Moxidectin: Absorption, Tissue Distribution, Excretion, and Biotransformation of ^{14}C-Labeled Moxidectin in Sheep

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The absorption, tissue distribution, excretion, and biotransformation of moxidectin, a novel endectocide that combats internal and external parasites in sheep, cattle, and horse, were studied in sheep. Following a single oral administration of a 1:1 mixture of ^{14}C- and deuterium-labeled moxidectin at 0.2 mg/kg of body weight, the highest residue concentrations among the edible tissues were observed in fat (249, 305, and 118 ppb; average of omental and back fat) at 1, 7, and 28 days postdose. The residue levels found in liver (135, 50, and 17 ppb), kidney (41, 22, and <4 ppb), and loin muscle were 25, 12, and <4 ppb, respectively. Fecal excretion was the major elimination pathway and accounted for 52%, while urinary excretion accounted for <1% of the administered dose. The extremely low residues in edible tissues precluded metabolite isolation in sufficient quantities for spectroscopic identification. Since the metabolite profile in feces is similar to that observed in liver, one major (monohydroxylated) and at least six minor (mono, dihydroxylated, O-demethylated) metabolites were characterized from feces by LC/MS and LC/MS/MS. Sheep liver in vitro metabolism studies permitted isolation of the major metabolite, whose identity was unequivocally established by LC/MS, LC/MS/MS, and NMR as the C-29/30 monohydroxymethyl derivative of moxidectin.

Keywords: *Moxidectin; sheep in vivo/in vitro metabolism; thermospray liquid chromatography/mass spectrometry*

INTRODUCTION

Moxidectin is a unique, new generation endectocide produced by a combination of fermentation and chemical synthesis. When the microorganism *Streptomyces cyaneogriseus* sp. noncyanogenus is grown under carefully controlled conditions, a compound called nemadectin is produced (Asato and France, 1990). After isolation and purification, nemadectin is chemically modified to moxidectin. Although these macrolides are closely related to other 16-membered lactones, namely avermectins (Albers-Schonberg et al., 1981) and milbemycins (Takiguchi et al., 1980), there are subtle structural differences. Nemadectin bears a hydroxyl group at the 23-position as well as a unique methylpentenyl side chain at C-25 and no C-13 disaccharide moiety. Moxidectin is the 23-(*O*-methyloxime) derivative of nemadectin. Moxidectin is being developed as a potent antiparasitic agent for the control and treatment of internal and external parasites in food-producing animals.

The exact mode of action or the anthelmintic activity of moxidectin has remained elusive. Identifying the mechanism is all the more challenging because moxidectin has been studied in various model systems in which the drug acts at multiple sites of target species possessing different sensitivities to the drug. In all probability, moxidectin, avermectin, and milbemycins share a common mode of action wherein they function either as a γ-aminobutyric acid (GABA) agonist or as a stimulator of GABA. GABA is also a neurotransmitter in mammals but is confined to the central nervous system. Since moxidectin essentially does not cross the blood–brain barrier, moxidectin is a highly effective parasiticide with a wide margin of safety for sheep and cattle.

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Moxidectin is marketed and formulated for species-specific use. While the active ingredient is the same in each formulation, livestock producers prefer different delivery systems for reasons of convenience, economy, and optimal effect. In cattle, moxidectin is applied either via injection or through a pour-on formulation, as an oral drench or injectable solution in sheep, as a tablet in dogs, and as a novel oral gel in horses.

The metabolism of moxidectin in cattle (Zulalian et al., 1994) and rat (Wu et al., 1993) has been reported recently. In this paper, we report the absorption, distribution, excretion, and biotransformation of ^{14}C-labeled moxidectin in sheep.

MATERIALS AND METHODS

Test Substance. The test substance used in this study was ^{14}C-labeled moxidectin (specific activity, 36.9 μCi/mg; radiopurity, >98%; and chemical purity, 99%). Moxidectin was prepared by chemically modifying its precursor nemadectin. Nemadectin was isolated from a fermentation using a mixture of ^{14}C-carboxyl-labeled acetate, propionate, and isobutyrate (Ahmed et al., 1993). This material was diluted in a 1:1 ratio with deuterium-labeled moxidectin (chemical purity, >99%) to yield a test substance with a specific activity of 18.7 μCi/mg. Moxidectin deuterium-labeled at the C-5 position (prepared from the corresponding 5-ketone followed by reduction of the ketone with sodium borodeuteride) was used in this study to serve as a mass marker in the structure elucidation of metabolites by mass spectrometry (Z. Ahmed and M. W. Bullock, American Cyanamid Co., private communication, 1991). The chemical structure and the metabolic pathway of moxidectin in sheep are shown in Figure 1.

