Determination of Abamectin and/or Ivermectin in Cattle Feces at Low Parts per Billion Levels Using HPLC with Fluorescence Detection

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Abamectin and ivermectin are potent antiparasitic animal health drugs that differ structurally by a double bond. Abamectin is a natural product produced by the soil microorganism *Streptomyces avermitilis*. Ivermectin is a unique hydrogenation product of abamectin. A sensitive HPLC-fluorescence method was developed to determine abamectin or ivermectin residues in bovine feces. The limit of quantitation is 2 ppb (ng/g) and the limit of detection is 1 ppb (wet weight basis). The range validated was from 2 ppb to 2 ppm (μg/g) with average recoveries of 84% for both abamectin and ivermectin. Abamectin and ivermectin can be analyzed simultaneously because the fluorescent derivatives of the two compounds are chromatographically well resolved on the C_{18} column used. The method was developed to support studies concerning the dissipation of incurred abamectin or ivermectin residues in cattle feces postdose and postdeposition.

Keywords: *Avermectins; abamectin; analytical method; feces; fluorescence; HPLC; ivermectin*

INTRODUCTION

Ivermectin (22,23-dihydroavermectin B_1) is a potent antiparasitic animal health drug introduced in 1981 and registered for use in cattle and other animals worldwide (Campbell, 1989; Campbell et al., 1983). It is prepared from abamectin (avermectin B_1), a natural product produced by the soil microorganism *Streptomyces avermitilis*, by reduction of the double bond at the 22–23 position (Figure 1). Abamectin is used as an antiparasitic agent in cattle. Abamectin and ivermectin are mixtures of homologues (B_{1a} and B_{1b}) which differ by a methylene unit (Figure 1). B_{1a} is the principal component (>80%) of the B_1 mixture. Both compounds are effective at extremely low dose levels (e.g. 200 μg/kg subcutaneously).

These drugs undergo little metabolism and most of the dose given to the animal is excreted, relatively unaltered, primarily in the feces (Halley et al., 1989). It is appropriate that a method be developed to assay drug levels in feces. The lack of a consistent methodology for avermectin assay in feces was highlighted recently in the proceedings of a symposium (Herd et al., 1993). The present paper addresses the analytical methodology that accurately measures abamectin and ivermectin residues in cattle feces. The method was developed to support studies concerning the dissipation of incurred abamectin or ivermectin residues in cattle feces postdose and postdeposition.

Nessel et al. (1989) published the first study on the determination of nonradiolabeled ivermectin in cattle feces in conjunction with a feedlot study. This method was based on an ivermectin tissue method (Tway et al., 1981; Downing, 1989) and had a limit of detection of 10 ppb wet weight. Unfortunately, the controls had a chromatographic interference at the HPLC retention time of interest. In 1990, an abstract reporting the determination of ivermectin in horse feces with a limit of detection of 50 ppb wet weight was published (Jernigan et al., 1990). The approach employed was based on a method for determining avermectins in plasma developed by Tolan et al. (1980), and the abstract was followed by a paper several years later (Sams, 1993). Another method based on the avermectin in plasma method was published by Sommer et al. in 1991 and 1992. The detailed method description was published in 1993 (Sommer and Steffansen, 1993). The authors reported a limit of detection of 50 ppb dry weight. The two most recently published methods for the measurement of ivermectin in feces, a Soxhlet/adapted mixer method (Lumaret et al., 1993) and a methanol extraction/internal standard method (Bernal et al., 1994), reported a limit of detection of 20 ppb wet weight.

No methods have been published for the determination of abamectin in feces.

Fecal material from pasture-grazed cattle is a very difficult matrix to assay. It contains partially digested plant particles, whose composition and particle size may vary with the season or location, as well as microbial and animal components which may vary with time and by animal and which complicate the assay. Initial experiments indicated that neither the ivermectin in tissue method nor the Sommer's method would provide the desired limit of detection in the most problematic cattle feces. The simple methanol extraction described by Bernal et al. (1994) also did not provide an adequate limit of detection when the above-mentioned pasture-grazed cattle feces was used as the matrix. Reported here is a comprehensive validated method, including noninterference studies, for the determination of both abamectin and ivermectin in a variety of feces from pasture-grazed cattle with a limit of detection of 1 ppb wet weight. The low limit of detection is achieved through the formation of fluorescent derivatives of ivermectin and abamectin (DeMontigny et al., 1990; Wehner et al., 1993) after extensive sample preparation.

