VETERINARY DRUG RESIDUES

A High-Throughput Analytical Method for Determination of Aminoglycosides in Veal Tissues by Liquid Chromatography/ Tandem Mass Spectrometry with Automated Cleanup

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A liquid chromatographic/tandem mass spectrometric (LC/MS/MS) method was developed for determining dihydrostreptomycin, gentamicin C1, and neomycin in veal kidney, liver, and muscle. The extraction prior to injection on the automated cleanup/analysis system is very simple, permitting preparation of 24 veal samples for analysis in half a day of work. The extracts are purified online on a reversed-phase column, with the help of an ion-pairing agent, and the analytes are separated on a Nucleosil C18 column prior to analyses by electrospray MS/MS. The cleanup is sufficient to minimize ion suppression/enhancement phenomena and permits quantification of the analytes extracted from veal tissues. Four secondary ions were measured for every analyte, which gives unambiguous identification of the compounds under analysis. Calibration curves were linear for all analytes between 50 and 5000 ppb, and recoveries in kidney were 76, 57, and 51%, respectively, for dihydrostreptomycin, gentamicin C1, and neomycin. Estimated limits of detection for kidney were, respectively, 0.1, 0.1, and 0.4 ppb. When compared to an LC method with fluorescence detection, the method gave equivalent results for kidneys incurred with neomycin. This rugged method has been applied to the analysis of more than 1000 veal samples over a 1-year period.

Aminoglycosides are broad-spectrum antibiotics widely used in human and veterinary medicine. Gentamicin, neomycin, dihydrostreptomycin, and streptomycin are the most commonly used aminoglycosides in food-animal production (1). In Canada, only neomycin has administrative maximum residue limits (MRLs) in cattle: 500 ppb in muscle and liver and 10 ppm in kidney. To our knowledge, when there are MRLs for these veterinary drugs in cattle in other countries, they are always ≥100 ppb. The presence of residues of these antibiotics in food can cause allergic reactions in sensitive persons and lead to the development of bacteria that are resistant to antibiotics (2).

Aminoglycosides are polar molecules with low volatility and lack useful chromophores. When administered to animals, they concentrate in the kidney, particularly in the cortex (1). Because they are not volatile, chemical analysis of these molecules in food is generally done by liquid chromatography (LC) rather than gas chromatography (3). And because of their hydrophilic character, ion pair agents are necessary to retain aminoglycosides on reversed-phase columns (4–24). For a long time, the most common detection mode was fluorescence after precolumn or postcolumn derivatization (4, 6, 7, 9–11, 13–16, 25–29).

Aminoglycoside extraction from animal tissues has been done using acids, generally trichloroacetic acid (TCA), especially after Posyniak at al. (29) showed the advantages of this approach. Ion-exchange chromatography is the most common cleanup mode (7, 14, 16, 23, 24, 28).

McLaughlin et al. (5, 8) pioneered the use of mass spectrometry (MS) for the analysis of aminoglycosides in animal tissues. In the last few years, more reports of the use of MS have appeared in the scientific literature for the analysis of these antibiotics in food, in general (17–21), and in animal tissues (22–24), in particular. LC with tandem mass spectrometry (LC/MS/MS) can unambiguously identify analytes, which is essential for regulatory analysis. It also has the advantage of being very sensitive and, because of its great selectivity, the samples do not require cleanup that is as extensive as for conventional LC methods with ultraviolet and fluorometric detection. In the case of aminoglycosides, MS has the further advantage that the derivatization of the chromophore-lacking analytes is not necessary.

Most methods reported for aminoglycosides in animal tissues analyze only 1 or 2 compounds.

To our knowledge, only 2 multiresidue LC/MS/MS methods have been reported for the analysis of aminoglycosides in animal tissues. Kaufmann and Maden (23) published a method that involves extraction with TCA followed by an anion-exchange step, in order to lower the ionic strength of the extract prior to combined weak

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cation-exchange and reversed-phase cleanup. The highly purified extracts produced minimized ion suppression during MS detection. Also, the Food Safety and Inspection Service of the U.S. Department of Agriculture (USDA-FSIS) reported a method (24) that also uses an acidic extraction with a buffer and TCA. The extract is then neutralized and purified with a weak cation-exchange solid-phase extraction column. The eluate from this column is evaporated, reconstituted in a solution of the ion-pair agent, and then analyzed by ion-pair reversed-phase LC/MS/MS. These 2 methods have the advantages of being multiresidue, sensitive, and highly selective because of the use of MS/MS detection. However, they both involve lengthy sample preparation steps that are not easy to automate. Because sample throughput per working day is an important factor in our laboratory, we have developed an automated LC/MS/MS method for the analysis of dihydrostreptomycin, gentamicin C1, and neomycin in veal tissues with online cleanup. We started with the USDA-FSIS method but took a completely different approach for cleanup. Using this approach, 1 person in our laboratory was able to easily prepare 24 extracts for automated analysis in half a day, compared to 12 extracts in a full day using the USDA-FSIS method. This permits us to run high sample throughput regulatory programs with minimum of manpower, with method that can screen, quantify, and confirm the identity of the analytes simultaneously.

