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Quantitative determination of the macrolide antibiotic tulathromycin in plasma and broncho-alveolar cells of foals using tandem mass spectrometry

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Abstract

The long-acting antibiotic tulathromycin is on the marked for treatment of pulmonary infection of cattle, swine and horses. To measure disposition and distribution of tulathromycin in foals, a high throughput method was developed for horse plasma (calibration range: 0.006–0.8 μg/mL) and broncho-alveolar cells (calibration range: 0.1–4.0 μg/10^9 cells) using tandem mass spectrometry. Tulathromycin was extracted from plasma and broncho-alveolar fluid using cation exchange cartridges with acetonitrile/ammonia (95:5, v/v). The chromatography was performed isocratically with a mobile phase consisting of 5 mM ammonium formiate buffer/acetonitrile (30:70, v/v). The mass spectrometer operated in selected ion mode with atmospheric pressure chemical ionization to monitor the respective MH^+ ions *m/z* 577.3 for tulathromycin and *m/z* 679.3 for the internal standard roxithromycin. All quality parameters fulfilled the international criteria for bioanalytical method validation and were successfully applied to the determination of tulathromycin in plasma of foals and broncho-alveolar cells. In foals, tulathromycin after intramuscular administration was rapidly absorbed, widely distributed and slowly eliminated. It cumulated manifold in broncho-alveolar cells.

Keywords: Tulathromycin; Drug assay; Pharmacokinetics; Broncho-alveolar cells; LC–MS/MS

1. Introduction

Tulathromycin (Fig. 1; $C_{41}H_{79}N_3O_{12}$; *M*: 806.1 g/mol), a novel long-acting macrolide antibiotic of the triamilide group has been launched on the marked for treatment of bacterial respiratory infections of cattle and swine in 2004 [1,2]. Recently, the drug was also successfully used in combination with rifampicin in treatment of abscessing pneumonia of fouls caused by *Rhodococcus equi* [3].

Tulathromycin after intramuscular administration is rapidly and nearly completely absorbed from the injection site to reach maximal serum concentrations within 1 h. It is widely distributed and accumulates in lung tissue. As shown by in vitro studies, tulathromycin cumulates also in leukocytes and macrophages to concentrations several times above the serum levels. Its elimination is extremely slow with half-lives of 4–6 days [1,4,5]. Metabolic elimination via *N*-oxidation and *N*-demethylation is only of minor significance.

Tulathromycin exists in aqueous solutions at equilibrium as a mixture of two isoforms; 90% in form of a 15-limb lactone ring (isomer A), 10% as a 13-limb lactone (isomer B) [4]. Because equilibrium in aqueous solutions is reached within 48 h, validation of quantitative assays is focused on isomer A [1,4,5]. So far, a LC–MS/MS for the determination of tulathromycin in plasma and lung homogenates after solid-phase extraction has been published [4]. In this paper, we present the validation of a tandem mass spectrometric assay for quantification of tulathromycin in horse plasma and broncho-alveolar cells based on the internal standard method without usage of radio labeled reference compounds.

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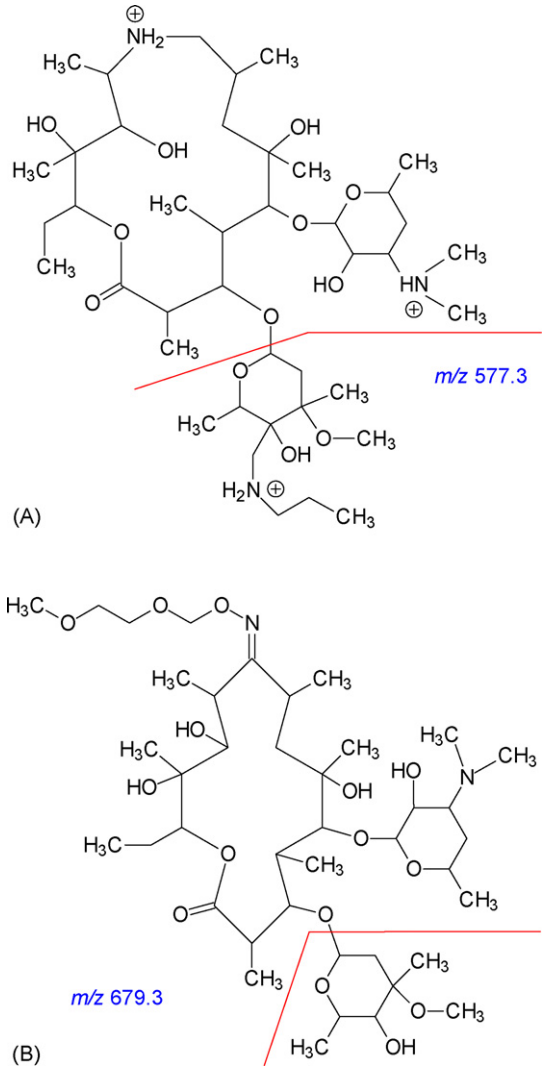

