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Determination of cefaclor in human plasma by a sensitive and specific liquid chromatographic–tandem mass spectrometric method

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Abstract

A sensitive and specific liquid chromatographic–tandem mass spectrometric method is described for the determination of cefaclor in human plasma. The plasma samples were treated by two sample preparation procedures, i.e. protein precipitation (PPT) and solid-phase extraction (SPE). The pretreated samples were analyzed on a C_{18} HPLC column interfaced with a triple quadrupole tandem mass spectrometer. Positive electrospray ionization (ESI) was employed as the ionization source. The analyte and internal standard ampicillin (for PPT) or cefetamet (for SPE) were detected by use of selected reaction monitoring (SRM) mode. The lower limit of quantitation obtained as a result of the PPT procedure was 100 ng/ml. The intra- and inter-run precision, calculated from quality control (QC) samples was less than 12% for cefaclor. The accuracy as determined from QC samples was within ±3% for the analyte. The SPE procedure could provide the lower limit of quantitation of 2 ng/ml. The precision and accuracy were measured to be below 7.1% and between −3.6% and 1.1%, respectively, for all QC samples. The method was applied for the evaluation of the pharmacokinetic profiles of cefaclor sustained-release formulation.

Keywords: Cefaclor

1. Introduction

Cefaclor is a second generation semi-synthetic, orally administration cephalosporin antibiotic. It has a broad antibacterial spectrum against various gram-positive bacteria and gram-negative bacteria such as *Haemophilus influenzae* and *Klebsiella* species. Cefaclor is extensively absorbed from the gastrointestinal tract after oral administration [1,2]. Peak concentrations in serum are attained within 30–60 min. The plasma half-life after oral administration is 0.5–0.7 h. The conventional immediate release formulation is administered three times daily. Plasma samples collected 0–6 h after administration could be sufficient for pharmacokinetic studies. The microbiological agar diffusion assay methods [3,4] and HPLC–UV methods [5–7] have been previously reported for the pharmacokinetic studies.

Cefaclor advanced formulation (AF), a new sus-

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tained-release formulation of cefaclor, provides a lower but more sustained plasma level than the immediate release formulation. It makes twice-daily dosing possible [8–10]. To support pharmacokinetic studies of cefaclor AF, an analytic method with sufficient sensitivity and specificity is needed to monitor plasma concentrations until 12 h after oral administration.

In this paper, we report a liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method which provides reliable and sensitive quantitation of cefaclor in human plasma to an lower limit of quantitation (LLOQ) of 2.0 ng/ml using 0.5 ml of plasma. This assay method was successfully applied to a pharmacokinetic study after single as well as multiple dose administration of 375 mg cefaclor AF to healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Cefaclor (99.5%) was obtained from Eli Lilly (Indianapolis, IN, USA). Ampicillin trihydrate (internal standard A, I.S. A) and cefetamet (I.S. B) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) (Fig. 1). Methanol was of HPLC-grade, and other chemicals used were of analytical grade. Blank (drug free) human plasma was obtained from Shenyang Blood Donor Service (China). Distilled water, prepared from demineralized water, was used throughout the study.

2.2. Instrumentation

A Finnigan TSQ™ tandem mass spectrometer equipped with an electrospray ionization (ESI) source (San Jose, CA, USA) and a Shimadzu LC-10AD pump (Kyoto, Japan) were used for LC–MS–MS analyses. The data processing was carried out using Finnigan LCQuan data analysis program.

2.3. Chromatographic conditions

Chromatographic analyses were performed using a Diamonsil C_{18} column (250×4.6 mm, 5 μm; Dikma, Beijing, China) and a SecurityGuard C_{18} guard column (4×3.0 mm I.D.; Phenomenex, Torrance, CA, USA). The mobile phase consisted of methanol–water–formic acid (80:20:1, v/v), delivered at a flow-rate of 0.55 ml/min.

