JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Residue Depletion of Tilmicosin in Cattle after Subcutaneous Administration

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A study of tissue residue depletion of tilmicosin in cattle was conducted after a single subcutaneous injection at the therapeutic level of 10 mg per kg body weight. Eighteen cross cattle were treated with the tilmicosin oil formulation (30%). Three treated animals (two males and one female) were selected randomly to be scarified at 1, 7, 14, 28, and 35 days withdrawal after injection. Samples of the injection site and of muscle, liver, kidney, and fat were collected. Tilmicosin residue concentrations were determined using a high-performance liquid chromatography (HPLC) method with a UV detector at 290 nm. Using a statistical method recommended by the Committee for Veterinary Medical Products of European Medical Evaluation Agency, the withdrawal time of 34 days was established when all tissue residues except samples in the injection site were below the accepted maximum residue limits.

KEYWORDS: Tilmicosin; residue; depletion; cattle; withdrawal time

INTRODUCTION

Tilmicosin [20-deoxy-20-(3,5-dimethyl)piperidin-1-yl] desmycosin, a semisynthetic macrolide antibiotic derived from tylosin, is composed of *cis*- and *trans*-piperidinyl isomers in an approximate ratio of 85:15. The structure of tilmicosin is shown in **Figure 1**. Tilmicosin has in vitro activity against Gram-positive organisms and mycoplasma and is active against certain Gram-negative organisms, such as *Haemophilus somnus*, *Pasteurella haemolytica*, and *Pasteurella multocida*. Other Gram-negative organisms tested, including *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella*, and *Serratia* species, are very sensitive to tilmicosin. Some strains of *Actinomyces* also are extremely sensitive to tilmicosin. In vitro, more than 90% of *P. multocida* and/or *P. haemolytica* isolates tested were sensitive to tilmicosin at a concentration of <6.25 μg mL^{-1} (*1*, *2*). These bacterial species are often isolated from pneumonic lung tissue in young calves with a pneumoenteritis complex and in feedlot cattle with respiratory disease. Many different studies show it can be used effectively and safely in the treatment of respiratory diseases in cattle (*1*, *3*−*8*), calves (*9*−*11*), sheep (*6*, *12*, *13*), and pigs (*14*−*16*).

Tilmicosin residue in bovine muscle, liver, kidney, or fat has been determined by liquid chromatographic methods with UV detection. The detection wavelengths are in the range of 280−290 nm (*17*−*23*).

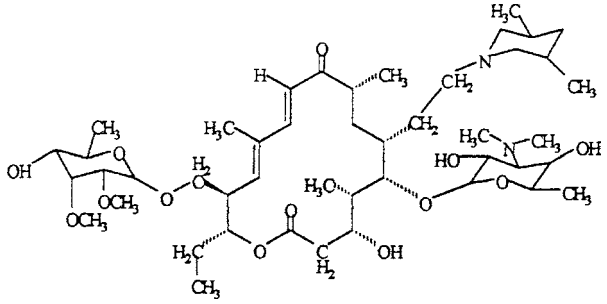

Figure 1. Molecular structure of tilmicosin.

Table 1. MRLs for Tilmicosin in Bovine Tissues (mg kg^{-1})[a]

	People's Republic of China	European Union	United States
muscle	0.1	0.05	0.1
fat	0.1	0.05	
liver	1	1	1.2
kidney	0.3	1	

[a] Blank cells, not recommended.

Tilmicosin injection has been approved in several countries to control *Pasteurella* diseases in bovine. However, although many countries recommended the maximum residue limits (MRLs) shown in **Table 1** for the injection formulation in cattle (*24*−*26*), at present, there are few reports on tilmicosin residue depletion in bovine tissues.

The aim of the present work was to study the depletion profiles of tilmicosin in cattle tissues and to verify the suggested

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10.1021/jf060863x CCC: $33.50 © 2006 American Chemical Society
Published on Web 06/13/2006

withdrawal time of 28 days after receiving a single subcutaneous injection at the therapeutic dose of 10 mg kg^{-1} body weight (*27*).

