Absorption, Tissue Distribution, Metabolism, and Excretion of Moxidectin in Cattle

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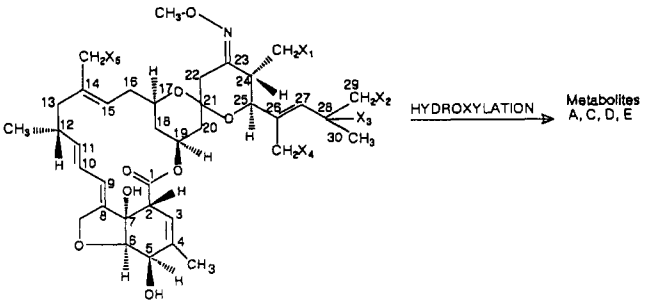
The absorption, tissue distribution, metabolism, and excretion of moxidectin, a new endectocide for the control of internal and external parasites in cattle and sheep, was studied in cattle. Following a single subcutaneous dose of ^{14}C- and ^{2}H-labeled moxidectin of 0.2 mg/kg of body weight, highest ^{14}C residues were present in abdominal fat (898, 636, and 275 ppb) and back fat (495, 424, and 186 ppb) at 7, 14, and 28 days posttreatment, respectively. Lower residues were detected in liver (109, 77, and 31 ppb), kidney (42, 38, and 13 ppb), and loin muscle (21, 10, and 4 ppb), respectively. The administered radioactivity was excreted primarily in the feces, with only 3% of the dose being eliminated in the urine. The HPLC/^{14}C profiles of the residues extracted from the tissues, fat, and feces were qualitatively similar and showed moxidectin was the major component of the residue. Only two metabolites were present that were more than 5% (2 ppb) of the total liver residues after 28 days. These were identified as the C-29/30 and the C-14 monohydroxymethyl metabolites by LC/MS and LC/MS/MS analysis of the metabolites isolated from the feces. Proton NMR analysis of the authentic compounds prepared *in-vitro* from cattle liver microsomal incubation and rat liver homogenate incubation with ^{14}C-labeled moxidectin confirmed the mass spectral results. By LC/MS and LC/MS/MS, several other mono- and dihydroxylated and O-demethylated metabolites were also identified.

Moxidectin is a semisynthetic derivative of nemadectin (Asato and France, 1990), a macrocyclic lactone produced by fermentation in a culture of *Streptomyces cyanogriseus*. It is active at extremely low dosages against a wide variety of nematode and arthropod parasites and is currently being marketed in various countries as an injectable and a pour-on product for beef cattle, as an oral drench and injectable product for sheep, and in tablet form for dogs. The chemical structure for moxidectin (Figure 1) is related to that of the milbemycins (Mishima et al., 1983; Takiguchi et al., 1980) and avermectins (Albers-Schonberg et al., 1981), which have a novel mode of action against a broad spectrum of nematode and arthropod parasites of animals (Putter et al., 1981). The common features of these compounds are a fused cyclohexene–tetrahydrofuran ring system, a bicyclic 6,6-membered spiroketal, and a cyclohexene ring fused to the 16-membered macrocyclic ring. Moxidectin is the 23-(*O*-methyloxime) derivative of nemadectin and structurally differs from ivermectin (Fisher and Mrozik, 1989) in that it has no sugar moiety at the C-13 position and has the unsaturated side chain at the C-25 position.

The exact mechanism of action of moxidectin has not been fully determined, but it would appear to be multifaceted. γ-Aminobutyric acid (GABA) is an important compound involved in the transmission of nerve signals and has a central role in this mode of action. Laboratory studies have demonstrated that moxidectin acts as an agonist to GABA receptors and stimulates the binding of this neurotransmitter. Paralysis of the parasite and eventual death and expulsion result. Such activity can also interfere with parasite reproduction as demonstrated by markedly reduced oviposition in engorged female ticks on cattle and sheep. Alternatively, the glutamate–chloride channel may also function as a central role in the mode of action of related milbemycins. Arena et al. (1992) have shown avermectin-sensitive chloride currents in *Xenopus oocytes* injected with *Caenorhabditis elegans* RNA.



Compound		X_1	X_2	X_3	X_4	X_5
Moxidectin		H	H	H	H	H
Metabolite A		OH	OH	H	H	H
	or	H	OH	H	H	OH
	or	OH	H	H	OH	H
	or	H	H	H	OH	OH
Metabolite C		OH	H	OH	H	H
	or	H	H	OH	H	OH
Metabolite D		H	OH	H	H	H
	and	H	H	H	OH	H
	or	H	H	OH	H	H
Metabolite E		H	H	H	H	OH

Figure 1. Metabolic pathway for moxidectin in cattle.

The absorption, tissue distribution, metabolism, and excretion of moxidectin were studied in cattle to evaluate the toxic potential of the residual tissue concentration of moxidectin and its metabolites. Comparative metabolism studies were also conducted in sheep (Afzal et al., 1994) and the laboratory rat (Wu et al., 1993). Comparative *in-vitro* studies were conducted with cattle liver microsomes and rat liver homogenates (Zulalian et al., 1992), and pharmacokinetic studies were conducted in cattle, sheep, and the laboratory rat. The cattle study described herein was conducted with ^{14}C-labeled and deuterium-labeled moxidectin following a single subcutaneous in-

0021-8561/94/1442-0381$04.50/0

jection of 0.2 mg/kg of body weight. NMR and mass spectrometric examinations were carried out on the metabolites isolated from the *in-vitro* metabolism studies for confirmation of structure and comparison to the metabolites isolated from the cattle study.

