



Pharmacology

Ceftriaxone pharmacokinetics by new simple and sensitive ultra-high-performance liquid chromatography method



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ABSTRACT

Ceftriaxone is a cephalosporin antibiotic with a potent antimicrobial activity and excellent penetration in most body fluids such as pleural, peritoneal, spinal and brain. These facts contribute to the application of ceftriaxone in the treatment of bacterial peritonitis, an abdominal disorder in veterinary medicine, with potential risk of death. The determination of ceftriaxone levels in plasma and peritoneal fluid may be used to assess the pharmacokinetic profile at various instances of administration and allows observing if the concentrations needed are being achieved. Therefore a method was developed and validated for the determination of ceftriaxone in plasma and peritoneal fluid which after was applied in a pharmacokinetic profile study. The bioanalytical method validation was performed according to widely acceptable experiments. Two horses were used as a model of the method applicability; ceftriaxone was intraperitoneally administered to these animals as a single dose. The plasma and peritoneal fluid analysis were performed using an UHPLC system in reverse phase chromatography mode in fully validated conditions. The methods have shown linearity between 0.49 and 500 µg/mL for plasma, and between 0.24 and 500 µg/mL for peritoneal fluid. The quantitative analysis of ceftriaxone in these matrices allows monitoring of the therapy. This method showed improved sensitivity as well as the quantitation in peritoneal fluid.

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1. Introduction

Ceftriaxone is a cephalosporin antibiotic with a potent antimicrobial activity against the majority of Gram-negative and Gram-positive bacteria (Reiner et al., 1980). The stability against beta-lactamase activity and relatively long elimination half-life of ceftriaxone make this cephalosporin a good choice for the treatment of patients with a number of infections (Owens and Dash, 2003), including intra-abdominal infections (Yellin et al., 2002). According to Ringger et al. (1998) and Lamb et al. (2002), ceftriaxone has excellent penetration in most body fluids such as pleural, peritoneal, spinal and brain, and this fact contributes to the use of ceftriaxone in the treatment of bacterial peritonitis, or in its prophylaxis. This abdominal infection requires the drug to reach the peritoneal fluid.

Ceftriaxone is not absorbed after oral administration and thus, only used intravenously, intramuscularly (Lamb et al., 2002), or by intraperitoneal administration (Albin et al., 1986), which is particularly relevant in patients receiving peritoneal dialysis (De Vin et al., 2009).

The therapeutic drug monitoring using plasma matrix is well established (Mcwhinney et al., 2010; Verdier et al., 2011), but the determination of ceftriaxone concentration in peritoneal fluid may provide

additional information to ensure treatment is successful. Based upon the target pathogen in the treatment of peritonitis, reaching the desired concentration of the therapeutic drug in the peritoneal fluid is essential for treatment.

Bioanalytical methods for the quantification of ceftriaxone in plasma, urine, fat tissue, bones or cerebrospinal fluid with application in vivo studies were available (Bowman et al., 1984; Gergs et al., 2014; Kratzer et al., 2014; Mcwhinney et al., 2010; Page-Sharp et al., 2016; Payasi et al., 2010; Ringger et al., 1996, 1998; Schleibinger et al., 2015; Steib et al., 1993; Sun et al., 2012; Verdier et al., 2011). High performance liquid chromatography (HPLC) systems with different detectors were used.

The aim of this study was to develop and validate a method for the determination of ceftriaxone in plasma and peritoneal fluid. This method was used to determine the concentration of ceftriaxone in peritoneal and plasma horse samples in order to allow comparison between those matrices.

2. Material and methods

2.1. Chemical and solutions

Ceftriaxone sodium and cefoperazone sodium (internal standard, I.S.) were purchased from Sigma-Aldrich (New Jersey, USA). The HPLC

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grade methanol and ammonium acetate were purchased from J.T. Baker (Mexico City, Mexico). The ultrapure water was obtained from a Millipore Milli-Q System and used throughout the analysis.

The stock solution of ceftriaxone was prepared in ultrapure water (Milli-Q System) at concentration 5 mg/mL, while the work solution of cefoperazone (internal standard, I.S.) was prepared in ultrapure water (Milli-Q System) at concentration 1 mg/mL. Both solutions were stored at $-80\text{ }^{\circ}\text{C} \pm 2$. The ceftriaxone stock solution was used in dilutions to obtain calibration curve standards and quality control samples in plasma and peritoneal fluid.