MATERIALS AND METHODS

Solvents and Reagents. The solvents used were of liquid chromatography (LC) grade, available from commercial sources. Tilmicosin reference standard was a gift from Lilly Co. (Greenfield, IN). Tetrahydrofuran, methanol, and acetonitrile were from Sigma Chemical Co. (St. Louis, MO). Dibutylamine, ammonium acetate, and phosphoric acid (85%) were purchased from Beijing Chemical Reagent Co. (Beijing, People's Republic of China). The water used was prepared with a Milli-Q system (Millipore, Bedford, MA). Dibutylammonium phosphate (DBAP) solution was prepared by the addition of 168 mL of dibutylamine to 700 mL of phosphoric acid (85%). The solution was allowed to cool and was adjusted to pH 2.5 using phosphoric acid (85%), and the volume was made up to 1 L with water. Monobasic potassium phosphate buffer was prepared by dissolving 13.61 g of monobasic potassium phosphate in 800 mL of water, adjusting the pH to 2.5 with phosphoric acid (85%), and making up the volume to 1 L with water. A 0.1 mol L^{-1} amount of ammonium acetate−methanol−acetonitrile solution was prepared by dissolving 7.71 g of ammonium acetate in 200 mL of methanol and 790 mL of acetonitrile and making up the volume to 1 L with acetonitrile. The tilmicosin injection containing 300 mg per mL was produced by Jining Medicine Corp. (Shandong Province, People's Republic of China).

Standards. A stock solution of 2 mg mL^{-1} was prepared by dissolving 200 mg of tilmicosin standard in 100 mL of acetonitrile. The working standard solutions of 0.02, 0.1, 0.5, 5, 10, and 50 μg mL^{-1} were prepared in acetonitrile.

Apparatus. LC equipment included a Waters 2695 separations module and a Waters 2996 photodiode array detector with an autosampler (Waters Co., Milford, MA). The chromatographic column was a reversed phase column (Extend-C_{18}, Zorbax column, 4.6 mm i.d. × 250 mm, 5 μm, Agilent Co., Palo Alto, CA). Solid phase extraction (SPE) cartridges (Bond Elut C_{18}, 500 mg/6 mL, Waters Co.) were used to clean up tissue samples.

Animal Treatment. To obtain the data about the residue of tilmicosin depletion in cattle, a residue depletion study was conducted. Eighteen head of cattle (12 males and six females) with an average weight of 225 kg were fed a nonmedicated ration and were administered tilmicosin subcutaneously on both sides of the neck at a single dose rate of 10 mg tilmicosin free base equivalents per kg body weight. Three animals (two males and one female) were killed at 1, 7, 14, 21, 28, and 35 days withdrawal after injection. Samples of the injection site and of muscle (breast and hind thigh), liver, kidney, and fat were collected from each animal and stored at −20 °C until they were processed.

Fortification. A fortifying test was performed by adding a microvolume of an aqueous standard solution containing tilmicosin to each portion of the weighed samples. The fortification levels for each tissue were 0.05, 0.5, and 5.0 μg g^{-1}. The sample extraction procedure is described below.

Sample Extraction. The sample extraction for tilmicosin from fortified and incurred tissues was performed following two reports with some modification (*17*, *19*). Briefly, bovine tissues (muscle, liver, kidney, and fat) were minced and homogenized in a homogenizer for 2 min. Five grams of homogenate was accurately weighed into a polypropylene centrifuge tube. Ten milliliters of acetonitrile was added and shaken for 20 min. Centrifugation was performed for 10 min at 3500 rpm. The supernatant was removed into a 100 mL polypropylene centrifuge tube, and 5 mL of monobasic potassium phosphate buffer and 8.0 mL of acetonitrile were added to the tissue pellet. The mixture was shaken for 20 min and centrifuged as before. Supernatants were combined, and 40.0 mL of water was added. The mixture solution was centrifuged at 3500 rpm for 10 min. The supernatant was subjected to SPE cleanup.

