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# LC/MS/MS measurement of gentamicin in bovine plasma, urine, milk, and biopsy samples taken from kidneys of standing animals

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**Abstract**

Methods for the measurement of gentamicin concentration in several bovine tissues were developed and validated. A novel liquid chromatographic (LC) technique employed trifluoroacetic acid in the mobile phase so that all gentamicin components co-eluted. Analytes were ionized by positive-ion pneumatically assisted electrospray and detected by selected reaction monitoring (SRM) with an LC-tandem mass spectrometer (LC/MS/MS). Calibration of plasma and urine samples was based on tobramycin internal standard. Calibration of milk and kidney samples was based on external standard, due to variability of tobramycin response in these matrices. The extraction technique employed treatment with aqueous trichloroacetic acid to both precipitate protein and liberate gentamicin from the matrix. Milk samples had to be defatted by centrifugation prior to extraction. Urine samples were further cleaned up with C-18 solid phase extraction (SPE). These methods were validated for use in several residue depletion studies (reported elsewhere) to monitor the depletion of gentamicin in tissues under various dosing conditions. The plasma method was calibrated from 1 to 5000 ng/mL in two ranges, with a limit of quantitation (LOQ) in the low range calculated at 3.3 ng/mL. The milk method was calibrated from 2.5 to 2500 ng/mL with an LOQ calculated at 4.5 ng/mL. The urine method was designed for use at low levels, and was calibrated from 1 to 100 ng/mL with an LOQ of 3.8 ng/mL. The kidney method was primarily designed for analysis of small samples (approximately 100 mg). This method was calibrated from 10 to 50,000 ng/g with an LOQ of 26 ng/g. Published by Elsevier B.V.

*Keywords:* Gentamicin; Liquid chromatography-tandem mass spectrometry; Residue analysis; Quantification; Plasma; Urine; Kidney; Milk

## 1. Introduction

A set of methods was developed for measuring the concentration of gentamicin in bovine plasma, urine, milk, and kidney tissues after various dosing regimes. The goal of method development was to combine a relatively simple extraction procedure with liquid chromatography/tandem mass spectrometry (LC/MS/MS) for detecting gentamicin at relatively low levels. A series of interrelated residue distribution and depletion studies (to be reported elsewhere) called for methods with the following performance requirements: limit of quantitation (LOQ) in the 1–5 ng/mL range in plasma, urine, and milk; limit of quantitation in the 25–50 ng/g range in kidney; ability to work with very small kidney samples (approximately 100 mg); simple and reliable for application to large numbers of samples; and fast enough to provide results in 24 h.

Quantitative analysis of the antibiotic gentamicin poses several challenges to the residue chemist. Gentamicin consists of several components, each of which lacks chromophoric groups for spectroscopic detection. Analysis by liquid chromatography (LC) is only feasible with post-column derivatization or mass spectrometry. Gentamicin is not retained on conventional C-18 bonded silica columns without an ion pair agent, but these agents can suppress

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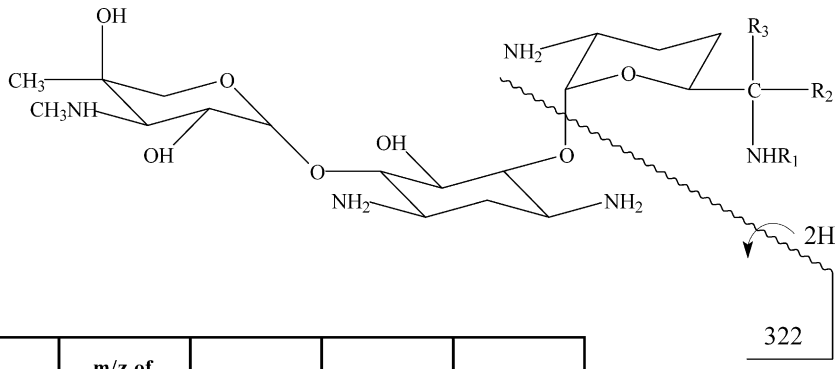


| Component | m/z of MH+ion | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| | | | | |
| C1 | 478 | $CH_3$ | H | $CH_3$ |
| C2, C2a | 464 | H | $(CH_3)$ | $(CH_3)$ |
| C1a | 450 | H | H | H |

Fig. 1. Structure and fragmentation of gentamicin components.

ionization and foul the LC/MS interface. Only fluorinated ion-pair agents are compatible with C-18 LC/MS analysis of aminoglycosides [1], although these can still create memory effects due their moderate volatility. A recently published method used hydrophilic interaction chromatography (HILIC) with LC/MS as an alternative to fluorinated ion pair agents [2].

Gentamicin consists of four components at three different molecular weights (Fig. 1). The relative composition is allowed to vary within predetermined limits [3]. In the present study, complete separation of gentamicin components was not considered necessary because the goal was to measure total gentamicin. Gentamicin is stable in aqueous acid and base solutions and is insoluble in organic solvents other than methanol. A mobile phase was developed using methanol combined with aqueous trifluoroacetic acid (TFA) so the four gentamicin components coeluted. A similar aminoglycoside (tobramycin) nearly co-eluted under these conditions, so it was chosen as an internal standard (Figs. 1 and 2).

