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Review

Current methodologies for the analysis of aminoglycosides

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Abstract

The aminoglycosides are a large and diverse class of antibiotics that characteristically contain two or more aminosugars linked by glycosidic bonds to an aminocyclitol component. Structures are presented for over 30 of the most important members of this family of compounds. The use of aminoglycosides in clinical and veterinary medicine and in agriculture is described. Qualitative methods for aminoglycoside analysis include X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). The major part of this article comprises a comprehensive review of quantitative methods for the determination of aminoglycosides. These are microbiological assay, radiochemical assay, radioimmunoassay, enzyme immunoassay, fluoroimmunoassay and other immunoassays, spectrophotometric and other non-separative methods, gas chromatography (GC), thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE). Simple spectrophotometric methods may be adequate for the assay of bulk pharmaceuticals and their formulations. Microbiological assays make useful semi-quantitative screening tests for the analysis of veterinary drug residues in food, but rapid enzyme immunoassays are more suitable for accurate measurements of aminoglycosides in complex matrices. Automated immunoassays are the most appropriate methods for serum aminoglycoside determinations during therapeutic drug monitoring. HPLC techniques provide the specificity and sensitivity required for pharmacokinetic and other research studies, while HPLC–MS is employed for the confirmation of veterinary drug residues. The potential for further development of chromatographic and CE methods for the analysis of biological samples is outlined.

Keywords: Reviews; Aminoglycosides

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1. Introduction

1.1. Classification and structure

The aminoglycosides are a large and diverse class of antibiotics [1] that characteristically contain two or more aminosugars linked by glycosidic bonds to an aminocyclitol component. The cyclitol is 2-deoxystreptamine in most cases, one exception being streptomycin, which has a streptidine moiety (Fig. 1).

The aminoglycosides may be categorised according to the pattern of substitution of the cyclitol (Table 1). The most important subclasses are the 4,5-disubstituted deoxystreptamines (Fig. 2), which include the neomycins, and the 4,6-disubstituted deoxystreptamines (Fig. 3), which include the gentamicins, kanamycins, and tobramycin. The structures of some other important aminoglycosides are shown in Fig. 4. These include apramycin (a 4-monosubstituted 4-deoxystreptamine), streptomycin (a 4-monosubstituted streptidines), and hygromycin B (a 5-monosubstituted (+)-*N*-methyl-2-deoxystreptamine). Spectinomycin is included although it is not strictly an aminoglycoside as it has neither an aminosugar nor a glycosidic bond. It contains the aminocyclitol *N,N′*-dimethyl-2-*epi*-streptamine linked at both the 4- and 5-positions to a single carbohydrate moiety.

1.2. Source

Many aminoglycosides occur naturally as products of various Actinobacteria (Actinomycetes), particularly members of the genera *Streptomyces* (in which case they are named -mycins) and *Micromonospora* (-micins) [2]. These organisms often produce a number of structurally related antibiotics simultaneously [3] and the therapeutic product may contain a mixture of active compounds, e.g. the gentamicin C complex. Some aminoglycoside antibiotics are semi-

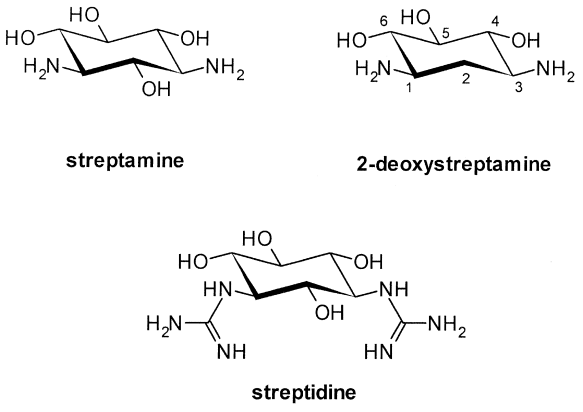


Fig. 1. Aminocyclitol components of aminoglycosides. Numbering of the ring atoms, shown for 2-deoxystreptamine, begins with the nitrogen-bearing carbon atom having the *R* configuration.

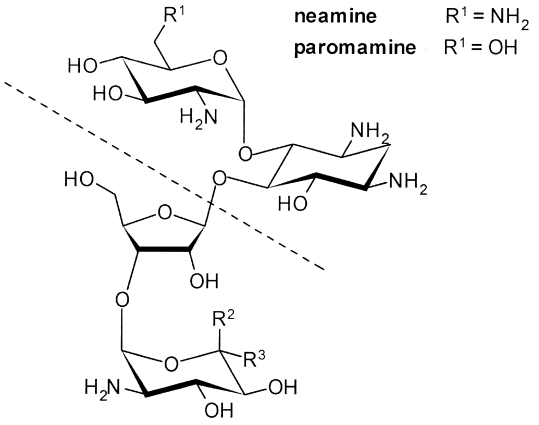
Table 1
Nomenclature and sources of aminoglycosides

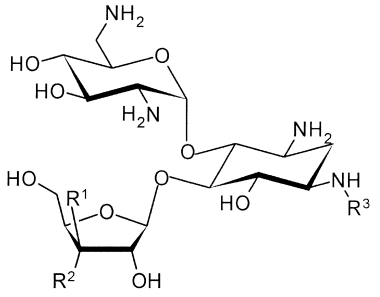
Aminoglycoside	Systematic name	Source
4,5-Disubstituted deoxystreptamines		
Butirosin A	(*S*)-O-2,6-Diamino-2,6-dideoxy-α-D-glucopyranosyl-(1→4)-O-[β-D-xylofuranosyl-(1→5)]-N1-(4-amino-2-hydroxy-1-oxobutyl)-2-deoxy-D-streptamine	Mucoid strains of *Bacillus circulans*; major component of butirosin
Butirosin B	(*S*)-O-2,6-Diamino-2,6-dideoxy-α-D-glucopyranosyl-(1→4)-O-[β-D-ribofuranosyl-(1→5)]-N1-(4-amino-2-hydroxy-1-oxobutyl)-2-deoxy-D-streptamine	Mucoid strains of *B. circulans*; minor component of butirosin
Neomycin B	O-2,6-Diamino-2,6-dideoxy-α-D-glucopyranosyl-(1→4)-O-[O-2,6-diamino-2,6-dideoxy-β-L-idopyranosyl-(1→3)-β-D-ribofuranosyl-(1→5)]-2-deoxy-D-streptamine	*Streptomyces fradiae*; major component of neomycin complex
Neomycin C	O-2,6-Diamino-2,6-dideoxy-α-D-glucopyranosyl-(1→4)-O-[O-2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl-(1→3)-β-D-ribofuranosyl-(1→5)]-2-deoxy-D-streptamine	*S. fradiae*; minor component of neomycin complex
Paromomycin I (neomycin E)	O-2-Amino-2-deoxy-α-D-glucopyranosyl-(1→4)-O-[O-2,6-diamino-2,6-dideoxy-β-L-idopyranosyl-(1→3)-β-D-ribofuranosyl-(1→5)]-2-deoxy-D-streptamine	Various *Streptomyces*; minor component of neomycin complex
Paromomycin II (neomycin F)	O-2-Amino-2deoxy-α-D-glucopyranosyl-(1→4)-O-[O-2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl-(1→3)-β-D-ribofuranosyl-(1→5)]-2-deoxy-D-streptamine	Various *Streptomyces*; minor component of neomycin complex
Ribostamycin	O-2,6-Dimino-2,6-dideoxy-α-D-glucopyranosyl-(1→4)-O-[β-D-ribofuranosyl-(1→5)]-2-deoxy-D-streptamine	*S. ribosidificus*
4,6-Disubstituted deoxystreptamines		
Amikacin	(*S*)-O-3-Amino-3-deoxy-α-D-glucopyranosyl-(1→6)-O-[6-amino-6-deoxy-α-D-glucopyranosyl-(1→4)]-N^1-(4-amino-2-hydroxy-1-oxobutyl)-2-deoxy-D-streptamine	Semi-synthetic; derived from kanamycin A
Arbekacin (habekacin)	(*S*)-O-3-Amino-3-deoxy-α-D-glucopyranosyl-(1→6)-O-[2,6-diamino-2,3,4,6-tetradeoxy-α-D-*erythro*-hexopyranosyl-(1→4)]-N^1-(4-amino-2-hydroxy-1-oxobutyl)-2-deoxy-D-streptamine	Semi-synthetic; derived from kanamycin B
Dibekacin	(*S*)-O-3-Amino-3-deoxy-α-D-glucopyranosyl-(1→6)-O-[2,6-diamino-2,3,4,6-tetradeoxy-α-D-*erythro*-hexopyranosyl-(1→4)]-2-deoxy-D-streptamine	Semi-synthetic; derived from kanamycin B
Gentamicin C_1	(*R*)-O-2,6-Diamino-2,3,4,6-tetradeoxy-6-(methylamino)-α-D-*erythro*-hexopyranosyl-(1→4)-O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1→6)]-2-deoxy-D-streptamine	*Micromonospora purpurea* or *M. echinospora*; major component of gentamicin C complex
Gentamicin C_{1a}	O-3-Deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1→6)-O-[2,6,-diamino-2,3,4,6-tetradeoxy-α-D-*erythro*-hexopyranosyl-(1→4)]-2-deoxy-D-streptamine	*M. purpurea* or *M. echinospora*; major component of gentamicin C complex
Gentamicin C_2	(*R*)-O-2,6-Diamino-2,3,4,6-tetradeoxy-6-methyl-α-D-*erythro*-hexopyranosyl-(1→4)-O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1→6)]-2-deoxy-D-streptamine	*M. purpurea* or *M. echinospora*; major component of gentamicin C complex
Gentamicin C_{2a}	(*S*)-O-2,6-Diamino-2,3,4,6-tetradeoxy-6-methyl-α-D-*erythro*-hexopyranosyl-(1→4)-O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1→6)]-2-deoxy-D-streptamine	*M. purpurea* or *M. echinospora*; minor component of gentamicin C complex
Gentamicin C_{2b} (micronomicin)	O-2-Amino-2,3,4,6-tetradeoxy-6-(methylamino)-α-D-*erythro*-hexopyranosyl-(1→4)-O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1→6)]-2-deoxy-D-streptamine	*M. sagamiensis* var. *nonreductans*; minor component of gentamicin C complex
Isepamicin	(*S*)-O-6-Amino-6-deoxy-α-D-glucopyranosyl-(1→4)-O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1→6)]-N^1-(3-amino-2-hydroxy-1-oxopropyl)-2-deoxy-D-streptamine	Semi-synthetic; derived from gentamicin B
Kanamycin A	O-3-Amino-3-deoxy-α-D-glucopyranosyl-(1→6)-O-[6-amino-6-deoxy-α-D-glucopyranosyl-(1→4)]-2-deoxy-D-streptamine	*Streptomyces kanamyceticus*; major component of kanamycin complex
Kanamycin B (bekanamycin)	O-3-Amino-3-deoxy-α-D-glucopyranosyl-(1→6)-O-[2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl-(1→4)]-2-deoxy-D-streptamine	*S. kanamyceticus*; minor component of kanamycin complex
Kanamycin C	O-3-Amino-3-deoxy-α-D-glucopyranosyl-(1→6)-O-[2-amino-2-deoxy-α-D-glucopyranosyl-(1→4)]-2-deoxy-D-streptamine	*S. kanamyceticus*; minor component of kanamycin complex
Netilmicin	O-3-Deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1→6)-O-[2,6,-diamino-2,3,4,6-tetradeoxy-α-D-glycero-hex-enopyranosyl-(1→4)]-2-deoxy-N^1-ethyl-D-streptamine	Semi-synthetic; derived from sisomicin
Sisomicin	O-3-Deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl-(1→6)-O-[2,6,-diamino-2,3,4,6-tetradeoxy-α-D-glycero-hex-enopyranosyl-(1→4)]-2-deoxy-D-streptamine	*M. inyoesis*
Tobramycin	O-3-Amino-3-deoxy-α-D-glucopyranosyl-(1→6)-O-[2,6-diamino-2,3,6-trideoxy-α-D-ribo-hexopyranosyl-(1→4)]-2-deoxy-D-streptamine	*S. tenebrarius*; component of nebramycin complex
Other important aminoglycosides		
Apramycin	O-4-Amino-4-deoxy-α-D-glucopyranosyl-(1→8)-O-(8*R*)-2-amino-2,3,7-trideoxy-7-(methylamino)-D-glycero-α-D-*allo*-octadialdo-1,5:8,4-dipyranosyl-(1→4)-2-deoxy-D-streptamine	*Streptomyces tenebrarius*; component of nebramycin complex
Destomycin A	O-6-Amino-6-deoxy-L-glycero-D-galacto-heptopyranosylidene-(1→2-3)-O-β-D-talopyranosyl-(1→5)-2-deoxy-N^1-methyl-D-streptamine	*S. rimofaciens*

