Glucuronidation of Monohydroxylated Warfarin Metabolites by Human Liver Microsomes and Human Recombinant UDP-Glucuronosyltransferases


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Received August 8, 2007; accepted October 4, 2007

ABSTRACT

Our understanding of human phase II metabolic pathways which facilitate detoxification and excretion of warfarin (Coumadin) is limited. The goal of this study was to test the hypothesis that there are specific human hepatic and extrahepatic UDP-glucuronosyltransferase (UGT) isozymes, which are responsible for conjugating warfarin and hydroxylated metabolites of warfarin. Glucuronidation activity of human liver microsomes (HLMs) and eight human recombinant UGTs toward (R)- and (S)-warfarin, racemic warfarin, and major cytochrome P450 metabolites of warfarin (4', 6-, 7-, 8-, and 10-hydroxywarfarin) has been assessed. HLMs, UGT1A1, 1A8, 1A9, and 1A10 showed glucuronidation activity toward 4', 6-, 7-, 8-, and 10-hydroxywarfarin with \( V_{\text{max}} \) values ranging from 59 to 480 \( \mu M/min/mg \) protein. Tandem mass spectrometry studies and structure comparisons suggested glucuronidation was occurring at the C4', C6-, C7-, and C8-positions. Of the hepatic UGT isozymes tested, UGT1A9 exclusively metabolized 8-hydroxywarfarin, whereas UGT1A1 metabolized 6-, 7-, and 8-hydroxywarfarin. Studies with extrahepatic UGT isoforms showed that UGT1A8 metabolized 7- and 8-hydroxywarfarin and that UGT1A10 glucuronidated 4', 6-, 7-, and 8-hydroxywarfarin. UGT1A4, 1A6, 1A7, and 2B7 did not have activity with any substrate, and none of the UGT isoforms evaluated catalyzed reactions with (R)- and (S)-warfarin, racemic warfarin, or 10-hydroxywarfarin. This is the first study identifying and characterizing specific human UGT isoforms, which glucuronidate major cytochrome P450 metabolites of warfarin with similar metabolic rates known to be associated with warfarin metabolism. Continued characterization of these pathways may enhance our ability to reduce life-threatening and costly complications associated with warfarin therapy.

Warfarin (Coumadin) is a coumarin anticoagulant drug that is used worldwide to manage thromboembolic disease. Despite difficulties in effective patient management, which can be attributed to a very narrow therapeutic range, considerable genetic variations, slow onset of action, and many known drug-drug interactions, warfarin remains one of the most commonly prescribed cardiovascular medications (Bauer, 2006; Rettie and Tai, 2006). Warfarin is primarily administered as an oral medication consisting of a racemic mixture of enantiomers. The (S) enantiomer exhibits approximately 2 to 5 times more anticoagulant activity than the (R) enantiomer in humans (Breckenridge, 1977; Chan et al., 1994; Pitsiu et al., 2003). Both enantiomers inhibit the regeneration of reduced vitamin K by blocking vitamin K epoxide reductase complex, thereby reducing the formation of active clotting factors II, VII, IX, and X (Hirsh et al., 2001; Holbrook et al., 2005; Wajih et al., 2005; Rettie and Tai, 2006). It is readily absorbed, and peak systemic concentrations occur within 60 to 90 min (O’Reilly et al., 1963; Holford, 1986; Chan et al., 1994). Approximately 99% of warfarin is systemically bound to serum albumin. The bound state is thought to be pharmacologically inactive and protected from biotransformation and excretion, but formation of the bound state is reversible (Yacobi and Levy, 1977; Chan et al., 1994).