Experimental

Apparatus

(**a**) *LC/MS/MS system*.—Waters 2695 liquid chromatograph with quartenary pump; Waters (Micromass) Quattro II triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) and Mass Lynx system software; Waters 590 Programmable LC Pump (Waters Mass Spectrometry Systems, Pointe Claire, Québec, Canada); and 2 Supelpro 2-position valves, 6 port, with electrical actuator (Supelco Canada, Oakville, ON, Canada).

(**b**) *LC column*.—Nucleosil C18, 3 μ, 2 × 50 mm (Phenomenex, Torrance, CA).

(**c**) *Trap column*.—Eclipse XDB-C18, 3.5 μ , 2.1 × 15 mm (Agilent Technologies, Palo Alto, CA).

(**d**) *Gilson ASPEC XLi sample processor*.—Mandel Scientific Co. Inc. (Guelph, ON, Canada).

(**e**) *Centrifuge*.—Model J6-MC (Beckman Coulter Canada Inc., Mississauga, ON, Canada).

(**f**) *Mechanical shaker*.—Model S1103 (Eberbach Corp., Ann Arbor, MI).

(**g**) *Homogenizer*.—Polytron Model PT 10-35 with a PTA 10 TS generator (Brinkmann Instruments Canada, Mississauga, ON, Canada).

(**h**) *Filters*.—13 mm polytetrafluoroethylene (PTFE) 0.45 μ (Canadian Life Science, Peterborough, ON, Canada).

(**i**) *Vortex*.—Genie 2 (Fisher, Nepean, ON, Canada).

(**j**) *pH Meter*.—Accumet 950 (Fisher).

Reagents

(**a**) *Standards*.—Dihydrostreptomycin [No. 20300, United States Pharmacopeia (A&C American Chemicals Ltd, Montréal, QC, Canada)]; streptomycin (No. 1623003, USP); neomycin (No. 45800, USP); gentamicin (No. 1289003, USP); tobramycin (No. T-1783, Sigma-Aldrich, St. Louis, MO).

(**b**) *Water*.—All water used was treated on a Millipore Milli-Q system (Billerica, MA).

(**c**) *Acetonitrile*.—LC grade (EMD Chemicals Inc., Gibbstown, NJ).

(**d**) *Methanol*.—LC grade (Fisher).

(**e**) *Disodium ethylenediamine tetraacetate dihydrate (Na_2EDTA dihydrate)*.—Fisher.

(**f**) *Heptafluorobutyric acid (HFBA) 99%*.—Sigma-Aldrich.

(**g**) *Hydrochloric acid (HCl)*.—EMD Chemicals Inc.

(**h**) *Potassium phosphate monobasic (KH_2PO_4)*.—Fisher.

(**i**) *TCA*.—Fisher.

(**j**) *Extraction solution (10 mM KH_2PO_4, 0.4 mM EDTA, 2% TCA)*.—Dissolve 2.72 g KH_2PO_4 in 2 L water. Adjust pH to 4.0 with 1 M HCl. Add 0.3 g Na_2EDTA dihydrate and 40 g TCA. This solution is stable for 2 months at room temperature.

(**k**) *Trap column loading solution (20 mM HFBA, 5% methanol)*.—Dilute 2.6 mL HFBA 99% and 50 mL methanol to 1 L with water. This solution is prepared weekly.

(**l**) *100 mM HFBA*.—Dilute 6.57 mL HFBA 99% to 500 mL with water. This solution is stable for 2 months at room temperature.

Preparation of Standard Solutions

(**a**) *Stock standard solutions (dihydrostreptomycin, gentamicin, neomycin, streptomycin, and tobramycin; 1000 μg/mL each)*.—Dissolve 100 mg of each aminoglycoside with water in a 100 mL plastic volumetric flask. Keep refrigerated for 6 months.

(**b**) *Tobramycin working solution, 10 μg/mL*.—Dilute 100 μL stock solution with water in a 10 mL volumetric flask. Transfer to a 15 mL polypropylene centrifuge tube. Prepare daily.