2.2. Chromatographic conditions and instrumentation

The UHPLC Waters Acquity H-Class UPLC® system equipped with UV-Vis detector was used. The chromatographic analysis was performed on an Acquity HSS T3 C₁₈ (2.1 × 100 mm; 1.8 μm) column protected by VanGuard guard column HSS C18 (2.1 × 5 mm; 1.8 μm), both were placed into the column oven at 40 °C. The mobile phase was a mixture of methanol: ammonium acetate 20 mM (21:79, v/v) in isocratic mode, with detection at 260 nm. The flow rate was 0.4 mL/min and the sample injection volume was 1 μL. The run time was 8 minutes. The ratio of peak area of the analyte to the I.S. was used for the drug quantitation.

2.3. Sample processing

In order to perform the analysis, 100 μL of plasma or peritoneal fluid (blank, sample, calibration standard, or quality control) plus 10 μL of I.S. were deproteinized by adding 100 μL of cold methanol. This procedure was followed by 1 min of vortex and centrifugation at 10640×g and 4 °C for 15 min. Then the supernatant were filtrated through PTFE syringe filters (0.22 μm; 13 mm). The final solution was transferred to maximum recovery vial and stored at 10 °C inside the sample manager until the injection.

2.4. Bioanalytical method validation

The validation procedure was based on the (U.S.) FDA Guidance for Industry (2001) and (Brazilian) ANVISA resolutions: 899 (2003) and 27 (2012). The evaluated parameters were linearity, precision, accuracy, selectivity, stability, carryover, recovery, and lower limit of quantitation.

For linearity, analyses of the analyte were conducted in three replicates, at seven concentration levels for plasma and eight concentration levels for peritoneal fluid. The calibration curves were generated from the ceftriaxone to I.S. peak area ratios, by least-squares linear regression and $1/Y^2$ weighting factor. The standard concentrations for plasma were 0.49, 0.98, 1.95, 7.81, 31.25, 125, and 500 μg/ml, while the standard concentrations for peritoneal fluid were 0.24, 0.49, 0.98, 1.95, 7.81, 31.25, 125, and 500 μg/ml. ANOVA ($p < 0.05$) was used to test the linearity of calibration curve.

Precision and accuracy were assessed for four concentration levels or quality control (QC), namely the lower limit of quantification (LLOQ) QC, low QC, mid QC and high QC, for which the chosen levels were 0.49, 0.98, 31.25, and 400 μg/ml, respectively for plasma, and 0.24, 0.49, 31.25, and 400 μg/ml, respectively for peritoneal fluid. Accuracy was evaluated as the deviation of the mean analytical result from the theoretical value, while precision was evaluated as the relative standard deviation (R.S.D.) of measurements at each level. Intra-run precision and accuracy were calculated for five replicates in the same day. On the other hand, Inter-run precision and accuracy were calculated for 15 replicate determinations at each QC level, analyzed over three non-consecutive days. All QC samples, plasma and peritoneal fluid, were prepared on the day of analysis. The data acceptance criterion for precision was R.S.D. less than 15%, except for LLOQ, where it should not exceed 20%. For accuracy, acceptable values were 85–115% of the theoretical value, except at LLOQ, where 80–120% was acceptable as well.

The method selectivity was demonstrated by the absence of interfering peaks on the chromatogram at the retention times of ceftriaxone, when both blank (blank sample) or a blank spiked with I.S. (zero sample) were analyzed, either in plasma or peritoneal fluid.

The stability of ceftriaxone was evaluated under several storage conditions, including short-term room temperature, long-term frozen, freeze and thaw cycles and post-preparative. All stability tests were performed at the low and high QC levels for both matrices.

Ceftriaxone was considered stable if the measured analyte concentration of stored samples were 85–115% of the measured analyte concentration of fresh samples.

2.5. Method application to a pharmacokinetic profile study

In order to show the method application, a pharmacokinetic profile study was carried out in a 370 kg gelding and 350 kg mare. They were housed in boxes with free access to food and water. The preclinical study protocol complied with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications no. 8023, revised 1978), and was approved by the Research Ethics Committee of the UNESP – College of Veterinary Medicine and Animal Sciences (CEUA process 105/2013).

The animals were submitted to laparoscopic procedure in order to implant a Tenckhoff peritoneal catheter, which was used for a single dose of 25 mg/kg of ceftriaxone through intraperitoneal route.

