Development and Validation of an HPLC/MS/MS Method for the Confirmation of Eprinomectin Marker Residue in Bovine Liver Tissue

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A liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) method has been developed and validated to detect and confirm the marker residue of eprinomectin (B_{1a} component) in bovine liver when present at levels ≥2400 ng/g. Sample extracts prepared for assay by the LC/fluorescence determinative method (prior to the derivatization step) and standard solutions were used to validate the confirmatory method. The chromatographic retention time of the eprinomectin B_{1a} component and the relative ion intensities of the parent ion ($[M + H]^+$ at *m/z* 914) and three product ions (*m/z* 896, 468, and 330) generated by fragmentation of the parent ion are compared to those determined from standards.

Keywords: *Eprinomectin; residue; confirmatory; bovine liver; LC/MS/MS*

INTRODUCTION

Eprinomectin is the semisynthetic avermectin, 4″-deoxy-4″-(*epi*-acetylamino)avermectin B_1, prepared by conversion of the 4″-hydroxy group of avermectin B_1 (abamectin) to a 4″-*epi*-*N*-acetyl substituent. Like abamectin, eprinomectin is a mixture of two active constituents, >90% 4″-deoxy-4″-(*epi*-acetylamino)avermectin B_{1a} (Figure 1; R = C_2H_5) and <10% 4″-deoxy-4″-(*epi*-acetylamino)avermectin B_{1b} (Figure 1; R = CH_3). Eprinomectin is a novel avermectin for the control of parasites in beef and dairy cattle.

The determinative method used to measure levels of eprinomectin in bovine tissue is described in the accompanying paper (Payne et al., 1997). The marker residue of eprinomectin is the B_{1a} component, the target tissue in cattle is liver, and the marker residue tolerance (R_m) is established as 4800 ng/g. Ivomec Eprinex Pour-On for Beef and Dairy Cattle was recently approved by the U.S. Food and Drug Administration (FDA) with a zero milk discard time and a zero slaughter withdrawal time. During the drug development process a confirmatory method was developed and validated but did not proceed to an FDA method trial because of the zero slaughter withdrawal time.

Because a confirmatory method must be definitive for the analyte, a very high degree of structural specificity is required (Sphon, 1978). This has led to mass spectrometry being widely accepted as the method of choice.

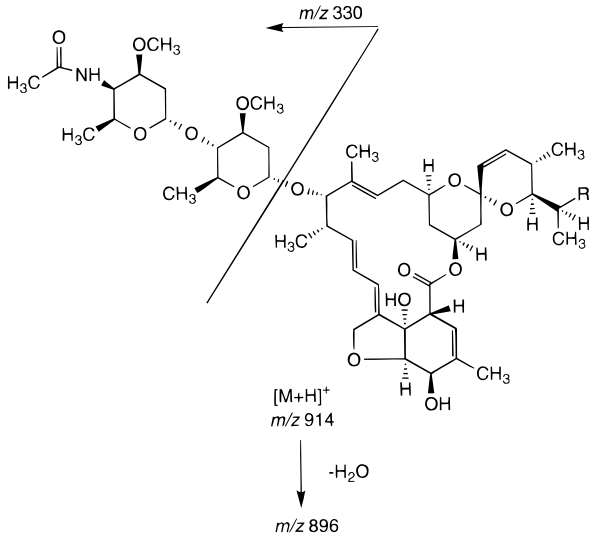

Figure 1. Eprinomectin. Structures of B_{1a} (R = C_2H_5) and B_{1b} (R = CH_3) components and identities of parent ion $[M + H]^+$ at *m/z* 914 and product ions at *m/z* 896 and 330.

