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Short communication

Determination of neomycin by LC–tandem mass spectrometry using hydrophilic interaction chromatography

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Abstract

A specific, and automated method was developed to quantitate neomycin in human serum. Samples were prepared with an automated solid phase extraction (SPE). The hydrophilic interaction chromatography (HILIC) was used for additional sample cleanup and baseline separation. The analyte neomycin was detected with electrospray ionisation tandem mass spectrometry (ESI–MS–MS). Using a volume of 500 μl biological sample the lower limit of quantification was 100 ng/ml. The described HILIC–MS–MS method is suitable for clinical and pharmcokinetical investigations of neomycin.

Keywords: LC–MS–MS; Hydrophilic interaction; HILIC; Neomycin; Aminoglycoside

1. Introduction

Neomycin (Fig. 1) belongs to a group of broad-spectrum aminoglycoside antibiotics which are widely used in clinical therapy of serious infections. It inhibits the growth of both Gram-positive and Gram-negative bacteria [1]. Neomycin has 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 of neomycin may cause ototoxicity and nephrotoxicity [2,3]. To exclude these adverse effects after topic application of neomycin it could be important to control the serum concentration of the drug. The 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 aminoglycoside neomycin has a strong hydrophilic behaviour. Therefore stationary reversed phases can only be used for HPLC separation in combination with ion-pair chromatography [4,5]. Furthermore fluorescence detection after post column derivatisation or electrochemical detection were described for neomycin [6–8]. However, these chromatographic methods lack specificity, sensitivity or robustness and require elaborate sample preparation.

For electrospray mass spectrometric detection ion pair chromatography is not ideal because it reduces the sensitivity of mass spectrometry. Normal-phase liquid chromatography (NPLC) should be suitable for the separation of strong hydrophilic compounds. However, the typical non aqueous eluents used for NPLC

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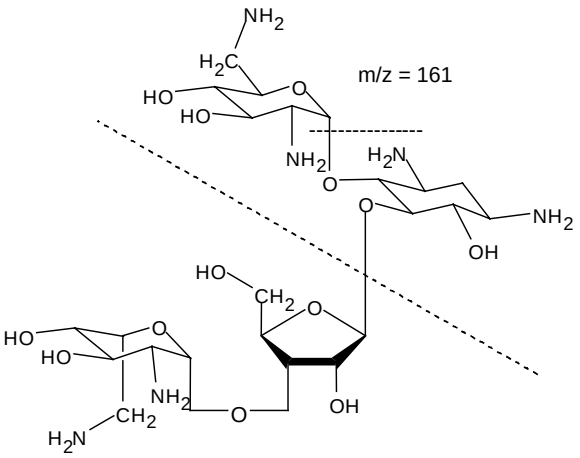

Fig. 1. Chemical structure of neomycin and its main MS–MS fragment (*m*/*z* 161).

are not well compatible with the electrospray process [9]. An alternative to NPLC is hydrophilic interaction chromatography (HILIC). Similar to NPLC the elution is promoted by the use of polar mobile phases, but HILIC is unique in that 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 [10–12]. The used stationary HILIC phase is a zwitterionic silica gel. The properties of the mobile phase buffers also greatly impact the separations. Ammonium acetate at a concentration of 5–10 mM were described to facilitate optimal HILIC retention, reproducibility and durability. Therefore the HILIC technique is suitable for the analysis of polar compounds (e.g. folates [13], carbohydrates [11], peptides [12] or natural products [14]) with MS–MS detection. This paper describes an application of the HILIC technique to determine the aminoglycoside neomycin in human serum.

2. Experimental

2.1. Chemicals

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 MΩ) was obtained using an ion exchange system RS 40 E, SG Ionenaustauscher (Barsbüttel, Germany).

Oasis MCX cartridges, 1 cc, 30 mg (Waters, Milford, MA, USA) were used for solid phase extraction.

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, GB) 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 ion as the precursor ions, scanning product ions from *m*/*z* 100 to 650. For positive ionisation a capillary voltage of 3500 V and ion source temperature of 100 °C were applied. The desolvation gas flow (nitrogen) was 600 l/h at 300 °C.

