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Tissue Depletion Profile of Ivermectin in Rabbits

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Abstract

Avermectins are largely used in different animal species. Ivermectin, the most popular avermectin, is used against a wide spectrum of endo and ectoparasites. Some mange mites are particularly sensitive to ivermectin. *Notoedres cati* var. cuniculi, the agent of rabbit mange, is particularly sensitive to ivermectin. Pharmacokinetic and residual studies of ivermectin in rabbits, however, are scarce. The objective of the present paper was to study the tissue residue profile of ivermectin (IVM) after subcutaneous (s.c.) administration of a 1% w/w solution. Sixteen young healthy male rabbits received 200 µg/kg of a 1% w/w ivermectin formulation subcutaneously. Groups of four treated animals were sacrificed at 10, 20, 30 and 40 days after injection. Samples of liver, fat, kidney and muscle tissue were obtained. IVM concentrations were determined by liquid chromatography with fluorescence detection after automatic solid phase extraction with SPE C_{18} cartridges.

Ivermectin was detected after subcutaneous administration until 40 days. Muscle samples showed the lowest IVM concentrations throughout the study. The highest IVM concentrations at all sampling times were measured in liver and fat tissues. Nevertheless, IVM concentrations in all the tissues analyzed were below the accepted maximum residue limits recommended by the European Union at 27 days post treatment.

Keywords: *Ivermectin; Tissues; Residues; Withdrawal Time; Rabbit*

Abbreviations

MLs: Macrocyclic Lactones; IVM: Ivermectin; DRM: Doramectin; MRL: Maximum Residue Limit; HPLC: High-Performance Liquid Chromatography; LOQ: Limit of Quantitation; LOD: Limit of Detection; SD: Standard Deviation; CV: Coefficient of Variation

Introduction

The avermectins are naturally occurring compounds derived from *Streptomyces avermitilis* [1], belonging to the macrocyclic lactones family widely used in livestock, humans and a variety of exotic pets including ferrets, rabbits, rodents, birds and reptiles as potent parasiticides. Ivermectin (IVM) is one of the most useful member of the macrocyclic lactones [2,3], endectocide of wide spectrum of activity at low doses rates with high potency and low mammalian toxicity [4] widely used for treatment and prevention of internal and external parasites in food-producing animals [2,3]. Ivermectin is effective when it is applied orally, parenterally or topically. Its absorption is rapid

by any of these routes of administration [1]. These hydrophobic drugs are distributed throughout the body bound to lipoproteins in the circulating blood [5]. They have a strong affinity for adipose tissue, which constitutes the main storage site for the drug. MLs are characterized by a unique long mean residence time in the host associated with a long-lasting activity when compared to other anthelmintics such as benzimidazoles. Although the activity of these compounds was first established in laboratory animal parasite systems, their use in rodents and other exotic pets is extra-label, and treatment protocols are often established through empirical clinical experience rather than controlled studies. The pharmacokinetic and the residual profiles of the semi-synthetic derivative of avermectin B1, IVM, have been extensively studied in different species [3,6,7]. It is particularly used to control ectoparasites, such as mite and mange infestations in rabbits. *Sarcoptes scabiei, Psoroptes cuniculi, Notoedres cati* var. cuniculi ear mange mites, are the predominant parasites of rabbits [8,9]. Mange mite infestation decreases rabbit productivity, growth rates and efficiency of feed conversion. Affected animals stop feeding and this predisposes them to secondary bacterial infection. Use of the macrocyclic lactones (MLs) (ivermectin, DRM, selamectin or moxidectin) for treatment of ear mites in rabbits has been reported [8,10,11].

In contrast, pharmacokinetic and depletion of residue in tissue data for ivermectin in rabbits are still very limited [12], although rabbits are considered not only companion animals but also minor species in commercial animal production. Residue studies are of fundamental importance in public health. Consumer safety is based on a series of measures including maximum residue limits (MRLs) and acceptable daily intakes (ADI) as the most important. Therefore, the tissue residue profile should be evaluated in this species as well. In accordance with the Notes for Guidance on Risk Analysis Approach for Residues of Veterinary Medicinal products in Food of Animal origin [13] and the Guideline on safety and residue data requirements for pharmaceutical veterinary medicinal products intended for minor use or minor species (MUMS)/limited market [14] it was possible to extrapolate the MRLs to all mammalian food producing species. The availability of safe and effective veterinary medicinal products for MUMS will improve both animal welfare, as well as animal health and public health. Food derived from minor species, such as rabbits, usually constitutes a small proportion of the diet of the average consumer. It may, nevertheless, constitute a major portion of the intake of animal derived products in certain geographic areas or for certain subpopulations and therefore consumer safety must not be compromised. If identical MRLs were obtained in cattle (or sheep), pigs and chicken (or poultry), which represent major species with different metabolic capacities and tissue composition, the same MRLs can also be set for ovine, equidae and rabbits, considering a possible extrapolation all food-producing mammalian animals. Thus, the European Union set ivermectin MRLs (22,23-dihydroavermectin B1a as marker residue) in edible tissues of food-producing mammals as follows: 100 ng/g in liver and fat, and 30 ng/g in kidney and muscle [15].

