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Enantioselective analysis of R- and S-propafenone in plasma by HPLC applying column switching and liquid–liquid extraction

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ABSTRACT

Two HPLC methods are described for the enantioselective analysis of R- and S-propafenone in plasma. In a column switching approach, centrifuged plasma was injected onto a silica-based strong acid cation-exchanger and the fraction containing propafenone was switched on-line onto an enantioselective Chiralcel column for separation of the enantiomers. In another approach, propafenone was extracted from plasma by liquid–liquid extraction at pH 11.4. The extracted components were transferred into aqueous medium and separated on a Chiralcel ODR. Both methods were validated and showed comparable performance. Within-day and between-day precision was better than 5% for both methods. Linear calibration functions were obtained ($r^2 > 0.999$), and the limit of detection for each enantiomer was 0.2 μg/mL for column switching and 0.55 μg/mL for liquid–liquid extraction. The analysis methods were applied for the determination of the effect of physical exercise on the enantiomeric ratio of R- and S-propafenone in plasma of healthy volunteers. During exercise, the concentration of both enantiomers reached a maximum, followed by a significant decrease during recovery.

1. Introduction

Propafenone (2-(2-hydroxy-3-(propylamine)-propoxy)-3-phenylpropiophenone) [Fig. 1] is a chiral drug applied in the treatment of supraventricular and ventricular arrhythmias. It shows moderate β-blocking and channel blocking activities. Both enantiomers are equally potent in their activity as sodium channel blockers [1], but the β-blocking activity of the S-enantiomer is about 100 times higher [2,3]. Propafenone is administered as racemic mixture in therapeutic formulations.

Enantioselective analysis of propafenone is preferably performed by chromatographic methods such as thin-layer chromatography [4,5], capillary electrophoresis [6–8] and HPLC [9–17]. Especially HPLC offers several methodical alternatives for separation of the propafenone enantiomers. For instance, a chiral counter ion can be added to the eluent [14,16] or the analyte is derivatised with a chiral reagent before analysis [15].

Another way is the enantioselective separation on a chiral stationary phase, either polysaccharides such as cellulose and amylose derivatives [9–11,13,18,19] or protein-based adsorbents [20]. These adsorbents can be operated in the normal-phase mode applying mixtures of a non-polar alkane and a polar modifier such as ethanol or in the polar-organic mode with acetonitrile and methanol as eluent. Some adsorbents can also be operated under reversed-phase conditions applying aqueous eluents, which is an advantage for the analysis of aqueous samples. For analysis of propafenone in plasma it is necessary to remove proteins of high molecular weight and other interfering components, which may disturb enantioselective separation and accelerate column degradation. In most cases this is performed before chromatographic separation applying liquid–liquid extraction [9,10,15,17,19] or solid-phase extraction [12].

In a study the effect of exercise on the concentration of R- and S-propafenone in plasma was examined. Healthy volunteers were subjected to physical exercise and blood samples were taken in defined intervals and analysed. This work describes the enantioselective analysis of the plasma samples by HPLC and reports some of the results of the study. Separation of R,S-propafenone was performed on an enantioselective Chiralcel cellulose column in reversed-phase modus. For analysis of the samples, two different methodical approaches were elaborated. A column switching method allows the injection of untreated plasma samples on the first column and separation of the enantiomers in the switched fraction on the enantioselective second column. In another approach, sample preparation was carried out by liquid–liquid extraction and the extract was injected directly onto the enantioselective column.

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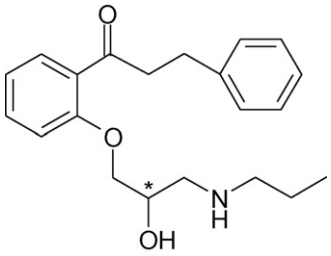

Fig. 1. Structure of R,S-propafenone.

