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Direct enantioselective determination of (R)- and (S)-propranolol in human plasma. Application to pharmacokinetic studies

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Abstract: In order to examine possible drug interactions of (R)- and (S)-propranolol a randomized, double blind, cross-over study has been performed, administering orally single doses of 40 mg (R,S)- and of 20 mg (S)-propranolol.HCl three times daily over a week to reach steady state conditions. After the first single dose of 40 mg (R,S)-propranolol.HCl, the $AUC_{0-\infty}$ and C_{max} values of the (S)-isomer were greater than those of the (R)-isomer: the ratio of $AUC_{(S)}$ over $AUC_{(R)}$ was 1.77 ($P < 0.05$) and that of C_{max} 1.57 ($P < 0.01$). When (S)-propranolol.HCl was given as a single 20 mg dose, the $AUC_{(S)}$ value was a factor of 0.55 lower than after administration of 40 mg (R,S)-propranolol.HCl. At steady state, the AUC of (S)-propranolol was 1.52 times higher ($P < 0.01$) than that of the (R)-isomer after administration of 40 mg racemate, and comparing the (S)-isomer, the ratio was 1.21. Following administration of the first single dose of 40 mg of the racemate, the mean (SD) clearance of the (R)- and (S)-isomers was 110 (84) and 61 (37) ml min^{-1} kg^{-1}, respectively; at steady state these values were 89 (55) and 57 (37) ml min^{-1} kg^{-1}, respectively. Respective values for (S)-propranolol after single isomer administration (20 mg) were 86 (36) and 57 (25) ml min^{-1} kg^{-1} in single dose and steady state situations. The data are based on the quantitative analysis of (R)- and (S)-propranolol in plasma. A sensitive enantioselective LC-bioassay based on the formation of the (R)- and (S)-propranolol-oxazolidine-2-one and resolution of these derivatives on a (R,R)-dinitrobenzoyl-diaminocyclohexane ((R,R)-DNB-DACH) chiral stationary phase was developed, using dichloromethane–methanol (99.75:0.25, v/v) as mobile phase, with fluorimetric detection.

Keywords: *Propranolol enantiomers; stereoselective pharmacokinetics; enantioselective LC-bioassay; oxazolidine-2-one-derivative; fluorimetric detection.*

Introduction

Propranolol (see Fig. 1) is a lipophilic, non cardioselective beta-blocking drug which is widely used in the treatment of cardiovascular disorders such as hypertension and angina pectoris. The drug is administered as a racemic mixture, i.e. a 1:1 mixture of the (R)- and (S)-enantiomers, although it is well known that the pharmacodynamic profiles of (R)- and (S)-propranolol differ significantly [1]. The beta-blocking activity resides predominantly in (S)-propranolol that is more than 100 times more potent in blocking β-receptors than the (R)-enantiomer [2, 3]. For structurally analogous beta-blocking drugs, similar stereoselective differences in activity have been found and prognosticated [4]. In contrast (R)- but not (S)-propranolol inhibits the conversion of thyroxine to triiodothyronine [5], whereas, the antiarrhythmic class 1-activity [6, 7] and decrease of intraocular pressure are similar for both the (R)- and (S)-enantiomers [8]. However, several studies have shown that also disposition and metabolism kinetics of the propranolol enantiomers are stereoselective [9–12].

In order to elucidate the pharmacodynamic and pharmacokinetic behavior of (R)- and (S)-propranolol one needs these drugs in optically pure form, but also as reference compounds for analytical purposes. Within the last few years several chromatographic methods have been developed to resolve mixtures of (R)- and (S)-propranolol but also of other beta-blocking drugs into its stereoisomers [4]. "Indirect enantioseparation techniques" involving a derivatization step with an optically pure chiral derivatizing agent (CDA) forming diastereo-isomers, have been widely adopted. CDAs

