ELSEVIER

Journal of Chromatography B, 718 (1998) 95–102

JOURNAL OF CHROMATOGRAPHY B

Confirmation of spectinomycin in milk using ion-pair solid-phase extraction and liquid chromatography–electrospray ion trap mass spectrometry

Mary C. Carson*, David N. Heller

Center for Veterinary Medicine, US Food and Drug Administration, 8401 Muirkirk Road, Laurel, MD 20708, USA

Received 16 March 1998; received in revised form 19 June 1998; accepted 6 July 1998

Abstract

A confirmation procedure is described for residues of spectinomycin in bovine milk. Spectinomycin is extracted from raw milk using ion-pair reversed-phase solid-phase extraction. The extracts are ion-pair chromatographed on a polymeric reversed-phase column and analyzed on a quadrupole ion trap mass spectrometer equipped with an electrospray interface. MS–MS data are acquired in the scan mode of product ions deriving from m/z 333, the protonated molecular ion. The estimated limit of confirmation is between 0.05 and 0.1 μg/ml. The procedure was validated with control milk, fortified milk (0.1–5.0 μg/ml), and milk from cows dosed with spectinomycin. Published by Elsevier Science B.V.

Keywords: Spectinomycin

1. Introduction

Spectinomycin is an aminocyclitol antibiotic that is widely used for veterinary purposes. It is approved in the US as oral or injectable forms for disease treatment in dogs, poultry (except laying hens), and young swine [1]. The established US tolerance for negligible residues in the edible tissues of turkey and chicken is 0.1 parts per million (ppm) [2]. Spectinomycin sulfate has recently been approved as a subcutaneous (s.c.) injectable for the treatment of bovine respiratory disease in non-lactating cattle with a tolerance of 4.0 ppm for residues in kidney [3]. In setting the tolerance for residues in tissue, no portion of the total allowable daily (human) intake was set aside for milk. Therefore, in the US there is no tolerance for residues of spectinomycin in milk. However, in order to minimize consumer exposure, the US Food and Drug Administration desired a method capable of monitoring residues as low as 0.1 ppm in milk.

The purpose of this study was to develop a regulatory confirmatory procedure for residues of spectinomycin in milk. Previous workers in this laboratory, Schermerhorn et al., developed a determinative procedure employing liquid chromatography with electrochemical detection [4]. This procedure had a lower limit of quantitation of 0.1 ppm. For regulatory purposes, a confirmatory procedure of high specificity was needed to complete the method for spectinomycin residues in milk.

Some work on spectinomycin confirmation had been published at the time this study began [5,6].

*Corresponding author.

0378-4347/98/$19.00 Published by Elsevier Science B.V.
PII: S0378-4347(98)00331-4

Neither of these procedures extracted spectinomycin from milk with high recovery. They also employed tandem mass spectrometry (MS–MS) on triple quadrupole instruments. We wished to use the newly available ion trap methodology whose lower cost makes it more accessible to regulatory laboratories.

The sample extraction method of Schermerhorn et al. [4] was tested for suitability for ion trap MS–MS confirmation. The extracts were chromatographed on a polymeric reversed-phase column using a volatile ion-pair agent, heptafluorobutyric acid (HFBA), in the mobile phase. The extraction worked, even when vastly simplified, but the limit of confirmation (LOC) was higher than desired. There was also a significant matrix effect on the signal which caused variable response. A suitable extraction would enrich the concentration of spectinomycin residues while removing more of the matrix.

At this time (early 1997), Kwok et al., gave a preliminary report on a procedure for aminoglycoside (including spectinomycin) confirmation on a triple quadrupole instrument using ion-pair solid-phase extraction (SPE) [7]. Using this information and conditions from our HFBA chromatographic mobile phase, we also developed an efficient ion-pair SPE procedure for spectinomycin in milk.

