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LC-MS/MS method for the simultaneous estimation of Cefepime and Tazobactam in dog plasma

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A high performance liquid chromatography (HPLC) method with tandem mass spectrometric detection (MS/MS) has been developed and validated for the simultaneous quantification of cefepime and tazobactam in dog plasma. The method was developed on amide column with isocratic elution. The developed method is simple and economic in terms of sample preparation. The method is specific, sensitive, accurate, precise and robust. The method was successfully applied for pre-clinical pharmacokinetic studies in dogs. The Tmax was found to be 0.5 h, the mean Cmax and $AUC_{(0-12)}$ displayed dose proportionate response.

Keywords: Cefepime, Tazobactam, Liquid chromatography, Mass spectrometry, Bioanalysis

Introduction

Cefepime is chemically ([6R,7R,Z]-7-[2-(2-aminothiazol-4-yl)-2- (methoxyimino) acetamido]-3-[1-methyl pyrrolidinium-1yl] methyl)-8-oxo-5-thia-1-aza-bicyclo[4.2.0] oct-2-ene-2 carboxylate. Cefepime is a fourth-generation cephalosporin antibiotic. Cefepime has an extended spectrum of activity against Gram-positive and Gram-negative bacteria, with greater activity against both types of organism than third-generation agents. Tazobactam is chemically (2S,3S,5R)-3-methyl-7-oxo-3-(1H-1,2,3-triazol-1-ylmethyl)-4-thia-1 azabicyclo [3.2.0] heptane-2-carboxylic acid 4,4-dioxide. Tazobactam is an active pharmaceutical ingredient that inhibits the action of bacterial beta-lactamases, especially those belonging to the SHV-1 and TEM groups. It is used as its sodium salt, tazobactam sodium. Tazobactam is in combination with the extended spectrum β-lactam antibiotic piperacillin in the drug piperacillin/tazobactam, one of the popular antibiotic treatment for nosocomial pneumonia caused by Pseudomonas aeruginosa. Tazobactam broadens the spectrum of piperacillin by making it effective against organisms that express β-lactamase and would normally degrade piperacillin (1).

Cefepime - tazobactam combination indicates as a parenteral therapy for the treatment of moderate to harsh infections due to susceptible beta-lactamase producing microbial organisms. Cefepime - tazobactam combination is mainly pointed if the cefepime treatment is not effective. Cefepime - combination is arranged for the treatment of basic skin and skin structure infections, urinary tract infections (UTI) and difficult intra-abdominal infections in adults as well as children. General use of 3rd generation cephalosporins and piperacillin-tazobactam have led to an increase in strains with multiple extended-spectrum β-lactamases (ESBLs) and Class C β-lactamases that have upper minimum inhibitory concentrations (MIC) for piperacillin-tazobactam and high-stage resistance to 3rd and 4th generation cephalosporins. Under this situation, carbapenems are generally used as empiric remedy for the cure of Gram-negative infections. This has given increase to multiple mechanisms of carbapenem resistance in Enterobacteriaceae and *P. aeruginosa* leading to the use of compromised therapies – colistin and tigecycline. Cefepime and tazobactam have approximately 20 years record of medical use. Both the agents have every time confirmed satisfactory safety and effectiveness in various indications. Taking in to account that the combination safety report would remain comparable to the individual components, cefepime-tazobactam combination would give a considerably positive benefit: risk proportion for the treatment of UTI and other indications involving certain multidrug-resistant (MDR) Gram-negative pathogens, agreeable only to carbapenems, thereby minimizing the therapeutic-belief on carbapenems. Looking at the safety and combined effective advantage of these two agents, a high proportion cefepime-tazobactam

combination is currently under clinical development. This combination is being developed to treat the complicated urinary-tract infection (cUTI) [including pyelonephritis] and hospital-acquired bacterial pneumonia (HABP) / ventilator-associated bacterial pneumonia (VABP).

A literature survey shown that several chromatographic methods are reported for the determination of cefepime alone or in combination with other drugs in formulation as well as in different biological matrices (2-17). Also several liquid chromatographic methods are reported for the determination of tazobactam and in combination with other drugs in formulation as well as in different biological matrices (18-29). Few methods are reported for simultaneous determination of cefepime and tazobactam in various dosage forms but none is reported in biological matrix (30-34). The objective of this study was to develop and validate a bio-analytical method for simultaneous determination of cefepime and tazobactam in beagle dog plasma. The developed method would be then applied for the pre-clinical study samples for the assessment of pharmacokinetics of these important drugs in dogs when administered by intravenous route in various ratios.

