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Stereoselective propranolol metabolism in two drug induced rat hepatic microsomes

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Subject headings propranolol; enantiomers; rat hepatic micro some; phenobarbital; β-naphthoflavone

Abstract

AIM To study the influence of inducers BNF and PB on the stere oselective metabolism of propranolol in rat hepatic microsomes.

METHODS Phase I metabolism of propranolol was studied by using the microsomes induced by BNF and PB and the non-induced microsome as the control. The enzymatic kinetic parameters of propranolol enantiomers were calculated by regression analysis of Lineweaver-Burk plots. Propranolol concentrations we re assayed by HPLC.

RESULTS A RP-HPLC method was developed to determine propranol ol concentration in rat hepatic microsomes. The linearity equations for R(+)pr opranolol and S(-)propranolol were A = 705.7C+311.2C (R = 0.9987) and A = 697.2C+311.4C (R=0.9970) respectively. Recoveries of each enant iomer were 98.9%, 99.5%, 101.0% at 60 μmol/L, 120 μmol/L, 240 μmol/L respectively. At the concentration level of 120 μmol/L, propranolol enantiomers were metabolized at different rates in different microsomes. The concentration ratio R(+)/S(-) of control and PB induced microsomes increased with time, whereas that of microsome induced by BNF decreased. The assayed enzyme parameters were: 1. Km. Control group: R(+)30±8, S(-)18±5; BNF group: R(+) 34±3, S(-)39±7; PB group: R(+)38±17, S(-) 36±10. 2. Vmax. Control group: R(+)1.5±0.2, S(-)2.9±0.3; BNF group: R(+)3.8±0.3, S(-)3. 3±0.5 ; PB group: R(+)0.07±0.03, S(-)1.94±0. 07. 3. Clint. Control group: R(+)60 ±3, S(-) 170±30; BNF group: R(+)111.0±1, S(-) 84± 5; PB group: R(+)2.0±2, S (-)56.0±1. The enzyme parameters compared with unpaired *t* tests showed that no stereoselectivity was observed in enzymatic affinity of three microsomes to enantiomers and their catalytic abilities were quite different and had stereoselectivities. Compared with the control, microsome induced by BNF enhanced enzyme activity to propranolol R(+)enantiomer, and microsome induced by PB showed less enzyme activity to propranolol S(-)-enan tiomer which remains the same stereoselectivities as that of the control.

CONCLUSION Enzyme activity centers of the microsome were changed in composition and regioselectivity after the induction of BNF and PB, and the stereoselectivities of propranolol cytochrome P450 metabolism in rat hepatic microsomes were likely due to the stereoselectivities of the catalyzing function in enzyme. CYP-1A subfamily induced by BNF exhibited pronounced contribution to propranolol metabolism with stereoselectivity to R(+)-enantiomer. CYP-2B subfamily induced by PB exhibited moderate contribution to propranolol metabolism, but still had the stereoselectivity of S(-)-enantiomer.

INTRODUCTION

Propranolol is a nonselective β-adrenergic blocking agent and widely used in clinic as a racemic mixture of R(+) and S(-) enantiomers. It is extensively metabolized and only a small amount of the drug is excreted unchanged[1,2]. As a beta blocking agent, the optical isomers of propranolol exert different beta receptor blocking and membrane stabilizing effects[3], therefore its stereoselective metabolism is of clinical importance. Propranolol is metabolized into a number of products *in vivo*. These products arise from naphthalene-ring hydroxylation[1], N-dealkylation of the isopropanolamine side-chain and side-chain o-glucuronidation[4,5]. When the influence by the hepatic blood flow[6] and oxygen delivery[7] *in vivo* is not considered, the metabolism by monooxygenation is mainly responsible for propranolol elimination in hepatic microsomes and O-glucuronidation was shown to be a minor pathway *in vivo*[2] and *in vitro*[5].

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The oxidative metabolism of propranolol is catalyzed by cytochrome p-450. Exper iments by Otton SV *et al*[8] and Ishida R *et al*[9] indicated that multiple isozymes were involved in popanolol metabolism in rat liver microsomes. Nelson *et al*[10] have observed that stereoselectivity of pro pranolol metabolism in 9000 g liver supernatant differs depending on the positions of metabolism. Although the metabolic fate of propranolol in rat has been studied extensively, the impact of PB and BNF induction on stereoselective propranolol metabolism in rat hepatic microsome was rarely reported. This experiment studied the stereoselective metabolism of propranolol in rat hepatic micro somes induced by BNF and PB and the enzymatic parameters were compared with that of the control.