(**c**) *Mixed spiking solutions*.—The concentration of the mixed spiking solution(s) of dihydrostreptomycin, gentamicin, and neomycin will depend upon the range of the calibration curve. In the 50–1000 ppm range, a 10 μg/mL solution will do; dilute 100 μL stock solution with water in a 10 mL volumetric flask. Transfer to a 15 mL polypropylene centrifuge tube. Prepare daily.

Sampling

All tissues were obtained from regulatory programs in Québec, and incurred samples used were positive monitoring samples. They were kept frozen at –20°C until analysis. Most of the results reported in this paper were obtained using tissues that were not homogenized before weighing. For regulatory programs, the whole tissues are homogenized prior to analysis.

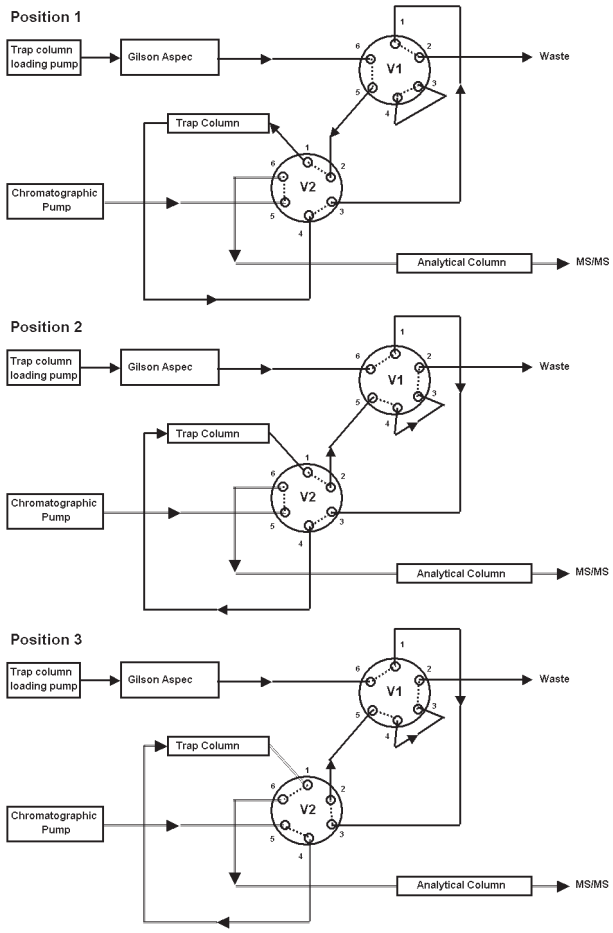

Figure 1. Automated cleanup and chromatography system.

Sample Extraction

Weigh 2 g sliced tissue into a 50 mL polypropylene conical tube. Add the appropriate volume of mixed spiking solution to 2 g of 3 negative samples in order to build the calibration curve. Add 100 μL tobramycin working solution to every sample. Wait 10 min. Add 10 mL extraction solution and homogenize with the Polytron for 30 s. Shake 10 min at high speed on the Eberbach mechanical shaker and then centrifuge at 4550 × *g* for 10 min. Transfer the supernatant to another 50 mL polypropylene conical tube. Add 10 mL extraction solution, free the tissue plug from the bottom of the conical tube by shaking vigorously, and shake 10 min at high speed on the Eberbach mechanical shaker. Centrifuge at 4550 × *g* for 10 min, then combine the 2 fractions of supernatant. Add 52 μL HFBA 99%. Filter 3 mL of the extract on a PTFE 0.45 μ filter directly into a test tube that fits in the sample holder of the Gilson ASPEC sample processor.

Automated Cleanup and Chromatography

The next part of the analysis is done automatically on the system depicted on Figure 1. Table 1 shows which of the valve positions given in Figure 1 is used at different times. This table also gives the proportion of the different solvents delivered by the chromatographic pump. Flow of the trap column loading pump is always 0.3 mL/min, while the flow of the chromatographic pump is always 0.2 mL/min. All the gradients are linear. At time zero, the Gilson ASPEC sample processor injects 100 μL of the extract into the trap column loading solution; the analytes are retained on the trap column (position 1). After 3 min, the flow of the loading solution is reversed and, for 2 min, the impurities trapped at the head of the trap column are diverted to waste (position 2). Then the chromatographic pump delivers the gradient given in Table 1 in order to elute the analytes from the trap column towards a Nucleosil C18, 3 μ, 2 × 50 mm column connected to the ESI source of the mass spectrometer (time: 5–17 min, position 3). Acetonitrile is used to completely wash the columns to remove hydrophobic impurities between each run. Putting both the trap and analytical columns online (position 3) increases the pressure by about 20%, and no pressure spikes in the chromatograms were observed when valve 2 was activated. The whole process, from one injection to the next, takes 30 min. A chromatogram of spiked kidney is shown in Figure 2.

