Determination of Ivermectin Levels in Swine Tissues at the Parts per Billion Level by Liquid Chromatography with Fluorescence Detection

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Ivermectin levels in swine tissues have been determined by an HPLC/fluorescence detection method, originally used to determine levels in sheep and cattle tissues. The method involves homogenization, centrifugation, and several liquid-liquid solvent partition steps. The recovery of ivermectin at levels between 6 and 150 ppb in kidney, liver, fat, and muscle averaged 85%, with a quantitation limit of 5 ppb and a limit of detection of 1 ppb. Good agreement was observed between this method and a reverse isotope dilution method. The method was further used to determine ivermectin levels in edible swine tissues, where swine were dosed by subcutaneous injection and in feed. The ivermectin distribution pattern showed high levels in fat and liver and an overall rapid depletion in all tissues. The suggested withdrawal time for swine dosed at 2 ppm for 7 days in feed is 5 days, and that for swine dosed at 0.4 mg/kg subcutaneously is 18 days.

INTRODUCTION

Ivermectin, a mixture of two homologues, containing not less than 80% 22,23-dihydroavermectin B_{1a} (H_2B_{1a}) and not more than 20% 22,23-dihydroavermectin B_{1b} (H_2B_{1b}) (Fisher and Mrozik, 1989), is a complex macrocyclic disaccharide. This compound is derived from a naturally occurring fermentation product, which is chemically reduced to form ivermectin. The structures of H_2B_{1a} and H_2B_{1b} are shown in Figure 1. These compounds differ only by one methylene unit ($-CH_2-$) at the C-25 position; B_{1a} contains a *sec*-butyl group, and B_{1b} contains an isopropyl group. Ivermectin is an exceptionally potent drug which exhibits a broad spectrum of activity against nematodes and arthropod parasites in cattle (Scott et al., 1985), goat (Swan and Gross, 1985), pig (Blue et al., 1986), horse (Campbell and Benz, 1983), and dog (Campbell, 1987). This compound has shown efficacy against various stages of parasites when given orally or when injected in different animal species often at low doses (as low as 0.05 mg/kg) (Campbell, 1987).

It is essential, with a drug that is so extensively used in food-producing animals, to assess the tissue distribution of the residue to determine the withdrawal time required to satisfy regulatory requirements. The total tissue residue includes the parent drug, its metabolites, and any tissue-bound residue. Work by Chiu et al. (1985, 1988) has demonstrated that the total radioactivity in edible tissues (liver, kidney, muscle, and fat) of cattle, sheep, swine, and rats was quantitatively extractable with organic solvents. Thus, bound residue is not a concern with ivermectin. They have also shown that the metabolism of ivermectin is quantitatively different in swine from that in cattle, sheep, or rats. However, the unaltered parent drug was shown to be the major residue component in all four animals, and thus H_2B_{1a} is the marker substance for residue assays in all animal species.

Earlier work has shown that ivermectin residues, even immediately following dosing, are in the low parts per billion (ng/g) range (Downing, 1989). To obtain a complete picture of the ivermectin distribution profile and disposition, an analytical method with high selectivity and high sensitivity was required. Analytical methods sensitive enough to detect and quantify ivermectin levels in parts per billion were developed in the early 1980s (Tolan et al., 1980; Tway et al., 1981, 1984; Chiu et al., 1985, 1988; Nordlander and Johnson, 1990). An assay based on derivatization of ivermectin to a fluorescent product and separation and quantitation by HPLC fluorescence detection was developed and used in this laboratory to determine residue levels in sheep and cattle tissues (Tway et al., 1981).

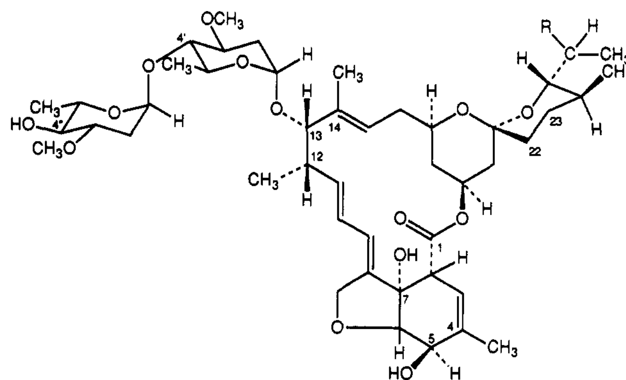

Figure 1. Structure of ivermectin. This modified fermentation product consists of two components, 22,23-dihydroavermectin B_{1a} (H_2B_{1a}, R = C_2H_5, ≥80%) and 22,23-dihydroavermectin B_{1b} (H_2B_{1b}, R = CH_3, ≤20%).