2.4. Mass spectrometric conditions

The HPLC system was connected to the mass spectrometer via an ESI source. The mass spectrometer was operated in the positive ion detection mode with the spray voltage set at 4.5 kV. Nitrogen was used as the sheath gas (80 p.s.i) and auxiliary gas (3 l/min) for nebulization. The heated capillary temperature was set to 280 °C. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of ~1.4 mTorr. Quantitation was performed using selected reaction monitoring (SRM) of the transitions of m/z 368→m/z 174 for cefaclor, m/z 350→m/z 106 for ampicillin (I.S. A), and m/z 398→m/z 241 for cefetamet (I.S. B), respectively, with a scan time of 0.3 s per transition. The

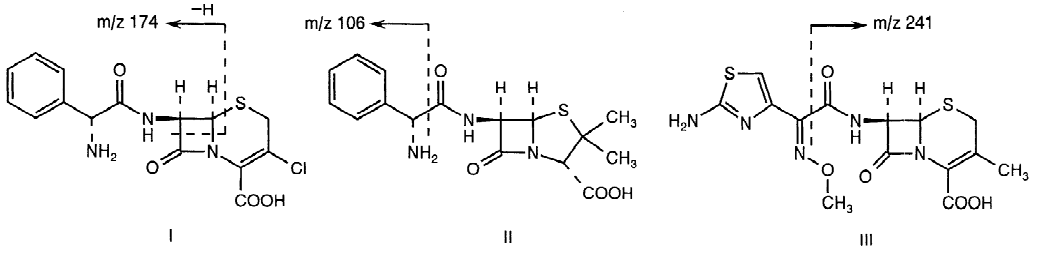

Fig. 1. Structures of cefaclor (I), ampicillin (II, internal standard A) and cefetamet (III, internal standard B).

optimized collision energy of 20 eV was used for the analyte and I.S. compounds.

2.5. Sample preparation

2.5.1. Protein precipitation procedure

To a 0.5-ml aliquot of plasma were added 100 μl of internal standard A (10 μg/ml ampicillin prepared in methanol). The sample mixture was deproteinized with 1 ml of formic acid–methanol (1:100, v/v) and the precipitate was removed by centrifugation at 2000 *g* for 10 min. Then 250 μl of the supernatant were transferred into a glass test tube containing 250 μl of methanol–water–formic acid (70:30:1, v/v), and vortex-mixed. A 20-μl aliquot of the sample solution was subjected to LC–MS–MS analysis.

2.5.2. Solid-phase extraction procedure

To a 0.5-ml aliquot of plasma were added 100 μl of internal standard B (2 μg/ml cefetamet, freshly prepared in water every day). The samples were acidified by addition of 100 μl of 5% acetic acid solution and transferred to Supelclean LC-18 solid-phase extraction tubes (3 ml; Supelco, Bellefonte, PA, USA). The cartridge was pre-treated sequentially with 2×1 ml of methanol and 2×1 ml of water. After loading the plasma sample, the cartridge was washed with 2×1 ml of water. Cefaclor and I.S. were eluted with 1 ml of methanol containing 1% formic acid. The eluate was evaporated to dryness at 40 °C under a gentle stream of nitrogen, and the residue was reconstituted by addition of 100 μl of the mobile phase. A 20-μl aliquot of the solution was injected into the LC–MS–MS system.

2.6. Calibration standards and quality control samples

Blank plasma samples were adjusted to pH≤4.5 with glacial acetic acid and stored frozen at −20 °C until needed. Working standard solutions (100 μl) of cefaclor were added to the acidified blank plasma (0.5 ml) to make a calibration standard over the range of 2–8000 ng/ml. Quality control (QC) samples were made using the pooled plasma at 5, 24, 100, 200, 800 and 6400 ng/ml. The spiked samples were then treated following the protein precipitation procedure or SPE procedure described above.

2.7. Method validation

Plasma samples were quantified using the ratio of the peak area of cefaclor to that of I.S. as the assay parameter. Peak area ratios were plotted against concentrations and cefaclor concentrations were calculated using weighted ($1/x^2$) least squares linear regression.

To evaluate linearity, plasma calibration curves of each preparation procedure were prepared and assayed in triplicate on 3 separate days. Accuracy and precision were also assessed by determining QC samples at three concentration levels (Tables 1 and 2, six samples each) on 3 different validation days. The accuracy was expressed by (mean observed concentration)/(spiked concentration)×100% and the precision by relative standard deviation (RSD).

The SPE recoveries of cefaclor at three QC levels were determined by comparing the peak area ratios of analyte to internal standard in sample that had been spiked with analyte prior to extraction with samples to which the analyte had been added post-extraction. The internal standards were added to both sets of samples post-extraction.

