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Journal of Chromatography B, 857 (2007) 9–14
JOURNAL OF CHROMATOGRAPHY B
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Quantitative determination of tilmicosin in canine serum by high performance liquid chromatography–tandem mass spectrometry

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Received 9 May 2007; accepted 22 June 2007
Available online 29 June 2007

Abstract

A highly sensitive and quantitative LC/MS/MS assay for the determination of tilmicosin in serum has been developed and validated. For sample preparation, 0.2 mL of canine serum was extracted with 3 mL of methyl *tert*-butyl ether. The organic layer was transferred to a new vessel and dried under nitrogen. The sample was then reconstituted for analysis by high performance liquid chromatography–tandem mass spectrometry. A Phenomenex Luna C8(2) analytical column was used for the chromatographic separation. The eluent was subsequently introduced to the mass spectrometer by electrospray ionization. A single range was validated for 50–5000 ng/mL for support of toxicokinetic studies. The inter-day relative error (inaccuracy) for the LLOQ samples ranged from −5.5% to 0.3%. The inter-day relative standard deviations (imprecision) at the respective LLOQ levels were ≤10.1%.

Keywords: Tilmicosin; Canine; Liquid chromatography/mass spectrometry

1. Introduction

Tilmicosin, a macrolide antimicrobial, is a semi-synthetic derivative of tylosin, and is indicated for the prevention and control of respiratory diseases associated with *Mannheimia haemolytica* in cattle and sheep and *Actinobacillus pleuropneumoniae* in swine [1]. While analytical methods have been demonstrated for the determination of tilmicosin in recent years, the vast majority of method development has centered around residue determinations in various tissues. A recent review [2] of analytical methods published in the past two decades for the determination of macrolide antimicrobials clearly emphasizes this point. While there were many methods that included the determination of tilmicosin in various tissues [3–10], only five methods were recognized for the quantitation of tilmicosin in plasma or serum [11–15]. Many methods that have been published for biological fluids utilize either liquid chromatography with UV detection or microbiological analysis, obtaining various limits of quantitation. A swine serum method [12] has been reported to have a lower limit of quantitation (LLOQ) of 25 parts per billion (ppb) using 2 mL of serum. A method for determining concentrations of tilmicosin in elk serum [14], which was an adaptation of an earlier published method [3], obtained an LOQ of 10 ppb but required 5 mL of serum. Liquid chromatography coupled with a tandem mass spectrometer (MS) was used to determine concentrations in several tissues and confirm identity of tilmicosin [6], but this method did not take full advantage of the sensitivity of LC/MS/MS as the LLOQ was established at the maximum residue limits (MRLs) in the various tissues analyzed (1 mg/kg for liver and kidney; 0.05 mg/kg for fat and muscle). Thus, the method that we report here offers significant advantages over previous methods in terms of sample size and sensitivity to support toxicokinetic studies.

2. Experimental

2.1. Reagents and standards

HPLC grade methanol, acetonitrile, and water and ACS grade sodium carbonate were obtained from Fisher (Pittsburgh, PA, USA). Formic acid (96%), methyl *tert*-butyl ether (99+%), and

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doi:10.1016/j.jchromb.2007.06.017

