JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 2179−2191 **2179**

An Analytical Method for the Analysis of Tulathromycin, an Equilibrating Triamilide, in Bovine and Porcine Plasma and Lung

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Tulathromycin is a novel member of the triamilide class of antibiotics that was developed as a safe and effective single-dose treatment of bovine and porcine respiratory disease. An accurate and precise analytical method was developed for the extraction and measurement of tulathromycin in livestock plasma and lung homogenates. Analytes were solid-phase extracted onto a weak cation exchanger after aqueous dilution of samples and addition of heptadeutero-tulathromycin as an internal standard. Following HPLC with a narrow bore C8 column, quantitative detection of tulathromycin was accomplished by monitoring the transition of a doubly charged precursor ion to a singly charged product ion by tandem mass spectrometry using a triple quadrupole mass spectrometer. Procedures were validated for a dynamic range of 0.1 to 25 ng on column. Observed accuracies were between 90 and 110% of nominal and precision (RSD) varying 7% or less. Response and stability experiments showed that deuterated tulathromycin did not parallel the chemical behavior of tulathromycin. Applicability of the method to livestock studies was tested with plasma and lung samples from cattle and swine dosed with tulathromycin at multiple doses. The results demonstrated that the analytical method performed well in a range of sample concentrations spanning over 3 orders of magnitude and provided dose−exposure relationships for cattle and swine.

KEYWORDS: Triamilide antibiotics; tulathromycin; mass spectrometry; swine; swine plasma; swine lung; cattle; cattle plasma; cattle lung; pharmacokinetics

INTRODUCTION

Tulathromycin is a novel triamilide (*1*) antibiotic suitable for the treatment of respiratory disease in cattle and swine. Macrolide antibiotics approved for use in livestock include erythromycin, tylosin, spiramycin, and tilmicosin. Typically, repeated administration of these products over several days is required to achieve therapeutic or preventative efficacy. Prolonged exposure of lung tissues to macrolides are important for the treatment and prevention of bovine respiratory disease, but single administration therapy is desirable for livestock producers who wish to minimize animal handling and maximize compliance. Of the macrolides, tilmicosin is the only agent used in cattle with an indication of single injection therapy for respiratory disease. Tulathromycin has been developed for both swine and cattle to provide high clinical efficacy against respiratory disease after a single parenteral administration of 2.5 mg/kg.

To characterize a drug's disposition in animals and its relationship to efficacy, an accurate, precise and robust analytical method is required. Tulathromycin is structurally related to the di-basic azalide antibiotic azithromycin (*2*). The methods reported for the analysis of azithromycin in biological matrixes involve basification with carbonate, extraction by an organic solvent, concentration, reconstitution with an HPLC mobile phase, separation by HPLC, and detection either by an electrochemical detector (EC) (*3*) or by atmospheric pressure chemical ionization (APCI) mass spectrometry (*4*) with selected ion monitoring. More convenient methods for extracting mac-

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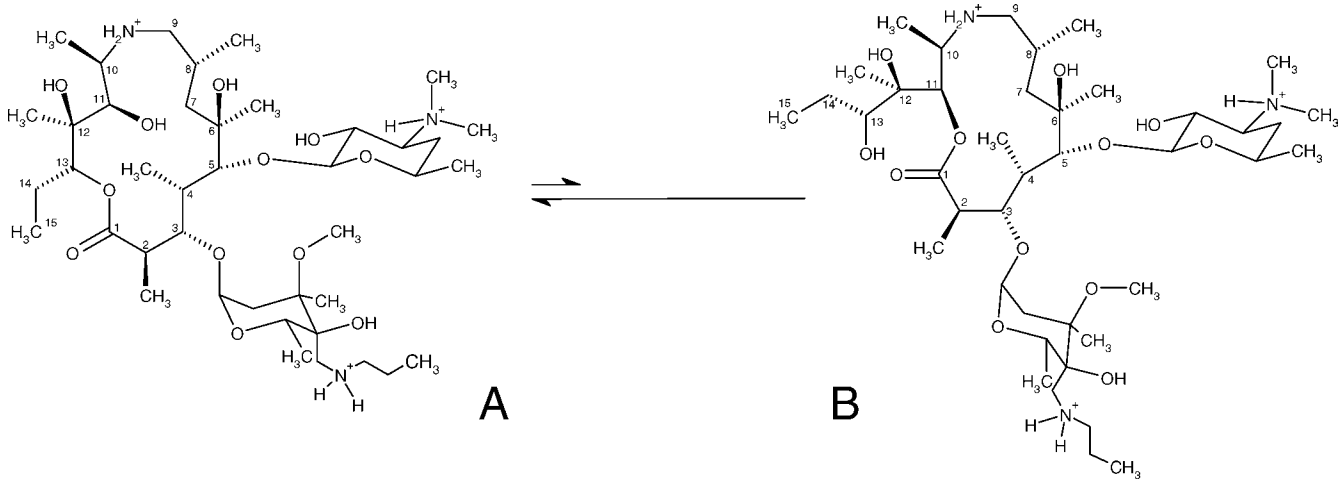
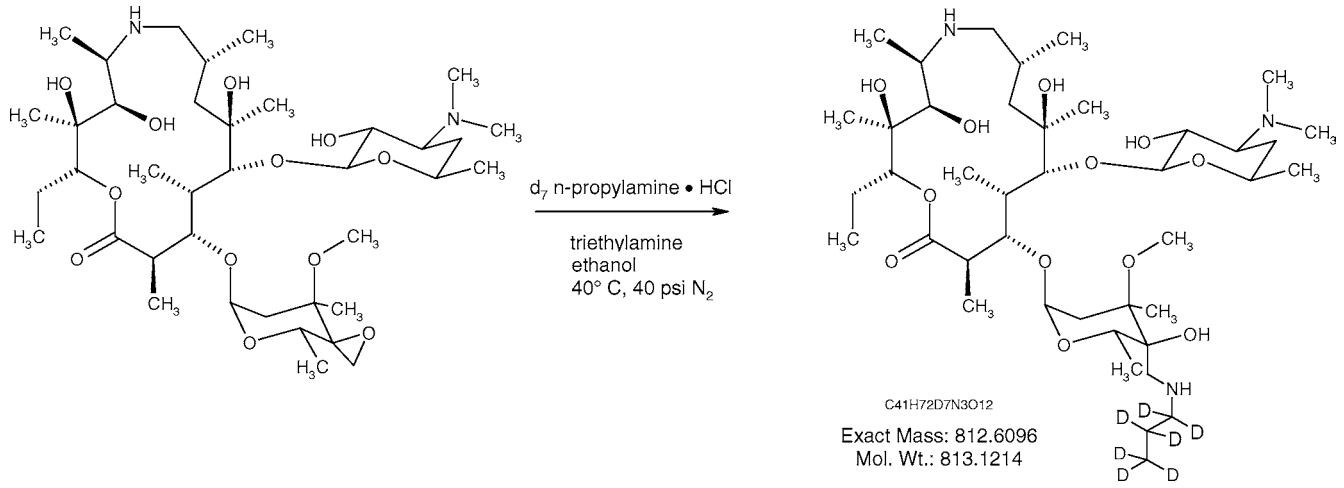
10.1021/jf0351624 CCC: $27.50
Published on Web 03/27/2004

Scheme 1. The Isomerization of Tulathromycin in Aqueous Media[a]



[a] At Equilibrium, Isomers A and B Represent 90 ± 3% and 10 ± 3% of the Total Tulathromycin Content.

Scheme 2 The Synthesis of Heptadeuterated Tulathromycin (Isomer A)



rolide antibiotics (*5*) from tissues have appeared recently employing solid-phase extraction (SPE) (*6*). Use of reverse-phase SPE requires analyte basification (e.g., extraction into aqueous buffer at pH 10) (*7*). Application of solid-phase extraction by strong ion exchangers has also been reported (*8*, *9*), taking advantage of the charged amino group(s) on the carbohydrate fragments found in macrolide antibiotics. Most of these methods for tissue analysis report multiple sample cleanup and extraction steps. Our objective for the analysis of tulathromycin was to minimize the required steps for sample preparation and to take advantage of the sensitivity and specificity of tandem mass spectrometry.

In aqueous media, tulathromycin exists as an equilibrated mixture (*10*−*12*) of 15-membered and 13-membered isomers (Isomers A and B, respectively) (*11*, *12*) (**Scheme 1**). Because tulathromycin is manufactured as Isomer A, providing an aqueous formulation of unchanging composition required a kinetic adjustment. Preparation of tulathromycin dosage formulations, therefore, involves steps to rapidly achieve the equilibrated ∼9:1 isomeric composition (*10*, *11*). On the other hand, tulathromycin analytical reference solutions are typically prepared without accelerating the equilibrium toward the composition endpoint. As a result, the amounts of isomer B are expected to increase (∼0−10%) over the life of the reference solution. We needed to define the resulting bias, if any, upon the assay.

Finally, we needed to demonstrate the practical dynamic range requirements for the assay as well as to provide time−concentration profiles to aid in the design of definitive pharmacokinetic studies in cattle and swine.

Our report describes a rapid new assay utilizing aqueous dilution of plasma or lung homogenate, followed by cation exchange SPE. Detection via electrospray ionization and tandem mass spectrometry afforded the sensitivity, selectivity, and accuracy required to measure tulathromycin in cattle and swine plasma and lung homogenate.

