Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 862 (2008) 257–262
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Simple and sensitive method for quantification of low tobramycin concentrations in human plasma using HPLC–MS/MS

Milly E. Attema-de Jonge [a,*], Judith M. Bekkers [a], Heleen M. Oudemans-van Straaten [b], Rolf W. Sparidans [c], Eric J.F. Franssen [a]

[a] *Department of Pharmacy, Onze Lieve Vrouwe Gasthuis, Oosterpark 9, 1091 AC Amsterdam, The Netherlands*
[b] *Department of Intensive Care, Onze Lieve Vrouwe Gasthuis, Oosterpark 9, 1091 AC Amsterdam, The Netherlands*
[c] *Utrecht University, Faculty of Science, Department of Pharmaceutical Sciences, Section of Biomedical Analysis, Division of Drug Toxicology, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*

Received 11 July 2007; accepted 10 December 2007
Available online 4 January 2008

Abstract

After oral administration of tobramycin, as part of selective decontamination of the digestive tract (SDD) in critically ill patients, absorption of tobramycin from the gut into the blood may take place. To quantify low concentrations of tobramycin in human plasma, we developed and validated a simple (sample pre-treatment consisting of protein precipitation with acetonitrile using 200 μl plasma), rapid (runtime 3 min using a Pathfinder MR reversed-phase column) and sensitive (concentration range of 0.05–1.0 mg/l using MS/MS detection) method.

Keywords: Tobramycin; Sisomicin; HPLC–MS/MS; Selective decontamination of the digestive tract

1. Introduction

Tobramycin is an aminoglycoside antibiotic widely used against Gram-negative bacterial infections. In combination with polymyxin E and amphotericin B, tobramycin is regularly used for local application in the oral cavity and stomach as part of selective decontamination of the digestive tract (SDD). The approach aims to eradicate colonisation of aerobic potentially pathogenic micro-organisms from the oropharynx, stomach, and gut, while leaving the indigenous anaerobic flora largely undisturbed. Treatment of intensive care unit (ICU) patients with SDD has been shown to reduce the number of Gram-negative and fungal infections and to reduce ICU and hospital mortality [1–3].

If the gut barrier function is intact, the three orally administered antibiotics exert their effect in the digestive tract and are not absorbed. However, several conditions in the critically ill, such as sepsis or shock, can increase intestinal permeability [4,5]. This may result in the permeation of the locally administered antibiotics from the gut into the blood. Permeation will most likely occur with tobramycin, since its molecular weight is the lowest. If tobramycin would indeed attain significant concentrations in the blood, the drug may cause damage to the kidney and the internal ear [6]. To investigate if and to what extent tobramycin is absorbed from the digestive tract in critically ill patients treated with SDD, a sensitive method for the quantification of low concentrations tobramycin in plasma is necessary.

Determination of aminoglycosides using conventional high-performance liquid chromatography (HPLC) with spectroscopic detection is difficult because of the lack of strong chromophores in the molecule structures. Therefore, traditionally, analysis of aminoglycosides has been performed using pre-column derivatization with ultraviolet or fluorescent detection [7–14] or post-column derivatization with fluorescent detection [7,15]. However, derivatization methods require complicated sample preparation procedures and incomplete or unstable derivatization may be a source of error in the method. Moreover, the sensitivity of these techniques is relatively low. An alternative for the detection of aminoglycosides is mass spectrometry. This detection technique is highly selective and specific, and therefore permits the use of short run times and minimal sample clean-up procedures. Mass spectrometric detection (MS) [16,17]

* Corresponding author. Tel.: +31 205993026; fax: +31 205993355.
E-mail address: m.e.attema-dejonge@olvg.nl (M.E. Attema-de Jonge).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.12.008

