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Sensitive liquid chromatography–tandem mass spectrometry method for the determination of cefixime in human plasma: Application to a pharmacokinetic study

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Abstract

A sensitive and selective liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method was developed to determine cefixime ((6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(carboxymethoxyimino)acetamido]-8-oxo- 3-vinyl-5-thia-1-azabicyclo-[4,2,0]-oct-2-ene-2-carboxylic acid) in human plasma. After a simple protein precipitation using acetonitrile, the post-treatment samples were analyzed on a C_8 column interfaced with a triple quadrupole tandem mass spectrometer. Positive electrospray ionization was employed as the ionization source. The mobile phase consisted of acetonitrile–water–formic acid (40:60:0.5, v/v/v). The analyte and internal standard cefetamet were both detected by use of selected reaction monitoring mode. The method was linear in the concentration range of 0.05–8.0 μg/ml. The lower limit of quantification was 0.05 μg/ml. The intra- and inter-day relative standard deviation across three validation runs over the entire concentration range was less than 12.7%. The accuracy determined at three concentrations (0.05, 0.80 and 7.2 μg/ml for cefixime) was within ±2.0% in terms of relative error. Each plasma sample was chromatographed within 3.5 min. The method herein described was successfully applied for the evaluation of pharmacokinetic profiles of cefixime capsule in 24 healthy volunteers.

Keywords: Cefixime

1. Introduction

Cefixime ((6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(carboxymethoxyimino)acetamido]-8-oxo- 3-vinyl-5-thia-1-azabicyclo-[4,2,0]-oct-2-ene-2-carboxylic acid), is an orally absorbed third generation cephalosporin antibiotic. It has a broad antibacterial spectrum against various gram-positive bacteria and gram-negative bacteria, including *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Escherichia coli*, and *Klebsiella pneumoniae* resistant to ampicillin, cephalexin, cefaclor, and trimethoprim-sulfamethoxazole [1,2]. It was not hydrolyzed by the common plasmid or by chromosomal β-lactamases that inactivate the oral penicillins and cephalosporins [3]. These in vitro advantages may provide cefixime feasibility to treat some of the more difficult respiratory and urinary tract infections [4].

Up to now, the determinations of cefixime in plasma have mainly been focused on microbiological [5] and high-performance liquid chromatographic (HPLC) techniques [6–12]. Due to poor selectivity, microbiological methods are only used for pharmacodynamic study now. Tokuma et al. [6] and Liu et al. [7] developed a sensitive HPLC–UV method to determine plasma and urine concentration of cefixime with a lower limit of quantification (LLOQ) of 0.05 μg/ml by using a double column and double pump HPLC switching system, whereas the chromatographic run time of one sample was more than 15 min. Nowadays liquid chromatography–tandem mass spectrometry (LC–MS–MS), due to its higher sensitivity and selectivity, has been applied to the quantification of cephalosporin antibiotic in biological samples [13].

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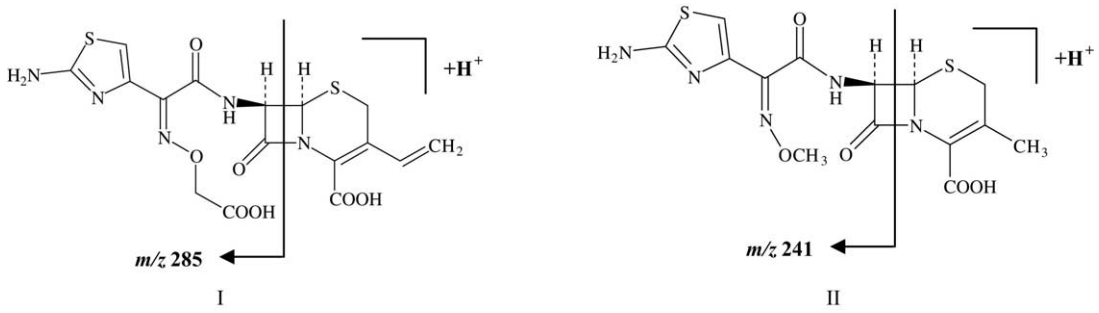

Fig. 1. Structures of cefixime (I) and cefetamet (II, internal standard).