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; GlcUA, glucuronic acid; HLM, human liver microsome; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS/MS, tandem mass spectrometry; MRM, multiple reaction monitoring; P450, cytochrome P450.
Variations in hepatic metabolism are thought to be a major determinant for warfarin response variations. Warfarin is readily oxidized via hepatic cytochrome P450s to produce 4', 6-, 7-, 8-, and 10-hydroxywarfarin. The monohydroxylated derivatives have little anticoagulant activity and are excreted through urine. (S)-Warfarin is predominantly metabolized via CYP2C9 to produce 6- and 7-hydroxywarfarin (Rettie and Tai, 2006). (R)-Warfarin is thought to be predominantly metabolized via CYP1A2, CYP3A4, CYP2C9, CYP2C18, and CYP2C19. CYP1A2 and/or CYP2C19 primarily produce 6-, 7-, and 8-hydroxywarfarin, whereas CYP3A4, CYP2C9, and CYP2C18 produce 4' and 10-hydroxywarfarin (Rettie and Tai, 2006) in the presence of (R)-warfarin. Despite having a vast understanding of warfarin metabolism via cytochrome P450 oxidation, little progress has been made in developing more efficacious anticoagulant drugs or therapeutic strategies that reduce adverse side effects associated with warfarin administration.

Glucuronidation is one of the main phase II metabolic pathways whereby xenobiotics, such as drugs and natural compounds present in the diet, are biotransformed into polycarboxylic conjugates. These conjugates are more water-soluble than the parent compounds and are easily excreted in bile or urine. The reaction is mediated by a family of enzymes, UDP-glucuronosyltransferases (UGTs), that catalyze the transfer of glucuronic acid (GlcUA) from UDP-GlcUA, to a wide range of structurally unrelated molecules bearing hydroxyl, carboxyl, amine, or thiol groups (Mackenzie et al., 2005). In humans, up to 20 different UGT isoforms belonging to the subfamilies 1 and 2 have been characterized following expression of their corresponding cDNA in heterologous cells (Ritter, 2000). Studies have shown that these isoforms present distinct, but frequently overlapping, substrate specificities (Radominska-Pandya et al., 1999). It is known that warfarin, as well as the monohydroxylated derivatives of warfarin, can be potential substrates for phase II conjugating enzymes (Jansing et al., 1992; Guo et al., 2006). However, human data are very limited with regard to warfarin and glucuronidation of hydroxylated warfarin derivatives is catalyzed by several UGT1A isoforms. These reactions are controlled by both hepatic and extrahepatic pathways, as well as specific substrate–enzyme interactions. Future studies continuing to describe these pathways may enhance our ability to reduce warfarin toxicity and develop new anticoagulant therapies.

**Materials and Methods**

**Materials.** All chemicals used for this study were of at least reagent grade. 4'-Hydroxywarfarin, 6-hydroxywarfarin, 7-hydroxywarfarin, 8-hydroxywarfarin, (R)-warfarin, (S)-warfarin, racemic warfarin, and UDP-glucuronic acid were purchased from Sigma-Aldrich (St. Louis, MO). Ethyl alcohol (95%) was purchased from AAPER (Shelbyville, KY). Unless otherwise specified, all other chemicals and reagents were purchased from Sigma-Aldrich. Recombinant human UGT 1A1, 1A3, 1A4, and 1A6–1A10 were produced in baculovirus-infected insect cells as described previously (Kurkela et al., 2003; Kuuranne et al., 2003). These preparations were shown to contain similar amounts of protein by Western blot analysis using an anti-His antibody directed at the His-tag modification present on each of these recombinant isoforms. The human UGT2B7 isoform was expressed in HEK293 cells (Coffman et al., 1997). UGT2B4, -2B15, and -2B17, which primarily catalyze the glucuronidation of bile acids and steroid hormones, rarely glucuronidate drugs and were not investigated. Recombinant human CYP2C9 expressed in baculovirus-infected insect cells was purchased from BD Biosciences (San Jose, CA). HLMs were obtained from a liver donated from a 67-year-old female who died of a stroke on December 31, 1999. Each enzyme tested in this study is known to be active toward substrates specific for that isoform.