MATERIALS AND METHODS

Chemicals and solutions

R(+) and S(-)-propranolol (hydrochlor ide), β-naphthoflavone (BNF), phenobarbital (PB) NADP and NADPH were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Tris-hydroxymethyl aminomethane (Gibco BRL) and bovine serum albumin (Serva) were purchased from Shanghai Reagent Station. All other chemicals were obtained from the common commercial sources.

Tris-HCl buffer (0.1 mol/L, pH 7.4): 1.21 g of Tris-hydroxymeth yl aminomethane was dissolved in 60 mL of water. The solution was adjusted to pH 7.4 by concentrated hydrochloride acid and then diluted with water to the desired volume of 100 mL. This solution was used to prepare rat hepatic microsome.

Ammonium acetate buffer: 4.0 g of ammonium acetate was dissolved in 10 mL glacial acetic acid and then diluted with water to the desired volume of 1000 mL (pH 4.0). This solution was used to prepare mobile phase.

Preparation of hepatic microsomes

Sprague-Dawley rats (male, 160 g - 200 g) were divided into three groups. One group received i.p. injection of sodium PB dissolved in physiological saline (0.9% NaCl) (80 mg/kg·d) for 3 days, another group, BNF in cornoil (80 mg/kg·d) for 3 days and the last group received nothing as the non-treated control. About 24 h after the last treatment and with no food supplied for 16 h before taking the livers, the rats were sacrificed by decapitation. Liver samples were excised and perfused by the ice-cold physiological saline to remove blood and homogenized in ice-cold Tris-HCl buffer. Hepatic microsomes were prepared with the ultracentrifugation method described by Gibbson GG *et al*[11]. All manipulations were carried out in a cold bath. Pellets were re-suspended in sucrose-Tris buffer (pH 7.4) (95:5) and immediately stored at -30 ℃.

Protein concentrations of the microsomal preparations were measured by the method of Lowry *et al*[12] using arystalline bovine serum albumin as the protein standard.

Incubation of propranolol and rat hepatic microsomes

0.5 mL incubation mixture containing 1 mg/mL microsomal protein per milliliter (85 mmol/L Tris-HCl buffer (pH 7.4), 50 mmol/L nico tinamide, 15 mmol/L $MgCl_2$, 3 mg/mL DL-isocitric acid tri-sodium salt, 0.4 units/mL isocitric dehydrogenase) was used Phase I metabolism was performed with 0.5 mL of the mixture bubbled with oxygen for 1 min and R(+) or S(-)propranolol enantiomer as the substrate. After 5min pre-incubation under air at 37 ℃, reaction was started by adding 10 μL of NADPH regenerating system (10 mg NADP and 3 mg NADPH in 100 μL of 1% $NaHCO_3$). The reaction was stopped after the indicated time by adding 0.5 mL of methanol and centrifuged at 4000 r/min for 10 min. 10 μL of the supernatant was sampled into HPLC.

HPLC procedure for propranolol determination in rat hepatic microsomes

A HPLC procedure was established to assay propranolol enantiomers in rat hepatic microsomes. After the termination of the reaction with methanol, 10 μL of the sample was applied to a reversed phase column (Shim-pack CLC-ODS 15 cm×0.6 cm id, 10 μm particle size). Propranolol was monitored with a UV detector at 290 nm. The mobile phase was made up with ammonium acetate buffer (pH 4.0)-methanol (50:50). The flow rate was 1.0 mL/min. Figure 1 shows the typical elution of propranolol in incubation solution.

Statistical analysis of the data

The maximum velocity (Vmax) and the Michaelis-Menten constant (Km) values for propranolol were determined by regression analysis of Lineweaver-Burk plots. The $\bar{x}\pm s$ of three determinations of Vmax and Km was calculated for each substrate and metabolic reaction. Intrinsic clearance was calculated by the ratio of Vmax/Km. The statistical difference between propranolol enantiomers was tested using an unpaired *t* test.

