ASSESSMENT OF AMOXYCILLIN SUPPOSITORIES

by

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STUDY OBJECTIVES

Amoxycillin is a broad spectrum penicillin antibiotic; suitable for paediatric use. It is used for the treatment of urinary tract infections, otitis media, respiratory tract infections, enteric infections and endocarditis, for prophylaxis of endocarditis and before surgery such as tonsillectomy. Although several studies have been performed to investigate the release of amoxycillin from various dosage forms such as capsules, suspensions and parenterals, no studies have been performed on the release of amoxycillin from a suppositories.

The objectives of this study were therefore:

(1) To develop and validate a suitable High Performance Liquid Chromatographic (HPLC) method with the necessary sensitivity to accurately and precisely quantitate amoxycillin trihydrate in aqueous solution and human serum; and to develop an efficient technique for the pretreatment of serum prior to HPLC analysis.

(2) To formulate a 250 mg amoxycillin suppository in a fatty suppository base.

(3) To utilize an appropriate dissolution technique for the measurement of the amount of amoxycillin released from a suppository base during *in vitro* testing and determination of the effects of ageing on drug release; to make use of Differential Scanning Calorimetry (DSC) to investigate any potential interaction between amoxycillin and a suppository base.

(4) To determine the stability of amoxycillin in aqueous solution and stored biological fluids.

(5) To determine the bioavailability of amoxycillin following the administration of amoxycillin as single oral and rectal doses.

ABSTRACT

The investigations in this dissertation have been conducted to investigate the formulation and analysis of a paediatric amoxycillin suppository. The oral administration of antibiotics to young children can at times be problematic. Compliance is sometimes poor because of a sore throat, nausea, vomiting, a high fever or a dislike for the taste or smell of the medicine. In such cases the rectal administration of an antibiotic could provide an alternative route of administration that avoids some of the problems that affect oral administration. Difficulties associated with rectal administration are bioavailability, local irritation, acceptability to patients and rejection of the dosage form. Few data, however, are available on the usefulness in children of suppositories in general, and antibiotic suppositories in particular.

The areas of investigation have included the formulation of an amoxycillin suppository in various fatty bases, the quantitation of amoxycillin in both aqueous solution and human serum, assessment of stability of amoxycillin in stored aqueous and biological samples, *in vitro* drug release testing of suppositories, and bioavailability and pharmacokinetics following administration to human subjects of capsule, suppository, oral suspension and rectal suspension dosage forms.

Suppositories containing 250 mg amoxycillin were prepared in theobroma oil and in the semisynthetic bases *Witepsol W35, Suppocire A32, Novata BD* and *Novata 299*. The *in vitro* release characteristics of amoxycillin from these lipophilic suppository formulations were investigated using the USP rotating basket method. The dissolution of a drug from a solid dosage unit is an important parameter affecting drug bioavialability.

High Performance Liquid Chromatography (HPLC) was used as the main analytical technique. An original HPLC method for analysis of amoxycillin in aqueous solution, using ultraviolet detection at 230 nm was developed. The validated method was applied to the determination of the stability of aqueous amoxycillin solutions, and was utilized to determine the amount of drug released during dissolution testing.

Differential scanning calorimetry (DSC) is a technique commonly used in preformulation studies. Dissolution testing was used in conjunction with DSC to select a suppository base suitable for formulation with amoxycillin trihydrate.

An HPLC method for analysis of amoxycillin in human serum using UV detection at 230 nm is presented. The method involves a solid phase extraction procedure followed by chromatography on a reversed phase column. The limit of sensitivity of 0.3 μg/mL in serum is sufficiently sensitive to monitor serum concentrations of amoxycillin in humans after the administration of a single 250 mg oral dose.

Pharmacokinetic parameters were calculated from data obtained following the administration of a capsule and oral suspension. These parameters were consistent with previously published results. Following administration of a lipophilic suppository and a rectal suspension, to human volunteers, it was concluded that amoxycillin trihydrate is not readily absorbed from the rectum. Further investigations into the modification of the suppository dosage form with absorption enhancers to improve rectal absorption of amoxycillin, as well as elucidation of the mechanism of absorption of the drug, could assist in improving this formulation so that it is suitable for paediatric use.

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CHAPTER ONE
AMOXYCILLIN MONOGRAPH

1.1 INTRODUCTION

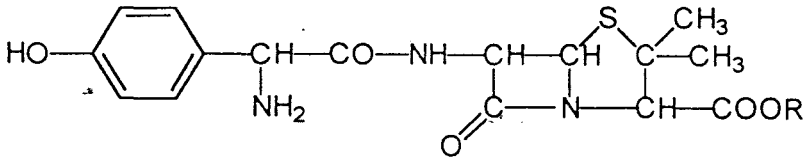
Amoxycillin is a broad spectrum, semisynthetic penicillin antibiotic related to ampicillin [1,2]. It was first described in 1970 and was originally marketed by Beecham Pharmaceuticals in 1972 [3]. Amoxycillin is listed on the World Health Organization's list of essential drugs [3]. Amoxycillin is used as the trihydrate in oral dosage forms and as the sodium salt in parenteral products.

1.2 PHYSICOCHEMICAL PROPERTIES

1.2.1 DESCRIPTION

Amoxycillin is described by several chemical names:

a) (6R)-6-[α-D-(4-hydroxyphenyl)-glycylamino]penicillanic acid,
b) D(-)-α-amino-*p*-hydroxybenzylpenicillin,
c) 6-[D-(-)-α-amino-*p*-hydroxyphenylacetamido penicillanic acid,
d) hydroxyampicillin, and
e) 4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,6-[(amino-4-hydroxyphenyl acetyl)amino]-3,3-dimethyl-7-oxo-2S[-2α,5α,6ß(S*)].



R=H	Anhydrous amoxycillin	$C_{16}H_{19}N_3O_5S$	MM 365.41
R=H.3H_2O	Amoxycillin trihydrate	$C_{16}H_{19}N_3O_5S.3H_2O$	MM 419.45
R=Na	Amoxycillin sodium	$C_{16}H_{19}N_3NaO_5S$	MM 387.39

Figure 1.1 Structure of amoxycillin

Amoxycillin occurs as either the trihydrate or the sodium salt. Amoxycillin trihydrate is an off-white crystalline powder, while amoxycillin sodium is a white or almost white powder, sometimes with a pinkish tinge [1]. Freshly prepared aqueous solutions may show a transient pink colouration. Both compounds are amorphous and hygroscopic and have a bitter taste and slightly sulphurous odour that is typical of many penicillins.

1.2.2 DISSOCIATION CONSTANT AND PARTITION COEFFICIENT

Amoxycillin has three ionizable groups: the baroxyl, amino and phenol groups. The dissociation constants (pK_a) for these groups in water are 2.4, 7.4 and 9.6 respectively [3,4]. The apparent isobutanol:water partition coefficients (P) [3] are listed in Table 1.1.

Table 1.1 Isobutanol:water partition coefficient

Aqueous phase	P
pH 1-3	0.79
pH 5-6	0.13
pH 9-9.5	0.25

The logP value for partitioning into n-octanol has been calculated as 0.87 [5]. These values can be used to predict the partitioning of amoxycillin between a lipophilic medium such as a semisynthetic suppository base and the aqueous rectal environment, during dosage form development.

1.2.3 SOLUBILITY

The solubility of the trihydrate in water varies with pH [3].The pH-solubility profile of amoxycillin trihydrate was determined, at 37°C and ionic strength 0.5, to be a U-shaped curve with minimum solubility at pH close to the isoelectric point [8]. Van't Hoff plots indicated a linear relationship between the equilibrium solubilities of amoxycillin to temperature in the range 20° to 50°C [8]. Table 1.2 lists the solubilities of amoxycillin in a variety solvents [3,4,6,7].

Table 1.2 Solubility of amoxycillin

Solvent	Solubility (mg/mL)	
	Trihydrate	Sodium
Water	1-10	)1000
Ethanol	1-10	10-33
Methanol	1-10	-
Chloroform	(0.1	(0.1
Diethyl ether	(0.1	(0.1
Acetone	1.0	0.1-1.0
Acetonitrile	Insoluble	-

1.2.4 SYNTHESIS

Amoxycillin is obtained directly by chemical synthesis, or semisynthetically from the compound 6-aminopenicillanic acid (6-APA) [4]. The route of synthesis which is generally used for large scale manufacture is illustrated in Figure 1.2.

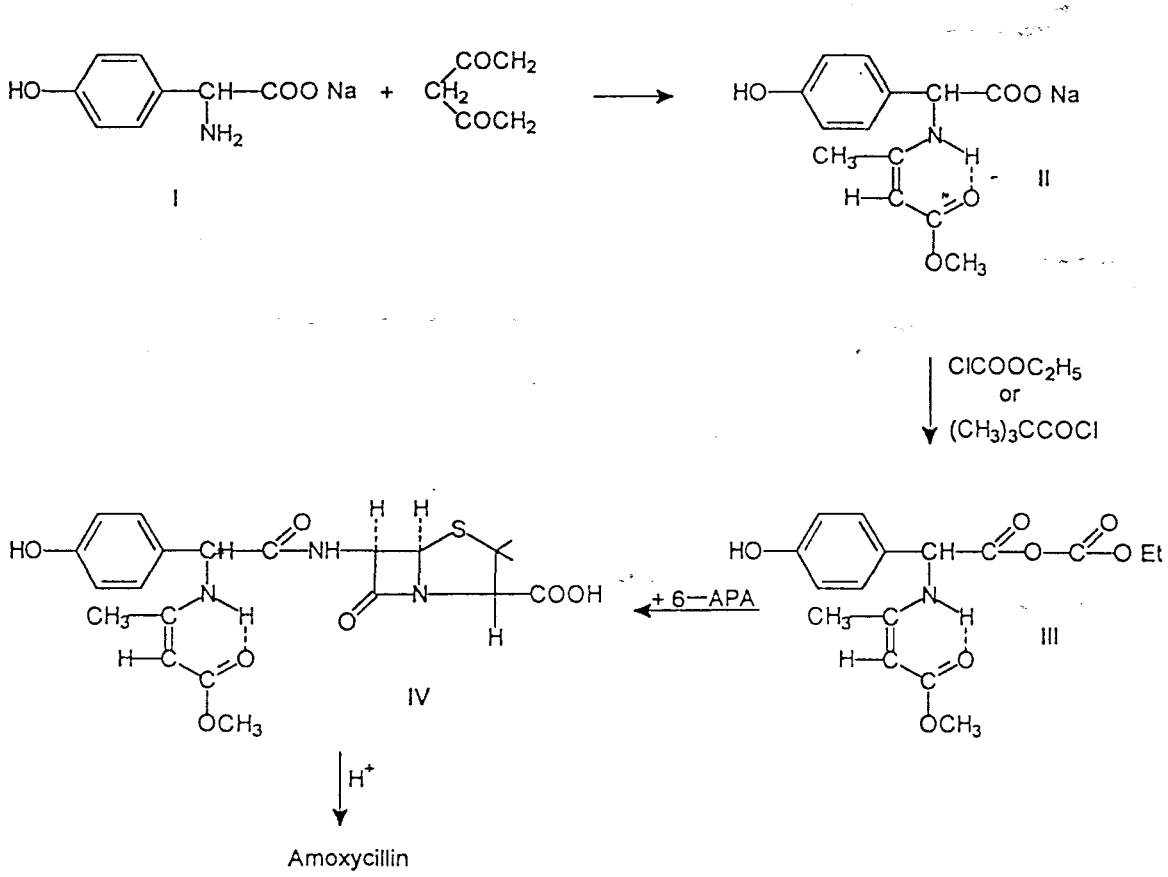


Figure 1.2 The synthesis of amoxycillin

This method involves protection of the amino group of D-(-)-*p*-hydroxyglycine (I) as an enamine by reaction with methylacetoacetate to give the Dane salt (II). This is isolated as a solid by solvent removal, suspended in a solvent such as acetone or dichloromethane and reacted with ethylchloroformate or pivaloylchloride between -10°C and -50°C in the presence of a basic catalyst such as N-methylmorpholine to form the mixed anhydride (III). A solution of the triethylamine salt of 6-APA in dichloromethane is added and reacts to give the N-protected amoxycillin (IV). The enamine is then hydrolysed at about 0°C by the addition of water and hydrochloric acid, which adjusts the pH to 1.5-2.5, and the dichloromethane layer is removed. Subsequent adjustment of the aqueous layer to about pH 5 (the isoelectric point) results in crystallization of amoxycillin trihydrate. Most major manufacturers use potassium rather than sodium for preparation of the Dane salt, and pivaloylchloride rather than ethylchloroformate for the mixed anhydride (III) [3].

1.2.5 STEREOCHEMISTRY AND STRUCTURE ACTIVITY RELATIONSHIP

Amoxycillin has the S,R,R configuration at carbons 2,5,6 respectively that is common to all penicillins. In addition, the carbon at the 10 position adjacent to the aromatic ring has the R configuration, formerly called D-(-) [9]. This structure, depicted in Figure 1.3, is numbered in accordance with the CA index name. Conventional penicillin numbering, which is used in much of the literature on amoxycillin, has S at 1, $C(CH_3)_2$ at 2, CH(COOH) at 3 and N at 4.

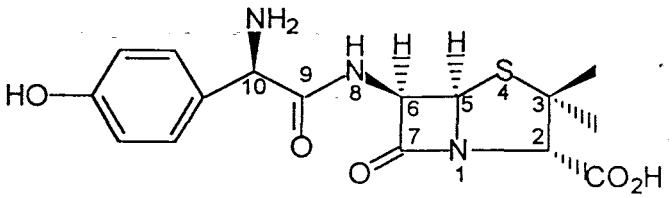


Figure 1.3 Stereochemistry of amoxycillin

The S epimer of amoxycillin at C10 has been synthesized, but has inferior antibacterial activity. The synthesis was further extended to include isomers (6 in all) at the C10, with an ortho, meta and para-hydroxy functional group on the aromatic ring. The two most active isomers *in vitro* had the R configuration on the C10 position and were meta and para-hydroxy substituted compounds. *In vivo* the R *p*-hydroxy isomer resulted in the highest blood levels, and as a result, this has been

used as the active amoxycillin [9].

1.2.6 SPECIFIC OPTICAL ROTATION

The specific optical rotation of a 0.25% w/v solution of the sodium salt, in a 0.4% w/v solution of potassium hydrogen phthalate was +240° to +290°, when calculated with reference to the anhydrous substance. Calculated on the anhydrous basis in a 0.2% w/v solution of the trihydrate in water, the specific optical rotation was +290° to +315° [9].

1.2.7 ULTRA VIOLET ABSORPTION SPECTRUM

The ultraviolet (UV) absorption spectra of amoxycillin indicate a phenolic type chromophore [9]. The single phenyl ring responsible for the UV absorption means that spectra are commonly detected in the region of 210-240 nm. Detection limits of the drug are generally in the order of 0.5-1 μg/mL [10]. The molar absorbance values of amoxycillin trihydrate in a variety of solvents are listed in Table 1.3 [3,9].

Table 1.3 Ultraviolet spectra of amoxycillin trihydrate

Solvent	Wavelength (nm)	Molar Absorbance (m^2/mol)
Ethanol	230 (max)	10850
	274 (max)	1400
	281 (sh)	1160
0.1 M HCl	229 (max)	9500
	272 (max)	1080
	278 (sh)	920
0.1 M KOH	248 (max)	82200
	291 (max)	3000
	325 (sh)	750

The UV spectra of amoxycillin trihydrate in mobile phase (methanol:phosphate buffer pH 7, 5:95) are shown in Figure 1.4. These spectra correspond with those recorded in the literature [3,9]. The UV spectrum of amoxycillin trihydrate was recorded on a Beckman DU-68 Spectrophotomer (Beckman Instruments Inc, Fullerton, CA).

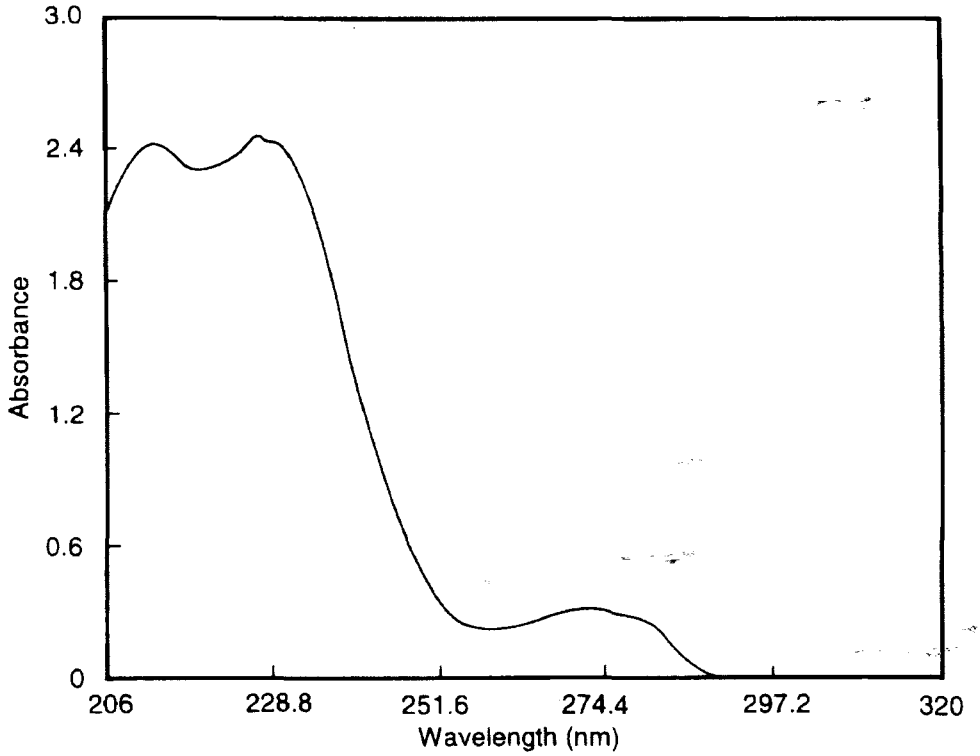


Figure 1.4 The ultraviolet spectrum of amoxycillin trihydrate in mobile phase

1.2.8 INFRARED SPECTRA

The frequencies and functional group assignments of the principal peaks in the infrared (IR) spectra of amoxycillin trihydrate and amoxycillin sodium salt are shown in Table 1.4 [3,5,9].

Table 1.4 IR spectra of amoxycillin sodium and amoxycillin trihydrate

Assignment of Amoxycillin Sodium	**Frequency (cm^{-1})**
Phenol and water OH, amine and amide NH stretches	3366 (broad)
Methyl CH stretch	2969
ß-lactam CO stretch	1764
Amide I, CO stretch	1671
COO^- asymmetric stretch	1601
Amide II, NH bend CN stretch combination band and benzene ring C=C stretch	1513
Gem dimethyl CH deformation	1457
COO^- symmetric stretch and phenol OH combination band	1398
Fused thiozolidine ß-lactam skeletal mode	1321
Phenol CO and Amide III, NH bend CN stretch in plane combination bands	1248
Benzene ring CH in plane deformation	1173, 1126
Assignment of Amoxycillin Trihydrate	**Frequency (cm^{-1})**
Water of crystallization, OH stretch	3520
Amide NH and phenol OH stretch	3458, 3175
Benzine ring CH stretch	3046
Methyl CH stretch	2964
ß-lactam CO stretch	1775
Amide I, CO stretch	1686
COO^- asymmetric stretch and NH_3^+ asymmetric deformation	1580
Benzene ring C=C stretch	1517
Amide II, NH bend CN stretch combination band and NH_3^+ symmetric deformation	1482
Gem dimethyl CH deformation and phenol OH combination band	1396, 1378
COO^- symmetric stretch	1327
Fused thiazolidine ß-lactam skeletal mode	1314
Amide III, NH bend CN stretch in plane combination band	1283
Phenol CO combination band	1250
Benzine ring CH in plane deformation	1143, 1120

1.2.9 pH

The pH of a 10% w/v solution of amoxycillin sodium is in the range 8.0 to 10.0. The pH of a 0.2% w/v aqueous solution of amoxycillin trihydrate is in the range 3.5 to 6.0 [7,8].

1.3 STABILITY

Amoxycillin in aqueous solution can degrade either by hydrolysis or by dimerization (Figure 1.5) [10,11]. The relative contributions of the two major reactions to the overall degradation process are concentration and pH dependent. Hydrolysis is predominant at lower concentrations, while

dimerization occurs at higher concentrations [10,12]. The degree of hydrolysis increases with increasing pH values while the degree of dimerization decreases [10]. In solutions with a pH between 8 and 10, hydrolysis is predominant at lower concentrations, while dimerization occurs at higher concentrations [10,11].

Kinetic studies of the degradation of amoxycillin in dilute aqueous solution (0.4-4 mg/mL) have been conducted over the pH range 0.3 to 10.5 at 35°C [13] and pH 1.1 to 10.8 at 35°C [14]. At constant pH, degradation followed first order or pseudo first order kinetics with a minimum degradation rate occurring at pH 6 [3,8,13,14].

The degradation behaviour of amoxycillin follows the same general pattern as other penicillins. In acid, both the cation and the zwitterion species undergo degradation at different rates with the rate constant of the zwitterion being about 15 fold faster than that of the cation [14]. In alkaline media, the anionic forms of amoxycillin degrade at different rates with the monohydrogen amoxycillinate being about 7 fold faster than that of the amoxycillinate. However at pH values between 5.5 and 6.5 where specific acid-base catalysed reactions are no longer dominant reactions, the zwitterion (amoxycillin trihydrate) and monohydrogen amoxycillinate undergo degradation at similar rates [14]. The pH of maximum stability of amoxycillin is 5.77 and the rate of degradation does not increase until the pH is below 5.5 or above 6.5 [14].

In acid, amoxycillin hydrolyses to form amoxypenicillenic acid which absorbs at 230 nm [8,9]. Additional breakdown products are the penillic acid, penamaldic acid, penicilloic acid, penilloic acid, penicillamine and ultimately penilloaldehyde [3,9]. Epimerization of penicilloic acids in aqueous solution occurs at C5 (refer to Figure 1.3) [3,9]. The instability of penicillins in acidic solution is well known, and is as a result of intramolecular attack of the side chain amide on the ß-lactam moeity. Introduction of an electron-withdrawing group may be expected to reduce such a reaction rate [13]. The electron withdrawing effect of the protonated α-amino group in the side chain of the amoxycillin molecule thus increases its stability, as a result of the electron attracting effect decreasing electron density, which subsequently decreases electron availability. Therefore amoxycillin and other aminopenicillins are markedly stable to acids [13]. Under acidic conditions the degradation half life of amoxycillin is approximately 15-20 hours [1,2,13], ampicillin approximately 12 hours [1,2] and penicillin G only 7 minutes [13].

As with other penicillins, amoxycillin hydrolyses in aqueous alkali media by opening of the ß-

lactam ring to form penicilloic acid [3,8,12]. Penicilloic acid ultimately loses carbon dioxide (CO_2) and forms penilloic acid. The amino group in the side chain, which is electron withdrawing, makes amoxycillin more susceptible to alkali hydrolysis. The penicilloic acid has been isolated as the monosodium salt [3], which retained the 5R configuration of the parent penicillin. Penicilloic acid can also be obtained by reaction of amoxycillin with ß-lactamase enzymes [3,9].

In addition to hydrolysis, a base catalysed self-aminolysis reaction can occur. Degradation at higher amoxycillin concentrations (25 to 125 mg/mL) at pH 8.6 to 10 and 35°C gives non-first order kinetics, indicative of a dimerization reaction [3,12]. When two molecules of amoxycillin interact, dimerization proceeds through nucleophilic attack by the side chain amino group of one molecule on the ß-lactam carbonyl of another [11,12]. The reacting molecules are subject to general base catalysis by the amino and phenolic hydroxyl groups in other molecules [3,10]. Dimerization occurs more readily with amoxycillin than ampicillin, because the basic nature of the amino group on the side chain increases when the phenol group is present in a dissociated form. Thus the rate of dimerization of amoxycillin at pH 9 is greater than that of other aminopenicillins due, in part, to this effect of the phenolic group [3,10].

Although the semi-synthetic penicillins are made from 6-APA, it is not a degradation product [9].

Unbuffered solutions of amoxycillin sodium are most stable at pH 5.8 [8]. Degradation of this compound is subject to general acid-base catalysis in citrate and phosphate buffer solutions [13,14]. The degradation is appreciably catalysed by monohydrogen and dihydrogen phosphate ions. Citric acid, dihydrogen citrate and monohydrogen citrate ions catalyse the hydrolysis of amoxycillin, while the unprotonated citrate ion has no catalytic effect [14]. Optimum stability conditions for aqueous solutions of amoxycillin are therefore in the range of pH 5.8 to 6.5 using an unprotonated citrate buffer [8,14].

The stability of different concentrations of amoxycillin sodium salt in water and various intravenous fluids has been investigated [12]. A limit of 10% inactivation was imposed. Degradation is faster at higher (5% w/v) amoxycillin concentrations than lower (1% or 2% w/v) concentrations. Stability in potassium and sodium chloride solutions was similar to that in water. Solutions in lactate and bicarbonate showed intermediate stability and stability was poor in dextran, dextrose 5% w/v and sorbitol 30% w/v solutions [8,12].

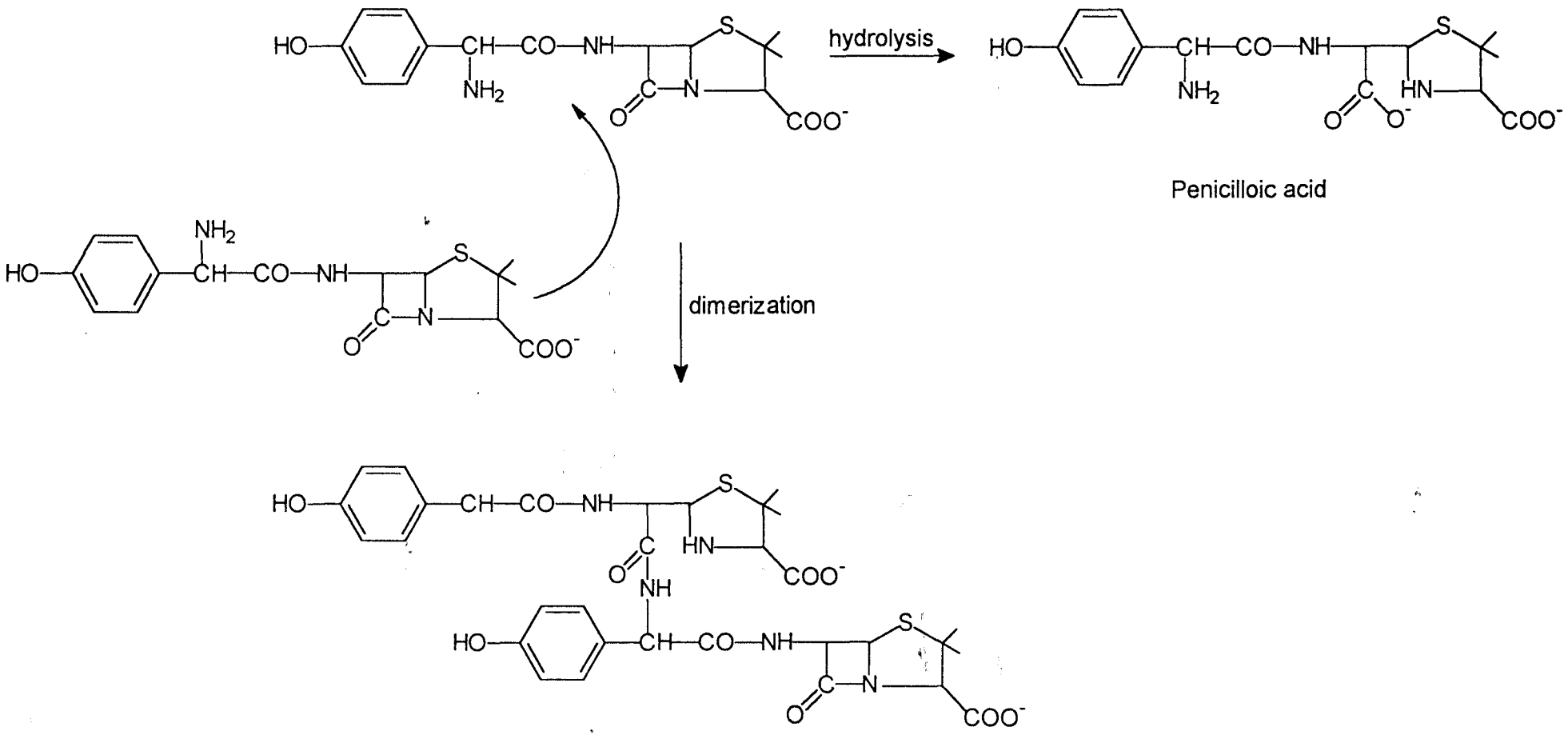


Figure 1.5 Degradation of amoxycillin

In the study of decomposition of amoxycillin in the solid state, it was demonstrated that the zwitterion form of amoxycillin trihydrate was more stable than amoxycillin sodium (anionic form) [15]. The $t_{10\%}$ values of amoxycillin trihydrate and amoxycillin sodium were calculated to be 3.2 years and 1.25 years respectively at 20°C. Solid state degradation of amoxycillin trihydrate results in formation of the penicilloic acid, piperazine-2,5-dione and unidentified compounds tentatively ascribed to amoxycillin oligomers and their penicilloates [3,15]. These degradation products may be present as impurities in samples of amoxycillin trihydrate, with the amoxylloate being the main impurity [16,17]. Samples of the sodium salt also contain piperazine-2,5-dione, the amoxycillin dimer and to a lesser extent higher oligomers such as the trimer and tetramer [16,17].

1.4 CLINICAL PHARMACOLOGY

1.4.1 MODE OF ACTION

The penicillin nucleus consists of a thiazolidine ring connected to a ß-lactam ring to which is attached a side chain. The structure of the side chain determines most of the pharmacological and antibacterial properties of a specific penicillin. The benzyl ring in the amino group side chain of amoxycillin extends the range of antimicrobial activity to include the Gram negative bacteria, since it allows the amoxycillin to penetrate the outer membrane of these bacteria [4]. It is therefore more active than benzylpenicillin against some Gram negative bacilli.

Amoxycillin kills bacteria by interfering with the final stage of peptidoglycan synthesis of the bacterial cell wall. Peptidoglycan is a heteropolymeric structure that provides the cell wall with its mechanical stability. The final stage in the synthesis of peptidoglycan involves the completion of cross-linking when the terminal glycine residue of the pentaglycine bridge is linked to the fourth residue of the pentapeptide, d-alanine. The transpeptidase enzyme that performs this step is inhibited by penicillins and cephalosporins. As a result the bacterial cell wall is weakened, the cell swells and then ruptures [4]. The action depends on the penicillin's ability to reach and bind to membrane-bound proteins known as penicillin-binding-proteins that are located beneath the cell wall. These proteins assist in maintaining cell wall structure, in cell wall synthesis and in cell division, and appear to possess transpeptidase and carboxypeptidase activity. Bacterial surface enzymes called autolysins also appear to be involved in the lethal effect of penicillins, particularly for Gram positive bacteria. In Gram negative bacilli, osmotic rupture of the cells may occur once the cell wall has been weakened [6]. It has been reported that amoxycillin predominantly inhibits

side-wall synthesis in susceptible bacteria, while ampicillin mainly inhibits cross-wall synthesis [6].

The action of amoxycillin is weakened by penicillinase and other beta-lactamase enzymes that are produced during the growth of micro-organisms such as staphylococci [6].

The inhibiting effect of amoxycillin on cell wall synthesis in susceptible bacteria is expressed in terms of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). If local amoxycillin levels exceed these values for a given bacterium, then no increase in dose can be expected to improve clinical outcome [4].

1.4.2 SPECTRUM OF ACTIVITY

Amoxycillin has no other clinical pharmacological effect apart from its antibacterial action [4]. Amoxycillin is bactericidal for both Gram positive and Gram negative organisms. It is active in low concentrations against Gram positive cocci, except penicillin-resistant staphylococci, and against Gram positive aerobic and anaerobic bacilli [1,2,4,6,9,18]. It is also active against various Gram negative cocci and bacilli [2,6,18]. Table 1.5 lists some of the organisms which are sensitive to amoxycillin.

Synergy occurs between amoxycillin and clavulanic acid against bacteria usually resistant to amoxycillin due to the production of certain beta-lactamases. Amoxycillin may be administered in combination with clavulanic acid as the potassium salt. Clavulanic acid is a beta-lactamase inhibitor that widens amoxycillin's antibacterial spectrum to organisms usually resistant due to their production of beta-lactamases. The combination should however be reserved for the treatment of infections caused by such amoxycillin resistant organisms. It is administered by mouth in a ratio of 2 or 4 parts amoxycillin trihydrate to 1 part clavulanic acid, or intravenously in a ratio of 5 parts amoxycillin sodium to 1 part clavulanic acid [6].

Table 1.5 Examples of the antibacterial spectrum of amoxycillin

Organism	MIC (μg/mL)
*Staphylococcus aureus, Staphylococcus aureus**	0.1
Streptococcus faecalis	250
Streptococcus pneumoniae	0.5
Streptococcus pyogenes	0.02
Streptococcus viridans	0.01
Corynebacterium diphtheriae	0.01
Listeria monocytogenes	0.02
Clostridium tetani	0.1
Clostridium welchii	0.05
Brucella abortus	0.05
Bordetella pertussis	0.25
Haemophilus influenzae	0.5
Neisseria gonorrhoeae	0.25
Neisseria meningitidis	0.02
Pasteurella septica	0.02
Escherichia coli	0.5
Salmonella typhi	5.0
Shigella sonnei	1.25
Klebsiella aerogenes	2.5
Proteus mirabilis	250
Proteus vulgaris	2.5
Pseudomonas aeruginosa	250
Serratia marcescens	)500
Vibrio cholreae	100

* Penicillinase producing strain

1.4.3 INDICATIONS

As amoxycillin is the 4-hydroxy analogue of ampicillin and there are few clinical differences between the two penicillins, it is used for the treatment of similar infections [6].

Amoxycillin has been used successfully in the treatment and prevention of various infections. These include urinary tract infections, otitis media, respiratory tract infections, mouth infections, bacterial meningitis, Gram negative septicaemia and other surgical infections, enteric infections and ulcers, gynaecological infections and sexually transmitted diseases such as gonorrhoea and chancroid and endocarditis. It is also used for the prophylaxis of infective endocarditis and for prophylaxis in surgical procedures such as tonsillectomy [4,6,9]. Amoxycillin has been used as an alternative to chloramphenicol in the treatment of infections caused by *Salmonella* species [6].

1.4.4 RESISTANCE

Penicillinase-producing strains of bacteria such as *Staphylococcus aureus* are resistant to amoxycillin as a result of beta-lactamase activity [2]. Complete cross resistance has also been reported between amoxycillin and ampicillin [6]. The rising prevalence of ampicillin resistance among previously susceptible strains of bacteria, such as the Enterobacteriaceae, has reduced the clinical usefulness of amoxycillin.

It can no longer be recommended for first choice or sole use in life-threatening infections due to enteric organisms. Amoxycillin was initially very effective against *Haemophilus influenzae*, but now, particularly in children, 6-12% of organisms isolated are resistant to amoxycillin. Similarly, an increasing percentage of *Salmonella spp*, *E coli*, *Proteus mirabilis* and *Shigella spp* are not sensitive to amoxycillin. Amoxycillin is not usually effective against *Pseudomonas* or *Acinetobacter*. Infections due to *Klebsiella* and *Providencia spp* should not be treated with amoxycillin as they possess intrinsic resistance to the drug [4]. Supra infections with non-susceptible organisms may occur particularly with prolonged use [6].

1.4.5 CONTRAINDICATIONS

Contraindications to the administration of amoxycillin include penicillin hypersensitivity, glandular fever, lymphatic lymphoma and bacterial resistance [4].

1.4.6 DRUG INTERACTIONS

No potentially hazardous interactions have been reported. The simultaneous use of amoxycillin and an oral contraceptive might be expected to cause breakthrough bleeding or pregnancy on rare occasions, because of reduced absorption of the oral contraceptive as a result of diarrhoea. Serum concentrations of amoxycillin are markedly increased after administration of probenicid, which blocks active secretion of the drug by the kidneys [2,4,6]. No technical interferences with clinical pathology tests have been reported [4]. Instability of amoxycillin trihydrate has been identified in infusion fluids containing substances that catalyze the degradation of penicillins. These include carbohydrates and polyhydric alcohols such as macrogols.

1.4.7 ADVERSE REACTIONS

As with all penicillins, anaphylactic shock may occur on very rare occasions and is more likely to occur following parental administration [4]. Convulsions may occur if large intravenous doses are given [4].

Acute overdosage produces very high urinary concentrations. Problems are unlikely if adequate fluid intake and urinary output are maintained; however crystalluria is a possibility [4]. Amoxycillin is removed to a certain extent by dialysis [6].

In addition to anaphylactic shock, amoxycillin may cause hypersensitivity reactions, such as rash and fever, similar to those induced by benzylpenicillin and ampicillin. The incidence of these reactions is probably no greater than with other penicillins. Amoxycillin should be discontinued in the event of any hypersensitivity reaction [4].

Penicillin allergy may be caused when either the penicillins or their rearrangement products formed *in vivo* react with proteins, forming a penicilloyl protein. Polymer formation may be an antigenic determinant. Thus the pathogenesis of this allergy may be attributed to lymphocyte function, which may explain its significantly high frequency in glandular fever.

These allergic reactions occur in sensitised persons. Skin rashes are among the most common side effects and are either urticarial or maculopapular. The urticarial reactions are typical of penicillin hypersensitivity, while the erythematous maculopapular eruptions are characteristic of ampicillin and amoxycillin and often appear more than 7 days after commencing treatment.

Most patients with infectious mononucleosis develop a skin rash when treated with amoxycillin, and patients with lymphatic leukaemia also appear to have a higher incidence of skin rashes [4,6].

Gastrointestinal side effects, such as nausea, vomiting and particularly diarrhoea, may occur during the course of treatment, usually following oral administration. Approximately 5% of patients or more will suffer these effects, with a higher incidence in children. The incidence of diarrhoea is lower with amoxycillin than with ampicillin and other penicillins. Diarrhoea is a result of depletion of sensitive bacteria from the bowel flora by unabsorbed antibiotic. The increasing resistance of Enterobacteriaceae to amoxycillin may eventually reduce the incidence of amoxycillin-

associated diarrhoea [4,6]. Amoxycillin has been associated with a small number of cases of pseudomembranous colitis [4,6].

1.4.8 HIGH RISK GROUPS

Because of its lack of teratogenicity and mutagenic potential, there appears to be no significant risk to the foetus from amoxycillin when administered during pregnancy, although there is the possibility of sensitising the foetus if used during the second and third trimesters. Amoxycillin can be given safely throughout pregnancy at the normal adult dose, if an antimicrobial agent has to be used for the treatment of asymptomatic bacteriurea or simple cystitis, bronchitis or the prophylaxis of endocarditis [4,6]. The small amount of amoxycillin secreted in maternal milk rarely causes problems in the infant. It can therefore be used safely during lactation [4].

1.5 PHARMACOKINETICS OF AMOXYCILLIN

1.5.1 DOSAGE

The usual adult oral dose of amoxycillin trihydrate is the equivalent of 250 mg to 500 mg amoxycillin three times daily. Amoxycillin is administered via the parenteral route as amoxycillin sodium and in moderate infections the equivalent of 500 mg amoxycillin may be given intramuscularly or intravenously every 8 hours. If pain is experienced with an intramuscular injection, this can be prepared using a 0.5% w/v solution of procaine hydrochloride or a 1% w/v solution of lignocaine hydrochloride. In severe infections the equivalent of 1 g amoxycillin may be given every 6 hours by slow intravenous injection over 3 to 4 minutes, or by infusion over 30 to 60 minutes [6]. In renal failure, the usual adult dose can normally be used, although the dose of amoxycillin may have to be reduced owing to the prolonged plasma elimination half life [4].

Children up to 10 years of age may be given the equivalent of 125 mg to 250 mg orally, three times daily. Where indicated, a parenteral dose of 50-100 mg/kg body weight can be given daily in divided doses [4]. Doses of amoxycillin should be reduced to 20 to 50 mg/kg daily in divided doses for infants under 20 kg body weight [4,6].

Higher doses of amoxycillin, often in short courses have been investigated for numerous conditions [6]. Amoxycillin has been given as a single 3 g dose, often with probenicid (1 g), in the treatment

of uncomplicated gonorrhoea, where gonococci have maintained sensitivity. This regimen, followed by a course of tetracycline has often been used for the treatment of pelvic inflammatory disease, or sexually transmitted diseases where the aetiological agent is unknown [6].

A single dose of 3 g may also be used for the treatment of uncomplicated urinary tract infections and for the prophylaxis of endocarditis in susceptible patients about 1 hour before procedures such as dental extractions. A high dose of amoxycillin (3 g) twice daily may be used in patients with severe or recurrent respiratory tract infections [6].

1.5.2 ABSORPTION

Amoxycillin is rapidly absorbed after oral administration [4,5]. The 90% bioavailability of amoxycillin following oral administration appears to be much higher than anticipated from its physicochemical properties and the pH partition theory [3-5].

It is more completely absorbed than ampicillin and is reported to produce peak plasma concentrations (C_{max}) that are 2 to 2.5 times higher than those observed after administration of equivalent doses of ampicillin [1,2,4,6]. Peak plasma concentrations of 4 to 6 μg/mL have been observed 1 to 2 hours (t_{max}) after a dose of 250 mg with detectable amounts present for up to 8 hours after administration, although there is considerable variation between individuals [4,5,6,20].

Following intramuscular injection of amoxycillin (500 mg), a C_{max} of 14 μg/mL has been observed at a t_{max} of 1 hour [4,6]. However, in general, similar concentrations are achieved with both parenteral and oral administration [6].

Oral studies in the rat showed that the plasma level curve is best fitted to a combined zero and first order kinetic function [21,22]. The same model also gives a good fit for human plasma level data. This appears to be consistent with a study of absorption kinetics in humans which provided evidence for saturated carrier mediated absorption. Carrier mediated absorption may explain the unexpectedly high oral bioavailability of amoxycillin [3,21,22].

Amoxycillin is resistant to inactivation by acidic gastric secretions, therefore more of the total oral dose reaches the small intestine [4,6]. Neither the presence of food in the stomach, nor co-administration with clavulanic acid appear to have a significant influence on the absorption of

amoxycillin [2,6,19].