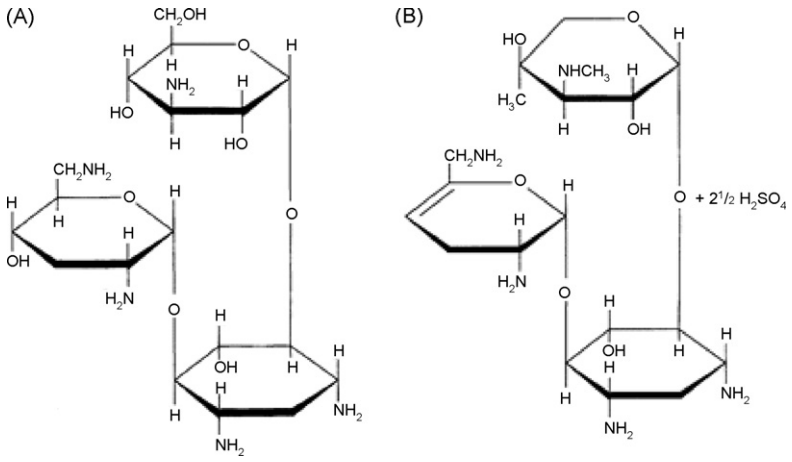

Fig. 1. Chemical structures of (A) tobramycin and (B) sisomicin sulphate.

and tandem mass spectrometric detection (MS/MS) [18–22] of aminoglycosides in biological matrices has been described previously, two of these focussing on tobramycin [18,22].

Aminoglycosides are extremely hydrophilic compounds because they possess a high number of amino and hydroxyl moieties on the carbon chain (Fig. 1). Due to their highly polar characteristics, the use of simple chromatographic methods for separation are not applicable: aminoglycosides are positively charged at the pH range employed in reversed-phase HPLC and are not retained on conventional C-18 bonded silica columns without an ion pairing reagent. Ion-pair chromatography has been described to prolong the retention of aminoglycosides [7,15–17,20–23]. Although electrospray ionization (ESI) MS detection of ion pairs is not ideal because the sensitivity of mass spectrometry will be reduced (due to suppression of ionization), volatile fluorinated ion-pair agents are compatible with ESI-MS [16,17,20–22]. An alternative for separation of hydrophilic compounds is hydrophilic interaction chromatography (HILIC). A recently published method used HILIC with MS/MS detection for tobramycin as an alternative for fluorinated ion-pair agents [18].

Published chromatographic methods for the quantification of tobramycin in human plasma report a LLQ suitable for regular therapeutic drug monitoring: 0.1 [18], 0.15 [22], 0.2 [12,13], 0.3 [14], 0.5 [9], 0.6 [23], 0.9 [11], 1.0 [10], and 2 mg/l [15]. In this article we present a simple, rapid and, above all, sensitive method (LLQ 0.05 mg/l) for the quantification of tobramycin in plasma using HPLC–MS/MS.

2. Experimental

2.1. Chemicals

Tobramycin (Fig. 1A) was purchased from BUFA (Uitgeest, The Netherlands; purity 91.4%). The internal standard (IS) sisomicin sulphate (Fig. 1B) originated from Sigma–Aldrich (Zwijndrecht, The Netherlands). Acetonitrile (HPLC-grade) was obtained from J.T. Baker (Deventer, The Netherlands). Ammonium acetate was purchased from Merck Generics (Bunschoten, The Netherlands). For selectivity and specificity tests, acetaminophen, polymixin B sulphate, amphotericine B and morphine hydrochloride originated from BUFA (Uitgeest, The Netherlands), and caffeine originated from Sigma–Aldrich (Zwijndrecht, The Netherlands). In-house distilled water was used throughout the analysis. Drug-free human plasma originated from Stichting Sanquin Bloedvoorziening (Amsterdam, The Netherlands).

2.2. Chromatographic and mass spectrometric conditions

A Thermo Finnigan (San Jose, CA, USA) Surveyor HPLC system was used, equipped with a Surveyor autosampler. Separation was carried out on a Pathfinder MR (C20C4) column, 150 mm × 4.6 mm, particle size 3.5 μm (Shimadzu, 's-Hertogenbosch, The Netherlands). A mixture of 95% (v/v) 2 mM ammonium acetate (pH 3.2) and 5% (v/v) acetonitrile was used as mobile phase. The flow rate was 0.5 ml/min. The column temperature was maintained at 60 °C. The column outlet was directly coupled to the ESI sample inlet of a Thermo Finnigan TSQ Quantum Discovery MAX triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The temperature of the heated capillary (ion tube) was set at 330 °C. The capillary voltage was 35 V. The electrospray source was operated in the positive ion mode. A source voltage of 3500 V was applied to the ESI needle, the tube lens offset voltage was 90 V. Nitrogen gas served as both sheat gas and the auxiliary gas, and argon served as the collision gas. Nitrogen sheath gas pressure was 49 (arbitrary units) and auxiliary gas pressure was 10 (arbitrary units). Collision gas was typically maintained at 1.5 mTorr.

