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Stereoselectivity and interaction between the glucuronidation of S-(−)- and R-(+)-propranolol in rat hepatic microsomes pretreated with different inducers

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Phase II glucuronidation metabolism of side-chain propranolol was studied using microsomes from rats treated with the inducers β-naphthoflavone (BNF) or dexamethasone (Dex). The glucuronide concentrations of propranolol enantiomers were assayed by RP-HPLC. The kinetic constants of glucuronidation, K_m, V_{max} and Cl_{int} were determined. There are significant differences between the R- and S-enantiomeric glucuronide in K_m, V_{max} and Cl_{int} $P < 0.05$, $P < 0.01$ and $P < 0.05$ in control microsome.There are significant differences in K_m and Cl_{int} ($P < 0.01$ or $P < 0.001$) but no significant differences in V_{max} ($P > 0.05$) between R and S-enantiomeric glucuronide in the microsomes induced with Dex and BNF. The formation of S-(−)-propranolol glucuronide was inhibited by R-(+)-propranolol from the rat microsomes pretreated with BNF and Dex. The glucuronidation metabolism of propranolol enantiomers exhibited the stereoselectivity in rat hepatic microsomes induced with BNF or Dex. Multiple UGT1A and 2B may be involved in stereoselective O-glucuronidation of propranolol enantiomers in rat liver microsomes. The glucuronides produced were in favor of the R-enantiomer. There is an interaction between the glucuronidation of R- and S-enantiomer.

1. Indroduction

Propranolol [1-isopropylamino-3-(1-naphthyloxy)-2-propanol], available commercially as the racemic mixture, is a nonselective β-adrenergic blocking agent used in the treatment of hypertension, angina pectoris and cardic arrhythmias. Since (−)-propranolol is about 100 times more potent as a β-blocker than its optical antipode, significant differences in their disposition may be clinically important. The primary metabolic pathways of propranolol are glucuronidation, side-chain oxidation and ring oxidation. These products arise from naphthalenering hydroxylation, N-dealkylation of the isopropanolamine side-chain and side-chain O-glucuronidation. The stereoselectivity of propranolol glucuronidation in dogs and humans (Silber et al. 1982; Wilson et al. 1984) was published but in microsomes from rat treated with the inducers data is scarce (Thompson et al. 1981). The uridine diphosphate (UDP)-glucuronosyltransferases (UGT) are a family of enzymes that catalyze the covalent addition of glucuronic acid to a wide range of lipophilic chemicals, with the formation of a β-D-glucuronide product. UGTs play a major role in the detoxification of many exogenous and endogenous compounds. This study investigated the stereoselective propranolol side-chain glucuronidation and the interaction between R-(+)- and S-(−)-propranolol in rat hepatic microsomes pretreated with β-naphthoflavone (BNF) or Dexamethasone (Dex).

2. Investigations and results

2.1. Time-courses for propranolol glucuronidation in the hepatic microsomes

For the time dependent experiments, 75 μmol/L of racemic propranolol was incubated for 5, 10, 15, 30, 60 min. Then the studies were carried out according to Chapter 4.4. A stereoselective glucuronidation was observed. The R(+)-isomer glucuronide was formed faster and to a higher extent than the S(−)-isomer (Fig. 1). The R/S ratios of

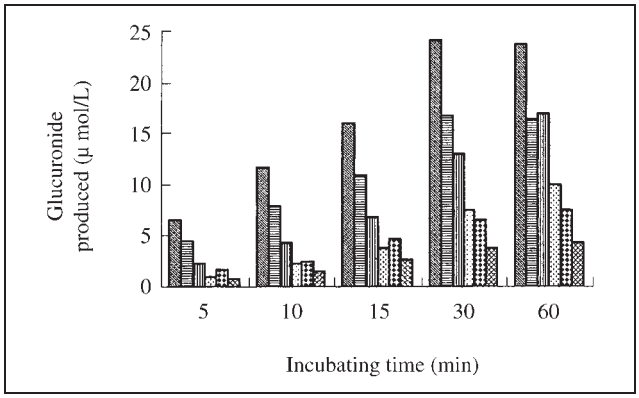

Fig. 1: Time-course for incubating racemate propranolol
Dex/R-PL-Glu
Dex/S-PL-Glu
BNF/R-PL-Glu
BNF/S-PL-Glu
Contrs/R-PL-Glu
Contrs/S-PL-Glu