1.5.3 DISTRIBUTION

Amoxycillin is extensively distributed in body tissues and fluids, with levels adequate for antibacterial activity being achieved in most regions. Amoxycillin diffuses across the placenta, but concentrations in umbilical cord blood have been found to be a fraction of those in maternal blood. Concentrations in amniotic fluid are variable, but are less than 50% of maternal blood levels. Only a small amount appears to be excreted in breast milk. It penetrates well into purulent and mucoid sputum and middle ear fluid, but only low concentrations have been found in ocular fluid. Amoxycillin is unlikely to pass into the central nervous system unless the meninges are inflamed. High concentrations have been reported in bile [3,4,6]. Amoxycillin concentrations in the interstitial fluid peak around 1 hour after the serum peak, according to skin window tests [3,4,5,6].

The volume of distribution is approximately 0.31 L/kg body weight (range 0.2-0.4 L/kg) [4,5]. The use of ultrafiltration to determine the extent of binding of amoxycillin to serum proteins in humans revealed that the drug is 20% bound, leaving 80% of the penicillin as unbound active drug [2,6].

1.5.4 METABOLISM

Presystemic metabolism is negligible due to the highly polar nature of the drug. Approximately 10-20% of absorbed drug is metabolized via hydrolysis of the ß-lactam ring to the corresponding penicilloic acid, which is excreted in the urine in a ratio of about 2 to 1 of the 5R to the 5S isomer [3,4,6,20]. Major metabolites are thus the respective penicilloic acids, generated by cleavage of the ß-lactam ring, and α-amino substituted derivatives of α-amino penicillins [20]. Interindividual variability in the amount of penicilloic acids excreted suggests their enzymatic, rather than spontaneous formation [20].

Enterohepatic circulation of the antibiotic is low, as hepatic metabolism is a relatively unimportant route of amoxycillin elimination.

1.5.5 ELIMINATION

Plasma clearance ranges from 3 to 5 mL/min/kg [5]. About 60% of the oral amoxycillin dose is excreted unchanged in the urine in 6 hours by glomerular filtration and active tubular secretion. Urinary concentrations of 0.3 to 1.3 g/L have been reported following a dose of 250 mg [4,5,6,20]. After parenteral administration, 75% is excreted via the kidneys in the following 6 hours [4,5].

The serum half life of amoxycillin in healthy volunteers ranges between 1 and 2 hours [4,5,6]. Subjects with a creatinine clearance less than 13 mL/minute who are in renal failure have a serum half life of around 7 hours [4,6]. The need for penicillin plasma level monitoring has been emphasised in neonates and the elderly where the elimination half life may be longer because of incomplete renal function [3,4,6,20].

Amoxycillin displays the linear kinetics characteristic of penicillins, that is increasing the dose of amoxycillin results in a corresponding increase in serum concentrations [2]. However, recent studies [21,22] using a rat model have investigated the non-linearity of disposition kinetics which are occasionally observed following intravenous administration. The intravenous study indicated that non-linearity arises from renal elimination mechanisms, possibly saturation of active tubular secretion or reabsorption at high doses.

CHAPTER TWO
RECTAL DOSAGE FORMS

2.1 INTRODUCTION

2.1.1 RECTAL DOSAGE FORMS

Suppositories are medicated solid dosage forms, which are inserted into body orifices other than the mouth, where they melt, soften or dissolve to exert a localized or systemic effect. The term suppository usually refers to the rectal dosage form. Suppositories vary in size and shape; those intended for adult use are generally about 2 g in size, and those for paediatric use about 1 g. They are conventionally bullet-shaped, torpedo-shaped or conical with a rounded apex.

Suppositories have a wide range of applications and can be an effective alternative when oral therapy is not possible. The potential uses of suppositories as convenient delivery systems are expanding and now include anti-malarial drugs [23,24], controlled-release and sustained-release suppositories [25], analgesic suppositories that allow patients a pain-free sleep [26], insulin suppositories for diabetics [27] and antimicrobial suppositories [28]. It is conceivable that any natural or synthetic drug can be formulated into a suppository by careful selection of the base, the right form of the drug itself and possible modification of the formulation to include absorption promoting agents or adjuvants.

Although the oral route is considered the most practical and acceptable, as well as the most economical means of administering a drug, a suppository could be an alternative treatment option if oral medication is contra-indicated, or it is not possible for a patient to take solids or even liquids by mouth. In addition the rectal route is convenient for administering drugs to patients who are unwilling or unable to swallow medication, for example unconscious patients, mentally disturbed patients, infants and children, patients who are vomiting, patients with pathological conditions of the alimentary tract and patients recovering from surgery who are unable to tolerate oral therapy [27]. Suppositories are used to exert a localized effect on the rectum as well as to avoid the disadvantages of oral medication. Drugs known to irritate the stomach, such as aminophylline [26] and non-steroidal anti-inflammatory drugs (NSAIDs), can be given in suppository form to minimize the effect of local gastric irritation, although the systemic effects of the NSAIDs will occur irrespective of the route of administration [27].

The use of suppositories, rather than oral medicine does not only have patient-orientated advantages, but also has physiological and metabolic benefits. Drugs administered rectally are not inactivated by the enzymatic activity or acidic conditions in the stomach and upper intestine and the "first-pass" effect of the liver enzymes may be partially bypassed [27].

Suppositories may be used to exert a localized effect on the bowel or rectum, for example the laxatives glycerol and bisacodyl exert their effect by irritating the mucosa of the rectum. Their formulation into suppositories enables this effect to be exerted locally without having to pass through the alimentary canal, causing undesirable gastric effects.

Rectal suppositories are often used for their local effect to relieve the pain, itching, irritation and inflammation associated with haemorrhoids and other rectal conditions. They contain combinations of local anaesthetics, anti-inflammatory agents, astringents, analgesics, soothing emollients and protective agents.

2.1.2 RECTAL ABSORPTION

Absorption across the rectal mucosa occurs in much the same manner as in other parts of the gastrointestinal tract [26]. However the dose of a drug administered rectally may differ from the dose of the same drug given orally, because drug absorption from the rectum is sometimes slower and more variable than with other types of dosing, due to either physiological factors or the physicochemical nature of the drug and suppository base.

2.1.2.1 PHYSIOLOGICAL FACTORS

The rectum is 15 to 20 cm long and has no villi, thus there is a relatively small surface area available for drug absorption [29]. As the rectum is covered by only 1 to 3 mL of mucous and does not receive any liquid through the intestinal wall, dissolution of water-soluble drugs may be hindered [8,29]. Since the pH of the rectal fluid is neutral and has little buffering capacity, the ionic form in which a drug is absorbed will generally not be changed [8,29]. The presence of faecal matter retards drug absorption and may lead to expulsion of the suppository [29]. Enzymatic processes in the lining of the rectum occur to a lesser extent than in the upper gastrointestinal tract, thereby minimising possible enzymatic degradation which occurs to some drugs prior to absorption [29].

Although opinions differ, it is thought that suppositories generally remain in the lower to middle section of the rectum, and do not migrate higher into the region of the superior rectal vein [8,29]. The site of drug absorption in the rectum and subsequent transport into the inferior, middle or superior rectal veins will determine whether a drug is transported directly to the general circulation, or via the liver [29]. The capillaries in the lower and middle sections of the rectum join to form the inferior and middle rectal veins which drain into the inferior vena cava, and from there into the general circulation. Any drug absorbed in these regions will bypass the liver and it has been suggested that at least 50% of a rectal dose can be assumed to avoid the first-pass effect in this way [26,30]. Higher up in the rectum the superior rectal vein drains into the hepatic portal system. Drugs which are absorbed in this zone will pass through the liver and be metabolized by the liver enzymes. Avoidance of the hepatic portal system could be advantageous to patients with liver disease, as drug metabolism by the liver and possible subsequent damage to the liver by drugs is partially averted [26,27,31].

2.1.2.2 PHYSICOCHEMICAL FACTORS

The relative solubility of the drug in lipid and water as well as the particle size of the drug can affect absorption [8,29]. The smaller the particle size, the more readily the particle will dissolve and the greater the chance for rapid absorption. The partition coefficient of the drug and the lipophilic or hydrophilic nature of the suppository base will regulate the partitioning of the drug between the suppository and the rectal environment. The ability of the base to melt, soften or dissolve at or below body temperature and its ability to release the drug substance will also play a role in determining the release characteristics of the drug from a specific base and subsequent absorption [29]. Chemical interaction between the base and drug will inhibit release and drug absorption will be impaired [29].

2.1.3 SUPPOSITORY BASES

Suppository bases play a critical role in the release of the drug and subsequent availability of the drug either for absorption to achieve systemic effects, or for localised effects.

Suppository bases have been classified according to their physical characteristics as either

(a) water-soluble or water-miscible bases that dissolve or disperse in rectal secretions and

include glycerol-gelatine bases and macrogols such as polyethylene glycol (PEG), or

(b) fatty or oleaginous bases which melt at body temperature and include theobroma oil (cocoa butter) and semi-synthetic mixtures of mono-, di- and triglycerides, such as *Witepsol* and *Suppocire*, or

(c) miscellaneous bases, generally combinations of lipophilic and hydrophilic bases [29].

The ideal suppository base should remain solid at room temperature, but soften, melt or dissolve at or slightly below body temperature so that the drug it contains may be readily available soon after insertion to exert its effect [29]. The base should be innocuous, non-sensitizing, physiologically inactive, not cause local irritation, nor itself be absorbed through the intestinal wall. It should be neutral and chemically inert and should not form complexes with the active ingredients. The base should be compatible with a wide range of drugs and should be readily moulded into stable, rigid shapes that retain uniform drug release characteristics on storage. The interval between melting and solidification temperatures of the final composition should be adequate; if it is too short there is difficulty in pouring into moulds, if it is too long there may be sedimentation of suspended solid material. Most fats have a broad melting range, but those selected for suppository bases usually melt within 3°C. Ideally the base should also contract on cooling to allow easy withdrawal of the suppositories from the mould during extemporaneous manufacture [8]. The final product should be odourless, opaque and have a smooth uniform surface and it should retain its hardness and melting point over the range of temperatures at which it is likely to be stored [32].

2.1.3.1 WATER SOLUBLE AND WATER MISCIBLE BASES

2.1.3.1.1 GLYCEROL-GELATIN

Glycerol-gelatin bases are most frequently used for the preparation of vaginal suppositories or pessaries. The water soluble base dissolves slowly in the mucous secretions of the rectum or vagina [8,29]. The solution time depends on the quality and content of the gelatin and the age of the base, the relative properties of the constituents and reaction with the active drug [8,26].

Glycerol-gelatin suppositories have several disadvantages, which cause them to be used less frequently than other types of suppository bases. They are difficult to prepare and handle and lubrication of the mould with a mineral or vegetable oil is necessary to prevent adhesion to the

mould [26]. They must be protected from heat and are hygroscopic, therefore they must be protected from atmospheric moisture in order for them to maintain their shape and consistency [26,29]. The suppositories support microbial growth so the inclusion of preservatives such as methyl and propyl hydroxybenzoates may be necessary [8]. Glycerol-gelatin suppositories are also less frequently used than fatty base suppositories because they have a laxative action which has been attributed to the hygroscopic nature of the base causing dehydration with consequent irritation of the rectal tissue [29].

2.1.3.1.2 MACROGOLS

The macrogols or polyethylene glycols (PEG) are polymers of ethylene oxide and water, prepared to various chain lengths, molecular weights and physical states. Various admixtures of high and low molecular weight polymers may be combined to prepare suppository bases of desired consistency, melting point ranges, physical characteristics and dissolution rates [8,29].

PEG with molecular weights of 200 to 600 are clear colourless liquids and molecular weights of greater than 1000 occur as wax-like solids. Hardness increases with increasing molecular weight. High molecular weight polymers form hard bases that disintegrate and release their drug slowly. Softer, less brittle preparations that disperse and liberate the drug quickly are obtained by mixing the high with medium, or medium and low molecular weight polymers, or by addition of plasticisers such as hexane-1,2,6-triol [26].

PEG suppositories generally have a melting point above 42 °C. The macrogols thus present fewer storage problems than bases that melt at lower temperatures and they are therefore satisfactory in hot climates, and administration is easy because they are not slippery to handle [26,29].

The macrogols have the advantages of inertness, stability, high water absorbing capacity and freedom from rancidity. They are therefore useful vehicles for drugs such as chloral hydrate and ichthammol which tend to lower the melting point of other bases [8]. The macrogols do not melt at body temperature, but rather gradually dissolve and disperse in the body fluids resulting in a slow release of medication from the base and a more prolonged action than is found with fatty bases [26]. However their good solvent properties can result in retention of the drug in the liquified base, resulting in reduction in therapeutic activity [26]. Substances that are incompatible with macrogols include penicillin, bacitracin, benzocaine, phenols, quinine salts, salicylic acid, sorbitol

and sulphonamides [8]. Suspended solid materials may display crystal growth in macrogol bases; such crystals may show delayed dissolution and cause irritation of the rectum, as well as making the product brittle [26].

Unlike glycerol-gelatin, macrogols do not stick to the mould, since they contract on cooling, thus no lubrication is required. However macrogol suppositories may be brittle unless the molten base is poured into the mould at as low a temperature as possible. Brittleness can be reduced by the addition of surfactants or plasticisers such as castor oil or propylene glycol [26].

Because of their high molecular weights, macrogols form solutions of high viscosity when they disperse in the body, and leakage from body cavities is minimised [8,26]. PEG suppositories are hygroscopic and irritating to the rectal mucosa due to dehydration. Discomfort can be reduced by advising a patient to dip the suppository in water before use or incorporating about 20% of water in the mass [26,29]. However products that contain water sometimes fracture on storage. The dehydrating effect may also be reduced by application of coatings of cetyl or stearyl alcohol, although these may retard dissolution as a result of their lipophilic nature [8].

2.1.3.2 FATTY BASES

2.1.3.2.1 THEOBROMA OIL

Theobroma oil or cocoa butter is considered an ideal suppository base, melting just below body temperature, between 30 to 36°C, yet maintaining its solidity at normal room temperature [26]. Theobroma oil shows ready liquification on warming and rapid setting on cooling. It is miscible with many excipients and is bland, a valuable feature when a suppository is intended to soothe irritation [26].

However, because of its triglyceride content, theobroma oil exhibits marked polymorphism [8,26,29]. When melted and cooled it solidifies in different crystalline forms depending on the temperature of melting, rate of cooling and size of the mass, so great care must be taken to avoid overheating the base.

Theobroma oil has the disadvantage that it shrinks only slightly on solidification necessitating a mould lubricant such as alcoholic solutions of soft soap and glycerol, silicones or sodium lauryl

sulphate in aqueous solution or in propylene glycol. A further undesirable property is that sometimes base escapes from the rectum or vagina due to its low viscosity when melted [26].

The melting point of theobroma is reduced by the addition of drugs such as volatile oils or chloral hydrate. If the melting point is lowered to such an extent by an excipient that the suppositories are too soft for use, solidifying agents such as cetyl ester wax (about 20% w/w) or beeswax (3% to 5% w/w) may be added [8,26,29]. If the softening point of theobroma suppositories is too low for hot climates, white beeswax may be added to the suppositories to raise the softening point [26,29].

Theobroma has a poor water absorbing capacity that can be enhanced by addition of emulsifying agents or surfactants such as cholesterol (2% w/w), emulsifying wax (up to 10% w/w), polysorbates (5% to 10% w/w) or wool fat (5% to 10% w/w) [8,26]. However, addition of surfactants may lead to drug-base interactions, affect the release of drug from the suppository, or influence absorption.

2.1.3.2.2 SYNTHETIC FATS

To produce an alternative base free from the disadvantages of theobroma, it has been necessary to use semisynthetic methods, because theobroma is the only natural fat with suitable properties for a suppository [26]. More modern bases, the hard fats, were developed in Germany in the 1940's and have slowly displaced theobroma oil by virtue of their superior properties and relative ease of high speed manufacture [26].

The hard fats are mixtures of triglyceride esters of saturated C_{10}-C_{18} fatty acids with varying proportions of mono- and diglycerides. They are virtually ubiquitous nowadays because of their chemical and physical stability, broad compatibility with nearly all active compounds, neutrality towards mucous membranes and their favourable processing properties even in high performance machines.

The semisynthetic fats are hard, brittle and unctuous to touch, and melt to a colourless or slightly yellow liquid when warmed [33]. They produce suppositories that are white and almost odourless and have a very attractive, clean and polished appearance [26]. They are available in various grades with different melting ranges, hydroxyl values and other physicochemical characteristics. Examples include the fractionated palm kernel oils *Extracoa* and *Supercoa* and the hard fats *Massa*

Estarinum, Massuppol, Suppocire and *Witepsol* [26]. Synonyms include adeps solidus, semi-synthetic glycerides, massa esterinica, adeps neutralis, synthetic fats, and hydrogenated vegetable oils. Special grades, identified by the manufacturers by means of suitable letters or numbers, may contain additives such as beeswax, lecithin, polysorbates, ethoxylated fatty alcohols and ethoxylated partial fatty glycerides [8,32,33].

Additives may also be incorporated during formulation if required. Water is undesirable as an additive because it enhances oxidation, crystallisation and the potential for chemical reaction between suppository constituents. In low concentration it plays little part in drug release and it can serve as a medium for microbial growth [8,33].

The most common method of manufacture involves hydrolysis of natural vegetable oils such as coconut or palm kernel oil, and then isolation of the free saturated acids by fractional distillation. Various fractions of the C_{12} to C_{18} series are re-esterified under controlled conditions with glycerol to yield a mixture of tri-, di- and monoglycerides, thus providing a series of bases with a range of physical characteristics appropriate for differing requirements [26,33]. Further diversification is gained by the inclusion of adjuvants as well as by blending available grades [32]. *Suppository Base IV Novata* and *Witepsol* are manufactured by this method.

An alternative procedure involves the direct hydrogenation of vegetable oils, followed by vacuum dehydration, analytical verification, grading, and finally heat treatment. Thus part of the triglyceride esters are split onto mono- and diglycerides (partial esters) and fatty acids are released. The liberated free fatty acids participate in trans-esterification reactions with available hydroxyl groups, resulting in mixed ester formation [32,33]. Controlled modification of this process yields a wide range of materials and subsequent blending of selected fractions, or the incorporation of adjuvants gives the desired grades [26]. *Suppocire* is an example of a base manufactured using this method.

The pharmacopoeial specifications for semisynthtic bases are listed in Table 2.1 [7,33].

Table 2.1 Pharmacopoeial specifications for semisynthetic fats

Test	Value
Melting point*	33-36°C
Acid value	≤0.5
Hydroxyl value	≤50
Iodine value	≤3
Peroxide value	≤6
Saponification value	225-245
Unsaponifiable matter	≤0.5%
Alkaline impurities	+
Decomposition products	+
Ash	≤0.05%

*Melting point ranges are defined by several pharmacopoeias as ranging between 33°C and 36°C, though proprietary grades may melt outside this range [32].

The iodine value is an inverse measure of the extent of hydrogenation and partial ester content is determined by the hydroxyl value [32]. The hydroxyl value of a base is determined by the proportions of mono- and di-glycerides it contains and is an indication of its potential reactivity. High hydroxyl values indicate that the base can adsorb water more readily and is therefore less suitable for formulations containing drugs that are easily hydrolysed [33]. Hydroxyl values are related to hydrophilic properties which in turn can modify both release and absorption rates of drugs from bases. Bases with high hydroxyl values produce irritancy and have longer solidification times, but give products with better release from moulds and improved elasticity to counteract the risk of brittleness incurred with a rapid cooling rate [8,33].

The large range of suppository bases available has reduced the incidence of chemical reaction between the hard fats and drugs. For example, the risk of hydrolysis of aspirin may be reduced by the use of a base with a low hydroxyl value and additionally by minimization of the water content of both base and aspirin [33].

The semi-synthetic suppository bases display varying degrees of solubility in certain solvents. They are practically insoluble in water and ethanol (90%), but are freely soluble in carbon tetrachloride,

chloroform, toluene and xylene. Solubility in ether and petroleum spirit varies between grades from slightly soluble to very soluble [33].

Hard fat bases are superior to theobroma oil in that they do not exhibit polymorphism and their solidification is not affected by overheating [8,26,32]. Hard fat bases are resistant to oxidation and hydrolysis. Peroxide values are a measure of the resistance of the base to oxidation, and are a guide to the onset of rancidity [33]. Water content is usually low and deterioration due to the absorption of water is infrequent [26,33]. Because they usually contain a portion of partial glycerides some of which, for example glyceryl monostearate, are water/oil emulsifying agents, their emulsifying and water absorbing capacities are good [8,26].

Mould lubrication is unnecessary since these bases show marked contraction on cooling [8,26]. A tendency to fracture due to brittleness on cooling when poured into chilled moulds can be overcome by including 0.05% of the surfactant polysorbate 80 [8,26].

On prolonged storage, semi-synthetic suppository bases have been shown to be subject to hardening and lengthening of the melting time [8]. The degree of hardening may be controlled by storage in a cold place, but melting characteristics, hardness and drug release profiles alter with time, and the melting point may rise by 0.5°C after storage for several months [33]. Due to the complexity of bases it is difficult to elucidate the mechanisms which induce these changes on ageing.

2.1.3.3 MISCELLANEOUS BASES

These include chemical or physical mixtures of the water-soluble or water-miscible materials and contain mainly non-ionic surfactants, either alone or mixed with vegetable oils or waxy solids. Some are preformed emulsions, generally of the water in oil (w/o) type or they may be capable of dispersing in aqueous fluids. The most frequently used surfactants are the polysorbates, polyoxyl stearates and sorbitan fatty acid esters. These bases can be handled at high temperatures, are non-toxic and do not support microbial growth. Hydrophilic bases are compatible with a wide range of drugs, but have a potential for interaction because of their surfactant content. Such interactions may increase or decrease the rate or extent of drug absorption [8].

2.1.4 SELECTION OF A SUPPOSITORY BASE

The release and absorption rates of drugs from different bases is dependent on the nature of the base. Theobroma oil melts quickly at body temperature, but since the resulting oil is insoluble with the body fluids, fat-soluble drugs will remain in the oil and have little tendency to partition into the aqueous physiological fluids. In the case of water-soluble drugs incorporated into theobroma oil or semisynthetic fats, the reverse is generally true, and the drugs will partition into the aqueous rectal fluid more readily. Fat-soluble drugs seem to be better released from bases of glycerol-gelatin or macrogols, both of which dissolve slowly in body fluids. When irritation or inflammation is to be relieved, theobroma oil appears to be the superior base because of its inherent emollient or soothing, spreading action [29].

Selection of a suitable base cannot be made in the absence of knowledge of the physicochemical properties and intrinsic thermodynamic activity of the drug substance which is to be incorporated into the suppository. Other drug-related factors that can affect release and subsequent absorption which must be considered include the particle size distribution of insoluble solids, the oil-water partition coefficient and the dissociation constant. The displacement value should also be known, as well as the ratio of drug to base.

Properties of the suppository base which may or may not be modified by the drug, or which may influence drug release are the melting characteristics, chemical reactivity and rheology [33]. Most manufacturers market a series of grades with slightly different melting point ranges and degrees of hardness. Fatty suppository bases intended for systemic use should liquefy at just below body temperature whereas softening or dispersion may be adequate for suppositories intended for local action or modified release. High melting point bases may be indicated for fat soluble drugs that tend to depress the melting point, and for suppositories used in warm climates [26,33]. Drugs which dissolve in the base when hot may create problems if they deposit as crystals of different form or increased size on cooling or storage [33]. Low melting point bases, particularly those which melt to liquids of low viscosity, can be of value when large volumes of insoluble solids are to be incorporated [26,33], although there is a risk of sedimentation in such instances.

An important factor during processing is the time required for setting. This is affected by the temperature difference between melting and solidification points [33]. Semi-synthetic bases are more fluid than theobroma when melted so thickeners such as magnesium stearate or bentonite may

be added to reduce sedimentation [8,26]. However this is seldom a problem since the difference between melting and setting points is small, seldom over 3°C, a prime requirement for the efficient manufacture of suppositories. Hence they set relatively quickly, the risk of sedimentation is low and they are easier to administer [8,26,32]. When the setting point of a base is below the melting point, the suppositories soften quickly when handled and become too slippery to administer [26].

The rheology of a suppository base may influence drug release, and may itself be influenced by the addition of active ingredients. The viscosity of molten base can affect the uniform distribution of suspended solids during manufacture and it may also influence the release and absorption of the drug in the rectum [33]. Reduction in particle size of insoluble solids is the method used to minimize sedimentation of drug during manufacture.

Additives are sometimes included to modify rheological properties and to maintain homogeneity, but their effect on drug release should first be assessed. Release from a base in which the viscosity has been enhanced by an added thickening agent may be variable and related to the aqueous solubility of the drug itself [33].

2.2 FORMULATION OF AMOXYCILLIN SUPPOSITORIES

2.2.1 DESCRIPTION OF SUPPOSITORY BASES USED

The bases used in this study were *Novata BD* (Henkel, RSA), *Novata 299* (Henkel, RSA), *Witepsol W35* (Hüls, RSA) and *Suppocire A32* (Hüls, RSA). Theobroma oil (Elabtech, RSA) was used as a well known base for comparative purposes. Products were used as received from the manufacturer or distributor with no further purification or adjunct addition. The characteristics of these bases are listed in Table 2.2 [33].

The bases each differed slightly in terms of physicochemical properties, widening the scope of options for a base suitable for formulation with amoxycillin trihydrate in a paediatric suppository.

Amoxycillin trihydrate, compacted (Clinimed, East London, RSA), the commonly used oral form of the drug, was used in the formulation of the amoxycillin suppositories.

Table 2.2 Characteristics of suppository bases used in investigations

Parameter	THEOBROMA	*Novata BD*	*Novata 299*	*Suppocire A32*	*Witepsol W35*
ACID VALUE	<4	≤0.3	≤0.3	≤0.5	≤0.3
HYDROXYL VALUE	-	≤15	≤5	32 (20-30)	40-50
IODINE VALUE	35-40	≤3	≤3	≤2	≤3
MELTING POINT	30-36	33.5-35.5	33.5-35.5	35-36.5	33.3-35.5
SAPONIFICATION POINT	188-196	230-245	253-250	225-245	225-235
SOLIDIFICATION POINT	-	30-32	31.5-33.5	-	27-32

2.2.2 MANUFACTURE OF SUPPOSITORIES

The volume of a suppository from a particular mould is uniform but its weight will vary because the densities of medicaments usually differ from the density of the base with which the mould was calibrated [26]. To prepare suppositories accurately, allowance must be made for the change in the density of the mass due to added medicaments. The most convenient way of making this allowance is to use the displacement value, defined as the number of parts by weight of the medicament that displaces one part by weight of the base.

Displacement values of amoxycillin for the various suppository bases were determined [26]. Six suppositories of base only were prepared and weighed (*a* mg) and six suppositories containing *x*% of medicament were prepared and weighed (*b* mg). The amount of base (*c* mg) and medicament (*d* mg) in the 6 suppositories was calculated.

$$c = (100-x)/100.b$$

$$d = x/100.b$$

The weight of base displaced by *d* mg of medicament was equal to *(a-c)* mg.

The amoxycillin trihydrate powder was size reduced. The mass of base required was calculated using the displacement values and the suppository base was heated to 60°C to melt. The suppositories were produced manually on a small scale by homogenising 250 mg amoxycillin base (290 mg of the trihydrate) per suppository into the melted suppository base. The melt was poured

into 1 g stainless steel moulds, and the suppositories were left to set at room temperature.

The suppositories were stored in a dark cupboard at room temperature (21 °C) until required. Fresh suppositories were manufactured prior to the *in vitro* dissolution release experiments and for the purposes of the *in vivo* pilot study.

CHAPTER THREE
THE *IN VITRO* ANALYSIS OF AMOXYCILLIN

3.1 INTRODUCTION

The quantitative analysis of amoxycillin and other ß-lactam antibiotics has been accomplished using a variety of techniques including microbiological assays [1,2,18], immunoassays [7,34], chemical assays such as the iodometric assay [35] or the hydroxylamine assay [35], spectrophotometry [7,36,37] and high performance liquid chromatography (HPLC) [16,17,34-36,38-41]. HPLC is superior with respect to specificity, rapidity of analysis, precision and simultaneous analysis of compounds.

HPLC has been used for the detection of amoxycillin in biological samples [42,43] and the separation of amoxycillin from other drugs [38,44]. A number of reports in the literature refer to its use in the determination of amoxycillin in pharmaceutical dosage forms [17,36] and it has been widely used by manufacturers for the assay of amoxycillin in bulk raw material.

A summary of techniques used for the analysis of amoxycillin in pharmaceutical dosage forms and raw materials follows.

3.1.1 THE ANALYSIS OF AMOXYCILLIN IN PHARMACEUTICAL DOSAGE FORMS AND RAW MATERIAL

3.1.1.1 REQUIREMENTS OF A SUITABLE METHOD

A suitable analytical technique for amoxycillin should be able to quantify the drug and indicate stability of the compound as a raw material and in the dosage form. The ideal assay should be precise, accurate, sensitive, rapid and specific with regard to metabolites and coadministered drugs. It should also be capable of processing small sample volumes, be inexpensive and simple to perform.

3.1.1.2 MICROBIOLOGICAL ANALYSIS

The agar diffusion plate and turbidometric assay have traditionally been used for routine

monitoring of amoxycillin and other antibiotics [1,2,7,18,34,35]. These microbiological assays are versatile, simple, inexpensive and provide high capacity throughput.

The potency of a sample of an antibiotic is determined by comparing the dose that inhibits the growth of a suitable susceptible micro-organism with the dose of a standard preparation of that antibiotic that produces the same degree of inhibition [7]. However the lowest detectable concentration is only 0.1 to 1 μg/mL in most microbiological assays and this sensitivity is inadequate for pharmacokinetic investigations [24]. Furthermore microbiological assay is relative rather than absolute, lacks accuracy and is not as reproducible as HPLC, immunoassays or other chemical methods. Microbiological assay has a long turn-around time of 4 to 8 hours before the desired information can be obtained. These assays lack specificity as the presence of other antimicrobial agents interferes with the results of the test. This technique cannot differentiate between microbiologically active metabolites and drug.

Microbiological assays are thus generally reserved for measuring the potency of those antibiotics which, because of their complex composition, cannot be measured adequately by chemical or physical means [35]. HPLC has virtually replaced all microbiological techniques, but these methods are still of value if the structure of an antibiotic is not fully known and no realistic choice of a chemico-physical method can be made [35]. Antibiotics assayed by biological techniques include tetracyclines, aminoglycosides and macrolides [7,35].

3.1.1.3 IMMUNOASSAY

The immunoassay technique is less frequently employed to measure antibiotic levels. Immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies and are used to detect or quantify either the antigens or antibodies in these complexes [35].

The amount of complex formed is measured by a variety of techniques which include the use of a labelled or an unlabelled antigen or antibody. Because antigen-antibody reactions are stoichiometric, the determination of either free or bound labelled antigen results in a direct calculation of the antibiotic level [34]. Labelling immunoassay techniques may use either a specific radioactive isotope, or a non-isotopic molecule as the label. Radioimmune assays are sensitive, specific, accurate and require only a small volume of sample but require the use of radioactive

isotopes which in turn need special precautions. Non-isotopic immune assays such as enzyme immunoassays (eg ELISA) or immunoelectrophoretic methods have the same advantages as isotopic immune assays, are more rapid and do not use radioactive materials. The use of immunoassay is however limited, as commercial tests are only available for a few antibiotics and few laboratories have the necessary expertise to raise their own antisera or prepare labelled antibiotics. Immunoassays are essentially applied to the quantitation of aminoglycosides and vancomycin [34].

3.1.1.4 CHEMICAL ASSAY

The official compendia specify methods for the analysis of amoxycillin in pharmaceutical preparations which include chemical, spectrophotometric and HPLC techniques. The iodometric assay is provided by the United States Pharmacopoeia (USP) [35] for the assay of most pharmacopoeial penicillin antibiotics, including amoxycillin. The British Pharmacopoeia (BP) [7] assay of amoxycillin sodium and amoxycillin trihydrate in bulk is based on a potentiometric titration following reaction with mercury nitrate.

3.1.1.5 ULTRAVIOLET SPECTROPHOTOMETRIC ANALYSIS

The BP [7] adopts a spectrophotometric method of assay for amoxycillin in pharmaceutical dosage forms that involves complexation of the drug with an imidazole-mercury reagent and measurement of absorbance of the complex at 325 nm [7]. This colorimetric method described by the BP was adopted from the procedure developed by Bundgaard and Ilver [37]. It measures penicillenic acid mercuric mercaptides of the penicillins formed in quantitative yield in an imidazole and mercuric chloride solution. This method permits detection of penicillins at a 0.5 μg/mL level, is specific and can be used to assess stability since the intact penicillin is required for penicillenic acid formation.

The hydroxylamine assay with spectrophotometric detection at 480 nm [35] may be used as a method of analysis of antibiotics such as cephalosporins and penicillins that possess a ß-lactam structure.

An alternative spectrophotometric method has been proposed by Buurs and Bundgaard [45], for the quantitative determination of ampicillin, amoxycillin and cyclacillin. The method involves the conversion of the aminopenicillins to the corresponding piperazine-2,5-dione derivatives by treatment with a diethanolamine-zinc ion solution, and subsequent treatment of these derivatives

with sodium hydroxide to give a highly absorbing compound with λ_{max} at 322 nm. This method is free of interference from degradation products, and was used to assess the stability of aminopenicillins.

3.1.1.6 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography (HPLC) which was developed in the 1960's has made an important contribution to the analysis of antibiotics and is the chromatographic procedure primarily used for the quantitation of antibiotics as it is sensitive, selective, accurate and rapid. The versatility of HPLC and its application to an enormous variety of compounds have allowed its use in clinical laboratories for the monitoring of a number of therapeutic agents. HPLC is also extensively used to quantify drugs in pharmaceutical formulations and is a powerful tool used in the development of new drugs, particularly for pharmacokinetic investigations and metabolism studies as its selectivity permits separation of drug, prodrug, metabolites and closely related compounds [34,36,38]. Unlike the responses in microbiological assays, those in a validated HPLC assay are not subject to random error, thus confirmation of the validity of each estimate is thought to be unnecessary [46]. A reversed phase HPLC method with UV detection at 230 nm is specified by the USP for determination of amoxycillin in bulk raw material and in pharmaceutical preparations [35].

To confirm the supremacy of HPLC as an analytical technique for penicillin antibiotics, several pharmacopoeial methods and reversed phase HPLC were compared for three penicillins; phenoxymethyl penicillin potassium, benzylpenicillin sodium and ampicillin trihydrate [47]. Of the six methods investigated, which included a microbiological test, an iodometric test, two spectrophotometric tests, mercury titration and HPLC, the HPLC assay was superior in terms of selectivity, reproducibility and analytical time requirement [47].

The quantitative determination of penicillins, their degradation products and impurities is one of the more difficult problems in pharmaceutical analysis, because of the need to separate the parent compounds from precursors, impurities and various degradation products and the fact that they are relatively unstable in aqueous solutions. Fong *et al* [38] developed an HPLC method for the determination of amoxycillin as well as degradation products such as the penicilloic acid, *p*-hydroxyphenylglycine, 6-aminopenicillanic acid and several unidentified materials present in an amoxycillin capsule.

Amoxycillin and its decomposition products, amoxylloates, amoxycillin oligomers and amoxycillin piperazine-2,5-dione, have been separated by reversed phase HPLC with gradient elution [16,17]. The results of a comparison of HPLC with mercurimetric titration corresponded well for amoxycillin trihydrate, which has the penicilloate as the main impurity [17].

Five isocratic HPLC methods were examined by Zhu *et al* [48] for the separation of amoxycillin and its related substances on C_{18} and C_8 columns. These methods included two applications reported in the literature [36], a method from the USP [35] and two methods developed by manufacturers of amoxycillin. Of these methods, the USP method was the most selective, and based on the USP method a gradient method was developed for the analysis of amoxycillin and related substances.

As the choice of suitable conditions for an HPLC procedure is governed by the polarity and ionizable groups of the drug molecule, Huang *et al* [44] studied the separation of amoxycillin and four other ß-lactam antibiotics to investigate their retention behaviour on C_{18} and phenyl columns. In the absence of ion-pairing agents the retention times were shortest between pH 4 and 6. The addition of tetraethylammonium acetate (TEA), an ion pairing agent, to the mobile phase did not result in significant ion pair formation, except at pH values higher than 5.5. A strong ion-pairing effect was noted at pH values higher than 6 when tetrabutylammonium phosphate (TBA) was added to the mobile phase, and the retention was decreased at pH values lower than 4. In contrast, heptane sulphonic acid (HSA) showed an ion-pair retention effect at pH values lower than 5.

The molecular structures and pK_a values were used to account for the retention behaviour of amoxycillin and the other ß-lactam antibiotics in various mobile phases [44]. Amoxycillin has a carboxylic acid group on the ß-lactam ring with a pK_a value of 2.4 and an amino function with pK_a of 7.4 [4]. In addition amoxycillin has a phenolic substitution with a pK_a of 9.6, as well as higher polarity than other similar ß-lactam antibiotics. Amoxycillin shows a V-shaped plot of capacity factor versus pH on a C_{18} column [44]. Amoxycillin is totally ionized between pH 4 and 6 and shows its shortest retention times in this range [43]. With a pH approaching 2.4 the carboxylic acid is only half ionized, so the retention time increases significantly at pH values below this. Similarly as the pH approaches 7.4 the amino group is only half ionized leading to increased retention times [44].

3.2 METHOD DEVELOPMENT

Previously published methods, listed in Table 3.1, were referred to for development of a suitable HPLC method. However several problems were encountered when attempting to duplicate methods reported in the literature. It was often difficult to reproduce results due to instrumental variation or the unavailability of specific reagents or analytical columns. A further problem encountered which made it difficult to duplicate methods, was incomplete reporting of the experimental conditions, column temperature, the way in which the mobile phase was prepared and type and concentration of buffer used. Method validation procedures were occasionally not performed or were inadequately dealt with.

Table 3.1 HPLC methods used for *in vitro* analysis of amoxycillin

Ref	Stationary Phase	Mobile Phase	λ	Internal Standard
16,17	C_8	Gradient elution, methanol, 0.2 M potassium phosphate buffer pH 7, water	274	sulphadimidine
35	C_{18}	Acetonitrile:potassium phosphate buffer pH 5, 4:96	230	-
36	C_8	Methanol:phosphate buffer pH 5, 6:94	254	phenoxyacetic acid
38	C_{18}	Gradient elution, phosphate buffer pH 5.9, methanol, acetonitrile	220	-
39	C_8	Methanol:Sörensen buffer pH 7, 38:62	240	phenoxymethyl penicillin potassium
40	C_{18}	Methanol:phosphate buffer:water, 15:1:84	235	-
41	C_{18}	0.05 M potassium phosphate buffer pH 5.9	228	-
48	C_{18}	0.02 M phosphate buffer pH 5:methanol, 93:7 or 0.1 M phosphate buffer pH 4.5:methanol, 95:5	230	-

Using the details listed in Table 3.1 as guidelines, as well as the knowledge of the physicochemical properties of amoxycillin trihydrate and the variables of an HPLC system which can be modified, various columns and mobile phases were investigated in order to optimize the analysis of amoxycillin trihydrate using the HPLC system.

3.2.1 EXPERIMENTAL

3.2.1.1 REAGENTS

All the chemicals used were at least of analytical reagent quality. Methanol (MeOH) was distilled-in-glass UV grade (Burdick and Jackson Laboratories, Muskegon, Michegan, USA) and HiPerSolv® acetonitrile (AcN) (BDH Laboratory Supplies, Poole, England) was of HPLC grade. Amoxycillin trihydrate, compacted (batch 94154303) was donated by Clinimed (East London, RSA). Salicylic acid, crystalline chloramphenicol and crystalline furosemide were obtained from Sigma Chemical Company (Midrand, RSA). Benzoic acid was obtained from E Merck (Johannesburg, RSA) and sulphadimidine from Maybaker (Port Elizabeth, RSA). Sodium hydroxide pellets and phosphoric acid (85%) were from Holpro Analytics (Pty) Ltd (Johannesburg, RSA).

Water used for buffer preparation, extraction and chromatography (HPLC grade) was purified by a reverse osmosis Milli-RO 15 Water Purification System (Millipore, Bedford, MA, USA) that consisted of a Super-C® carbon cartridge, two Ion-X® ion exchange cartridges and an Oganex-Q® cartridge. The water was filtered through a 0.22 μm Millipak® stack filter prior to use.

3.2.1.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM

Two modular HPLC systems, System A and System B, were used for the *in vitro* analysis of amoxycillin.

SYSTEM A

A model M 6000A Solvent Delivery System (Waters Associates, Milford, MA, USA), a Rheodyne injector, Model 7125 (Rheodyne, California, USA), with a Pressure Lok® 100μl Syringe, Model 160225RN (Dynatech Precision Sampling Corporation, Louisiana, USA) and a Hewlett Packard 1040A Diode-Array HPLC-Detection System (Hewlett Packard Company, CA, USA) were initially used to develop an analytical method. Various reversed phase columns, discussed in Section 3.3.3.4 were used in this system at ambient temperature.

SYSTEM B

A model M 6000A Solvent Delivery System (Waters Associates, Milford, MA, USA) with a Waters Intelligent Sample Processor (WISP) Model 710B Automated Sample Injection System (Milford, MA, USA), a Waters M 490 Programmable Multiwavelength Detector (Waters Associates, MA, USA) and model 561-3002 strip-chart recorder (Perkin-Elmer Corp, Norwalk, Conn, USA) were used for the *in vitro* analysis of amoxycillin. Separation was achieved on a Nova-Pak® C_{18} 60 Å 4 μm 3.9x150 mm HPLC cartridge column (Waters Associates, Milford, MA, USA). An Upchurch guard column kit, part no 1603, with Perisorb RP-18 pellicular packing material (C603) (Upchurch Scientific, WA, USA) was used. An in-line mobile phase filter, model A340 (Upchurch Scientific, WA, USA) was installed between the pump and the autosampler.

3.2.1.3 ULTRAVIOLET DETECTION

The most frequently used detector in the HPLC analysis of antibiotics is the fixed or variable ultraviolet (UV) photometer [49]. Penicillins do not have specific chromophores and eluents must be monitored at low wavelengths where many endogenous compounds interfere [34]. Most of the solvents used in HPLC have broad windows in the UV-visible region, making them compatible with the UV detectors even at low wavelengths.

A Hewlett Packard 1040A Diode-Array HPLC-Detection System with accessories was used for method development. Diode-array detection is advantageous for analytical method development because it allows simultaneous monitoring at several wavelengths and has the ability to acquire spectral information without stopping flow. Higher sensitivity was achieved because of the variable bandwidth option which allowed the optimization of signal to noise ratios in relation to wavelength as well as time.