Table 1. Continued

Aminoglycoside	Systematic name	Source
Other important aminoglycosides (continued)		
Fortimicin A	2,6-Diamino-2,3,4,6,7-pentadeoxy-β-L-lyxo-heptopyranosyl-(1→3)-4-amino-1-[(aminoacetyl)methylamino]-1,4-dideoxy-6-O-methyl-1L-chiro-inositol	*Micromonospora olivoasterospora*; major component of fortimicin complex
Fortimicin B	2,6-Diamino-2,3,4,6,7-pentadeoxy-β-L-lyxo-heptopyranosyl-(1→3)-4-amino-1,4-dideoxy-6-O-methyl-1-(methylamino)-1L-chiro-inositol	*M. olivoasterospora*; major component of fortimicin complex
Hygromycin B	O-6-Amino-6-deoxy-L-glycero-D-galacto-heptopyranosylidene-(1→2-3)-O-β-D-talopyranosyl-(1→5)-2-deoxy-N^3-methyl-D-streptamine	*S. hygroscopicus*
Kasugamycin	2-Amino-4-[(carboxyiminomethyl)amino]-2,3,4,6-tetradeoxy-D-arabino-hexose-(1→3)-1D-chiro-inositol	*S. kasugaensis*
Spectinomycin	[2*R*-(2α,4aβ,5aβ,6β,7β,8β,9α,9aα,10aβ)]-decahydro-4a,7,9-trihydroxy-2-methyl-6,8-bis-(methylamino)-4H-pyrano[2,3-b][1,4]benzodioxin-4-one	*S. spectabilis*
Streptomycin	O-2-Deoxy-2-(methylamino)-α-L-glucopyranosyl-(1→2)-O-5-deoxy-3-C-formyl-α-L-lyxo-furanosyl-(1→4)-N,N′-bis(aminoiminomethyl)-D-streptamine	Certain strains of *S. griseus*
Dihydro-streptomycin	O-2-Deoxy-2-(methylamino)-α-L-glucopyranosyl-(1→2)-O-5-deoxy-3-C-(hydroxymethyl)-α-L-lyxofuranosyl-(1→4)-N,N′-bis(aminoiminomethyl)-D-streptamine	Semi-synthetic derivative of streptomycin; also produced by *S. humidus*

synthetic derivatives of natural fermentation products, e.g. amikacin (derived from kanamycin A), arbekacin and dibekacin (from kanamycin B), isepamicin (from gentamicin B), netilmicin (from sisomicin), and dihydrostreptomycin (from streptomycin) although the last is also produced naturally. The total chemical synthesis of many of the aminoglycosides has been achieved [4] but fermentation remains the most economical route for their production. Table 1 lists the sources of important aminoglycoside antibiotics together with their chemical nomenclature.





	R^1	R^2	R^3
neomycin B	NH_2	CH_2NH_2	H
neomycin C	NH_2	H	CH_2NH_2
paromomycin I	OH	CH_2NH_2	H
paromomycin II	OH	H	CH_2NH_2

	R^1	R^2	R^3
ribostamycin	H	OH	H
xylostasin	OH	H	H
butirosin A	OH	H	
butirosin B	H	OH	

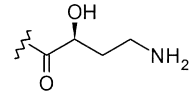


Fig. 2. Structures of deoxystreptamine aminoglycosides, 4,5-disubstituted.

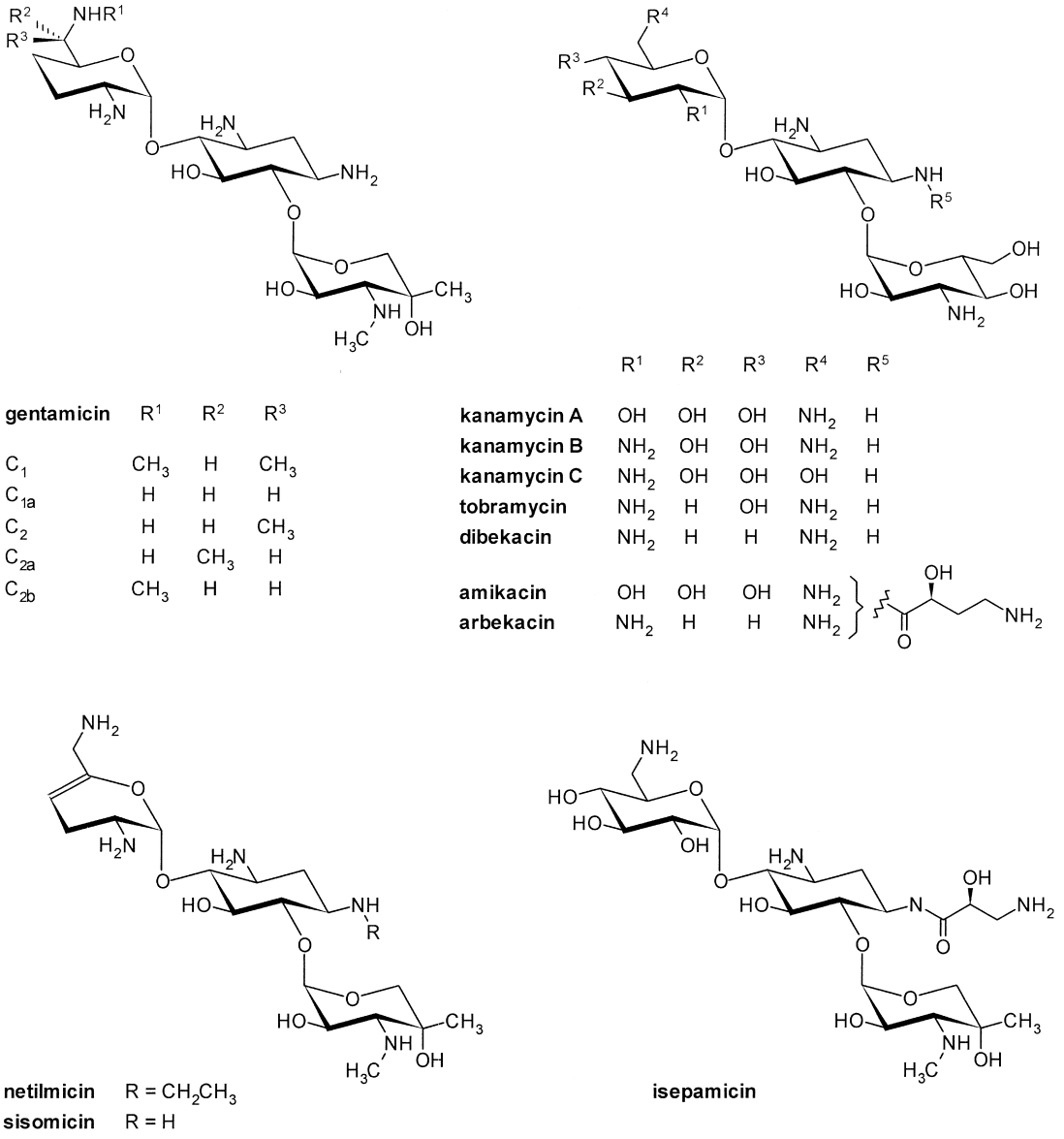
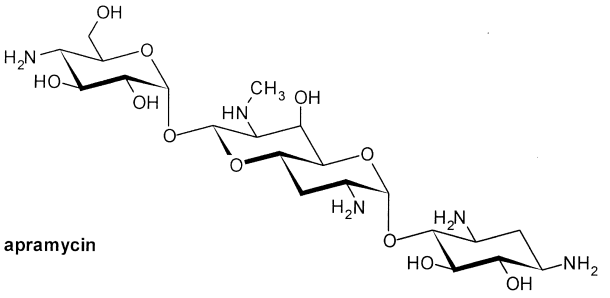


Fig. 3. Structures of 4,6-disubstituted deoxystreptamine aminoglycosides.

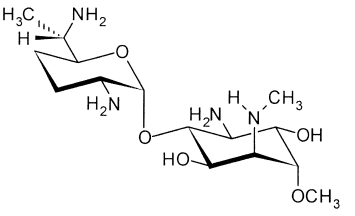
1.3. Use as therapeutic agents in clinical and veterinary medicine

The aminoglycosides are broad-spectrum antibiotics that have bactericidal activity against some Gram-positive and many Gram-negative organisms. They are not active against anaerobic bacteria, possibly because their uptake is blocked under these conditions [5]. Their clinical use has been limited by side effects of nephrotoxicity and ototoxicity [6,7], and by the emergence of resistant strains of organisms that produce aminoglycoside-modifying enzymes [8].

