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Enantioselective determination of propafenone and its metabolites in human plasma by liquid chromatography–mass spectrometry

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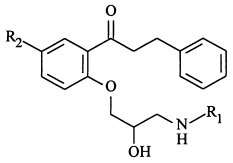
Abstract

A sensitive and stereospecific method was developed to determine propafenone (PPF), 5-hydroxypropafenone (5-OHP) as well as their glucuronide and sulfate conjugates in human plasma. Quantitative analyses and preparative isolations of PPF and 5-OHP were performed on a Nucleosil C_{18} column after liquid–liquid extraction. Afterwards the enantiomeric ratios of PPF and 5-OHP were determined on a Chiral-AGP column with ion trap mass spectrometric detection in the selected reaction monitoring (SRM) mode via electrospray ionization (ESI). The enantiomers of PPF and 5-OHP were separated with different mobile phases. For PPF enantiomers, the mobile phase consisted of 10 m*M* ammonium acetate buffer (pH 5.96)–1-propanol (100:9, v/v), at a flow-rate of 0.5 ml/min; And for 5-OHP enantiomers, the mobile phase was 10 m*M* ammonium acetate buffer (pH 4.1)–2-propanol (100:0.9, v/v), at a flow-rate of 0.6 ml/min. The SRM transitions *m/z* 342 to *m/z* 324 and *m/z* 358 to *m/z* 340 were monitored for detection of enantiomers of PPF and 5-OHP, respectively. Linear calibration curves were obtained in the concentration range of 20–1600 ng/ml for each enantiomer of PPF and 20–500 ng/ml for the 5-OHP enantiomer. The limits of quantification for each enantiomer of PPF and 5-OHP were found to be 20 ng/ml. Precision and accuracy were within 11% over the calibration range for each of the analytes. Incubation of the plasma samples with β-glucuronidase/arylsulfatase and the use of the specific β-glucuronidase inhibitor saccharo-1,4-lactone allows the quantitation of both the glucuronide and sulfate conjugates of the enantiomers. The method was applied to human plasma samples from ten Chinese male volunteers after oral administration of 300 mg racemic propafenone.

Keywords: Enantiomer separation; Propafenone; 5-Hydroxypropafenone

1. Introduction

Propafenone (PPF, Fig. 1) is a class Ic antiarrhythmic agent that is clinically used as a racemic mixture of *S*-(+)- and *R*-(−)-PPF. Both enantiomers are



	R_1	R_2
PPF	$-CH_2CH_2CH_3$	-H
5-OHP	$-CH_2CH_2CH_3$	-OH
Li-1115	$-CH_2CH_3$	-H

Fig. 1. Structures of propafenone, 5-hydroxypropafenone and Li-1115 (I.S.)

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equally potent sodium channel blockers. But only the *S*-enantiomer exerts a modest degree of β-blockade [1,2]. Studies on the metabolism of PPF in man [3] and dog [4] revealed that PPF is extensively metabolized. The urinary and biliary metabolites were almost exclusively conjugated. PPF, hydroxylated PPF derivatives and *O*-methylated catechol-like derivatives were the main metabolites identified after enzymatic conjugate cleavage. And the major metabolite, 5-hydroxypropafenone (5-OHP, Fig. 1), also possesses antiarrhythmic properties [5]. The stereoselective pharmacokinetics of PPF have been studied by several investigators [1,6]. The results showed that, after racemic PPF is administered, *R*-PPF is cleared faster than *S*-PPF, leading to higher plasma levels of *S*-PPF. However, there are no data reported on the stereoselective pharmacokinetics of its metabolites.