2. Experimental

2.1. Reagents and chemicals

House deionized water was further purified by Milli-Q Plus (Millipore, Bedford, MA, USA). Methanol was HPLC grade (Burdick & Jackson, Muskegan, MI, USA). Spectinomycin hydrochloride analytical reference standard was from US Pharmacopeia (Rockville, MD, USA). Adspec™ sterile solution (spectinomycin sulfate), was a gift of The Upjohn Company (now Pharmacia & Upjohn; Kalamazoo, MI, USA). All other chemicals were analytical reagent grade. Stock standard spectinomycin solution was prepared in water at 100 μg free base per milliliter and stored refrigerated. Chromatographic standards were prepared daily in 8 m*M* aqueous HFBA at eight concentrations ranging from 0.125 to 12.5 μg/ml (equivalent to original milk concentrations ranging from 50 ppb to 5 ppm).

2.2. Animal treatment

Control milk was obtained from an individual cow prior to drug treatment and from several bulk tanks from different regions of the US. Milk containing incurred residues of spectinomycin was produced by either infusing a solution of spectinomycin into the udder (intramammary infusion, IMM) or by administering a single s.c. dose of Adspec to a lactating dairy cow. The IMM incurred residue milk was from a previous study and had been stored at −80°C before analysis in this study. The s.c. incurred residue milk was analyzed fresh within 48 h of milking.

2.3. Extraction and cleanup

Negative and positive (fortified) control samples were prepared with each sample set. Milk (5.0 ml) was Vortex mixed with 0.25 ml 30% (w/v) trichloroacetic acid (TCA) and centrifuged at 2200 *g* for ~10 min. A portion (2.5±0.1 ml) of the aqueous middle layer was carefully removed, avoiding the lower protein pellet and the upper fat layer, and transferred to tubes containing 2 ml 20 m*M* aqueous HFBA. The contents were mixed and applied to 3 ml Sep-Pak Vac tC_{18} cartridges (Waters Corporation, Milford, MA, USA) that had been conditioned with 2.5–3 ml MeOH and 2×1.5 ml 8 m*M* HFBA. The ion-paired extracts were allowed to gravity filter through the cartridges, and then ~15 inches Hg vacuum was applied for a few minutes to dry the cartridges. The cartridges were eluted with 2×1.5 ml methanol–20 m*M* aqueous HFBA (4:1, v/v) into tubes marked at 1 ml. The volumes of the extracts were reduced to approximately 0.5 ml under a stream of zero-grade nitrogen at 50°C in a TurboVap LV Evaporator (Zymark Corporation, Hopkinton, MA, USA) and brought back to the 1 ml mark with water. The extracts were sonicated for 5 min and filtered through 0.2 μm 13 mm PVDF syringe filters (Whatman) into autosampler vials. Extracts not analyzed the same day were stored refrigerated or at −10°C.

2.4. Liquid chromatographic system

The LC system consisted of a Hewlett-Packard 1050 quaternary pump equipped with a 1050 autosampler, solvent helium sparging, and inline filter frit

between the pump and autosampler. The column was a PLRP-S 2.1×150 mm, 5 μm particle size (Polymer Labs, Amherst, MA, USA). Mobile phase A was methanol, B was water, C was 20 m*M* HFBA in water, and D was not used. The flow-rate was 0.23 ml/min of an isocratic composition of A–B–C (40:20:40, v/v). The injection volume for all standards and extracts was 20 μl. Total run time was 4–5 min. At the end of the day the column was flushed with methanol–water (80:20, v/v).

2.5. Ion trap mass spectrometer set-up

The Finnigan LCQ ion trap mass spectrometer with electrospray interface (ESI) was calibrated according to the manufacturer's specifications. It was operated in positive ion MS–MS mode using a tune file that had been optimized while infusing a 100 μg/ml solution of spectinomycin at 5 μl/min into the mobile phase at 0.23 ml/min. The optimization resulted in the following ESI source settings: Sheath gas (N_2) flow-rate, 86; auxiliary gas flow-rate, 43; spray voltage, 5.50 kV; capillary temperature, 250°C; capillary voltage, 23.00 V; tube lens offset, 31.00 V. Waveform and source collision induced dissociation (CID) were left off.

