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Hydrophilic interaction chromatography combined with tandem-mass spectrometry to determine six aminoglycosides in serum

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Abstract

A specific and automated method was developed to quantitate the aminoglycosides amikacin, gentamicin, kanamycin, neomycin, paromomycin, and tobramycin simultaneously in human serum. Samples were prepared with an automated solid phase extraction (SPE). The hydrophilic interaction chromatography (HILIC) was used for separation of analytes from endogenous compounds and baseline separation. The aminoglycosides were detected with electrospray ionisation tandem mass spectrometry (ESI-MS–MS). Using a volume of 500 μl biological sample the lower limits of quantification were 100 ng/ml or better. The described HILIC–MS–MS method is suitable for therapeutic drug monitoring and for clinical and pharmcokinetical investigations of the aminoglycosides.

Keywords: Hydrophilic interaction; Aminoglycosides; Antibiotics

1. Introduction

Aminoglycosides belong to broad-spectrum antibiotics which characteristically contain two or more aminosugars linked by glycosidic bonds to an aminocyclitol component. The most important subclasses of aminoglycosides are the 4,5-disubstituted deoxystreptamines, which include neomycin and paromomycin, and the 4,6-disubstituted deoxystreptamines, which include amikacin, gentamicin, kanamycin, and tobramycin. Aminoglycosides are widely used in clinical therapy of serious infections with a good clinical effectiveness, a low rate of true resistance and low cost. They inhibit the growth of some gram-positive and many gram-negative bacteria [1]. Aminoglycosides have a narrow therapeutic range. To achieve a maximum effect against bacterial infections serum levels of the aminoglycoside have to be controlled. A too high plasma level may cause ototoxicity and nephrotoxicity [2–7]. To exclude these adverse effects it is important to control the serum concentration of the drugs. The therapeutic range of amikacin is 5–25 μg/ml and the serum concentrations of the aminoglycosides gentamicin, kanamycin, neomycin, paromomycin, and tobramycin should be between 2 and 10 μg/ml. Therapeutic drug monitoring has been used extensively to assist dosing and target concentrations have been advocated [8]. Besides aminoglycosides are frequently used in treatment of animal diseases. Therefore, a residue control in edible animal tissues is necessary.

Automated immunoassays are the most appropriate methods for serum aminoglycoside determinations during therapeutic drug monitoring. But immunoassays are not available for all aminoglycosides and therefore several chromatographic methods are used. Simple chromatographic methods are not applicable due to the lack of volatility, chromophore, and strong hydrophility of aminoglycosides. Therefore, stationary reversed phases can only be used for HPLC-separation in combination with ion-pair chromatography [3,4,9,10]. Furthermore fluorescence detection after postcolumn derivatisation or electrochemical detection

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were described for neomycin and gentamicin [11–14]. However, these chromatographic methods lack specificity, sensitivity or robustness and require elaborate sample preparation.

Ion pair chromatography is not ideal for electrospray mass spectrometric detection because the sensitivity of mass spectrometry is reduced. A LC–MS method for the determination of kanamycin using phenylisocyanate as a derivatisation reagent was described [15]. The determinations of aminoglycosides in animal tissues, of gentamicin in wastewater and of tobramycin in serum by ion pair chromatography with volatile reagents and MS detection were reported in literature [16–20]. Normal-phase liquid chromatography (NPLC) ought to be suitable for the separation of strong hydrophilic compounds. However, the typical non aqueous eluents used for NPLC are not well compatible with the electrospray process [21]. Hydrophilic interaction chromatography (HILIC) is an alternative to NPLC. Similar to NPLC the elution is promoted by the use of polar mobile phases, but HILIC is unique because the presence of water in the mobile phase is crucial for the establishment of a stagnant enriched aqueous layer on the surface of the stationary phase into which analytes may selectively partition [22–24]. The used stationary HILIC phase is a zwitterionic silica gel. Therefore, the HILIC technique is suitable for the analysis of polar compounds (e.g. folates [25], carbohydrates [23], peptides [24] or natural products [26]) with MS–MS detection.

