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SUBSTRATE STEREOSELECTIVITY AND ENANTIOMER/ENANTIOMER INTERACTION IN PROPRANOLOL METABOLISM IN RAT LIVER MICROSOMES

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Abstract—The substrate stereoselectivity and enantiomer/enantiomer interaction of (*S*)- and (*R*)-propranolol for the formation of their metabolites were investigated in rat liver microsomal fractions. The enantiomers of primary metabolites of propranolol, 4-, 5-, 7-hydroxy- and *N*-desisopropylpropranolol were separated and assayed by an HPLC method employing a chiral ovomucoid column. Regioselective substrate stereoselectivity ($R < S$ for 4- and 5-hydroxylations; $R > S$ for 7-hydroxylation; $R = S$ for N-desisopropylation) was observed in the formation of propranolol metabolites when the individual enantiomers or a racemic mixture of propranolol were used as substrates. Concentration-dependent metabolic inhibition of propranolol enantiomers by their optical isomers was also observed. In addition, the inhibition of propranolol 4-, 5- and 7-hydroxylations between the enantiomers showed a typical competitive nature. These findings suggested that the propranolol enantiomers competed for the same enzyme, probably a cytochrome P450 isozyme in the CYP2D subfamily.

Propranolol, a β-adrenergic receptor blocking agent, is widely used as a racemic mixture of two optical isomers, (*R*)-(+)- and (*S*)-(−)-propranolol, in the treatment of hypertension, cardiac arrhythmias and other diseases. The *S*-enantiomer is 60–100 times more potent as a β-blocker than the *R*-enantiomer and is probably responsible for almost all of its pharmacological effects [1, 2]. The pharmacokinetics of the *R*- and *S*-enantiomers are different after giving racemic propranolol to humans [3–6], and the differences are more pronounced in the rat [7–10], probably because of a large difference in plasma protein binding between the enantiomers in the rat [8–10]. The urinary excretion of propranolol metabolites after administration of each enantiomer [11] and of the racemate [12, 13] in the rat was reported to be stereoselective. Stereoselective metabolism was also reported *in vitro* when racemic propranolol was incubated with the 9000 *g* supernatant fraction from rat liver [12, 13].

Recently we have demonstrated the regio- and stereoselectivity of cytochrome P450 (P450‡)-mediated propranolol metabolism (4-, 5- and 7-hydroxylations and N-desisopropylation) using 15 species of P450 purified from rat liver microsomes [14]. All of these isozymes exhibited more or less enzyme activity for each metabolic pathway. With each purified P450 species, stereoselectivity was different for the oxidation sites of propranolol, and also the regioselectivity differed between the two propranolol enantiomers. In addition, stereoselectivity was sometimes altered when the substrate concentration was varied. Therefore, the regio- and stereoselective propranolol metabolism by rat liver microsomes was suggested to result from the combined activities of multiple species of P450, each isozyme possessing its own stereoselectivity.

P450BTL, an isozyme belonging to the CYP2D subfamily [15], exhibited high activities of propranolol hydroxylation and showed regio- and stereoselectivity similar to that of liver microsomes at a substrate concentration of 5 μM, whereas other constitutive P450 species showed a selectivity different from that observed in the microsomes [14]. This observation suggests that P450BTL is the major species in propranolol metabolism at low substrate concentrations. The propranolol enantiomers may be metabolized by the enzyme at different rates and the enantiomers may compete for each metabolic step, giving rise to the possibility of an enantiomer/enantiomer interaction.

In the present study, we developed an HPLC method without derivatization using a chiral ovomucoid column and fluorescence detection to determine enantiomers of propranolol metabolites in rat liver microsomal fractions. By using this analytical technique, the regio- and stereoselectivity and also the enantiomer/enantiomer interaction of propranolol metabolism in rat liver microsomes were investigated.

MATERIALS AND METHODS

Chemicals. Propranolol hydrochloride (racemate) and procainamide hydrochloride were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). (*R*)- and (*S*)-propranolol were separated

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‡ Abbreviations: P450, cytochrome P450; X-OH-P, X-hydroxypropranolol; NDP, *N*-desisopropylpropanolol.