MATERIALS AND METHODS

Reagents. Tulathromycin Isomer A was prepared as reported earlier (*1*). Isomer B was obtained as described in the patent literature (*12*). All solvents were HPLC grade. Reagents were USP/NF or reagent grade.

Synthesis of a Heptadeutero Tulathromycin Derivative. The heptadeuterated standard was prepared as outlined in **Scheme 2**.

The preparation of the epoxide has been reported previously (*11*). The epoxide (2.0 g) was dissolved in 20 mL ethanol then mixed with d_7-*n*-propylamine hydrochloride (5 g, CDN Isotopes, Quebec, Canada) and triethylamine (37 mL). The mixture was stirred under a positive pressure of nitrogen (40 psi) and heated to 40 °C over a period of 5 days. The reaction progress was monitored by TLC and MS. Upon completion, the excess d_7-*n*-propylamine hydrochloride was filtered, and the filtrate was concentrated on a rotary evaporator. To the resultant off-white foam was added water (60 mL) and methylene chloride (30

mL). The pH of the aqueous layer was adjusted to 6.7 by the addition of 3 N HCl. The phases were separated, the aqueous phase was combined with fresh methylene chloride (30 mL), the pH was adjusted to 9.0 by the addition of potassium carbonate powder, and the methylene chloride phase was separated, dried over sodium sulfate, filtered, and concentrated. The resulting solid was purified by flash column chromatography using a 30- × 3-cm slurry packed silica gel column and a mobile phase consisting of hexanes, diethylamine, and acetonitrile mixed in volumetric proportions of 10:3:1, respectively. The product was loaded on the column in a solution prepared by dissolving it in a minimal amount of methylene chloride and then diluting with mobile phase. Fractions (15 mL) were collected at a flow rate of about 33 mL/min, those containing the desired product were combined and concentrated. The resulting material was re-processed similarly. The final residue (1.69 g) from the combined fractions was dissolved in *n*-heptane (10 mL), where a fine white powder precipitate formed during overnight stirring at ambient temperature. The final yield was 1.3 g of 98% purity, as demonstrated by HPLC with a parent ion of 813.6 ($M + H^+$) by MS analysis. Subsequent HPLC/MS/MS characterization showed the ratio of tulathromycin/d_7-tulathromycin to vary between 0.015 and 0.019.

Dose Formulation. Tulathromycin Isomer A (38.74 g) was slowly dissolved in a mixture prepared by combining citric acid (7.19 g), water (119.18 g), and 10% HCl (8.82 g). The resulting solution was adjusted to pH 7 by the addition of 10% HCl (2.98 g), sparged with nitrogen, and heated to 70 °C for 100 minutes to form the equilibrated mixture of Isomers A and B. Upon cooling to room temperature, propylene glycol (187 g) was stirred into the solution and the pH adjusted from 7.65 to 5.53 with 10% HCl (15.3 g). Liquefied phenol (1.072 g) and monothioglycerol (1.851 g) were dispersed in the solution, the pH was adjusted to 5.4 with 10% HCl (0.58 g), and water (14.95 g) was added to bring the total weight to 397.50 g. The process was completed by filtering the solution in a laminar flow hood through a 0.22-μm GV Durapore Stericup sterile filter unit into sterilized flint Type I vials (10, 20, and 50 mL), sparging with sterile nitrogen, stoppering with rubber stoppers, and sealing with aluminum crimps. All components except the tulathromycin were USP/NF grade. The potency and equilibrium composition of the formulation were verified by HPLC by a modification of a procedure reported for azithromycin (*13*).

Equilibration in Bovine Plasma. Stock solutions (100 μg/mL) of tulathromycins A and B were prepared separately in a mixture of 50 mM K_2HPO_4 (pH 6.3) and acetonitrile (3:1, v/v). Fresh blank bovine plasma was obtained from Charles River Pharm Services (Wilmington, MA). Immediately after the preparation of each stock solution, 2-mL portions of plasma were fortified with 60 μL of each stock solution, and the mixtures were kept at room temperature. Fortified plasma portions were sampled (200 μL aliquots) 0, 1, 4, and 24 h after fortification with isomer A and 0, 1, 4, and 48-h after fortification with isomer B; each sample was assayed promptly after collection. Samples were prepared by adding 200 μL of acetonitrile, vortex mixing for about twenty seconds, clarifying by centrifugation, and diluting 100 μL of the supernatant with 100 μL of 10 mM acetic acid. Analysis was conducted by injecting 20 μL onto an HPLC/MS system. Components were resolved using a 5-μm Waters Symmetry C18 column (2.1- × 150-mm). Mobile phase (0.3 mL/min), was delivered in a linear gradient activated one minute postinjection, ramping from 95% 5 mM ammonium acetate (pH 4.0) to 10% 5 mM ammonium acetate over eleven minutes. Acetonitrile comprised the organic portion of the mobile phase. After each analysis, mobile phase composition was returned to its initial point over 2 min and was held constant for at least 6 min before subsequent injections. For work demonstrating the tulathromycin equilibrium in bovine plasma, a Sciex API 150 mass spectrometer with an APCI source was operated in the positive-ion mode. The flow of the nebulizer gas was set at 12 L/min and the curtain gas at 9 L/min. The needle current (NC) was set at 3.5 μA, nebulizer probe temperature at 450 °C, and the orifice voltage was 35 V. All other voltages were set to maximize transmission and optimize sensitivity. Selected ion monitoring at *m*/*z* 807 was performed.

HPLC/MS/MS. A BAS PM-80 gradient pump equipped with an LC-26A on-line vacuum degasser, a Thermo Separations Sample Sentinel autosampler, model SS-2001, with 20 μL loop and a BDS Hypersil C8 column (30- × 2-mm, Keystone Scientific, part number 035−45−2) was used for the separation of tulathromycin from plasma and lung extracts. Autoinjector flushing was carried out with a 1:1 mixture of 20 mM ammonium acetate (pH 4.0) and acetonitrile. Isocratic analyses were performed with a mobile phase consisting of 82% 0.02 M aqueous ammonium acetate (pH 4.0) and 18% acetonitrile (0.25 mL/min) (the mobile phase proportions indicate the component volumes mixed, e.g., 500 mL aqueous with 110 mL acetonitrile.) A MicroMass QuattroLC tandem quadrupole mass spectrometer with an electrospray ionization source operated in the positive ion mode was used as the HPLC detector for the quantitation of tulathromycin in plasma and lung extracts. Instrumental parameters (**Table 1**) were optimized for detecting the transition of the doubly charged molecular ion to a product ion representing the loss of the modified cladinose moiety for each analyte (**Figure 1**). The doubly charged molecular ion [MH_2^{2+}] was *m*/*z* 403.9 for tulathromycin and 407.5 for tulathromycin-d_7. Product ions were the same at *m*/*z* 577.6 for tulathromycin and its d_7 analogue. Dwell time (0.30) and the collision offset (15) were the same for both analytes.

Table 1. Optimized Instrumental Variables for the Detection of Tulathromycin in Plasma and Lung Extracts

variable	value
nebulizer	96.6 L/h
desolvation gas	428.9 L/h
ion mode	positive
capillary voltage (ESI)	0.75 kV
acquisition time	6.5 min
interchannel delay	0.03 s
cone voltage	22 V
source block temp.	100 deg C
desolvation temp.	350 deg C
CID gas	Ar
appox. gas cell pressure	1.2e-3 mBar
multiplier setting	650
LM res 1	13
HM res 1	13
LM res 2	13
HM res 2	13

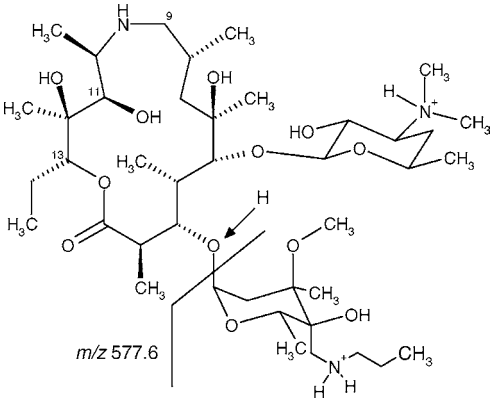


Figure 1. Tulathromycin Isomer A and major fragment ions.

Solutions for HPLC/MS/MS Analyses. Stock solutions (100 μg/mL) of tulathromycin and tulathromycin-d7, were prepared in 75% 50 mM K_2PO_4 and 25% acetonitrile (apparent pH adjusted to 6). Working solutions of tulathromycin were prepared by dilution of the stock solution to concentrations of 10 and 2.2 μg/mL. The internal standard working solution was prepared at a concentration of 50 ng/mL.

System check solutions within the dynamic range of the assay were prepared from the stock and/or working solutions of both tulathromycin and tulathromycin-d_7 by dilution of the appropriate aliquots to 10 mL with the HPLC mobile phase. Concentrations of tulathromycin in the check solutions are representative of the expected concentrations of drug and internal standard in the extracts from the quality control samples.