In this paper, we demonstrate the application of this assay method to swine tissues. The assay was shown to work well for porcine liver, kidney, muscle, and fat tissue samples with no significant modification from the earlier published method.

The method was validated with control, fortified, and incurred residue samples. The chromatograms of the control tissues (liver, kidney, fat, and muscle) had no peaks that would interfere with the ivermectin peaks. Control animal tissues spiked in the 6-150 ppb range gave average recoveries for H_2B_{1a} of 85%. The detection limit of this method is approximately 1 ppb (S/N >3), and the quantitation limit is 5 ppb.

This method was also applied to incurred residue samples from two swine studies. In the first study, ivermectin was administered orally at 2 ppm in feed for a period of 7 days. Animals were sacrificed while on the drug and at 0.5, 1, 1.5, 2, 3, 5, and 7 days following withdrawal of the medicated feed. In the target tissue,

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liver, ivermectin levels depleted from an average 38 ppb on drug to 3 ppb at 5 days. In the second study, a subcutaneous injection of 0.4 mg of ivermectin/kg of body weight was administered. Animals were sacrificed 1, 3, 5, 7, 10, 14, and 28 days after dosing. The ivermectin levels in the liver averaged 67 ppb on day 1 and depleted to 0 ppb by day 28.

EXPERIMENTAL PROCEDURES

A. Analytical Method. *Reagents.* All reagents and standards were the same as used in the previously published method (Tway et al., 1981). All aqueous solutions were prepared with distilled water, which was further purified by use of a Millipore Milli-Q water system consisting of one organic adsorption cartridge, three mixed-bed ion exchangers, and a 0.22-μm finishing filter in series. Dissolved oxygen in the chromatographic mobile phase was removed by bubbling with helium or nitrogen. A standard curve was run with each set of samples.

Apparatus. The apparatus used in the ivermectin injection study was the same as that used in the cattle and sheep tissue assay method (Tway et al., 1981); the apparatus used in the ivermectin in-feed study is described below. Tissues were homogenized with a Polytron (Brinkmann Instruments) Model 27-11-200-5 with PTA Model 27-11-330-3 generator. Worldwide Monitoring or Analytichem International vacuum manifolds were used throughout the work. The ultrasonic bath used for dissolving the samples was made by Branson (Model 520). After the derivatization reaction, samples were cleaned up by silica columns (Bond Elut, 1000 mg, 6 mL, part no. 6061406, Analytichem International). Chromatographic equipment consisted of a Spectra-Physics Model SP8800 liquid chromatographic pump connected to a Waters Wisp autosampler Model 710B and Kratos-Schoeffel Instrument Model FS950 fluorescence detector with a Spectra-Physics 4200 integrator. The column heater was a Fiatron CH-30 with a TC-55 controller. A 5 μm, 4.6 mm i.d., C_{18} standard Brownlee Laboratories guard column (Spheri-5 RP-18 OD-GU obtained from Rainin Instrument Co., Inc.) was used before the Chromega Bonded (E.S. Industries) C_{18} (15 cm × 4.6 mm i.d.) analytical column. The chromatographic conditions were as follows: column temperature, 30 °C; mobile phase, methanol–water (93:7); flow rate, 1.6 mL/min; retention time of the ivermectin fluorescent product, 12–14 min. The fluorescence detector configuration was the following: excitation wavelength filter 365 nm; emission filter, 418-nm cutoff filter.

Extraction and Detection of Ivermectin. The extraction of ivermectin residue from a tissue sample is difficult because of the lipophilicity of the ivermectin and its low levels of residue in tissue. The extraction and detection procedures employed in the ivermectin injectable and in-feed studies were similar to the cattle and sheep tissue method (Tway et al., 1981).

