Simultaneous High-Performance Liquid Chromatographic Analysis of Cefprozil Diastereomers in a Pharmacokinetic Study

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Cefprozil, a new oral cephalosporin, consists of a 90:10 cis:trans isomer mixture. Sensitive, specific and reproducible high performance liquid chromatographic methods have been developed for the simultaneous quantification of the two stereoisomers of cefprozil in plasma and urine samples from human and rats. Cephalexin acted as the internal standard. Plasma protein was precipitated with acetonitrile and trichloracetic acid with subsequent extraction of acetonitrile. After vortexing and centrifuging, the aqueous phase was injected onto a reverse phase C8 column. Urine samples were acidified with sodium acetate buffer (pH 3.8) and then directly injected onto a reverse phase C18 column. The detector was set at 280 nm. These methods were applied to determine protein binding of both isomers in human and rat sera, and to perform a pharmacokinetic study in human. Results showed that both isomers bound moderately to serum proteins with no interference by the other isomer. The pharmacokinetic study in human indicated that cefprozil was well absorbed and the cis and trans isomers have similar pharmacokinetics.

KEY WORDS: cefprozil; stereoisomer; *cis; trans;* high performance liquid chromatography (HPLC); plasma; urine.

INTRODUCTION

Cefprozil is an oral cephalosporin with a broad antimicrobial spectrum (1–4). It is reported to be more active than either cefaclor or cephalexin against many gram-positive bacteria. Its activity is comparable to that of cefaclor and greater than that of cephalexin against most gram-negative bacteria (1–4). It is more resistant to hydrolysis by some beta-lactamases than cefaclor (2). Cefprozil consists of a mixture of *cis* and *trans* stereoisomers (Fig. 1) at a ratio of approximately 90:10. Both isomers exhibit antimicrobial activities (5). A bioassay has been developed for measuring the concentration of cefprozil in plasma (3). However, because the two isomers are both microbiologically active (5), the bioassay does not distinguish with respect to the measurement of each isomer in biological fluids. A high-performance liquid chromatographic assay was later developed to determine combined isomer levels in biological fluids (6). Since the clinical dosage form of cefprozil contains up to 10% of the trans isomer, it was necessary to determine separately the pharmacokinetics of the cis and trans isomers in animals and human. The present paper describes the development of assays for the determination of the *cis* and *trans* isomers in plasma and urine and their validation with regard to sensitivity, accuracy, precision, and specificity. These assays were then applied in protein binding and pharmacokinetic studies.

MATERIALS AND METHODS

Reagents

The *cis* and *trans* isomers of cefprozil and an internal standard, cephalexin, were obtained from the Bristol-Myers Squibb Pharmaceutical Research Institute, Syracuse, NY. Trichloroacetic acid, sodium acetate, methanol, tetrahydrofuran, glacial acetic acid, and acetonitrile were purchased from Fischer Scientific Company, Fair Lawn, NJ. Sodium dodecyl sulfate was purchased from BDH Chemical Limited, Poole, England. All chemicals were reagent grade.

Sample Processing

A 0.1-ml aliquot of a 1000 μg/ml cephalexin solution was added to each 0.5 ml of plasma standard or sample. Plasma proteins were precipitated by adding 0.15 ml of 10% trichloroacetic acid (TCA) and 0.5 ml of acetonitrile. To separate acetonitrile, 1.5 ml dichloromethane was added, followed by vortexing and centrifugation. Approximately 200 μl of the aqueous supernatant was transferred to an injection vial insert and 60 μl was injected onto the HPLC column.

Each 5 ml of urine sample was buffered with an equal volume of sodium acetate buffer (20 m*M*, pH 3.8). Then 0.1 ml of 1500 μg/ml cephalexin (internal standard) and 0.15 ml of 5% TCA were added to 0.5-ml aliquots of buffered urine standard and sample. The mixture was then vortexed. From this, 200 μl was transferred to an injection vial insert and 10 μl injected onto the HPLC column.

Chromatographic Conditions

The HPLC system consisted of a pump (Waters Model 590), an autosampler (Waters 710B WISP), and a UV detector (Applied Biosystems Spectroflow Model 783) set at 280 nm. The chromatographic separation for the plasma and urine assays was accomplished on reverse-phase columns, the C8 Zorbax (Mac-Mod Analytical Inc.) and C18 Partisil 5 ODS-3 RAC (Whatman Inc.), respectively. A precolumn (0.4 × 4.5 cm) packed with C18/Corasil 37 to 50-μm particles (Waters Associates, Inc.) was placed before the inlet junction of each analytical column.

