Stereoselective Glucuronidation of Propafenone and Its Analogues by Human Recombinant UGT1A9

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Stereoselective glucuronidation of propafenone and its β-blocker analogues by human recombinant UGT1A3 and UGT1A9 from the recombinant baculovirus in insect sf9 cells was studied. The glucuronides produced in incubation mixtures were assayed by HPLC equipped with UV detector, and identified by β-glucuronidase. The stereoselective glucuronidation was measured by pre-column 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocynate (GITC) derivatization HPLC method for propafenone and esomolol. In all of ten β-blocker drugs studied, six showed the glucuronidation activity with UGT1A9, while four with UGT1A3. From roughly quantitative stereoselective glucuronidation study of racemic β-blocker analogues by UGT1A9, propranolol had a high ratio of the ratios of *S*- to *R*-isomer glucuronide (*S*-G/*R*-G), about 4.3, the ratios of terbutaline, atenolol and esomolol were 3.3, 3.1 and 2.8 respectively, sotalol and propafenone were 2.3 and 2.0. In a word, *S*-isomers of these drugs were glucuronidated by human UGT1A9 much faster than their antipodes.

Key words propafenone; β-blocker analogue; UGT1A9; glucuronidation; stereoselectivity

Most of chiral drugs are used as their racemic mixtures in clinical practice. Two enantiomers of a chiral drug generally differ in pharmacokinetic properties. Stereoselective metabolism of drugs is most commonly the major factor to stereoselectivity in pharmacokinetics. Propafenone (PPF, Fig. 1) is a chiral antiarrhythmic drug used clinically as a racemic mixture.[1] The stereoselective disposition of racemic propafenone has been reported in humans.[2] Although both enantiomers are equally potent in their activity as sodium channel blockers, the *S*-enantiomer exhibits β-blocking activity approximately 100 times higher than *R*-enantiomer.[3] The main metabolic pathways of propafenone *in vivo* and *in vitro* involve cytochrome P450 (CYP)1A2 and CYP3A4 mediated *N*-desalkylation, CYP2D6 mediated 5-hydroxylation and uridine 5′-diphosphate glucuronosyltransferases (UGTs) mediated glucuronidation.[4—7] Glucuronidation involves the transfer of the uridine 5-diphosphoglucuronic acid (UDPGA) to endogenous or exogenous substrate molecules containing a suitable group, the conjugates formed are more hydrophilic than the parent aglycones, so that they are more readily excreted from the body through urine or bile.

For stereoselective metabolism mediated by CYP450s, it has been reported that propafenone had the stereoselectivity of *S*-isomer in metabolism *via* CYP1A2 and CYP3A4 at high concentrations, while the opposite situation was observed at low concentrations.[4] *S*-Enantiomer had faster metabolite formation than *R*-enantiomer with CYP2D6.[8] Others reported

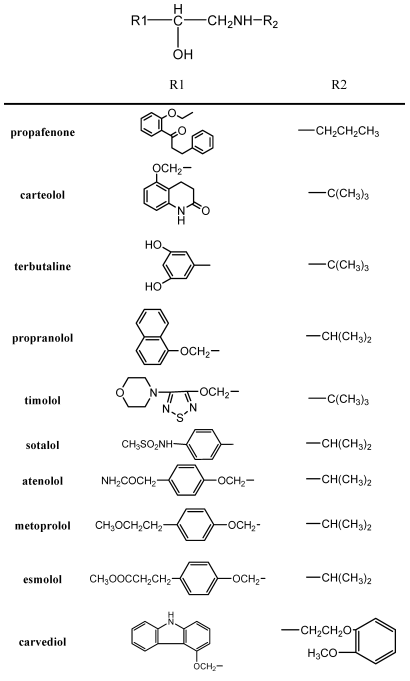

Fig. 1. The Chemical Structures of Propafenone and β-Blocker Analogues

the ratio of intrinsic clearance (Cl_{int}) for the glucuronide conjugate of *R*-propafenone was 1.20±0.12 fold greater than for the conjugate of the *S*-enantiomer in healthy Chinese volunteers.[9,10] And Hofmann *et al.* also studied the stereoselective glucuronidation of *S*- and *R*-propafenone in human plasma after oral administration, the concentration of *R*-PPFG was slightly greater than *S*-PPFG.[11] The related data of stereoselective glucuronidation used by recombinant human UGT proteins is few.

