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Short communication

Liquid chromatographic method for the simultaneous determination of cefalexin and trimethoprim in dog plasma and application to the pharmacokinetic studies of a coformulated preparation

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Abstract

A liquid chromatographic method is described for the simultaneous determination of cefalexin and trimethoprim in dog plasma. A simple protein precipitation procedure was adopted for the sample preparation with satisfactory extraction recoveries for both analytes. Chromatographic separation of the analytes was achieved on a C_{18} column using a mixture of 2 mol/l formate buffer (pH 3.5), methanol and acetonitrile (22:7:7, v/v/v) containing a 0.002 mol/l sodium dodecyl sulfate as mobile phase and detection was performed at 240 nm. The linearity was obtained over the concentration ranges of 1.0–100.0 μg/ml for cefalexin and 0.5–50.0 μg/ml for trimethoprim. For each level of QC samples including the lower limit of quantification, both inter- and intra-day precisions (R.S.D.) were ≤14.0% for cefalexin and ≤11.4% for trimethoprim, and accuracy (RE) was −1.4% for cefalexin and −3.0% for trimethoprim. The present LC method was successfully applied to the pharmacokinetic studies of coformulated cefalexin dispersible tablets after oral administration to beagle dogs.

Keywords: Cefalexin; Trimethoprim; Liquid chromatography; Dog plasma

1. Introduction

Cefalexin and trimethoprim have been individually used in clinical trials for the treatment of respiratory, urinary tract and other infections over several decades [1–3]. It was found that the combination of cefalexin and trimethoprim (5:1) exhibited synergistic action against various clinical isolates and could expand antibacterial spectrum and enhance the antibacterial activity [4]. The results showed that cefalexin–trimethoprim (5:1) could enhance the antibacterial activities 2–32 times against *Serratia* spp., *Citrobacter freunddi*, *Proteus vulgaris*, *Protues rnirabilis*, *Morganella morganii* and *Haemophilus influenzae*. The combination could improve the antibacterial activities against some resistant-*Enterobacters* and MRSA, decreasing MIC_{50} of *Enterobacter aerogenes* from 256 to 8 mg/l, and from 256 to 2 mg/l, respectively. The synergistic rates were from 92.3 to 56.3%.

For the convenience of clinical uses, a combination tablet formulation containing 125 mg of cefalexin and 25 mg of trimethoprim each tablet, namely coformulated cefalexin dispersible tablets, was developed in our laboratory. On the basis of the clinical daily dosage intervals of the individual drugs, i.e., four times for cefalexin and twice for trimethoprim, dosage interval of every 5 h for the coformulated tablets was designed in order to keep a steady concentration ratio of the two drugs in plasma within a proper range. To demonstrate whether the dosage interval was reasonable or not, pharmacokinetic studies in dogs should be performed and therefore a simple and rapid analytical method for the simultaneous determination of cefalexin and trimethoprim in dog plasma was required.

Several liquid chromatographic methods (LC) are available for the determination of cefalexin [5,6] or trimethoprim [7–12] in biological samples with UV [5–7,9–12] and/or MS [8] detection (and references cited therein). To the best of our knowledge, no LC method for the simultaneous determination of cefalexin and trimethoprim in biological samples has been published. On the basis of the structures of the analytes of interest, it can be found that it was a tough task to achieve the purpose because of

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the great differences of the analytes in physico-chemical properties, i.e., acid–base property and solubility, which brought us a big challenge for both extraction and chromatographic determination of the two drugs in dog plasma.

The present study describes a simple LC method for the simultaneous determination of cefalexin and trimethoprim in dog plasma. The present method provides a simple sample preparation with satisfactory extraction recoveries for both analytes and satisfactory chromatographic separation with a reasonable run-time, good precision and accuracy. This method has been applied to the single- and multiple-dose pharmacokinetic studies of the coformulated formulation in beagle dogs.

