Received: 28 November 2021 | Revised: 28 March 2022 | Accepted: 4 April 2022

DOI: 10.1002/dta.3270

SHORT COMMUNICATION

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A single LC-MS/MS validated method for tulathromycin quantification in plasma, seminal plasma, and urine to be applied in a pharmacokinetic study in bull

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Abstract

Tulathromycin is a macrolide antibiotic generally used for the treatment of respiratory diseases in cattle and swine. This work proposes an improvement of a previously published LC-MS/MS method for tulathromycin determination in pig serum, here validated in three different bull matrices: plasma, seminal plasma, and urine. The approach is based on a quick protein precipitation with acetonitrile, filtration, and sample dilution before injection, allowing to rapidly process large batches of samples. Analytes separation was obtained using a BEH C18 (50 × 2.1 mm, 1.7 μm) column, maintained at 40°C with a chromatographic run of 5 min. The method was fully validated over concentration ranges suitable for field levels of tulathromycin found in each matrix (0.01–1 μg/ml for plasma, 0.05–5 μg/ml for seminal plasma, and 0.1–10 μg/ml for urine), showing good linearity during each day of testing (R^2 always >0.99). Accuracy and precision were within ±15% at all QC concentrations in all the three matrices. Furthermore, the use of tulathromycine-d7 as internal standard mitigated the potential impacts of matrix effect. The validated technique was successfully applied to samples collected during a pharmacokinetic study in bulls, allowing to monitor tulathromycin concentrations over time in the three matrices. To our knowledge, this is the first validated approach for LC-MS/MS quantification of tulathromycin in seminal plasma and urine.

KEYWORDS
LC-MS, plasma, seminal plasma, tulathromycin, urine

1 | INTRODUCTION

Tulathromycin is a semi-synthetic macrolide antibiotic approved in the European Union and the United States for the treatment of respiratory disease in bovine (BRD, caused by *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*) and swine (SRD, caused by *Actinobacillus pleuropneumoniae*, *P. multocida*, *Mycoplasma hyopneumoniae*, *Haemophilus parasuis*, and *Bordetella bronchiseptica*.[1,2] Tulathromycin (MW: 806.1) is an effective protein synthesis inhibitor that targets the 50s ribosomal subunit of bacteria and stimulate the dissociation of peptidyl-tRNA from the ribosome during the translocation process. This inhibition prevents the bacteria from being able to make

vital proteins and stops them growing and multiplying.[1,2] Following administration at the recommended dosage (2.5 mg/kg b.w., single subcutaneous injection in cattle, and intramuscular in pigs), the drug shows a rapid absorption, a high bioavailability, significant tissue distribution, and a long plasma elimination half-life.[2–4]

Over the past years, several liquid chromatography mass spectrometry methods (LC-MS/MS) have been developed to determine tulathromycin in different biological matrices, including plasma,[3,5–10] serum,[11,12] synovial fluid,[13] milk,[14] and other tissues.[15–18] However, to date, no LC-MS/MS approaches have been validated in seminal plasma and urine. This could be useful to obtain information on its concentrations when used to treat genital tract infections, allowing to develop an appropriate and effective antibiotic therapy in animals. This work proposes the validation of a single LC-MS/MS approach, previously developed in pig serum,[12] for tulathromycin quantification in bull plasma, seminal plasma, and urine, to be applied in the context of a pharmacokinetic study in bulls.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Acetonitrile and formic acid were obtained from Merck Company (Darmstadt, Germany). Ultra-pure water was produced in-house (Millipore, Milano, Italy). Analytical standards of tulathromycin (molecular structure is shown in Figure 1) and tulathromycin-d7 were purchased from Toronto Research Chemicals (North York, ON, Canada). Stock solutions (500 μg/ml) of both compounds were prepared by dissolving 5 mg of pure powder in 10 ml of acetonitrile and stored at −20°C in the dark.

2.2 | Sample preparation

Plasma, seminal plasma, and urine, previously thawed at room temperature (22°C), were extracted with the technique described by Zhou et al.,[12] with slight modifications.

Briefly, 180 μl of acetonitrile and 20 μl of internal standard tulathromycin-d7 (1 μg/ml in acetonitrile) were added to 200 μl of sample. The tube was then agitated on vortex mixer for 30 s, centrifuged at 21,000×*g* for 10 min at 4°C, and the supernatant was filtered through a 0.22 μm nylon syringe filter. A 100 μl aliquot of the purified sample was diluted into a LC vial with an equal amount of 0.1% formic acid aqueous solution, and 10 μl was finally injected.

