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Selective method for the determination of cefdinir in human plasma using liquid chromatography electrospray ionization tandam mass spectrometry

Zhang-jing Chen*, Jing Zhang, Ji-cheng Yu, Guo-ying Cao, Xiao-jie Wu, Yao-guo Shi

Institute of Antibiotics, Huashan Hospital, Fudan University, 12 Wulumuqi Zhong Road, Shanghai 20040, China

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Abstract

A sensitive and selective liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method was developed for the determination of cefdinir in human plasma. After a simple protein precipitation using trichloracetic acid, the post-treatment samples were applied to a prepacked RP18 Waters SymmetryShield column interfaced with a triple quadrupole tandem mass spectrometer. Positive electrospray ionization was employed as the ionization source. The mobile phase consisted of methanol–water–formic acid (25:75:0.075, v/v/v). The analyte and I.S. cefaclor were both detected by the use of selected reaction monitoring mode. The method was linear in the concentration range of 5–2000 ng/ml. The lower limit of quantification was 5 ng/ml. The intra- and inter-day relative standard deviation across three validation runs over the entire concentration range was less than 4.3%. The accuracy determined at three concentrations (36, 360 and 1800 ng/ml for cefdinir) ranged from 99.6 to 106.7% in terms of recovery. The chromatographic run time for each plasma sample was less than 3 min. The method herein described was successfully applied for the evaluation of pharmacokinetic profiles of cefdinir capsule in 12 healthy volunteers.

Keywords: Cefdinir; LC; MS/MS; Human plasma; Method validation

1. Introduction

Cefdinir(6R-[6α,7β(Z)]-7)[[(2-amino-4-thiazolyl)(hydroxyimino)acetyl]amino]-3-ethenyl-8-oxo-5-thia-1azabicyclo [4.2.0]oct-2-ene-2-carboxylic acid; FK482, BMY-28488, CI983, PD134393) is an extended-spectrum, oral administration, third-generation cephem antimicrobial angent first synthesized in 1988 [1] and subsequently approved by the US Food and Drug Administration in 1997 for the treatment of community-acquired infection. The empirical formula of Cefdinir is $C_{14}H_{13}N_5O_5S_2$ with a molecular weight of 395.42 [2].

As with other cephalosporins, bactericidal activity of cefdinir results from the inhibition of cell wall synthesis. Cefdinir is stable in the presence of some, but not all, β-lactamase enzymes. As a result, many organisms resistant to penicillins and some cepholosporins are susceptible to cefdinir [3]. It has structural similarities to cefixime but with a hydroxyimino-aminothiazole side chain substituting the carboxymethoxyimino moiety (present in most orally-active cephalosporins) at the 7-position, thus sterically hinders the entry of the molecule into the active site of TEM-1 β-lactamase, and also enhances its activity against Gram-positive bacteria [4].

Cefdinir may be taken without regard to food. Following a single administration of 100 mg of cefdinir, C_{max}, T_{max}, $t_{1/2}$, and $AUC_{0-\infty}$ of cefdinir were 0.79 ± 0.19 μg/ml, 4.3 ± 0.5 h, 1.48 ± 0.12 h, and 4.04 ± 0.72 μg·h/ml, respectively [5]. Renal CL in healthy subjects was 89.2 ± 6.0 ml/min, and 30.8 ± 8.2% of each administered dose was excreted unchanged in the urine during the first 24 h.

Liquid chromatography is widely used for the quantitative determination of pharmaceutical compounds with UV, fluorescence or electrochemical detection. LC–MS with electrospray ionization (ESI) provide a rugged, sensitive and widely used technique to mass select a parent and a characteristic product ion of an analyte, making it a highly specific method for the determination of pharmaceutical compounds in human plasma.

Cefdinir were quantitated by either reversed-phase high-performance liquid chromatography (RP-HPLC) [6–9] or microbiological assay [10]. Up to now, there has no publica-

* Corresponding author. Fax: +86 2162488290.
E-mail address: chenzhangjing@fudan.edu.cn (Z.-j. Chen).

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tion on the determination of cefdinir using LC–MS/MS. As a result of lower oral dose, the plasma concentration of cefdinir is relatively lower than that of other cephalosporins such as cefaclor, thus LC–MS/MS may provide a highly sensitive assay for the quantitative determination of cefdinir in human plasma. In this paper, an LC/ESI-MS/MS method was developed to analyze cefdinir in human plasma using cefaclor as an internal standard, and simple precipitation of plasma proteins with trichloracetic acid (TCA) as a sample pretreatment procedure.