2. Experimental

2.1. Chemicals and samples

Racemic propafenone and S-(−)-propanolol and were from Sigma–Aldrich (St. Louis, MO, USA). Potassium hydroxide, sodium hydroxide, acetic acid, dichloromethane, and diethylether of analytical grade, acetonitrile and methanol of chromatographic grade were obtained from VWR (Darmstadt, Germany). Phosphoric acid of analytical grade was from Roth (Karlsruhe, Germany). Double distilled water was further purified by an Elgastat UHQ water system (Elga, High Wycombe, UK).

Venous blood samples, taken from the test persons, were centrifuged for separation of plasma and stored at −20 °C until analysis. Immediately prior to use, plasma samples were thawed at ambient temperature and centrifuged for 5 min at 13,000 g. The clear supernatant was used for analysis.

2.2. Analysis of propafenone by column switching

2.2.1. Standard solutions

Stock solutions of racemic R,S-propafenone were prepared by dissolving the drug in methanol to a final concentration of 22 μg/mL. Propafenone free plasma was spiked with propafenone standard solutions in the range of 1.1–10.0 μg/mL ($n = 5$). Control standard solutions at concentrations of 2.2 and 5.0 μg/mL were prepared in water.

2.2.2. Two-dimensional column switching system

The two-dimensional HPLC column switching system is shown in Fig. 2. Eluent was delivered by a Merck/Hitachi ternary gradient pump model L-6200 (VWR, Darmstadt, Germany). Samples were injected onto column 1 by a Merck/Hitachi autosampler model AS-4000 (VWR) equipped with a 100 μL injection loop. Column switching was performed by means of an electrically driven switching valve model ELV 7000 (VWR, Vienna, Austria).

Table 1
Eluent and linear gradient programme for column switching using a reversed-phase column in the first dimension.

Pump 1
Eluent A = 0.025 M KCl + 0.03 M H_3PO_4, adjusted to pH 2.4 with KOH
Eluent B = acetonitrile
Eluent C = methanol

Pump 2
Eluent = 0.25 M $HCLO_4$ + 0.05 M H_3PO_4 + 44% acetonitrile, adjusted to pH 2.5 with NaOH

t (min)	%A	%B	%C	Comment
0.0	100	0	0	Start of cycle: injection of sample
4.0	100	0	0	
10.0	70	30	0	
12.5	67	33	0	Pump 2 stopped, column 1 connected to column 2
15.5	67	33	0	Column 2 disconnected, pump 2 and data acquisition started
22.0	0	50	50	Cleaning of column 1
24.0	100	0	0	Reequilibration of column 1
30.0	100	0	0	End of cycle

Eluent for the second dimension was delivered by an isocratic Merck/Hitachi HPLC pump model L-6000 (VWR). Separation of the transferred fractions was performed on a Chiralcel ODR enantioselective column (Daicel Chemicals, Llkirch, France) of dimensions 250 mm × 4.6 mm i.d. The column was thermostatted at 15 °C by a column thermostat (Thermotechnik Products, Langenzersdorf, Austria). Detection of the effluent was performed by a Merck/Hitachi diode array detector model L-4500 (VWR). Data acquisition and processing was performed by chromatographic software (VWR).

2.2.3. Column switching using a reversed-phase column in the first dimension

A Brownlee C8 column (PerkinElmer, Montreal, Quebec, Canada) of dimensions 50 mm × 4 mm i.d., particle size 7 μm, was used as column 1. Eluent and gradient programme are described in Table 1. Flow rate of pump 1 was set to 1.3 mL/min and reduced to 0.5 mL/min in the time interval of column switching (12.5–15.5 min). Flow rate of pump 2 was 1.3 mL/min.

