Stereoselective Metabolism of Racemic Carvedilol by UGT1A1 and UGT2B7, and Effects of Mutation of these Enzymes on Glucuronidation Activity

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Carvedilol, an α- and β-adrenergic blocking drug, is mainly metabolized by CYP2D6, UGT1A1, UGT2B4 and UGT2B7. This drug is administered orally as a racemic mixture of R(+)- and S(−)-enantiomers. It has been reported that CYP2D6 prefers metabolizing S-carvedilol to R-carvedilol stereoselectively. On the other hand, stereoselective metabolism of carvedilol by UGTs is still unclear. Moreover, we have reported that patients with chronic heart failure who had polymorphism in CYP2D6, UGT1A1 and/or UGT2B7 had lower metabolic activity and/or oral clearance than did patients with no polymorphism. The aim of this study was to clarify stereoselective metabolism of carvedilol by UGT1A1 and UGT2B7 and to determine by using a recombinant enzyme-introduced mutation whether genetic mutation in UGT1A1 and UGT2B7 causes reduction in metabolic activity for carvedilol. A glucuronidation assay using human liver microsomes and recombinant UGT1A1 and UGT2B7 expressed in HeLa cells demonstrated that UGT1A1 prefers metabolizing R-carvedilol to S-carvedilol. On the other hand, UGT2B7 prefers metabolizing S-carvedilol to R-carvedilol. Moreover, G71R mutation of UGT1A1 reduced both affinity and capacity but did not affect stereoselective metabolism. On the other hand, both A71S and H268Y mutations of UGT2B7 reduced capacity but did not affect affinity and, as a result, the efficiency of metabolism was remarkably reduced. However, as in the case of UGT1A1, neither of the mutations affected stereoselective metabolism.

Key words carvedilol; UGT1A1; UGT2B7; polymorphism; glucuronidation; metabolism

Carvedilol is an antihypertensive and antianginal drug that favorably combines β-adrenergic blocking and vasodilating activities as a result of α-blocking action.1,2) Recently, several studies has proved that carvedilol improves left ventricular ejection in patients with chronic heart failure (CHF) and that it reduces mortality in patients with mild to severe CHF. However, it is recommended that the dose of carvedilol be gradually and carefully increased for treatment of CHF because of its negative inotropic activity.2–7) Therefore, it is important that the metabolic enzymes that affect individual variation of carvedilol pharmacokinetics are precisely identified in order to achieve safe usage of this drug.

Carvedilol is administered orally as a racemic mixture of R(+) - and S(−)-enantiomers. These enantiomers differ in pharmacological properties with both R(+) - and S(−)-enantiomers exhibiting approximately equal α-blocking activity and S(−)-enantiomer exhibiting β-blocking activity.8,9) After oral administration of carvedilol, the area under the time–concentration curve (AUC) of R(+) -enantiomer has been reported to be much larger than that of S(−)-enantiomer.10–12) It is known that carvedilol is metabolized through multiple pathways. The enzymes that are involved in its metabolism are cytochrome P450 (CYP) 2D6 and CYP2C9 as phase I enzymes and UDP-glucuronosyl transferase (UGT) 1A1, UGT2B4 and UGT2B7 as phase II enzymes.13,14) It has been reported that both CYP2D6 and CYP2C9 metabolized S(−)-carvedilol more rapidly than R(+) -carvedilol and that the rate of carvedilol metabolism by CYP2D6 was greater than that by CYP2C9.13) Moreover, it has been reported that clearance of carvedilol in poor metabolizers in CYP2D6 was lower than that in extensive metabolizers, though there was no difference in clearance between poor and extensive metabolizers in CYP2C9.12,15,16)

On the other hand, stereoselective metabolism of carvedilol by the three isoforms of UGT is still unclear. It is thought that the main pathway is direct glucuronidation of carvedilol because the main metabolite in plasma and urine was found to be the glucuronide of unchanged carvedilol (22% and 32%, respectively).17,18) Therefore, it is very important to clarify the stereoselective metabolism by UGTs.

The major metabolite of carvedilol in plasma was reported to be O-glucuronide of unchanged carvedilol.19) Ohno et al. reported that the three UGT isoforms were capable of conjugating carvedilol into two forms of its glucuronide (G1 and G2).14) UGT2B4 formed both glucuronides, whereas UGT1A1 (G2) and UGT2B7 (G1) formed either one.14) Therefore, it is possible that these two metabolites are O-glucuronides derived from R(+) - and S(−)-enantiomers, namely, diastereomers.

