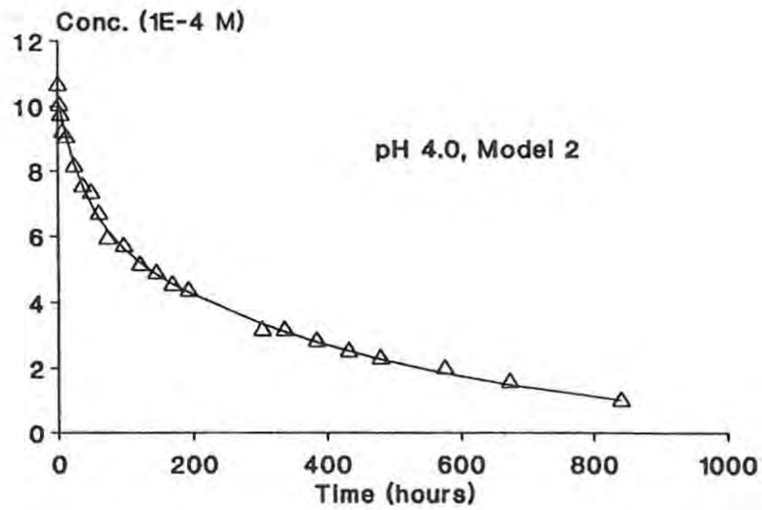


Figure 5.36a, b and c Experimental and fitted data for Model 1 (a), Model 2 (b) and Model 3 (c) at pH 3.0 (37°C with μ = 1.0).





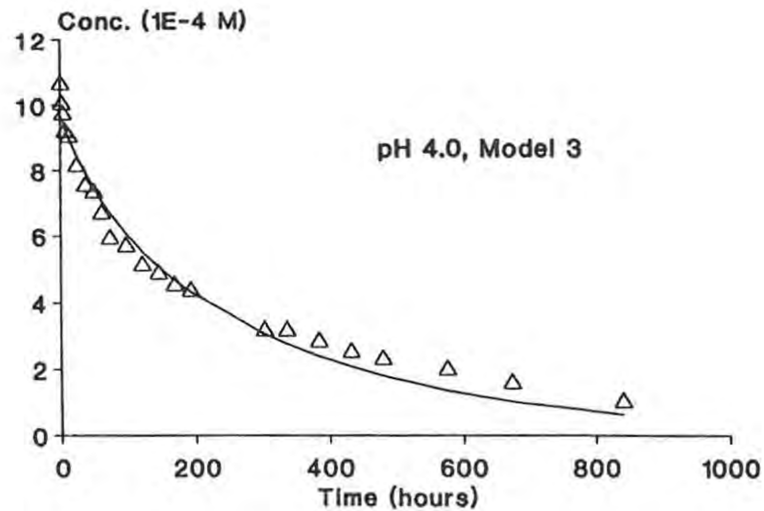
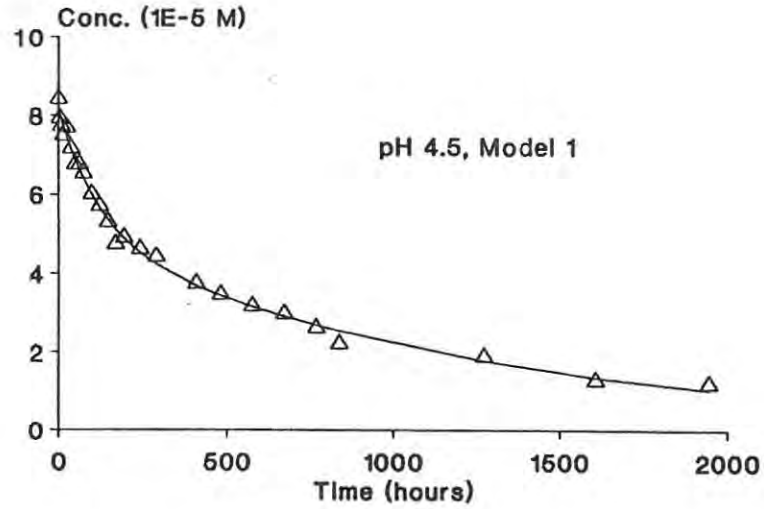
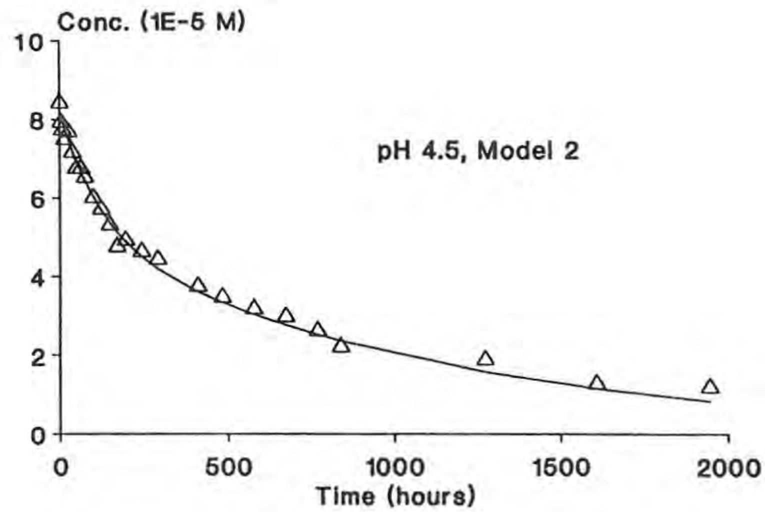


Figure 5.37a, b and c Experimental and fitted data for Model 1 (a), Model 2 (b) and Model 3 (c) at pH 4.0 (37°C with μ = 1.0).





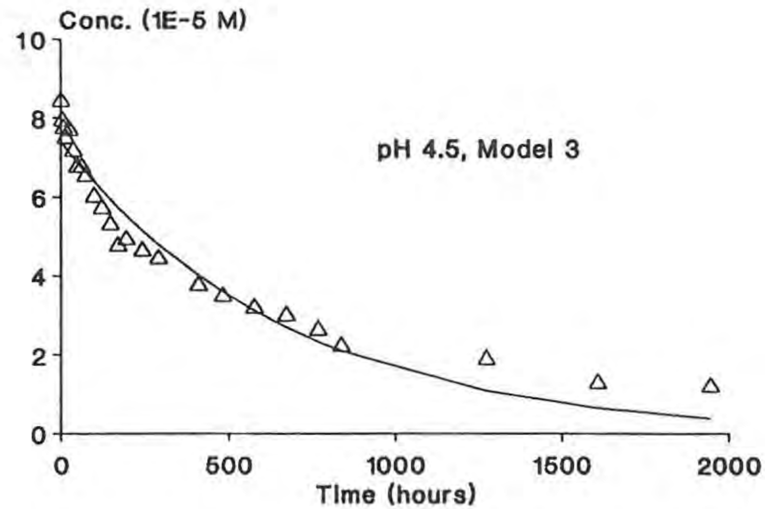


Figure 5.38a, b and c Experimental and fitted data for Model 1 (a), Model 2 (b) and Model 3 (c) at pH 4.5 (37°C with μ = 1.0).

Figure 5.39a

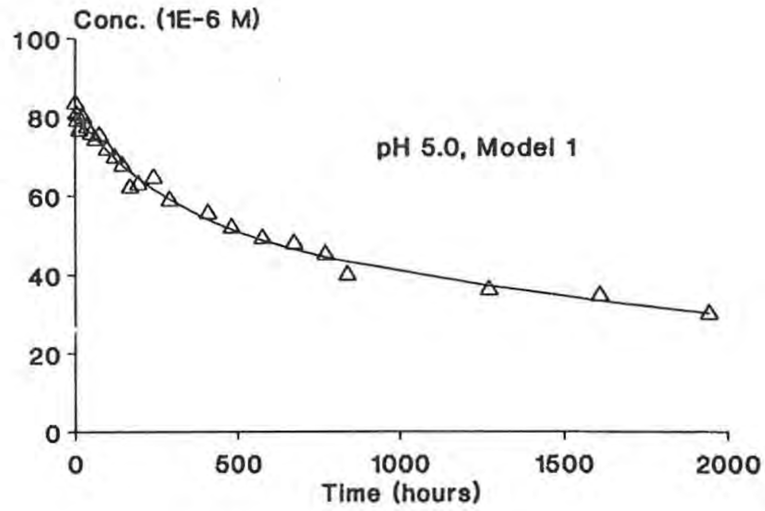


Figure 5.39b

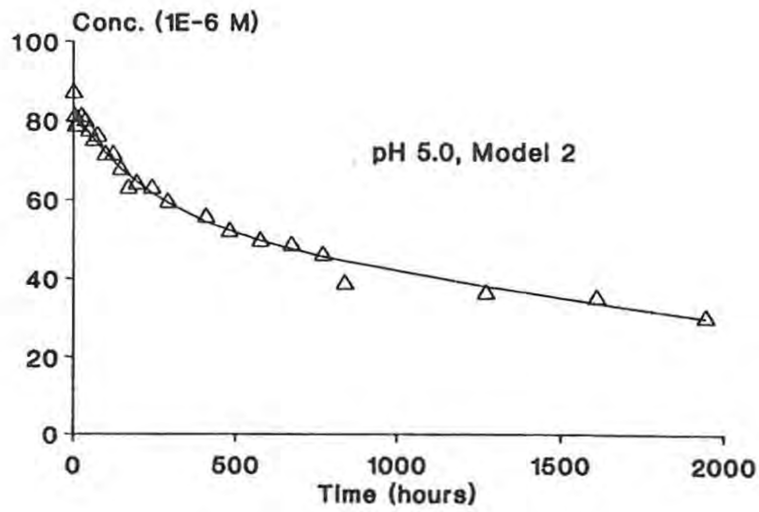


Figure 5.39c

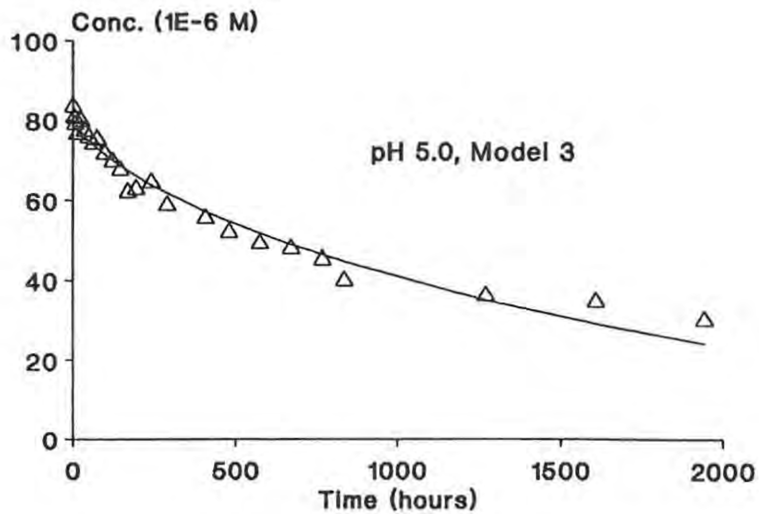


Figure 5.39a, b and c Experimental and fitted data for Model 1 (a), Model 2 (b) and Model 3 (c) at pH 5.0 (37°C with μ = 1.0).

further distinction as to the correct scheme cannot be made. Complete elucidation of the pathway cannot be accomplished without isolating degradation compounds for use as standards. After determining the concentration *vs.* time profiles of degradation products, simultaneous modelling of parent compound and product profiles will provide further evidence for the correct degradation pathway. Additional evidence for the correct pathway could also be obtained by utilising pure degradation compounds to determine the "initial rate" of each individual reaction and subsequently determine the magnitude of each rate constant (213).

5.3 CONCLUSION

In conclusion, the degradation characteristics of josamycin in aqueous media have been established by investigating the effect of pH, ionic strength and buffer concentration on the reaction rate. The pH-rate profile was typically V-shaped demonstrating specific acid catalysis over a wide range of acidic pH's with very rapid degradation at the lower pH region. Catalysis in alkaline media was found to be more complex and josamycin also undergoes rapid degradation at high pH. On the other hand, degradation due to catalysis by water is extremely small. Degradation in acidic media also exhibited a significant primary salt effect which was described by the modified Debye-Hückle equation up to an ionic strength of 1.0. Conversely, a negligible secondary salt effect was observed in McIlvaine's buffer. Adjustment of the reaction medium to constant ionic strength was therefore essential for accurate quantitation of the pH-rate profile whilst buffer concentration was not a significant factor.

Over the physiological pH range the stability of josamycin varied greatly from being rapidly degraded in simulated gastric fluid at pH 1.0 where 50% was degraded in about 20 mins (μ = 1.0), to being highly stable at pH 6.5. Degradation in gastric fluid could therefore be extensive after an oral dose whilst insignificant degradation would occur in the small intestine. However, josamycin was found to be significantly more stable than the extensively used macrolide - erythromycin base. Comparison of rates obtained in these studies with those obtained by Atkins *et al.* (190) show that at pH 4.0, josamycin is approximately 45 times more stable than erythromycin whilst at pH 7.0, it is about 5 times more stable. Boggiano and Gleeson (50) showed that 90% of an oral dose of erythromycin could be inactivated in the stomach in 5 mins (pH 1.0) whilst under similar conditions inactivation of 90% of an oral dose of josamycin would take more than 60 mins. Although bioavailabiltiy problems associated with acid degradation should be significantly less with josamycin than with

erythromycin, results from this study indicate that the bioavailabiltiy of josamycin could still be significantly compromised by *in-vivo* acid degradation. In this regard the gastric pH and gastric residence time of dissolved drug will play an important role in determining bioavailability.

Utilisation of HPLC, thermospray mass-spectrometry and mathematical modelling provided significant insight into the acid degradation pathway of josamycin. Furthermore, the biphasic nature of the first-order plots of acid degradation profiles could be adequately accounted for by the degradation pathways described by schemes 2 and 3. These pathways were found to be feasible by the use of non-linear curve fitting techniques. Results suggest that josamycin initially undergoes a rapid reversible acid catalysed isomerisation reaction to an equilibrium with its isomer, together with slower acid catalysed hydrolysis of the glycosidic linkage between the mycaminose and mycarose moieties of one or both isomers. Various reaction pathways were proposed, two of which were found to be feasible. However, elucidation of the correct pathway will require further investigation utilising pure samples of degradation products.

Table 5.1 Apparent First-Order Degradation Rate Constants for Acid and Alkali Degradation of Josamycin under all Conditions Investigated.

		Apparent Rate Constants	
pH	μ	K_{init} hr^{-1} (mean, n=3)	K_{term} hr^{-1} (mean, n=3)
1.0	0.1*	2.6264	1.1292
1.0	0.2	3.2801	1.4524
1.0	0.5	5.2178	1.9936
1.0	1.0	7.2716	3.2321
1.2	0.06*	2.5935	6.4926×10^{-1}
1.2	1.0	5.1403	1.6301
1.2	SGF**	2.1652	6.9040×10^{-1}
1.5	0.03*	0.9880	3.0612×10^{-1}
1.5	1.0	1.6927	7.5932×10^{-1}
2.2 - 0.04M	1.0	6.4383×10^{-1}	1.4142×10^{-1}
2.2 - 0.05M	1.0	6.6555×10^{-1}	1.4443×10^{-1}
2.2 - 0.10M	1.0	5.7853×10^{-1}	1.5398×10^{-1}
2.2 - 0.20M	1.0	2.9541×10^{-1}	1.4853×10^{-1}
3.0	1.0	1.3150×10^{-1}	3.0211×10^{-2}
4.0	1.0	7.0922×10^{-3}	2.2714×10^{-3}
4.5	1.0	2.9964×10^{-3}	8.6099×10^{-4}
5.0	1.0	1.2571×10^{-3}	4.1639×10^{-4}
5.5	1.0	3.1480×10^{-4}	----
6.0	1.0	1.9421×10^{-4}	----
6.5	1.0	1.4609×10^{-4}	----
7.0	1.0	2.1734×10^{-4}	----
7.5	1.0	4.0846×10^{-4}	----
7.5	SIF***	3.6834×10^{-4}	----
8.0	1.0	9.1814×10^{-4}	----
8.5	1.0	2.0877×10^{-3}	----
9.0	1.0	5.5226×10^{-3}	----
10.0	1.0	4.1124×10^{-2}	4.1124×10^{-2}
11.0	1.0	3.1083×10^{-1}	1.7970×10^{-1}
12.0	1.0	1.4842	8.1972×10^{-1}

* Hydrochloric acid without adjustment of ionic strength.
** USP simulated gastric fluid.
*** USP simulated intestinal fluid.

