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Residue Depletion of Eprinomectin in Bovine Tissues after Subcutaneous Administration

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A study of the tissue depletion of eprinomectin (EPR) subcutaneously administered to cattle at a dose of 500 mg per kg of body weight was carried out. EPR concentrations were determined in muscle, liver, kidney, and fat. Twenty-four parasite-free cross cattle were treated with the EPR injectable oil formulation. Three treated animals (two males and one female) were selected randomly to be sacrificed at 1, 3, 7, 14, 21, 28, 42, and 56 days withdrawal after injection. EPR residue concentrations were determined using HPLC with fluorescence detection. Muscle samples showed the lowest EPR concentrations throughout the study period. The highest EPR concentrations at all sampling times were measured in liver tissue, indicating that liver is a target tissue for EPR. EPR concentrations in all of the tissues analyzed were below the accepted maximum residue limits recommended by the European Union at 8 days posttreatment.

KEYWORDS: Eprinomectin; subcutaneous administration; depletion; cattle; HPLC

INTRODUCTION

Eprinomectin [4′-(epiacetylamino)-4′-deoxyavermectin B1] is a semisynthetic member of the avermectin family of macrocyclic lactones. It was developed by Merck & Co., Inc. and marketed as a pour-on for use in beef and dairy cattle at a dose of 500 $\mu g \cdot kg^{-1}$ of body weight due to the most potent broad-spectrum activity against gastrointestinal nematodes, lice, mange mites, and flies (*1*−*5*). It consists of a mixture of two homologous components, B1a (not less than 90%) and B1b (not more than 10%), which differ by a single methylene group at the 25-carbon positon. The structures are shown in **Figure 1**.

Because EPR has a favorable partitioning profile between serum and milk (*6*), it is the only endectocide approved for use during lactation with a zero milk withdrawal period. However, it is used only as a pour-on formulation. Recently, Alvinerie reported that subcutaneous administration of endectocides led to a higher bioavailability of the drug, compared with that of a pour-on application (*7*). Moreover, some studies revealed the tremendous anthelmintic potential of eprinomectin against gastrointestinal nematodes in cattle when used as an injectable formulation (*8*−*10*). Alvinerie and co-workers (*11*−*12*) found that the area under the concentration−time curve (AUC) after subcutaneous administration of 0.2 $mg \cdot kg^{-1}$ eprinomectin (68.5 ± 3.2 $ng \cdot day^{-1} \cdot mL^{-1}$) and the AUC ($72.3 \pm 11.15$ $ng \cdot day^{-1} \cdot mL^{-1}$) for goats after a pour-on administration of 0.5 $mg \cdot kg^{-1}$ eprinomectin was similar, which showed that subcutaneous administration is 2.5 times more effective than pour-on administration concerning the amount of drug present in the animal body. Shi et al. (*13*) reported that the main pharmacokinetic parameters were the elimination half-life of 26.22 h, the AUC of 1.19 $mg \cdot h^{-1} \cdot L^{-1}$, the peak EPR concentration in plasma

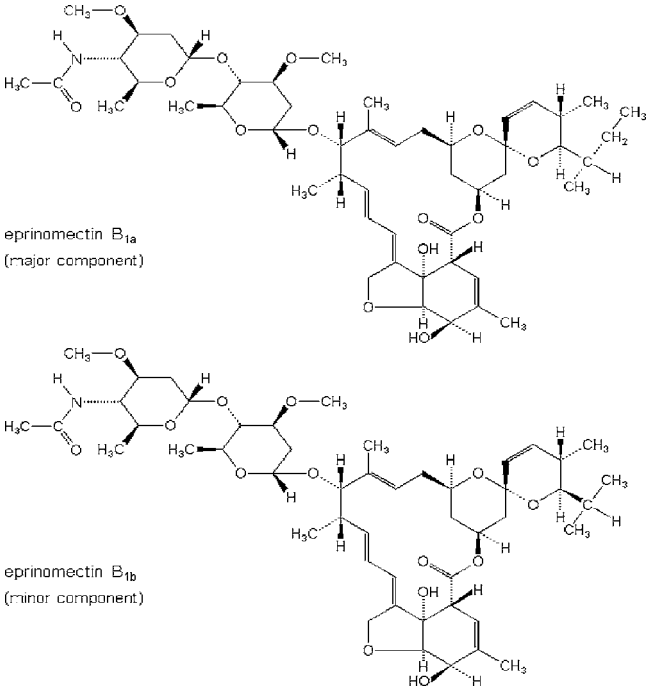

Figure 1. Structure of eprinomectin (B1a and B1b).

