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High-performance liquid chromatographic determination of (*S*)- and (*R*)-propranolol in human plasma and urine with a chiral β-cyclodextrin bonded phase

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Abstract

The determination of propranolol enantiomers in microsamples of human plasma and urine by HPLC using a chiral stationary phase is described. After extraction from 200 μl of plasma or urine with racemic alprenolol as internal standard (I.S.), the enantiomers are separated on a β-cyclodextrin column with a polar organic mobile phase and determined by fluorescence detection. The retention times of I.S. and propranolol enantiomers are about 12–13 min and 16–18 min, respectively. Peak resolutions are 1.4 for I.S. and 2.2 for propranolol. The use of alprenolol as I.S. improves significantly the coefficients of variation (C.V.: 0.6–4.2%). Sensitivity is approximately 1.5 ng/ml per propranolol enantiomer. The assay is applied to pharmacokinetic studies of racemic propranolol in human biological fluids. The (*S*)-propranolol levels are always higher than the (*R*)-antipode concentrations in plasma and urine.

1. Introduction

Propranolol, a racemic β-adrenergic blocking agent, is widely used in the treatment of cardiovascular diseases (hypertension, angina pectoris, and cardiac arythmia) and the prophylaxis of acute myocardial infarction. The single enantiomers of propranolol have been shown to exhibit large differences in pharmacological activities and pharmacokinetic profiles: (*S*)-propranolol is about 100 times more active than its (*R*)-antipode against β-receptors [1]. Other studies have shown that the metabolism of racemic propranolol in man is also stereoselective [2]. Therefore, it is suitable to analyze the respective propranolol enantiomer levels in biological fluids rather than the total propranolol concentration in order to establish their individual therapeutic profiles.

In the literature, two different approaches to the enantioselective determination of racemic propranolol are described: immunochemical assays and physicochemical assays. Most of the immunoassays cannot distinguish between the two enantiomers, except for the two enantioselective techniques performed either by radioimmunoassay [3] or by enzyme-linked immunosorbent assay (ELISA) [4]. Most of the physicochemical assays were performed by HPLC using either chiral stationary phases or

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chiral derivatization reagents. Several optically active derivatization reagents such as: *R*(+)-phenylethylisocyanate (PEIC) [5–7], (−)menthyl chloroformate [8] and (*R*,R)-O,O-diacetyl tartaric acid anhydride (DATAAN) [9], have been used to convert propranolol into diastereomeric derivatives which can be separated on reversed-phase columns [10]. A number of chiral stationary phases (CSP) have been applied to the direct separation of propranolol enantiomers [11–18]. A commonly used CSP for separating β-adrenergic blocking agents is the α_1-acid glycoprotein (AGP) column [11]. However, the overall stability of the AGP columns is still less than desired and their cost is high [18]. Also the Pirkle-type 1A CSP, i.e. (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine, has been applied to separate propranolol enantiomers [12], but this method required condensation of propranolol with phosgene, which is toxic. Derivatized cellulose columns (Chiralcel) [13,14] as well as a recently developed cellulase CSP immobilized on polymer beds [15], can also separate some β-blocking agents; however these CSPs presented a great difference between the (*S*)- and (*R*)-enantiomer peak heights of standard racemic propranolol and of some other β-blockers. Recently, Armstrong and co-workers [16–18] reported the direct liquid chromatographic separation of racemic β-adrenergic blocking agents in aqueous solution with UV detection on different cyclodextrin chiral stationary phases using either hydroorganic mobile phases [16] or a polar organic solvent mixture [17,18]. However, neither of these methods has yet been applied to the determination of propranolol enantiomers in biological fluids.

α-, β- and γ-Cyclodextrins (CDs) are three native cyclic oligosaccharides composed of respectively, six, seven and eight α-D-glucose units linked through the 1,4 position [19]. The CD molecule has a stereospecific, toroidal structure which can be represented schematically as a truncated cone. The interior cavity of the CD molecule is relatively hydrophobic, and a variety of water-soluble and insoluble compounds can fit into this cavity to form inclusion complexes [19].