In vitro, the stereoselectivity glucuronidation of propafenone was evaluated *via* human UGT1A3 and UGT1A9 expressed with the recombinant baculovirus in sf9 cells.[12] One of the most important metabolism pathways of propafenone analogues, such as propranolol, carvedilol, is glucuronidation mediated by UGTs, especially UGT1A9.[13,14] However, there are not sufficient reports about the stereoselective glucuronidation of chiral drugs by recombinant UGTs. In this study, human UGT1A3 and UGT1A9 are used to screen ten β-blocker analogues glucuronidation. Chemical structures of these drugs are shown in Fig. 1.

Experimental

Chemicals and Solutions Alamethicin, UDPGA, β-glucuronidase, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocynate (GITC), *R*- and *S*-propafenone, propafenone, esomolol, atenolol, propranolol, carvedilol, sotalol, metoprolol, bisoprolol, terbutalin and timolol were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bac-to-Bac baculovirus expression system and *Spodoptera frugiperda* (Sf9) insect cells were supplied by Invitrogen™. All other chemicals were obtained from the common commercial sources.

Preparation of the Human UGTs Which Were Expressed with the Recombinant Baculovirus in sf9 Cells Sf9 insect cells, were cultured at 27 °C in Grace's medium containing 10% fetal bovine serum, 100000 U/l penicillin and 100000 U/l streptomycin. The recombinant baculovirus (Bacmid-UGT1A3 and Bacmid-UGT1A9) were amplified from the monolayer cultures of Sf9, transfected of Sf9 cells at 27 °C in Grace's medium, incubated for 72 h, and then harvested the cells. Cells were disrupted by a standard optimized sonication method.[15] The concentration of protein was

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determined by the Lowry's method.[16)]

Incubation of Propafenone and Its Analogues with UGT Enzymes Enantiomeric, racemic propafenone or other β-blocker analogues were added as substrates into 100 μl of the incubation solution with 100 μM concentrations, respectively, which contained 100 mmol/l Tris–HCl (pH 7.81), 10 mmol/l $MgCl_2$, 10 mmol/l alamethicin, and 1.0 mmol/l UGT1A3 or 1.0 mg/ml UGT1A9. After preincubation at 37 °C water bath for 5 min, 5 μl 5.0 mmol/l UDPGA was added as a cofactor to start the reaction. Blank controls without UDPGA were incubated at the same time. Enzymatic reaction was stopped with an equal volume of cold methanol at the designated time. Then the incubation solution was centrifuged at 10000 rpm for 10 min, a 20 μl aliquot of the supernatant was analyzed by HPLC system. β-Glucuronidase was used to identify glucuronides. For this purpose, an aliquot of glucuronidation incubates was heated at 100 °C for 5 min, centrifuged at 10000 rpm for 10 min and divided into two parts. One part was treated with 0.2 ml of KH_2PO_4 buffer (0.1 mol/l, pH 5.0) containing 200 U of β-glucuronidase for 2 h at 37 °C. The other part containing no β-glucuronidase was treated in parallel. After incubation, both parts were centrifuged at 10000 rpm for 10 min. An aliquot of 20 μl of each supernatant was analyzed by HPLC as described below. Once we confirmed the metabolite peaks in chromatograms and the glucuronidation activity, the enzymatic kinetic studies were performed using the same batch of stable expressed human UGT1A9.

Extraction and Derivatization of Propafenone and Esomolol[17,18)] Each recamic propafenone and esomolol incubation sample (100 μl) was immediately mixed with 100 μl of CH_3CH_2Cl. The mixture was vortexed for 5 min and then centrifuged at 10000 rpm for 10 min. The organic layer was transferred to another tube and evaporated to dryness under a gentle stream of N_2. Fifty microliters of GITC (1.02 mg/ml in acetonitrile) and 5 μl of triethylamine (TEA) (1.25% in acetonitrile) were added to the residue. The reaction was made for 45 min at 35 °C. After chiral derivatization was completed, the reaction mixture was evaporated to dryness under a gentle N_2 stream, and the residue was reconstituted with 100 μl of mobile phase. Finally, an aliquot of 20 μl of the resulting solution was injected into the HPLC system. Other β-blocker analogues were not derivatizated with GITC for the *R*- and *S*-conjugates of these compounds were clearly separated on HPLC chromatogram while propafenone and esomolol conjugates were not.

