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**ANALYSIS OF THE AMINOGLYCOSIDES
NEOMYCIN AND STREPTOMYCIN**

by

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B.Sc. (Hons)

**A Doctoral Thesis submitted in partial fulfilment
of the requirements for the award of the Doctor of
Philosophy of Loughborough University**

August 1998

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Department of Chemistry**

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ABSTRACT

The aim of the study was the determination of neomycin and streptomycin aminoglycoside antibiotics in bovine kidney tissue at trace levels. The aminoglycosides contain no chromophore making detection difficult by conventional spectrophotometric detection and are highly polar making separation from tissue samples a multistep clean-up procedure.

The approach taken in the study was to simplify the clean-up procedure by replacing the conventional liquid-liquid extraction steps which can result in considerable dilution of the analyte and be labour intensive. Matrix solid phase dispersion for extraction was examined as an alternative clean-up procedure. This was investigated and evaluated in conjunction with either ion-exchange, ion-pair chromatography and pulsed amperometric detection or with capillary electrophoresis and indirect UV detection.

The direct detection of the aminoglycosides was achieved with the pulsed amperometric detection coupled with post-column addition of alkali to increase sensitivity. Detection by pulsed amperometric detection was used with ion-exchange and ion-pair chromatography. Initially, separation of the aminoglycosides was carried out by ion-exchange chromatography with gradient elution. This method worked well for standard samples but did not work with samples obtained from the clean-up procedure due to differences in the pH of the sample resulting in variable retention times. The ion-pair chromatography method developed was more successful and gave good separation of neomycin and streptomycin samples obtained from the sample clean-up procedure.

Capillary electrophoresis was investigated as an alternative separation technique. Detection by indirect UV gave good results with similar sensitivity to pulse

amperometric detection. Methanol was added to the buffer to separate the aminoglycosides from the bovine kidney tissue extracts.

The recovery of neomycin and streptomycin from bovine kidney tissue by MSPD was then evaluated by both the ion-pair chromatography and pulsed amperometric detection or capillary electrophoresis and indirect UV detection methods.

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LIST OF ABBREVIATIONS

Ministry of Agriculture fisheries and food	MAFF
Maximum residue limit	MRL
Ultra violet	UV
Thin layer chromatography	TLC
High performance thin layer chromatography	HPTLC
High performance liquid chromatography	HPLC
Liquid chromatography	LC
o-phthaldehyde	OPA
Gas liquid chromatography	GLC
Gas chromatography	GC
Mass spectrometer	MS
Ion exchange chromatography	IEC
Pentafluoropropionic acid	PFFA
Heptafluorobutyric acid	HFBA
Trifluoroacetic acid	TFA
Federal drugs administration	FDA
Pulsed Amperometric detection	PAD
Limit of detection	LOD
Capillary zone electrophoresis	CZE
Capillary electrophoresis	CE
Cetyltrimethylammonium bromide	CTAB
Solid phase extraction	SPE
Matrix solid phase dispersion	MSPD
Cyanopropyl sorbent	CN
Carboxymethyl	CBA
Internal surface reversed-phase	ISRP

Covariance	CV
Relative standard deviation	RSD
Standard deviation	SD
Regression	r
Parts per million	ppm
Parts per billion	ppb

Introduction

CHAPTER ONE

CHAPTER ONE

1. *Introduction*

The analysis of consumable animal tissue is carried out for the screening of particular types of drugs that may be regarded as a possible risk to humans. A variety of different sample extraction and assay techniques are therefore employed depending on the family of drug. There may only be trace levels of the drug in the tissue under examination which could be the specified limit for human consumption therefore, these techniques have to be quite precise and sensitive.

The aim of the research project was to explore and finally develop an on-line method for the extraction and analysis of a group of antibiotics. The aminoglycosides, comprising mainly of neomycin and streptomycin are considered by The Ministry of Agriculture Fisheries and Food (MAFF) as a possible risk to humans on prolonged ingestion. This method was then to be implemented for use by MAFF to determine residue levels of the aminoglycoside in animal tissue.

The goal was to use minimal sample clean-up (reduce the number of extraction and clean-up steps) prior to analysis and to use an assay technique that required minimal time and could easily be employed by analysts at MAFF on a daily basis.

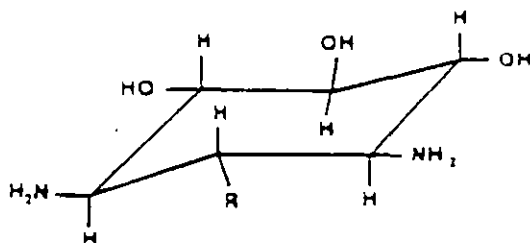
An aminoglycoside is a broad term which denotes a carbohydrate containing an aminosugar linked via a glycosidic bond to an aglycone moiety known as an "aminocyclitol" (Figure 1.1.) Aminoglycoside antibiotics are subdivided further

into two main groups, those containing streptamine and those containing 2-deoxystreptamine. Those containing 2-deoxystreptamine form the largest and most important group and are further divided depending on the number and location of the substituents attached to the deoxystreptamine unit. The neomycin and the paromomycin substituents are at adjacent positions [4, 5] however, the gentamicin and kanamycin substituents are on non-adjacent positions of the aminocyclitol [4, 6]. Streptomycin and dihydrostreptomycin, where streptidine is the aminocyclitol moiety, are included in the streptamine antibiotics.

Figure 1.1 Aminocyclitol moiety of aminoglycoside ¹

R = OH streptamine

R = H 2-deoxystreptamine



During the course of the research project neomycin and streptomycin were chosen as representatives of each sub-group of the aminoglycosides, those containing streptamine and those containing the deoxystreptamine respectively.

The drugs act by interfering with microbial protein synthesis and are uniformly bactericidal.² The aminoglycosides act directly on the bacterial ribosome to inhibit the initiation of protein synthesis and to interfere with the fidelity of translation of the genetic message. They bind to the 30S ribosomal subunit to

form a complex that is unable to initiate proper amino acid polymerisation.^{3,4,5,6} The aminoglycoside antibiotics are important therapeutic agents used to treat food-producing animals for antibacterial prophylaxis as well as for growth promotion.^{7,8} The aminoglycosides approved for use in food producing animals, include dihydrostreptomycin, gentamicin, neomycin and streptomycin.⁹ There is a high incidence of toxic side effects causing damage to the vestibular and auditory functions (tissue damage resulting in irreversible loss of hearing), also nephrotoxicity.^{10,11} This suggests there is a need for the levels of these aminoglycosides to be monitored in consumable animal tissue. There is therefore a risk to the consumer from the consumption of aminoglycoside drug residues in foods like milk and meat.^{8,12,13}

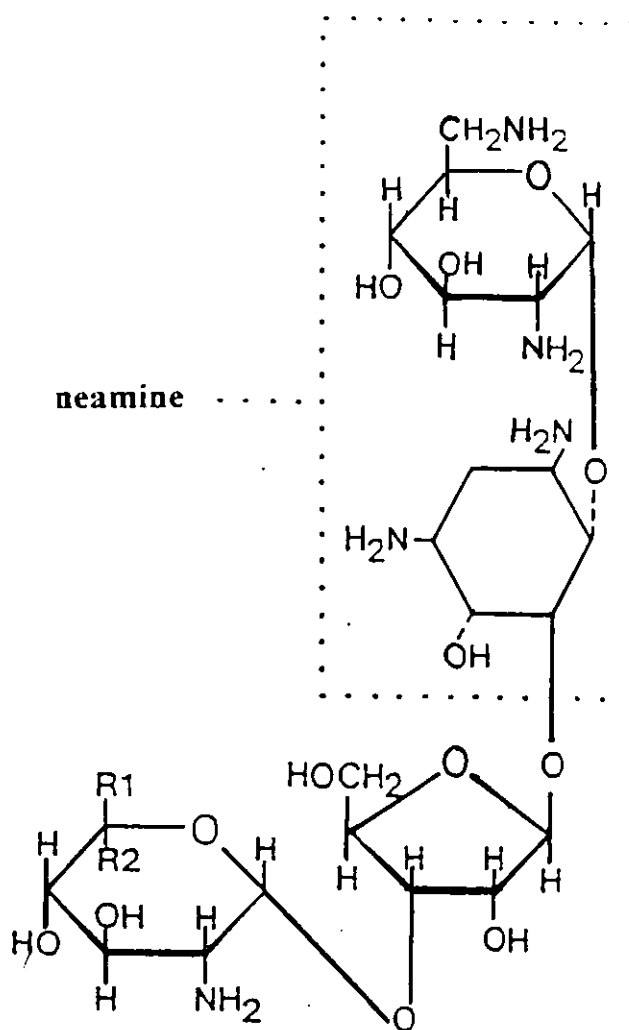
1.1 Neomycin

Neomycin belongs to the aminoglycoside sub-group containing streptamine which is the largest and most important group. The substituents in the case of neomycin are attached at adjacent positions. The neomycin complex, comprises neomycin A, B and C. The principal components of the neomycin mixture are neomycin B (I) and neomycin C(II) (Figure 1.2.), which are isomers together with a small quantity of neamine, a degradation product. Neomycin was first obtained from cultures of *Streptomyces fradiae* by Waksman *et al.* in 1949¹⁴ and subsequently from other *Streptomyces* species, including *S. coeruleoprunus* and also from a *Micromonospora* species. Neomycin B is isolated by adsorption from the culture broth on to activated carbon followed by elution with acidified ethanol. The antibiotic is then separated from the accompanying neomycin C by chromatography over alumina.¹⁵ Neomycin is active against gram positive and gram negative bacteria, mycobacteria and actinomycetes. In medicine it has found use in the treatment

of bacterial infections of the eye and skin and also for the sterilisation of the intestine. It also has a use in veterinary medicine for the treatment of enteric, systemic and reproductive infections, together with local infections of the ear, eye and skin caused by a variety of gram negative and gram positive organisms.

Figure 1.2 The structure of neomycin sulphate

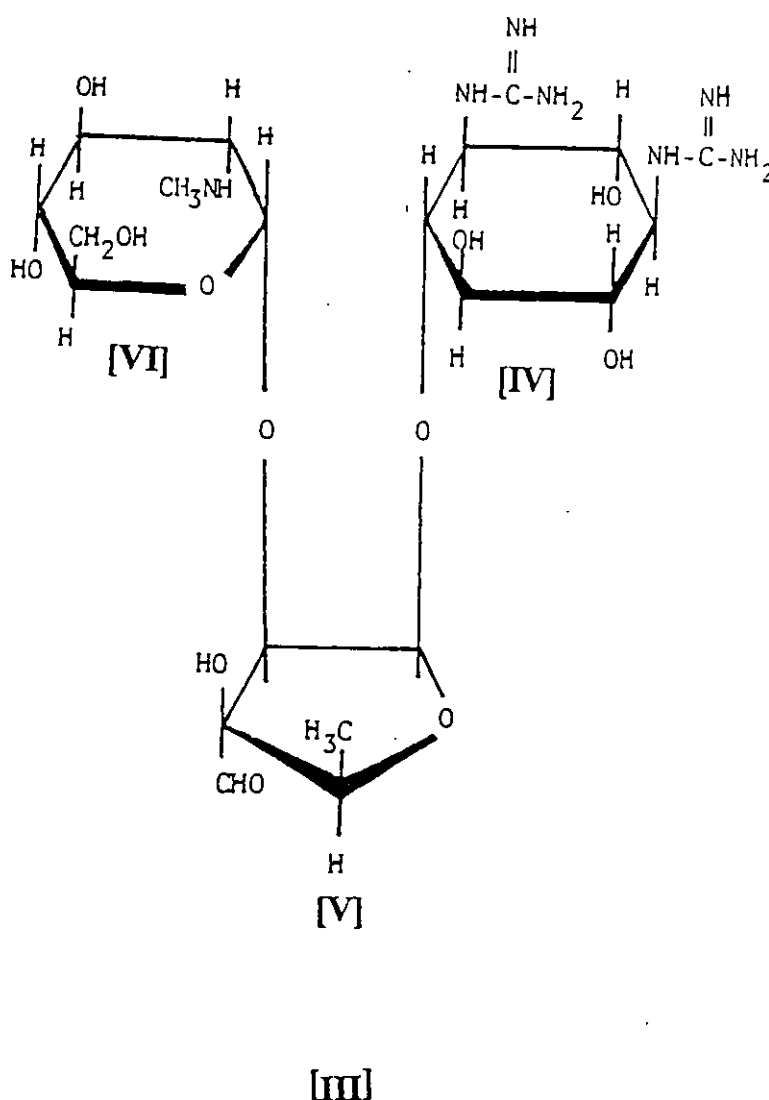
neomycin B (I)	$R_1 = H$	$R_2 = CH_2NH_2$
neomycin C (II)	$R_1 = CH_2NH_2$	$R_2 = H$



1.2 Streptomycin

Streptomycin, belongs to the aminoglycoside sub-group containing 2-deoxystreptamine, where streptidine is the aminocyclitol moiety. Streptomycin is an optically active, base, $C_{21}H_{39}N_7O_{12}$, possessing an aldehydic carbonyl group. Streptomycin [III] is made up of 3 components streptidine [IV] (the aminocyclitol), streptose [V], and N-methyl-L-glucosamine [VI], linked together by glycosidic bonds (Figure 1.3.)

Figure 1.3 Structure of streptomycin



Streptomycin is isolated from cultures of *Streptomyces griseus*. Streptomycin is active against a range of gram positive, gram negative bacteria and mycobacteria, particularly against *Mycobacterium tuberculosis*. It also finds a use in veterinary medicine in the treatment of leptospira, chronic respiratory disease, infectious sinusitis of turkeys and infectious synovitis of chickens.¹⁶

1.3 Pharmacokinetics and Bioavailability

Studies have been carried out to determine the toxicity effects of various aminoglycosides and the mechanisms causing these effects. As briefly mentioned in the introduction these drugs are considered as high risk compounds to the consumer because they can become prolonged residues, due to their strong affinity for tissue depots and binding sites.¹⁷ Oehme¹⁸ reported that dihydrostreptomycin is a persistent residue in tissues. Neomycin was found to be the second most frequent residue in the kidneys of slaughtered cattle.¹⁹

Toxicity of the drug has to be investigated prior to its release for use. Initially, toxicology involves the administration of increasing single doses of drug to groups of animals to establish the toxic doses of single administrations. The next stage of toxicity testing is to dose animals with amounts of drug much lower than the established limit. This method may introduce a considerable error in the amount of drug actually dosed. The error can be caused by a variety of factors:

- The individual animals may not eat and drink to the same extent.
- If the drug is not thoroughly mixed into the diet, then some animals may receive more than others.

- The animals may be selective in picking out food with or without the drug.

The fact that the drug is given in the diet and only dosed with food may impart quite different absorption characteristics compared with the acute dosing regimen, where the drug would be given without food.

The main factors determining the levels of drug achieved in plasma and tissue following oral dosing, are absorption, distribution, elimination and the various physiological, pathological conditions which in turn affect these parameters. A pharmacokinetic study of the drug would give a good indication of what the animal does to the drug after ingestion, which includes all aspects of absorption, distribution, metabolism and excretion and the various physiological pathological conditions which in turn affect these parameters.²⁰

To establish the bioavailability of the drug in the body a variety of pharmacokinetic studies are carried out to provide a mathematical disposition of the active drug in the body. The distribution of the drug will depend on its route of administration. A single intravenous injection results in mono-exponential decay as the drug is eliminated from plasma by natural processes. The rate of elimination can be described with a characteristic half-life.

For an oral dose the drug will have to be absorbed through the stomach or gut prior to entry to the plasma. The level of the drug in plasma is therefore dependent on the ability of the drug to be absorbed through the gut. The absorption process is also represented as exponential. The serum half-life of the aminoglycosides streptomycin and neomycin after oral dosing is about 2.5 hours in young adults, this will be increased in the new born and in adults above 40 years. In renal function impairment, the half-life may be increased to

2-5 days.²¹

An important concept in pharmacokinetics is the idea of distribution of the drug in the body tissues other than the plasma, including the target tissue. This is referred to as the volume of distribution, where the rest of the body is described by the volume it would have to be if the concentration was the same as the plasma.

A polar, water soluble drug can be directly excreted from the body via the urine. However, drugs that are lipophilic, are not water soluble and therefore have to be metabolised by the body in the liver into a reduced lipophilic more polar, water soluble form that can be excreted by the kidney. Williams²² classified the stages of metabolism into two phases. In the first phase, the drug is attached by a single enzyme to affect a simple change in its structure. Such reactions are usually oxidation's, reductions or hydrolysis which usually result in a structure with a convenient functional group or handle used in the second phase. The second phase involves a synthetic step where a water-soluble function is added to form a water-soluble less lipophilic conjugate easily excreted by the body usually via the urine.

A number of pharmacokinetic studies have been carried out to determine the bioavailability of neomycin and streptomycin in animals and humans.

Jayachadran²³ and co-workers, carried out the pharmacokinetics of streptomycin with reference to its distribution in plasma, milk and uterine fluid of female buffaloes, after a single muscular injection (10 mg/kg). The drug was detectable in the plasma, milk and uterine fluid for 30, 8 and 12 hours respectively. A therapeutic concentration of the drug was maintained for 6 to 7 hours in the plasma and for around 1 hour only in the uterine fluid. A

therapeutic level could not be achieved in milk at anytime. The results indicated that the drug could be used clinically by the intramuscular route against streptomycin susceptible systemic infections but not those in the uterus and mammary gland.

The disposition of oral neomycin in calves was determined using ^{14}C labeled neomycin by Ascbacher and Feil.²⁴ The influences of the age, diet, and method of administration were observed. All calves were killed (96 hr) after a single oral dose of ^{14}C neomycin of approximately 30 mg/kg. As determined by urinary excretion, absorption of neomycin was greater in 3 day old calves dosed similarly. In calves dosed at 3 days of age, ^{14}C concentration in the kidneys represented 55 ± 4.9 ppm of neomycin equivalents. The next highest concentration occurred in the livers, which contained less than 5 % of the levels in kidneys. Isolation and characterisation by positive-ion fast atom bombardment mass spectrometry and NMR spectrometry of ^{14}C compounds in kidneys of calves dosed at 3 days of age indicated that at least 90 % of the ^{14}C was present as neomycin. Neomycin was also the major ^{14}C compound of 70 to 80 % in the feces of all calves.

The disposition and absorption kinetics of neomycin were studied in healthy ruminating dairy calves approximately 3 months old.²⁵ The calves were treated with single intravenous (12 mg/kg), intramuscular (24 mg/kg) and oral (96 mg/kg) doses of neomycin. Blood samples were collected from each calf 96 hours after administration. Plasma concentrations of neomycin were determined by HPLC. The mean of some selected parameters were $t_{1/2}$ lambda 37.48 ± 2.02 hr, Clt (clearance time) = 0.25 ± 0.04 l/h/kg, Vd (SS) (volume of distribution at the steady state) = 1.17 ± 0.23 l/kg, and MRT (mean residue time) = 4.63 ± 0.87 h for the intravenous data $t_{1/2} = 11.5 \pm 3.8$ h, MRT abs =

0.960 \pm 1.001 h, F (bioavailability) = 127 \pm 35.2 %, and Clt/F = 0.199 \pm 0.047 l/h/kg for the intramuscular data, respectively. Only one single calf absorbed neomycin to any significant degree (F = 0.0042) after a single oral dose. Selected mean parameters determined after repeat oral dosing were: F = 0.45 \pm 0.45 % Cmax (maximum plasma concentration amount / volume) = 0.26 \pm 0.37 mg/ml, and tmax (time to maximum concentration or effect) = 2.6 \pm 2.9 hr.

Errecalde *et al.*²⁶ determined the pharmacokinetics and bioavailability of neomycin in sheep following intravenous, intramuscular, subcutaneous and intratracheal administration of 10 mg/kg. A rapid distribution phase (t $\frac{1}{2}$ alpha, 3.16 min) was followed by a slower elimination phase (t $\frac{1}{2}$ beta, 1.98 h). The apparent volume of distribution was 304.69 ml/kg. Absorption half-lives were 18.62, 21.22, 40.83 min and elimination half-lives 2.68, 2.82 and 2.5 hr after intramuscular, subcutaneous and intratracheal administration, respectively. Bioavailabilities between 0.74 and 0.85 were obtained for the 3 routes of administration. Based on the bioavailability and disposition kinetics of neomycin, Errecalde concluded that a twice daily 1 M dosage regimen should both be practical and adequate to maintain plasma neomycin concentrations within the pharmacologically active but non-toxic range.

The deposition of neomycin in chickens was also investigated by Atef and El. Gendi.²⁷ Neomycin sulphate was administered in chickens via intravenous, oral and intramuscular routes in a dose of 20 mg/kg. Following a single intravenous injection, the blood concentration curve revealed a biexponential decline with an elimination half life value (t $\frac{1}{2}$ beta) of 5.7 \pm 0.46 hours. The lower apparent volume of distribution of neomycin in chickens (0.8 \pm 0.02 l/kg) indicated its lower distribution in tissues than in blood. Neomycin

could not be detected in blood in chickens after oral administration. The bioavailability of neomycin after intramuscular injection amounted to $67.19 \pm 7.16 \%$ with a maximum blood concentration of $17.33 \pm 0.28 \text{ mg/ml}$, about 40 minutes after administration.

The bioavailability of aminoglycosides neomycin and streptomycin was investigated in horses.²⁸ Single doses (10 mg/kg) of each aminoglycoside were given by the intravenous and intramuscular routes and at a later time, seven intramuscular doses were injected at 12 hr intervals. The pharmacokinetic behavior of the 2 aminoglycosides was similar, a rapid distribution phase followed by a relatively short half life. The half life of neomycin ($2.10 \pm 0.97 \text{ hr}$) was shorter than that of streptomycin ($3.40 \pm 0.42 \text{ hr}$). The apparent volume of distribution of neomycin and streptomycin did not vary significantly (P greater than 0.05) and numerically was the same as the extracellular fluid volume. Following intramuscular injection, each aminoglycoside was rapidly and completely absorbed from the injection site, although neomycin showed wide individual variation in the fraction absorbed. The administration of multiple doses did not change either the bioavailability or the apparent half life from the values obtained after a single dose. The only pharmacokinetic difference found between these aminoglycosides that is of clinical importance lies in the rate of their elimination. From this investigation Baggot concluded²⁸ a dosage interval for neomycin based on the half life should be 8 hours but, due to the relatively greater toxicity of this aminoglycoside an interval of 12 hours was suggested.

In terms of their bioavailability aminoglycoside antibiotics are poorly absorbed from the gastrointestinal tract,²⁹ do not penetrate well into the cerebrospinal fluid, are minimally bound to plasma proteins and are rapidly excreted by the

kidney.³⁰ It is evident from the chemical properties of aminoglycosides their polarity and reduced lipophilicity contributes to their pharmacokinetic properties. They tend to concentrate in the kidney and are excreted by glomerular filtration which guarantees direct excretion by the kidney, avoiding any form of metabolism prior to excretion. The organ which will contain the highest concentration of the aminoglycoside will be the kidney. Therefore analysis of the kidney for the aminoglycoside will be a good indicator of levels of the aminoglycoside in the animal.

1.3.1 Concentration and excretion

Permissible maximum residue levels (MRL), the amount of residue permitted in edible tissue from treated animals, for drug residues in animal products are set by a number of organisations including the E.E.C.¹²

The MRL values are used by the UK Veterinary Products Committee, to set withdrawal periods, so that the processes of excretion and metabolism of the drug reduce its concentration in animal tissue to safe levels before slaughter.¹² The MRLs for a drug vary depending on the species and tissue type. For neomycin the MRL is 500 µg/kg in meat and for streptomycin or dihydrostreptomycin is 1000 µg/kg in meat (Table 1.1).¹² It is important to monitor the MRLs to ensure the safety of consumers.

In order to monitor compliance with these regulations it was therefore important to develop methods for the determination of these drugs at low concentrations in animal tissue, milk and eggs. Animal products are highly complex in their makeup and can vary in composition depending on the

species and tissue type. This provides extra problems for the analytical chemist.¹² Withdrawal times after oral treatment are generally short, whereas for intramuscular treatment they are long (Table 1.2.) neomycin is not approved for parental use in food-producing animals because an extensive withdrawal interval is required.³¹

TABLE 1.1 MRLS USED BY THE UNITED KINGDOM VETERINARY PRODUCTS COMMITTEE TO SET WITHDRAWAL PERIODS¹²

Compound	MRL (µg/kg)		
	Meat	Milk	Eggs
Dihydrostreptomycin	1000	200	500
Neomycin	500	150	200
Streptomycin	1000	200	500

TABLE 1.2 TOLERANCE AND WITHDRAWAL TIME FOR AMINOGLYCOSIDES IN FOOD-PRODUCING ANIMALS³²

Drug	Species	Tolerance (ppm)	Dose	Withdrawal time, days, edible tissue.
Streptomycin	Calves	_b	0.1-1.5 g/gal for 5 days	2
	Swine	tissue 0	0.5-1.5 g/gal for 4 days	_b
	Chickens	tissue 0	0.5-1.5 g/gal. for 5 days	4
Neomycin	Calves	tissue 0.25 milk 0.15	35-140 g/ton feed for subtherapeutic use.	_b
Dihydrostreptomycin	Calves	tissue 0	5 mg/lb/day for 3-5 days IM	30
		milk 0	500 mg bolus	10

_b results not yet known, IM - intramuscular

1.4 Chemical Nature

The degradation of neomycin was studied by Rinehart³³ as a means of establishing the structures of the neomycin components. Figure 1.4 illustrates the route by which complete degradation of the antibiotic was achieved.

Figure 1.4 Hydrolytic degradation of neomycin c

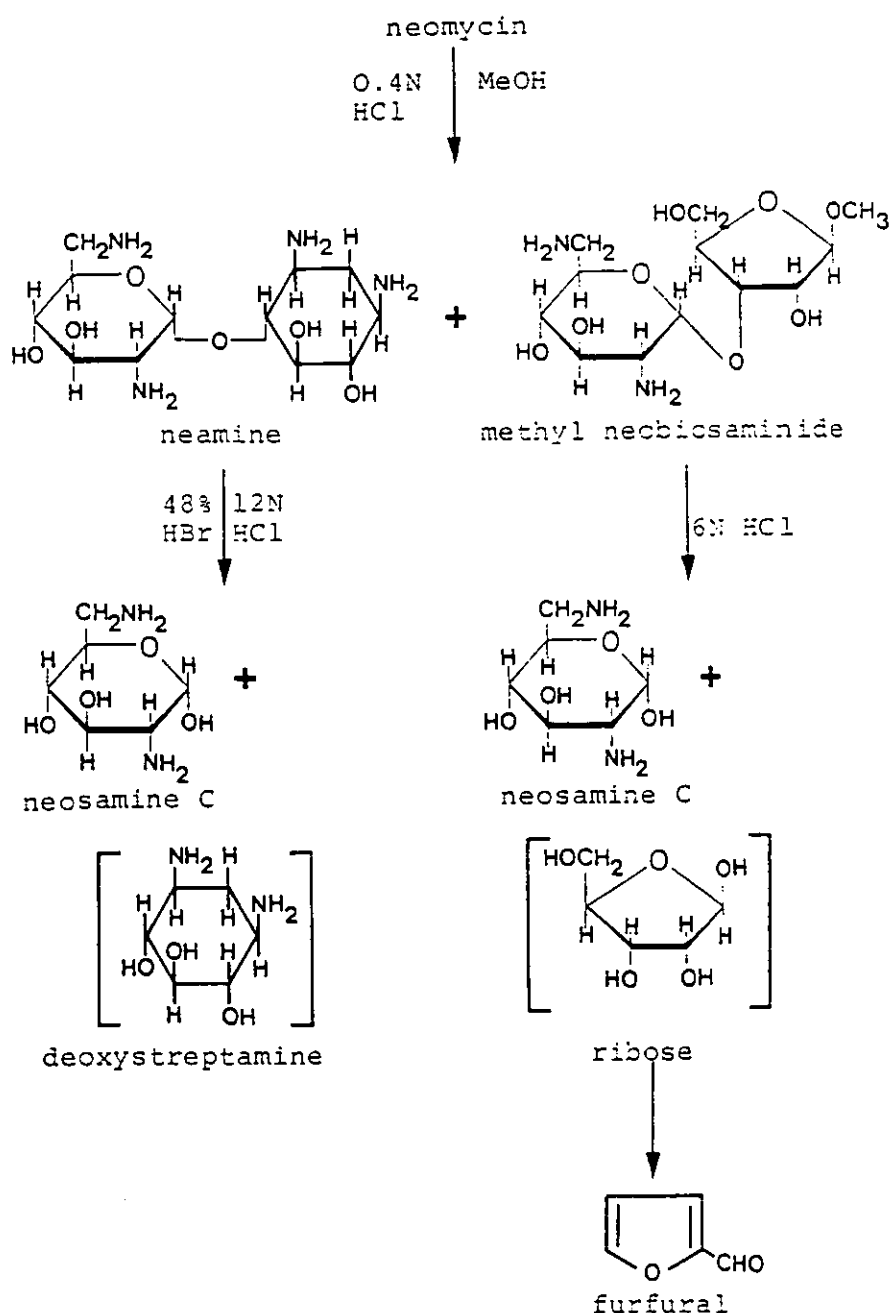


Figure 1.5 The degradation reactions of streptomycin.



Both streptomycin and neomycin are stable to heat, acid and base, Table 1.3. Also they are highly water soluble, polar, basic and very lipophobic in nature.^{1, 48,55} These last few features contribute to difficulties encountered in their isolation and impose limits on the techniques that can be used to isolate them from tissue extracts. Also the absence of a strong chromophoric group results in a very low UV absorption, weaker in neomycin than streptomycin, virtually eliminating the possibility of their photometric detection in analytical chromatography.^{48,55}

TABLE 1.3 GENERAL CHARACTERISTICS OF THE AMINOGLYCOSIDES NEOMYCIN AND STREPTOMYCIN USED IN FOOD PRODUCING ANIMALS.

Aminoglycoside	Solubility			Stability			UV, nm (in water)	LD50 mg/kg (given IV)	pKa
	Water	Methanol	Other ^a	Heat	Acid	Base			
Streptomycin	good	fair	poor	+	+	+	200	200	8
Dihydro streptomycin	good	fair	poor	+	+	+	200	200	
Neomycin B	good	fair	poor	+	+	+	200	36	7.5

^a ethanol, hexane, benzene, petroleum, ether

IV - Intravenous injection

1.5 Analytical methods used for the determination of neomycin and streptomycin

The British Pharmacopoeia,⁴⁰ contains specifications for the purity and assay of neomycin sulphate that are based on a combination of chromatographic tests for related substances, including TLC and ion-exchange column chromatography. The British Pharmacopoeia method for the determination of

streptomycin sulphate is a microbiological assay.⁴⁰ These procedures are time consuming and as far as the microbiological assay is concerned, not very precise.

The most important points that determine the value of a residue method are its specificity, sensitivity, accuracy, precision and practicability. The method must be specific for the drug being analysed and sensitive enough to measure legal levels of the drug in a tissue. It is sometimes necessary to derivatise the drug in some way to enhance its sensitivity.¹² The method must be able to accurately recover the drug from tissues (biological matrices) with a good degree of precision. The method must be practical and economical to run, work well with real samples, be reasonably fast and able to be carried out routinely by analytical chemists working in a analysis laboratory.

The following literature review outlines the attempts carried out over the years to achieve the criteria outlined above in the analysis of neomycin and streptomycin. A variety of different analytical techniques were investigated. The main aim of the methods was the ability to eventually analyse levels of neomycin and streptomycin in biological matrices at levels equivalent to or lower than the set MRLs (maximum residue limit).

1.5.1 Immunoassay

Immunoassay techniques are important for the specific determination of hormones, drugs, vitamins, and other compounds at nanogram and lower levels. In these techniques, an antigen and a specific antibody react to form a complex or precipitate.⁴¹ Immunoassays and related competitive binding

assays are widely used now in the clinical chemistry laboratory. Immunoassay techniques generally involve a competitive reaction between an analyte antigen and a standard antigen that has been tagged, for limited binding sites on the antibody to that antigen, the tag may be a radio-active tracer, an enzyme, or a fluorophore.

Immunoassay techniques cited in literature used for the analysis of neomycin and streptomycin are fluorescence polarisation immunoassay and solid-phase enzyme immunoassay. The principle behind polarisation immunoassay is outlined in the following sentence. If a molecule is excited using polarised light, then the emitted light will be polarised in the same plane as the excited light, provided that the molecule does not rotate in the time between excitation and emission. In solution, the free rotation of the molecule reduces the polarisation of the emitted light and hence solutions do not usually exhibit fluorescence polarisation. When the fluorescent agent is bound to macromolecules, the rotation is slowed and the fluorescence remains polarised. When used in an immunoassay the fluorescent labeled molecules displaced from an antibody, have reduced polarisation fluorescence. Thus, this method can be used without separating free and bound fractions, making the technique much simpler to use or to automate. Schwenger and Anhalt⁴² used fluorescence polarisation immunoassay technique to analyse streptomycin. Fluorescein labeled streptomycin was used as the tracer, and antiserum specific for streptomycin was raised in rabbits. Tracer, sample and diluted antiserum were combined, and the polarisation of tracer fluorescence was determined in a specially designed fluorometer. Due to the design of the instrument, the possibility of fluorescent interference was eliminated. The coefficient of variation within the run was 4 % (n=5) and between runs was 5 % (n=5). Later work using fluorescence polarisation immunoassay has compared the

effectiveness of this technique for streptomycin and neomycin analysis in comparison with other drugs⁴³ and ways in which the procedure could be improved to increase sensitivity.^{44,45}

On reference to the literature, the more commonly used immunoassay technique used for the analysis of aminoglycosides is that which uses an enzyme label - ELISA kits (enzyme-linked immunosorbent assays). The drug molecule is covalently linked to a stable enzyme such as glucose-6-phosphate dehydrogenase to provide the tracer. When the immunoassay procedure is carried out, the unbound tracer remains active, whereas the bound tracer loses its enzyme activity. Hence, rather than a separation step, the enzyme activity is measured after adding a suitable substrate. The sensitivity of the standard enzyme labeled assays is of the order of $\mu\text{g/ml}$ rather than ng/ml . For enzyme labeled assays, the special reagents are the drug-enzyme complex and the antiserum; for the specific drugs, these and other standards are supplied in kit form. One advantage of enzyme-labeled assays is that all the steps of the assay can be performed in the same vessel, making it a homogeneous assay. A development of the enzyme labeled technique was to immobilise the enzyme on a solid support in the reaction vessel so that the complete assay can be carried out by monitoring the solution. The method has been most highly developed in the range of ELISA (enzyme linked immunosorbent assays) kits, which have been used in the analysis of aminoglycosides. Yao and Mahoney⁴⁶ developed an enzyme-linked immunosorbent assay (ELISA) for the detection of aminoglycoside antibiotics including neomycin and streptomycin in fermentation broths.

Schnappinger *et al.*⁴⁷ developed an enzyme immunoassay for the detection of streptomycin and dihydrostreptomycin in milk at levels as low as 6 and

0.8 ng/ml respectively. Polydonal and antisera against streptomycin were prepared by using streptomycin-oxime derivative coupled to bovine serum albumin for the immunisation of rabbits. The specificity and sensitivity of these antibodies were tested in a competitive assay using a streptomycin-enzyme conjugate in a double antibody solid phase technique. The only detectable cross-reactivity of the assay system with other aminoglycoside antibiotics and other substances similar in structure was shown to be with dihydrostreptomycin of about 148.7%. The detection limit in buffer solution was 0.6 ng/ml for streptomycin and 0.4 ng/ml for dihydrostreptomycin.

1.5.2 Microbiology

Microbiological methods are often used for the detection of antibiotics. Traditionally they have been used for the detection of aminoglycoside residues in edible products of animal origin however, these methods lack specificity.¹

The principle of a microbiological assay is based on the comparison of the inhibition of the growth of a micro-organism produced by a known concentration of a standard preparation of the antibiotic, with that produced by the sample being tested.⁴⁸ These assays provide a measure of the biological activity of the antibiotic, whereas non-microbiological methods measure concentration. The traditional microbiological assays for example, agar-diffusion methods on solid media or turbidimetric methods, require long incubation times i.e. 10 - 24 hr's. The major drawbacks of these methods are their lack of speed and the lack of specificity due to the aspecific nature of the bacterial response. Neomycin has been determined by a number of microbiological assays.^{49,50,51,52} Quantitation of neomycin by microbiological

methods is also complicated because neomycin B and C do not exhibit the same antimicrobial effect.⁵³ In the microbiological assay the potency varies with the micro-organism used and with the experimental conditions. The most reliable results are only obtained when the same isomer B/C ratio is present in the test and the reference material. However, the strength of these microbial methods is that a number of samples can be analysed simultaneously.

Stahl and Kratzer modified microbiological methods so that they could be used to determine levels of aminoglycosides in feeds, supplements, premixes, oils, suspensions, boluses, and antibiotic impregnated paper.^{49,50} A buffered monolayer plating medium was used in place of the water-prepared medium, this resulted in a more sensitive standard response line using the organism *Staphylococcus epidermis*. Results for 50 commercial complete feeds and fifty commercial premixes received by Stahl over 5 years produced an overall mean recovery of 101 % with a mean percentage recovery range of 80 - 112 %. A statistical analysis of the results from these commercial, complete feeds and premixes, ranging in concentration from 47 g/ton to 70 g/ton indicated the assay has little, if any concentration related bias. The precision and accuracy of the method were supported by laboratory studies of 20 assays that produced a mean recovery of 101 % and a standard deviation of 3 %. Microbiological assays have also been developed for the detection of aminoglycoside residues in milk.⁵¹ Yadavalle, Singh and Anand used *B. stearothersophilus* as a test organism in the detection of streptomycin residues in milk using an agar-well assay technique. In this method the standardised spore suspension of *B. stearothersophilus*, was added at 2 % to the antibiotic medium. The inoculated medium was poured into standard assay plates and wells were bored into the solidified agar. Diluted streptomycin was added to each well and the plates incubated. The diameters of the zones of inhibition were measured. As an

alternative Simpson and Kobas⁵⁴ used a carbon dioxide gas sensor in conjunction with a microbiological assay to determine levels of neomycin. The method was based on the antibiotic's inhibition of carbon dioxide production by a suspension of *Escherichia coli*. This was measured with the potentiometric gas sensor, after a 2 hour incubation of the antibiotic in nutrient broth at 37 °C. Linear log dose-response curves were obtained for the antibiotics, streptomycin and neomycin, over the ranges of 0.33 - 16.67 µg/ml and 0.17 - 3.33 µg/ml, respectively.

1.5.3 Thin layer chromatography

Thin layer chromatography and paper chromatography are used extensively for the analysis of the aminoglycosides to separate and identify the aminoglycoside from other antibiotics and to separate individual components and degradation products of the aminoglycoside. A complete review of these TLC and paper chromatography methods for the aminoglycosides neomycin and streptomycin has been edited by Heyes⁵⁵ for neomycin and by Mossa *et al.*⁴⁸ for streptomycin.

Vega and co-workers⁵⁶ achieved the analysis of streptomycin and neomycin in biological samples as dansyl derivatives by using TLC. This allowed the compounds to be distinguished both from interferences and from each other. The extract was derivatised with dansyl chloride and sodium bicarbonate solution. Chromatography was performed on plates coated with silica gel. Development was first carried out with 20 % disodium monohydrogen phosphate, pH 4.5. After drying the plate was then chromatographed to a similar distance with n-butanol-diethyl ether-20 % aqueous dipotassium

monohydrate phosphate (35:10:20 v/v/v). Quantitation of the aminoglycosides was carried out by densitometry with TLC/HPTLC scanner. The minimum detectable quantity of dansyl streptomycin was 25 ng and for dansyl neomycin 20 ng. Detectability was therefore achievable in the ng range. The different colours of the fluorescence of the aminoglycosides allows them to be identified and distinguished from each other and other compounds present. However, within the paper, the authors did not specify what types of biological samples could be analysed, nor did they mention animal meat as a possibility for extraction. As with other TLC and HPLC techniques, some form of derivatisation of the aminoglycoside is required. This method had not been used quantitatively for the analysis of neomycin sulphate at the low levels required for residue analysis.

Roets and co-workers⁵⁷ determined the relative amounts of the B and C components of neomycin by TLC using fluorescence detection. Separation was carried out on the underivatised neomycin with a mobile phase, which contained methanol-20 % sodium chloride solution (15:85 v/v). Fluorescence detection was performed after derivatisation on the plate with 4-chloro-7-nitro-2-oxa-1,3-diazole (NBD-Cl). The different components were quantified by densitometry using the fluorescence mode. For an application of 4 µg sample to the plate, the limit of detection was about 0.04 µg.

Funk *et al.*⁵⁸ were also able to determine the amount of neomycin B and C and neamine quantitatively by TLC. Optimum separation was achieved on silica gel 60 F plates. Methanol-25 % ammonia solution-acetone-chloroform (35:20:20:5 v/v/v/v) was used as the mobile phase. Post-chromatographic derivatisation was performed with fluorescamine. It was possible to detect neomycin B and C and neamine simultaneously. The derivatives obtained were

visible as bright pale blue fluorescent zones on a dark background under long wavelength UV light. Funk claimed this method sensitive enough to determine the individual neomycin components quantitatively at the low concentrations found in neomycin raw materials. The detection limits for neomycins B and C and neamine were 20 ng substance/chromatographic zone.

Medina and Unruh⁵⁹ optimised the conditions for the isolation of the related aminoglycoside hygromycin B with a co-polymeric bonded solid-phase silica column followed by TLC separation and detection of its fluorescence derivative after reaction with fluorescamine. Serum and plasma samples fortified with hygromycin B were acidified and passed through co-polymerised solid-phase columns previously conditioned with phosphate buffer. Hygromycin B was trapped in the columns, eluted with diethylamine-methanol and analysed by TLC using acetone-ethanol-ammonium hydroxide as the developing solvent. Hygromycin B bands were derivatised at acidic pH with fluorescamine and visualised under UV light, detection limit of the drug was 50 ng. Hygromycin B added to bovine plasma was detectable at 25, 50, 100, 250 and 500 ng/ml (ppb). Hygromycin B added to swine serum was detected at 50 ng/ml. However, the serum had to be deproteinised with trichloroacetic acid or acetonitrile prior to solid phase extraction to gain accurate values. Neomycin and gentamicin (100 ng/ml aqueous solutions) could also be isolated with copolymeric solid phase columns at a level of 50 ng.⁵⁹ Gentamicin, neomycin, spectinomycin, hygromycin B and streptomycin could also be separated, allowing multiresidue detection of these aminoglycosides. This chromatographic method allowed rapid analysis of 12 samples in an 8 hour period.

1.5.4 Gas liquid chromatography

Gas liquid chromatography (GLC) methods are not usually suitable for these antibiotics because aminoglycosides are involatile. Tsuji and Robertson,⁶⁰ achieved the separation of neomycin B, neomycin C and neamine as the trimethylsilyl ethers. Margosis and Tsuji⁶¹ later recommended a number of improvements to optimise the analysis of neomycin by GLC. The improvements to the assay included modifications of the injection port to prevent sample decomposition by contact of the injected material with metal or Teflon and the addition of an accurate volume of silylating reagent to each sample and standard to overcome inconsistent derivatisation.

Murata *et al.* reported the examination of neomycin B by a combined GC-MS procedure.⁶² Neomycin was separated from kanamycin as the trimethyl silyl derivatives. The resulting mass spectrum of the neomycin derivative exhibited a low intensity molecular ion peak at m/e 1550 which indicated that all active hydrogens of both hydroxy and amine groups were completely silylated.

1.5.5 Ion-exchange chromatography

Ion-exchange chromatography is a technique that has been used in the separation of various aminoglycosides. In ion-exchange chromatography (IEC) ionic or ionisable groups are permanently present on the surface of the stationary phase. In the absence of the solute, these groups are all masked by a counter-ion, which is present in the mobile phase in a constant concentration. Retention is based on an opposite charge between the solute ion and the ionic groups on the stationary phase. The counter-ion has a charge similar to that of

the solute ions. Therefore, there are anion-exchange columns with positive groups on the surface and cation-exchange columns with negative group. Ion-exchanger resins are defined by the variation of their exchanging properties with pH. Strong ion-exchangers contain strong acid or basic groups that are fully ionised over a wide range of pH. A strong cation-exchange resin contains sulphonic acid exchange sites that are fully ionised above pH 2. A strong anion-exchange resin contains quaternary ammonium exchange sites that are fully ionised up to pH 10. Weak cation and anion-exchangers contain, respectively, carboxylic acid and amino groups. The weak exchangers have a higher exchange capacity than the strong types although they only possess a narrow ionisation range with pH (Table 1.4.) The hydroxy groups on the neomycin, as with carbohydrates, can be deprotonated at high pH to provide oxyanions suitable for anion exchange. At low pH, the amine group can be protonated for cation-exchange.

TABLE 1.4 OUTLINING THE DIFFERENT KIND OF EXCHANGERS USED IN ION-EXCHANGE CHROMATOGRAPHY

Exchanger Strength	Cation	Anion
Strong	Sulphonate $-\text{SO}_3^-$	Quaternary amines $-\text{NR}_3^+$
Weak	Phosphonate $-\text{PO}_3^{2-}$	Tertiary Amines $-\text{NR}_2^+$
	Carboxylate $-\text{COO}^-$	Secondary Amines $-\text{NR}^+$

The stationary phases are composed of different kinds of material. Pellicular materials, the particles of which consist of a hard glass core covered by a thin layer of an ion-exchange resin may be used if a moderate efficiency and a small ion-exchange capacity are acceptable, but not if the column is required to have a high stability. A column containing glass beads covered with a styrene-divinylbenzene cross-linked copolymer backbone to which the functional (ion-

exchange) groups are chemically bonded may be employed over the entire range 1-13.

Microparticulate stationary phases may also be used. These are either based on organic resins or on inorganic oxides. Here, the functional group is an ionic one. Resin based materials offer a greater chemical stability with a large pH range, whereas silica based materials are mechanically more stable and allow a wider range of solvents to be used. All microparticulate phases offer a high column efficiency and a large ion-exchange capacity.