The stability of cefaclor in the reconstituted solution obtained by PPT procedure was assessed by placing QC samples at three concentrations under ambient conditions for 24 h. The stability of cefaclor when SPE eluates were concentrated was determined by placing QC samples at three concentrations at 40 °C under a gentle stream of nitrogen for 1 h.

2.8. Application of the assay

To demonstrate the reliability of this method for the study of pharmacokinetics of cefaclor AF, it was

Table 1
Summary of precision and accuracy from QC samples prepared by PPT procedure ($n=3$ day, six replicates per day)

Added, *C* (ng/ml)	Found, *C* (ng/ml)	Intra-run, RSD (%)	Inter-run, RSD (%)	Relative error, (%)
100.0	102.8	6.0	4.9	2.8
800.0	817.2	6.6	8.3	2.1
6400.0	6353.5	3.7	11.4	−0.7

Table 2
Summary of precision and accuracy from QC samples prepared by SPE procedure ($n=3$ day, six replicates per day)

Added, C (ng/ml)	Found, C (ng/ml)	Intra-run, RSD (%)	Inter-run, RSD (%)	Relative error, (%)
5.00	5.06	6.0	1.2	1.1
24.0	23.6	7.1	6.3	−1.5
200.0	192.8	6.9	7.0	−3.6

used to determine cefaclor concentrations in plasma samples 0–12 h after administration of 375 mg cefaclor AF to 20 healthy volunteers. Blood samples were drawn at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8 and 12 h after oral administration and were immediately centrifuged to separate the plasma fractions. The obtained plasma samples (2 ml) was acidified with glacial acetic acid (200 μl) to pH<4.5 to increase the stability of cefaclor [10] and stored at −20 °C until analysis.

3. Results and discussion

3.1. LC–MS–MS optimization

To determine cefaclor using the SRM mode, full scan and product ion spectra of cefaclor and two internal standard compounds were investigated under the present HPLC conditions. Since cefaclor and I.S. compounds are all amphoteric, containing a carboxyl group and an amino group, the possibility of using positive or negative ion detection was first evaluated. It was found that positive ESI could offer higher sensitivities than negative ESI. By positive ESI, the analyte and each I.S. form predominately protonated molecules $[M+H]^+$ in full scan spectra. Fig. 2 displays product ion spectra of $[M+H]^+$ ions from three compounds. Several fragment ions were observed in the product ion spectra. The major fragment ions at m/z 174, 106 and 241 were chosen in the SRM acquisition for cefaclor, ampicillin and cefetamet, respectively.

3.2. Preparation of plasma samples

Sample preparation is a critical step for accurate and reliable LC–MS–MS assays. Currently, the most widely employed biological sample preparation methodologies are protein precipitation (PPT), solid-phase extraction (SPE), and liquid–liquid extraction