The Experiment Method consisted of the following conditions: acquire time, 5.00 min; flow diverted from MS to waste, 0.00–2.30 min and 4.00–5.00 min; scan power, MS^2; parent ion, 333.10; isolation width, 1.0 a.m.u.; relative collision energy, 22.0 (arbitrary units); polarity, positive; scan mode, full; range, *m/z* 90.0–317.00; microscans/scan, 3; maximum ion injection time, 300 ms. Spectra were generally determined by averaging across the retention window (start of peak to end of peak) for spectinomycin.

In between analysis sets (approximately 45 injections), the ESI heated capillary was cleaned with a fine wire and 50% methanol following the manufacturer's directions. This process took approximately 5 min. The capillary temperature was reduced to 200°C at the end of the day.

2.6. Confirmation criteria

Four criteria had to be met for spectinomycin presence to be confirmed in a sample extract. (1) The retention time in the sample was within 0.2 min of

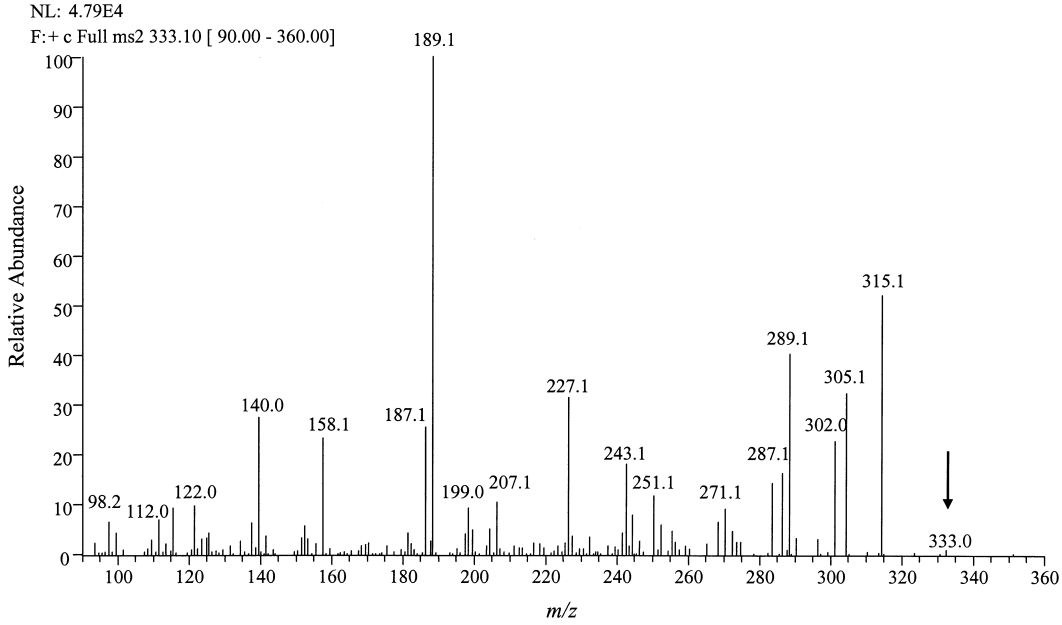

Fig. 1. Ion trap product ion spectrum (averaged) from a high level (10 μg/ml) of chromatographed spectinomycin standard. Relative collision energy was selected to optimize reproducibility of the spectra. Under the conditions selected, little or no precursor *m/z* 333.1 remains (position shown by arrow). With a precursor ion of 333, *m/z* 90 is the lower instrumental limit for acquisition on the LCQ.

spectinomycin's retention time in a contemporaneous standard. (2) The spectinomycin peak in the reconstructed ion chromatogram (RIC, consisting of m/z 333 fragment ions m/z 140, 186–190 and 227) was present at a S/N ratio of at least 3/1 (estimated by visual inspection of the RIC). (3) The spectrum obtained from the putative spectinomycin peak visually matched the spectrum obtained from a contemporaneous standard. (4) The quality assurance positive and negative control samples confirmed and failed to confirm, respectively, for the presence of spectinomycin.