Previously described methods for separation of PPF enantiomers in plasma have employed HPLC coupled with chiral derivatization, for instance, with isocyanates [1,7]. But such derivatization has its limitations. In particular, the differences in the rate of the derivatization and enantiomeric purity of the reagents are problems that sometimes hamper accurate analysis. For direct HPLC analysis of chiral drugs, various chiral-differentiating stationary phases have been developed, which allow us to avoid pre-column derivatization. Chiralcel OD column and Chiralcel AD column have been introduced [8] to separate the enantiomers of PPF, but 5-OHP enantiomers were separated only on the Chiralcel OD column, whereas the resolution factor, R_s was 1.0. In addition, an HPLC–UV method was reported for the simultaneous assay of PPF and 5-OHP enantiomers in human plasma, in which the separation was carried out on a silica gel column using *N-tert*-Boc-(L)-proline as the chiral additive to the mobile phase [9]. In this paper we describe a direct stereoselective method for the separation of enantiomers of PPF and 5-OHP on a Chiral-AGP column. Detection was performed by an ion trap mass spectrometric method in the selected reaction monitoring (SRM) mode via positive-ion electrospray ionization (ESI) with a sensitivity in the nanogram per millilitre range. The method was demonstrated to be sufficiently sensitive for stereoselective pharmacokinetic studies of PPF, 5-OHP and their conjugated metabolites, using 2-ml plasma samples.

2. Experimental

2.1. Reference compounds and chemicals

S-(+)- and *R*-(−)-PPF hydrochloride, (±)-PPF hydrochloride, (±)-5-OHP hydrochloride and Li-1115 (2'-(2-hydroxy-3-ethylamino-propoxy)-3-phenyl propiophenone hydrochloride) were obtained from Knoll AG (Ludwigshafen, Germany). β-D-Glucuronidase (with arylsulfatase activity, partially purified, G-0751) and saccharo-1,4-lactone were purchased from Sigma (St. Louis, MO, USA). Methanol and acetonitrile were of HPLC-grade, and other chemicals used were of analytical grade. Diethyl ether, 2-propanol and 1-propanol were distilled before use.

Standard stock solutions of (±)-PPF, *S*/*R*-PPF and (±)-5-OHP were prepared in methanol to give a final solution concentration of 1 mg/ml. The working standard solutions were prepared by appropriate dilutions of stock solutions with 2% phosphoric acid for racemic determination, or with mobile phase used for enantiomeric determination. The solutions were stable for more than 3 months when stored at 4°C.

2.2. Equipment and chromatographic conditions

The HPLC instrument (Shimadzu, Kyoto, Japan) consisted of a LC-10AD pump, a LC-10A UV–Vis detector and a C-R6A Chromatopac integrator. Three HPLC systems were used. System I was used for the analysis and preparative isolation of the racemates of PPF and 5-OHP before/after enzymatic hydrolysis, respectively. Li-1115 (Fig. 1) was used as the internal standard. The achiral separation was performed on a Nucleosil C_{18} Column (300×4.0 mm I.D., 7 μm), supplied by Knauer (Berlin, Germany). The mobile phase consisted of methanol–acetonitrile–ammonium dihydrogen phosphate buffer, 60 m*M*, pH 3 (3:2:5, v/v/v), with a flow-rate of 1.2 ml/min. The HPLC eluates were monitored at 220 nm. System II was used for the analysis of PPF enantiomers. *S*- and *R*-PPF were separated using a

Chiral-AGP column (150×4.0 mm I.D., 5 μm) with a Chiral-AGP guard column (10×3 mm I.D.), supplied by ChromTech (Haegersten, Sweden). The mobile phase consisted of 10 m*M* ammonium acetate buffer (pH 5.96)–1-propanol (100:9, v/v), at a flow-rate of 0.5 ml/min. The enantiomers were detected with a commercially available ion trap-based HPLC–MS system. System III was used for the analysis of 5-OHP enantiomers and identical to System II except that the mobile phase was 10 m*M* ammonium acetate buffer (pH 4.1)–2-propanol (100:0.9, v/v), at a flow-rate of 0.6 ml/min. All columns used in the experiment were operated at 20°C.

