Exploring strategies to individualize treatment with aminoglycosides and co-trimoxazole for MDR Tuberculosis
Dijkstra, Jacob Albert

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Quantification of Amikacin and Kanamycin in Serum Using a Simple and Validated Liquid Chromatography-Tandem Mass Spectrometry Method

J.A. Dijkstra
M.G.G. Sturkenboom
K. van Hateren
R.A. Koster
B. Greijdanus
J.W.C. Alffenaar
ABSTRACT

Amikacin and kanamycin are frequently used in the treatment of multidrug resistant tuberculosis. The commercially available immunoassay is unable to analyze kanamycin and through levels of amikacin. The objective was therefore to develop a LC-MS/MS method for the quantification of amikacin and kanamycin in human serum.

The presented LC-MS/MS method meets the recommendations of the FDA with a low lower limit of quantification (LLOQ) of 250 ng/mL for amikacin and 100 ng/mL for kanamycin. No statistical significant difference was found between the LC-MS/MS and the immunoassay of amikacin (Architect, P = 0.501).

The lower LLOQ of amikacin and the ability to analyze kanamycin makes the LC-MS/MS method the preferred method for analyzing these aminoglycosides.

INTRODUCTION

Tuberculosis (TB) is an infectious disease that kills almost 2 million patients every year. Multidrug resistant tuberculosis (MDR-TB) is caused by strains of Mycobacterium tuberculosis resistant to at least two first line drugs: rifampin and isoniazid. Annually, 500,000 patients get infected with an MDR-TB strain. The aminoglycosides amikacin and kanamycin are both frequently used for the treatment of MDR-TB, since minimal inhibitory concentrations (MIC) range from 1,000 to 4,000 ng/mL. However, a reliable, robust method without extensive sample pre-processing to measure both aminoglycosides in plasma or serum is not available until now.

The effective pharmacodynamic parameter for aminoglycosides is the peak serum concentration (C_{max}) over MIC. To reduce the emergence of resistance and to optimize the efficacy, a C_{max}/MIC ratio of ≥ 8 is preferred. Common side effects associated with aminoglycosides therapy are ototoxicity and nephrotoxicity, both possibly related to the treatment duration and the cumulative dose. Furthermore, a study with human subjects showed a significant correlation between the average trough level (C_{min}) of aminoglycosides and auditory toxicity. This effect could be more important in patients with TB, whom are often treated for months with aminoglycosides. Although evidence is limited, monitoring and minimizing trough levels seems justifiable.

Therefore, both C_{max} and C_{min} are important parameters in therapeutic drug monitoring (TDM) of aminoglycosides. TDM may help to optimize the dose if measured serum concentrations are not within the desired therapeutic range. To enable TDM, a specific and sensitive method of analysis is required. There is no commercially available immunoassay for the quantification of kanamycin. Furthermore, the immunoassay available for amikacin has a high lower limit of quantification (LLOQ) of 1500 ng/mL.

In addition, the available immunoassay is less versatile than newer methods of analysis, such as liquid chromatography tandem mass spectrometry (LC-MS/MS). The Stop TB Partnership proposed in the Global Plan to Stop TB 2011-2015 that laboratory strengthening is one of the key components in the fight against TB. With one single LC-MS/MS, TDM could be performed for many drugs, improving efficacy and reducing toxicity, thereby hopefully increasing compliance.

Literature about analysis of amikacin and kanamycin in serum using LC-MS/MS is scarcely available. A published LC-MS/MS method used solid-phase extraction (SPE) with different types of columns, but this is a time-consuming method with runtimes up to 12 minutes. Furthermore, no internal standard (IS) was used. The authors used only external calibrations.

Baietto et al. developed a sample preparation method without SPE for LC-MS/MS. Unfortunately, this method was not validated for kanamycin. Moreover, this method requires an additional dilution step and used quinoxaline as IS, which is not structurally related to amikacin.
or kanamycin. Therefore, both described methods are less able to compensate for sample and matrix variability.