Several methods utilizing mass spectrometric detection for the confirmation of compounds related to avermectin in bovine tissue or milk have been reported. A chemical ionization method using mass spectrometry/mass spectrometry (MS/MS), with sample introduction via a direct exposure probe, gave a detection limit of 8−10 ppb for ivermectin in bovine liver tissue (Tway et al., 1984). A liquid chromatography/mass spectrometry (LC/MS) method with thermospray ionization was used to confirm moxidectin residues at the 250 ppb level (spiked or incurred) in cattle fat (Khunachak et al., 1993), and the use of a particle beam interface with LC/MS enabled Heller and Schenck (1993) to detect ivermectin at 15 ppb in liver and at 2 ppb in milk. For confirmatory methods based upon chromatography/mass spectrometry, FDA requirements for confirmation of the presence

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of residues are the application of criteria to the chromatographic and spectrometric measurements (Sphon, 1978). These are based on comparisons with measurements determined from injections of standard solutions of the analyte. First, the chromatographic retention time of the analyte in samples should match (±10%) that of the analyte in standards. Second, the relative intensities of ions characteristic of the structure should match (±10% absolute units) the relative intensities determined for standards. For example, if a relative ion intensity determined from the standards is 40%, then the value determined for the sample should be within the range 30−50%. A minimum of three (preferably four) ions, of which one should be the molecular ion, should be monitored by mass spectrometry. A total of four (preferably five) criteria is therefore set and each of the criteria must be met for the presence of the analyte to be confirmed.

This paper describes the development and validation of an LC/MS/MS method to detect and confirm the marker residue of eprinomectin in bovine liver tissue when present at levels ≥2400 ng/g.

MATERIALS AND METHODS

Avermectins. Abamectin, doramectin, eprinomectin, ivermectin, and moxidectin were supplied by Merck Research Laboratories, Rahway, NJ. Sample extracts of control, fortified, and incurred tissues were prepared according to the determinative method by the Department of Agricultural and Veterinary Analytical Research and Development, Merck Research Laboratories, Rahway, NJ.

Instrumentation. High-performance liquid chromatography (HPLC) was performed using a Waters 600-MS liquid chromatograph equipped with a Rheodyne 8125 injector (100 μL loop), a Waters 486-MS UV−vis detector, and a Hewlett-Packard 3390A integrator. The column was an Abzelute ODS -DB (50 × 3 mm i.d.; 5 μm particles) equipped with an Abzelute guard disk. Mass spectrometry was performed on a Finnigan MAT TSQ 700 fitted with a Finnigan MAT electrospray interface.

Experimental Methods. Injections (10 μL) of methanolic solutions of standards and sample extracts were made manually. The mobile phase was water (adjusted to pH 5 with acetic acid)/acetonitrile (30:70 v/v), at a flow rate of 200 μL/min. The column was held at 40 °C, and UV detection was at 245 nm. Eprinomectin B_{1a} eluted at approximately 8 min. The mass spectrometer was operated in the MS/MS multiple reaction monitoring mode under positive ionization conditions. The electrospray needle voltage was 5.5 kV, and the capillary temperature was 225 °C. Nitrogen at 60 psi was used as the sheath gas; no auxiliary gas was used. The resolution of Q1 was approximately 2.4 amu, while Q3 was set at unit mass resolution; the scan width for each product ion was 0.3 amu. The $[M + H]^+$ ion of eprinomectin B_{1a} at *m/z* 914 was selected by Q1 and was fragmented by collision-induced dissociation with argon at a collision energy of 11.6 eV; the collision gas pressure was approximately 2 mTorr. The ions at *m/z* 914, 896, 468, and 330 were monitored by Q3, each with a dwell time of 0.48 s; total scan time was 2.0 s. The mass spectrometer was tuned to give a product ion mass spectrum in which the ion at *m/z* 914 was at 100% relative abundance, while the ions at *m/z* 896, 468, and 330 were each in the range 40−60% relative abundance. Automatic integration of the areas under the peaks in the mass chromatograms was performed under data system control, using Finnigan MAT ICIS software (version 8.1.1). Analyses were performed over 3 days, and at the end of each day the column (with guard disk) was regenerated by back-flushing sequentially with methanol, acetonitrile, tetrahydrofuran, acetonitrile, and methanol. Each solvent was pumped for 10 min at a flow rate of 1.0 mL/min. The column was then reversed to the normal direction of flow and was equilibrated with the mobile phase.