Table 1. Retention times, calibration curves and lower limits of detection of propranolol metabolites

Compound	Retention time (min)	Calibration curve	r	Limit of sensitivity (μM)†
(*R*)-5-OH-P	7.8*	$y = 77.8x - 0.0252$	0.9998	0.002
(*S*)-5-OH-P	8.8	$y = 76.9x - 0.0460$	0.9997	0.002
(*S*)-4-OH-P	11.0	$y = 7.46x - 0.0885$	0.9967	0.02
(*R*)-4-OH-P	12.1	$y = 7.16x - 0.101$	0.9959	0.02
(*S*)-NDP	8.5	$y = 164x - 0.0343$	0.9998	0.001
(*R*)-NDP	10.0	$y = 154x - 0.0272$	0.9997	0.001
(*S*)-7-OH-P	12.5	$y = 62.3x - 0.147$	0.9995	0.003
(*R*)-7-OH-P	15.0	$y = 54.9x - 0.131$	0.9995	0.003

* The mobile phase used for the enantiomers of 4- and 5-OH-P was 20 mM potassium phosphate buffer (pH 6.8):acetonitrile:di-*n*-butylamine (90:10:0.27, by vol.). The mobile phase used for the enantiomers of NDP and 7-OH-P was 20 mM potassium phosphate buffer (pH 6.8):acetonitrile:di-*n*-butylamine (89:11:1.0, by vol.).

† The values were determined at a signal-to-noise ratio of 3:1.

according to the method of Yost and Holtzman [16]. 4-Hydroxypropranolol (4-OH-P) hydrochloride was provided by Sumitomo Chemical Industries (Osaka, Japan). *N*-Desisopropylpropanolol (NDP) hydrochloride was provided by the ICI Pharmaceuticals Co. (Macclesfield, U.K.). 5- and 7-hydroxypropranolol (5-OH-P and 7-OH-P, respectively) were synthesized as hydrochlorides according to the method of Oatis *et al.* [17]. Glucose 6-phosphate glucose 6-phosphate dehydrogenase and NADPH were purchased from the Oriental Yeast Co., Ltd (Tokyo, Japan). All other chemicals and solvents used were of analytical grade.

Preparation of hepatic microsomes. Male Wistar rats (2 months old) were obtained from Takasugi Experimental Animals (Kasukabe, Japan). Hepatic microsomal fractions were prepared according to the method of Omura and Sato [18]. Protein concentrations were assayed by the method of Lowry *et al.* [19].

Incubation of propranolol with liver microsomes. Incubation of propranolol with liver microsomes was performed in a 1-mL incubation mixture containing 0.5 mg microsomal protein, 10 mM glucose 6-phosphate, 2 U/mL glucose 6-phosphate dehydrogenase, 0.5 mM NADPH, 8 mM $MgCl_2$, an appropriate concentration of a substrate and 0.15 M potassium phosphate buffer (pH 7.4). Five micromolar (*R*)- or (*S*)-propranolol, or 10 μM racemic propranolol was used as a substrate. Kinetic parameters were determined employing propranolol enantiomers at 10 different concentrations ranging from 0.1 to 5 μM as substrates. Inhibition experiments were performed by using (*R*)-propranalol as a substrate and (*S*)-propranolol as an inhibitor, and *vice versa*, at concentrations of 0, 0.5, 1 and 2 μM of each. After 5-min pre-incubation under air at 37°, incubation was started by adding NADPH, and carried out for 1 min. The reaction was stopped by adding 1 mL of 1 N NaOH including sodium bisulfite (25 mg/mL) as an antioxidant to the reaction mixture to avoid degradation of 4-OH-P.