2.3 | Instrumental conditions

The apparatus consisted of a Waters Acquity UHPLC binary pump (Waters, Milford, MA, USA) and thermostated autosampler, held at

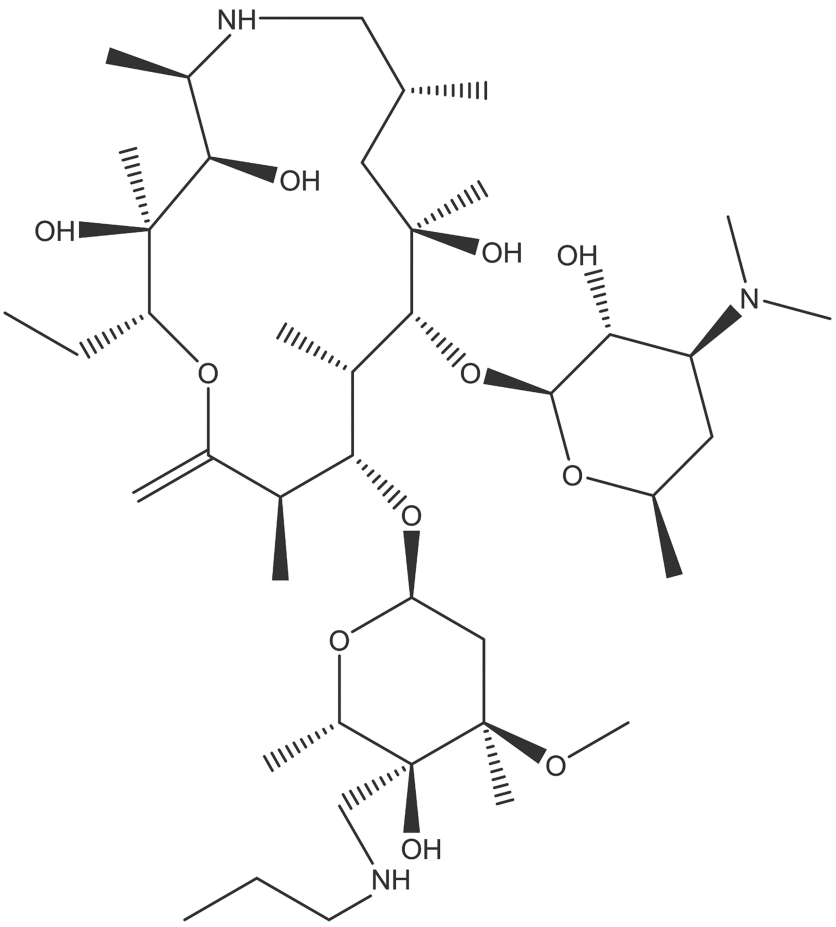

FIGURE 1 Molecular structure of tulathromycin

20°C. Chromatographic separation was obtained with a Waters Acquity BEH C18 (50 × 2.1 mm, 1.7 μm) column (Waters, Milford, MA, USA), maintained at 40°C to lower system backpressure. The mobile phase was a mixture of 0.1% formic acid in water (A) and acetonitrile (B), switching from 90:10 to 50:50 and back to 90:10 (V_A:V_B) over a 5 min run at 0.3 ml/min.

The LC was coupled to a Waters Quattro Premier XE triple quadrupole mass spectrometer (Waters, Milford, MA, USA), equipped with an electrospray ionization source (ESI), with capillary voltage set at +3.0 kV, source temperature at 120°C, and desolvation temperature at 400°C. Desolvation and cone gas flow were 600 and 100 L/h, respectively, and argon was used as collision gas. The instrument operated in MRM mode, monitoring the following transitions obtained from doubly charged precursor ions: 403.7 > 576.9 *m/z* (cone voltage 25 V; collision energy 16 eV) and 403.7 > 229.9 *m/z* (25 V; 15 eV) for tulathromycin quantification and confirmation, respectively; 407.3 > 236.9 *m/z* (25 V; 16 eV) for tulathromycin-d7. Data acquisition and processing were carried out with MassLynx 4.1 software (Waters, Milford, MA, USA).

