HPLC−Fluorescence Method for the Determination of Eprinomectin Marker Residue in Edible Bovine Tissue

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A method was developed and validated to determine eprinomectin marker residue in bovine liver, kidney, muscle, and fat. The overall percent recovery (± CV) was 93 ± 12% ($n = 66$) for liver, 100 ± 13% ($n = 34$) for muscle, 87 ± 13% ($n = 42$) for kidney, and 95 ± 11% ($n = 42$) for fat. The limit of detection was 1 ng/g, the lower limit of quantitation was 2 ng/g, and the upper limit of quantitation was 5000 ng/g (μg/kg). Accuracy, precision, linearity, selectivity, and ruggedness were demonstrated. For the determination, tissue is mixed with sodium sulfate, homogenized, and extracted. The reconstituted extract is loaded onto an aminopropyl cartridge. After solvent exchange, a portion of the eluate is derivatized precolumn via automated addition of TFAA in acetonitrile and analyzed using fluorescence detection. The method is rapid, sensitive, and selective and provides for determination of eprinomectin marker residue in edible bovine tissue from the low parts per billion (ng/g) level to the parts per million level. The method has been successfully performed by several different analysts.

Keywords: *Avermectins; tissue residue; fluorescence; eprinomectin; HPLC; SPE*

INTRODUCTION

Eprinomectin [MK-0397 or 4″-(*epi*-acetylamino)-4″-deoxyavermectin B_1] has recently been approved for use in the United States as a beef and dairy topical endectocide under the tradename Ivomec Eprinex Pour-On for Beef and Dairy Cattle (NADA 141-079). World-wide registration is being pursued for this product. It has already been approved for use in New Zealand and Mexico in addition to the United States.

Eprinomectin is a member of the avermectin class of compounds. It is derived from the natural product avermectin B_1 (abamectin), as is the widely used parasiticide ivermectin. Unlike ivermectin, eprinomectin is approved for use in all classes of cattle, including dairy cattle and nonruminating calves. This is due in part to the safety profile of the compound and the low eprinomectin residues found in milk as compared to ivermectin. Ivomec Eprinex Pour-On for Beef and Dairy Cattle can be used in the United States with zero milk discard and a zero slaughter withdrawal period.

The selection of eprinomectin for the development program at Merck & Co., Inc., was the result of a large screening process based on efficacy, pharmacokinetics, and milk-to-plasma partitioning behavior (Shoop et al., 1996a). Eprinomectin exhibited the most potent efficacy of any avermectin tested against adult endoparasites of sheep in addition to a very favorable milk-to-plasma partition ratio (M/P = 0.17). Subsequent testing in cattle confirmed the efficacy of eprinomectin against endoparasites of cattle as well as three major groups of bovine ectoparasites (Shoop et al., 1996b).

Eprinomectin, like abamectin, is a mixture of two homologs (Figure 1). It is composed of not less than 90% of the B_{1a} component and not more than 10% of the B_{1b} component. These homologs differ by only one methylene unit ($-CH_2-$) at the 25-carbon position, wherein the B_{1a} component contains a *sec*-butyl group and the B_{1b} component contains an isopropyl group. Radiolabeled metabolism studies in rats have demonstrated that eprinomectin is not metabolized to any great extent (<10% metabolized; Zeng et al., 1996). The marker residue in cattle is the parent B_{1a} component, and liver is the target tissue (N. Narasimhan, Merck & Co., unpublished results).

To determine eprinomectin marker residue in the edible tissues of treated cattle, a rapid, sensitive HPLC−fluorescence method was developed and is the subject of this paper. The method was validated with a limit of quantitation (LOQ) of 2 ng/g (ppb) and a limit of detection (LOD) of 1 ng/g (ppb) in all four tissues tested. The low level of quantitation and detection is the result of the formation of a fluorescent derivative of eprinomectin (Figure 1) utilizing the inherent structure of the compound similar to that described for other avermectins (deMontigny et al., 1990; Payne et al., 1995; Tolan et al., 1980; Tway et al., 1981).