Table 5.2 Percentage of Intact Josamycin Remaining after Inoculation of Simulated Gastric Fluids at pH 1.0, 1.2 and 1.5 and McIlvaine's Buffer at pH 2.2 and 3.0 and USP Simulated Intestinal Fluid (pH 7.5).

	Percentage of Intact Josamycin Remaining.					
Time (mins)	pH 1.0*	pH 1.2 (SGF**)	pH 1.5*	pH 2.2***	pH 3.0***	pH 7.5 (SIF****)
0	100	100	100	100	100	100
10	65	70	83	--	--	--
20	52	58	80	83	92	--
30	44	48	70	--	--	--
40	37	42	60	70	90	--
50	30	--	--	--	--	--
60	26	34	55	60	88	99

* Hydrochloric acid without ionic strength adjusted.
** USP simulated gastric fluid.
*** McIlvaine's buffer (μ = 1.0).
**** USP simulated intestinal fluid.

Table 5.3 Mass-Spectral Data for Josamycin, P1, P2 and P3 with the Respective Thermospray Experimental Conditions.

	Thermospray Experimental Conditions						Mass-Spectral Data			
Sample	Fila-ment	Elec. Energy x+800V	Stem Temp. °C	Tip Temp. °C	Vapour Temp. °C	Source Temp. °C	*m/z*	Mol. Mass	Proposed Structure of Ion	Abundance
J*	on	50	104	150	240	274	828.65	827	$[J + 1]^+$	62872
							829.65		$[J + 2]^+$	26856
							768.70		$[J(-)CH_3COOH + 1]^+$	8180
							769.70		$[J(-)CH_3COOH + 2]^+$	3910
P1*	on	250	104	172	232	240	829.15	827	$[P1 + 1]^+$	2725
							830.05		$[P1 + 2]^+$	1397
							769.05		$[P1(-)CH_3COOH + 1]^+$	2343
							770.05		$[P1(-)CH_3COOH + 2]^+$	1043
P2**	on	155	90	134	238	240	600.50	599	$[P2 + 1]^+$	12303
							601.50		$[P2 + 2]^+$	4002
							540.45		$[P2(-)CH_3COOH + 1]^+$	7468
							541.45		$[P2(-)CH_3COOH + 2]^+$	2485
P3**	on	155	90	134	238	240	600.50	599	$[P3 + 1]^+$	33240
							601.50		$[P3 + 2]^+$	10416
							540.45		$[P3(-)CH_3COOH + 1]^+$	12365
							541.45		$[P3(-)CH_3COOH + 2]^+$	3655

* Direct injection technique.

** Spectra obtained after chromatogrphic separation of P2 and P3

Table 5.4 Parameter and Curve Fitting Data for Model 1 (mean data, n=3).

			Model 1 ($P1 \rightleftharpoons J \rightarrow P2 \rightleftharpoons P3$)			
pH	μ	J_0 (M)	k_1 (hr^{-1})	k_{-1} (hr^{-1})	k_2 (hr^{-1})	Sum of Sq. Deviations.
1.0	0.1*	7.6982×10^{-4}	2.2273	7.7292	1.4216	1.1951×10^{-9}
1.0	0.2	8.4861×10^{-4}	3.0991	11.513	1.8762	6.6420×10^{-10}
1.0	0.5	8.6705×10^{-4}	4.4236	11.567	2.8228	2.0658×10^{-9}
1.0	1.0	8.2716×10^{-4}	7.5480	25.060	4.0612	2.0025×10^{-10}
1.2	0.06*	8.9014×10^{-4}	2.6968	5.8358	1.0869	2.5805×10^{-9}
1.2	1.0	9.4815×10^{-4}	6.2844	12.170	2.6313	1.4539×10^{-10}
1.2	SGF**	8.7450×10^{-4}	1.6690	4.0921	9.5440×10^{-1}	1.1401×10^{-9}
1.5	0.03*	8.7037×10^{-4}	1.1510	3.1334	4.3927×10^{-1}	3.7170×10^{-9}
1.5	1.0	8.5671×10^{-4}	1.4698	3.8816	1.0344	1.8768×10^{-9}
2.2	1.0	9.2652×10^{-4}	4.2510×10^{-1}	2.3416	2.3804×10^{-1}	5.5766×10^{-9}
3.0	1.0	9.1345×10^{-4}	7.1180×10^{-2}	2.3790×10^{-1}	3.6211×10^{-2}	6.2262×10^{-9}
4.0	1.0	1.0173×10^{-3}	6.8608×10^{-3}	1.5745×10^{-2}	3.2458×10^{-3}	8.6744×10^{-11}
4.5	1.0	8.3183×10^{-5}	2.5389×10^{-3}	5.5777×10^{-3}	1.2380×10^{-3}	9.5945×10^{-11}
5.0	1.0	8.0913×10^{-5}	1.2290×10^{-3}	2.5696×10^{-3}	4.2185×10^{-4}	9.7282×10^{-11}

Table 5.5 Parameter and Curve Fitting Data for Model 2 (mean data, n=3).

			Model 2 ($P2 \leftarrow J \rightleftharpoons P1 \rightarrow P3$)				
pH	μ	J_0 (M)	k_1 (hr^{-1})	k_{-1} (hr^{-1})	k_2 (hr^{-1})	k_5 (hr^{-1})	Sum of Sq. Deviations.
1.0	0.1*	8.0416×10^{-4}	2.6303	5.2218	0.6940	1.2818	1.6040×10^{-9}
1.0	0.2	8.4895×10^{-4}	3.6390	9.1440	1.2705	1.7293	7.3845×10^{-10}
1.0	0.5	8.6587×10^{-4}	5.0211	10.423	2.2466	1.2861	2.1468×10^{-9}
1.0	1.0	8.2757×10^{-4}	8.4234	22.999	3.2464	2.3761	2.4676×10^{-10}
1.2	0.06*	8.9329×10^{-4}	2.9881	6.0672	9.6252×10^{-1}	3.0264×10^{-1}	2.6103×10^{-9}
1.2	1.0	9.4899×10^{-4}	6.8244	11.203	2.3068	5.6447×10^{-1}	1.5206×10^{-10}
1.2	SGF**	8.7914×10^{-4}	1.8862	3.7059	7.5462×10^{-1}	4.3397×10^{-1}	1.1537×10^{-9}
1.5	0.03*	8.7239×10^{-4}	1.2426	3.2600	4.1214×10^{-1}	1.0155×10^{-1}	3.8865×10^{-9}
1.5	1.0	7.6412×10^{-4}	1.5101	3.8909	9.7026×10^{-1}	2.1477×10^{-1}	2.0938×10^{-9}
2.2	1.0	9.2610×10^{-4}	4.6085×10^{-1}	2.1444	2.2717×10^{-1}	4.5769×10^{-2}	5.6081×10^{-9}
3.0	1.0	9.0627×10^{-4}	6.1314×10^{-2}	1.5296×10^{-1}	3.1033×10^{-2}	1.0451×10^{-2}	5.4696×10^{-9}
4.0	1.0	1.0221×10^{-3}	7.6924×10^{-3}	1.6333×10^{-2}	3.0035×10^{-3}	5.9734×10^{-4}	8.6994×10^{-9}
4.5	1.0	8.3135×10^{-5}	3.2179×10^{-3}	6.6508×10^{-3}	1.2742×10^{-3}	2.6667×10^{-4}	1.4154×10^{-10}
5.0	1.0	8.1501×10^{-5}	1.0871×10^{-3}	3.6037×10^{-3}	4.9261×10^{-4}	6.7565×10^{-6}	9.8888×10^{-10}

Table 5.6 Parameter and Curve Fitting Data for Model 3 (mean data, n=3).

			Model 3 ($J \rightleftharpoons P1 \rightarrow P2 \rightleftharpoons P3$)			
pH	μ	J_0 (M)	k_1 (hr^{-1})	k_{-1} (hr^{-1})	k_2 (hr^{-1})	Sum of Sq. Deviations
1.0	0.1*	7.5645×10^{-4}	2.3147	6.1422	3.6000×10^{-1}	6.7817×10^{-9}
1.0	0.2	8.0188×10^{-4}	3.0112	7.0548	4.0381×10^{-1}	5.6154×10^{-9}
1.0	0.5	8.1121×10^{-4}	4.5538	8.7624	3.6780×10^{-1}	1.0496×10^{-8}
1.0	1.0	7.8698×10^{-4}	7.0080	18.657	8.3394×10^{-2}	5.4324×10^{-9}
1.2	0.06*	8.1238×10^{-4}	1.9948	4.5387	3.6000×10^{-1}	2.3025×10^{-8}
1.2	1.0	8.7055×10^{-4}	5.0441	13.844	3.6000×10^{-1}	2.4377×10^{-8}
1.2	SGF**	8.2147×10^{-4}	1.6745	3.6016	3.6000×10^{-1}	1.2280×10^{-8}
1.5	0.03*	8.0355×10^{-4}	7.7868×10^{-1}	1.8891	3.6000×10^{-1}	2.0047×10^{-8}
1.5	1.0	7.2298×10^{-4}	1.9553	7.2408	3.6000×10^{-1}	9.7785×10^{-9}
2.2	1.0	8.8632×10^{-4}	4.0449×10^{-1}	1.3934	2.4221	8.8500×10^{-9}
3.0	1.0	8.6891×10^{-4}	6.2202×10^{-2}	1.6858×10^{-1}	1.8000×10^{-1}	1.6404×10^{-8}
4.0	1.0	9.6302×10^{-4}	5.8824×10^{-3}	1.2936×10^{-2}	5.0000×10^{-7}	4.4807×10^{-8}
4.5	1.0	7.4854×10^{-5}	3.0335×10^{-3}	5.0000×10^{-1}	5.0000×10^{-4}	6.7670×10^{-10}
5.0	1.0	7.9097×10^{-5}	1.1083×10^{-3}	5.0000×10^{-1}	1.0000×10^{-4}	2.4603×10^{-10}

* Hydrochloric acid without adjustment of ionic strength.

** USP simulated gastric fluid.

CHAPTER 6

BIOAVAILABILITY STUDIES ON JOSAMYCIN

Josamycin is reported to exhibit significant advantages over other macrolide antibiotics *e.g.*, it does not induce resistance in susceptible micro-organisms (13,19,214), has excellent tissue penetration and distribution (48,61), is relatively acid stable compared to erythromycin and is extremely well tolerated with a low incidence of side-effects (28). However, it has never achieved significant usage in this country possibly due to poor clinical efficacy. The bioavailability of josamycin from the dosage form available commercially in South Africa (Josamycin 200 mg tablets) was therefore investigated. Poor results (*vide infra* 6.2.2), however, initiated *in-vitro* investigations into the possibility that tablet dissolution characteristics and the intrinsic dissolution and solubility characteristics of josamycin were responsible for poor bioavailability. Dissolution and solubility studies on raw material indicated that the absorption of josamycin may indeed be dissolution and solubility limited whilst dissolution studies on the Josamycin 200 mg tablets indicated that formulation factors could further adversely influence bioavailability. Studies were therefore undertaken to investigate the bioavailability of josamycin without the influence of dissolution and tablet formulation factors by administering a solution and subsequently the powder as a suspension. Furthermore, bioavailability from a different tablet formulation marketed in some European countries (Josacine 500 mg tablets) was also investigated.

To-date, the majority of bioavailability and pharmacokinetic data for josamycin in humans have been derived utilising microbiological assay techniques with either *Micrococcus luteus* or *Sarcinia lutea* as test organisms (Table 1.2). These non-specific methods have continued to be used despite the fact that metabolites of josamycin have been reported to be microbiologically active (72). A major factor contributing to the continued use of microbiological techniques has been the lack of a simple and specific HPLC assay. As discussed in chapter 3, HPLC assays described in the literature for the determination of josamycin in biological fluids are either inadequate for bioavailability and pharmacokinetic studies or require complex and expensive equipment. Only one of the pharmacokinetic studies reported in the literature utilised a specific HPLC assay (47). However, in this study limited data on study design and pharmacokinetics were presented. Furthermore, the elimination half-life determined was 12.7 hrs which is approximately 4 - 11 times greater than those determined by microbiological methods. Moreover, the serum concentration *vs.* time profile of the total concentration of josamycin plus the major metabolite (JM-O_1) more closely reflected the serum concentration *vs.* time profiles obtained by microbiological methods in other studies. This suggests that pharmacokinetic data for josamycin

calculated from data obtained by microbiological assay techniques may be erroneous and reflect the pharmacokinetics of josamycin including metabolite rather than parent only. A confusing picture of the pharmacokinetics of josamycin therefore exists. Pharmacokinetic parameters obtained after the analysis of biological samples with the specific assay described in chapter 3 were therefore determined in order to clarify the ambiguities in the literature.

Furthermore, multiple dose studies have also had variable results. Fraschini *et al.* (48) found that after dosing with 1 gram twelve hourly for seven days there was no difference between $AUC_{0-\infty}$ for the first and last doses, although these subjects were bronchopneumopathic. In two different studies lasting four and eight days also with a dosing regimen of 1 gram twelve hourly, Wildfeuer *et al.* (5) found that the C_{max} after the last dose in both studies was double that for the first dose whilst Okolicsanyi *et al.* (49) found the C_{max} after dosing with 1 gram twelve hourly for 4 days to be over four times the C_{max} after the initial dose. Also, Strausbaugh *et al.* (28) showed varying degrees of accumulation after multiple dosing with dosing intervals of between 6 and 12 hours. In all studies, however, dosing intervals were large compared to the half-life of about 2 hrs as determined in each study and it is surprising that accumulation occurred during any of these dosing regimens. Little is known as to possible reasons for any accumulation although Bergan *et al.* (36) has shown that concentrations attained with doses ranging from 250 mg to 2 gm were dose dependent. Consequently, a multiple dose study was conducted to determine any changes in pharmacokinetic parameters compared with single dose parameters and to attempt to determine any possible causes thereof.

6.1 BIOAVAILABILITY TRIALS

6.1.1 Study Design

Healthy male subjects who volunteered for the studies and who were found to be in good health by physical examination, haematological testing and urinalysis were used in the trials after their informed consent had been given. Haematological tests included urea, serum creatinine, urates, total bilirubin, SGOT and SGPT, glucose, ESR and total blood count. Urinalysis included pH, protein, glucose and blood together with microscopic examination. Subjects with a history of any previous liver disorder, allergy to macrolide antibiotics, were excessively overweight or had received any drug therapy during the preceding two weeks were excluded. Demographic data

of the subjects are tabulated in table 6.1.