Plasma Assay Procedure. System check solutions were prepared containing 1125, 526.5, and 37.5 ng/mL tulathromycin and 125 ng/

mL each of the internal standard. Quality control (QC) samples were prepared directly from either the stock solutions or the working solutions (75−225 μL volumes) by adding them to plasma at a final volume of 50 mL. The three fortifications represented tulathromycin concentrations in plasma of 450, 225, and 15 ng/mL. Each QC sample was subdivided into several 0.6 mL or 1.2 mL portions and stored at −20 °C. A QC sample containing 2000 ng/mL tulathromycin was prepared to test the analysis of over-range samples by dilution. This sample was then diluted 5× with control plasma before sample analysis. Quality control, blank plasma, and test samples were thawed at room temperature and then vortex mixed. Calibration standards were prepared by the serial dilution of bovine plasma containing 500 ng/mL of tulathromycin with bovine plasma. Resultant calibration standards contained 500, 400, 250, 100, 50, 10, 5, and 2 ng/mL tulathromycin. Aliquots (500 μL) of each calibration standard, quality control sample, plasma blank, and unknown were transferred to labeled 13- × 100-mm polypropylene tubes along with 500 μL of the internal standard working solution. A nonfortified blank was prepared by adding 500 μL of 50 mM K_2HPO_4 (pH 6.8) instead of the internal standard working solution. Each extract sample was briefly vortex mixed. Samples containing suspended particulates were centrifuged at approximately 215*g* (IEC Centra GP8) for approximately 5 min. SPE cartridges (CBA, 500-mg bed mass, 3-mL tube volume, Bond Elut, Varian) were preconditioned with 2.0 mL of acetonitrile followed by 2.0 mL of pH 6.8, 50 mM K_2HPO_4. A weak vacuum was applied to the SPE manifold. All extracts were decanted onto the SPE cartridges. SPE tubes were rinsed sequentially with 2.0 mL of pH 6.8, 50 mM K_2HPO_4, 2.0 mL of H_2O, and 2.0 mL of acetonitrile. Analytes were eluted with 2 × 2.0 mL, and 1 × 1.0 mL (5.0 mL total) of freshly prepared 5% NH_4OH/95% acetonitrile into labeled 13- × 100-mm polypropylene tubes. Collected extracts were evaporated to dryness (TurboVap LV evaporator; Zymark) under a stream of nitrogen at 50−55 °C. Extract residues were reconstituted with 0.200 mL mobile phase by vortex mixing for approximately 2 min in a multitube vortex mixer. Reconstituted samples were centrifuged at about 215*g* for approximately 5 min, and resulting supernatants were transferred to plastic autosampler vials.

Lung Homogenate Assay Procedure. System check solutions were prepared containing 1000, 500, and 10 ng/mL of tulathromycin, each mixed with 125 ng/mL of the internal standard. Blank tissue (from NTX animals) and test samples were thawed at room temperature. Aliquots of blank lung homogenate (∼200 mg) were weighed into 16.5- × 101-mm polypropylene tubes for the preparation of three blanks, quality control samples, and the primary calibration extract. Quality control samples for lung homogenate were prepared by spiking 200-mg aliquots of blank lung homogenate with tulathromycin (40−200-μL volumes of the stock solution or working solutions). Over-range, upper limit, and lower limit of quantitation samples were also prepared providing extract concentrations of 4000, 1000, and 4 ng/mL representing tulathromycin lung residues of 100 000, 25 000, and 100 ng/g, respectively. The high, middle and low quality control samples provided extract concentrations of 800, 400, and 8 ng/mL equivalent to 20, 10, and 0.2 μg/g, respectively, of tulathromycin lung residues. The primary calibration extract (1000 ng/mL) was prepared by mixing 200 mg of blank lung homogenate, 5 μg of tulathromycin (50 μL of the tulathromycin stock solution), and 0.04 M H_3PO_4 (4.95 mL), followed by agitation with a Polytron probe, centrifugation and decanting. Calibration standards were prepared from the 1000 ng/mL primary calibration extract by serial dilution with the extract from blank lung homogenate. Eight calibration solutions were thus prepared containing tulathromycin at concentrations of 1000, 800, 500, 200, 100, 20, 10, and 4 ng/mL. Replicate processing of lung homogenate samples is diagrammed in **Scheme 3**. Triplicate ∼200-mg portions of each unknown were mixed with 5 mL of 0.04 M H_3PO_4 in 16.5- × 101-mm polypropylene tubes. Samples were homogenized (Polytron probe) and centrifuged for 10 min at ∼4500*g*, and the supernatants were decanted from the remaining pellets. Extract aliquots (0.250 mL) were transferred into 13- × 100-mm polypropylene tubes, 0.250 mL of 0.1 M K_2HPO_4 (pH 6.8) was added to adjust the pH of the sample mixture, followed by the addition (0.5 mL) of the internal standard working solution. Control extract was mixed with 0.5 mL of 50 mM K_2HPO_4 (pH 6.8) in place of the internal standard. SPE cartridges were preconditioned as described for the plasma sample assay. Samples were loaded onto the SPE cartridges, which were sequentially rinsed with 2.0 mL of 50 mM K_2HPO_4 (pH 6.8), 2.0 mL of H_2O, and 2.0 mL of acetonitrile. Tulathromycin was eluted from the SPE cartridges as described for the plasma samples. Extracts were concentrated under N_2, residue reconstituted as described for the plasma samples, and analyzed using HPLC/MS/MS.

Quantitative Relationships. The chromatographic peaks generated by the MRM were integrated using MassLynx 3.1 software to produce peak areas. The ratio of the drug peak area to the internal standard peak area was determined, and quantitative results were calculated applying 1/concentration^2 weighting to the quadratic regression procedure.

Validation Exercises. Calibration samples were prepared in duplicate at eight concentrations. Accuracy and precision was also determined from the replicate analysis of the QC samples ($n = 6$ at each concentration). Intra-assay and inter-assay precision were determined from the relative standard deviations of the quality control samples. Accuracies were characterized with the inter-assay results of the calibration samples (which had to be within ±20% of nominal) as well as the intra and inter-assay data from the QC samples (which had to be within ±15% of the nominal concentration for the high and mid QC or ±20% for the low QC). Accuracy results were estimated by comparing mean measured concentrations with the appropriate nominal concentrations. Quality control samples were also prepared at a concentration exceeding the range of the standard curve. They were diluted 5-fold with blank matrix before analysis to characterize sample dilution integrity. Limits of quantitation were tested with six replicates prepared at both the highest and lowest concentration of the calibration curve samples. Assay specificity was tested by the analysis of blank matrix from six different sources. Response recoveries were estimated by comparing data from normal quality control samples with those of response recovery samples (blank sample extracts reconstituted with system check solutions). Analyte stability under the assay conditions was tested with storage experiments at ambient, refrigerated, and freezer temperatures, as well as freeze/thaw cycling. Stability in lung homogenate was investigated with pooled samples from treated animals where the initial concentration was determined immediately after sample blending. All of the validation exercises were performed with plasma and lung homogenate from cattle. Cross validation for swine samples was by preparation of QC samples in the swine biomatrixes and comparing the results to cattle QC samples in the same run.

Sample Analysis. Before the start of each run and after completion, system check solutions were injected into the HPLC/MS/MS system to monitor system stability. Samples were injected in random order between runs. A sample run consisted of duplicate calibration samples, duplicates of each QC sample, control samples with and without the internal standard, and the study samples. Lung homogenate samples were analyzed in triplicate, and the final concentration was their average. Sample RSD had to be 15% or less indicating adequate homogeneity. Lung samples not meeting the homogeneity requirement were re-blended in whole and the analysis repeated. Samples found to be above the dynamic range of the standard curve were reanalyzed after dilution. In this case, over-range QC samples were also included in the run.

Animal Studies. Crossbred beef calves (240−310 kg) and crossbred swine (15 to 20 kg) were acclimated for 84 and 10 days, respectively, prior to initiating the study. On day 5, animals were weighed, and 20 animals from each species were selected on the basis of body weight and general health. Selected animals were randomly assigned to treatment groups as shown in **Table 2**. Animals were fed appropriate diets that met or exceeded NRC requirements (cattle, 12.5% protein, (70:30 concentrate: roughage), up to 4 kg daily; swine, 21% protein, ad libitum) and were provided unrestricted access to water.

On day 0, calves were weighed and administered individually calculated doses by subcutaneous injection in the right neck; injection volumes were limited to 10 mL per site. Doses for treatment groups T1 and T2 were administered as single injections; the T3 dose required two injections (10 mL in first injection, remainder in second injection). Swine were injected (0.21−0.99 mL) intramuscularly in the right side of the neck. Each syringe was weighed with contents and after injection