Objective of the Study

Therefore, the objective of the present paper was to study the tissue residue profile of IVM after subcutaneous (s.c.) administration of a 1% w/w solution.

Materials and Methods

Reagents and chemicals

Ivermectin (IVM) pure reference standard (97% purity) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and methanol solvents used during the extraction and drug assay were of HPLC grade and purchased from J. T. Baker (Phillipsburg, NJ, USA). The N-methylimidazole and trifluoroacetic anhydride used for derivatization reaction were from Sigma-Aldrich Chemical Company (USA). Solid phase extraction (SPE) columns (Strata, C_{18}, 100 mg, 1mL) and analytical column (Luna C_{18}) were obtained from Phenomenex (USA).

Animals

Twenty (20) New Zealand white male rabbits weighing 3.2-4.8 kg were used in this trial. Animals were clinically healthy and parasite free and did not receive any drug treatment for at least 30 days prior to the study. During 10 days' acclimation period and a subsequent period, the rabbits were fed pelleted feed concentrate three times per day with free access to water. The animals were housed in cages under a 12h light/dark cycle.

Drug administration and sample collection

Four animals were kept as untreated controls and sixteen animals received a single subcutaneous (dorsocervical area) injection of 1% w/w IVM (Ivomec®, Merial) at a dose rate of 200 µg/kg. After IVM administration, all animals were euthanized with lethal IV doses of penthobarbital (Euthanyle®, Brouwer, Argentina) in groups of four according to the following schedule: 10, 20, 30 and 40 days' post – treatment, in order to collect tissue samples. Four rabbits used as control (free IVM) were euthanized before experiment. Tissues collected were the complete organs in the case of liver and kidney, and samples for skeletal muscle, and adipose tissue. Tissue samples were collected according to standard operating procedures, placed in plastic bags, heat sealed, uniquely identified, and stored at –20ºC until extraction and assay.

The protocol followed the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science societies -FASS) and was approved by the Experimental Ethics Committee of the Faculty of Veterinary Science, UNLP, Argentina.

Ivermectin analysis

Ivermectin tissue analysis was performed by high-performance liquid chromatography (HPLC) with automated solid phase extraction and fluorescence detection following an adapted version of the methodology previously described [1-3]. Briefly, tissue sample (muscle, liver, kidney and fat) were thinly sliced and aliquots of 2 g were homogenized in 1 mL of acetonitrile (Ultra Turrax T25 basic, IKA). The homogenate was mixed 20 minutes sonicated 10 minutes (Ultrasound Bath) and centrifuged at 2000 x g for 2 minutes at 4°C. The clear supernatant was transferred to a new tube and the extraction procedure was repeated once again. The total supernatant obtained was placed on the appropriate rack of Aspec XL automatic solid phase extraction apparatus (Gilson, France). Automatic sample preparation was a modified procedure based on that reported by Alvinerie., *et al* [16]. This was performed using SPE C_{18} cartridges (Strata C_{18}, 100 mg, 1 mL, Phenomenex), which were conditioned with 2 mL of methanol, and followed by 2 mL of water HPLC quality. All samples were applied to cartridges, washed with 1 mL of water followed by 1 mL of methanol/water (1:3, v/v), dried with air during 2 minutes and finally eluted with 3 mL of methanol HPLC grade. The eluted volume was evaporated at 60°C to dryness in a vacuum concentrator (AVC 2-25CD Christ, Germany). Fluorescent derivative was obtained by dissolving the dry residue in 100 µL of a mixture of N-methylimidazole solution in acetonitrile (1:1, v/v). To initiate the derivatization, 150 µL of a solution of trifluoroacetic anhydride in acetonitrile (1:2, v/v) was added. After completion of the reaction (< 30s) and mixing, the solution was transferred to autosampler polypropylene vials and an aliquot of 100 µL was injected into the chromatographic system. HPLC analyses were carried out within 4 h to avoid the degradation of the fluorescent derivatives.