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of 20 ng·mL^{-1}, and the peak time of 15.36 h after sheep were administered subcutaneously at a single dose of 200 μg·kg^{-1} of body weight, which showed that EPR was distributed widely and eliminated slowly. But until now no tissue residue data have been available concerning the EPR injectable preparation. The aim of the present work was to study the depletion profiles of EPR in cattle tissues to establish the withdrawal period after subcutaneous administration.

MATERIALS AND METHODS

Reagents, Chemicals, and Materials. The EPR injectable oil formulation (1%, w/v) used in this study was provided by Zhejiang Haizheng Co., Zhejiang Province, P.R. China. The EPR standard (95.3% purity; 90.4% B1a and 4.9% B1b) was supplied by Merck Research Laboratories (Rahway, NJ). 1-Methylimidazole was purchased from Fluka Co. (Steinheim, Germany), and trifluoroacetic anhydride was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Acetonitrile and methanol were obtained from Dikma Technology Inc. (Richmond Hill, U.S.A.). Milli-Q organic-free water (Millipore, Bedford, MA) was used. All reagents were analytical grade. Solid-phase extraction cartridges (Bond Amino cartridges, 500 mg/6 mL, Agilent Technologies, U.K.) were used to clean up tissue samples.

Standards. A stock solution of 1 mg·mL^{-1} was prepared by dissolving 100 mg of EPR standard in 100 mL of acetonitrile. The working standard solutions of 2, 5, 10, 20, 50, 100, and 200 ng·mL^{-1} were prepared in acetonitrile.

Animal Treatment. The trial was conducted in 27 adult parasite-free cross cattle (18 males, 9 females) with an average weight of 300 kg. Animals were housed in a barn with concrete-floored pens. During acclimatization for 3 weeks, and the subsequent treatment periods, they were fed drug-free balanced rations ad libitum with free access to water and the health of the animals was monitored by a professional veterinarian. The experimental animals (n = 24, 16 males and 8 females) were weighed and treated on the same day with EPR injected subcutaneously at 500 μg·kg^{-1} of body weight on the left side of the neck. Three animals were kept as untreated controls. Three animals of the treated group (2 males, 1 female) were killed at 1, 3, 7, 14, 21, 28, 42, and 56 days withdrawal after injection. Samples of muscle, liver, kidney, and fat were collected from each animal and stored at −20 °C until they were processed. The untreated animals were sacrificed on day 21 posttreatment to obtain blank tissues.

Sample Preparation. The extraction of EPR from fortified and incurred tissue samples was performed following a little modification of the technique described by Payne et al. (*14*). Briefly, bovine tissues (muscle, liver, kidney, and fat) were minced and homogenized in a homogenizer for 2 min. An amount of 5 g of homogenate was mixed with 5 g or more of anhydrous sodium sulfate in a polypropylene centrifuge tube. A volume of 30 mL of methylene chloride/acetone (1:1, v/v) was added. The mixture was shaken on a shaker for 5 min and centrifuged at 3000 rpm for 5 min. The supernatant was transferred into a heart-shaped flask. The remaining material was re-extracted twice by shaking with 15 mL aliquots of methylene chloride/acetone (1:1, v/v) solution. The combined extract was evaporated to dryness, and the residue was reconstituted in 10 mL of methylene chloride and was loaded onto an amino extraction cartridge conditioned with 10 mL of methylene chloride. The cartridge was rinsed with 10 mL of methylene chloride and 10 mL of toluene. Before adding 10 mL of ethanol/ethyl acetate solution (1:1, v/v) to elute the sample the cartridge was dried for 1−2 min with vacuum. The collected eluate was evaporated to dryness and reconstituted in 5 mL of methanol. A portion of the sample solution (0.5 mL) was evaporated to dryness.

Derivatization. This procedure mainly followed the reported derivatization method (*15*). A 200 μL portion of methylimidazole/acetonitrile (2:7, v/v) was added to the dry tube, which was stoppered and vortexed for 2 min. Another 200 μL portion of trifluoroacetic anhydride/acetonitrile (2:7, v/v) was added, and the tube was stoppered and vortexed for 2 min. A 45 μL portion of glacial acetic acid was added, and the tube was stoppered and vortexed for 1 min. The tube was incubated in an oven for 30 min at 65 °C, cooled in a refrigerator (4 °C) for 3 min, and then left at room temperature (18 °C) for 12 min. The derivatized sample solution was filtered through a 0.45 μm filter before an aliquot (20 μL) was subjected to high-performance liquid chromatography (HPLC).

