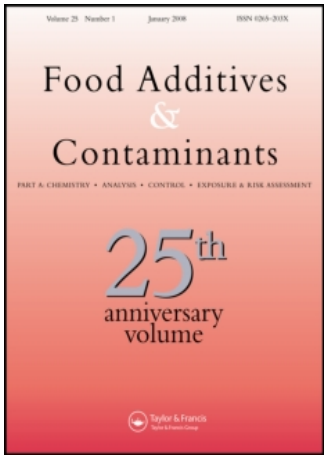

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Pattern of ivermectin (sheep) and doramectin (cattle) residues in muscular tissue from various anatomical locations

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Pattern of ivermectin (sheep) and doramectin (cattle) residues in muscular tissue from various anatomical locations

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Abstract
This trial reports comparative drug residual concentrations in muscular tissue obtained from various anatomical locations after subcutaneous administration of ivermectin (IVM) to sheep and topical treatment with doramectin (DRM) to calves at recommended therapeutic dose rates. Seven muscle samples from different anatomical locations (rhomboideus, supraspinatus, semitendinosus, gluteus medius, longissimus dorsi thoracis, intercostales and diaphragma) were collected at several post-treatment sampling times. Samples were frozen at –20°C until analyzed by HPLC. The highest IVM residual concentrations in muscular tissue from the different locations were found at 15 days post-treatment in sheep. Although the highest IVM mean concentrations were measured at 15 ($16.8 \pm 5.17\,ng\,g^{-1}$) and 20 ($10.5 \pm 4.06\,ng\,g^{-1}$) days post-administration in the intercostales muscles, at 30 days post-administration, the IVM concentration in this location was similar to that measured in the rhomboideus and diaphragma muscles. DRM residual concentrations were quantified in muscular tissue from all anatomical locations after topical administration to calves. Maximum residue level was observed at 10 days post-treatment in all anatomical sites. The diaphragma muscle showed the highest DRM residue levels at 2 ($22.0 \pm 4.35\,ng\,g^{-1}$), 5 ($45.2 \pm 3.78\,ng\,g^{-1}$) and 10 ($57.9 \pm 9.57\,ng\,g^{-1}$) days post-treatment in calves. These results demonstrated that the pattern of residue depletion from muscular tissue may differ according to its anatomical locations and/or physiological role. This should be considered in implementing residue control strategies in meat safety assurance for human consumption.

Keywords: *Drug residues, muscle, anatomical locations, ivermectin, doramectin, sheep, cattle*

Introduction

Veterinary drugs are widely used to protect animal health, prevent production loss and ensure a safe food supply. The fate of chemical residues from veterinary drugs in animal tissues destined to human consumption is an important issue in food safety. To reach the required food protection level, reliable data must be available for adequate risk evaluation and subsequent action. Veterinary drugs used in livestock require withdrawal times after treatment to avoid residual concentration above the defined maximum residues level (MRL). Concentrations attained in the tissues depend on the ability of the drug to penetrate capillary endothelium and diffuse across cell membranes. This tissue distribution process is a key issue in the pharmacokinetic behaviour of the drug, where the lipophilicity of the compound plays a major role (Baggot 1977).

Ivermectin (IVM) and doramectin (DRM) are avermectin endectocide compounds with exceptional potency and broad nematode and arthropod spectrum of activity, extensively used in livestock (McKellar and Benchaoui 1996). The pharmacokinetic behaviour of IVM and DRM in ruminant species are characterized by a prolonged residence time, as a consequence of high tissue distribution and low metabolic rates (Lanusse et al. 1997; Lifschitz et al. 2000; Hennessy and Alvinerie 2002). They have a high affinity for adipose tissue, the main storage site for these drugs (Chiu et al. 1986). Thus, edible fat-containing tissues are important reservoirs for avermectin and related compounds in ruminants, which accounts for the

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extended persistence of antiparasitic activity. On the other hand, the amount and quality of fat stores within the muscle tissue are important for the overall acceptability of most forms of consumed meat (German 1990). Fat content is affected by age, breed, nutrition and animal management. In cattle, for example, an increase in feeding level or castration, induces a higher fat content in muscles (Geay and Renand 1994; Haurez and Joulie 1994). Moreover, it has been demonstrated that different muscles in the carcass showed different growth patterns, amount of fat and connective tissue (Bosselmann et al. 1995; O'Neill et al. 2004) and fat content is much higher in oxidative than in glycolytic muscles (Wood and Warris 1992; Touraille 1994). Therefore, the pattern of residues distribution for highly lipophilic drugs may vary among muscles from different anatomical areas.

