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# Quantitation of spectinomycin residues in bovine tissues by ion-exchange high-performance liquid chromatography with post-column derivatization and confirmation by reversed-phase high-performance liquid chromatography–atmospheric pressure chemical ionization tandem mass spectrometry

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**Abstract**

Determinative and confirmatory methods of analysis for spectinomycin residue in bovine kidney, liver, muscle and fat have been developed. The determinative method is a single-column HPLC ion-exchange procedure that incorporates a two-step post-column oxidation of the secondary amines to primary amines followed by derivatization with *o*-phthalaldehyde. The method was validated in all tissues to a low-end concentration of 0.10 μg/g (limit of quantitation) and to a high-end of 10 μg/g for kidney, which is the rate-limiting tissue for residues of spectinomycin. The recovery of spectinomycin from all tissues was >80% and the variability (R.S.D.) was generally <10%. For liver, an alternative reversed-phase HPLC separation was required for incurred-residue samples. The confirmatory method employed an atmospheric pressure chemical ionization–MS–MS approach utilizing a rapid reversed-phase HPLC system with a mobile phase of methanol and 1% acetic acid. The protonated molecular ion for spectinomycin at *m/z* 333 produced four diagnostic reaction–product ions at 98, 116, 158 and 189 for confirmation. The method was validated to a lower limit of confirmation of 0.10 μg/g. 

*Keywords:* Spectinomycin; Aminocyclitols; Antibiotics; Aminoglycosides

## 1. Introduction

Spectinomycin is an aminocyclitol antibiotic produced by *Streptomyces spectabils* and may be regarded as part of a subclass of aminoglycoside antibiotics. It is bacteriostatic and exerts its antibacterial effect by binding to the 30S ribosome, which disrupts bacterial protein synthesis. Spectinomycin is primarily effective against Gram-negative bacteria such as *Pasteurella haemolytica, Pasteurella multocida and Haemophilus somnus*. It has limited activity against a number of Gram-positive bacteria. Generally, it is not active against anaerobic bacteria.

Spectinomycin has good activity against various organisms that are responsible for respiratory diseases in food-producing animals such as cattle and swine. When used for this indication, spectinomycin must be detectable in the various edible tissues of these animals for the purposes of regulating its use. Thus, in the development of spectinomycin for such

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use, analytical methods were required to not only detect and quantify spectinomycin at the residue level, but also confirm its identify whenever the concentration of it was found to be above the concentration deemed to be safe for human consumption (MRL or tolerance).

Structurally, spectinomycin has no chromophore that would provide reasonable detection in the normal operating range of UV detectors. HPLC chromatographic detection of UV-transparent molecules such as spectinomycin offer an interesting challenge to the analytical chemist, particularly when the molecule needs to be detected as a residue in a complex biological matrix and, thus, must be either derivatized for detection or detected by non-UV techniques. Several approaches have been reported in the literature. Myers and Rindler developed a post-column derivatization technique for spectinomycin bulk-drug analysis that was based on the derivatization of primary amines with *o*-phthalaldehyde (OPA), which has often been used for protein and peptide analysis [1]. For spectinomycin, which has two secondary amines, oxidation to primary amines with perchlorate was required before the reaction with OPA proceeded smoothly. However, their analysis was not applied to spectinomycin at the residue level. Haagsma et al. [2] recently adapted this approach for the detection of spectinomycin in swine, chicken and calf plasma. Other approaches have included precolumn derivatization with such agents as 2-naphthalenesulfonyl chloride, for bulk-drug applications [3], and 2,4-dinitrophenylhydrazine, for spectinomycin residue in plasma [4]. Fluorescamine derivatization has been used for thin-layer chromatography (TLC) detection [5], but not for HPLC.

There have also been other detection methods reported, such as the electrochemical detection method for spectinomycin residue in milk [6] and pulsed amperometric detection of spectinomycin in tissues [7]. There have also been confirmatory methods reported using particle beam LC–MS [8], ion spray LC–MS [9] and ion spray LC–MS–MS [10].

We have developed a modification of the post-column OPA method of Haagsma et al. [2] for the quantification of spectinomycin in bovine kidney, liver, muscle and fat that has the advantage of simplified ion-exchange chromatography. In addition, we have also discovered that a dihydrospectinomycin metabolite, which interferes with the analysis of parent spectinomycin in liver, can be resolved by a reversed-phase HPLC separation. The tissue extract that is prepared for this quantitative ion-exchange HPLC method can also be used in a rapid reversed-phase HPLC atmospheric pressure ionization with a collision-induced dissociation (MS–MS) procedure for confirmation based on the detection of four reaction product ions. These methods have been validated to a limit of quantitation (LOQ) of 0.10 μg/g, with an estimated limit of detection of 0.025 μg/g, and a limit of confirmation (LOC) of 0.10 μg/g.

