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LC–MS/MS method for the simultaneous determination of clarithromycin, rifampicin and their main metabolites in horse plasma, epithelial lining fluid and broncho-alveolar cells

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ABSTRACT

Clarithromycin (CLA) is a well established macrolide antibiotic which is frequently used in therapy of airway diseases in foals. It is extensively metabolized by CYP3A4 resulting in the antimicrobial active metabolite 14-hydroxyclarithromycin (OH-CLA). Rifampicin (RIF) is often comedicated to prevent resistance and augment therapy. RIF is a known inducer for metabolizing enzymes and transporter proteins. Therefore, comedication might bare the risks of pharmacokinetic drug interactions which were investigated in a clinical trial. As no adequate method to determine CLA, RIF and their main metabolites OH-CLA and 25-O-desacetylrifampicin (DAc-RIF) were described so far, we developed a selective and sensitive assay to measure concentrations of all four substances simultaneously in plasma, epithelial lining fluid (ELF) and broncho-alveolar cells (BAC) of foals. Drugs were measured after extraction with methyl *tert*-butyl ether using roxithromycin as internal standard and liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) for detection. The chromatography was done isocratically using 25 mM ammonium acetate buffer (pH 4)/acetonitrile (45%/55%, flow rate 200 μl/min). The MS/MS analysis was performed in the positive ion mode (*m*/*z* transitions: CLA, 748.5–590.1; OH-CLA, 764.1–606.1; RIF, 823.1–791.2; DAc-RIF, 781.1–749.1 and 837.3–679.2 for the internal standard). The method was validated according to selectivity, linearity, accuracy, precision, recovery, matrix effects and stability. The validation ranges for all substances were 2.5–25 for the low and 25–250 ng/ml for the high validation range. The described assay was shown to be valid and successfully applied to measure disposition of CLA, OH-CLA, RIF and DAc-RIF in plasma, ELF and BAC of foals in a clinical trial.

1. Introduction

The macrolide antibiotic drug clarithromycin ($C_{38}H_{69}NO_{13}$; *Mr*: 747.95 g/mol, Fig. 1) and the ansamycine derivative rifampicin ($C_{43}H_{58}N_4O_{12}$; *Mr*: 822.94 g/mol, Fig. 1) are frequently used in severe lung infections with *Mycobacterium avium* complex [1] and *Rhodococcus equi* in man and domestic animals, respectively [2]. After oral administration, clarithromycin (CLA) and rifampicin (RIF) both penetrate from blood via the broncho epithelial cells into the epithelial lining fluid (ELF) and accumulate in broncho-alveolar cells (BAC) reaching intracellular concentrations manifold above plasma levels [3,4]. However, these pulmonary penetration mechanisms are still poorly understood. Due to their pharmacokinetic properties CLA and RIF appear suitable to obtain deeper insight in penetration and pulmonary accumulation of drugs. CLA is predominately metabolized by CYP3A4 [5,6] and a substrate of the efflux pump ABCB1 [7]. Chronic treatment with RIF causes a strong induction of CYP3A4 [8] and drug transporting proteins such as ABCB1 [9]. On the contrary, single dose RIF inhibits ABCB1 [10]. Under consideration of the high expression levels of CYP3A4 and ABCB1 in the intestine and the known expression of several transporter proteins including ABCB1 within the pulmonary tissue, pharmacokinetic interactions between CLA and RIF may occur.

To evaluate the influence of RIF comedication on plasma pharmacokinetics and distribution of CLA into its pharmacologically relevant compartments, namely ELF and BAC obtained by broncho-alveolar lavage (BAL), we initiated a clinical trial including both drugs. In order to quantify concentrations of CLA, 14-hydroxyclarithromycin (OH-CLA), RIF and its main metabolite

Abbreviations: BAC, broncho-alveolar cells; BAL, broncho-alveolar lavage; CL, Aclarithromycin; DAc-RIF, 25-O-desacetylrifampicin; ELF, epithelial lining fluid; LC–MS/MS, liquid chromatography tandem mass spectrometry; LLOQ, lower limit of quantification; MRM, multiple reaction monitoring; OH-CLA, 14-hydroxyclarithromycin; RIF, rifampicin.