2. Experimental

2.1. Materials

Cefdinir (98.6% purity, Fig. 1) was a gift from Xi'an-Janssen Pharmaceutical Ltd. (Xi'an, China). Cefaclor (internal standard, 94% purity, Fig. 1) was obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol (HPLC grade) was obtained from Sigma–Aldrich Laborchemikalien GmbH (Germany). Acetic acid and ammonium acetate (HPLC grade) were obtained from Tedia Company (Fairfield, OH 45014). Formic acid, ammonium formate and TCA were of analytical grade. Formic acid and TCA were commercially obtained from Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China) while ammonium formate from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). A Milli-Q® (Millipore, France) water purification system was used to obtain the purified water for the HPLC analysis.

2.2. Instrumentation

A Thermo Finnigan TSQ Quantum tandem mass spectrometer equipped with an electrospray ionization (ESI) source (San Jose, CA, USA), a Waters 2690 Alliance high-performance liquid chromatography system (Waters, Milford, MA, USA) were used for LC–MS/MS analysis. Data acquisition was performed with Xcalibur 1.2 software (Thermo Finnigan). Peak integration and calibration were carried out using Finnigan LCQuan software.

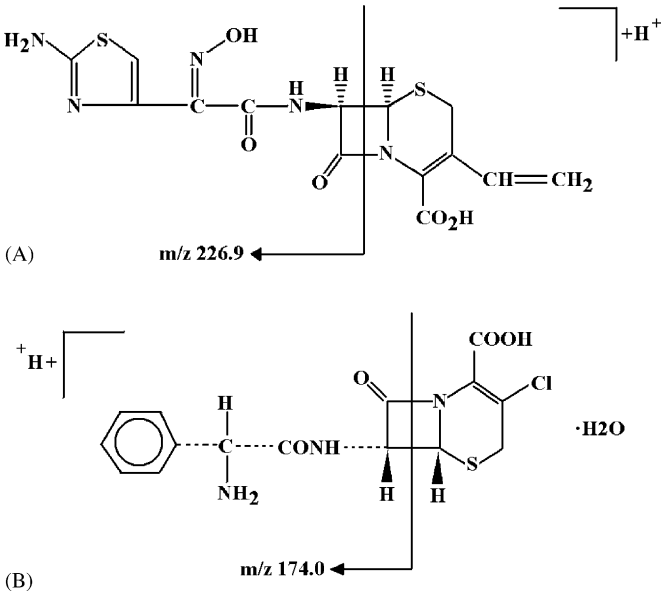

Fig. 1. Structural formulate of cefdinir (A) and cefaclor (B). Based on the chemical stability of these compounds and the mass charge ratio (*m/z*) of the fragment ion, we concluded that the fragmentation site should be the β-lactam ring indicated above.

2.3. LC–MS/MS conditions

The chromatographic separation was achieved on a prepacked RP_{18} Waters SymmetryShield column (5 μm, 50 mm × 2.1 mm, Waters, Milford, MA, USA) with a 0.5 μm high pressure biocompatible inline filter (UpchurchScientific, Oak Harbor, WA, USA), using a mobile phase of methanol–water–formic acid (25:75:0.075, v/v/v) degassed online. The liquid flow-rate was set at 0.2 ml/min. Separations were performed at room temperature.

Mass spectrometer was operated in the positive mode. Quantification was performed using selected reaction monitoring (SRM) of the transitions of *m/z* 396.1 → *m/z* 226.9 for cefdinir and *m/z* 368.1 → *m/z* 174.0 for cefaclor (I.S.), respectively, with a scan time of 0.5 s per transition. Fig. 2 shows the product ion spectra of $[M+H]^+$ of cefdinir and cefaclor.

In order to optimize all the MS parameters, standard solutions (1 μg/ml) of the analyte and I.S. were infused into the mass spectrometer. The signal was optimized on the total ion current in MS mode, producing a spray voltage of 4.5 kV, and a transfer capillary temperature of 350 °C. Nitrogen was used as the sheath gas (30 psi) and auxiliary gas (8 U, arbitrary units). For collision-induced dissociaton (CID), argon was used as the collision gas at a pressure of approximately 1 mTorr. The optimized collision energy of 20 and 15 eV was chosen for cefdinir and cefaclor, respectively.