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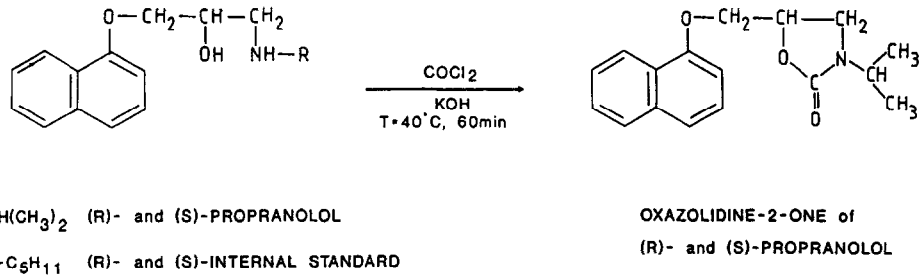

Figure 1
Formulae and reaction scheme of (R,S)-propranolol and "(R,S)-n-pentyl-propranolol" with phosgene leading to the corresponding (R,S)-oxazolidine-2-one-derivatives.

such as (R)-(+)- and (S)-(−)-1-phenylethyl isocyanate [13–16], (R)-(+)-1-phenylethyl isothiocyanate [17], (R)-(+)-1-(1-naphthyl)-ethyl isocyanate [18, 19], (−)-N-trifluoroacetyl-propylchloride [20], (R,R)-O,O'-diacetyl-tartaric acid anhydride [21, 22], 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate [23], (+)-1-(9-fluorenyl)-ethylchlorformate [24], (−)-menthyl chloroformate [25], t-BOC-L-leucine anhydride [26, 27], flunaproxen- and naproxenisocyanate [28] have widely proved their usefulness to separate enantiomers of beta-blocking drugs with an amino-alcohol structure. However, this technique puts high demands on the quality (i.e. optical purity) of the chiral reagent.

In contrast to the "indirect" method the "direct enantioseparation technique" is based on a reversible formation of diastereomeric molecule associates between the enantiomers of the analyte ((R)- and (S)-selectand, (R)-SA and (S)-SA) and chiral host moleçules (e.g. (R)-selector (R)-SO) representing the chiral stationary phase (CSP) [29–32]. Many beta-blocking drugs and particularly (R,S)-propranolol can also be resolved directly without any derivatization of the molecule [33].

In the following the most successful CSPs for resolving (R,S)-propranolol and other beta-blockers are briefly mentioned. Particularly "protein type" CSPs generated by immobilizing proteins as α_1-acid glycoprotein (chiral AGP), ovomucoid (OVM column) avidin or cellulase onto silica gel have been reported to resolve (R,S)-propranolol quite well [34–37]. However, the efficiency of the columns is often relatively poor which might be a limiting factor for trace analysis. Chemi- or physisorbed chiral cellulose-tris-(3,5-dimethylphenyl)-carb-amate onto wide pore silica-gel, commercial available as Chiralcell OD, resolves (R,S)-propranolol highly efficiently [33], but the stability of this column in the course of bioanalytical work might limit its use. (R,S)-propranolol has also been separated using cyclodexrin [38] and benzoxycarbonyl glycyl proline [39] as chiral selector, also as chiral mobile phase additive.

Interestingly, it has been shown that (R,S)-propranolol can also be resolved by LC on a simple brush type CPS but only as oxazolidine-2-one derivatives (see Fig. 1). First these enantiomers were resolved on a first generation "Pirkle column" containing (R)-N-(3,5-dinitrobenzoyl)phenyl glycine as the chiral selector [40–42]. Recently, Gasparrini and coworkers [43], and Uray and Lindner [44] using 3,5-dinitrobenzoylated (R,R)-diaminocyclohexane ((R,R)-DACH-DNB) and (S,S)-diphenylethanediamine (DPEDA) derivatives as SOs, respectively, accomplished similar resolutions.

