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Achiral and chiral high-performance liquid chromatographic methods for clinafloxacin, a fluoroquinolone antibacterial, in human plasma

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Abstract

Achiral and chiral HPLC methods were developed for clinafloxacin, a quinolone antimicrobial agent. For achiral assay, analytes were isolated from plasma by precipitating plasma proteins. Separation was achieved on a C_{18} column using an isocratic eluent of ion pairing solution–acetonitrile (80:20, v/v) at 1.0 ml/min with UV detection at 340 nm. The ion pairing solution was 0.05 *M* citric acid, 1.15 m*M* tetrabutylammonium hydroxide and 0.1% ammonium perchlorate. Inter-assay accuracy was within 4.9% with an inter-assay precision of 3.7% over a quantitation range of 0.025 to 10.0 μg/ml. For chiral assay, analytes were isolated from plasma by solid-phase extraction. Separation was achieved on a Crownpak CR(+) column using an isocratic eluent of water–methanol (88:12, v/v) containing 0.1 m*M* decylamine at 1.0 ml/min with UV detection at 340 nm. Perchloric acid was added to adjust pH to 2. Inter-assay accuracy was within 3.5% with a inter-assay precision of 5.4% over a quantitation range of 0.040 to 2.5 μg/ml.

Keywords: Clinafloxacin

1. Introduction

Clinafloxacin (CF, CI-960, PD 127391) is a broad spectrum antibacterial agent of the quinolone carboxylic acid category. This compound is a potent inhibitor of bacterial DNA gyrase, an enzyme that regulates supercoiling and uncoiling of bacterial DNA which is required for synthesis, replication, repair and transcription [1,2]. CF is an extremely potent member of the quinolone class of antimicrobial agents demonstrating activity against Gram-negative organisms, Gram-positive organisms and strict anaerobes. It is effective against multiple drug-resistant organisms and is even active against known quinolone-resistant strains [3,4]. Oral and intravenous (i.v.) formulations of CF are currently being developed for use in the treatment of serious infections in hospitalized patients. CF is being developed as a racemate because preclinical studies found similar pharmacological and toxicological profiles for both enantiomers (data on file, Parke-Davis, Ann Arbor, MI, USA).

Plasma concentrations and pharmacokinetic parameters of antibacterial agents can be integrated with minimum inhibitory concentration (MIC) values to make assessments or predictions of the

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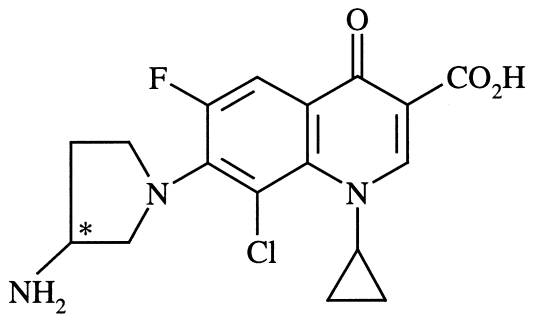

Fig. 1. Structure of CF. Chiral center indicated with and asterisk.

outcomes of therapy [5]. In order to support clinical development and fulfil regulatory requirements, achiral and chiral analytical methods for CF were required. This report describes development of achiral and chiral high-performance liquid chromatographic assays for CF in human plasma. Both methods have been applied to human plasma samples and are currently being used to characterize the pharmacokinetics of CF. The chemical structure of CF is shown in Fig. 1.

2. Experimental

2.1. Chemicals

CF hydrochloride [CF, CI-960, (±)7-(3-amino-1-pyrrolidinyl)-8-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid, monohydrochloride], PD 137951 (*R*-CF; enantiomeric purity >99%), PD 134066 (*S*-CF; enantiomeric purity >85%), PD 118012 [internal standard (I.S.) for achiral assay, 1-cyclopropyl-6,8-difluoro-1,4-dihydro-7-[3-[(methylamino)methyl]-1-pyrrolidinyl]-4-oxo-3-quinolinecarboxylic acid] and PD 138312 [I.S. for chiral assay, *R*-7-[3-(1-amino-1-methyl-ethyl)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid, monohydrochloride] were obtained from Parke-Davis Pharmaceutical Research (Ann Arbor, MI, USA). Water and anhydrous citric acid were purchased from EM Science (Gibbstown, NJ, USA). Acetonitrile, methanol and 60% perchloric acid were supplied by Mallinckrodt (Paris, KY, USA). Tetrabutylammonium hydroxide (TBAH, 1 *M* in methanol) and ammonium perchlorate (99.8%) were obtained from Aldrich (Milwaukee, WI, USA). *n*-Decylamine (95%) was supplied by Sigma (St. Louis, MO, USA). Heparinized human plasma was purchased from Interstate Blood Blank (Memphis, TN, USA). All chemicals were HPLC or reagent grade unless noted otherwise.