A recent study described the simultaneous analysis of nine second-line anti-tuberculous drugs. Unfortunately, amikacin was not included in the method validation. Furthermore, the lower limit of quantification of kanamycin (LLOQ) was 2500 ng/mL and therefore relatively high.

To enable the use of TDM in the therapy with amikacin or kanamycin, a suitable, sensitive and robust method is required to analyse amikacin and kanamycin over a wide concentration range. The objective of this study is to develop and validate a simple and fast LC-MS/MS method for the quantification of amikacin and kanamycin in human serum for routine patient care and clinical studies.

MATERIALS AND METHODS

Analysis

Kanamycin and amikacin were purchased from Sigma Aldrich (St. Louis, MO, USA). Heptafluorobutyric acid anhydride (HFBAA) was obtained from Fluka (Sigma Aldrich, St. Louis, MO, USA). Water was purified with a Milli-Q system (Millipore Corporation, Billerica, MA, USA). Buffer solution consisted of 1% HFBAA in purified water. The anhydride formulation was already available in our laboratory. Trichloroacetic acid (TCA) was used in sample processing and was purchased from Merck (Whitehouse Station, NJ, USA). Eluent for liquid chromatography consisted of buffer solution, purified water and methanol (Lichrosolv®, Merck, Whitehouse Station, NJ, USA). IS stock solution consisted of 5000 ng/mL apramycin (Sigma Aldrich, St. Louis, MO, USA) in HFBAA (1%).

To 100 μL serum, 50 μL TCA was added. In addition, 50 μL IS stock solution was added. After each addition the sample was vortexed for 1 minute. The subsequent sample was centrifuged (5 minutes at 11,000 rpm) and the supernatant was transferred into a clear glass vial with a silicone/PTFE septum.

A tandem mass spectrometer was used to perform the analysis. The mass spectrometer was a Finnigan TSQ Quantum Discovery Max (TSQ Quantum, Thermo Fisher, San Jose, CA, USA), supplied with a Finnigan Surveyor MS Pump Plus and a Finnigan Autosampler Plus. Positive electron spray ionization was performed at 3500V. The liquid chromatography (LC) system was equipped with a Thermo Scientific Hypurity C18, 5.0 * 2.1 mm column with a particle size of 3 μm. Separation took place with gradient elution and a flow of 300 μL/min at approximately 100 bar. The gradient elution used is shown in table 1.

The autosampler was configured to an injection volume of 5 μL and a tray temperature of 10 °C. Nitrogen was used as sheath and auxiliary gas and argon was used as collision gas. Sheath gas pressure was set to 35 arbitrary units, auxiliary gas to 5 arbitrary units. Used mass transitions are shown in table 2.

Table 1. Gradient elution

<table>
<thead>
<tr>
<th>Minutes</th>
<th>HFBA (% in water)</th>
<th>Water</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5%</td>
<td>77.5%</td>
<td>17.5%</td>
</tr>
<tr>
<td>0.50</td>
<td>5%</td>
<td>50%</td>
<td>45%</td>
</tr>
<tr>
<td>1.20</td>
<td>5%</td>
<td>43%</td>
<td>52%</td>
</tr>
<tr>
<td>5.51</td>
<td>5%</td>
<td>77.5%</td>
<td>17.5%</td>
</tr>
<tr>
<td>6.00</td>
<td>5%</td>
<td>77.5%</td>
<td>17.5%</td>
</tr>
</tbody>
</table>

Table 2. MS/MS conditions and mass transitions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tube lens (V)</th>
<th>Collision energy (eV)</th>
<th>Mass transition (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>109</td>
<td>33</td>
<td>586.2 → 163.0</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>100</td>
<td>27</td>
<td>485.3 → 163.1</td>
</tr>
<tr>
<td>Apramycin (IS)</td>
<td>109</td>
<td>14</td>
<td>540.3 → 378.2</td>
</tr>
</tbody>
</table>
The immunoassay analysis for amikacin was performed using a validated immunoassay method (Architect, Abbott Diagnostics, Chicago, IL, USA). This method was validated based on the FDA guidelines [16]. The LLOQ of this method was 1500 ng/mL. Accuracy and precision was determined on 4 concentration levels (11,500, 6,500, 15,000 and 27,500 ng/mL). The bias varied from -1.5% to 6.7% over all concentration levels. Within-run CV varied from 0.5% – 8.5% and between-run from 0.0 – 2.4%.