MS/MS Conditions

The LC/MS/MS system was operated in the positive ESI mode, the capillary voltage was 1.0 kV, and the cone voltages and collision energy for each analyte are listed in Table 2. The extractor and radio frequency lens voltages were 6 and 0.1 V, respectively; the source and desolvation temperatures were 120 and 350°C, respectively; and nitrogen was used as the desolvation gas at 400 L/h. Argon collision gas pressure was 3 × 10^{-3} mbar, dwell times were of 100 ms, and both the low mass and high mass resolutions were set to 10.5 on the Quattro II mass spectrometer, which corresponds approximately to unit resolution.

Calculations and Quality Control

In order to take into account ion suppression/enhancement effects, the calibration curve was made of spiked tissues of the same kind (kidney, liver, or muscle) as the tissue under assay. Peak areas of the most intense daughter ion of each analyte were used for calculation of the concentration, the other secondary ions serving only for identification purpose. The calibration method was external standard, using the mean of each of the 3 points of the calibration curve injected before and after the samples. For quality control of this automated analysis, tobramycin was added to every sample, either under assay or used as calibration standard, and the relative standard deviation (RSD) of the areas of tobramycin for all extracts had to be <20%. Also, the ratios of the areas of every analyte over the area of tobramycin were recorded in control charts and used as another quality control tool. Identification criteria were based on the identification points system of the European Community commission (30).

Table 1. LC gradient parameters

Time, min	Valve position	100 mM HFBA, %	Water, %	Methanol, %	Acetonitrile, %
0.0	1	20	75	5	0
3.0	2	20	75	5	0
5.0	3	20	75	5	0
5.5	3	20	40	40	0
15.0	3	20	0	80	0
17.0	3	20	0	80	0
17.1	3	20	0	0	80
17.5	2/3[a]	20	0	0	80
24.0	2	20	0	0	80
24.1	2	20	75	5	0
28.0	1	20	75	5	0
30.0	1	20	75	5	0

[a] Valve 2 is switched every 30 s in order to minimize carryover.

Preventive Maintenance

In order to avoid problems with the LC system, the trap column should be changed after every 500 injections or if the pressure exceeds 800 psi (a new column will give a reading of about 300 psi). The analytical column is changed after every 1500 injections or when the quality of the neomycin peak has deteriorated. This column can be regenerated by washing with tetrahydrofuran to extend its lifetime.

Results and Discussion

Extraction

Many solutions have been reported in the scientific literature for the extraction of aminoglycoside antibiotics from biological matrixes. The first truly multiresidue methods that came to our knowledge was from the USDA-FSIS (24). We chose to work with the extraction solution from this method (10 mM KH_2PO_4 with 0.4 mM EDTA and 2% TCA)

