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Determination of propafenone and its main metabolite 5-hydroxypropafenone in human serum with direct injection into a column-switching chromatographic system

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Abstract

Column-switching chromatographic systems using conventional reversed-phase Separon SGX C_{18} and restricted access media LiChrospher ADS RP-18 precolumns were applied for the determination of propafenone and its main metabolite 5-hydroxypropafenone in human serum samples. The LiChrospher ADS RP-18 precolumn has been found to be more suitable for the sample clean-up. Serum samples were directly injected into the chromatographic system. Proteins and other endogenous compounds were removed by washing with 10% 2-propanol in water and the analytes separated on the Gromsil ODS AB analytical column. The chromatograms were detected at 246 nm. The method validation confirms the suitability of the column-switching system for the quantitation of propafenone and its metabolite. The presented assay shows good linearity with high correlation coefficients (0.992–0.999), high recoveries (96.6±6.1–103.5±5.8) and excellent values of the repeatabilities (1.23–4.5%). The limits of quantitation are 25–40 ng/ml for the injection volume of 50 μl. The complete analysis including the precolumn reconditioning and the sample clean-up requires 26 min, the sample throughput is approximately four samples in an hour.

Keywords: Propafenone; 5-Hydroxypropafenone

1. Introduction

Propafenone 2-(2-hydroxy-3-(propylamino)-propoxy)-3-phenylpropiophenone (Fig. 1) belongs to the group of drugs used in the treatment of supraventricular and ventricular arrhytmias [1]. Propafenone (PPF) is metabolized, besides a minor dealkylation pathway, mainly via 5-hydroxylation forming an active metabolite 5-hydroxypropafenone (5OHPPF) presenting antiarrhytmic and electrophysiologic activity comparable with that of the parent compound [2]. The therapeutic plasma concentration of propafenone is in the range of 100–2000 ng/ml [1].

Several methods have been described for the determination of PPF and its metabolite in human plasma. Most of the authors used a liquid–liquid extraction (LLE) as the sample clean-up step [3–7]. A simple solid-phase extraction (SPE) as the clean-up procedure was published for the determination of PPF derivatives in liver microsomes [8] and serum samples [9]. The stabilities of the PPF intravenous solutions were tested by Dupuis et al. [10].

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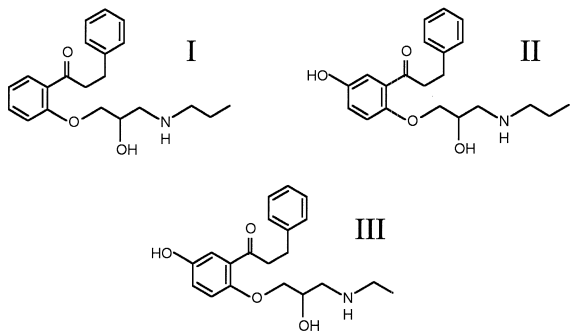

Fig. 1. The structures of propafenone (I), its metabolite 5-hydroxypropafenone (II), and internal standard (III).

Described methods apply labour and time-consuming LLE and off-line SPE clean-up procedures. The column-switching chromatographic system using conventional reversed-phase and/or restricted access media (RAM) precolumns allows the direct injection of the serum sample with full automation of the procedure.

A wide variety of RAM sorbents, reviewed in [11–14], has been developed for sample clean-up. The use of these materials in a single-column mode [15–17] shows two main drawbacks – a limitation of the mobile phase composition by non-denaturing conditions and the poor detection limits caused by the low sample amount [20]. These limitations are overcome by applying the column-switching systems.

The LiChrospher ADS RP-18 precolumn presents a type of packing having the external surface covered with glycerylpropyl-(diol)-phase and the internal one with alkyl chains [18]. The ADS RP-18 (or RP-4, RP-8 resp.) have been applied in column-switching assays for selected drugs in human serum samples [18–24] and as a tool for removing derivatization agents in the post-column derivatization reactor [25,26]. The properties of the material allow direct injection of at least 50 ml of plasma with no change in column efficiency and back pressure [27], however its lifetime is mainly determined by obstruction of the column frits by proteins.