## 2. Experimental

### 2.1. Materials and reagents

Acetonitrile, dichloromethane and methanol were of analytical-reagent or HPLC grade from Baxter Healthcare, Burdick and Jackson, Muskegon, MI, USA. Boric acid (granular), citric acid (granular), monobasic potassium phosphate, potassium hydroxide (pellets), phosphoric acid (85% solution), sodium hydroxide (pellets), sodium hydroxide (50% solution), sodium sulfate (anhydrous, granular), sulfuric acid (concentrated) and trichloroacetic acid (crystals) were all of analytical-reagent grade from Mallinckrodt (Paris, KY, USA). HPLC water was either deionized or Milli-Q 18W, processed through a Milli-Q Plus 4 stage system, Model ZD40 115 84 (Millipore, Milford, MA, USA). Fluoraldehyde (OPA) was the Reagent Solution from Pickering Laboratories (Mountain View, CA, USA). Sodium hypochlorite was obtained as the commercial 5.25% solution, Clorox Bleach, from Clorox (Oakland, CA, USA).

### 2.2. Solutions

Various solutions were prepared as follows: Boric acid buffer (0.4 *M*, pH 10.2–10.5) was prepared with 24.4 g of boric acid dissolved in 1000 ml of deionized water and basified with 22.0 g of potassium hydroxide pellets. The mixture was stirred on a hot plate, using low heat to facilitate the dissolution.

Citric acid extraction buffer (0.025 *M*, pH 4.0) was prepared with 5.25 g of citric acid dissolved in 1000 ml of deionized water and the pH was adjusted to 4.0 with a 50% sodium hydroxide solution. Citric acid elution buffer (0.05 *M*, pH 2.6) was prepared with 10.5 g of citric acid dissolved in 1000 ml of deionized water and the pH was adjusted to 2.6 with a 50% sodium hydroxide solution. Trichloroacetic acid (10%, w/v) was prepared with 100 g of trichloroacetic acid dissolved in 1000 ml of deionized water. Potassium phosphate buffer (0.025 *M*, pH 6.7) was prepared with 3.4 g of monobasic potassium phosphate dissolved in 1000 ml of deionized water; the pH was adjusted to 6.7 with a 50% sodium hydroxide solution. Potassium phosphate buffer (0.1 *M*, pH 7.0) was prepared by dissolving 6.8 g of monobasic potassium phosphate in 500 ml of deionized water and adjusting the pH to 7.0 with a 50% sodium hydroxide solution. Sodium hydroxide (1 *M*) was prepared using 40 g of sodium hydroxide pellets dissolved in 1000 ml of deionized water. Sodium sulfate buffer (0.5 *M*, pH 2.6) was prepared by dissolving 142 g of sodium sulfate in 2000 ml of Milli-Q water, adjusting the pH to 2.6 with concentrated sulfuric acid and suction-filtering it through a 0.2-mm nylon filter (Rainin). Sodium sulfate buffer (0.05 *M*, pH 2.6) was prepared by diluting the 0.5 *M* sodium sulfate buffer 1:10 (v/v) with Milli-Q water and adjusting the pH to 2.6 with concentrated sulfuric acid. Sodium hypochlorite (0.13%) was prepared by diluting 25 ml of Clorox Bleach with 500 ml of deionized water followed by 10 ml of 0.1 *M* monobasic potassium phosphate buffer, pH 7. The volume was brought to 1000 ml with deionized water. Sulfuric acid (0.1%) was prepared by adding 1 ml of concentrated sulfuric acid to 1000 ml of Milli-Q water.

### 2.3. Spectinomycin standard solutions

Approximately 5 mg of spectinomycin sulfate reference standard (Pharmacia and Upjohn Control Reference Standard) was accurately weighed into a 100-ml volumetric flask and diluted to volume with 0.05 *M* citric acid buffer, pH 2.6. Appropriate dilutions were made with 0.05 *M* citric acid buffer, pH 2.6, to obtain the desired concentration range for the external standard curve, nominally 10 to 0.05 μg/ml, corrected to spectinomycin free base equivalents on the basis of the potency of the reference standard.

### 2.4. Bovine tissues

Control (spectinomycin-free) bovine liver, kidney, muscle and fat were obtained from several non-treated cattle. Biologically incurred-residue samples of the tissues were obtained from several cattle (steers and heifers) that had been treated subcutaneously with spectinomycin sulfate sterile solution (ADSPEC) for five consecutive days at a dose at 15 mg/kg.

### 2.5. Tissue sample preparation

Approximately 5 g of ground tissue were weighed accurately into a 50-ml polypropylene centrifuge tube. Then, 25 ml of 0.025 *M* citric acid buffer, pH 4.0, were added followed by 1 ml of 10% trichloroacetic acid and 10 ml of dichloromethane. The tube was tightly capped and manually shaken until the tissue was dislodged from the bottom of the tube. The sample was placed on a platform shaker and shaken at high speed (240 cpm) for 20 min. The sample was centrifuged for 10 min at 2800 RCF, then the supernatant was transferred to a clean 50 ml polypropylene centrifuge tube. The pH of the solution was adjusted to 6.6 to 6.8 with 1 *M* sodium hydroxide. The sample was centrifuged for 10 min at 2800 RCF.