We have reported that individual variation in plasma concentrations of carvedilol in patients with CHF was very large and that patients with polymorphism in CYP2D6, UGT1A1 and/or UGT2B7 had lower metabolic activity and/or oral clearance than did patients with no polymorphism.19,20)

The aim of this study was to clarify which of R(+) -carvedilol and S(−)-carvedilol is the origin of the two glucuronides (G1 and G2) and to determine by using a recombinant enzyme-introduced mutation whether genetic mutation in UGT1A1 and UGT2B7 causes reduction in metabolic activity for carvedilol.
MATERIALS AND METHODS

Chemicals and Reagents  (±)-Carvedilol was kindly supplied by Daiichi Pharmaceutical Co. (Tokyo, Japan). R(+) and S(-)-carvedilol were kindly supplied by Roche Diagnostics Co., Ltd. (Basel, Switzerland). Alamethicin was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). UDP-glucuronic acid trisodium salt was purchased from Wako Pure Chemicals (Osaka, Japan). Human liver mixed pool microsomes were purchased from In Vitro Technologies, Inc. (Baltimore, Maryland, U.S.A.). Microsomes of S9 cells expressing recombinant UGT1A1 and UGT2B7, human UGT1A1 western blotting kit, and human UGT2B7 western blotting kit were purchased from GENTEST (MA, U.S.A.). Flecainide acetate was kindly supplied by Eisai Co. (Tokyo, Japan). All other reagents were of the highest grade available.

Assay for Carvedilol Glucuronidation  Carvedilol glucuronidation using human liver mixed pool microsomes was assayed by a previously reported method with minor modification. The reaction mixture contained carvedilol, 0.5 mg/ml (identification of glucuronides) or 0.025 mg/ml (analysis for kinetic parameters) microsomal proteins, 10 mM MgCl2, 2 mM UDP-glucuronic acid, and 12.5 µg/ml alamethicin in 50 mM Tris–HCl buffer (pH 7.4) in a final volume of 200 µl. Final concentrations were 10 µM for RS-carvedilol and 5 µM for R- or S-carvedilol. After preincubation at 37 °C for 5 min, the reaction was initiated by the addition of UDP-glucuronic acid. The mixture was incubated for 4 h (identification of glucuronides) or 15 min (analysis for kinetic parameters) at 37 °C. Then the reaction was terminated by the addition of glucuronidase (15 min) (identifying glucuronides) or 10 min (analysis for kinetic parameters) microsomal proteins, 10 mM potassium dihydrogen phosphate (39 : 61) and column temperature was 40 °C. In all conditions, the flow rate was 1.0 ml/min. Excitation and emission wavelengths of 240 nm and 340 nm, respectively, were used for fluorometric detection.

For quantification of carvedilol glucurononide, the first of the above conditions was used for HPLC. Carvedilol was used as the standard assuming the same fluorescence intensities of carvedilol and its glucuronide because the glucuronic acid-connected site of carvedilol was not related with resonance. The lowest measurable concentration was 0.5 nm. Coefficient of variation was less than 5%.

Cell Culture  HeLa cells were routinely maintained in a culture dish (Griner bio-one, Tokyo, Japan) and the stock cells were subcultivated before reaching confluence. The growth medium was Dulbecco’s modified Eagle’s medium (DMEM, Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (ICN, Biochemicals, Aurora, OH, U.S.A.). The cells were incubated in 5% CO2 at 37 °C. The cells were harvested with 0.25% trypsin and 0.2% EDTA, resuspended, and seeded into a new culture dish. Cells between 20th and 26th passages were used in this study.