International organizations have established MRL values for endectocide molecules in marker tissues, such as fat, liver, kidney and muscle. Considering that meat is the main edible tissue destined for human consumption, the current study provides muscle residue kinetic data, using these molecules as a model of lipophilic compounds widely used in livestock, which may be valuable in assuring food safety. The trial presents comparative drug residual concentrations in muscular tissue from different locations of the carcass after subcutaneous administration of IVM in sheep and topical treatment with DRM in calves at therapeutically recommended dose rates.

Materials and methods

Animal trials

Patterns of IVM and DRM residues in muscular tissues were characterized in treated sheep (IVM) and cattle (DRM) sacrificed at different times post-treatment within an experimental design where animal tissues were also used for other scientific purposes. Thus, the scheme for animal sacrifice was adapted to different experimental requirements to take advantage of the available biological material. Animals received food and water ad libitum during the whole experimental period.

In Experiment 1: IVM muscle residues in sheep, 12 adult male Corriedale sheep (38.3 ± 5.23 kg) were subcutaneously treated with a commercial formulation of IVM at $200\,\mu g\,kg^{-1}$. Four animals were randomly assigned for sacrifice at: 15, 20 and 30 days post-treatment. In Experiment 2: DRM muscle residues in cattle, 12 Holstein calves (180 ± 30.9 kg) were treated with a commercial formulation of DRM by topical (pour-on) administration at the recommended dose rate ($500\,\mu g\,kg^{-1}$). Four calves were randomly sacrificed at 2, 5 and 10 days post-treatment. In both experiments, animals were stunned by captive bolt and immediately exsanguinated according to ethical guidelines (American Veterinary Medical Association 2001). Muscle samples (200–300 g), without any visible fat, from different anatomical locations were collected at the above indicated sampling times: Rhomboideus (neck), supraspinatus (shoulder), semitendinosus (leg), gluteus medius (leg), longissimus dorsi thoracis (rack), intercostales externi/interni (rib) and diaphragma. Muscle samples from untreated sheep/cattle were used as blank controls for development of the analytical method. Blank and experimental samples were frozen at -20°C until analyzed by high performance liquid chromatography (HPLC).

Muscle samples analysis

Drug analysis. IVM and DRM were analyzed in sheep and cattle muscles by HPLC with fluorescence detection, as previously described (Lifschitz et al. 2000).

Muscle samples were thinly sliced, 1 g was placed into a 5-ml plastic tube and spiked with 50 µl of the internal standard (IS) abamectin (2 ng per 10 µl). Drug molecules were extracted by addition of 0.5 ml acetonitrile for 10 min under a high-speed vortexing shaker (Multi-tube Vortexer; VWR Scientific Products, West Chester, PA, USA). After mixing, the sample was sonicated (Ultrasound Bath, Lab-Line Instrument, Inc., Melrose Park, OL, USA) and centrifuged (BR 4i Centrifuge, Jouan®, Saint Herblain, France) at 2000 *g* for 10 min at 5°C. The clear supernatant was transferred to a tube and the procedure repeated. The total supernatant was transferred to C_{18} cartridges (100 mg, 1 ml; Lichrolut®, Merck, Darmstadt, Germany) using a manifold vacuum (Baker spe-24G, Phillipsburg, PA, USA). The cartridges were previously conditioned with 2 ml methanol, followed by 2 ml water. All samples were applied and then sequentially washed with 1 ml of water, 1 ml methanol/water (1:4, v/v), dried with air for 5 min and eluted with 1.5 ml methanol. The eluted volume was evaporated (60 °C) to dryness in a vacuum concentrator (Speed-Vac®; Savant, Los Angeles, CA, USA) and the dry residue dissolved in 100 µl of a *N*-methylimidazole (Aldrich, Milwaukee, WI, USA) solution in acetonitrile (1:1, v/v). To initiate the derivatization, 150 µl trifluoroacetic anhydride (Aldrich) solution in acetonitrile (1:2, v/v) were added (De Montigny et al. 1990). After completion of the reaction, an aliquot (100 µl) of this solution was injected in a Shimadzu chromatography system