This contribution is dealing with the column-switching chromatographic systems applying conventional reversed-phase Separon SGX C_{18} and restricted access media LiChrospher ADS RP-18 precolumns. The different clean-up effects of both precolumns with the focus on protein recovery, preconcentration ability and the selectivity of the separation will be discussed and the validation parameters data will be presented.

2. Experimentals

2.1. Chemicals and reagents

The standards of propafenone, 5-hydroxypropafenone and the internal standard 5-hydroxy-2-(2 -hydroxy- 3 -(ethylamino)propoxy)- 3 -phenylpropiophenone (LU46532) (Fig. 1) were obtained from Knoll, Ludwigshafen, Germany. Acetonitrile, methanol, 2-propanol, water (all of gradient grade), triethylamine (≥99.5%), phosphoric acid and sodium hydrogenphosphate (analytical grade) were purchased from Merck, Darmstadt, Germany, the control serum samples from Imuna, Šarišské Michal′any, Slovakia.

The stock solutions of all analyzed compounds were prepared in methanol at concentrations of 0.1 mg/ml and the working solutions diluted with water.

2.2. Chromatographic equipment and conditions

The chromatographic system included the DAD and the quaternary pump HP 1100 (Hewlett-Packard, Waldbronn, Germany), an autosampler Basic-Marathon (Spark, Emmen, The Netherlands), a HPLC pump (Knauer, Berlin, Germany), a UV Variable Wavelength Detector (Knauer, Berlin, Germany) and an Automated Switching Valve equipped with Rheodyne valve 7010 (Waters, Milford, USA). The Gromsil ODS AB (250×4.6 mm, 5 μm) analytical column was supplied by Watrex, Bratislava, Slovakia. The LiChrospher ADS RP-18, 25×4 mm, 25 μm (Merck, Darmstadt, Germany) or the Separon SGX C_{18}, 30×3 mm, 5 μm (Tessek, Prague, Czech Republic) were used as precolumns in the column switching systems, respectively. The serum samples were centrifuged in the Laboratory centrifuge MPW-300 (Mechanika Precyzyjna, Warsaw, Poland).

Mobile phase I – a washing mobile phase – consisted of 10% 2-propanol in water, the flow-rate was 1 ml/min. Mobile phase II – an eluting mobile

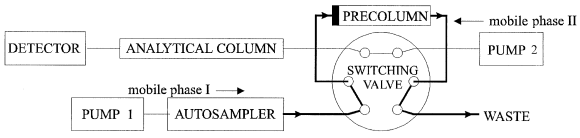

Fig. 2. The column-switching chromatographic system.

phase – consisted of 28% acetonitrile in 0.3% triethylamine solution, pH=3.5, adjusted with phosphoric acid and the flow-rate was 1.2 ml/min. The chromatograms were detected with DAD at 246 nm and UV spectra were scanned from 200–400 nm.

2.3. Protein recovery determination

The extraction precolumn was directly connected to the UV detector. An amount of 50 μl of the serum sample was injected onto the precolumn, the signal at 280 nm was registered for at least 10 min, and the matrix peak area was evaluated. The following solvents were tested as mobile phase I: water, 10% solutions of methanol, acetonitrile, *n*-propanol, 2-propanol in water and 10% 2-propanol in phosphate buffer (pH=7.2).

2.4. Sample clean-up procedure

An amount of 10 μl internal standard solution (c=50 μg/ml) was added to the 0.5 ml serum sample (final concentration of 1 μg/ml) and the sample was centrifuged at 3000 min^{-1} for 5 min. An amount of 50 μl serum sample was directly injected into the stream of mobile phase I. The instrumentation set-up (LOAD position) is given in Fig. 2 and the timetable of the analysis in Table 1.

3. Results and discussion

3.1. The sample clean-up

In this paper, the column-switching chromatographic system using ADS RP-18 or SGX C_{18} precolumns is presented for the serum sample clean-up and analysis. The efficiency of the removal of the proteins and the endogenous compounds was evaluated by the protein recovery and the selectivity of the separation. The solvents were tested as mobile phase I are listed in Section 2.3.