The SPE cartridge placed into a vacuum manifold system was conditioned with 10 mL of methanol followed by 10 mL of water prior to addition of the extracted supernatant. After the extract was drained through the cartridge by applying a vacuum, the cartridge was washed with 10.0 mL of water and 10.0 mL of acetonitrile. The SPE cartridge was dried for at least 3 min. Tilmicosin was eluted from the cartridge with 2.5 mL of 0.1 mol L^{-1} ammonium acetate−methanol−acetonitrile solution. The collected eluate was evaporated to dryness under a nitrogen stream at 30 °C in a water bath and then reconstituted in 1 mL of mobile phase. The processed sample solution was filtered with a 0.2 μm syringe filter and injected into the HPLC system.

Table 2. Average Accuracy and Precision of Tilmicosin in Fortified Tissue Samples ($n = 5$)

	μg g^{-1}		%	
samples	added	detected	average recovery	CV[a]
muscle	0.05	0.046	89.2	9.2
	0.5	0.458	91.6	7.5
	5.0	4.625	92.4	6.0
liver	0.05	0.425	85.0	10.1
	0.5	0.437	87.4	8.9
	5.0	4.411	88.1	6.7
kidney	0.05	0.039	79.2	8.9
	0.5	0.407	81.4	6.4
	5.0	4.105	82.1	5.6
fat	0.05	0.041	82.2	9.2
	0.5	0.387	77.4	5.9
	5.0	4.234	84.7	6.3

[a] CV, coefficient of variation.

LC Analysis. The analysis of standards, fortified samples, and incurred samples were performed at room temperature. The mobile phase was prepared by adding 135 mL of acetonitrile, 55 mL of tetrahydrofuran, and 25 mL of 1 M DBAP to 700 mL of water and diluting with water to the volume scale of 1000 mL; the flow rate was 1.0 mL min^{-1}, the injection volume was 100 μL, and the detection wavelength was 290 nm.

Calculations. The concentration of tilmicosin in tissue samples can be calculated by the following equation:

$$\text{concn } (\mu\text{g g}^{-1}) = \frac{(S - B) \times V \times F}{K \times M}$$

where S = combined area of the *cis*- and *trans*-tilmicosin peaks from the chromatogram; B = intercept of the standard curve; K = slope of the standard curve; M = weight of the tissue sample; V = final volume of sample extract; and F = dilution factor.

Data Analysis. The withdrawal time 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 European Union (EU) MRLs (*28*).

RESULTS AND DISCUSSIONS

LC Method Validation. In residue analysis and depletion study, it is very important to select an analytical method. The critical parameters for a method are linearity, accuracy, and precision. The method linearity was determined over the concentration range of 0.02−50 μg mL^{-1}. The method correlation coefficient is 0.9997. On the basis of signal-to-noise ratios of 3:1 and 6:1, the limits of detection (LODs) and the limits of quantitation (LOQs) were 0.01, 0.025, 0.02, and 0.02 μg g^{-1} and 0.02, 0.05, 0.04, and 0.04 μg g^{-1}, respectively, for bovine muscle, liver, kidney, and fat. The typical chromatograms for tilmicosin standard solution and extracts of control and fortified tissues are shown in **Figure 2**. Method accuracy and precision for the bovine tissues also were studied by fortifying the control tissues at the levels of 0.05, 0.5, and 5 μg g^{-1}. The recovery data were presented in **Table 2**. For all tested samples, the recoveries of tilmicosin were in the range of 79.2−92.4%, with coefficients of variation (CVs) of 5.6−10.1%. These data met