The mobile phase for plasma assay was acetonitrile:glacial acetic acid:water (17:2:81, v/v/v). The apparent pH of the mobile phase was 2.7 ± 0.1. The mobile phase could be stored at room temperature for 1 week. The flow rate was 1.5 ml/min.

The mobile phase for the urine assay was acetonitrile:methanol:tetrahydrofuran:trichloroacetic acid:glacial acetic acid:sodium acetate trihydrate:sodium dodecyl sulfate:water (25%, v/v:3%, v/v:0.925%, v/v:0.075, w/v:0.25%, v/v:0.077%, w/v:0.134%, w/v:70.75%, v/v). The mobile

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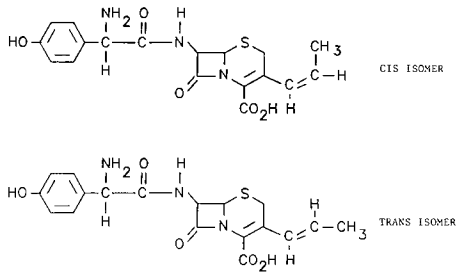

Fig. 1. Structures of the *cis* isomer and the *trans* isomer of cefprozil.

phase was prepared by dissolving 1.54 g sodium acetate trihydrate and 2.67 g sodium dodecyl sulfate in 1000 ml water, adding 5.0 ml glacial acetic acid, 30 ml 5% trichloroacetic acid, 500 ml acetonitrile, 60 ml methanol, and 18.5 ml tetrahydrofuran, and diluting this to 2000 ml with water. The addition of glacial acetic acid and trichloroacetic acid moved the peaks of interest away from the endogenous substances and provided desirable peak shapes. The mobile phase could be stored at room temperature for 2 weeks. The flow rate was 2.0 ml/min.

Data Processing

The analog output of the UV detector was digitized by an analog-to-digital converter and recorded for each chromatogram on an HP-3357 computer system. The peak height ratio of the appropriate isomer of cefprozil to that of the internal standard (cephalexin) was calculated for each chromatogram using in-house software. The least-squares linear regression of the peak height ratio on concentration for each standard, weighted by the inverse of the standard's nominal concentration, was computed. Outlier rejection of assay standards was done by the procedure discussed by Prescott (7). Unknown sample concentrations were calculated by inverse prediction from the regression line.

Assay Validation

Specificity was assessed by examining peak interference from endogenous matrix components. This was assessed by inspecting chromatograms of paired blank and spiked samples. Heparinized plasma or urine from 10 individuals were processed, both as blank samples and as samples spiked at the lower limit of quantitation, defined as 0.10 and 5.0 μg/ml for each isomer of cefprozil in plasma and urine, respectively. Statistical significance of the difference between peak heights for blank and those for spiked samples was assessed by a paired t test at the 5% significance level.

Accuracy and precision were determined by assaying 10 replicate samples at two different concentrations of spiked plasma or buffered urine in a blinded manner, on 3 different days. The respective concentrations of each isomer were about 3 and 20 μg/ml in the spiked plasma samples and approximately 50 and 400 μg/ml in the spiked buffered urine samples. The deviation of the mean predicted concentration from the nominal was taken as the estimate of accuracy. Estimates of intra- and interassay precision were calculated across the 3 assay days using the VARCOMP procedure in the software package, SAS. The intraassay precision was calculated as the relative standard deviation (%RSD) of the predicted concentrations for each set of replicates. The interassay precision was calculated as %RSD based on the daily means, overall grand mean, and intraassay precision estimate. The overall precision was calculated as the %RSD of the individual assay means relative to the overall grand mean.

The stabilities of the isomers of cefprozil during storage at −20°C and after freeze–thaw cycles were evaluated with spiked plasma and urine samples that were divided into aliquots and frozen. Plasma samples for both isomers were spiked at 2 and 25 μg/ml; urine samples were spiked at 56 μg/ml. The stabilities of the two isomers, prior to injection, were also evaluated by repeatedly injecting a sample and determining peak height as a function of time.

Protein Binding

Binding of the two cefprozil isomers to human and rat serum proteins was determined using an ultrafiltration technique (8). Fresh human serum samples containing 2, 5, 10, and 20 μg/ml of either the *cis* isomer or the *trans* isomer alone or in 50:50 combination were prepared. Fresh rat serum samples were prepared identically except using isomer levels of 2, 5, 10, 50, and 100 μg/ml. Samples were incubated at 37°C for 15, 30, and 60 min, after which 1.0 ml was transferred to a micropartition system and centrifuged (1000g, 37°C, 5 min) to obtain approximately 100 μl of ultrafiltrate. The concentrations of both isomers in serum and ultrafiltrate were determined using the described plasma assay procedures. The percentage of protein binding was estimated as [1 − (drug concentration in ultrafiltrate)/(drug concentration in serum)] ×100%.