Material and methods

Chemicals and standards

Cefepime for injection and tazobactam sodium were obtained from Aurobindo Pharma Limited, India. Commercially available Cefotaxime sodium injection (Alkem Laboratories, India) was used. The chemical structures for these compounds are shown in Figure 1. All the solvents and reagents used were of either HPLC or LC-MS grade. Acetonitrile (HPLC gradient grade, Rankem), ammonium formate (LC-MS grade, Fluka), formic acid (LC-MS grade, Fluka) were used. Ultra pure water was obtained through Purelab classic (US Filters). Drug free heparinized dog plasma was obtained from healthy beagle dogs.

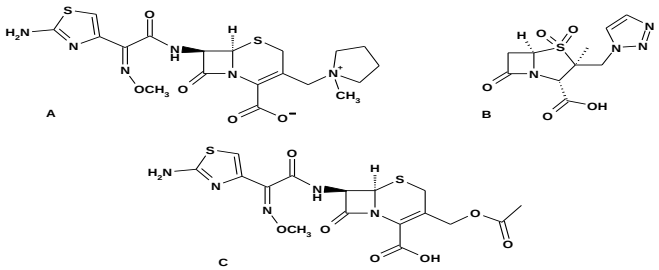

Figure 1. Chemical structures of Cefepime (A), Tazobactam (B) and Cefotaxime (C)

Instrument and conditions

High performance liquid chromatograph (Agilent 1100 series, Agilent Technologies) coupled with triple quadrupole mass spectrometer (API 3000, AB Sciex) was used for conducting bio-analysis. Agilent 1100 series HPLC consisted low pressure quaternary pump, degasser, thermostated auto-injector and thermostated column compartment. API 3000 mass spectrometer consisting of turbo-ion spray source interface was used for MS/MS analysis in positive ion mode.

The LC column used was Unisol Amide, 100 x 4.6 mm, 3 micron (Agela Technologies). The buffer solution was 25 mM ammonium formate in water pH adjusted to 3.2 with formic acid. A mixture of buffer and acetonitrile (25:75 v/v) was used as mobile phase. The flow rate was set at 1 mL/min. Column oven and auto-injector was set at 30°C and 10°C respectively. Injection volume was 3 µL and run time 4.5 minutes.

Standard and sample preparation

Standard solution preparation for calibration standards

Aqueous solutions containing cefepime and tazobactam were prepared in the range of 10 to 2000 µg/mL and were stored at -70°C. These solutions were diluted with drug-free plasma to yield calibration standards containing cefepime and tazobactam in the range of 0.5 to 100 µg/mL.

Internal standard (is) solution preparation

Aqueous solution of Cefotaxime was prepared at 5 µg/mL and stored at -20°C.

Quality control (QC) samples preparation

Aqueous solutions containing cefepime and tazobactam were prepared at 25, 250 and 1500 µg/mL and were stored at -70°C. These solutions were diluted with drug-free plasma to yield samples containing cefepime and tazobactam at 1.25 (LQC), 12.5 (MQC) and 75 (HQC) µg/mL; these QC samples were stored at -70°C.

Plasma bank preparation

In a 1.5 mL centrifuge tube 475 µL of heparinized blank plasma was mixed with 25 µL of water. In another centrifuge tube 50 µL of this mixture was mixed with 750 µL of acetonitrile and 200 µL of water. This mixture was vortexed for 30 seconds and centrifuged for 3 min at 4000 g to remove precipitated proteins. The supernatant was used for analysis.

Plasma blank with IS preparation

Identical to the above procedure except addition of 200 µL of IS solution instead of water.

Study sample preparation

In a 1.5 mL centrifuge tube 50 µL of dog plasma was mixed with 750 µL of acetonitrile and 200 µL of IS solution. This mixture was vortexed for 30 seconds and centrifuged for 3 min at 4000 g to remove precipitated proteins. The supernatant was used for analysis.

Method validation

The method was validated as per the FDA and EMA guidelines on the subject to demonstrate the suitability of the method for intended purpose (35-36). The validation was assessed for selectivity, matrix effect, sensitivity (Lower limit of quantification, LLOQ), linearity, accuracy, precision, recovery, dilution effect, carry over and stability parameters. The stability studies included short term stability (bench top stability), post-preparative stability (auto-injector stability), freeze thaw stability and long term stability.

