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Liquid chromatographic analysis of propafenone enantiomers in human plasma

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SUMMARY

A convenient and sensitive high-performance liquid chromatographic method for analysis of the enantiomers of propafenone (PPF) in human plasma was developed. Racemic propafenone and (−)-ephedrine (internal standard) were first extracted from plasma samples into a mixture of hexane–2-propanol–heptafluorobutanol (95:5:1.25, v/v). After evaporation of the organic layer, the samples were derivatized with *R*(−)-naphthylethyl isocyanate. The derivatization reached its maximum within 30 s at room temperature with an efficiency of 93.9 ± 2.8% (mean ± S.D.). The formed diastereomers were subsequently separated on a silica column with a mobile phase of hexane–2-propanol–isobutanol (96:2:2, v/v) at a flow-rate of 1.5 ml/min. The ultraviolet detection wavelength was set at 220 nm. Using 1 ml plasma, the detection limit was 6.25 ng/ml for the propafenone enantiomers. The assay was successfully employed to measure propafenone enantiomers in plasma samples of a healthy subject after oral administration of a single 150-mg dose of the racemate.

INTRODUCTION

Propafenone (PPF, Fig. 1) is a type IC antiarrhythmic drug [1] which is marketed as a racemic mixture. Although the two PPF enantiomers are virtually equipotent sodium channel blocking agents, β-adrenoceptor blockade has been attributed mainly to the (+)-enantiomer [2].

Similar to most of the available β-adrenoceptor blocking agents, PPF contains an isopropanolamine side-chain in its structure (Fig. 1). Therefore, chiral reagents utilized for derivatization and separation of the enantiomers of β-blockers may be employed for this drug. Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) is a chiral reagent which has been used for derivati-

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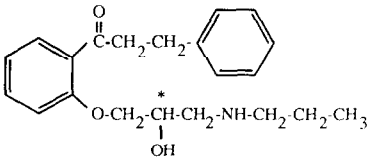

Fig. 1. Structure of propafenone; the asterisk indicates the asymmetric center.

zation and separation of the enantiomers of several β-adrenoceptor blockers [3]. Recently, Kroemer et al. [2] applied this reagent for high-performance liquid chromatographic (HPLC) analysis of the PPF enantiomers in plasma after multiple-dose administration of the racemate. The reported HPLC assay, however, utilizes a multi-step extraction process which results in loss of 60% of the drug [2]. Furthermore, the detection limit of the assay, 100 ng/ml [2], limits its utility for single-dose pharmacokinetic studies where lower concentrations are expected [4].

Our laboratory, very recently, reported the application of (−)-menthyl chloroformate for separation of the enantiomers of β-adrenoceptor agonists and antagonists by liquid chromatography [5,6]. In a preliminary experiment, this derivatization method was applied to PPF, and the resultant diastereomers were successfully separated on a silica column. However, similar to the results obtained with GITC [2], the sensitivity of the method was unsuitable for single-dose pharmacokinetic studies.

Blaschke and Walther [7] utilized fractional crystallization to preparatively resolve the enantiomers of PPF as their diastereomeric salts with di-O,O′-*p*-toluoyltartaric acid; the purity of the obtained enantiomers was then confirmed by derivatization of the enantiomers with *R*(+)-phenylethyl isocyanate and liquid chromatographic separation of the formed derivatives. The chromatographic method, however, was not applied to measurements of the enantiomers in biological fluids.

In this article a sensitive and convenient HPLC method for measurement of the propafenone enantiomers in human plasma, based on derivatization with *R*(−)-1-(1-naphthyl)ethyl isocyanate (NEIC), is reported.

EXPERIMENTAL

Materials

Powders of racemic PPF·HCl (lot 127F0782) and the internal standard (I.S.), (−)-ephedrine sulfate (lot 71621A), were purchased from Sigma (St. Louis, MO, U.S.A.) and Merck Chemical Division (Merck & Co., Rahway, NJ, U.S.A.), respectively. A small amount of pure (−)-PPF was kindly supplied by Dr. Dan M. Roden of the Vanderbilt University Department of Pharmacology (Nashville, TN, U.S.A.). Racemic bupranolol hydrochloride was a generous gift from Dr. F.M. Pasutto of the University of Alberta Faculty of Phar-

macy (Edmonton, Canada). NEIC and heptafluoro-1-butanol (HFB) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Isobutanol and 2-propanol (Fisher Scientific, Itasca, IL, U.S.A.) and hexane (Baxter Healthcare, Muskegon, MI, U.S.A.) were high-purity HPLC grade. All other reagents and solvents were analytical-reagent grade.

