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Stereoselective glucuronidation of carvedilol by Chinese liver microsomes*

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Abstract: Objective: To study the stereoselective glucuronidation of carvedilol (CARV) by three Chinese liver microsomes. Methods: The metabolites of CARV were identified by a hydrolysis reaction with β-glucuronidase and HPLC-MS/MS. The enzyme kinetics for CARV enantiomers glucuronidation was determined by a reversed phase-high pressure liquid chromatography (RP-HPLC) assay using (S)-propafenone as internal standard after precolumn derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylisothiocyanate. Results: Two CARV glucuronides were found in three Chinese liver microsomes incubated with CARV. The non-linear regression analysis showed that the values of K_m and V_{max} for (S)-CARV and (R)-CARV enantiomers were (118±44) μmol/L, (2500±833) pmol/(min·mg protein) and (24±7) μmol/L, (953±399) pmol/(min·mg protein), respectively. Conclusion: These results suggested that there was a significant (P<0.05) stereoselective glucuronidation of CARV enantiomers in three Chinese liver microsomes, which might partly explain the enantioselective pharmacokinetics of CARV.

Key words: Carvedilol (CARV), Derivatization, Stereoselectivity, Enzyme kinetics, Chinese liver microsomes
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INTRODUCTION

Glucuronidation, catalyzed by uridine 5′-diphosphate (UDP)-glucuronosyltransferase (UGT), is one of the most common phase II biotransformations for a large number of therapeutic drugs. UGTs are not only involved in the metabolism of many drugs but also capable of biotransforming endogenous substrates and several xenobiotics (Green and Tephly, 1996; Radominska-Pandya *et al*., 1999). UGTs convert lipophilic molecules into more polar and hydrophilic glucuronides facilitating their elimination via bile, feces, and urine.

Carvedilol (CARV, Fig.1), administered orally as a racemic mixture of the (R)- and (S)-enantiomers, is an α1, β1, and β2 adrenoceptor antagonist (Ruffolo *et al*., 1993; van Zwieten, 1993), and has been clinically used to treat chronic heart failure as well as hypertension, angina pectoris, and cardiac arrhythmias (Packer *et al*., 1996; Eggertsen *et al*., 1987; Nägele *et al*., 2000; Nahrendorf *et al*., 1992). It is highly lipophilic and eliminated predominantly by hepatic metabolism, with renal excretion accounting for only 0.3% of the administered dose (Neugebauer *et al*., 1987). The drug is absorbed rapidly from the gastrointestinal tract after oral administration; however, the amount of unchanged drug excreted in the feces was 23% of the administered dose probably because of its incomplete intestinal absorption (Fujimaki *et al*., 1990). The two enantiomers exhibit different pharmacological effects, i.e., the β-receptor blocking activity of the (S)-CARV is about 200 times higher than that of (R)-CARV, whereas both enantiomers are equipotent α-blockers (Sponer and Muller-Beckmann, 1983; Morgan, 1994). In addition,

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the orally administered CARV undergoes stereoselective first-pass metabolism, and the maximal plasma concentration of (R)-enantiomer with low β-blocking activity is approximately 2-fold higher than that of (S)-enantiomer with high β-blocking activity (Fujimaki *et al*., 1990).

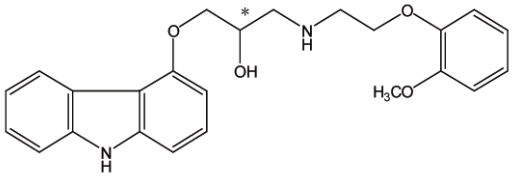

Fig.1 Chemical structure of CARV
The asterisk (*) indicates the chiral center

With regard to conjugation reactions, glucuronidation is the major metabolic pathway of CARV (22% in plasma and 32% in urine) in humans (Neugebauer *et al*., 1987; Neugebauer and Neubert, 1991). There have been few publications about the stereoselective glucuronidation of CARV in vitro, especially in human liver microsomes (HLMs). We, therefore, explore in the present study whether there is a stereoselectivity of CARV glucuronidation in HLMs.

MATERIALS AND METHODS

Materials

Racemic (rac)-CARV (purity>99.6%, HPLC) was kindly supplied by Tianheng Pharmaceutical Co. (Ningbo, China). 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosylisothiocyanate (GITC), UDP-glucuronic acid (UDPGA), alamethicin, (S)-propafenone hydrochloride, β-D-glucuronidase from *Escherichia coli*, and 4-trifluoromethyl-7-hydroxyl coumarin glucuronide were purchased from Sigma-Aldrich (St Louis, MO, USA). 7-Hydroxy-4-trifluoromethylcoumarin (7-HFC) was obtained from Acros Organics (Belgium). All other chemicals and organic solvents were of the highest quality commercially available.