In a previous study the authors determined neomycin in serum of patients after systemic uptake [27]. This paper describes a similar application of the HILIC technique to determine additionally and simultaneously the aminoglycosides amikacin, gentamicin, kanamycin, paromomycin, and tobramycin in human serum in and below the therapeutical range. Using a sample volume of 500 μl a good response over the range of 100–5000 ng/ml serum was demonstrated. Samples with higher analyte concentration can be diluted.

2. Experimental

2.1. Chemicals

Amikacin sulphate, gentamicin sulphate, kanamycin sulphate, paromomycin sulphate, and tobramycin sulphate were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Neomycin sulphate was provided by Schur Pharma (Düsseldorf, Germany). Acetonitrile LiChrosolv (for chromatography), methanol LiChrosolv (for chromatography), formic acid (p.a.) and ammonium acetate (p.a.) were purchased from MERCK (Darmstadt, Germany). Pure water (18 M) was obtained using an ion exchange system RS 40 E, SG Ionenaustauscher (Barsbüttel, Germany).

Oasis MCX cartridges, 1 cm^3, 30 mg, (Waters, Milford, MA, USA) were used for solid phase extraction (SPE). MCX is a mixed mode cation exchange/reversed phase sorbent.

Table 1
Relevant data on MS analysis of aminoglycosides

Substance	Molecular weight	Cone voltage	Collision energy	Used transitions
Amikacin	585	28	22	586 → 163
			17	586 → 425
Kanamycin	484	25	17	485 → 163
Paromomycin	615	32	21	616 → 163
Gentamicin	477	26	21	478 → 157
Tobramycin	467	22	16	468 → 163
Neomycin	614	38	30	615 → 161
			21	615 → 293

2.2. LC–MS–MS analysis—apparatus and chromatographic conditions

2.2.1. Mass spectrometer

The MS–MS system used was an Quattro micro (Micromass, Manchester, UK) equipped with an electrospray interface (ESI). Full-scan mass spectra were acquired by continual infusion of standard solution (concentration 1000 ng/ml with 10 μl/min). The product ion mass spectra were obtained by choosing the molecular ions as the precursor ions and scanning product ions from *m/z* 100 to 650. For positive ionisation a capillary voltage of 3200 V and ion source temperature of 100 °C were applied. The desolvation gas flow (nitrogen) was 600 l/h at 300 °C.

The analytes were determined by using the multiple reaction monitoring mode (MRM) with the specific transitions shown in Table 1. The collision gas was argon.

The MassLynx Data System was applied for MS control and QuanLynx for peak area evaluation, regression analysis of standard curves and calculation of concentrations.

2.2.2. LC system

The LC equipment consisted of a Dionex P580 HP-Gradient pump and a Dionex ASI 100 T autosampler (Idstein, Germany) with a Chromeleon Chromatography Data System (Dionex Softron, Idstein, Germany). Separation was performed on a zwitterionic ZIC-HILIC column, 100 mm × 2.1 mm (SeQuant; Umea, Sweden) with a SecurityGuard C18, 4 mm × 2 mm i.d. (Phenomenex, Aschaffenburg, Germany). The following gradient was applied, with solvent A (5/95/0.2, v/v/v) and solvent B (95/5/0.2, v/v/v) each being a mixture of acetonitrile, 2 mM ammonium acetate and formic acid.

	Time (min)					
	0.0	0.6	1.6	7.0	7.5	10.8
A (%)	0	0	90	90	0	0
B (%)	100	100	10	10	100	100

The flow rate was 0.6 ml/min.

2.3. Sample preparation

SPE of the aminoglycosides in human serum samples was performed with Oasis MCX cartridges, a Gilson Automatic Sample Processor for Solid Phase Extraction ASPEC XL and sampler software 735 (ABIMED, Langenfeld, Germany). All liquids and air were pressed through the cartridges. This is in contrast to most other tools which draw the liquids through the cartridges. Due to the reproducibility of SPE and the good precision and accuracy of the method, an internal standard was not necessary, therefore, external calibrations were used.