MATERIALS AND METHODS

^{14}C-Labeled Moxidectin. Nemadectin, the precursor to moxidectin, was multiply-labeled with ^{14}C by incorporation of a mixture of carboxyl-^{14}C-labeled acetate, propionate, and isobutyrate (Z. Ahmed and M. W. Bullock, American Cyanamid, private communication, 1991) and chemically modified using a semisynthetic reaction sequence to produce ^{14}C-labeled moxidectin with a radiopurity of 96% by HPLC and TLC analysis, a chemical purity of 97% by HPLC analysis, and a specific activity of 34.1 μCi/mg.

^{2}H-Labeled Moxidectin. Moxidectin labeled at the 5-α position with deuterium was prepared via oxidation of the C-5 hydroxyl group to a ketone followed by reduction with sodium borodeuteride (Ahmed and Bullock, private communication, 1991). The chemical purity was 95% as determined by HPLC.

Dose Formulation. A formulation of 1% (w/v) active ingredient was prepared from a mixture of ^{14}C-labeled moxidectin and ^{2}H-labeled and unlabeled moxidectin. The concentration of moxidectin in the injectable solution was 9.33 mg/g (161.3 ± 4.1 μCi/g) as determined by HPLC analysis and liquid scintillation counting. The specific activity of moxidectin was 16.6 μCi/mg. The ^{2}H-labeled moxidectin was used to serve as a mass marker in the identification of the metabolites by mass spectrometry. A blank formulation was prepared for treatment of the control steer. The density of the injectable solution for the subcutaneous dosing was 1.04 g/mL.

Handling and Treatment of Steers. Hereford steers weighing between 212 and 243 kg were used for the metabolism study. After a period of adaptation and observation for any clinical signs of disease, the steers were placed in individual pens and the activity of each steer was restricted (as would be in the metabolism stall) by the use of a halter attached to a rope tied to the stanchion. Animals were fed a roughage mixture (70% corn silage and 30% chopped alfalfa hay) *ad libitum* plus 1.0 kg of 20% protein supplement per head per day. Water was supplied *ad libitum*. Three animals were randomly assigned to the treatment group with sacrifice times of 7, 14, and 28 days postdose and one animal was assigned to the control with a sacrifice time of 6 days postdose. The steers were weighed on the day before and on the day they were placed in metabolism stalls, and the average weight of each steer on these two days was used for the determination of the drug dosage. Each steer in the treatment group received a single subcutaneous injection of the 1% injectable solution (4.29–5.02 g) equal to 0.2 mg of moxidectin/kg of body weight. The control steer received 4.40 g of the blank formulation. The dose was administered anterior to the left shoulder using an 18-gauge, 1.5-in. needle.

Collection of Samples. Total daily urine and feces outputs were collected and recorded for each steer starting 1 day prior to treatment and continuing until the time of scheduled sacrifice. Urine was collected from each steer using a canvas collection bag, patterned after one obtained from Colorado State University for use in cattle. The collection device was placed around the prepuce area of the steer and sutured with nylon to the adjacent abdominal skin. Tygon flexible plastic tubing was connected from the collection device to a 5-gal glass bottle for urine collection. A stainless steel collection pan lined with a sheet of plastic and placed on the floor at the back of the metabolism stall was used for collection of the feces. The total daily collection was mixed thoroughly with a wooden paddle. The fecal mixture was then placed in a heavy-duty plastic bag, and the feces were further mixed by kneading the plastic bag over the feces. Approximately 2 kg of feces from each steer, randomly collected from different areas of the already well-mixed sample, was provided for radioanalysis. At sacrifice, blood, liver, kidney, fat (omental and back), skin with associated fat and muscle from an area surrounding the injection site (15 cm wide and 2.5 cm deep), and loin muscle from the right side of the carcass were collected from each steer. A number of other biological samples were also collected as listed in Table I. Bile was collected directly from the gall bladder at sacrifice.

Sample Preparation and Radioanalysis Procedures. All tissues, thoracic and abdominal viscera, and skin with associated fat and muscle were ground with dry ice and stored frozen, and the dry ice was allowed to dissipate. The radioactive residues were determined by homogenizing a 5.0-g aliquot of the ground sample with 5.0 g of water and combusting 1.0-g aliquots of the homogenate (equal to 0.5 g of tissue) in triplicate. Blood, bile, gastrointestinal contents, and feces were analyzed by combusting 0.5-g aliquots in triplicate. Urine was analyzed by counting 1.0-mL aliquots directly in liquid scintillation cocktail. Radioactivity was measured in a Beckman LS 5801 or Beckman LS 9800 liquid scintillation counter. Sample combustions were performed in a Model 306 Packard oxidizer.

Extraction of ^{14}C Residues. The extractions of the ^{14}C residues from the tissues and the feces were performed in a homogenizer using 10 mL of solvent/g of sample. The fat, edible tissues, and injection site samples were extracted (3×) with acetonitrile. The postextracted solids from the liver were extracted (2×) with methanol/water (90:10 v/v/). The feces were extracted (3×) with methanol/water (95:5 v/v). Urine samples (100-mL aliquots) were extracted with diethyl ether (4 × 150 mL) followed by methylene chloride (4 × 150 mL). The bile (40–80 mL) was extracted (4 × 100 mL) with diethyl ether. The acetonitrile extracts of the tissues, fat, and injection site samples were extracted (3×) with hexane using a solvent ratio of 1:3 (v/v) hexane/acetonitrile for further cleanup. Aliquots of the extracts, the aqueous phase, and the postextracted solids were analyzed for radioactivity by liquid scintillation counting and combustion. The aqueous and organic fractions from the extractions of the tissues, feces, urine, and bile were concentrated preparatory to HPLC and TLC analysis. Bile and urine were also analyzed directly by HPLC. The methanol/water extracts of the feces were extracted with diethyl ether for isolation of the metabolites for mass spectral analysis. Samples of control feces, urine, and fat were fortified with ^{14}C-labeled moxidectin, and the fortified samples were processed as described for the treated samples.