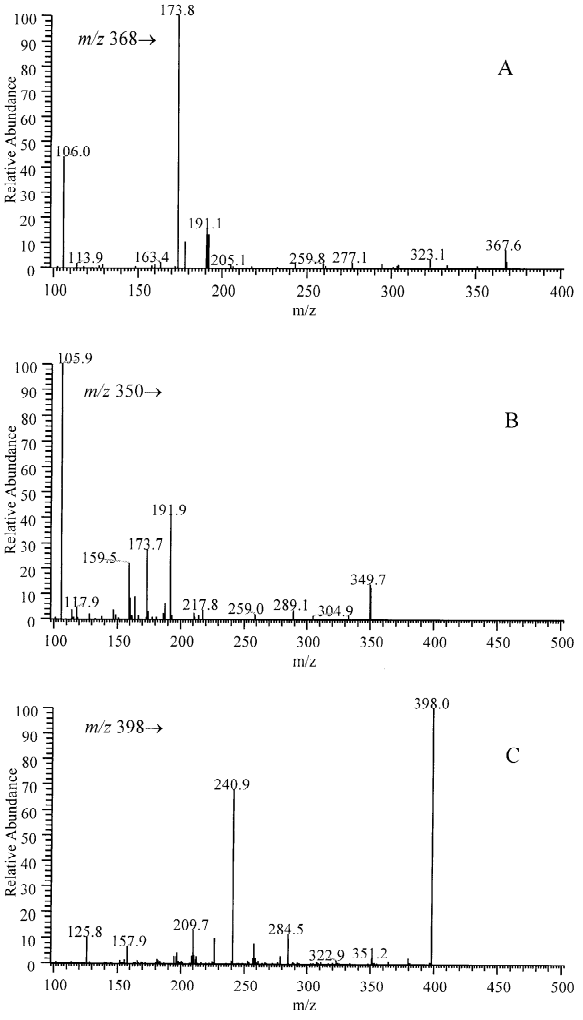

Fig. 2. Full-scan product ion spectra of $[M+H]^+$ of (A) cefaclor, (B) ampicillin and (C) cefetamet.

(LLE). The polar and amphoteric character of cefaclor makes it difficult to extract from plasma with organic solvents. Thus, the plasma samples containing cefaclor were prepared by PPT or SPE procedure.

As PPT procedure has the advantages of simplicity and universality for drug molecules in plasma, our initial approach of developing an assay for cefaclor in plasma was based on protein precipitation. The solvent selected was methanol instead of acetonitrile, which ensured that the organic content of the sample was approximately equal or less than that of the mobile phase to avoid chromatographic peak distortion. It was found that the supernatant easily formed a jelly after plasma proteins were precipitated, which could be attributed to the acidification of plasma samples before storing. To avoid jelly formation, the supernatant had to be diluted further by methanol–water–formic acid. In the PPT procedure, ampicillin served as internal standard because its chromatographic behavior was close to that of the analyte.

The PPT procedure minimized sample processing time due to simplicity. The preparation of a batch (16 plasma samples) could be completed within 15 min. The method provided LLOQ of 100 ng/ml, which was more sensitive than that of HPLC reported in the literature, but still not sufficient for the determination of trough concentrations after administration of cefaclor sustained-release formulation. For this reason, we developed a more sensitive sample preparation procedure with SPE. The SPE could greatly reduce the plasma background, compared with the PPT procedure. To obtain high sensitivity, the SPE eluate had to be concentrated, which increased the time of sample preparation. An LLOQ of 2 ng/ml was thus achieved.

In the SPE procedure, ampicillin was not retained on Bond-Elut C_{18} solid-phase extraction cartridges. So cefetamet was chosen as the internal standard to obtain good precision and accuracy, although it increased chromatographic time.

To reduce analytical time, the plasma samples collected 0–6 h after administration of cefaclor sustained-release formulation were prepared by the PPT procedure, and plasma samples 8–12 h after administration and plasma samples with a concentrations of <100 ng/ml were treated by the SPE procedure in the pharmacokinetic study.

3.3. Method validation

3.3.1. Specificity

Potential interference from endogenous compounds was investigated by the analysis of six different sources of human plasma. Representative chromatograms of a blank plasma sample, a blank plasma sample spiked with cefaclor at the LLOQ and I.S., and a volunteer sample for each preparation procedure are shown in Figs. 3 and 4. No interferences from endogenous substances with analyte or I.S. were detected.

3.3.2. Linearity and lower limit of quantitation

Visual inspection of the plotted triplicate calibration curves and correlation coefficients >0.99 confirmed that the calibration curves were linear over the concentration ranges 100–8000 ng/ml for the PPT procedure and 2–200 ng/ml for the SPE procedure.

The limit of quantitation (LOQ) is defined as the lowest concentration on the calibration curve for which an acceptable accuracy of $\pm 15\%$ and a precision below 15% were obtained. The current assay had an LLOQ of 100 ng/ml for PPT procedure and 2 ng/ml for SPE procedure.

3.3.3. Precision and accuracy

The method showed very good precision and accuracy. Tables 1 and 2 summarize the intra- and inter-assay precision and accuracy for cefaclor from QC samples prepared by PPT and SPE procedures, respectively. The results were calculated using one-way ANOVA. For PPT procedure, the intra- and inter-assay precision were measured to be below 7 and 12%, respectively. The inter-assay accuracy ranged from -0.7% to 2.8%. For SPE procedure, the intra- and inter-assay precision were less than 7.1%. The accuracy ranged from -3.6% to 1.1%.