Table: Enzyme kinetic parameters in glucuronidation of propranolol in rat hepatic microsomes ($x \pm s$, $n = 3$)

Group	Enantiomer	K_m ($\mu mol \cdot L^{-}1$)	V_{max} ($\mu mol \cdot g^{-1} \cdot min^{-1}$)	$Cl_{int}*10^{-4}$ ($L \cdot min^{-1} \cdot g^{-1}$ protein)
Control	R(+)	$1639 \pm 147^{*}$	$0.609 \pm 0.028^{**}$	$3.7 \pm 0.1^{*}$
	S(−)	1127 ± 140	0.368 ± 0.036	3.3 ± 0.1
BNF	R(+)	$1922 \pm 125^{**\triangle\triangle\triangle}$	$1.305 \pm 0.105^{\#\#\#\triangle\triangle}$	$6.8 \pm 0.1^{***\#\#\#\triangle\triangle\triangle}$
	S(−)	$2801 \pm 240^{\#\#\#\triangle\triangle}$	$1.494 \pm 0.123^{\#\#\#\triangle}$	$5.3 \pm 0.2^{\#\#\#\triangle\triangle\triangle}$
Dex	R(+)	$831.4 \pm 42.9^{***\#\#\#}$	$0.817 \pm 0.051^{\#\#}$	$9.8 \pm 0.1^{***\#\#\#}$
	S(−)	1167 ± 26.7	$0.932 \pm 0.004^{\#\#}$	$8.0 \pm 0.2^{\#\#\#}$

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with S(−)-propranolol, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$, compared with control group with unpaired T-test $^{\triangle}$ $P < 0.05$, $^{\triangle\triangle}$ $P<0.01$, $^{\triangle\triangle\triangle}$ $P<0.001$, compared with dexamethasone group with unpaired T-test

propranolol glucuronide were almost constant for all the three groups of microsomes. The amount of glucuronides produced decreased in the following order: Dex group > BNF group > control.

2.2. Enzyme kinetics for propranolol glucuronidation in the hepatic microsomes

Racemic propranolol was added to the incubation media as a substrate at concentrations from 28.13 μmol/L to 4780 μmol/L for each enantiomer and the samples were incubated for 15 min. Then the experiments were carried out according to Chapter 4.4. The data showed that the formation of glucuronide was favored for the R-(+)-propranolol enantiomer when racemic propranolol was used as a substrate and production of the glucuronide was reduced for both enantiomers at high substrate concentrations (Fig. 2). The enzyme kinetic parameters of propranolol enantiomer glucuronides are listed in the Table 1.

There are significant differences between R and S-enantiomeric glucuronides regarding K_m, V_{max} and Cl_{int} ($P < 0.05$, $P < 0.01$ and $P < 0.05$) in control microsomes. There are significant differences in K_m and Cl_{int} ($P < 0.01$ or $P < 0.001$) but no significant differences in V_{max} ($P > 0.05$) between R and S-enantiomer glucuronides in the microsomes induced with BNF or Dex. And the glucuronidation metabolism of RS-propranolol is favored for R-(+)-propranolol.

2.3. Inhibition of S-(−)-propranolol glucuronide produced in rat microsome by R-(+)-propranolol

246.1, 492.2, 1195, 2390 μmol/L of R-(+)-propranolol were incubated with S-(−)-propranolol. The concentration of S-(−)-propranolol was set from 87.9 to 2390 μmol/L. The inhibition experiments were carried out according to Chapter 4.4 and incubating time was 15 min. The results show that the formation of S-(−)-propranolol glucuronide was inhibited by R-(+)-propranolol and the inhibitory constant (K_i) is 171.2 μmol/L and 421.3 μmol/L, respectively, from the rat microsomes pretreated with BNF or Dex.