Dose Formulation. Moxidectin was formulated as a liquid formulation at a nominal concentration of 0.2% (w/v). The formulated dose solution was stored in a refrigerator at approximately 4 °C prior to use. The stability of the active ingredient in the formulation was verified for the period from the time of dose preparation to the completion of dose

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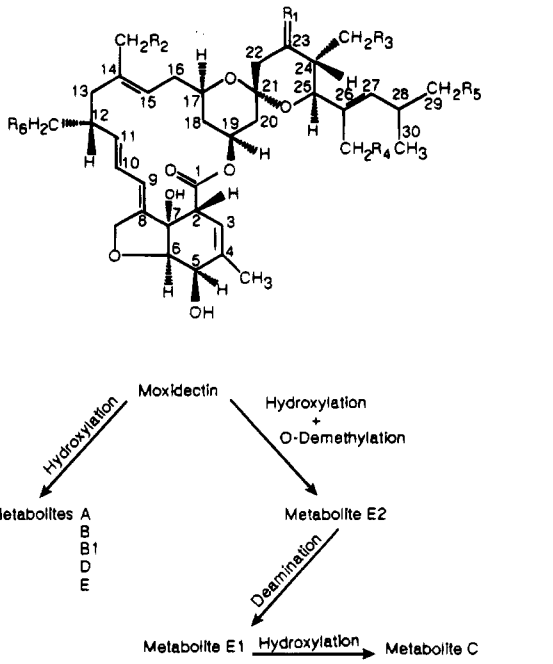

	R_1	R_2	R_3	R_4	R_5	R_6
Moxidectin	N-OCH_3	H	H	H	H	H
Metabolite E	N-OCH_3	OH	H	H	H	H
Metabolite A	N-OCH_3	OH	H	H	OH	H
Metabolite B	N-OCH_3	H	H	OH	H or OH	OH
Metabolite B1	N-OCH_3	H	H	OH	OH	H
Metabolite C	O	H	H	H	OH	H
Metabolite D	N-OCH_3	H	H	H	OH	H
Metabolite E1	O	H	H	H	H	H
Metabolite E2	N-OH	OH	H	H	H	H

Figure 1. Chemical structure and metabolic pathway of moxidectin in sheep.

administration by high-performance liquid chromatography (HPLC). Individual animals were orally dosed to provide moxidectin at 0.2 mg/kg of body weight based on the average of body weights taken before treatment. The control animal was dosed with a placebo (blank) formulation in a volume approximately equivalent to that given to the treated animals.

Animal Handling. Twelve sheep (growing wethers) weighing 30–45 kg at study initiation were obtained from Mr. Geyer, Jr., of Harleysville, PA; nine of the sheep were selected following a period of adaptation for any clinical signs of disease. The animals were placed in individual stainless steel metabolism cages, and individual feed consumption was recorded daily. American Cyanamid sheep feed pellets 76000P (13% crude protein), manufactured by Pennfield Corp., Lancaster, PA, were fed *ad libitum* as a complete feed. Water was supplied *ad libitum*. Each animal was identified by an individually numbered ear tag. One sheep was selected as a control. Of the remaining eight animals, three each were assigned to withdrawal times of 1 and 7 days postdose and two animals to a 28-day withdrawal time, at which time they were sacrificed. Blood, bile, muscle, liver, kidney, and omental and back fat were collected from all animals at all time points. To determine the mass balance from the three animals sacrificed at 7 days postdose, in addition to the edible tissues and fluids described above, the following biological samples were collected: tongue, thyroid, esophagus, pancreas, spleen, adrenals, bladder, diaphragm, abdominal fat, lungs, heart, gastrointestinal (G.I.) contents (feed, feces, and rinses from G.I. tract), G.I. tract (rumen, reticulum, omasum, abomasum, small and large intestine) without contents, skin (whole animal), and carcass. Urine and feces were collected separately from all animals on a daily basis until the time of sacrifice, and the output from each animal was recorded. Urine was collected by using a vinyl collection bag around the prepuce area of the sheep and sutured to the adjacent abdominal skin. The total amount of individual daily urine was recorded, the bag contents were stirred inside a collection bottle, and a 1-L sample was collected. The total amount of fecal material was mixed thoroughly with a wooden paddle and then placed in a plastic bag; the plastic bag was kneaded over the feces. The entire daily feces output was collected and recorded.

Sample Preparation and Radioanalysis. Frozen tissue/organ samples were ground with dry ice in a Hobart food chopper (Model 84181-D, Hobart Manufacturing Co., Troy, OH). To prevent contamination, control tissues were ground first, followed by the treated tissues in the order of expected increasing tissue residues (e.g., muscle, kidney, liver, back fat, and omental fat). The same approach was used for the preparation of feces, G.I. contents, carcass, organs/glands, and skin samples (day 7). The processed samples were placed in a freezer (−20 °C) in loosely closed containers to allow dissipation of carbon dioxide prior to analysis (1–2 days).