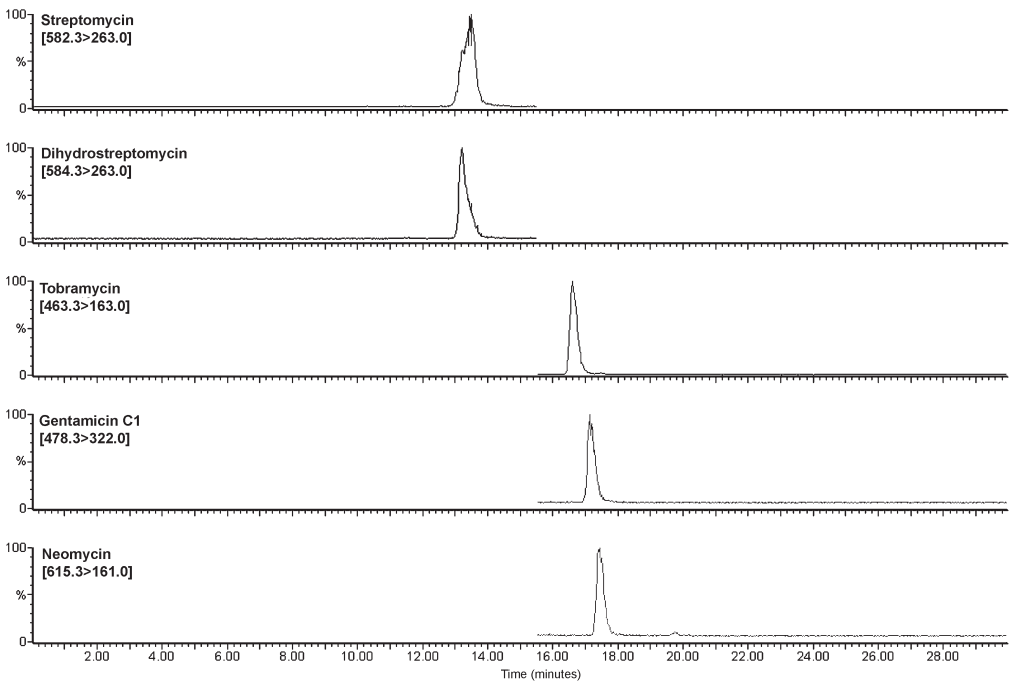

Figure 2. Chromatogram of a kidney tissue spiked at 50 ppb with aminoglycosides.

Table 2. Parameters of MS transitions

Analyte	Transition[a]	Cone voltage, V	Collision energy, eV
Dihydrostreptomycin	584.3 to 263.0	55	28
	584.3 to 204.0	55	55
	584.3 to 246.0	55	35
	584.3 to 221.0	55	40
Streptomycin	582.3 to 263.0	60	28
	582.3 to 204.0	60	55
Gentamicin C1	478.3 to 322.0	30	15
	478.3 to 160.0	30	20
	478.3 to 163.0	30	20
	478.3 to 157.0	30	20
Neomycin	615.3 to 161.0	35	35
	615.3 to 293.0	35	22
	615.3 to 323.0	35	22
	615.3 to 163.0	35	35
Tobramycin	468.3 to 163.0	30	25
	468.3 to 324.0	30	15

[a] The first transition listed for each analyte is the most intense and is used for quantitation.

after having studied the influence of the TCA concentration on the efficiency of this extraction, reported in Figures 3 and 4 as the MS/MS signal. Figure 3 shows the signal from kidney samples spiked at 50 ppb with dihydrostreptomycin, gentamicin, and neomycin. It can be seen that a plateau is reached before 2% TCA, which is consistent with the results reported by Kaufmann and Maden (23). A similar observation can be seen in Figure 4 for 2 kidneys incurred with gentamicin and neomycin, respectively, at 200 and 290 ppb; at 2% TCA, a plateau has already been reached.

In order to check that the efficiency of the extraction was adequate, we measured the extraction recovery coefficient by computing the ratio of the signal of 500 ppb spiked kidney and liver samples over the signal of a negative sample of the same tissue to which the 500 ppb spiking solution was added just prior to injection on the automated cleanup/analysis system (Table 3). Recovery values ranged from 66 to 96%, and they are generally higher than those reported by Kaufmann and Maden (23) for the extraction of aminoglycosides from pork muscle with 5% TCA. Their results also show lower recovery for neomycin than for other analytes.

Automated Cleanup and Chromatography

Most of the chromatographic cleanups of aminoglycoside extracts have been done by ion-exchange processes. Having highlighted some drawbacks of this approach, Posyniak et al. (29) successfully applied reversed-phase chromatography with ion-pairing to the cleanup of animal tissue extracts. Since, in our experience, reversed-phase processes are more easy to automate than ion-exchange processes, we adopted the former approach. The most widely used ion-pair agents for analysis of aminoglycosides are alkylsulfonates. Unfortunately, these molecules are not compatible with MS detection because of their low volatility, so fluorinated carboxylic acids are used for LC/MS applications (31). Among these compounds, HFBA has been shown to produce the least ion suppression (17). For developing an automated cleanup, the idea was to retain the analyte/HFBA complex on a short reversed-phase column (the trap column), then backflushing towards the analytical column and the MS/MS analyzer while diverting to waste the impurities trapped at the head of the trap column, and finally eluting the analytes to the analytical column with a gradient. We first used an Eclipse XDB-C18, 3.5 μ, 2.1 × 30 mm column (Agilent Technologies) as the trap column, but the backpressure became too high, so we switched to an identical but shorter column (15 mm). We then optimized the trap column cleanup parameters: methanol (0 to 20%) and HFBA (10 to 40 mM) concentrations for sample loading, flow of the loading solution (0.2 to 0.5 mL/min), loading time (1 to 3 min), and backflushing time (1 to 3 min) before the start of the gradient. For optimizing the injection volume, we measured the RSD of the method (on 10 negative samples spiked at 50 ppb) at 50, 100 and 200 μL of injection volumes. The RSD was best at 100 μL. From 100 to 200 μL, the MS/MS signal increase was much lower for all of the analytes than the factor of 2 theoretically expected. This might be due to less available binding sites with higher extract volumes and to more ion suppression.