MATERIALS AND METHODS

Materials. The analytical reference standard of eprinomectin used in the study was obtained from Chemical Data, Merck & Co., Inc., Rahway, NJ. All solvents used were of HPLC or Optima grade. They were obtained from the following suppliers: acetone, triethylamine, and phosphoric acid from Fisher Scientific, Fair Lawn, NJ; acetonitrile, methanol, methylene chloride, ethyl acetate, hexane, and toluene from Baxter, McGaw Park, IL; and ethyl alcohol from Quantum Chemical Co. Anhydrous sodium sulfate was obtained from Aldrich Chemical Co., Milwaukee, WI, or EM Science, Gibbstown, NJ. 1-Methylimidazole (1-MIM) was obtained from Aldrich Chemical Co. Trifluoroacetic anhydride (TFAA) was obtained from Pierce, Rockford, IL. Aminopropyl NH_2 solid phase extraction (SPE) columns (Mega Bond Elut), 1 g/6 cm^3, were purchased from Varian, Harbor City, CA. Worldwide Monitoring, Horsham, PA, NH_2 cartridges were also used. Solutions were prepared using Milli-Q water. The control bovine tissue was provided by Branchburg Farm, Merck & Co., Inc., Somerville, NJ, or was purchased at local supermarkets. Incurred tissue was obtained from animals on trials conducted at Merck Research Laboratories.

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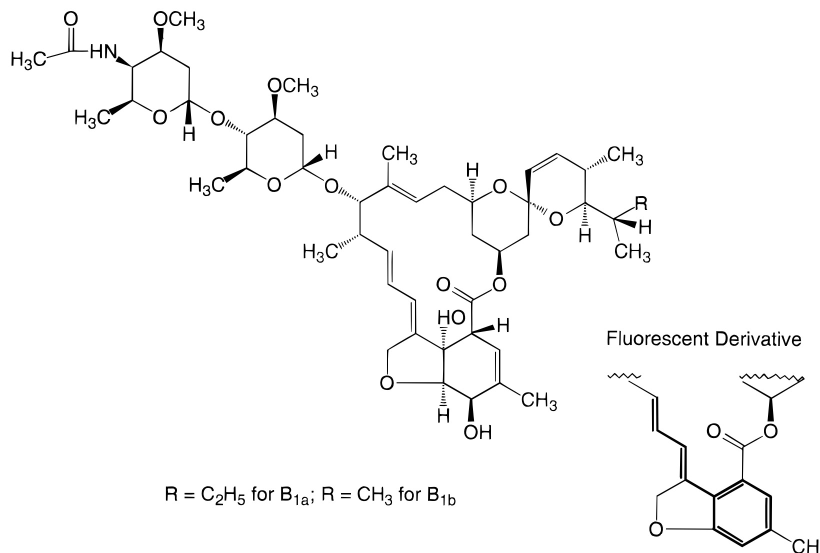

Figure 1. Structures of eprinomectin and its fluorescent derivative.

HPLC Conditions. The HPLC systems used consisted of the following instruments: a Shimadzu RF-551 (Columbia, MD) or Waters 470 (Milford, MA) fluorescence detector, a Shimadzu SIL-10A with a SCL-10A system controller or a Thermo Separation Product (TSP, San Jose, CA) AS300 autoinjector, a Shimadzu LC-10AD or TSP P4000 liquid chromatographic pump, a FIAtron CH-30 column heater at 35 °C with a TC-55 controller (Oconomowoc, WI), and a PE-Nelson 900 series A/D box (Cupertino, CA). Zorbax RX-C8 (25 cm × 4.6 mm i.d.) analytical columns (several different lots) from MacMod, Chadds Ford, PA, were used. The mobile phase used was methanol/acetonitrile/water/triethylamine/phosphoric acid (55:30:15:0.1:0.1) at a flow rate of 1 mL/min. The fluorescence detector settings were excitation wavelength of 365 nm, emission wavelength of 470 nm, sensitivity on high, gain of 1×, bandwidth of 15 or 18 nm, and response time of 1.5 s.