Standard curve

Standards were prepared by adding 0.5, 1, 2.5, 5, 10, 20 and 30 ng of IVM to test-tubes, evaporating to dryness at 60ºC and dissolving and derivatising as described above. Linear regression analysis using a least-square fit was performed.

Chromatographic conditions

The chromatographic system consisted of an isocratic pump (Gilson Inc. 307), an automatic injector (Gilson Inc. 234), a Fluoro Monitor IM III Detector (Sp Thermo Separation products) set at an excitation wavelength of 365 nm and an emission wavelength of 475 nm, and Eppendorf CH-30 Column Heater (set at a 30ºC). The system is controlled through the Unipoint® Gilson system. A C_{18} column (Luna 5 µm, 4.6 mm x 150 mm, Phenomenex, Torrance, CA, USA) was eluted with a mixture of acetic acid 2%, methanol, acetonitrile (4:32:64) at a flow rate of 1.5 $mL.min^{-1}$. Identification of IVM in rabbit tissues was accomplished by comparison with the retention times of the reference standards. The precision of the extraction procedure and chromatography technique was evaluated by processing as replicates in six different occasions, aliquots of pooled different tissue samples containing known amounts of IVM.

Method validation

The method for the identification of IVM in tissue rabbits was validated in terms of the analytical parameters of linearity, precision, accuracy, limit of quantitation (LOQ) and detection (LOD) and selectivity following the guideline for the validation of analytical methods used in residue depletion studies, from the International Cooperation on Harmonization of Technical Requirements for the Registration of Veterinary Medicinal Products [17].

Linearity was determine using seven concentrations of IVM standard (0.5, 1, 2.5, 5, 10, 20 and 30 ng mL^{-1}) injected three times onto the chromatography system. Linear regression analysis was used to determine the slope and correlation coefficient of the calibration curve.

Firstly, tissue samples from untreated animals (blank tissues) were analysed to confirm IVM absence and the specificity of the analytical methods and then blank tissue samples were fortified with IVM ranging between 0.5 and 30 ng g^{1}. IVM concentrations were determined from peak areas and the use of calibration curves obtained by running tissue samples from rabbits not treated with IVM (i.e. rabbits control) that were spiked with known concentrations of IVM. For tissue specimens as determined using the linear least squares regression procedure, a linear relationship existed in the calibration curve of IVM over the range of 0.5 and 30 ng g^{1} for muscle, liver, kidney and fat.

Precision and accuracy of the method were determined by evaluation of replicates of IVM-free samples (n = 6) fortified with IVM at different concentrations (0.5, 5, 10, 20, 30 ng g^{-1}). Precision was expressed as coefficient of variation (% CV). Accuracy, defined as the closeness between the experimentally measured and the true value, was determined by the recovery percentage [18].

The LOQ was calculated as the lowest IVM concentration (n = 6) on the standard curve that could be quantitated with precision not exceeding 20% and accuracy within 20% of nominal. The LOD was estimated through the analysis of 10 aliquots of control tissue (free of IVM). The noise of the base-line was measured; the average and the standard deviation were calculated, the LOD corresponds to three of those SD (sign/noise ≥ 3/1).

Selectivity is the ability of the method to distinguish between the analyte being measured and other different substances which might be present in the sample being analysed. The selectivity of the method was determined by comparing the chromatograms of IVM-free tissue samples with the chromatograms of each tissue fortified with IVM. The lack of interferences in the separation suggests a high specificity of the chromatographic method and a good selectivity of the extraction procedure.

Tissue concentrations were expressed as nanograms per gram.

Withdrawal time

Withdrawal period estimation was done according to the Guideline on determination of withdrawal periods for edible tissues made by Committee for Medicinal Products for Veterinary Use (CVMP) [19].

The withdrawal periods for edible tissues of rabbits (muscle, liver, kidney and fat) were estimated by linear regression analysis of the log transformed tissue concentrations and determined at the time when the upper one-sided 95% tolerance limit for the residue was below the MRLs, with a confidence of 95%. IVM concentrations in function of time found in muscle, kidney, liver and fat were plotted and analyzed with the program WT version 1.4 in order to recommend a period of withdrawal time for this experimental formulation.

Results

No adverse response was observed clinically for the treatment during the study.

The development and validation were successfully accomplished. This method performed accurately and reproducibly over a range of 0.5 to 30 ng g^{-1} for IVM.