1.5.5.1 Columns

Maehr and Schaffner ⁶³ described the column chromatographic separation of neomycin B, C and neamine on an ion-exchange column. The ion-exchange resin used was DOWEX (OH-form) and water was the eluting solvent. The column elute was monitored by reaction with ninhydrin. This method was applied to the quantitative determination of neomycin C and neamine in commercial samples of raw materials.⁶⁴ Roets and Vanderhaeghe⁶⁵ also examined neomycin by chromatography on DOWEX column, the column elute was measured conductometrically. Minor components present in commercial neomycin have been separated by column chromatography on a carboxylic cation-exchange resin, (Amberlite CG-50). The components were eluted from the resin with ammonium hydroxide solution.⁶⁶

Vanderhaughe *et al.*⁶⁷ reported a method that determined the amount of neomycin B and C in neomycin sulphate powders using ion-exchange chromatography on a strong alkaline ion-exchange resin with water as the

mobile phase in combination with refractometric detection. The use of an internal standard (+) - α -terhalose allowed the quantitation of absolute amounts of neomycin B and C. Commercial neomycin samples of different origin were assayed and the quantity of several minor components estimated. This method had the advantage of avoiding pre- or post-column derivatisation. The peak area of the neomycin components over the internal standard peak area was plotted vs the calculated amount of free base present in the sample solution. In the 7 - 70 mg range of neomycin free base the calibration curve was linear. However, it was observed during the course of the investigation that the resin in the hydroxide form deteriorated rapidly.

St. John and co-workers⁶⁸ used a column of cation exchange resin Amberlite IRC-50 to separate streptomycin from fermentation broths, the antibiotic was determined colorimetrically. This method was also applied for the determination of streptomycin in animal feed⁶⁹ and in formulations⁷⁰ Galina and co-workers⁷¹ reported a comparative study of carboxylic cation exchangers in respects of the sorption and desorption of streptomycin.

1.5.5.2 Ion-Exchange Chromatography

Aminoglycosides behave chromatographically much the same as aminosugars. They are anionic at very high pH and thus are weakly retained on the high-capacity strong anion exchange column. Dionex (Sunnyvale, CA, USA) developed an anion-exchange method for the separation of neomycin in a topical lotion. Anion exchange separation was carried out on a pellicular CarboPac PA1 analytical column using a dilute sodium hydroxide gradient. This separation technique had been previously commonly used for

carbohydrate determination.^{72,73,74,75,76}

1.5.6 Atomic absorption spectroscopy

Abu-El-Wafa *et al.*⁷⁷ carried out co-ordination chemical studies of some polymeric transition metal complexes with neomycin and examined their biological-activity uses. This involved the indirect determination of neomycin by atomic absorption spectroscopy using carbonate as an auxiliary ligand. Determination of neomycin indirectly in tablets (500 - 100 mg/tablet) and ointments (2.5 mg/10 g) was carried out this way. The metal (II)-neomycin complex can be formulated as $[M(\text{neomycin})(\text{H}_2\text{O})_x]_n$; where $X = 4$ in case of Co (II), Ni (II) or Cu (II). This indicates that neomycin behaves as a dianionic bidentate ligand towards one metal (II) ion.

Abu-El-Wafa *et al.*⁷⁷ also by carrying out the structural elucidation of the copper (II) streptomycin complex used the indirect estimation of streptomycin by atomic absorption spectroscopy.

With the exception of TLC,^{56,59} the methods reviewed so far gas chromatography, ion-exchange and atomic absorption spectroscopy have only been able to determine the levels of neomycin and streptomycin in uncomplicated matrices and further evidence is needed to suggest these methods could be used to detect these aminoglycosides in biological matrices.

1.5.7 High performance liquid chromatography

Chromatography is the common laboratory method for both quantitative and qualitative analysis of drugs. This is because chromatographic methods can combine a powerful separation step with a sensitive and specific detection

system. The basic principle underlying all types of chromatography is that the system consists of two components, one stationary and one mobile. The mobile phase is always a fluid, the stationary phase may be a liquid or solid. If the stationary phase is a solid, the affinity for this phase may be governed by several different factors, including absorption and the ability of the pores in the solid matrix to accommodate the molecules to be separated; this accommodation may depend on the analyte's shape as well as its size. It is important to distinguish between a support, which is always a solid, and the stationary phase, which may be the support itself, or may be a coating on the support, or an adsorbed liquid. The early chromatography columns were based on absorption chromatography. In addition to adsorbent phase, ion-exchange phases were also developed to separate ionisable drugs using their charge refer to section 1.5.5.2.

Reversed-phase chromatography was tried on high performance liquid chromatography (HPLC) columns, by impregnating a sorbent support with a non-volatile organic phase such as paraffin oil. This had the disadvantage that the stationary phase was gradually stripped from the column and the characteristics of the column gradually changed from partition behavior to the familiar adsorbent behaviour.²⁰ The real breakthrough in HPLC came with the introduction of packing materials in which a stationary phase was covalently linked to the silica support. This enabled reproducible and durable reversed-phase columns to be manufactured. The usual method is to react the silica with organochlorosilane or a similar reagent to make a stationary phase of any desired polarity. The most popular supports have been where the stationary phase is octadecyl or octyl. The former is suitable for all applications where it is important to not have any residual interaction with silicon atoms, which are sterically shielded. Octyl phases are less lipophilic and retain drug molecules

less strongly.²⁰ The phase should be capped with silylating agent such as trimethyl chlorosilane after the main derivatisation to ensure the chromatography is not partly of the absorption type.

These columns are suited to drug analysis in biological fluids. The reversed-phase mode means that the mobile phase is aqueous which is a more appropriate medium for analysing an aqueous sample. The use of a partition system rather than an absorption system gives sharper peaks with less tailing.⁷⁸ HPLC can be used for involatile drugs unlike GC.

In reversed-phase column chromatography the separation is achieved by controlling the partition between the organic stationary phase and the mobile aqueous phase. The mobile phase is water, the addition of a water miscible organic solvent, such as methanol, ethanol, or acetonitrile, may be used to modify the polarity of the mobile phase. Over the short range of concentrations usually used for the organic modifier, the retention of the analyte will be proportional to the logarithm of the percentage amount of modifier in the mobile phase; over wider ranges the relationship is more likely to be quadratic.

For drugs which are weak acids (organic acids) or weak bases (organic bases) the partition can be adjusted by altering the pH of the aqueous phase. For example the addition of small amounts of acid (acetic or phosphoric) to the aqueous phase will cause increased retention of organic acids as the ionisation is suppressed and decreased retention of bases as the ionic form is preferred. A more precise control of pH can be achieved with buffered mobile phases using acetate or phosphate buffers in combination with methanol or acetonitrile.

A method for the extraction of charged compounds from aqueous phases is the

technique of ion-pairing, which is also used in altering partition coefficients in HPLC. A counter-ion is added to the solution containing the ion to be extracted and an association complex with reduced charge is formed; this entity is then extractable with an organic solvent. Therefore, for organic acids, a suitable counter-ion would be tetramethylammonium, while for organic bases, such ions as heptane sulphonate.

A major advantage of HPLC over other chromatographic approaches such as GLC for antibiotic residue analysis is that frequently little or no sample preparation is required as an aqueous sample can often be injected directly. Another advantage is that procedures can often be partially or completely automated.⁷⁹

An extensive amount of work has been carried out in this area to determine levels of aminoglycosides (refer to sections 1.5.7 (a) and (b)) in lotions and complex biological matrices. Attempts have been made to achieve levels at or below the accepted MRL. The following pages will give a brief insight to the various methods.

As mentioned in section 1.4 the chemical nature of streptomycin and neomycin, that they have no or very weak chromophores, limits their detection by conventional UV photometric detection. Therefore the aminoglycoside is usually derivatised with a fluorophore or chromophore to make it detectable by UV or fluorescence spectrophotometry. Derivatisation of the aminoglycoside can be either pre-column or post-column, both require different separation systems.

High performance liquid chromatographic methods for the assay of a variety of

aminoglycosides including neomycin and streptomycin may be categorised into the following two classes.

- (a) Reversed-phase ion-pair chromatography with post-column derivatisation.
- (b) Pre-column derivatisation followed with normal phase chromatography

(a) Methods using reversed-phase ion-pair chromatography with post-column derivatisation.

On reference to the literature ion-pair HPLC seems to be the preferred method for the separation of highly polar and ionic compounds, including many pharmacologically, and clinically important drugs. The main advantages of ion-pair HPLC are the ease of adjusting the operating parameters to optimise a given separation (pH, nature of the pairing ion, percentage of organic modifier, ionic strength) and the high-speed determination of compounds.¹

Shaikh *et al.*^{1, 80,81,82,83} carried out an extensive study into the analysis of neomycin in biological matrices. They⁸⁰ described a liquid chromatographic method for the determination of neomycin in animal tissues, bovine and porcine kidney and muscle tissue. Sample preparation involved homogenisation of the fortified tissue extract in phosphate buffer followed by centrifugation and heating to precipitate the protein. The HPLC method consisted of an ion-pair mobile phase containing 0.01 M 1-pentane sulphonate, 0.056 M sodium sulphonate, 0.007 M acetic acid and methanol-water (1.5:98.5 v/v). Separation was carried out on a reversed-phase ODS column, followed by post-column derivatisation of the amine groups in the molecule with o-phthaldehyde (OPA) reagent, and by fluorometric detection at 340 nm excitation and 455 nm emission. Under these conditions the detection limit of

neomycin was about 3.5 ng. The overall recovery of neomycin from kidney tissues spiked at 1 - 30 ppm was 96 %. Shaikh found this HPLC assay could also be used on skimmed milk,⁸¹ plasma and urine samples.⁸²

Shaikh *et al.*⁸¹ went on to report a method for the determination of neomycin in milk. Whole or skimmed milk was defatted by centrifugation at 4 °C and deproteination was carried out by the addition of trichloroacetic acid followed by centrifugation. The neomycin was then determined directly in the supernatant by HPLC and post column derivatisation. The HPLC conditions used were equivalent to those used in the previous method.⁸⁰ The overall recovery of neomycin was 94 % in whole milk spiked at 0.15 - 10 ppm and 99 % in shelf milk spiked at 0.15 - 5 ppm. The method was then used to detect neomycin in milk obtained from cows dosed intramuscularly with neomycin (10 mg/kg). The neomycin concentrations obtained in milk at 8 and 24 hours after dosing were 0.3 and 0.2 µg/ml respectively.

Shaikh *et al.*⁸² then adapted this method for the determination of neomycin in plasma and urine. The samples were obtained from animals injected intramuscularly with neomycin. The same procedure was used for sample preparation as that used for fortified milk.⁸¹ Then under the same HPLC conditions used for the previous two methods, the resultant supernatant was analysed. The overall average recovery of neomycin was 97 and 113 % from plasma spiked at 0.25 - 1.0 µg/ml, using standard curves prepared in plasma extract and in water, respectively, and 94 % for urine spiked at 1 - 10 µg/ml using a standard curve prepared in water.

Shaikh modified the mobile phase conditions used for the separation of neomycin in the previous methods.^{80,81,82} The continual use of aqueous salt

mobile phase containing a very small percentage of organic solvent, wore out the column quickly, as it required frequent regeneration for the analysis of tissue extracts. Also as the equilibration of ion-pair mobile phases on reversed-phase columns takes longer than for organic solvent systems, a mobile phase with a low percentage of organic modifier requires a longer time to equilibrate. This can result in a variable retention times if complete equilibration of the column has not been achieved. Therefore Shaikh *et al.*⁸³ developed an improved liquid chromatographic method for determination of neomycin B in kidney tissue following intramuscular administration. The composition of the mobile phase was modified by increasing the methanol concentration from methanol-water 1.5:98.5 v/v to 18:82 v/v, decreasing the sodium sulphate concentration from 0.056 M to 0.0056 M, and changing the analytical column from Supelcosil LC-8-DB to Spherisorb-ODS. Under these new conditions the problems mentioned previously were resolved. The extraction and deproteinisation step remained unchanged to the earlier method⁸⁰ for extracting neomycin from bovine kidney. Under these conditions Shaikh *et al.*⁸³ achieved average recoveries of neomycin B from kidney tissues spiked at 3, 6 and 12 ppm, of 103, 99 and 104 % respectively, similar to recoveries obtained for the earlier method.⁸⁰

Agarwal⁸⁴ used a HISEP column for the determination of neomycin in milk. The milk is passed directly through an amberlite CG-50 ion exchange resin column, and the neomycin which is retained on the column is then derivatised with OPA reagent. The derivatised neomycin was eluted from the column with potassium borate buffer/methanol and analysed by reversed-phase HPLC using an HISEP column. The HISEP column is made of silica based material containing hydrophobic regions shielded by a hydrophilic network, which shields the large protein molecules from contact with the inner hydrophobic

regions. As a result of this protein molecules are unretained by the column and pass through. Drugs, however penetrate the hydrophilic network and are retained on the column. These compounds are then eluted by adjusting the mobile phase. Fluorometric detection was carried out at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. Neomycin was determined at levels ranging from 0.1 to 5 ppm, with recoveries ranging from 94 to 102 %. The detection limit achieved was 50 ppb. The advantage of Agarwal's over Shaikh's method was the lack of sample preparation prior to analysis, avoiding excessive sample handling.

Unlike neomycin, streptomycin has a weak chromophore. Therefore, early HPLC methods used for the analysis of streptomycin sulphate at high levels involved no form of derivatisation of streptomycin sulphate to improve its UV absorption. UV detection was carried out at low wavelengths, of 195 nm. At this wavelength range problems can arise from UV absorption by other contaminants and limits the use of various mobile phases, this in turn limits sensitivity.

Whall⁸⁵ developed a method for the determination of streptomycin sulphate and dihydrostreptomycin sulphate by ion-pair reversed-phase HPLC. This method used a microparticulate reversed-phase column and a mobile phase made up of 0.02 M sodium hexanesulphonate and 0.025 M tribasic sodium phosphate in acetonitrile-water (8:92 v/v) at pH 6, with detection by UV absorption at 195 nm. Trace level assay linearity for streptidine, streptomycin A, and streptomycin B was established over 1.0 - 3.0 and 3 - 10 mg/ml concentration ranges. With slight modification to the mobile phase by reducing the ion-pair concentration and increasing the amount of acetonitrile, 0.016 M sodium hexanesulphonate, 0.02 M tribasic sodium phosphate in acetonitrile-

water pH 3.5 (17:83 v/v), and by employing refractive index detection. It was claimed that neomycin could also be analysed by this method. However, they did not report any evidence for the trace level analysis of neomycin.

Keiji et al.⁸⁶ slightly modified the method developed by Whall so that streptomycin could be determined in serum by HPLC. With an ion-pair mobile phase, the separation was carried out on a reversed-phase LiChrosorb RP-18 column, with detection by UV absorption at 195 nm. Clean-up in the method was achieved using a Sep-Pak C18 cartridge to retain cell debris. The calibration graph of serum streptomycin concentrations was linear over the range 5 - 50 µg/ml. Streptomycin was added to a serum at the level 20 µg/ml and its concentration determined to be 18.9 µg/ml.

It is evident from the levels of streptomycin detected by the methods of Whall⁸⁵ and Keiji⁸⁶ that direct detection of the aminoglycoside by UV, avoiding derivatisation, limits the sensitivity of the method making it inappropriate for use on samples containing streptomycin at residue levels. Therefore to achieve greater sensitivity it is necessary to derivatise streptomycin with chromophoric reagents to enhance its detection and thereby improve sensitivity. This has been achieved by Gerhardt.^{87,88}

Gerhardt et al.⁸⁷ reported a sensitive method to monitor streptomycin and dihydrostreptomycin levels in animal tissues, pork and bovine, muscle and kidney. This method used a novel strong cation-exchange clean-up followed by on-line column enrichment to increase sensitivity. The ion-pair mobile phase contained 10 mM 1-hexanesulphonic acid and 0.4 mM 1,2-naphthoquinone-4-sulphonic acid potassium salt with water-acetonitrile (83:17 v/v) at a pH of 3.3. The separation was carried out on a LC8-DB column. To enhance the sensitivity of the streptomycin and dihydrostreptomycin post-column derivatisation of

both compounds was carried out using 1,2-napthoquinone-4-sulphonic acid for detection by fluorescence, with the excitation wavelength of 347 nm, and the emission wavelength of 418 nm. Clean-up of the sample was then further enhanced and modified. A perchloric acid solution was used to precipitate the proteins. The extract was then loaded onto the cation-exchange and solid-phase extraction column. The drugs were eluted with pH 8 phosphate buffer followed by on-line column enrichment of the eluent to increase sensitivity. The recoveries were 61 % for streptomycin and 55.3 % for dihydrostreptomycin with samples fortified at 40, 80 ppb and 200, 400 ppb of streptomycin and dihydrostreptomycin respectively. The detection limits were 10 ppb for streptomycin and 20 ppb for dihydrostreptomycin.

Gerhardt et al.⁸⁸ went on to use this method for the analysis of streptomycin and dihydrostreptomycin in milk. Under the same conditions,⁸⁷ the recovery of analytes was in the range of 50 - 65 % for skimmed or partially defatted fluid milk samples fortified with 20, 40 ppb and 100, 200 ppb of streptomycin and dihydrostreptomycin, respectively. Recoveries from homogenised whole milk were lower. Limits of quantitation were 10 ppb for streptomycin and 20 ppb for dihydrostreptomycin, which was a thousand fold lower in the concentration measured compared to Keiji's earlier method using direct UV detection of the streptomycin.

(b) Methods using Precolumn derivatisation followed by normal phase chromatography

Tsuji et al.⁸⁹ reported a HPLC method that separated neomycin B, C and neamine as their 2,4-dinitrophenyl (DNP) derivatives on a silica column. This method did not allow simultaneous determination by the isocratic elution of

the components, but involved the use of two slightly different isocratic systems or a gradient system. However, Helboe and Kryger⁹⁰ report an isocratic method version of the Tsuji method⁸⁹ allowing simultaneous determination of neomycin B, C and related substances i.e., neamine, as their 2,4-dinitrophenyl derivatives, on a Zorbax SIL column.

Tsuji and Binns⁹¹ went on to report a HPLC method for the determination of neomycin in petroleum-based ophthalmic and topical ointments and in veterinary formulations. Neomycin assay interferences were from other drug components and were eliminated by a methanol wash and/or a partitioning method. The amino groups in neomycin were derivatised with 2,4-dinitrofluorobenzene (DNFB) followed by normal phase HPLC on a LiChrosorb column, with a mobile phase tetrahydrofuran-chloroform-water-glacial acetic acid (392:598:8:2 v/v/v/v) and detection at 254 nm. The average recovery of neomycin from spiked samples was around 100 % at a level ranging from 22.45 - 37.57 mg/g of neomycin sulphate added to the topical ointments.

A later paper by Tsuji and Jenkins⁹² reported a HPLC method for the assay of neomycin sulphate involving precolumn derivatisation with 2-naphthalenesulphonyl chloride (NSCl) instead of 2,4-dinitrofluorobenzene. The use of NSCl as a derivatising agent allowed for all six primary amines on the neomycin molecule to be derivatised, which was confirmed by mass spectroscopy. The derivatisation was followed by extraction into chloroform and chromatography using a normal phase silica column. The mobile phase contained chloroform-methanol-acetic acid (950:23:25 v/v/v). UV detection was carried out at 254 nm. The standard curve for the HPLC assay of neomycin sulphate was linear over the range 0.02 - 0.4 mg/ml. The average sensitivity of

the NSCI HPLC method fell between those of the microbiological and DNFB-HPLC methods. Also a good correlation of the neomycin C content in neomycin was obtained between the NSCI-HPLC and DNFB-HPLC as used in the previous assay method.⁹¹ However, these methods had not been reported for use on biological matrices.

A normal phase HPLC method for the determination of neomycin in the perilymph collected from the inner ear was reported by Harada et al.⁹³ The method attempted to determine the amount of neomycin that permeated from the middle ear into the inner ear through the round window membrane. The samples, containing the aminoglycoside streptomycin, dihydrostreptomycin or neomycin, were derivatised with benzoyl chloride in pyridine. This introduced the UV-absorbing benzoyl chromophores into both the hydroxyl and amino groups in the form of ester and acid amide linkages, respectively. This led to the introduction of 11 to 13 chromophores into the neomycin molecule, which allowed the determination of a small amount of neomycin in perilymph. No pretreatment of the perilymph was carried out as derivatisation of neomycin could be successfully performed, and any contamination removed in the washing steps of the derivatisation procedure. The derivatives were dissolved in chloroform and injected onto the HPLC column, Zorbax SIL packed with silica gel. The mobile phase contained n-hexane-tetrahydrofuran (50:50 v/v). UV detection was carried out at 230 nm. After topical applications of neomycin on the round window membrane of the guinea pigs, perilymph samples were collected at various time intervals and directly benzoylated. The results of the study showed the permeable property of the round window membrane to neomycin. A standard curve made for the quantitation of neomycin showed a curve linear up to 100 µg of neomycin injected. The lower limit was about 10 ng of neomycin, which was the lowest level of neomycin reported by HPLC

methods using precolumn derivatisation followed by normal phase chromatography.

The HPLC methods summarised above all involve some form of derivatisation, which is a tedious, long process dependent on achieving optimal experimental conditions, i.e. temperature, to ensure complete derivatisation of the aminoglycoside. Also the use of hazardous material like DNFB,⁹¹ which would preferably be avoided. The purpose behind the present project was to investigate and develop a HPLC assay method for MAFF which avoided derivatisation and the tedious sample clean-up steps summarised for each of the HPLC methods.

Other forms of detection which avoid any modification by derivatisation of the aminoglycoside for UV or fluorescence, therefore need to be explored. Therefore alternative approaches to detection could involve mass spectrometry and electrochemical detection.

1.6 Liquid chromatography - mass spectrometry

Detection of the aminoglycosides with or without the aid of derivatisation can be carried out by mass spectrometry, which is principally used to confirm the identity of analytes. In this respect it is very specific. However, the use of mass spectrometry in conjunction with other techniques is costly, the sophisticated instrumentation requiring costly maintenance.

Mass spectrometry was probably firstly used in the analysis of drugs in biological fluids as a device for characterising highly purified metabolites, and

then as a very specific detector, only used for evaluating the specificity of simple, routine procedures. As the need arose for the analysis of less volatile drugs, highly polar drugs such as polypeptides, the advantages of linking HPLC to mass spectrometers (MS) became attractive. However linking of the HPLC to the mass spectrometer brought along its own set of problems, the removal of large amounts of polar solvents, and maintaining polar solutes in the gas phase as ions. The choice of buffers used in the mobile phase is selective to avoid the use of involatile buffers that will not vaporise thereby interfering with the signal and tarnishing the inside of the MS.

Attempts to provide an HPLC-MS interface included enrichment of the effluent using a membrane separator,^{94,95,96,97} and transport of the effluent through vacuum locks on a wire, belt, or ribbon.^{98,99}

There are interface methods that can be used to obtain mass spectra of molecules which may have low molecular weights but have numerous polar functionality's, or have high molecular weights, field desorption is one such technique. Here sharp needles are grown on a wire supported between two posts on the probe. A solution containing a small amount of the sample is deposited on the wire. The field created at the tips of the sharp needles is high enough to cause the discharge of an electron from the sample into the vacant orbitals of the metal from which the wire is made. This leads to the production of the positive ions (M^+). Recently four methods in the interface for the conversion of the molecule to ions have been developed which allow the molecular weight determination of large polar molecules. These are laser desorption, fast atom bombardment (FAB), secondary ion MS (SIMS), and californium plasma desorption. All these methods are based upon giving a large pulse of energy to the sample. The effect of this is to put a relatively large

amount of energy into translational modes involving sample molecules.

Therefore, intermolecular bonds involving the sample for example hydrogen bonds are broken in preference to covalent bonds, and the sample is desorbed from its environment into the gas phase. In laser desorption, the energy is provided by a laser beam, whereas in SIMS or FAB it is provided by a beam of ions of atoms, respectively, of large translational energies. The sample may be bombarded in its solid state, but more commonly as in FAB, it is dissolved in a matrix of low viscosity. When the fast xenon atoms impact into the solution of the sample in the matrix, the sample is desorbed often as an ion.

A number of studies have been carried out and papers published on the detection of aminoglycosides by mass spectrometry after separation by HPLC.

Samain and co-workers^{100,101} described the use of perfluorinated counter ions for the combination of ion-pair HPLC and off-line field desorption M.S. in the separation of aminoglycoside antibiotics. The perfluorinated carboxylic acids investigated were heptafluorobutyric, pentafluoropropionic and trifluoroacetic acid. The use of these volatile counter ions allowed easy recovery of pure micro samples of antibiotics after chromatography which were readily analysable by field desorption-MS. They reported that no ions were observed for streptomycin due to the presence of 2 guanidine residues. Neomycin, however, recorded satisfactory FD mass spectra with a mass/charge ratio (m/z) of 615, compared to the published molecular weight at 614, on using μg amounts of injected and recovered material. It was found that good results could be obtained on 1 μg samples when sample handling was reduced to a minimum. They found that the perfluorinated carboxylic acids exhibited the same properties as conventional perhydrogenated ion-pairing ions, such as the alkylsulphonates used in the chromatographic separation of aminoglycosides. The chromatographic conditions could be easily changed to suit a given

separation by varying the solvent composition and changing the nature and the concentration of the pairing ion. The column efficiency increased with increasing concentration of the pairing ions. The best results were obtained with pentafluoropropionic acid (PFPA). Unlike the other aminoglycosides, streptomycin exhibited a higher efficiency with heptafluorobutyric acid (HFBA) than with PFPA. However, due to the acidity of the pairing-ion and its effect on the column Samain made a compromise between efficiency and acidity. Trifluoroacetic acid (TFA) was not found to be effective for retaining the aminoglycosides, with the exception of the gentamicins. Samain made an attempt to account for the chromatographic behavior of the aminoglycosides with perfluorinated pairing ions. The mechanism was described as a concerted ionic and hydrophobic interaction, which is highly dependent on the structure of the aminoglycoside and the chain length of the pairing ion.

Henion and McLaughlin¹⁰² described studies that extended the work of Samain and co-workers^{100,101} and developed an LC-MS assay technique to confirm aminoglycoside residues in bovine tissues. A reversed-phase ion-pair method for the separation of four aminoglycosides: spectinomycin, hygromycin B, streptomycin and dihydrostreptomycin was developed using the volatile ion-pairing agents, PFPA and HFBA, and was optimised for detection with an ion-spray HPLC-MS interface. With the postcolumn addition of a strong base the method was also used in conjunction with pulsed amperometric detection (PAD) see later section 1.7. Their results also showed that bovine kidney fortified at levels of 20 ppm could be extracted by matrix solid-phase dispersion, a relatively new sample preparation technique dealt with in more detail in section 1.10.3. They found that increasing the concentration of PFPA in the eluent decreased the ion-spray sensitivity. However, a concentration of at least 10 mM was necessary for reasonable chromatographic resolution on the

columns tested. A concentration of 20 mM PFPA in acetonitrile-water (8:92 v/v) gave the best separation efficiency and resolution on the Spherisorb ODS-2 column. Use of the PAD for screening and quantification in the sub-ppm range, needed further clean-up of the bovine kidney extracts and further concentration of the aminoglycosides. This was also the case with the ion-spray HPLC-MS method.

McLaughlin and Henion¹⁰³ continued their work to improve and modify the assay, to achieve the analysis of a wide range of aminoglycosides and also, carry out the detection of these aminoglycosides at levels set by the Federal Drug Administration FDA. To date they have reported¹⁰³ an ion spray high-performance liquid chromatographic/tandem mass spectrometric (HPLC/MS/MS) method capable of determining the following six aminoglycosides in bovine kidney: spectinomycin, hygromycin B, streptomycin, dihydrostreptomycin, gentamicin C complex and neomycin B. Tobramycin was used as the internal standard. The modified matrix solid-phase dispersion technique is covered in section 1.10.3 of this chapter. This method used a gradient HPLC separation with mobile phases containing 20 mM PFPA and acetonitrile. Protonated molecules served as precursor ions for collision-induced dissociation (CID) and 3 product ions were chosen for each analyte for selected reaction monitoring (SRM) where possible. A validation study was conducted for the confirmation of dihydrostreptomycin, neomycin B and four major components of the gentamicin C complex through SRM HPLC/MS/MS analysis of negative control, spiked (dosing 3 Holstein steers each with a different aminoglycoside) bovine kidney samples. The use of 'up-front' CID can provide an improved ion current signal for the analytes, but the high matrix interferences from the tissue samples requires the selectivity of MS/MS. This selectivity of SRM LC/MS/MS was apparent from absence of

matrix interferences for any of the analytes. Five of the six compounds of interest could be determined at levels set by the FDA. All compounds except spectinomycin could be detected in bovine kidney tissue at or below the regulatory level of concern. Streptomycin fortified at 1 ppm had a mean recovery of 67 %, dihydrostreptomycin spiked at 0.8 ppm had a mean recovery of 75 % and neomycin spiked at 1.0 ppm had a mean recovery of 46 %. These recoveries were lower than those obtained by Shaikh^{80,83} and Gerhardt⁸⁷ using the conventional postcolumn derivatisation for fluorescence detection and sample clean-up using a precipitation and centrifugation technique.

In a recent paper Aschbacher and Feil²⁴ carried out an investigation into the metabolism of neomycin in calves using a combination of these techniques. This was achieved by a study into the disposition of oral neomycin in calves using ¹⁴C-labeled neomycin. The influences of age, diet and method of administration were observed. All calves were killed after a single oral dose of ¹⁴C neomycin (approximately 30 mg/kg) and the distribution of ¹⁴C in excreta and tissues was determined. Extraction of neomycin from the kidney tissue involved the use of a hot-base method or the matrix solid-phase dispersion method (covered in more detail in section 1.10.3). The samples in each case were concentrated further. The concentrated extracts from both extraction procedures were then chromatographed on a cation exchange column (CG-50). A TLC system was used for final clean-up of kidney tissue extracts. The silica gel in the radioactive area was scraped from the plate and eluted. The isolated radioactivity compound was then characterised by positive-ion fast atom bombardment mass spectrometry (+ FAB MS) and NMR spectroscopy. A similar extraction and clean-up procedure was used to recover ¹⁴C compounds from faeces. Radioassays were carried out on the samples of urine and blood plasma for ¹⁴C directly in "ecolite" scintillation cocktail. Chromatography was

also carried out on the samples, it involved the use of a C8 reversed-phase column followed by post-column derivatisation of the neomycin by o-phthaldehyde and monitored with a fluorescence detector (excitation 350 nm, emission 450 nm). Extraction of kidney tissue by either the hot-base method or the matrix solid phase dispersion MSPD method extracted 90 to 95 % of the ^{14}C present. Recoveries were based on combustion analysis of the residue after extraction. The study found that faeces were the major route of excretion in all calves, kidneys had the highest ^{14}C concentration. The next highest concentration was in livers. The kidney residues of neomycin equivalents observed in this study exceeded the current tolerance in the US for neomycin in edible tissue at 0.25 ppm. Therefore the results indicated that the use of neomycin in veal production at recommended levels would cause kidney residues of above the 0.25 ppm limit.

1.7 Liquid chromatography - pulsed amperometric detection

An alternative approach which has been used for the HPLC detection of carbohydrates, amino acids and alkanolamines is pulsed amperometric detection.^{104,105,106,107} This method avoids the need for derivatisation or expensive equipment like mass spectrometry. Pulsed amperometric detection (PAD) involves the redox reaction of the analyte at a working electrode. The current produced in the redox reaction is measured by comparison with a reference and is proportional to the amount of analyte present. Electrochemical detection depends on the property of analytes to be oxidised or reduced, the resulting electron flow being the basis of the detection principle. These electrochemical detectors are based on the fact that the current is measured as a function of time with a constant potential applied at a fixed electrode exposed to a moving fluid. The drug in the detector cell is converted to its oxidised

form, yielding one or more electrons per molecule reacted. The oxidised form is usually unstable, and reacts further to form a stable compound which passes out of the cell. The instantaneous current is proportional to the concentration of drug in the cell. Improvements in design and theoretical considerations have made electrochemical detectors more widely used in drug analysis and their sensitivities rival those of fluorescence detectors under favorable conditions.²⁰ However, compounds, containing multiple hydroxyl groups, such as carbohydrates, alcohols and similar compounds lead to the loss of electrode activity of the working electrode surface, due to the accumulation of strongly adsorbed reaction products.¹⁰⁸ Adsorbed reaction products can be oxidatively desorbed quite efficiently from these electrodes by application of a large positive potential excursion, which causes the formation of surface oxide.^{109,110,111,112,113} The oxide is cathodically dissolved by a negative potential excursion to clean and restore the native reactivity of the clean metal surfaces. The pulsed electrochemical detector (PAD) uses these sequence of events in its operation by incorporating the desorption and cleaning step using multiple potentials which are applied in a repeating sequence.^{72,108,114,115,116,117,118,119,120}

All aldehydes, simple alcohols, glycols, polyalcohols and carbohydrates can be detected by PAD^{114,115,116} at gold and platinum electrodes in alkaline media (pH greater ~ 12). However, use of gold electrodes has the distinct advantage that detection can be achieved without simultaneous reduction of dissolved oxygen.¹⁰⁹ The maximum anodic signal for polyalcohols and carbohydrates in 0.1 M sodium hydroxide is obtained at $E = +0.1 - +0.2$ V and a value in this range is chosen for the measuring potential E_1 in the PAD waveform (Figure 1.6). The rates of anodic mechanisms at gold electrodes decline with increasing solution acidity, and virtually no response for alcohols is obtained for $\text{pH} < \text{or} = 12$. The enhancement of oxidation rates by high alkalinity results from H^+

production in reaction steps leading to the rate determining step.¹²¹

Aminoglycosides have the same organic groups as carbohydrates and therefore the PAD is demonstrated¹²² to be applicable for the detection of aminoglycosides.

Conventional commercial electrochemical detectors operate by applying a single fixed potential to the working electrode in a flow-through cell. As mentioned earlier compounds containing multiple hydroxyl groups, such as carbohydrates and similar compounds, lead to the loss of electrode activity of the working electrode surface, due to the accumulation of strongly adsorbed reaction products. PAD avoids this using multiple potentials which are applied in a repeating sequence. The detector measures the current over a very short sampling period. Then pulsing at a large positive or negative potential results in the removal of the reaction products from the surface of the working electrode.

The working electrode is pulsed with three different potentials for defined times:

The first potential (E1), the measuring potential, is applied to the electrode. A delay is incorporated to allow the surface to become fully charged and the analyte current is subsequently measured.

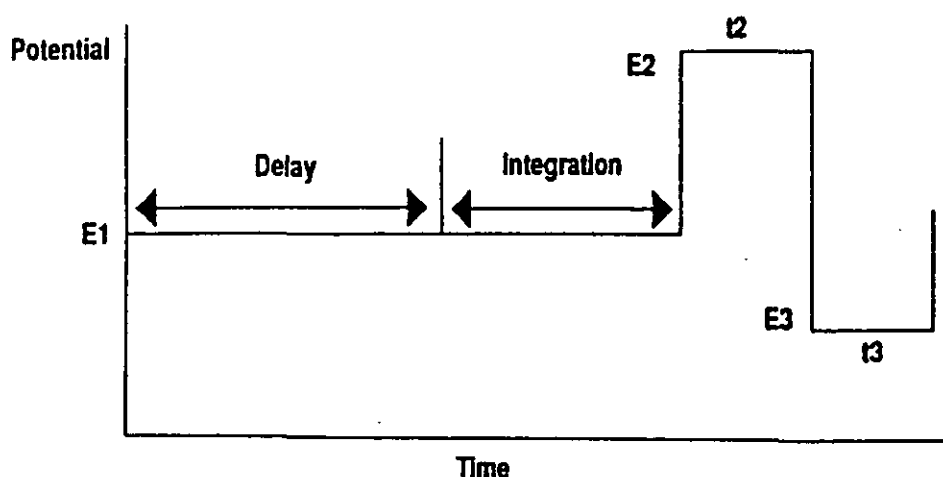
The second potential (E2), the cleaning potential, is large and positive. It is used to desorb the surface contaminants, which are now present on the electrode.

The third potential (E3), is large and negative. This potential is applied to remove the surface oxide produced during the cleaning pulse and leave the surface ready for use.

These potentials can be summarised in the form of a three step waveform (Figure 1.6)

In order to be able to select a suitable measuring potential value the analyte is examined by using a technique known as cyclic voltammetry. In cyclic voltammetry, the applied potential is slowly scanned back and forth between a positive and a negative potential limit. The resulting current across the cell is plotted on the y-axis with oxidation (anodic) currents up and reduction (cathodic) currents down.

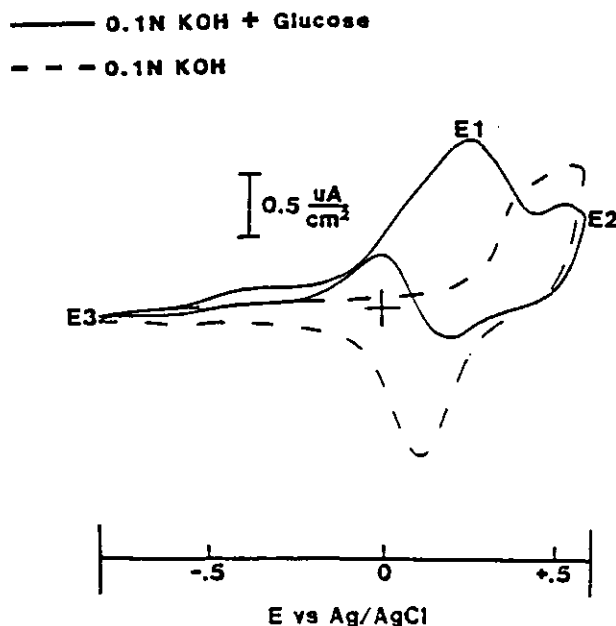
Figure 1.6 Pulsed amperometric detection with a 3 step waveform



An example of the traces obtained for a carbohydrate is shown in Figure 1.7 for glucose on a gold electrode in 100 mM potassium hydroxide and for a background scan of 100 mM potassium hydroxide solution without glucose present. It shows oxidation of the potassium hydroxide solution at the gold surface beginning at about +0.25 volts and peaking at +0.5 volts. The reduction of the surface gold oxide back to gold occurs on the reverse scan, with a peak at about +0.1 volts. With glucose added to the solution, glucose oxidation peaks

at about +0.25 volts. On the reverse scan the current reverses from negative to positive at the onset of the gold oxide reduction. This implies that the glucose oxidation occurring at the bare surface, was inhibited upon the formation of gold oxide. As soon as the reduction of the gold oxide back to gold begins, glucose oxidation also begins. It may appear that maximum detector sensitivity would be obtained at a potential of + 0.25 volts. However at this point the detector 'noise' is increasing rapidly. Therefore the maximum sensitivity (on a signal to noise basis) is achieved at an potential of + 0.05 volts to + 0.1 volts, the potential chosen for the current measurement of glucose is + 0.1 volts. Bearing this in mind attempts have been made to detect aminoglycosides in this way by Johnson and Polta¹²² and the application chemists at Dionex.¹²³

Figure 1.7 Cyclic voltammetry of glucose on a gold electrode



Polta and Johnson¹²² reported the development of a PAD method for the sensitive detection of several nebramycin aminoglycosides following separation on a neutral polystyrene column. The eluent used was 0.25 M sodium hydroxide, which was also appropriate as the electrolyte for the electrochemical detection. Sample concentrations were detected at 1 ppm in 50 µl samples (50 ng) after the use of a coupled pre-column of HPLC-CS1 for on-line preconcentration. Determination of nebramycin factors in fermentation broths and of tobramycin and apramycin in blood serum were carried out. The blood serum was analysed with deproteination and filtration as the only pre-injection steps.

Dionex reported a method¹²³ that determined neomycin B and C in topical lotions, using high performance anion exchange chromatography on a CarboPac PA1 column using a dilute 10 mM sodium hydroxide eluent. The addition of 0.5 M sodium hydroxide postcolumn increased the sensitivity of the detector, and the PAD electrochemically oxidises the neomycin at a pH of about 13 using a gold electrode. The PAD response for neomycin B was linear over the range 10 - 1000 ng. The minimum detection limit was 5 ng. However so far none of these methods applied to trace levels of neomycin in tissue.

More recently Schermerhorn *et al.*¹²⁴ developed a method that determined the amount of a related aminoglycoside spectinomycin residues in bovine milk using liquid chromatography with electrochemical detection. The method was capable of quantifying spectinomycin in raw bovine milk, and was developed and validated for the 100 - 400 µg/ml range. Sample preparation procedure involved centrifugation of the milk at -4 °C and removal of the top fat layer. The defatted milk was deproteinated by precipitation with 30 % trichloroacetic acid and centrifugation. The supernatant was washed sequentially with

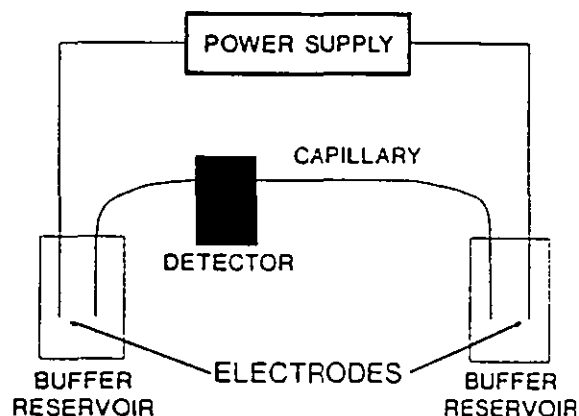
dichloromethane, hexane and ethyl acetate. An aliquot of the separated aqueous layer was prepared for HPLC analysis by mixing with the ion-pair 1-decanesulphonic acid and filtering. The analyte was separated from the matrix components using an ion-pair mobile phase, buffer-acetonitrile (84:16 v/v), the ion-pair buffer was 0.02 M citric acid and 0.002 M 1-decanesulphonic acid sodium salt solution adjusted to pH 6.1 with 50 % sodium hydroxide. Separation was carried out on a reversed-phase column. The analyte was then quantified with a electrochemical detector at the following detector settings $E_1 = 650 \text{ mV}$, $E_2 = 850 \text{ mV}$. Mean recoveries were 80 % for milk fortified at 100 ng/ml, 76 % for milk fortified at 200 ng/ml, and 77 % for milk fortified at 400 ng/ml. The LOD for this method was approximately 50 ng/ml. This method was claimed to be simple, rapid and direct, 6 - 10 samples could be prepared in a normal eight hour working day by a single analyst. However, this method has been applied to determine levels of the aminoglycoside at the microgram and not nanogram residue level.

1.8 Capillary electrophoresis

An alternative method that could potentially be used for aminoglycosides is capillary electrophoresis as under appropriate conditions the aminoglycosides can be ionised. Capillary electrophoresis as an instrumental method is relatively new but has attracted considerable interest. The capillary electrophoresis system consists of a buffer filled capillary placed between two buffer reservoirs, and a potential field, which is applied across this capillary (Figure 1.8). Ions in the sample are drawn through the capillary to the detector. An important phenomenon which takes place in capillary electrophoresis is electroosmosis, this refers to the flow of buffer solution in a applied potential field. Capillaries used for capillary electrophoresis are made of fused silica,

containing surface silanol groups. The silanol groups become ionised in the presence of electrophoretic medium resulting in the walls having a net negative charge.

Figure 1.8 A Basic Capillary Electrophoresis system.



Positively charged cations in the buffer will associate with the anionic species on the capillary wall. When a voltage is applied the cationic species migrate towards the cathode and because they are solvated this has the effect of dragging the solvent molecules within the cations. This flow of liquid through the capillary is called electroosmotic flow. Therefore most anions, although attracted to the anode, are swept towards the cathode with the bulk flow of the liquid in the capillary because migration is less than flow, this is referred to as the cathodic mode. Therefore under these conditions, cations with the highest charge/size ratio migrate and are detected first, all neutral components are then migrated at the electroosmotic front as their charge/size ratio is zero and finally anions with lower charge/size ratio migrate earlier than those with greater charge/size ratio. The migration of analytes is towards the cathode, and hence a detector is placed at this end. Injection of solutes is performed at the anodic end by pressure or voltage.

In the case of some compounds that are not separated well in the cathodic mode as they are attracted to the capillary wall, an alternative mode of separation can be achieved by capillary electrophoresis. The flow of the ions can be reversed so that the bulk flow of the solvent is towards the anode. This electroosmotic flow can be achieved by the addition of a surfactant additive. The surfactant additive is added to the electrolyte, it shields the negative charge of the surface silanol groups of the capillary tube from the normal negative charge and changes it to the reversed positive charge. Now negatively charged anions in the buffer will associate with the cationic species on the capillary wall. When a voltage is applied the anionic species migrate towards the anode. This results in the effective mobility of the analytes to detection at the anode instead of typically towards the cathode, this is referred to as the anionic mode.

Almost all organic drugs can exist as acids or bases and therefore the pH has a major effect on the selectivity of the separation. For specific compounds, it is useful to set the pH near the pKa value because any changes around this value have the greatest effect on mobility and therefore the separation can be fine tuned most effectively by changes in pH.¹²⁵ Further changes in selectivity can be achieved¹²⁶ in capillary electrophoresis by complexation, by the addition of organic solvents¹²⁷ by changing temperature,¹²⁸ and most significantly by the use of micelles.

1.8.1 Application of Capillary Electrophoresis

During the course of the project, a paper by Ackermans and Everaerts reported a method for the determination of aminoglycosides by capillary zone electrophoresis CZE.¹²⁹ Detection of the aminoglycosides was achieved by indirect UV in the anionic mode by reversed electroosmotic flow achieved by the addition of a surfactant additive referred to as FC 135 to the background electrolyte of 0.01 M imidazole. The addition of a surfactant additive FC 135 to the electrolyte was claimed to shield the negative charge of the surface silanol groups of the capillary tube from the normal negative charge and change it to the reversed positive charge, which reversed the electroosmotic flow.

Ackerman¹²⁹ found that using the conventional cathodic mode produced very bad peak shapes, due to strong attractive forces between the highly positively charged components and the negatively charged capillary wall, and low resolution was the result. Therefore the alternative anodic mode with reversed electroosmotic flow was used to suppress the attractive forces between the analytes and the capillary wall. Under these conditions the effective mobilities of thirteen aminoglycosides were measured as a function of pH. At an optimum pH of about 5, aminoglycosides could easily be determined.

Ackerman also found that by applying CZE with indirect UV detection in the anionic mode and reversed electroosmotic flow coupled with micellar electrokinetic capillary chromatography with the cationic surfactant cetyltrimethylammonium bromide, both charged and neutral antibiotics can be determined in pharmaceuticals. Cetyltrimethylammonium bromide acts as a micelle-forming surfactant in the background electrolyte¹²⁹. Neomycin and hydrocortisone were determined in otosporin ear drops and the values obtained agreed with the labeled values of 5.00 mg/ml the labeled value for

neomycin and 10 mg/ml the labeled value for hydrocortisone.¹²⁹

Other work was also reported in this area, by Flurer and Wolnik.¹³⁰ They reported a method for the determination of the aminoglycoside gentamicin sulphate in injectable solutions by CE. A borate buffer 0.15 M at pH 9.4 was used to separate the individual components, and their detection by direct UV detection at 195 nm. This method provided, qualitative and quantitative information without resorting to alteration of CE conditions to the anionic mode or indirect UV detection as used in the Ackerman method¹²⁹ which could not produce resolution of the individual components of gentamicin. It was claimed that the use of total peak area for quantitation of gentamicin base in injectable solutions lead to values that agreed well with those obtained with the accepted U.S.P. (U.S.Pharmacopoeia) microbial assay,¹³¹ and demonstrated reproducibility over different-capillary/buffer combinations.