Apparatus

The HPLC instrument (Waters, Milford, MA, U.S.A.) consisted of a 501 pump, a 712 WISP autosampler, a 484 tunable absorbance detector and a 745 integrator. The detector was set at a wavelength of 220 nm.

Chromatography

Diastereomeric derivatives of PPF enantiomers and I.S. were analyzed at ambient temperature utilizing a 10 cm×4.6 mm I.D. analytical column containing 5-μm silica packing material (Partisil 5; Whatman, Clifton, NJ, U.S.A.) and a 5 cm×4.6 mm I.D. refillable guard column (Supelco, Bellefonte, PA, U.S.A.) packed with pellicular normal-phase media (Pellicular silica; Whatman). The mobile phase consisted of hexane–2-propanol–isobutanol (96:2:2, v/v) which was pumped at a flow rate of 1.5 ml/min.

Underivatized racemic PPF was analyzed utilizing a reversed-phase chromatographic system; a 10 cm×4.6 mm I.D. analytical column containing 5-μm octadecylsilane packing material (Partisil 5 ODS3; Whatman) and a mobile phase of water–methanol–acetic acid–triethylamine (50:50:0.1:0.05, v/v) were used. The detection system and flow-rate were similar to the assay of the diastereomers. Under these conditions, the drug eluted as a symmetric peak at 6.7 min.

Standard solutions

The Chiral reagent (NEIC) was purified as follows: commercially available NEIC was first diluted (10%, v/v) in hexane. This solution was then passed through a 5-cm guard column packed with silica material; the solution in a glass syringe was manually forced through the column with a rate of approximately 1 ml/min. The eluent was diluted further with hexane (0.1%, v/v) and stored in amber containers at −30°C. Under these conditions, the reagent solution was stable for several months. Working solutions were prepared daily immediately before use by diluting (0.005%, v/v) the reagent solution with hexane–2-propanol (95:5). The process of purification resulted in elimination of late-eluting peaks in the chromatogram.

Bupranolol base solution was prepared as follows. Bupranolol hydrochloride powder (5 mg) was dissolved in 1 ml distilled water to which was added 0.5 ml saturated sodium carbonate. Bupranolol base was then extracted into 25 ml of hexane.

Stock solutions of racemic PPF (equivalent of 25 μg/ml base) and I.S. (1 μg/

ml) were prepared in water and stored at 4 °C. Varying volumes of the PPF stock solution were added to drug-free plasma to produce final concentrations (based on each enantiomer) of 6.25, 12.5, 25, 50, 125 and 500 ng/ml.

Calibration curves were constructed by plotting the peak-area ratios of (+)- or (−)-PPF to I.S. versus the concentrations of PPF in plasma.

Sample preparation

To 1.0 ml plasma in a glass tube were added 100 μl of I.S. (1 μg/ml) and 200 μl saturated sodium carbonate, resulting in a pH of 10.4. Following addition of 4 ml of a hexane–2-propanol–HFB (95:5:1.25) mixture, the samples were vortex-mixed for 30 s and centrifuged for 5 min at 1800 *g*. The organic layer was then transferred to clean glass tubes and evaporated under a nitrogen stream. The residue was dissolved in 100 μl of the reagent solution by a brief (5 s) vortex-mixing of the samples. The samples were kept at room temperature for 3 min, then 100 μl of bupranolol solution were added to remove the unreacted NEIC. The solvent was evaporated to dryness under a nitrogen stream. Following dissolving the residue in 400 μl hexane, 200 μl of a 0.1 *M* solution of hydrochloric acid were added, and excess bupranolol was extracted into the aqueous layer by vortex-mixing for 15 s. After centrifugation for 5 min at 1800 *g*, 100 μl of the upper layer were injected into the chromatograph.