Fig. 1. Chemical structure of tulathromycin (A) and the internal standard roxithromycin (B) with the respective fragment ions.

2. Methods

2.1. Assay of tulathromycin in plasma and broncho-alveolar cells

2.1.1. Materials

Tulathromycin and roxithromycin were provided as white crystalline powders by Pfizer, (Karlsruhe/Main, Germany) and by Sigma–Aldrich (Steinheim, Germany), respectively. Ammonium formiate and formic acid were purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol (LC–MS Chromasolv®) were obtained from Riedel-de Haën (Seelze, Germany). Deionized water was prepared by the SG 2800 Reinstwassersystem RF 40 EZ (Hamburg-Barsbüttel, Germany).

2.1.2. Sample preparation

0.5 mL of the respective plasma sample (sample from the study, calibrator or quality control sample) was spiked with 0.5 mL de-ionized water (pH 6.0), 25 μL internal standard (5 μg/mL roxithromycin) and 50 μL saturated sodium carbonate solution. The analytical compounds were extracted from plasma using the Gilson Aspec XL system equipped with the 735 sampler software V 3.10 (Abimed, Langenfeld, Germany) and cation exchange cartridges Oasis MCX 1 cm^3 (Waters, Milford, USA). The cartridges were preconditioned in two steps with 1 mL acetonitrile and with 1 mL 5 mM potassium bishydrogen phosphate solution (pH 6.2) before loading with 0.5 mL of the sample. After washing with 1.0 mL potassium bishydrogen posphate solution and with 1.0 mL deionized water, elution was performed twice with 0.5 mL acetonitrile/ammonia (95:5, v/v). Finally, the cartridges were washed with 1.0 mL water (pH 6.0) and dried using pressured air. The eluates were evaporated to dryness under a gentle air stream (50 °C), redissolved in 100 μL mobile phase of which 20 μL were injected for tandem mass spectrometric analysis.

To 0.5 mL broncho-alveolar cell suspension (10^9 cells/mL), 0.05 mL saturated sodium carbonate solution, 0.025 mL internal standard solution (5 μg/mL roxithromycin) and 1.0 mL de-ionized water (pH 6.0) were added. Then, the sample was homogenized for 10 min using an ultrasonic homogenizer (Qalilab, Merck Eurolab, Bruchsal, Germany) and centrifuged at 10,000 × *g* for 10 min to separate 0.5 mL of the supernatant. To the residue, 0.5 mL acetonitrile was added, mixed for 1.0 min and centrifuged at 10,000 × *g* for 10 min to separate 0.75 mL supernatant. The supernatants were combined and subjected to solid phase extraction as described for plasma.

2.1.3. Chromatography and mass spectrometry

The liquid chromatography–tandem mass spectrometry (LC–MS/MS) system consisted of a binary pump series 1100 (Hewlett Packard, Waldbronn, Germany), an autosampler series 200 equipped with a cooling Peltier track which was set to 15 °C (Perkin-Elmer, Applied Biosystems, Darmstadt, Germany), the column thermostat L-5025 (Merck-Hitachi, Darmstadt, Germany) temporized at 30 °C and the PE Sciex API 2000 mass spectrometer with atmospheric pressure chemical ionization (APCI) interface (Heated Nebulizer, Applied Biosystems). Chromatography was performed without analytical column using formiate buffer (pH 3.0)/acetonitrile (30:70, v/v) for 2 min (flow rate 0.25 mL/min). The interface and mass spectrometer were protected against micro-particles using three 0.5 μm Frit Micro Filter (PEEK, Supelco, Bellefonte, USA).

