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Short communication

Determination of cefazedone in human plasma by high performance liquid chromatography–tandem mass spectrometry: Application to a pharmacokinetic study on Chinese volunteers

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ABSTRACT

A rapid, sensitive and simple high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method was developed for determination of cefazedone in human plasma using metronidazole as internal standard (IS). The chromatographic separation was achieved on an Ultimate XB-CN column (2.1 mm × 150 mm, 5 μm) with an isocratic mobile phase of acetonitrile and 20 mM ammonium acetate in 0.1% formic acid in water (15:85, v/v). Detection was performed using electrospray ionization in positive ion multiple reaction-monitoring mode (SRM), monitoring the transitions *m/z* 548.2 → 344.1 for cefazedone and *m/z* 172.2 → 128.1 for IS. Calibration curves were linear over a wide range of 0.20–401.12 μg/mL for cefazedone in plasma. The lower limit of quantification (LLOQ) was 0.20 μg/mL. The intra- and inter-day precisions were less than 7.2%. The average recovery of cefazedone was 90.8–91.0%. The validated method was successfully applied to the pharmacokinetic study of cefazedone in Chinese healthy volunteers following intravenous (IV) administration of 500, 1000 and 2000 mg cefazedone injection.

1. Introduction

Cefazedone, (6R,7R)-7-(2-[3,5-dichloro-4-oxo-1(4H)-pyridyl]-acetamido)-3-([(5-methyl-1,3,4-thiadiazol-2-yl)-thio]methyl)-8-oxo-5-thia-1-azabicyclo[4,2,0]oct-2-ene-2-carboxylic acid, is a cephalosporin antibiotic with activity against Gram-positive and some Gram-negative bacteria [1–3]. It can be used for a variety of indications, including urinary tract infections, respiratory tract infections, biliary tract and other abdominal infections, surgical and dermatological infections, gynaecological infections [4–7]. It plays an important role in antibiotic prophylaxis as a basic cephalosporin. Cefazedone exhibits a high protein binding (>90%). However, it has a short half-life of approximately 1.6 h. Cefazedone is eliminated primarily through the kidney by glomerular filtration or tubular secretion. After intravenous administration of 1000 mg cefazedone, the peak plasma concentration reaches 144 μg/mL [8].

So far as we know, there is only one HPLC method [9] reported to determine cefazedone in human plasma up to now. The plasma samples were prepared by solid phase extraction (SPE) including complicated condition, wash and elution procedures. A large volume of 500 μl plasma was used and a volume of 20 μl of elute was injected into the column. The reported method required long analysis time over 8 min. In this study, we developed a simple, sensitive and fast LC–MS/MS method to determine cefazedone in human plasma, with a LLOQ of 0.20 μg/mL. The method was fully validated and successfully applied to the pharmacokinetic study in Chinese healthy volunteers after IV administration of 500, 1000 and 2000 mg cefazedone injection.

2. Experimental

2.1. Chemicals and reagents

Cefazedone (Batch No. 081104, 96.7% purity) reference standard was provided by Shandong Luoxin Pharmaceutical Co., Ltd. (Shandong, China). Metronidazole (Batch No. 100191-200305, 100.0% purity), the internal standard, was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). HPLC grade acetonitrile was purchased from Fisher Scientific (Emerson, IA, USA). Methanol, formic acid and ammonium acetate were of analytical grade and purchased from Research Center for the Chemical Reagent Engineering and Technology of Guangdong Province (Guangzhou, PR China). Water was distilled and purified using a Milli-Q Water Purification Sys-

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tem (Millipore, Bedford, MA, USA). Drug-free human plasma from healthy volunteers was provided by the Blood Center of Tongji Hospital of Tongji Medical College (Wuhan, PR China) and was stored at −20 °C.

2.2. High performance liquid chromatography and mass spectrometry

The chromatography was performed on Shimadzu ultra fast liquid chromatography (UFLC) system (Chiyoda-Ku, Kyoto, Japan) equipped with two LC-20AD pumps, DGU-20A3 on-line degasser, SIL-20AC XR autosampler, CTO-20AC column thermostat. The chromatographic separation was achieved on an Ultimate XB-CN column (2.1 mm × 150 mm, 5 μm, MD, USA) protected by a Phenomenex ODS guard column (4.0 mm × 3.0 mm i.d., 5 μm, Torrance, CA, USA). The mobile phase was a mixture of acetonitrile and 20 mM ammonium acetate in 0.1% formic acid (15:85, v/v), which was pumped at a flow rate of 0.3 mL/min. The column temperature was set at 35 °C. The total run time of each sample analysis was 4.5 min.

