A robust LC–MS/MS method for amikacin: application to cellular uptake and pharmacokinetic studies

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Background: Aminoglycosides are last-resort antibiotics for bacterial infections due to concerns of nephrotoxicity. A robust method is needed to correlate the magnitude of drug accumulation in the kidneys and the onset of nephrotoxicity. **Materials & methods:** A LC–MS/MS assay was developed, circumventing common limitations associated with conventional assays. To demonstrate its applicability, renal cellular uptake and rat pharmacokinetic studies were performed with amikacin. **Results:** To improve elution, the mobile phases were optimized with 60 mM ammonium hydroxide (pH = 11.2). An extended quantifiable range was achieved with different ionization modes. Kidney cells incubated with escalating amikacin concentrations showed increased uptake. Single-dose pharmacokinetics of amikacin were reasonably characterized. **Conclusion:** This assay will facilitate future studies on improving amikacin-associated nephrotoxicity.

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Multidrug resistance in Gram-negative bacteria such as *Pseudomonas aeruginosa* causes potentially life-threatening infections. The incidence of such infections has been rapidly increasing worldwide [1]. Many first-line antibiotics are no longer effective in treating these infections, leading to very poor patient outcomes [2]. Additionally, challenges in developing new antibiotics have further hindered combat of these infections. As a result of these obstacles, there has been a renewed interest in older drugs such as aminoglycosides.

The aminoglycosides are a class of broad-spectrum antibiotics that are highly effective against Gram-negative bacteria *in vitro*. However, these agents have been traditionally associated with severe nephrotoxicity, thus limiting their overall clinical use. Previous research has established that aminoglycosides accumulate in the kidneys via renal reabsorption and this accumulation is believed to play a key role in aminoglycoside-associated nephrotoxicity [3]. However, the correlation between the magnitude of accumulation and the onset of nephrotoxicity has not been fully established. Therefore, it is important to understand this relationship quantitatively in order to ultimately mitigate the associated nephrotoxicity.

In order to further examine aminoglycoside accumulation in the kidneys, developing a robust assay is necessary. Conventional assays such as immunoassays generally lack specificity due to the limitations of antibody binding as well as sensitivity [4]. On the other hand, LC is a popular assaying method due to its ability to separate and identify different but structurally-similar compounds [5]. Previously, successful assays have been developed for aminoglycosides, which used ion-pairing techniques for polar compounds when other more conservative methods failed [6]. Other current methods have several constraints including long running times [7], use of unconventional agents (e.g., perfluoropentanoic acid [8] or aminoethane [9]) in the mobile phases, and requires chemically modifying aminoglycosides if UV detection methods are used [10]. Therefore, this study seeks to develop an assay that is quick, simple and robust in order to facilitate *in vitro* as well as *in vivo* pharmacokinetic studies. To facilitate this objective, we focused on optimizing the mobile phases. Reagents were selected to reduce potential concerns associated with alternative strategies, such as using ion-pairing reagents (e.g., heptafluorobutyric acid [HFBA]) [11].

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In the clinical setting, gentamicin and amikacin are two of the most commonly administered aminoglycosides. Amikacin is semisynthetically derived from kanamycin and has been found to be effective against a wide range of strains of *Enterobacteriaceae* and *Pseudomonas* [12]. Amikacin is also believed to be less nephrotoxic than gentamicin [13]. While this assay was originally developed for amikacin, it can be easily extended to quantify several other aminoglycosides including tobramycin, kanamycin, neomycin and plazomicin. An illustration of the applicability of this assay is demonstrated by the quantification of amikacin concentrations in cell lysate and in rat serum samples.

Materials & methods

Chemicals & reagents

Amikacin sulfate (USP) powder was purchased from LKT Laboratories (MN, USA). Tobramycin (USP) powder, kanamycin sulfate (USP) powder and neomycin sulfate (USP) powder were purchased from Sigma-Aldrich (MO, USA). Plazomicin was obtained from Achaogen (CA, USA). LC–MS-grade water and acetonitrile were obtained from EMD Millipore (MA, USA). HPLC-grade ammonium hydroxide was purchased from Honeywell Fluka (NJ, USA). Heparin lock solution was obtained from SAI chemicals (IL, USA). Amikacin sulfate (USP) for injection was purchased from Henry Schein (OH, USA). PRiME MCX SPE cartridges (1 ml, 30 mg) were purchased from Waters (MA, USA). Chromatography grade methanol, isopropyl alcohol, formic acid, phosphoric acid and ammonium hydroxide for SPE were purchased from EMD Millipore (MA, USA). Chromatography grade ammonium formate was purchased from Sigma-Aldrich (MO, USA). Sterile rat serum was obtained from Equitech-Bio Inc. (TX, USA).