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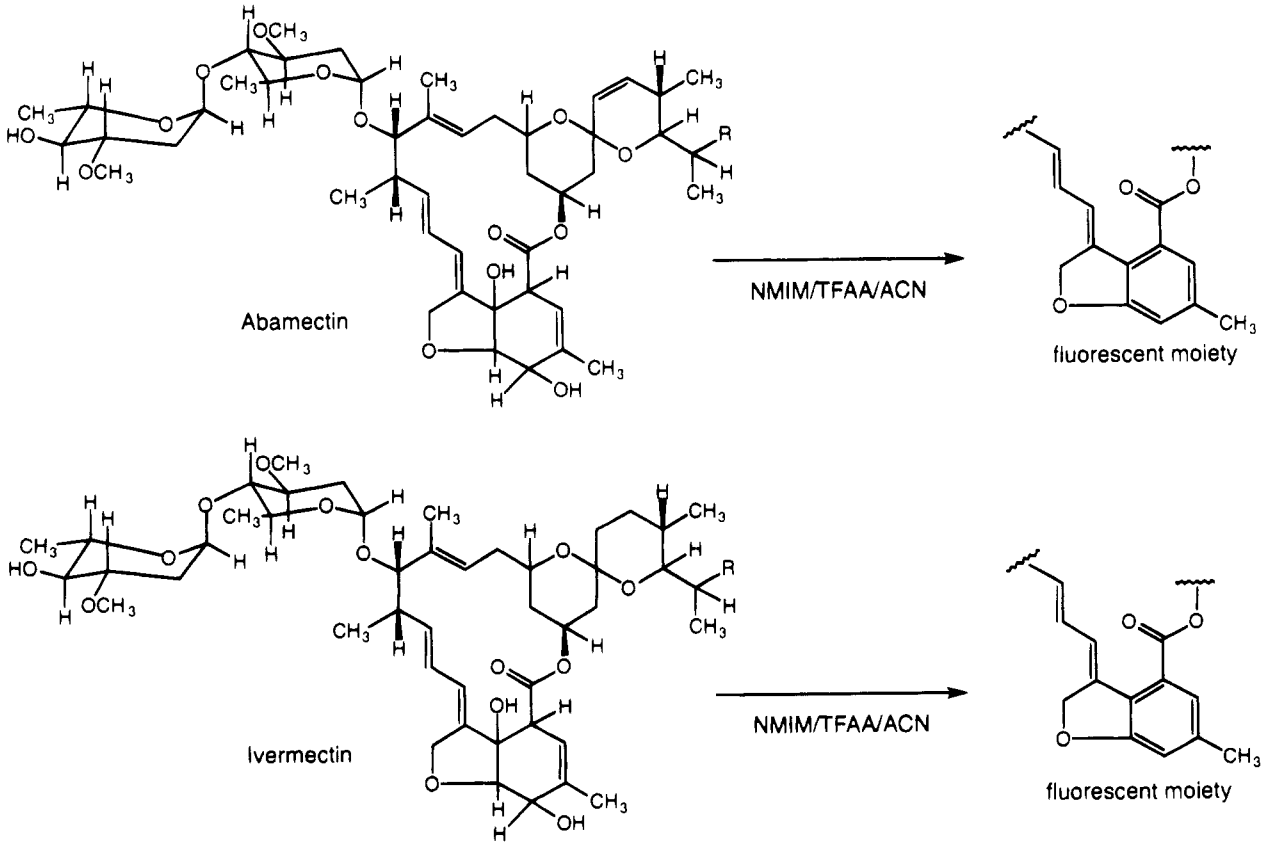

Figure 1. Structures of abamectin and ivermectin and their respective fluorescent derivatives. Each compound is a mixture of not less than 80% of the B_{1a} homolog (R = C_2H_5) and not more than 20% of the B_{1b} homolog (R = CH_3).

MATERIALS AND METHODS

Materials. The standards used in this study were obtained from Chemical Data, Merck & Co., Inc. (Rahway, NJ). All solvents used were of HPLC grade either from Fisher (Pittsburgh, PA) or from Baxter (McGaw Park, IL). Milli-Q water was used. Feces from pasture-grazed cattle were obtained from Branchburg Farm, Merck & Co., Inc. (Somerville, NJ) or Missouri Farm, Merck & Co., Inc. (Fulton, MO). Solid phase extraction columns (C_{18}, 1 g, 6 cm^3) were obtained from Millipore (Milford, MA). 1-Methylimidazole (NMIM) was obtained from Aldrich (Milwaukee, WI), and trifluoroacetic anhydride (TFAA) was obtained from Pierce (Rockford, IL).

