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DETERMINATION OF TOBRAMYCIN IN HUMAN SERUM USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY AND COMPARISON WITH A FLUORESCENCE POLARIZATION ASSAY

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OVERVIEW

- A rapid LC/MS/MS method for the analysis of Tobramycin in human serum is presented.
- A volatile ion-pair reagent is used to promote the retention of Tobramycin on a small C_{18} cartridge
- The assay is precise (intra- and inter- assay CV<6%) and accurate.
- Comparison with an existing fluorescence polarization assay shows good agreement for serum samples from 108 individuals.

INTRODUCTION

The aminoglycoside antibiotic Tobramycin is widely used against gram negative bacterial infections and is particularly useful for the treatment of Pseudomonas aeruginosa in patients with Cystic Fibrosis. It has a narrow therapeutic range and dosage alterations based on the results of drug monitoring have been found to improve efficacy and minimize toxicity. Microbiological assays and HPLC assays with fluorescence or electrochemical detection have previously been used for the measurement of Tobramycin in serum but these have largely been replaced with immunoassays which offer the advantages of speed and simplicity[1-4]. However, immunoassays can suffer from poor accuracy due to the specificity of the antibodies used[5] and because of variable interference between individuals[6]. LC/MS/MS has been used for the trace analysis of Gentamycin in animal products[7,8] but has not previously been considered for routine TDM assays of aminoglycosides. We have now developed a rapid, accurate and precise LC/MS/MS procedure for the analysis of Tobramycin in human serum that uses an MS compatible ion-pair reagent. The method is suitable for use in a routine laboratory and may also be applicable to other aminoglycoside antibiotics.

METHODS

Patient Samples

The use of patient samples for this study was approved by the Wythenshawe Hospital local ethical committee. Venous blood was collected from 109 patients attending the cystic fibrosis clinic using serum Vacutainer tubes (Beckton Dickinson, Oxford, UK).

Sample analysis by Fluorescence Polarization

For comparative purposes, Tobramycin concentrations in patient samples were measured on a Cobas Integra analyzer using a fluorescence polarization immunoassay (FPIA) as directed by the manufacturer (Roche Diagnostics, Lewes, UK).

Internal Standard and Calibrators

The non-prescribed aminoglycoside Sisomycin (Sigma-Aldrich Company Ltd) was dissolved at a concentration of 25g/L in water for use as internal standard. Calibrators were prepared by diluting an aqueous stock solution of Tobramycin (Eli Lilly and Company) into pooled drug-free serum to give final concentrations of 0, 0.1, 0.5, 1.0, 10.0, 20.0 and 50.0 mg/L. A series of Tobramycin calibrators was also purchased from Roche Diagnostics. Pooled patient samples were used to prepare quality control material for the precision studies.

Mass Spectrometry

A Waters® Micromass® Quattro micro™ tandem mass spectrometer operated in positive electrospray ionization mode was used for all analyses. The optimum MS/MS conditions were determined by infusing standard solutions of Tobramycin and Sisomycin into a stream of mobile phase flowing into the source of the mass spectrometer. The cone voltage and collision energy were adjusted to maximize the intensities of the precursor and major product ions respectively. The optimized conditions are shown in Table 1 and example spectra are shown in Figure 1.

Compound	Precursor Ion (m/z)	Product Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)	Dwell Time (s)
Tobramycin	467.8	163.0	22	22	0.25
Sisomycin (IS)	447.8	160.0	22	22	0.25

Table 1. Optimized MS/MS conditions for the analysis of Tobramycin and Sisomycin.

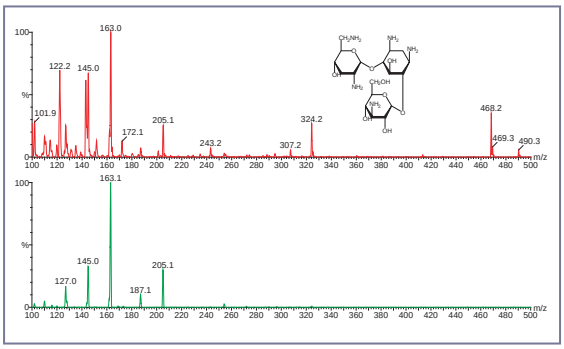

Figure 1. Positive ionization electrospray MS (upper) and MS/MS (lower) spectra for Tobramycin. Conditions were optimized to maximize the intensity of the precursor ion (m/z 467.8) and the major product ion (m/z 163.0).

