Journal of Chromatography, 491 (1989) 355–366
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4735

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF (*R*)- AND (*S*)-PROXYPHYLLINE IN HUMAN PLASMA

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(First received September 6th, 1988; revised manuscript received February 23rd, 1989)

SUMMARY

A reversed-phase high-performance liquid chromatographic assay has been developed for determination of (*R*)-(−)- and (*S*)-(+)-proxyphylline in human plasma. The procedure is based on liquid–solid extraction of proxyphylline from plasma followed by derivatization of extracted proxyphylline with (−)-camphanoyl chloride. The ratio between the enantiomers is calculated from the peak areas of the corresponding diastereoisomeric proxyphylline camphanates after injection into the liquid chromatograph. The recovery of proxyphylline from plasma was 88% (coefficient of variation = 4%) and proxyphylline was detectable from a plasma concentration of 0.2 μg/ml. Three different plasma extraction procedures for proxyphylline using Extrelut, Bond Elut, and Chem Elut columns have been developed and compared, and the rate of derivatization of the proxyphylline enantiomers with camphanoyl chloride has been studied.

INTRODUCTION

Proxyphylline, (±)-3,7-dihydro-7-(2-hydroxypropyl)-1,3-dimethyl-1*H*-purine-2,6-dione (Fig. 1), a bronchodilator used in the treatment of bronchial asthma, is marketed as a racemic mixture of two optical isomers, (*R*)-(−)- and (*S*)-(+)-proxyphylline [1]. Previously, we have reported on the optical separation, the absolute configuration, the synthesis and the biological activity of the enantiomers of proxyphylline and a high-performance liquid chromatographic (HPLC) method for the analysis of diastereoisomeric derivatives [2–4]. Both (*R*)- and (*S*)-proxyphylline were equally potent as inhibitors of cyclic nucleotide phosphodiesterase, isolated from human lung tissue [2]. Oth-

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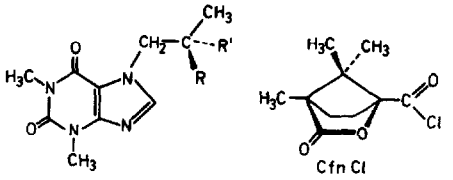

Fig. 1. Chemical structures of (*R*)-(−)-proxyphylline (R=OH, R′=H), (*S*)-(+)-proxyphylline (R=H, R′=OH), (*R*)-proxyphylline camphanate (R=OCfn, R′=H), (*S*)-proxyphylline camphanate (R=H, R′=OCfn) and (−)-camphanoyl chloride (Cfn Cl).

ers have examined the pharmacokinetics of racemic proxyphylline employing a simple, achiral liquid chromatographic method for the analysis of the drug from serum, after precipitation of the plasma proteins [5,6]. The metabolites have been investigated using urine analysis [7,8].

A chromatographic procedure allowing quantitative analysis of the proxyphylline enantiomers in plasma is necessary for examination of the pharmacokinetics and the metabolism of the stereoisomers. We have previously reported an HPLC method describing the separation of (*R*)- and (*S*)-proxyphylline as diastereoisomeric esters with (−)-camphanic acid [4]. The use of (−)-camphanoyl chloride as chiral derivatizing reagent requires anhydrous conditions since traces of water will destroy the acid chloride, causing reduced yields and possible kinetic resolution. Thus, we have extracted the plasma by a liquid–solid extraction method which ensures a dry proxyphylline fraction.

EXPERIMENTAL

Chemicals

All solvents were of analytical or HPLC quality and only distilled water was used.

(±)-Proxyphylline was purchased through Norsk Medisinaldepot (Oslo, Norway). (*S*)-(+)- and (*R*)-(−)-proxyphylline were obtained by synthesis from theophylline and (*R*)-(+)- and (*S*)-(−)-propylene oxides [2,3], respectively, and by resolution of racemic proxyphylline via corresponding camphanates [2]. The proxyphylline enantiomers had optical purities exceeding 98% determined by HPLC and NMR spectroscopy.

