STUDIES ON THE STABILITY AND ACTIVITY OF POLYMYXIN B SOLUTIONS

WIPAWEE SAOHIN

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

School of Pharmacy
The Robert Gordon University
Aberdeen
United Kingdom

SEPTEMBER 1997

To my parents,

for their love, support and understanding.

Acknowledgements

I am deeply indebted to Prof. R. M. E. Richards for his supervision, encouragement, patience and support throughout my study. I am also grateful to Prof. R. B. Taylor for his supervision and advice in the analytical work and to Prof. K. E. Kendle and Mr. Alex Wilson for their statistical advice.

My thanks are also due to Dr. D. K. L. Xing, Dr. J. Z. Xing and Miss Vivienne Hamilton for technical assistance in the microbiological work, Mr. Raymond Reid, Mrs. Lynn Hardie and Mrs. Ann Low in the analytical work. Support from academic and technical staff in the School of Pharmacy was invaluable throughout this work. I also wish to thank all friends, for their assistance and encouragement, in particular Juraporn, whose companionship made my stay here more meaningful.

Finally, my gratitude goes to the Royal Thai Government for the financial support for this study.

Abstract

The correlation between the chemical stability and the microbiological activity of polymyxin B in phosphate buffer pH 6.0 and in proprietary eye drops was evaluated. High Performance Liquid Chromatography (HPLC) was used to quantify the amount of the main components in samples stored at 43,50,55 and 60°C for a period of 500 h. The data indicated that there are significant differences in chemical stability among the different proprietary eye drops. The accurate decomposition rate constants and shelf-lives (t_{90}) at 4°C of two of the six formulated eye drops and the standard polymyxin B solution stored in glass containers at pH 6.0 were established.

It was concluded that microbiological assay by agar diffusion was unsuitable for determining the activity of control polymyxin B in phosphate buffer and polymyxin B in eye drops. Killing time determinations for polymyxin B against cell suspensions of *P. aeruginosa NCTC 6750* were consequently used. Thioglycollate broth containing *p*- aminobenzoic acid (PABA) 0.16 %w/v and magnesium sulphate 1 %w/v was used as an inactivating recovery medium. The effect of preservatives and of second antibacterials contained in the eye drops were tested individually and combined with polymyxin B. Thiomersal 0.001 %w/v, trimethoprim 0.02 %w/v and thiomersal 0.001 %w/v plus trimethoprim 0.02 %w/v did not have an effect on the activity of polymyxin B 2000 U/ml. Neomycin was an exception and at the concentrations in the range 0.0192 to 0.16 %w/v exhibited an antagonistic effect. Chemical interaction between polymyxin B and neomycin could not be detected and

it was considered that the inhibitory effect of neomycin may be the result of competition between polymyxin B and neomycin for the same binding sites on the cell surface.

Gentamicin is active against *P. aeruginosa NCTC 6750* and at concentrations of 0.075 and 0.036 %w/v it exhibited an additive effect with polymyxin B 2000 U/ml against the test organism.

The results obtained with the samples stored at 55 ^{o}C for a period of 500 h demonstrated the critical effect of pH. At a pH of 6.0 microbiological activity and chemical stability appeared optimal. The chemical stability data of five eye drop samples correlated with microbiological activity data. Exceptions were polymyxin B in one eye drop sample and control polymyxin B. These extensively decomposed samples showed good antibacterial activity which appeared to result from the activity of decomposition products. Chemical stability data for standard polymyxin B solution at pH 6.0 also correlated to microbiological activity data over the temperature range of 92 - 115 ^{o}C. The polymyxin B retained detectable microbiological activity when the amount of PB_1 was greater than 20%. It is suggested that the decomposition products which occurred at these higher temperatures did not possess antibacterial activity.

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Chapter 1 Introduction

1.1 A review of the polymyxins

1.1.1 History

The polymyxins are a group of closely related decapeptide antibiotics produced by species of Bacillus and with selective action against Gram-negative bacilli. They were isolated independently in 1947 in a laboratory in Britain and two laboratories in the United States. The British investigators called the antibiotic 'aerosporin' since they identified the bacillus as *Bacillus aerosporus*. The American investigators identified the bacillus as *Bacillus polymyxa* and called the antibiotic polymyxin. Comparative studies in the two laboratories proved that the 'two bacilli' was the same organism and the name polymyxin was agreed [1].

1.1.2 Structure

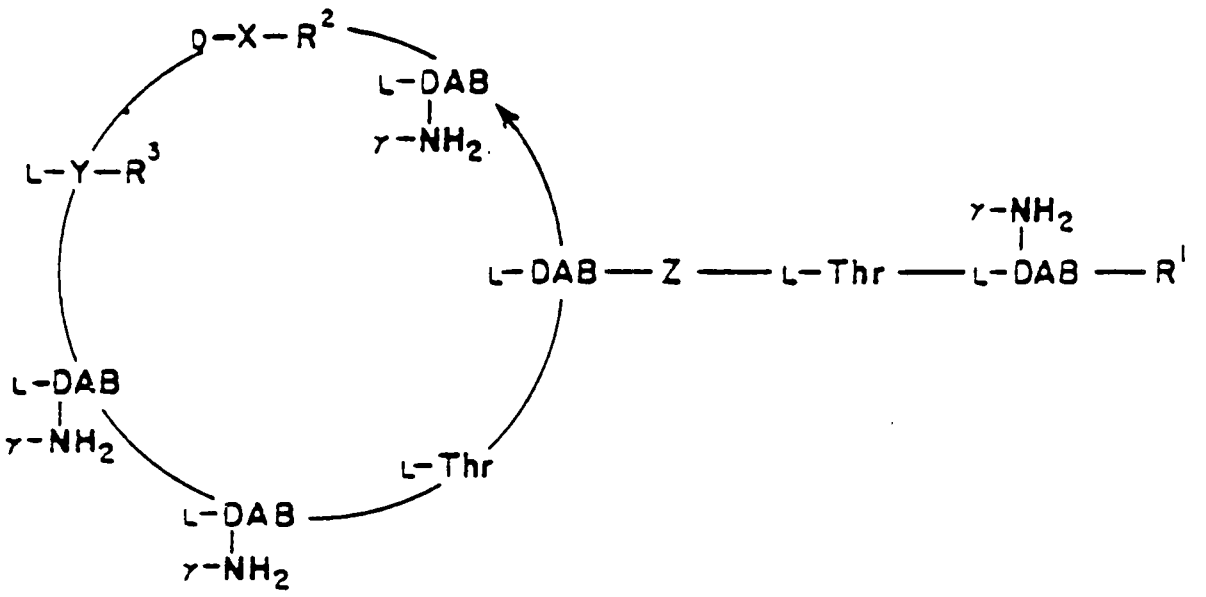
The polymyxin group comprises of polymyxins, colistins and circulins which have similar chemical structure. For polymyxins, eleven components have been distinguished (A, B, C, D, E, F, K, M, P, S, T) and these can be separated further into (for example) B_1 and B_2 [2]. Polymyxin A is the antibiotic originally named aerosporin and polymyxin D is the polymyxin isolated by Stansly *et al* in 1947 [via 1]. Only two of polymyxins have found clinical usefulness, namely polymyxin B,

usually in the form of sulphate, and polymyxin E (colistin), either as the sulphate or sodium sulphomethate. The major constituents of commercial polymyxin B are polymyxin B_1 , B_2 and B_3 [3].

The polymyxins have a general structure composed of a cyclic heptapeptide moiety and a side chain consisting of a tripeptide acylated at the N-terminus. They differ from each other in amino acid composition and each polymyxin consists of a mixture of polypeptides with an identical amino acid composition but with a different terminating fatty acid as shown in Fig.1.1 and 1.2 [4,5]. Thus, each polymyxin may be subdivided corresponding to at least three fatty acid components : 6-methyloctanoic acid, 6-methylheptanoic acid or isooctanoic acid and n-octanoic acid. The characteristic feature of polymyxins, in addition to the hydrophobic fatty acid moiety, is their strong basicity due to four or five unmasked amino acid groups from 2,4-diaminobutyric acid [6].

For polymyxin B, important things to note are the cyclic portion (7α) made up of seven amino acid residues and the acyl tripeptide tail. Of the ten amino acid residues present, all except phenylalanine are of the L-configuration. Of the six diaminobutyric acid residues, five provide a free side chain amino group and the sixth holds together the cyclic moiety and acyl tripeptide segment. Two hydrophilic side chains are provided by the threonine residues. At ordinary pH the antibiotic will be a polycation. The acyl group and amino acid groups of residues 1 and 3 of polymyxin B need to be intact for biological activity. Neutral amino acid substitution at locations corresponding to residues 8, 9 and 10 in the cyclic portion

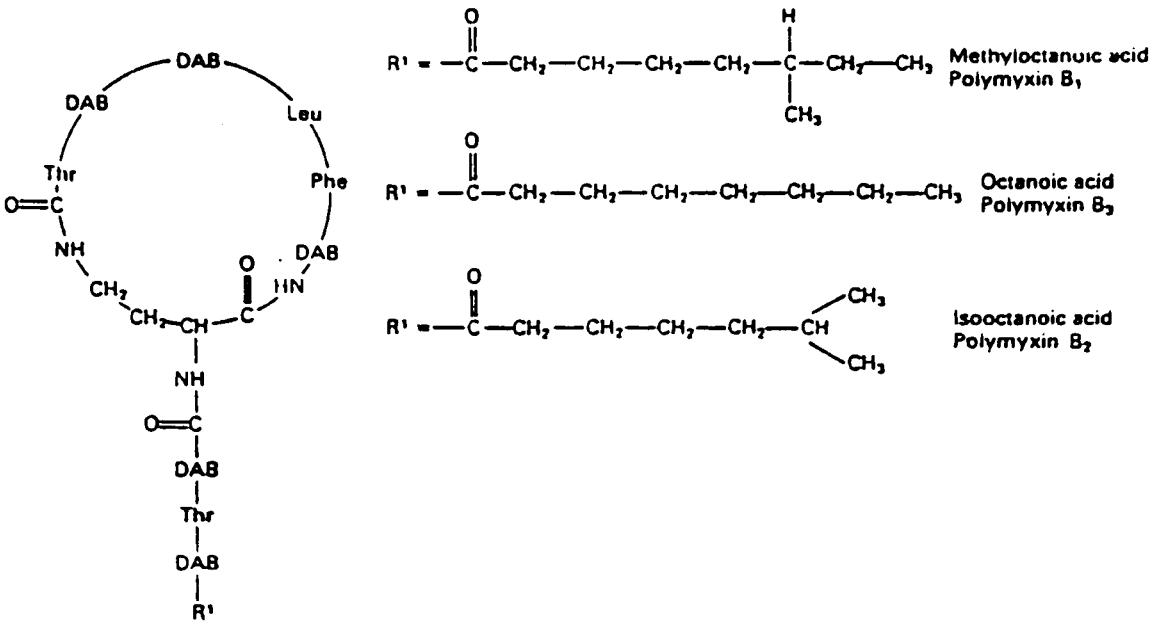
of such molecules seems permissible. The residue at location 6 needs to be of the D-configuration. If the L-diaminobutyric acid at position three is replaced either by the D-diaminobutyric acid or a neutral D-amino acid, then residues 1 and 2 of polymyxin B become dispensable. A C_{7-9} acyl group would still seems to be essential[7].



	R1	R2	R3	X	Y	Z
POLYMYXIN B_1	MOA	$-CH_2-C_6H_5$	$-CH_2-CH(CH_3)_2$	Phe	Leu	L-Dab
POLYMYXIN B_2	MHA	$-CH_2-C_6H_5$	$-CH_2-CH(CH_3)_2$	Phe	Leu	L-Dab
POLYMYXIN D_1	MOA	$-CH_2-CH(CH_3)_2$	$-CH(OH)-CH_3$	Leu	Thr	D-Ser
POLYMYXIN D_2	MHA	$-CH_2-CH(CH_3)_2$	$-CH(OH)-CH_3$	Leu	Thr	D-Ser
COLISTIN A (=polymyxin E_1)	MOA	$-CH_2-CH(CH_3)_2$	$-CH_2-CH(CH_3)_2$	Leu	Leu	L-Dab
COLISTIN B (=polymyxin E_2)	MHA	$-CH_2-CH(CH_3)_2$	$-CH_2-CH(CH_3)_2$	Leu	Leu	L-Dab
CIRCULIN A	MOA	$-CH_2-CH(CH_3)_2$	$-CH(CH_3)-CH_2-CH_3$	Leu	Ile	L-Dab

MOA = (+)-6-methyloctanoic acid
MHA = (+)-6-methylheptanoic acid
DAB = α-γ-diaminobutyric acid

Fig. 1.1 The structures of the known members of the polymyxin group.



Polymixins. DAB = α,γ-diaminobutyric acid (all linked through the α-group except where shown); Leu = leucine; Phe = phenylalanine; Thr = threonine.

Fig.1.2 Structure of polymyxin B.

1.1.3 Chemical properties

The polymyxins (molecular weight 1000-2000) were shown to be stable in the pH range 2.0-7.0 even when heated but unfortunately the time and temperature of heating were not stated [8]. Under more acid or alkaline conditions the polymyxins lost their activity after 36 hours at room temperature [8]. The same workers showed that pepsin, trypsin, erepsin and pancreatin were not found to inactivate the polymyxins, but lipase decomposes polymyxin B and a polymyxin related compound known as circulin. They also showed that polymyxins were adsorbed onto paper and Seitz filters.

Polymyxin B sulphate is a white or buff-coloured, hygroscorpic powder, odourless or with a slight odour. The B.P. specifies that it contains not less than 6500 units

per mg and the U.S.P. specifies not less than 6000 units of polymyxin B per mg. Both specifications are for the dried substance. One unit of polymyxin B is contained in 0.000119 mg of the second International Standard Preparation (1969) of polymyxin B sulphate which contains 8403 units per mg. The commercially available forms of polymyxin B sulphate are generally less pure than the International Standard Preparation and doses are sometimes stated in term of pure polymyxin base : 100 mg of pure polymyxin B is considered to be equivalent to 1 million units (1mega unit)[9,10]. Polymyxin B sulphate is highly soluble in water and methanol ; solutions are fairly stable and is stated to withstand heating to 60°C for one hour [1]. Its aqueous solutions are slightly acidic or nearly neutral . The B.P. states that a 2% solution in water has a pH of 5 to 7, and the U.S.P. states that a 0.5% solution has a pH 5.0 to 7.5, and when refrigerated, is stable for at least 6 months. Alkaline solutions are not stable. Moisture content should not be more than 6%, determined by drying over phosphorous pentoxide at 60°C under vacuum. The dissociation constant (pKa) is 8.9[9,10].

1.1.4 Mechanism and site of action

The polymyxins are bactericidal for a broad range of Gram-negative bacteria but they have less effect on Gram-positive bacteria. They cause leakage of small molecules, such as phosphates and nucleosides from sensitive bacteria. The extent of the leakage is proportional to the killing effect of the drug. Alterations in membrane permeability is one of the earliest changes caused by the polymyxins, and

their effect on the outer cellular functions, such as respiration and ATP levels, are thought to be entirely secondary to membrane damage.

It is likely that the polymyxins are not very active against Gram-positive bacteria because the thick cell wall prevents access to the cytoplasmic membrane. In causing a change in the cytoplasmic membrane permeability of Gram-negative bacteria, the polymyxins must either first disrupt the permeability of the outer membrane or pass through the outer membrane and interact with the cytoplasmic membrane [11]. Kubesh and Beggs [12] proposed that polymyxin B crosses the outer membrane and subsequently disrupts the cytoplasmic membrane, possibly by causing interdigitation of some domains of gel phase lipid containing saturated chains. At low concentrations, polymyxin B causes changes in the morphology of the outer membrane and causes selective release of proteins that are located in the periplasmic space between the inner and outer membranes. The drug can also alter respiration either directly by interacting with the cytoplasmic membrane, which is the site of electron transport and oxidative phosphorylation, or indirectly by perturbation of the outer membrane structure.

Polymyxins are cyclic polycationic peptides with a fatty acid chain attached to the peptide through amide linkage. The heptapeptide polar head and nonpolar fatty acid chain confer amphipathic properties which permit the polymyxins to interact with both lipopolysaccharides and phospholipids [13]. The heptapeptide head binds to a high-affinity binding site on lipopolysaccharide that is also a binding site for calcium and magnesium ions, which are known to play important roles in stabilising

the structure of membranes particularly the outer membrane of Gram-negative bacteria. It is proposed that polymyxins may act by competitively displacing calcium and magnesium ions from negatively charged phosphate groups on membrane lipids. This would be the effect of the charged cycloheptapeptide while the insertion of the fatty acid side chain into the core of the membrane would perturb the normal packing of the phospholipids. The two interactions together could disrupt the membrane organisation to an extent that would increase permeability to polar or charged molecules [14,15]. The effects of the polymyxins are shown in Fig. 1.3 [16].

PB_1 and PB_2 differ only in the fatty acid residue terminating the tripeptide chain. PB_1 terminates in a methyloctanoic acid residue and PB_2 terminates in a methylheptanoic acid residue. Thus, PB_1 and PB_2 have a similar antibiotic mechanism and may be expected to have a similar quantitative antibiotic effect [15].

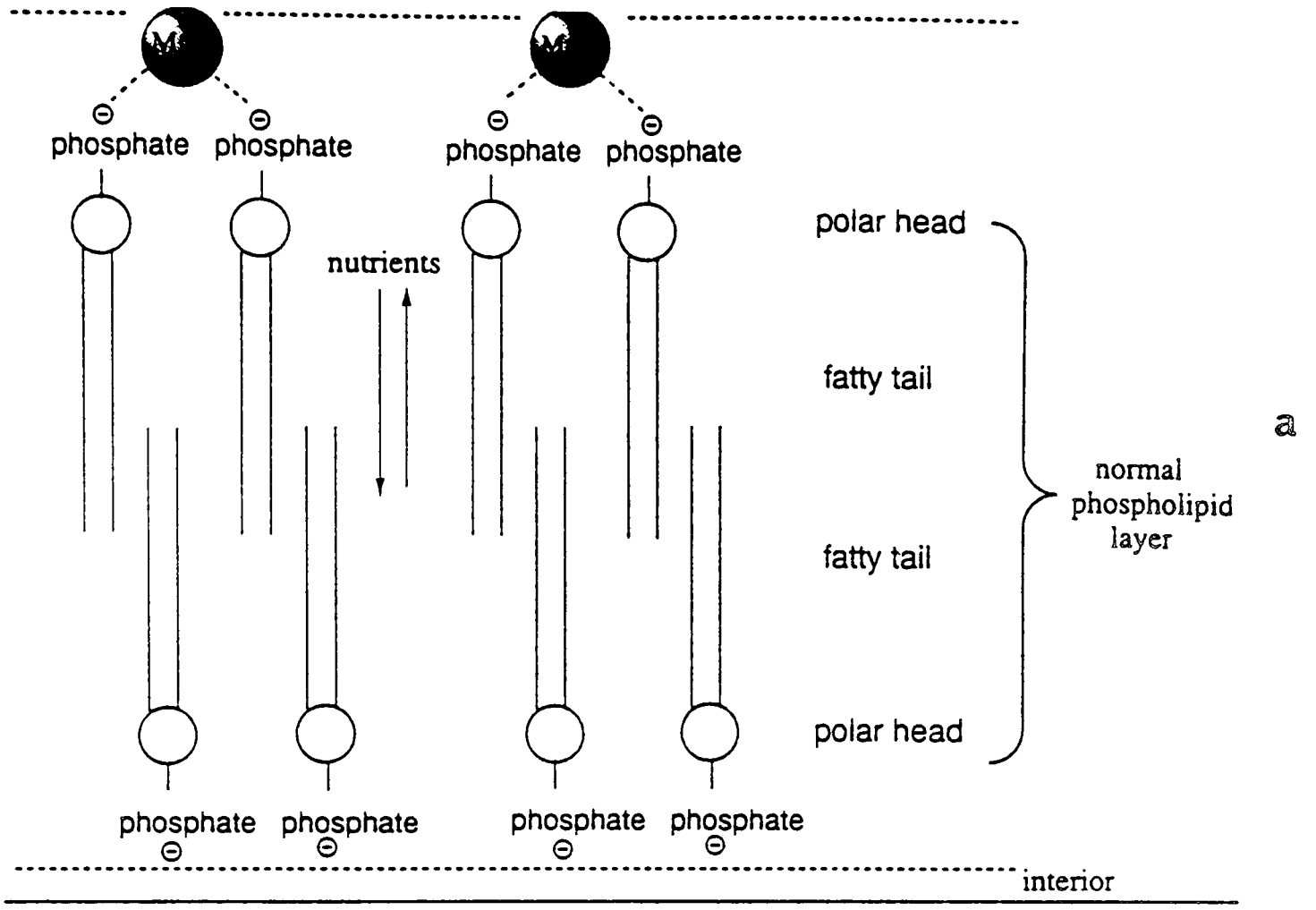


Fig. 1.3 Effects of the polymyxins on the cell outer membrane,

a) normal membrane, b) membrane plus polymyxin.

1.1.5 Antimicrobial activity and resistance

The antimicrobial activity of five members (A to E) of the polymyxin group is of a similar nature. However, they are all nephrotoxic although this effect is much reduced with polymyxin B and polymyxin E (colistin). The spectrum of clinical action of the polymyxins is limited to Gram-negative bacilli. Gram-positive bacteria and obligate anaerobic bacteria are resistant. *Escherichia coli, Klebsiella pneumoniae,* and the following species *Enterobacter*, *Salmonella, Shigella, Haemophilus, Pasturella* and *Bordetella* are usually susceptible. Classical *Vibrio cholerae* is sensitive. *Proteus*, *Providencia*, *Serratia* and *Neisseria* species are usually resistant. Some fungi such as *Coccidiodes immitis* are susceptible but most are resistant [9,11]. Minimum inhibitory concentrations of polymyxin B sulphate for the most sensitive species generally range from about 0.01 to 4 μg per ml [9].

Polymyxins have been reported to demonstrate antimicrobial synergy with a variety of other agents, including chloramphenical, tetracyclines, sulphonamides and trimethoprim. Polymyxins have been shown to be synergistic with trimethoprim and sulphamethoxazole against several multiply drug-resistant, Gram-negative bacilli (e.g. *Serratia marcescens*, *Pseudomonas cepacia* and *Pseudomonas maltophilia*). Synergism has also been demonstrate with rifampicin against multiresistant *Serratia* species. The mechanism of the synergism is probably the same as that observed with polymyxin B and tetracyclines against fungi at

concentrations where each drug alone has no effect. The polymyxin B increases the permeability of the yeast cell membrane to tetracyclines, which then inhibits protein synthesis and causes cell death [9,11]. Richards and Xing found [17] that subinhibitory concentrations of combinations of any two of ciprofloxacin, colistin (or polymyxin B), sodium sulphadiazine and p-aminobenzoic acid had synergistic inhibitory activity against *P. aeruginosa* and had synergistic or additive activity against *Staphylococcus aureus*. In addition sulphadiazine plus either ciprofloxacin or polymyxin showed markedly enhanced killing activity against both *P. aeruginosa* and *S. aureus*. p-Aminobenzoic acid plus either ciprofloxacin or polymyxin also demonstrated enhanced killing activity against *P. aeruginosa* but the combinations were less effective in enhancing activity against *S. aureus.* Ciprofloxacin in combination with polymyxin had a marked synergistic effect against *P. aeruginosa* but only a slight effect against *S. aureus.*

P. aeruginosa has been found to be resistant to polymyxin B in vitro [18-20]. It is suggested that polymyxin-susceptible cell populations adapt to polymyxin by the rapid alteration of the cell envelopes of the entire cell population. Cell envelope phospholipases and protease may play a major role in an adaptive response and the cation content of the cell envelope may be a critical controlling factor in the process [19]. Bell and Hancock [21] proposed that overall expression of divalent cation-regulated outer membrane protein H_1 of *P. aeruginosa* is associated with resistance to polymyxin B. Protein H_1 is believed to act by replacing divalent cations at binding sites on lipopolysaccharide, thereby preventing disruption of the sites and

subsequent self-promoted uptake of the antibiotic. In addition, loss of hydroxy fatty acid from lipopolysaccharide may perturb outer membrane hydrophobicity and is a contributing factor to polymyxin B adaptive resistance [20].

1.1.6 Clinical use

The polymyxins are used parenterally almost exclusively to treat serious *P.aeruginosa* or other Gram-negative bacillary infections caused by strains resistant to other drugs or in patients who cannot tolerate the preferred drugs. Because of their severe toxicity (neurotoxicity, nephrotoxicity and neuromuscular blockade),they are not the drug of first choice for the treatment of any specific infection[9,11]. The advent of carbenicillin derivatives like ticarcillin, the ampicillin derivatives like ureidopenicillins and also piperacillin and extended spectrum cephalosporins means that severe Gram-negative infections including those caused by *P. aeruginosa* can now be treated with less toxic agents than the peptide antibiotics or even the aminoglycosides. The use of parenteral polymyxins has therefore declined. They can be used to treat bacteraemia or urinary tract infection but are not effective in the treatment of deep tissue infections or infections in granulocytopenic patients [3,11].

Polymyxin B is the most commonly used polymyxin. It is used topically, often in combination with other antibiotics to extend the antimicrobial spectrum, in the treatment of eye, ear and skin infections due to susceptible organisms [9].

Polymyxin B has been combined with many other antibacterial agents including neomycin, bacitracin, oxytetracycline, trimethoprim, framycetin, gramicidin, chloramphenicol, kanamycin, nystatin and tyrothricin. Polymyxins have been formulated in various topical dosage forms such as ointments, creams, gels, solutions, aerosols, powders, eye and ear drops both in the form of solutions and suspensions. For example, eye drops of polymyxin B with neomycin and gramicidin have been used prophylactically to prevent infections in patients undergoing ocular surgery and conjunctival inflammatory disorders. More recently, trimethoprim with polymyxin B drops (Polytrim^{R}) have become available for superficial bacterial eye infections. They are intended for use in bacterial infection of the eye including blepharoconjunctivitis, conjunctivitis, keratitis and corneal ulceration, and for prophylaxis in eye surgery and trauma. Polymyxin B complements the action of trimethoprim which is active against most common Gram-positive and Gram-negative organism except *P. aeruginosa*. The combination provides useful broad-spectrum antibiotic activity. A combination of both drugs produced identical clinical and microbiological responses when compared with a solution containing trimethoprim , polymyxin B and sulphacetamide. The sulphadiazine component was not thought to be essential to produce the desired clinical and microbiological results. The antibacterial spectrum

of trimethoprim and polymyxin B eye drops resembles that of polymyxin B and bacitracin eye ointment (Polyfax^{R}) and polymyxin B, gramicidin and bacitracin eye drops (Neosporin^{R}). It has no advantage over them except as an alternative in a patient hypersensitive to one of the other constituents. Its activity against *P. aeruginosa* gives it a slight advantage over chloramphenicol. The preservative in polymyxin eye drops (thiomersal) can cause keratoconjunctivitis, particularly in soft contact lens wearers. This disadvantage and the expense means that the cheaper chloramphenicol eye drops remain the eye drops of choice in the routine management of bacterial infections [22-25].

Trimethoprim and polymyxin B are also combined in ophthalmic ointment. There was no statistically significant difference between trimethoprim-polymyxin B ophthalmic ointment and chloramphenicol ophthalmic ointment with regard to eradication of organisms or clinical improvement in the treatment of bacterial conjunctivitis and the prophylaxis of infection in patients undergoing cataract surgery [26-27]. A combination of hydrocortisone acetate-polymyxin B showed no significant difference in therapeutic efficacy from a combination of hydrocortisone butyrate-oxytetracycline in the local treatment of acute external otitis except for *S.aureus* which was cultured from 17% of the patients [28].

Polymyxin B-trimethoprim cream has been used in the treatment of superficial infection of the skin and pyoderma. A combination of 0.17% mg/g trimethoprim

and 10,000 IU/g polymyxin B was more effective than a 20 mg/g fusidic acid cream in reducing the signs of crust and tenderness , alleviation severity of pyogenic infection of the skin and reducing the overall severity score at the end of the two weeks study in 87 patients [29]. Polymyxin B is also combined with neomycin and bacitracin in an ointment used in the treatment of skin infections [30,31]. Other dosage forms such as sprays and aerosols are effective in the treatment of wound infections [32,33].

1.1.6 Analytical methods for polymyxin B

Various analytical methods such as countercurrent distribution, spectrophotometric or fluorimetric methods, column chromatography, thin layer chromatography (TLC), gas chromatography, high performance liquid chromatography (HPLC) and electrophoresis have been used to separate various components of polymyxins [34,35].

Spectrophotometric and fluorometric methods

Several spectrophotometric and fluorometric methods have been developed for polymyxin B. Both methods involve treatment of polymyxin with some reagents to make a colour complex or fluorescence derivatives that can be detected at a specific wavelength. Many reagents such as bromthymol blue, sodium hypobromite,

α-aminocarboxylic acid with sodium hypobromite, ninhydrin and fluorescamine have been used [36-40]. The disadvantages of these methods are non-specificity and the incapability of differentiating various components of polymyxin B [37]. However, they are useful for the quantification of polymyxin B components after separation by other methods e.g. TLC and electrophoresis.

Column chromatography

Haemers and Moerloose [41] reported a column chromatography determination of polymyxin B sulphate . Polymyxin B was taken up by a weakly acid polysaccharide ion-exchange resin with carboxyl groups in citrate buffer, pH 6.2, and eluted by a continuous ionic strength gradient at the same pH. The antibiotic was determined in the eluate with ninhydrin by means of an Auto-Analyzer. This was applied to polymyxin B bulk samples, tablets, hydrophilic ointments and aerosol powders. Good correlation between chemical and biological results in preparations where polymyxin B was partially degraded was obtained. This method can be considered as a specific and stability-indicating assay for polymyxin B sulphate and it was accepted as a valuable substitute for the microbiological method. The procedure offered greater accuracy and reproducibility (standard deviation 2.8%) than the microbiological method. However, the complication of the method and the long retention time (2.4 h) makes it not suitable for routine use. In addition this method cannot provide the information on separation and quantification of individual components of polymyxin B.

Thin-layer chromatography (TLC)

TLC is the method recommended in The British Pharmacopoeia for the identification of polymyxin B [42]. Different media and mobile phases have been used by several workers in order to separate its individual components [35]. Thomas *et al* [43] used pre-coated silica gel plates and the mobile phase consisted of acetonitrile-hydrochloric acid. Prior to chromatography, the plates were placed in a filter-paper lined chromatographic tank that had dried at 110°C for 30 minutes. The samples were applied to the plates and developed. The plates were then removed from the tank and the separated components detected with ninhydrin reagents. The ninhydrin-treated chromatoplates were scanned at 570 nm, using the thin-layer chromatography accessory of a fluorescence spectrophotometer. Thomas, A.H. and Thomas, J.M. [44] applied the use of the Optomax (a modular image analysis system) for the measurement of zones of inhibition of growth and ninhydrin zones. The relative proportions of PB_1 and PB_2 were determined in samples of polymyxin after TLC separation on the basis of antimicrobial activity and ninhydrin reactivity. The incomplete separation of polymyxin and the lack of sensitivity for the minor components meant that only the two major components could be quantified.

Gas-liquid chromatography (GLC)

GLC has been used to measure the fatty acids present in polymyxin B in order to discover the relative composition of the material and it is included in the monograph

for colistin in the British Pharmacopoeia [45]. The details of fatty acids assay were as follows. Polymyxin was hydrolysed with hydrochloric acid, the hydrolysate was extracted with ethyl ether and the fatty acids were separated with GLC on a column consisting of 20% diethylene glycol and 3% phosphoric acid on chromosorb G [42,45,46].

High-performance liquid Chromatography (HPLC)

Analysis of the polymyxin by high-performance liquid Chromatography was developed in recent years. There are quite a few publications in the literature, but different HPLC systems were used according to different conditions. In general, the columns were packed with Spherisorb ODS 5 μm diameter particles. The samples were dissolved in the mobile phase and were injected on to the column. The detector was operated from 185 to 220 nm and the peak areas were measured with an electronic integrator. The results are given as the percentage of the total area produced by each component [3,5,6,34,44,47-56].

The compendial methods of quantitative analysis for polymyxin B in the British , European and United States Pharmacopoeias are currently microbiological. These microbiological methods are capable of great sensitivity but their disadvantage is the lack of reproducibility of the potency determinations between laboratories, especially where the dose-response lines of the standard and test are not parallel. If suitable chemical methods are available they are usually more reliable, reproducible

and precise [5]. Both gel electrophoresis and TLC have proved invaluable in demonstrating the heterogeneous nature of many antibiotics [44]. The assay of fatty acid by gas-liquid chromatography is a reproducible and reliable method for determining the relative proportions of the main polymyxin components present in a sample. However, because of the hydrolysis and extraction procedures, it would be difficult to adapt this method to the quantitative determination of the polymyxin on a mass basis. High performance liquid chromatography provides the most information on the composition of the polymyxin sample and it would be the method best suited to monitoring the composition of polymyxin in order to ensure that material of a known composition is used. However, because of the complex nature of the polymyxin, it is difficult to suggest a suitable chemical assay to replace the microbiological assay. In the case of antibiotics, a chemical method is valueless if the results differ from those obtained by the microbiological method [43]. It is suggested that in the first instance, a comparison of the HPLC assay with the microbiological assay should be carried out [34].

Only two publications reported chemical stability studies of polymyxin B. Taylor *et al* [55] used HPLC assay to carry out a stability study and showed that polymyxin B alone in aqueous solution was not stable for long periods of time (shelf-life (t_{90}) = 130 days at 4^{o}C). Dreyer-van der Glas *et al* [56] developed a stability indicating method of analysis for polymyxin B in eye and ear drops and they demonstrated a shelf-life (t_{90}) of at least 18 months at 4^{o}C. These two investigations yielded

different shelf-lives of polymyxin B and only a chemical assay was used to determine the rate constants.

Both a microbiological assay and a chemical assay were used in three publications. Good correlation between both methods were obtained by comparison of column chromatography data with microbiological data both in terms of total polymyxin B [41] and TLC data with microbiological data in terms of PB_1 and PB_2 [44]. However, the information of individual components of polymyxin B was not clearly demonstrated. Thomas *et al* [42] used a microbiological assay, TLC,GLC, and HPLC to examine the potency and composition of polymyxin B and polymyxin E from different sources. It was found that there was no correlation between the total proportion of the two major components in each polymyxin determined by either GLC or HPLC and the microbiological potency. Although a microbiological assay and chemical assay were used in this study, the stability of polymyxins was not investigated. There are no reports in the literature concerning the relationship between chemical stability and microbiological activity of polymyxin preparations.

1.2 Purposes of this work

The purpose of the present work was to develop HPLC and microbiological methods capable of determining the chemical stability and microbiological activity of polymyxin B in solutions. It was intended to develop both methods for standard material and various eye drop formulations also containing other added ingredients (trimethoprim, neomycin, gramicidin, metaoxedrine, thiomersal and benzalkonium chloride). It was also intended to use both methods to monitor the concentration of polymyxin B components and their microbiological activity during a decomposition process. This would allow correlation of microbiological activity data with chemical stability data which has not been found in the literature. Such correlation would add significantly to existing knowledge of the overall action of the drug and would possibly provide new insights into its stability in formulations.

Chapter 2 HPLC assay development

2.1 Introduction

Analysis of polymyxin B by high performance liquid chromatography(HPLC) has been developed using several different systems. Polymyxin B components were first separated by using gradient elution [47]. An isocratic HPLC method is preferred over a gradient one due to the fact that laboratory automation can be more easily and inexpensively accomplished with an isocratic system [48]. The first isocratic HPLC method for the analysis of PB_1 and PB_2 was reported by Fong and Kho [48]. The method uses a 25 cm Hypersil-ODS (5 μm) column, a mobile phase containing 22.5 % acetonitrile in an aqueous phase with tetramethylammonium chloride (TMAC), a flow rate of 1.0 ml/minute and a wavelength of 220 nm detection. Complete resolution of PB_1 and PB_2 and their separation from all other components and/or impurities was achieved in less than 23 minutes. The effect of mobile phase components on the separation of polymyxin B, the linearity and the lower limit of detection were investigated. Terabe *et al* [48] successfully separated seventeen components of the polymyxins (M_1, M_2, D_1, D_2, C_1, C_2, S_1, E_1, E_2, B_1, B_2, B_3, F_1, F_2, F_3, T_1, T_2) using Nucleosil $5C_{18}$ column with tartrate buffer-acetonitrile containing sodium 1-butanesulphonate and sodium sulphate as the mobile phase. Kimura *et al* [50] used different system to separate polymyxin A, B, D, E, K, M and P. A porous styrene-divinylbenzene copolymer column and methanol : 0.2 M potassium chloride/hydrochloric acid buffer (pH 2.0) (1:1) as a mobile phase were

employed. Thomas *et al* [43] used a Spherisorb C_{18} column and a mobile phase consisting of tartrate buffer (0.005 M), pH 3.0, sodium butane 1-sulphonate (0.05 M), sodium sulphate (0.2 M) and acetonitrile (18%v/v). The different proportions of polymyxin B components from different sources was shown. Fisher and Raja [5] suggested that the mobile phase used by Terabe *et al* and Thomas *et al* causes many problem owing to crystallisation of solutes in pump heads, valves and injectors which is unsatisfactory and occasionally expensive. They used a column packed with 5 μm Hypersil C_{18} and water-methanol-methanesulphonic acid (250+250+5) as the eluent and claimed that there was no potential problem of crystallisation of buffer from the mobile phase, making it ideal for routine use. Similar to Thomas *et al*, the polymyxin B was divided into three groups according to the difference in proportion of PB_1 and PB_2. Whall [51] reported the complete separation of the three major components using Altex-Ultrasphere ion-pair column and a mobile phase containing acetonitrile-phosphate buffer. This system was simple and rapid. Linearity and limit of detection were also reported. At approximately the same time, Elverdam [52] developed the system to separate polymyxin B into ten to thirteen components using a chemically bonded C_{18} stationary phase and a mobile phase consisting of a mixture of acetonitrile/formate and acetate buffer. Recently, separation of polymyxins by micellar electrokinetic capillary chromatography has been reported [6]. The method developed is useful for both qualitative and quantitative measurements on polymyxins. The variations between the relative peak areas of the main peaks from difference sources was described.