Construction of Expression Plasmids of UGTs  UGT1A1-Wild-Type (wt)-pcDNA3.1(+) Construct: A full-length clone of UGT1A1 in pReceiver-B02a vector was purchased from GeneCopoeia (Maryland, U.S.A.). The construct was sequenced using an ABI3100 sequencer (Applied Biosystems, Foster City, CA, U.S.A.). The coding sequence was found to be identical to the published sequence of UGT1A1 (Accession Number: NM_000463) with a mutation (12C>G, D4G). Then the mutation was repaired using mega primer methods.21,22) The forward primer was 5’-TGGGT-TGAAGGCTCTCAAGG-3’ and the reverse primer was 5’-CGTCCGCCCTGGGAC-TGAAGGCTCTCAAGG-3’ containing a mutation-repaired site (underlined). Amplification was performed for 1 cycle of 98 °C for 30 s, 16 cycles of 98 °C for 10 s, 52 °C for 60 s, and 72 °C for 60 s using pReceiver-B02a-UGT1A1 construct (50 ng) containing a mutation, 2.5 units of Pyrobest DNA polymerase (TaKaRa, Tokyo, Japan), 0.4 mM dNTP mix and 0.4 µM of the primers. The PCR fragments were purified and used as a mega primer for the next PCR. The other primer (reverse primer) was 5’-TGCTAGT- TATTGCTCAGCGGT-3’ and the reverse primer was 5’- CTGGTG-TGAAGGCTCTCAAGG-3’ containing a mutation-repaired site (underlined). Amplification was performed for 1 cycle of 98 °C for 30 s, 18 cycles of 98 °C for 10 s, 52 °C for 60 s, and 72 °C for 60 s using pReceiver-B02a-UGT1A1 construct (50 ng) containing a mutation, 2.5 units of Pyrobest DNA polymerase (TaKaRa, Tokyo, Japan), 0.4 mM dNTP mix and 0.4 µM of the primers. The PCR fragments were purified and used as a mega primer for the next PCR. The other primer (reverse primer) was 5’-TGCTAGT- TATTGCTCAGCGGT-3’ and the reverse primer was 5’- TGCTAGT- TATTGCTCAGCGGT-3’ containing a mutation-repaired site (underlined). Amplification was performed for 1 cycle of 98 °C for 30 s, 18 cycles of 98 °C for 10 s, 52 °C for 60 s, and 72 °C for 60 s, and other conditions were the same. The PCR fragment obtained was purified and digested with XbaI and XhoI. The fragment was subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, U.S.A.) digested with NheI and XhoI.

UGT1A1-G71R-pcDNA3.1(+) Construct: The UGT1A1 cDNA fragment obtained was subcloned into pcDNA3.1(+) digested with XbaI and XhoI. A mutation (211G>A, G71R) was introduced into this construct using the mega primer method.22,23) The forward primer was 5’-AGGGGCAAC- AACAGATGG-3’ and the reverse primer was 5’-GCGTCTCTGAGCTCTTAAC-3’ containing a mutation-repaired site (underlined). Amplification was performed for 1 cycle of 98 °C for 30 s, 18 cycles of 98 °C for 10 s, 52 °C for 60 s, and 72 °C for 210 s, and other conditions were the same. The PCR fragment obtained was purified and digested with XbaI and XhoI. The fragment was subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, U.S.A.) digested with NheI and XhoI.

For identification and quantification of two carvedilol glucuronides, three HPLC conditions were used: A) the mobile phase was a mixture of acetonitrile and 50 mM potassium dihydrogenphosphate (22:78) containing a final concentration of 5 mM tetra-n-butylammonium chloride and column temperature was 40 °C, B) the mobile phase was a mixture of methanol and 50 mM potassium dihydrogenphosphate (39:61) and column temperature was 50 °C, and C) the mobile phase was a mixture of acetonitrile, methanol and 50 mM potassium dihydrogenphosphate (18:20:62) and column temperature was 40 °C. In all conditions, the flow rate was 1.0 ml/min. Excitation and emission wavelengths of 240 nm and 340 nm, respectively, were used for fluorometric detection.

For quantification of carvedilol glucurononide, the first of the above conditions was used for HPLC. Carvedilol was used as the standard assuming the same fluorescence intensities of carvedilol and its glucuronide because the glucuronic acid-connected site of carvedilol was not related with resonance. The lowest measurable concentration was 0.5 nm. Coefficient of variation was less than 5%.
15 s, 47 °C for 60 s, and 72 °C for 180 s, and other conditions were the same. The PCR fragment obtained was purified and digested with XbaI and XhoI. The fragment was subcloned into pcDNA3.1(+) digested with NheI and XhoI.

UGT2B7-H268Y-pcDNA3.1(+) Construct: A full-length clone of UGT2B7 in pME18SFL3 vector was purchased from TOYOBO Co. (Osaka, Japan). As a result of sequencing the clone of UGT2B7 in pME18SFL3 vector was purchased from TOYOBO Co. (Osaka, Japan). As a result of sequencing the clone of UGT2B7 in pME18SFL3 vector with StuI and NorI. The fragment was subcloned into pcDNA3.1(+) digested with EcoRV and NotI.