2.3. MS conditions

Mass spectrometric detection was carried out by an ion trap-based Finnigan LCQ system (Finnigan MAT, San Jose CA, USA), using an electrospray ionization source operating in positive ion mode. The spray was generated with aid of a sheath gas (nitrogen, 99.99% purity) at a flow-rate of 50 units (ca. 0.75 l/min) and an auxiliary gas (nitrogen) at a flow-rate of 10 units (ca. 0.15 l/min). Ionization was performed applying the following parameters: spray voltage, 5.6 kV; capillary temperature, 180°C; and capillary voltage, 29 V. Ions were collimated applying a tube lens offset of 30 V, an octapole 1 offset of −2.50 V, a lens voltage of −16.0 V, an octapole 2 offset of −5.5 V and octapole amplitude of 400 V (peak to peak). Selected reaction monitoring (SRM) was employed using helium (99.999% purity) as collision gas. Precursor to product ion transitions were monitored for m/z 342 to m/z 324 for the PPF enantiomers, and m/z 358 to m/z 340 for the 5-OHP enantiomers.

2.4. Sample preparation

2.4.1. Analysis of unconjugated PPF and 5-OHP in plasma

The plasma concentrations of the sum of *S*- and *R*-PPF and 5-OHP were determined as published [10]. To 1 ml of plasma were added 100 μl internal standard solution (2 μg/ml Li-1115 in 2% phosphoric acid) and 100 μl 2% phosphoric acid. The mixture was made alkaline (100 μl ammonium hydroxide–ammonium chloride buffer, pH 11), extracted into 3 ml of diethyl ether and back-extracted into 200 μl phosphoric acid (1 *M*), from which 50-μl aliquots were applied to HPLC system I.

2.4.2. Analysis of conjugates of PPF and 5-OHP in plasma

As the reference substances of conjugated metabolites are not available, their concentrations were measured by an indirect method after a specific enzymatic hydrolysis. To quantify the glucuronide conjugates, to 0.5 ml of plasma were added 100 μl of internal standard and 200 μl of phosphate buffer (0.1 *M*, pH 4.5). The mixture was shaken and treated with 100 μl β-glucuronidase (5000 units/ml in 50 m*M* phosphate buffer, pH 5.0) for 24 h at 37°C. After hydrolysis, PPF and 5-OHP were determined by HPLC as described in Section 2.4.1. For the exclusive determination of the sulfate conjugate, to 0.5 ml of plasma were added 100 μl of internal standard, 200 μl of phosphate buffer (0.1 *M*, pH 4.5) and 100 μl saccharo-1,4-lactone (4 mg/ml), a specific β-glucuronidase inhibitor. The mixture was vortex-mixed for 30 s, then treated with 100 μl β-glucuronidase for 24 h at 37°C. After hydrolysis, PPF and 5-OHP were determined by HPLC as described in Section 2.4.1.

2.4.3. Analysis of the enantiomers of PPF and 5-OHP

With the method described as 2.4.1 determining the sum of *S*- and *R*-PPF and 5-OHP in plasma before/after enzymatic hydrolysis by HPLC system I, the column eluates containing 5-OHP (t_R, 7.9 min, fraction collected between 7.6 and 8.1 min) and PPF (t_R, 17.2 min, fraction collected between 16.7 and 17.7 min) were manually collected from the detector outlet into tubes, respectively. This procedure was repeated 2–3 times. Then the collected eluates containing 5-OHP and PPF were combined respectively and concentrated under vacuum to remove methanol and acetonitrile. The remaining buffer salts were adjusted to pH 9 with ammonium hydroxide–ammonium chloride buffer and extracted with 3 ml diethyl ether. The organic layer was evaporated to dryness at 37°C under a gentle stream of nitrogen. The resulting residues were dissolved in 50–100 μl

Table 1
Precision and accuracy for the analysis of 5-OHP enantiomers in plasma (in prestudy validation, $n=15$)

	Concentration (ng/ml)	Concentration determined (ng/ml)	Within-run CV (%)	Between-run CV (%)	RE (%)
R-5-OHP	20.0	19.4	7.2	5.1	−3.1
	100.0	100.2	8.5	5.2	0.2
	500.0	502.9	4.9	5.5	0.6
S-5-OHP	20.0	20.3	6.8	4.9	1.3
	100.0	97.4	7.2	10.8	−2.6
	500.0	504.2	3.7	4.6	0.8

of the mobile phase used for the chiral separation. A 20-μl aliquot was injected into the Chiral-AGP column.