2. Experimental

2.1. Reagents and chemicals

Cefalexin reference standard (99.3% purity) was from Shandong Xinhua Pharmaceutical Company, Ltd. (Zhibo, China), trimethoprim reference standard (99.4% purity) was from the Sixth Pharmaceutical Factory of Northeast (Shenyang, China) and norfloxacin reference standard (99.7% purity) was from Funing Pharmaceutical Factory (Shenyang, China). Coformulated cefalexin dispersible tablets (label claim: 125 mg cefalexin and 25 mg trimethoprim) were from Shenyang Pharmtech Institute of Pharmaceuticals (Shenyang, China). Cefalexin capsules (label claim: 125 mg) and trimethoprim capsules (label claim: 25 mg) containing only the indicated drug substance without any pharmaceutical excipients were prepared in our laboratory and used as the reference preparations for the pharmacokinetic studies. Blank dog plasma was from the Laboratory Animal Center in Shenyang Pharmaceutical University. Methanol and acetonitrile of HPLC grade were from Tianjin Concord Science and Technology Company, Ltd. (Tianjin, China). Sodium dodecyl sulfate (SDS) and formic acid were analytical-grade chemicals from Shenyang Reagent Company (Shenyang, China).

2.2. Instrumentation

Chromatographic separation was performed with an HP 1100 chromatographic system equipped with a G1310A isocratic pump, a G1314A variable UV–vis detector (Agilent, USA), a 7725i manual injector with a 20 μl loop (Rheodyne, USA). ChemStation A.09.03 (Agilent, USA) was used for data processing and collecting. A Shimadzu UV-2201 double-beam spectrophotometer (Shimadzu, Japan) was used for scanning and selecting the detection wavelength.

2.3. Preparation of calibration standards and quality control samples

A mixed stock solution containing 1 mg/ml cefalexin and 0.5 mg/ml trimethoprim was prepared in mobile phase. A standard solution of norfloxacin at 2 mg/ml (internal standard) was prepared in mobile phase. The two solutions were found to be stable for at least 3 days at −4 °C. Mixed calibration standards containing cefalexin at the levels of 1.0, 2.0, 10.0, 20.0, 50.0 and 100.0 μg/ml and trimethoprim at the levels of 0.5, 1.0, 5.0, 10.0, 25.0 and 50.0 μg/ml were prepared by spiking appropriate amount of the mixed stock or diluted solutions of both analytes in blank dog plasma. Quality control (QC) samples were prepared at low, medium and high levels of cefalexin (1.0, 20.0 and 100.0 μg/ml) and trimethoprim (0.5, 10.0 and 50.0 μg/ml) using the pooled blank plasma. The spiked samples were then treated following the sample preparation procedure as indicated in Section 2.4.

2.4. Sample preparation

Aliquots of dog plasma (200 μl) were placed in microcentrifuge tubes, 50 μl of norfloxacin solution (2 mg/ml) and 200 μl of mobile phase were added and briefly vortexed. Acetonitrile (200 μl) were added and vortexed for 1 min and then centrifuged at 2000 × *g* for 10 min at room temperature. The supernatant was transferred to HPLC vials and capped. A 20 μl aliquot was injected onto the LC system for analysis.

2.5. Chromatographic conditions

An analytical column, Diamonsil™ C_{18} column (250 mm × 4.6 mm, 5 μm) from Dikma Technologies (Beijing, China) and a DL-II type guard column packed with YWG-C_{18} (10 mm × 4.0 mm, 10 μm) from Tianjin Chromatographic Science and Technology Company (Tianjin, China) were used for chromatographic separation. Mobile phase consisted of a mixture of 2 mol/l sodium formate buffer (pH 3.5)–methanol–acetonitrile (22:7:7, v/v/v) containing 0.002 mol/l SDS at a flow rate of 1.3 ml/min. The detection was made at 240 nm. Chromatography was performed at room temperature and the run time was less than 15 min.

2.6. Method validation

Validation runs were conducted on 3 separate days. Each validation run consisted of a set of calibration standards at six concentrations over the concentration range (each in triplicate) and QC samples at three concentrations ($n = 6$ at each concentration). The concentrations of the analytes in plasma samples were determined by back-calculation of the observed peak area ratios of the individual analytes and internal standard from the best-fit calibration curve. During routine analysis, each analytical run included a set of calibration standards, a set of QC samples in duplicate and plasma samples to be determined.

Selectivity was investigated by comparing chromatograms of blank plasma, calibration standard plasma sample spiked with cefalexin (1.0 μg/ml), trimethoprim (0.5 μg/ml) and norfloxacin (2 mg/ml, I.S.), and plasma sample at 5.5 h after an oral dose of two coformulated cefalexin dispersible tablets to a beagle dog at a steady plasma concentration state.

Precision and accuracy were determined by the analysis of low, medium and high QC samples and were calculated by analysis of variance. Relative standard deviation (R.S.D.) were calculated from the QC values and used for the estimation of intra- and inter-day precision. Relative error was used for the

evaluation of the accuracy and expressed as the percent deviation between the mean concentration relative to the nominal concentration.