Assay of propranolol metabolites. Propranolol metabolites in the reaction mixture were assayed by a reported method [20] with modifications. The amounts of procainamide employed as internal standard for the assay of enantiomers were 250 ng for 4-OH-P and 5-OH-P, and 6.25 ng for 7-OH-P and NDP. Propranolol metabolites were extracted with ethyl acetate (5 mL), and the organic phase was evaporated to dryness. The residue was dissolved in 100 μL of the HPLC mobile phase described below and subjected to HPLC analysis.

HPLC conditions. The HPLC apparatus consisted of a JASCO model PU-880 pump (Japan Spectroscopic Co., Tokyo, Japan), a model RF-535 fluorescence HPLC monitor (Shimadzu, Kyoto, Japan), and a chiral ovomucoid column (ULTRON ES-OVM, 5 μm, 15 cm × 4.6 mm i.d., Chromato Packing Center, Kyoto, Japan). The mobile phase for the determination of the enantiomers of 4-OH-P and 5-OH-P (mobile phase A) was 20 mM phosphate buffer (pH 6.8):acetonitrile:di-*n*-butylamine (90:10:0.27, by vol.). The mobile phase for the determination of the enantiomers of 7-OH-P and NDP (mobile phase B) was 20 mM phosphate buffer (pH 6.8) : acetonitrile : di-*n*-butylamine (89:11:1, by vol.). A flow rate was 1.0 mL/min. Fluorescence detection was performed at an excitation/emission wavelength of 315/430 nm for 4-OH-P and 5-OH-P, and 300/350 nm for 7-OH-P and NDP.

Data analysis. Enzyme kinetic parameters (K_m, V_{max} and K_i) were analysed by non-linear least squares regression based on a simplex method [21]. Best fitting of the data was performed by weighting the reciprocal of their square. Statistical significance was calculated by Student's *t*-test.

RESULTS

HPLC procedure for the determination of the enantiomers of propranolol metabolites

In aliquots (10–20 μL) the enantiomers of 4-OH-P and 5-OH-P, and of 7-OH-P and NDP were separately measured using different mobile phases

Table 2. Inter-assay reproducibility of the assay of propranolol metabolites

Concentration (μM)	Coefficient of variation (%)*							
	(*R*)-4-OH-P	(*S*)-4-OH-P	(*R*)-5-OH-P	(*S*)-5-OH-P	(*R*)-7-OH-P	(*S*)-7-OH-P	(*R*)-NDP	(*S*)- NDP
0.0025			4.6	6.2			1.8	2.5
0.005			3.6	3.0			4.8	8.5
0.01			6.4	4.3			3.4	3.1
0.0125					2.8	2.7		
0.02			5.1	6.0			2.4	2.5
0.025	6.8	5.8			4.4	6.5		
0.04			5.3	4.4			8.8	7.7
0.05	8.7	8.7			5.6	5.7		
0.1	3.8	5.2			1.0	1.4		
0.2	7.3	5.2			5.8	5.4		
0.4	4.0	4.6						

* Values were obtained from five determinations.

and detection wavelengths. The sequence of elution of the individual enantiomers was confirmed using microsomal samples formed from optically pure enantiomers of propranolol. In the mobile phase A, the retention times were: procainamide (internal standard), 3.9 min; (*R*)-5-OH-P, 7.8 min; (*S*)-5-OH-P, 8.8 min; (*S*)-4-OH-P, 11.0 min; (*R*)-4-OH-P, 12.1 min; racemic 7-OH-P, 40 min; racemic propranolol, 110 min. NDP was not detectable at the wavelength used. In the mobile phase B, the retention times were: procainamide, 4.0 min; racemic 5-OH-P, 6.5 min; (*S*)-NDP, 8.5 min; (*R*)-NDP 10.0 min; (*S*)-7-OH-P, 12.5 min; (*R*)-7-OH-P, 15.0 min; racemic propranolol, 25 min. 4-OH-P was not detectable at the wavelength used. Blank microsomal extracts yielded no interfering peaks derived from endogenous substances. Table 1 shows retention times, sensitivities of the method and results obtained for the calibration curves of the enantiomeric propranolol metabolites. The calibration curves exhibited excellent linearities (Table 1), with correlation coefficients of at least 0.999 for the enantiomers of 5-OH-P, 7-OH-P and NDP, and with those of at least 0.995 for 4-OH-P within the concentration range evaluated (4-OH-P, 0.025–0.4 μM; 5-OH-P, 0.0025–0.04 μM; 7-OH-P, 0.0125–0.2 μM; NDP, 0.0025–0.04 μM). The lowest detection limits defined as three times the levels of baseline noise are also listed in Table 1. The coefficients of inter-assay variation were smaller than 10% for all of the enantiomeric metabolites tested (Table 2).