High-Performance Liquid Chromatography. The HPLC system consisted of a Spectra-Physics (SP) Model SP8700XR liquid chromatographic pump, a SP Model SP8780 autosampler, a Waters Model 470 fluorescence detector, a Varian Model 4270 integrator, and a Fiatron CH-30 column heater with a TC-55 controller set at 35 °C. A Rainin RP_{18} guard column cartridge (7 μm, 15 mm × 3.0 mm i.d.) was used before an ES Industries MC_{18} analytical column (3 μm, 150 mm × 4.6 mm i.d.). The premixed mobile phase used 5% water in methanol at a flow rate of 1.2 mL/min. The injection volume was 50 μL. The fluorescence detector was set with an excitation wavelength of 365 nm, an emission wavelength of 470 nm, and a time constant of 1.5 s. Under these conditions, the B_{1a} components of abamectin and ivermectin eluted at approximately 10 and 14 min, respectively. The smaller B_{1b} component peaks elute 1–1.5 min before their respective B_{1a} counterparts.

Method. A flow diagram for the method is presented in Figure 2. Feces samples were analyzed in sets which normally consisted of 10 samples. For each sample, 15 mL of 30% acetone in water (v/v, freshly prepared) was added to 10 g of feces in a 50 mL centrifuge tube. The tubes were shaken for 20 min at high speed on a table shaker (Eberbach) and then sonicated for 10 min (Branson Series 7000 sonicator). Fifteen milliliters of isooctane was added to the mixture, and the tubes were again shaken and sonicated and then centrifuged (5 min at approximately 2000 rpm). The upper, isooctane layer was carefully transferred to a clean 50 mL tube. The isooctane extraction was repeated an additional three times. During the last two extractions, the tubes were shaken for only 10 min and sonicated for 5 min. The combined isooctane extracts were evaporated to dryness under a stream of nitrogen in a water bath (~70 °C).

The residue was reconstituted in 6 mL of methanol by vortexing and sonicating. Forty milliliters of water was mixed with the methanol for loading on a C_{18} solid phase extraction (SPE) column conditioned sequentially with 6 mL of methanol and 6 mL of water and fitted with a 75 mL reservoir containing a 20 mm frit. The mixture was loaded at a flow rate of about 2 mL/min. The tubes and then the columns were washed with 20 mL of 20% methanol in water and then 5 mL of isooctane to remove residual water. The columns were eluted with 10 mL of methanol into 15 mL centrifuge tubes. Much of the color remained on the SPE column, but the methanol extract was still green to dark green. The methanol was evaporated to dryness and then reconstituted in 1 mL.

Six milliliters of water was mixed with the 1 mL of methanol. Then, 5 mL of hexane was added. The tubes were shaken for 1 min by hand and then centrifuged for 5 min at 2000 rpm. The hexane layer was transferred to a clean 15 mL centrifuge tube, and the hexane extraction was repeated two more times. The hexane extracts were relatively clear as most of the color remained in the aqueous phase. The combined hexane extracts were evaporated to dryness.

The residue from the hexane partition was reconstituted in 2.0 mL of acetonitrile. Exactly 1.0 mL was removed to a separate tube and stored frozen to serve as a reserve sample in case the fluorescence derivatization reaction failed.

Preparation of Standards. Five standards ranging in concentration from 1 to 12 ng/mL were prepared by diluting aliquots of a working standard solution of the combined abamectin/ivermectin standard to 1 mL of acetonitrile and