The aim of this study is to present an improved analysis of (*S*)- and (*R*)-propranolol enantiomers in microsamples of human plasma and urine utilizing racemic alprenolol as an internal standard and a chiral β-cyclodextrin column with polar organic mobile phase and fluorescence detection. The assay was applied to pharmacokinetic studies in man after oral administration of the racemic drug.

2. Experimental

2.1. Chemicals

Racemic (*R*,*S*)-, (*S*)- and (*R*)-propranolol hydrochloride, (*R*,*S*)-4-methylpropranolol, (*R*,*S*)-pronethalol, (*R*,*S*)-practolol, were purchased from Cambridge Research Biochemicals (Gadbrook Park Northwich, Cheshire, UK). Racemic alprenolol hydrochloride used as internal standard (I.S.), (*R*,*S*)-oxprenolol, (*R*,*S*)-metoprolol, (*R*,*S*)-pindolol, (*R*,*S*)-acebutolol, (*R*,*S*)-verapamil, amiodarone, were obtained from Sigma (St Louis, MO, USA). All solvents were of HPLC grade (Merck, Darmstad, Germany). Other chemicals (Merck) were of analytical purity. Deionized water was purified by Milli Q.UV Plus system (Millipore, Milford, USA).

2.2. Apparatus and chromatographic conditions

The liquid chromatographic system consisted of a Varian Model 5000 (Walnut Creek, CA, USA), a Waters 470 scanning fluorescence detector (Milford, USA), a Shimadzu CR-6A integrator (Kyoto, Japan) and a Rheodyne injector, Model 7125, fitted with a 50-μl loop. An analytical column (250 × 4.6 mm I.D.) packed with β-cyclodextrin bonded phase, 5 μm particle diameter (Cyclobond I) was purchased from Advance Separation Technologies (Astec) (Whippany, NY, USA). A guard column (15 × 3.2 mm I.D.) packed with 7 μm, wide pore, spherical C_{18} silica (Brownlee Labs, CA, USA) was connected between the injector and the analytical column.

The mobile phase of acetonitrile–absolute ethanol–glacial acetic acid–triethylamine

(96:4:0.4:0.3, v/v), was degassed by sonication and pumped at a flow-rate of 1 ml/min. Injection volumes of samples and standards were performed with the 50-μl sample loop. Column temperature was ambient (18–21°C). The fluorescence detector was set at 222 nm (excitation) and 340 nm (emission).

2.3. Preparation of standards

Four stock standard solutions were obtained by dissolving individually racemic, (*S*)- and (*R*)-propranolol hydrochloride and racemic alprenolol hydrochloride (I.S.) in methanol at 1.0 mg/ml (free base). Stock solutions were stable for 1 month at 4°C. Standard calibration solutions were prepared by spiking drug-free human plasma or urine with stock standard solution further diluted to achieve final concentrations of 25, 50, 100, 200, 500 and 1000 ng/ml of racemic propranolol base and stored at −20°C. An I.S. working solution at 15 μg/ml was obtained by dilution of an aliquot of racemic alprenolol stock solution with methanol.

2.4. Extraction procedure

The extraction procedure for racemic propranolol in biological fluids was previously described with slight modifications [7]. Briefly, 200-μl samples of thawed plasma, urine or standard calibration solution were added into a labelled 11 × 100 mm centrifuge glass tube, then followed successively by 200 μl of internal standard solution in methanol, 200 μl of concentrated ammonium hydroxide (25%) and 2 ml of a diethyl ether–methanol mixture (90:10, v/v). For urine, the last solvent mixture was replaced by 2 ml of diethyl ether alone. The tubes were tightly capped and mixed by vortex-mixing for 1.5 min. After centrifugation for 5 min at 1500 *g*, the upper organic layer was transferred into a 5-ml glass tube and completely evaporated under a stream of nitrogen at room temperature. A 200-μl volume of mobile phase was added into each tube and vortex-mixed for 30 s. A 50-μl aliquot of the extract was then injected onto the column.

2.5. Validation of the method

Precision of the assay was tested by comparing the results of six plasma and two urine samples from two healthy volunteers obtained by the present method with those achieved by HPLC after chiral derivatization with *R*(+)-phenylethylisocyanate as previously described [7].

