Ivermectin in Reindeer Feces: Determination by HPLC

Kjetil Åsbakk,* Heidi R. Bendiksen, and Antti Oksanen

Department of Arctic Veterinary Medicine, The Norwegian School of Veterinary Science, Stakkevollveien 23B, N-9005 Tromsø, Norway

An assay method for the determination of ivermectin in reindeer feces was developed. Ivermectin was quantified by high-performance liquid chromatography and fluorescence detection after extraction (acetone, isooctane), cleanup (C-18 solid-phase extraction column), and derivatization to a fluorescent derivative using *N*-methylimidazole and trifluoroacetic anhydride. Concentration calculations were based on calibration lines found from analyses of standards prepared in feces. Abamectin was used as internal standard. Ivermectin was determined over a wide concentration range in a single sample preparation and HPLC run for each sample. The recovery from fortified samples was greater than 95% over the concentration range 5−2000 ng/g wet weight feces.

Keywords: *Ivermectin; HPLC; analysis; reindeer; feces; dung*

INTRODUCTION

Ivermectin (22,23-dihydroavermectin B_1) is a potent antiparasitic animal health drug in worldwide use after its introduction to the market in 1981. The drug is a synthetic derivative of abamectin, a natural fermentation product of the soil microorganism *Streptomyces avermitilis*. Abamectin and ivermectin are members of the avermectin subfamily of the macrocyclic lactones, a class of highly lipophilic substances with a correspondingly low solubility in water. Ivermectin is a mixture of homologous products with B_{1a} and B_{1b} components differing from each other by a single methylene unit. B_{1a} is the principal component of the mixture (>80%). B_{1a} and B_{1b} have very similar biological and toxicological properties and can, for all practical purposes, be considered equivalent (Campbell, 1989).

In Finland, Sweden, and Norway, ivermectin is widely used for the treatment of warbles, throat bots, and nematodes in reindeer (Haugerud et al., 1993). In Finland, more than 80% of the overwintering animals are treated with ivermectin once a year, in early winter (Anon, 1993). The proportion of animals treated in Norway and Sweden has been lower. The standard procedure is to give one subcutaneous injection of 0.2 mg/kg body mass. Also oral administration methods are in use (Oksanen et al., 1995). Studies in several animal species show that ivermectin undergoes little metabolism, and most of the dose given to the animal is excreted, mostly unaltered, primarily in the feces (Halley et al., 1989; Campbell, 1989; Andrew and Halley, 1996).

Analytical procedures for the determination of low concentrations of ivermectin are numerous (e.g., Tway et al. 1981; Pivnichny et al. 1983; Schnitzerling and Nolan 1985; Montigny et al. 1990; Sams 1993; Bernal et al. 1994; Payne et al. 1995; Reising 1998). These include HPLC procedures for determination of ivermectin in cattle feces. The feces of reindeer, especially during the winter season, is different from the feces of many other ruminants in that it consists of very dry and hard, small (≈12 × 7 mm) pellets. Because of the distinctive dry character of the reindeer feces, procedures developed for cattle feces could not be applied without modifications. Here we report an assay method for the determination of ivermectin in reindeer feces.

* Author to whom correspondence should be addressed (telephone +47 77665400; fax +47 77684411; e-mail Kjetil.Aasbakk@veths.no).

MATERIALS AND METHODS

Animals and Samples. Fecal samples were obtained from three male reindeer calves (body mass ≈ 50 kg each) never treated with ivermectin or other pharmaceuticals. Feces was also obtained from a male reindeer calf that had been treated with ivermectin by subcutaneous injection with Ivomec veterinary injection 10 mg/mL, (Merck, Sharp & Dohme B. V., Haarlem, Holland) at a dose of 0.2 mg/kg body mass. The samples were stored at − 20 °C in plastic bags for subsequent analysis (up to 2 months, Payne et al., 1995).

Internal Standard. Abamectin (Enzec injection solution; Janssen GMBH, Neuss, Germany) (content according to declaration: abamectin 10 mg/mL in glycerol formal), diluted in glycerol formal (98%, Sigma-Aldrich, Steinheim, Germany) to 10 ng/mL, was used as internal standard for HPLC.