The present study was undertaken to develop a sensitive and rapid LC–MS–MS method for the determination of cefixime in human plasma using cefetamet as the internal standard (Fig. 1). The sample preparation procedure was simple and rapid, requiring only precipitation of proteins with 0.8 ml of acetonitrile and the run time of each sample was 3.5 min, which were suitable for the analysis of large batches of samples. The method was successfully applied to a pharmacokinetic study of cefixime after an oral administration of 200 mg cefixime to 24 healthy volunteers.

2. Experimental

2.1. Materials

Cefixime (99.0% purity) and cefetamet (internal standard, 98.0% purity) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol and acetonitrile (HPLC-grade) were purchased from Kangkede Chemical (Tianjin, China). Formic acid (analytical grade) was from Shenyang Chemical Co. (Shenyang, China). Heparinized 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 Thermo Finnigan TSQ API II tandem mass spectrometer equipped with an electrospray ionization (ESI) source (San Jose, CA, USA), a Shimadzu LC-10AD pump (Kyoto, Japan) and Agilent 1100 Autosampler (Agilent, Wilmington, DE, USA) were used for LC–MS–MS analysis. Data acquisition was performed with Xcalibur 1.1 software (Thermo Finnigan). Peak integration and calibration were carried out using Finnigan LCQuan software.

2.3. LC–MS–MS conditions

The chromatographic separation was achieved on a Zorbax SB C_8 column (150 mm × 4.6 mm i.d., 5 μm, Agilent, Wilmington, DE, USA) with a 4 mm × 3.0 mm i.d. SecurityGuard C_{18} (5 μm) guard column (Phenomenex, Torrance, CA, USA), using a mobile phase of acetonitrile–water–formic acid (40:60:0.5, v/v/v), which was degassed by supersonic procession before use. The liquid flow-rate was set at 0.5 ml/min. The column temperature was maintained at 20 °C.

Mass spectrometer was operated in the positive mode. Quantification was performed using selected reaction monitoring (SRM) of the transitions of *m/z* 454 → *m/z* 285 for cefixime and *m/z* 398 → *m/z* 241 for cefetamet (I.S.), respectively, with a scan time of 0.3 s per transition. Fig. 2 shows the product ion spectra of $[M + H]^+$ of cefixime and cefetamet.

In order to optimize all the MS parameters, a standard solution (1 μg/ml) of the analyte and I.S. was infused into the mass spectrometer. For both cefixime and cefetamet, the following optimized parameters were obtained. The spray voltage was set at 4.5 kV. Nitrogen was used as the sheath

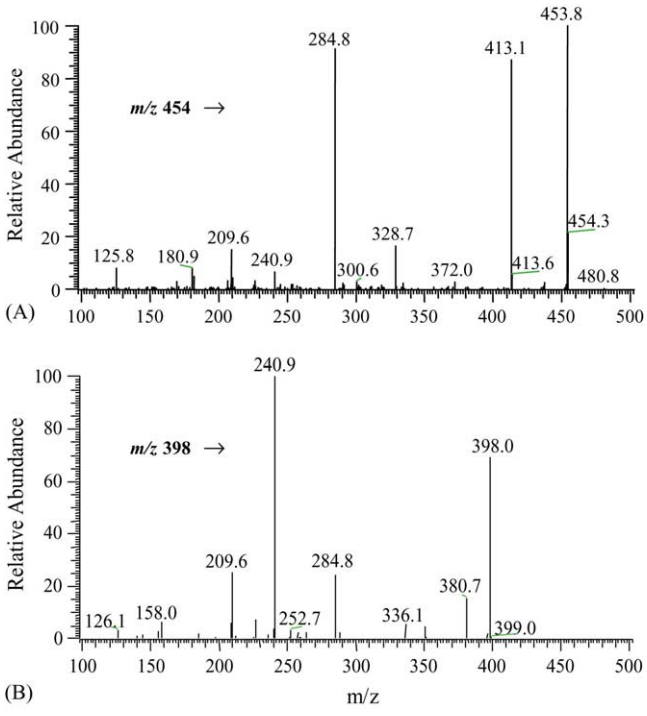

Fig. 2. Full-scan product ion spectra of $[M + H]^+$ of cefixime (A) and cefetamet (B).