HPLC Analysis Incubation mixtures were quantified by using an HPLC system composed of a LC-10AT *VP* with SPD-10AT *VP* (Shimadzu, Japan). A 5 μm reverse phase column Diamonsil^{TM} C_{18} 250 mm×4.6 mm was used as analytical column. The mobile phases and UV detection wavelength for different drugs were respectively: 1, Methanol–buffer (10 mM KH_2PO_4, pH 2.5 solution) (50 : 50) and 254 nm for propafenone; methanol–H_2O–CH_3COOH (70 : 30 : 0.05) and 254 nm for propafenone GITC derivative. 2, Methanol–buffer (30 : 70) and 280 nm for esomolol; methanol–H_2O–CH_3COOH (70 : 30 : 0.05) and 248 nm for esomolol GITC derivative. 3, Methanol–buffer (10 : 90) and 275 nm for atenolol, methanol–buffer (50 : 50) and 275 nm for carvedilol, methanol–buffer (10 : 90) and 228 nm for sotalol, methanol–buffer (30 : 70) and 297 nm for timolol, methanol–buffer (40 : 60) and 222 nm for bisoprolol, methanol–buffer (50 : 50) and 290 nm for propranolol, methanol–buffer (10 : 90) and 221 nm for terbutaline, methanol–buffer (30 : 70) and 223 nm for metoprolol. The flow rate was 1.0 ml/min for all and injection volume was 20 μl. The chromatographic data were recorded and processed using a HS2000 Station Version 4.0 (Empire Science, Hangzhou, China).

Enzymatic Kinetic and the Ratio of *S*-isomer glucuronide (*S*-G) to *R*-isomer glucuronide (*R*-G) Analysis For enzymatic kinetic studies, substrate concentrations and velocity data were fitted to the standard Michaelis–Menten model, the maximum velocity (V_{max}) and Michaelis–Menten constant (K_m) values for propafenone and esomolol were determined. The $x \pm s$ of three determinations of V_{max} and K_m were calculated for each substrate. Intrinsic clearance Cl_{int} was calculated by the ratio of V_{max}/K_m. All statistical differences were tested by unpaired *t* test.

Propafenone and esomolol glucuronidation enzymatic kinetic parameters were calculated by substrate disappearance and GITC derivatization. The other drugs conjugates formation were calculated only based on glucuronide peak area under UV detector for the low substrates conversion rates, and it was hard to measure these conjugates formation rates for lacking of conjugates standards.

Results

The Glucuronidation Activity of Propafenone and Its Analogues In order to affirm the glucuronidation activity of propafenone and its analogues, incubation mixtures with the substrate were hold at 37 °C water bath for 12 h and then subjected to HPLC system, respectively. Metabolite peaks were observed compared with the parallel blank incubation mixtures without UDPGA, the cofactor of glucuronidation reaction (Fig. 2). Then these metabolite peaks were identified by β-glucuronidase as mentioned above. They all can be hydrolyzed completely by β-glucuronidase after 37 °C water bath, the metabolite peaks disappeared in HPLC chromatogram after hydrolysis with β-glucuronidase. They were able to recover all of the conjugates as the parent drugs. All other drugs glucuronidation were identified by the same method, and it was surprising that propafenone glucuronide showed a longer retention time than parent drug, this may be related to its specific chemical structure.

A series of drugs were investigated here for their glucuronidation activity with human UGT1A3 and UGT1A9. With the incubation, we did not detect any metabolite peaks in the chromatograms of carvedilol, timolol, metoprolol and bisoprolol in contrast to the blank control incubation. In propafenone and propranolol incubation chromatograms, the metabolite peaks were observed only with UGT1A9, while no metabolite peaks detected with UGT1A3. Esomolol, sotalol, terbutaline and atenolol incubation chromatograms all had metabolite peaks detected both with UGT1A3 and UGT1A9. These results display that both UGT1A3 and UGT1A9 have no glucuronidation activity toward carvedilol, timolol, metoprolol and bisoprolol. Only UGT1A9 has activity toward propafenone and propranolol. The others can be catalyzed by both UGT1A3 and UGT1A9. Representative HPLC chromatograms of β-blockers incubation with UGT1A9 were shown in Fig. 2.