The Millipore Waters M 490 Programmable Multiwavelength Detector was then used in the stability and dissolution studies once a method had been developed.

3.2.1.4 COLUMN SELECTION

Each stationary phase imparts a unique selectivity towards sample components. The choice of an analytical column is based on physical properties of the analyte, such as molecular weight,

solubility and ionic character. Strongly polar substituents (alkali, acids or ionizable groups) may require ion-exchange chromatography, while weakly polar substituents (alkyl groups, halogens) may be analyzed by reversed phase partition chromatography, and substituents of intermediary polarity can be analyzed by either partition or adsorption chromatography. A large number of HPLC methods use reversed phase partitioning, possibly because of the great stability and ease of use of non-polar coated silica (C_2, C_8, C_{18}) stationary phase [49]. Moreover, relatively polar antibiotics may be analyzed on these stationary phases either by ion pairing chromatography or by suppression of ionization.

The retention of a drug on the HPLC column is also a function of column packing and column dimensions. The smaller the microparticle, the better the resolution and sensitivity. The 10 μm particles provide adequate resolution for most analytical separations, while particles of 5 μm and smaller are used for more demanding tasks. Smaller particles sizes give more theoretical plates and better sensitivity, but higher backpressure than the larger particles. The number of theoretical plates is an indication of bandspreading. The sample capacity of a column increases with the volume of the stationary phase, the column length and the column internal diameter. Different brands of C_{18} and C_8 columns have very different selectivities and retention characteristics that often cannot be predicted accurately by examining the specifications supplied by manufacturers, necessitating in-house method validation [50].

Three types of analytical columns were compared to determine which provided the most favourable retention of amoxycillin during the development of the HPLC method. The columns used were a C_8 column (4.5x250 mm, custom packed in our laboratory with Techsil® C_8), a C_{18} cartridge column (Nova-Pak®) and a C_{18} end-capped column (Nova-Pak®).

3.2.1.5 MOBILE PHASE SELECTION AND PREPARATION

The mobile phase moves the sample components through the chromatographic column. In addition the mobile phase interacts with the solute molecules and often the stationary phase itself. It is those solute-mobile phase-stationary phase interactions that make HPLC a powerful separation technique. Alkyl bonded stationary phases are essentially used with buffer and methanol or acetonitrile as mobile phases. The organic modifier content in the mobile phase depends on the polarity of the drug being investigated and on the type of the reversed phase packing. For any given stationary phase, a strong solvent will cause the solute to partition into the mobile phase and consequently

the retention time will be short. A weak solvent will cause the solute to favour the stationary phase and the retention time will be longer. The pH of the buffer component is selected to provide sufficient retention and complete separation, in the case of ionizable drugs. The retention times of weak acids decrease with increasing pH values while those of weak bases increase with increasing pH [51].

The mobile phase should not alter the stationary phase by reacting with it or changing its structure and it should be chemically pure to prevent trace impurities collecting on the column. The presence of UV-active impurities is undesirable therefore HPLC or spectroscopic quality solvents must be used. The solvent should exhibit a low absorbance at the operating wavelength. Water and acetonitrile can be used at wavelengths down to 195 nm. The solvent viscosity must be taken into account when selecting a mobile phase. As columns age and particles accumulate on the inlet frit, backpressure will increase. If the pressure is initially high because of the solvent viscosity, the upper pressure limit of the chromatographic method will be reached as columns age. If a lower viscosity solvent is selected this will be avoided and the column will be able to be used for a longer time before the backpressure limit is exceeded.

The mobile phases used in several published HPLC techniques were investigated (Table 3.1). When none of these methods proved suitable without major alterations, an original mobile phase was developed.

The establishment of the mobile phase was conducted with simple aqueous solutions of amoxycillin trihydrate in water, to ensure that a single peak corresponding to the antibiotic was monitored. Retention times from the time of injection, rather than capacity factor were measured, as it was difficult to identify a non-eluting peak or the solvent front. A mobile phase of weak eluotropic strength was used as a starting point (acetonitrile:water, 75:25). The mobile phase was then adjusted to reduce the retention time for the peak of interest to approximately 10 minutes. However, due to the high polarity of amoxycillin, the drug tended to be eluted from these systems more rapidly than desired, therefore further modifications of the mobile phase were considered.

The ways to improve the definitive mobile phase are numerous and include slight variations of pH, an increase or decrease of ionic strength and changes in the amount of organic modifier in the mobile phase. In order to determine an optimum mobile phase system, it was decided to experiment both with organic phases, and various buffer compositions, as well as different ratios

of organic phase to aqueous phase, in separate experiments.

Ionization control by means of a buffer was considered when the drug appeared to be poorly retained with weakly eluting mobile phases. Modifiers of mobile phase pH or ionic strength such as buffers or salts, as well as counter ions influence the mobile phase selectivity. Different pH values and concentrations of salts were tested until a suitable retention time and symmetrical peaks were obtained.

3.2.1.6 GUARD COLUMNS AND PRE-COLUMNS

The use of a guard column (part number 1603, Upchurch Scientific, WA, USA) dry packed with Perisorb RP-18 pellicular packing material (part number C603, Upchurch Scientific, WA, USA) was initiated because both biological samples and samples containing fatty material from molten suppositories were to be injected onto the analytical column. The guard column served to trap particulate matter and high molecular weight constituents such as protein and fat globules, thereby prolonging the life of the analytical column. Samples were loaded onto the analytical column, both with and without the guard column in line, and it was confirmed that the guard column made no significant contribution to dead volume or extra column effects. When the guard column lost efficiency or caused the backpressure in the system to rise when it became blocked, the frits and column packing were replaced.

Since the mobile phase was continuously recycled, an in-line filter (model A430, Upchurch Scientific, WA, USA) was installed between pump and injector in order to protect the system from sample contaminants in the mobile phase. The frit of the in-line filter was replaced if the filter became less efficient or caused the backpressure to rise.

3.2.1.7 INTERNAL STANDARD

Internal standards are needed when an analytical method results in highly variable recovery of the analyte, most often in multi-step preparations. The internal standard compensates for varying injection volumes and day to day instrumental changes [138]. The internal standard should have physicochemical properties and chromatographic behaviour close to those of the drug to be measured [49]. The introduction of an internal standard implies that the entire developed procedure should be suitable for both molecules.

The drugs mentioned in the literature for use as internal standards in the assay of amoxycillin include phenoxymethylpenicillin potassium [39], phenoxyacetic acid [38], hydroflumethiazide [42], benzoic acid [43] and sulphadimidine [16,17]. Chloramphenicol has been used in the assay of benzylpenicillin, and benzyl alcohol in ampicillin assay [131]. Perusal of the literature revealed that furosemide and salicylic acid have absorption spectra similar to that of amoxycillin [5], suggesting that they may exhibit similar chromatographic behaviour.

3.2.1.8 PREPARATION OF STOCK SOLUTIONS

Stock solutions of 1.0 mg/mL amoxycillin were prepared by accurately weighing 116 mg of amoxycillin trihydrate and dissolving this in HPLC grade water, with the aid of the ultrasonic cleaner (model 8845-30, Cole-Parmer Instrument Company, Illanois, USA) and making up to volume with HPLC grade water in a 100 mL volumetric flask.

Stock solutions of 1.0 mg/mL salicylic acid were prepared by accurately weighing 100 mg of salicylic acid and dissolving this in HPLC grade water and making up to volume with HPLC grade water in a 100 mL volumetric flask.

Serial dilutions were made with HPLC grade water to produce the concentrations required.

3.2.2 OPTIMISATION OF CHROMATOGRAPHIC CONDITIONS

3.2.2.1 DETECTOR WAVELENGTH (λ)

Amoxycillin was monitored at 200, 230 and 254 nm using diode array detection (Figure 3.1). From Figure 3.1 it can be seen that a wavelength of approximately 200 nm gives the highest absorptivity, but many endogenous compounds also tend to absorb at this wavelength. It was decided to set the detector wavelength to 230 nm as amoxycillin also displays relatively high absorptivity at this wavelength, as discussed in Section 1.2.5. Some published methods have used alternative wavelengths [16,17,36,38,39], but detection in this region has been employed by several authors [15,40,41], and it was shown to be suitably sensitive for the purposes of this *in vitro* study.

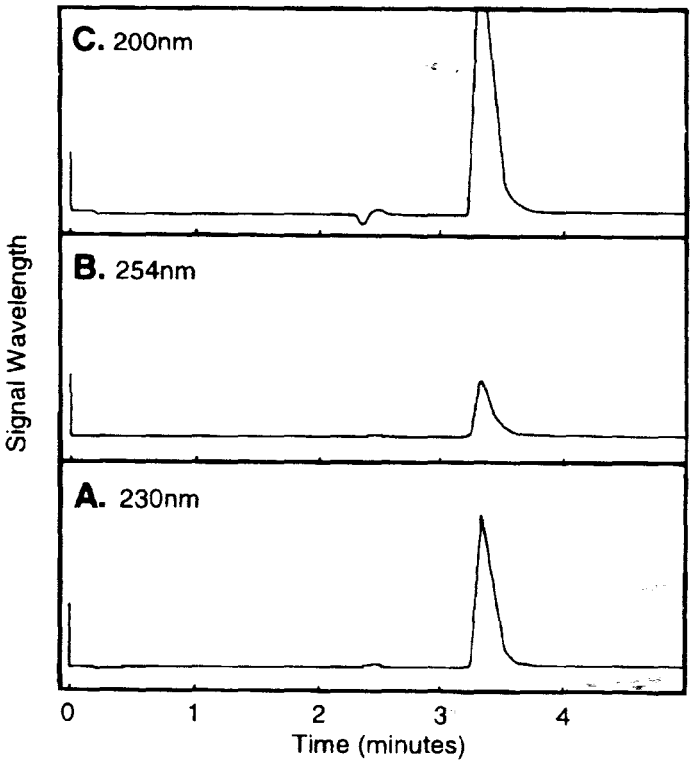


Figure 3.1 Chromatogram of amoxycillin monitored at 200, 230 and 254 nm using diode array detection

3.2.2.2 MOBILE PHASE SELECTION

The effects of different organic modifiers, buffer concentration, pH and ratios of organic to inorganic phase on the retention of amoxycillin on a C_{18} Nova-Pak® column were investigated, and the results are summarized in Table 3.2.

The mobile phase was prepared by pipetting a specific volume of methanol or acetonitrile into a 1 litre volumetric flask and making up to volume with phosphate buffer solution. A 0.05 M phosphoric acid solution was prepared by adding 7 mL phosphoric acid (85%) to HPLC grade water in a 2 litre volumetric flask, then making to volume. Sodium hydroxide pellets were then added to adjust the buffer to the pH required for the mobile phase. The mobile phase was degassed

and filtered through a 0.45μm membrane filter (type HVLP, Millipore Corp., Bedford, MA, USA) prior to use, by aspiration with an Eyela® aspirator (model A-35, Tokyo Rikakikai Co Ltd, Tokyo, Japan).

Table 3.2 Retention time of amoxycillin using various mobile phase combinations

Mobile phase	**Ratio**	**Retention time (min)**
AcN:water	75:25	1.4
AcN:water	50:50	1.6
AcN:water	25:75	1.6
AcN:0.05 M Phosphate buffer pH 2.4	25:75	2.5
AcN:0.05 M Phosphate buffer pH 7	25:75	2.4
AcN:0.05 M Phosphate buffer pH 3	10:90	3.2
AcN:0.05 M Phosphate buffer pH 7	10:90	3.3
AcN:0.05 M Phosphate buffer pH 5	10:90	3.4
MeOH:water	20:80	2.7
MeOH:water	10:90	3.4
MeOH:0.05 M Phosphate buffer pH 3	10:90	5.7
MeOH:0.05 M Phosphate buffer pH 7	10:90	8.2
MeOH:0.05 M Phosphate buffer pH 5	10:90	4.8
MeOH:0.05 M Phosphate buffer pH 3	5:95	11.4
MeOH:0.05 M Phosphate buffer pH 7	5:95	12.0
MeOH:0.05 M Phosphate buffer pH 5	5:95	7.2
MeOH:0.05 M Phosphate buffer pH 7	8:92	8.4
MeOH:0.05 M Phosphate buffer pH 7	6:94	11.1

Altering the ratio of acetonitrile:water did not appear to have a marked effect on retention time whereas decreasing the organic fraction slightly increased the retention time by a few seconds.

Changing the methanol:water ratio made a more noticeable difference, with a change from 2.7 to 3.5 minutes, as the methanol portion was decreased from 20 to 10%, although only two different ratios were monitored. When a buffer was used in the mobile phase, a similar trend was observed, with the methanol:buffer systems showing a longer retention time than acetonitrile:buffer systems

with the same pH. It was decided to concentrate on a methanol:buffer system for this reason. A difference in solubility in these two organic phases may account for this result. Amoxycillin trihydrate is insoluble in acetonitrile, but is soluble 7.5 mg/mL in methanol [6,7].

Since amoxycillin is an amphoteric compound, with pK_a values of 2.4, 7.4 and 9.6, the retention time was best controlled by a buffered system, as proposed by Huang *et al* [44]. A phosphate buffer system was used. The pH values reported are those of the buffer solutions before mixing with the organic phase.

A 10:90 mixture of methanol:0.05 M phosphate buffer (pH 7), gave a retention time of 6.9 minutes. As the fraction of methanol was decreased, an 8:92 mixture, a 6:94 mixture and a 5:95 mixture gave increasing retention times of 8.4 minutes, 11.1 minutes and 12 minutes respectively.

The retention times, for a 10:90 mobile phase mixture, with a buffer pH of 3, 5 and 7 were 5.7, 4.8 and 6.9 minutes respectively. These results supported the findings of Huang *et al*, that when the pH of the mobile phase was close to the pK_a values of amoxycillin, the drug was retained on the column for a longer time, since around these pH values amoxycillin is approximately 50% ionized and is therefore less polar, and tends to be bound to the stationary phase. Between pH 4 and pH 6 amoxycillin is totally ionized and is therefore not well retained, corresponding with the V-shaped curve described by Huang *et al* [44].

The effect of buffer molarity was investigated, using a 10:90 methanol:phosphate buffer pH 7 mobile phase and a Nova-Pak® C_{18} analytical column. The retention times which resulted when buffers of 0.005, 0.025, 0.05 and 0.1 molarity were used are summarized in Table 3.3.

Table 3.3 The effect of buffer molarity on retention time of amoxycillin

Molarity (M)	**Retention Time (min)**
0.005	5.7
0.025	7.3
0.05	8.2
0.1	9.2

The higher the molarity of the phosphoric acid solution used in the buffer, the longer was the retention time of amoxycillin when a 10:90 methanol:phosphate buffer pH 7 mobile phase was used.

3.2.2.3 INTERNAL STANDARD

Several drugs were tested as potential internal standards for the amoxycillin assays which were to be performed. 0.5 mg/mL solutions of benzoic acid, chloramphenicol, sulphadimidine, furosemide and salicylic acid were tested using mobile phases of 10:90 and 5:95 methanol: phosphate buffer, with a buffer pH of either 3, 5 or 7.

Only salicylic acid and benzoic acid were detectable under these conditions. The effect of various methanol:0.05 M phosphate buffer mobile phases on the retention of these compounds on a Nova-Pak® C_{18} column is shown in Table 3.4.

Table 3.4 Retention time of amoxycillin, salicylic acid and benzoic acid

Mobile phase	Retention time (min)		
	Amoxycillin	Salicylic acid	Benzoic acid
5:95 pH 7	12.0	9.1	5.0
5:95 pH 5	7.2	9.8	6.5
5:95 pH 3	11.4	*	*
10:90 pH 7	8.2	6.8	4.2
10:90 pH 5	4.8	6.6	*
10:90 pH 3	10.4	*	*

* No peaks eluted within 20 minutes

As the fraction of organic phase was decreased, the retention times of both amoxycillin and the internal standards under investigation increased. When the ratio of methanol to phosphate buffer was kept constant, at 5:95, and the pH changed, the sequence of retention times changed. At pH 5, the salicylic acid eluted after the amoxycillin, whereas the benzoic acid still eluted earlier. At pH 3 no peaks appeared within 20 minutes for either benzoic acid or salicylic acid. This could possibly be explained by the state of ionization of these drugs; pK_a values are 4.2 for benzoic acid and 3 and 13.4 for salicylic acid. At pH 7 retention times were 12, 9.1, and 5 minutes for

amoxycillin, salicylic acid and benzoic acid respectively. The retention times of the mobile phases with a 10:90 methanol:phosphate buffer combination were slightly lower than the 5:95 combination.

The retention times of benzoic acid under the mobile phase conditions tested, were not suitable for the purposes of this study. Since the salicylic acid peak eluted near to the amoxycillin peak but was well separated from it, under the mobile phase conditions tested, it was chosen as the internal standard for the HPLC method for *in vitro* analysis of amoxycillin.

3.2.2.4 COLUMN CHOICE

A comparison of the reversed phase columns described in Section 3.2.1.4, using the mobile phase combinations listed in Table 3.5, revealed that a C_{18} column retained amoxycillin and salicylic acid to a greater extent than a C_{18} end-capped or a C_8 column. Optimum retention was achieved on a C_{18} column with a methanol:phosphate buffer pH 7 mobile phase.

Table 3.5 Retention time (min) of amoxycillin and salicylic acid on a C_{18}, a C_8 and a C_{18} end capped column

Column	Mobile phase	Amoxycillin	Salicylic acid
C_{18}	50:50 MeOH:water[a]	1.6	1.1
	10:90 MeOH:water[a]	3.4	1.2
	10:90 MeOH:0.05 M Phosphate buffer pH 3	10.4	*
	10:90 MeOH:0.05 M Phosphate buffer pH 5	4.8	6.6
	10:90 MeOH:0.05 M Phosphate buffer pH 7	8.2	6.8
C_8	50:50 MeOH:water[a]	1.4	1.0
	10:90 MeOH:water[a]	2.4	1.4
	10:90 MeOH:0.05 M Phosphate buffer pH 3	4.9	*
	10:90 MeOH:0.05 M Phosphate buffer pH 5	2.4	4.2
	10:90 MeOH:0.05 M Phosphate buffer pH 7	3.9	3.5
C_{18} end capped	50:50 MeOH:water[a]	1.1	0.9
	10:90 MeOH:water[a]	1.7	1.1
	10:90 MeOH:0.05 M Phosphate buffer pH 3	5.9	*
	10:90 MeOH:0.05 M Phosphate buffer pH 5	2.0	6.0
	10:90 MeOH:0.05 M Phosphate buffer pH 7	7.4	5.7

[a] pH of MeOH:water mixtures not measured * no peaks seen in 20 minutes

3.2.2.5 CHROMATOGRAPHIC CONDITIONS

HPLC	System B
Mobile Phase	Methanol:0.05 M phosphate buffer pH 7 (5:95)
Flow Rate	1.0 mL/min
Attenuation	0.05 AUFS
Recorder Input	10 mV
Injection Volume	10 μL
Temperature	Ambient
Retention Time	Amoxycillin 12 minutes
	Salicylic acid 7 minutes

A typical chromatogram showing amoxycillin and the internal standard, using the chromatographic conditions listed above, is depicted in figure 3.2.

3.2.2.6 CONCLUSION

The effects of the parameters involved in HPLC analysis were identified by systematic examination of detector wavelength, mobile phase composition and stationary phase. By optimizing these chromatographic conditions, an HPLC method which provided distinctly resolved peaks, retention times within the required ranges and minimal baseline noise was developed. A suitable internal standard, salicylic acid, was identified.

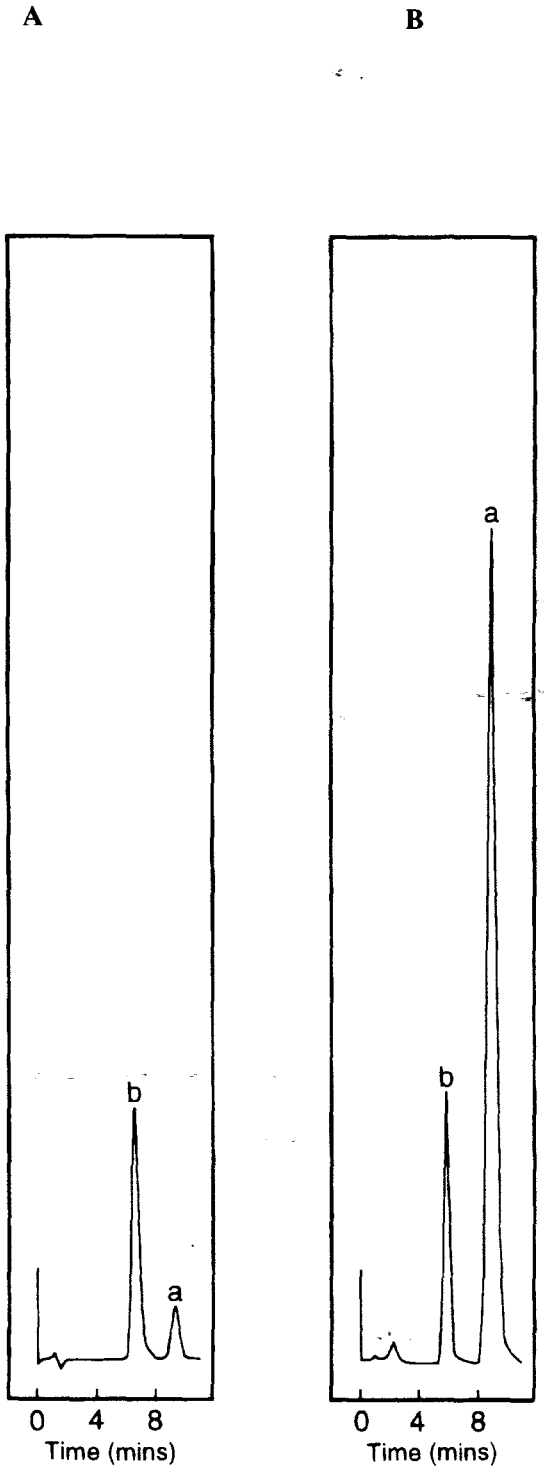


Figure 3.2 Typical chromatograms of low (A) and high (B) concentrations of amoxycillin (a) with salicylic acid (b)

3.3 METHOD VALIDATION AND STABILITY OF AMOXYCILLIN TRIHYDRATE IN AQUEOUS SOLUTION

3.3.1 INTRODUCTION

Following optimization of an HPLC system for the *in vitro* analysis of amoxycillin, it was necessary to validate the method in terms of linearity, accuracy and precision and to determine the limit of quantitation of the method. A series of aqueous samples was processed in order to validate these parameters.

An investigation of stability is also a crucial part of method development. A study was conducted to establish the stability of amoxycillin trihydrate in unbuffered aqueous solution. The results of this study were useful in determining the length of time for which samples could be left in the autosampler during an extended run. The results obtained after storage at room temperature (21°C), in a refrigerator (4°C) and in a freezer (-15°C) allowed a decision to be made on the best way of storing samples that could not be assayed immediately. Samples were stored both with and without internal standard in order to determine whether it was feasible to add internal standard before storage, or if it was necessary to add the internal standard immediately before assay due to reaction between amoxycillin and salicylic acid.

Amoxycillin degrades either by hydrolysis or dimerization depending on the pH and the concentration of a solution (Section 1.3). Hydrolysis is predominant at low concentrations and higher pH values, whereas dimerization occurs more readily at high concentrations in acidic solutions.

3.3.2 EXPERIMENTAL

3.3.2.1 REAGENTS

All reagents have been detailed in Section 3.2.1.1. All the chemicals used were at least of analytical reagent quality.

3.3.2.2 CHROMATOGRAPHIC CONDITIONS

The chromatographic conditions have been described in Section 3.2.2.5.

3.3.2.3 CALIBRATION CURVES

Calibration curves were constructed on the days that samples were assayed. Fresh stock solutions of amoxycillin trihydrate and salicylic acid were prepared as described in Section 3.2.1.8 on each assay day. Concentrations of 0.005, 0.01, 0.02, 0.05, 0.07 and 0.1 mg/mL were obtained by serial dilution of the stock solution of 1.0 mg/mL amoxycillin trihydrate with HPLC grade water. Before making to volume, salicylic acid (1.0 mg/mL) was added to each dilution to give a final internal standard concentration of 0.01 mg/mL. Each calibration sample was assayed six times. The linear regression line is shown in Figure 3.4.

3.3.2.4 LINEARITY

Linearity of the ratio of amoxycillin peak height to internal standard peak height versus concentration, over the concentration range studied was established. The linearity data are tabulated in Table 3.6.

3.3.2.5 PRECISION AND ACCURACY

The precision of the assay was determined by calculating the percent relative standard deviation (RSD) of the peak height ratios of six replicate samples of standards (Table 3.6). Accuracy was assessed at two concentrations from three samples prepared by an independent analyst, by comparing the mean concentrations found with the actual concentrations. Sample concentrations were calculated by interpolation of peak height ratios from the calibration curve. The results are summarized in Table 3.7.

3.3.2.6 LIMIT OF QUANTITATION AND DETECTION

The limit of quantitation was determined by diluting the amoxycillin stock solution until the peak on the HPLC trace was detectable but no longer measurable. The lower limit of quantitation was taken to be twice this value.

3.3.2.7 pH

The pH of solutions of amoxycillin, salicylic acid and a mixture of amoxycillin and salicylic acid was determined. The results are summarized in Table 3.10.

3.3.2.8 STABILITY

An aliquot of the amoxycillin trihydrate stock solution was diluted to 0.005 and 0.1 mg/mL. Salicylic acid (0.01 mg/mL) was included in one batch of both of the dilutions, but was excluded from the other batch. 10 mL aliquots of each solution were set aside for storage at -15°C, 4°C and 21°C in transparent glass screw-cap tubes.

On Day 1, Day 2, Day 3, Day 7 and Day 14 samples were allowed to thaw naturally to room temperature and were assayed in triplicate. In the case of the amoxycillin sample stored without internal standard, salicylic acid was added just prior to the assay procedure.

Fresh calibration curves were made up on each day of sampling. The concentration of drug remaining in solution after storage was calculated from the peak height ratio. The results were evaluated to determine whether there was a significant change in concentration on storage under the various conditions over the 14 day period.

3.3.2.9 STATISTICAL INTERPRETATION OF THE DATA

The statistical procedure used was adapted from the study described by Timm *et al* [52]. In this study a procedure was developed for investigating the stability of drugs based on sound experimental design and the use of a statistical procedure which allowed conclusions to be made concerning stability with an acceptable degree of certainty (95%). The relative difference in response between stored samples and the initial concentration and a 90% confidence interval for the true change in response was calculated. This confidence interval allowed the detection of a pharmacokinetically relevant degradation. It is argued that this approach is superior to stability test procedures based on the Student's *t* test.

The measurements of peak height or peak height ratios, when the internal standard was used, are referred to as the response in the study. For the purpose of the calculations in the statistical

treatment described, it was necessary that the analytical methods used resulted in a relationship between response and concentration that could be described by a linear curve starting at the origin. The linearity results are shown in Tables 3.9 and 3.10 in Section 3.3.3.1.

The measured percentage difference (D) between stored samples and original concentrations was calculated. The true percentage difference in response after storage (Δ), which is equal to the percentage difference in concentration, may differ from D because of the imprecision of the analytical procedure. In other words Δ and D become identical only in the absence of measurement errors. Therefore a 90% confidence interval for Δ was calculated. The true change of response on storage is contained by the lower limit (LL) and the upper limit (UL) of the confidence interval with a probability of 90%. That is, there exists a probability of 5% that $\Delta \leq$ LL and a probability of 5% $\Delta \geq$ UL (Figure 3.3).

This statistical interpretation distinguishes between significant and relevant instability. A change of response during storage is statistically significant if the value $\Delta = 0\%$ lies outside the confidence interval. Three possibilities are considered:

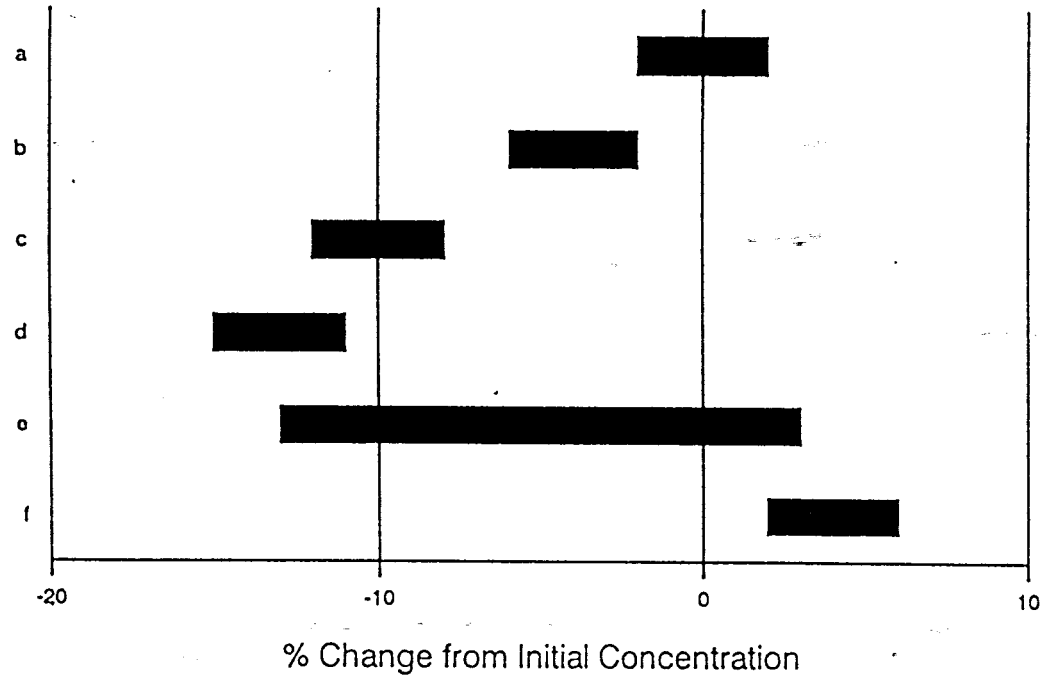
(a) a significant decrease, LL $\leq$ UL $\leq$ 0%,
(b) no significant change, LL $\leq$ 0% $\leq$ UL,
(c) a significant increase, 0% $\leq$ LL $\leq$ UL.

However a significant degradation is only important when it reaches pharmacokinetically relevant proportions. A substance should be denoted as stable only if a relevant degradation can be excluded with high certainty. Timm *et al* considered a degradation of Δ = -10% to be relevant. Thus if Δ = -10% falls within the 90% confidence interval for Δ, the substance is not denoted as stable. There are therefore three additional possibilities:

(a) no relevant decrease, -10% $\leq$ LL $\leq$ UL,
(b) a possibly relevant decrease, LL $\leq$ -10% $\leq$ UL,
(c) a relevant decrease, LL $\leq$ UL $\leq$ -10%.

Figure 3.3 shows the various combinations arising from these definitions. The bars above the axis characterise the ranges of the 90% confidence intervals for the true percentage response difference, Δ, between stored and freshly prepared samples.

The drug is classified as stable in cases (a) and (b), as no relative instability is observed. In case (c) a relevant degradation of 10% or more cannot be excluded with a statistical probability of 95% and in case (d) a significant and relevant decrease of concentration on storage is observable, both cases indicating the compound is not stable according to the definition. Example (e) leads to the decision that the compound is not stable. However the wide confidence interval indicates poor precision. Case (f) indicates that the compound is stable. However the experiments should be repeated because of the possibility of a systematic error [52].



(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 3.3 Key to definitions

3.3.3 RESULTS

3.3.3.1 LINEARITY AND CALIBRATION CURVES

A calibration curve was constructed by linear regression of a plot of peak height ratio of amoxycillin to that of the internal standard versus amoxycillin concentration. The linear regression equation was *y=31.8195x-0.0026* with a correlation coefficient of 0.9998. The calibration curve for aqueous amoxycillin is shown in Figure 3.4.

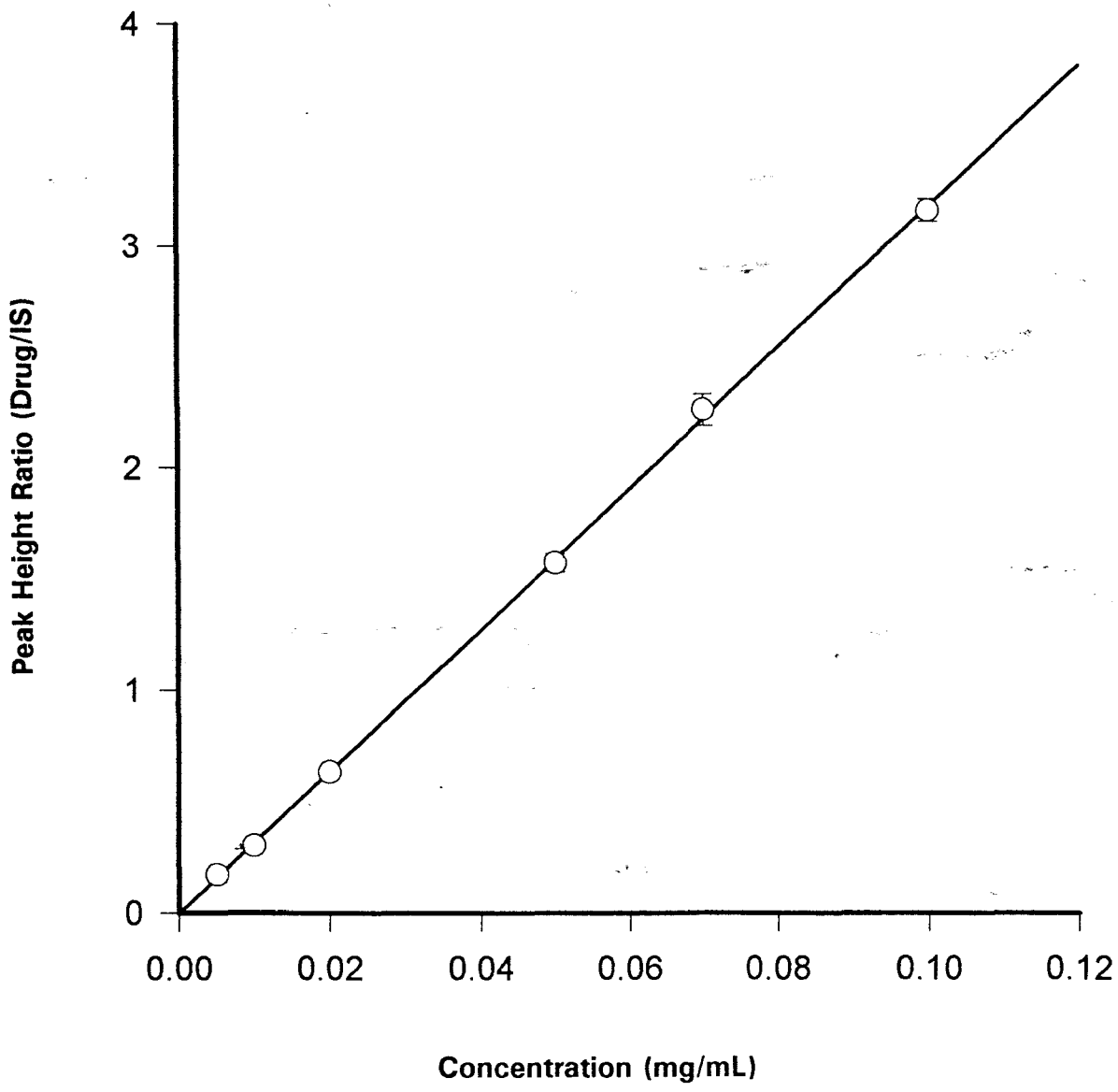


Figure 3.4 Calibration curve for aqueous amoxycillin (y=31.8195x-0.0026, R^2=0.9998)

The validation data for linearity and precision are shown in Table 3.6.

Table 3.6 Linearity and precision data for aqueous amoxycillin solutions

Calibration Standards		
Concentration (μg/mL)	**Peak Height Ratio Mean ± SD (n=6)**	**%RSD**
0.1	3.16 ± 0.05	1.6
0.07	2.26 ± 0.07	3.1
0.05	1.57 ± 0.04	2.5
0.02	0.63 ± 0.02	3.2
0.01	0.30 ± 0.01	3.3
0.005	0.17 ± 0.01	5.9

3.3.3.2 PRECISION AND ACCURACY

Results from the precision study are tabulated in Table 3.6. The method of analysis was found to be reproducible with RSD values less than 6% at all concentrations.

Results from the accuracy study are tabulated in Table 3.7. The percentage bias was calculated as the difference between the calculated concentration and spiked concentration as a percentage of the spiked concentration. The difference between the calculated and spiked concentrations was less than 3% in both instances.

Since the criteria for acceptable precision and accuracy were taken to be $\leq 10\%$ RSD and $\leq 10\%$ bias [132], these results were considered acceptable for the purposes of this study.

Table 3.7 Calculation of spiked concentration for accuracy determination

Spiked Concentration (μg/mL)	**Calculated Concentration (μg/mL), Mean ± SD (n=3)**	**Percentage Bias**
0.035	0.0360 ± 0.0000	2.8%
0.080	0.0799 ± 0.0002	-0.2%

3.3.3.3 LIMIT OF QUANTITATION AND DETECTION

The limit of detection of aqueous amoxycillin was 0.001 mg/mL, determined by a 10 μL injection volume and a sensitivity of 0.05 AUFS. This peak was detectable, but not measurable. The lower limit of quantitation was found to be 0.002 mg/mL with a signal to noise ratio of 3.

3.3.3.4 pH

The pH values of aqueous amoxycillin, salicylic acid and amoxycillin with salicylic acid were recorded and are shown in Table 3.8. All solutions were acidic, and the addition of salicylic acid to the amoxycillin solutions decreased the pH of these solutions slightly, from pH 4.46 to pH 4.12. The implications of the effect of salicylic acid on amoxycillin are discussed in Section 3.3.3.5.

Table 3.8 pH values of aqueous solutions

Solution	pH
Amoxycillin 1.0 mg/mL	4.46
Salicylic acid 1.0 mg/mL	2.50
Amoxycillin with salicylic acid	4.12

3.3.3.5 STABILITY

Calibration curves were constructed in a similar manner to that described in Section 3.3.2.3, on each day of the stability study. The details of the calibration curves used for the determination of the concentrations of aqueous solutions of amoxycillin stored both with and with out salicylic acid are shown in Tables 3.9 and 3.10.

Table 3.9 Linearity data for amoxycillin solutions stored with internal standard

Day	Slope	Y-intercept	Correlation Coefficient
0	31.8195	-0.0026	0.99983
1	34.6255	-0.0238	0.99965
2	34.1911	-0.0106	0.99983
3	34.1764	-0.0101	0.99988
7	38.5732	0.0026	0.99993
14	342.5597	-0.0205	0.98560

Table 3.10 Linearity data for amoxycillin solutions stored without internal standard

Day	Slope	Y-intercept	Correlation Coefficient
0	38.8125	-0.0106	0.99972
1	34.8342	0.0354	0.99950
2	34.8234	0.0310	0.99934
3	42.5597	-0.0205	0.98560
7	34.6579	-0.0199	0.99934
14	33.6808	0.1650	0.99986

The stability of the aqueous amoxycillin samples of high and low concentration, stored at 21°C, 4°C and -15°C for two weeks without an internal standard is indicated by the change in concentration of these solutions, as shown in Tables 3.11 and 3.12.

Table 3.11 Stability data for aqueous amoxycillin 0.005 mg/mL stored over a 14 day period without internal standard

Day	Concentration (mg/mL), Mean ± SD (n=3)		
	21°C	4°C	-15°C
0	0.0052 ± 0.0001	0.0052 ± 0.0001	0.0052 ± 0.0001
1	0.0051 ± 0.0000	0.0058 ± 0.0006	0.0051 ± 0.0000
2	0.0051 ± 0.0000	0.0051 ± 0.0000	0.0051 ± 0.0000
3	0.0056 ± 0.0000	0.0045 ± 0.0000	0.0056 ± 0.0000
7	0.0043 ± 0.0000	0.0043 ± 0.0000	0.0053 ± 0.0000
14	0.0012 ± 0.0000	0.0028 ± 0.0010	0.0034 ± 0.0000

Table 3.12 Stability data for aqueous amoxycillin 0.1 mg/mL stored over a 14 day period without internal standard

Day	Concentration (mg/mL), Mean ± SD (n=3)		
	21°C	4°C	-15°C
0	0.1028 ± 0.0000	0.1028 ± 0.0000	0.1028 ± 0.0000
1	0.1007 ± 0.0000	0.1004 ± 0.0006	0.1024 ± 0.0006
2	0.0900 ± 0.0012	0.0903 ± 0.0010	0.0979 ± 0.0017
3	0.1006 ± 0.0000	0.0981 ± 0.0006	0.0914 ± 0.0027
7	0.0952 ± 0.0000	0.0963 ± 0.0000	0.0843 ± 0.0006
14	0.0827 ± 0.0000	0.0925 ± 0.0000	0.0716 ± 0.0006

The stability of the aqueous amoxycillin solutions of high and low concentration, stored at 21°C, 4°C and -15°C for two weeks with an internal standard is demonstrated by the change in concentration of these solutions, as shown in Tables 3.13 and 3.14.

Table 3.13 Stability data for amoxycillin 0.005 mg/mL stored for a 14 day period with internal standard

Day	Concentration (mg/mL), Mean ± SD (n=3)		
	21°C	4°C	-15°C
0	0.0051 ± 0.0000	0.0051 ± 0.0000	0.0051 ± 0.0000
1	0.0053 ± 0.0000	0.0056 ± 0.0005	0.0053 ± 0.0000
2	0.0045 ± 0.0000	0.0050 ± 0.0005	0.0053 ± 0.0000
3	0.0054 ± 0.0000	0.0051 ± 0.0005	0.0054 ± 0.0000
7	0.0051 ± 0.0000	0.0042 ± 0.0000	0.0042 ± 0.0000
14	0.0021 ± 0.0006	0.0045 ± 0.0000	0.0049 ± 0.0006

Table 3.14 Stability data for amoxycillin 0.1 mg/mL stored for a 14 day period with internal standard

Day	Concentration (mg/mL), Mean ± SD (n=3)		
	21°C	4°C	-15°C
0	0.1001 ± 0.0005	0.1001 ± 0.0005	0.1001 ± 0.0005
1	0.1052 ± 0.0000	0.1052 ± 0.0000	0.1018 ± 0.0000
2	0.0983 ± 0.0006	0.0997 ± 0.0006	0.0993 ± 0.0006
3	0.1017 ± 0.0058	0.0983 ± 0.0006	0.0970 ± 0.0000
7	0.0998 ± 0.0024	0.0986 ± 0.0002	0.0850 ± 0.0000
14	0.0657 ± 0.0012	0.0960 ± 0.0006	0.0674 ± 0.0007

The percent of drug remaining was calculated from the measured percentage difference in response (Table 3.15 and 3.16). The results which were calculated as greater than 100 % were as a result of the differences in the daily calibration curves.

