Short communication

The novel sensitive and high throughput determination of cefepime in mouse plasma by SCX-LC/MS/MS method following off-line μElution 96-well solid-phase extraction to support systemic antibiotic programs

Wei Bu *, Holly Sexton, Xiaoqing Fan, Patricia Torres, Paul Houston, Irwin Heyman, Liang Liu

Anacor Pharmaceuticals, 1020 East Meadow Circle, Palo Alto, CA 94303, United States

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ABSTRACT

A sensitive and high throughput off-line μElution 96-well solid-phase extraction (SPE) followed by strong cation exchange (SCX) liquid chromatography with tandem mass spectrometry (LC/MS/MS) quantification for determination of cefepime has been developed and validated in mouse plasma. Using the chemical analog, ceftazidime as an internal standard (IS), the linear range of the method for the determination of cefepime in mouse plasma was 4–2048 ng/mL with the lower limit of quantitation level (LLOQ) of 4 ng/mL. The inter- and intra-assay precision and accuracy of the method were below 9.05% and ranged from 95.6 to 113%, respectively, determined by quality control (QC) samples at five concentration levels including LLOQ. After μElution SPE, 71.1% of cefepime was recovered. The application of the validated assay for the determination of cefepime in mouse pharmacokinetics (PK) samples after intravenous (IV) and subcutaneous (SC) doses was demonstrated.

1. Introduction

Cefepime ((6R,7R,Z)-7-(2-(2-aminothiazol-4-yl)-2-(methoxy-imino)acetamido)-3-((1-methylpyrrolidinium-1-yl)methyl)-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylate, **I**, Fig. 1), a fourth-generation cephalosporin with a broad antibiotic spectrum was first marketed in 1994 and has been used in the treatment of various infectious diseases [1]. The quantitative determinations of cefepime in biological matrices have been proposed by several approaches, including the second-derivative spectrophotometry [2], micellar capillary electrokinetic chromatography [3], polarographic technique [4], bioassay [5], and HPLC-UV methods [6–10]. All these methods showed low sensitivity with quantitation limits over 100 ng/mL. Since cefepime remains an important benchmark for new antibiotic drug development, there is increasing need for sensitive and high throughput determination of cefepime in biological matrices to support preclinical, clinical pharmacokinetic, and toxicokinetic studies. In this paper, a sensitive and high throughput LC/MS/MS method is presented. The validated method achieved a lower limit of quantitation (LLOQ) of 4 ng/mL. The application of this method in cefepime mouse pharmacokinetic study is demonstrated.

2. Experimental

2.1. Materials

Cefepime (**I**) and ceftazidime (**II**) reference standards, as shown in Fig. 1, were purchased from US Pharmacopoeia (USP). The purity of 100% was used for both **I** and **II**, according to USP certificates. Oasis HLB μElution plates were products of Waters Co. (Milford, MA, USA). Control drug-free mouse plasma (K_2EDTA as anticoagulant) was purchased from Bioreclamation (Hicksville, NY, USA). Ammonium acetate crystal was purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA) and ammonium formate was a product of Alfa Aesar (Ward Hill, MA, USA). Formic acid was purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC-grade water and methanol were products of Honeywell International Inc (Morristown, NJ, USA) and HPLC-grade acetonitrile was purchased from CCI (Columbus, WI, USA). Dimethyl sulfoxide (DMSO) was a product of Fisher Scientific (Pittsburgh, PA, USA).