Mass spectrometric detection was performed on an API 4000 LC/MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization source in the positive ion mode. Quantification was performed using multiple reaction-monitoring mode, monitoring the transitions m/z 548.2 → 344.1 for cefazedone and m/z 172.2 → 128.1 for IS with a dwell time of 200 ms per transition. The working parameters maintained were as following: curtain gas (CUR) 10 psi, GAS1 55 psi, GAS2 60 psi, collision activated dissociation (CAD) Medium, ion spray voltage 5500 V, heater temperature 600 °C, respectively. The declustering potential (DP), collision energy (CE), entrance potential (EP), collision cell exit potential (CXP) were optimized as 75 V, 27 V, 10 V, 9 V for cefazedone; 48 V, 20 V, 10 V, 10 V for IS. All data were acquired and processed by the Analyst 1.5 software (Applied Biosystems, Foster City, CA, USA).

2.3. Preparation of stock solution and standards

Stock solutions of cefazedone and IS were prepared in water and methanol, respectively. Standard working solutions of cefazedone range from 2.0 to 4011.2 μg/mL were prepared by serial diluting the stock solution with water. Internal standard working solution (14.0 μg/mL) was prepared by diluting the IS stock solution with methanol. All solutions were stored at −20 °C when not in use.

Calibration standards of cefazedone were prepared at the concentration levels of 0.20, 0.81, 3.03, 10.11, 40.11, 120.34, 320.90 and 401.12 μg/mL by spiking 20 μl of standard solutions in 200 μl blank plasma. Quality control (QC) samples were prepared in the same way as the calibration samples, to achieve low, middle and high concentrations of cefazedone in plasma at 0.40, 10.11 and 320.90 μg/mL. The spiked QC samples were stored at −80 °C for stability studies.

2.4. Sample preparation

To an aliquot of 200 μl plasma, 20 μl of IS working solution and 20 μl water was added and vortex-mixed for 30 s. The mixture was precipitated with 800 μl methanol, vortex-mixed for 1 min, and then centrifuged at 13,000 rpm for 10 min. To 100 μl aliquot of supernatant, 200 μl water was added and mixed well. 200 μl of mixture was transferred into autosampler vials and 5 μl was injected into the LC–MS/MS system.

2.5. Method validation

2.5.1. Specificity and matrix effect

The specificity of the method was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma samples. Each blank sample was tested for interferences using the precipitation procedure and LC–MS/MS conditions described above. The plasma samples studied for specificity were normal human plasma. The matrix effect was evaluated by comparing peak areas of cefazedone and IS spiked in protein precipitated samples with those of analytes in neat solution at equivalent concentration. Matrix effect was evaluated at three QC concentration levels of 0.40, 10.11 and 320.90 μg/mL.

2.5.2. Linearity and lower limit of quantification

The calibration curve was constructed by plotting the peak area ratios (y) of cefazedone to IS against the spiked concentrations (x) of cefazedone. Linearity was assessed by weighted ($1/x^2$) least squares regression analysis. LLOQ was defined as the lowest concentration on the calibration curve. Each LLOQ sample should be obtained with an acceptable accuracy (RE) within ±20% and a precision (RSD) not greater than 20% [10].

2.5.3. Precision, accuracy and recovery

The intra-day accuracy and precision were assessed by analyzing five replicates of low, middle, high QC samples on the same day. The inter-day accuracy and precision were determined by analyzing the QC samples at three levels on three consecutive days. Accuracy and precision were evaluated in terms of relative error (RE) and relative standard deviation (RSD), respectively. The recovery was determined by comparing the peak areas of cefazedone in spiked plasma samples with those of samples to which the same amounts of cefazedone was added after protein precipitation. Recovery was performed at three QC levels (n = 5).

2.5.4. Stability

The stability of cefazedone was fully evaluated under different storage and handling conditions. Post-preparative stability was assessed by analyzing the processed QC samples kept in an autosampler at 4 °C for 12 h. For short-term and long-term stability, QC samples were exposed at room temperature for 12 h and stored at −80 °C for 40 days, respectively. To determine the freeze–thaw stability, the frozen QC samples were thawed at room temperature and re-frozen for 24 h. After completion of the three cycles, these samples were analyzed and compared with the nominal values.