2.5. Validation of the enantioselective HPLC–MS assay procedure

The method for the determination of the sum of *S*- and *R*-PPF and 5-OHP in human plasma has been previously validated [10]. In our experiments, each analytical run included a set of calibration samples, a set of QC samples in duplicate at three concentration levels (20, 200 and 1000 ng/ml (±)-PPF, and 10, 100 and 500 ng/ml (±)-5-OHP) and unknowns. QC samples were used to determine the accuracy and precision of the assay and as a criteria for quality assurance during the analysis of the unknowns.

The calibration curves for the determination of PPF and 5-OHP enantiomers were prepared by analyzing spiked plasma samples. The spiked plasma samples at three concentration levels (see Tables 1 and 2) were used as QC samples and analyzed by the enantioselective HPLC–MS system.

To determine PPF enantiomeric ratios, pure enantiomers of PPF were combined to obtain six mixtures of known enantiomeric ratios in the range 2:1, 1:1 and 1:2 (S/R), containing *S*- and *R*-PPF at total concentrations of 1.2 μg/ml and 2.4 μg/ml. The plasma samples spiked with the series of standard solutions were analyzed with the achiral and chiral HPLC systems consecutively. A weighted ($1/y$) least squares linear regression was used to construct a calibration curve for the peak-height ratio *S*-PPF to *R*-PPF vs. S/R concentration ratio. Additionally, calibration curves of 5-OHP enantiomers were obtained after analyzing plasma samples spiked with the standard solutions of 5-OHP by the achiral and chiral HPLC systems consecutively. Five concentrations over the range 20–500 ng/ml for each enantiomer were used for the calibration curve. Absolute peak-heights were measured and plotted against the concentration of each enantiomer using weighted ($1/y$) least squares linear regression analysis.

During prestudy validation, the calibration curves were defined in three runs based on triplicate assays of the spiked plasma samples, and QC samples were determined in replicates ($n=5$) on the same day. Overall assay performance was assessed by calculat-

Table 2
Precision and accuracy for the analysis of PPF enantiomers ratios in plasma (in prestudy validation, $n=15$)

Total concentration (ng/ml)	S/R-PPF ratios	S/R-PPF ratios determined	Within-run CV (%)	Between-run CV (%)	RE (%)
1200.0	0.50	0.48	5.1	4.9	−4.67
	1.00	1.04	2.4	4.3	3.83
	2.00	2.00	1.7	2.9	0.08
2400.0	0.50	0.47	2.5	4.4	−6.00
	1.00	0.99	3.7	3.5	−0.67
	2.00	1.97	1.8	2.7	−1.67

ing the accuracy and within-run and between-run precision of QC samples analyzed. During routine analysis each analytical run included a set of calibration samples, a set of QC samples in duplicate and unknowns.

2.6. Application of the analytical method

Ten healthy Chinese male volunteers participated in a pharmacokinetic study after giving informed consent based on explanation of the objective of the study and possible risks. They had not suffered from any illness or taken any other medications in the preceding two weeks.

Volunteers received 300 mg PPF hydrochloride tablets (Shandong, China, lot 940813, 50 mg/tablet) around 7 a.m., with 200 ml of water after an overnight fast. Blood samples (5 ml) were collected into heparinized tubes at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5.5, 7, 9, 12 h after administration, and immediately centrifuged. Plasma was separated and stored at −20°C until assayed.