Table 1. Recoveries of EPR from Bovine Tissues Fortified at Levels of 20, 50, and 100 ng·g^{-1} (n = 4)

		interassay		intra-assay	
tissue	added (ng·g^{-1})	recovery (%)	CV[a] (%)	recovery (%)	CV (%)
muscle	20	82.5	7.2	84.5	8.6
	50	86.4	6.5	87.4	7.3
	100	87.2	8.3	88.5	8.9
liver	20	70.5	4.5	73.5	5.6
	50	77.2	5.6	74.8	6.8
	100	84.7	6.9	88.2	7.9
kidney	20	73.5	8.9	72.5	10.3
	50	74.4	7.6	76.2	8.7
	100	76.9	7.2	80.3	8.8
fat	20	82.5	8.5	81.5	9.6
	50	85.8	5.6	87.2	7.8
	100	82.3	6.1	85.5	7.2

[a] CV, coefficient of variation.

Table 2. EPR Residue Concentrations (ng·g^{-1}) in Bovine Tissues after Subcutaneous Administration at a Dose of 500 μg·kg^{-1} of Body Weight

withdrawal time (day)	animal no.	sex	muscle	liver	kidney	fat
1	1	male	37.5	1689.3	311.5	223.7
	2	male	56.2	1136.5	237.8	279.8
	3	female	28.6	1455.6	283.5	201.2
3	4	male	22.6	1125.3	229.7	186.2
	5	male	17.8	944.6	168.6	199.3
	6	female	26.4	1032.4	237.5	125.9
7	7	male	12.6	822.7	156.3	39.5
	8	male	7.5	696.3	176.5	61.4
	9	female	9.4	900.1	146.2	55.8
14	10	male	5.2	586.9	122.2	35.5
	11	male	4.3	555.3	141.6	24.6
	12	female	3.9	512.5	117.3	28.6
21	13	male	3.8	448.2	101.6	22.8
	14	male	2.0	479.3	83.4	20.3
	15	female	2.9	421.9	75.8	16.9
28	16	male	ND[a]	265.4	49.5	7.5
	17	male	ND[a]	221.7	36.3	11.2
	18	female	ND[a]	282.5	31.2	8.3
42	19	male	ND[a]	49.6	10.7	5.6
	20	male	ND[a]	86.5	8.3	7.1
	21	female	ND[a]	103.7	18.6	4.6
56	22	male	ND[a]	27.6	2.7	ND[a]
	23	male	ND[a]	19.5	ND[a]	ND[a]
	24	female	ND[a]	15.3	ND[a]	ND[a]

[a] ND, not detected.

HPLC Analysis. The analysis of standards, fortified samples, and incurred samples was performed using an HPLC system at room temperature. The HPLC system included a Waters 2695 separations module and a Waters 2475 fluorescence detector with an autosampler (Waters Co., Milford, MA). The chromatographic column was a reversed-phase column (Inertsil ODS, 4.6 mm i.d. × 250 mm, 5 μm, GL Sciences Inc., Tokyo, Japan). The mobile phase was methanol/water (97:3, v/v) at a flow rate of 1 mL per min. The fluorescence detector settings were an excitation wavelength of 365 nm and an emission wavelength of 475 nm.

Calibration. The calibration curves were prepared with the peak areas and the working standard solution concentrations. A volume of 0.5 mL of the working standard solutions of 2, 5, 10, 20, 50, 100, and