The use of gentamicin in humans can lead to nerve and kidney damage, so knowledge of its depletion in animal tissues is important to ensure proper dosing of animals raised for human food. Kidney tissue binds gentamicin very strongly, and residues persist in kidney for many months after dosing. The U.S. tolerance for gentamicin in swine kidney is 400 ng/g; gentamicin is not approved by the U.S. Food and Drug Administration (FDA) for use in dairy cattle [4].

Figs. 1 and 2 show the molecular ions selected in the first stage of MS/MS along with the product ions formed by collision-induced dissociation (CID). One ion per component was chosen for selected reaction monitoring (SRM). The three ion chromatograms resulting from the four gentamicin components were summed before integration and calibration.

LC/MS/MS methods have been developed for determination of gentamicin in wastewater [5], human serum [2], swine and calf tissues [6], and for confirming the presence of gentamicin in milk [7] and kidney [8]. Other methods for gentamicin residues were based on post-column derivatization and HPLC [9], pre-column derivatization and HPLC [10,11], and cylinder plate microbiological assay [12]. In the absence of complex tissue matrices, quantitation of gentamicin in water was possible as low as 0.2 ng/mL in water [5]. However, the LOQs in tissues have generally been higher than the needs of the current studies, e.g., 100 ng/mL in human serum [2], 25 ng/g in animal tissues [6], 15 ng/mL in milk [9], 70 ng/mL in urine [10], 25 ng/mL in milk [12], or 250 ng/mL in urine [13].

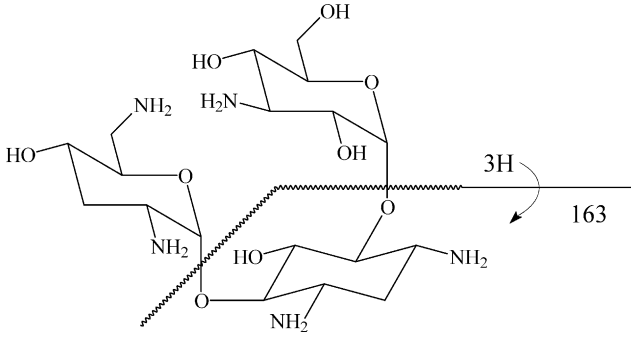


Fig. 2. Structure and fragmentation of tobramycin.

## 2. Experimental

### 2.1. Apparatus

The instrument was a Sciex API2000 benchtop triple quadrupole interfaced to two Perkin Elmer Series 200 mi-

cro pumps and a Perkin Elmer Series 200 autosampler. The LC column was a Waters ODS-AM, 3 mm × 150 mm, with 3.5 μm silica. The method used two benchtop centrifuges: Centra GP8-R, with 218A rotor capable of 5000 RPM (Thermo IEC), and Spectrafuge microfuge capable of 14,000 RPM for 1.5 mL tubes (Labnet). Solutions of aminoglycoside antibiotics must not be stored in glass, because they will bind tightly to the glass. Polypropylene volumetric flasks, centrifuge tubes (15 and 50 mL capacity, 17 mm × 100 mm Falcon tubes, #2059 with snap tops), and conical autosampler vials with 350 or 750 μL capacity were used. Eppendorf 1.5 mL centrifuge tubes (Brinkmann) were used with the microfuge. All liquids were measured and transferred with calibrated variable pipetters fitted with disposable polypropylene pipet tips (Eppendorf). An Omni TH tissue homogenizer fitted with 110 mm plastic probe tips was used for blending kidney samples. Each sample was processed with a dedicated plastic probe tip. These probe tips are designed to be disposable; in this study, they were washed in a dishwasher and rinsed in methanol before reuse. Disposable PVDF syringe filters in polypropylene housings, 0.2 μ, 13 mm diameter were used (Whatman). The solid phase extraction (SPE) cartridges for the urine extraction SPE cartridges were Bond-Elut C-18, 500 mg, 3 mL (Varian #12102028).

### 2.2. Reagents and standards

Trifluoroacetic acid (TFA) and trichloroacetic acid (TCA) were obtained from Sigma Chemical Co. HPLC Grade Methanol was obtained from Burdick and Jackson. Water processed with the Milli-Q system to give resistivity >18 MΩ cm (Millipore) was used for all subsequent references to water. Gentamicin (lot 10K1510) and tobramycin were obtained from Sigma Chemical. U.S. Pharmacopeia (USP) was also a source of gentamicin standard used in this study (lot K).

### 2.3. Solutions

A 0.11 M aqueous TFA solution was prepared by dissolving 8.5 mL TFA in 1 L water (8.5 mL, TFA density of 1.48 g/mL, TFA molecular weight of 114 g/mol). Mobile phase solution A was prepared by combining 1 L of 0.11 M TFA with 1 L methanol and filtering through 0.22 μ nylon membranes. This mobile phase was stored at room temperature for up to 6 months. The precipitation solution, 30% TCA in water, was prepared by dissolving 75 g TCA in 250 mL water in a glass bottle (stable 6 months in refrigerator). The SPE elution solution for urine extracts was 1.5% ammonia in methanol, prepared by adding 5 mL concentrated ammonium hydroxide in water (30%) and 95 mL methanol (stable 1 week).