Sample Preparation and Derivatization for HPLC. The procedure is a modification of the method by Payne et al. (1995). The feces was thawed at room temperature, and to 1.0 g (wet weight) were added 1.0 mL (10 ng) of internal standard solution and 15 mL of 30% acetone (Merck, Darmstadt, Germany) in deionized water. After the solution was mixed extensively (1 min vortexing, end-over-end rotation 45 rpm for 20 min) and sonicated (10 min, Branson 5210 sonication bath), 15 mL of isooctane (Merck) was added. After this solution was mixed (end-over-end rotation 45 rpm for 20 min, 10 min sonication), the water and isooctane phase was separated (centrifugation 5 min at 500*g*), and the isooctane (upper) layer was collected. The extraction with isooctane was repeated additional three times. The collected isooctane was evaporated to dryness in a Büchi RE 111 Rotavapor (Büchi laboratoriumstechnik AG, Flawil, Switzerland) at 70 °C (water bath) and finally under a stream of N_2. The dry residue was reconstituted in 6 mL of methanol (Merck) (could now be stored at −20 °C) and mixed thoroughly with 40 mL of deionized water. The resulting preparation was applied, at a flow rate of 2 mL/min, on a Supelclean C-18 solid-phase extraction (SPE) tube (6 cm^3, Supelco Inc., Bellefonte, PA) conditioned sequentially with 6 mL of methanol and 6 mL of deionized water and fitted with a 75-mL reservoir. Unbound material and water were washed

10.1021/jf980919q CCC: $18.00
Published on Web 02/25/1999

out with 20 mL 20% methanol followed by 5 mL of isooctane. The column was eluted with 10 mL of methanol. After complete removal of the methanol from the eluate (evaporation in a heating block at 60 °C under a stream of N_2), the residue was reconstituted in 1 mL of methanol and mixed (1 min vortexing) with 6 mL of deionized water and 5 mL of *n*-hexane (Merck). After centrifugation (5 min, 500*g*), the hexane (upper) layer was collected. The extraction with hexane was repeated two additional times. The collected hexane was evaporated to dryness at 60 °C (heating block) under a stream of N_2 and reconstituted in 1 mL of acetonitrile (Merck) (could now be stored at −20 °C). Half of this preparation (at room temperature) was transferred to a glass tube capped with a rubber/Teflon septum screwcap (the other half was stored at −20 °C as a reserve), and 50 μL of *N*-methylimidazole (NMIM, Sigma-Aldrich) was added through the septum. The tube was cooled on ice while the derivatization reagent was prepared.

The derivatization reagent was prepared as follows: 500 μL of trifluoroacetic anhydride (TFAA, > 99%, Sigma-Aldrich) was added to 1.0 mL of acetonitrile in a glass tube that had been cooled on ice (care was taken to avoid condensation of water in the tube). The tube was capped and cooled on ice.

To the cold sample was added (through screwcap septum) 150 μL of the cold derivatization reagent. The tube was allowed to reach room temperature, 1.8 mL of acetonitrile was added, and the preparation was ready for analysis by HPLC.

Acetone, acetonitrile, isooctane, methanol, and *n*-hexane were all of chromatography grade. All glassware had been heated (30 min in a ceramic oven at 500 °C) to remove organic matter. Other tubes and vessels used were new and thoroughly cleaned, finally with deionized water.

General Safety Considerations. All work was performed in a fume hood, particularly work with hexane and TFAA. All reagents were handled with gloves. The chemicals were allowed to reach room temperature prior to use (to avoid condensation of water). TFAA is a corrosive and dehydrating agent, and residual water or methanol will quench the derivatization reaction.

HPLC Analysis. The HPLC system consisted of a CTO-10A column oven, DGU-4A degassing unit, LC-10AD pump, RF-10A spectrofluorometric detector, CBM-10A communication bus module (all Shimadzu Corp., Kyoto, Japan), and the Class LC-10 workstation software package (Shimadzu) on a standard personal computer. The column system was a Hewlett-Packard (HP) Hypersil BDS C18, 5 μm, 125 × 4 mm column equipped with a HP LiChrospher 100 RP-18, 5 μm, 4 × 4 mm guard column. The column oven was set to 35 °C. The mobile phase was 5% water in methanol, at a flow rate of 1.2 mL/min. The injection volume was 50 μL. The fluorescence detector was set to excitation wavelength 365 nm and emission wavelength 470 nm, with a time constant of 1.5 s.