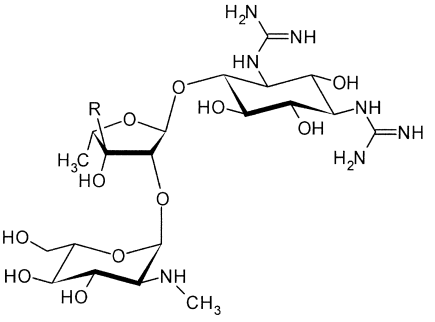
Streptomycin, discovered in 1944, was initially successful in the treatment of tuberculosis and other serious infections, but after several years of wide-



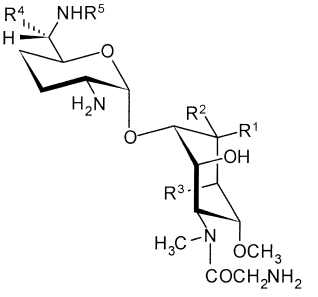
apramycin



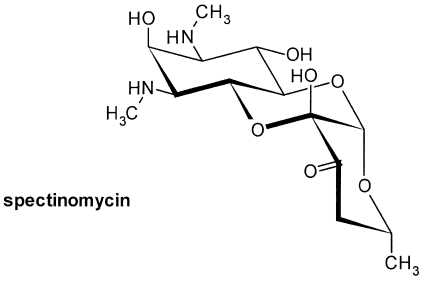
fortimicin B



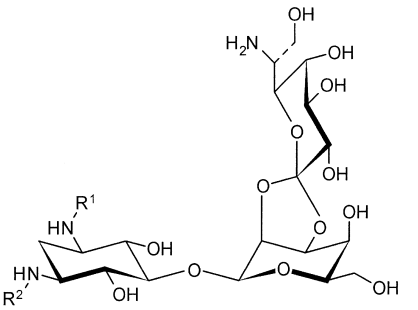
streptomycin R = CHO
dihydrostreptomycin R = CH_2OH

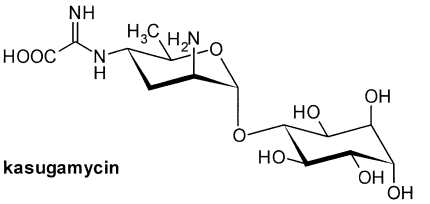


	R^1	R^2	R^3	R^4	R^5
fortimicin A	NH_2	H	OH	CH_3	H
istamycin A	NH_2	H	H	H	CH_3
istamycin B	H	NH_2	H	H	CH_3



spectinomycin





kasugamycin

	R^1	R^2
hygromycin B	H	CH_3
destomycin A	CH_3	H

Fig. 4. Structures of some other important aminoglycosides.

spread use resistant strains began to appear in hospital patients in the late 1950s. The same pattern was repeated with kanamycin during the following decade. This stimulated a search for new aminoglycosides and semi-synthetic derivatives that retained activity against the resistant organisms. During the 1970s, these efforts were rewarded with the discovery of many novel agents, of which several were successfully developed for therapeutic use. However, no aminoglycosides have moved into development since 1980 and it seems reasonable to conclude that there is little potential for improving upon the properties of existing antibiotics of this class [9].

Despite the introduction of newer, less toxic antimicrobials, aminoglycosides continue to serve a useful role as therapeutic agents [10,11]. Their clinical importance is being reassessed in the light of the increasing resistance of pathogenic organisms to other antimicrobials [12]. Gentamicin is currently the first choice aminoglycoside for the treatment of serious infections, alternatives being amikacin, netilmicin, and tobramycin [13]. They are all poorly absorbed from the gut and are administered by intramuscular or intravenous injection. Monotherapy is indicated for less serious infections, e.g. those of the urinary and biliary tracts. Combination therapy with a penicillin may be used to treat more serious infections, e.g. bacterial endocarditis, hospital-acquired pneumonia, and septicaemia. Streptomycin still has a role in the treatment of multidrug-resistant tuberculosis. Neomycin may be given orally for sterilisation of the gut prior to surgery. Spectinomycin is indicated for the treatment of gonorrhoea when penicillin is inappropriate. Arbekacin has been used to combat the increasing problems caused by methicillin-resistant *Staphylococcus aureus* (MRSA) [14].

In veterinary medicine and animal husbandry, aminoglycosides are widely used in the treatment of bacterial infections, e.g. bacterial enteritis (scours) and mastitis, and have been added to feeds for prophylaxis and to promote growth [15,16]. No aminoglycosides are currently permitted for use as growth promoters in the EU. Those most commonly used as therapeutic agents are gentamicin, neomycin, dihydrostreptomycin and streptomycin. Others may include apramycin, hygromycin B, and spectinomycin.

1.4. Use in agriculture

Kasugamycin has been used for many years in Japan for crop protection. It has a narrow spectrum of activity against phytopathogenic microbes and certain strains of pseudomonads, and has been applied in the control of *Magnaporthe grisea* (*Pyricularia oryzae*), the rice blast fungus [2,17].

1.5. Need for analytical methodologies

In a broad sense, methods of analysis have applications in the characterisation of new aminoglycosides from bacterial fermentations. Such techniques include thin-layer chromatography (TLC) of fragments released by chemical degradation, X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS).

Quantitative analyses of aminoglycosides in blood are routinely used in therapeutic drug monitoring as a guide to dosing, to prevent toxicity and to ensure efficacy [7]. Automated immunoassays (Sections 3.3–3.7) are the most appropriate methods for these serum aminoglycoside determinations. Levels of aminoglycosides are also measured in biological fluids and tissues during pharmacokinetic and other research studies. For these applications, the methodologies must provide high specificity and sensitivity, and it is advantageous if any metabolites can also be detected. High-performance liquid chromatography (HPLC, Section 3.12) currently best meets these requirements.

Sensitive methods are necessary for the analysis of veterinary drug residues, including aminoglycosides, in food products of animal origin. They are used by statutory agencies responsible for ensuring food safety and for enforcing the regulations governing the use of drugs in food-producing animals. Microbiological assays (Section 3.1) are preferred as screening tests for residue analysis because they can detect all of the different classes of antibiotics used and are simple and inexpensive to perform. Gas chromatography–mass spectrometry (GC–MS, see Section 3.10) and more commonly HPLC–MS (see Section 3.12) are the techniques employed for confirmation of veterinary drug residues.

Analytical methodologies are applied to bulk pharmaceuticals and their formulations for stability studies, quality control procedures, and for authenti-

cation purposes. In many such cases the analyte is present in a simple matrix without interfering compounds. Simple non-specific spectrophotometric methods (see Section 3.9) are often the most appropriate in these situations.

2. Qualitative methods for identification/ characterisation of aminoglycosides

2.1. X-ray crystallography

Preparations of purified aminoglycosides tend to form amorphous solids that are not amenable to X-ray diffraction studies. For this reason, the stereochemistry of most of the aminoglycosides has been derived by chemical and NMR spectroscopic methods. However, the structures of kanamycin A [18], streptomycin [19], spectinomycin [20], apramycin [21], kasugamycin [22], and fortimicin B [23] have been confirmed by X-ray studies.

2.2. Nuclear magnetic resonance spectroscopy

Proton- and ^{13}C-NMR spectoscopic methods have been particularly useful for determining the structural configuration of aminoglycosides [8,24,25].

NMR spectroscopy has been used recently to study the interaction between aminoglycosides and RNA [26–30], yielding important information about the mechanism of action of these drugs on the ribosome. Similar techniques have also been used to look at the interaction between aminoglycoside-modifying enzymes and their substrates [31–34]. Such studies provide a structural basis for the design of novel antimicrobial agents that are resistant to inactivation by these bacterial enzymes.

2.3. Mass spectrometry

MS has also been applied to the structural analysis of aminoglycosides [35–38] and their enzyme-modified products [8,32]. A recent study showed the utility of fast-atom bombardment (FAB) or electrospray ionisation (ESI) in combination with tandem MS for determining the positional isomers of aminoacyl derivatives of kanamycin A [39]. Californium-252 plasma desorption MS has also been applied to the identification of a range of aminoglycosides [40]. The authors suggested that the technique could be developed into a multiresidue method for the identification of aminoglycosides in food products of animal origin. A combination of FAB-MS and NMR spectroscopy has been used to analyse residues of neomycin and its metabolites in calf kidney after oral dosing [41].

MS is well established as a powerful detection method when coupled with gas chromatography (GC–MS) or liquid chromatography (LC–MS) (see Sections 3.10 and 3.12).

3. Quantitative methods for the determination of aminoglycosides

3.1. Microbiological assay

Microbiological assays (bioassays) historically have been used for the assay of aminoglycosides and other antibiotics. They are the methods of choice when an estimate of biological potency is required. Potency is estimated by comparing the inhibition of growth of a sensitive microorganism produced by known concentrations of the antibiotic being examined and a reference substance. Microbiological assays are relatively inexpensive to perform, require little in the way of sophisticated equipment other than that used for handling the microorganisms, and are suitable for testing large numbers of samples.

Microbiological assay is the method prescribed by British, European and US pharmacopoeias for the determination of aminoglycosides in bulk pharmaceuticals and their formulations. The official assay may be performed either by agar diffusion or turbidimetrically [42,43]. These methods were recently tested for neomycin assay in a multicentre study [44]. Of the six laboratories that returned results using the agar diffusion method, two failed to achieve the required precision and the accuracy ranged from 96.9 to 115.3% of the target value for a neomycin B standard. Three laboratories returned results from the turbidimetric method: all achieved acceptable precision and the accuracy varied from 99.8 to 112.2% for the same standard. It was noted that the reproducibility of the microbiological assays deteriorated as the content of neomycin C in the

sample increased. Because of these problems, the authors recommended that the microbiological assay of neomycin be replaced in official monographs by a liquid chromatographic method [45] (see Section 3.12.1).

Inaccuracy was also reported in the determination of tobramycin by agar diffusion assay when compared with HPLC and enzyme-linked immunosorbent assay (ELISA) [46]. The microbiological technique overestimated the actual quantity of tobramycin in pure solutions, particularly at or near the detection limit of 5 μg/ml when the error was of the order of 500%. Another disadvantage with the standard agar diffusion method is the length of time before the results can be read (18–24 h). Incorporation of the redox indicator triphenyltetrazolium chloride in the agar medium allowed measurement of the inhibition zones after 7-h incubation [47].

Microbiological assays, when combined with suitable extraction techniques, may be used for the determination of aminoglycosides and other drugs in animal feeds [48–50]. Aminoglycosides are very hydrophilic compounds that do not bind strongly to proteins in the matrix. They are easily extracted with aqueous solutions from finely ground feeds. Spectinomycin was extracted from dry meal and pelleted feeds using a trifluoroacetic acid solution (containing 40% methanol to increase wetting efficiency) with an average recovery of 98% [51]. The Association of Official Analytical Chemists (AOAC) cylinder-plate assay for neomycin has been the subject of two validation studies. The analyses showed considerable variation in recovery, ranging from 69 to 129% of the target value [52]. The analytical performance was not substantially improved by the use of more than two replicate plates [53].

A single procedure has been developed to separate mixtures of antibiotics in animal feeds and to enable their identification [54]. The system used a solvent pre-wash step to differentiate groups of antibiotics, agarose gel electrophoresis for their separation and identification, and bioautography using a self-contained modification of the agar diffusion assay for detection of the antibiotics. Limits of detection were 0.25 μg/ml for hygromycin B, 8.0 μg/ml for neomycin, 1.0 μg/ml for streptomycin, and 32.0 μg/ml for spectinomycin. Electrophoresis–bioautography has been used similarly to determine seven aminoglycosides with detection limits of 0.08–0.3 μg/ml and the methodology applied to the determination of residual drugs in bovine kidney [55].

