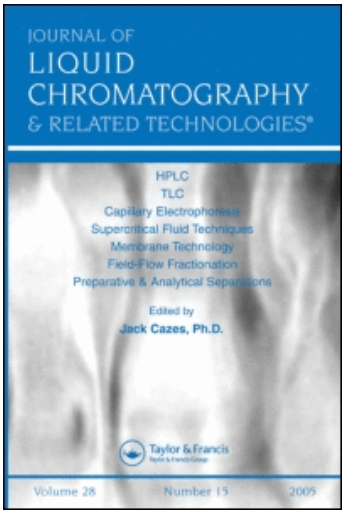

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Development and Validation of a Method for Determination of Tilmicosin Residues in Equine Plasma and Tissues Using HPLC

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Abstract: A sensitive liquid chromatographic method with UV detection has been developed for the analysis of tilmicosin concentrations in equine tissues and plasma. Tilmicosin is extracted from plasma or tissues using acetonitrile and a phosphate buffer. The extract is centrifuged, filtered, and cleaned up on a conditioned C_{18} solid phase extraction cartridge. Tilmicosin is eluted with ammonium acetate in methanol and diluted with ammonium acetate. It is analyzed by reversed phase liquid chromatography with UV detection at 287 nm. The method was shown to be suitable for detecting tilmicosin in equine plasma, muscle, liver, kidney, and lung tissues. The limit of detection of the method was 13 ng/mL in plasma and 181 ng/g in lung tissue.

Keywords: Horses, HPLC-UV, Pharmacokinetics, Plasma, Tilmicosin, Tissue

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INTRODUCTION

Tilmicosin is a macrolide antibiotic formed from a chemical modification of tylosin. Micotil® (Elanco Animal Health, Guelph, ON) is licensed for subcutaneous injection for the treatment of respiratory disease in sheep and cattle and is used extensively in North American feedlots. Pulmotil® (Elanco Animal Health, Guelph, ON) is licensed as a feed additive for the prevention and control of respiratory disease in swine. Tilmicosin has been tested and used in various other species including rabbits and poultry[1,2] and is licensed for use in these species in Europe. Tilmicosin is efficacious for the treatment of respiratory disease due to its accumulation in pulmonary tissues, its long elimination half-life, and antimicrobial spectrum of activity that includes most Gram-positive aerobes, Gram-negatives associated with respiratory disease, some anaerobes, and *Mycoplasma* spp.[3] With these characteristics, tilmicosin would be ideal for treatment of equine bacterial respiratory infections.

Experimental study of the use of tilmicosin in horses requires that the concentration in plasma be determined in order to determine the plasma pharmacokinetics. Since tilmicosin has a large volume of distribution and is known to selectively accumulate in tissues, especially the lung, it is also necessary to determine the concentration of tilmicosin in other tissues. The concentration of tilmicosin in these tissues could be used to determine potential dosing regimens, as well as estimations of withdrawal intervals, since the horse is used as a food animal in some countries. An HPLC method for measuring tilmicosin residues in animal tissues has been described for bovine and porcine kidney, liver, and muscle,[4] but there has been no method described for equine plasma, serum, or lung tissues. This paper describes the analytical method that was adapted from the method of Chan et al.,[4] validated for the determination of tilmicosin residues in equine plasma and lung tissue, and used to determine the concentrations of tilmicosin residues in equine liver, kidney, plasma lung, and muscle tissues in a pharmacokinetic study.[5]

EXPERIMENTAL

Apparatus

Solid phase extraction (SPE) cartridges, C_{18} 500 mg (6 mL capacity) were obtained from Varian (Harbor City, CA).

HPLC System

Analysis was conducted using a Waters 590 pump, a Waters 490 Programmable Detector set at 287 nm, and a Waters 712 WISP Autosampler. The liquid chromatography (LC) column was an Inertsil ODS-2, 5 μm, 150 × 4.6 mm column (Lablink inc. Rockford, IL) with a guard column, Inertsil ODS-2, 5 μm, 4.0 × 10 mm (Lablink inc. Rockford, IL). Separation was achieved under isocratic conditions with the mobile phase at room temperature and a flow rate of 1 mL/min.

Reagents

Ammonium acetate, ammonium formate, ortho-phosphoric acid, sodium phosphate, and trifluroacetic acid were of reagent grade and all solvents used in this study including acetonitrile and methanol were of HPLC grade and were obtained from VWR Canlab (Mississauga, ON). The tilmicosin standard was generously provided by Elanco Animal Health (Guelph, ON). Tylosin tartrate standard was obtained from Sigma-Aldrich (Oakville, ON). Water was obtained from a reverse osmosis still.