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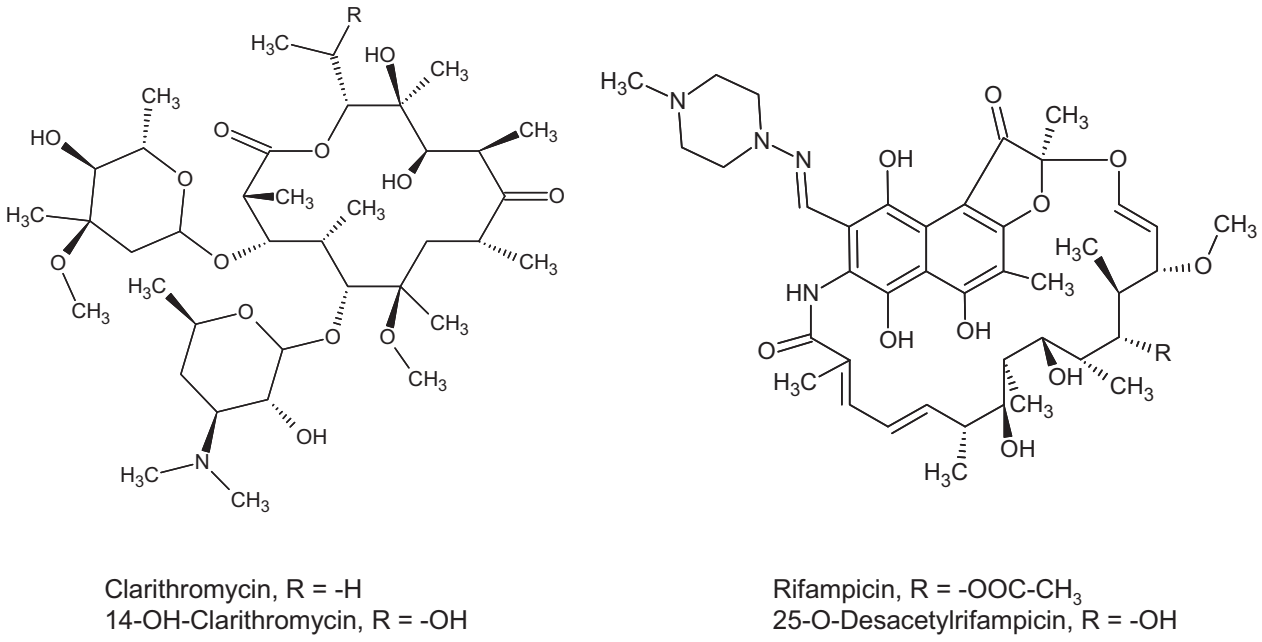

Fig. 1. Structural formulas of clarithromycin (left) and rifampicin (right) with their main metabolites.

25-O-desacetylrifampicin (DAc-RIF) simultaneously in all mentioned biological matrices there was the need for a sensitive quantification method. Several methods for the quantification of CLA in human plasma using HPLC with fluorescent [11], UV [12,13], electrochemical [14–16] and mass spectrometry detection [17–20] have been described. However, these assays do either lack adequate sensitivity [12–17,20] or do not include the simultaneous determination of the parent compounds and their main metabolites. A very recent report by de Velde et al. described the simultaneous quantification of all four substances in human plasma [20]. Nevertheless, this LC–MS/MS method was not appropriate for our study because of the following reasons: (1) the lower limit of quantification (LLOQ) of the mentioned method was 100 ng for CLA and OH-CLA. Thus, the plasma concentrations could be monitored for about three half-lives (~15 h) considering that CLA was expected to reach peak plasma concentrations in our foals of about 0.9 μg/ml [3]. This was supposed to be not enough for a comprehensive pharmacokinetic analysis of our study. (2) Because our study investigated the influence of the enzyme inducer rifampicin on the disposition of clarithromycin, the serum concentrations of the drug were expected to be markedly lower after co-medication. Therefore, a method with a higher sensitivity was needed. (3) While the study of de Velde et al. exclusively validated determination of the analytes in plasma, we were also interested in the pulmonary disposition of the drugs. Due to substantial differences between plasma, BAL and BAC (e.g. high concentrations of inorganic salts in BAL buffer, detergent in BAC lysate) and that determination of intracellular drug concentrations in BAC requires an adequate sample preparation (i.e. cell lysis) it was necessary to develop a new quantification method for these matrices. Finally, the drug levels in BAL and BAC were also expected to be very low [3,4].

Consequently, there was the need for a new and more sensitive LC–MS/MS method to be applicable for studies on plasma pharmacokinetics and pulmonary distribution.

This paper describes the development and validation of this method for the quantitative determination of CLA, RIF and their metabolites OH-CLA and DAc-RIF simultaneously in plasma, ELF and BAC and its successful application in a clinical trial in foals.

2. Materials and methods

2.1. Reagents

Acetonitrile was purchased in LC–MS quality (Chromasolv®, Sigma–Aldrich, Taufkirchen, Germany). Deionized water (conductance: ≤0.055 μS/cm, pH 5.0–6.0) was generated with the system SG 2800 (Hamburg, Germany). The internal standard roxithromycin as well as CLA and RIF were purchased from Sigma–Aldrich, methyl *tert*-butyl ether from Merck (Darmstadt, Germany) and DAc-RIF from Synfine research (Richmond, Canada). Dulbecco's phosphate buffered saline used as BAL buffer was from PAA Laboratories (Pasching, Austria). Stock solutions of CLA, RIF and DAc-RIF were prepared in ammonium acetate buffer (50 mM, pH 5.5)/acetonitrile (1/1, v/v) and stored at −20 °C. Working solutions were made weekly from stock solutions by dilution with ammonium acetate buffer and stored at 4 °C. All other chemicals were of analytical grade.