The protein recovery on the ADS RP-18 precolumn is approximately 100% and it does not depend strongly on the elution solvent composition [18]. The best purification of the serum sample on ADS RP-18 precolumn was observed using 10% 2-propanol in water. Several types of mobile phase I (various organic modifiers in water or phosphate buffer, pH=7.4; water) were presented in Refs. [18,19,23]. It was impossible to use them in this assay due to the presence of the large interfering peak eluting with the peak of 5OHPPF (organic modifier in phosphate buffer, pH=7.4) or not sufficiently removing all endogenous compounds (water, methanol and acetonitrile in water).

The SGX C_{18} is the conventional precolumn packed with the octadecylsilane stationary phase. Proteins are partially denatured and adsorbed on the surface resulting in lower protein recoveries. The protein recoveries, determined as described above, varied from 83.9±1.3% for water to 90.4±1.4% for 10% acetonitrile in water and 87.5±3.5% for 10% 2-propanol in water. Mobile phase I consisting of 10% 2-propanol in water was chosen because of the relatively high protein recovery and the cleanest chromatogram obtained in the column-switching mode.

Table 1
The timetable of the analysis

Time (min)	Autosampler	Switching valve	Action
0	INJECT	LOAD	Injection of the 1st sample
10		INJECT	Analyte transfer
12		LOAD	Reconditioning precolumn
15	INJECT	LOAD	Injection of the 2nd sample

The highest possible recoveries of proteins for both tested precolumns were achieved after 10 min washing with mobile phase I. The removal of approximately 97% of all eluted proteins was removed in the first fraction (1–2.5 min) and the content did not exceed 0.4% in the fraction eluted in 7.5–10 min. The longer washing step did not improve the efficiency of the protein removing on both precolumns.

The cleaned serum sample was transferred from the precolumn into the analytical column changing the switching valve position. The dependences of the change in the switching time (time between the changing of the switching valve position to the inject position and back to the load position) on the peak areas (shown in Fig. 3(a and b)) were measured. The peak areas of the compounds reached the plateau at the switching times of 0.9 and 1 min, respectively when the transfer of all compounds was completed. The switching time of 2 min was set for the final design of the column-switching assay.

3.2. Preconcentration ability of the precolumns

The standard solutions of PPF and 5OHPPF were injected into the single column chromatographic system with ADS RP-18 and SGX C_{18} precolumns, respectively, directly connected to the DAD. The dependencies of the capacity ratios on the content of the 2-propanol in mobile phase I are shown in Fig. 4(a and b). PPF and 5OHPPF retain strongly on both studied precolumns at low concentration of the organic modifier in mobile phase I and thus these columns show good preconcentration ability.

3.3. Chromatographic separation

The chromatographic separation of the standards at 246 nm is shown in Fig. 5. PPF and its metabolite 5OHPPF are weak bases (pK=8.8) showing relatively strong interactions between their amino-groups and the residual silanols of the ODS packing material, therefore an addition of triethylamine into mobile phase II was necessary even though the analytical column designed especially for the analysis of basic compounds has been used.

The chromatograms of serum blank and spiked samples directly injected into the column-switching