Calibration Lines and Quantitation. Ivomec veterinary injection (of the same batch as that used for treatment of reindeer calf as described in the "Animals and Samples" section; content according to declaration: ivermectin 10 mg, glycerol formal 0.4 mL, propylene glycol to 1 mL) was diluted serially in glycerol formal and added to 1.0-g portions (wet weight) of feces from an untreated animal to give a range of standards with 2, 10, 100, 250, 500, 1000, and 2000 ng of ivermectin/portion. Each portion contained 10 ng of internal standard. The standards were run through the sample preparation procedure and by HPLC as detailed above. For each concentration, three parallels were made (each going through the entire sample preparation procedure) and run on HPLC. Three linear calibration lines, for the concentration ranges 2−100, 100−500, and 500−2000 ng/portion, respectively, were found by the least-squares method from the resulting average values. The construction of the calibration lines and the quantitative calculations were based on the measured peak heights for the $B1_a$ components of abamectin and ivermectin. The quantitative calculations were performed by the internal standard method according to the equation (eq 1):

$$C_s = \left(\frac{R_{f_1} H_i}{H_a} + R_{f_2}\right) \frac{W_a}{W_s} D_f \quad (1)$$

where C_s is the concentration of ivermectin (ng/g of feces wet weight); R_{f_1} and R_{f_2} are the response factors representing the calibration line used (see Results); H_i is the height of ivermectin $B1_a$ peak; H_a, the height of internal standard (abamectin) $B1_a$ peak; W_a, the weight (ng) of internal standard; W_s, the sample wet weight; D_f, the dilution factor for sample injected.

The concentration in dry feces (ng of ivermectin/g of feces dry weight) was calculated as

$$C_d = \frac{C_s 100}{D} \quad (2)$$

where C_s is defined as above and D is the feces dry weight as a percentage of wet weight.

Recovery Calculation. Portions (1.0 g) of feces from untreated animals, fortified with different concentrations of ivermectin (ranging from 5 to 2000 ng/portion) were analyzed by HPLC, and recovery was calculated as follows

$$\% \text{ recovery} = \frac{C_s 100}{C_f} \quad (3)$$

where C_s is defined as above and C_f is the fortified concentration (ng/g feces wet weight).

Dry Weight Determination. Feces was dried in an oven between 80 and 90 °C until a constant weight was obtained (24 h), and the dry weight was determined.

RESULTS

Chromatography of the standards (feces from untreated animal fortified with ivermectin, each containing internal standard) (Figure 1a,b) gave major peaks representing the $B1_a$ components of abamectin and ivermectin at approximate retention times of 4.9 and 7.3 min, respectively. Their smaller $B1_b$ components eluted a little under 1 min earlier. Chromatography of feces from untreated animals, to whom no abamectin or ivermectin had been added, showed that there were no extraneous peaks interfering with the $B1_a$ component of ivermectin or abamectin (Figure 1c). Identical results (no interfering peaks) were obtained with feces from all the three untreated animals. The individual peaks were well-resolved even at the highest fortification levels (Figure 1b). Concentrations as low as 2 ng/g wet weight portion, corresponding to ∼ 3 ng/g dry weight (mean dry weight was 68% of the wet weight), gave distinct peaks and could well be determined.

The assay procedure was established so that ivermectin could be determined in a single sample preparation and HPLC run for each sample. The relationship between concentration ratio (concentration of ivermectin/concentration of abamectin) and height ratio (peak height ivermectin $B1_a$/peak height abamectin $B1_a$) increased with increasing concentration of ivermectin (Figure 2). Therefore, as a compromise between accuracy and simplicity, three different calibration lines were constructed (Figure 3) to enable determination of concentrations over the whole concentration range relevant for reindeer feces (up to 2 μg of ivermectin/g wet weight). The calculated response factors (R_{f_1}, R_{f_2}) are given in the legend to Figure 3. In concentration calculations, the response factors for the 0−100 ng/g calibration line were used for height ratios (see above) lower than 7.8. For height ratios between 7.8 and 33.0, the response factors for the 100−500 ng/g calibration