A CBA solid-phase extraction (SPE) column was prepared by washing the cartridge with 3 ml of methanol followed by 3 ml of 0.025 *M* potassium phosphate buffer, pH 6.7, using 5 inches of vacuum (as measured by the manifold vacuum gauge) to pull the solvents through the cartridge. The sample extract was transferred as aliquots to the SPE cartridge and pulled through the cartridge using a vacuum to maintain a flow-rate of about 1–2 ml/min. After all of the sample had passed through the cartridge, the cartridge was washed with 10 ml of 0.025 *M* potassium phosphate buffer, pH 6.7. The SPE cartridge was then placed in the empty sample tube and centrifuged for 10 min at 2800 RCF to remove residual wash solution.

A clean 100×13 mm glass tube was placed into

the vacuum manifold and the analyte was eluted from the SPE cartridge with 3 ml of 0.05 *M* citric acid buffer, pH 2.6. The SPE eluent was quantitatively transferred to a 5.0-ml volumetric flask and brought to volume with 0.05 *M* citric acid buffer, pH 2.6. Approximately 0.75 ml of the final sample solution was transferred into an autoinjector vial and 20, 50 or 100 μl were injected into the HPLC system.

### *2.6. Ion-exchange HPLC with post-column derivatization*

The HPLC ion-exchange system consisted of the following components: A quaternary HPLC pump, an autoinjector, a Chrompack Ionosphere C column (150×4.6 mm, Chrompack, Raritan, NJ, USA), a mixing tee and a fluorescence detector that was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The mobile phase contained 20% acetonitrile throughout. The ionic gradient ran from 80% 0.05 *M* sodium sulfate to 55% 0.05 *M*–25% 0.50 *M* sodium sulfate in 20 min, followed by a washout, first with 80% 0.50 *M* sodium sulfate, then with 80% 0.1% sulfuric acid. The flow-rate was 0.80 ml/min throughout the run.

### *2.7. Reversed-phase HPLC with post-column derivatization for liver analysis*

The reversed-phase chromatography was accomplished with a Zorbax SB $C_{18}$ column (250×4.6 mm) with a water–acetonitrile gradient containing 0.2% heptafluorobutyric acid ion-pairing agent.

### *2.8. Post-column derivatization*

The post-column oxidation/derivatization system consisted of a binary gradient pump for the oxidizing solutions, an isocratic pump for the OPA solution and two mixing coils, a 1.0-ml volume coil in a 70°C water bath for the oxidizing step, followed by a 0.5-ml coil in a 26°C bath for the derivatizing step. The hypochlorite and boric acid buffers were introduced post-column at a ratio of 50:50 at 1.0 ml/min. The OPA solution was introduced after the reaction coil at a flow-rate of 0.5 ml/min.

### *2.9. Solutions of antibiotics for interference testing*

An aqueous solution containing ceftiofur, erythromycin, lincomycin, neomycin, penicillin, sulfadimethoxine and tylosin was prepared to deliver approximately 5 μg of each, in 100 μl, to a 5-g spectinomycin-fortified control kidney sample, to provide a residue concentration of 1 μg/g for each antibiotic.

### *2.10. Reversed-phase HPLC–atmospheric pressure chemical ionization (APCI) MS–MS*

The reversed-phase chromatography was accomplished using a Zorbax SB $C_{18}$ column (150×2.1 mm) with an isocratic mixture of methanol–1% acetic acid (8:92, v/v). The flow-rate was 0.17 ml/min. A divert-valve was employed to divert column effluent to waste until about 30 s prior to, and resuming about 30 s after, the elution of the spectinomycin component.

### *2.11. Mass spectrometer*

The mass spectrometer used was a Finnigan TSQ 7000, operating in the Q1–Q3 product ion mode, fitted with the Finnigan APCI source. The corona voltage was set to 4.7 kV, the vaporizer temperature was set at 400°C, the capillary temperature was set at 150°C and the electron multiplier was set at variable settings, but nominally at 1300 V. Q1 was set to $m/z$ 333 for the protonated ion for parent spectinomycin. Q3 was set to scan the selected fragment ions at $m/z$ 98, 116, 158, 189 and 333 over 1 s. The collision gas was argon, with the cell pressure set at 2.5 mT. The collision cell offset was −30 V.

## 3. Results and discussion

### *3.1. Quantitative assay chromatography*

The original Haagsma method [2] for spectinomycin in tissues was performed with a dual column system that required two switching valves and a timing mechanism to produce optimum ion-exchange separation of spectinomycin from the various endogenous tissue components. However,

this column-switching approach was eliminated with the implementation of a more careful ion-strength gradient that efficiently resolves spectinomycin from all of these substances with a variable retention time that was dependent upon the condition of the column.