***In-Vitro* Experiments.** Microsomes from steer liver were prepared according to the procedure of Miwa et al. (1982). The washed microsomal pellet was resuspended in 0.25 M sucrose and stored at −78 °C at a protein concentration of 32.1 mg/mL. Steer liver microsomes and a commercially available preparation of S9 liver homogenates from Aroclor 1254 induced rats were incubated with ^{14}C-labeled moxidectin to generate metabolites for isolation and identification by mass spectrometry and for comparison to the *in-vivo* metabolites. Preparative-scale incubations were also conducted for the isolation and structural determination of the *in-vitro* metabolites by proton NMR analysis. For the preparative-scale incubation with steer liver microsomes, ^{14}C-labeled moxidectin (1.0 mg, specific activity 8.3 μCi/mg) in 3.0 mL of methanol was added to a vial containing nonlabeled moxidectin (14.3 mg) and Pluronic surfactant F-68 (BASF Wyandotte Corp., Parsippany, NJ) and diluted to 4.0 mL with methanol. This solution was divided into four equal portions and placed in 250-mL flasks. To each flask were added 50 mL of 1 M potassium phosphate buffer (pH 7.4), steer liver microsomes equivalent to 120 mg of protein, 10 mL of 0.075 M glucose 6-phosphate (0.858 g/40 mL), 5 mL of 5.7 nM β-nicotinamide adenine dinucleotide phosphate (84.4 mg/20 mL), 100 units of type IX glucose-6-phosphate dehydrogenase, and 20 mL of distilled water. The samples were aerobically incubated with mechanical shaking at approximately 37 °C for 1 h, after which time additional steer liver microsomes were added, equivalent to 120 mg of protein in 25 mL of 0.1 M potassium phosphate buffer (pH 7.4). For the experiment conducted with S9 rat liver homogenates, ^{14}C-labeled moxidectin (28 mg, 0.31 μCi/mg) and the surfactant (0.56 g) were dissolved in methanol (6.0 mL). To four 125-mL flasks were added 1.0 mL of the methanol solution (4.7 mg of moxidectin) and 45 mL of a cofactor mix [prepared from 125 mL of 0.2 M sodium phosphate buffer (pH 7.4), 0.78 g of β-nicotinamide adenine dinucleotide phosphate, 0.38 g of glucose 6-phosphate, 0.65 g of potassium chloride, and 0.42 g of magnesium chloride hexahydrate, diluted to 225 mL with water],

5 mL of the rat liver homogenate, and 10 mL of 0.15 M potassium chloride containing 0.1 g of β-adeninine dinucleotide, reduced form.

After incubation overnight at 37 °C, the samples from the respective experiments were extracted with diethyl ether. The ether extracts were concentrated to dryness. The residual radioactivity was taken up in methanol for radioassay and HPLC analysis in mobile phase systems I and II. The extracts were chromatographed on C_{18} reversed-phase TLC plates to separate moxidectin from the metabolites. The metabolites from the analytical-scale incubations were isolated as a mixture and analyzed by mass spectrometry. The metabolites from the preparative-scale incubations were separated by TLC on normal-phase plates, purified by HPLC, and analyzed by proton NMR.

TLC Procedures. For comparison of the radioactivity profiles by TLC, the extracts of the feces and tissues were chromatographed on 20 × 20 cm precoated silica gel 60 plates (EM Laboratories Inc., Elmsford, NY) using the solvent system of methylene chloride/ethyl acetate/formic acid (340:60:80 v/v/v) and on 20 × 20 cm LKC18F reversed-phase plates (Whatman Chemical Separation, Inc., Clifton, NJ) using the solvent system of acetonitrile/water (180:20 v/v). Solvent migration was 15 cm. An incremental multiple development TLC procedure was used to separate and isolate the metabolites using Whatman $PLKC_{18}F$ plates developed with acetonitrile/water (360:40 v/v) and Whatman PK6F silica gel plates developed with ethyl acetate/hexane (250:100 v/v). Radioactive zones on the TLC plates were located by means of autoradiography on XAR double-coated medical X-ray film (Eastman Kodak, Rochester, NY).

High-Performance Liquid Chromatography (HPLC). The moxidectin-derived residues in the urine, bile, and extracts of tissues, fat, injection site, and feces were routinely quantitated by HPLC on a 4.6 mm × 25 cm APEX C_{18} reversed-phase column followed by liquid scintillation of the collected fractions. Mobile phase system I, a gradient of 40:60 methanol/water to 90:10 methanol/water over a period of 50 min followed by 90:10 methanol/water for another 25 min, was used for these analyses. The flow rate was 1.0 mL/min, and the run time was 75 min. Fractions were collected at 0.5- or 1.0-min intervals in a fraction collector and analyzed for radioactivity following the addition of 5 mL of liquid scintillation cocktail to each vial. The column eluate was monitored at 242 nm and the UV profile recorded.

A 4.6 mm × 25 cm SUPELCOSIL C_8-DB reversed-phase column and mobile phase system II, a combined isocratic–gradient mobile phase of methanol/water, both containing 0.1 M ammonium acetate, were also used for the chromatographic comparison of the radioactivity profiles of the *in-vivo* and *in-vitro* metabolites. The HPLC run time was 75 min. For the first 20 min the mobile phase was 70:30:0.1 M ammonium acetate. This was followed by two gradients. The first gradient was 25 min to 75:25:0.1 M ammonium acetate, and the second was 15 min to 90:10:0.1 M ammonium acetate. For the final 15 min, the mobile phase was 90:10:0.1 M ammonium acetate.

