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Residue Depletion of Tilmicosin in Chicken Tissues

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A high-performance liquid chromatography (HPLC) method with detection at 290 nm was modified and validated for the determination of tilmicosin residues in broiler chicken tissues. The limits of detection (LOD) of the method were 0.01 μg/g for muscle and 0.025 μg/g for liver and kidney. Average recoveries ranged from 80.4 to 88.3%. Relative standard deviation values ranged from 5.2 to 12.1%. Residue depletion of tilmicosin in broiler chickens was examined after dosing over a 5-day period by incorporation of the drug into drinking water at 37.5 and 75.0 mg/L. Tilmicosin concentrations in liver and kidney were highest on day 3 of medication and on day 5 in muscle, in both low- and high-dose groups. The residue levels in both groups were significantly higher in liver than in kidney or muscle. A minimum withdrawal time of 9 days was indicated for residue levels in muscle, liver, and kidney tissues below the maximum residue level (MRL).

KEYWORDS: Tilmicosin; residue; liquid chromatography; depletion; chicken tissue

INTRODUCTION

Tilmicosin is a semisynthetic macrolide antibiotic with a wide range of veterinary uses for the treatment of bacterial and mycoplasma infections. Tilmicosin is composed of *cis*- and *trans*-pyrimidinyl isomers in a ratio of about 85:15. The structure of tilmicosin is shown in **Figure 1**. Tilmicosin has a spectrum of antimicrobial activity that includes *Pasteurella* spp., *Mycoplasma* spp., and a variety of Gram-positive organisms (*1*). Tilmicosin has a stronger antimicrobial activity than tylosin against *Pasteurella haemolyticus*, *Pasteurella multocida*, and *Mycoplasma* (*2*−*4*). Tilmicosin has been approved in China to treat *Pasteurella* diseases in swine and bovine and *Mycoplasma* infections in chickens.

Administering veterinary drugs to animals without an appropriate withdrawal period may lead to violative residues in tissues. Tilmicosin residues in animal tissues have been determined by liquid chromatographic (LC) methods (*5*−*9*), liquid chromatography−mass spectrometry (LC-MS; *10*), and liquid chromatography−atmospheric pressure chemical ionization mass spectrometry (LC-APCIMS; *11*).

We describe a modified LC method for determining tilmicosin residues in chicken tissues such as muscle, liver, and kidney. The method was applied in a residue depletion study of tilmicosin in broiler chickens.

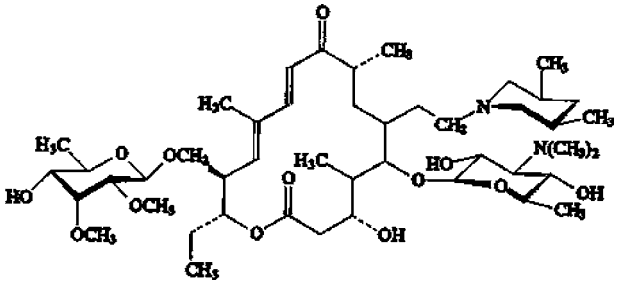

Figure 1. Molecular structure of tilmicosin.

MATERIALS AND METHODS

Solvents and Reagents. The solvents used were of LC grade, available from commercial sources. Water for HPLC analysis was Milli-Q filtered. 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, China). Dibutylammonium phosphate (DBAP) solution was prepared by the addition of 168 mL of dibutylamine to 700 mL of phosphoric acid. The solution was allowed to cool and was adjusted to pH 2.5 using phosphoric acid, 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 to pH 2.5 with phosphoric acid, and making up the volume to 1 L with water. A 0.1 mol/L 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 solution (25%) was produced by Jining Medicine Corp. (Shandong, China). Tilmicosin reference standard was

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a gift from Lilly Co. Stock solution of 2 mg/mL was prepared by dissolving 200 mg of tilmicosin standard in 100 mL of acetonitrile. The working standard solution of 1 μg/mL was prepared in acetonitrile.