RESULTS

Validation of HPLC

Linearity Drug-free microsomes were spiked with increasing concentrations of propranolol enantiomers (10 μmol/L - 620 μmol/L). The solution was constituted according to “Incubation of propranolol with rat hepatic microsomes” with no occurrence of metabolism reaction. Propranolol enantiomers were assayed by HPLC preciously described. Standard calibration curves were

constructed by performing a linear regression analysis of the peak area (Y) of propranolol enantiomers versus their concentrations (X), i.e., R(+)propranolol: Y = 705.7+311.2X, r = 0.9987; S(-)propranolol: Y = 697.2+311.4X, r = 0.9970. The limit of detection (single-to-noise ratio = 3) for propranolol was 3 μmol/L.

Precision and accuracy The spiked drug-free microsomes at 3 concentration levels (60 μmol/L, 120 μmol/L and 240 μmol/L) were assayed following the procedure of 2.1.1. Results were listed in Table 1.

Table 1 Accuracy and precision to assay propranolol in rat liver microsome

Target concentrations (μmol/L)	Recovery (%)	Precisions (RSD, %)	
		Intra-assay (n = 3)	Inter-assay (n = 3)
60	98.8	5.1	5.6
120	99.5	3.5	4.8
240	101.0	3.2	5.3

Concentration-time curves and variation of the ratio of R(+)/S(-) propranolol concentration in microsomes after incubation of different time Phase I metabolism was performed with 0.5 mL of the mixture and 60 μmol of propranolol enantiomers as the substrate. The incubation procedure was carried out according to 1.3. and 1 mL of methanol was added to stop the reaction at 0, 40, 80, 160, 320 min respectively. The mixtures were then analyzed by HPLC. Results are shown in Figure 2 and Table 2.

Table 2 Ratio of R(+)/S(-) propranolol concentration in incubation m edia at different incubation time

Group	Ratio of R(+)/S(-) propranolol					
	0	5	10	15	20	30 (min)
Control	0.989	9.99±0.07	1.01±0.10	1.02±0.02	1.04±0.04	1.07±0.02
BNF	0.989	0.94±0.05	0.93±0.06	0.93±0.04[a]	0.95±0.05[c]	0.91±0.05[bc]
PB	0.989	1.05±0.06	1.04±0.08	1.05±0.10	1.09±0.05	1.09±0.06

Values were obtained from propranolol concentration at 120 μmol/L for each enantiomer, BNF and PB: microsomes from the rats induced with BNF (β-naphthoflavone) or (phenobarbital) 80 mg/(kg·d), ip, 3 d, respectively. $\bar{x}$±s, n = 3. [a]P<0.05, [b]P<0.01, compared with control; [c]P<0.05, compared with PB by unpaired t test.

It was indicated that at the propranolol concentration level of 120 μmol/L, propranolol enantiomers were metabolized in different rate in different microsomes. The ratio of R(+)/S(-) propranolol concentration in incubation media in control and PB group increased, whereas that in BNF group dec reased. The ratio of R(+)/S(-) propranolol concentration in BNF group was signi ficantly different with the corresponding ratio in control group or PB group at 15, 20 and 30 min (P<0.05, 0.01).

Enzymatic kinetic parameters for propranolol metabolism in liver microsomes from control, BNF and PB induced rats The enzymatic kinetic parame ters of propranolol enantiomers were calculated by Lineweaver-Burk method with the substrate concentrations of 20 μmol/L-600 μmol/L in three forms of rat hepatic microsomes after 10 min incubation (1.3).The results were listed in Table 3.

Table 3 Enzymatic kinetic parameters in propranolol enantiomer metab olism *in vitro* in rat hepatic microsomes induced by β-naphthoflavone or ph enobarbital

Group	Enantiomer	Km μmol/L	V_{max} mmol/g/min	Clint L/min/g protein	R(+)Vmax: S(-)Vmax
Control	R(+)	30±8	1.5±0.2[b]	60±3[b]	0.5
	S(-)	18±5	2.9±0.3	170±30	
BNF	R(+)	34±3	3.8±0.3[fh]	111.0±1[afh]	1.14
	S(-)	39±7[d]	3.3±0.5[g]	84±5[eh]	
PB	R(+)	38±17	0.07±0.03[ef]	2.0±2[cf]	0.038
	S(-)	36±10[d]	1.94±0.07[e]	56.0±1[e]	

Clint (intrinsic clearance) is the ratio of V_{max}/Km, mean± s, n = 3. [a]P<0.05, [b]P<0.01, [c]P<0.001, compared with S(-) propranolol; [d]P<0.05, [e]P<0.01, [f]P<0.001, compared with corresponding enantiomer in control group; [g]P<0.01, [h]P<0.001, compared with corresponding enantiome r in PB group with unpaired t test.