The mass spectrometer was used in the multiple reaction monitoring (MRM) mode in the positive ion mode. The *m/z* transition was monitored for tulathromycin at 806.4–577.3 and for roxithromycin at 837.3–679.3. The optimized APCI and MS/MS parameters are given in Table 1.

The chromatograms were evaluated with the internal standard method using peak-area ratios for calculation. The device-specific software Analyst 1.2 (Applied Biosystems) and the program package Microsoft® Office Excel 2003 (Microsoft® Cooperation, Redmond, USA) and SPSS (Apache Software Foundation, Chicago, USA), were used for data acquisition and statistical evaluation.

Table 1
APCI and MS/MS parameters for determination of tulathromycin in plasma and bronchial cells

APCI parameters	MS/MS parameters
Nebulizer current: 2 μA	Declustering potential: 30 V
Nebulizer gas: 70 psi	Focusing potential: 290 V
Auxiliary gas: 35 psi	Entrance potential: 10 V
Temperature: 350 °C	Collision-activated dissociation gas: 3 psi
Curtain gas: 15 psi	Collision energy: 30 V

Nitrogen was used as nebulizer, auxiliary and curtain gas (1 psi = 6894.8 Pa).

2.1.4. Validation procedures

Validation of the quantitative assay was performed according to international recommendations [6–8]. Because isotope labeled tulathromycin was not available, we used roxithromycin as an internal standard. Roxithromycin is very similar in chemical structure and physicochemical properties to tulathromycin (Fig. 1).

Selectivity was verified by comparing chromatograms of five pooled blank plasma samples with five plasma samples which were spiked with 0.1 μg/mL tulathromycin and the internal standard roxithromycin. LC–MS/MS detection at *m/z* transition 806.4–577.3 and 837.3–679.3 was specific for tulathromycin and roxithromycin, respectively.

The quantitative assay for tulathromycin was validated between 0.006 and 0.8 μg/mL for plasma samples and 0.1 and 4.0 μg/mL for broncho-alveolar cell suspensions. One calibration set for validation consisted of seven calibrator samples, one double-blank sample without tulathromycin and internal standard, one blank sample spiked with the internal standard and three quality control samples with low, medium and high concentrations of tulathromycin.

Recovery of tulathromycin was assessed by comparing the peak area of the tulathromycin signal after extraction from plasma and broncho-alveolar fluid, respectively, with the peak area of tulathromycin which has been dissolved in acetonitrile. Six independently prepared samples sets with 0.16, 0.3 and 0.7 μg/mL tulathromycin in plasma and 0.2, 1.0 and 3.6 μg/mL tulathromycin in broncho-alveolar fluid were measured.

Stock solution and working solution stability of tulathromycin and the internal standard were verified by daily measurement of three aliquots of the stock and working solutions for 2 weeks before and during the validation procedure. The working solutions were prepared weekly and stored at room temperature; stock solutions with tulathromycin and the internal standard were stored in darkness at 4–6 °C.

Post-preparative stability in open sample vials was assessed in the autosampler at 15 °C by repeated tracing of the respective MS signals of freshly extracted plasma samples (0.016, 0.3 and 0.7 μg/mL, five times) and broncho-alveolar fluid (0.2, 1.0 and 3.6 μg/mL) in mobile phase over a period of 8 h.

S*hort-term stability* in serum and broncho-alveolar fluid was assessed by thawing three aliquots of each quality control samples (low, middle and height concentration) and keeping them at room temperature for 6 h. After that, the samples were worked off and measured as described above.

L*ong-term stability* in serum and broncho-alveolar fluid was assessed also with quality control samples of the respective concentrations by comparing the MS signals from samples after 1 day and 4 week of thawing in comparison with deeply frozen (−20 °C) samples.

2.1.5. Freeze–thaw stability was not evaluated

Accuracy and *precision* in plasma were assessed with five sets of calibrants containing 0.006, 0.02, 0.1, 0.2, 0.4, 0.6 and 0.8 μg/mL tulathromycin and five sets of quality control samples spiked with 0.016, 0.3 and 0.7 μg/mL tulathromycin. For broncho-alveolar fluid, the calibrants were set to 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 μg/mL tulathromycin and the quality controls to 0.2, 1.0 and 3.6 μg/mL.

All samples were prepared using pooled horse plasma and broncho-alveolar fluid, respectively, and stored at −20 °C until use on test days. Accuracy was assessed by comparison of the concentrations in quality control- and calibration samples with the respective nominal concentrations and expressed as relative error. Precision was evaluated by measuring of six separately prepared calibration- and quality control samples, respectively, and expressed as relative standard deviation of the mean concentrations.