The blood and peritoneal fluid samples were collected at times of 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 12, 16, and 24 hours post-dose into tubes containing EDTA. The plasma was separated by centrifugation at 2000×g for 5 min and stored at $-80\text{ }^{\circ}\text{C}$ until analysis was performed.

After analysis of the concentrations, the results were used to construct a concentration versus time profile, which were expressed as individual values for each animal. Non compartmental pharmacokinetic analysis was performed to obtain the parameters elimination half-life, volume of distribution and clearance. Statistical moments approach was used to calculate mean residence time. The extraction rate (ER) was calculated to improve the understanding of the elimination process for this animal model. It is calculated as the ratio between cardiac output (CO) of the animal used and the obtained CI ($ER = CO/CI$) (Toutain and Bousquet-Mélou, 2004).

3. Results and discussion

3.1. UHPLC bioanalytical method validation

The bioanalytical method for ceftriaxone quantitation in plasma and peritoneal fluid by UHPLC was validated according to the aforementioned guides. The selectivity was found to be adequate as no interfering peaks were observed at the same retention time of the analyte in plasma or peritoneal fluid. Blank subtraction was used to enhance peak integration of ceftriaxone from peritoneal fluid. The chromatograms show ceftriaxone at 0.87 minutes and I.S. at 5.50 minutes in plasma and peritoneal fluid (Fig. 1).

The calibration curves were linear from 0.49 to 500 μg/ml for plasma ($y = 1.007x - 0.151$, $r = 0.996$), and from 0.24 to 500 μg/ml for peritoneal fluid ($y = 1.456x - 0.149$, $r = 0.992$). The linearity tests met the acceptance criteria, with precision and accuracy in accordance with the established limits (Table 1). The established linear range of this method is superior to published ranges for both horse (Ringger et al., 1996, 1998) and human (Gergs et al., 2014; Mcwhinney et al., 2010; Page-Sharp et al., 2016; Schleibinger et al., 2015; Sun et al., 2012; Verdier et al., 2011; Zhao et al., 2014). The linear range for peritoneal fluid would be useful for the monitoring of treatment with ceftriaxone for bacterial peritonitis.

Tests of precision and accuracy were adequate according to analysis of the proposed quality controls: Low QC, Mid QC and High QC (Table 2).