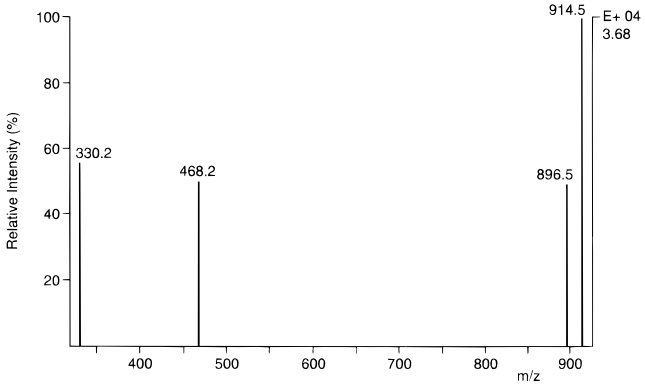

Figure 2. Typical MS/MS spectrum from injection of a 5.5 ng/μL standard of eprinomectin B_{1a}.

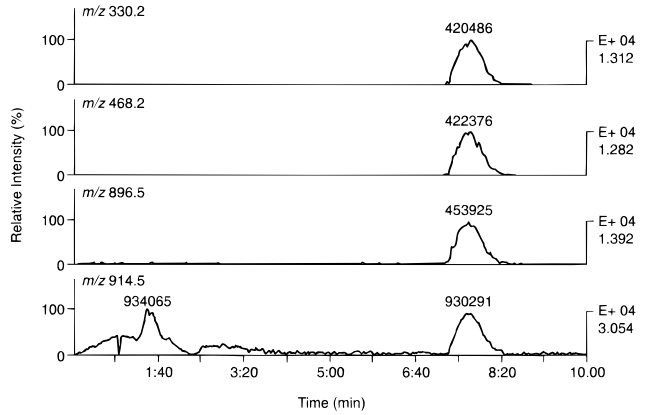

Figure 3. Typical ion chromatograms from injection of an extract of fortified bovine liver (2990 ng/g of eprinomectin B_{1a}).

RESULTS AND DISCUSSION

Analytical Method Development. An LC/MS/MS method using isocratic elution from a short HPLC column, electrospray ionization, and multiple reaction monitoring in the MS/MS mode with positive ion detection was developed. This method employs sample extracts prepared by the determinative method (prior to the derivatization step for HPLC/fluorescence assay) and standard solutions. Using a 10 μL injection volume, at least 30 matrix samples could be injected without any loss of chromatographic peak integrity. The efficiency of the column did degrade slowly, which required that the column be regenerated at the end of each day. Successful regeneration of the column was achieved by back-flushing with a series of organic solvents (see Materials and Methods) and was demonstrated by the injection on successive days of 27 matrix samples (plus 27 standards) and 40 matrix samples (plus more than 20 standards). No significant deterioration of the peak shape was observed in either set of injections, and the retention time drifted only about 1 min over an entire injection set.

The parent ion, $[M + H]^+$ at *m/z* 914, is fragmented by collision-induced dissociation to yield three product ions (*m/z* 896, 468, and 330). The proposed identities of the product ions at *m/z* 896 and 330 are depicted in Figure 1; the identity of the ion at *m/z* 468 is unknown. A typical MS/MS spectrum of a 5.5 ng/μL standard of eprinomectin B_{1a} is shown in Figure 2. Representative selected ion chromatograms from the LC/MS/MS analysis of an extract from bovine liver fortified at 2990 ng/g are given in Figure 3.

Method Validation Performance. To address issues encountered during the development work and

Table 1. Relative Ion Intensities from Triplicate Injections of an Incurred Sample Extract and Injections of a Bracketing Standard (Criteria Were Met)

			incurred				standard			
sample code	level (ng/μL)	equiv concn in tissue (ng/g)	*m/z* 914	*m/z* 896	*m/z* 468	*m/z* 330	*m/z* 914	*m/z* 896	*m/z* 468	*m/z* 330
STD[a]	5.5	2208					100	47.9	45.1	49.8
LC6225ARI	1.7	693	100	48.7	45.9	50.4				
			100	51.2	44.7	51.4				
			100	54.3	47.8	53.1				
STD	5.5	2208					100	47.3	44.2	47.3
mean			**100**	**51.4**	**46.1**	**51.6**	**100**	**47.6**	**44.7**	**48.6**

[a] STD, standard.