Concentration Dependent and Concentration–Time Experiments of Propafenone Glucuronidation with UGT1A9 For concentration dependent experiments with UGT1A9, *R*- or *S*-propafenone was added into incubation mixtures with concentration ranging from 40 to 1200 μM. Then experiments were carried out according to "Incubation of Propafenone and Its Analogues with UGT Enzymes." For each concentration, three samples were carried out in parallel and incubated for 120 min.

For enantiomeric incubations with UGT1A9, the *R*-propafenone glucuronide decreases gradually when the substrate concentration comes about to 800 μM. However when the substrate concentration is over 1200 μM, the *S*-propafenone glucuronide still doesn't decrease in enantiomeric incubations for UGT1A9 (seen in Fig. 3). For time-dependent experiments, 100 μM enantiomeric propafenone was incubated with 1.0 mg/ml UGT1A9 for 30, 60, 90, 120 and 150 min. Then the studies were carried out according to "Incubation of Propafenone and Its Analogues with UGT Enzymes." The stereoselective glucuronidation of enantiomeric propafenone is obvious, the ratio of *S*-PPF to *R*-PPF glucuronide formation rate is approximate 2.2 at 30 min, while 1.9 at 150 min.

Enzymatic Kinetic Study for Propafenone and Esomolol Glucuronidation *via* UGT1A9 Calibration curves were constructed by performing a regression linear analysis of the peak area *versus* the concentrations. The calibration curve of each compound was linear over the concentration