2.2. Preparation of standards and quality control samples

Stock solutions of cefepime and ceftazidime were prepared separately by dissolving accurately weighed amounts of each reference standard in DMSO to yield the concentration of 1.0 mg/mL. Two separate cefepime stocks were prepared for standards and quality control (QC). The 200 μg/mL cefepime standard, QC and the ceftazidime internal standard (IS) sub-stocks were prepared by diluting stock solutions with methanol–water (1:1, v/v).
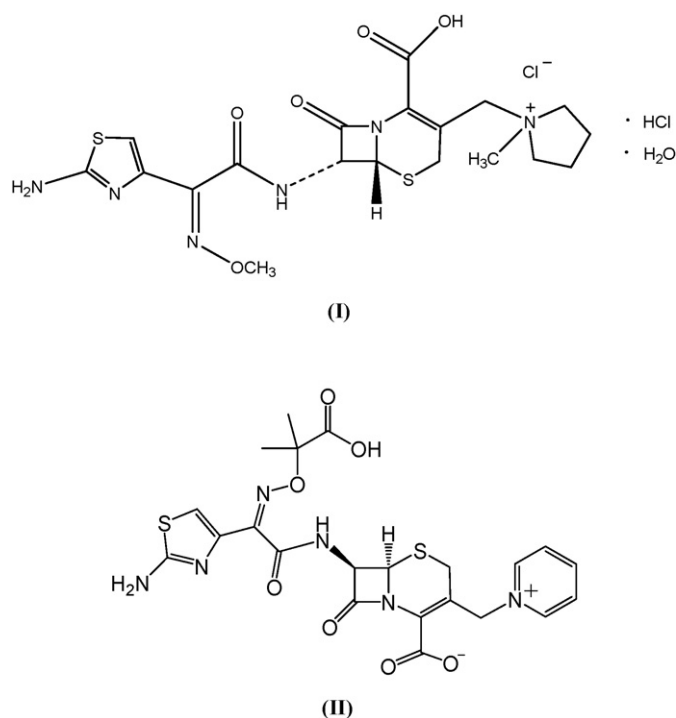
Fig. 1. Chemical structures of cefepime (**I**) and ceftazidime (**II**).

Standard samples of cefepime at 10 concentrations (4–2048 ng/mL) and QC samples at 5 concentrations (4, 12, 60, 300 and 1500 ng/mL) were prepared by serial dilutions of standard sub-stock solution and QC sub-stock with drug-free mouse plasma. An internal standard working solution (ISWS) containing 500 ng/mL of ceftazidime was prepared by transferring 125 μL of IS sub-stock into a 50 mL volumetric flask and bringing to volume with 5 mM ammonium acetate buffer. QC samples were stored at −70 °C until analysis.

2.3. Plasma sample extraction

Study samples and QC samples were thawed at room temperature, mixed thoroughly by vortexing, centrifuged at $1310 \times g$ (25 °C) for 5 min. For analyte stability concern, calibration standards (freshly prepared), QCs, and study samples were placed on ice prior to and during extraction. To a 96-well plate, 100 μL aliquots of plasma standards, QCs, and experimental samples were added to the designated wells followed by the addition of 100 μL of IS WS (except the double blanks where 100 μL of 5 mM ammonium acetate buffer were added). The plate was capped with a 96-well mat, and vortexed for 1 min at medium speed. 200 μL of the mixture from each well was transferred, using a 12-channel pipette, to an Oasis HLB μElution SPE 96-well plate pre-conditioned with 200 μL of methanol and equilibrated with 200 μL of 5 mM ammonium acetate buffer. The loaded samples were drawn under vacuum that was adjusted to an optimized pressure. The wells in the plate were next washed with 100 μL of HPLC water to remove matrix interferences. After drying the plate under vacuum for approximate 1 min, the retained analyte and IS were eluted to a 1.2 mL 96-well receiving plate with 2×50 μL of methanol under vacuum. The receiving plate was then capped, vortexed briefly, and 10 μL of extract was injected for LC/MS/MS analysis.

2.4. SCX chromatographic conditions

The chromatographic separation was performed on a Zorbax 300-SCX column, 2.1 mm × 50 mm, 5 μm (Agilent Technologies,

Santa Clara, CA, USA). An aqueous 25 mM ammonium formate buffer was prepared by dissolving ammonium formate in HPLC water and adjusting pH with formic acid to 2.79. This buffer was used for further preparations of solvent A and B. A mobile phase gradient program with solvent A (combination of 25 mM ammonium formate buffer, pH 2.79 and acetonitrile, 95:5, v/v) and solvent B (combination of addition of another 500 mM ammonium formate to pre-pared pH 2.79, 25 mM ammonium formate buffer and acetonitrile, 70:30, v/v) was applied at a flow rate of 0.5 mL/min. The gradient program started with 5% B followed by a linear increase in B to 90% (from 0 to 1.0 min) and held at 90% for 0.5 min (from 1.0 to 1.5 min). Mobile phase B was increased to 100% within 0.1 min, held at 100% for another 0.4 min (from 1.6 to 2.0 min) and then reduced linearly to the initial condition (5% B) within 0.1 min. This condition was held until the end of the run. The total run time was 3.5 min.