Good Laboratory Practices, the experimental design of the validation study incorporated the following elements. Triplicate injections of a midlevel standard (5.5 ng/μL) were made at the start of each day of analysis, to set initial retention time windows and relative ion intensities and to demonstrate that the system was in stable operation. It was decided to test the method initially at about the $0.5R_m$ level (2400 ng/g), and during the development and initial validation phases, triplicate injections of the fortified residue extracts (fortified, control liver from untreated animals) and incurred residue extracts (liver from treated animals) were made between single, bracketing injections of the midlevel standard. This was done to assess whether averaging the results from multiple analyses would improve method performance for samples containing the marker residue at levels substantially below the R_m.

Daily Schedules. Samples of each type, *i.e.*, extracts from samples of control liver from untreated animals ("control"), extracts from samples of liver from treated animals ("incurred"), and extracts from samples of fortified, control liver from untreated animals ("fortified") were assayed on each of the three days of analysis.

Assay of Mixed Animal Drug Standard. Injection of a high-level, mixed standard of products in the avermectin class (approximately 2500 ng/μL each of abamectin, doramectin, ivermectin, and moxidectin) produced no significant response for any of the characteristic ions in the retention time window where ions from a standard of eprinomectin B_{1a} appeared. This demonstrated the noninterference of these drugs in the assay.

Assay of Control Samples. Five control samples were assayed. They produced no response for any of the characteristic ions in the retention time window where ions from a standard of eprinomectin B_{1a} appeared. This demonstrated the specificity of the method and the noninterference of the matrix.

Assay of Incurred Samples. Six incurred samples (three samples from each of two different animals) were assayed. These samples had been previously assayed by the determinative method, which determined levels of the marker residue in the extracts in the range 1.6−2.2 ng/μL. These values correspond to an original level in tissue of approximately 647−860 ng/g. Although the use of samples from tissue containing incurred residue approximately in the range 2400−9600 ng/g was desirable, no incurred samples were ever available that contained the marker residue in this relatively high range. Two incurred samples were assayed on each of the three days of analysis, using the same HPLC column each day. Triplicate injections of each sample were made and were preceded and followed, immediately when possible, by a single injection of a midlevel standard (5.5 ng/μL).

Retention Time Criterion. For the incurred samples, the interday RSD of the retention time was 2.68% (n = 18) over the three days of analysis. For the 10 injections of standards used to bracket the incurred samples, the interday RSD of the retention time was 2.47% (n = 10). The mean retention times of the incurred samples were within ±10% of those of the standards on each day and clearly met the criterion for chromatographic reproducibility.

Relative Ion Intensity Criterion. A representative example of the relative ion intensities determined from triplicate injections of an incurred sample (LC6255ARI; marker residue concentration about 693 ng/g in tissue) and the bracketing standards are shown in Table 1. The mean relative ion intensities of the four characteristic ions determined from the standards are displayed immediately to the right of the means determined from the triplicate assays of the sample, all in bold type. For this sample, each mean relative ion intensity was within ±10% (absolute units) of the value determined for the bracketing standards; the criteria for confirmation by mass spectrometry were clearly met.

In the analysis of four of six incurred samples with marker residue concentrations in the range 647−820 ng/g, each of the relative ion intensities of each of the triplicate injections met the criteria. For the two samples that failed (concentration about 707 and 860 ng/g), the results did provide additional evidence for the presence of the marker residue even at levels below 1000 ng/g.

Assay of Fortified Samples. Ten different fortified samples were assayed over 3 days. Because incurred samples were available only at low levels, fortified samples were prepared to cover a wider range of residue levels. The samples had been previously assayed by the determinative method, which measured levels of eprinomectin B_{1a} in the range 2.1−7.5 ng/μL. These values correspond to a fortified level in tissue of approximately 828−2990 ng/g.

Retention Time Criterion. Three injections of 9 of the 10 fortified samples were made, and 1 sample was assayed on each of 2 days, for a total of 33 injections. The interday RSD of the retention time was 3.20% (n = 33) over the three days of analysis. For the 16 injections of standards used to bracket the incurred samples, the interday RSD of the retention time was 3.46% (n = 16). The retention times of the fortified samples, over all levels, met the retention time criterion.