gas (80 psi) and auxiliary gas (3 l/min). 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 approximately 1.9 mTorr. The optimized collision energy of 18 eV was chosen for both cefixime and I.S.

2.4. Sample preparation

To a 0.5 ml aliquot of plasma sample, 100 μl of internal standard (20 μg/ml cefetamet) and 100 μl of methanol were added. The sample mixture was deproteinized with 0.8 ml of acetonitrile and vortex-mixed for approximate 1 min, allowed to stand for 5 min, and the precipitate was removed by centrifugation at 2000 × *g* for 5 min. Then 200 μl of supernatant was transferred and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dry residue was reconstituted in 150 μl of the mobile phase, and then vortex-mixed. A 20 μl aliquot of the resulting solution was injected onto the LC–MS–MS system for analysis.

2.5. Preparation of standard and quality control samples

Stock solution of cefixime was prepared in methanol at the concentration of 400 μg/ml. Stock solution of I.S. was prepared in methanol at the concentration of 400 μg/ml and diluted to 20 μg/ml with methanol. Calibration curves were prepared by spiking 100 μl of the appropriate standard solution to 0.5 ml of blank plasma. Effective concentrations in plasma samples were 0.05, 0.10, 0.20, 0.80, 2.0, 4.0 and 8.0 μg/ml for cefixime. The quality control (QC) samples were separately prepared in blank plasma at the concentrations of 0.05, 0.80 and 7.2 μg/ml, respectively. The spiked plasma samples (standards and quality controls) were then treated following the protein precipitation procedure on each analytical batch along with the unknown samples.

2.6. Method validation

Plasma samples were quantified using the ratio of the peak area of cefixime to that of I.S. as the assay parameter. Peak area ratios were plotted against cefixime concentrations and standard curves in the form of $y = A + Bx$ were calculated using weighted ($1/x^2$) least squares linear regression.

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

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

According to the literatures reported by Knoller [8] and Falkowski [9], cefixime in plasma was found to be stable at 37 °C for at least 24 h and at −18 °C for at least 6 months. Stability of processing (three freeze–thaw cycles) and that processed plasma samples subjected to 24 h at ambient temperature was assessed by analyzing replicates ($n = 3$) of QC samples. The results were compared with those QC samples freshly prepared, and the percentage concentration deviation was calculated.

2.7. Pharmacokinetic study

To demonstrate the reliability of this method for the study of pharmacokinetics of cefixime, it was used to determine cefixime concentrations in plasma samples 0–24 h after administration of 200 mg cefixime to 24 healthy volunteers in a pharmacokinetic study approved by the Ethics Committee. The mean age of 24 male healthy volunteers was 21.1 ± 0.8 years, and the mean weight was 59.5 ± 5.9 kg. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Serial blood samples (4 ml) from a suitable antecubital vein were collected into sodium heparin-containing tubes before and 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 13.0 and 24.0 h after the administration of cefixime. Plasma was separated by centrifugation at 2000 × *g* for 10 min and stored frozen at −20 °C until analysis.