As mentioned by Taylor *et al* [55], most of the information appearing in the literature is concerned with separation. There are only three papers which describe the quantitative analysis of polymyxin B. Whall [51] reported the linearity and detection limit of polymyxin B. Fong and Kho [48] also described the linearity and detection limit of polymyxin B, but only two components are resolved and the quantitation is in terms of the sum of these peaks. The publication by Fisher and Raja [5] estimates the relative proportions of each of the major components by peak normalisation.

Two recent publications have described HPLC as stability indicating methods of analysis of polymyxin B. Dreyer-van der Glas *et al* [56] used a 60-RP-select B column and a mobile phase containing 0.01 M diethylamine (adjusted to pH 2.5 with phosphoric acid and 0.064 M Na_2S0_4) : acetonitrile , 78 : 22 v/v to assay polymyxin B eye and ear drops during a stability study. The influences of pH and ionic composition of the solution on the quantitative determination method of polymyxin B was not clearly described. Taylor *et al* [55] used two systems consisting of 3 μm ODS Hypersil and two mobile phases called solvent A and solvent B differing in composition of phosphate buffer, acetonitrile, perchloric acid and pH. Solvent A which containing 24:76 acetonitrile : aqueous 0.05 M disodium hydrogen phosphate adjusted to pH 2.9 was used to determined the percentage of polymyxin B remaining during an accelerated stability study. The quantitation of polymyxin B was based on the remaining peak height of PB_1 and PB_2 which were in good agreement. The chromatographic variables affecting the separation, the validation of the assay and the specificity of the assay for polymyxin B in the presence of trimethoprim,

propamidine and dibromopropamidine were reported. However, the specificity of solvent A for the assay of polymyxin B in various eye drops formulations was not reported. It is suggested that solvent B may be suitable for use in the assay of polymyxin B in the presence of co-formulated antibacterial drugs since the inclusion of perchloric acid would improve the resolution and it is less critical than solvent A in routine use. The effect of chromatographic variables of solvent B on the separation was not studied.

It was intended to investigate further the effect of chromatographic variables of solvent B on the separation of polymyxin B components. The results obtained from this investigation is necessary for mobile phase optimisation. It was also intended to report the quantitative aspects of assay validation for polymyxin B in the presence of the other ingredients by using the developed mobile phase. This would allow the appropriate chemical assay for polymyxin B to be used in the proposed stability study of the drug in various eye drop formulations.

2.2 Materials and methods

Chemicals and Reagents, apparatus and chromatographic conditions described below were employed in all experiments in Chapters 2 and 3.

2.2.1 Chemicals and Reagents

Water used in the chromatography was purified by a Millipore Milli-Q system. HPLC grade acetonitrile(ACN) was obtained from Rathburn Chemicals. Polymyxin B sulphate (PMB), trimethroprim (TMP), neomycin sulphate (N), gramicidin (G), metaoxedrine or L-phenylephrine hydrochloride (M), benzalkonium chloride (BZK) and sodium hydroxide were purchased from Sigma. Thiomersal (TMS) was supplied by Aldrich Chemical. Disodium hydrogen phosphate (DSHP), perchloric acid, ammonia (SG 0.88, SLR grade), orthophosphoric acid, potassium dihydrogen orthophosphate (SLR grade) were supplied by Fisons. Polymyxin B eye drops were purchased from Wellcome (sample 1), Seng Thai (sample 2), Dispersa (sample 3) and Alcon (sample 4-6). (Table 2.7)

2.2.2 Apparatus

The HPLC system employed in this work comprised a Jasco PU 880 pump and UV 2550 detector, a Rheodyne Model 7125 injector incorporating a 20 µl loop and a BBC Goerz Servogor Type SE 210 chart recorder. The chromatographic column (100 X 4.6 mm) was slurry packed in the laboratory with 3 µm ODS Hypersil.

2.2.3 Chromatographic conditions

The mobile phase was filtered through a 0.45 µl Millipore filter Type HVLP prior to use. The column flow-rate was maintained at 1.5 ml/min. All measurements were

made at room temperature. The column was equilibrated with the mobile phase for about 30 minutes at the flow rate used for this work. The detector wavelength was set at 200 nm using the sensitivity from 0.08 to 1.28 AUFS, at a recorder input of 10 mV. Recorder chart speeds between 0.2 and 0.5 cm/min were used.

2.2.4 Separation development

As the separation of polymyxin B in this work was based on a reverse phase separation, the magnitude of the separation depended on many factors, particularly the composition of the mobile phase which was, in this study, composed of acetonitrile as an organic modifier, pH , buffer and perchloric acid as an ion pairing agent. In the present work, each of these chromatographic variables was modified and the resultant effect on the separation of polymyxin B components was observed.

The separation parameters (capacity factor ; k' and resolution ; R_s) were calculated from the following equations [57].

$$k' = (t_r - t_o) / t_o \qquad (1)$$

Where the retention time of solute is t_r and the retention time of solvent is t_o.

$$R_s = t_{R2} - t_{R1} / 0.5 (W1 + W2) \qquad (2)$$

Where t_{R2} is the retention time of solute 2, t_{R1} is the retention time of solute 1, W1 is the peak width of solute 1 and W2 is the peak width of solute 2.

2.2.4.1 Effect of acetonitrile concentration

The mobile phase was prepared by dissolving 3.58 g of DSHP and 3.85 g of perchloric acid (60 %w/v) in a mixture of water and acetonitrile to make the concentration of 0.01 M DSHP and 0.2702 % w/v perchloric acid. The volumes of acetonitrile and water were varied to obtain the concentrations of acetonitrile ranging from 25 to 50 % v/v. The pH was adjusted to 3.0 by addition of concentrated ammonia.

The sample solution was prepared by weighting out accurately about 0.02 g of polymyxin B sulphate (8090 U/mg) in a 10 ml volumetric flask and dissolving this in water to make a concentration of 16,250 U/ml. After the sample was injected at different concentration of acetonitrile, the retention time (t_r), separation and capacity factor (k') of the three components of polymyxin B (PB_1, PB_2 and PB_3) were recorded.

2.2.4.2 Effect of pH

A mobile phase consisting of 30:70 acetonitrile : aqueous 0.01 M DSHP and 0.2702 % w/v perchloric acid was used in this experiment. The mobile phase was prepared by dissolving 3.58 g of DSHP and 3.85 g of perchloric acid (60 % w/v) in a mixture of 300 ml acetonitrile and 700 ml water. The pH was adjusted by adding ammonia or orthophosphoric acid to obtain a range of pH from 2.0 to 5.5.

Samples containing 16,250 U/ml of polymyxin B were prepared by the method described in 2.2.4.1. After the sample was injected at different pH, the retention time (t_r), and capacity factor (k') of the three components of polymyxin B (PB_1, PB_2 and PB_3) were recorded. The separation among the individual peaks was also qualitatively observed

2.2.4.3 Effect of buffer concentration

The solvent system consisted of 30:70 acetonitrile : aqueous DSHP and 0.2072 % w/v perchloric acid was used and prepared as described in 2.2.4.2. This solvent was recycled and the buffer concentration increased from 0.005 to 0.020 by addition of solid DSHP.

Samples containing 16,250 U/ml, prepared as described in 2.2.4.1 were injected at each concentration of buffer after equilibration. The retention time (t_r) and capacity factors (k') of the three components of polymyxin B (PB_1, PB_2 and PB_3) were recorded and the separation among the peaks observed qualitatively.

2.2.4.4 Effect of perchloric acid

To confirm and extend the results of Taylor *et al* [54] concerning the effect of perchloric acid on the separation of polymyxin components, the effect of addition of perchloric acid was studied over an extended range of concentration. Since this resulted in considerable increases in retention, the concentration of acetonitrile was adjusted as appropriate to maintain the same overall separation time.

The solvent system consisted of 28:72 acetonitrile : aqueous 0.01 M DSHP was prepared by dissolving 3.58 g of DSHP in a mixture of 280 ml acetonitrile and 720 ml water. Perchloric acid was added to provide a range of concentrations from 0.0348 to 0.6 %w/v. The pH was adjusted to 3.0 by addition of ammonia. Perchloric acid concentration was further increased from 0.6 to 5.0 %w/v by using mobile phase containing 40:60 acetonitrile : aqueous 0.01 M DSHP, pH 3.0 adjusted by addition of ammonia.

Samples containing 16,250 U/ml polymyxin B was prepared as the same method as 2.2.4.1 and injected at each concentration of perchloric acid. The retention time (t_r), and capacity factor (k') of the three components of polymyxin B (PB_1, PB_2 and PB_3) were recorded. The resolution between PB_1 and PB_3 was also measured at each perchloric acid concentration.

2.2.5 Quantitative aspects - assay validation

Using the solvent system containing 40:60 acetonitrile : aqueous 0.01 M DSHP , pH 3.0 and 4.4 %w/v perchloric acid, the quantitative aspects and assay validation were carried out as follows.

2.2.5.1 Linearity of detector

The concentration of polymyxin B in commercial eye drops used in stability study varies from 5.000 to 16,250 U/ml and the viscosity of the eye drops make it difficult to draw a sample for injection directly from the formulation. The concentration of polymyxin B, therefore, was determined by comparing the peak height of a 1:10 dilution of the formulation with that of a freshly prepared aqueous standard.

Five concentrations of aqueous fresh polymyxin solutions were prepared ranging in concentration from 162.5 to 1625 U/ml. These concentrations were chosen since the estimated range of drug concentrations to be determined during proposed stability studies was from 1,625 to 16,250 U/ml. The peak height of each component was measured at 0.16 AUFS following injection of 20 µl of each concentration.

2.2.5.2 Accuracy

Since no extraction procedure is involved in this assay the accuracy of the method will be dictated largely by the specificity. The following procedures validate the specificity of the separation with respect to the other drugs and preservatives present in the formulations being determined. The overall accuracy of the assay is also determined by a standard addition method.

1) Specificity with respect to other species

Solutions of polymyxin B (16,250 U/ml), trimethoprim (0.1%w/v), neomycin (0.5%w/v), gramicidin (0.0025%w/v), benzalkonium chloride (0.004%w/v), thiomersal (0.005%w/v) and metaoxedrine (0.12%w/v) were prepared in water. Solutions were also prepared of polymyxin B (16,250 U/ml) combined with the above drugs at the following concentrations : trimethoprim (0.1%w/v), neomycin (0.5%w/v), gramicidin (0.0025%w/v), benzalkonium chloride (0.01%w/v), thiomersal (0.005%w/v) and metaoxedrine (0.12%w/v). Since gramicidin is insoluble in water, the first solution of gramicidin was made in ethanol and diluted with water to get the required concentration. These solutions were injected and the retention time of polymyxin B and the other species were measured.

2) Percentage recovery

Four different eye drops formulations of sample 1,2,3 and 4 were spiked with known concentration of standard polymyxin B as the method described below:

2.1 *Standard* : Standard polymyxin B concentration 16,250 U/ml was prepared by the same method as described in 2.2.4.1 and further diluted ten times by water to produce the concentration 1,625 U/ml.

2.2 *Diluted eye drops* : Eye drops were diluted ten times by water to get the concentration about 1,000 U/ml (sample 1), 500 U/ml (sample 2), 1,500 U/ml (sample 3), and 1,625 U/ml (sample 4).

2.2 *Diluted eye drops plus standard polymyxin B* : 0.5 ml of standard solution (16,250 U/ml) and diluted eye drops were adjusted to 5 ml by water to achieve the concentration of polymyxin B in sample about 1,161.8 U/ml (sample 1), 661.8 U/ml (sample 2), 1,661.8 (sample 3) and 1,786.8 U/ml (sample 4).

The concentration of PB_1, PB_2 and PB_3 of diluted eye drops plus standard were determined by comparing the peak height with diluted standard. The differences between assayed concentration and expected concentration were described in term of percentage accuracy.

2.2.5.3 Precision

The within day precision of the assay with respect to retention time and peak height was determined by dissolving 0.02 g polymyxin B (8090 U/mg) in water (16,250 U/ml) and diluting this solution 1 to 100 with water to provide the upper and lower concentrations expected during the proposed stability study. Replicate 20 µl injections (10) of these solutions were made and the relative standard deviations of both retention times and peak heights for each of the polymyxin components were calculated.

2.2.5.4 Limits of detection

An aqueous polymyxin sample of concentration 16,250 U/ml was made by the same method as described in 2.2.4.1. It was diluted and injected until the ratio of the height of the PB_1 peak to the baseline noise level was equal to 2.0.

2.2.6 Chemical assay of polymyxin B eye drops

Six selected commercial polymyxin B eye drops (sample 1-6, Table 2.7) were diluted ten times by water. The external standard concentration 16,180 U/ml was freshly prepared by dissolving 0.0200 g of polymyxin B sulphate (8090 U/mg) in 10 ml of water and diluting ten times by water. Quantitative determination of polymyxin B in the eye drops was based on peak height comparison of PB_1 and PB_2 of standard and samples. While it is accepted that this does not give absolute values of the concentrations of the individual components it allows comparisons among the different samples. Only PB_1 and PB_2 were used as they clearly separated from the other components and were not affected by decomposition products [55].

2.3 Results and discussion

2.3.1 Separation development

2.3.1.1 Effect of acetonitrile concentration

The effects of altering acetonitrile concentration are summarised in Table 2.1 and Figure 2.1 Increasing acetonitrile concentration resulted in critically decreased retention time and separation of polymyxin B components. In system consisting of 0.01 M DSHP, pH 3.0 and 0.2702 %w/v perchloric acid, changing acetonitrile concentration from 30 to 32 %v/v decreased retention time of peak PB_1 from 34 to 15 minutes and the capacity factor which indicates separation was much decreased. The separation was strongly dependent on the concentration of acetonitrile in the mobile phase correlating with the results of earlier reports [5,48,52]. This finding is also consistent with the principle of reverse phase separation that changing the polarity of the mobile phase (by increasing the ratio of organic solvent to water) will shift the distribution of solutes towards the mobile phase, and their retention will decrease [57]. The best separation of PB_1, PB_2 and PB_3 from this mobile phase system was obtained at acetonitrile concentration 30%v/v but the retention times were still quite long (34, 29 and 20 minutes for PB_1, PB_2 and PB_3,respectively). Acetonitrile concentration 30 %v/v was used to investigate the effect of the other chromatographic variables.

Table 2.1 Effect of acetonitrile concentration on retention time and separation

ACN(%v/v)	0.01 M DSHP(%v/v)	t_r (min) PB_2	PB_3	PB_1	Separation
50	50	-	-	*1.2*	t_r value too short, only PB_1 appeared.
40	*60*	-	*2.2*	*2.6*	t_r value too short, PB_3 too close to PB_1.
32	*68*	*9.5*	*13.0*	*15.0*	Three peaks were separated.
30	*70*	*20.0*	*29.0*	*34.0*	The best separation
28	*72*	*25.0*	*38.0*	*45.0*	Similar to 30:70 but longer t_r
25	*75*	-	-	-	No peak appeared after 1.4 h.

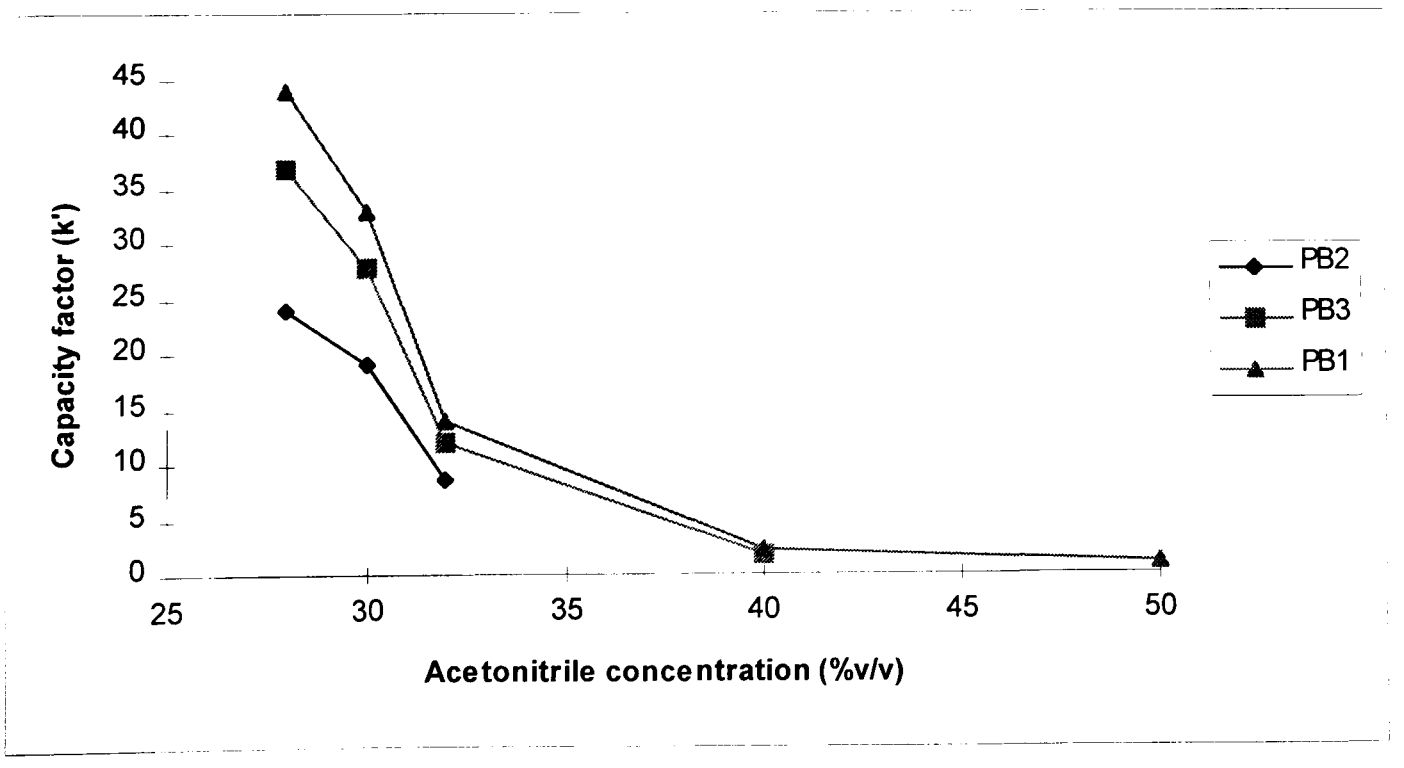


Fig. 2.1 Effect of acetonitrile concentration on capacity factor(k').

2.3.1. 2 Effect of pH

The effects of varying pH are summarised in Table 2.2 and Figure 2.2. Increase in pH from 2.0 to 2.8 resulted in decreased retention times of polymyxin peaks. In contrast, retention time was increased when pH was increased from 3.0 to 5.0. However, between pH 2.8 to 4.0, effect of pH was not critical and the capacity factor (k') of three major components were satisfactory (4.5 to 9.0). Therefore, the pH of the mobile phase should be adjusted within this range of pH. The pH of 3.0 as used before is thus suitable for this solvent system.

Table 2.2 Effect of pH on retention time (t_r) and separation

pH	t_r(min.) PB_2	PB_3	PB_1	Separation
2.0	11.0	16.0	19.0	Good separation (pH 2.0 -3.0)
2.3	9.0	13.0	15.0	
2.5	6.5	9.5	11.0	
2.8	5.5	7.5	9.0	
3.0	5.6	8.2	9.6	
3.6	5.5	8.0	8.5	PB_3 was close to PB_1 (pH 3.6 -4.4)
4.0	5.5	8.5	10.0	
4.15	7.5	11.0	13.0	
4.4	9.0	12.0	14.5	
5.0	10.0	16.0	18.0	All peaks were not sharp and had bad shape.

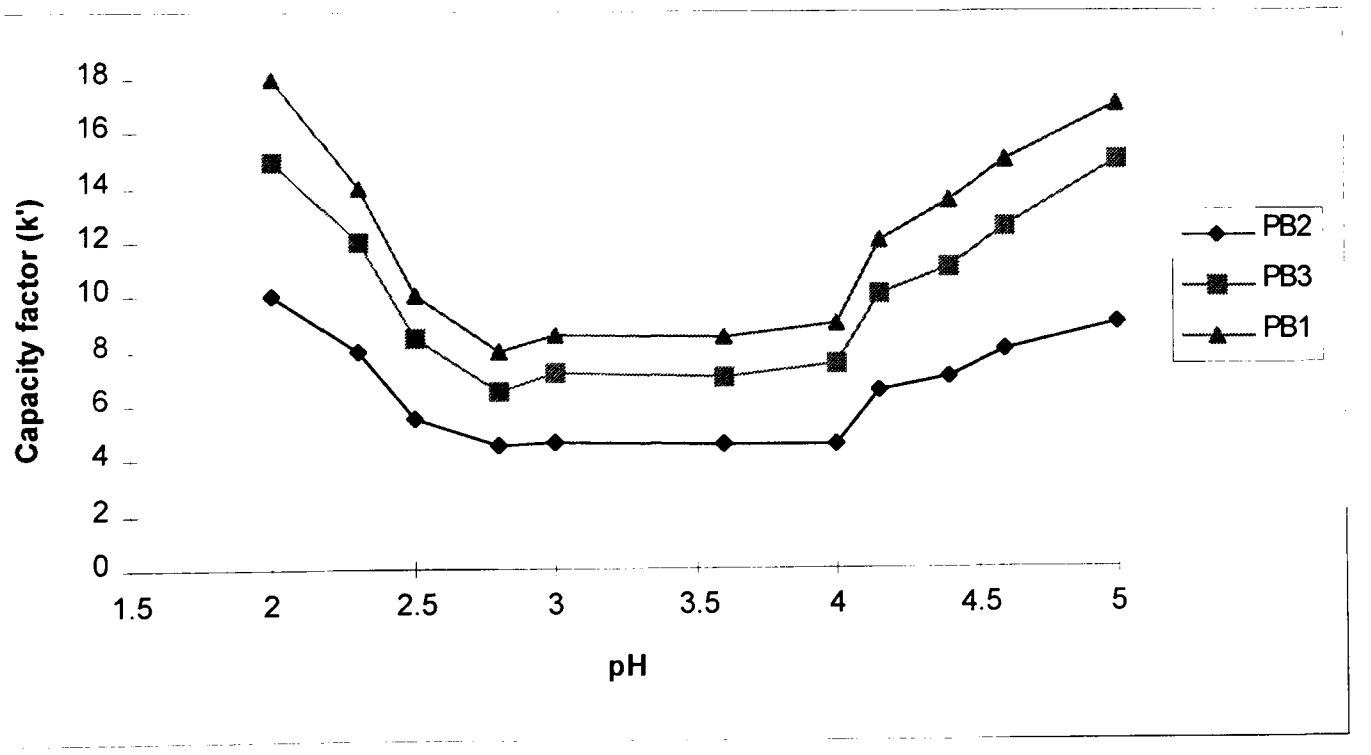


Fig. 2.2 Effect of pH on capacity factor(k').

2.3.1.3 Effect of buffer concentration

As seen in Table 2.3 and Fig. 2.3, the investigation of effect of buffer concentration was difficult because the wavelength of detection had to be increased due to the increased absorbance of DSHP at the higher concentrations. From the previous experiments, adequate separation was obtained from a system consisting of 30:70 acetonitrile : aqueous 0.01 M DSHP, pH 3.0 and 0.2072 %w/v perchloric acid. At the higher wavelengths used PB_2 and PB_3 could not be detected. Although it was not possible to determine the effect of buffer concentration at wavelength 200 nm in this experiment, the overall data show that buffer concentration has little effect on the retention. 0.01 M DSHP is presumably the suitable concentration for this solvent system.

Table 2.3 Effect of buffer concentration on retention time(t_r) and separation

DSHP(M)	t_r(min.) PB_2	PB_3	PB_1	wavelength (nm)	separation
0.0050	16.0	24.0	28.0	200	Three peaks were separated but not sharp. (0.0050-0.0075)
0.0075	10.0	16.0	19.0	200	
0.0085	-	-	18.0	209	Only PB1 appeared. (0.0085-0.0175)
0.0100	-	-	16.0	210	
0.0115	-	-	18.5	214	
0.0125	-	-	15.0	214	
0.0150	-	-	15.5	216	
0.0175	-	-	14.0	218	

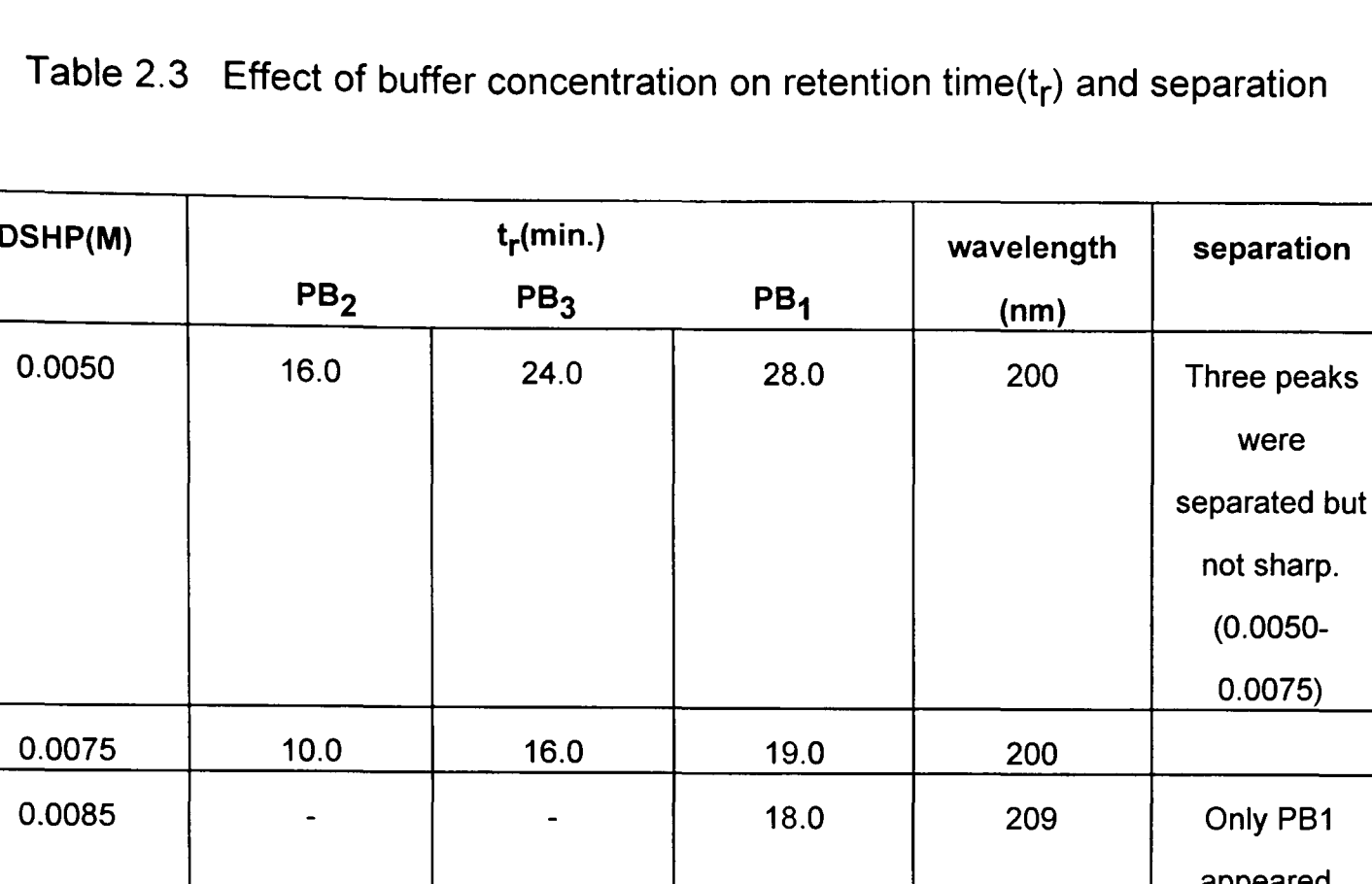


Fig. 2.3 Effect of buffer concentration on capacity factor(k').

2.3.1.4 Effect of perchloric acid

Based on knowledge gained as to the effect of the variables acetonitrile concentration, pH and buffer concentration, a solvent system containing 28:72 acetonitrile : aqueous 0.01 M DSHP, pH 3.0 was used to study the effect of perchloric acid on the separation. 28%v/v of acetonitrile was initially used since the lower ratio of acetonitrile would improve the separation of polymyxin B components as discussed in 2.3.1.1 and perchloric acid was another factor to increase the separation. From the previous data, the resolution between peak PB_1 and PB_2 was usually appreciable at low concentrations of perchloric acid while peak PB_3 was always close to PB_1. It was found that the presence of perchloric acid was important in increasing the resolution between peak PB_1 and PB_3. Up to concentration 0.6000%w/v, the resolution between PB_1 and PB_3 was not adequate and the retention time was still too long (Table 2.4 a). To decrease retention time, acetonitrile was increased to 40%v/v and perchloric acid was then increased up to 5.0%w/v (Table 2.4 b). The best separation was obtained between 4.0 - 5.0%w/v perchloric acid as this range of concentration gave the highest resolution between PB_1 and PB_3. In order to reduce the analysis time, the effects of concentration in the range 4.1 to 4.5%w/v were investigated further as shown in Table 2.4 c. In subsequent experiments in chapter 2, 3 and 9, a perchloric acid concentration of between 4.4 and 4.5 %w/v was used since this presented adequate resolution between components PB_1 and PB_3 and gave the lowest obtainable analysis time.

Table 2.4 a Effect of perchloric acid concentration 0.0348 to 0.6000 %w/v on retention time(t_r), separation and resolution of peak PB_1 and PB_3

Mobile phase = 28:72 acetonitrile : aqueous 0.01 M DSHP, pH 3.0

perchloric acid(%w/v)	t_r(min.) PB_2	PB_3	PB_1	separation	resolution PB_1 and PB_3
0.0348	2.0	-	3.0	PB_1 and PB_2 were eluted	-
0.1849	5.0	6.5	7.5	PB_1, PB_2 and PB_3 were eluted but PB_3 was close to PB_1 (0.1849 - 0.6000)	-
0.2055	7.0	9.5	11.0		-
0.2704	9.5	13.5	15.0		0.5
0.4000	15.0	22.0	25.5		0.8
0.5000	19.0	29.0	34.0		0.71
0.6000	25.0	37.0	43.0		0.77

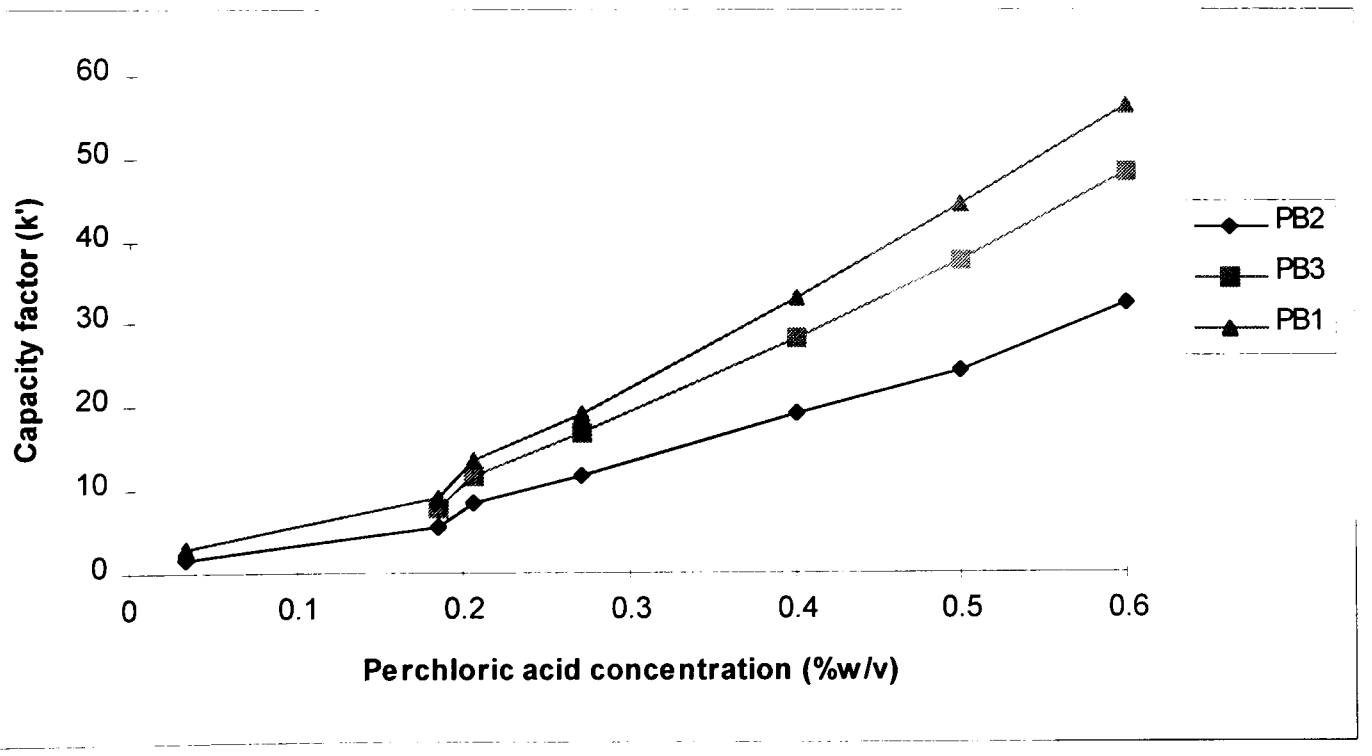


Fig. 2.4 a Effect of perchloric acid concentration 0.0348 to 0.6000 %w/v on capacity factor(k').

Mobile phase = 28:72 acetonitrile : aqueous 0.01 M DSHP, pH 3.0

Table 2.4 b Effect of perchloric acid concentration 2.0 - 5.0 %w/v on retention time and resolution of peak PB_1 and PB_3

Mobile phase = 40:60 acetonitrile :aqueous 0.01 M DSHP , pH 3.0

perchloric acid(%w/v)	t_r(min.) PB_2	PB_3	PB_1	resolution ($PB_1$and PB_3)
2.0	3.6	4.6	5.2	1.6
3.0	4.8	6.4	7.4	0.8
3.5	4.2	7.2	8.2	1.5
4.0	6.4	9.0	10.6	1.8
4.5	7.8	11.2	13.2	1.67
5.0	9.8	14.4	17.6	1.58

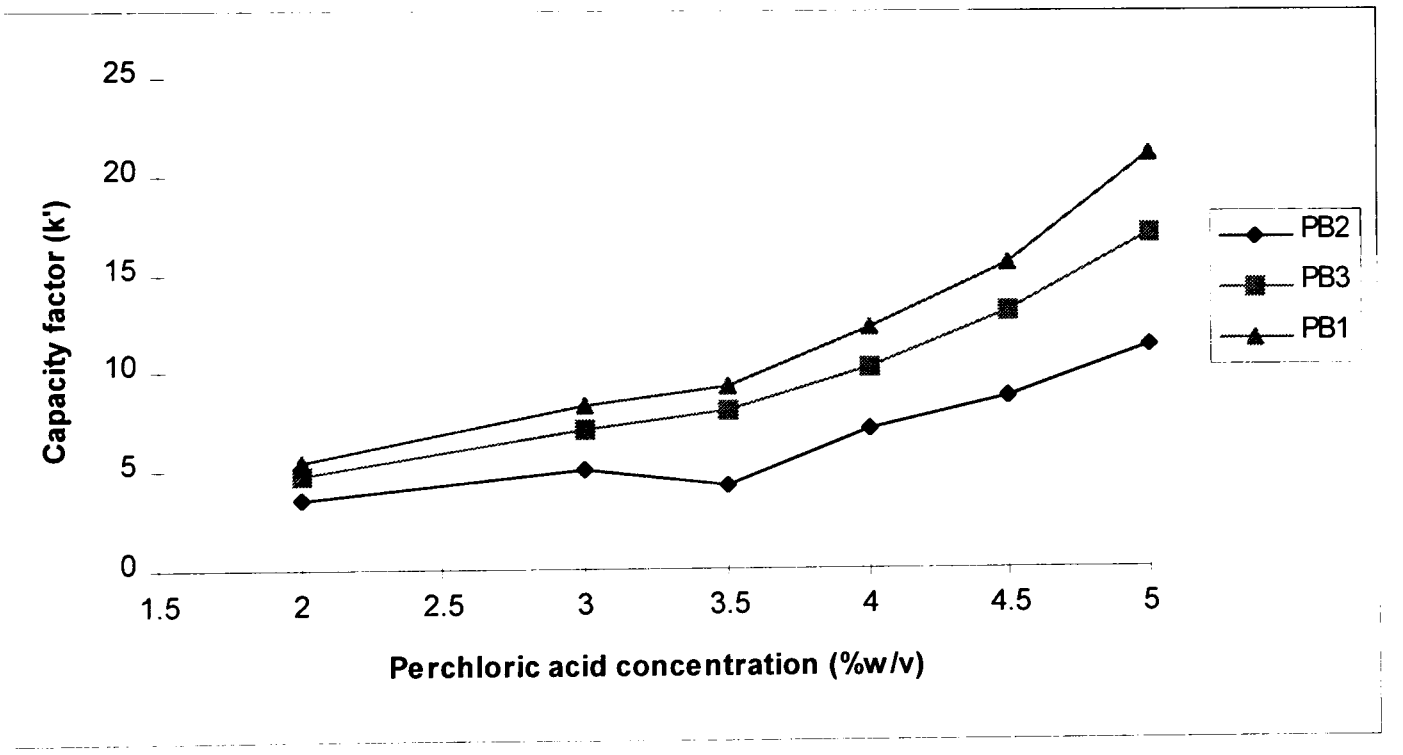


Fig. 2.4 b Effect of perchloric acid concentration 2.0 to 5.0 %w/v on capacity factor(k').