**Human Liver and Recombinant UGT Isoform Incubations.** HLM (50 μg) or UGT recombinant membrane protein (5 μg) were incubated in 100 μM Tris-HCl, pH 7.4–7.5/5 mM MgCl2/5 mM saccharolactone with 100 to 2000 μM substrate, in a total volume of 30 μl. Substrates were added in dimethyl sulfoxide with a final concentration of 2%, and controls omitting substrates were run with each assay. No additional detergents or other activators used in the incubations. Reactions were started by the addition of UDP-GlcUA (4 mM) and incubated for up to 180 min at 37°C. The rate of glucuronidation of hydroxywarfarin was linear for up to 3 h (data not shown). The reactions were stopped by addition of 40 μl of ethanol. Each sample was centrifuged at 14,000 rpm for 8 min to spin down the protein, and 60 μl of the supernatant was transferred to an autosampler vial for analysis as described above. All incubations were performed in duplicate, and corresponding error was calculated.

**TABLE 1**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Q1</th>
<th>Q3</th>
<th>CE</th>
<th>EP</th>
<th>DP</th>
<th>CXP</th>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>Hydroxywarfarin glucuronides</td>
<td>501</td>
<td>50–550</td>
<td>25</td>
<td>10</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>Warfarin Glucuronides</td>
<td>485</td>
<td>50–550</td>
<td>25</td>
<td>10</td>
<td>52</td>
</tr>
<tr>
<td>MRM</td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>Warfarin</td>
<td>309</td>
<td>163</td>
<td>45</td>
<td>10</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>Warfarin Glucuronide</td>
<td>485</td>
<td>163</td>
<td>45</td>
<td>10</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>4', 6-, 7-, and 8-Hydroxywarfarin</td>
<td>325</td>
<td>179</td>
<td>20</td>
<td>10</td>
<td>52</td>
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<tr>
<td>3</td>
<td>10-Hydroxywarfarin</td>
<td>325</td>
<td>251</td>
<td>31</td>
<td>10</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>10-Hydroxywarfarin glucuronide</td>
<td>501</td>
<td>251</td>
<td>31</td>
<td>10</td>
<td>52</td>
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<tr>
<td>Neutral loss</td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>Loss of glucuronic acid</td>
<td>Loss of 176</td>
<td>30</td>
<td>10</td>
<td>52</td>
<td>5–20 (2-s cycle)</td>
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</tbody>
</table>

CE, collision energy; EP, entrance potential; DP, declustering potential; CXP, collision cell exit potential; Q1, quadrupole 1; Q3, quadrupole 3; amu, atomic mass unit.
Recombinant Cytochrome P450 Incubations. Recombinant CYP2C9 was expressed from human CYP2C9 (Arg144) cDNA using a baculovirus expression system. Microsomes also contained cDNA-expressed human cytochrome P450 reductase and human cytochrome b5. CYP2C9 (50 pmol) was incubated in 400 μl of a Tris-HCl buffer, pH 7.5, containing 1.3 mM NADP⁺, 3.3 mM MgCl₂, 3.3 mM glucose 6-phosphate, and 0.4 U/ml glucose-6-phosphate dehydrogenase with 100 μM (S)-warfarin. Some incubations also contained 100 μM 8-hydroxywarfarin. All cytochrome P450 reactions were incubated for 60 min at 37°C. Reactions were terminated by the addition of 400 μl of ethanol. Protein was pelleted by centrifugation, and supernatant was transferred to autosampler vials and stored at −80°C before analysis.