The trials consisted of 5 phases and were conducted with two different sets of six subjects. The first set (subjects 1 - 6) took part in phases 1 and 2 which were conducted in a parallel fashion, whilst the second set (subjects 7 - 12) took part in phases 3, 4 and 5, with phases 3 and 4 designed as a randomised two-way cross-over followed by phase 5. The dosage regimens were as follows:-

Phase 1:- 5 x Josamycin 200 mg tablets (Yamanouchi Pharmaceutical Co., Tokyo, Japan) as a single oral dose.

Phase 2:- 1 gram of josamycin powder as an aqueous suspension prepared by mixing the powder in 30 mls of de-ionised water.

Phase 3:- 2 x Josacine 500 mg tablets (Pharmuka, Gennevilliers, France) as a single oral dose.

Phase 4:- 1 gram of josamycin as a solution dissolved in 10 mls of McIlvaine's Buffer of pH 4.0.

Phase 5:- 1 gram of josamycin as a solution dissolved in 10 mls of McIlvaine's Buffer of pH 4.0 administered orally every two hrs for five doses. Due to the variability in accumulation after six and twelve hour dosing intervals as cited in the literature (Table 1.2), a dosing interval close to the terminal elimination half-life observed in Phase 4 was utilised).

Trial subjects were asked to refrain from ingesting any alcoholic beverages for 48 hrs prior to the commencement of each study, or any food or caffeine containing beverages from 10.00 pm on the evening before each study. Subjects were also asked to ensure that they had an adequate fluid intake during the day prior to each study and to consume 250 mls of water at least 2 hrs before the start of each study in order to aid phlebotomy.

For single dose studies, subjects were asked to report to the trial centre at 7.30 am on the morning of the trial. On arrival, a blank urine specimen was collected both for analysis and for the preparation of standards and *in-vitro* controls. Thereafter, a winged infusion set (Terumo Corporation, Tokyo, Japan) was inserted into an arm vein and held in position for the duration of the trial. After withdrawal of a blank sample, the catheter was flushed at five minute intervals for 15 mins with 0.5 mls of a 100 IU/ml solution of heparin in normal saline. Thereafter, the catheter was flushed every hour and immediately after the withdrawal of a blood sample. From previous trials it was found that frequent flushing of the catheter with heparin immediately after insertion together with a "stabilisation period" of half an hour prior to the commencement of regular sampling considerably reduced the incidence of blockages during the trial period. This significantly improved the efficacy of sample

withdrawal which was vital during the trials described due to the short time between samples (10 mins) during the initial part of each study. Subjects were also asked to keep warm and to minimise movement of the forearm, in particular flexion and extension of the wrist.

Subjects were administered their respective doses together with 250 mls of water after which they maintained a slightly supine position for 2 hrs after administration of the dose. Thereafter, subjects were free to adopt any position whilst remaining in bed for the following 4 hrs. After this time, subjects were no longer confined to bed but were forbidden to pursue any form of sport or strenuous exercise. A standard light breakfast consisting of 30 g of cereal, two teaspoons of sugar, a helping of skimmed milk, three lightly buttered slices of toast with jam and 250 mls of orange juice was served 2.5 hrs after dosing. A standard fat-free lunch of chicken, vegetables, fruit salad and orange juice was served 5.5 hrs after dosing. Subjects were encouraged to drink water after the initial post-dose urine collection time so as to enable the collection of urine at two hourly intervals.

For the multiple dose study, subjects were asked to refrain from ingesting food and caffeine from 2.00 pm and to report to the trial centre at 11.30 pm. Dosing was commenced at 12.00 am and the blank blood sample and trough samples taken by venipuncture. The procedure for insertion of an indwelling catheter as described above was followed prior to the last dose at 8.00 am and subsequent samples were withdrawn via the catheter.

6.1.2 Sample Collection and Storage

Blood samples (10 mls) were taken during phases 1 and 2 at 0, 0.17, 0.33, 0.5, 0.67, 0.83, 1.0, 1.33, 1.67, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0 and 8.0 hrs after dosing whilst blood samples were taken during phases 3 and 4 at 0, 0.17, 0.33, 0.50, 0.67, 0.83, 1.0, 1.33, 1.67, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 and 12.0 hrs after dosing. Blood samples during phase 5 were taken prior to the first, third, fourth and fifth dose and thereafter as in phases 3 and 4. During phases 3 and 4, duplicate samples were withdrawn at randomly selected sampling times for use as *ex-vivo* controls. Whole blood samples were allowed to clot for 30 min after which they were centrifuged at 1600 x g for 20 mins. The serum was then harvested and 1.5 mls of each sample pipetted into a clean test-tube for the initial analysis. The remaining serum was pipetted into a second test-tube for any repeat analysis which may have been required. Samples were stored at -15°C until analysed.

Urine collection periods were 0-2, 2-4, 4-6, 6-8, 8-10, 10-12 and 12-24 hrs after dosing. Urine collected during and at the end of each collection period was

pooled, the volume and pH noted and a sample pipetted into a test-tube and stored at -15°C until analysed.

6.1.3 Sample Analysis

The concentration of josamycin in serum and urine was determined by HPLC as described in 3.3.1.6 and 3.5.1 respectively. Initially a calibration range for serum of 0.1 - 2.0 μg/ml was used, however, the concentrations in numerous samples from phase 1 were below this lower limit. These samples were therefore re-analysed using a calibration curve over the concentration range of 0.025 - 2.0 μg/ml, which was used for all subsequent analysis of serum samples from single dose studies. Serum samples obtained after multiple dosing were analysed utilising a calibration range of 0.05 - 5.0 μg/ml and all samples with concentrations above this range were diluted accordingly with blank serum and re-analysed. Urine samples above 5 μg/ml were also diluted accordingly with blank urine and re-analysed.

Serum and urine standards and *in-vitro* controls were prepared prior to the commencement of analysis and stored at -15°C until required. The feasibility of this is discussed in 3.6.2. *In-vitro* controls were prepared by an independent person and the concentrations made known to the analyst only after analysis was complete. Results of *in-vitro* and *in-vivo* controls are tabulated in table 6.2.

6.2 RESULTS AND DISCUSSION

6.2.1 Calculation of Non-Compartmental Parameters

Oral bioavailability, which can be defined as the rate and extent to which a drug is absorbed after oral administration, can be assessed in terms of C_{max}, t_{max} and AUC. C_{max}, t_{max}, $AUC_{0\text{-}last}$ (where $AUC_{0\text{-}last}$ is the area under the concentration *vs.* time curve from time zero to the last measurable concentration for single dose studies) and $AUC_{0\text{-}\tau}$ for the multiple dose study were calculated by the computer program BIOPAK (Statistical Consultants, INC., Lexington, Kentucky, U.S.A.). The elimination half-life was determined for the single dose solution, the powder and the multiple dose solution from the terminal elimination rate constants which were calculated by linear regression of the terminal linear portion of the log concentration *vs.* time curves by BIOPAK. Results are tabulated in table 6.3. The highly variable profiles obtained after dosing

with the two tablet preparations precluded the determination of the elimination rate constants (k_{el}) from these profiles and the half-life of josamycin for these studies could not be assessed. The relatively few data points during the absorption phase following solution and powder administration precluded the calculation of absorption rates for these studies. Similarly, the highly erratic absorption after tablet administration also precluded the calculation of absorption rates for these studies.

The classical parameter used in comparing the extent of absorption from different formulations after single dose administration is the area under the concentration *vs.* time curve from time zero to time infinity - $AUC_{0-\infty}$ (215). However, as determination of this parameter requires calculation of k_{el}, its use for comparative purposes in this study is not possible as k_{el} cannot be reliably determined for most of the profiles obtained after tablet administration. Alternatively, AUC can be calculated as AUC_{0-t} (where AUC_{0-t} is the area under the concentration *vs.* time curve from time 0 to a specific sampling time (t)) or as AUC_{0-last}. AUC_{0-last} has generally been found to be the most accurate way of estimating $AUC_{0-\infty}$ under such circumstances (216). AUC can be calculated by both the linear and logarithmic trapezoidal rules. Chiou (217) has discussed the merits of each method and recommends that the linear trapezoidal rule be used for the pre-peak portion of the curve and the logarithmic trapezoidal rule be used for the post-peak portion in order to minimise error. However, due to the highly erratic absorption from the tablet formulations, the linear trapezoidal rule was considered to be the most appropriate and used throughout. As two different sets of subjects were used for the five phases, comparative statistical testing of parameters between each phase is not strictly valid. However, general comparisons between phases can give a good indication of the performance of each formulation.

Values for k_{el} were in good agreement with those obtained by other investigators utilising microbiological techniques (Table 1.2). This enhances the validity of elimination rates obtained by microbiological methods and indicates that this parameter was not significantly affected by microbiologically active metabolites. However, results obtained in the studies cited herein were in sharp contrast with those of Fourtillan *et al.* (47) who used an HPLC assay employing fluorescence detection. Comparison between concentrations determined by HPLC in studies conducted herein and those determined in other studies using microbiological methods is difficult. A comprehensive study in which the concentrations determined for a common sample by both analytical techniques are compared, is therefore required before the effect of microbiologically active metabolites on concentration can be determine.

6.2.2 Comparative Bioavailability of Solution, Powder and Tablets

Serum concentration *vs.* time profiles and cumulative urinary excretion profiles for the single dose solution (phase 3) are depicted in figures 6.1 and 6.2 respectively, for the powder (phase 2) in figures 6.3 and 6.4 respectively, for Josamycin 200 mg tablets (phase 1) in figures 6.5 and 6.6 respectively, and for Josacine 500 mg tablets (phase 4) in figures 6.7 and 6.8 respectively. Non-compartmental parameters for each phase are tabulated in table 6.3 and urinary excretion data are tabulated in table 6.4.

Comparison of mean C_{max}, t_{max} and $AUC_{0\text{-}last}$ indicate that the bioavailability of josamycin was greatest from the solution followed by the powder followed by the two tablet formulations. All bioavailability profiles showed distinct lag times. For the solution and powder, the lag time probably reflects the gastric transit time as josamycin, with a pKa of 7.1 will be highly ionised in acidic gastric fluid resulting in insignificant absorption occurring from the stomach. This is in agreement with the pH-partition theory which states that only the non-ionised form of dissolved drug will be absorbed through a biological membrane by diffusion - the major mechanism for drug absorption (218). Ionised molecules have, however, been shown to be absorbed to a significant extent through the intestinal mucosa but by virtue of the exceedingly large surface area available for absorption in the intestine (160). The small surface area of the stomach, however, was evidently insufficient to facilitate any significant absorption of ionised molecules. Absorption therefore occurred only once josamycin had reached the small intestine where ionisation will be considerably reduced. Absorption from the solution and powder commenced after only a short lag time as fluids are rapidly expelled from the stomach (166,219). The lag times for the tablets, however, were in most cases significantly longer than for the solution and powder, presumably due to the lag time in disintegration and dissolution.

Absorption of the solution was extremely rapid and complete within 0.33 to 0.50 hrs after dosing (Figure 6.1 and table 6.3) with the actual period of absorption lasting for approximately 10 to 20 mins. These data show that josamycin is inherently well absorbed. The short absorption phase was followed by a rapid distribution phase (*vide infra* 6.2.3.2). The mean C_{max} was 1.640 $\pm$0.670 μg/ml which was attained after a mean t_{max} of 0.39 $\pm$0.08 hrs with a mean $AUC_{0\text{-}last}$ of 1.510 $\pm$0.687 $\mu g.hr.ml^{-1}$. Josamycin was rapidly eliminated with the mean terminal elimination half-life ($t\frac{1}{2}$) calculated to be 1.81 $\pm$0.41 hrs.

Absorption from the powder administered as an aqueous suspension was less rapid and less extensive than from the solution (Figure 6.3) with a mean C_{max} of only 0.755 $\pm$0.420 μg/ml attained after a mean t_{max} of 0.50 $\pm$0.14 hrs. The mean $AUC_{0\text{-}last}$ was only 0.725 $\pm$0.278 $\mu g.hr.ml^{-1}$. The t_{max} for the powder was slightly longer than for the solution, and furthermore, comparison of C_{max} and $AUC_{0\text{-}last}$ values for the