The linearity of each calibration curve was determined by plotting the peak-area ratio (y) of the analytes to internal standard versus the nominal concentration (x) of the analytes. The calibration curves were obtained by weighted ($1/x^2$) linear regression analysis.

The extraction recoveries of the analytes were determined at low, medium and high concentrations (1.0, 20.0 and 100.0 μg/ml for cefalexin and 0.5, 10.0 and 50.0 μg/ml for trimethoprim) by comparing the responses from plasma samples spiked before extraction with the corresponding standard solutions in mobile phase without extraction.

The stability of the processed samples was determined by repeated analysis of QC samples with the analytes at high, medium and low levels after extraction and exposure to ambient temperature over a time period of 12 h.

2.7. Application to the pharmacokinetic studies

The present LC method was successfully applied to the single- and multiple-dose pharmacokinetic studies of coformulated cefalexin dispersible tablets in beagle dogs.

For single-dose pharmacokinetic studies, six beagle dogs (13.7 ± 0.4 kg) were used from the Laboratory Animal Center in Shenyang Pharmaceutical University. This study was based on a single-dose, randomized, three-treatment, three-period crossover design. In the morning of phase I, after an overnight fast (12 h), six dogs were given single dose of either two coformulated cefalexin dispersible tablets (test preparation) or two cefalexin capsules (reference preparation) or two trimethoprim capsules (reference preparation). No other food was allowed until 4 h after dose administration while water intake was free. About 1.5 ml of blood samples were collected from the foreleg vein into heparinized test tubes before (0 h) and at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 5.0, 7.0 and 12.0 h after dosing. Plasma was separated by centrifugation at $2000 \times g$ for 10 min at room temperature and kept frozen at −20 °C until analysis. After a washout period of 7 days, the study was repeated in the same manner to complete the crossover design.

For multiple-dose pharmacokinetic studies, eight beagle dogs (13.9 ± 0.5 kg) from the same source were used after an overnight fast (12 h). Two coformulated cefalexin dispersible tablets were administered at 7:00, 12:00, 17:00 and 22:00 for a total of 5 days. On Day 5 multiple blood samples were collected before dosing and at 0.5, 1.0, 2.0, 3.0, 5.0, 5.5, 6.0, 7.0, 8.0, 10.0, 10.5, 11.0, 12.0, 13.0 and 15.0 h after dosing. Plasma was separated by centrifugation at $2000 \times g$ for 10 min at room temperature and kept frozen at −20 °C until analysis.

3. Results and discussion

3.1. Method development

The present LC method was developed for the pharmacokinetic studies of the coformulated cefalexin dispersible tablets in dogs. The primary challenge for the purpose was the great differences of the two drugs in physico-chemical properties, which presented great difficulty in both extraction and chromatographic separation of the analytes of interest. Cefalexin is an amphiprotic compound with a carboxyl group and primary amine group and exhibits a stronger acid property while trimethoprim is a weakly base. In the initial period of the study, based on the extraction procedure in the references, liquid–liquid extraction and solid-phase extraction were tried but failed to co-extract the analytes satisfactorily from the dog plasma. After that, a protein precipitation procedure with perchloric acid, methanol or acetonitrile was tested, and finally a protein precipitation with acetonitrile as protein-precipitating solvent was found to be suitable for the purpose. In the procedure, the supernatant after centrifugation was directly injected onto the LC system, which simplified the process and minimized the possible loss of the analytes. The present procedure for the sample preparation produced a clean chromatogram for a blank plasma sample and yielded satisfactory recoveries for the analytes from the dog plasma.

For the chromatographic conditions, a C_{18} column (250 mm × 4.6 mm I.D., 5 μm) was used for the chromatographic separation because of its wide availability and applicability in ordinary laboratories. Besides, other chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes of analytes as well as short run time. Taking into account the great difference of the analytes in acid–base property and solubility, a mixture of a weakly acidic formate buffer, methanol and acetonitrile containing a low level of SDS as an ion-pair reagent was tested to find the optimum composition and SDS level. After a series of adjustment and trials, a mixture of 2 mol/l sodium formate buffer (pH 3.5), methanol and acetonitrile (22:7:7, v/v/v) containing a 0.002 mol/l SDS was found to be optimal for the purpose and finally adopted as the mobile phase for the chromatographic separation. To find a proper internal standard for the present work, several compounds were tried and norfloxacin was finally found to be suitable for our work.