Sample Preparation for LC/MS/MS Analysis

Patient samples, calibrators or QCs were prepared for LC/MS/MS analysis in deep-well microtitre plates as shown in Figure 2.

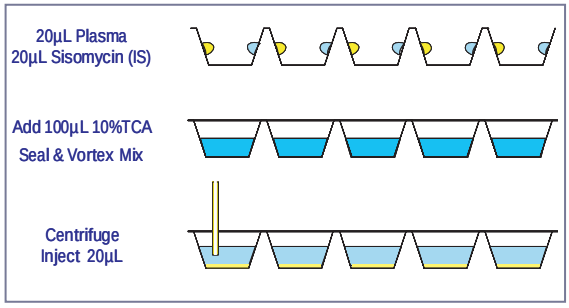

Figure 2. Sample preparation for the LC/MS/MS analysis of Tobramycin using a single 96-well microtitre plate.

HPLC

A Waters Alliance® HT HPLC System was used for all analyses. Prepared samples were analyzed using a a Waters C_{18} guard cartridge eluted with a step gradient containing HFBA ion-pair reagent to promote the retention of Tobramycin (Table 2). The cycle time was approximately 2.5 min., injection to injection.

Time (min)	%A	%B	%C
0	70	20	10
0.6	0	100	0
1.0	70	20	10
1.4	70	20	10

Table 2. Solvent conditions used for the LC/MS/MS analysis of Tobramycin. Each time point represents a step change from the previous solvent composition. A=2mM ammonium acetate, 0.1% formic acid in water; B=2mM ammonium acetate, 0.1% formic acid in methanol, C=10% heptafluorobutyric acid (HFBA) in A.

RESULTS

The use of an ion-pair reagent allows Tobramycin to be retained on a small C_{18} cartridge and washed free from potential interferences. Switching to a mobile phase with a high methanol content and no ion-pair reagent then elutes the drug and internal standard, both with a retention time of 1.05 minutes (Figure 3).

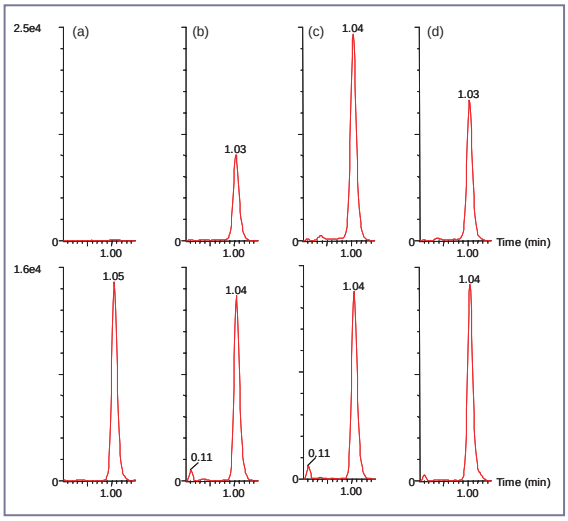

Figure 3. LC/MS/MS chromatograms for Tobramycin (upper traces; m/z 467.8>163.0) and for Sisomycin internal standard (lower traces; m/z 447.8>160.0) extracted from (A) zero calibrator (B) 4.0 mg/L calibrator (C) 10.0 mg/L calibrator and (D) a patient sample found to contain 7.0 mg/L Tobramycin.

In addition to Tobramycin, the patient cohort was being treated with one or more of the drugs shown in Table 3 using standard treatment regimes for the drugs in question. The selectivity of the LC/MS/MS method was demonstrated by the absence of any interfering peaks.

Maxalon	Tazocin	Meropenem	Tacrolimus	Mycophenolate
Prednisolone	Ranitidine	Septrin	Colistin	Vitamin E
Ventolin	Creon	Erythromycin	Aminophylline	Hydrocortisone
Allopurinol	Losec	Diltiazam	Doxazosin	Nystatin
Dnase	Ceftazadine	Flucloxacilin	Seretide	Betnesol
Ibuprofen	Erythromycin	Paracetomol	Ursodeoxycholic acid	

Table 3. Drugs found not to interfere with the LC/MS/MS assay for Tobramycin.

A calibration curve was constructed by plotting the Tobramycin:internal standard peak area ratio against Tobramycin concentration (Figure 4). The curve was linear over the calibration range up to 50 mg/L and there was good agreement between the commercial and in-house calibrators (Figure 4).