Pharmacokinetic Application

To demonstrate application of this assay, serial blood and urine samples were collected from nine healthy male volunteers receiving a 1000-mg oral dose of cefprozil with 200 ml of water after an overnight fast. Noncompartmental analysis was performed to describe the pharmacokinetics of both isomers of cefprozil. The following parameters were calculated using standard methods (9,10): maximum concentration in plasma (C_{MAX}), time to C_{MAX} (T_{MAX}), area under the plasma concentration–time curve (AUC), plasma elimination half-life ($T_{1/2}$), mean residence time in the body (MRT), renal clearance (CLR), and percentage of dose excreted in the urine as unchanged form (%UR).

RESULTS AND DISCUSSION

Plasma and Urine Assays

Typical HPLC chromatograms obtained from human plasma samples, both blank and drug-spiked, are shown in Fig. 2. The *cis* isomer, the *trans* isomer, and the internal standard (cephalexin) separated clearly. Endogenous substances did not interfere at the retention times of the peaks of interest. Those retention times for the *cis* isomer, the internal standard, and the *trans* isomer were approximately 14, 17, and 20 min, respectively. The peak height ratio varied linearly with concentration ($r > 0.999$) between 0.1 and 25 μg/ml. The values of mean (SD) slopes for the *cis* and *trans*

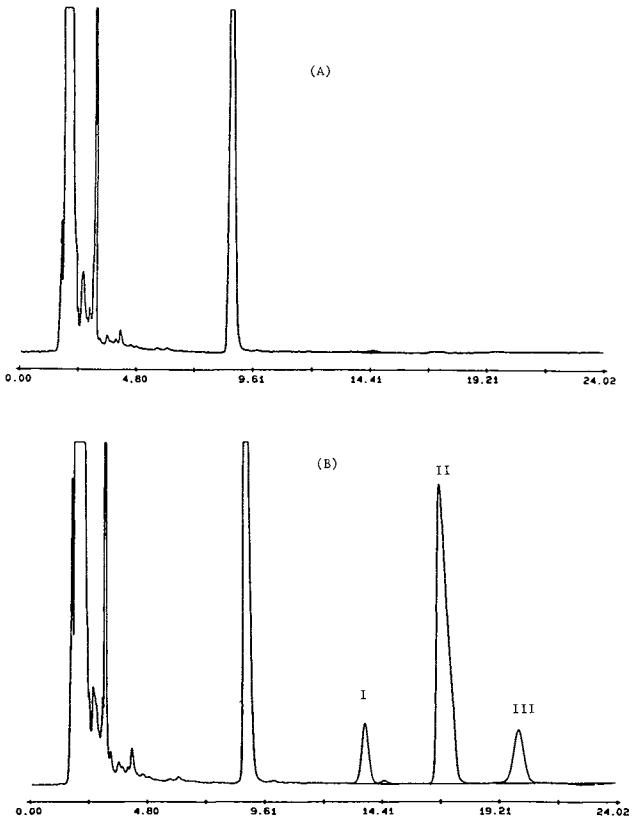

Fig. 2. Representative HPLC chromatograms of human plasma. (A) Blank human plasma. (B) Plasma standard at 1.5 μg/ml: (I) the *cis* isomer of cefprozil; (II) internal standard (cephalexin); (III) the *trans* isomer of cefprozil.