Extraction procedure:

Condition: 1 ml methanol and 1 ml water, pushed with 1 ml of air.
Load: Mixture of 0.5 ml serum and 0.5 ml 9% formic acid, pushed with 1 ml of air.
Wash: 1 ml 50% methanol in 9% formic acid and 0.7 ml water, pushed with 1 ml of air.
Elute: 0.8 ml 50% methanol in 25% ammonia, pushed with 1 ml of air.

Eluates were evaporated to dryness at 70 °C in an air stream with a Techne DRI Block SC-3 (thermo-DUX, Wertheim, Germany) and redissolved in 100 μl of a mixture of solvents A and B and 20 μl were injected. The capacity of an ASPEC XL Sample Processor for the described automated SPE methods was five samples per hour.

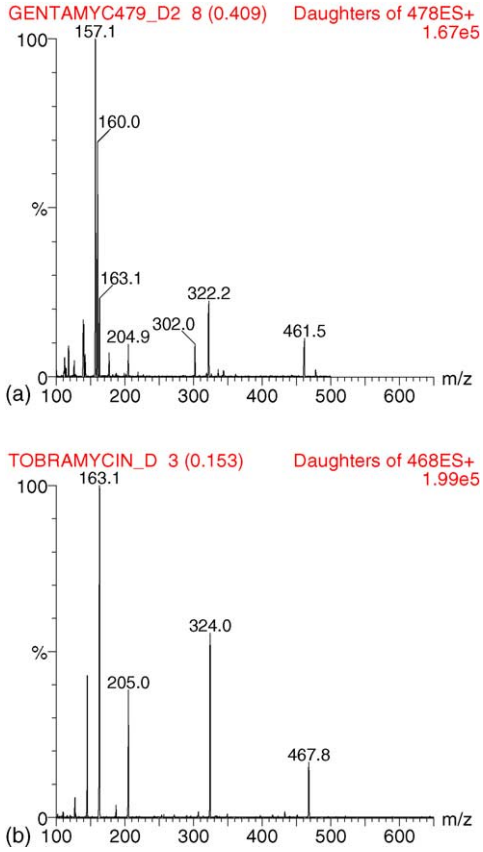

Fig. 1. Product ion mass spectrum of aminoglycosides (1 μg/ml, flow rate 10 μl/min); molecular ions were chosen as the precursor ion (a) gentamicin, and (b) tobramycin.

3. Results and discussion

3.1. Mass spectrometry

The mass spectra of the aminoglycosides revealed base peaks corresponding to the molecular ions $(M + H)^+$. The optimised cone voltage are given in Table 1. The ionisation of the polar analytes was difficult, in comparison with other drugs the sensitivity, i.e. the ratio of signal to concentration, is very small [28]. Using the continual infusion method (10 μl/min) a standard solution with a concentration of more than 0.5 μg/ml aminoglycoside was necessary to obtain sufficient mass spectra.

The fragment ions and the optimised collision energy are shown in Table 1; the product ion mass spectra are presented in Fig. 1a and b. The aminoglycosides showed a similar fragmentation which resulted from a rearrangement of glycoside bindings. Fragment ions with the highest intensity were chosen for quantification. The dwell time of the eight measured transitions was 0.15 s. A good sensitivity and enough points for the peaks were achieved.

3.2. Chromatography

Because of the high selectivity of the MS–MS method in many cases a complete LC separation of analytes and matrix is not necessary. However, to achieve high-quality analytical data for biological samples containing low levels of analytes sufficient chromatographic retention of the analyte is preferred to minimise signal suppression and other matrix effects. Besides, a simultaneous measurement of similar analytes like neomycin and paromomycin with a difference in their molecular weights of only one Dalton and with equal product ions in the MS–MS mode without complete LC separation is not possible. Due to their strong hydrophilic behaviour, the aminoglycosides showed no retention on a stationary reversed phase packing material if an ammonium acetate-acetonitrile eluent was used. NPLC and ion-pair chromatography are suitable for the separation of neomycin and other strong hydrophilic compounds [9–12]. NPLC is not compatible with the electrospray process. Some ion-pair chromatography methods with volatile reagents and MS detection for the determination of gentamicin, streptomycin, neomycin or tobramycin were described [16–20]. HILIC shows separation similar to NPLC but it is possible to use water and volatile buffering agents, which are compatible for mass spectrometry. The separation mechanism of HILIC is opposite to that of RPLC [22,26]. Using a solvent with 95% acetonitrile a complete retention of the hydrophilic analytes was observed for more than 20 min. Using a solvent with 90% water, the hydrophilic analytes eluted with the front. Therefore the HILIC gradient was started with a mobile phase