2.2.4. Column switching using an ion-exchange column in the first dimension

Two strong acid cation-exchanger were used for column switching: Partisil SCX, a strong acid cation-exchanger with ligands of benzenesulfonic acid and 10 μm particle size (Whatman, Kent, UK), and a cation-exchanger with ligands of propylsulfonic acid and

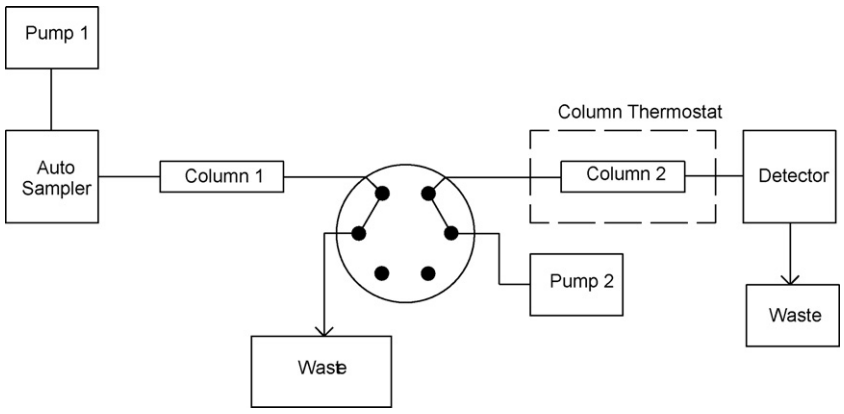

Fig. 2. Scheme of the HPLC system for column switching.

Table 2
Eluent and linear gradient programme for column switching using a cation-exchanger (propylsulfonic acid) in the first dimension.

Pump 1
Eluent A: 0.1 M KCl + 1.0 mL CH_3COOH/L + 1.0 mL H_3PO_4/L, adjusted to pH 5.8 with KOH
Eluent B: 1.0 M KCl + 1.0 mL CH_3COOH/L + 1.0 mL H_3PO_4/L, adjusted to pH 5.8 with KOH
Eluent C: acetonitrile

Pump 2
Eluent: 0.25 M $HCLO_4$ + 0.05 M H_3PO_4 + 44% acetonitrile (%, v/v), adjusted to pH 2.5 with NaOH

t (min)	%A	%B	%C	Comment
0	100	0	0	Start of cycle: injection of sample
2	100	0	0	
6	80	20	0	
8	67	30	3	Pump 2 stopped, column 1 connected to column 2
12	42	50	8	Column 2 disconnected, pump 2 and data acquisition started
18	5	80	15	Cleaning of column 1
20	100	0	0	Reequilibration of column 1
30	100	0	0	End of cycle

40 μm particle size (Varian, Palo Alto, CA, USA). Eluent and linear gradient programme of the HPLC method are shown in Table 2. Flow rate of pumps 1 and 2 was set to 1.3 mL/min and detection of the effluent was performed at 211 nm.

2.3. Analysis of propafenone using liquid–liquid extraction for sample preparation

2.3.1. Standard solutions for liquid–liquid extraction

Stock solutions of racemic R,S-propafenone were prepared by dissolving the drug in methanol to a final concentration of 20 μg/mL. Propafenone free plasma was spiked with propafenone standard solutions in the range of 0.2–5.0 μg/mL ($n = 5$). Control standard solutions at concentrations of 0.5 and 2.0 μg/mL were prepared in water. The internal standard S-propanolol was dissolved in methanol and a working standard solution of 10 μg/mL was prepared in water.

2.3.2. Extraction

To 1.0 mL sample was added 100 μL of 1 M NaOH and 100 μL of the internal standard S-propanolol. The sample solution was thoroughly mixed and extracted with two 1.5 mL portions of dichloromethane + diethylether = 2 + 3 (%, v/v). The combined organic extracts were acidified with a 0.5 mL portion of 0.25 M perchloric acid and 0.02 M phosphoric acid of pH 2.4, adjusted with NaOH. The organic solvent was evaporated on a rotary evaporator (Büchi, Buchs,Switzerland) at 35 °C. The volume of the residue was adjusted to 0.5 mL with water and used for analysis.