2.5. ESI-MS/MS conditions

An AB Sciex API 4000 linear ion TRAP quadrupole mass spectrometer (4000 Q TRAP), operated in positive turbo electrospray ionization (ESI) mode, was used for mass detection and analysis. Multiple reaction monitoring (MRM) was used to monitor the product ion transitions of m/z $481.0 \rightarrow 86.2$ and $547.4 \rightarrow 467.9$ for cefepime and ceftazidime, respectively. Dwell time for both transitions was 150 ms. The ESI ion source temperature was at 550 °C. Other optimized MS/MS parameters were: curtain gas flow: 20 psi; collisionally activated dissociation (CAD) gas setting: medium; ion spray voltage: 5500 V; ion gas 1 and 2: 50 psi; entrance potential: 10 V; collision cell exit potential: 10 V; declustering potential: 76 V for cefepime and 74 V for ceftazidime; and collision energy: 25 eV for cefepime and 15 eV for ceftazidime.

2.6. Drug administration and plasma sample collection

The pharmacokinetics of cefepime was evaluated in healthy female CD-1 mice following intravenous (IV) via tail vein and subcutaneous (SC) administrations. A total of 24 animals for two dose groups were used for this study. The animals were administered single does of cefepime, dissolved in 0.9% sodium chloride (saline), at 25 and 50 mg/kg for IV and SC, respectively. Plasma samples were collected from each of three animals per time point at the following time points: 5, 15, 30 min, 1, 2, 4, 7 and 24 h post-dose for the IV group; 15, 30 min, 1, 2, 4, 6, 8 and 24 h post-dose for the SC group.

2.7. Pharmacokinetics analysis

The plasma concentration-versus-time data of cefepime from the mean of three animals was analyzed to determine the pharmacokinetic parameters using a two-compartmental model in WinNonlin Professional version 5.2 (Pharsight, Mountain View, CA). The maximum concentration (C_{max}), half-life ($t_{1/2}$), area under the curve (AUC), clearance (CL), volume distribution at steady state (V_{ss}) and bioavailability were evaluated. The area under the plasma concentration-versus-time curve, from 0 to infinity (AUC_{0-inf}) was calculated using the linear trapezoidal-rule option in WinNonlin. Plasma concentrations below the limit of quantification (BLQ) were recorded as zero.

## 3. Results and discussion

3.1. Sample extraction optimization

In the initial effort of developing a routine sensitive LC/MS/MS method for the determination of cefepime in mouse plasma, poor retention and extremely low recovery of cefepime was experienced. To achieve better recovery and improve the sensitivity,

a μElution solid-phase extraction (SPE) technique was adapted for the sample clean-up and enrichment. The unique design of Oasis hydrophilic hydrophobic bond (HLB) μElution SPE with small amount (2.5 mg) cartridges allows large volume (750 μL) of sample loaded, yet, very small volume of elution solvent (25 μL) applied yielding significant sample enrichment. The retention capacity depends on the polarity and ionization state of the compounds. It is recommended by the manufacturer that for basic analyte the pH be adjusted to at least 2 units above its pKa value. In the case of cefepime containing polar quaternary amine, with pK_a of approximate 3.0 [11,12], the pH of plasma sample was adjusted to approximate 6.4, with 5 mM ammonium acetate aqueous solution resulting in 71% recovery of cefepime with a satisfied LLOQ of 4 ng/mL. To minimize the loss of cefepime during SPE process, only water (100 μL) was used as the washing solvent to remove salts and other water-soluble impurities.