Stereoselectivity of the formation of propranolol metabolites in rat liver microsomes

Rat liver microsomal metabolism of propranolol was examined using 5 μM of the individual enantiomers and 10 μM of racemic propranolol (Fig. 1). Propranolol 4-hydroxylase activity was higher for (*S*)-propranolol than for (*R*)-propranolol as a substrate (Fig. 1A). When racemic propranolol was incubated, 4-OH-P was formed preferentially from the *S*-enantiomer as compared with the *R*-enantiomer (Fig. 1A). Similar results were observed in propranolol 5-hydroxylase activity (Fig. 1B). As for 7-hydroxylation, (*R*)-propranolol was a preferential substrate as compared with (*S*)-propranolol (Fig. 1C) for the individual enantiomers and the racemate. Substrate stereoselectivity was not observed in propranolol N-desisopropylation (Fig. 1D). These regioselective substrate stereoselectivities in aromatic hydroxylations of propranolol agreed with *in vivo* observations after administration of propranolol enantiomers [11] and of racemic propranolol [13] and *in vitro* observations of incubated racemic propranolol with the liver 9000 *g* fraction [13].

The rates of formation of 4-, 5-, 7-OH-P and NDP (only from (*S*)-propranolol) for the racemate were lower than the respective values obtained after incubation of the individual enantiomers. These findings suggested that the metabolism of each enantiomer was inhibited by its optical antipode in propranolol racemate as a substrate.

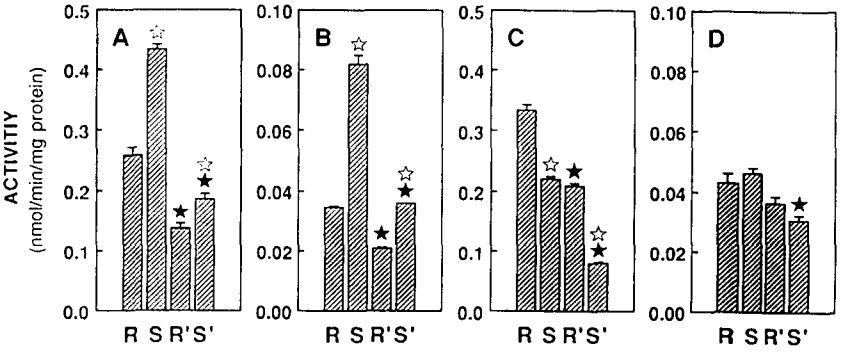

Fig. 1. Substrate stereoselectivity of the formation of propranolol metabolites after incubation of the enantiomers or the racemate in rat liver microsomes. (A), (B), (C) and (D) show propranolol 4-, 5- and 7-hydroxylations and N-desisopropylation, respectively. Abbreviations indicate substrates and products: R, *R*-metabolites formed from 5 μM (*R*)-propranolol; S, *S*-metabolites from 5 μM (*S*)-propranolol; R', *R*-metabolites from 10 μM racemic propranolol; S', *S*-metabolites from 10 μM racemic propranolol. Results represent means ± SE for four different rat liver microsomes. (☆) *S* and *S'* significantly different from *R* and *R'*, respectively ($P < 0.01$). (★) *R'* and *S'* significantly different from *R* and *S*, respectively ($P < 0.01$).