Preparation of HLMs

Human liver microsome (HLM) samples from 3 patients (HLM1, HLM2, and HLM3) were obtained from Sir Run Run Shaw Hospital, Zhejiang Univesity, China. Informed consent was obtained from each patient prior to study entry. The present study was approved by the Ethics Committee of School of Pharmaceutical Sciences, Zhejiang University, China. Pathologically and histologically normal liver samples used in the study were obtained from normal portions of removed tissues. All fresh samples were rapidly frozen in liquid nitrogen and stored at −80 °C before use. The preparation of hepatic microsomes and the determination of protein concentrations in the microsomal preparations were performed according to the methods of Gibbson and Shett (1994) and Lowry *et al*.(1951), respectively.

Determination of enzyme activities of microsomes

The activities of HLMs were determined by using HPLC with 7-HFC as a substrate. The incubates consisted of 1.0 g/L microsomes, alamethicin (25 μg/mg protein), 10 mmol/L magnesium chloride, and 50 μmol/L 7-HFC in 50 mmol/L Tris-HCl buffer, pH 7.4. The reactions were initiated by adding 2 mmol/L UDPGA and incubated for 25 min at 37 °C after the incubation mixture was preincubated for 5 min at 37 °C. The reactions were terminated with 100 μl of ice-cold methanol. The incubation mixture was vortexed and centrifuged (9300×g) at 4 °C for 10 min, and the supernatants were analyzed by HPLC. The formation of glucuronide was quantitated by comparing with the known concentration of 4-trifluoromethyl-7-hydroxyl coumarin glucuronide. Chromatography was performed using a Shimadzu 10A HPLC system equipped with a Shimadzu VP-ODS column (150 mm×4.6 mm) (Shimadzu, Japan). The eluates were monitored at 325 nm. Mobile phases A and B were 0.01 mol/L KH_2PO_4 (pH 3.0) and 90% acetonitrile, respectively. The mobile phase B was set at 30% from 0.01 to 7 min, 65% from 8 to 15 min with a flow rate of 1.0 ml/min for the analysis of 7-HFC and its glucuronide.

Identification of CARV glucuronides

The metabolites (M) CARV glucuronide M1 and M2 formed by incubation with HLMs were identified by β-glucuronidase hydrolysis and HPLC-MS/MS. For the β-glucuronidase hydrolysis, a CARV glucuronidation incubate, in a total volume of 100 μl, was terminated by heating at 90 °C for 1 min and centrifuged (9300×g) for 20 min. The supernatant was incubated with 400 units of β-glucuronidase

dissolved in 0.05 mol/L sodium acetate buffer (pH 5.0) at 37 °C for 2 h. A control incubation without β-glucuronidase was performed simultaneously. The hydrolysis for both samples was stopped by adding 100 μl of ice-cold methanol. After the removal of proteins by centrifugation, the supernatant was subjected to HPLC for the analysis of CARV glucuronides. The mixture of acetonitrile and 10 mmol/L KH_2PO_4 solution (30:70, v/v, pH 3.0) was used as mobile phase with a flow rate of 1.0 ml/min and the excitation and emission wavelengths were set at 284 and 343 nm, respectively (Shimadzu, RF-10AXL Fluorescence Detector, Japan).

Detection of CARV glucuronides was performed with the injection of 10 μl of the condensed centrifugal supernatant onto a Waters Acquity UPLC system (Waters Corporation, Milford, MA) equipped with a Photodiode Array Detector and a TQ Detector (Waters Corporation). The mobile phase consisted of acetonitrile and 5 mmol/L ammonium formate solution (30:70, v/v, pH 3.0) with a flow rate of 0.5 ml/min. The metabolite was detected at 242 nm. The compounds were ionized in the electrospray ionization (ESI) mode and detected in the selected ion recording (SIR) mode. MS/MS conditions used were as follows: 3 kV of capillary voltage, 45 V of cone voltage, 120 °C of source temperature, 20 V of collision voltage, and 0.09 ml/min of collision gas flow.

Stereoselective CARV glucuronidation by HLMs

Rac-CARV was incubated with HLMs. The standard incubation mixture contained 12~240 μmol/L of rac-CARV, 1.0 g/L of HLMs, 10 mmol/L $MgCl_2$, 2 mmol/L UDPGA and alamethicin (25 μg/mg protein) (Ohno *et al*., 2004) in 50 mmol/L Tris-HCl buffer (pH 7.4) in a final volume of 100 μl. CARV was dissolved in DMSO/0.05 mol/L NaOH (50:50, v/v). The final concentration of DMSO in the reaction mixture was 1.5% (v/v) (Ohno *et al*., 2004), and the 1.5% DMSO had no significant difference on reducing the activity of UGTs compared to 1% DMSO (Xie *et al*., 2006). After a preincubation at 37 °C for 5 min, the reaction was initiated by the addition of UDPGA. For kinetic experiments, rac-CARV was used at concentrations of 12, 24, 48, 96, 144, and 216 μmol/L, and the substrates were incubated for 20 min. For the time-dependent experiments, the substrate concentration used was 48 μmol/L. After incubating for the indicated time, the reaction was terminated by adding 1 ml chloroform. Then 20 μl of 350 μmol/L (S)-propafenone solution was added as the internal standard. The mixture was vortexed for 3 min and then centrifuged at 3000×g for 5 min. The organic layer was transferred to a clean tube and dried by a gentle stream of air. One hundred microlitres of 3 mmol/L GITC solution (in acetonitrile) and 100 μl of 1.0% triethamine (in acetonitrile) were added and the tube was capped and allowed to react for 30 min at 35 °C. Chiral inversion did not occur during the derivatization reactions. After evaporation of organic solvents, the residues were reconstituted with 100 μl of mobile phase, from which 20 μl was injected into the HPLC system.