Solutions. For the 50:50 ethanol/ethyl acetate solution, equal volumes of ethanol and ethyl acetate are mixed thoroughly. For the 50:50 methylene chloride/acetone solution, equal volumes of methylene chloride and acetone are mixed thoroughly. For the 30% 1-MIM in acetonitrile solution, 3 mL of 1-MIM is mixed with 7 mL of acetonitrile. For the 30% TFAA in acetonitrile solution, 3 mL of TFAA and 7 mL of acetonitrile are mixed. The solutions should be made the day of use.

Preparation of Standards. The eprinomectin standard solutions are prepared in acetonitrile and stored at or below −10 °C, at which they are stable for several months. For low-level detection, a 100 ng/mL working standard solution corrected for purity and a standard curve of 2−20 ng/mL are recommended. The working standard is prepared by serial dilution of an approximately 100 μg/mL stock solution, and the standard curve is prepared from aliquots of the working standard solution.

Sample Preparation Procedure. A 5 g liver tissue sample is mixed with 5 g or more of anhydrous sodium sulfate, homogenized, and extracted with 30 mL of a 50:50 methylene chloride/acetone solution using a Polytron homogenizer (Brinkmann Instruments, Littau, Switzerland). The Polytron probe is cleaned thoroughly between samples with water, methylene chloride, and acetone. The homogenate is shaken on a table shaker for 5 min and centrifuged at ~750*g* for 5 min. The supernatant is decanted into a separate tube and the macerate re-extracted twice by shaking with 15 mL aliquots of the 50:50 methylene chloride/acetone solution. The combined methylene chloride/acetone extract is evaporated to dryness and the residue reconstituted in 10 mL of methylene chloride and loaded by gravity onto a preconditioned NH_2 column cartridge. The SPE column is conditioned with 10 mL of methylene chloride prior to loading. The column is rinsed with 10 mL of methylene chloride and then 10 mL of toluene. After the toluene rinse, the SPE column is dried for 1−2 min using vacuum prior to elution. The sample is eluted with 10 mL of the 50:50 ethanol/ethyl acetate solution. The ethanol/ethyl acetate eluate is evaporated to dryness and reconstituted in 3−10 mL of methanol, depending on the residue level expected. If low or unknown eprinomectin marker residue levels are expected, then the residue should be reconstituted in 3 mL of methanol and later diluted if necessary. This is a stopping point in the method. Sample extracts may be stored frozen (<−10 °C) for up to 6 weeks at this point. These methanol solutions can also be used in the confirmatory assay (Ballard et al., 1997).

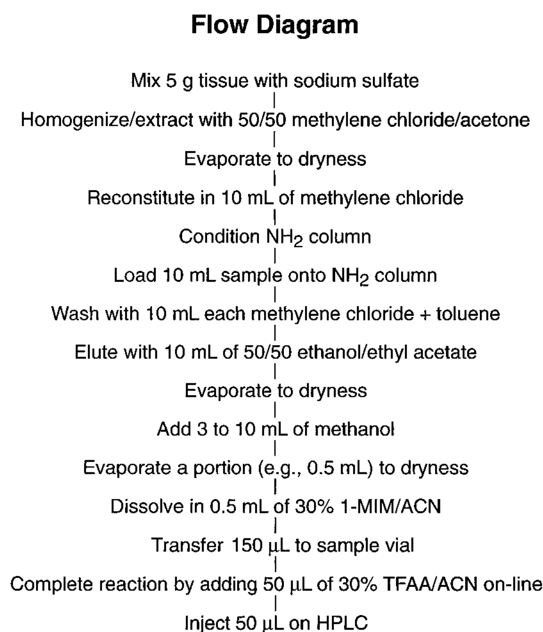

Figure 2. Flow diagram of the procedure to determine eprinomectin marker residue in edible bovine tissue.

A portion of the sample, for example, 0.5 mL, is evaporated to dryness. The residue is dissolved in 0.5 mL of the 30% 1-MIM in acetonitrile solution. One hundred fifty microliters of this solution is transferred to an HPLC vial for analysis. The reaction to form a fluorescent derivative is initiated on-line by adding the 30% TFAA in acetonitrile and mixing just before injection. The analysis is completed via reversed-phase HPLC and fluorescence detection. A flow diagram of the procedure is presented in Figure 2.