Pharmacokinetic parameters were determined from the plasma concentration–time data. The elimination half-life ($t_{1/2}$) was calculated with non-compartmental model of TopFit program (Version 2.0, Gustav Fischer Verlag, Stuttgart, Germany) 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 spectrometry

Because cefixime has both amino and carboxylic groups in its structure, it has mass spectrometric response either in the positive ionization or in the negative ionization, whereas the signal intensity obtained in the positive mode was much higher than that in the negative mode. Then the possibility of using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) sources under positive ion detection mode was evaluated during the early stage of method development. ESI spectra revealed higher signals for the protonated molecule of cefixime at *m*/*z* 454 compared to APCI source. Further assay development was therefore limited to the ESI source. The Q1 full scan spectra of cefixime and I.S. were dominated by protonated molecules $[M + H]^+$ and no

significant solvent adduct ions and fragments ions were observed. In the product spectra of $[M+H]^+$ ions for cefixime and I.S., along with the raising of the CID energy more fragment ions were observed, at the same time, the response of $[M+H]^+$ lowered significantly. When the CID energy was set at 18 eV, the main fragment ion at *m/z* 285 from cefixime showed a highest MS response. Meanwhile, the most abundant product ion from the I.S. was *m/z* 241. 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* 454 → *m/z* 285 (cefixime) and *m/z* 398 → *m/z* 241 (cefetamet) further improved the sensitivity. The fragmentations of cefixime and I.S. were showed in Fig. 1.

3.2. Chromatography

Although in the aspect of chromatographic separation the determination of the analyte was not interfered with by endogenous substances in the plasma, yet the ionization of the analyte, especially of low concentration, was easily suppressed, which resulted in the linearity of narrow concentration range. In order to avoid the ion suppression induced by endogenous substances, the influence of the mobile phase that composed of different percentage of organic phase to the ion suppression was evaluated during the experiment. As is well known, if the percentage of the organic phase was raised, the retention time of the analyte can be shortened, whereas significant ion suppression was observed. In the three compositions of mobile phase acetonitrile–water–formic acid (30:70:0.5, 40:60:0.5 and 50:50:0.5, v/v/v), it was found that only when the mobile phase consists of acetonitrile–water–formic acid (40:60:0.5), the plasma assay demonstrated good linearity between 0.05 and 8.0 μg/ml for cefixime. 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 3.5 min. The retention times were 3.2 min and 3.1 min for cefixime and cefetamet, respectively, and most of the endogenous substances have been eluted within 2 min.

3.3. Preparation of plasma samples

Sample preparation is a critical step for accurate and reliable LC–MS–MS assays. The most widely employed biological sample preparation methodologies currently are liquid–liquid extraction (LLE), protein precipitation (PPT), and solid-phase extraction (SPE). The polar, water-soluble and amphoteric character of cefixime makes it difficult to extract from plasma by techniques such as conventional liquid–liquid extraction which works with cefazolin, cephalothin, cefamandole, cefoxitin, cefuroxime, cefotaxime and cefoperazone, or by ion-pair extraction with quaternary ammonium salts which works with cephalothin.

PPT often provides higher recovery compared with LLE, especially for compounds which have high polarity. Thus, the plasma samples containing cefixime were prepared by protein precipitation procedure. Three kinds of precipitation reagents (methanol, acetonitrile and trichloroacetic acid) were investigated during the experiment. It was found that the supernatant was very difficult to be evaporated to dryness when using trichloroacetic acid, on the other hand, if the supernatant was directly injected onto the LC–MS–MS system for analysis, the peak shape was not good. In the case of methanol, the resulting supernatant was not so clear that it increased column pressure and gradually deteriorated chromatograms and the recovery was only about 80%. Using of acetonitrile could provide sharp peak shape and higher recovery (more than 90% at three concentrations).

The difference between percentages of the organic phase in the upper organic layer obtained after centrifugation (70%) and that in the mobile phase (40%) was very large, so in order to obtain symmetric chromatogram, 200 μl of supernatant were removed and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dry residue was reconstituted in 150 μl of the mobile phase.

3.4. Method validation

3.4.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Fig. 3 shows the typical chromatograms of a blank, a spiked plasma sample with cefixime (0.05 μg/ml) and I.S., and a plasma sample from a healthy volunteer 2 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 cefixime and cefetamet were 3.2 and 3.1 min, respectively.