The concentrations of (R)- and (S)-CARV in microsomal samples were measured by reversed phase (RP)-HPLC (LC-10A system, Shimadzu, Japan) after the precolumn derivatization with GITC. The detector wavelength was set at 250 nm. The mobile phase consisted of methanol-water-glacial acetic acid (64:36:0.05, v/v) with a flow rate of 1.2 ml/min at ambient temperature.

Statistical analysis

All results were expressed as the mean±*SD*. The maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) values for CARV enantiomer glucuronidation were estimated by non-linear regression analysis with Michaelis-Menten plots. Intrinsic clearance was calculated by the ratio of V_{max}/K_m. All statistical differences were tested by unpaired *t*-test using Prism V. 4.0 software (GraphPad Software Inc., San Diego, CA).

RESULTS

Activities of liver microsomes

The activity of HLMs using 7-HFC as substrates was 1.2349 nmol/(min·mg protein) for the formation of glucuronide.

Identification of CARV glucuronides formed by HLMs

Two metabolite peaks, one at a retention time of 7.37 min and the other at 8.21 min, were detected in HLM incubates and no interference in blank HLMs was observed (Fig.2). The identity of CARV glu-

curonides was confirmed by ESI in the positive ion mode. The MS showed an $[M+H]^+$ ion at *m/z* 583.5, corresponding to CARV glucuronide and a fragment ion at *m/z* 407.4, corresponding to the parent drug [CARV+H] with a loss of the glucuronic acid moiety (176 amu) (Fig.3). CARV glucuronides were sensitive to β-glucuronidase. The two metabolites were hydrolyzed to CARV after 2 h hydrolysis (Fig.4). These results indicate that the two metabolites were glucuronide conjugates.

Analytical method validation

1. Specificity

Chromatographic separation of (S)-CARV, (R)-CARV and (S)-propafenone was excellent, with the retention time at approximately 23 min, 29 min and 36 min, respectively. No interference in blank incubates or of commonly used drugs was observed. Typical chromatograms are shown in Fig.5. Quantification analysis was performed by internal standardization.

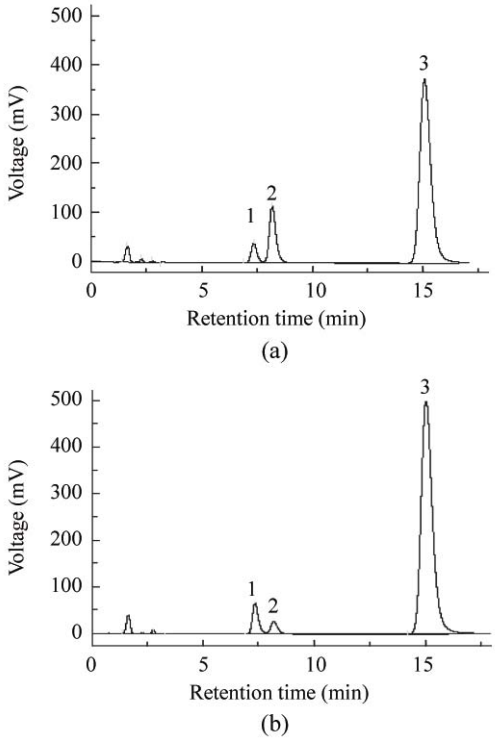

Fig.2 Representative HPLC chromatograms of CARV glucuronidation

HLM (a) and rat liver microsome (RLM) (b) incubated with 20 μmol/L CARV and UDPGA for 1 h, respectively. Peak 1: CARV glucuronide conjugates M1; Peak 2: CARV glucuronide conjugates M2; Peak 3: CARV