2.4. Preparation of stock solutions

Stock solution of cefdinir was prepared at the concentration of 500 μg/ml in 10 mM buffered solution of ammonium acetate and stored at −80 °C. Working solutions of cefdinir were prepared daily in 10 mM ammonium acetate buffered solution by appropriate dilution to the final concentration of 0.05, 0.10, 0.50, 1.00, 5.00, 10.00 and 20.00 μg/ml.

The internal standard stock solution was prepared by dissolving 10.6 mg of cefaclor in 10 ml 10 mM buffered solution of ammonium acetate to produce a concentration of 1000.0 μg/ml. This solution was also stored at −80 °C. Working solutions of internal standard were prepared daily in 10 mM ammonium acetate buffered solution to produce a final concentration of 5 μg/ml.

2.5. Calibration curves and quality control samples

Calibration curves were prepared by spiking 10 μl of the abovementioned working solutions to 90 μl blank plasma to produce the calibration curve points equivalent to 5, 10, 50, 100, 500, 1000 and 2000 ng/ml of cefdinir. The quality control (QC) samples were separately prepared in blank plasma at the con-

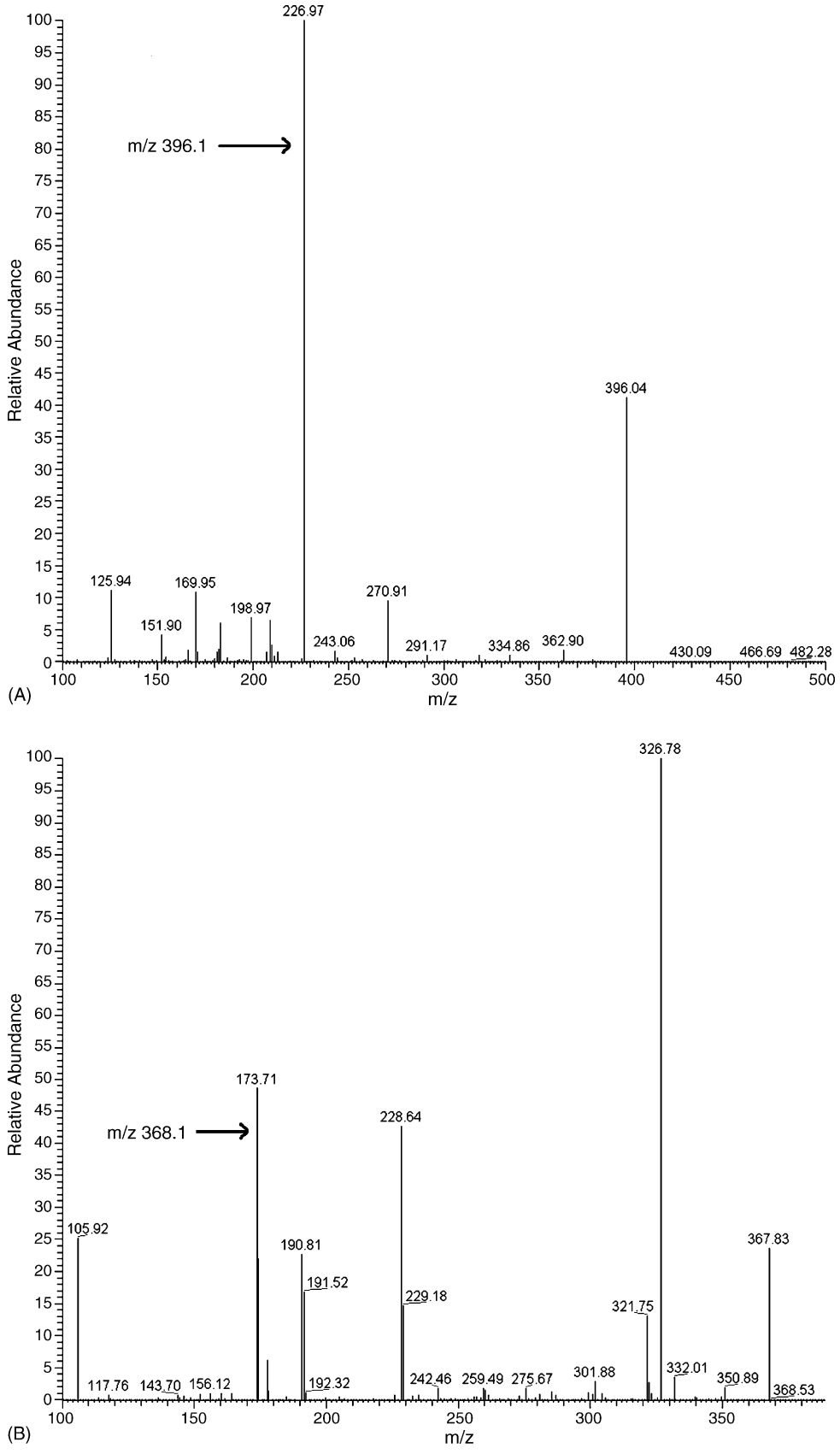

Fig. 2. Full-scan product ion spectra of $[M+H]^+$ of cefdinir (A) and cefaclor (B).

centrations of 36, 360 and 1800 ng/ml, respectively. The spiked plasma samples (standards and quality controls) were treated following the protein precipitation procedure on each analytical batch along with the unknown samples.

2.6. Pretreatment of plasma samples for LC–MS/MS analysis

To a 100 μl aliquot of plasma sample, 10 μl of I.S. (cefaclor 5 μg/ml) was added. The sample mixture was deproteinized with 40 μl of 10% TCA aqueous solution and vortex-mixed for approximately 1 min, allowed to stand for 5 min at ambient temperature and centrifuged for 10 min at 10,000 × *g*. Only 5 μl aliquot of the supernatant was injected into the HPLC column.

2.7. Method validation

The linearity of the method determining cefdinir in human plasma was tested at the concentration range of 5–2000 ng/ml. Calibration curves were prepared by measuring the peak area ratios (peak area analyte/peak area internal standard) versus the analyte concentrations in plasma, and fitted to the equation $y = a + bx$ by weighted least-squares regression (weighting = $1/x$). The standard curves were used to calculate concentrations of the analytes in unknown and QC samples from the measured peak area ratios.