To avoid matrix effects, all calibrator and quality control samples were prepared in pooled blank horse plasma and broncho-alveolar fluid, respectively. A formiate buffer containing mixture was used for elution and an internal standard for quantification.

2.2. Pharmacokinetics of tulathromycin in foals

Subjects: six healthy foals (three females, three males, age 50–70 days, body weight 120–140 kg) of German warm-blooded horses were included in the study after physical examination including sonography of the lung, routine clinical–chemical and hematological screenings. The foals were kept in barns at natural light rhythm and free access to standard pellets, hey, oats and tap water. They did not receive any other medication except for monthly de-worming. All clinical examinations were done in individual stables coved with straw. The study has been approved by the State Authority of Mecklenburg/Vorpommern.

Study design: a single dose of 2.5 mg/kg tulathromycin (Draxxin®, Pfizer, Karlsruhe, Germany) was injected into the semimembranosus or semitendinosus muscle (injection volume: 0.025 mL/kg). Blood samples from a jugular vein were collected in lithium heparin containing vials (Monovette® LH, Sarstedt, Nuernberg, Germany) before and 0.33, 0.66, 1, 2, 4, 6, 8, 12, 24, 48 72, 96, 120, 144, 168 and 192 h after injection using an indwelling venous catheter (Vygonuele® T, Vygon, Aachen, Germany) or cannulas for repeated punctures of the vein (Terumo®, Nueolus, BSN Medical, Hamburg, Germany). Plasma was separated by centrifugation at 2000 × *g* for 10 min (Hettich Zentrifuge Universal 32, Hettich, Tuttlingen, Germany) and was immediately shock-frozen in liquid nitrogen to be stored at least at −20 °C until quantitative analysis.

Broncho-alveolar cells were obtained by broncho-alveolar lavage (BAL) 24 h and 8 days after administration using a

flexible fiberscope (60512 VG Storz, Karl Storz, Tuttlingen, Germany) which was advanced in anesthetized subjects through the nose, trachea and a second-generation bronchus until it became wedged. The lavage was performed two times with 100 mL phosphate buffered saline (pH 7.4, temperature about 30 °C). The broncho-alveolar cell number was counted using a chamber according to Neubauer (Karl Hecht, Sondheim, Germany) and the microscope Laboval 4 (Zeiss, Jena, Germany). Cell sediments were prepared by centrifugation of the lavage fluid at 448 × *g* for 10 min and stored frozen using liquid nitrogen.