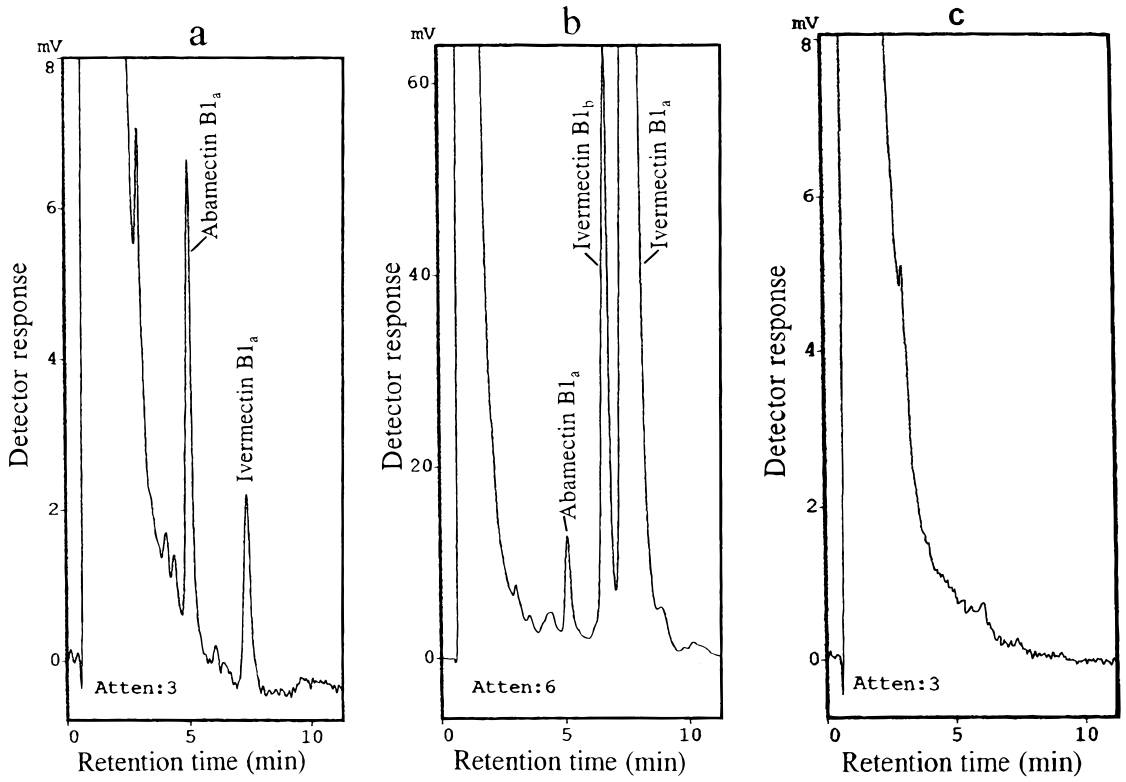

Figure 1. Chromatograms of standards. Feces portions (1 g wet weight) from untreated animal, each containing internal standard (10 ng of abamectin), fortified with 5 ng (a) and 2000 ng (b) of ivermectin/portion. Part c is of feces, without internal standard and ivermectin. Detector attenuation: see lower left corner.

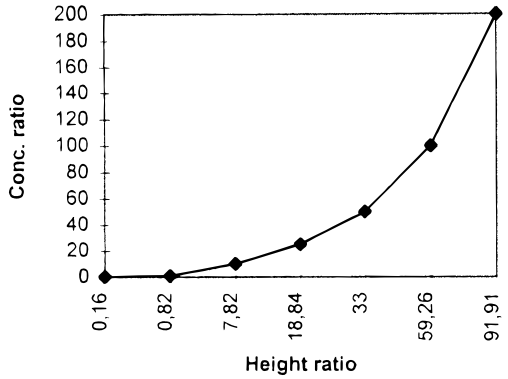

Figure 2. HPLC analysis of ivermectin in reindeer feces with abamectin as internal standard. The diagram shows the relationship between the concentration ratio (ivermectin to abamectin) and the height ratio (peak height of ivermectin $B1_a$ to peak height of internal standard $B1_a$) as the ivermectin concentration increases.

line were used, and for height ratios higher than 33.0 the response factors for the 500−2000 ng/g calibration line were used. For height ratios near the intersection points between the lines (height ratios 5.8−9.8 and 31−35), the concentration was determined as the mean of the values found from the calculations using the R_fs for each of the intersecting lines (see Figure 3a,b).