3. Results and discussion

3.1. ESI-ion trap MS–MS of spectinomycin

Spectinomycin was readily ionized by ESI and detected in the ion trap mass spectrometer. In full scan MS mode, the major ion detected was the protonated hydrated molecular ion, m/z 351, with the protonated molecular ion, m/z 333, present at about 30% relative abundance. A dimer of the hydrated form, m/z 701, was the third most abundant ion. These results are consistent with what other investigators have observed for ESI analysis of spectinomycin using other analyzers [8,5]. Spectinomycin exists in aqueous solution as a ketone hydrate [8]. In MS–MS mode, CID of m/z 351 produced m/z 333 with little further fragmentation. Since this was not sufficiently specific for regulatory confirmation, $[M+H]^+$ m/z 333 (less abundant but a more intuitive choice) was selected as the precursor ion for MS–MS analysis. Relative collision energy (RCE) optimized over a narrow range. Only the precursor was observed at 19%, and no ions at all were found at 30%, with maximum fragment ion response observed at 21–22%. Using a RCE of 22% produced the most reproducible fragment ion spectra, but left very little precursor ion (Fig. 1). Since a non-spectinomycin background ion, m/z 333 319 (transition from m/z 333 to 319), occasionally was strong enough to interfere with the analysis, only fragment ions from m/z 317 to m/z 90, the lower limit of the instrument, were scanned during residue analyses. The fragment ions observed were comparable to those reported using a triple quadrupole instrument [6], but the relative abundances of the ions were much different. Fig. 2 shows a proposed scheme for fragmentation of spectinomycin in an ion trap mass spectrometer. This scheme is based on MS^n experiments.

3.2. Extraction, analysis, and confirmation of spectinomycin in milk

Spectinomycin residues were initially analyzed using LC conditions described by Hornish and Wiest [6]. Spectinomycin peak shape was very poor (multiply split) when 20 μl of standard in either water, citrate buffer, or mobile phase (1% aqueous acetic acid–methanol, 92:8, v/v) were injected on the Zorbax SB C_{18} narrowbore column. The quality of the MS–MS spectra was also poor for concentrations at and below 200 ng/ml. Volatile ion-pair agents had been previously used by other investigators [5,8,9] for spectinomycin LC. Replacing acetic acid with HFBA increased spectinomycin retentivity, enabling use of a higher concentration of methanol, thereby improving peak shape, sensitivity, and electrospray performance. Quantitative reproducibility also improved, presumably due to more controlled ionization of the ion-paired spectinomycin in the ESI source. Chromatography was further enhanced by switching to a polymeric column. A postcolumn divert valve was used as described in [6]. Although not entirely ideal (peak tailing and occasional splitting still existed, and there was a small but consistent difference of retention times between standards and extract residues), these LC conditions were sufficient for residue confirmation. The quantitative response (sum of fragment ions m/z 140, 186–190 and 227) of the ion trap was highly variable from day to day, but within a day was linear from 500 to 12 500 ng/ml, with $R^2>0.995$ and residuals usually $<10\%$. From 125 to 500 ng/ml the response was slightly more variable, with R^2 usually near 0.98.

Milk samples were initially extracted as described by Schermerhorn et al. [4], with only the modification that the final extracts were mixed with HFBA rather than the nonvolatile sodium 1-decanesulfonate. Using this extraction, residues in milk were reliably confirmed for concentrations as low as 200 ng/ml (ppb). The power of the ion trap in MS–MS mode was such that even when the ex-

