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Research Article

Validated capillary electrophoresis assay for the simultaneous enantioselective determination of propafenone and its major metabolites in biological samples

A robust, inexpensive, and fully validated CE method for the simultaneous determination of the enantiomers of propafenone (PPF), 5-hydroxy-propafenone (5OH-PPF) and *N*-despropyl-propafenone (NOR-PPF) in serum and in *in vitro* media is described. It is based upon liquid–liquid extraction at alkaline pH followed by analysis of the reconstituted extract by CE in presence of a pH 2.0 running buffer composed of 100 mM sodium phosphate, 19% methanol, and 0.6% highly sulfated β-CD. For each compound, the *S*-enantiomers are shown to migrate ahead of their antipodes, and the overall run time is about 30 min. Enantiomer levels between 25 and 1000 ng/mL provide linear calibration graphs, and the LOD for all enantiomers is between 10 and 12 ng/mL. The assay is shown to be suitable for the determination of the enantiomers of PPF and its metabolites in *in vitro* incubations comprising human liver microsomes or single CYP450 enzymes (SUPERSOMES). Incubations with CYP2D6 SUPERSOMES revealed, for the first time, the simultaneous formation of the enantiomers of 5OH-PPF and NOR-PPF with that enzyme. CE data can be used for the evaluation of the enzymatic *N*-dealkylation and hydroxylation rates.

Keywords: *N*-Despropyl-propafenone / 5-Hydroxy-propafenone / *In vitro* metabolism / Serum / Stereoselective metabolism / Sulfated β-cyclodextrin

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1 Introduction

Propafenone (PPF) is a potent antiarrhythmic agent, which is used in the treatment of ventricular and supraventricular arrhythmias. It produces sodium channel- and β-blocking actions as well as weak calcium channel antagonism. PPF is a chiral synthetic compound (for chemical structure see Fig. 1) with a pK_a value of 8.8 and a log *P* value of 40. It is administered as a racemic mixture of *R*-PPF and *S*-PPF [1–3]. The metabolic pathway of PPF is illustrated in Fig. 1. In humans, PPF is extensively metabolized by ring hydroxylation, as the dominant metabolic pathway, and *N*-dealkylation, leading to pharmacologically active 5-hydroxy-propafenone (5OH-PPF) and *N*-despropyl-propafenone (norpropafenone, NOR-PPF), respectively. Hydroxylation is catalyzed by the polymorphically expressed cytochromal enzyme CYP2D6, such that poor metabolizers can be distinguished from extensive metabolizers, while, *N*-dealkylation is known to be mediated *via* CYP3A4 and CYP1A2 [4, 5]. The enantiomers of PPF were found to display stereoselective pharmacodynamic, distribution, and elimination characteristics. Although equipotent in the sodium channel-blocking activity, *S*-PPF is almost 100 times more potent at β-adrenergic receptors [4, 6]. In terms of distribution, the free fraction of the *R*-isomer is higher than that of the *S*-isomer. *In vivo*, the *R*-enantiomer of PPF shows a higher clearance than its antipode in extensive and poor metabolizers, with almost the same degree of stereoselectivity in both populations. This may be due to an enantiomer–enantiomer interaction at the level of metabolism in which *R*-PPF reduces the metabolism of *S*-PPF. This leads to an accumulation of the latter

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Abbreviations: HLM, human liver microsomes; **NOR-PPF**, *N*-despropyl-propafenone; **5OH-PPF**, 5-hydroxy-propafenone; **PPF**, propafenone

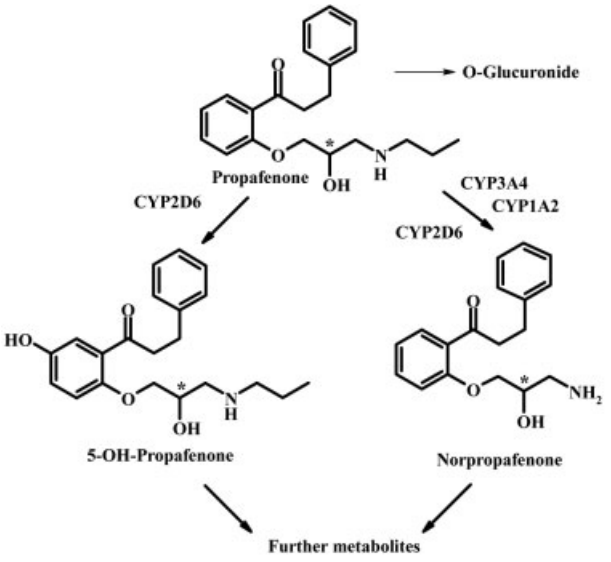

Figure 1. Chemical structures and metabolic pathway of PPF.

antipode which is responsible for the β-blocking effect. Therefore, the disposition of the individual enantiomers and their potential drug–drug interactions in humans are of clinical relevance [6]. The same might be true for the enantiomers of the active PPF metabolites, 5OH-PPF and NOR-PPF. No such information could be found in the literature.