(−)-Camphanoyl chloride was obtained from Fluka (Buchs, Switzerland). The derivatization reagent was prepared by dissolving (−)-camphanoyl chloride in diethyl ether (10 mg/ml), which had been dried over metallic sodium. Pyridine was dried over potassium hydroxide. The camphanoyl chloride solution and the pyridine were stored in capsulated vials. The reagents were used freshly prepared, i.e. within one day.

Caffeine-free human plasma and human blood were obtained from the Red Cross (Oslo, Norway). The plasma was stored in glass vials at −18°C.

High-performance liquid chromatography

The HPLC equipment was a Beckman 112 solvent-delivery module pump (Berkeley, CA, U.S.A.), a Rheodyne No. 7125 sample valve with a 20-μl loop (Berkeley, CA, U.S.A.), and LDC UV III Monitor 1203 detector (Riviera Beach, FL, U.S.A.), and Autolab minigrator from Spectra-Physics (Santa Clara, CA, U.S.A.) and a single-channel recorder from Cole-Parmer (Chicago, IL, U.S.A.). The detection wavelength was 254 nm, and the sensitivity setting was 0.016–0.128 a.u.f.s.

Proxyphylline and (*R*)- and (*S*)-proxyphylline camphanates were separated on a 250 mm × 4.6 mm I.D. RP-18 Spheri-5 column (5 μm particle size, C_{18} reversed phase; Brownlee Labs., Santa Clara, CA, U.S.A.). A 50 mm × 4.6 mm I.D. LC-18 guard column (40 μm particle size, pellicular packing material; Supelco, Bellefonte, PA, U.S.A.) was used between the injector and the main column. The mobile phase was methanol–water (15:14, v/v) with isocratic elution at a flow-rate of 1.0 ml/min performed at 20°C.

Standard solutions

Solutions of (±)-proxyphylline in mobile phase varying in concentration from 0.002 to 80 μg/ml were made from an aqueous stock solution of 1 mg/ml in water. These solutions were stable for three months when stored at +5°C.

The plasma solutions of (±)-proxyphylline were made by dilution of the stock solution (1 mg/ml) with plasma, giving concentrations of (±)-proxyphylline of 0.1–50 μg/ml. The plasma solutions were stored at −18°C. Solutions of racemic proxyphylline in human blood were used freshly prepared.

Separately, 1 mg (*R*)-(−)- and 1 mg (*S*)-(+)-proxyphylline were dissolved in 10 ml of water. Dilution with plasma gave solutions of (*R*)-(−)- and (*S*)-(+)-proxyphylline of 30 μg/ml of plasma, respectively.

Solutions of (*R*)- and (*S*)-proxyphylline camphanates were made in the mobile phase by dissolving derivatized (±)-proxyphylline [2] giving a mixture of the two esters and by dissolving separately derivatized (*R*)-(−)- and (*S*)-(+)-proxyphylline [2,3].

Plasma extraction procedures

Three solid-phase extraction procedures were worked out and tested with respect to recovery and derivatization of the eluates. The recoveries were calculated using a calibration curve of proxyphylline varying in concentration from 5 to 80 μg/ml.

The Extrelut method. The Extrelut material (Merck, Darmstadt, F.R.G.) was washed with ethanol and chloroform and dried at 60°C. Then 1 ml of plasma containing 10–50 μg/ml (±)-proxyphylline or 1 ml of human blood

containing 10 and 50 μg/ml was added to a 25 cm long glass column filled with silanized glass wool and 3 ml of pretreated Extrelut material. After 10 min, 8 ml of an organic solvent (see Table I) were added and the eluate was collected after another 2 min. The solvent (8 ml) was evaporated on a heating block at 90 °C under a stream of nitrogen, and the residue was dissolved in 1 ml of the mobile phase and injected into the liquid chromatograph. The organic solvents employed to study the recovery of proxyphylline are listed in Table I. Chloroform with 5% (v/v) methanol was used for the measurement of linearity and precision of the extraction procedure.