3. Results and discussion

3.1. Chromatography

When a Chiral-AGP column is used in bioanalysis, separation problems are frequently encountered due to the restricted choice of solvent conditions available. Also, the presence of co-extracted contaminants may cause problems in analyte retention, resolution, column stability and column lifetime. An additional problem is the possible co-elution of the enantiomers of the analyte of interest with those of structurally similar metabolites. Some of these problems associated with a Chiral-AGP column can be overcome by pre-isolation of the analytes from biological samples. In the present investigation, the determination of the enantiomers of PPF and 5-OHP before/after enzymatic hydrolysis was carried out in two steps. First, the concentrations of PPF and 5-OHP were determined on a Nucleosil C_{18} column and the HPLC eluates containing PPF and 5-OHP were separately collected. Secondly, PPF and 5-OHP were extracted from HPLC eluates, respectively, and injected onto a Chiral-AGP column for the determination of enantiomeric ratios. The effects of the mobile phase compositions on the separation of PPF and 5-OHP enantiomers on a Chiral-AGP column were investigated in the paper.

By optimizing the separation conditions of PPF enantiomers, it was observed that PPF enantiomers were incompletely separated using the mobile phase containing organic modifier acetonitrile or 2-propanol in 10 m*M* ammonium acetate buffer (pH 5.96), $R_s<1$. But 1-propanol can dramatically improve the separation. Concerning the organic modifier concentration in the mobile phase, it was found that the resolution of PPF was 2.4 at a concentration of 2.5% 1-propanol in 10 m*M* ammonium acetate buffer (pH 5.96), and 1.7 at 8.2% 1-propanol. The results show that the nature and concentration of the organic modifier in the mobile phase could strongly effect the enantioselectivity of PPF on a Chiral-AGP column.

By optimizing the separation conditions of 5-OHP enantiomers, it was found that the resolution for 5-OHP increased from 2.1 to 2.3 on increasing the pH from 4.1 to 4.5 with 0.9% 2-propanol in 10 m*M* ammonium acetate buffer, whereas the retention time changed from 9.4 min to 18.7 min for the *R*-enantiomer, and from 11.7 min to 22.3 min for the *S*-enantiomer. The results indicate that changing the pH resulted in small effects on the separation, but strongly affected the retention time of enantiomers.

As the mobile phases should not contain non-volatile buffer in the HPLC–MS system, the choice of buffer is restricted while separating the enantiomers on a Chiral-AGP column. In addition, due to different chemical properties, the enantiomers of PPF and those of 5-OHP had to be separated with different mobile phases (system II and III) to obtain the best resolution and as low a retention as possible (see Figs. 2 and 3).

3.2. Enantiomeric elution order

The elution order of PPF enantiomers on the Chiral-AGP column was determined after injection of each reference substance. *S*-PPF is eluted first under the given conditions.