Gentamicin stock solution was prepared at 100 μg/mL (ppm) nominal concentration. The USP standard was dried before weighing according to package instructions at 100 °C in a vacuum oven at <5 Torr for 3 h. The Sigma standard concentration was calculated by adjusting for purity and converting from the sulfate salt to free base. Approximately 0.015 g gentamicin sulfate were weighed in a plastic boat, quantitatively transferred to a 100 mL plastic volumetric flask, dissolved and diluted to the mark with water. The stock solutions were stored in the refrigerator for up to 6 months. Diluted gentamicin standards were prepared serially in water at the following concentrations: 100, 50, 10, 5, and 1 μg/mL, and 500, 100, 50, and 10 ng/mL. If the stock solution was not exactly 100.0 ppm gentamicin free base, the volume of the first dilution was adjusted so the first dilution was exactly 50.0 μg/mL.

Tobramycin stock solution was prepared at 1 mg/mL nominal concentration by weighing 10 ± 2 mg and dissolving in 10 mL water in a 15 mL polypropylene centrifuge tube. A higher level internal standard solution was prepared at 15 ppm by diluting 600 μL of the 1 mg/mL stock solution into 40 mL water in a 50 mL polypropylene centrifuge tube. A lower level internal standard solution was prepared by diluting 60 μL of the 1 mg/mL tobramycin stock solution into 40 mL water in a 50 mL polypropylene centrifuge tube. Alternatively, 4 mL of the 15 ppm solution were diluted to 40 mL with water in a 50 mL polypropylene tube. These solutions were stored in the refrigerator for 6 months.

### 2.4. Sample storage

Although aminoglycosides are stable when stored in aqueous solution at 4–8 °C, samples from animals were stored at <−60 °C to avoid tissue decomposition.

### 2.5. Plasma extraction

Thawed samples were vortexed, and 500 μL were transferred to Eppendorf tubes. Plasma injection standards and fortified samples (for quality control, QC) were prepared by adding appropriate volumes (25–75 μL) of the diluted gentamicin standards to control plasma to provide concentrations ranging from 1 to 5000 ng/mL. Blanks were prepared from 0.5 mL control plasma. All samples were spiked with 100 μL tobramycin 1.5 ppm internal standard, equivalent to 300 ng/mL. Tubes were briefly vortex-mixed and 100 μL of the precipitation solution (30% TCA) were added. Tubes were vortex-mixed for 30 s to thoroughly break up any clumping precipitate. Samples were centrifuged in the microfuge for 5 min at 14,000 RPM (20,000 RCF), resulting in a clear layer above a solid white precipitate. Using disposable plastic tips and a variable pipettor, 300 μL of supernatant solution were transferred to conical polypropylene autosampler vials. The vials were capped and stored in the refrigerator prior to analysis. Extracts were stable for many months prior to analysis. For low range samples, 60 μL were injected and the standard curve was prepared from 1 to 100 ng/mL. For other samples, 20 μL were injected and the standard curve was prepared from 25 to 5000 ng/mL.

### 2.6. Urine extraction

After thawing, samples were vortexed. One milliliter aliquots were transferred to 15 mL polypropylene centrifuge tubes. Fortified samples were prepared by adding an appropriate volume of a diluted gentamicin solution to 1 mL urine and vortexing briefly. For example, for 16 ng/mL, 160 μL of a 100 ng/mL standard solution were added, or for 100 ng/mL, 100 μL of a 1000 ng/mL standards solution were added. No gentamicin was added to blanks. Then, 200 μL 1.5 ppm tobramycin internal standard solution (equivalent to 300 ng/mL) were added to all fortified and blank samples (excluding control samples to be used for the standard curve). Tubes were vortexed briefly to mix the contents.

Injection standards were prepared by adding gentamicin and tobramycin to extracts of control urine *after* the extraction (see Section 3). Each sample batch required extraction of sufficient control urine samples to enable preparation of these spiked standards. For example, to prepare a 10 ng/mL equivalent standard, the following were added to a dried control urine extract, giving a final volume of 1.0 mL: 100 μL of 100 ng/mL gentamicin, 200 μL of 1.5 μg/mL tobramycin, 500 μL water, and 200 μL 30% TCA in water.

The SPE cartridges (Bond-Elut C-18, 500 mg, 3 mL) were conditioned with 3 mL methanol and 3 mL water. Then, 200 μL 30% TCA precipitation solutions were added to each sample, followed by brief vortexing. Samples were loaded on the SPE cartridges and passed through at about 1 mL/min. SPE cartridges were dried under vacuum for 1 min, then washed with 3 mL water, then 3 mL methanol, drying for 1 min under vacuum after each wash. Analytes were eluted into clean 15 mL polypropylene tubes with 3 mL 1.5% ammonia in methanol. The extracts were evaporated to dryness under nitrogen in a water bath at 45 °C. All test samples, fortified samples and blanks were dissolved in 800 μL water and 200 μL 30% TCA, with thorough vortex mixing.

After all extracts were diluted to 1.0 mL, they were filtered using 0.22 μm pore size 13 mm disposable syringe filters. Filtrate was collected in polypropylene autosampler vials. Injection volume for LC/MS was 100 μL.

