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Sensitive high-performance liquid chromatographic assay for aminoglycosides in biological matrices enables the direct estimation of bacterial drug uptake

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Abstract

Following the development of a sensitive high-performance liquid chromatographic (HPLC) assay for gentamicin in biological matrices, the utility of this assay for the determination of other clinically important aminoglycosides (neomycin, netilmicin and sisomicin) in bacterial culture media or plasma is demonstrated. The high sensitivity of the assay enables direct measurement of the aminoglycoside content of bacterial cells cultured in the presence of unlabelled drug.

Keywords: Aminoglycosides; Neomycin; Netilmicin; Sisomicin

1. Introduction

The aminoglycosides are a class of broad spectrum antibacterial drugs widely used in the treatment of serious infections. Because the margin between their therapeutic and toxic dose is small, monitoring of plasma concentrations is indicated, especially in infants or patients with renal impairment. Gentamicin and netilmicin are important members of this group of therapeutic agents. Neomycin, although considered too toxic for systemic use in humans, is used in topical preparations and in agriculture. Because of the latter, it is necessary to have sensitive methods that are capable of detecting aminoglycoside residues in fluids and tissues of farm animals [1,2].

We recently developed a method for the fluorimetric high-performance liquid chromatographic (HPLC) assay of gentamicin [3] that employed solid-phase extraction on carboxypropyl (CBA)-bonded silica, pre-column derivatisation with 9-fluorenylmethyl chloroformate (FMOC-Cl) and reversed-phase HPLC. FMOC-Cl reacts with both primary and secondary amines (Fig. 1) and is therefore expected to label all the amino groups of the aminoglycosides. It was shown that FMOC-Cl provided greater reagent stability and detection sensitivity than other methods using alternative derivatising agents [3], most commonly *o*-phthaldialdehyde (OPA). A minor drawback to the use of FMOC-Cl is that, unlike OPA, the reagent itself is fluorescent.

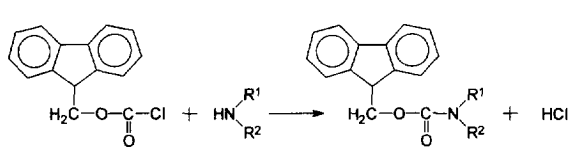

Fig. 1. Reaction of amines with FMOC-Cl.

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However, excess reagent is easily separated from the aminoglycoside derivatives by reversed-phase HPLC. Most of the literature concerning HPLC analysis of aminoglycosides in biological matrices is limited to gentamicin [2–4]. Published assays for neomycin, netilmicin and sisomicin [5–9] employ pre- or post-column derivatisation with OPA and suffer from the same disadvantages of this reagent, including poor stability, the unpleasant and toxic nature of the thiol co-reagent (usually 2-mercaptoethanol) and the varying fluorescent properties of its aminoglycoside derivatives [4]. The fluorimetric HPLC assay of gentamicin [3] is now extended to the determination of neomycin, netilmicin and sisomicin in biological fluids. Such a procedure is likely to be useful for the applications described above, namely therapeutic drug monitoring and drug residue analysis in food-producing animals.

In studies of the antibacterial action of aminoglycosides, it is important to quantify the amount of bacterial drug uptake. This has traditionally been done using radiolabelled aminoglycosides [10,11], but such an approach is expensive and relies on the particular compound under study being available in labelled form. Previous attempts at using non-isotopic methods to measure the aminoglycoside content of bacterial cells [12] employed an indirect microbiological assay and were of limited success. This paper demonstrates for the first time the use of a highly sensitive HPLC assay for the direct determination of aminoglycoside (netilmicin) in cells of the bacterium *Enterococcus faecalis*.

2. Experimental

2.1. Chemicals and reagents

Stock solutions (10 mg/ml) of neomycin sulphate (Sigma N-1876; minimum 85% neomycin B, remainder neomycin C) and sisomicin sulphate (Sigma S-8757) were prepared in water. Netilmicin sulphate was obtained as Netillin injection (Schering-Plough, Mildenhall, UK) and contained the equivalent of 10 mg of netilmicin base per millilitre of aqueous solution. 9-Fluorenylmethyl chloroformate (FMOC-Cl) was supplied by Sigma. Acetonitrile and methanol were obtained from Rathburn (Walkerburn, UK). Water was glass-distilled and further purified for HPLC with a Milli-Q system (Millipore, Watford, UK). Iso-sensitest broth was purchased from Oxoid (Basingstoke, UK) and was supplemented with 0.25% (w/v) D-glucose monohydrate. Solid-phase extraction (SPE) columns (3 ml) that were packed with 100 mg of Isolute carboxypropyl (CBA)-bonded silica (Jones Chromatography, Hengoed, UK) were supplied by Crawford Scientific (Strathaven, UK). Phosphate buffer (pH 7.4) was prepared by mixing appropriate volumes of 20 m*M* sodium dihydrogen phosphate and sodium hydrogen phosphate solutions. Borate buffers were prepared by adjusting the pH of boric acid solutions with potassium hydroxide (45%, w/v).