Automated Fluorescent Derivatization Reaction. The on-line derivatization procedure is described for the Shimadzu

SIL-10A autoinjector system. Any system capable of accurately measuring and mixing solutions, reagents, and solvents with water excluded (under anhydrous conditions) can be used. The presence of water or alcohol in the reaction loop or vial can interfere with the derivatization reaction.

For the external standards injected before and after the samples, 5, 15, 25, 35, and 50 μL of the working standard solution are aliquoted by the autoinjector into separate HPLC vials. Acetonitrile is added to make to a total volume of 50 μL in each vial. The autoinjector then aliquots 150 μL of the 30% 1-MIM solution to each of the standards vials and mixes the contents. The sample extracts are already in 150 μL of the 30% 1-MIM solution. The preceding procedure can be run individually or in a batch. However, the next step, namely the addition of the 30% TFAA in acetonitrile solution, must be accomplished immediately before injection onto the HPLC because the fluorescent derivative of eprinomectin is not stable enough to allow batch analysis. The autoinjector aliquots 50 μL of the 30% TFAA in acetonitrile solution, mixes, and then injects 50 μL of the sample or standard within 2−7 min of the addition of the TFAA solution. The time between the addition of the TFAA and injection should be consistent for all samples and standards.

The automated derivatization procedure has been done manually, but it is tedious and time-consuming since each individual sample and standard must be derivatized and injected separately.

Quantitation. Using the peak height and concentration of the five or more external standards injected before and after the samples, calculate a linear regression equation. All five standards are run before and after the samples to ensure the stability and suitability of the system. A typical analysis set is 12 samples including 1 control and at least 1 method recovery (fortified or spiked) sample. If more than 16 samples are to be analyzed in 1 set, an additional complete standard series should be run in the middle of the sample set. Using the slope (S) and intercept (I) from the linear regression equation, calculate the unknown sample concentrations in a 0.2 mL final volume using the following equation:

$$\text{unknown concn in ng/g} = \frac{(\text{peak height} - I) \times 0.2\ \text{mL}}{S \times 5\ \text{g} \times \text{fraction of sample used}} \quad (1)$$

The fraction of the sample used depends on the aliquot of methanol extract taken for derivatization. For controls and low-level samples the fraction would be 0.5/3 or 0.167 (0.5 mL aliquot taken from 3 mL). For higher level samples the fraction could be 0.5/10 or 0.05 (0.5 mL taken from 10 mL). Smaller fractions can be taken or dilutions can be made prior to the derivatization step if necessary.

Notes to Analyst and Safety Considerations. Anhydrous sodium sulfate is added to the tissue to help remove moisture from the tissue. The preferred consistency of the tissue/sulfate mixture is that of a “freeze-dried” or powdery texture. It is recommended to use fresh sodium sulfate or to store the sodium sulfate under dry conditions, such as in a desiccator. Care should be taken when handling TFAA. It is a strong acid, oxidizer, and dehydrating agent. It is recommended that TFAA be handled in a fume hood and be stored in a sealed container containing desiccant. Avoid contact with skin. In case of skin contact, rinse immediately and thoroughly with water. Personal protective equipment such as gloves, safety glasses, and lab coats should be worn at all times in the laboratory.

RESULTS AND DISCUSSION

Accuracy and Recovery. Percent recoveries generated during the validation of the method are presented in Table 1. The method was successfully validated as measured by recoveries of between 80 and 110% by two analysts for the liver and muscle tissues and by three analysts for kidney and fat. The average recovery for the target tissue, liver, was 93% over a fortification range from 2 to 5000 ng/g. The average recovery for kidney tissue was 87% over a fortification range of 2−5000 ng/g. The average recoveries for muscle and fat tissue were 100 and 95%, respectively, over a fortification range of 2−2500 ng/g. The average recovery of eprinomectin marker residue over all fortification levels in four tissues was 93 ± 14% (n = 184).