The *in-vitro* metabolites were purified by HPLC on the C_8-DB reversed-phase column using mobile phase system III, 70:30 methanol/water for 20 min followed by a gradient over 25 min to 75:25 methanol/water. Fractions were collected manually or with the aid of the fraction collector. The isolates were concentrated to dryness in a SpeedVac SC 100 (Savant Instruments, Inc., Farmingdale, NY) and submitted for proton NMR analysis.

Isolation of Metabolites. The ether extract of the methanol/water extract of the steer feces (day 2) was concentrated to dryness. The residue was taken up in 7.5 mL of methanol and applied to three Whatman $PLKC_{18}F$ reversed-phase silica gel plates. The plates were developed in four stages using an incremental multiple development with acetonitrile/water (360:40 v/v) wherein the solvent was allowed to migrate only a prescribed distance from the spotting origin at each development stage. The solvent migration distances were 3.75, 7.5, 12.5, and 15 cm, respectively. Following autoradiography of the TLC plates, the silica gel scrapings of bands of similar R_f (9.0, 8.5, 8.0, and 5.5) from each plate were combined and extracted with acetone to recover the radioactivity.

The reversed-phase TLC isolate R_f 9.0 was further resolved into three components (R_f 4.2, 3.8, and 2.8) following chromatography on Whatman PK6F silica gel plates developed with ethyl acetate/hexane (250:100 v/v) using the incremental multiple development procedure. The isolate R_f 2.8 from this normal-phase TLC was the major component of this mixture. The reversed-phase TLC isolate R_f 8.0 was resolved into four components (R_f 5.5, 5.0, 4.5, and 2.8) after normal-phase TLC. The isolate of R_f 5.0 was the major component of the mixture. The two remaining reversed-phase TLC isolates (R_f 8.5 and 5.5) each appeared as one component, with R_f 2.8 and 7.2, respectively, after normal-phase TLC. The normal-phase TLC isolates were analyzed using HPLC mobile phase systems I and II for correlation of the isolated metabolites to the components in the radiometabolite mixture followed by mass spectrometry.

The radioactivity from the analytical-scale *in-vitro* experiments were resolved into two discrete bands of radioactivity (R_f 3.5 and 6.0) following chromatography on reversed-phase silica gel plates developed with a single pass of acetonitrile/water (180:20 v/v). The R_f 3.5 isolate was moxidectin. The R_f 6.0 isolate contained a mixture of metabolites and was analyzed by LC/mass spectrometry. The radioactivity from the preparative-scale *in-vitro* experiments was resolved into four discrete bands of radioactivity (R_f 9.0, 7.0, 6.0, and 3.5) following chromatography on reversed-phase silica gel plates. The three zones of radioactivity due to the metabolites (R_f 9.0, 7.0, and 6.0) were isolated. The two minor isolates (R_f 7.0 and 9.0) were examined by mass spectrometry without further purification. The major isolate (R_f 6.0) was further resolved into nine discrete radioactive bands following chromatography on normal-phase silica gel plates developed with ethyl acetate/hexane (250:100 v/v) using the incremental multiple development procedure. The three major bands of radioactivity from the normal-phase TLC, located at R_f 5.5, 5.0, and 4.8, respectively, were isolated for HPLC cleanup and NMR analysis. The minor bands, located at R_f 7.0, 6.5, 5.9, 4.0, 3.0, and 2.0, respectively, were isolated for mass spectral analysis.

Mass Spectrometry. Structural characterizations of the metabolites by thermospray liquid chromatography/mass spectrometry (LC/MS) and thermospray liquid chromatography/tandem mass spectrometry (LC/MS/MS) were performed on a Finnigan-MAT TSQ 70 triple-stage quadrupole system equipped with a Finnigan-MAT thermospray accessory. The detailed conditions for the LC/MS and LC/MS/MS are described elsewhere (Stout et al., 1994).

Proton NMR. NMR spectra were obtained on a Bruker AM-500 NMR spectrometer in deuteriated chloroform. Chemical shifts were referenced with respect to the chloroform resonance in the spectrum placed at 7.26 ppm. This implies that the shifts relative to TMS were ~0 ppm. The instrument tuning and shimming were optimized to yield good-quality spectra. For data acquisition a 30° flip pulse was used. A relaxation delay of 3 s was employed. Typical 1024 scans were coherently signal averaged using 32K complex data points both spin time and the frequency domains, respectively.

RESULTS AND DISCUSSION

Distribution and Excretion of Radioactive Residues. The major edible tissues/organs for the meat-consuming public are the liver, kidney, muscle, and fat. These are also the edible tissues/organs specifically monitored by the U.S. FDA on animal health drugs. Of the tissues listed in Table 1, following the subcutaneous dosing of steer with ^{14}C-labeled moxidectin equal to 0.2 mg/kg of body weight, the highest residue levels, expressed as ppb equivalents of moxidectin, were detected in the omental fat and back fat with depletion half-lives of 12 and 14 days, respectively. Lower residues were detected in the liver, kidney, and muscle. Muscle contained the lowest residue among the edible tissues. The steady decline in the magnitude of the ^{14}C residues at each sacrifice interval provides convincing evidence for the lack of accumulation of the residues in the edible tissues. The depletion half-lives for the residues in the other edible tissues were 9 days for muscle and 11 days for liver and kidney. The lowest residues were detected in the brain,