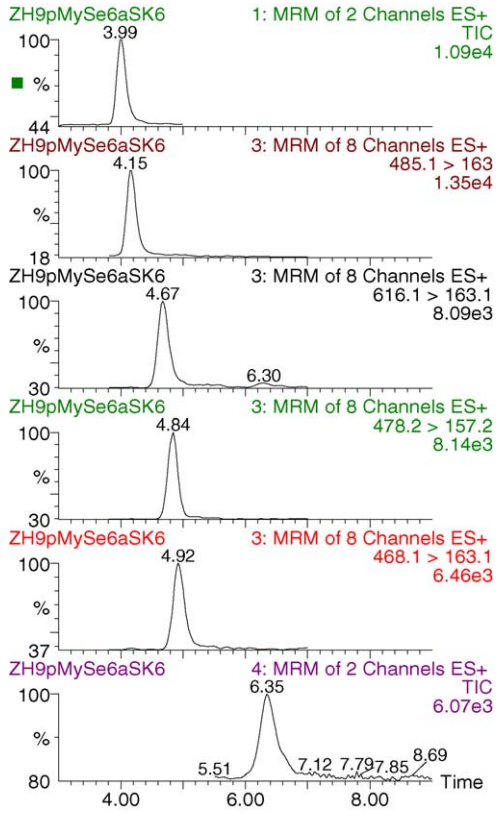

Fig. 2. MRM chromatograms of an aminoglycosides standard sample. Blank serum spiked with 500 ng/ml. Specific transition of amikacin, kanamycin, paromomycin, gentamicin, tobramycin, and neomycin (from top to bottom).

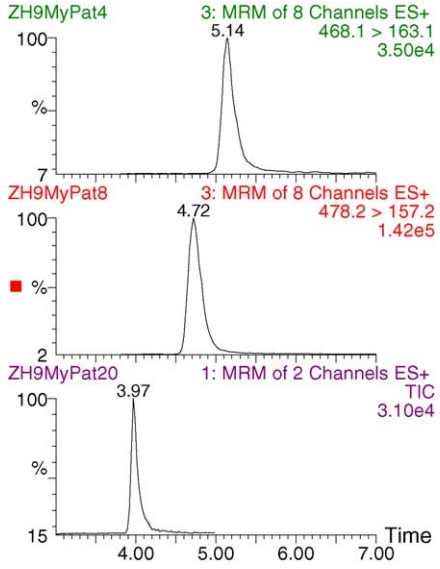

Fig. 3. MRM chromatograms of serum samples of patients after treatment with tobramycin (patient with 2.6 μg/ml), gentamicin (patient with 6 μg/ml) and amikacin (patient with 3.2 μg/ml) (from top to bottom). No peaks showed up at all at any of the other transitions of interest.

containing 95% acetonitrile. To achieve a separation of paromomycin and neomycin and to get small and high peaks a gradient from 5 to 90% ammonium acetate within 60 s was advantageous (Fig. 2).

The use of a buffered mobile phase is crucial to achieve acceptable repeatability for a LC separation of charged compounds, since electrostatic interactions between the solute and the stationary phase are controlled by the buffer. Its concentration should be low to avoid ionisation suppression in the ESI [26]. Using a 2 mM ammonium acetate solution the ion suppression was low and the repeatability was acceptable. The retention times were about: amikacin 4.0 min, kanamycin 4.2 min, paromomycin 4.7 min, gentamicin 4.8 min, tobramycin 4.9 min, and neomycin 6.5 min and the overall chromatographic cycle time was 10 min (Fig. 2). Using zwitterionic ZIC-HILIC columns problems with the constancy of the retention times were sometimes found. Analytes injected on new columns had up to 100% longer retention times than the same analytes after 20 injections on this column. But then the retention times are stable or decrease only slightly (up to 10% after 100 injections). To remove remaining organic solvent and polar impurities 200 μl of 0.5 M sodium chloride solution were injected after 25 samples. One time a week or after about 250 injections the column was washed for 15 min with a 0.5 M sodium chloride solution. After this procedure the retention times were slightly longer (up to 10%). Small and symmetrical peaks were observed for the aminoglycosides. To exclude interferences from the biological matrix, chromatograms of two transitions for amikacin and neomycin were controlled separately. No interferences and a low background noise were found (Figs. 2 and 3). The specifity of the described method is excellent.