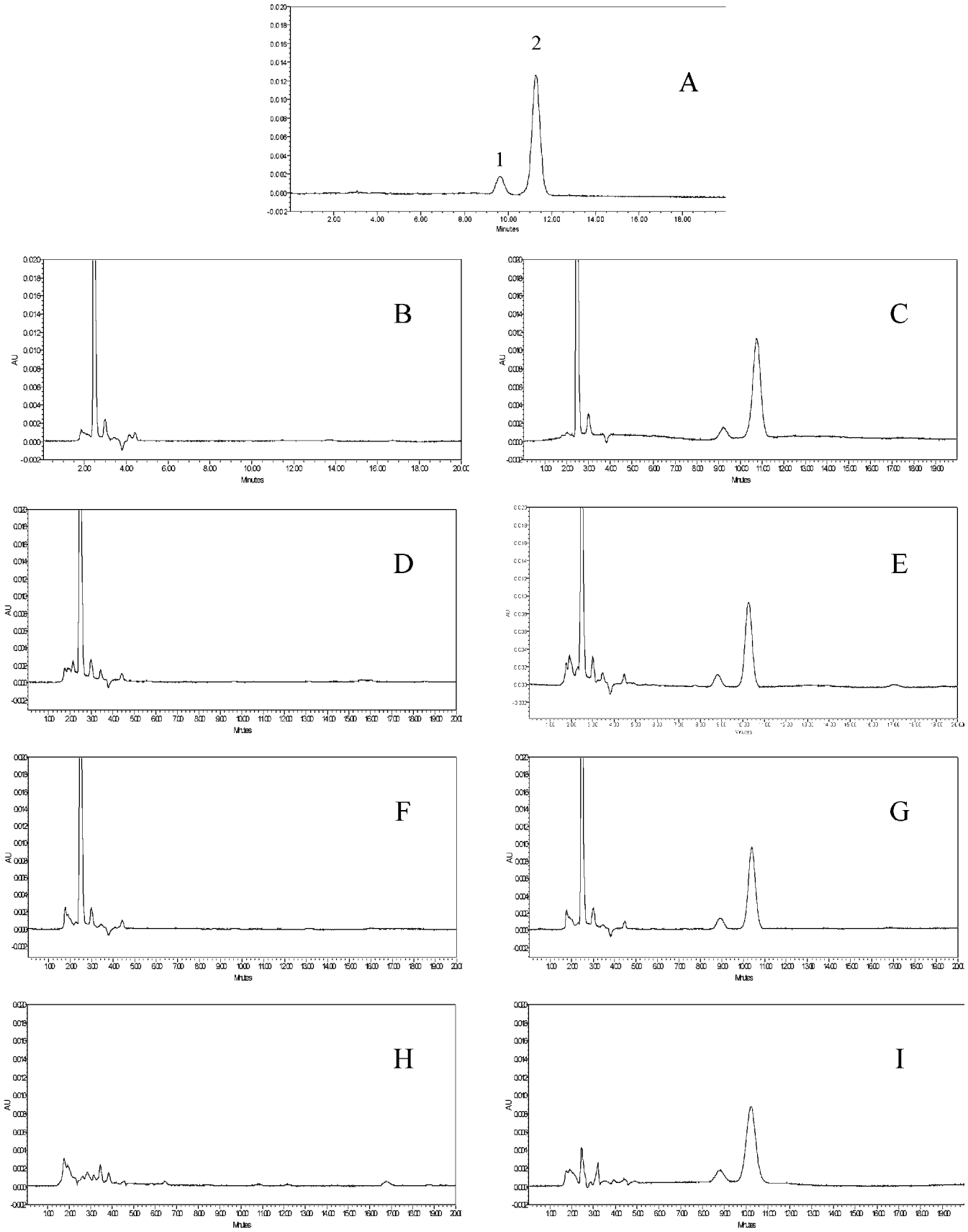

Figure 2. Chromatograms of (**A**) tilmicosin standard (0.5 μg mL^{-1}), (**B**) control bovine muscle, (**C**) fortified bovine muscle (0.5 μg g^{-1}), (**D**) control bovine liver, (**E**) fortified bovine liver (0.5 μg g^{-1}), (**F**) control bovine kidney, (**G**) fortified bovine kidney (0.5 μg g^{-1}), (**H**) control bovine fat, and (**I**) fortified bovine fat (0.5 μg g^{-1}). Peaks: 1, *trans*-tilmicosin isomer; 2, *cis*-tilmicosin isomer.

the acceptance criteria that for method accuracy an average recovery was between 70 and 110% and for method precision a CV was 15% or less for each group of samples.