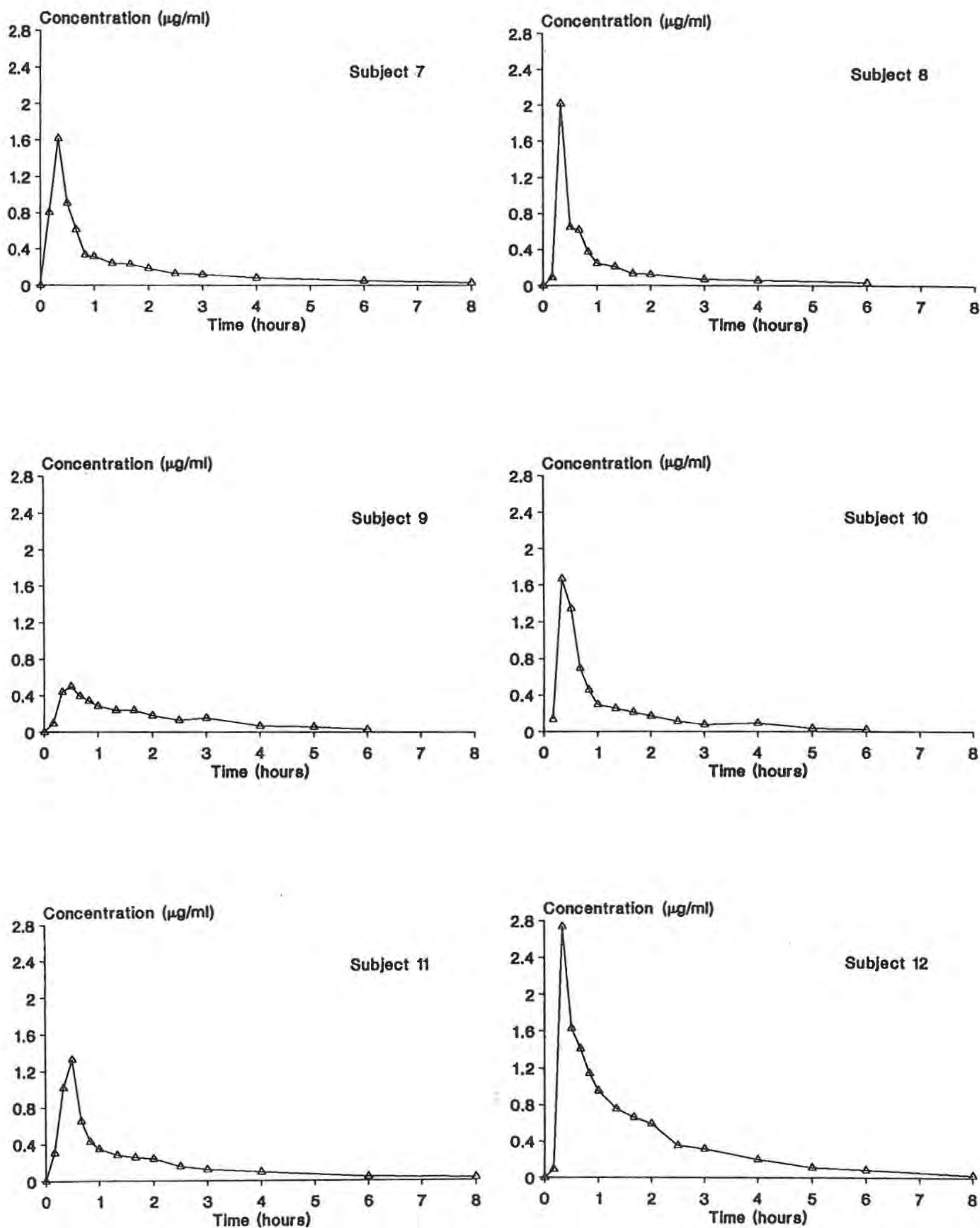
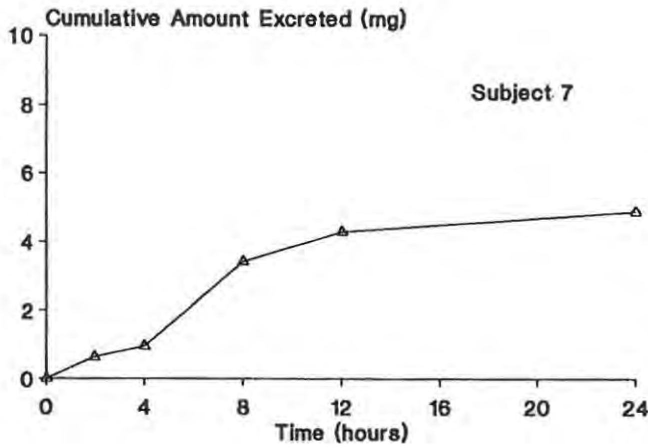
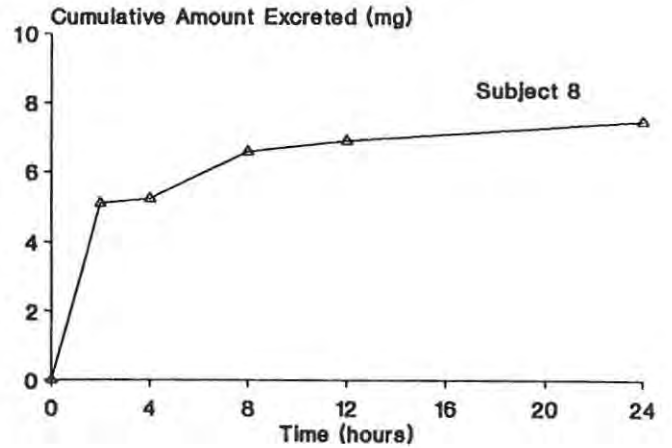
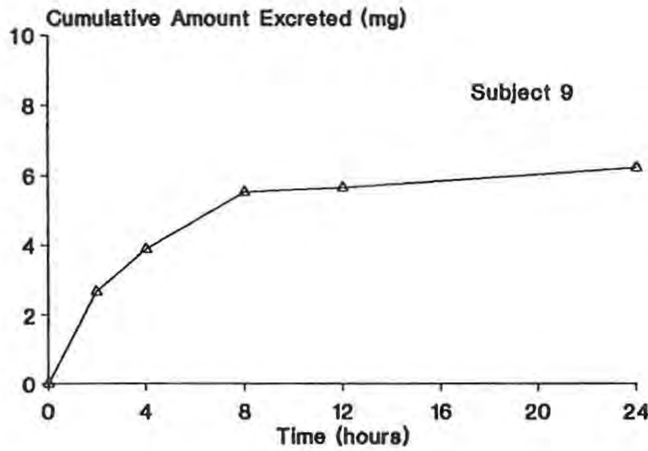
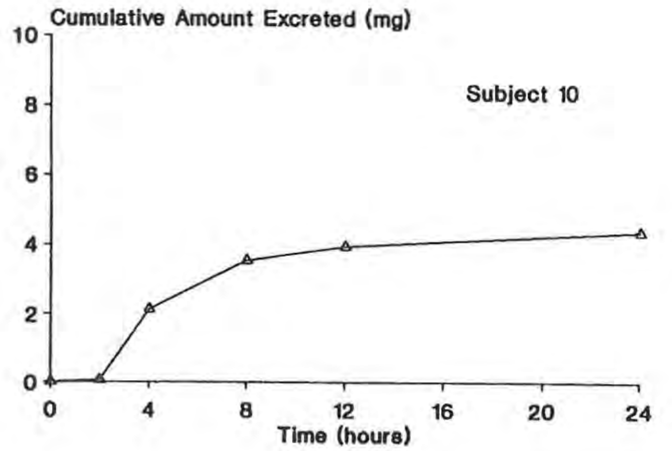


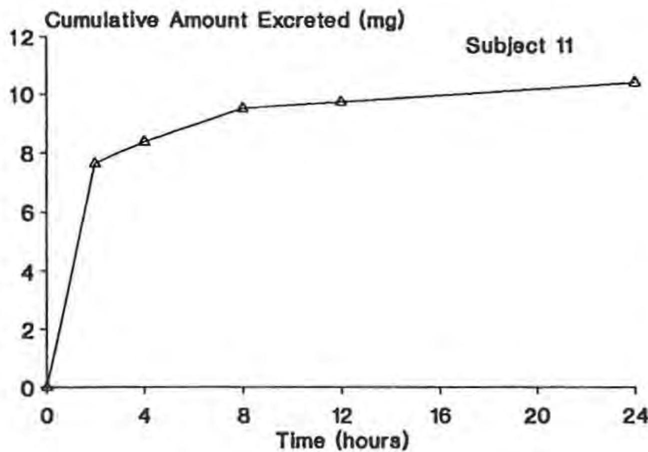
Figure 6.1 Serum concentration *vs.* time profiles for subjects 7 to 12 after the administration of a single oral 1 gram dose of josamycin as a solution.











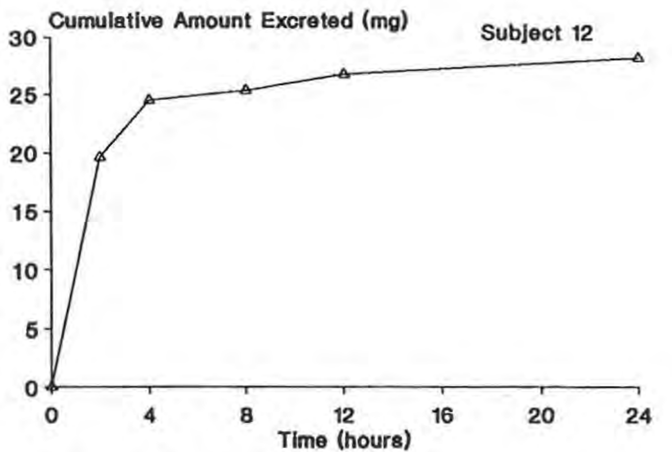


Figure 6.2 Cumulative urinary excretion profiles for subjects 7 to 12 after the administration of a single oral 1 gram dose of josamycin as a solution.

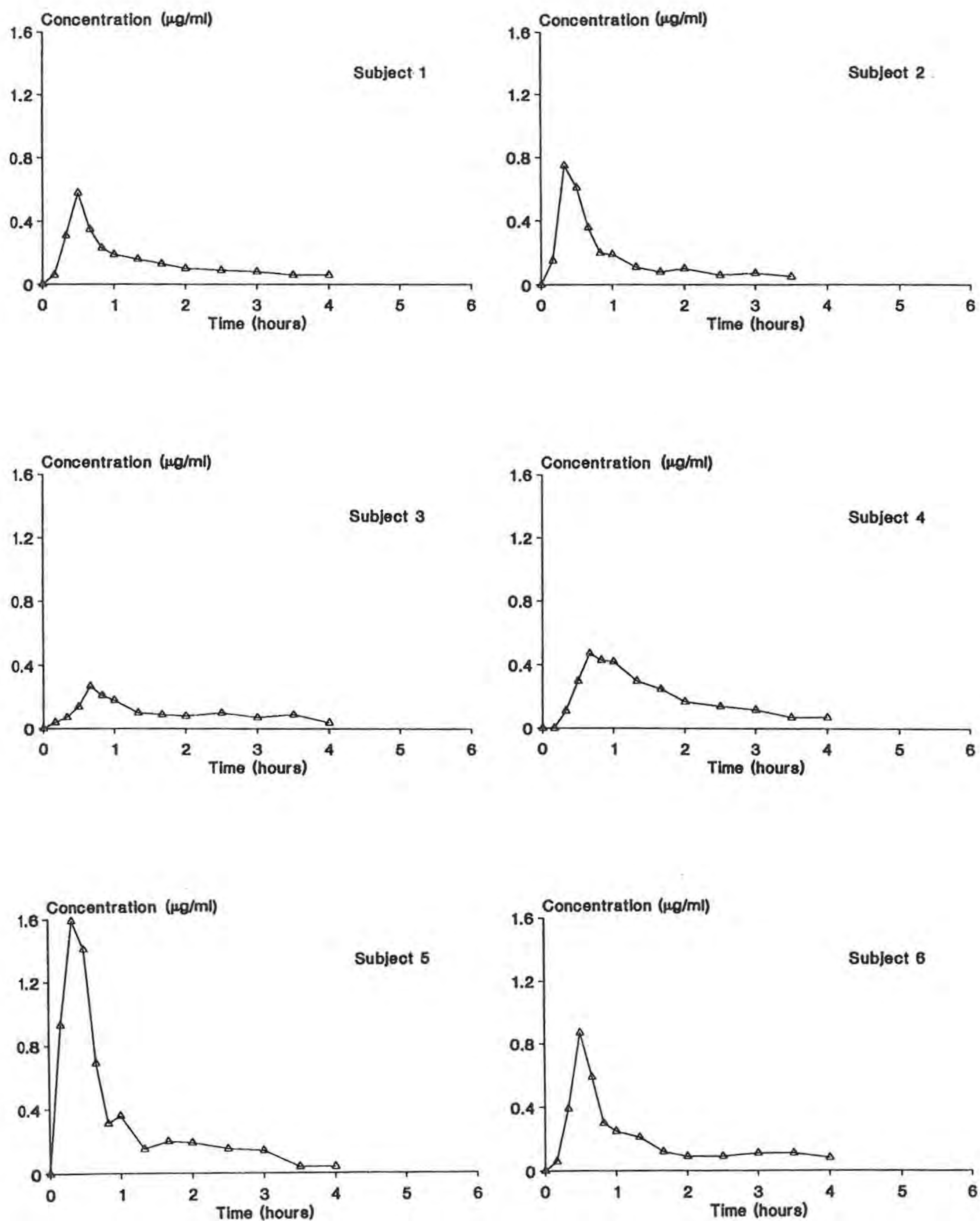
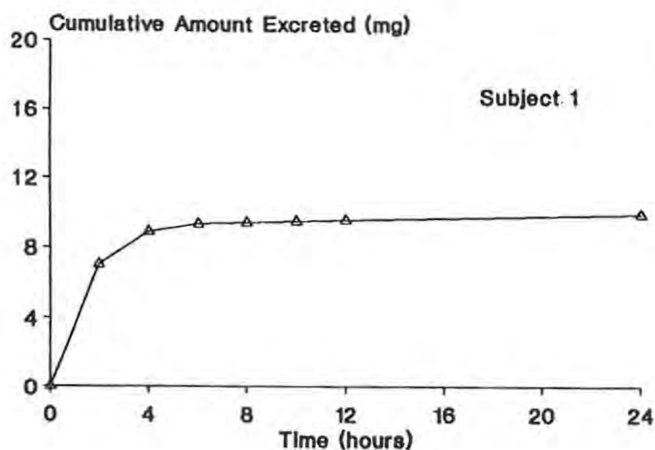
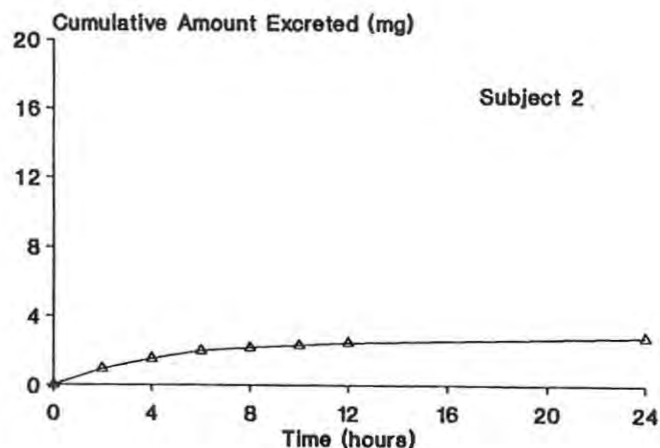
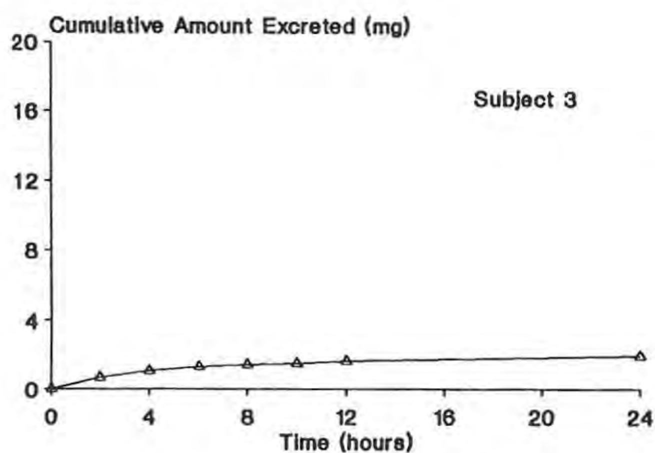
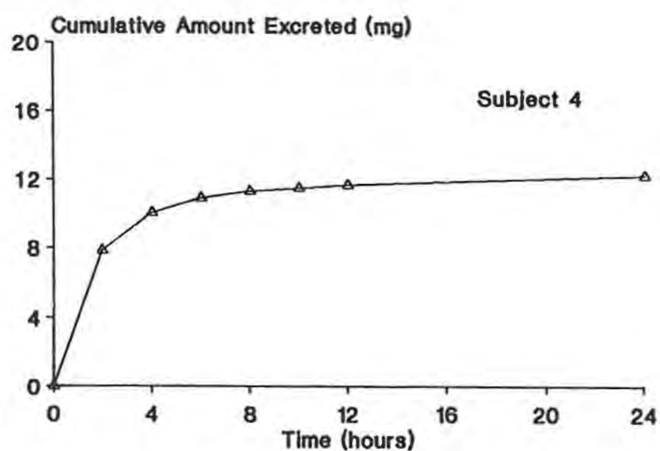


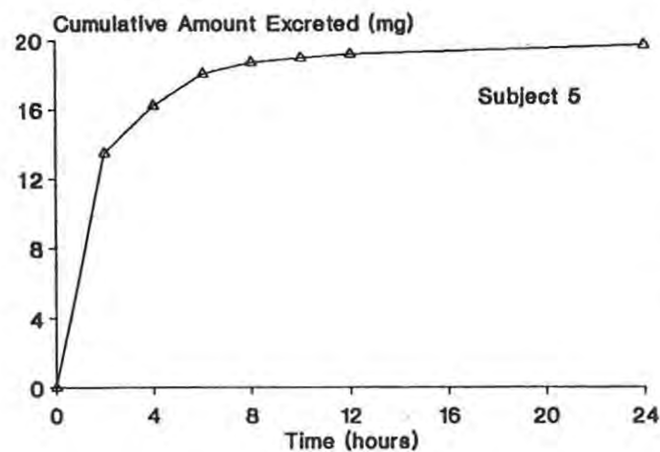
Figure 6.3 Serum concentration *vs.* time profiles for subjects 1 to 6 after the administration of a single oral 1 gram dose of josamycin as a powder in suspension.