Total carbon-14 residues in whole blood, feces, and G.I. contents were determined in triplicate by combustion of 0.5-g samples in a sample oxidizer (Packard 306 sample oxidizer, Packard Instruments Co., Downers Grove, IL) and quantitation of liberated $^{14}CO_2$ by liquid scintillation counting (LSC). The radioactivity in bile was determined by measuring 0.25-mL aliquots (undiluted), in triplicate, directly into 20-mL scintillation vials. After sonication for about 1 h, the vials were cooled and then counted by LSC (Beckman LS 5801 or Beckman LS 9800, Beckman Instruments, Inc., Fullerton, CA). The ground fat was analyzed by weighing 0.5-g aliquots (undiluted), in triplicate, directly into 20-mL scintillation vials, sonicated for approximately 10 min, and counted in a liquid scintillation counter. Subsamples (5-g aliquots) of the ground tissue, organs/glands, and carcass were homogenized with 5 g of distilled water using a Williams Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY). A subsample of skin was prepared for combustion analysis by the random collection of 2 g each from three different areas of the skin, and the composite sample was homogenized with 6 g of water. A 0.5-g aliquot of homogenate, equivalent to 0.25 g of tissue, was combusted in triplicate using the same procedure as described earlier for feces. To validate the detection limit of the radioassay, recoveries of radioactivity from the sample oxidizer were measured by assay of control samples of each tissue with an added known amount of ^{14}C-labeled moxidectin. Recoveries in this study ranged from 90 to 102%. The efficiency of the sample oxidizer was routinely checked by combusting an aliquot of the SPEC-CHEC-^{14}C radiocarbon standard (Packard Instrument Co.). The resultant efficiencies averaged >95%. In the computation of the raw data, a reagent dpm value was used to determine the average dpm value for an untreated (control) sample. For the treated samples, the average dpm value of the control sample was subtracted from the dpm values of the treated samples.

Extraction of Radioactive Moxidectin-Related Residues from Liver, Kidney, Muscle, and Fat Tissue. Processed (ground) tissue samples (20–100 g) were homogenized with high purity grade acetonitrile (Baxter Health Care Corp., Muskegon, MI) using a Brinkmann Polytron homogenizer equipped with a Model PT20A "saw-tooth" generator. The tissue to acetonitrile ratio used for extraction was 1:10 (w/v) for the first homogenization. The supernatant was filtered through a 7.0-cm Whatman qualitative filter paper (Whatman International Ltd., Maidstone, England). The homogenization was repeated two additional times using a tissue to acetonitrile ratio of 1:5 (w/v). To remove undesirable fats and lipids, acetonitrile extracts were combined and extracted twice with hexane in a separatory funnel using a hexane to acetonitrile ratio of 1:4 (v/v). The postextracted solid (PES) was allowed to dry overnight, weighed, and combusted in triplicate aliquots to determine the residual activity. The hexane and acetonitrile

extracts were concentrated separately on a rotary evaporator at reduced pressure. The residue from each fraction was reconstituted with the respective solvents and filtered into two separate 25-mL volumetric flasks to remove any suspended particles. Reconstitution was aided by vortex mixing several times to allow complete dissolution of moxidectin-derived residue. The final volumes of both the hexane and acetonitrile extracts were brought to 25 mL, and the radioactivity was quantitated by counting triplicate aliquots (0.25 mL) in a liquid scintillation counter.

This procedure was used to extract liver, muscle, and kidney tissue samples from day 1 and day 7 postdose and liver sample from day 28. Kidney and muscle samples from day 28 postdose were not extracted because the residue levels found in both tissues were below the detection limit of 4 ppb. For the extraction of all omental and back fat samples, a 1:1 mixture of hexane and acetonitrile was substituted for 100% acetonitrile to help solubilize fat and avoid emulsion problems.

PES from liver was re-extracted twice using methanol/water (95:5), filtered, and concentrated to dryness. The residue was reconstituted in methanol and triplicate aliquots counted by LSC as described above. The PES was dried and weighed, and triplicate aliquots were combusted to determine the percentage of unextractable residue.

Moxidectin-Related Residue Analysis. To quantitate the level of moxidectin-related residue in the liver, kidney, muscle, and fat tissues from this study, an HPLC method was developed.

HPLC was carried out on a Waters Maxima 820 Chromatography Workstation consisting of a Waters 600 E System Controller and a Waters 490 E programmable multiwavelength detector (Waters Millipore Corp., Morristown, NJ). A linear gradient at 1.0 mL/min of methanol:water (40:60) from time 0 to methanol/water (90:10) over 50 min was used and then isocratically maintained for an additional 25 min. A Supelco reversed-phase C_{18} column (5 μm, 4.6 × 250 mm) in conjunction with a SUPELCOGUARD LC_{18} guard column (2 cm, Supelco, Inc., Bellefonte, PA) was used for gradient analysis. Detection of moxidectin-related residues was accomplished at a wavelength of 240 nm.

The radioactivity eluting from the column was fractionated and collected (1.0 mL/fraction) using a FRAC-100 fraction collector (Pharmacia Fine Chemicals, Piscataway, NJ). The radioactivity in each fraction was quantitated by LSC after addition of 5 mL of Beckman READY-SOLV CP scintillation fluid to each fraction.

