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Short communication

HPLC method for simultaneous determination of cefprozil diastereomers in human plasma

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Abstract

A high-performance liquid chromatography method was developed for the determination of cefprozil diastereomers in human plasma. Cefprozil exists as *cis* and *trans* isomer at the ratio of 90:10. Plasma samples were prepared by protein precipitation using acetonitrile, trichloroacetic acid and methylene chloride. After the mixtures were vortexed and centrifuged, the aqueous supernatant was injected into a reversed-phase C_8 column. The mobile phase consisted of acetonitrile, glacial acetic acid and distilled water at the volume ratio of 5.5:1.75:92.75 (pH 2.7). The signals were monitored with UV detection at 280 nm. The calibration curves of *cis* and *trans* isomer were linear in concentration ranges of 0.1–25 and 0.02–2.5 μg/mL with the correlation coefficient of 0.9999 and 0.9989, respectively. After oral administration of cefprozil in humans, C_{max} and T_{max} of total cefprozil were 18.80 ± 2.14 μg/mL and 2.06 ± 0.62 h. This method was sensitive with excellent selectivity and reproducibility, and successfully applied to a bioavailability study of cefprozil in healthy subjects.

Keywords: Cefprozil; Stereoisomer; *Cis*; *Trans*; HPLC; Pharmacokinetic studies in human

1. Introduction

Cefprozil is a novel third generation oral cephalosporin possessing a cephem nucleus [1–2]. It has a broad in vitro spectrum of antimicrobial activity by inhibiting bacterial peptidoglycan cell wall synthesis [3–7] and penicillinase resistant against both Gram-positive and -negative bacteria [8].

Cefprozil consists of *cis* and *trans* isomer in an approximately 90:10 ratio (Fig. 1). Since both isomers exhibit antimicrobial activities [9], it is necessary to determine the pharmacokinetics of each isomer separately. A bioassay has been developed for the determination and quantification of cefprozil diastereomers separately [10]. Although this assay suggested a highly specific and precise bioanalytical method, there were some limitations such as lack of stability and validation data. Run times were longer than 20 min. Moreover, the low limit of quantification was established to be 100 ng/mL for both isomers. However, as the levels of *trans* isomer in plasma are about one-tenth of those of *cis* isomer, a suitable quantification method needs to be developed to lower the limit of quantification and improve the sensitivity of *trans* isomer.

In this study, we developed a simple, specific and reliable analytical method to determine cefprozil isomers in plasma using cephaloridine as an internal standard with UV detection.

2. Experimental

2.1. Materials

The *cis* and *trans* isomer of cefprozil were kind gifts of Bristol-Myers Squibb Company (Seoul, Korea). Trichloroacetic acid, glacial acetic acid, methylene chloride and cephaloridine as an internal standard were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). All chemicals were reagent grade.

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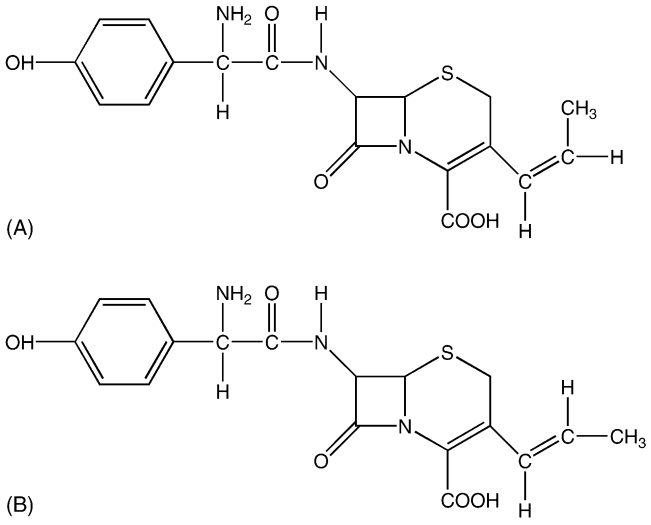

Fig. 1. Chemical structures of *cis*-cefprozil (A) and *trans*-cefprozil (B).

2.2. Preparation of standard solutions

A stock solution of cefprozil (1 mg/mL) was prepared by dissolving it in methanol and kept at −20 °C. Standard solutions of cefprozil in human plasma were prepared by spiking with an appropriate volume (<10 μL/mL) of the diluted stock solutions, giving final concentrations of 0.1, 0.5, 1, 5, 15, 25 and 0.02, 0.05, 0.1, 0.5, 1.5, 2.5 μg/mL (*cis* and *trans* isomer, respectively). The internal standard solution was prepared to give final concentration of 10 μg/mL.