2.2. Sample preparation

Deeply frozen samples (plasma, ELF or BAC) were carefully thawed using a cold water bath and immediately frozen after sample removal. Due to high differences in concentrations of the analytes within the different specimens a low and a high validation range had to be defined and samples were processed for both concentration ranges as follows. For determination of CLA, OH-CLA, RIF and DAc-RIF, 0.2 ml plasma or ELF were mixed with 0.3 ml ammonium acetate (50 mM, pH 5.5) and 25 μl roxithromycin solution as internal standard (concentration 200 ng/ml for low and 2 μg/ml for high validation range). Then, the samples were extracted with 5 ml methyl *tert*-butyl ether for 15 min at room temperature. After centrifugation at 2980 × *g* for 2 min, the organic layer was separated, mixed with 50 μl butylhydroxytoluene solution (1%) as antioxidant agent and evaporated under a gentle air stream at room temperature. The residue was dissolved in 100 μl mobile phase (25 mM ammonium acetate (pH 4)/acetonitrile, 45%/55%) of which 5 μl were injected into the chromatographic system.

For analysis of BAC, to 0.2 ml suspension (cell density 3.2×10^6–2.8×10^7 cells/ml) 25 μl sodium dodecyl sulfate solution

(2%, m/m) were added and suspension was left for cell lysis at room temperature for 5 min. Then, the samples were homogenized for 5 min using an ultrasonic bath (Qualilab, Merck Eurolab, Bruchsal, Germany). Afterwards, 0.3 ml ammonium acetate (50 mM, pH 5.5) and 25 μl roxithromycin solution (200 ng/ml for low and 2 μg/ml for high validation range) were added and mixed intensively. Further preparation followed the same protocol as described for plasma and ELF.

2.3. LC–MS/MS analysis

The LC–MS/MS system consisted of the Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled with the API4000 mass spectrometer equipped with the Analyst 1.4 software (AB Sciex, Darmstadt, Germany).

Chromatography was performed isocratically using 25 mM ammonium acetate buffer (pH 4.0)/acetonitrile (45%/55%) as mobile phase at a flow rate of 200 μl/min and the reverse phase column XTerra® MS (C_{18}, 2.1 mm × 100 mm, particle size 3.0 μm, Waters, Milford, USA). To avoid contamination by particles, the chromatographic flow was filtered through a 0.5 μm filter device (PEEK, Supelco, Bellefonte, USA). The mass spectrometer was equipped with the TurboIon® interface operated in the positive ion mode monitoring the following *m*/*z* transitions: CLA, 748.5–590.1; OH-CLA, 764.1–606.1; RIF, 823.1–791.2; DAc-RIF, 781.1–749.1 and 837.3–679.2 for the internal standard roxithromycin. The following parameters were applied to the TurboIon® interface: temperature, 300 °C; gas 1, 60 psi; gas 2, 50 psi; collision-activated dissociation (CAD) gas, 8 psi (all nitrogen) and voltage, 4500 V. The detailed mass spectrometry parameters are given in Table 1. The chromatograms were evaluated with the validated Analyst 1.4 software using the internal standard method and peak-area ratios for calculation (linear regression, 1/*x* weighting).

2.4. Determination of OH-CLA

As OH-CLA was commercially not available, we used a quantification method based on the parent compound. To characterize the mass transition of OH-CLA for a MRM experiment, drug containing horse plasma was extracted as mentioned before and monitored for the parent ion of OH-CLA using the high sensitive API4000 QTRAP mass spectrometer (AB Sciex, Darmstadt, Germany). In the positive ionization mode 764.1 *m*/*z* was identified as the respective protonated species of OH-CLA and used for development and optimization of a highly sensitive MRM experiment (Table 1). The resulting mass transition (*m*/*z* 764.1–606.1) for the metabolite measured in biological samples was matched to the daily calibration curve of CLA. To avoid over- or underestimation of OH-CLA by this approach (e.g. by different slopes of the calibration curves), the signal to concentration response for CLA and its metabolite were determined. For this purpose, four dilution series of drug containing plasma were prepared (each consisting of seven values, dilution factors: 0–100) and measured as mentioned before. The resulting calibration curves were compared with respect to the slopes of the analytes. The mean ratio of the slopes of the signal to concentration curves between CLA and OH-CLA was 1.80 ± 0.15. Consequently, all estimated concentration values of OH-CLA generated by this approach (based on the measured peak area ratio of OH-CLA to the internal standard) were divided by this correction factor to conclude on the concentration of the metabolite. Due to this indirect approach, quantitative determination of OH-CLA could not be validated.