To evaluate linearity, plasma calibration curves were prepared and assayed in duplicate on three different days. The accuracy and precision were also assessed by determining QC samples at three concentration levels on three separate validation days. The accuracy was expressed by (mean observed concentration)/(spiked concentration) × 100% and the precision was expressed by relative standard deviation (R.S.D.).

Absolute recoveries of cefdinir at three QC levels were measured by assaying the samples as described above and comparing the peak areas of both cefdinir and I.S. with those obtained from direct injection of the compounds dissolved in the supernatant of the processed blank plasma.

To evaluate the three freeze–thaw cycle stability and room temperature matrix stability, six replicates of QC samples at three levels were subjected to three freeze–thaw cycles or were stored at room temperature for 24 h before processing, respectively. Six replicates of QC samples were processed and stored in autosampler for 24 h, then assayed to assess processed sample stability. Stability is considered acceptable if the mean value is within 15% of the theoretical value at each concentration.

2.8. Pharmacokinetic study

The method was applied to a pharmacokinetic study of cefdinir approved by the Ethics Committee, in which 12 healthy volunteers were participated and administrated 200 mg of cefdinir. Cefdinir plasma concentrations were determined after 0–12 h of administration. The mean age of 12 male healthy volunteers was 27.50 ± 2.40 years, and the mean weight was 66.71 ± 3.67 kg. All the volunteers were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Three milliliters of venous blood were withdrawn from each volunteer before and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 h after the administration of cefdinir. Blood samples were transferred immediately to heparinized tubes, centrifuged for 10 min at 10,000 × *g* (at 20 °C). The separated plasma were transferred to Eppendorf tubes and stored at −40 °C until the day of analysis.

Pharmacokinetics parameters were determined from the plasma concentration-time data. The elimination half-life ($t_{1/2}$) was calculated using the one-compartmental model of DAS Pharmacokinetic Program (version 1.0, Anhui, China) on a personal computer. The area under the plasma concentration–time curve from zero to the last measurable plasma concentration point (AUC_{0-t}) was calculated by the linear trapezoidal method. Extrapolation to time infinity ($AUC_{0-\infty}$) was calculated as follows: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$, where C_t is the last measurable plasma concentration and k_e is the elimination rate constant.