B. Animal Treatment. *Ivermectin in Feed.* Pigs of a commercial crossbreed assigned to receive medication were fed, ad libitum, a ration containing 2 ppm of ivermectin for a period of 7 days. Five swine (barrows and gilts) were slaughtered at each time point: on drug and 0.5, 1, 1.5, 2, 2.5, 3, 5, and 7 days off drug; the kidneys, liver, muscle from the hindquarters, and backfat were collected for residue analysis. On-drug samples were collected 4 h following the last feeding on the seventh day of medication. Five animals were given no medication; these animals were used as controls for the study, and the tissues were collected in the same manner as for the dosed samples.

Ivermectin Injection. In this study 35 animals were given subcutaneous injection of 0.4 mg of ivermectin (formulation 40% v/v glycerol formal in propylene glycol)/kg of body weight. This level is higher than the recommended dose for swine of 0.3 mg/kg. At each withdrawal time, 1, 3, 5, 7, 10, 14, and 28 days postdose, five animals (barrows and gilts) were slaughtered, and their muscle, liver, kidneys, and fat were collected. In addition, tissue was taken from beneath the site of the subcutaneous injection and surrounding area. Similarly, tissues were collected from the control animals. All tissues from both the oral (in-feed) and the injection studies were frozen and stored at −20 °C until assayed.

Table I. Summary of Recoveries from Swine Tissues by the Ivermectin HPLC Fluorescence Method[a]

fortification level, ppb	liver	fat	kidney	muscle
6.3		80		
7.5	[80]^2			
10	[87]^3	[97]^3	[87]^3	[92]^3
12.5	[85]^2	[80]^2	[91]^3	
15	67			
17.5	63			
20	[78]^3	[93]^3	[87]^3	[90]^3
25			80	
40				86
50	[81]^3	[87]^3	[89]^3	[86]^3
62.5	86			
100	[82]^3	[86]^3	[84]^6	[86]^6
150		87		
av[b]	79	87	86	88
SD	±8	±6	±4	±3

[a] [ ]^x, x is the number of individual determinations used to calculate the average recovery value. [b] Overall average = 85.

Table II. Comparison of Chemical Assay and RIDA Results

	swine tissue at 14 days postdose	
	liver	fat
chemical assay H_2B_{1a}, ppb	42	85
RIDA[a] H_2B_{1a}, ppb	47	95

[a] Performed by Animal Drug Metabolism and Exploratory Group, MSDRL, Rahway, NJ.

Table III. Average Residue Level, Range, and Standard Deviation from the Swine Ivermectin in Feed Study

time postdose, days	liver, ppb	fat, ppb	kidney, ppb	muscle, ppb
control	0	0	0	0
on drug	38 ± 5 (32–45)	100 ± 16 (80–120)	24 ± 2 (20–26)	14 ± 3 (10–17)
0.5	NA[a]	NA	NA	8 ± 4 (3–14)
1.0	22 ± 11 (11–40)	73 ± 32 (46–120)	13 ± 7 (4–23)	7 ± 3 (5–12)
1.5	19 ± 8 (12–32)	48 ± 12 (36–65)	8 ± 3 (6–14)	5 ± 1 (4–7)
2.0	14 ± 4 (11–20)	41 ± 8 (32–49)	7 ± 1 (6–9)	5 ± 1 (4–6)
3.0	12 ± 5 (6–17)	26 ± 8 (19–39)	4 ± 1 (3–6)	3 ± 1 (2–4)
5.0	3 ± 2 (1–5)	13 ± 5 (7–30)	2 ± 1 (1–2)	1 ± 1 (1–2)
7.0	NA	7 ± 6 (1–5)	NA	NA

[a] NA, not assayed.

RESULTS AND DISCUSSION

The earlier assay method used for the determination of ivermectin in cattle and sheep (Tway et al., 1981) has been validated for use with porcine tissues. Table I summarizes recovery studies performed on samples fortified at various levels with ivermectin. Recoveries for the various tissues, the standard deviation for the particular tissue, and the range of values for each tissue are as follows: liver, 79 ± 8%, 63–87%; fat, 87 ± 6%, 80–97%; kidney, 86 ± 4%, 80–91%; muscle, 88 ± 3%, 86–92%. The overall average for all the fortified tissues was 85%.