For analytical chromatography, we started from the USDA-FSIS method (24), which uses ion-pair reversed-phase separation on an Xterra MS C_{18}, 3.5 μ, 2.1 × 100 mm column (Waters, Milford, MA), with HFBA as the counter-ion. The backpressure was too high with the Xterra column inline with the trap column, so we switched to a shorter column, Nucleosil C_{18}, 3 μ, 2 × 50 mm. We then slightly modified the gradient to take the column change into account. We measured the loss of analytes due to the cleanup process for spiked

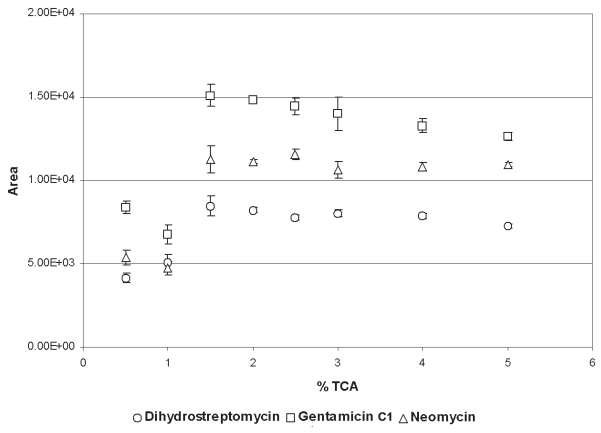

Figure 3. Effect of TCA concentration (%) on the MS/MS signal of kidneys spiked at 50 ppb with the different aminoglycosides. Each point resuts from the average of 3 independent extractions.

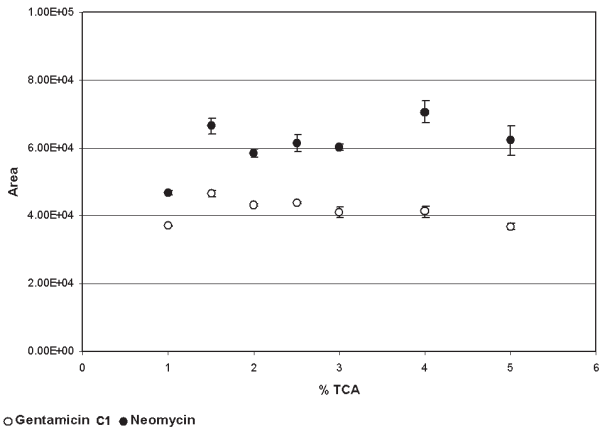

Figure 4. Effect of TCA concentration (%) on the MS/MS signal of kidneys incurred with gentamicin (200 ppb) and neomycin (290 ppb). Each point results from the average of 2 independent extractions.

kidneys and livers. To calculate this loss, we compared the signals from 2 negative samples, the first spiked at 500 ppb. Both extracts were injected on the automated cleanup system, and a fraction was manually collected after cleanup on the trap column. The fraction from the second sample was then spiked with the same solution as for the first sample, and 100 μL from both fractions was injected into the LC/MS/MS system without cleanup on the trap column. The results are shown in Table 3. Cleanup recovery is high for dihydrostreptomycin and streptomycin, but lower for the more strongly retained gentamicin C1 and neomycin. This suggests that some of these analytes remain on the trap column, and this part of the method could probably be further optimized. However, elution of all the analytes is reproducible, as will be shown in *Method Performance* below.

Mass Spectrometry

Because gentamicin and neomycin are thermolabile molecules, ESI is expected to give better results than atmospheric pressure chemical ionization (APCI). Indeed, Heller et al. (17) have shown that the signal given by aminoglycosides extracted from milk was higher using positive-mode ESI than with APCI. Using this interface, we have optimized the detection parameters of the mass spectrometer by injecting standard solutions of the different aminoglycosides in the mobile phase. The precursor ion was $[M + H]^+$ for each analyte, and we measured 4 secondary ions for dihydrostreptomycin, gentamicin C1, and neomycin and 2 for streptomycin and tobramycin. The different precursor and secondary ions are shown in Table 2.