Microbiological assays in a variety of formats are also used as screening tests for the detection of veterinary drug residues in foods of animal origin [16,56,57]. The procedure commonly used for screening meat samples in the EU is the four-plate test (FPT). The minimal inhibitory concentration (MIC) of neomycin detectable in the FPT was 0.2 μg/g tissue [16]. This method was used to screen nearly 5000 meat samples from retail outlets in the EU for the presence of antibiotic residues. Positive results were obtained in 2% of samples, most of which were confirmed as containing tetracycline antibiotics, and no aminoglycoside residues were reported [58]. No significant differences were found between monolayer and bilayer plates used in the AOAC method for the analysis of antibiotic residues in meat extracts and monolayer plates were recommended as being more efficient [59]. Where combinations of penicillin-G and an aminoglycoside such as neomycin or dihydrostreptomycin have been used in animal husbandry, it may be useful to be able to determine the residue levels of the individual drugs in food products. Simple modification of an agar diffusion method, in which the sample was incubated with penicillinase prior to assay, allowed various aminoglycosides to be quantified in the presence of penicillin-G [60].

In therapeutic drug monitoring and pharmacokinetic investigations, microbiological assays have been widely used to measure levels of aminoglycosides in human serum or plasma [6,61]. Although bioassays used to be popular for monitoring aminoglycosides during therapy, there has been a massive decline in their use over the past 20 years due to the introduction of the more convenient enzyme-multiplied immunoassay technique (EMIT) (see Section 3.4.2) and more recently the fluorescence polarisation immunoassay (FPIA) (see Section 3.5) [62]. The current situation is illustrated by the fact that, for gentamicin, bioassay was used by only two out of the 280 laboratories that participated in the UK National External Quality Assessment Scheme (UK NEQAS) for Antibiotic Assays during 1997–98. The remainder employed immunoassays, with FPIA accounting for 84% of all returns [63].

Agar diffusion assay was used for the determination of isepamicin in plasma in a recent pharmacokinetic study [64]. Comparison with HPLC and radioimmunoassay (RIA) methods showed that results obtained with the microbiological assay were 20–30% lower than those by HPLC and RIA whereas the last two were in good agreement. Microbiological assay suffered from non-specificity which would be a problem in the case of combination therapy using an aminoglycoside plus another antibiotic, or in the situation where active metabolites of the drug are present. Microbiological assay also had the disadvantages of a narrow linear range (0.5–24 μg/ml isepamicin) and poor sensitivity (limit of quantification 0.5 μg/ml). In a study to determine the effectiveness of experimental routes of drug delivery in the treatment of eye infections, bioassays were used to quantify tobramycin in aqueous humour [65]. The optimal sensitivity of 0.24 μg/ml tobramycin was obtained by applying the sample in wells rather than on paper discs, and by using thinly poured agar plates. Sensitivity was found to be dependent on the ionic strength of the sample, and improved with increasing salt concentrations. It was suggested that the higher solute content reduced ionic interactions between the drug and the agar medium, thereby enhancing antibiotic diffusion. The concentration of cations in the agar medium is thought to affect both the rate of diffusion of aminoglycosides and the growth of test organisms. Since agar is highly variable in its composition, this is likely to contribute to the reliability and accuracy problems with of this type of microbiological assay [66].

3.2. Radiochemical assay

The Charm II Tests (Charm, Malden, MA, USA) are a series of microbial receptor assays that are widely used for the rapid detection and identification of β-lactam antibiotics, sulphonamides, tetracyclines, macrolides, aminoglycosides, novobiocin, and chloramphenicol in milk [67]. A modification of the procedure may also be used for screening these drugs in other dairy products, meats, feeds, eggs and urine. The methodology is based on competition between the drugs in the sample and radiolabelled tracers for specific receptor sites. The receptors are present on the surface of microbial cells that are added to the sample. The amount of radiolabelled tracer binding to the receptor sites is measured using a dedicated analyser or liquid scintillation counter. The Charm II tests can detect gentamicin, streptomycins, and neomycin with good sensitivity, e.g. gentamicin at 0.02 μg/ml in milk and 0.4 μg/g in tissue and streptomycin at 0.15 μg/g in tissue, fish and eggs [56]. In a comparison with thin-layer chromatography–bioautography for screening meat samples, Charm II Tests were found to be faster, more convenient, and more sensitive [68].

3.3. Radioimmunoassay

RIA has been used successfully for the determination of aminoglycosides in biological fluids [6,61]. The technique involves competition between free drug in the sample and a fixed amount of ^{125}I-labelled drug (tracer), for a limited number of antibody binding sites. Separation of bound and free drug traditionally has been achieved by adding a second antibody directed against the first antibody to precipitate the antigen–antibody complex. Following centrifugation and removal of the supernatant (free drug), the amount of radioactivity in the precipitate is measured using a gamma counter. Most RIAs are now automated with separation assisted by the use of antibody bound to a solid phase, commonly the polystyrene assay tubes or magnetic particles. Increasing the amount of free drug in the sample causes less tracer to bind to the antibody and the measured radioactivity to decrease. The methodology is highly sensitive and precise, but the equipment is costly and there are problems associated with handling radioactivity. Like all immunoassays, there may be cross-reactivity between different aminoglycosides, and the technique depends on the availability of a suitable antibody and tracer, or the expertise, facilities and time required to produce them. The RIA used to measure plasma isepamicin in a recent pharmacokinetic study was very sensitive (limit of quantification 1 ng/ml), had a reasonable linearity range (100-fold), and was highly specific (<0.4% cross-reactivity with gentamicin) [64]. By suitably varying the assay conditions, plasma isepamicin concentrations could be determined up to 72 h after the drug was administered.

3.4. Enzyme immunoassay

Enzyme immunoassays (EIAs) may be classified as heterogeneous (the enzyme-labelled antigen or antibody is separated from the antigen–antibody complex before the enzyme activity is measured in either fraction), or homogeneous (the enzyme activity of the labelled antigen is measured in the presence of the antigen–antibody complex).

3.4.1. Heterogeneous EIA

Heterogeneous EIAs are best exemplified by ELISA. These assays are commonly performed as quantitative tests using 96-well microtitre plates, but rapid qualitative tests have also been developed that use test tube, dipstick and membrane (immunofiltration) formats [56,69]. ELISAs may be competitive or non-competitive. Competitive assays may be described as antigen-capture or antibody-capture depending on whether the solid phase is coated with antibody or antigen, respectively. In the former, competition occurs between antigen in the sample and tracer (enzyme-labelled antigen) for a limited number of immobilised antibody binding sites. In the latter, soluble antigen in the sample competes with immobilised antigen for binding the primary antibody. In this case the tracer is an enzyme-labelled secondary antibody that is directed against the primary antibody. The amount of tracer that is immunospecifically bound is determined by addition of a chromogenic enzyme substrate. In all competitive ELISAs the intensity of the colour formed in the final step is inversely proportional to the concentration of antigen in the sample. The antibody-capture method has the advantage that a conjugated reagent specific to each assay is not required.

Non-competitive ELISAs usually involve binding of the antigen in the sample to an excess of immobilised antibody. The extent of binding is measured with a second (enzyme-labelled) antibody directed against a different epitope. In this procedure, which is also known as a sandwich ELISA or two-site immunometric assay, the measured signal is directly proportional to the amount of antigen in the sample.

ELISAs that have been developed for drugs and other small-molecular-mass compounds are mostly of the competitive type because such molecules generally possess only one antibody binding site. Examples of competitive ELISAs that have been developed for aminoglycosides are given in Table 2. The gentamicin ELISA used to screen bacterial fermentations for the production of aminoglycosides is typical of the standard antigen-capture technique [70]. The antibody employed as immunosorbent showed cross-reactivity with other aminoglycosides, but this was a useful attribute for this application. An ELISA was reported for the determination of streptomycins in milk in which a double antibody solid-

Table 2
Competitive microtitre plate ELISAs for aminoglycosides[a]

Type	Analyte	Sample (pretreatment)	Enzyme label	Substrate	Sensitivity	Ref.
Antigen-capture	Gentamicin	Fermentation broth (none)	ALP	PNPP	10 pg/ml	[70]
Antigen-capture, DASP	Streptomycin, Dihydrostreptomycin	Milk (defatted, diluted 1/10)	HRP	TMB	6, 0.8 ng/ml respectively (milk)	[71]
Antigen-capture, DASP	Gentamicin Neomycin Streptomycin	Milk (defatted, diluted 1/10) or kidney (deproteinised extract, diluted to 2% w/v)	HRP	TMB	0.7 ng/ml (milk), 4 ng/g (kidney) 3.6 ng/ml (milk), 25 ng/g (kidney) 5.1 ng/ml (milk), 28 ng/g (kidney)	[72]
Antigen-capture	Streptomycin	Honey (purified by C_{18} solid-phase extraction and immunoaffinity chromatography)	HRP	TMB	10 ng/g (honey)	[74]
Antibody-capture	Streptomycin, DHS	Milk (defatted)	ALP	PNPP	1.6 ng/ml (milk)	[76]
Antibody-capture	Spectinomycin	Liver, kidney (2.5% w/v aqueous extract)	ALP	PNPP	0.5 ng/ml (tissue extract)	[79]
Antibody-capture	Gentamicin	Human serum (diluted 1/1000)	HRP	OPD[b]	0.5 ng/ml (diluted serum)	[77]
Antibody-capture	Spectinomycin	Chicken plasma (diluted 1/20)	β-Gal	MUG	2 ng/ml (diluted plasma)	[78]

[a] Abbreviations: DASP, double antibody solid-phase; DHS, dihydrostreptomycin; ALP, alkaline phosphatase; HRP, horseradish peroxidase; β-Gal, β-galactosidase; PNPP, *p*-nitrophenyl phosphate; TMB, 3,3′,5,5′-tetramethylbenzidine; OPD, *o*-phenylenediamine; MUG, 4-methylumbelliferyl-β-galactoside.

[b] This was not stated, but detection of the coloured product at 490 nm suggested that OPD was used as substrate.

phase (DASP) technique was used [71]. The DASP technique significantly reduces the consumption of specific antiserum since most of the reagent used to coat a solid phase by non-specific adsorption remains unbound and is discarded. The assay was specific for streptomycin and dihydrostreptomycin, and their recoveries from milk were generally >80%. Similar ELISAs have recently been developed for the detection of gentamicin, neomycin and streptomycin in milk and kidney [72]. Limits of detection for gentamicin, neomycin and streptomycin were far below the corresponding maximum residue levels (MRLs) of 100, 500, and 200 μg/l in milk and 1000, 5000 and 1000 μg/kg in kidney that are permitted in the European Union [73]. The gentamicin ELISA showed 25% cross-reactivity with sisomicin, which has a closely related structure. Similarly, dihydrostreptomycin had high cross-reactivity (150%) in the streptomycin ELISA. No other cross-reactions between aminoglycosides were detected in the three assays. However, the assays were only suitable for screening purposes because of their inaccuracy (recoveries ranged from 47 to 78% in milk and 70 to 96% in kidney) and poor reproducibility (relative standard deviation, RSD, ranged from 23 to 60% for milk and 10 to 38% for kidney analyses). The assay developed by Schnappinger et al. was recently applied to the screening of streptomycin residues in honey [74]. The two-stage sample preparation procedure eliminated interference from the sample matrix with excellent recovery of streptomycin, and therefore allowed the required sensitivity to be achieved.