**Analytical method validation**

The method was validated based on to the Guidance for Industry Bioanalytical Method Validation, published by the Food and Drug Administration (FDA). Since the FDA guidelines lack requirements on the stability, a maximum bias of 15% was used according the EMA guidelines.

The calibration line consisted of 8 concentration levels: 250-500-2,500-5,000-10,000-15,000-20,000-25,000 ng/mL for amikacin and 100-500-2,500-5,000-10,000-15,000-20,000-25,000 ng/mL for kanamycin. The upper limit was chosen as the linearity of the calibration line was not sufficient above 25,000 ng/mL. Stock standards and working solutions were diluted with serum to get the desired concentrations. Samples with concentrations above 25,000 ng/mL were diluted to obtain a concentration within the limits of the calibration line. Four QC samples were used at LLOQ (100 and 250 ng/mL for kanamycin and amikacin), LOW (500 ng/mL), MED (10,000 ng/mL) and HIGH (20,000 ng/mL). Calibration lines, correlation coefficients and regression coefficients were calculated using one-way ANOVA. Concentrations of the samples were calculated based on the peak height ratio with the internal standard with the compiled formula.

Selectivity was determined by analyzing six serum samples, each obtained from a different pool of serum. The extent of ion suppression or enhancement was tested, during a constant infusion of amikacin or kanamycin with IS, by injecting six pooled serum samples. Accuracy was evaluated by replicate analysis of the QC samples at all four concentration levels (LLOQ, LOW, MED and HIGH) on three consecutive days. The mean difference between the nominal concentration and the experimental result should be <15% (LLOQ <20%) [16]. Precision was determined using the measurements originating from the accuracy evaluation. The coefficient of variation (CV) was calculated at each individual concentration level. The CV should be lower than 15% (LLOQ < 20%).

Recovery was determined by comparing the mean peak area of spiked blank serum with the peak area of the spiked extract serum. Recovery was determined at LOW, MED and HIGH levels of both amikacin and kanamycin. Matrix effect was assessed by dividing the peak area of spiked extract of blank serum by the peak area of spiked extraction fluid. Matrix effects were also determined at LOW, MED and HIGH concentration levels of amikacin and kanamycin. Both recovery and matrix effects were determined in five-fold.

Blank serum was spiked with amikacin or kanamycin at two concentration levels, LOW and HIGH. Stability was determined after three freeze-thaw cycles by measuring the spiked samples and assessing the difference with the nominal concentration. Short-term stability over 24 hours was tested at room temperature. The post-preparative stability was tested by analyzing spiked samples that had been placed in the autosampler for 24 hours. Concentrations were compared with calibration standards, which were freshly prepared on the day of the analysis. The mean of each stability test should be within 15% of the nominal concentration, according to EMA guidelines. To assess the dilution integrity, a solution of 25,000 ng/mL amikacin and kanamycin in serum was diluted in tenfold with serum to a 2,500 ng/mL solution and subsequently analyzed on three subsequent days.
Comparison between immunoassay and LC-MS/MS methods

To compare both methods, 17 clinical samples were measured with the new LC-MS/MS method and with the immunoassay. Only amikacin was compared, since the immunoassay is unable to analyze kanamycin.