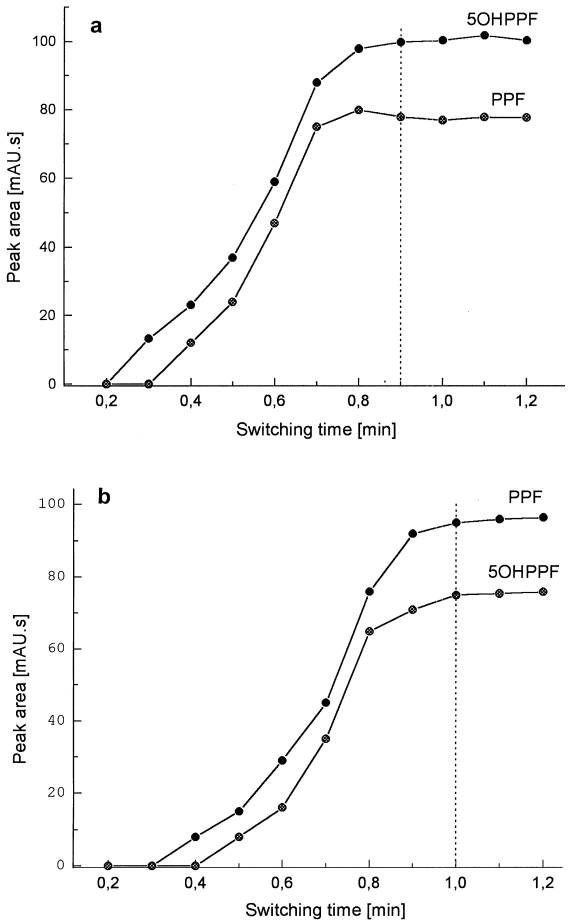

Fig. 3. The dependences of peak areas of PPF and 5OHPPF on switching time for LiChrospher ADS RP-18 (a) and Separon SGX C_{18} (b) precolumns. Chromatographic conditions: analytical column: Cromsil ODS AB, 250×4.6 mm, 5 μm. Mobile phase I: 10% 2-propanol in water, F=1 ml/min. Mobile phase II: 28% MeCN in 0.3% TEA, pH=3.5, F=1.2 ml/min. Detection: DAD, 246 nm.

system are illustrated in Fig. 6(a and b). The chromatograms without the interfering peaks document the excellent efficiency of the sample clean-up using the ADS RP-18 precolumn. A small peak eluting at 13.8 min does not disturb the quantitation of PPF.

The sample clean-up using the Separon SGX C_{18} precolumn was not sufficient compared to the results of the analysis with the ADS RP-18 precolumn. The determination of the 5OHPPF peak area could be influenced by a small peak eluting close to it. These

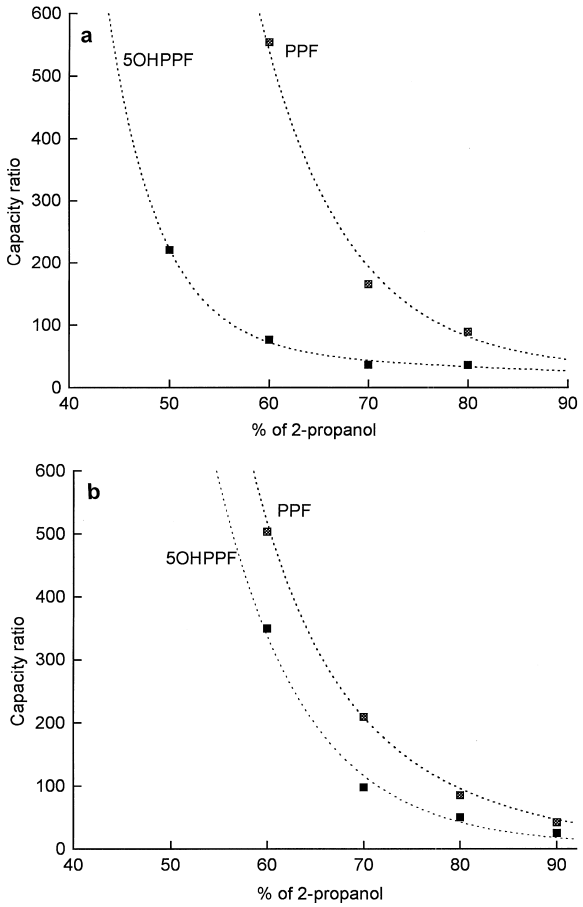

Fig. 4. The dependencies of the capacity ratios on the 2-propanol content for LiChrospher ADS RP-18 (a) and Separon SGX C_{18} (b) precolumns. Chromatographic conditions: Mobile phase: 2-propanol in water, $F=1.0$ ml/min. Detection: DAD, 246 nm.

two peaks are separated with the resolution of $R_{ij}=1.0$ at the metabolite concentrations close to the LOQ. The 5OHPPF peak becomes broader at higher metabolite concentrations, and in this case there is no possibility of resolving them. The resolution of the peaks has not been satisfactory improved thus decreasing the acetonitrile content in mobile phase II, even the analysis time has been extended. Therefore, the use of the SGX C_{18} is limited due to the inaccuracy of the metabolite determination.

