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Determination of tobramycin in serum using liquid chromatography–tandem mass spectrometry and comparison with a fluorescence polarisation assay

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Abstract

We have developed a tandem mass spectrometry (LC–MS–MS) method for measuring tobramycin concentrations in serum samples and have compared it with a fluorescence polarisation immunoassay. After protein precipitation with acetonitrile supernatant was injected into the LC–MS–MS system. A C_{18} cartridge (4×2 mm) was eluted with a step gradient of 20–100% methanol containing HFBA. The retention times were, tobramycin 1.05 min and sisomycin 1.05 min. The MRM transitions were: m/z 467.8>163 (tobramycin) and m/z 447.8>160 (sisomycin). The limit of quantification was 0.15 mg/l and the assay was linear up to 50 mg/l. Assay precision was <6% within and between batch.

Keywords: Tobramycin

1. Introduction

Tobramycin, an aminoglycoside antibiotic, is widely used against Gram negative bacterial infections and is particularly useful for the treatment of *P. aeruginosa* in patients with cystic fibrosis. It has a narrow therapeutic range and monitoring of the drug is required to reduce serious side effects such as nephro and ototoxicity [1–4]. Dosage alterations based on the results of drug monitoring have been found to improve efficacy and minimise toxicity [5].

Methods for measuring tobramycin in serum samples include microbiological assays, immunoassays and HPLC [6,7]. Microbiological assays are slow and can be imprecise at lower concentrations, whereas HPLC methods, although exhibiting greater accuracy and precision, require extensive clean-up and derivatisation steps. This makes both of these approaches impractical for routine use, where a rapid sample turnaround is often required for dosing purposes. Immunoassays, which can be performed quickly on routine analysers, have therefore become increasingly popular and are now the preferred method in the majority of laboratories participating in the UK external quality assessment scheme (UKNEQAS) [8]. HPLC methods for tobramycin are necessarily complex because of a lack of chromophores on the molecule which necessitates derivatisation for use with fluorescence [9], electrochemical

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[10] or UV detectors [11], but the outcome has been an increase in assay complexity and time. Chromatographic assays do offer advantages over immunoassays, particularly in regard to antibody specificity [12] and the lack of accuracy shown with immunoassays caused by variable interference between individuals [13]. It has recently been demonstrated that aminoglycoside antibiotics can be measured without derivatisation when tandem mass spectrometry is used as a detector with liquid chromatography (LC–MS–MS) [14,15]. Both of these studies measured gentamicin in animal tissues and milk and neither study measured tobramycin. Tobramycin is structurally similar to gentamicin and should therefore be detectable using LC–MS–MS. We have therefore developed a method for tobramycin in human serum using LC–MS–MS in an attempt to reduce sample size and also to increase the speed of the assay.

2. Materials and methods

2.1. Patient samples

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

2.2. Sample analysis by fluorescence polarisation

For comparative purposes, tobramycin concentrations were measured on a Cobas Integra analyser using fluorescence polarisation (Roche Diagnostics, Lewes, UK) according to the manufacturers instructions. Fluorescein labelled tobramycin when bound to antitobramycin monoclonal antibody rotates slowly in solution compared to unbound label and can emit polarised light when irradiated, whereas the unbound label does not. The amount of fluorescence polarisation is therefore proportional to the drug concentration. The assay requires a 3-μl sample although a dead volume of approximately 100 μl is required in the sample cup. The range of the assay is from 0.04 to 10 mg/l measuring at 485 nm excitation and 515 nm emission. Samples were analysed daily with quality controls and the assay was calibrated once every week.

2.3. Internal standards and calibrators

Sisomycin was purchased from Sigma–Aldrich (Poole, UK). Tobramycin (99% pure) was a gift from Eli Lilly (Indianapolis, USA). Tobramycin was dissolved in deionised water and a series of calibrators were prepared in pooled serum by dilution of this stock standard to give 0, 0.1, 0.5, 1.0, 10.0, 20.0 and 50.0 mg/l. The working serum based standards were stored in aliquots at −30 °C. A series of tobramycin calibrators were also purchased from Roche Diagnostics. Pooled patient samples were used to prepare quality control material for the precision studies.The precipitating solution was prepared by adding trichloroacetic acid (TCA) to water to give a concentration of 100 g/l. An internal standard was prepared by dissolving sisomycin in water (25 mg/l). HPLC grade acetonitrile and heptafluorobutyric acid (HFBA) was supplied by VWR (Poole, UK).