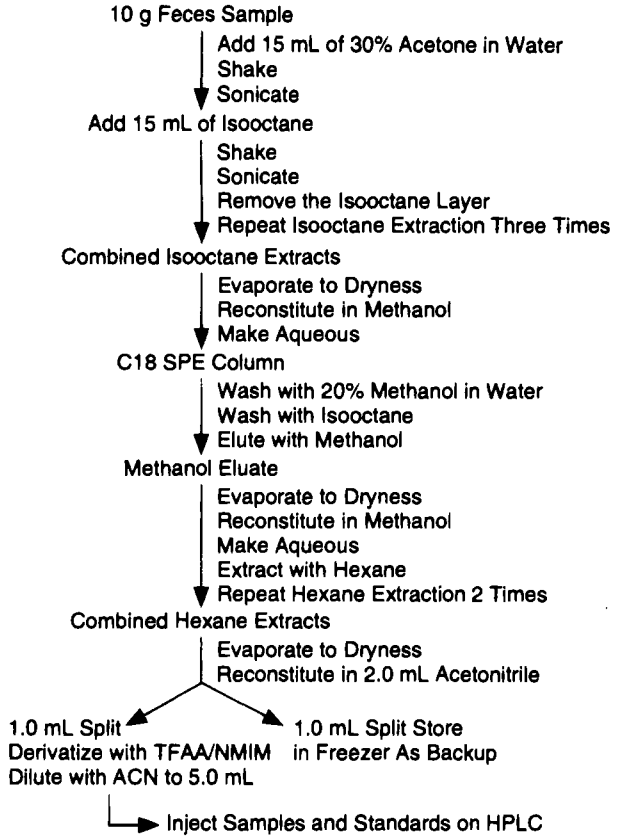

Figure 2. Flow diagram of the analytical procedure to determine abamectin and ivermectin residues in feces from pasture-grazed cattle.

derivatizing as described below. The working standard solutions used for the fortifications and for the standard curve were made by diluting a stock solution of approximately 400 μg/mL of B_{1a} with acetonitrile. The stock solution was made by weighing an appropriate amount of the analytical standard in glycerol formal and diluting with acetonitrile.

Derivatization for Fluorescence Detection. To each sample and standard was added 0.1 mL of NMIM. A fresh solution (1:2) of TFAA in acetonitrile was prepared by adding 2 mL of TFAA to 4 mL of acetonitrile in a 15 mL centrifuge tube. The samples, standards, and derivatizing reagent (acetonitrile/TFAA) were chilled for 10 min in a Kryorack (Streck Laboratories Inc., Omaha, NE). An ice bath can also be used. To each sample and standard was added 0.3 mL of the derivatizing reagent. A white vapor is produced immediately after the addition of the derivatizing reagent when the derivatization is working properly. The tubes were vortexed and allowed to come to room temperature for 10 min before 3.6 mL of acetonitrile was added to make the final volume of the standards and samples 5 mL. Aliquots of the solutions were placed in HPLC vials for analysis. The five standards were injected before and after the samples to assess the performance of the HPLC. Higher fortification levels were diluted appropriately using acetonitrile to fall within the range of the standard curve.

Care was taken to avoid the presence of water before injection on the HPLC; therefore, the extracts were not diluted with mobile phase, which contains water, before analysis. The trifluoroacetylated fluorescent derivative formed is sensitive to hydrolysis, resulting in the formation of two derivatized products rather than one when water is present (Hampton et al., 1993).

Stopping Points and Safety Considerations. The procedure generally requires approximately 10 h in experienced hands before HPLC analysis. The samples can be stored frozen overnight in methanol after the initial extraction or in acetonitrile after the hexane partition or derivatization reaction. The samples should not be stored frozen in hexane. Care should be taken in the handling of TFAA, which is corrosive and a dehydrating reagent and is quite reactive. TFAA should be handled with gloves and in a fume hood. Residual water or methanol will quench the derivatization reaction, so steps should be taken to ensure that evaporation is complete and that the TFAA is anhydrous before derivatization is attempted.

Dry Weight Determination. The dry weight of the feces was determined by drying in an oven between 80 and 90 °C until a constant weight was obtained.

Quantitation. Percent recoveries were determined by linear regression analysis with comparison to the external standards of analyte derivatized with the samples using the following equations:

$$\text{concn of analyte in sample (ng/mL)} = (\text{peak height from HPLC analysis} - \text{intercept from regression equation})/\text{slope} \quad (1)$$

$$\text{concn of analyte in sample (ng/g)} = [\text{concn (ng/mL) from eq 1} \times \text{final volume (mL) (e.g. 5 mL)}]/[\text{wt of sample (e.g. 10 g)} \times \text{fraction taken (e.g. 0.5)}] \quad (2)$$

$$\%\ \text{recovery} = [\text{concn of analyte in sample (ng/g) from eq 2}/\text{fortified concn (ng/g)}] \times 100 \quad (3)$$

Table 1. Average Abamectin and Ivermectin Validation Recoveries

	abamectin % recovery		ivermectin % recovery	
fortification level	mean ± %RSD, n	range	mean ± %RSD, n	range
2 ng/g B_{1a}	83 ± 10, 9	70–95	83 ± 11, 10	70–95
10 ng/g B_{1a}	83 ± 12, 8	65–96	83 ± 13, 8	64–100
100 ng/g B_{1a}	89 ± 3, 5	84–91	89 ± 10, 5	75–101
2000 ng/g B_{1a}	84 ± 6, 4	82–89	84 ± 6, 5	76–89
5 ng/g B_{1b}	86 ± 0, 4	86	98 ± 0.6, 3	98–99
100 ng/g B_{1b}	86 ± 4, 4	82–90	96 ± 5, 5	93–104