The precolumns were washed with methanol and water after use and were kept in mobile phase I overnight. The backpressure of the precolumns was monitored. No differences in precolumn backpressures were observed for ADS RP-18. The total serum sample volume was approximately 20 ml during this study. However, a slight increase (20%) of the backpressure for the conventional SGX C_{18} after 8 ml of the total serum sample volume noticed shorter life span of this precolumn. The behaviour and the stability of ADS RP-18 precolumn were discussed in [27] and the lifetimes for the conventional precolumn are summarised in [12].

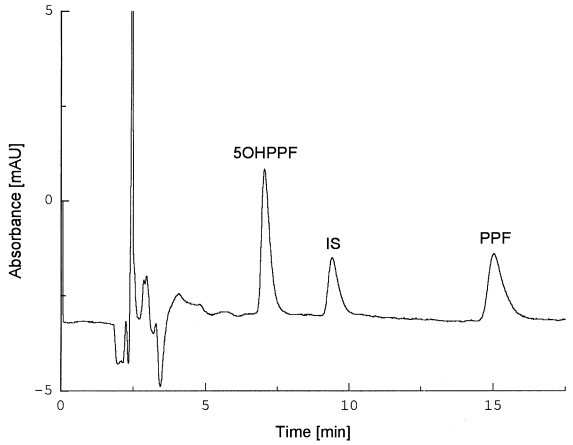

Fig. 5. Chromatographic separations of standard solution containing 2.12 μg/ml of 5OHPPF, 2.24 μg/ml of PPF and 0.92 μg/ml of I.S. Sample clean-up: column-switching system using LiChrospher ADS RP-18 precolumn. Chromatographic conditions: as in Fig. 3.

3.4. Validation of the assay

The main validation parameters (listed in Table 2) were evaluated to check the suitability of the chromatographic assay developed for the determination of PPF, its metabolite and internal standard. The repeatabilities of the retention times were determined from 20 injections of the standard and control serum samples at different concentrations. The repeatabilities of the injection were calculated at two concentration levels. The values lower than 5% are better than those observed by LLE or SPE sample clean-up procedures [3,8]. The repeatability of the retention time of the internal standard is 0.74% and 0.88%, the repeatability of its injection is 1.52% and 1.97% for ADS RP-18 and SGX C_{18}, respectively.

The parameters of the calibration curves and their correlation coefficients show very good linearity in