The Bond Elut method. The plasma samples were extracted using a Vac Elut solid-phase extraction system with 1-ml C_{18} Bond Elut extraction columns (Analytichem, Harbor City, CA, U.S.A.). The extraction manifold was connected to a vacuum source (1.3 MPa), and ten samples were extracted simultaneously. A 500-ml vacuum flask was placed between the vacuum pump and the Vac Elut to collect the washing and waste materials. Each column was conditioned with two column volumes of methanol followed by two column volumes of water. At this point, the vacuum was discontinued to avoid dehydration of the column. With the vacuum off, 1 ml of plasma containing proxyphylline (10 or 50 μg/ml) was added to each column. The vacuum was then reapplied to 1.3 MPa, and the column was washed with two column volumes of water.

A glass vial was placed in the Vac Elut rack under each column to collect the eluates. Chloroform (400 μl) was added to each Bond Elut column with the vacuum off. After 1 min the vacuum was reapplied to 1.3 MPa to draw the chloroform into the collection vials. The vacuum was then turned off. This process was carried out twice.

TABLE I

RECOVERY OF PROXYPHYLLINE FROM HUMAN BLOOD USING THE EXTRELUT METHOD WITH DIFFERENT ELUENTS

Eluent	Recovery (%)
Chloroform with 5% (v/v) methanol[a]	94
Chloroform with 5% (v/v) ethyl acetate[a]	93
Dichloromethane with 5% (v/v) methanol[a]	91
Dichloromethane with 5% (v/v) ethyl acetate[b]	91
Chloroform with 5% (v/v) acetone[a]	90
Chloroform with 5% (v/v) acetonitrile[a]	87
Chloroform with 5% (v/v) tetrahydrofuran[b]	84
Chloroform[b]	78
Dichloromethane[b]	70

[a]50 μg/ml proxyphylline.
[b]10 μg/ml proxyphylline.

The eluates were evaporated to dryness on a heating block at 90 °C under a stream of nitrogen. The residues were dissolved in 1 ml of the mobile phase and the solution was thoroughly mixed before injection into the liquid chromatograph.

C_2 and C_8 Bond Elut columns were tested without showing increased recovery. Different organic solvents (chloroform, dichloromethane, diethyl ether and chloroform with 5% (v/v) ethyl acetate) were used to elute the proxyphylline. Of these solvents chloroform was found to be the solvent of choice with respect to recovery and the purity of the extracts.

The Bond Elut–Chem Elut method. C_{18} Bond Elut columns (1 ml) with luer stopcocks were placed on a Vac Elut manifold and pretreated with methanol and water as described for the previous method. Plasma samples containing proxyphylline (0.2–50 μg/ml) were added, and the columns were washed with water as described above. The Bond Elut columns were then connected via an adapter to the top of the Chem Elut 1000M columns (Analytichem), to which 200 μl of water had been added. The Chem Elut columns contained diatomaceous earth and had a sample capacity of 0.2 ml of aqueous solution. The columns were eluted with two 400-μl volumes of chloroform. The Bond Elut columns were removed, and the Chem Elut columns were eluted with two 750-μl volumes of chloroform with the vacuum on (1.3 MPa). The eluates were collected in glass tubes and evaporated to dryness on a heating block at 90 °C under a stream of nitrogen. The residues were dissolved in 1 ml of the mobile phase and injected directly, or derivatized with the camphanoyl chloride reagent prior to HPLC analysis.

Derivatization

Derivatization vessels (1 ml) containing racemic or enantiomeric proxyphylline were heated to 90 °C under a stream of nitrogen to remove traces of water. The hot vessels were supplied with Mininert valves (Supelco), and 1 μl of pyridine and 100 μl of the camphanoyl chloride reagent were injected through the valves. The reaction mixtures were allowed to stand for 16 h at 20 °C. Diethyl ether and pyridine were then evaporated on a heating block at 90 °C under a stream of nitrogen, and the residues were dissolved in 0.6 ml of the mobile phase and injected into the liquid chromatograph.

The completeness of this derivatization procedure was investigated by adding (±)-proxyphylline (5–50 μg) to the derivatization vessels both as chloroform solutions and as plasma extracts.