2.6. Pharmacokinetic study

The whole pharmacokinetic study followed the Good Clinical Practice (GCP) guidelines [11]. Thirty healthy volunteers, including 15 males and 15 females, were enrolled in this trial after physical examination and laboratory tests. The clinical protocol was approved by the local ethics committee, and all volunteers signed the informed consent. All volunteers were randomly divided into three groups, as group low, middle and high dose (five males and five females per group). All volunteers were IV administered a single dose of 500, 1000, 2000 mg cefazedone injection dissolved in physiologic saline, respectively. Blood samples were collected in heparin containing tubes before dosing and at 0.1, 0.2, 0.33, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8 and 12 h after drug administration. In the multiple-dose study, 10 volunteers in middle-dose group received cefazedone injection twice a day for 5 consecutive days (the single-dose study as the first day). Blood samples were collected on the morning of day 3, 4 and 5 before dosing to determine if the plasma concentration had reached steady state. On the day 5, blood samples were collected at the same time as that of the single-dose

Table 1
Stability of cefazedone under various storage conditions ($n = 3$).

Storage conditions	Nominal concentration (μg/ml)	Mean measured concentration (μg/ml)	RSD (%)	RE (%)
Autosampler for 12 h	0.40	0.39 ± 0.50	3.8	−3.0
	10.11	10.26 ± 0.44	4.3	1.5
	320.90	303.10 ± 16.49	5.4	−4.9
Room temperature for 12 h	0.40	0.40 ± 0.02	6.2	−1.5
	10.11	10.08 ± 0.18	1.8	−0.2
	320.90	310.17 ± 5.32	1.7	−3.3
3rd freeze and thaw	0.40	0.41 ± 0.01	2.7	0.5
	10.11	9.96 ± 0.29	2.9	−1.5
	320.90	307.83 ± 4.07	1.3	−4.1
−80 °C for 40 days	0.40	0.42 ± 0.02	5.2	4.5
	10.11	10.47 ± 0.33	3.2	3.5
	320.90	301.47 ± 4.18	1.4	−6.1

study. All samples were separated immediately by centrifugation at 3000 rpm for 10 min and stored at −80 °C until analysis.

Pharmacokinetic parameters were calculated using DAS program (Version 2.0, Sun Ruiyuan, China). The peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}) were noted directly. The area under the plasma concentration versus time curve from zero to the last measurable concentration (AUC_{0-t}) was calculated using the linear trapezoidal rule. The AUC from zero to infinity ($AUC_{0-\infty}$) was calculated as: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$, where C_t is the last measurable plasma concentration. The elimination rate consent (k_e) was determined by linear regression of the terminal points of the logarithms plot of plasma concentration against time. The elimination half-life ($t_{1/2}$) was calculated as $0.693/k_e$. C_{av} was the average steady-state plasma concentration and AUC_{ss} was the AUC at steady state. AUC_{ss} was also calculated according the trapezoidal rule. C_{av} was calculated as AUC_{ss}/τ, where τ is the administration interval.

3. Results and discussion

3.1. Method development

In order to optimize mass spectrometric conditions, the full scans were carried out in positive ion detection mode. The solutions containing cefazedone and IS were directly infused into the mass spectrometer, respectively. Cefazedone standard solutions diluted with methanol, water and water containing 0.1% formic acid were evaluated for MS scan. The results revealed that cefazedone in 0.1% formic acid solution provided the best signals. The precursor ions were not observed when infusing solutions of cefazedone in methanol and water with a concentration of 1 μg/mL, respectively. The predominately SRM transitions m/z 548.2 → 344.1 for cefazedone and m/z 172.2 → 128.1 for IS were selected for quantitative analysis.

Various mixtures of solvents such as methanol and acetonitrile, with typical buffers such as formic acid and ammonium acetate were tested. The addition of formic acid to the mobile phase could enhance the MS ionization efficiency of cefazedone. The addition of 20 mM ammonium acetate to the mobile phase was found to be crucial to improve the peak shapes of cefazedone and IS. A mixture of acetonitrile and 0.1% formic acid containing 20 mM ammonium acetate (15:85, v/v) was adopted in this study, which provided symmetric peak shapes of cefazedone and IS as well as high sensitivity. Three types of columns, Ultimate C18, Lichrospher C18 and Ultimate XB-CN, were compared. Both cefazedone and IS had poor retention on the Ultimate C18 (2.1 mm × 150 mm, 5 μm, MD, USA). Lichrospher C18 (2.1 mm × 150 mm, 5 μm, Merck, Darmstadt, Germany) gave poor signal intensity and peak shape of the cefazedone. The results show that Ultimate XB-CN column (2.1 mm × 150 mm, 5 μm, MD, USA) provided best retention and chromatographic separation. In this study, we have tried rabeprazole and metronidazole for IS selection, which can be easily obtained in our laboratory. Rabeprazole has the similar retention time to cefazedone, but it was unstable in the supernatant of the plasma sample after addition of water. Metronidazole was selected as the internal standard for its similar chromatographic retention and mass spectrometric behavior to cefazedone. The retention times of cefazedone and IS was 3.19 and 2.44 min, respectively. The total run time was only 4.5 min per sample.