3. Results and discussion

3.1. Mass spectrometer

ESI is a "soft" ionization technique that produces high mass-to-charge $[M+H]^+$ parent ion with minimal fragmentation of the analytes. Cefdinir and I.S. both gave protonated parent ion $[M+H]^+$ in the MS mode. The major ions observed were *m/z* 396.1 for cefdinir (Fig. 2A) and *m/z* 368.1 for the I.S. (Fig. 2B). The most intense product ion observed in the MS/MS spectra was *m/z* 226.9 for cefdinir and *m/z* 174.0 for the I.S. Additional tuning of the ESI source such as capillary temperature, flow of sheath and auxiliary gas (N_2) and spray voltage onto the transition *m/z* 396.1 → *m/z* 226.9 (cefdinir) and *m/z* 368.1 → *m/z* 174.0 (cefaclor) further improved the sensitivity. The corresponding spectra of cefdinir and the I.S. (cefaclor) are shown in Fig. 2.

3.2. Chromatography

In order to avoid ion suppression induced by endogenous substances, the influence of the mobile phase composed of different percentage of organic phase to ion suppression was evaluated during the experiment. When the percentage of organic phase reduced gradually below 50%, it was found that the composition of mobile phase methanol–water–formic acid (25:75:0.075, v/v/v) could provide good linearity between 5 and 2000 ng/ml for cefdinir. In positive ion mode, the presence of a low amount of formic acid in the mobile phase can improve the detection response of the analytes. Meanwhile, under the present chromatographic conditions, the run time of each sample was 3 min. The retention times were 2.4 and 2.2 min for cefdinir and cefaclor, respectively.

3.3. Pretreatment of plasma samples

Protein precipitation (PPT) is one of the most widely used biological sample pretreatment methodologies at the present. It

often provides rapid and higher recovery for the preparation of biological samples. Thus, all plasma samples containing cefdinir were prepared by protein precipitation procedure. Three types of precipitation reagents (methanol, acetonitrile, and TCA) were investigated during the experiment. The supernatant was directly injected into the LC–MS/MS system for analysis. TCA proves to be the best among the three reagents in terms of peak shape and the convenient of operation.

3.4. Method validation

3.4.1. Selectivity

The combination of HPLC with ESI-MS/MS leads to high selectivity and sensitivity. To test the specificity, six batches of blank human plasma and the corresponding spiked plasma were compared. Fig. 3 shows the typical SRM chromatograms of a blank, a spiked plasma sample with cefdinir (5 ng/ml) and I.S.