2.3. Preparation of the samples

A 10 μL aliquot of a 1000 μg/mL cephaloridine solution was added to each 1 mL of plasma standard or sample. Plasma proteins were precipitated by adding 0.3 mL of 10% trichloroacetic acid and 1 mL of acetonitrile. To remove acetonitrile, 3 mL of methylene chloride was added, followed by vortexing and centrifugation. Approximately, 200 μL of the aqueous supernatant was transferred to an injection vial and 50 μL was injected onto the HPLC column.

2.4. Chromatographic conditions

All experiments were performed using an HPLC system (Waters Model 2690 Separations Module) equipped with a pump and an autosampler (Milford, MA, USA), a Waters Model 2487 Dual Wavelength Absorbance Detector (Milford), Micromass Masslynx (Version 3.5 software with LC mode, Milford), C_8 Luna column (Phenomenex, CA, USA) and column inlet filter (3 mm × 0.5 μm, Shiseido, Tokyo, Japan). The plasma samples were separated by isocratic elution with acetonitrile–glacial acetic acid–distilled water, 5.5:1.75:92.75 (v/v), adjusted to pH 2.7, at a flow rate of 1 mL/min at 4°C. The eluates was monitored with an UV detector at 280 nm.

2.5. Validation

The chromatographic method was validated based on five repetitions for five different days to determine specificity, sensitivity, linearity, precision and accuracy of the HPLC method.

2.5.1. Specificity

Specificity was assessed by examining peak interference from endogenous substances. This was assessed by inspecting chromatograms of blank and spiked plasma samples.

2.5.2. Sensitivity

Sensitivity was determined in terms of limit of quantification (LOQ) which was taken as the lowest concentration in the calibration range. It was defined yielding a precision <20% relative standard deviation (RSD), an accuracy between 80 and 120% of the theoretical value, and the ratio of signal to noise better than 10.

2.5.3. Linearity

Linearity was determined by a calibration curve with the peak area ratio of standard cefprozil to cephaloridine in the concentration ranges of 0.1–25 and 0.02–2.5 μg/mL for *cis* and *trans* isomer, respectively.

2.5.4. Precision and accuracy

The inter- and intra-day assay were used to validate the RSD and standard errors of mean precision and accuracy of the assay by analyzing plasma samples spiked with cefprozil diastereomers at six different concentrations.

2.5.5. Robustness

The study of robustness was carried out to evaluate the influence of the mobile phase. In order to determine the influence of the acetonitrile/glacial acetic acid solution mixing ratio on retention times, we varied the content of acetonitrile between 3 and 8% (v/v). All glacial acetic acid solution consisted of glacial acetic acid and distilled water at the volume ratio of 1.75:92.75 to adjust pH 2.7.

2.5.6. Matrix effect

Matrix effects were determined by comparing the peak area of the analytes in the samples spiked post protein precipitation to that of each analyte obtained in neat solutions.

2.5.7. Stability

Freeze-thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature and refreezing at −70 °C for 24 h. The short-term stability was examined by keeping plasma samples at room temperature for approximately 24 h. The long-term stability was tested after storage at −70 °C for 8 weeks. Stock solution

stability was evaluated using samples in the mobile phase left at room temperature for 6 h and autosampler stability of cefprozil was tested by analysis of processed samples which were stored in the autosampler tray for 24 h. Each stability was determined at low and high concentrations in three replicates.