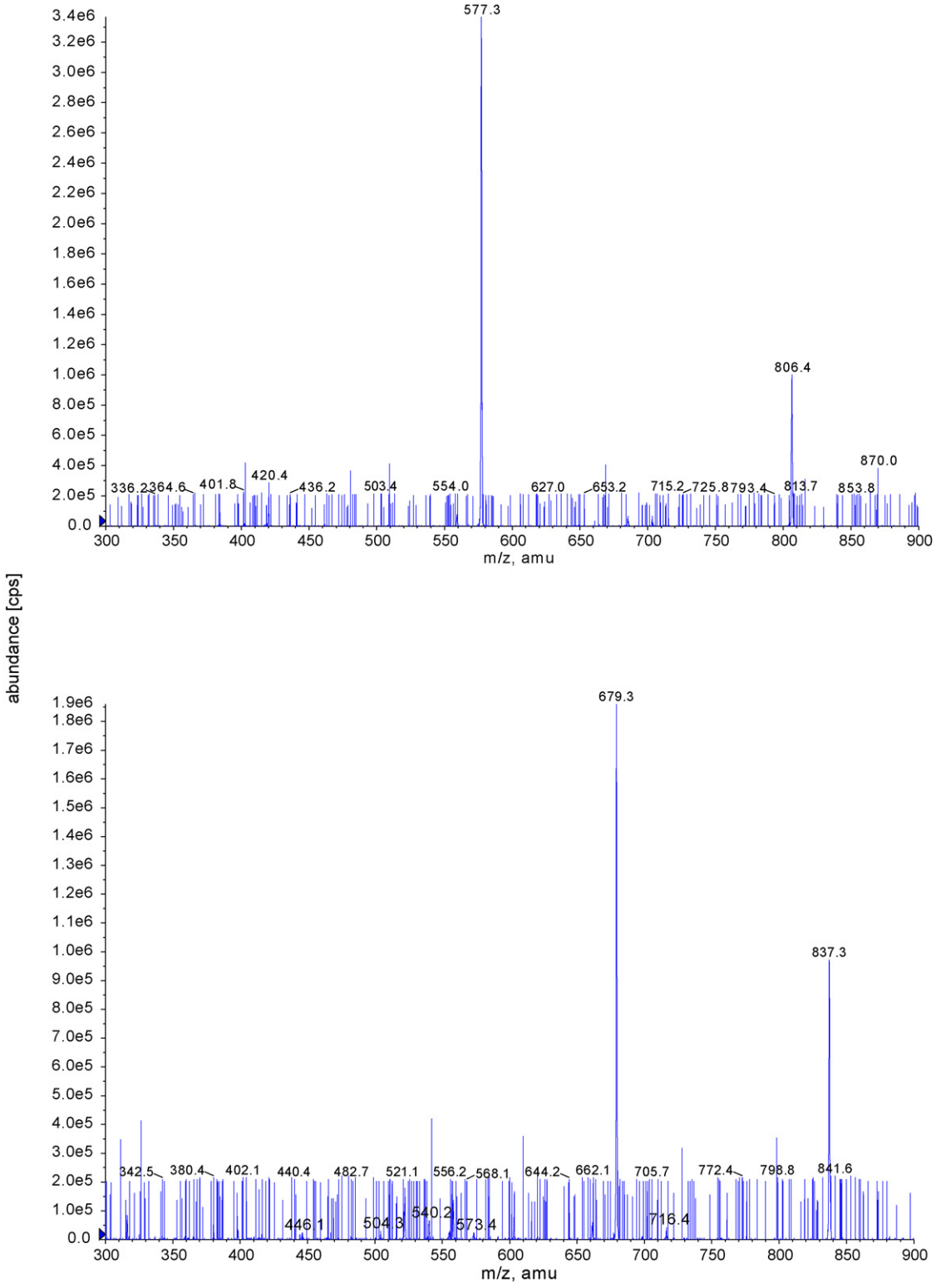

Fig. 2. Mass spectra of tulathromycin (A) and the internal standard roxithromycin (B) as obtained by positive atmospheric pressure chemical ionization.

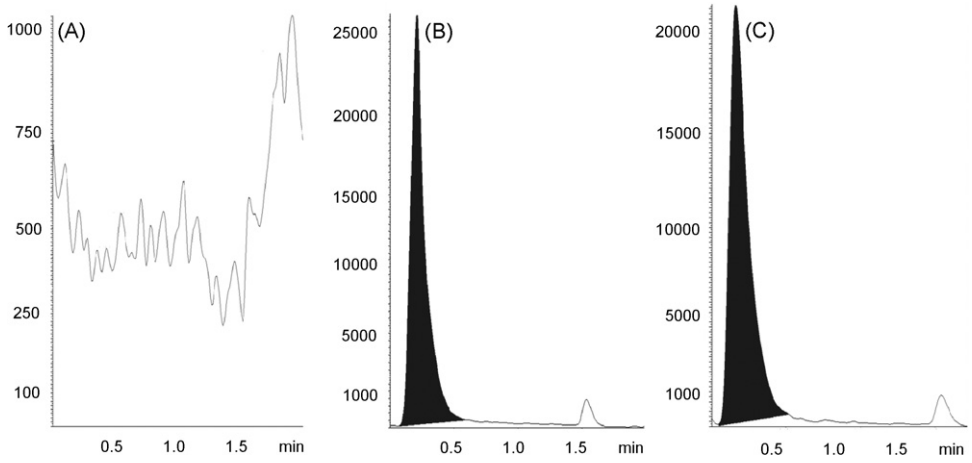

Fig. 3. Typical mass chromatograms of extracts from a plasma blank sample without tulathromycin and the internal standard roxithromycin (A) and from a plasma sample spiked with 0.1 μg/mL tulathromycin (B) and 0.05 μg/mL roxithromycin (C).