In this paper, plasma samples were pretreated with methanol for protein precipitation. Protein precipitation has advantages in that it can simplify the sample preparation procedure and provide excellent reproducibility. It was found that it was necessary to add water to the supernatant of the plasma sample after protein precipitation. Cefazedone was unstable in the supernatant without water. Our stability studies show that cefazedone was stable for 12 h when diluted with water after protein precipitation.

3.2. Method validation

3.2.1. Specificity and matrix effect

Fig. 1 shows the typical chromatograms of blank plasma, spiked plasma sample with cefazedone and IS, and plasma sample from a volunteer after administration. The retention times of cefazedone and IS were 3.19 and 2.44 min, respectively. No significant interference from endogenous substances was observed at the retention time of cefazedone and IS.

Matrix effect was determined at three concentration levels of 0.40, 10.11 and 320.90 μg/mL. The results varied from 86.6% to 100.3%, which were within the acceptable limits (85–115%) [10].

Table 2
Mean pharmacokinetic parameters of cefazedone after single dose of 500, 1000 and 2000 mg and multiple dose of 1000 mg to Chinese healthy volunteers, respectively ($n = 10$).

Parameters	500 mg	1000 mg	2000 mg	1000 mg (ss)
$t_{1/2}$ (h)	1.9 ± 0.5	1.8 ± 0.3	2.1 ± 0.3	1.8 ± 0.3
C_{max} (μg/ml)	87.1 ± 36.4	152.0 ± 19.2	272.3 ± 38.9	129.2 ± 22.7
T_{max} (h)	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.33 ± 0.0
AUC_{0-12} (μg h/ml)	145.2 ± 68.6	245.6 ± 38.5	513.9 ± 127.5	207.3 ± 37.1
$AUC_{0-\infty}$ (μg h/ml)	147.8 ± 70.4	247.9 ± 40.8	521.8 ± 134.5	209.0 ± 38.2

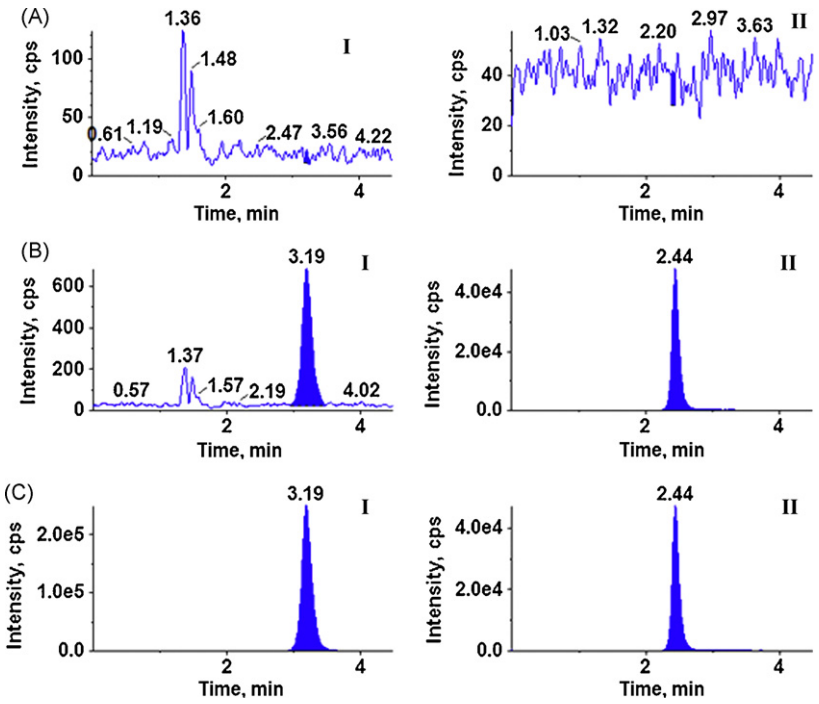

Fig. 1. Typical SRM chromatograms of (A) blank human plasma; (B) blank plasma spiked with cefazedone at LLOQ (0.20 μg/mL) and IS (1.4 μg/mL); (C) volunteer plasma after 1 h single dose of 1000 mg cefazedone. Peak I: cefazedone, Peak II: IS.