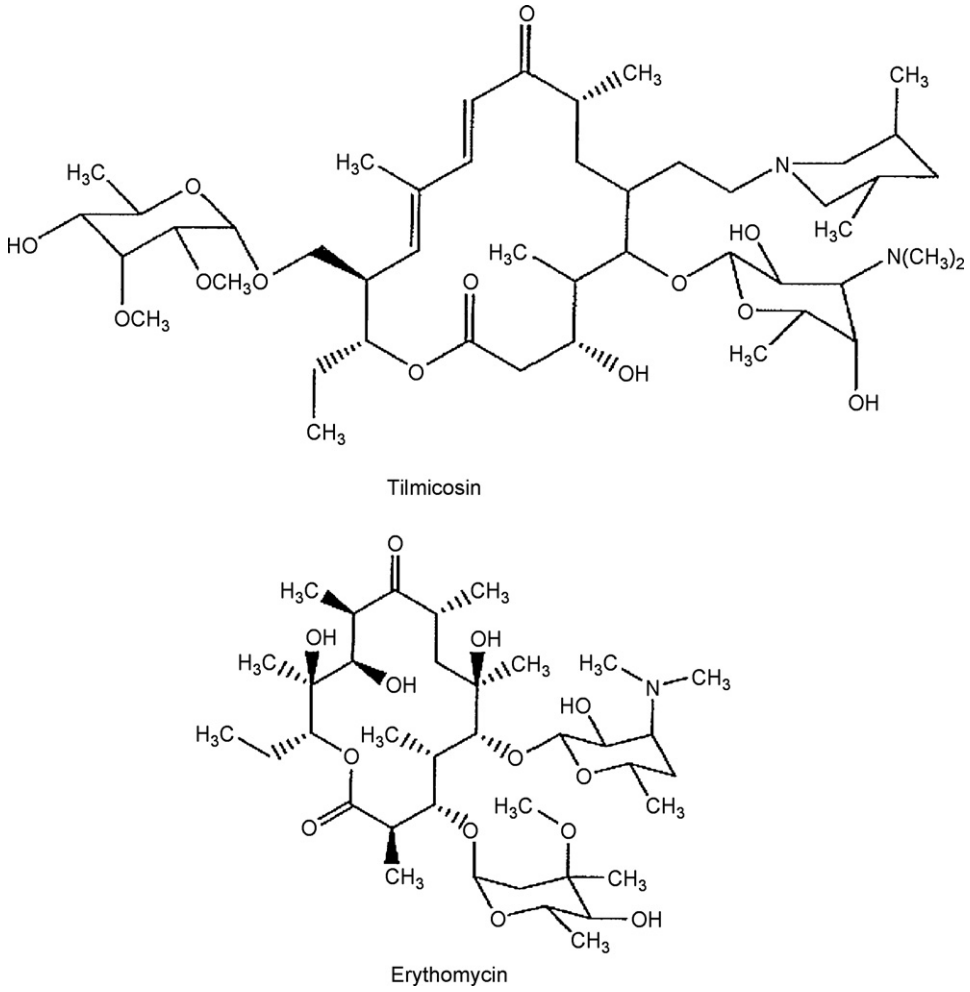

Fig. 1. Chemical structure of tilmicosin and erythromycin (internal standard).

ACS grade ammonium acetate and ammonium formate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tilmicosin (purity = 90.7%) was provided by Eli Lilly and Co. (Indianapolis, IN, USA). Erythromycin (purity = 95.6% Factor A), used as the internal standard, was obtained from MP Biomedicals, Inc. (Aurora, OH, USA). The structures of both tilmicosin and erythromycin can be found in Fig. 1.

2.2. Equipment

The HPLC system consisted of a LC-10ATvp pump, a SCL-10Avp controller, a FCV-10ALvp switching valve, and a SIL-10ADvp autosampler from Shimadzu (Duisberg, Germany). The HPLC was coupled to an Applied Biosystems Sciex API 3000 triple-quadrupole mass spectrometer (Concord, Canada) for tandem mass spectrometric detection. The chromatographic separation was performed using a Phenomenex (Torrance, CA, USA) Luna C8(2) (30 mm × 2.0 mm, 3 μm particle size) analytical column.

2.3. Quality control samples and standard solutions

Quality control (QC) samples were prepared just prior to assay by fortifying serum with the appropriate QC working solutions. For the canine serum the QC concentrations were prepared at 50, 150, 2500, 4500, and 5000 ng/mL.

Stock solutions for both tilmicosin and erythromycin (internal standard) were prepared separately in acetonitrile at concentrations of 200 μg/mL. The tilmicosin stock solution was subsequently diluted with [acetonitrile:water:formic acid (20:80:0.1)] to prepare working standard solutions for the canine serum assay at concentrations of 500, 1000, 2000, 5000, 10,000, 20,000, and 50,000 ng/mL. To 200 μL of serum, 20 μL of each appropriate working standard solution was added for final serum concentrations of 50, 100, 200, 500, 1000, 2000, and 5000 ng/mL for the canine serum assay. The internal standard stock solution was diluted with dilution solution to 2000 ng/mL as the working solution for the assay.

2.4. Sample preparation

A 200 μL aliquot of serum was fortified with 20 μL of appropriate internal standard solution. To the sample, 40 μL of ACN:MeOH:0.1 M NH_4OAc, pH 6.3–6.4 (30:20:50 v/v/v) was added followed by 20 μL of saturated sodium carbonate solution. The sample was then extracted with 3 mL of *tert*-butyl methyl ether, vortexed for 5 min, and centrifuged for 5 min at

Table 1
Accuracy and precision[a]

Concentration (ng/mL)	n	Mean ± SD (ng/mL)	Relative error (%)	CV (%)
Canine serum				
50	6	50.2 ± 3.72	0.3	7.4
2500	6	2422 ± 64.6	−3.1	2.7
5000	6	4702 ± 176	−6.0	3.8

[a] For canine assay, data from one validation batch with six replicates.