Mobile phase = 40:60 acetonitrile :aqueous 0.01 M DSHP , pH 3.0

Table 2.4 c Effect of perchloric acid concentration 4.10 - 4.50 %w/v on retention time(t_r) and resolution of peak PB_1 and PB_3

Mobile phase = 40:60 acetonitrile: aqueous 0.01 M DSHP , pH 3.0

perchloric acid(%w/v)	t_r(min.) PB_2	PB_3	PB_1	resolution (PB_1 and PB_3)
4.10	5.9	6.6	7.6	1.25
4.20	5.2	7.0	8.2	1.50
4.30	5.4	7.4	8.6	1.50
4.35	5.8	7.8	9.2	1.33
4.40	6.0	8.4	10.0	2.00
4.42	6.6	9.2	10.8	2.00
4.46	7.0	9.8	11.6	2.00
4.50	7.3	11.8	12.6	2.13

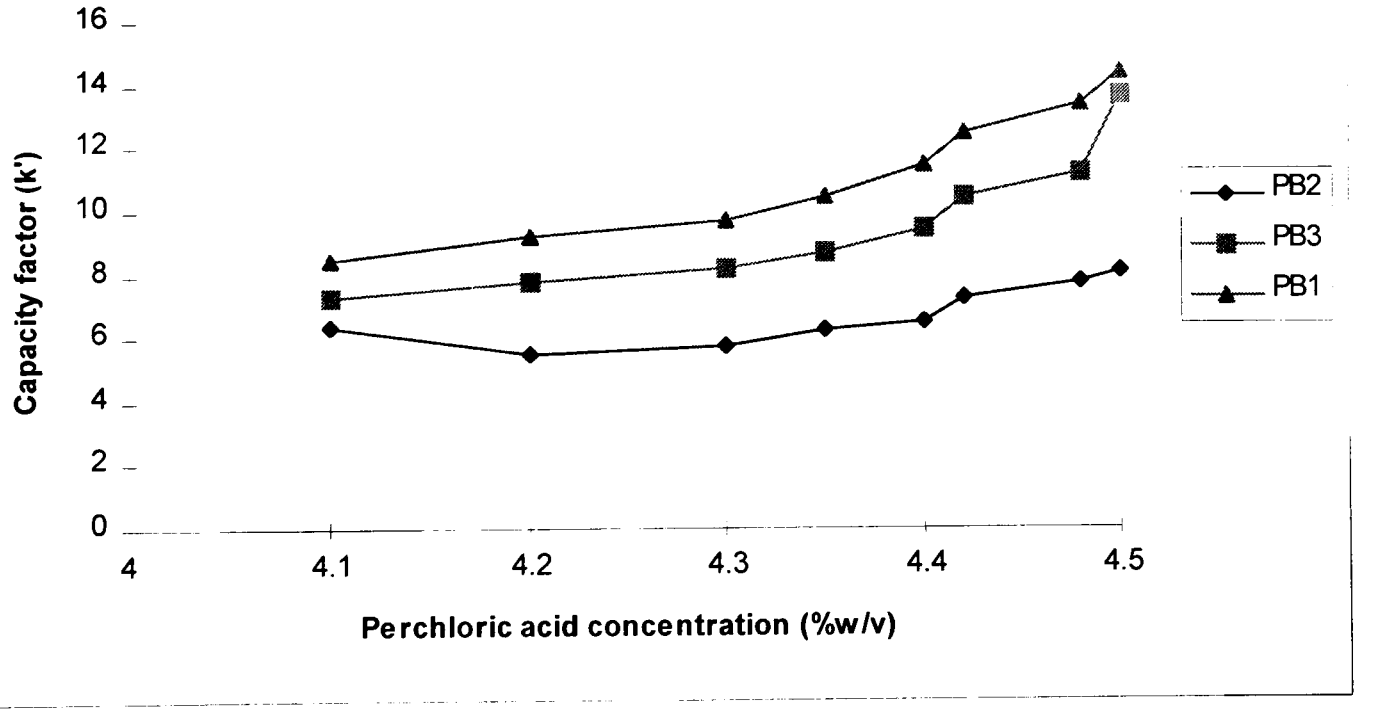


Fig. 2.4 c Effect of perchloric acid concentration 4.10 - 4.50 %w/v on capacity factor(k').

Mobile phase = 40:60 acetonitrile: aqueous 0.01 M DSHP , pH 3.0

2.3.1.5 General conclusion

Based on the data obtained from the investigation of these several chromatographic variables, the optimum mobile phase for the separation of polymyxin B components was 40:60 acetonitrile : aqueous 0.01 M DSHP, pH 3.0 and 4.4 %w/v perchloric acid. In this system, the use of high ratio of organic solvent (40%v/v) was necessary to prevent excessively long retention times. The effect of acetonitrile was critical and perchloric acid was the key factor for improving resolution. The effect of pH between 2.8 to 4.0 was not critical and buffer concentration had little effect on retention above 0.008M. By varying the concentration of perchloric acid, retention increased as the amount of perchloric acid increased. It was not clear how perchloric acid acts as ion pairing agent in this system. A possible mechanism of separation is that the polymyxin which is protonated at low pH forms ion pairs with perchlorate anions. The ion pairs behave as if they are non-ionic polar molecules, soluble in organic solvent. They are formed in the mobile phase and travel through the system as neutral species. Separation may occur by partitioning of these neutral ion pairs between the mobile phase and the C-18 [57].

This mobile phase allowed short analytical times (within 13 minutes for all three major polymyxin B components) and satisfactory degree of separation (the resolution(Rs) of polymyxin B components was equal or more than 2). A specimen chromatogram of polymyxin B obtained using this mobile phase system is shown in Fig. 2.5. The quantitative aspects of the assay and their validation and when applying this solvent system to samples are discussed below in 2.3.2.

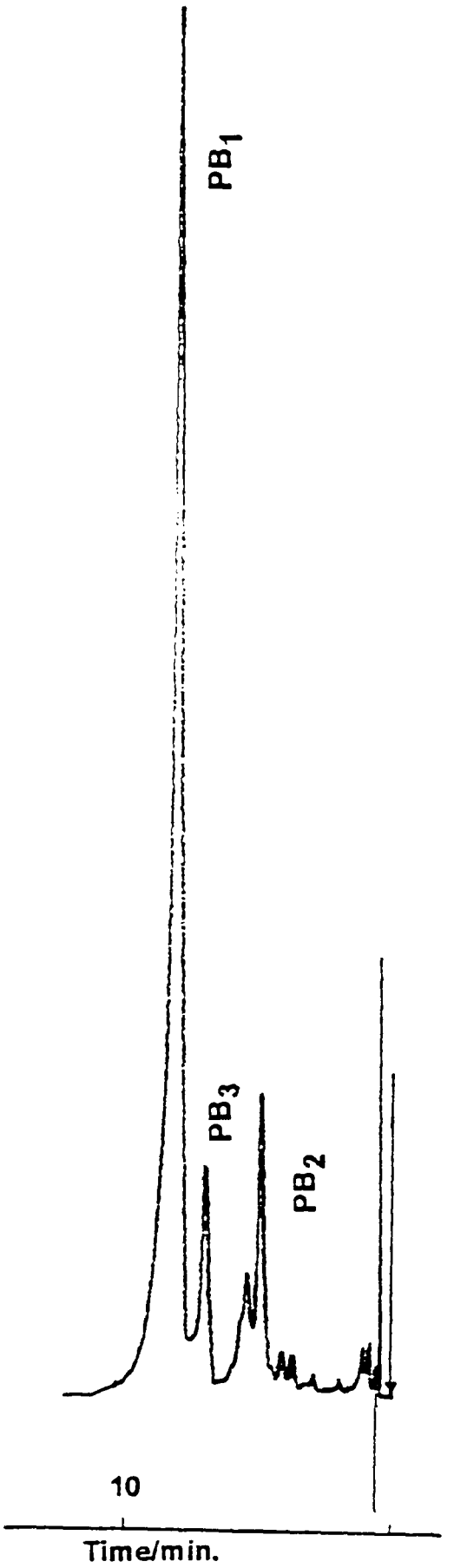


Fig. 2.5 Representative chromatogram of 16,250 U/ml polymyxin B using mobile phase containing 40:60 ACN : aqueous 0.01M DSHP, pH 3.0 and 4.4 %w/v perchloric acid (chart speed = 0.2 cm/min., 0.08 AUFS).

2.3.2 Quantitative aspects - assay validation

2.3.2.1 Linearity of detector

Least-squares regression of the three components peak height on total polymyxin B sulphate concentration (C ; U/ml) yield the following equations:

Peak PB_1

Peak height (cm) = 936.8C + 0.9184 (r^2 = 0.9981)

Peak PB_2

Peak height (cm) = 197.1C + 0.1229 (r^2 = 0.9994)

Peak PB_3

Peak height (cm) = 165.2C + 0.0808 (r^2 = 0.9989)

The relative standard deviation of slopes (RSD) of PB1, PB2 and PB3 were 2.5, 1.4 and 1.8 %, respectively. The high correlation coefficient and the small RSD show that the relationship between the peak height of these components and total polymyxin B concentration is linear although it is important to realise that the concentrations quoted refer to the total polymyxin and that these regression equations cannot be used directly to estimate individual polymyxin components.

2.3.2.2 Accuracy

Figure 2.6 is a composite chromatogram to show the retention pattern for the additional components and polymyxin. All potential components of the eye drop

formulations other than neomycin are eluted before the first polymyxin peak. Neomycin was eluted with a retention time very much greater than that of PB_1.

Table 2.5 shows the results of the spiking procedure. The concentrations of polymyxin added to four formulations were found by determining the concentration in the formulation alone and after addition of a known concentration. These are shown in Table 2.5 expressed as percentages of the amounts added. These recoveries vary for different samples between 80.38 to 99.65 %.

The stability indicating aspects of the specificity with respect to polymyxin i.e. the absence of interference by the products produced during decomposition has already been established by Taylor *et al* [55] and as recommended in that publication peak heights of PB_1 and PB_2 were used to determine polymyxin chemical stability.

Table 2.5 Percentage accuracy of eye drop preparations spiked with standard polymyxin B

Sample	**Concentration of polymyxin added standard determined expressed as a percentage of the theoretical amount added**		
	PB_2	**PB_3**	**PB_1**
1	86.00	86.94	80.38
2	94.10	94.75	96.74
3	98.41	93.93	93.52
4	90.09	99.65	99.76

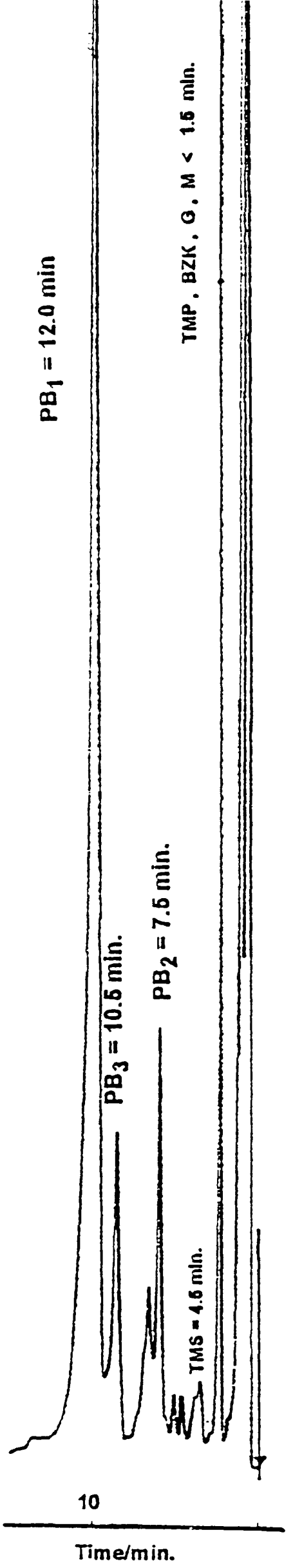


Fig. 2.6 Chromatogram and retention times of polymyxin B and the other species in eye drops preparations (chart speed = 0.2 cm/min., 0.08 AUFS).

2.3.2.3 Precision

The relative standard deviations of retention times and peak heights are shown in Table 2.6. The RSD for t_r varies between 1.07 to 2.56 %. These values are higher than normal and may reflect the complex chromatographic system. The RSD for peak height are also high varying from 1.90 to 7.03. Both of these are considered to be adequate for the proposed stability study. The day to day precision was not evaluated since fresh standards were required to be prepared for quantification over an extended period of time.

Table 2.6 RSD % of retention time and peak height at low and high concentration

Polymyxin B conc.(U/ml)	RSD % (t_r) (n = 10)			RSD% (Pk. Ht.) (n = 10)		
	PB1	PB2	PB3	PB1	PB2	PB3
162.5	2.56	1.75	2.26	5.83	6.58	7.03
16250	1.07	1.63	1.53	1.90	2.77	2.23

2.3.2.4 Limits of detection

The smallest concentration detected on the above criterion was 0.3236 U/ml. This represents a conservative limit of quantification in the region of 1 U/ml which is more than adequate for estimation of polymyxin B concentration during a stability study in which the anticipated concentrations will lie between 162.5 to 16,250 U/ml.

2.3.2.5 Ruggedness

The ruggedness of the method was investigated by changing variables slightly and examining the effects on retention time and peak heights. The results confirmed the information obtained during assay development that the critical parameter in the method was the proportion of acetonitrile in the mobile phase. The separation could tolerate appreciable alterations in the remaining chromatographic variables.

2.3.3 Chemical assay of polymyxin B eye drops

It is found that polymyxin B from different sources show different relative peak heights, for the different samples tested. This is demonstrated in specimen chromatograms shown in Figure 2.7. This is consistent with literature information [5,6,43,52]. From the eye drops assayed, polymyxin B can be subdivided into three groups according to the proportions of the individual components. The first group

comprises samples 1 and 2 which have the same proportion as the standard. The second group is sample 3 and the third group are sample 4, 5 and 6.

Table 2.7 List of samples

Sample	Ingredient
1 (*Wellcome**)	PMB 10,000 U/ml TMP 0.1% (Preservative : TMS 0.005%)
2 (*Seng Thai* *)	PMB 5000 U/ml N 0.2% Gramicidin 0.0025% **
3 (*Dispersa**)	PMB 15,000 U/ml N 0.35% (Preservative : BZK 0.01%)
4 (*ALcon**)	PMB 16,250 U/ml N 0.35% (Preservative : BZK 0.004%)
5 (*Alcon**)	PMB 16,250 U/ml N 0.35% (Preservative : BZK 0.004%)
6 (*Alcon**)	PMB 16,250 U/ml N 0.35% Phenylephrine hydrochloride 0.12% (Preservative : BZK 0.004%)

* Manufacturer

** Preservative is not stated

Sample 1,4 and 6 from U.K. Sample 2, 3 and 5 from Thailand

PMB = polymyxin B , TMP = Trimethoprim, TMS = Thiomersal , N = Neomycin

BZK = Benzalkonium chloride

Table 2.8 shows the apparent concentrations of the individual components obtained by direct comparison of the corresponding peaks of sample and standard. These values show more clearly the differences in relative amounts of each component compared with the polymyxin used as standard. It can be seen that there is an apparent difference in correspondence between the labelled amount , based on microbiological assay and the chemical concentration of the individual molecular component as determined by comparison with the polymyxin standard. It is therefore of importance to investigate further means for the chemical determination of individual components.

Table 2.8 Apparent Concentrations of polymyxin B mean ± S.D. according to the PB_1 and PB_2 measurements obtained from HPLC assay in eye drops

sample	labelled concentration (U/ml)	assayed concentration (U/ml) ; n =7 PB_1	PB_2	% labelled amount PB_1	PB_2
1	10,000	**13,635.27** (1711.14)	**12,816.26** (2178.71)	**136.35** (17.11)	**128.16** (21.78)
2	5,000	**6,332.92** (1064.92)	**5,954.48** (890.97)	**126.66** (21.30)	**119.09** (17.82)
3	15,000	**6,273.42** (744.58)	**36,859.17** (4079.12)	**41.82** (4.96)	**245.73** (27.19)
4	16,250	**15,997.50** (1033.17)	**75,365.33** (6154.88)	**98.45** (6.35)	**463.79** (37.87)
5	16,250	**13,648.00** (1862.63)	**61,171.54** (4599.45)	**83.99** (11.46)	**376.44** (28.30)
6	16,250	**14,647.75** (2177.78)	**63,699.38** (5903.57)	**90.14** (13.40)	**391.99** (36.33)

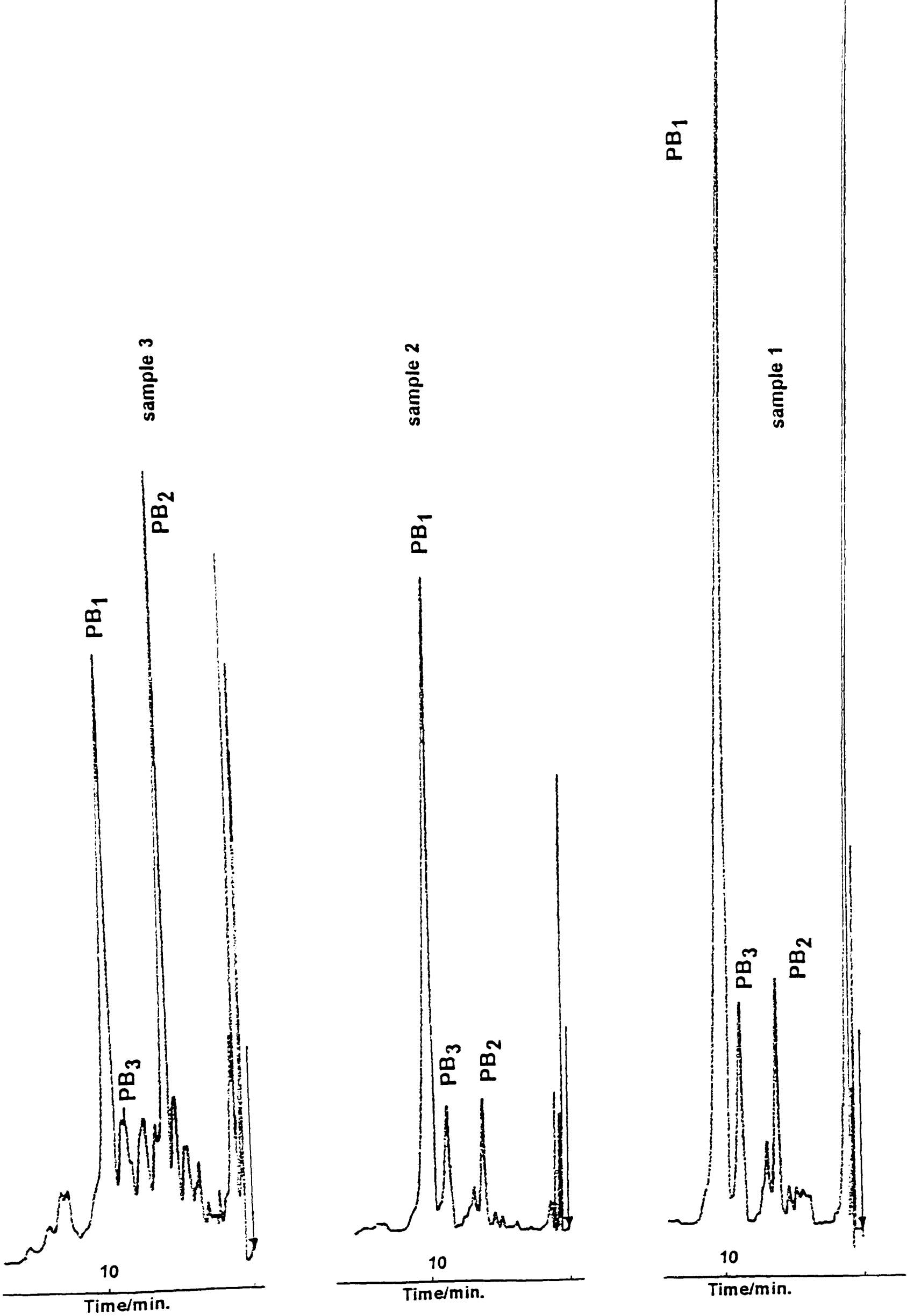
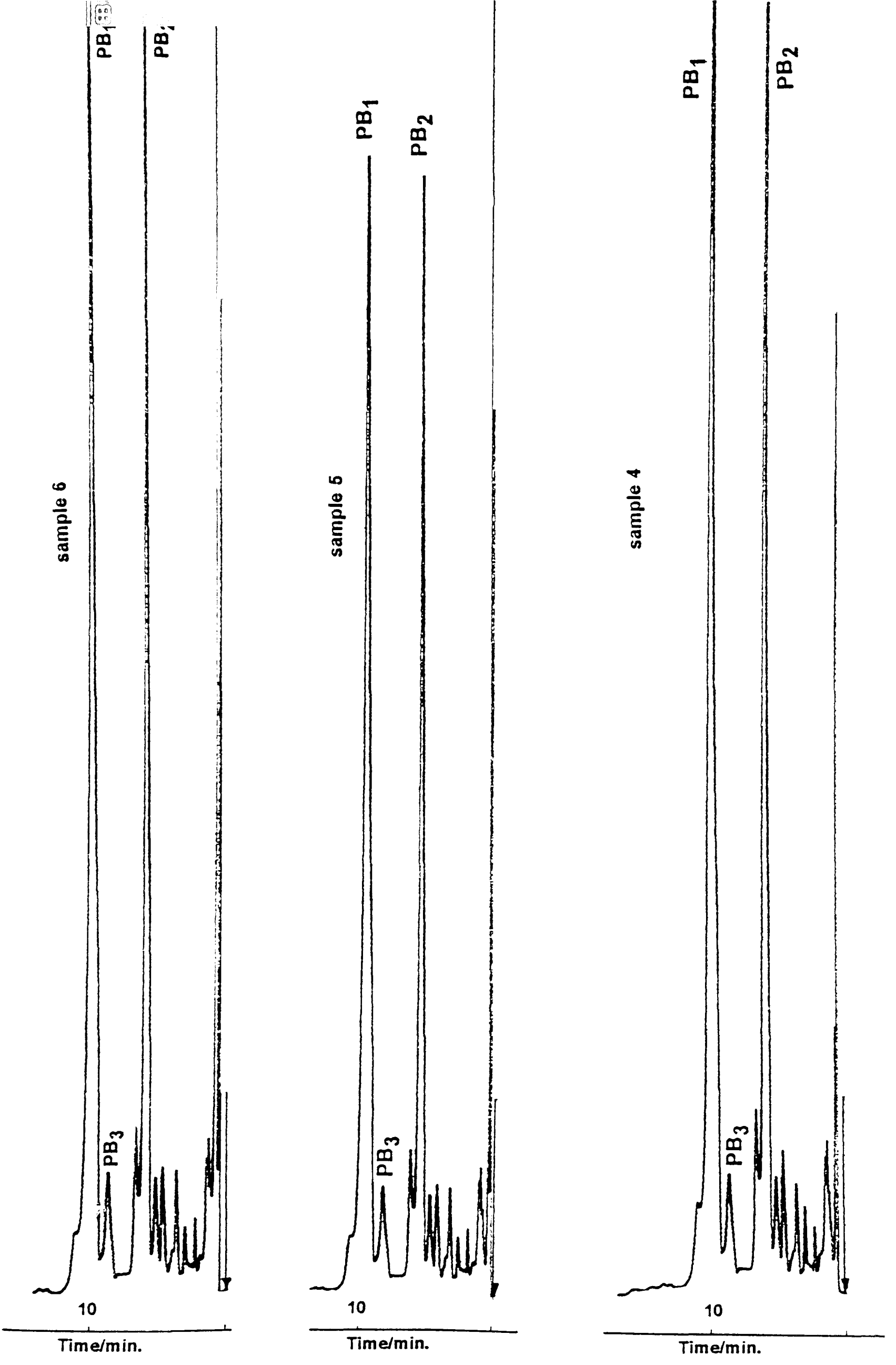


Fig. 2.7 Representative chromatograms of polymyxin B eye drops assayed by developed mobile phase (chart speed = 0.02 cm/min., 0.08 AUFS).


sample 6
PB1
PB2
PB3
10
Time/min.
sample 5
PB1
PB2
PB3
10
Time/min.
sample 4
PB1
PB2
PB3
10
Time/min.

2.4 Conclusion

The HPLC assay for the quantitative determination of polymyxin B in the eye drops in combination of other ingredients has been developed and validated. The proposed mobile phase for stability study should compose of 40:60 acetonitrile : aqueous 0.01 M disodium hydrogen phosphate , pH 3.0 and 4.4 %w/v perchloric acid. This mobile phase allows short analytical time and the inclusion of perchloric acid improves the resolution and specificity of the polymyxin components. The disadvantage of this system is that concentration of perchloric acid has to be modified according to the column condition. The specificity of polymyxin B in the presence of other substances has been established and the use of this system for the stability determination of this drug in formulations has been validated. Additional work is required to allow quantification of individual components.

Chapter 3

Chemical Stability Studies of Polymyxin B solutions

3.1 Introduction

This chapter describes accelerated chemical stability studies of polymyxin B in six commercial eye drops and polymyxin B alone in aqueous solution pH 6.0 using a developed stability-indicating HPLC method. It was intended to establish decomposition rate constants at elevated temperatures and estimate shelf-lives of test samples at low temperature (4°C). Three consecutive experiments were designed and performed to achieve suitable conditions for all samples. Tested temperatures included 32, 43, 50, 55, 60, 65 and 75°C. Temperatures higher than 60°C were not suitable for sample 2, 3 and polymyxin B alone as rapid decomposition of polymyxin B in these samples occurred. The effect of container, the duration of sampling and the duration of study were also tested. The original containers and plastic vials were used in the first and second experiment and it was found that there was some leaking of samples especially at high temperatures. The most suitable procedure is described below :

3.2 Methods

Chemicals and reagents, apparatus and chromatographic conditions were as described in Chapter 2.

The standard aqueous solution was prepared by dissolving 0.20 g of polymyxin B sulphate (8090 U/mg) in 100 ml phosphate buffer pH 6.0 (see chapter 4 for preparation) to give a concentration of 16,250 U/ml and diluting ten times with HPLC water to give 1625 U/ml. The diluted standard solution was then transferred to 10 ml plastic vials and frozen.

Phosphate buffer pH 6.0 was used since it is recommended in the BP[44] to be used as a solvent for polymyxin B. It is also important for stability reasons to maintain the pH of polymyxin B solutions stored in glass containers especially at high temperatures.

Test samples were six selected commercial polymyxin B eye drops all have original pH range between 5.41 to 6.92 (sample 1-6, Table 2.7 , see Table 9.3, chapter 9 for the pH of each sample) and polymyxin B solution concentration 16,250 U/ml (PMB) prepared by the same method as standard solution. They were stored in completely closed volumetric flasks in 43**,50*,55* and 60*^{o}C thermostatted ovens for a period of 500 h (** = normal oven, * = GLC oven). The samples were not stored in their original packages as the preliminary stability study showed that there was some leaking of the solutions from the containers during storage at high temperatures.

Samples were withdrawn from the stored preparations at appropriate intervals for assay. When taking measurements, an individual standard solution vial was removed from the freezer, thawed and injected into the HPLC system. Samples were removed from the heat sources and cooled immediately under running tap water for a few minutes. They were diluted ten times by HPLC water and injected into HPLC system. All dilutions were kept at room temperature and analysed within 8 h. At least triplicate injections of the standard solution were made to ensure the reproducibility of results. Each sample was injected once and compared with the nearest standard. The results were based on the comparison of the percentage peak height remaining of PB_1 and PB_2 in the samples during decomposition relative to the original peak height produced by the standard.

Possible interference of the decomposition products of the other antibacterials and preservatives in formulations was tested by placing eye drops concentration of aqueous solutions of trimethoprim, neomycin, gramicidin, benzalkonium chloride, thiomersal and metaoxedrine (0.1%w/v, 0.5%w/v, 0.0025%w/v, 0.004%w/v, 0.005%w/v and 0.12%w/v, respectively) stored in volumetric flasks in 55°C GLC oven during the time of study. They were taken from the stored solutions, diluted ten times with HPLC water and then injected into HPLC system at the same time as the other samples.

3.3 Results and discussion

3.3.1 Temperature profiles

The chemical stabilities of all samples at 43, 50, 55 and 60°C are represented by plotting the natural logarithm of the percentage remaining of PB_1 and PB_2 at each temperature against time (Fig. 3.1). As the degradation pattern of PB_2 is similar to PB_1 , only PB_1 plots are demonstrated. Since the decomposition of polymyxin B in aqueous solution follows first - order kinetics [55], the decomposition rate constants at each temperature were calculated from the plots based on equation (1) [58].

$$\ln[C] = \ln[Co] - kt \qquad (1)$$

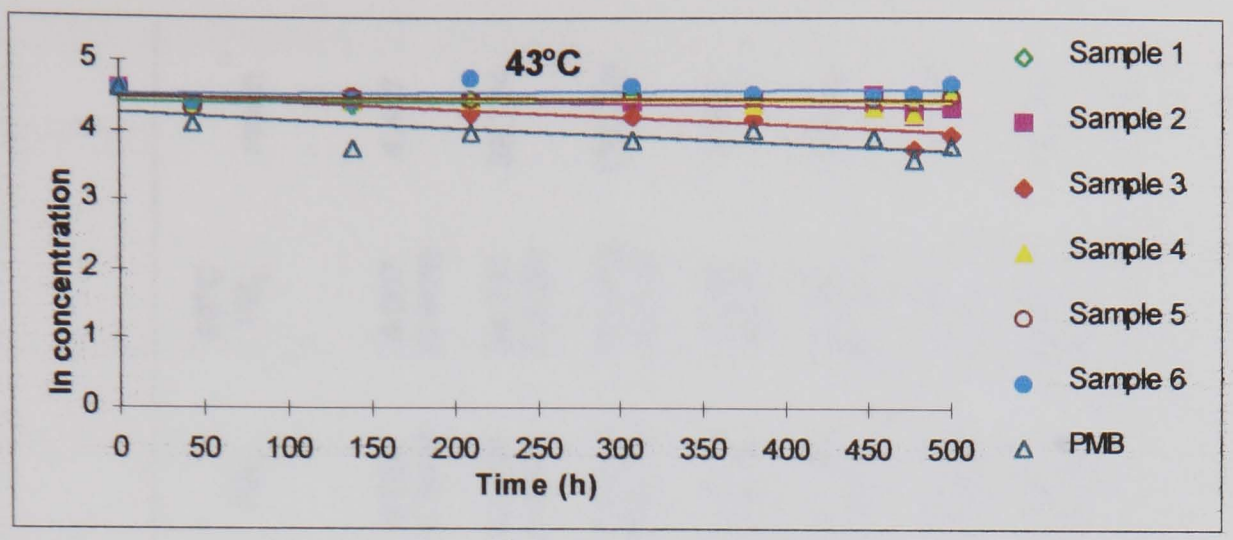
Where the initial concentration of polymyxin B is [Co], the time-dependent concentration is [C] and time is t.

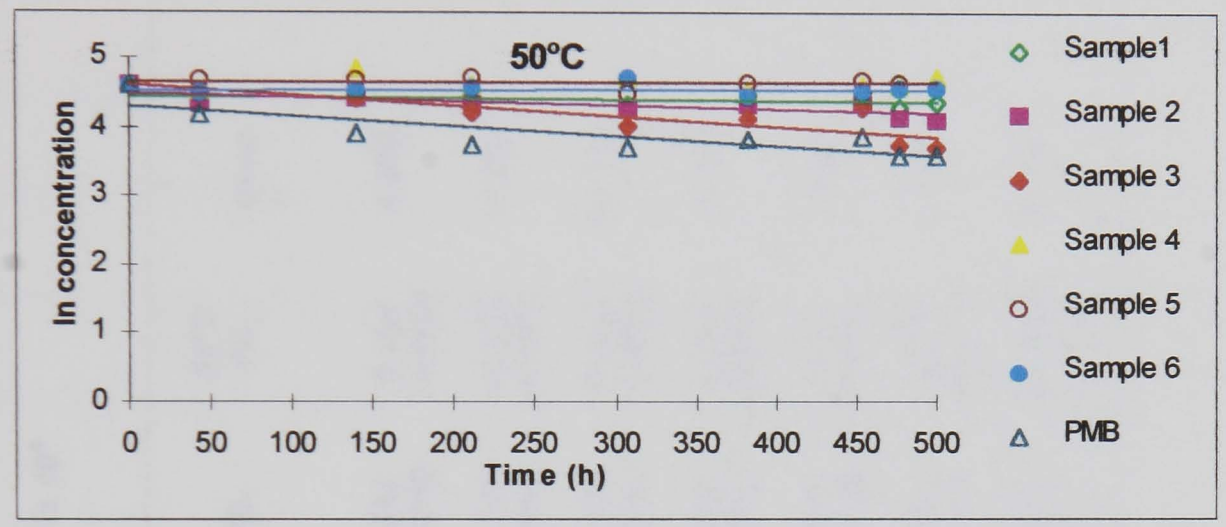
The calculated decomposition rate constants are shown in Table 3.1.

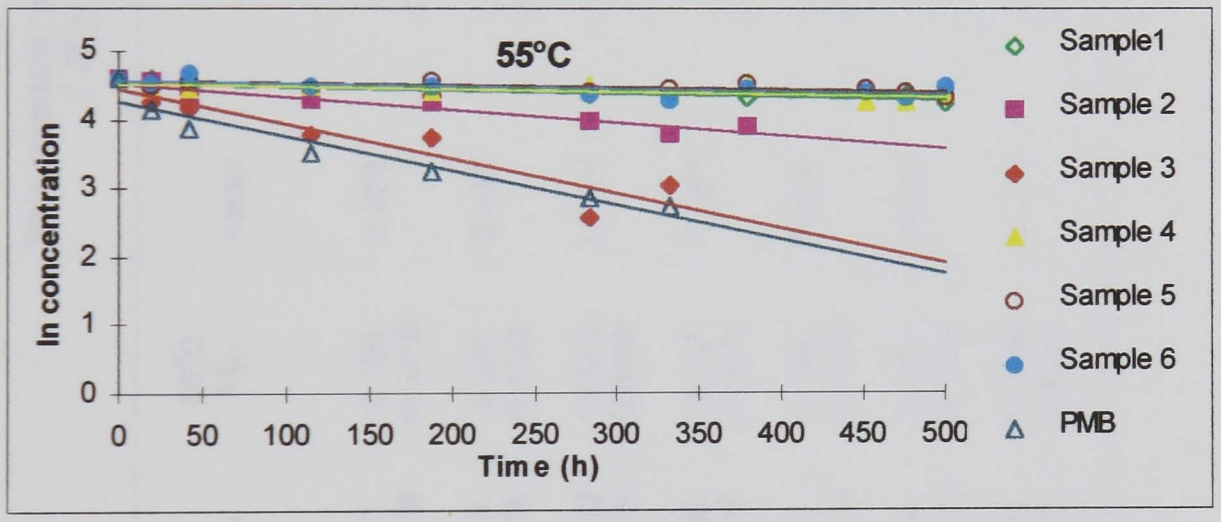
The data indicate that there was no significant degradation of PB_1 and PB_2 in sample 1, 4, 5 and 6 at all accelerated temperatures over this duration of storage. The rate constants estimated from the regression equations of these samples were not reliable because they were stable in these temperatures and the study time period was too short to accurately estimate small decomposition rate constants.

In contrast, regression analysis of sample 2, 3 and polymyxin B alone for all temperatures showed significant time - dependent changes in the percentage remaining of PB_1 and PB_2. The correlation coefficients of the plots increased as the

temperature increased. The overall results show that sample 1, 4, 5 and 6 were more stable than sample 2, 3 and polymyxin B alone in aqueous solution pH 6.0 (PMB) under the conditions for this study.







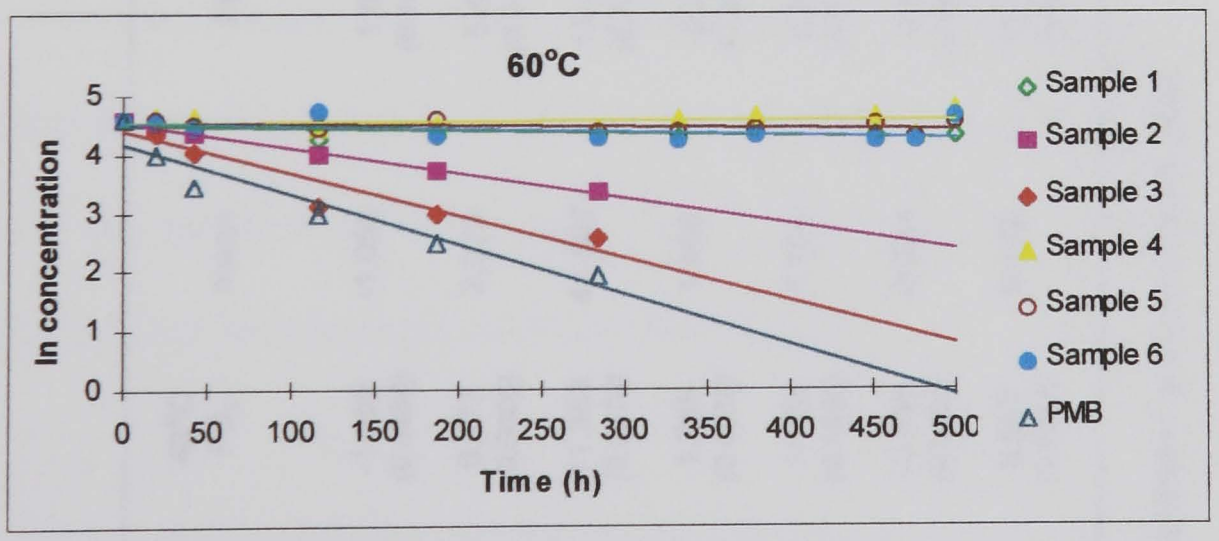


Fig. 3.1 First-order plots of PB_1 in sample 1-6 and polymyxin B aqueous solution pH 6.0 (PMB) after storage at 43, 50, 55 and 60°C for 500 hours.