Tilmicosin Residue Depletion. At present, only a few studies on tilmicosin residue or metabolism are reported. The authors focused on metabolic route, metabolite, residue in secretion, or

Table 3. Tilmicosin Residue Concentrations (ng g^{-1}) in Bovine Tissues after Subcutaneous Administration at a Single Dose of 10 mg kg^{-1} Body Weight

withdrawal time (day)	animal no.	sex	injection site	muscle	liver	kidney	fat
1	1	male	72.13	0.85	3.55	7.47	1.23
	2	male	63.15	0.69	4.78	8.74	0.99
	3	female	89.68	1.25	6.42	9.11	0.67
7	4	male	26.47	0.06	2.98	1.15	0.47
	5	male	21.58	0.04	3.42	1.79	0.39
	6	female	16.87	0.03	2.19	0.69	0.22
14	7	male	17.58	0.05	1.99	0.36	0.11
	8	male	8.52	<LOQ	2.21	0.55	0.06
	9	female	6.48	0.02	1.54	0.23	0.04
21	10	male	2.57	<LOD	1.37	0.24	<LOD
	11	male	3.33	<LOD	1.09	0.12	<LOD
	12	female	2.11	<LOD	0.98	0.09	<LOD
28	13	male	1.27	<LOD	0.92	0.13	<LOD
	14	male	1.75	<LOD	0.63	<LOQ	<LOD
	15	female	0.68	<LOD	0.73	<LOD	<LOD
35	16	male	0.53	<LOD	0.45	0.09	<LOD
	17	male	<LOQ	<LOD	0.38	<LOQ	<LOD
	18	female	<LOD	<LOD	0.12	<LOD	<LOD

effect of different administration ways (*29*−*32*). In this depletion study, shown in **Table 3**, at 1 day withdrawal, tilmicosin residue levels were highest at the injection site (63.15−89.68 μg g^{-1}), the second in kidney (7.47−9.11 μg g^{-1}), and lowest in muscle (0.68−1.25 μg g^{-1}) and fat (0.67−1.23 μg g^{-1}) after injection at a subcutaneous dose of 10 mg kg^{-1} body weight. After 7 days withdrawal, tilmicosin residue concentrations at the injection site (16.87−26.47 μg g^{-1}), in muscle (0.03−0.06 μg g^{-1}), kidney (0.69−1.15 μg g^{-1}), and fat (0.22−0.47 μg g^{-1}) were decreased very quickly. Twenty-one days posttreatment, the tilmicosin concentrations in all muscle and fat samples dropped below the LOD of the method (**Table 1**). At 35 days withdrawal, the tilmicosin concentrations in two out of three samples at the injection site and in kidney were below the LOQ. However, in liver, the tilmicosin residue was eliminated slowly, which was similar to the result reported in our previous study (*33*).

Tilmicosin residue concentrations in all tissues (muscle, liver, kidney, and fat) examined dropped below the EU-set MRLs of 0.05, 1, 1, and 0.05 mg kg^{-1}, 28 days after the end of the subcutaneous administration. This period, however, is not validated for use as a withdrawal period when diseased animals were used; the number of animals per slaughter day was small, and the levels of tilmicosin on the final sampling day were still above the MRL, which rendered an extrapolation inevitable. In this research, this period cannot be used for the samples collected from the injection site.