A 0.2 M ammonium formate solution (pH 5.0) was prepared by dissolving 12.62 g ammonium formate in approximately 800 mL water and adjusting the pH to 5.0 with trifluroacetic acid. It was mixed and made up to volume with water in a 1000 mL volumetric flask.

Elution solution for eluting adsorbed tilmicosin and tylosin from the C_{18} SPE cartridge was prepared by dissolving 0.771 g ammonium acetate in approximately 80 mL methanol and making it up to volume with methanol in a 100 mL volumetric flask. A 0.1 M ammonium acetate solution was prepared in the same way using water instead of methanol.

A 0.1 M phosphate buffer (pH 2.5) was prepared by dissolving 6.90 g monobasic sodium phosphate in approximately 400 mL water. The pH was adjusted to 2.5 with ortho-phosphoric acid. The solution was mixed and made up to volume in a 500 mL volumetric flask.

The mobile phase was prepared by mixing 0.2 M ammonium formate solution (pH 5.0), water, methanol, and acetonitrile. The exact composition was varied according to the tissue being analyzed (see Table 1).

Preparation of Standard Solutions

Stock standard solutions of tilmicosin (TIL) and tylosin tartrate (TYL) (1 mg/mL) were prepared by dissolving 113.1 mg tilmicosin (88.4%

Table 1. Mobile phase composition used for the analysis of tilmicosin residues in equine plasma and tissues

Matrix	Mobile phase composition				AUFS	Injection volume (μL)
	A (%)	B (%)	C (%)	D (%)		
Plasma	20	32	24	24	0.01	50
Muscle	20	32	24	24	0.04	50
Liver	30	25	25	20	0.02	20
Lung	30	25	25	20	0.02	20
Kidney	30	25	25	20	0.02	20

A: 0.2 M Ammonium formate (pH 5); B: Water; C: Acetonitrile; D: Methanol.
AUFS – Absorbance units full scale.

purity) or 113.3 mg tylosin tartrate (88.3% purity) in 80 mL methanol. The solution was thoroughly mixed and brought up to volume in a 100 mL volumetric flask. The stock solutions were stored at −20°C, and new solutions were prepared every 6 months.

Working standard solutions were prepared from the stock solutions by using the appropriate dilution with methanol. The working standards consisted of 5 μg/mL (TIL 5) and 50 μg/mL (TIL 50), for tilmicosin and 50 μg/mL (TYL 50) and 100 μg/mL (TYL 100) for tylosin, and were also stored at −20°C and replaced each time a new stock standard was prepared.

Sample Preparation

The method of sample preparation varied slightly depending on the tissue of interest. The concentrations of tilmicosin used in the standard curve reflect the concentrations of tilmicosin found in the tissue following administration of a 10 mg/kg intramuscular dose used by Clark, Dowling et al.[5]

5.00 ± 0.05 mL of thawed plasma test samples were accurately measured into individual 50 mL polypropylene centrifuge tubes. Equivalent volumes of drug free (negative control) plasma were measured into four individual centrifuge tubes and fortified with appropriate amounts of tilmicosin to prepare the calibration standards shown in Table 2. The internal standard (50 μL) tylosin (TYL 50 or TYL 100), was added to each sample. The samples were allowed to sit on the bench top for 15 minutes. Each sample was mixed with 20 mL acetonitrile, 5 mL of 0.1 M phosphate buffer (pH 2.5), and vortexed for 1 minute. The treated

Table 2. Standards solutions used to prepare calibration curves for the determination of tilmicosin residues in equine plasma, kidney, liver, lung and muscle tissues

	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6	Standard 7	Standard 8
[ng/g or ng/mL]	40	150	250	500	1000	1500	2000	3000
5.0 mL Plasma	40 µL TIL5	15 µL TIL50	25 µL TIL 50	50 µL TIL 50				
2.5 g Lung				25 µL TIL 50	50 µL TIL 50		100 µL TIL 50	150 µL TIL 50
2.5 g Liver				25 µL TIL 50	50 µL TIL 50		100 µL TIL 50	150 µL TIL 50
2.5 g Kidney				25 µL TIL 50	50 µL TIL 50		100 µL TIL 50	150 µL TIL 50
5.0 g Muscle			25 µL TIL 50	50 µL TIL 50	100 µL TIL 50	150 µL TIL 50		

TIL 5: 5 ng/mL Tilmicosin standard working solution; TIL 50: 50 ng/mL Tilmicosin standard working solution.

plasma samples were centrifuged at 2000 g for 5 minutes, after which they were filtered through a Whatman GF/B, 5.5 cm filter paper, and diluted with approximately 70 mL of water ready for SPE cleanup.