The standard curve was generally prepared by injecting from five to seven standards, two or three times each. When the standard curve ranged from 0.05 to 10.0 μg/ml, a $1/x^2$ weighting was employed to preserve linearity at the low end of the curve. Each standard response was evaluated with regards to its deviation from the theoretical value. When a standard response varied by more that 10% (15% for the lowest standard), it was discarded from the data set and the curve was recalculated. In most cases, no standards were discarded on this basis.

### 3.2. Quantitative assay for kidney

Kidney is the rate-limiting tissue for cattle treated with ADSPEC and generally contains five to ten times as much parent spectinomycin residue as liver [11]. The proposed European Union (EU) MRL for spectinomycin in kidney is 5.0 μg/g and the US tolerance is 4.0 μg/g. As a result, the method was evaluated for kidney at concentrations ranging from 0.10 μg/g, expected to be the low-end concentration required to conduct residue depletion studies, to 10.0 μg/g (twice the MRL), to ensure method performance at high concentrations.

Five sets of bovine kidney samples, which included four samples at each of three fortification concentrations (0.10, 2.5 and 10.0 μg/g) and a control/blank sample were prepared and analyzed in separate analytical runs, to evaluate the repeatability of the assay as well as to assess the day-to-day variability. The combined data are summarized in Table 1. Except for the high R.S.D. at 0.1 μg/g, which was driven largely by the high responses of one of the sample sets at 0.1 μg/g, all gave acceptable within-day R.S.D. values (<12% when this sample was excluded). For the entire data set at 0.10 μg/g, the overall R.S.D. was 18.7%.

The data at 2.5 and 10 μg/g demonstrated a within-day R.S.D. <10% and a day-to-day R.S.D. <10%. Thus, the variability of the method was within acceptable limits. The overall mean recovery of the method was 84.4% across the three concentrations studied, with a range from 81.2% at 2.5 μg/g to 93.9% at 0.10 μg/g.

Table 1
Validation data for the quantitative method for spectinomycin in various tissues

| Tissue | Fortification concentration (μg/g) | *n* | Recovery (%) | R.S.D. (%) |
|---|---|---|---|---|
| Kidney | 0.10 | 19 | 93.9±17.6 | 18.7 |
| | 2.5 | 20 | 81.2±4.1 | 5.0 |
| | 10.0 | 20 | 84.5±5.3 | 6.3 |
| | 2.0[a] | 18[a] | 83.5±10.1[a] | 12.1[a] |
| | 4.0[a] | 19[a] | 82.7±8.5[a] | 10.3[a] |
| | 8.0[a] | 19[a] | 85.6±5.7[a] | 6.7[a] |
| Liver | 0.10 | 6 | 89.6±9.2 | 10.3 |
| | 2.5 and 5.0 | 9 | 85.0±5.1 | 6.0 |
| Muscle | 0.10 | 11 | 89.4±11.1 | 12.4 |
| | 0.66 | 11 | 90.2±1.6 | 1.8 |
| Fat | 0.10 | 9 | 88.8±7.8 | 8.8 |
| | 0.66 and 0.99 | 8 | 92.5±5.7 | 6.2 |

[a]Overall combined results from a four-laboratories method trial.

A second series of control kidney samples fortified at 2.0, 4.0 and 8.0 mg/g was prepared and assayed during the course of a method trial that involved four laboratories. These results are also presented in Table 1. The mean recoveries at the three concentrations tested across the four laboratories were very consistent, ranging from 82.7% at 4.0 mg/g to 85.6% at 8.0 mg/g. The R.S.D. values ranged from 6.7% at 8.0 μg/g to 12.1% at 2.0 μg/g.

Typical chromatograms of a control/blank kidney sample fortified with spectinomycin at 2.5 μg/g, an incurred-residue kidney sample containing 0.1 μg/g of spectinomycin and a non-fortified control/blank kidney sample are illustrated in Fig. 1. The same results were obtained when a mixture of seven antibiotics, namely ceftiofur, erythromycin, lincomycin, neomycin, penicillin, sulfadimethoxine and tylosin, which were also added at a concentration of 1 μg/g each. Thus, there were no interferences in the method as a result of the presence of these antibiotics.

In addition to the fortified samples, several samples of biologically incurred residue obtained from a recently completed field study were analyzed in replicates by four different laboratories, to evaluate the method with incurred-residue kidney samples as