2.3.3. Chromatography

The extracted samples were analysed using the following HPLC system: Eluent was delivered by a Merck/Hitachi gradient HPLC pump model L-6200A (VWR). Isocratic elution was performed by an aqueous-organic eluent of 0.25 M perchloric acid and 0.02 M phosphoric acid, adjusted to pH 2.4 with sodium hydroxide, and acetonitrile. They were mixed together at a ratio of 6:4 (%, v/v). Flow rate was set to 0.7 mL/min. Samples were injected by a Merck/Hitachi autosampler model AS-4000 (VWR) and injection volume was set to 100 μL. Separation of the enantiomers was performed on a Chiralcel ODR column of dimensions 250 mm × 4.6 mm i.d. and protected by a 35 mm × 4 mm i.d. Brownlee C8 column (PerkinElmer) of 7 μm particle size. The columns were thermostatted to 15 °C by a column thermostat (Thermotechnic products). Absorption of the effluent was monitored by a Merck/Hitachi UV-detector model L-4200 (VWR) at 211 nm. Data acquisition was performed by a Nelson series 760 interface (PerkinElmer).

3. Results and discussion

3.1. Enantioselective separation system

Separation of the propafenone enantiomers was performed on an enantioselective cellulose column in reversed-phase mode applying an aqueous-organic eluent. Detection of the effluent was carried out by UV-measurement. R,S-propafenone shows a maximum of absorption of low intensity at 250 nm. Below 215 nm intensity of absorption is much higher so that wavelength of detection was set to 211 nm.

The enantioselective separation column is susceptible towards proteins of high molecular weight and other interfering components of low molecular weight. Therefore, to remove these compounds, sample preparation techniques are essential.

In that study column switching and liquid–liquid extraction were applied for sample clean-up.

3.2. Column switching using a reversed-phase column in the first dimension

In a first approach we used a reversed-phase column for on-line deproteination of plasma in the first dimension.

Eluent and separation column of the first dimension were adjusted to the analysis conditions of the enantioselective column in such a way that the solvent of the switched fractions did not disturb or reduce resolution of the enantiomeric species. In addition, peak broadening of the analyte on the first column was reversed on the enantioselective column during fraction transfer. Using a Brownlee C8 column in the first dimension, the concentration of the organic modifier in the switched fraction was about 10% lower than applied for enantioselective separation thus achieving a peak compression effect on the second column, and the peak of R,S-propafenone was adsorbed in a small zone on top of the enantioselective column.

Separation efficiency and results of analysis were reproducible, but after analysis of a dozen plasma samples, resolution of the enantiomeric separation decreased severely. This was mainly due to blocking of the enantioselective sites of the Chiralcel column by concomitant compounds in the transferred fraction and excessive cleaning of the column was necessary to restore the previous enantiomeric separation efficiency. Because of that reason, we searched for an alternative separation system in the first dimension.

3.3. Column switching using an ion-exchange column in the first dimension

In another approach, several types of silica-based strong acid ion-exchange adsorbents were tested for their applicability as separation column in the first dimension. The adsorbents differed mostly in the nature and length of the spacer, fixing the functional group to the carrier.

Ion-exchange adsorbents carrying phenylsulfonic acid as functional group exhibit non-polar and ion-exchange interactions to propafenone. Elution of the analyte may therefore be controlled by the counter ion and the organic component in the eluent.

Although applying a gradient for elution, the elution profile of R,S-propafenone was broad with strong tailing (Fig. 3A). Increasing the part of the organic component in the eluent resulted in a smaller peak, but ionic equilibrium was distorted and peak splitting was