Many enantioselective methods based upon HPLC have been developed for the determination of the enantiomers of PPF and its metabolites. In these assays, enantiomeric separation was obtained as diastereoisomers using chiral derivatization reagents [3, 7–9], with chiral mobile phases [10, 11], and with chiral stationary phases [12–18]. These approaches were either used to separate PPF enantiomers only [7–10], to determine the enantiomers of PPF and 5OH-PPF [11–13, 18] and to monitor the enantiomers of NOR-PPF under separate conditions [12, 15]. Moreover, only few communications describe validated assays for these compounds in biological samples, including plasma [7, 8, 13, 14, 17, 18] and urine [9]. No HPLC paper could be found that reports the simultaneous determination of the enantiomers of PPF, 5OH-PPF and NOR-PPF.

CE has gained a significant degree of acceptance in the analytical laboratories owing to its many advantageous features such as high efficiency, high resolution, rapid analysis, and low consumption of samples and reagents. Enantioselective CE separations are obtained by adding chiral selectors, including plain and derivatized CD, to the running buffer and were found to be highly effective for analysis of the enantiomers of drugs and metabolites in body fluids [19, 20]. CE represents an attractive alternative to chromatographic methods for enantioseparation of chiral analytes. PPF enantiomers were shown to be separable using an alkaline borate buffer containing uncharged mono-3-*O*-phenylcarbamoyl-β-CD as chiral selector [21] and at low pH with triethanolamine phosphate buffers comprising various negatively charged modified β-CDs [22]. In the latter approach, the combined use of carboxymethyl-β-CD and randomly sulfated β-CD (ratio 4/1 w/w) permitted the simultaneous separation of the enantiomers of PPF, 5OH-PPF and NOR-PPF in a single 100 min run. Furthermore, CE with sulfated β-CD was used to show that PFF belongs to the rather few examples of chemical compounds for which the sign of rotation reverts when the free base is transformed into the hydrochloride salt with preservation of the absolute configuration. For the free base, (+)-PPF and (−)-PPF were reported to possess the *S*- and *R*-configuration, respectively, whereas for the hydrochloride salt, (−)-PPF and (+)-PPF have *S*- and *R*-configurations, respectively [22].

No CE-based assays for the determination of PPF enantiomers in biological samples have been reported thus far. Thus, the use of enantioselective CE for that purpose was investigated in our laboratory. This paper reports the development and validation of a sensitive, selective, reproducible, and inexpensive CE method for the simultaneous enantioselective determination of PPF and its main metabolites in human serum and microsomal preparations. The assay is shown to be suitable to screen for and to quantify the formation of enantiomers of PPF metabolites in *in vitro* samples prepared with single human cytochromes (SUPERSOMES™), human liver microsomes (HLM), and human liver S9 fraction. Experiments performed *in vitro* are useful to estimate the *in vivo* metabolic pathways.

2 Materials and methods

2.1 Chemicals, reagents, and standard solutions

Racemic PPF, *S*-PPF and *R*-PPF (all as hydrochlorides) were kindly obtained from Knoll (Ludwigshafen, Germany). Racemic 5OH-PPF (as hydrochloride) and racemic NOR-PPF (as fumarate salt) were kindly donated by Dr. Ute Hofmann (Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie, Stuttgart, Germany). Atenolol was used as internal standard and obtained from the Inselspital Pharmacy (Bern, Switzerland). Methanol (HPLC grade), sodium dihydrogen phosphate, orthophosphoric acid, dichloromethane (HPLC grade), and sodium carbonate were purchased from Merck (Darmstadt, Germany). Sulfated β-CD (7–11 mol sulfate/mol β-CD) sodium salt was obtained from Sigma-Aldrich (Schnelldorf, Germany). Double-distilled water was used throughout the study. Microsomes from baculovirus-infected cells expressing CYP2D6*1, CYP3A4, CYP2A6, CYP1A1,

CYP1A2, CYP2C9*1, CYP2C19, and CYP2B6 (SUPERSOMES), a mixed gender pool of HLM, containing CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP4A11, and FMO with a protein concentration of 20 mg/mL, and pooled human liver S9 fraction (20 mg/mL protein concentration) were obtained from Gentest (Woburn, MA, USA). All SUPERSOMES contained coexpressed human P450 reductase, and CYP3A4, CYP2C9*1, and CYP2C19 also human cytochrome b_5. Microsomes were stored at −80°C and thawed on ice before use. NADPH regenerating system solutions A and B were also from Gentest and kept at −20°C. HSA (5% solution) was from ZLB Bioplasma (Bern, Switzerland). Serum of six healthy subjects was used to prepare blank and fortified serum samples. Standard stock solutions (100 μg/mL as free base of each compound) were prepared separately in methanol and stored at −20°C. Working solutions were prepared daily from the stock solutions by dilution with a mixture of 0.004 M HCl in water and methanol (90:10 v/v).

2.2 CE instrumentation and analytical conditions

All CE analyses were performed on a BioFocus® 3000 (BioRad Laboratories, Hercules, CA, USA) that featured a 50 μm id fused-silica capillary of 36 cm total (31.4 cm effective) length and an on-column UV variable wavelength detector which was set at 195 nm. For solute identification purposes, the fast scanning mode (range: 195–320 nm, scanning interval: 5 nm) was employed. Data were acquired and processed by means of a BioFocus operating software version 5.2. If not stated otherwise, the applied voltage was 13 kV and the carousels and capillary temperatures were controlled at 25 and 20°C, respectively. Each capillary was conditioned with 1.0 M NaOH, 0.1 M NaOH, distilled water, and running buffer for 30, 30, 10, and 10 min, respectively. To assure reproducibility, the capillary was washed at the beginning of every working day with 0.1 M NaOH, distilled water, and running buffer for 10, 10, and 3 min, respectively. Before each analysis, the capillary was flushed with running buffer for 2 min. After use the capillary was flushed with water for 10 min and stored in water. If not stated otherwise, the running buffer was composed of 100 mM sodium phosphate, pH 2.0, 19% methanol, and 0.6% sulfated β-CD. The samples were hydrodynamically injected for 9 psi × s (injection volume: 26.36 nL, calculated with the CE Expert software, version 1 (Beckman, Fullerton, CA, USA)).