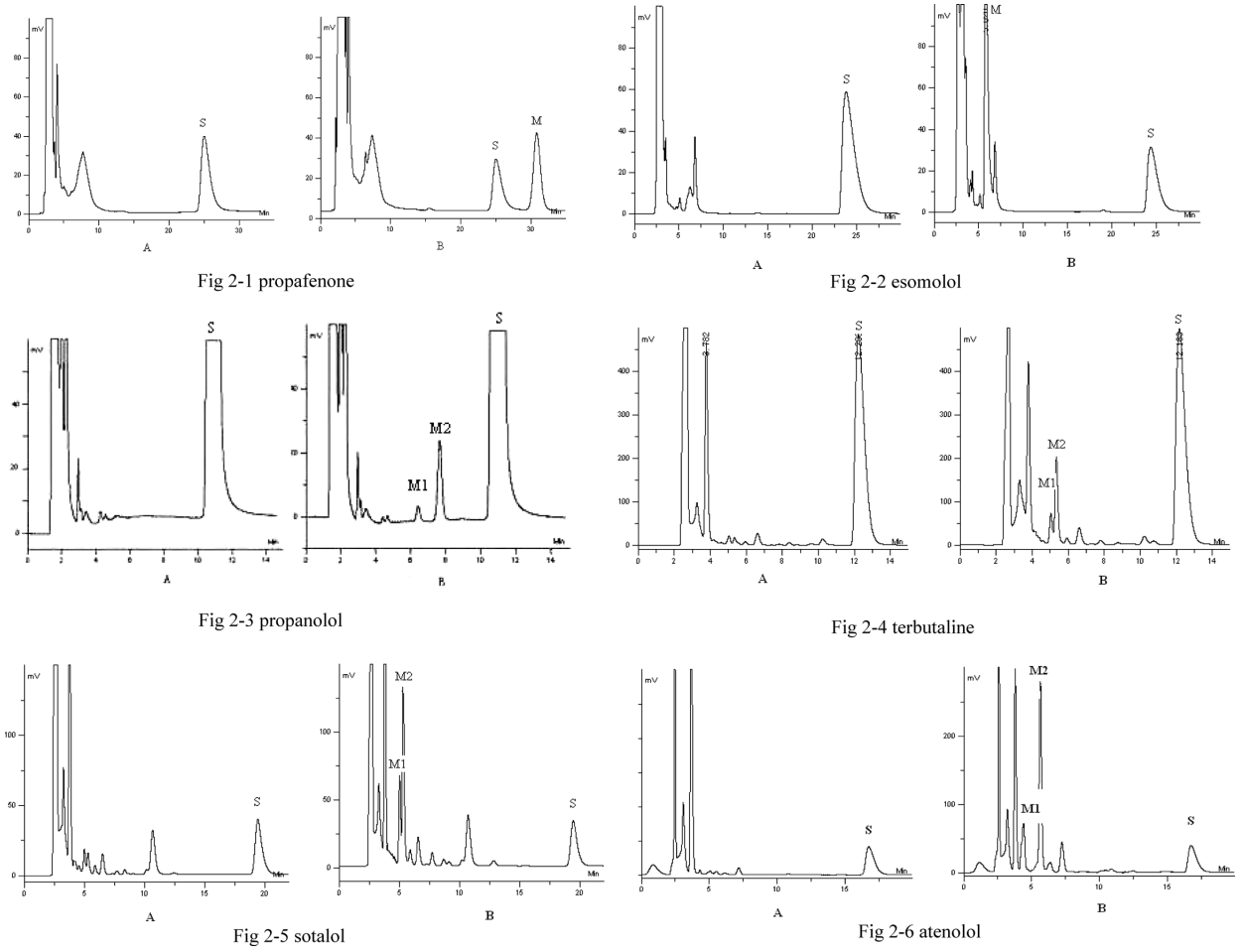

Fig. 2. The Representative Chromatograms for Drugs Glucuronidation Activity by UGT1A9, Peak S: Substrate, Peak M: Metabolite
A: blank control (substrate was incubated without UDPGA overnight). B: Substrate was incubated with UGT1A9 overnight.

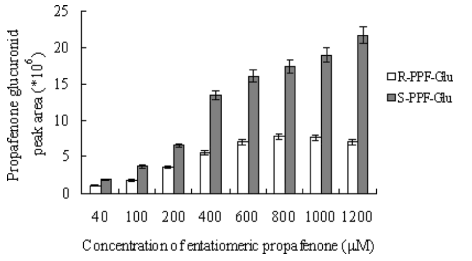

Fig. 3. Concentration Course of Enantiomeric Propafenon Incubated with UGT1A9 for 120 min

ranges. For example, enantiomeric propafenone *R*-PPF: $y(\mu M)=0.00001440x+0.0043$, $r=0.9998$; *S*-PPF: $y(\mu M)=0.00001457x-0.4871$, $r=0.9997$; GITC derivatization propafenone, *R*-PPF: $y(\mu M)=0.00002080x-0.1549$, $r=0.9997$; *S*-PPF: $y(\mu M)=0.00002080x-0.1487$, $r=0.9995$. GITC derivatization esomolol, *R*-esomolol: $y(\mu M)=0.00002353x-8.4755$, $r=0.9995$; *S*-esomolol: $y(\mu M)=0.00002497x-7.8437$, $r=0.9994$.

For enzymatic kinetic studies with UGT1A9, *R*-, *S*-propafenone, racemic propafenone or racemic esomolol was added into incubation mixtures as a substrate with concentration ranging from 40 to 375 μM. Then the experiments were carried out according to "Incubation of Propafenone and Its Analogues with UGT Enzymes" and "Extraction and Derivatization of Propafenone and Esomolol." For each concentration, three samples in parallel were measured. These samples were incubated for 60 min. The enzymatic kinetic parameters of propafenone and esomolol glucuronidation were listed in Table 1 and Table 2.

It can be seen from Table 1 that for propafenone incubations with UGT1A9, there is significant difference on K_m and V_{max} between *R*- and *S*-propafenone in the enantiomeric and racemic glucuronidation mediated by human UGT1A9. Comparing the values of Cl_{int} from enantiomeric incubations with those from racemic incubations for each enantiomer, there is significant difference for *R*- and *S*-propafenone ($p<0.001$). The values of V_{max} of racemic incubations greatly decreased compared with those of enantiomeric incubations for each enatiomer. The Cl_{int} ratio of *S*-propafenone to *R*-propafenone is about 2.21 in enantiomeric incubations. The Cl_{int} ratio of *S*-propafenone to *R*-propafenone is about 2.01 in racemic incubations, it doesn't change too much.