This assay exhibited a linear dynamic range between 0.5 and 30 ng mL^{-1}. A linear relationship was obtained across one dynamic range with r values ranged from 0.997832 to 0.998658 for IVM (Figure 1). The linearity was between r = 0.99648 to 0.99850 values ranged over 0.5 and 30 ng g^{-1} of ivermectin concentration range in all tissues assayed.

The specificity of the method was demonstrated by the absence of interferences and the adequate symmetry of chromatograms. The percentage of recovery ranged from 87.27 to 101.13% for all tissues analyzed and different drug concentrations (Table 1). The limit of detection (LOD) was established in 0.18 ng g^{-1} for adipose tissue, 0.26 ng g^{-1} for liver, 0.43 ng g^{-1} for kidney and 0.37 ng g^{-1} for muscle. The LOQ was 0.5 ng g^{-1} similar for these tissues. The validated analytical methodology showed satisfactory results of sensitivity, precision and accuracy intra-day and inter-day; that allow its use for the detection and quantification of tissue residues of IVM in rabbits.

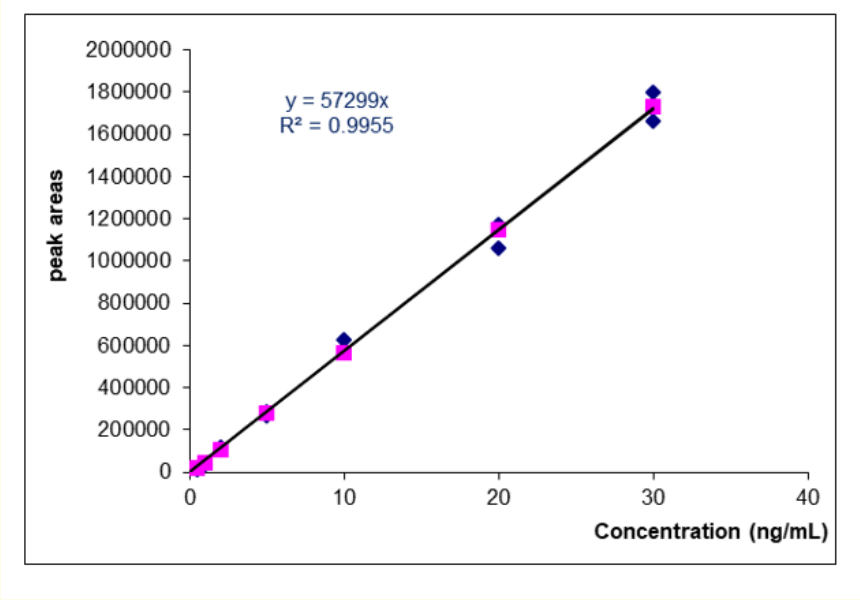

Figure 1: *Calibration curve of ivermectin standard.*

The validated analytical methodology showed satisfactory results of sensitivity, precision and accuracy that allow its use for the detection and quantification of tissue residues of IVM in rabbits.

IVM tissue concentrations

Ivermectin was detected in all the tissues evaluated, which demonstrated its high. lipophilicity and extensive distribution. In figure 2 the mean ± SD tissue concentrations of IVM in muscle, kidney, liver and fat at different days after the end of treatment are presented. The higher concentrations were found in adipose tissue (386.58 ± 32.44 ng g^{-1}) and liver (188.13 ± 32.57 ng g^{-1}) at 10 days, the drug concentrations in both tissues maintained for a period of 40 days' post treatment with 3.50 ± 0.38 ng g-1 and 2.64 ± 0.50 ng g^{-1}, respectively. The lowest concentrations of IVM in tissues were observed in muscle samples (Figure 2).

			Intra-day		Inter-day (over 3 days)	
Tissue	**r**	**ng.g^{-1}**	**Recovery (%) n = 6**	**Precision (%) n = 6**	**Recovery (%)**	**Precision (%)**
Muscle	0.99783 (0.5 - 30 ng g^{-1})	0.5	101.13	2.99	97.56	10
		5	89.99	6.03	93.45	2.44
		10	108.23	11.47	102.12	3.56
		20	99.18	3.24	97.26	4.13
		30	99.46	1.42	97.33	1.41
Kidney	0.99850 (0.5 - 30 ng g^{-1})	0.5	98.59	0.21	100	2.2
		5	92.44	3.35	93.33	2.47
		10	95.12	2.29	89.99	4.54
		20	90.57	1.65	87.58	5.34
		30	98.07	0.68	96.67	2.99
Liver	0.99843 (0.5 - 30 ng g^{-1})	0.5	95.63	5.28	91.23	3.28
		5	88.53	3.03	87.69	1.97
		10	93.11	2.72	89.99	5.12
		20	92.39	1.54	90.67	2.98
		30	95.49	4.77	96.34	5.15
Fat	0.99648 (0.5 - 30 ng g^{-1})	0.5	95.63	5.28	91.23	3.28
		5	88.53	3.03	87.27	1.97
		10	93.11	2.72	89.99	5.12
		20	92.39	1.54	90.67	2.98
		30	95.49	4.77	96.34	5.15