Selected reaction monitoring (SRM) was used for drug quantification. Precursor ions were determined from spectra obtained during the infusion of standard solutions using an infusion pump connected directly to the electrospray source. Each of the precursor ions was subjected to collision-induced dissociation to determine the product ions. The transitions of the protonated precursor/product ion pairs that were used for recording the selected-ion mass chromatograms, were 468.2/163.0 for tobramycin and 448.2/160.0 for sisomicin, using collision energies of 23 and 25 V, respectively. An Xcalibur software package

(version 1.4, Thermo Finnigan) was used for instrument control, data acquisition and processing.

2.3. Sample collection, pre-treatment and processing

Whole blood samples were collected in critically ill patients receiving 4 times daily SDD (100 mg tobramycin, in the form of tobramycin sulphate, in each oral dose). Samples were collected in heparin Vacutainer tubes, cooled and centrifuged at $2800 \times g$ for 10 min. The plasma was removed and stored in polypropylene tubes at −20 °C until analysis (polypropylene tubes were used for storage because of the strong tendency of aminoglycosides to bind to glassware [21]).

For analysis, 50 μl of the IS solution (containing approximately 3 mg/l sisomicin in water) was added to 200 μl of plasma. After vortex-mixing for 10 s, 300 μl of acetonitrile was added. The mixture was vortex-mixed for 30 s, and then centrifuged at $2800 \times g$ for 10 min. The supernatant was transferred into a glass vial and a volume of 10 μl was injected onto the analytical column.

Ion suppression due to matrix effects was determined by evaluating the effect of injection of a pre-treated drug-free plasma sample onto the chromatographic column on the signal produced by the continuous infusion of a standard tobramycin or sisomicin solution into the electrospray source.

2.4. Preparation of stock solutions, working solutions and plasma standards

Two fresh stock solutions of tobramycin were prepared independently in water at a concentration of approximately 100 mg/l. One solution was used to spike the plasma calibration samples and the other was used to prepare the quality control (QC) samples. The stock solutions were diluted further with water to obtain working solutions. After spiking 900 μl of drug-free human plasma with 100 μl of the working solutions, the following tobramycin concentrations in plasma were obtained: 0.05, 0.1, 0.3, 0.5 and 1 mg/l. QC samples were prepared in a similar way, resulting in concentrations of 0.05 (LLQ), 0.1 (low), 0.3 (medium) and 0.8 (high) mg/l. Volumes of 200 μl of each calibration sample were processed as described for the plasma samples.

2.5. Validation procedures

2.5.1. Linearity

Calibration standards were prepared and analysed in duplicate in three independent runs. Calibration curves (area ratio to the IS versus nominal analyte concentration) were fitted by least-squares linear regression. To assess linearity, deviations of the mean calculated concentrations over three runs should be within ±15% from nominal concentrations. At the LLQ level a deviation of ±20% was permitted.

2.5.2. Accuracy and precision

Inter-assay accuracy and intra- and inter-assay precisions of the method were determined by assaying five replicates of each of the QC samples with analyte concentrations in the LLQ, low, medium and high concentration ranges in three separate analytical runs. The QC samples were prepared from a different drug-free biological batch compared to the calibrators.

Inter-assay accuracy was determined as the percentage difference between the mean concentration after three analytical runs and the nominal concentration. Accuracy should be within 15% except at the LLQ concentration, where it should be less than 20%.

The coefficient of variation (CV) was used as a measure for intra- and inter-assay precision. Precision should not exceed 15% CV except for the LLQ where it should not exceed 20% CV.

2.5.3. Specificity and selectivity

Out of six batches drug-free human plasma, double blank samples (no analyte, no IS), blank samples (no analyte, with IS) and LLQ samples were prepared, processed and analysed to determine whether endogenous plasma constituents interfered with the assay. Interference may occur when co-eluting endogenous compounds produce ions at the same *m*/*z* values that are used to monitor the analyte or IS.