Extraction efficiency

Plasma samples ($n=4$) containing 25 and 250 ng/ml PPF were extracted according to the above extraction method without addition of I.S. Exact volumes (3 ml) of the extraction solvent were taken, and the samples were processed according to the procedure described in the *Sample preparation* section. The peak areas of the derivatized enantiomers in these samples were then compared to those obtained from reference samples with comparable concentrations. Reference sample preparation was similar to that of the extracted samples with one difference; instead of addition of PPF standard solution to the plasma, PPF was added to the extraction solvent before evaporation.

Because PPF is extracted as its base into the organic solvent, and the derivatization reaction may be affected by presence of salt, the extraction efficiency experiments were conducted using PPF free base.

Derivatization efficiency

Spiked plasma samples ($n=6$) containing 500 ng/ml PPF enantiomers and no I.S. were subjected to the above extraction procedure. An exact volume (3 ml) of the extraction solvent was evaporated, and the residue was derivatized with NEIC according to the above method. The unreacted racemic PPF was then extracted into 0.5 ml of 0.1 *M* hydrochloric acid by vortexing-mixing for 30 s and centrifugation for 5 min. A 100-μl aliquot of the acidic solution was injected into the HPLC system developed for analysis of the racemic PPF. The

peak areas of the racemic PPF in these derivatized samples were then compared with those obtained after injection of the underivatized samples prepared in a similar manner in the absence of the reagent.

Accuracy and precision

On different days, spiked plasma samples ($n=6$) containing 6.25–500 ng/ml PPF enantiomers were subjected to the assay procedure, and the concentrations of the individual enantiomers were determined from the standard calibration curves. The accuracy was calculated as the difference between the calculated and added concentrations, whereas the precision was evaluated by the intra- and inter-day coefficients of variation (C.V.).

Identification of derivatives

PPF·HCl powder (2 mg) was first dissolved in 0.5 ml distilled water, and 0.5 ml saturated sodium carbonate solution was subsequently added. The PPF free base was then extracted into 8 ml of the extraction solvent after vortex-mixing for 1 min and centrifugation for 5 min. The residue after evaporation was derivatized with 1 ml of a 0.1% solution of NEIC in hexane–2-propanol (95:5, v/v). The unreacted PPF, if any, was extracted into 1 ml of 1 *M* hydrochloric acid after vortex-mixing for 1 min and centrifugation for 5 min. After injection of the organic layer into the HPLC system, the derivatives of PPF were collected from the eluent. The residue remaining after evaporation of the mobile phase was then subjected to a Kratos positive-ion low-resolution fast atom bombardment mass spectrometric (FAB-MS) system, using a 3-nitrobenzyl alcohol–2-hydroxyethyl disulfide matrix.

Order of elution of the derivatized enantiomers

This was determined by subjecting the racemate and the available pure enantiomer of PPF [(−)-PPF] to the assay procedure and comparison of the HPLC retention times.

Application to pharmacokinetic studies

A single 150-mg tablet of racemic PPF·HCl (Rythmol; Knoll Pharmaceuticals, Mississauga, Canada) was administered orally to a healthy 32-year-old male volunteer. Blood samples (8 ml) were collected into citrated tubes through a forearm vein at 0, 0.5, 1, 2, 4 and 8 h after the drug administration. Resultant plasma samples were kept frozen at −30°C for two days; they were then subjected to the aforementioned procedure for determination of the individual PPF enantiomers.

RESULTS AND DISCUSSION

NEIC is a chiral reagent which has been used previously for separation of the enantiomers of a number of β-adrenoceptor blocking agents [8,9]. The

naphthyl moiety of NEIC also contributes strong UV and fluorescence characteristics to the derivatized molecule. In the present study, this reagent was successfully employed for precolumn derivatization of relatively low concentrations of PPF enantiomers, subsequent separation and UV detection of the formed diastereomers (Fig. 2). Under the stated conditions, the formed diastereomers of (—)- and (+)-PPF and I.S. eluted at 8.3, 10.1 and 16.4 min, respectively (Fig. 2); complete baseline resolution was obtained for the two diastereomers of PPF.

The reported HPLC method for measurement of the underivatized racemic PPF [10] has utilized UV detection. This is because underivatized PPF, unlike most β-blockers, lacks detectable fluorescence properties. Because of the naphthyl moiety of the derivatization reagent, however, the derivatives of PPF enantiomers are expected to exhibit fluorescence activity. Interestingly, when derivatized samples were subjected to fluorescence detection, no peaks related to the enantiomers of PPF could be detected in the chromatogram. Nevertheless, UV detection of the derivatives resulted in a very satisfactory sensitivity (Fig. 2).