The CE methods reported do not require sample derivatisation unlike the majority of the HPLC methods discussed. Both methods reported provide quantitative as well as qualitative information and require a relatively short analysis time. The use of less reagent and the equilibration of experimental conditions is much quicker. However, unlike the HPLC separation methods there was no evidence to suggest capillary electrophoresis could be used to analyse trace levels of aminoglycosides extracted from animal tissue which was a area that needed to be investigated.

1.9 Other methods

Gala¹³² took an alternative approach to the analysis of aminoglycosides, this involved the use of a fast spectrofluorimetric method for the determination of neomycin using a stopped-flow mixing technique. The method involves

reaction of neomycin with o-phthaldehyde (OPA) in the presence of N-acetylcysteine, which is used as the thiol reagent. OPA was chosen as a reagent as it reacts easily and rapidly with primary amines in combination with a thiol in an alkaline medium to give highly fluorescent substituted isoindoles. The calibration graph was linear over the range 0.07 - 70 µg/ml of neomycin. The mid-range relative standard deviation was less than 3 %. The proposed method was applied to the determination of the aminoglycoside in pharmaceutical samples, with a recovery of 98.1 - 103.6 % for neomycin.

Halvatzis used a continuous-flow method for the determination of streptomycin at 2.00 - 30.0 µg/ml.¹³³ The method is based on the chemiluminogenic (CL) oxidation of the analytes by N-bromosuccinimide in alkaline solution. The procedure is automated and solutions can be analysed at a rate of 250 h⁻¹ with a relative error of about 1.6 %. When applied to commercial formulations, the procedure was relatively free from interferences from common excipients. The accuracy of the continuous-flow CL method for the determination of streptomycin was examined by performing recovery experiments on solutions prepared from various pharmaceutical preparations. Mean recoveries were 95.5 % (range 93.3 - 96.8 %) of streptomycin at 5 µg/ml.

Aman et al. has determined streptomycin spectrophotometrically.¹³⁴ Streptomycin is reacted with ninhydrin in alkaline medium to give a violet colour having maximum absorbance at 560 nm. The reaction is specific for streptomycin with 0.1 µg/ml as minimum limit of detection. It provides the basis for a new spectrophotometric determination of pure streptomycin and in the presence of the organic compounds. The method like the other methods discussed in this section, is applicable to the determination of streptomycin from pharmaceutical preparation.

1.10 Sample preparation approaches to extraction of aminoglycosides

This section outlines methods and techniques used for the extraction of drugs from biological fluids and tissue at trace levels. This is further detailed in the extraction of aminoglycosides particularly in kidney tissue, milk and plasma which have been cited in literature.^{24,80,82,87,102,103,135} From a general overview of the sample preparation methods in terms of the problems encountered and the progress made, an attempt can then be made in the practical work to develop a suitable sample preparation method, which can be used on-line with the analysis by HPLC.

Residue analysis requires the isolation of the analyte aminoglycoside from a more complex matrix than is usual for other applications. It also requires a greater degree of sensitivity in the detection method. Therefore to achieve the detection of the analyte residues, the clean-up involved in the sample preparation has to be thorough to avoid contamination of the sample for analysis. Also, concentration of the analyte prior to analysis will increase the sensitivity of the analyte.

The first problem in the extraction of the drug from the matrix is to separate the drug from as much of the endogenous material as possible. The ease with which samples can be analysed increases with the degree of fluidity, cerebrospinal fluid generally being the easiest fluid to handle, while tissue the most difficult. To increase the fluidity, solids, and semisolids submitted for analysis are often subjected to mechanical procedures. These procedures may affect the sample in several undesirable ways, some leading to actual changes in concentration of the drug in the sample (temperature effects, conjugate

hydrolysis) and some actually making subsequent handling more difficult (foaming, emulsions). The solvent used as the medium for such procedures is critical and Maikel¹³⁶ has reviewed the general advantages and disadvantages of most often used aqueous systems Table 1.5. the eventual aim is to extract as much of the drug as possible, but leaving behind the undesired interfering material. This is most commonly done by a combination of steps where the polarity of the extracting solvent or the pH of the aqueous phase is adjusted. Sample extraction techniques are primarily protein precipitation and liquid-liquid extraction.

In protein precipitation concentrated acid or solvent (for example trifluoroacetic acid (TFA) or acetonitrile) will precipitate serum proteins from a plasma matrix; the proteins are then removed by filtration or centrifugation. Protein precipitation has two major drawbacks:¹³⁷

1. Strongly protein-bound analytes may also precipitate, resulting in low, irreproducible recoveries;
2. The sensitivity decreases because precipitation requires sample dilution; and many interfering constituents may remain in solution.

Liquid-liquid extraction is a selective reproducible technique particularly suited for non-polar drugs. Solvents, such as ethyl ether and ethyl acetate can often isolate non-polar drugs from serum. However, scaling up and automation can present problems. Also, a small choice of solvents makes liquid-liquid extraction of limited use for the extraction of polar drugs and metabolites.

TABLE 1.5 CHARACTERISTICS OF AQUEOUS SOLVENTS USED FOR PREPARING BIOLOGICAL SAMPLES PRIOR TO SOLVENT EXTRACTION³⁶

Solvent	Advantages	Disadvantages
Distilled Water	Relatively good solvent. Does not destroy tissue constituents. Final pH near 7.0.	Degree of ionisation may vary. Does not denature enzymes consistently.
Dilute acid (<0.5 N)	Relatively good solvent. Denatures many enzymes. Final pH < 7.0. Foaming is minimal.	Considerable protein denaturation. Compounds may be acid sensitive.
Strong acid (>0.5 N)	Good solvent. Denatures all enzymes. Precipitates proteins. Final pH < 4.0.	Clumping and aggregation may occur. Compounds may be acid sensitive. Tissue constituents may break down.
Dilute alkali (<0.5 N)	Relatively good solvent. Denatures many enzymes. Final pH > 7.0.	Considerable protein denaturation. Compounds may be alkali sensitive. May cause foaming (soaps).
Strong alkali (>0.5 N)	Relatively good solvent. Denatures all enzymes. precipitates proteins. Final pH > 10.0.	Clumping and aggregation may occur. Compounds may be alkali sensitive. Tissue constituents may break down. Foaming generally serious .

The extraction method required depends on firstly the characteristic chemical properties of the analyte and secondly the biological matrix from which the analyte is being extracted from i.e. milk, kidney tissue or muscle tissue. It was noted earlier (section 1.4) that the chemical, polar, basic and hydrophilic nature of aminoglycosides like neomycin and streptomycin excludes them from the conventional method of organic solvent/liquid extraction. Both neomycin and streptomycin are insoluble in organic solvents and freely soluble in water. Therefore alternative approaches needed to be investigated.

1.10.1 Conventional methods used in the extraction of aminoglycosides from animal tissue, plasma and urine

The main drawbacks to the present existing clean-up extraction procedures were extensive sample handling requiring long and tedious steps to defat, deproteinate and centrifuge the sample in order to separate the neomycin or streptomycin from the tissue homogenate. This results in a high degree of sample manipulation, which can lead to poor recoveries for those analysts not familiar with these procedures. They also take time and do not lend themselves to automation.

1.10.1.1 Tissue Samples

Moats presented an extensive literature review of residue analysis methods of antibiotics with reference to the aminoglycosides.¹³⁸ The review covers the different extraction methods used for the different drugs and by different analysts. The disadvantages and advantages of each sample extraction method were also discussed. Moats listed approaches that have been used for the extraction and/or deproteinisation methods used for antibiotic residue analysis Table 1.6.¹³⁸

TABLE 1.6 APPROACHES USED FOR THE EXTRACTION PROCEDURES USED IN AMINOGLYCOSIDE RESIDUE ANALYSIS.

Approach	Compound / Substrate	Reference
Trichloroacetic acid with sodium hydroxide	Kanamycin/Tissue	139
	Tobramycin/Kidney	140
Buffer extract/heat	Neomycin /Tissues	80
Strong Alkali (sodium hydroxide)	Tobramycin/Kidney	141
Solid phase extraction	Drugs/Enzymatically liquefied tissue	142

After trying a number of the approaches mentioned in table 1.6 Moats concluded that extraction/deproteinisation with water-miscible organic solvents was simple, and broadly applicable for antibiotics. Moats found that the advantages of extraction/deproteinisation with water-miscible organic solvents are:

1. The procedure was rapid and simple and, with pH adjustment, appeared to be universally applicable
2. Recoveries were generally consistent and high (near 100%)
3. Because residues were uniformly distributed in the melange formed by addition of organic solvents, an aliquot of filtrate could be taken as equivalent to a given amount of original sample.

Disadvantages cited with the use of organic solvents:

1. They were somewhat toxic
2. Organic solvents are usually, but not always removed from extracts prior to reversed-phase chromatography

3. Water-insoluble interferences co-extracted
4. Aminoglycosides solubility in solvents is poor and therefore could not be extracted this way

Moats found that the simplest approach was to concentrate the analyte directly from the filtrate on the LC column and then to elute with a solvent gradient for analysis. For this approach to be successful, the sample must be injected in a solvent with no eluting strength at all. Most compounds were not retained on reversed-phase packings from the filtrates because of the high organic solvent concentrations. The organic solvent could be removed by evaporation or by extraction with a water-immiscible organic solvent. Petz,¹⁴³ Malisch and Huber¹⁴⁴ found that many compounds could be recovered in the organic layer formed after salt (sometimes methylene chloride) was added to acetonitrile filtrates. This procedure will not work with very polar compounds like the aminoglycosides. The compounds of interest can be eluted as narrow bands by using a solvent gradient. Narrow fractions can be collected by a procedure termed "heart-cutting"¹⁴⁵ and rechromatographed under different conditions. Concentration directly on the LC column offers some advantages over use of disposal cartridges.

1. Results were reproducible since the same column was used repeatedly
2. No activation was required
3. There was no contamination from the column
4. Column efficiency was much greater so that more precise fractionation was possible

In a typical example Shaikh et al.⁸⁰ used buffer extraction and heat to extract neomycin from bovine and porcine kidney and muscle tissue. The tissue was

homogenised in potassium phosphate buffer, followed by centrifugation. The supernate was then heated to precipitate the protein and extract the neomycin.

Gerhardt et al.⁸⁷ carried out the clean-up and extraction of streptomycin and dihydrostreptomycin from bovine and pork, muscle and kidney tissue by deproteination using perchloric acid followed by further clean-up on a solid phase extraction column. Dilute perchloric acid was added to the tissue followed by homogenisation and then centrifugation. The supernate was then passed through a cation-exchange solid-phase extraction column and rinsed with water for further clean-up. Streptomycin and dihydrostreptomycin were then eluted off by 0.2 M phosphate buffer and analysed by HPLC.

1.10.1.2 Extraction of aminoglycosides from plasma and Urine

Plasma contains large amounts of protein. There is often a strong affinity between such proteins and drugs, and straight removal of protein in plasma by example ultrafiltration or dialysis could also remove a large fraction of the drug. Any direct measurement of drug could miss the total drug present and measure only "free" drug. The free drug concentrations are extremely low and it is usual to measure the total drug in the plasma sample. To obtain a protein-free aqueous solution precipitated and the filtrate isolated. The protein is denatured by the precipitation and its drug-binding ability is destroyed. Popular acidic reagents for protein precipitation include trichloroacetic acid, perchloric acid, and tungstic acid. The main drawback here is that such strong acids could have detrimental effects on the drug to be extracted. The instability of drugs at low pH can be overcome by using organic solvents to denature and precipitate proteins. Dell¹⁴⁶ recommended methanol or ethanol, with at least

two volumes of ethanol being necessary to precipitate all plasma proteins. Proteolytic enzymes can also be used to denature proteins. This procedure would avoid the possibility of damage to the analyte using chemical-type denaturation. Such procedures are generally found in the preparation of tissue for drug analysis, but the enzyme subtilisin has been successfully used for the digestion of plasma proteins. Osselton¹⁴⁷ showed that the better recoveries of drug from tissue using enzyme hydrolysis compared with direct extraction was also obtained in the analysis of plasma.

Urine unlike plasma, is generally free of protein and lipids and therefore does not require deproteinisation or lipid clean-up and can be directly extracted for the drug. The normal type of compounds found in urine are water soluble. One of the greatest difficulties that arises, is the volume of urine that may be produced over fixed time intervals. For most analytical tests it is usual to perform the analysis on a fixed volume of sample; with urinary excretion, it is the amount of substance excreted in a certain interval which is of interest, not its concentration, and the amount is obtained by multiplying the volume of the concentration. Problems arise if the urine volume is large, then the sample becomes too dilute and the method may be operating near its L.O.D., and the inevitable errors at the low concentrations will be multiplied on adjustment for the volume. Urine has a wide range of pH values, predicted to a great extent on the diet, or medication. The nature of the excreted drug could depend on the pH at this time. Weakly basic drugs are more efficiently excreted in acid urine, whereas weakly acidic drugs are more excreted in alkaline urine.¹⁴⁸

Shaikh⁸² Cleaned-up plasma and urine samples by deproteination using trichloroacetic acid and centrifugation from which neomycin was extracted in the supernate. Determination of a related aminoglycoside Spectinomycin, in

turkey plasma by Tyczkowski and co-workers¹⁴⁹ involved deproteinisation by trifluoroacetic acid in acetonitrile and centrifugation.

1.10.1.3 Extraction of aminoglycosides from milk

Milk is not a very usual fluid for the analysis of drugs, but it is occasionally of interest when trying to establish whether drugs may be transferred from mother to infant by this route. The main problem with the extraction procedure would be the removal of fats present in the milk.²⁰ Most authors seem to be able to adapt existing methods for urine or serum.^{81,82,88} For the extraction of neomycin from milk by Shaikh et al.,⁸¹ firstly clean-up of the milk was carried out by centrifugation at 4°C, this removed the fat, followed by deproteination by the addition of trichloroacetic acid and centrifugation again. Gerhardt *et al.*⁸⁸ extracted streptomycin and dihydrostreptomycin from milk using the same clean-up procedure for animal tissue with the exclusion of the solid phase extraction step, discussed in section 1.10.1.

Agarwal⁸⁴ in his approach to the determination of neomycin in milk used an Amberlite CG-50 ion-exchange resin column to isolate the neomycin from the milk avoiding deproteination or defatting steps. The milk was passed directly through the ion-exchange column, the neomycin retained on the column was then derivatised in situ with OPA reagent. The derivatised neomycin was then eluted from the column with potassium borate buffer/methanol and analysed by HPLC. The use of the HISEP (internal surface reversed-phase) column which contains hydrophobic regions shielded by a hydrophilic network excluding the large protein molecules from contact with the inner hydrophobic regions. Thus protein molecules are passed through unretained on the column

which allowed for separation, allowing for further isolation of the derivatised neomycin from unnecessary contaminant protein molecules.

1.10.2 The use of solid phase extraction cartridges for sample clean-up.

An alternative to deproteinisation and liquid - liquid extraction is solid phase extraction (SPE). However, this technique is sometimes used in combination with deproteinisation^{87,88} or liquid-liquid extraction. SPE involves a liquid and a solid phase. In sorbent extraction the solid phase has a greater attraction for the isolate than the solvent in which the isolate is dissolved. As the sample solution passes through the sorbent bed, the isolate concentrates on this surface, while the other sample components pass through the bed.¹⁵⁰ Very selective extractions resulting in highly purified and concentrated isolates can be achieved by choosing sorbents with an attraction for the isolate but not for the sample components.

Compared with the other two techniques of deproteinisation and liquid-liquid extraction SPE typically provides the cleanest samples for analysis, as it avoids the formation of emulsions, and, in most cases, reduces the volume of solvents required for efficient isolation of the compounds of interest. However, the use of SPE for the isolation of drugs, environmental contaminants and natural compounds from tissues has so far required sample homogenisation and removal of tissue debris prior to column application. Addition of homogenates directly to the top of a column invariably leads to the cessation of flow because of the plugging of the frit or upper layers of the column packing. Solid phase extraction cartridges can be used for the direct extraction of the drug from plasma or serum requiring no prior sample handling.

It appears that most of the methods isolate the neomycin from the matrix by employing its basicity, releasing neomycin from the matrix by lowering the pH.

1.10.3 Matrix solid-phase dispersion

The sample preparation techniques covered so far in section 1.10.1, of sample homogenisation, centrifugation, precipitation, etc., remain labour intensive and contribute, through sample manipulation and the entrainment or electrostatic binding of some drugs to tissue debris, to lower than ideal recoveries. Therefore an alternative sample preparation technique had to be employed.

A relatively new sample preparation method based on solid phase dispersion of tissue for the subsequent isolation of drugs was reported by Barker et al.¹⁵¹ By blending tissues with a polymeric phase bound to a solid support one obtains a semi-dry material from which one can isolate drugs in a stepwise fashion based on the solubility characteristics of the drugs in this matrix. This method avoided the conventional methods of tissue extraction discussed earlier. It claimed to encompass sample homogenisation and cellular disruption, exhaustive extraction, fractionation and partition in a single process. The sample tissue containing the residue is ground with irregular shaped particles of silica or a polymer based solid support, for example C18. This reduces the sample tissue to a cluster of cells thereby achieving disruption of the tissue sample. This can produce a sample mixture material, which is packed into a column from which dispersed sample matrix components can be selectively isolated by elution. Barker demonstrated the applicability of this approach for multidrug residue extraction from a single sample for compounds

representing the organophosphate, benzimidazole, anthelmintic and β -lactum antibiotic drug classes. Barker originally used a lipophilic solid phase packing material (C18)¹⁵² to disperse tissues onto the solid support (C18), and produce a semi-dry, easy to handle column packing material from which individual drugs could be isolated from a single sample.

Samples were prepared in the following way: spiked tissue was blended with the C18 packing material producing a semi-dry homogenate. This was added to a syringe barrel and compressed. Hexane, benzene, ethyl acetate and methanol were eluted through the packing respectively, the four eluted fractions were evaporated to dryness, reconstituted and analysed for the drug under investigation. This indicated the levels of each of the compounds examined in each of the fractions giving an idea of the recoveries obtained. The organophosphates fractions were analysed by GC and show recoveries of greater 75 % in hexane and benzene fraction. No further sample clean-up was required for the analysis of the organophosphates. Analysis of the ethyl acetate by HPLC showed that all of the benzimidazoles examined were eluted in this fraction with recoveries ranging from 63-85 % with no interfering substances. Analysis of the methanol fraction by HPLC indicated the elution of the β -lactams in this fraction, which was determined to consist mainly of proteins, produced recoveries ranging from 60-86 %. Barker claimed the range of compounds examined suggested the method could provide a technique for multiresidue analysis of drugs and their metabolites in tissues. This approach eliminated the need to conduct tissue homogenisation, precipitation, centrifugation pH adjustments and sample transfers. Barker found that other various phases available C3, C8, C3 sulphonic and other polymer phases could be useful in more specific applications for other drugs. Barker admitted that the extraction of drugs from tissues from animals actually administered these

drugs was at that time outside the scope of the research.

Barker later published a review on data obtained up to 1993 examining the application of matrix solid phase dispersion (MSPD) to the isolation of drug and pollutant residues from a variety of biological matrices. Table 1.7 shows the application of this method to a variety of different compounds representing several major drug classes from a variety of tissues and sample matrices.

The extraction methods for the various drug classes summarised in table 1.7 provided recoveries of greater than 60 % for the individual compounds, whether the method was for a given compound or several compounds, over the range of concentrations examined. The limits of detection obtained for these

TABLE 1.7 A LISTING OF COMPOUND CLASSES FOR WHICH MSPD EXTRACTION METHODOLOGY HAS BEEN ESTABLISHED.

Compound	Matrix	Recovery (n≥20)	MSPD wash and eluting solvent
Neomycin	Bovine kidney ¹⁵³	88.6 ± 4.6	Cyanopropyl: wash; hexane, ethyl acetate, methanol, water; elute 0.1 M HCL
Ivermectin	Bovine liver ¹⁵⁴	74.9 ± 7.3	Wash; hexane, DCM-ethyl acetate (3:1), acetonitrile; elute methanol
Penicillin	Bovine muscle ¹⁵¹	86.3 ± 6.1	Wash; hexane, benzene, ethyl acetate; elute, methanol
Cephapirin	Bovine muscle ¹⁵¹	72.4 ± 26.5	Wash; hexane, benzene, ethyl acetate; elute, methanol
Chloramphenicol	Milk ¹⁵⁵	68.7 ± 8.3	Wash; hexane, benzene; elute, ethyl acetate
Sulfonamides	Swine muscle ¹⁵⁶	95.1 ± 15.1	Wash; hexane; elute, DCM

drugs were at or below the action levels established for the various drugs by the different regulatory agencies at the time of publication. In most cases, no clean-up steps were required post-elution and concentration. The extracts, analysed by HPLC or GC, were relatively free of contaminating co-extractants. The samples were assayed directly after elution, reducing the degree of sample manipulation required and the time necessary to obtain results. The MSPD approach required a minimum of 8 mls of solvent and could be performed within 30 minutes, ready for analysis.

The MSPD process is different from SPE, possessing elution and retention properties that appear to be a mix of partition, adsorption and paired ion/paired component chromatography that is somewhat unique. These properties are effected by the following variables

1. The solid support and the bound phase utilised
2. The nature of the sample matrix
3. The ratio of sample to solid support
4. The solvent elution sequence performed
5. The use of matrix modifiers, by blending the sample in the presence of acids, bases, salts, chelators, preservatives or other modifiers
6. The use of various solid support combinations or tandem column configurations

As briefly outlined in table 1.7 Schenck¹⁵³ modified the matrix solid-phase dispersion technique, to extract neomycin from bovine kidney tissue, in combination with HPLC separation using post-column derivatisation with OPA and fluorescence detection, recoveries at a fortification level of 2.5 to 10 ppm gave a mean recovery of 88.6 % (RSD 4.6 %) compared to conventional

methods where recoveries have been achieved at 97 - 113 % at fortification levels of 0.1 to 10 ppm. The sample preparation procedure started with the mixing of the spiked bovine kidney with cyanopropyl (CN) sorbent by a pestle and mortar to achieve a uniform homogeneous mixture. The cellular components and drug residues were dispersed over a large surface area in a thin film, which optimises the sample extraction efficiency. The tissue-cyanopropyl homogenate was then transferred to a syringe barrel fitted with a frit and compressed to a smaller volume column. A sequence of solvents were then washed through the tissue-cyanopropyl homogenate to remove tissue/cell contents that need to be separated from the drug for its extraction. Lipids are first eluted from the tissue-cyanopropyl matrix complex with hexane. The other tissue components i.e., phospholipids, amino acids, sugars, proteins and peptides, were then eluted from the tissue-cyanopropyl complex by a sequence of more polar solvents starting with ethyl acetate, methanol and ending with methanol/water (50:50 v/v). The volume of solvent used for extraction was typically small. The drug neomycin was finally eluted, firstly with a 1 ml aliquot of water and then subsequent aliquots of 0.05 M sulphuric acid. Potassium phosphate buffer and ion-pair concentrate were then added to the collected aliquots containing the neomycin, the final sample was now ready for analysis by HPLC. Using the same MSPD procedure for the extraction of neomycin from bovine kidney Schenck firstly tried other solid support sorbents, C18, CH, and cyclohexyl bonded phases, but found that the CN sorbent produced the most consistent recoveries.

Henion¹⁰³ produced a slightly modified version of his original MSPD method¹⁰² and that employed by Schenck¹⁵³ for the extraction of aminoglycosides from bovine tissue. The amount of CN sorbent used for 0.5 g amount of homogenised tissue was increased from 2 g to 3 g. It was claimed this provided

better clean-up. Concentration of the MSPD elute was also carried out, with filtration of the elute followed by evaporation in a speed vac. This allowed the original elute to be concentrated by a factor of 53. Therefore the detection of neomycin and streptomycin was achieved at or below the regulatory level specified of 0.25 ppm for neomycin and 0.5 ppm for streptomycin, which was quite an improvement to his previous method¹⁰² which managed recovery of the aminoglycosides at only 20 ppm.

Aschbacher and Feil²⁴ in their investigation into the deposition of neomycin in calves used a combination of MSPD and a hot base method (section 1.6) for the extraction of neomycin from animal tissue. In the hot-base method a sample of the kidney tissue was suspended in a centrifuge bottle in an aqueous solution containing 1 M sodium hydroxide and 0.06 M CaCl_2 , which was placed in a boiling water bath and then centrifuged. The procedure was repeated two or more times with removal of the supernatant in each case. The combined supernatants were then evaporated. In the MSPD method the original sample tissue used was larger in weight in comparison to the amount used by Henion,¹⁰³ 2 g of tissue were ground with 30 g of cyanopropyl sorbent for sample handling. The samples in each case were further concentrated. On comparison between the two extraction techniques, Aschbacher found the hot-base extraction resulted in a large volume of extract, which on concentration contained an appreciable amount of radioactivity due to the ^{14}C labeled neomycin administered to the calves. Concentration of the extract from the MSPD extraction produced a small amount of material with a loss of less than 10 % of the ^{14}C present. The MSPD extraction worked well with wet tissue at a ratio of tissue:CN sorbent of 1:6.

The studies carried out in this area so far appear to suggest that MSPD is a

good technique for the extraction/clean-up of the aminoglycosides neomycin and streptomycin from animal tissue, encompassing all the steps in the conventional methods of tissue disruption, removal of tissue contaminants by using various solvents for clean-up and finally elution of the aminoglycoside off the sorbent for analysis.

1.11 Aims and Objectives

An assay was required for the analysis of residues levels of neomycin and associated aminoglycosides like streptomycin in bovine tissue at the Ministry of Agriculture Fisheries and Food (MAFF). Neomycin and its associated aminoglycosides were considered a possible risk to the consumer of animal tissue on prolonged ingestion. Assays that had been developed to date involved the use of long extraction and clean-up procedures which accounted for the insolubility of the aminoglycosides in solvent and also its polarity.^{81,82,87,88} The majority of analysis was carried out by ion-pair liquid chromatography with detection by UV or fluorescence. However detection by these photometric methods was only achievable by derivatisation of aminoglycoside with a highly chromophoric compound (section 1.5.7), as neomycin in particular was not detectable by UV or fluorescence due to a lack of chromophores.

Attempts had been made at the Ministry of Agriculture to implement the method developed by Shaikh⁸⁰ for the detection of neomycin in bovine kidney using a postcolumn derivatisation procedure with OPA. However, there were problems with the derivatisation reaction reproducibility and the method was not able to achieve a sensitivity which could detect neomycin at 0.5 ppm, the MRL. This attempt was abandoned and they suggested that the project should

avoid derivatisation altogether and try an alternative detection method other than direct photometric UV which was too insensitive. Alternative modes of detection which had already been discussed in the literature were mass spectrometry (section 1.6) and pulsed amperometric detection (section 1.7). During the project mass spectrometric detection was unavailable for use, although during the course of the project papers were published^{102,103} that had detected the aminoglycosides neomycin and streptomycin using mass spectrometry at residue levels in bovine tissue. Neomycin had been detected electrochemically using the PAD, this had been reported by Dionex¹²³ with separation by anion exchange chromatography. This assay worked well with topical solutions, however analysis of residues in tissue samples had not been reported. This method was therefore selected as a starting point for the development of a method for the analysis of bovine kidney tissue samples spiked with the aminoglycoside.

The sample preparation MSPD method developed by Schenck¹⁵³ for the extraction of neomycin from bovine tissue samples would be investigated for its implementation as the main clean-up method for the tissue samples prior to detection. It was hoped the method could be used to extract and clean-up the tissue sample sufficiently to allow for detection of the aminoglycoside by the PAD at the MRL. Minimal sample handling was to be aimed for so that the elute obtained from the sample preparation stage could be analysed directly by the HPLC-PAD system, with no prior sample manipulation avoiding changes in pH. To achieve direct injection of the extracted sample onto the column for an on-line method, the solute used to extract the analyte has to be compatible with the mobile phase for good reproducible chromatography. Insolubility of the sample in the mobile phase can result in tailing and column blockage also a difference in pH between the sample solute and mobile phase can result in

shifts in the retention time of the analyte. A mobile phase system therefore has to be selected that is robust enough to separate the aminoglycoside in the solute reproducibly. The eventual aim was to develop a generic extraction and analysis method, that could be used on a group of aminoglycosides, which if possible was suitable for automation.

Experimental

CHAPTER TWO

CHAPTER TWO

2. *Experimental*

2.1 Reagents

2.1.1 Test compounds

Neomycin sulphate, streptomycin sulphate, glucosamine and glucose were obtained from Sigma (Poole, Dorset, UK.)

2.1.2 Solvents

Methanol, acetonitrile, ethyl acetate, hexane and ethanol were of HPLC grade supplied by FSA Laboratory Supplies (Loughborough, Leics., U.K.).

2.1.3 Mobile phase and buffer compounds

Pentafluoropropionic acid (PFPA) was obtained from the Aldrich Chemical Company (Aldrich Chemical Company, Gillingham, Dorset, England) 1-heptane sulphonic acid sodium salt and 1-hexane sulphonate sodium salt were from BDH Ltd Laboratory Supplies, (Poole, England).

Sodium acetate, sodium chloride, potassium chloride, potassium dihydrogen orthophosphate, sodium hydrogen orthophosphate, 46% sodium hydroxide, and buffer tablets (pH 4 and 7) were from FSA Laboratory Supplies

(Loughborough, Leics., U.K.). Acetic acid, orthophosphoric acid, sulphuric acid and hydrochloric acid of AR grade from BDH. Cetyl trimethyl ammonium bromide (CTAB) AR grade was from FSA Laboratory Supplies (Loughborough, Leics., UK.), Imidazole AR grade was from BDH.

2.2 Material used for the sample preparation stage

The plastic syringe barrels at 5 ml, 10 ml and 30 ml from plastic syringes (BD Plastipak), used during the MSPD sample preparation procedure for holding the sorbent-tissue mix.

2.2.1 Solid phase extraction cartridges

Silica (unbonded, activated silica), CN (cyanopropyl), C18 (octadecyl), SCX (propylbenzene sulphonyl, hydrogen form) and ODS at 100 mg/1 ml Bond Elute solid phase extraction cartridges (Analytichem International, Harbour City, CA, USA.) CBA (carboxymethyl, hydrogen form) at 100 mg/1 ml and 10 ml International Sorbent Technology cartridges, Jones Chromatography, (Hengoed, Mid Glamorgan. UK.) used during the sample preparation developmental stage to determine the sorbent best suited for the retention and elution of the aminoglycoside in the MSPD extraction procedure, refer to chapter 3 section 3.2.

2.2.2 Matrix

The solid phase sorbents used were CN (cyanopropyl), end-capped silica, particle size 30 - 75 μm , 94 % purity and silica, both from International Sorbent

Technology, Jones Chromatography, (Hengoed, Mid Glamorgan, UK.)

2.3 Chromatographic and Electrophoretic Methods

2.3.1 Chromatography

2.3.1.1 Ion-exchange chromatography

The HPLC separations were carried out by Dionex series 4000i metal-free quaternary gradient (GPM) pump (Dionex Corporation Ltd., Sunnyvale, California, USA), a Dionex metal-free rotary injection valve (LCM-2) fitted with a 25 µl sample loop. The separations were carried out on a Dionex CarboPac PA1 column (250 x 4 mm id) and CarboPac PA guard column (25 x 3 mm id) for anion-exchange and a IonPac CS3 column (250 x 4 mm id) and IonPac CG3 guard column (25 x 3 mm id) for cation-exchange chromatography. The columns were kept at ambient temperature by enclosing them in the compartment holding the pump. Chromatograms were recorded on a Linseis chart recorder as well as a SP4270 Spectra Physics integrator (Spectra Physics, San Jose, CA, USA).

2.3.1.2 Ion-pair chromatography

The HPLC separations were initially carried out using a Waters model 6000A solvent delivery system (Waters Associates, Milford, MA, USA.) this was subsequently replaced by a LC10AD solvent delivery module (Shimadzu

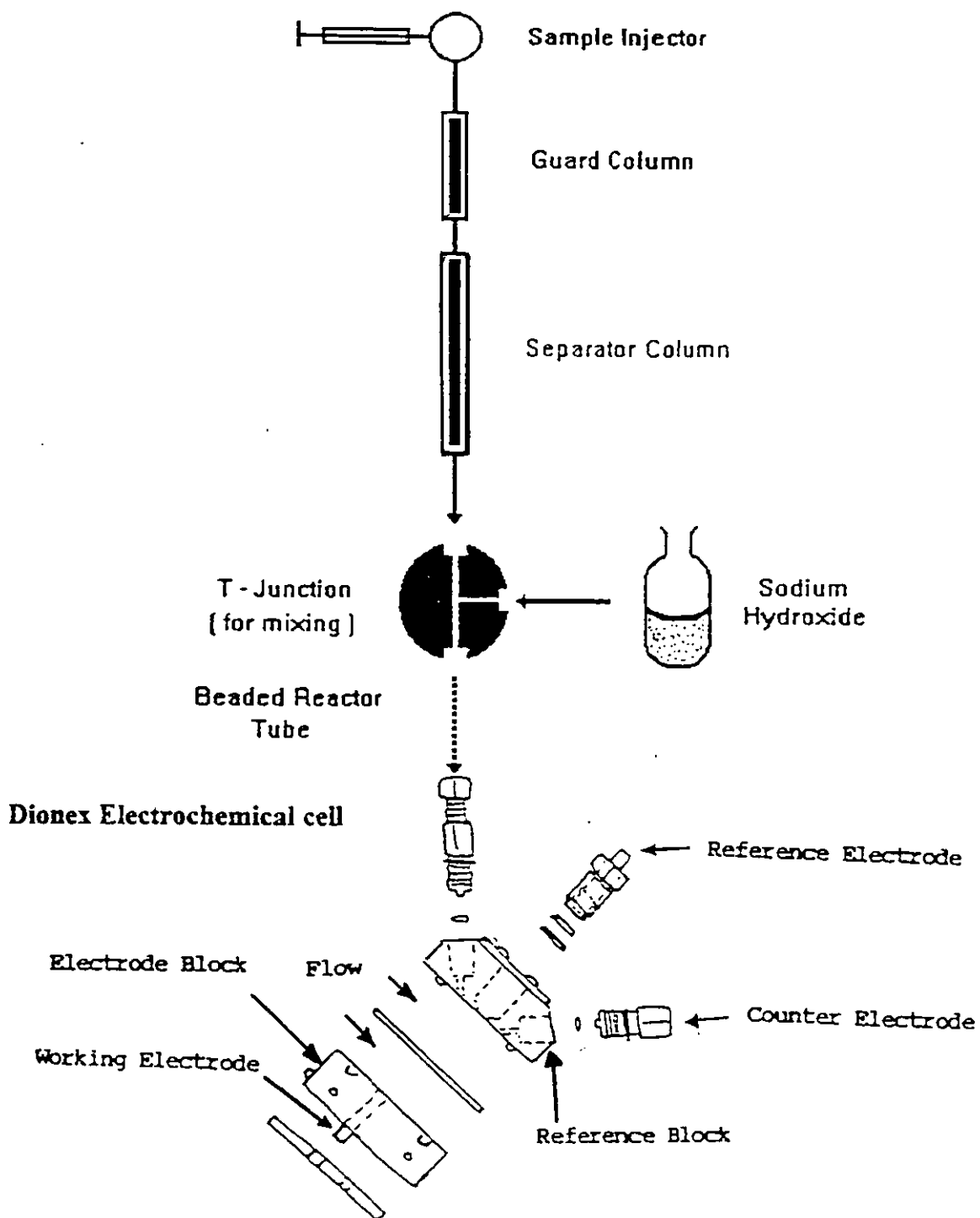
Corporation, Analytical Instruments Division, Kyoto, Japan) which was used for the majority of the chromatography. The samples were injected using a Rheodyne 7125 injection valve (Rheodyne Inc., Colati, CA, USA) fitted with a 20 μ l sample loop. The separations were carried out on a Hypersil-ODS 5 μ m (100 x 5 mm id) column, Capcell Pak C18 5 μ m (250 x 5 mm id) column Shiseido Co. Ltd. (Yokohana, Japan), Kromasil 100-5 C18 5 μ m (250 x 4.6 mm id) column Hichrom Ltd. (Reading, UK), Spherisorb ODS (150 x 4.6 mm id) column Spherisorb C6 (250 x 4.5 mm id), PLRP-S (150 x 4.6 mm id) column porous polystyrene-divinylbenzene copolymer, 5 μ m (Polymer Laboratories Church Stretton, UK) and Regis SPS-5PM-100-C18 5 μ m (250 x 4.6 mm) column (Regis, Illinois, USA). The temperature of the column was maintained at 40°C by a Shimadzu C10-6A column oven (Shimadzu Co., Kyoto, Japan). The chromatograms were recorded and integrated on a SP4270 Spectra Physics integrator (Spectra Physics, San Jose, CA, USA).

2.3.2 Pulsed amperometric detector

The pulsed amperometric detector (PAD) Dionex Co. (Sunnyvale, CA, USA) with a flow cell volume of 3.5 μ l was used in conjunction with a gold working electrode with a surface area of 1.54 cm². The applied potential for oxidation (E1) was 0.1V with a pulse duration of 480 ms. Potentials used for cleaning the working electrode (E2 and E3) were 0.6 V and -0.8 V with pulse duration's of 120 and 300 ms, respectively. The response time was set at 1 sec. The detector range used was from between 100 nA to 1K nA. A post column LC500 isocratic pump (Applied Chromatography Systems Ltd., Luton, UK) which was later replaced by the Dionex series 4000i metal free quaternary gradient pump, supplied sodium hydroxide to the HPLC column effluent. The two streams

were joined at a T or Y shaped mixing junction. A 130 cm² Dionex beaded reactor tube was used to mix the sodium hydroxide with the effluent prior to entry to the PAD cell (Figure 2.1.)

Figure 2.1 A Diagram of the Layout for the HPLC and PAD



2.3.3 Capillary electrophoresis

Initial work on capillary zone electrophoresis (CE) was carried out on the ATI Unicam model 310 Crystal Capillary Electrophoresis System, and a Unicam 4225 UV detector. This was later replaced by the Beckman P/ACE capillary electrophoresis system 2050, with a Beckman P/ACE UV detector. Silica capillary tubes with lengths of 55 cm (50 μ m id) and 50 cm (70 μ m id) from injector to detector were used in the Unicam and Beckman systems, respectively.

2.3.4 pH Measurements

The pH measurements were made on a PHM 64 research pH meter (AS Copenhagen, Denmark) with a Corning 500 combination electrode, calibrated against aqueous buffer solutions (pH 4.00 and 7.00).

2.4 Procedure

2.4.1 Mobile phase preparation

2.4.1.1 Ion-exchange chromatography

The eluents for anion exchange chromatography were prepared by dilution of a 2 M solution of sodium hydroxide to the required concentrations for the mobile phase and for postcolumn addition. The mobile phase used in the study was a 10 mM sodium hydroxide. The mobile phase originally used for cation

exchange was a 10 mM sodium hydroxide and water gradient as used in anion exchange, however, other alternatives were studied later. A mobile phase containing 0.6 M sodium acetate at a pH of 6.0, prepared by adding glacial acetic acid to adjust the pH, was used in conjunction with either water or 0.15 M sodium hydroxide in an isocratic and gradient study.

2.4.1.2 Ion-pair chromatography

The mobile phases were prepared by adding the ion-pairing reagent, 1-heptane sulphonic acid sodium salt, 1-hexane sulphonic sodium salt or pentafluoropropionic acid (PFPA) to the corresponding amount of solvent, methanol or acetonitrile. This solution was then made up to the required concentration with deionised water. For some studies the pH of the mobile phase was adjusted by the addition of phosphoric acid (pH 6.0) or acetic acid (pH 5.5) dependent upon the mobile phase. Most of the work used the mobile phase composition methanol-water (40:60 v/v) or acetonitrile-water (27:73 v/v) containing 20 mM PFPA in each eluent.

2.4.2 Buffer preparation for the Capillary electrophoresis

The buffer was prepared by diluting a 0.1 M imidazole solution to the required concentration. The surfactant cetyltrimethylammonium bromide was weighed out to the required concentration of 400 µg/ml and added to the buffer. The pH of the buffer was adjusted to 5.5 by the addition of glacial acetic acid.

2.5 Standard methods

2.5.1 Cyclic voltammetry

The voltammetry was carried out on a PAR model 174 polarographic analyser and HR2000 recorder (EG & G Princetown Applied Research, NJ, USA). A gold working electrode was used, with a surface area of 12.6 mm². The reference electrode was silver chloride with an auxiliary platinum electrode. Cyclic scans of 0.5 mM neomycin sulphate and 0.5 mM streptomycin sulphate in 0.15 M sodium hydroxide (equivalent to the composition of the eluent upon entry to the PAD cell) were carried out. The potential range scanned was from - 0.4 V to + 0.2 V. A cyclic scan of a blank 0.15 M sodium hydroxide was carried out at the same potential range.

2.5.2 Ion-exchange chromatography

Refer to section 2.3.1.1. for the equipment used in this procedure. The mobile phase used in the study was 10 mM sodium hydroxide-water gradient, initially at 0:100 v/v and changing to 40:60 v/v over the 10 min period of the gradient program. To re-equilibrate the column after the gradient, water was run through for 5 min. The flowrate of the mobile phase was set at 1 ml/min. The two streams, one from the column and the other from the postcolumn LC 500 isocratic pump (Applied Chromatographic systems Ltd., Luton, UK) supplying 1M sodium hydroxide at a flowrate of 1 ml/min, were joined at a Dionex T shaped junction. A 130 cm² Dionex beaded reactor tube was used to mix the sodium hydroxide with the effluent from the column, prior to entry to the PAD cell.

2.5.3 Ion-pair chromatography

The HPLC separations were carried out on a Shimadzu LC10AD solvent delivery module. 10 µl of the sample was injected onto a Rheodyne 7125 injection valve fitted with a 20 µl sample loop. Detection was carried out by the Dionex PAD set under the same conditions to that used in anion-exchange chromatography. The separation was carried out on a Kromasil 100-5C18 5 µm (250 x 4.6 mm id) column kept at a temperature of 40°C in the Shimadzu C10-6A column oven. The mobile phase contained acetonitrile-deionised water at 27:73 v/v for the separation of neomycin sulphate or 18:82 v/v for the separation of streptomycin sulphate with 20 mM PFPA. The two streams, one from the column and the other from the postcolumn Dionex series 4000i metal-free quaternary pump supplying 0.5 M sodium hydroxide at a flowrate of 0.4 ml/min were joined at a Y or T shaped junction. A 130 cm² Dionex beaded reactor tube was used to mix the sodium hydroxide with the effluent from the column prior to entry to the PAD cell detector.

2.5.4 Capillary electrophoresis

Initial work on capillary electrophoresis was carried out on a ATI Unicam model 310 Crystal capillary electrophoresis system, with a Unicam 4225 UV detector set at 214 nm. This was later replaced by Beckman P/ACE capillary electrophoresis system 2050, and a Beckman P/ACE UV detector set at 214 nm. The potential of 30 kV applied across the capillary tube was reversed, the samples were therefore injected at the cathode and detection was carried out at the anode. The capillary tube was dipped in buffer at each end. The buffer contained 0.01 M imidazole, 400 µg/ml of cetyltrimethylammonium bromide

(CTAB) adjusted to a pH of 5.5 with the addition of glacial acetic acid. The samples also contained 400 µg/ml of CTAB. To enable complete separation of neomycin sulphate and streptomycin sulphate from the bovine kidney sample contaminants, methanol was added to the buffer, to give methanol-buffer at 20:80 v/v. Interpretation and integration of the chromatograms was carried out on the system Gold software on the Beckman CE instrument and 4880 system software on the Unicam CE instrument.

2.5.5 Sample preparation

A bovine kidney purchased from the butchers was kept in a freezer at a temperature of -20°C. A few grams of the kidney were homogenised at low speed in a Kenwood blender, aliquoted into 15 ml glass vial sample bottles which were stored at -20 °C. A 0.5 g amount of the tissue homogenate was weighed out into mortar. The tissue was then spiked with the appropriate volume of neomycin sulphate or streptomycin sulphate standard stock solution to provide a certain concentration. This was carried out using either an 20, 50 or 200 µl Epindorf pipette. The tissue was then left at room temperature and allowed to equilibrate for at least 15 min. End-capped cyanopropyl (CN) silica (2 gm, 30 - 75 µm) was added to the tissue and ground (blended) manually with a pestle for a few minutes. The resulting mixture was transferred through a plastic funnel into an empty 10 ml plastic syringe barrel, cotton wool plugs were placed at the top and bottom of the column mixture. This mixture was compressed to a volume of 3.5 ml. The syringe barrel containing the mixture was then placed on a Dionex On-Guard sample preparation vacuum manifold. The mortar was rinsed with 3 ml of water and then 3 ml of hexane, which were separately eluted through the matrix column

via a vacuum at a rate of two drops per sec. Successive 5 ml aliquots of ethyl acetate, methanol and methanol-water (50:50 v/v) were poured onto the top of the column matrix and eluted through at the same rate. Each solvent wash was eluted through separately and complete elution of the solvent occurred before the start of the next solvent wash. After all the solvents were eluted the vacuum was set at a maximum for 30 sec to dry the column. 0.05 M sulphuric acid (8 ml) was poured into the column, allowed to soak in for a few minutes and then eluted through slowly at a rate of one drop every two seconds. The elute was collected in a 12 ml plastic centrifuge tube. The elute was then neutralised to a pH of 7 by the addition of 0.5 M and 0.1 M sodium hydroxide.

A CBA 100 mg/10 ml solid phase extraction cartridge was placed on the vacuum manifold, and equilibrated by eluting through firstly 0.05 M sulphuric acid (3 ml), followed by buffer pH 7 (5 ml). The neutralised solution containing the analyte, neomycin sulphate or streptomycin sulphate, was then slowly eluted through the equilibrated CBA cartridge at a rate of one drop every three seconds. The analytes were trapped on the solid phase extraction cartridge and were eluted by the addition of 0.05 M sulphuric acid. The acid was poured into the CBA cartridge and left to soak in for 3 min, and then eluted through the CBA sorbent at a rate of one drop every three seconds, the first 1 ml elute was collected and analysed by HPLC or CE.