The aim of this contribution was to set up a rapid, sensitive, reliable and rugged enantioselective HPLC-bioassay for (R)- and (S)-propranolol using (R,R)-DACH-DNB [43] as chiral stationary phase which proved to be a highly stable packing material. The method involves a derivatization step (Fig. 1) of plasma sample extracts containing propranolol enantiomers and a structurally similar internal standard using phosgene in toluene as reagent. The reaction of the aminoalcohol group to the corresponding oxazolidine-2-one derivatives is fast and quantitative. Subsequently, this method was applied for comparative pharmacokinetic studies in humans, administering (S)- or (R,S)-propranolol.HCl to 10 volunteers in both single dose and steady state régimes, by dosing three times daily for a period of 7 days.

Experimental

Instrumentation and chromatographic conditions

The chromatographic system consisted of an LC Pump Model 410 (Kontron, Switzerland), a Rheodyne injector Model 7125 fitted with a 20-μl loop, a fluorescence detector Model 820-FP (Jasco, Japan) λ_{Ex} = 290 nm, λ_{Em} = 330 nm. Chromatograms were recorded on an Integrator HP 3396 (Hewlett–Packard, Germany). Enantioseparations were carried out on a 250 × 4 mm i.d. column packed with Lichrosorb Si 100, R,R-DACH DNB CSP 5 μm (gift from F. Gasparrini [43]) connected to a pre-column 10 × 4 mm i.d. packed with Lichrosorb Si 60 5 μm (Merck, Darmstadt, Germany). The mobile phase was dichloromethane–methanol (99.75:0.25, v/v) used at a flow rate of 1.0 ml min^{-1}. Determination and validation of extraction yield was carried out on a Chiralcell OD analytical column, 250 × 4.6 mm i.d. with a mobile phase of hexane–isopropanol–triethylamine (79.9:20:0.1, v/v/v).

Chemicals

Racemic (R,S)-propranolol hydrochloride (USP 23 quality) was supplied by Schweizerhall (Basel, Switzerland). Optically pure (R)- and (S)-propranolol.HCl was prepared according to a method developed by Lindner [45] but can also be purchased from Aldrich (Milwaukee, USA). The optical purity of (R)-propranolol.HCl was >99.5% and of (S)-propranolol.HCl > 99.6%. Potassium hydroxide, n-hexane, 1-butanol, 20% phosgene in toluene, diethylether (all pro analysi grade), dichloromethane and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany). The dosage forms of the drugs (hard gelatine capsules) containing 20 mg (S)- or 40 mg (R,S)-propranolol.HCl were manufactured at the department of Pharmaceutical Technology, Institute of Pharmaceutical Chemistry (Karl-Franzens-University of Graz, Austria).

Synthesis of the internal standard

The internal standard, racemic (R,S)-1-n-pentylamino-3-(1-naphthoxy)-2-propranol HCl internally termed (R,S)-n-pentylpropanolol (see also Fig. 1) was synthesized according to the following procedure: 4.5 g (30 mmol) 1-naphthol, 3.3 ml epichlorhydrin (33 mmol) and 6.5 g (32.5 mmol) of anion-exchange resin in the OH form (Merck, Germany) in 60 ml toluene were refluxed for 5 h. After filtration and evaporation of the organic solvent the oily and brown residue was taken up in toluene and reevaporated to remove excess reagent. The main product was 1-chloro-3-(1-naphthoxy)-2-propane-epoxyd. To convert the by product 1-chloro-3-(1-naphthoxy)-2-propanol to the epoxide, the residue was dissolved in 40 ml toluene and shaken with 20 ml of 30% aquous KOH for 15 min. After washing the organic phase with water and drying with Mg_2SO_4 the solvent was evaporated and the crude (R,S)-3-(1-naphthoxy)-1,2-propane epoxide could be obtained (4.8 g yield, 80%). Without further purification 2.4 g (12 mmol) of this product were refluxed with 6.8 ml n-pentylamine (40 mmol) for 3 h to form the corresponding aminoalcohol. After evaporating the excess of amine the orange and the oily residue was stirred with cyclohexane/ether (9/1). The crystallized, white residue was taken up in diethylether (30 ml), and 2 ml aqueous HCl concentration were added to transfer the base to the hydrochloride salt. After removing the ethereal phase the hydrochloride salt of (R,S)-n-pentylpropranolol crystallized after several hours. The yield was 1.5 g (62%) after recrystallization from ethanol/acetone (m.p. 161°C). The NMR data were (CDCl3, free base): 8.5–6.8 (m naphthyl); 4.0 (m CH20, CHO), 3.4 (m OH, NH); 2.7 (m (CH2)2N); 1.0 (m C4H9).