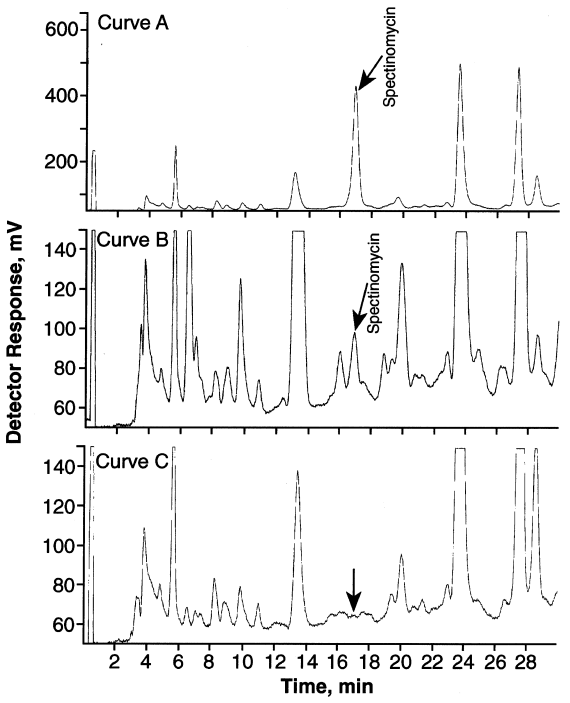


Fig. 1. Sample chromatograms for spectinomycin residue in bovine kidney: Fortified control kidney at 2.5 μg/g (curve A), incurred-residue kidney at 0.10 μg/g (curve B) and control/blank kidney (curve C).

well. These data, presented in Table 2, demonstrate the utility of the method for incurred residues of parent spectinomycin.

### 3.3. Quantitative assay for liver, muscle and fat

In a similar fashion, the method was applied to liver, muscle and fat, although at concentrations that were more in line with the respective MRLs and observed incurred-residue concentrations. The data are presented in Table 1.

Table 2
Analysis of spectinomycin incurred-residue in bovine kidney

| Concentration range (μg/g) | *n* | Concentration found (μg/g) | R.S.D. (%) |
|---|---|---|---|
| 0.30 to 5.6[a] | 33[a] | 0.32–5.56 | 1.1–11.0 |
| 2.45[b] | 20[b] | 2.09±0.35 | 16.9 |
| 7.65[c] | 20[c] | 6.43±0.96 | 14.9 |

[a]Samples from eleven animals were assayed in triplicate.
[b]Subsamples from one animal were analyzed five times in four different laboratories.
[c]Subsamples from another animal were analyzed five times in four different laboratories.

#### 3.3.1. Liver

Control/blank liver samples were fortified with spectinomycin at 0.10, 2.5 and 5 μg/g. The results are presented in Table 1. The recovery and variability at all concentrations were acceptable (recovery >80%, R.S.D. <10%). Thus, the modified method performed well for parent spectinomycin fortified in control bovine liver.

However, problems were encountered with incurred-residue liver samples during residue-depletion studies. An alternative approach for incurred-residue in liver was required to resolve parent spectinomycin from other substances in liver, as described in Section 3.4 below.

#### 3.3.2. Muscle

Control/blank bovine muscle samples were fortified with spectinomycin at 0.10 and 0.66 μg/g. The recoveries at 0.10 and 0.66 μg/g were 89.4 and 90.2%, respectively, with acceptable R.S.D. values, as shown in Table 1. Thus, the method performed well for parent spectinomycin fortified in control bovine muscle. There were no problems encountered with incurred-residue muscle samples obtained from non-injection sites, as far as could be determined. Injection-site muscle appeared to contain a dihydroxy-spectinomycin metabolite that did produce a false-positive for samples examined several days after dose administration, but at concentrations below the MRL for muscle. [11]

#### 3.3.3. Fat

Control/blank bovine fat samples were fortified with spectinomycin at 0.10 and 0.66–0.99 μg/g. The recoveries were 88.8% at 0.10 μg/g and 92.5% in the 0.66–0.99 μg/g samples, respectively, with acceptable R.S.D. values, as shown in Table 1. Thus, the method performed well for parent spectinomycin fortified in bovine fat. There were no problems encountered with incurred-residue fat samples, as far as could be determined.

### 3.4. Reversed-phase method for liver

During the course of a residue depletion study for ADSPEC, the ion-exchange method produced inordinately high concentrations of parent spectinomycin compared to what had been observed in a radiolabeled study [11]. The results of this radiolabeled residue study indicated that the primary residue in liver following the subcutaneous administration of spectinomycin sulfate was dihydrospectinomycin, and less than 5% of the residue was detected as parent spectinomycin [11]. Analysis of these incurred-residue liver samples by a modification of the HPLC–MS–MS method described below, demonstrated that an anomer of the principal liver residue metabolite, dihydrospectinomycin, eluted at nearly the same time as parent spectinomycin. Thus, the integration of the ''spectinomycin'' peak was overestimated for spectinomycin itself. While careful attenuation of the ion-strength gradient was partially successful for the resolution of these components, complete resolution was not always achieved.

A reversed-phase separation with the ion-pairing reagent, heptafluorobutyric acid, in acetonitrile and water, was developed that successfully resolved these components, in addition to an endogenous component of liver itself. A sample chromatogram is provided in Fig. 2. This method was validated with control liver samples fortified with nominal concentrations of spectinomycin at 1.0, 1.9 and 3.9 μg/g. The recoveries were 81.0% (R.S.D.=4.8%), 80.6% (R.S.D.=2.8%) and 80.4% (R.S.D.=2.1%), respectively.