Isolation and Purification of In Vivo Metabolites. For the isolation of metabolites from feces, fecal samples (109–200 g) from day 2 through day 7 were individually extracted with a Brinkmann Polytron homogenizer using methanol/water (95:5). The ratio of feces to solvent used was 1:10 (w/v). Each fecal extract was filtered on a 7.0-cm Whatman qualitative filter paper. The PES from each extract was homogenized twice using methanol/water (95:5). The methanol/water fecal extracts from day 2 through day 7 were concentrated separately at reduced pressure on a rotary evaporator. The residue from each extract was reconstituted in methanol and transferred by filtration into a 50-mL volumetric flask or a measuring cylinder. Triplicate aliquots of 0.1 mL were then counted by LSC using 10 mL of scintillation fluid. PES from each extract from day 2 through day 7 were dried, weighed, and combusted in triplicate. The reconstituted fecal extracts from day 4 through day 7 were composited and concentrated to dryness at reduced pressure on a rotary evaporator. A schematic of the steps involved in the isolation and purification of fecal metabolites is illustrated in Figure 2.

Isolation and Purification of In Vitro Metabolites. All reagents were purchased from Sigma Chemical Co. A sheep liver microsomal pellet was prepared following the procedure reported by Miwa et al. (1982). Fresh sheep liver (110 gm) was cut into small pieces and homogenized with a Brinkmann Polytron homogenizer using 330 mL of Tris buffer (0.05 M; pH 2.5) containing 1.15% KCl. The homogenized tissue sample was centrifuged using a DuPont RC-5 Superspeed refrigerated centrifuge (DuPont Co., Wilmington, DE) for 30 min at 10000*g*. The supernatant fraction was recentrifuged (Beckman L8-M

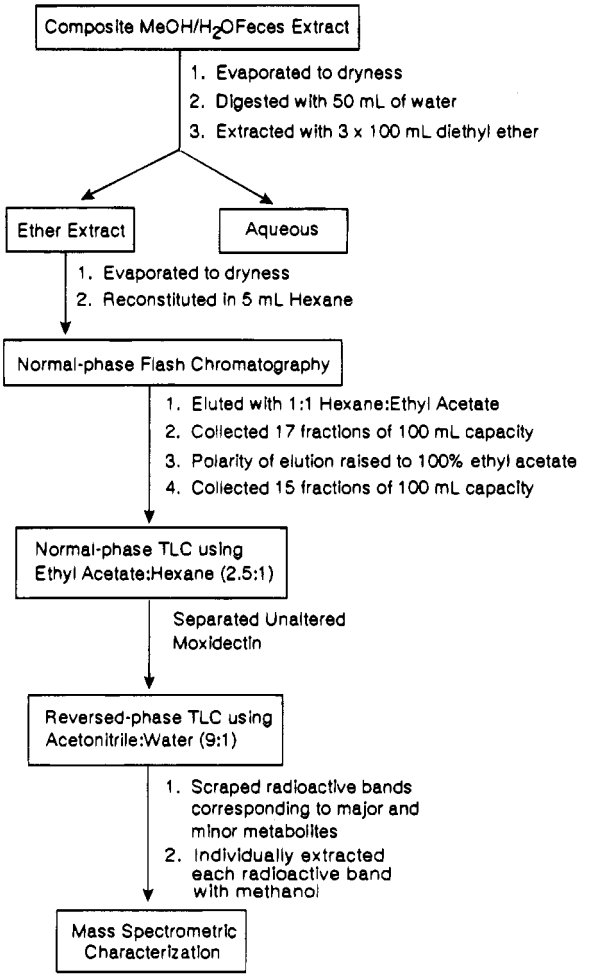

Figure 2. Schematic of the steps involved in the isolation and purification of fecal metabolites.

Ultra centrifuge) for 60 min at 105000*g*. The generated microsomal pellet was washed by resuspension in 1.15% KCl containing 10 mM EDTA. Centrifugation for another 60 min at 105000*g* yielded a washed microsomal pellet. The resultant pellet was resuspended in 0.25 M sucrose solution and stored overnight at −78 °C at a protein concentration of approximately 60 mg/mL. Into a 200-mL Erlenmeyer flask was transferred 760 μL of an acetonitrile solution of substrate (specific activity of 33.36 μCi/mg; equivalent to 1.0 mg of ^{14}C-labeled moxidectin), 4.93 mg of nonradiolabeled moxidectin analytical standard, and 100 mg of PLURONIC-Polyol F-68. The solution was evaporated to dryness by a gentle stream of nitrogen and the substrate reconstituted in 0.5 mL of methanol. The estimated specific activity of the tracer was 12 489 dpm/μg. To the 0.5-mL solution of substrate was added the sheep liver microsomal pellet (equivalent to 240 mg) in 10 mL of 1 M potassium phosphate buffer at pH 7.4, 20 mL of 0.05 M glucose 6-phosphate, 10 mL of 10 mM NADP, and 1 mL of glucose-6-phosphate dehydrogenase (100 units). The final volume was adjusted to 90 mL with water and aerobically incubated at 37 °C for 30 min using a mechanical shaker. Thereafter, additional protein, equivalent to 240 mg in 40 mL of 0.1 M potassium phosphate buffer at pH 7.4, was added and the sample incubated for an additional 1.5 h. The incubation mixture was then extracted with 3 × 150 mL of diethyl ether. The ether-extractable fractions were combined, washed with 2 × 50 mL of saturated NaCl solution, dried over sodium sulfate, and concentrated at reduced pressure on a rotary evaporator. The residue was then reconstituted in methanol in a 25-mL volumetric flask. Triplicate 50-μL aliquots of ether-extractable fraction and 1-mL aliquots of the aqueous layer were counted by LSC. The organic fraction was purified further by reversed-phase and normal-phase preparative TLC plates as described earlier for in vivo metabolites. The minor