Scheme 3. Processing of Lung Homogenate

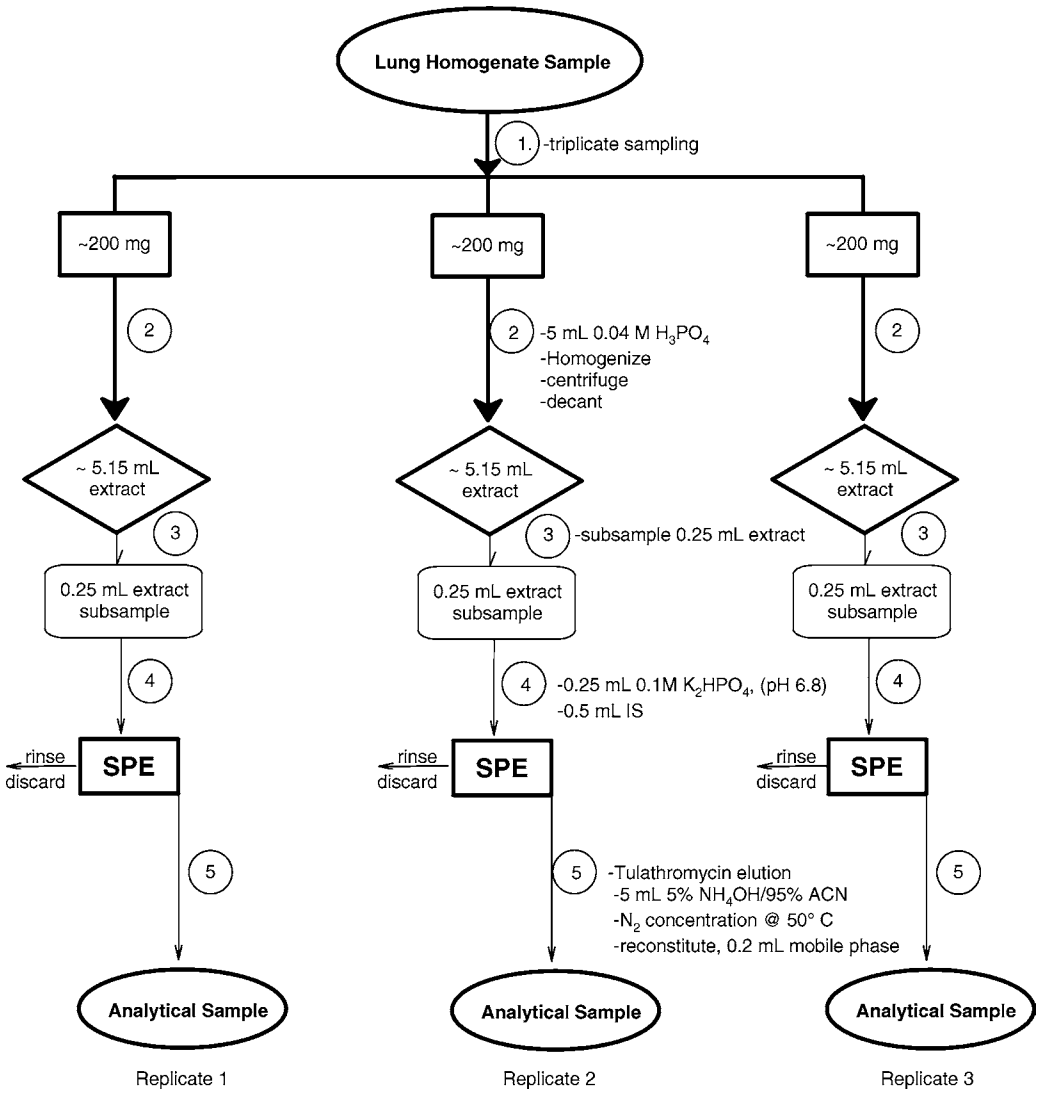


Table 2. Experimental Design for Cattle and Swine Studies

treatment	target dose[a] (mg/k)g	study duration (d)	total animals	animals per sex	
				castrate	female
NTX[b]		7	2	1	1
T1	1.25	7	6	3	3
T2	2.50	7	6	3	3
T3	5.00	7	6	3	3

[a] Dose delivered as a subcutaneous injection in cattle and as a single intramuscular dose in swine. Actual doses delivered were determined by calculating the difference in syringe weight before and after injection. [b] NTX = no treatment administered; control animals.

to determine the actual dose delivered. Doses were selected to bracket the intended commercial dose of 2.5 mg/kg.

Blood was collected from each animal via jugular venipuncture into heparinized vacutainers at 0 (pre-dose), 0.25, 0.5, 1, 2, 4, 8, 24, 48, 72, 120, and 168 h post-dose. Plasma was separated by centrifugation, poured into two plastic vials, and stored frozen.

On day 7, animals were euthanized, exsanguinated, and eviscerated. Lung tissue (a composite of samples from all lobes, ~500 g from cattle and ~250 g from swine) was collected from each animal and homogenized in a food processor, and the homogenate was subsampled and frozen.

Pharmacokinetic Analysis. Depletion of plasma tulathromycin from treated animals was used to calculate AUC_{0-168} (AUC from time 0 to 168 h.), $AUC_{0-\infty}$, C_{max}, T_{max}, k_{el} and apparent $t_{1/2}$. Pharmacokinetic parameters were estimated by noncompartmental analysis (WinNonlin Professional Network Edition Version 2.1). The mean (apparent)

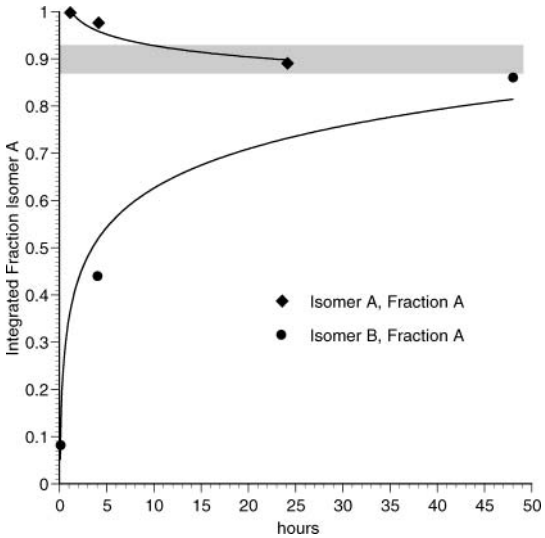


Figure 2. The fraction of isomer A observed over time in experiments spiking bovine plasma with Isomer A (◆) or Isomer B (●). The shaded area represents the endpoint composition reported for aqueous systems.

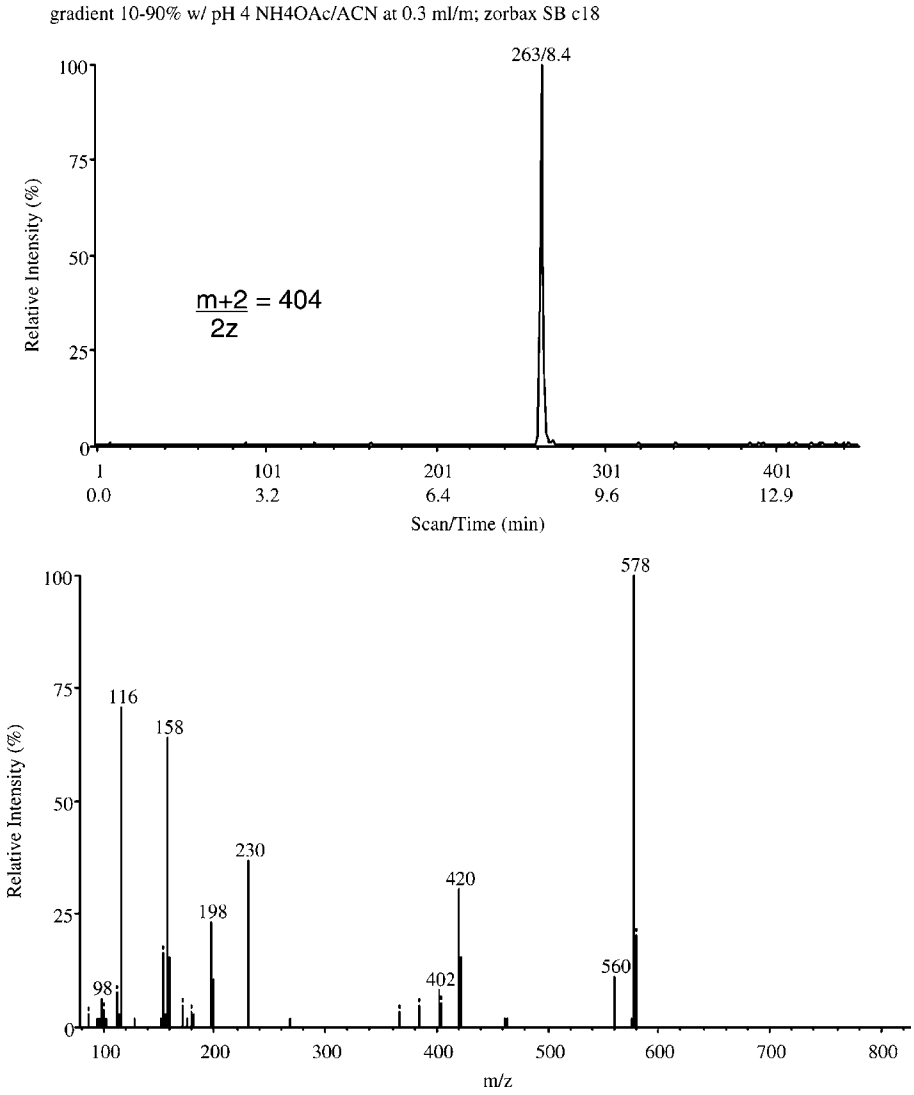


Figure 3. Chromatogram from a solution of the 15 membered isomer monitoring the precursor ion at *m/z* 404 (top) and the spectra of its product ions (bottom) obtained on a Sciex API III+ mass spectrometer.

elimination half-life was calculated as the harmonic mean (ln(2)/mean k_{el}).

Tulathromycin concentrations in plasma were summarized as geometric means from all animals in each treatment group at each time point along with the 99% upper and lower confidence limits of the drug concentration. Geometric means and 99% confidence bounds were calculated with log transformed data ($y = \ln(x)$) and the final results obtained by back transformation ($\exp(y)$).