Preparation of internal standard working solutions

Aliquots of a concentrated stock solution of (R,S)-n-pentyl-propranolol.HCl ((R,S)-I.St.) (200 μg ml^{-1}) in methanol/H_2O (20/80) were diluted with aqua bidest. to a concentration of 2 μg ml^{-1}.

Extraction and derivatization procedure

To a 1 ml plasma sample 0.5 ml of 0.1 M KOH and 30 μl (corresponding to 60 ng of (R,S)-I.St.) of the working solution of the I.St. were added and vortexed for 5 s. The mixture was extracted for 10 min by a shaking apparatus with 7 ml n-hexane containing 1% (v/v) n-butanol, centrifuged for 5 min and 6 ml of the organic phase were transferred to a centrifuge tube and evaporated to dryness by a steam of nitrogen at 40°C. The residue was taken up in 1 ml diethylether, which was stored over a 2% aqueous solution of KOH, followed by the addition of 100 μl toluene containing 20%

phosgene. The mixture was vortexed in stoppered tubes and incubated for 3 h at 40°C. After blowing off the organic solvent by nitrogen the residue was redissolved in 100 μl mobile phase of which 20 μl were injected into the HPLC system.

Protocol for the pharmacokinetic study

According to a double-blind, cross-over protocol, 10 healthy volunteers (after giving a written informed consent) were randomized to take oral doses of either 40 mg racemic propranolol.HCl or 20 mg (S)-propranolol.HCl. Blood samples were drawn at 0, 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 8 h post dose by an indwelling cannula. The respective racemate or isomer were taken orally three times daily over 1 week, and on day 8 the same blood sampling procedure was repeated. After a wash-out period of 7 days subjects were crossed over, and the single dose and steady state procedures described above were repeated under the same conditions.

Plasma was separated by centrifugation and stored at −10°C prior to analysis. All plasma concentrations of (R)- and (S)-propranolol of each sample were assayed.

The $AUC_{0-\infty}$ after the single dose ($AUC_{0-\infty}$) and $AUC_{0-\tau}$ in steady state (AUC_{SS}) were obtained from the plasma concentration data using the trapezoidal rule. Total body clearance (Cl) was calculated from the relationship

$$Cl/F = D/AUC$$

where D is the dose and the bioavailability (F) assumed to be 1. The terminal half-life was calculated from the terminal slope of each profile. Statistical analysis was carried out using the Wilcoxon signed-rank test.

Results and Discussion

Chromatography

Chromatograms of typical plasma samples of volunteers and spiked blank plasma are shown in Fig. 2. For the conditions chosen the absolute retention times of (R)- and (S)-propranolol-oxazolidine-2-one were 8.8 min and 10.8 min and those of (R)- and (S)-n-pentylpropranolol-oxazolidine-2-one were 7.8 min and 9.8 min. The elution order and retention time were examined with authentic reference standard. The α values for the enantiomers of propranolol and I.St. were 1.27 and 1.30, respectively. Chromatograms of blank plasma were free of interfering and late eluting peaks.

Validation

For experiments of recoveries and within-day reproducibilities 1 ml aliquots of blank plasma (obtained from a local hospital) were spiked with 20, 60 and 120 ng (R,S)-propranolol.HCl and 60 ng (R,S)-internal standard. To determine extraction yields the spiked plasma samples were extracted, and after blowing off the solvent the residue was taken up in mobile phase and without derivatization injected onto a Chiralcell OD analytical column (for chromatographic conditions see Experimental). Day-to-day reproducibility of the total analysis method was determined by comparing the slopes of 18 two-point calibration curves of quality control plasma samples containing 60 ng (R,S)-propranolol.HCl/60 ng (R,S)-internal standard and 120 ng (R,S)-propranolol.HCl/60 ng (R,S)-internal standard; they were made together with each series of authentic blood samples (30 samples a day) in the course of the pharmacokinetic study. All drug analysis was made over a period of 2 months and the ruggedness of the method was monitored by a chart. Statistical data of the assay are shown in Table 1.