(Shimadzu Corporation, Kyoto, Japan). A mobile phase of water/methanol/acetonitrile (6:40:54, v/v) was pumped into the system through a C_{18} column (BDS Hypersil Thermo, 5 µm, 4.6 × 250 mm) placed in an oven at 30 °C. Fluorescence detection (Spectrofluorometric detector RF 10; Shimadzu, Kyoto, Japan) was performed at 365 nm excitation and 475 nm emission wavelength.

Validation procedure

A complete validation of the analytical procedures for the extraction and quantification of IVM (sheep) and DRM (cattle) in muscular tissue was performed. The linearity of the method was tested after elaboration of analytical calibration curves for each compound in muscle. Blank muscle samples were fortified with each analyte in two ranges of calibration: 0.1–5 and 5–100 ng g^{-1}. The extraction efficiency of the analytes was determined by comparison of the peak areas from fortified blank muscle samples (0.2, 5 and 50 ng g^{-1}, $n = 5$) with the peak areas from direct injections of equivalent quantities of standards. Precision and accuracy (intra- and inter-assay) of the method were determined by evaluation of replicates of drug-free muscle ($n = 5$) fortified with each compound at three different concentrations (0.2, 5, 50 ng g^{-1}). Precision and accuracy were expressed as coefficient of variation (% CV) and relative error (% RE), respectively. The theoretical LOD was defined as the mean ($n = 5$) baseline noise/IS peak area ratio plus three standard deviations (SD). The limit of quantification (LOQ) was calculated as the lowest drug concentration ($n = 5$) on the standard curve that could be quantitated with precision not exceeding 20% and accuracy within 20% of nominal.

Data analysis

Once the concentration values (expressed as ng g^{-1}) for each drug (Experiments 1 and 2) in the different muscle locations were determined, the area under the concentration vs. time curve (AUC) was calculated by the trapezoidal method (Gibaldi and Perrier 1982), using the PkSolution 2.0 program (Summit Research Services, Ashland, OH, USA). The AUC value (expressed as ng day^{-1} g^{-1}) for each location was considered as an indicator of the total drug availability within each muscle. The values for muscle concentrations and AUC are presented as means ± SE (four animals). The concentrations found in the different muscles at each sampling time and AUC values were compared by analysis of variance with paired data (ANOVA), using the Instat 3.0 Software (Graph Pad Software, San Diego, CA, USA). The Tuckey range test was used to indicate the order of significance when a significant F value was obtained. A value of $p < 0.05$ was considered statistically significant. Since ANOVA assumes populations with equal SDs, when differences among the SD of the concentrations at the different times were significant (Bartlett test), concentration data were logarithmically transformed.

Results and discussion

In Experiment 1, IVM residual concentrations were detected in all muscle locations in the carcass of sheep treated subcutaneously. Concentrations measured at the different sampling times are presented in Figure 1. High variability in IVM concentrations was observed among animals. The highest IVM residual concentrations in all muscle locations were found at 15 days post-treatment (Figure 1a). At this time, the highest IVM mean concentration was measured in the intercostales muscles with a mean value of 16.8 ± 5.17 ng g^{-1}, while the lowest mean concentration was measured in supraspinatus with a mean residue level of 8.8 ± 2.58 ng g^{-1}. The differences observed in IVM concentrations measured at day 15 post-treatment in the intercostales vs. gluteus medius, intercostales vs. longissimus dorsi and intercostales vs. supraspinatus muscles reached statistical significance. At 20 days post-administration (Figure 1b), the intercostales muscles showed the highest IVM mean residue concentrations with a value of 10.5 ± 4.06 ng g^{-1}. The lowest concentration was measured in gluteus medius (3.9 ± 1.95 ng g^{-1}). Similarly, at 20 days post-treatment, IVM concentrations in the intercostales muscles were significantly higher ($p < 0.05$) than those observed in gluteus medius, longissimus dorsi and supraspinatus muscles (Figure 1b). At 30 days post-administration, the diaphragma, rhromboideus and intercostales muscles showed very similar mean concentrations (3.14, 3.22 and 3.11 ng g^{-1}, respectively). These muscular locations had the highest mean values of IVM residues, while the lowest concentration corresponded to the semitendinosus muscle (1.26 ± 0.92 ng g^{-1}). At the assayed times post-treatment, the differences found were consistent among muscular locations with the highest mean concentration measured at the intercostales muscles.