Table 1. Eprinomectin Marker Residue Tissue Method Validation Results[a]

fortification level (ng/g)	av % recovery	% RSD[b]	N
liver			
2	89	8.4	7
10−15	99	13	6
100	89	12	24
500	95	9.1	6
1300	95	7.3	5
2500	99	10	10
5000	94	15	8
overall	93	12	66
muscle			
2	99	7.3	8
10−15	99	11	7
100	107	11	7
500	111	12	6
2500	84	7.5	6
overall	100	13	34
kidney			
2	84	12	14
10−20	81	14	5
100−250	91	10	13
2500	84	15	7
5000	91	8.3	3
overall	87	13	42
fat			
2	99	11	14
10−20	97	8.5	11
100−250	92	9.9	12
2500	87	12	5
overall	95	11	42

[a] Values for the liver and muscle summarize the results of validations conducted by two analysts. For kidney and fat, three analysts performed the assays. [b] % RSD equivalent to CV.

Precision and Repeatability. The overall coefficient of variation (CV or % RSD) over all fortification levels, analysts, and tissues was 14% (n = 184). Analysis for the eprinomectin marker residue fortified in each tissue had an overall CV of 13% or less (Table 1) with an average of 12% and a range of 11−13%. The interset variability at each fortification level (intermediate precision) ranged from 7.3 to 15%. The average interset variability over all tissues and all fortification levels was 11%. The range of the intraset variability (repeatability) ranged from a low of 1.5% to a high of 16% and averaged 8.1%. Repeated derivatization of the same sample solution resulted in a % RSD of between 4 and 6%, indicating that the derivatization reaction and injection may contribute to up to approximately half of the variability. The within-laboratory coefficient of variation was 15% or less for all tissues fortified from 2 ng/g to 2500 or 5000 ng/g, indicating that the method performs reproducibly throughout the fortification range.

Linearity and Range. All coefficients of determination of the standard curve linear regression equations were 0.98 or greater, demonstrating good linearity of the derivatization reaction and detection system. Since the higher fortifications are diluted to fall within the range of the standards, the effective linear concentration range of the method is from 2 to 5000 ng/g for liver and

kidney and from 2 to 2500 ng/g for muscle and fat. The highest incurred eprinomectin tissue marker residues encountered in field trials (L. D. Payne, unpublished data) were less than $^1/_3$ the highest validated concentration for liver and less than $^1/_{20}$ the highest validated concentration for the other tissues. The tolerance (R_m) for eprinomectin marker residue in liver is 4800 ng/g in the United States. The average liver method recovery ranged from 89 to 99%, and the within-laboratory coefficient of variation was 15% or less.

Ruggedness. Ruggedness (robustness) tests were performed using liver and muscle tissue. The source and quantity of sodium sulfate (Na_2SO_4) used, the age of the control tissue, and the source, size, and loading of the NH_2 cartridges were tested. Different lots of sodium sulfate from two manufacturers were used successfully. Neither the source, size of the crystals, nor amount (5 or 10 g) of sodium sulfate used significantly affected the recovery values. Old tissue (tissue that had been purchased earlier and had been through several freeze/thaw cycles) was compared with recently purchased tissue. The average recoveries were comparable; however, the older tissue produced greater % RSD than the newer tissue. Using 2−30 mL initial extractions of methylene chloride/acetone instead of 1−30 and 2−15 mL extractions produced comparable recoveries.

The size, manufacturer, and loading of the aminopropyl SPE column cartridge were investigated. The recoveries did not vary significantly. Use of World-Wide Monitoring columns resulted in slightly higher recoveries, but the reproducibility was worse. Smaller Bond Elut columns (500 mg vs 1 g of packing) produced comparable recoveries, as did loading the cartridges with vacuum. It was found critical, however, to dry the cartridge for 1−2 min with vacuum prior to elution.

The chromatographic ruggedness of the method was also investigated. The mobile phase consists of methanol/acetonitrile/water (55:30:15) with 0.1% each of triethylamine (TEA) and phosphoric acid (H_3PO_4). This mobile phase was compared to mobile phases prepared without TEA and/or phosphoric acid. Both additives were required for good peak shape. The absence of TEA lengthened the retention time of derivatized eprinomectin marker residue from about 9 min to about 14 min. The peak width at baseline doubled, and the peak asymmetry greatly increased. The absence of H_3PO_4 caused great distortion of the peak, which eluted a grossly fronting peak on the shoulder of the solvent front. The absence of both TEA and H_3PO_4 resulted in no observable peak. Derivatized eprinomectin marker residue may elute under these conditions as a broad late eluting peak. Therefore, both TEA and H_3PO_4 are required components of the mobile phase. Different lots of the analytical column packing did not affect the results.