The standards for the calibration lines were prepared in feces in the same manner as the samples analyzed for the recovery studies. The relative recovery was from ∼95 to ∼116% (Table 1), with standard deviations ranging from 1.1 to 8.0%. The recovery was relatively uniform throughout the range of fortifications levels.

To see how the derivatized samples were influenced by storing, derivatized samples were placed in tightly capped vials in the dark at room temperature and analyzed after 12 days. All peak heights were reduced with time, but the relative reduction was nearly identical for the $B1_b$ components of both ivermectin and abamectin, and thus concentrations determined after HPLC runs of 12-day-old samples were nearly the same as the concentrations determined after analysis of freshly prepared samples. After 12 days, all peak heights were reduced with ∼ 50%, and thus analysis of samples with low concentrations of ivermectin (<10 ng/g) had to be performed with freshly derivatized samples.

To see whether the assay method was capable of recovering incurred ivermectin, feces from a reindeer treated with ivermectin by standard procedure was analyzed (Figure 4). The concentration was determined to 632 ng/g dry weight.

DISCUSSION

The reindeer feces has a great capability of absorbing liquid, and the modifications consisted mainly of the use of large volumes of liquids in the early steps of the preparation procedure. We also used abamectin as internal standard. The derivatization procedure that we used (acetylation reaction converting ivermectin to a fluorescent derivative with *N*-methylimidazole and trifluoroacetic anhydride) can be affected by residual water that leads to decreased yields. Reising (1998) found that the yields of derivatives of ivermectin and abamectin were identical under the various circumstances that they tested, and they found that the use of abamectin as an internal standard eliminated derivative yield as a source of analytical variation. We found that the use of abamectin as internal standard also allowed the determination of ivermectin from chromatography results from relatively old (12 days) derivatized samples that had been stored tightly closed in the dark.

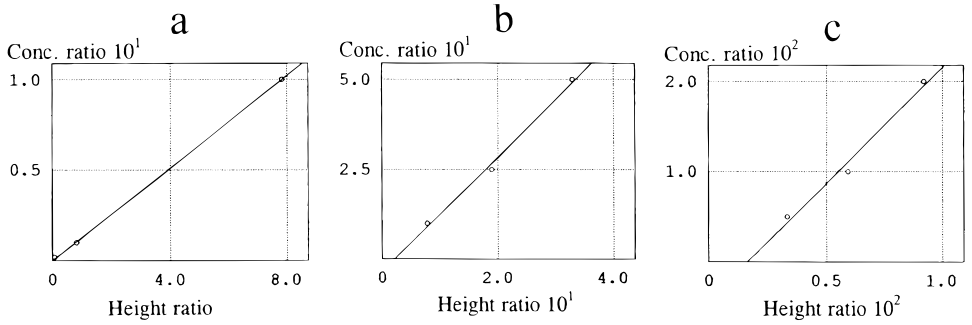

Figure 3. Calibration lines for ivermectin in reindeer feces, with abamectin as internal standard, for the concentration ranges 2−100 (a, $R_{f_1} = 1.28999$, $R_{f_2} = 0.0305967$), 100−500 (b, $R_{f_1} = 1.60141$, $R_{f_2} = -3.47313$), and 500−2000 (c, $R_{f_1} = 2.57454$, $R_{f_2} = -41.5477$) ivermectin/1 g wet weight. The lines were found from analysis of feces portions (1 g wet weight) from an untreated animal, each containing abamectin (10 ng/portion), and 2, 10, or 100 ng of ivermectin (a), 100, 250, or 500 ng of ivermectin (b), and 500, 1000, or 2000 ng of ivermectin (c).

Table 1. Recovery of Ivermectin from Reindeer Feces

fortification level (ng/g wet weight)	% recovery	SD (%)
5	116.0	4.6[a]
10	109.2	8.0[a]
20	97.0	nd[b]
100	95.4	1.1[a]
250	106.8	nd[b]
500	96.4	2.6[a]
1000	110.1	5.7[a]
2000	97.8	nd[b]

[a] $n = 6$. [b] Not determined ($n = 2$).