UGT2B7-wt-pcDNA3.1(+) Construct: A mutation (802C to the published sequence of UGT2B7 (Accession Number: NM_001074) with a mutation (802C → T). The forward primer was 5′-ATTTCAATTTCCATCCATCTTACC-A-3′ and the reverse primer was 5′-TGTTAAGAGTGGA-TGGAAACTGAAA-T-3′ containing a mutation-repaired site (underlined).

UGT2B7-A71S-pcDNA3.1(+) Construct: UGT2B7 H268Y in pcDNA3.1(+) vector was repaired using a QuickChange Site-Directed Mutagenesis Kit (STRATAGENE, CA, U.S.A.) according to the manufacturer’s instructions. The forward primer was 5′-ATTTCATTTCCATCCATCTTACC-A-3′ and the reverse primer was 5′-TGTTAAGAGTGGA-TGGAAACTGAAA-T-3′ containing a mutation-repaired site (underlined).

Expression of UGTs in HeLa Cells and Preparation of Membrane Fraction Expression of each UGT isoform in HeLa cells was done using the procedure described by Gana- et al.24,25) HeLa cells expressing each UGT variant were separated from HeLa cells expressing each UGT variant by 10% SDS-polyacrylamide gel electrophoresis. The gel was transferred onto a nitrocellulose membrane and probed with rabbit anti-human UGT (1A1 or 2B7) antisera (1:500 dilution). An anti-rabbit IgG goat antibody conjugated with horseradish peroxidase was used as the second antibody, and the resulting immunocomplexes were visualized using ECL Western blotting detection reagents (GE Healthcare Bio-Sciences KK, Tokyo, Japan) and exposed on X-ray films and quantified by Luminous Imager version 2.0 (Aisin Cosmos R&D Co., Aichi, Japan).

Statistical Analysis Data are expressed as means±SD. Comparisons of pharmacokinetics parameters between UGT variants were made by student’s t-test. Statistical analyses were performed using StatView ver. 5.0 (SAS institute Inc., NC, U.S.A.). Kinetic parameters (Km and Vmax) for carvedilol glucuronidation were calculated by fitting Michaelis–Menten equation using Origin 6.1J (Origin Lab Corporation, MA, U.S.A.). A p value below 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Identification of Carvedilol Glucuronides Neugebauer et al. reported that the glucuronide of unchanged carvedilol in humans was O-glucuronide by LC/MS assay (Fig. 1).13 Schaefer et al. reported that structures of several carvedilol glucuronides which were metabolites in dog and rat, were identified by NMR and MS assay and that only O-glucuronides (diastereomers) were formed under CO2 free condition in vitro. Moreover, we confirmed that the retention times of carvedilol glucuronides conjugated using rat and human liver microsomal proteins were the same (data not shown).24,25) On the other hand, it has been reported that the glucuronide of unchanged carvedilol was two species14 and we confirmed the two glucuronides by HPLC (Fig. 2A). Since enantiomers have the same physicochemical properties except for optical rotatory power, separation of them is not possible by ordinary reverse-phase HPLC. On the other hand, diastereomers have different physicochemical properties. Therefore, it is possible that these glucuronides were derived from R- and S-carvedilol, namely a pair of diastereomers, but it is still precisely unknown. We determined metabolites of R-carvedilol, S-carvedilol and racemic carvedilol using pooled human liver microsomes by HPLC with different three elution conditions and analyzed each retention time and peak area. Retention times of metabolites of racemic
carvedilol were at 15.8 min and 18.6 min with condition A (Fig. 2). The former matched the retention time of the metabolite of R-carvedilol, and the latter matched that of S-carvedilol. Furthermore, when a mixture of metabolites of R-carvedilol and racemic carvedilol was determined by HPLC, two peaks were completely overlapped and the peak area of 15.8 min was larger than that in the case of only racemic carvedilol. On the other hand, when a mixture of metabolites of S-carvedilol and racemic carvedilol was determined by HPLC, two peaks were observed similarly, and the peak area of 18.6 min was larger than that in the case of only racemic carvedilol. In the same way, this experiment was performed with other two conditions. The results were the same in all elution conditions. Retention times of metabolites of racemic carvedilol were at 14.9 min and 19.3 min with condition B, and were at 8.9 min and 10.1 min with condition C, respectively (data not shown). In both cases, the first peak was overlapped with the peak of a metabolite of R-carvedilol and the second peak was overlapped with the peak of a metabolite of S-carvedilol completely. Therefore, it was proved that the two glucuronides of racemic carvedilol were a pair of diastereomers and that the former eluted one was derived form R-carvedilol and the latter eluted one was derived from S-carvedilol in these conditions.