It can be seen from Table 2 that for esomolol, there is significant difference on K_m between *R*-esomolol and *S*-esomolol in the racemic incubations. However, the values of V_{max} of racemic incubations have no significant difference. Comparing the values of Cl_{int} from racemic incubations for each enantiomer, there is significant difference for *R*- and *S*-esomolol ($p<0.001$). And the Cl_{int} ratio of *S*-esomolol to *R*-isomer is 2.74 in racemic incubations.

Table 1. Enzymatic Kinetic Parameters of Propafenone Glucuronidation with the Recombinant UGT1A9 ($x\pm s$, $n=3$)

	Enantiomeric propafenone		Racemic propafenone	
	$R(+)$	$S(-)$	$R(+)$	$S(-)$
K_m (μM)	188.15±23.45†	176.15±5.99†	221.32±31.41	210.86±17.63
V_{max} (μmol/g/min)	793.71±64.19**,††	1649.57±136.76††	269.23±25.17*	525.63±45.94
Cl_{int} (μl/min/mg)	4.25±0.19*,††	9.41±1.09††	1.25±0.09*	2.51±0.47

* $p<0.01$, ** $p<0.001$ compared with that of *S*-(−)-propafenone with unpaired *t*-test; $\dagger p<0.01$, $\dagger\dagger p<0.001$ compared with that of racemic incubations with unpaired *t*-test.

Table 2. Enzymatic Kinetic Parameters of Esomolol Glucuronidation with the Recombinant UGT1A9 ($x\pm s$, $n=3$)

	Racemic esomolol	
	$R(+)$	$S(-)$
K_m (μM)	508.27±29.60*	247±30.27
V_{max} (μmol/g/min)	1607.06±59.96*	2129±73.11
Cl_{int} (μl/min/mg)	3.18±0.30**	8.71±0.13

* $p<0.01$, ** $p<0.001$ compared with that of *S*-(−)-esomolol with unpaired *t*-test.

β-Blocker Analogues Stereoselective Glucuronidation by Human UGT1A9 and Structure–Metabolism Relationships (SMR) For time-dependent experiments, 200 μM of racemic propafenone or esomolol was incubated with the recombinant UGT1A9 for 30, 60, 90, 120, 180 and 240 min. Then the studies were carried out according to "Incubation of Propafenone and Its Analogues with UGT Enzymes." And the samples were processed according to "Extraction and Derivatization of Propafenone and Esomolol." The residue substrate after incubation was derivatizated with GITC. The ratio of *R*- to *S*-isomer increased with time both racemic propafenone and esomolol (Fig. 4). It indicates the disappearance rate of *S*-isomer is greater than *R*-isomer, and *S*-propafenone or *S*-esomolol glucuronide is also greater than *R*-isomer conjugates, respectively.

According to "The Glucuronidation Activity of Propafenone and Its Analogues," propafenone, esomolol, sotalol, atenolol, terbutaline and propranolol are the substrates for UGT1A9, and the rest four drugs stereoselective glucuronidation were not quantitatively determined for the low rates of substrate disappearance rates and there were no metabolite standard samples. In order to roughly determine the glucuronidation stereoselectivity, 200 μM of each four drugs was incubated with UGT1A9 for 120 min, the ratio of *S*-G to *R*-G were roughly measured by HPLC equipped with UV detector only based on peak area. Results show that the ratios of *S*-G to *R*-G for sotalol, atenolol, terbutaline and propranolol are approximately 2.3, 3.1, 3.3 and 4.3, respectively. The metabolite peaks of *R*- and *S*-isomer were validated by enantiomeric isomer standards.

From roughly quantitative stereoselective glucuronidation studies of racemic β-blocker analogues by UGT1A9, it can be classified three levels according to the ratio of *S*- to *R*-isomer glucuronide (*S*-G/*R*-G). Propranolol had high ratio of *S*-G/*R*-G (4.3), terbutaline, atenolol and esomolol had the middle ratios of *S*-G/*R*-G (3.3, 3.1, 2.8), while sotalol and propafenone had the low ratios of *S*-G/*R*-G (2.3, 2.0).