To calculate the recovery, spiked plasma and urine samples (100 and 500 ng/ml racemate) were analyzed and the peak heights were compared with those of corresponding non-extracted standard propranolol diluted in mobile phase.

2.6. Application of the method

To test the applicability of the described method for pharmacokinetic studies, two healthy volunteers (one male, 42 years and one female, 24 years) received orally a single dose of racemic propranolol tablets in the morning. One volunteer received 80 mg, the other 40 mg of racemic drug. Blood samples were taken at timed intervals, i.e. 2, 4, and 8 h after dosing. The plasma was separated by centrifugation and stored at −20°C before use. Urine samples were collected 24 h after drug intake and an aliquot was stored at −20°C until analysis.

2.7. Calculation

The analysis of each propranolol enantiomer in the sample was based on the peak-height ratio of (*S*)- or (*R*)-propranolol to the corresponding isomer of the internal standard. The ratio values found were reported on a standard calibration curve performed under the same conditions with standard calibration solutions at the range of the concentrations studied.

3. Results and discussion

3.1. Chromatography

Typical chromatograms of drug-free human plasma and urine, standard calibration plasma

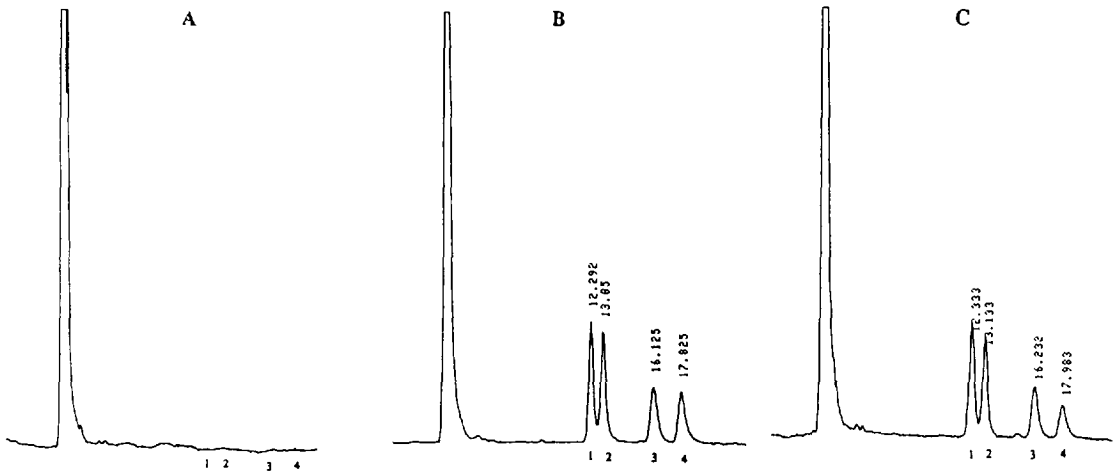

Fig. 1. Chromatograms of propranolol and internal standard (I.S.) enantiomers in human plasma after extraction. (A) Blank plasma; (B) human plasma standard spiked with 100 ng/ml racemic propranolol, i.e. 50 ng/ml of each enantiomer; (C) plasma of one healthy volunteer after oral administration of 80 mg racemic drug. Peaks: 1,2 = (*S*)- and (*R*)-alprenolol (I.S.), respectively; 3,4 = (*S*)- and (*R*)-propranolol respectively.

and urine and treated subject plasma and urine are shown in Figs. 1 and 2. The first set of peaks, at retention times of about 12.3 and 13.1 min corresponds to the (*S*)- and (*R*)-alprenolol enantiomers (I.S.), respectively. The second set of peaks, at retention times of about 16.1 and 17.8 min corresponds to the (*S*)- and (*R*)-propranolol enantiomers, respectively.

The resolution factor (R_s) and the separation factor (α) of propranolol were 2.18 and 1.2, respectively and those of alprenolol (I.S.) were 1.38 and 1.08, respectively. The peak form of both compounds was sharp and symmetric.

The peak-height ratios of (*S*)/(*R*) standard propranolol enantiomers and of (*S*)/(*R*) I.S. enantiomers were 1.1 ± 0.05 ($n = 6$).

