Depletion of Moxidectin Tissue Residues in Sheep

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The pattern of tissue depletion of moxidectin (MXD) subcutaneously administered to sheep was characterized in this study. MXD concentration profiles were determined in muscle, fat, and liver and at the site of injection following administration of a formulation combining MXD (0.5% w/v) with a standard 6 in 1 clostridial vaccine. Thirty-five (35) parasite-free Lincoln sheep were treated with the MXD injectable formulation at a dose rate of 0.2 mg of MXD/kg of live weight, administered subcutaneously on the inner surface of the thigh. Treated animals were sacrificed in randomly selected groups of six sheep weekly from day 21 until day 49 post-treatment. Three nontreated animals were sacrificed to obtain blank tissue samples to validate the analytical methodology. MXD concentration profiles were determined by a validated HPLC analytical method using fluorescence detection. MXD has an adequate pattern of absorption, based on the low residual concentrations found in the injection site area at all sampling intervals. Muscle samples showed the lowest MXD concentrations throughout the study period. The highest MXD concentrations at all sampling times were measured in the adipose tissue, indicating that fat is a target tissue for MXD. MXD concentrations in all of the tissues analyzed were below the accepted maximum limit of residue at 21 days post-treatment.

Keywords: *Moxidectin; tissue residues; sheep*

INTRODUCTION

Moxidectin (MXD) is a milbemycin endectocide compound produced by a combination of fermentation and chemical synthesis. MXD is obtained by chemical modification of nemadectin, the natural compound produced when *Streptomyces cyaneogriseus* is grown under controlled culture conditions (Takiguchi et al., 1980). MXD is active at extremely low dosages against a wide variety of nematode and arthropod parasites and is currently marketed as injectable and pour-on formulations for use in beef cattle and as oral and injectable formulations for sheep (McKellar and Benchaoui, 1996). Milbemycins and avermectins share a common mode of action, based on a high-affinity binding to glutamate-gated chloride channels, producing a slow and irreversible increase in membrane conductance (Arena et al., 1995; Shoop et al., 1995).

The increasing use of preventive medicine practices against the most common infectious and parasitic diseases affecting sheep flock has encouraged the need of developing more practical delivering systems to reduce the handling inconveniences, which derive from the use of both antiparasitic treatment and vaccines at different moments of the flocks' production cycle. Combining MXD with a 6 in 1 clostridial vaccine requires the evaluation of the pharmacokinetic behavior of the drug to prove if the presence of the biological fraction interferes with absorption of MXD from the site of injection. The goal of this study was to characterize the depletion of tissue residues of MXD after its subcutaneous (SC) administration to sheep.

MATERIALS AND METHODS

Dose Formulation. The formulation used in this study is a commercially available product containing a combination of MXD 0.5% w/v with a 6 in 1 clostridial vaccine, containing toxoids of *Clostridium septicum*, *Clostridium novyi*, *Clostridium tetani*, and *Clostridium perfringens* and anacultures of *Clostridium chauvoei* and *Corynebacterium pseudotuberculosis* (Eweguard, Fort Dodge Animal Health).

Handling and Treatment of Sheep. The trial was conducted in 35 adult female parasite-free Lincoln sheep (purchased from a farm in the area of Tandil, province of Buenos Aires, Argentina) with an average weight of 38.0 ± 4.97 kg. Animals were housed in a barn with concrete-floored pens, 2 weeks before the onset of the study for a period of acclimation. The health of the animals was monitored prior to and throughout the experimental period. Animals were fed on high-quality hay prior to and during the trial. Water was available ad libitum. The experimental sheep ($n = 30$) were weighed with a conventional livestock scale and treated the same day with MXD injected SC at 200 μg/kg of body weight in the inner face of the hind limb (left side leg). Five animals were kept as untreated control.

Tissue Sample Collection. Three untreated animals from the control group were sacrificed on day 20 post-treatment to obtain "blank" tissues to validate the analytical methodology. All experimental animals (treated group) were killed at different times post-treatment to characterize the profile of MXD tissue residues. Six animals were randomly chosen to be slaughtered at each of the following days post-treatment: 21, 28, 35, 42, and 49. The animals were stunned by captive

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bolt and exanguinated immediately. At sacrifice, liver and muscle samples (between 90 and 160 g) and whatever available amount of abdominal fat were obtained; these tissue samples were cut into pieces of 7−10 cm wide, long, and thick. Additionally, tissue samples of the area surrounding the site of injection (including subcutaneous fat adequately and muscle) were obtained. Injection site samples were obtained from areas previously shaved and marked; at sacrifice, the skin was removed from the underlying fascia by dissection. A 100−180 g cylindrical tissue sample was obtained (∼10 cm diameter and ∼2.5 cm in depth). The weight of the collected tissue sample was recorded. The samples were placed in labeled plastic bags and rapidly (within an hour of their collection) transported with dry ice from the abattoir to the analytical laboratory. Samples were immediately frozen until analyzed by high-pressure liquid chromatography (HPLC).