### 2.7. Kidney extraction

This procedure was designed specifically for analysis of small samples of kidney cortex (outer layer) taken during surgery from standing animals. Control tissue consisted of kidney cortex that had been previously homogenized with a Polytron probe and preweighed into 17 mm × 100 mm Falcon tubes. Biopsy injection standards and fortified samples were prepared from these 100 mg samples of control kidney cortex homogenate by adding appropriate volumes (10–50 μL) of the diluted gentamicin standards to provide concentrations ranging from 10 to 25,000 ng/mL.

Yellow fat tissue was removed from red kidney biopsy tissue with clean razor blades before weighing. Test samples were weighed to 0.1 ± 0.01 g, if possible, but samples as small as 50 mg were analyzed. Kidney biopsy tissues were also weighed in 17 mm × 100 mm polypropylene tubes, and tissue weight was recorded. All tubes were centrifuged briefly to push the tissue to the bottom of the tube.

For sample batches covering higher ranges, expected to be >500 ng/g, 20 μL of 15 ppm tobramycin internal standard were added (equivalent to 3000 ng/g). Lower level batches were prepared by adding 20 μL of 1.5 ppm tobramcyin solution (equivalent to 300 ng/g). Then, after adding 500 μL methanol to each tube, samples were homogenized with the plastic probe tips for 1 min until an emulsion formed. For biopsy tissue samples, the probe tip was removed and reserved for the second homogenization step. Tubes were centrifuged at 3000 RPM for 15 min on the bench-top centrifuge (2000 RCF, not to exceed 3000 RCF). Methanol was poured off to waste and 400 μL water was added. The 30% TCA precipitation solution was added (100 μL) to all samples. For standards, fortified and control samples made up with pre-homogenized tissues, extracts were vortexed for 30 s to thoroughly break up the pellet. Kidney biopsy samples were rehomogenized for 30 s using the dedicated plastic homogenizer probe tip.

The emulsion was poured into a 1.5 mL Eppendorf tube and centrifuged at 20,000 RCF for 15 min. After centrifugation, the supernatant was filtered using disposable PVDF syringe filters, 0.2 μm pore size 13 mm disposable syringe filter, directly into 750 μL capacity polypropylene autosampler vials. Injection volume for LC/MS was 40 μL. The calibration curve was prepared by external standard, so the tobramycin served as a surrogate to verify that the extraction proceeded properly. Section 3 describes problems using internal standard calibration with kidney (and milk as well).

### 2.8. Milk extraction

Milk injection standards and fortified samples (for quality control, QC) were prepared by adding appropriate volumes (25–50 μL) of the diluted gentamicin standards to provide concentrations ranging from 2.5 to 2500 ng/mL. Blanks were prepared from 0.5 mL control milk. Milk was extracted in the same manner as plasma, with three modifications. First, although tobramycin was added to the milk sample, it was not used in measuring concentration (see Section 3). Second, samples were centrifuged to separate the upper fat layer. Samples of the skimmed milk (1 mL) were withdrawn after pressing the fat layer aside. Third, a modification to the chromatography was employed to flush strongly retained co-extractants to waste after the gentamicin peak had eluted. The initial isocratic conditions were the same as for plasma, urine and kidney analysis. After 6.5 min, the column was flushed for 3 min with 75% acetonitrile. The column was re-equilibrated in the starting mobile phase for 10 min before the next injection. If this step was not carried out (milk extracts only), gentamicin's absolute response degraded slowly across the sample set, causing problems for external standard calibration. This was probably due to suppression by late-eluting

Table 1
API2000 tuning parameters

| Tune parameters | *m/z* (478.2 → 322.1) | *m/z* (464.2 → 322.1) | *m/z* (450.2 → 322.1) | *m/z* (468.2 → 163.1) |
|---|---|---|---|---|
| DP (declustering potential) (V) | 70 | 65 | 60 | 40 |
| FP (focusing potential) (V) | 310 | 340 | 340 | 350 |
| EP (RF-only focusing quadrupole array) (V) | 12 | 11 | 11 | 11 |
| CEP (collision entrance potential) (V) | 20 | 18 | 20 | 18 |
| CE (collision energy) (V) | 21 | 21 | 21 | 31 |
| CXP (collision exit potential) (V) | 18 | 18 | 18 | 8 |
| Dwell time (ms) | 100 | 100 | 100 | 100 |

compounds, which could be removed with the column flush. Injection volume for milk extracts was 30 μL.

### 2.9. Liquid chromatography

Except for the milk procedure described above, the mobile phase was a combination of mobile phase solution A (95%) and acetonitrile (5%) at 220 μL/min. Column temperature was ambient for validation of the plasma and urine method, but was set to 30 °C for the kidney validation and all subsequent work. Column backpressure was roughly 1700 psi at 30 °C and 2100 psi at ambient temperature. If backpressure exceeded 2750 psi, typically the column was replaced because peak shape degraded as backpressure increased.

### 2.10. Data acquisition and processing

The LC/MS data system was Analyst v.1.3.1. The mass spectrometer used the Turbo IonSpray source in positive-ion mode. Unit mass resolution was used in Q1 and low resolution in Q3. Tuning parameters were optimized manually to give the values shown in Table 1. Source parameters were optimized as follows: CUR (curtain gas) 30, IS (ion spray voltage) 5500, GS1 (nebulizing gas) 55, GS2 (turbo gas) 55, Ihe (interface heater) on, CAD (collision gas pressure) 5, TEM (turbo gas temperature) 370.