Cell cultures

Rat kidney proximal tubule (NRK-52E) cells were purchased from the ATCC (VA, USA). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with fetal calf serum from Thermo Fisher Scientific (MA, USA) and penicillin, in a humidified atmosphere of 37°C and 5% CO₂. Hank's balanced salt solution was purchased from Thermo Fisher Scientific.

Animals

Female Sprague–Dawley rats (225–250 g) were purchased from Envigo (NJ, USA). Housing and care of the animals followed all guidelines set forth by the Institutional Animal Care and Use Committee of the University of Houston.

Instrumentation & conditions

Ultra Performance Liquid Chromatography

Amikacin, tobramycin, kanamycin, neomycin and plazomicin were resolved using the Waters Ultra Performance Liquid Chromatography Acquity™ system with the following conditions: Acquity Ultra Performance Liquid Chromatography BEH C18 column (2.1 × 150 mm internal diameter, 1.7 μm) from Waters (MA, USA) maintained at 50°C, autosampler temperature of 20°C, flow rate of 0.25 ml/min and injection volume of 0.01 ml. The aqueous mobile phase (A) was ammonium hydroxide in water, and the organic mobile phase (B) was ammonium hydroxide in 100% acetonitrile. The following gradient was used, initial: 2% B, 0–0.5 min: 2% B, 0.5–0.6 min: 2–15% B, 0.6–1.6 min: 15–45% B, 1.6–2.1 min: 45–95% B, 2.1–2.7 min: 95% B, 2.7–2.8 min: 95–2% B, 2.8–6 min, 2% B. The initial period from 0–0.5 min and the last period from 2.8–6 min were the equilibrium times. The aqueous mobile phase was optimized by running a series of experiments with varying concentrations of ammonium hydroxide (10%) in water, from 10–80 mM ammonium hydroxide in water. A similar concentration of ammonium hydroxide in acetonitrile was used for the organic mobile phase.

Mass Spectrometry

An API 5500 QTrap Triple-Quadrupole mass spectrometer from Applied Biosystems/MDS SCIEX (CA, USA) that has a TurboIonSpray™ source was used to distinguish the analytes. The following settings were used: ion-spray voltage of 5.5 kV, ion source temperature of 400°C, gas 1 of 20 psi, gas 2 of 20 psi, curtain gas of 30 psi and the collision gas setting of medium. For initial method development, the multiple reactions monitoring (MRM) method in positive-ion mode was used. Multiple MRM transitions were examined for each analyte. The best transition pairs were m/z 586.0 → 264.0 for amikacin, m/z 469.1 → 163.1 for tobramycin, m/z 485.0 → 163.0 for kanamycin, m/z 614.9 → 456.0 for neomycin and m/z 593.3 → 434.2 for plazomicin. Additional parameters for each aminoglycoside are detailed in Table 1, including declustering potential, collision energy and collision cell exit potential. The aminoglycoside concentrations were determined by the Analyst 1.5.2 software (CA, USA). In

Table 1. Optimized MS parameters in positive ion mode.

Aminoglycoside	Declustering potential (volts)	Collision energy (volts)	Collision cell exit potential (volts)
Amikacin	157	40	4
Tobramycin	204	25	10
Kanamycin	200	25	10
Neomycin	250	30	12
Plazomicin	250	40	17

Table 2. Optimized MS parameters in negative ion mode.

Aminoglycoside	Declustering potential (volts)	Collision energy (volts)	Collision cell exit potential (volts)
Amikacin	-180	-36	-18
Tobramycin	-180	-31	-8

later method development, negative ion mode was also explored. The transition pairs were m/z 584.1 $\rightarrow$ 405.0 for amikacin and m/z 466.4 $\rightarrow$ 160.9 for tobramycin. Additional parameters for these aminoglycosides in negative mode are detailed in Table 2.

Preparation of standards & quality control samples

Cell lysate

Standard stock solutions of amikacin were first prepared in water. The working solutions for the standard curve were then obtained by serial dilution of the prepared stock solution in blank cell lysate. The final standard curve concentrations were 0.016, 0.031, 0.063, 0.125, 0.25, 0.5, 1, 2, 4 and 8 mg/l. A standard stock solution of the internal standard was prepared by dissolving the necessary amount of tobramycin in water, and diluting the solution to a final concentration of 10 mg/l. The concentrations of 0.125, 1 and 2 mg/l were used for quality control (QC).