3300 × g. The ether layer was transferred by pipette to a 15 mL glass tube and evaporated to dryness at approximately 37 °C under nitrogen gas flow.

The sample was then reconstituted with 1 mL of reconstitution solution, vortexed, and centrifuged for 5 min at approximately 3300 × g. A 100 μL sample aliquot was transferred to HPLC autosampler vials containing 900 μL reconstitution solution and then analyzed by LC/MS/MS.

2.5. Chromatographic and mass spectrometric conditions

For the chromatography, two mobile phases were employed. Mobile phase A was 0.5% formic acid in 3 mM ammonium formate water solution. Mobile phase B was 0.5% formic acid in ACN. The flow rate was 0.4 mL/min. The analytes were eluted by gradient LC where the initial step was composed of 15% mobile phase B and increased linearly to 50% mobile phase B at 0.5 min. The composition was switched back to the original conditions at 2.6 min and held until 5 min. The column eluent was diverted to waste before 0.9 min and after 4.1 min. An injection of 3 μL was made for each sample.

To perform MS/MS analysis, m/z transitions of 869.6 → 174.1 for tilmicosin and 734.5 → 158.0 for erythromycin were monitored using total ion monitoring. The TurboIonSpray source was operated in positive ion mode. The following settings were used for acquisition: capillary = 4500 V; source temperature = 500 °C; orifice = 40 V; focusing ring = 200 V; dwell time = 300 ms.

2.6. Data analysis

Peak integration of tilmicosin and the internal standard were performed using MacQuan version 1.7.1 software. Calibration curves were obtained by plotting the peak area ratio of tilmicosin to internal standard against the amount of tilmicosin added. A weighted ($1/x^2$, where x is concentration) least squares regression analysis was used to obtain a linear equation over the standard curve range. The origin was not used in the standard curve calculations.

3. Results and discussion

3.1. Selectivity

Selectivity was achieved by independent separation mechanisms of chromatography and tandem mass spectrometry. The sample was initially separated by reversed-phase HPLC and subsequently introduced to the mass spectrometer where the sample was further separated and detected by mass-to-charge ratio (m/z). Six independent sources of serum were analyzed. Representative chromatograms of matrix blank and blank with internal standard can be seen in Figs. 2 and 3, respectively, for canine serum. No significant interferences were observed for tilmicosin (retention time: ∼2.2 min) or erythromycin (retention time: ∼2.3 min).

3.2. Linearity

The validation batch included two sets (by duplicate injection) of calibration standards. One set of the calibration standards was run at the beginning of the analytical batch, and the other set was run at the end of the batch.

The peak area ratio of tilmicosin to the internal standard is related to concentration using a linear fit, with $1/x^2$ (where x is concentration) weighting. The standard curves had a mean slope of 0.00133 and a corresponding intercept of −0.00065. Sufficient linearity was observed for curve range of 50–5000 ng/mL. The correlation coefficient (r) for the calibration curve was 0.9977.

3.3. Accuracy and precision

The accuracy and precision results for the determination of tilmicosin in canine serum are shown in Table 1 for the canine serum assay. The validation batch included six replicates at each concentration of the QC samples.

The inter-day relative error (inaccuracy) for the LLOQ samples ranged from −5.5% to 0.3%. The inter-day relative standard deviations (imprecision) at the respective LLOQ levels were ≤10.1%. At tilmicosin concentrations above the respective LLOQ, the inaccuracy ranged from −7.5% to 4.3% and the imprecision was ≤4.1% for the assay.

3.4. Recovery

Extraction efficiency was evaluated at 150, 2500, and 4500 ng/mL of tilmicosin for the canine serum assays. Included in Table 2 is the extraction efficiency data for the canine serum assays. Average extraction efficiencies of both tilmicosin and internal standard were approximately 90% for this assay.

3.5. Stability

Tilmicosin stability was evaluated for stock solutions and in matrix. Table 3 is a summary of the stability results gener-