Table 3.15 Percent amoxycillin remaining in solution after storage for 14 days without internal standard

Day	21°C		4°C		-15°C	
	0.005 mg/mL	**0.1 mg/mL**	**0.005 mg/mL**	**0.1 mg/mL**	**0.005 mg/mL**	**0.1 mg/mL**
0	100%	100%	100%	100%	100%	100%
1	98%	98%	108%	98%	96%	100%
2	98%	88%	96%	88%	96%	95%
3	108%	98%	85%	95%	106%	89%
7	83%	93%	81%	94%	100%	82%
14	23%	80%	49%	90%	64%	70%

Table 3.16 Percent amoxycillin remaining in solution after storage for 14 days with internal standard

Day	21°C		4°C		-15°C	
	0.005 mg/mL	**0.1 mg/mL**	**0.005 mg/mL**	**0.1 mg/mL**	**0.005 mg/mL**	**0.1 mg/mL**
0	100%	100%	100%	100%	100%	100%
1	104%	105%	109%	105%	104%	102%
2	88%	98%	98%	100%	104%	99%
3	106%	101%	100%	98%	106%	97%
7	100%	100%	82%	99%	82%	85%
14	39%	66%	88%	96%	94%	67%

The fact that an apparent increase occurred showed that it was necessary to differentiate between the true percentage response after storing (Δ) and the measured percentage difference in response (D). The significance and relevance of each change in concentration was determined, using the method of Timm *et al* [52], and these results are depicted in Figures 3.5 to 3.8.

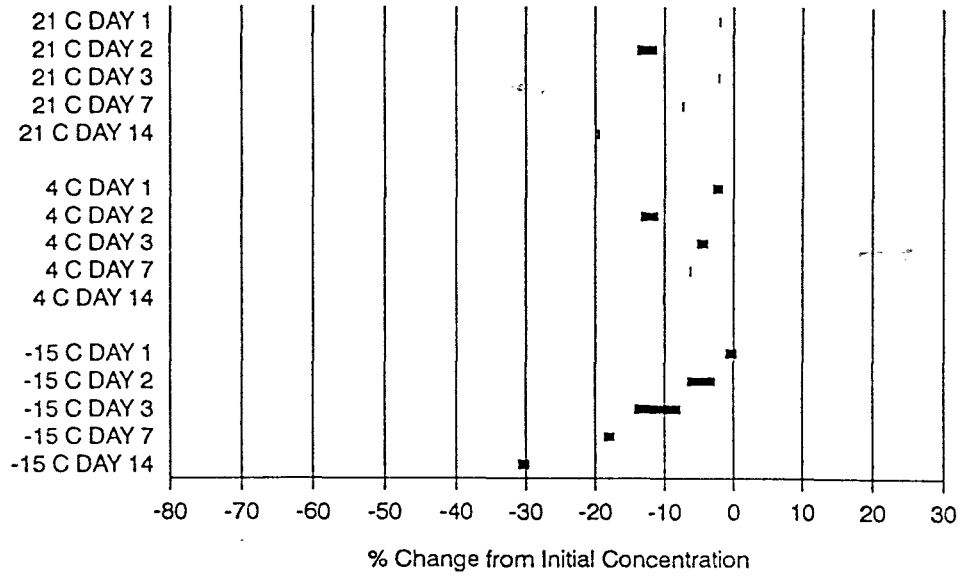


Figure 3.5 Percent change from initial concentration of an aqueous solution of 0.1 mg/mL amoxycillin stored over a 14 day period without internal standard

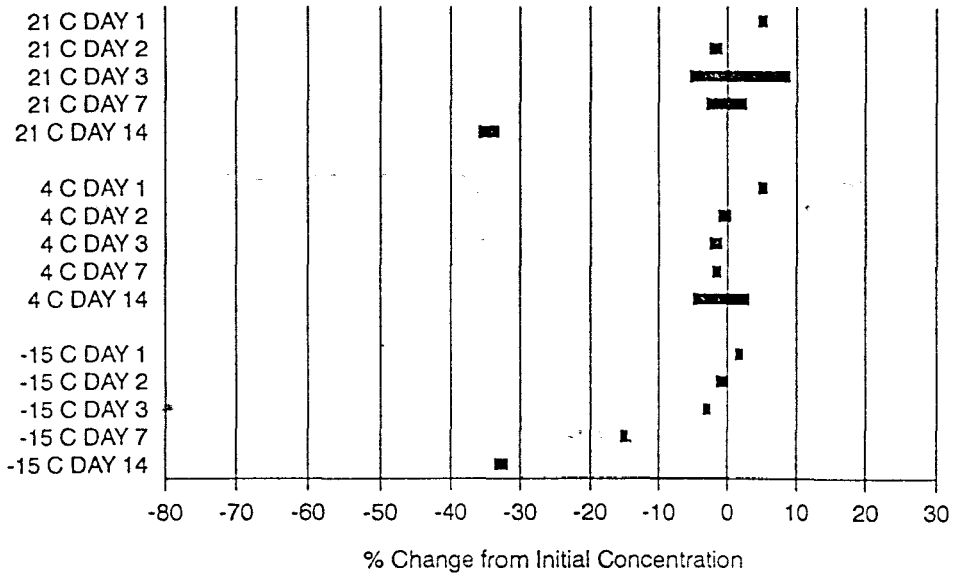


Figure 3.6 Percent change from initial concentration of an aqueous solution of 0.1 mg/mL amoxycillin stored over a 14 day period with internal standard

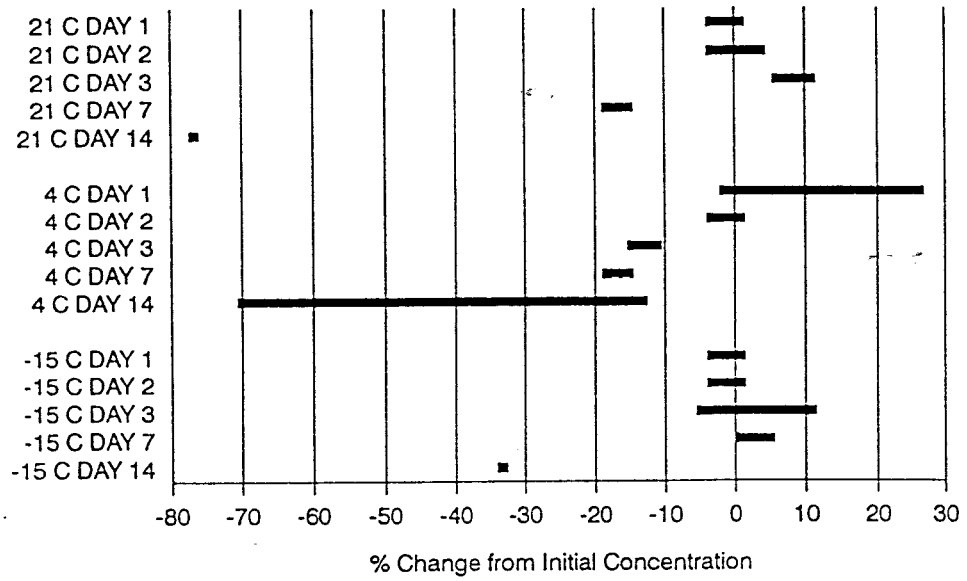


Figure 3.7 Percent change from initial concentration of an aqueous solution of 0.005 mg/mL amoxycillin stored over a 14 day period without internal standard

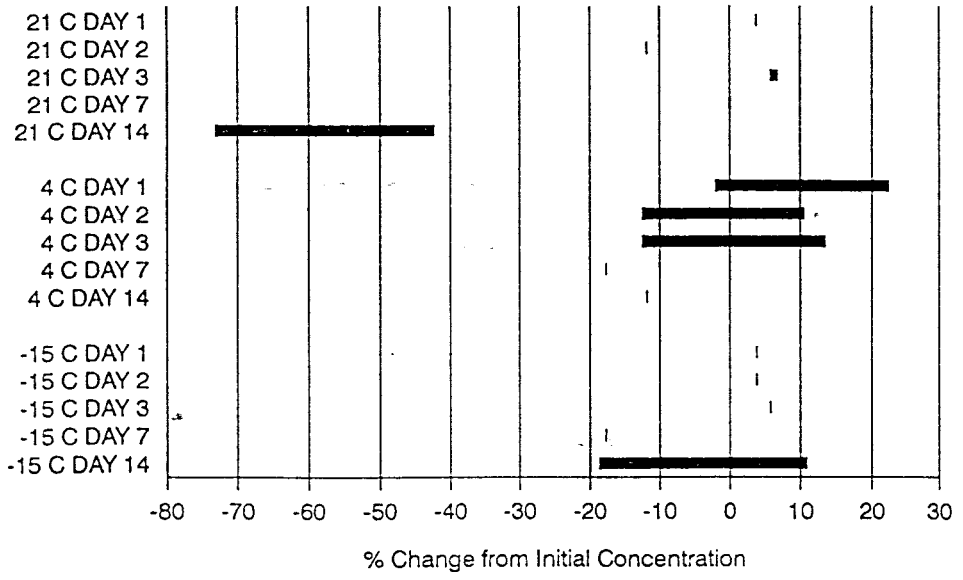


Figure 3.8 Percent change from initial concentration of an aqueous solution of 0.005 mg/mL amoxycillin stored over a 14 day period with internal standard

The solutions used in the study were slightly acidic, with a pH of approximately 4.5 for amoxycillin solutions, and a slightly lower pH of 4.1 for the amoxycillin solutions to which salicylic acid (pH 2.5) had been added (Table 3.8). In this pH range and for the two concentrations studied, there is likely to be a combination of dimerization and hydrolysis reactions since the solutions were acidic, which is conducive to dimerization, but of relatively low concentrations, which is more favourable for degradation by hydrolysis [10,13,14].

All samples were stable for the first 24 hours of storage, according to the definition of Timm *et al* [52], as shown in Figures 3.5 to 3.8, with either a change of response that was not significant and not relevant (a), or a decrease of response that was significant but not relevant (b), or an increase of response that was significant (f). This result meant that it was not necessary to store samples below room temperature, if they were to be assayed within a day of preparation. This also meant that samples could be left in the autosampler for 24 hours if necessary. This result supports those of Tsuji *et al* [13] and Sutherland and Rolinson [1], who observed a degradation half life of approximately 15 to 20 hours, at lower pH, but otherwise similar conditions. The lower pH conditions used by these researchers, being pH 2.35 and 1.5 respectively, would cause acid hydrolysis to occur to a greater extent than at the pH values observed in this stability study. This could explain why most samples were still stable under all conditions after 3 days, with the exception of the 0.1 mg/mL amoxycillin solution stored at room temperature and at 4°C, and the dilute amoxycillin solution which was refrigerated. After seven days discernable degradation was noted in all solutions.

Solutions at lower concentrations seem to be more affected by the presence of salicylic acid. Although salicylic acid has been shown to be stable when stored on its own, the presence of amoxycillin may cause it to degrade. There may be a salicylic acid degradation peak occurring at the same time as the major amoxycillin peak, which has more of an obvious effect on the smaller amoxycillin peaks.

The increase in the concentration of the frozen 0.005 mg/mL solutions at Day 14 (Table 3.13) can be explained by the fact that the test tubes containing amoxycillin 0.005 mg/mL and SA cracked while thawing and some of the sample was lost.

If it were necessary to store aqueous solution of amoxycillin for a prolonged period of time, it would probably be necessary to buffer the solutions to a pH of approximately 6, which is the pH

of maximum stability for amoxycillin in solution [14].

3.3.4 CONCLUSION

It is possible to leave aqueous samples of amoxycillin trihydrate at room temperature in an autosampler for a day, while analysis is in progress, without special storage conditions. Although the samples appear to be stable for longer periods, when presented as percent drug remaining (Tables 3.15 and 3.16), storage of solutions for longer periods would necessitate freezing and possibly the addition of a buffer, since aqueous solutions of amoxycillin are not very stable according to the stringent requirements of Timm *et al.* To avoid potential interactions between salicylic acid and amoxycillin, the internal standard should be added immediately before analysis.

CHAPTER FOUR
IN VITRO RELEASE OF AMOXYCILLIN FROM SUPPOSITORY BASES

4.1 INTRODUCTION

The absorption of drug from a suppository base entails release from the vehicle and subsequent diffusion of the drug through the rectal mucosa to available absorption sites. Drug release from suppository bases into the rectal environment is influenced partially by factors related to the suppository base such as drug-vehicle interactions, suppository base composition, melting point, crystalline form of the base and viscosity of the base at body temperature, as well as characteristics of the drug such as solubility, partition coefficient and drug permeability in the vehicle, solubility of drug in the rectal fluid, particle size of the drug and drug concentration. The fraction of drug substance in relation to the total suppository weight may influence the rate of absorption due to an improved rectal spreading of the melted suppository mass when the fraction of drug substance is low [53]. The dissolution rate might also be influenced by the area of contact between the suppository and the rectal fluid and is regulated by the volume of fluid. As the spread of the suppository mass is limited to between 5 cm to 7 cm into the rectal cavity, a formulation should be designed that utilizes the available absorption area as effectively as possible [54,55].

Pharmacopoeial specifications state, as part of the definition of rectal suppositories, that suppositories should soften, melt or dissolve at body temperature [8]. This statement is however meaningless unless a standard method of determining these qualities is given. Release rates of drugs may influence the bioavailability of drugs from suppositories but as different methods give different results, suppositories may be accepted as suitable despite not adequately melting, softening or dissolving in the rectal environment. This suggests that quality control of suppositories by *in vitro* release tests is needed for the bioavailability certification. An *in vitro* dissolution technique should discriminate between dissolution profiles from suppositories of different composition, be reproducible and enable *in vitro-in vivo* correlation in humans.

A number of studies have been performed for bioavailability determination of rectally administered drugs, but there are no official tests or guidelines for drug release from suppositories or dissolution from rectal dosage systems *in vitro*. The only official guideline is a disintegration test for suppositories and pessaries in the British Pharmacopoeia [7], which determines whether these

disintegrate or soften within a prescribed time, when placed in a liquid medium under the prescribed experimental conditions.

Vastly varied systems have been used to investigate *in vitro* drug release and techniques that are in use differ mainly in the extent to which they mimic *in vivo* conditions. Two basic systems are employed; those that use membranes and those that do not. The former may consist of a sample chamber separated from a reservoir by a membrane; or it may utilize dialysis tubing or a natural membrane [64,65,67,68]. The latter systems may range from simple placement of a suppository in a beaker or flask, to use of existing tablet dissolution apparatus [35,56-58].

4.1.1 SIMPLE *IN VITRO* METHODS

Baichwal *et al* [56] explored the release of salicylic acid, copper sulphate and boric acid from fatty suppository bases, using an apparatus claimed to simulate body conditions. The suppository was supported on a cotton plug in a glass funnel, mounted in a hot water jacket thermostatically controlled to 37 ± 0.5°C. Normal saline was allowed to flow onto the suppository at a rate of 100 drops/minute. The suppository was immersed in normal saline at all times. Samples were withdrawn periodically over 1 hour and analyzed to determine the amount of medicament released. The release of drug from the bases was linear after an initial non-linear period, and the drug release was related inversely to the consistency of the bases.

In their investigation of sustained release sodium diclofenac suppositories, Toshiaki Nishihata *et al* [57] used a simple *in vitro* dissolution technique. Each test suppository was wrapped with two sheets of gauze and immersed in a beaker containing 100 ml saline warmed to 38°C. Aliquots (100μl) were withdrawn at designated time intervals, passed through a 0.5 μm millipore filter and analyzed. Since the data obtained from this dissolution technique were comparable to the apparent urinary excretion rate of sodium diclofenac, it was suggested that the method used was suitable for the relative estimation of sustained release of sodium diclofenac from a suppository containing lecithin in the rectal lumen. It was also proposed that the permeating rate of rectal fluid into the suppository's matrix may regulate the release of diclofenac from the suppository, as shown by this dissolution method.

4.1.2 METHODS BASED ON USP APPARATUS

The apparatus most commonly used by researchers to measure dissolution appear to be the dissolution apparatus for tablets and capsules described in the USP as Apparatus I, the basket apparatus; Apparatus II, the paddle apparatus; and Apparatus III, the flow-through cell apparatus [35], or modifications thereof.

Several comparative studies of rectal formulations have been performed using the basket, the paddle and flow-through methods [58-60]. One of the aims of these studies was to determine whether these *in vitro* dissolution methods established for oral dosage forms, are applicable to rectal dosage forms. The dissolution medium used was either water or phosphate buffer (pH 7.4), deaerated and maintained at 37 ± 0.5°C. Samples were collected manually. In these studies [58-60] Apparatus I was used unmodified with a rotation speed of 100 rpm, and a basket of mesh size 0.42 mm. Apparatus II, the paddle method, consisted of a stainless steel net (mesh width 1 mm) placed between the paddle and the suppository. A metal helix was used around the suppository to prevent it from floating up to the interface of the dissolution medium after insertion into the beaker. The rotation speed of the paddle was 50 rpm. The flow through cells used in these studies had a diameter of 12 mm. The flow rate of the non-circulated, deaerated water or buffer was 16 ml/minute, or 8 ml/minute when it was necessary to decrease the flow rate to prevent certain fatty bases from clogging the filter. The suppository was placed in the first chamber and an upward stream of water flowed past the suppository, through the second chamber, through the filter into the collecting bottle. Dissolving suppositories stayed in chamber 1, while melting suppositories which are less dense than the fluid, flowed into chamber 2, where the melt was collected on the top. There was continuous contact between dissolution fluid and melted mass. Unlike the other techniques, the agitation was constant throughout the whole experiment. Six suppositories were used for each dissolution technique. These methods were used by the authors to determine the release of ibuprofen, paracetamol and dextropropoxyphene. The concentration of drug in the test samples was determined spectrophotometrically.

From these studies [58-60], it could be concluded that the release pattern of drug from different suppositories is determined by both composition and dissolution technique. Hydrophilic suppositories appear to be less sensitive to the dissolution technique and gave more consistent results than the melting suppositories. Therefore the basket, paddle and flow-through techniques could be considered equivalent for dissolving suppositories. However, when it was necessary to

predict a rank order between different suppository compositions, the basket method proved more suitable. The lipophilic suppositories deform and spread, and as the different techniques permit various kinds of spreading, different *in vitro* patterns may result. The drug was more easily dispersed by the paddle than the basket. When the basket was used to test dissolution of melting suppositories, the melted fat sometimes escaped from the basket through the gap between basket and holder, resulting in faster dissolution rates. The different spreading conditions of the molten base in the flow cell show that this apparatus would result in more rapid dissolution than the other methods. There is a constant flow of fresh dissolution medium past the melting base, compared to the beaker techniques where the volume of dissolution medium was constant. The flow-through technique could be applied to all tested compositions, if the flow rate is kept low (0.8 mL/minute).

The basket, paddle and flow-through methods have been employed by numerous other researchers. Kellaway and Marriott [61] used a modified rotating basket apparatus to investigate the release of prednisolone from water miscible suppositories made from polyethylene glycol. A rotating basket (125 rpm) in a dissolution medium of 2 litres deionised water, maintained at 37 ± 0.2 °C was used. Samples were withdrawn at intervals from a fixed point in the medium. The release was found to be linear.

Hosny *et al* [62] also used a modification of the USP rotating basket to assess the *in vitro* release of ampicillin from various suppository bases. They used a dissolution medium of 200 mL distilled water maintained at 37 ± 0.5 °C. Using this method, the authors were able to demonstrate the effect of ageing on various suppository formulations.

Roseman *et al* [63] developed a flow-through technique in which the suppository was enveloped by a bed of glass beads. This continuous flow bead-bed dissolution apparatus controlled the interface exposed for dissolution for the fatty suppository bases used in this study, and allowed direct contact between suppository and dissolution medium. This proposed bead-bed dissolution apparatus was claimed to offer an improved means of measuring drug release from suppositories.

Gjellan *et al* [53] used a flow-through cell to investigate the *in vitro* release rate and bioavailability of paracetamol and codeine from hard fat suppositories. The dissolution profiles correlated with the plasma concentration profiles obtained. The lower flow rate of 8 mL/min reflected *in vivo* conditions more approximately than the higher rate of 16 mL/min. A change in flow rate indicated a change in agitation and amount of vehicle available. The dissolution data seemed to indicate that

the rate-controlling step for paracetamol and codeine is the diffusion process through the melt to the interface between fat and dissolution medium.

4.1.3 METHODS UTILIZING MEMBRANES

One difficulty in testing for drug release from a suppository is that on softening, melting, deformation or disintegration, a non-constant interfacial area is exposed to the dissolution medium. The variability of the interfacial area leads to poor test reproducibility, since release rates are dependant on this factor. Membranes have been used to limit the surface area, on the supposition that when the suppository softens, it will spread over the entire membrane, controlling the area exposed to the dissolution liquid.

Othman and Muti [64] used a dialysis method to investigate the *in vitro* release characteristics of indomethacin from different suppository formulations. Dialysing bags prepared from cellophane dialysis tubing (100x35 mm) were tied with cotton thread and soaked overnight in a phosphate buffer solution (pH 8). The bags were rinsed twice and filled with 20 mL phosphate buffer and one suppository. The bags were suspended in a 500 mL wide-mouth bottle containing 400 mL phosphate buffer solution. The bottle was placed in a water bath at 37°C and stirred with a magnetic stirrer at a slow and constant speed. At time intervals a sample was withdrawn for UV spectrophotometric analysis. The dissolution fluid in the bottle was kept constant by addition of phosphate buffer after each sample had been taken.

De Muynck and Reman [65] made use of a dialysis method to determine the influence of fat composition on the *in vitro* release of indomethacin suppositories. Dialysis tubing was washed with boiling water and distilled water. Before use the remaining water was removed and one end of the tubing tied. A suppository was inserted and the 5 cm dialysis tube sealed. An 8 g weight was connected to the end and the tubing was immersed in the 900 mL of 0.1 M buffer solution pH 7.2 [35] heated to 37 ± 0.2°C which was used as the dissolution medium. The dialysis tubing was placed in such a way that the lowest knot was 3 cm above the paddle and 2 cm from the side wall of the dissolution vessel. A USP dissolution vessel equipped with a paddle, rotating at 50 rpm at a height of 1.5 cm above the bottom of the vessel was used. These researchers [65] used the dialysis tubing method as described by Aoyagi *et al* [66] because the correlation between this *in vitro* method and the bioavailability in rabbits and pigs, as animal models, was found to be good. The small amount of water in the rectal compartment and the spreading after melting, according

to the surface active properties appear to be quite well simulated by this system. The polyethylene glycol (PEG) suppositories behaved quite differently from the fatty bases (*Suppocire AP, Mesuro PS, Witepsol H15*). Due to the osmotic effect of PEG the dialysis tubing was filled with dissolution fluid. The indomethacin was completely dissolved in the resultant mixture of PEG and dissolution medium. This phenomenon was not observed with the fatty suppositories, although the indomethacin release was not significantly different from that of the PEG formulation after 480 minutes.

Masaru Yamazaki *et al* [67] used a modification of the dialysis membrane method, in an attempt to overcome some of the difficulties which occur when using this apparatus. This revised apparatus included a rack assembly made of stainless steel and a 1000 mL tall beaker. The rack assembly involved two agitator rings, a bent wire and a hanging clip. The rings were held vertically at 4.5 cm intervals by the wire. One litre of 0.05 M phosphate buffer was placed in the flask and maintained at 37 ± 0.2°C. The dialysis tubing was cut into 17 cm sections, soaked in purified water for 24 hours and rinsed. As manual methods had proved inefficient, a film squeegee was used to remove any remnant water. Instead of tying the tubing with thread, which wrinkles the end of the tubing and causes the surface area of the tubing to be irregular and the inside liquid at the bottom of the tubing not to be stirred satisfactorily, a snap closure was used with a 5 g weight. A suppository was placed into the dialysis tubing, which was suspended from the rack assembly. The rack assembly was immersed in the beaker and moved vertically, avoiding any horizontal movement or deviation of the axis from the vertical. The water pressure expelled any air remaining in the tubing.

Uekama *et al* [68] examined the *in vitro* release of morphine from *Witepsol H15* hollow type suppositories, using a suppository release apparatus prepared as described by Muranishi [69]. Each suppository was placed in a cylindrical chamber, which was lined with a 30 μm membrane filter to eliminate diffusion of the suppository base. The chamber was lowered into a flask containing a degassed, isotonic phosphate buffer maintained at 37°C. The receptor phase was agitated with a magnetic stirrer at 100 rpm. A steel rod in the suppository chamber was rotated at 25 rpm. A 1 mL sample was withdrawn at predetermined intervals from the receptor phase and 1 mL of the buffer medium used to replace the sampling volume. The *in vitro* results correlated with *in vivo* bioavailability in rabbits.

4.1.4 COMPARISON OF MEMBRANE AND NON-MEMBRANE METHODS

Ermis and Tarimci [70] performed *in vitro* release and diffusion rate tests for ketoprofen in polyethylene glycol bases according to the USP XXII basket method [35] and the Muranishi [69] method respectively. This study effectively demonstrated the difference between membrane and non-membrane methods. In *in vitro* tests, each suppository was placed in the basket which was lowered into a vessel containing 500 mL phosphate buffer solution, pH 7.2. The basket was rotated at 50 rpm at a temperature of 37 ± 0.5°C. Samples were withdrawn at set times and assayed to obtain a dissolution profile. To compensate for sampling phosphate, buffer was immediately added to the dissolution medium. A modification of the Muranishi apparatus [69] was used for diffusion rate tests. A suppository was placed in the test solution (pH 7.2 phosphate buffer) in a cylindrical cell which was fitted with a 3 mm millipore filter, and stirred with a rod at 25 rpm. The test solution in the glass vessel to which the cell was connected was agitated at 250 rpm with a magnetic stirrer. Drug release across the membrane was determined spectrophotometrically. Release profiles obtained by the basket method and diffusion rates from the Muranishi method differed significantly, as in the Muranishi method the drug had to diffuse across a membrane which exposed a constant surface area to the aqueous medium. However in the basket method the drug was released directly into the aqueous medium from the surface of the suppository. Both methods displayed slower release for lipophilic bases than hydrophilic bases.

Aoyagi *et al* [66] investigated inter-laboratory reproducibility of release tests for fatty and water soluble suppositories. For the fatty suppositories (*Witepsol*), Muranishi [69] and dialysis tubing methods were employed. For the water soluble suppositories (PEG), modified Muranishi [69] and paddle methods were used. The Muranishi method was modified such that the suppositories were not stirred with a steel rod, and no membrane was used. The release rates from the fatty suppositories were determined by a dialysis tubing method in 900 mL of the test medium and in 500 mL by the Muranishi method. For the dialysis method one of the tube ends was tied, after washing in distilled water. The tubing was soaked in test liquid, then used after the test fluid had been squeezed out manually. A suppository was placed in the tubing and submerged in 900 mL test fluid, with a lead weight. For the Muranishi method, a membrane filter was attached to the base of a cylindrical cell, which was immersed in the test fluid. A test suppository was stirred with a steel rod at 10 rpm. The test medium in the outer vessel was agitated at 100 rpm. The opinion at the end of this study was that while both paddle and modified Muranishi methods may be used as a quality control for water soluble suppositories, neither Muranishi nor dialysis methods should

be employed for fatty suppositories unless reproducibility is substantially improved. This could be explained by the difficulty in achieving the exact set of the Muranishi cell at the specified position, and the difficulty in completely removing the test medium from the dialysis tubing by manual methods. In the presence of test fluid, the suppository did not spread well in the dialysis tube thus possibly causing a delay in release.

Vidras *et al* [71] used the USP rotating basket apparatus (Method A and B) and dialysis (Method C) to investigate indomethacin release from various bases. For Method A, a suppository was placed in the basket, which was lined with filter paper, and lowered into a vessel containing 600 mL phosphate buffer, pH 8. The filter paper was used as a barrier to the diffusion of the suppository base. The basket was rotated at 100 rpm at a constant temperature of 37 ± 0.5°C. The same apparatus and conditions were used for method B, except that the suppository was placed in a cellophane membrane before exposure to the buffer solution. For Method C the dialysing bags were prepared from dialysing tubing tied with plastic cord, and soaked overnight in phosphate buffer solution. The bags were rinsed twice, filled with 20 mL of buffer solution and a suppository and suspended in a 500 mL wide-mouth bottle containing 400 mL phosphate buffer, kept in a waterbath at 39 ± 0.5°C and agitated with a magnetic stirrer. In all experiments 5 mL samples were withdrawn for analysis, and were replaced with 5 mL phosphate buffer to compensate for sampling. The resulting data indicate that the total amount of indomethacin was released about 10 times faster from the polyethylene glycol base than from the fatty bases, when the USP basket apparatus was used. The researchers concluded that the USP apparatus cannot be used to determine release from water soluble suppositories without the use of membranes or filter media. The inability of the USP dissolution apparatus to be used with water soluble bases led to the introduction of membranes to hinder the erosion process. However the use of cellophane membranes and the dialysis technique (methods B and C), may represent a further slow step in the overall release of indomethacin which may preclude a comparison of techniques. Therefore, although a membrane may control the interfacial area, it does introduce an additional physical process of membrane transport, which may mask the real release characteristics of particular drug-suppository base combinations.

Fassihi *et al* [72] made use of a combination of aspects of several methods to determine theophylline release rates from polyethylene glycol suppositories. The system incorporated a membrane, bead-bed and basket. The basket was modified to keep constant hydrodynamic conditions and to provide better and more precise control over the interfacial area for drug

dissolution. The setup consisted of a stainless steel 20 mesh screen, chemically resistant glass beads of 3-6 mm diameter and a dialysing membrane. After soaking the cellophane dialysing bags overnight in distilled water, a suppository and 15 mL distilled water were placed in each. The bag was placed in a basket, which was half filled with glass beads, then the rest of the basket was filled with beads and the basket was mounted and lowered into the 1 litre beaker containing 500 mL distilled water at 37±0.5°C.

4.1.5 ANIMAL MODELS

Some investigators have used live tissue, in order to simulate a living system as closely as possible. Izgu and Gunger [73] studied the *in vitro* rate of absorption of paracetamol using isolated rectums of rabbits as dialysis membranes. An isotonic buffer solution (100 mL pH 7.4) was placed in a special glass funnel which had a heated outer jacket. The terminal 10 cm section of the rabbit colon was used. The intestine was tied 0.5 cm from the colon end and a suppository, two glass beads and 2 mL of the buffer solution were inserted and the other end of the bag was tied. The dialysis bag, which remained viable during the test, was suspended in the buffer solution in the funnel. The solution was stirred with a propeller-type stirrer at about 120 rpm. From the *in vivo-in vitro* correlation obtained, it was concluded that the isolated rectums of albino rabbits can be used successfully as dialysis membranes in the investigation and control of formulation parameters of suppositories in respect to the absorption characteristics.

4.1.6 CONCLUSION

When appraising the suitability of a dissolution test, it is necessary to consider the physicochemical properties of the drug as well as the behavioural changes which occur in the rectal dosage form in an *in vitro* method. The release pattern of a drug is influenced by both the suppository composition and the dissolution technique adopted. Factors such as flow rate and temperature of the dissolution solvent must be considered, as they can influence drug release trends.

Non-membrane techniques, such as the USP basket, paddle and flow-through cell, appear to be equivalent when used to assess hydrophillic suppositories [58,59,60] as these water soluble bases are less sensitive to the type of experimental equipment used, than the lipophilic suppository bases, and give more consistent results. Vidras *et al* [71] have suggested that the USP dissolution apparatus should be modified with membranes, if they are to be used for hydrophilic suppositories.

Lipophilic suppositories deform and spread when subjected to dissolution tests, resulting in different *in vitro* release patterns, because the methods allow various kinds of spreading [58,59,60]. The flow-through cell is a more rapid technique than the basket or paddle, because of different spreading conditions and the constant flow of fresh dissolution medium past the melting base. The flow-through cell can be applied to all types of suppository bases, if the flow rate is kept low at 0.8 mL/minute.

The results of membrane and non-membrane methods are not always comparable. Membrane-type techniques have been employed in an attempt to regulate the interfacial area of the suppository exposed to the dissolution medium, when the suppository melts, softens, deforms or disintegrates during dissolution testing. Aoyagi *et al* [66] claim that membrane-based methods such as the Muranishi cell and dialysis methods are not reproducible when used to analyze release from fatty suppositories, since in such membrane methods, the drug has to first diffuse across the membrane before it reaches the test medium. The use of a membrane in a test system introduces an additional physical process of membrane transport to the release profile. On the other hand for non-membrane methods, such as the basket and paddle, the drug is released directly into the aqueous medium from the surface of the suppository. It has been postulated [74] that when a membrane is used with a fatty base, a lipophilic barrier is formed through which the drug has to first diffuse before entering the aqueous medium.

4.2 METHOD

4.2.1 EXPERIMENTAL

4.2.1.1 REAGENTS

Suppositories were manufactured as outlined in Section 2.2.2. All other chemicals are listed in Section 3.2.1.1.

4.2.1.2 HPLC SYSTEM

HPLC analysis was performed on System B (Section 3.2.1.2) using the validated HPLC method described in Section 3.3.

4.2.1.3 STOCK SOLUTIONS

Stock solutions were prepared as described in Section 3.2.1.8.

4.2.1.4 CALIBRATION CURVES

Fresh stock solutions of amoxycillin and salicylic acid were prepared for each analytical run. A calibration curve was constructed prior to each dissolution run. Six calibration standards of 0.002, 0.005, 0.02, 0.05, 0.07 and 0.1 mg/mL were prepared by serial dilution of amoxycillin stock solutions. Before making up to volume, salicylic acid was added to each standard to give a final internal standard concentration of 0.01 mg/mL. Each calibration standard was assayed six times. The linearity of peak height ratio to concentration was established.

4.2.1.5 PROCEDURE

Six amoxycillin suppositories formulated with *Novata BD, Novata 299, Suppocire A32, Witepsol W35* and theobroma were weighed prior to dissolution. The mean mass values are shown in Table 4.2.

The *in vitro* dissolution of amoxycillin from the suppositories was examined by means of the basket apparatus (Apparatus I USP) [35].

Based on the recommendations for oral dosage forms, 900 mL deaerated HPLC-grade water, at 37 ± 0.5°C, was used as the dissolution medium. The speed of rotation was 100 rpm and the mesh width of the basket was 40 mesh.

The dissolution tests were performed on six separate dosage units for each type of base. Three 500 μL samples of test solution were collected manually after 5, 10, 15, 30, 60, 90, 120, 180 and 240 minutes and filtered through a 0.2 μm filter. 100 μL of each sample was mixed with 200 μL of the internal standard solution. The amount of dissolved amoxycillin was quantitated by the validated HPLC method described in Section 3.3, with UV detection at 230 nm.

The suppositories were tested within one week of manufacture, and again at one month, to determine whether ageing had an effect on the amount of drug released from the suppositories.

A mass balance was carried out on each suppository melt remaining in the baskets after the 240 minute dissolution run, in order to assess and account for any drug that had not been released from the base and dissolved during the dissolution test. The basket containing the melt was left to stand in a beaker containing 200 mL water, kept at approximately 40°C for 10 minutes, until the base had remelted. The contents of the beaker were then sonicated (Ultrasonic Cleaner, Model 8845-30, Cole-Parmer Instrument Company, Illanois, USA) to dissolve any amoxycillin before the base resolidified. An aliquot was removed and filtered through a 0.2 μm filter. A 0.1 mL sample was then mixed with internal standard solution and made up to 10 mL with water and an aliquot was analyzed in triplicate.

4.2.1.6 STATISTICAL INTERPRETATION OF THE DATA

The statistical method of Timm *et al* [52], as described in Section 3.3.2.9, was adapted for the purposes of this dissolution study, in order to determine whether ageing had an effect on the release characteristics of amoxycillin from the suppository bases.

4.2.2 RESULTS

4.2.2.1 LINEARITY AND CALIBRATION CURVES

Calibration curves were constructed as described in Section 4.2.1.5 and linearity was established over the concentration range 0.005 to 0.1 mg/mL on each day that a different type of suppository was studied. The linearity data are described in Table 4.1.

Table 4.1 Linearity data from calibration curves constructed for each types of suppository base assayed

Suppository Assayed	Slope	Y-intercept	Correlation Coefficient
t=0 months			
Novata BD	39.4349	-0.0042	0.99935
Novata 299	41.2065	0.0020	0.99999
Witepsol	41.3188	-0.0018	0.99996
Suppocire	38.4954	0.0151	0.99923
Theobroma	36.1980	0.0062	0.99995
t=1 month			
Novata BD	36.3844	0.0275	0.99990
Novata 299	41.1459	0.0239	0.99515
Witepsol	36.3844	0.0275	0.99990
Suppocire	41.1459	0.0239	0.99515
Theobroma	40.2583	0.0238	0.99981

4.2.2.2 AMOUNT OF DRUG RELEASED FROM SUPPOSITORIES

The mean masses of the suppositories used in the dissolution tests are listed in Table 4.2.

Table 4.2 Mean mass of suppositories used for dissolution tests

Suppository	t=0 months Mean Mass (g) ± SD (n=6)	t=1 month Mean Mass (g) ± SD (n=6)
Novata BD	1.13 ± 0.01	1.13 ± 0.02
Novata 299	1.13 ± 0.02	1.11 ± 0.01
Witepsol	1.12 ± 0.00	1.14 ± 0.01
Suppocire	1.12 ± 0.02	1.13 ± 0.02
Theobroma	1.13 ± 0.01	1.05 ± 0.08

The amount of amoxycillin released from each suppository during the 240 minute dissolution period was calculated for both the freshly made and the one month old suppositories, and the results have been summarised in Table 4.3.

Table 4.3 Mass of amoxycillin released from suppository bases within 240 minutes, at t=0 and t=1 month

Time (min)	Mean Mass (mg) ± SD (n=3)				
	Novata BD	**Novata 299**	**Witepsol W35**	**Suppocire A32**	**Theobroma**
t=0 months					
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5	18.90 ± 4.55	10.35 ± 1.87	7.35 ± 1.05	1.35 ± 1.24	2.25 ± 0.75
10	45.45 ± 6.61	24.00 ± 3.20	17.70 ± 2.71	3.30 ± 3.05	4.50 ± 0.99
15	90.45 ± 6.61	43.35 ± 6.17	28.35 ± 2.40	5.55 ± 4.24	6.00 ± 1.09
30	141.15 ± 16.67	82.20 ± 23.97	54.75 ± 10.79	10.20 ± 8.44	9.00 ± 1.14
60	170.70 ± 17.16	120.45 ± 27.37	92.85 ± 18.46	29.85 ± 24.67	14.10 ± 1.77
90	201.15 ± 14.07	145.35 ± 33.34	106.80 ± 20.03	52.95 ± 44.76	16.50 ± 2.18
120	203.85 ± 10.89	201.15 ± 32.48	108.90 ± 21.52	77.85 ± 63.58	18.00 ± 1.51
180	205.65 ± 9.77	198.75 ± 34.10	114.75 ± 25.25	111.00 ± 71.09	20.25 ± 1.48
240	218.93 ± 20.45	213.00 ± 41.10	126.30 ± 30.78	111.00 ± 71.09	22.05 ± 1.87
Mass balance	8.61 ± 6.67	48.33 ± 16.64	89.13 ± 19.65	120.83 ± 54.44	220.53±21.90
% Total drug*	91%	104%	86%	92%	97%
t=1 month					
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5	15.60 ± 1.23	13.05 ± 0.94	6.60 ± 1.09	5.13 ± 0.03	1.32 ± 0.58
10	37.35 ± 4.21	26.25 ± 1.84	14.10 ± 1.35	7.52 ± 0.16	3.28 ± 0.18
15	64.35 ± 9.94	39.30 ± 0.73	21.90 ± 0.93	8.73 ± 0.07	5.11 ± 0.49
30	150.60 ± 24.42	71.55 ± 3.36	44.55 ± 1.68	10.47 ± 0.09	9.28 ± 1.63
60	219.30 ± 17.41	123.00 ± 3.45	62.55 ± 2.03	12.94 ± 0.32	17.13 ± 4.64
90	223.20 ± 20.43	147.75 ± 4.64	67.95 ± 3.97	15.83 ± 0.04	21.90 ± 6.16
120	232.80 ± 18.26	168.30 ± 4.59	74.40 ± 3.54	17.56 ± 0.89	25.03 ± 6.80
180	232.80 ± 18.12	170.70 ± 4.61	76.95 ± 3.89	19.35 ± 0.03	28.98 ± 13.42
240	248.15 ± 23.90	186.30 ± 7.91	79.95 ± 6.56	19.91 ± 0.01	29.27 ± 9.85
Mass balance	12.45 ± 14.18	60.89 ± 7.85	154.77 ± 23.58	203.28 ± 6.16	206.97±20.40
% Total drug*	104%	99%	94%	89%	94%

* % Total drug = 100(Mass of drug released by 240 minutes + Mass balance)/250.

In vitro release dissolution profiles from the various bases are shown in Figures 4.1-4.5. In the 4 hour dissolution of newly made suppositories, 87.57 ± 8.18 % amoxycillin was released from the *Novata BD* base, 85.20 ± 16.42 % from the *Novata 299*, 44.40 ± 28.43 % from the *Suppocire A32*, 50.40 ± 12.31 % from the *Witepsol W35* and 8.82 ± 0.75 % from theobroma oil. When one month old suppositories were tested, 99.66 ± 6.63 % was released from *Novata BD*, 74.52 ± 3.16 % from *Novata 299*, 7.98 ± 0.27 % from *Suppocire A32*, 31.98 ± 2.07 % from *Witepsol W35* and 11.22 ± 4.04 % from theobroma oil.

The amount of drug which was recovered when a mass balance was performed approximately accounted for the amoxycillin which was not released from the suppository bases during the dissolution period of 240 minutes, as shown in Table 4.3. In total, 91% and 104% of the drug in the *Novata BD* new and old suppositories respectively was accounted for, 104% and 99% of the drug in the *Novata 299*, 86% and 94% of the drug in the *Witepsol W35*, 92% and 89% of the drug in the *Suppocire A32* and 97% and 94% of the drug in the theobroma suppositories.

The statistical method used by Timm *et al* [52] to describe stability data was adapted to describe the change in the amount of amoxycillin present in the different suppositories from the time of manufacture (t=0 months) to one month later (t=1 month). This is represented in Figure 4.6 as the percent change from initial amount of amoxycillin released during the 240 minute dissolution period.