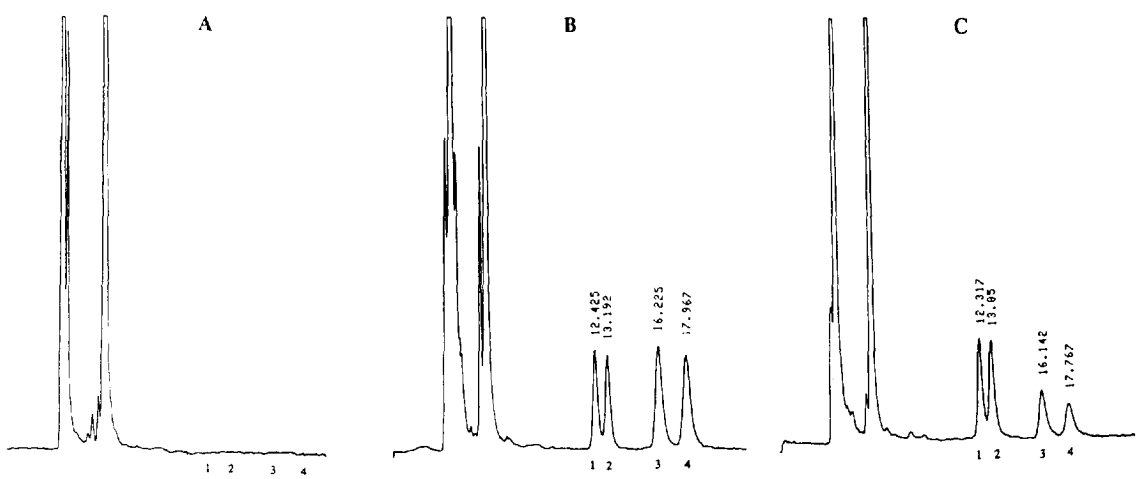

Fig. 2. Chromatograms of propranolol and internal standard (I.S.) enantiomers in human urine after extraction. (A) Blank urine; (B) human urine standard spiked with 200 ng/ml racemic propranolol, i.e. 100 ng/ml of each enantiomer; (C) urine of one healthy volunteer after oral administration of 80 mg racemic drug. Peaks: 1,2 = (*S*)- and (*R*)-alprenolol (I.S.), respectively; 3,4 = (*S*)- and (*R*)-propranolol respectively.

No endogenous interfering peaks were observed with drug-free human plasma and urine at the retention times of propranolol and I.S. (Figs. 1 and 2). Other racemic β-blockers such as practolol, oxprenolol, acebutolol, metoprolol, pindolol or other cardiovascular drugs such as racemic verapamil, amiodarone diluted in mobile phase at 10 μg/ml and subsequently directly injected onto the column, were shown not to interfere with the assay.

3.2. Statistical data

The detection limit (signal-to-noise ratio >3) of the assay after extraction was about 1.5 ng/ml for each propranolol enantiomer.

The standard calibration curves of plasma and urine exhibited good linearity for (*S*)- and (*R*)-propranolol over the range of concentrations tested (12.5–500 ng/ml enantiomer) with correlation coefficients greater than 0.999 for both enantiomers (Table 1).

The intra-day coefficients of variation (C.V.) determined from replicate injections ($n = 6$) of 2 plasma and urine standards (50 ng/ml and 250 ng/ml enantiomers) and of 2 plasma and urine samples from 2 healthy volunteers varied between 0.6 and 2.8% (Tables 1 and 2).

The inter-day C.V. ($n = 6$) of the same standards and the same plasma and urine samples varied between 1.7% and 4.6% (Tables 1 and 2).

The recovery of propranolol enantiomers from the two plasma and urine standards with 100 ng/ml and 500 ng/ml of racemic drug varied between 94 and 98% (Table 1) and that of the I.S. enantiomers was about 86% for the (*S*)-isomer and 88% for the (*R*)-isomer.

A comparison between 16 concentrations of (*S*)- and (*R*)-propranolol in six plasma and two urine samples from two healthy volunteers determined with this direct HPLC method using the chiral β-cyclodextrin column with the results obtained by HPLC after chiral derivatization with (*R*)-phenylethylisocyanate [7] gave congruent results with a correlation coefficient $r = 0.997$ and regression line $y = 0.96x - 0.029$ where x is the concentration determined by direct HPLC and y is the concentration determined by indirect HPLC (Fig. 3).