To demonstrate that the chemical assay method was

Table IV. Average Residue Level, Range, and Standard Deviation from the Swine Ivermectin Injection Study

time postdose, days	liver, ppb	fat, ppb	kidney, ppb	muscle, ppb	injection site, ppb
control	0	0	0	0	NA[a]
1	67 ± 35 (37–125)	74 ± 32 (38–122)	47 ± 28 (19–91)	24 ± 10 (11–29)	12500 ± 5270 (6500–21000)
3	69 ± 9 (59–82)	110 ± 17 (84–125)	48 ± 15 (34–71)	32 ± 8 (22–43)	5100 ± 5182 (620–14000)
5	53 ± 10 (44–68)	91 ± 9 (77–98)	32 ± 7 (23–40)	20 ± 3 (17–24)	1110 ± 583 (280–1830)
7	41 ± 17 (26–70)	73 ± 24 (48–109)	23 ± 8 (12–34)	13 ± 3 (11–17)	2300 ± 3264 (196–8000)
10	23 ± 7 (11–30)	47 ± 11 (30–59)	14 ± 8 (5–23)	9 ± 2 (5–10)	2500 ± 3887 (80–9400)
14	13 ± 3 (8–16)	24 ± 8 (15–36)	5 ± 2 (3–7)	4 ± 2 (3–7)	230 ± 229 (4–550)
28	0	0	0	0	0

[a] NA, not applicable.

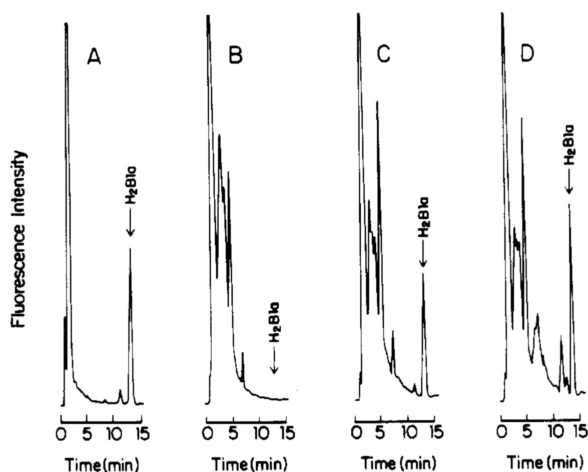


Figure 2. Typical chromatograms for ivermectin in swine liver tissue. (A) Ivermectin standard, 25 ppb; (B) control liver; (C) control liver fortified with 20 ppb of ivermectin; (D) on-drug liver (ivermectin feed study).

capable of recovering incurred as well as fortified ivermectin residues from tissues, porcine liver and fat samples from animals that had been dosed subcutaneously with radioactive ivermectin were assayed. The results are shown in Table II. The chemical assay recovered 89% of the value found by the reverse isotope dilution assay (RIDA) (Chiu et al., 1985) in each case. Thus, it is clear that the chemical assay method is applicable to authentically incurred ivermectin samples.

Typical LC fluorescence chromatograms of an ivermectin standard, control liver, liver fortified with 20 ppb of ivermectin, and an on-drug liver sample (feed study) are shown in Figure 2. For the ivermectin standard two peaks were observed; the first peak corresponds to H_2B_{1b}, and the second (larger) peak corresponds to H_2B_{1a}. For the control tissue sample, no peaks were present in the chromatogram which might interfere with the determination of ivermectin.

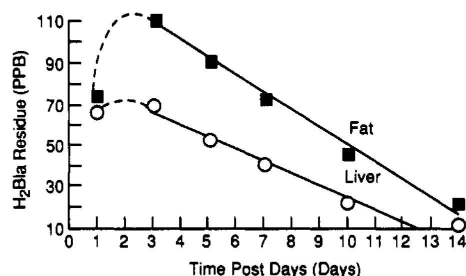
Under the LC conditions, a constant background noise at the retention time of ivermectin (equivalent to 0.3 ppb) limited the lowest detectable level to 1 ppb (3× background). The method had a lower limit of reliable measurement of approximately 5 ppb. At this level an LC peak with $S/N = 15$ was generally observed. For ivermectin levels calculated in this work, all peaks observed are reported down to a level of 1 ppb. Where no peaks were observed, the level was reported as 0.