High-Performance Liquid Chromatography-UV/Vis Analysis. High-performance liquid chromatography (HPLC) methods were elaborated for the initial separation and identification of warfarin glucuronides. Analyses were performed using an HP 1050 HPLC system equipped with a UV-Vis diode array detector. Instrument operation and data acquisition were controlled through the Agilent ChemStation software package (Agilent Technologies, Santa Clara, CA). Samples were separated using a Supelcosil LC-18 (25 cm × 4.6 mm, 5 μm) column warmed to 37°C. The solvent system consisted of 0.1% acetic acid in water (A) and methanol (B) at a flow rate of 1 ml/min. The separation of warfarin and metabolites was achieved using the following elution gradient: 100% A (5 min), linear gradient from 100% A to 100% B (5–25 min), and 100% B (25–30 min). The column was then re-equilibrated at initial conditions for 10 min between runs. The elution of each warfarin metabolite was monitored at 313 nm. Primary standards for the glucuronidated monohydroxylated warfarin metabolites were not available; therefore, product concentrations were semiquantified using the responses for external standards of each warfarin substrate. It has been shown previously that the addition of the glucuronic acid moiety does

Fig. 1. Glucuronidation activities of human liver microsomes and human recombinant UGTs toward native warfarin and its hydroxylated derivatives. Glucuronidation activities UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, and 1A10 were measured using membrane fractions of recombinant UGTs expressed as His-tag proteins in baculovirus-infected Sf9 insect cells (5 μg). The substrate and cosubstrate (UDP-GlcUA) concentrations were 750 μM and 4 mM, respectively. Specific activities are expressed in micromolar per milligram of protein per minute and shown with S.E.s of the mean based on two experiments.
not alter the extinction coefficients from that of the unreacted substrate (Doerge et al., 2000). A minimum detection limit (0.3 nmol \( \pm \) 9.6% relative standard deviation) was determined by measuring 3 times the S.D. of the 7-hydroxwarfarin low level standard (1 nmol). Detection limit studies were conducted with eight independent analyses conducted over 4 days.

**Steady-state parameters for glucuronidation of warfarin metabolites by UGT isoforms**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>UGT Isoform</th>
<th>( V_{\text{max}} ) ( \mu M/\text{min/mg protein} )</th>
<th>( K_m ) ( \mu M )</th>
<th>( V_{\text{max}}/K_m ) ( \times 10^{-3} \text{ min}^{-1} \text{mg}^{-1} \text{protein} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-OH-warfarin</td>
<td>UGT1A10</td>
<td>0.11 ( \pm ) 0.01</td>
<td>480 ( \pm ) 130</td>
<td>0.23 ( \pm ) 0.06</td>
</tr>
<tr>
<td>7-OH-warfarin</td>
<td>UGT1A1</td>
<td>0.045 ( \pm ) 0.003</td>
<td>59 ( \pm ) 24</td>
<td>0.76 ( \pm ) 0.31</td>
</tr>
<tr>
<td></td>
<td>UGT1A10</td>
<td>0.030 ( \pm ) 0.003</td>
<td>165 ( \pm ) 41</td>
<td>0.18 ( \pm ) 0.05</td>
</tr>
<tr>
<td>8-OH-warfarin</td>
<td>UGT1A8</td>
<td>0.050 ( \pm ) 0.003</td>
<td>225 ( \pm ) 65</td>
<td>0.22 ( \pm ) 0.06</td>
</tr>
<tr>
<td></td>
<td>UGT1A9</td>
<td>0.057 ( \pm ) 0.003</td>
<td>97 ( \pm ) 36</td>
<td>0.59 ( \pm ) 0.22</td>
</tr>
<tr>
<td></td>
<td>UGT1A10</td>
<td>0.78 ( \pm ) 0.03</td>
<td>346 ( \pm ) 57</td>
<td>2.3 ( \pm ) 0.4</td>
</tr>
</tbody>
</table>

*Parameters determined from the fit of initial velocities to a Michaelis-Menten kinetic scheme using the software GraphPad Prism.*