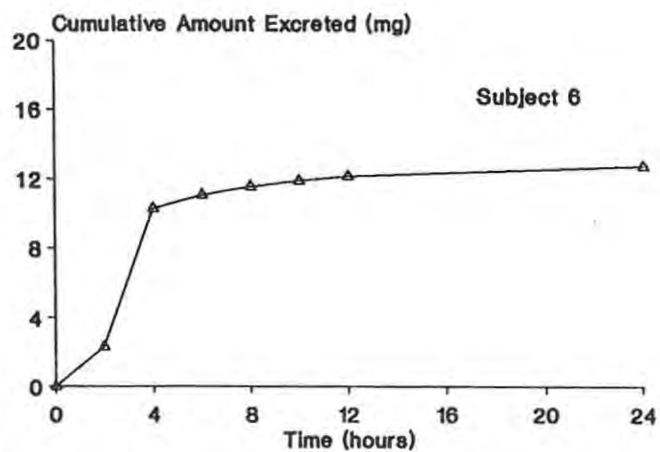


Figure 6.4 Cumulative urinary excretion profiles for subjects 1 to 6 after the administration of a single oral 1 gram dose of josamycin as a powder in suspension.

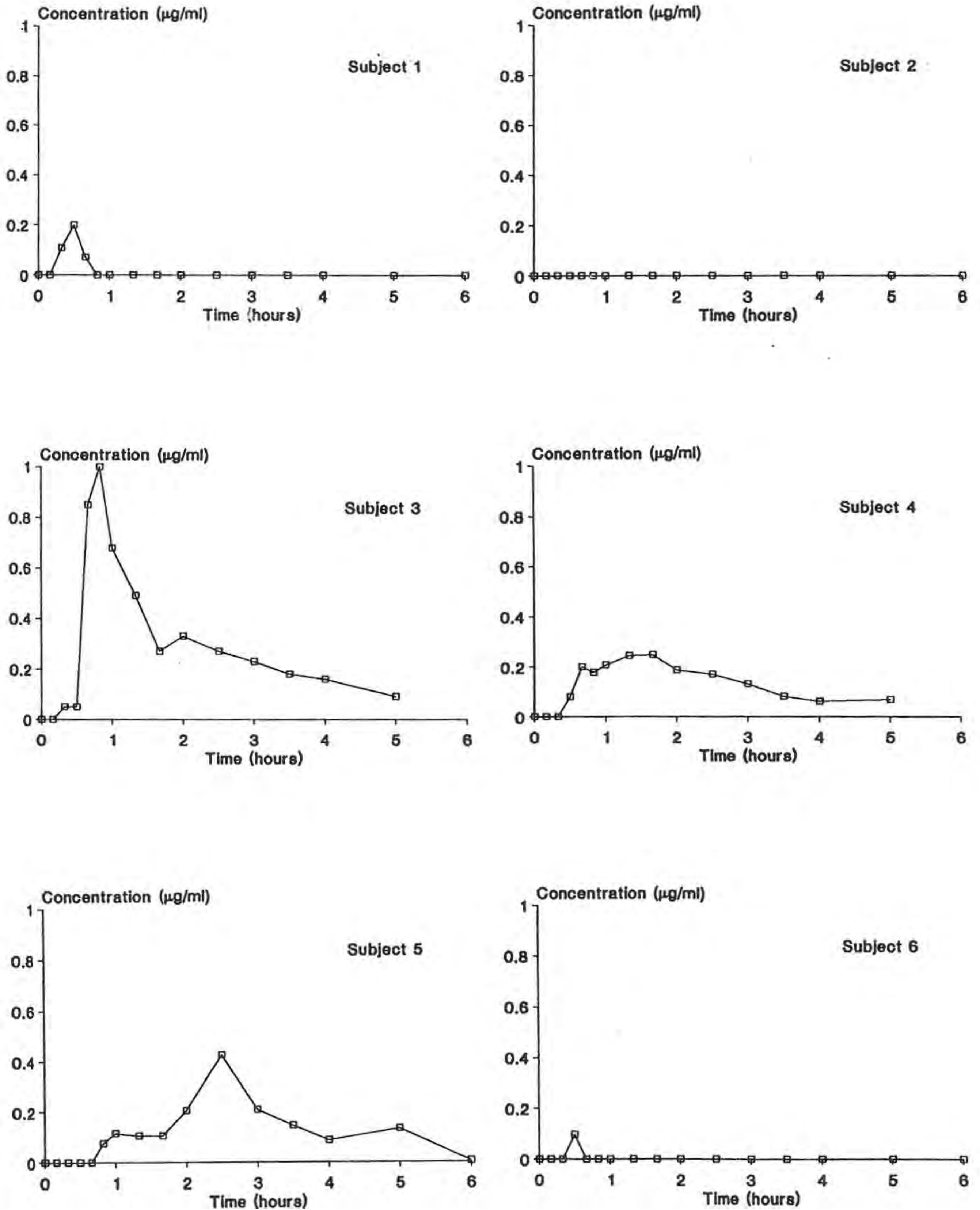


Figure 6.5 Serum concentration *vs.* time profiles for subjects 1 to 6 after the administration of a single oral 1 gram dose of josamycin consisting of five Josamycin 200 mg tablets.

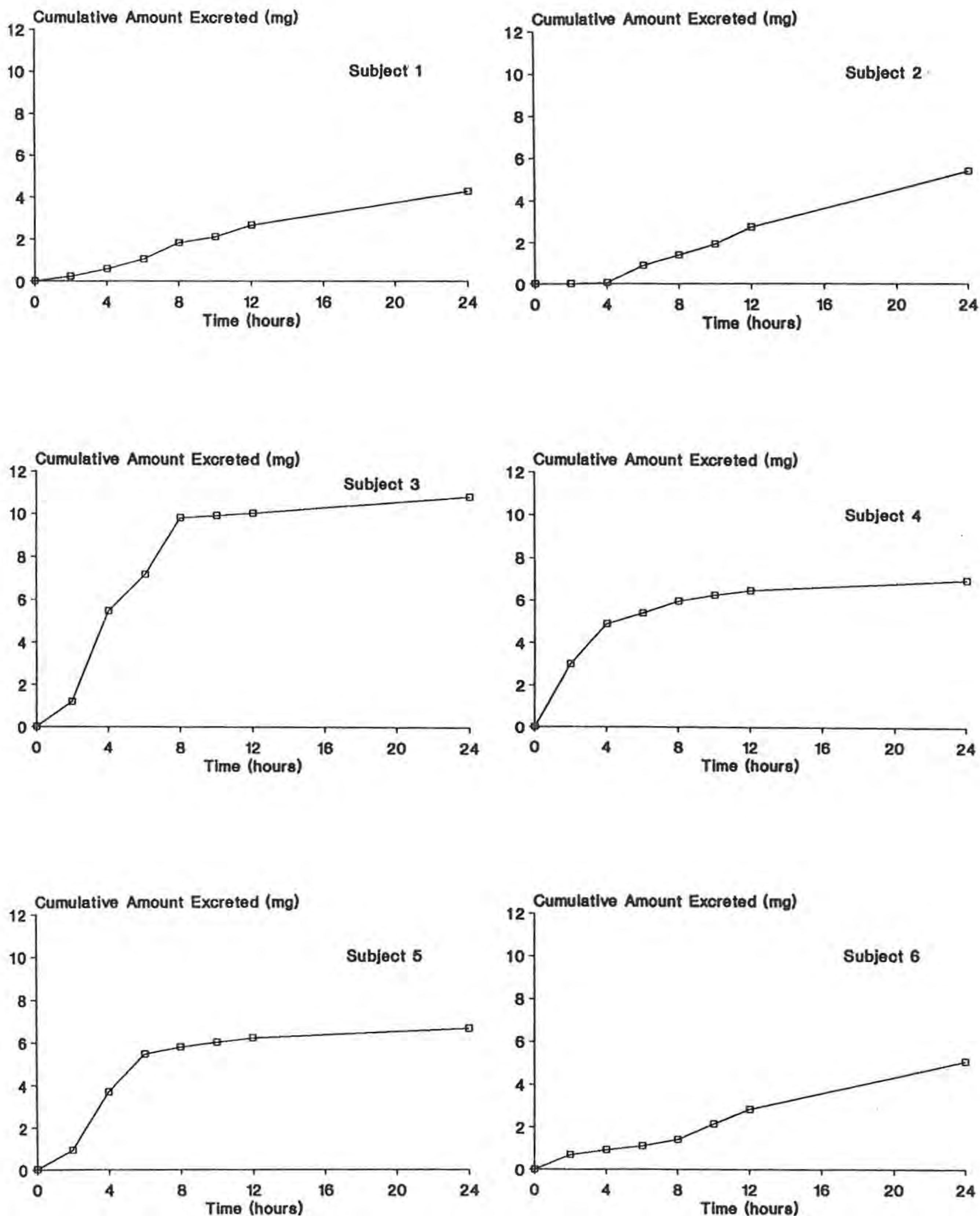
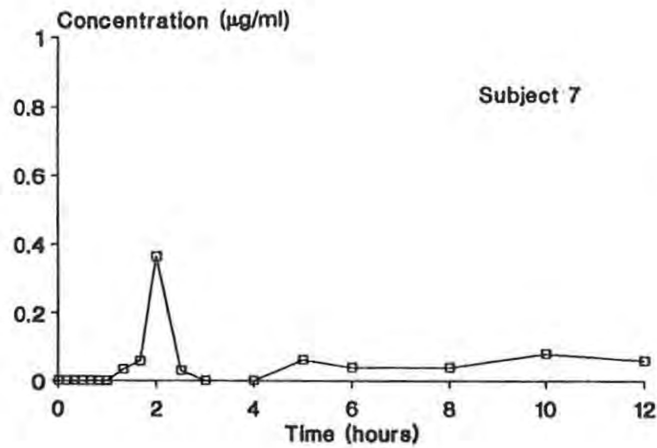
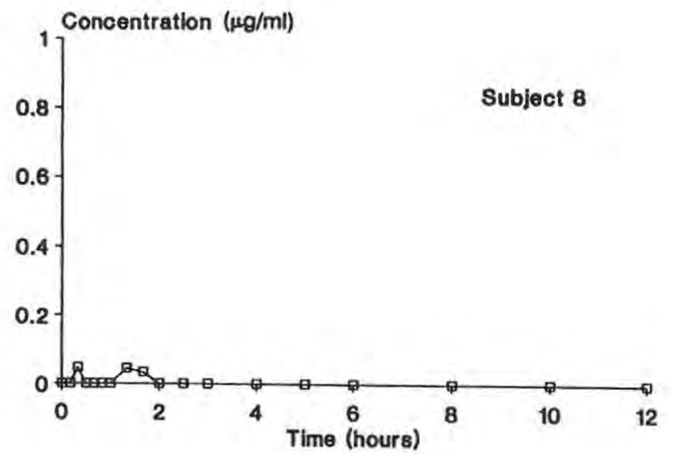
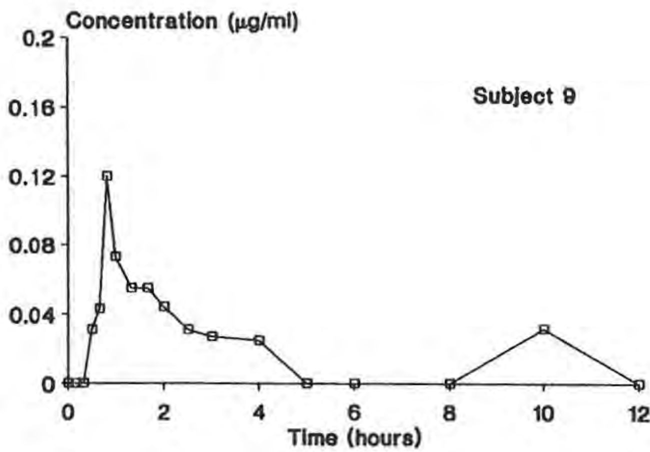
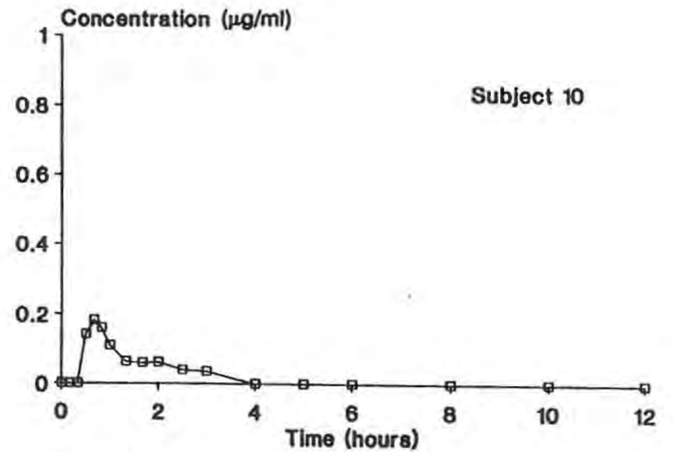


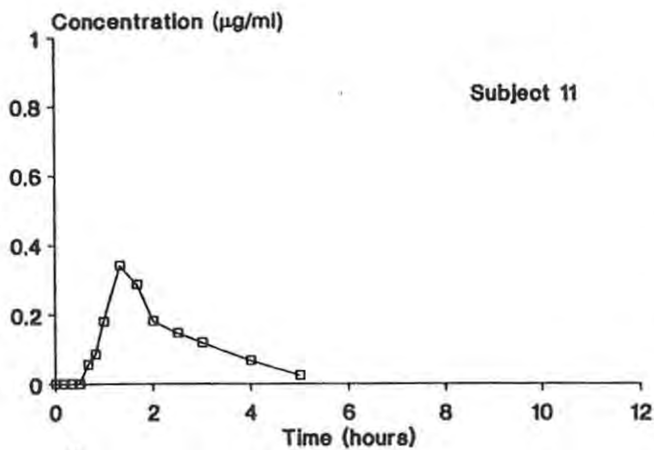
Figure 6.6 Cumulative urinary excretion profiles for subjects 1 to 6 after the administration of a single oral 1 gram dose of josamycin consisting of five Josamycin 200 mg tablets.











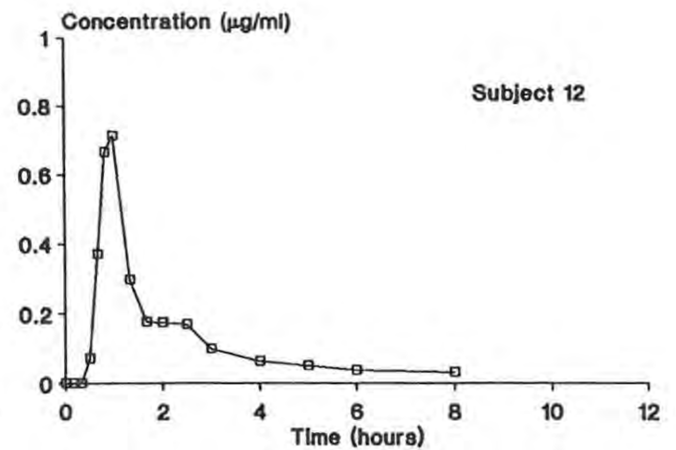


Figure 6.7 Serum concentration *vs.* time profiles for subjects 7 to 12 after the administration of a single oral 1 gram dose of josamycin consisting of two Josacine 500 mg tablets.