2.2.1. Pharmacokinetic and statistical evaluation

Maximum plasma concentrations (C_{max}) and the time of C_{max} (t_{max}) were taken from the plasma concentration–time curves. The area under the serum concentration–time curve ($AUC_{0-\infty}$) was assessed up to the last sampling time above the limit of quantification (AUC_{0-t}) and was extrapolated to infinity ($AUC_{t-\infty}$). Elimination half-life ($t_{1/2}$) was estimated by log–linear regression analysis. The apparent distribution volume V_{ss} was calculated based on a three-compartment open model. Arithmetic means ± standard deviations are given. Calibration curves were evaluated using linear regression analysis weighted by $1/x$ (x = concentrations). The F-test was used to evaluate variance homogeneity [9].

3. Results

3.1. Quantitative analysis of tulathromycin

Tulathromycin and roxithromycin showed under the conditions of APCI mass spectra with main peaks of the protonated molecules at m/z 806.4 and 837.3, respectively. Under optimized conditions, m/z transition was monitored from 806.4 to 577.3 for tulathromycin and for the internal standard from 837.3 to 679.3 (Fig. 2). We have tested three different commercially available MS-RP-18 columns of two manufacturers to optimize the chromatographic conditions. However, pattern and reproducibility of the tulathromycin and roxithromycin peaks were best without separation column. The mass spectrometer was therefore protected using a triple polyterafluorethylene filter system. Under these conditions, the mean retention time of tulathromycin and the internal standard was 0.17 min after isocratic elution with ammonium formiate buffer/acetonitrile (30:70, v/v; pH 3.0). Therefore, one analytical run required two minutes, i.e., the method enables a daily throughput of about 700 samples.

3.1.1. Selectivity, recovery and stability

Selectivity was verified by absence of a tulathromycin-specific signal by measurement of blank plasma and broncho-alveolar fluid samples from five foals (Fig. 3).

With samples of the low, middle and height segment of the appropriate calibration range and biological matrix *short term- long term- and post-preparative stability* was investigated. In all three cases only small (maximal deviation 12.3%) but no significant (five-fold measuring) lowering of the MS-signal in comparison with the initial values could be observed.

The recovery varies between 64.4 and 72.1% for plasma and between 71.4 and 80% for broncho-alveolar fluid (Table 2).

3.1.2. Linearity, accuracy and precision

Linearity of the calibration curve for plasma was shown between 0.006 and 0.8 μg/mL. The linear regression analysis (weighted by 1/concentration) showed a homoscedastic distribution of the individual residuals. The coefficients of correlation ranged between 0.9959 and 0.9996. The calibration curve for lavage fluid was linear between 0.1 and 4.0 μg/mL. The individual residues showed a homoscedastic distribution with coefficients of correlation between 0.9979 and 0.9998. For both calibration functions, the F-test revealed no significant difference between the variances.

Within-day accuracy and within-day and between-day precision for plasma samples and broncho-alveolar fluid were within the accepted limits [6–8]. The relative errors and relative standard deviations, respectively, were lower than 15% or lower than 20% for values near to the limit of quantification (Table 2).

3.2. Pharmacokinetics of tulathromycin

Tulathromycin was rapidly absorbed after intramuscular administration and reached maximum serum concentrations between 0.24 and 0.76 ng/mL within about 1 h. The drug was widely distributed with volumes of distribution at equilibrium ranging between 12.7 and 18.2 L/kg and slowly eliminated with half-lives between 101 and 158 h (Fig. 4; Table 3). On the assumption that the cytoplasmatic volume of 10^9 packed broncho-alveolar cells is about one millilitre, the intracellu-

Table 2
Recovery, accuracy and precision of calibration curves and quality control of the LC–MS/MS assay for tulathromycin in horse plasma (A and B) and broncho-alveolar cells (C and D)

	Concentration (μg/mL)						
	0.006	0.02	0.1	0.2	0.4	0.6	0.8
(A) Calibration curve (plasma; $n = 5$)							
Between-day accuracy (%)	13.4	−10.6	0.6	3.1	2.5	−0.3	−1.4
Between-day precision (%)	8.1	5.9	5.1	10.5	4.8	6.5	2.4

	Concentration (μg/mL)		
	0.016	0.3	0.7
(B) Quality controls (plasma; $n = 5$)			
Between-day accuracy (%)	−1.7	3.7	−0.5
Within-day accuracy (%)	12.8	−0.8	−2.2
Between-day precision (%)	3.5	8.2	7.3
Within-day precision (%)	14.6	3.8	9.3
Recovery (%)	64.4	63.7	72.1