Neomycin B and neomycin C are stereoisomers that are detected simultaneously with this method. We checked and verified that neomycin A was not present in significant amount in either the calibration standard used or in incurred veal samples. Gentamicin consists of 4 compounds: C1, $C1_a$, and stereoisomers C2 and $C2_a$. Our high-throughput method was primarily developed for the analysis of veal tissues, and gentamicin is not approved for use in veal raised for their meat in Canada. Because one of the objectives in developing this multiresidue method was to determine if gentamicin was being used illegally in veal production, only gentamicin C1, which is the major compound, was monitored in this study.

Method Performance

The method performance was evaluated for veal kidney, liver, and muscle, even though we are not likely to find aminoglycosides residues in muscle. We measured overall

Table 3. Extraction, cleanup, and overall percentage recoveries from tissues spiked at 500 ppb

Tissue	Dihydrostreptomycin	Gentamicin C1	Neomycin	Streptomycin	Tobramycin
Kidney, extraction, assay No. 1	78.3	69.5	67.6	76.0	75.2
Kidney, extraction, assay No. 2	86.3	73.5	71.0	94.8	85.7
Kidney, extraction, average	82.3	71.5	69.3	85.4	80.5
Kidney, cleanup, assay No. 1	90.7	74.9	78.0	98.1	83.6
Kidney, cleanup, assay No. 2	92.8	85.2	68.9	100.8	84.1
Kidney, cleanup, average	91.8	80.1	73.5	99.5	83.9
Kidney, overall (average)	76	57	51	85	68
Liver, extraction, assay No. 1	89.5	85.3	61.7	86.0	79.3
Liver, extraction, assay No. 2	103.1	77.1	69.5	85.0	84.8
Liver, extraction, average	96.3	81.2	65.6	85.5	82.1
Liver, cleanup, assay No. 1	92.1	71.5	80.2	95.1	86.1
Liver, cleanup, assay No. 2	88.5	66.6	71.2	87.7	71.0
Liver, cleanup, average	90.3	69.1	75.7	91.4	78.6
Liver, overall (average)	87	56	50	78	65

Table 4. Relative standard deviation of the quantification ions of kidneys spiked at 50 ppb, with and without internal standard

	Repeatability RSD[a], %	Reproducibility RSD[b], %	Interday RSD[c], %
Dihydrostreptomycin with internal standard	2	13	9
Dihydrostreptomycin without internal standard	4	16	11
Gentamicin C1 with internal standard	5	14	10
Gentamicin C1 without internal standard	3	12	11
Neomycin with internal standard	15	19	17
Neomycin without internal standard	14	18	18

[a] On 10 different spiked portions of the same kidney.
[b] On 10 spiked kidneys from different animals.
[c] Combined data of the first 2 batches (each batch was normalized by dividing each individual datum by the mean of the batch).

method recovery for kidney and liver only, and these results are given in Table 3. Values reported are the averages of 2 assays only, since the process of measurement of the cleanup recovery was particularly laborious without a fraction collector. It can be seen that at least half of each analyte is recovered over the whole analytical process; this is acceptable for a multiresidue method, as long as it is reproducible, since we use spiked tissues as calibration points.

The goal of this method development was to be able to quantify from a concentration of 50 ppb, which corresponds to half the reporting limit we use for dihydrostreptomycin and gentamicin (the MRL for neomycin is higher, but the performance of the 3 analytes was verified at the same concentrations for practical purposes). We measured the RSD of the method at this level 10 times from the same spiked negative kidney, from 10 different spiked negative kidneys, and from the combination of the 2 sets (Table 4). The RSDs were <20% in all cases, which demonstrates that quantification is possible at 50 ppb for all of the analytes. The calculation of the RSD from a batch of spiked samples made of negative tissues of different origins is very important because it shows that the cleanup of the extract is sufficient so that ion suppression/enhancement effects will not make impossible the quantification using a calibration curve made of spiked tissue from another animal of the same species. It can be seen from Table 4 that the RSD values of the batches from different origins are higher than those of batches from the same tissue; this is to be expected, since the exact composition of the kidney will vary slightly from one animal to another so that the co-extracts present will be in different proportion, and this will reflect on the ion suppression/enhancement phenomena. However, the fact that the RSD values in Table 4 are all within 20%, the required intralaboratory RSD of the European Community commission at this concentration level (30), gives assurance that no unacceptable bias will be introduced by the fact of using tissue from another animal for calibration. The RSD values calculated from the combination of the 2 batches give an estimate of the interday precision and are also <20%. Table 5 gives the reproducibility and interday RSD for liver and muscle.