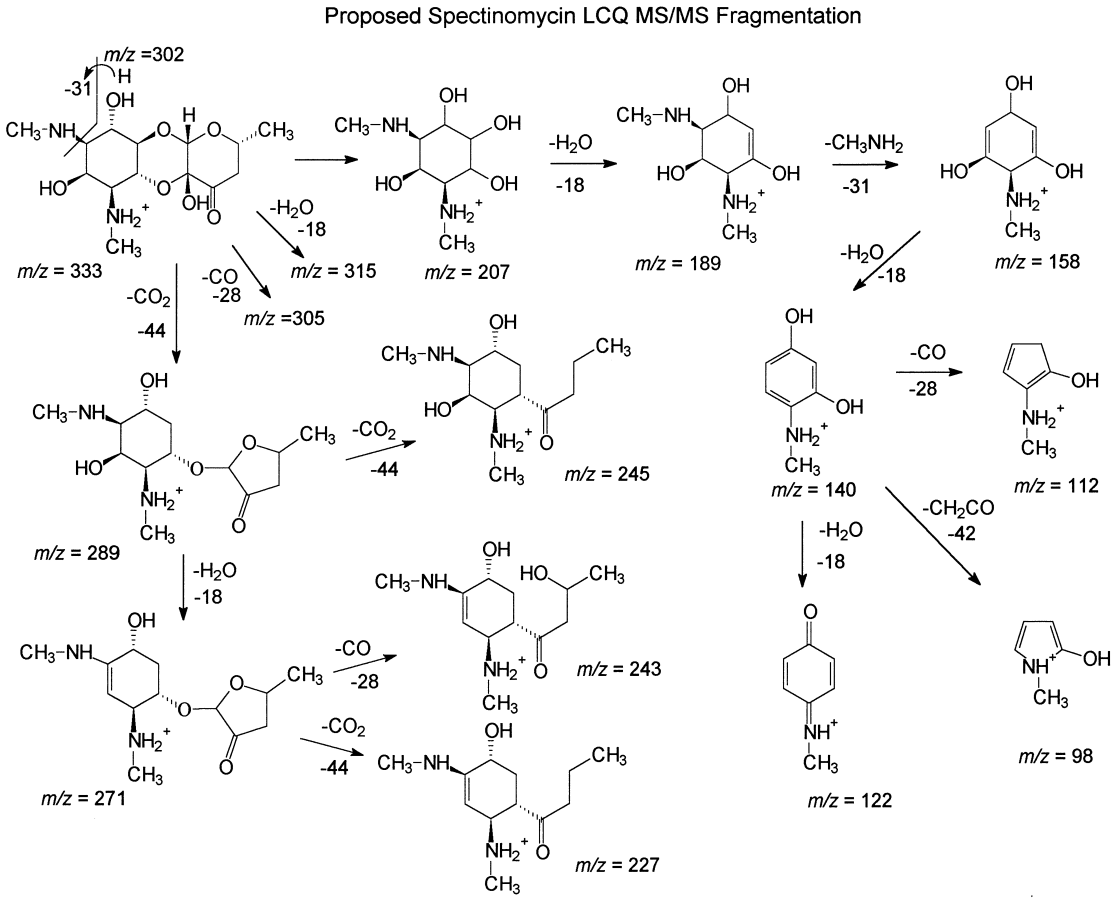

Fig. 2. Proposed spectinomycin fragmentation in ion trap MS–MS. Fragmentation scheme is based on ions observed in MS–MS–MS experiments.

traction procedure was severely abbreviated (milk mixed with TCA and methylene chloride, centrifuged, and a portion of the aqueous layer mixed with HFBA and analyzed), residues of spectinomycin at 200 ppb confirmed. However, below 200 ppb the spectra became erratic in both standards and extracts (change of base peak, multiple missing diagnostic ions) and confirmation was not always possible. Quantitation with these extracts was also not reliable, due to a pronounced and variable positive matrix effect on absolute response.

A major limitation of the approach in [4] was the lack of a concentration step. Attempts at modification of an ion-exchange SPE procedure developed for spectinomycin residues in tissue [10] were unsuccessful, due to very low apparent recovery. Haagsma and co-workers [11,12] published poultry plasma procedures employing sodium dioctylsulfosuccinate ion-pair SPE on C_{18} cartridges, establishing the utility of ion-pair SPE for residual spectinomycin analysis. Most recently, an abstract appeared outlining the use of HFBA ion-pair reversed-phase SPE for MS–MS (triple quadrupole) confirmation of multiple aminoglycosides and spectinomycin in a variety of matrices, including milk [7].