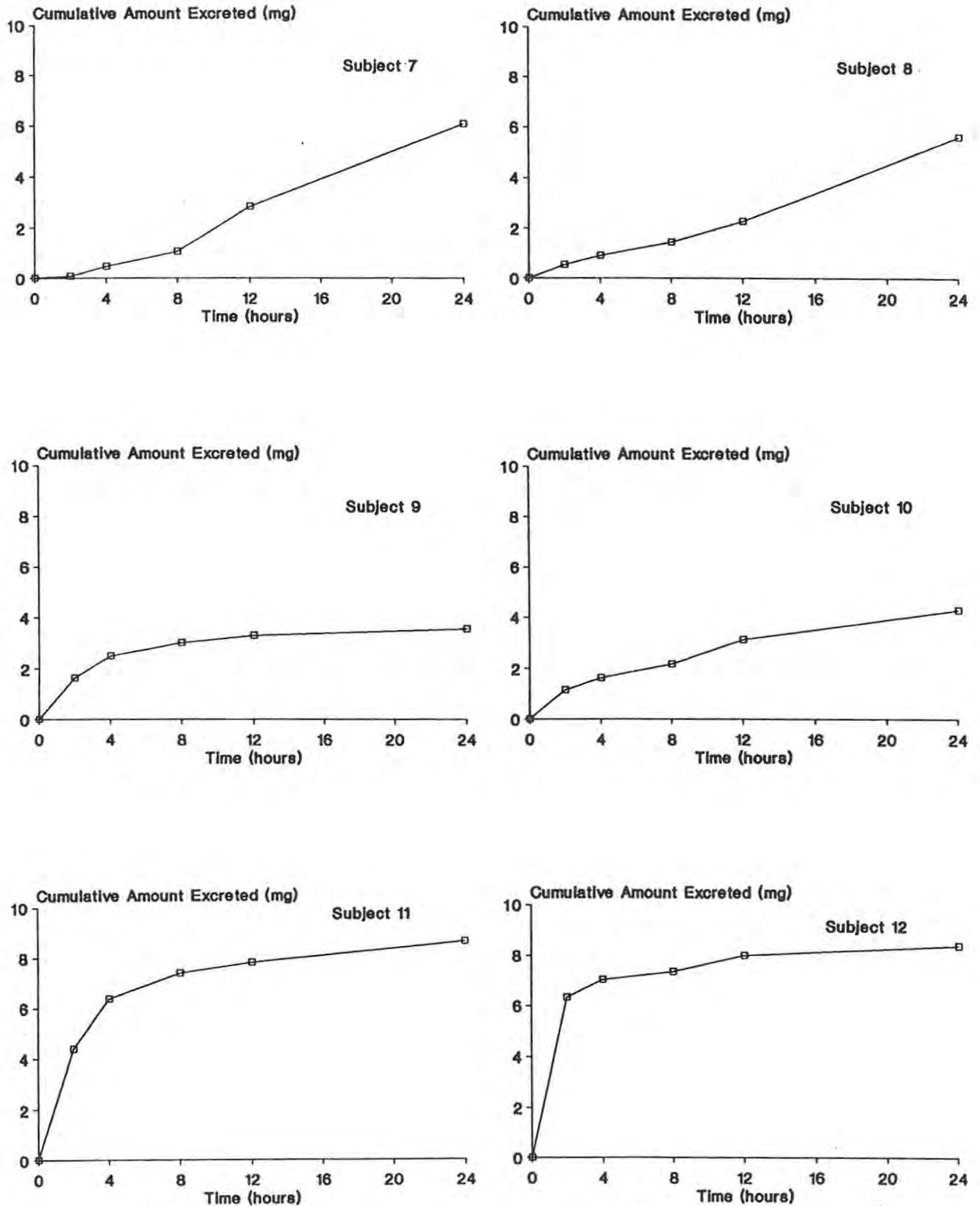


Figure 6.8 Cumulative urinary excretion profiles for subjects 7 to 12 after the administration of a single oral 1 gram dose of josamycin consisting of two Josacine 500 mg tablets.

powder with those of the solution indicate that the overall bioavailability of the powder was approximately half that of the solution.

Cumulative urinary excretion profiles for the solution and powder (Figures 6.2 and 6.4, Table 6.4) showed that 1.03 ±0.83% and 0.99 ±0.61% of the dose was excreted in the urine within 24 hrs for the solution and powder respectively. Urinary excretion was initially rapid and reached a plateau after about 4 - 8 hours. One subject, however, had an inexplicable delay in urinary excretion after dosing with the solution (Subject 10). These results are similar to those obtained by other investigators (28,36,48) and indicate that urinary excretion is only of minor importance in the elimination process. Despite the lower $AUC_{0\text{-}last}$ for the powder compared with the solution, the fraction of the dose excreted in the urine was similar in each instance. This, however, is not surprising as the fraction excreted unchanged in the urine is extremely small and therefore prone to considerable variation.

Bioavailability from Josamycin 200 mg tablets was generally extremely poor and variable with mean C_{max}, t_{max} and $AUC_{0\text{-}last}$ values of 0.328 μg/ml, 1.2 hrs and 0.468 $\mu g.hr.ml^{-1}$ respectively. Only subject 3 exhibited rapid absorption. Subjects 4 and 5 showed significant but slow and erratic absorption whilst for subjects 1 and 6 only very low concentrations were reached. For subject 2, no josamycin was detected in serum throughout the eight hour sampling period. The large RSD% values of 100.1%, 64.8% and 108.1% for C_{max}, t_{max} and $AUC_{0\text{-}last}$ respectively amply illustrate the highly variable absorption from these tablets. Serum concentration *vs.* time profiles for subjects 1 and 6 suggest that absorption was complete within 30 min whilst that for subject 2 suggests that absorption did not occur at all. However, the cumulative urinary excretion profiles of these subjects suggest otherwise. Urinary excretion profiles obtained for subjects exhibiting rapid absorption after administration of the solution and powder illustrate that urinary excretion is fairly rapid, and complete within 4 - 8 hrs once absorption has ceased. However, after dosing with 5 x 200 mg Josamycin tablets, subjects 1, 2 and 6 exhibited prolonged urinary excretion which continued with almost zero-order characteristics over the entire 24 hr urine collection period. These urine profiles therefore indicate that for subjects 1, 2 and 6, josamycin was absorbed over a prolonged period although this was not evident from serum concentration *vs.* time profiles. Absorption was therefore extremely slow over the major part of the study period for subjects 1 and 6 and over the entire study period for subject 2 with the rate of absorption generally insufficient to raise serum concentrations above the detection limit.

Bioavailability from Josacine 500 mg tablets was also poor with mean C_{max}, t_{max} and AUC_{0-last} values of 0.294 μg/ml, 1.03 hrs and 0.434 $\mu g.hr.ml^{-1}$ respectively. Comparison of AUC_{0-last} values shows that josamycin was slightly less bioavailable from the Josacine 500 mg tablets than from the Josamycin 200 mg tablets and only about 1/4 as bioavailable as the solution. Absorption was, however, less erratic than from the Josamycin 200 mg tablets with RSD% values of 74.3%, 51.7% and 76.9% for C_{max}, t_{max} and AUC_{0-last} respectively. As for the Josamycin 200 mg tablets, urinary excretion profiles obtained after administration of Josacine 500 mg tablets could be divided into two categories *i.e.*, those from subjects who showed relatively good absorption profiles and whose cumulative urinary excretion profiles attained a plateau after about 8 hours (Subjects 9, 11 and 12), and those with poorer absorption profiles and whose cumulative urinary excretion profiles were almost linear over the entire 24 hr urinary collection period (Subjects 7, 8 and 10). Again, the prolonged urinary excretion is evidence of a prolonged absorption period. For subjects 7 and 9, further evidence of a prolonged absorption phase was seen in the form of a slight late increase in serum concentration at 10 hrs after tablet administration. Similar and more pronounced artifacts of this nature have been observed by a number of investigators and have been ascribed to entero-hepatic recycling (220,221), site-specific absorption at more than one site along the gastro-intestinal tract (222) and a second dissolution phase effected by an outpouring of bile at some point in time subsequent to dosing (222). Second absorption peaks coinciding with food intake *i.e.* after standard meals at 2.5 and 5.5 hrs after dosing, which would indicate entero-hepatic recycling, were not observed. The slight increases in serum concentrations at 10 hrs for subjects 7 and 9 are therefore unlikely to be due to entero-hepatic recycling but rather to the passage of unabsorbed josamycin past a second absorption site or late disintegration of tablet particles and dissolution of unabsorbed drug possibly by bile salts.

Since josamycin is highly soluble at gastric pH's, josamycin powder should dissolve rapidly in the acidic environment of the stomach. This suggests that powder should exhibit similar absorption profiles to the solution. However, bioavailability from the powder was about 50% of the solution and absorption limiting processes appear to be operative during absorption from the powder. For basic drugs such as josamycin which are soluble at gastric pH's but poorly soluble at intestinal pH's, the possibility exists that precipitation of dissolved drug will occur once the gastric contents leave the stomach and are neutralised in the small intestine. However, the solution formulation administered was prepared using McIlvaine's buffer of pH 4.0 which could have ensured that josamycin remained in solution once in the small intestine by buffering the intestinal fluid to an adequately low pH. Absorption from this preparation would then have been completely unaffected by any dissolution

process. For the powder, however, dissolution may well have occurred in the stomach but followed by precipitation in the intestinal tract. As dissolution of the powder would then be the only additional factor affecting absorption from the powder compared to the solution, dissolution appeared to play a significant role in determining the bioavailability of josamycin from the powder. The intrinsic dissolution rate and the solubility of josamycin have both been found to be sufficiently low at higher intestinal pH's to significantly influence absorption (4.1.2.4 and 4.2.2). These factors may therefore explain the diminished bioavailability of the powder by causing dissolution rate limited and solubility limited absorption. Furthermore, the intrinsic dissolution rate and solubility are both highly dependent on pH. Their effect on absorption, therefore, would be significantly influenced by small changes in intestinal pH, which could subsequently vary significantly between individuals. A greater degree of variability was obtained for C_{max} and t_{max} after administration of the powder than after administration of the solution and this could well be due to inter-subject variation in intestinal pH.

Comparing the Josamycin 200 mg tablet and Josacine 500 mg tablet profiles with those of the solution and powder, it is immediately evident that absorption from both tablet preparations was severely restricted by formulation factors. As stated in 4.4, dissolution from Josamycin 200 mg tablets should be rapid if complete disintegration of the tablet and granules occurs in the acidic environment of the stomach. Apart from a short lag-time, the serum concentration *vs.* time profiles should be affected minimally by disintegration and dissolution. This appeared to be the case only for subject 3, with the remaining subjects exhibiting erratic absorption presumably due to erratic dissolution. Erratic dissolution *in-vivo* may have occurred if intact tablets or granules became imbedded in gastric mucous (223), or more likely, intact tablets and granules having passed through the pyloric sphincter into the more alkaline intestinal tract. In this environment then, dissolution would be highly pH dependent and range from rapid at pH 5.0 to extremely slow above pH 6.0 as illustrated in figure 4.19. In recent years numerous reports have appeared in the literature on the gastric transit time of solutions and solids, a short summary of which is given in table 6.5. Davis *et al.* (187) have found that particles 5 mm in diameter can pass through the pyloris in as little as 10 min after administration on a fasting stomach. As the dose used in this study consisted of multiple (5) small tablets (4 mm dia. x 6 mm long) it is highly likely that one or more intact or partially disintegrated tablets passed through the pyloris together or at varying times (167) resulting in a diminished dissolution rate and subsequently the diminished and erratic bioavailability observed. During *in-vitro* tablet dissolution studies at higher intestinal pH's, a portion of the dose remained entrapped in the tablet matrix. This could also be expected to

occur *in-vivo* if intact tablets or granules pass into the small intestine, and will also lead to reduced bioavailability. Furthermore, slow dissolution and incomplete release of josamycin from the tablet matrix could be responsible for a very prolonged absorption phase which could account for the linear nature of the urinary excretion profiles observed in subjects 1, 2 and 6.

The poor disintegration and dissolution characteristics of Josacine 500 mg tablets at gastric, but particularly at intestinal pH's (Figure 4.23) can also help to explain the extremely poor bioavailability observed in the majority of subjects. Josacine tablets are approx. 13 mm x 7 mm and it is unlikely that intact tablets will pass through the pyloris. However, Davis *et al.* (165,187) have found that tablets 8 mm and 5 mm in diameter can have gastric transit times as low as 30 min and 10 min respectively. As disintegration of Josacine tablets is slow (39 - 60 min, Table 4.6) it is likely that partially disintegrated tablets passed through the pyloris, after which disintegration and dissolution continued in the intestinal tract. Dissolution and absorption would then be extremely slow and highly pH dependent resulting in poor and erratic bioavailability. Furthermore, *in-vitro* studies showed that a large proportion of the dose could remain entrapped in the granule matrix for an extended period of time. As for Josamycin 200 mg tablets, this could account for the prolonged absorption in subjects 7, 8 and 10 as reflected by the urinary excretion profiles, and also for the slight increases in serum concentrations observed after 10 hrs in subjects 7 and 9. Any factors which enhance disintegration of these tablets will also enhance dissolution and subsequently absorption. Graham *et al.* (223) found that tablets coming to rest in the gastric antrum were ground by the antro-pyloric pump and subject to more intense agitation than elsewhere in the stomach. Absorption would therefore be maximised if the tablets came to rest in the gastric antrum which may have been the case in subjects exhibiting better absorption profiles after administration of these tablets (*e.g.* subjects 11 and 12).

The instability of josamycin at gastric pH's could also have contributed to the diminished bioavailability of the powder and tablet formulations. The pH of the gastric contents would have risen immediately after administration of the solution due to buffering by the McIlvaine's buffer (pH 4.0) used in the formulation and since solutions are rapidly expelled from the stomach (166,219), josamycin administered as a solution would have been exposed to low pH's for only a limited period of time. Thus, acid degradation of the solution is likely to have been minimal. After administration of the powder, however, no such buffering action would have been present and some acid degradation may have occurred before the complete dose and co-administered water were expelled into the duodenum. After administration of the tablets, it is likely that the co-administered water was rapidly expelled into the

duodenum leaving the tablets to disintegrate and dissolve in a small residual volume of highly acidic gastric fluid. Josamycin dissolving from the tablets would then have been exposed to low pH's (pH 1→2.5) for a considerable period of time, resulting in possibly significant degradation and reduced bioavailability from the tablets. For the powder and tablets, the high variability of gastric emptying, and thus of the gastric residence time of dissolved josamycin, could have a significant impact on bioavailability, and compounded with other factors, contribute to poor and erratic absorption.

Factors such as hepatic first-pass metabolism, intestinal metabolism (which is most prevalent in the duodenum, the most likely site of absorption for josamycin, and which includes oxidation (224) by which josamycin is metabolised (71)), and intestinal blood flow can all reduce bioavailability and cause inter-individual variation in serum concentration profiles (224). The extent of hepatic and intestinal first-pass metabolism can in part be determined by the absorption rate of the drug and can decrease with increasing absorption rate probably due to saturation of enzyme systems after rapid absorption (160,224). The effect of these two factors on the bioavailability of josamycin is therefore likely to have been lowest after administration of the solution. However, their effect is likely to have been increasingly prevalent for the powder and tablets. Highly lipid-soluble or poorly water soluble drugs can be particularly affected by intestinal blood flow as capillary fluid can rapidly become saturated by such drugs if the intestinal blood flow is low. Since josamycin is highly lipid soluble and poorly water soluble, intestinal blood flow could also have played a role in determining the inter-individual variability in bioavailability from each formulation.