Table 3.1 Decomposition rate constants of the main components of polymyxin B in sample 1-6 and PMB at 43, 50, 55 and 60°C

Sample	Rate constant : $h^{-1} \times 10^4$ (R^2)											
	43°C			**50°C**			**55°C**			**60°C**		
	PB_1	PB_2	mean	PB_1	PB_2	mean	PB_1	PB_2	mean	PB_1	PB_2	mean
1	-0.461 *(0.0061)*	-1.656 *(0.0835)*	-1.059	1.661 *(0.1013)*	2.785 *(0.3275)*	2.223	4.553 *(0.6758)*	5.164 *(0.6377)*	4.859	4.887 *(0.5404)*	4.007 *(0.4470)*	4.447
2	3.173 *(0.2927)*	0.973 *(0.0600)*	2.073	5.489 *(0.5403)*	5.098 *(0.1703)*	5.489	21.329 *(0.9095)*	14.325 *(0.4557)*	17.827	42.716 *(0.9839)*	34.731 *(0.9162)*	38.724
3	11.421 *(0.6561)*	11.953 *(0.8578)*	11.687	15.010 *(0.7355)*	16.910 *(0.8889)*	15.960	60.575 *(0.9151)*	70.431 *(0.9713)*	65.503	128.486 *(0.9970)*	136.962 *(0.9750)*	132.724
4	1.647 *(0.0606)*	1.285 *(0.0530)*	1.466	-0.266 *(0.0025)*	-0.067 *(0.0002)*	-0.167	3.674 *(0.4084)*	4.281 *(0.3997)*	3.977	-0.270 *(0.0014)*	7.399 *(0.091)*	3.565
5	0.456 *(0.0147)*	1.753 *(0.1880)*	1.104	0.381 *(0.0119)*	0.148 *(0.0015)*	0.264	3.286 *(0.6112)*	4.103 *(0.7485)*	3.694	3.239 *(0.2979)*	3.398 *(0.3579)*	3.318
6	-1.559 *(0.0564)*	-0.129 *(0.0005)*	-0.844	-0.450 *(0.0127)*	0.487 *(0.1095)*	0.0185	3.957 *(0.4536)*	3.639 *(0.4014)*	3.798	4.824 *(0.2611)*	3.442 *(0.1576)*	4.133
PMB	10.596 *(0.4881)*	8.667 (0.4278)	9.632	13.497 *(0.6022)*	11.938 *(0.7009)*	12.718	49.426 *(0.9367)*	43.249 *(0.8849)*	46.338	78.311 *(0.9137)*	74.257 *(0.8837)*	76.284

R^2 = correlation coefficient of the first order plots

3.3.2 The interference of decomposition products of other antibacterials and preservatives

It was reported earlier that the HPLC method used in this study was specific to identify PB_1 and PB_2 from the decomposition products of polymyxin B itself and from other antibacterials and preservatives in eye drop formulations. The interference of the decomposition products was also tested in this study. No additional HPLC peaks were noted in stored samples at the same retention times of PB_1 or PB_2 after storage at 55°C for 500 h. It can be concluded that the interference of both these substances and their decomposition products was avoided.

3.3.3 Shelf-life determination

The effect of temperature on the rate of decomposition may be described by an equation (2) proposed by Arrhenius [58].

$$\ln k = \ln A - Ea/RT \qquad (2)$$

where k is rate constant, A is frequency factor, Ea is the activation energy, R is the gas constant (equal to 8.314 $Jmol^{-1} K^{-1}$) and T is absolute temperature.

Once the rate constant at 4°C is obtained by extrapolation from Arrhenius plot, the shelf-life (t_{90}) can be calculated using equation (3) [58].

$$t_{90} = 0.105/k \qquad (3)$$

where k is the observed first order rate constant at 4°C.

Only the data from sample 2, 3 and polymyxin B alone (PMB) were reliable to determine shelf-life (t_{90}). The activation energy (Ea) of polymyxin B in sample 2, 3 and PMB were estimated from slope of the Arrhenius plots of the data obtained at accelerated conditions (Fig. 3.2 a). The decomposition rate constants used in the plots were an average of decomposition rate constants of PB_1 and PB_2. All plots show good linearity. Estimated Ea of polymyxin B in sample 2, 3 and PMB were 154.37, 132.44 and 114.24 $kJmol^{-1}$, respectively. They were all higher than that calculated by Taylor *et al* [55] which was 64.8 $kJmol^{-1}$. The estimated shelf-lives from this study and those obtained from Taylor *et al* and Dreyer van der Glas *et al* [56] are shown in Table 3.2.

The t_{90} at 4°C of polymyxin B in these three samples were much higher than the previous studies especially polymyxin B alone in buffer pH 6.0 which was nearly 20-times greater than Taylor's value. Similar to Taylor's study, t_{90} estimation was based on extrapolation from the measurement of polymyxin B at elevated temperatures but with higher temperatures. The t_{90} from these two studies should be similar. The higher t_{90} of polymyxin B in these samples may result from the higher temperatures used. The high temperatures may cause the breakdown of the main components (PB_1 and PB_2) and produce the small amount of decomposition products underneath these two peaks which could be detected by HPLC at the same time as the original PB_1 and PB_2. This suggestion correlates to the chromatograms of all decomposed samples at the end of the experiment which were similar to the original except the decreasing of peak height of PB_1 and PB_2. The high temperatures may also affect degradation mechanism of polymyxin B. As

mentioned by Pearlman and Nguyen [59] that extrapolation of stability data of protein drugs to lower temperatures should be limited to the temperature range over which the same degradation pathway is operative. Arrhenius approach should be conducted in the temperature range below 40°C. Polymyxin B, as a decapeptide drug, may be considered to have similar properties to some of protein drugs which degradation mechanisms have been reported to vary as a function of temperature. As there are no reports in the literature regarding degradation mechanism of polymyxin B, this suggestion cannot be excluded. Another possible reason may result from the effect of the other ingredients added in the formulations.

Both this study and that of Taylor *et al* [55] study obtained good correlation between decomposition rate constant and temperature of polymyxin B alone in aqueous solution pH 6.0. Arrhenius plots of two set of data as shown in Fig 3.2 b also demonstrate an acceptable correlation coefficient. The estimated Ea was 81.80 $kJmol^{-1}$. The t_{90} at 4°C extrapolated from the plots was about 312 days or 10 months. This t_{90} was more reliable than the t_{90} estimated from this study and this may indicate that the extrapolated t_{90} would be more accurate if an increased number of temperatures are used.

Dreyer van der Glas *et al* [56] also studied chemical stability of polymyxin B in eye and ear drops. Different methods and different samples were used. Polymyxin B content in samples stored at -18, 4 and 21°C was analysed by HPLC for a period of 25 months. The t_{90} values were estimated when polymyxin B content dropped to 90%. The disadvantage of this method is that the measurement of drug content

during a long period of time may cause the variability of the results. The t_{90} obtained was much lower than those obtained from this study and Taylor *et al.* Taylor and Shivji [60] suggested that in order to estimate decomposition rate constant and subsequently shelf-life, it is necessary to decompose the drug by 75 percent.

While it is accepted that extrapolation from higher temperatures has advantages over direct measurement at storage temperatures due to the long time scales required for the determination of decomposition rate constants, it is important to choose suitable temperatures and stability indicating methods of analysis. In this study the both factors were carefully chosen. However, the extremely high t_{90} when they were applied to polymyxin B samples indicate that both have to be modified. In the case of polymyxin B formulations where the original drug is combined with many ingredients, it is very difficult to find the most suitable stability study method for all samples.

Although these three stability studies obtained different t_{90} values of polymyxin B, there is a good agreement on the effect of pH on the stability between two studies. Taylor concluded that, at 37°C, below pH 7, pH has little effect upon the decomposition but above this value, increase of pH markedly accelerates the reaction. This is consistent with Dreyer van der Glas's findings which showed that at 21°C, percentage remaining of polymyxin B formulated at pH 7.1 significantly decreased from 100% to about 30% within 15 months but those formulated at pH 4.7 and 6.6 remained higher than 90% over a period of 20 months.

Table 3.2 Estimated shelf-life (t_{90}) of polymyxin B at 4°C

Sample	t_{90} at 4°C (day)			
	1) This study[a]	*2) Taylor et al*[b]	*1) and 2)*	*3) Dreyer et al*[c]
2	90570	-	-	-
3	5878	-	-	-
polymyxin B new formulation[d]	-	-	-	540
polymyxin B alone in aqueous solution pH 6.0	2544	130	312	-

[a] estimated from accelerated stability study of at 43, 50, 55 and 60°C, samples were made up in phosphate buffer pH 6.0.

[b] estimated from accelerated stability study at 32, 37, 43 and 52°C, sample were made up in disodium hydrogen phosphate buffer (50 mM) , pH 6.0.

[c] estimated by storage polymyxin B formulation at 4°C over a period of 25 months.

[d] Polymyxin B sulphate 1,000,000 Units, Borax 60 mg, Boric acid 1.95 g, Benzalkonium chloride 10 mg, Sodium acetate 100 mg and Sterile water to 100 ml.

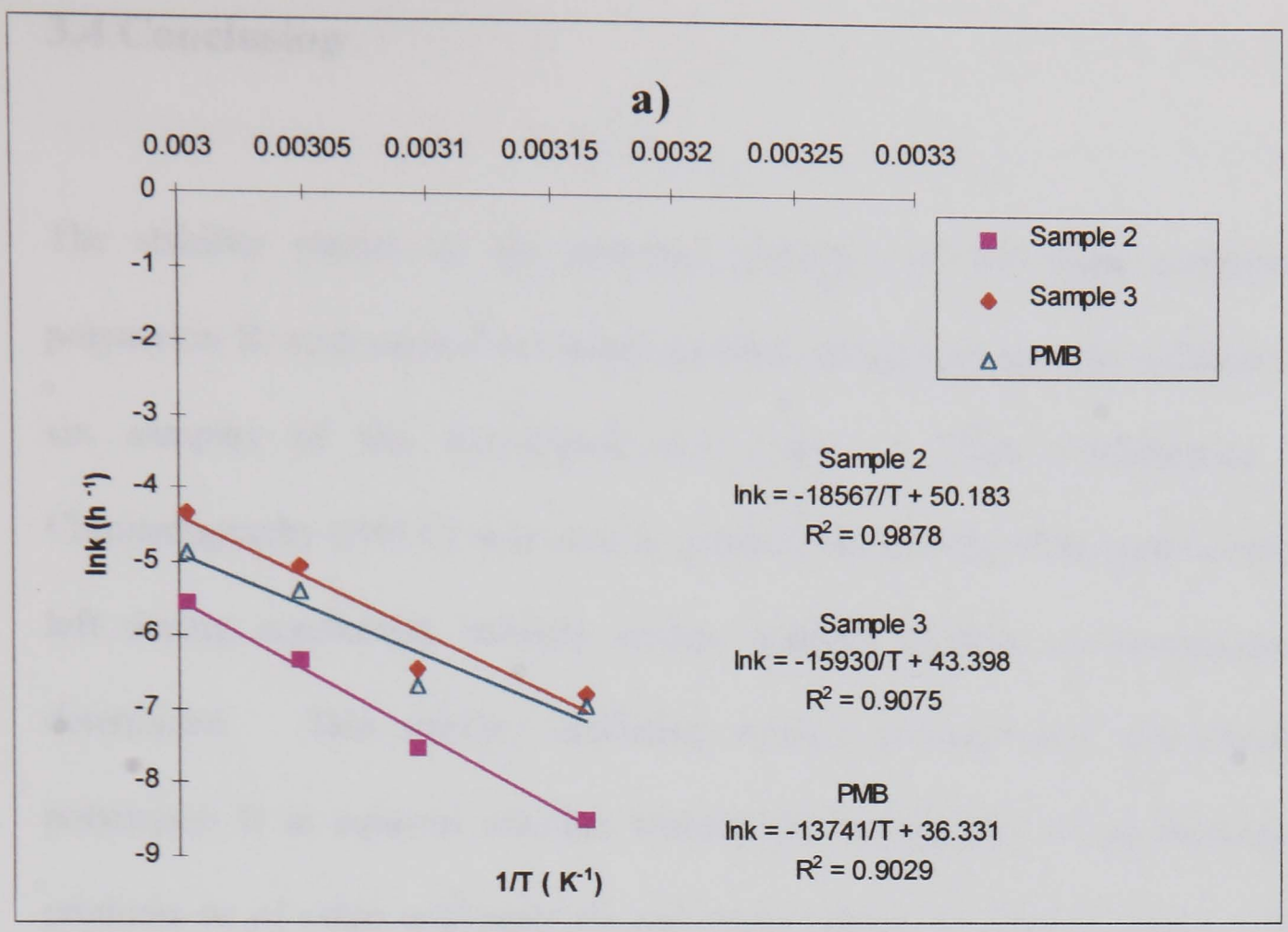


Fig. 3.2 Arrhenius plots of sample 2, 3 and PMB using data from this study **a)** and combined data of PMB from this study and Taylor *et al* **b)**.

3.4 Conclusion

The stability studies of the chemical stabilities of the main components of polymyxin B were carried out using standard material in aqueous solution and the six samples of the formulated eye drops. High Performance Liquid Chromatography (HPLC) was used to quantify the amount of the main components left during accelerated stability studies without absolute concentrations being determined. This stability indicating method allowed rapid determination of polymyxin B in aqueous solution without the interference of the decomposition products or of other antibacterials and preservatives in the eye drops. The data indicate that there are significant differences in chemical stability among the different formulated eyedrops. The exact reasons for this are not clear at present. The elevated temperatures used in this study were not suitable for all samples. The accurate decomposition rate constants and shelf-lives (t_{90}) at 4°C of sample 2, 3 and polymyxin B alone in aqueous solution pH 6.0 stored in glass containers in which rapid decomposition process occurred were established. The combined data from this study and Taylor *et al* [55] both carried out in elevated temperatures shows significant correlation of decomposition rate constants of polymyxin B in aqueous solution pH 6.0 and temperature in the range of 32 to 60°C. Shelf-life estimation from this study yielded higher t_{90} value than those obtained in previous studies which may result from the higher temperatures used and the effect of other added ingredients in the formulations. Further investigations on the degradation mechanism of polymyxin B at various temperature and also the effect of other substances in the formulations on the stability of polymyxin B are needed. The

differences between decomposed samples in terms of microbiological activity which will be used to correlate with chemical stability data is described in chapter 9 and 10.

Chapter 4

Materials and microbiological methods

This chapter describes materials and microbiogical methods used in the following chapters.

4.1 Materials

4.1.1 Chemical and reagents

The distilled water used in the microbiological experiments was prepared using a glass still Model A45, Aquatron Ltd. . Polymyxin B sulphate (PMB), trimethoprim (TMP), neomycin (N), gramicidin (G), metaoxedrine or phenylephrine hydrochloride (M), benzalkonium choride (BZK), lecithin and sodium hydroxide were obtained from Sigma. Thiomersal was purchased from Aldrich Chemical. Magnesium sulphate and potassium dihydrogen phosphate were supplied by Fisons Scientific Apparatus, Loughborough, Leics, UK. Polysorbate 80 was purchased from BDH Laboratory Supplies. Polymyxin B eye drops were purchased from Wellcome (sample 1), Seng Thai (sample 2), Dispersa (sample 3) and Alcon (sample 4, 5 and 6).

(see Table 2.7, chapter 2 for detail)

Phosphate buffer pH 6.0 was used as the solvent for the test substances and was prepared by the method described in the European Pharmacopoeia [61] by mixing 50.0 ml of 0.2 M potassium dihydrogen phosphate with 5.70 ml of 0.2 N sodium hydroxide. Distilled water was added to produce 200.0 ml.

4.1.2 Media

4.1.2.1 Nutrient agar

Nutrient agar (*Oxoid. Basingstoke, Hampshire, UK) prepared as slopes was used to maintain the viability of stock cultures. Slopes were separately inoculated with broth culture of each test organism, incubated for 24 h at 37°C and then stored at 2-8 °C. The slope cultures were used as the source of inoculum for each experiment. Fresh slope cultures were prepared every 4 weeks.

Nutrient agar was also used as the growth medium for determining the number of colony-forming units (CFU). See chapters 7 to 9.

The formula is :	"Lab-Lemco" Powder	1.0 g
	Yeast Extract	2.0 g
	Peptone	5.0 g
	Sodium chloride	5.0 g
	Agar	15.0 g
	Distilled water to	1000 ml
	pH 7.2 - 7.6	

The nutrient agar was prepared by suspending 28 g in 1 l of distilled water and bringing to the boil until the granules were dissolved completely. Sterilization was by autoclaving at 121 °C for 15 min.

4.1.2.2 Medium B agar

Medium B agar [43] is a specialist medium for culturing *Bordetella bronchiseptica* NCTC 8344.

The formula is :	Pancreatic digest of casein	17 g
	Papaic digest of soya bean	3 g
	Sodium chloride	5 g
	Dipotassium hydrogen orthophosphate	2.5 g
	D-glucose	2.5 g
	Agar	15 g
	Polysorbate 80	10 g
	Distilled water to	1000 ml

The agar was prepared by dissolving all the ingredients in 1 L of distilled water and bringing to the boil until the granules were dissolved completely. The polysorbate 80 was added to a hot solution of the other ingredients. Sterilization was by autoclaving at 121 °C for 15 min.

4.1.2.3 Nutrient broth

Oxoid* nutrient broth was used for culturing *P. aeruginosa NCTC 6750.* The formula and the directions for preparation are the same as for nutrient agar except that there is no agar.

4.1.2.4 Thioglycollate broth

Oxoid* thioglycollate broth USP alternative was used as inactivating medium for thiomersal. Magnesium sulphate and p-aminobenzoic acid (PABA) concentration 1 % and 0.16 % respectively were added for the inactivation of polymyxin B and trimethoprim, respectively.

The formula is : L-cystine	0.5 g
Sodium chloride	2.5 g
Glucose	5.5 g
Yeast extract	5.0 g
Pancreatic digest of casein	15.0 g
Sodium thioglycollate	0.5 g
Distilled water to	1000 ml
pH 6.9 - 7.3	

The broth was prepared by suspending 29 g of granules in 1 l of distilled water and bringing to the boil in order to dissolve the medium completely. Sterilization was by autoclaving at 121 ^{o}C for 15 min.

4.1.3 Test organisms

Bordetella bronchiseptica NCTC 8344 and *Pseudomonas aeruginosa NCTC 6750* were obtained from the National Collection of Type Cultures, Public Health Laboratory Service, Colindale Avenue, London NW9.

4.1.4 Apparatus

Autoclave (Dataclave^{R})	Southtrim Autoclave Limited Valley Road Bradford, West Yorkshire BD1 ARJ England
Bench autoclave	Adelphi Manufacturing Co. Ltd. 20/21 Duncan Terrace London N1 887, England
Centrifuge (IEC Centra-4B)	International Equipment company
Water bath	Grant Instruments Ltd.
UV/VIS Spectrophotometer (Model SP8-400)	Cecils Instruments
Membrane filters 0.2 μm pore size (Aerodisc^{R} PF)	Gelman Sciences

4.2 Microbiological methods

4.2.1 Microbiological assay

4.2.1.1 Inoculum preparation

B. bronchiseptica NCTC 8344 was incubated at 37°C overnight on an agar plate of medium B. A suspension of the test organism was prepared by washing the growth off the nutrient agar and diluting with normal saline solution to give an opacity of 0.20 at 500 nm. It was further diluted to prepare a suspension having approximately 10^7 CFU/ml.

4.2.1.2 Assay procedure

Molten medium B was added to petri dishes in 20 ml quantities and inoculated with 0.1 ml of *B. bronchiseptica* suspension (10^7 CFU/ml) at a temperature not exceeding 50 °C. When the agar was set, wells were cut and the plugs removed. The test solutions were introduced to the wells and the dishes were maintained at room temperature for about 2 h to allow diffusion of the solutions into the agar. After incubation at 37°C for 18 h, the diameter of the zones of inhibition was measured in 3 directions.

4.2.2 Killing time determinations

4.2.2.1 Inoculum preparation

A sample of a slope culture of *P. aeruginosa* NCTC 6750 was inoculated into nutrient broth and incubated at 37°C for 18 h. The 18 h cultures were centrifuged (4000 g, 10 min). The cell pellets were washed in 0.9% sodium chloride (NSS), recentrifuged for 5 min and resuspened in 4 ml NSS.

4.2.2.2 Procedure for determining microtitre counts

The following procedure was used in 7.2.3 , chapter 8 and chapter 9.

Standard solutions of polymyxin B were freshly prepared by dissolving 0.0131 g of polymyxin B sulphate (7760 U/mg) in 10 ml of phosphate buffer pH 6.0 to produce a concentration of 10,000 U/ml. Further dilutions were made using phosphate buffer pH 6.0 to produce concentrations of 5000, 4000, 2000, 1000, 500, 250 and 125 U/ml. When 4000 U/ml was needed, a standard solution was prepared by dissolving 0.0131 g polymyxin B in 25 ml of solvent. These solutions were sterilized by filtering through 0.2 μ membrane filters. The subsequent procedure was similar to that described by Richards and Xing [62]. A 150 μl volume of each standard solution was mixed with 150 μl of cell suspension in the first row

(designated N) of a microtiter plate to yield final concentrations of ~ 10^8 - 10^9 CFU per ml. The wells in row 1-6 of the microtiter plate were primed with 200 µl of appropriate inactivating recovery medium, and 10 µl samples from row N were added to row 1 and diluted through six serial 20-fold dilutions by adding 10 µl samples from row 1 to row 2, from row 2 to row 3 and so on to row 6. Each dilution was plated in triplicate on overdried nutrient agar for counting. Samples were thoroughly mixed by being drawn into a pipette tip and expelled four times before either being plated for counting or being diluted further. At the contact times of 10, 20, 30,40.50 and 60 min, 10 µl samples were transferred and diluted through another set of rows 1-6. Samples of each dilution were again transferred to overdried nutrient agar plates in triplicate and incubated at 32°C for 15 h. The number of CFU per 10 µl were determined for each sample. The highest countable number of CFU per 10 µl (15-30 CFU) for each series of dilutions was the count recorded. The number of CFU per milliliter was calculated for each row of dilutions, i.e. neat 10 µl drop, number of colonies times 10^2; row 1, number of colonies times 2 x 10^3, etc., to row 6, number of colonies times 6.4 x 10^9. The lower limit of sensitivity of the counting method was 10^3 CFU/ml. Cell suspensions (150 µl) mixed with either water or phosphate buffer pH 6.0 (150 µl) were used as the controls. The killing curves were plotted from log CFU/ml of *P. aeruginosa* against time. The dotted lines represented the concentration of the test bacteria below the detection limit of sensitivity (10^3 CFU/ml). The standard deviation (SD) of each count was also calculated. SD of some figures were omitted from the graphs in order to clarify the killing curves.

Chapter 5

Effect of added ingredients in polymyxin B eye drops on *B. bronchiseptica NCTC 8344*

5.1 Introduction

The compendial methods of quantitative analysis for polymyxin B in the British, European and United States Pharmacopoeias are currently microbiological [44,61.63]. The suggested method in the British Pharmacopoeia is by using the agar plate diffusion method which is suitable for polymyxin B alone. However, the selected polymyxin B eye drops contain other ingredients which may affect the growth of the test organism. For example the six eye drop formulations of polymyxin B contain one or more of the following antibacterials ; 0.1%w/v trimethoprim, 0.35%w/v neomycin , 0.0025%w/v gramicidin and either of the following preservatives ; 0.01% or 0.004%w/v benzalkonium chloride or 0.005%w/v thiomersal. In addition one preparation contains 0.12%w/v metaoxedrine (phenylephrine hydrochloride). Therefore the effect of these substances on the inhibition of the test organism was investigated using the same agar plate diffusion method as for determining the potency of polymyxin B eye drops.

5.2 Methods

Inoculum preparation and assay procedure were as described in 4.2.1. except that preparation and distribution of the test solutions in the wells were as follow :

Preparation of test solutions :

The solutions of polymyxin B combined with each of the other ingredients were made in phosphate buffer pH 6.0 at the appropriate concentrations. For the polymyxin B plus trimethoprim solution ,10% of 0.05 M of lactic acid was used to increase the solubility of trimethoprim. For gramicidin which is insoluble in water, the highest concentration made in a mixture of water and ethanol was 0.00008 %w/v. Therefore only dilute concentrations of gramicidin (0.00001 to 0.00008 %w/v) were investigated. Eye drop concentrations of polymyxin B plus the other substances were then diluted accordingly to provide the concentrations of polymyxin B used in the microbiological assay (160, 80, 40 and 20 U/ml). These were chosen from the determinations of the diameter of the zones of inhibition produced by polymyxin B concentrations ranging from 2.5 to 160 U/ml. The log concentration range from 20 to 160 U/ml plotted against the diameters of their zones of inhibition gave a straight line (Fig. 5.1). A transformation of concentration to log concentration is necessary to achieve a linear relationship. This range of concentration is within the recommended potency of polymyxin B suitable for microbiological assay [44].

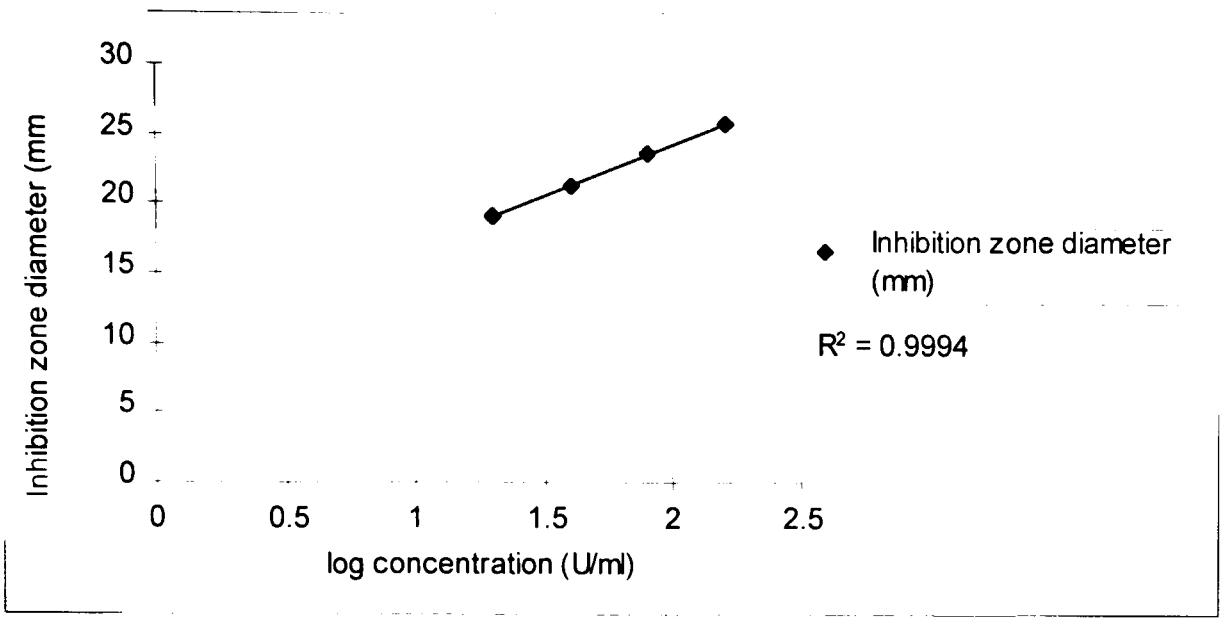


Fig. 5.1 Plot of log concentration and inhibition zone diameter of polymyxin B 20 to 160 U/ml against *B. bronchiseptica NCTC 8344*

The distribution of the test solutions in the wells were as follows :

Trimethoprim, neomycin, benzalkonium chloride,metaoxedrine and thiomersal

Dishes	Solutions					
1	1	6	11	2	7	12
2	2	7	12	3	8	13
3	3	8	13	4	9	14
4	4	9	14	5	10	15
5	5	10	15	1	6	11

The number of the test solutions indicating the concentrations used are given in Tables 5.1-5.4.

Gramicidin

Dishes	Solutions				
1	1	6	11	16	0
2	2	7	12	13	0
3	3	8	9	14	0
4	4	5	10	15	0

The number of the test solutions indicating the concentrations used are given in Table 5.5.

The effect of preservatives and the other ingredients in polymyxin B eye drops on *B. bronchiseptica* was investigated. *Trimethoprim, neomycin, benzalkonium chloride, thiomersal, metaoxedrine* (L - *phenylephrine hydrochloride) and gramicidin* are contained in the various polymyxin B eye drops at the following concentrations ; 0.1%w/v trimethoprim (sample 1), 0.35 %w/v neomycin (samples 2, 3, 4, 5 and 6), 0.004 %w/v benzalkonium chloride (samples 4, 5 and 6), 0.01 %w/v benzalkonium chloride (sample 3), 0.005 %w/v thiomersal (sample 1), 0.12 %w/v metaoxedrine (sample 6) and 0.0025 %w/v gramicidin (sample 2). In the microbiological assay of polymyxin B, the eye drops were diluted to achieve concentrations of 20, 40 and 80 U/ml of polymyxin B. The inhibition zone diameters of these dilutions were then compared with the inhibition zone diameters of freshly prepared standard solutions of polymyxin B at the same concentrations.

In this experiment, eye drop concentrations of test substances were prepared and diluted to the same dilutions as the polymyxin B eye drops. The inhibition zone diameters of the solutions were measured and compared with standard solutions of polymyxin B.

5.3 Results and discussion

Trimethoprim

Table 5.1 shows that inhibition zones were produced by 0.1 % w/v trimethoprim (concentration in the eye drops). Trimethoprim 0.1 % w/v combined with polymyxin B 10,000 U/ml also produced inhibition zones which were significantly different from those produced by polymyxin B 10,000 U/ml (t - test, p = 0.05). In contrast, there was no significant differences between the inhibition zones of polymyxin B alone and polymyxin B plus trimethoprim in dilute concentrations (t - test, p = 0.05). It can be concluded that trimethoprim 0.1 %w/v addded to the antibacterial activity of polymyxin B 10,000 U/ml. When this combination was diluted to give concentrations of polymyxin B of 160, 80, 40 and 20 U/ml, then the diluted trimethoprim did not add to the inhibition zone diameter of polymyxin B. Thus trimethoprim at these dilute concentrations would not adversely affect the biological assay of polymyxin.

Neomycin

The data in Table 5.2 shows that 0.5 % w/v neomycin produced inhibition zones against *B.bronchiseptica* and the inhibition zone diameters of 0.5 % w/v neomycin plus polymyxin B 16,250 U/ml (concentration in eye drops) were significantly different from those produced by polymyxin B alone 16,250 U/ml (t - test, p = 0.05). That is neomycin added to the effect of polymyxin B at the concentrations used in the eye drops. However, the dilute concentrations of neomycin had no effect on *B.bronchiseptica* in medium B and the inhibition zones from diluted concentrations

of neomycin plus polymyxin B were no different from those of polymyxin B alone (t - test, p = 0.05). Consequently, these dilute concentrations of neomycin can be used in the biological assay of polymyxin B without influencing the inhibition zones.

Benzalkonium chloride and metaoxedrine

The results are summarised in Table 5.3. Benzalkonium chloride 0.004 %w/v had no effect and the combination of benzalkonium chloride 0.004 %w/v with polymyxin B 16.250 U/ml showed no significant difference from the effect of the polymyxin B alone. This indicates that benzalkonium chloride at concentrations below 0.004 %w/v did not have antibacterial activity against *B. bronchiseptica* and would not interfere with the biological assay of polymyxin B.

Similar results were obtained with metaoxedrine. The eye drop concentration of metaoxedrine produced a small inhibition zone against *B. bronchiseptica* but the inhibition zone diameter of the combination of metaoxedrine with polymyxin B at the eye drop concentration was not significantly different from the inhibition zone diameter of the eye drop concentration of polymyxin B alone. Consequently, metaoxedrine was assumed to have no significant antibacterial activity against *B. bronchiseptica* at concentrations below 0.12 %w/v.

Thiomersal

Thiomersal is different from both benzalkonium chloride and metaoxedrine (Table 5.4). Thiomersal both alone and in combination with polymyxin B produced zones of inhibition against *B. bronchiseptica* in agar medium B. The significant difference of inhibition zone diameter of polymyxin B alone from polymyxin B plus thiomersal and the consistent increase in inhibition zone from low concentration to high concentration of thiomersal indicated that thiomersal inhibited the growth of *B. bronchiseptica* at a concentration as low as 0.00002 %.

Gramicidin

The other ingredient examined in this series of investigations was gramicidin. Since gramicidin is insoluble in water but freely soluble in alcohol, the solution of gramicidin was prepared in 20 %v/v ethanol. As shown in Table 5.5, this concentration of ethanol did not have any inhibitory effect on *B. bronchiseptica*. The results with gramicidin were not consistent although repeat determinations were made. However, the overall data suggested that gramicidin did not show significant growth inhibition against *B. bronchiseptica*.

Table 5.1 Effect of trimethoprim on the effect of polymyxin B against *B. bronchiseptica*

Antimicrobial agent	**Inhibition zone diameter(mm) : mean(SD) , n = 8**				
	Eye Drop concentration	**Diluted concentration**			
PMB(U/ml)	**(10,000)^{5}**	**(160)^{4}**	**(80)^{3}**	**(40)^{2}**	**(20)^{1}**
	30.7(0.02)	19.3(0.04)	17.8(0.03)	16.5(0.03)	15.5(0.00)
TMP(%w/v)	**(0.1)^{10}**	**(0.0016)^{9}**	**(0.0008)^{8}**	**(0.0004)^{7}**	**(0.0002)^{6}**
	30.9(0.08)	n	n	n	n
PMB(U/ml) +TMP(%w/v)	**(10,000 +0.1)^{15}**	**(160+0.0016)^{14}**	**(80 +0.0008)^{13}**	**(40+0.0004)^{12}**	**(20+0.0002)^{11}**
	31.9(0.09)	18.9(0.03)	17.8(0.03)	16.5(0.03)	15.3(0.03)
t-test (p=0.05)	**5 and 15**	**4 and 14**	**3 and 13**	**2 and 12**	**1 and 11**
	+	-	-	-	-

PMB = polymyxin B

TMP = trimethoprim

n = no inhibition zone

\+ = significant difference

\- = no-significant difference

Table 5.2 Effect of neomycin on the effect of polymyxin B against *B. bronchiseptica*

Antimicrobial agent	**Inhibition zone diameter(mm) : mean(SD), n = 8**				
	Eye drop concentration	Diluted concentration			
PMB(U/ml)	**(16,250)^{5}**	**(160)^{4}**	**(80)^{3}**	**(40)^{2}**	**(20)^{1}**
	31.6(0.04)	19.8(0.03)	18.7(0.00)	17.4(0.02)	16.2(0.01)
N(%w/v)	**(0.5)^{10}**	**(0.008)^{9}**	**(0.004)^{8}**	**(0.002)^{7}**	**(0.001)^{6}**
	21.4(0.08)	n	n	n	n
PMB(U/ml) +N(%w/v)	**(16,250+0.5)^{15}**	**(160+0.008)^{14}**	**(80+0.004)^{13}**	**(40+0.002)^{12}**	**(20+0.001)^{11}**
	33.0(0.03)	19.8(0.03)	18.9(0.01)	17.3(0.00)	16.1(0.03)
t-test (p = 0.05)	**5 and 15**	**4 and 14**	**3 and 13**	**2 and 12**	**1 and 11**
	+	-	-	-	-

PMB = polymyxin B

N = neomycin

n = no inhibition zone

\+ = significant difference

\- = no-significant difference

Table 5.3 Effect of benzalkoniumchloride and metaoxedrine on the effect of polymyxin B against *B. bronchiseptica*

Antimicrobial agent	Inhibition zone diameter of eye drop concentration(mm) mean(SD), n = 8
(PMB 16,250 U/ml)[5]	31.8(0.05)
(BZK 0.004 %w/v)[10]	8.0*
(PMB 16,250 U/ml + BZK 0.004 %w/v)[15a]	32.0(0.07)
t-test (p = 0.05) 5 and 15a	-
(PMB 16,250 U/ml)[5]	36.7(0.09)
(M 0.12% w/v)[10]	10.4(0.03)
(PMB 16,250 U/ml + M 0.12 %w/v)[15b]	36.7(0.05)
t-test (p = 0.05) 5 and 15b	-

PMB = polymyxin B

BZK = benzalkonium chloride

M = metaoxedrine

\+ = significant difference

\- = no-significant difference

* = the diameter of the well ie no observed inhibition

Since eye drop concentration of polymyxin B plus benzalkonium chloride and polymyxin B plus metaoxedrine were not significantly different from eye drop concentration of polymyxin B alone, the effect of diluted concentration of benzalkonium chloride and metaoxedrine on the effect of polymyxin B against *B. bronchiseptica* was not investigated.