Using a mixed linear model, a priori contrasts among least squares means were used to assess treatment differences in each pharmacokinetic variable. A significant overall *F*-test ($P \leq 0.05$) for treatment effects was taken as evidence of a dose-related effect.

Prior to analysis, the variance distribution of each variable was examined using Proc Univariate in SAS. For C_{max}, and T_{max}, a log transformation was used to reduce skewness. Additionally, homogeneity of variance across treatment groups was tested using Bartlett's test. The variables AUC_{0-168} in cattle and C_{max} in swine had heterogeneous within treatment variances ($P < 0.005$); therefore the mixed model analyses for these variables was modified to allow for estimation of different treatment group variances.

RESULTS AND DISCUSSION

Equilibration of Tulathromycin Isomers in Cattle Plasma. Characterization of the tulathromycin equilibrium (*10*, *11*) led to the assumption that in aqueous formulation as well as under physiological conditions both isomers would be present even if only one isomer was originally present. Hence, dosage formulations are prepared with an equilibration step. Notably, composition at equilibrium was insensitive to changes in pH and the presence of organic cosolvents. Under physiological conditions, however, specific binding, metabolism, or other enzymatic processes may disproportionate the equilibrium composition. To test for gross specific effects, we added tulathromycin isomer A or isomer B to fresh bovine plasma (which was never frozen) and analyzed the fortified plasma for its isomeric composition. The results are shown in **Figure 2** where the relative amount of isomer A is plotted versus time for both experiments.

These results demonstrate little or no influence of the biological matrix on the equilibrium composition. Neither isomer appears to be preferentially scavenged by a specific process under the reported experimental conditions. The measured analytical response, therefore, will always be that of the predominant species (~90%) isomer A. Isomer A is the only isomer practical for use as an analytical reference. Reverse reaction rates from isomer B have been determined to be about 7-fold faster (*11*), hence reference solutions prepared from isomer B are of limited usefulness.

Detection and Chromatography of Tulathromycin. Under the conditions investigated, monitoring the doubly charged

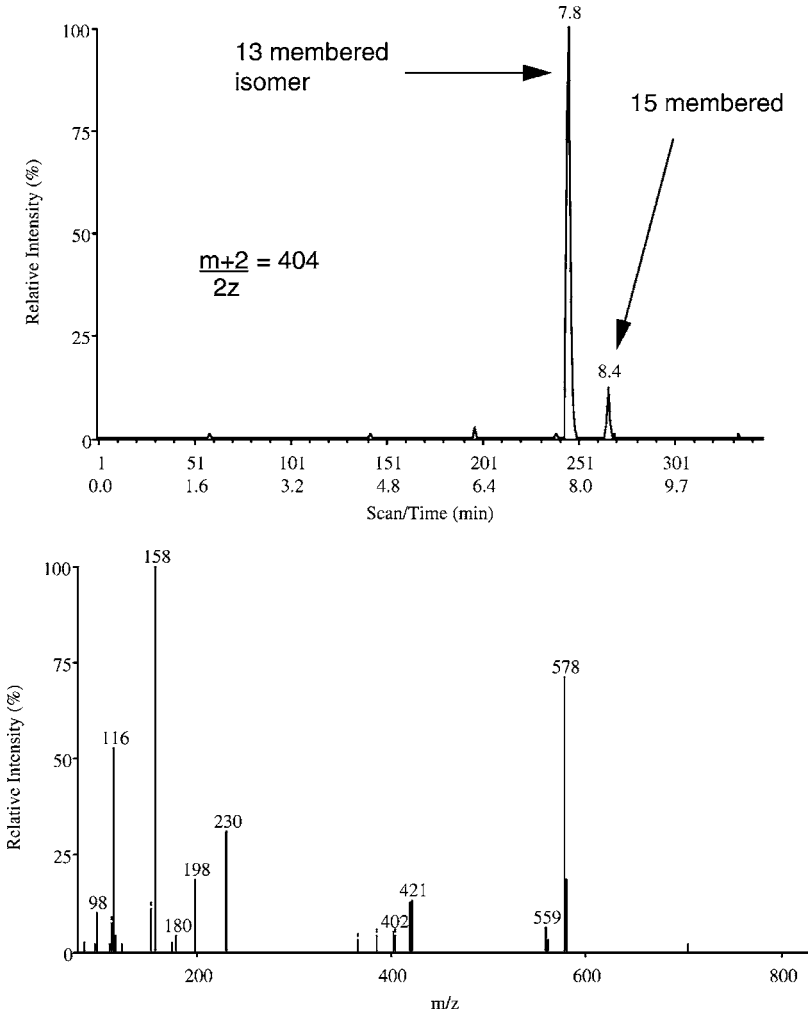


Figure 4. Chromatogram from a solution of the 13 membered isomer monitoring the molecular ion at *m/z* 404 (top) and the spectra of its product ions (bottom) obtained on a Sciex API III+ mass spectrometer.

parent by MS/MS provided an effective choice for quantitative analysis. Following multiply charged species is much more common in the analysis of proteins than it is for smaller organic molecules. However, a similar approach was reported for the confirmation of tilmicosin (*14*) in bovine tissues. The transition of the doubly charged ion at *m/z* 404 to a product ion of m/z 578 appeared to be consistent for both of its isomers. Examples from the chromatography of standards of both isomers are shown in **Figure 3** (isomer A) and **Figure 4** (isomer B).

Chromatography for the quantitative method was adjusted such that both isomers coeluted with a retention time of about 1.7 min and a total run time of 6.5 min (**Figures 5**−**7** for plasma samples). Lung homogenate chromatograms were similar and are not shown here.

Some carry-over is apparent in the chromatogram of control samples (**Figure 7**) but was not considered problematic because the ion counts representing the analytes are at least 100-fold less than those observed in samples containing analytes.

Stock Solution Stability. Tulathromycin stock solution stability was monitored over a 99 day period. When not in use, the solutions were stored refrigerated (4−8 °C), and at the end of 99 days, the peak areas from replicate injections ($n = 6$) from the aged and freshly prepared solutions were compared. The difference in the mean peak area of the aged solution was insignificant (mean ± SD, fresh vs aged, 742 000 ± 10 000 vs 740 000 ± 20 000). Heptadeutero tulathromycin showed a −3.7% change under similar conditions after seventeen days (1 970 000 ± 30 000 vs 1 896 000 ± 13 000). Therefore, differences, if any, in total peak area response due to contributions from isomer B, as the equilibrium endpoint is approached with time, are inferred to be negligible. Under these conditions, the MS/MS response from each regio-isomer is similar, with no need to separate them chromatographically for accurate quantitation.

Plasma Assay Validation Summary, Calibration Samples. In four separate runs with plasma, the quadratic regression of peak area ratios versus concentration resulted in excellent linearity. Correlation coefficients varied from 0.9984 to 0.9999. Inter-assay precision (%RSD) of the plasma calibration samples was 2.3% or less for samples fortified with 10−500 ng/mL tulathromycin. Greater variation, 7.3%, was observed for the lowest calibration standard (5 ng/mL fortification). Inter-assay accuracy of the calibration sample concentrations varied between 98.5 and 101% over the dynamic range. Tulathromycin concentration in the extracts from the plasma calibration samples ranged from 5 to 1250 ng/mL, resulting in a dynamic range of 0.1−25 ng tulathromycin injected (20μL) on column. Limits of quantitation were demonstrated with plasma samples fortified with tulathromycin at 500 ng/mL and 2 ng/mL (accuracy ± precision (RSD) of 90.2 ± 1.2% and 110± 6%, respectively). All results were well within the calibration sample accuracy acceptance guideline set for these experiments at ± 20% of nominal. Limits of detection were governed by the fraction of