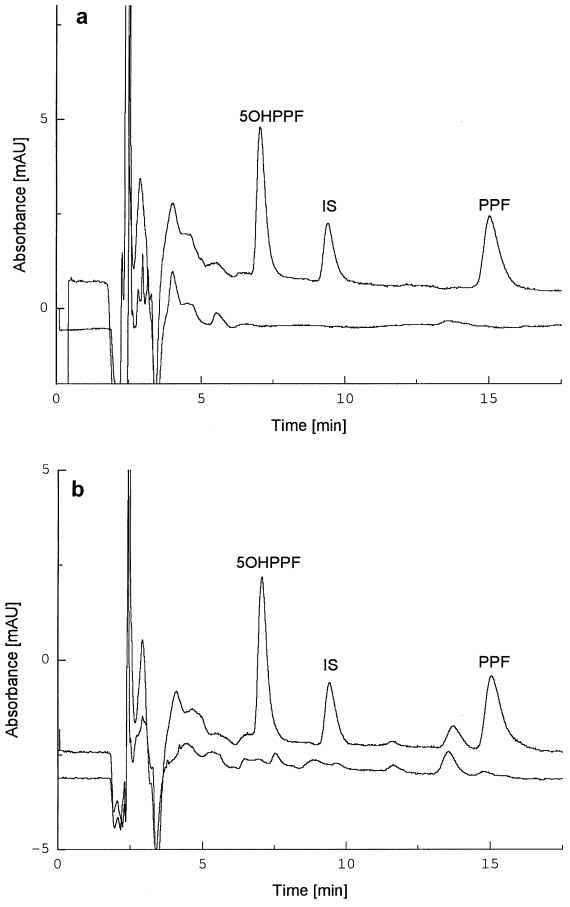

Fig. 6. Chromatographic separations of blank and fortified serum sample containing 2.12 μg/ml of 5OHPPF, 2.24 μg/ml of PPF and 0.92 μg/ml of I.S. (a) Column-switching system using LiChrospher ADS RP-18 precolumn. (b) Column-switching system using Separon SGX C_{18} precolumn. Chromatographic conditions: as in Fig. 3.

the concentration range from the limit of quantitation to 4 μg/ml for both precolumns. The high values of correlation coefficients indicate good correlation between the drug concentration and the peak area. Moreover, the *F*-values lower than the critical ones confirmed the suitability of the chosen calibration model.

Relative recoveries were calculated as the ratio between the relative response of the analyte (ratio of the analyte and I.S. peak area) in the fortified serum sample and the relative response of the standard solution injected into the column-switching system. The recoveries of the sample clean-up procedure do not differ significantly from 100% (the recoveries for the internal standard were 98.4±3.9 on ADS RP-18 and 99.5±5.5 on SGX C_{18}). Although the recovery data are excellent, the addition of an internal standard makes this assay suitable for the manual injection of the samples when the repeatability could be influenced by laboratory staff.

The limit of quantitation (LOQ) was calculated from the peak height based on a signal-to-noise ratio of ten. LOQ achieved in this work correspond to the limits published for the conventional sample clean-up assays [1,8,9] and they are lower than the therapeutic plasma concentrations (100–2000 ng/ml [1]).

4. Conclusion

A selective column-switching method for the determination of PPF and its metabolite 5-hydroxypropafenone was developed and sample clean-up procedures using both the restricted access media LiChrospher ADS RP-18 and the conventional Separon SGX C_{18} precolumns were compared. It was demonstrated that the use of restricted access media precolumn provides selective removing of proteins and endogenous compounds from the sample matrix.

The most important advantages of the presented assay are: (1) the direct injection of the serum sample into the chromatographic system; (2) the small amount of the sample (few 100 μl only); (3) the improvement in the method of precision and recovery; (4) the avoidance of manual purification procedures apart from the centrifugation of the sample prior to the analysis; (5) the full automation.

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Table 2
Validation parameters for the column-switching chromatographic system using LiChrospher ADS RP-18 and Separon SGX C_{18} precolumns

Compound precolumn	5OHPPF		PPF	
	ADS RP-18	SGX C_{18}	ADS RP-18	SGX C_{18}
Repeatability				
– of retention times[a]	0.86%	0.92%	0.53%	0.76%
– of the injection[b]				
2 μg/ml	1.23%	1.28%	1.48%	1.76%
0.25 μg/ml	2.42%	2.35%	3.28%	4.5%
Day-to-day precision[c]				
2 μg/ml	3.59%	4.12%	4.36%	4.89%
0.25 μg/ml	5.12%	4.57%	6.77%	6.25%
Calibration curve[d]				
– intercept	−0.23±0.31	2.85±0.46	1.23±0.87	3.02±1.59
– slope	51.07±0.48	55.09±0.75	72.55±1.59	69.27±3.12
–correl. coeficient	0.999	0.995	0.998	0.992
–*F*-test[e]	2.12	3.61	2.85	4.29
LOQ (50 μl injected)	25 ng/ml	35 ng/ml	30 ng/ml	40 ng/ml
Recovery				
2 μg/ml	98.3±4.2	97.5±5.4	96.6±6.1	101.2±6.9
0.25 μg/ml	101.0±6.2	103.5±5.8	99.5±8.2	101.5±9.3

[a] Calculated as relative standard deviation (RSD) for 20 determinations.
[b] Calculated as RSD from six measurements.
[c] Calculated as RSD from three different days.
[d] Duplicate measurements of six calibration standards.
[e] F_{crit}=4.53.

of Medicine of Commenius University, Bratislava, Slovakia for providing standard samples.

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