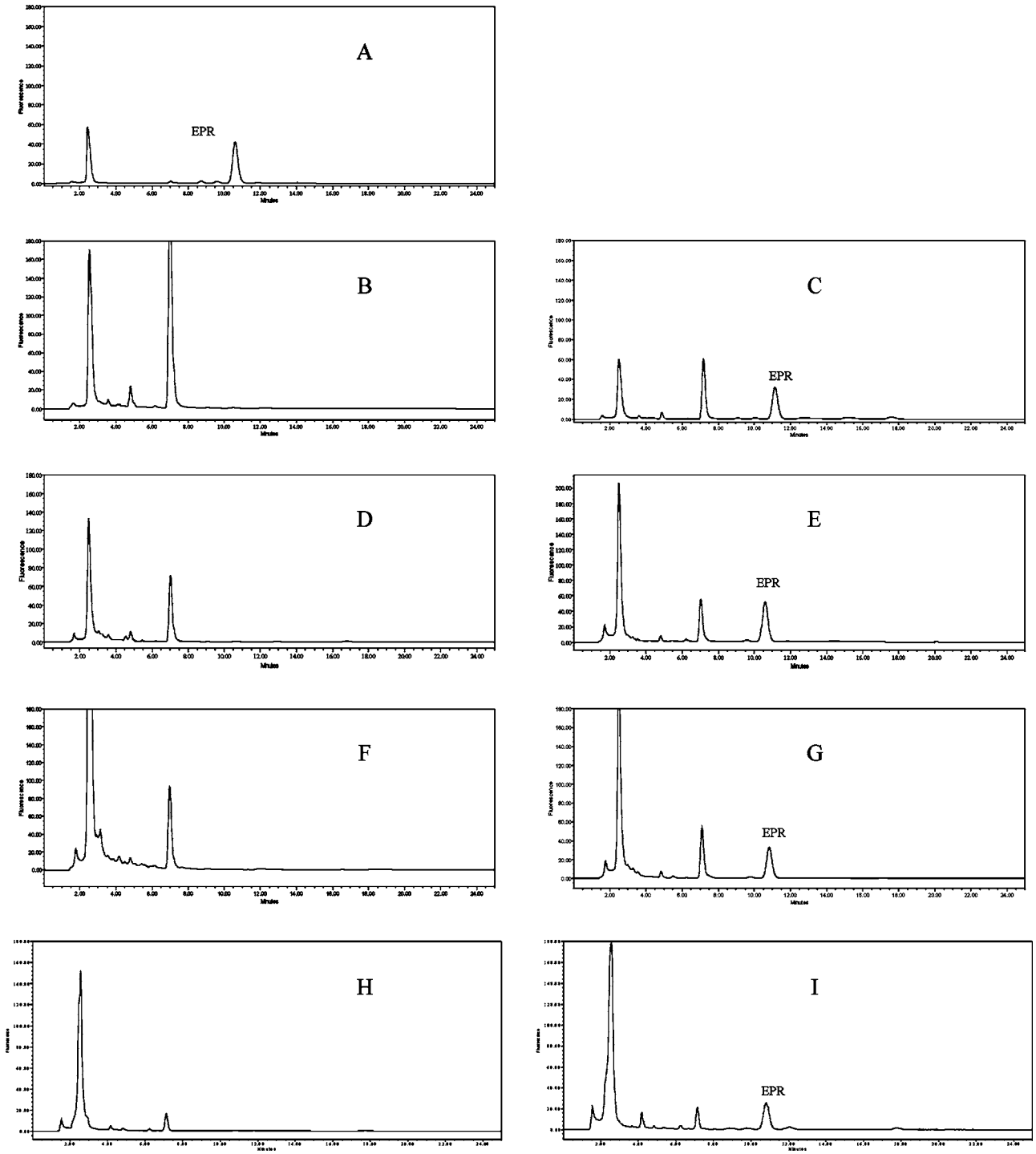

Figure 2. Chromatograms of (**A**) EPR standard (20 ng·mL^{-1}); (**B**) control bovine muscle; (**C**) fortified bovine muscle (20 ng·g^{-1}); (**D**) control bovine liver; (**E**) fortified bovine liver (20 ng·g^{-1}); (**F**) control bovine kidney; (**G**) fortified bovine kidney (20 ng·g^{-1}); (**H**) control bovine fat; (**I**) fortified bovine fat (20 ng·g^{-1})

200 ng·mL^{-1} was evaporated to dryness and derivatized as described above.

Data Analysis. The withdrawal period was estimated by linear regression analysis of the log-transformed tissue concentrations and determined at the time when the one-sided 95% upper tolerance limit was below the MRLs (*16*).