Relative Quantification of Expression of UGT1A1, UGT2B7 and Mutant Variants in HeLa Cells

Relative quantification of the expression of UGT1A1 wild type and G71R in HeLa cells was performed by Western blotting. Results of Western blot analysis of UGT1A1 in the membrane fraction and α-tubulin in whole cell homogenates are shown in Fig. 3. The immunoblot bands showed that UGT1A1-wt and G71R protein expression was observed and that HeLa cells had no UGT1A1 protein. Alfa-tubulin protein expression was observed in all preparations. Relative expression levels of UGT1A1-wt and G71R variant normalized with that of α-tubulin are shown in Fig. 3. Expression level of UGT1A1-G71R to wt was 0.95 ± 0.13.

Results of Western blot analysis of UGT2B7 in the membrane fraction and α-tubulin in whole cell homogenates are shown in Fig. 4. The immunoblot bands showed that UGT2B7 wild type, A71S and H268Y protein expression was observed and that HeLa cells had no UGT2B7 protein.
Protein expression of α-tubulin was observed in all preparations. Relative expression levels of UGT2B7 wild type, A71S and H268Y normalized with that of α-tubulin are shown in Fig. 4. Expression levels of UGT2B7 A71S and H268Y to wild type were 0.85±0.18 and 1.07±0.28, respectively.

**Kinetic Parameters for Carvedilol Glucuronidation by Human Liver Microsomal Proteins and Membrane Fraction Expressing UGTs**

The formation rate of carvedilol glucuronide as a function of substrate concentration was measured using human liver microsomal proteins (Fig. 5). When racemic carvedilol was used as substrate, the rate of S-carvedilol glucuronidation was much higher than that of R-carvedilol (Fig. 5, Table 1). However, when each enantiomer was used as substrate, the difference between the rates of R- and S-carvedilol glucuronidation was not observed. Moreover, $V_{max}$ and $V_{max}/K_m$ of both R- and S-carvedilol glucuronidation were much higher than those in the case of racemic carvedilol. Therefore, it was suggested that R- and S-carvedilol inhibited the glucuronidation each other. It was reported that plasma concentration of S-carvedilol was lower.

**Table 1. Kinetic Parameters of Carvedilol Glucuronidation by Human Liver Microsomal Proteins**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Parameter</th>
<th>R-Carvedilol glucuronide</th>
<th>S-Carvedilol glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Racemic carvedilol</td>
<td>$K_m$ ($\mu$M)</td>
<td>5.9±1.3</td>
<td>9.2±0.7*</td>
</tr>
<tr>
<td></td>
<td>$V_{max}$ (pmol/min/mg protein)</td>
<td>31.1±1.9</td>
<td>106.1±5.8*</td>
</tr>
<tr>
<td></td>
<td>$V_{max}/K_m$ (nl/min/mg protein)</td>
<td>5364±753</td>
<td>11580±751*</td>
</tr>
<tr>
<td>R- or S-Carvedilol</td>
<td>$K_m$ ($\mu$M)</td>
<td>87.6±23.2</td>
<td>35.8±16.0*</td>
</tr>
<tr>
<td></td>
<td>$V_{max}$ (pmol/min/mg protein)</td>
<td>399.6±76.8</td>
<td>258.8±79.5</td>
</tr>
<tr>
<td></td>
<td>$V_{max}/K_m$ (nl/min/mg protein)</td>
<td>4640±452</td>
<td>7524±999*</td>
</tr>
</tbody>
</table>

$K_m$ values are converted into each enantiomer concentration. Each value represents the mean±S.D. of three measurements. *p<0.05, **p<0.01 versus R-carvedilol glucuronidation.
than that of R-carvedilol in volunteers after administering racemic carvedilol.\textsuperscript{16} Difference of the potency of inhibitory effect between R- and S-carvedilol is a possible reason for the difference of plasma concentration of R- and S-carvedilol.