Table 5.4 Effect of thiomersal on the effect of polymyxin B against *B. bronchiseptica*

Antimicrobial agent	**Inhibition zone diameter (mm) : mean(SD) , n = 8**				
	Eye drop concentration	**Diluted concentration**			
PMB(U/ml)	**(10,000)^{5}**	**(160)^{4}**	**(80)^{3}**	**(40)^{2}**	**(20)^{1}**
	29.8(0.03)	19.7(0.06)	17.8(0.05)	15.4(0.04)	12.9(0.04)
TMS(%w/v)	**(0.005)^{10}**	**(0.00008)^{9}**	**(0.00004)^{8}**	**(0.00002)^{7}**	**(0.00001)^{6}**
	31.6(0.06)	16.1(0.27)	12.5(0.09)	7.9(0.06)	n
PMB(U/ml) +TMS(%w/v)	**(10,000+0.005)^{15}**	**(160+0.00008) ^{14}**	**(80+0.00004)^{13}**	**(40+0.00002)^{12}**	**(20+0.00001)^{11}**
	32.2(0.06)	22.6(0.08)	20.9(0.05)	18.8(0.02)	16.7(0.02)
t-test (p = 0.05)	**5 and 15**	**4 and 14**	**3 and 13**	**2 and 12**	**1 and 11**
	+	+	+	+	+

PMB = polymyxin B

TMS = thiomersal

n = no inhibition zone

\+ = significant difference

\- = no-significant difference

Table 5.5 Effect of gramicidin on the effect of polymyxin B against *B. bronchiseptica*

Antimicrobial agent	Inhibition zone diameter(mm) : mean(SD) , n = 8			
PMB(U/ml)	**(160)[1]**	**(80)[2]**	**(40)[3]**	**(20)[4]**
	21.7(0.02) 20.0(0.04)	*19.4(0.04)* 18.3(0.02)	*16.1(0.02)* 15.3(0.03)	*11.7(0.06)* 12.1(0.06)
PMB(U/ml) **+20%v/v Ethanol**	**(160)[5]**	**(80)[6]**	**(40)[7]**	**(20)[8]**
	21.4(0.02) 19.8(0.05)	*20.5(1.75)* 17.5(0.04)	*16.4(0.04)* 15.3(0.03)	*12.2(0.01)* 12.7(0.05)
G(%w/v) **+20%v/v Ethanol**	**(0.00008)[9]**	**(0.00004)[10]**	**(0.00002)[11]**	**(0.00001)[12]**
	14.2(0.01) 12.0(0.02)	*12.4(0.10)* 9.3(0.00)	*9.3(0.01)* 9.1(0.02)	*n* 8.1(0.01)
PMB(U/ml)+G(%w/v) **+20%v/v Ethanol**	**(160+0.00008)[13]**	**(80+0.00004)[14]**	**(40+0.00002)15**	**(20+0.00001)[16]**
	21.1(0.05) 20.1(0.03)	*19.2(0.02)* 17.9(2.61)	*17.2(0.02)* 13.5(0.03)	*14.7(0.08)* 13.5(0.03)
Ethanol	**(20%v/v)[0]**			
	n n			
t-test (p = 0.05)	**1. PMB alone and PMB + 20 %v/v Ethanol**			
	1 and 5	2 and 6	3 and 7	4 and 8
	- -	- +	- -	+ +
	2. PMB + 20 %v/v Ethanol and PMB + G + 20 %v/v Ethanol			
	5 and 13	6 and 14	7 and 15	8 and 16
	- -	- -	- +	+ +
	3. PMB alone and PMB + G + 20 %v/v Ethanol			
	1 and 13	2 and 14	3 and 15	4 and 16
	+ -	- +	- +	+ +

PMB = polymyxin B

G = gramicidin

n = no inhibition zone

\+ = significant difference

\- = no-significant difference

5.4 Conclusion

The antibacterial activity of the additional chemicals present in commercial polymyxin B eye drop formulations was investigated. At the concentrations used in the microbiological assay of polymyxin B, none of these substances, except thiomersal, had any effect on the test organism (*B. bronchiseptica*). The microbiological assay of the polymyxin B for one of the eye drop formulations which was to be used in the stability determinations was slightly modified from the agar plate diffusion method of the British Pharmacopoeia [44]. The BP method could be applied to the polymyxin B eye drops containing trimethoprim, neomycin , benzalkonium chloride, metaoxedrine and gramicidin at concentrations of 0.1 %w/v, 0.5 %w/v, 0.004 %w/v, 0.12 %w/v and 0.0025 %w/v, respectively but not for the eye drops containing thiomersal. In order to assay the eye drops containing 0.005 %w/v of thiomersal, thiomersal has to be added to the standard solution at the same concentration that it is present in the eye drops in order to avoid the enhanced effect on the test organism produced by the thiomersal.

Chapter 6

Microbiological assay of polymyxin B solutions

6.1 Introduction

This chapter describes the microbiological assay of both undecomposed and decomposed polymyxin B solutions. The method used was the agar plate diffusion as suggested in the British Pharmacopoeia with addition of 0.005 % thiomersal in freshly prepared standard polymyxin B solution for sample that contained thiomersal. Barnard [15] has reported that an improved turbidimetric assay method has enabled a comparative study to be made of the antibiotic potencies of PB_1 and PB_2 in the traditional agar plate diffusion assay and the nutrient broth turbidimetric assay. The PB_1 and PB_2 fractions were shown to be of similar potency when assayed turbidimetrically while PB_2 was more potent on agar plate diffusion assay than PB_1. These results from agar plate diffusion assay are consistent with those of Thomas *et al* [42]. Barnard suggested that any standard used to establish the potency of a batch of polymyxin B sulphate should be as similar as possible to the test material in the contents of PB_1 and PB_2 . This could be a problem when comparing the activity of partially decomposed polymyxin B formulations in which the ratio of PB_1 to PB_2 was different. Therefore it is necessary to determine

whether such a microbiological assay procedure can be used to determine the antibacterial activity of polymyxin B solutions during a stability study.

6.2 Methods

Only two undecomposed eye drops were assayed. Sample 1 and Sample 4 represented the formulations containing thiomersal and without thiomersal, respectively. For decomposed samples : sample 1, 2, 3, 4, 5, 6, and polymyxin B alone prepared in phosphate buffer at pH 6.0 (PMB) stored at 60°C for 500 h were assayed. (see Table 2.7 for details of sample 1 -6)

Inoculum preparation and assay procedure were as described in 4.2.1 except that the preparations and the arrangement of standard and unknown solutions were as follows:

Preparation of standard solutions

Standard solution for sample 1 were prepared by making 10,000 U/ml polymyxin B and 0.005 % w/v thiomersal in phosphate buffer at pH 6.0 and then diluting to achieve the concentrations 80 (S_3), 40 (S_2) and 20 (S_1) U/ml polymyxin B.

Standard solutions for the others samples were prepared by making 16,250 U/ml of polymyxin B and diluting to achieve the concentrations of 80 (S_3), 40 (S_2) and 20 (S_1) U/ml polymyxin B.

Preparation of unknown solutions

Eye drop samples were diluted with phosphate buffer at pH 6.0 to get the concentrations 80 (U_3), 40 (U_2) and 20 (U_1) U/ml polymyxin B.

Assay procedure

The assay was carried out by the same method as described in 4.2.1 but the arrangement of standard and unknown solutions were as follows :

3 X 3 Latin Square

Dishes	Solutions					
1	U1	S1	U2	S2	U3	S3
2	S1	U2	S2	U3	S3	U1
3	U2	S2	U3	S3	U1	S1
4	S2	U3	S3	U1	S1	U2
5	U3	S3	U1	S1	U2	S2
6	S3	U1	S1	U2	S2	U3

S = standard U = unknown

Statistical analysis of the results and the calculation of potency were carried out according to the methods described in the European Pharmacopoeia [61] and Finney [64].

6.3 Results and discussion

The microbiological assay of polymyxin B in two undecomposed eye drops (sample 1 and sample 4) was carried out. The results are shown in Table 6.1 and 6.2. The assay was based on the parallel-line model in which the two log-dose response lines of the preparation being examined and of the standard preparation should be parallel. They should be rectilinear over the range of doses used in the calculation (20 to 80 U/ml). These conditions should be verified by validity tests for a given probability. The validity tests used in these experiments was the F test [61,64] at the 95 % level of significance. The most important parameters are regression, parallelism and linearity. The assay is valid if

1) the regression which indicates the slope of the log-dose response line is highly significant.

2) the deviation from parallelism of the regression curves is non-significant.

3) the deviation from linearity of the regression curves is non-significant.

The validity of the assays of undecomposed sample 1 and sample 4 was determined as shown in Table 6.1 and 6.2. Figs. 6.1 and 6.2 show the parallel lines resulting from plotting log concentration against inhibition zone diameter of standard and samples. The estimated potency of polymyxin B in both samples was higher than the stated label potency especially sample 4. The estimated potency of sample 1 was within the range of the USP limits for a similar preparation containing polymyxin and neomycin (90 to 125 % labelled amount) but the estimated potency of sample 4 was much higher than the standard. The proportion of individual components of polymyxin in sample 1 was the same as for the standard but for

sample 4 the proportion of PB_1 to PB_2 was different from the standard, as evidenced from the chromatograms in Fig. 2.5 and Fig. 2.7. The high estimated potency of sample 4 may result from this difference in proportion of PB_1 to PB_2 between the standard solution and the sample. It is also possible that the original potency of both of the two samples was much higher than the stated label amounts.

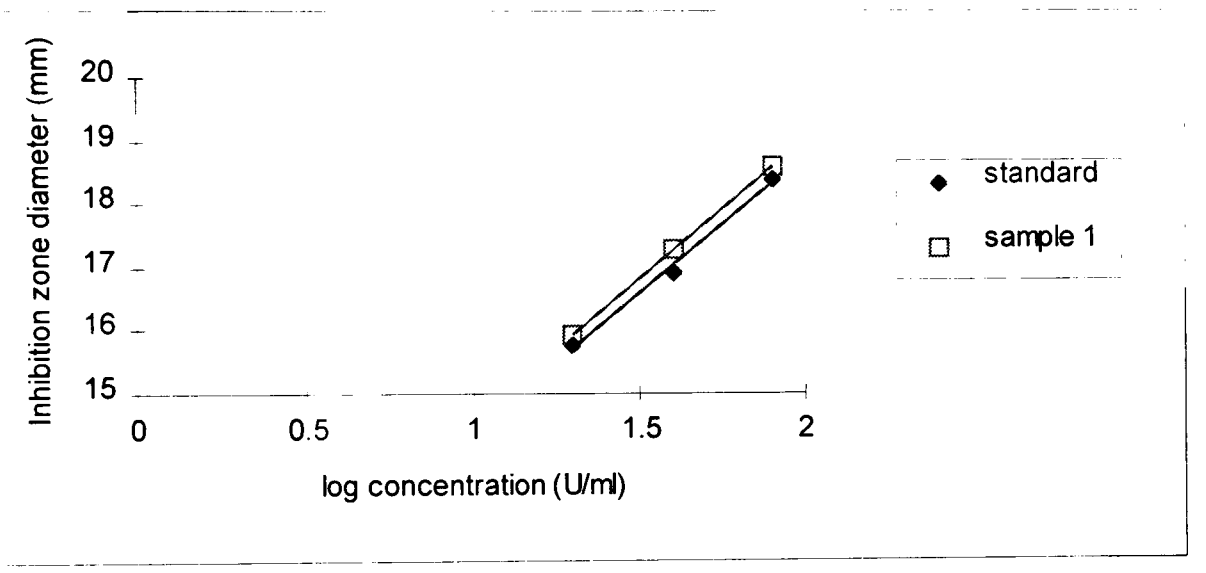


Fig. 6.1 Plot of log concentration and inhibition zone diameter for the assay of undecomposed sample 1

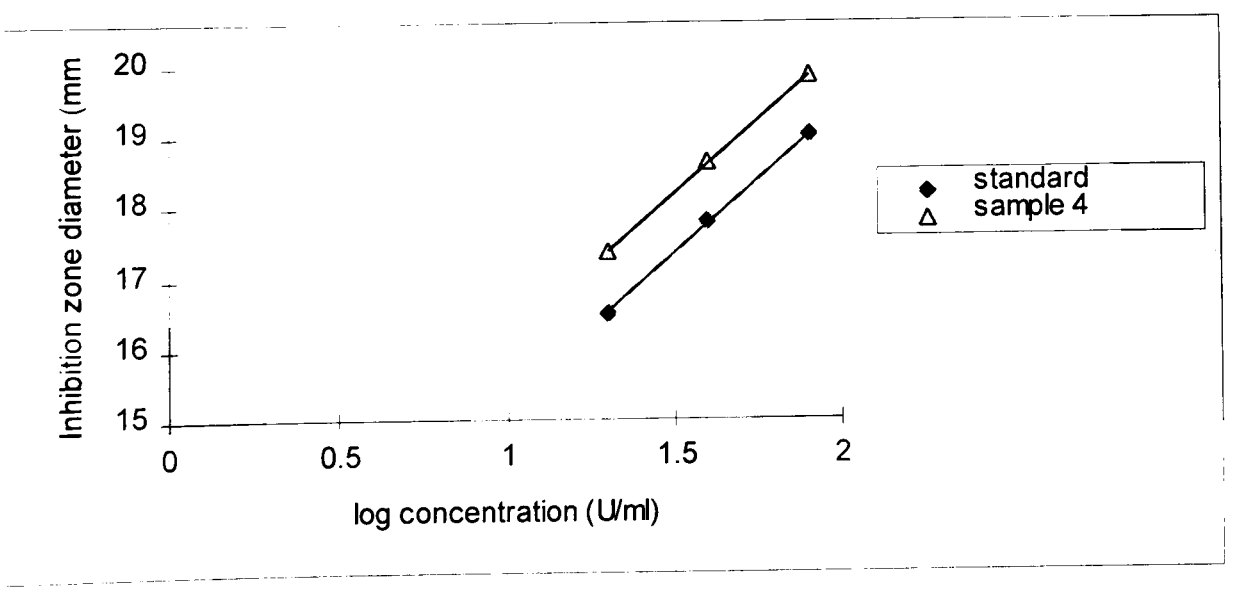


Fig. 6.2 Plot of log concentration and inhibition zone diameter for the assay of undecomposed sample 4

Table 6.1 Microbiological assay of undecomposed sample 1

Inhibition zone diameter (mm) : mean(SD)	
S1	15.8 (0.03)
S2	16.9 (0.02)
S3	18.4 (0.01)
U1	15.9 (0.03)
U2	17.3 (0.02)
U3	18.6 (0.02)

Analysis of variance

Source	Degree of freedom	Sum of square	Mean square	F ratio
Preparations	1	3.50189×10^{-3}	3.50189×10^{-3}	7.40157
Regression	1	0.283562	0.283562	599.335
Parallelism	1	6.25104×10^{-6}	6.25104×10^{-6}	1.32121×10^{-2}
Linearity	2	6.14792×10^{-4}	3.07396×10^{-4}	0.64971
Doses	5	287685		
Error	18	8.51631×10^{-3}	4.73128×10^{-4}	
Total	23	0.296202		
Stated label potency	10,000 U/ml			
Estimated potency	11,340.90 U/ml (113.41 % labeled amount)			
Fiducial limits	10,290.70 - 12,521.60 U/ml (102.91-125.22 % labeled amount)			

Table 6.2 Microbiological assay of undecomposed sample 4

Inhibition Zone Diameter (mm) : mean (SD)	
S1	16.5 (0.03)
S2	17.8 (0.03)
S3	19.0 (0.03)
U1	17.4 (0.03)
U2	18.6 (0.03)
U3	19.8 (0.03)

Analysis of variance

Source	Degree of freedom	Sum of square	Mean square	F ratio
Preparations	1	592	592	66.9873
Regression	1	3725.11	3725.11	421.512
Parallelism	1	4.15955×10^{-2}	4.159559×10^{-2}	4.70670×10^{-3}
Linearity	2	7.72559	3.86276	0.437091
Doses	5	4324.87		
Error	30	265.125	8.8375	
Total	35	4590		
Stated label potency	16,250 U/ml			
Estimated potency	25,517.54 U/ml (157.03 % labeled amount)			
Fiducial limits	22,691.50 - 28,955.55 U/ml (139.64 - 178.19 % labeled amount)			

The results from the microbiological assays of the decomposed samples are not shown because the relative potency of all samples obtained from parallel line assays (3 doses level) were not reliable. There was a significant departure from parallelism between the log dose - response line of standard and test. This was also reported by Thomas *et al* [42] when the higher range of doses (160, 80 and 40 U/ml) were used. The relative potency of decomposed samples compared to freshly prepared standard varied from 1.14 to 140.73. Thus the relative potencies of all samples were recalculated using only two doses that gave parallel lines. This is according to the European Phamacopoeia that at least three different doses of the reference substance and three doses of the antibiotic to be examined should be used initially. If the relationship between the logarithm of concentration of the antibiotic and the diameter of the zone of inhibition has been shown to be linear for a given system, assays may be carried out using only two concentrations of the reference substance and two of the antibiotic to be examined [61]. However, the relative potency of all samples was still higher than the relative potency of the standard . It was concluded from these overall results that the microbiological assay was not suitable for monitoring the potency of polymyxin B in the stability study. Therefore killing time determinations for polymyxin B eye drops and the standard material against *Pseudomonas aeruginosa NCTC 6750* were developed as a possible alternative. These are described and used in chapters 7-9.

Chapter 7

Effect of inactivating recovery medium on polymyxin B activity

7.1 Introduction

Killing time determinations were investigated in order to attempt to use this method of assessment to differentiate between the microbiological activity of polymyxin B in different samples. The killing curves were obtained using a microdilution viable counting method. In this method the dilutions of antibacterial test samples combined with the test organism were made using medium containing suitable inactivating agents for polymyxin B and any other ingredients that may affect the test bacteria. The numbers of bacteria remaining at specified time was calculated from these dilutions.

The first objective of this section was to find a suitable inactivating recovery medium for using in the killing time determinations for polymyxin B in phosphate buffer pH 6.0. The second objective was to select an appropriate concentration of polymyxin B for use in the first row of wells in the microtiter plate.

The effects of two inactivating agents for polymyxin B (lecithin and magnesium sulphate) using *P. aeruginosa NCTC 6750* as the test organism were investigated. Lecithin at a concentration of 0.125 %w/v has been used to inactivate cationic antibacterials agents in lozenges and also solutions of polymyxin B [17,62,65]. In this study, a lower concentration of lecithin (0.025 %w/v) was used. Polysorbate 80 cannot be used to help solubilize the lecithin in this study because polysorbate 80 is synergistic with polymyxin against *P. aeruginosa*. The surface active agent causes the polymyxin damaged cells to lyse. However, without the polysorbate 80 it is difficult to get the lecithin into solution.

Several workers have noted that the effect of polymyxin - e.g. binding to membrane and lipids, changes in membrane permeability, inhibition of growth - can be antagonized by divalent cations and the presence of Mg^{2+} and Ca^{2+} has been reported to inhibit antibacterial activity of polymyxin B [14,66-68]. It is also known that Mg^{2+} and Ca^{2+} have an important role in stabilizing the outer membrane of Gram-negative bacteria [14]. In this work it was decided to investigate two concentrations of magnesium sulphate (0.5% and 1%w/v) and to compare the results with lecithin. The effectiveness of the selected inactivating agent was tested when it was incorporated in water, nutrient broth and thioglycollate broth.

The killing time determinations of different concentrations of polymyxin B prepared in water and phosphate buffer pH 6.0 using an appropriate inactivating recovery medium were also investigated .

7.2 Methods

P. aeruginosa NCTC 6750 was used as the test organism. Inoculum preparation for all experiments in this chapter was as described in 4.2.2.1.

7.2.1 Calibration curve

The cell suspension from 4.2.2.1 was diluted with normal saline solution to give five suspensions having absorbance readings in the range 0.05 to 0.25 at a wavelength of 500 nm. Dilutions for viable counting were made by further diluting each of the cell suspensions to produce a cell concentration of approximately 10^2 CFU/ml. Dilutions of each absorbance reading were made to produce approximately 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 CFU/ml and were pipetted in 0.1 ml volumes onto the surface of overdried nutrient agar plates and spread evenly with sterile angled glass rods. This procedure was carried out in triplicate and after incubation at 32°C for 15 h, the number of colony forming units (CFU) per 0.1 ml were counted and recorded. The dilution giving a countable number of CFU's (50-200) was counted and the CFU of the original culture calculated. The calibration curve was plotted for log viable count per ml (log CFU/ml) versus absorbance readings.

7.2.2 Effect of lecithin and magnesium sulphate

Three types of test sample containing lecithin and magnesium sulphate were prepared as shown in Table 7.1. The final concentrations of lecithin, magnesium sulphate and polymyxin B were 0.025 %w/v, 1.0 %w/v and 1000 U/ml, respectively. The concentration of 1000 U/ml was used for all test samples. When these samples were diluted in microtiter plates, the concentration of polymyxin B in row 1, in which it was inactivated was about 400 U/ml. The original concentration of polymyxin B may be 25 % higher than stated on the label and for this reason it was not possible to know the exact concentration of polymyxin B in the diluted eye drops. That is the concentration in row 1 could be as high as 500 U/ml. Therefore the concentration of 1000 U/ml was considered to be high enough to test the effectiveness of the inactivating recovery medium. For every test sample, the bacterial suspension was the last addition to be made to the first row of wells. At the contact times of 2 and 12 min, 0.1 ml of each sample was withdrawn and suitably diluted for the viable count. The subsequent procedure was as described in 7.2.1.

Table 7.1 Components of the test samples used for testing the effect of magnesium sulphate and lecithin on the effect of polymyxin B on the viability of cell suspensions of *P. aeruginosa NCTC 6750*

No.	Bacteria suspension :absorbance 0.175 - 0.185 (ml)	Magnesium sulphate* and lecithin** (ml)	Polymyxin B solution : 10,000 U/ml (ml)	Water (ml)	Nutrient broth (ml)
1. Bacteria + Inactivator	0.05	4.5	-	0.5	-
2. Bacteria + PMB	0.05	-	0.5	-	4.5
3. Bacteria + Inactivator + PMB	0.05	4.5	0.5	-	-

* = Magnesium sulphate 1 g in 89 ml nutrient broth

** = Lecithin 0.025 g in 89 ml nutrient broth

7.2.3 Effect of magnesium sulphate 0.5 and 1 % w/v

The objective of this section was to determine whether magnesium sulphate 1 %w/v is a suitable concentration for using to inhibit polymyxin B at the concenrations used. Two types of test samples containing magnesium sulphate were prepared as shown in Table 7.2. The final concentration of polymyxin B was 1000 U/ml. For every test sample, the bacterial suspension was added last in order. At the contact times of 2, 12 and 22 min, 0.1 ml of each sample was withdrawn and diluted for viable counting using the procedure described in 7.2.1.

Table 7.2 Components of the test samples used for testing the effect of different concentrations of magnesium sulphate on the effect of polymyxin B on the viability of cell suspensions of *P.aeruginosa NCTC 6750*

No.	Bacteria suspension: absorbance 0.175-0.185 (ml)	Magnesium sulphate* (ml)	Polymyxin B solution :10,000 U/ml (ml)	Water (ml)	Nutrient broth (ml)
1. Bacteria + MgS04	0.05	4.5	-	0.5	-
2. Bacteria + PMB	0.05	-	0.5	-	4.5
3. Bacteria + MgS04	0.05	4.5	0.5	-	-
4. Control	0.05	-	-	0.5	4.5

* 0.5 g in 89 ml nutrient broth to make final concentraion to 0.5 %

or 1.0 g in 89 ml nutrient broth to make final concentraion to 1.0 %

7.2.4 Killing curves for different concentrations of polymyxin B using magnesium sulphate 1 %w/v solution as an inactivating recovery medium

Different concentrations of standard polymyxin B solutions ranging from 125 U/ml to 5000 U/ml were made in phosphate buffer as described in 4.2.2.2. Test solutions (150 µl) were mixed with 150 µl of cell suspension in row N of the microtiter plates. The wells in rows 1-6 were primed with magnesium sulphate 1 %w/v solution. Killing time determinations of polymyxin B at concentrations of 2500, 2000, 1000, 500, 250, 125 and 62.5 U/ml were then carried out following the methods described in 4.2.2.2.

7.2.5 Killing curves for different concentrations of polymyxin B when prepared in water and phosphate buffer pH 6.0

Phosphate buffer pH 6.0 was used in this study as a solvent for polymyxin B as recommended by the British Phamacopoeia [43]. This pH was found to be suitable for polymyxin B solution [55,56]. The objective of this section was to test whether there was any difference between the antibacterial activity of polymyxin B prepared in water and in phosphate buffer pH 6.0. The preparation of standard solutions of polymyxin B ranged from 125 U/ml to 5000 U/ml and the killing time determinations were as described in 4.2.2.2. The inactivating recovery medium was magnesium sulphate 1 %w/v solution .

7.2.6 Effect of magnesium sulphate 1 %w/v when prepared in water, nutrient broth and thioglycollate broth

Thioglycollate broth containing magnesium sulphate 1% w/v was prepared as described in 4.1.2.4. Thioglycollate broth was expected to be suitable for determining the killing times of polymyxin B in sample 1 which contains thiomersal. It would also have to be suitable for polymyxin B in the other samples. In order to compare the effectiveness of magnesium sulphate 1 %w/v when prepared in water, nutrient broth and thioglycollate broth, the three different solutions were prepared and added to rows 1-6 of the microtiter plates. In the previous section the results had shown that the most suitable concentration of polymyxin B was 2000 U/ml. Therefore, standard polymyxin B 4000 U/ml was prepared in phosphate buffer. This was diluted to 2000 U/ml in the first row of wells in the microtiter plate and used as the test sample. Preparation of polymyxin B 4000 U/ml and killing time determinations were carried out as described in 4.2.2.2.

7.3 Results and discussion

7.3.1 Calibration curve

Fig. 7.1 shows good correlation between absorbance readings and log viable counts for *P. aeruginosa NCTC 6750.* The concentration of bacteria used in the killing time determinations should be approximately 10^8 to 10^9 CFU/ml. Therefore bacterial suspensions in the range of absorbance between 0.175 to 0.185 should be used for the subsequent experiments.

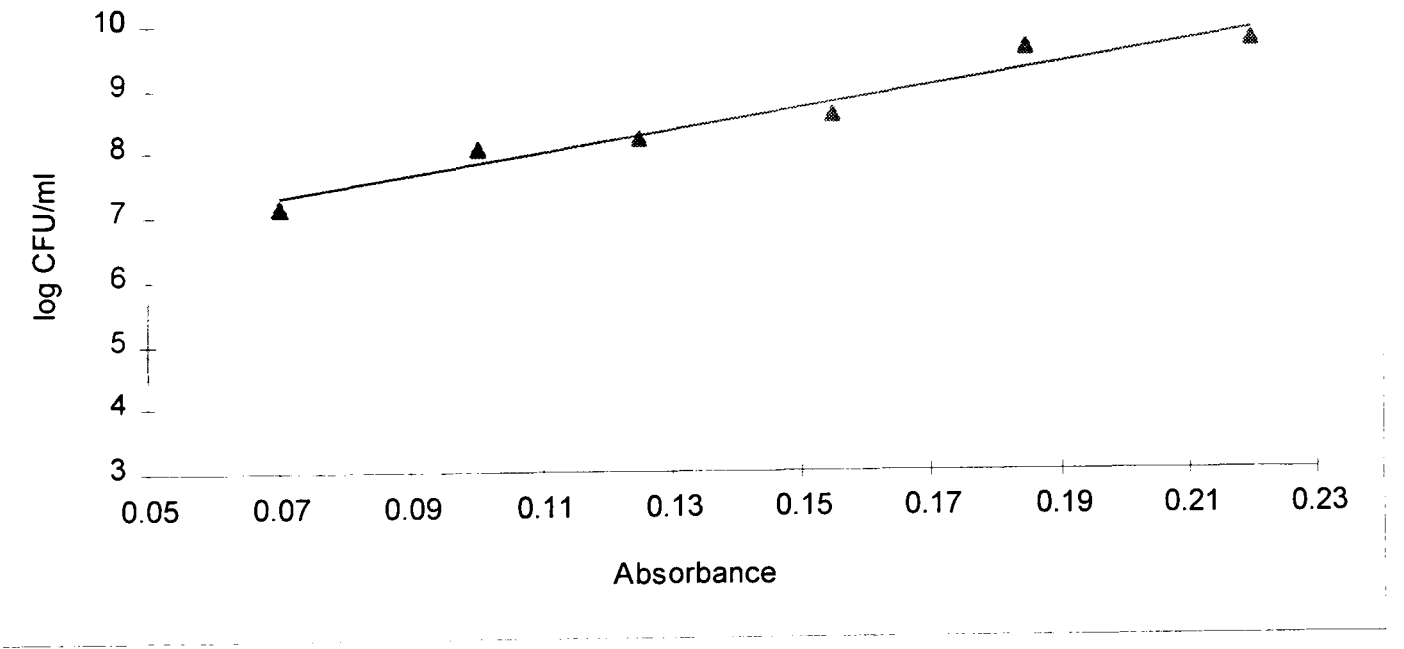


Fig. 7.1 Calibration curve of log CFU per milliliter against corresponding absorbance readings at 500 nm for cell suspensions of *P. aeruginosa* NCTC 6750.

7.3.2. Effect of lecithin and magnesium sulphate

Tables 7.3, 7.4 and Figs. 7.2, 7.3 give the CFU/ml of *P.aeruginosa* at 2 min and 12 min in three different types of sample. The differences between the numbers of bacteria at 2 and 12 min were analysed by the Student t-test (p=0.05). Lecithin 0.025 %w/v had some inhibitory activity on the activity of polymyxin compared with the activity of polymyxin in buffer and this is seen at 2 and 12 min. Although the number of bacteria in the lecithin 0.025 %w/v did show a slight decrease between the 2 min and 12 min sample, this was considered to be the result of experimental error and not a killing effect of the lecithin on the *P. aeruginosa* cells. The t-test of bacterial numbers at 2 and 12 min in the presence of magnesium sulphate 1 % w/v showed no significant difference. Although polymyxin B in the presence of magnesium sulphate 1 %w/v had a slight effect on the number of bacteria, the overall data showed that magnesium sulphate 1 %w/v was much more effective than lecithin 0.025 %w/v as an inactivator of polymyxin B 1000 U/ml.

Table 7.3 Effect of lecithin 0.025 %w/v on the effect of polymyxin B 1000 U/ml on the viability of cell suspensions of *P. aeruginosa NCTC 6750*

	CFU/ml : mean x 10^6 (SD x 10^6) , n=3		
Sample	2 min	12 min	t-test between 2 min and 12 min (p = 0.05)
1.Bacteria + Lecithin	1.75 (0.12)	1.44 (0.19)	+
2. Bacteria + PMB	0.57 (0.05)	*	0
3. Bacteria + PMB + Lecithin	1.45 (0.14)	0.0165 (0.07)	+

- = non - significant

0 = t-test was not performed

+ = significant

* = less than 10^3 CFU/ml

Table 7.4 Effect of magnesium sulphate 1 %w/v on the effect of polymyxin B 1000 U/ml on the viability of cell suspensions of *P.aeruginosa NCTC 6750*

	CFU/ml : mean x 10^6 (SD x 10^6), n=3		
Sample	*2* min	*12* min	*t-test between 2* min *and 12* min *(p=0.05)*
1. Bacteria + $MgSO_4$	2.10 (0.38)	1.77 (0.10)	_
2. Bacteria + PMB	5.70 (0.05)	*	0
3. Bacteria + PMB + $MgSO_4$	1.41 (0.16)	0.90 (0.00)	+

- = non - significant

0 = t-test was not performed

+ = significant

* = less than 10^3 CFU/ml

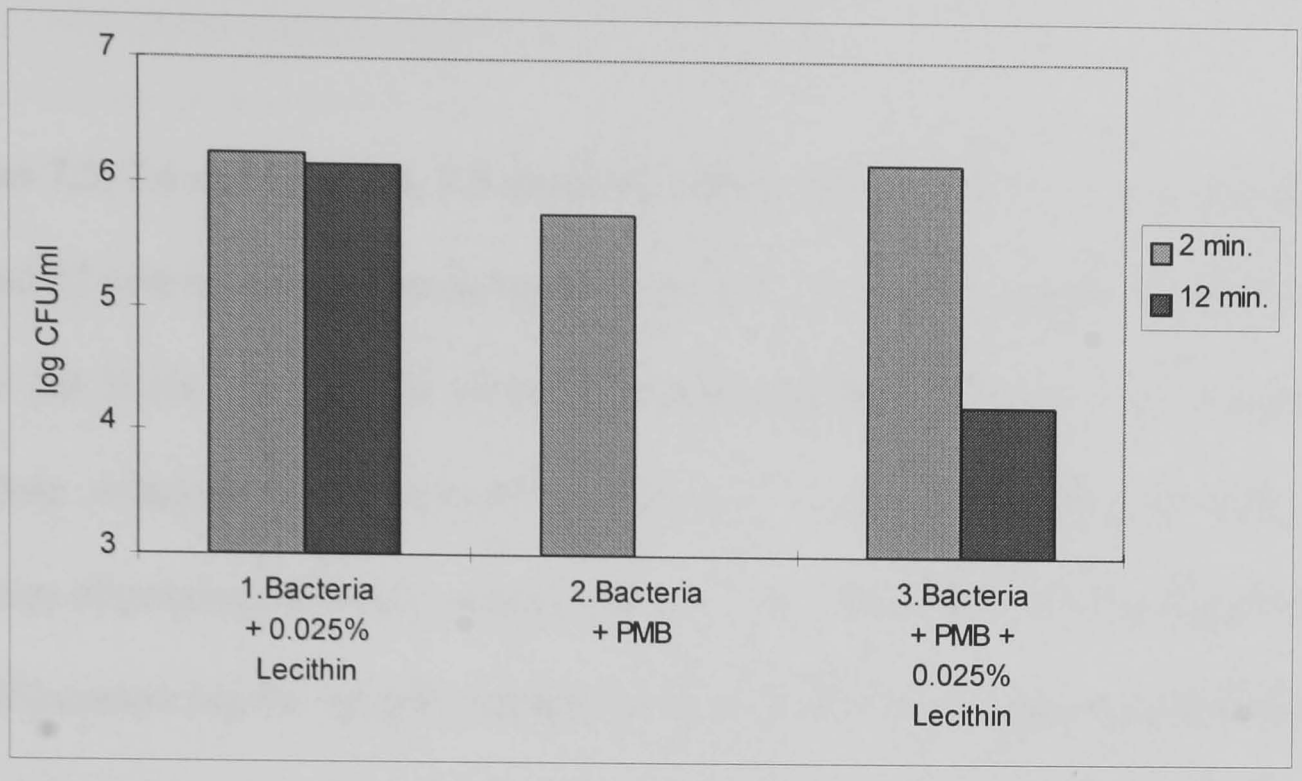


Fig. 7.2 Effect of lecithin 0.025 %w/v on the effect of polymyxin B 1000 U/ml on the viability of cell suspensions of *P.aeruginosa NCTC 6750.*

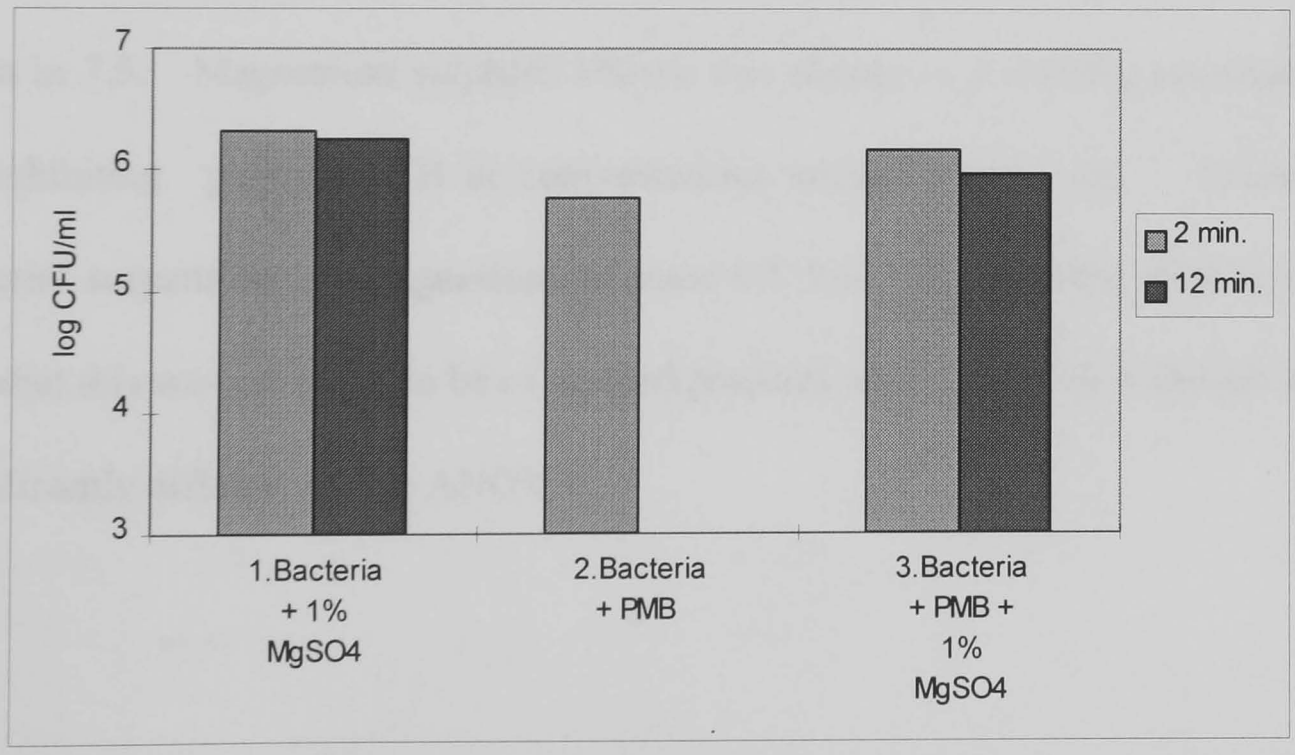


Fig. 7.3 Effect of magnesium sulphate 1 %w/v on the effect of polymyxin B 1000 U/ml on the viability of cell suspensions of *P.aeruginosa NCTC 6750.*

7.3.3 Effect of magnesium sulphate 0.5 and 1.0 %w/v

Tables 7.5, 7.6 and Figs. 7.4, 7.5 show the number of CFUs of *P. aeruginosa* at 0, 2, 12 and 22 min in three different types of samples, i.e, either magnesium sulphate 0.5 % or 1.0 %w/v, polymyxin 1000 U/ml and combinations of the two magnesium sulphate solutions with polymyxin. Similar results were obtained with both samples of polymyxin alone (sample No. 2). An analysis of variance (ANOVA ; p = 0.05) comparing the samples containing bacteria plus magnesium sulphate (sample No. 1) and bacteria plus magnesium sulphate and polymyxin B (sample No. 3) indicate that only samples contain magnesium sulphate 1.0 %w/v showed no significant reduction in the number of bacteria. This was slightly different from the results shown in Fig. 7.3 but in this test the inoculum was 100 times larger and the reduction in numbers shown in 7.3 would have a very small effect on the results given in 7.5. Magnesium sulphate 1%w/v was chosen as a suitable concentration for inhibiting polymyxin B at concentrations around 1000 U/ml. Counts on bacterial suspensions in magnesium sulphate 0.5 %w/v did increase slightly (Fig. 7.4) but this was not taken to be of marked practical significance even though it was significantly different by the ANOVA.