Table 1
Mass spectrometry parameters for detection of clarithromycin, rifampicin and their main metabolites in the positive multiple reaction monitoring mode. Dwell time was in each case 200 ms. DP, declustering potential; EP, entrance potential; CE, collision energy and CXP, collision cell exit potential (all in V); CLA, clarithromycin; OH-CLA, 14-hydroxyclarithromycin; RIF, rifampicin; DAc-RIF, 25-O-desacetylrifampicin.

Compound	Q1 mass	Q3 mass	DP	EP	CE	CXP
CLA	748.5	590.1	76	10	27	18
OH-CLA	764.1	606.1	70	10	27	16
RIF	823.1	791.2	81	10	25	26
DAc-RIF	781.1	749.1	56	10	19	24
ROX	837.4	679.2	85	10	31	20

2.5. Validation

The developed assay was validated according to the recent FDA guideline for bioanalytical method validation [21]. Selectivity of the LC–MS/MS method was confirmed for plasma, ELF and BAC by comparing blank biological matrices and drug containing samples.

Linearity of the method was confirmed by adding increasing amounts of CLA, RIF or DAc-RIF to drug-free plasma, BAL buffer and suspension of THP-1 cells (monocyte cell line to mimic BAC). The calibration curves (each $N = 6$) consisted of a double blank matrix sample without analyte and without internal standard, a blank matrix sample spiked with internal standard only and seven (low validation range) or six (high validation range) calibrants. The validation range for all analytes in all matrices was 2.5–25 ng/ml for the low and 25–250 ng/ml for the high concentration range.

For evaluation of accuracy and precision, quality control samples (QC) spiked with CLA, RIF and DAc-RIF in plasma, BAL buffer and THP-1 cell suspension were prepared. The spiking concentrations were 5, 10 and 25 ng/ml for the low and 50, 100 and 250 ng/ml for the high validation range.

Between-day accuracy and precision was assessed by comparing the measured concentrations in QC samples (six separately prepared sets measured on different days) with the respective nominal concentrations, expressed as relative error (accuracy) and the respective coefficients of variation of the mean values (precision). Within-day accuracy and precision was determined by six-fold measuring of the respective quality control samples on one day.

Recovery of CLA, RIF and DAc-RIF from plasma was assessed by comparing the peak areas obtained from accordingly six extracted matrix samples with peak areas from samples obtained without extraction (diluted stock solution). The spiking concentrations were identical to those of the QC samples of the low and high validation range. Because real blank matrix was not available for BAL and BAC and due to the similar aqueous consistence of all matrices, the recovery was exclusively determined for plasma assuming only small differences between the matrices.

Detection of significant matrix effects (i.e. ion suppression), which are very common when using electrospray ionization sources, was performed as follows: peak areas obtained from extracted blank matrix samples which were afterwards spiked with the respective QC sample concentrations were compared with peak areas from samples without any matrix (diluted stock solution). Matrix effects were expressed as ratio of the analytical signals.

Stability of CLA, RIF and DAc-RIF was determined with respect to short-term, post-preparative and freeze–thaw stability in biological samples by using in each case six QC sample sets. Short-term (bench-top) stability was tested after storing these samples at room temperature for 2 h prior to extraction. Post-preparative stability was assessed by storing extracts in the autosampler at 5 °C for 24 h. To assess freeze-and-thaw stability, the samples were thawed and frozen up to three times before extraction. In parallel to assessment of the recovery, all stability tests were exclusively performed in plasma, assuming the worst case scenario for drug stability. Working solution stability of CLA, RIF, DAc-RIF and roxithromycin was tested during storage at 4 °C for one week. In each case, stability

was assumed if the drug content after the given storage conditions was within the acceptable range of accuracy, i.e. ±15%.

2.6. Measurement of biological samples

On each day of analysis, calibration curves were freshly prepared using the respective biological blank matrix as mentioned above. QC samples represented at least 10% of all analytical samples and were measured during the entire analytical run. The criterion of acceptance for an analytical run was if at least 4 of 6 of all QC samples were within an accuracy range of ±15% of the nominal values as suggested by the FDA guideline [21].

2.7. Clinical study protocol

2.7.1. Animals

Nine healthy foals of warm-blooded horses of the Oldenburger trait were included in the study which was conducted under the same conditions as recently reported [22].

