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Rapid and simple method for determination of cephradine in human plasma using liquid chromatography–tandem mass spectrometry (LC–MS/MS): Application to the bioequivalence study

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ABSTRACT

A rapid and simple procedure was developed for the determination of cephradine in human plasma using liquid chromatography coupled with electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS). After trichloroacetic acid (TCA) precipitation of proteins from plasma samples, cephradine and cefaclor (the internal standard; IS) were eluted on a CN column. The isocratic mobile phase used consisted of acetonitrile–water–formic acid (25:75:0.1, v/v/v). Cephradine and the IS were both detected in multiple reaction monitoring (MRM) mode at the transitions: m/z 350.0 → 90.8 for cephradine and m/z 368.1 → 106.0 for the IS, respectively. The calibration curve was linear over the concentration range from 0.05 to 50 μg/ml, and correlation coefficients were greater than 0.996. The coefficient of variation of assay precision was less than 9.36%, and its accuracy ranged from 87.92% to 111.16%. The chromatographic run time for each plasma sample was less than 3 min. The developed method was successfully applied to a bioequivalence study of cephradine in healthy male volunteers.

1. Introduction

Cephradine (6R,7R)-7-[[(2R)-2-amino-2-(1-cyclohexa-1,4-dienyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, a member of the first generation cephalosporins, is widely used clinically due to its activity against Gram-positive and Gram-negative microorganisms, attributed to its inhibition of bacterial cell wall synthesis. Cephradine is used to treat infections caused by bacteria, including upper respiratory infections, ear infections, skin infections, and urinary tract infections.

Cephradine is rapidly absorbed after oral administration in the fasting state. Following single dose of 500 mg to normal adult volunteers, its average peak serum concentration reaches approximately 16.5 μg/ml within an hour. In vitro studies performed using an ultracentrifugation technique showed that cephradine in serum does not pass across the blood–brain barrier to any appreciable extent. The presence of food in the gastrointestinal tract delays absorption, but does not affect the total amount of cephradine absorbed. Furthermore, over 90% of the drug is excreted unchanged in urine within 6 h of administration [1].

Several analytical methods have been reported for the determination of cephradine in pharmaceutical preparations and biological fluids. These methods include; spectrophotometry [2,3], spectrofluorometry [4,5], luminescence [6,7], capillary electrophoresis [8], and high-performance liquid chromatography [9–11]. However, most of these methods required laborious sample pretreatment and a long analysis time, and thus, are not convenient for analyzing the large numbers of samples generated during pharmacokinetic research.

Furthermore, no study has been previously performed on plasma cephradine determination in human plasma by LC–MS/MS. Therefore, we developed a rapid quantification method for quantifying cephradine in human plasma based on liquid chromatography/electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS). The devised method was found to be more selective and reliable, faster, and more straightforward than other reported methods.

This analytical procedure was fully validated and successfully used to assess the bioequivalence of two marketed pharmaceutical formulations of 500 mg cephradine capsule.

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2. Experimental

2.1. Materials and reagents

Cephradine standard was supplied from Choongwae Pharma Corporation (Seoul, Korea). Cephradine standard was checked by HPLC and was found to be >99% pure. Cefaclor (IS) standard and trichloroacetic acid (TCA) was used in preparation of the protein precipitant, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetonitrile of HPLC grade were purchased from J.T. Baker (Philipsburg, NJ, USA). Water was produced by a Milli-Q water system (Millipore Corp., Bedford, MA, USA) and all other chemicals and solvents were of the highest analytical grade available. The test medication, Il-sung cephradine capsule [500 mg cephradine capsule, Il-sung Pharm. Co. Ltd. (Seoul, Korea)] and the reference medication, Yuhan cephradine capsule [500 mg cephradine capsule, Yuhan Pharm. Co. Ltd. (Seoul, Korea)] were supplied in the form of capsules.

2.2. Instrumentation and analytical conditions

The liquid chromatography was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Inc., Palo Alto, CA, USA). The analytical column was a Capcell Pak CN (50 mm × 2.0 mm I.D., 3 μm, Shiseido, Tokyo, Japan). A mixture of acetonitrile–water–formic acid (25:75:0.1, v/v/v) was used as mobile phase at a flow rate of 0.2 ml/min for isocratic elution. The temperatures of the autosampler and column oven were 4 and 40 °C, respectively.