3.2. LC/MS/MS conditions

The quaternary amine group contained in cefepime hampers its retention on a reversed-phase column. The significant retention improvement of cefepime was eventually achieved with SCX-LC/MS/SM. The effect of buffer concentration, pH and organic percentage in the mobile phases was investigated and optimized on the retention of cefepime. With 25 mM ammonium formate, pH 2.79, in both mobile phases as a starting point, the best retention of cefepime was obtained at a combination of additional 500 mM ammonium formate and 30% acetonitrile in mobile phase B. The
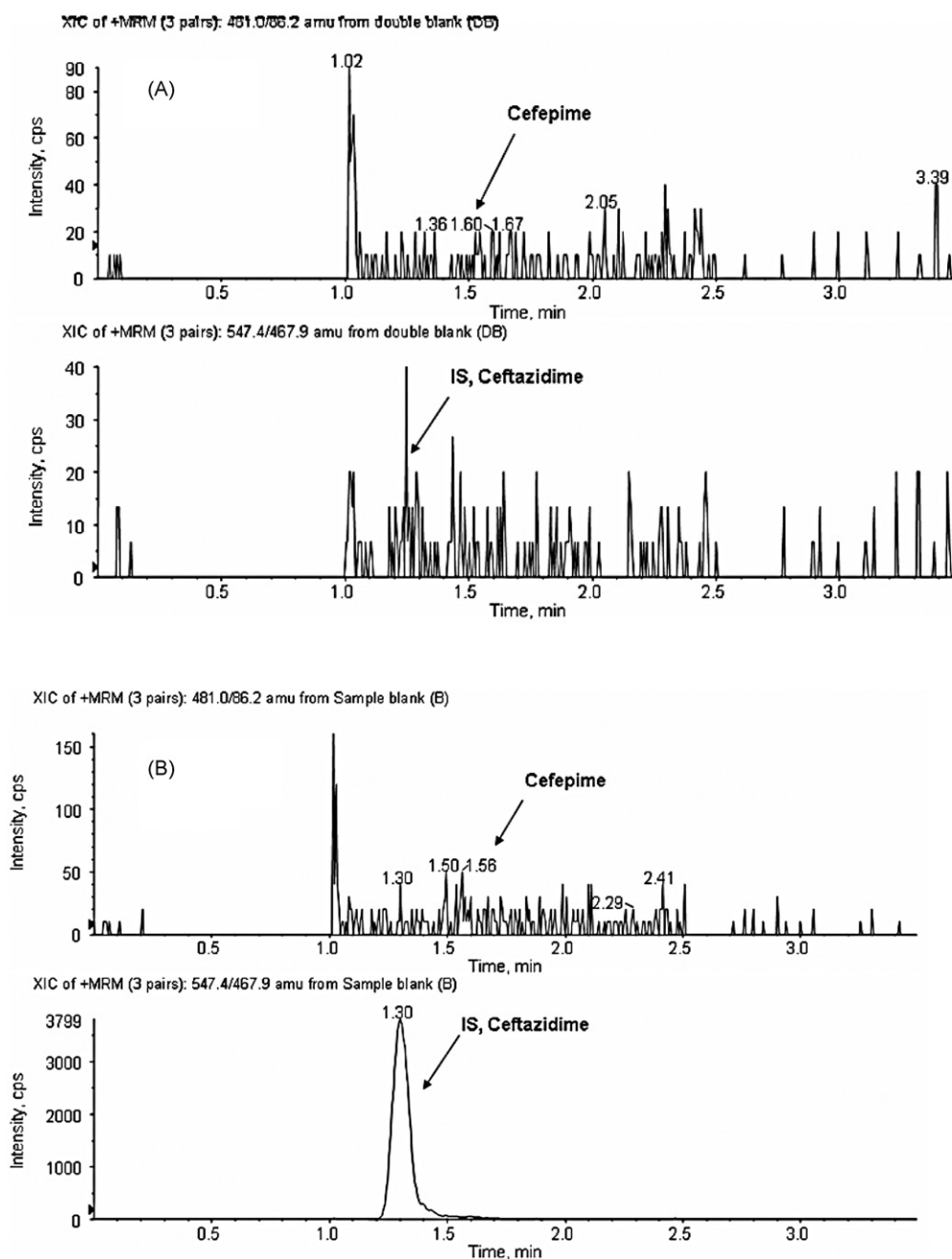
Fig. 2. Representative chromatograms of a double blank matrix without IS (A), blank matrix with IS (B), an LLOQ sample at 4.0 ng/mL (C) and an ULOQ sample at 2048 ng/mL (D).
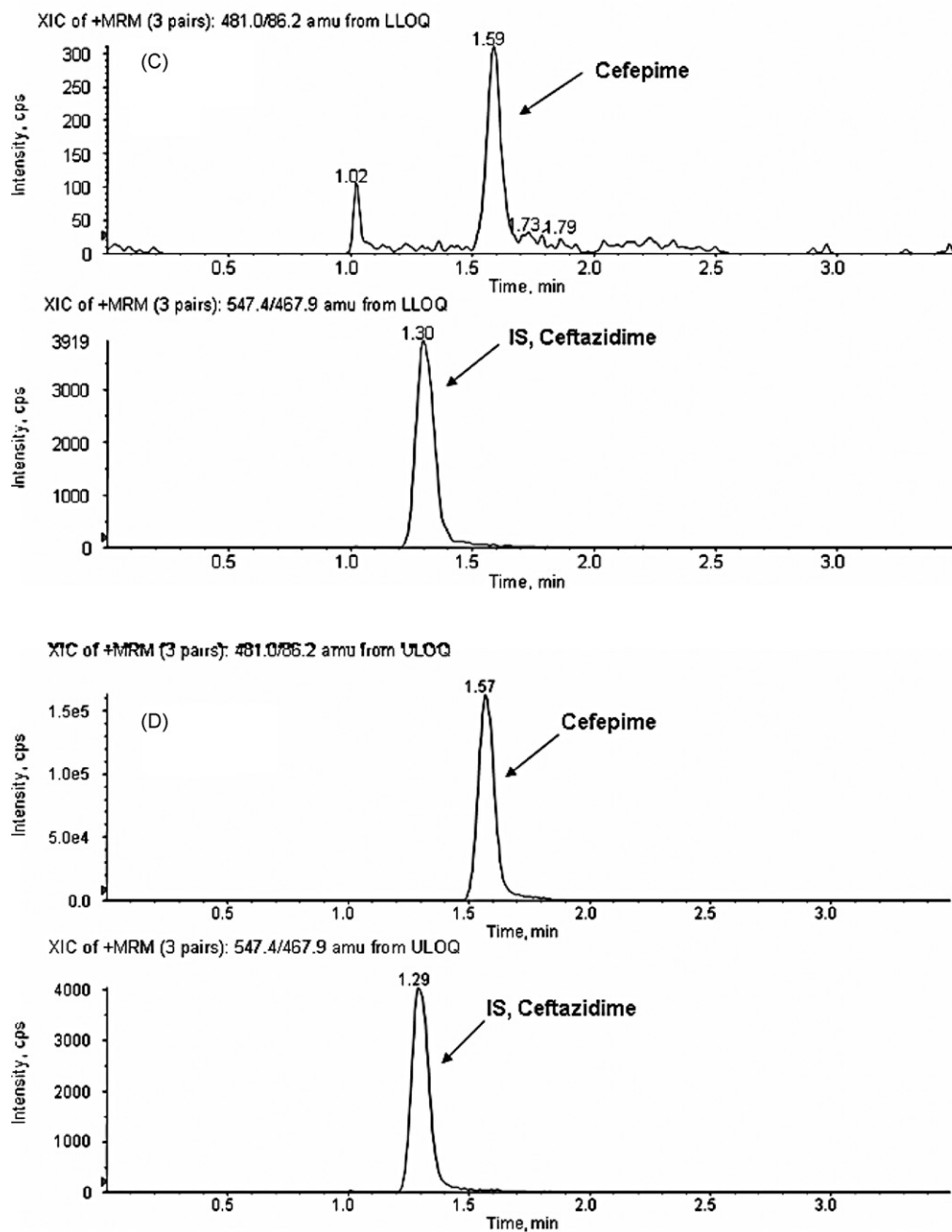
XIC of +MRM (3 pairs): 481.0/86.2 amu from LLOQ