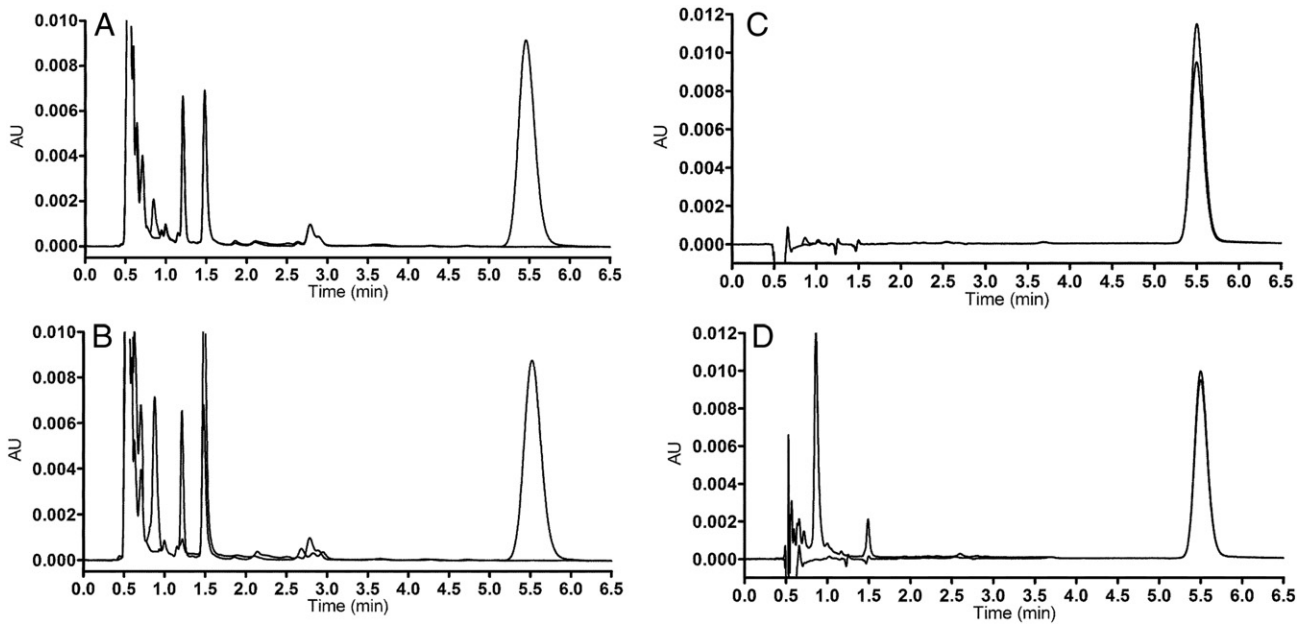


Fig. 1. Representative chromatograms of ceftriaxone at retention time of 0.87 minutes and I.S. at retention time of 5.50 minutes. (A) Blank plasma plus ceftriaxone in the lower limit of quantitation. (B) Blank plasma plus animal plasma sample at time 10 hours after ceftriaxone administration. (C) Zero peritoneal fluid plus lower limit of quantitation. (D) Zero peritoneal fluid plus peritoneal fluid sample at 8 hours after ceftriaxone administration.

Table 1
Precision and accuracy of calibration curve in horse plasma and peritoneal fluid plasma (n = 3).

Plasma			Peritoneal fluid		
Concentration (µg/mL)	Precision (%)	Accuracy (%)	Concentration (µg/mL)	Precision (%)	Accuracy (%)
-	-	-	0.24	3.41	104.03
0.49	7.20	102.15	0.49	10.05	97.35
0.98	1.12	100.03	0.98	9.98	94.66
1.95	6.20	95.52	1.95	3.08	102.53
7.81	5.22	95.35	7.81	10.34	105.41
31.3	0.70	102.59	31.3	6.21	98.78
125	6.44	106.83	125	6.43	96.57
500	3.62	103.91	500	5.29	104.04

The lower limit of quantitation (LLOQ) was established as 0.49 µg/ml and 0.24 µg/ml for plasma and peritoneal fluid, respectively, based on the aforementioned criteria of precision and accuracy (Table 2) and their signal-to-noise ratio were higher than 5-fold. Our LLOQ is lower compared to other published methods for horse plasma (Ringger et al., 1996, 1998) and it is fully validated. Furthermore, it has better quantitation limit of several published HPLC methods for human plasma (Gergs et al., 2014; Mcwhinney et al., 2010; Page-Sharp et al., 2016; Schleibinger et al., 2015; Verdier et al., 2011; Zhao et al., 2014). Once the quantitation of ceftriaxone in peritoneal fluid can be performed through this method, that is fully validated, this is the first method to

Table 2
Precision (expressed as R.S.D.) and accuracy of quality controls of ceftriaxone plasma and peritoneal fluid.

Ceftriaxone Statistics		Plasma concentration µg/mL				Peritoneal fluid concentration µg/mL			
		0.49	0.98	31.3	400	0.24	0.49	31.3	400
		LLOQ	Low QC	Mid QC	High QC	LLOQ	Low QC	Mid QC	High QC
Intra-run	n	5	5	5	5	5	5	5	5
	Precision (%)	5.55	1.67	8.29	3.56	11.03	8.48	11.98	8.93
	Accuracy (%)	115.31	102.89	97.92	94.83	99.03	101.02	89.00	110.79
Inter-run	n	15	15	15	15	15	15	15	15
	Precision (%)	7.46	3.90	8.60	6.50	6.69	1.66	7.63	9.64
	Accuracy (%)	108.50	104.69	93.45	94.30	99.58	104.41	96.26	110.04

quantitate ceftriaxone in this biological matrix, which is a very important place of ceftriaxone treatment for bacterial peritonitis.

The recovery test also showed the acceptance criteria for precision and accuracy (Table 3), demonstrating that the method of processing the sample was adequate for the analysis of ceftriaxone in plasma and peritoneal fluid.

The stability assays demonstrated that prepared ceftriaxone samples were stable for 4 hours inside the auto sampler at 10 °C, and the matrix sample was stable 18 hours over the bench at room temperature, and for 14 days at -80 °C. Moreover, ceftriaxone was stable after three freeze and thaw cycles with a 24 hour interval for each thawing in both matrices (Table 4). Those evaluations are crucial, since they ensure the integrity of the drug after all storage conditions that may be necessary between the sampling and analysis.