2.5.6 Fortification of bovine kidney at 10 ppm of aminoglycoside

A 2.5 g amount of homogenised bovine kidney was spiked at 10 ppm with the aminoglycoside. This was ground with 10 g of cyanopropyl silica (CN) with the aid of a pestle and mortar. The matrix mixed with the kidney was then packed

into a 30 ml plastic syringe barrel with cotton wool plugs top and bottom and compressed. For the clean-up stage the volume of each solvent used was increased five-fold from the original amount in the previous method. The following solvents were eluted through in succession, 10 mls of water, 15 mls of hexane and 27 mls of ethyl acetate, methanol and finally methanol-water (50:50 v/v). Neomycin sulphate was collected off the column matrix by the elution of 50 mls of 0.05 M sulphuric acid through the column matrix, at 1 drop/sec into a 100 ml plastic beaker. The pH of this sample was changed to 7 by the addition of 0.5 M sodium hydroxide. All 50 mls of the sample analyte were then eluted through a preconditioned CBA cartridge, 1 ml of 0.05 M sulphuric acid was then eluted through slowly at 1 drop/sec. The eluted 1 ml of sample analyte was injected (10 μ L) onto the column.

Development of a Sample Preparation Procedure.

CHAPTER THREE

CHAPTER THREE

3. *Development of a sample Preparation Procedure*

This chapter discusses the development of a suitable sample preparation procedure for the extraction of neomycin and streptomycin from bovine kidney tissue for subsequent analysis by HPLC, in conjunction with the PAD, or by capillary electrophoresis, which are discussed in the following chapters.

The anion exchange procedure which is used in the determination of the two aminoglycosides involved the use of a CarboPac PA1 column using a dilute sodium hydroxide gradient. Detection was carried out by the PAD with the addition of sodium hydroxide postcolumn to increase the sensitivity of the detector. This method was reported by the application chemists at Dionex who had used it to determine levels of neomycin in topical lotions.¹²³ At this point in the project this HPLC method was the only method in the literature which allowed for detection of the aminoglycoside without the need for derivatisation. Due to this fact and the availability of the PAD detector it was decided to proceed with this method for the sample analysis. The method was optimised by washing the column out with water for an additional 5 minutes at the end of the gradient to re-equilibrate the column prior to the next injection. Also to improve baseline noise and thereby improve sensitivity a beaded coil reactor tube was placed after the post column mixing junction to aid mixing of the column eluent and postcolumn sodium hydroxide. The method was found to be linear over the range 10 - 100 ppm with a detection limit of 1 ppm. Initially during the developmental phase of the sample preparation the anion-exchange HPLC method proved good for the determination of recoveries of the aminoglycoside from the SPE cartridge. However as neomycin was eluted from the SPE cartridge in acid the elute had to be neutralised prior to injection

as direct injections of the acidic elute lead to shifts in the retention time. Therefore the anion-exchange HPLC method was abandoned in favour of a reversed-phase ion-pair HPLC system whose mobile phase allowed for direct injection of the acidic elute with no shifts in the retention (refer to chapter 6).

3.1 Matrix solid phase dispersion

The presence of basic amino groups and several hydroxyl groups contributes to the hydrophilicity of the aminoglycosides, which are all readily soluble in water, with limited solubility in methanol and poor solubility in more hydrophobic organic solvents.^{48,55} Because of these chemical properties it has proven difficult to extract the aminoglycosides, neomycin and streptomycin, in a concentrated and purified form from animal tissue using conventional techniques of sample preparation, such as solvent extraction with a low polarity organic solvent. The usual methods used for the extraction of aminoglycosides involve the use of multiple clean-up steps, often involving centrifugation, resulting in a high amount of sample handling. These techniques also tend to involve an extensive amount of time as has already been outlined in the Introduction chapter.^{56,80,81,82,83,87,88} Alternative techniques were therefore investigated. A method called matrix solid phase dispersion (MSPD) has been reported to provide a quick alternative to usual techniques of tissue extraction.¹⁵² An MSPD method published by Barker *et al.*¹⁵² and modified by Schenck¹⁵³ for the extraction of neomycin, demonstrated that biological matrices could be homogenised and ground with irregular shaped particle matrices like C18 bonded (40 µm, octadecylsilyl derivatised) silica, silica (Si) or cyanopropyl bonded silica (CN). By this process the sample tissue is then reduced to clusters of cells. The resulting homogenate mixture can be packed into a column and various residues and the analytes can then be

selectively eluted (refer to chapter 1 section 1.10.3 for more detail). However, before elution of the analyte under investigation, a considerable amount of clean-up of the tissue sample takes place, before the final analyte sample is able to produce reasonable chromatography. For this Schenck¹⁵³ has previously used hexane as the initial solvent to extract lipophilic residues, and fats from the biological matrix. Then the ethyl acetate, methanol, and water was used to remove residual hexane and lipids.

This MSPD method looked suitable for investigation as a sample preparation method it could be applied to the extraction of aminoglycosides from bovine kidney tissue as Schenck had demonstrated. This method would avoid the long sample preparation procedures outlined in the introduction chapter (section 1.10.1.) The MSPD method outlined by Schenck¹⁵³ was therefore applied to the extraction of bovine kidney samples to test its efficiency and try to achieve recoveries at or below those quoted by Schenck. In addition, a concentration step was added to the procedure to increase the probability of detection of the neomycin and streptomycin at or below the MRL. The next stage was to find a suitable sorbent matrix for the MSPD procedure and the concentration step.

3.2 Examination of materials as a suitable sorbent matrix

As part of the development of the MSPD technique for the extraction of neomycin and streptomycin from control bovine kidney tissue, a range of Bond Elute solid phase extraction cartridges (100 mg/ml) containing different stationary phases were examined to determine a suitable sorbent matrix type for both the retention and elution of neomycin, under specific trial conditions.

Solid phase extraction cartridges are often used for sample clean-up, refer to

section 1.10.2. Historically, reversed-phase separations using C18 bonded silica provided the broadest applicability. C18 SPE cartridges have been used extensively, to clean-up plasma and tissue samples containing various aminoglycosides.

Kurosawa and co-workers¹⁵⁷ used a C18 Sep-pak cartridge to clean-up serum samples containing streptomycin. Using ion-pair extraction, sodium-1-hexanesulphonate (50mM) and tribasic sodium phosphate (25 mM), to separate the hydrophilic compound from the biological components. The results showed pH 2 allowed for maximum recovery of streptomycin. Haagsma¹⁵⁸ like Kurosawa used ion-pair solid phase extraction of a C18 column for sample clean-up of calf and chicken plasma samples containing spectinomycin. The solid phase column was treated with ion-pair reagent sodium dioctylsulphosuccinate. The non-polar part of the dioctylsulphosuccinate ion (DOSS⁻) is attached to the SPE column, allowing spectinomycin to form an ion-pair with the negative DOSS ions. The ion-pair DOSS⁻ spectinomycin⁺ may be subsequently eluted with an organic solvent such as methanol. During the method development on using C8, C18, phenyl and high-hydrophobic C18 SPE columns, Haagsman found recovery from the high-hydrophobic C18 was best. The extracts were cleaned up by washing the column with citric acid solution. Gerhardt^{87,88} also used C18 SPE cartridges for the sample clean-up of streptomycin and dihydrostreptomycin in animal tissue. In this case the extract was loaded onto the column after deproteinisation using perchloric acid. The column was washed with water to remove further contaminants and finally streptomycin and dihydrostreptomycin were eluted off with 2 M phosphate buffer pH 8. Medina and Unruh⁵⁹ used copolymeric bonded silica with hydrophobic and ionic functions (C8/sulphonic) as SPE cartridges for clean-up of a related aminoglycoside hygromycin in bovine plasma and swine serum. The spiked plasma was acidified and loaded onto the acidified column. The column was then rinsed with deionised water, followed by propanol and the hygromycin eluted with diethylamine-methanol (5:95 v/v) and the eluent

applied to a TLC plate.

The hydrophobic nature of the C18 bonded phase requires a conditioning step with a wetting solvent, such as methanol or acetonitrile. Once conditioned, the sorbent must not dry out before loading the sample otherwise the recovery of the compounds will be reduced. Residual surface silanols on C18 bonded silica pose problems for basic compounds, the silanols can affect the recovery of the basic compounds detrimentally by interacting through ion-exchange, which may prevent complete elution. In addition, the pH sensitivity of silica-based sorbents restricts their usable range to approximately pH 2-8. Low pH solutions will hydrolyse the bonded phase and solutions at elevated pH will dissolve the base. Another limitation of C18 sorbents is that polar analytes may be insufficiently retained. This reduced retention can result in lower recoveries because of analyte breakthrough during the sample-loading step.

The solid phase extraction cartridges Si, C18, SCX and CN were investigated for their trapping and eluting ability of neomycin sulphate due to their different sorbent properties. The Si sorbent for its ability to retain polar compounds due to polar interactions between the Si and the compound. Silica is regarded as the most polar sorbent therefore trapping of the compound by silica may be irreversible. Cyanopropyl (CN) sorbent is a medium polarity sorbent, therefore it can be used as a polar sorbent that is less retentive for very polar isolates that might be retained irreversibly on the most polar sorbents such as silica. C18 is the most non-polar sorbent available and has been used in the clean-up of aminoglycosides. C18 is regarded as the least selective sorbent, as it retains almost anything from aqueous matrices, however, it does not retain very polar molecules for example carbohydrates. Finally propylbenzenesulphonyl (SCX hydrogen form) is a strong cation-exchanger with a very low pKa. The non-polar character of the SCX, due to the presence of the benzene ring on its surface, is important when the sorbent is used as an ion-exchanger from aqueous solvent systems. In Schenck's paper the CN

matrix was found to work well for trapping neomycin sulphate in tissue samples whilst various solvents were eluted through to clean-up the tissue sample, elution of neomycin from the CN matrix was achieved with 0.05 M sulphuric acid.

The following procedure was carried out for each SPE cartridge (for full details of the method see 2.5.5). An aqueous solution (1 ml) containing neomycin sulphate (20 ppm) was placed on top of the SPE column, which had been prewashed with water to wet the sorbent. The neomycin sulphate solution was loaded onto the column and the elution from the loading collected for analysis. To imitate Schenk's MSPD method sulphuric acid (0.05 M) was then used to elute the neomycin off the SPE cartridge. The acidic elute fractions (2 mls) were then neutralised with sodium hydroxide prior to analysis. The monitoring of the trapping and release of neomycin sulphate on the SPE cartridges was carried out using anion exchange chromatography in conjunction with PAD detection (refer to chapter 5). This procedure was carried out once for each SPE cartridge, the results obtained for each SPE are outlined in Table 3.1. The recovery of neomycin from the cartridge was determined by the amount obtained in the acidic elute. The amount of neomycin sulphate in the elute was determined by calibrating with a single standard equivalent concentration of neomycin expected for 100 % full recovery. The calibrating standard was injected alongside the sample elute.

Results showed that with the C18 cartridge and to a lesser extent the cation exchange SCX SPE cartridge, neomycin was not fully retained on the sorbent. Neomycin in water was completely retained by Si and CN sorbent SPE cartridges (refer to Table 3.1). Although the Si sorbent retained neomycin, on washing the cartridge with sulphuric acid, there was no trace of neomycin in the elute which indicated that neomycin was irreversibly retained. Neomycin was also completely retained by the CN sorbent, but was eluted by sulphuric

acid, however, the values showed poor recoveries (33 %).

TABLE 3.1 RECOVERIES OF NEOMYCIN FROM VARIOUS SOLID-PHASE EXTRACTION CARTRIDGES.

Trapping effect of various sorbents in the form of solid phase extraction cartridges on neomycin sulphate (20 ppm) from an aqueous. Elution of the neomycin off the solid phase extraction cartridge was carried out with 0.05 M sulphuric acid (2 mls). The concentration was monitored by anion exchange chromatography and PAD detection as outlined in section 2.5.2.

Sorbent phase	n (number of assays)	Neomycin Sulphate (%)	
		Amount retained on the column after loading.	Eluted off the SPE cartridge with sulphuric acid
CN	1	94	33
Si	1	100	0
C18	1	5	5
SCX	1	75	7.6

Silica has a very acidic and polar nature, and the amino and hydroxyl groups on the analyte are the most sensitive to polar interactions with the silica substrate. Therefore the polar characteristics of the silanol groups will interact through hydrogen-bonding with the amino and hydroxyl groups on the aminoglycoside molecule. Because of these strong interactions subsequent elution of the aminoglycoside molecules, such as neomycin is very difficult. However, CN as a medium polarity sorbent has less retention for polar isolates like aminoglycosides but still gave good trapping of the neomycin on the

sorbent. To improve the initial recovery of neomycin sulphate from CN sorbent, which was poor at 33 % for a standard cartridge, an endcapped CN cartridge was used instead of the standard cartridge. On the endcapped CN cartridge, many of the remaining silanol groups on the silica are deactivated to produce a surface whose principal properties are due to the functional group with minimal interactions from the silica substrate. Therefore an investigation was carried out to monitor the trapping and elution properties of the endcapped CN sorbent with regard to the aminoglycoside neomycin sulphate.

3.2.1 Investigation of bulk endcapped cyanopropyl sorbent

The endcapped cyanopropyl (CN) sorbent was not available prepacked into a cartridge so bulk endcapped CN sorbent was therefore investigated as a possible matrix for the MSPD step of the sample preparation stage. A bed of CN sorbent was placed into a plastic syringe barrel. The matrix column was compressed and 1 ml of a 10 ppm aqueous standard solution of neomycin sulphate was loaded onto the column. In this case the trapping of the neomycin sulphate was monitored using the ion-pair HPLC system (chapter 2 section 2.5.3) which had been developed at this stage of the project. Analysis of the elute obtained from loading the neomycin sulphate onto the cartridge showed no trace of neomycin sulphate as the pulsed amperometric detector indicated no response for neomycin. Therefore the neomycin sulphate had been fully retained on the matrix column. To elute the neomycin as the sulphate, the matrix column was slowly washed through with sulphuric acid (0.05 M). The acid elute was analysed. The resulting chromatogram showed a peak corresponding to neomycin sulphate. The amount recovered was calculated at 94 %. Therefore it was apparent that the endcapped CN sorbent could work

well as a matrix for MSPD in trapping and releasing neomycin and possibly other aminoglycosides under appropriate pH conditions. This study compared well with other reports of MSPD for neomycin using cyanopropyl as the trapping sorbent and sulphuric acid (0.05 M) as the elute, Schenck¹⁵³ found percentage recoveries of 88 % for neomycin from the CN sorbent in the MSPD procedure used.

The next stage of this investigation was to see whether CN sorbent could be used as the matrix solid phase dispersion medium, for sample extraction from bovine kidney tissue samples spiked with neomycin sulphate.

3.2.2 Initial investigation into the extraction of neomycin sulphate from bovine kidney samples using cyanopropyl sorbent matrix in MSPD

The intention was to develop a technique that could be used to extract and analyse neomycin sulphate as well as other aminoglycosides in bovine kidney tissue at very low concentration levels equivalent to or below the set MRLs. As the project progressed, streptomycin sulphate was also incorporated into the extraction and analysis techniques under development.

Bovine kidney was the sample tissue of interest because this was the principal location of the aminoglycosides in the organism, as discussed in the introduction. Due to the aminoglycoside's polar nature they are soluble in aqueous solution and are therefore excreted directly out of the body in urine via the kidney. Shaikh,⁸⁰ Gerhardt⁸⁷ and Schenck¹³⁵ looked at levels of neomycin and streptomycin in bovine and porcine kidney.

The efficiency and development of the sample preparation procedure was monitored by using ion-pair chromatography with PAD detection as outlined in section 2.5.3 of the experimental chapter.

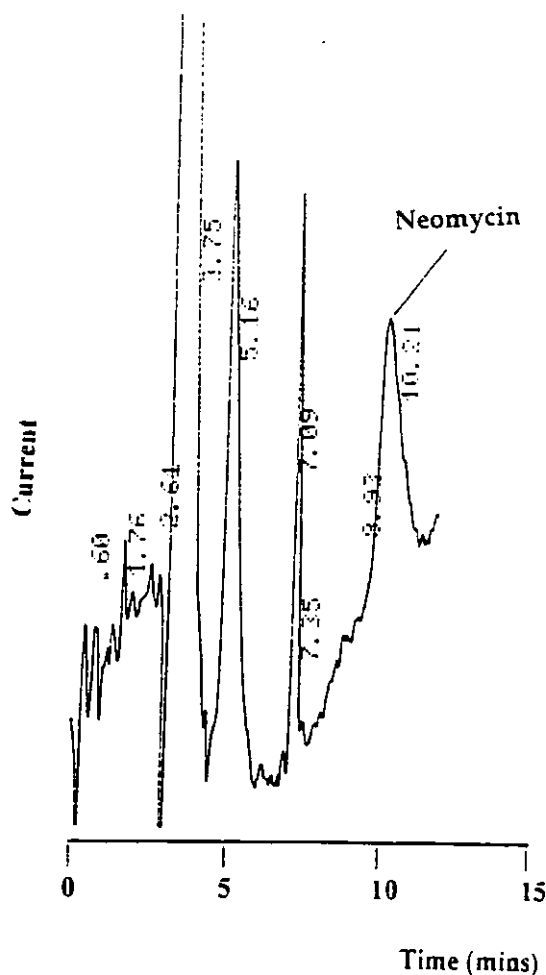
A more detailed account of the steps used in the MSPD technique for the extraction of the aminoglycoside from the tissue are outlined in the experimental chapter 2 section 2.5.5. A sample of bovine kidney (0.5 g) was spiked at 100 ppm of neomycin sulphate prior to the MSPD sample extraction procedure. The bovine kidney was then ground with the cyanopropyl (CN) sorbent matrix (2 g) and the CN sorbent tissue mix were transferred into a plastic syringe barrel, with cotton wool placed at both ends of the matrix mix. The mixture in the syringe barrel was compressed to the 3.5 ml mark on the 5 ml syringe barrel. The sorbent tissue column was washed with, 2 mls deionised water, 3 mls hexane and 5 mls of ethyl acetate, methanol and methanol-water (50:50 v/v), in succession to cleanup the tissue. Finally the volume of sulphuric acid used for the elution of the aminoglycoside from the matrix tissue mix was varied to determine the quantity required for complete elution of the aminoglycoside in the smallest volume. Water (1 ml) was initially eluted through the matrix tissue column after the organic washes and injected, this gave no response. This was followed by two successive 4 ml aliquots of 0.05 M sulphuric acid, the first 4 ml aliquot produced a clearly definable neomycin sulphate peak (Figure 3.1.)

A second 4 ml aliquot of the acid was eluted through the matrix tissue column, this upon injection produced no response. The amount of neomycin sulphate recovered by the first 4 mls of sulphuric acid was calculated at 71 %. This procedure was repeated again on a bovine kidney tissue sample spiked at 100 ppm neomycin sulphate. After clean-up of the tissue with the succession of

organic solvents outlined earlier the neomycin sulphate was eluted directly off by a 4 ml amount of sulphuric acid. The recovery achieved in this case was 102 %. It was therefore apparent that MSPD method worked well for the extraction of neomycin sulphate from bovine kidney tissue spiked at 100 ppm. Schenck¹⁵³ had managed to achieve a mean recovery of 88 % of neomycin sulphate from bovine kidney tissue spiked at 2.5 - 10 ppm. The advantage of MSPD as an

Figure 3.1 The chromatogram of an acid extract from MSPD of bovine kidney sample spiked at 100 ppm neomycin sulphate.

Injection volume 10 μ l. Mobile phase conditions: 20 mM PFPA, Methanol-water (40:60 v/v) at 1 ml/min with postcolumn 1 M sodium hydroxide at 1 ml/min. PAD settings: E1 0.1 V(480 ms), E2 0.6 V(120 ms) and E3 -0.8 V(300 ms). Amplitude sensitivity 300 nA.



extraction technique is that the final eluted fractions should contain only polar basic analytes as the non-polar extracts are eluted early on by the solvents used for clean-up and the polar neutrals such as glucose are eluted by the methanol-water solvent mixture. It is important that the neutral sugars are removed from the sample, avoiding interference by the neutral sugar in the detection of the final eluent containing the aminoglycoside by the PAD.

MSPD was found to work well as a method for sample clean-up and preparation prior to analysis by HPLC and later Capillary electrophoresis, attempts to recover lower levels of neomycin sulphate and streptomycin sulphate from bovine kidney tissue are discussed in the ion-pair HPLC and capillary electrophoresis chapters.

3.2.3 Investigation into the extraction of neomycin sulphate from bovine kidney samples using silica sorbent matrix in MSPD

From the investigation of the different sorbent Bond Elute cartridges, silica appeared to have similar properties to cyanopropyl (CN) sorbent with a high trapping ability, however the results appeared to indicate (Table 3.1) an irreversible trapping of neomycin sulphate. Silica is a much cheaper alternative as a sorbent matrix to CN, and therefore, if a method could be found which worked well in the MSPD sample preparation, it would provide a lower cost technique for use on a large number of samples. Therefore an investigation was carried out into the ability of bulk silica sorbent to retain and release neomycin sulphate.

The sample preparation used paralleled that in the previous section when

cyanopropyl was used as the sorbent. Bovine kidney tissue was spiked with 100 ppm neomycin sulphate, ground with the silica and the tissue matrix mix washed with the solvents for clean-up as previously listed in section 3.2.2 and then sulphuric acid (0.05 M) eluted through the tissue matrix mix to collect any of the trapped neomycin. The separation of neomycin was carried out by the ion-pair chromatography assay method with pulsed amperometric detection (chapter 2, section 2.5.3.)

The acid eluent, obtained from the MSPD step, was injected onto the HPLC column but there was no response from the detector repeating the effect on the bond elute. It appeared that neomycin could not be eluted from the silica with acid but was fully retained by the acidic surface. The conclusion from this investigation was that silica was an unsuitable sorbent for use in MSPD for the extraction of aminoglycosides from bovine kidney tissue. These results confirmed those in section 3.2 of this chapter on the ability of various solid phase extraction cartridges to retain and elute neomycin sulphate.

Trials were subsequently carried out under the experimental conditions outlined in section 3.2.2 of this chapter to attempt the extraction of aminoglycosides neomycin sulphate and streptomycin sulphate at concentration levels close to the MRL. Refer to chapter 7 for the results. Early on in the trial problems were encountered with the chromatography and sensitivity of the detector, these problems are tackled in the followings pages of this chapter.

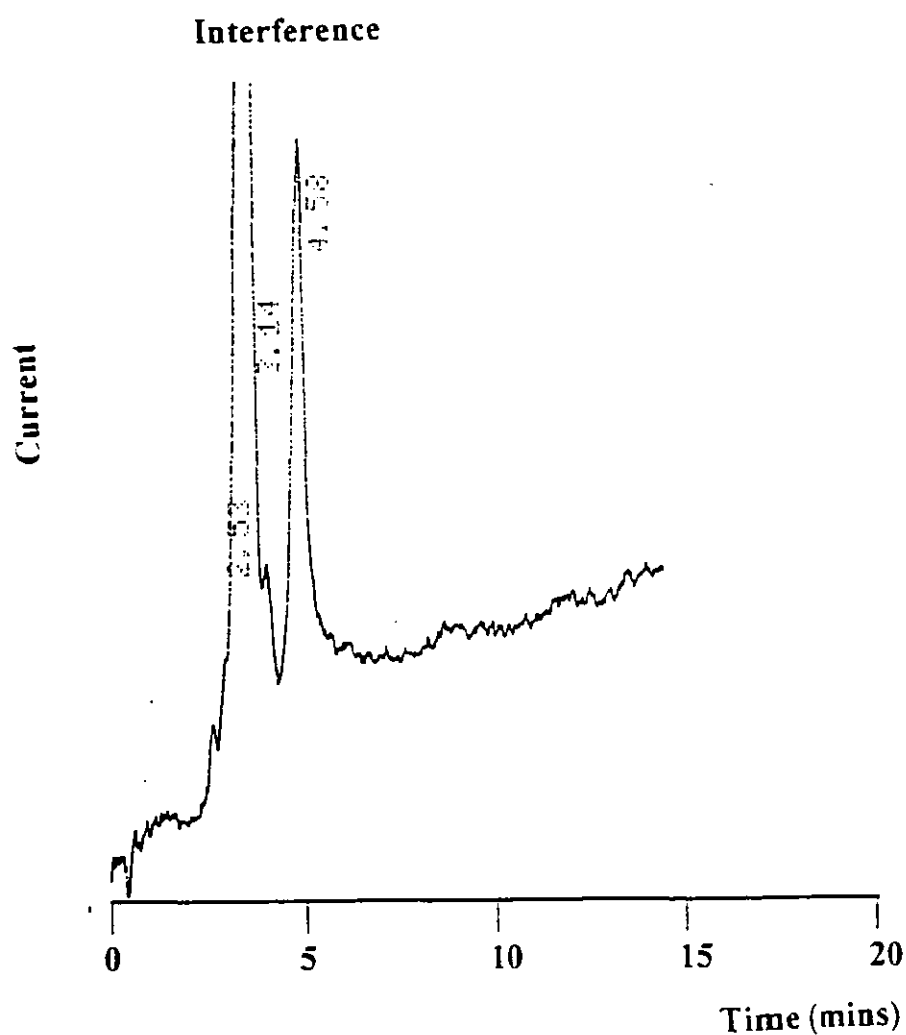
3.3 Interference peaks

Chromatograms of a spiked bovine kidney after MSPD, (Figure 3.1), had interference peaks eluting slightly earlier than the neomycin. The interference peaks were eluted at retention times ranging from three to five minutes. It was assumed these peaks represented the bovine kidney tissue residues, which were not completely removed by the MSPD step and could include native aminosugars. Extraction of a blank kidney tissue was carried out to determine the origin of the interference peaks (Figure 3.2). The mobile phase conditions had changed as the mobile phase system containing acetonitrile was found to give a relatively stable baseline and better peak shape for neomycin in comparison to the methanol mobile phase used in the case of Figure 3.1.

To ensure that the interference on the chromatogram was from the bovine kidney sample and not from impurities from the sorbent or chemicals used in the MSPD procedure a series of investigations were carried out. In each case analysis was carried out by ion-pair chromatography in conjunction with the PAD under the conditions specified in the experimental chapter (section 2.5.3.).

Figure 3.2 A chromatogram of an acid extract from MSPD on CN from a blank untreated bovine kidney.

Injection volume 10 μ l. Mobile phase conditions: 20 mM PFPA, acetonitrile-water (28:72 v/v) at 1 ml/min and postcolumn 0.5 M sodium hydroxide at 0.3 ml/min. Column temperature 40°C. PAD Settings : Refer to Figure 3.1
Amplitude sensitivity 300 nA.



3.3.1 Blank injections

A number of blank solvent and reagent injections were carried out, and each allowed to run for 30 minutes. The chromatogram for each run was observed for any response.

Injection of deionised water and sulphuric acid (0.05 M) gave no response. The mobile phase of 20 mM PFPA and acetonitrile-water (28:72 v/v) upon injection gave no response either. Therefore from these results it was apparent that the interference was not due to impurities in the deionised water, sulphuric acid or mobile phase.

3.3.2 Solubility of the cyanopropyl sorbent after treatment with various solvents

The next step was to investigate whether the elution of methanol, deionised water or sulphuric acid, through the cyanopropyl sorbent during MSPD attacked the surface of the sorbent particles causing dissolution of the sorbent resulting in contamination of the sample analyte. Therefore the MSPD procedure was repeated, with no kidney tissue or spiked neomycin sulphate.

The bulk cyanopropyl sorbent was ground in a mortar after the addition of 1 ml of deionised water and then transferred to a plastic syringe barrel, to replicate the procedure used during the MSPD procedure. The solvents used in the MSPD step of the sample preparation stage were eluted through successively, 2 mls deionised water, 3 mls of hexane and 5 mls of ethyl acetate, methanol and methanol-water at 50:50 v/v, and finally 1 ml of deionised water.

The final stage replicated the elution of the sample analytes from the matrix column by the elution of 4 mls of 0.05 M sulphuric acid through the matrix column. The elutes obtained were injected onto the HPLC. A large peak at a retention time of 3.23 min showed up on the chromatogram (Figure 3.3.) upon the injection of the sulphuric acid elute.

For comparison, to determine whether the surface of other bulk sorbents was stripped by the solvent clean-up procedure in MSPD this procedure was repeated with the silica sorbent. Again upon injection of the acidic elute a large peak appeared at a retention time of 3.06 min, (Figure 3.4.) The results obtained from the investigation appeared to indicate that something was stripping off the sorbent and contaminating the sample but that this was not a function of the presence of the cyano bonded phase.

3.3.3 Clean-up of cyanopropyl sorbent matrix with sulphuric acid prior to use in MSPD.

Sulphuric acid (0.05 M) was eluted through the cyanopropyl (CN) sorbent, which was packed into a plastic syringe barrel. Injection of each acid elute onto the HPLC column indicated that at least 10 mls of sulphuric acid were required to successfully elute the contaminant off the CN sorbent matrix. The CN sorbent was then flushed with deionised water followed by buffer at pH 7 until the eluent was free of acid. The sorbent was then left to dry in an oven overnight at 50 °C.

To examine whether this procedure had successfully eliminated the contamination, a standard MSPD sample preparation procedure was carried

out in duplicate, one used the pre-treated CN sorbent (treated earlier with sulphuric acid to remove any source of contamination) the other used CN sorbent directly from the bulk container.

Figure 3.3 Chromatogram of an acidic extract using MSPD treated cyanopropyl silica.
Injection volume 10 µl. Mobile phase conditions: refer to Figure 3.2.
PAD Settings: refer to Figure 3.1. Amplitude sensitivity 300 nA.

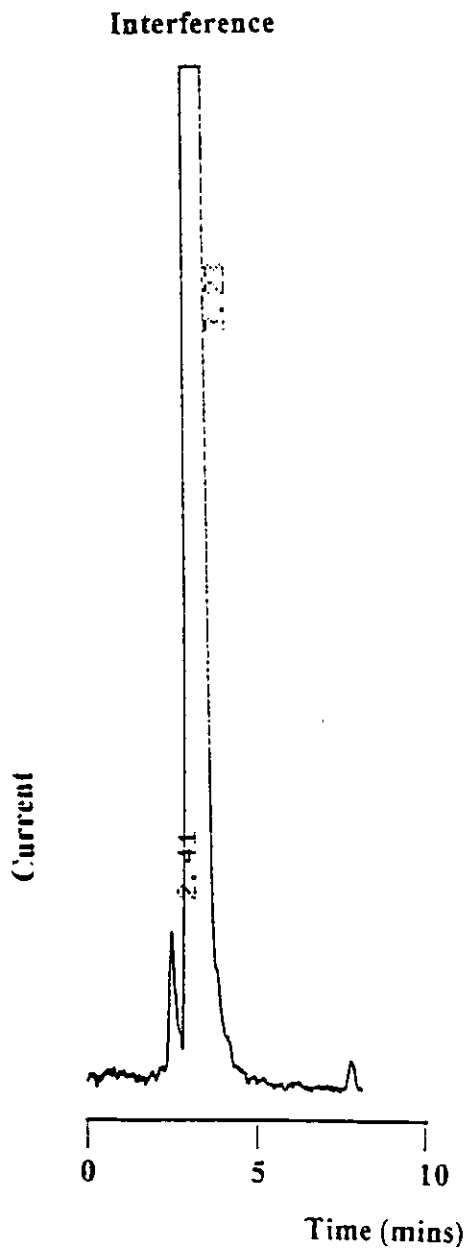
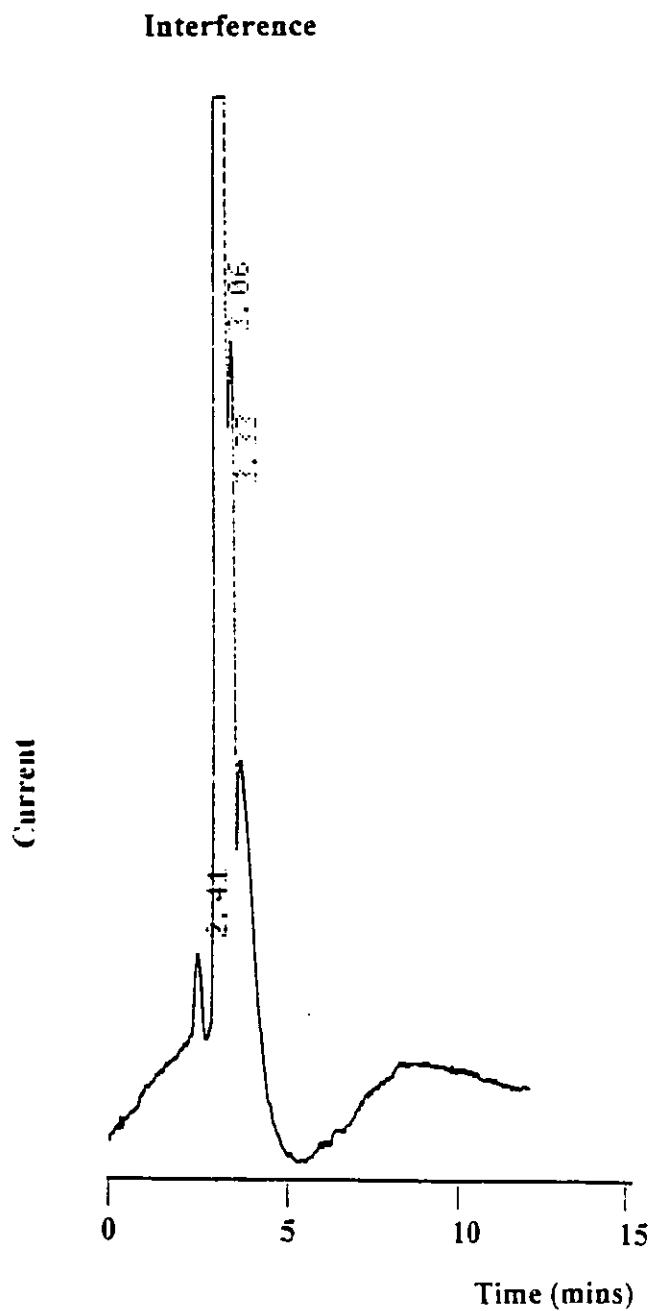


Figure 3.4 Chromatogram of an acidic extract using MSPD treated silica.

Injection volume 10 µl. Mobile phase conditions: refer to Figure 3.2.

PAD Settings : Refer to Figure 3.1. Amplitude sensitivity 300 nA.



Both bovine kidney tissue homogenates were spiked at 200 ppm neomycin sulphate. Both samples underwent the standard MSPD sample preparation procedure outlined in the standard method section of the experimental chapter (section 2.5.5). The sample analyte was eluted off the sorbent matrix column of each sample with 5 mls of 0.05 M sulphuric acid.

On injection of the acid elute from each sample onto the HPLC column a large peak appeared at a retention time of 3 mins for both samples, the extraction procedure using the pre-treated cyanopropyl (Figure 3.5.) and the untreated cyanopropyl (Figure 3.6.) Therefore it was apparent from these results that the acid wash procedure had not been successful enough as a method to remove the contaminant causing the large peak. Also pre-treating the cyanopropyl sorbent had affected its trapping and eluting properties, because the chromatogram obtained from the acidic elute of the pretreated cyanopropyl matrix (figure 3.5) showed no neomycin peak.

3.3.4 Discussion

The results from the investigation into the origin of the interference indicated that some contaminants were stripping from the sorbent into the final elute as a result of the solvent and acid wash of the sorbent. Barker¹⁵¹ washed bulk C18 sorbent with successive 50 mls each of hexane, ethyl acetate and methanol to remove contaminants inherent in manufacture prior to the extraction procedure. Therefore it is possible that the contaminants still present in the bulk cyanopropyl (CN) are due to the manufacturing process, and a great deal of pretreatment of the sorbent is required with large amounts of solvent prior to its use.

Figure 3.5 Chromatogram of an acidic extract from MSPD treated bovine kidney tissue spiked at 200 ppm neomycin sulphate, using pre-treated cyanopropyl sorbent.

Injection volume 10 µl. Mobile phase conditions: refer to Figure 3.2.

PAD Settings: Refer to Figure 3.1. Amplitude sensitivity 100 nA.

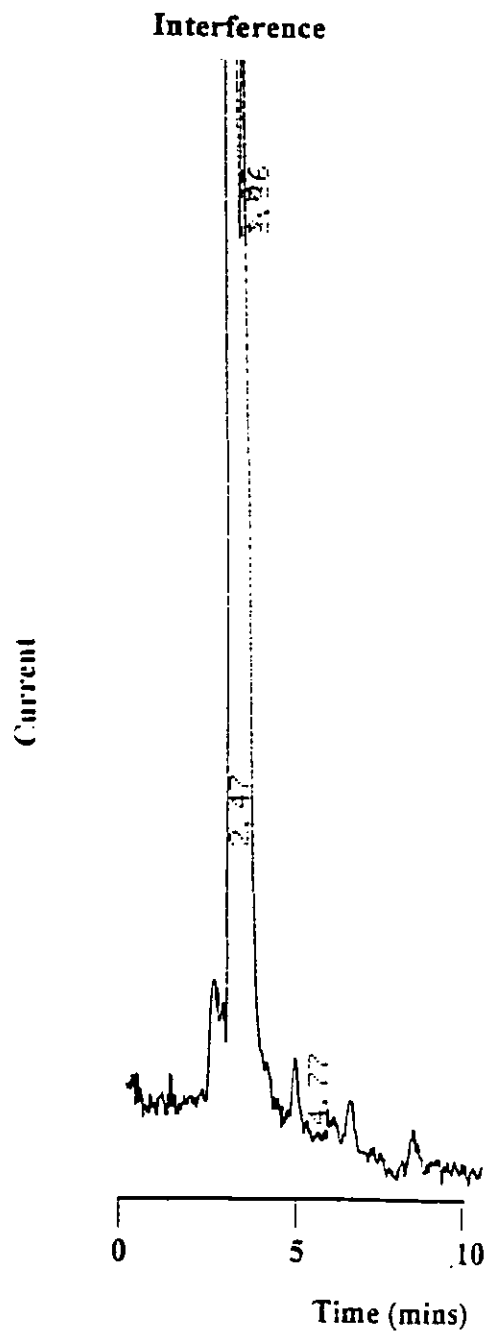
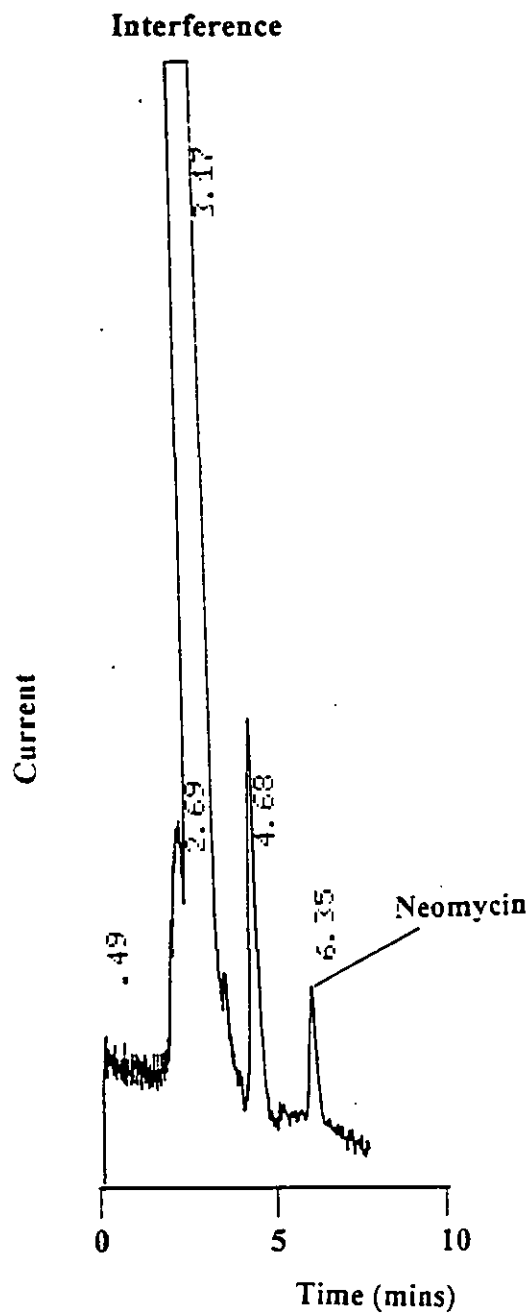


Figure 3.6 Chromatogram of acidic extract obtained from MSPD treated bovine kidney tissue spiked at 200 ppm, using untreated cyanopropyl sorbent.

Injection volume 10 µl. Mobile phase conditions : refer to Figure 3.2.

PAD Settings: Refer to Figure 3.1. Sensitivity 100 nA.



The contamination peak could also be the result of the functional groups stripping of the reversed-phase column resulting in the gradual degradation of the column as a result of the acidic mobile phase due to the acidic ion-pair PFPA (20 mM). Silica based columns are pH sensitive. A low pH mobile phase (= 2.0) will hydrolyse the bonded phase and strip of the functional groups and a high pH (= 8.0) will dissolve the silica. However, counter measures were taken to prevent degradation of the reversed-phase column, the column was cleaned by washing it overnight with acetonitrile-water (50:50 v/v). There was no appearance of the interference peaks on the injection of sulphuric acid (0.05 M) or mobile phase (section 3.3.1) onto the reversed-phase column under the ion-pair mobile phase conditions used for the study. This indicated that the interference peaks were not a result of column degradation. As discussed earlier in this section the interferences were from contaminants that had stripped off the CN sorbent by the solvent and acid washes that the sorbent was exposed to during the MSPD procedure. It was concluded that pretreatment of the CN sorbent with large amounts of solvent and acid washes to remove the contaminants was too time consuming and was not carried out as part of the MSPD procedure. The interference peaks were therefore accepted as part of the chromatographic profile.

3.4 Preconcentration

An additional step was needed after the MSPD procedure to reduce the volume in which the neomycin or streptomycin was reconstituted prior to injection. The detection limit of the procedure was 5 ng (10 µl injection of a 5 ppm solution of neomycin sulphate), the limit of quantification was not measured. A large volume of sulphuric acid (8 ml) was required to elute the aminoglycoside from the tissue matrix column. This further diluted the

aminoglycoside. Therefore an extra step was added to concentrate the aminoglycoside back to at least half its original spiking concentration and therefore improve sensitivity. This procedure had the advantage of at least concentrating the analyte after its dilution in the MSPD procedure thereby allowing for the analysis of neomycin spiked at 20 ppm. This would give a final response equivalent to 10 ppm which was the limit of quantification using this method. It was therefore necessary to locate a sorbent which would efficiently trap neomycin but only hold it weakly so that it could be readily released. Various solid phase extraction cartridges were therefore investigated for preconcentration of the analyte aminoglycoside.

Development of the preconcentration step was monitored by ion-pair HPLC and PAD detection under the conditions specified in the experimental chapter (section 2.5.3). Due to early work (section 3.2) it was evident that the sorbent cartridges tested earlier in i.e., C18, Si, SCX gave poor results showing no evident ability to trap or release the aminoglycoside neomycin. A sorbent was required that not only effectively retained and eluted the aminoglycoside from the aqueous solution obtained in the MSPD stage but also required a minimal amount of elution solvent to yield the aminoglycoside.

The intention was to examine a weak acid CBA (carboxymethyl) SPE cartridge as a possible means of further concentrating the sample analyte. Above a pH of 4.8 CBA carries a negative charge that can be used to retain cationic isolates, such as the amino groups on the aminoglycoside. On dropping the pH below 4.8 the CBA is neutralised allowing the retained aminoglycoside to be eluted from the sorbent. Because of its weak cation exchange characteristics irreversible retention of the cation onto the CBA sorbent should not be a problem. Furthermore, only a small amount of acid at a pH of 2 or below

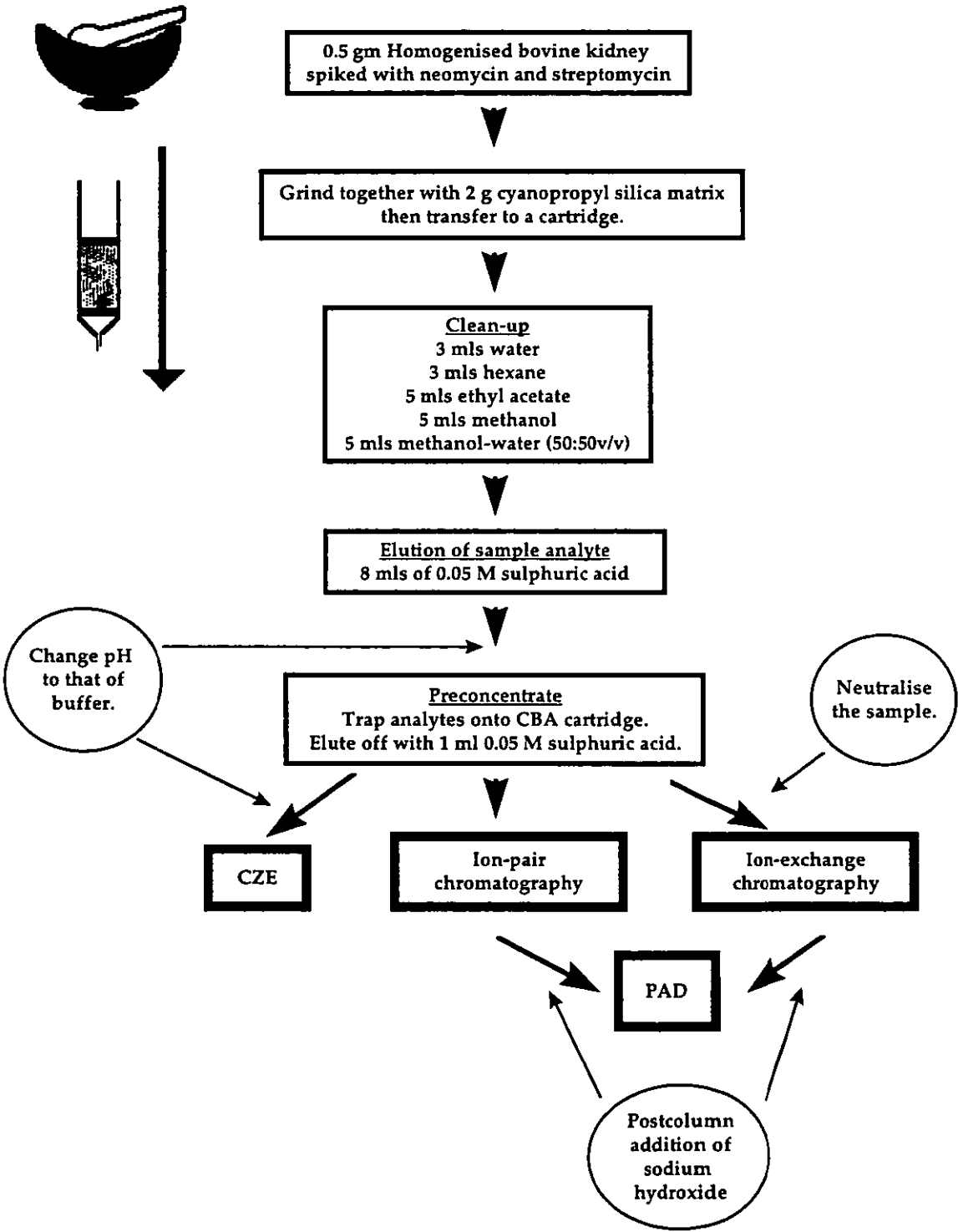
would be required for elution of the aminoglycoside.