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

Animal Treatment. To obtain the data about the residue of tilmicosin depletion in broiler chickens, a residue depletion study was conducted. One hundred and forty broiler chickens, aged 3 weeks and weighing 400−500 g, were used in this study. During acclimatization for 3 weeks, and the subsequent treatment periods, they were fed drug-free balanced rations ad libitum with free access to water. The animals were randomly divided into two groups of 70 animals each and administered tilmicosin in drinking water at concentrations of 37.5 or 75.0 mg/L for 5 days. Six chickens of each group, three males and three females, were slaughtered on days 3 and 5 after dosing and 0.25, 0.5, 1, 2, 3, 5, 9, and 14 days into withdrawal, respectively. Samples of muscle, liver, and kidney were collected from each animal and stored at −20 °C until they were processed.

Fortification. To test the stability of the HPLC system and the feasibility of the determination method for tilmicosin in chicken tissues, the recoveries were carried out on muscle, liver, and kidney at 0.05, 0.5 and 5.0 μg/g fortification levels. To obtain the within-day and day-to-day precisions, five replicates of fortified samples were extracted and analyzed at each of the three fortification levels under reproducibility conditions.

Sample Preparation. Chicken tissues (muscle, liver, and kidney) 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 the 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 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 3min. Tilmicosin was eluted from the cartridge with 2.5 mL of the 0.1 mol/L 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.

LC Analysis. The analysis of standards, fortified samples, and incurred samples was 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, the injection volume was 100 μL, and the wavelength was 290 nm.

Calculations. The concentration of tilmicosin in tissue sample can be calculated with the equation

$$\text{concn } (\mu g/g) = \frac{(S - B)VF}{KM}$$

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

Statistical Analysis. Student's t test was used to test parameters for significant difference between the two dose groups after dosing.

RESULTS AND DISCUSSION

Analytical Performance. A key point of drug residue analysis is the sample extraction and cleanup step required to separate the compound of interest from biological matrix components. This HPLC method is a modification in sample treatment of a previously reported method (*5*). In the sample extraction and cleanup procedure, Carolyn et al. (*6*) extracted samples twice with methanol and monobasic potassium phosphate buffer, and Wayne et al. (*5*) extracted twice with acetonitrile and monobasic potassium phosphate buffer. We found that extraction with methanol may result in matrix interferences, whereas extraction efficiency was increased by the addition of potassium phosphate buffer. In our method, we extracted first with acetonitrile followed by potassium phosphate solution and obtained high tilmicosin recoveries with no matrix interferences.

Use of the SPE columns eliminated the need for chlorinated solvents in sample cleanup, and with our choice of eluting solvent no interfering substances were detected.

Typical LC chromatograms for tilmicosin standards and extracts of control and fortified tissues are presented in **Figure 2**. The linear correlation coefficient for the standard curve (range = 0.05−25.0 μg/mL) was 0.9999. The limits of detection were 0.01 μg/g for tilmicosin in muscle and 0.025 μg/g in liver and kidney at a signal to noise ratio of 3:1. Recoveries of tilmicosin are shown in **Table 1**. The within-day recoveries of tilmicosin were 85.1−88.3% in muscle, 82.6−86.4% in liver, and 80.4−84.5% in kidney. The within-day coefficients of variation (CVs) ranged from 5.2 to 11.8% for all samples. The between-day recoveries for all samples were 80.2−87.5%, with CVs of 7.6−12.1%. The results indicate that the proposed method is suitable for monitoring residues of tilmicosin in animal tissues.

Residue Depletion Study. During and after treatment, residue levels were highest in liver and lowest in muscle (**Figure 3**), which suggests that liver should be the target tissue for tilmicosin residues in broiler chickens. There were no differences between the tilmicosin levels in muscle, liver, and kidney of each group on days 3 and 5 ($P > 0.05$). Those results further revealed tilmicosin was absorbed and distributed rapidly in the animal's body. The depletion curve shown in **Figure 3** indicated tilmicosin residue was eliminated from muscle very quickly, but from liver very slowly.