2.4. Sample preparation for LC–MS–MS

Serum samples or calibrators (20 μl) were added to the internal standard (20 μl) in deep well microtitre plates and then precipitating solution (100 μl) was added. The plate was sealed with thermosealing film and vortex mixed vigorously for 30 s using a multivortex mixer to disperse the precipitated material. After centrifugation at 800 *g* for 5 min, the sealed plate was transferred to the autosampler for analysis.

2.5. High-performance liquid chromatography

Chromatography was performed using a Waters 2795 Alliance HT LC system (Waters, Watford, UK). Supernatant (20 μl) was directly injected from the 96 well microtitre plate onto two SecurityGuard C_{18} cartridge columns in series, 4.0×2.0 mm, particle size and porosity not stated by manufacturer (Phenomenex, Macclesfield, UK). The following solvent conditions were used [where A=water containing

2 m*M* ammonium acetate, 0.1% (v/v) formic acid, B=methanol containing 2 m*M* ammonium acetate, 0.1% (v/v) formic acid) and C=A containing HFBA (heptafluorobutyric acid) (10 m*M*)]: 20% B and 10% C for 0.6 min, step to 100% B for 0.4 min, step back to 20% B and 10% C to re-equilibrate the column. The run time was set to 1.4 min to give a cycle time of approximately 2.5 min injection to injection, thereby allowing a total re-equilibration time of approximately 1.7 min. The column was maintained at room temperature and the eluent was connected directly to the electrospray probe of the mass spectrometer with no splitting or solvent diversion.

2.6. Mass spectrometry

A Quattro micro tandem mass spectrometer fitted with a Z Spray ion source was used for all analyses (Micromass, Manchester, UK). The instrument was operated in electrospray positive ionisation mode and was directly coupled to the HPLC system. System control and data acquisition was performed with MASSLYNX NT 4.0 software with automated data processing using the MASSLYNX QUANLYNX programme provided with the mass spectrometer. Calibration curves were constructed using linear least squares regression and $1/x$ weighting was used to ensure maximum accuracy at the lower tobramycin concentrations.

To tune the mass spectrometer, a solution of tobramycin or sisomycin (1 mg/l) in 50% aqueous methanol containing 2 m*M* ammonium acetate and 0.1% formic acid, was infused into the ion source, and the cone voltage optimised to maximise the intensity of the $(M+H)^+$ precursor ions for tobramycin or sisomycin (m/z 467.8 and 447.8, respectively). The collision energy was then adjusted to optimise the signal for the most abundant product ions (m/z 163 and 160, respectively). Typical tuning conditions were as follows; electrospray capillary voltage 1.0 kV, sample cone voltage 22 V, and collision energy 22 eV at a collision gas pressure $1.8 \cdot 10^{-3}$ mBar argon. Sample analyses were performed in the multiple reaction monitoring (MRM) mode of the mass spectrometer with a dwell time of 0.25 s per channel using the following transitions; m/z 467.8$>$ 163 (tobramycin) and m/z 447.8$>$160 (sisomycin).

3. Results

Sample preparation using TCA resulted in a clear, colourless supernatant that gave clean chromatograms with no interfering compounds present (Fig. 1). Elution of tobramycin (1.05 min) and sisomycin (1.05 min) allowed an injection-to-injection cycle time of 2.5 min. In addition to tobramycin the patient cohort was being treated with one or more of the drugs shown in Table 1 using standard treatment regimes for the drugs in question. The selectivity of the method was demonstrated by the absence of any interfering peaks, from parent drug or metabolite, with retention times similar to tobramycin.

Quantitation was performed by integrating the area under the extracted ion chromatograms for tobramycin and internal standard for a series of serum calibrators. A calibration curve was constructed by plotting the tobramycin:internal standard peak area ratio against tobramycin concentration for in house and Roche calibrators. The curve was linear over the calibration range up to 50 mg/l and showed a good correlation with the stated values for the calibrators ($R^2=0.9986$, $y=0.021x+0.005$). The limit of detection was 0.1 mg/l (the tobramycin concentration equivalent to three times the signal-to-noise value for the zero calibrator) and the limit of quantification (RSD$<$20%), derived from the precision profile curve, was 0.15 mg/l (Fig. 2).

The stability of the extracted materials in the supernatant was tested by repeat analysis of an extracted sample (tobramycin concentration 6.3 mg/l) over a 12-h period. No systematic loss in sensitivity was observed in the peak area ratio (analyte:internal standard) and the RSD for this ratio was 6%.