The immunoassay analysis for amikacin was performed using a validated immunoassay method (Architect, Abbott Diagnostics, Chicago, IL, USA). This method was validated based on the FDA guidelines [16]. The LLOQ of this method was 1500 ng/mL. Accuracy and precision was determined on 4 concentration levels (11,500, 6,500, 15,000 and 27,500 ng/mL). The bias varied from -1.5% to 6.7% over all concentration levels. Within-run CV varied from 0.5% – 8.5% and between-run from 0.0 – 2.4%.

Clinical pilot

Routine TDM for TB drugs is performed for all patients with MDR-TB in the Tuberculosis Centre Beatrixoord, University Medical Center Groningen (Haren, the Netherlands). Routine TDM consists of sampling at 0, 1, 2, 3, 4 and 7 hours after administration. Medical charts were reviewed to detect patients that received amikacin or kanamycin as part of their TB treatment at our TB center and that were subjected to routine TDM. This study was evaluated by the local ethics committee (IRB 2013-492) and was according to the Dutch law allowed due to its retrospective nature.

Statistics

Validation parameters were calculated using a validated Excel sheet (Microsoft, Redmond, WA). Both the test for normality (Shapiro-Wilk test) as the Wilcoxon Signed Rank Test were performed using SPSS 20 (SPSS, Virginia, IL).

RESULTS

Method validation

Three chromatograms of the LOW concentration (500 ng/mL) of amikacin, kanamycin and apramycin are displayed in figure 1. The retention times of amikacin, kanamycin and apramycin were determined: 2.53, 2.62 and 3.33 minutes. At these retention times, no other peaks are observed in pooled serum samples. The validated LLOQs were 250 ng/mL for amikacin and 100 ng/mL for kanamycin. Ion suppression or enhancement was tested using the constant infusion test.[18] No ion suppression or enhancement was observed at the retention times of amikacin and kanamycin as displayed in figure 1.

Calibration of both amikacin and kanamycin was performed with eight concentration levels. Resulting calibration lines are displayed in table 3. Accuracy was determined by analyzing five different spiked samples at the four different QC concentration levels. Calculated biases were -8.8 – +7.0% and -6.7 – +5.6% for amikacin and kanamycin, respectively. All results are displayed in table 4. Precision was determined using data originating from the accuracy evaluation. The CV of the within-run and the between-run were calculated and are displayed in table 4. The CV varied between 1.7% – 6.7% for amikacin and 0.0% – 4.9% for kanamycin.
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Figure 1. Three chromatograms of a LOW (500 ng/mL) concentration of amikacin, kanamycin and apramycin and the ion suppression test

Table 3. Calibration curves

<table>
<thead>
<tr>
<th>Compound</th>
<th>Slope (± st. dev)</th>
<th>Intercept (± st. dev)</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin</td>
<td>0.761 ± 0.0168</td>
<td>-0.0545 ± 0.0105</td>
<td>0.995</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>0.997 ± 0.0143</td>
<td>-0.00390 ± 0.00391</td>
<td>0.997</td>
</tr>
</tbody>
</table>
The recovery of amikacin and kanamycin varied between 93.6 – 98.8%, as displayed in Table 4. Matrix effects were determined in five-fold and varied between 98.5 – 103.0%.

Stability of both analytes was assessed using the above proposed method. Measured concentrations differed from the nominal concentration with a maximal difference of -11.7 – +1.2% at room temperature, 1.3 – 7.4% in the autosampler and -2.6 – 10.8% after three freeze-thaw cycles.

Dilution integrity was assessed on three different days in fivefold. The bias found was -8.0% and 5.0% for amikacin and kanamycin, respectively. Within-run CV was 2.6%, between-run CV varied from 1.7% to 2.9%.

Comparison between immunoassay (Architect) and LC-MS/MS

Results of both methods for quantification of amikacin are compared using the Wilcoxon Signed Rank Test, since the data was not normally distributed (P < 0.001, Shapiro-Wilk test). Test results from the immunoassay which were below the detection limit, were assumed to be 0. No significant difference was found (P = 0.501, n = 17). Therefore, no significant difference was found between the outcomes of both methods. However, the LLOQ of amikacin is on the LC-MS/MS lower than on the immunoassay.