3.2. Selectivity

The results for selectivity are shown in Fig. 1. The retention times were approximately 5.3 min for cefalexin, 10.5 min for trimethoprim and 13.0 min for norfloxacin. Fig. 1 indicates no significant interferences from endogenous substances in plasma with the analytes and internal standard.

3.3. Linearity

To evaluate the linearity of the LC method, calibration curves were determined in triplicate on 3 separate days. Representative regression equations for the calibration curve were $y = 7.68 \times 10^{-3}$ ($\pm 2.91 \times 10^{-4}$)$x + 6.41 \times 10^{-4}$ ($\pm 3.03 \times 10^{-3}$) ($r = 0.9975$) for cefalexin and $y = 2.58 \times 10^{-2}$ ($\pm 9.47 \times 10^{-3}$)$x - 4.34 \times 10^{-3}$ ($\pm 3.10 \times 10^{-3}$) ($r = 0.9996$) for trimethoprim. Satisfactory linearity was observed over the

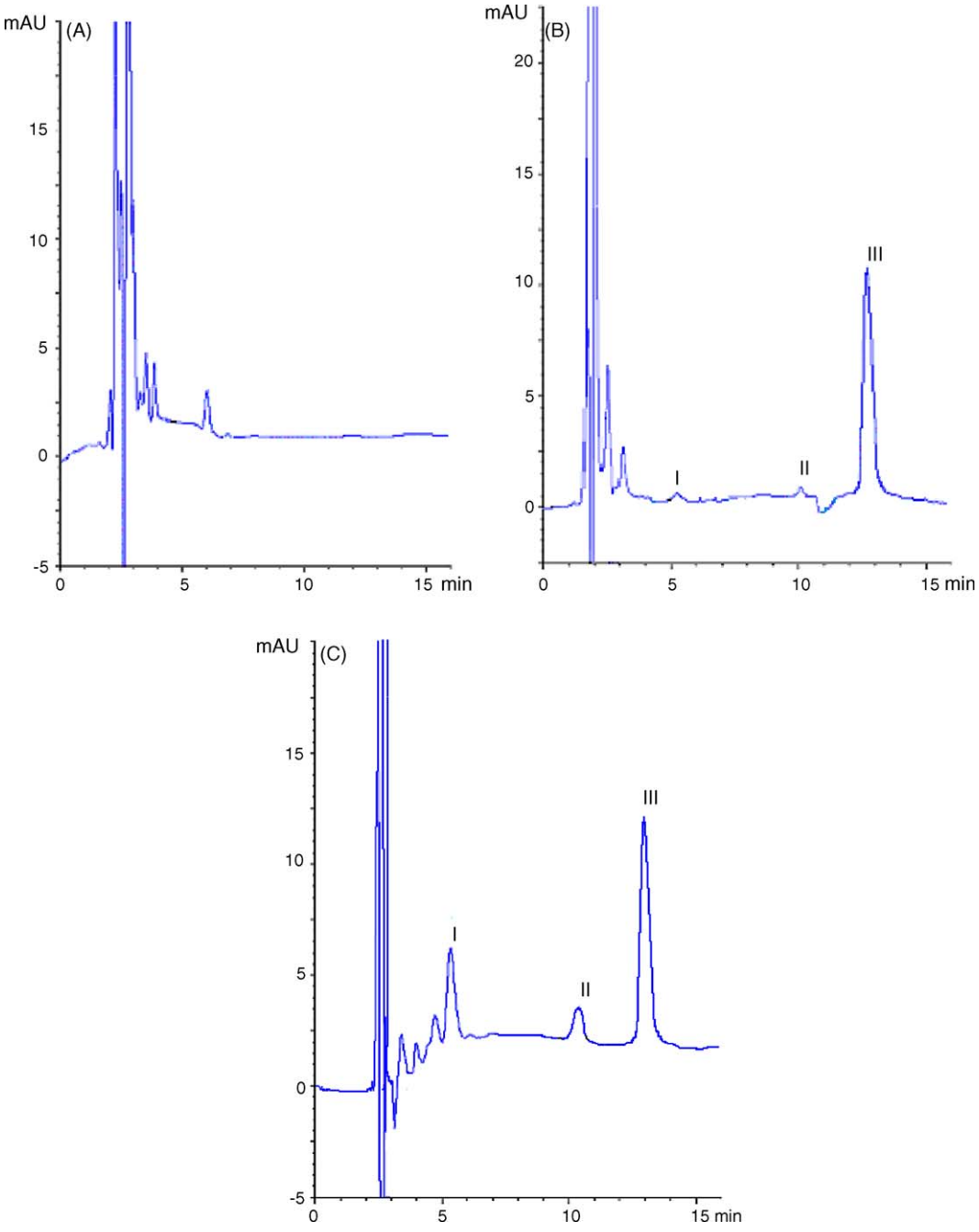

Fig. 1. Representative liquid chromatograms of: (A) blank dog plasma sample; (B) blank plasma sample spiked with cefalexin (1.0 μg/ml), trimethoprim (0.5 μg/ml) and norfloxacin (2 mg/ml); (C) plasma sample of 16.0 μg/ml cefalexin and 3.5 μg/ml trimethoprim at 5.5 h after an oral dose of two coformulated cefalexin dispersible tablets to a beagle dog at a steady state. Peaks: I—cefalexin (t_R = 5.3 min), II—trimethoprim (t_R = 10.5 min), III—norfloxacin (t_R = 13.0 min).

concentration ranges of 1.0–100.0 μg/ml for cefalexin and 0.5–50.0 μg/ml for trimethoprim.

3.4. Lower limit of quantitation

The lower limit of quantitation (LLOQ) was defined as the lowest concentration having a signal to noise ratio of at least 10:1 and acceptable precision and accuracy within 20%. LLOQ was found to be 1.0 μg/ml for cefalexin and 0.5 μg/ml for trimethoprim. The chromatogram and precision and accuracy at LLOQ levels of the analytes are shown in Fig. 1B and Table 1, respectively.

3.5. Precision and accuracy

The results for the accuracy and intra- and inter-day precision of the method are shown in Table 1. As shown in Table 1, for each QC level (including LLOQ) of the analytes, the R.S.D. values for the inter- and intra-day precisions were ≤14.0% for cefalexin and ≤11.4% for trimethoprim, and the relative error for