### 3.5. Confirmatory method for spectinomycin in tissues

For regulatory needs, a confirmatory method for parent spectinomycin was required that must perform at a concentration $\geq R_m$ (tolerance) or ≥MRL, preferably well below these concentrations, since acceptable performance below the $R_m$/MRL would provide a high level of confidence that the assay will confirm parent spectinomycin in violative samples, defined as tissue samples that contain marker residue at a concentration $>R_m$/MRL. The objective was, therefore, to provide validation data for confirmation above a concentration of 2.5 mg/g of parent spectinomycin in fortified control/blank kidney, which was approximately $\frac{1}{2}$ of the anticipated MRL [11], at 10 mg/g (2×MRL) in fortified control/blank kidney, and in several incurred-residue samples across this concentration range, as well as below the $R_m$/MRL.

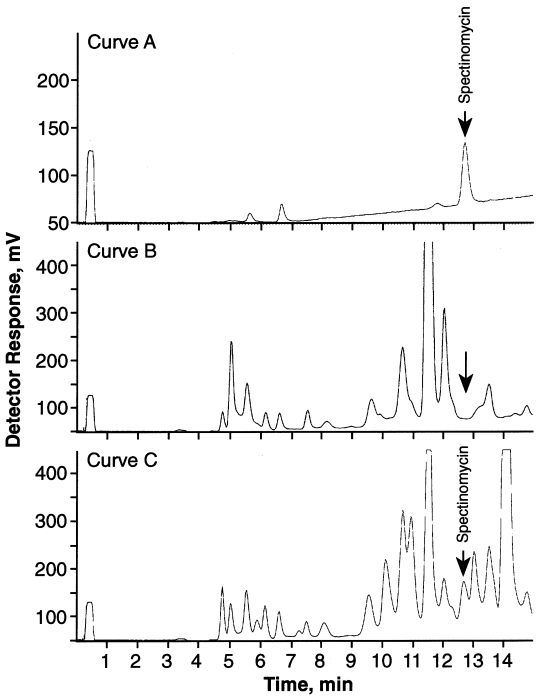


Fig. 2. Sample chromatograms for spectinomycin by reversed-phase analysis: A 0.50-μg/ml standard (curve A), control/blank liver (curve B) and incurred-residue liver containing spectinomycin at 0.6 μg/g (curve C).

During initial attempts to analyze the samples for parent spectinomycin by thermospray HPLC–MS, the only fragment ion observed was at $m/z$ 207, which indicated the actinamine moiety resulting from the hydrolysis of spectinomycin in the thermospray source. The same observation was made when spectinomycin was analyzed by electrospray HPLC, although the intensity of $m/z$ 207 was very weak. These gentle techniques, when limited to single quadrupole mass spectrum analysis, would not provide sufficient information by themselves for the confirmation of parent spectinomycin in tissue samples. These observations indicated that either interface conditions more conducive to fragment ion generation were needed, or a highly selective frag-

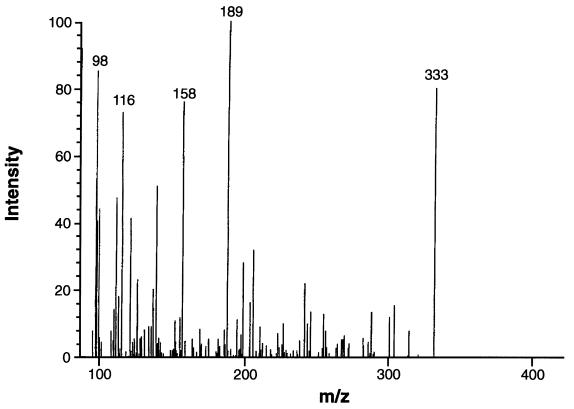


Fig. 3. APCI-CID reaction product spectrum of $m/z$ 333 for spectinomycin.

mentation technique, such as collision-induced dissociation mass spectrometry (CID–MS or MS–MS) would be needed to confirm parent spectinomycin residue.

There have been several papers published on confirmatory methods employing CID–MS. These include clenbuterol in retinal tissue [12], sarafloxacin in catfish tissue [13] and aminoglycosides in bovine kidney [10], among others. This technique has therefore been accepted by regulatory agencies for confirmatory analyses of drug residues in food-producing animal tissues. Spectinomycin undergoes CID–MS to generate a spectrum of reaction product ions as illustrated in Fig. 3. The selection of four of the most intense reaction product ions at $m/z$ 98, 116, 158 and 189, derived from $m/z$ 333, as shown in Fig. 4, should be more than adequate for the confirmation of parent spectinomycin following literature recommendations [14,15].

The development and validation of the confirmatory analysis for parent spectinomycin employed the APCI interface between the HPLC column and the triple-quadrupole mass spectrometer. Quadrupole 1 (Q1) was set to selectively pass only ions at $m/z$ 333 throughout the chromatographic run. Argon gas was introduced into the collision cell (Q2) to collide with and fragment the incoming ions from Q1. The third quadrupole (Q3) was set for selected ion monitoring at $m/z$ 98, 116, 158, 189 and 333. This technique is now known as reaction product ion monitoring, RPM.