Table 7.5 Effect of magnesium sulphate 0.5 %w/v on the effect of polymyxin B 1000 U/ml on the viability of cell suspensions of *P. aeruginosa NCTC 6750*

CFU/ml : mean $x10^8$ (SD x 10^8) , n=3					
No.	0 min	2 min	12 min	22 min	ANOVA (p=0.05)
1. Bacteria + 0.5% MgSO4	1.04 (0.17)	1.04 (0.14)	1.38 (0.15)	1.25 (0.22)	+
2. Bacteria + PMB	1.04 (0.17)	*	*	*	0
3. Bacteria + PMB + 0.5 % MgSO4	1.04 (0.17)	0.62 (0.12)	0.64 (0.12)	0.39 (0.12)	+

Table 7.6 Effect of magnesium sulphate 1.0 %w/v on the effect of polymyxin B 1000 U/ml on the viability of cell suspensions of *P. aeruginosa NCTC 6750*

CFU/ml : mean $x10^8$ (SD x 10^8) , n =3					
No.	0 min	2 min	12 min	22 min	ANOVA (p=0.05)
1. Bacteria + 1.0% MgSO4	1.04 (0.17)	1.17 (0.07)	1.13 (0.17)	1.36 (0.35)	-
2. Bacteria + PMB	1.04 (0.17)	*	*	*	0
3. Bacteria + PMB + 1.0 % MgSO4	1.04 (0.17)	0.94 (0.07)	0.84 (0.17)	0.82 (0.35)	-

- = non - significant

0 = ANOVA was not performed

+ = significant

* = less than 10^3 CFU/ml

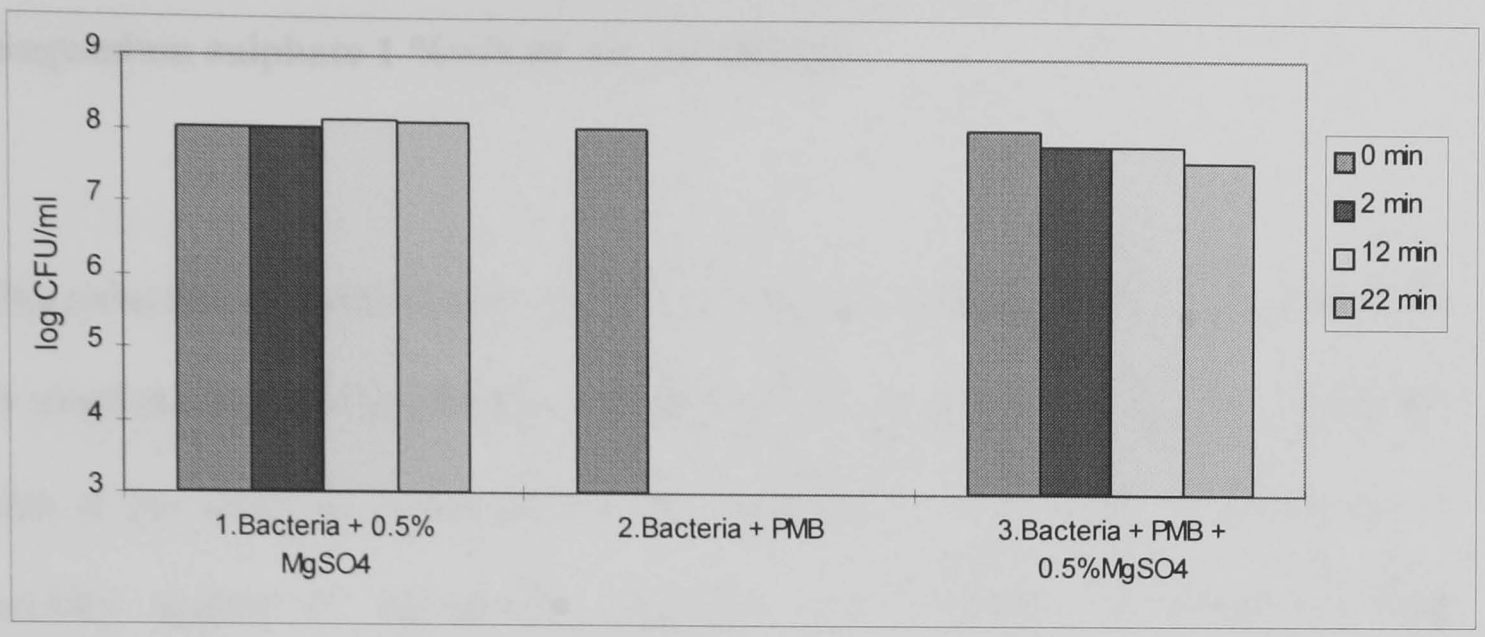


Fig. 7.4 Effect of magnesium sulphate 0.5 %w/v on the effect of polymyxin B 1000 U/ml on the viability of cell suspensions of *P. aeruginosa NCTC 6750.*

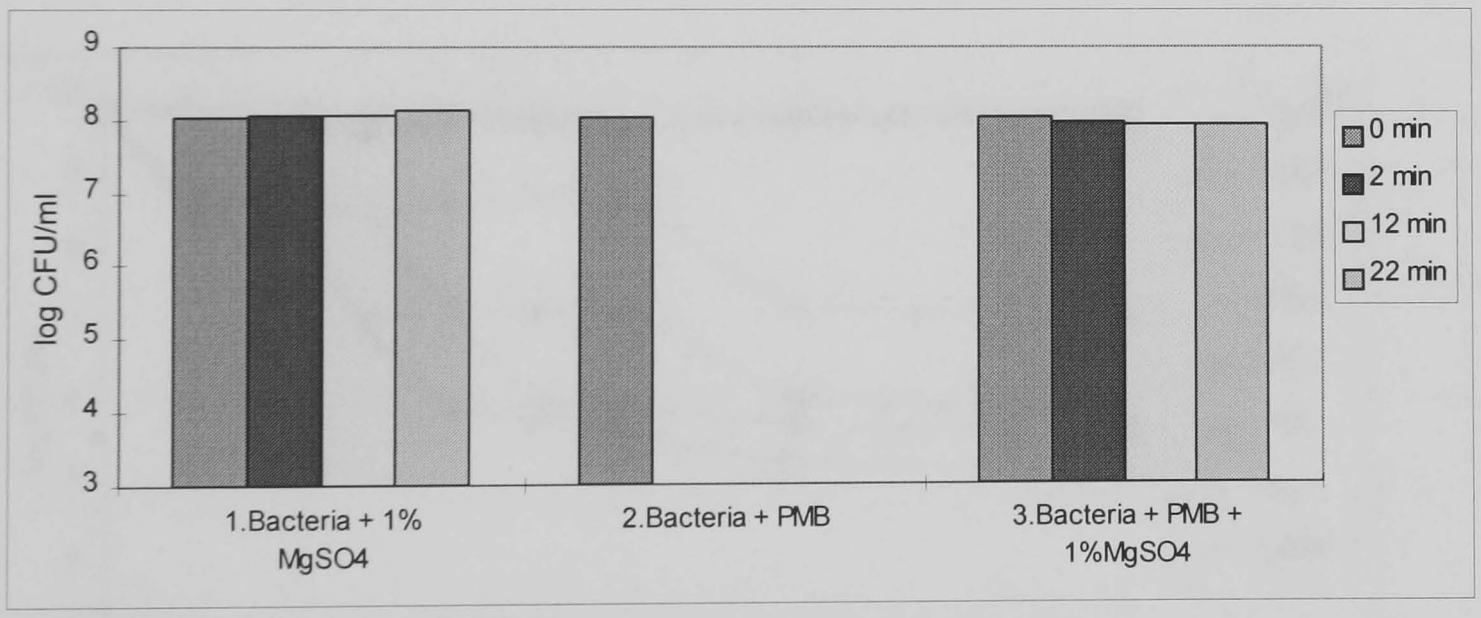


Fig. 7.5 Effect of magnesium sulphate 1 %w/v on the effect of polymyxin B 1000 U/ml on the viability of cell suspensions of *P. aeruginosa NCTC 6750.*

7.3.4 Killing curves of different concentrations of polymyxin B using magnesium sulphate 1 %w/v as an inactivator

The reduction in viable counts of the test organism with time produced by polymyxin B solutions prepared in phosphate buffer pH 6.0 are shown in Fig. 7.6. It appears that at the range of concentrations between 62.5 to 250 U/ml, the polymyxin B activity against *P. aeruginosa* was not very marked. The effect of these concentrations was similar to the control (water). Concentrations from 500 to 2500 U/ml produced a greater reduction in the colony count. The greatest reduction in colony count was ~ 5 log cycle over 60 min. The concentration of 2000 U/ml was chosen for comparing the killing time of various polymyxin B samples.

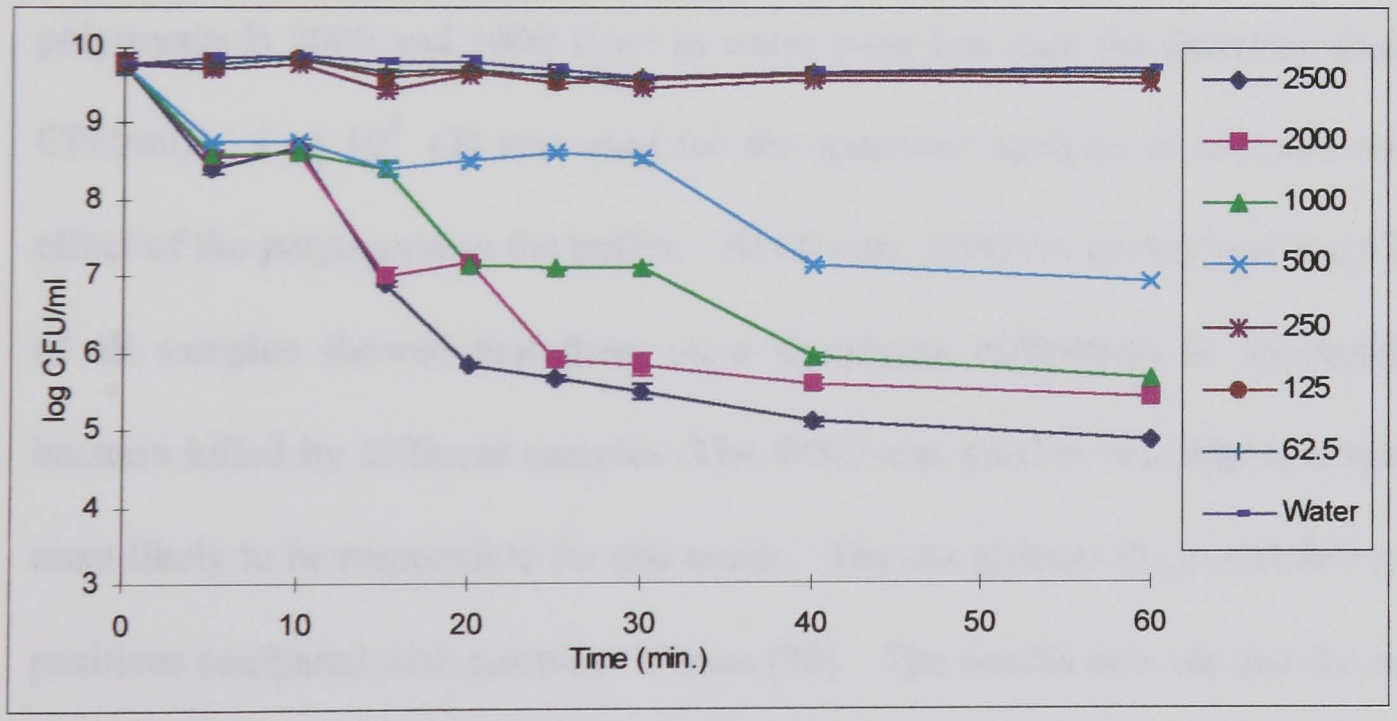


Fig. 7.6 The reduction in CFU per milliliter of cell suspensions of *P. aeruginosa NCTC 6750* produced by polymyxin B (U/ml) at graded concentrations.

7.3.5 Killing curves of graded concentrations of polymyxin B when prepared in water and phosphate buffer pH 6.0

The killing curves of graded concentrations of polymyxin B ranging from 62.5 to 2000 U/ml in water and phosphate buffer pH 6.0 are shown in Fig. 7.7 and 7.6. Analysis of variance (ANOVA, p=0.05) of all contact times and log CFU/ml showed significant differences between polymyxin B prepared in water and buffer at the concentrations of 500, 250, 125 and 62.5 U/ml. Tukey's Wholly Significance difference (WSD) was used to investigate this further. Both figures show that the differences in antibacterial activity of polymyxin B prepared in water and buffer started to become obvious after the contact time of 50 min. In Fig. 7.6, the number of bacteria at 50 min was not determined. Therefore, the contact time of 60 min was chosen to test for differences. The number of bacteria after 60 min contact with polymyxin B 2000 and 1000 U/ml in water were less than the detection limit (10^3 CFU/ml). Log 10^3 (3) was used for the statistical analysis to compare with the effect of the polymyxin in the buffer. At 60 min, ANOVA (p=0.05) of log CFU/ml of all samples showed that there were significant differences in the number of bacteria killed by different samples. The WSD was used to test which sample was most likely to be responsible for this result. The test reduces the possibility of false positives compared with pairwise 't' tests [70]. The results indicate that the number of bacteria after 60 min contact with polymyxin B 2000, 1000 U/ml in water were lower than those in contact with other samples. From this it appears that polymyxin B may have more activity when prepared in water. Polymyxin B 2000 and 1000 U/ml prepared in water reduced the number of bacteria from about 10^9 to about 10^3

CFU/ml after 50 min while the number of bacteria in the polymyxin B solutions prepared in buffer remained at concentration greater than 10^5 CFU/ml at 60 min.

Phosphate ion in the buffer may affect the activity of polymyxin B against *P. aeruginosa*. Polymyxin B is able to mimic 'head and tail' features of phospholipids and this allows polymyxin B to become incorporated within the membrane. The heptapeptide core serves as a polar head to complement the polar glycerol-derived phosphate moiety of the phospholipid unit. The fatty acid tail which extends from the cyclic core serves as a hydrophobic tail similar to that of the unsaturated and saturated long chain aliphatic fatty acids which comprise the tail of the phospholipids [16]. The presence of high concentrations of phosphate ion may prevent the binding of the polar head of polymyxin B to the phospholipid and therefore reduce the activity of polymyxin B. However, it is proposed to use phosphate buffer pH 6.0 as a solvent for polymyxin B in all experiments in this study. This is in order to ensure that the pH remains constant at the high temperatures used in the stability studies of polymyxin B solutions. The buffer should counteract any changes of pH which might be produced by the release of alkaline substances from the glass containers. Thus the use of phosphate buffer pH at 6.0 as a solvent for polymyxin was considered necessary.

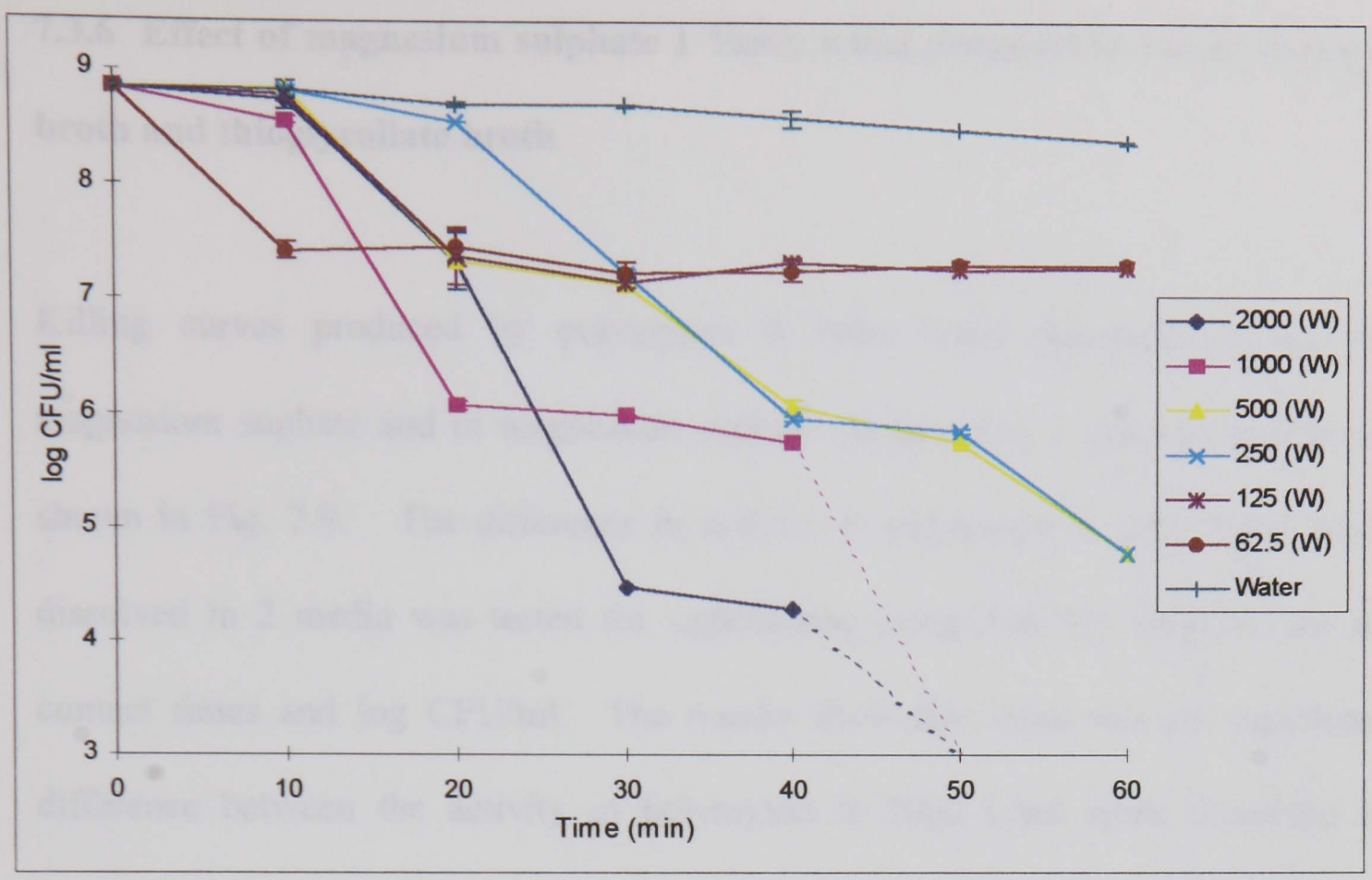


Fig. 7.7 Killing curves of graded concentrations of polymyxin B (U/ml) prepared in water (W) against cell suspensions of *P. aeruginosa NCTC 6750.*

7.3.6 Effect of magnesium sulphate 1 %w/v when prepared in water, nutrient broth and thioglycollate broth

Killing curves produced by polymyxin B 2000 U/ml dissolved in aqueous magnesium suphate and in magnesium suphate dissolved in 2 different media are shown in Fig. 7.9. The difference in activity of polymyxin B 2000 U/ml when dissolved in 2 media was tested for significance using ANOVA (p=0.05) for all contact times and log CFU/ml. The results show that there was no significant difference between the activity of polymyxin B 2000 U/ml when dissolved in magnesium sulphate 1 %w/v aqueous solution, magnesium sulphate 1 %w/v nutrient broth and magnesium sulphate 1 %w/v thioglycollate broth (Fig. 7.8). This also indicates that the inactivating effectiveness of magnesium sulphate 1 %w/v was not affected by the substances present in nutrient broth or thioglycollate broth.

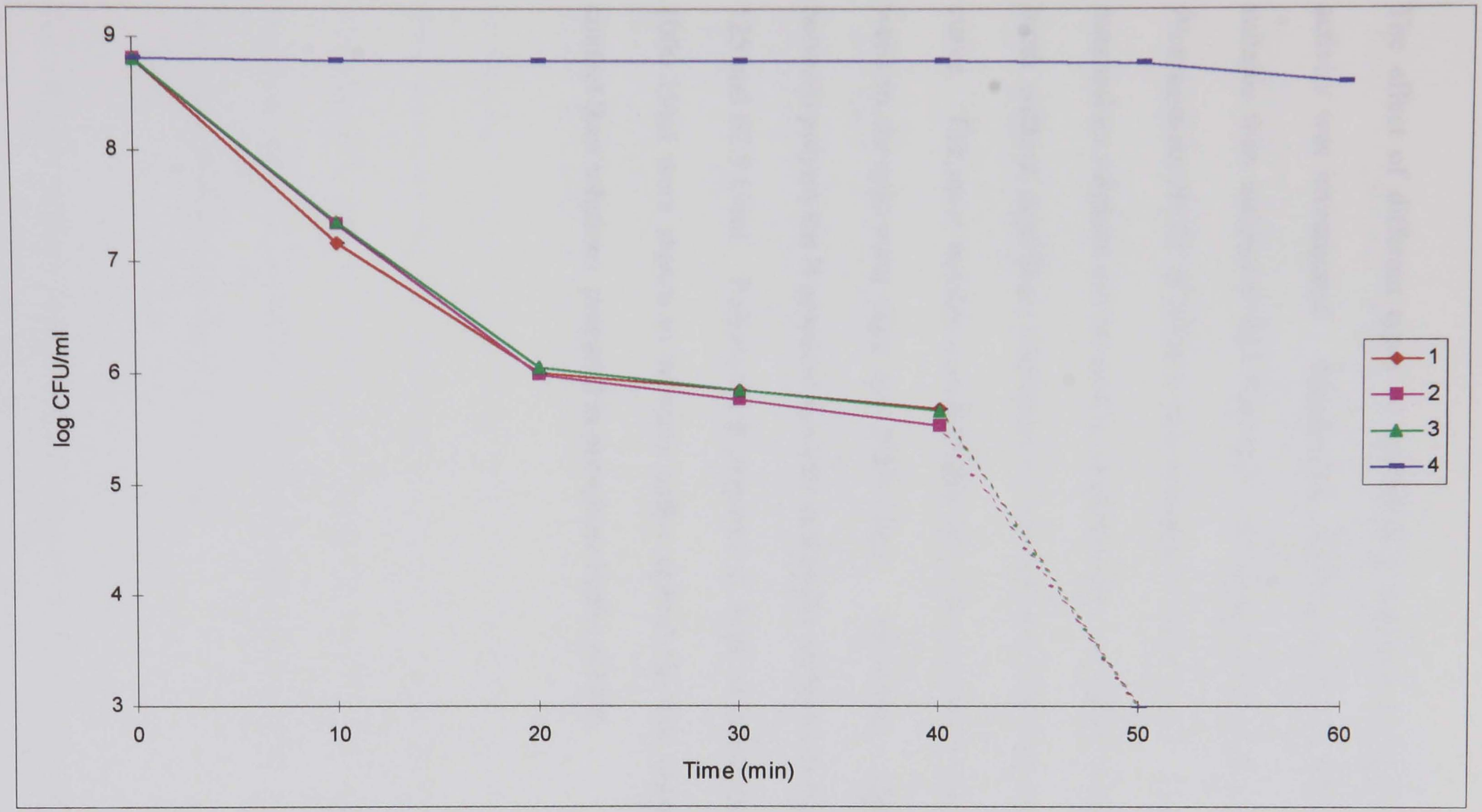


Fig. 7.8 Effect of different types of reaction mixture on the effect of 2000 U/ml polymyxin B against cell suspensions of *P. aeruginosa NCTC 6750*

1 = Magnesium sulphate 1 %w/v aqueous
2 = Magnesium sulphate 1 %w/v nutrient broth
3 = Magnesium sulphate 1 %w/v thioglycollate broth
4 = Control (no polymyxin)

7.4 Conclusions

The effect of different types of inactivating recovery solutions on polymyxin B activity was investigated. Magnesium sulphate (1 %w/v) was found to be more suitable than lecithin (0.025 %w/v) for inactivating polymyxin B activity against *P.aeruginosa NCTC 6750* at a concentration of 1000 U/ml. This concentration of magnesium sulphate can be used in aqueous solution, nutrient broth or thioglycollate broth without significant differences in the effect of polymyxin B on the killing curve. The most suitable concentration of polymyxin B for use in the first row of wells in the microtiter plate was 2000 U/ml. There were significant differences between polymyxin B prepared in water and buffer at the concentrations of 500, 250, 125 and 62.5 U/ml. Polymyxin B prepared in water at concentrations of 2000 and 1000 U/ml were shown to be more active against the test organism after 60 min contact than solutions prepared in phosphate buffer pH 6.0.

Chapter 8

Effect of preservatives and second antibacterials present in eye drops on polymyxin B bactericidal activity against *P. aeruginosa NCTC 6750*

8.1 Introduction

The effects of preservatives and second antibacterials present in eye drops on polymyxin B activity against *B. bronchiseptica NCTC 8344* were investigated in chapter 5. This organism was chosen as a test bacteria as suggested in the British Pharmacopoeia for the microbiological assay of polymyxin B. However, the results in chapter 6 indicate that the microbiological assay was not suitable for monitoring the potency of polymyxin B in the stability study. Therefore, killing time determinations for polymyxin B against *P. aeruginosa NCTC 6750* were investigated as the test system. *P. aeruginosa NCTC 6750* was used as the test organism since the bactericidal activity of polymyxin B against this organism has been reported frequently [9,11,17,65,66].

The objective of this chapter was to determine whether or not the preservatives and second antibacterials present in polymyxin B eye drops had effects on polymyxin B activity against *P. aeruginosa NCTC 6750.* Previous results (chapter 7) indicated

that magnesium sulphate 1 %w/v was suitable for inactivating polymyxin B activity and it could be used effectively at this concentration in aqueous solution, nutrient broth or thioglycollate broth. In this chapter, thioglycollate broth containing *p*-aminobenzoic acid (PABA) 0.16 %w/v and magnesium sulphate 1 %w/v was used as an inactivating recovery medium. Thioglycollate broth and PABA were expected to inhibit the activity of thiomersal and trimethoprim present in sample 1 (Table 8.1). This concentration of PABA was used following Richards *el al* findings that 12.5 μg of TMP could be inhibited by 50 μg of PABA [71]. PABA has been found to enhance the activity of other antibacterials due to its ability to increase the bacterial cell envelope permeability[17,65,66]. Richards and Xing [17] reported that subinhibitory concentrations of polymyxin B and PABA had synergistic inhibitory activity against *P. aeruginosa.* The killing time determinations were carried out at 37°C over a period of 5 h. The conditions were different from this current study in which test samples were maintained at room temperature for 1 h. Under these conditions PABA was not expected to have an effect on the activity of polymyxin B since this was not a suitable environment for cell growth and PABA can only affect the cell envelope permeability of dividing cells [65].

Test substances included thiomersal (TMS), trimethoprim (TMP), neomycin (N) and gentamicin (G). Thiomersal and trimethoprim were used as the preservative and the second antibacterial in sample 1. Neomycin (sample 2, 3, 4, 5 and 6) and trimethoprim (sample 1) had been included in the eye drops in order to extend the spectrum of activity of the formulations. Gentamicin was not included in any of the test samples but its activity was tested in this investigation because it is, like

neomycin, an aminoglycoside. The concentrations investigated were the concentrations of gentamicin included in eye drops of the British National Formulary [72].

The specific effect of other ingredients present in some of the test samples (benzalkonium chloride, gramicidin and metaoxedrine) were not tested. Benzalkonium chloride, as a quaternary ammonium compound, is active against Gram-positive bacteria and less active against Gram-negative organisms, especially *P. aeruginosa* [73]. It is used as a preservative for samples 3, 4, 5 and 6. High concentrations of benzalkonium chloride (0.1 %w/v) have been used as a disinfectant for *P. aeruginosa* [74,75]. However, *P. aeruginosa* is often highly resistant to quaternary ammonium compounds (QACs). A factor of considerable importance for bacterial resistance is the cation content of the bacterial outer cell membrane. The Mg^{2+} content having the greatest influence in *P. aeruginosa* cells [16]. Mg^{2+} was added to the recovery medium for determining the killing times of polymyxin B samples. In addition, the concentration of benzalkonium chloride in the test samples was diluted as low as 0.001 or 0.002 %w/v. Both the low concentrations of benzalkonium chloride and the high concentrations of Mg^{2+} would effectively inactivate the activity of benzalkonium chloride on *P. aeruginosa.*

Gramicidin was present in sample 2 in order to extend the spectrum of activity against Gram-positive bacteria. It is primarily active against many species of Gram-positive bacteria but many of the Gram-negative bacterial species are unaffected by gramicidin. This is due to the outer layer of the bacterial cell envelope

preventing the compound gaining access to and exerting its action on the bacterial cytoplasmic membrane[9,11,16].

Metaoxedrine or phenylephrine hydrochloride was present in sample 6 at concentration of 0.12 %w/v. It is employed in eye drops as a mydriatic in concentrations of up to 10 %w/v [9]. There is no reported activity of this substance against *P. aeruginosa* . Consequently, it was not expected that metaoxedine would have an effect on the activity of polymyxin B against *P. aeruginosa.*

8.2 Methods

P. aeruginosa NCTC 6750 was used as the test organism. Inoculum preparation for all experiments in this chapter were as described in 4.2.2.1.

The test solutions (150 µl) were mixed with 150 µl of cell suspensions in the first row of wells in the microtiter plates. The wells in rows 1 to 6 of the microtiter plates were primed with 200 µl of thioglycollate broth containing PABA 0.16 %w/v and magnesium sulphate 1 %w/v. Killing time determinations were carried out as described in 4.2.2.2.

The solutions of polymyxin B in combination with other substances at the concentrations contained in the eye drops were diluted to give 4000 U/ml of polymyxin B (see Table 8.1) and tested by the same procedure as that used for

polymyxin B alone. The concentrations given in the last column of Table 8.1 were diluted by a factor of 2 in the first row of wells in the microtiter plates. Phosphate buffer pH 6.0 was used as the control. The preparations of polymyxin B in combination with the other substances are given in Table 8.1.

Table 8.1 Ingredients of the test eye drops

Sample	Ingredients	Concentration when polymyxin B is diluted to 4000 U/ml
1 (*Wellcome**)	PMB 10,000 U/ml TMP 0.1 %w/v (Preservative TMS 0.005 %w/v)	 0.04 %w/v 0.002 %w/v
2 (*Seng Thai* *)	PMB 5000 U/ml N 0.2 %w/v Gramicidin 0.025 %w/v **	 0.16 %w/v 0.02 %w/v
3 (*Dispersa**)	PMB 15,000 U/ml N 0.35 %w/v (Preservative BZK 0.01 %w/v)	 0.08 %w/v 0.002 %w/v
4 (*ALcon**)	PMB 16,250 U/ml N 0.35 %w/v (Preservative BZK 0.004 %w/v)	 0.08 %w/v 0.0010 %w/v
5 (*Alcon**)	PMB 16,250 U/ml N 0.35 %w/v (Preservative BZK 0.004 %w/v)	 0.08 %w/v 0.0010 %w/v
6 (*Alcon**)	PMB 16,250 U/ml N 0.35 %w/v Metaoxedrine or Phenylephrine hydrochloride 0.12%w/v (Preservative BZK 0.004%w/v)	 0.08 %w/v 0.030 %w/v 0.0010 %w/v

PMB = polymyxin B, TMP = trimethoprim,

TMS = thiomersal, N= neomycin, BZK = benzalkonium chloride

* Manufacturer

** Preservative is not stated

Sample 1, 4 and 6 were obtained from U.K.

Sample 2, 3 and 5 were obtained from Thailand

8.2.1 Effect of thiomersal and trimethoprim

Test solutions were prepared as shown in Table 8.2.

Table 8.2 Components of the test samples used for testing the effect of thiomersal (TMS) and trimethoprim (TMP) on the effect of polymyxin B (PMB) on the viability of cell suspensions of *P. aeruginosa NCTC 6750*

Sample	PMB* (ml)	TMS* (ml)	TMP* (ml)	Phosphate buffer pH 6.0 to (ml)
1. PMB 4000 U/ml	4	-	-	10
2. TMS 0.002 %w/v	-	1	-	10
3. PMB 4000 U/ml + TMS 0.002 %w/v	4	1	-	10
4. PMB 4000U/ml + TMP 0.04 %w/v	4	-	1	10
5. PMB 4000 U/ml + TMS 0.002 %w/v + TMP 0.04 %w/v	4	1	1	10
6. TMS 0.002 %w/v + TMP 0.04 %w/v	-	1	1	10
7. TMP 0.04 %w/v	-	-	1	10

PMB* = 0.0329 g of polymyxin B sulphate (7690 U/mg) in 25 ml of phosphate buffer pH 6.0 (10,000 U/ml)

TMS* = 0.02 g of thiomersal in 100 ml of phosphate buffer pH 6.0 (0.02 %w/v)

TMP* = 0.1 g of trimethoprim in 25 ml of phosphate buffer pH 6.0 (0.4 %w/v)

8.2.2 Effect of neomycin

8.2.2.1 Killing time determinations

Test solutions were prepared as shown in Table 8.3.

Table 8.3 Components of the test samples used for testing the effect of neomycin (N) on the effect of polymyxin B (PMB) on the viability of cell suspensions of *P. aeruginosa NCTC 6750*

Sample	PMB* (ml)	N* (ml)	Phosphate buffer pH 6.0 to (ml)
1. N 0.32 %w/v	-	5	10
2. N 0.16 %w/v	-	2.5	10
3. N 0.08 %w/v	-	1.25	10
4. N 0.0384 %w/v	-	0.6	10
5. PMB 4000 U/ml	4	-	10
6. PMB 4000 U/ml + N 0.32 %w/v	4	5	10
7. PMB 4000 U/ml + N 0.16 %w/v	4	2.5	10
8. PMB 4000 U/ml + N 0.08 %w/v	4	1.25	10
9. PMB 4000 U/ml + N 0.0384 %w/v	4	0.6	10

PMB* = 0.0329 g of polymyxin B sulphate (7690 U/mg) in 25 ml of phosphate buffer pH 6.0 (10,000 U/ml)
N* = 0.4706 g of neomycin sulphate (680 μg/mg) in 50 ml of phosphate buffer pH 6.0. (0.64 %w/v)

8.2.2.2 Possible chemical interaction between polymyxin B and neomycin

Killing time determinations showed that neomycin alone at the concentrations tested was not active against *P. aeruginosa* but the combination of all concentrations of neomycin with polymyxin B 2000 U/ml seemed to produce a slower reduction in the viable counts of the test organism with time than polymyxin B alone. Therefore tests were carried out to determine whether there was a chemical interaction between these two substances which could reduce the amount of available polymyxin B.

Initial measurements were carried out by determining absorbances at 2 wavelengths, 200 and 220 nm. The test solutions were prepared as follows:

The calibration curves for polymyxin B concentrations ranged from 10 to 250 U/ml (200 nm) and 50 to 250 U/ml (220 nm) were plotted from the absorbances of polymyxin B solutions against concentrations. The preparation of polymyxin B at these concentration were described in Table 8.4.

The test solutions used for testing the chemical interaction between polymyxin B and neomycin were prepared as indicated in Table 8.3. The solutions were diluted 2-times with phosphate buffer pH 6.0 and then 10-times with the same solvent to give suitable concentrations for determining the absorbance readings at 200 nm. The absorbance readings were carried out in triplicate. The same procedure was also carried out at a wavelength of 220 nm except that at this wavelength the test solutions were only diluted 2-times.

Table 8.4 Components of polymyxin B solutions used in calibration curves at the wavelength of 220 nm

Polymyxin B concentration (U/ml)	PMB* (ml)	Phosphate buffer pH 6.0 to (ml)
100	0.1	10
200	0.2	10
500	0.5	10
1000	1.0	10
1500	1.5	10
2000	2.0	10
2500	2.5	10

PMB* = 0.0329 g of polymyxin B sulphate (7690 U/mg) in 25 ml of phosphate buffer pH 6.0 (10,000 U/ml)

All solutions were diluted ten-times with buffer to achieve the concentrations of 10, 20, 50, 100, 150, 200 and 250 U/ml for determining the absorbance measurements at 200 nm.

The above results were confirmed by obtaining complete spectrum at the range 200 - 300 nm.

The test solutions were prepared as shown in Table 8.5.