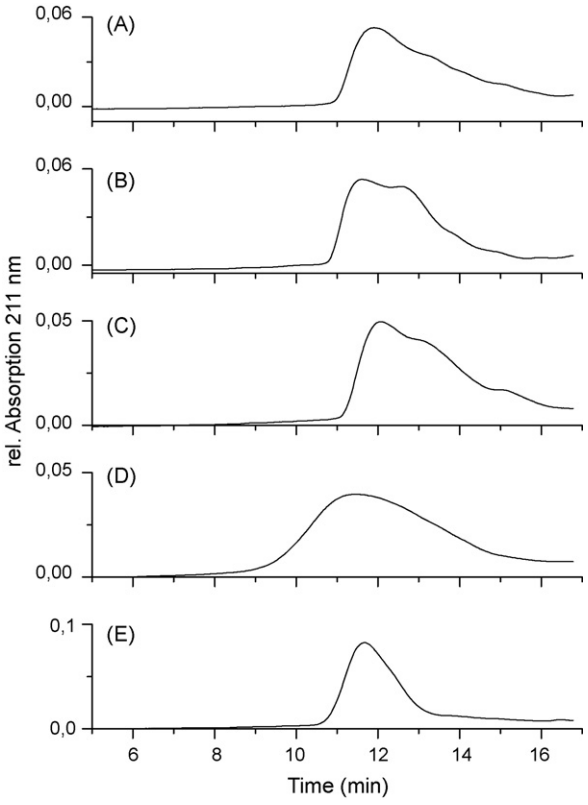

Fig. 3. Effect of eluent composition on the elution profile of propafenone on an ion-exchange column. Eluents: A = 0.1 M KCl + 1.0 mL CH_3COOH/L + 1.0 mL H_3PO_4/L, pH 5.6 with KOH; B = 1.0 M KCl + 1.0 mL CH_3COOH/L + 1.0 mL H_3PO_4/L, pH 5.6 with KOH; C = ACN + MeOH = 1 + 1 (%, v/v); D = ACN; E = MeOH. Flow rate = 1.3 mL/min; column dimension = 50 mm × 4 mm i.d.; columns A–D: Partisil SCX; column E: Bond Elut PRS; linear gradient: 0–4.0 min: 100% A – 80% A + 20% B. 4.0–16.0 min: (A, E) 80% A + 20% B – 70% B + 30% C; (B) 80% A + 20% B – 60% B + 40% C; (C) 80% A + 20% B – 75% B + 25% C; (D) 80% A + 20% B – 70% B + 30% D.

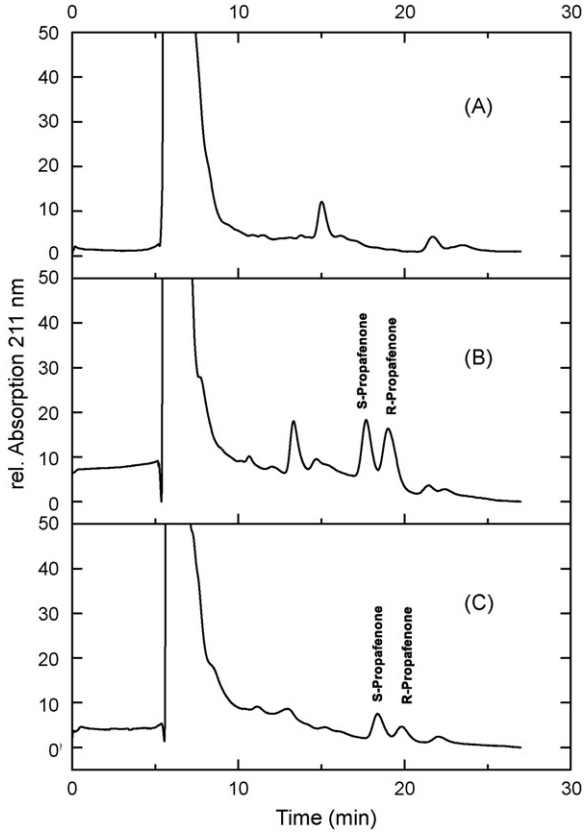

Fig. 4. Typical chromatograms showing the enantioselective separation of R,S-propafenone on a Chiralcel ODR column after column switching: (A) blank plasma, (B) spiked blank plasma (0.7 μg/mL each enantiomer), (C) sample (R-propafenone = 0.32 g/mL, S-propafenone = 0.42 μg/mL).

observed (Fig. 3B). On the other side, increasing the concentration of the counter ion did not improve peak shape (Fig. 3C). On that type of ion-exchange adsorbent the best results were obtained applying acetonitrile as organic modifier. A nearly symmetric, but still broad peak was obtained (Fig. 3D).