Muscle, (5.00 ± 0.5 g) or 2.50 ± 0.05 g liver, lung, or kidney of finely diced thawed test samples were accurately weighed into individual 50 mL polypropylene centrifuge tubes. Equivalent amounts of drug free (negative control) tissue samples were weighed into four individual centrifuge tubes. Appropriate amounts of the tilmicosin standard solutions were added to the four drug free tissue samples to prepare the calibration standards shown in Table 2. For muscle tissues, 200 μL of TYL 50 was added to provide a constant concentration of 4000 ng/g TYL in each sample, while for liver, kidney, and lung tissue a 200 μL of TYL 100 was added to each sample to provide a constant concentration of 8000 ng/g. The samples were allowed to sit on the bench top for 15 minutes. Ten mL of acetonitrile was added to each tissue sample and homogenized (Polytron Model PT 10-35 (Brinkman Instruments Ltd., Rexdale, Ont)) at high speed for 20 seconds. The homogenizer probe was rinsed with 2×2 mL aliquots of acetonitrile into the sample tube. The homogenized samples were placed on a bench top shaker for 5 minutes and then centrifuged at $2000 \times$ g for 5 minutes. The supernatant was decanted and collected in a clean labeled centrifuge tube. The tissue plug was resuspended in 10 mL acetonitrile and 5 mL of phosphate buffer, vortexed, and placed on the bench top shaker at high speed for a further 5 minutes. This sample was then centrifuged at $2250 \times$ g for 5 minutes and the supernatant from this extraction was combined with that from the first extraction. The combined extracts were centrifuged at $2000 \times$ g for 5 minutes, after which they were filtered through a Whatman GF/B, 5.5 cm filter paper and diluted with approximately 70 mL of water ready for SPE cleanup.

SPE Cleanup

The SPE cartridges were conditioned with 10 mL methanol followed by 10 mL water (it is important that the columns not be allowed to dry out after this point). The extracted solutions obtained from tissue or plasma were loaded onto the columns and allowed to drain under gravity. The cartridges were then washed with 20 mL water and dried for 5 minutes under a vacuum of 20 mm/Hg. Tilmicosin and tylosin (the internal standard) were eluted from the SPE cartridge into a 2 mL volumetric flask using 2×750 μL of elution solution. The eluate in the volumetric flask was brought up to 2 mL with 0.1 M ammonium acetate. The final solution was prepared for HPLC analysis by mixing and filtering through a 0.45 μm LC13 polytetrafluoroethylene (PTFE) Acrodisc syringe filter.

HPLC Analysis

HPLC analysis was conducted under isocratic conditions using the solvent combinations shown in Table 1. Chromatographic separation was achieved on an Inertsil ODS-2 column at a mobile phase flow rate of 1 mL/min with UV detection at 287 nm.

Validation of the Analytical Method

Selectivity

The selectivity of the method (i.e., the ability of the test method to detect truly negative samples as negative) was demonstrated by analyzing control (drug free) tissues obtained from six different horses (the horses represented both sexes and a variety of ages and breeds with a known history that they had never been treated with a macrolide antimicrobial) using the developed analytical method.

Since the assay was to be used in a controlled setting for pharmacokinetic analysis of tilmicosin in equine plasma and tissues, the assay was not evaluated further for interference with other commonly used veterinary pharmaceuticals, since the subjects of the study would not be receiving any other medications.

Quantitative Analysis

Peak heights of the peak responses of tilmicosin and tylosin in the extracts from the fortified control tissue or plasma standards and the test samples were measured using digital calipers. Standard calibration curves were generated from four negative control matrix samples individually fortified with working solutions according to Table 2, to cover the linear range of the analytical method. The samples were analyzed as previously described. An unweighted least squares regression curve was generated by plotting the response ratio (i.e., detector response [peak height] of tilmicosin/detector response [peak height] of tylosin) vs. concentration of tilmicosin added to the control tissue or plasma. For all matrices the relationship between tilmicosin concentration and response ratio was linear ($R > 0.99$). Response ratios obtained for any test samples found to contain tilmicosin were measured and used to calculate the concentrations of tilmicosin in those test samples.