Table 1
Accuracy and precision for the determination of cephalexin and trimethoprim in dog plasma ($n = 3$ days, six replicates per day)

Drugs	Nominal concentration (μg/ml)	Found concentration (μg/ml)	Intra-day R.S.D.[a] (%)	Inter-day R.S.D. (%)	Relative error (%)
Cephalexin	1.00	1.01	6.3	14.0	1.0
	20.00	19.80	6.1	12.0	−1.0
	100.0	98.60	4.4	12.4	−1.4
Trimethoprim	0.50	0.51	5.1	6.4	2.0
	10.00	9.70	7.9	11.4	−3.0
	50.00	49.50	5.5	10.1	−1.0

Relative error (%) = 100 × (mean concentration − nominal concentration)/nominal concentration.
[a] Relative standard deviation.

accuracy was −1.4% for cefalexin and −3.0% for trimethoprim, indicating the acceptable accuracy and precision of the method developed.

3.6. Extraction recovery

The results of extraction recovery are shown in Table 2. For the high, medium and low (LLOQ) levels of both analytes, the extraction recoveries ranged from 82.7 to 92.9%.

3.7. Stability

The stability of cefalexin and trimethoprim in the supernatant after protein precipitation was determined. Both analytes were found to be stable for at least 12 h after sample preparation at ambient temperature within accuracy of 11.0% at three levels of QC samples.

3.8. Application to the pharmacokinetic studies

The mean plasma concentration–time curves for the single-dose and multiple-dose pharmacokinetic studies of coformulated cefalexin dispersible tablets following oral administration to beagle dogs are shown in Figs. 2 and 3, respectively. Fig. 2 shows the comparable pharmacokinetic behavior between the coformulated formulation and the individual drug formulations in terms of the maximum concentration (C_{max}), half-life ($t_{1/2}$) and the area under the curve (AUC_{0-t}). For cefalexin, Cmax, $t_{1/2}$ and AUC_{0-t} are 18.97 μg/ml, 2.04 h and 64.12 μg h/ml for the test formulation and 15.55 μg/ml, 2.11 h and 56.06 μg h/ml for the reference formulation. For trimethoprim, the correspond-

Table 2
Extraction recoveries of cephalexin and trimethoprim in dog plasma ($n = 3$)