Peaks shape was better on an ion-exchange adsorbent with propylsulfonic acid as active group. Here ion-exchange mechanisms are the dominating retention principle. The peak of R,S-propafenone was much smaller and symmetric (Fig. 3E). After injection of plasma, the plasma proteins of high molecular weight eluted within 2.0 min from the ion-exchange column and the enantiomers of R,S-propafenone in the switched fraction were well separated on the enantioselective column (Fig. 4B).

3.4. Validation of the column switching method

Representative chromatograms for blank plasma, spiked plasma and sample are shown in Fig. 4. The chromatograms show that no endogenous compounds are interfering with the detection of the propafenone enantiomers.

Plasma calibration curves were constructed by plotting the peak area against the concentration of each propafenone enantiomer. Linearity of the calibration functions, expressed by the correlations coefficients, was better than 0.9992. Limit of quantitation was 0.2 μg/mL for each enantiomer at an injection volume of 50 μL and repeatability was better than 5% (Table 3). Between-day precision was determined on 4 days.

3.5. Liquid–liquid extraction and chromatography

Although analysis of the propafenone enantiomers could be performed by the column switching method, it was found however, that concentration of the enantiomers in some plasma samples was lower than the estimated limit of quantitation. Because sample amount could not be increased as desired without overloading the column, a more sensitive enrichment method was required for these samples. This was aspired by liquid–liquid extraction.

Plasma was extracted by a solvent mixture of dichloromethane and diethylether. Recovery of R,S-propafenone depends on pH of the solution to be extracted. In the pH range from 7 to 12 recovery of R,S-propafenone increases with pH (Fig. 5) and reaches its

Table 3
Validation data for the enantioselective analysis of (R)- and (S)-propafenone in plasma applying column switching and liquid–liquid extraction.

	Column switching	Liquid–liquid extraction
Within-day precision		
Concentration (μg/mL)	2.20	2.0
n	4	4
C.V. (%)[a]	*R* = 4.2, *S* = 2.4	*R* = 4.6, *S* = 4.5
Between-day precision		
Concentration (μg/L)	2.20	2.0
n	4	4
C.V. (%)	*R* = 3.7, *S* = 3.2	*R* = 4.1, *S* = 3.2
Linearity (r^2)	>0.9992	>0.9990
LOQ[b] (μg/mL)	0.20	0.055

[a] C.V. = coefficient of variation.
[b] LOQ = limit of quantitation for each enantiomer at S/N = 6.

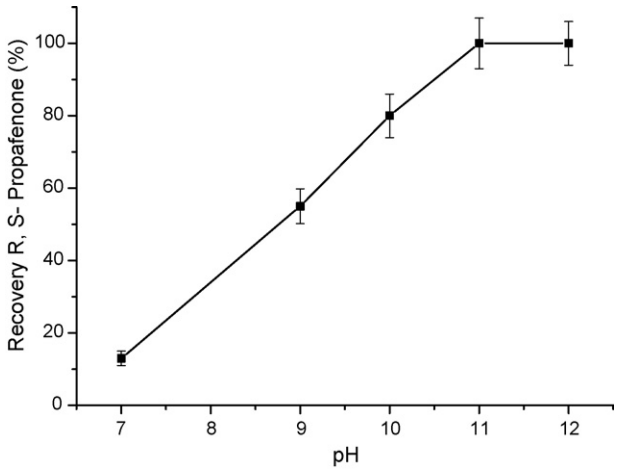

Fig. 5. Recovery of R,S-propafenone after liquid–liquid extraction of plasma in dependence on pH. Extraction solvent: dichloromethane + diethylether = 2 + 3 (%, v/v); plasma + extraction solvent = 2 + 3 (%, v/v); number of extraction steps: 2.