**Fig. 2.** Steady-state glucuronidation of hydroxywarfarins for selected recombinant UGT isoforms. Glucuronidation activities of recombinant proteins were measured by incubating membrane fractions containing recombinant UGT1A10 (5 \( \mu g \)) with increasing concentrations (shown in the figure) of substrate at a constant concentration of UDP-GlcUA (4 mM). Diamonds, 6-hydroxywarfarin incubations; squares, 7-hydroxywarfarin incubations; circles, 8-hydroxywarfarin incubations. Curve fits and kinetic constants were determined using GraphPad Prism 4 software, and the resulting parameters are included in Table 2.
Glucuronidation of Warfarin Metabolites by Human UGTs

**Enzyme Kinetics.** Kinetic parameters and $V_{\text{max}}$ and $K_m$ values, were determined by incubating the UGT membrane protein (5 μg) in the presence of varying concentrations of substrate (100–2000 μM) at a fixed concentration of UDP-GlcUA (4 mM) for 90 min. All other conditions were identical to those of the screening experiments. Assuming Michaelis-Menten kinetics, the parameters were determined using Prism4 software (GraphPad Software, San Diego, CA).

**Statistics.** Cytochrome P450 inhibition studies were analyzed by analysis of variance and tested for significance using a $p$ value of $<$0.05.

**Results**

Glucuronidation of Warfarin and Its Hydroxylated Derivatives by Human Hepatic Microsomes and Recombinant UGT Isoforms. Eight human recombinant UGTs expressed as His-tag proteins in baculovirus-infected SF9 insect cells from the UGT1A family and UGT2B7 overexpressed in HEK293 cells were evaluated for their ability to glucuronidate warfarin and racemic mixtures of hydroxylated warfarin derivatives. Racemic incubations represented clinical dosing because warfarin is usually administered as a racemic mixture of its two enantiomeric forms. Both the $(R)$- and $(S)$-enantiomers of native warfarin were commercially available and tested as specific substrates. During preliminary assessments, a series of screening studies were purposely designed at a high substrate concentration (750 μM) to maximize the potential of forming and identifying any potential glucuronide conjugate. Screening data with $(R)$-, $(S)$-, and racemic warfarin, as well as five racemic monohydroxylated warfarin metabolites (4′-, 6-, 7-, 8-, and 10-hydroxy) are shown (Fig. 1).

Neither enantiomerically pure isofrom of warfarin served as a direct substrate for human UGTs (Fig. 1). This is important new information indicating that native warfarin biotransformation is exclusively dependent on the oxidative metabolism catalyzed by P450s. Two other monohydroxylated warfarin derivatives, 4′- and 10-hydroxy, showed very little or no activity with any isofrom under experimental conditions tested (Fig. 1). The two major products of CYP2C9 metabolism, 6- and 7-hydroxywarfarin, were glucuronidated by UGT1A1, -1A3, and -1A10 and to a lesser extent -1A8 (Fig. 1). UGT1A10 glucuronidated both 6- and 7-hydroxywarfarin with similar efficiency ($<0.04 \mu M/mg$ protein/min). 7-Hydroxywarfarin was a slightly better substrate for UGT1A1 ($<0.06 \mu M/mg$ protein/min), but this isofrom’s activity toward 6-hydroxywarfarin was lower ($<0.01 \mu M/mg$ protein/min). UGT1A3 and -1A8 also glucuronidated 7-hydroxywarfarin with low efficiency ($<0.005 \mu M/mg$ protein/min). 8-Hydroxywarfarin seemed to be the best substrate for glucuronidation (Fig. 1). UGT 1A1, -1A3, -1A8, 1A9, and -1A10 showed activity with 8-hydroxywarfarin, with UGT1A10 showing the highest activity ($>0.6 \mu M/mg$ protein/min). This compound was also a very good substrate for UGT1A8 and -1A9 ($<0.1 \mu M/mg$ protein/min). UGT1A1 and -1A3 had much lower activity toward 8-hydroxywarfarin ($<0.05 \mu M/mg$ protein/min, respectively). Human recombinant UGT1A4, 1A6, 1A7, and 2B7 did not show any measurable activity toward any substrate tested (Fig. 1).