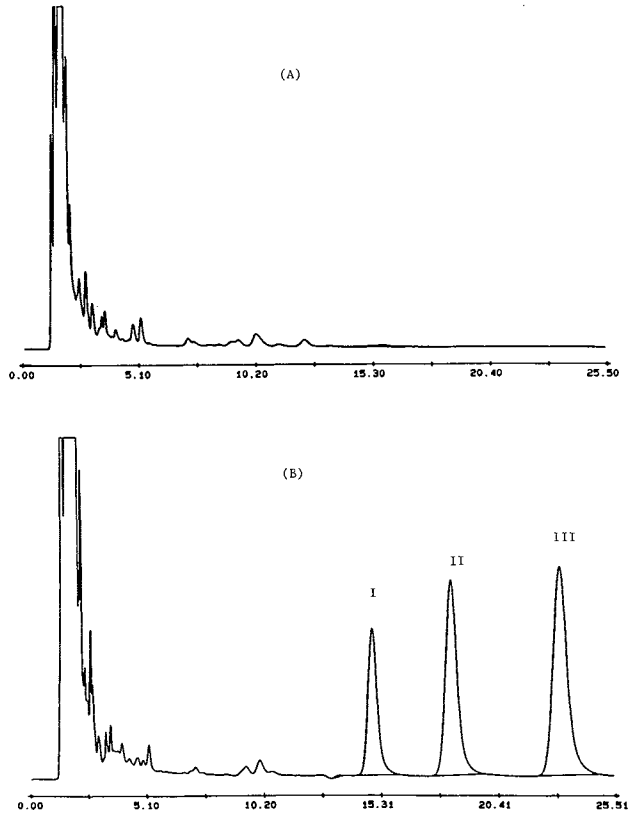

Fig. 3. Representative HPLC chromatograms of human urine. (A) Blank buffered human urine. (B) Buffered urine standard at 50 μg/ml: (I) the *cis* isomer of cefprozil; (II) the *trans* isomer of cefprozil; (III) internal standard (cephalexin).

isomers in the human plasma were 0.12503 (0.00611) and 0.12154 (0.00663), respectively; the mean intecepts were of 0.02172 (0.01091) and 0.02382 (0.00866), respectively.

The lower limit of quantification was established as the lowest standard curve concentration, 0.1 μg/ml, for both isomers. A statistically significant ($P < 0.001$) difference between mean peak height values was observed for 10 spiked individual subject plasma and 10 corresponding blank plasma samples. The mean predicted concentration for the 10 spiked samples was 0.09, with 10% relative standard deviation (RSD) for the *cis* isomer and 0.09 with 11% RSD for the *trans* isomer, indicating the absence of interfering endogenous substances in the tested individual.

Typical HPLC chromatograms obtained for human urine samples, blank and drug-spiked, are shown in Fig. 3. Again, the *cis* isomer, the *trans* isomer, and the internal standard (cephalexin) all separated distinctly and endogenous substances did not interfere. The respective retention times for the *cis* isomer, the *trans* isomer, and the internal standard were 15, 18, and 23 min. The peak height ratio varied linearly with concentration ($r > 0.999$) over the range of 5–500 μg/ml. The values of mean (SD) slopes for the *cis* and *trans* isomers in the buffered human urine were 0.01432 (0.00023) and 0.01799 (0.00038), respectively; the mean intercepts were of 0.02044 (0.02419) and 0.02413 (0.02399), respectively.

Assay results for the accuracy and precision assessments are summarized in Table I. For plasma, the assay precision for both isomers at different concentrations was better than 4% as indicated by the within-day and between-day errors. For urine, the assay precision for the isomers at different concentrations was better than 2%. These data show that the assays were accurate, precise and reproducible. The values of mean (SD) slopes for the *cis* and *trans* isomers in the buffered human urine were 0.01432 (0.00023) and 0.01799 (0.00038), respectively; the mean intecepts were of 0.02044 (0.02419) and 0.02413 (0.02399), respectively.

The lower limit of quantification was established at 5 μg/ml for both isomers in the urine assay. Again, a statistically significant ($P < 0.001$) difference between mean peak height values was observed for 10 spiked individual subject urine and 10 corresponding blank urine samples. The mean predicted concentration for the 10 spiked samples was 5.0 with 8.4% of RSD for the *cis* isomer and was 5.4 with 8.9% of RSD for the *trans* isomer.

Stabilities of the two cefprozil isomers were high. Both were stable at −20°C for at least 38 days in human plasma and 47 days in buffered human urine. The isomers showed no substantial loss of concentration (more than 10%) through three freeze–thaw cycles in either matrix.

Finally, isomer stabilities in the autosampler at room

Table I. Accuracy and Precision of Assays for the *cis* and the *trans* Isomers of Cefprozil in Human Plasma and Urine (N = 10)

Matrix	Isomer	Concentration (μg/ml)	Deviation from nominal concentration (%)	Within-day error (% RSD)	Between-day error (% RSD)
Plasma	*cis*	3.0	+2.5	1.4	1.9
	cis	20.0	−7.4	1.2	1.3
	trans	3.0	+1.4	3.1	1.3
	trans	20.0	−2.2	1.2	1.0
Urine	*cis*	50.0	+5.7	1.7	1.1
	cis	400.0	−2.7	1.5	0.1
	trans	50.0	+7.2	1.5	1.5
	trans	400.0	−3.6	1.5	0.1

temperature were demonstrated for both human plasma (up to 78 hr) and buffered human urine (up to 28 hour).