Pharmacokinetic data

Ten healthy volunteers whose mean (SD) weight was 72 (8) kg participated in this study. Figure 3(A) and (B) shows the mean (SD) plasma concentration of (R)- and of (S)-propranolol from the single dose studies. The mean (SD) pharmacokinetic data are summarized in Table 2. Figure 3(A) and Table 2 reveal that after oral single-dose administration of racemic propranolol the C_{max} of the (S)-isomer is higher than that of the (R)-isomer ($P < 0.01$). Table 2 and Fig. 3(B) indicate that the C_{max} for (S)-propranolol after oral single-dose administration of 40 mg racemate is higher than after a single dose of the pure (S)-isomer ($P < 0.05$). The ratio of $AUC_{(S)}$ over $AUC_{(R)}$, also termed eudismic ratio, was 1.77(0.75).

The mean (SD) plasma concentrations of (R)- and (S)-propranolol at steady state administering (R,S)-propranolol and (S)-propranolol are shown in Figure 4(A) and (B), respectively, the mean pharmacokinetic data

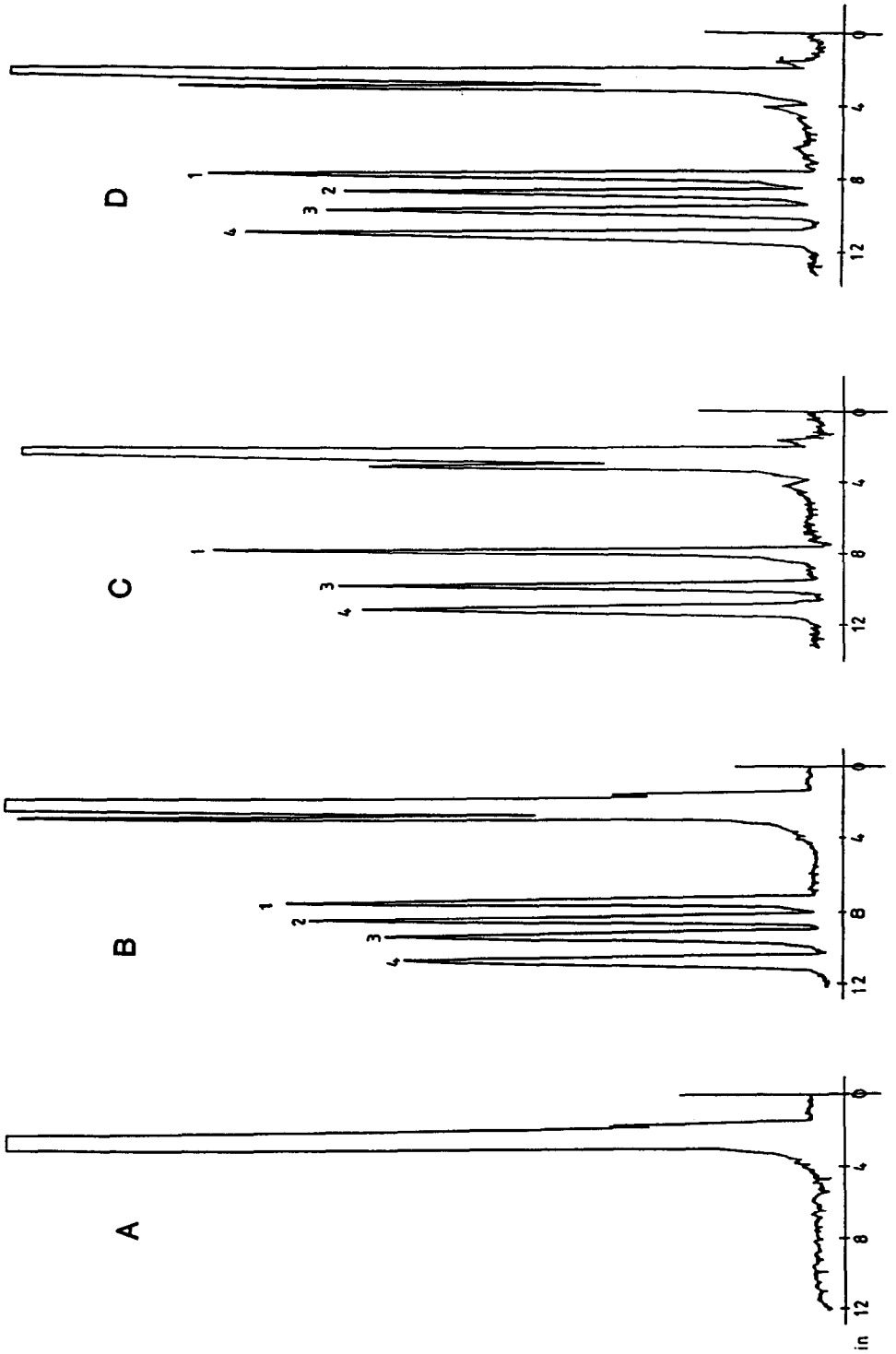

Figure 2
Typical chromatograms of plasma (serum) extracts of (A) blank plasma, (B) spiked plasma containing 30 ng of each enantiomer, (C) spiked sample containing 60 ng (R,S)-n-pentyl-propranolol and 30 ng (S)-propranolol and (D) real sample. Peaks 1 and 3 correspond to oxazolidine-2-ones of (R)- and (S)-n-pentylpropranolol, and peaks 2 and 4 to (R)- and (S)-propranolol, respectively. For HPLC conditions see Experimental.