Selectivity. Controls were free from interferences. Figure 3 depicts typical chromatograms that result from the use of the method. Five macrocyclic lactone compounds and one additional anthelmintic were tested for interference in the eprinomectin chromatographic system as well as through the sample preparation procedure: ivermectin, abamectin, emamectin benzoate, moxidectin, doramectin, and coumaphos. Ivermectin, abamectin, emamectin benzoate, moxidectin, and doramectin are members of the avermectin/milbemycin class of compounds and, except for emamectin benzoate, are used as antiparasitics for cattle. Emamectin benzoate is a crop protection agent (Prabhu et al., 1991).

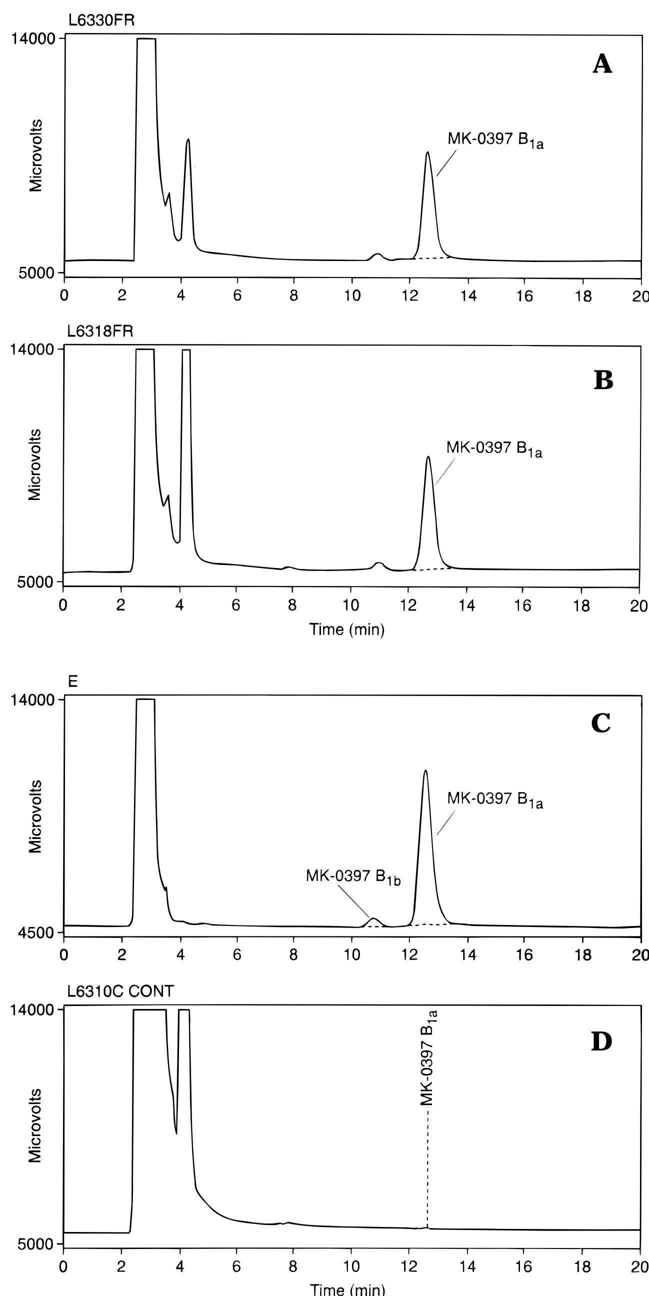

Figure 3. Typical chromatograms: (A) fortified tissue at ∼100 ng/g; (B) incurred tissue at ∼85 ng/g; (C) standard at ∼20 ng/mL; (D) control tissue.