We evaluated several reversed-phase (polymeric and silica-based C_{18}) SPE cartridges for their ability to extract spectinomycin from milk. The HFBA conditioning and extraction conditions used were based on the LC mobile phase we had established. Although all the cartridges tested produced extracts

that were 'confirmable' at 100 ppb, Sep-Pak Vac tC_{18} cartridges gave the highest recoveries. Typical results using HFBA ion-pair SPE with Sep-Pak Vac tC_{18} cartridges are shown in Figs. 3 and 4. Control milk samples from various sources were tested. Ion [333]301 was consistently observed in the milk extracts, and ion [333]315 was also found, but otherwise there were no major interferences found. Several classes of veterinary drugs (aminoglycosides, β-lactams, tetracyclines, fluoroquinolones and others) were also tested and did not interfere. The procedure was validated using control milk, milk fortified at a wide range of concentrations, and milk from cows dosed with spectinomycin. The results are summarized in the first five columns of Table 1. All control milk failed to confirm, all samples containing >100 ppb confirmed, and most samples containing 100 ppb confirmed without difficulty on the first analysis. Spectinomycin presence was confirmed for 58 h following dosing in milk from a cow dosed s.c. and was also confirmed in milk following IMM dosing.

This procedure was developed primarily as a qualitative confirmation of residue presence. However, since the linearity of response for standards was good, we also evaluated the ability of the ion trap, when used with this extraction, to determine the quantitative amount of residue present. These results are summarized in the last four columns of Table 1. These results do not quite meet the US Food and Drug Administration guidelines [13] (recovery between 80 and 110%, and relative standard deviation below 10%) for methods for residues with tolerances at and above 100 ppb, but they do not fail to meet the guidelines by a large margin. Minor modifications to instrumental conditions and the extraction

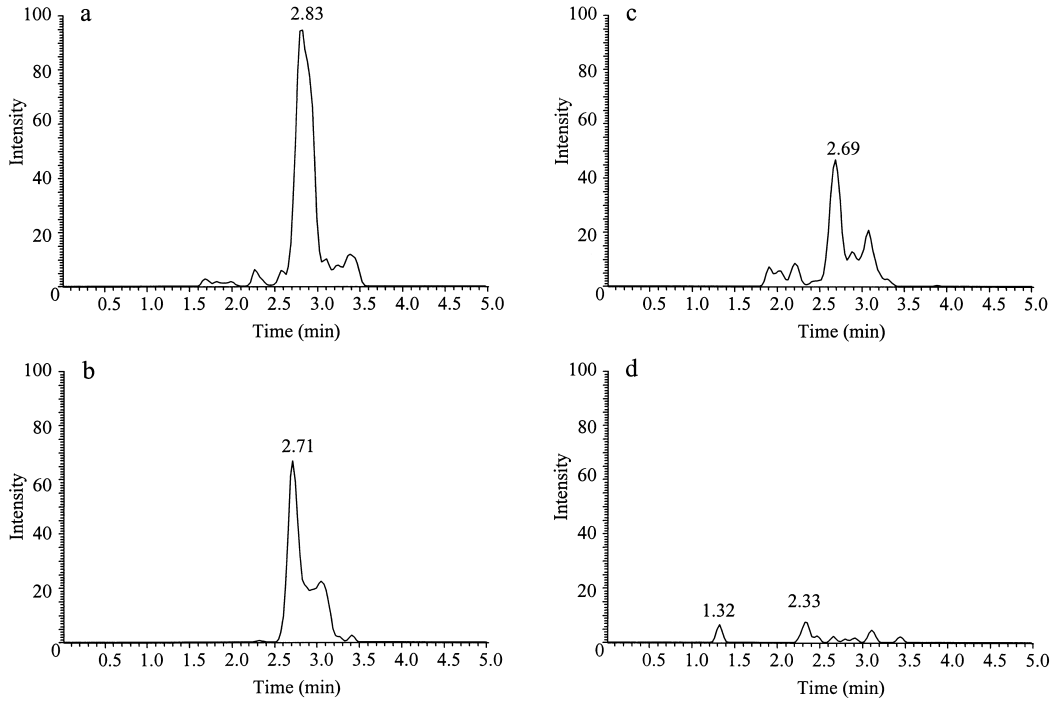

Fig. 3. Reconstructed ion chromatograms for 250 ng/ml (100 ppb equivalent) spectinomycin standard (a), 100 ppb fortified milk sample (b), milk from dosed cow, 48 h post-s.c. dose (c), and control milk sample (d). Chromatograms are the combined response from m/z 333 product ions m/z 140, 186–190, and 227. All chromatograms are normalized to the same scale, with 100%=14 000 units. Instrumental conditions and sample preparation are described in the text.