3.2. Application to a pharmacokinetic profile study

Fig. 2 shows the plasma and peritoneal fluid concentration-time curves for ceftriaxone administration in two horses based on the validated bioanalytical method. The resulting concentrations allowed construction of the profiles of plasma and peritoneal fluid concentration versus time profiles of ceftriaxone. The half-life was 2.2 h for the gelding and 3.1 h for the mare. This may be explained by the difference between their volume of distribution, the results for which were 64 L and 97 L, respectively. The clearances were 20.2 L/h and 21.8 L/h for the gelding and the mare, respectively. Based on their weight, the extraction rate was

Table 3
Recoveries at low and high QC levels for ceftriaxone in plasma and peritoneal fluid.

QC level	Statistics	Plasma	Peritoneal fluid
Low QC	R.S.D. (%)*	4.5/9.9	4.5/17.9
	Recovery (%)	107.5	108.1
High QC	R.S.D. (%)	5.0/6.2	5.0/13.2
	Recovery (%)	99.3	98.4
Total	Recovery (%)	103.4	103.25

* R.S.D. (relative standard deviation); R.S.D. of three replicates of processed QC samples/
R.S.D. of three replicates of unprocessed QC samples.

calculated as 0.016 and 0.017, respectively, which means that the observed clearance is very low (Toutain and Bousquet-Mélou, 2004). Therefore, the clearance is not responsible for the longer half-life in the mare. The mean residence time was 3.7 h for the gelding and 4.7 h for the mare, probably reflecting their different half-lives. It is certain that a higher number of subjects would be necessary to better characterize these parameters. However, the objective of this study was to demonstrate the application of the bioanalytical method for the determination of ceftriaxone in both intended matrices.

4. Conclusion

As a result of this work, we developed and validated a bioanalytical method for the determination of ceftriaxone in plasma and peritoneal fluid. The method, which was applied in a pharmacokinetic profile study of ceftriaxone administered intraperitoneally in horses, demonstrated adequate sensitivity. Moreover, when compared with the published methods in the literature, most of which are for human data, our method showed certain advantages. These advantages are mainly about the ceftriaxone quantitation in peritoneal fluid, which were not found in the current survey, and the improved sensitivity, as discussed above. Finally, this is the first full validated bioanalytical method for ceftriaxone quantitation in plasma and peritoneal fluid using ultra-high-performance liquid chromatography.

Declarations

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Table 4
Stability of ceftriaxone in plasma and peritoneal fluid under several conditions and time intervals.

Statistics	Plasma		Peritoneal fluid	
	Low QC	High QC	Low QC	High QC
Short-term stability, 18 h at room temperature				
N	3	3	3	3
R.S.D.* (%)	5.2	3.0	6.2	8.2
Accuracy (%)	93.78	99.99	102.65	112.06
Freeze and thaw cycles stability, 3 cycles h at –80 °C				
N	3	3	3	3
R.S.D.* (%)	2.5	8.0	1.3	13.8
Accuracy (%)	98.67	94.08	99.18	102.39
Long-term stability, 14 days at –80 °C				
N	3	3	3	3
R.S.D.* (%)	5.7	10.7	2.9	14.7
Accuracy (%)	96.22	102.22	108.50	102.36
Post-preparative stability, 4 h at 10 °C				
N	3	3	3	3
R.S.D.* (%)	3.1	5.2	4.0	8.8
Accuracy (%)	99.62	97.84	92.45	101.87

* Relative standard deviation.

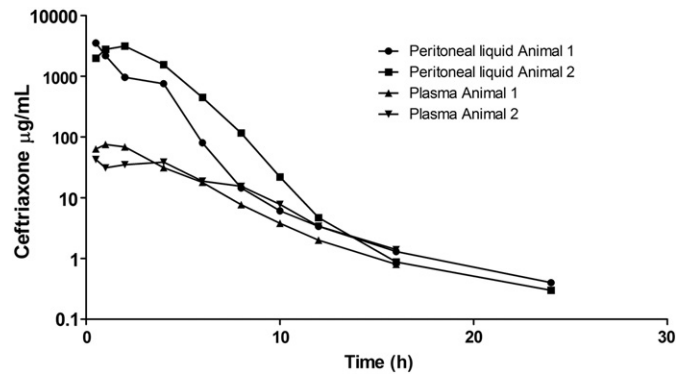


Fig. 2. Pharmacokinetic profile after single intraperitoneal dose of ceftriaxone in two horses (dose = 25 mg/kg).

fundors had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Competing Interests: The authors confirm that this article content has no conflict of interest.

Ethical Approval: The protocol for animal use was approved by the Research Ethics Committee of the UNESP - College of Veterinary Medicine and Animal Sciences (CEUA process 105/2013).

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