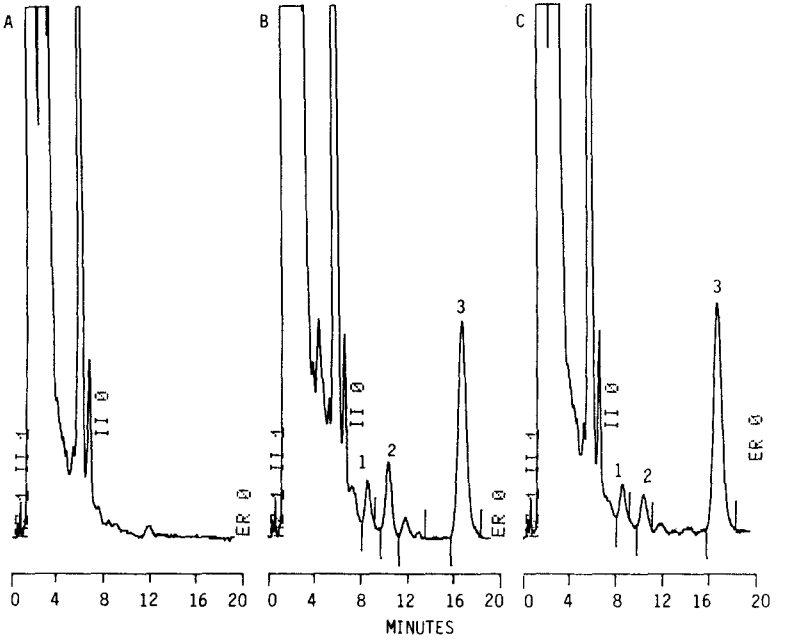

Fig. 2. Chromatograms of a blank plasma (A), a sample 0.5 h after oral administration of 150 mg racemic PPF to a healthy subject (B) and a blank plasma sample spiked with 6.25 ng/ml of each PPF enantiomer (C). The 0.5-h sample contained 9.61 and 15.8 ng/ml of the (−)- and (+)-enantiomers, respectively. Peaks 1 and 2 are derivatized (−)- and (+)-PPF, respectively; peak 3 is derivatized internal standard. The detector attenuation was set at 8.

To optimize derivatization, NEIC was dissolved in the following selected solvents: acetonitrile, acetone, methanol, 2-propanol, chloroform, hexane and ethyl acetate, each in the presence and absence of basic modifiers such as pyridine or triethylamine. Among the solvents tested, the most satisfactory result was obtained when NEIC was dissolved in hexane without the addition of basic modifiers. At room temperature, the reaction yield for both PPF enantiomers and I.S. increased with time and reached its maximum within approximately 40 min (Fig. 3). However, when a mixture of hexane–2-propanol (95:5) was used as a vehicle for the reagent, the reaction reached its maximum within 30 s (Fig. 3), exhibiting results similar to those observed 40 min after derivatization in hexane alone. Hence, this mixture was selected as the vehicle for the reaction. Using the optimum conditions, the derivatization yield, based on residual analysis of PPF, was $93.9 \pm 2.83\%$ (mean $\pm$ S.D.).

The following major ions, *m/z* (%), were observed when the PPF derivatives were subjected to FAB-MS: 539 (68, MH^+), 521 (32, loss of water), 342 (30, loss of isocyanate) and 155 (100, $C_{12}H_{11}$). A molecular ion of 539 indicates that the derivatization of PPF with NEIC takes place on a 1:1 molar basis; no peak was observed at *m/z* 737 (expected MH^+ of a double derivative). Furthermore, presence of an ion at *m/z* 521 suggests that the alcohol group is intact in the derivatized molecule. The derivatization of the amine group was further supported by the fact that the derivatives could be extracted into an organic solvent in the presence of an acidic medium. This conclusion is in agreement with that of Blaschke and Walther [7] who reported that phenylethyl isocyanate reacted only with the amine group of PPF. Furthermore, it has been reported [9] that at room temperature NEIC reacts with the secondary amine group located on the side-chain of β-blocking agents, leaving the alcohol functional group intact.