Table 1. Total Radioactive Residue Levels (Parts per Billion)[a] in Tissues and Fluids from Sheep Dosed Orally with Moxidectin at 0.2 mg/kg of Body Weight

	days postdose		
	1	7	28
loin muscle	25	12	<4
kidney	41	22	<4
liver	135	50	17
back fat	220	287	113
omental fat	277	322	123
blood	5	<2	<2
bile	177	47	16
carcass	ND[b]	94	ND
skin	ND	170	ND
organs/glands	ND	135	ND
G.I. contents	ND	8	ND

[a] Average values for 3, 3, and 2 animals sacrificed at 1, 7, and 28 days, respectively. [b] ND, not determined.

in vitro metabolites were amenable to mass spectrometric characterization without any further purification. The radioactive fractions corresponding to the major metabolite were concentrated to dryness using a SPEEDVAC SC100 (Savant Instruments, Inc., Farmingdale, NY) and purified further by HPLC following the gradient method described earlier. The fractions corresponding to the major metabolite were pooled and rotary evaporated to dryness at reduced pressure repetitively until a symmetrical peak was obtained. This purified major metabolite was characterized by LC/MS, LC/MS/MS, and NMR spectroscopy.

Mass Spectrometric Analysis (LC/MS and LC/MS/MS) of Moxidectin and Metabolites. 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 thermospray TSQ triple-stage quadrupole system equipped with a Finnigan-MAT thermospray LC/MS accessory as previously reported (Stout et al., 1994).

NMR Analysis. Proton NMR (500 MHz) spectra were acquired in $CDCl_3$ using a Bruker AM 500 superconducting spectrometer (Bruker Instruments, Inc., Manning Park, Billerica, MA) equipped with a Fourier-transform accessory and a 5-mm probe head. The instrument tuning and shimming were optimized to yield good quality spectra. For data acquisition, a 30° flip pulse was used and a relaxation delay of 3 s was employed. An appropriate number of scans (1024–4096) were coherently signal averaged using 32K complex data points in both the time and frequency domains, respectively.

RESULTS AND DISCUSSION

Radioactive Tissue Residue Distribution and Excretion. Table 1 summarizes the typical distribution of total radioactivity expressed as nanograms equivalents per gram (ppb) in selected tissues and fluids. These results indicated that fat was the target tissue. The residue levels found in omental and back fat were comparable, indicating a uniform dispersion of moxidectin throughout the sheep body fat. The residue depletion half-lives determined earlier in this laboratory were 13.5 days for omental fat and 15.0 days for back fat (S-S. Wu, American Cyanamid, private communication, 1990). The validated detection limit of the radioassay was 4 ppb for liver, kidney, and muscle and 2 ppb for blood and omental and back fat. A mass balance was determined for three animals sacrificed at 7 days postdose. The total recovery of radioactivity for sheep sacrificed at day 7 from feces, urine, tissues/organs, skin, carcass, G.I. contents, blood, and bile averaged 93.2% (Table 2). Fecal excretion was the major elimination pathway, accounting for 52% of the

Table 2. Mass Balance of Total ^{14}C-Labeled Moxidectin Administered for Sheep Sacrificed 7 Days after Treatment

	% dose recovered		
sample	sheep 1	sheep 2	sheep 3
feces	47.3	29.9	52.4
urine	0.5	0.5	0.6
liver	0.6	0.5	0.4
kidney	<0.1	<0.1	<0.1
loin muscle[a]	<0.1	<0.1	<0.1
omental fat[a]	3.4	6.2	2.4
back fat[a]	0.9	1.5	0.6
bile	<0.1	<0.1	<0.1
blood	NM[b]	NM	NM
carcass	25.1	32.0	18.7
skin	9.1	13.5	6.7
organs/glands	5.8	11.0	8.1
G.I. contents	0.6	0.2	0.6
total recovery	93.5	95.5	90.6
av recovery	93.2 ± 2.5		

[a] The percentage of dose recovered was calculated on the basis of total sample collected. [b] NM, not measurable; radioactivity found was below the validated sensitivity limit.

administered radioactivity. Urinary excretion accounted for <1% of the administered dose in all three animals. The carcass, skin, and G.I. contents accounted for 19–32, 7–14, and 0.2–0.6% of the administered dose, respectively. The residual body parts (head and feet) were not analyzed.

Extractability of Carbon-14 Residues from Tissues. The distributions of total carbon-14 residues in acetonitrile, hexane, methanol/water, and postextracted solid are expressed both as percent of the total dpm recovered and parts per billion (ppb).