Sensitivity of the Method: Limit of Detection, Limit of Quantification

The limit of detection (LOD) of the method for tilmicosin was calculated for only plasma and lung tissue since the method had previously been

validated for kidney, muscle, and liver tissues. The LOD was calculated by analyzing five sets of matrix fortified calibration standards over three days, in the region at which the assay was anticipated to be used (i.e., for plasma, 10, 20, 40, 60, 250, 400 ng/mL and 100, 200, 400, 600, 1200, 2500 ng/g for lung tissue). An unweighted linear least squares regression equation was generated for each set of standards as follows:

$$\mathbf{RR}_{\mathrm{PLASMA}} = 0.0485 \pm 0.0239 + (0.00560 \pm 0.000087)[\mathrm{TIL}] \quad (1)$$

$$\mathbf{RR}_{\mathrm{LUNG}} = 0.0035 \pm 0.0241 + (0.000385 \pm 0.0000147)[\mathrm{TIL}] \quad (2)$$

Where, $\mathbf{RR}_{\mathrm{PLASMA}}$ and $\mathbf{RR}_{\mathrm{LUNG}}$ represent the response ratio for tilmicosin to tylosin in plasma and lung, respectively, and [TIL], the concentration of tilmicosin added to plasma or tissue.

The LOD, defined as the concentration at which the signal-to-background noise ratio (S/N) was equal to 3 times the standard error of the y-intercept, was estimated to be 13 ng/mL for plasma and 181 ng/g for lung tissue. The limit of quantification (LOQ) of the analytical method, defined as the concentration at which the signal-to-background noise was equal to 10 times the standard error of the intercept was calculated to be 42 ng/mL and 603 ng/g, respectively, for residues in plasma and lung tissue.

Matrix Effects

To demonstrate whether the detector response for tilmicosin and tylosin could be affected by the presence or absence of the matrix, the detector responses measured for tilmicosin in the matrix matched calibration standards were compared with the responses obtained from the chemical standard solutions at equivalent concentrations dissolved in the elution solution and analyzed under similar experimental conditions (Table 3).

Recovery, Inter-Assay, and Intra-Assay Precision for Plasma and Lung Tissue

The absolute recoveries of tilmicosin from plasma and tissue were calculated by comparing the detector responses obtained from a set of negative control tissues or plasma fortified for the typical calibration curve (matrix fortified samples) (Table 3), with the detector responses obtained from chemical standards that were added to negative control tissue extract that had passed through the extraction procedure (matrix-matched samples).

Intra-assay precision and accuracy was determined by preparing three replicate sets of matrix fortified samples at 3 concentrations within

Table 3a. Absolute recovery, matrix effect, intra-day, inter-day precision and accuracy of the method for determination of tilmicosin residues in equine plasma

Concentration of tilmicosin added to control plasma (ng/mL)	Detector response in arbitrary units			Matrix effect (matrix factor [MF])	Absolute recovery (%)
	Chemical standard (CS)	Matrix-matched standard (MMS)	Matrix-fortified standard (MFS)	MMS/CS	MFS/MMS
40	8.3	7.8	7.6	0.94	97
150	26.6	25.2	25.9	0.95	100
250	47.3	45.2	41.4	0.96	92
500	97.2	89.4	85.5	0.92	96
				0.94 ± 0.02	96 ± 3

	Mean concentration ±S.D. of TIL measured for intra-assay precision	Concentration of tilmicosin in ng/mL measured experimentally from 3 replicate experiments for inter-assay precision			Mean concentration ±S.D. (%RSD) measured for inter-assay precision	Mean accuracy (%) (inter-assay)
	DAY 0	DAY 1	DAY 2	DAY 3		
60	68 ± 9 (13%)	60	79	67	69 ± 10 (14%)	−15
250	273 ± 6 (2%)	242	273	231	249 ± 22 (8%)	0
400	421 ± 9 (2%)	376	411	356	381 ± 28 (7%)	+5

Table 3b. Absolute recovery, matrix effect, intra-day, inter-day precision and accuracy of the method for determination of tilmicosin residues in lung tissue