Tilmicosin pharmacokinetics had been reported in cattle (*12*−*14*), goats (*15*), sheep (*13, 16*), and pigs (*17*−*18*). The major pharmacokinetic characteristics for tilmicosin are rapid absorption after oral or subcutaneous injection, long elimination half-time in serum, rapid and extensive penetration from blood into milk, high concentration in milk and lung, and slow elimination from milk for at least 1 day. In addition, the apparent distribution volume is very large (>2.0 L/kg). Tilmicosin is available for the treatment of bacterial diseases and *Mycoplasma* infections in chickens (*19*−*21*) by medicating the animals' drinking water for several days. The only reported study (*22*) that determined lung tissue and airsac concentrations of tilmicosin during administration over 3 days in drinking water indicated that tilmicosin was detected in lung and airsac tissues within 6 h of its being offered in drinking water, and after 24 h, the concentration in the airsac exceeded that in the lung. After the drug had been administered at a level of 75.0 mg/L in drinking water for three consecutive days, the concentrations in lung and airsac (1.53 and 2.38 μg/g) were higher than that in muscle (1.44 μg/g) and significantly lower than those in liver and kidney (15.75 and 6.31 μg/g) in our study ($P < 0.05$). After 2 days of withdrawal, the concentration in lung (0.87 μg/g) was higher

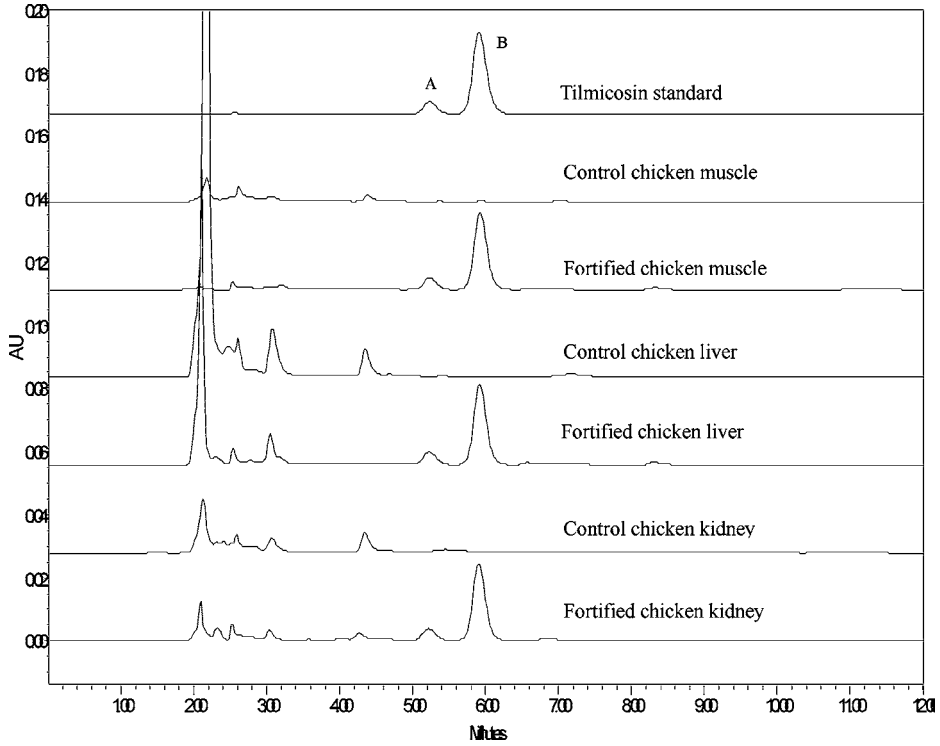

Figure 2. Chromatograms of tilmicosin standard (1.0 μg/mL); control chicken muscle, liver, and kidney; and fortified chicken muscle, liver, and kidney (1.0 μg/g). Peaks: A, *trans*-tilmicosin; B, *cis*-tilmicosin.