Molecular ions from all gentamicin components ($MH^+$ shown in Fig. 1) were dissociated to yield product ions at *m/z* 322. Tobramycin ($MH^+ = m/z$ 468) was dissociated to a product ion at *m/z* 163 (Fig. 2). Integration of peaks was carried out following two smoothing steps with a bunching factor of 2. At very low levels, the baseline was adjusted manually to distinguish low-intensity gentamicin peaks from chemical noise. The calibration curve used tobramycin as the internal standard, with quadratic fit and $1/x$ weighting.

### 2.11. System suitability and quality control

Before analyzing test samples, a standard was injected from the middle of the calibration range to verify that the retention time and instrumental response were within the expected range. For the set to be considered valid, the levels measured for standards could not deviate >20% from their nominal levels. The calibration range was narrowed until no more than two standards were excluded. For example, if the lowest standard was excluded, the LOQ for that set was raised to the next higher standard. At least one QC sample should have shown <15% error at each fortified level. For the purposes of the related animal studies, measured values found to be less than the value calculated for LOQ (Tables 2–5) were reported as "<LOQ".

## 3. Results and discussion

Early in method development it became apparent that standard curves had to be prepared in matrix, due to matrix effects on gentamicin LC/MS ionization. We found that ion suppression was a factor in all matrices, although the SPE cleanup of urine gave cleaner extracts and less suppression than the other tissue extracts. Our approach to managing matrix effects were: (1) to use a properly matched control tissue for injection standards; (2) to show that tobramycin and gentamicin recoveries and ionization efficiencies were similar, to enable internal standard calibration; (3) if this was not the case, to use external standard calibration with matrix extracted standards. In either case, whether internal or external standard calibration was used, the net effect was a recovery compensation. Losses during extraction were factored out by either internal standard calibration or by using matrix-extracted external standard calibration.

Tobramycin proved to be a valid internal standard in plasma and urine, showing similar absolute recoveries in these matrices. Unfortunately, as the study progressed to kidney and milk samples, it became apparent that tobramycin response varied depending on the source of milk and the composition of the kidney control tissue. These variations, which did not occur for gentamicin, meant that the tobramycin internal standard introduced bias into the results for kidney and milk. Therefore, external standard calibration was required for kidney and milk analysis. The Ionspray™ interface (pneumatically assisted electrospray) gave consistent response from gentamicin over many runs of milk and kidney tissues. At such times when sensitivity for gentamicin did degrade, it could be restored by cleaning the interface and replacing the Ionspray needle.

The TCA added to release gentamicin and precipitate proteins was found to have a beneficial effect on chromatography. Peak focusing occurred when TCA was present in the injection solution at about 6%. The injection solution,

Table 2
Validation data for plasma method

| Fortification level | Average | | |
|---|---|---|---|
| | *n* | % Accuracy | % CV |
| 7.5 ng/mL, 60 μL injected | 17 | 92 | 8 |
| 15 ng/mL, 60 μL injected | 20 | 96 | 8 |
| 150 ng/mL, 60 μL injected | 3 | 88 | 8 |
| 7.5 ng/mL, 20 μL injected | 4 | 91 | 20 |
| 15 ng/mL, 20 μL injected | 4 | 103 | 17 |
| 150 ng/mL, 20 μL injected | 6 | 107 | 6 |
| 1500 ng/mL, 20 μL injected | 4 | 101 | 3 |
| 60 μL injections | 40 | 94 | 8 |
| 20 μL injections >100 ng/mL | 10 | 104 | 6 |
| Average of analyses of control plasma (60 μL only) | 21 | 0.4 ng/mL | |
| Standard deviation of analyses of control plasma | | 0.3 ng/mL | |
| Limit of quantitation (average + 10 × standard deviation) | | | 3.3 ng/mL |

Table 3
Validation data for urine method

| Fortification level | Average | | |
|---|---|---|---|
| | *n* | % Accuracy | % CV |
| 8 ng/mL | 6 | 99 | 9 |
| 16 ng/mL | 6 | 101 | 6 |
| 32 ng/mL | 6 | 93 | 3 |
| Overall | 18 | 98 | 8 |
| Average of analyses of control urine | 16 | 0.7 ng/mL | |
| Standard deviation of analyses of control urine | | 0.3 ng/mL | |
| Limit of quantitation (average + 10 × standard deviation) | | | 3.8 ng/mL |

Table 4
Validation data for biopsy method

| Fortification level | Average | | |
|---|---|---|---|
| | *n* | % Accuracy | % CV |
| 75 ng/g | 6 | 96 | 6 |
| 150 ng/g | 24 | 99 | 14 |
| 1000 ng/g | 8 | 104 | 7 |
| 10000 ng/g | 6 | 101 | 9 |
| Overall | 55 | 100 | 12 |
| Average of analyses of control kidney | 31 | 2.7 ng/g | |
| Standard deviation of analyses of control kidney | | 2.3 ng/g | |
| Limit of quantitation (average + 10 × standard deviation) | | | 26 ng/g |