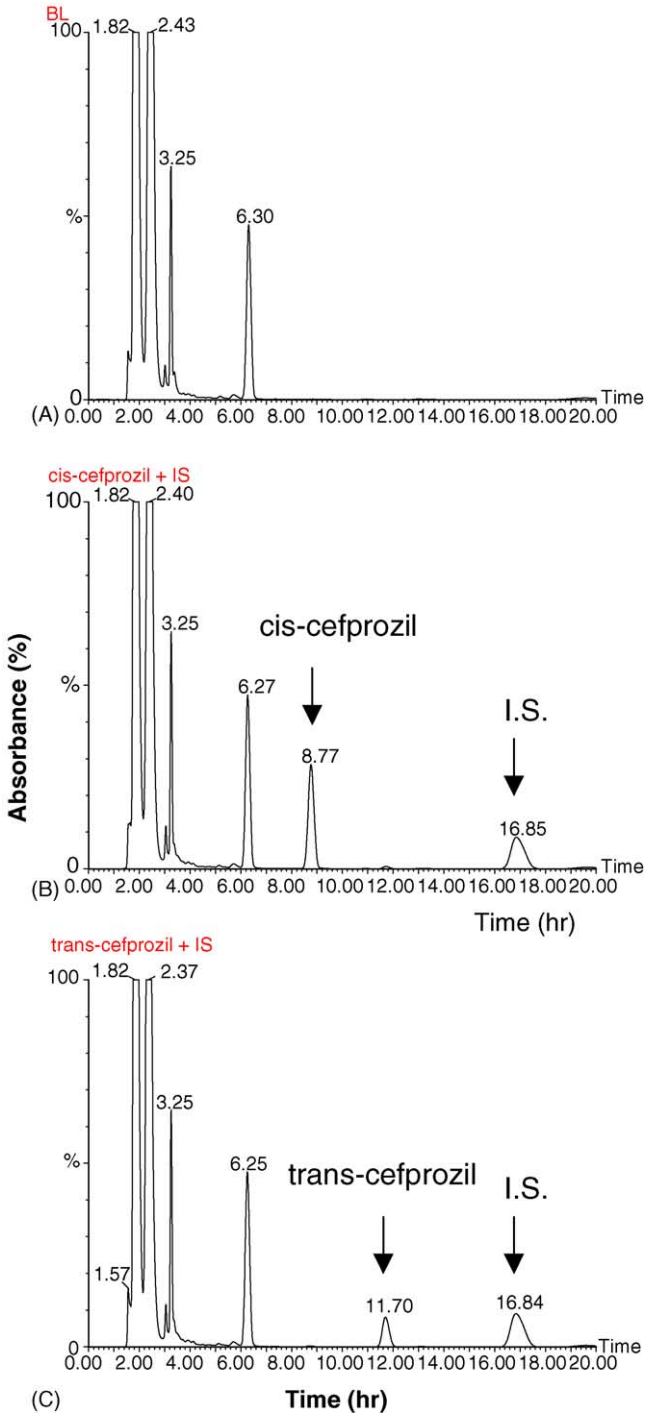

Fig. 2. Representative chromatograms of human blank (A), a plasma containing *cis*-cefprozil and cephaloridine (I.S.) (B) and a plasma containing *trans*-cefprozil and cephaloridine (I.S.) (C).

Table 1
Inter- and intra-day precision and accuracy of *cis*-cefprozil ($n = 5$)

Concentration (μg/mL)	Precision (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.1	7.4	9.8	107.2	95.3
0.5	3.1	7.6	98.5	103.2
1	3.0	0.2	95.5	103.5
5	8.8	9.0	104.0	100.0
15	7.8	6.8	98.1	99.3
25	7.0	7.4	100.5	100.3

2.6. Preparation of biological samples

Eight healthy male volunteers participated in the study. The aim of this study was to assess the bioavailability of cefprozil after an oral dose of 1000 mg of the drug.

The subjects were administered with the drug at 8 a.m. Blood samples were collected in heparinized tubes at 0, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h following each administration. The samples were centrifuged and plasma was separated and stored at −20 °C until analysis. All the samples of each subject were assayed in the same chromatographic session.

3. Results and discussion

3.1. Chromatography

Representative chromatograms of human blank and spiked samples of plasma are shown in Fig. 2. No interfering peaks were observed near the retention time of *cis*, *trans* isomer and the internal standard in blank human plasma. The retention time for *cis*, *trans*-cefprozil and the internal standard were approximately 8.5, 11.5 and 16.5 min, respectively. The chromatographic run time was 20 min for plasma sample analysis.

3.2. Validation

3.2.1. Specificity

As mentioned above, there were no peaks of interfering with cefprozil diastereomers and I.S. at their retention times in the blank and spiked samples.

Table 2
Inter- and intra-day precision and accuracy of *trans*-cefprozil ($n = 5$)

Concentration (μg/mL)	Precision (%)		Accuracy (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.02	5.1	12.9	98.4	87.2
0.05	3.5	6.5	102.0	97.5
0.1	7.3	9.2	99.0	94.9
0.5	4.6	6.8	100.2	103.8
1.5	4.3	6.8	99.9	98.9
2.5	2.7	5.1	100.0	100.3

Table 3
Influence of the amount of acetonitrile in the mobile phase on the retention times

Acetonitrile (%)	Glacial acetic acid solution (%)	Retention time (min)		
		Cis-cefprozil	*Trans*-cefprozil	I.S.
8	92	6.7	6.9	9.5
7	93	8.1	8.9	12.2
5.5	94.5	8.5	11.5	16.5
4	96	9.0	12.7	18.4
3	97	10.5	16.2	>22

Table 4
Assessment of matrix effect ($n = 5$)

	Concentration (μg/mL)	Peak area ratio (protein precipitation/neat × 100)
Cis-cefprozil	0.1	89.6
	0.5	95.2
	1	86.8
	5	90.7
	15	82.9
	25	87.5
Trans-cefprozil	0.02	77.3
	0.05	70.5
	0.1	79.7
	0.5	82.5
	1.5	97.2
	2.5	97.4

3.2.2. Sensitivity

LOQ of *cis* and *trans* isomer in human plasma were 100 and 20 ng/mL, respectively.