XIC of +MRM (3 pairs): 547.4/467.9 amu from LLOQ

XIC of +MRM (3 pairs): 481.0/86.2 amu from ULOQ

XIC of +MRM (3 pairs): 547.4/467.9 amu from ULOQ

Fig. 2. (*Continued*).

MRM transitions were determined and the corresponding mass spectrometry parameters were optimized by tuning the instrument with the infusion of cefepime and IS solutions dissolved in HLPC water/ACN, 50:50 (v/v). A gradient elution program was developed to obtain the best retentions of cefepime and IS with a short run time of 3.5 min.

3.3. Method validation

3.3.1. Specificity, sensitivity and relative matrix effect

Specificity of the method was assessed by extracting and analyzing double drug-free mouse plasma. As shown in Fig. 2, there was no interference peaks at the retention times of either analyte or internal standard.

Despite the minor loss of cefepime in the course of μElution SEP, approximately 25 times better sensitivity was achieved over the published methods [2–10] with lower limit of quantitation (LLOQ) of 4.0 ng/mL. The LLOQ was determined based on a signal-to-noise (S/N) ratio of at least 10. The accuracy and precision at LLOQ determined in the intra-assay ($n = 6$) were 113% and 4.56% (Table 1), respectively. The representative chromatograms of double blank sample, blank sample, cefepime at LLOQ and at upper limit of quantitation (ULOQ) are shown in Fig. 2.

The variation due to use of different source of matrix was defined as relative matrix effect. The relative matrix effect was evaluated by analyzing triplicate post-extraction spiked cefepime LLOQ samples of six different lots of mouse plasma and cefepime spiked in extraction solvent at LLOQ level. The precision (% CV) of each triplicate set ranged from 2.01 to 5.73% using cefepime peak area and

Table 1
Intra-assay and inter-assay precision and accuracy.

Assay type	Nominal concentration (ng/mL)	n	Mean calculated concentration (ng/mL)	Precision (% CV)	Accuracy (% nominal)
Intra-assay (mouse)	4	6	4.54	4.56	113
	12	6	13.1	7.57	109
	60	6	62.7	4.84	104
	300	6	291	4.86	96.9
	1500	6	1639	6.55	109
Inter-assay (mouse)	4	15	4.17	8.60	104
	12	15	12.6	9.05	105
	60	15	59.4	7.78	98.9
	300	15	287	5.03	95.6
	1500	15	1547	8.40	103

Table 2
Mean pharmacokinetic parameters of cefepime following single intravenous and subcutaneous administrations of cefepime in mice.

Dosing route	Dose (mg/kg)	n	C_{max} (µg/mL)	T_{max} (h)	AUC_{0-inf} (h*µg/mL)	CL (mL/h/kg)	Terminal $t_{1/2}$ (h)	V_{ss} (mL/kg)	Bioavailability (%)
IV	25	3	51.9	0.083	14.3	1744	0.685	351	na
SC	50	3	35.4	0.25	22.0	2255[a]	1.07	643[b]	77

n: number of animals; na: not applicable; IV: intravenous; SC: subcutaneous.

[a] CL/F.
[b] V_{ss}/F.

1.02–4.37% using peak area ratio of cefepime/IS, which was comparable with 3.88% using peak area and 2.44% using area ratio of cefepime/IS spiked in extraction solvent. The results of this relative matrix effect study result indicate there was no relative matrix effect between different sources of mouse plasma.

3.3.2. Precision and accuracy

The intra-assay precision and accuracy was evaluated by analyzing within the same run six replicate QC samples at each of five concentration levels. The intra-assay precision ranged from 4.56% to 7.57% and the accuracy, expressed as percentage of nominal values, ranged from 96.9% to 113% (Table 1). The inter-assay precision determined by analyzing triplicate QC samples at each of five concentration levels for four sets of runs (including one intra-assay), were between 5.03% and 9.05% and inter-assay accuracy ranged from 95.6% to 105% (Table 1).

3.3.3. Calibration reproducibility

In each of the validation sessions, fresh calibration samples at 10 concentration levels were prepared and analyzed as described above. The calibration curves were linear using weighted (1/concentration) least-squares linear regression mode ($y = ax + b$, weighing $1/x$) over a concentration range of 4.0–2048 ng/mL, with correlation coefficients (r) equal to or greater than 0.9990. The standard errors of mean slope and intercept were 0.0054 and 0.0055, respectively.