2.3 Sample preparation

The extraction of PPF and its metabolites from human serum, serum albumin solutions, and *in vitro* samples was essentially carried out according to the method of Bohm *et al.* [17]. 0.5 mL aliquots of sample were pipetted into 15 mL centrifuge glass tubes. Then 20 μL of a 10 μg/mL (or 100 μg/mL for some of the screening data) methanolic solution of atenolol (internal standard), 200 μL of 10% sodium carbonate in water (pH of the sample = 10), and 7 mL of dichloromethane were added. The tubes were tightly capped and shaken horizontally for 20 min. After centrifugation for 10 min at 1600 × *g*, the organic phase was transferred into a conical glass tube and completely evaporated at 37°C under a gentle stream of air. The residue was reconstituted with 50 μL of a mixture composed of 0.004 M HCl and methanol (90:10), vortex-mixed for 30 s, and injected into the capillary.

2.4 Data evaluation and method validation

Data were evaluated using peak heights and quantification of each enantiomer was based upon internal calibration (ratio of peak heights of enantiomer and internal standard). Six calibrator samples (serum or 5% human albumin solution) were freshly prepared for each calibration covering a concentration range of 25–1000 ng/mL for each enantiomer. Method validation was carried out according to the FDA guidance for industry bioanalytical method validation [23] using calibrators and controls prepared with human albumin solution. For each compound, five replicate spiked samples were assayed between and within day at three different concentration levels (50, 100, and 500 ng/mL of each enantiomer). The assay precision was expressed as RSD. Accuracy was calculated as the deviation of the mean from nominal concentration. The LOQ was defined as the lowest concentration of each analyte at which the percent deviation from the nominal concentration (accuracy) and the RSD were within ±20% and less than 20%, respectively, considering at least five times the response compared to the blank response. The recovery of each compound was determined by comparing the peak height with that obtained after the injection of the same amount of drug dissolved in the sample medium.

2.5 Microsomal incubations

Incubation mixtures (500 μL containing 25 μL of solution A and 5 μL of solution B) comprised substrate (racemic PPF, *S*-PPF or *R*-PPF), 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.1 M potassium phosphate buffer (pH 7.5), and methanol (<0.5%, originating from substrate solution). For the incubations with CYP2C19 and CYP2C9, 50 mM phosphate buffer (pH 7.4) and 100 mM Tris buffer (pH 7.5) instead of the 100 mM pH 7.4 phosphate solution were used, respectively. The mixtures were first preincubated for 10 min at 37°C in an Eppendorf Thermomixer followed by addition of ice-cold SUPERSOMES (12.5 or 25 pmol for kinetic and screening data, respectively) and incubation for 30 min

(kinetic data) or 3 h (screening data) at 37°C without shaking. The reactions were terminated on ice and samples were immediately pretreated for CE analysis as described above. For HLM and S9 fraction the incubation times were 3 h and 0.5 mg/mL of protein was used. All metabolite formation rates were expressed as picomol of enantiomer formed *per* hour *per* picomol of P450 enzyme used. Experiments were carried out in duplicate and the mean values are reported.

3 Results and discussion

3.1 CE separations in absence and presence of the chiral selector

The separation of PFF and its two metabolites was first studied in an achiral system using phosphate buffers at low pH in which the compounds of interest are cations. Keeping the applied voltage, the capillary temperature, and the injection time constant at 11 kV, 25°C, and 5 psi × s, respectively, best resolution was obtained with a 50 mM sodium phosphate buffer at pH 2. Sensitivity, however, was noted to be rather low (about 200 ng/mL). An increase of the sample injection to 18 psi × s provided sensitivity at the cost of resolution. With an increase of the buffer concentration to 100 mM, a reduction of the capillary temperature to 20°C, and an increase of the applied voltage to 13 kV, however, sharp, symmetrical peaks with complete separation of the analytes (R_s>2.5 at concentrations up to at least 10 μg/mL) were obtained (Fig. 2A). The run time was found to be less than 10 min, the current was about 53 μA, and the detection limit for each compound was determined to be about 50 ng/mL. The configuration used does not exhibit any appreciable electroosmosis.