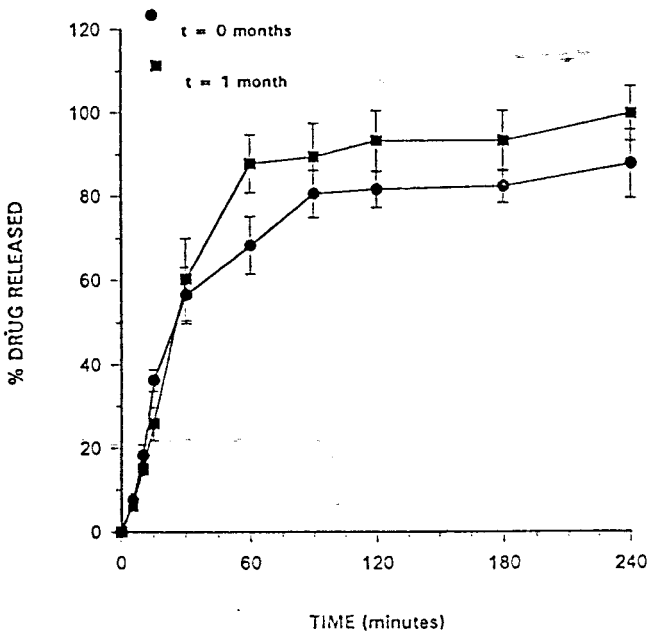


Figure 4.1 Amoxycillin released from Novata BD suppository base, at t=0 months and t=1 month after manufacture

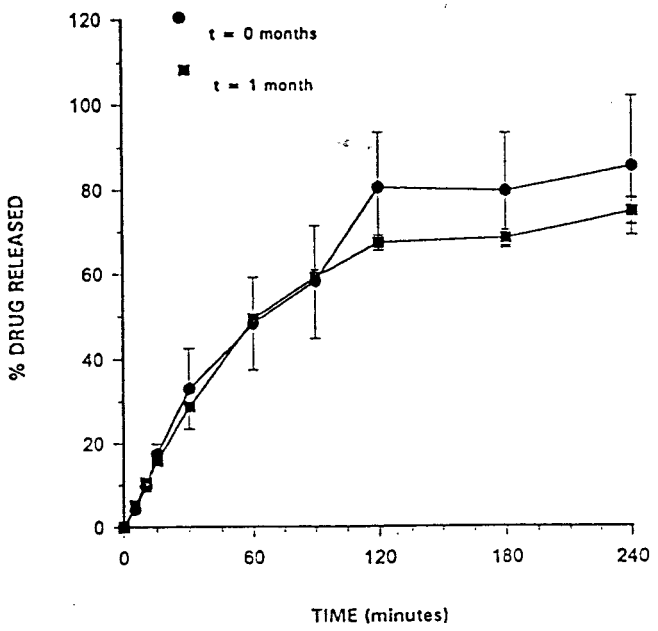


Figure 4.2 Amoxycillin released from Novata 299 suppository base, at t=0 months and t=1 month after manufacture

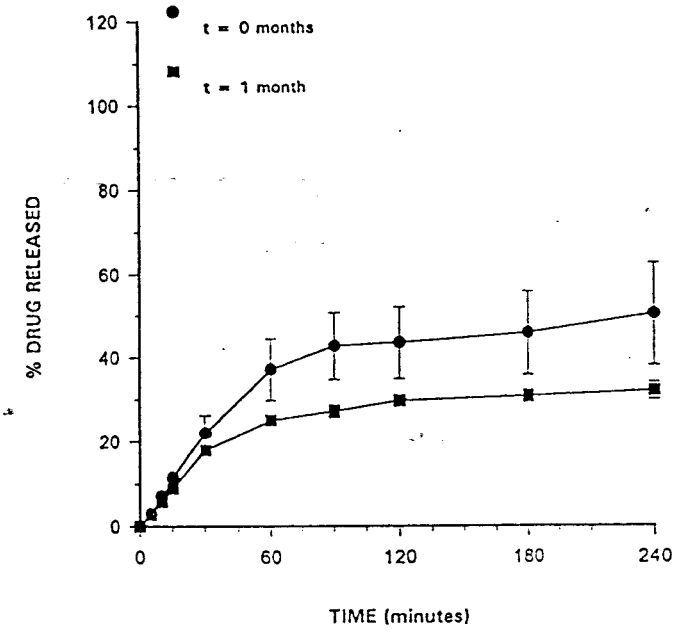


Figure 4.3 Amoxycillin released from Witepsol W35 suppository base, at t=0 months and t=1 month after manufacture

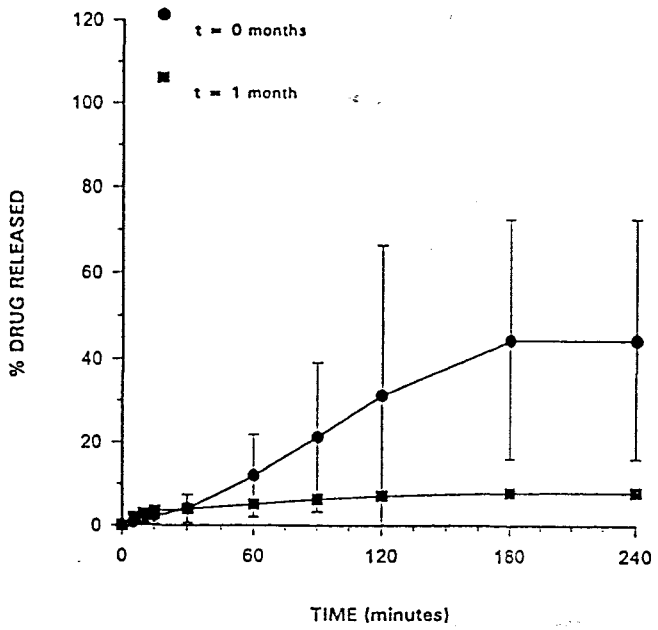


Figure 4.4 Amoxycillin released from Suppocire A32 suppository base, at t=0 months and t=1 month after manufacture

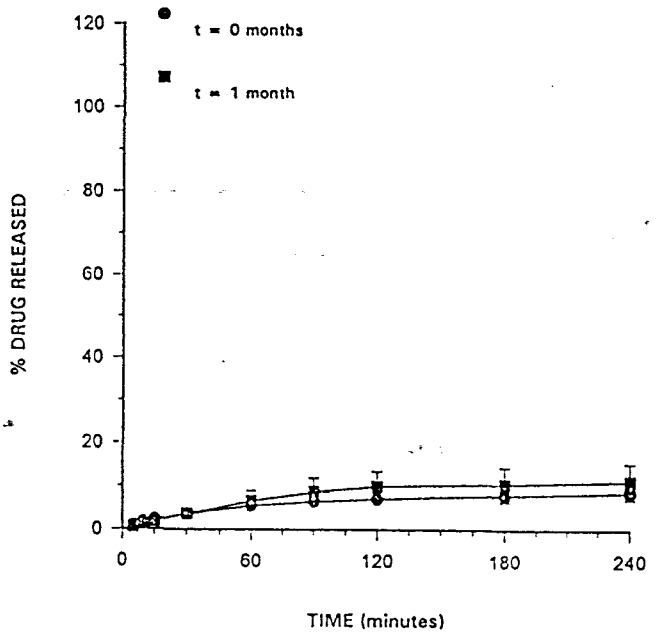


Figure 4.5 Amoxycillin released from theobroma suppository base, at t=0 months and t=1 month after manufacture

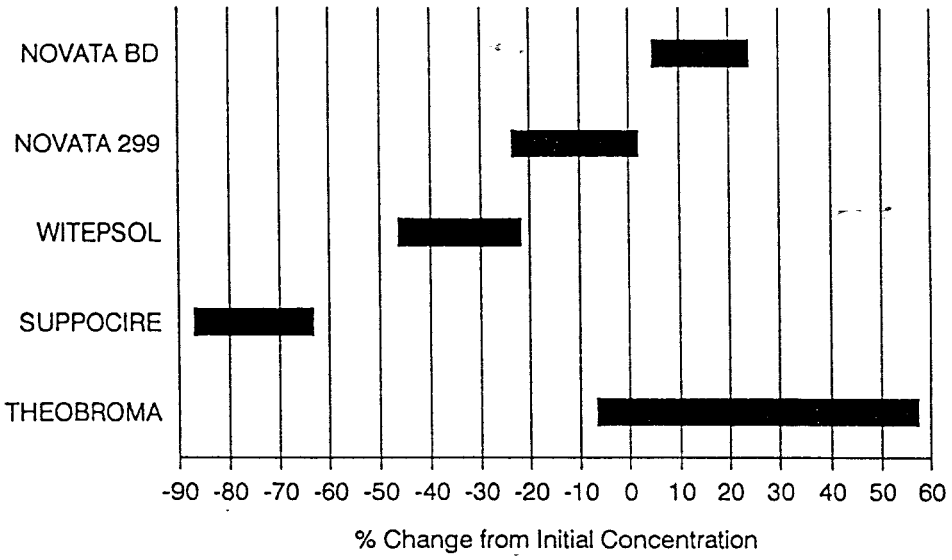


Figure 4.6 Percent change from initial amount of drug released from suppository bases, after storage for one month

4.2.2.3 DISCUSSION

The different brands of semisynthetic bases showed different dissolution profiles. Each brand contains an unique combination of tri-, di- and mono-glycerides which confer certain characteristics to that base. The nature of the base clearly has an effect on the dissolution rate and the amount of drug which is released. Theobroma, being primarily a mixture of fatty acid triglycerides, also shows a different dissolution profile.

It would appear that the hydroxyl value of a suppository base is of some significance to the release of the drug from a suppository. This finding is consistent with that of Othman and Muti [64]. The synthetic suppository bases are mixtures of fatty acid esters with certain amounts of glycerides. The hydroxyl value of a base is determined by the presence of mono- and diglycerides and therefore represents the availability of free hydroxyl groups. The potential reactivity of a base is usually indicated by the magnitude of its hydroxyl value, with a high hydroxyl value indicating that the base can adsorb water more readily and is therefore less suitable for formulations containing drugs that are easily hydrolysed [8]. Amoxycillin, which is susceptible to hydrolysis may interact with

these hydroxyl groups and degrade.

Drug release from bases with lower hydroxyl values (eg the *Novata* bases) was shown to be faster and more complete than from those with a higher hydroxyl value (Figures 4.1 and 4.2). These results could be accounted for on the basis of simple partitioning between aqueous and lipid phases [64]. The partitioning of the drug when using bases with high hydroxyl values, such as the *Witepsol* and *Suppocire* (Figures 4.3 and 4.4), appears to favour the lipid phase.

Consideration of hydroxyl value alone could not account for these dissolution results however, since *Novata BD*, despite having a higher hydroxyl value than *Novata 299*, showed a slightly higher rate and extent of release. Examination of the melting point showed that *Novata BD* has a slightly lower solidification point (30-32°C) than *Novata 299* (31.5-33.3°C), which could result in a faster release of the drug [64].

On prolonged storage, semi-synthetic suppository bases have been shown to be subject to hardening and lengthening of the melting time [8]. The degree of hardening may be remedied by storage in a cold place, but melting characteristics, hardness and drug release profiles alter with time, and the melting point may rise by 0.5°C after storage for several months [33]. Storage can thus result in a marked reduction in drug release from suppositories [62] and this effect is more pronounced in those bases which have a very low hydroxyl value. This could explain, in part, why after storage for a month, less amoxycillin was released when *Novata 299*, with a hydroxyl value of $\leq$ 5 was used, whereas the amount of amoxycillin released from *Novata BD*, which has a hydroxyl value of $\leq$ 15, increased.

The greatest quantity of amoxycillin released in 240 minutes was released from the *Novata* bases, and the release rate for the first hour of dissolution was also highest with these bases. Both newly prepared and aged *Novata* bases showed a rapid release and dissolution of drug over the first 60 minutes, then the drug seemed to be retained in the matrix and released at a slower rate between 1 and 4 hours. Within the first hour of dissolution, 68% of the drug in the fresh *Novata BD* suppositories had dissolved, 88% from the aged *Novata BD* suppositories and almost 50% from both fresh and aged *Novata 299* suppositories. No more than 40% was released from the *Witepsol* suppositories and the fresh *Suppocire* suppositories, while not even 10% of the drug in the theobroma suppositories and the aged *Suppocire* suppositories was dissolved within the same time.

4.2.3 CONCLUSION

The USP basket apparatus was successfully used to compare the release of amoxycillin from *Novata BD*, *Novata 299*, *Witepsol W35*, *Suppocire A32* and theobroma suppository bases. This *in vitro* assessment of the suppositories enabled the identification of the base with the best release characteristics. The *Novata* bases showed the highest rate and extent of drug-release over the dissolution period. For this reason these bases were considered to be most appropriate for the formulation of a paediatric amoxycillin suppository.

CHAPTER FIVE
DIFFERENTIAL SCANNING CALORIMETRY

5.1 INTRODUCTION

Differential Scanning Calorimetry (DSC) is a technique of thermal analysis in which the difference in energy inputs into a substance and reference material is measured as a function of temperature, whilst the substance and reference are subjected to a controlled temperature programme [75]. DSC has been recognised for its usefulness in the pharmaceutical field, where it is used in the determination of melting and boiling points, stability and drug-excipient compatibility, thermal kinetic parameters, drug purity and detection of polymorphism.

5.1.1 USES OF DSC

5.1.1.1 DETERMINATION OF INCOMPATIBILITIES WITHIN A FORMULATION

One of the factors on which the stability of a formulation depends, is the compatibility of the active drug with the other components of the mixture. The formulation of a stable and effective dosage form requires careful selection of the excipients used to facilitate administration, promote constant release and bioavailability of the drug and to protect the active moeity from the environment.

Although usually regarded as inert, excipients can readily interact with drugs and affect the solid state stability of a drug, either directly as a chemical reaction between the drug and excipients or, most often, indirectly by adsorption of moisture and/or catalysis [76]. The evaluation of drug-excipient compatibility is therefore an essential aspect of any preformulation study prior to large scale development trials, thereby improving the efficiency of dosage form development.

In preformulation studies it is possible to derive information about potential physical or chemical incompatibilities between the active ingredients and the so-called inert excipients. These reactions may or may not lead to the inactivation of the active ingredient in the formulation. Additional information regarding the effects of storage at elevated temperatures, the necessary physicochemical parameters of a drug substance, its kinetic rate profile and physical characteristics can also be obtained. The identification of hygroscopic differences during preformulation is also important, as moisture is considered to be the most deleterious environmental factor causing

instability in solid dosage forms.

Ideally the method used should be relatively fast and require only small amounts of drug substance. Results should enable accurate prediction and be easily interpreted [77]. Unless an incompatibility is obvious, it is necessary to carry out stability studies that usually take weeks or months. The two commonly employed compatibility screening techniques are quantitative assay after isothermal stress testing of drug-excipient mixtures, and thermal analysis using either DSC or differential thermal analysis (DTA) [78,79].

Thermal analysis has significant advantages over the conventional technique of isothermal stress testing [80]. No long term storage of mixtures and subsequent chromatographic analysis is required, and only a few milligrams of drug are needed per individual experiment. Thermal analysis can therefore be valuable during the early stages of a preformulation programme where time is of the essence, only small amounts of drug are available or no chromatographic methods of analysis have been developed. Using thermal analysis it is possible to obtain stability data rapidly.

According to the literature [76,78,79,81], DSC has become one of the screening methods of first choice for studying drug-excipient interactions since it allows the fast evaluation of possible incompatibilities within the formulation derived from appearance, shift or disappearance of melting endotherms or exotherms and/or variations in the corresponding enthalpy [76]. In general DSC can distinguish between those excipients that are unlikely to cause problems and those that may cause problems in the formulation. This allows a more rational approach to be established in early formulation designs.

DSC data may be used to predict compatibility from the differences in the heats of melting of the drug alone and in a mixture with excipients [77]. The method assumes that when an incompatibility occurs, the melting peak area and heat of melting of drug in the mixture will be decreased. The melting peak and heat of melting stay the same if the drug-excipient mixture is compatible. The potential for incompatibility is proportional to the decrease in the heat of melting.

Thermal analysis does not replace the chemical methods for determination of the concentration of a drug in a dosage form or stability tests involving long-time observation. It does however represent a valuable tool in the first step of a formulation since it can provide an early alert to

compatibility problems and indicate the best direction to pursue for a successful dosage form [76,78,81].

There are however a lot of problems associated with DSC which do not make the technique applicable on its own. It must be kept in mind that the results of such tests may have no or hardly any connection with results under normal conditions. Predictions are difficult as degradation reactions or transformations shown in accelerated thermal analyses may not appear at all at room temperature [78]. Several workers have found that the results obtained by DSC screening are not conclusive and frequently require further investigation. Reasons for this include the unrealistically high temperatures and heating rates used, the lack of moisture stress and difficulty in interpreting thermograms [77,78,80]. The shortcomings of DSC are the inability to give good estimates of the extent and significance of the destabilizing effects and the inability to determine stabilizing effects. An additional disadvantage of DSC screening is the uncertainty as to whether or not the observed interaction is chemical in nature.

Several researchers have suggested that to substantiate DSC findings, other more direct and conclusive techniques have to be used in conjunction, such as the quantitative assay of mixtures of the active ingredient after isothermal stress tests [78,80,82]. In its simplest form, isothermal stress testing involves the exposure of binary drug-excipient mixtures to elevated temperatures and moisture levels to accelerate drug aging and drug-excipient interactions [80]. After a specific storage time, the samples can then be analyzed by visual comparisons as well as by chromatography.

In summary DSC can be used as a quick screening tool for preformulation studies to study the potential incompatibilities of ingredients in the solid state.

5.1.1.2 PURITY DETERMINATION

DSC provides a rapid and accurate method for purity determination. However it is limited to those compounds that do not decompose on melting. There is also the possibility that the impurity may form a solid solution and thus invalidate the analysis [83].

5.1.1.3 IDENTIFICATION OF SOLVATES

During the process of crystallization, solvent may become incorporated into the crystal structure. Hydrates form when water is the solvent of crystallization. Water is a highly polar molecule and can therefore interact strongly with ions or polar molecules by ion-dipole or dipole-dipole interactions respectively. Due to such interactions, water may become adsorbed onto the surface of a crystal or may occupy a position within the lattice.

The presence of solvent in the crystal structure usually modifies the pharmaceutical properties such as solubility, dissolution rate, bioavailability, chemical stability, powder flow and compaction. Therefore the characterization and analysis of the solvent of crystallization are important in preformulation studies [84].

DSC has been widely used for the identification of solvates. The desolvation endotherm in DSC includes such steps as the breakage of the solvate bonds and vaporization of the solvent. Upon desolvation the crystal structure may rearrange through the breakage and formation of intermolecular forces which include van der Waals interactions and/or hydrogen bonds, in which case the rearrangement of the desolvated structure will also be included in the desolvation process. The area under the desolvation peaks in the DSC curve yields the enthalpies of the desolvation transition [84].

5.1.2 INTERPRETATION OF RESULTS

When two substances are mixed, the purity of each may be reduced and generally broader, slightly lower melting endotherms result. If the curve of the mixture is a simple superposition of those of the single components, an incompatibility is highly improbable. If the solid-solid interaction is extremely weak or non-existent, the reduction of the melting point is usually inconsequential. On the other hand, any large shift in melting point signifies that a strong solid-solid interaction has occurred, although it does not necessarily indicate an incompatibility [76,80].

A peak which is smaller than anticipated could be indicative of an interaction while extra thermal effects, or a component peak which has disappeared completely may also indicate incompatibility. If one of the components degrades before the melting point of the other one it may be possible that the latter substance reacts with the decomposition products. More reliable conclusions can be made

if duplicate samples of the mixtures are stored in the DSC holders at elevated temperatures for a few weeks [78].

5.1.3 DSC FOR SUPPOSITORIES

DSC is useful as a support technique for the development of suppositories. The development of procedures as both predictive and ongoing physical stability tests, for both bases and formulated products, can be undertaken. The initial choice of the suppository base is easily made with DSC. The checking of manufacturing reproducibility is also efficient. Furthermore the identity and uniform distribution of the drug substance may be determined [85].

The ideal melting point for suppositories is approximately 32°C. Triglyceride suppository bases generally exhibit a broad, irregularly shaped DSC endotherm peak at around 30-38°C. This peak corresponds to the melting temperature of the base. The broad shape is attributed to the fact that the triglycerides are a heterogenous mixture of compounds of similar properties, but with a polydisperse molecular mass distribution [81].

Fatty suppositories are complex mixtures with different crystalline modifications, and undergo slow transitions into the higher melting form during storage. Elevation of melting ranges and large increases in melting times of suppositories after storage result in a reduction of the release rate of drug *in vitro* and an unpredictable response *in vivo* [8,62]. If the melting point of the mass increases to above 37°C, this may lead to products which become ineffective on storage. The hardening effect which may result in little or no suppository melting, can cause local irritation, a defaecatory reflex, or bowel obstruction [86].

As the suppositories age, some endotherms reduce in size or disappear and are replaced by others due to the decomposition products. Storage for a short time at elevated temperatures successfully simulates the effects of ageing on the DSC thermograms. Comparing the results of thermal analysis with physical hardening data allows prediction of shelf-life hardening [86].

5.2 THE APPLICATION OF DSC TO AMOXYCILLIN SUPPOSITORIES

In this investigation, DSC was used as a rapid screening tool during the preformulation studies of amoxycillin suppositories, in order to detect potential interactions and incompatibilities between

amoxycillin trihydrate and the semisynthetic bases which were used to formulate amoxycillin suppositories.

5.2.1 EXPERIMENTAL

5.2.1.1 REAGENTS

Amoxycillin trihydrate, compacted (Clinimed, East London, RSA), the *Novata BD, Novata 299, Witepsol W35* and *Suppocire A32* suppository bases described in Section 2.2.1 and amoxycillin suppositories from the batches manufactured for dissolution testing (Section 4.2.1.1) were used in the DSC analysis.

5.2.1.2 PROCEDURE

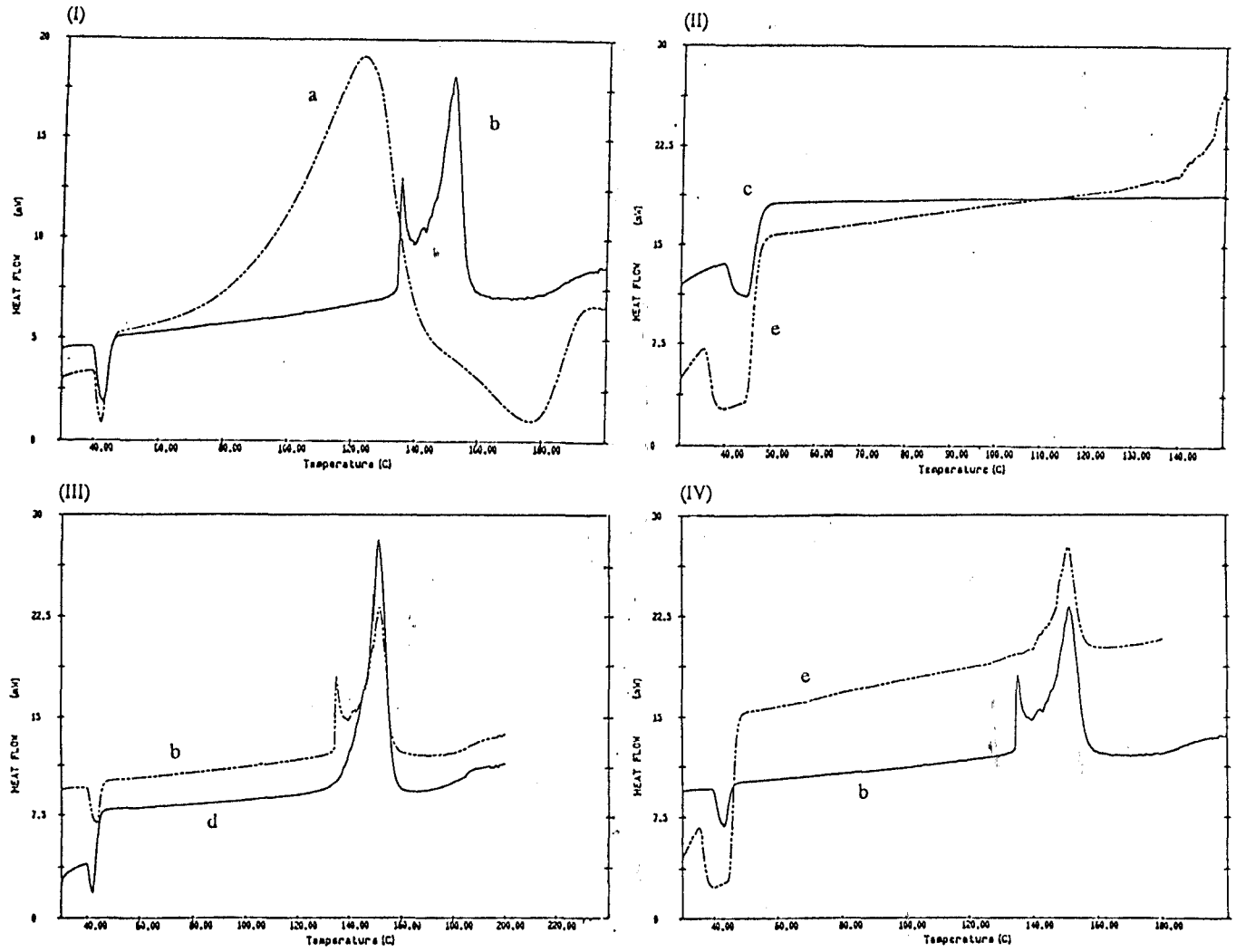
Tests were carried out on each of the unformulated suppository bases, amoxycillin trihydrate powder, freshly manufactured suppositories, suppositories which had been stored at room temperature for one month and physical mixtures of unmelted base and drug. The whole suppositories and flakes of base were size reduced with a stainless steel blade. Physical mixtures were made up by homogenizing amoxycillin powder with base, using a blade, in a ratio of 1 to 3, which was the ratio of drug to base in the suppositories.

A Perkin-Elmer DSC7 Differential Scanning Calorimeter (Norwalk, Connecticut, USA) was used to investigate the thermal properties of the suppository bases and amoxycillin. The instrument was calibrated using indium and all experiments ran at a rate of 10°C per minute.

Samples weighing between 8 and 12 mg were placed in aluminium pans and compared to an empty reference pan. The aluminium sample pan lids were not crimped in place, but were placed loosely on top of the sample pan. The test runs were carried out at a rate of 10°C per minute, over a temperature range of 50°C to 200°C under nitrogen purge.

5.2.2 DSC RESULTS

Figures 5.1 and 5.2 illustrate examples of the DSC thermograms obtained after heating various samples from 50°C to 200°C.



(a) amoxycillin trihydrate powder

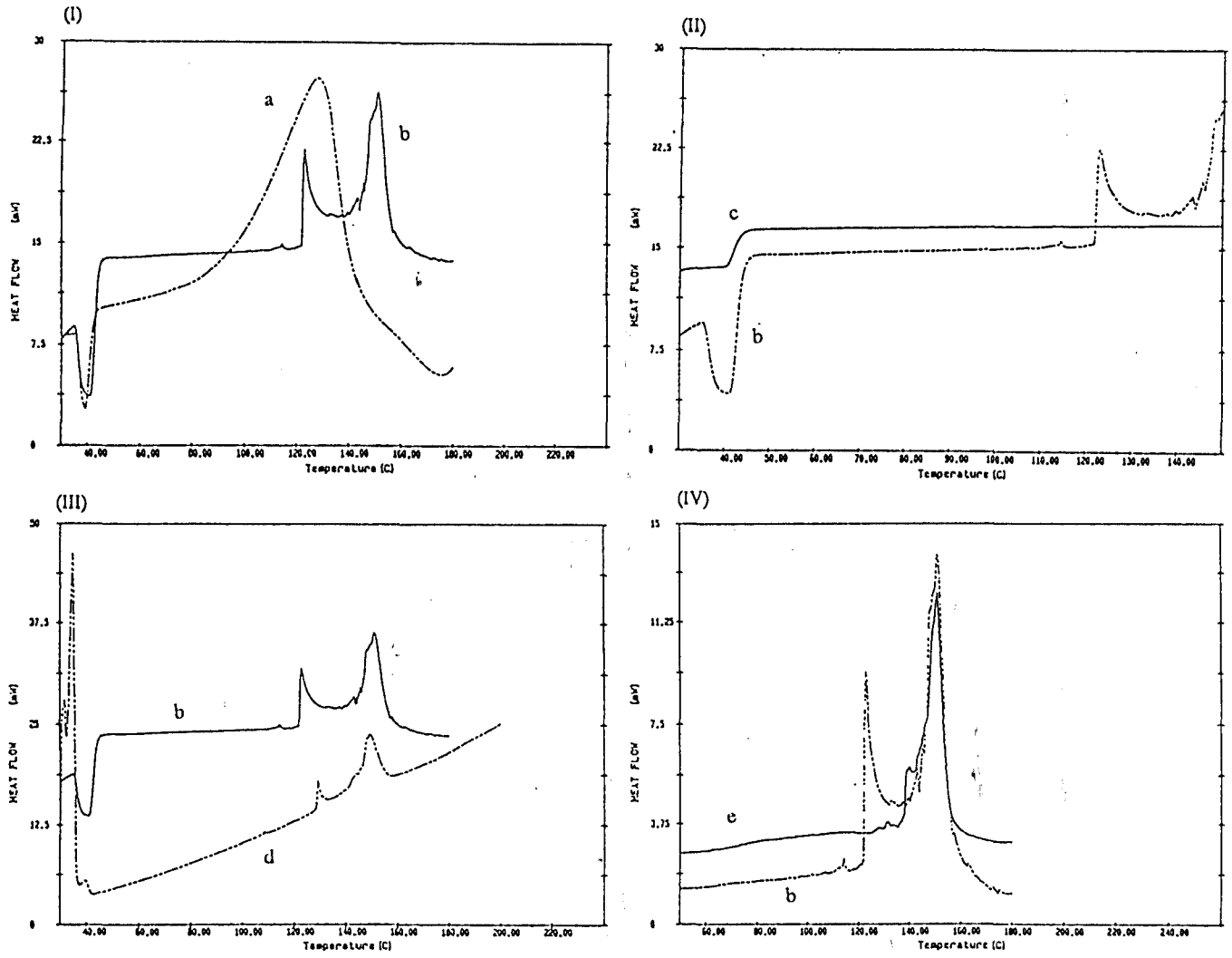
(b) one month old amoxycillin suppository

(c) suppository base

(d) physical mixture of amoxycillin and base

(e) newly manufactured amoxycillin suppository

Figure 5.1 DSC thermograms of amoxycillin trihydrate and Suppocire A32 suppository base



(a) amoxycillin trihydrate powder
(b) one month old amoxycillin suppository
(c) suppository base
(d) physical mixture of amoxycillin and base
(e) newly manufactured amoxycillin suppository

Figure 5.2 DSC thermograms of amoxycillin trihydrate and Novata BD suppository base

Amoxycillin trihydrate exhibited a broad endotherm, which peaked at approximately 120°C (Figure 5.1(Ia) and 5.2(Ia)).

The broad, irregular endotherm, occurring from 30°C to 38°C, which is typical of semisynthetic suppository bases [81] was not obvious in these thermograms, because of the limitation of the instrument not to respond to temperatures below 40°C. The unformulated suppository bases did not produce any endotherms beyond 40°C when analyzed (Figure 5.1(IIc) and 5.2(IIc)).

The physical mixtures of amoxycillin and suppository base exhibited a single endotherm peak at approximately 150°C in the case of the mixtures containing *Suppocire A32, Witepsol W35* and *Novata 299*, illustrated in Figure 5.1(IIId), whereas the mixture containing *Novata BD* exhibited an endotherm at approximately 130°C as well as an endotherm at approximately 150°C, illustrated in Figure 5.2(IIId).

All of the freshly made amoxycillin suppositories exhibited a single, irregularly shaped endotherm, which occurred at 150°C. This endotherm was roughly superimposable over that of the physical mixture. In comparison, the amoxycillin suppositories which had been stored for a month exhibited an additional, lower melting point endotherm. Figures 5.1(IV) and 5.2(IV) are typical examples of these endotherms.

5.2.3 DISCUSSION

The endotherms which resulted when the physical mixtures of amoxycillin and suppository base and the amoxycillin suppositories were analyzed suggest a number of possible alternatives. These DSC results could indicate degradation of amoxycillin or suppository base, or an interaction between amoxycillin and suppository base, or a transformation in the polymorphic form of amoxycillin.

The single broad endotherm of amoxycillin trihydrate (Figures 5.1(Ia) and 5.2(Ia)) and the absence of endotherms in the results from the unformulated bases (Figure 5.1(Ic) and 5.2(Ic)) suggest that the separate and individual degradation of these substances under DSC conditions was not responsible for the endotherms which resulted when these substances were combined.

When amoxycillin and a suppository base were combined in a physical mixture, melting

endotherms appeared and possibly shifted, indicating a conceivable interaction between these compounds. Such an interaction may have resulted in degradation of the amoxycillin, explaining the shift in the endotherm at 150°C (Figures 5.1(III) and 5.2(III)), and the slightly smaller endotherm at 120°C (Figure 5.2(III)).

The theory of an interaction between the drug and the suppository base is supported by the fact that the melting endotherms of an amoxycillin suppository were further altered by ageing of the suppository (Figure 5.1(iv) and Figure 5.2(iv)), suggesting increased interaction on storage. Each of the newly manufactured amoxycillin suppositories exhibited an endotherm at approximately 150°C, while the suppositories that had been stored for a month exhibited an additional endotherm at approximately 120°C.

If these results do indicate an interaction between drug and base, this interaction may be due to a chemical reaction, such as a reaction between the free hydroxyl groups of the suppository base and reactive functional groups on the amoxycillin molecule, or it may be catalyzed by the elevated temperatures of the DSC analysis.

It is known that on storage, the semisynthetic suppository bases generally exhibit higher melting times and increased melting ranges due to hardening of the bases [81]. The difference between the endotherms of a newly manufactured suppository and one which has been stored for some time could be due to some endotherms becoming smaller or disappearing, to be replaced by others due to decomposition products.

Finally, the shift in endotherm may be due to a change in the crystalline form of amoxycillin, caused by an interaction between amoxycillin and the base.

While the shift in endotherm signifies that either degradation, transformation or some sort of interaction has occurred, it does not necessarily indicate an incompatibility. When the DSC results are compared with the results of the *in vitro* release experiments, it becomes apparent that a reaction or interaction which has occurred during storage, has caused a change in the amount of drug released from each type of suppository. The hypothetical interaction or reaction may have been favourable, as in the case of *Novata BD*, where drug release improved on storage (from 87% to 99%); or it may have been unfavourable, as in the case of *Novata 299*, *Witepsol W35* and *Suppocire A32*, where the amount of drug released decreased on storage (85% to 74%, 50% to

31%, and 44% to 7% respectively). Although not obvious in the DSC results, these latter bases with many free hydroxyl groups could have interacted more readily with amoxycillin. The potential interaction did not cause degradation of the amoxycillin, as approximately 100% of the drug was accounted for.

The greater the stress to which a sample is exposed, the greater is the risk of altering that sample. The interactions or transformations which were seen under the accelerated conditions therefore may not be comparable with the results that occur at room temperature, since these results may not appear at all under normal conditions.

5.2.4 CONCLUSION

Thermal analysis proved useful for the determination of possible interactions or reactions between amoxycillin and semisynthetic suppository bases. However the fact that the transformations or interactions which occur under accelerated conditions are not necessarily comparable with the results that occur at room temperature, should be kept in mind when interpreting the results.

When analyzed separately, amoxycillin and the suppository base do not degrade under DSC conditions. However in a physical mixture of drug and base, the shift of the amoxycillin endotherm from 120°C to 150°C illustrated in the DSC results indicates that some sort of interaction occurs between amoxycillin and the suppository bases under accelerated thermal analysis. Degradation or another polymorphic form of the drug may be occurring as a result of this interaction.

An interaction between substances generally results in a decrease in size and melting point of an endotherm, but an interesting result was the development of a large, second endotherm. The appearance of this second endotherm may be interpreted to support the change, after storage for a month, in drug release from the various suppositories during dissolution testing. This endotherm may indicate an interaction between the suppository base and amoxycillin. This interaction may be favourable and result in improved drug release, as in the case of the *Novata BD* suppositories, or it may be unfavourable and cause decreased drug release, as was observed with the *Novata 299, Witepsol W35* and *Suppocire A32*.

CHAPTER SIX
DETERMINATION OF AMOXYCILLIN IN BIOLOGICAL FLUIDS

6.1 INTRODUCTION

The analysis of amoxycillin in biological samples requires a selective, sensitive, accurate and rapid technique. The antibiotic may be present in a complex biological matrix of plasma, serum, urine, tissue or bile from which its concentration must be selectively determined [34]. The optimal antibiotic level should exceed the minimum inhibitory concentration by as large a margin as possible to ensure that a sufficient level of antibiotic is present in infected tissues. Routine monitoring of antibiotics generally involves quantitation of drug levels in the microgram concentration range, as the administered doses are high and antibiotics are generally metabolized to only a small extent [34]. An analytical technique must be sensitive enough to determine drug concentrations in this range. Pharmacokinetic and other *in vivo* studies generate a large number of samples which necessitates the development of an assay method with a high sample throughput requiring a minimum of supervision for routine determinations. The choice of a method should ultimately be determined by these considerations as well as an evaluation of factors such as the stability and recovery of the compound of interest and the precision of the assay.

Several methods used to monitor amoxycillin have been discussed in Section 3.1.1. The conventional method of monitoring antibiotics in body fluids has been microbiological assay [1,2]. However, the microbiological assays available are tedious and have limited selectivity, specificity and accuracy at lower concentration levels. These considerations have prompted the development of other methods for the assay of antibiotics such as immunoassays, gas-liquid chromatography, high performance thin layer chromatography, derivative spectrophotometry, spectrophotometry and fluorimetry [34,86].

These methods are seldom used since HPLC has proved to be superior in terms of selectivity, sensitivity, accuracy, speed of analysis and specificity.

6.1.1 HPLC ANALYSIS OF AMOXYCILLIN IN BIOLOGICAL FLUIDS

Perusal of the literature has revealed that HPLC has been used increasingly for the determination of antibiotics in body fluids as it is uniquely suited to the separation of closely related compounds

when compared to nonselective techniques such as bioassays and immunoassays. HPLC permits the simultaneous assay of a drug, its prodrug or its metabolites, whether they are active or inactive [87]. This allows direct comparison of pharmacokinetics or penetration into tissues following drug administration [34]. HPLC has unlimited potential; it can deal with a variety of drugs, and both low and high molecular weight compounds. Reversed phase chromatography, which is most commonly used, affords a great selectivity by the choice of the mobile phase composition and column packing type.

However, HPLC does have a limited capacity as samples cannot be concurrently assayed, as with bioassays and immunoassays [34]. Sample cleanup is required in most instances, so that the drug is extracted from the biological matrix. A further shortcoming is the lack of versatility when different drugs have to be regularly analyzed as changing from one method to another takes some time. Furthermore HPLC requires expensive equipment, although this is partly compensated by its low running costs [34].

Numerous HPLC methods have been described for the analysis of amoxycillin in biological fluids (Table 6.1) [34]. Using UV detection, limits of quantitation of 0.5 μg/mL and lower have been determined in plasma, serum and urine [42,43,49,89-91].

To improve assay performance and obtain lower limits of detection, pre- or post column derivatization has been used [92-94], as well as column switching [94] and ion pairing [43,94-96]. Amoxycillin does not have intrinsic fluorescent properties, but post-column derivatization with fluorescamine allows fluorescent detection to be used [92,94]. These HPLC derivatization methods are sensitive and specific, but require lengthy sample workup and often complex instrumentation.

The selection of adequate separation for HPLC analysis of a compound is dependent on the polarity and ionizable functional groups of the drug, and the biological matrix from which the drug is to be separated. The chromatography of amoxycillin has been performed on C_8 and C_{18} alkyl-bonded phases with mixtures of acetonitrile or methanol and phosphate buffers as the mobile phase. A summary of selected methods is shown in Table 6.1. The organic modifier content in the mobile phase depends on the polarity of the drug and on the type of reversed phase packing. The choice of pH for the extraction technique and separation stages is critical. The pH of the buffer component of the mobile phase is a determinant in the retention of ionizable drugs.

Table 6.1 Examples of HPLC methods used for *in vivo* analysis of amoxycillin

Ref	Body fluid or tissue	Sample treatment	Stationary phase	Mobile phase	Derivatization	λ (nm)	Limit of detection
42	Chinchilla middle ear fluid, plasma	Perchloric acid precipitation	C_8	MeOH-phosphate buffer pH 3-AcN	-	UV 230	0.5 μg/mL
43	Plasma	Baker-10 C_{18} SPE cartridge, IPA	C_{18}	MeOH-phosphate buffer pH 6-IPA	-	UV 229	0.1 μg/mL
89	Plasma	Sep-Pak C_{18} SPE cartridge	C_8	MeOH-phosphate buffer pH 7	-	UV 225	0.5 μg/mL
90	Plasma, saliva, urine	Perchloric acid precipitation	C_8	MeOH-phosphate buffer pH 4.6	-	UV 225	0.5 μg/mL
91	Plasma, middle ear fluid	Bond-Elute C_{18} SPE cartridge	C_8	AcN-phosphate buffer pH 5-IPA	-	UV 210	0.125 μg/mL
92	Urine	Dilution	C_{18}	MeOH-acetic acid	Post-column fluorescamine	Fluorescence 395/485	2.5 μg/mL
93	Plasma, urine	Perchloric acid precipitation, dichloroethane	C_8	MeOH-phosphate buffer pH 8	Post-column imidazole and mercuric chloride	UV 310	0.025 μg/mL
94	Plasma, urine	Perchloric acid precipitation (plasma)	C_{18}	MeOH-phosphate buffer pH 7.4-IPA	Column switching, post-column fluorescamine	Fluorescence 372.5/470	0.01 μg/mL
95	Urine	Dilution, filtration	C_{18}	MeOH-phosphate buffer-IPA	Post column degradation with sodium hypochlorite	UV 270	1 μg/mL

Table 6.1 Continued

Ref	Body fluid or tissue	Sample treatment	Stationary phase	Mobile phase	Derivatization	λ (nm)	Limit of detection
96	Bovine milk	Dilution	Phenyl	AcN-MeOH-phosphate buffer-IPA	-	200-340 UV photodiode array	100 ppb
97	Serum	Direct injection	Silica	AcN-phosphate buffer pH 2	-	-	-
98	Plasma	C_8 AASP SPE cartridge with on-line elution	C_{18}	MeOH-phosphate buffer pH 7.6-IPA	-	UV 234	0.025 μg/mL
99	Urine	Sep-Pak SPE C_{18} cartridge, IPA	C_{18}	AcN-phosphate buffer pH 7.1	-	UV 229	7.5 μg/mL
100	Urine	-	-	-	Derivative spectrophotometry	UV 240-340	-
101	Catfish, salmon tissue	Trichloroacetic acid precipitation, Sep-Pak cartridge	C_{18}	AcN-phosphate buffer pH 5.6	Precolumn trichloroacetic acid and formaldehyde	Fluorescence 358/440	0.002 μg/mL
102	Bovine milk	Et_4NCl and AcN deproteinization	C_{18}	AcN-phosphate buffer gradient	-	UV 214	2-5 ppb

IPA = Ion Pairing Agent SPE = Solid Phase Extraction

6.1.2 SAMPLE PREPARATION FOR HPLC

6.1.2.1 INTRODUCTION

Biological fluids are complex mixtures of proteins, carbohydrates and lipids. The biological sample is usually pretreated prior to chromatography to deplete the sample of proteins and to extract the compound of interest quantitatively.