**Kinetic Analysis.** Kinetic analyses revealed UGT1A10 universally accepted the racemic 6-, 7-, and 8-hydroxywarfarins as substrates, whereas the remaining UGTs were more selective (Fig. 2; Table 2). Despite variations in the $V_{\text{max}}$ and $K_m$ values, the catalytic efficiencies toward the 6- and 7-hydroxylated warfarins were similar, whereas the efficiency toward 8-hydroxywarfarin was approximately 10-fold higher (Table 2). Compared with UGT1A10, 7-hydroxywarfarin glucuronidation by UGT1A1 was 4-fold more efficient than a higher $V_{\text{max}}$ and lower $K_m$ (Table 2). The turnover rates for 8-hydroxywarfarin by UGT1A8 and UGT1A9 were similar, although UGT1A9 displayed a 2-fold lower $K_m$, resulting in a more efficient enzyme toward this substrate (Table 2).

![Fig. 3. Reverse phase-HPLC chromatograph of product ion (m/z 501) experiments. Tracings represent organic-soluble metabolites generated during incubation of human liver microsomes with UDP-glucuronic acid (4 mM) and 750 μM of each substrate (green, 4′-hydroxywarfarin; red, 6-hydroxywarfarin; blue, 7-hydroxywarfarin; black, 8-hydroxywarfarin; pink, 10-hydroxywarfarin). No specific metabolites were generated when 10-hydroxywarfarin (750 μM), racemic warfarin (750 μM), (S)-warfarin (750 μM), or (R)-warfarin (750 μM) were used as substrates (data not shown). Each substrate was incubated individually for 180 min. All other incubation conditions are noted under Materials and Methods.](Image)
Product Confirmations/MS Spectral Interpretation.

Metabolite identification by LC-MS/MS confirmed that 4’-, 6-, 7-, and 8-hydroxywarfarin served as substrates for human UGTs, but not 10-hydroxywarfarin, racemic warfarin, (S)-(-)-warfarin, or (R)(+)-warfarin (Figs. 3 and 4). The presence of $m/z$ 325 in product ion analyses suggested the loss of glucuronic acid (Fig. 4), and identification of other fragment ions has been proposed (Fig. 5).

For each of the hydroxywarfarins, formation of two possible regioisomeric products is possible. Glucuronidation can occur at either the 4-hydroxyl group of the original warfarin skeleton or at the P450 oxidation site (C4’, C6-, C7-, and C8-positions). Examination of the MS/MS spectrum for 4’-hydroxywarfarin glucuronide (Fig. 4A) revealed a peak at $m/z$ 339, which corresponds to the mass of the side chain plus glucuronic acid (Fig. 5A). This suggests that glucuronidation occurred at the C4’-position. The MS/MS spectra for 6-hydroxywarfarin glucuronide (Fig. 4B) and 7-hydroxywarfarin glucuronide (Fig. 4C) are virtually identical with regard to the masses of the fragment ions, although there are slight differences in relative abundance of the fragments. The base peak in the MS/MS spectrum appears at $m/z$ 355, which corresponds to loss of the side chain from the glucuronidated hydroxycoumarin skeleton (Fig. 4, B and C). The MS/MS spectrum of 8-hydroxywarfarin glucuronide (Fig. 4D) was different from the spectra of 6- and 7-hydroxywarfarin glucuronide. The base peak of the 8-hydroxywarfarin glucuronide was $m/z$ 325 rather than 355 (Fig. 4D). Although these data allow for conclusive identification of the hydroxywarfarin glucuronides, they do not allow for specific assignments of product regiochemistry.

Hydroxywarfarin glucuronides demonstrated a propensity to undergo in-source fragmentation during MS/MS analysis. MRM experiments (data not shown) and neutral loss studies (data not shown) were designed to assess whether additional metabolites were formed but not identified during product ion scans (Fig. 3). Neither study identified major glucuronidated metabolites other than those observed in product ion scans (Fig. 3). MRM studies showed a small degree of in-source fragmentation.