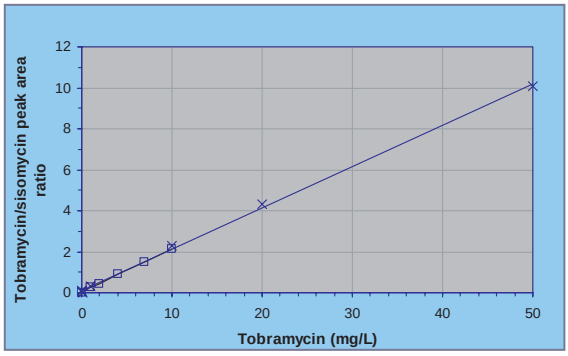

Figure 4. Calibration curve for the LC/MS/MS analysis of Tobramycin using Roche calibrators (p) 0, 1, 2, 4, 7 and 10 mg/L and in-house calibrators (X) 0, 0.1, 0.5, 1.0, 10.0, 20.0, and 50.0 mg/L.

The limit of detection was 0.1 mg/L (the Tobramycin concentration equivalent to 3 times the signal to noise value for the zero calibrator) and the limit of quantification, derived from the precision profile, was 0.2 mg/L (CV<20%; Figure 5).

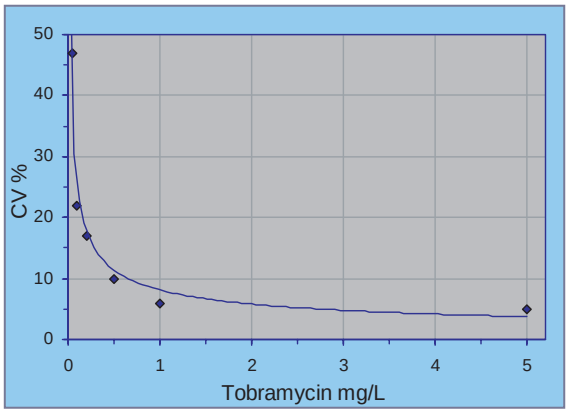

Figure 5. Precision profile for Tobramycin by LC/MS/MS. CV was calculated from 10 replicate injections of single extracts of serum calibrators.

The within day and between day precision for Tobramycin assessed on three separate pools ranging from 1.0 mg/L to 8.4 mg/L was <6% (Table 4). The mean recovery of Tobramycin across a range of concentrations between 2.0 mg/L and 9.0 mg/L was 99.5% (range 93% - 105%).

QC	Tobramycin Concentration (mg/L)			
	Intra-assay (N=15)		Inter-assay (N=15)	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
Low	1.0 ± 0.1	5.8	1.1 ± 0.1	6.0
Medium	4.2 ± 0.2	3.6	4.2 ± 0.2	5.0
High	8.4 ± 0.2	2.7	8.3 ± 0.4	4.0

Table 4. Analytical imprecision of the LC/MS/MS assay.

The stability of the extracted materials in the supernatant was tested by the analysis of replicate extractions of a serum sample over a 12-hour period. No systematic loss in sensitivity was observed in the peak area ratio (analyte/internal standard) and the CV for this ratio was 6% (Figure 6).

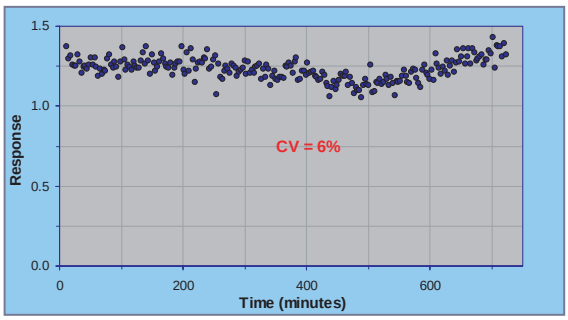

Figure 6. Replicate extractions of a single serum sample were prepared. Each extract was analyzed by LC/MS/MS for Tobramycin with an injection interval of 2.5 min. Injections were made over a 12-hour time period.

The time taken to process a routine batch of 20 samples together with appropriate controls and calibrators was 1.5 hours.

Comparison of the Tobramycin concentration measured in the samples from 109 cystic fibrosis patients using FPIA and LC/MS/MS, showed good agreement between the two methods (Figures 7 and 8).