	Concentration (μg/mL)					
	0.1	0.5	1.0	2.0	3.0	4.0
(C) Calibration (broncho-alveolar fluid; $n = 5$)						
Between-day accuracy (%)	7.6	−2.1	−7.5	−2.1	0.5	2.5
Between-day precision (%)	4.9	7.2	6.7	3.9	2.5	2.0

	Concentration (μg/mL)		
	0.3	1.6	3.6
(D) Quality controls (broncho-alveolar fluid; $n = 5$)			
Between-day accuracy (%)	2.8	−2.4	−2.4
Within-day accuracy (%)	6.1	−4.0	−6.6
Between-day precision (%)	8.8	4.9	3.7
Within-day precision (%)	13.7	7.2	3.3
Recovery (%)	71.4	78.5	80.0

Accuracy is given in percent of the respective nominal concentration and precision as standard deviation in percent of the respective concentration mean.

lar concentrations of tulathromycin in broncho-alveolar cells 24 and 192 h after single dose intramuscular administration of the drug were 1.24 ± 0.41 times and 18.8 ± 8.15 times, respectively, above the respective plasma concentration at that time points.

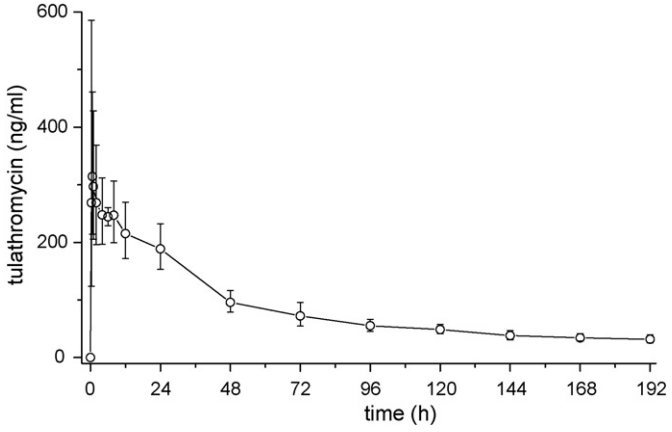

Fig. 4. Mean (geometric means) plasma concentrations–time curve of tulathromycin after a single intramuscular dose of 2.5 mg/kg tulathromycin in six healthy foals.

Table 3
Pharmacokinetic parameters and concentration of tulathromycin in broncho-alveolar cells 24 and 192 h after intramuscular administration of 2.5 mg/kg in six healthy foals

$AUC_{0-\infty}$ (μg × h/mL)	21.9 ± 3.37
C_{max} (ng/mL)	410 ± 192
C_{24h} (ng/mL)	192 ± 39.7
C_{192h} (ng/mL)	32.3 ± 7.07
t_{max} (h)	3.89 ± 3.85
$t_{1/2}$ (h)	117 ± 20.6
V_{ss} (L/kg)	15.2 ± 2.5
BAZ (24 h) (ng/10^9 cells)	247 ± 74
Cell/plasma ratio (24 h)	1.24 ± 0.41
BAZ (192 h) (ng/10^9 cells)	605 ± 336
Cell/plasma ratio (192 h)	18.7 ± 8.15

AUC: area under the concentration–time curve; C_{max}: maximum plasma concentration; C_{24h}: plasma concentration 24 h after administration; C_{192h}: plasma concentration 192 h after administration; t_{max}: time at the maximum plasma concentration; $t_{1/2}$: half-life; V_{ss}: volume of distribution at equilibrium; BAZ: broncho-alveolar cells. Arithmetic means and standard deviations are given.

4. Conclusions

The paper presents the result of a successful development and validation of a high-sensitive, selective LC–MS/MS method for

the quantitative determination of tulathromycin in horse plasma and broncho-alveolar cells without chromatographic separation and use of radio-labeled reference compounds. The validated range was 0.006–0.8 μg/mL for plasma and 0.1–4.0 $\mu g/10^9$ broncho-alveolar cells with linear correlation between peak area of the *m*/*z* signal and tulathromycin concentration. All quality parameters fulfilled the international criteria for bioanalytical method validation in pharmacokinetic, bioavailability and bioequivalence studies.

In foals, tulathromycin after intramuscular administration is rapidly absorbed, widely distributed and slowly eliminated. It cumulates manifold in broncho-alveolar cells.

Conflict of interest

All authors have no conflict of interest.

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