Techniques for sample pretreatment prior to HPLC assay include simple dilution of sample in an appropriate solvent, protein precipitation, filtration, solvent extraction and use of cartridges of various types that allow pre-chromatography or enrichment of the sample [49]. Generally the type of pretreatment and the degree of sample cleanup is dictated by the efficiency and selectivity of the technique used for analysis. The more specific and efficient the chromatographic procedure, the less sample extraction and sample cleaning are necessary. In addition to cleaning up the sample, the extraction steps are also necessary to improve the detection limit of the assay by concentrating the drug [49].

6.1.2.2 DIRECT INJECTION

Direct injection of biological samples is the simplest and most rapid way of introducing them onto an HPLC system. However direct injection results in a rapid increase in backpressure and a deterioration of column performance due to precipitation of proteins on the column. Beyond the deleterious effects these biological materials have on the injector, the column packing material and the pump, their presence will frequently interfere with the separation of the antibiotic under study. Consequently, some form of sample preparation is almost always required [49,86].

A method for direct application of untreated serum or urine samples onto silica columns, to reduce the number of pretreatment steps was proposed by Admovics [96]. This simple approach did not lead to the rapid deterioration of the column as might be expected, as the eluents were almost totally aqueous and thus did not precipitate serum proteins onto the column. However this method was only suitable for the detection of plasma concentrations between 20 and 100 μg/mL which are not sufficiently sensitive for most kinetic studies. In addition if the aqueous mobile phase contained organic modifiers at concentrations greater than 15%, precipitation of proteins occurred.

6.1.2.3 DILUTION

Dilution is mainly applicable to biological fluids in which the antibiotic concentration is high, and there is little or no protein present, for example urine and bile. In these body fluids, dilution by an appropriate solvent is often sufficient to eliminate background interferences [49], although in urine samples the many endogenous compounds present in high concentrations may produce a multitude of chromatographic peaks such that resolution of the compound under investigation from these endogenous materials becomes a problem [87].

The dilution solvent may be water, but because the ionic strength may affect the retention time of an antibiotic, it may be useful to adjust the ionic strength of the diluting solvents to those of the biological samples under investigation [49]. If dilution alone is used prior to chromatography, a guard column must be installed in front of the analytical column [87].

Simple dilution should not be applied to serum or plasma. As with direct injection, eluents which contain a high proportion of organic modifier will, after numerous injections, cause precipitation of the serum or plasma protein, followed by obstruction and rapid deterioration of the chromatographic column.

The determination of amoxycillin in urine using HPLC has included diluting the urine sample and then subjecting it to analysis [89,91,94]. However, at 230 nm which is the wavelength often used for detection of amoxycillin, endogenous compounds have a high molar absorptivity, suggesting that this approach lacks adequate selectivity [89,98].

6.1.2.4 SOLVENT EXTRACTION

Organic solvents of low polarity can be used to selectively extract some drugs from biological matrices by solvent partitioning, for example interfering urine components have been extracted into n-amyl alcohol-dichloroethane [92] in an attempt to decrease the sensitivity limit. The most widely used solvents are di-ethyl ether, ethyl acetate, methylene chloride and chloroform [87]. The compound of interest is removed from the biological matrix by use of a suitable solvent system and specific pH conditions, thereby allowing selective extraction of the desired components and leaving behind unwanted materials. The solvent is then removed by evaporation and the dried residue is reconstituted in a small volume of the mobile phase or a similar solvent for injection onto the

HPLC system. Extraction methods for isolating and concentrating antibiotics from a sample matrix may vary from a simple one step extraction to complex back extractions requiring several manipulations.

Liquid-liquid extraction works well for lipophilic substances such as chloramphenicol [87]. Penicillinase-resistant penicillins such as isoxazolyl penicillins and nafcillin can be extracted from body fluids into organic solvents of medium polarity, such as chloroform and methylene chloride. These penicillins are concentrated in the aqueous phase while the plasma or serum lipids are extracted into the organic phase [34].

However the measurement of penicillins such as amoxycillin and ampicillin in biological fluids is more challenging since their amphoteric nature makes extraction into organic solvents difficult [34,43,97]. Amoxycillin exists as a zwitterion over almost the entire pH range [98] and is a hydrophillic molecule with relatively high water solubility. The drug is also slightly soluble in polar solvents such as ethanol, methanol and acetone. Amoxycillin is unstable in the strongly acidic solutions required for the drug to be present in the unionized form (pH values below 2.4) [43]. As a consequence of these physicochemical properties it is almost impossible to extract amoxycillin from plasma by liquid-liquid extraction [43].

6.1.2.5 DEPROTEINIZATION

Polar compounds are often directly determined in plasma and serum following precipitation of proteinaceous material [34]. This technique is used to deproteinize the sample and to solubilize the drug [87]. Either acids or organic solvents may be used as precipitants [49,91]. Acids such as perchloric acid, trichloroacetic acid and trifluoroacetic acid and organic solvents such as acetonitrile, methanol, dimethylformamide and 2-propanol have been used. These techniques involve mixing a small amount of plasma or serum (50-100μl) with the same volume or excess of the deproteinizing agent [49].

Protein precipitation by addition of perchloric acid has been described by several authors [42,90,93,94]. Vree *et al* [90] circumvented the problem of the difficult extractability of amoxycillin by directly injecting onto the chromatograph the supernatant obtained after deproteinization of the sample with perchloric acid. This method is rapid and suitable for immediate analysis of plasma, urine and saliva samples, and is ideal for drug monitoring purposes.

A post-column derivatization procedure using imidazole and mercuric chloride was reported by Carlqvist and Westerlund [93]. The plasma and urine samples were purified by protein precipitation with perchloric acid, followed by extraction with dichloroethane. This method allowed a sensitivity of 200 ng/mL in urine and 25 ng/mL in plasma, but the column had to be repacked every 15 injections due to column deterioration.

A change in pH, caused by acid precipitation, may cause degradation of the sample. Amoxycillin is unstable under acidic conditions and must be analyzed immediately after preparation, thus precluding automated injection [34,93,98]. In order to enhance the stability of amoxycillin, the samples may be neutralized prior to injection. Although neutralization may improve stability when a large number of samples are being analyzed, reinjection of the samples the following day is not reliable. Erdman *et al* [42] used this method of neutralization after precipitation, before injecting middle ear effusion and plasma samples onto HPLC columns. Potassium chloride was added to precipitate the unreacted perchlorate.

The risk of preparing an unstable sample by protein precipitation is minimised if the precipitation is performed with the same organic solvent as is used in the mobile phase, instead of an acid [49]. Organic solvents, although less effective precipitants, are mostly employed for highly protein bound antibiotics to avoid co-precipitation [103]. The relative order of their protein precipitating capability is acetonitrile > acetone > ethanol > methanol, which is approximately inversely related to their polarity [103]. Relatively high organic solvent volumes must be added to ensure complete precipitation of proteins [103]. Sample dilution which occurs with the addition of the precipitating agent may be problematic if low concentrations of antibiotics are to be measured. Therefore excess solvent can be back extracted. For example if acetonitrile is used, methylene chloride is added to remove the remaining acetonitrile from the supernatant [49]. The use of the organic solvents methanol and acetonitrile, to precipitate protein in biological samples containing ß-lactam antibiotics has been described by several researchers [104-107].

The suitability of protein precipitation by acids or organic solvents, for the assay of large numbers of samples is limited since the column performance tends to deteriorate after the direct injection of between 30 to 40 samples [43,90,93]. The column life may be extended to some extent, when deproteinized samples are injected, by adding a short guard column packed with large-sized molecules of alkyl bonded phase [34].

As an alternative to precipitation, proteins may be removed by ultrafiltration through filters with a 25000 to 50000 molecular mass cut-off, although small protein molecules and other interfering materials may still pass through the filter [49,87,98]. This is a relatively simple method, and has several advantages over precipitation, but it should be kept in mind that analysis of ultrafiltrate will provide a measure of non-protein-bound drug, as opposed to total drug concentration [87,103]. It is also necessary to determine whether drug is adsorbed to the membrane material.

Blanchard [103] performed an evaluation of various techniques for deproteinizing plasma samples. Several types of protein precipitants were assessed, as well as protein removal by ultrafiltration. Ultrafiltration, by means of centrifugation in ultrafiltration cones, provided nearly complete removal of plasma proteins. The protein precipitation data indicated that only very small quantities of 10% trichloroacetic acid and 6% perchloric acid were needed to remove more than 98% of the plasma protein. At a 1:1 (v/v) ratio of precipitant to plasma only methanol and saturated ammonium sulphate failed to remove more than 90% of the plasma protein.

6.1.2.6 SOLID PHASE EXTRACTION (SPE)

A variety of analytes can be isolated from complex matrices using bonded phase extraction techniques. These methods are rapid and can often avoid the use of solvent extraction and protein precipitation. By avoiding the use of highly acidic protein-free filtrate, the resultant drug stability allows assay using autoinjection. Solid phase extraction has offered a potential solution to the non-extractability of amoxycillin.

A sorbent with strong affinity for organic compounds will retain and concentrate those compounds from very dilute samples [108]. The first step of the bonded phase extraction method involves the activation of the extraction material then application of the diluted sample onto the bonded phase resin, to retain analytes together with interfering compounds. Following this a selective solvent is usually used to remove interferences and then another solvent is chosen to wash out the target analytes.

SPE has gained popularity in sample preparation due to its better selectivity and easy operation [91]. SPE has a number of advantages compared to traditional solvent extraction: it is simple, inexpensive and uses very little solvent. SPE does however have some limitations. The interaction between sample matrix and analytes often results in low recovery and solid and oily compounds

in a sample matrix may plug the SPE cartridge, or can block pores in the sorbent so that it becomes overloaded [108]. Sorbents suffer from high carry over values, and batch to batch variation of the sorbents leads to poor reproducibility. The SPE technique is limited to semi-volatile compounds, with boiling temperatures substantially above that of the solvents [108].

Only a few reports have detailed SPE methods for extracting amoxycillin from human biological fluids [43,89,91,98,99]. Lee and Brooks [89] performed a study which detailed a sensitive and selective HPLC assay for the determination of amoxycillin in human plasma using such a bonded phase extraction. Yuan *et al* [91] used SPE for measuring amoxycillin in middle ear fluid and plasma. Chulavatnatol and Charles [99] described a selective and precise method for assaying amoxycillin in urine which used a SPE process combined with ion pairing and Jonkman *et al* [43] used similar principles for determination of the drug in plasma. No internal standard was used in either study. Krauwinkel *et al* [98] were the first to report the use of SPE followed by on-line elution to the HPLC column, for the determination of amoxycillin in human plasma.

6.1.2.7 ION PAIRING

Polar ionized compounds are often difficult to isolate from biological fluids for subsequent analysis. Although it is often possible to adjust the pH of weak acids or bases to give the unionized, extractable form, the drastic extraction conditions needed for those compounds with pK_a values outside the range 3-9 can lead to problems of solute stability [109]. Additionally, for aprotic molecules and materials that are ionized at all pH values, pH adjustment techniques are not possible. Although the primary equilibrium may involve the formation of an ionized solute, the physicochemical nature of this ionized species can be altered by introducing a second equilibrium, leading to the formation of ion pairs. Ion pairs possess a low aqueous solubility and they tend to move from areas of high polarity to areas of low polarity. Ion pair formation can be highly specific and the combination of ion pair formation and distribution can contribute great selectivity to an extraction procedure [109].

Amoxycillin, a polar compound, elutes early in reversed phase HPLC systems, often resulting in blank chromatograms [93]. Because it is an amphoteric compound, it can be considered a potentially good candidate for ion pairing processes [43,94,109].

Tetrabutyl ammonium (TBA) ions have been shown to be a very good counter ion for the

formation of ion pairs with acidic groups in bulky molecules [43,109]. Jonkman *et al* [43] described a quantitative method for the determination of amoxycillin in plasma, which used ion pair extraction on octadecyl disposable columns with TBA as the counter ion and methanol as the eluent. The TBA-amoxycillin ion pair was retained by the C_{18} extraction column and hydrophilic components of the plasma were washed from the column with a phosphate buffer that contained TBA to keep the amoxycillin in the ion paired state. The TBA-amoxycillin ion pair was then eluted from the column with methanol.

Carlqvist and Westerlund [94] described the determination of amoxycillin in plasma and urine based on ion pairing principals. Clean-up of the biological fluids was obtained by a heart cut from the first column, where the mobile phase contained hexyl sulphate. In the second column, the organic anion was exchanged for a large quarternary ammonium compound, amoxycillin was then retained as an ion pair. Adequate selectivity and sensitivity was obtained by post column derivatization with fluorescamine.

6.1.2.8 CONCLUSION

Direct injection and simple dilution have a detrimental effect on the analytical column, acidic protein precipitating agents are unsuitable since they catalyze degradation of amoxycillin, and liquid-liquid extraction is difficult because of the polar nature of the drug. From this examination of the methods available for the preparation of biological samples prior to HPLC analysis, it would appear as if the most appropriate and efficient method would be either protein precipitation or solid phase extraction using cartridges or a combination of the two techniques.

6.2 METHOD DEVELOPMENT

6.2.1 EXPERIMENTAL

6.2.1.1 REAGENTS

The analytical reagents used are as listed in Section 3.2.1.1. Additional chemicals which were used were ceftazidime pentahydrate (Glaxo Pharmaceuticals, RSA), cephalexin hydrate (Sigma Chemical Company, RSA) and sodium dihydrogen phosphate (Merck, RSA). Drug free serum was obtained from the South African Blood Transfusion Service (PE, RSA). All reagents were at least of

analytical grade.

6.2.1.2 HPLC SYSTEM

The modular HPLC system described in Section 3.2.1.2 (System B) was optimized for *in vivo* analysis of amoxycillin (System C).

SYSTEM C

An Iso-Chrom LC pump (Spectra-Physics, San Jose, CA, USA) with a Waters Intelligent Sample Processor (WISP) Model 710B Automated Sample Injection System (Waters Associates, Milford, MA, USA), a Water 490 Programmable Multiwavelength Detector (Waters Associates, Milford, MA, USA) and a model 561-3002 strip-chart recorder (Perkin-Elmer Corp., Norwalk, Conn., USA) were used. Separation was achieved on an Inertsil ODS-2 4.6x250 mm HPLC column (GL Sciences Inc., Tokyo, Japan), maintained at various controlled temperatures. A guard column kit, part number 1603, with Perisorb RP-18 pellicular packing material (C-603) (Upchurch Scientific, WA) was used. An analytical filter, model A340 (Upchurch Scientific, WA) was used in-line between the pump and autosampler.

6.2.1.3 COLUMN SELECTION

As discussed in Section 3.2.1.4, the analytical column is an integral component of an HPLC system. The polar nature of the serum matrix made it necessary to review the stationary phase in order to optimise retention of amoxycillin and the internal standard, and minimise retention of unwanted serum contaminants.

6.2.1.4 MOBILE PHASE SELECTION AND PREPARATION

The variables involved in mobile phase selection and preparation were discussed in Section 3.2.1.5. The mobile phases described in several published HPLC analyses (Table 6.1) were examined, and compared with the mobile phase used for *in vitro* work. None provided the desired conditions, and several alterations were made to the organic phase, pH and temperature, in order to optimise the mobile phase.

6.2.1.5 PREPARATION OF STOCK SOLUTIONS

Stock solutions of amoxycillin 1.0 mg/mL were prepared as described in Section 3.2.1.8.

A stock solution of 0.02 M potassium dihydrogen phosphate pH 4.5 used for extractions was prepared by accurately weighing 2.72 g KH_2PO_4 and dissolving it in HPLC-grade water, then making to volume in a 1 litre volumetric flask.

Stock solutions of 0.2 mg/mL sulphadimidine, the internal standard were made up by accurately weighing 20 mg sulphadimidine and dissolving it in KH_2PO_4 0.02 M, then making to volume in a 100 mL volumetric flask. The ultrasonic cleaner (Model 8845-30, Cole-Parmer Instrument Company, Illanois, USA) was used to ensure rapid and complete solution of the drug. A 10 mL aliquot was then further diluted to 100 mL with KH_2PO_4, to yield a 0.02 mg/mL solution.

6.2.2 SOLID PHASE EXTRACTION METHOD DEVELOPMENT

6.2.2.1 METHOD OBJECTIVES

When deciding on a choice of extraction method several objectives, including the required purity of the isolate, the final concentration of the isolate, the final solvent required and a definition of the actual isolates, should be examined [110]. The definition of the isolate must include all species that are to be extracted, including both amoxycillin and the internal standard, sulphadimidine.

The required purity of the isolate has a major influence on the complexity of the extraction procedure developed [110]. If the isolate is extracted prior to analysis, the selectivity of the final analytical technique will largely determine the selectivity of the extraction method. For example, if the final analysis uses highly selective detection, the extraction method may be required to provide only a gross cleanup of major matrix contaminants. Conversely a relatively non-selective final analysis may require that the extraction method be highly selective for the isolate, in order to remove analytical interferences.

The choice of extraction method may also be affected by the desired final solvent for the purified isolate. For example if analysis of the purified isolate requires an aqueous environment, a non-polar sorbent for isolate retention may not be the optimum choice, because aqueous solvents often

lack the strength to elute the isolate from the sorbent. An ion-exchange or polar interaction would be a better choice in such a case.

6.2.2.2 ISOLATE CONSIDERATIONS

The skeletal structure and functional groups of the isolate molecule must be examined as these may indicate the type of interactive mechanisms that can be used for the selective retention of the isolate on a SPE cartridge.

The presence of polar atoms indicates a potential for retention by polar interactions. Amine or hydroxyl groups are important because these will hydrogen bond or interact ionically not only with the functional groups on sorbents, but also with the polar silica substrate. Other ionizable groups on the isolate molecule indicate that a potential for retention by ion exchange exists. Based on the dissociation constants of these groups, an ion exchange molecule can be selected that will be the best choice for retention and subsequent elution. Amoxycillin has polar oxygen, nitrogen and sulphur atoms, which are not localized, but spread over the molecular structure, suggesting that the drug may be retained by polar interactions. However since it also has ionizable hydroxyl, amine and carboxylic acid groups it would seem that ion-exchange extraction methods would be appropriate for the retention and isolation of the drug. If the isolate has areas of carbon/hydrogen content only, the isolate would probably be retained by non-polar interactions; the aromatic ring of amoxycillin adds a further possibility of non-polar interactions.

The solubility characteristics of the isolate should be known. The best elution solvents are those in which the isolate is highly soluble. Amoxycillin is soluble 1 in 400 of water and 1 in 200 of methanol but insoluble in acetonitrile [6,7]. Solubility information also helps in selecting solvents for elution of interfering material from sorbent material that retains the isolate. Solvents in which the isolate is not soluble are excellent for washing remaining matrix interferences from the retention sorbent without eluting the isolate.

The stability of the isolate in various solvents should also be examined as well as any pH requirements to ensure isolate stability. For example, amoxycillin undergoes hydrolysis in water and methanol and is most stable at pH 7 [43]. If the isolate is stable in a limited number of solvents, or within a limited pH range, extraction mechanisms appropriate to these limitations should be chosen. Penicillins are unstable compounds, sensitive to nucleophilic and electrophilic

attacks, and can undergo degradation during the analysis process. Degradation can be prevented by rapid sample pretreatment and storage of clinical samples at -70°C or below [34,94].

Other considerations are protein binding and adsorptive properties of the isolate. Some isolates have a tendency to adsorb to certain materials or surfaces. Often when retention of a drug on a non-polar sorbent is either irreproducible or weaker than anticipated, the problem is binding of the drug to proteins that pass through the sorbent unretained.

6.2.2.3 MATRIX CONSIDERATIONS

The nature of the sample matrix may also influence the choice of sorbents and procedures. For example blood is a polar, aqueous matrix, consisting of a high percentage of proteins, fats, salts and a variety of polar substances, all of which may influence the resultant retention. It is important to identify any major matrix components that are similar in structure to the compound of interest or share chemical properties with the isolate, and to identify in what way these substances differ from the drug so that efficient separation can be achieved.

6.2.2.3.1 SERUM, PLASMA AND WHOLE BLOOD

Plasma and serum are commonly extracted by non-polar or ion-exchange SPE. Sample pretreatment usually involves dilution with an equal volume of water or a suitable buffer before applying the sample to the extraction sorbent. Protein binding of isolates may be a problem and should be considered if recoveries of aqueous standards are high but recoveries from samples are low [110].

Whole blood is handled in a similar way to serum and plasma samples. The difference is the presence of the whole red blood cells, which are chemically active, in that many drugs and natural products are taken up into the cells and are therefore not available to the sorbent surface unless the cell is disrupted by the addition of an organic solvent or dilution with buffers. The difficulty of protein binding common in serum and plasma is even greater in whole blood samples [110].

6.2.2.3.2 URINE

The extraction of urine is usually by means of non-polar and/or ion-exchange methods. Urine

samples may be pretreated by diluting with at least an equal volume of water or appropriate buffer before applying the sample to the sorbent. The high and variable salt content of the urine matrix often complicates the use of ion-exchange as the first extraction mode. This problem may be bypassed by first retaining the isolate on a non-polar sorbent, allowing easy removal of the salt through an aqueous wash, followed by isolute elution and subsequent use of an ion-exchange compound. Urinary pigments are also present at high levels in the matrix. Some of these will retain on ion-exchangers, others on non-polar sorbents [110].

6.2.2.3.3 PROTEINS

Proteins are often a major hinderance in the development of an HPLC method. The important properties of proteins are their solubility characteristics which are limited to very polar solvents, their molecular weights which range into millions, their sensitivity to pH and their often globular nature. Most commercially available sorbents are of porosity in the 60 to 100 Å range. Therefore, proteins above 15000 to 20000 molecular weight tend to be too large to penetrate most pores of the sorbent, and are exposed to a minimum of the available active functional groups on the sorbent surface. For this reason, most proteins pass unretained through such sorbents, especially non-polar ones such as C_{18}. Retention of the isolates on these non-polar sorbents from polar sample matrices, followed by washing the sorbent with large amounts of water or buffers is one of the best approaches for removing large amounts of protein from samples.

When a drug is protein bound, its active sites that would normally interact with the sorbent to produce retention are not available for interaction with the sorbent. In addition, if the protein to which the drug is bound is not retained on the sorbent, the drug is carried through the sorbent bed by the protein instead of being retained. Protein binding should be suspected whenever drug standards retain well on the sorbent, but drug present in the sample matrix does not. Amoxycillin is 20% protein bound [2,6].

There are a few effective strategies for overcoming protein binding, thus freeing the drug for retention in the desired manner. The first approach is a change in the pH of the sample. Protein binding is often pH dependent and changing the pH may release the isolate. The addition of protein denaturing agents such as urea or methanol, or precipitants such as acetonitrile, methanol or perchloric acid may also free drug bound to proteins. In some cases protein precipitation may precipitate the isolate from solution, therefore this approach should be used with caution.

6.2.2.4 SORBENT TESTING

The sorbent should be systematically tested in order to select the most appropriate sorbent type and method of extraction. The retention of standard solutions similar to the sample matrix should be optimised. The elution of standards should then be optimised and a wash solvent identified that will remove unwanted contaminants and matrix components, but not elute the isolate. The blank matrix should be tested by pretreating the matrix as planned with wash solvent and eluent. The solvent yielding the cleanest eluent would be the most selective for the isolate of interest. Finally spiked samples can be tested.

Various types of SPE cartridges were tested, and the results are shown in Table 6.2.

The drug was not retained on the C_8, C_{18} end-capped, cyano or phenyl columns under the standard extraction method applied (Section 6.2.2.11). Of the C_{18} cartridges, the best yield of drug and the cleanest eluent was obtained when the Sep-Pak® C_{18} cartridges were used.

Table 6.2 Retention of amoxycillin on various SPE cartridges

SPE cartridge	Extent of amoxycillin retention
Isolute® C_{18}	+
Supelco® C_{18}	+
Sep-Pak® C_{18}	++
Isolute® C_8	-
Isolute® C_{18} end-capped	-
Isolute® CN	-
Isolute® phenyl	-

\- = Drug not retained
\+ = Small amount of drug retained
++ = Large amount of drug retained

6.2.2.5 SAMPLE PREPARATION AND LOADING

Sample preparation prior to loading onto the SPE cartridge can involve simple dilution with an appropriate solvent in order to reduce viscosity, or addition of a buffer to control pH and possibly suppress ionisation, or protein precipitation to remove some of the protein from the sample before extraction.

Several techniques of extracting amoxycillin from human plasma and serum by protein precipitation with either methanol or acetonitrile were examined. Spiked samples were precipitated with either methanol or acetonitrile, but the resultant chromatograms were not sufficiently clean, and the amoxycillin peak was obscured. After several injections the supernatant from the precipitated samples tended to cause an increase in column backpressure. Precipitation with acids was not attempted because of the well documented instability of amoxycillin following acid precipitation [42,90,93,94]. Precipitation alone did not yield the clean extracts necessary to achieve the sensitivity required for the assay.

Precipitation followed by solid phase extraction was used, to prevent clogging of the columns and to release protein-bound drug. This was achieved by adding an equal volume of acetonitrile or methanol to a 1 mL serum sample and then mixing with a vortex mixer (Maxi Mixer®, Model M-16710-12, Thermolyne Corporation, Iowa, USA), followed by centrifugation at 2000 x G for 10 minutes, with a General Purpose Centrifuge (Model HN-SII, Interantional Equipment Company, MA, USA). The supernatant was then extracted. Precipitation with acetonitrile resulted in cleaner extracts than with methanol although the amount of drug recovered was low. This may have been due to the drug being trapped in the precipitate, or the fact that the eluotropic strength of the protein-free solution containing acetonitrile or methanol was sufficient to prevent retention of amoxycillin on the SPE cartridge. Recovery did not increase when the eluotropic strength was decreased by diluting the supernatant with water.

Simple dilution of the sample with an aqueous solution containing an internal standard, before loading it onto the extraction cartridge was attempted. Solutions of different pH were compared as sample diluents, but the pH of the buffer did not significantly influence the amount of drug recovered, possibly because the use of a buffer to suppress ionisation would be unsuccessful as amoxycillin is amphoteric. The addition of an ion pairing agent to the diluting solution, as suggested by some researchers [6,14] did not result in a noticeably greater retention of the drug on the column or higher recoveries. It was decided to dilute the sample with KH_2PO_4 (0.02 M, pH4.5), as described by Lee and Brooks [89]. The internal standard was dissolved in the KH_2PO_4 solution.

Sample loading is a function of the number of active sites available on the packing material. The amount of sample that can be loaded depends on the concentration of all components in the sample, solvent strength and choice of packing material. Overloading of the packing material results in

breakthrough and poor results, as the packing material in the cartridge has a finite capacity. When the amount of sample loaded onto the packing material exceeds the packing material's capacity, the excess sample elutes, resulting in poor separation, poor sample recovery and lack of reproducibility.

When following the method of Lee and Brooks [89], recovery was poor when a 2 mL diluted sample was loaded onto a 1 mL cartridge. However, recovery increased when the same sample was loaded onto a 3 mL cartridge, confirming that the former method had overloaded the 1 mL cartridge. By halving the volume of solvents used in cartridge preparation, the volume of the sample loaded and the wash and elution volumes, recovery of amoxycillin from a 1 mL cartridge was optimised.

6.2.2.6 EXTRACTION CARTRIDGE PREPARATION

The preparation of non-polar C_{18} extraction cartridges prior to application of the biological samples involves the wetting of the hydrophobic stationary phase, followed by conditioning. The wetting solvates and opens the hydrocarbon chains increasing the surface area available for interaction with the compound of interest and creates the proper phase interface between sorbent and sample. The cartridge is solvated with a suitable solvent such as methanol, ethanol, propanol or acetonitrile with typical solvation volumes 1 mL/ 100 mg sorbent. The organic solvent is discarded as waste.

Conditioning the cartridge pre-equilibrates the cartridge and provides optimum conditions for retention of the analyte by removing excess solvating solvent from the stationary phase. The cartridge is usually flushed with a volume of water or buffer, equal to the volume used for solvation and this is discarded to waste. The cartridge should not be allowed to dry out prior to application, or breakthrough and variable recovery may result.

Various solvation and conditioning steps were attempted, to determine the optimum sequence for cartridge preparation. Acetonitrile and methanol have been described most often as solvating agents [43,91,99,101]. Both solvents resulted in similar recoveries of drug and volumes slightly greater or less than those recommended did not alter the results noticeably so long as the solvation was balanced by the conditioning step. The solvating step was essential; if it was omitted, the aqueous conditioning solution was unable to pass through the cartridge.

The conditioning step was equally important; if it was omitted, and the sample was loaded immediately after the column had been wetted, the solvating solvents tended to precipitate protein in the sample, blocking the inlet frit of the cartridge. Water and several buffers were used to condition the cartridges. Retention was better when pH control was used. The most favourable results were achieved when either 0.02 M KH_2PO_4 pH 4.5 or a phosphate buffer of pH 7 were used.

The final wetting and conditioning steps adopted were solvation with 2 mL methanol, followed by pre-equilibration with 400 μL KH_2PO_4 pH 4.5 (0.02 M).

6.2.2.7 DETERMINATION OF THE WASH SEQUENCE

There are two possible strategies for isolation and cleanup of sample components. The chromatographic conditions can be chosen so that the compound of interest will either be unretained while the matrix interferences are adsorbed or retained, while the matrix interferences pass through the cartridge unretained. The first strategy is often used when the desired sample component is present in high concentration, and the compound of interest is eluted before the remaining compounds. The second method is used when the compound of interest is present at low concentration, or multiple components of differing polarities need to be isolated. The compound of interest is retained, while unwanted components are eluted with a solvent which is weak in relation to the compound of interest. The compound of interest is then eluted with a solvent of higher eluotropic strength.

The latter approach was used in this study. Various wash sequences were investigated, in order to optimize the retention of amoxycillin and the internal standard, and ensure that as many endogenous serum contaminants were removed as possible. It was not possible to elute the drug with an organic solvent, or a mixture of aqueous and organic solvents, directly after loading the sample, since the organic portion tended to react with residual proteins on the column, causing precipitation and blockage of the SPE cartridge. Careful use of the wash solvents was important. If the cartridge was washed with a small volume ($<$ 1 mL) of water, the drug was removed along with some of the serum contaminants, whereas a small volume of KH_2PO_4 or phosphate buffer pH 7 removed interferences, but did not elute amoxycillin. If a large volume of either was used, the amoxycillin was eluted along with contaminating interfering compounds. A compromise was reached by washing first with 600 μL KH_2PO_4 ph 4.5 (0.02 M) to remove most of the

interferences, then washing with 200 μl water to remove further contaminants, without eluting the drug.

6.2.2.8 DETERMINATION OF THE ELUTION MIXTURE

The small amount of sample contaminants that remained on the extraction cartridge required the selective elution of amoxycillin from the cartridge. Water, buffer, methanol and acetonitrile were used in various combinations to find the most efficient elution mixture. Acetonitrile proved to be a poor eluent, more than likely because amoxycillin is insoluble in acetonitrile whereas buffers generally promoted retention. Solutions of water and methanol achieved the most efficient elution profile. Water alone did not have sufficient eluotropic strength to elute the maximum amount of amoxycillin, even though it was able to wash a small amount from the cartridge. Methanol, being a "stronger" solvent was able to elute a satisfactory amount of drug, but this yield was improved by the addition of water to the elution mixture. The recovery of drug improved as the fraction of water was increased, for example more drug was recovered with an 80:20 mixture of water:methanol than a 20:80 mixture.

Different volumes of the elution mixture were tested. The most effective elution possible requires a minimum of two bed volumes of eluting solvent, for example a 100 mg sorbent bed requires at least 240μl of solvent for quantitative isolute elution [34].

A final elution mixture of 1 ml of 15:85 methanol:water was employed and the eluent was injected directly onto the analytical column. Smaller elution volumes resulted in higher concentrations of analytes, which were however accompanied by greater interference from contaminants, so it was decided to analyze more dilute eluents, rather than overload the analytical column with unnecessary contamination. It was noted that a number of serum contaminants were eluted by the same solvents that were used to elute the amoxycillin. These contaminants could not be removed by selective washing, without significant loss of the drug, therefore they were allowed to be eluted with the amoxycillin, and the mobile phase composition was altered to control the separation of these compounds from the drug.

Evaporation and reconstitution of samples was investigated. Samples were evaporated to dryness in a rotary vacuum centrifuge (Savant, Hicksville, NY, USA) and reconstituted in a small volume of mobile phase. The aim of this step was to concentrate the final sample for analysis, and reduce

the size of the solvent front. However since detection of amoxycillin at the lower limits of the concentration range, with a final elution volume of 1 mL and injection of a 200 μL aliquot was adequate, it was decided that the time-consuming evaporation step that also has stability implications, was unnecessary.

6.2.2.9 FLOW RATE

Flow rate also affects column efficiency. If the sample flows through the SPE cartridge too quickly it will not have enough time to interact with the packing material and some of the sample will elute with the solvent. This commonly causes poor separation, low retention and poor sample recovery.

The flow rate was determined by measuring the time taken for one SPE cartridge volume (1 mL) of solvent to move through the cartridge. The flow rate chosen for column preparation was gravity (approximately 0.3 mL/minute). The wash solvent was allowed to flow with gravity, but the column was dried on the vacuum manifold, at a flow rate of 1 mL/minute before the elution solvent was applied. The flow rate of the elution solvent was maintained at 1 mL/minute.

6.2.2.10 INTERNAL STANDARD

Although chromatographic conditions for the separation of amoxycillin and salicylic acid as internal standard had been optimised in Chapter 3, salicylic acid was not suitable as an internal standard under the chromatographic conditions used for *in vivo* analysis, since the salicylic acid peak was obscured by serum contaminants.

Cefadroxil, 60 μg/mL [91], hydroflumethiazide, 50 μg/ml [42] and benzoic acid, 0.025 mM [43] have been used as internal standards in other *in vivo* analyses. Benzoic acid, salicylic acid, ceftazidime, cephalexin and sulphadimidine were tested as potential internal standards. Aqueous solutions of these drugs were assayed, using HPLC System C, to determine their elution time in relation to amoxycillin.

Aqueous samples of salicylic acid, benzoic acid and sulphadimidine were detected, but detectable amounts of aqueous ceftazidime and cephalexin were not eluted after 40 minutes. Spiked serum samples were diluted with KH_2PO_4 pH 4.5 (0.02 M) containing each of the potential internal standards, and extracted. Benzoic acid and salicylic acid eluted before amoxycillin, amongst the

large interfering serum peaks, and were therefore not considered suitable. Sulphadimidine was eluted after amoxycillin, was clear of any other serum peaks and was therefore selected as the internal standard for this *in vivo* method.

6.2.2.11 FINAL SERUM EXTRACTION METHOD

The solid phase extraction method described by Lee and Brooks [89] was adapted for the purposes of this study. Serum was used instead of plasma, since the recovery of amoxycillin from serum was greater than from plasma. The final serum extraction method involved activation of the extraction material (Sep-Pak Vac® C_{18} cartridges) with 2 mL methanol followed by 400 μL of 0.02 M KH_2PO_4 pH 4.5; dilution of a 1 mL serum sample with 1 mL 0.02 M KH_2PO_4 pH 4.5 containing 0.01 mg/mL internal standard; application of 1 mL of the diluted plasma onto the extraction cartridge; clean-up with 600 μL 0.02 M KH_2PO_4 pH 4.5 and 200 μL water; and elution with 1 mL of water:methanol (85:15). Each SPE cartridge was used once only, then discarded.

6.2.3 OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS

6.2.3.1 COLUMN SELECTION

Various extraction combinations, including the method of Lee *et al* [89] were carried out using HPLC System B with a Nova-Pak® C_{18} 4 μm 3.9x150 mm column. However optimal resolution was difficult to achieve because all analytes, including amoxycillin, eluted rapidly and close together despite use of varying mobile phase composition. Decreasing the amount of methanol in the mobile phase did not significantly improve the chromatogram. It was decided that a longer column would improve resolution and allow for better retention characteristics. An Inertsil ODS-2 column (GL Sciences Inc., Tokyo, Japan) was tested using the mobile phase described in Section 3.2.2.5. The resolution improved significantly, but the column backpressure was over 3000 psi and too high for use with the autosampler, therefore it was decided to change the mobile phase.

6.2.3.2 MOBILE PHASE PREPARATION AND SELECTION

The major interferences on the chromatogram (Figure 6.1) were two large serum peaks, S2 which was eluted just after the solvent front and had a more or less constant retention time, and S1 which tended to elute at the same time as amoxycillin. The aim of these mobile phase manipulations was

to optimize the separation of amoxycillin from these serum peaks.

The mobile phase was prepared by the method described in Section 3.2.2.2. A methanol-buffer combination resulted in a higher backpressure than an acetonitrile-buffer combination, therefore it was decided to change the organic phase from methanol to acetonitrile. Using a mobile phase of 5:95 acetonitrile:0.05 M phosphate buffer pH 7 as a reference standard, the effect of various changes in mobile phase on the retention of amoxycillin, internal standard and serum peaks of a serum sample extracted using the method described in Section 6.2.2.11, was investigated. The results are shown in Table 6.3.

As anticipated, an increase in the proportion of acetonitrile decreased the retention time of all components. The maximum amount of acetonitrile which still allowed separation of all components was 10% v/v. A decrease in the amount of acetonitrile increased the retention times of the analytes. This improved separation to some extent, but the long retention times often resulted in very broad peaks which were poorly resolved. At ambient temperature, the best resolution was provided by a 5:95 mixture (pH 7).

The pH of the mobile phase also influenced retention and resolution. Optimum resolution was achieved with a pH 8 buffer. However, since the analytical column manufactures advocate the use of buffers within the range 2 to 7 to prolong column life, it was decided to compromise with a buffer of pH 7, which still provided adequate resolution.

6.2.3.3 EFFECT OF TEMPERATURE

A column heater was used, when it was noted that increasing the temperature tended to lower the backpressure of the analytical column as well as decrease the retention times of both the drug and serum peaks. In most cases a temperature increase caused the serum peak S1 to be eluted faster than the drug peak, so that the amoxycillin eluted beyond the major interfering serum peaks. The effect of temperature on retention times is summarised in Table 6.3. It was decided to use the analytical column at a constant temperature of 25°C.

Table 6.3 The effect of mobile phase composition and temperature of analytical column

Mobile Phase Composition[a]	pH	Column temperature	Retention time (min)		
			S1 RT[b]	Amoxycillin RT[c]	Resolution[d]
5:95	7	25°C	14.8	17.2	yes
5:95	7	50°C	11.4	16.4	yes
4:96	7	25°C	11.0	17.4	yes
4:96	7	50°C	11.2	16.4	yes
6:94	7	25°C	12.8	9.2	no
6:94	7	50°C	8.8	9.2	no
7.5:92.5	7	25°C	14.0	6.4	no
7.5:92.5	7	50°C	6.0	6.0	no
10:90	7	25°C	7.2	4.4	no
10:90	7	50°C	5.6	4.0	no
5:95	8	25°c	14.8	22.0	yes
5:95	8	50°C	10.0	14.8	yes
5:95	6	25°C	14.0	6.4	no
5:95	6	50°C	9.6	6.0	no
5:95	5	25°C	15.6	5.2	no
5:95	5	50°C	10.0	4.4	no
5:95	3	25°C	18.0	9.2	no
5:95	3	50°C	10.8	6.0	no
4:96	8	25°C	18.4	32.0	yes
4:96	8	50°C	10.4	18.8	yes

[a] Mobile phase composition is represented by ratio of acetonitrile to phosphate buffer
[b] Retention time of serum peak S1, in minutes.
[c] Retention time of amoxycillin, in minutes.
[d] Resolution of amoxycillin peak from the major serum interferences S1 and S2.

6.2.3.4 FINAL CHROMATOGRAPHIC CONDITIONS

HPLC	System C
Mobile Phase	Acetonitrile:0.05 M phosphate buffer pH 7 (5:95)
Flow Rate	1.0 mL/min
Attenuation	0.02 AUFS
Recorder Input	10 mV
Injection Volume	100 μL
Temperature	25°C
Retention Time	Amoxycillin 18 minutes, sulphadimidine 22 minutes

Typical chromatograms of blank serum and serum spiked with amoxycillin are shown in Figure 6.1. These chromatograms were generated using the conditions listed above.