The rate of reaction between the proxyphylline enantiomers and camphanoyl chloride was investigated by derivatization of 50 μg of racemic proxyphylline as described above. The reaction mixtures were, however, allowed to stand at 20 °C for different periods of time (5 min to 24 h) before evaporation of the pyridine and the diethyl ether (90 °C, nitrogen). The residues were dissolved in mobile phase and analysed by HPLC. The quantitative relationship

between the remaining proxyphylline and the corresponding camphanates, and hence the amount derivatized (*S*)-(+)- and (*R*)-(−)-proxyphylline, was calculated from the peak areas.

Plasma extracts containing (*S*)-(+)- and (*R*)-(−)-proxyphylline, respectively, were derivatized to reveal possible racemization during the procedure.

RESULTS AND DISCUSSION

High-performance liquid chromatography

The chromatographic system with a C_{18} column and methanol–water as the mobile phase effected a complete separation of proxyphylline, (*R*)-proxyphylline camphanate and (*S*)-proxyphylline camphanate (Fig. 2). This chromatographic system is preferred to that described previously [4] because of the simpler composition of the mobile phase, resulting in approximately the same α values ($\alpha = 1.17$ compared with $\alpha = 1.16$ for the previous method).

The retention times were 4.4, 12.0 and 13.7 min for proxyphylline, (*R*)-proxyphylline camphanate and (*S*)-proxyphylline camphanate, respectively. The intra-assay variation in retention times showed a coefficient of variation (C.V.) of 0.4% for proxyphylline and 0.2% for the camphanates ($n = 18$). The detection limit of proxyphylline was 0.4 ng at 254 nm and a signal-to-noise ratio of 2. The detection limit of the proxyphylline camphanates was 1 ng (sig-

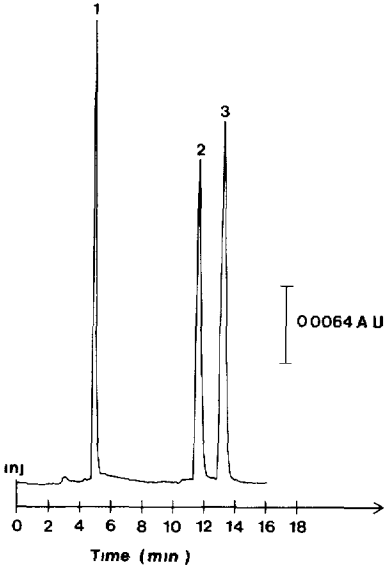

Fig. 2. (±)-Proxyphylline, (*R*)-proxyphylline camphanate and (*S*)-proxyphylline camphanate separated on a C_{18} column with methanol–water (15.14) as the mobile phase. Peaks: 1 = proxyphylline; 2 = (*R*)-proxyphylline camphanate; 3 = (*S*)-proxyphylline camphanate.

nal-to-noise ratio = 2) at 254 nm and 0.5 ng at 272 nm. The response factors (area/mol) were found to be 1.2, 1.0 and 1.0 for proxyphylline, (*R*)-proxyphylline camphanate and (*S*)-proxyphylline camphanate, respectively.

A calibration curve of proxyphylline dissolved in mobile phase (5–80 μg/ml) was linear, with a regression coefficient of 0.9999 ($y = 1.13 + 1.16x$, where y is the peak area and x is the concentration of proxyphylline).

Plasma extraction methods

The methods were designed to produce anhydrous eluates of proxyphylline. Precipitation of the proteins followed by extraction of proxyphylline into an organic solvent was cumbersome owing to the high polarity and the water solubility of the drug.

A work-up procedure of proxyphylline based on Extrelut columns gave sufficiently dry extracts. Different eluents were tested for the extraction of proxyphylline from human blood, as shown in Table I. Chloroform with 5% (v/v) methanol was preferred and was also used for plasma extractions. The method gave a satisfactory recovery and precision of proxyphylline extracted from plasma (Table II). Plasma with proxyphylline concentrations of 20, 30, 40 and 50 μg/ml were extracted in triplicate, giving a regression coefficient of 0.994 when the area of proxyphylline was plotted against the plasma concentration (Fig. 3). This method was, however, time-consuming owing to the need for pretreatment of the Extrelut material, the low rate of elution and the evaporation of the 8-ml chloroform extracts. Quantification of traces of underivatized proxyphylline was difficult owing to impurities from plasma.