To determine the elution order of 5-OHP enantio-

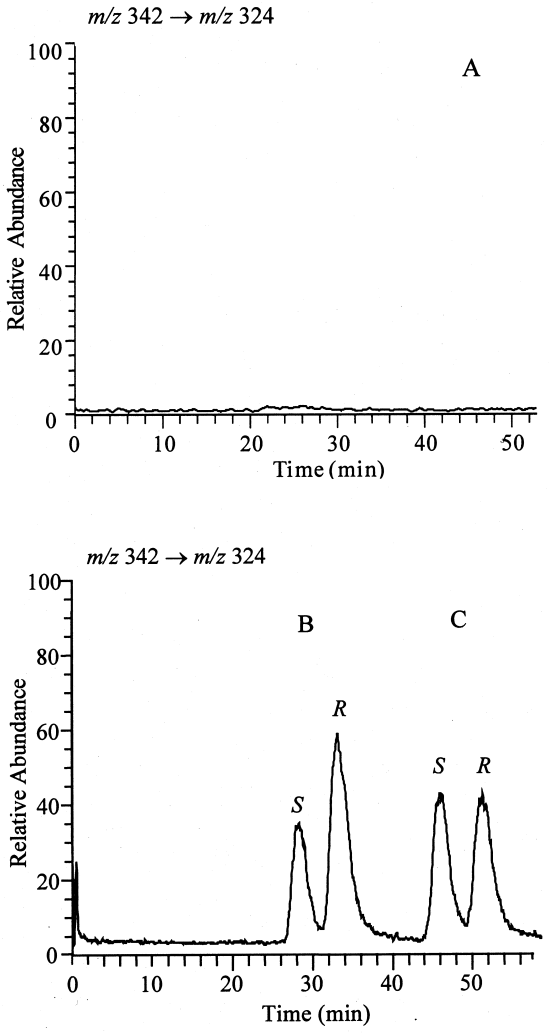

Fig. 2. SRM chromatograms of PPF enantiomers. (A) eluate from blank plasma analyzed by achiral HPLC; (B) eluate from the achiral HPLC analysis of a plasma sample spiked with 400 ng/ml of *S*-PPF and 800 ng/ml of *R*-PPF; (C) eluates from the achiral HPLC analysis of an enzyme-treated plasma sample obtained from a volunteer, 2 h following a single oral administration of 300 mg propafenone hydrochloride (samples injected at an interval of 15 min).

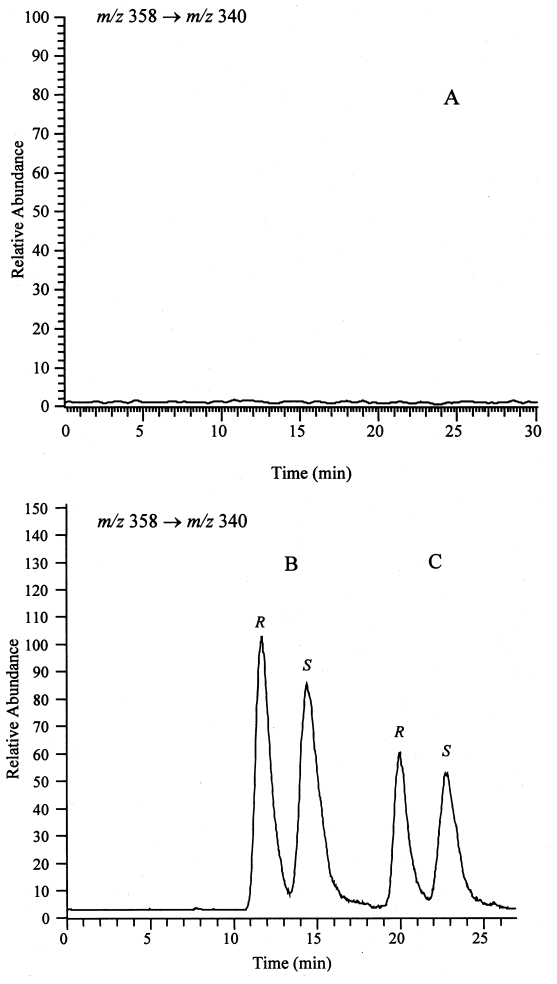

Fig. 3. SRM chromatograms of 5-OHP enantiomers. (A) eluate from blank plasma analyzed by achiral HPLC; (B) eluate from the achiral HPLC analysis of a plasma sample spiked with 250 ng/ml of each enantiomer of 5-OHP; (C) eluates from the achiral HPLC analysis of a plasma sample obtained from a volunteer, 2 h following a single oral administration of 300 mg propafenone hydrochloride (samples injected at an interval of 8 min).

mers, which were not available as references, each of the PPF enantiomers was administered to the male rat. A 2-ml plasma sample 1 h after administration was extracted and chromatographed as described above. The metabolite 5-OHP isolated from plasma by the achiral method was injected onto a Chiral-AGP column. We observed that *R*-5-OHP is eluted before the *S*-enantiomer on a Chiral-AGP column. Furthermore, *R*-5-OHP after administration of *S*-PPF was not detected, and vice verse. So the results show that racemization did not happen during storage, sample preparation and the metabolic process.