The within- and between-day precisions for tobramycin assessed on three separate pools, is shown in Table 2. The pools were made from samples routinely analysed for tobramycin within our laboratory and the same pools were used for both within- and between-day analyses. Within-day (RSD$<$6%) and between-day precisions (RSD$\leq$6%) were both acceptable. The mean recovery of tobramycin across a range of concentrations between 2.0 and 9.0 mg/l was 99.5%, range 93–105%. The time taken to process a batch of 20 samples including controls and calibrators was 1.5 h.

In order to examine any suppression of ionisation,

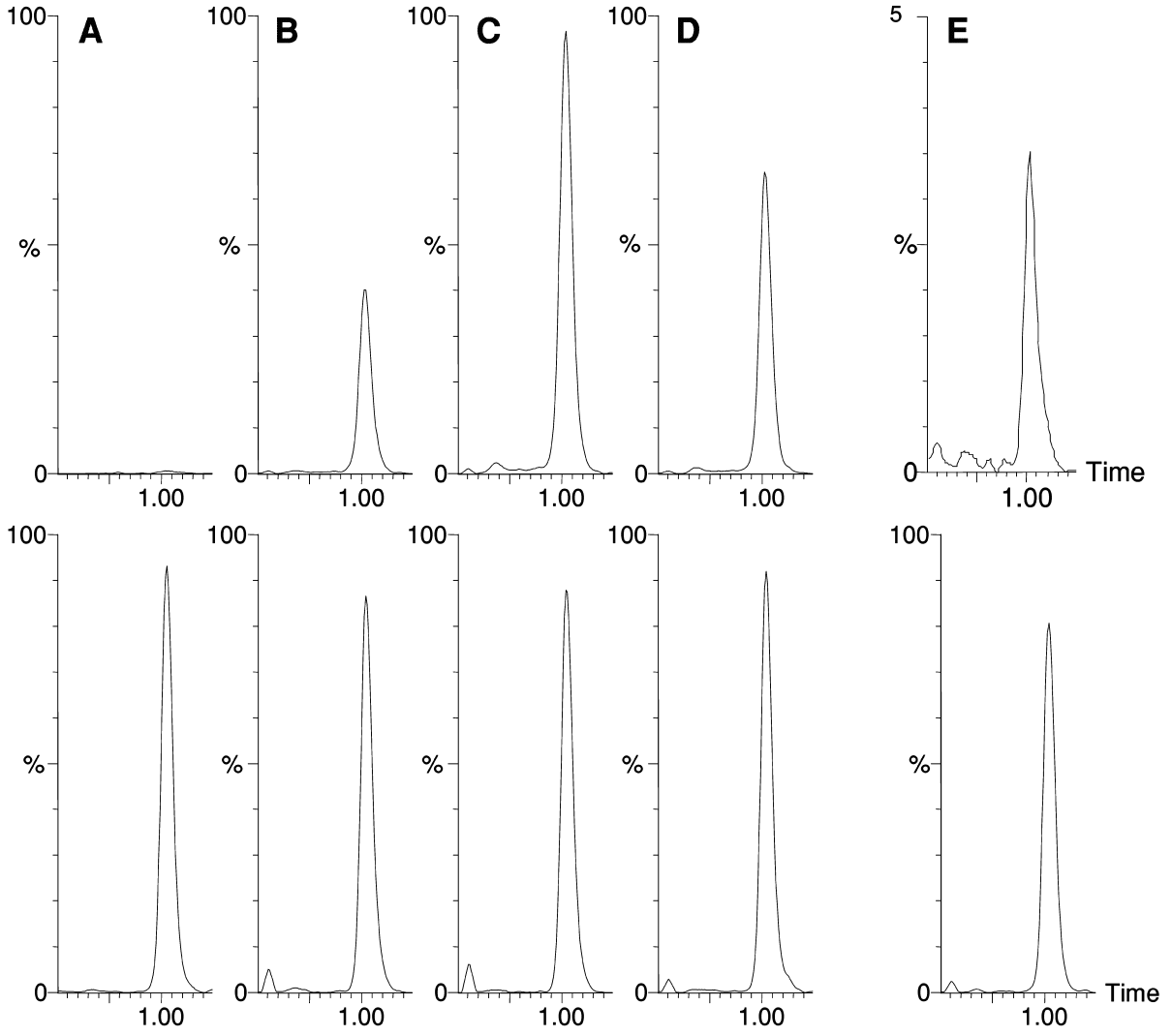

Fig. 1. LC–MS–MS chromatograms for tobramycin (upper traces; m/z 467.8>163) and for sisomycin internal standard (lower traces; m/z 447.8>160). (A) Zero calibrator; (B) 4.0 mg/l calibrator; (C) 10.0 mg/l calibrator, (D) and (E) patient samples found to contain 6.0 mg/l and 0.2 mg/l tobramycin, respectively. Chromatogram (E) is shown using a different scale to highlight the peak shape near the limit of quantification.