Km of propranolol enantiomers in control group had no stereoselectivity (P>0.05), whereas Vmax and Clint had stereoselectivity of S (-)-propranolol (P<0.01). For BNF induced microsome, Km and Vmax had no stereoselectivity between R (+), S(-)-propranolol (P>0.05), and Clint had significant differenc e between the two enantiomers (P<0.05). For PB group, Km had no stereosele ctivity (P>0.05), and Vmax, Clint had stereoselectivity of S(-)-proprano lol (P<0.001).

Comparing the enzymatic parameters of R(+)-propranolol among three microsomes, Km had no statistical difference (P>0.05), whereas Vmax and Clint had stat istical differences (P<0.05, 0.01 or 0.001); compared with the control group, Vmax for BNF group increased 2.5 times and that for PB group decreased 20 times; clint for BNF and PB group increased or decreased 1.8 and 30 times, respectively. With the same way to compare those parameters of S(-)-propranolol, Kms for BNF and PB group increased 2.2 and 2.1 times, respectively, but had n o statistical difference with each other; Vmax for PB group decreased about 1.5 times and that for BNF group nearly remained the same, in addition, no statisti cal difference was found between PB and BNF group; Clint for BNF and PB group decreased 2 times and 1.5 times respectively and there was significant difference between BNF and PB group.

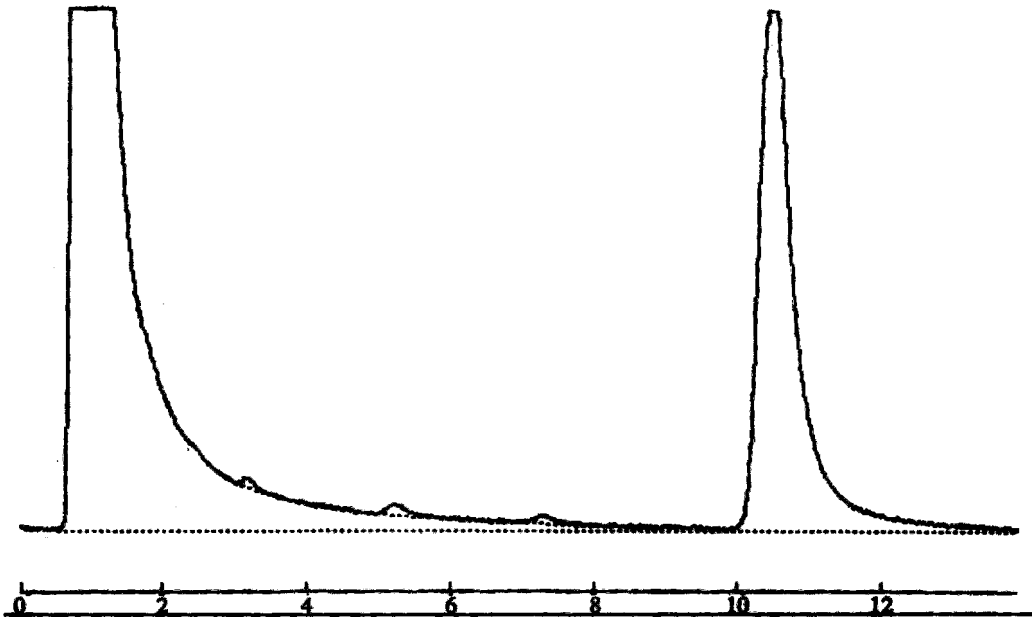

Figure 1 Chromatograms of propranolol after incubati on with rat hepatic microsome. A Shim-pack CLC-ODS column (15cm×0.6cm i.d.) was used. The mobile phase was constituted with ammonium acetate buffer (pH4.0)-methanol (50:50) with flow rate at 1.0mL / min. Propranolol was monitored at 290nm. Propranolol: ^{t}R=10.1min.