3.3. Quantification

The calibration graphs were generated from MRM of increasing amounts of drug standard in blank serum samples. A quadratic calibration graph was constructed using least-squares regression of quantities versus peak area. Using a sample volume of 500 μl a good response over the range of 100–5000 ng/ml serum was demonstrated. Samples with higher analyte concentration can be diluted. The correlation coefficient of regression lines was 0.993 or higher. The precision and accuracy of the method was assessed by the determination of six concentrations in six independent series of spiked serum samples as shown in the Table 2. The accuracy

Table 2
Precision and accuracy of the analytical method for aminoglycosides (six independent sets of spiked serum samples 100–5000 ng/ml)

Analyte	Precision (%)	Accuracy (%)
Amikacin	0.5–9.4	94.4–102.3
Gentamicin	6.1–11.4	92.4–104.8
Kanamycin	0.5–12.7	95.4–101.7
Neomycin	0.3–11.6	94.5–104.1
Paromomycin	0.3–10.2	96.0–101.6
Tobramycin	0.4–9.9	91.3–101.5

of added aminoglycosides ranged from 91 to 105%. The coefficient of variation ranged from 0.3 to 13%. The lower limit of quantification, i.e. a coefficient of variation <15% for six repeated measurements, was 100 ng/ml.

Typical chromatograms obtained from extracted serum samples are illustrated in Figs. 2 and 3. Recovery of about 100% from the serum matrix was found irrespective of the concentration.

It is possible to use one of the six analytes as an internal standard for the other five analytes.

3.4. Therapeutic drug monitoring

3.4.1. Objectives

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. One aim of the drug monitoring in our clinical study was to show that in most patients the systemic uptake of Neomycin in the urinary tract via resorption was lower than 100 ng/ml serum. The other objective of the study was to compare the results of an automated immunoassay with HILIC–MS–MS. For that blood samples of patients after treatment with amikacin, tobramycin and gentamicin were measured twice. In the chromatograms of the patients no peaks showed up at all at any of the other transitions of interest (Fig. 3).

Immunoassay methods are described briefly: amikacin, gentamicin, and tobramycin were analysed by Fluorescence Polarisation Immunoassays (FPIA) from ABBOTT GmbH & Co. KG (Wiesbaden, Germany). Amikacin was analysed with the analyser TDxFlx, Gentamicin and Tobramycin were analysed with the analyser AxSym, both from ABBOTT. Using a sample volume of 50 μl serum the lower limits of quantification were 0.8 μg/ml for amikacin 0.3 μg/ml for gentamicin and 0.18 μg/ml for tobramycin.

Blood samples of patients with kanamycin and paromomycin were not available.

3.4.2. Results

The drug monitoring in the clinical study with Neomycin showed that in most patients the systemic uptake of Neomycin in the urinary tract via resorption was lower than 100 ng per ml serum [27].

The concentration of amikacin, tobramycin and gentamicin in the blood samples of patients measured with the immunoassay method and with HILIC–MS–MS were similar. Differences were smaller than 25% and should not have clinical relevance. For 20 of the 25 samples the difference was smaller than 15%. No systematic deviation was observed.

4. Conclusion

HILIC combined with tandem mass spectrometry is a powerful and robust technique for highly specific simultaneous quantitating of the aminoglycosides amikacin, gentamicin, kanamycin, neomycin, paromomycin, and tobramycin in biological matrices. HILIC is better compatible with MS–MS than ion-pair chromatography. Furthermore the assays require a simple automatic off-line sample preparation. The sensitivity is sufficient for therapeutic drug monitoring, for clinical and pharmacokinetic questions and to estimate the possibility of adverse effects.

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