2.2. Equipment

Bond-Elut C_{18} cartridges (100 mg sorbent, 1.0 ml cartridge volume) and Vac-Elut, vacuum manifold, were purchased from Varian (Sunnyvale, CA, USA). A TurboVap LV nitrogen evaporator was purchased from Zymark (Hopkinton, MA, USA). A Scientific Products Megafuge 1.0 centrifuge was obtained from Baxter (McGaw Park, IL, USA).

2.3. Achiral method

2.3.1. Chromatographic equipment and conditions

The HPLC system consisted of a SP 4400 integrator and SP 8800 solvent delivery system from Spectra Physics (San Jose, CA, USA) and a Model 712 WISP autosampler, Model 480 spectrophotometer, and TCM column oven from Waters (Milford, MA, USA). Separation was achieved on a BDS-Hypersil C_{18} column, 5 μm particle size, 250×4.6 mm (Keystone Scientific, Bellefonte, PA, USA) using an isocratic eluent of ion pairing solution–acetonitrile (80:20, v/v) at 1.0 ml/min with UV detection at 340 nm. The aqueous ion pairing solution was 0.05 *M* citric acid, 1.15 m*M* TBAH and 0.1% ammonium perchlorate and had a final pH of 4. The column temperature was 25°C.

2.3.2. Quality control samples

Quality control (QC) samples were prepared at 0.075, 3.0 and 7.2 μg/ml CF in human plasma from a 150 μg/ml and 75 μg/ml CF stock solution in HPLC-grade water. These solutions were stored frozen at −20°C. Plastic containers were used to prepare QCs because CF has been found to bind to glass.

2.3.3. Plasma processing

Calibration standards, 0.025 to 10.0 μg/ml, were prepared by adding 0.025 ml of a working solution,

0.200 to 40.0 μg/ml CF in HPLC-grade water, to 0.200 ml of blank human plasma. Final calibration standard concentrations were based on a 0.200 ml plasma volume. QC samples and unknowns were prepared by combining 0.200 ml sample with 0.025 ml of HPLC-grade water and 0.025 ml I.S. solution (15 μg/ml). A 0.050-ml aliquot of precipitating agent (acetonitrile–perchloric acid, 4:1, v/v) was added to each sample followed by centrifugation at 10 687 *g* for 10 min. A 150-μl aliquot of supernatant was injected for chromatographic analysis. All preparations containing CF or I.S. were processed under sodium light illumination due to the instability of these compounds under normal laboratory light conditions.

2.3.4. Recovery from human plasma

Recoveries of CF and I.S. were determined by comparing peak heights of standards prepared by precipitation method to controls at the same concentration prepared in water. Percent recoveries for CF at 0.100, 1.0 and 5.0 μg/ml and I.S. at 1.9 μg/ml were determined.

2.4. Chiral method

2.4.1. Chromatographic equipment and conditions

The HPLC system was the same as for the achiral method except an ISS 200 autosampler from Perkin-Elmer (Norwalk, CT, USA) and a Model 486 Spectrophotometer from Waters were used. Liquid chromatographic separation was achieved on a Daicel Crownpak CR(+) chiral column, 5 μm particle size, 150×4.0 mm (Chiral Technologies, Exton, PA, USA) using an isocratic eluent of HPLC-grade water–methanol (88:12, v/v) containing 0.1 m*M* decylamine at 1.0 ml/min. Perchloric acid was added to adjust pH to 2. The column temperature was 35°C. Column effluent was monitored spectrophotometrically at 340 nm.