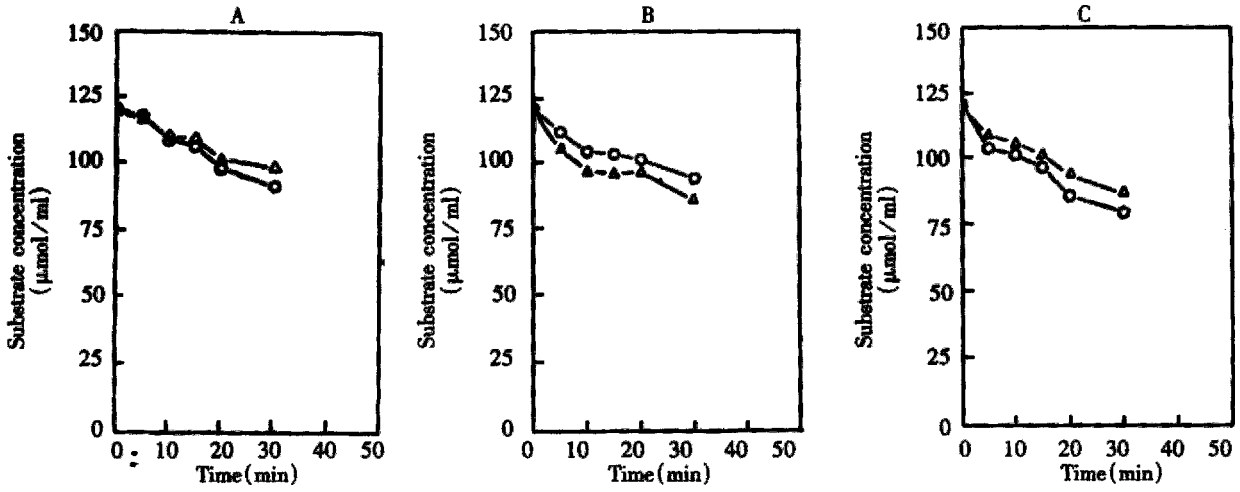

Figure 2 Concentration time curves for R(+) and S(-)-propranolol metabolism in rat hepatic microsomes.
A. Microsome of control. B. Microsome induced by BNF. C. Microsome induced by PB. △—△: R(+)-propranolol. ○—○: S(-)-propranolol.

DISCUSSION

In this *in vitro* study, stereoselectivity of propranolol occurred in catalyzing velocity and intrinsic clearance in control group, and no stereoselectivity was observed in enzyme affinity to the substrate. The introduction of BNF and PB caused changes in the composition of CYP subfamilies and therefore influenced the stereoselective catalyzing ability of microsome to propranolol metabolism, or even reversed the sequence of stereoselectivity, whereas the affinity of enzyme to substrate remained nearly the same and had no stereoselectivity. This phenomenon indicated that, regio-structure of binding site in the activity center of enzyme was almost unchanged, and that of the catalyzing site was significantly changed in propranolol metabolism in rat hepatic microsomes after the introduction of PB and BNF, the influence of BNF and PB induction had reversed effect on the catalyzing stereoselectivity of microsome to propranolol.

BNF is an inducer of CYP-1A subfamily[13-15] and PB is that of CYP - 3A[15], *CYP-2B* subfamily[16,17] (IIB1 and IIB2[18]). Different kinds of cytochrome P-450 may be involved in propranolol metabolism, depending on the metabolic positions[10]. CYP-1A is suggested to catalyze 4, 5-

hydroxylation and *N-desisopropylation* stereoselectively[19,20]. CYP-1A2 accounts for about 10% to 15% of the total CYP content of human liver and is the major enzyme involved in the metabolism of propranolol[21]. Another subfamily CYP-2D6 mainly catalyzes 4, 5 and 7-hydro xylation stereoselectively[22,23] and it has been confirmed that CYP- 2D6 does not contribute to N-desisopropylation of propranolol[8]. N- desisopropylation in propranolol enantiomer metabolism is mainly mediated by CYP-1A2[24,25]. Masubuchi Y *et al*[26] reported that there is competition between enantiomers of propranolol for the enzyme, probably the same enzyme, a cytochrome P450 isozyme in the CYP-2D subfamily. All of these showed that different cytochrome subfamilies have different functions in metabolism of propranolol enantiomers and the optical isomers of propranolol have different stereoselectivities in metabolism. Our results indicated that CYP-1A was involved in propranolol metabolism and showed the stereoselectivity of R(+)-enantiomer in general. CYP-3A, CYP-2B subfamily does not play a main role in propranolol metabolism *in vitro*, though it showed the stereoselectivity of S(-)-enanti omer.

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