2.2. High-performance liquid chromatography

The HPLC system comprised a Waters Model 510 pump, a Rheodyne Model 7125 valve fitted with a 20-μl loop, a stainless steel column (200×4.6 mm ID) that was slurry packed with 3 μm ODS Hypersil (Shandon HPLC, Runcorn, UK). Detection was by a Varian Model 2070 spectrofluorimeter (excitation 260 nm, emission 315 nm) connected to a Goertz Metrawatt Model SE120 chart recorder for manual peak-height measurement.

The mobile phase was acetonitrile–water (90:10, v/v), degassed prior to use by vacuum filtration through a 0.2-μm filter. Aminoglycoside–FMOC derivatives were separated at room temperature (20–25°C) using a flow-rate of 1.0 ml/min.

2.3. HPLC assay of neomycin, netilmicin and sisomicin

Mixtures of neomycin, netilmicin and sisomicin were prepared in 0.37 *M* borate buffer (pH 8.5). In experiments performed during the optimisation of derivatisation conditions, 0.5 ml of the drug mixture (each component at 5 μg/ml) was added to 0.5 ml of FMOC-Cl (2 m*M* in acetonitrile) in a 2-ml glass vial and reacted in the dark at room temperature for 15 min. The reaction was stopped by adding 50 μl of glycine (0.1 *M*), then 100 μl of the reaction mixture was used to load the loop (20 μl injected). Linearity of the assay was assessed for each aminoglycoside by triplicate analyses of mixtures of neomycin,

netilmicin and sisomicin at 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 μg/ml each in borate buffer, as above.

2.4. Determination of neomycin, netilmicin and sisomicin in Iso-sensitest broth or plasma

Mixtures of neomycin, netilmicin and sisomicin (1 μg/ml each) were prepared in Iso-sensitest broth and pooled human plasma by dilution of the stock solutions.

2.4.1. Solid-phase extraction

A CBA-bonded SPE column was conditioned with 1 ml of methanol followed by 1 ml of phosphate buffer. Iso-sensitest broth or pooled human plasma (1 ml) containing the analytes was applied to the column, which was then washed with 2 ml of phosphate buffer followed by 4 ml of borate buffer (0.2 *M*, pH 9). The column was dried by application of a volume of air (30 ml) and then was eluted into a 2-ml glass vial with 1 ml of acetonitrile–borate buffer (0.2 *M*, pH 10.5) (1:1, v/v) followed by a further volume of air.

2.4.2. Derivatisation

Eluate (1 ml) was adjusted to pH 8.9 by the addition of 0.2 ml of boric acid (0.8 *M*) and derivatised with 0.2 ml of FMOC-Cl (7 m*M* in acetonitrile) at ambient temperature for 15 min. The reaction was stopped by adding 50 μl of glycine (0.1 *M*) and 20 μl of the reaction mixture was injected by total loop filling as before.

2.5. Netilmicin uptake study

2.5.1. Cell culture

Enterococcus faecalis NCTC 5957 was obtained from the National Collection of Type Cultures (Public Health Laboratory Service, London, UK). The minimal inhibitory concentration (MIC) of netilmicin with this strain was 60 μg/ml using a standard broth dilution method with an inoculum of 10^5 cfu/ml [13].

For the uptake studies, 4 ml of an 18 h broth culture of *E. faecalis* were inoculated into 96 ml of Iso-sensitest broth at 37°C in a 250-ml conical flask and incubated in a shaking water bath at 37°C for 4 h. Volumes (10 ml) of this log-phase culture were then inoculated into 250 ml conical flasks containing 90 ml of Iso-sensitest broth plus netilmicin (15 μg/ml final concentration) and incubated as before. At intervals (0, 1, 2, 4 and 24 h), portions (10 ml) of the cultures were removed into centrifuge tubes on ice and centrifuged (10 000 *g*, 5 min, 4°C) to pellet the cells. Cells were washed twice by vortex-mixing in 10 ml of water and recentrifuging as above. The washed pellets were resuspended in 2 ml of water in pre-weighed glass vials; 0.5-ml volumes were transferred into test tubes containing 0.5 ml of 2 *M* NaOH plus 50 μl of internal standard (20 μg/ml sisomicin) for alkali digestion and the remainder was dried to a constant weight in a 60°C oven for the determination of dry cell weight. Cells were digested by placing the stoppered test tubes in a water bath at 100°C for 15 min, then were neutralised by the addition of 1 ml of 1 *M* HCl and diluted to 10 ml with phosphate buffer. Both netilmicin and sisomicin were stable to alkali digestion under these conditions (data not shown).