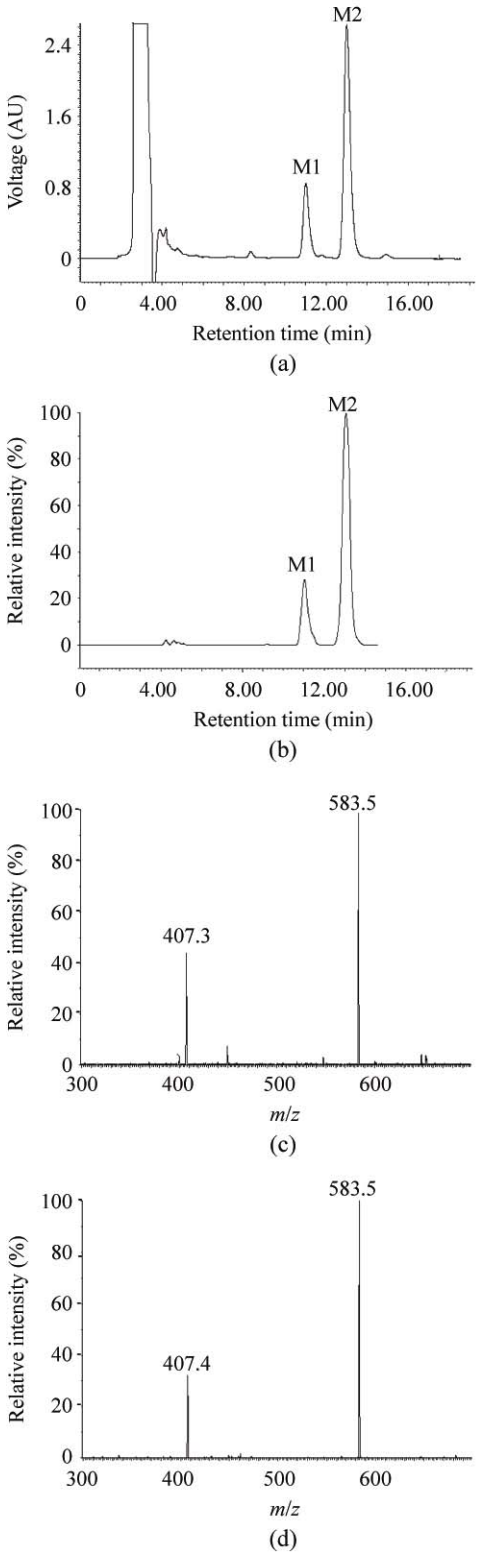

Fig.3 The chromatogram with photodioxde array detector (a) and total ion chromatogram (b) of glucuronide conjugates M1 and M2 generated by glucuronidation of rac-CARV in HLMs. MS/MS spectra for M1 (c) and M2 (d)

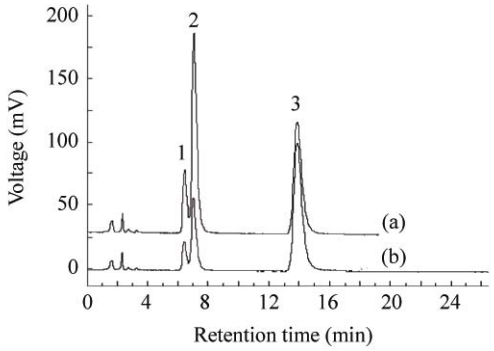

Fig.4 Representative HPLC chromatograms of CARV glucuronides in HLM before (a) and after (b) hydrolysis with β-glucuronidase for 2 h
Peak 1: CARV glucuronide conjugates M1; Peak 2: CARV glucuronide conjugates M2; Peak 3: CARV

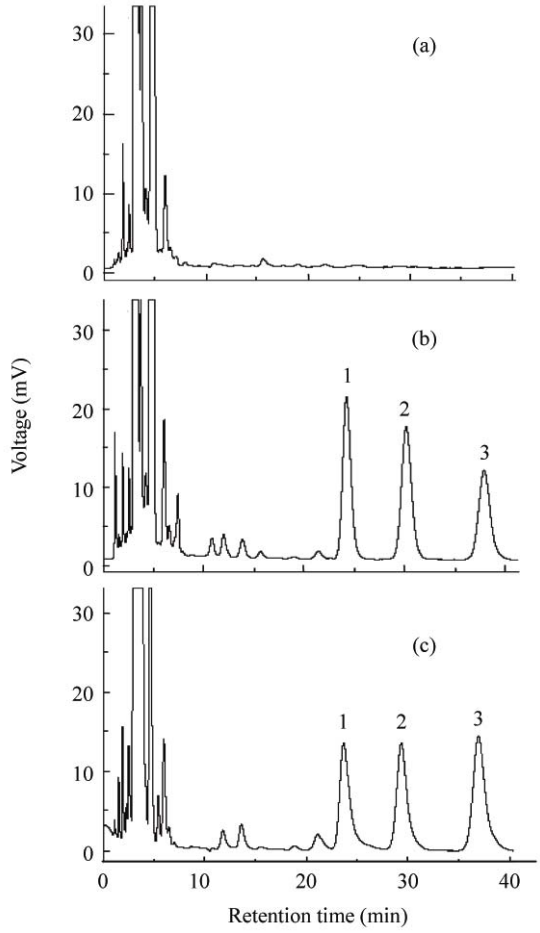

Fig.5 Representative HPLC chromatograms of GITC-derivatives of (a) blank microsomal incubate; (b) blank microsomal incubate spiked with references of CARV and internal standard; and (c) microsome incubated with 72 μmol/L of CARV for 20 min
Peak 1: (S)-CARV; Peak 2: (R)-CARV; Peak 3: (S)-propafenone