Selectivity

The selectivity of the method was assessed by analyzing six blank plasma samples. The response of the interfering substances or background noise at the retention time of cefepime and tazobactam are acceptable if they are less than 20% of the response of the lowest concentration calibration standard. The response of the interfering substances or background noise at the retention time of internal standard are acceptable if it is less than 5% of the response of the internal standard in calibration standard.

Matrix effect

The matrix effect is investigated to ensure that precision, selectivity and sensitivity are not compromised by the matrix. Matrix effect was determined by analysing set of six LQC's and HQC's prepared in six different blank matrices. % Accuracy and % CV was determined for both QC's.

Linearity, Precision and Accuracy

Linearity was tested by determining response of ten non-zero standards in the concentration range of 0.5 to 100 µg/mL**.** The acceptance limit of accuracy for each of the back calculated concentrations is $\pm$15% of the respective nominal concentration except at LLOQ, where it must be within $\pm$20% of the nominal concentration.

Precision and accuracy was evaluated by analyzing QC samples against freshly prepared calibration standards. Intra-day and inter-day precision and accuracy was determined by analyzing three sets of QC samples, two sets on single day and a set on another day. Accuracy of at least 67% QC samples must be within $\pm$15% of the respective nominal value except at LLOQ, where it must be within $\pm$20% of the nominal concentration. The accuracy of the 50% of the QC's injected at each level must be within $\pm$15 % of the respective nominal concentration except at LLOQ, where it must be within $\pm$20% of the nominal concentration. The precision (% CV) determined at each QC level must be $\leq$ 15%, except for LLOQ where it must be $\leq$ 20%.

Recovery

The recovery of cefepime and tazobactam was evaluated by preparing set of six samples at each QC concentrations in blank matrix and another set in water. The peak area of analytes and IS from both sets were compared to calculate recovery.

Stability

Stability of cefepime and tazobactam in dog plasma was assessed at three freeze–thaw cycles (-70°C to ambient temperature). Long term stability was evaluated by storing frozen plasma samples containing both analytes at -70°C for 4 weeks. Bench-top stability was assessed for 4 h at room temperature. Post preparative stability was assessed at 10°C for 20 h. All the stability was evaluated using six replicates of the LQC and HQC. The analytes are considered to be stable in the matrix if the accuracy is within $\pm$15% of the nominal concentration and % CV $\leq$ 15%.

PK application

The validated method was applied to determine the drug plasma concentrations from pre-clinical pharmaco-kinetics studies conducted in healthy male adult beagle dogs. The animals were administered the cefepime - tazobactam mixture at dose of 30 and 60 mg/kg each through 0.5 h infusion. Blood was collected in heparin containing tubes at prior to dosing (-0.25h) and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0 and 12 h post administration. The blood samples were stored on ice bath until centrifugation to obtain plasma. Blood samples were centrifuged at 4000 g for 10 min to separate plasma. The plasma aliquots were stored at -70°C until analysis in polypropylene tubes.

Method development

Mass spectrometry parameters of cefepime, tazobactam and IS were optimized by continuously injecting 10 µg/mL solution in to the turboionspray source using syringe pump. The declustering potential, focusing potential and entrance potential were optimized to achieve maximum response of the parent ion of both analytes and IS. Collision energy was adjusted to achieve maximum response of at least one daughter ion for each compound. The optimized MS parameters are presented in Table 1. The daughter ion of cefepime at m/z 396.1 arises due to the loss of N-Methyl pyrrolidine; the daughter ion of cefotaxime (IS) at m/z 324.4 arises due to loss of 2-amino thiazole and methoxy group. In tazobactam fragment m/z 168.1 represents 3-(1*H*-1,2,3-triazol-1-yl)-2-methyl butanoic acid which is formed due to loss of (4*R*)-4-(dioxido-λ^6-sulfanyl)-azetidin-2-one.

Table 1. Mass Spectrometer Parameters.

	Cefepime	**Tazobactam**	**Cefotaxime (IS)**
Ion spray voltage (Volts)	5000	5000	5000
Source temperature (°C)	500	500	500
MRM transition (amu)	481.3 > 396.1	301.2 > 168.1	456.3 > 324.4
Declustering potential (Volts)	24	28	47
Focusing potential (Volts)	195	175	250
Entrance potential (Volts)	10	10	10
Collision energy (Volts)	12	25	23
Collision cell exit potential (Volts)	10	10	10
Dwell time (msec)	300	300	300