Sample Extraction/Derivatization. The chemical extraction and derivatization of MXD from spiked and experimental tissue samples were performed following an adaptation of the technique described by De Montigny et al. (1990) and Alvinerie et al. (1995). Samples of the different sheep tissues were homogenized by using a blender, and a 1 g aliquot of each tissue sample was combined with 100 μL of internal standard [abamectin (ABM), 100 ng/mL] and then mixed with 1 mL of acetonitrile, vortexed (30 s), and shaken vigorously during 20 min. The mixture was sonicated in an ultrasound bath (Transonic 570/H, Elma) for 10 min at 35 °C and centrifuged during 10 min at 2000*g*. The procedure was repeated twice, and the combined supernatants were collected, mixed with 2 mL of hexane (HPLC grade), and shaken for 10 min. The upper *n*-hexane phase was discarded, and 2 mL of deionized water was added to the remaining acetonitrile phase (lower). The mixture was injected to a Supelclean LC_{18} cartridge (Supelco, Bellfonte, PA); the cartridge was flushed with 2 mL of water/methanol (75:25). The analytes were eluted with 1 mL of methanol and concentrated to dryness under a stream of nitrogen. The reconstitution was done using 100 μL of an *N*-methylimidazole solution in acetonitrile (1:1). The derivatization was initiated by adding 150 μL of trifluoroacetic anhydride solution in acetonitrile (1:2) and then injected into the HPLC system. The solvents used for sample extraction and drug analysis were of HPLC grade (Baker, Phillipsburg, NJ).

Drug Analysis. MXD tissue concentrations were determined by HPLC. HPLC analysis was undertaken using a reverse phase C_{18} column (Phenomenex, 3 μm, 4.6 mm × 150 mm) and an acetonitrile/methanol/water (64:32:4) mobile phase at a flow rate of 1.5 mL/min. Detection was done with a fluorescence detector reading at an excitation wavelength of 365 nm and an emission wavelength of 475 nm (RF-10 spectrofluorometric detector, Shimadzu, Kyoto, Japan). Pure reference standards (provided by Fort Dodge Laboratories, Princeton, NJ) were used to prepare calibration curves in a range between 0.5 and 100 ng/mL. Calibration curves were prepared for the different tissues spiked with known amounts of MXD, using least squares linear regression analysis. Calibration curves were established and correlation coefficients and coefficient of variations (CV) calculated. Experimental MXD concentrations were determined by calculating the ratio between the areas under the peaks of MXD and ABM (internal standard), using CR10 software (Shimadzu) and interpolating these areas on the calibration lines prepared for each tissue.

Validation Procedures. A complete method validation was performed as follows: linearity, recovery percentages, method precision, and theoretical detection and quantification limits for liver, fat tissue, muscle, and injection site samples were carried out before the start of analysis of experimental samples.

(a) Linearity. MXD was identified with the retention times of a pure reference standard (91.8% pure). Linearity was established to determine the concentration−detector response relationship. The linearity was determined by injection of spiked MXD standards in muscle, liver, administration site, and adipose tissue at different concentrations (triplicate determinations). Calibration curves were prepared using the least squares linear regression analysis. Correlation coefficients (r) and CVs were calculated following routine procedures.