Protein Binding

The two isomers were stable at 37°C in human and rat sera for up to 1 hr. Lack of nonspecific binding by the two isomers to the filtration unit was then established. The results of the protein binding study are listed in Table II. The estimates for protein binding of the *cis* isomer to human serum alone and in 50:50 combination with the *trans* isomer were 36.2 and 35.9%, respectively. The estimates for protein binding of the *trans* isomer alone and in 50:50 combination with the *cis* isomer, were 44.3 and 46.4%, respectively. These values are similar to those reported for cefaclor (11). The estimates for protein binding of the *cis* isomer in rat serum alone and in combination with the *trans* isomer were 57.6 and 57.3%, respectively. The estimates for protein binding of the *trans* isomer in rat serum alone and in combination with the *cis* isomer were both 63.6%. The presence of the other isomer did not affect the extent of protein binding for either isomer in either serum, nor was the extent of protein binding of either isomer concentration or time dependent.

Pharmacokinetics

The HPLC assay was also used to characterize the pharmacokinetic properties of the cefprozil isomers in nine healthy, young volunteers. The plasma concentration profiles of the isomers for a representive subject following a single 1000-mg dose of cefprozil are shown in Fig. 4. The profiles of both isomers indicate a rapid increase in plasma concentrations and a smooth monophasic decline.

After 1 hr, mean plasma concentration of the *cis* isomer peaked at 14 μg/ml; the plasma concentration profile declined with a mean terminal half-life of 1.4 hr. The mean values of MRT, AUC and CLR were 2.9 hr, 43 hr · μg/ml, and 234 ml/min, respectively. Approximately 67% of the administered *cis* dose was recovered in urine as intact form. The majority of the compound was found in urine within 4 hr after dosing. These findings are similar to those reported in previous studies (6,12–14). The levels of *trans* isomer in plasma were about one-tenth of those observed for the *cis*

Table II. Mean (±SD) Protein Binding of Cefrpozil in Human and Rat Sera (N = 12)

Species	Isomer	*cis:trans* ratio	Protein binding (%) at concentration (μg/ml) of 2	5	10	20	50	100
Human	*cis*	100:0	35.3	37.5	37.1	34.7		
			(5.5)	(5.9)	(4.0)	(3.4)		
	cis	50:50	35.0	38.1	36.9	33.7		
			(5.1)	(2.3)	(4.4)	(3.7)		
	trans	0:100	44.9	44.8	44.6	43.0		
			(1.4)	(2.9)	(3.7)	(2.7)		
	trans	50:50	47.0	48.5	47.4	42.8		
			(2.6)	(1.5)	(2.8)	(1.2)		
Rat	*cis*	100:0	63.0	60.3	60.2		56.0	48.3
			(6.9)	(1.9)	(0.6)		(2.3)	(2.0)
	cis	50:50	62.3	60.3	58.8		54.5	50.4
			(6.9)	(1.5)	(1.1)		(2.0)	(1.1)
	trans	0:100	62.6	65.8	66.8		65.5	59.5
			(3.8)	(1.4)	(0.9)		(0.8)	(2.1)
	trans	50:50	63.7	66.7	66.4		62.8	58.4
			(7.7)	(2.5)	(2.6)		(2.6)	(1.4)

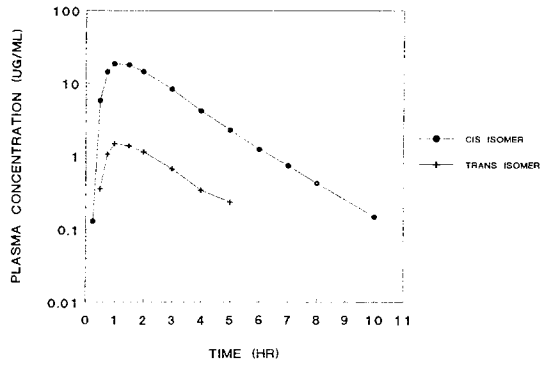

Fig. 4. Plasma levels of the *cis* and the *trans* isomers of cefprozil in one volunteer after a single 1000-mg oral dose of cefprozil.

isomer. The mean pharmacokinetic parameters found for the trans isomer are as follows: T_{MAX} = 1.5 hr; C_{MAX} = 1.2 μg/ml; $T_{1/2}$ = 1.3 hr; MRT = 3.0 hr; AUC = 3.4 hr · μg/ml; CLR = 277 ml/min; and %UR = 54%. The above results suggest that the pharmacokinetics of the *cis* and *trans* isomers of cefprozil are virtually identical in human.

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