Native and modified CDs, including sulfated CDs, provide good chiral recognition with many different optically active compounds [24–26]. As sulfated β-CD was previously reported to provide enantioselectivity for the enantiomers of PPF and 5OH-PPF [22], its use was investigated with the 100 mM phosphate buffer at pH 2 together with a sample injection of 5 psi × s and application of 13 kV (normal polarity). With 0.3% sulfated β-CD and sampling of each racemate in a separate experiment, complete resolution of the enantiomer pairs was observed. Analysis of the mixture however provided incomplete separation between *R*-PPF and *S*-5OH-PPF. The use of 0.6% sulfated β-CD together with 15% v/v methanol [27, 28] resulted in complete separation of all compounds. For gaining sensitivity, sample injection was increased to 9 psi × s, which in turn decreased the resolution between *R*-PPF and *S*-5OH-PPF. Finally, with 19% of methanol, complete resolution between all analytes (R_s>1.5 for enantiomer concentrations up to 10 μg/mL) was obtained (Fig. 2B, for enantiomer assignment see below). The run time was about 30 min, the current was 35–40 μA, and the detection limit for all enantiomers was found to be about 250 ng/mL. It is important to realize that normal polarity was used in all experiments. Thus, all compounds of interest were detected as slightly positively charged complexes in an environment without any appreciable electroosmosis. Compared to the approach described by Chankvetadze *et al.* [22], in which PPF, 5OH-PPF and NOR-PPF enantiomers were shown to be separable using a low-pH triethanolamine phosphate buffer containing carboxymethyl-β-CD and sulfated β-CD (ratio 4/1 w/w), the run time with our system is more than three-fold quicker.

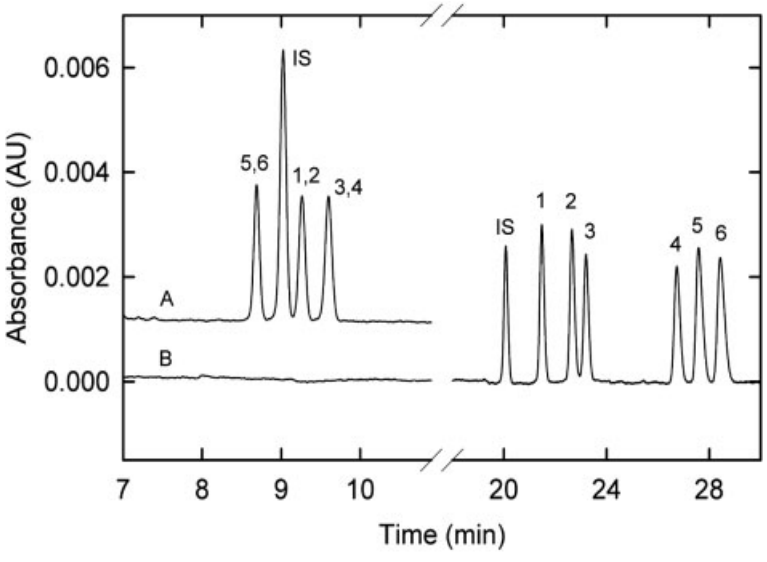

Figure 2. Electropherograms obtained with standards for (A) analysis of 1 μg/mL amounts in absence of a chiral selector and (B) analysis of about 5 μg/mL of each enantiomer in presence of 0.6% sulfated β-CD. Data of graph A are plotted with a *y*-axis offset of 0.0015 AU. Experimental conditions as described in the text. Key: 1, *S*-PPF; 2, *R*-PPF; 3, *S*-5OH-PPF; 4, *R*-5OH-PPF; 5, *S*-NOR-PPF; 6, *R*-NOR-PPF; IS, atenolol.

3.2 Method development

The extraction of PPF and its metabolites from biological fluids was studied using different solvents, including hexane, diethylether, dichloroethane, dichloromethane, ethyl acetate, and chloroform, and selected binary mixtures of these solvents. Considering the least interferences and the highest recoveries, dichloromethane was chosen to extract the samples. Sample analysis occurred in a pH 2.0 running buffer composed of 100 mM sodium phosphate, 19% methanol, and 0.6% sulfated β-CD and the sample was injected for 9 psi × s. Typical data obtained for human serum blank and serum blank fortified with 500 ng/mL of each compound are presented in Fig. 3. The blank is shown to provide a clean, interference-free electropherogram (Fig. 3A), and the data obtained with 500 ng/mL of each enantiomer (Fig. 3B) reveal that sufficient enantiomeric resolution is given for the entire calibration range used (up to 1000 ng/mL). The LODs (S/N = 3/1) for all six compounds were found to be between 10 and 12 ng/mL. Comparable results were also obtained with the 5% human albumin solution and the matrix used in microsomal preparations. Since the recoveries obtained with serum and albumin solution were found to be equal, the latter matrix was used to validate the method.

The migration order of the PPF enantiomers was established *via* analysis of a mixture of a pure enantiomer and the racemate. *S*-PPF was thereby determined to be detected first (data not shown), which is in agreement with the findings of Chankvetadze *et al.* [22]. Compared to *R*-PPF, *S*-PPF is thus forming a weaker complex with sulfated β-CD. The enantiomers of 5OH-PPF and NOR-PPF could be properly assigned *via* analysis of microsomal CYP2D6 preparations that contained the pure PPF enantiomers as well as the racemate as substrates (Fig. 4). It was thereby assumed that there is no change of configuration occuring during metabolization. The data presented in Fig. 4 clearly reveal that the *S*-enantiomers of both metabolites are detected ahead of their antipodes. Atenolol was selected as internal standard because atenolol was detected shortly before the first enantiomer of interest and without enantiomeric separation (Fig. 2B). Furthermore, atenolol showed a favorable extraction behavior (Fig. 3B).