a series of six serum samples were spiked with tobramycin at concentrations of 2.3, 4.5 and 9.0 mg/l, and prepared in the standard way. In addition, a series of aqueous standards were prepared in triplicate at identical concentrations. There was no difference in the area counts or the ratios between serum samples and aqueous tobramycin solution indicating that there was no evidence of ion suppression. Bland Altman analysis [16] of the tobramycin concentration measured in the samples from 109 cystic fibrosis patients, by FPIA and LC–MS–MS, showed good agreement between the two methods with the LC–MS–MS assay having a small positive bias (0.25 mg/l, 95% confidence interval 0.16–0.34) (Fig. 3). The regression line (Passing and Bablock) was LC–MS–MS = 1.07 ± 0.01(FPIA) -0.02 ± 0.07, $R^2 = 0.98$, $S_{y/x} = 0.4$. Samples from the UKNEQAS [8] were also analysed by LC–MS–MS and the

Table 1
Drugs found not to interfere with the LC–MS–MS assay for tobramycin

Metoclopramide
Hydrochloride
Piperacillin
Meropenem
Tacrolimus
Mycophenolate mofetil
Prednisolone
Ranitidine
Co-trimoxazole
Colistin
Vitamin E
Ventolin
Creon
Ursodeoxycholic acid
Aminophylline
Hydrocortisone
Allopurinol
Omeprazole
Diltiazam
Doxazosin
Nystatin
Dnase
Ceftazadime
Flucloxacilin
Fluticasone propionate
Betamethasone
Ibuprofen
Erythromycin
paracetamol

results were compared with spiked target values and also method group means. The range of concentration measured was 0.2–15.4 mg/l ($n=22$) and the

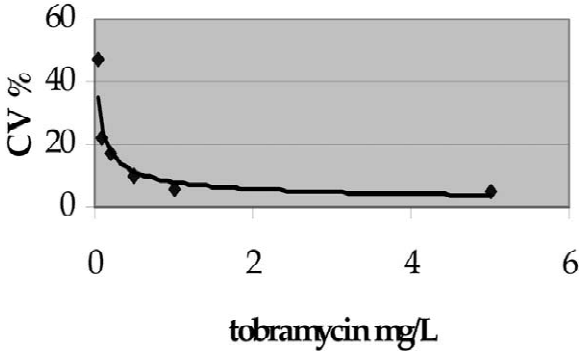

Fig. 2. Precision profile for tobramycin by LC–MS–MS. The RSD value was calculated from ten replicate injections of single extracts of serum calibrators.

Table 2
Analytical imprecision of LC–MS–MS assay; the minimal concentration of the low, medium and high samples was 1.0, 3.9 and 8.0 mg/l

	Tobramycin concentration (mg/l)			
	Intra-assay ($n=15$)		Inter-assay ($n=15$)	
	Mean±SD	RSD (%)	Mean±SD	RSD (%)
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±0.04	4.0

regression equations were LC–MS–MS = 0.92±0.02 (NEQAS target value) −0.02±0.2, $R^2=0.99$, $S_y/_x=0.5$ and LC–MS–MS = 1.0±0.02 (NEQAS method mean) −0.02±0.2, $R^2=0.99$, $S_y/_x=0.4$.

4. Discussion

Aminoglycoside drugs are traditionally difficult to retain on conventional HPLC columns because of their highly polar characteristics. We had tried several different HPLC columns that utilise modern embedded phase technology and which allow the use of highly aqueous mobile phases, but without success. We therefore decided to use an ion pair reagent to prolong the retention of tobramycin on the column and thereby minimise interference in the assay.