In Experiment 2, DRM concentrations were quantified in muscular tissue from all anatomical locations at different times after topical administration to calves (Figure 2). The maximum residue level was determined at 10 days post-treatment in all muscle samples assayed (Figure 2c). At 2 days post-treatment, significantly higher DRM mean concentrations were measured in diaphragma (22.0 ± 4.35 ng g^{-1}) compared to those recorded in

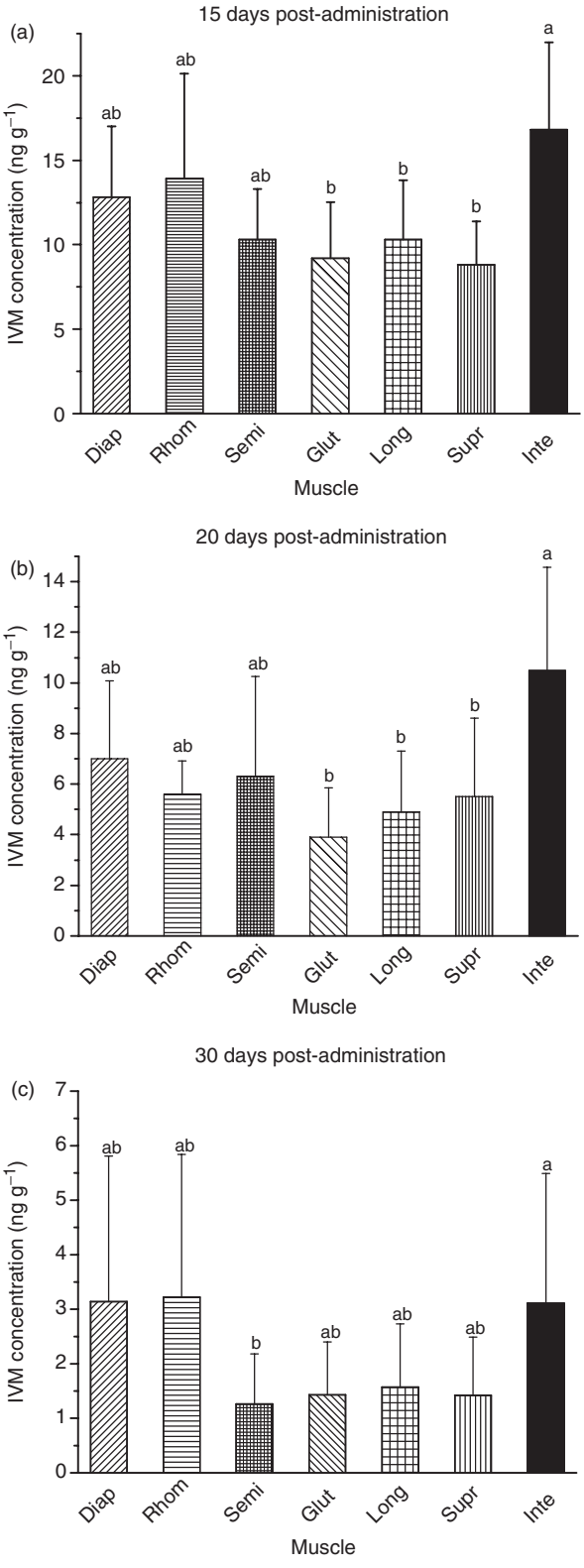

Figure 1. Ivermectin (IVM) concentrations (mean ± SE) (ng g^{-1}) in different muscles (diaphragm (Dia), rhomboideus (Rhom), semitendinosus (Semi), gluteus medius (Glut), longissimus dorsi thoracis (Long), supraspinatus (Supr) and intercostales