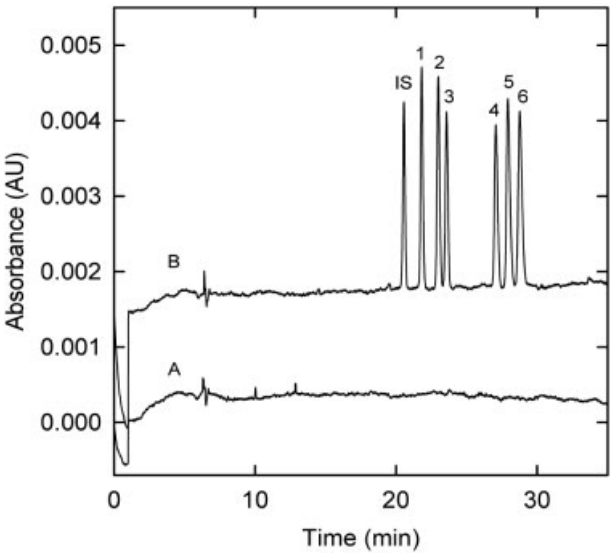

Figure 3. Electropherograms obtained for analysis of extracts prepared from (A) blank human serum and (B) human serum spiked with 500 ng/mL of each enantiomer. Experimental conditions as described in Sections 2.2 and 2.3. Current was 36 μA. Key as for Fig. 2.

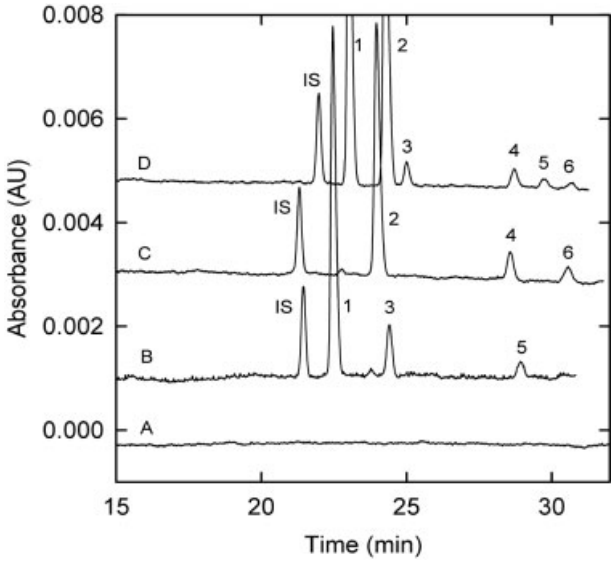

Figure 4. Electropherograms obtained with extracts prepared from 0.5 h incubations with 12.5 pmol CYP2D6 containing (A) no substrate, (B) 5 μM *S*-PPF, (C) 5 μM *R*-PPF, and (D) 10 μM racemic PPF. Experimental conditions as for Fig. 3 and key as for Fig. 2.

3.3 Method validation

Detection times of all enantiomers were found to be reproducible. RSD values (n = 5) for 50, 100, and 500 ng/mL enantiomer levels ranged between 0.89 and 2.83%. Calibration curves for all enantiomers were found to be linear in the concentration range of 25–1000 ng/mL (Table 1). Equations obtained with linear regression analysis manifested small intercepts and regression coefficients close to unity. RSD values for intraday and interday determinations were found to be in the range of 2.9–14.7% (Table 2). These values document a high precision of the assay. They fulfill the validation criteria of an analytical method designed for pharmacokinetic and drug metabolism stud-

Table 1. Calibration data obtained with human albumin solutions[a)]

Enantiomer	Slope	*y*-Intercept	r^2
S-PPF	2.12×10^{-3} (4.20)	0.049 (12.35)	0.998
R-PPF	2.18×10^{-3} (4.81)	0.052 (7.39)	0.997
S-5OH-PPF	1.50×10^{-3} (5.80)	1.30×10^{-3} (12.70)	0.996
R-5OH-PPF	1.50×10^{-3} (4.12)	5.41×10^{-3} (4.63)	0.998
S-NOR-PPF	1.16×10^{-3} (9.64)	1.94×10^{-3} (13.63)	0.996
R-NOR-PPF	9.80×10^{-4} (8.4)	1.43×10^{-3} (12.70)	0.998

a) Concentrations (ng/mL) and peak height ratios were taken as *x* and *y*, respectively. Data represent mean (RSD,%) with $n = 5$.

ies for which RSD values <15% are acceptable [23]. Also, the accuracy was found to be in the range of 88.3–109.6% (Table 2), data that are acceptable for testing drug content in biological samples. Although the recoveries for the extraction with dichloromethane are not too high (51.2–66.5%, Table 2), they are reproducible and were found to provide adequate LOQ values. Interday precision data for 25 ng/mL enantiomer levels were found to be <19% and accuracy values were in the range of 97.5–112.9%. Thus, 25 ng/mL was taken as LOQ for all six compounds.

As was discussed previously [29], the standards dissolved in methanol and kept at −20°C are stable for at least 1 month. The stability of the processed quality control samples was assessed with 500 ng/mL samples. Differences between nominal and determined concentration of less than 10% and high precision with RSD values below 4.5% ($n = 3$) were noted for samples kept at ambient temperature for at least 10 h, analyzed after four freeze-thaw cycles, and determined after 5 h storage in the autosampler of the CE instrument. These data confirm the stability of the samples.