2.7.2. Study design

Foals were administered either CLA (7.5 mg/kg body weight, twice daily, Klacid Saft Forte®, 250 mg/5 ml, Abbott GmbH&Co. KG, Wiesbaden, Germany) or RIF (10 mg/kg body weight, twice daily, Rifa® 600 tablets, Gruenenthal, Aachen, Germany) or a combination of both drugs for three days. Plasma concentration–time profiles of CLA, OH-CLA, RIF and DAc-RIF were measured on day 3. Venous blood was sampled for 48 h in lithium heparin containing vials (Monovette® LH, Sarstedt, Nuernberg, Germany) after last administration of study medication. Plasma was separated by centrifugation at 2000 × *g* for 10 min (Hettich Zentrifuge Universal 32, Hettich, Tuttlingen, Germany) and stored at least at −80 °C until quantitative analysis

2.7.3. Broncho-alveolar lavage (BAL)

ELF and BAC were obtained by BAL performed 12 h after last drug administration. BAL was conducted as described recently [22]. Aliquots were stored until quantitative analysis at least at −80 °C.

2.8. Pharmacokinetic evaluation

Maximum plasma concentrations (C_{max}) were taken from the plasma concentration–time curves. The area under the serum concentration–time curve ($AUC_{0-12\,h}$) was calculated using the trapezoidal rule. Concentrations in ELF were assessed by normalizing with urea concentrations in plasma and in the broncho-alveolar lavage fluid. Concentrations within BAC were calculated assuming a mean macrophage cell volume of 1.2 μl/10^6 cells in foals as described previously [22].

3. Results and discussion

3.1. LC–MS/MS analysis

Roxithromycin was used as the internal standard because of its structural similarity and comparable chemical properties to CLA (roxithromycin: pK_a: 8.8, log *P*: 2.75; CLA pK_a 8.99, log *P* 3.16). In the positive ionization mode, all compounds generated higher signals for the protonated molecule peaks ($[M+H]^+$) than for the respective molecular ion produced by hydride abstraction ($[M-H]^-$) in the negative mode. The TurboIon® source resulted in markedly higher signals than the Heated Nebulizer® (APCI) interface. Therefore, we used the TurboIon® source operated in the positive MRM mode for our assay. The respective mass to charge transitions were manually generated and optimized in order to obtain maximum mass peak intensities and are in good agreement with the literature [20] (Table 1).

Isocratic elution with the reversed phase column resulted in short retention times of 1.5 min, 1.2 min, 1.65 min, 1.4 min and 1.45 min for CLA, OH-CLA, RIF, DAc-RIF and roxithromycin (Fig. 2). Thus, the analytical run was finished after 3 min which enables a high throughput of about 300 samples per day. From the chromatographic point of view, the separation of the analytes was poor but compensated by compound-specific tandem mass spectrometric detection. Moreover, interferences between the analytes were not observed.

3.2. Validation

The analytical method was shown to be selective for CLA, OH-CLA, RIF and DAc-RIF as concluded from the absence of analytical signals in different blank matrix samples and interferences between the analytes and the internal standard (Fig. 2).

There was a sufficient linear correlation between the analyte concentration and the analytical signal for all analytes in all matrices observed. Correlation coefficients (*r*) of all calibration curves were at least 0.9914 or better. However, it was necessary to define two validation ranges (2.5–25 ng/ml and 25–250 ng/ml) to ensure adequate linearity. The lowest concentration value of our analytical range (2.5 ng/ml) was the LLOQ of our method. According to the bioanalytical guideline from the FDA, the analytical signal was at least >5 times above blank matrix for all analytes at this concentration.

Within-day as well as between-day accuracy and precision for both calibration ranges for all analytes in plasma, ELF and BAC were within the stipulated range of ±15% of the nominal concentrations and <15% for the respective coefficients of variation of the mean values (Table 2).

All investigated analytes can be reliably extracted by liquid extraction using methyl *tert*-butyl ether resulting in mean recovery rates of 79.9 ± 2.1% for CLA, 68.0 ± 5.4% for RIF and 45.0 ± 5.9% for DAc-RIF with good homogeneity among the entire validation range (Table 3).

Matrix effects were not observed as concluded from the absence of significant ion suppression, i.e. signal intensity remained nearly unchanged compared to matrix free samples (Table 3). Mean recovery rates for the analytical signal were 105 ± 8.4% for CLA, 110 ± 10.4% for RIF and 111 ± 5.5% for DAc-RIF. However, there was a slight but not significant increase in signal intensity observed for the low validation range as most likely caused by enhanced formation of ions in the ion source by acidic constituents of the biological matrix. Given that the accuracy and precision for all analytes was within 15% among the entire concentration range (Table 2), this weak influence of the matrix seems to be of subordinate significance in our method.

All investigated compounds were shown to be stable in all matrices at room temperature for at least 2 h (102 ± 4.8% for CLA, 111 ± 2.7% for RIF and 110 ± 5.4% for DAc-RIF) and for at least 24 h at 5 °C in the autosampler (101 ± 0.8% for CLA, 96.3 ± 4.6% for RIF and 98.8 ± 1.6% for DAc-RIF). The results for freeze–thaw stability are given in Table 3 and indicate that all analytes may undergo up to three freeze–thaw cycles without impaired stability. These stability data are in good agreement with the literature [17,20]. Moreover, working solutions of CLA, RIF and DAc-RIF and roxithromycin were stable when stored for one week at 4 °C.