externi/interni (Inte)) at 15 (a), 20 (b) and 30 (c) days after IVM administration by the subcutaneous route (200 µg kg^{-1}) to sheep. [a,b]Values with different superscripts are statistically different ($p < 0.05$).

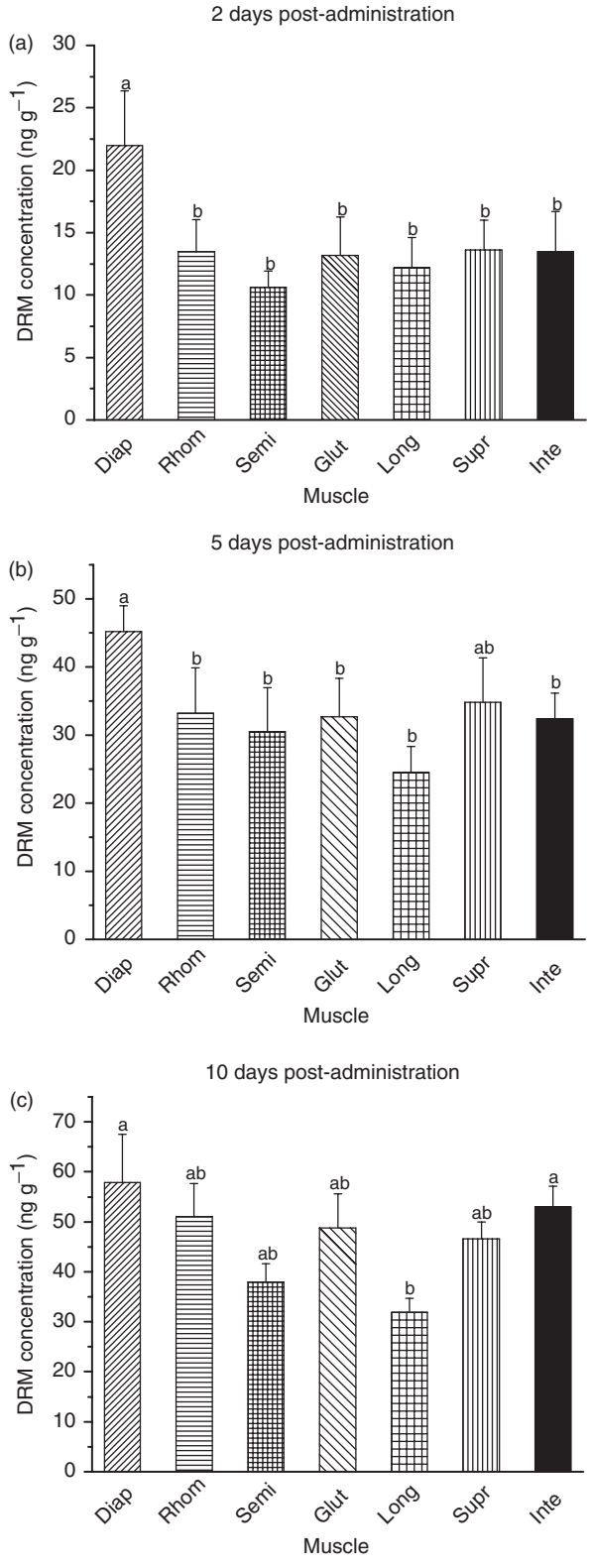

Figure 2. Doramectin (DRM) concentrations (mean ± SE) (ng g^{-1}) in different muscles (diaphragm (Dia), rhomboideus (Rhom), semitendinosus (Semi), gluteus medius (Glut), longissimus dorsi thoracis (Long), supraspinatus (Supr) and intercostales externi/interni (Inte)) at 2 (a), 5 (b) and 10 (c) days after DRM administration by the topical (''pour on'') route (200 µg kg^{-1}) to cattle. [a,b]Values with different superscripts are statistically different ($p < 0.05$).