Relative Ion Intensity Criterion. Six fortified samples (concentration range 966−2990 ng/g in tissue) assayed over the three days met all criteria. Several relative intensities determined for one low-level sample (fortified at 897 ng/g) were outside the ±10% criterion, but when

the means of triplicate injections were evaluated, the criterion was met for all four ions. Unexpectedly, 9 of 12 values determined for individual injections of 1 sample (LC6256F5RIN; fortified at 2990 ng/g) failed to meet the ±10% criterion, and 3 of 4 means from the triplicate injections also failed. However, sample LC6256F5RIN was reassayed successfully on day 3 of analysis when all 12 individual injection values then met the criterion. Two other, low-level fortified samples analyzed on day 3, LC6256F1 (943 ng/g) and LC6256F2 (828 ng/g), had one and two individual injections, respectively, in which three of four values were outside the ±10% criterion. Evaluation of the means from the triplicate injections brought sample LC6256F1 into compliance, but sample LC6256F2 continued to show three of four mean values just outside the ±10% criterion (+13.8%, +12.2%, +10.4%).

Nonconforming Results. Inspection of the data revealed that the nonconforming values determined for samples LC6256F5RIN and LC6256F2 were outside the ±10% limit due to a combination of elevated relative ion intensities observed for the samples and depresssed relative intensities determined for the bracketing standards. Together, the two effects were sufficient to cause the criterion to be exceeded.

A second validation trial was then performed with extracts from both incurred and fortified samples which had higher levels of marker residue than were available for the first trial. All sample preparation, LC/MS/MS parameters, and other conditions were as described under Materials and Methods, except that the collision energy was set at 12.8 eV and the mass spectrometer tuning gave the product ion at *m*/*z* 468 in 10−20% relative intensity. Triplicate injections of the midlevel standard (5.5 ng/μL) were again made at the start of each day of analysis to set retention time windows and relative ion intensities and to demonstrate system suitability and stability. In this trial, a single injection of each sample was bracketed by single injections of the standard.

Retention Time Criterion. All of the incurred samples and the fortified samples met the retention time criterion on both days of analysis.

Relative Ion Intensity Criterion. On day 1 of analysis, all criteria were met for each of the 10 fortified samples (2390−4680 ng/g in tissue) that were injected, and 7 of 9 incurred samples (1230−1770 ng/g in tissue) also met all of the criteria. However, a single relative intensity from each of two fortified samples (LS1A3, LS2A10; 1560 and 1240 ng/g in tissue, respectively) was outside the ±10% criterion (+16.9 and −22.0%, respectively). On day 2 of analysis, 9 of 11 incurred samples (1160−1440 ng/g in tissue) and 4 of 5 fortified samples (all at 2300 ng/g in tissue) met all of the criteria. For the nonconforming incurred samples, two relative intensities from one sample (ID38; 1380 ng/g in tissue) and one from a second sample (ID37; 1350 ng/g in tissue) were outside the ±10% criterion (+18.8%, +22.0%, and +10.8%, respectively). For the nonconforming fortified sample (ID8; 2300 ng/g), a single relative intensity did not meet the ±10% criterion (−11.7%). Each of the five samples that had failed to meet the criteria was reassayed in triplicate on day 3 of analysis, and each individual ion ratio (as well as the means) of every injection then met the criteria.

To summarize, the data from the first validation trial indicate that extracts from samples containing marker residue below about 1000 ng/g will not consistently meet the ±10% relative ion intensity criteria, but taking the mean values determined from triplicate injections does somewhat improve method performance at this low level ($0.2R_m$). The combined data from the two trials show that the majority of samples will meet the criteria. In a very few cases, some samples containing marker residue approximately in the range 1200−3000 ng/g may fail but will meet the criteria when reassayed on a regenerated column. Overall, the method provides a successful confirmation of eprinomectin marker residue at ≥2400 ppb in bovine liver tissue.

ABBREVIATIONS USED

LC/MS/MS, liquid chromatography/mass spectrometry/mass spectrometry; HPLC, high-performance liquid chromatography; FDA, Food and Drug Administration; LC/MS, liquid chromatography/mass spectrometry; MS/MS, mass spectrometry/mass spectrometry; R_m, marker residue tolerance; RSD, relative standard deviation.

Ivomec is a registered trademark and Eprinex is a trademark of Merck & Co., Inc., Whitehouse Station, NJ 08889.

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