3.3. Mass spectrometry

The major product ion fragment of PPF is formed with a loss of 18 u (H_2O), yielding the product ion used for quantitation at m/z 324. Another, competing fragment of PPF is formed by the loss of 77 u (phenyl), exhibiting a minor fragment ion at m/z 265. Fig. 4A shows a full scan MS–MS spectrum of PPF using the protonated molecule (m/z 342) as the precursor ion. Similar to PPF, the major fragment of 5-OHP after the parent ion (m/z 358, MH^+) colliding with background gas was also formed by the loss of water (18 u) (Fig. 4B). Therefore, the major fragment ion (m/z 340) was used for detection.

The mass spectrometer's parameters (capillary voltage, tube lens offset, octapole 1 offset, lens voltage and octapole 2 offset) were set so as to optimize the abundance of the major fragment ion (m/z 324 or m/z 340) by the semi-automatic tune method.

As PPF and 5-OHP in plasma were isolated and purified with an achiral HPLC system, the matrix interference should be negligible during subsequent chiral HPLC–MS analyses. Therefore, according to the resolution of enantiomers, PPF fractions obtained by an achiral HPLC were injected onto a Chiral-AGP column at an interval of 15 min, 5-OHP was injected at an even shorter interval of 8 min to reduce analysis time (see Figs. 2 and 3).

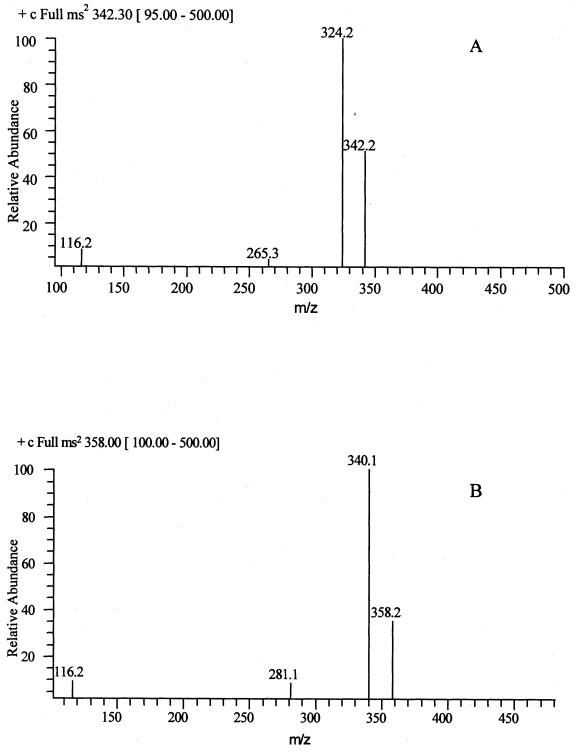

Fig. 4. Full scan MS–MS spectra. (A) PPF (m/z 342) dissolved in the mobile phase for system II; (B) 5-OHP (m/z 358) dissolved in the mobile phase for system III.

3.4. Method specifications

The validity of the sample pretreatment with an extraction efficiency of about 80% has been previously presented [10].

For (±)-PPF and (±})-5-OHP in plasma, linear calibration curves could be established in the range of 10–2000 ng/ml and 5–1000 ng/ml (calculated as free base), respectively. Accuracy and precision were within 9% over the calibration ranges.

For both enantiomers of PPF and 5-OHP, a chiral HPLC–MS analysis showed good linearity throughout the concentration ranges of 20–1600 ng/ml and 20–500 ng/ml, respectively ($r>0.99$). Precision and accuracy data derived from QC results are presented in Tables 1 and 2.

The limit of quantitation (LOQ), i.e., with an accuracy≤15%, and a precision≤15%, was defined at the lowest concentration for each calibration curve. These limits are sufficient for pharmacokinetic study.