Table 5
Validation data for milk method

| Fortification level | Average | | |
|---|---|---|---|
| | *n* | % Accuracy | % CV |
| 7.5 ng/mL fortified | 12 | 101 | 14 |
| 15 ng/mL fortified | 12 | 96 | 8 |
| 150 ng/mL fortified | 12 | 95 | 9 |
| 1500 ng/mL fortified | 4 | 98 | 6 |
| Overall | 40 | 97 | 10 |
| Average of analyses of control milk | 29 | 0.3 ng/mL | |
| Standard deviation of analyses of control milk | | 0.4 ng/mL | |
| Limit of quantitation (average + 10 × standard deviation) | | | 4.5 ng/mL |

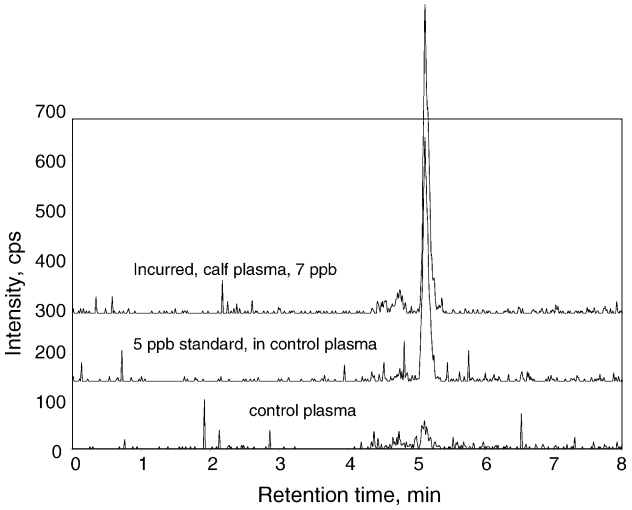


Fig. 3. Comparison of control bovine plasma, gentamicin standard prepared in plasma, and incurred gentamicin from calf dosed with gentamicin via mother's milk. Each chromatogram is the sum of three ions, *m*/*z* (478 + 464 + 450) → 322.

therefore, was prepared with 6% TCA added. Interestingly, the peak focusing effect for tobramycin was dependent on injection volume. If injection volume was <20 μL with the 3 mm × 150 mm LC column used for method validation, tobramycin eluted early with a broad, asymmetric shape, even though gentamicin retention time and peak shape was unaffected. Gentamicin peak shape remained very sharp (except for heavily used columns) with injection volumes of 20 μL (high level plasma), 30 μL (milk), 40 μL (kidney biopsy), 60 μL (low level plasma) or 100 μL (urine). Retention time was correspondingly longer for larger injection volumes.

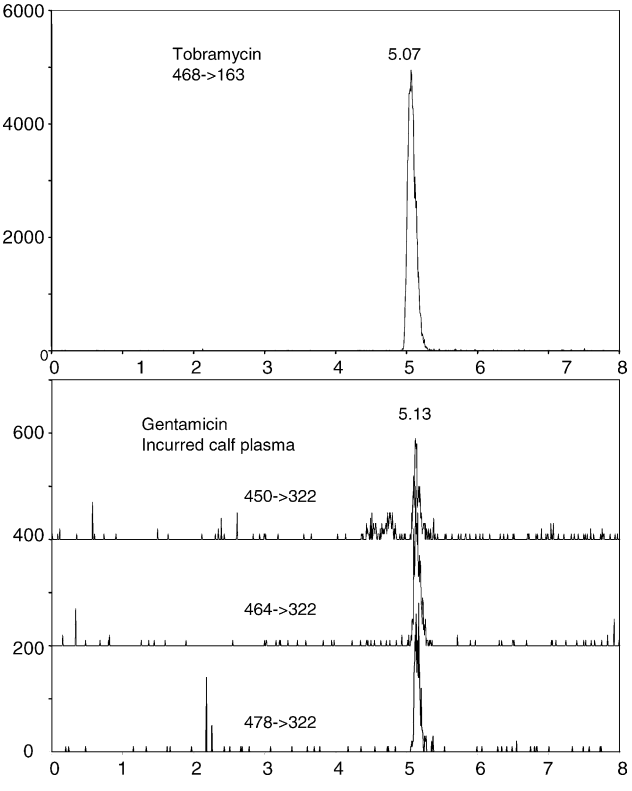


Fig. 4. Individual ions chromatograms from analysis of bovine plasma (as in Fig. 3) tobramycin level 300 ng/mL, incurred gentamicin measured at 7 ng/mL.

Initial work for method development was carried out with a short, narrow bore C-18 LC column (2 mm × 50 mm). This resulted in gentamicin retention time of about 3 min, allowing injections every 5 min. For high level plasma samples this LC system worked adequately. However, as work progressed to lower concentrations, an interference SRM trace for 450 → 322 (gentamicin C1a) was observed. The interference was separated from gentamicin using the larger column used for the remainder of the study (Figs. 3 and 4).

Trichloroacetic acid (TCA) has been used for protein precipitation in previous gentamicin methods [6,9]. Addition of a high concentration of TCA to 0.5 mL plasma followed by high-speed centrifugation at 14,000 RPM (20,000 RCF) yielded an extract that could be injected directly on the LC/MS.