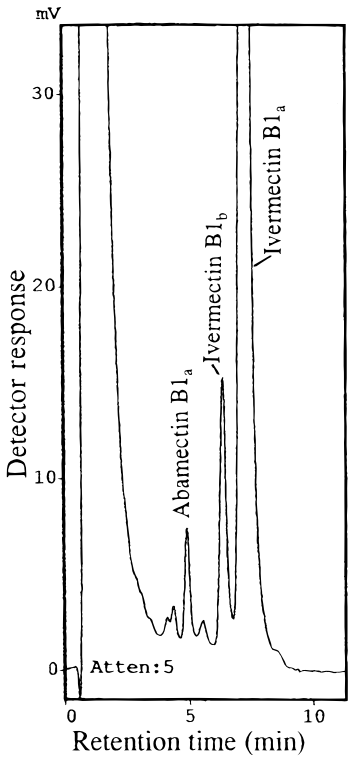

Figure 4. Chromatogram of feces (1.0 g portion, wet weight, containing internal standard 10 ng) from reindeer treated with ivermectin (subcutaneous injection of Ivomec veterinary injection 10 mg/mL at a dose of 0.2 mg/kg body mass), sample from day 2 after treatment. Concentration of ivermectin calculated to 632 ng/g of feces dry weight.

The use of the commercially available Ivomec veterinary injection for the construction of calibration lines implied that approximate and no exact concentrations could be determined. The simplicity of the assay method (determination of all relevant concentrations from one sample preparation and one HPLC run, using three different calibration lines) also contribute in the direction that only approximate concentrations can be determined. If concentrations should be determined relative to a single calibration line, this line would have to span a narrow concentration range, and for each of the samples with higher concentrations, at least two sample preparations would be necessary to allow the use of the internal standard method. For the purpose of determining approximate concentrations and for the comparison of different samples, we feel that the use of Ivomec veterinary injection as analytical standard is satisfactory.

In the preliminary work with the assay method, we found calibration lines from the analysis of standards (ivermectin/abamectin) that had not been prepared in feces (run through the preparation procedure starting with the C-18 column). This gave calibration lines that gave higher recovery of ivermectin from the feces over the whole range of fortification levels (data not shown), and it appeared that the height ratio relative to the concentration ratio was dependent on whether feces was present or not. Klotz et al. (1990) and Okonkwo et al. (1993) reported that ivermectin binds avidly to plasma proteins. The finding concerning the calibration lines may indicate a difference between the degree to which ivermectin and abamectin bind to feces components. To avoid problems associated to this, the internal standard was added to the feces as the first step of the sample preparation procedure, and the standards were run through the same procedure as the samples. The assay method was not subject to interference by extraneous peaks originating from the feces itself or from the sample preparation procedure.

Analyses of feces fortified with ivermectin demonstrated that approximate ivermectin concentrations over the range 2 ng to 2 μg/g on a wet weight basis can be determined in a single sample preparation and HPLC run. The analyses also demonstrated high relative recovery rate of ivermectin from feces and good reproducibility for the assay. The assay recovers ivermectin incurred on reindeer at a concentration level comparable to levels in feces from cattle treated with ivermectin (Herd et al., 1996). Levels of ivermectin added to feces at low concentration (2 ng/g wet weight) could be recovered and detected by the assay, indicating good extraction efficiency. Obviously, due to possible differences in binding of ivermectin to plasma proteins and feces components as outlined above, the possibility exists that the extraction efficiency differs between feces

fortified with ivermectin and feces from animals incurred with ivermectin. However, since the assay method is intended for determination of approximate concentrations and for comparison between samples, we find it justifiable to leave this question concerning extraction efficiency open for future study. We will in our next report use the assay to determine ivermectin in feces from reindeer treated with the drug and relate the findings to studies concerning the dung fauna of coprophageous insects. The assay method may also prove useful for other feces matrixes (e.g., goat, sheep) with consistency related to reindeer feces.

ACKNOWLEDGMENT

We thank the following people for their contribution to the work: Tor Hemmingsen, Department of Mathematics and Natural Sciences, Stavanger College, School of Science and Technology, Stavanger, Norway; Arne C. Nilssen, Tromsø Museum, N-9037 Tromsø, Norway; Willy Hemmingsen, Institute for Biology, University of Tromsø, N-9037 Tromsø; Rolf E. Haugerud, Nordic Council for Reindeer Research, c/o The Norwegian School of Veterinary Science, N-9005 Tromsø. We also thank Dietrich Barth, Merial, Lauterbach, Germany, for providing us with Enzec (Abamectin) solution.