The extraction efficiencies of carbon-14 residues from both abdominal and back fat tissues (1, 7, and 28 days postdose) were in the range 97–99% with negligible radioactivity in the PES. The extraction recoveries from liver (in acetonitrile and methanol/water fractions) for days 1, 7, and 28 ranged from 88 to 92%. Similarly, the extractability of the carbon-14 residue from kidney (hexane and acetonitrile fractions) ranged from 77 to 80%. The radioactivity in the PES ranged from 20 to 23% (5 and 13 ppb), respectively, at days 1 and 7 postdose. Since the detection limit of the radioassay was 4 ppb, no efforts were made to release the radioactivity from the postextracted solid. The initial residue levels found in loin muscle at 1 and 7 days postdose were 25 and 12 ppb, respectively. Extraction of the day 1 muscle sample with acetonitrile gave a recovery of 68%. However, extraction with methanol/water for the day 7 sample gave 100% recovery, indicating that the residue was extractable with a more polar solvent.

Residue Profile of Carbon-14 Moxidectin Equivalents in Tissues. A comparison of the carbon-14 residue profiles in extracts of liver, omental fat, and feces (day 7) is shown in the HPLC chromatograms in Figure 3. The residue profiles in extracts of back fat (days 1, 7, and 28), omental fat (days 1 and 28), liver (days 1 and 28), and kidney and muscle (days 1 and 7) were similar. Extraction of day 28 kidney and muscle samples was not pursued because the residue levels found were below the detection limit of 4 ppb.

Quantitation and comparison of the components of the residue in each tissue was accomplished by HPLC. A comparison of the residue levels of parent and its metabolites in edible tissues at day 7 are summarized in Table 3. The HPLC chromatograms clearly indicated

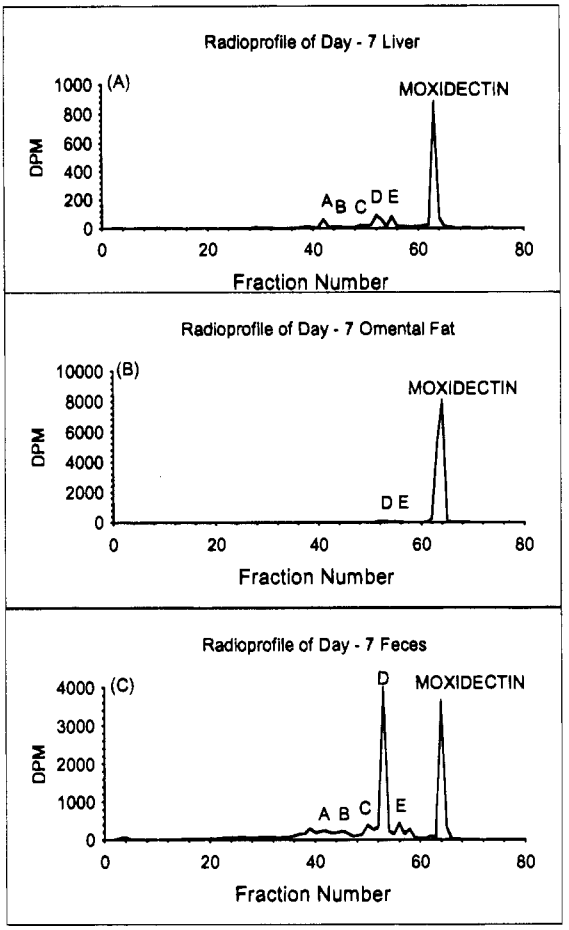

Figure 3. Comparison of the carbon-14 residue profiles (day 7) in (A) liver, (B) omental fat, and (C) feces.

that the major component of the carbon-14 residue in the tissues corresponded to the unaltered parent compound, accounting for up to 88–93% of the residue in the target tissue (fat). To compare the metabolite profiles in tissues and feces, metabolites common to tissues and feces were designated by letters (A–E). Further purification of these components resulted in separation of other minor components which were identified as 1 and 2 following the letter designation (e.g., E1, E2, etc.). A minimum of six metabolites of moxidectin were detected in the tissues, of which only two (metabolites D and E) individually accounted for more than 10% of the TRR at any time point in any tissue. Metabolite D, the more abundant of the two, accounted for 15 ppb (8% of total residue) in liver at 1 day postdose, declining to 2 ppb (12% of total residue) by 28 days postdose. No other metabolite accounted for more than 10% of the TRR at any of the sacrifice intervals.

Characterization of In Vivo Metabolites by LC/MS and LC/MS/MS. *Metabolites D and E.* The radioactive band isolated from reversed-phase TLC, when analyzed by LC/MS, indicated one major component (metabolite D) and a minor component (metabolite E).

The major component (metabolite D) was identified by LC/MS and LC/MS/MS of the daughter ion *m/z* 656 as a monohydroxylated derivative of parent (16 amu higher). The structurally significant fragment ion doublets at *m/z* 528, 529 and *m/z* 498, 499 indicated that the hydroxyl group is lost with the side chain. The minor component (metabolite E) was identified by LC/MS as another monohydroxylated derivative giving characteristic fragment ion doublets at *m/z* 544, 545, indicating that the site of hydroxylation was on a methyl of the macrocyclic ring.