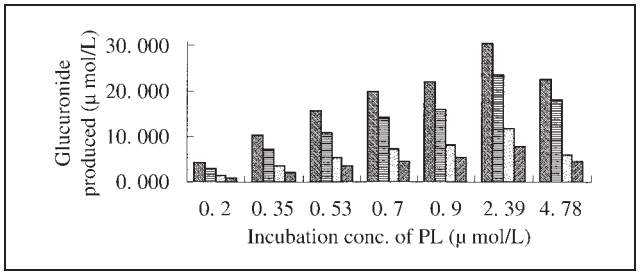

Fig. 2: Concentration-course for incubating racemate propranolol for 15 min
Dex/R-PL-Glu
Dex/S-PL-Glu
BNF/R-PL-Glu
BNF/S-PL-Glu

3. Discussion

3.1. Inducible UGT isozymes and stereoselectivity

Uridine diphosphate glucuronosyltransferase (UGT) is localized to the endoplasmic reticulum and spans the membrane with a type I topology. UGTs structures of functional sites may be changed by induction with different inducers. Considerable variation in xenobiotic conjugation is observed as a result of altered expression of UGTs. Accordingly, the induction of UGT is highly dependent on the nature of inducers under consideration. For our study, the UGT catalyzing ability was enhanced but selectivity was decreased and the enzyme affinity to S-(−)-and R-(+)-enantiomer was reversed comparing the microsomes pretreated with BNF or Dex with control. The results from the kinetic and time-dependent experiments show that the activity of UGT in rat hepatic microsomes is inducible and the activity in microsomes induced with Dex and BNF is more active than that from control. The contents of propranolol glucuronides produced after incubating with the microsome pretreated with Dex for 5-60 min were 3.1–4.6-fold and 3.8–5.9-fold more than that of control and 1.4–3.0-fold and 1.6–4.5-fold more than that of the microsome pretreated with BNF for R- and S-enantiomer glucuronides, respectively (Fig. 1).

Glucuronidation is a phase II metabolic reaction catalyzed by large families of different isoenzymes. At this time, over 35 different UGT gene products have been described from several different species. UGTs have been divided into two distinct subfamilies based on sequence identities, UGT1 and UGT2. It was reported that most UGT1A isoforms and UGT2B were up-regulated by dexamethasone, but to different degrees (Jemnitz et al. 2000, 2002; Li et al. 1999; Strasser et al. 1998). Induction of UGT enzyme with BNF results in the induction of a form of the transferase that has a specificity for 3-hydroxy benzo[a]pyrene likely as substrate and rat liver microsomal UGT activity is induced by treatment with BNF, an effect correlating with the induction of UGT 1A6 and 1A7 (Auyeung et al. 2001; Kessler et al. 2002; Metz et al. 1998; Saarikoski et al. 1998). Therefore, the induction of non-cytochrome P450 enzymes responsible for drug metabolism particularly the UGT impose another level of control on the overall metabolic fate of a drug. The UGT subfamily contributed pronouncedly to propranolol glucuronidation exhibiting stereoselectivity depending on substrate concentration. During glucuronidation of propranolol enantiomers in rat hepatic microsome stereoselectivity occurs and may involve UGT 1A and 2B although the percentage contri-