Detection of cephradine and IS was performed on triple quadrupole mass spectrometer API 2000 (Applied Biosystems/MDS SCIEX, Concord, ON, Canada) equipped with a TurboIonSpray ionization source operating in ESI positive ion mode. In order to optimize MS parameters, a solution of cephradine and IS was infused into the mass spectrometer and its mass spectrum obtained. Full-scan mass spectra of cephradine and the IS showed the protonated molecules ($[M+H]^+$) at m/z 350.0 and 368.1, respectively. Fragmentation of the protonated precursors showed that the most abundant ion in the full-scan MS/MS spectrum of cephradine was at m/z 90.8 and of the IS was at 106.0. Optimized parameters were as follows: curtain gas, 10 psi; nebulizer gas, 20 psi; turbo gas, 40 psi; IonSpray voltage, 5500 V; and source temperature 350 °C. The MRM mode was chosen to monitor the transitions of the protonated forms of cephradine at m/z 350.0 → 90.8 and IS at m/z 368.1 → 106.0, respectively. Data acquisition and analysis were processed using Analyst software (version 1.4.2).

2.3. Preparation of standard solutions and quality control samples

Standard stock solutions of cephradine and IS were both prepared in methanol at the concentration of 1000 μg/ml. Working standard solutions were serially diluted with methanol in the range from 0.5 to 500 μg/ml. The solution of IS was prepared in methanol with a concentration of 10 μg/ml. All solutions were stored at −70 °C until required. Calibration samples were prepared by spiking cephradine in blank plasma at 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25 and 50 μg/ml. Quality control (QC) samples with concentrations of 0.2, 20 and 40 μg/ml were prepared separately in the same manner. The calibration and QC samples were freshly prepared on each day of the analysis.

2.4. Sample preparation

Human plasma samples were stored at −80 °C and frozen plasma samples were thawed at room temperature before analysis. The IS solution (20 μl, 10 μg/ml in methanol) was added to 200 μl of plasma sample and the mixture was precipitated with 100 μl of 10% TCA aqueous solution. The mixture was vortex for 5 min and centrifuged (Eppendorf 5417R, Eppendorf AG, Germany) at 14,000 rpm for 5 min. Then supernatant was transferred into autosampler vials and aliquots (10 μl) were injected into the LC–MS/MS system using a temperature-controlled autosampling device (set at 4 °C).

2.5. Method validation

The method was validated for specificity, selectivity, linearity, sensitivity, accuracy, precision, recovery, matrix effect, process efficiency and stability according to the US Food and Drug Administration (USFDA) guidelines for Industry (Bioanalytical Method Validation) [12].

2.5.1. Specificity, linearity, precision and accuracy

The selectivity of the method towards endogenous plasma matrix components was assessed in double blank human plasma (not containing analyte and IS). Double blank plasma samples from six different volunteers were tested for the presence of endogenous compounds, which might interfere with analytes, using the described procedures and chromatographic/spectroscopic conditions. And then, it is compared with those obtained with a solution of the analyte at a concentration near the LLOQ. LLOQ was defined as ten times the signal to noise ratio (S/N).

2.5.2. Linearity

Linearity was determined to assess the performance of the method. The linearity of the calibration curves, ranging from 0.05 to 50 μg/ml was validated with nine different calibration curves. Calibration curves were constructed by using weighted ($1/x^2$) linear least-squares regression analysis plotting of peak–area ratio (cephradine/IS) versus the analyte concentrations. Calibration curves had to have correlation coefficients (r^2) of 0.99 or better.

2.5.3. Accuracy and precision

To evaluate the intra- and inter-day accuracy and precision of this method, plasma samples were analyzed at five concentration levels (0.05, 0.5, 5, 25 and 50 μg/ml). At least five replicates of each point were analyzed to determine the intra-day accuracy and precision. This process was repeated on five consecutive days in order to determine the inter-day accuracy and precision. Intra- and inter-day assay accuracies were expressed as percentages of theoretical concentration, as accuracy (%) = (found concentration/nominal concentration) × 100 and precisions were defined as coefficient of variation (C.V.). The acceptance criterion recommended by USFDA for each back-calculated standard concentration was a 15% deviation from the normal value except at the LLOQ, which was set at 20% [12].