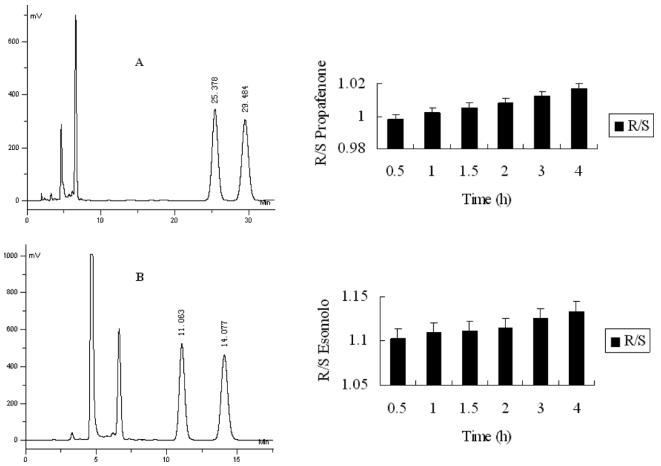

Fig. 4. The Representative Chromatograms of Propafenon (A) and Esomolol (B) after Derivatizated with GITC

Time dependent course of *R*/*S* ratio of racemic substrate incubated with UGT1A9.

Discussion

The UGT1 locus is highly conserved between species. UGT1A is a subfamily of UGT1 gene complex that is located at chromosome 2q37. As one of the isoforms, UGT1A9 is mainly expressed in liver and renal tissues. UGT1A9 has been reported to catalyze the glucuronidation of hydroxyl, carboxyl and hydroxylamine compounds.[19] It was reported that UGT1A9 was mainly responsible for the stereoselective glucuronidation of *S*-etodolac and *S*-propranolol compared with *R*-isomer.[13,20] Other recombinant human UGTs are not studied in this experiment now and they may also contribute to the stereoselective glucuronidation of propafenone and other β-blocker analogues, such as, carvedilol glucuronidation *via* UGT2B7, propranolol glucuronidation also *via* UGT1A10.[13,14]

A set of drugs were then screened for human UGT1A9 glucuronidation activity. The pronounced UGT1A9 stereoselectivity bias for *S*-isomer that we observed (Figs. 2, 4, Tables 1, 2) has not been reported previously. Propafenone and β-blocker analogues are widely used drugs, and one of their important metabolism pathways is glucuronidation. Previous studies have evaluated propranolol glucuronidation by human UGT1A9, but other drugs less to be investigated. And the study may deepen our limited understanding of SMR (structure–metabolism relationships) between UGT1A9 and the stereoselectivity bias of drug glucuronidation. It is also noteworthy that other β-blocker analogues whose chemical structures are similar, show differently glucuronidation activity with human UGT1A3 and UGT1A9. Especially, atenolol, esomolol and metoprolol are highly similar, but only atenolol and esomolol can be catalyzed by UGTs. These findings suggest that a few structure differences could determine whether a compound is the substrate of UGTs.

In concentration dependent experiments, *R*-propafenone has the substrate inhibition effect when the substrate concen-

tration reaches to about 800 μM for the enantiomeric incubation mixtures with UGT1A9. However in *S*-propafenone incubation mixtures with UGT1A9, this effect is not shown even when the concentration of substrate is at 1200 μM. Comparing the kinetic parameters obtained from enatiomeric incubation mixtures with those from racemic incubation mixtures with UGT1A9, the values of K_m of *R*- and *S*-propafenone change little. The values of V_{max} in racemic incubations decrease greatly for two enantiomers.

The present study mainly tries to explore detailed information on stereoselective metabolism of propafenone mediated by human UGT1A9. In conclusion, it is demonstrated that recombinant human UGT1A9 have glucuronidation activity with six drugs and four with UGT1A3 *in vitro*. Human UGT1A9 often shows a preference for *S*-isomer of a β-blocker analogue chiral drug over the other. The results enrich new information about the substrates specificity of different human UGTs. Others reported the rate for *R*-propafenone glucuronide formation was greater than *S*-enantiomer in human,[9—11] which were quite different from this study. It may indicate that other UGTs excluding UGT1A9 will play an important role *in vivo*. Detection of the different activity and stereoselectivity of the recombinant enzymes to chiral drugs *in vitro* can provide some efficient information to explain the profile of drug metabolism *in vivo*.

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