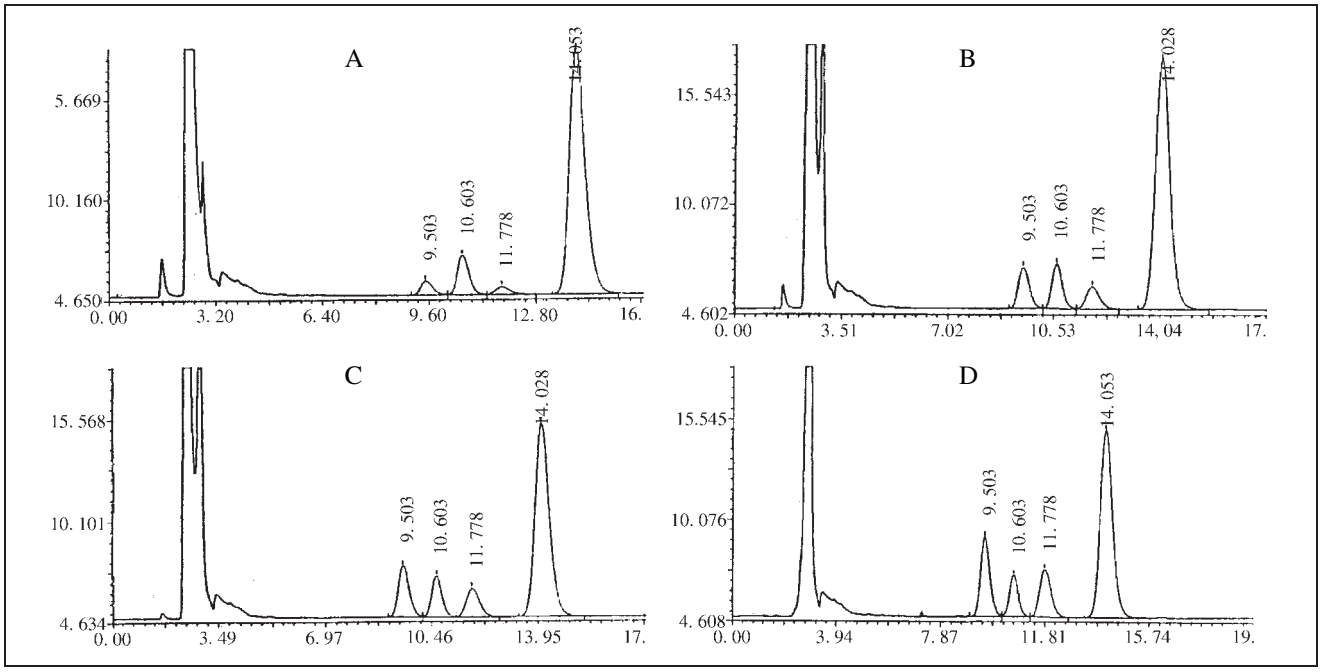

Fig. 3: Chromatograms of propranolol glucuronides after propranolol incubated for indicated time. A: 3 min; B: 5 min; C: 10 min; D: 20 min. 9.5 min: R(+)-propranolol glucuronide 10.6 min: p-nitrobenzonic acid (internal standard); 11.8 min: S(−)-propranolol glucuronide; 14.0 min: RS-propranolol

bution of each UGT1A and 2B could not be estimated. The glucuronide produced was in favor of the R-enantiomer.

3.2. Interaction between R- and S-propranolol

The enantiomers of a racemic drug may differ in metabolic behavior as a consequence of stereoselective interaction with hepatic microsomes. The results from the kinetic and time-dependent experiments in this study show that the glucuronidation of propranolol enantiomers in rat hepatic microsomes occurs stereoselectively because of the different enzyme affinities for the enantiomers of the drug (Table). (R)-(+)-Propranolol was glucuronidated 1.4-fold and 1.7-fold faster than the (S)-(−) enantiomer at 175.8 μmol/L substrate concentrations, and 1.2-fold and 1.1-fold faster at 3.585 mmol/L substrate concentrations in rat liver microsomes induced with BNF or Dex, respectively. So the formation of glucuronide favored R-(+)-propranolol enantiomer with concentration dependency. Glucuronidation of R-(+)- or S-(−)-propranolol was inhibited by itself at higher concentration of substrates. The ratio of R-(+)- to S-(−)-propranolol glucuronide decreased with increasing concentration of substrates. The molecular base for this phenomenon was the stereoselectivity in affinity of the substrate to the enzyme activity centers instead of the catalyzing sites.

When the R-antipode was co-incubated with S-propranolol, the metabolic formation of S-(−)-propranolol glucuronide was inhibited by R-(+)-propranolol with an inhibitory constant (Ki) of 171.2 μmol/L and 421.3 μmol/L, respectively, from the rat microsomes pretreated with BNF or Dex. So the S-(−)-propranolol glucuronidation was inhibited by R-(+)-propranolol.