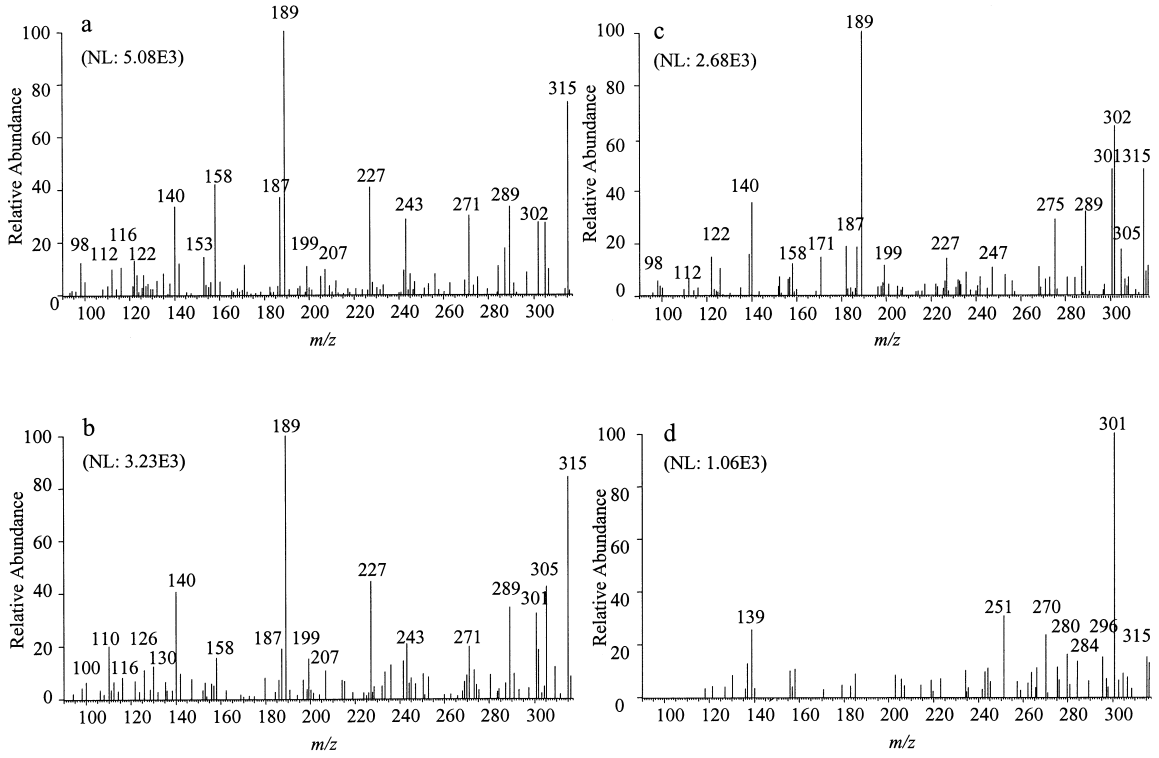

Fig. 4. Averaged spectra from the same analyses depicted in Fig. 3. Product ion spectra were only acquired up to m/z 317 due to lack of specific spectinomycin ions and presence of nonspecific background ions at higher masses. The NL number in parentheses is the intensity of the base peak. The incurred residue spectrum shown (c) is typical of data collected at or just above the lower limit of confirmation of the method.

process may be sufficient to decrease the variability of the assay and increase the recovery at the lower concentrations.

4. Conclusions

Spectinomycin is readily detected using ESI ion trap MS–MS. Use of the ion-pair agent HFBA enables reversed-phase SPE of spectinomycin residues from milk. This procedure meets qualitative specificity criteria for regulatory confirmation with an LOC between 50 and 100 ppb. Quantitatively, the ion trap response was more variable than that normally observed for UV–visible or fluorescence detectors, and some of the recoveries were slightly low. However, the quantitative data suggest that with additional refinement, this procedure has the potential to meet guidelines for regulatory determination in the US. With minor modifications and further validation, this procedure could serve as a complete method.