2.5.4. Recovery, matrix effect and process efficiency

Recovery, matrix effect and process efficiency were evaluated at concentrations of 0.2, 20 and 40 μg/ml. Each set of samples was analyzed in triplicate. They were analyzed in:

A: standards extracted from spiked matrix;
B: standards spiked after post-extraction matrix;
C: standards in the pure solution.

The recovery was determined by comparing the peak areas of set A to the peak areas of set B. These set B represented 100% recovery. The matrix effect, the possible suppression or enhancement of ionization induced by the endogenous substances, was assessed by comparing the peak areas of set B with set C. The process efficiency,

defined as the overall extractability of the assay method, was calculated by comparing the peak area of set A with set C at the same concentrations.

2.5.5. Stability

Stabilities of cephradine in plasma were examined under different storage conditions described below using triplicates of QC samples. Test conditions included three freeze–thaw cycles, room temperature (25 °C) for 4 h, 4 °C for 24 h and −80 °C for 1 week. The autosampler stability was conducted by reanalyzing extracted QC samples kept under the autosampler conditions (4 °C) for 24 h. These results were compared with freshly prepared QC samples. For all assays, analyte was considered stable if the deviation was not greater than 15% from the initial values.

2.6. Bioequivalence study

The present method was applied to evaluate the bioequivalence of two capsule formulations of cephradine in 24 healthy adult Korean male volunteers with an average age of 24.3 years and an average of weight 65.8 kg. All of the participants signed a written consent form after they had been informed of the nature and details of the study in accordance with the Korean Guidelines for Bioequivalence Test [13]. Each volunteer was administrated a single dose of 500 mg cephradine orally in a two-period randomized crossover design with a 1 week washout period between doses. At 7.00 a.m., their median cubital vein was cannulated and 7 ml blood samples were drawn into heparinized tubes. The doses were taken at 8.00 a.m. of each dosing day along with 240 ml of tap water. At 4 h after the oral administration, all of the subjects were given standardized meals. The subjects were not allowed to remain in the supine position or to sleep until 8 h after the oral administration. Venous blood samples were collected in heparinized vacutainers before and 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4, 5 and 6 h after drug administration. All blood samples were centrifuged immediately, the plasma separated and stored at −80 °C until LC–MS/MS analysis.

Pharmacokinetic parameters including extrapolated AUC_{inf} (the area under the plasma concentration versus time curve from time 0 to infinity), AUC_{6h} (the area under the plasma concentration versus time curve from time 0 to 6 h), C_{max} (peak plasma concentration), T_{max} (time to C_{max}) and $t_{1/2}$ (elimination half-life) were calculated using BA Calc 2002 [13]. For the purpose of bioequivalence analysis AUC_{6h} and C_{max} were considered as primary variables. Bioequivalence of two formulations was assessed by means of an analysis of variance (ANOVA) for crossover design and calculating 90% confidence intervals (CIs) of the ratio of test/reference using log-transformed data. This study was approved by the Korean Food and Drug Administration (KFDA) and Kyung-Hee University Ethical Committee.

3. Results and discussion

3.1. Mass spectrometry

Mass spectrometric parameters were optimized to achieve the maximum abundances of product and fragment ions. Since cephradine and cefaclor (IS) each possess amino and carboxylic groups, it has mass spectrometric response either in the positive ionization or in the negative ionization, although signal intensity in positive mode is much higher than that in the negative mode. The precursor ions of cephradine and IS were formed using declustering potentials of 11 and 16 V, respectively, and their precursor ions were fragmented at collision energies of 69 and 33 eV by collision-activated dissociation with nitrogen as the collision gas at a pressure setting of 7 (arbitrary unit) on the instrument. Both quadrupoles were maintained at unit resolution. The product ion mass spectra and the postulated fragmentation patterns of cephradine and IS are illustrated in Fig. 1.