A work-up procedure of proxyphylline from plasma based on Bond Elut columns (C_{18}) was less time-consuming, and only 0.8 ml of chloroform extract had to be evaporated. The method gave a lower recovery than the Extrelut

TABLE II

RECOVERIES OF PROXYPHYLLINE FROM PLASMA USING THE EXTRELUT, THE BOND ELUT AND THE BOND ELUT-CHEM ELUT METHODS

The eluent was chloroform with 5% (v/v) methanol for the Extrelut method and chloroform for the Bond Elut and the Bond Elut–Chem Elut methods.

Plasma concentration of proxyphylline (μg/ml)	Extrelut			Bond Elut			Bond Elut–Chem Elut		
	Recovery (%)	C.V. (%)	*n*	Recovery (%)	C.V. (%)	*n*	Recovery (%)	C.V. (%)	*n*
10				88	8	6	77	9	10
30							88	4	10
50	98	5	10	91	3	10	86	4	10
							88	2	6

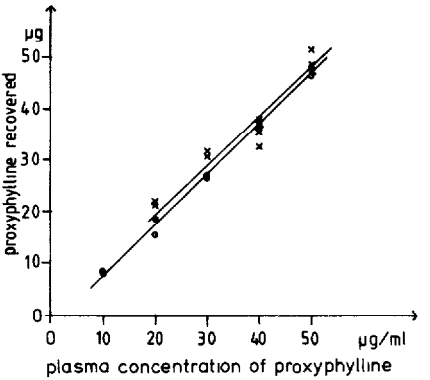

Fig. 3. Amount of proxyphylline recovered from 1 ml of plasma using the Extrelut method (×) and the Bond Elut-Chem Elut method (○), plotted against the plasma concentration of proxyphylline.

method. The recovery and the precision of proxyphylline extracted from plasma (10 and 50 µg/ml) on C_{18} Bond Elut columns are shown in Table II.

The use of C_{18} Bond Elut columns exclusively gave eluates with a relatively high content of water. Additional reduction of the water content in the chloroform eluates was obtained by passing the C_{18} Bond Elut eluates through Chem Elut columns. This procedure gave dry chloroform extracts with a slight reduction in recovery. This combination of columns had the advantage of giving a chromatogram with fewer interfering peaks, permitting the analysis of underivatized proxyphylline. The recovery and precision of proxyphylline extracted from plasma using this method are shown in Table II. Plasma with proxyphylline concentrations of 10, 20, 30, 40 and 50 µg/ml was extracted in duplicate, giving a regression coefficient of 0.999 when the area response was plotted against the plasma concentration as shown in Fig. 3. Proxyphylline from plasma with a concentration of 0.2 µg/ml was detectable. This is less than 2% of the therapeutic plasma concentration of the drug [9].

The Chem Elut columns were replaced by 1-ml silica columns to remove the water content in the Bond Elut eluates. The recovery of proxyphylline was, however, only 3% when using the same amounts of chloroform as for the Bond Elut–Chem Elut procedure.

Extraction of proxyphylline from human blood was not investigated employing Bond Elut or Chem Elut columns.

The derivatization method

Derivatization of (±)-proxyphylline with excess (−)-camphanoyl chloride under anhydrous conditions with pyridine as catalyst yielded diastereoisomeric esters which were separated by HPLC and thin-layer chromatography (TLC) [2,3]. The rate of esterification, investigated by derivatization of ra-

cemic proxyphylline, differed for the proxyphylline enantiomers. (*R*)-(−)-Proxyphylline reacted faster than the (*S*)-enantiomer (Fig. 4): it was completely derivatized after 30 min whereas the (*S*)-enantiomer required several hours (94% of the (*S*)-enantiomer was derivatized after 2 h). Thus, to ensure reliable quantification of (*R*)-(−)- and (*S*)-(+)-proxyphylline, the reaction mixture was allowed to stand overnight before removal of the solvent.