RESULTS AND DISSCUSION

Formulation Selection. The therapeutic efficacy of endectocides depends on the formulation of the dosage form, route of administration, bioavailability, pharmacokinetic behavior, and metabolism patterns. Among avermectin analogues, ivermectin and doramectin are developed as injectable formulations, which have excellent efficacy against endoparasites and ectoparasites. EPR injection has the same effect as them. Pan et al. (*10*) reported that the EPR injectable formulation administered subcutaneously at a low dose of 200 μg·kg^{-1} of body weight to beef cattle was very safe and highly efficient against *Haematopinidae eurysternus*. EPR killed all lice in 7 days and prevented reinfection in 42 days. In this research the EPR formulation subcutaneously injected to the experimental cattle was well tolerated. No adverse reactions at the site of the subcutaneous injection or indirectly caused by the drug were observed.

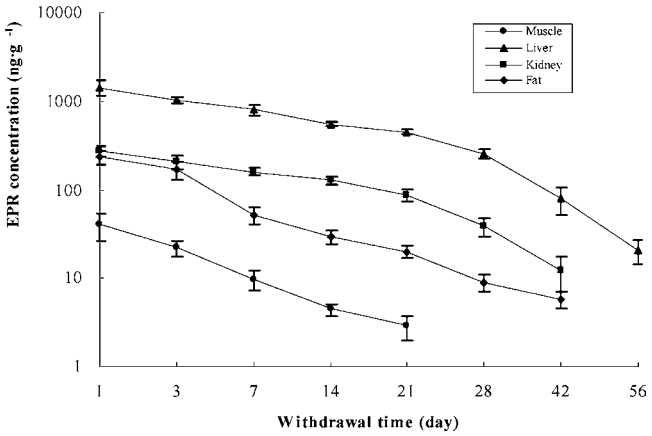

Figure 3. Residue depletion curves of EPR from bovine muscle, liver, kidney, and fat after subcutaneous injection at a single dose of 500 μg per kg of body weight.

Analytical Method Feasibility. The analytical method was performed following a combination of sample extraction and derivatization in different reports (*14*, *15*). Both of them are validated to analyze hundreds of bovine tissue samples. In the present study the combined analytical method is demonstrated to be reliable and stable. The standard curves were linear from 2 to 200 ng·mL^{-1} ($R^2 = 0.9988$). The limit of detection (LOD) and the limit of quantitation (LOQ) for the method are 1 ng·g^{-1} and 2 ng·g^{-1}, respectively. The accuracy and precision of the method were determined using bovine muscle, liver, kidney, and fat samples fortified at levels of 20, 50, and 100 ng·g^{-1}. Interassay mean recovery of the analytes was between 70.5% and 87.2% with coefficients of variation (CVs) of 4.5−8.9%. Intra-assay mean recovery of the analytes was between 72.5% and 87.4% with coefficients of variation (CVs) of 5.6−10.3% (**Table 1**). The chromatograms of the standard, control, and fortified bovine tissues are shown in **Figure 2**. The lack of interferences in the chromatographic separation demonstrates a high specificity of the analytical method and a good selectivity obtained in the extraction procedure.

Residue Depletion. The incurred bovine tissue samples were collected from 24 herds of cattle, which were divided into eight groups (each of 2 males and 1 female) randomly. The concentrations of EPR measured in muscle, liver, kidney, and fat tissues from cattle administered subcutaneously at a dose of 500 μg·kg^{-1} of body weight and slaughtered at 1, 3, 7, 14, 21, 28, 42, and 56 days posttreatment are summarized in **Table 2**. At 1 day postadministration the peak concentrations in all tissues (muscle, 28.6−37.5 ng·g^{-1}; liver, 1136.5−1689.3 ng·g^{-1}; kidney, 237.8−311.5 ng·g^{-1}; fat, 201.2−279.8 ng·g^{-1}) were determined. The results showed liver should be considered to be the target tissue for EPR in cattle. At 56 days posttreatment the concentrations of EPR in liver tissue ranged from 15.3 to 27.6 ng·g^{-1}, which were detected in kidney tissue of one animal and not detected in fat tissue. Moreover, as shown in **Table 2**, there were no significant differences between the data obtained from male and female animals at each sampling time point. The depletion curves, shown in **Figure 3**, were constructed using the average residue concentrations in bovine tissues and the withdrawal day. A comparison of four curves showed the EPR residues in bovine liver and fat were eliminated very slowly.