2.5.2. Solid-phase extraction and derivatisation

Diluted cell digest (5 ml) was applied to a conditioned CBA-bonded SPE column, which was then washed with 1 ml of phosphate buffer followed by 2 ml of borate buffer (0.2 *M*, pH 9). The procedures for column drying, elution and derivatisation were as before (see Section 2.4).

3. Results and discussion

3.1. Optimisation of neomycin, netilmicin and sisomicin HPLC assay

Neomycin, netilmicin and sisomicin (5 μg/ml each) were reacted with FMOC-Cl as described above, but the reaction time, pH, concentrations of acetonitrile and FMOC-Cl were independently varied in turn. The effects of these parameters on the peak heights of the three analytes are shown in Fig. 2.

Optimal reaction of neomycin, netilmicin and sisomicin with FMOC-Cl occurred under conditions similar to those reported previously for gentamicin [3] and which were discussed in that communication. Derivatisation of neomycin showed greater dependence on the reaction conditions than was the case for netilmicin and sisomicin. This is reflected in the

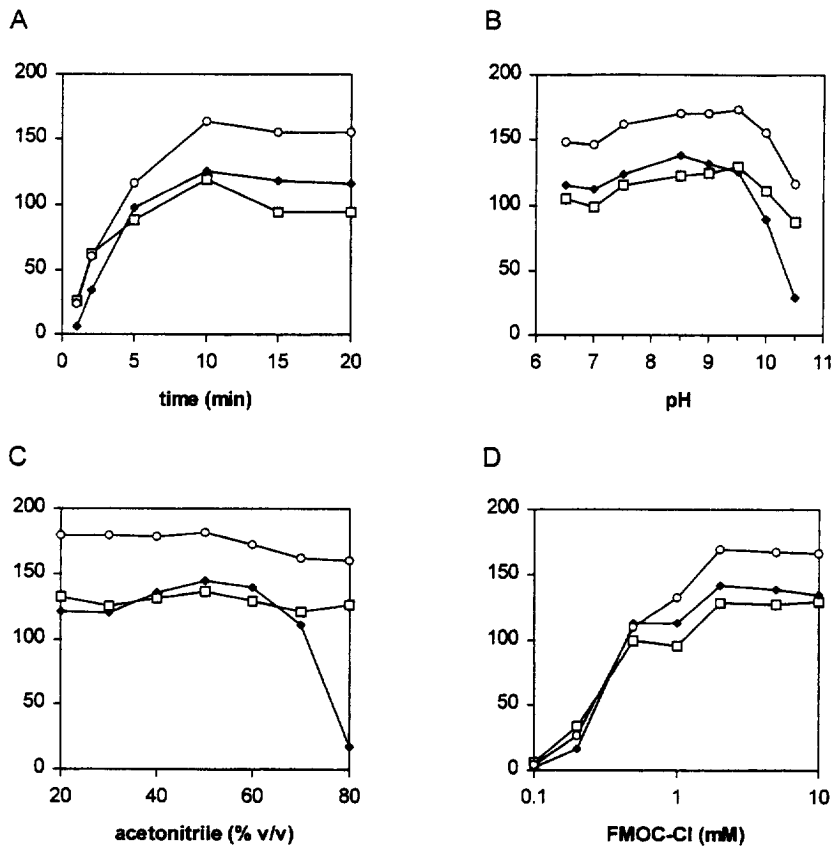


Fig. 2. Effect of (A) reaction time, (B) pH, (C) acetonitrile concentration and (D) FMOC-Cl concentration on the derivatisation of aminoglycosides with FMOC-Cl. Conditions as in Section 2.3. Results are plotted as peak heights (mm) with detector settings; gain×1, attenuation, 256. Legend: ○=Neomycin, ♦= netilmicin and □=sisomicin.

structural differences between these aminoglycosides (Fig. 3).