The analyte neomycin was measured employing the multiple reaction monitoring mode (MRM) with the specific transitions between *m*/*z* 615 (parent ion) and 161, and *m*/*z* 293 and 455 with argon as collision gas. The sum of the three fragment ions was used for determination of analyte concentration.

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. HPLC system

The HPLC equipment consisted of a Dionex P580 HP-Gradient pump and an autosampler Dionex ASI 100 T (Idstein, Germany) with a Chromeleon Chromatography Data System (Dionex Softron, Idstein, Germany). The chromatographic 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 mobile phase gradient was applied with solvent A (5/95/0.2, v/v/v) and solvent B (95/5/0.2, v/v/v) of a mixture of acetonitrile, 10 mM ammonium acetate and formic acid:

	Time (min)					
	0.0	0.1	0.6	5.8	6.3	8.8
A (%)	20	20	80	80	20	20
B (%)	80	80	20	20	80	80

The flow rate was 0.6 ml/min and the retention time of neomycin was about 5.7 min.

2.3. Sample preparation

SPE of neomycin 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 the most other tools which draw the liquids through the cartridges. Due to the reproducibility of SPE, an internal standard was not necessary and, therefore, external calibrations were used.

Extraction procedure:

- Condition: 1 ml methanol and 1 ml water, pushing with 1 ml of air.
- Load: mixture of 0.5 ml serum and 0.5 ml 9% formic acid, pushing with 1 ml of air.
- Wash: 1 ml 50% methanol in 9% formic acid and 0.7 ml water, pushing with 1 ml of air.
- Elute: 0.8 ml 50% methanol in 25% ammonia, pushing 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 eluents A and B.

The capacity of an ASPEC XL Sample Processor for the described automated solid phase extraction methods was five samples per hour.

3. Results and discussion

3.1. Mass spectrometry

The mass spectrum of neomycin revealed a base peak at *m*/*z* 615 or 308, respectively, corresponding to the molecular ions $(M+H)^+$ and $(M+2H)^{2+}$ (Fig. 2).

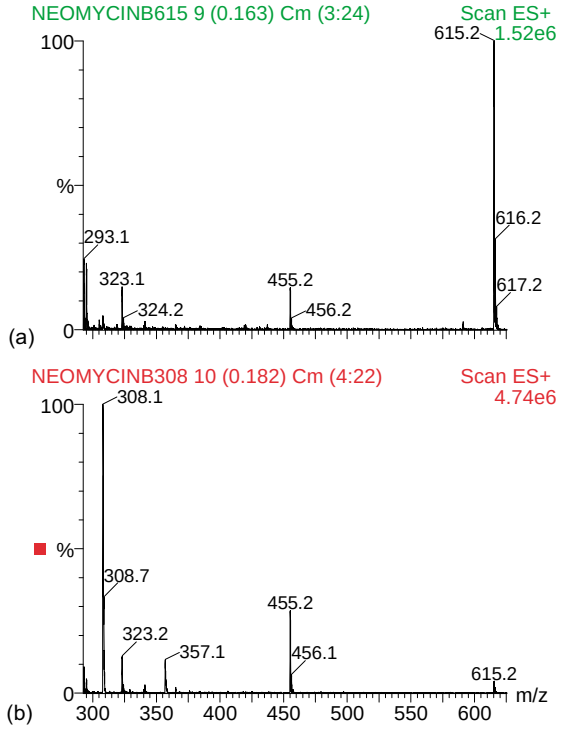

Fig. 2. Mass spectrum of neomycin (1 μg/ml, flow rate 10 μl/min): (a) cone voltage 38 V, molecular ion $(M+H)^+$ *m*/*z* 615; (b) cone voltage 18 V, molecular ion $(M+2H)^{2+}$ *m*/*z* 308.

The maximum intensity of the single charged ion was found using a cone voltage of 38 V. Applying a lower cone voltage (18 V) the double charged molecular ion showed the most intensive signal.