Although the plasma procedure was quick, the extracts were fairly dirty. Column performance degraded after several hundred injections, evidenced by broad, asymmetrical peaks and higher back-pressure. The problem appeared to affect the LC column throughout its length, because frequent guard column changes did not prevent degradation of the analytical column. As a result, the plasma method was performed without using a guard column, hence the difference in retention times for plasma versus other tissues reported here. As a last resort, the life of the LC column dedicated to plasma could be extended by reversing its direction.

Quantitative performance of the plasma method below 25 ng/mL was improved by tripling the volume injected from 20 to 60 μL, as shown in Table 2. For 20 μL injections the coefficients of variation (CV) at 7.5 and 15 ng/mL were nearly at the acceptance limit of 20%. When 60 μL was injected the CV improved to 8%, so this volume was used for low range analyses. The LOQ for 60 μL injections was calculated to be 3.3 ng/mL (Table 2).

It was very important to divert early-eluting compounds to waste until about 1 min before gentamicin eluted. If the divert valve was switched to the source prematurely, significant fouling of the LC/MS interface occurred. The interface spray shield became much dirtier after analysis of plasma and milk samples than the other tissue matrices.

Column temperature regulation was used to eliminate occasional retention time drift, which contributed to peak shape variations and less precise quantitation.

It was discovered that heavily used columns tended to produce ghost peaks in the analysis of control samples. This was an important issue for the biopsy procedure, so a specific analytical-guard column combination was dedicated to low levels samples.

Early procedures for urine analysis were based on the plasma procedure, by simple TCA treatment, but this did

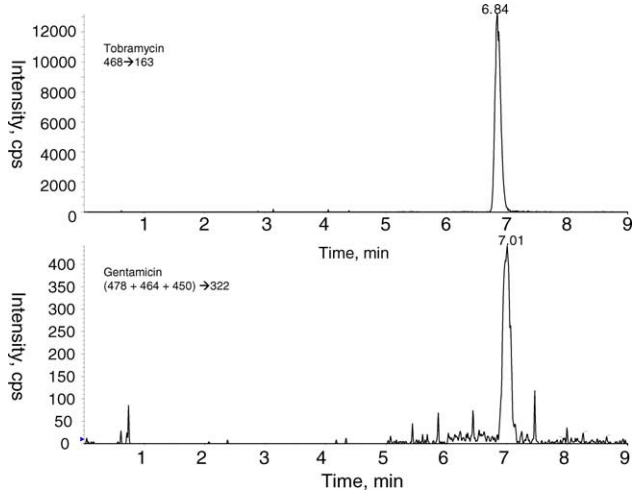


Fig. 5. Reconstructed, smoothed ion chromatogram for sum of gentamicin components in a 16 ng/mL fortified control urine extract, with tobramycin internal standard equivalent to 300 ng/mL.

not yield consistent results. This procedure apparently left too many salts and other solutes, which led to suppression of ionization. Therefore, a published method for gentamicin in urine based on C-18 solid phase extraction [13] was modified to be compatible with the LC procedure. This modified method worked successfully (Fig. 5). Although the procedure uses C-18 bonded silica, the retention of gentamicin is determined by free silanols, not by the C-18 group. This operating mode is more like a weak-cation exchange cleanup than a conventional reversed phase cleanup.

The goal was to measure accurately down to 1 ng/mL in urine if possible. However, some gentamicin was always lost during extraction of matrix standards. To give better standard curves at low urine concentrations, injection standards were prepared by adding gentamicin and tobramycin to extracts of control urine *after* the extraction. This technique does not introduce significant error because the absolute recovery of gentamicin and tobramycin were very similar (76% for gentamicin at 8–32 ng/mL compared to 77% for tobramycin at 300 ng/mL). Thus, this technique provided about 30% more standard than would have otherwise been present if the standards had been added to urine before extraction. The benefit was sharper peaks and more consistent standard curves in the 1–10 ng/mL range in urine.

### 3.1. Validation

Method performance was characterized by assessing accuracy, precision, and limit of quantitation (Tables 2–5). Use of internal standard (plasma, urine) or matrix-matched, extracted external standards (milk, kidney) meant that measurements were corrected for losses during extraction. Absolute recovery was assessed in separate experiments by comparing the responses from control extracts with standards added before or after extraction. LOQ was calculated from the apparent measured value of blanks, as the mean + 10*x* standard deviation.

Some blanks were injected after very high-level standards to assure that no carryover took place into subsequent low level samples. These blanks were not used in the calculation of LOQ. Carryover due to the autosampler and LC system was <0.5%.

### 3.2. Freezer stability in plasma

An experiment evaluated the stability of gentamicin in plasma at <−60 °C. Fifty-three plasma samples were re-analyzed 16 months after a first analysis. Concentrations ranged from 10 to 80 ng/mL. On average, the second analysis was 93% of the first analysis, although the CV of the ratio was 26%. It should be noted that the first analysis was done with the shorter LC column, which could have led to slightly higher values due to the unresolved interference.