A rapid test for streptomycins in milk has been developed using a related enzyme-linked immunofiltration assay (ELIFA) format [75]. This employed a nylon membrane as the support for a DASP similar to that used in the ELISA described earlier [71]. Subjective detection limits were 5 ng/ml for streptomycin and 2 μg/l for dihydrostreptomycin. No cross-reactivity was observed with nine different aminoglycosides at high concentrations. This sensitive test was simple to perform, could be completed within 10 min, and because no equipment was required it would be suitable for screening milk samples in the field.

Microtitre plates with a specially modified surface (Nunc-Immuno™ MaxiSorp™, Nalge Nunc, Rochester, NY, USA) were used to develop a competitive antibody-capture ELISA for streptomycins in milk [76]. Small molecules in general adsorb very poorly to solid phases. This proprietary modification, which results in a polystyrene surface with high affinity for polar groups, enabled dihydrostreptomycin to be used directly as immunosorbent. Streptomycin and dihydrostreptomycin gave similar responses in the test, but little (<2%) cross-reactivity with other aminoglycosides was observed. Reproducibility of this ELISA appeared to be good although the results were only reported semi-quantitatively.

Conjugating the antigen to a carrier protein has also been used to facilitate its adsorption to the solid phase. Care must be taken not to use the same carrier and cross-linking chemistry that was used during the production of the antiserum otherwise high background signals are likely to be obtained. Examples of this strategy include ELISAs for gentamicin in human serum [77] and spectinomycin in chicken plasma [78]. The gentamicin ELISA showed good reproducibility (RSD<12%), accuracy (recovery varied from 91 to 106%) and correlated well with a fluorescence polarisation immunassay technique. Cross-reactivity of the primary antiserum with other aminoglycosides was less than 1%. Dilution of serum by 1/1000 was necessary to reduce interference by the sample matrix on the assay measurements [77]. Performance of the spectinomycin ELISA was similar, with RSD<12% and recovery of spectinomycin in spiked plasma varying from 97 to 110%. However, interference from the sample matrix did not appear to be as high with this method, which may be due to the use of a fluorogenic enzyme substrate. It was observed that spectinomycin bound strongly to protein which prevented accurate measurement of the total drug in the sample. This problem was overcome by the incorporation of sufficient streptomycin in the assay buffer to saturate the plasma protein drug binding sites [78]. No such difficulties were reported during the development of a spectinomycin ELISA for the analysis of residues in food animal tissues, in which a simple aqueous extraction provided recoveries of 83–99% [79].

Heterogeneous, competitive ELISAs for gentamicin and tobramycin have been developed by Bayer Diagnostics for the automated Technicon Immuno 1™ system (Bayer, Tarrytown, NY, USA).

The methodology involves the mixing of sample (drug), tracer (drug–enzyme conjugate), and monoclonal anti-drug antibody that is labelled with fluorescein. Antigen–antibody complexes are captured on magnetic solid-phase particles coated with anti-fluorescein antibodies, allowing the rapid separation of bound and free tracer. Bayer gentamicin and tobramycin immunoassays are currently used by some clinical chemistry laboratories in the UK and, although not as accurate as FPIA, appear to be reliable [63].

A non-competitive sandwich ELISA in the form of a dipstick test for gentamicin in milk has been reported [80] despite theory suggesting that such two-site immunoassays are not possible for small molecules. The test detected gentamicin at concentrations of 0.1 μg/ml or above and, because of its simplicity, could be used for field studies.

A non-competitive EIA for tobramycin has recently been reported which, although described as an ELISA, used a non-immunological coating reagent, Alcian blue, to capture the antigen non-specifically onto the microtitre plate surface [46]. Tobramycin could be detected at 0.05 μg/ml with a working range up to 6 μg/ml. The anti-tobramycin antibody showed strong cross-reactivity with the closely related aminoglycoside, kanamycin. Results from the analysis of pure kanamycin solutions were not as accurate as by HPLC but more accurate than by a microbiological assay. It was also reported that solid phase coated with Alcian blue could bind a wide range of molecules including peptides and phospholipids. The tobramycin ELISA was not tested on samples with complex matrices, for example tissue extracts or plasma. It is doubtful whether this assay would be suitable for such applications since many molecules in the sample would compete with the analyte for the non-specific binding sites.

3.4.2. Homogeneous EIA

Homogeneous EIAs are best exemplified by the enzyme multiplied immunoassay technique (EMIT). EMIT® assays were developed and first introduced in 1972 by Syva (now part of Dade Behring, Deerfield, IL, USA). This major breakthrough in immunoassay technology was successfully applied to therapeutic drug monitoring in the late 1970s and caused a rapid decline in the use of bioassays for monitoring aminoglycosides [62]. The EMIT principle involves competition between drug in the sample and drug labelled with the enzyme glucose-6-phosphate dehydrogenase (G6PDH) for antibody binding sites. Enzyme activity decreases when the labelled drug is bound to the antibody because of steric hindrance and is inversely related to the concentration of drug in the sample. Enzyme activity is measured spectrophotometrically by the conversion of nicotinamide adenine dinucleotide (NAD) coenzyme from the oxidised form (NAD^+) to the reduced form (NADH) [81]. Reagent kits are available for amikacin, gentamicin, netilmicin and tobramycin EMIT assays which may be used with the Viva automated dedicated EMIT analyser (Dade Behring) or with automated clinical chemistry analysers from different manufacturers. Now largely superseded by FPIAs (see below), EMIT aminoglycoside assays are still used by several clinical chemistry laboratories in the UK for routine therapeutic drug monitoring [62]. The EMIT gentamicin assay was found to be rapid, very precise, reliable and correlated well with FPIA and two other commercial automated immunoassays over the concentration range 0.3–16.3 μg/ml [82].

Substrate-labelled immunofluorescent assay (SLIFA) is analogous to EMIT but in this case the enzyme substrate generates a fluorescent product [56]. Miles Labs. (Elkhart, IN, USA) developed aminoglycoside SLIFAs for use with the Ames Fluorostat [83] but it is not clear to what extent, if any, these assays are still in use.

3.5. Fluoroimmunoassay

Heterogeneous fluoroimmunoassay (FIA) is analogous to RIA except that the label is a fluorophore such as rhodamine or fluorescein rather than a radioisotope. Competitive FIAs for gentamicin and tobramycin are currently available in a multilayer dry-film format for use with the Opus™ Plus automated immunoassay analyser (Dade Behring). The Opus multilayer film system was originally developed by PB Diagnostics and first introduced in 1990. It consists of a transparent polyester base on top of which are an agarose signal layer containing immobilised monoclonal anti-drug antibody and rhodamine-labelled drug (tracer), an iron oxide opti-

cal screen, a top coat containing buffers, detergents etc. and finally a grooved sample applicator [84]. Sample spread over the top layer mixes with buffer and surfactant, and low-molecular-mass components diffuse downwards through the iron oxide layer. When liquid meets the signal layer, dried antibody and tracer are reconstituted and competition occurs between drug from the sample and tracer for antibody binding sites. Tracer not bound by the immobilised antibody diffuses upwards into the iron oxide layer which screens it from detection by the fluorimeter positioned below. The Opus assay for serum gentamicin, although reliable and reproducible, performed less well than two alternative automated immunoassays [82]. According to the UK NEQAS, the Opus assay is used by few laboratories and was the least accurate method for monitoring gentamicin [63].

Homogeneous FIA is exemplified by the fluorescence polarisation immunoassay (FPIA). Abbott (Abbott Park, IL, USA) introduced the TDx® system in 1981 and rapidly became the market leader in therapeutic drug monitoring. This batch analyser system, and its updated version the TDxFLx® (with random access capability), uses FPIA methodology for a wide range of analytes, including the aminoglycosides amikacin, gentamicin, netilmicin and tobramycin. The principle of FPIA is as follows. Sample (drug), tracer (fluorescein-labelled drug), and anti-drug antibody are incubated together in the reaction cell until competition for the limited number of antibody binding sites reaches equilibrium (3 min). Illumination of the reaction cell with vertically-polarised light causes the fluorescein label to emit light at longer wavelength that is detected through another vertical polarising filter. Free tracer molecules rotate rapidly in solution; their emitted light is orientated in different planes from the incident light and is not detected. Tracer that is bound to antibody has a much slower rotation; its emitted light is in almost the same plane as the incident light and is therefore detected. Increasing the concentration of drug in the sample limits the binding of tracer to the antibody and therefore causes a decrease in the measured fluorescence [85]. The Abbott TDx system has been widely used and evaluated for serum aminoglycoside analyses and found to be accurate, precise (RSDs$<$5%), rapid and simple to use. Roche Diagnostics (a division of Hoffmann-La Roche, Basel, Switzerland) also developed FPIA methodology for their Cobas Fara™ II centrifugal clinical chemistry analyser. Cobas-Fp™ assays had similar performance to Abbott TDx for the analysis of serum amikacin and gentamicin, but were even more rapid (1 min per test) and cost effective [86]. Sensitivity of the TDx FPIA for serum tobramycin (working range 0.1–10.0 μg/ml) could theoretically be increased 10-fold by using a reverse dilution technique. Normal operation of the TDx system causes sample to be diluted during the automated procedure, but if buffer is replaced by sample in the predilution well of the instrument, dilution of the sample is avoided. Using such a modification it was possible to quantify serum tobramycin down to 0.025 μg/ml with good precision and little interference, with the exception of lipaemic samples which gave falsely elevated results [87]. FPIA has been shown to be suitable for the analysis of other biological fluids. Performance of the netilmicin TDx assay was found to be similar for serum, peritoneal dialysate and urine samples and the results correlated well with an HPLC method [88]. In another study, FPIA using the TDx system was found to be suitable for the measurement of aminoglycosides in cerebrospinal fluid [89]. Using a modified calibration procedure with the TDxFLx system, another group were able to quantify very low concentrations (down to 0.02 μg/ml) of tobramycin in bronchoalveolar lavage fluid with good accuracy and precision [90]. FPIA has also been used to quantify gentamicin residues extracted from tissues of food-producing animals using a one-step alkali digestion procedure [91]. The method provided good recovery and reproducibility (RSDs$<$10%) and a sensitivity of 17 ng/g (ppb) for kidney tissue.