In comparison with other drugs the sensitivity, i.e. the ratio signal to concentration is very small [15]. Using the continual infusion method (10 μl/min) a standard solution with a concentration of more than 0.5 μg/ml neomycin was necessary to receive sufficient mass spectra. The most intensive product ion mass spectrum was obtained by choosing the molecular ion $(M+H)^+$ with *m*/*z* 615 as the precursor ion. The fragment ions observed at *m*/*z* 161, 163, 293, 323 and 455 (Fig. 3). Fragments result from a rearrangement of glycosidic bindings (Fig. 1). Normally fragment ion with the highest intensity (*m*/*z* 161) is chosen. However, concerning an improved specificity and sensitivity in cases of a complex matrix, incomplete chromatographic separation or low sensitivity additional transitions are analysed. The sum of the

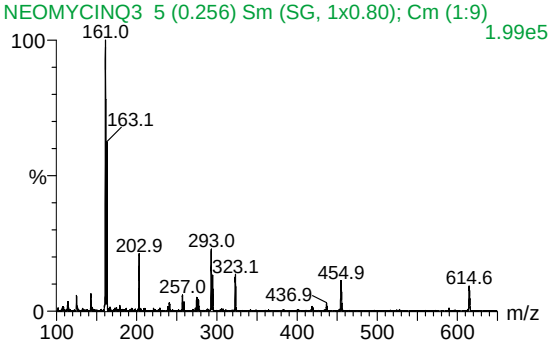

Fig. 3. Product ion mass spectrum of neomycin (1 μg/ml, flow rate 10 μl/min); molecular ion *m/z* 615 was chosen as the precursor ion.

three transitions between *m/z* 615 and 161, and *m/z* 293 and 455 was used to determine the concentration of neomycin in serum.

3.2. Chromatography

Because of the high specificity of the MS–MS method a complete chromatographic separation of analytes and matrix is not necessary. However, to achieve high-quality analytical data for samples with low levels of analytes originally in biological fluids, sufficient chromatographic retention of the analyte is preferred to minimise signal suppression and other matrix effects. Caused by strong hydrophilic behaviour of neomycin the analyte showed no retention of on a stationary reversed phase (RP) using an ammonium acetate–acetonitrile–eluent system. Normal-phase liquid chromatography (NPLC) and ion-pair chromatography are suitable for the separation of neomycin and other strong hydrophilic compounds [4–7]. But both techniques are not well compatible with the electrospray process. Hydrophilic interaction chromatography (HILIC) shows similar separation to NPLC but it is possible to use the same eluent systems like for reversed phases chromatography with water and volatile buffering agents, which are suitable for mass spectrometry. The separation mechanism of HILIC is opposite to that of RPLC [10,14]. Using a solvent with 80% acetonitrile a complete retention of the hydrophilic analyte was observed for more than 30 min. Using a solvent with 80% water the hydrophilic analyte eluted with the front. Therefore the gradient for the HILIC was started with a mobile phase containing