maximum at pH above 11. Therefore, after addition of 1 M NaOH, extraction of plasma was carried out at pH 11.4. Measurement of pH before and after extraction showed that variations of pH were small and within 0.1 pH units ($n = 12$). The chromatographic analysis of the extracts was performed by means of a one-dimensional HPLC system. The enantioselective column was protected by a Brownlee C8 precolumn and operated in isocratic elution mode. Enrichment by liquid–liquid extraction improved sensitivity by a factor of 4 compared to the column switching approach (Table 3).

3.6. Validation of the liquid–liquid extraction method

S-propanolol was added as internal standard for extraction. Recovery of liquid–liquid extraction was 98.2 ± 5.1% for R-propafenone ($n = 5$) and 100.2 ± 3.2% for S-propafenone ($n = 5$) at the 2.0 μg/ml level.

Representative chromatograms for blank plasma, spiked blank plasma and sample are shown in Fig. 6. The internal standard S-propanolol eluted before the enantiomers of R- and S-propafenone on a free place in the chromatogram and the separation of the propafenone enantiomers is not disturbed by other compounds.

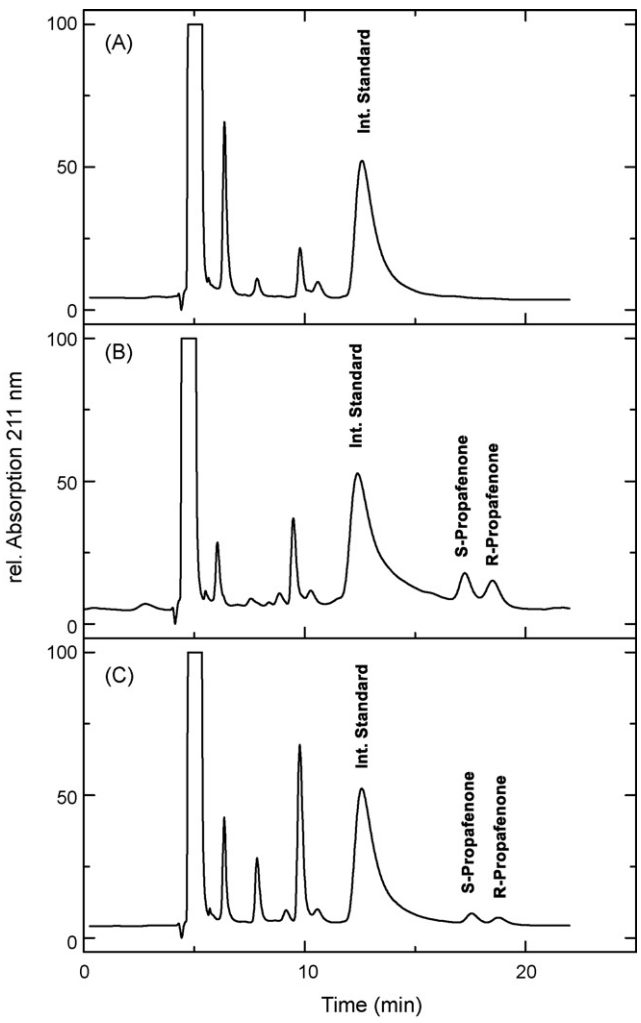

Fig. 6. Typical chromatograms showing the enantioselective separation of R,S-propafenone on Chiralcel ODR after liquid–liquid extraction: (A) blank plasma, (B) spiked plasma (1.1 μg/mL each enantiomer), (C) sample (R-propafenone = 0.42 μg/mL, S-propafenone = 0.63 μg/mL).