The enantiomers peaks were assigned based on the references (Yang *et al*., 2004; Neugebauer *et al*., 1990) and the experiment performed by our lab. Briefly, 600 μl of 1 mmol/L CARV was evaporated to dryness, 600 μl of 1.0% triethamine and 600 μl of 3 mmol/L GITC were added to the residue and then the derivertization was carried out according to the procedures described in the part of **Stereoselective CARV glucuronidation by HLMs** of MATERIALS AND METHODS. The mixture was divided equally into 10 test-tubes and each dissolved in 400 μl of mobile phase. Injection was repeated and two peaks were collected from post-columns, with about 60~70 ml of each peak obtained. After evaporated to dryness, 200 μl of water and 200 μl of 6 mol/L HCl were added to the residues and hydrolyzed at 80 °C for 8 h. The hydrolytes were adjusted to pH 7 with NaOH and extracted twice with 500 μl of CH_2Cl_2, and then the organic phase was combined and dried. The residues were reconstituted with 150 μl of methanol and the optical rotation was determined with a JASCO P-1020 automatic digital polarimeter (JASCO, Tokyo, Japan). The results indicate that the peaks 1 and 2 were S-(−)-CARV-GITC and R-(+)-CARV-GITC, respectively, which is in consistence with the published literatures (Yang *et al*., 2004; Neugebauer *et al*., 1990).

2. Linearity, precision and accuracy

The calibration curves were found to be linear over the concentration range of 6.0~120.0 μmol/L for both (R)- and (S)-CARV with 100 μl incubate solution. Peak area ratios (y) of the (R)- and (S)-CARV vs the internal standard were measured and plotted against the concentration (x) of (R)- or (S)-CARV. The regression equations of the calibration curves of (R)- and (S)-CARV were y=0.0173x+0.0252 (r= 0.9980) and y=0.0174x+0.0511 (r=0.9974). The lower limit of quantification (LLOQ) was 6.0 μmol/L with of 5.6% RSD (n=5) for (R)-CARV and 10.0% RSD (n=5) for (S)-CARV. The lower limit of the detection (LLOD) of (R)- and (S)-CARV was 0.6 μmol/L (S/N=3).

The precision and accuracy of the assay were examined by adding the known amounts of (R)- and (S)-CARV to blank HLMs. For intra-day precision and accuracy tests, five replicates of quality control samples at each concentration were assayed on the same day. The inter-day precision and accuracy were evaluated in five different days. The results obtained

are summarized in Table 1. The intra-day and inter-day precisions were within 10% RSD.

Table 1 Precision for assay of (R)- and (S)-CARV in HLM incubates (RSD, *n*=5)

Conc. spiked (μmol/L)	Intra-day (%)		Inter-day (%)	
	(S)-	(R)-	(S)-	(R)-
12	9.6	7.4	8.4	9.1
54	3.9	3.7	7.4	7.7
108	1.4	6.8	2.5	2.3

The internal standard, (S)-propafenone, also had a good recovery (Table 2). The peak area ratios of (R)- and (S)-CARV to the internal standard were compared with calibration curves of (R)- and (S)-CARV, respectively. The average recovery of this analytical method was 100.3%.

Table 2 Recovery and accuracy for assay of (R)- and (S)-CARV in HLM incubates (mean±*SD*, *n*=5)

Con. spiked (μmol/L)	Extraction recovery (%)			Accuracy (%) (method recovery)	
	(S)-	(R)-	IS	(S)-	(R)-
12	82.1±5.5	79.9±1.3	87.9±5.0	94.9±5.4	89.0±9.1
54	77.2±1.3	75.5±3.7	87.4±1.5	103.4±1.1	102.9±3.8
108	82.0±5.0	80.9±7.1	86.6±1.1	109.7±2.3	102.2±7.0

IS: Internal standard

Time-dependent curves and kinetic parameters for CARV enantiomer glucuronidation

The concentrations of reacted (R)- and (S)-CARV were significantly different at 10, 20, 30 min after the rac-CARV was incubated in HLM incubates (Fig.6). This indicates that the disposition velocity of (S)-CARV was faster than that of (R)-CARV and the stereoselective glucuronidation was time-dependent in HLMs.

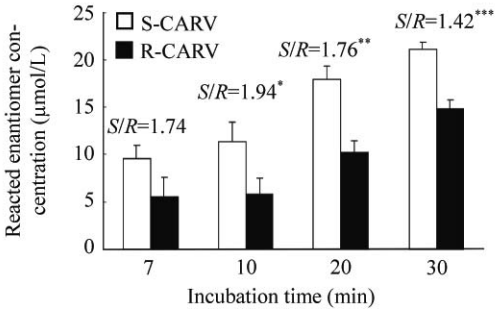

Fig.6 Difference between the glucuronidation after incubating 24 μmol/L of (S)- and (R)-CARV with HLMs for 7, 10, 20, 30 min

Data are expressed as mean±*SD* of three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

CARV glucuronidation followed Michaelis-Menten kinetics for the three Chinese liver microsomes investigated (Fig.7), in which all curves were linear (r=0.97~0.99). Derived K_m and V_{max} values for the three livers were (118±44) μmol/L, (2500±833) pmol/(min·mg protein) for (S)-CARV and (24±7) μmol/L, (953±399) pmol/(min·mg protein) for (R)-CARV. The apparent kinetic constants for CARV enantiomer glucuronidation were summarized in Table 3.