Table 1. Total ^{14}C Residues in Tissues, Blood, and Bile from Steers Dosed Subcutaneously with ^{14}C-Labeled and ^{2}H-Labeled Moxidectin, Expressed as Parts per Billion Equivalents of Moxidectin

	days posttreatment		
tissue	7	14	28
abdominal fat	898	636	275
back fat	495	424	186
kidney	42	38	13
liver	109	77	31
muscle	21	10	4
adrenals	88	120	29
bile	159	82	42
bladder	33	61	21
blood	10	7	3
brain	7	3	<2
carcass	115	67	42
esophagus	80	67	61
GI contents	22	17	2
heart	94	91	37
injection site	383	192	96
intestines (large)	139	167	65
intestines (small)	111	100	19
lungs	32	161	12
pancreas	83	69	32
stomach compartments	74	42	30
spleen	8	26	10
thyroid	403	90	127
thymus	197	83	48
tongue	120	90	61

indicating that the migration of the moxidectin-derived residues in blood to the brain was minimal. For the subcutaneously treated steers, the major route of excretion of the administered radioactive dose was via the feces, 32.2% after 7 days, 41.3% after 14 days, and 58.1% after 28 days. The excretion of radioactivity in feces peaked within 3 days (5–8% of the dose). Low levels of radioactivity (1–3% of the dose) were excreted daily thereafter. Only 3% of the radioactivity was recovered in the urine 28 days postdose. The overall recoveries of the administered radioactivity were 70.7–76.9% after all of the collected repositories in the steer were analyzed. The residual body parts (head, feet, and tail) and bone knuckles were not analyzed.

Extraction and Residue Profiles in Tissues. The radioactive residues in the edible tissues, fat, and injection site were essentially all extractable (89–99%), and very little remained in the postextracted solids after 28 days (11%, <3 ppb). Acetonitrile extracted between 67 and 95% of the total radioactive residues in the tissues. Some of the acetonitrile-soluble radioactivity was partitioned into hexane from cleanup of the extracts of fat (4–11%), tissues (3–6%), and injection site (5–8%). Additional radioactivity (8–16%) was extracted from the liver with methanol/water. Thus, there were very few, if any, intractable covalently bound residues in the edible tissues. For the abdominal fat control fortified with ^{14}C-labeled moxidectin, 99.7% of the radioactivity was extracted. The acetonitrile and hexane extracts of fat, the acetonitrile extracts of the tissues and injection site, and the methanol/water extract of the day 7 liver were analyzed by reversed-phase HPLC to quantitate the components of the residue and compare the radioactivity profiles of the tissues and feces.

As an aid to the discussion of the HPLC results, the individual radioactive peaks that were detected and quantitated in the tissue extracts, feces, urine, and bile were identified by letter (A–G) or number (1–12). All components in the bile, urine, and feces that were identical by retention time to the components in the tissue extracts were thus individually identified by letter (A–G), and all

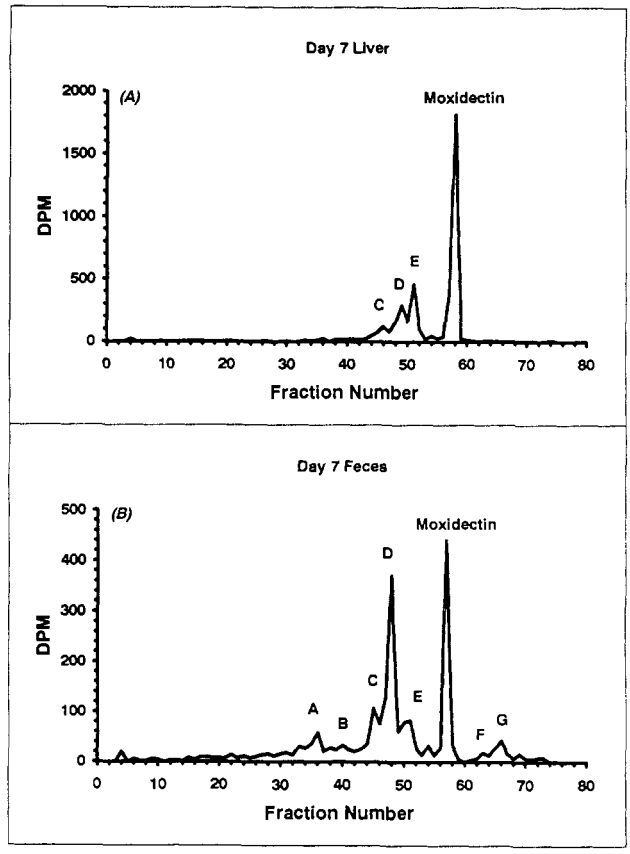


Figure 2. Radioactivity profile of (A) liver and (B) feces.

other radiocomponents in the feces, bile, and urine were identified by number. Thus, for the moxidectin-derived residues, the very polar metabolites (**1–4**) eluted between 0 and 15 min, the polar metabolites (**5–9**) eluted between 15 and 35 min, moxidectin, metabolites A–E, **10**, and **11** eluted between 35 and 60 min, and the nonpolar metabolites F, G, and **12** eluted between 60 and 75 min. Except for the nonpolar group, all of the metabolite groups were more polar than moxidectin, thus eluting before the parent drug. All of the radioactivity profiles for the hexane and acetonitrile extracts of day 7 fat and injection site and the acetonitrile extracts of liver, kidney, and muscle showed moxidectin as the most significant component of the total radioactive residue. Figure 2 shows a comparison of the radioactivity profile of the acetonitrile extract of liver and the methanol/water extract of feces. The radioactivity profiles of the extracts of the edible tissues and injection site for the day 14 and day 28 samples were comparable to the day 7 chromatograms. The radioactivity profile of the methanol/water of the liver was comparable to that of the acetonitrile extract.