During chromatography development, columns of various stationary phases like C18, C8, amide and HILIC were attempted with different mobile phases containing varying proportions of buffer solution and organic solvents. The objective was to separate two analytes and IS peaks devoid of matrix effect. Protein precipitation technique was used as sample preparation procedure due to economic factor. This procedure leads to presence of higher proportion of organic phase in sample to be injected on LC. Amide stationary phase LC column with isocratic elution was found to best suitable for application providing sufficient retention of the analytes and no matrix effect. The mobile phase used was a mixture of 25 mM ammonium formate in water pH 3.2 with formic acid and acetonitrile (25:75; v/v). The higher percentage of organic phase in mobile phase also helped to achieve required mass spectrometer sensitivity. The mobile phase was passed through column at 1 ml/min, the HPLC column was maintained at 30°C, the injection

volume was 3 µL and overall run time was 4.5 min. The samples were maintained at 10°C in auto-injector. Carryover effect was investigated by injecting blank plasma preparation after highest concentration calibration standard; the resultant chromatograms did not reveal any carryover effect. Chromatograms of blank matrix, blank matrix with IS and study sample are shown in Figure 2, 3 and 4.

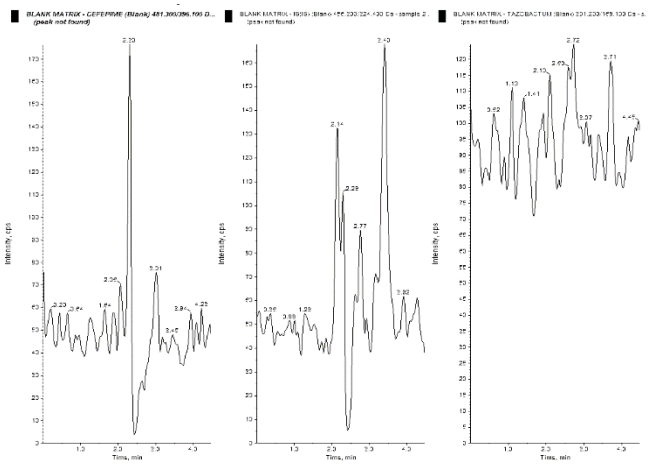

Figure 2. Representative chromatogram of blank matrix.

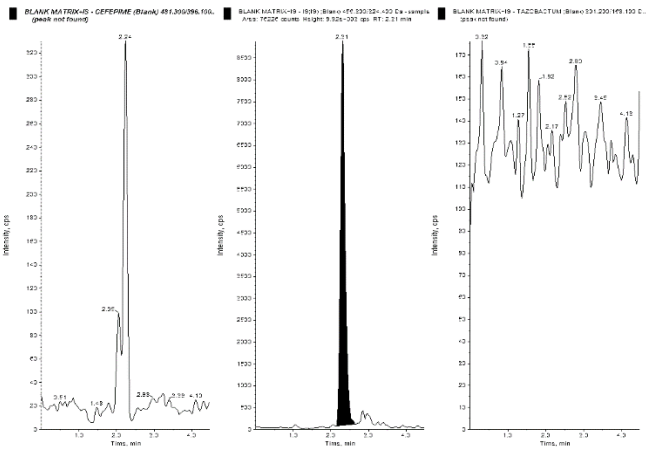

Figure 3. Representative chromatogram of blank matrix with IS.

Cefepime Cefotaxime (IS) Tazobactam

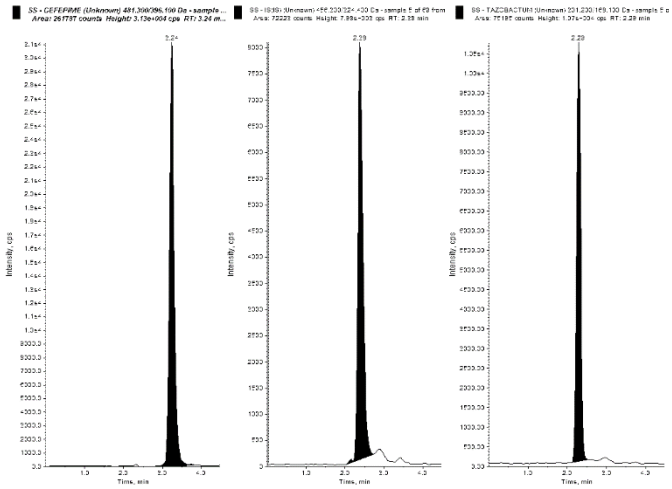

Figure 4. Representative chromatogram of study sample.