3.3.4. Extraction recovery

The recovery of cefepime from mouse plasma by µElution SPE was evaluated by comparing peak area ratios (analyte/internal standard) of pre-spiked with post-spiked QC samples. The internal standard was spiked after the extraction in both cases. Despite the minor loss of cefepime due to its high polarity, 71% recovery was achieved which led to a satisfied LLOQ of 4 ng/mL.

3.3.5. Stability

After the µElution SPE process, the post-extract cefepime samples were found stable for at least 3 days in the auto-sampler at 4 °C with percentage of loss ranging from −5.5% to −4.4% for all QC samples tested at low quality control (LQC) and high quality control (HQC) levels. After 13-day long-term storage (−70 °C) and three freeze/thaw cycles, cefepime in mouse plasma demonstrated acceptable stability with percentage loss ranging from −8.20% to 7.7% for LQC and HQC samples. The results indicated that the mouse plasma samples were stable for at least 13 days at −70 °C.

3.4. Pharmacokinetic results

The plasma concentration–time profiles of cefepime in female CD-1 mice, following single IV and SC administrations of cefepime saline solution at 25 and 50 mg/kg, are illustrated in Fig. 3. The mean pharmacokinetic parameters estimated using a two-compartmental model by WinNonlin 5.2 are summarized in Table 2. Following single IV 25 mg/kg dosing of cefepime saline solution, the maximum plasma concentration (C_{max}) at 5 min was 51.9 µg/mL. The clearance was 1774 mL/h/kg, AUC_{0-inf} was 14.3 h*µg/mL and the terminal half-life was 0.685 h. Following a single SC administration of 50 mg/kg, the C_{max} of 35.4 µg/mL was at 0.25 h. The bioavailability of SC was 77%.

It is well recognized and widely agreed that the bioanalytical methodology is crucial for pharmacokinetic studies. Sensitive assays enable determination of complete data ensuring accurate pharmacokinetic modeling. With the sensitive assay developed in this study, our pharmacokinetic study of cefepime in mice showed a definitive two-compartmental profile (Fig. 3). In contrast, the pharmacokinetic profiles of cefepime in previously reported studies in *Escherichia coli* inoculated mice following SC dosing [13,14] were
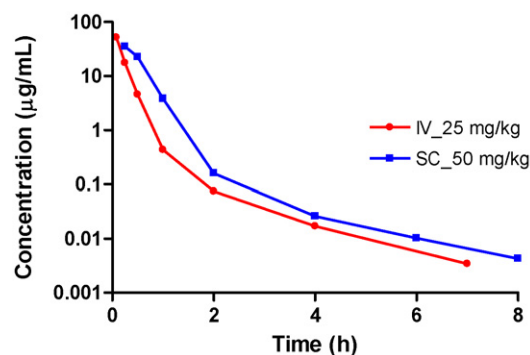

Fig. 3. Plasma concentration–time profiles of cefepime in female CD-1 mice following a single IV dose of 25 mg/kg and SC dose of 50 mg/kg.

not well defined due to the limits of insensitive bioanalytical techniques with LLOQ of 100 ng/mL. The undetermined data at later time points of low dose group [14] or high variability of data at later time points [13] had significant impact on the accuracy of data fitting to the pharmacokinetic models and terminal elimination rates.

4. Conclusion

A rapid, sensitive and selective μElution 96-well solid-phase extraction (SPE) followed by strong cation exchange chromatography/tandem mass spectrometry (SCX-LC/MS/MS) method has been developed and validated for the determination of cefepime in mouse plasma.

The SPE method was efficient for the clean-up and enrichment of cefepime plasma samples with a recovery of 71%. The method was linear ($r > 0.9990$) over the concentration range of 4.0–2048 ng/mL. The intra- and inter-day assay accuracy (% of nominal) and precision (% CV) were, respectively, within ±15% (±20% at the LLOQ) and ≤15% (≤20% at the LLOQ). There was no matrix effect in six different sources of mouse plasma. The validated method was successfully utilized to accurately analyze mouse experimental pharmacokinetic study samples and a pharmacokinetic report was generated.

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