Table 1
Statistical data of the assay

	Spiked amounts of (R,S)-propranolol.HCl [ng ml^{-1}] plasma		
	20	60	120
Extraction yield (mean ± SD*, n = 6)	95 ± 5%	95 ± 5%	95 ± 5%
Recovery (mean ± SD, n = 9)	80 ± 5%	80 ± 5%	80 ± 5%
Within-day reproducibility RSD (n = 6)	3.6%	4.8%	6.1%
	60		120 ng ml^{-1}
Day-to-day reproducibility RSD† (n = 18) (judged by slopes of calibration curves)	2.7%		
	5, 10, 30, 60, 100, 200 ng ml^{-1}		
Linearity	(R)-propranolol	r‡ = 0.996	$y = -0.5 + 0.901x$
	(S)-propranolol	r = 0.995	$y = 0.7 + 0.804x$
Limit of determination (ng ml^{-1} plasma), signal:noise level 7:1	(R)-propranolol	0.4 ± 0.2 ng	
	(S)-propranolol	0.5 ± 0.2 ng	

*Standard deviation.
†Relative standard deviation.
‡Linear regression analysis.

Table 2
Mean (SD) pharmacokinetic data after oral single dose administration of 40 mg (R,S)-propranolol.HCl and of 20 mg (S)-propranolol.HCl (n = 10)

	Racemic drug		(S)-isomer
	(R)-propranolol	(S)-propranolol	(S)-propranolol
C_{max} (ng ml^{-1})	44 (8)*	22 (14)	134 (7)
$t_{1/2}$ (h)	3.3 (2.5)	5.1 (5.9)	2.9 (1.3)
$AUC_{0-\infty}$ (ng h ml^{-1})	77 (68)	121 (91)	66 (37)
Cl (ml min^{-1} kg^{-1})	110 (84)	61 (37)	86 (36)
$C_{max(S)}/C_{max(R)}$	1.57 ($P < 0.01$)		
$AUC_{(S)}/AUC_{(R)}$	1.77 ($P < 0.05$)		
$AUC_{(S)rac}/AUC_{(S)o.p.d.}$†		1.83 ($P < 0.05$)	

*Values in brackets represent standard deviation SD.
†Optically pure drug.