Sample preparation

Protein precipitation is a faster, straightforward and inexpensive method for extraction of analytes from biological matrices such as serum, plasma, feces, tissue fluids etc. This is preferred method when sample concentration enrichment is not required. In the present study the doses of both drugs are quite high and hence expected plasma levels were also high. Thus protein precipitation technique was found to be most suitable. Crashing out the proteins was achieved by adding acetonitrile.

Results and discussion

System suitability was carried out at the beginning of each run to verify the reproducibility of the method. The system suitability was verified by injecting 6 replicates of calibration standard (25 µg/mL) and determining the % CV of the response for both analytes. The % CV of the response for cefepime and tazobactam was found to be within the acceptable range (≤10%) in all runs. None of the plasma obtained from 6 different animals showed presence of any interference at the retention time and mass transition of analytes and IS. A set of six LLOQ in drug free matrix was prepared and analyzed against freshly prepared calibration standards to determine % accuracy and % CV The detector response was found to be accurate and precise at 0.50 µg/mL of cefepime and 0.44 µg/mL of tazobactam. The method was found to be sensitive enough to determine the plasma

concentrations during elimination phase. The results are reported in Table 2.

Table 2. LLOQ data.

	Nominal Conc. (µg/mL)	Found Conc. Mean (µg/mL) (n = 6)	Accuracy (%)	Precision (%)
Cefepime	0.50	0.46	92.00	6.15
Tazobactam	0.44	0.45	102.27	7.68

The developed method did not show any ion suppression or enhancement effect as the concentration determined at two levels in six different plasma preparations was found to be accurate and precise. The results are reported in Table 3.

Table 3. Matrix effect.

	Nominal Conc. (µg/mL)	Found Conc. Mean (µg/mL) (n = 6)	Accuracy (%)	Precision (%)
Cefepime	1.25	1.21	96.80	2.81
	75.02	78.26	104.32	3.68
Tazobactam	1.11	1.11	100.00	4.08
	66.67	67.96	101.93	4.07

Linearity, precision and accuracy **(**LPA) was carried out by preparing three batches of calibration standards, LLOQ, LQC, MQC and HQC; two batches were analyzed on single day and third batch on another. A batch contained plasma blank, plasma blank with IS, calibration standards and six replicates each of LLOQ, LQC, MQC and HQC samples.

Correlation coefficient, mean of found concentration, intraday and inter-day % accuracy and % CV were determined. The results are presented in Table 4 and Table 5. Mean absolute recovery of cefepime, tazobactam determined at three concentrations was found to be consistent. Recovery was found to be 114.2±12.4% and 88.1±2.6% for cefepime and tazobactam respectively. Recovery for IS was found to be 87.5%. Cefepime and tazobactam were found to be stable in dog plasma at all the studied conditions. The stability study results are presented in Table 6 and Table 7.

Table 4. Intra and Inter-day LPA data for cefepime.

Analysis	Calibration range (µg/mL)	Correlation coefficient	QC conc. (µg/mL)	Found conc. Mean±SD (µg/mL) (n = 6)	Precision %	Accuracy %
Intra-day	0.5-100.46	Batch-1 0.9979	0.50	0.521±0.05	9.46	104.00
			1.26	1.18±0.09	7.61	93.65
		Batch-2 0.9979	12.56	12.08±1.10	9.10	96.18
			75.34	79.28±8.05	10.15	105.23
Inter-day	0.5-100.46	Batch-3 0.9928	0.50	0.51±0.05	9.72	102.00
			1.26	1.20±0.14	11.32	95.24
			12.56	11.77±1.14	9.66	93.71
			75.34	72.75±8.14	11.19	96.56

Table 5. Intra and Inter-day LPA data for tazobactam.

Analysis	Calibration range (µg/mL)	Correlation coefficient	QC conc. (µg/mL)	Found conc. Mean±SD (µg/mL) (n = 6)	Precision %	Accuracy %
Intra-day	0.44-88.16	Batch-1	0.44	0.47±0.05	10.03	106.82
		0.9995	1.10	1.09±0.06	5.59	99.09
		Batch-2	11.02	10.61±0.49	4.64	96.28
		0.9979	66.12	67.64±3.17	4.68	102.30
Inter-day	0.44-88.16	Batch-3	0.44	0.48±0.04	8.75	109.09
			1.10	1.16±0.11	9.18	105.45
		0.9987	11.02	10.99±0.76	7.00	99.73
			66.12	68.62±3.76	5.48	103.78

Table 6. Stability data of cefepime.