3.4.2. Linearity of calibration curves and lower limits of quantification

Visual inspection of the plotted duplicate calibration curves and correlation coefficients >0.99 confirmed that the calibration curves were linear over the concentration ranges 0.05–8.0 μg/ml for the analyte. Typical standard curve was $y = 2.342 \times 10^{-3} + 0.247x$. Where y represents the ratios of cefixime peak area to that of I.S. and x represents the plasma concentrations of cefixime.

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 0.05 μg/ml in 0.5 ml plasma sample. Under present LLOQ of 0.05 μg/ml, the cefixime concentration can be determined in plasma samples until 24 h after a single oral dose of 200 mg cefixime, which is sensitive enough to investigate the pharmacokinetic behaviors of cefixime, to establish the relationship between dose and pharmacological effect in human.

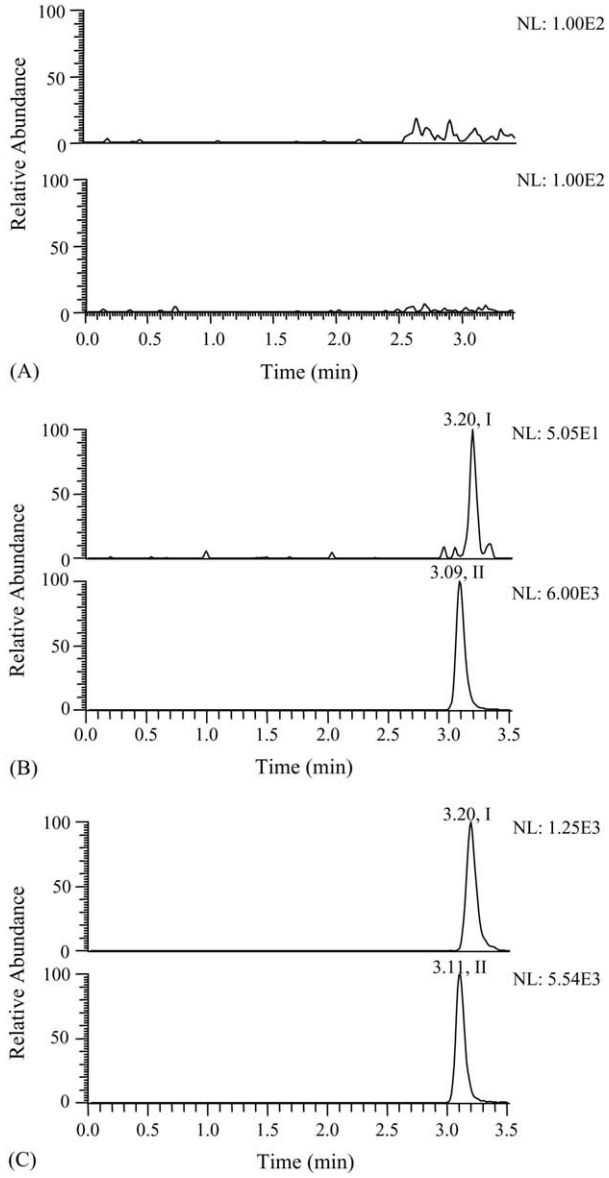

Fig. 3. Representative SRM chromatograms of cefixime (I) and I.S. (cefetamet, II) in human plasma samples. (A) A blank plasma sample; (B) a blank plasma sample spiked with cefixime at the LLOQ of 0.05 μg/ml and I.S. (4 μg/ml); (C) plasma sample from a volunteer 2.0 h after administration of 200 mg of cefixime.

3.4.3. Precision and accuracy

Table 1 summarizes the intra- and inter-day precision and accuracy for cefixime evaluated by assaying the QC samples. The precision was calculated by using one-way ANOVA. In this assay, the intra-run precision was 7.2% or less, and the inter-run precision was 12.7% or less for each QC level of cefixime. The accuracy was within ±2.0%. The results above demonstrated that the values were within the acceptable range and the method was accurate and precise.