Estimation of the Withdrawal Time. Tilmicosin residue levels in muscle, liver, kidney, and fat samples were estimated (95% tolerance limit and 95% confidence) to fall below the MRLs after a withdrawal time of 20.54, 33.39, 23.24, and 23.57 days, respectively (**Figures 3**−**6**). Because these time points do not make up full days, the withdrawal times have to be rounded up to the next day. However, the longest withdrawal time of 34 days has to be selected as the conclusive withdrawal time to guarantee consumer safety (*28*).

Moreover, when considering the establishment of withdrawal periods for parenterally administered drugs, it is important to take into account the residues of the intramuscular or subcutaneous injection site. In this study, the tilmicosin residues in muscle samples at the injection site were eliminated very slowly and the estimated withdrawal time for them was 56.84 days (**Figure 7**), which was much longer than the other periods described above. The approach recommended by EMEA (*28*) may be adopted as follows: "For drugs where the target tissue or one of the target tissues is muscle, national authorities should set withdrawal periods on the basis of the MRL for muscle. The injection site and its residues would be treated as 'normal' muscle and the withdrawal time based on residues depletion to below the MRL at the injection site. Where muscle is not a

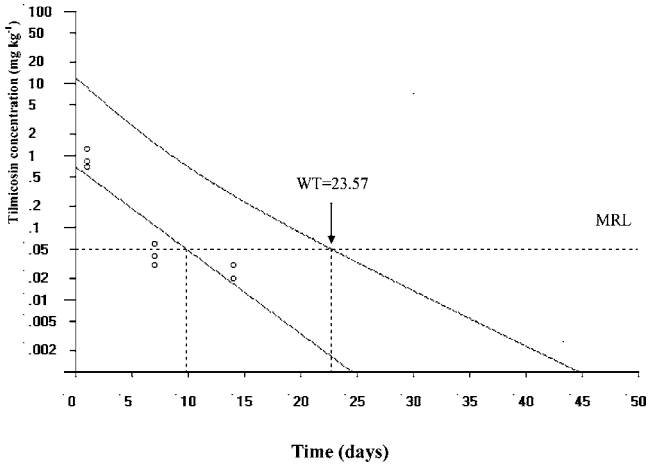

Figure 3. Plot of withdrawal time calculation for bovine muscle at the time when the one-sided 95% upper tolerance limit was below the EU MRL (0.05 mg kg^{-1}).

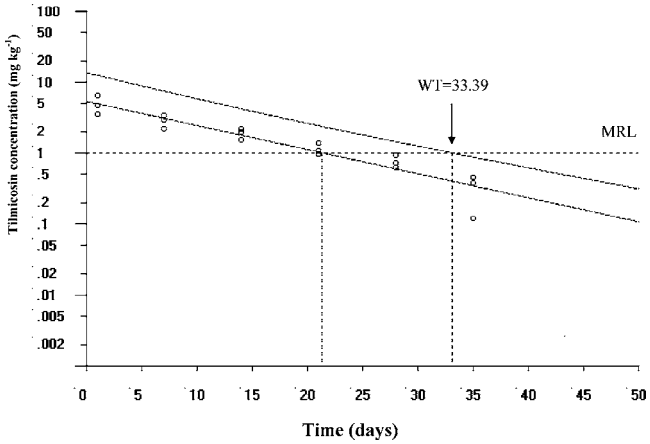

Figure 4. Plot of withdrawal time calculation for bovine liver at the time when the one-sided 95% upper tolerance limit was below the EU MRL (1 mg kg^{-1}).

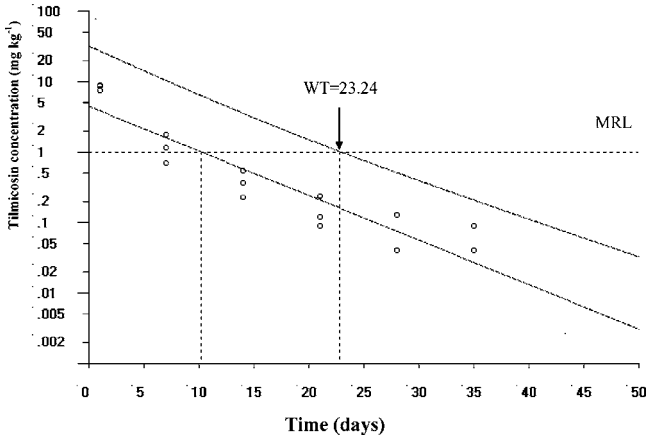

Figure 5. Plot of withdrawal time calculation for bovine kidney at the time when the one-sided 95% upper tolerance limit was below the EU MRL (1 mg kg^{-1}).