Serum

Standard stock solutions of amikacin were prepared similarly as above. The standard curve was then obtained by spiking blank rat serum samples with 0.25, 0.5, 1.25, 2.5, 5, 10, 20, 40 and 80 mg/l of amikacin and a SPE procedure (details below) was used to prepare the standards for the assay. Similarly, a standard stock solution of the internal standard was prepared by dissolving the necessary amount of tobramycin in water, and spiking blank rat serum to a final concentration of 10 mg/l. The QC samples were prepared at low, medium and high amikacin concentrations (0.5, 5 and 40 mg/l).

Preparation of samples for quantification

Cell lysate

To prepare the cell lysate samples, 400 μ l of LC–MS-grade acetonitrile was added to 100 μ l of each cell lysate sample. The samples were vortexed and centrifuged at 11,000 RCF at 4°C for 15 min. The supernatants were recovered, blown to complete dryness under ambient air and stored at -80°C. Just before LC–MS/MS quantification, the dried samples were reconstituted with 100 μ l of LC–MS-grade water. The amikacin concentrations in these reconstituted samples were measured using the LC–MS/MS method and normalized to the total protein concentration of the samples.

Serum

Extraction by protein precipitation (as described above) was initially attempted but satisfactory recovery was not observed. Therefore, the serum samples obtained were prepared for the assay using the following SPE procedure. Each cartridge was conditioned with 1 ml of methanol followed by 1 ml of water. From each serum sample, 50 μ l were mixed with 50 μ l of 10 mg/l tobramycin (internal standard) in water and 100 μ l of 4% phosphoric acid. This mixture was then passed through the cartridge. The cartridge was then washed twice, first with 1 ml of 100 mM ammonium formate in 2% formic acid, then with 1 ml of 3:1 methanol: water (v/v). The final sample was then eluted with 1 ml of 1:1:1 isopropyl alcohol: methanol: water: concentrated ammonium hydroxide (v/v/v/v). The

final eluted sample was then dried under ambient air and was reconstituted with 200 µl of 95:5 water: methanol prior to assaying.

Method validation

Calibration curve & LLOQ

The calibration curves were prepared as described above. To determine linearity of the standard curve, the peak area ratio of amikacin versus tobramycin (internal standard) was plotted against the concentrations of known standards. A least-square linear regression method of $1/x^2$ weighing was used to calculate the slope, intercept and correlation coefficient of the linear regression equation. The LLOQ was defined by the following conditions as provided by the US FDA guidelines: peak response $\geq$ ten-times that of the blank response, precision within 20% and accuracy between 80 and 120% [14].

Precision & accuracy

Intraday/interday precision and accuracy were determined by running five replicates of the three concentrations described above on the same day, and on five different days, respectively. Subsequent standard curves in each experimental run were evaluated for reasonable variation before being used to quantify samples. This was done for both ion modes.

Extraction recovery & matrix effect

For both cell lysate and serum, extraction recovery was determined by comparing the peak areas obtained from blank serum samples spiked with the analyte and the internal standard before and after extraction. Similarly, the matrix effect was determined by comparing the peak area of spiked samples to the peak area of corresponding neat solutions.

Cellular uptake study

NRK-52E cells were grown to confluence in six-well plates. The DMEM complete growth medium was removed, the cells were washed once with fresh DMEM complete growth medium. The cells were then incubated with increasing concentrations (50, 200, 400 and 1600 mM) of amikacin for 1 h at 37°C in 5% CO₂. After incubation, the cells were washed with cold (4–7°C) Hank's balanced salt solution and recovered twice in 100 µl of deionized water. The cell suspensions were then sonicated for 30–45 min in a water sonicator set to 15–20°C, followed by centrifugation at 11000 RCF at 4°C for 15 min. After centrifugation, the supernatants were aliquoted. The total protein concentrations from each well were determined using the Pierce BCA protein assay kit. The cell lysate samples were stored at -80°C until assayed for drug content.

Pharmacokinetic study

Prior to the experiments, catheters were surgically placed in the jugular veins of the Sprague–Dawley rats to facilitate blood sampling. This study was approved by the Institutional Animal Care and Use Committee of the University of Houston. Each animal was given a single amikacin dose (100 mg/kg) subcutaneously (n = 4). Five blood samples were obtained serially from each animal, at 0.5, 1, 2, 4 and 6 h post dosing. Each blood sample was allowed to clot on ice, then centrifuged at 11,000 RCF at 4°C for 15 min. The serum samples were stored at -80°C until the LC–MS assay was performed. The average serum concentration at each time point was calculated after assaying. To derive the best-fit pharmacokinetic parameter estimates, a one-compartment bolus model with first order absorption was fit to the average concentration-time profiles using ADAPT 5 (CA, USA).