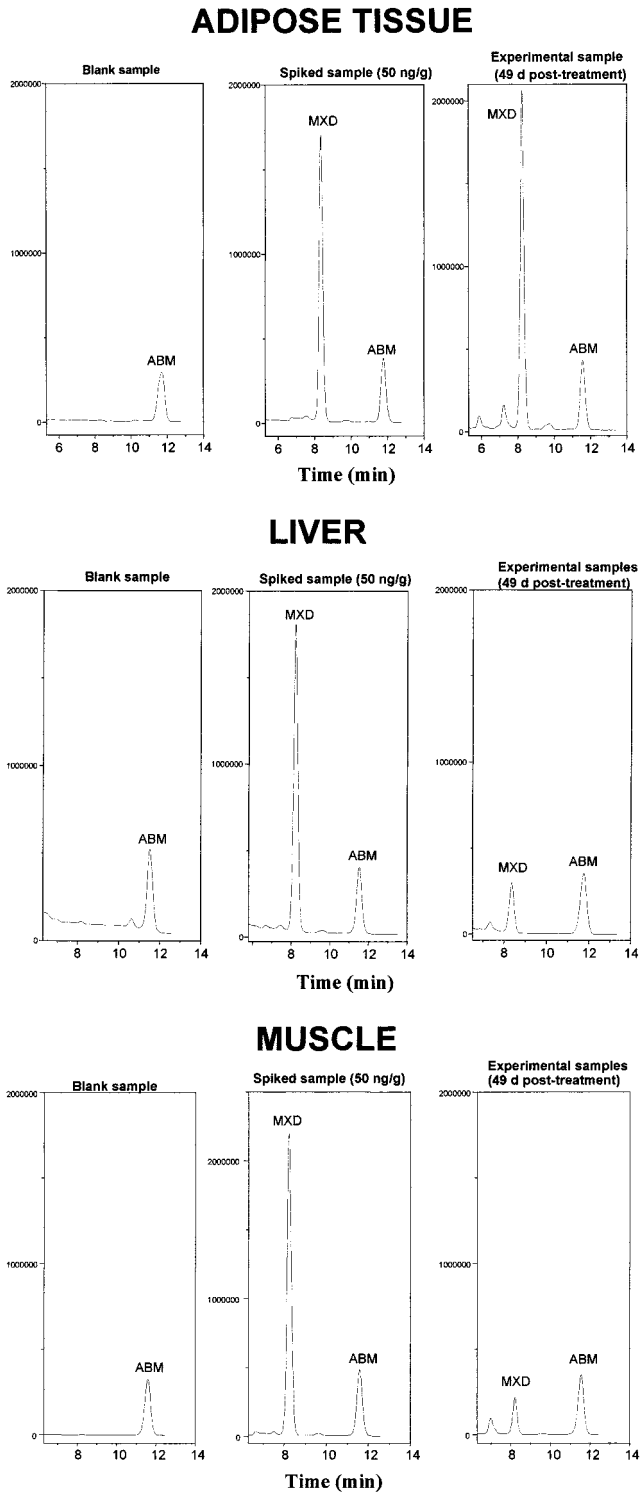

Figure 1. Typical chromatograms for MXD in (A, top) adipose tissue, (B, middle) liver, and (C, bottom) muscle. Blank tissue sample fortified with ABM as internal standard, MXD-spiked sample (50 ng/g), and experimental sample obtained at 49 days postadministration of MXD (200 μg/kg) are shown for each tissue.

(b) Recovery. Drug recovery was calculated by comparison of the peak areas obtained from spiked tissue standards and those obtained from direct injection of standards of MXD in methanol using ABM as internal standard. Percentages of MXD recovery from the different tissue samples were obtained

Table 1. Validation of the Analytical Methodology Used To Determine MXD Concentrations from Muscle, Administration Site, Liver, and Adipose Tissue of Sheep

	tissue			
parameter	muscle	administration site	liver	adipose tissue
limit of quantification (ng/g)	0.15	0.08	0.14	0.14
recovery (%) ± SD (*n*)[a]	86.1 ± 6.53 (6)	77.5 ± 1.71 (6)	93.7 ± 13.1 (6)	92.8 ± 12.2 (6)
linearity (*r*)	0.999	0.996	0.999	0.998
precision (CV) (%)	3.25	2.36	7.18	7.36

[a] *n*, number of samples.

Table 2. Animal Body Weights and MXD Tissue Concentrations Measured at 21 Days after Its SC Administration (200 μg/kg) to Sheep

	animal no.							
	53	54	57	60	80	84	mean	SD
animal wt[a] (kg)	43.5	32.3	41.5	36.9	42.2	47.9	40.72	4.08
muscle MXD concn (ng/g)	8.85	7.8	5.25	5.1	12.2	7.35	7.76	1.86
liver MXD concn (ng/g)	21.3	21.0	14.6	25.1	36.4	20.1	23.08	5.11
administration site MXD concn (ng/g)	27.8	77.9	32.5	9.59	123	37.9	51.45	32.67
adipose tissue MXD concn (ng/g)	161	279	229	65.6	300	238	212.10	65.87

[a] Weight was recorded the day of treatment.