Table 1. Recoveries and Precisions of Tilmicosin in Fortified Tissue Samples ($n = 5$)

sample	added (μg/g)	within-day av recovery (%)	within-day CV[a] (%)	day-to-day av recovery (%)	day-to-day CV (%)
muscle	0.05	88.3	11.8	87.2	12.1
	0.5	86.2	7.7	84.6	9.6
	5.0	85.1	8.3	87.5	8.8
liver	0.05	86.4	9.8	83.1	8.0
	0.5	82.6	5.9	81.4	10.5
	5.0	84.7	7.6	82.6	7.6
kidney	0.05	80.4	10.5	80.2	9.7
	0.5	84.5	5.2	83.6	11.2
	5.0	84.2	7.4	83.8	9.9

[a] CV, coefficient of variation.

than that in muscle (0.26 μg/g) ($P > 0.05$) but lower than those in liver (1.23 μg/g) ($P > 0.05$) and kidney (6.59 μg/g) ($P < 0.05$), and the concentration in airsac (2.86 μg/g) was higher than those in muscle and kidney ($P > 0.05$) but lower than that in liver ($P < 0.05$). These results showed the airsac is another target tissue for tilmicosin residues in broiler chickens besides the liver. However, there was no other relative report about tilmicosin pharmacokinetics and metabolism in poultry. Our study indicated the distribution and elimination characteristics of tilmicosin in the chicken's body, which may provide a scientific basis for administering tilmicosin in practice and recommending a rational withdrawal period and safety assurance for food consumption.

According to the veterinary drug residue regulations of the Chinese Ministry of Agriculture and the European Union, the maximum residue levels (MRLs) of tilmicosin in broiler chicken muscle, liver, and kidney are 0.075, 1.0, and 0.25 μg/g, respectively (*23*, *24*). The recommended withdrawal time is 10 days. In this study, in the high-dose group, tilmicosin residue

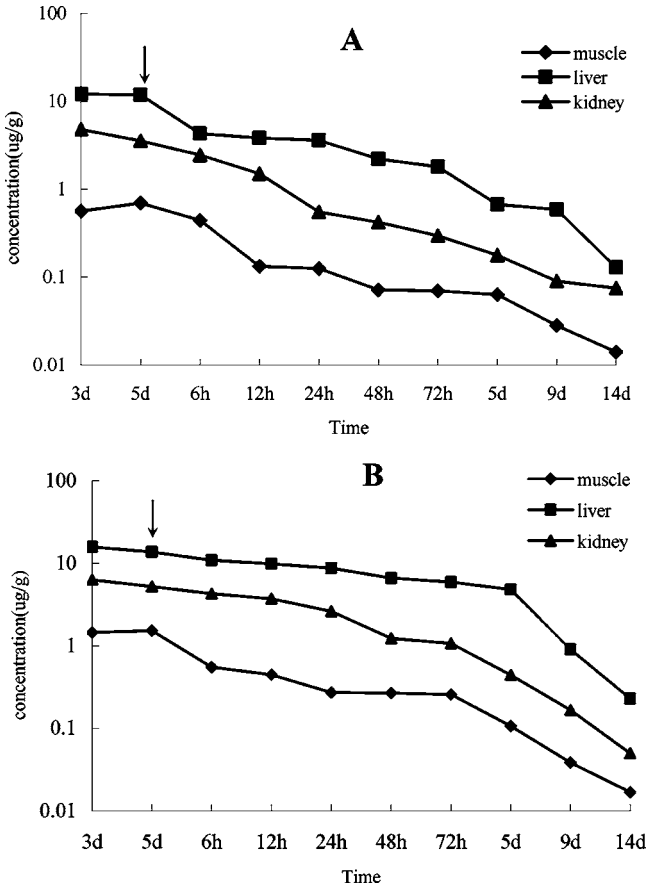

Figure 3. Depletion curves of tilmicosin from tissues: (**A**) chickens dosed at a concentration of 37.5 mg/L in drinking water; (**B**) chickens dosed at a concentration of 75.0 mg/L in drinking water; (↓) time at which tilmicosin was removed from drinking water.

in muscle decreased to the approved level after 2 days of withdrawal, in liver after 9 days, and in kidney after 5 days. These results were in accord with the regulated requirement above.

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