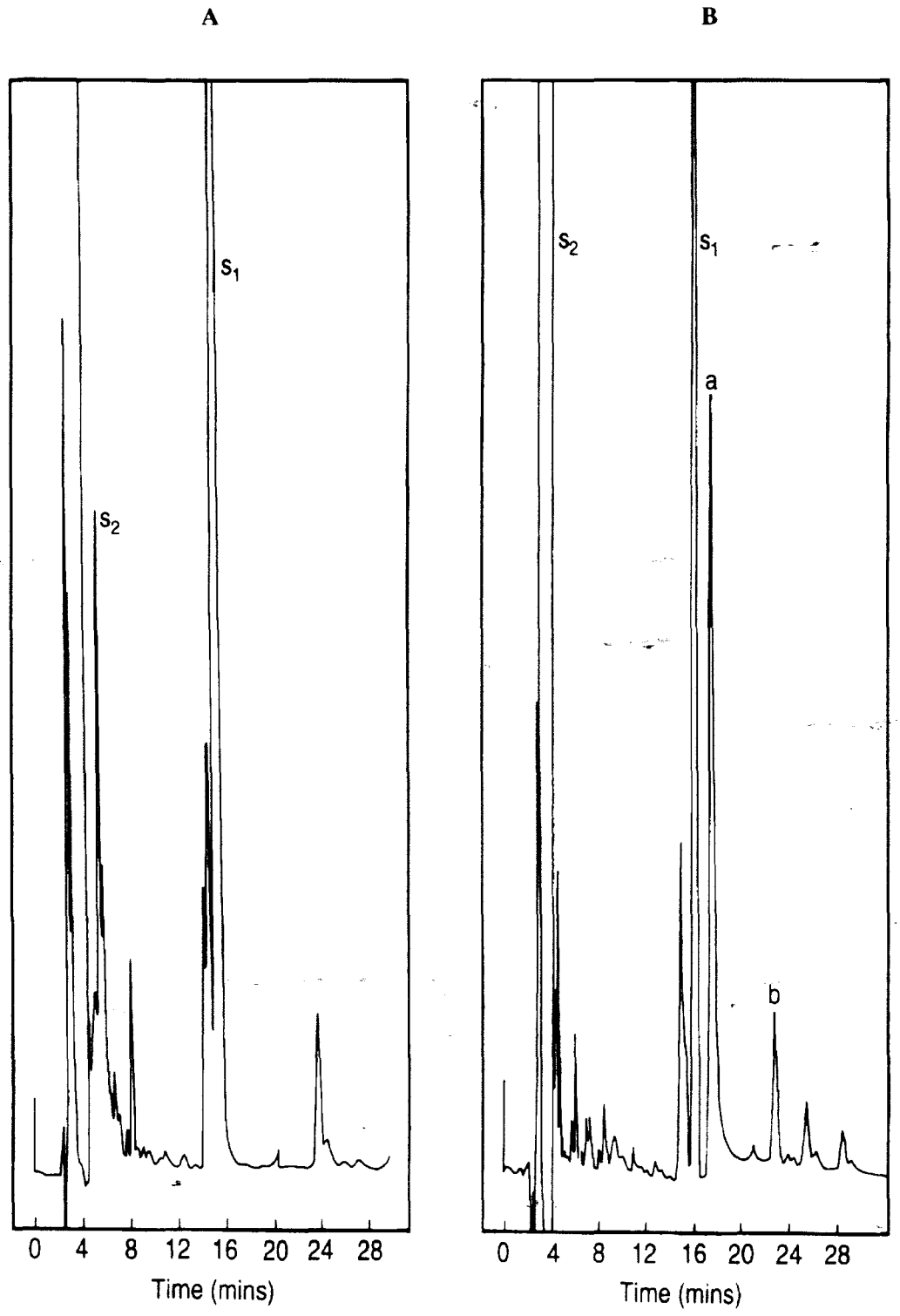


Figure 6.1 Typical chromatogram of (A) blank serum and (B) spiked serum, with (a) amoxycillin, (b) sulphadimidine and serum peaks S1 and S2

6.3 METHOD VALIDATION AND *IN VIVO* STABILITY

6.3.1 INTRODUCTION

The development of an analytical method for *in vivo* samples involves validation of the method and an assessment of the stability of the drug in biological samples, as well as the in-process stability of extracted samples. Thus a study was conducted to validate the assay and to determine the stability of amoxycillin trihydrate in serum and the stability in the aqueous-methanolic eluent after solid phase extraction.

The stability of amoxycillin has been discussed (Sections 1.3 and 3.3.1). The results of this *in vivo* stability study were important in determining optimum storage conditions and the length of time for which serum samples could be stored after donation. The investigation into in-process stability allowed a decision to be made about the length of time for which samples could be refrigerated or left in the autosampler during an extended run.

6.3.2 EXPERIMENTAL

6.3.2.1 REAGENTS

All reagents and equipment used have been described in Sections 3.2.1.1 and 6.2.1.1.

6.3.2.2 CHROMATOGRAPHIC CONDITIONS

Chromatographic conditions were as listed in Section 6.2.3.4.

6.3.2.3 EXTRACTION PROCEDURE

The extraction procedure was as described in Section 6.2.2.11.

6.3.2.4 CALIBRATION CURVES

Calibration curves were constructed on the days that samples were assayed. Aqueous stock solutions of amoxycillin trihydrate 1.0 mg/mL and sulphadimidine 0.1 mg/mL were prepared on

each assay day as described in Section 6.2.1.5. The stock solution of amoxycillin trihydrate was used to spike blank, drug-free serum to achieve a concentration of 6 μg/mL. Concentrations of 4.8, 2.4, 1.2 and 0.6 μg/mL were obtained by the serial dilution of this serum stock solution. Six samples of each concentration were extracted, and then assayed. The calibration curve is shown in Figure 6.2.

6.3.2.5 LINEARITY

Linearity over the concentration range studied was established by the extraction of standards prepared as described in Section 6.3.2.4. Six samples of each concentration were extracted and assayed. The results are summarized in Table 6.4.

6.3.2.6 PRECISION AND ACCURACY

The precision of the assay was determined by calculating the percent relative standard deviation (RSD) of the peak height ratios (drug to internal standard) of six replicate samples of standards. The results are shown in Table 6.4.

Accuracy was assessed by analysis of three replicate serum samples at two concentrations, that had been prepared by an independent analyst. The mean measured concentrations were compared with the known spiked concentrations. Sample concentrations were calculated by interpolation of peak height ratios from the calibration curve. The results are shown in Table 6.5.

6.3.2.7 PERCENTAGE RECOVERY

The percentage recovery from spiked serum samples was assessed by comparing peak heights of samples with the peak heights of aqueous reference samples containing amoxycillin. Replicate samples of two serum concentrations were extracted. Reference samples were prepared by diluting the amoxycillin aqueous stock solution to concentrations equivalent to 100% recovery. The peak heights of amoxycillin in the test samples were then compared to those of the reference samples.

6.3.2.8 LIMIT OF QUANTITATION AND DETECTION

The limit of quantitation was determined by diluting spiked serum until the amoxycillin peak on

the HPLC trace was detectable but no longer measurable. The lower limit of quantitation was taken to be twice this value, based on a signal to noise ratio of 3.

6.3.2.9 STABILITY

A blank serum sample was spiked with amoxycillin trihydrate stock solution and this spiked serum was diluted to 1.2 and 6.0 μg/mL by serial dilution. Aliquots of each solution were set aside for storage at -70°C, -15°C, 4°C and 21°C.

On Day 1, Day 3, Day 7 and Day 14 spiked serum samples were allowed to thaw to room temperature, then these were extracted and assayed in triplicate. The sample stored at 21°C was only assayed on Day 1. The concentration of amoxycillin in each sample was calculated, as well as the percentage drug remaining.

Aliquots of the amoxycillin eluents of samples of both concentrations which were extracted on Day 0 were stored at room temperature and at 4°C, and were assayed again at 12, 24 and 48 hours to determine in-process stability. The concentration of drug was calculated, as well as the percentage of drug remaining after storage.

6.3.2.10 STATISTICAL INTERPRETATION OF THE DATA

The statistical procedure used was that described in Section 3.3.2.9.

6.3.3 RESULTS AND DISCUSSION

6.3.3.1 LINEARITY AND CALIBRATION CURVES

A calibration curve was constructed by linear regression of a plot of peak height ratio versus time after the extraction of replicate serum standards. The validation data are shown in Table 6.4. The linear regression equation was $y=0.5046x-0.1579$ with a correlation coefficient of 0.9997. The calibration curve for amoxycillin in serum is shown in Figure 6.2.

Table 6.4 Linearity and precision data for amoxycillin in serum

Calibration Standards		
Concentration (μg/mL)	**Peak Height Ratio Mean ± SD (n=6)**	**%RSD**
0.6	0.17 ± 0.01	5.9%
1.2	0.50 ± 0.03	6.0%
2.4	1.10 ± 0.06	5.5%
4.8	2.48 ± 0.18	7.3%
6.0	3.06 ± 0.15	4.9%

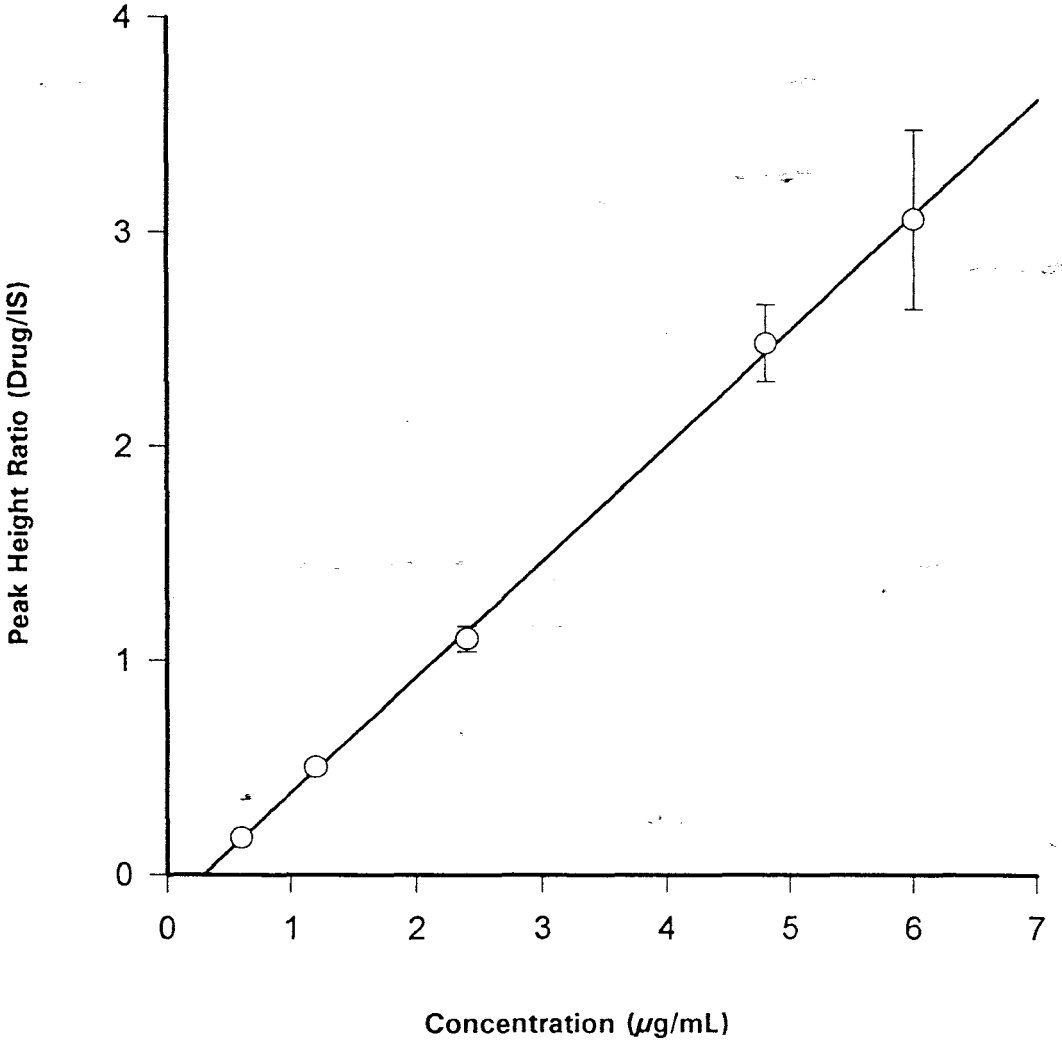


Figure 6.2 Calibration curve of amoxycillin in serum ($y = 0.5046x - 0.1579$; $R^2 = 0.9997$)

6.3.3.2 PRECISION AND ACCURACY

The extraction method was found to be precise, with RSD values less than 8% at all concentrations (Table 6.4) and accurate, with a percentage bias ≤3% (Table 6.5). The percentage bias was calculated as the difference between calculated concentration and spiked concentration as a percentage of the spiked concentration. The assay results for precision and accuracy are acceptable according to the definition of Peng and Chiou [132], since both %RSD and percentage bias are ≤10%.

Table 6.5 Accuracy data for the extraction of amoxycillin from serum

Spiked Concentration (μg/mL)	Calculated Concentration (μg/mL) Mean ± SD (n=3)	Percentage bias
3.6	3.52 ± 0.08	-2.3%
5.4	5.38 ± 0.10	-0.4%

6.3.3.3 LIMIT OF QUANTITATION

The detection limit of amoxycillin in serum was 0.3 μg/mL, determined with a 200 μL injection volume and a sensitivity of 0.02 AUFS. This corresponds with an on-column load of 30 ng. The lower limit of quantitation was taken to be 0.6 μg/mL, with a 60 ng on-column load.

6.3.3.4 PERCENTAGE RECOVERY

Results from percentage recovery studies are shown in Table 6.6. The mean percentage recoveries at the upper and lower limits of the concentration range were 81% and 86% respectively.

Table 6.6 Percentage recovery data after the extraction of amoxycillin from serum

Spiked Sample Concentration (μg/mL)	Reference Samples MH* ± SD (n=3)	Extracted Samples MH* ± SD (n=3)	Percentage Recovery
0.6	4.67 ± 0.58	4.00 ± 0.00	86%
6.0	96.67 ± 2.31	78.00 ± 9.54	81%

* Mean peak height of amoxycillin

6.3.3.5 STABILITY

Calibration curves were constructed in a similar manner to that described in Section 6.3.2.4 on each day of the stability study, and the results are tabulated in Table 6.7.

Table 6.7 Linearity data from spiked serum

Day	Slope	Y-intercept	Correlation Coefficient
0	0.5402	-0.1579	0.9997
1	0.3487	-0.1452	0.9987
3	0.2957	0.1601	0.9914
7	0.3247	0.0773	0.9917
14	0.8859	-0.1878	0.9997

The concentration of amoxycillin remaining in the serum samples after storage was calculated from the calibration data. The stability data of the serum samples containing amoxycillin, stored at 21°C, 4°C, -15°C and -70°C for two weeks, are shown below in Tables 6.8 and 6.9.

Table 6.8 Stability data for amoxycillin 1.2 μg/mL in serum stored over a 14 day period

Day	Concentration (μg/mL), Mean ± SD (n=3)			
	21° C	4° C	-15° C	-70° C
0	1.20 ± 0.09	1.20 ± 0.09	1.20 ± 0.09	1.20 ± 0.09
1	0.85 ± 0.10	0.88 ± 0.03	1.15 ± 0.06	1.15 ± 0.06
3	-	0.85 ± 0.18	1.08 ± 0.02	1.15 ± 0.06
7	-	0.66 ± 0.05	1.08 ± 0.13	1.10 ± 0.17
14	-	0.27 ± 0.03	1.06 ± 0.05	1.10 ± 0.26

Table 6.9 Stability data for amoxycillin 6.0 μg/mL in serum stored over a 14 day period

Day	Concentration (μg/mL), Mean ± SD (n=3)			
	21° C	4° C	-15° C	-70° C
0	5.90 ± 0.79	5.90 ± 0.79	5.90 ± 0.79	5.90 ± 0.79
1	4.92 ± 0.19	5.11 ± 0.10	5.90 ± 0.05	5.90 ± 0.05
3	-	4.22 ± 0.77	5.93 ± 0.06	5.92 ± 0.08
7	-	2.38 ± 0.03	5.91 ± 0.01	5.90 ± 0.10
14	-	0.40 ± 0.05	5.88 ± 0.03	5.89 ± 0.07

The percent of amoxycillin remaining in each serum sample was calculated from the measured percentage difference in response. The results are shown in Table 6.10.

Table 6.10 Percent drug remaining in serum after storage

Day	21° C		4° C		-15° C		-70° C	
	1.2 μg/mL	6.0 μg/mL	1.2 μg/mL	6.0 μg/mL	1.2 μg/mL	6.0 μg/mL	1.2 μg/mL	6.0 μg/mL
0	100%	100%	100%	100%	100%	100%	100%	100%
1	71%	83%	73%	87%	96%	100%	96%	100%
3	-	-	71%	72%	90%	101%	96%	100%
7	-	-	55%	40%	90%	100%	92%	100%
14	-	-	23%	7%	88%	100%	92%	100%

The significance and relevance of each change in concentration was determined using the method of Timm *et al* [52]. The results from the determination of percent change from initial concentration of amoxycillin 1.2 μg/mL and 6.0 μg/mL, after storage at 21°C, 4°C, -15°C and -70°C for 14 days, are depicted in Figures 6.3 and 6.4 respectively.

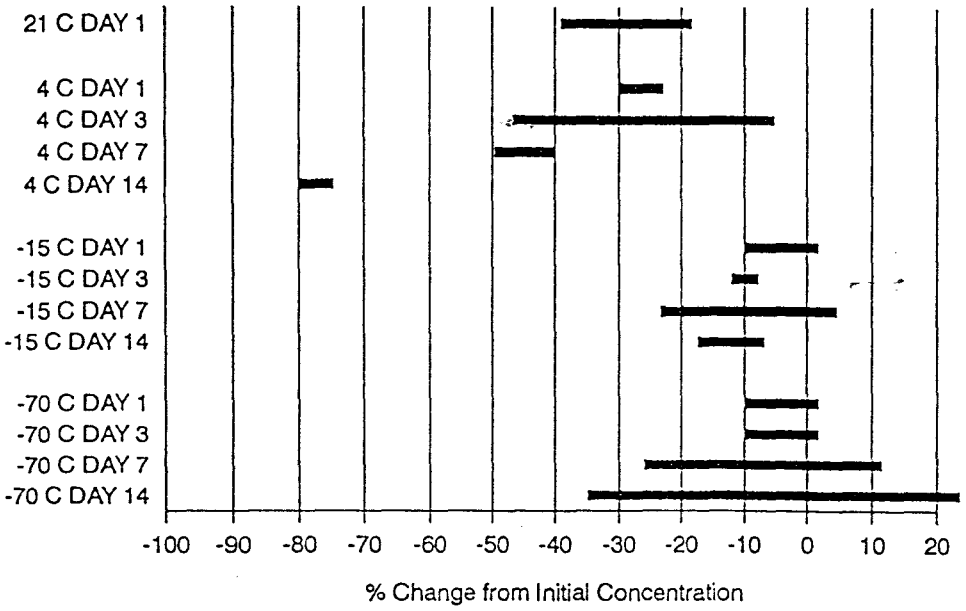


Figure 6.3 Percentage change from initial concentration of a spiked serum sample containing amoxycillin 1.2 μg/mL

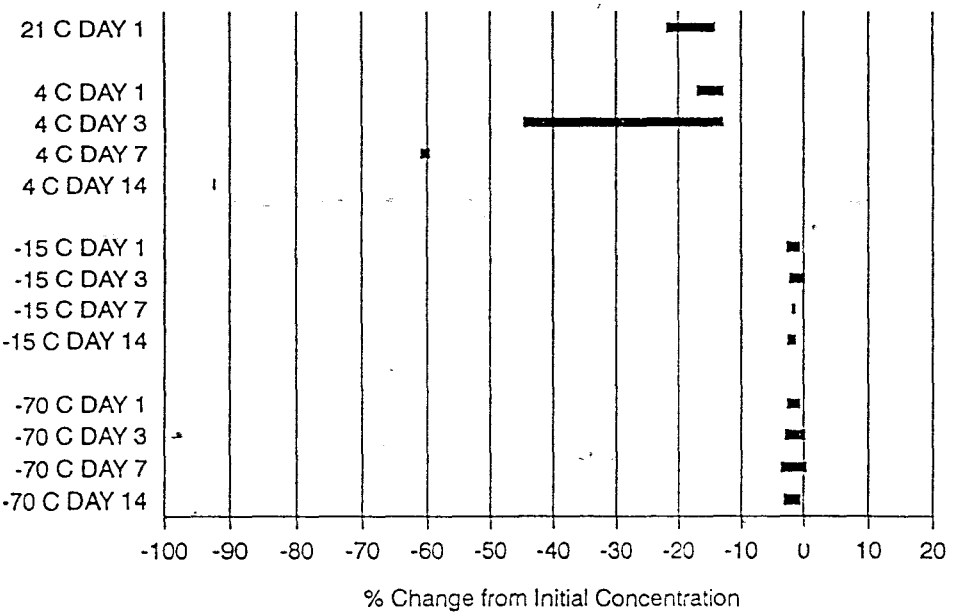


Figure 6.4 Percentage change from initial concentration of a spiked serum sample containing amoxycillin 6.0 μg/mL

After storage for 24 hours at room temperature, both the 1.2 μg/mL and 6.0 μg/mL serum samples showed a significant and relevant change in concentration, indicating that the serum samples were unstable under these conditions. Similarly, all solutions stored in the refrigerator, were unstable after 24 hours.

The 6.0 μg/mL samples which were stored at -15°C and -70°C were stable for two weeks when stored at both -15°C and -70°C, with the results indicating a change from initial concentration which was either not significant and not relevant (a) or significant but not relevant (b) (Figure 3.3).

The 1.2 μg/mL samples were stable after being frozen for 24 hours, but after three days, only the sample stored at -70°C was stable. After this time all other samples were unstable, according to the definition of Timm *et al* [52]. In the case of the samples stored at -70°C for 7 and 14 days, and the sample stored at -15°C for 7 days, it was decided that the drug was not stable (e), but the confidence interval was wide, indicating poor precision and the need for processing a higher number of replicates.

In summary, serum samples should be stored at -15°C or below, immediately after donation. The samples should be extracted within three days of donation.

In order to determine the in-process stability of the extraction procedure, samples of the eluent obtained, by extraction of the spiked serum sample, were stored at room temperature and 4°C for 48 hours, in order to determine the change in amoxycillin concentration. Each sample was analyzed in triplicate. The results are shown in Tables 6.11 and 6.12.

Table 6.11 In-process stability data for amoxycillin 1.2 μg/mL following extraction

Time (Hours)	Concentration (μg/mL), Mean ± SD (n=3)	
	21° C	4° C
0	1.21 ± 0.09	1.21 ± 0.09
12	1.22 ± 0.04	1.25 ± 0.01
24	1.11 ± 0.02	1.22 ± 0.08
48	0.81 ± 0.18	1.18 ± 0.02

Table 6.12 In-process stability data for amoxycillin 6.0 μg/mL

Time (Hours)	Concentration (μg/mL), Mean ± SD (n=3)	
	21° C	4° C
0	5.90 ± 0.79	5.90 ± 0.79
12	5.47 ± 0.16	5.99 ± 0.11
24	5.18 ± 0.19	5.41 ± 0.10
48	5.06 ± 0.12	5.55 ± 0.13

The percentage amoxycillin remaining in the extracts was calculated from the measured percentage difference in response, and the results are shown in Table 6.13.

Table 6.13 Percent drug remaining in extracts of amoxycillin

Time (hours)	21° C		4° C	
	1.2 μg/mL	6.0 μg/mL	1.2 μg/mL	6.0 μg/mL
0	100%	100%	100%	100%
12	101%	93%	103%	102%
24	92%	88%	101%	93%
48	67%	86%	98%	94%

The significance and relevance of any change in response was determined, and these results are depicted in Figure 6.5.

The lower concentration samples were stable for 48 hours following extraction when stored at 4°C, and stable for 24 hours when stored at 21°C. The solutions containing a high concentration of drug were stable for only 12 hours at 4°C and unstable under the other storage conditions, according to the definition of Timm *et al* [52]. These results imply that extracted samples should be assayed immediately after extraction, and should not be left for long periods in the autosampler during a run. If rapid analysis is not possible, they can be refrigerated until use, for up to 12 hours.

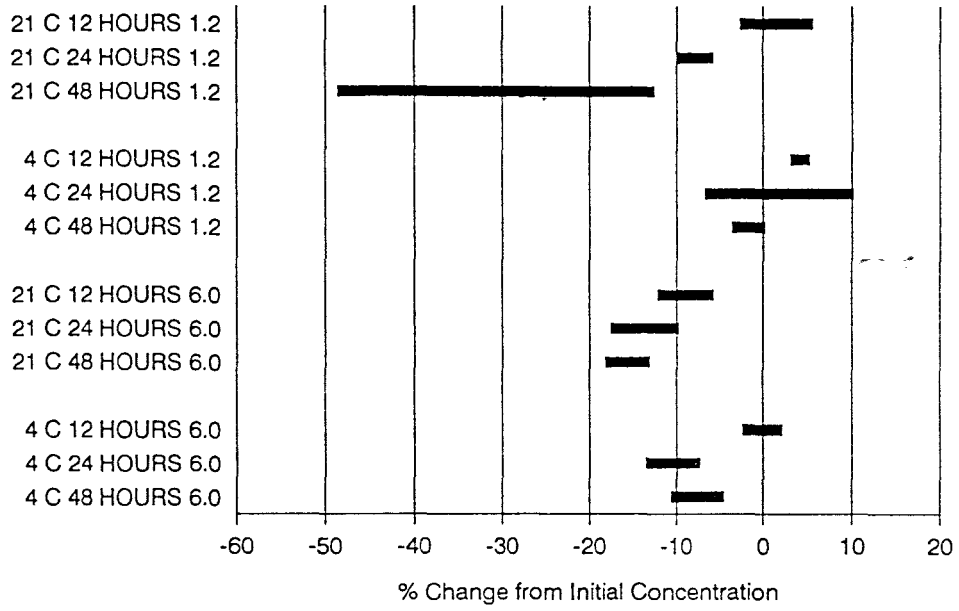


Figure 6.5 Percentage change from initial concentration of extracted serum samples

6.3.4 CONCLUSION

A validated method suitable for pharmacokinetic studies was developed for the analysis of amoxycillin in serum. This method involved sample preparation by solid phase extraction, followed by analysis using high performance liquid chromatography. The method was found to be precise and accurate, with a limit of quantitation of 0.6 μg/mL, and provided a recovery of drug from serum samples of greater than 80%. This method was used to determine the stability of amoxycillin in serum under various storage conditions. The relative instability of amoxycillin in serum meant that samples had to be assayed immediately, or could be frozen for a maximum of three days before analysis if this was not possible. The results of the in-process stability investigation suggested that once a sample had been extracted it could not be left in the autosampler for extended periods, but could be stored at 4°C for up to 12 hours.

CHAPTER SEVEN
BIOAVAILABILITY OF AMOXYCILLIN IN RECTAL DOSAGE FORMS

7.1 INTRODUCTION

7.1.1 OPTIMISING RECTAL ABSORPTION

The factors that control the rate of absorption from the rectum are not fully understood, although the absorption mechanism of most drugs from the colorectum is thought to be similar to that in the upper part of the gastrointestinal tract. Some types of drugs such as antibiotics and antitumour agents seem to have a different mechanism according to the absorption site within the gastrointestinal tract [111]. The suggestion of a different absorption mechanism has been proposed to account for the relatively low bioavailability of rectally administered bacampicillin compared to that of an oral dose [112].

The luminal surface of the rectum is covered by a membrane formed by a layer of cells consisting of columnar epithelium, endocrine and goblet cells [113]. After rectal administration, drugs may be absorbed either across the epithelial cells or via the tight junctions connecting the mucosal cells [113]. It has been recognized that drug absorption after rectal administration occurs according to the pH partition theory [114]. There is no experimental proof of carrier mediated drug transport, thus colorectal absorption is a simple diffusion process through the lipoidal membrane [27,114], with maximal absorption occurring at a pH conducive to the formation of non-ionised drug molecules [31].

There are several possible methods of improving the rectal absorption of drugs which are poorly absorbed due to their ionic structure, low lipophilicity or high molecular weight. These methods include the addition of surfactants, non-surfactants, solvents, cosolvents as permeation enhancers and the use of prodrugs.

The high sensitivity of the colorectal mucous membrane to membrane active adjuvants may be exploited for the formulation design of poorly absorbed drugs [27,114]. The use of surfactant and non-surfactant adjuvants to enhance drug absorption is well documented in the literature [27,114,115]. Surfactants seem to stimulate rectal absorption [113], although many, such as sodium

lauryl sulphate, appear to damage the rectal membrane causing a lasting effect on rectal drug absorption which may limit their use in formulation design [113,115].

The enhancing action of non-surfactant adjuvants such as salicylic acid or salicylate has been observed [115]. Nishihata *et al* [115] described studies of salicylate as an adjunct to enhance the rectal absorption of theophylline. The absorption of theophylline depended on the simultaneous absorption of salicylate, and increased with increasing concentrations of salicylate [114]. Unlike sodium lauryl sulphate which causes damage to the rectal epithelium and alters subsequent absorption, the effect of salicylate is seen only when the compound is included in the formulation [115]. The mechanism of the enhancement of rectal absorption of theophylline by salicylate is still unclear, but it is possible that salicylate reduces the lipophilicity or increases the permeability of the membrane, perhaps by interacting with calcium or magnesium ions [113,115].

During studies of the mechanism involved in the adjuvant action of salicylate, the enhancing effects of certain agents, or co-adjuvants, on the promoting action of salicylates was observed, increasing the options available for devising efficient drug delivery systems [116]. Sodium chloride, for example, facilitated the adjuvant action of sodium salicylate in enhancing rectal ampicillin absorption in rats after administration of a micro-enema (0.25 mL/kg) [116].

Murakami *et al* [117] investigated the effect of co-administration of dicloxacillin sodium and other isoxazolyl penicillins on the rectal absorption of ampicillin sodium in healthy volunteers, using suppositories with *Witepsol H15* as a base. Isoxazolyl penicillins were shown to enhance the urinary recovery of rectally administered ampicillin, possibly as a result of the formation of penicillin-calcium complexes resulting in improved mucosal permeability [117,118].

Bergström *et al* [119] investigated the clinical efficacy of suppositories containing sodium ampicillin using sodium decanoate as an absorption promoter. The clinical effect of rectally administered ampicillin was similar to that of orally administered amoxycillin, when used for the treatment of otitis media in children, which reflects adequate rectal absorption of ampicillin.

When fatty suppositories or aqueous microenemas are unfavourable vehicles due to poor dissolution and release characteristics of a drug, the addition of water miscible solvents such as polyethylene glycol or propylene glycol may be used to improve absorption [31].

Sjövall *et al* [120] investigated the prodrug approach to enhancing the rectal absorption of ampicillin. Rectal administration of the prodrug bacampicillin resulted in mean ampicillin bioavailability of 30% relative to an oral suspension, indicating incomplete rectal absorption of bacampicillin despite oral and rectal absorption rates being similar [112,118,121]. However bacampicillin shows greatly improved rectal absorption when compared to ampicillin, probably because despite adequate water solubility, it has greater lipophilicity [121].

Bioavailability depends not only on the formulation, but also on the administration route. In many suppositories the drug is in suspension in the vehicle which means that absorption by the rectal route is governed by particle size, solubility in water and interfacial tensions [18]. The factors which influence the rate and extent of drug absorption when a drug is administered in a suppository dosage form have been discussed in Section 2.1.2.2. Apart from these factors which may affect drug release and dissolution, potential barriers to the passage of dissolved drug into venous blood or lymph also exist in the unstirred water layer, the mucous layer, the cellular structure of the mucous membrane and the walls of lymph vessels and blood capillaries [24].

7.1.2 ORAL ABSORPTION OF AMOXYCILLIN AND OTHER ß-LACTAMS

Although amoxycillin is completely ionized at all pH values and has low lipid solubility, aminopenicillins such as amoxycillin are well absorbed from the alimentary tract [2].

The mechanism by which amino-ß-lactams are absorbed is not clearly understood. Sjöval *et al* [120] observed that oral amoxycillin is better absorbed than would be anticipated if passive diffusion were the only absorptive process. It has been postulated, mainly from *in situ* rat gut experiments, that amoxycillin and various ß-lactams are absorbed by an active and consequently saturable carrier mediated absorption mechanism [120,122,123,124]. This suggests that dose-dependent absorption of amoxycillin supports the concept of a capacity-limited and probably specialized transport system in man. The results of Tsuji *et al* [122] and Arancibia *et al* [125] supported these findings.

Mizen *et al* [124] observed that the uptake of amoxycillin was facilitated, whereas cyclacillin was absorbed via an active transport process from the gastrointestinal tract after oral dosing. Tsuji *et al* [122] have suggested that a specialized transport mechanism that deviates from the pH-partition hypothesis may contribute to the absorption of zwitterionic antibiotics. This mechanism is thought

to occur in addition to the simple diffusion transport responsible for the absorption of unionized species of the monobasic ß-lactam antibiotics. This deviation can be explained by passive transport of the undissociated molecule through both the aqueous diffusion barrier layer adjacent to the gastrointestinal membrane surface and the lipid barrier of the gastrointestinal membrane, suggesting two compartment diffusion [122]. However, the gastrointestinal absorption of amino-ß-lactams, which have very low lipid solubility and are completely ionized in all alimentary tract areas, cannot be predicted by this mechanism of passive diffusion of the unionized species. These results also suggest that there may be a specific area in the alimentary tract of mammals for the absorption of ß-lactam antibiotics [122].

It has been shown that the permeability of the rat small intestine to amoxycillin depends on the concentration of the drug in the perfusate [123]. At higher concentrations (> 100 μg/mL) passive diffusion is the dominant absorption mechanism. The relation between amount absorbed and the initial drug concentration suggests that the absorption of amoxycillin follows the simultaneous kinetics of a simple diffusion process predominant at high concentrations, and a Michaelis-Menten process which can only be seen at low concentrations [123,126]. Thus it is suggested that facilitated diffusion and not active transport is involved in the absorption of small doses of amoxycillin. Tsuji *et al* [123] did not demonstrate the nature of the transport mechanism, but showed that a saturable, rate-limiting step in the absorption process of amoxycillin must be considered. Torres-Molina *et al* [21] were in agreement with the saturable absorption theory, although the saturation phenomena did not completely explain their findings and a limiting step which prevents carrier saturation seemed to be in operation [21].

It has been suggested that active transport of some ß-lactams takes place via a dipeptide carrier system [127], in an active window of the small intestine [126]. Muranushi *et al* postulated that two different carrier-mediated transport systems may exist for oral cephem antibiotics, an H^+ ion gradient dependent and an independent mechanism [127]. Their study concluded that ceftibutem is absorbed mainly through the oligopeptide transport route in the small intestine, in which the driving force is the transmembrane H^+ ion gradient represented by the existence of an acidic microclimate pH at the surface of the small intestine [127].

Amoxycillin pharmacokinetic data could be described using a single compartment open model with first order absorption [122,128,129]. Couet *et al* [126] have proposed a pharmacokinetic model for a different amino-ß-lactam, cefatrizine, which incorporated a saturated rate of absorption. This

Michaelis-Menten type model incorporated a time factor to predict the reduction in the extent of absorption observed *in vivo*. This model is also applicable to other amino-ß-lactam antibiotics and drugs that are absorbed by a carrier-mediated transport mechanism in the small intestine [126].

7.2 PHARMACOKINETIC PARAMETER ESTIMATION

Parameters such as C_{max}, t_{max} and area under the plasma concentration time curve (AUC^{∞}) were used to define the rate and extent of oral bioavailability. C_{max} and t_{max} were derived directly from the serum concentration time curve.

AUC was calculated using the linear trapezoidal method:

$$AUC = \sum \left(\frac{C_n + C_{n-1}}{2}\right) x \left(t_n - t_{n-1}\right) \qquad \text{Eq. 7.1}$$

where C_n = plasma concentration at time (t_n).

The AUC calculated was the area under the serum curve to the last measurable concentration. To obtain the total area under the curve from time zero to infinity (AUC^{∞}), Equation 7.2 was used to calculate the area from the last measurable concentration to infinity and this was added to the AUC obtained from Equation 7.1.

$$AUC^{n\to\infty} = \frac{C_n}{k_{el}} \qquad \text{Eq. 7.2}$$

where C_n = the last measurable concentration, and
k_{el} = the slope of the terminal portion of a semilogarithmic concentration-time plot.

The elimination rate constant (k_{el}) describes the total removal of drug from the body as a result of biotransformation and excretion, if the drug follows one compartment kinetics. The k_{el} was calculated by linear regression of the terminal slope of a semilog plot of serum concentration versus time. Calculation of the absorption rate constant (k_a) was based on the method of Wagner-Nelson [130].

The serum half life ($t_{1/2}$) of a drug is a function of its elimination and distribution. The $t_{1/2}$ was calculated using Equation 7.3.

$$t_{\frac{1}{2}} = \frac{0.693}{k_{el}} \qquad \text{Eq. 7.3}$$

The apparent clearance was reported as a ratio of clearance and bioavailability (F). An estimate of clearance was calculated by use of the following equation:

$$\frac{Cl_{TOT}}{F} = \frac{DOSE}{AUC^{\infty}} \qquad \text{Eq. 7.4}$$

where F = absolute bioavailability.

The volume of distribution ($VD_{(area)}$) was calculated using Equation 7.5.

$$\frac{VD_{area}}{F} = \frac{DOSE}{k_{el} \times AUC^{\infty}} \qquad \text{Eq. 7.5}$$

where F= absolute bioavailability.

7.3 PILOT STUDIES

Two pilot studies were performed to assess the bioavailability of amoxycillin in oral and rectal dosage forms. In Study 1, an amoxycillin capsule (250 mg) was administered to a single volunteer to determine whether the extraction technique and HPLC method were suitable for the determination of amoxycillin in serum. Amoxycillin suppositories (250 mg) were administered to two volunteers in Study 2, in order to determine the extent of rectal absorption of the drug.

7.3.1 EXPERIMENTAL

7.3.1.1 HPLC SYSTEM

Drug analysis was performed by HPLC using the system described in Section 6.2.3.4.

7.3.1.2 STOCK SOLUTIONS

Aqueous and serum stock solutions were prepared as described in Section 6.2.1.5.

7.3.1.3 CALIBRATION CURVES

Calibration curves were constructed on the days on which samples were analyzed. Calibration standards were prepared as described in Section 6.3.2.4. The linearity data for these analyses are shown in Tables 7.1 and 7.4.

7.3.2 *IN VIVO* TRIAL

7.3.2.1 VOLUNTEERS, DOSING AND SAMPLING SCHEDULE

All human studies were conducted in accordance with the Declaration of Helsinki and its subsequent Amendments [131], following approval by the Rhodes School of Pharmaceutical Sciences Departmental Ethics Committee.

A pilot study (Study 1) using one female volunteer was conducted to establish the effectiveness of the analytical method for measuring serum concentrations of amoxycillin, after the oral administration of a commercially available amoxycillin trihydrate capsule formulation (Amoxil® 250 mg, Smith-Kline Beecham).

A pilot study (Study 2) using one male and one female volunteer was conducted to determine the amoxycillin concentrations which could be expected in serum following the administration of a suppository containing 250 mg amoxycillin, formulated in either *Novata BD* or *Novata 299* semisynthetic bases. Amoxycillin suppositories were prepared immediately prior to use using *Novata BD* and *Novata 299* semisynthetic bases, as described in Section 2.2.2.

The subject in Study 1 received one capsule containing 250 mg amoxycillin, and the subjects in Study 2 were dosed with a suppository containing 250 mg amoxycillin. Subjects fasted overnight and ingested 200 mL water at the time of dosing. Suppositories were inserted to a standard depth.

Blood samples were withdrawn at 0, 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240, 360, 480 and 720 minutes after dosing. Additional samples taken at two predetermined times were used as *ex vivo* standards for quality control purposes.

7.3.2.2 COLLECTION AND STORAGE OF BLOOD SAMPLES

An indwelling 3.3 mm cannula (Mandrin Vasocan®, B Braun, Melsunger, Germany) was inserted into a suitable vein in the forearm and secured with Micropore® surgical tape (3M Medical Products Division, Johannesburg, SA) to allow complete mobility of the forearm.

A 10 mL blood sample was withdrawn directly from the butterfly by syringe aspiration and the catheter needle was closed with a sterile stylet (Mandrin Vasofix® Vasocan®, B Braun, Melsunger, Germany) to prevent coagulation of blood inside the cannula. The blood sample was transferred to a sterile evacuated blood collecting tube (Radem Medical (Pty) Ltd, Sandton, RSA) for serum collection. The tube was stoppered and blood samples were left to stand for 30 minutes to clot. The serum was separated by centrifugation at 3000 rpm (1600xG) for 20 minutes. One millilitre of serum was transferred to a clean, labelled tube for analysis and the remaining serum was transferred into a second labelled glass tube for storage. Both samples were frozen at -15°C until analyzed. Analysis was performed within 2 days of sampling in all cases, as stability had been established for that period (Section 6.3.3.5).

7.3.2.3 ANALYSIS OF SERUM

One millilitre aliquots of serum were allowed to thaw at room temperature and were mixed thoroughly on a vortex mixer (Maxi Mixer®, Model M-16710-12, Thermolyne Corporation, Iowa, USA) before being analyzed. The samples were prepared for analysis using the method described in Section 6.22. An aliquot (200 μL) of the final extract was injected onto the HPLC system.

Assay performance was continuously monitored during the period of analysis of the biological samples by inclusion of quality control samples containing known concentrations of the analyte.

These *in vitro* samples were stored under identical conditions to those of the trial samples.

Additional blood was obtained from all trial subjects at predetermined sample times and were analyzed at different times during the course of the analytical run. The *ex vivo* samples were stored under identical conditions to those of the trial samples.

Trial samples, *in vitro* standards and *ex vivo* standards were analyzed in a randomized order.

7.3.3 RESULTS OF *IN VIVO* STUDIES

7.3.3.1 ANALYTICAL

The linearity data from the calibration curves constructed for Study 1 and Study 2 are shown in Table 7.1.

Table 7.1 Calibration data from peak height ratios

Study	Slope	Y-intercept	Correlation Coefficient
Study 1	0.5007	3.9525	0.9985
Study 2	0.4862	2.1978	0.9982

The results of the quality control samples analyzed to assess the performance of the assay indicated that all *in vitro* and *ex vivo* standards had a RSD of less than 9% and the percent bias was not greater than 7%. The results shown in Tables 7.2 and 7.3 indicate that the performance of the assay procedure was consistent and precise throughout the run, and acceptable for the determination of pharmacokinetic parameters [132].

Table 7.2 *In vitro* standards for Study 1 and Study 2

Standard concentration (μg/mL)	Study 1			Study 2		
	Mean concentration (μg/mL) ± SD (n=3)	% RSD	% Bias	Mean concentration (μg/mL) ± SD (n=3)	% RSD	% Bias
6.0	5.78 ± 0.07	1.20	-3.66	5.79 ± 0.39	6.68	-3.50
1.2	1.28 ± 0.04	3.12	6.67	1.13 ± 0.10	8.76	-5.83

Table 7.3 *Ex vivo* standards for Study 1 and Study 2

Sample time (min)	Concentration (μg/mL)							
	Study 1				Study 2			
	Subject sample	Mean ± SD (n=3)	% RSD	% Bias	Subject sample	Mean ± SD (n=3)	% RSD	% Bias
30	0.65	0.62 ± 0.05	8.23	-4.62	0.00	0.00	-	-
120	2.92	2.91 ± 0.16	5.33	-0.34	0.00	0.00	-	-

7.3.3.2 *IN VIVO* STUDY 1

The concentration of amoxycillin measured in serum at predetermined time intervals following a single 250 mg oral dose of amoxycillin, is shown in Table 7.4.

