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A Simple and Sensitive Assay for Cefepime in Human Plasma Using High Performance Liquid Chromatography

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A simple and sensitive assay method was developed for cefepime in human plasma using high performance liquid chromatography (HPLC). Cefepime and cefadroxil (the internal standard) were extracted from heparinized human plasma by simple deproteination with perchloric acid. The extract was injected into an Atlantis dC18 column (250×4.6 mm; particle size 5 μm, Waters) and the column was eluted with methanol and 0.01 M dihydrogen phosphate at pH 3.0 (15 : 85 v : v) as a mobile phase at a flow rate of 0.7 mL/min. Linearity was confirmed for the range 0.25 to 200 μL/mL and the limit of quantitation was 0.25 μL/mL. The retention times were 10.2 min and 13.4 min for cefepime and cefadroxil, respectively. This method was successfully applied to a pharmacokinetic study of cefepime in plasma from bone marrow transplant patients.

Key Words: Cefepime, Cefadroxil, HPLC

INTRODUCTION

Cefepime is a fourth generation cephalosporin antibiotic with a quaternized *N*-methyl-pyrrolidine moiety at the 3' position conferring zwitterionic properties. This property allows the molecule to penetrate the outer cell membrane of Gram-negative bacteria and resist degradation by several plasmid and chromosomally mediated beta-lactamases. Cefepime has a broad spectrum of activity against Gram-positive and Gram-negative pathogens, and is particularly more active against Enterobacteriaceae than cefotaxime and ceftazidime are (Grassi & Grassi, 1993). As cefepime is one of the major antibiotics used to combat serious infections, determining its optimal dosing regimen is important economically as well as therapeutically. Currently, in Korea, injections of 2 g cefepime are given twice daily for treating infections in immunocompromized patients, but it is not certain whether twice- or thrice-daily injections are more cost-effective. Thus, clear understanding of the pharmacokinetics of cefepime in critically ill patients will help clinicians and health insurance policy-makers to draw reasonable conclusions as to its use.

There are several methods for assaying cefepime in biological fluids using conventional (Barbhaiya et al, 1987; Elkhaili et al, 1997; Ip et al, 1998; Calahorra et al, 1999; Valassis et al, 1999) and column-switching (Cherti et al, 2001) high performance liquid chromatography (HPLC). During a pharmacokinetic study of cefepime in a group of bone marrow-transplant patients, we first planned to measure its concentration by conventional reverse-phase columns, using the extraction procedure and mobile phase conditions described in the above reports. When tried various combinations of sample preparation methods and mobile phase conditions, we were unable to separate non-specific peaks from that of cefepime, which has a rather short HPLC retention time for its polar nature.

Fortuitously, we had the good fortune to test another kind of separation column using a modified condition of HPLC, as reported by Valassis et al (Valassis et al, 1999). This allowed us to establish a method that proved simple and sensitive enough to apply in laboratories equipped with conventional HPLC systems.

METHODS

Chemicals and columns

Cefepime was a kind gift from Bristol Myers Squib (Seoul, Korea). Cefadroxil, the internal standard, and perchloric acid were purchased from Sigma Co. (Seoul, Korea). HPLC grade methanol (Fisher, Seoul, Korea) and analytical grade ammonium dihydrogen phosphate (Merck Co., USA) were used for the mobile phase. An Atlantis dC_{18} column (250×4.6 mm, particle size 5 μm, Waters) was used for the separation, and a guard column (μ-Bondapak C_{18} Guard-Pak, Waters) was placed before the inlet of the separation column.

Sample preparation

Plasma samples for standard and quality control (QC) were prepared by spiking stock solutions of cefepime into blank plasma. Three hundred microliters of internal stan-

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ABBREVIATIONS: HPLC, high performance liquid chromatography, QC, quality control; CV, coefficient of variation; LOQ, limit of quantitation.

dard solution (Cefadroxil 10 μL/mL distilled water) was mixed with 100 μL of heparinized plasma, and then 100 μL of perchloric acid solution (60% in distilled water) were added for deproteination. After being vortexed for 30 s, the mixture was centrifuged at 2500 *g* for 10 min. One hundred microliters of the supernatant were transferred to the auto sampler vial, and 20 μL was injected into the column.

Equipment and chromatographic condition

The HPLC system consisted of a 510 pump (Waters, Milford, MA, USA), an auto sampler (Triathlon, Nederland), a UV/visible 486 detector (Waters, Milford, MA, USA), a model WTC-120 column oven (Waters, Milford, MA, USA) and an integrator (Spectra-Physics, USA).