Analytes	Spiked concentration (μg/ml)	Recovery (%)	R.S.D.[a] (%)
Cephalexin	1.0	85.1 ± 7.2	8.5
	20.0	82.7 ± 1.5	1.8
	100.0	83.6 ± 4.8	5.7
Trimethoprim	0.5	89.9 ± 2.8	3.1
	10.0	88.3 ± 6.5	7.4
	50.0	92.9 ± 5.5	5.9

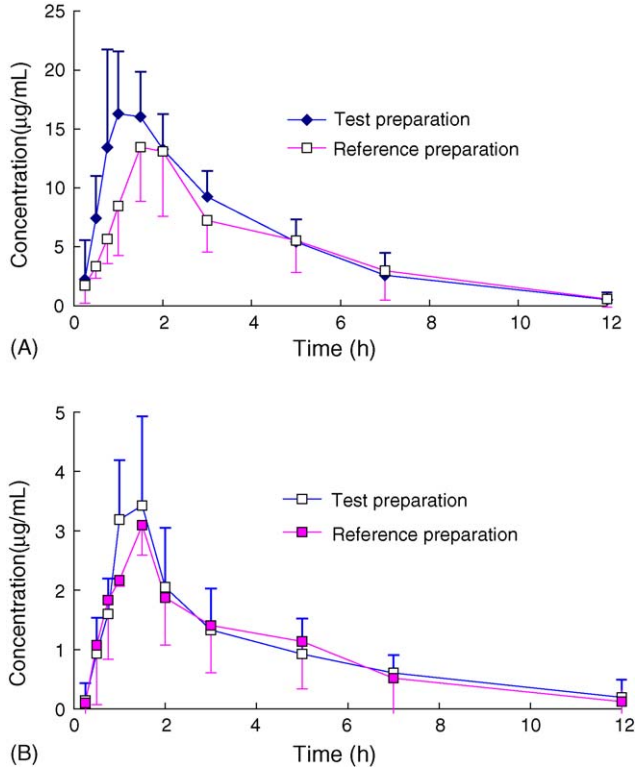

Fig. 2. Mean plasma concentration–time curves of cefalexin (A) and trimethoprim (B) after single-dose oral administration of two coformulated cefalexin dispersible tablets (test preparation) and two cefalexin capsules (reference preparation) and two trimethoprim capsules (reference preparation) to six beagle dogs.

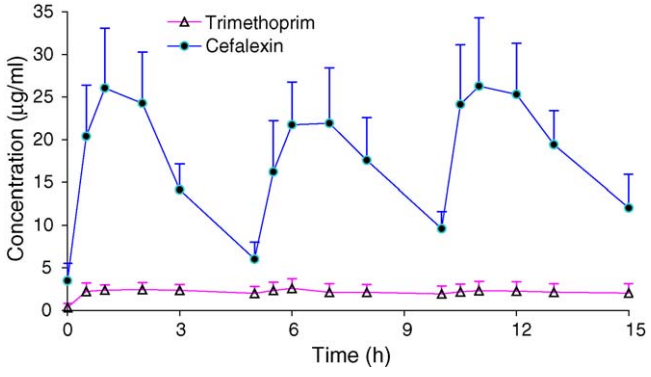

Fig. 3. Mean steady plasma concentration-time curves of cefalexin and trimethoprim after multiple-dose oral administration of two coformulated cefalexin dispersible tablets to eight beagle dogs.

ing parameters are 3.77 μg/ml, 5.27 h and 11.30 μg h/ml for the test formulation and 3.82 μg/ml, 5.03 h and 10.18 μg h/ml for the reference formulation. Fig. 3 indicates the reasonable concentration ratio of cefalexin to trimethoprim in steady-state dog plasma ranging from 4 to 16. The obtained results provide the key bases for the further development of the coformulated preparation.

4. Conclusion

A simple liquid chromatographic method was developed for the simultaneous determination of cefalexin and trimethoprim in dog plasma. To the best of our knowledge, the present study is the first report for the purpose. For the analytes of great differences in acid–base property and solubility, the present method succeeded in adopting a simple sample preparation that achieved satisfactory extraction recovery and facilitated its application in the pharmacokinetic studies of the indicated coformulated formulation. It also provided a satisfactory chromatographic determination of the analytes with acceptable selectivity, precision and accuracy, and a reasonable run-time. It has been successfully used for the pharmacokinetic studies of the coformulated cefalexin dispersible tablets.

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