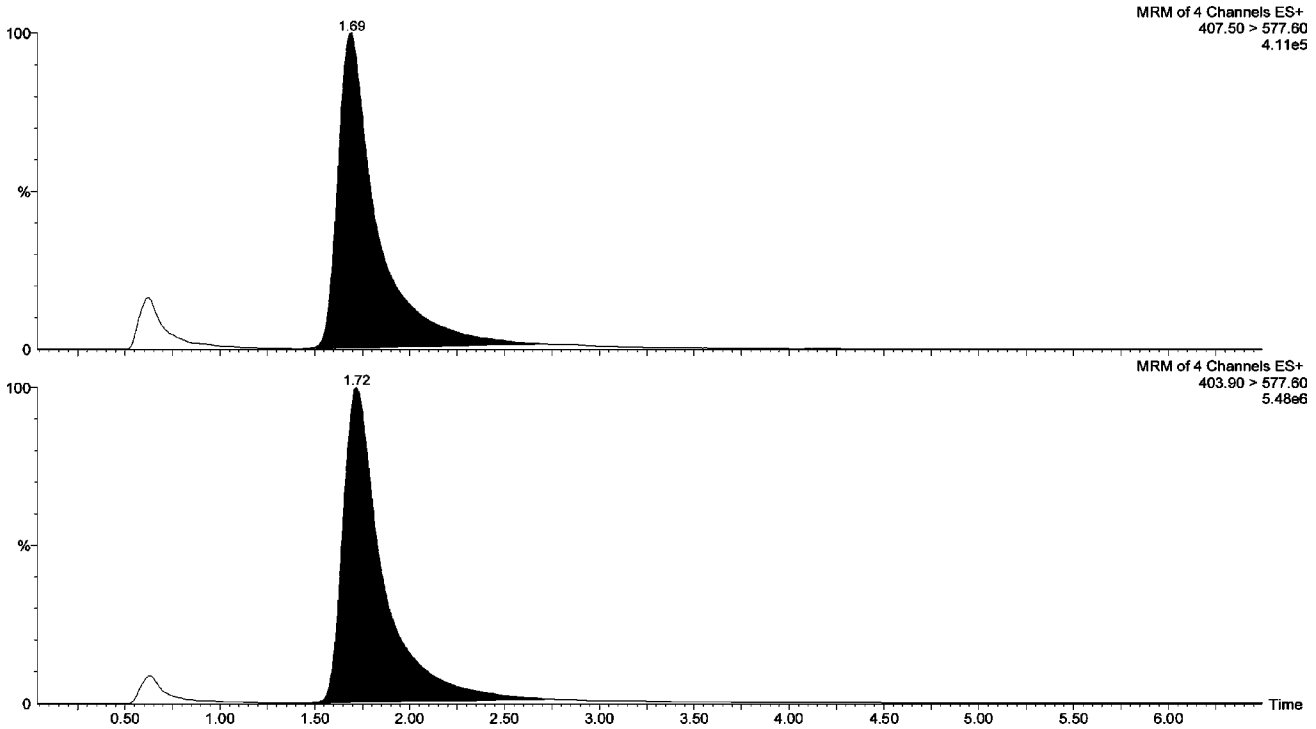


Figure 5. A representative chromatogram of the 500 ng/mL calibration standard used in the analysis of plasma containing the internal standard (top trace) and tulathromycin (bottom trace).

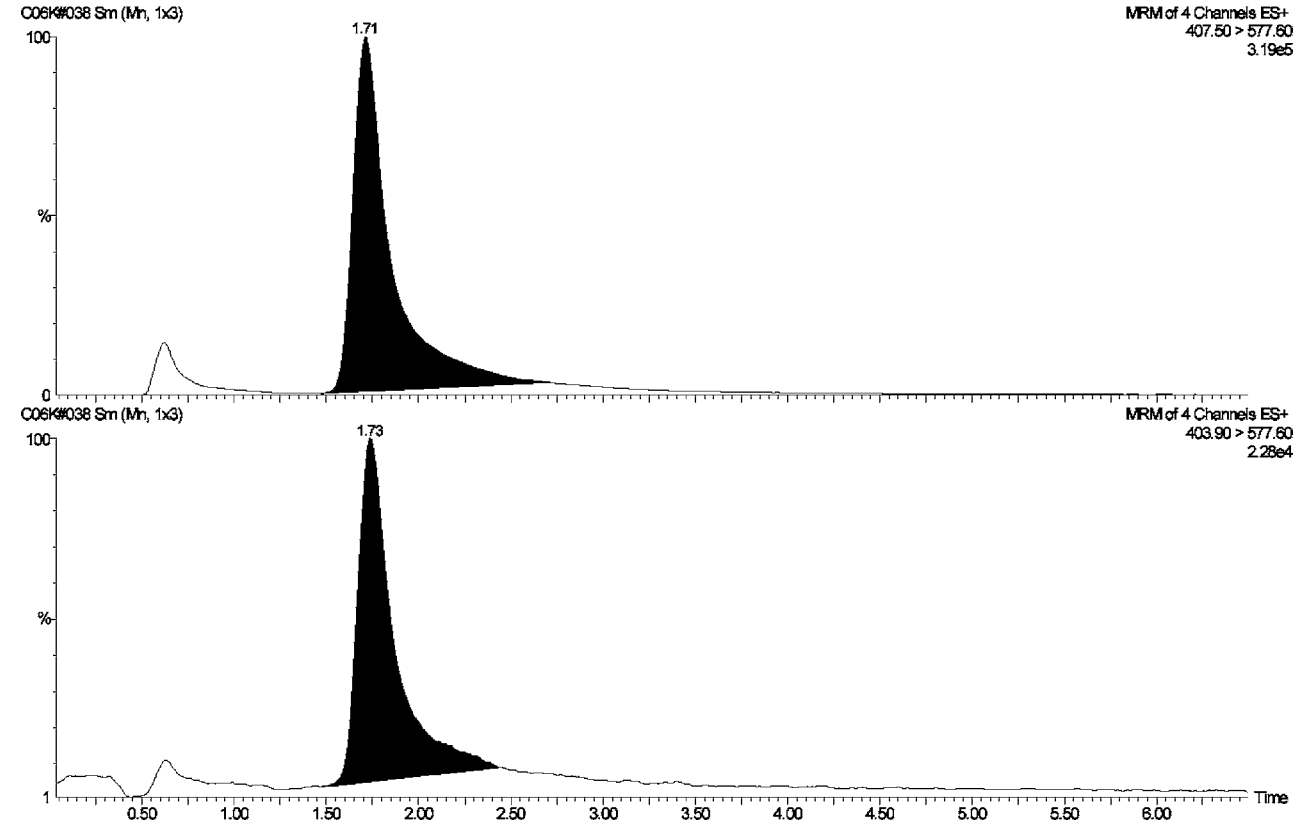


Figure 6. A representative chromatogram of the 2 ng/mL calibration standard used in the analysis of plasma containing the internal standard (top trace) and tulathromycin (bottom trace).

nondeuterated drug in the internal standard, and in this study, were extrapolated at about 0.7 ng/mL.

Plasma Quality Control Samples. The nominal concentrations of the QC samples were 450, 225, and 15 ng/mL. Four runs consisted of replicate ($n = 6$) analyses of each QC sample. Intra-assay accuracy varied over a 5.5% range (97.5−103%) and precision (RSD) from 0.6% to 2.9%. The mean inter-assay QC concentrations from all four runs were determined as 456, 224, and 15.4 ng/mL indicating accuracies ± RSD of 101.3 ± 1.6, 99.5 ± 2.4, and 102.5 ± 1.7%, respectively.

Replicate analysis of the plasma over-range sample gave a measured concentration 96.5% of nominal (2.1% RSD). Analy-

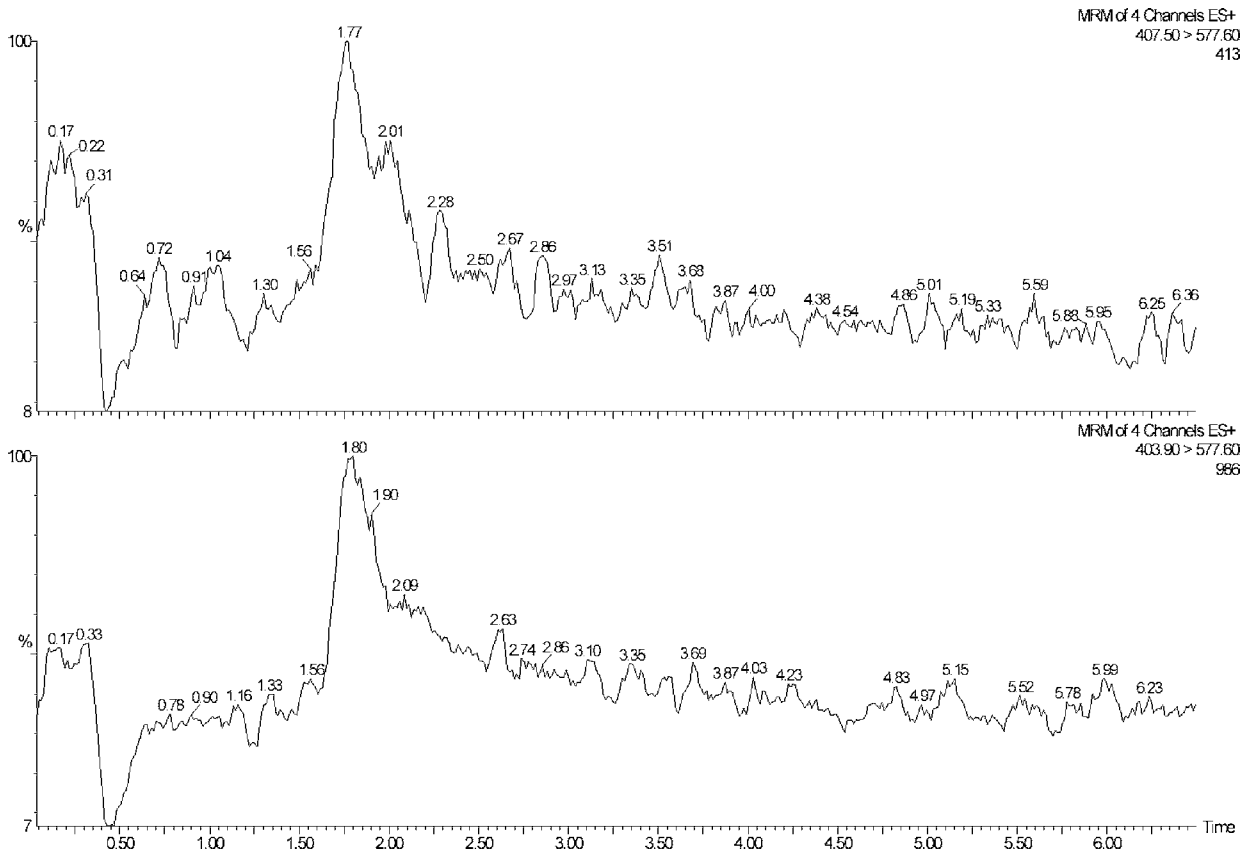


Figure 7. A representative chromatogram of a blank plasma extract (internal standard, top trace and tulathromycin, bottom trace).

sis, therefore, with up to a 5× sample dilution is appropriate for samples initially found to contain more than 500 ng/mL tulathromycin.