In comparison with the EPR concentrations in muscle (8 ± 3 ng·g^{-1}), liver (977 ± 136 ng·g^{-1}), kidney (181 ± 62 ng·g^{-1}), and fat (34 ± 15 ng·g^{-1}) obtained from cattle following a topical dose of 5-^{3}H-EPR at 500 μg·kg^{-1} of body weight at 7 days postadministration (*17*), the determined results in the present study showed no significant difference. At 28 days postadministration the EPR concentrations in liver (185 ± 55 ng·g^{-1}), kidney (30 ± 10 ng·g^{-1}), and fat (5 ± 2 ng·g^{-1}) were lower than those (liver (256.5 ± 31.4 ng·g^{-1}), kidney (39.0 ± 9.4 ng·g^{-1}), and fat (9.0 ± 1.9 ng·g^{-1})) in this trial except for the EPR concentration in muscle (2 ± 0 ng·g^{-1}), which was below the LOD (1 ng·g^{-1}). The reason may be due to the formulation of the dosage form and route of administration.

The Committee for Veterinary Medicinal Products of the European Union has established the maximum residue limits (MRLs) for EPR in cattle (*18*). The MRLs for EPR are 50, 1500, 300, and 250 μg·kg^{-1} in muscle, liver, kidney, and fat, respectively. As shown in **Table 2** and **Figure 4**, at 3 days posttreatment the EPR concentrations in all tissues from cattle subcutaneously administered at a dose of 500 μg·kg^{-1} of body weight were below the accepted MRLs. However, due to the

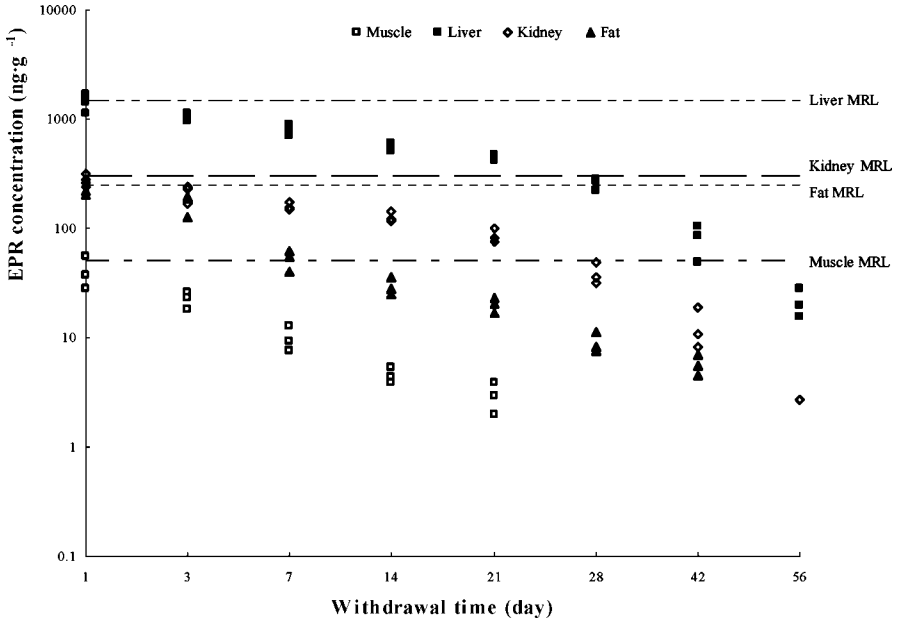

Figure 4. Comparison of EPR residues in individual animals from bovine muscle, liver, kidney, and fat with the EU MRLs (50, 1500, 300, and 250 ng·g^{-1}, respectively).

high interindividual variability and limited test animal numbers, to avoid potential hazards to human health, the withdrawal periods were estimated by the statistical method stated in the guidance (*16*), which were 4 days for muscle, 7 days for liver, 8 days for kidney, and 7 days for fat. Moreover, most public reports did not including the residue data on the injection site which is often much higher than kidney, fat, and muscle samples from remote sampling points, even higher than the target tissue liver at 1−3 days posttreatment. To guarantee consumer safety, the drug residues at the injection site may be fully considered when the withdrawal periods and MRLs were set.

In conclusion, the residues in bovine tissues are eliminated slowly after cattle were administered subcutaneously at a dose of 500 $\mu g \cdot kg^{-1}$ of body weight. A withdrawal period of 8 days is proposed to avoid the presence of violative residues in bovine edible tissues.

ABBREVIATIONS USED

EPR, eprinomectin; HPLC, high-performance liquid chromatograph; CV, coefficient of variation; SD, standard deviation; *R*, correlation coefficient; LOD, limit of detection; LOQ, limit of quantitation; MRL, maximum residue limit.

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