2.4 | Method validation

After defining the retention time of tulathromycin and tulathromycin-d7 through the injection of pure standards, the selectivity of the method was assessed analyzing ten blank samples of each matrix collected from 10 different bulls, to verify the absence of chromatographic signals within the same time window.

During each day of validation, 7-point (plus a blank) matrix-matched calibration curves were freshly prepared at suitable concentration ranges (0.01–1 μg/ml for plasma, 0.05–5 μg/ml for seminal plasma, and 0.1–10 μg/ml for urine) spiking 200 μl aliquots of each matrix with 20 μl of corresponding tulathromycin working solutions and adding 20 μl of tulathromycin-d7. In parallel, quality control samples (QCs) were prepared in triplicates at three different levels, chosen accordingly to each matrix concentration range: 0.02, 0.1, and 0.5 μg/ml for plasma; 0.1, 0.5, and 2.0 μg/ml for seminal plasma; and 0.2, 1.0, and 5.0 μg/ml for urine.

Peak area ratios between tulathromycin and the internal standard were plotted against their concentration, and a linear least square regression model was applied. All the calibration standards had to be within ±15% of the nominal value, and the resulting coefficient of determination (r^2) was considered acceptable if ≥0.99.

Although the focus of our experiment was to validate the method over concentrations ranges suitable for the analysis of samples collected during the pharmacokinetic study, we also assessed the potential performances of the method at lower concentrations. For each matrix, pooled sample replicates spiked at levels below the lowest calibrator were analyzed to verify if they could produce a chromatographic response with a signal-to-noise (S/N) ratio of ≥10 and a coefficient of variation (CV%) of <15%.

Accuracy, expressed as relative difference between measured value and expected concentration, was evaluated at each QC concentration and was considered acceptable if within ±15% of the nominal value. Precision, defined as the coefficient of variation (CV%) among repeated individual measures, had to be <15% for each QC level.

Potential matrix effect was verified by the post-column infusion technique[19]: During the injection of a blank sample of each matrix in the LC-MS/MS system, standard solution first of tulathromycin and then of tulathromycine-d7 were directly and continuously infused in the MS interface, to evaluate the stability of the produced signals.

2.5 | Application to a pharmacokinetic study

The trial was approved by the Committee for Animal Welfare of the University of Bologna Prot. No. 0005783. Before the beginning of the study, drug free matrices were collected from different healthy bulls and made available for the analytical laboratory for method development. The validated method was used to determine the pharmacokinetic profile of tulathromycin in bull plasma, seminal plasma, and urine. Four adult healthy bulls received a single subcutaneous dose (2.5 mg/kg of body weight; day 0 time 0) of tulathromycin (Draxxin, Zoetis Italy, Rome). Two of the bulls received the dose at the base of the left ear and two in the middle of the left side of the neck. Samples of blood (in lithium heparin), semen, and urine were collected at 0, 12, 24, 48, 72, 96, 144, 192, and 240 h after tulathromycin administration.[20] All the samples were immediately refrigerated, centrifuged for 30 min at 600×*g*, and stored at −80°C until analysis in LC-MS/MS. During the analysis of each batch of the collected samples, a calibration curve and quality control samples were freshly prepared for each matrix as performed for validation, to confirm linearity, accuracy, and precision of the method. Drug-free samples were also injected before and after each series to confirm the absence of carry-over.

3 | RESULTS AND DISCUSSION

3.1 | Method validation

The present method for tulathromycin determination in three bull matrices was validated in accordance with in house criteria based in part on the current European Medicines Agency (EMA) guidelines on bioanalytical method validation.[21]

The injection of pure standards of tulathromycin and tulathromycin-d7 allowed to define their retention time which was 1.23 min for both compounds. Singly charged and doubly charged precursor ions of target analytes can be both produced in ESI-MS. During method development, we observed stronger relative abundance for the doubly protonated ions and therefore decided to monitor the 403.7 > 576.9 *m/z* (quantification) and 403.7 > 229.9 *m/z* (confirmation) transitions for tulathromycin, and 407.3 > 236.9 *m/z* for tulathromycin-d7. For each matrix, the analysis of 10 blank samples did not show chromatographic interferences at the retention time

of the monitored transitions proving the good selectivity of the method, as shown in Figure 2.