The major and minor sheep fecal metabolites were proposed to be hydroxylated on the isopropyl methyl group of the side chain and C-14 methyl of the macrocyclic ring, respectively, on the basis of observed LC polarity and relative losses of H_2O from the $(M + H)^+$ ion of the side chain.

Metabolite E1. The minor sheep feces metabolite (metabolite E1) was identified by LC/MS as the 23-keto derivative of moxidectin. The identity of the 23-keto compound was confirmed by comparison with a synthetic reference standard whose LC/UV retention time and LC/MS were identical to those of metabolite E1.

Ion doublets (one due to the deuterium label in moxidectin) at *m/z* 611, 612 and *m/z* 628, 629 correspond to $(M + H)^+$ and $(M + NH_4)^+$ ions, respectively. Successive losses of water gave ion doublets at *m/z* 593, 594 and *m/z* 575, 576. The characteristic fragment ion doublet at *m/z* 481, 482 was due to the loss of the side chain (112 amu) from *m/z* 593, 594, respectively.

Metabolites C and B1. The radioactive band isolated from reversed-phase TLC when analyzed by LC/MS consisted of a mixture of two components. The less polar component was the major metabolite (metabolite C) and generated ion doublets at *m/z* 644, 645 corresponding to the $(M + NH_4)^+$ ion of the hydroxylated 23-keto compound. This assignment was rationalized on the basis of a much larger $(M + NH_4)^+$ ion than the $(M + H)^+$ ion for the 23-keto compound and the fact that the 23-keto compound has already been determined as a sheep feces metabolite.

By MS/MS, the daughter ion spectrum of *m/z* 644, 645 corresponds very closely with that of the $(M + NH_4)^+$ ion of 23-keto compound, allowing for +16 amu shifts in ions at *m/z* 593 and 575. The key fragment ion at

Table 3. Comparison of the Carbon-14 Residue Profile in the Tissues of Sheep, 7 Days Postdose, Expressed as Parts per Billion Equivalents and Percentage of the TRR

component[a]	retention time,[a] min	liver,[b] ppb (%)	kidney,[c] ppb (%)	loin muscle,[d] ppb (%)	abdominal fat,[c] ppb (%)	back fat,[c] ppb (%)
moxidectin	63–64	26 (51)	13 (52)	12 (92)	365 (95)	342 (91)
metabolite A	38–40	<1 (<2)	<1 (<4)	<1	<1 (<1)	<1 (<1)
metabolite B	41–42	3 (6)	<1 (<4)	<1	<1 (<1)	<1 (<1)
metabolite B1	46–47	<1 (2)	<1 (<4)	<1	<1 (<1)	<1 (<1)
metabolite C	50–51	2 (4)	<1 (<4)	<1	<1 (<1)	<1 (<1)
metabolite D	52–53	6 (12)	3 (12)	<1	5 (1)	7 (2)
metabolite E	55–56	3 (6)	1 (4)	<1	4 (1)	5 (1)
metabolite E1	58–59	<1 (<2)	<1 (<4)	<1	<1 (<1)	3 (<1)

[a] Based on HPLC analysis on a Supelco C_{18} reversed-phase column. Total radioactive residue (TRR) levels in liver, kidney, loin muscle, and abdominal and back fat were 51, 25, 13, 385, and 374 ppb, respectively. [b] Acetonitrile and methanol/water fractions. [c] Acetonitrile fraction. [d] Methanol/water fraction.

m/z 481 is present in the daughter ion spectra of both compounds (metabolites C and E1). To obtain this same fragment ion from the metabolite, the hydroxyl group must be lost with the side chain. Consequently, metabolite C is a side-chain hydroxylated 23-keto compound.

The more polar minor metabolite (metabolite B1) generated a base peak in its mass spectrum at m/z 654, 655, which corresponds to the $(M + H - H_2O)^+$ ion of a dihydroxy metabolite, and an ion with m/z 689, 690 corresponding to $(M + NH_4)^+$. The key ion of structural significance in the MS/MS of the daughter ion (m/z 654, 655) is at m/z 374. This ion is 18 amu lower than the m/z 392 ion generated for the dihydroxy metabolite and can only arise if H_2O is lost from m/z 392 ion. The m/z 392 ion localizes both hydroxyls to the region of the molecule between C-13 and C-29. This fragment ion includes C-14, C-24, and the side chain. Ions of weak intensity at m/z 528, 529 and the cluster at m/z 496 and 498 are from moxidectin and indicate that the macrocyclic regions of the molecule are unaltered. These data indicate that both hydroxyl groups are on the side chain.