Initial studies were carried out on the relative recovery ability of the CBA solid phase extraction cartridge of 100 mg sorbent mass with a cartridge volume of 1 ml using standard solutions of neomycin sulphate and streptomycin sulphate. Firstly the cartridge was preconditioned by running through 0.05 M sulphuric acid followed by pH 7 buffer solution. The buffer was eluted through until the elute washings were neutral (pH 7), the CBA cartridge was now ready for use. A known concentration of neomycin sulphate and streptomycin sulphate standard solution, adjusted to pH of 7, was loaded onto the cartridge. At this stage the two analytes should have been trapped by the sorbent. To ensure this was the case the elute obtained from loading the standard solution onto the cartridge was assayed. The detector showed no response therefore it appeared both neomycin and streptomycin had been bound to the sorbent. To elute the analytes 0.5 ml aliquots of 0.05 M sulphuric acid were eluted through very slowly, at a flowrate of a drop every 3 secs and the eluent collected for injection. Injections of the aliquots showed that at least 1 ml of sulphuric acid was required to completely elute both neomycin sulphate and streptomycin sulphate off the CBA sorbent. Recovery of both analytes was calculated at 80 %.

The next stage of the study was to couple the preconcentration step involving the CBA cartridge to the MSPD sample preparation procedure discussed earlier, so that further concentration of the aminoglycosides was achieved prior to detection. Again the same procedure carried out with the CBA cartridge outlined earlier was repeated using the elute containing the aminoglycoside obtained from a spiked bovine kidney sample after the MSPD procedure. Following the MSPD sample preparation procedure the acidic elute, containing the analytes collected from the CN sorbent tissue mix column, was neutralised

to a pH of 7 by the addition of 0.1 M sodium hydroxide and then loaded onto the preconditioned CBA 100 mg/ml cartridge. The analytes were then eluted off the CBA cartridge with sulphuric acid (0.05 M). The first millilitre eluted through the CBA cartridge was assayed. Using this procedure (as outlined in Figure 3.7) recovery of both neomycin sulphate and streptomycin sulphate was obtained as well as further concentration of the aminoglycosides in the MSPD elute prior to injection. The final concentration of the aminoglycoside sample on concentration after MSPD on the CBA SPE cartridge was half the original spiking concentration. Therefore the aminoglycoside samples concentration was halved by the sample preparation procedure prior to detection.

Figure 3.7 The following diagram outlines each step of the sample preparation stage prior to injection.



Pulsed Amperometric Detection

CHAPTER FOUR

CHAPTER FOUR

4. *Pulsed Amperometric Detection*

4.1 Introduction

Carbohydrates like aminoglycosides are nonchromophoric but can be detected with good sensitivity by the PAD at ppm levels. As mentioned in the introduction, the PAD has been reported to be used for the direct detection of the aminoglycosides, avoiding a derivatisation step which would be needed for conventional UV detection. Previous studies had been carried out using the PAD as a detector for aminosugars and related compounds for example amino acids, alkanolamines by Johnson^{105,106,107} carbohydrates Quigley¹⁰⁴, and neomycin by the application chemists at Dionex.¹²³ These studies are covered in more detail in the introduction chapter. Aminoglycosides have properties related to carbohydrates, therefore the conditions and settings selected for pulsed amperometric detection of carbohydrates can be similarly used for the aminoglycosides.

Glucose is a good example of a compound easily detected by the PAD.^{72,73,74,75,76, 159,160,161,162} With all carbohydrate related compounds a high pH is required to increase the sensitivity of the PAD for detection of the compound as this results in an increase in the rate of oxidation of the compound. The enhancement of oxidation rates by high alkalinity results from H^+ produced in the reaction steps leading to the rate-determining step.¹⁰⁹

The PAD electrochemically oxidises neomycin at a pH of about 13 using a gold electrode.¹²³ As the pH of the mobile phase is below 12 in the anion exchange

chromatography of neomycin developed by Dionex,¹²³ sodium hydroxide had to be added as a postcolumn solution to raise the pH and improve sensitivity on the PAD.

4.2 Cyclic voltammetry of neomycin sulphate and streptomycin sulphate

The optimum detection, cleaning and regeneration pulses as mentioned in the introduction (section 1.7) were determined for the aminoglycosides using cyclic voltammetry. In cyclic voltammetry,¹⁰⁹ the applied potential is slowly scanned back and forth between a positive and a negative potential. The resulting current is plotted on the y-axis against the scanning potential on the x-axis. Once scans have been obtained for the sample and for the background, for example the mobile phase eluent, the optimum pulse sequence can be chosen by studying the two traces to select the position of maximum current difference.

A cyclic voltammetry scan of the sample neomycin sulphate at a concentration of 0.5 mM in 0.15 M sodium hydroxide and streptomycin sulphate at a concentration of 0.5 mM in 0.15 M sodium hydroxide were carried out, (refer to Figures 4.1 and 4.2.) This was to determine the maximum current (signal) obtained for oxidation of the aminoglycoside at a pH equivalent to 13, the optimum pH for oxidation. Finally a background scan of 0.15 M sodium hydroxide without either aminoglycoside was measured (Figure 4.3.), to determine the optimum difference in current between the background and the aminoglycoside scan. The concentration of the sodium hydroxide was equivalent to that entering the PAD cell after mixing with the column effluent.

Neomycin and streptomycin sulphate voltammograms (figure 4.1 and 4.2)

showed that broad oxidation peaks are in the region from - 0.15 V to + 0.15 V with a maximum at - 0.025 V. To obtain the maximum sensitivity (on signal to noise basis) the initial applied potential chosen for monitoring in flow was + 0.1 V as this would give the maximum signal. The cleaning potential chosen was large and positive, + 0.6 V and to reduce the gold electrode back to gold a large negative potential was applied, - 0.8 V.¹²³ These conditions were equivalent to those used for the detection of carbohydrates.¹⁰⁷

Figure 4.1 Voltammetric scan of 0.5 mM neomycin sulphate in 0.15 M sodium hydroxide pH 13.

Superimposed over background scan of 0.15 M sodium hydroxide (- - -)

Neomycin scan (----)

Scan range -0.4 V to + 0.2 V, X = 50 mv/cm, Y = 0.1 mv/cm

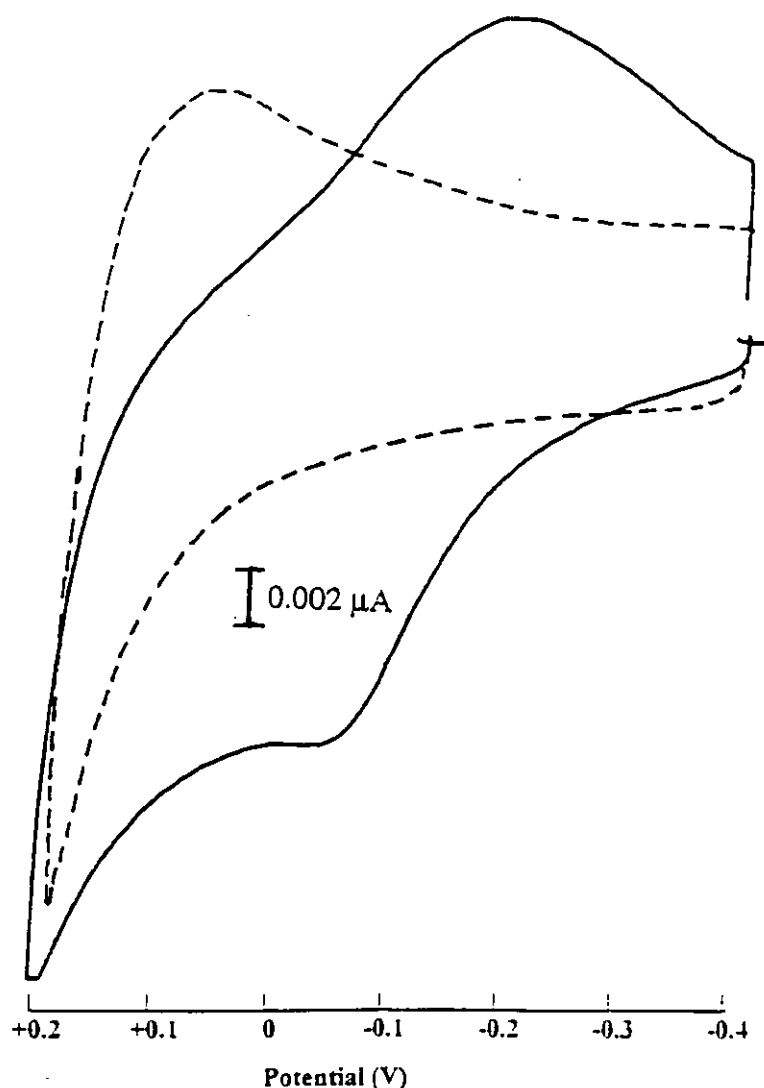


Figure 4.2 Voltammetric scan of 0.5 mM streptomycin sulphate in 0.15 M sodium hydroxide pH 13.

Superimposed over the background scan of sodium hydroxide 0.15 M (---)

Streptomycin scan (—)

Scan range -0.4 V to +0.2 V, X = 50 mv/cm, Y = 0.1 mv/cm

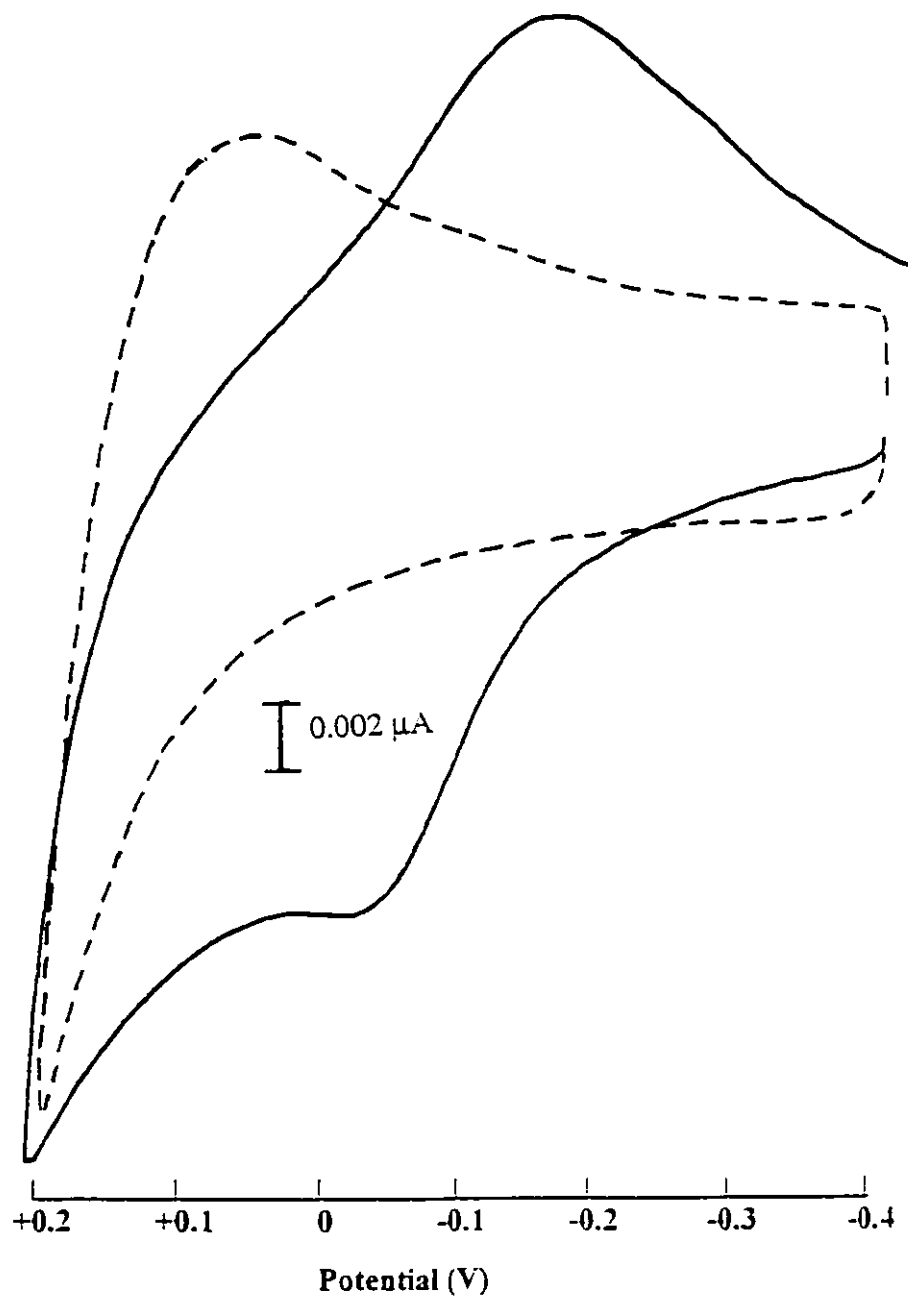
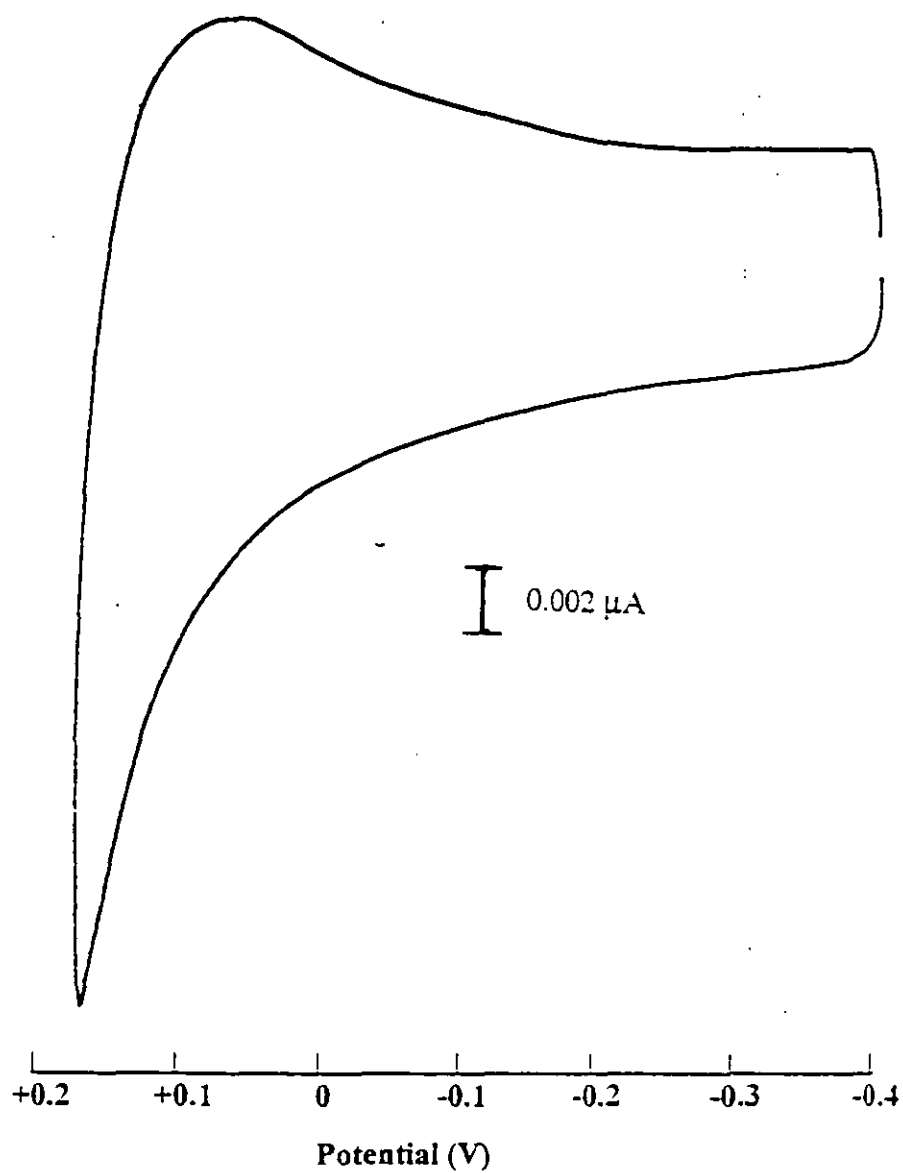


Figure 4.3 Voltammetric background Scan of Sodium hydroxide 0.15 M pH 13.

Scan range -0.4 V to +0.2 V, X = 50 mv/cm, Y = 0.2 mv/cm



4.3 Trial for an optimum E1 measuring potential

The E1 measuring potential on the PAD was varied from the initial value of +0.1 V to confirm that this potential represented the optimum response and magnitude of the neomycin sulphate peak.

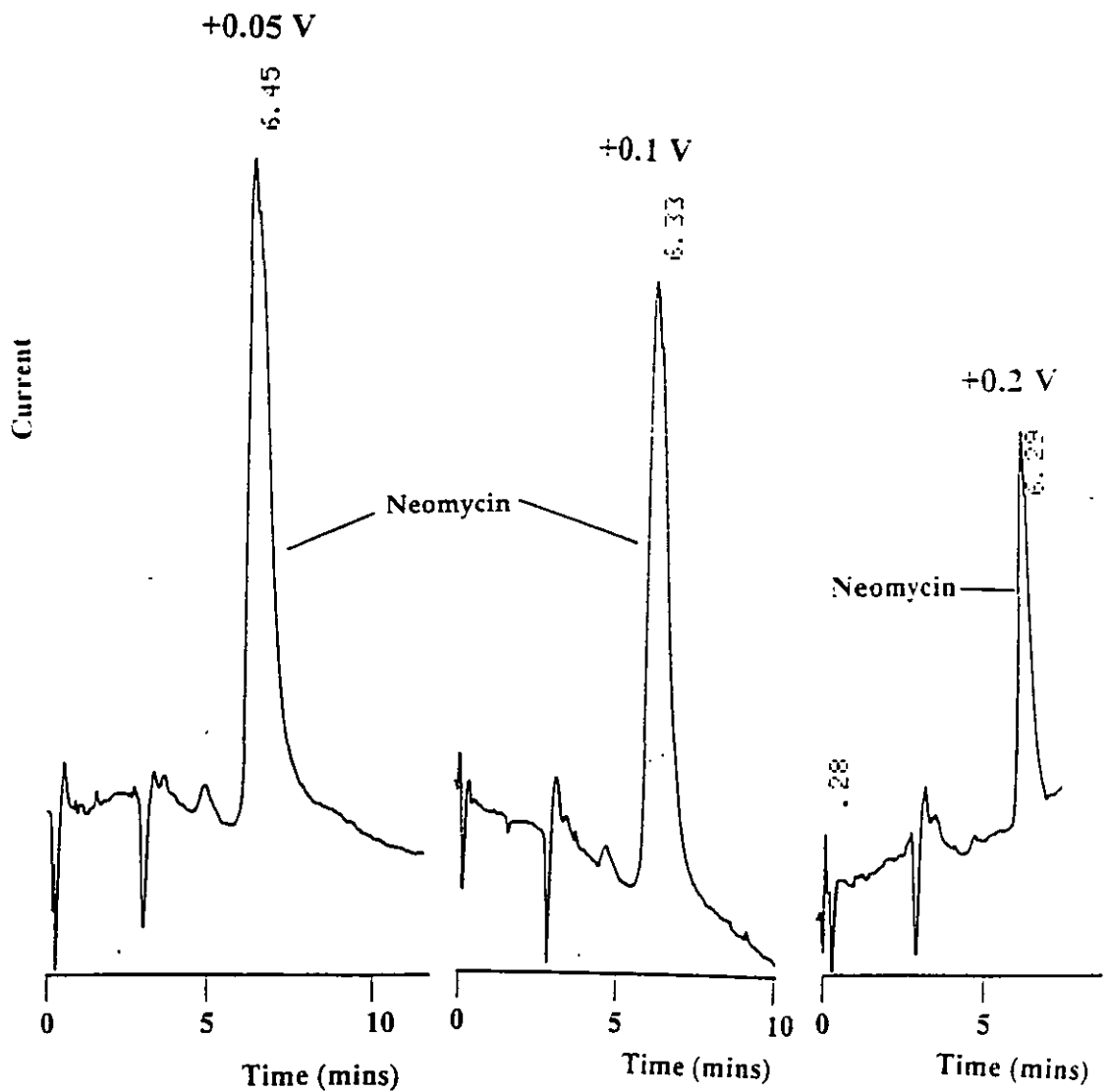
The other pulse sequence on the PAD was set equivalent to the settings just outlined, the cleaning E2 at + 0.6 V and regenerating E3 at - 0.8 V pulse. The measuring potential was varied from the range + 0.05 V to + 0.2 V. This range represented the region of the scan which showed the maximum current signal (Figure 4.1, 4.2). The experiment was carried out by the ion-pair chromatography assay method outlined in the experimental chapter (section 2.5.3). Solutions of neomycin sulphate standard (100 ppm) were injected and the measuring potential (E1) on the PAD was changed for each injection. The response and peak area of each injection was compared with the corresponding E1 potential (Figure 4.4.)

From the results it is apparent that there is not a great difference in the magnitude of response between the potential settings + 0.1 V and + 0.05 V. A potential of + 0.2 V for E1 in comparison with the E1 potential setting of + 0.1 V shows a 40 % drop in the response. Therefore it is clear from these results that a measuring potential set at + 0.1 V would give the optimum response value and this value was used in all subsequent work.

Figure 4.4 The following chromatograms were obtained for neomycin sulphate (100 ppm) at the following *EI* potential settings.

Injection volume 10 µl. Mobile phase conditions: 20 mM PFPA, methanol-water (40:60 v/v) at 1 ml/min, postcolumn 0.5 M sodium hydroxide at 1 ml/min. Column temperature at 45 °C. Amplitude sensitivity 300 nA.

EI potential setting



Ion-Exchange Chromatography of Aminoglycosides

CHAPTER FIVE

CHAPTER FIVE

5. *Ion-Exchange Chromatography of Aminoglycosides*

The second part of the study examined the separation methods that could be employed. Initially ion-exchange separations were examined as these had been reported previously.¹²³ Neomycin has two accessible functional groups, the amino and hydroxyl (refer to Figure 1.2 in the introduction), that allow both anion and cation exchange chromatography to be exploited. Hydroxyl groups can be deprotonated at high pH to provide oxyanions, suitable for anion exchange separation. At low pH the amine group can be protonated and the ammonium ion form separated by cation exchange chromatography.

Significant advances in the chromatographic separations of simple and complex carbohydrates have resulted from the development of new, highly efficient stationary phases¹⁶³ for ion-exchange chromatography. These polymer-based materials are robust and tolerant of the alkaline mobile phases desired for optimum sensitivity in the various forms of pulsed electrochemical detection. Anion-exchange liquid chromatography with pulsed amperometric detection (LC-PAD) has been applied for the determination of alcohols and carbohydrates,^{72,74,104,118} Amines,¹⁶⁴ and aminosugars,^{75,76,122,165} and several sulphur compounds.¹⁶⁶ LaCourse¹⁶² analysed carbohydrates by anion-exchange chromatography with detection by PAD, using an HPIC-AS6A anion-exchange column, and a sodium hydroxide gradient for separation of the carbohydrates. The increase in sodium hydroxide concentration from 1-100 mM is considered typical of pH gradient separations of carbohydrates. Quigley and Englyst¹⁰⁴ used an anion exchange CarboPac PA1 column with detection by the PAD, for the separation of neutral sugars and aminosugars. Separation of neutral sugars

in the absence of aminosugars was achieved using isocratic elution with 40 mM sodium hydroxide-water 23:73 v/v from 0 to 3.5 mins, a gradient of 40 mM sodium hydroxide-water from 23:77 v/v to 1:99 v/v from 3.5 to 4.5 min, and isocratic elution with 40 mM sodium hydroxide-water 1:99 v/v from 4.5 to 30 min at a flowrate of 1 ml/min, followed by at least 6 mins to re-equilibrate with the starting conditions before injection of the next sample. Isocratic separation of neutral and aminosugars was achieved with 40 mM sodium hydroxide-water 16:84 v/v. After 40 minutes the column was purged with 100 mM sodium hydroxide for 5 minutes followed by 6 minutes re-equilibration with starting conditions before the next injection. Aminoglycosides behave chromatographically much the same as aminosugars, are anionic at very high pH and are thereby retained on the high capacity anion exchange column.

A method had been reported by Dionex in a technical report,¹²³ which separated neomycin sulphate B and C by high performance anion exchange chromatography (HPAEC) on an anion exchange column under alkaline gradient conditions (section 1.7). Combining this with detection by the pulsed amperometric detector (PAD) resulted in an analytical method with sensitivity equal to or greater than the published methods for aminoglycoside detection using UV, but without the need for derivatisation. It was decided to examine the reported method by Dionex¹²³ to determine its value and robustness for use with the extracted aminoglycoside from bovine kidney.

5.1 Anion exchange chromatography

The method outlined by Dionex for the analysis of neomycin sulphate¹²³ involved a 10 mM sodium hydroxide - water gradient with post column addition of 0.5 M sodium hydroxide. Increasing the pH of the mobile phase

gradually as a gradient results in increasing the degree of deprotonation of the aminoglycoside converting it to the oxyanion making it anionic and therefore retained on the anion exchange column for longer. Separation of the aminoglycoside was carried out on an anion exchange CarboPac PA1 column. In the present work this method was slightly modified by an increase in the concentration of the postcolumn sodium hydroxide to 1 M as this was found to give the maximum amount of response on the PAD detector. Initially successive injections of neomycin on the CarboPac PA1 column were found to result in large shifts in the retention time of neomycin. It became apparent that the column needed to equilibrate back to the original starting conditions of the gradient prior to injection. Therefore after each injection the column was flushed with water for 5 minutes prior to the next injection. These changes achieved a stable retention time and better peak shape. The slightly modified method is outlined in the standard method section of the experimental chapter as the standard method for the analysis of neomycin sulphate by anion exchange chromatography (section 2.5.2). The instrumental set-up is outlined in Figure 2.1 of the experimental chapter.

5.1.1 Standard injections

Injections (25 µl) of neomycin sulphate at concentrations ranging from 1 to 100 ppm were made onto the anion exchange column to examine the chromatography and sensitivity of the method at a range of concentrations of the aminoglycoside. An injection of a 10 ppm standard solution produced a very good response, (Figure 5.1.) Because the sample reagent of neomycin sulphate obtained from Sigma for the project contained 92 % neomycin B and 8 % neomycin C, two peaks appeared, firstly a small peak representing neomycin

C tailing onto the large neomycin B peak at a retention time of 9.2 mins.

Further injections were carried out at concentrations less than 10 ppm to determine the sensitivity of the detector. The lowest concentration that could be detected by the detector was 1 ppm, (Figure 5.2), with a signal to noise ratio of 1:5. However, to detect ppb levels, the sensitivity of the detector would have to be increased. Increasing the sensitivity of the detector amplifier to 30 nA range also resulted in further magnification of the baseline noise which in turn lead to problems with resolution of the analyte peak.

5.1.2 Calibration graph

After the establishment of the high performance anion exchange chromatography method the next stage was to determine this methods precision and linearity. This was carried out for all the assay methods developed during the course of the project. A standard linearity check of detection was carried out with a standard range from 1 to 100 ppm to determine the suitability of the system for the range of concentrations under analysis.

Figure 5.1 A chromatogram of neomycin sulphate at 10 ppm.

Injection volume 25 μ l. Mobile phase conditions: 100 mM sodium hydroxide-water gradient, water (100 %) for 5 mins followed by 100 mM sodium hydroxide-water (to 40:60 %) over 10 mins at 1 ml/min. Postcolumn 1 M sodium hydroxide at 1 ml/min. PAD Settings: E1 +0.1 V (300 ms), E2 +0.6 V (119 ms) and E3 -0.8 V (299 ms). Amplitude sensitivity 100 nA.

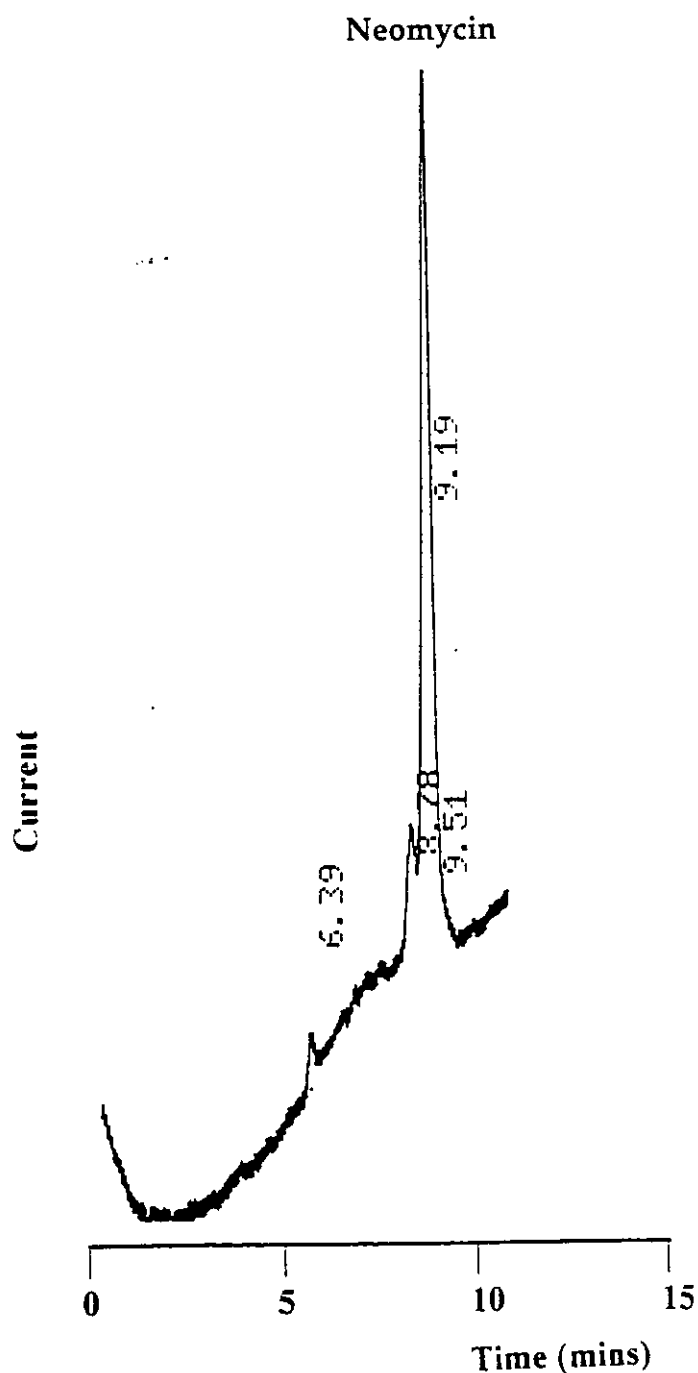
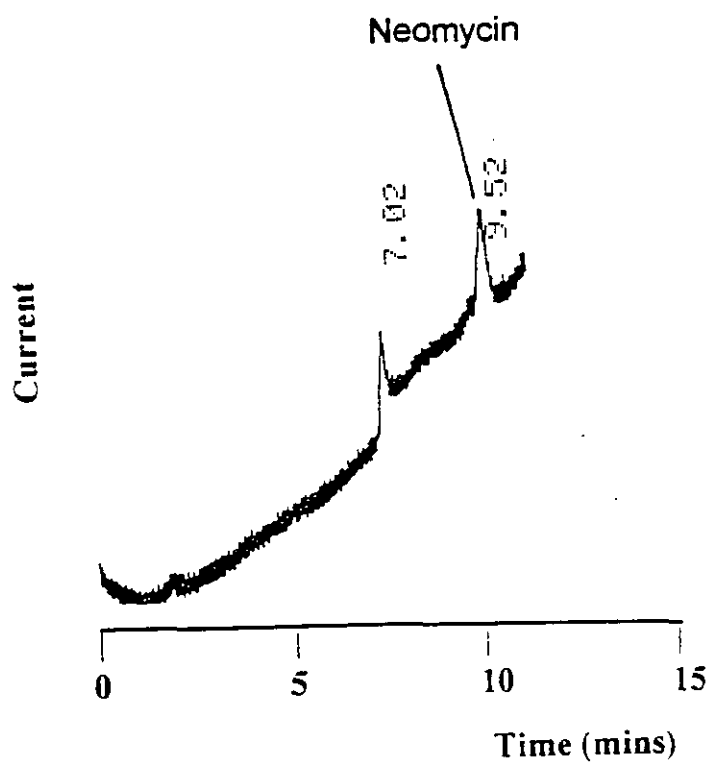


Figure 5.2 A chromatogram of neomycin sulphate at 1 ppm.

Injection volume 25 μ l. Mobile phase conditions: refer to figure 5.1

PAD Settings: Refer to Figure 5.1. Amplitude sensitivity 100 nA.



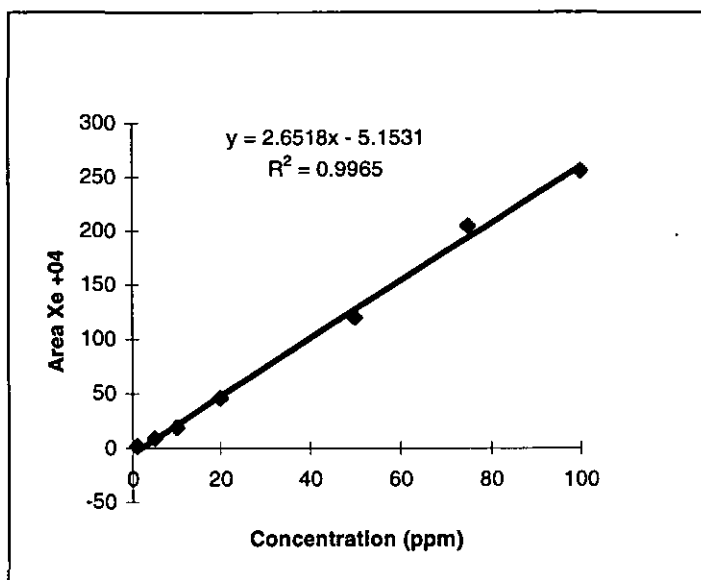
Standard solutions of neomycin sulphate were prepared at concentrations ranging from 1-100 ppm. Duplicate injections of each concentration were made and peak areas obtained corresponding to each standard (Table 5.1.) The peak areas obtained for each standard were plotted to represent a calibration graph ranging from 1ppm to 100 ppm (Figure 5.3). On reference to the graph the working range for sample analysis appeared to be achievable from 5 ppm upwards to 100ppm (Figure 5.3).

TABLE 5.1 TABLE CONTAINING THE PEAK AREAS CORRESPONDING TO EACH NEOMYCIN SULPHATE CONCENTRATION FOR THE CALIBRATION GRAPH.

Mobile phase conditions refer to figure 5.1.

Concentration of neomycin sulphate (ppm)	Area E + 04
1	1.67
5	8.79
10	18.89
20	46.03
50	119.80
75	204.74
100	256.14

Figure 5.3 A calibration graph of neomycin sulphate ranging from 1 to 100 ppm
(Data Table 5.1)



5.1.3 Reproducibility

The reproducibility of the assay outlined in section 2.5.2 of the Experimental chapter was determined by carrying out 6 replicate injections of a 5 ppm standard of neomycin sulphate. The areas obtained are displayed in Table 5.2. The standard deviation of the areas obtained was calculated as an indication of reproducibility for system suitability.

TABLE 5.2 A TABLE OF THE PEAK AREAS OBTAINED ON REPEAT INJECTIONS OF 25 μ L INJECTION VOLUMES OF 5 PPM NEOMYCIN SULPHATE.

For separation conditions refer to figure 5.1.

Area n = 6
15109620
15752124
14548977
15975625
15226543
14262816
Mean: 15145951
SD: 663313.66
RSD: 4%

The RSD of 4 % showed that the reproducibility of the injections was good considering the samples were injected manually.

5.2 Examination of acidic analyte solutes

The overall aim of the project was to develop a method in which the solute neomycin sulphate could be eluted off a CN sorbent/tissue matrix column packing (or from the CBA column after further concentration) as a solution that could be injected directly onto an anion exchange column for further analysis.

However, for this method to operate the anion exchange gradient system set-up on the CarboPac PA1 column, would have to be able to accept the neomycin sulphate in a solution of dilute sulphuric acid (0.05 M). The alternative would involve prior neutralisation of the acidic sample extract containing neomycin sulphate. However, this was to be avoided as it would result in further sample handling and dilution of the sample. At this point in the project the intention was to produce an on-line sample extraction assay method which avoided any sample pH manipulation prior to injection.

Neomycin sulphate standard solutions prepared in sulphuric acid (0.05 M) and solutions of neomycin sulphate eluted from a cyanopropyl solid phase extraction cartridge by sulphuric acid, were injected onto the CarboPac PA1 column. There was no response corresponding to neomycin sulphate, but a large peak appeared in both cases at a retention time of 2 minutes. It appeared that the pH change of the sample had resulted in a shift in the retention time of neomycin sulphate. Further injections of acidic neomycin sulphate standards produced either no response or peaks of varying retention times from 2 to 14 minutes. It was likely that the eluent condition at the start of the gradient had little buffer capacity and therefore neomycin eluted directly as a cation without interaction with the ion-exchanger.

A different gradient system was therefore investigated, starting under acidic conditions at 100 % 0.8 M glacial acetic acid and switching over to 150 mM sodium hydroxide-0.8 M glacial acetic acid at 50:50 v/v over a 10 minute period. It was hoped these mobile phase conditions would stabilise the neomycin in the acid state prior to separation by the alkali gradient. Injection of acidic neomycin sulphate on the anion CarboPac PA1 column, under these mobile phase conditions, produced a peak at a retention time of 10 mins.

However, on just running the gradient with no injection, a peak appeared at the same retention time. The reason for this may have been the retention and later elution of the glacial acetic acid or possibly there may have been an impurity present in the mobile phase used in the gradient.

Therefore it was apparent that the injection of acidic neomycin sulphate onto the anion CarboPac PA1 column produced irreproducible results. With the gradient system that was proposed there was no buffer or pH control in the initial stages the protonated acidic neomycin was unretained. Therefore as an alternative cation exchange chromatography was investigated.

A good solution would have been to buffer the eluent throughout the gradient, however this would have an adverse effect on the PAD as the buffer would reduce the pH of the eluent even after postcolumn addition of sodium hydroxide resulting in varying sensitivity of the detector.

5.3 Cation exchange chromatography coupled with the PAD

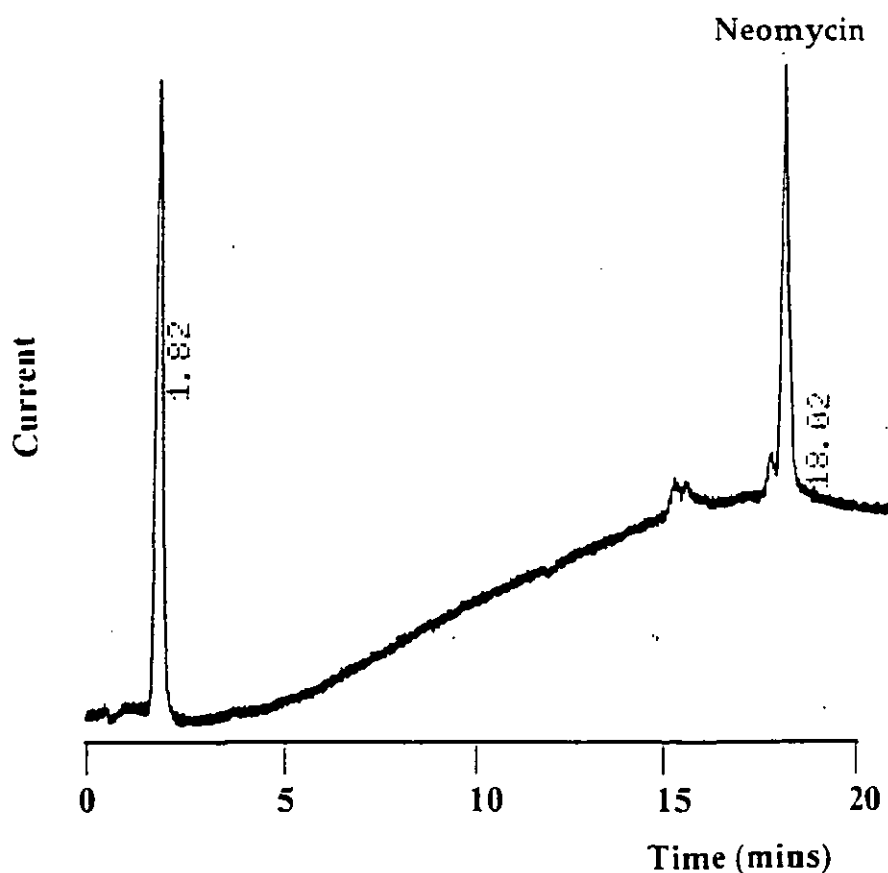
In order to undergo cation exchange chromatography the components of the sample are separated by the protonation of the amino groups present and detection by the PAD is achieved by oxidation of the hydroxy groups after the pH has been adjusted to 13. The cation-exchange separation was performed using a cation Dionex IonPac CS3 separator column with an IonPac CG3 guard column, under the conditions specified for the anion exchange chromatography of neomycin sulphate using the 10 mM sodium hydroxide gradient. This sodium hydroxide gradient was used to determine its effect on the separation of neomycin on a cation exchange column. Injection of a standard solution of neomycin sulphate produced a retention time of 12 mins.

However on injection of a acidic standard solution of neomycin sulphate, the neomycin sulphate peak appeared at a retention time of 18 mins (Figure 5.4.) However, further injections of the neutral and acidic standard solutions of neomycin sulphate, resulted in a different retention time for each neomycin sulphate injection ranging from between 12 to 20 mins.

Figure 5.4 Chromatogram of 50 ppm neomycin sulphate solution (prepared in 0.05 M sulphuric acid) injected onto a Cation exchange CS3 column.

Mobile phase conditions: refer to figure 5.1

PAD Settings: Refer to Figure 5.1. Sensitivity 100 nA.



A pH of below 7 would be required for the protonation of the amine functional group on the aminoglycoside to occur forming the cation for cation exchange to occur on the column. Alternative mobile phase systems were investigated using milder pH conditions.

Using 0.6 M sodium acetate - water (20:80 v/v) at a pH of 5 in an isocratic run, injection of a neomycin sulphate standard prepared in water or acid (0.05 M sulphuric acid) resulted in no evident chromatography or response. Previous work carried out in this area of study involving ion-exchange coupled with the PAD for the analysis of aminoglycosides, found the sodium acetate gradient system detailed in Table 5.3 would separate a related aminoglycoside spectinomycin from its related impurities with improved resolution.¹⁶⁷ It was hoped that this gradient could also separate neomycin sulphate. Increasing the sodium acetate concentration would decrease the retention time of the neomycin, due to the competition of the acetate ions with the neomycin ions for sites on the column. However, upon injection of a standard solution of neomycin sulphate solution there was no evident chromatography or response.

TABLE 5.3 SODIUM ACETATE GRADIENT PROGRAMME⁶⁷ PREPARED FOR THE ANALYSIS OF NEOMYCIN SULPHATE ON THE CATION EXCHANGE CS3 COLUMN.

Time (Mins)	0.6 M Sodium acetate (%)	Water (%)
0	20	80
5	70	30
20	90	10
21	20	80

5.4 Conclusion

High performance ion-exchange chromatography in conjunction with the PAD detector initially appeared to provide a good technique for the separation of neomycin sulphate at a reasonably good sensitivity. This is especially the case with anion-exchange chromatography utilising the standard gradient program of sodium hydroxide outlined in the standard method section (section 2.5.2). However, this technique failed when used to separate neomycin sulphate in the acidic samples obtained from the MSPD sample preparation procedure under development for the extraction of neomycin sulphate from bovine kidney tissue.

Cation exchange chromatography as an alternative was found to separate neomycin sulphate using the sodium hydroxide gradient used for anion exchange separation. However, it failed when applied to the separation of neomycin sulphate as acidic samples, resulting in inconsistent results with varying retention times on each injection. Mobile phases containing sodium acetate were investigated, sodium acetate is retained strongly by the column it was hoped it would allow faster elution of the neomycin sulphate. However using mobile phases containing sodium acetate produced no evident separation.

Because of the basic nature of neomycin sulphate any sample preparation method is almost certainly going to release neomycin by and in an acidic solution from a matrix mix. Ion-exchange chromatography was therefore considered to be incompatible with the MSPD. As a result an alternative method for HPLC would have to be investigated, one which will be able to

reproducibly separate neomycin sulphate in an acidic solution with good chromatography.

**Separation of Neomycin and Streptomycin by Ion-Pair
Chromatography Coupled with Pulsed Amperometric Detection**

CHAPTER SIX

CHAPTER SIX

6. *Separation of neomycin and streptomycin by ion-pair chromatography coupled with Pulsed Amperometric Detection*

As discussed in the previous chapter neomycin extracted from bovine kidney tissue by MSPD in acid could not be separated directly by ion-exchange chromatography without neutralisation and hence dilution, it was therefore decided to examine ion-pair chromatography. Ion-pair chromatography has been examined previously for aminoglycosides.^{80-88,100-103} Polar compounds like neomycin and streptomycin that would normally be soluble only in aqueous phases can be made more soluble in organic solvents by the formation of a hydrophobic "ion-pair" with an aqueous counter-ion. The ion-pair is then able to partition between the organic and aqueous layers in the same manner as a neutral molecule and hence be retained by a reversed mode of separation.