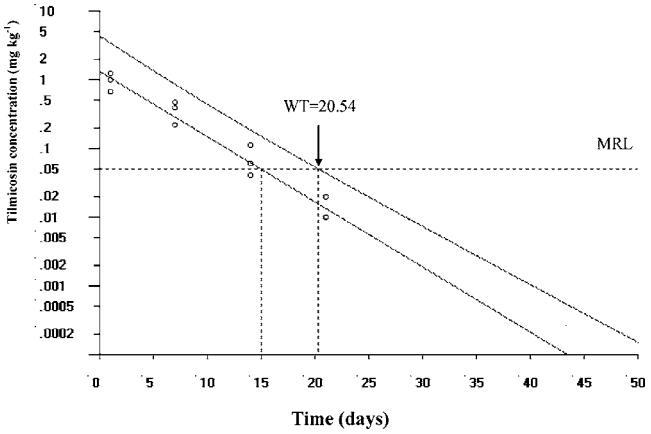

Figure 6. Plot of withdrawal time calculation for bovine fat at the time when the one-sided 95% upper tolerance limit was below the EU MRL (0.05 mg kg^{-1}).

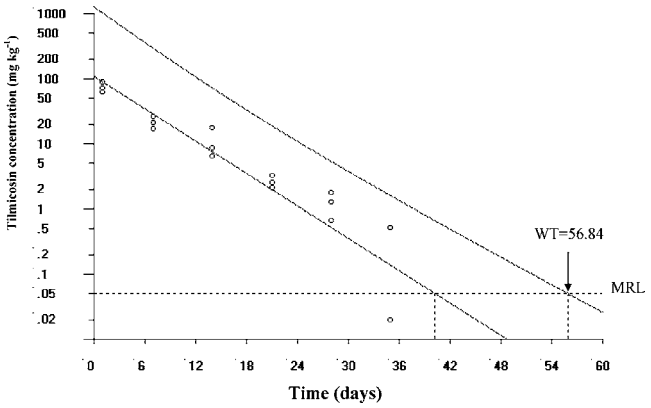

Figure 7. Plot of withdrawal time calculation for bovine muscle at the injection site at the time when the one-sided 95% upper tolerance limit was below the EU MRL (0.05 mg kg^{-1}).

target tissue and hence there is no MRL for muscle, national authorities should ensure that the withdrawal time is established to ensure that the ADI is not exceeded when the usual food package is consumed. Here, the usual food intake package of 300 g of muscle would be considered to include the injection site."

In conclusion, the results of the present study clearly indicate that a 35 day treatment of the cattle with tilmicosin at a single subcutaneous dose of 10 mg tilmicosin per kg body weight results in residue levels that are eliminated quite slowly. The period for the concentrations of tilmicosin residues below the existing EU MRLs in all examined bovine tissues was 34 days by using the statistical method suggested by EMEA. A longer 57 day withdrawal time was estimated for the tilmicosin residue at the injection site. These results indicate that all factors, which probably result in drug residues in animal bodies, which cause risks to human health, should be fully considered when the withdrawal time is set by every country.

ACKNOWLEDGMENT

We thank the cooperation of other faculty members in the Department of Pharmacology and Toxicology of the College of Veterinary Medicine at China Agriculture University.

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Received for review March 28, 2006. Revised manuscript received May 11, 2006. Accepted May 12, 2006. This research is supported by National Science Fund for Distinguished Young Scholars (Grant 30325032).

JF060863X