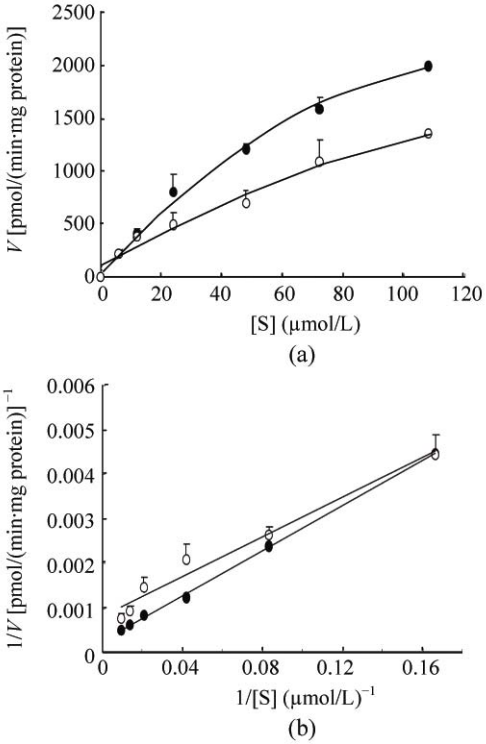

Fig.7 Glucuronidation kinetics of (S)- and (R)-CARV incubated with HLM1. (a) Michaelis-Menten plot (*n*=3); (b) Lineweaver Burk plot (*n*=3). Closed circle (●) for (S)-CARV, open circle (○) for (R)-CARV

Table 3 Enzymatic parameters of CARV enantiomer glucuronidation in HLMs

Enzyme source		K_m	V_{max}	V_{max}/K_m
HLM1	(S)-	84.3	3333.3	39.5
	(R)-	28.0	1250.0	44.6
HLM2	(S)-	102.5	1666.7	16.2
	(R)-	16.2	500.0	30.8
HLM3	(S)-	168.5	2500.0	14.8
	(R)-	28.5	1111.1	39.0
Mean	(S)-	118±44*	2500±833*	23±13
	(R)-	24±7	953±399	38±7

Units: K_m (μmol/L); V_{max} [pmol/(min·mg protein)]; V_{max}/K_m [μl/(min·mg protein)]. $^{*}P<0.05$, comparing (S)-CARV with (R)-CARV

DISCUSSION

The identifications of CARV glucuronides M1 and M2 formed by the incubation with HLMs were confirmed by β-glucuronidase hydrolysis and HPLC-MS/MS. CARV has three potential conjugation sites (Ohno *et al*., 2004), the aliphatic secondary hydroxyl group at the chiral center, the aliphatic secondary amino group, and the carbazolyl amine moiety, and therefore it may be glucuronidated to form N- and/or O-glucuronides (Fig.1). A previous study has showed that the two glucuronides formed with rat liver microsomes (RLMs) were identified as diastereomeric O-linked glucuronic acid conjugates (Schaefer, 1992). In our study, we observed an interesting phenomenon that when incubated with HLMs and RLMs, respectively, the glucuronidation profiles of CARV happened to be similar, that is, both revealed two metabolite peaks with the same retention time but with reverse contents ratios (Fig.2). Moreover, by screening with five human recombinant UGTs, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7 (data not shown), we found that CARV was not metabolized by UGT1A4, which was evidence for its possible O-linked glucuronidation: UGT1A4 preferentially catalyzes N-linked glucuronidation (Sorich *et al*., 2006). We thus suggest that the metabolites may be O-glucuronides.

Chiral chromatography is very useful for the study of enantioselective metabolism of racemic drugs in vitro and in vivo (Yao *et al*., 2000; Zeng *et al*., 1999; Yao and Zeng, 2001). It is important to establish an enantioselective method for the simultaneous analysis of (S)- and (R)-CARV in order to understand the interaction between (S)- and (R)-CARV enantiomers. The principle of resolving two enantiomers of CARV is that the second amine function group in the CARV structure reacts with GITC and forms diastereomers that can be separated on the ordinary analytical column. The derivatization reaction is simple and efficient for the separation of CARV enantiomers.

The enantioselective pharmacokinetics of CARV has been widely investigated in rat, monkey, man and congestive heart failure (CHF) patients (Yang *et al*., 2004; Saito *et al*., 2006; Stahl *et al*., 1993; Fujimaki *et al*., 1990; 1991; Neugebauer *et al*., 1990). All results have led to the conclusion that (S)-enantiomer has greater intrinsic clearance in the liver.

The oxidation of CARV in humans is mainly catalyzed by a hepatic cytochrome P450 2D6 (CYP2D6) (Honda *et al*., 2005; Zhou and Wood, 1995; Fujimaki, 1994; Oldham and Clarke, 1997). Honda *et al*.(2005) further investigated the effect of CYP2D6*10 on the pharmacokinetics of (R)- and (S)-CARV in healthy Japanese volunteers. The stereoselectivity of CYP2D6 is in favor of (S)-CARV by CYP2D6 in vivo and in vitro.