Table 3. Animal Body Weights and MXD Tissue Concentrations Measured at 28 Days after Its SC Administration (200 μg/kg) to Sheep

	animal no.							
	56	65	66	77	79	81	mean	SD
animal wt[a] (kg)	42.9	26	35.9	35.5	31.2	40.7	35.37	4.51
muscle MXD concn (ng/g)	12.2	3.14	1.64	4.8	3.29	6.3	5.23	2.68
liver MXD concn (ng/g)	27.1	14	13.2	17.9	19.7	21.6	18.92	3.88
administration site MXD concn (ng/g)	19.2	7.25	2.58	12.7	13	13.2	11.32	4.27
adipose tissue MXD concn (ng/g)	244	163	85.9	222	173	296	197.32	56.68

[a] Weight was recorded the day of treatment.

Table 4. Animal Body Weights and MXD Tissue Concentrations Measured at 35 Days after Its SC Administration (200 μg/kg) to Sheep

	animal no.							
	51	73	75	76	78	83	mean	SD
animal wt[a] (kg)	40.5	36.1	31.1	28	31.3	34.5	33.58	3.45
muscle MXD concn (ng/g)	12.5	0.74	1.94	1.04	3.59	1.64	3.58	2.98
liver MXD concn (ng/g)	36.8	2.95	11.3	3.53	12.2	12.3	13.18	7.87
administration site MXD concn (ng/g)	36.6	1.8	14.2	3.2	65.4	3.83	20.84	20.11
adipose tissue MXD concn (ng/g)	387	7.25	119	46	137	20.8	119.51	94.99

[a] Weight was recorded the day of treatment.

in the range between 0.5 and 50 ng/mL (triplicate determinations). The mean percentage of recovery and the CV were calculated.

(c) Precision. The interassay precision of the extraction and chromatography procedures was evaluated by processing replicate aliquots of each tissue (quadruplicate determinations) containing known amounts of MXD on different days.

(d) Detection and Quantification Limits. The limit of drug detection was established with injection of tissue blanks fortified with the internal standard and measuring the baseline noise at the time of retention of the MXD peak. The mean baseline noise at the MXD retention time plus three standard deviations was defined as the detection limit. The mean baseline noise plus six standard deviations was defined as the theoretical quantification limit.

RESULTS AND DISCUSSION

The analytical method was validated appropriately. Calibration curves were prepared in the ranges between 0.50 and 10 ng/g and between 10 and 100 ng/g (ng/g = ppb) using the least squares linear regression analysis, and the linearity of the analytical method was corroborated at the mentioned concentration ranges in the different tissues analyzed. The percentages of MXD recovery from muscle, liver, administration site, and adipose tissue ranged from 77.5 to 93.7%. The theoretical quantification limit obtained for the different tissues ranged between 0.08 and 0.15 ng/g. Blank, MXD-spiked, and experimental chromatograms for adipose tissue, liver, and muscle are shown in Figure 1. The interassay precision of the analytical method showed a CV < 7.36% for the different tissues analyzed. Data on the validation of the analytical method for either tissue are summarized in Table 1.

MXD was detected in all of the tissues analyzed up to 49 days post-treatment after SC administration to sheep; this is in agreement with the high lipophilicity and extensive tissue distribution of this endectocide compound. Higher MXD concentrations were obtained in the adipose tissue than in the different tissues analyzed between 21 and 49 days post-treatment. The MXD tissue concentrations measured at 21, 28, 35, 42, and 49 days post-treatment are shown in Tables 2, 3, 4, 5, and 6, respectively.

A critical aspect of drug residue analyses is the sample extraction and preparation steps required to isolate the drug residue from complex biological matri-

Table 5. Animal Body Weights and MXD Tissue Concentrations Measured at 42 Days after Its SC Administration (200 μg/kg) to Sheep

	animal no.							
	59	67	69	70	72	74	mean	SD
animal wt[a] (kg)	38.3	42.1	36.1	39.8	42.8	35.4	39.08	2.48
muscle MXD concn (ng/g)	0.28	2.09	0.22	0.47	0.4	0.28	0.62	0.49
liver MXD concn (ng/g)	1.33	6.32	0.19	5.44	2.8	2.65	3.12	1.84
administration site MXD concn (ng/g)	0.63	4.14	ND[b]	3.51	2.58	1.26	2.42	1.18
adipose tissue MXD concn (ng/g)	2.75	76.5	0.65	7.11	35.1	16.1	23.04	21.84

[a] Weight was recorded the day of treatment. [b] ND, not detected.