3.3.4. Extraction recovery and storage stability

The SPE recoveries of cefaclor at concentrations of 5.0, 24.0 and 200 ng/ml were determined to be 60.6 ± 3.3, 57.0 ± 4.4, and $56.8\pm4.8\%$, respectively; and the extraction recoveries of I.S. (cefetamet) were $63.6\pm6.4\%$ at concentrations of 400 ng/ml.

According to Kovach et al. [11], cefaclor in plasma showed significant degradation at room tem-

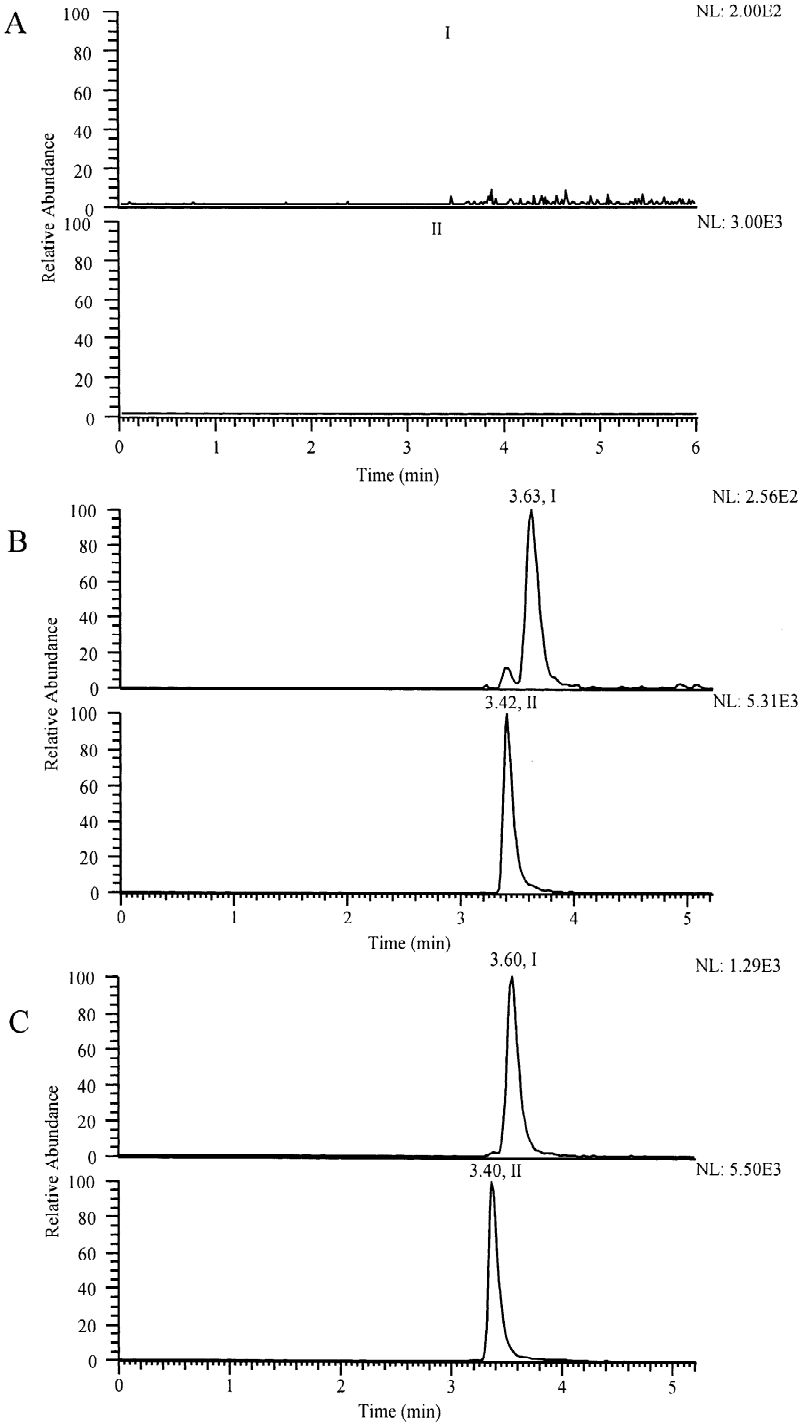

Fig. 3. Representative SRM chromatograms of cefaclor plasma samples prepared by PPT procedure. (A) A blank plasma sample; (B) a blank plasma sample spiked with cefaclor at the LLOQ of 100 ng/ml and ampicillin (I.S., 2 μg/ml); (C) plasma sample from a volunteer 5.0 h after administration of 375 mg of cefaclor AF. Peak I, cefaclor; peak II, ampicillin.