The mobile phase of methanol and 0.01 M dihydrogen phosphate at pH 3.0 (15 : 85, v : v) was delivered at a flow rate of 0.7 mL/min, and the effluent was monitored at 270 nm. The temperature of the analytical column was maintained throughout at 40°C.

Specificity and linearity

The specificity was checked by testing for the presence of interfering peaks in the plasma samples of six different healthy subjects. The standard plasma samples, which had been spiked with cefepime stock solution, had final concentrations of 200, 50, 20, 10, 5, 1, 0.5 and 0.25 μg/mL. Measurement of five sets of standard samples indicated linearity in this range. Typical chromatograms of blank plasma and spiked samples are shown in Fig. 1.

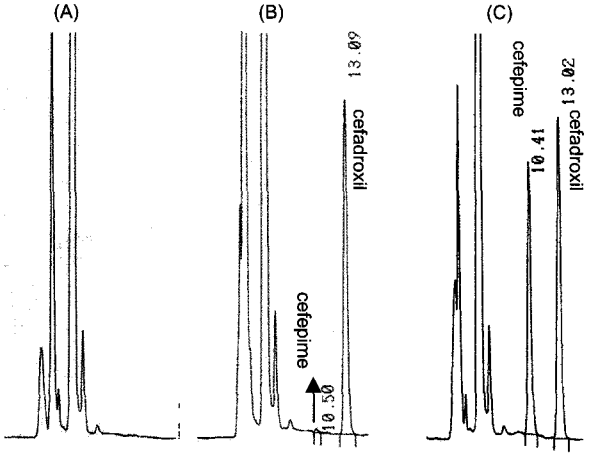

Fig. 1. Representative chromatogram of blank plasma (A) and plasma spiked with cefepime 0.25 (B) and 20 μg/mL (C).

Accuracy, sensitivity and limit of quantitation

To check the accuracy and sensitivity, QC samples (2, 15 and 30 μg/mL) were used. The mean percentage differences between the nominal and calculated concentrations were evaluated. Intra-batch accuracy was tested by measuring six sets of QC samples in a day and inter-batch accuracy was tested by measuring each set for six consecutive days.

Recovery

Spiked plasma samples of 2, 15 and 30 μg/mL were measured by injecting six aliquots for each concentration level. The calculated concentrations were compared with those obtained from direct injection of aqueous solutions of the same nominal concentrations.

Benchtop stability

As cefepime is known to decompose by prolonged exposure to room temperature (Elkhaili et al, 1997), the aim of this test was to confirm its stability during the time required for HPLC assays. Freezethaw stability was estimated by measuring concentration changes after four consecutive freezing (−20°C) and thawing of the QC samples. Short-term stability at room temperature (20°C) was estimated by exposing the samples for 4 h. Except for this stability test, all assay procedures including thawing were carried out at 0~4°C.

RESULTS

Specificity and linearity

No peaks overlapping those of cefepime and cefadroxil were observed in six different subject plasma samples. Cefepime and cefadroxil had retention times of 10.5 min and 13.0 min, respectively. The mean and standard deviations of the intercept and slope of linear equations calculated from the five standard sets were: y (peak area ratio)=0.0600 (S.D. 0.0026)×concentration (μg/mL)−0.0086 (S.D. 0.0108) with a mean correlation coefficient r=0.9998 (S.D. 0.0002).

Table 1. Accuracy and sensitivity of the method to assay cefepime in human plasma during a six-day validation trial

Spiked (μg/mL)	Intra-batch (n=6)			Inter-batch (n=6)		
	Measured (Mean±S.D.)	C.V. (%)	Relative error (%)	Measured (Mean±S.D.)	C.V. (%)	Relative error (%)
0.25	0.29±0.03	9.8	16.0	0.29±0.03	8.8	14.7
0.5	0.54±0.03	4.7	7.3	0.52±0.04	7.3	3.8
2	2.0±0.1	3.9	−1.3	2.1±0.1	6.6	3.3
15	15.5±0.3	1.7	3.3	15.4±0.4	1.9	2.3
30	30.7±0.4	1.3	4.9	31.1±0.6	1.8	3.5

Table 2. Recovery of cefepime and internal standard (cefadroxil: I.S.) tested by the peak area ratios