Acknowledgements

We are grateful to Dr. B. Lamar Lee of the Pharmacia & Upjohn Company for the gift of Adspec sterile solution and to Mr. Herbert Righter for dosing the cows to generate the incurred residue milk used in this study.

Table 1
Confirmatory assay validation results and quantitative performance of the assay with control, fortified, and residue incurred milk samples

Sample	Confirmation results				Quantitative results			
	n	C[a]	FTC[b]	?[c]	*n*	Average found ppb	R.S.D.[d] (%)	Recovery (%)
Control milk (Sources: USDA, OR, 6 states)	18		18		18	N.d.		
100 ppb fortified	30	26		4	15	72.7	15	73
200 ppb fortified	6	6			6	139	13	69
500 ppb fortified	6	6			6	460	18	92
1 ppm fortified	6	6			6	927	11	93
2 ppm fortified	6	6			6	1740	12	87
5 ppm fortified	6	6			6	4140	5.9	83
10 h post-s.c. dose incurred[e]	3	3			3	2080	0.89	N.a.[f]
24 h post-s.c. dose incurred	3	3			3	644	15	N.a.
34 h post-s.c. dose incurred	3	3			3	222	4.5	N.a.
48 h post-s.c. dose incurred	3	3			3	70.2	5.7	N.a.
58 h post-s.c. dose incurred	3	3			3	53.3	8.5	N.a.
72 h post-s.c. dose incurred	3		1	2	3	18.2	21	N.a.
52 h post-IMM dose incurred[g]	3	3			3	537	20	N.a.
72 h post-IMM dose incurred	3	3			3	280	5.1	N.a.

[a] Confirms: spectrum at retention time window for spectinomycin visually matches standard's spectrum.
[b] Fails to confirm.
[c] Evidence spectinomycin present, but not an unambiguous match (usually two or more ions exceed m/z 189 intensity).
[d] Replicates of incurred residue samples were analyzed on the same day; replicates of fortified samples were analyzed over three or more days.
[e] Fresh milk from a cow treated with a subcutaneous dose of spectinomycin.
[f] Not applicable.
[g] Milk from a cow treated by intramammary infusion with spectinomycin. The milk had been stored frozen for more than three years.

References

[1] 21 CFR §520.2122, §520.2123, §522.2120, US Government Printing Office, Washington, DC, 1997.
[2] 21 CFR §556.600, US Government Printing Office, Washington, DC, 1997.
[3] US Federal Register, US Government Printing Office, Washington, DC, Vol. 63, No. 84, 1998, p. 24 106.
[4] P.G. Schermerhorn, P.-S. Chu, P.J. Kijak, J. Agr. Food Chem. 43 (1995) 2122.
[5] L.G. McLaughlin, J.D. Henion, P.J. Kijak, Biol. Mass Spectrom. 23 (1994) 417.
[6] R.E. Hornish, J.R. Wiest, Proceedings 44th ASMS Conference on Mass Spectrometry, Portland, OR, 1996, p. 979.
[7] D. Kwok, P. Chow, B. Mori, M. Yong, Proc. 45th ASMS Conference on Mass Spectrometry, Palm Springs, CA, 1997, p. 960.
[8] L.G. McLaughlin, J.D. Henion, J. Chromatogr. 591 (1992) 195.
[9] G. Inschauspé, D. Samain, J. Chromatogr. 303 (1984) 277.
[10] R.E. Hornish, J.R. Wiest, J. Chromatogr. A 812 (1998) 123.
[11] N. Haagsma, J.R. Keegstra, P. Scherpenisse, J. Chromatogr. 615 (1993) 289.
[12] N. Haagsma, P. Scherpenisse, R.J. Simmonds, S.A. Wood, S.A. Rees, J. Chromatogr. B 672 (1995) 165.
[13] General Principles for Evaluating the Safety of Compounds Used in Food-producing Animals, Parts V-2 and V-3, US Food and Drug Administration, Center for Veterinary Medicine, Rockville, MD, 1994.