Table 8.5 Components of the test samples used for the absorbance scanning from 200 to 300 nm

Sample	PMB* (ml)	N* (ml)	Phosphate buffer pH 6.0 to (ml)
1. PMB 2000 U/ml	2.5	-	12.5
2. PMB 2000 U/ml + N 0.08 %w/v	2.5	1.6	12.5
3. PMB 2000 U/ml + N 0.16 %w/v	2.5	3.2	12.5

PMB* = 0.0329 g of polymyxin B sulphate (7690 U/mg) in 25 ml of phosphate buffer pH 6.0 (10,000 U/ml)

N* = 0.4706 g of neomycin sulphate (680 μg/mg) in 50 ml of phosphate buffer pH 6.0. (0.64 %)

The absorbances of the test samples were scanned at the wavelength of 200 to 300 nm using a UV-Visible Recording Spectrophotometer Type UV-160A (Shimadzu). The spectrum of sample No.1 was superimposed with sample No.2 and 3.

8.2.3 Effect of gentamicin

Test solutions were prepared as described in Table 8.6.

Table 8.6 Components of the test samples used for testing the effect of gentamicin (G) on the effect of polymyxin B (PMB) on the viability of *P. aeruginosa NCTC 6750*

Sample	PMB* (ml)	G* (ml)	Phosphate buffer pH 6.0 to (ml)
1. G 0.60 %w/v	-	5.0	10
2. G 0.30 %w/v	-	2.5	10
3. 0.15 %w/v	-	1.25	10
4. 0.072 %w/v	-	0.6	10
5. PMB 4000 U/ml	4	-	10
6. PMB 4000 U/ml + G 0.60 %w/v	4	5.0	10
7. PMB 4000 U/ml + G 0.30 %w/v	4	2.5	10
8. PMB 4000 U/ml + G 0.15 %w/v	4	1.25	10
9. PMB 4000 U/ml + G 0.072 %w/v	4	0.6	10

PMB* = 0.0329 g of polymyxin B sulphate (7690 U/mg) in 25 ml of phosphate buffer pH 6.0 (10,000 U/ml)

G* = 0.4601 g of gentamicin sulphate (652 μg/mg) in 25 ml of phosphate buffer pH 6.0 (1.2 %w/v)

8.3 Results and discussion

8.3.1 Effect of thiomersal and trimethoprim

The killing curves of all samples are shown in Fig. 8.1. Analysis of data was carried out using ANOVA (p=0.05) at all contact times and log CFUs/ml. The killing curves of thiomersal 0.001 %w/v, trimethoprim 0.02 %w/v and thiomersal 0.001 %w/v plus trimethoprim 0.02 %w/v were not significantly different from the control (plots No. 2, 7, 6 and 8). Thiomersal is known to be mainly a bacteriostatic agent and trimethoprim is a slowly bactericidal agent. *P. aeruginosa* is inherently resistant to trimethoprim due to the inability of the agent to penetrate the cell envelope and gain access to the dihydrofolate reductase enzyme [9,16]. As expected, thiomersal and trimethoprim alone at these concentrations were shown to be inactive against *P. aeruginosa*. The combinations of either thiomersal and trimethoprim or both together did not produce a significant effect on the activity of the polymyxin B 2000 U/ml. This is shown in plots No. 1, 3, 4 and 5.

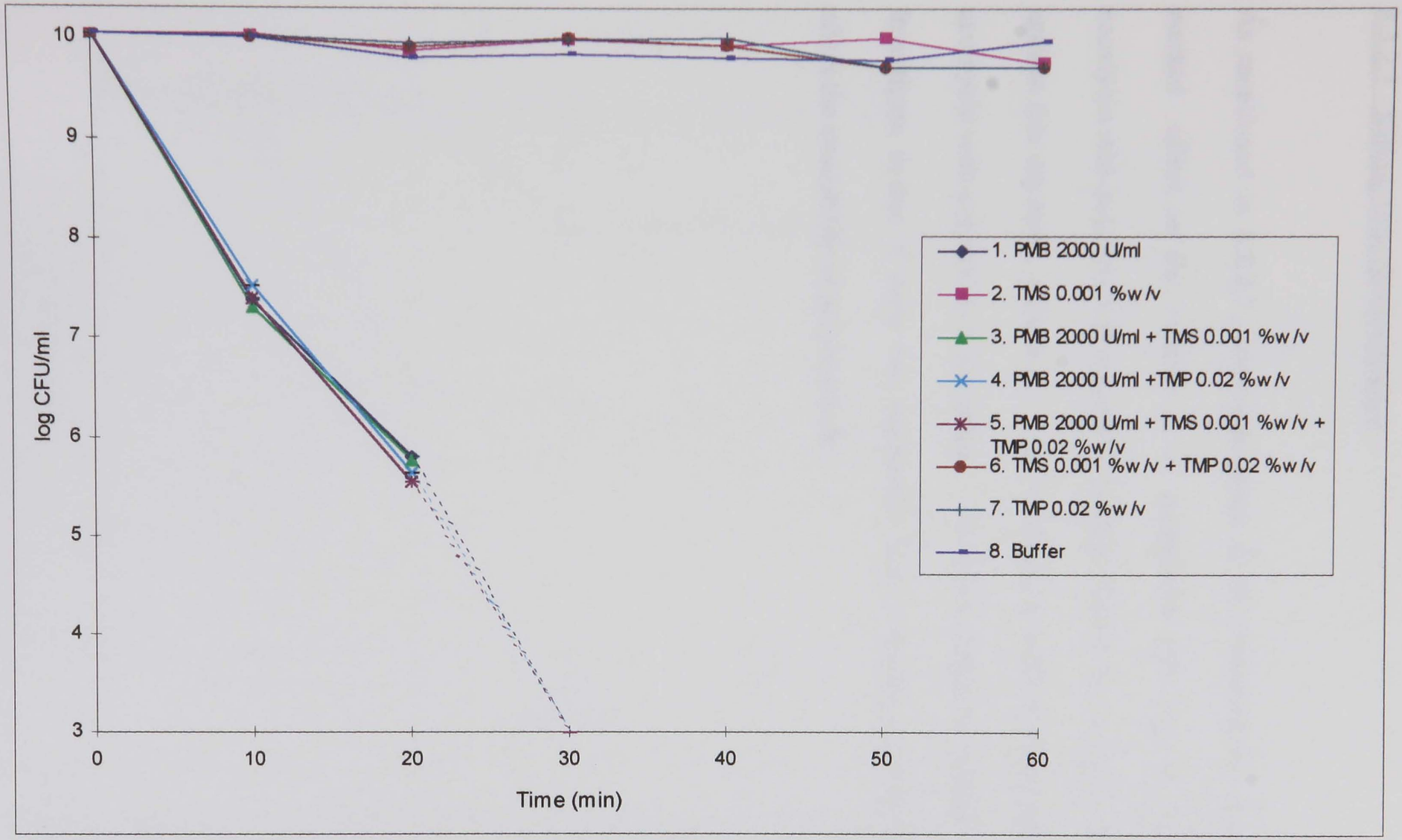


Fig. 8.1 Reduction in the CFU/ml with time for cell suspensions of *P. aeruginosa NCTC 6750* produced by polymyxin B (PMB), thiomersal (TMS), trimethoprim (TMP) and the combinations of these three substances.

8.3.2 Effect of neomycin

8.3.2.1 Killing time determinations

As mentioned in 8.2.2.2., neomycin alone at all concentrations did not show a marked effect on the viability of *P. aeruginosa* cells but the combination of neomycin with polymyxin B apparently slightly reduced the activity of polymyxin B against this organism. (Fig.8.2). Any such effect could contraindicate the use of neomycin with polymyxin B eye drops. Therefore chemical methods were used to investigate further if these two substances have a chemical interaction that may affect the availability of polymyxin B.

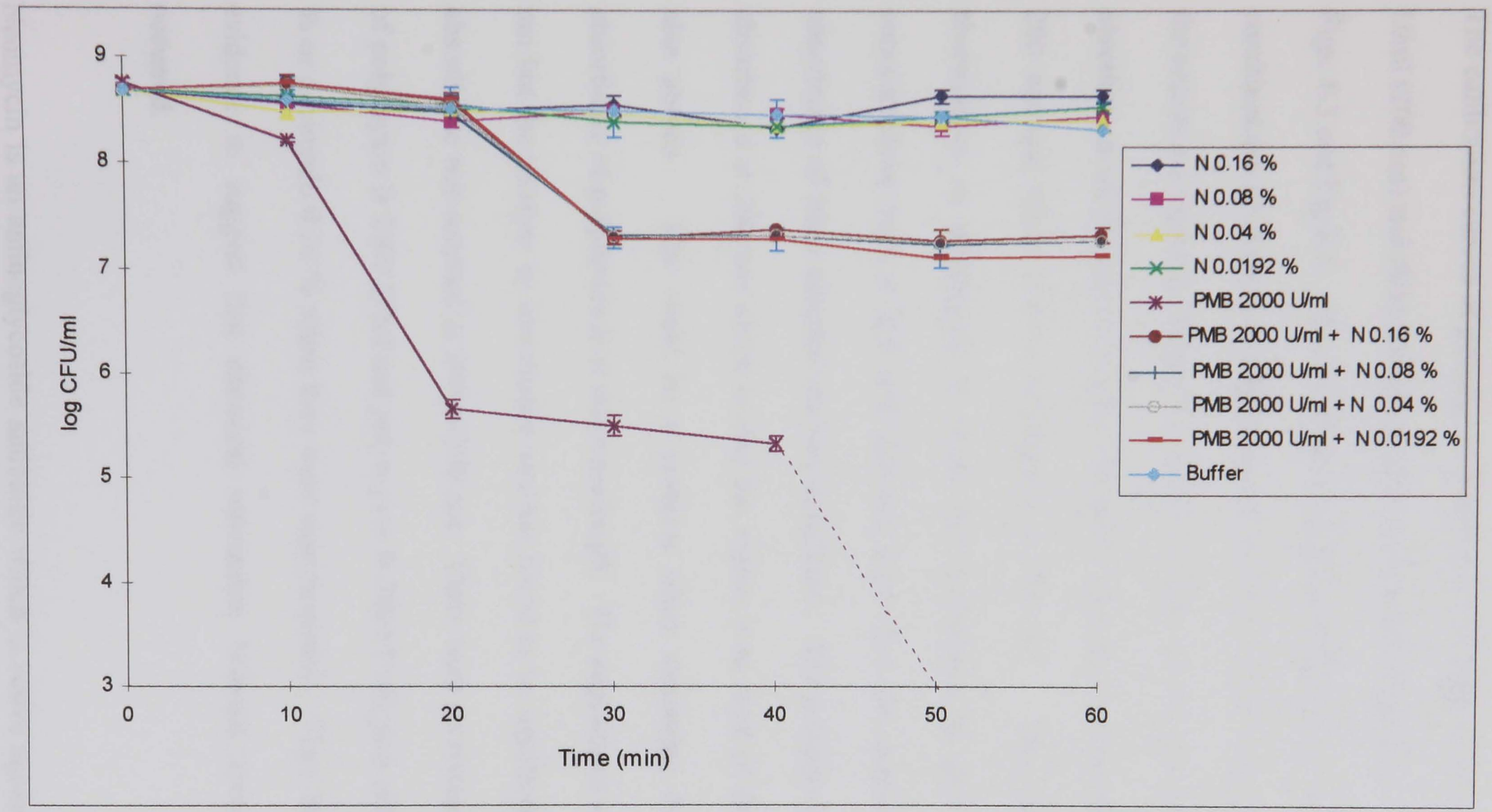


Fig. 8.2 Reduction in the CFU/ml with time for cell suspensions of *P. aeruginosa NCTC 6750* produced by polymyxin B (PMB), neomycin (N) and the combinations of these two substances.

8.3.2.2 Chemical interaction between polymyxin B and neomycin

The calibration curves of polymyxin B at the concentrations ranging from 10 to 250 U/ml (200 nm) and 50 to 250 U/ml (220 nm) gave straight lines. These are shown in Figs. 8.3 and Fig 8.4. The absorbance readings of neomycin, polymyxin B and the combinations of these two substances at 200 nm and 220 nm were carried out and the results are shown in Tables 8.7 and 8.8. There was about a 15 % decrease in the absorbances of polymyxin B plus neomycin compared with polymyxin B alone at 200 nm and about 1 to 5 % increase at 220 nm. The Student t-test of the absorbances of polymyxin B alone and polymyxin B plus neomycin at all concentrations both at 200 and 220 nm show that the decrease/increase in the absorbance of these samples was not significant. The polymyxin B has maximum absorbance at 200 nm which is also the region were most of the other substances also absorb. This could be a problem when measuring the changes in the absorbance of polymyxin B at this wavelength. The experiment was repeated at 220 nm but the increase in absorbance was not found to be significant. Therefore the absorbance was scanned at 200 to 300 nm. There were no changes in the spectrum of polymyxin B 2000 U/ml and polymyxin B 2000 U/ml plus either neomycin 0.08 % or neomycin 0.16 % when they were superimposed. Thus, there was no strong evidence to suggest that chemical interaction between these two substances occurred.

Neomycin is an aminoglycoside antibiotic which is active against many strains of Gram-negative bacteria but not against *P. aeruginosa* [1,9,76]. It can act as an

inhibitor of bacterial protein synthesis but also produce effects on the bacterial cell membrane. Neomycin , being cationic under physiological conditions, is accepted by anionic sites on the cell surface. In Gram-negative bacteria, anionic binding sites include the polar heads of phospholipids and lipopolysaccharide [1,16]. These are the same binding sites as for polymyxin B which also behaves as a cation [77]. Neomycin may compete with polymyxin B and therefore reduce polymyxin B activity on *P. aeruginosa*. This may be the explanation for the slight decrease in acitivity observed in Fig. 8.2.

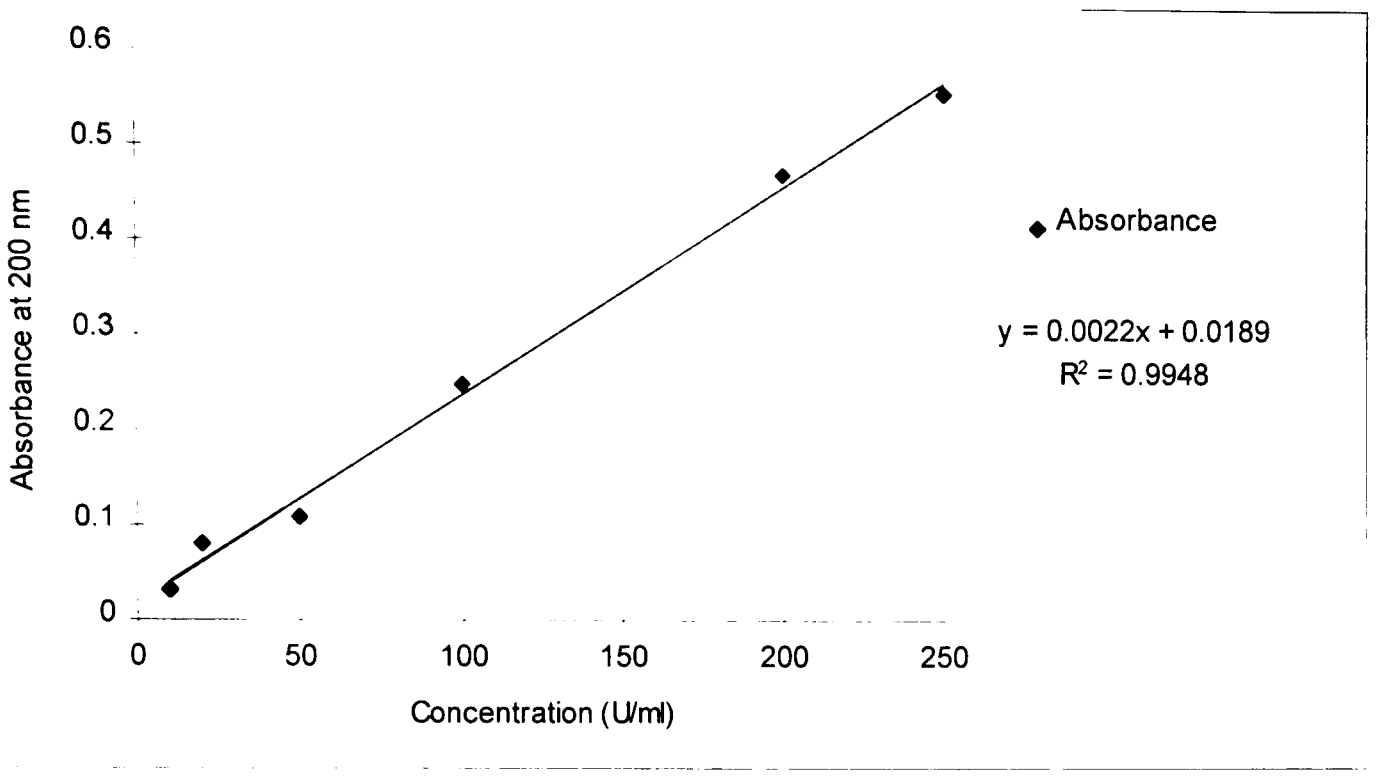


Fig. 8.3 Calibration curve of polymyxin B (10 - 250 U/ml) for absorbance readings at 200 nm.

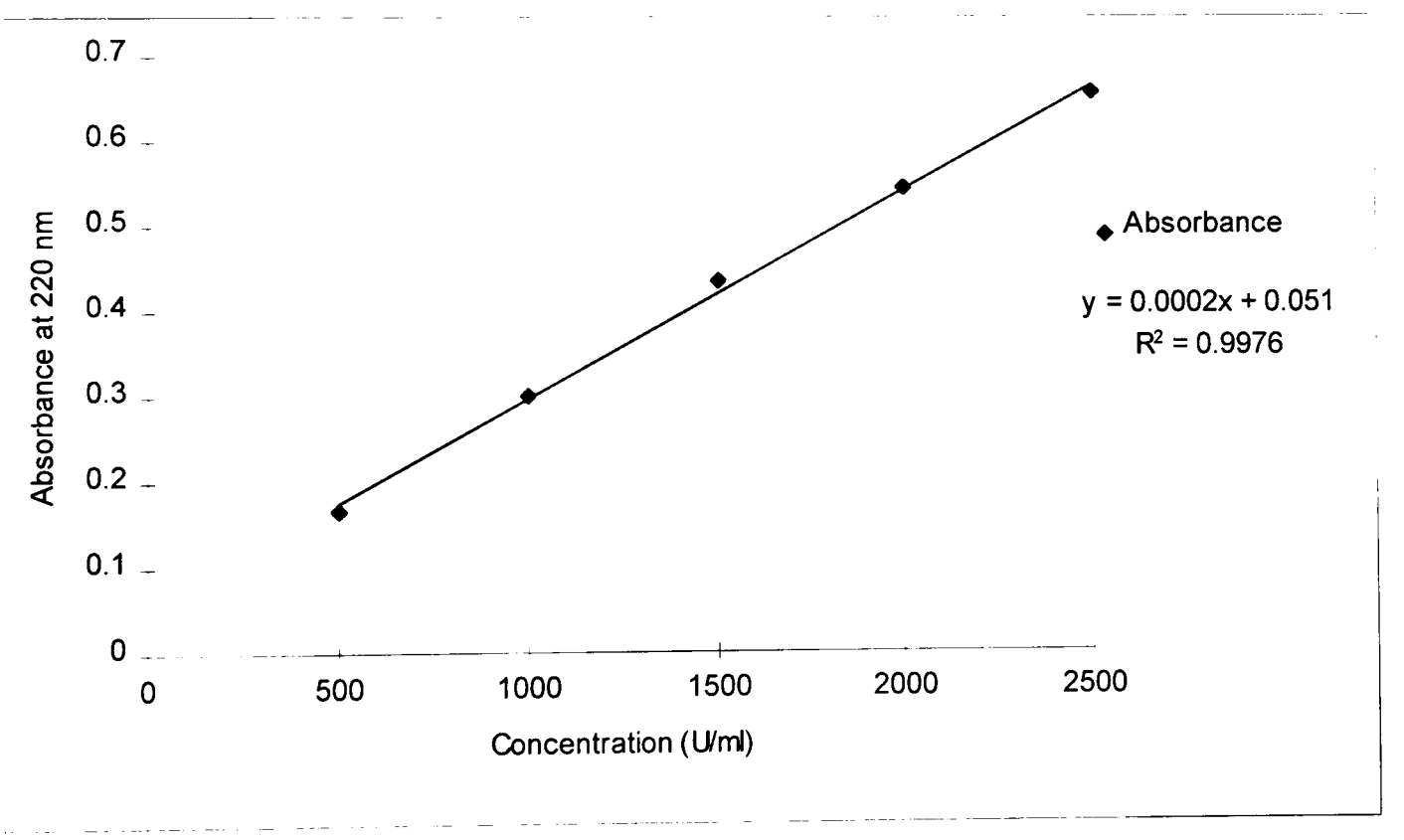


Fig. 8.4 Calibration curve of polymyxin B (500 - 2500 U/ml) for absorbance readings at 220 nm.

Table 8.7 Absorbance readings of polymyxin B (PMB), neomycin (N) and the combination of these two substances at 200 nm

Sample	Absorbance at 200 nm Mean ; n=3 (SD)	% decrease compared with PMB 200 U/ml	t-test of absorbance compared with PMB 200 U/ml (p= 0.05)
1. N 0.016 %w/v	0.0677 (0.0025)		
2. N 0.008 %w/v	0.0677 (0.0025)		
3. N 0.00384 %w/v	0.0667 (0.0029)		
4. N 0.00192 %w/v	0.0650 (0.0050)		
5. PMB 200 U/ml	0.5417 (0.0029)		
6. PMB 200 U/ml + N 0.016 %w/v	0.4577 (0.0025)	15.5082	-
7. PMB 200 U/ml + N 0.008 %w/v	0.4760 (0.0069)	12.1236	-
8. PMB 200 U/ml + N 0.00384 %w/v	0.4583 (0.0029)	15.3852	-
9. PMB 200 U/ml + N 0.00192 %w/v	0.4683 (0.0029)	13.5391	-

\+ = significant difference

\- = no-significant difference

Table 8.8 Absorbance readings of polymyxin B (PMB), neomycin (N) and the combination of these two substances at 220 nm

Sample	Absorbance at 220 nm Mean ; n=3 (SD)	% increase compared with PMB 200 U/ml	t-test of absorbance compared with PMB 2000 U/ml (p= 0.05)
1. N 0.16 %	0.0550 (0.0021)		
2. N 0.08 %	0.0290 (0.0014)		
3. N 0.0384 %	0.0210 (0.0014)		
4. N 0.0192 %	0.0175 (0.0035)		
5. PMB 2000 U/ml	0.5325 (0.0035)		
6. PMB 2000 U/ml + N 0.16 %	0.5600 (0.0013)	5.1643	-
7. PMB 2000 U/ml + N 0.08 %	0.5575 (0.0106)	4.6948	-
8. PMB 2000 U/ml + N 0.0384 %	0.5500 (0.0025)	3.2864	-
9. PMB 2000 U/ml + N 0.0192 %	0.5425 (0.0035)	1.8779	-

+ = significant difference

- = no-significant difference

8.3.3 Effect of gentamicin

Fig. 8.5 and 8.6 show killing curves of gentamicin, polymyxin B and the combinations of both substances. The data was analysed by using ANOVA (p=0.05) of all contact times and log CFUs/ml. The results indicated that gentamicin at all concentrations except 0.018 and 0.009 %w/v showed more activity against *P. aeruginosa* than that of polymyxin B 2000 U/ml. The highest concentration of G (0.15 %w/v) reduced the number of bacteria from ~ 10^8 CFU/ml to less than 10^3 within 20 min. The other concentrations killed the test organism from ~ 10^8 CFU/ml to ~ 10^6 CFU/ml after contact time of 50 min. The combination of polymyxin B 2000 U/ml plus gentamicin 0.15, 0.075 or 0.036%w/v were more active against *P. aeruginosa* than polymyxin B 2000 U/ml plus gentamicin 0.018 or 0.009 %w/v, gentamicin 0.018 and gentamicin 0.009 %w/v which were not significantly different from PMB 2000 U/ml alone. Gentamicin itself is more active than other aminoglycosides against *P. aeruginosa* and its activity against *P. aeruginosa* is ten-times more active than neomycin [1]. Unlike neomycin which did not show a significant effect against *P. aeruginosa* , gentamicin caused a greater effect on cell viability at concentrations of 0.15, 0.075, 0.036 %w/v than polymyxin B 2000 U/ml. Gentamicin 0.15 %w/v plus polymyxin B 2000 U/ml had a similar effect to gentamicin 0.15 %w/v alone. However, gentamicin at concentrations of 0.075 and 0.036 %w/v combined with 2000 U/ml also added to the activity of the individual antibiotics against *P. aeruginosa*. Gentamicin , like neomycin, can bind to multiple sites on the cell surface which are also the binding sites for polymyxin B [77]. Gentamicin itself has antimicrobial activity. Even if it displaced some

polymyxin B the overall effect was either indifferent or additive. Neomycin has no little or no activity against *P. aeruginosa* and therefore the overall effect of combinations was inhibition.

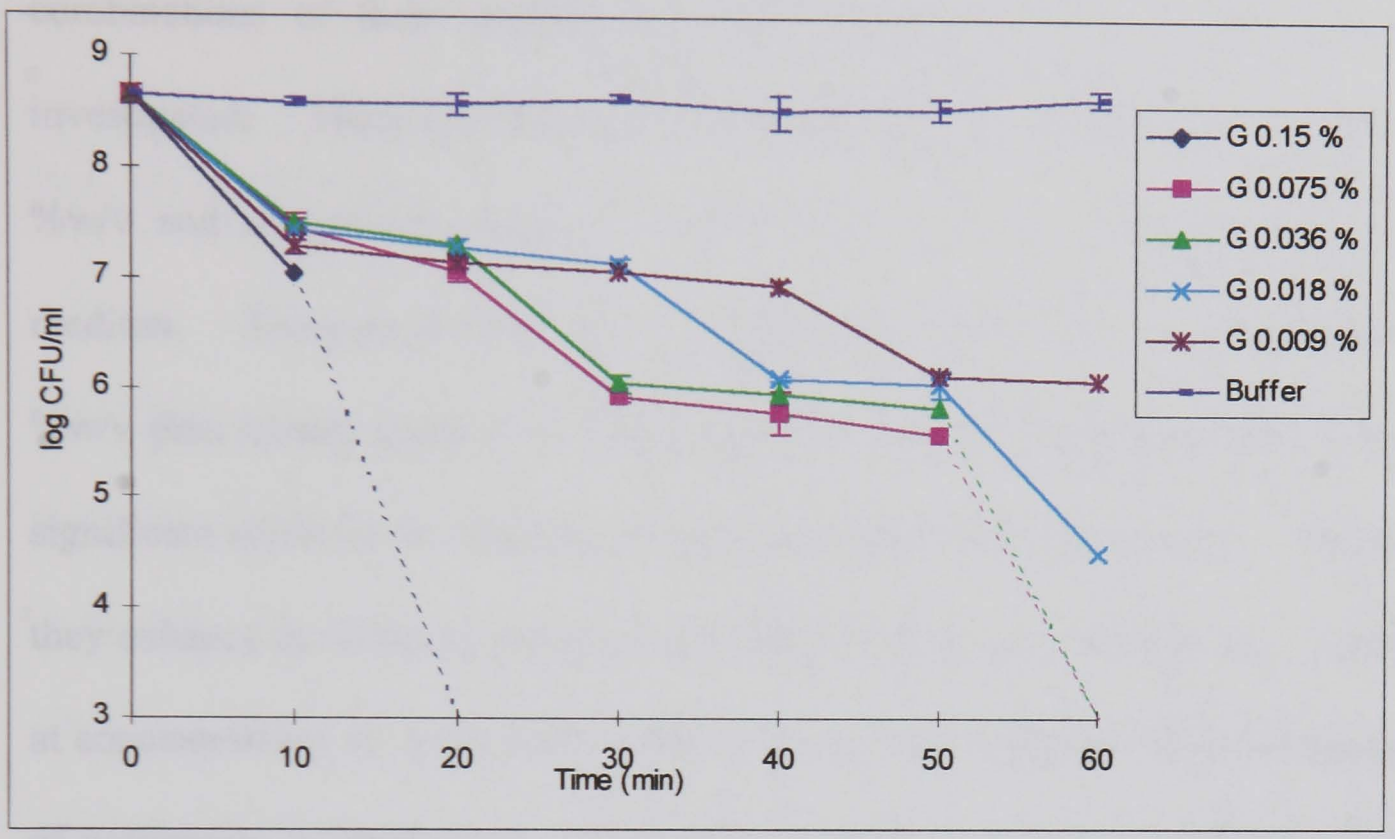


Fig. 8.5 Reduction in the CFU/ml with time for cell suspensions of *P. aeruginosa NCTC 6750* produced by gentamicin (G).

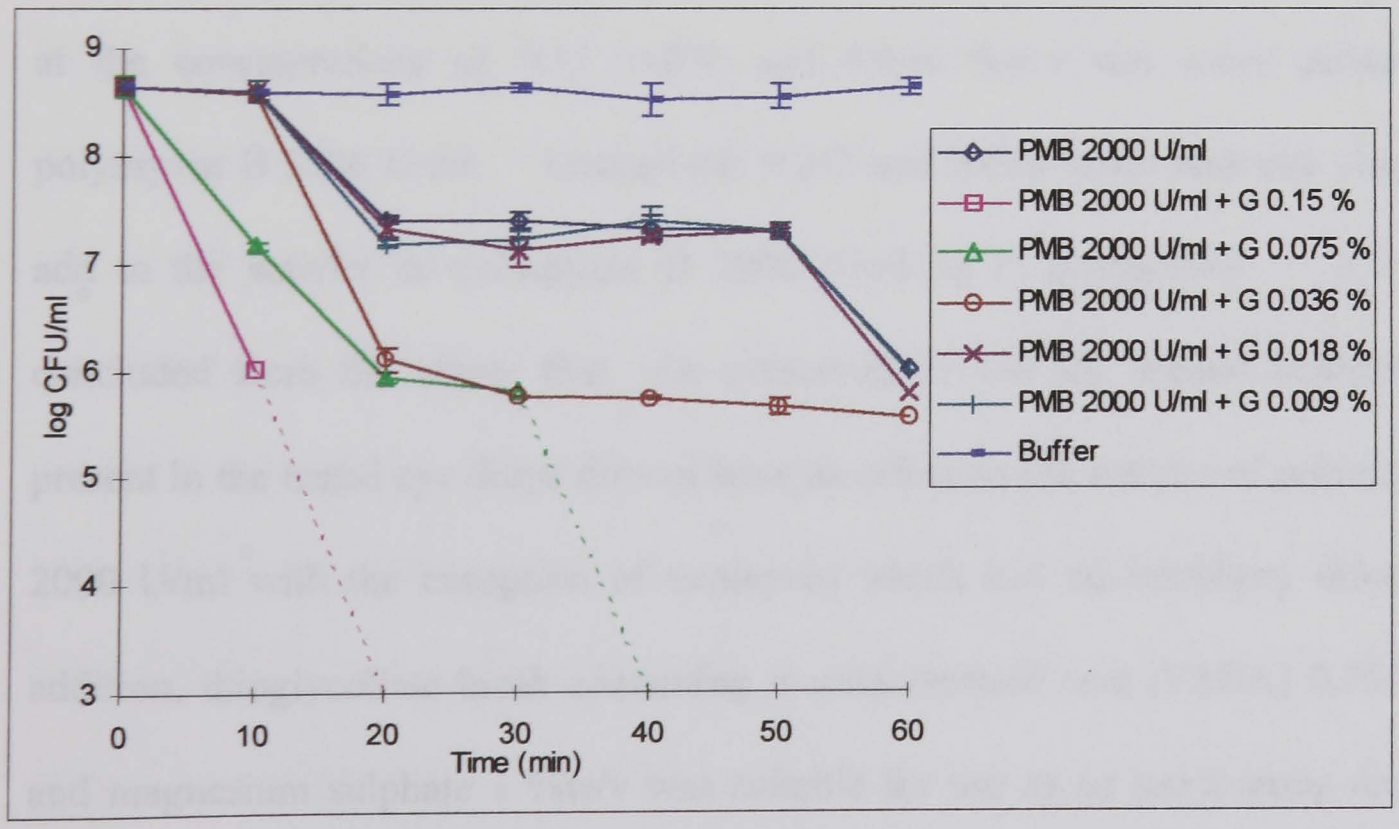


Fig. 8.6 Reduction in the CFU/ml with time for cell suspensions of *P. aeruginosa NCTC 6750* produced by polymyxin B (PMB) and polymyxin B plus gentamicin (PMB +G).

8.4 Conclusion

The effects of thiomersal, trimethoprim, neomycin and gentamicin singly and the combinations of these substances with polymyxin B on *P. aeruginosa* were investigated. Thioglycollate broth containing *p*-aminobenzoic acid (PABA) 0.16 %w/v and magnesium sulphate 1 %w/v was used as an inactivating recovery medium. Thiomersal 0.001 %w/v, trimethoprim 0.02 %w/v and thiomersal 0.001 %w/v plus trimethoprim 0.02 %w/v when mixed in this medium did not show a significant effect on the viability of cell suspensions of *P. aeruginosa.* Neither did they enhance the effect of polymyxin B 2000 U/ml on the test organism. Neomycin at concentrations of 0.16, 0.08, 0.04 or 0.0192 %w/v slightly inhibited the activity of polymyxin B 2000 U/ml. The results with gentamicin were different from the results with the other antibacterials. Although neomycin and gentamicin have the same mode of action, only gentamicin is active against *P. aeruginosa.* Gentamicin at the concentrations of 0.15, 0.075 and 0.036 %w/v was more active than polymyxin B 2000 U/ml. Gentamicin 0.075 and 0.036 %w/v was also shown to add to the activity of polymyxin B 2000 U/ml on *P. aeruginosa.* It can be concluded from the above that the preservatives and the second antibacterials present in the tested eye drops did not have an effect on the activity of polymyxin B 2000 U/ml with the exception of neomycin which had an inhibitory effect. In addition, thioglycollate broth containing *p*-aminobenzoic acid (PABA) 0.16 %w/v and magnesium sulphate 1 %w/v was suitable for use as an inactivating recovery medium for determining killing times for eye drops containing the tested concentrations of thiomersal, trimethoprim and neomycin.

Chapter 9

Effect of heat on polymyxin B stability and activity

9.1 Introduction

This chapter was to determine the correlation between chemical stability and microbiological activity of polymyxin B standard material in aqueous solution and proprietary eye drops. As discussed in chapter 6 the microbiological assay was not considered suitable for determining the differences in microbiological activity of polymyxin B samples in the stability study. Therefore killing time determinations for polymyxin B in the presence of other antibacterials and preservatives contained in the formulations were developed as described in chapter 7 and 8. Although most of the ingredients in the proprietary eye drops did not affect polymyxin B activity against *P. aeruginosa NCTC 6750*, neomycin which was present in sample 2, 3, 4, 5 and 6 slightly reduced the effect of polymyxin B. The method developed for determining the killing times of the test samples were used also for samples containing neomycin. The inactivating recovery medium used was thioglycollate broth containing *p*-aminobenzoic acid (PABA) 0.16 %w/v and magnesium sulphate 1 %w/v which was expected to inhibit any activity of thiomersal and trimethoprim against the test organism. The HPLC assay developed in chapter 2 was used in the investigations described in this chapter to determine the amount of polymyxin B left after heat treatment.

The major peak (PB_1) was used to represent the amount of effective polymyxin B in the samples. Test samples were six eye drops (sample 1-6) and polymyxin B in phosphate buffer pH 6.0 which were similar to those used in the stability study in chapter 3. They were stored at 43, 50, 55 and 60 °C over a period of 500 h. Since all the samples stored at 60°C were used in the microbiological assay in chapter 6, the samples stored at 55°C were used for the killing time determinations described in this chapter. It was expected that the results would represent the microbiological activity of the undecomposed polymyxin B remaining in the eye drop formulations and the buffer. In addition, polymyxin B standard material was prepared in phosphate buffer pH 6.0 and heated at a series of higher temperatures (92-94 and 115 °C). Killing time determinations and HPLC assays for these samples were also carried out.

9.2 Methods

9.2.1 Killing time determinations

P. aeruginosa NCTC 6750 was used as the test organism. Inoculum preparation for all experiments in this chapter were as described in 4.2.2.1

The test solutions (150 µl) were mixed with 150 µl of cell suspension in the first row of wells in the microtiter plates. The wells in rows 1 to 6 of the microtiter plates were primed with 200 µl of thioglycollate broth containing PABA 0.16 %w/v

and magnesium sulphate 1 %w/v. Phosphate buffer pH 6.0 was used as the control. Killing time determinations were carried out as described in 4.2.2.2.

The term " activity " used in the following sections refers to microbiological activity of polymyxin B against *P. aeruginosa NCTC 6750* .

The preparations of the test samples were as follows:

9.2.1.1 Polymyxin B in phosphate buffer pH 6.0 heated in a water bath (92-94 ^{o}C)

Standard solutions of polymyxin B 4000 U/ml were prepared by dissolving 0.0521 g of polymyxin B sulphate (7690 U/mg) in 100 ml of phosphate buffer pH 6.0. Ten milliliters of this solution was filled into each of 6 screwcap bottles, respectively. The inner rubber lining of the metal caps were further lined with aluminum foil in order to prevent the adsorption and or absorption of the polymyxin B solution onto and into the rubber.

The water bath was set at the highest temperature and the water was covered with polypropylene spheres in order to help maintain the temperature. The sample bottles and a bottle of 10 ml buffer were placed in the water bath when the temperature of water was at the required temperature and constant. A thermometer was placed in a bottle of buffer and the time was noted when the temperature had reached the required temperature and remained constant (92-94 ^{o}C). At time intervals of 30, 60, 90, 120 and 150 min, one bottle of sample was removed from the

water bath and cooled down to room temperature. The pH of each sample was determined before and after heating.