In summary, josamycin is intrinsically well absorbed from solution. However, bioavailability was reduced when administered as a powder and further diminished from the two tablet preparations studied. The intrinsic dissolution, solubility, tablet dissolution and acid degradation characteristics are most likely responsible for both the variable and diminished bioavailability of josamycin after administration as a powder and from the tablet formulations. Various attempts have been made to improve the absorption of drugs which are poorly bioavailable due to poor solubility and dissolution characteristics. For instance, improved bioavailability was obtained by formulating etoposide (155) and methyltestosterone (225) in "drink ampoules" in order to administer both drugs as solutions. Unfortunately this is not a suitable option for routine administration of josamycin due to its extremely unpleasant taste. However, numerous other possibilities exist such as complexation with cyclodextrins (226), administration in lipid vehicles such as oleic acid (227) and surfactants such as polyethylene glycol and the polysorbates (228). The ideal formulation for josamycin,

however, would appear to be one which reduces gastric acidity thus minimising degradation, and which reduces the pH of the intestinal tract to prevent precipitation thus circumventing any dissolution problems. Manipulation of the gastric pH with sodium bicarbonate has been successful in increasing the bioavailability of erythromycin acistrate presumably by reducing acid degradation (229). Buffering both the gastric and intestinal contents to about pH 2 - 4 using a preparation containing buffer salts could well be an effective way to increase the bioavailability of josamycin from a solid dosage form, and warrants further investigation. Further bioavailability studies should include monitoring the gastric and intestinal pH *in-vivo* to determine the correlation between bioavailability and gastric and intestinal pH. Data collected in such studies would be useful in determining the most suitable preparation of josamycin. Monitoring the gastric and intestinal pH's can be accomplished using either radiotelemetry *e.g.* the Heidelberg capsule (Heidelberg International, Atlanta, GA, U.S.A.), or by placing pH sensitive electrodes in the gastrointestinal tract *e.g.* the Proxima apparatus (M&B, Casalecchio di Reno Bo, Italy)

Josamycin was extremely well tolerated and only one subject reported slight gastro-intestinal side effects following the final dose of the multiple dose study. However, symptoms were mild and withdrawal from the trial was not necessary.

6.2.3 Comparison of Single and Multiple Dose Solution Profiles

Non-compartmental parameters for the multiple dose study are tabulated in table 6.3. After multiple dosing the mean C_{max} obtained was 9.135 $\pm$1.407 μg/ml after a mean t_{max} of 0.58 $\pm$0.16 hrs. The mean $AUC_{0\rightarrow\tau}$ was 11.425 $\pm$1.953 μg.hr.ml^{-1} and the mean k_{el} was 0.176 $\pm$0.026 hr^{-1} corresponding to a mean terminal half-life of 4.02 $\pm$0.53 hrs. All parameters (besides k_{el}) are markedly greater than after the single dose solution. The most significant being the $AUC_{0\rightarrow\tau}$ which is about 7 times greater than the single dose $AUC_{0\rightarrow\infty}$ and the t½ which was about twice as long after multiple dosing. To determine possible causes of these apparent non-linear kinetics, pharmacokinetic parameters were determined for both single and multiple dose profiles by non-linear curve fitting to the mathematical models described below.

6.2.3.1 Pharmacokinetic Modelling

Semilogarithmic plots of serum concentration *vs.* time data of single and multiple dose solution studies exhibit a distinct distribution phase and a log-linear elimination phase in all but one profile (multiple dose study, Subject 10). This suggests that josamycin exhibits kinetics as described by an open two-compartment model (2BCM). Each single dose profile was therefore fitted to a 2BCM with first-

order absorption and elimination as described by equation 6.1 (230) with a lag time.

$$C_t = \frac{D.F.k_a}{Vd} \cdot [(\frac{k_{21}-\alpha}{(k_a-\alpha) \cdot (\beta-\alpha)}) \cdot e^{-\alpha \cdot (t-t_{lag})}$$

$$+ (\frac{k_{21}-\beta}{(k_a-\beta) \cdot (\alpha-\beta)}) \cdot e^{-\beta \cdot (t-t_{lag})} \qquad \text{Eq. 6.1}$$

$$+ (\frac{k_{21}-k_a}{(\alpha-k_a) \cdot (\beta-k_a)}) \cdot e^{-k_a \cdot (t-t_{lag})}]$$

where

C_t	= serum concentration at time t
D	= dose administered
F	= bioavailability factor
k_a	= first-order absorption rate constant
Vd	= volume of distribution of the central compartment
k_{21}	= distribution rate constant for the transfer of drug from the peripheral to central compartment
α	= slope of the distribution line
β	= slope of the terminal elimination line
t_{lag}	= the lag time

Multiple dose profiles were fitted to equation 6.2 (230) with a lag time, which is an expanded version of equation 6.1 and incorporates terms describing the accumulation of drug on multiple dosing and takes into account the number of doses administered and the dosing interval.

$$C_t = \frac{D.F.k_a}{Vd} \cdot [(\frac{1-e^{-n \cdot \alpha \cdot \tau}}{1-e^{-\alpha \cdot \tau}}) \cdot (\frac{k_{21}-\alpha}{(k_a-\alpha) \cdot (\beta-\alpha)}) \cdot e^{-\alpha \cdot (t-t_{lag})}$$

$$+ (\frac{1-e^{-n \cdot \beta \cdot \tau}}{1-e^{-\beta \cdot \tau}}) \cdot (\frac{k_{21}-\beta}{(k_a-\beta) \cdot (\alpha-\beta)}) \cdot e^{-\beta \cdot (t-t_{lag})} \qquad \text{Eq. 6.2}$$

$$+ (\frac{1-e^{-n \cdot k_a \cdot \tau}}{1-e^{-k_a \cdot \tau}}) \cdot (\frac{k_{21}-k_a}{(\alpha-k_a) \cdot (\beta-k_a)}) \cdot e^{-k_a \cdot (t-t_{lag})}]$$

where

n	= number of doses administered
τ	= dosing interval

Initial estimates for each parameter were obtained by fitting the appropriate equation to each profile using the Simplex curve fitting option in the computer program MINSQ (Micromath Scientific Software, Salt Lake City, Utah, U.S.A.). Final curve fitting was carried out on a CDC Cyber 179 series 825 computer utilising the nonlinear curve fitting program NONLIN (Research Biostatistics, The Upjohn

Company, Kalamazoo, MI, U.S.A.). Simulated serum profiles after five 1 gram doses two hourly were generated for each subject using equation 6.2 with parameters determined from single dose profiles for comparison with the actual multiple dose profiles obtained.

6.2.3.2 Results and Discussion

Plots of experimental and fitted data for single dose solution and multiple dose solution with the simulated profiles are depicted in figures 6.9 and 6.10 respectively. Parameter estimates obtained after optimisation of curve fitting for each study are tabulated in tables 6.6 and 6.7.

Curve fitting was initially carried out on single dose solution data with all parameters unrestrained. Serum profiles were well described by the 2BCM. Final parameter estimates close to the initial estimates were obtained for all parameters except k_a. The final estimates of k_a throughout, were found to be variable and dependent largely on the initial estimate, and in the case of subjects 7 and 12 on the upper limit. For these two subjects k_a increased to the upper limit of 80.0 hr^{-1} during the first run. The upper limit was subsequently set to 20.0 hr^{-1} but restraining k_a had a negligible effect on the remaining parameters and no significant effect on the goodness of fit as determined by the sum of weighted squared deviations. This variability can be expected due to the very rapid absorption rates being characterised by only two or at the most, three data points. Final estimates of k_a therefore cannot be regarded as definitive. Estimates of k_{el}, however, were found to be particularly robust after rigorous testing by varying initial estimates and limits of each parameter, and were in good agreement with values of k_{el} determined by linear regression of the terminal elimination phase.

Estimates of k_a, Vd, k_{21}, α and k_{el} were all substantially lower after multiple dosing than after a single dose. However, F was similar for both single and multiple dose regimens. Since no IV study was conducted, the absolute bioavailability could not be determined. However, the estimated values obtained according to computer fitting of oral data (F = 0.51 $\pm$0.20 and 0.54 $\pm$21 for single and multiple dose respectively) were realistic and similar to those for the absolute bioavailability of erythromycin which is reported to be between 30% and 65% (231). A comparison of the actual and simulated profiles (Figure 6.10) gives an indication of the extent to which the change in parameters resulted in an increased accumulation above that which was expected.

The Vd after single dosing was 161.7 $\pm$49.5 l. Thus, the distribution of josamycin is extensive and extends to the "deep" tissue in the central compartment (230). This value is consistent with the excellent tissue penetration properties of

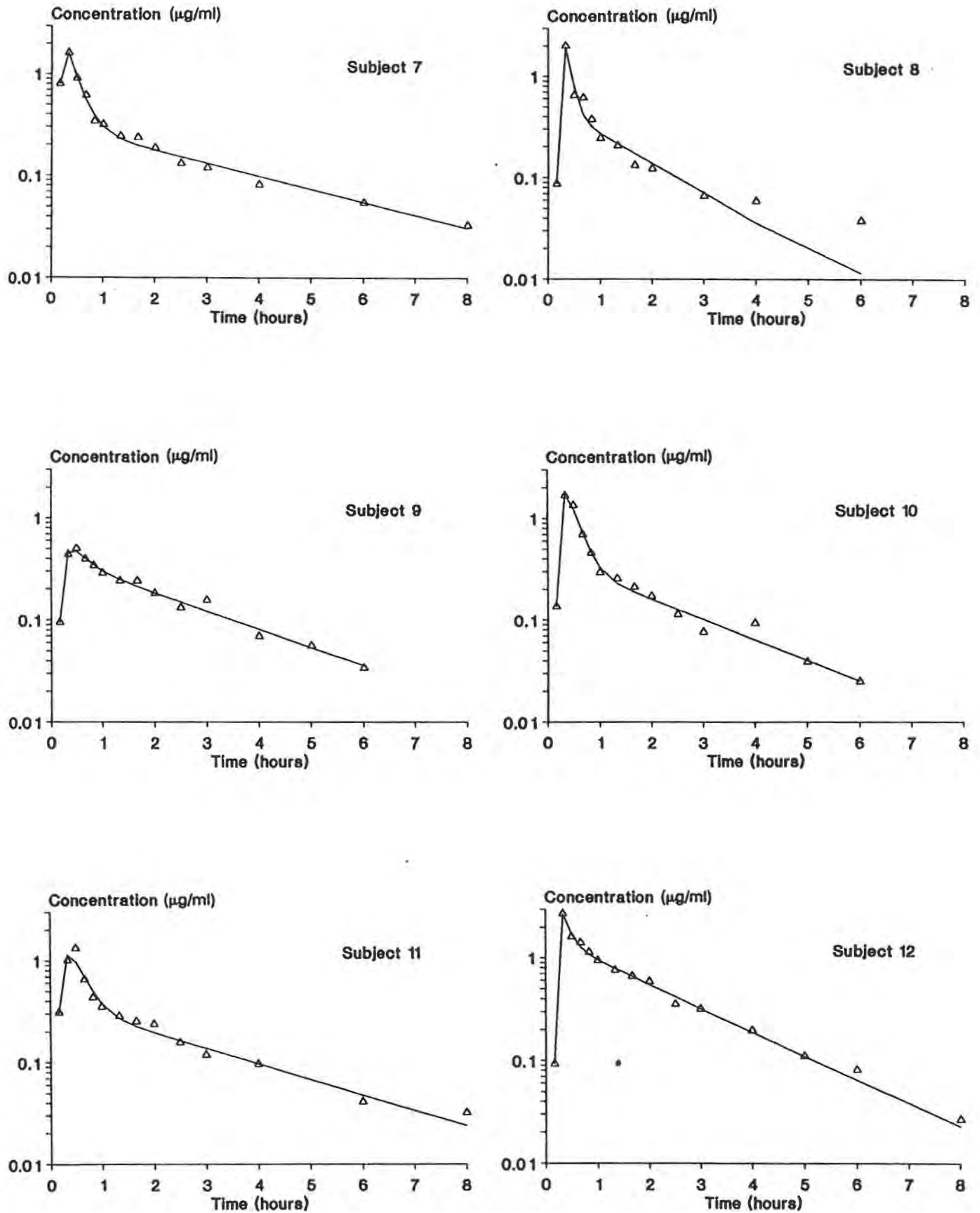


Figure 6.9 Semilogarithmic plots of experimental and fitted serum data for subjects 7 to 12 after the administration of a 1 gram dose of josamycin as a solution.

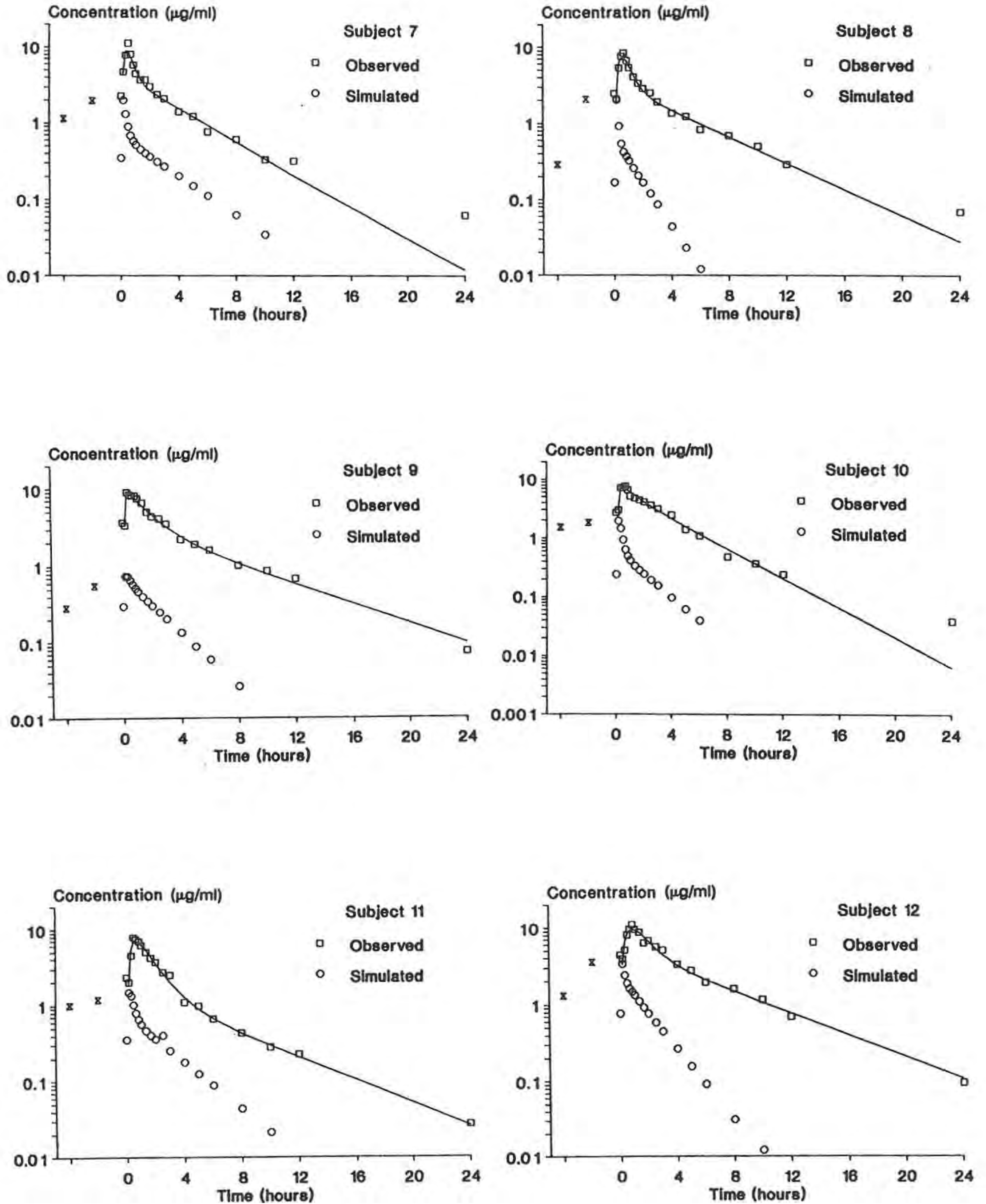


Figure 6.10 Semilogarithmic plots of experimental (□) and fitted serum data for subjects 7 to 12 after multiple dosing with 1 gram doses of josamycin in solution showing troughs (⋈) before the third and fourth doses together with the simulated serum concentration *vs.* time curve (○) for the fifth dose using single dose parameters in equation 6.2.

josamycin (48,61,63,232). Extensive distribution is also demonstrated by the rapid distribution constant α (6.50 $\pm$1.74 hr^{-1}) in comparison with the much slower transfer of josamycin from the peripheral to the central compartment (k_{21} = 1.46 $\pm$0.64 hr^{-1}). However, after multiple dosing Vd was significantly lower at 34.3 $\pm$11.9 l indicating that distribution of the last dose was restricted to the total body water (230), probably due to saturation of the peripheral compartment. This is particularly evident in subject 10 in whom there was a negligible distribution phase after the last dose with the profile approaching that of a one compartment model. Further evidence of saturation of the peripheral compartment is the substantial decrease in α and k_{21} indicating increasingly sluggish distribution as the concentration gradient between the two compartments decreased. The surprisingly high C_{max} and $AUC_{0\rightarrow\tau}$ values after multiple dosing can therefore be explained in part by a decrease in the volume of distribution due to saturation or partial saturation of the peripheral compartment. Bergan *et al.* (36) proposed that the peripheral compartment is saturated with a dose above 1250 mg. Although only 1 gram doses were administered in this multiple dose study, saturation of the peripheral compartment can be explained by the short dosing interval. Since elimination was incomplete at the end of each dosing interval, the total amount in the body increased after each dose. Accumulation in this manner was sufficient to saturate the peripheral compartment, resulting in the decrease in volume of distribution and concentrations above the simulated levels.