2.4.2. QC samples

Aliquots (0.090, 0.75 and 3.0 ml) of a 100 μg/ml CF stock solution in HPLC-grade water were diluted with blank human plasma to prepare QC samples at racemate concentrations of 0.09, 0.75 and 3.0 μg/ml, respectively. Concentrations of each enantiomer in the QC samples were 0.045, 0.375 and 1.5 μg/ml, respectively. Aliquots of QC samples were stored frozen at −20°C.

2.4.3. Processing of plasma

Plasma calibration standards were prepared by adding 0.100 ml of a working solution, 0.8 to 20.0 μg/ml racemic CF in HPLC-grade water, to 1.0 ml of blank human plasma. Final plasma calibration standard concentrations were based on a 1.0 ml plasma volume. Since each standard consisted of a 1:1 mixture of *R*- and *S*-CF, the concentration range of each enantiomer over the standard curve was 0.040 to 2.5 μg/ml. QC samples and unknowns were prepared for analysis by combining 1.0 ml sample with 0.100 ml of HPLC-grade water and 50 μl I.S. solution (20 μg/ml), and 1-ml decylamine (0.25 m*M* in water).

C_{18} cartridge conditioning solutions, wash solution and elution solution described below contained 0.25 m*M* decylamine. C_{18} cartridges were conditioned with two 1-ml volumes of acetonitrile, four 1-ml volumes of acetonitrile–water (50:50, v/v) followed by two 1-ml volumes of water. The sample was added to the cartridge and the cartridge was washed with 1-ml of water–acetonitrile (95:5, v/v). The compounds were then eluted with 1-ml methanol–water (40:60, v/v). Samples were evaporated under nitrogen in a water bath (≈45°C) and reconstituted in 200 μl water–methanol (85:15). A-125 μl aliquot was injected onto the HPLC system for chromatographic analysis. All preparations containing CF or I.S. were processed under sodium light illumination due to the instability of these compounds under normal laboratory light conditions.

2.4.4. Recovery from human plasma

Recovery of racemic CF from human plasma was determined at concentrations of 0.2, 1.0 and 3.0 μg/ml. Recovery of I.S. was determined at 10 μg/ml. Recovery was assessed using the achiral human plasma chromatographic conditions. Ratios of peak-heights for CF and I.S. from solid-phase extraction (SPE) samples to mean peak-heights of nonextracted standards in water were used to calculate percent recoveries.

2.5. Storage stability

Storage stability of CF in human plasma was evaluated by analyzing QC samples stored at −20°C over a period of 11 weeks. CF concentrations in QC samples, analyzed following frozen storage, were compared to the concentrations of the QC samples determined during validation. Additional stability data were obtained by subjecting QC samples to three freeze–thaw cycles. QC samples were analyzed after the third freeze–thaw cycle and concentrations were compared to mean concentrations obtained for the QC samples during the validation.

2.6. Data analysis

Calibration curves were characterized by assaying each calibration standard in triplicate on three separate occasions. Linear regression analysis of calibration standard data was performed by regressing peak height ratios on concentrations of drug in matrix. Slopes, intercepts and coefficients of determination were determined and evaluated for linearity and reproducibility.

2.7. Accuracy and precision

QC samples were assayed in triplicate on three separate occasions. Accuracy was expressed as the percent deviation of the mean observed concentration ($n=9$) from theoretical value. Precision was expressed as coefficient of variation of the observed concentration, expressed as a percentage (C.V., %). These samples were also assayed in duplicate for each assay during unknown sample analysis in order to confirm the validity of each analytical run and to assess long-term stability.

2.8. Lower limit of quantification (LOQ)

The LOQ was determined by evaluating calibration standards during the validation process and selecting the lowest concentration of calibration standards tested that possessed acceptable accuracy and precision, generally 10%.

2.9. Racemization

Injection solvent (water–methanol, 85:15), stock solution and human plasma containing single CF enantiomers was stored at room temperature under sodium lights for up to 72 h (injection solvent and stock solution) and up to 24 h (plasma) to assess racemization in matrix during storage and sample processing. Aliquots of plasma containing individual CF enantiomers were also placed in long term storage at −20°C for continuing stability assessments. Racemization was checked using the chiral chromatographic method described in Section 2.4.1.