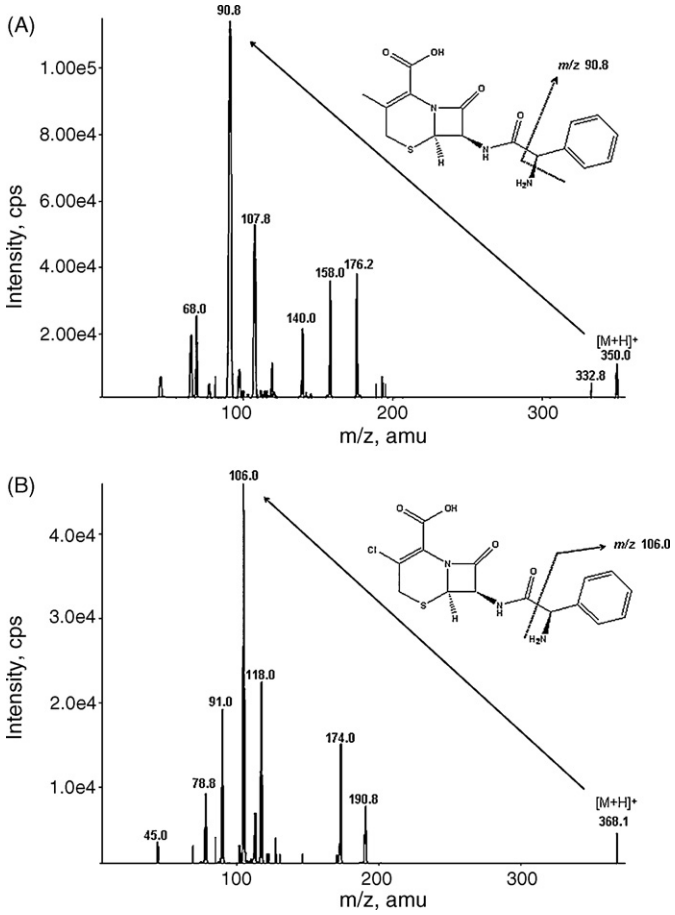

Fig. 1. Product ion mass spectra of the $[M+H]^+$ ions of (A) cephradine and (B) cefaclor (IS).

3.2. Chromatography

Various mobile phases and columns were investigated to reduce the ion suppression induced by endogenous substances. Due to similar structures for cephradine and IS, it was essential to have a chromatographic separation of the drugs so as to minimize any interference during quantitation. Chromatographic analysis of cephradine and IS was initiated under isocratic conditions to obtain adequate response, sharp peak shape and a short run time. Thus, separation was attempted using various combinations of methanol/acetonitrile, acidic buffers and additives like formic acid on different reversed-phase columns [Capcell Pak MGII C18 (50 mm × 2.0 mm I.D., 3 μm), Capcell Pak UG120 C18 (50 mm × 2.0 mm I.D., 3 μm), Hydrosphere C18 (50 mm × 2.0 mm I.D., 3 μm), Capcell Pak C8 (50 mm × 2.0 mm I.D., 3 μm), YMC-Pack C8 (50 mm × 2.0 mm I.D., 3 μm), YMC-Pack Pro C8 (50 mm × 2.0 mm I.D., 5 μm) and Capcell Pak CN (50 mm × 2.0 mm I.D., 3 μm)]. Best results in terms of reproducibility, complete separation and peak shape were obtained with Capcell Pak CN (50 mm × 2.0 mm I.D., 3 μm) column compared to others and hence was selected for further study. The analytes showed poor reproducibility for proposed linear range compared with Capcell Pak CN column that offered superior peak shape, efficient separation, desired linearity and reproducibility for cephradine and IS from endogenous plasma matrix.