Table 1: *Ivermectin recovery and precision intra-day and inter-day from tissue samples spiked with IVM.*

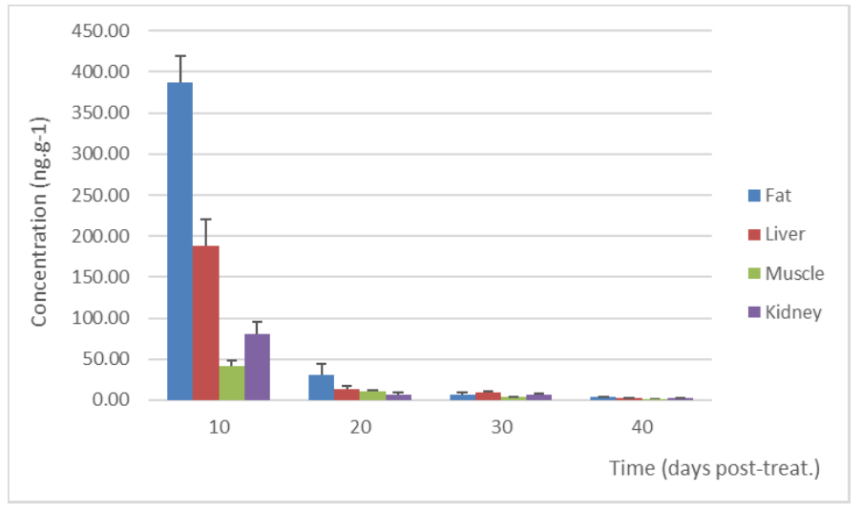

Figure 2: *Mean ± SD tissue concentrations of IVM in rabbits after subcutaneous administration at a single dose of 200 µg kg^{-1} body weight.*

Linear regression analysis of the logarithmic transformed data can be considered for the calculation of the withdrawal periods. Using this approach, the withdrawal time is determined as the time when the one-sided, 95% upper tolerance limit of the regression line with a 95% confidence level is below the MRL. According to the residual concentrations found, optimal withdrawal times for edible tissues were 22 days for liver, 25 days for fat, 18.44 days for muscle and curiously an extensive time for kidney, 27.51 (Figure 3).