To investigate the potential interference of co-medication with the quantification of the analyte, frequently co-medicated drugs were added to LLQ samples at therapeutic relevant concentrations. The samples were then processed and assayed according to the described method. The following drugs were tested in a concentration of 1 mg/l: caffeine, acetaminophen, morphine, polymixin B and amphotericine B. Areas of peaks co-eluting with the analyte peaks should not exceed 20% of the area at the LLQ level. At the IS retention time the interference should not exceed 5% of the IS peak area.

2.5.4. Stability

The stability of tobramycin was investigated in plasma and in the final extract. Analytes were considered to be stable in plasma when 80–120% of the initial concentration was found. The long-term stability of tobramycin in plasma at −20 °C was studied by re-analysing previously measured low and high QC samples. Stability of freshly prepared low and high QC samples was assessed after three freeze (−20 °C)–thaw cycles. The concentrations of the analytes were related to the initial concentration as determined for the samples that were freshly prepared and processed immediately. The processed sample stability of tobramycin in the final extract after 24 h at room temperature was also assessed at low and high QC levels. The measured concentrations of the analytes in the stored processed samples were related to the measured concentrations of the same QC samples immediately after processing.

Stability of tobramycin in the stock solutions stored at 2–8 °C was also assessed, both when stored in glass and in polypropylene tubes. Stock solutions were considered to be stable when 95–105% of the original concentration was found.

3. Results and discussion

3.1. Sample pre-treatment

Sisomicin was used as internal standard because it is structurally similar to tobramycin and has similar physicochemical properties. Both compounds should therefore behave in a similar manner during sample processing.

For sample pre-treatment we focussed on non-labor-intensive methods to accelerate sample processing. Because aminoglycosides are not extensively bound to any particular plasma protein, a protein precipitation method could be validated using acetonitrile as precipitation agent. Protein precipitation recovery data for tobramycin using acetonitrile have been published previously, and were in the range of 93–105% [11,22]. This simple sample clean-up procedure was found to yield sufficiently clean extracts to allow proper quantification at the imposed LLQ and to allow reasonable HPLC column life-time.

3.2. Chromatography

The past years, new reversed-phase columns that utilise modern polar embedded phase technology, and which allow the use of highly aqueous mobile phases, have been introduced. The high hydrophilicity of tobramycin encouraged us to use one of these new reversed-phase columns with embedded polar groups (Pathfinder MR) for separation. Separation on this column is possible with a 100% polar mobile phase. The mobile phase used in our method consisted of a combination of 95% (v/v) 2 mM ammonium acetate and 5% (v/v) acetonitrile. This mixture allowed a better performance of the ion spray in MS/MS detection compared with 100% ammonium acetate. However, both the 100% ammonium acetate and the 95:5 (v/v) mixture with acetonitrile did not give any retention to the analytes.

Representative chromatograms of tobramycin and sisomicin in a QC sample are shown in Fig. 2. Both tobramycin and sisomicin had no retention and eluted at the same time (retention time of 2.2 min). Despite the fact that no retention was obtained for both tobramycin and sisomicin, signal suppression and matrix effects at the recorded retention times were acceptable considering the small and symmetrical peaks obtained and the high sensitivity of the method. Moreover, because of the high selectivity of the MS/MS method, HPLC separation of tobramycin and sisomicin is not necessary. The method allowed an injection-to-injection cycle of 3 min.

It was observed that column performance decreased after about one hundred injections. Column degradation was evidenced by a reduction of sensitivity or even disappearance of peaks. This could be explained by the relatively dirty extracts that were injected onto the column. Therefore, after each 25th sample, the column was washed with mobile phase and acetonitrile to remove remaining impurities. Heavily used columns tended to produce ghost peaks in the analysis of control samples. Also decrease of retention times was seen. Therefore, such a column was then replaced.