RESULTS

Feces samples were fortified with 2, 10, 100, or 2000 ng/g (ppb) of abamectin and/or ivermectin on a wet weight basis. Table 1 summarizes the validation results. Average method recoveries of the B_{1a} component of both abamectin and ivermectin ranged from 83% to 89%. Average method recoveries of the B_{1b} component of abamectin and ivermectin were 86% and 97%, respectively. The relative standard deviation was less than 10% at the two higher fortification levels and less than 20% at the two lower fortification levels for the B_{1a} components. The relative standard deviation of the B_{1b} components was 5% or less.

There was no significant difference in the recoveries between the two types of feces tested. The percent moisture values were 87% and 86% for the Branchburg and Missouri feces, respectively.

Lack of interference in the method was demonstrated by the lack of extraneous peaks in the chromatography of the control samples. Abamectin and ivermectin were chromatographically well resolved (Figure 3). Linearity of the quantitation is demonstrated by the uniformity of the recoveries throughout the range of concentrations of fortifications. The limit of detection (LOD) of the method was 1 ppb (ng/g) on a wet weight basis; the 10 g sample size equivalent of the lowest standard used in the standard curve, i.e. 1 ng/mL, is equivalent to 1 ng/g in a 10 g sample: 1 ng/g × 10 g (sample size)/5 mL (final volume) × 0.5 (fraction analyzed) = 1 ng/mL. The limit

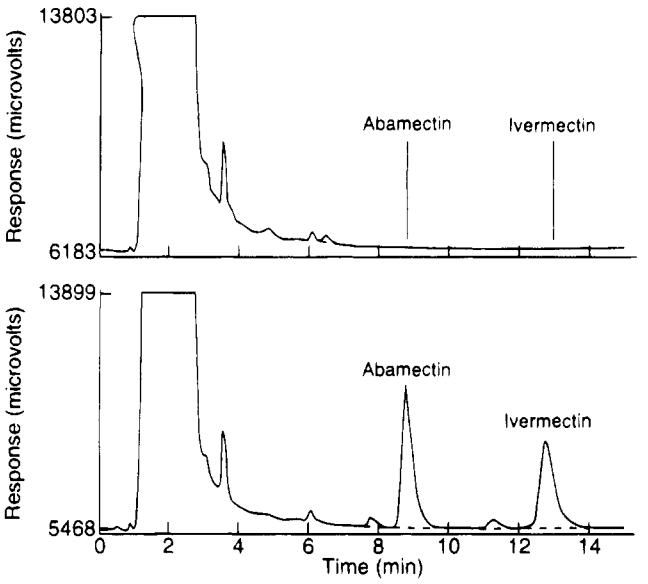

Figure 3. Chromatograms of control feces (upper) and feces fortified at the limit of quantitation (lower).

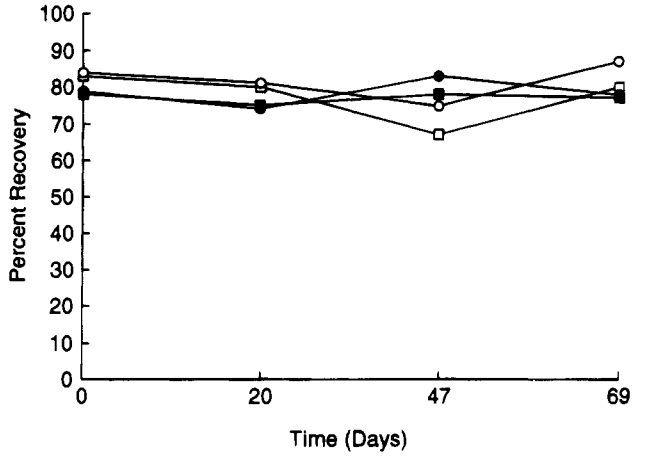

Figure 4. Freezer storage stability of abamectin and ivermectin fortified residues in feces (●, 10 ng/g abamectin; ■, 10 ng/g ivermectin; ○, 100 ng/g abamectin; □, 100 ng/g ivermectin).

of quantitation (LOQ) was 2 ppb, the lowest concentration validated for both abamectin and ivermectin (Figure 3).