Concentration of tilmicosin added to control lung tissue (ng/g)	Detector response in arbitrary units			Matrix effect (Matrix factor [MF])	Absolute recovery (%)
	Chemical standard (CS)	Matrix-matched standard (MMS)	Matrix-fortified standard (MFS)	MMS/CS	MFS/MMS
500	10.2	8.3	3.3	0.81	40
1000	19.4	18.0	7.5	0.93	42
2000	36.7	30.2	16.3	0.82	54
3000	57.0	49.1	25.7	0.86	52
				0.86 ± 0.05 (6%)	47 ± 7 (15%)

	Concentration of tilmicosin in ng/g measured experimentally from 3 replicate experiments for intra-day and inter-day precision and accuracy evaluation				Mean concentration ±S.D. (%RSD)	Mean accuracy (%) (inter-assay)
	DAY 0	DAY 1	DAY 2	DAY 3		
600	630 ± 32 (5%)	889	593	550	677 ± 185 (27%)	−13
1200	1311 ± 52 (4%)	1541	1262	1107	1303 ± 220 (17%)	−9
2500	2584 ± 53 (2%)	2953	2532	2225	2570 ± 365 (14%)	−3

the linear range of the standard curve (i.e., low end, mid-point, and high end). These replicate sets of samples were analyzed on the same day (Day 0).

Inter-assay precision and accuracy was determined by preparing and analyzing the same set of matrix fortified samples at the same 3 concentrations on three separate days (Days 1, 2, and 3).

Stability Studies

Stability of Standard Solutions and Tissue Extracts During Analysis. The stability of the standard solutions and the tissue extracts during analysis under typical laboratory room temperature conditions was evaluated by analyzing tissue extracts containing the chemical standards over three cycles of analysis time (i.e., over a 9 hour period).

Stability of Tilmicosin in Tissues Under Freezer Storage Conditions. The stability of control tissue matrices fortified with tilmicosin (250 ng/mL for plasma and 1000 ng/g for lung tissue) and frozen at −20°C was also monitored over a period of 12 months for the plasma and 4 months for the lung to evaluate the effects of freezing storage and freeze/thaw cycles on the stability of tilmicosin.

Verification of the Accuracy of the Method for Analyzing "Unknown Samples"

Once the precision and the accuracy of the method had been estimated and demonstrated to be acceptable, the accuracy of the analytical method to measure the concentration of tilmicosin in "unknown samples" was verified by using the described procedure to analyze tissue samples prepared by the laboratory quality manager or designate, randomized, coded, and presented "blind" to an analyst familiarized with the method.

Application of the Validated Method for Pharmacokinetic Studies

Phase I Single Dose Study

Six foals (approximately 6 months old and ranging in weight from 130 to 245 kg) recently acquired from a Pregnant Mare Urine (PMU) ranch in western Canada were entered into the study. They were administered 10 mg/kg subcutaneous (s.c.) in the neck with Pulmotil® [Elanco Animal Health, Guelph, Canada]. Blood samples were collected from a jugular catheter immediately prior to drug administration and at 15, 30, 45, 60, 90, 120, 150, 180, 240, 300, and 360 min, 7, 8, 12, 24, 36, 48, 60, 72, 84,

Table 4. Concentrations of tilmicosin found in a fully randomized blind-fortified set of samples

Sample identification numbers	Concentration of tilmicosin added to drug-free plasma (ng/mL)	Concentration of tilmicosin experimentally measured in blind-fortified sample (ng/mL)	Average determination	Accuracy (%) 100 * ([Added concn−mean concn]/Added concn)
1	30	28		−7
12	40	36		−10
2, 11	60	57, 62	60	0
3, 10	100	94, 100	97	−3
4, 9	150	141, 138	140	−7
5, 8	300	288, 277	283	−6
6, 7	400	376, 398	387	−3
	Concentration of tilmicosin added to drug-free lung tissue (ng/g)	Concentration of tilmicosin experimentally measured in blind-fortified sample (ng/g)	Mean concentration ±S.D. (%RSD) measured in ng/g	Mean accuracy (%)
2, 6, 10	600	574, 469, 532	525 ± 53 (10%)	−13
12, 8, 4	1600	1617, 1616, 1777	1670 ± 93 (6%)	+4
3, 11, 7	2400	2330, 2610, 2313	2418 ± 167 (7%)	+1
5, 9, 1	2800	3078, 2162, 2790	2677 ± 468 (17%)	−4

and 96 h. Blood samples were collected into EDTA tubes and immediately centrifuged. The plasma was harvested and frozen at −80°C until analyzed.