The coefficient of determination (r^2) always ≥0.99 and calibration standards always within ±15% of the nominal value during each day of validation proved the linearity of the method in the three matrices. Moreover, the analytical response of all reinjected samples was within ±15% that of the first injection, indicating the stability of the analyte for at least 16–24 h at autosampler conditions. Accuracy and precision, intended as measured-expected concentration relative difference and coefficient of variation (CV%), respectively, were always within ±15% at all QC concentrations in all the three matrices both in intra-day and inter-day analysis (data are reported in Table 1). Below the calibration range, the lowest tested concentration producing a quantifiable signal was 0.01 μg/ml for plasma and 0.02 μg/ml for seminal plasma and urine.

During the post-column infusion test, no ionization suppression or enhancement was observed in the monitored transitions around the retention time of target analytes, demonstrating the absence of

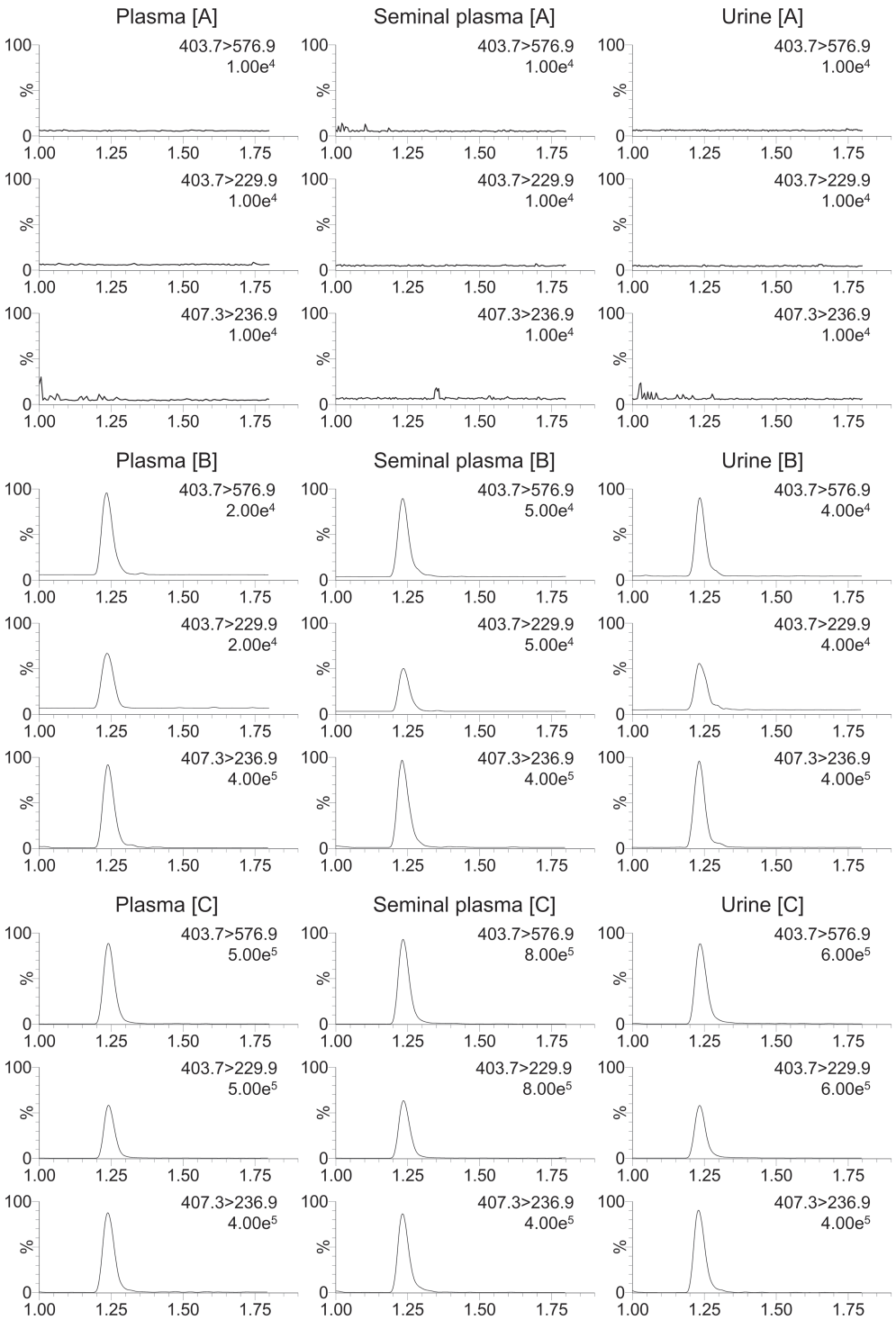

FIGURE 2 Chromatograms of the 403.7 > 576.9 *m/z* and 403.7 > 229.9 *m/z* transitions monitored for tulathromycin and the 407.3 > 236.9 *m/z* transition monitored for tulathromycin-d7, obtained for each matrix after injection of