2.10. Application of method

The suitability of the methods for investigating the pharmacokinetics of CF and the enantiomers was assessed by analyzing plasma samples from a healthy human subject who received a single 1 h i.v. infusion of 400 mg CF. Heparinized blood samples were obtained prior to dosing and at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, 9, 12, 24 for 36 h postdose relative to the start of infusion. Plasma was harvested by centrifugation and stored at −20°C until analysis. Plasma concentration–time data were analyzed using noncompartmental methods.

3. Results and discussion

3.1. Data collection and calculation

Peak-height ratios of calibration standards were proportional to CF concentration with both methods over the ranges tested. The calibration curves were linear and were well described by least squares regression lines with mean ($n=3$) coefficients of determination of ≥ 0.998 for both the achiral and chiral methods. The mean slope ($\pm$S.D.) for the achiral method was 0.770 (± 0.020). The mean slope ($\pm$S.D.) for the chiral method was 2.22 (± 0.095) and 1.92 (± 0.082) for the *R* and *S* isomers, respectively. A weighting factor of $1/(\text{concentration})^2$ was chosen for the calibration curves to achieve homogeneity of variance. The mean back calculated concentration of the calibration standards following analysis in triplicate on three separate occasions were

within 10% of theoretical values with relative standard deviations (R.S.D.s) less than 10%.

3.2. Chromatographic specificity

Chromatograms representing the separation of the analytes from matrix are shown in Figs. 2 and 3. No interfering human plasma components eluted at the retention times for CF (≈6.0 min) or I.S. (≈7.9 min) in blank control human plasma with the achiral method (Fig. 2). The CF enantiomers were completely separated (α=1.2) in our chiral system. Decylamine was added to the mobile phase to decrease the retention of the CF enantiomers in order

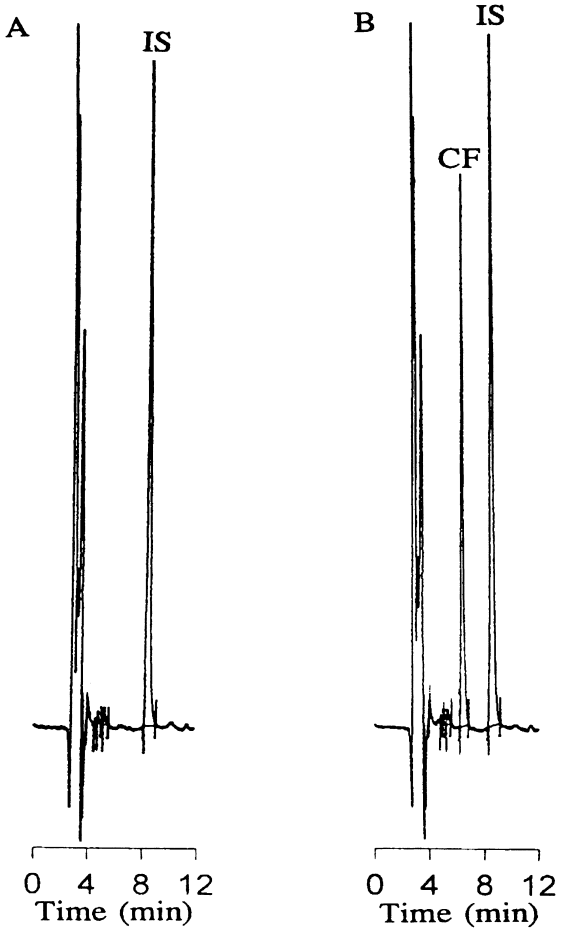

Fig. 2. Chromatograms of extracted human plasma obtained (A) prior to dosing and (B) 6 h after intravenous administration of 400 mg CF using achiral separation conditions (sample CF concentration=0.922 μg/ml). I.S.=Internal standard.