rhomboideus ($13.5 \pm 2.52\,ng\,g^{-1}$), supraspinatus ($13.6 \pm 2.40\,ng\,g^{-1}$), semitendinosus ($10.6 \pm 1.31\,ng\,g^{-1}$), gluteus medius ($13.2 \pm 3.06\,ng\,g^{-1}$), longissimus dorsi ($12.2 \pm 2.41\,ng\,g^{-1}$) and intercostales ($13.5 \pm 3.21\,ng\,g^{-1}$) muscles (Figure 2a). Similarly, at five days post-administration, the highest DRM residue levels ($45.2 \pm 3.78\,ng\,g^{-1}$) were detected in the diaphragma, significantly higher that other muscular locations, except for supraspinatus muscle (Figure 2b). At 10 days post-administration, the highest ($57.9 \pm 9.57\,ng\,g^{-1}$) DRM mean concentration was also found in the diaphragmatic area. However, significant differences were observed among mean concentrations of diaphragma vs. longissimus dorsi and intercostales ($53.0 \pm 4.12\,ng\,g^{-1}$) vs. longissimus dorsi (Figure 2c).

The values of AUC in muscular tissue are good indicators of drug availability at different locations, which is a valid and useful facility in interpreting residue depletion results. AUC values for IVM and DRM in each muscle location were calculated (Table I). When the AUCs for IVM were compared, differences between intercostales and gluteus medius were obtained, indicating some variation in the distribution process among anatomical locations. AUC values calculated for DRM in each muscle reached statistical differences. The AUC value for the diaphragma was significantly higher than that obtained for the other muscles, which may indicate a greater distribution of DRM into this muscle. This is consistent with the results obtained for the comparison of DRM concentrations among the different muscles.

In the present study, fat content in muscles from different locations was not determined, although it has been demonstrated that different muscles in the carcass contain different amounts of fat (Bosselmann et al. 1995; O'Neill et al. 2004; Von Seggern et al. 2005). It is known that avermectins have a high affinity for adipose tissue, which constitutes the main storage site for these antiparasitic molecules. In fact, data obtained in our laboratory demonstrated that when IVM was administered subcutaneously in sheep, the concentrations measured were much higher in fat than in liver or muscle (Figure 3, unpublished data). Thus, a differential IVM residual pattern among different muscle locations could be explained by the affinity of this molecule to distribute in the infiltrated fat of muscles. Therefore, the highest IVM concentration found in intercostales muscles could be due to its higher fat content. Although the fat content was not determined, macroscopic inspection of muscles showed a higher fat infiltration in the intercostal area.

Both IVM and DRM are highly lipophilic compounds and are extensively distributed from the bloodstream to different tissues. However, our hypothesis does not explain the results obtained for DRM, with the highest concentration measured in the diaphragma, a less fatty muscle (macroscopically) compared to others, which is consistent with its physiological role. In this case, other reasons could be implicated in the varying distribution among muscles, such as blood flow and muscle irrigation.

Few reports are available on variations in drug residue concentrations according to sampled

Table I. Comparison of the concentration of ivermectin (IVM) and doramectin (DRM) residues, expressed as area under the concentrations vs. time curve (AUC) ($ng\,day\,g^{-1}$) (mean ± SEM), in muscles from different anatomical location after subcutaneous administration of IVM to sheep ($200\,\mu g\,kg^{-1}$) and topical administration of DRM to cattle ($500\,\mu g\,kg^{-1}$).