A limited freezer storage stability study was performed. Abamectin and ivermectin were stable in frozen feces for up to 7 weeks (Figure 4). At the 5-week time point, the average recovery of ivermectin at 100 ng/g stored frozen in feces was slightly below 70% but was acceptable at the 10-week time point.

Two structurally related animal health drugs were tested for noninterference in the method. Moxidectin is a milbemycin, and doramectin is an avermectin. Neither compound interfered in the chromatographic analysis of abamectin or ivermectin (Figure 5).

DISCUSSION

The method presented here is a sensitive, reliable means of measuring the concentration of abamectin and/or ivermectin in fresh–frozen cattle feces. The method was also tested with dry, aged (weathered) feces. When a lower quantity (4 g) was used, results were satisfactory. At the LOQ (2 ppb), established in wet feces, the signal to noise ratio was greater than 50. Therefore, the limits of detection and quantitation could probably

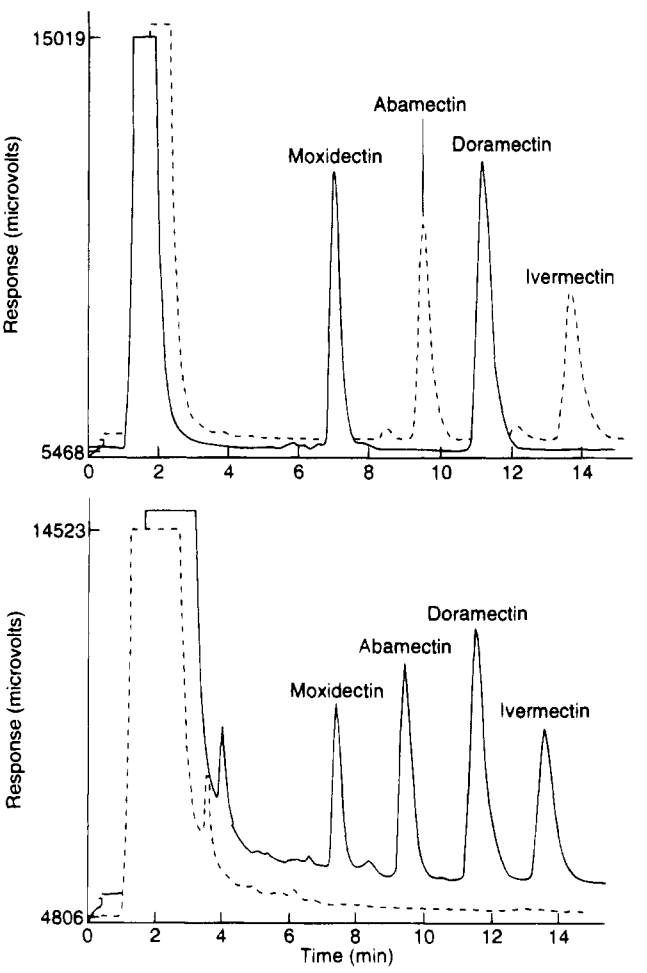

Figure 5. Chromatograms (upper) of 8 ng/mL standards of abamectin and ivermectin (- - -) and moxidectin and doramectin (—) and chromatograms (lower) of control feces (- - -) and feces fortified with 10 ng/g each of moxidectin, abamectin, doramectin, and ivermectin (—).

be lower; however, the method was not investigated at lower levels. This method is the most sensitive (lowest limit of detection) of those reported to date for the determination of ivermectin in feces. This is the first report of a method for the determination of abamectin in feces. The combination of HPLC with fluorescence detection makes this sensitivity possible.

The one potential disadvantage to the method is that it requires approximately 10 h to complete before HPLC analysis. This is attributable to the complexity of the matrix and the very low level of detection. The method was designed to work with a variety of feces and accurately quantitate two very potent antiparasitic agents, abamectin and ivermectin, which occur at low levels.

Two structurally related antiparasitic drugs, doramectin and moxidectin, are chromatographically well resolved on a reversed-phase C_{18} analytical column, under isocratic conditions, and consequently do not interfere with the analysis of abamectin and ivermectin.

Recently, the method was transferred to a Merck Research Laboratory in Europe (Kathrinenhof, Germany), where it has been validated successfully with two additional types of cattle feces obtained in Europe. This validation was in support of field trials performed in Europe. The results of the additional validation of the method and of the field trials will be reported elsewhere.

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