Phase II – Multiple Dose Study

Following the Phase I study all foals had a minimum of a two week washout period. The six foals were each administered 10 mg/kg s.c. tilmicosin every 72 h for a period of two weeks and individually sacrificed at 4, 16, 26, 40, 50, and 64 h post treatment. Blood samples were collected immediately prior to euthanasia. A post mortem examination was performed and samples of the lung, liver, kidney, and muscle tissue were collected, immediately frozen, and stored at −80°C until analyzed (Table 5).

RESULTS AND DISCUSSION

One of the reasons why tylosin, with similar chemical and biological properties as tilmicosin, was selected as the internal standard was because it was known to be efficiently extracted according to the method of Chan et al.[4] It was selected after analyzing all the pretreatment (drug free) plasma and tissue samples to confirm that they were free from tylosin drug residues. Figure 1a is a typical LC-UV chromatogram of an extract obtained from a drug free (negative control) equine lung tissue containing the internal standard, tylosin. Figure 1b is a typical LC-UV chromatogram of an extract obtained from a drug free lung tissue sample fortified with tilmicosin and also the same amount of the internal standard, tylosin. Tilmicosin and tylosin eluted from the analytical column with a retention time of approximately 6 and 7 minutes, respectively. Tilmicosin was resolved from tylosin and matrix coextractives. Tylosin, the internal standard was, however, not completely resolved from matrix coextracives but this did not impact significantly on the ability to consistently integrate the peak for response ratio measurements. Because the molar absorptivity for tilmicosin is about 4 times greater than that for tylosin, the concentration of tylosin for each matrix was chosen, such that the response ratio was about 1:1 when the concentration of tilmicosin in plasma, muscle, and the other tissues (kidney, liver, and lung) were 150 ng/mL, 1000 ng/g, and 2000 ng/g, respectively.

Selectivity

All the negative control samples (plasma, muscle, kidney, liver, and lung) analyzed showed no detectable concentrations of tilmicosin, regardless of

Table 5. Concentrations of tilmicosin measured in a disposition study after 10 mg/kg multiple dose s.c. administration

Time of sample collection/euthanasia (h)	Foal number	Plasma concentration (ng/mL)	Liver concentration (ng/g)	Kidney concentration (ng/g)	Lung concentration (ng/g)	Muscle concentration (ng/g)
4	3	94.2	560	1367	1189	342
16	7	144.1	1400	4877	2783	589
26	11	85.6	946	2973	1333	881
40	5		595	2093	1587	240
50	1		288	865	859	420
64	9		336	1034	782	170

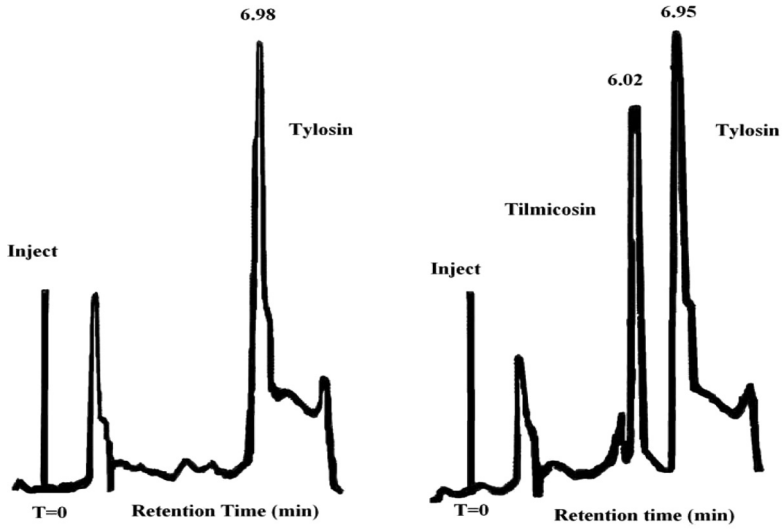

Figure 1. A typical LC-UV chromatogram of an extract obtained from (a) a negative control lung tissue containing the internal standard Tylosin and (b) a negative control lung tissue fortified with tilmicosin at a concentration of 2000 ng/g and containing the same amount of internal standard.

the source of the sample. The results indicated that the analytical method was able to detect truly negative samples as negatives regardless of the age, breed, or sex of the horse from which the sample was taken. It was, therefore, concluded that the method was selective.