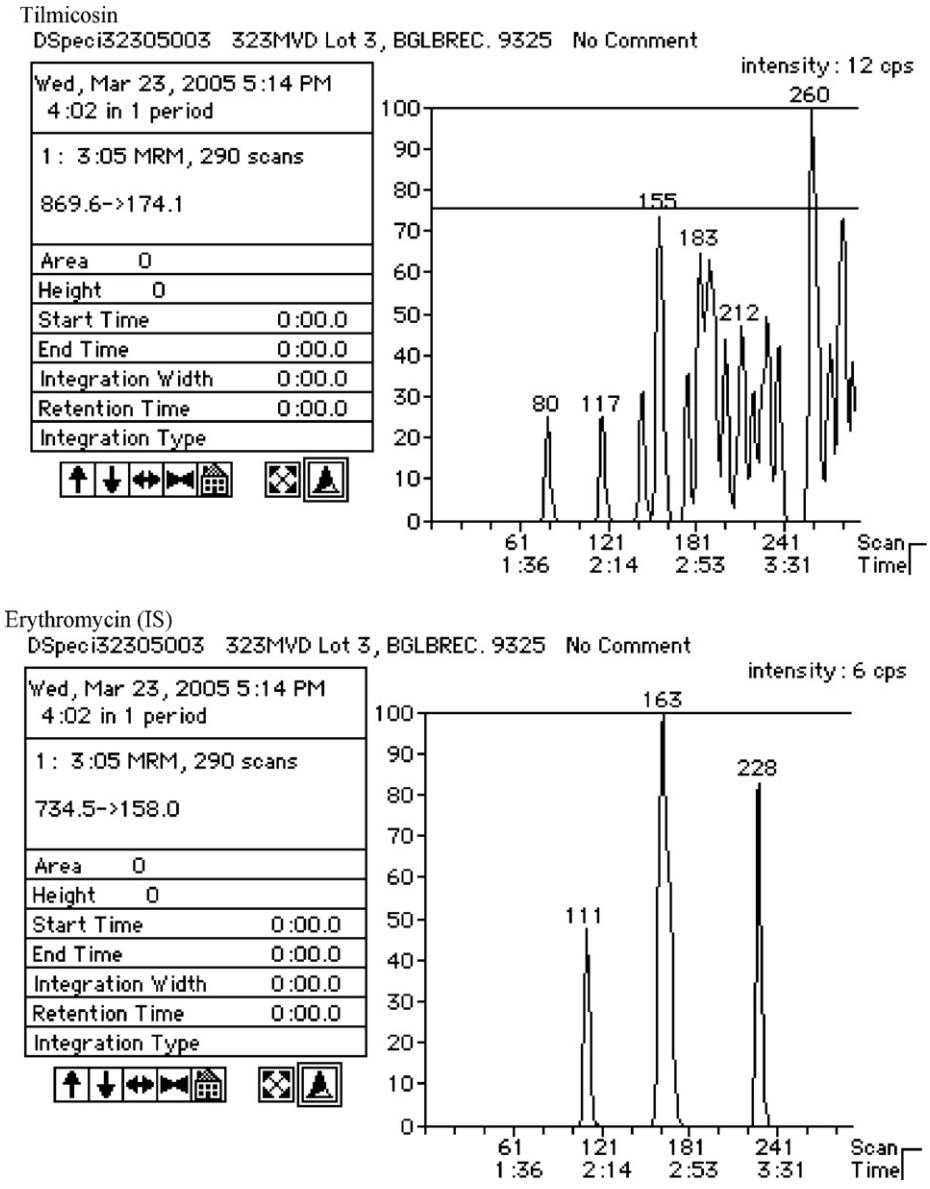

Fig. 2. Chromatogram of extracted tilmicosin naïve (blank, no IS) canine serum. The numbers above each peak represent *m*/*z* values.

Table 2
Extraction efficiency from canine serum

Concentration in serum (ng/mL)	Recovery (%)
Canine serum	
150	89.7
2500	91.3
4500	92.8
(I.S.) 10000	93.3

Table 3
The stability data of tilmicosin in canine serum

Concentration (ng/mL)	*n*	Accuracy (%)
Bench-top stability (~21 h in canine serum at ambient temperature)		
150	3	3.2
4500	3	−6.6
Freeze–thaw stability (three cycles in canine serum at −80 °C)		
150	3	0.4
4500	3	−2.3
Long-term storage stability (92 days in canine serum at −80 °C)		
150	3	0.2
4500	3	−1.4