To simplify the implementation of this method, samples of kidney extracts that were prepared for the determinative method were used in the development of this confirmatory method. Thus, no special manipulations or extra sample preparation steps were necessary for this confirmatory assay. However, the HPLC conditions utilized in the determinative method were not compatible with HPLC–MS interfaces because the mobile phases in that method utilized non-volatile inorganic buffer salts. Therefore, a modified chromatographic method was developed that utilized a $C_{18}$ reversed-phase column and an

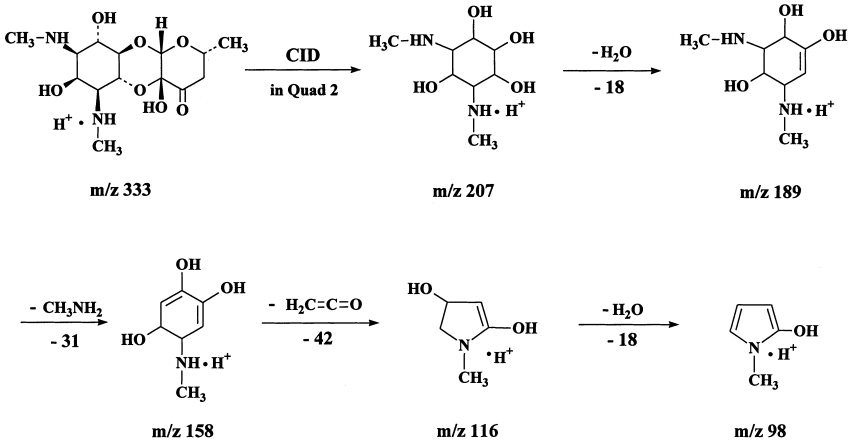


Fig. 4. Proposed CID fragmentation scheme for $m/z$ 333 for spectinomycin.

acetic acid (1%)–methanol mobile phase. This combination produced a very fast chromatography, with a retention time of 2.4 to 2.9 min for parent spectinomycin, as illustrated in Fig. 5 for an incurred-residue kidney sample containing spectinomycin at 0.18 μg/g, which gave a retention time of 2.40 min. The relative intensities, or abundances, of the ions are also shown. Because mass spectrometry, especially CID–MS, is a highly specific technique, less stringent separation/resolution requirements were needed for successful HPLC–MS, i.e. that might otherwise be inadequate for conventional HPLC detectors.

The qualitative confirmatory analysis for spectinomycin residue in tissue samples must meet three criteria, as outlined for MS methods such as employed in the confirmatory method for pirlimycin in bovine milk and liver [16]. The criteria applied to spectinomycin are:

(1) Observation of peak area responses above a signal-to-noise ($S/N$) ratio of 3:1 for the four ions $m/z$ 98, 116, 158 and 189, for the confirmation of spectinomycin. The minimum acceptable $S/N$ ratio for each ion may be obtained from three times (3×) the baseline noise for a control kidney sample analysis, or from a ''clean'' peak-free area of the chromatogram that was identified as a suitable background area.

(2) The appearance of the spectinomycin response in the RIC for the sample should be at the appropriate HPLC retention time ($t_R$ within 0.2 min, or 12 s) relative to a spectinomycin standard.

(3) The relative abundances (RAs) of $m/z$ 98, 116, 158 and 189 (relative to the base peak) for spectinomycin in the kidney sample must not differ by more than ±10% from an external spectinomycin standard or from the average of external spectinomycin standards, using standard solutions that have a spectinomycin concentration that is close to that in the sample, obtained just before and/or after the sample by the bracketing technique. For chemical ionization techniques and identification with more than three ions, the acceptable RA variability may be extended to ±15% [14,15].

The analytical observations for the confirmation of spectinomycin residue are illustrated in Fig. 5, which shows the reconstructed ion chromatograms at $m/z$ 98, 116, 158 and 189 for an incurred-residue kidney