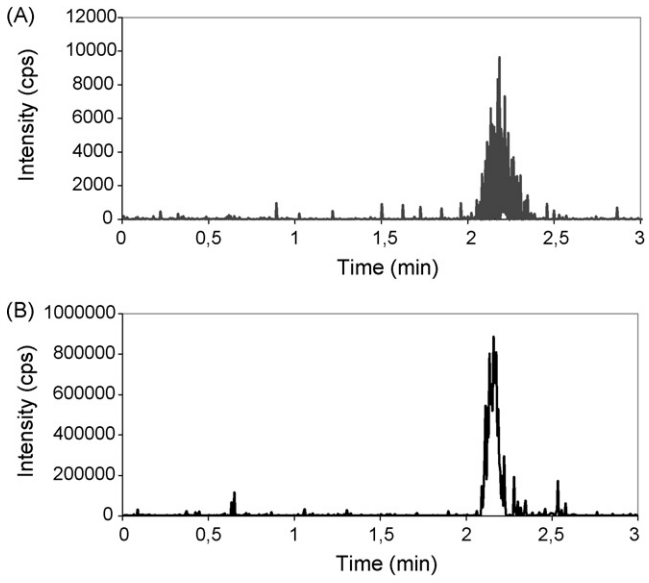

Fig. 2. Extracted single ion chromatograms of tobramycin (A, 54 μg/l) and sisomicin (B, approximately 1 mg/l) in a processed quality control sample at the LLQ.

3.3. Mass spectrometry

In Fig. 3, mass spectra of tobramycin and sisomicin are shown. In the mass spectra, peaks corresponding to the pro-

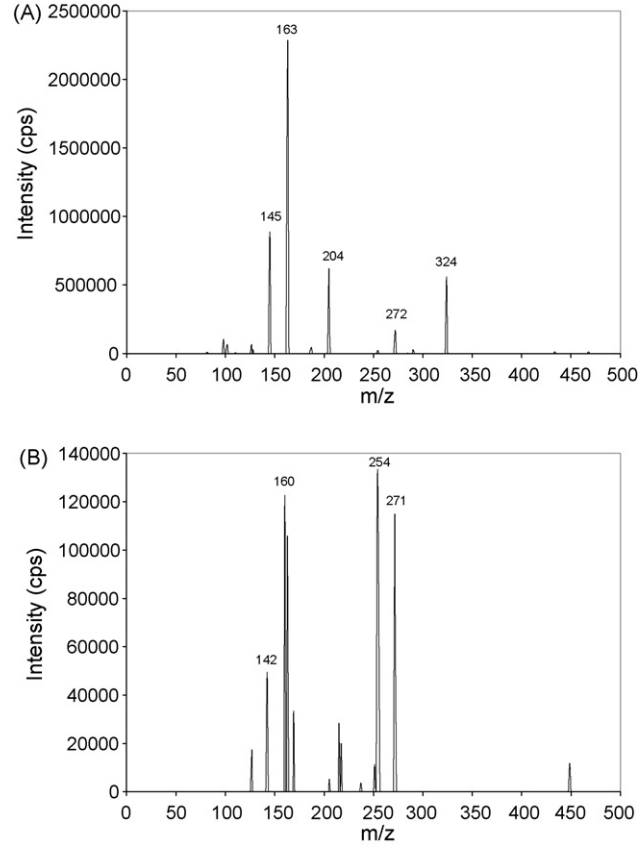

Fig. 3. Mass spectra of tobramycin (A, parent *m/z* 468) and sisomicin (B, parent *m/z* 448).

Table 1
Intra- and inter-assay performance data of tobramycin at four concentration levels in three analytical runs

Nominal concentration (mg/l)	Measured concentration (mg/l)	Inter-assay accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
0.053	0.052	−8.5	7.2	11.5
0.107	0.106	−3.8	5.6	8.3
0.320	0.302	−7.9	10.0	10.2
0.853	0.855	1.6	11.4	10.8

tonated molecular ions $(MH)^+$ are observed. Abundant product ions of tobramycin were seen at *m/z* ratios of 324.1, 163.0 and 145.1, and for sisomicin at *m/z* ratios of 271.4, 253.9 and 160.2. These product ions are a result of cleavage of the glycosidic bonds and the subsequent loss of the aminosugar rings.

During usage of the developed method, the ion tube tended to get polluted with brown material. The pollution could be the result of the injection of the relatively dirty extracts on the chromatographic column. As a result of pollution of the ion tube, ghost peaks were produced in the analysis of control samples. Therefore, the ion tube was cleaned or replaced frequently, and a divert valve was used to divert compounds, eluting before and after tobramycin and sisomicin, to waste.