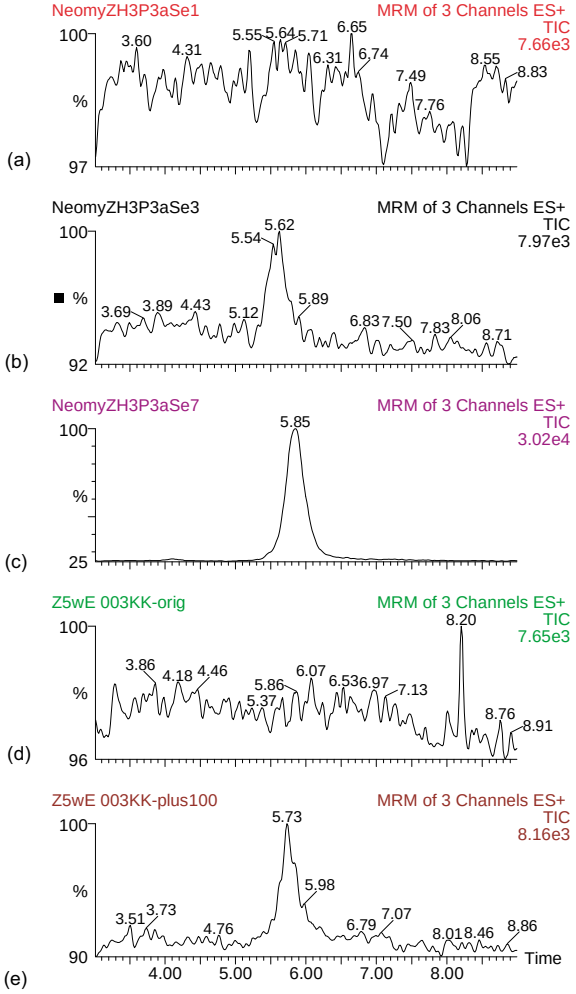

Fig. 4. MRM chromatograms of neomycin extracted from serum. MRM was performed by monitoring the sum of transitions between *m/z* 615 and 161, and *m/z* 293 and 455: (a) blank serum; (b) blank serum spiked with 100 ng/ml neomycin (lowest point of the standard curve); (c) blank serum spiked with 2000 ng/ml neomycin; (d) serum of a patient after topic application of neomycin; (e) serum of a patient after topic application of neomycin spiked with 100 ng/ml neomycin.

80% acetonitrile. To achieve small and high peaks a steep gradient from 20 to 80% ammonium acetate within 30 s was advantageous (Fig. 4).

Typically, the use of buffered mobile phase is crucial for the achievement of acceptable reproducibility for chromatographic separations of charged species, since electrostatic interactions between the solute and the stationary phase impacting retention are influenced

Table 1
Precision and accuracy of the analytical method for neomycin from six independent sets of spiked serum samples

	Found concentration of neomycin (ng/ml)						*r*
	100[a]	200[a]	500[a]	1000[a]	2000[a]	5000[a]	
SC	108	186	528	905	2077	4989	0.9994
SC	110	191	480	953	2082	4983	0.9995
SC	101	204	495	967	2035	4995	0.9999
SC	103	188	561	871	2079	4990	0.9992
SC	99	209	500	949	2045	4994	0.9998
SC	85	198	563	1095	1844	5026	0.9985
Mean	101	196	521	957	2027	4996	
S.D.	8.0	8.5	32.3	70.1	83.8	14.0	
CV (%)	7.94	4.33	6.19	7.33	4.13	0.28	
Accuracy (%)	100.8	97.9	104.2	95.7	101.3	99.9	

SC: standard curve; S.D.: standard deviation; CV: coefficient of variation; *r*: coefficient of correlation.
[a] Concentration of neomycin (ng/ml) added.

and controlled by the buffer. Its concentration should be low to avoid ionisation suppression in the ESI [14]. Using a 10 mM ammonium acetate solution the ion suppression was low and the reproducibility was acceptable.

The retention time of neomycin was 5.7 min and the overall chromatographic cycle time was 9 min. Small and symmetrical peaks were observed for neomycin. To exclude interferences from the biological matrix, chromatograms of three transitions were controlled separately. Using only one transition and measurement without sufficient chromatographic separation can lead to false results. No interferences and a low background noise were found. The best sensitivity was achieved by monitoring the sum of three fragment ions.

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. The correlation coefficient of regression lines was 0.9985 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 Table 1. The accuracy for added neomycin ranged from 95.7 to 104.2%. The coefficient of variation ranged from 0.3 to 7.9%. The lower limit of quantification, i.e. a coefficient of variation <10% for six repeated measurements, was 100 ng/ml. Typical chromatograms obtained from extracted serum samples are illustrated in Fig. 3. Recovery of about 100% from the serum matrix was found irrespective of the concentration. Day-to-day precision data were obtained over a period of five working days by taking aliquots of serum with 185, 680 and 3200 ng/ml neomycin, respectively, and by processing them daily. Adequate coefficients of variation were found: 7.6% (lowest concentration), 7.4% (medium concentration) and 6.6% (highest concentration).

The drug monitoring in our clinical study has shown that in most patients the systemic uptake of neomycin in the urinary tract via resorption was lower than 100 ng/ml serum.

4. Conclusion

HILIC combined with tandem mass spectrometry is a powerful and robust technique for highly specific quantitating of neomycin in biological matrices. Furthermore the assays require a simple automatic off-line sample preparation. The sensitivity is sufficient for clinical and pharmacokinetic questions and to estimate the possibility of adverse effects.

Acknowledgements

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