Catalytic activity was not observed with the membrane fraction from pcDNA3.1(+) -transfected HeLa cells (data not shown). This result agreed with the results of Western blot analysis shown above. The formation rate of carvedilol glucuronide as a function of substrate concentration was measured in the membrane fraction expressing UGT1A1 (Fig. 6). In both wild type and G71R, concentration dependency was observed, and the rate of R-carvedilol glucuronidation was much higher than that of S-carvedilol glucuronidation. Kinetic parameters were calculated using the Michaelis–Menten equation (Table 2). $K_m$ value of R-carvedilol glucuronidation of wild-type was 4.8±0.8 μM (9.6±0.8 μM as racemic carvedilol). Ohno et al. reported that the $K_m$ value of G2 (R-carvedilol glucuronide in this study) formation was 55.1±6.6 μM in microsomes expressing recombinant UGT1A1 in S9 cells,\textsuperscript{14} and that value is 6-times larger than the value in this study. Since S9 cells were derived from insects, addition of a sugar chain to UGT as a posttranslational modification might be different from that in human cells. This might be responsible for different affinities to R- carvedilol. Moreover, Ohno et al. reported that formation of G1 (S-carvedilol glucuronide in this study) was not detected,\textsuperscript{14} but we detected the formation of S-carvedilol glucuronide. However, the amount was very small compared with that of R-carvedilol glucuronide. This tendency did not change either in the case of racemic carvedilol and enantiomers. These findings suggest that UGT1A1 metabolized R-carvedilol stereoselectively.

$V_{max}$ and $V_{max}/K_m$ were normalized by relative expression level measured using Western blot analysis. When each enantiomer was used as substrate, $K_m$ value was high and normalized $V_{max}$ value in G71R variant was low compared with those in the wild type. The catalytic efficiencies (normalized $V_{max}/K_m$ for R- and S-carvedilol were reduced to 18.7% and 6.3%, respectively. These results suggest that G71R mutation caused reduction of affinity and rate of glucuronidation of both R- and S-carvedilol. When racemic carvedilol was used as substrate, normalized $V_{max}$ and $V_{max}/K_m$ values were much higher than those in the case of using enantiomer.

These results demonstrated that UGT1A1 metabolized R-carvedilol stereoselectively and that G71R mutation caused reduction of the catalytic activity of UGT1A1 without any effect on stereoselectivity. Moreover, it was suggested that R- and S-carvedilol inhibited glucuronidation by UGT1A1 each other. It has been reported that UGT1A1-G71R activities toward bilirubin, 17β-estradiol and SN-38 were reduced to 32.2%, 23.6% and 47.1% of UGT1A1-wt activity, respectively.\textsuperscript{26–28} The results obtained in this study using carvedilol were similar.

The formation rate of carvedilol glucuronide as a function of substrate concentration was measured in the membrane fraction expressing UGT2B7 variants (Fig. 7). In all variants, concentration dependency was observed, and the rate of S-carvedilol glucuronidation was higher than that of R-carvedilol glucuronidation, in contrast with UGT1A1. Ki-
Table 3. Kinetic Parameters of Carvedilol Glucuronidation by Membrane Fractions Expressing UGT2B7 Wild-Type, 71S and H268Y

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolites</th>
<th>R-Carvedilol glucuronide</th>
<th>S-Carvedilol glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino acid change</td>
<td>Wild-type</td>
<td>A71S</td>
</tr>
<tr>
<td>Racemic carvedilol</td>
<td>$K_m$ ($\mu$m)</td>
<td>18.9±5.9</td>
<td>23.6±6.9</td>
</tr>
<tr>
<td></td>
<td>Normalized $V_{max}$ (pmol/min/mg protein)</td>
<td>0.55±0.10</td>
<td>0.35±0.06*</td>
</tr>
<tr>
<td></td>
<td>Normalized $V_{max}/K_m$ (nl/min/mg protein)</td>
<td>14.9±1.8</td>
<td>7.7±1.7*</td>
</tr>
<tr>
<td>R- or S-Carvedilol</td>
<td>$K_m$ ($\mu$m)</td>
<td>49.4±11.5</td>
<td>47.1±16.5</td>
</tr>
<tr>
<td></td>
<td>Normalized $V_{max}$ (pmol/min/mg protein)</td>
<td>3.28±0.14</td>
<td>1.62±0.42*</td>
</tr>
<tr>
<td></td>
<td>Normalized $V_{max}/K_m$ (nl/min/mg protein)</td>
<td>68.8±14.6</td>
<td>35.4±3.7*</td>
</tr>
</tbody>
</table>