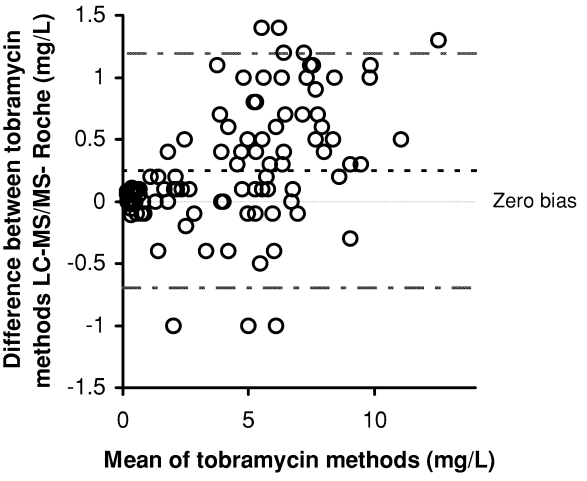

Fig. 3. Bland Altman difference plot of tobramycin concentrations measured by fluorescence polarisation (Roche Integra) and LC–MS–MS.

HFBA was chosen because it has been shown to exhibit better retention of aminoglycosides on the analytical column and is also better at enhancing the electrospray process than other ion pair reagents [14]. The mobile phase used a step gradient with a decreasing concentration of HFBA as solvent B does not contain this component. Tobramycin and sisomycin are therefore eluted into the mass spectrometer at nearly zero HFBA concentration. Nevertheless, we did see some loss of sensitivity in the assay caused by signal suppression, but the limit of quantification was still more than adequate for monitoring tobramycin concentration in serum samples, and was still less than that of a recently published method using derivatisation with ultraviolet detection [11].

We have developed an assay for the rapid analysis of tobramycin in serum samples by the use of limited sample clean-up, a small chromatography column and a highly specific detector. The small analytical column provides sufficient separation to remove interfering material, thereby reducing ion suppression, and the detector specificity allows simultaneous measurement of superimposed peaks. In this way sample preparation has been kept to a minimum and the throughput of the assay has been increased. We have also demonstrated that the injection of relatively large amounts of extract (20 μl) does not impair the detector, as shown by the steady detector response over a 12-h period. This is largely due to the dual-orthogonal design of the ion source causing diversion of potential contaminants away from sensitive parts of the instrument.

We used sisomycin as an internal standard because it is a structurally similar to tobramycin and should therefore behave in a similar manner during processing, it also has the advantage of not being a prescribed drug in the UK. The excellent accuracy of the method is shown by the agreement between the LC–MS–MS method and the FPIA assay and also with assigned values from UKNEQAS samples. Our results also show that lengthy sample clean-up procedures are not necessary and that the use of crude sample extracts provides the required accuracy and precision.

The use of deep well microtitre plates which fit directly onto the autosampler has further saved time by allowing the processing and injection of samples without having to transfer into autosampler vials, thus removing a transfer step. Reducing sample transfer steps also significantly reduces the chance of error. Direct injection of the supernatant from the sample block was accomplished by judicious positioning of the injector needle, this prevented contamination from the protein precipitate formed during the sample preparation. Using this procedure, 20 samples can be processed and are ready for injection onto the LC–MS–MS within 30 min. Assay time and hence result turnaround was further reduced by optimising the chromatography time. This has resulted in injection to injection times of 2.5 min and has enabled the processing of approximately 20 samples per hour, including result generation. The LC–MS–MS system can be safely left in standby mode when not in use and can be ready for operation in less than ten min, during which time sample preparation can commence.

Production of suitable calibrators can often provide problems when developing assays. We have shown that good results with the LC–MS–MS procedure were achieved when either in-house or commercially available calibrators designed for immunoassays were used. This will be advantageous to laboratories unable to make their own calibrators and will further save processing and analysis time.

The small sample size that we have developed should prove useful for monitoring paediatric patients and will also be sparing of expensive calibrator and QC materials.

Although we have developed and validated an assay for the measurement of tobramycin it should also be possible to simultaneously measure other aminoglycoside antibiotic drugs in serum samples. This will however require further validation. LC–MS–MS is an expensive technique in terms of procurement costs but it is extremely cost effective to run. The high capital costs of LC–MS–MS would probably make it an extremely expensive option to replace the FPIA method. Nevertheless, an increasing number laboratories are now using LC–MS–MS, to perform assays for immunosuppressive drugs and neonatal screening, and it is for these laboratories that it should prove a useful addition to their assay range.

5. Conclusion

We have developed an LC–MS–MS method for the measurement of tobramycin that is quick, precise and robust and will provide a fast turnaround of results for the requesting physician.

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