The nature of the counter-ion can be selected to provide optimum chromatographic retention and selectivity. Also the nature of the organic solvent in the mobile phase influences the selectivity of the system, whereas the concentration of organic solvent determines the degree of retention of solutes as with conventional reversed-phase separations. Considering these points in this study a variety of ion-pair solvent systems were investigated by reference to previous work discussed in the introduction chapter.^{80-83,85,87,88,100-103} The intention was to develop a method that would efficiently analyse neomycin and streptomycin and could be used as a standard assay technique in conjunction with acid solutions obtained from the sample preparation MSPD step for extracting the two analytes neomycin and streptomycin from the bovine kidney sample.

6.1 Investigation of ion-pair solvent systems

The neomycin molecule is very polar and is poorly retained on reversed-phase C18 columns.¹⁶⁸ The retention times of polar molecules on reversed-phase columns can be increased by adding ion-pairing agents, sodium salts of alkylsulphonic acids in the case of basic compounds, to the mobile phase. The polar molecules will form a more non-polar alkylsulphonic ion-pair. The neomycin-alkylsulphonate ion-pair binds strongly to the C18 and is not readily eluted. Adding sodium sulphate to the mobile phase produces sulphate anions which may compete with the alkylsulphonate anions for the neomycin, therefore making a less lipophilic analyte. Retention time of neomycin sulphate can effectively be changed by varying the sodium sulphate concentration in the mobile phase.

A variety of ion-pair solvent systems were tried in order to find one that could produce good chromatography of a sample containing neomycin and streptomycin in a 0.05 M sulphuric acid solution.

A number of different methods were examined (Table 6.1). In the solvent systems used detection was carried out by PAD, therefore sodium hydroxide solution was added postcolumn to improve the detection response of the aminoglycoside (as in ion-exchange chromatography chapter 5). These different solvent systems were tested on a 5 μ m Hypersil ODS column.

The mobile phase conditions used by Whall⁸⁵ for the chromatography of neomycin using 0.16 M 1-hexane sodium sulphonate salt and 0.02 M tribasic sodium phosphate should have produced a well resolved peak at a retention time of 8 minutes. In his method Whall used refractive index detection.

However, there was no response for neomycin sulphate even after 45 minutes with a mobile phase containing the ion-pair reagent heptane sulphonic acid sodium salt. This suggested that the neomycin was strongly retained on the column but the reason was not clear. With hexane sulphonic acid sodium salt as the ion pair at a mobile phase pH of 6 the neomycin peak appeared at a retention time of 2.5 minutes close to the solvent front but the peak was broad and tailing. Decreasing the pH of the mobile phase to 4 increased the protonation of the neomycin resulting in increased binding of the neomycin with the ion-pair. This increased the retention time of neomycin on the column to 10 minutes, however, the peak was still very broad. When the ion pair reagent was used in the mobile phase the sensitivity of the PAD detector decreased over a period of a day. The surface of the electrode became tarnished by the ion-pair and therefore would need regular cleaning in an assay.

Perfluorinated carboxylic acids e.g. pentafluoropropionic acid (PFPA) as pairing ions for the chromatography of aminoglycoside antibiotics exhibit the same properties as conventional perhydrogenated pairing ions e.g. alkylsulphonates as discussed earlier. Perfluoroalkonic acids are commonly used to supply pairing ions, especially in the separation of peptides and proteins.^{169,170} The only promising solvent system encountered which produced reasonable results was that developed and used by Samain^{100,101} and Henion^{102,103} for the LCMS method. They used pentafluoropropionic acid (PFPA) as the counter-ion because of its high volatility, which made it easily removable from the mobile phase after separation by the column and prior to detection by mass spectrometry. The initial work carried out was based on the method reported by Henion.¹⁰²

TABLE 6.1 ION-PAIR MOBILE PHASE SYSTEMS INVESTIGATED FOR USE IN THE ANALYSIS OF NEOMYCIN AND STREPTOMYCIN SAMPLES EXTRACTED FROM ANIMAL TISSUE, WITH DETECTION BY PAD.

Mobile Phase system		Flowrate (ml/min)		Neomycin retention t_R	Based on Reference
Ion-pair reagent	Solvent	Mobile phase	1 M sodium hydroxide Postcolumn		
0.01M 1-heptane sulphonic acid sodium salt pH 5.5 Acetic acid	Water	1.5	1	No response	80
0.16 M 1-hexane sodium sulphonate salt 0.02 M tribasic sodium phosphate	MeCN - Water (17:83 v/v/)	1	0.5	2.5 min (small broad peak)	85
20 mM PFPA acid	MeCN - Water (8:92 v/v)	1	1	1.5 min	100
20 mM PFPA acid @ 40°C	MeOH - Water (40:60 v/v)	1	1	6 min	modification of 100

MeCN - Acetonitrile MeOH - Methanol

PFPA - pentafluoropropionic acid

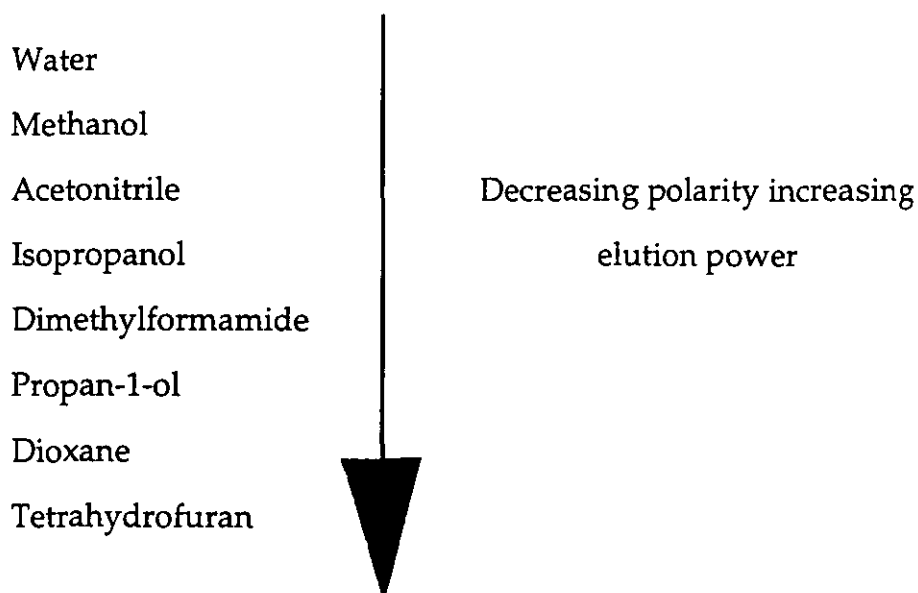
For the chromatography of the aminoglycosides streptomycin, dihydrostreptomycin, spectinomycin and hygromycin detected by the PAD, Henion found the highest efficiency and resolution were achieved using acetonitrile-water 8:92 v/v in 20 mM PFPA on the Spherisorb ODS-2 column. Using the same solvent system specified by Henion acetonitrile-water (8:92v/v) and 20 mM PFPA, neomycin sulphate was found to elute far too rapidly with a retention time of 1.4 minutes at the solvent front suggesting it

had not been retained by the column. Reducing the amount of solvent in the mobile phase to acetonitrile-water 4:96 v/v still did not effect the retention of neomycin by the column which still came off with the solvent front.

6.2 Methanol with ion-pairing reagent PFPA in the mobile phase

The next option was to see whether the use of methanol in place of acetonitrile on the Hypersil ODS column would elute neomycin sulphate at a reasonable retention time with a good peak shape. Methanol is a weaker reversed-phase eluent than acetonitrile, Table 6.2. Samain^{100,101} carried out work into the use of various perfluorinated counter ions in the mobile phase for the chromatography of aminoglycosides, in the studies carried out methanol was used and proved to work well as an organic modifier.

TABLE 6.2 SOLVENTS USED IN THE MOBILE PHASE FOR THE REVERSED-PHASE SEPARATION HAVE THE FOLLOWING ELUTION POWER.



On reference to Samains paper¹⁰⁰ the mobile phase composition used as a starting point was 0.05 M PFPA (50 mM) methanol-water 60:40 v/v, as this

proved to give a reasonable capacity factor for the chromatography of neomycin. However under these conditions neomycin appeared at a retention time of 2.65 minutes, which was too short and close to the solvent front. At this solvent composition methanol-water 60:40 v/v and the ion-pair PFPA at a concentration of 20 mM neomycin still eluted too early at a retention time of 3.5 minutes close to the solvent front. To determine the amount of methanol required in the mobile phase for the chromatography of neomycin at a reasonable retention time the amount of methanol was gradually decreased from the methanol-water composition of 60:40 v/v. When the methanol in the mobile phase was reduced to methanol:water 50:50 v/v neomycin eluted at 6 minutes with a retention factor (k) of 1. The methanol in the mobile phase was further decreased to methanol:water 40:60 v/v and under these mobile phase conditions neomycin eluted at a retention time of 10 minutes with a retention factor of 2.33. Under these mobile phase conditions neomycin eluted at a suitable retention time and with a good peak shape (Figure 6.1). A retention factor between 1 and 5 is preferred, if the retention factor values are too low, then the level of separation may be inadequate as the compounds pass through the column too rapidly with no stationary phase interactions. High retention factor values result in long analysis times which needs to be avoided. This study was carried out with the column maintained at a suitable temperature of 35 °C in a column oven. This improved peak shape and reduced the retention time. The concentration of PFPA was maintained at 20 mM throughout the study as mobile phases containing higher concentrations of the PFPA lead to the rapid tarnishing of the working electrode in the PAD. This resulted in decreasing sensitivity which increased the number of times the working electrode had to be cleaned and reduced the number of assays between cleaning. Therefore at this stage of the project a mobile phase composition of methanol-water at 40:60 v/v with PFPA at 20 mM and the column maintained

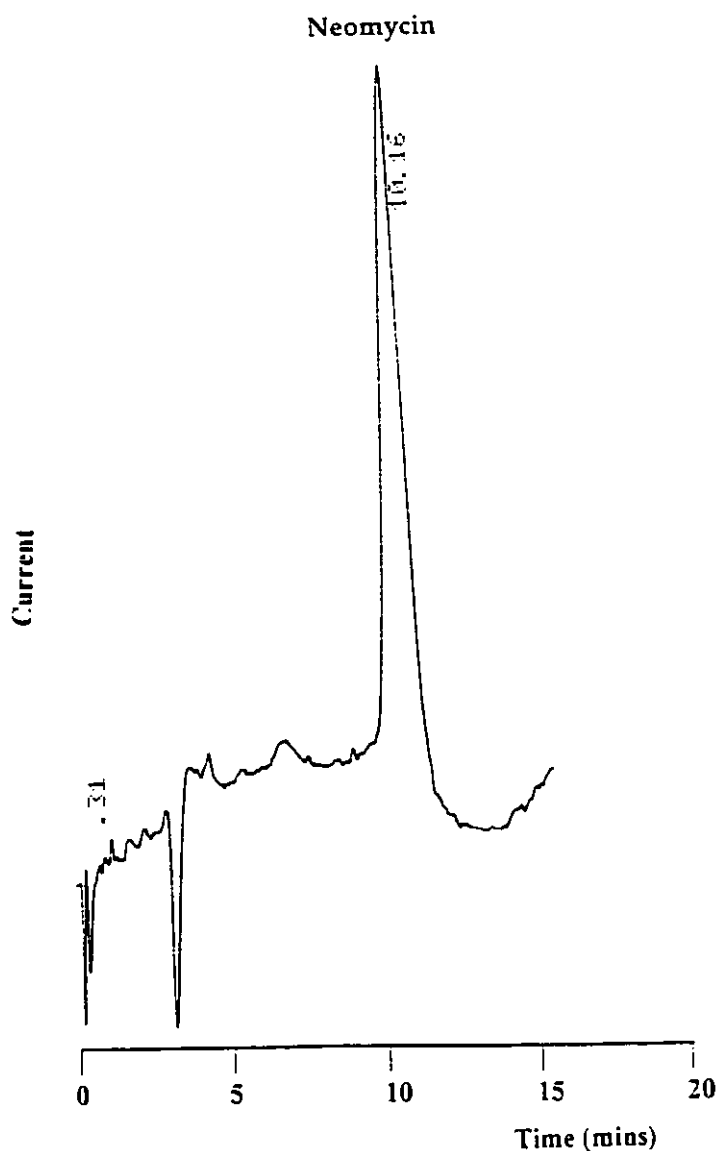
at 35-40 °C proved an adequate mobile phase system for the chromatography of neomycin sulphate.

Figure 6.1 Chromatogram of 140 ppm neomycin sulphate standard.

10 µl injection volume. Mobile phase: 20 mM PFPA, methanol-water (40:60) at 1 ml/min, postcolumn 1 M sodium hydroxide at 1 ml/min. Column temperature 35 °C.

PAD settings: E1 +0.1 V (480 ms), E2 +0.6 V (120 ms), E3 -0.8 V (300 ms).

Amplitude sensitivity at 1 KnA.



An added advantage of using an acidic counter ion, such as PFPA, in the mobile phase, is the capability to inject acidic solutions of neomycin sulphate or free neomycin directly onto the column with no change in the retention time, which is partly due to the buffering ability of the acidic ion-pair PFPA at low pH. This separation method is therefore compatible with extraction.

The next stage is to determine the sensitivity, linearity and reproducibility of the assay at low concentrations similar to those that might be obtained in trace analysis.

6.3 Investigations to improve detection sensitivity

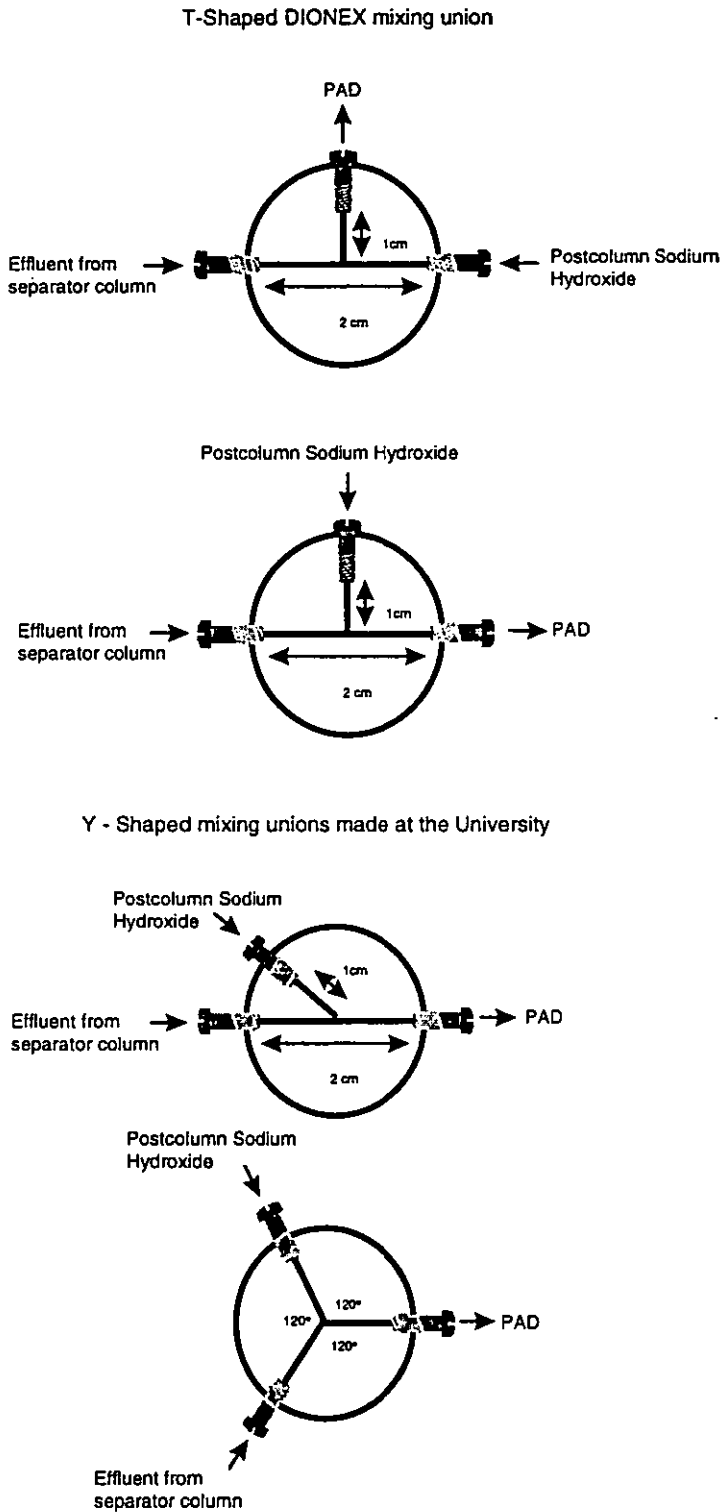
Injections of standard solutions containing neomycin sulphate at progressively lower concentrations down to 1 ppm, were made using the modified solvent system of PFPA and methanol discussed in the previous section. However, reasonable separation and detection of neomycin sulphate at 1 ppm or even 5 ppm was not achievable due to the baseline noise. This prevented clear identification of the neomycin peak. Increasing the sensitivity of the detector amplifier just increased the noise level on the baseline. Attempts were made to determine the cause of the baseline noise. Initially it was considered that the noise may have been produced by the pump pulsations, as the PAD is a reaction detector it is sensitive to pump pulsations. The LC pumps used for the delivery of the eluent (LC10AD Shimadzu) and the post-column sodium hydroxide (LC500 isocratic pump later replaced by the Dionex series 4000i pump) were not found to be the cause of the noise. They were tested by delivering a premixed mixture of the mobile phase and postcolumn sodium hydroxide directly to the detector cell using each pump, the LC10AD Shimadzu pump (usually used for eluent delivery) and the LC500 isocratic

pump (usually used for sodium hydroxide delivery). The baselines obtained in each case were stable at sensitivity ranges as high as 300 nA. Although noise was still present it was much lower in magnitude than the noise obtained earlier with postcolumn addition of the sodium hydroxide. This suggested that incomplete or irregular mixing was the likely source of baseline noise. The postcolumn flowrate of the sodium hydroxide should be as constant as the eluent flow from the column to keep the relationship of eluent and postcolumn solute constant during the whole analysis. This helps to reduce baseline noise and drift.¹⁷¹ Mixing chambers were considered but avoided as it would result in an increase in the dead volume.

Different angled mixing unions T-shaped and Y-shaped (Figure 6.2), were investigated. However, the angle at which the postcolumn sodium hydroxide solute hit the mobile phase leaving the column had no effect on the baseline noise. Eventually it was decided to use the original T type mixing union provided by Dionex.

In order to increase the mixing efficiency and give a more homogeneous mixture to the cell a beaded reactor tube, 130 cm² in length with an internal diameter of 1 mm as suggested by Dionex, was fitted between the mixing junction and the detector cell. This change resulted in a more stable baseline but the baseline noise was still problematic.

Figure 6.2 *Mixing Unions used to mix the Mobile phase with the postcolumn sodium hydroxide.*



Methanol unlike acetonitrile is electroactive at the potential of +0.1 V used to oxidise the analytes. This is another contributing factor to the spiky noisy baseline. Methanol was added to the postcolumn sodium hydroxide reagent in the same proportion as the column eluent methanol-water 40:60 v/v. This was intended to make the viscosity of the two streams similar and so aid mixing. It also minimised any changes resulting from differences in the organic content and oxidation of methanol. However, the concentration of sodium hydroxide had to be increased from 1 M to 2 M to compensate for the dilution by methanol. As a result of these changes a more stable baseline was achievable and the sensitivity of the detector could be increased to the 100 nA range. However, peak shape was still poor and the baseline still relatively noisy at higher sensitivities. The temperature of the column was increased to 50 °C to improve peak shape as the column was deteriorating (Figure 6.3.) However, the baseline sloped over-time and tailing of peaks started to occur.

The peak shapes started to deteriorate as the efficiency of the column decreased. The problem was identified as column deterioration, due to the use of the acidic eluents (pH 2). Therefore the original Hypersil C18 (5 µm) (100 x 5 mm id) column was replaced with a Kromasil C18 (5µm) (250 x 4.6 mm id) column. Separation was carried out under the same HPLC conditions but with this new column neomycin sulphate eluted at a longer retention time of 22.5 minutes.

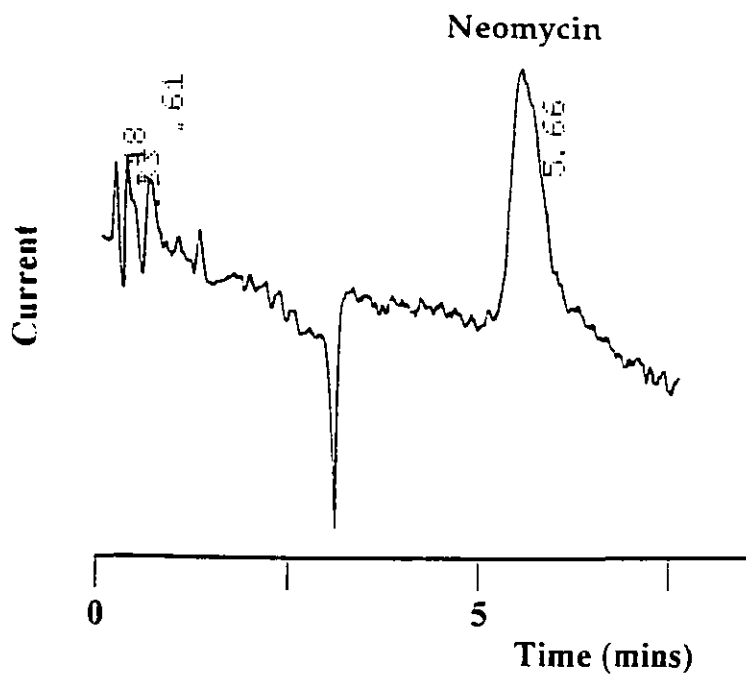
To avoid column degradation, a solution would be to use a buffered mobile phase which would maintain the pH of the mobile phase within the pH limits required (pH 2 - 8) for a longer column life. However, buffering of the mobile phase would also buffer the effect of the sodium hydroxide added postcolumn to achieve the pH required for PAD detection, thereby reducing the sensitivity

of the detector. Also the buffer salts would result in early tarnishing of the electrode surface reducing its sensitivity. Therefore the mobile phase was not buffered.

Figure 6.3 Chromatogram of neomycin sulphate 5 ppm.

10 μ l injection volume. Mobile phase: 20 mM PFPA, methanol - water (40:60 v/v) at 1 ml/min, Postcolumn reagent 2 M sodium hydroxide, methanol - water (40:60 v/v) at 0.5 ml/min. Column temperature 50 $^{\circ}$ C.

PAD settings refer to Figure 6.1. Amplitude sensitivity 100 nA.



6.4 Acetonitrile with ion-pairing reagent PFPA in the mobile phase

A retention time of 22 minutes was achieved for neomycin sulphate with methanol as the organic modifier on the new Kromasil C18 column. It was hoped that acetonitrile as the organic modifier, being a stronger reversed-phase eluent, would provide a shorter retention time for neomycin but not as close to the solvent front as observed earlier with the Hypersil ODS column. The aim was to achieve the separation of neomycin and streptomycin at a reasonable retention time (retention factor between 1-5). Long retention times would result in extended overall run times, which would be problematic in the analysis of a large number of samples. Also the use of acetonitrile in place of methanol would avoid the problem of oxidation of the mobile phase and hopefully reduce the background signals (section 6.3).

Initial investigations used a small proportion of acetonitrile in the mobile phase, acetonitrile-water (10:90 v/v), 20 mM PFPA with postcolumn addition of sodium hydroxide. Injection of a neomycin sulphate standard produced no response within a one hour period. The column was then placed in a column oven and the temperature kept at 40 °C to reduce the retention time. In this case on injection neomycin eluted at a retention time of 25 minutes. For comparison, other standard samples were injected. Glucosamine produced a peak at a retention time of 4.15 min, and streptomycin sulphate produced a peak at a retention time of 16 minutes.

To reduce the retention time and achieve a reasonable retention factor the amount of acetonitrile in the mobile phase was increased to acetonitrile-water, 20:80 v/v with 20 mM PFPA. Under these mobile phase conditions

streptomycin sulphate eluted at a retention time of 4 minutes (retention factor = 0.33) which was too early for separation of the aminoglycoside from the solvent front. Neomycin sulphate eluted at a reasonable retention time of 11 minutes (retention factor = 2.64) for the analysis of samples, however, this retention time could be shortened so that analysis could be achieved within 10 minutes. The aim was to develop a mobile phase system that would separate both neomycin and streptomycin at reasonable retention times for sample analysis. However, these results proved that separate solvent systems would have to be used for the separation of the aminoglycosides. To reduce the retention time of neomycin sulphate to below 10 minutes the acetonitrile in the mobile phase was increased to acetonitrile-water 25:75 v/v with 20 mM PFPA. Under these conditions the retention time of neomycin sulphate was 9 minutes (retention factor = 2). However, slightly increasing the acetonitrile in the mobile phase to acetonitrile-water 27:73 v/v reduced the retention time of neomycin further to 7 minutes (retention factor = 1.33). This was selected as the solvent system for the separation of neomycin sulphate as neomycin eluted at the shortest retention time allowable for sample analysis. To increase the retention time of streptomycin sulphate from 4 minutes to a more reasonable retention time and retention factor the acetonitrile in the mobile phase was decreased to acetonitrile-water, 17:83 v/v which produced a retention time of 6.5 minutes (retention factor = 1.2) for streptomycin. Also this ensured that the streptomycin would not co-elute with the interfering co-extractives which eluted at retention times ranging from 2 - 4, refer to chapter 3 section 3.3 for discussion of interfering co-extractives.

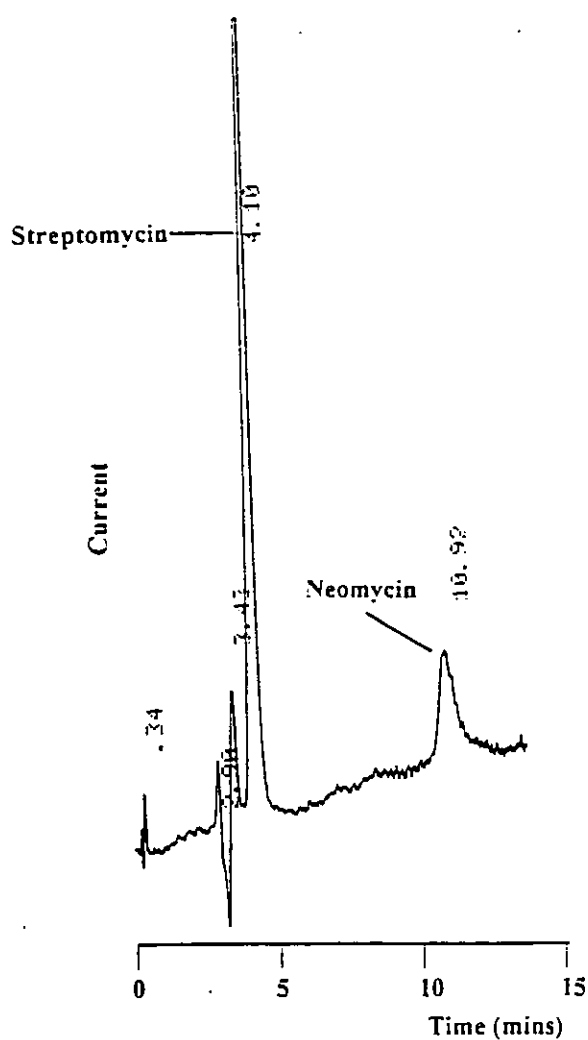
With acetonitrile in the mobile phase in place of methanol the baseline was stable and did not slope. However, on reaching a more sensitive amplitude setting of 100 nA the baseline was still not stable enough to achieve detection of

neomycin sulphate at ppb levels as problems were still present with baseline noise.

As in the case of the previous mobile phase containing methanol, acetonitrile was added to the postcolumn reagent, 0.5 M sodium hydroxide solution, to determine whether this would improve mixing of the two streams (the effluent mobile phase from the column and postcolumn sodium hydroxide) prior to detection. The postcolumn reagent sodium hydroxide was made up in acetonitrile-water (20:80 v/v) and the concentration of sodium hydroxide was increased to 2 M as before. Surprisingly at a sensitivity of 1 KnA the baseline appeared noisier with the addition of acetonitrile in the postcolumn reagent than without its addition. Therefore this approach was abandoned.

It appeared that bubble formation in the detector cell, after mixing of the two streams was a cause of the noise. A back pressure was applied to the cell outlet to prevent the formation of bubbles, by inserting a narrow bore tube, 0.1 mm id and 10 cm in length, after the cell. The pressure applied on the cell was approximately 300 psi but this varied from day to day. A fairly stable baseline at a amplitude sensitivity of 100 nA was then achieved (Figure 6.4.)

Figure 6.4 Chromatogram of a standard solution of 10 ppm neomycin sulphate and 20 ppm streptomycin sulphate. 10 μ l injection volume. Mobile phase: 20 mM PFPA, acetonitrile - water (20:80 v/v) at 1 ml/min, postcolumn 1 M sodium hydroxide at 0.5 ml/min. Column temperature 40 $^{\circ}$ C. PAD settings refer to Figure 6.1. Amplitude sensitivity 100 nA.



The response of the PAD detector was lower for the aminoglycosides when acetonitrile was used in the mobile phase in place of methanol in the amount acetonitrile-water (20:80 v/v). Increasing the proportion of acetonitrile results in the passivation of the gold electrode as acetonitrile is strongly adsorbed at the electrode surface.¹⁰⁶ The adsorbed acetonitrile has been reported to block surface sites required for the pre-adsorption step in the anodic detection of alcohols.^{106,172} However, with acetonitrile in the mobile phase the chromatography and resolution is much improved and the baseline is more stable. The Limit of detection of neomycin calculated from figure 6.5 (b) was found to be 5ppm.

Therefore in conclusion acetonitrile was used in favour of methanol as the organic solvent for the mobile phase. The difference can be seen in a comparison of the mobile phases used, that which contained methanol Figure 6.5 (a) and that which contained acetonitrile Figure 6.5 (b).

The final eluent separation conditions used for further studies were 20 mM PFPA, acetonitrile-water at 27: 73 v/v for neomycin sulphate separation and at 17:83 v/v for streptomycin separation, at 1 ml/min with post column addition of 0.5 M sodium hydroxide at 0.7 ml/min. The column was kept at 40 °C. A 10 µl amount of sample was injected onto a 20 µl injection loop. The PAD settings: E1 at +0.1 V (pulse duration of 480 ms), E2 at +0.6 V (pulse duration of 120 ms) and E3 at -0.8 V (pulse duration of 300 ms). The response time was set at 1 sec.

Figure 6.5 Comparison of a Chromatogram of a standard solution of 140 ppm neomycin sulphate separated by a mobile phase containing (a) methanol and (b) acetonitrile (10 μ l injection volume)

Figure 6.5 (a) Mobile phase: methanol-water (40:60 v/v) at 1 ml/min, postcolumn 1 M sodium hydroxide at 0.5 ml/min. Column temperature 40 °C. PAD settings refer to Figure 6.1. Sensitivity 1 KnA.

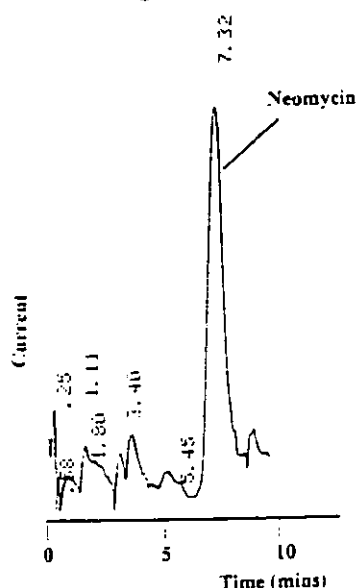
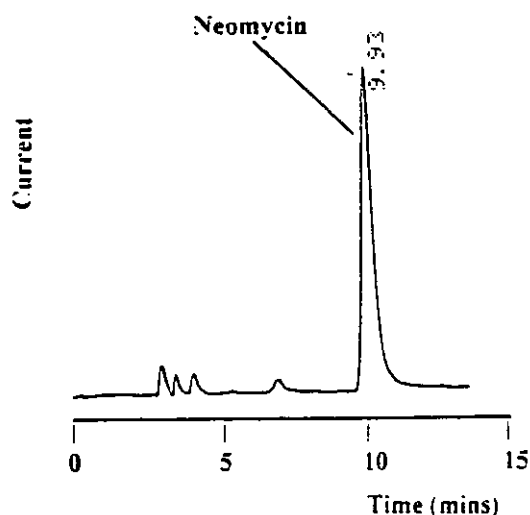


Figure 6.5 (b) Mobile phase: acetonitrile-water (20:80 v/v) at 1 ml/min, Postcolumn 1 M sodium hydroxide at 0.5 ml/min. Column temperature 40 °C. PAD settings refer to Figure 6.1. Amplitude sensitivity 1 KnA.



6.5 Repeatability and linearity check.

A repeatability and linearity check was carried out on the system with the final mobile phase conditions outlined in section 6.4. Standard solutions of neomycin and streptomycin sulphate were prepared and injected.

6.5.1 Linearity check

An investigation was carried out to establish whether a linear calibration range could be achieved for neomycin sulphate and streptomycin sulphate.

Standard solutions of neomycin sulphate and streptomycin sulphate were prepared at concentrations ranging from 5 - 100 ppm and duplicate injections of each standard were made, the results are outlined in Table 6.3.

The peak height of each peak was used instead of the peak area for the calibration. The peak area of chromatograms at low concentrations could not be integrated very well.

From the peak heights obtained for neomycin sulphate and streptomycin sulphate, a calibration graph was plotted. The graphs were linear from 10 ppm up to 100 ppm for both neomycin sulphate (Figure 6.6) and streptomycin sulphate (Figure 6.7.) Both aminoglycosides exhibited good regression over the concentration range measured. The regression in each case was above that of 0.98 which is the acceptance limit for application in a assay.

TABLE 6.3 TABLE OF THE MEAN PEAK HEIGHTS OBTAINED FOR THE STANDARDS OF NEOMYCIN SULPHATE AND STREPTOMYCIN SULPHATE.

Concentration of aminoglycosides (ppm)	Peak Height (mm)	
	Streptomycin	Neomycin
10	3.8	1.6
20	8	3.4
50	20.7	10.2
75	31.8	16.2
90	37.8	20.1
100	41.4	22.5

Figure 6.6 Calibration graph of neomycin

Injection volume 10 μ l. using the mobile phase: acetonitrile-water 27:73 v/v 20 mM PFPA at 40 $^{\circ}$ C, postcolumn 0.5 M sodium hydroxide flowrate 0.3 ml/min.

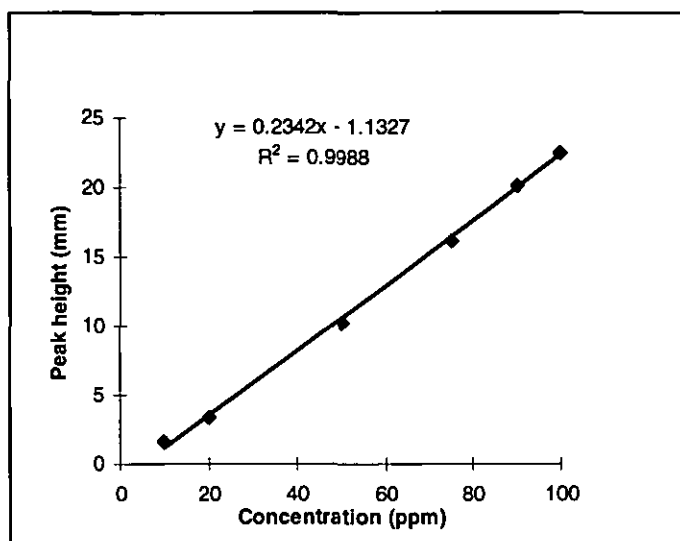
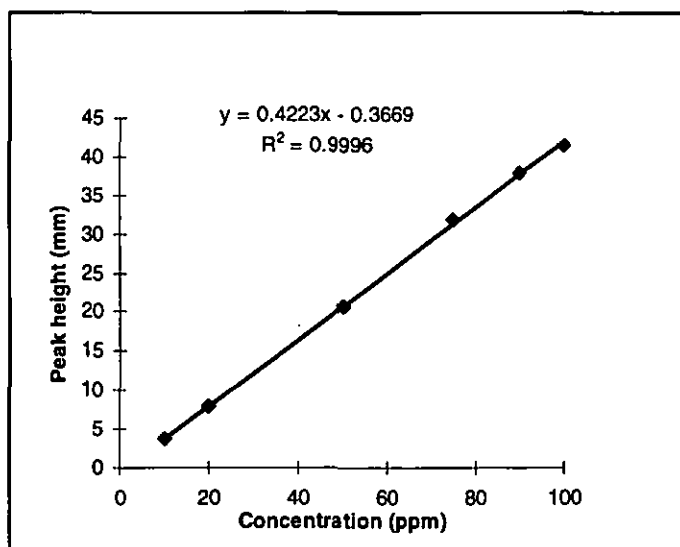


Figure 6.7 Calibration graph of streptomycin

10 μ l Injection volume. using the mobile phase: acetonitrile-water 17:83 v/v 20 mM PFPA at 40 $^{\circ}$ C, postcolumn 0.5 M sodium hydroxide at 0.3 ml/min.



6.5.2 Precision study of streptomycin sulphate and neomycin sulphate at 10 ppm

A precision study was carried out to test the reproducibility of the ion-pair chromatography assay method outlined in section 6.4 for both streptomycin sulphate and neomycin sulphate. Standard solutions of 10 ppm neomycin and streptomycin sulphate were repeatedly injected onto the column. The standard solutions were all prepared in 0.05 M sulphuric acid to resemble the conditions of the aminoglycosides after their extraction from the bovine kidney tissue following the sample preparation stage. The areas obtained for each respective peak were measured, and the standard deviation calculated.

6.5.2.1 Streptomycin sulphate precision at 10 ppm

Refer to table 6.4

TABLE 6.4 PEAK AREAS FROM REPLICATE INJECTIONS OF STREPTOMYCIN SULPHATE 10 PPM PREPARED IN SULPHURIC ACID (0.05 M).

Injection volume 10 µl. Mobile phase conditions: 20 mM PFPA, acetonitrile-water (17:83 v/v) flowrate of 1 ml/min and postcolumn 0.5 M sodium hydroxide flowrate of 0.3 ml/min. Column temperature 40 °C.

Peak Area
5824970
6493115
5445175
5828376
5652048
5975539
Mean: 5.87 E+ 06
SD: 355288.7
RSD 6 %

6.5.2.2 Neomycin sulphate precision at 10 ppm

Refer to Table 6.5.

TABLE 6.5 PEAK AREAS FROM REPLICATE INJECTIONS OF NEOMYCIN SULPHATE AT 10 PPM PREPARED IN SULPHURIC ACID (0.05 M).

Injection volume 10 µl. Mobile phase conditions: 20 mM PFPA, acetonitrile/water (27:73 v/v) flowrate of 1 ml/min and postcolumn 0.5 M sodium hydroxide flowrate of 0.3 ml/min. Column temperature 40 °C.

Area
4461668
4851315
3139116
3034888
3719572
Mean: 3.84 E+ 06
SD: 800494.71
RSD 21 %

The precision for this method was poor at this concentration, the reason for this could be under filling of the injection loop resulting in varying injection amounts. The use of an internal standard might have overcome the differences in the injection volume. However, at a concentration of 10 ppm the reproducibility of streptomycin sulphate is better than neomycin sulphate, as determined by the standard deviation which is higher for neomycin. Because the reproducibility of the neomycin at 10 ppm is so poor, the injection of acidic samples extracted from bovine kidney tissue containing neomycin at concentrations equivalent to 10 ppm, would give unreproducible results that could not be relied on for accurate percentage recovery calculations.

The next stage in the work was to evaluate the recovery of neomycin and streptomycin from spiked bovine kidney samples using the MSPD extraction procedure outlined in chapter three and using the mobile phase system in section 6.4. Prior to that an internal surface reversed-phase column was evaluated using the mobile phase system outlined in section 6.4 to determine whether further sample cleanup could be achieved by this column.

6.6 Use of an internal surface reversed-phase column as part of the sample preparation procedure.

As an alternative method of sample preparation an internal surface reversed-phase (ISRP) column was examined. ISRP supports allow the analysis of serum and plasma samples by HPLC without requiring prior removal of large biopolymers, proteins. The large biopolymers, such as proteins cannot enter the pores of ISRP supports and are not adsorbed by the ISRP outer surfaces; therefore proteins pass right through the ISRP HPLC columns.¹⁷³ The internal-surface reversed-phase particle was invented at Purdue university by Pinkerton.^{174,175,176} The ISRP packing has two stationary phases one on the outside of the particles and the other on the inner wall of pores. The outer surface is a hydrophobic polyoxyethylene polymer, covalently bonded to the silica surface. The inner surface, is a common hydrophobic reversed-phase as in this case C18 also bonded to the Silica surface, but below the polymer. The outer phase forms a semipermeable surface that prevents large biopolymers from reaching the inner phase. Small molecules, however, can and do interact with either the outer or the inner phase. They are retained by a unique mechanism, a combination of hydrogen-bonding at the outer polyoxethylene surface and hydrophobic interaction at the inner reversed-phase surface.

Therefore it was hoped that by using this column, injection of the sample analyte directly after the initial MSPD stage would result in rapid elution of the bovine kidney tissue matrix co-extractives which cause the interference peaks in the profile of the chromatogram and retention of the analyte. A separation was carried out using a mobile phase which contained 20 mM PFPA and acetonitrile-water (20:80 v/v) on a Regis C18 5 μ m (250 x 4.6 mm) column kept at 40°C. Sodium hydroxide 0.5 M was added postcolumn. Injection of a standard solution of 50 ppm streptomycin sulphate and neomycin sulphate prepared in water at pH 7 on the Regis column produced well resolved peaks for both analytes at retention times of 3.9 min and 5.8 min, respectively. A standard of 50 ppm neomycin sulphate and streptomycin sulphate prepared in sulphuric acid (0.05 M) injected onto the Regis column produced neomycin sulphate and streptomycin sulphate peaks with a broader profile.

The HPLC conditions were slightly altered to increase the retention time of the analytes in particular streptomycin as it was eluting too early and also hopefully improve peak shape. Therefore the acetonitrile in the mobile phase was reduced (acetonitrile-water, 15:85 v/v). Injection of a standard solution of neomycin sulphate and streptomycin sulphate prepared in sulphuric acid onto the column resulted in neomycin and streptomycin peaks at retention times of 13.5 min and 5.0 min, respectively.

A bovine kidney tissue extract fortified with neomycin and streptomycin sulphate at a 50 ppm level was injected directly onto the column using these conditions (Figure 6.8). The contaminant peaks came off at a slightly later retention time of ~ 4 min in comparison to the separation involving the conventionally used Kromasil C18 column. The contaminant peaks were also a lot broader not allowing either the neomycin or streptomycin peak to be

resolved and identified. To confirm the presence of neomycin and streptomycin an aliquot of the sample extract was diluted and spiked with 50 μ l of a 1000 ppm standard of neomycin sulphate and streptomycin sulphate. Two large peaks appeared at retention times equivalent to those of the peaks assumed to be neomycin and streptomycin.

In comparison to the C18 columns the Regis column provided no real benefit in the separation of the aminoglycoside, from spiked bovine kidney tissue. The interference peaks were no better resolved from the aminoglycoside peaks. Like the tissue matrix co-extractives causing the interference peaks, the aminoglycosides are also relatively large in size and also polar in nature, their behavior on the column is similar to the co-extractives. The broadness of the peaks and slightly extended retention times may be due to the acetonitrile-water PFPA acidic mobile phase. As with most silica based columns the working range can only be between pH 2 to 8, outside these limits results in column degradation. However the working range for ISRP columns is from pH 6 to 7.5 which is very limited. Therefore the mobile phase used which has a pH of at least 2 is outside the working range of the column. The large peak which is referred to as the interference peak in figure 6.8 could possibly be the surface of the column eluting due to the effect of the acidic mobile phase.

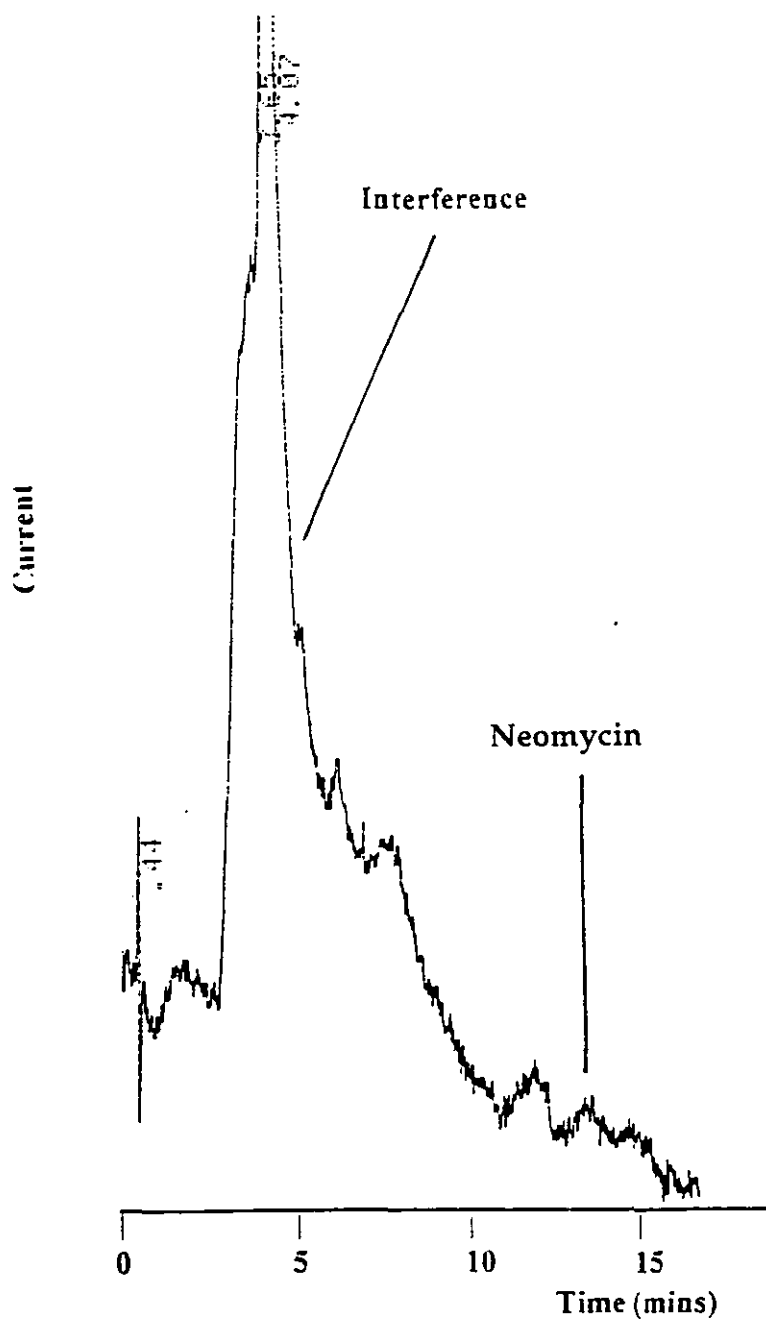
Figure 6.8 Chromatogram of bovine kidney extract spiked with 50 ppm neomycin and streptomycin sulphate.