Table 1
Accuracy and precision for the analysis of cefixime in human plasma (in prestudy validation, $n = 3$ days, six replicates per day)

Added C (μg/ml)	Found C (μg/ml)	Intra-run RSD (%)	Inter-run RSD (%)	Relative error (%)
0.050	0.049	7.0	10.5	−2.0
0.80	0.81	7.2	12.7	1.4
7.20	7.11	6.2	3.2	−1.2

Table 2
Stability data of cefixime in human plasma under various storage conditions ($n = 3$)

Storage conditions	Added C (μg/ml)	Found C (μg/ml)	Inter-run RSD (%)	Relative error (%)
Three freeze/thaw cycles	0.050	0.047	13.7	−5.2
	7.20	6.38	2.1	−11.4
24 h at room temperature	0.050	0.047	9.6	−6.4
	7.20	6.32	1.7	−12.2

3.4.4. Recovery and stability

The recovery of cefixime, determined at three concentrations (0.05, 0.80, 7.2 μg/ml), were 95.4 ± 5.4%, 93.9 ± 6.6% and 91.1 ± 5.8% ($n = 6$), respectively. The recovery of cefetamet was investigated as 93.1 ± 4.7% ($n = 6$).

The results of stability experiments showed that no significant degradation occurred at ambient temperature for 24 h and during the three freeze-thaw cycles for cefixime plasma samples. Stability data of cefixime in human plasma were shown in Table 2. Standard solutions of cefixime and cefetamet were shown to remain stable for at least 20 days at 4 °C. The results were obtained by comparing with those solutions freshly prepared, and the percentage concentration deviation were within ±5%.

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

The method was applied to determine the plasma concentration of cefixime after an oral administration of 200 mg cefixime to 24 volunteers. The mean plasma concentration–time curve of cefixime was shown in Fig. 4. The main

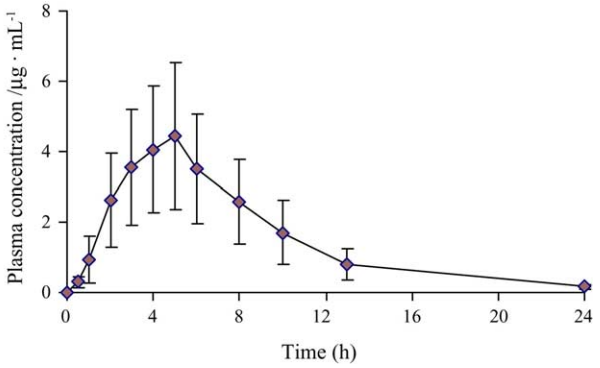

Fig. 4. Mean plasma concentration–time profile of cefixime after an oral administration of 200 mg cefixime to 24 healthy volunteers. Each point represents the mean ± SD ($n = 24$).

pharmacokinetic parameters of cefixime in 24 volunteers were calculated. After oral administration of 200 mg cefixime, the median of T_{max} and C_{max} were found to be 4 h (range 3–6 h) and 4.33 μg/ml (range 1.52 to 8.81 μg/ml), respectively. Plasma concentrations declined with $t_{1/2}$ of 4.19 ± 0.67 h. The AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 36.74 ± 15.98 μg h/ml and 37.75 ± 16.27 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 cefixime in human plasma was readily applicable to the clinical pharmacokinetic study for cefixime.

4. Conclusions

A sensitive LC–MS–MS method for the quantification of cefixime in human plasma was developed and validated. It was then successfully applied for the evaluation of pharmacokinetic profiles of cefixime capsule in 24 healthy volunteers. The method is rapid, sensitive and highly selective with a LLOQ of 0.05 μg/ml using 0.5 ml human plasma. The determination of one plasma sample needs 3.5 min. These results indicated that it is suitable for routine analysis of large batches of biological samples.

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