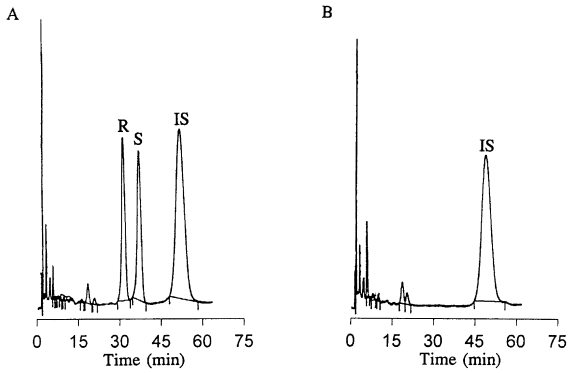

Fig. 3. Chromatograms of extracted human plasma obtained (A) 6 h after intravenous administration of CF and (B) prior to dosing using chiral separation conditions. R=*R*-CF (0.447 μg/ml), S=*S*-CF (0.475 μg/ml), I.S.=internal standard.

to achieve a reasonable analysis time while maintaining the percentage of methanol in the mobile phase within the recommended range (≤15%). SPE was used to remove interferences and concentrate the sample. No human plasma components eluted at the retention times of PD 137951 (*R* isomer, 31.3±1.0 min), PD 134066 (*S* isomer, 36.9±1.0 min), and PD 138312 (I.S.) (54.7±1.0 min) in blank human plasma samples with the chiral method (Fig. 3). Chromatograms of plasma extracts from more than 30 subjects (achiral) and 13 subjects (chiral) were free from interfering peaks.

Table 1
Accuracy and precision for achiral and chiral human plasma QCs

	n	Mean concentration (μg/ml)		% C.V.	% Deviation from nominal
		Nominal	Observed		
Achiral					
	9	0.075	0.0734	3.68	−2.1
	9	3.00	2.93	2.73	−2.3
	9	7.20	7.55	2.38	4.9
Chiral					
R-CF					
	9	0.045	0.0461	4.1	2.4
	9	0.375	0.362	3.9	−3.5
	9	1.50	1.46	3.4	−2.7
S-CF					
	9	0.0450	0.0465	5.4	3.3
	9	0.375	0.362	3.9	−3.5
	9	1.50	1.46	3.4	−2.7

3.3. Accuracy and precision

Assay precision and accuracy were determined by assaying three QC samples in triplicate in three separate batch runs. Achiral assay precision (C.V., %) and accuracy (% deviation from nominal) for CF was 3.7% and within ±4.9%, respectively (Table 1). Chiral assay precision for *R*- and *S*-CF was 4.1% and 5.4%, respectively. Chiral assay accuracy for *R*- and *S*-CF was within ±3.5%.

3.4. LOQ

The LOQ was defined as the lowest concentration on the standard curve. The LOQ in human plasma, as determined by the precision of replicate standards, was 0.0250 μg/ml for CF using the achiral method, which had a precision (C.V., %) of 6.6% and accuracy (% deviation from nominal) of 2.8%. For the chiral CF assay, replicate standards produced a precision of 3.7% and 5.7% at 0.040 μg/ml, the