The high reactivity of NEIC requires certain precautions to avoid formation

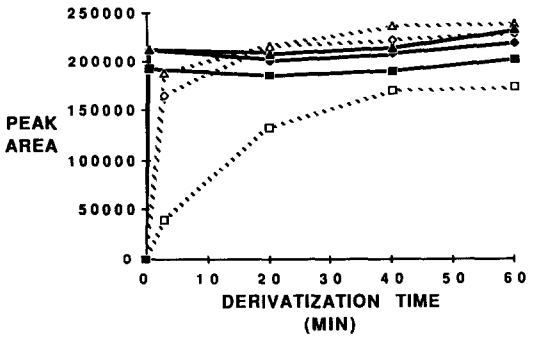

Fig. 3. Effect of time on the derivatization yield of (−)-PPF (◇, ◆), (+)-PPF (△, ▲), and I.S. (□, ■) in the presence (filled symbols; continuous lines) and absence (open symbols; broken lines) of 2-propanol.

of by-products and/or degradation products that may interfere with the chromatographic peak(s) of interest. For instance, although the derivatization was very rapid when the reagent was dissolved in hexane–2-propanol, this mixture could not be used to store the reagent; with time several interfering and late-eluting chromatographic peaks would develop. To avoid this, the reagent was dissolved in hexane and stored in light-protected containers; working solutions were prepared in hexane–2-propanol immediately before derivatization. Using this method, resultant chromatograms (Fig. 2) were free of interfering peaks. Furthermore, no late-eluting chromatographic peaks were observed.

Various structurally related compounds (Table I) were evaluated for their suitability as internal standard. Although derivatization was apparently successful for all drugs studied, only ephedrine exhibited suitable chromatographic characteristics as an internal standard (Fig. 2). Since ephedrine contains a secondary alcohol and amine group in its side-chain, it is expected to undergo a derivatization reaction similar to that of PPF.

Initially the plasma samples were extracted utilizing hexane–2-propanol (95:5). Although this mixture was appropriate for extraction of PPF, the peak areas of the derivatized I.S. were not reproducible. Addition of HFB to the extraction solvent, however, resulted in a significant increase in the peak area of I.S. with subsequent increase in assay reproducibility. HFB has been used previously to enhance the extraction yield of the hydrophilic β-blocker, atenolol from plasma [11]. Utilizing a solvent of hexane–2-propanol–HFB (95:5:1.25), the extraction efficiency (mean $\pm$ S.D.) for PPF was 82.1 $\pm$ 2.2

TABLE I

CHROMATOGRAPHIC CHARACTERISTICS OF THE STRUCTURALLY RELATED DRUGS

Drug	Retention time (min)	
	First peak	Second peak
Alprenolol	4.8	5.2
Bupranolol	3.1	3.4
(−)-Ephedrine	16.2[a]	–
Metoprolol	7.8[b]	–
Methoxamine	11.1	13.6
Mexiletine	4.6	5.6
Pindolol	13.2	13.8
Propranolol	5.9	6.5
Oxprenolol	4.9[b]	–
S-Timolol	5.4[a]	–
Tocainide	3.1	4.0

[a]Pure enantiomer.
[b]One peak was observed.

and 86.2±2.9% at plasma concentrations of 25 and 250 ng/ml, respectively. This is in contrast to the low extraction yield of 40% which was reported by Kroemer et al. [2] after multi-step extraction of PPF.

The relationship between (−)- or (+)-PPF/I.S. peak-area ratios and PPF plasma concentrations were linear ($r>0.998$) over a wide concentration range: 6.25–500 ng/ml. The typical equations describing this relationship were $y=0.0112x-0.0417$ for (−)-PPF and $y=0.0120x-0.0319$ for (+)-PPF, where y and x are the peak-area ratios and plasma concentrations, respectively.