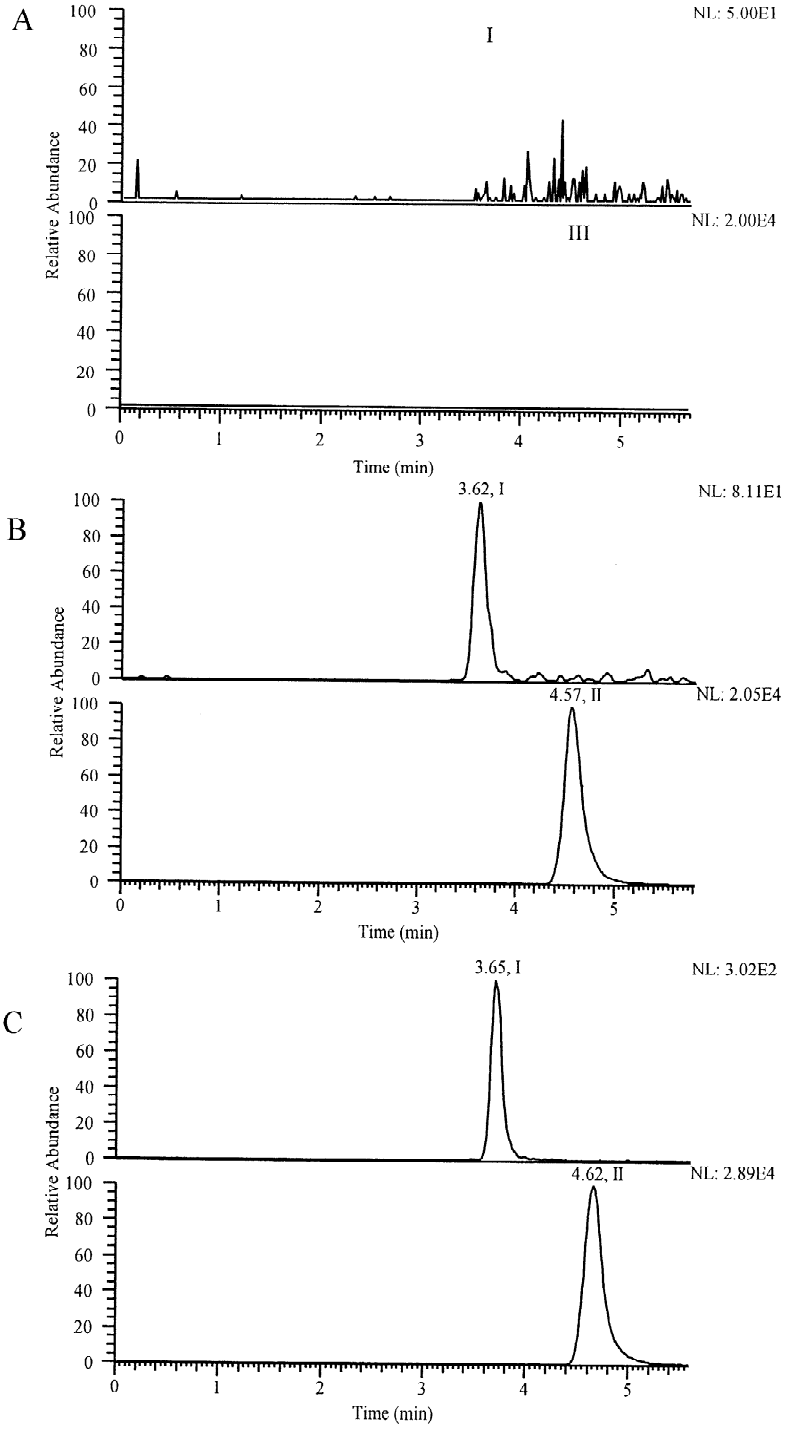

Fig. 4. Representative SRM chromatograms of cefaclor plasma samples prepared by SPE procedure. (A) A blank plasma sample; (B) a blank plasma sample spiked with cefaclor at the LLOQ of 2.0 ng/ml and cefetamet (I.S., 400 ng/ml); (C) plasma sample from a volunteer 12.0 h after administration of 375 mg of cefaclor AF. Peak I, cefaclor; peak II, cefetamet.