However, when it comes to the conjugation metabolism, the predominant glucuronidation, only a few reports (Ohno *et al*., 2004; Takekuma *et al*., 2006; 2007) are available. The glucuronidation of CARV is catalyzed at least by three recombinant UGT isoforms: UGT1A1, UGT2B4, and UGT2B7 (Ohno *et al*., 2004), and the polymorphisms of UGT1A1, UGT2B7, and CYP2D6 strongly affect the pharmacokinetics and disposition of carvedilol in the Japanese (Takekuma *et al*., 2006; 2007). However, there have been no studies reported on the stereoselectivity of CARV glucuronidation, and our work is the first to study the stereoselective glucuronidation of CARV by HLMs.

CONCLUSION

In summary, the chiral HPLC method described in this study is suitable for the study of the stereoselective glucuronidation of CARV in HLMs. There were two CARV glucuronides found in HLMs but the content ratios were different from those in RLMs. Stereoselective glucuronidation of CARV was observed to be time-dependent and to favor the S-isomer in HLMs. These findings strongly suggest that the stereoselective pharmacokinetics of CARV in several species including humans could be explained not only by its stereoselective oxidation, but also by its stereoselective glucuronidation in HLMs.

References

Eggertsen, R., Sivertsson, R., Andren, L., Hansson, L., 1987. Acute and long-term hemodynamic effects of carvedilol, a combined beta-adrenoceptor blocking and precapillary vasodilating agent, in hypertensive patients. *J. Cardiovasc. Pharmacol.*, **10**(Suppl. 11):97-100.

Fujimaki, M., 1994. Oxidation of R(+)- and S(−)-carvedilol by rat liver microsomes. Evidence for stereoselective oxidation and characterization of the cytochrome P450 isozymes involved. *Drug Metab. Dispos.*, **22**(5):700-708.

Fujimaki, M., Murakoshi, Y., Hakusui, H., 1990. Assay and disposition of carvedilol enantiomers in humans and monkeys: evidence of stereoselective presystemic metabolism. *J. Pharm. Sci.*, **79**(7):568-572. [doi:10.1002/jps.2600790704]

Fujimaki, M., Shintani, S., Hakusui, H., 1991. Stereoselective metabolism of carvedilol in the rat. Use of enantiomerically radiolabeled pseudoracemates. *Drug Metab. Dispos.*, **19**(4):749-753.

Gibbson, G.G., Shett, P., 1994. Introduction to Drug Metabolism, 2nd Ed. Blackie Academic and Professional, London, p.217-221.

Green, M.D., Tephly, T.R., 1996. Glucuronidation of amines and hydroxylated xenobiotics and endobiotics catalyzed by expressed human UGT1.4 protein. *Drug Metab. Dispos.*, **24**(3):356-363.

Honda, M., Nozawa, T., Igarashi, N., Inoue, H., Arakawa, R., Ogura, Y., Okabe, H., Taquchi, M., Hashimoto, Y., 2005. Effect of CYP2D6*10 on the pharmacokinetics of R- and S-carvedilol in healthy Japanese volunteers. *Biol. Pharm. Bull.*, **28**(8):1476-1479. [doi:10.1248/bpb.28.1476]

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**(1):265-275.

Morgan, T., 1994. Clinical pharmacokinetics and pharmacodynamics of carvedilol. *Clin. Pharmacokinet.*, **26**(5):335-346.

Nägele, H., Bohlmann, M., Eck, U., Petersen, B., Rodiger, W., 2000. Combination therapy with carvedilol and amiodarone in patients with severe heart failure. *Eur. J. Heart Fail.*, **2**(1):71-79. [doi:10.1016/S1388-9842(99)00071-9]

Nahrendorf, W., Rading, A., Steinig, G., van der Does, R., Schlote, A., 1992. A comparison of carvedilol with a combination of propranolol and isosorbide dinitrate in the chronic treatment of stable angina. *J. Cardiovasc. Pharmacol.*, **19**(Supp1. 1):114-116.

Neugebauer, G., Neubert, P., 1991. Metabolism of carvedilol in man. *Eur. J. Drug Metab. Pharmacokinet.*, **16**(4):257-260.

Neugebauer, G., Akpan, W., von Mollendorff, E., Neubert, P., Reiff, K., 1987. Pharmacokinetics and disposition of carvedilol in humans. *J. Cardiovasc. Pharmacol.*, **10**(Supp1. 11):85-88.

Neugebauer, G., Akpan, W., Kaufmann, B., Reiff, K., 1990. Stereoselective disposition of carvedilol in man after intravenous and oral administration of the racemic compound. *Eur. J. Clin. Pharmacol.*, **38**(Supp1. 2):108-111. [doi:10.1007/BF01409476]

Ohno, A., Saito, Y., Hanioka, N., Jinno, H., Saeki, M., Ando, M., Ozawa, S., Sawada, J., 2004. Involvement of human hepatic UGT1A1, UGT2B4, and UGT2B7 in the glucuronidation of carvedilol. *Drug Metab. Dispos.*, **32**(2):235-239. [doi:10.1124/dmd.32.2.235]

Oldham, H.G., Clarke, S.E., 1997. In vitro identification of the human cytochrome P450 enzymes involved in the metabolism of R(+)- and S(−)-carvedilol. *Drug Metab. Dispos.*, **25**(8):970-977.