The yield of derivatization was investigated by derivatizing 5–50 μg of pure, racemic proxyphylline (Table III). HPLC analysis, by comparison of the peak areas of the camphanates and the remaining proxyphylline, showed that more than 99% of the proxyphylline was esterified. The ratio between HPLC areas of the (*R*)- and (*S*)-esters was 1.06 (C.V.=4%, n=10). The derivatization

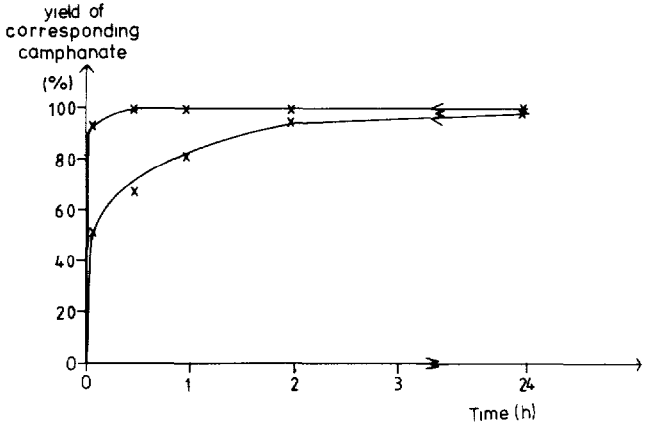

Fig. 4. Yields of (*R*)- and (*S*)-proxyphylline camphanates as a function of time after derivatization of 50 μg of racemic proxyphylline with camphanoyl chloride in heated vessels (90°C) followed by further time at 20°C. The upper graph shows the yield of (*R*)-proxyphylline camphanate, the lower (*S*)-proxyphylline camphanate.

TABLE III

DERIVATIZATION OF (±)-PROXYPHYLLINE WITH (−)-CAMPHANOYL CHLORIDE

The derivatization was carried out in hot vessels (90°C) followed by 16 h at 20°C. The maximum yield of each camphanate is 50%.

Amount of proxyphylline to be derivatized (μg)	n	Remaining proxyphylline (%)	Yield (%)	
			(*R*)-Camphanate	(*S*)-Camphanate
5	2	0	52.8	47.2
10	3	0	51.7	48.3
25	2	0.6	50.4	49.0
50	3	0.02	50.7	49.3

TABLE IV

DERIVATIZATION OF PROXYPHYLLINE EXTRACTED FROM PLASMA USING THE BOND ELUT-CHEM ELUT METHOD

The derivatization of proxyphylline with camphanoyl chloride was carried out in hot vessels (90°C) followed by 16 h at 20°C. The maximum yield of each camphanate is 50% when derivatizing (±)-proxyphylline.

Enantiomer	Concentration (μg/ml)	n	Remaining proxyphylline (%)	Yield (%)	
				(*R*)-Camphanate	(*S*)-Camphanate
(±)-Proxyphylline	50	10	1.5	50.0	48.5
(*R*)-Proxyphylline	30	6	1.2	98.6	0.2
(*S*)-Proxyphylline	30	4	2.8	0.7	96.5