High (450 ng/mL) and mid (225 ng/mL) QC samples were stored at ambient temperature for 168 h testing analyte stability. The measured concentrations were 91.7 and 87.9%, respectively, of nominal. Both results were within the assay guidelines.

A series of four freeze−thaw cycles were also conducted with the high and mid QC samples. Accuracy in both cases was 94%.

Processed extracts from QC samples were stored in autoinjector vials at ambient temperature for 95.5 and 143.5 h before analysis with freshly prepared calibration curve samples. Samples stored for 95.5 h produced results within the assay QC guideline (accuracies of 108, 104, and 105% for high, mid, and low QC samples, respectively). By 143.5 h, however, the mid QC sample at 80.5% was below the accuracy acceptability range of ±15%.

Peak area ratios from analytes in QC samples were not the same as their corresponding response recovery samples. Mean ratios for the high (450 ng/mL), mid (225 ng/mL), and low (15 ng/mL) QC samples were 13.49, 6.518, and 0.4456, respectively (RSDs 0.8, 3, and 2%, respectively). Those for the response recovery samples were 9.725, 4.741, and 0.3330 (RSDs 1, 0.9, and 7%, respectively). The relative response (response recovery sample ratio mean/QC sample ratio mean) of the internal standard in the high, mid, and low QC samples was therefore 72.1, 72.7, and 74.7%, respectively.

Recoveries in QC samples based on peak areas alone were 67% for tulathromycin and 49% for heptadeutero-tulathromycin. Variability (RSD, $n = 18$) in the peak areas from heptadeutero-tulathromycin was 17% in the response recovery samples and 28% in the QC samples. Tulathromycin peak area variability ($n = 6$) in the high, mid, and low response recovery samples was 15, 4, and 12%, respectively, and 10, 36, and 29% from the corresponding QC samples. Because of the variability in peak areas, determination of absolute recovery is, at best, approximate, and no absolute recovery guidelines were set for this study.

Plasma Samples from Cattle and Swine. Samples from cattle and swine (480) were analyzed in twelve batches. Assay performance reflected the validation exercises. The inter-assay accuracy of the calibration samples remained in the range of 98.9−101.8% with RSDs no greater than 3.9%. All QC samples gave results well within our guidelines. Maximum concentrations in cattle and swine plasma were 1740 and 2440 ng/mL, respectively. Minimum observed concentrations were 14.7 and 2.92 ng/mL in cattle and swine, respectively. Cattle ($n = 16$) and swine ($n = 19$) plasma samples gave initial results above the dynamic range of the assay. These samples were re-assayed after dilution. The summary results are shown in **Figures 8** and **9** for cattle and swine, respectively.

Lung Homogenate Validation Summary, Calibration Samples. Three runs were carried out with lung. Correlation coefficients were similar for each run (0.99987, 0.99987, and 0.99929). Inter-assay precision (%RSD) of the calibration sample extracts was 2.3% or less for extracts containing 10−1000 ng/mL tulathromycin and 5.3% for the 4 ng/mL extract. The calibration sample accuracy range was 98.8−101.4%. Tulathromycin concentration in the reconstituted extracts was the same as that for plasma (dynamic range of 0.1−25 ng on column). Limits of quantitation were demonstrated with lung homogenate extract fortified with tulathromycin at 1000 and 4 ng/mL. Results (replicates of 6) from these samples resulted in accuracy ± precisions (RSDs) of 100.4 ± 0.7% and 110 ± 7%, respectively.

Lung Homogenate Quality Control Samples. QC samples at 800, 400, and 8 ng/mL tulathromycin (representative of lung residues of 20 000, 10 000, and 200 ng/g, respectively) produced

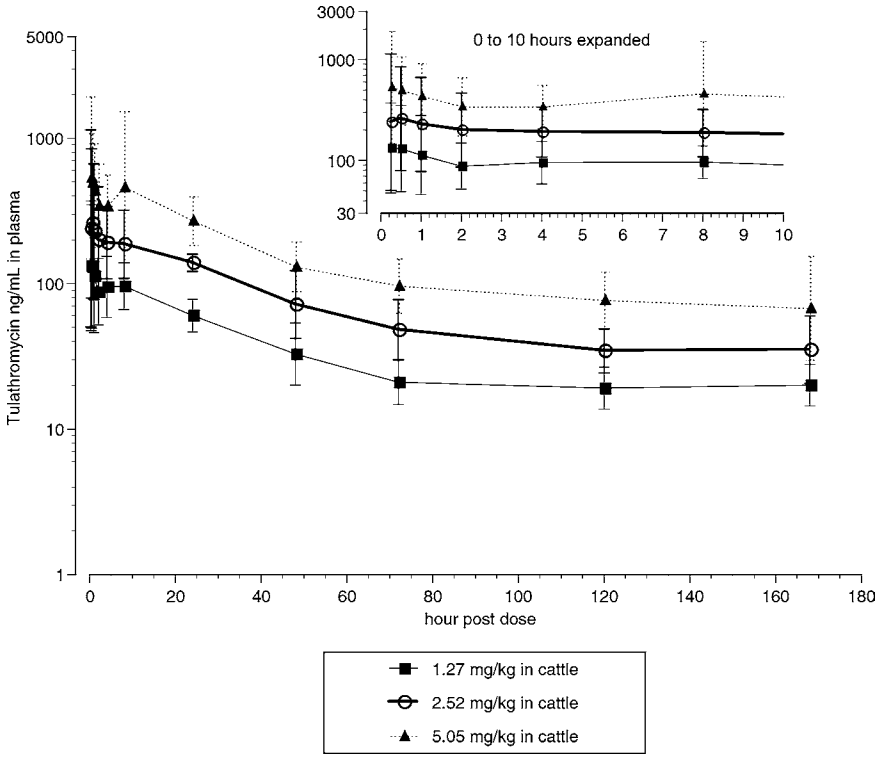


Figure 8. Plot of composite drug concentrations in plasma from cattle dosed with tulathromycin.

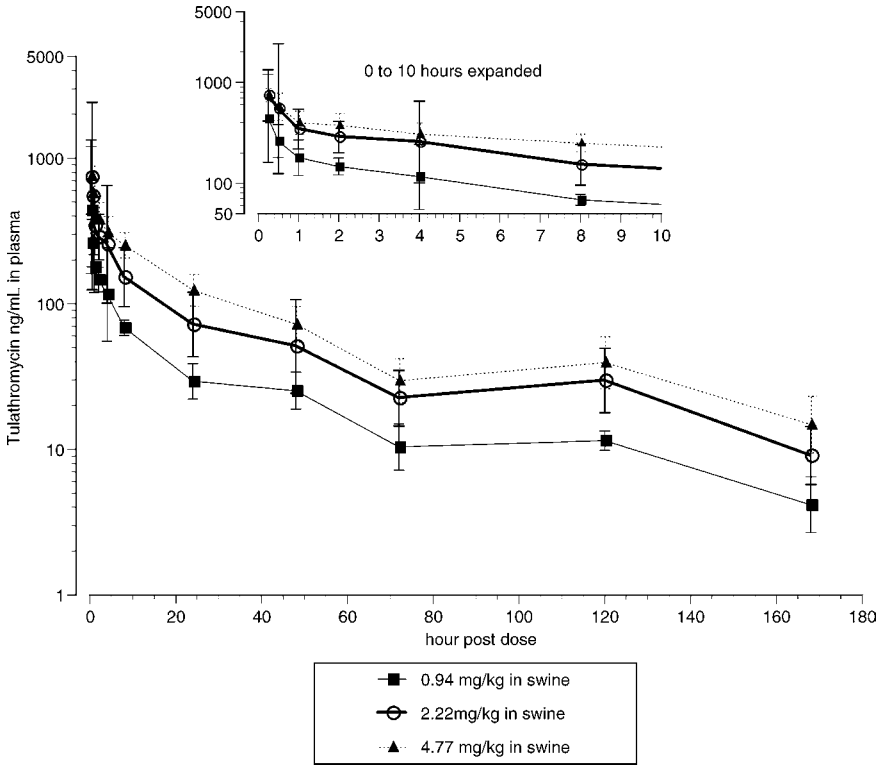


Figure 9. Plot of composite drug concentrations in plasma from swine dosed with tulathromycin.

accuracy ± precisions (RSDs) of 101± 2%, 106.8 ± 1.9%, and 107 ± 3%, respectively (n = 18 at each level).

Analyte stability in lung homogenate was tested directly with tulathromycin-incurred samples, thereby avoiding any bias that may be introduced by fortification of control tissue samples. Immediate analysis of the composite established the initial concentration as 3700 ng/g. Portions were kept at room temperature for 24 and 72 h. After 24 h the concentration was 3600 ± 400 ng/g (mean ± SD, n = 6) whereas after 72 h, the concentration dropped to 3000 ± 500 ng/g, 81% of the original. Freeze−thaw cycling with the incurred composite sample showed little change after five cycles.