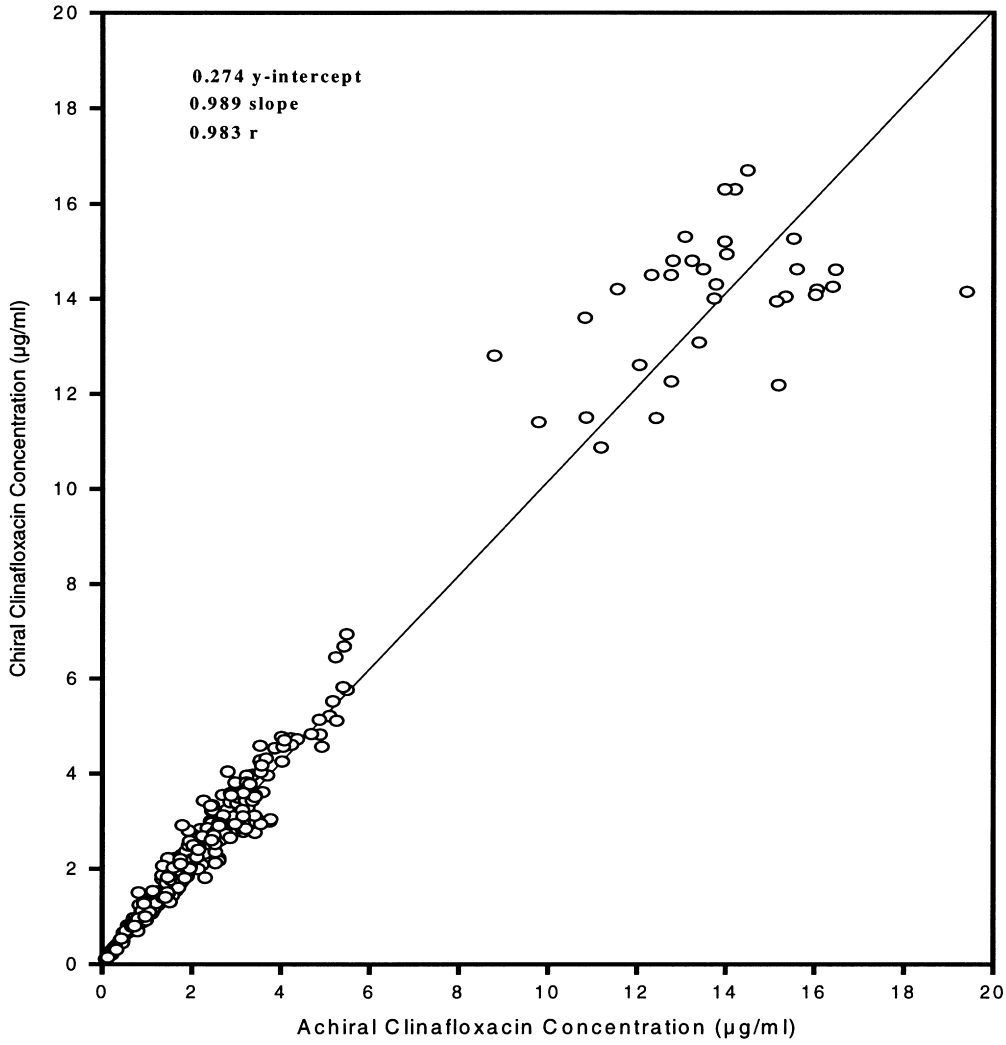

Fig. 4. Comparison of plasma CF concentrations determined by achiral procedure and chiral procedure (sum of *R* and *S* isomer concentrations). Regression line shown on graph.

LOQ, for *R*- and *S*-CF, respectively. The LOQ for the chiral method is higher than that of the achiral method due to broader peak shape. However, the sensitivity of the method was adequate for characterizing the chiral pharmacokinetics.

3.5. Recovery

Recovery (C.V., %) of CF and I.S. from human plasma using the achiral method was 67.4 (2.2)% and 58.4 (1.5)%, respectively. The extraction efficiencies (C.V.%) of CF and I.S. from human plasma using SPE were 107 (7.5)% and 102 (2.0)%, respectively. The extraction efficiency was greatly enhanced by the addition of decylamine to the extraction solutions. Without ion-pairing agent the recovery of CF was less than 10%.

3.6. Storage stability

Storage stability of QC samples has been evaluated for CF after 11 weeks of frozen storage at −20°C. Measured concentrations of CF in QC samples following frozen storage ranged from 91.2 to 104% of mean values obtained during validation, indicating that CF is stable for at least 11 weeks in frozen human plasma. Additional storage stability of the QC samples was evaluated after subjecting them to three freeze–thaw cycles. Measured concentrations of CF in QC samples after three freeze–thaw cycles ranged from 95.4 to 103% of mean values