Finally, the developed method was shown to be specific, sensitive, precise and accurate for the quantification of CLA, RIF and its metabolite DAc-RIF in plasma, ELF and BAC of foals. The developed LC–MS/MS assay has a substantially lower LOQ than previously described methods (factor 2–5) [17,19,20]. Therefore, the method allows quantification of the analytes in plasma as well as in pulmonary specimens even after induction of metabolizing enzymes,

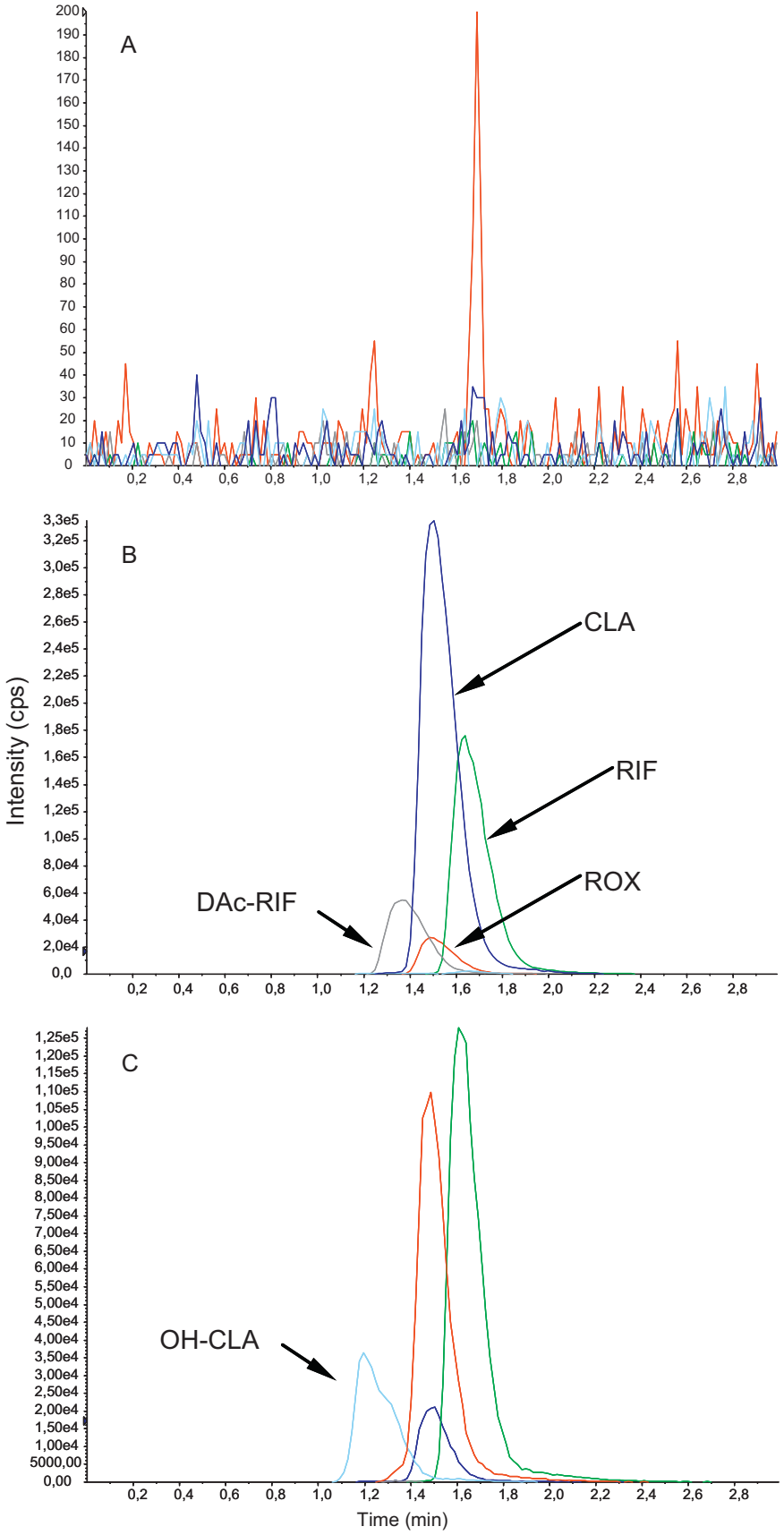

Fig. 2. Total ion chromatograms of a blank plasma (A), of a plasma calibrator spiked with 50 ng/ml clarithromycin, rifampicin, 25-O-desacetylrifampicin and the internal standard roxithromycin (B), and a plasma sample of one foal from the clinical study (C). All chromatograms were obtained by monitoring the *m*/*z* transitions 748.5/590.1 (clarithromycin), 764.1/606.1 (14-hydroxyclarithromycin), 823.1/791.2 (rifampicin), 781.1/749.1 (25-O-desacetylrifampicin) and 837.3/679.2 (roxithromycin) in the positive ion mode. CLA, clarithromycin; OH-CLA, 14-hydroxyclarithromycin; RIF, rifampicin; DAc-RIF, 25-O-desacetylrifampicin; ROX, roxithromycin.