Coumaphos, a thiophosphate, is registered for use in cattle as an anthelmintic and ectoparasiticide. The results can been seen in Figure 4. All of the macrocyclic lactone compounds had much longer retention times than eprinomectin. A gradient was required to elute them from the analytical column in a reasonable amount of time. None of the compounds tested interfered with the detection of the eprinomectin peak. Coumaphos did not produce a peak response under the chromatographic conditions of this method.

These compounds were also tested in the sample preparation procedure in various combinations with eprinomectin to detect any effect on eprinomectin marker residue recoveries due to the presence of these compounds. None of the compounds tested interfered with the recovery of eprinomectin marker residue except when emamectin benzoate was fortified concurrently at a high level (500 ng/g). In that case eprinomectin marker residue recoveries were slightly reduced. It is

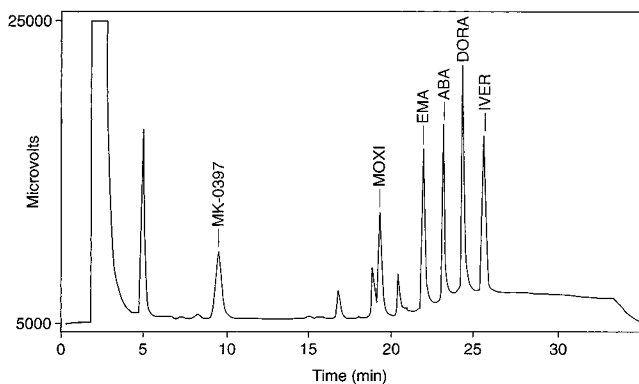

Figure 4. Noninterference testing. MOXI, moxidectin; EMA, emamectin; ABA, abamectin; DORA, doramectin; IVER, ivermectin. Gradient elution: MK-0397 conditions for 9 min, to 95% organic over 13 min, hold for 8 min. Reequilibration: 20 min between runs.

possible that the interference may be due to the NH_2 cartridge capacity or some interaction between emamectin benzoate and eprinomectin. However, since emamectin benzoate is not registered for use in cattle, this potential effect on eprinomectin marker residue recoveries is not relevant.

A minor metabolite of eprinomectin observed in rats (Zeng et al., 1996), L-653,649 [4‴-(*epi*-amino)-4″-deoxyavermectin B_1], was also tested for noninterference. L-653,649 ($t_r \sim 26$ min) does not interfere with the determination of eprinomectin marker residue ($t_r \sim 9$ min). It elutes well after the eprinomectin marker residue with a selectivity of 3.2 under the isocratic chromatographic conditions specified in the method.

Extraction Efficiency. This procedure has been used to analyze tissues for incurred eprinomectin marker residue from two radiolabeled tissue residue studies. The ratios of marker residue (the B_{1a} component of eprinomectin) to total radioresidues averaged 83% for liver, 85% for kidney, 92% for fat, 71% for application or dose site, and 69% for muscle. These ratios correspond favorably with the estimation of the B_{1a} component contribution to the total radioresidue based on metabolism results (N. Narasimhan, Merck & Co., unpublished results). In a second study, the method was used to determine the incurred marker residue concentration in veal liver. The ratios of marker to total radioresidues averaged 90% (7% RSD). This again was in line with metabolism data verifying that the extraction procedure outlined here extracts incurred eprinomectin residues from bovine tissues with high efficiency.

Limits of Detection and Quantitation. The LOD of the method was determined using standard solutions and was set conservatively at 1 ng/g equivalent in tissue. The signal/noise ratio at this concentration is >10. The LOQ is the lower limit at which adequate (precise and accurate) quantitation of eprinomectin residues can be obtained. Since the lowest fortification level in the method validation was 2 ng/g and the average recovery at this level was 89% with 10% RSD for liver, 84% with 12% RSD for kidney, 100% with 13% RSD for muscle, and 95% with 11% RSD for fat, the data support a LOQ of 2 ng/g. The upper validated limit of quantitation was 5000 ng/g (μg/kg) for liver and kidney and 2500 ng/g for muscle and fat.