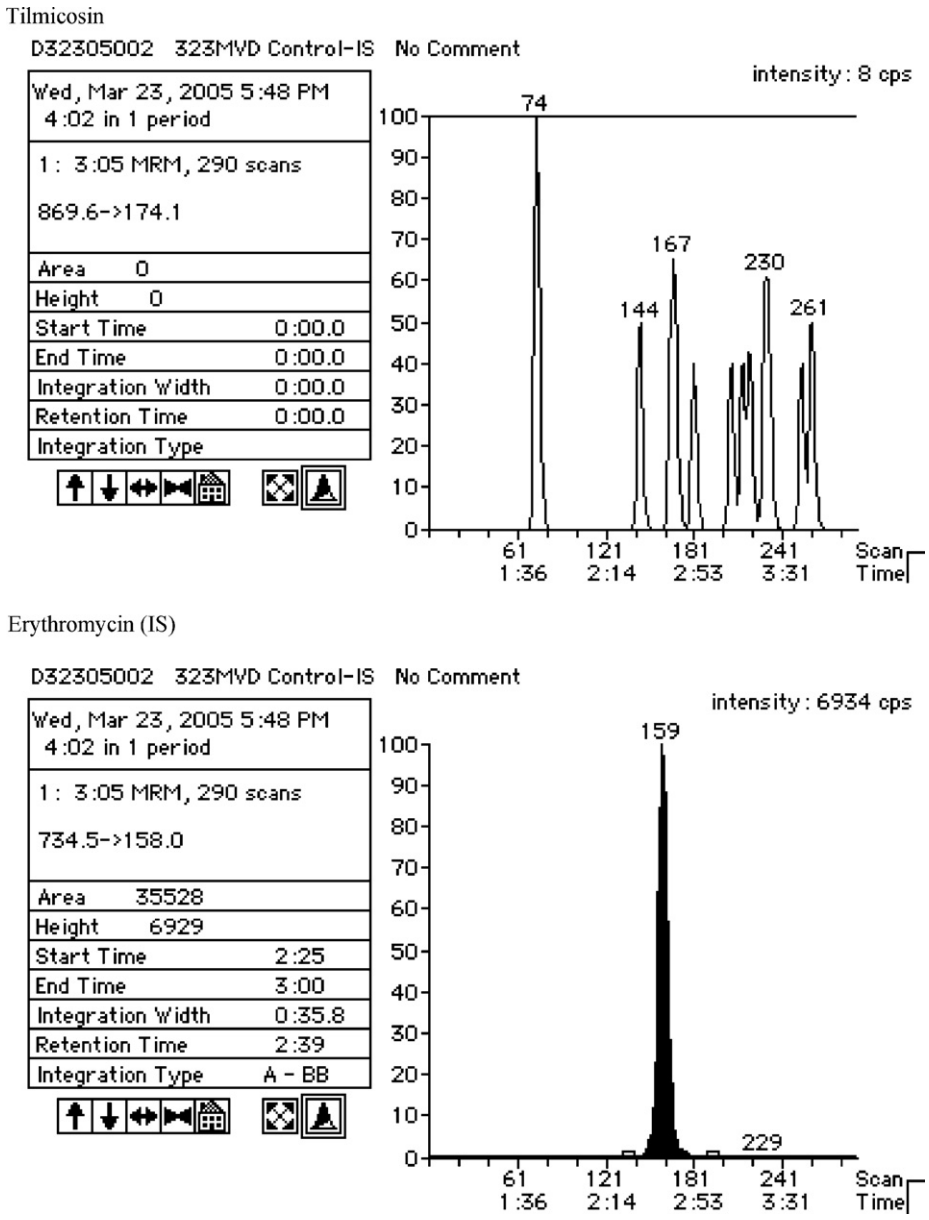

Fig. 3. Chromatogram of extracted tilmicosin naïve canine serum with internal standard (erythromycin). The numbers above each peak represent *m/z* values.

ated for tilmicosin in canine serum. Standard and working stock solution stability was demonstrated for 42 days when stored at approximately 5 °C. Tilmicosin stability in canine serum was demonstrated for approximately 21 h at ambient temperature, for three freeze–thaw cycles when frozen at −80 °C, and for 92 days when stored at −80 °C.

3.6. System suitability sample preparation and test

Two separate solutions each containing 50 ng/mL of tilmicosin and 200 ng/mL of the internal standard were prepared to evaluate system suitability. The system suitability samples were prepared by adding 100 μL of the 500 ng/mL tilmicosin working solution and 100 μL of the 2000 ng/mL internal standard working solution to 800 μL of reconstitution solution in an autosampler vial.

Each of the two solutions was injected in triplicate before the batch analysis. The samples exhibited acceptable peak shape for the analyte and internal standard. The CV% of the areas of tilmicosin and internal standard peaks was within the acceptance criteria of less than or equal to 15%. Typical retention times for tilmicosin and the internal standard were approximately 2.3 and 2.5 min, respectively.

4. Conclusions

A validation of tilmicosin in canine serum was conducted which provided good sensitivity, linearity, accuracy, and precision over a range of concentrations providing analysis over two orders of magnitude. The sample preparation and chromatographic separation combined with tandem mass spectrometric detection provide sufficient selectivity, minimize the

effects of matrix on the results, and use a relatively small amount of sample compared to currently published methods [11–15].

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