Stereoselectivity of kinetics for propranolol metabolism

The kinetic parameters for propranolol ring-hydroxylations were obtained after incubation of the individual propranolol enantiomers as substrates in rat liver microsomes. Propranolol 4- and 5-hydroxylations in rat liver microsomes have been reported to be biphasic [22]. We employed 0.1–5 μM as substrate concentrations, because enzyme activities in this concentration range mainly reflect reaction velocity of high-affinity components, which are important in the *in vivo* metabolism of propranolol. The rates of formation of 4-, 5- and 7-OH-P were expressed by a single Michaelis–Menten equation. The kinetic parameters calculated are listed in Table 3. Propranolol N-desisopropylation was not saturable in this concentration range and no enantiomeric difference ($P > 0.05$) in V_{max}/K_m was observed.

No significant difference was observed in K_m value between the individual enantiomers for any metabolic pathway determined. However, the V_{max} values of (*S*)-propranolol 4- and 5-hydroxylase activities were 70 and 134%, respectively, higher than those of the corresponding activities for (*R*)-propranolol (Table 3). On the other hand, the V_{max} value of (*R*)-propranolol 7-hydroxylase activity was 73% higher than that of (*S*)-propranolol 7-hydroxylase activity. Consequently, the two propranolol enantiomers are recognized kinetically as two different substrates for propranolol 4-, 5- and 7-hydroxylations.

Inhibition of ring-hydroxylations and N-desisopropylation of propranolol enantiomers by their optical antipodes

Figure 2A shows the inhibition of 4, 5, 7-hydroxylations and N-desisopropylation for (*R*)-propranolol metabolism by (*S*)-propranolol, the reverse is shown in Fig. 2B. Inhibitor concentrations ranged from one-half (0.5 μM) to twice (2.0 μM) the fixed substrate concentration (1.0 μM). Concentration-dependent inhibition of metabolic reactions of propranolol enantiomers by their optical antipodes was observed for all the pathways tested. (*R*)- and (*S*)-propranolol 4-hydroxylations were inhibited by 56% and 50% of control by (*S*)- and (*R*)-propranolol, respectively. Similar results were obtained for propranolol 5- and 7-hydroxylase activities. The inhibition of N-desisopropylation of propranolol enantiomers by their optical antipodes was smaller than that of the ring-hydroxylations.

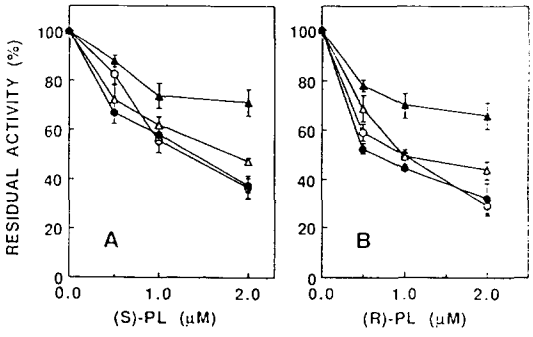

Fig. 2. Inhibition of ring-hydroxylations and N-desisopropylation of propranolol enantiomers by their optical antipodes in rat liver microsomes. (A) and (B) show the inhibition of (*R*)-propranolol [(R)-PL] ring-hydroxylations by (*S*)-propranolol [(S)-PL] and *vice versa*, respectively. Values were obtained for 4- (○), 5- (●), 7- (△) hydroxylations and N-desisopropylation (▲). The substrate concentration was 1 μM. Data are per cent of activity in the case of no inhibitor. Each value represents the mean ± SE of three determinations for different rat liver microsomes.

Competitive inhibition of the enantiomer/enantiomer interaction for propranolol ring-hydroxylations

The kinetic mechanism of inhibition between (*R*)-

Table 3. Kinetic parameters of the formation of the individual enantiomers of 4-, 5- and 7-hydroxypropranolol in rat liver microsomes at low substrate concentrations