3.2.2. Calibration curves and LLOQ

Five calibration curves were performed during the method validation. Good linearity was showed over the concentration range of 0.20–401.12 μg/mL for cefazedone in plasma. The average calibration curve of cefazedone in human plasma was $y = (0.127 \pm 0.013)x + (0.000549 \pm 0.00091)$, $r = (0.9989 \pm 0.0003)$. The LLOQ was evaluated by analyzing six replicates of the lowest standard on the calibration curve. The LLOQ was found to be 0.20 μg/mL with an accuracy of 3.1% and precision of 4.9%.

3.2.3. Accuracy, precision and recovery

The intra- and inter-day precisions ranged from 2.2% to 7.3% and 1.7% to 6.0% at three QC levels, respectively. The relative error values were −2.7%, 4.3% and −6.0% for intra-day accuracy, and −1.4%, 2.5% and −6.3% for inter-day accuracy at three concentration levels of 0.40, 10.11 and 320.90 μg/mL, respectively. The values are well within the acceptable criteria where the precision should not exceeding 15% and accuracy within ±15% of the actual value. The recoveries of cefazedone from human plasma were 90.8 ± 3.8%, 91.0 ± 2.9% and 91.0 ± 4.2% at concentration levels of 0.40, 10.11 and 320.90 μg/mL, respectively. The recovery of IS from human plasma was also evaluated and found 91.3 ± 3.5%.

3.2.4. Stability

Stability was performed under different experimental conditions that samples might be exposed to. The results were obtained by comparing the freshly prepared samples with those handled as described above. The results are presented in Table 1. All the values were within ±15% of the nominal values which indicated a good stability of analytes in human plasma.

3.3. Application to a pharmacokinetic study

The validated method was applied to determine the human plasma concentration of cefazedone following single-dose intravenous drop infusion of 500, 1000 and 2000 mg and multiple-dose intravenous drop infusion of 1000 mg twice daily for 5 days in Chinese volunteers. The mean plasma concentration–time profiles are presented in Fig. 2. The main pharmacokinetic parameters are summarized in Table 2. The results were similar to the literature values [8,9]. Calibration standards of cefazedone were prepared at the concentration levels of 0.20, 0.81, 3.03, 10.11, 40.11, 120.34, 320.90 and 401.12 μg/mL. We compared the main pharmacokinetic data calculated by this calibration curve with that calculated using the calibration curve constructed without 320.90 μg/mL. The average of C_{max}, AUC_{0-12} and $AUC_{0-\infty}$ are 100.0%, 100.0% and 100.0% of that calculated using the latter calibration curve, respectively. It shows that there is almost no difference between the two calibration curves.

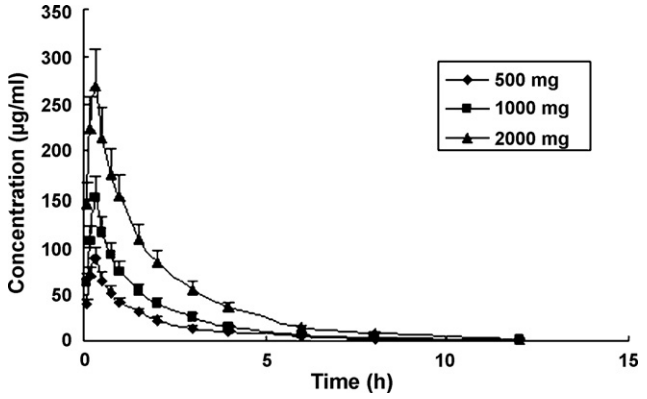

Fig. 2. Mean plasma concentration–time profiles of cefazedone after single dose of 500, 1000 and 2000 mg cefazedone injection to Chinese healthy volunteers ($n = 10$).

4. Conclusion

A rapid, sensitive and accurate method has been developed for determination of cefazedone in human plasma by LC–MS/MS with simple protein precipitation process and fully validated. Calibration curves were linear over a wide range of 0.20–401.12 μg/mL for

cefazedone in plasma with a LLOQ of 0.20 μg/mL. The method has been successfully applied to the pharmacokinetic study of cefazedone.

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