3.3. Species differences in enantioselectivity for propranolol glucuronidation

Silber et al. (1982) reported the ratio of V_{max}/K_m for the glucuronide conjugate of S-(−)-propranolol was 2.1- to 4.9-fold greater than for the conjugate of the R-(+)-enantiomer in human. Our experiments show that the ratio of V_{max}/K_m for the glucuronidation of R(+)-propranolol was from 1.2-fold and 1.3-fold greater than for the glucuronidation of the S(−)-enantiomer in rat liver microsomes induced with BNF or Dex, respectively. Narimatsu et al. (2000) found that cytochrome P450 2D enzymes cause reversed enantioselectivity of the oxidation of propranolol between human and monkey liver microsomes. These results indicate that there are species differences in enantioselectivity for propranolol metabolism by UGT and CYP.

In conclusion, UGT1A and 2B are involved in stereoselective O-glucuronidation of propranolol enantiomers in rat liver microsomes and the glucuronides produced were in favor of the R-enantiomer. There is an interaction between R- and S-propranolol in stereoselective propranolol glucuronidation.

4. Experimental

4.1. Chemicals and solutions

Dexamethasone(Dex), β-naphthoflavone(BNF), NADPH, Triton X-100, BSA, Uridine 5-Diphosphoglucuronic Acid (UDPGA), Tris [hydroxy methyl] aminomethane (Tris), β-glucuronidase, p-nitrobenzonic acid, (R,S)-propranolol, R(+) and S(−)-propranolol were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Propranolol glucuronide was biosynthesized according to Luan et al. (2001). All other chemicals were obtained from the common commercial sources.

4.2. Preparation of hepatic microsomes

Sprague-Dawley rats (male, 190 ~ 210 g) were divided into three groups. One group received three daily intraperitoned injections of 80 mg/kg BNF (dissolved in oil); the second group received Dex (132 mg/kg.d, ig.) three times daily and the third group was used 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 ice-cold physiological saline to remove blood and homogenized in ice-cold Tris buffer. Hepatic microsomes were prepared with the ultracentrifugation method described by Gibbson et al. (1994). All manipulations were carried out in cold bath. Pellets were resuspended in sucrose-Tris buffer (pH 7.4) (95 : 5,v/v) and immediately stored at −80 °C. Protein concentration of the microsomal preparations were measured by the method of Lowry et al. (1951) using BSA as standard.

4.3. HPLC Analysis

R- and S-propranolol enantiomers glucuronides were quantified using an HPLC system composed of a LC-10AT VP with SPD-10A VP (Shimadzu, Japan). UV detection wavelength was set at 290 nm. A 5-μm reverse phase column (C_{18} 250 cm × 4.6 mm) was used. The mobile phase was a mixture of 67 mmol/L KH_2PO_4 buffer-methanol (55 : 45, v/v, pH 3.5) with a flow rate of 1.0 mL/min. Column temperature was set at 35 °C and the injection volume was 20 μL.

4.4. Incubation of rat hepatic microsomes with propranolol

The microsomal preparations were suspended in phosphate buffer (pH 7.8) to obtaine a suspension of microsomes (2 mg protein/ml) as the incubation media which contained 0.04% Triton X-100, 50 mmol/L Tris-HCl buffer, 5mmol/L $MgCl_2$ and racemic propranolol as substrate. The total volume of incubation solution was 0.5 ml. Samples of each concentration were prepared in duplicate. After 5 min preincubation at 37 °C, the glucuronidation reaction was started by addition of UDPGA (final concentration 3 mmol/L) then the samples were incubated for the indicated time at 37 °C in a shaking water bath, the reaction was stopped by addition of 1.0 ml of 2 mol/L TCA. Then p-nitrobenzoic acid was added as an internal standard. The pH of the mixture were adjusted to about 7 using 2 mol/L sodium hydroxide then centrifuged at 8000 r/min for 10 min to remove proteins. The aliquot of 20 μl supernatant was injected into the HPLC system. Analytical procedure followed Luan et al. (2001) (Fig. 3).

4.5. Statistical analysis of the data

The maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) values for propranolol enantiomers were determined by regression analysis of Eadie-Hofstee plots. The $\bar{x} \pm s$ values of three determinations of V_{max} and K_m were calculated for each substrate and metabolic reaction. Intrinsic clearance was calculated by the ratio of V_{max}/K_m. All statistical differences were tested by the unpaired t test.

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