3.6. Direct chemiluminescence immunoassay

This is analogous to RIA, except the label is a chemiluminescent acridinium ester. Signal is generated by cleavage of the ester bond, releasing *N*-methylacridone which decomposes with the emission of an intense flash of light [92]. The ACS:180, launched in 1991 by Chiron Diagnostics (East Walpole, MA, USA and recently acquired by Bayer) was the first automated immunoassay system to use this methodology. Heterogeneous competitive assays are available for amikacin, gentamicin and tobramycin and are currently used by some clinical chemistry

laboratories for therapeutic drug monitoring. Sample (drug), drug–acridinium ester (tracer) and solid-phase antibody reagent are incubated together for about 7 min. Following the separation step, in which the antibody-coated ferric oxide microparticles are sedimented in a magnetic field, the amount of antibody-bound tracer is measured upon addition of an oxidising reagent by photomultiplier detection of the flash emission within 5 s. Sensitivity is at least as high as RIA and better than most other non-isotopic immunoassays [93].

3.7. Nephelometric and turbidimetric immunoassays

Homogeneous immunoassays for proteins and other macromolecules have been developed using the principle that large, multivalent antigens form insoluble complexes with antibodies that can be detected by their optical properties. In nephelometry, a beam of light is shone into the sample and the amount of light scattered by the immune complexes is measured at an angle from the beam. Turbidimetry is similar except that in this case the amount of light transmitted through the solution and not lost due to adsorption, scattering or reflection is measured. These techniques have been developed into competitive assays for small analytes by using tracer that consists of latex particles coated with analyte molecules. Formation of insoluble complexes between anti-analyte antibody and tracer is inhibited by the presence of univalent analyte in the sample [92].

Examples of automated immunoassays currently available for monitoring gentamicin and tobramycin using these methodologies are as follows. The Array® rate nephelometric assay (Beckman Coulter, Fullerton, CA, USA), the particle-enhanced turbidimetric inhibition immunoassay (PETINIA) for the Dimension® system (Dade Behring, Newark, DE, USA), and the latex agglutination (LA) assay for the Technicon Immuno 1™ system (Bayer). The Dimension assay for serum gentamicin was very precise, linear to 12 μg/ml, had a sensitivity of 0.3 μg/ml, and correlated well with FPIA [94]. The antibody reagent was derived from a monoclonal anti-gentamicin antibody that had minimal cross-reactivity with structurally related aminoglycosides. The Technicon latex agglutination assay was reported to have similar performance and gave comparable results to FPIA [82]. It has been suggested that the latex agglutination reaction does not require bridging of the particles by divalent antibody to form a classical immune complex. Colloidal stability of the analyte-modified latex particles may instead be decreased by specific binding of antibody causing agglutination without cross-linking [94].

3.8. Immunohistochemical techniques

In order to study the distribution of drugs in tissues, methods are required that can detect the analyte in situ. Such studies are particularly relevant for drugs that are known to concentrate in specific organs causing damage to particular cells, for example the ototoxic and nephrotoxic effects of aminoglycosides.

Gentamicin was localised in inner ear fluid compartments using an immunohistochemical method related to the heterogeneous non-competitive EIAs described above [95]. Cochlea tissue sections were placed onto nitrocellulose membranes and gentamicin was adsorbed to the surface of the membrane corresponding to its location in the tissue. Incubation of the membrane with anti-gentamicin antibody followed by a secondary peroxidase-labelled antibody and a chromogenic substrate allowed gentamicin to be detected at concentrations down to 10 μg/ml. Alternatively, use of a chemiluminescent substrate increased the sensitivity of detection 100-fold.

Tobramycin has been quantified with extremely high sensitivity in renal tubular epithelial cells of kidney following an immunostaining technique [96]. Tissue sections were incubated with anti-tobramycin antiserum followed by a secondary antibody labelled with colloidal gold. Detection of the gold label by photo-thermal microscopy allowed the quantification of tobramycin in individual cells down to 10 zmol (10^{-21} mol).

3.9. Spectrophotometric and other non-separative physicochemical methods

Simple, non-specific methods may be appropriate when there is a requirement for the assay of aminoglycosides in the absence of interfering compounds, for example the quality control of bulk and formulated pharmaceuticals, and dissolution studies.

Aminoglycosides absorb light poorly in the UV–Visible range and various indirect spectrophotometric methods have been developed using different derivatisation reagents giving limits of detection of around 1 μg/ml [97–103]. Streptomycin, however, have been determined directly with comparable sensitivity using derivative spectroscopy [104,105].

Aminoglycosides have been determined by stopped-flow fluorimetry using *o*-phthaldialdehyde as derivatisation reagent, with a sensitivity of 0.02 μg/ml [106]. The interaction of lanthanide ions (Eu^{3+}) with aminoglycosides has also been used as a basis for their fluorimetric determination at levels of 5–100 μg/ml [107].

Kanamycin was determined by its inhibitory effect on the chemiluminescent reaction between lucigenin and hydrogen peroxide in basic solution [108]. Precise determinations were possible over a very wide linear dynamic range (0.06 pg/ml–6 μg/ml) using a flow-injection system. Flow-injection chemiluminescence has also been used to measure streptomycin at levels of 2–30 μg/ml [109]. The method was based on the chemiluminogenic oxidation of streptomycin by *N*-bromosuccinimide in alkaline solution.

Titration with tetrabutylammonium hydroxide or barium acetate has also been reported for the assay of netilmicin in acetic acid solution [110,111].

3.10. Gas chromatography

Few GC methods have been developed for the analysis of aminoglycosides [15,61,112,113]. These compounds are non-volatile and require lengthy derivatisations at elevated temperatures with silylating agents to make them amenable to analysis. GC is the prescribed method of the US Pharmacopoeia for the assay of spectinomycin and a modification of this has been reported [114]. Spectinomycin was derivatised with hexamethyldisilazane then analysed by packed-column GC with flame ionisation detection. The method was linear over the concentration range 75–125% and the limit of quantification for the determination of impurities was 0.1%. Aminoglycosides and a range of other drug residues in meat were simultaneously determined by a packed-column GC method [115]. Meat was homogenised and deproteinised with trichloroacetic acid. The crude extract was purified sequentially on Amberlite XAD-2 and activated carbon columns, then derivatised with a 1:1:1 mixture of *N,O*-bis(trimethylsilyl)-acetamide (BSA), 1-(trimethylsilyl)-imidazole (TMSI), and trimethylchlorosilane (TMCS). Recoveries for aminoglycosides from meat ranged from 82 to 94% and the limits of detection were 25–50 μg/kg using a flame ionisation detector.

A chemometric experimental design has been used to optimise the derivatisation of gentamicin and kanamycin prior to analysis by GC–MS [112]. In this case the aminoglycosides were derivatised using a two-step procedure involving trimethylsilylation of the hydroxyl groups with TMSI and acylation of the amino groups with *N*-(heptafluorobutyryl)-imidazole (HFBI). Following liquid–liquid extraction the derivatives were analysed by capillary GC–MS using on-column injection and the detector in electron ionisation mode. Gentamicin was separated into three major components (C_1, C_{1a} and C_2). Specific fragment ions were selected for monitoring each analyte because the limited range of the MS system prevented detection of the molecular ions. This resulted in a substantial loss in sensitivity compared to electron-capture detection, although the actual sensitivities were not reported. The method showed good linearity between 30 and 200 μg gentamicin and 10 and 200 μg kanamycin.

3.11. Thin-layer chromatography

TLC, or planar chromatography, has been used as a qualitative method for the identification of aminoglycosides and for routine quality testing. Examples are the methods prescribed by the British and European Pharmacopoeias for the identification and quality control of kanamycin and neomycin [116,117].

Advances in TLC technology, including automation and the application of scanning densitometry, have occurred during the past decade which have increased the speed and reliability of this technique and extended its use to quantitative determinations. Many of these advances in TLC instrumentation have been introduced by Camag (Muttenz, Switzerland). High-performance thin-layer chromatography (HPTLC) is a term that has been introduced to describe the improved sensitivity, resolution and

accuracy that may be obtained using thinner layers (100–200 μm) of stationary phases with smaller (e.g. 5 μm), more uniform particle sizes comparable to those used in HPLC.

A mixture of nine aminoglycosides was almost fully separated by HPTLC [118]. Gentamicin was partially resolved into three peaks (C_{1a}, C_2 and C_1) and impurities in a netilmicin preparation were also detected by varying the composition of the solvent system. Detection limits ranged from 4 to 100 ng per spot using a ninhydrin reagent and scanning densitometry. Neomycin A, B and C were quantified in pharmaceuticals by HPTLC [119]. Fluorodensitometry of the fluorescamine derivatives gave a detection limit of 20 ng per zone. The relative amounts of neomycins B and C in commercial samples were determined in a similar study [120]. Detection was performed after derivatisation with 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl). Sensitivity for the assay of neomycin C content was about 1% (0.04 μg for an application of 4 μg sample to the plate). HPTLC was used to determine amikacin in parenteral dosage forms [121]. Quantification of the ninhydrin derivative at 498 nm was very precise (RSD<2%) and was linear over the range 0.5–3 ng/ml. In a comparative study of methods for the determination of gentamicin in pharmaceuticals, an automated HPTLC procedure using ninhydrin derivatisation was found to be faster (total analysis time of 45 min compared with 100 min) but 10 times less sensitive than HPLC [122].

TLC has been tried in the past as an alternative to microbiological assay for the determination of aminoglycosides in biological samples, but its sensitivity was comparatively poor [113]. Gentamicin has been recently quantified in plasma and urine by HPTLC [123]. Fluorodensitometry of the gentamicin NBD-Cl derivatives gave a linear quantification range of 40–200 ng per spot, which equated to 20–100 μg/ml in plasma and 10–50 μg/ml in urine. This is above the therapeutic range for gentamicin of 5–12 μg/ml [7] and the method would not be suitable for monitoring plasma levels without modification.

Sensitivity can be improved by pre-treatment of the sample prior to TLC analysis. Thus, solid-phase extraction of serum on C_{18} cartridges followed by HPTLC and fluorodensitometry allowed the detection of netilmicin at concentrations down to 0.2 μg/ml [124]. Post-chromatographic reaction with a mixture of diphenylborinic anhydride and salicylaldehyde produced a highly fluorescent netilmicin derivative. The assay had good precision (RSD<5%) but a limited linear range of 1–5 μg/ml. Comparison with FPIA revealed a reasonably good correlation, although the HPTLC method was less sensitive and subject to greater interference by other drugs and also heparin. In another study, hygromycin B was adsorbed and concentrated from deproteinised bovine plasma by solid-phase extraction on silica gel copolymerised with C_8 and benzene sulphonic acid functional groups prior to TLC [125]. Hygromycin B was quantified by fluorodensitometry following derivatisation with fluorescamine. The lowest amount of hygromycin B detectable was 5 ng per spot, giving a limit of detection in plasma of 25 ng/ml, which is similar to some immunoassays. This method was further developed for the analysis of hygromycin B in plasma, serum or milk [126]. The same copolymeric solid-phase extraction columns were used to extract the drug from the biological fluid prior to further purification by affinity chromatography on agarose–lysozyme columns. Lysozyme, which normally catalyses hydrolysis of (β1→4) glycosidic bonds, presumably binds aminoglycosides at the active site without hydrolysis taking place. Affinity chromatography proved useful in reducing the amount of fluorescent derivatives from plasma that interfered in the TLC analysis, but gave incomplete recovery of hygromycin B from milk.