The ivermectin levels in liver, kidney, muscle, and fat tissues at various withdrawal times found in the study in which the animals were dosed in feed are shown in Table III. These values are the average levels found for five animals at each withdrawal time in each tissue. A rapid depletion of ivermectin (H_2B_{1a}) in porcine tissues from swine fed for 7 days at the recommended use rate (2 ppm) was observed. The fat tissues generally contained the highest level; it averaged 100 ppb on drug and depleted to an average residue of 13 ppb 5 days off drug. Liver contained the second highest ivermectin levels: 38 ppb on drug which depleted to 3 ppb or below at 5 days postdose. The kidney and muscle tissues had lower residue levels which depleted to 2 ppb or less at 5 days after withdrawal time. All of the control liver, fat, kidney, and muscle tissues contained no detectable levels of ivermectin. Levels below 2 ppb were negligible and are well below the quantitation limit for the method.

Similar trends were also observed in the study in which the animals were dosed subcutaneously at 0.4 mg/kg, although the initial ivermectin levels were higher and depleted more slowly. In this study, in addition to liver, fat, kidney, and muscle tissue, ivermectin levels were also determined from injection site tissue. The results of this study are reported in Table IV. Again, the values are an average of five animals sacrificed at each withdrawal time for each tissue. The liver had an average ivermectin level at 3 days postdose of 69 ppb, which depleted to 13 ppb at 14 days postdose and to nondetectable level at 28 days postdose. Ivermectin levels in all four tissues peaked at approximately 3 days after injection. Fat tissue samples again had the highest ivermectin concentration among tissues; kidney and muscle had lower levels at all times postdose. This ivermectin distribution pattern among edible tissues is similar to those found in cattle, sheep, and rats (Downing, 1989). In both studies, the interanimal difference is larger at shorter time periods, where the decay is predominantly exponential.

The injection site had very high levels at early time points with an average level value of 12.5 ppm (μg/g) at 1 day postdose. However, the levels decreased rapidly and by 28 days were nondetectable.

The concentration–withdrawal time curves for ivermectin in-feed and injection studies from liver and fat tissues are shown in Figure 3. In the subcutaneous study, the ivermectin levels in both fat and liver remained high for 2–3 days and then declined linearly with time from day 3 to day 14. Ivermectin levels in fat in the in-feed study



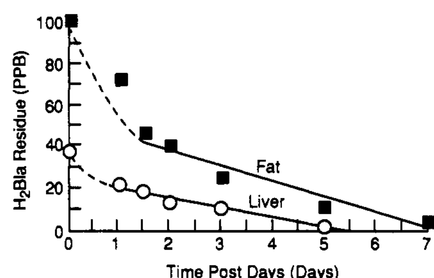


Figure 3. Regression curve for the depletion of H_2B_{1a} in swine liver and fat tissues.

decreased exponentially with time from day 0 to day 1.5 after withdrawal of drug and then followed a linear decrease. In the in-feed study, swine kidney and muscle showed similar behavior to fat and liver.

The average ivermectin levels in liver and fat have a half-life, $t_{1/2}$, of 2 and 6 days in the in-feed study and in the subcutaneous study, respectively. The half-life is defined as the time required for maximum ivermectin levels to decrease by half. Excellent linear regression curves were obtained in both studies by using the points in Figure 3 connected by the solid line. The correlation coefficients of the depletion curve in the feed study were 0.97 and 0.92 and those in the injection study were 0.94 and 0.99 for liver and fat tissues, respectively.

In conclusion, the assay method for sheep and cattle tissues (Tway et al., 1981) works equally well on porcine samples. Examination of the data shows a rapid depletion of ivermectin levels in all cases. Fat and liver generally contained the highest and longest lived levels. The fat levels were generally higher than the liver levels at all time points in both the in-feed and the injection studies. Their depletion patterns are essentially the same in both tissues. Because of the ivermectin residues found in the liver, and its level of consumption in the diet, liver is considered the target tissue for the analysis. The permitted concentration of H_2B_{1a} in swine liver was derived from toxicity and metabolism data and has been determined to be 20 ppb (*Code of Federal Regulations*, 1989). Thus, statistical analysis (Food and Drug Administration, 1986) of the ivermectin depletion data using the upper tolerance limit with 95% confidence limits suggests a 5-day withdrawal period for swine dosed at 2 ppm of ivermectin in feed. In the study in which swine were dosed subcutaneously at 0.4 mg/kg, the levels depleted more slowly, and an 18-day withdrawal period is calculated on the basis of the same statistics.

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