Ion suppression due to matrix effects appeared to decrease the MS signal from 2.2 to 3.0 min after injection, with a maximum suppression at 2.6 min. Ion suppression appeared to have little or no effect on the detection of tobramycin and sisomicin, most likely because the analytes eluted before significant suppression was observed. The absence of significant ion suppression after 3 min after injection allowed a run time of 3 min.

3.4. Validation procedures

3.4.1. Linearity

The assay was linear over the validated concentration range of 0.05–1.0 mg/l. The deviations from the nominal concentrations were <12.5% at all concentrations except the LLQ, and <20% for the LLQ. The calibration curves of the three validation runs were described with the equations $y = 8.1126x - 0.0275$, $y = 7.186x + 0.3828$ and $y = 8.107x - 0.3946$. Correlation coefficients of the calibration curves were 0.995, 0.975 and 0.964, respectively.

3.4.2. Accuracy and precision

The intra-assay and inter-assay performance data are presented in Table 1. Accuracies were within ±9% for the LLQ and within ±8% for the other concentrations. The inter-assay precision, expressed as CV, was <12% for all concentrations tested. The mean intra-assay precision also did not exceed 12%.

3.4.3. Specificity and selectivity

As could be expected with the use of a highly specific detection technique such as HPLC–(ESI)MS/MS, no chromatographic interferences were found from the co-medicated drugs tested in drug-free human plasma: LLQ samples spiked with the tested drugs could be quantified within the required 20% deviation. Moreover, SRM chromatograms of six batches of drug-free human plasma also contained no endogenous peaks co-eluting with tobramycin or sisomicin. LLQ samples, prepared in these six batches of human plasma, could be quantified within the required 20% deviation from the nominal concentration (range deviation 1.1–12.3%).

3.4.4. Stability

In Table 2, results of the stability tests for tobramycin are summarized. Tobramycin appeared to be stable in plasma at −20 °C for at least 1 month. This is consistent with a previous report [11]. Tobramycin was also stable in plasma after three additional freeze–thaw cycles. The stability of tobramycin in the final extract was guaranteed for at least 24 h at room temperature. Tobramycin in stock solution was stable for at least 1 month at a temperature of 2–8 °C, both when stored in glass as well as in polypropylene containers (data not shown).

3.5. Patient samples

The developed assay is successfully applied in our hospital to monitor unintended permeation of tobramycin from the gut into the blood in ICU patients treated with SDD. High-throughput analysis of tobramycin is possible because of both the simple sample pre-treatment procedure and the short run-time. Results obtained in 12 ventilated patients acutely admitted to the ICU (receiving 4 times daily SDD) in the first 24-h of treatment are shown in Fig. 4. From these results it is concluded

Table 2
Stability data of tobramycin

Matrix and conditions	Nominal concentration (mg/l)	Deviation (%)	CV (%)	No. of replicates
Plasma, 3 freeze–thaw cycles	0.109	19.6	6.1	3
	0.870	−7.7	15.0	3
Plasma, 1 month at −20 °C	0.109	−10.0	5.0	3
	0.870	−5.2	5.6	3
Final extract, 24 h at room temperature	0.105	−7.7	16.6	3
	0.841	1.2	5.0	3

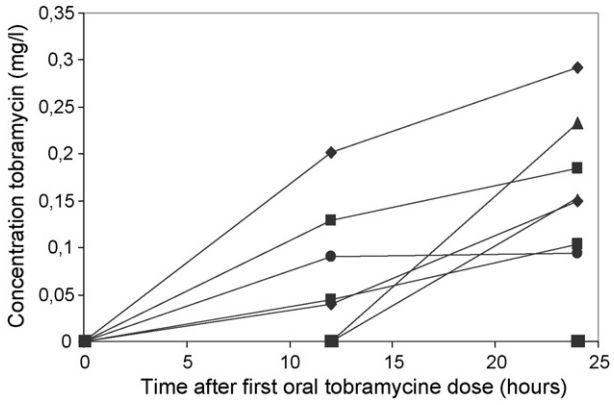

Fig. 4. Plasma concentrations of tobramycin in 12 critically ill patients in the first 24 h after treatment with 'selective decontamination of the digestive tract' (orally 4 times daily 100 mg tobramycin sulphate).

that absorption of tobramycin from the gut into the blood takes place in selected critically ill patients. The results also suggest that tobramycin concentrations rise over time. The clinical implication of sustained low tobramycin plasma concentrations after oral tobramycin treatment remains to be investigated.