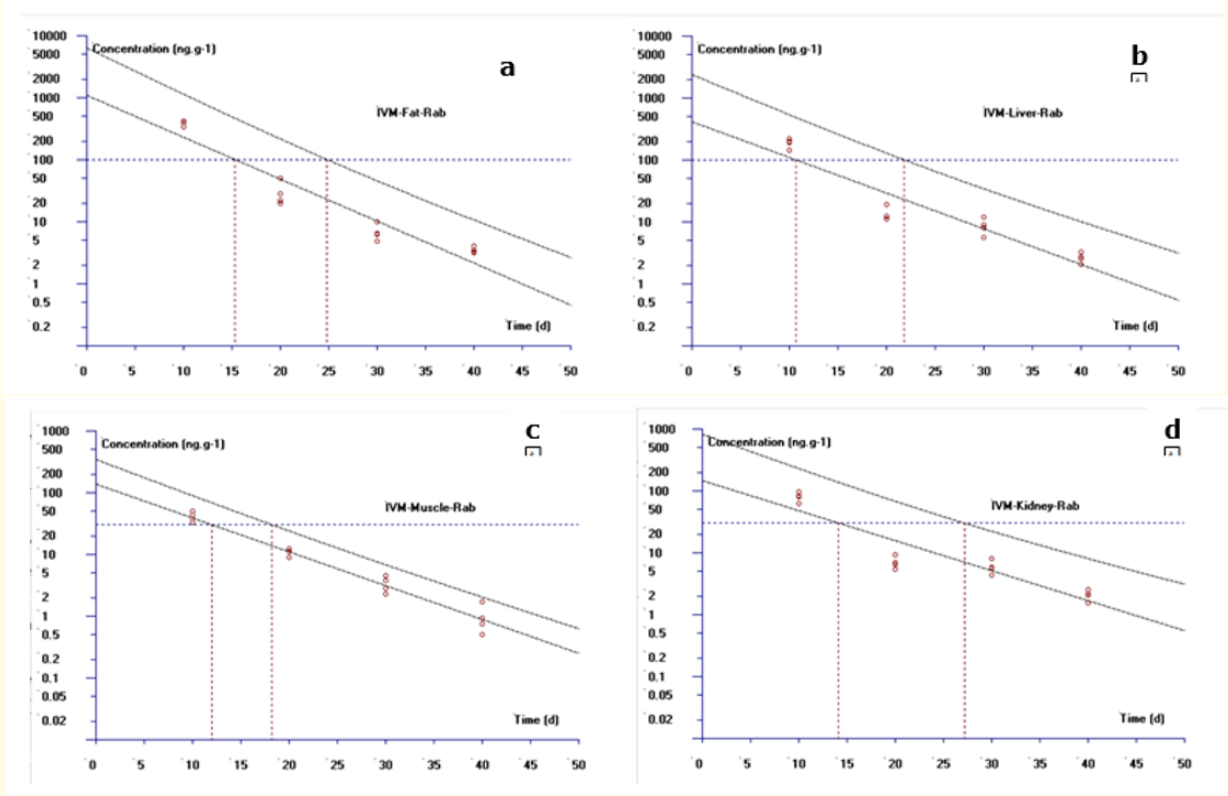

Figure 3: *Withdrawal times of IVM, calculated by linear regression analysis for Fat (a), Liver (b), Muscle (c) and Kidney (d) (logarithmic transformed data).*

Discussion and Conclusion

The available information reporting tissue residue profiles after IVM treatment is limited in rabbits compared to other species. Macrocyclic lactones endectocides, as ivermectin, are lipophilic compounds, so high concentrations will be found in edible tissues, particularly in those with a high fat content [20].

The available information about tissue residue profiles of IVM after its use in rabbit's treatments is scarce compared to other species [21,22]. Whereby edible tissues containing veterinary drug residues, can pose risks to human health, including direct toxic effects, allergic reactions, and increased parasite resistance [23].

The residue depletion profile observed in the present study was consistent with that previously described by us in chicken poultry [3] and other authors in different animal's species, as in cattle [7,24], sheep [24], goats [5] and hens [22], who reported that IVM produced higher concentrations in fat than in other edible tissues.

In the present study at 20 days post-treatment, IVM concentrations in all tissues dropped below the MRL. However, they were measurable up to 40 days after treatment above the LOQ of our analytical methodology. Our results do not agree with Shen., *et al*. [25], who analyzed the depletion profiles of doramectin (DRM) administered subcutaneously to rabbits at a rate of 400 μg kg^{-1} of body weight. It is strange because the waiting times are similar to ours. Shen., *et al.* (2009) reported that at 24 days post treatment, the DRM concentrations in all muscle samples dropped below the LOQ of the method and at 35 days post treatment, the DRM concentrations examined in all kidney samples and in four of five liver samples dropped below the LOQ of the method. But notably, at 46 days post treatment, the DRM residue concentration in one of five fat samples remained at a level higher than the LOQ (1 ng g^{-1}) of the method, indicating that DRM residue was eliminated slowly in fat tissues.

As we mentioned, the higher concentrations were found in adipose tissue and liver at 10 days, the drug concentrations in both tissues maintained for a period of 40 days' post treatment This finding is very logical since IVM is a molecule that have a strong affinity for adipose tissue and undergoes extensive hepatic metabolism, mainly by hydroxylation processes.

An interesting finding was that kidney tissue showed residual concentrations until day 27.51. Despite it is known that IVM is eliminated mainly by faecal excretion (90%), and less than 2% by urine, Al-Jassim., *et al.* [26] reported nephrotoxicity after IVM administration in rabbits, like vacuolation of cortical tubules and atrophy of glomeruli. This damage in kidney may explain the difficulty for IVM excretion, and consequently the longer withdrawal time needed for this tissue.

We can conclude that the present findings corroborate in rabbits what was previously described in other species: IVM liposolubility explains the high concentrations found in fat and liver. The withdrawal periods for rabbit muscle, liver, kidney, and fat tissues were 18, 22, 27 and 25 days, respectively. So, the longest withdrawal time, 27 days, should be selected as the withdrawal period to guarantee consumer safety most reliably.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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