Table 6. Animal Body Weights and MXD Tissue Concentrations Measured at 49 Days after Its SC Administration (200 μg/kg) to Sheep

	animal no.							
	58	82	68	63	61	64	mean	SD
animal wt[b] (kg)	46.1	41.8	39.2	41.1	40.1	35.9	40.70	2.30
muscle MXD concn (ng/g)	9.29	5.85	6.6	1.34	1.34	0.73	4.19	3.06
liver MXD concn (ng/g)	13	3.53	8.38	3.68	3.53	1.77	5.65	3,36
administration site MXD concn (ng/g)	9.43	9.12	23.8	3.67	3.98	1.49	8,58	5.54
adipose tissue MXD concn (ng/g)	155	47.2	108	49.2	56.6	22	73.00	39.00

[a] Weight was recorded the day of treatment.

ces. In the current trial the extraction and chromatographic analyses of MXD in the different tissues were performed following internationally accepted validation criteria. The lack of interferences in chromatographic separation (Figure 1) demonstrates a high specifity of the chromatographic method and a good selectivity obtained in the extraction procedure.

The MXD formulation SC injected to the experimental sheep was well tolerated. No adverse reactions in the site of subcutaneous injection were observed. The low MXD residual concentrations measured at the site of SC injection correlate with an adequate pattern of MXD absorption from the site of injection in sheep. The tissue residues pattern obtained in the current trial are in agreement with those obtained by Afzal et al. (1994). The lowest MXD residue concentrations were observed in muscle tissue at all of the post-treatment intervals, whereas the highest MXD concentrations were found in the adipose tissue. This result confirms that, as it is in cattle, the adipose tissue should be considered to be the target tissue for MXD in sheep.

The high affinity of MXD for adipose tissue may facilitate its deposit in this tissue, which may act as a drug reservoir that contributes to its long persistence in the body. Distribution of MXD into adipose tissue largely accounts for the large volume of distribution values obtained for this compound compared with other endectocide drugs (Lanusse et al., 1997). MXD is a highly lipophilic substance that is extensively distributed from the bloodstream to different tissues, and a large volume of distribution (13.6 L/kg) has been reported after SC administration to cattle (Lanusse et al., 1997). Its high lipophilicity accounts for a wide tissue distribution and long residence in plasma in sheep and cattle (Alvinerie et al., 1996; Lanusse et al., 1997). The large tissue distribution of MXD is in agreement with the high availability of MXD obtained in the gastrointestinal mucosal tissues, lungs, and skin and with the extended detection of MXD concentrations for up to 58 days post-treatment in those tissues, which represent the most important sites of endo- and ectoparasite location (Lifschitz et al., 1999). Higher MXD concentrations were measured in different tissues at 49 days post-treatment compared with those obtained at 42 days postadministration; this was particularly clear for adipose tissue. Nutritional status and body composition have been shown, among other factors, to substantially affect the systemic availability of ivermectin in cattle (Lifschitz et al., 1997) and sheep (Bogan and McKellar, 1988). In the current trial, animals killed at each post-treatment sampling point were randomly chosen; unfortunately, some of the animals assigned to be sacrificed at day 49 post-treatment had higher body weights than those sacrificed at day 42 postadministration. A difference in body condition (fatty condition) may have contributed to the longer pesistence of higher MXD concentrations observed at 49 days post-treatment in the different tissues under study.

The Committee for Veterinary Medicinal Products has established the maximum residues limits (MRLs) for MXD in sheep. The MRLs for MXD were 500, 100, and 50 μg/kg in fat, liver, and muscle, respectively (CVMP, 1996) As shown in the current trial, the concentrations of MXD at 21 days post-treatment in adipose tissue, liver, and muscle were below the established MRLs.

In conclusion, the adipose tissue should be considered as a site of sequestration for SC administered MXD using the formulation investigated in sheep. The SC administration of a single dose of MXD to adult sheep results in MXD tissue concentrations below the established MRL in all of the tissues analyzed at 21 days post-treatment. The fact that MXD was combined with the mentioned 6 in 1 clostridial vaccine did not affect the drug's pharmacokinetic behavior, showing patterns similar to those observed with conventional injectable formulations.

ABBREVIATIONS USED

MXD, moxidectin; ABM, abamectin; SC, subcutaneous; HPLC, high-pressure liquid chromatograph; MRL, maximum residue level; CV, coefficient of variation; *r*, correlation coefficient; SD, standard deviation.

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