3.4 *In vitro* metabolism of PPF

The formation of metabolites was investigated using HLM. PPF and its metabolites were resolved from interfering compounds of the microsomal matrix as blank runs showed (Fig. 5A). A typical electropherogram obtained after a 3-h incubation of 100 μM racemic PPF with HLM is depicted in Fig. 5B. From these data, it can be concluded that the two NOR-PPF enantiomers are produced in equal quantities, which means that there is no apparent stereoselectivity for that metabolite. For 5OH-PPF, however, *R*-5OH-PPF appears to be formed in a higher amount than *S*-5OH-PPF. In addition to 5OH-PPF and NOR-PPF, four other metabolites (denoted with u_1 to u_4) appeared in the electropherogram shown in Fig. 5B. A similar pattern suggesting a lower enzyme activity compared to HLM was obtained with the S9 fraction (data not shown). Incubation with HLM and S9 are configurations with many different active enzymes. For elucidation of the impact of single enzymes, *in vitro* studies with 25 pmol of various

Table 2. Interday, intraday, and recovery data for determination of PPF, 5-OH-PPF and NOR-PPF enantiomers

Enantiomer	Concentration (ng/mL)	Interday data ($n = 5$)		Intraday data ($n = 5$)		Recovery ($n = 10$)	
		Variability (RSD,%)	Accuracy (%)	Variability (RSD,%)	Accuracy (%)	%	±SD
S-PPF	50	11.4	101.1	12.4	109.6	57.9	4.5
	100	8.6	105.4	2.9	105.1	60.3	8.1
	500	6.6	99.1	7.6	103.8	63.7	5.2
R-PPF	50	11.6	98.5	9.4	98.3	58.9	6.8
	100	9.4	96.5	7.9	102.8	63.1	9.5
	500	7.3	97.6	7.8	94.7	66.5	3.9
S-5OH-PPF	50	13.7	95.5	6.4	97.1	53.8	4.6
	100	6.8	93.1	13.1	92.5	54.5	6.7
	500	6.7	98.9	8.5	98.4	54.5	6.8
R-5OH-PPF	50	14.1	99.6	14.2	99.1	58.5	5.9
	100	14.7	97.3	11.9	96.3	57.8	6.2
	500	8.6	97.1	12.1	98.7	61.7	4.6
S-NOR-PPF	50	10.8	100.3	10.5	102.1	55.5	9.3
	100	14.5	97.1	7.8	88.3	51.2	6.7
	500	10.2	95.3	12.2	100.5	65.9	4.6
R-NOR-PPF	50	14.1	97.1	9.9	107.6	57.2	7.6
	100	12.9	95.8	9.1	91.5	53.3	7.3
	500	7.1	96.7	13.6	100.3	65.6	4.5

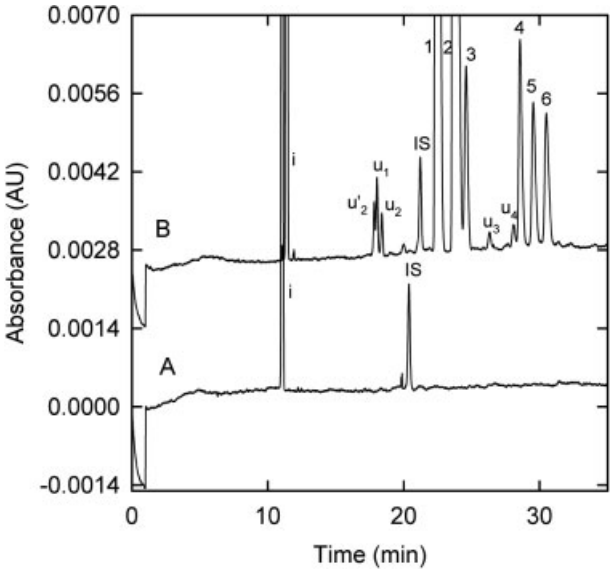

Figure 5. Electropherograms obtained with extracts prepared from 3 h incubations with HLM containing (A) no substrate and (B) 100 μM racemic PPF. Experimental conditions as for Fig. 3. Peak i is unrelated to PPF, u_1, u_2, u_2', u_3 and u_4 are unidentified PPF metabolites. Other key as for Fig. 2.

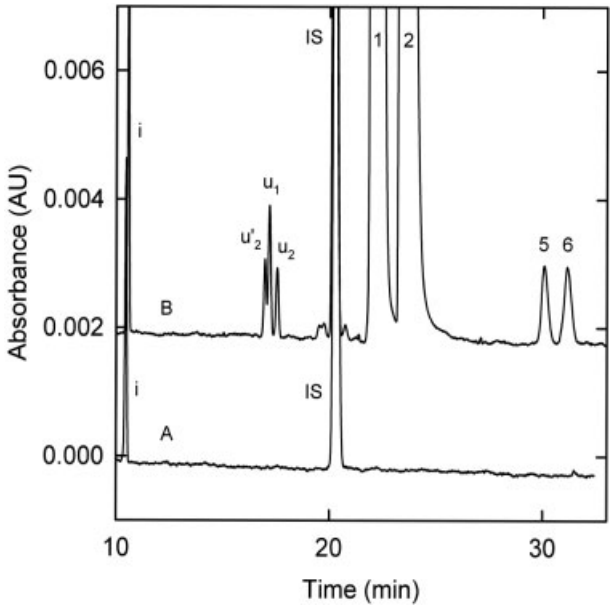

Figure 6. Electropherograms obtained with extracts prepared from 3 h incubations with 25 pmol CYP3A4 containing (A) no substrate and (B) 100 μM racemic PPF. 100 μg/mL IS solution was used. Key as for Figs. 2 and 5.