3.3. Optimization of mobile phase

According to Amstrong et al. [18], the elution of cyclodextrin bonded phase columns can be performed in three different modes: normal mobile phase, reversed or hydroorganic mobile

Table 1
Precision, linearity and recovery of propranolol enantiomer standards ($n = 6$)

Enantiomer	Precision (C.V.%)				Linearity of enantiomers between 12.5 and 500 ng/ml [a]	Recovery (%)	
	50 ng/ml		250 ng/ml			50 ng/ml	250 ng/ml
	Intra-day	Inter-day	Intra-day	Inter-day			
Plasma							
(*S*)-Propranolol	1.20	3.83	0.96	1.50	$r = 0.9994$ $y = 0.0095x + 0.012$	96.3 ± 1.2	95,5 ± 1.5
(*R*)-Propranolol	2.65	3.57	1.62	1.84	$r = 0.9991$ $y = 0.0093x + 0.011$	95.1 ± 1.2	94.6 ± 1.7
Urine							
(*S*)-Propranolol	1.23	3.05	1.75	3.42	$r = 0.9998$ $y = 0.0099x + 0,008$	98.2 ± 0.8	97.1 ± 1.0
(*R*)-Propranolol	1.96	3.15	0.68	3.67	$r = 0.9996$ $y = 0.0095x + 0.009$	97.6 ± 0.9	96.2 ± 1.2

[a] y = peak-height ratio; x = concentration (ng/ml).

Table 2
Intra-day and inter-day precision of propranolol enantiomers in human plasma and urine samples

Enantiomer	Subject 1			Subject 2		
	Concentration (ng/ml)	C.V. ($n = 6$) (%)		Concentration (ng/ml)	C.V. ($n = 6$) (%)	
		Intra-day	Inter-day		Intra-day	Inter-day
Plasma						
(*S*)-Propranolol	48.7	1.80	3.50	37.2	1.62	3.29
(*R*)-Propranolol	34.5	2.33	4.11	23.7	2.81	4.27
Urine						
(*S*)-Propranolol	47.9	0.68	4.34	30.7	1.11	3.72
(*R*)-Propranolol	33.3	1.46	4.67	22.5	1.65	4.21

phase and polar organic solvent mixture. Only the two last modes can separate propranolol and some other β-blocking agents. For the polar organic mobile phase, consisting in general of acetonitrile, methanol, acetic acid and triethylamine at different concentrations, the role of each polar solvent has been previously explained [17,18]. However, using the reversed-phase mode, the separation of (*S*)- and (*R*)-propranolol is weaker ($R_s = 1.4$ and $\alpha = 1.04$) and is achieved with a mobile phase of methanol–1% triethylammonium acetate in water (pH 4.1) (25:75, v/v), but on two Cyclobond I columns connected in series [16].

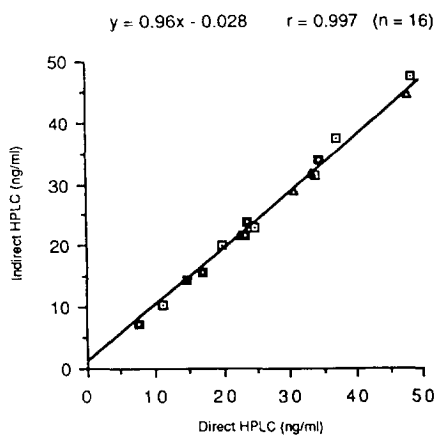

Fig. 3. Correlation between plasma and urine propranolol enantiometer concentrations determined by direct HPLC and indirect HPLC. (□ and △) (*S*)-Propranolol concentrations (ng/ml) in plasma and urine respectively. (■ and ▲) (*R*)-Propranolol concentrations (ng/ml) in plasma and urine respectively; *x*-axis = direct HPLC or HPLC with chiral β-cyclodextrin column; *y*-axis = indirect HPLC or HPLC after chiral derivatization.