Results

Method optimization

The pH adjustment of the mobile phases with ammonium hydroxide allowed the aminoglycosides to be retained in its unionized form, which resulted in acceptable chromatography peaks, as seen in Figure 1. The final concentration of ammonium hydroxide was 60 mM in the aqueous mobile phase and for the organic mobile phase, which gave a final pH of 11.0–11.2. To further optimize the chromatography, a gradient that used a very low acetonitrile percentage (2%), for the first 0.6 min allowed for the separation of the aminoglycosides. The acetonitrile percentage was gradually increased to 95% to obtain symmetrical and sharp peaks. Representative chromatograms of all five aminoglycosides are shown in Figure 1. MRM scans were used to identify pseudomolecular and corresponding

Figure 1. Representative chromatograms of amikacin (A), tobramycin (B), kanamycin (C), neomycin (D) and plazomicin (E).

daughter ions, which allowed for subsequent identification of the peaks in the chromatograms. Representative MS² mass chromatograms for the analytes in positive ion mode are shown in Figure 2, and MS² mass chromatograms for amikacin and tobramycin in negative ion mode are shown in Figure 3.

Assay performance & validation

The linear range of amikacin standards in cell lysate was 0.016–8 mg/l and in serum was 0.25–80 mg/l. The R^2 value for the standard curves were >0.98 in all runs. The interday and intraday variability for QC samples were within 15%, which were well within the acceptable range recommended by FDA for assay validations. Subsequent standard curves used in all experimental runs were also checked with linear regression analysis for appropriate variability, which were $<15\%$ in all experiments.

Figure 2. MS2 full scans for amikacin (A), tobramycin (B), kanamycin (C), neomycin (D) and plazomicin (E) in positive ion mode.

Extraction recovery & matrix effect

The extraction recoveries of QC samples were 65–70% in cell lysate, and 57–77% in serum. With regard to the matrix effect, all the calculated values were <15% in cell lysate and <20% in rat serum. Overall, matrix effect for amikacin and the internal standard in either cell lysate or serum was not significant.

Amikacin cellular uptake kinetics

In view of the lower concentrations generally seen in cellular uptake studies, assaying the samples using positive ion mode yielded better results because of its significantly lower LLOQ. This lower LLOQ reduces the need for more complex sample preparation, and therefore protein precipitation is sufficient. In Figure 4, intracellular concen-

Figure 3. MS2 full scans for amikacin (A) and tobramycin (B) in negative ion mode.

Figure 4. Cellular uptake of amikacin in NRK-52E cells.
Note: 100 μM = 64 mg/l.

trations were successfully quantified and demonstrated increasing uptake with escalating amikacin concentration exposures.

Table 3. Best-fit pharmacokinetic parameters.

Parameter	Best-fit estimates
R^2	0.997
k_a (h^{-1})	1.20
k_{el} (h^{-1})	6.77
$t_{1/2}$ (h)	0.102
V (l/kg)	0.279
$\text{AUC}_{0 \rightarrow \infty}$ (mg.h/l)	53.0

R^2 : Coefficient of correlation; k_a : Absorption rate constant; k_{el} : Elimination rate constant; $t_{1/2}$: Terminal elimination half-life; V : Volume of distribution; $\text{AUC}_{0 \rightarrow \infty}$: Area under the concentration-time curve from 0 h to infinity.

Amikacin pharmacokinetics

Since higher concentrations were expected from the serum samples, using negative ion mode yielded a more efficient work flow (i.e., reduced need in diluting the samples to the linear detection range). The average serum drug concentration–time profile observed was reasonably well characterized by the one-compartment bolus model ($R^2 = 0.99$). The best-fit parameter estimates are shown in Table 3.

Discussion

Current methods for the quantification of aminoglycosides are limited. Developing a more efficient and robust method is vital for future studies to understand renal drug uptake/accumulation. Traditional chromatographic assays for the quantification of any aminoglycoside generally involve a lengthy derivatization process, or using unconventional reagents to resolve particular challenges associated with the chemical properties of aminoglycosides. For example, a recent successful assay opted for HFBA as an ion-pairing reagent to overcome retention time issues with the aminoglycosides [11]. However, HFBA could potentially contaminate the MS and may not be ideal for other assays being run on the same machine, especially those run in the negative mode. More conservative reagents are preferred with regards to the longevity of our shared mass spectrometer. Additionally, our method has a lower limit of detection (LLOQ), which is deemed desirable depending on the application of the assay.