single enzyme SUPERSOMES and 100 μM racemic PPF were conducted. CYP3A4 could thereby be shown to produce NOR-PPF, and two of the unidentified metabolites, namely u_1 and u_2 (Fig. 6). Incubation with single enantiomers revealed that u_2 and u_2' are the *R*- and *S*-

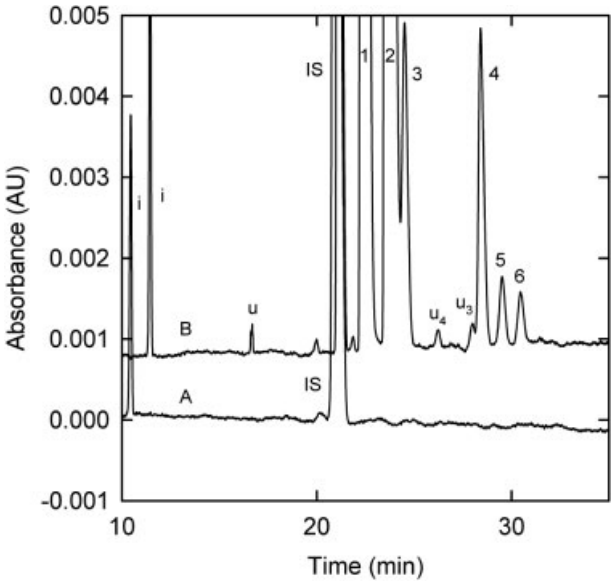

Figure 7. Electropherograms obtained with extracts prepared from 3 h incubations with 25 pmol CYP2D6 containing (A) no substrate and (B) 100 μM racemic PPF. 100 μg/mL IS solution was used. Key as for Figs. 2 and 5.

enantiomers of this metabolite, respectively (data not shown). CYP2D6 was found to form 5OH-PPF and, to a lesser extent, also NOR-PPF (Fig. 7). Small amounts of the unidentified metabolites were also detected under these conditions (Fig. 7), but not for the 30-min incubations with smaller amounts of enzyme and substrate (Fig. 4D). Metabolite u in Fig. 7 might be identical to metabolite u_1 of Figs. 5 and 6. No further investigations were undertaken to confirm this assumption. Metabolite u_4 was determined to be formed by CYP2A6 (Fig. 8). CYP1A1 and CYP1A2 produced NOR-PPF and no PPF metabolites were detected in presence of CYP2C9, CYP2C19, and CYP2B6 (data not shown).

According to the recommendations of the manufacturer of the microsomal preparations, metabolite production is expected to be linear with respect to an enzyme concentration up to at least 100 pmol/mL and for 30- and 20-min incubations for CYP2D6 and CYP3A4, respectively. Our preliminary studies using 5 μM of racemic PPF showed that production of 5OH-PPF and NOR-PPF using 12.5 pmol of each enzyme was linear using an incubation time of 30 min. In order to check the applicability of the developed CE method for *in vitro* studies, PPF at two different concentrations (5 and 25 μM) for each enantiomer were incubated with CYP2D6 and CYP3A4 SUPERSOMES, as described in Section 2.5. These enantiomer concentrations were selected because Zoble *et al.* [30] showed that PPF plasma concentrations of equal to or higher than 1.5 μg/mL (4.39 μM) could suppress pre-

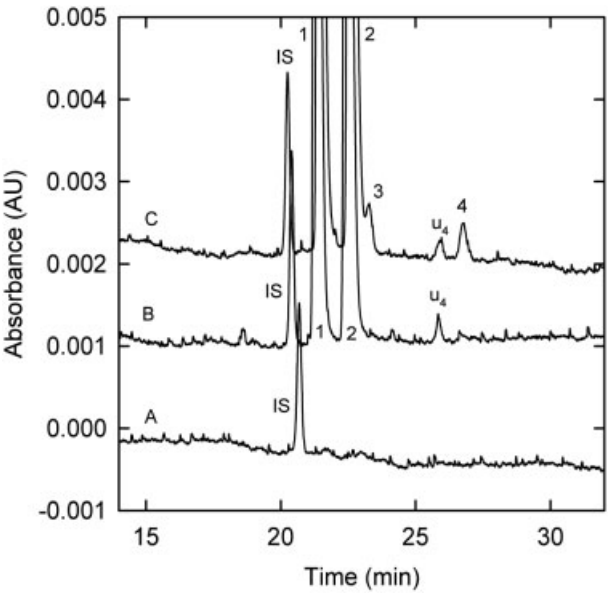

Figure 8. Electropherograms obtained with extracts prepared from 3 h incubations with 25 pmol CYP2A6 containing (A) no substrate and (B) 100 μM racemic PPF. Data obtained in panel C were obtained with the extract of B that was fortified with a small amount of racemic 5OH-PPF. Key as for Figs. 2 and 5.

mature ventricular beats, couplet beats (pairs), and ventricular tachycardia beats in patients with ventricular arrhythmia. The concentration of each enantiomer was determined using the enantioselective CE assay (Fig. 4), and the metabolic formation rates were calculated (Table 3). To the best of our knowledge, this is the first report on investigating PPF metabolite formation in incubation media containing SUPERSOMES. CYP2D6 was determined to produce both major metabolites of PPF (Figs. 4 and 7), whereas CYP3A4 is producing NOR-PPF only (Table 3). This is the first account for the formation of NOR-PPF *via* the CYP2D6 pathway.