9.2.1.2 Polymyxin B in phosphate buffer pH 6.0 heated in an autoclave (115 °C)

A standard solution of polymyxin B was prepared and filled into screwcap bottles as described in 9.2.1.1.

The bench autoclave was switched on and the temperature allowed to rise to 100 °C (~ 30 min). It was then switched off and the samples were placed in the chamber. The autoclave was switched on again to allow the temperature and pressure to reach 115 °C and 10 lb/inch^2 (about 5 min). The time was set for 30 min* after the above conditions had been achieved and the autoclave was switched off to cool down for 30 min. Samples were then removed and cooled down to room temperature. This procedure was repeated for 60, 90 and 120 min*. Each time a control bottle of 10 ml phosphate buffer pH 6.0 was autoclaved with the test samples. The pH of each samples was measured before and after autoclaving.

9.2.1.3 Polymyxin B in phosphate buffer pH 6.0 and polymyxin B eye drops heated at 55 °C

Six samples of polymyxin B eye drops (sample 1-6) and standard polymyxin B (16250 U/ml) prepared in phosphate buffer pH 6.0 were used as test samples. They were heated in the oven at 55 °C over a period of 500 h. Chemical stability studies of these samples were carried out as described in chapter 3. They were kept in the

freezer (-20°C) and thawed 30 min before using for killing time determinations. Dilutions of test samples were made in phosphate buffer pH 6.0 to give a concentration of 4000 U/ml of polymyxin B. This was further diluted to 2000 U/ml in the first row of wells of the microtiter plates. The pH of test samples before and after heating were also measured.

9.2.2 HPLC assay of heated polymyxin B

Chemicals and reagents, apparatus and chromatographic conditions were as described in chapter 2.

Freshly prepared standard polymyxin B was prepared by dissolving 0.0133 g of polymyxin B sulphate (7760 U/mg) in 25 ml phosphate buffer pH 6.0 to get the concentration of 4000 U/ml. All samples from 9.2.1 were kept in the freezer (-20°C) and thawed 30 min before injecting undiluted and in duplicate into the HPLC system. The percentage of polymyxin B remaining in the test samples was calculated by comparing the peak height of peak PB_1 of the test samples to the peak height of freshly prepared standard polymyxin B.

The term 'percentage remaining' used in the following sections refers to the percentage remaining of peak PB_1.

9.3 Results and discussion

9.3.1 Polymyxin B sulutions in phosphate buffer pH 6.0 heated either in a waterbath (92-94 °C) or autoclave (115°C)

Fig. 9.1 and 9.3 show killing curves and chromatograms of polymyxin B heated at 92-94 °C in a waterbath. The results indicate that heating polymyxin B at 92-94 °C reduced the activity of polymyxin B against *P. aeruginosa* as a function of time. Microbiological activity data is similar to chemical stability data which showed that the percentage remaining decreased as the heating time increased. ANOVA (p=0.05) of all contact times and log CFUs/ml indicated that only polymyxin B heated for a period of 150 min showed no significant effect on the test organism. The percentage of polymyxin B remaining as determined by the HPLC assay was less than 10 percent. Polymyxin B heated for shorter periods (30, 60, 90, and 120 min) still possessed marked activity. The percentage remaining ranged from 43.60 to 20.12 percent polymyxin. After heating for 120 min at 92-94 °C , about 20 percent of the original polymyxin B reduced the number of *P. aeruginosa* from 10^{11} CFU/ml to 10^{7} CFU/ml within 60 min. It could be concluded that polymyxin B in phosphate buffer pH 6.0 retained sufficient microbiological activity if the percentage remaining was more than 20. This represent a concentration of 400 U/ml which in initial experiments showed comparable activity.

Chromatograms of autoclaved polymyxin B in phosphate buffer pH 6.0 are shown in Fig. 9.4. Killing curves of all autoclaved samples were not significantly different

from the control (ANOVA, p=0.05). This indicated that autoclaving at 115°C for 30 min and longer destroyed all antibacterial activity. The amount of polymyxin B, as determined by HPLC, was also extensively decreased by the same heat treatment. Correlation between microbiological activity data and chemical stability data is consistent with the similarity of the results obtained at 92-94 °C.

The pH of all polymyxin B samples heated in the waterbath and autoclave remained close to 6.0 after heat treatment as shown in Table 9.2. Therefore pH was not a factor in the polymyxin B activity and stability in this study.

From the chromatograms in Figs. 9.3 and 9.4, the obvious peaks of decomposition products appeared near the solvent front. These new compounds did not seem to have activities against *P. aeruginosa* since the activity of polymyxin B after heat treatment appeared to depend on the amount of the remaining PB_1

Table 9.1 Percentages remaining of the main component (PB_1) of polymyxin B (4000 U/ml) prepared in phosphate buffer pH 6.0 and exposed to a series of elevated temperatures for fixed times.

The results were obtained from the comparison of peak height of untreated polymyxin B at the same concentration as the test samples.

Heat treatment sample	Duration (min)	% remaining of PB_1
Water bath (92-94°C)	30	43.60
	60	34.76
	90	24.70
	120	20.12
	150	<10
Autoclave (115°C)	30	Extensively decomposed.
	60	All main peaks disappeared.
	90	
	120	

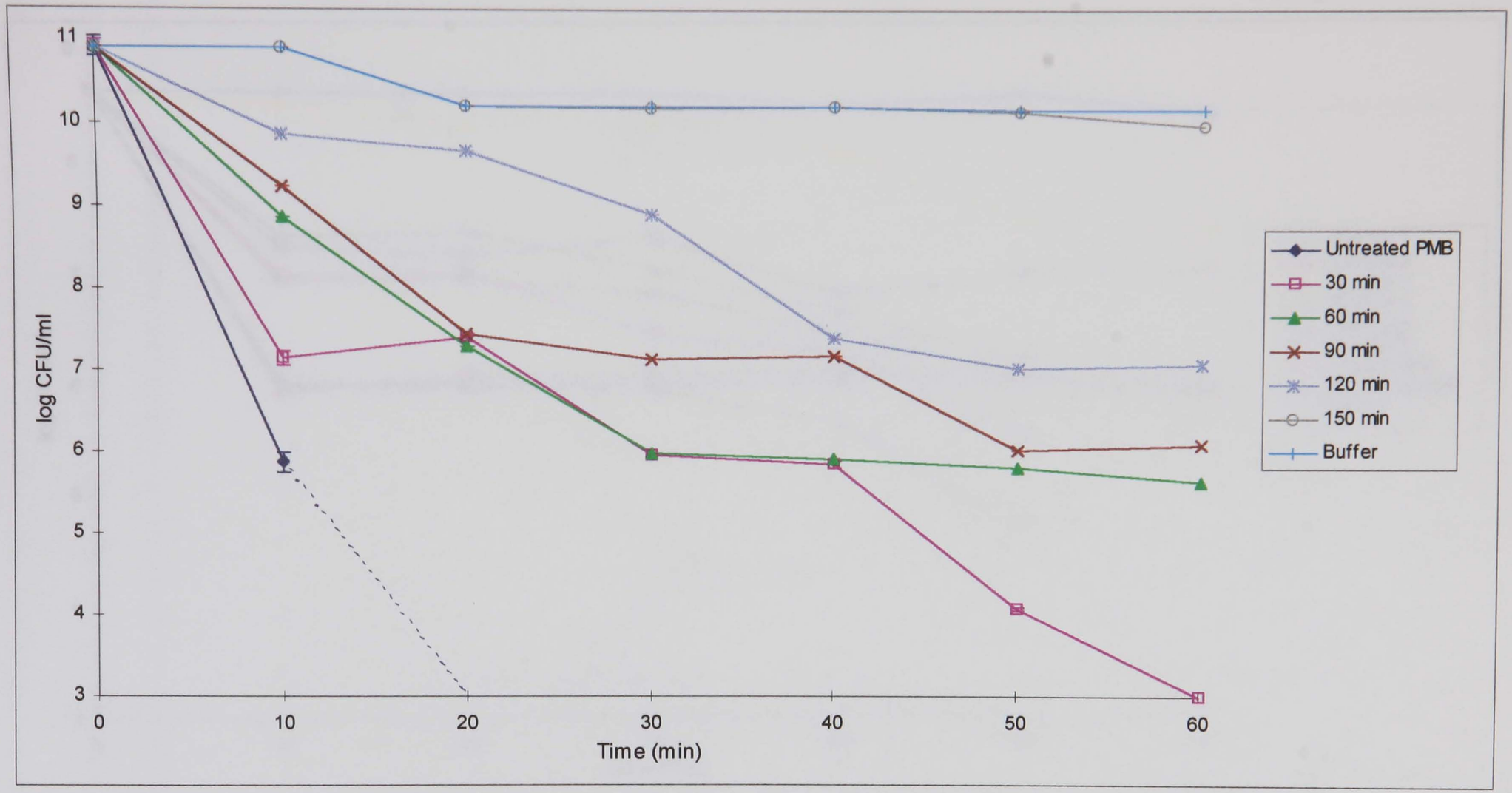


Fig. 9.1 Reduction in the CFU/ml with time for cell suspensions of *P. aeruginosa NCTC 6750* produced by polymyxin B (PMB) 2000 U/ml untreated and heated in a waterbath at 92-94°C.

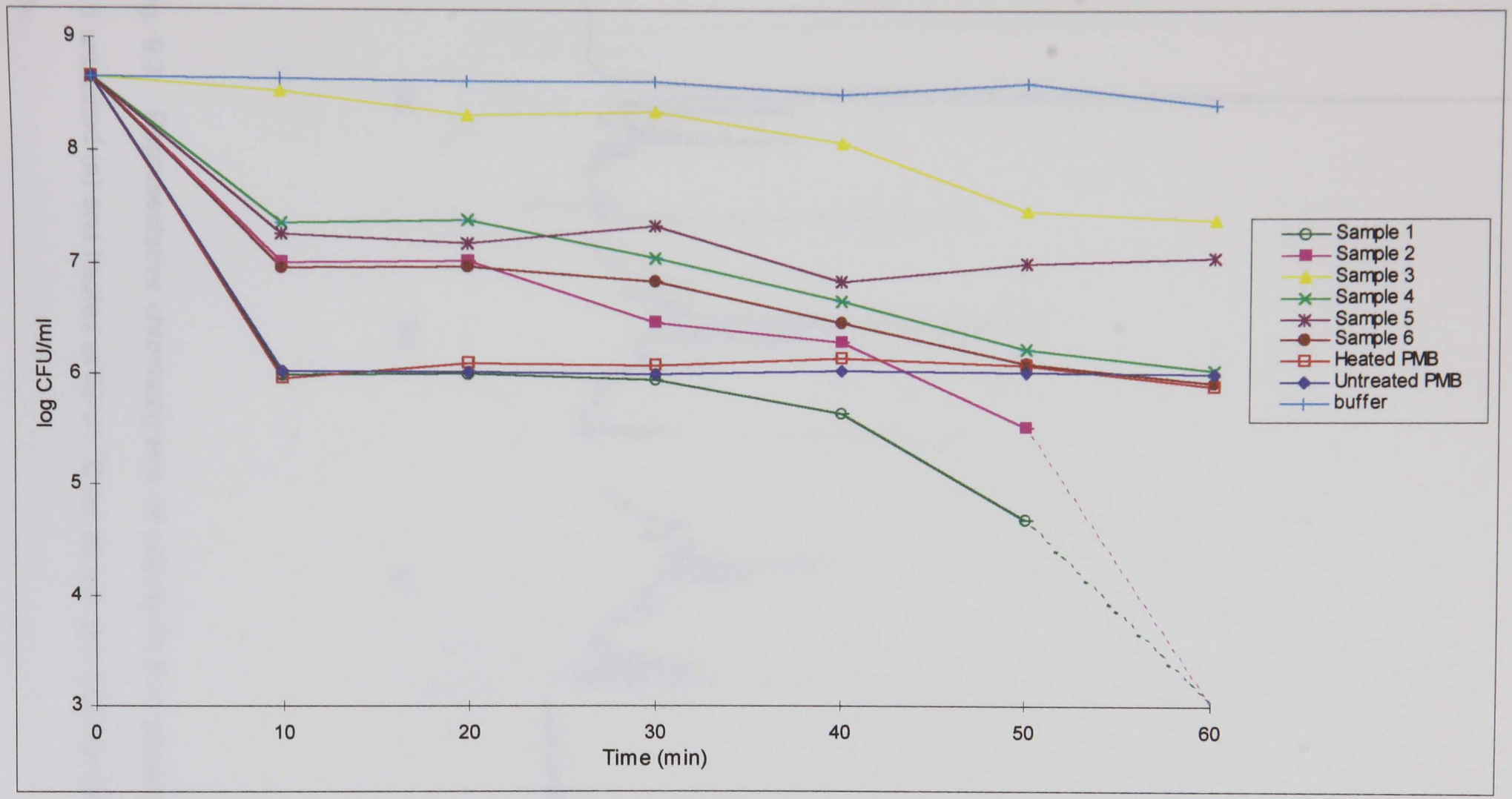
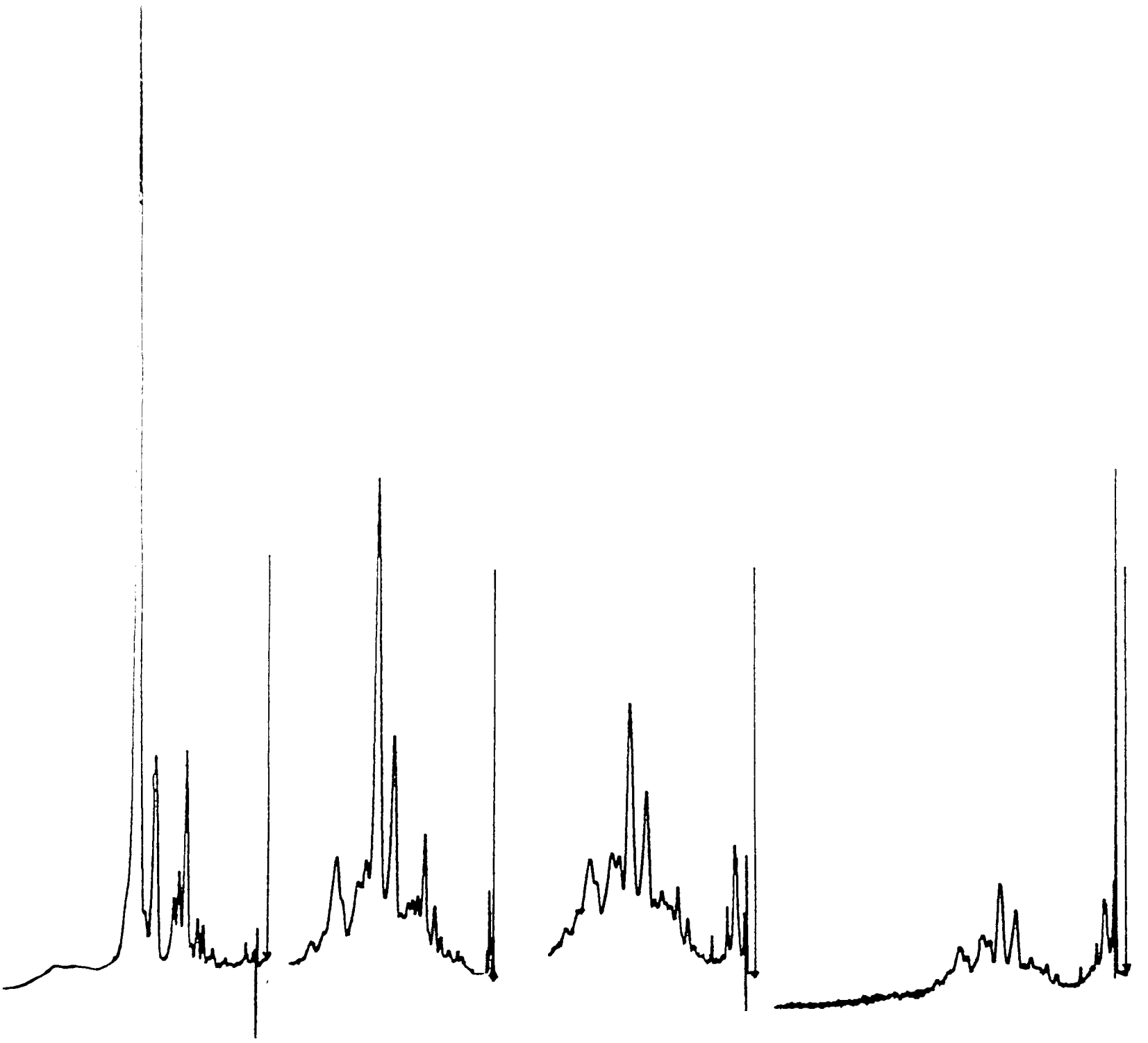
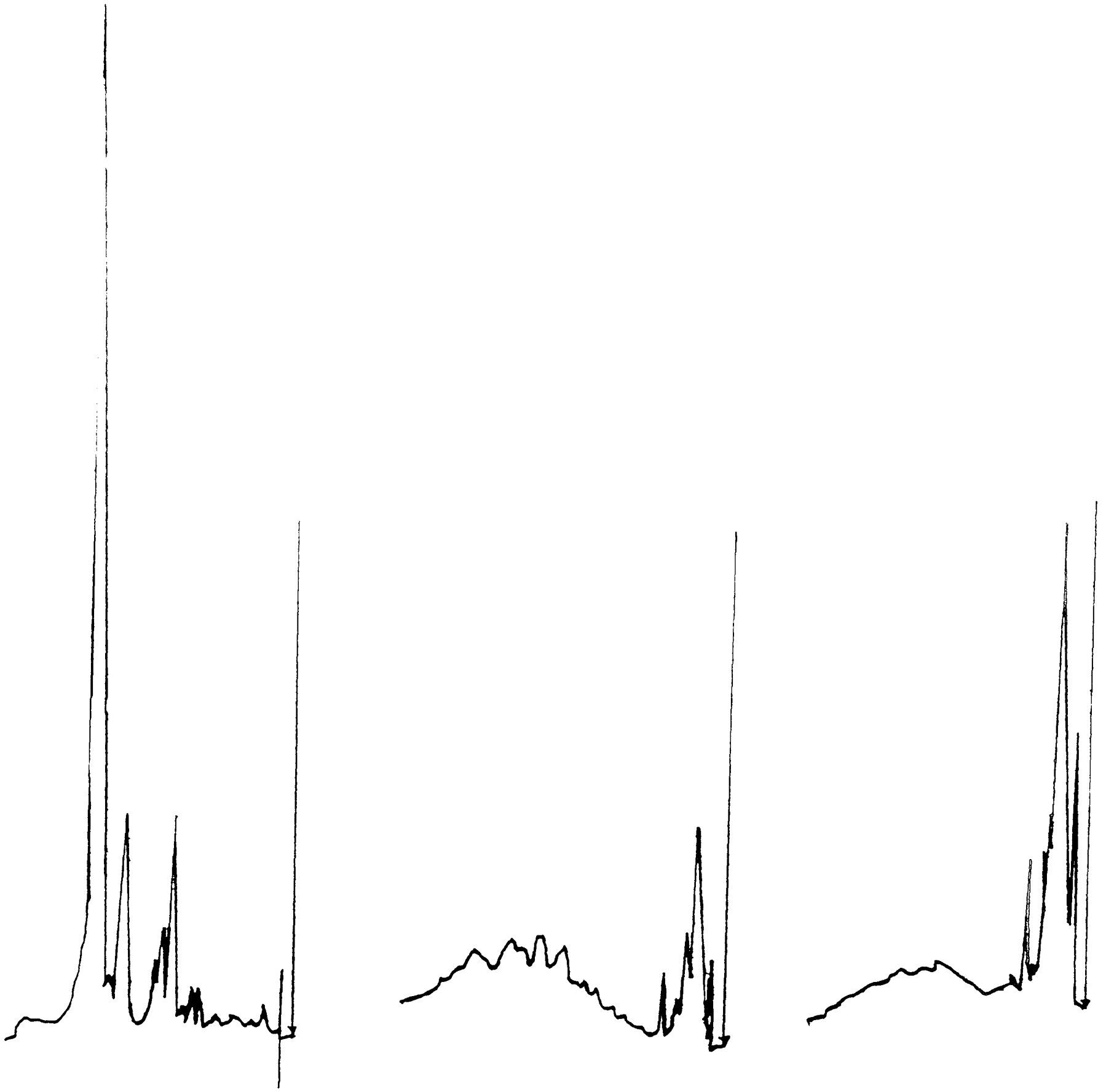


Fig. 9.2 Reduction in the CFU/ml for cell suspensions of *P. aeruginosa NCTC 6750* produced by sample 1-6 and polymyxin B stored at 55°C over a period of 500 h. All samples were diluted to get 2000 U/ml of polymyxin B.



(a) (b) (c) (d)

Fig. 9.3 Representative chromatograms of polymyxin B in phosphate buffer pH 6.0 untreated (a) and heated at 92-94 °C for 30 min (b), 120 min (c) and 150 min (d).



(a) (b) (c)

Fig. 9.4 Representative chromatograms of polymyxin B in phosphate buffer pH 6.0 untreated (a) and heated at 115 °C for 30 min (b) and 120 min (c).

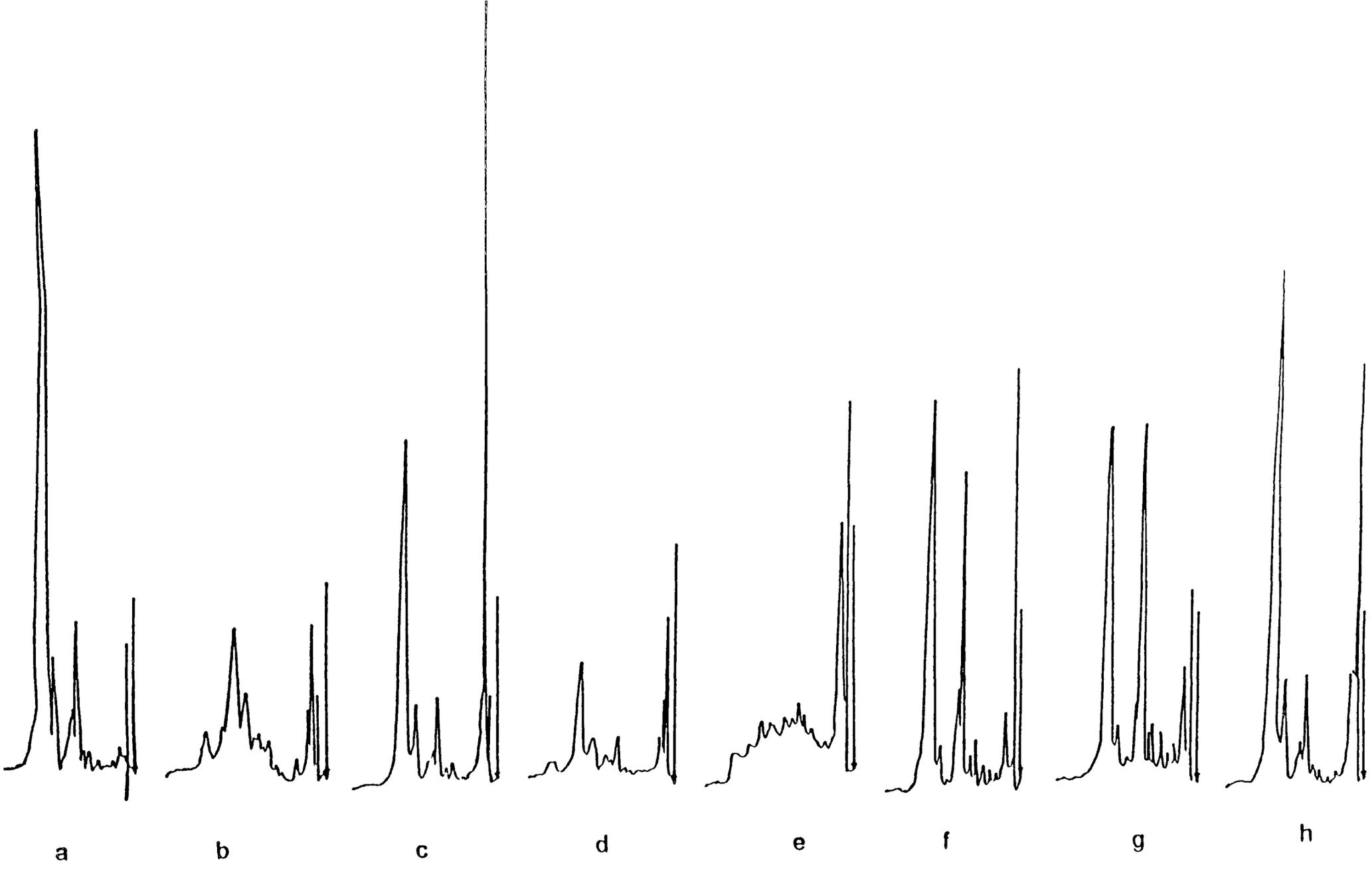


Fig. 9.5 Representative chromatograms of samples untreated and heated at 55°C over a period of 331.5 h.
(a) polymyxin B in phosphate buffer pH 6.0 untreated (b) polymyxin B in phosphate buffer pH 6.0
(c) sample 1 (d) sample 2 (e) sample 3 (f) sample 4 (g) sample 5 (h) sample 6 .

Table 9.2 The pH of polymyxin B 4000 U/ml in phosphate buffer pH 6.0 after exposure to elevated temperatures for fixed times

Temperature (°C)	Duration (min)	pH
92-94 °C	30	6.03
	60	6.03
	90	6.05
	120	6.05
	150	6.10
115 °C	30	6.05
	60	6.05
	90	6.08
	120	6.09

Table 9.3 The pH of polymyxin B eye drops and polymyxin B 16,250 U/ml alone in phosphate buffer pH 6.0 before and after storage at 55°C over a period of 500 h

Sample	pH	
	Before	After
1	5.67	6.18
2	6.10	6.27
3	6.92	7.24
4	5.45	5.89
5	5.41	5.85
6	5.48	5.69
PMB	6.00	6.25

9.3.2 The stability of polymyxin B in phosphate buffer pH 6.0 and polymyxin B eye drops stored at 55°C

The activity of polymyxin B as determined by the reduction in CFU/ml produced by storing polymyxin B eye drops and polymyxin B in phosphate buffer pH 6.0 over a period of 500 h as compared with the activity of freshly prepared polymyxin B is shown in Fig. 9.2. The data was analyzed using ANOVA (p=0.05) of all the contact times and log CFUs/ml.

From the killing curves in Fig. 9.2, four groups of samples can be classified. Sample 1 and sample 2 exhibited the most activity against *P. aeruginosa*. They showed a similar initial killing pattern to freshly prepared polymyxin B and also the heated polymyxin B but after 50 min contact they appaeared to be more active than the other solutions. Between 50 min and 60 min contact sample 1 and 2 had reduced the initial inoculum of almost 10^9 CFU/ml to less than 10^3 CFU/ml. Thus microbiological activity of sample 1 correlates well to the chemical stability data in chapter 3. However, sample 2 did not appeared to be as stable chemically but still exhibited high activity against the test organism. It is not clear why sample 2 was so active against the test organism unless some of the activity originated from degradation products.

The second catagory of solutions included freshly prepared polymyxin B and heated polymyxin B. Both had an initial rapid killing effect at 10 min which was similar to sample 1. Unlike sample 1 however these two samples of polymyxin solutions did

not exhibit further marked activity after 10 min. Killing curves obtained using both samples were not significantly different (ANOVA, p=0.05). This is a perplexing result because it indicated that heating polymyxin B at 55 °C for 500 h did not significantly affect the activity of polymyxin B in phosphate buffer at pH 6.0. The same heat treatment greatly reduced the concentration of polymyxin B as determined by HPLC (chapter 3). In fact simple polymyxin solution showed the highest degree of decomposition. For both polymyxin B prepared in phosphate buffer pH 6.0 without any other ingredients and also for sample 2, the activities may result from the decomposition products which obviously appeared from 331.5 h as shown in Fig. 9.5.

The third group included samples 4, 5 and 6 which exhibited medium level activity against *P. aeruginosa* over the period of 60 min. The microbiological data correlated to chemical stability data which showed that these three samples were very stable in the conditions used in the study. The decomposition rate constants were much lower than sample 2, 3 and polymyxin B alone. Sample 4, 5 and 6 were all produced by the same company and contained the same original concentration of polymyxin B. They were expected to have similar chemical stability and microbiological activity.

Sample 3 was the least active sample. Similar results were obtained in the chemical stability investigation. This chemical data is presented in chapter 3. It was shown to have a high rate of decomposition.

It was assumed in this investigation that the concentration of polymyxin B in each sample was equal to the stated label concentration. On this basis the concentration of all the diluted samples used for killing time determinations would be expected to be 2000 U/ml. Up to 30 percent overage of polymyxin is allowed by the various compendia. Thus it is highly likely that all the eye drops contain higher concentrations of polymyxin B than the concentration stated on the label. Although some polymyxin B decomposed during the stability study, the amount of effective polymyxin B was high enough to show similar activity to that of freshly prepared polymyxin B. It is difficult to compare polymyxin B activity among these samples if the original concentrations of polymyxin B in the test samples were higher than the stated label concentrations. Another factor is the different ratios of polymyxin B that could be present. The killing curves indicate the degree of lost activity once it has dropped lower than 100 percent of the labelled concentration.

It can be seen from Fig. 9.2 that sample 3 showed the greatest loss of antibacterial activity. Samples 1, 2, 3, 4, 5 and 6 all retained marked activity after the same heat treatment. It is also obvious that pH plays an important role in polymyxin B stability and activity. The original pH of sample 3 was higher than the other samples (6.92). After heating the pH was even more alkaline (7.24). A high degree of decomposition of polymyxin B in sample 3 was shown in the data presented in chapter 3. This is consistent with earlier work [9,10,54,55] that polymyxin B in solution above pH 7 is not stable. In the case of sample 3 the pH could be expected to have an adverse effect on polymyxin B activity.

The sample of solution of polymyxin B in phosphate buffer pH 6.0 which had been heated at 55°C did not significantly lose activity compared with freshly prepared polymyxin B. The pH of polymyxin B solution changed only slightly after storage. Samples 1, 2, 4, 5, 6 also demonstrated marked activity after similar heat treatments. The pH before and after the stability study was close to 6.0. Perhaps it was not surprising that the chemical composition and microbiological activity of these samples remained high. The exceptions were the findings of the chemical assay for sample 2 and for the sample polymyxin B in phosphate buffer pH 6.0. Both indicated extensive chemical decomposition but both still possessed good antibacterial activity. Although the reason for this is not obvious from this study, the results emphasize the critical effect of pH on polymyxin B stability and microbiological activity in aqueous solution. There is still an unsolved question about the effect of decomposition products.

9.3 Conclusion

Microbiological activity data for polymyxin B in phosphate buffer pH 6.0 correlated to chemical stability data over the temperature range of 92-115 °C. Heating polymyxin B in phosphate buffer pH 6.0 at 92-94 °C in a waterbath and at 115 °C in an autoclave from 30 to 150 min reduced the activity and the amount of the main component (PB_1) of polymyxin B as function of time. The microbiological activity of polymyxin B remained sufficient to reduce an inoculum of 10^{11} CFU/ml

P. aeruginosa NCTC 6750 to 10^7 CFU/ml within 60 min even when the percentage of PB_1 reduced to about 20 percent of the labelled amount.

It is difficult to correlate the microbiological activity data and chemical stability data for polymyxin B in phosphate buffer pH 6.0 with the polymyxin B eye drops (sample 1-6) heated at 55 °C over a period of 500 h. The difficulties are related to the lack of knowledge of the original concentration of polymyxin B in the samples and the microbiological activity of the decomposition products of polymyxin B.

The results obtained at 55 °C indicated the critical effect of pH on the microbiological activity and chemical stability of polymyxin B in aqueous solution. It is suggested that the decomposition products of polymyxin B which occurred at 55 °C in the solutions of pH 6.0 possessed microbiological activity. It is also suggested that at the same pH but higher temperatures (92-115 °C) the decomposition products were further degraded and did not show activity against the test organism. Further investigation on the microbiological activity of decomposition products of polymyxin B which occur at various pH and temperatures would seem to be needed in order to offer a possibility of clarifying this complex situation.

Chapter 10 Overall conclusions

10.1 Correlation between chemical stability and microbiological activity

The present work attempted to correlate chemical stability and microbiological activity of polymyxin B standard material and polymyxin B in proprietary eye drops. An HPLC method and a microbiological method were developed. It was postulated that the chemical data and microbiological data would correlate. However, only the results obtained at high temperatures (92-115 ^{o}C) were in agreement with this hypothesis.

The results presented in this thesis indicate that the decomposition rate constants of polymyxin B in phosphate buffer pH 6.0 correlate over the temperature range from 32 to 60^{o}C. Differences in the chemical stability of the different proprietary eye drops were observed. The microbiological activity was determined by killing time determinations. The results obtained at 55 ^{o}C showed that polymyxin B eye drops buffered at pH close to 6.0 retained a high proportion of the main component (PB_1) and possessed marked microbiological activity after heating for 500 h. These results are consistent to previous findings [9,10,55,56]which concluded that pH 6.0 was important for maintaing polymyxin B stability in aqueous solution. However, one eye drop sample and the polymyxin B standard material both prepared in buffer at a pH close to 6.0 exhibited marked microbiological activity although chemical

analysis indicated a high degree of decomposition. This may result from the antibacterial activity of the decomposition products of polymyxin B which were formed.

The stability/activity results at higher temperatures (92-115 °C) demonstrated a correlation between the chemical stability data and microbiological activity data. Heating polymyxin B in phosphate buffer pH 6.0 at these high temperatures reduced both the activity against *P. aeruginosa NCTC 6750* and the percentage of the main component (PB_1) as a function of time. The polymyxin retained observable antibacterial activity when the percentage PB_1 remaining was greater than 20%. It is suggested that further degradation took place at these temperatures and that these products did not possess antibacterial activity.

The overall results suggest that the decomposition products of polymyxin B produced at 55 °C or lower, in aqueous solution at pH 6.0 possess antibacterial activity and that pH is critical to the maintaining of the microbiological activity and stability of polymyxin B. The HPLC and microbiological methods used in the current study do not provide precise information which would confirm this. There are a number of limitations. Polymyxin B itself is a complex substance containing at least 13 components. Each component could decompose to various decomposition products depending on many factors such as pH and temperature. The current HPLC assay cannot determine the amount of the individual components and their decomposition products. It is difficult to explain the chemical stability

results of polymyxin B without more information on the decomposition kinetics of the individual components of polymyxin B.

It is also important to investigate whether or not the decomposition products exhibit microbiological activity. The microbiological assay by the agar plate diffusion was not suitable for monitoring the potency of polymyxin B in the stability study. Therefore, killing time determinations for polymyxin B standard material and polymyxin B eye drops were developed. The advantage of this method is that the activity of each undecomposed and decomposed sample could be easily distinguished from the killing curves. However, the killing curve represents the activity of total polymyxin B but the activity of the individual components cannot be shown by this method. PB_2 has been shown in other studies to be more active than PB_1 against *B. bronchiseptica* when determined by the agar plate diffusion assay [15.42]. However, there is no information about the activity of the individual components of polymyxin B against *P. aeruginosa*. Because of the heterogeneous component of polymyxin B, the ratio of each component in each sample could well be different. When the decomposition process occurs, the amount of active polymyxin B and its decomposition products in each test sample is likely to be in different proportions. In addition, the lack of knowledge of the activity of the decomposition products of polymyxin B makes the comparison between the activity of decomposed samples difficult. It cannot be finally concluded from this study that the decomposition products of polymyxin B possess antibacterial activity. A difficulty was also encountered with a lack of absolute consistency with the response of polymyxin B activity obtained between experiments.

The variation of polymyxin B activity as shown in chapter 9 and also chapter 7 and 8 may result from many factors. Possible explanations involve Mg^{2+} and phosphate ions. Mg^{2+} has been reported as a major cation in *P. aeruginosa* walls [78]. Within the outer membrane , the Mg^{2+} ions associate electrostatically with the phosphate residues attached to the lipid A and core oligosaccharide regions of the lipopolysaccharide (LPS) molecules [79]. Polymyxin B, as a polycationic compound, is able to disrupt the outer membrane permeability barrier by displacing the Mg^{2+} binding sites on LPS. The positively charged groups of polymyxin interact with the negatively charged phosphates [77]. It has been suggested that phosphate availability itself may influence polymyxin susceptibility in *P. aeruginosa*, perhaps by modulating LPS phosphorylation, but results on this aspect are contradictory [75]. In the present study phosphate buffer was used as a solvent for polymyxin B. The extra amount of phosphate in the system tended to reduce the activity of polymyxin B on the test organism as shown in chapter 7. This is consistent with the comment by Edwards [80] that soap, phosphatides and phosphate ions all antagonise the drug's activity. Polymyxin B could bind to phosphate groups available both in the solution and at the binding sites on LPS. In addition Mg^{2+} was added in the medium as an inactivator in the killing time determination of polymyxin B. Like phosphate ions, the Mg^{2+} is known to inhibit polymyxin B activity [14,66-68]. It is likely that both ions inhibit the polymyxin's activity by competing for the same binding sites. The effective amount of polymyxin B would therefore depend on the available binding sites on LPS. This may have varied between experiments. The

reason for this variation is not clear at present. This may lead to the observed inconsistency in the determination of polymyxin B activity.

10.2 Further work

In order to extend the understanding of the chemical stability and microbiological activity of polymyxin B, further work should include :

- investigations of the degradation mechanisms of polymyxin B which occur at various pHs and temperatures,
- development of quantitative methods of analysing the decomposition products of polymyxin B by more highly resolving techniques,
- investigations of the microbiological activity of the decomposition products of polymyxin B which occur at various pHs and temperatures.

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