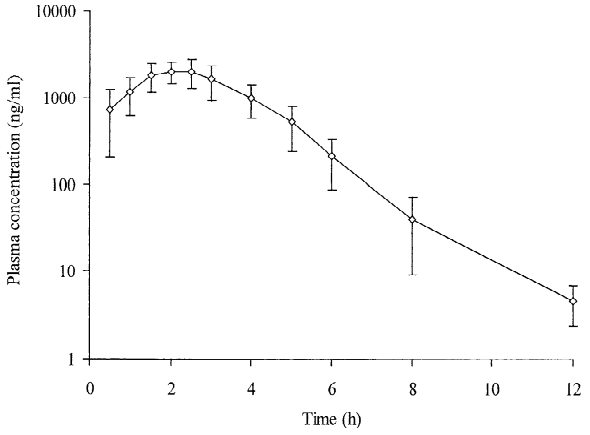

Fig. 5. Mean plasma concentration-time profile of cefaclor after an oral administration of 375 mg cefaclor AF to 20 healthy volunteers. Each point represents the mean±SD (n=20).

perature and at −20 °C. However, acidification of the plasma (pH≤4.5) retards the degradation of cefaclor in frozen plasma for at least 4 months. In our experiment, blood samples collected from volunteers were centrifuged within 20 min. The obtained plasma samples (2 ml) were acidified by glacial acetic acid (200 μl) and stored at −20 °C until analysis. The stability of cefaclor under process conditions was evaluated. It was found that cefaclor and internal standards were stable in a reconstitution solution of methanol–water−formic acid (pH<4) for at least 24 h at room temperature. The analyte was also found to be stable over 1 h in acidic methanol at 40 °C under a gentle stream of nitrogen.

3.4. Pharmacokinetic study

The mean plasma concentration−time curve of cefaclor after an oral administration of cefaclor AF (375 mg) to 20 healthy volunteers is shown in Fig. 5. The mean plasma concentration of cefaclor at 12 h following administration was 4.6±2.2 ng/ml, which was higher than the LLOQ.

4. Conclusions

A sensitive LC–ESI-MS–MS assay for cefaclor in human plasma has been developed. The two sample preparation procedures, PPT and SPE, were applied to the analyte in plasma. The PPT procedure with a LLOQ of 100 ng/ml provided simple preparation and reduced the preparation time, which was more sensitive than the reported methods [3–7]. The SPE procedure provides much higher sensitivity with the LLOQ of 2 ng/ml and was successfully applied to determination of trough concentrations of cefaclor AF.

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References

[1] G.D. Sides, T.R. Franson, K.A. DeSante, H.R. Black, Clin. Ther. 11 (1988) 5.
[2] H. Sourgen, H. Derendorf, H. Schifferen, Int. J. Clin. Pharmacol. Ther. 35 (1997) 374.
[3] T. Mazzei, A. Novelli, S. Esposito, P. Periti, J. Chemother. 12 (2000) 53.
[4] P. Cole, Clin. Ther. 19 (1997) 617.
[5] K. Lindgren, J. Chromatogr. 413 (1987) 351.
[6] S. Gupta, B.B. Prasad, J. Pharm. Biomed. Anal. 23 (2000) 307.
[7] M. Tutunji, O. Jarrar, M. Musameh, S.M. Alam, Quamruzaman, R. Dham, J. Clin. Pharm. Ther. 26 (2001) 149.
[8] A. Alanis, K.A. Longest, J.E. Senetar, W.H. Dere, Postgrad. Med. J. 68 (1992) S24.
[9] S.T. Haines, M.A. Kraynak, Ann. Pharmacother. 28 (1994) 1353.
[10] J.H. Satterwhite, B.J. Cerimele, D.L. Coleman, B.L. Hatcher, J. Kisicki, K.A. DeSante, Postgrad. Med. J. 68 (1992) S3.
[11] P.M. Kovach, R.J. Lantz, G. Brier, J. Chromatogr. 567 (1991) 129.