It was originally planned to use streptomycin as an internal standard for dihydrostreptomycin, and tobramycin as an internal standard for gentamicin and neomycin. However, the

Table 5. Relative standard deviation of the quantification ions for series of livers and muscles spiked at 50 ppb, with and without internal standard

	Liver		Muscle	
	Reproducibility RSD[a], %	Interday RSD[b], %	Reproducibility RSD[a], %	Interday RSD[b], %
Dihydrostreptomycin with internal standard	10	5	6	3
Dihydrostreptomycin without internal standard	14	9	6	15
Gentamicin C1 with internal standard	17	16	9	6
Gentamicin C1 without internal standard	16	13	9	14
Neomycin with internal standard	18	12	16	9
Neomycin without internal standard	19	10	14	14

[a] On 10 spiked tissues from different animals.
[b] Calculated from the first 5 portions of footnote a, plus 5 different spiked portions of the same animal analyzed another day.

Table 6. Comparison with a liquid chromatographic method with fluorometric detection[a] for samples incurred with neomycin

	Concn[b], ppb	
Sample	Mass spectrometry	Fluorescence
1	556	453
2	748	682
3	83	92
4	162	187
5	165	199

[a] Ref. 7.

[b] Each result is the mean from 2 different extractions. Significant figures are not representative of the precision of the method.

use of streptomycin was shown to be impractical, since its molecular weight and retention time were too close to those of dihydrostreptomycin and both compounds share the same secondary ions, so that streptomycin produces significant interference on the dihydrostreptomycin signal. Tobramycin, on the other hand, seemed to be a good internal standard; it eluted close to gentamicin and neomycin, it had a similar recovery percentage in spiked samples, and its use as an internal standard had been reported in methods for aminoglycosides in animal tissues (8, 32). However, RSD values for 10 spiked kidneys analyzed on the same day were higher when using tobramycin as an internal standard (*see* Table 4). This result is in agreement with that of Heller et al. (22), who were able to use tobramycin as an internal standard for gentamicin in an LC/MS/MS method when applied to plasma and urine, but not when the matrix was milk or kidney because the signal of tobramycin varied depending on the composition of the kidney while the signal of gentamicin did not change. It was, therefore, decided not to use tobramycin as an internal standard, but it was still included in our analyses as a quality control tool. The ideal internal standards would be, of course, isotope-labelled aminoglycosides, but none could be found on the market.

The limits of detection (LODs), estimated as signal-to-noise ratio = 3, are, respectively, 0.1, 0.1, and 0.4 ppb for dihydrostreptomycin, gentamicin C1, and neomycin in kidney. They are, respectively, 0.2, 0.2, and 0.5 ppb in liver and 0.2, 0.4, and 0.7 ppb in muscle. Linearity was checked in the 0–5000 ppb range for gentamicin C1, neomycin, and dihydrostreptomycin. For every analyte, the correlation coefficient was >0.995 for both assays performed.

Since no certified samples were available for aminoglycosides, we checked exactness by comparing results from this method with those obtained with an LC method with fluorometric detection (7) for veal kidney samples incurred with gentamicin and neomycin. The fluorometric method was reported for gentamicin, but it proved to work as well for neomycin in our laboratory. Data for neomycin are given in Table 6. Each entry in this table is the mean of 2 analyses of the incurred sample. A *t*-test showed that we cannot reject the null hypothesis, at the 95% confidence level, that the mean difference between the 2 techniques is equal to zero. For gentamicin, only 2 incurred samples were available, so no statistical test was performed. However, it was obvious that results from LC/MS/MS were lower than those from LC. This situation results from the fact that, in the LC method, gentamicin C1, $C1_a$, C2, and $C2_a$ are all measured (7), while the MS method only measures gentamicin C1 as stated earlier.

While optimizing different extraction and cleanup parameters (TCA concentration, trap column loading time, etc.), we did not identify any parameters that should be critically controlled. Robustness was further proven by the fact that more than 4000 extracts were injected on the system over a 1-year period without a significant problem or degradation in the quality of the chromatograms, as long as maintenance conditions reported in the *Experimental* section were observed. This method can be extended to include more analytes; it has already been shown to work for streptomycin and tobramycin in this paper, and preliminary work in our laboratory for spiked spectinomycin and quinolones, and for incurred tetracyclines, has been successful.

Conclusions

We have developed a rugged, high sample throughput, and highly automated LC/MS/MS method for the analysis of dihydrostreptomycin, gentamicin C1, and neomycin in veal tissues. The sample extraction is fast and simple, which permits preparation of 24 samples in half a day, and the automated cleanup is sufficient to minimize ion suppression/enhancement phenomena and permit quantification. Estimated LODs range from 0.1 to 0.7 ppb, depending on the tissue and the analyte.

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