Spiked value (μg/mL)	Mean Peak Area (S.D.): Direct injection of spiked mobile phase		Mean Peak Area (S.D.): Injection of extracted plasma		Mean Peak Ratio (S.D.): Extracted plasma/ Mobile phase	
	Cefepime	I.S.	Cefepime	I.S.	Cefepime	I.S.
2 (n=6)	34383 (1785)	305363 (8339)	31707 (1465)	289112 (4140)	0.92 (0.06)	0.95 (0.03)
15 (n=6)	274767 (2855)	300964 (9207)	261991 (2139)	284374 (3143)	0.95 (0.02)	0.95 (0.03)
30 (n=6)	561801 (14104)	303039 (7768)	537533 (3971)	285983 (3119)	0.96 (0.03)	0.94 (0.03)

Table 3. Benchtop stability of cefepime. Freezethaw stability was measured after three cycles of freezing (−20°C) and thawing (+20°C)

Spiked	Measured	At 20°C		Freeze-thaw[2]
		0 h	4 h	
2	Mean±S.D.	1.97±0.08	1.90±0.08	1.94±0.06
	CV (%)	3.93	4.26	3.03
	RE (%)	−1.33	−4.92	−3.25
15	Mean±S.D.	15.50±0.27	13.73±0.28	15.06±0.12
	CV (%)	1.71	2.03	0.82
	RE (%)	3.33	−8.47	0.41
30	Mean± S.D.	31.47±0.40	26.97±0.25	30.40±0.32
	CV (%)	1.26	0.93	1.06
	RE (%)	4.91	−10.11	1.34

Accuracy, sensitivity and limit of quantitation

The intra- and inter-day accuracy and precision of the QC samples and the limit of quantification (LOQ) are shown in Table 1. The coefficients of variation (CVs) of QC samples were less than 4% with relative errors of less than 5%. At the LOQ, the CVs and relative error (RE) were greater than those of the QC samples, but they were less than 20%.

Recovery

The results of recovery tests using plasma aliquots and a mobile phase are shown in Table 2. Six pairs of directly injected mobile phase and extracted plasma were compared. The mean recovery of cefepime ranged from 92 to 96 % and that of the internal standard (cefadroxil) was about 95%.

Benchtop stability

Concentrations measured after three freezethaw cycles and 4 h exposure to room temperature are shown in Table 3. At the highest concentration tested (30 μg/mL), 4 h exposure to room temperature resulted in about a 10% decrease in the measured concentration.

Clinical application

We successfully applied the current method to the assay of one hundred plasma samples obtained from fifty patients. Plasma concentrations sampled near the peak and trough times in five patients are shown in Fig. 2.

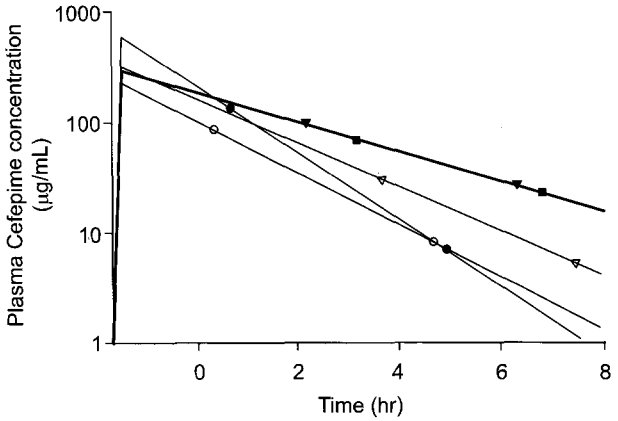

Fig. 2. Predicted pharmacokinetic profiles of cefepime in five bone marrow transplant patients. Each patient's plasma cefepime concentration is shown by a different symbol, and the solid lines have been predicted based on plasma concentrations (two points per patient).

DISCUSSION

Unlike other typical pharmacokinetic studies, a population pharmacokinetic study protocol generally allows fewer than three instances of blood sampling per drug dosage. With such sparse data, concentrations near trough levels cannot be regarded as trivial and high sensitivity of assay is needed. Although HPLC methods together with simple extraction process have been reported, the LOQ was either excessive for such a population study (2.52 μg/mL, (Valassis et al, 1999) or was not mentioned (Ip et al, 1998). Some methods with an LOQ of 0.5 μg/mL required more than two steps in the sample extraction process with organic solvents (Elkhaili et al, 1997; Calahorra et al, 1999). Cherti et al, (Cherti et al, 2001) recently reported a column-switching method, however this approach requires a specially equipped switching system as well as higher column costs.

The analytical column used in this study shows increased retention times and enhanced resolution of polar compounds such as cefepime, therefore is a simple, sensitive and low cost assay for cefepime, using a conventional HPLC system.

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