Of the tissue metabolites, C–E were more predominant in the liver (acetonitrile and methanol/water extracts) and less predominant in the fat, injection site, kidney, and muscle. Moxidectin was stable by the procedure used to extract the tissues. Of the seven tissue metabolites, D and E were the major metabolites in liver (day 7). Trace amounts of metabolites A, B, F, and G were detected in the tissues at all sampling intervals. The residue levels of moxidectin and the metabolites in selected tissues at 7, 14, and 28 days after treatment, expressed as ppb equivalents and percent of the total residue, were determined. Of the total radioactive residue in liver after 7 days, moxidectin accounted for 47.7% (52 ppb), metabolite C accounted for 3.7% (4 ppb), metabolite D accounted for 9.2% (10 ppb), metabolite E accounted for 11.9% (13 ppb),

Table 2. Quantitation of ^{14}C Residues in Selected Cattle Tissues, Expressed as Parts per Billion Equivalents and Percent of Residue, 28 Days Posttreatment with Moxidectin at 0.2 mg/kg of Body Weight

component	approx retention time[a] (min)	liver[b] ppb	liver[b] %	kidney[b] ppb	kidney[b] %	muscle[b] ppb	muscle[b] %	abdominal fat[c] ppb	abdominal fat[c] %	back fat[c] ppb	back fat[c] %
total residue		31		13		4		275		186	
moxidectin	56–58	11	35.5	10	76.9	2	50	250	90.0	159	85.5
metabolite A[d]	36–38	<1	2.1	<1	<1	<1	<1	<1	<1	<1	<1
metabolite B	38–40	<1	1.1	<1	<1	<1	<1	<1	<1	<1	<1
metabolite C	44–46	<1	2.2	<1	<1	<1	<1	<1	<1	<1	<1
metabolite D[e]	47–49	2	6.5	<1	2.7	<1	3.1	<1	<1	<1	<1
metabolite E[e]	50–52	2	6.5	<1	4.5	<1	4.9	<1	<1	<1	<1
metabolite F[f]	62–64	<1	<1	<1	<1	<1	2.6	<1	<1	2	1.1
metabolite G[f]	65–67	<1	<1	<1	<1	<1	<1	<1	<1	2	1.1

[a] Retention times based on HPLC/^{14}C analysis using mobile phase system I. [b] Acetonitrile extract. [c] Acetonitrile and hexane extracts. [d] Mixture of at least two dihydroxylated metabolites. [e] Mixture of two monohydroxylated metabolites. [f] May be fatty acid esters of moxidectin or its metabolites.

and the remaining metabolites were less than 1%. After 14 days, moxidectin accounted for 40.3% (31 ppb), D accounted for 3.9% (7 ppb), and E accounted for 11.7% (9 ppb). The results for liver, fat, and the other tissues 28 days posttreatment (Table 2) showed that the residue levels of all metabolites were 2 ppb or less (<7%), and the only significant component of the residues in the tissues and fat was moxidectin, which accounted for 36–91% (4–275 ppb) of the residue. Thus, there was no accumulation of moxidectin-derived metabolites in the tissues, and the residue levels of moxidectin deplete with time.

The identification of moxidectin in omental fat and back fat was subsequently confirmed by LC/MS analysis of the extracts of fat. The key features in the mass spectrum of the moxidectin isolated from the fat were the ion doublets at m/z 640/641, 622/623, and 528/529, which were observed at the retention time of the moxidectin standard. These ions correspond to the $(M + H)^+$, the $(M + H - H_2O)^+$ and a fragment ion of the $^{14}C/^{2}H$-labeled moxidectin. The half-lives for the clearance of moxidectin in the target tissue (omental and back fat) were 12 and 15 days, respectively. The half-lives for moxidectin clearance were 12 days in kidney, 9 days in liver, and 8 days in muscle.

Extraction and Residue Profiles in Feces, Bile, and Urine. For the steer sacrificed 7 days posttreatment, 91–97% of the radioactivity in the feces from day 1 to day 7 was extracted vs 98.9% for the control feces fortified with ^{14}C-labeled moxidectin. The feces samples from the steers sacrificed 14 and 28 days posttreatment were not extracted. For the day 1 urine, 76.8% of the radioactivity was extracted vs 98.4% for the fortified control. For the bile, 36–53% of the radioactivity was extracted with diethyl ether and the remainder was water-soluble. The feces extract contained moxidectin and all of the metabolites found in the liver (Figure 2). Moxidectin accounted for 26% of the residue at day 2 and 22% at day 7. Metabolite D was the major metabolite, accounting for 25–34% of the extractable residue. The other fecal metabolites were minor and individually amounted to less than 10% of the residual radioactivity. Moxidectin was recovered unchanged from the fortified control feces sample.

The urine contained a minimum of seven metabolites of which three were major. These were A (48%), **2** (13.9%), and B (10.3%). Moxidectin accounted for less than 0.1% of the residue in day 1 urine. Moxidectin was stable in the fortified control urine and in the fortified control urine following extraction with ether. The bile contained moxidectin and the full complement of the tissue metabolites in addition to the very polar and polar metabolites. The major metabolite in bile was **6**, accounting for 19–23% of the residue. This metabolite was not detected in the urine or the feces. Moxidectin accounted for 13–15% of the ^{14}C residue in the bile collected 7, 14, and 28 days posttreatment. Moxidectin and metabolites A–E were extracted from the bile with diethyl ether; the remaining metabolites were water-soluble. After mild acid hydrolysis of the water-soluble bile metabolites and extraction with ether, 76% of the radioactivity was ether-soluble. HPLC analysis of the ether extract showed one major product corresponding to moxidectin, thus suggesting that the water-soluble bile metabolites are conjugates. These results show that moxidectin is absorbed from the injection site into the bloodstream, it is stored in the fat, and it is metabolized in the liver. Moxidectin and its metabolites are excreted in the bile and ultimately eliminated from the body via the feces.