10 μ l injection volume. Mobile phase: 20 mM PFPA, acetonitrile-water (15:85 v/v) at 1 ml/min, postcolumn 0.5 M sodium hydroxide at 0.3 ml/min.

Column temperature 40 °C.

Column: Regis SPS C18 %5 μ m (250 X 4.6 mm)

PAD settings: refer to Figure 6.1. Amplitude sensitivity 100 nA.



6.7 HPLC method selected for the separation of neomycin sulphate and streptomycin sulphate extracted from bovine kidney samples

Bovine kidney tissue was spiked with neomycin and streptomycin sulphate followed by the MSPD extraction procedure outlined in the standard method section of chapter 2 (section 2.5.5). The final sample elute containing neomycin sulphate and streptomycin sulphate obtained from the MSPD extraction procedure was then injected directly onto the HPLC for analysis. As discussed earlier in section 6.4 of this chapter, separation of both neomycin sulphate and streptomycin sulphate extracted from bovine kidney samples could not be carried out using the same amount of percentage organic modifier acetonitrile in the mobile phase. The mobile phase used on the C18 Kromasil column for the separation of neomycin (acetonitrile-water, 27:73 v/v) when used for the separation of a kidney extract containing neomycin and streptomycin resulted in good resolution of the neomycin peak ($t_R = 7$ mins) however the streptomycin peak ($t_R = 4$ mins) eluted too early along with the interfering peaks and was not resolved sufficiently for accurate measurement (Figure 6.9.) refer to section 6.4.

The separation of streptomycin sulphate by HPLC was therefore carried out using a mobile phase containing less acetonitrile (acetonitrile-water, 17:82 v/v) so that the column retained streptomycin long enough for it to be separated from the early contaminant peaks. Under these mobile phase conditions the streptomycin sulphate peak was well resolved ($t_R = 6.5$ mins) and separated from the interfering peaks of the spiked bovine kidney extract (Figure 6.10.)

Recovery and reproducibility of the MSPD extraction and assay procedure are detailed in chapter 7.

Figure 6.9 Chromatogram of extract of bovine kidney sample spiked with 40 ppm neomycin sulphate and streptomycin sulphate.

10 μ l injection volume. Mobile phase: 20 mM PFPA, acetonitrile-water (27:73 v/v) at 1 ml/min, postcolumn 0.5 M sodium hydroxide at 0.3 ml/min.

Column temperature 40 °C.

PAD settings refer to figure 6.1. Amplitude sensitivity 100 nA.

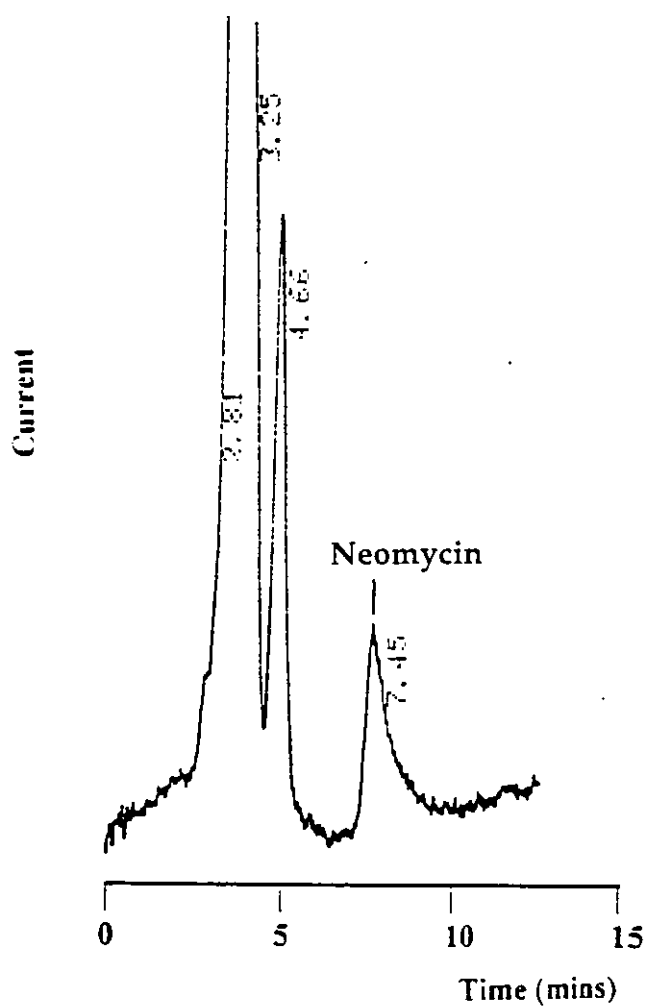
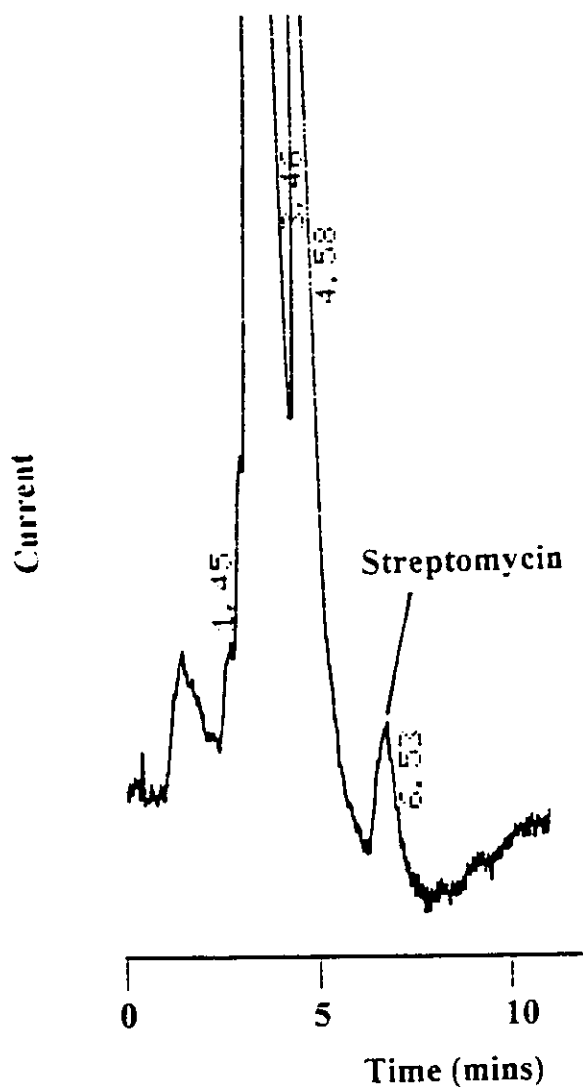


Figure 6.10 Chromatogram of extract of bovine kidney sample extract spiked with 20 ppm streptomycin sulphate

Injection volume 10 μ l. Mobile phase: 20 mM PFPA, acetonitrile-water (17:82 v/v) at 1 ml/min, postcolumn 0.5 M sodium hydroxide at 0.3 ml/min.

Column temperature 40°C.

PAD settings refer to figure 6.1. Amplitude sensitivity 30 nA.



**Sample Preparation Combined with Separation by Ion-Pair HPLC
for the Determination of Neomycin and Streptomycin in Bovine
Kidney Tissue**

CHAPTER SEVEN

CHAPTER SEVEN

7. *Sample Preparation Combined with Separation by Ion-Pair HPLC for The Determination of Neomycin and Streptomycin in Bovine Kidney Tissue*

7.1 Introduction

The next stage in the project was to examine the combination of the MSPD procedure discussed and developed in chapter 3 and the finally selected HPLC methods in chapter 6 section 6.7. The eventual aim was to develop a group method for analysing levels of different aminoglycosides in bovine kidney tissue.

The sample preparation method involving MSPD discussed in chapter 3 was therefore evaluated using the ion-pair HPLC and PAD assay method outlined in chapter 6 section 6.7.

7.2 Recovery of neomycin from bovine kidney tissue prior to pre-concentration

The spiked bovine kidney samples were cleaned up using the MSPD procedure outlined in section 2.5.5 of chapter 2, however the concentration step which used the CBA cartridge was excluded from this procedure. This was to determine the amount of the aminoglycoside that could be recovered from the MSPD extraction procedure without any form of concentration prior to detection. The volume of sulphuric acid (0.05 M) used to elute the neomycin off

the tissue matrix column was varied for each extraction. The concentration of the neomycin in the acidic extract was then determined using the HPLC method which used the mobile phase of methanol:water 40:60 v/v PFPA (20 mM) (section 6.2) because this experiment was carried out at the stage of the project when this HPLC system was developed. The amount of the aminoglycoside spiked onto the kidney was calculated using equation 7.1 and the recovery of the aminoglycoside was calculated using equation 7.2. The concentration of the aminoglycoside in the extract was determined and calibrated from single standards of the aminoglycoside injected alongside the samples. The single standard concentration used for calibration was equivalent to that of the expected concentration of the aminoglycoside in the sample extract for full recovery.

The results indicated that recovery of the aminoglycoside from the bovine kidney tissue was occurring, however, the variance in the results was quite large and the recovery obtained was not reproducible.

Equation 7.1 Equation used for the fortification of the kidney tissue

$$\text{Amount Spiked} = \frac{C_{std} \times V_{std}}{W_{spt}}$$

(ug/g)

C_{std} - Standard concentration used for spiking ($\mu\text{g/ml}$ or ppm)

V_{std} - volume of standard spiked (mls)

W_{spt} - weight of tissue (g)

Equation 7.2 Equation used for the recovery calculation of the aminoglycoside from the kidney tissue

$$\% \text{ Recovery} = \frac{\text{Asp}}{\text{Astd}} \times \text{Cstd} \times \text{DF} \times \frac{100}{\text{F}}$$

Asp - Area of sample peak

Astd - Area of standard peak

DF - Dilution factor :
 amount of acidic extract containing the aminoglycoside / amount of tissue fortified

F - Fortification (µg/ml)

TABLE 7.1 RECOVERIES OBTAINED ON THE EXTRACTION OF NEOMYCINSULPHATE FROM 0.5 GMS OF BOVINE KIDNEY TISSUE SPIKED AT 100 PPM (FOUR EXPERIMENTS).

10 µl injection volume. Mobile phase conditions: 20 mM PFPA, methanol-water (40:60 v/v), postcolumn 1 M sodium hydroxide. Column temperature 40 °C. Column Hypersil C18 (100 X 5 mm id).

Volume of acidic extract used to extract neomycin sulphate Prior to injection (mls)	Expected neomycin concentration for full recovery (µg/ml)	Amount of neomycin present in the acidic extract obtained from the tissue matrix (µg/ml)	Recovery (%)
6	12	15	125
4	8	5.7	71
4	8	8.2	102
4	8	4	50

Using the quantity of bovine kidney outlined by the method^{102,135} of 0.5 g the suggested amount of acid used to extract the neomycin was 8 mls. The volume of acid used for the elution was varied to determine whether a smaller amount than 8 mls could be used to elute the neomycin so reducing dilution of the analyte further. On reference to Table 7.1 a larger volume of acid 6 mls gives a higher yield of the aminoglycoside. The last sample has a poorer recovery yield of 50 %. In this case the remaining 50 % of neomycin could have been washed off the CN sorbent during the washing procedure or possibly the tissue was not adequately mixed with the CN sorbent which prevented some of the neomycin from having contact with the CN sorbent.

The noisy baseline apparent on the chromatograms produced peaks with areas that were not reproducible because the additional spikes resulted in changing peak shapes (refer to Figure 3.1 chapter 3). The excessive noise on the baseline was due to problems with the methanol-water mobile phase discussed in more detail in chapter 6. Therefore for an improved more stable baseline further work was carried out using an acetonitrile-water mobile phase.

Further concentration of the acidic extracts containing the aminoglycoside obtained from the MSPD stage was required to determine the amount of the aminoglycoside in kidney tissue spiked at levels of 10 ppm (section 6.3) where the sensitivity of the method would be a limiting factor. The amount of sulphuric acid required to elute the aminoglycoside off the CN matrix was at least 4 mls (Table 7.1), this amount of solute diluted the analyte further and the PAD sensitivity was not enough to detect such low levels of the aminoglycoside. Therefore an extra step was added to concentrate the aminoglycoside back to at least half its original spiking concentration thereby increasing the concentration of the final extract prior to injection to give a

larger detection response.

In the next series of experiments further concentration of the sample extract from the MSPD procedure was carried out with the aid of the CBA SPE cartridge. The pH of the sample extract was changed to 7 prior to its elution through the CBA cartridge as above a pH of 4.8 CBA carries a negative charge that can be used for retaining the amino groups on the aminoglycoside (section 3.4). The aminoglycoside was then eluted off the CBA cartridge by 0.05 M sulphuric acid (1 ml). The addition of acid neutralises the CBA allowing the retained aminoglycoside to be released. Only a small amount of acid is required for elution of the aminoglycoside (chapter 3 section 3.4). The overall sample preparation procedure as modified is outlined in the standard method section of the experimental chapter (section 2.5.5.)

Using this modified sample preparation method bovine kidney samples were then spiked at progressively lower concentrations of neomycin sulphate (Figure 7.1) and streptomycin sulphate to determine the limit of analysis achievable.

7.3 Blank samples

For a control, to ensure that no interfering substances in the bovine kidney sample or the sample preparation procedure were eluted with the analytes, analysis was carried out on an untreated bovine kidney sample.

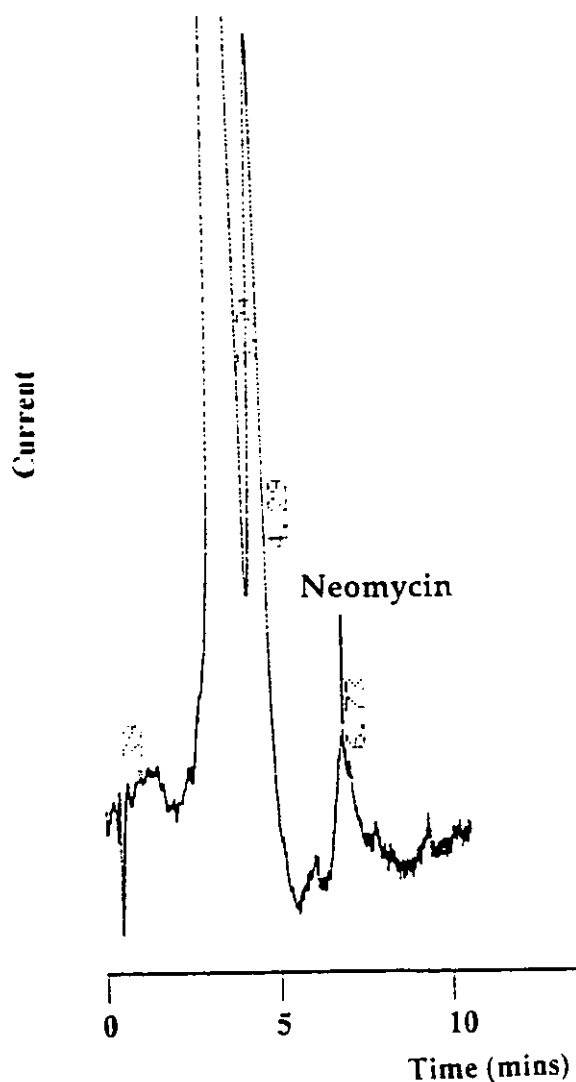
The untreated bovine kidney sample underwent the same sample preparation procedure outlined in the standard method section of the experimental chapter (section 2.5.5), undergoing MSPD and further pre-concentration of the elute.

Figure 7.1 Chromatogram of extract from bovine kidney tissue spiked with 20ppm neomycin sulphate using MSPD and CBA solid phase extraction cartridge.

10 μ l injection volume. Mobile phase: 20 mM PFPA, acetonitrile - water (27:73 v/v) at 1 ml/min, postcolumn 0.5 M sodium hydroxide at 0.4 ml/min. Column temperature 40 °C.

PAD settings: E1 +0.1 V (480 ms), E2 +0.6 V (120 ms), E3 -0.8 V (300 ms).

Amplitude sensitivity at 100 nA.



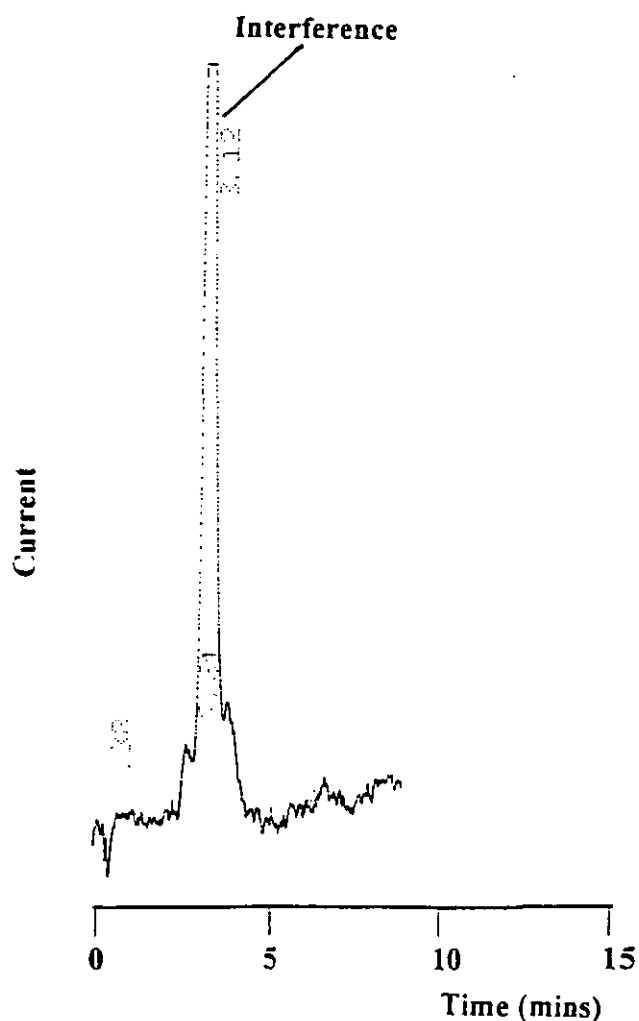
A 10 µl injection of the final 1 ml elute obtained from the pre-concentration step was analysed. The mobile phase conditions were equivalent to those outlined in the standard method section of the experimental chapter (section 2.5.3.)

The only peaks present are those representing the tissue matrix co-extractives at retention times ranging from 3 - 5 minutes (Figure 7.2) as discussed earlier in chapter 3. These peaks were also present in the chromatograms of the spiked bovine kidney sample (Figure 7.1.)

Figure 7.2 Chromatogram of extract control bovine kidney tissue with no added aminoglycoside after undergoing the sample preparation procedure of MSPD with the CBA SPE.

Mobile phase refer to 3.2.

PAD settings refer to Figure 3.1. Sensitivity at 300 nA.



7.4 Recovery of neomycin and streptomycin from bovine kidney samples using MSPD followed by preconcentration of the aminoglycosides with the CBA solid phase extraction cartridge

Recoveries of neomycin sulphate and streptomycin sulphate from bovine kidney tissue spiked at various concentrations are outlined for neomycin (Table 7.2) and streptomycin (Table 7.3). Good recoveries of neomycin sulphate from bovine kidney tissue fortified at 10 ppm were not achievable using 0.5 gms of tissue, as the amount of neomycin sulphate extracted after preconcentration was below the limit of detection 5 ppm (section 3.4 and 6.4) using the mobile phase developed of acetonitrile-water (section 6.7).

Henion¹⁰³ modified the MSPD extraction procedure he used for extraction of aminoglycosides by using a larger amount of tissue with a corresponding increase in the amount of sorbent and solvent used for the extraction procedure. He found this gave him a more efficient extraction procedure. Also Aschbacher and Feil²⁴ used a larger amount of tissue in there MSPD method to determine the low levels of neomycin in calf tissue, they increased the amount of sorbent and solvent in relation to the amount of tissue used. This approach was then taken to determine low levels of the aminoglycoside by extraction from a larger amount of tissue. A larger amount of kidney tissue was spiked (2.5 g) so that the corresponding amount of aminoglycoside extracted for analysis would be larger than that obtained from a smaller amount of spiked kidney tissue (0.5 g). Also the amount of cyanopropyl sorbent was increased to 10 g. The amount of solvents used for the clean-up were increased five-fold to correspond to the five-fold increase in tissue spiked for analysis. This was to ensure clean-up of the extract was equivalent to that achieved prior to the scale-up. The quantity of bovine kidney and solvents used for tissue spiked at a

10 ppm level of neomycin sulphate are outlined in the standard method section of the experimental chapter (section 2.5.6.)

Whilst the initial developmental work for the extraction of the aminoglycoside from the bovine kidney tissue was carried out on neomycin sulphate, it was assumed that the same method for extraction and analysis could be extended to streptomycin sulphate and this was tested. However the recovery results for the extraction of streptomycin sulphate from spiked bovine kidney tissue were generally poorer (range 35 - 60%).

TABLE 7.2 RECOVERY OF NEOMYCIN SULPHATE FROM BOVINE KIDNEY TISSUE SPIKED AT VARIOUS CONCENTRATIONS 50-10 PPM,

MSPD method as detailed in chapter 2 (section 2.5.4 & 2.5.5)

Injection volume 10 μ l. Mobile phase: 20 mM PFPA acetonitrile-water 27:73 v/v at 1 ml/min, postcolumn 0.5 M sodium hydroxide at 0.3 ml/min. Column temperature 40 °C. Column Kromasil C18 (250 x 4.6 mm id).

(a) Recovery values (b) Reproducibility

Table 7.2 (a)

Sample	Concentration at which the tissue sample is spiked with neomycin (ppm)	Volume of acidic extract containing the aminoglycoside prior to injection (mls)	Recovery of neomycin (%)
1	56	0.5	102
2	38	1	70
3.a	20	1	72
b	20	1	68
4.a	10	1	44
b	10	1	68

Table 7.2 (b)

Neomycin Concentration (ppm)	Mean	n	SD
38 & 56	86	2	22.6
20	70	2	2.83
10	56	2	17.0

TABLE 7.3 RECOVERY OF STREPTOMYCIN SULPHATE FROM BOVINE KIDNEY TISSUE SPIKED AT 20 PPM.

MSPD method as detailed in chapter 2 (section 2.5.5.)

Injection volume 10 μ l. Mobile phase: 20 mM PFPA, acetonitrile-water (17:83 v/v) flowrate 1 ml/min, postcolumn 0.5 M sodium hydroxide at 0.7 ml/min.

Column Kromasil C18 (250 \times 4.6 mm id)

Sample	Volume prior to injection (mls)	Recovery of streptomycin (%)
5.a	1	60
5.b	1	53
6.a	1	35
6.b	1	46

Mean: 49 (n = 4)

SD: 10.66

The low recovery values obtained for the extraction of neomycin sulphate and streptomycin sulphate from bovine kidney tissue spiked at the 20 to 40 ppm level are considered primarily due to the sample handling problems during the extraction process. The additional sample handling in the concentration step which involved changing the pH of the sample extract prior to loading the sample onto the CBA cartridge, also probably resulted in some loss of the aminoglycoside. Further loss in recovery of neomycin sulphate from bovine kidney spiked at 10 ppm (refer to Table 7.2 sample 4) can be attributed to the excessive amount of sulphuric acid (50 mls) required to elute the aminoglycoside from the 10 g tissue CN matrix mix. The amount of acid used was scaled up in proportion to the corresponding amount of cyanopropyl sorbent that was used, this amount of acid (50 mls) was required to ensure a complete wash of the tissue matrix. This resulted in further dilution of the sample, with a greater risk in loss of the sample on the walls of the flasks on transference from one container to another during the sample handling. Also there was proof of poor peak precision of neomycin at 10 ppm (concentration equivalent to the working standard used to calculate the recovery) as a result of baseline noise (refer to chapter 6) which suggested unreliable results.

7.5 Conclusion

The recoveries obtained in Table 7.2 and 7.3 for both neomycin and streptomycin suggest the MSPD method does not work effectively at extracting aminoglycosides from bovine kidney tissue spiked at levels lower than 50 ppm. Recovery from tissue spiked at 10 ppm of the neomycin sulphate is quite low. The maximum recovery achievable using this method at that concentration level was 68 %. Therefore to attempt to achieve decent recovery of the aminoglycoside at fortified levels lower than 10 ppm and even close to the

MRL would appear doubtful. Using the present ion-pair HPLC assay method, acetonitrile-water 20 mM PFPA with postcolumn addition of sodium hydroxide, in order to achieve reasonably reliable recovery results from bovine kidney tissue spiked at concentrations below 10 ppm, a stabler noise free baseline would be required so that the detector can be optimised at higher sensitivities. As presently the maximum sensitivity range achievable on the detector is 100 nA.

Concentration of the extract containing the aminoglycoside prior to injection could be improved by the use of a CBA cartridge comprising of a smaller amount of matrix allowing for elution of the aminoglycoside into a smaller volume of sulphuric acid. However, the time was not available to explore this.

Another alternative is the use of a greater weight of bovine kidney in the extraction procedure allowing the extraction of more of the aminoglycoside spiked at a lower level. However, this approach has a disadvantage in that the amount of materials used in the MSPD procedure have to be increased in line with the quantity of bovine kidney tissue used. Therefore the quantity and cost of the bulk cyanopropyl sorbent and solvent used is increased.

In conclusion with reference to the results (Tables 7.2 & 7.3) the MSPD method is not robust enough for the analysis of trace levels of the aminoglycosides in kidney tissue, as the results achieved at tissue spiked at 20 ppm are quite poor and variable. Also this extraction method does not appear to lend itself to automation or on-line use with the HPLC and detection system.

7.6 A comparison of the results obtained with previous work reported in literature

Previous studies show better overall percentage recoveries at lower levels of tissue spiking in the HPLC assay methods using fluorescence detection of derivatised aminoglycosides. Gerhardt⁸⁷ achieved recoveries from bovine kidney spiked at 40 ppb streptomycin and 80 ppb dihydrostreptomycin of 59.2 % with a s.d. of 7.58 using a reversed-phase ion-pair HPLC assay method with post-column derivatisation using 1,2-napthoquinone-4-sulphonic acid.

However, the recovery of neomycin from bovine kidney tissue at ppb levels has not been reported. Shaikh⁸³ achieved recoveries of neomycin sulphate from bovine kidney tissue spiked at 1, 2 and 4 ppm levels of 115 % (n=5), 97.6 % (n=5) and 79.8 % (n = 4), respectively, with s.d. of 3.1, 2.3, and 2.3 respectively. Shaikh used a reversed-phase ion-pair mobile phase HPLC method, post-column derivatisation with o-phthaldehyde reagent, and fluorometric detection. The sample preparation procedure used in each case was the conventional buffer, centrifugation liquid extraction outlined in the introduction chapter (section 1.10.1.)

Schenck¹⁵³ using MSPD in conjunction with a reversed-phase ion-pair HPLC method with post-column derivatisation of the aminoglycoside with o-phthaldehyde reagent, and fluorometric detection as discussed in chapter one (section 1.5.6) was only able to achieve recoveries of neomycin from bovine kidney tissue spiked at ppm levels, 2.5, 5.0 and 10 ppm with recoveries of 87.5 %, 86.1 % and 91.9 % respectively. If the time was available Schencks method would have been repeated without fluorescence detection but with the PAD as an alternative, to discover whether the recoveries obtained at the levels he spiked could be achieved.

Previous studies that have avoided any form of derivatisation of the aminoglycoside, show less promising results in comparison to those achieved by Gerhardt, Shaikh and Schenck. Henion *et al.*¹⁰² used a reversed-phase ion-pair HPLC assay method for the separation of aminoglycosides, streptomycin, dihydrostreptomycin using volatile ion-pairing agents i.e. PFPA, optimised for detection with ion-spray HPLC-MS interface (refer to chapter 1 section 1.6.) Henion also used pulsed amperometric detection in conjunction with the ion-pair HPLC assay for the method development stage. Extraction of aminoglycosides from bovine kidney tissue was achieved using MSPD, the lowest level of recovery achieved was at 20 ppm of streptomycin and dihydrostreptomycin, however no percentage recoveries values were quoted.

It appeared Henion came to similar levels of limitation, with detection of the extracted aminoglycoside from spiked bovine kidney tissue only achievable at a 20 ppm level. Further sample clean-up and concentration of the aminoglycosides would be necessary to reach the ppb concentration levels, quoted by the fluorescence detection methods discussed earlier.^{83,87}

Henion went on to further modify the MSPD extraction procedure using a larger amount of cyanopropyl sorbent matrix to provide a better sample clean-up. By evaporation of the elute in a speed vac he achieved further concentration of the MSPD elute by a factor of 53. An ion spray high performance liquid chromatographic/tandem mass spectrometer (HPLC/MS/MS) method was used for detection. A gradient HPLC separation was developed to separate a whole group of aminoglycosides.¹⁰³ The mean recovery of streptomycin spiked at 1 ppm and dihydrostreptomycin spiked at 0.8 ppm was 67 % with a s.d. of 7.8.

The detection of aminoglycosides at ppb levels, avoiding derivatisation for fluorescence detection, can be achieved by HPLC-MS with a modified MSPD extraction procedure as developed by Henion.¹⁰³ Although further concentration of the aminoglycosides by solid phase extraction cartridges as in the case of the CBA cartridge appears to be a good possibility for further development, the sample handling involved would appear to result in loss of some of the analyte. The use of an internal standard would provide a better indication of recoveries but more time would have been required to investigate this particular approach.

Recovery of the aminoglycosides at ppb levels presently seems only achievable using the methods involving derivatisation and fluorescence detection and in most cases using sample preparation procedures involving protein precipitation and liquid extraction in conjunction with centrifugation. The main disadvantage to these techniques is the length of time required to extract and then derivatise the analyte for a single analyst which limits the number of samples that can be analysed.

**Separation of Neomycin and Streptomycin by Capillary Zone
Electrophoresis with Indirect UV Detection**

CHAPTER EIGHT

CHAPTER EIGHT

8. *Separation of Neomycin and Streptomycin by Capillary Zone Electrophoresis with Indirect UV Detection*

8.1 Introduction

Because of problems with the HPLC method alternate detection and separation methods were considered. During the course of the study a paper was published by Ackermans *et al.*¹²⁹ that claimed to have developed a method for detecting aminoglycosides by capillary zone electrophoresis.¹²⁹ As the ion-pair HPLC method with detection by PAD appeared to have reached its limit, in terms of the concentrations detectable, CZE was an alternative technique to investigate. The main advantage of CZE is the need for less solvents and the ease with which the capillary tube stabilises under different conditions in comparison to HPLC. Also the resulting peaks produced are sharper however sensitivity on small samples is a problem.

Ackerman¹²⁹ found reversed electroosmotic flow in the anionic mode worked for the separation of aminoglycosides, with detection by indirect UV, this is discussed in more detail in the introduction, chapter 1 (section 1.8.) He had attempted separation of aminoglycosides in the cationic mode, however, he found the aminoglycosides showed very bad peak shapes, reportedly due to the strong attractive forces between the highly positively charged components and negatively charged capillary wall. Nonetheless, in the present study initial work was carried out under normal cationic mode to test Ackermans theory. Ackerman used imidazole as a background electrolyte cation, to aid indirect UV detection, this was buffered by adding acetic acid as a counter ion to reach

the desired pH. In indirect UV detection the mobile phase or in this case the electrophoretic medium is UV absorbing because of the addition of imidazole, by using this type of eluent, non-absorbing sample components will be detected as negative peaks because there will be less light absorbed when they pass through the detector cell. The aminoglycosides displace imidazole to preserve electrical neutrality.

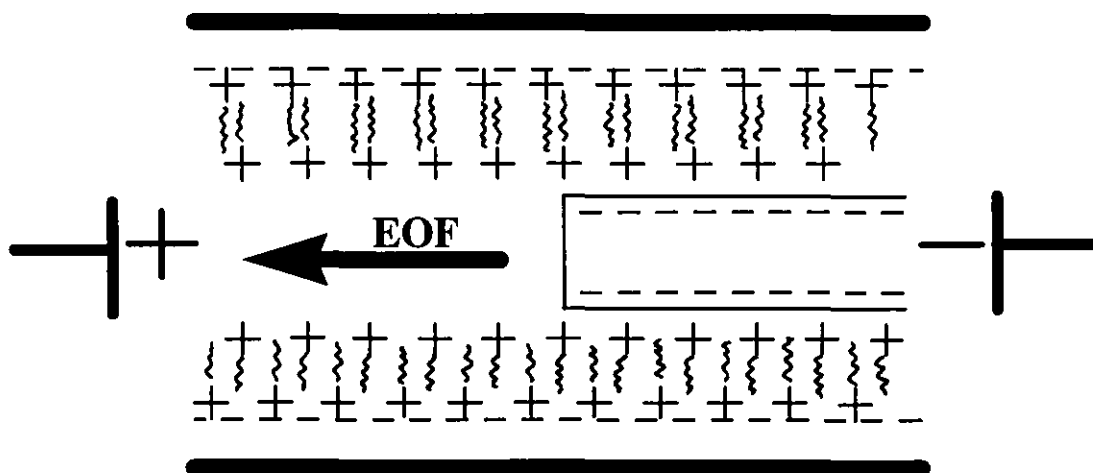
In the present study 0.01 M imidazole was buffered to a pH of 5 using acetic acid and indirect UV detection was carried out at 214 nm, a voltage of 30 kV was applied across the capillary tube replicating the conditions used by Ackerman. Under these conditions an injection of neomycin sulphate produced a peak at a migration time of 2 minutes. However, on injection of deionised water or the buffer solution a peak appeared at the same migration time. Further injections of neomycin and streptomycin give two broad peaks, however, replicate injections of the same standards produced a large degree of variance in the migration time in the two peaks and in some cases there was no response. These results confirmed the problems encountered in Ackermans original work under these conditions. It is true that the reason for the poor separations was the strong attractive forces between the highly positively charged aminoglycosides and negatively charged capillary wall. Therefore further work was carried out in the anionic reversed mode as outlined by Ackerman.¹²⁹

8.2 Anionic (reversed) mode

The electroosmotic flow in the capillary tube was reversed, so that the electroosmotic flow was towards the anode. This was achieved by adding a cationic surfactant to the buffer.¹²⁹ This surfactant produced a positively

charged layer on top of the negative ionic charge on the wall of the capillary tube (Figure 8.1.) This had the effect of shielding the negative charge of the capillary wall with positive cations. Now the capillary wall has a net positive charge, the anions in the buffer are attracted to the cationic layer, therefore when a voltage is applied the anionic species migrate towards the anode dragging the solvent molecules with them. The potential across the capillary tube is therefore reversed so that detection now takes place at the anode. Ackerman in his method used a additive referred to as FC 135 to reverse the charge on the capillary wall. In the present study cetyltrimethylammonium bromide (CTAB) was found to work just as well.

Figure 8.1 The layout of ions in the capillary tube in the anionic mode



8.3 Effect of cetyltrimethylammonium bromide (CTAB) concentration on the neomycin and streptomycin peak shape

Using buffer containing imidazole and varying the concentration of CTAB from 100 - 400 $\mu\text{g/ml}$, standard solutions of neomycin and streptomycin were injected onto the capillary and gave peaks at 4.3 mins and 3.1 mins, respectively. The peak width of both neomycin sulphate and streptomycin sulphate improved by increasing the CTAB concentration in the buffer and standards from 100 $\mu\text{g/ml}$ to 400 $\mu\text{g/ml}$. Increasing this amount further to 1000 $\mu\text{g/ml}$ resulted in much broader peaks. Therefore it appeared that the optimum concentration of CTAB was 400 $\mu\text{g/ml}$. Changing the concentration of CTAB produced no measurable change in the migration time of the neomycin or streptomycin peak in the buffer (100 - 1000 $\mu\text{g/ml}$).

8.4 The effect of pH on neomycin and streptomycin

The pH of the buffer, 0.01 M imidazole plus 400 $\mu\text{g/ml}$ CTAB, was changed by the addition of formic acid to achieve a pH of 4 or the addition of acetic acid to achieve a pH of 5 or 6. Injections of a standard solution at 100 ppm neomycin sulphate and streptomycin sulphate were made with the buffer at a pH of 4, 5 and 6.

With the buffer at pH 4, injection of the standard solution produced broad neomycin and streptomycin peaks. However, with the buffer at a pH of 5 or 6 injection of the standard solution produced tall narrow peaks for both neomycin sulphate and streptomycin sulphate (Figure 8.2.)

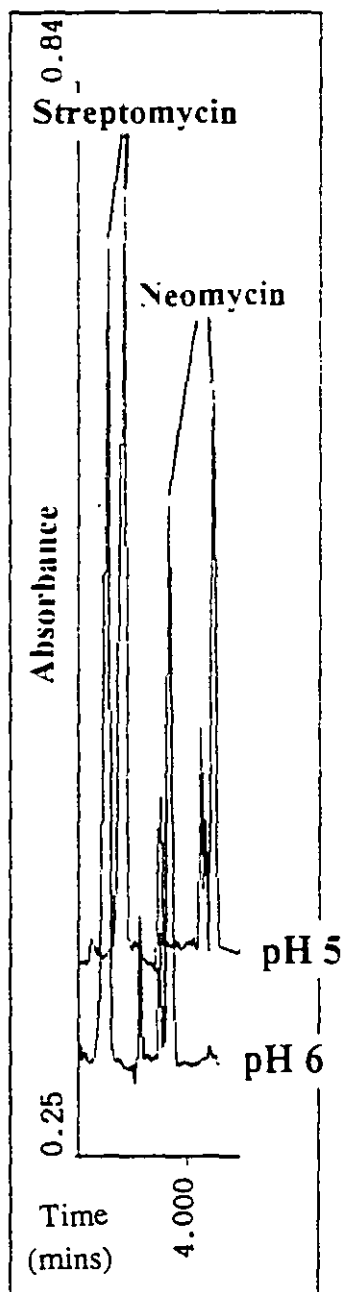
The migration time of neomycin sulphate and streptomycin sulphate decreased

as the pH of the buffer was increased further, partially deprotonating the aminoglycoside molecule rendering it more anionic, therefore it is attracted to the anode more rapidly and migrates quicker towards the anode.

Figure 8.2 Electropherograms of neomycin and streptomycin peaks at a pH of 5 and 6.

Buffer: 0.01 M imidazole, 400 µg/ml. Voltage (anionic mode) 30 kV.

Detector at 214 nm.



8.5 Affect of imidazole concentration on absorption of neomycin and streptomycin

The main problem with the detection of aminoglycosides, which has already been covered in the introduction, is their inability to absorb energy in the UV region. In the present method UV detection was carried out indirectly.

Imidazole acted as an UV absorbing co-ion, the principal component of the background electrolyte. Because there is a need to maintain electrical neutrality, the non-absorbing aminoglycosides displace the imidazole forming zones of non-absorbing ionic species. The analytes were therefore revealed by decreases in light absorption due to charge displacement of the absorbing imidazole co-ion.

Foret *et al.*¹⁷⁷ showed that for indirect photometric detection, the highest sensitivity could be achieved for sample ions having an effective mobility close to the mobility of the absorbing co-ion. The best sensitivities were also obtained in low concentration background absorption at a given detection wavelength. This was apparent on comparing the effect on the neomycin peak, when the imidazole in the buffer was changed from 0.01 M to 0.02 M the size of the peak appeared to half although there was no change in the migration time or resolution of the peak.

8.6 CZE conditions

The following conditions were therefore selected for the separation of neomycin sulphate and streptomycin sulphate.

Capillary:	Silica	Length	55 cm (Unicam)	50 cm (Beckman)
		id	50/70 micron	

Voltage applied: Anionic mode 30 kV

Detector wavelength: 214 nm

Buffer: 0.01 M Imidazole
400 µg/ml CTAB
pH 5 adjusted with the addition of acetic acid

Electropherograms obtained on the injections of standard solutions ranging from 10 - 200 ppm of neomycin sulphate and streptomycin sulphate containing 400 µg/ml of CTAB (Figure 8.3 and 8.4) showed that neomycin and streptomycin could be separated and resolved at 10 ppm.

Figure 8.3 Electropherogram of a standard solution of 7 ppm neomycin sulphate and 10 ppm streptomycin sulphate

Buffer: 0.01 M imidazole, 400 µg/ml, pH 5. Voltage (anionic mode) 30 kV.
Detector at 214 nm.

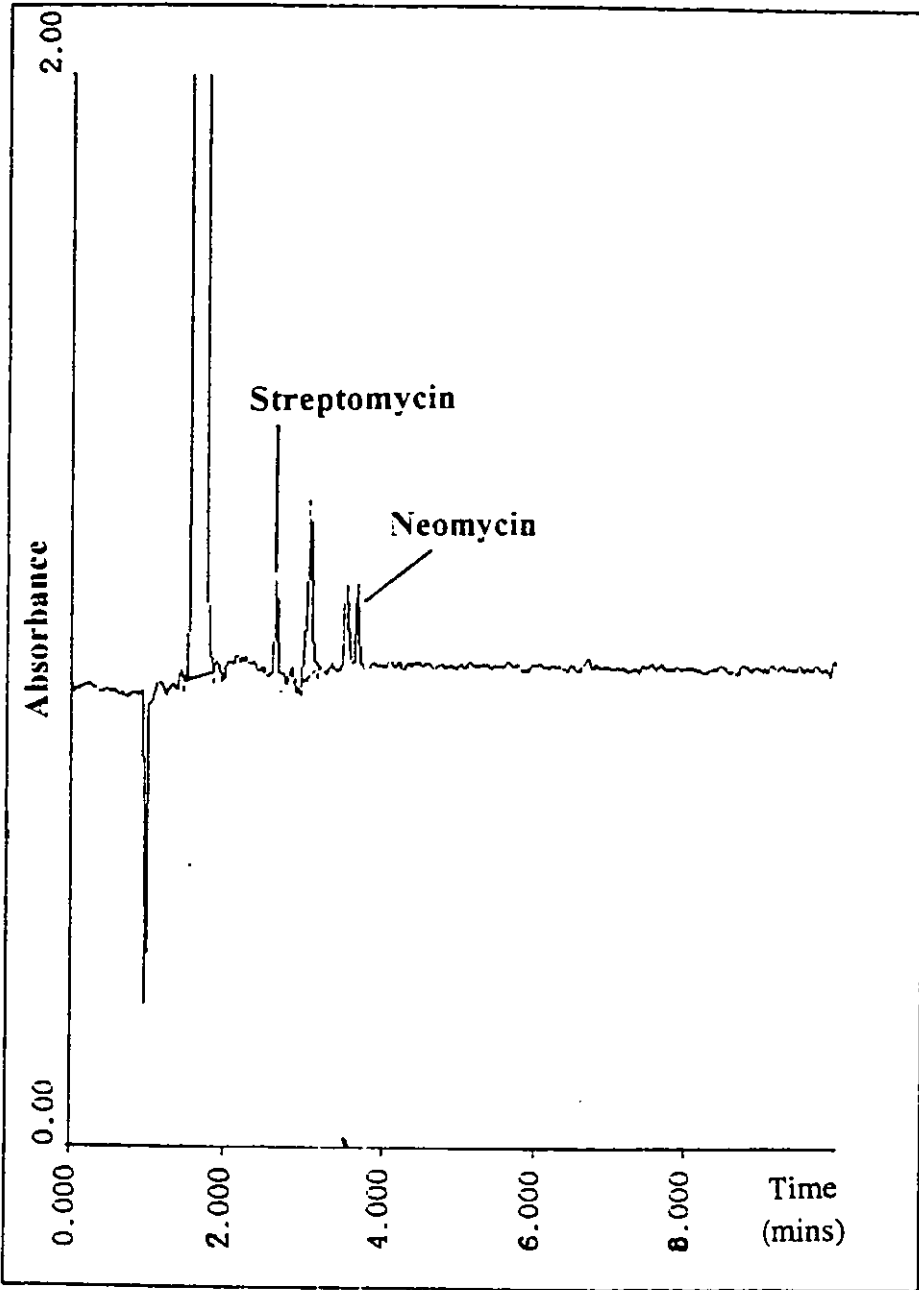
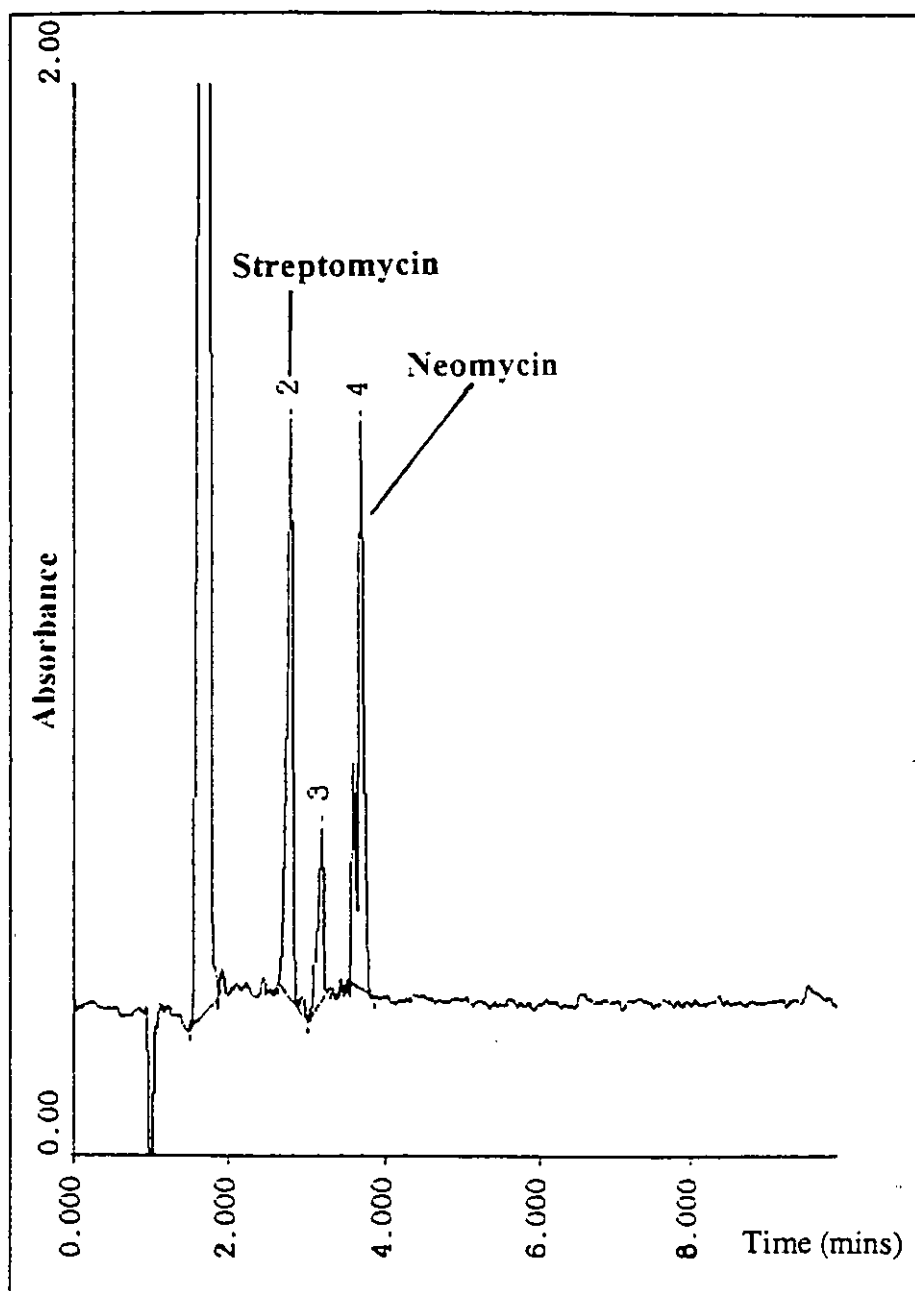


Figure 8.4 Electropherogram of a standard solution of 140 ppm neomycin sulphate and 200 ppm streptomycin sulphate

CE conditions, refer to Figure 8.3.