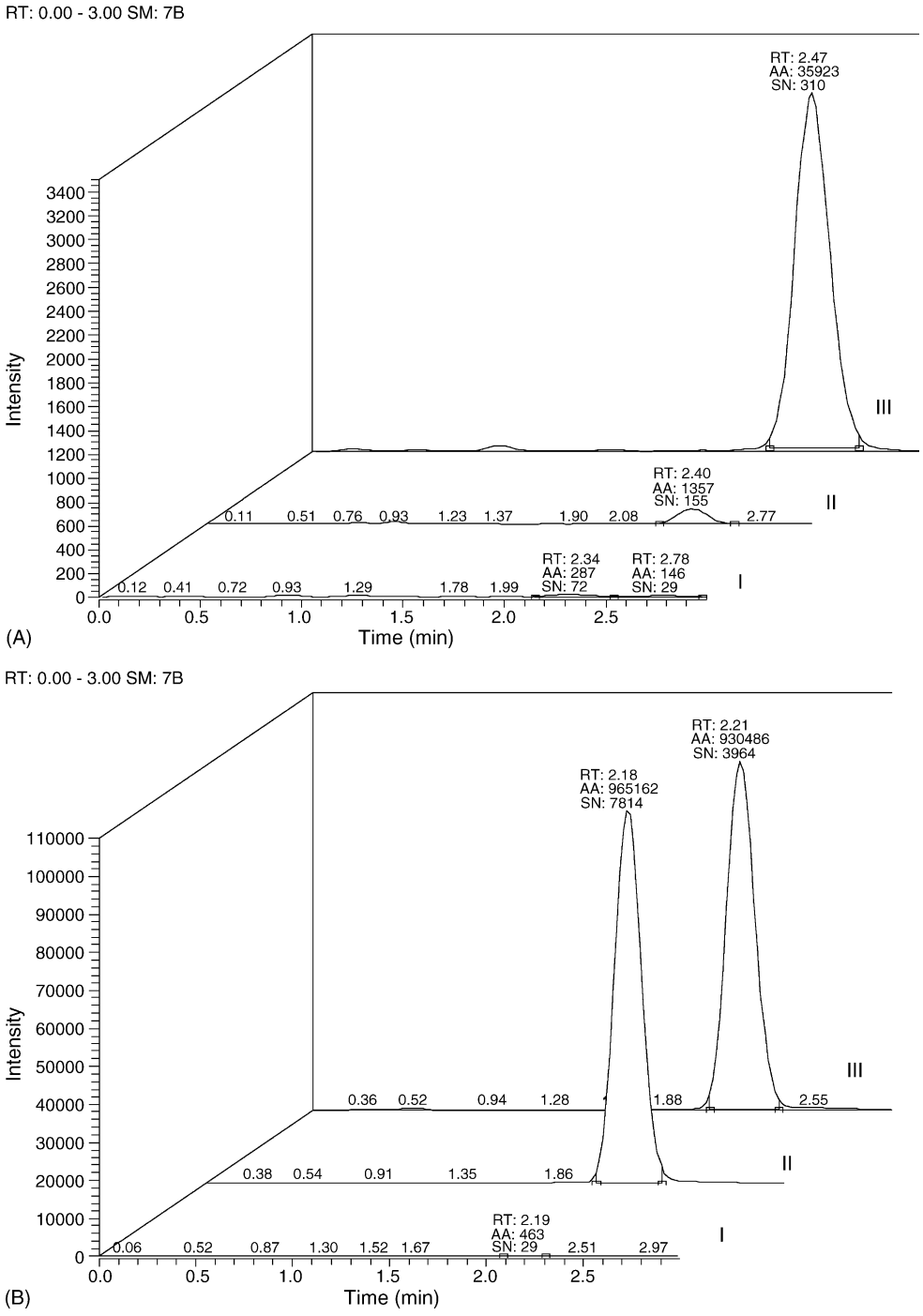

Fig. 3. Representative SRM chromatograms of cefdinir (A) and I.S. (cefaclor, B) in human plasma samples. (I) A blank plasma sample; (II) a blank plasma sample spiked with cefdinir at the LLOQ of 5 ng/ml and I.S. (5 μg/ml); (III) plasma sample from a volunteer 10 h after administration of 200 mg of cefdinir.

(5 μg/ml), and a plasma sample from a healthy volunteer 10 h after an oral administration. There was no significant interference or ion suppression from endogenous substances observed at the retention times of the analytes. Typical retention times for cefdinir and cefaclor were 2.4 and 2.2 min, respectively.

3.4.2. Linearity of calibration curves and lower limit of quantification

Linearity was tested for the range of concentrations 5–2000 ng/ml, employing standard calibration curves of at least 7 points (non-zero standards). The calibration range was selected according to the concentrations anticipated in the samples to be determined. In addition, a blank (non-spiked sample) and a zero plasma samples (only spiked with I.S.) were also analyzed to confirm the absence of interferences. These two samples were not used to construct the calibration curves. The method exhibited a good linear response for the range of concentrations from 5–2000 ng/ml. Correlation coefficient (r) and the coefficient of determination (r^2) were greater than 0.999. Typical standard curve was $y = -2.6907 \times 10^{-3} + 6.3843x$ where y represents the ratios of cefdinir peak area to that of I.S. and x represents the plasma concentrations of cefdinir.

The lower limit of quantification was defined as the lowest concentration on the calibration curve for which an acceptable accuracy of ±15% and a precision below 15% were obtained. The present LC–MS/MS method offered an LLOQ of 5 ng/ml in 100 μl plasma sample. Under the present LLOQ of 5 ng/ml, the cefdinir concentration can be determined in plasma samples until 12 h after a single oral dose of 200 mg cefdinir, which is sensitive enough to investigate the pharmacokinetic profiles of cefdinir, to establish the relationship between dose and pharmacological effect in human.

3.4.3. Precision and accuracy

The intra-day precision and accuracy of the assay was evaluated by analyzing six spiked samples of cefdinir at each QC level (36, 360 and 1800 ng/ml). Intra-day precision (R.S.D.%) of the method range from 2.2 to 4.3%, while the intra-day accuracy ranged from 100.7 to 106.7% for each QC level of cefdinir.

The inter-day precision and accuracy was determined over three days by analyzing 54 QC samples. The inter-day precision range from 2.9 to 3.3%, while the inter-day accuracy ranged from 99.6 to 104.6% for each QC level.