Table 3
Mean (SD) pharmacokinetic data at steady state following 22 (three times daily over a week) oral administrations of 40 mg (R,S)-propranolol.HCl and 20 mg (S)-propranolol.HCl (n = 10)

	Racemic drug		(S)-isomer
	(R)-propranolol	(S)-propranolol	(S)-propranolol
C_{max} (ng ml^{-1})	20 (15)*	27 (18)	25 (14)
$t_{1/2}$ (h)	2.4 (0.8)	2.8 (1.0)	3.8 (2.8)
AUC_{SS} (ng h ml^{-1})	84 (67)	119 (79)	98 (45)
Cl (ml min^{-1} kg^{-1})	89 (55)	57 (35)	57 (25)
$C_{max(S)}/C_{max(R)}$	1.35 ($P < 0.01$)		
$AUC_{(S)}/AUC_{(R)}$	1.52 ($P < 0.01$)		
$AUC_{(S)rac}/AUC_{(R)o.p.d.}$		1.21 n.s†	

*Values in brackets represent standard deviation SD.
†Not significant.

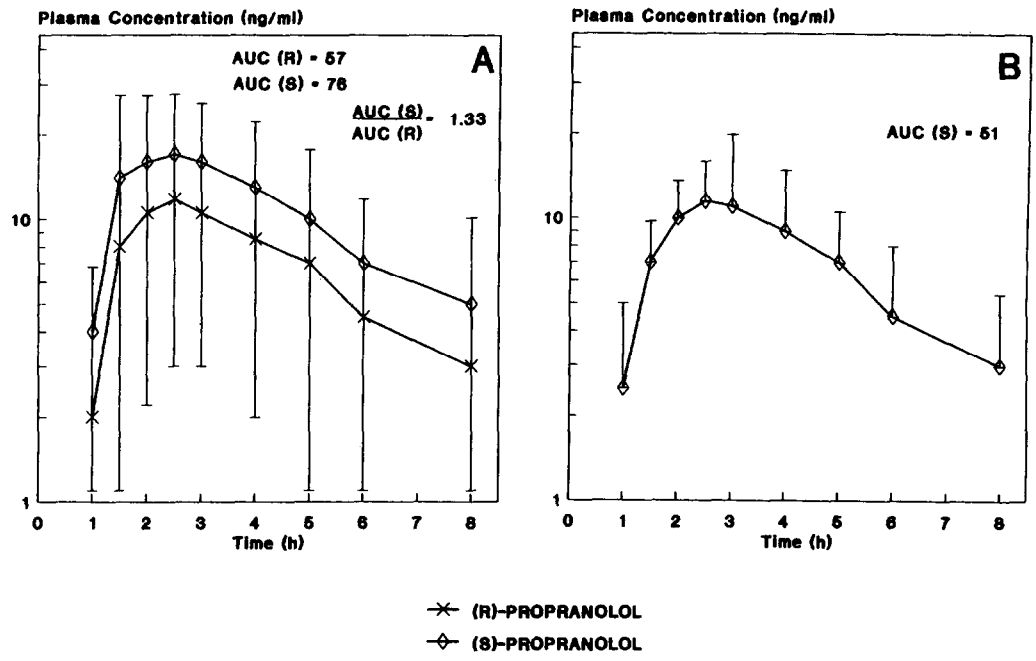

Figure 3
Mean plasma concentration–time curves (AUC_{0-8}) of (R)- and (S)-propranolol of 10 healthy volunteers after the first oral dose of (A) 40 mg (R,S)-propranolol.HCl and of (B) 20 mg (S)-propranolol.HCl.