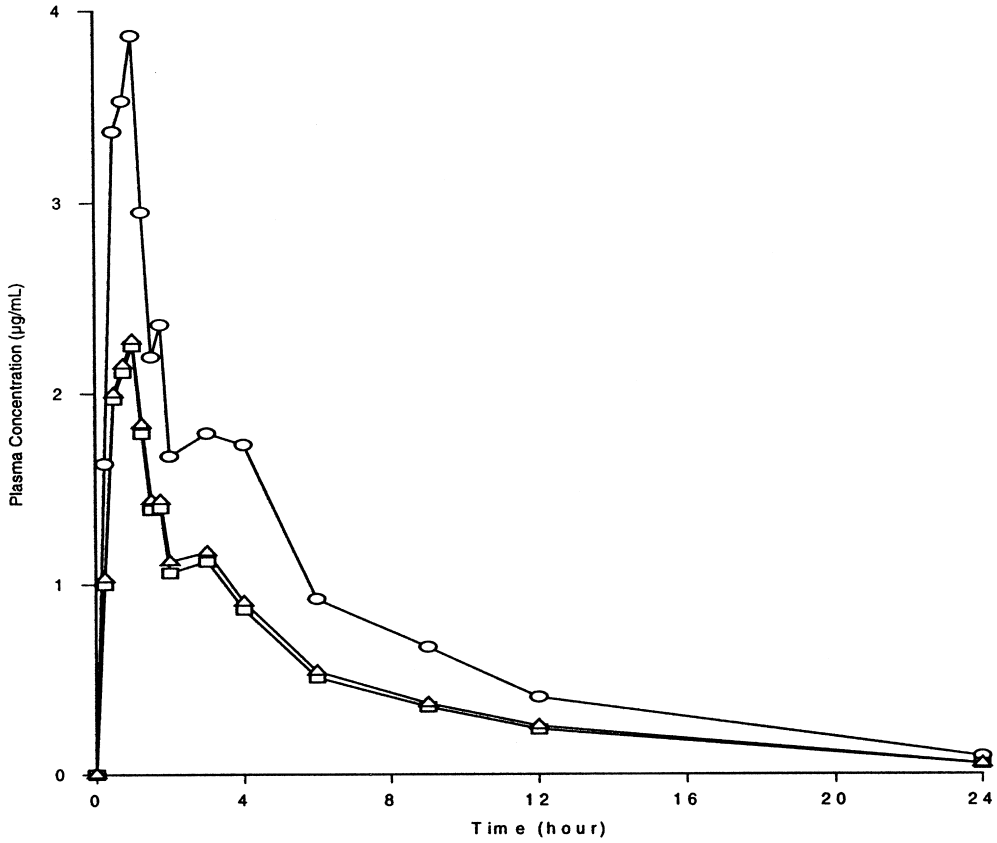

Fig. 5. Plasma concentration–time profile of *R*-CF (△), *S*-CF (□) and racemic CF (○) obtained after a healthy volunteer was administered a single 400 mg i.v. dose of CF by 1-h infusion. *R*-CF: C_{max}=1.92 μg/ml, $AUC_{(0-\infty)}$=9.31 μg h/ml, $t_{1/2}$=5.4 h; *S*-CF: C_{max}=1.95 μg/ml, $AUC_{(0-\infty)}$=9.73 μg h/ml, $t_{1/2}$=5.4 h; racemate CF: C_{max}=3.87 μg/ml, $AUC_{(0-\infty)}$=19.0 μg h/ml, $t_{1/2}$=5.4 h.

obtained during validation, indicating that CF is stable in human plasma after repeated freezing and thawing.

3.7. Racemization

The racemization of *R*- and *S*-CF was evaluated in stock solution, human plasma and injection solvent at room temperature and in human plasma stored for six months at −20°C. For the *R*-CF solutions, no peaks were observed at the retention time of *S*-CF over the course of the study, indicating that there was no conversion of the *R* isomer to the *S* isomer. For *S*-CF, prior to beginning the inter-conversion study, an impurity was observed at the same retention time as *R*-CF. Since this was the only *S*-CF available the inter-conversion study was conducted with this material. The impurity was 13% of the total area. The impurity did not increase or decrease over the course of the study in stock solution, human plasma, or injection solvent at room temperature or frozen human plasma, indicating that there was no inter-conversion between the *S* isomer and *R* isomer.

3.8. Method comparison

A comparison of total CF concentration in human plasma samples determined achirally and chirally is illustrated in Fig. 4. There was good correlation between the two methods ($r=0.983$) and a slope not significantly different from 1.0 ($p>0.05$) (slope= 0.989).

3.9. Application

Fig. 5 depicts plasma concentration–time profiles of CF and enantiomers obtained following administration of a single 400 mg CF dose over 1 h by i.v. infusion. The concentrations were above the LOQ for 24 h which was adequate for determination of exposure. The overall *R*:*S* ratio of the enantiomers was 0.98 indicating there was no relevant stereoselective difference in this healthy volunteer.

4. Conclusions

Reversed-phase HPLC methods to quantitate CF and CF enantiomers have been validated in human plasma. The methods have proven to have adequate accuracy, precision and sensitivity to determine plasma concentrations in clinical pharmacokinetic studies after therapeutic doses.

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