After 48 h at room-temperature, extracts from QC samples appeared stable with sample accuracies from 101.6 to 109.2%

As with plasma, peak area ratios in the QC samples did not correspond to those in the response recovery samples. Recovery of the internal standard relative to tulathromycin was 83%. Unlike plasma, the variability in the peak areas was small with

Table 3. The Concentration of Tulathromycin Found in Bovine and Porcine Lung Homogenate after 7 Days

cattle no.	treatment	dose (mg/kg)	tulathromycin (ng/g)
699	NTX		0.00
715	NTX		0.00
708	T1	1.24	1210
714	T1	1.29	1550
716	T1	1.24	1730
718	T1	1.27	1230
722	T1	1.30	1690
730	T1	1.25	1650
700	T2	2.52	2310
702	T2	2.54	3170
712	T2	2.49	2880
719	T2	2.53	2750
726	T2	2.52	2910
731	T2	2.50	2510
703	T3	5.07	4520
721	T3	5.04	6490
724	T3	5.01	5050
725	T3	5.05	6090
728	T3	5.05	4250
729	T3	5.07	4890
swine no.			
775	NTX		0.00
783	NTX		0.00
761	T1	0.80	414
770	T1	1.01	627
772	T1	0.98	713
774	T1	0.99	540
777	T1	0.94	720
784	T1	0.94	600
762	T2	2.27	925
768	T2	2.18	1200
773	T2	2.26	1310
776	T2	2.21	1240
782	T2	2.24	1320
788	T2	2.16	1010
764	T3	4.82	2100
765	T3	4.75	1870
771	T3	4.72	1470
780	T3	4.78	2100
781	T3	4.84	2250
785	T3	4.73	1830

RSDs < 5% from the response recovery samples and < 10% for the extracted samples. Peak area recoveries in the lung homogenate QC samples were 54% for heptadeutero-tulathromycin (~15% higher than for plasma). Recoveries of tulathromycin were 66 and 67% for the high and mid QC samples, respectively, and 61% in the low QC sample.

Lung Homogenate Samples from Cattle and Swine. Cattle and swine samples were analyzed in separate batches of 20 samples. Excellent sample homogeneity was observed in all samples confirming the adequacy of the sample collecting and blending procedure. RSD's of <15% were observed in the triplicate analysis of each sample, and no sample required repeat processing and analysis. The results are shown in **Table 3**.

General Analytical Conclusions. The assay provides simple sample prep procedures with relatively short HPLC run times enabling high sample capacity and high sample throughput. Selectivity is defined by the LC/MS/MS detection of the unique transition of *m*/*z* 404−578 (doubly charged precursor ion to a singly charged product ion with a higher mass). Analysis of actual study samples shows the method is well suited for tulathromycin drug disposition studies in livestock.

There are apparent differences in the properties of tulathromycin and its heptadeuterated isomer. These differences appear in response recovery tests and analyte stability tests. Drug and internal standard were expected to behave near identically in the assay procedures. However, comparison of peak area ratios of QC sample extracts with response recovery samples indicated significant differences as reported in the validation results for plasma and lung. The lowered response of the d_7 isomer, relative to tulathromycin, in the QC samples can be related to extractability as well as to ionization suppression or enhancement effects. We propose that elution of the basic drug by NH_4OH from the weak cation exchange SPE may be influenced by a secondary isotope effect on the basicity of the heptadeuterated propylamino group. Suppressed ionization in the extracted samples may also contribute but is less likely because all the samples contained similar matrix elements and were subject to identical chromatographic environments. Analyte ionization suppression and/or enhancement by matrix elements and chromatographic conditions are well-known (*15*−*17*), and the procedures here are consistent with practices equalizing these effects in all samples. A rigorous experimental investigation, however, is beyond the scope of the data and intent of this study.

Stability testing also indicated a difference in behavior between the drug and its deuterated isomer. Drug to internal standard peak area ratios should compensate for losses due to degradation of analyte as long as the same process acts equivalently on the internal standard. Decreasing tulathromycin concentrations during stability tests imply its degradation to a greater extent than its heptadeutero isomer. Hydrolytic degradation at neutral and acidic pH is primarily through acetal cleavage at the C-1″ position (modified cladinose acetal) and is influenced by the propylamino group (*18*). If a similar mechanism predominates here, then the observed differences in stability could also implicate a secondary deuterium isotope effect on the positively charged nitrogen.

Pharmacokinetics. The actual doses delivered were calculated based on the data recorded from each syringe measurement (**Table 3**, average doses in **Table 4**). Pharmacokinetic parameters obtained over 7 days are summarized in **Table 4** for cattle and swine. Apparent elimination half-lives from plasma are 54 h in swine and 110 h in cattle (~2.3 and 4.6 days, respectively).

Table 4. Mean ± SD Pharmacokinetic Variables Obtained from Cattle and Swine

treatment group	dose (mg/kg)	$AUC_{(0-168}$ h) (ng · h/mL)	T_{max}[a] (h)	C_{max}[a] (ng/mL)	$t_{1/2}$[b] (h)	lung conc[a] (ng/g)	lung conc /C_{last}
cattle T1	1.27 ± 0.03	5800 ± 700	0.35 ± 0.14	150 ± 90	110 ± 50	1500 ± 200	80 ± 20
cattle T2	2.517 ± 0.019	12 000 ± 2000	0.71 ± 3	300 ± 400	110 ± 40	2700 ± 300	80 ± 30
cattle T3	5.05 ± 0.02	27 000 ± 6000	1.7 ± 9	700 ± 600	100 ± 25	5200 ± 900	80 ± 40
swine T1	0.94 ± 0.07	3900 ± 300	0.29 ± 0.1	500 ± 400	54 ± 12	590 ± 110	140 ± 20
swine T2	2.22 ± 0.04	9000 ± 1600	0.33 ± 0.13	1100 ± 700	57 ± 14	1160 ± 160	130 ± 40
swine T3	4.77 ± 0.05	12 600 ± 1300	0.25 ± 0	760 ± 70	59 ± 9	1900 ± 300	130 ± 20

[a] Calculated as geometric means. [b] Calculated as the harmonic mean.

Study duration was therefore less than 5× the estimated half-lives, which, in practice (*19*), suggests terminal elimination may not have been reached. Calculated $AUC_{0-\infty}$ values were on the average 45 and 13% greater than AUC_{0-168} for cattle and swine, respectively, also indicative of insufficient data in the terminal elimination phase. Therefore, $AUC_{0-\infty}$, a primary parameter of systemic exposure, could not be estimated with confidence and is not reported here. As a result of this preliminary investigation, definitive studies were planned and executed (*20*, *21*) where study duration was increased, resulting in data adequately representative of the terminal elimination phases in plasma as well as lung.

Dose Response of Tulathromycin. In this study, we were able to look at the dose response of cattle and swine to tulathromycin AUC_{0-168} and lung residues after 168 h. In both cattle and swine, AUC_{0-168} increased with increasing dose (**Table 4**). Mean cattle plasma AUC_{0-168} values (**Table 4**) for the T1, T2, and T3 groups increased in the relative proportion of 0.48, 1.0, and 2.3, while swine plasma AUC_{0-168} values increased in the relative proportion of 0.43, 1.0, and 1.4. Lack of proportionality in swine from the T3 group is shown by only a 1.4-fold increase in plasma AUC after a 2.15-fold increase in dose. The differences in the AUCs are significant for each treatment group (cattle $P \leq 0.0028$, swine $P \leq 0.0006$). Variability in plasma C_{max} was high, and C_{max} was not used as a marker of proportionality.

For both cattle and swine (**Table 4**), tulathromycin residues in the lung increased with dose. The calculated geometric means for cattle lung for T1, T2, and T3 increased in proportions of 0.56−1.0−1.93 (the dose proportion was 0.505−1.0−2.01). Geometric means in swine lung increased in proportions of 0.51−1.0−1.64 (the dose proportion was 0.42−1.0−2.15). The differences in drug residues in the lung were significant (cattle $p \leq 0.0022$, swine $p \leq 0.0004$) for each treatment group.

After 7 days, the concentration of tulathromycin in lung homogenate was about 80-fold greater than plasma for cattle and at least 130-fold greater for swine. High distribution into respiratory tissue was expected for this class of drug (*22*−*24*), and the results suggest this to be the case for tulathromycin. The variables that most adequately reflect dose proportionality are plasma AUC and drug-lung residues 7 days post dose. The variable expected to most adequately reflect the efficacy of the drug is lung AUC, which was not incorporated into the study design of this preliminary investigation. However, subsequent studies (*20*, *21*), whose design was refined using the data from this study, address the pharmacodynamic relationships between lung AUC and the MIC of pertinent respiratory pathogens.

The overall absorption and distribution of the drug appear constant in the dose range studied because the T_{max}, apparent half-life, and the drug concentration ratio of lung to plasma 7 days post dose show little change for all three doses.

ACKNOWLEDGMENT

We wish to thank Tim Alberts, Mike Beasley, Bruce Cheesman, Brad Colglazier, Jack Drumb, Diane Gilbert, Brian Milner, Bill Russell, Renee Hassfurther, David O. Lewis, Sr., and Pat Taube at the Pfizer, Terre Haute, Indiana animal research facility for their contributions to the "in-life" portions of this study. We wish to thank Yingjie Deng, Brian Lewis, and Don Grey at BASi for their help in conducting the analyses of plasma and tissue samples.

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Received for review October 9, 2003. Revised manuscript received February 18, 2004. Accepted February 19, 2004.

JF0351624