Table 2
Within-day and between-day accuracy and precision data for low and high validation range expressed as relative error (accuracy) and coefficients of variation (precision) of nominal and respective mean concentrations of clarithromycin (CLA), rifampicin (RIF) and 25-O-desacetylrifampicin (DAc-RIF) in horse plasma. Precision and accuracy were calculated in each case from $N = 6$ values.

Quality control	Analyte	Concentration (ng/ml)	Precision (%)		Accuracy (%)	
			Within day	Between day	Within day	Between day
Q1-low	CLA	5	4.3	10.0	5.4	6.2
	RIF	5	4.1	6.9	2.0	−0.2
	DAc-RIF	5	11.2	5.8	−11.8	−11.7
Q2-low	CLA	10	4.0	6.5	2.2	0.1
	RIF	10	1.6	7.4	−1.7	0.1
	DAc-RIF	10	2.8	3.7	−3.4	0.6
Q3-low	CLA	25	4.2	6.2	−3.3	1.7
	RIF	25	2.6	9.0	7.4	−2.2
	DAc-RIF	25	2.1	6.2	8.1	−2.0
Q1-high	CLA	50	4.3	8.9	−1.5	8.6
	RIF	50	8.0	9.4	−1.1	2.1
	DAc-RIF	50	6.0	8.5	3.7	3.1
Q2-high	CLA	100	4.2	5.1	6.6	2.6
	RIF	100	8.9	8.8	−2.1	1.2
	DAc-RIF	100	5.7	8.9	0.9	0.4
Q3-high	CLA	250	7.6	5.1	−1.6	−0.1
	RIF	250	7.5	6.6	9.3	−1.4
	DAc-RIF	250	7.5	8.2	6.6	−0.1

Table 3
Data for matrix effects, recovery and freeze–thaw stability of clarithromycin (CLA), rifampicin (RIF) and 25-O-desacetylrifampicin (DAc-RIF) determined in low and high quality control samples in horse plasma. Matrix effects, recovery rate and stability data were calculated in each case from $N = 6$ values.

Quality control	Analyte	Concentration (ng/ml)	Matrix effects (%)	Recovery (%)	Freeze–thaw stability (%)		
					1st cycle	2nd cycle	3rd cycle
Q1-low	CLA	5	115	81.3	106	115	94.6
	RIF	5	123	69.0	113	109	107
	DAc-RIF	5	119	41.5	101	107	106
Q2-low	CLA	10	109	77.4	96.0	101	94.1
	RIF	10	118	63.3	96.7	102	101
	DAc-RIF	10	114	35.8	98.5	105	110
Q3-low	CLA	25	112	82.8	91.7	94.8	88.4
	RIF	25	117	62.6	88.3	101	106
	DAc-RIF	25	115	45.4	91.8	101	102
Q1-high	CLA	50	103	78.0	94.3	98.1	93.9
	RIF	50	104	71.9	91.6	95.6	92.4
	DAc-RIF	50	109	49.7	89.5	88.1	102
Q2-high	CLA	100	98.6	79.4	90.7	91.7	84.9
	RIF	100	102	76.4	87.6	93.2	90.5
	DAc-RIF	100	107	45.0	89.2	90.2	93.5
Q3-high	CLA	250	93.4	81.0	91.9	90.9	81.9
	RIF	250	97.9	64.9	105	107	98.1
	DAc-RIF	250	105	52.5	106	106	106

i.e. lower systemic and pulmonary drug exposure. Moreover, our approach to quantify the OH-CLA without reference compound but based on the quantification of the parent compound was successful as concluded from very similar pharmacokinetics in a human study resulting in comparable ratios of maximum plasma concentration for parent compound to metabolite with regard to the administered dosage [23].

3.3. Application of the method

The validated analytical assay enabled the quantitative determination of CLA, RIF and their active metabolites OH-CLA and DAc-RIF simultaneously in plasma, ELF and BAC of foals collected during our clinical trial.

Plasma concentration–time profiles for one exemplary foal are shown in Fig. 3. During monotherapy with CLA, concentrations of CLA and its metabolite were significantly higher in ELF and even higher in BAC compared to plasma levels (Table 4), which is in

Table 4
Pharmacokinetic parameters for clarithromycin (CLA), 14-hydroxyclarithromycin (OH-CLA), rifampicin (RIF) and 25-O-desacetylrifampicin (DAc-RIF) determined in plasma, epithelial lining fluid (ELF) and broncho-alveolar cells (BAC) for one exemplary foal.