3.5. Application of the analytical method in pharmacokinetic studies

After a single oral administration of 300 mg racemic PPF to ten healthy subjects, the stereochemical composition of unconjugated as well as glucuronide- and sulfate-conjugated PPF and 5-OHP in plasma was determined by the method. The concentrations of the separate enantiomers were calculated from the S/R peak height ratios and the total concentration. Fig. 5 shows mean plasma concentration–time curves of PPF enantiomers, PPF

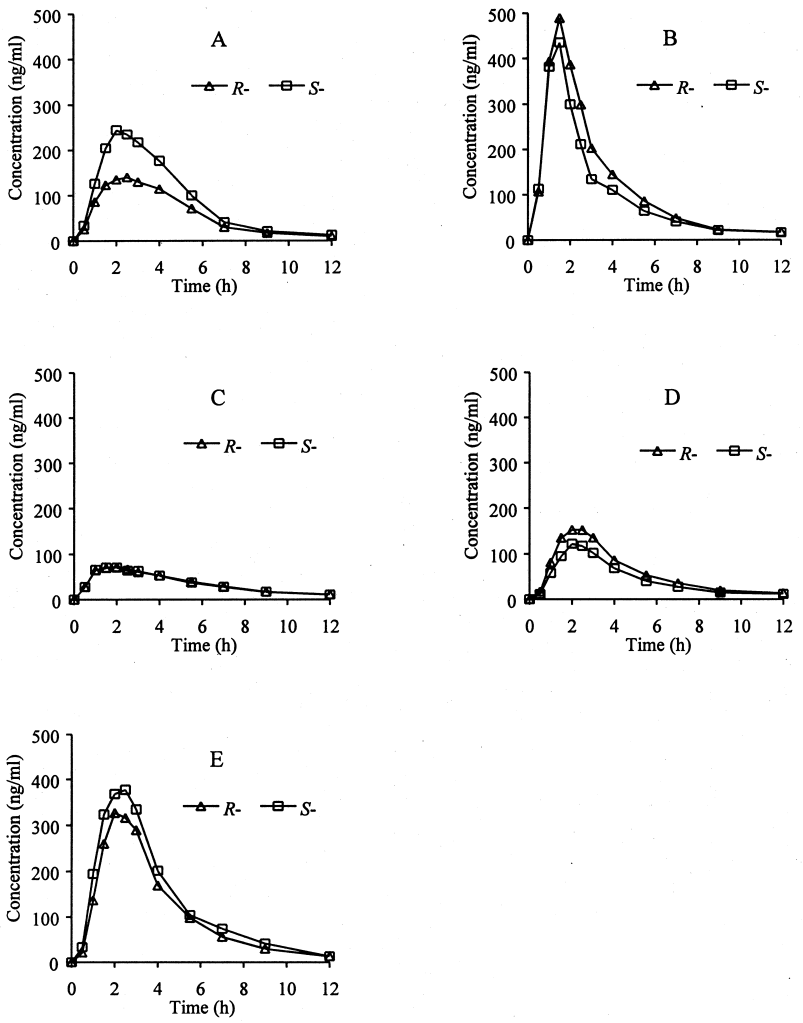

Fig. 5. Mean plasma concentration–time profiles of (A) PPF enantiomers; (B) PPF glucuronide diasteroisomers; (C) 5-OHP enantiomers; (D) 5-OHP sulfate enantiomers; and (E) 5-OHP glucuronide diasteroisomers after an oral administration of 300 mg propafenone hydrochloride to ten healthy volunteers.

glucuronide diasteroisomers, 5-OHP enantiomers, 5-OHP sulfate enantiomers, and 5-OHP glucuronide diasteroisomers after administration ($n=10$).

4. Conclusion

A sensitive, reproducible and accurate detection method of enantiomers of PPF and its 5-hydroxyl metabolite in plasma was developed by coupling HPLC with MS analyses in the present study. With this method, it is possible to determine the enantiomeric composition of unconjugated and conjugated PPF and 5-OHP using 2-ml plasma samples after a single oral dose of racemic PPF.

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