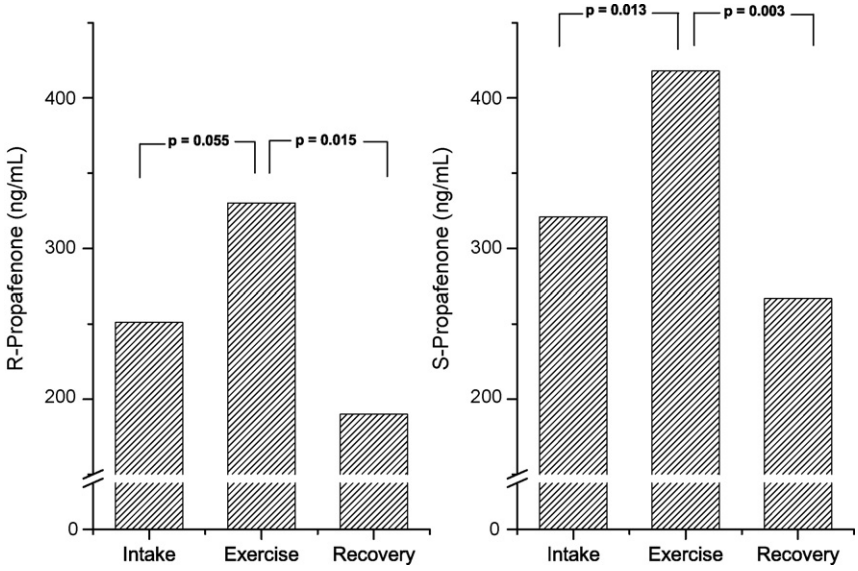

Fig. 7. Plasma levels of R- and S-propafenone after oral intake, exercise and recovery.

Plasma calibration curves were constructed by plotting the ratio of the peak area of S-propanolol to the individual propafenone enantiomer against the concentration of the propafenone enantiomer. Linearity of the calibration functions, expressed by the correlations coefficients, was better than 0.9990. Limit of quantitation was 0.055 μg/mL for each enantiomer and repeatability was better than 5% (Table 3). Between-day precision was determined on 4 days.

4. Application

The effect of exercise on the plasma concentration of R- and S-propafenone was examined in a double-blind, randomized, crossover study in 12 healthy males. Mean age of the test persons was 25 years and mean body weight was 76 kg. Four hours following oral intake of a single dose of 600 mg of racemic propafenone, exercise was performed on a bicycle ergometer for 10 min, followed by 15 min of recovery. Blood samples were taken 1 min before the end of each section (oral intake, exercise, recovery).

Analysis results were subjected to statistical analysis applying a paired sample *t*-test. Clearance of the S-enantiomer of propafenone is considerably reduced in the racemic mixture resulting in significantly higher plasma concentrations of S-propafenone (Fig. 7). The highest concentrations of the R- and S-enantiomers were found during exercise. Differences between the individual sections were significant ($p < 0.05$) with the exception of the transition from intake to exercise for the R-enantiomer ($p = 0.055$). Before and after exercise, concentrations of the R- and S-enantiomers differed significantly (p for $R = 0.012$, $S = 0.022$). A detailed discussion in connection with further parameters is described elsewhere [21].

5. Conclusions

Two HPLC methods were described for the enantioselective analysis of R,S-propafenone in plasma. The methods have been proved to be useful for the enantioselective analysis of propafenone in plasma samples for the pharmacokinetic study of 12 volunteers, which were subjected to exercise.

The liquid–liquid extraction method requires the addition of an internal standard and the limit of quantitation can be easily adjusted by the sample volume used for extraction. In addition, analysis can be performed on a simple one-dimensional (one-column) HPLC system. On the other side, the extraction method is more laborious than the column switching method, which can be easily automated. Here sample preparation is part of the separation process and no internal standard is required, but limit of quantitation is higher, which may be a problem for low concentrations of analyte. Validation results are comparable for both methods.

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