LITERATURE CITED

Andrew, N. W.; Halley, B. A. Stability of ivermectin in rumen fluids. *J. Vet. Pharmacol. Ther.* **1996**, *19*, 295−299.

Anon. Kertomus Paliskuntain yhdistyksen toiminnasta vuodelta 1992. (Yearly report from the Finnish Association of Reindeer Herding Cooperatives). *Poromies* **1993**, *2*, 7−25 (in Finnish).

Bernal, J. L.; Nozal, J. D.; Salas, M.; Galante, E.; Lumaret, J. P. HPLC determination of residual ivermectin in cattle dung following subcutaneous injection. *J. Liq. Chromatogr.* **1994**, *17* (11), 2429−2444.

Campbell, W. C., Ed. *Ivermectin and abamectin;* Springer-Verlag: New York, 1989.

Halley, B. A.; Jacob, T. A.; Lu, A. Y. H. The environmental impact of the use of ivermectin: environmental effects and fate. *Chemosphere* **1989**, *18*, 1453−1563.

Haugerud, R. E.; Nilssen, A. C.; Rognmo, A. On the efficacy of ivermectin against the reindeer sinus worm *Linguatula arctica* (Pentastomida), with a review on ivermectin in reindeer. *Rangifer* **1993**, *13* (3), 157−162.

Herd, R. P.; Sams, R. A.; Ashcraft, S. M. Persistence of ivermectin in plasma and faeces following treatment of cows with ivermectin sustained-release, pour-on or injectable formulations. *Int. J. Parasitol.* **1996**, *26* (10), 1087−1093.

Klotz, U.; Ogbuokiri, J. E.; Okonkwo, P. O. Ivermectin binds avidly to plasma proteins. *Eur. J. Clin. Pharmacol.* **1990**, *39* (6), 607−608.

Montigny, P. D.; Shim, J. S. K.; Pivnichny, J. V. Liquid chromatographic determination of ivermectin in animal plasma with trifluoroacetic anhydride and *N*-methylimidazole as the derivatization reagent. *J. Pharmaceut. Biomed. Anal.* **1990**, *8* (6), 507−511.

Okonkwo, P. O.; Ogbuokiri, J. E.; Ofoegbu, E.; Klotz, U. Protein binding and ivermectin estimations in patients with onchocerciasis. *Clin. Pharmacol. Ther.* **1993**, *53* (4), 426−430.

Oksanen, A.; Norberg, H.; Nieminen, M.; Bernstad, S. Influence of route of administration on the plasma concentrations of ivermectin in reindeer. *Res. Vet. Sci.* **1995**, *58*, 286−287.

Payne, L. D.; Hicks, M. B.; Wehner, T. A. Determination of abamectin and/or ivermectin in cattle feces at low parts per billion levels using HPLC with fluorescence detection. *J. Agric. Food Chem.* **1995**, *43*, 1233−1237.

Pivnichny, J. V.; Shim, J. S. K.; Zimmerman, L. A. Direct determination of avermectins in plasma at nanogram levels by high-performance liquid chromatography. *J. Pharm. Sci.* **1983**, *72* (12), 1447−1450.

Reising, K. P. Ivermectin quantitation with an abamectin internal standard. *J. AOAC Int.* **1998**, *81* (2), 477−483.

Sams, R. Chemical assay of avermectins by high performance liquid chromatography with fluorescence detection. *Vet. Parasitol.* **1993**, *48* (1−4), 59−66.

Schnitzerling, H. J.; Nolan, J. Normal phase liquid chromatographic determination of nanogram quantities of ivermectin in cattle blood or plasma. *J. Assoc. Off. Anal. Chem.* **1985**, *68* (1), 36−40.

Tway, P. C.; Wood, J. S.; Downing, G. V. Determination of ivermectin in cattle and sheep tissues using high-performance liquid chromatography with fluorescence detection. *J. Agric. Food Chem.* **1981**, *29*, 1059−1063.

Received for review August 18, 1998. Revised manuscript received December 18, 1998. Accepted December 23, 1998. This work was supported by grants from the Reindeer Research Fund under the auspices of the Norwegian Ministry of Agriculture (RF sak 16/96).

JF980919Q