3.12. High-performance liquid chromatography

HPLC, because of the specificity and sensitivity that may be achieved, is the analytical technique most used in pharmacokinetic [127] and drug stability studies. When coupled with mass spectrometry (MS), it becomes the most powerful method for the confirmation of antibiotic residues in food [113,128–131]. The literature contains a vast number of HPLC methods for the analysis of aminoglycosides and other antibiotics. Several conclusions may be drawn from this fact. HPLC methods are relatively easy to develop and are very suitable for the analysis of small-molecular-mass compounds. Some of the published methods, however, are not robust and are

therefore not transferable to different laboratories without modification. Continual development of HPLC as an analytical technique has resulted in a wide range of columns from different manufacturers exhibiting varying selectivities. Separations using different column types have been combined with various detection systems, resulting in the publication of a large number of methods. Advances in sample preparation procedures, particularly the introduction of disposable solid-phase extraction (SPE) columns, have also been reported and have greatly improved the extraction of antibiotics from biomatrices [132].

Analysis of aminoglycosides by HPLC has been dealt with in a number of reviews, which adequately cover the literature up to 1995 [15,61,113,133,134]. The present work will review those methods that have been developed and applications reported since then.

HPLC methods may be subdivided by the type of separation (normal-phase, reversed-phase, ion-pair, or ion-exchange) and by the mode of detection (ultraviolet (UV) absorption, fluorescence, electrochemical or MS detection).

3.12.1. Ion-exchange and ion-pair HPLC

Aminoglycosides are polybasic cations at low pH and have either been separated on strong cation-exchange (SCX) columns [135–137] or more commonly by ion-pair HPLC, typically on C_{18} reversed-phase columns using alkylsulphonate ion-pair reagents [45,64,138–148]. Details of these methods are given in Table 3. Ion-pair is generally preferred to ion-exchange HPLC because of the higher efficiencies obtainable, easier control over selectivity and resolution, and greater stability and reproducibility of reversed-phase columns [149]. Aminoglycosides lack chromophores and are therefore not amenable to direct UV or fluorescence detection. They may be analysed using electrochemical [150] or MS [128,129] detectors. A volatile mobile phase is required for MS detection, which may be achieved using ion-exchange with perfluorinated carboxylic acids and ammonium salts, or ion-pairing with a volatile reagent such as heptafluorobutyric acid [113,137,139]. Aminoglycosides may be derivatised after separation with UV absorbent or fluorescent agents, which allows analysis with more generally available spectrophotometric detectors. This approach has been widely adopted using *o*-phthaldialdehyde (OPA) [64,135,136,144–147,151], 1,2-naphthoquinone-4-sulphonic acid (NQS) [138,140,143, 148] and ninhydrin [142] as derivatisation reagents.

3.12.2. Reversed-phase HPLC

An alternative strategy is to use pre-column derivatisation, which has several advantages [152,153]. The derivatives formed are invariably less polar than the parent aminoglycosides and are amenable to separation by the preferred technique of reversed-phase HPLC [154–161] (see Table 3 for details). No extra pump, mixing apparatus and reaction coil are required for the derivatisation reagent. Sensitivity is higher because derivatisation is complete and the chromatographic peak is not diluted or chromatographically degraded prior to detection. It has also been suggested that the pre-column derivatisation technique is more likely to remove interfering substances [153]. Pre-column derivatisation with OPA plus 2-mercaptoethanol is not recommended because the derivatives formed are not stable. Stability may be improved by using 2-mercaptopropionic acid as the thiol [134]. More suitable reagents that have been used for pre-column derivatisation of aminoglycosides include 1-fluoro-2,4-dinitrobenzene (DNFB) [154,157] and 2,4,6-trinitrobenzene-1-sulphonic acid (TNBS) [155] for UV detection, and 9-fluorenylmethyl chloroformate (FMOC-Cl) [156,158,159] which is fluorescent.

3.12.3. Sample preparation for HPLC analysis

Biological samples contain many matrix components that may interfere with the HPLC analysis of aminoglycosides. The aim of sample preparation is to extract, and where necessary to concentrate, the analyte from the matrix, leaving behind proteins and other components that may interfere with subsequent derivatisation, separation, and detection steps. In order to minimise the potential for interference it is sensible to select different principles for the extraction and analytical separations, for example ion-exchange extraction followed by reversed-phase HPLC.

The use of SPE columns and cartridges has greatly increased the convenience and performance of sam-

Table 3
Recent HPLC methods for the analysis of aminoglycosides[a]

Type	Analyte	Detection, derivatisation	Column (5-μm unless stated)	Mobile phase	Sensitivity	Ref.
IE	Spectinomycin	Fl. 340/460 nm, Post-col. OPA–ME	Spherisorb SCX, 250×4.6 mm	Potassium phosphate (0.15 *M*), pH 3.5–acetonitrile (80:20)	70 ng/ml (tissue extract)	[135]
IE	Spectinomycin	Fl. 340/460 nm, Post-col. OPA–ME	Spherisorb SCX, 250×4.6 mm	Sodium sulphate (0.1 *M*) pH 2.6–acetonitrile (80:20)	100 ng/ml (plasma extract)	[136]
IE	Spectinomycin	Fl. 340/455 nm, Post-col. OPA–ME	IonoSpher 5C, 150×4.6 mm	Sodium sulphate (0.05–0.19 *M* gradient)–acetonitrile (80:20)	100 ng/ml (tissue extract)	[137]
IP	Neomycin	Pulsed electrochemical	8-μm PLRP-S 1000Å, 250×4.6 mm	1-Octanesulphonate (6.5 m*M*), sodium sulphate (0.49 *M*), potassium phosphate (10 m*M*, pH 3)	750 ng/ml (aqueous soln.)	[45]
IP	Gentamicin	Electrochemical- or thermospray-MS	Symmetry C_{18}, 150×3.9 mm	Trifluoroacetic acid (0.11 *M*, pH 3.6)–acetonitrile (97:3–80:20 gradient)	ND	[141]
IP	Spectinomycin	Electrospray-MS–MS	PLRP-S, 150×2.1 mm	Heptafluorobutyric acid (8 m*M*) in water–methanol (60:40)	125–250 ng/ml (milk extract)	[139]
IP	DHS	Fl. 270/420 nm, Post-col. NQS	Supelcosil ABZ$^+$Plus, 150×4.6 mm	1-Octanesulphonate (40 m*M*), NQS (0.4 m*M*), pH 3.2–acetonitrile (68:32)	7 ng/ml (milk extract)	[138]
IP	Streptomycin	Fl. 260/435 nm, Post-col. NQS	3-μm Hypersil BDS, 100×4 mm	1-Heptanesulphonate (10 m*M*), NQS (0.4 m*M*) in 20% acetonitrile, pH 3.3–acetonitrile (97:3)	50 ng/ml (milk extract)	[140]
IP	DHS	Fl. 375/420 nm, Post-col. NQS	Supelcosil ABZ$^+$Plus, 150×4.6 mm	1-Octanesulphonate (40 m*M*), NQS (0.4 m*M*), pH 3.2 –acetonitrile (68:32)	400 ng/ml (tissue extract)	[143]
IP	Streptomycin, DHS	Fl. 263/435 nm, Post-col. NQS	4-μm Superspher RP select B, 125×3 mm	1-Hexanesulphonate (10 m*M*), NQS (3.5 m*M*),triethylamine (0.15%), acetic acid (0.9%), pH 3.3–acetonitrile (88:12)	120 ng/ml (milk extract)	[148]
IP	DHS	Fl. 305/500 nm, Post-col. ninhydrin	Supelcosil LC-ABZ, 150×4.6 mm	1-Octanesulphonate (40 m*M*), 1,2-ethanedisulphonate (20 m*M*), ninhydrin (5 m*M*), pH 3.2–triethylamine (0.3%) in acetonitrile–methanol (63:19:18)	375 ng/ml (milk extract)	[142]
IP	Gentamicin	Fl. 340/430 nm, Post-col. OPA–ME	Spherisorb ODS2, 150×4.6 mm	1-Pentanesulphonate (11 m*M*), sodium sulphate (5.6 m*M*), acetic acid (0.1%) in water–methanol (82:18)	54 ng/ml (milk extract)	[144]
IP	Isepamicin	Fl. 338/450 nm, Post-col. OPA–ME	Hypersil ODS, 150×4.6 mm	1-Hexanesulphonate (10 m*M*), sodium acetate (0.1 *M*), acetic acid (17 m*M*) in water–methanol (86:14)	100 ng/ml (plasma)	[64]
IP	Paromomycin	Fl. 340/455 nm, Post-col. OPA–ME	Kromasil C_{18}, 250×4.6 mm	1-Pentanesulphonate (20 m*M*), sodium sulphate (0.2 *M*), pH 6.8–8.5 gradient	ND	[145]
IP	Various	Fl. 355/415 nm, Post-col. OPA–ME	TSK ODS 120T, 150×4.6 mm	1-Pentanesulphonate (10 m*M*), sodium sulphate (15 m*M*), acetic acid (10 m*M*)–tetrahydrofuran (97:3)	150–220 ng/ml (feed extract)	[146]
IP	Paromomycin	Fl. 340/440 nm, Post-col. OPA–ME	Inertsil C_8, 250×4.5 mm	1-Heptanesulphonate (1.2 m*M*), sodium sulphate (0.2 *M*), acetic acid (0.1%)	500 ng/ml (aqueous soln.)	[147]
RP	Spectinomycin	APCI-MS–MS	Zorbax SB-C_{18}, 150×2.1 mm	Acetic acid (1%)–methanol (92:8)	50 ng/ml (tissue extract)	[137]
RP	Paromomycin	UV 350 nm, Pre-col. DNFB	Zorbax SB-C_{18}, 250×4.6 mm	Water, pH 3–methanol (36:64)	150 ng/ml (plasma extract)	[154]
RP	Tobramycin	UV 365 nm, Pre-col. DNFB	4-μm Nova-Pak C_{18}, 150×3.9 mm	Tris (17 m*M*), sulphuric acid (20 m*M*)–acetonitrile (45:55)	3 μg/ml (aqueous soln.)	[157]
RP	Amikacin	UV 350 nm, Pre-col. TNBS	Zorbax SB-C_8, 150×4.6 mm	Potassium phosphate (20 m*M*)–acetonitrile–methanol (45:41:14), pH 7.7	ND	[155]