Clinical pilot

Full pharmacokinetic concentrations curves were obtained of 2 patients using amikacin and 4
patients using kanamycin, as shown in figure 2. This population consisted of five male patients and one female patient with a mean age of 33.7 (range 19 - 67). The mean BMI was 20.2 (range 13.0 – 26.5).

**DISCUSSION**

*Method validation*

This is the first LC-MS/MS method in literature to analyze amikacin and kanamycin in serum with minimal sample preprocessing. This LC-MS/MS method is validated on accuracy, precision, recovery, dilution integrity, matrix effect, autosampler stability, bench top stability and
freeze-thaw stability, based on the FDA guidelines. Concerning the stability, the FDA guidelines do not contain exact limits. Therefore, the limits of the EMA guidelines were applied.

The requirements which were demanded for this method complicated the development. In order to minimize the total processing time, SPE was avoided and a simple sample pre-processing method was developed. Furthermore, gradient elution was optimized to shorten the time of analysis. However, this method still has an adequate LLOQ for TDM and provides high sensitivity and selectivity.

The LLOQs are 250 ng/mL for amikacin and 100 ng/mL for kanamycin. These LLOQs are comparable to a previous study (100 ng/mL), but significantly lower in comparison with the method developed by Baietto et al. (2,340 ng/mL) and another recently published method (2.5 ng/mL). Furthermore, the LLOQ of amikacin was lower than the LLOQ of the immunoassay. The LLOQs found in this study are sufficient to detect any clinically relevant trough levels. The ability to measure below 1500 ng/mL makes this method suitable for detecting trends in rising trough levels early and to adjust the dose accordingly.

Another advantage of this method is that other second line TB drugs, such as linezolid, clarithromycin, and moxifloxacin, can be monitored using LC-MS/MS. This in contrast to the immunoassay, which provides only the quantification of amikacin. As described in the Global Plan to Stop TB, laboratory strengthening is one of the core components. With this single LC-MS/MS, TDM of these anti-TB drugs can be performed to monitor efficacy and possibly reduce toxicity. Further research could elucidate how to use dried blood spot to monitor aminoglycoside concentrations; simplifying storage and transport of blood samples, as already done for linezolid and moxifloxacin.

The sample processing method used in this study is less time-consuming than the SPE methods previously described. The recoveries found in this study were higher than in the study of Baietto (85.2%), who used similar sample preprocessing. The method of Baietto required an extra dilution step, which may introduce an additional error.

During this study, apramycin was used as IS. Since apramycin is structurally related to amikacin and kanamycin (figure 3a, b and c), it is our opinion that apramycin is suitable as IS. Apramycin is neither registered within the European Union nor in the United States for human use. A structural analogue as internal standard may not compensate as well for ion suppression effects as a deuterated internal standard. This may be because of the different retention time and ionization characteristics. However, the ion suppression tests showed that there was less than ±3% ion suppression, indicating that the developed method is free from ion suppression and that the used internal standard is suitable for its use.

Identical samples were measured with the immunoassay (Architect) and the new LC-MS/MS method. A Wilcoxon Signed Rank test has been used to compare the two methods. With this statistical test, no significant difference has been found in the measured concentrations.

The newly validated LC-MS/MS enables the use of TDM with amikacin or kanamycin. Moreover, the new method is more sensitive in comparison with the immunoassay, providing information about trough levels.

**CONCLUSION**

This new method enables one to analyse amikacin and kanamycin both with one single method of analysis in a robust and reliable way. Moreover, the LLOQ of this method is sufficient to measure trough levels of both aminoglycosides.
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Figure 3a. Chemical structure of apramycin

Figure 3b. Chemical structure of amikacin

Figure 3c. Chemical structure of kanamycin
REFERENCES