a blank sample [A], a sample at the lowest concentration tested (0.01 μg/ml for plasma and 0.02 μg/ml for seminal plasma and urine) [B] and a sample collected during the pharmacokinetic study [C]. Measured concentrations in [C] were 0.25 μg/ml in the plasma sample, 0.47 μg/ml in the seminal plasma sample, and 0.37 μg/ml in the urine sample

TABLE 1 Intra- and inter-day accuracy and precision data obtained for tulathromycin in bull plasma, seminal plasma, and urine at three different QC concentrations in triplicates during three separated days of validation

	Plasma		Seminal plasma		Urine	
	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
	QCL (0.02 μg/ml)		QCL (0.1 μg/ml)		QCL (0.2 μg/ml)	
Day 1 ($n = 3$)	−3.3	3.0	−6.0	4.9	2.8	3.6
Day 2 ($n = 3$)	5.0	4.8	9.5	5.3	−2.5	9.8
Day 3 ($n = 3$)	−4.8	5.9	4.2	7.0	−3.0	5.4
Inter-day ($n = 9$)	−1.1	6.2	2.6	8.3	−0.9	6.5
	QCM (0.1 μg/ml)		QCM (0.5 μg/ml)		QCM (1.0 μg/ml)	
Day 1 ($n = 3$)	−2.0	2.3	−0.3	6.7	3.2	2.3
Day 2 ($n = 3$)	3.0	2.1	−3.5	1.9	−2.1	6.3
Day 3 ($n = 3$)	−0.3	2.9	4.7	3.9	−2.7	6.2
Inter-day ($n = 9$)	0.2	3.1	0.3	5.4	−0.6	5.3
	QCH (0.5 μg/ml)		QCH (2.0 μg/ml)		QCH (5.0 μg/ml)	
Day 1 ($n = 3$)	1.6	1.0	3.4	2.6	1.9	4.8
Day 2 ($n = 3$)	−2.5	1.4	−6.7	5.8	0.4	0.6
Day 3 ($n = 3$)	0.6	2.1	−0.5	2.1	0.9	4.4
Inter-day ($n = 9$)	−0.1	2.3	−1.3	5.5	1.0	3.3