	Drug residues (AUC) ($ng\,day\,g^{-1}$)			
	IVM		DRM	
	Mean	SEM	Mean	SEM
Diaphragma	205.0^{ab}	50.1	424.1^{a}	40.5
Rhomboideus	197.0^{ab}	66.1	$294.3^{b\star}$	39.5
Semitendinosus	157.0^{ab}	35.0	$243.3^{b\star}$	33.4
Gluteus medius	128.6^{a}	34.1	$285.9^{b\star}$	38.6
Longissimus dorsi thoracis	147.4^{ab}	36.7	$208.3^{b\star\star}$	23.1
Supraspinatus	136.5^{ab}	31.2	$289.7^{b\star}$	29.7
Intercostales externi/interni	$262.0^{b\star}$	75.2	$295.8^{b\star}$	18.4

[a,b]Values with different superscripts in the same column are statistically different at $\star p < 0.05$ or $\star\star p < 0.01$.
SEM = standard error of the mean.

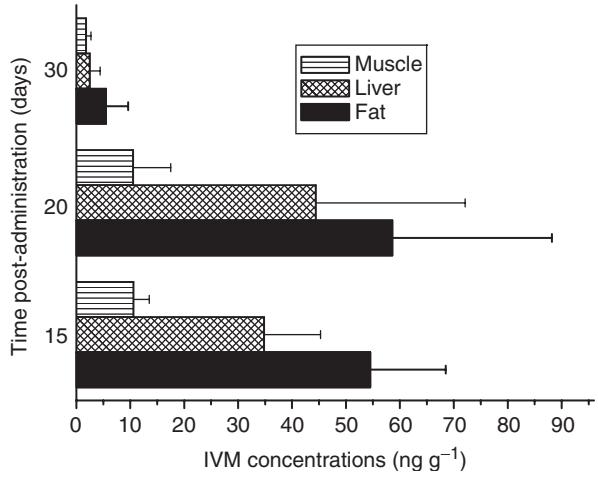

Figure 3. Comparison of ivermectin (IVM) residues in muscular tissue (mean ± SE) ($ng\,g^{-1}$) with those measured in fat and liver at 15, 20 and 30 days (unpublished data obtained in our laboratory) in IVM-treated sheep.

muscles. There have been some studies on the influence of muscle location (different fat proportions) on natural hormone concentration patterns for meat quality or residue control, as lipophilic hormones reach higher levels in fat than in muscle (Gaiani and Chiesa 1986; Tsujioka et al. 1992). Fritsche et al. (1998) determined 11 steroid hormones in three different muscles in bulls and steers. They reported statistical differences only for the epitestosterone level in the extensor carpi ulnaris muscle of steers and a tendency for cortisol content to be higher in this muscle compared to others in the same animal. Testosterone levels in four samples of diaphragm and subfascial muscle from bulls showed a tendency to be higher in the diaphragm, which was explained by the higher blood perfusion rate (Hoffmann and Rattenberger 1977), consistent with the data reported in this article for DRM residual concentrations.

In conclusion, the pattern of IVM and DRM residues depletion from muscular tissue showed some variation according to its anatomical location and/or physiological role.

Different MRLs for endectocide drugs have been set in bovine and ovine species by regulatory agencies. The European Union has set MRLs in muscle, fat, liver and kidney for DRM in ovine species (EMEA, 2006). Meanwhile, fat, liver and kidney (not muscle) are the target tissues for IVM in both species (EMEA, 2004). In the US, a tolerance level for DRM in cattle muscle ($30\,ng\,g^{-1}$) has been established by the Food and Drug Administration (FDA, 2006). According to this, the residual concentration profiles measured in cattle muscle after DRM topical administration were above the FDA-recommended MRL values in all muscles at 5 (except for longissimus muscle) and 10 days post-treatment. The reported DRM concentration values exceeding the recommended MRL for muscular tissue and the observed variation among muscle from different anatomical locations should be evaluated to establish safe withdrawal periods after topical treatment. However, the high variability in concentration values observed among animals must be considered in assessing the practical relevance of the reported residual data.

IVM and DRM were used as models of lipophilic molecules widely used in livestock production. Therefore, these results need to be confirmed for other drugs where muscle is a marker tissue. The results reported here on the pattern of distribution of drug residues in muscular tissues from different locations in the carcass may be valuable when considering the implementation of residue control strategies in meat for human consumption. Additionally, the information described here may be helpful in designing trials to assess tissue-residue profiles for the establishment of safe post-treatment withdrawal periods in drug-treated livestock.

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