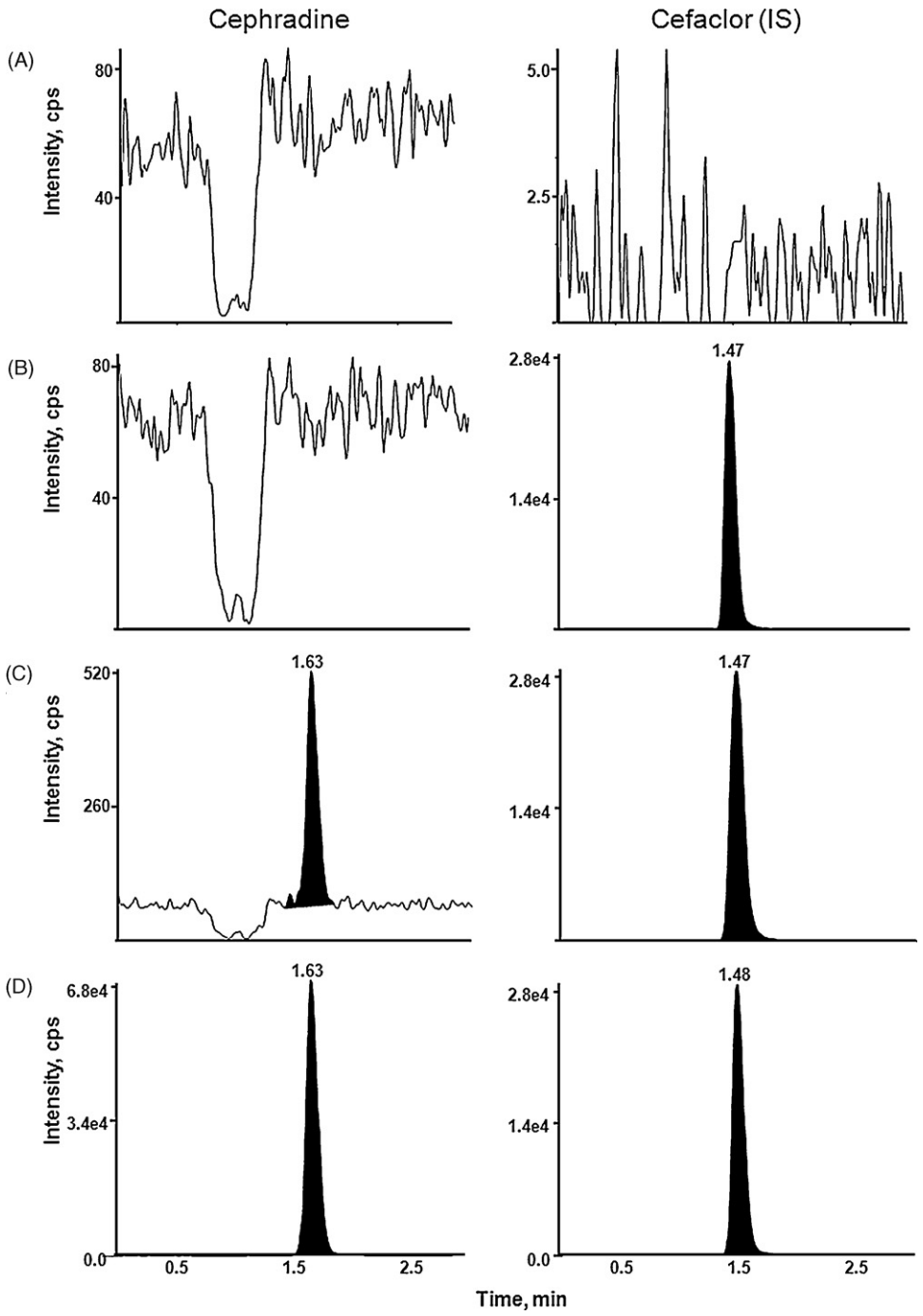

Fig. 2. Representative MRM chromatograms of (A) double blank human plasma, (B) blank human plasma spiked with IS, (C) blank human plasma spiked 0.05 μg/ml (LLOQ) of cephradine and IS, (D) plasma sample 0.5 h after an oral dose of 500 mg cephradine capsule to a volunteer (measured concentration 1.10 μg/ml).

Table 1
Intra- and inter-day assay precision and accuracy for LC–MS/MS assay of cephradine in human plasma ($n = 5$).

Nominal concentration (μg/ml)	Intra-day			Inter-day		
	Measured concentration (μg/ml) (mean ± SD)	C.V. (%)	Accuracy (%)	Measured concentration (μg/ml) (mean ± SD)	C.V. (%)	Accuracy (%)
0.05	0.05 ± 0.01	9.36	100.48	0.05 ± 0.01	8.97	103.30
0.5	0.55 ± 0.00	0.58	109.20	0.56 ± 0.01	1.57	111.16
5	5.02 ± 0.08	1.51	100.44	5.03 ± 0.04	0.81	100.69
25	21.98 ± 0.15	0.67	087.92	22.10 ± 0.18	0.80	088.38
50	46.56 ± 0.54	1.16	093.12	46.31 ± 0.38	0.81	092.62

Table 2
Recovery, matrix effect and process efficiency ($n = 3$).

		RE[a] (%)	ME[b] (%)	PE[c] (%)
Cephradine concentration (μg/ml)	0.2	79.10	86.36	68.31
	20	84.18	80.52	68.02
	40	85.80	81.60	70.02
	Mean	83.13	82.80	68.78
IS (cefaclor) concentration (μg/ml)	1	85.72	84.22	72.09

[a] Recovery was calculated using the following formula: RE (%) = $A/B \times 100$.
[b] Matrix effect was calculate using the following formula: ME (%) = $B/C \times 100$.
[c] Process efficiency was calculate using the following formula: PE (%) = $A/C \times 100$.