Analyte	Plasma		ELF	BAC
	$AUC_{0-12\,h}$ (μg × h/ml)	C_{max} (μg/ml)	C (μg/ml)	C (μg/ml)
Phase I: CLA alone				
CLA	4.07	0.61	7.96	92.3
OH-CLA	1.12	0.13	0.17	2.54
Phase II: RIF alone				
RIF	169	17.9	5.02	3.63
DAc-RIF	0.16	0.02	0.72	0.83
Phase III: CLA + RIF				
CLA	0.25	0.05	0.54	6.23
OH-CLA	0.68	0.08	0.05	0.64
RIF	181	17.3	2.29	5.11
DAc-RIF	0.21	0.03	0.37	1.27

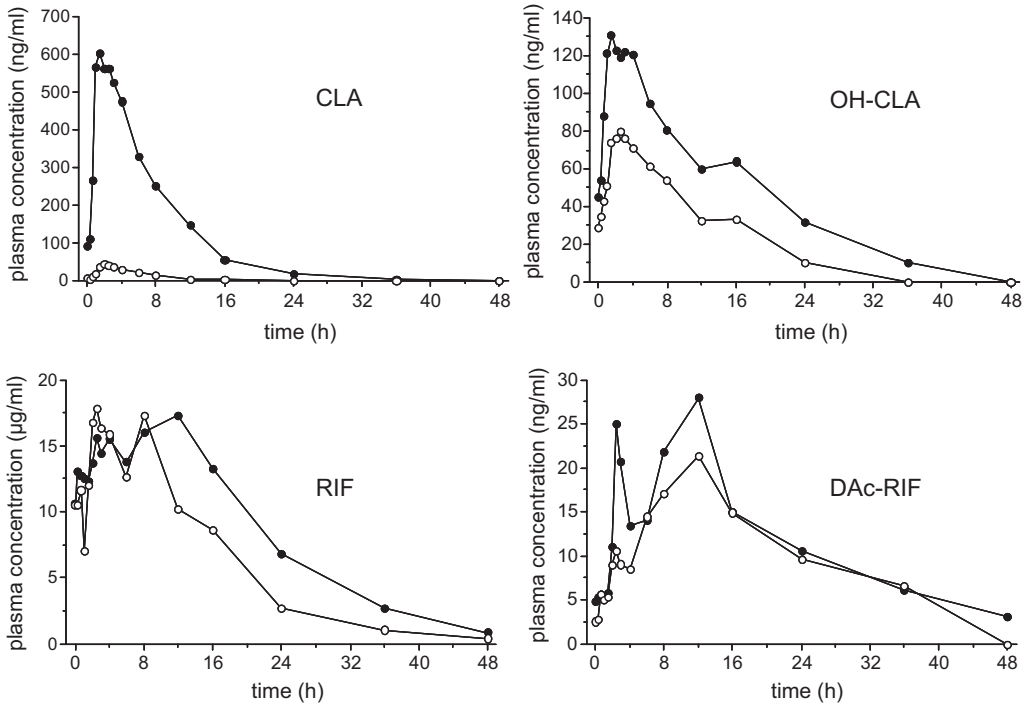

Fig. 3. Plasma concentration–time profiles of one exemplary foal. Closed symbols indicate monotherapy of clarithromycin (7.5 mg/kg body weight, twice daily) or rifampicin (10 mg/kg body weight, twice daily); open symbols indicate combined therapy. CLA, clarithromycin; OH-CLA, 14-hydroxyclarithromycin; RIF, rifampicin; DAc-RIF, 25-O-desacetylrifampicin.

good agreement with the literature [3]. Chronic comedication with RIF substantially lowered plasma AUC, maximum plasma levels and pulmonary availability of CLA and OH-CLA (Table 4). However, the ELF to plasma and the BAC to plasma ratio remained nearly unchanged. On the other side, CLA had no significant influence on plasma concentrations and pulmonary distribution of RIF and DAc-RIF.

4. Conclusion

The described analytical method was shown to be selective and sensitive for the quantitative determination of CLA, RIF and DAc-RIF in horse plasma, ELF and BAC. The method validation indicated good linearity, within-day and between-day precision and accuracy, stability and recovery for CLA, RIF and DAc-RIF. All quality parameters fulfilled the international criteria for bioanalytical method validation. OH-CLA was successfully quantified using a method based on the parent compound. The developed method was successfully applied to analyze a pharmacokinetic study.

Our data indicated that comedication of CLA and RIF leads to considerable pharmacokinetic interactions resulting in remarkably lowered concentrations of CLA and OH-CLA in plasma and in turn in ELF and BAC.

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical approval

The corresponding Federal Authority of Mecklenburg/Vorpommern (Landesveterinär–und Lebensmitteluntersuchungsamt) has been notified. Reference is LALLF M-V/TSD/7221.3-1.1-066/08.

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