In this work, we have chosen ethanol rather than methanol for the preparation of the polar organic mobile phase, because ethanol is less polar than methanol, thereby facilitating the search for the optimum peak resolution and retention time. Indeed, after comparing the effects of several solvent mixtures constituted of different concentrations of acetonitrile, methanol or/and ethanol, acetic acid and triethylamine on the resolution and retention time of racemic propranolol and racemic alprenolol used as I.S., we found that only the mobile phase described here allowed the simultaneous separation of these two racemates with good resolution and short retention times. The short chromatographic run time (about 19 min) is also an interesting condition for routine therapeutic monitoring of propranolol. In addition, the proposed mobile phase can also separate two other racemic β-blockers, pindolol and metoprolol, which both had retention times (about 19–21 min for (*S*)- and (*R*)-pindolol and 20–24 min for the other) shorter than those reported by others [17] and presented good peak resolutions (1.6 and 3.0, respectively).

We also tried to replace the triethylamine of the mobile phase by different concentrations of diethylamine; however, the resolution of pro-

pranolol and I.S. was largely reduced and their peaks were not symmetric.

To preserve the column efficiency, it is recommended to wash the column for ca. 1 h with methanol alone after analysis of about 30 samples with this polar organic mobile phase.

3.4. Choice of internal standard

Racemic alprenolol has been chosen as internal standard (I.S.) because of its sufficient resolution, its shorter retention time than that of propranolol and also because of the absence of interference with plasma and urine endogenous peaks. The racemic 4-methylpropranolol [7] and racemic pronethalol [6] previously used as I.S., have not been chosen because of weak (4-methylpropranolol) or no separation (pronethalol) on the β-cyclodextrin column with the mobile phase used here.

An assay was carried out without I.S. by the same procedure; the linearity and reproducibility were less satisfactory with $r = 0.98$ and C.V.s between 10 and 17% (versus $r = 0.999$ and C.V. $< 3\%$ with the use of I.S.), because it is difficult to measure exactly the volatile solvent volume. The possible presence of alprenolol at therapeutic levels (5–10 ng/ml) in the plasma does not interfere with this analysis because the detection limit of alprenolol (I.S.) at the wavelengths used in the present method was 100 ng/ml per enantiomer.

3.5. Method validation

The excellent recovery (94–98%) obtained in this work confirms the results of the extraction technique previously described [7]. In this procedure, the plasma is first precipitated with methanol present in the I.S. solution in order to release the propranolol bound to proteins. Then, the extraction of unbound propranolol with methanol–diethyl ether (10:90, v/v) in the presence of ammonium hydroxide is rapidly achieved by vortex-mixing. Methanol is added to the diethyl ether to avoid the formation of a protein emulsion which is one of the causes of the extraction losses [7].

The good correlation between the results ($n = 16$) obtained by direct HPLC using the chiral β-cyclodextrin column and those obtained by indirect HPLC after chiral derivatization with (*R*)-phenylethylisocyanate is shown in the Fig. 3. The advantages of this direct determination of propranolol enantiomers over the indirect techniques are the simplicity and the precision of the results. The indirect techniques require derivatization procedures, which are time-consuming and sometimes reduce the method accuracy or cause peak inversion.

3.6. Application of the method

In spite of a preliminary study on the pharmacokinetic profile of propranolol enantiomers in man, the results obtained in the present work appear interesting. The maximum plasma concentrations of both (*S*)- and (*R*)-propranolol for two healthy volunteers were found at 2 h after dosing, then they decreased in parallel at 4 and 8 h after dosing. The levels of both enantiomers in plasma and urine were dose-dependent. The (*S*)-propranolol concentrations in human plasma and urine samples were always higher than those of its (*R*)-antipode. The (*S*)/(*R*) concentration ratios in plasma varied between 1.3 and 1.5 and those in urine between 1.3 and 1.4. These values are consistent with those reported by others [2,20].

In conclusion, the liquid chromatographic micromethod described here is applicable to the direct determination of (*S*)- and (*R*)-propranolol in plasma and urine for routine therapeutic monitoring and pharmacokinetic studies. This chiral method will be a suitable tool for exploring the differences between the (*S*)- and (*R*)-propranolol levels in human biological fluids.

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