Substrate	Pathway	K_m (μM)	V_{max} (nmol/min/mg protein)
(*R*)-PL*	4-OH	0.101 ± 0.012†	0.258 ± 0.021
(*S*)-PL	4-OH	0.087 ± 0.004	0.438 ± 0.014‡
(*R*)-PL	5-OH	0.169 ± 0.021	0.0360 ± 0.0010
(*S*)-PL	5-OH	0.138 ± 0.004	0.0843 ± 0.0054‡
(*R*)-PL	7-OH	0.164 ± 0.035	0.359 ± 0.018
(*S*)-PL	7-OH	0.127 ± 0.022	0.208 ± 0.019‡
		V_{max}/K_m (μL/min/mg protein)	
(*R*)-PL	NDP	8.20 ± 0.31	
(*S*)-PL	NDP	8.46 ± 0.49	

* Abbreviations: PL, propranolol; X-OH, propranolol X-hydroxylation; NDP, propranolol N-desisopropylation.

† Each value represents the mean ± SE of three determinations from different rat liver microsomes.

‡ Significantly different from mean values for *R*-enantiomer ($P < 0.01$).

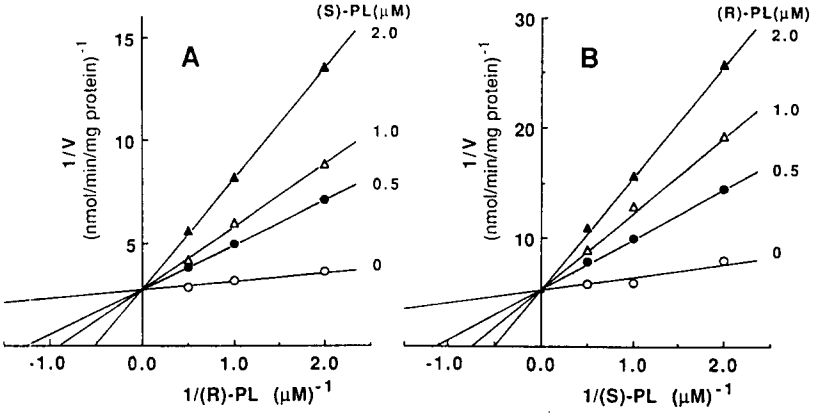

Fig. 3. Lineweaver–Burk plots showing the inhibition of 7-hydroxylase activities of propranolol enantiomers by their optical antipodes in rat liver microsomes. (A) and (B) show the inhibition of (*R*)-propranolol [(R)-PL] 7-hydroxylation by (*S*)-propranolol [(S)-PL] and *vice versa*, respectively. Illustrations show typical data for four different rat liver microsomes.

and (*S*)-propranolol hydroxylations was examined. Figure 3A shows typical Lineweaver–Burk plots for the rates of formation of 7-OH-P from (*R*)-propranolol (0.5–2.0 μM) in the presence of various concentrations of (*S*)-propranolol (0–2.0 μM), and Fig. 3B shows the situation *vice versa*. The metabolic interaction between (*R*)- and (*S*)-propranolol 7-hydroxylations showed a typical competitive pattern. The inhibitory constant (K_i) of (*R*)-propranolol 7-hydroxylation by (*S*)-propranolol and that of (*S*)-propranolol 7-hydroxylation by (*R*)-propranolol were calculated to be 0.237 ± 0.038 and 0.211 ± 0.019 μM, respectively (Table 4). These values were close to the K_m values when the inhibitors were subjected to microsomal metabolism as substrates (Table 3). The metabolic interactions of (*R*)- and (*S*)-propranolol for 4- and 5-hydroxylations were also competitive (data not shown). The K_i

Table 4. Inhibitory constants of (*R*)- and (*S*)-propranolol for 4-, 5- and 7-hydroxylations in rat liver microsomes

Substrate/inhibitor	Pathway	K_i (μM)
(*R*)-PL/(*S*)-PL*	4-OH	0.235 ± 0.044†
(*S*)-PL/(*R*)-PL	4-OH	0.201 ± 0.027
(*R*)-PL/(*S*)-PL	5-OH	0.239 ± 0.043
(*S*)-PL/(*R*)-PL	5-OH	0.214 ± 0.028
(*R*)-PL/(*S*)-PL	7-OH	0.237 ± 0.038
(*S*)-PL/(*R*)-PL	7-OH	0.211 ± 0.019

* Abbreviations as in the legend to Table 3.