To evaluate the robustness of the analytical method, the effects of the most important chromatographic parameters, e.g., flow rate, column temperature and mobile phase pH, were deliberately changed. Each parameter was studied at a high level (flow 0.3 ml/min; column temperature 45 °C; mobile phase pH 2.5), an intermediate level (flow 0.2 ml/min; column temperature 40 °C; mobile phase pH 3.5) and low level (flow 0.1 ml/min; column temperature 35 °C; mobile phase pH 4.5) with respect to the value specified in the analytical method. The flow 0.2 ml/min; column temperature 40 °C; mobile phase pH 3.5 (acetonitrile–water–formic acid (25:75:0.1, v/v/v)) was found most suitable for eluting cephradine and IS at 1.63 and 1.47 min, respectively, which resulted in a total analytical run time of 3 min. Representative chromatograms of cephradine and IS at the LLOQ level of 0.05 μg/ml in human plasma are shown in Fig. 2.

3.3. Sample preparation

Protein precipitation is one of the most widely used biological sample pretreatment methodologies, and often allows rapid and high recoveries for the preparation of biological samples. Furthermore, the polar, water-soluble, and amphoteric characters of cephradine make it difficult to extract from plasma by techniques, such as, conventional liquid–liquid extraction. Thus, all plasma samples containing cephradine were prepared using a protein precipitation procedure. Various type of precipitation reagents (methanol, acetonitrile, perchloric acid, and TCA) were evaluated during the experiment. In the event, TCA was chosen as a precipitant because it provided good signal intensities, running time and peak shapes.

3.4. Validation

3.4.1. Specificity and selectivity

LC–MS/MS has high specificity because only ions derived from analytes of interest are monitored. Using six different lots of human plasma, double blank plasma, blank plasma, and plasma sample spiked with 0.05 μg/ml cephradine and IS were analyzed. A comparison of the chromatograms of the blank and spiked human plasma samples (Fig. 2) indicated the endogenous substances did not interfere with retention times of analyte or IS.

3.4.2. Linearity

To evaluate the linearity of the devised method, the ten-point calibration curves were determined on nine independent runs. The mean equation of the calibration curves obtained was $y = (0.000267 \pm 0.000002)x + (0.00427 \pm 0.00101)$, and correlation coefficients (r^2) were greater than 0.996 for all curves and the within- and between-run C.V.s of the response factors for the concentrations assayed were <15%. The calibration curves showed good linearity within the range 0.05–50 μg/ml.

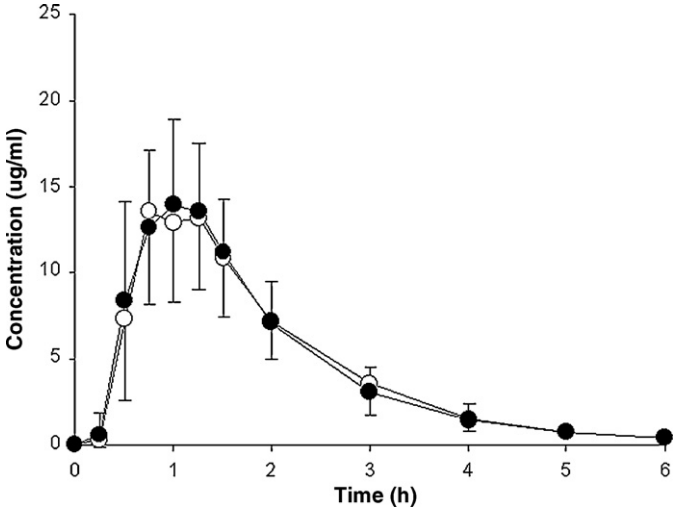

Fig. 3. Mean plasma concentration–time profile of cephradine from 24 healthy volunteers after oral administration of a single 500 mg cephradine capsule (●, reference formulation; ○, test formulation).

3.4.3. Precision and accuracy

Cephradine plasma samples at five concentration levels (0.05, 0.5, 5, 25, and 50 μg/ml) were analyzed for accuracy and precision. Assay performance data for cephradine are summarized in Table 1. For the devised assay, intra-day C.V. was <9.36%, inter-day C.V. was <8.97%, intra-day accuracy ranged from 87.92% to 109.20%, and inter-day accuracy from 88.38 to 111.16%. The data obtained for cephradine was within the acceptable precision and accuracy limits required to meet current guidelines for bioanalytical methods [12].