3.2.3. Linearity

The calibration equation for this method is as follows: $y = 0.2499\ (\pm 0.0184)x + 0.0092\ (\pm 0.0067)$, $y = 0.3596\ (\pm 0.0183)x + 0.0013\ (\pm 0.0014)$ (*cis* and *trans* isomer, respectively). The calibration curves of *cis* and *trans* isomer were linear in the concentration ranges of 0.1–25 and 0.02–2.5 μg/mL with a correlation coefficient (r^2) of 0.9999 (±0.0001) and 0.9989 (±0.0017) (*cis* and *trans* isomer, respectively).

3.2.4. Precision and accuracy

The inter-assay precision and accuracy were determined by analyzing five calibration curves on five different days. The intra-assay precision and accuracy were determined by analyzing five replicates of samples on the same day. The results of inter- and intra-day precision and accuracy of *cis* and *trans* isomer in human plasma are presented in Tables 1 and 2, respectively. As shown in Table 1, the precision of *cis* isomer of intra- and inter-day in plasma were varied from 3.0 to 8.8% and from 0.2 to 9.8%, respectively. The intra- and inter-day accuracy ranged from 95.5 to 107.2% and from 95.3 to 103.5%, respectively. As shown in Table 2, the precision of *trans* isomer of intra- and inter-day were from 2.7 to 7.3% and from 5.1 to 12.9%, respectively. The intra- and inter-day accuracy ranged from 98.4 to 102.0% and from 87.2 to 103.8%, respectively. The accuracy and precision for each concentration, excluding the LOQ, should be <15% and should be <20% at the LOQ [11].

3.2.5. Robustness

Table 3 shows the influence of the mobile phase on the retention times of cefprozil diastereomers and internal standard. These results indicate that small changes in the percentage of acetonitrile had a strong influence on the retention times. As the amount of acetonitrile increased, the retention times were shorter. However, when the ratio of acetonitrile and glacial acetic acid solution was 8:92, endogenous matrix components interfered with peaks. Therefore we chose the mobile phase consisted of acetonitrile, glacial acetic acid and distilled water at the ratio of 5.5:1.75:92.75.

3.2.6. Matrix effect

As shown in Table 4, little matrix effect was observed when samples were deproteinized by this method.

Table 5
Stability of the samples ($n = 3$)

	Sample concentration (μg/mL)	Freeze-thaw stability (%)	Short-term stability (%)	Long-term stability (%)	Stock solution stability (%)	Autosampler stability (%)
Cis-cefprozil	0.5	101.3	95.8	96.1	101.1	93.8
	25	99.8	92.2	99.4	98.9	104.7
Trans-cefprozil	0.02	98.9	89.8	91.6	106.2	96.7
	2.5	100.4	93.0	98.9	96.4	101.5

Table 6
Pharmacokinetic parameters of *cis*, *trans* and total cefprozil ($n = 8$)

	AUC (μg·h/mL)	C_{max} (μg/mL)	T_{max} (h)	K_e (h^{-1})	$t_{1/2}$ (h)
Cis-cefprozil	65.03 ± 11.24	17.27 ± 2.09	2.06 ± 0.62	0.40 ± 0.08	1.80 ± 0.35
Trans-cefprozil	5.76 ± 1.36	1.63 ± 0.30	2.13 ± 0.79	0.50 ± 0.07	1.40 ± 0.23
Total cefprozil	70.84 ± 12.38	18.80 ± 2.14	2.06 ± 0.62	0.42 ± 0.07	1.69 ± 0.31