Table 7.4 Concentration of amoxycillin measured in serum after oral administration of a single capsule containing 250 mg amoxycillin

Time (min)	Concentration (μg/mL)
0	0.00
15	0.27
30	0.65
45	1.49
60	3.04
75	3.67
90	4.26
105	3.21
120	2.92
150	2.20
180	2.33
240	1.07
360	0.65
480	0.27
720	0.00

The serum concentration time profile and semilogarithmic serum time profile from Study 1 are shown in Figure 7.1.

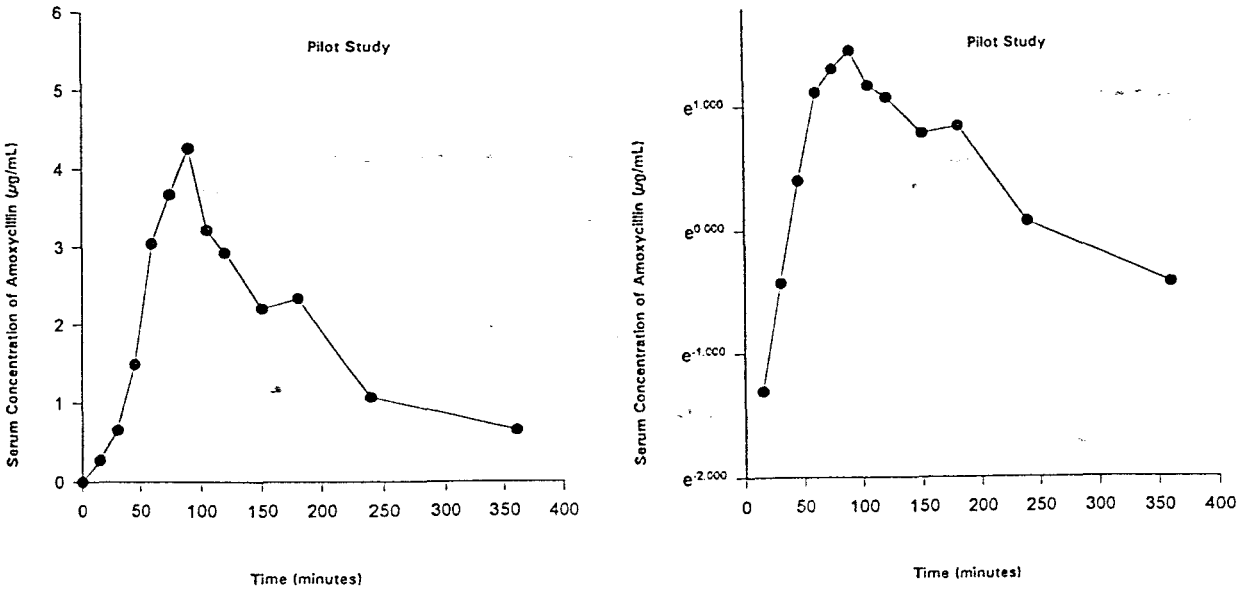


Figure 7.1 Serum concentration time profile and semilogarithmic serum concentration time profile showing amoxycillin following administration of a single amoxycillin capsule (250 mg)

7.3.3.3 PHARMACOKINETICS

Pharmacokinetic parameters were determined following the administration of a single amoxycillin capsule (250 mg) to a 25 year old female volunteer, of mass 53 kg and height 1.6 m. These parameters are shown in Table 7.5.

Table 7.5 Pharmacokinetic parameters from Study 1

Parameter	Subject
C_{max} (μg/mL)	4.26
t_{max} (hr)	1.50
AUC^{∞}	10.3850
k_a (hr^{-1})	0.7948
k_{el} (hr^{-1})	0.5663
Clearance/F (L/kg/hr)	0.5047
$VD_{(area)}$/F (L/kg)	0.8911
$t_{1/2}$ (hr)	1.22

The C_{max} of 4.26 μg/mL was reached within 1 to 2 hours after dosing, and was slightly lower than the average peak plasma concentration of 5 μg/mL that has been previously reported [4,6]. The $t_{1/2}$ of 1.22 hours approximated the previously quoted value of 1 hour [4]. The $VD_{(area)}$ of 0.89 L/kg was larger than the 0.3 Lkg^{-1} reported in the literature [4].

These results demonstrate that the HPLC method was suitable for the determination of amoxycillin following an oral dose, and sensitive enough to be applied to the measurement of pharmacokinetic parameters.

7.3.3.4 *IN VIVO* STUDY 2

No amoxycillin was detectable in the serum of the volunteers that had been administered a suppository.

Bioavailability would mainly be influenced by drug availability or release of drug from the dosage form, and by the characteristics of the particular absorption sites encountered following oral and rectal administration. The poor rectal absorption of amoxycillin necessitated further investigations to determine whether the absence of amoxycillin in serum after the administration of a rectal

suppository was due to formulation inadequacies or absorption factors.

7.3.4 CLINICAL TRIAL

7.3.4.1 INTRODUCTION

In order to determine whether amoxycillin was absorbed rectally, without the influence of suppository dosage form variables, a commercially available amoxycillin suspension was administered both orally and rectally in a crossover study. It was assumed that a suspension would be more bioavailable than a suppository. By using an aqueous enema or microenema from which drugs are readily available for absorption, it may be possible to circumvent the process of drug release and apply the drug directly to potential absorption sites in the rectal wall. A solution would have been preferable to a suspension, since dissolution into rectal fluid would be unnecessary, but preparation of a small volume solution containing 250 mg amoxycillin was not possible due to the limited solubility of the drug (1-10 mg/mL) [4,6].

The clinical trial was conducted in accordance with the Declaration of Helsinki and its subsequent Amendments [131], following approval by the Rhodes University Ethical Standards Committee. A copy of the trial protocol is appended (Appendix 1).

7.3.4.2 VOLUNTEERS

Three healthy, adult Caucasian male volunteers participated in the study. Inclusion and exclusion criteria are listed in Section 2.4 of the protocol. Demographic details of subjects are listed in Table 7.6. Each volunteer was provided with details of the trial (Appendix 2) and was required to sign an informed consent form (Appendix 3).

Table 7.6 Demographic data of subjects

Parameter	Subject 1	Subject 2	Subject 3	Mean ± SD
Mass (kg)	78.2	84.4	92.8	85.1 ± 7.3
Height (m)	1.82	1.83	1.76	1.80 ± 0.03
Age (years)	23	19	22	21.3 ± 2.1

A complete medical history was taken, and a physical evaluation conducted at the time of the initial visit and on the day following the completion of the trial. The haematological examination, clinical chemistry and urinalysis were performed by Drs du Buisson and Partners, Grahamstown. These parameters were evaluated and subjects with abnormal results were not admitted into this study. The medical practitioner in attendance completed a declaration to the effect that the volunteers were in good health and able to participate in the trial (Appendix 4).

7.3.4.3 TREATMENTS

Each subject received a single dose of 250 mg Amoxil SF® suspension (250 mg/5 mL) (Smith-Kline Beecham, RSA) rectally, or 250 mg Amoxil SF® suspension orally. One hour prior to the rectal dose, a Lenolax® enema (Lennon Ltd, Port Elizabeth, RSA) was self-administered by each volunteer, in order to evacuate the bowel prior to administration of the rectal suspension. The rectal suspension was administered by means of an enema bottle. Subjects were asked to lie on their stomachs for two hours following administration of the rectal suspension, after which they were allowed to sit up. The subjects were requested to report any loss of dosage after administration.

Since amoxycillin has a serum half life of approximately one hour [5], a washout period of 48 hours was considered sufficient for the purposes of this trial, as after 6.62 half lives more than 99% of the drug is removed from the serum [133].

7.3.4.4 DOSAGE AND SAMPLING SCHEDULE AND COLLECTION AND STORAGE OF BLOOD SAMPLES

The dosage and sampling schedules were as listed in Section 2.11 of Appendix 1. Blood samples were collected and stored following the procedure described in Section 7.3.2.2.

7.3.4.5 ANALYSIS OF SERUM

Serum samples, *in vitro* and *ex vivo* quality control standards were analyzed as previously described in Section 7.3.2.3 using solid phase extraction followed by HPLC.

7.3.5 RESULTS

7.2.5.1 ANALYTICAL RESULTS

The linearity data from the calibration curves that were constructed on each day of assay are shown in Table 7.7.

Table 7.7 Calibration data from peak height ratios of drug/internal standard

Dosage Form Administered	Slope	Y-intercept	Correlation Coefficient
Rectal Suspension	0.6467	0.0760	0.9979
Oral Suspension	0.7533	0.2413	0.9949

The *in vitro* and *ex vivo* quality control results are shown in Tables 7.8 and 7.9 respectively. The term subject sample in Table 7.8 refers to the concentration measured following the analysis of a subject serum sample for the first time. The *ex vivo* samples were analyzed in triplicate and are reported as a mean concentration.

Table 7.8 *In vitro* standards for oral and rectal study

Standard concentration (μg/mL)	Oral study			Rectal study		
	Mean concentration (μg/mL) ± SD (n=3)	% RSD	% Bias	Mean concentration (μg/mL) ± SD (n=3)	% RSD	% Bias
6.0	5.38 ± 0.28	5.15	-10.33	6.10 ± 0.09	1.50	1.67
1.2	1.15 ± 0.10	8.73	-4.17	1.21 ± 0.13	10.44	0.83

Table 7.9 *Ex vivo* standards for oral and rectal studies

Sample time (min)	Concentration (μg/mL)							
	Oral study				Rectal study			
	Subject sample	Mean ± SD (n=3)	% RSD	% Bias	Subject sample	Mean ± SD (n=3)	% RSD	% Bias
Subject 1								
30	1.22	1.22 ± 0.07	5.91	0.00	0.00	0.00	-	-
180	2.33	2.41 ± 0.10	4.18	3.43	0.00	0.00	-	-
Subject 2								
45	2.97	2.74 ± 0.28	10.17	-7.74	0.00	0.00	-	-
150	2.55	2.39 ± 0.16	6.52	-6.27	0.00	0.00	-	-
Subject 3								
75	4.56	4.21 ± 0.47	11.24	-7.68	0.00	0.00	-	-
240	0.53	0.60 ± 0.03	4.22	13.20	0.00	0.00	-	-

The *ex vivo* and *in vitro* quality control samples used to assess the precision of the analytical method indicated that the method was consistent for the duration of the run. The results show RSD values less than 12% and a percent bias less than 14% for these quality control samples. Although these values are slightly greater than the 10% suggested by Peng and Chiou [132], they are acceptable for the purposes of this study since low concentrations (0.6 to 6.0 μg/mL) of the drug were analyzed.

7.3.5.2 CLINICAL TRIAL

The serum concentrations following oral dosing, are presented in Table 7.10 and the serum concentration time curves are depicted in Figure 7.2. The mean serum concentrations of the three subjects are shown in Figure 7.3.

Table 7.10 Concentration of amoxycillin measured in serum of three volunteers after the oral administration of a single dose of 250 mg amoxycillin suspension

Time (min)	Concentration (μg/mL)			
	Subject 1	Subject 2	Subject 3	Mean ± SD
0	0.00	0.00	0.00	0.00 ± 0.00
15	0.31	0.21	0.10	0.21 ± 0.11
30	1.22	2.33	4.09	2.55 ± 1.45
45	1.33	2.97	4.25	2.85 ± 1.46
60	4.41	3.82	4.35	4.19 ± 0.32
75	4.88	4.56	4.56	4.67 ± 0.18
90	5.00	4.46	4.30	4.58 ± 0.37
105	4.88	3.61	3.93	4.14 ± 0.66
120	3.98	3.56	3.66	3.73 ± 0.22
150	3.29	2.55	2.29	2.71 ± 0.52
180	2.33	1.80	1.64	1.92 ± 0.36
240	0.74	1.01	0.53	0.76 ± 0.24
360	0.00	0.00	0.00	0.00 ± 0.00
480	0.00	0.00	0.00	0.00 ± 0.00
720	0.00	0.00	0.00	0.00 ± 0.00

In the oral dose study, absorption of the drug was fairly rapid, with the C_{max} occurring between 75 minutes to 90 minutes after ingestion of the suspension, with concentrations ranging between 4.56 μg/mL and 5.00 μg/mL. Detectable amounts were present up to 4 hours following administration. In previously reported studies, a peak serum concentration of 5 μg/mL occurred from between 60 to 120 minutes after ingestion of a 250 mg oral dose, and amoxycillin was detectable in the serum for up to 8 hours [4,5,6].

Following administration of the rectal suspension, no amoxycillin was detectable in the serum.

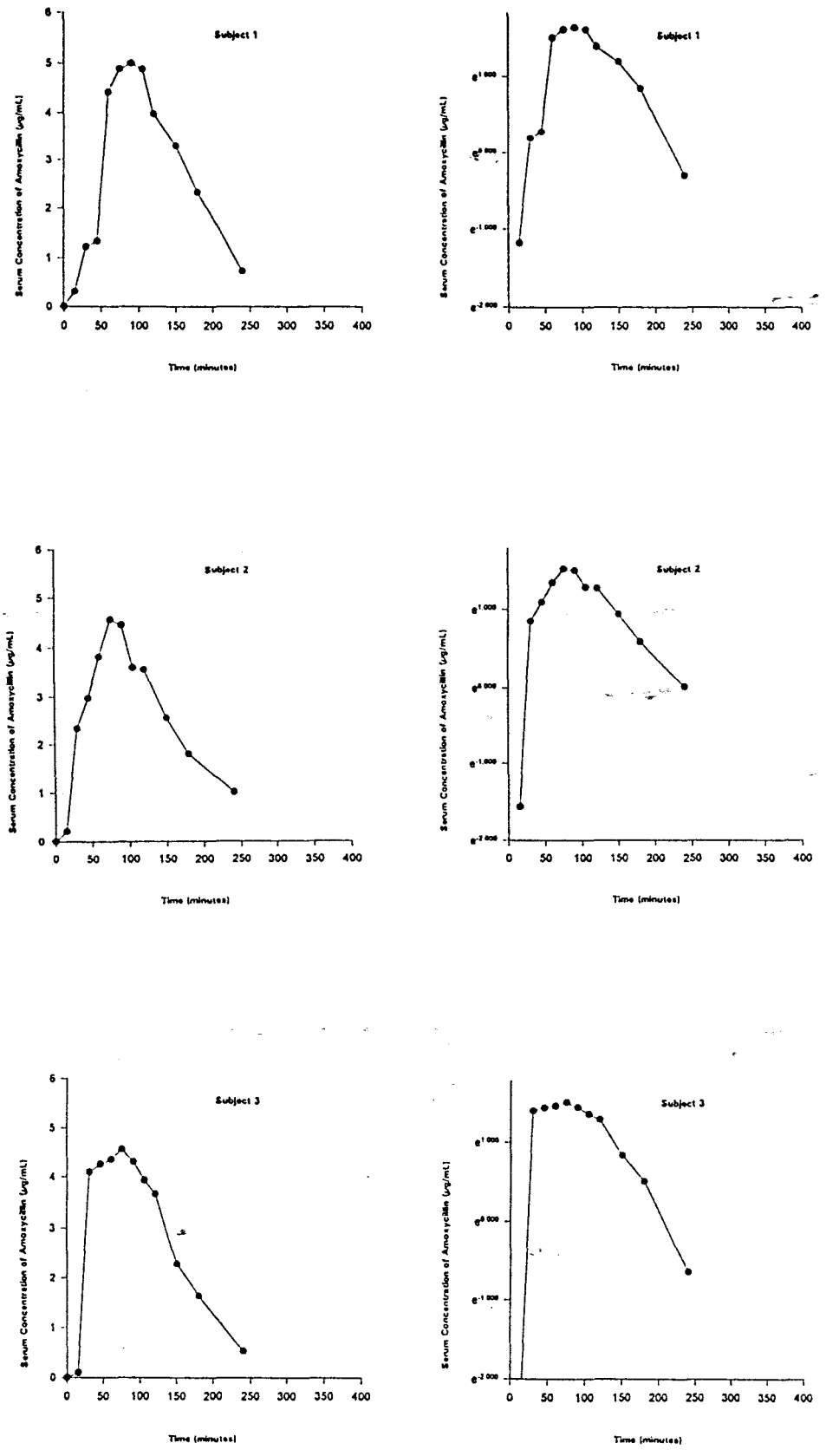


Figure 7.2 Serum concentration time profiles and semilogarithmic serum time profiles for subjects 1 to 3 showing amoxycillin following administration of a single (250 mg) oral dose of amoxycillin suspension

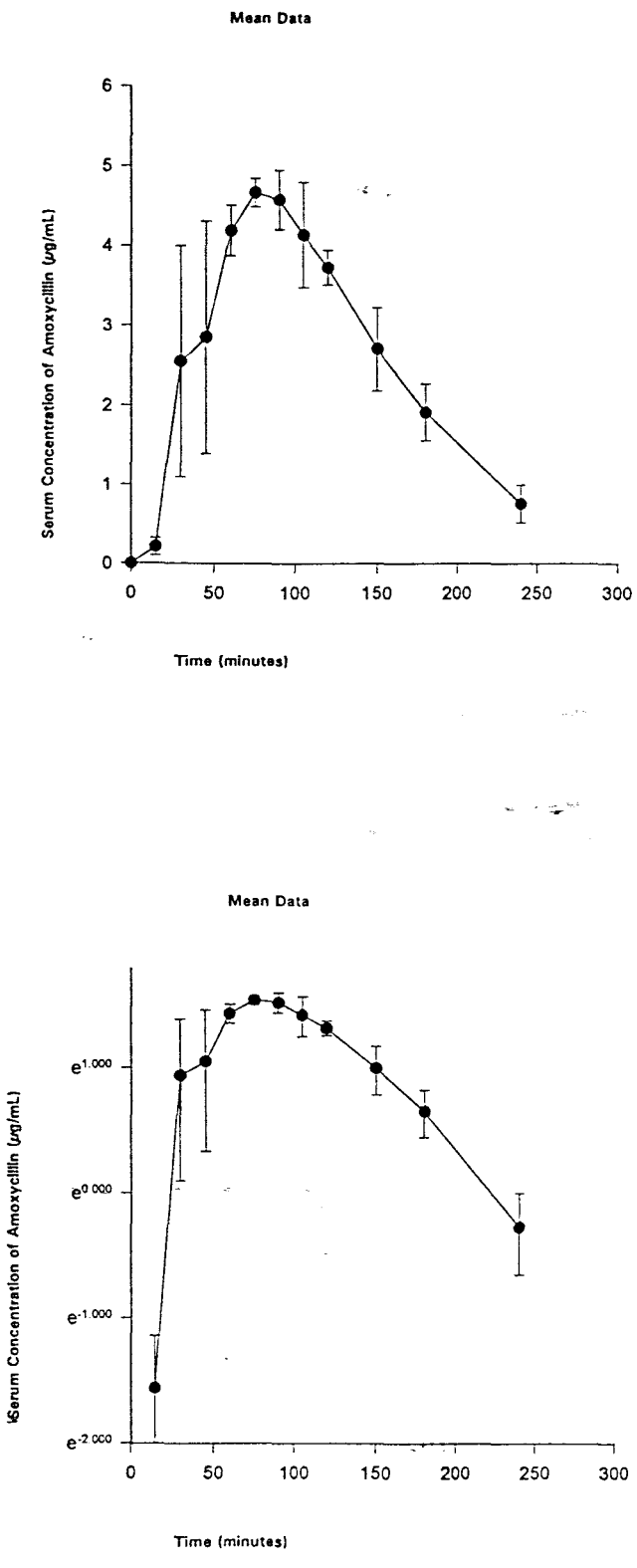


Figure 7.3 Mean serum concentration time profile and semilogarithmic mean serum concentration time profile following administration of a single (250 mg) oral dose of amoxycillin suspension

7.3.5.3 DATA ANALYSIS

The pharmacokinetic parameters were calculated as described in Section 7.2. Results are presented in Table 7.11.

Table 7.11 Pharmacokinetic parameters

Parameter	Subject 1	Subject 2	Subject 3	Mean ± SD
C_{max} (μg/mL)	5.00	4.56	4.56	4.70 ± 0.25
t_{max} (hr)	1.50	1.25	1.25	1.33 ± 0.14
AUC^{∞}	11.3522	11.4686	10.8516	11.2241 ± 0.3278
k_a (hr^{-1})	0.7363	0.8176	1.0860	0.8800 ± 0.1830
k_{el} (hr^{-1})	0.8167	0.6297	0.9593	0.8019 ± 0.1653
Clearance/F (L/kg/hr)	0.3053	0.2870	0.2759	0.2894 ± 0.0148
$VD_{(area)}$/F (L/kg)	0.3739	0.4558	0.2876	0.3724 ± 0.0841
$t_{1/2}$ (hr)	0.8485	0.1005	0.7224	0.8905 ± 0.1925

7.2.6 DISCUSSION

No amoxycillin was detectable in serum following the administration of a suppository containing 250 mg amoxycillin. This negative result suggested that either the semisynthetic suppository base used to formulate the suppository was preventing efficient drug release, or detectable amounts of amoxycillin were not absorbed through the rectal mucosa. In order to discern which of these factors was inhibiting absorption, a clinical trial was conducted, in which an oral and a rectal suspension were administered.

In order to allay the subjects' apprehension, the rectal study was conducted before the oral study. A fleet enema was used to evacuate the bowel prior to administration of the rectal suspension, so that the presence of faecal matter would not interfere with drug absorption, or cause premature expulsion of the suspension. The rectal suspension was conveniently administered with a fleet enema bottle. Experiments prior to the trial confirmed that the complete 250 mg per 5 mL dose was delivered. The subjects were asked to lie on their stomachs for two hours after dosing with the rectal suspension, and no subject reported leakage of the dosage form from the rectum during or after this time. These factors ensured minimal loss of the dosage form and it is therefore unlikely that the lack of amoxycillin in the serum was due to an inadequate quantity of amoxycillin present in the rectum.

The extraction process was sensitive and precise and allowed measurement of amoxycillin in serum following the administration of a single 250 mg dose of oral suspension to three subjects. Pharmacokinetic parameters were calculated from these data.

The mean C_{max} of 4.67 μg/mL was slightly lower than the average of 5 μg/mL quoted in previous reports [4,5,6]. The range quoted for t_{max} is between 1 and 2 hours, which is consistent with the mean t_{max} of 1.25 hours [4,6]. The mean VD_{area} (0.3724 L/kg) is slightly greater than the previously reported value of 0.3 L/kg [4].

It was anticipated that an amoxycillin suspension would be more bioavailable than a capsule. When the data obtained after administration of a capsule (Study 1) and the oral suspension (Clinical Study) were compared, the mean C_{max} obtained after administration of the suspension was higher (4.67 μg/mL) than that following administration of the capsule (4.26 μg/mL), and the t_{max} occurred sooner (1.25 hours versus 1.5 hours) as expected. The serum half life of amoxycillin calculated following administration of the capsule (1.22 hours) was slightly longer than that observed following the oral suspension (0.89 hours). It is possible that this difference is consistent with normal intra- and inter-individual variation. The ratio of $AUC^{\infty}_{capsule}/AUC^{\infty}_{suspension}$ was 0.92, indicating that the suspension is a slightly more bioavailable dosage form than the capsule. It must be borne in mind, however, that data for Study 1 was obtained from only one subject.

A suspension was used to determine whether amoxycillin is absorbed rectally, since this should be more bioavailable than a suppository. The lack of detectable amoxycillin in the serum samples following administration of the rectal suspension may be attributed to the differences in structure and surface area of the upper gastrointestinal tract and colorectum. The upper gastrointestinal tract has a greater surface area than the colorectum since the colorectum has no microvilli. There is a minimal amount of fluid in the rectum for dissolution, thus given the limited solubility of amoxycillin it is unlikely that complete dissolution of the drug in the rectal fluid occurred. The lack of drug in the serum may also be due to different absorption mechanisms for amoxycillin in the upper part of the gastrointestine and in the colorectum [110]. It has been suggested that a specialized, saturable transport system for amoxycillin, which is probably carrier mediated exists in the upper gastrointestine of man [120,128,129]. The absorption of low concentrations of drug has been attributed to a Michaelis-Menten process, while passive diffusion is the dominant absorption mechanism at high concentrations [123,126]. The small dose of 250 mg used in this study was probably absorbed by facilitated diffusion following oral administration, but since this

transport mechanism may be absent in the colorectum, this may explain why amoxycillin was not absorbed in this region.

Since the absorption of amoxycillin is thought to be a facilitated process [21,123,126,127], the difficulties involved in rectal absorption of the drug may be overcome by addition of a chemical adjunct to the suppository that would promote drug partitioning into the rectal mucosa and affect the intrinsic diffusional barriers of the rectal membrane. Most chemical enhancers are active by spacial disruption of the normally ordered arrangement of the intercellular molecules [134]. Dimethylsulfoxide, for example, is an effective transdermal penetration enhancer which might be useful in promoting rectal absorption. Fatty acids such as oleic acid have been used as transdermal penetration enhancers, as they disturb the intercellular layers in the stratum corneum [134] and decanoic acid has been used to enhance the rectal absorption of phenolsulfonphthalein [135]. Surface active agents enhance the penetration of compounds primarily by adsorbing at interfaces and thus interacting with biological membranes. Sodium lauryl sulphate is an example of a surface active agent which has been associated with lipid modification and disorganization of the stratum cornea and enhanced permeation [134]. However sodium lauryl sulphate has been implicated in permanent damage to the rectal membrane causing a lasting effect on absorption [113,115], thereby discounting its usefulness as a permeation enhancer in rectal drug delivery systems. Isoxazolyl penicillins and salicylates have been successfully used as penetration enhancers in certain rectal dosage forms [115,116,117]. Further studies should focus on identifying a suitable penetration enhancer to be included in a rectal amoxycillin formulation.

In vitro studies using live tissue such as isolated intestinal and rectal membrane [73,135] could be used to simulate a living system as closely as possible in order to investigate absorption mechanisms in these regions of the gastrointestinal tract and to further elucidate the mechanism of absorption of amoxycillin. Following these investigations, *in vivo* studies on animal models such as rats, rabbits, pigs or dogs [66,68,116,117,135,136] would be useful to identify suitable penetration enhancers to include in a rectal delivery system for amoxycillin.

CHAPTER EIGHT
CONCLUSION

Amoxycillin is a broad spectrum, semisynthetic antibiotic, suitable for administration to children, and used for the treatment of infections such as otitis media, respiratory tract infections and urinary tract infections. It is also useful for prophylaxis prior to surgery. Administration of medication in a suppository dosage form may be a useful alternative when patients, especially children, are unwilling or unable to take oral medication.

Amoxycillin was formulated in several types of suppository bases, in an attempt to manufacture an antibiotic suppository containing 250 mg amoxycillin, suitable for paediatric use. *Novata BD, Novata 299, Witepsol W35, Suppocire A32* and theobroma are fatty suppository bases with slightly different physicochemical characteristics. *In vitro* dissolution testing differentiated between the release profiles of amoxycillin from these bases with the greatest amount of amoxycillin being released from the *Novata BD* suppositories; 85% of the 250 mg of drug was released from the newly made suppositories and 99% was released from those which had been stored for a month. The amount of drug released from the other types of suppository bases was lower; 85% and 74% were released from the *Novata 299* new and aged suppositories respectively, 44% and 7% from the *Suppocire A32*, 50% and 31% from the *Witepsol W35* and 8% and 11% from the theobroma suppositories. These results gave an indication of a change in drug release on storage and also allowed a decision to be made regarding the suppository bases most suitable for formulation with amoxycillin.

The DSC results suggest the possibility of a transformation or interaction between amoxycillin and the semisynthetic suppository bases. It is uncertain whether this interaction or transformation would also occur at room temperature, as it may be a result of the accelerated DSC conditions. However, a comparison of the endotherms of a newly manufactured suppository and one which had been stored for a month confirmed that a change did occur during storage. The development of a second endotherm on storage supports the change in drug release observed in the *in vitro* dissolution tests.

The extraction and HPLC methods developed and described in this thesis have the necessary accuracy, sensitivity and precision for the selective determination of amoxycillin in aqueous solution and serum, and are thus suitable for the elucidation of the pharmacokinetics of the drug in humans.

Various pharmacokinetic parameters were calculated from the oral studies following the administration of a commercially available amoxycillin capsule (250 mg) and amoxycillin suspension (250 mg). These values were comparable with pharmacokinetic values previously reported in the literature.

The mean C_{max} measured following administration of the oral suspension was slightly higher (4.67 μg/mL) than that obtained following administration of the capsule (4.26 μg/mL), and both were slightly lower than the 5 μg/mL quoted in previous reports [4,5,6]. The range quoted for t_{max} is 1 to 2 hours, which is consistent with the mean t_{max} of 1.25 hours observed after administration of the oral suspension, and the t_{max} of 1.55 hours observed after administration of the capsule. Amoxycillin is reported to have a half life of one hour [4,5,6]; the $t_{1/2}$ following administration of the capsule was slightly longer at 1.22 hours and that following administration of the oral suspension was shorter, at 0.89 hours. The ratio of $AUC^{\infty}_{capsule}/AUC^{\infty}_{suspension}$ was 0.92, indicating that the suspension was slightly more bioavailable than the capsule. The mean value for volume of distribution ($VD_{area}/F=0.37$ L/kg) calculated from the data of the clinical study was slightly greater than the previously reported value of 0.3 L/kg [4], but it must be borne in mind that the former value was calculated from oral and not IV data. The VD_{area}/F calculated for the single subject that took the capsule was larger than both of these values, at 0.89 L/kg. The mean clearance calculated following administration of the oral suspension (0.289 L/kg/hr) was approximately half of that calculated following administration of the capsule and the mean k_{el} and k_a were greater (0.802 hr^{-1} versus 0.566 hr^{-1} and 0.880 hr^{-1} versus 0.795 hr^{-1} respectively). These differences are more than likely a result of the small sample population used in these studies.

No rectal absorption of amoxycillin was observed, following the administration of amoxycillin suppositories and rectal suspension. The lack of absorption from either rectal formulation suggests an absorption related, rather than a formulation related obstacle. There is limited information on the rectal administration of amoxycillin, which is more than likely due to the intrinsic problem with rectal absorption of the drug. Suggestions of carrier mediated absorption following oral administration have been made, and it is possible that the transport mechanism of simple diffusion which is reported to take place in the colorectum is insufficient for the absorption of detectable amounts of amoxycillin. Further investigations into the use of permeation enhancers or prodrugs to improve rectal absorption of the drug should be pursued. In addition studies using *in vitro* membrane techniques and animal models should be undertaken to elucidate the absorption mechanism(s) of amoxycillin.

APPENDICES

APPENDIX 1

BIOAVAILABILITY OF AMOXYCILLIN TRIHYDRATE AFTER ORAL AND RECTAL ADMINISTRATION

CLINICAL TRIAL PROTOCOL

J.A. WEBSTER
SCHOOL OF PHARMACEUTICAL SCIENCES
RHODES UNIVERSITY
GRAHAMSTOWN

CONTENTS

1. INVESTIGATORS

1.1 STUDY SUPERVISORS

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1.2 MEDICAL PRACTITIONER

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Rhodes University
Grahamstown

1.3 REGISTERED NURSES

(a) Sr Penny Bernard
(b) Sr Lyn Hopewell

2. PROTOCOL

2.1 BACKGROUND INFORMATION

Amoxycillin is a broad spectrum ß-lactam antibiotic, used for the treatment of conditions such as otitis media, respiratory tract infections, urinary tract infections, sexually transmitted diseases such as gonorrhoea, enteric infections and endocarditis. It is also used for surgical prophylaxis and the prophylaxis of infective endocarditis.

The pharmacokinetics and bioavailability of amoxycillin after oral dosing have been well documented, but the bioavailability from a rectal dosage form has yet to be investigated. The usual adult oral dose is 250 to 500 mg of the trihydrate three times daily, while the paediatric dose is 125 to 250 mg three times daily. A peak plasma concentration of about 5 μg/mL is observed 1 to 2 hours after a dose of 250 mg, with detectable amounts present up to 8 hours.

Amoxycillin is contraindicated in patients with penicillin or cephalosporin hypersensitivity, glandular fever and lymphatic lymphoma. Gastro-intestinal side effects including diarrhoea, nausea

and vomiting may occur, particularly after oral dosing, but these effects are unlikely after a single dose.

2.2 OBJECTIVES

The main objective of this study is to determine the bioavailability of amoxycillin after rectal and oral administration.

2.3 STUDY DESIGN

Patients will receive a rectal dose of 250 mg amoxycillin suspension, then following a 48 hour washout period, they will receive an oral dose of 250 mg amoxycillin suspension.

2.4 STUDY POPULATION

2.4.1 NUMBER OF SUBJECTS

Three (3) healthy male volunteers will be included in the clinical trial.

2.4.2 INCLUSION CRITERIA

(a) Caucasian males between 18 and 45 years of age.
(b) Normal in terms of medical history and physical examination.
(c) Normal laboratory values (unless the clinical investigator considers an abnormality to be clinically irrelevant).

2.4.3 EXCLUSION CRITERIA

(a) Regular use of medication, abuse of alcoholic beverages or participation in another trial 60 days preceding the study.
(b) Treatment within the previous 3 months with any drug known to have a well defined potential of toxicity to any of the major organs.
(c) A major illness during 3 months preceding the study.
(d) History of hypersensitivity to penicillin or cephalosporin antibiotics.

2.5 MEDICAL AND CLINICAL LABORATORY EXAMINATION

The subjects will be examined before the study and assessed for their suitability to participate. They will undergo the same examinations within 1 to 2 days after the study. The examinations will be carried out by the attending physician or a registered nursing sister, and will include the following:

(a) Medical history and physical examination.
(b) Vital signs: general medical and cardiovascular system examination.
(c) Haematological status: haemoglobin, haematocrit, red blood cell count, white blood cell count, platelet count and differential count.
(d) Clinical chemistry: alanine transaminase, aspartate transaminase, alkaline phosphatase, glutamyl transferase, sodium and potassium chlorides, total protein, albumin, creatinine, glucose, total bilirubin, conjugated bilirubin and urea.
(e) Urinalysis and microscopic examination: pH, protein, glucose, blood and cell count.

2.6 WRITTEN CONSENT FORM

Preceding the trial, the nature, purpose and risk of participating will be explained to all subjects (Appendix 2). They will be informed that they may withdraw from the study at any time. They will sign a consent form in the presence of two witnesses (Appendix 3). Volunteers who are minors will obtain written consent from a parent or guardian.

2.7 DROP-OUTS

If any subject fails to complete the study due to a side effect, the reason must be specified by the medical practitioner in writing.

2.8 DOSAGE FORMS

Amoxil SF® suspension, containing the equivalent of 250 mg amoxycillin per 5 mL (SmithKline Beecham Pharmaceuticals (Pty) Ltd, RSA) will be administered as a 5 mL oral dose, and a 5 mL rectal dose, administered via an enema bottle.

2.9 STUDY PERFORMANCE

The study will be performed in the Biopharmaceutics Research Institute Clinic, Rhodes University, Grahamstown. Subjects will receive detailed, written instructions concerning the trial performance and restrictions (Appendix 2). Subjects will receive one dose of each treatment (Section 2.8) per trial phase.

On the first trial day, the subjects will report to the clinic at 06h30, having fasted from 24h00 of the previous day. On arrival, each volunteer will self-administer a fleet enema (Lenolax® Lennon Ltd, RSA). An indwelling cannula (Mandrin Vasocan®, Melsungen, Germany) will be inserted into an arm vein and a zero-time sample collected. At 08h00 the subjects will receive the dose of the preparation for that phase, with 200 ml water. A standard breakfast will be served 2 hours after drug administration. A standard lunch will be served 4 hours after dosing. Subjects will remain sedentary in bed for the first 5 hours after administration and then may be ambulatory.

On the second trial day volunteers will arrive at the clinic at 06h30 on the day of the trial, having fasted from 24h00 of the previous day. An indwelling needle will be inserted into an arm vein and a blood sample will be withdrawn. An oral suspension will then be administered at 08h00. Blood samples will be collected as in phase 1. All conditions pertaining to meals and confinement to the clinic are as listed in phase 1.

2.10 SUBJECT RESTRICTIONS

All subjects must refrain from taking medication, including OTC drugs for at least one week preceding phase one of the study and, except for the test drug, during the study. Ingestion of alcohol and caffeine containing beverages and foods will not be permitted 48 hours before the study, during the trial period and 24 hours thereafter. Strenuous exercise will not be allowed 24 hours before the trial, during the trial period or 24 hours thereafter. During the trial, fluid and food intake will be standardized to minimise individual variations.

2.11 BLOOD SAMPLING

On each trial day of phase one and phase two, blood samples (10 mL) will be collected before dosage administration, and at the following times after administration:

15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240, 360, 480 and 720 minutes.

At two sampling times, an additional 10 mL sample will be withdrawn. These samples will be used as *ex vivo* controls during analysis.

After each sample, the cannula will be sealed with a stylet (Mandrin Vasofix® Vasocan®, Melsunger, Germany) to prevent the blockage of the needle due to blood coagulation. Blood samples will be collected in sterile evacuated blood collection tubes for serum collection (Radem Medical (Pty) Ltd, Sandton, RSA).

2.11 ADVERSE REACTIONS

If any side effects or adverse reactions are reported, the medical practitioner will decide whether or not to withdraw the subject from the study and to initiate appropriate treatment if required. Any unusual events will be recorded.

2.12 DRUG ANALYSIS

2.12.1 SPECIAL INVESTIGATIONS

All clinical, haematological and urinalysis determinations will be performed by Drs du Buisson and Partners, Grahamstown.

2.12.2 DRUG ASSAY METHOD

Quantitative analysis of amoxycillin in serum samples will be performed at the Biopharmaceutical Research Group laboratory, Rhodes University, Grahamstown by means of high performance liquid chromatography.

APPENDIX 2

VOLUNTEER INFORMATION

1. PLACE

The trial will be conducted in the BRI Clinic, Rhodes University.

2. DATE

The initial phase will take place on Monday 16 December and the second phase on Wednesday 18 December 1996.

3. STUDY

The aim of this study is to determine and compare the rate and extent to which amoxycillin enters the blood stream after a single oral dose and a single rectal dose.

4. STUDY INFORMATION

Amoxycillin is a broad spectrum penicillin antibiotic. Amoxycillin is generally well tolerated and has few side effects. Side effects which may occur are diarrhoea, nausea and vomiting. It is essential that the volunteer is not allergic to penicillin, as amoxycillin may cause a hypersensitivity reaction in an allergic person.

5.

STUDY SUPERVISORS:	Miss J.A. Webster
MEDICAL PRACTITIONERS:	Dr A. Kench
NURSING SISTERS:	Sr Penny Bernard
	Sr Lyn Hopewell

6. CONDITIONS OF PARTICIPATION IN THE TRIAL

To participate in this trial a volunteer must fulfil the following requirements:

(a) Must be in good health; established on the basis of an interview, physical examination, urine and blood analysis.

(b) Must not have a history of gastrointestinal, hepatic, renal, cardiovascular, respiratory or endocrine disease.

(c) Must not have had any major illness for at least 3 months preceding the trial.

(d) Must not take any medication for one week preceding the trial and up to the last phase of the trial.

(e) Must refrain from ingesting any alcohol or caffeine containing beverages or food (eg Milo, coffee, tea, Coke, chocolate) for 48 hours before the start of each phase, until the end of that particular phase.

(f) Must follow all instructions pertaining to diet before and during each phase of the trial.

(g) Must refrain from any abnormal or strenuous exercise on the day before each phase and for the duration of that phase.

(h) Must have no history of asthma or related syndromes, or **penicillin allergy.**

7. CONDITIONS OF THE TRIAL

Phase 1

Volunteers will arrive at the clinic at 06h30 on the day of the trial, having fasted from 24h00 the previous day. An enema will be self administered. An indwelling needle will be inserted into an arm vein, by a qualified nursing sister, and a blood sample will be withdrawn. The rectal suspension will then be administered at 08h00 am, by a medical practitioner, to the volunteer. Blood samples will then be withdrawn at the specified times.

A standard breakfast will be served 2 hours after dosing. Volunteers will remain sedentary for the first 5 hours after dosing, after which they may be ambulatory. A standard lunch will be served 4 hours after dosing. The volunteers will have to remain at the clinic for the duration of the trial.

Phase 2

Volunteers will arrive at the clinic at 06h30 on the day of the trial, having fasted from 24h00 of the previous day. An indwelling needle will be inserted into an arm vein and a blood sample will be withdrawn. An oral suspension will then be administered at 08h00. Blood samples will be collected as in phase 1.

All conditions pertaining to meals and confinement to the clinic are as listed in phase 1.

8. BLOOD SAMPLING

Blood samples (10 mL) will be withdrawn at the following times after dosing for both phase 1 and phase 2:

0, 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240, 360, 480 and 720 minutes.

At two sampling times, an additional 10 mL sample will be withdrawn. These samples will be used as *ex vivo* controls during analysis. After each sample, the cannula will be sealed to prevent the blockage of the needle due to blood coagulation.

9. SIDE-EFFECTS AND ADVERSE REACTIONS

Subjects are asked to report the occurrence of any side-effects after administration of the respective dose. If any side effects or adverse reactions are reported, the medical practitioner will decide whether or not to withdraw the subject from the trial, or initiate the appropriate therapy.

10. ADDITIONAL REMARKS

(a) Volunteers may ask any questions regarding his personal involvement in the trial.

(b) The volunteers have the right to refuse to participate or may withdraw at any stage without jeopardising his standing.

(c) Volunteers must follow all instructions given either by the medical practitioner or supervisor of the trial.

11. REMUNERATION

Volunteers will receive a gratuity for participating in the trial.

APPENDIX 3

CONSENT FORM

I,..... hereby
give permission that the drug mentioned below may be administered to me.

I undertake to comply with all the relevant conditions contained in the volunteer information form and confirm that I accept any consequence arising as a result of my withholding or misrepresenting any information required of me. I have been fully informed by the medical practitioner regarding the possible side-effects of the drug.

The formulations which will be administered to me are:

(a) A 5 mL oral dose of Amoxil SF® suspension, manufactured by SmithKline Beecham Pharmaceuticals (Pty) Ltd.

(b) A 5 mL rectal dose of Amoxil SF® suspension, manufactured by SmithKline Beecham Pharmaceuticals (Pty) Ltd.

My consent is freely given and I realise that it may be withdrawn at any time. I also declare that I have made the necessary arrangements regarding attendance of lectures and other academic activities.

VOLUNTEER

NAME..... SIGNATURE.....

MEDICAL PRACTITIONER

NAME..... SIGNATURE.....

TRIAL SUPERVISOR

NAME..... SIGNATURE.....

APPENDIX 4

DECLARATION BY MEDICAL PRACTITIONER

I, Dr.....

have examined the volunteer

.....

and on the basis of an interview, physical examination, serum and urine analysis have found the said volunteer to be in good health and able to participate in the abovementioned trial.

Signed.....

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