The results of assay precision and accuracy, which were carried out over a one-week period, are reported in Table II. The wide range of the plasma concentrations employed in this assay necessitated the use of more specific calibration equations for determination of lower (6.25–50 ng/ml) PPF concentrations; values for the plasma concentrations of 125 and 500 ng/ml were not included in constructing the calibration curves for this range. This resulted in typical calibration curves of $y=0.0090x-0.0010$ for (−)-PPF and $y=0.0103x-0.0023$ for (+)-PPF for the concentration range 6.25–50 ng/ml ($r>0.999$). Using this method the accuracy (percentage error, Table II) was excellent even at very low concentrations. However, when only one calibration equation was used for the entire concentration range, the percentage error was higher at low concentrations. In general, the interday C.V.s and error values ranged from 3.2 to 12.5% and −2.38 to 5.28%, respectively (Table II). The intra-day C.V.s were less than 9.7 and 10.2% for the (−)- and (+)-enantiomers, respectively. Considering a signal-to-noise ratio of 5, a detection limit of lower than 6.25 ng/ml could be established. However, lower concentrations were associated with higher C.V. and percentage error.

Mobile phase selection was crucial to achieve desired sensitivity and resolution. Normal-phase solvents such as chloroform and ethyl acetate could not

TABLE II

ACCURACY AND PRECISION OF THE ASSAY ($n=6$)

The values in parentheses are inter-day coefficients of variation (%).

Added concentration (ng/ml)	Calculated concentration (ng/ml)		Percentage error	
	(−)-PPF	(+)-PPF	(−)-PPF	(+)-PPF
6.25	6.10 (12.2)	6.46 (12.5)	−2.4	3.3
12.5	13.0 (10.1)	12.7 (11.1)	4.3	1.6
25.0	24.6 (5.7)	23.7 (8.2)	−1.8	−5.3
50.0	50.1 (7.0)	50.2 (6.7)	0.22	0.40
125	124 (6.6)	125 (4.9)	−1.1	0.16
500	496 (3.3)	497 (3.2)	−0.73	−0.58

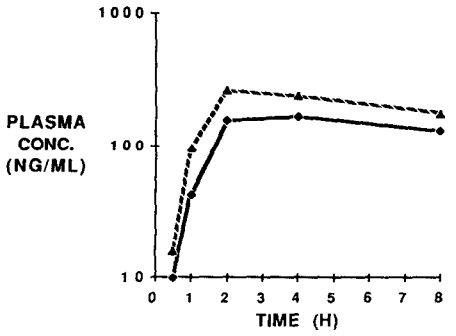

Fig. 4. Plasma concentration–time courses of (−)-PPF (♦) and (+)-PPF (▲) after oral administration of a single 150-mg dose of the racemate.

be used as, at the relatively low detection wavelength utilized in this assay, their use was associated with high baseline noise.

Preliminary experiments revealed that the presence of excess reagent in the samples resulted in the appearance of interfering peaks with time. This is important as several samples are usually prepared at once, and some of these samples are injected into the chromatograph several hours afterward. Ethanolamine has been previously used to react with excess phenylethyl isocyanate [12]; the resultant derivative eluted early in a reversed-phase chromatographic system. However, in the normal-phase assay employed in this study, use of ethanolamine resulted in the appearance of several late-eluting chromatographic peaks. Any structurally related compound with its derivatives eluting early in the chromatogram could be an alternative to remove excess derivatizing reagent. Among the tested compounds (Table I), bupranolol exhibited these characteristics. However, it must be realized that bupranolol may be replaced by any other similar drug (Table I) which its derivatives elute before the derivatized PPF enantiomers. Before injection of the samples into the chromatograph, a simple vortex-mixing in the presence of 0.1 *M* hydrochloric acid permits removal of underivatized bupranolol.

The assay was successfully utilized for analysis of plasma samples obtained from a healthy subject who was administered a single 150-mg tablet of PPF·HCl orally (Fig. 4). Consistent with a previous report [2], the plasma concentration–time courses of the enantiomers were stereoselective. In our study, the last plasma sample was obtained at 8 h. However, based on the concentrations of the PPF enantiomers in this sample (Fig. 4), the time courses of the enantiomers could have been followed for a substantially longer period of time in this subject. No significant change in the concentration of PPF was observed when spiked plasma samples were kept at −30°C and injected into the HPLC system over a one-week period. Hence, storage of the plasma samples obtained

from the volunteer for two days before analysis is not expected to have any significant impact on the outcome of the results.

In conclusion, a sensitive and convenient liquid chromatographic method is reported for determination of propafenone enantiomers in human plasma. The applicability of the assay to single-dose pharmacokinetic studies of the enantiomers of propafenone is also demonstrated.

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