4. Conclusions

We developed and validated a rapid, simple, sensitive and specific assay for the quantification of tobramycin in human plasma using HPLC–MS/MS, requiring 200 μl of plasma. A simple protein precipitation sample clean-up procedure was found to yield sufficiently clean extracts to allow for quantification down to concentrations of 0.05 mg/l. Detection of tobramycin using ESI-MS/MS allowed significant sensitivity and avoided any necessary laborious and hardly controllable derivatization steps.

Acknowledgements

We thank Arnold Kruise and Martijn Brancart for their skilful technical assistance.

References

[1] A. Liberati, R. D'Amico, S. Pifferi, V. Torri, L. Brazzi, Cochrane Database Syst. Rev. (2004) CD000022.
[2] E. de Jonge, M.J. Schultz, L. Spanjaard, P.M. Bossuyt, M.B. Vroom, J. Dankert, J. Kesecioglu, Lancet 362 (2003) 1011.
[3] C.P. Stoutenbeek, H.K. van Saene, R.A. Little, A. Whitehead, Intensive Care Med. 33 (2007) 261.
[4] H.M. Oudemans-van Straaten, P.G. Jansen, F.J. Hoek, S.J. van Deventer, A. Sturk, C.P. Stoutenbeek, G.N. Tytgat, C.R. Wildevuur, L. Eysman, J. Cardiothorac. Vasc. Anesth. 10 (1996) 187.
[5] S. Lal, A. Teubner, J.L. Shaffer, Aliment. Pharmacol. Ther. 24 (2006) 19.
[6] E.J. Begg, M.L. Barclay, Br. J. Clin. Pharmacol. 39 (1995) 597.
[7] D.A. Stead, J. Chromatogr. B 747 (2000) 69.
[8] N. Isoherranen, S. Soback, Clin. Chem. 46 (2000) 837.
[9] D.M. Barends, C.L. Zwaan, A. Hulshoff, J. Chromatogr. 225 (1981) 417.
[10] S.E. Bäck, I. Nilsson-Ehle, P. Nilsson-Ehle, Clin. Chem. 25 (1979) 1222.
[11] C.H. Feng, S.J. Lin, H.L. Wu, S.H. Chen, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 780 (2002) 349.
[12] P.M. Kabra, P.K. Bhatnagar, M.A. Nelson, J.H. Wall, L.J. Marton, Clin. Chem. 29 (1983) 672.
[13] D.B. Haughey, D.M. Janicke, M. Adelman, J.J. Schentag, Antimicrob. Agents Chemother. 17 (1980) 649.
[14] H. Fonge, E. Kaale, C. Govaerts, K. Desmet, A. van Schepdael, J. Hoogmartens, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 810 (2004) 313.
[15] J.P. Anhalt, S.D. Brown, Clin. Chem. 24 (1978) 1940.
[16] L.G. McLaughlin, J.D. Henion, J. Chromatogr. 91 (1992) 195.
[17] C. Lecároz, M.A. Campanero, C. Gamazo, M.J. Blanco-Prieto, J. Antimicrob. Chemother. 58 (2006) 557.
[18] R. Oertel, V. Neumeister, W. Kirch, J. Chromatogr. A 1058 (2004) 197.
[19] D.N. Heller, J.O. Peggins, C.B. Nochetto, M.L. Smith, O.A. Chiesa, K. Moulton, J. Chromatogr. B 821 (2005) 22.
[20] L.G. McLaughlin, J.D. Henion, P.J. Kijak, Biol. Mass Spectrom. 23 (1994) 417.
[21] M. Cherlet, S.D. Baere, P.D. Backer, J. Mass Spectrom. 35 (2000) 1342.
[22] B.G. Keevil, S.J. Lockhart, D.P. Cooper, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 794 (2003) 329.
[23] N.C. Megoulas, M.A. Koupparis, Anal. Bioanal. Chem. 382 (2005) 290.