The electropherogram (Figure 8.4) shows that neomycin sulphate has been clearly separated into the two peaks and these were assigned to isomers C and B using CZE. These isomers were identified earlier using high performance anion exchange chromatography (chapter 5).

Increasing the internal diameter of the capillary tube from 50 micron to 75 micron enabled larger samples to be injected and increased the detector pathlength. This increased the sensitivity producing larger neomycin and streptomycin peaks with no apparent loss in resolution, (Figure 8.5.)

Figure 8.5 Electropherograms of a standard solution containing both neomycin sulphate and streptomycin sulphate using a capillary tube at (a) 50 micron and (b) 75 micron

CE conditions: Buffer, 0.01 M imidazole, CTAB 400 $\mu\text{g}/\text{ml}$, pH 5

Figure 8.5 (a)

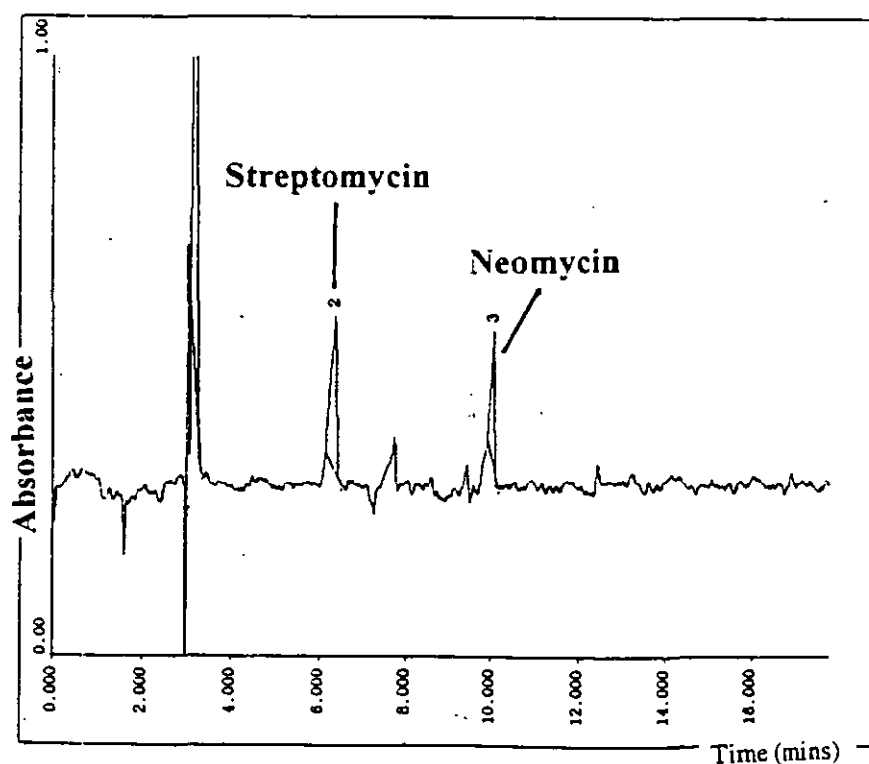
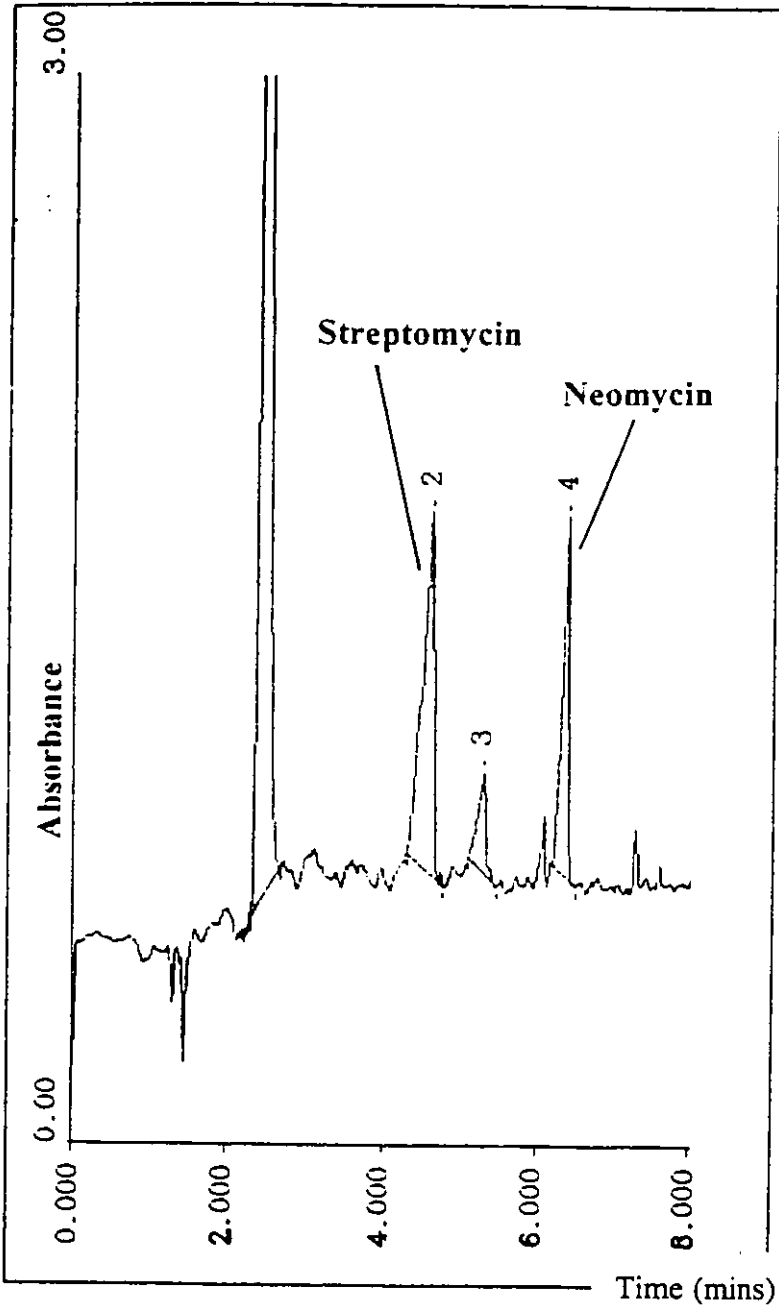


Figure 8.5 (b)



8.7 Calibration graph

A calibration graph was plotted to establish whether a linear calibration range could be produced for both neomycin sulphate and streptomycin sulphate under the CZE conditions outlined in the standard method section of the experimental chapter (figure 8.6 & 8.7).

Standard solutions containing both neomycin sulphate and streptomycin sulphate were prepared at concentrations ranging from 10 to 250 ppm. The peak areas obtained for each standard (Table 8.1) were used to plot a calibration graph for neomycin (Figure 8.6) and streptomycin (Figure 8.7.) Both neomycin and streptomycin exhibit good linearity at the concentration range plotted.

TABLE 8.1 CALIBRATION TABLE LISTING THE AREAS OBTAINED FOR EACH STANDARD CONCENTRATION OF NEOMYCIN SULPHATE AND STREPTOMYCIN SULPHATE.

CE conditions refer to figure 8.3 under the conditions outlined earlier in this chapter section 8.6.)

Neomycin Sulphate		Streptomycin Sulphate	
Concentration (ppm)	Area	Concentration (ppm)	Area
14	0.902	25	0.858
28	1.707	50	1.191
70	3.351	125	2.939
105	5.518	187.5	4.774
140	6.930	250	6.418

Figure 8.6 Calibration graph of neomycin sulphate at pH 5,
for the CZE conditions refer to section 8.6.

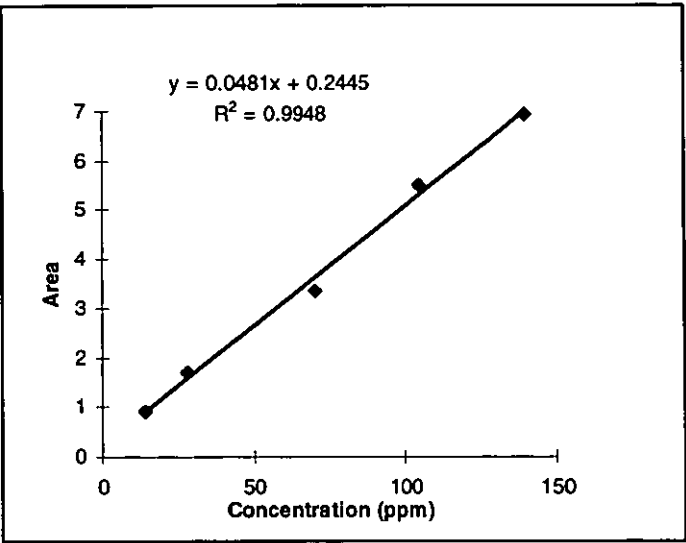
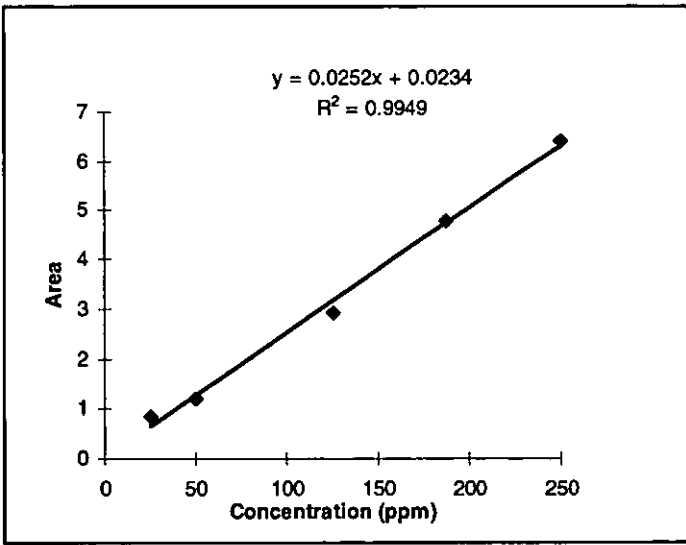


Figure 8.7 Calibration graph of streptomycin sulphate at pH of 5,
for the CZE conditions refer to section 8.6



8.8 The CZE separation of bovine kidney tissue extracts fortified with neomycin sulphate and streptomycin sulphate

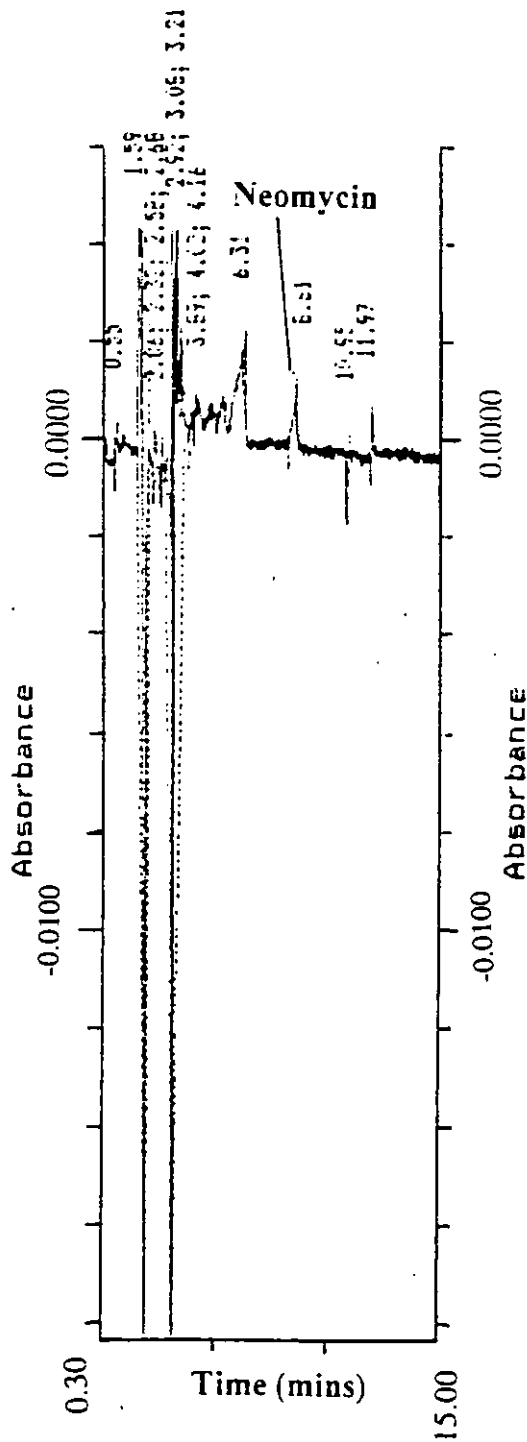
With reference to section 8.7, neomycin and streptomycin showed linearity over the concentration range measured. This method was therefore applied to bovine kidney spiked with neomycin and streptomycin at concentrations that were extracted within this calibration range. Extraction of both aminoglycosides from the bovine kidney was carried out by the MSPD sample preparation procedure outlined in the standard method section of the experimental chapter (section 2.5.5.)

The separation of analytes by CZE is very dependent upon the pH, not only of the buffer but also of the sample to be injected. This was tested by injecting a standard solution of neomycin sulphate 140 ppm prepared in 0.05 M sulphuric acid onto the capillary tube (Figure 8.8.) Two peaks appeared, a broad peak corresponding to neomycin sulphate at a migration time of 8.61 minutes and the other corresponding to sulphuric acid at a migration time of 6.31 minutes. In comparison a standard solution of 140 ppm neomycin sulphate prepared in deionised water at a pH of 5.50 was also prepared and injected. This produced two well resolved peaks corresponding to neomycin B and C. Therefore it was apparent from these results that neomycin extracted with sulphuric acid from bovine kidney tissue had to have its pH adjusted to that of the buffer (pH 5.5) by the addition of sodium hydroxide prior to injection, for good peak shape and separation of the two isomers neomycin B and C. CTAB (400 µg/ml) is also added to the samples prior to injection onto the capillary tube.

Figure 8.8 Electropherogram of a standard solution of 140 ppm neomycin sulphate prepared in 0.05 M sulphuric acid.

Buffer: 0.01 M imidazole - methanol (80:20 v/v), 400 µg/ml CTAB, pH 5.5. Voltage (anionic mode) 30 kV.

Detector wavelength 214 nm.



Preliminary studies were carried out including concentration of the aminoglycosides in the MSPD extract by the CBA cartridge. However, after a reasonable method had been developed, concentration of the aminoglycosides in the MSPD extract using the CBA cartridge was included in the sample preparation procedure. Both analytes neomycin sulphate and streptomycin sulphate were eluted off the CBA cartridge with 1 ml of 0.05 M sulphuric acid as in the standard method outlined in the experimental chapter (section 2.5.5.) The pH of this sample was changed to that of the buffer pH 5.5 and CTAB was added at 400 µg/ml. The sample was then made up to a volume of 2 mls and injected.

An extract from bovine kidney tissue spiked at 280 ppm neomycin sulphate prior to concentration on the CBA cartridge, was injected onto the CE column, buffer conditions 0.01 M imidazole, 400 µg/ml CTAB, pH 5.2. Under these conditions on injection of a standard solution of 35 ppm neomycin sulphate, neomycin was eluted at a migration time of 3.39 minutes. The electropherogram of the extracted bovine kidney tissue showed interference peaks at migration times ranging from 3.24 to 6.5 minutes (Figure 8.9.) It appeared that the neomycin peak had co-eluted with the interference peaks and was not sufficiently resolved for measurement.

The large interference peak also appeared in the electropherogram of a control bovine kidney sample. Chromatograms of extracted bovine kidney tissue samples separated by ion-pair chromatography, also showed large interference peaks, which were tissue matrix co-extractives that had not been removed by the MSPD clean-up extraction procedure. As it was felt that the MSPD extraction procedure could not be modified any further to increase the selectivity to remove the by-products interfering with the chromatography.

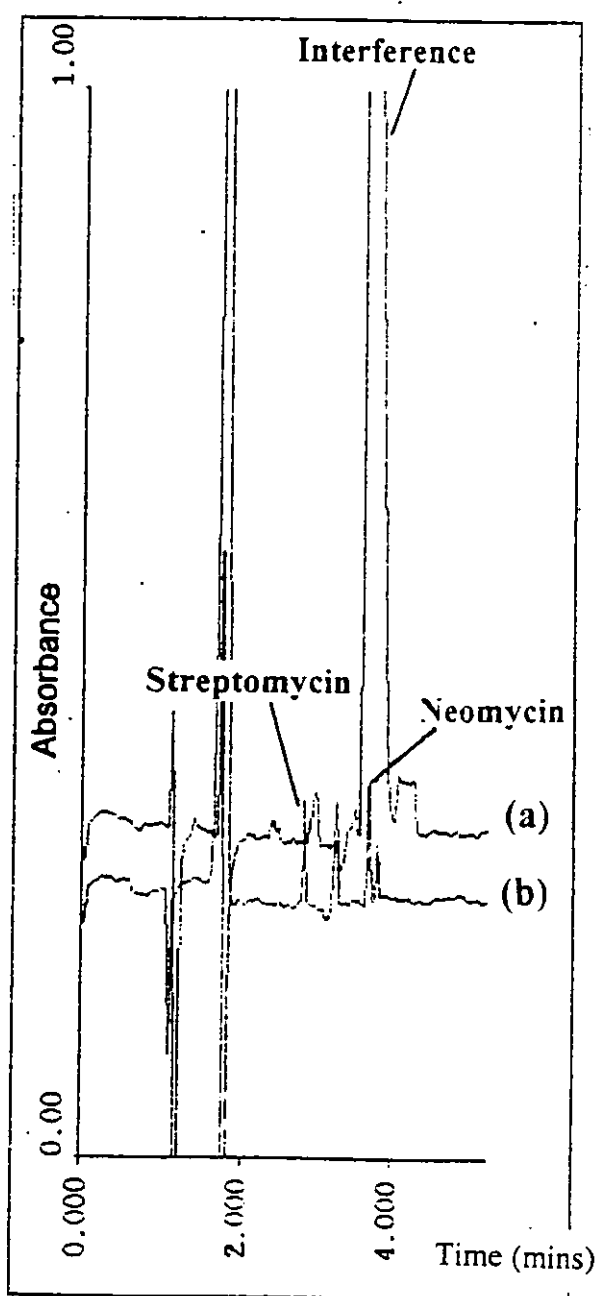
Attempts were therefore made with the electrophoresis method and therefore the buffer composition or its pH would be adjusted to separate the neomycin peak from the interfering peaks.

Figure 8.9 Electropherogram of

(a) Bovine kidney tissue fortified at 280 ppm neomycin sulphate.

(b) Standard solution of 35 ppm neomycin sulphate and 62 ppm streptomycin sulphate

CE conditions refer to Figure 8.4.



8.9 To improve the separation of the neomycin and biological background

8.9.1 Changes to the sample

8.9.1.1 pH

Initially the pH of the sample extracted spiked bovine kidney tissue was adjusted from a pH of 3 up to a pH of 10. Injection of the extract sample at each pH produced no overall effect on the contamination peak, it still co-eluted with the neomycin peak.

8.9.2 Changes to electrolyte composition.

Organic solvents tend to strongly reduce electroosmotic flow with resulting improved resolution.¹⁷⁸ Therefore it was assumed that addition of either methanol or acetonitrile to the buffer might narrow the width of the large contamination peak allowing the neomycin peak to be fully resolved and separated from it.

The percentage amount of acetonitrile and methanol was steadily increased in the buffer to determine the amount required to fully resolve the neomycin sulphate peak from the interference peaks in an extracted bovine kidney sample (Table 8.2.)

TABLE 8.2 MIGRATION OF NEOMYCIN SULPHATE IN RELATION TO THE INTERFERENCE FROM AN EXTRACTED BOVINE KIDNEY TISSUE SAMPLE ON ADDITION OF METHANOL OR ACETONITRILE TO THE BUFFER (CONDITIONS AS OUTLINED IN SECTION 8.6).

Solvent present in buffer (%)		Migration Time (minutes)	
Acetonitrile	Methanol	neomycin	Extract Interference
10	-	4.15	4.11 - 4.16
20	-	9.65	10.19 - 11.76
-	15	5.24	5.14 - 5.75
-	20	6.13	6.36 - 7.21
-	25	8.69	4.99 - 8.26

On reference to the results in table 8.2 it was evident that the optimum percentage concentration of the organic solvent methanol was 20 % methanol. This buffer produced a less extended migration time (Figure 8.10a) in comparison to acetonitrile (Figure 8.10b) at the same concentration. Therefore the buffer composition found to efficiently separate neomycin from the contaminants was 0.01 M imidazole - methanol (80:20 v/v), 400 µg/ml CTAB, pH 5.5, which was used for further work.

Figure 8.10 Electropherogram of 200 ppm neomycin sulphate, with the use of different organic modifiers in the electrophoretic medium. (a) organic modifier is methanol, (b) organic modifier is acetonitrile.

Figure 8.10 (a) Buffer: 0.01 M imidazole - methanol (80:20 v/v),
CTAB 400 µg/ml, pH 5.5. Voltage (anionic mode) 30 kV.
Detector wavelength 214 nm.

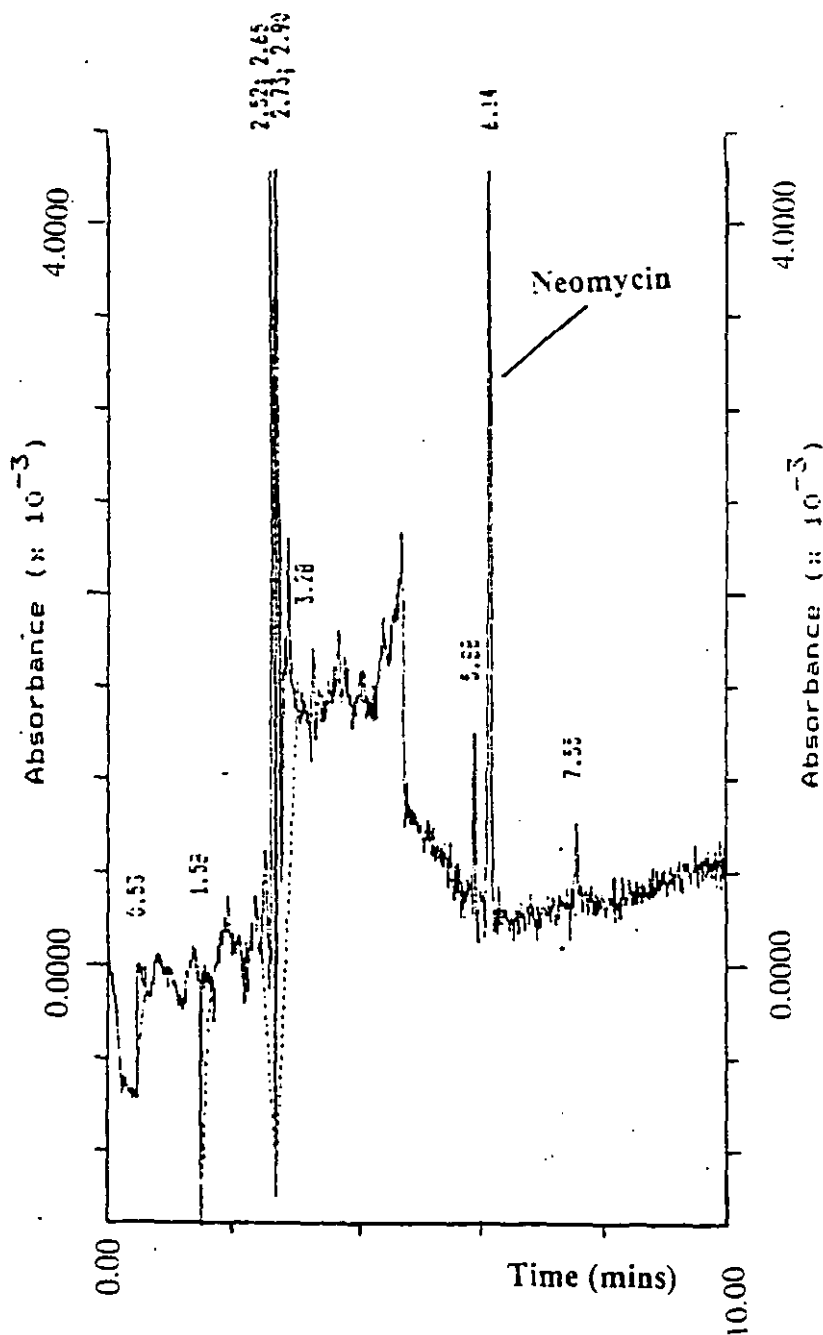
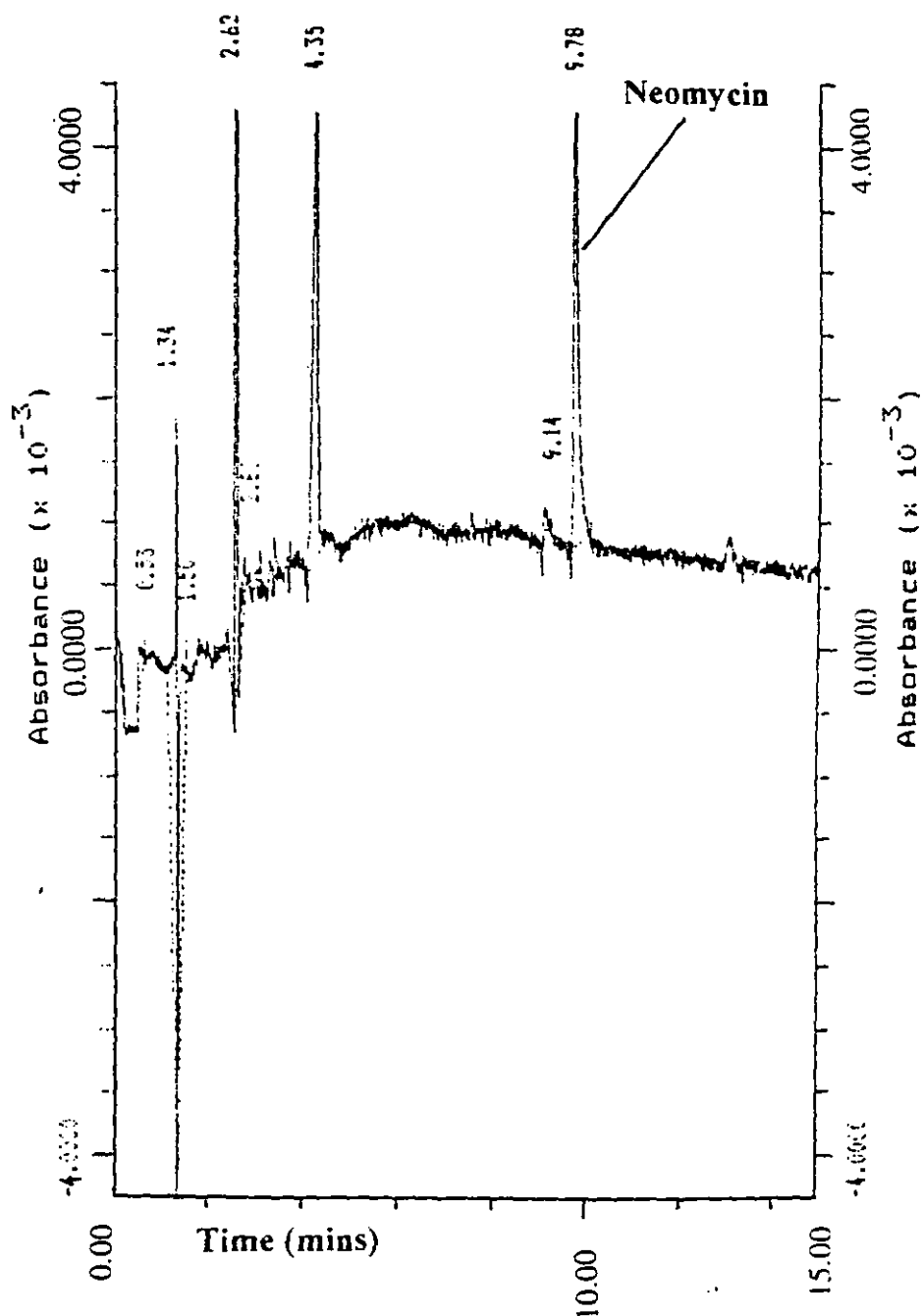


Figure 8.10 (b) Buffer: 0.01 M imidazole - acetonitrile (80:20 v/v),
 CTAB 400 µg/ml, pH 5.5. Voltage (anionic mode) 30 kV
 Detector wavelength 214 nm.



Investigations were then carried out to determine the lowest level of neomycin and streptomycin that could be analysed by CZE from spiked bovine kidney tissue. Bovine kidney samples were spiked with both neomycin and streptomycin at levels ranging from 20 to 500 ppm. The two aminoglycosides were then extracted using the sample preparation procedure outlined in the standard method section of the experimental chapter (section 2.5.5) ending with further concentration of the aminoglycosides in the extract using the CBA cartridge prior to injection. The sample extracts pH was changed to that of the buffer (pH 5.50) and CTAB (400 µg/ml) was added. The final 2 ml sample extract containing the analytes was separated using the CZE assay method outlined in the standard method section of the experimental chapter (section 2.5.4.)

8.10 Results

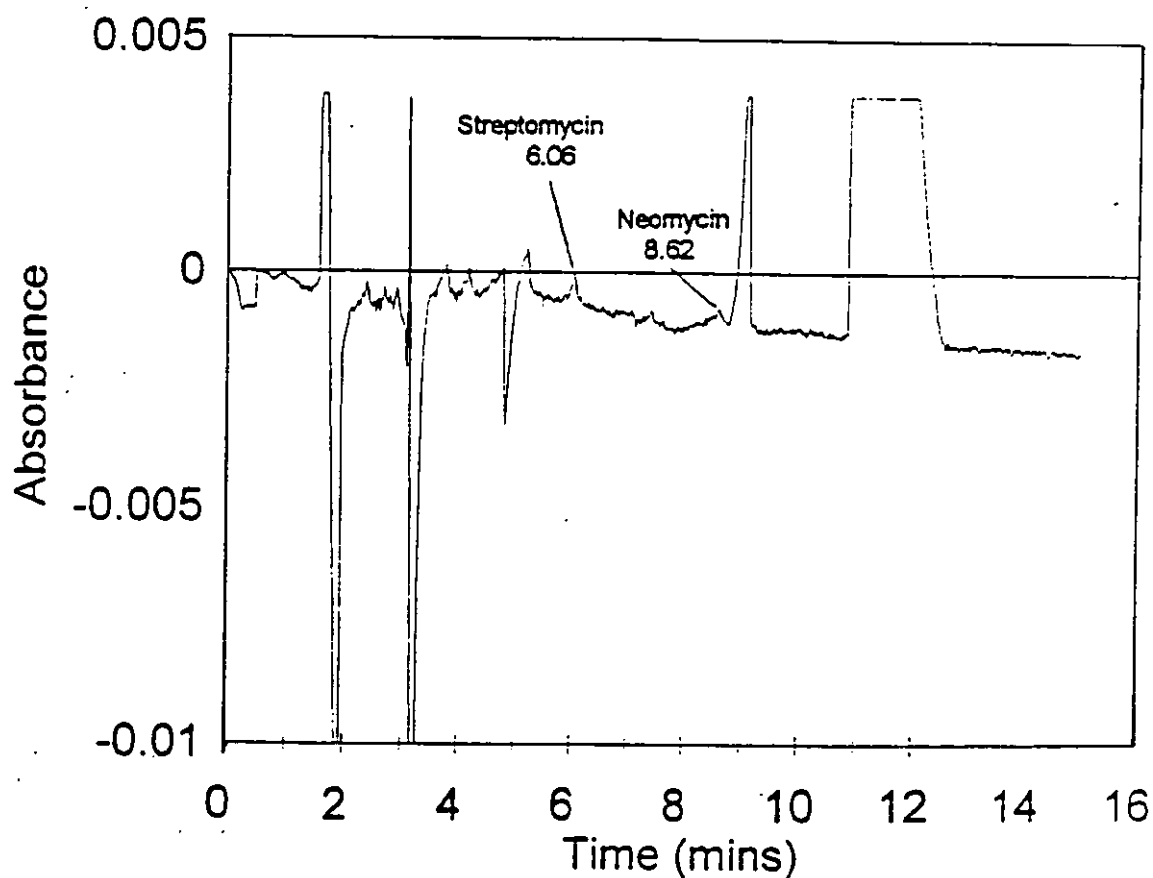
Following the MSPD extraction and CZE separation procedure outlined in the experimental chapter the lowest level at which neomycin and streptomycin could be reasonably recovered from spiked bovine kidney tissue was 500 ppm, there was no evidence of recovery from bovine kidney tissue spiked at 20 ppm. The recovery of neomycin from bovine kidney tissue spiked at 500 ppm was 23 % (Figure 8.11.). This result was poor in relation to the recovery results obtained for the MSPD sample extraction method used for the ion-pair HPLC assay with pulsed amperometric detection. Recovery of streptomycin from bovine kidney tissue fortified at 500 ppm was 37.6 % (Figure 8.11.) The poor recoveries in this case could be attributable to the sample handling during the extraction procedure. The samples were continually transferred from one container into another as the pH of the sample was adjusted to that of the electrolyte by the addition of sodium hydroxide. Also spiking the bovine

kidney at such a high concentration of 500 ppm could have overloaded the extraction procedure.

Ackerman¹²⁹ used the anionic mode and reversed electroosmotic flow to detect levels of neomycin in eardrops. The eardrop sample containing neomycin (5 mg/ml) was diluted to near the middle of a linear range (0.1 -1.0 mg/ml) of a calibration graph. However, analysis of neomycin at levels lower than this were not mentioned in Ackermans study.

Figure 8.11 Electropherogram of extract from kidney tissue fortified at 500 ppm neomycin sulphate and streptomycin sulphate.

CE conditions: Refer to figure 8.10 (a)



8.11 Conclusion

The poor recovery of both neomycin and streptomycin is probably due to the additional steps in the sample preparation procedure involving changes to the pH of the sample extract prior to injection. This has resulted in further loss and dilution of the sample analytes as the sample was transferred from one vessel to another. Further work needs to be carried out in this area to achieve a better recovery at lower levels. Possibly further concentration of both the analytes neomycin and streptomycin could be carried out on the actual capillary tube by what is referred to as sample stacking.¹⁷⁹ This basically takes advantage of conductivity differences between the sample zone and the background electrolyte. Conductivity of the injected sample is lower than that of the surrounding buffer, and hence results in concentration of the analyte zone.¹⁷⁹ The narrowing of the analyte zone can be attributed to the fact that the electric field at any point depends inversely on the specific conductivity. Therefore, the electric field strength increases in the sample zone of lower conductivity. The electrophoretic velocity increases at the higher field and hence the analyte zone becomes narrower, as a result the sample volume can be increased by a factor of ten without contributing to band broadening.¹⁸⁰ A simple stacking system was developed for the analysis of peptides or proteins in phosphate buffer at low pH, in which stacking of the analytes is achieved by sandwiching the sample between a zone of OH^- and H^+ ions. As OH^- and H^+ ions are migrating towards each other, zone of low conductivity is formed, as can be seen by a drop in electric current at the start of the separation. In addition, OH^- and H^+ may act as terminating ions, which leads to concentration of the analytes.¹⁷⁹

Possibly other buffer systems should be explored for future work, Flurer *et al*¹⁸¹

used a borate buffer for the separation of the antibiotic complex gentamicin sulphate, detection was carried out by direct UV detection, levels of detection were not revealed in this paper, however, concentrations quoted ranged from 88 - 108 mg/ml of gentamicin base.

Conclusion

CHAPTER NINE

CHAPTER NINE

9. *Conclusion*

The anion exchange HPLC method with detection of neomycin by the PAD gave very promising results. The limit of detection was 1 ppm and linearity between the range 1-100 ppm was good with a correlation coefficient of 0.9982 above the acceptable regression of 0.98 for application in a procedure. It was hoped this method could be used for the detection of the aminoglycosides in bovine kidney tissue. Concentration of the sample prior to injection would allow for detection of tissue spiked at levels equivalent to the MRL for neomycin at 500 µg/kg (0.5 ng/ml) and streptomycin at 1000 µg/kg (1 ng/ml). The MSPD sample preparation procedure developed by Schenck¹⁵³ appeared to work well, however, direct injection of the aminoglycoside in the solvent used to extract the analyte led to poor chromatography, because of problems with the effect of the acid sample solvent on the eluent pH. Reasonable chromatography of neomycin could be obtained if the extract was neutralised prior to injection but this would have added another manual step to the extraction procedure. No further work was therefore carried out using the anion exchange HPLC method.

An alternative HPLC system was developed using an ion-pair PFPA on a reversed-phase column. This HPLC system allowed direct injection of the acidic solute containing the aminoglycoside after extraction, with no change in pH. The limit of detection for this method was 5 ppm. Some problems with sensitivity were due to postcolumn mixing of the mobile phase containing solvent and the aqueous post column sodium hydroxide, resulting in baseline noise.

Using the MSPD extraction procedure with chromatography by the ion-pair HPLC system the recovery of neomycin and streptomycin was achievable with tissue spiked at 20 ppm with a mean recovery of 70 % (n=2) for neomycin and 49 % (n=4) for streptomycin. The lowest level at which extraction of neomycin sulphate from bovine kidney tissue was achievable was 10 ppm. At this 10 ppm level the MSPD procedure was modified by using 2 gms of bovine kidney for spiking in preference to 0.5 gms. The mean recovery of neomycin from bovine kidney tissue spiked at this level (10 ppm) was 56 % (n = 2).

Concentration of the aminoglycosides in the sample extract prior to injection using a CBA solid phase extraction cartridge was attempted but the sample concentration achieved was not high enough to allow tissue spiking and detection at ppb levels. Henion¹⁰³ achieved a 53 fold concentration of the aminoglycosides analysed using a Speed vac technique of evaporation and this technique could be an area for further developmental work.

The study demonstrated that it would also be possible to use CZE separation with indirect UV detection. Detection of both neomycin and streptomycin showed good linearity with regressions of 0.9948 for neomycin in the range 14 - 140 ppm and 0.9949 for streptomycin in the range 25 - 250 ppm above the acceptable limit for use in a procedure. Overall the sensitivity for CZE was similar to pulsed amperometric detection. The CZE method with modification to the electrolyte by the addition of methanol was shown to improve separation of neomycin from the co-eluting contaminant extracts obtained from the bovine kidney. However, the recovery of both neomycin and streptomycin from bovine kidney tissue was only detectable at the maximum concentration of 500 ppm at which the kidney was spiked in the study. At this concentration

recovery was extremely low due to additional sample pretreatment required after sample extraction by the MSPD method, which involved pH changes to the sample prior to injection.

In future work further development will be needed to analyse kidney samples spiked at lower concentrations. It will be necessary to examine the extract at each stage of the extraction and the sample handling procedure, to determine the point at which most of the aminoglycoside was lost.

Another approach which could be examined in future work is on capillary concentration in which concentration of the sample may be achievable on either the actual capillary tube or on a separate pre-capillary tube.¹⁷⁹

Concentration of the sample on the capillary tube, instead of the CBA solid phase extraction cartridge, would avoid the sample handling required that results in loss of the sample. The concentration technique most widely used in CE, work on the conductivity differences between the sample zone and the background electrolyte to focus the sample. When applying a sample dissolved in water or dilute buffer, a higher electrical field strength in the sample zone results in the concentration of the ionic analytes at the front of the zone. This can have the effect of decreasing the sample volume by a factor of 10 without resulting in band broadening, enabling an increase sample volume to be used. An alternative concentration technique using isotachopheresis can also concentrate dilute samples on the capillary tube by changing the concentration to that of the leading zone.¹⁷⁹ Further work could be carried out in this area to investigate which concentration technique would work well in the anionic mode with a reversed electroosmotic flow for the separation of both neomycin and streptomycin.

Once the developed assay method had achieved the recovery and sensitivity required for the determination of the analyte at trace levels the evaluation and validation must be done for the compound being studied in trace analysis because of the potential of other interfering extracts. The method can be evaluated by measuring the precision, accuracy and specificity. Accuracy is defined as the closeness of the measured value to the true value, whereas precision is the variation within replicate measurements. The precision of a method describes the agreement between replicate measurements. It has been suggested that the numerical value to describe this characteristic of a method should be referred to as imprecision, so that a high value would reflect high imprecision (bad) and a low value would reflect low imprecision (good). The imprecision then would be measured as the standard deviation or coefficient of variation of the results in a set of replicate measurements. The aim would be to then state the mean value and number of replicates as well as the design of the study, that is whether we are talking about within-day or between-day imprecision. The most meaningful value is the between-day precision because it is most relevant to the routine laboratory, which is what the method was intended for. Accuracy is the agreement between the best estimate of a quantity and its true value. A factor which may affect the accuracy when measuring real samples is the interference by endogenous compounds. Specificity is the ability of the analytical method to determine solely the component it sets out to measure and to remain unaffected by the presence of other substances. This is made possible with detection by mass spectrometry, where if the resources were available would be a good alternative to the PAD for detection of the aminoglycosides.

The sample preparation procedure and the assay used for analysis needs to be fully validated. Validation of the MSPD method in conjunction with the ion-

pair HPLC method was the next stage of the project. However the aim was to be able to achieve the extraction of neomycin and streptomycin at the MRL concentrations and then to validate the method at these concentration levels. However, validation of the method can be achieved by the extraction and analysis of at least six bovine kidney tissue samples spiked at the lowest concentration level where recovery was achievable which in the case of neomycin and streptomycin for this study was 20 ppm. The mean of the results and the standard deviation would give an indication of the spread of results at a given concentration level. Further analysis could be carried out on other biological matrices of the bovine; liver, muscle and fat, the areas of the animal where aminoglycoside deposition is most likely. Finally, analysis could be carried out on animals dosed with the aminoglycoside under question. If the method was robust and sensitive enough to detect the residue levels in the kidney tissue at the maximum residue limits referred to in table 1.1 the next stage would be to set up a calibration line over the expected concentration range. The amount for each standard would be spiked onto the tissue and extracted, the extracted aminoglycoside would give a true reflection of the amount representing a certain concentration in tissue. From this concentration line the amount of the aminoglycoside in the kidney of an animal dosed or fed the antibiotic, could be measured.

LIST OF PRESENTATIONS

1. Roger M. Smith, A. K. Ubhi and M. J. Shepherd, "Determination of the aminoglycoside antibiotic neomycin using pulsed amperometric detection", presented at 17th International Symposium on Column Liquid Chromatography, organised by the Fachgruppe Analytische Chemie, Gesellschaft Deutscher Chemie, in Hamburg 9th - 14th May 1993.
2. Roger M. Smith, A. K. Ubhi and M. J. Shepherd, "Determination of the aminoglycoside antibiotic neomycin using pulsed amperometric detection", presented at the Research and Development Topics in Analytical Chemistry meeting organised by the Analytical Division, Royal Society of Chemistry, Bradford, 13th - 14th July 1993.
3. A. K. Ubhi, Roger M. Smith, and M. J. Shepherd, "The determination of the aminoglycosides neomycin and streptomycin by capillary zone electrophoresis and ion-pair high performance liquid chromatography", presented at 20th International Symposium on Chromatography, organised by the Chromatographic Society, Bournemouth 20th - 24th 1994.
4. A. K. Ubhi, Roger M. Smith, and M. J. Shepherd, "The determination of the aminoglycosides neomycin and streptomycin by capillary zone electrophoresis and ion-pair high performance liquid chromatography", presented at Research and Developments Topics Meeting, organised by the Analytical Division, Royal Society of Chemistry at University of Hertfordshire, 18th and 19th July, 1994.

5. A. K. Ubhi, Roger M. Smith, and M. J. Shepherd, "The determination of the aminoglycosides neomycin and streptomycin by capillary zone electrophoresis and ion-pair high performance liquid chromatography", presented at Predoctoral Symposium, Autumn Meeting, Royal Society of Chemistry, Strathclyde, September 1994.

6. A. K. Ubhi, Roger M. Smith, and M. J. Shepherd, "The determination of the aminoglycosides neomycin and streptomycin by capillary zone electrophoresis and ion-pair high performance liquid chromatography", presented to Pharmaceutical Analytical Science Group, at Burleigh Court Loughborough, 4th October 1994 by A. K. Ubhi on the occasion of the award of a prize for the poster. A summary was repeated at the Royal Society of Chemistry, Joint Pharmaceutical Analysis Group meeting in London on 20th October.

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