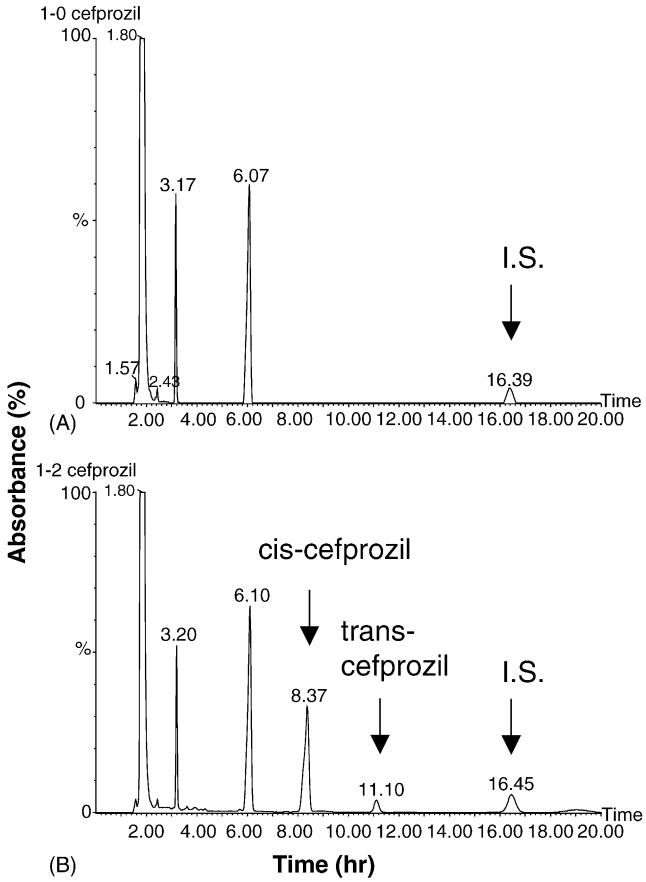

Fig. 3. Representative chromatograms of a volunteer plasma at 0 h (A) and 2 h (B) after administration of cefprozil.

3.2.7. Stability

The results are shown in Table 5. All samples carried out for stability analysis showed no significant degradation under the conditions previously described in the Section 2.

3.3. Subject samples

For pharmacokinetic purposes, plasma samples from eight subjects have been assayed with this method. The representative chromatograms of a volunteer plasma at 0 and 2 h after administration of cefprozil are shown in Fig. 3. As shown in Fig. 3, plasma concentrations of *cis* and *trans* isomer were 14.59 and 1.11 μg/mL, respectively. The mean plasma concentration–time profiles of *cis*, *trans* and total cefprozil are shown in Fig. 4.

Table 6 presents the mean values of pharmacokinetic parameters obtained after administration of cefprozil to eight subjects. The maximum plasma concentration of *cis* isomer was 17.27 ± 2.09 μg/mL at 2.06 ± 0.62 h after administration. The half-life of the drug and area under the curve (AUC) were 1.80 ± 0.35 h and 65.03 ± 11.24 μg·h/mL, respectively. These results are similar to those reported in previous studies [12–14]. The levels of *trans* isomer in plasma were about one-tenth of those of *cis* isomer. After 2.13 ± 0.49 h, plasma concentration of the *trans* isomer peaked at 1.63 ± 0.30 μg/mL with half-life of 1.40 ± 0.23 h and AUC of 5.76 ± 1.36 μg·h/mL.

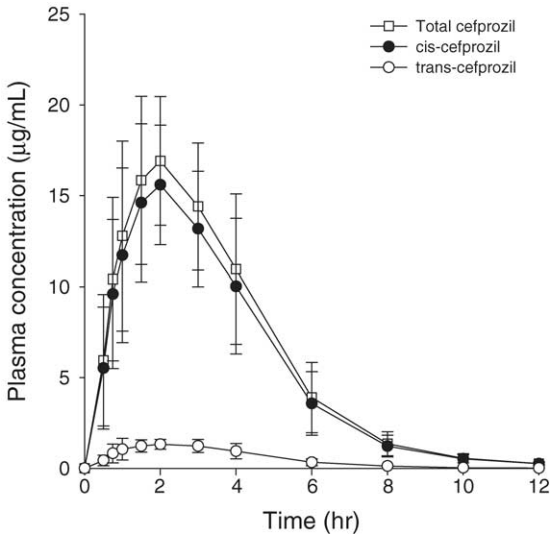

Fig. 4. The mean plasma concentration–time profiles of *cis*, *trans* and total cefprozil after administration of cefprozil to eight subjects.

4. Conclusion

We validated HPLC method for the quantification of cefprozil diastereomers in human plasma. The assay had a satisfactory linearity, accuracy and precision. Practical testing of the developed assay on samples from subjects also showed adequate sensitivity for clinical studies. The proposed technique could be tested for bioequivalence studies of cefprozil in the future.

Acknowledgements

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