3.4.4. Recovery, matrix effect, and process efficiency

Recovery, matrix effect, and process efficiency results are presented in Table 2. Mean recoveries of cephradine at QC concentrations after TCA protein precipitation from human plasma were 79.10 ± 0.64%, 84.18 ± 3.17%, and 85.80 ± 1.49%, respectively. The mean matrix effect at concentrations of cephradine (0.2, 20, and 40 μg/ml) and cefaclor were 82.80% and 84.22%, respectively, which is well within acceptable limits (<20%). These results indicate the presence of a minimal matrix effect in terms of the cephradine/cefaclor response ratio. The devised method was able to reduce the matrix effect and achieved sufficient LLOQ signal intensity. Process efficiency is determined by a combination of matrix effects and analyte recovery from the matrix by sample extraction. The devised method showed adequate efficiency for cephradine in plasma matrix (68–70%). Through overall good process efficiency, the sensitivity of the devised method was found to be high enough to analyze samples during the bioequivalence study.

Table 3
The main pharmacokinetic parameter of cephradine after single oral dose of 500 mg to healthy volunteers (mean ± S.D, $n = 24$).

Pharmacokinetic parameter	Reference drug (mean ± SD)	Test drug (mean ± SD)
AUC_{inf} (μg h/ml)	27.52 ± 4.21	27.92 ± 4.30
AUC_{6h} (μg h/ml)	26.99 ± 4.18	27.30 ± 4.40
C_{max} (μg/ml)	16.30 ± 4.28	16.48 ± 4.55
T_{max} (h)	1.20 ± 0.52	1.13 ± 0.40
$t_{1/2}$ (h)	0.85 ± 0.11	0.93 ± 0.22

3.4.5. Stability

To evaluate the stability of cephradine in human plasma, drug-free plasma samples were spiked at 0.2, 20, and 40 μg/ml. After extraction, the samples were arranged in an autosampler and analyzed. After three freeze–thaw cycles, the cephradine stabilities at 0.2, 20, and 40 μg/ml were 85.22%, 87.04% and 87.86%, respectively. In 4 h at room temperature, the cephradine stabilities were 93.81%, 95.24% and 92.14%, respectively, and after 24 h at 4 °C they were 97.2%, 94.74% and 89.81%, respectively. Moreover, in 1 week at −80 °C, the stabilities were 92.45%, 90.47% and 88.72%, respectively. Thus in the stability study, cephradine in human plasma was found to be stable for 4 h at room temperature, 24 h at 4 °C, and 1 week at −80 °C. The autosampler stability (stability in post-extraction matrix) was conducted by reanalyzing extracted QC samples kept under the autosampler conditions (4 °C) for 24 h. The stabilities of cephradine (at 0.2, 20, and 40 μg/ml) in post-extraction matrix were 93.53%, 92.37% and 92.29%, respectively. No significant deterioration was observed under any of the examined conditions.

3.5. Bioequivalence study

The devised method was successfully used to analyze approximately 600 plasma samples in a bioequivalence study of cephradine. The mean plasma concentration–time curve of cephradine, after oral administration of 500 mg to 24 healthy volunteers is shown in Fig. 3, and the pharmacokinetic parameters derived from these profiles are presented in Table 3. The 90% CIs for the ratios of $AUC_{6\,h}$ and C_{max} were $\log 0.9808 - \log 1.0409$ and $\log 0.9108 - \log 1.0842$, respectively, which met the bioequivalence criteria of $\log 0.80 - \log 1.25$. This study showed that the test formulations and reference formulations were bioequivalent. These pharmacokinetic data were similar to previous literature [14]. It indicates that this method is well suited for routine high-throughput analyses, such as in a pharmacokinetic study and a bioequivalence study.

4. Conclusions

Here, we describe a rapid and convenient method for the determination of cephradine levels in human plasma. The devised method was found to meet all USFDA guidelines for bioanalytical method validation [12]. In addition, it was successfully applied to bioequivalence studies of cephradine. Finally, when compared with previously described methods, the devised method reduced analysis time and improved LLOQ, and was found to be more capable of analyzing large sample numbers.

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