† Each value represents the mean ± SE of four determinations from different rat liver microsomes.

values for the inhibition of (*R*)-propranolol 4- and 5-hydroxylations by (*S*)-propranolol, and *vice versa* are also shown in Table 4. These values were almost equal to the K_m values for the corresponding reactions of the propranolol enantiomers (Table 3).

DISCUSSION

The enantiomeric interaction for glucuronidation of propranolol was observed in dog liver microsomes with (*S*)-propranolol being a non-competitive inhibitor of (*R*)-propranolol glucuronide formation [23]. The enantiomer/enantiomer interaction for propranolol oxidative metabolism in rat liver microsomes is reported for the first time. Racemic propranolol can be regarded as a combination of two drugs, (*S*)-propranolol and (*R*)-propranolol, that differ in kinetic property for P450-mediated metabolism (Table 3), and, therefore, possible drug/drug interactions arising from such a combination should be taken into account. Compared with the values for the individual enantiomers, low ring-hydroxylase activities for an equal concentration (5 μM) of the enantiomers included in racemic propranolol (10 μM, Fig. 1) suggested that ring-hydroxylations of each enantiomer were inhibited by the other enantiomer. Indeed, 4-, 5- and 7-hydroxylations of propranolol enantiomers were inhibited by their optical antipodes concentration dependently (Fig. 2).

Propranolol 4-, 5- and 7-hydroxylations were shown to be catalysed by a P450 isozyme(s) belonging to the CYP2D subfamily [24–27]. The propranolol ring-hydroxylations at a racemic propranolol concentration of 5 μM were almost completely inhibited by an antibody against P450BTL, a CYP2D subfamily isozyme [27], indicating the involvement of enzyme(s) with similar kinetic properties in the metabolism of propranolol enantiomers as two different substrates. Similar K_m values of propranolol hydroxylations for the individual enantiomers, and a pronounced difference in V_{max} make it likely that not the binding site of the enzyme for propranolol but the catalytic site was stereoselective, as pointed out in the study on stereoselective metabolism of bufuralol 1'-hydroxylation catalysed by CYP2D6 [28]. The competitive nature of the enantiomer/enantiomer interaction of the propranolol ring-hydroxylations (Fig. 3) suggests that both the enantiomers can compete for the same enzyme. Stereoselectivity and a competitive metabolic interaction between enantiomers were also reported for CYP2D6-dependent propafenone 5-hydroxylation on the basis of kinetic data in human liver microsomes and their mathematical modelling [29].

Stereoselectivity in the monooxygenation of propranolol was found to depend on the metabolic position of propranolol. Its major metabolic routes in the rat were aromatic 4- and 7-hydroxylations, and these pathways exhibited an opposite substrate stereoselectivity (Fig. 1). Similar metabolic rates between the enantiomers were obtained by the summation of four mean activities measured (0.669 and 0.769 nmol/min/mg protein for *R*- and *S*-enantiomer, respectively). Because P450-dependent monooxygenation was considered to be the major reaction in *in vivo* elimination of propranolol in the rat, the *in vitro* data obtained in the present study suggest that *in vivo* metabolism of propranolol in this species is not stereoselective. This agrees with the previous finding in the rat that the intrinsic clearance of propranolol does not differ significantly between the propranolol enantiomers [9, 10].

The pharmacokinetics of propranolol enantiomers were reported to be different in the absence and presence of the optical antipode [5, 8]. Ring-hydroxylations of the pharmacologically active enantiomer (*S*)-propranolol were inhibited by contaminant (*R*)-propranolol in the racemate. In other words, the interaction observed in this study suggests the usefulness of (*R*)-propranolol as an inhibitor of the first-pass metabolism of (*S*)-propranolol. If propranolol *S*-enantiomer is administered instead of its racemate, one must take into account an alteration in the pharmacokinetics of (*S*)-propranolol.

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