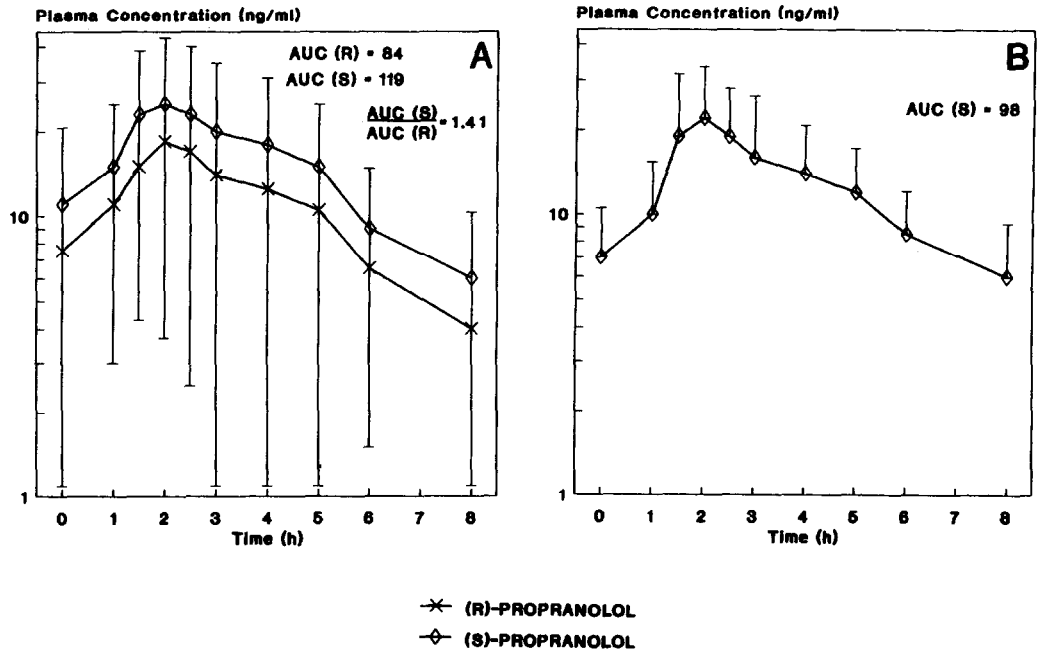

Figure 4
Mean plasma concentration–time curves (AUC_{0-8}) of (R)- and (S)-propranolol of 10 healthy volunteers at steady state after the 22nd oral application (three times daily over 1 week) of (A) 40 mg (R,S)-propranolol.HCl and of (B) 20 mg (S)-propranolol HCl, respectively (eighth day of the study).

are summarized in Table 3. The data in Table 3 demonstrate that the C_{max} and AUC are higher for (S)-propranolol than for (*R*)-propranolol ($P < 0.01$). The eudismic ratio of $AUC_{(S)}$ over $AUC_{(R)}$ was 1.52 (0.16).

Conclusion

HPLC-bioassay

A number of methods for quantitation of propranolol enantiomers in biological fluids have now been described in the literature; however, this method involving a non-chiral derivatization, has been found to be reliable, rugged and sensitive for the assay of (R)- with (S)-propranolol in plasma (serum) by HPLC. The enantiomeric derivatives are well separated on a new chiral stationary phase (R,R)-DACH-DNB [43]. The assay is sufficiently sensitive and reliable to monitor (R)- and (S)-propranolol down to levels less than 0.5 ng ml^{-1}. This guarantees quantitation down to four half-lives after the C_{max} following single oral dose administration. Preliminary experiments have shown that this method could be used for monitoring the individual enantiomers of other important beta-blocking drugs such as metoprolol and atenolol [46].

Pharmacokinetic results

As earlier findings have demonstrated the higher C_{max} plasma concentration and AUC values observed for (S)-propranolol compared to (R)-propranolol could be attributed to either higher oral absorption, to a lower volume of distribution or to a stereoselective hepatic metabolism [26]. In other words, the lower clearance values of the (S)-isomer could also be due to a reduced volume of distribution related to its higher plasma binding [12], since the terminal half-lives were comparable to each other. All these observations summarized in Tables 2 and 3 are derived from single dose and multiple dose studies, and are consistent with earlier reports [9–11]. Further work in the author's laboratories on the stereoselective pharmacokinetics and pharmacodynamics of (R)- and (S)-propranolol utilizing this analytical method is reported elsewhere [47].

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