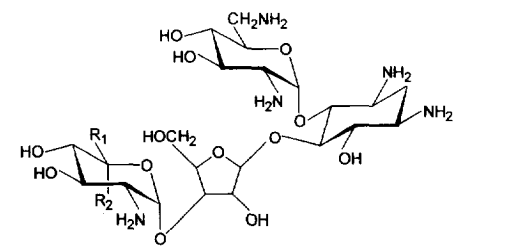
3.2. Linearity and sensitivity

The calibration lines for neomycin, netilmicin and sisomicin at 0.1–10 μg/ml are described in Table 1. The assay showed good linearity over the whole of this range. The limit of quantification for each drug was estimated by analysing mixtures of the aminoglycosides at progressively lower concentrations. Accurate measurement of peak height at very low drug concentrations was limited not by the signal-to-noise ratio but by the steeply sloping baseline, and the practical quantification limit was approximately 10 ng/ml.

3.3. Determination of neomycin, netilmicin and sisomicin in Iso-sensitest broth or plasma

3.3.1. Recovery

The recoveries of neomycin, netilmicin and sisomicin (1 μg/ml each) extracted from spiked Iso-sensitest broth or plasma, calculated by comparison with a solution of the drugs in eluent (1 μg/ml each) that had been derivatised directly, are shown in Table 2. All three aminoglycosides were extracted with high efficiency (92–107%) from the biological matrices by the CBA-bonded silica. A representative chromatogram is shown in Fig. 4. Peak identification was by comparison of the retention times with those of standards that had been derivatised and chromatographed individually.



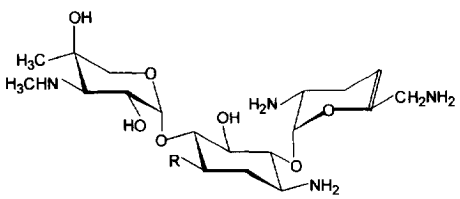


Fig. 3. Structures of the aminoglycosides.

3.3.2. Reproducibility

Reproducibility of the assay was assessed: (i) Intra-day by five consecutive analyses of mixtures of neomycin, netilmicin and sisomicin (1 μg/ml each) in Iso-sensitest broth and plasma and (ii) inter-day by analyses of the same mixtures on five separate occasions. The results are shown in Table 3.

The reproducibility was generally very good, but it was clear that by using one aminoglycoside as an internal standard in the analysis of another, the imprecision could be decreased substantially. For example, in the assay of netilmicin from Iso-sensitest broth, inter-assay variation was reduced from 14.1 to 2.3% when sisomicin was used as the internal standard. However, finding a suitable internal standard for the analysis of aminoglycoside mixtures can

Table 2
Recovery of aminoglycosides extracted from biological matrices

Aminoglycoside	Recovery (%)		n
	Iso-sensitest broth	Plasma	
Neomycin (1 μg/ml)	103.2 (6.5)[a]	106.7 (6.4)[a]	3
Netilmicin (1 μg/ml)	92.0 (3.2)	95.2 (3.4)	3
Sisomicin (1 μg/ml)	92.5 (4.9)	99.1 (4.0)	3

[a] Mean (standard deviation).

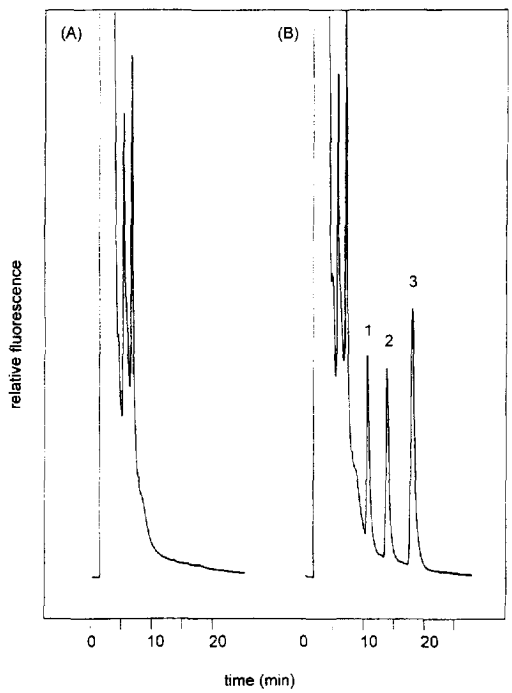


Fig. 4. HPLC of the aminoglycosides in Iso-sensitest broth. Iso-sensitest broth, (A) blank or (B) spiked with 1 μg/ml each of neomycin, netilmicin and sisomicin, was extracted and analysed as described in Section 2.4. Detector settings; gain×1, attenuation, 256. Peak identification: 1=Neomycin, 2=sisomicin and 3=netilmicin.