Fig. 4. Product ion ($m/z$ 501) spectra of the organic-soluble metabolites generated during incubation of human liver microsomes with UDP-glucuronic acid (4 mM) and 750 $\mu$M 4’-hydroxywarfarin (A), 6-hydroxywarfarin (B), 7-hydroxywarfarin (C), and 8-hydroxywarfarin (D). Each substrate was incubated individually for 180 min. All other incubation conditions are noted in Materials and Methods. MS/MS spectra are representative of the major products resolved in Fig. 3. Minor products were also observed in negative controls.
**Cytochrome P450 Warfarin Product Inhibition Study.**

To begin assessing the physiological significance of hydroxywarfarin glucuronidation, 6-hydroxylase activity of CYP2C9 was measured with \((S)\)-warfarin (100 \(\mu\)M) in the presence and absence of 8-hydroxywarfarin (100 \(\mu\)M). Reaction rates were approximately 6.0 ± 4.7 pmol product/nmol P450/min (mean ± S.D.) in the absence of 8-hydroxywarfarin. The addition of 8-hydroxywarfarin (100 \(\mu\)M) significantly inhibited this reaction by approximately 85% (Fig. 6, A and B).

**Discussion**

Phase II metabolism of warfarin and warfarin metabolites is important for proper detoxification and excretion. However, the specific human isozymes involved in glucuronidation and excretion of warfarin and the corresponding cytochrome P450 metabolites have never been characterized. In this report, we evaluate the ability of specific human recombinant UGT isoforms to metabolize warfarin and several hydroxywarfarin derivatives. These studies show that warfarin itself is not a substrate for any of the assayed UGTs, despite a hydroxyl group at the C4-position that could be a potential target for conjugation with glucuronic acid. Rather, warfarin becomes a substrate for human UGTs after being processed via oxidative biotransformation. A possible explanation for the lack of activity at the C4-position may be related to the formation of a cyclic hemiketal isomer within the active site of the UGT. This is analogous to what has been observed with CYP2C9 interactions (Heimark and Trager, 1985; He et al., 1999).

Previous studies support the finding that biotransformation by human UGTs is exclusively dependent on oxidative metabolism catalyzed by P450s. Isolated rat hepatocytes as well as in vivo rodent models produce glucuronides of hydroxywarfarin metabolites (Jansing et al., 1992; Guo et al., 2006). There is also one report indicating that hydroxywarfarin glucuronides are excreted in human urine (Kaminsky...
and Zhang, 1997), which is consistent with our unpublished observations showing that hydroxywarfarin glucuronides are a major metabolite excreted in human urine.

Of all the specific UGTs examined in the current study, the extrahepatic isoform, UGT1A10, exhibits the highest glucuronidation activity toward the largest number of monohydroxylated warfarin derivatives tested. UGT1A10 glucuronidates 4’, 6-, 7-, and 8-hydroxywarfarin at relatively high metabolic rates. UGT1A8, another extrahepatic isoform, has a similar metabolic capacity but exhibits more enzyme substrate specificity compared with the reactivity of UGT1A10. UGT1A8 only metabolizes 7- and 8-hydroxywarfarin. Of the hepatic UGTs tested, UGT1A1 and -1A9 are the only isoforms shown to be involved in the glucuronidation of hydroxywarfarins. It is noteworthy that UGT1A9 metabolizes 8-hydroxywarfarin with the highest efficiency as measured by $V_{\text{max}}/K_m$. UGT1A1 metabolized all three substrates, including the highly efficient glucuronidation of 7-hydroxywarfarin. Even though the extrapolation of $K_m$ and $V_{\text{max}}$ values derived in this study to in vivo processes is difficult, the reported metabolic parameters are comparable and/or much higher than values stated in previous reports measuring warfarin oxidation rates catalyzed by P450s (Rettie et al., 1992; Kaminsky et al., 1993; Sullivan-Klose et al., 1996; Zhang et al., 1999). This comparison as well as the fact that these products are known to be excreted in human urine (Kaminsky and Zhang, 1997) suggests that hydroxywarfarin conjugation via UGTs is important for human detoxification and excretion.