Packer, M., Bristow, M.R., Cohn, J.N., Colucci, W.S., Fowler, M.B., Gilbert , E.M., Shusterman, N.H., 1996. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. *N. Engl. J. Med.*, **334**(21):1349-1355. [doi:10.1056/NEJM199605233342101]

Radominska-Pandya, A., Czernik, P.J., Little, J.M., Battaglia, E., Mackenzie, P.I., 1999. Structural and functional studies of UDP-glucuronosyltransferases. *Drug Metab. Rev.*, **31**(4):817-899. [doi:10.1081/DMR-100101944]

Ruffolo, R.R.Jr, Boyle, D.A., Venuti, R.P., Lukas, M.A., 1993. Preclinical and clinical pharmacology of carvedilol. *J. Hum. Hypertens.*, **7**(Suppl. 1):2-15.

Saito, M., Kawana, J., Ohno, T., Kaneko, M., Mihara, K., Hanada, K., Suqita, R., Okada, N., Oosato, S., Naqayama, M., Sumiyoshi, T., Oqata, H., 2006. Enantioselective and highly sensitive determination of carvedilol in human plasma and whole blood after administration of the racemate using normal-phase high-performance liquid chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **843**(1):73-77. [doi:10.1016/j.jchromb.2006.05.018]

Schaefer, W.H., 1992. Formation of a carbamoyl glucuronide conjugate of carvedilol in vitro using dog and rat liver microsomes. *Drug Metab. Dispos.*, **20**(1):130-133.

Sorich, M.J., McKinnon, R.A., Miners, J.O., Smith, P.A., 2006. The importance of local chemical structure for chemical metabolism by human uridine 5-diphosphate-glucuronosyltransferase. *J. Chem. Inf. Model.*, **46**(6):2692-2697. [doi:10.1021/ci600248e]

Sponer, G., Muller-Beckmann, B., 1983. Studies on the mechanisms on the vasodilating activity of BM 14190. *Naungy-Schmiedeberg's Archies of Pharmacology*, **322**(Suppl.):R46.

Stahl, E., Henke, D., Mutschler, E., Spahn-Langguth, H., 1993. Saturable enantioselective first-pass effect for carvedilol after high oral racemate doses in rats. *Archiv Der Pharmazie*, **326**(3):123-125. [doi:10.1002/ardp.19933260302]

Takekuma, Y., Takenaka, T., Kiyokawa, M., Yamazaki, K., Okamoto, H., Kitabatake, A., Tsutsui, H., Suqawara, M., 2006. Contribution of polymorphisms in UDP-glucuronosyltransferase and CYP2D6 to the individual variation in disposition of carvedilol. *J. Pharm. Pharm. Sci.*, **9**(1):101-112.

Takekuma, Y., Takenaka, T., Kiyokawa, M., Yamazaki, K., Okamoto, H., Kitabatake, A., Tsutsui, H., Suqawara, M., 2007. Evaluation of effects of polymorphism for metabolic enzymes on pharmacokinetics of carvedilol by population pharmacokinetic analysis. *Biol. Pharm. Bull.*, **30**(3):537-542. [doi:10.1248/bpb.30.537]

van Zwieten, P.A., 1993. Pharmacodynamic profile of carvedilol. *Cardiology*, **82**(Suppl. 3):19-23.

Xie, S.G., Chen, Y.K., Chen, S.Q., Zeng, S., 2006. Glucuronidation of apigenin by the recombinant human UGT1A3. *Chin. J. Pharmacol. Toxicol.*, **20**(5):405-409 (in Chinese).

Yang, E., Wang, S., Kratz, J., Cyronak, M.J., 2004. Stereoselective analysis of carvedilol in human plasma using HPLC/MS/MS after chiral derivatization. *J. Pharm. Biomed. Anal.*, **36**(3):609-615. [doi:10.1016/j.jpba.2004.07.008]

Yao, T.W., Zeng, S., 2001. Stereoselective determination of p-hydroxyphenyl-phenylhydantoin enantiomers in rat liver microsomal incubates by reversed-phase high-performance liquid chromatography using β-cyclodextrin as chiral mobile phase additives. *Biomed. Chromatogr.*, **15**(2):141-144. [doi:10.1002/bmc.50]

Yao, T.W., Zhou, Q., Zeng, S., 2000. Stereoselective determination of propafenone enantiomers in transgenic Chinese hamster CHL cells expressing human cytochrome P450. *Biomed. Chromatogr.*, **14**(7):498-501. [doi:10.1002/1099-0801(200011)14:7<498::AID-BMC9>3.0.CO;2-D]

Zeng, S., Zhong, J., Pan, L., Li, Y., 1999. HPLC separation and quantitation of ofloxacin enantiomes in rat microsomes. *J. Chromatogr. B Biomed. Sci. Appl.*, **728**(1):151-155. [doi:10.1016/S0378-4347(99)00085-7]

Zhou, H.H., Wood, A.J., 1995. Stereoselective disposition of carvedilol is determined by CYP2D6. *Clin. Pharmacol. Ther.*, **57**(5):518-524. [doi:10.1016/0009-9236(95)90036-5]