Metabolites A and B. The radioactive band isolated from reversed-phase TLC when analyzed by LC/MS showed two peaks, the first of which was the major (metabolite A) while the other was the minor component (Metabolite B). Both peaks generated ion doublets at m/z 689, 690 $(M + NH_4)^+$, m/z 672, 673 $(M + H)^+$, and m/z 654, 655 $(M + H - H_2O)^+$, indicative of dihydroxylated metabolites. Only the first peak had sufficient response for subsequent characterization by MS/MS. The daughter ion spectrum of the $(M + H)^+$ ion is very similar to that of the monohydroxy metabolite (metabolite E) and can only occur if the hydroxyl group is at the C-14 methyl and the second hydroxyl group is on the side chain. In particular, ions at m/z 264, 232 indicate one hydroxyl group at the side chain. Ions at m/z 544, 545, the side-chain loss, are unaffected by the additional hydroxyl group and, therefore, indicate that the second one is on the side chain.

Considering the close similarity of the daughter ion spectrum to that of metabolite E, it is proposed that the one hydroxyl group is at the C-14 methyl while the other is in the side chain. The structure of metabolite B is proposed to have one hydroxyl group on a methyl of the macrocyclic ring and the other in the side chain.

Characterization of In Vitro Metabolites by Mass Spectrometry. LC/MS and/or LC/MS/MS of the sheep liver microsomal preparation revealed the presence of seven metabolites of moxidectin. The various components are discussed below in order of increasing LC retention time. Unlike feces metabolites, no ion doublets were detected in the in vitro microsomal metabolites because of the absence of the deuterium-labeled mass marker. Peak 1 eluting at 4 min 12 s gave a protonated molecular ion at m/z 672. LC retention time, MS data, and MS/MS data corresponded to the dihydroxy metabolite isolated from sheep feces (metabolite A). Peak 2 had a retention time of 5 min 53 s and gave a protonated molecular ion at m/z 642. LC retention time and MS/MS data corresponded to the polar metabolite characterized as the O-demethylated oximino oxygen derivative with the site of hydroxylation at the C-14 methyl group (metabolite E2). The key fragmentations were at m/z 530 (loss of unaltered side chain) and m/z 250 (14 amu lower than m/z 264, corresponding to monohydroxymethyl at C-14), showing demethylation at the oximino oxygen atom. This metabolite (metabolite E2) was not observed in sheep feces. Peak 3 (12 min 19 s) gave the same LC retention time, MS data, and MS/MS data as metabolite E. Peak 4 (13 min 31 s) matched the LC retention time, MS, and MS/MS data of the major sheep feces metabolite (metabolite D). Peaks 5 (15 min 20 s) and 6 (15 min 52 s) had retention times, MS data, and MS/MS data corresponding to the side-chain monohydroxylated metabolites of peaks 3 and 4 from the steer liver microsomal incubation (Stout et al., 1994). Peak 7 protonated molecular ion at m/z 656 (17 min 21 s) corresponded to the $(M + H)^+$ ion of a very minor mono-oxygenated metabolite not previously observed. Because of the weak intensity of the spectrum, no additional structural data were obtainable.

Proton NMR Analysis of the Major Metabolite. The proton NMR spectrum of the major metabolite (metabolite D) indicated that biotransformation of moxidectin resulted in the monohydroxylation of the C-29/C-30 methyl group. A comparison of the proton NMR spectrum of the major metabolite versus the NMR spectrum of moxidectin (Rajan et al., 1989) indicated two significant differences: (a) disappearance of the characteristic C-29 and C-30 methyl doublet centered at 0.95 and 1.05 ppm and (b) appearance of a new resonance signal centered at 3.45 ppm. Since the LC/MS and LC/MS/MS data of the major metabolite indicated that the site of hydroxylation was on the side chain of the moxidectin molecule and the proton NMR spectrum revealed the disappearance of the C-29/C-30 methyl group, one can conclude the site of hydroxylation to be at the C-29/C-30 methyl group inasmuch as the appearance of the new signal at 3.45 ppm coincided with the downfield chemical shift characteristic for CH_2 attached to an oxygen atom.

Conclusion. The absorption, tissue residue, excretion, and biotransformation were studied in sheep (a target food-producing species) following oral administration of moxidectin at 0.2 mg/kg of body weight. Fecal excretion was the major pathway of drug elimination, accounting for up to 52% of the administered dose. Urinary excretion was <1% of the dose. The highest levels of carbon-14 residues in tissue were observed in fat, identifying it as the target tissue. The depletion of residues in liver, kidney, muscle, and fat indicates that there is no evidence for bioaccumulation of residues in the edible tissues.

The extraction efficiencies of ^{14}C-derived residues in edible tissues ranged from 77 to 99% and in feces from 86 to 97%. Moxidectin was the major component of the total radioactive residue in both tissues and feces. In fat, the target tissue, moxidectin accounted for 87.9, 93.1, and 92.2% (average value of abdominal and back fat) of the total radioactive residue at 1, 7, and 28 days postdose, respectively. One major and at least six minor fecal metabolites were identified by LC/MS and/or LC/MS/MS. The major feces metabolite (metabolite D) corresponded to a monohydroxylated derivative of parent. The site of hydroxylation at the C-29/C-30 position in the major metabolite was unequivocally confirmed by proton NMR spectroscopy. In vivo metabolism of moxidectin was found to correlate closely with in vitro liver microsomes and demonstrated that hydroxylation is the principal route for the biotransformation of moxidectin in sheep.

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