Values of F were used to calculate clearance with the equation $Cl = F.D/AUC$. A considerable decrease in clearance from 4.71 $\pm$1.71 $l.kg^{-1}.hr^{-1}$ after single dosing to 0.63 $\pm$0.22 $l.kg^{-1}.hr^{-1}$ after multiple dosing was observed. This is reflected by a decrease in the terminal elimination rate from 0.45 $\pm$0.12 hr^{1} to 0.20 $\pm$ 0.05 hr^{-1} and would also have been responsible for the unexpectedly high serum concentrations observed after multiple dosing. Similarly, disproportionate serum levels have also been observed in multiple dose studies with erythromycin (233) which has been attributed to its action on cytochrome P-450 (234). Erythromycin, as well as other macrolide antibiotics, have a dual effect on the cytochrome P-450 drug metabolising enzymes and both induce certain species and subsequently inhibit their activity by forming a stable inactive metabolite-cytochrome P-450-Fe(II) complex (74,77-79). This may result in a decrease in clearance since macrolides are almost entirely eliminated by the liver and any changes in hepatic metabolism will significantly alter their pharmacokinetics. On the other hand, the AUC and C_{max} after multiple dosing with a new semi-synthetic macrolide, roxithromycin, was slightly lower than expected and in contrast to erythromycin, roxithromycin does not induce or inhibit cytochrome P-450 (235,236). Similarly, josamycin exhibits no inactivating effects with cytochrome P-450 and shows only weak inducing activity, probably due to steric hindrance of the N-dimethylamino group, since an unhindered readily accessible group is required for such activity (79).

The observed levels of josamycin being above the simulated levels therefore cannot be ascribed to cytochrome P-450 inhibition. However, the acid degradation product of josamycin - desmycarose josamycin, which has an unhindered N-dimethylamino group, has been shown to be capable of forming inactive complexes (79). It is possible that acid degradation products formed *in-vivo* may have contributed to a decrease in hepatic clearance although their effect would have been slight. A more likely explanation then, is that saturation of hepatic drug metabolising enzyme systems may have occurred. Bergan *et al.* (36) found that for josamycin over the dose range of 250 mg to 2 gm, the C_{max} attained was dose dependent. However, no change in the elimination half-life with dose was apparent. The maximum serum concentration attained in the Bergan studies was about 6 μg/ml which is lower than those obtained in the multiple dose solution study. A decrease in the elimination half-life therefore appears to occur only after relatively high serum concentrations have been reached. This also suggests that saturation of metabolic enzyme pathways occurred after multiple dosing. Further multiple dose studies with various doses below 1 gm administered two hourly would be useful to determine the serum concentrations at which enzyme saturation is attained.

In summary, the single dose pharmacokinetics of josamycin is well described by an open two-compartment model with inherent rapid absorption, rapid and extensive distribution followed by rapid elimination. After multiple dosing, however, the absorption rate, elimination rate, volume of distribution and clearance all decreased and resulted in unpredictably high serum concentrations. The non-linear kinetics observed was probably due to saturation of the peripheral compartment and, in contrast to erythromycin, simple saturation of enzyme systems occurred rather than going through a more complex mechanism of induction and inhibition of cytochrome P-450.

Table 6.1 Demographic Data of Subjects Used in Bioavailabiltiy Studies

No.	Name	Age (yrs)	Height (m)	Mass (kg)	No.	Name	Age (yrs)	Height (m)	Mass (kg)
1	G. B.	19	1.73	65	7	K. F.	25	1.80	92
2	M. M.	20	1.68	78	8	M. M.	21	1.68	78
3	R. G.	18	1.69	72	9	I. vd N.	19	1.75	71
4	G. S.	21	1.85	78	10	P. v M.	23	1.66	82
5	M. G.	19	1.70	75	11	R. B.	20	1.63	67
6	B. H.	18	1.74	68	12	A. F.	20	1.68	62

Table 6.2 *In-Vitro* and *Ex-Vivo* Control Results for the Analysis of Serum Samples from Phases 3, 4 and 5.

		Concentration Found (μg/ml)								
In-Vitro Sample	Spiked Conc. μg/ml	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Mean ±RSD%
IVA	1.270	1.322	1.437	–	–	–	–	–	–	1.292 ±7.53%
IVA		1.190	1.218	–	–	–	–	–	–	(n=4)
IVB	0.061	0.077	0.086	–	–	–	–	–	–	0.080 ±7.95%
IVB		0.071	0.086	–	–	–	–	–	–	(n=4)
IVC	1.670	–	–	1.410	1.579	1.483	1.650	–	–	1.604 ±8.08%
IVC		–	–	1.681	1.537	1.866	1.626	–	–	(n=16)
IVD	0.079	–	–	0.113	0.082	0.085	0.060	–	–	0.082 ±19.74%
IVD		–	–	0.087	0.092	0.074	0.061	–	–	(n=16)
IVE	7.540	–	–	–	–	–	–	7.478	7.347	7.766 ±4.86%
IVE		–	–	–	–	–	–	7.942	8.296	(n=4)
IVF	0.754	–	–	–	–	–	–	0.705	0.696	0.722 ±3.29%
IVF		–	–	–	–	–	–	0.757	0.731	(n=4)
Ex-Vivo Sample										
EVA		0.745	0.823	0.610	0.829	0.634	–			0.728 ±12.63% (n=5)
EVB		0.067	–	0.065	0.042	0.079	0.058			0.062 ±19.54% (n=5)
EVC		0.031	0.050	–	0.025	0.036	0.038			0.036 ±23.11% (n=5)
EVD		0.137	0.131	0.127	–	0.165	–			0.140 ±10.62% (n=4)
EVE		–	0.113	0.087	0.083	–	0.102			0.096 ±12.45% (n=4)
EVF		0.116	0.113	0.110	0.095	0.126	–			0.112 ±8.98% (n=5)

Table 6.3 Non-Compartmental Parameters Determined by BIOPAK after the Administration of Single Oral 1 Gram Doses of Josamycin as a Solution, Powder, Josamycin 200 mg Tablets and Josacine 500 mg Tablets, and after Multiple Dosing with 1 Gram of Josamycin as a Solution.

Formulation	Subject	C_{max} (μg/ml)	T_{max} (hr)	$AUC_{0\to last}$ (μg.hr.ml^{-1})	$AUC_{0\to\infty}$ (μg.hr.ml^{-1})	k_{el} (hr^{-1})	$t_{½}$ (hr)
Single Dose Solution	7	1.616	0.33	1.449	1.560	0.299	2.32
	8	2.014	0.33	1.068	1.200	0.288	2.41
	9	0.505	0.50	0.927	1.007	0.425	1.63
	10	1.669	0.33	1.276	1.328	0.478	1.45
	11	1.327	0.50	1.342	1.438	0.425	1.63
	12	2.735	0.33	2.997	3.051	0.499	1.39
	Mean	1.640	0.39	1.510	1.597	0.402	1.81
	±SD%	0.67	0.08	0.687	0.672	0.081	0.41
Single Dose Powder	1	0.580	0.50	0.572	0.707	0.445	1.56
	2	0.750	0.33	0.576	0.685	0.460	1.51
	3	0.270	0.67	0.403	0.524	0.332	2.09
	4	0.470	0.67	0.760	0.871	0.632	1.20
	5	1.590	0.33	1.284	1.403	0.336	2.06
	6	0.870	0.50	0.755	0.927	0.465	1.49
	Mean	0.755	0.50	0.725	0.853	0.445	1.65
	±SD%	0.420	0.14	0.278	0.279	0.100	0.32
Josamycin 5 x 200 mg tablets	1	0.198	0.50	0.057	–	–	–
	2	0.000	0.00*	0.000	–	–	–
	3	1.000	0.83	1.389	–	–	–
	4	0.248	1.67	0.643	–	–	–
	5	0.425	2.50	0.713	–	–	–
	6	0.096	0.50	0.008	–	–	–
	Mean	0.328	1.20	0.468	–	–	–
	±SD%	0.328	0.93	0.506	–	–	–
Josacine 2 x 500 mg tablets	7	0.364	2.00	0.693	0.341	–	–
	8	0.047	0.33	0.028	0.065	–	–
	9	0.120	0.83	0.154	0.221	–	–
	10	0.181	0.67	0.181	0.250	–	–
	11	0.340	1.33	0.595	0.650	–	–
	12	0.713	1.00	0.950	0.995	–	–
	Mean	0.294	1.03	0.434	0.420	–	–
	±SD%	0.218	0.53	0.334	0.312	–	–
Multi-Dose Solution.	7	11.162	0.50	10.485**	–	0.152	4.57
	8	8.357	0.67	9.480	–	0.149	4.64
	9	8.987	0.33	12.866	–	0.168	4.12
	10	7.649	0.67	10.537	–	0.227	3.06
	11	7.760	0.50	10.082	–	0.184	3.77
	12	10.896	0.83	15.087	–	0.176	3.93
	Mean	9.135	0.58	11.423		0.176	4.02
	±SD%	1.407	0.16	1.953		0.026	0.53

* Omitted for calculation of mean T_{max}.

** AUC values for multi-dose study calculated as $AUC_{0\to\tau}$.

Table 6.4 Percentage of Dose Excreted in Urine in 24 Hours for Each Single Dose Study.

	Percentage of Dose Excreted in Urine in 24 Hours			
Subject	Solution (subjects 7 - 12)	Powder (subjects 1 - 6)	Josamycin 200 mg Tablets (subjects 1 - 6)	Josacine 500 mg Tablets (subjects 7 - 12)
1 or 7	0.49	0.99	0.43	0.61
2 or 8	0.75	0.28	0.54	0.56
3 or 9	0.62	0.19	1.09	0.36
4 or 10	0.44	1.22	0.70	0.43
5 or 11	1.04	1.97	0.67	0.86
6 or 12	2.81	1.28	0.51	0.84
Mean	1.03	0.99	0.66	0.61
±SD	0.83	0.61	0.22	0.19

Table 6.5 Summary of Literature Data on Gastric Transit Times of Solutions and Solids.

	Gastric Emptying Half-Life (mins)			Gastric Retention Time of Tablet (mins)				
Subject No.	Solution[a,b]	Pellets[a,c]		Tablets[b] 5 mm dia.			Tablets[c]	Tablets[b]
		0.8-1.1 mm	0.71-1.44 mm	Day 1	Day 2	Day 3	8x4.5 mm	12 mm dia.
1	25	125	95	35	10	43	600	150
2	10	30	116	10	30	230	30	600
3	30	150	120	30	30	45	225	120
4	10	33	124	20	30	100	42	200
5	15	75	95	28	50	145	30	215
6		60	93				60	115
7			81				164	610
8			61					310
mean	18	79	99	25	30	113	164	290
Ref.	166	165	166	187			165	167

[a] Half-life for gastric emptying.
[b] Fasted.
[c] Non-fasted.

Table 6.6 Results of Non-Linear Curve Fitting with Equation 6.1 of Profiles Obtained after Dosing with 1 gram of Josamycin as a Solution.

Subject	k_a (hr^{-1})	F	Vd (l)	k_{21} (hr^{-1})	α (hr^{-1})	k_{el} (hr^{-1})	t-lag (hr)	t½ (hr)	Cl ($l.kg^{-1}.hr^{-1}$)	Sum of Sq. Dev.*	Corr. Coeff.
7	20.00	0.29	104.5	0.75	4.58	0.29	0.15	2.39	2.17	0.004273	0.998
8	15.10	0.54	104.4	1.46	10.0	0.66	0.17	1.05	6.48	0.019115	0.990
9	5.43	0.25	249.7	2.19	5.44	0.41	0.15	1.69	3.50	0.002612	0.993
10	6.84	0.77	173.1	0.96	6.86	0.46	0.17	1.51	7.03	0.004826	0.997
11	5.56	0.51	178.0	0.98	5.56	0.35	0.14	1.98	5.29	0.020309	0.963
12	20.10	0.72	160.6	2.44	6.56	0.53	0.17	1.31	3.81	0.007620	0.998
Mean	12.17	0.51	161.7	1.46	6.50	0.45	0.16	1.66	4.71	0.009793	0.990
±SD	6.46	0.20	49.5	0.64	1.74	0.12	0.01	0.44	1.71	0.007175	0.012

* Sum of Weighted (1/y) Squared Deviations.

Table 6.7 Results of Non-Linear Curve Fitting with Equation 6.2 to Profiles Obtained after Multiple Dosing with five 1 gram doses of Josamycin as a Solution.

Subject	k_a (hr^{-1})	F	Vd (l)	k_{21} (hr^{-1})	α (hr^{-1})	k_{el} (hr^{-1})	t-lag (hr)	t½ (hr)	Cl ($l.kg^{-1}.hr^{-1}$)	Sum of Sq. Dev.*	Corr. Coeff.
7	3.99	0.73	40.2	0.60	4.01	0.25	0.14	2.77	0.76	2.0920	0.963
8	2.63	0.29	20.6	0.39	2.61	0.20	0.17	3.47	0.39	1.9935	0.966
9	12.93	0.26	32.7	0.22	0.68	0.15	0.16	4.62	0.28	3.5032	0.959
10	1.05	0.76	20.0	0.73	8.01	0.29	0.17	2.39	0.87	1.5913	0.961
11	3.72	0.50	54.6	0.21	0.83	0.17	0.14	4.08	0.74	0.9727	0.970
12	1.15	0.71	37.4	0.30	1.77	0.16	0.17	4.33	0.76	6.1879	0.940
Mean	4.25	0.54	34.3	0.41	2.99	0.20	0.16	3.61	0.63	2.7234	0.960
±SD	4.04	0.21	11.9	0.20	2.50	0.05	0.01	0.81	0.22	1.7267	0.001

* Sum of Weighted (1/y) Squared Deviations.

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