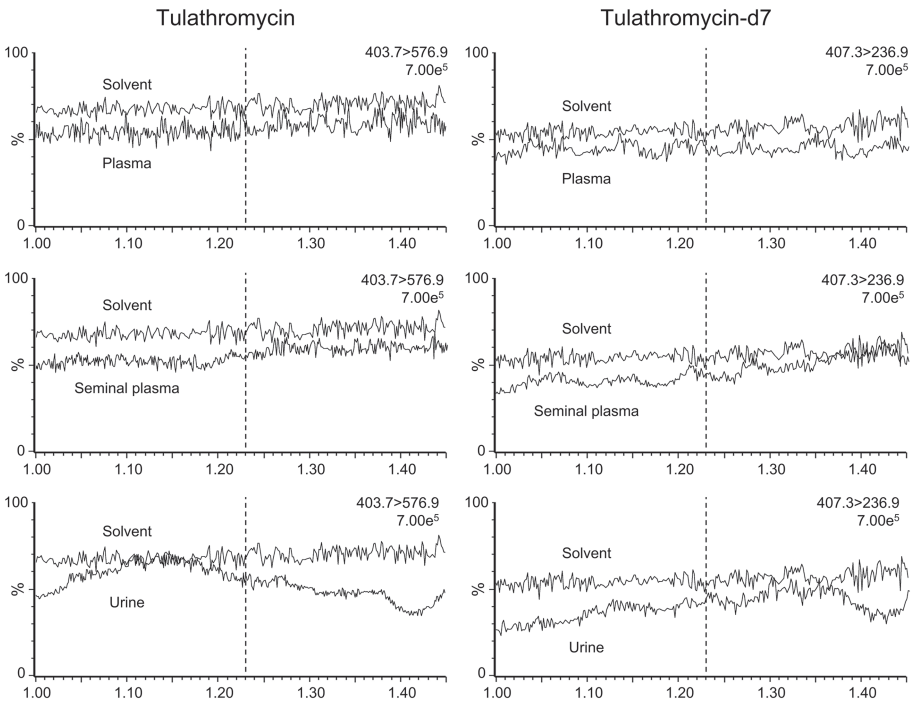

FIGURE 3 Evaluation of the matrix effect by acquisition of the signal generated by the simultaneous post-column infusion of tulathromycin or tulathromycin-d7 standard solution and injection of a blank sample of plasma, seminal plasma and urine, in comparison with the baseline response obtained injecting solvent. Dashed line indicates the expected retention time for target analytes

matrix effect (the acquisition for the quantification transition of tulathromycin and tulathromycin-d7 in each matrix is shown in Figure 3). Moreover, the use of tulathromycin-d7 instead of other structural analogs as internal standard was more effective in reducing potential matrix-related bias for the quantification of tulathromycin. No carry-over was observed injecting blank samples following the highest point of each calibration curve.

3.2 | Application to pharmacokinetic study

The method was successfully applied to plasma, seminal plasma, and urine samples collected during a pharmacokinetics study of tulathromycin in healthy bulls, proving that the validated range of concentrations was suitable for the detected levels of the drug. The robustness of the approach even during long analytical sessions was

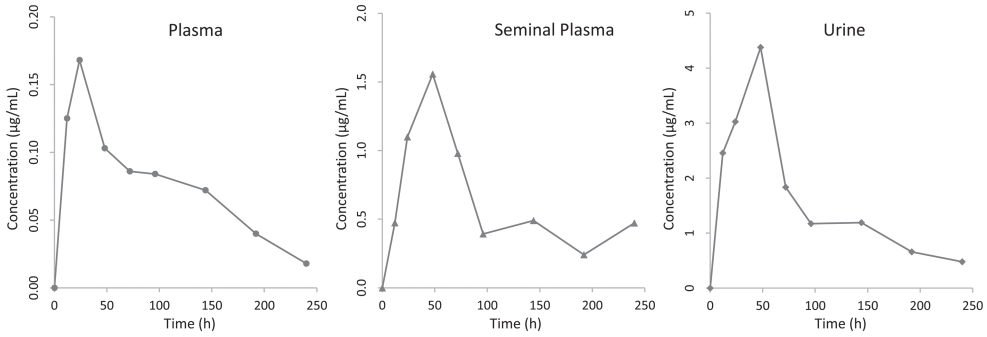

FIGURE 4 Concentration-time profile of tulathromycin in bull plasma, seminal plasma, and urine after subcutaneous administration at 2.5 mg/kg in one subject enrolled in the pharmacokinetic study

confirmed by drug-free, calibrators and quality control samples injected in between sample batches. Figure 4 shows sample concentration versus time curves for the three matrices in one of the bulls enrolled in the study. Data obtained from the pharmacokinetic study are available elsewhere.[20]

4 | CONCLUSIONS

The current study describes a single LC–MS/MS validated method for the quantification of tulathromycin in bull plasma, seminal plasma, and urine. The proposed approach provides a quick and easy sample preparation followed by a short analytical run time. Moreover, deuterated tulathromycin was first used as internal standard in the present work, contributing to a more reliable quantification of tulathromycin. This method was successfully used for the analysis of samples of three matrices collected during an experiment aimed at studying the pharmacokinetics of tulathromycin in bulls.

ACKNOWLEDGEMENTS

Open access funding enabled and organized by Projekt DEAL. Open access funding enabled and organized by Projekt DEAL.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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How to cite this article: Barbarossa A, Bardhi A, Gazzotti T, Mari G, Pagliuca G. A single LC-MS/MS validated method for tulathromycin quantification in plasma, seminal plasma, and urine to be applied in a pharmacokinetic study in bull. *Drug Test Anal*. 2022;14(8):1525-1531. doi:10.1002/dta.3270