Study	QC Conc. (µg/mL)	Found Conc. Mean (µg/mL) (n = 6	Accuracy (%)	Precision (%)	Stability period
Bench top stability	1.26	1.26	100.00	5.88	4 h
	75.34	74.75	99.22	5.95	
Post preparative stability	1.26	1.35	107.14	3.14	20 h
	75.34	85.13	112.99	5.73	
Freeze thaw stability	1.26	1.10	87.30	5.63	3 cycles
	75.34	69.04	91.64	8.50	
Long term stability	1.26	1.32	104.76	9.97	4 weeks
	75.34	78.75	104.53	7.52	

Table 7. Stability data of tazobactam.

Study	QC Conc. (µg/mL)	Found Conc. Mean (µg/mL) (n = 6)	Accuracy (%)	Precision (%)	Stability period
Bench top stability	1.10	1.10	100.00	4.88	4 h
	66.12	64.99	98.29	2.51	
Post preparative stability	1.10	1.13	102.73	1.82	20 h
	66.12	66.14	100.03	4.24	
Freeze thaw stability	1.10	1.12	101.82	5.50	3 cycles
	66.12	64.17	97.05	8.43	
Long term stability	1.10	1.08	98.18	9.31	4 weeks
	66.12	63.59	96.17	6.77	

Impact of dilution was assessed by preparing six replicates of plasma samples at higher concentration than the linearity range. At the time of analysis these samples were diluted appropriately with drug free plasma to achieve 50 µg/mL and then processed and analyzed. Results are reported in Table 8 and 9. The concentrations of the diluted samples were found to be accurate. The method was successfully developed, validated and applied for bioanalysis of plasma samples of pre-clinical pharmacokinetic studies in beagle dogs. The plasma concentration vs time profile is presented in Figure 5.

Table 8. Effect of dilution on cefepime.

Dilution factor	Conc. (µg/mL)	Found Conc. Mean (µg/mL)	Accuracy (%)
1:9	500.1	52.10	104.18
1:19	1000.2	55.54	111.06
1:39	2000.4	54.72	109.42

Table 9. Effect of dilution on tazobactam.

Dilution factor	Conc. (µg/mL)	Found Conc. Mean (µg/mL)	Accuracy (%)
1:9	444.4	44.53	100.20
1:19	888.8	47.07	105.92
1:39	1777.6	46.31	104.21

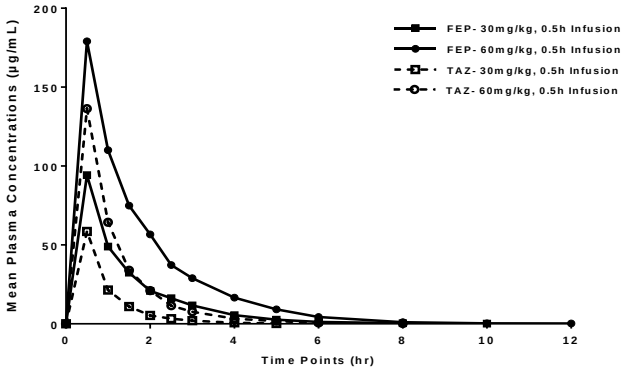

Figure 5. Mean plasma concentration vs time profile for cefepime and tazobactam at 30 and 60 mg/kg, IV in beagle dogs.

Pharmacokinetic parameters were calculated by non-compartmental analysis using Phoenix Winnonlin 6.7 software. The Tmax was observed to be 0.5 h at the end of infusion. The mean Cmax

at 30 and 60 mg/kg for cefepime were 94.25 and 179.0 µg/mL and for tazobactam were 58.52 and 136.40 µg/mL. The mean AUC_{0-12} at 30 and 60 mg/kg for cefepime were 123.4 and 280.4 µg.h/mL and for tazobactam were 49.8 and 140.5 µg.h/mL. Overall dose proportionate linear increase in Cmax and AUC were observed for both drug components.

Conclusion

The described method for simultaneous estimation of cefepime and tazobactam in dog plasma is validated as per FDA and EMA guideline and found to be accurate, precise having required sensitivity. The method is economic due to fast and easy sample preparation as well as small run time. The method is successfully applied for pre-clinical dog PK studies.

Financial and competing interest disclosure:

The study is a part of R&D expenses of Wockhardt Limited. The authors have not financial or other affiliations with any other organization.

Conflict of Interest:

None to declare.

Ethical conduct of research:

The authors state that they have obtained appropriate approvals from local ethics committee for animal experimental investigations.

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