### 3.3. Gentamicin composition

U.S. FDA regulations allow gentamicin composition to vary within limits. An experiment was conducted to compare the composition of gentamicin from the two suppliers used in this study. The relative responses for *m*/*z* 478, 464 and 450 (C1, C2 + C2a, and C1a) were 38%, 33%, and 29% (Sigma) versus 39%, 40%, and 21% (USP). Despite these differences, the total responses were nearly the same from each supplier, suggesting that the ionization efficiencies for each component were similar.

### 3.4. Kidney biopsy samples

Kidney biopsy samples were taken with an endoscope during surgery on a standing animal. This technique was developed at CVM by O.A. Chiesa [14] to reduce the number of animals for depletion studies, because tissue samples can be

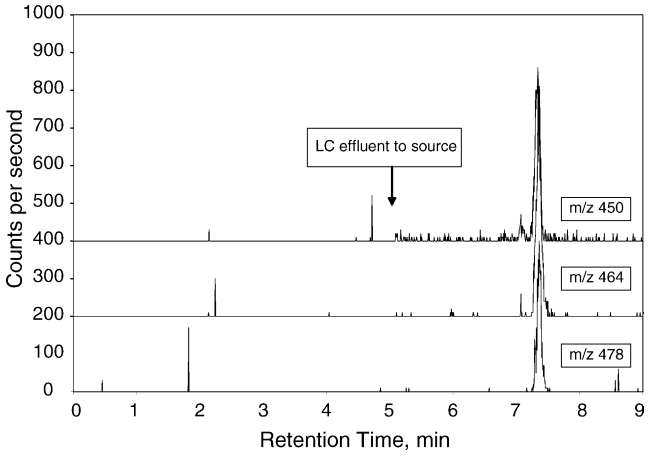


Fig. 6. Incurred gentamicin in biopsy taken from kidney cortex of standing animal, following dosing with gentamicin (see also Fig. 7).

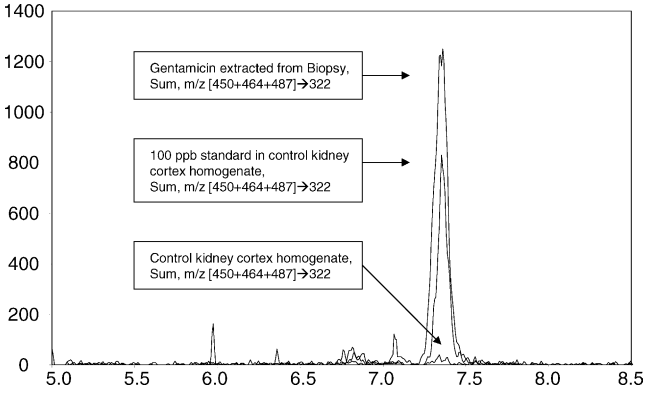


Fig. 7. Incurred gentamicin in biopsy taken from kidney cortex of standing animal, summed from Fig. 6 and compared to control sample and 100 ng/g standard from the same batch. The measured sample concentration was 186 ng/g, prior to correction for sample weight.

taken repeatedly without sacrificing the animal. The sample matrix is kidney cortex, the thin layer found on the kidney surface. Control kidney cortex, not whole kidney homogenate, was necessary for preparing the standard curve for biopsy analysis.

The biopsy method takes advantage of gentamicin's high affinity for kidney tissue. An experiment showed that blending in methanol, centrifuging, and discarding the supernatant, resulted in the loss of only about 10% of both gentamicin and tobramycin. When this methanol extraction was performed on samples fortified at 100–300 ng/g (see Section 2) the absolute recovery of gentamicin was 59% with 7% CV ($n = 6$) and tobramycin recovery was 55% with 5% CV ($n = 6$). If the methanol step was omitted, so that the tissue was homogenized directly in aqueous TCA, the absolute recovery of gentamicin was 67% with 10% CV ($n = 6$) and tobramycin recovery was 66% with 6% CV ($n = 6$).

However, the absolute recovery of tobramycin and gentamicin differed widely if whole kidney homogenate, which includes medulla tissue, was extracted. Medulla tissue is known to not bind gentamicin. There was a bias resulting from internal standard calibration with whole kidney homogenate, so external standard calibration was used with whole kidney homogenate control. As soon as control cortex homogenate was obtained, this matrix was used for matrix-matched extracted standards in subsequent analysis of kidney biopsy samples (Figs. 6 and 7). The application of the biopsy technique to monitoring the depletion of gentamicin from standing animals will be discussed in more detail in a future publication.

## 4. Conclusions

A set of LC/MS/MS methods for comprehensive analysis of gentamicin residues in various bovine tissues was developed and validated. The common element of each extraction was use of trichloroacetic acid to precipitate proteins and release bound gentamicin. Urine samples were further cleaned up by C-18 SPE. LC was carried out in all cases with a novel methanol:water:trifluoroacetic acid mobile phase. Matrix-matched tissue standards were required with all tissues to compensate for matrix effects on ionization. Each tissue was found to have unique characteristics that required some individual treatment. Internal standard calibration with tobramycin was feasible in plasma and urine. However, tobramycin internal standard was not usable in kidney or milk, due to differences in apparent recovery between gentamicin and tobramycin in those tissues. Quantitation in milk and kidney was carried out by external standard calibration.

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