Isolation and Identification of Metabolites. Because tissue residue levels in cattle treated with ^{14}C-labeled moxidectin were extremely low in all but the target tissue fat, where the predominant component of the residue was moxidectin, it was impractical to attempt isolation of the metabolites directly from tissue for structural identification. Since the low tissue residues prohibited use of the tissue extracts as a source for metabolite isolation and identification, the metabolites were isolated from the feces. The relevance of using the feces extracts lay in the fact that metabolite D was common to the metabolite in liver (Figure 2) and it amounted to 25–34% of the residue in the feces. The methanol/water extract of the residue in feces was extracted with diethyl ether to recover 75% of the residue; 25% remained in the aqueous phase. HPLC of these two fractions showed that moxidectin and all of the metabolites associated with the tissues were in the ether extract. The residual water-soluble radioactivity was also due to the polar metabolites and residual amounts of moxidectin and D which were not completely extracted. By reversed-phase TLC, all of the metabolites of moxidectin were shown to be more polar than moxidectin. The ^{14}C residue was separated into four discrete fractions (R_f 9.0, 8.5, 8.0, and 2.8) by preparative reversed-phase TLC using the incremental multiple development procedure. Two of the isolates (R_f 9.0 and 8.0) were further resolved by normal-phase TLC using the multiple development procedure into several discrete components, indicating there was a complex mixture of metabolites requiring characterization by mass spectrometry.

The structural characterization of moxidectin and the metabolites isolated from the feces was achieved by thermospray liquid chromatography/mass spectrometry (LC/MS) and thermospray liquid chromatography/tandem mass spectrometry (LC/MS/MS). The mass spectral data for moxidectin and the metabolites isolated from the feces are summarized in Table 3. The metabolites were identified as mono- and dihydroxylated derivatives of

Table 3. LC/MS and LC/MS/MS Data for Moxidectin and Metabolites Isolated from Cattle Feces Derived from ^{14}C- and ^{2}H-Labeled Moxidectin

metabolite	M + H	daughter ions A	B	C	structure
	640	528	218		moxidectin
		498			
		496			
A	672	544	264	392	dihydroxy metabolite CH_2OH at C-14 and OH on side chain
		514	232		
C	672	544	264		dihydroxy metabolite CH_2OH at C-14 or C-24 and OH on side chain
D	656	528	218		monohydroxy metabolite OH at C-29
		498			
		496			
	656	528	218		monohydroxy metabolite OH on side chain
		498			
		496			
	656				monohydroxy metabolite
E	656	544	264	376	monohydroxy metabolite
		514	234	344	CH_2OH at C-14
		512			

moxidectin. A detailed description relating to the interpretation of the mass spectral data is published elsewhere (Stout et al., 1994).

***In-Vitro* Metabolism.** In the analytical-scale experiment involving steer liver microsomes, a 7% yield of metabolites was obtained. The metabolites were isolated as a mixture, and the results for the LC/MS and LC/MS/MS analyses are summarized in Table 4, for comparison to the *in-vivo* metabolites (Table 3). The interpretation of the mass spectral data for the *in-vivo* metabolites is published elsewhere (Stout et al., 1994).

For the NMR analysis of the metabolites derived from the steer liver microsome *in-vitro* experiment, the only meaningful NMR data were obtained from the metabolite from the normal-phase TLC isolate R_f 5.0. Comparison of the 500-MHz spectrum of the metabolite to that of moxidectin shows the absence of the characteristic octet at 1.0 ppm, which is assigned to the methyl groups at C-29, C-30, C-12, and C-24. The NMR spectrum of the metabolite clearly showed a sextet which indicated that one of the methyl groups (C-29/30) from the isopropyl side chain was missing. It is inferred that substitution of a CH_2OH group for one methyl group on the isopropyl side chain has occurred. Homonuclear proton decoupling experiments were carried out to confirm this conclusion. The new resonance corresponding to the methylene group from the CH_2OH occurs around 3.5 ppm and overlaps the 17-position proton. Thus, the NMR data confirm the structural proposal from the mass spectrometry study (Table 3) that metabolite D, the major metabolite in feces and a major metabolite in liver, is hydroxylated at C-29/30.

For the metabolite derived from the rat liver homogenate *in-vitro* experiment (Zulalian et al., 1992), the NMR spectrum showed that the chemical shift for proton 15 was shifted downfield in comparison to the chemical shift for proton 15 in the NMR spectrum of moxidectin. The methyl groups at C-4, C-12, C-24, and C-26 and the two methyl groups at C-28 were intact in the spectrum of the metabolite. The absence of the methyl group at C-14 was not obvious as residual waterline resonates between 1.5 and 1.6 ppm. However, one can infer that it must be the site of modification for the corresponding hydroxymethyl metabolite. The new resonances corresponding to the hydroxymethyl group are suggested to occur between 3.6 and 3.8 ppm. Proton resonances corresponding to the 13-methylene and the 16-methylene show some differences compared to the moxidectin spectrum. Thus, the NMR data confirm the structural proposal from the mass spectrometry study (Table 3) that metabolite E, the major metabolite in liver and a minor metabolite in feces, is hydroxylated at the C-14 methyl group.