The results above demonstrated that the values were within the acceptable range, which establish that the deviation values should be within ±15% of the actual values, and the method was accurate and precise.

3.4.4. Recovery and stability

The absolute recovery of cefdinir, determined at three concentrations (36, 360 and 1800 ng/ml), were 80.0 ± 1.7%, 84.1 ± 2.4% and 76.3 ± 1.9% ($n = 6$), respectively. The recovery of cefaclor was investigated as 88.9 ± 4.1% ($n = 18$).

The stability of the analytes in human plasma under different temperature and timing conditions was evaluated as follows:

The stability of cefdinir plasma sample at three QC levels over three cycles of freeze (−40 °C) and thawing (room temperature) was assessed. The mean recoveries of the low, medium and high QC levels ranged from 101.9 to 105.8%, which indicated that the analyte is stable in human plasma for three cycles of freeze and thaw, when stored at −40 °C and thawed to room temperature.

For short-term stability determination, stored plasma aliquots were kept at ambient temperature for 12 h exceeding that expected to be encountered during the routine sample preparation. Samples were pretreated and analyzed as abovementioned. The mean recoveries of the low, medium and high QC levels ranged from 99.7 to 101.8%. These results indicated reliable stability behavior under the experimental conditions of the regular runs.

The post-preparative stability of QC samples kept in the autosampler for 12 h at 20 °C was also assessed. The mean recoveries of the low, medium and high QC levels ranged from 100.6 to 105.4%. These results showed that cefdinir and I.S. can remain stable at the autosampler temperature (20 °C) for at least 12 h, without showing significant loss in the quantified values, indicating that samples should be processed within this period of time.

3.4.5. Application of the method to a pharmacokinetic study in healthy volunteers

The method was applied to determine the plasma concentration of cefdinir after an oral administration of 200 mg cefdinir to 12 volunteers. The mean plasma concentration–time profile of cefdinir was best fitted to a one-compartment model using DAS Pharmacokinetic Program (Fig. 4). The main pharmacokinetic parameters of cefdinir in 12 volunteers were calculated. After oral administration of 200 mg cefdinir, the median of T_{max} and the mean of C_{max} were found to be 4 h (range 3–5 h) and 1.45 ± 0.32 μg/ml, respectively. Plasma concentrations declined with $t_{1/2}$ of 1.93 ± 0.29 h. The AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 6.99 ± 1.60 and 7.19 ± 1.62 μg·h/ml, respectively.

Because of the relatively short chromatographic run time and straightforward sample preparation procedure, a sample throughput of 120 per day was routinely achieved. This simple and selective method for the determination of cefdinir in human plasma was readily applicable to the clinical pharmacokinetic study for cefdinir.

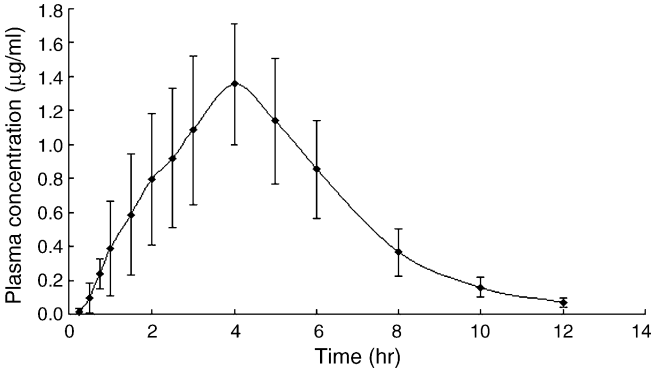

Fig. 4. Mean plasma concentration–time profile of cefdinir after an oral administration of 200 mg cefdinir to 12 healthy volunteers. Each point represents the mean ± S.D. ($n = 12$).

4. Conclusion

A rapid, sensitive and selective LC–MS/MS method for the quantification of cefdinir in human plasma, with a LLOQ of 5 ng/ml using 100 μl human plasma, was developed and validated. Prior to analysis, the plasma proteins are removed by protein precipitation using a TCA solution. Sample preparation is therefore simple and not labor intensive. The determination of one plasma sample needs 3 min. This method was successfully applied for the evaluation of pharmacokinetic profiles of cefdinir capsule in 12 healthy volunteers. These results indicated that it is suitable for routine analysis of large batches of biological samples.

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