Having 5 and 25 μM substrate concentrations, the rates of metabolite formation were found to range between 10.34 and 82.34 $pmolh^{-1}pmol^{-1}$ enzyme (Table 3). For the incubations with CYP2D6, the rates of metabolite formation were found to be essentially equal at the two substrate concentrations (Table 3). This does not come as a surprise because a low Michaelis–Menten constant K_m was reported for the *in vitro* formation of 5OH-PPF using HLM (K_m = 5.3 μM and 3.0 μM for *S*-PPF and *R*-PPF, respectively [31]), which suggests that saturation is reached. CYP2D6 incubation with the single enantiomers of PPF revealed that the formation of *S*-5OH-PPF and *S*-NOR-PPF was significantly faster (1.43-fold and 1.31-fold, respectively) than the production of their antipodes. This is in contrast to the incubation with racemic PPF for which the formation of the *S*-enantiomers was only slightly faster (1.15-fold and 1.08-fold, respectively). Furthermore, all formation rates were found to be smaller compared to those obtained with single enantiomers (Table 3). It appears that metabolite formation is somewhat hindered in presence of both enantiomers. This was previously reported and attributed to enantiomer–enantiomer interactions [6, 31, 32]. Similarly, in humans and when the enantiomers were administered separately, *S*-PPF was found to become faster metabolized than *R*-PPF [6, 32, 33]. Thus, our data appear to be in agreement with the literature.

For the incubations with CYP3A4, data obtained for the two concentration levels were found to be different (Table 3). More than two-fold higher formation rates were

Table 3. Formation rates of the metabolite enantiomers obtained with CYP2D6 and CYP3A4 SUPERSOMES[a)]

Substrate	Enzyme	Metabolite(s)	Rate of metabolite formation ($pmolh^{-1}pmol^{-1}$ enzyme) 5 μM of substrate[b)]		Rate of metabolite formation ($pmolh^{-1}pmol^{-1}$ enzyme) 25 μM of substrate[b)]	
			R-enantiomer	*S*-enantiomer	*R*-enantiomer	*S*-enantiomer
Racemic PPF	CYP2D6	*R*- and *S*-5OH-PPF	30.44	35.05	31.70	36.14
	CYP2D6	*R*- and *S*-NOR-PPF	21.31	23.19	21.17	22.92
	CYP3A4	*R*- and *S*-NOR-PPF	15.12	10.34	37.15	28.23
R-PPF	CYP2D6	*R*-5OH-PPF	54.01	–	53.43	–
	CYP2D6	*R*-NOR-PPF	33.22	–	35.23	–
	CYP3A4	*R*-NOR-PPF	13.19	–	43.38	–
S-PPF	CYP2D6	*S*-5OH-PPF	–	77.43	–	82.34
	CYP2D6	*S*-NOR-PPF	–	43.55	–	44.85
	CYP3A4	*S*-NOR-PPF	–	16.17	–	61.11

a) 30 min incubation with 12.5 pmol enzyme.
b) Concentration for each enantiomer.

noted for the 25 μM incubations. As the K_m value for the NOR-PPF formation was reported to be high (K_m = 116 μM [34]), no saturation is expected for the studied concentration levels. CYP3A4 incubation with the single enantiomers of PPF revealed that the formation of *S*-NOR-PPF was faster (1.23-fold and 1.41-fold for 5 and 25 μM substrate concentrations, respectively) than that of its antipode (Table 3). The opposite was found to be true for incubation with racemic PPF, indicating that the formation of *S*-NOR-PPF is much reduced in the presence of *R*-PPF. This reduction is stronger than that observed for the CYP2D6 pathway discussed above. It is interesting to note that Zhou *et al.* [35] reported opposite results using S9 incubations prepared from transgenic Chinese hamster CHL cell lines expressing liver CYP3A4.

4 Concluding remarks

This is the first report on chiral determination of PPF and its main metabolites in biological samples using enantioselective CE. To our knowledge, the assay described even represents the first approach for the simultaneous determination of the enantiomers of PPF, 5OH-PPF and NOR-PPF in such samples. The CE method is shown to be sensitive, selective, reproducible, and inexpensive. The assay is demonstrated to be suitable for the determination of enantiomers of the three compounds in *in vitro* incubations comprising HLM, S9 fraction, or SUPERSOMES. Incubations with CYP2D6 revealed, for the first time, the simultaneous formation of the enantiomers of 5OH-PPF and NOR-PPF with that enzyme. The assay is further shown to permit the evaluation of the enzymatic *N*-dealkylation and hydroxylation rates and has been applied to the determination of the *in vitro* kinetics of the PPF metabolism [36]. Furthermore, it might be suitable to evaluate stereoselective pharmacological aspects of the active metabolites of PPF.

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