The matrix factor (MF), defined as the ratio of the detector signal response of an analyte in the presence of matrix to that of the same analyte in the absence of matrix, is 0.94 ± 0.02 for plasma and 0.86 ± 0.05 for lung tissue. A $MF > 1$, $MF < 1$, and $MF = 1$ indicate matrix enhancement, suppression, and no effect, respectively. It is, therefore, important to recognize that the matrix can have an effect on signal responses generated from even a typical UV detector where the effects of matrix on analyte responses are hardly considered. There is a small matrix suppression effect for tilmicosin response in plasma while there is a significant effect in lung tissue, observations that are important in the calculation of absolute recoveries of the analytes of interest from the matrix under investigation.

Tables 2 and 3 summarize the results of experiments conducted to determine the recovery of tilmicosin added to control plasma and lung tissue using the described method. The method is able to recover approximately 96% of tilmicosin residues from plasma and 47% from lung tissue.

It is believed that the reason why the recovery of tilmicosin from lung tissue was low was because it is very difficult to efficiently homogenize lung tissue using the same equipment that was used for the other matrices primarily because lung tissue, unlike the other matrices, is spongy and air filled and is extremely difficult to homogenize. Nevertheless, despite the low absolute recovery, the results demonstrate that the method is capable of consistently and repeatably extracting about 50% from the matrix and that the analytical method is sufficiently sensitive to permit the measurement of its residual concentration in this matrix. Particularly for lung tissue, then, it was imperative that calibration curves generated from matrix fortified samples be used for quantitative analysis.

The accuracy of the method to measure the residual concentration of tilmicosin in ''unknown samples'' was verified by examination of blind fortified samples prepared by a laboratory quality manager (Table 4). These results confirmed that the method is suitable for quantifying tilmicosin concentrations in ''unknown samples''. The accuracy of the method for tilmicosin detection was better than 10% for plasma and 13% for lung tissue.

Tilmicosin plasma and tissue extracts obtained using the described procedure, held in the autosampler chamber at room temperature of about 23°C, and analyzed over three cycles of analysis time (an analysis cycle for 12 sample extracts including calibration standards would typically be 3 hours) were also found to be stable. There was less than 4% reduction in the measured tilmicosin concentration over that period of time. Additionally, tilmicosin tissue extracts obtained from control plasma and tissues fortified with tilmicosin and stored at −25°C were stable for 12 months in the case of plasma and at least 4 months for lung tissue.

The results of the evaluation of the operational parameters of the method described in this study demonstrated that it is accurate and suitable for determining tilmicosin concentrations in equine plasma and tissues obtained from a control study. It must be noted, that interference from common veterinary drugs that may be efficacious for the treatment of similar conditions in the horse under typical equine feedlot operation was not investigated, and the choice of tylosin as an internal standard was suitable because the study excluded the use of tylosin and any other veterinary drugs. The validated analytical method was used to investigate the pharmacokinetic parameters of tilmicosin in equine tissues and plasma.[5]

The absorption depletion profile plotted for the single dose s.c. administration of tilmicosin to foals (not shown) appeared to be multiphasic with no distinct time to maximum concentration (t_{max}), making it difficult to calculate meaningful c_{max} values. The mean highest concentration of tilmicosin residue measured in plasma was 258 ± 92 ng/mL at a mean time of 6 h.

The concentrations of tilmicosin residues in plasma under multiple dose s.c. administration were still relatively low compared to the concentrations found in animal tissues including lung. For example, the concentration of tilmicosin in kidney, lung, liver, and muscle tissue were, respectively, 34, 19, 10, and 4 times the concentration in plasma 16 h into the multiple dose administration study (Table 5). The disposition of tilmicosin in animal tissues found in this study resembles what we had observed in an earlier study[6] for tilmicosin disposition in elk tissues and plasma, and would tend to lend further support to the fact that the preferential accumulation in tissue is because tilmicosin is highly lipophilic.

The use of tilmicosin in horses is extra label (the US label for Micotil® states that the use of tilmicosin is contraindicated) and although horses can no longer be slaughtered for human consumption in the USA, this is common in other countries. There are no maximum residue limits (MRLs) defined for tilmicosin in countries where horses are slaughtered for food. From a food safety point of view then where the horse is slaughtered for domestic consumption or is involved in international commerce, the tissue data indicate that kidney and lung tissue would be the most appropriate target tissues for monitoring and regulating the use/misuse of tilmicosin in equine food production.

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