Other methods utilize UV technology; however, aminoglycosides do not have chromophores, and therefore detection by UV light requires derivatization to structurally modify the aminoglycosides [15]. Additionally, due to the high polarity of aminoglycosides, unconventional reagents such as trichloroacetic acid, monopotassium phosphate and EDTA are often used to improve sensitivity as well as retention times [16]. Finally, the derivatization process and the use of such reagents in the mobile phases typically requires very long preparation/assay time for each sample, limiting the efficiency of the assay workflow.

Clinically, aminoglycosides are typically quantified by immunoassays, such as ELISA and enzyme multiplied immunoassay technique (EMIT), which rely on the binding affinity of antibodies [4]. Immunoassays are known to be limited by the specificity of the antibody, which often can bind to other related molecules, particularly analyte metabolites. These methods also generally have a LLOQ around 1–3 mg/l and a substantial extent of interday/intraday variability (>20%), making them less ideal than chromatographic methods. Immunoassays could also be problematic when a complex matrix such as blood or serum is being tested, as such matrices contain numerous biological molecules that could potentially bind to the antibodies (with variable binding affinity) due to structural similarity.

We have developed a method that is simple and efficient. Our method uses traditional LC coupled with MS to distinguish the aminoglycosides, and thus can be applied to multiple aminoglycosides. We used ammonium hydroxide to increase the pH of the mobile phases, so that the retention time was significantly improved. This approach kept the aminoglycosides from ionizing, and the unionized form bound more readily to the hydrophobic packing material until eluted by the organic mobile phase. This eliminated the need for any derivatization process and lengthy sample run times. Our assay used more common reagents to achieve conditions compatible with the native form of aminoglycosides. Additionally, this method has a higher sensitivity than derivatization-based methods because a complete chemical reaction to create a synthetic chromophore cannot be routinely guaranteed. Collectively, these modifications significantly improve both the specificity and sensitivity of the assay. This assay is versatile to quantify amikacin in different biological matrices and can be further optimized depending on which ionization mode is used. In the cellular uptake study, the LC–MS assay run in positive ion mode was able to detect

very low levels of amikacin. This could be used for future *in vitro* drug uptake studies. In the pharmacokinetic study, the LC–MS assay run in negative mode was able to satisfactorily quantify serum concentrations of amikacin in a clinically relevant range at different time points post dosing. The area under the concentration-time curve (AUC) is a surrogate representing the overall systemic drug exposure and could be used to correlate to the human dosing equivalents for subsequent studies.

There are several limitations of this method. Only partial separation of the analytes was achieved with a handful of aminoglycosides. Considerations for the mobile phase were primarily based on improving the retention time. Amikacin and other aminoglycosides (polar by nature) were eluted quickly together with the solvent front, and posed a great challenge in analyte separation. The impact of high pH on assay sensitivity was not specifically examined. However, based on the results reported, assay sensitivity by MS was not deemed to be a major problem. Additional aminoglycosides including those with multiple components (e.g., gentamicin) will be examined in the future. In addition, the performance of the assay has only been examined in cell lysate and serum, and further validation of this method in other biological matrices (e.g., kidney tissues, epithelial lining fluid and urine) will be pursued to facilitate upcoming biodistribution and pharmacokinetic studies.

Conclusion

A robust LC–MS method was developed and validated to quantify amikacin in different biological matrices. The versatility of this method would facilitate *in vitro* studies characterizing amikacin uptake kinetics in renal cells and *in vivo* studies characterizing various pharmacokinetic profiles in the future.

Future perspective

Antibiotic resistance continues to be a leading cause of death globally. In view of the decreasing momentum in the antibiotic development pipeline, physicians resort to older drugs that are still effective but associated with significant toxicity in dire cases. There is an urgent need to mitigate nephrotoxicity for patients who require the life-saving aminoglycoside antibiotics. The amikacin assay developed in this paper is a first step toward meeting this need. Results from cellular uptake and animal pharmacokinetic studies using this assay will help to elucidate the magnitude of renal accumulation, and potentially open a pathway for developing interventions to mitigate nephrotoxicity.

Executive summary

- Antibiotic resistance is a global problem with poor clinical outcomes.
- Amikacin is used only as a last-resort for multidrug resistant infections due to associated nephrotoxicity.
- Circumventing limitations associated with conventional assays, a LC–MS/MS assay was developed.
- Using high pH mobile phases improves retention time and analyte resolution.
- Different ionization modes can be used to expand the quantifiable range.
- This assay does not require complex derivatization, only uses mild chromatographic reagents, and works with different biological matrices.
- This assay has been applied to cellular uptake and animal pharmacokinetic studies with satisfactory results.
- This assay will be used in future studies to facilitate a better understanding of amikacin uptake in the kidneys.

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Disclaimer

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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