$K_m$ values are converted into each enantiomer concentration. Each value represents the mean±S.D. of three measurements. *p<0.05, **p<0.01 versus wild-type.

dynamic parameters were calculated using the Michaelis–Menten equation (Table 3). The $K_m$ value of S-carvedilol glucuronidation of wild type was 18.5±4.5 $\mu$m (36.9±8.9 $\mu$m as racemic carvedilol concentration). This value was similar to the $K_m$ value (27.9 $\mu$m) of microsomes expressing recombinant UGT2B7 in S9 cells in a previous study. Moreover, Ohno et al. reported that formation of R-carvedilol glucuronide was not detected, but we detected the formation of R-carvedilol glucuronide. One possible reason is that sensitivity of our quantification (limit: 0.025 pmol) was higher than that in Ohno’s study (limit: 5.94 pmol). The fact that sensitivity of our quantification (limit: 0.025 pmol) was higher than that in Ohno’s study (limit: 5.94 pmol). The fact that UGT2B7 metabolized carvedilol stereoselectively.

$V_{max}$ and $V_{max}/K_m$ were normalized by relative expression level measured using Western blot analysis. When each enantiomer was used as substrate, A71S mutation reduced normalized $V_{max}$ value but no effect on $K_m$ value. On the other hand, H268Y mutation reduced $K_m$ and normalized $V_{max}$ values. A71S mutation reduced the normalized $V_{max}/K_m$ values of R- and S-carvedilol to 51.4% and 51.9% of the wild type, respectively. On the other hand, H268Y mutation reduced the $V_{max}/K_m$ values of R- and S-carvedilol to 25.4% and 28.7% of wild type, respectively. When racemic carvedilol was used as substrate, normalized $V_{max}$ and $V_{max}/K_m$ values were much higher than those in the case of enantiomer. Therefore, it was suggested that R- and S-carvedilol inhibited glucuronidation by UGT2B7 each other same as UGT1A1.

Exon 1 and exon 2 domains code substrate-binding sites in the UGT2B family. Recently, it has been reported that several amino acid residues have an important role in substrate recognition in UGT2B7. The binding site for opioids is within the 84 to 118 N-terminal amino acids. Moreover, replacement of tyrosine by leucine strongly reduced the catalytic activity. There is no report about the effect of A71S mutation on the activity of UGT2B7. A71S mutation exists in exon 1 and was replacement of the hydrophobic amino acid alanine by the hydrophilic amino acid serine. Therefore, we predicted that A71S mutation would reduce the affinity of UGT2B7. However, the $K_m$ value of UGT2B7 A71S variant was the same as that of wild type, whereas the $V_{max}$ value of UGT2B7 A71S variant was lower than that of wild type. Accordingly, this replacement might reduce the rate of turnover of glucuronidation.

There are some reports on the effects of H268Y mutation on catalytic activity of UGT2B7. This effect depends on the kind of substrate. $K_m/V_{max}$ values of UGT2B7 H268Y variant
toward morphine, buprenorphine were higher than those of wild type, whereas $K_m/V_{\text{max}}$ values of UGT2B7 H268Y variant toward naroxone, naltrexone, oxymorphone and AZT were lower than those of wild type. In this study, the $K_m/V_{\text{max}}$ value of UGT2B7 H268Y variant toward carvedilol was lower than that of wild type, though $K_m$ values of H268Y and wild type were not different.\(^{22,33}\)

Our results demonstrate that UGT2B7 prefers metabolizing S-carvedilol to R-carvedilol. However, because normalized $V_{\text{max}}/K_m$ values of both mutant variants toward R- and S-carvedilol were about 50% and 23% of the wild type, respectively, A71S and H268Y mutations did not affect stereoselective metabolism.

In conclusion, our results demonstrate that UGT1A1 prefers metabolizing R-carvedilol to S-carvedilol. On the other hand, UGT2B7 prefers metabolizing S-carvedilol to R-carvedilol. G71R mutation of UGT1A1 reduced both affinity and capacity but did not affect stereoselective metabolism. On the other hand, A71S mutations of UGT2B7 reduced capacity but not affinity and H268Y mutation reduced $K_m$ and normalized $V_{\text{max}}$ values, as a result, greatly reduced efficiency. However, same as in the case of UGT1A1, neither of the mutations affected stereoselective metabolism. Moreover, R- and S-enantiomer inhibited glucuronidation each other by both UGT1A1 and UGT2B7.

REFERENCES