Table 1
Calibration lines for the HPLC assay of neomycin, netilmicin and sisomicin

Aminoglycoside	Linear regression equation ($y=a+bx$)			n
	Slope (b)	Intercept (a)	Correlation (r^2)	
Neomycin	191.8 (10.7)[a]	−0.3 (14.6)[a]	0.9986	3
Netilmicin	229.5 (14.4)	7.7 (21.6)	0.9987	3
Sisomicin	167.1 (15.5)	13.0 (20.4)	0.9965	3

Results are expressed as peak heights (mm) with detector settings; gain×1, attenuation, 32.
[a] Mean (standard deviation).

Table 3
Reproducibility of the aminoglycoside HPLC assay

Aminoglycoside	Imprecision (coefficient of variation, %)				*n*
	Iso-sensitest broth		Plasma		
	Intra-day	Inter-day	Intra-day	Inter-day	
Neomycin (1 μg/ml)	5.7	15.8	4.5	3.5	5
Netilmicin (1 μg/ml)	2.9	14.1	3.5	2.4	5
Sisomicin (1 μg/ml)	3.1	15.9	3.5	2.5	5

be difficult. Gentamicin, for example, is a complex mixture with four major components (C_1, C_{1a}, C_2 and C_{2a}) detected by reversed-phase HPLC [3]. We have found that, under the assay conditions described above, gentamicin C_1 co-elutes with netilmicin, and gentamicin C_{1a} co-elutes with sisomicin. Neomycin was separated from all the gentamicin components, and may therefore be a suitable internal standard for use in the gentamicin assay. Simultaneous analysis of neomycin, netilmicin and sisomicin could be standardised using gentamicin C_2 or C_{2a}, but the pure components of gentamicin are not commercially available.

3.3.3. Netilmicin uptake by enterococci

The amount of netilmicin taken up by cells of *E. faecalis* incubated with the drug at the sub-inhibitory concentration of 15 μg/ml (1/4×MIC) is shown in Fig. 5. Rapid initial uptake, which occurred within the time taken to process the zero time sample, was followed by a more gradual increase in the amount of netilmicin associated with the cell mass, and reached a steady state by 4 h.

Although the amount of netilmicin taken up by the cells was small (at 4 h, this would be sufficient only to cause a 5% fall in the netilmicin concentration of the culture medium), the high sensitivity of the assay allowed duplicate determinations of uptake to be made using only 2.5 ml of bacterial culture. The limiting factor in these estimations is the measurement of dry cell mass.

Most of the netilmicin taken up could be removed by washing the cell pellets with 1 *M* NaCl instead of water (results not shown). This indicated that most of the drug was bound ionically to the bacterial cell surface and had not been internalised. Similar findings have been reported for enterococci incubated with labelled streptomycin at 200 μg/ml (1/2× MIC); in this case, the bound streptomycin could be displaced by washing with unlabelled drug [11].

4. Conclusions

It has been shown that a sensitive HPLC assay originally developed for gentamicin can be applied successfully to the analysis of the other aminoglycosides; neomycin, netilmicin and sisomicin, in biological matrices. The performance of the assay was further improved by using one aminoglycoside (e.g. sisomicin) as an internal standard in the determination of another (e.g. netilmicin). Because of its high sensitivity, the method may find other useful applications, for example, the quantification of aminoglycoside residues in foodstuffs. It has been

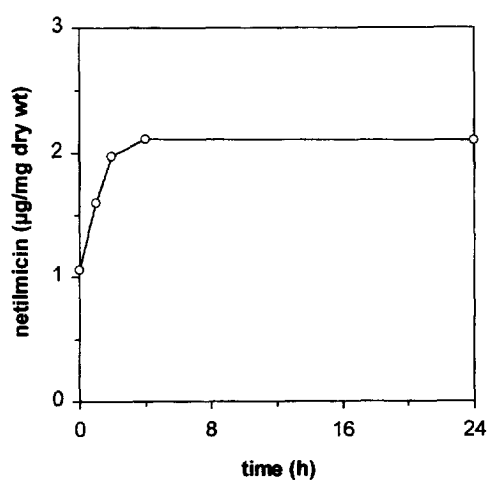


Fig. 5. Uptake of netilmicin by enterococci. A log-phase culture of *E. faecalis* was incubated in Iso-sensitest broth containing 15 μg/ml of netilmicin, as described in Section 2.5.

demonstrated for the first time that direct estimation of aminoglycoside uptake by bacterial cells is possible without the need for radiolabelled drug.

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