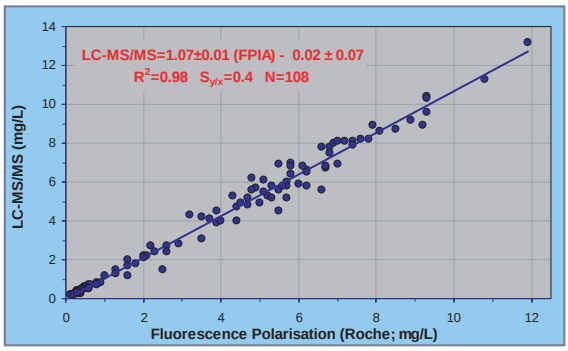

Figure 7. Passing - Bablok correlation between the serum Tobramycin concentrations determined by LC/MS/MS or by FPIA for 108 cystic fibrosis patients.

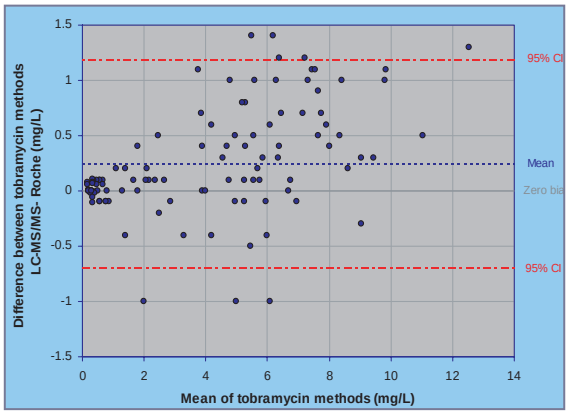

Figure 8. Bland Altman difference plot of Tobramycin concentrations measured by FPIA (Roche Integra) and LC/MS/MS.

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The low concentration of the volatile ion-pair reagent causes minimum suppression of ionization (Table 5) and can easily be washed from the LC system to allow rapid switching between different applications.

Matrix	Serum (Peak Area)			Water (Peak Area)		
Tobramycin (mg/L)	Tobramycin	Sisomycin	Ratio	Tobramycin	Sisomycin	Ratio
2.3	1713 ± 102	3426 ± 75	0.47 ± 0.03	1642 ± 112	3471 ± 69	0.47 ± 0.02
4.5	3313 ± 98	3528 ± 101	0.94 ± 0.04	3352 ± 98	3525 ± 15	0.98 ± 0.9
9.0	6552 ± 114	3475 ± 83	1.87 ± 0.01	6273 ± 568	3507 ± 112	1.76 ± 0.1

Table 5. Summary of the peak area counts and their ratios for Sisomycin and Tobramycin extracted from whole blood or water after the addition of Tobramycin at the indicated concentrations.

CONCLUSIONS

- LC/MS/MS provides a rapid, precise and robust method for the analysis of Tobramycin in human serum with minimal sample preparation.
- Commercial or "in-house" calibrators can be used.
- It is likely that the method could be extended to include the simultaneous analysis of other commonly used aminoglycoside antibiotics (e.g. amikacin and gentamycin).
- Low concentrations of volatile ion-pair reagents may prove a useful tool in the development of MS-compatible chromatography methods for polar compounds that are not easily retained on reversed phase columns using conventional mobile phases.

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REFERENCES

1. Maitra SK, YoshikawaTT, Guze LB, et al. Determination of aminoglycoside antibiotics in biological fluids. Clin Chem 1979; 25:1361-1367.
2. Soltes L. Aminoglycoside antibiotics , two decades of their HPLC bioanalysis. Biomed Chromatogr. 1999;13:3-10.
3. Lai F, Sheehan T. Enhancement of detector sensitivity and cleanup selectivity for tobramycin through pre-column derivatization. J Chromatogr 1992;18:173-9
4. Statler JA. Determination of tobramycin using high –performance liquid chromatography with pulsed amperometic detection. J Chromatogr 1990;27:244-6.
5. Isoheranen N, Soback S. determination of gentamicins C1, C1a and C2 in plasma and urine by HPLC. Clin Chem 2000;46:837-42.
6. Ezan E, Emmanuel A, Valente D, Grognet J-M. Effect of variability of plasma interferences on the accuracy of drug immnuassays. Ther Drug Monit 1997; 19:212-8
7. Heller DN, Clark SB, Righter HF. Confirmation of gentamicin and neomycin in milk by weak cation exchange extraction and electrospray ionization/ion trap tandem mass spetrometry. J Mass Spectrom 2000;35:39-49.
8. Cherlet M, De Baere S, De Backer P. Determination of gentamicin in swine and calf tissues by high performance liquid chromatography combined with electrospray ionization mass spectrometry. J Mass Spectrom 2000;35:1342-Figure 8. Bland Altman difference plot of tobramycin concentrations measured by fluorescence polarization (Roche Integra) and LC-MS/MS

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