Assay Timing and Splits. The procedure takes about 7 h to prepare 12 samples for HPLC analysis. A typical set for this method would contain 10 samples, a

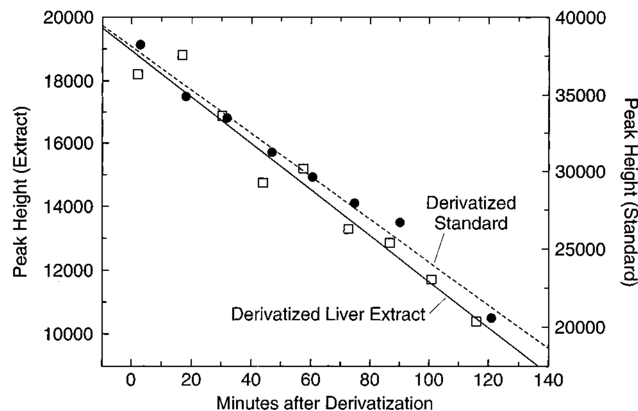

Figure 5. Degradation of the eprinomectin fluorescent derivative with time: (●) derivatized standard; (□) derivatized liver extract.

control, a spiked control (method recovery), and a set of 5 standards injected before and after the samples (a 10-point standard curve). Multiple aliquots from the methanol extract after the NH_2 cartridges cleanup can be used as splits. The extracts in methanol are stable when stored frozen (≤ -10 °C) for up to 6 weeks. This extract may be used to confirm eprinomectin marker residue determined to be above the R_m using an LC/MS/MS technique described in the subsequent companion paper (Ballard et al., 1997).

Freezer Storage Stability of Eprinomectin Residues in Tissue. Eprinomectin residues in bovine tissue were stable for up to 24 months when the tissues were stored frozen at or below −10 °C. The freeze/thaw stability of the residues in all four bovine tissues was also tested. The residues were stable for up to six freeze/thaw cycles, except possibly in muscle, which showed a slight decrease in eprinomectin marker residue levels after six cycles but not after three.

Fluorescent Derivative Stability. The fluorescent derivative of eprinomectin is not stable for long periods of time after preparation. This precludes derivatization in batches. Figure 5 shows the degradation of the derivatized standard and the derivatized eprinomectin in a liver extract with time. The eprinomectin peak response degrades to approximately 56% of the original peak height in about 2 h. Therefore, automated preparation and injection of the derivative is recommended. A window of approximately from 2 to 7 min after the addition of TFAA has been identified as the optimum time after derivatization for injection. There are several models of autoinjectors that can perform the precolumn derivatization reaction successfully.

Conclusion. Eprinomectin, marketed as Ivomec Eprinex Pour-On for Beef and Dairy Cattle, is a new animal health product for the treatment of both endo- and ectoparasites of cattle. The method reported herein is a rapid and sensitive procedure for the determination of eprinomectin marker residue in edible bovine tissues: liver, muscle, kidney, and fat. The method has a LOD of 1 ng/g and LOQ of 2 ng/g. The method was tested for ruggedness and found to be robust. Potential interferences from several other animal health compounds were investigated, and no interferences were found. The method adequately determines eprinomectin marker residue over a wide concentration range of 2−5000 ng/g. The overall method recovery over all edible tissues and all fortification levels was 93 ± 14%. Overall fortification recoveries for each tissue were 93% for liver, 100% for muscle, 87% for kidney, and 95% for

fat. The method can be performed in 1 day since 12 samples can be completed in 7 h or less with automated precolumn derivatization. The method was validated at the 2300 ppb eprinomectin marker residue level in a sponsor-monitored method trial and the data met the U.S. FDA Center for Veterinary Medicine (USFDA/CVM) performance criteria. This method for liver target tissue was also accepted by New Zealand Ministry of Agriculture and Fisheries (MAF) and the Committee for Veterinary Medicinal Products of the European Agency for the Evaluation of Medicinal Products (EMEA/CVMP).

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Received for review May 10, 1997. Revised manuscript received June 23, 1997. Accepted July 1, 1997.[⊗] We acknowledge the work provided by the contract chemists Dr. Leo Posner (Integro), Mrs. Irene Nwodili, and Dr. Ritu Singh (Manpower).

JF970393U

[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1997.