Table 4. LC/MS and LC/MS/MS Data for *In-Vitro* Steer Liver Metabolites Derived from ^{14}C-Labeled Moxidectin

LC/RT[a]	M + H	ions A	B	C	structure
4:14	672	544	264	392	dihydroxy metabolite CH_2OH at C-14, OH on side chain (metabolite A)
		514	232		
5:14	672	544	264		dihydroxy metabolite CH_2OH at C-14 or C-24, OH on side chain (metabolite C)
5:53	642	530	250	362	monohydroxy metabolite
		512		344	O-demethylated CH_2OH at C-14 or C-24
6:14	642				monohydroxy metabolite O-demethylated
9:56	672	528[b]	218	374[b]	dihydroxy metabolite both OH's on side chain
		498[b]			
		496[b]			
10:20	672				dihydroxy metabolite
12:20	656	544	264	376	monohydroxy metabolite
		514	234	344	CH_2OH at C-14
		512			(metabolite E)
	656				monohydroxy metabolite
13:32	656	528	218		monohydroxy metabolite OH at C-29 (metabolite D)
		498			
		496			
14:03	656				monohydroxy metabolite
15:21	656	528	218		monohydroxy metabolite OH on side chain
		498			
		512			
15:53	656	528	218		monohydroxy metabolite OH on side chain
		598			
		496			
24:49	640	528	218		moxidectin
		498			
		496			

[a] Retention time of peaks from Stout et al. (1994). [b] Daughter ions from M + H − H_2O.

CONCLUSION

Detectable ^{14}C residues were found in all tissues of steers following a single subcutaneous injection of a mixture of ^{14}C-labeled and ^{2}H-labeled moxidectin at a dosage rate of 0.2 mg/kg of body weight. There was a steady decline of the radioactive residues at each sacrifice interval, demonstrating that there was no accumulation of residues in the tissues. The major route for the elimination of the radioactivity was via the feces; only 3% of the dose was excreted in the urine by 28 days after treatment. Essentially all of the tissue residues were extractable. Moxidectin was the only significant component of the residue in the fat and the major edible tissues and accounted for 86–91% of the total residue in the target tissue (fat). No metabolite accounted for more than 10% or 2 ppb of the total radioactive residue 28 days after treatment. The results of the pharmacokinetic study with ^{14}C-labeled

moxidectin (Zulalian, unpublished data, 1991) showed that moxidectin is rapidly and completely absorbed from the injection site when administered by subcutaneous injection. Moxidectin is transported in the serum and is not associated with the cellular components of the blood. After the first 24 h postdose, moxidectin accounted for approximately 50% of the total ^{14}C residue in serum. Moxidectin and the related metabolites had a half-life of 3 days in serum. ^{14}C residue profiles of the extracts of the tissues, fat, and feces were qualitatively similar by C_{18} reversed phase HPLC. The total radioactive residues depleted with half-lives ranging from 9 (muscle) to 14 days (fat). Six metabolites were isolated from the steer feces and identified as monohydroxylated and dihydroxylated derivatives of moxidectin. The C-29/30 hydroxymethyl derivative was the major metabolite in feces and a major metabolite in liver. The C-14 hydroxymethyl metabolite was the major metabolite in liver and a minor metabolite in feces. The dihydroxylated metabolites were minor and individually accounted for less than 10% of the total residue in the feces. These were identical to the metabolites in the tissues. Moxidectin was also identified in the feces. The nonpolar metabolites (F, **12**, and G) were negligible. These may be fatty acid ester conjugates of moxidectin or the hydroxylated metabolites. The bile contained all of the metabolites detected in the tissues in addition to the polar metabolites. Some of these were partially characterized as conjugates of moxidectin.

The metabolism of moxidectin was also investigated *in vitro* with the aid of steer liver microsomes and rat liver homogenates. This resulted in the identification of at least 12 metabolites from the cattle metabolism study. The NMR spectra of the major metabolites derived from the *in-vitro* experiments led to the unequivocal identification of the C-29/30 hydroxymethyl metabolite and the C-14 hydroxymethyl metabolite found in the cattle metabolism study. Other metabolites isolated from the *in-vitro* experiments showed that they were identical to the minor monohydroxylated and dihydroxylated metabolites found in the steer feces. Also detected were O-demethylated metabolites and other mono- and dihydroxylated metabolites which were not detected in the cattle study. It is evident from these results that the principal route of the metabolism of moxidectin in cattle is hydroxylation as depicted in Figure 1. The primary sites for the hydroxylation of moxidectin were the C-14 methyl group and the methyl groups attached to C-28. However, there was some evidence for hydroxylation of the methine carbon at the allylic positions in the side chain. Of lesser importance was the hydroxylation of the C-24 methyl group. For the dihydroxylated metabolites, the hydroxylation was localized on the side chain and on the C-14 and C-24 methyl groups.

ACKNOWLEDGMENT

We thank Mr. J. Colavita, Mr. R. Search, Mr. P. D. Johnson, Mr. S. Biroc, and Ms. S. M. Paci for assistance in the treatment, handling, and collection of the biological samples. We are grateful to Mr. J. Cadet, Ms. L. Turi, and Mr. G. Szucsik for the preparation of the samples for radioanalysis and to Mr. A. Singh, Mrs. S. Singh, and Ms. F. J. Washington for technical assistance in radioanalysis and extraction of the tissues and feces. We are also grateful to Dr. S. Rajan for conducting and interpreting the proton NMR spectral analysis of the *in-vitro* metabolites.

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Received for review June 22, 1993. Accepted November 5, 1993.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, January 1, 1994.