Table 3. Continued

Type	Analyte	Detection, derivatisation	Column (5-μm unless stated)	Mobile phase	Sensitivity	Ref.
RP	Neomycin	Fl. 260/315 nm, Pre-col. FMOC-Cl	Supelcosil LC-304 (C_4), 250×4.6 mm	Sodium phosphate (11 m*M*), pH 5–acetonitrile (37:63)	170 ng/ml (muscle extract)	[156]
RP	Various	Fl. 260/315 nm, Pre-col. FMOC-Cl	3-μm Hypersil ODS, 200×4.6 mm	Water–acetonitrile (10:90)	10 ng/ml (aqueous soln.)	[158,159]
RP	Netilmicin	Fl. 337/437 nm, Pre-col. OPA–ME	10-μm LiChrosorb RP18, 300×4 mm	Acetic acid (10%), 1-heptanesulphonate (10 m*M*)–acetonitrile (60:40–50:50 gradient)	7 ng/ml (dilute deprot. plasma)	[151]
RP	Apramycin	Fl. 230/389 nm, Pre-col. OPA–ME	Nova-Pak C_{18}, 300×3.9 mm	Ammonium acetate or 1-octanesulphonate (5 m*M*) in water–acetic acid–acetonitrile (60:2:40)	23 ng/ml (dilute tissue extract)	[160]

[a] Abbreviations: IE, ion-exchange; IP, ion-pair; RP, reversed-phase; DHS, dihydrostreptomycin; Post-col., post-column; Pre-col., pre-column; APCI, atmospheric pressure chemical ionisation; OPA–ME, *o*-phthaldialdehyde–2-mercaptoethanol; NQS, 1,2-naphthoquinone-4-sulphonate; DNFB, 1-fluoro-2,4-dinitrobenzene; TNBS, 2,4,6-trinitrobenzene-1-sulphonic acid; FMOC-Cl, 9-fluorenylmethyl chloroformate; Tris, Tris(hydroxymethyl)aminomethane; ND, not determined; deprot., deproteinised.

ple preparation for HPLC analysis. Aminoglycosides are retained most successfully on weak cation-exchangers, or by ion-pairing on reversed-phase media [113,130–132]. Table 4 gives details of some recent pre-treatment procedures that have been used for aminoglycosides in plasma and various food products.

A column-switching arrangement was developed that allowed the extraction step to be performed automatically on-line [64]. Isepamicin was extracted from deproteinised plasma on a guard column containing normal-phase (cyano-bonded) silica then subjected to ion-pair chromatography on a reversed-phase (C_{18}) analytical column. A minimum of 60 plasma samples could be processed in this way before replacement of the guard column packing was required.

Future developments in the HPLC analysis of aminoglycosides may include greater use of restricted access, or shielded, stationary phases for the direct injection of serum and other biological samples. This column technology has been developed for the analysis of drugs in serum eliminating the need for laborious deproteinisation steps. A shielded hydrophobic phase column was used in the analysis of neomycin in milk [162]. The sample, however, had first been extracted on a weak cation-exchange column, which would be expected to remove proteins and fats. It is therefore not clear what advantage was gained by using a restricted access medium for the analytical separation in this case.

3.13. Capillary electrophoresis

Capillary electrophoresis (CE) is a general term covering the various electrophoretically driven capillary separation techniques of capillary zone electrophoresis (CZE), capillary isotachophoresis (CITP), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography (MECC) and capillary electrochromatography (CEC). CZE and MECC in particular are well established techniques that have been applied to the analysis of a wide range of biological substances with the advantages of very high resolution and minute sample volume requirement. Little has been published on the analysis of aminoglycosides using CE however, the main reason for this being the difficulty in detecting these compounds by conventional spectrophotometric means [163]. The problems of aminoglycoside detection discussed earlier (Section 3.12.1) are even more severe for CE because the very short path length used in CE spectrophotometric detectors decreases the concentration sensitivity that may be achieved.

Sensitivity is not a problem for the analysis of bulk pharmaceuticals and their formulations where the concentrations of aminoglycosides are in the 1–250 mg/ml range. Aminoglycosides were first determined by CZE using indirect UV detection with imidazole as background electrolyte [164]. Thirteen different aminoglycosides were analysed in the anionic mode (anode at the detection side) with

Table 4
Sample preparation for HPLC analysis of aminoglycosides[a]

Matrix	Analyte	Pre-treatment	Solid-phase extraction	Recovery (%)	Ref.
Plasma	Spectinomycin	Dilute, pH→5.5, centrifuge	Ion-pair (dioctyl sulphosuccinate) on high hydrophobic C_{18} (3 ml; J.T.Baker)	91–104	[136]
Plasma	Paromomycin	Deprot. (PCA), centrifuge, pH→7.8, deriv. (DNFB), LLE (toluene/acetonitrile)	None	87	[154]
Plasma	Netilmicin	Deprot. (TCA), centrifuge, pH→11, LLE (dichloromethane), deriv. (OPA), LLE (2-propanol)	None	ND	[151]
Plasma	Various	None	Ion-exchange on CBA (Isolute, 3 ml, 100 mg; International Sorbent Technology)	94–107	[158,159]
Tissue	DHS	Homogenise, deprot. (PCA), centrifuge, LLE (dichloromethane)	Ion-exchange on SCX (3 ml, 500 mg; Varian)	67–83	[138]
Tissue	Spectinomycin	Homogenise, deprot. (TCA), centrifuge, filter, pH→6.7	Ion-exchange on CBA (3 ml, 500 mg; J.T.Baker)	74–97	[135]
Tissue	Spectinomycin	Homogenise, deprot. (TCA), LLE (dichloromethane), centrifuge, pH→6.7, centrifuge	Ion-exchange on CBA (no details available)	81–94	[137]
Tissue	Streptomycin	Homogenise, deprot. (PCA), centrifuge, filter	(1) Ion-exchange on SCX (200 mg; Appl. Sep.) (2) Ion-pair (1-heptanesulphonate) on C_{18} (500 mg; J.T.Baker)	81	[140]
Tissue	DHS	Homogenise, deprot. (TCA), centrifuge, LLE (dichloromethane), homogenise, centrifuge, pH→5.5	Ion-pair (1-heptanesulphonate) on mixed-mode SAX/C_8 (Certify II, 6 ml, 500 mg; Varian)	73–83	[143]
Tissue	Neomycin	Homogenise, alkali digest, deprot. (PCA), pH→5	Ion-exchange on CBA (Accell™ Plus, 6 ml, 1 g,; Waters)	78–120	[156]
Tissue	Apramycin	Homogenise, LE (alkali/methanol), centrifuge, ion-pair LLE (ethyl acetate), LLE (dil. HCl), pH↑, LLE (toluene)	None	76–86	[160]
Tissue	Gentamicin	Homogenise, centrifuge, filter	Ion-exchange on mixed-mode CBA/C_{18} (300 mg; Chemical Separations)	>90	[161]
Milk	DHS	Deprot. (TCA), filter, pH→3.2, ultrafilter	None	90–97	[138]
Milk	Spectinomycin	Deprot. (TCA), centrifuge, dilute	Ion-pair (heptafluorobutyric acid) on trifunctional C_{18} (Sep-Pak Vac, 3 ml; Waters)	69–93	[139]
Milk	Streptomycin	None	(1) Ion-exchange on SCX (200 mg; Appl. Sep.) (2) Ion-pair (1-heptanesulphonate) on C_{18} (500 mg; J.T.Baker)	88	[140]
Milk	DHS	Deprot. (TCA), centrifuge, LLE (dichloromethane), neutralise, centrifuge, pH→6	Ion-pair (1-heptanesulphonate) on trifunctional C_{18} (Sep-Pak Vac, 6 ml, 1 g; Waters)	83	[142]
Milk	Gentamicin	Defat (centrifuge), deprot. (TCA), centrifuge	Reversed-phase on C_{18} (6 ml, 500 mg; Supelco)	72–88	[144]
Milk	DHS, Streptomycin	Defat (oxalic acid), centrifuge, deprot. (TCA), centrifuge, pH↑, centrifuge	Ion-pair (1-heptanesulphonate) on C_{18} (Chromabond ec, 500 mg; Macherey-Nagel)	78–107	[148]
Milk	Gentamicin	None	Ion-exchange on mixed-mode CBA/C_{18} (300 mg; Chemical Separations)	>90	[161]

[a] Abbreviations: DHS, dihydrostreptomycin; deprot., deproteinise; LLE, liquid–liquid extraction; LE, liquid extraction; OPA, *o*-phthaldialdehyde; PCA, perchloric acid; TCA, trichloroacetic acid; CBA, carboxylic acid-bonded weak cation-exchanger; SCX, strong cation-exchanger; ND, not determined.

electroosmotic flow reversed by the addition of the surfactant FC 135. Although the analyte peaks were very sharp, not all the components could be separated under optimal conditions (pH 5). Addition of cetyltrimethylammonium bromide (CTAB) to the electrolyte allowed the simultaneous determination

of neomycin and hydrocortisone in eardrops by combined CZE–MECC. Limits of detection were less than 50 μg/ml for aminoglycosides.

Borate complexation was used for the direct UV detection of aminoglycosides in a CZE method [165]. Twelve different aminoglycosides were almost completely separated by CZE in the cationic mode (cathode at the detection side) at pH 9. The method showed excellent precision (RSD=3%) using internal standardisation and the limit of quantification was again in the region of 50 μg/ml.

Derivatisation of aminoglycosides to allow more sensitive direct UV or fluorescence detection has also been reported in a MECC method [166]. Amikacin in plasma ultrafiltrate was derivatised with 1-methoxy-carbonylindolizine-3,5-dicarbaldehyde. The derivative was separated by MECC at pH 7 using sodiumdodecyl sulphate (SDS) as micellar reagent and detected by fluorescence. Sensitivity of this method was about 100-fold greater than for the non-derivatisation methods above, but the ability to separate different aminoglycosides appeared to be lost. Thus, amikacin, arbekacin, dibekacin and kanamycin derivatives had almost identical migration times by MECC, whereas the parent aminoglycosides were well separated by the CZE methods described above.

Electrochemical detection has been used with some success in the analysis of aminoglycosides by HPLC (see Section 3.12.1) and its suitability for use in CE has also been demonstrated. Seven aminoglycosides were separated by CZE in the cationic mode at high pH (100 m*M* NaOH as electrolyte) and detected down to 0.2–2 μg/ml using a nickel electrode in wall-jet configuration [167]. Similar CZE conditions were employed for the separation of a different mixture of seven aminoglycosides, but in this case detection was achieved below 5 μg/ml with greater stability, reproducibility and simplicity using a copper on-capillary electrode [168].

4. Conclusions

Despite the introduction of newer, less toxic antimicrobials, aminoglycosides continue to serve a useful role as therapeutic agents. The ongoing need for routine therapeutic drug monitoring is adequately met by automated immunoassays and it is difficult to foresee major changes in this area. Further development and commercialisation of simple visual immunoassay tests is desirable to facilitate the screening of aminoglycoside residues in milk and animal tissues on the farm and at slaughter. Microbiological assays however, because of their non-specificity and inaccuracy, cannot be recommended for analytical use when alternative methods of far superior performance are available. There is potential for further development of chromatographic and CE methods for the analysis of biological samples. In particular, greater use of restricted access columns for HPLC that allow direct injection of serum and other biological samples may be anticipated. As MS detection becomes more widely available and its quantitative performance improves, this will probably replace spectrophotometric and electrochemical detection in many applications. Perhaps the greatest scope for development in this direction will be in CE–MS.

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