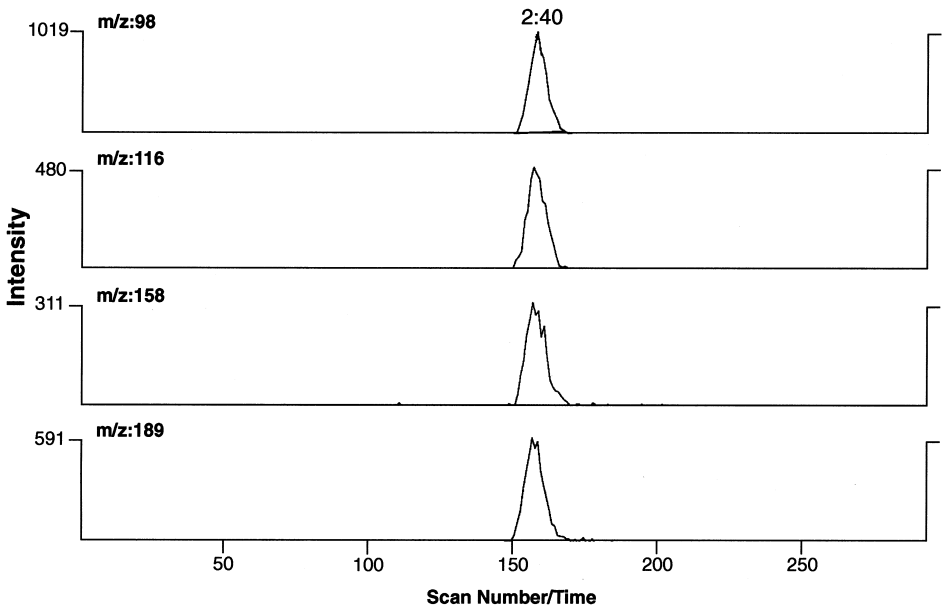


Fig. 5. HPLC–APCI-MS–MS reconstructed ion chromatograms of spectinomycin incurred-residue in bovine kidney at a concentration of 0.18 μg/g.

Table 3
Confirmatory analysis of spectinomycin in bovine tissues

| Tissue | Concentration range (μg/g) | *n* | Number of samples meeting confirmatory criteria | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Criterion 1 ($S/N>3$) (all ions) | Criterion 2 ($t_R$ standard±0.2 min) | Criterion 3 (±10% RA for four ions) (*m*/*z*) | | | |
| | | | | | 98 | 116 | 158 | 189 |
| Kidney | Fortified, 1.78–7.05 | 12 | 12 | 12 | 12 | 12 | 12 | 12 |
| | Incurred, 0.72–3.96 | 18 | 18 | 18 | 18 | 18 | 18 | 18 |
| | Incurred, 0.04–0.70 | 12 | 12 | 12 | 11[a] | 11[a] | 12 | 12 |
| Liver | Fortified, 1.0–5.0 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Muscle | Fortified, 0.1–0.6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Fat | Fortified, 0.1–1.1 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |

[a]One sample at 0.04 μg/g had RAs for two ions that were >10% variance relative to a standard.

sample containing spectinomycin at 0.18 μg/g. Evaluation of the observations of this type relative to the criteria for confirmation is presented in Table 3 for a series of eighteen fortified control kidney samples and 30 incurred-residue kidney samples. The control/blank kidney samples were fortified with spectinomycin at concentrations of 1.78, 3.63 and 7.05 μg/g. The incurred-residue kidney samples contained spectinomycin at concentrations ranging from 0.04 to 3.96 μg/g.

Two kidney samples that contained spectinomycin at 0.07 and 0.05 μg/g were confirmed since the three criteria were fully met for all of the four ions. One kidney sample that contained spectinomycin at a concentration of 0.04 μg/g failed criterion three. Therefore, the validated LOC for this method in bovine kidney was established as 0.05 μg/g. These data demonstrated that the confirmatory method positively confirmed parent spectinomycin in kidney down to a concentration of 0.05 μg/g, well below the concentration for which the assay will be used for regulatory purposes (see the discussion on $R_m$/MRL above).

To test the applicability of this confirmatory method in liver, muscle and fat, two control/blank samples of each tissue fortified with spectinomycin at two concentrations and one non-fortified control/blank sample were analyzed. These data (Table 3) demonstrate the utility of this method to positively confirm parent spectinomycin down to a concentration of 0.10 μg/g in these tissues.

## 4. Conclusions

An adaptation/modification of the Haagsma residue analysis procedure that reduces the two-column-switch analytical HPLC separation for parent spectinomycin to a single HPLC column analysis has been validated for use in bovine kidney, liver, muscle and fat. The modification preserved the extraction step and the post-column fluorescence derivatization detection step prescribed by the Haagsma procedure. The recoveries of spectinomycin from kidney fortified at 2.5 and 10.0 μg/g were 81.2% (R.S.D.=5.0%) and 84.5% (R.S.D.=6.3%), respectively. The LOQ was validated at 0.10 μg/g, with a recovery of 93.9% (R.S.D.=18.7%). The assay was not susceptible to the presence of other antibiotics and must be classified as a highly specific method.

An HPLC–APCI-MS–MS analytical method for the confirmation of parent spectinomycin in bovine kidney has been developed and validated. Three criteria were met for confirmation, i.e. $S/N>3$:1 for four reaction product ions, an HPLC retention time of spectinomycin in the sample within ±0.2 min relative to an external spectinomycin standard, and a relative abundance of four reaction product ions in the sample within ±10% relative to an external standard. The method was validated to an upper concentration of 7.05 μg/g. The method was shown to confirm parent spectinomycin residue in bovine kidney to a LOC of 0.05 μg/g. The method was also

shown to confirm parent spectinomycin in bovine muscle, liver and fat to a LOC of 0.10 μg/g.

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