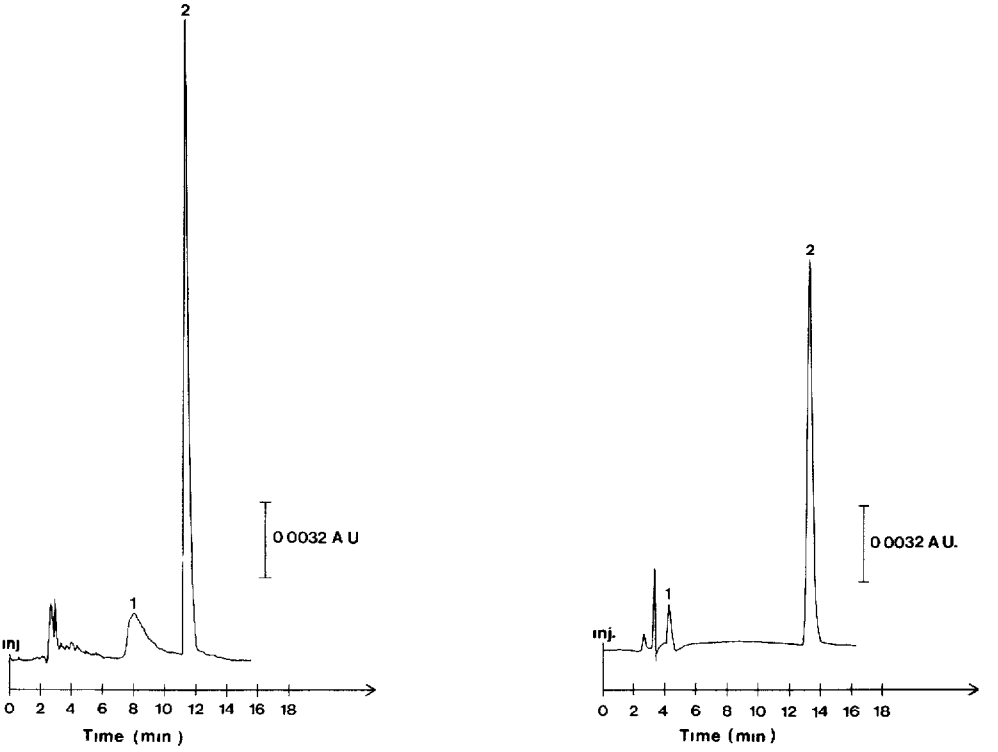

Fig. 5. HPLC profile of (*R*)-proxyphylline camphanate obtained from (*R*)-proxyphylline (30 μg/ml) in plasma using the Bond Elut-Chem Elut procedure. Peaks: 1 = residual pyridine; 2 = (*R*)-proxyphylline camphanate

Fig. 6. HPLC profile of (*S*)-proxyphylline camphanate obtained from (*S*)-proxyphylline (30 μg/ml) in plasma using the Bond Elut-Chem Elut procedure. Peaks: 1 = proxyphylline; 2 = (*S*)-proxyphylline camphanate.

was carried out in hot vessels (80–90°C). Reduced yields (94%, C.V.=3%, $n=10$) were obtained when the process was performed at 20°C.

The derivatization yield was linear in the range 2.5–25 μg of each enantiomer, with respect to the original amount of proxyphylline. When the area response of (*R*)-proxyphylline camphanate was plotted against the amount of (*R*)-(−)-proxyphylline, a regression coefficient of 0.9998 was obtained ($y=-0.88+0.97x$; y=peak area of the corresponding camphanate, x=amount of the proxyphylline enantiomer). When the area response of the (*S*)-camphanate was plotted against the amount of (*S*)-(+)-proxyphylline, the regression coefficient was 0.9997 ($y=-0.98+0.95x$).

Derivatization of (±)-proxyphylline (50 μg/ml from plasma) resulted in a yield of the corresponding esters of 98.5%. As shown in Table IV, the ratio between the HPLC areas of the (*R*)- and (*S*)-proxyphylline camphanates was 1.03. Kinetic resolution due to the difference in the rate of derivatization between the two enantiomers can thus be ignored.

No racemization occurred in plasma or during the derivatization procedure. HPLC analysis after derivatization of almost pure enantiomers extracted from plasma showed no change in enantiomeric composition (Table IV, Figs. 5 and 6).

Solutions of (*R*)-proxyphylline camphanate in methanol–water (15:14, v/v) kept at 20°C for one month did not reveal any racemization or hydrolysis.

CONCLUSION

This method is suitable for selective analysis of (*R*)-(−)- and (*S*)-(+)-proxyphylline in human plasma. The calibration curve is linear with respect to the plasma concentration of proxyphylline after therapeutic doses of the drug. The derivatization yields are 98–100% without racemization or kinetic resolution, and the HPLC method enables the study of underivatized proxyphylline as a control over the derivatization process. The method allows pharmacokinetic studies of the enantiomers of proxyphylline in human plasma.

ACKNOWLEDGEMENTS

The authors are indebted to John Remme and Finn Tønnesen for technical assistance.

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