It is very interesting that UGT1A8 and 1A10 react readily with hydroxywarfarin metabolites. UGT1A8 and 1A10 are known to be primarily expressed in human stomach and intestine and are thought to be important for detoxifying xenobiotics (Strassburg et al., 1997; Cheng et al., 1999; Fisher et al., 2001). Data presented in this report are consistent with published reports studying extrahepatic P450 oxidation of warfarin. For example, CYP2C19 is known to be expressed in human intestine and is known to specifically produce 8-hydroxywarfarin in the presence of (R)-warfarin (Rettie and Tai, 2006). The current study shows that 8-hydroxywarfarin is one of the primary metabolites of warfarin.
glucuronidated by UGT1A8 and UGT1A10. This suggests that UGTs expressed in human intestine work in concert with CYP2C19 to preferentially detoxify and remove (R)-warfarin. Perhaps intestinal glucuronidation of (R)-warfarin oxidation products contributes to the lower efficacy of this enantiomer (Breckenridge, 1977; Chan et al., 1994; Pitsiu et al., 2003).

LC-MS/MS analysis of product mixtures confirm that glucuronide conjugation does indeed occur for 4'-, 6-, 7-, and 8-hydroxywarfarin but not for racemic warfarin, (R)-warfarin, (S)-warfarin, or 10-hydroxywarfarin. These data show that warfarin oxidation at the 4'-, 6-, 7-, and 8-position is required for glucuronidation. Product ion scans of the desired monoglucuronides provide MS/MS spectra for species with appropriate mass (m/z 501 for the hydroxywarfarins) and in the case of 4'-hydroxywarfarin glucuronide allow for a specific regiochemistry assignment at the C4'-position. This finding differs from results obtained with in vivo and in vitro rodent models that assigned glucuronidation at the C4-position (Jansing et al., 1992; Guo et al., 2006). The reason for this difference is unknown but is possibly due to differences between rodent and human metabolic systems.

Assignment of product regiochemistry is not as straightforward for the 6-, 7-, and 8-hydroxywarfarin glucurononides. The presence of two reactive hydroxyl groups on the coumarin skeleton (Fig. 5) limits MS/MS spectral information because potential fragment ions have the same mass regardless of which hydroxyl group (4- or 6-, 7-, or 8-hydroxy) is glucuronidated. The lack of UGT activity with warfarin, (R)-warfarin, (S)-warfarin, and 10-hydroxywarfarin (Fig. 1; Jansing et al., 1992) suggests that the hydroxyl group located at the C4-position is sterically hindered and less reactive with human UGTs. Information provided by these structure comparisons suggests that glucuronidation is occurring at the C6-, C7-, and C8-positions. Figure 7 shows a representative scheme indicating which UGTs act on specific hydroxylated warfarin metabolites and where it is thought that this action takes place in humans.

Although it is widely recognized that oxidation by P450s inactivates warfarin, this is the first report suggesting that variations in UGT loci and metabolism need to be considered as important variables while adjusting warfarin dosing algorithms. Our in vitro data showing significant inhibition (approximately 85%) of CYP2C9 catalytic activity toward (S)-warfarin in the presence of 8-hydroxywarfarin highlight the potential importance of this pathway. Thus, CYP2C9 inhibition would be avoided by removing the P450 products through UGT-mediated conjugation with glucuronic acid. Alternatively, it is possible that hydroxywarfarin glucuronides may inhibit P450 activity. Glucuronides inhibiting P450 activity is a relatively new concept and is considered an unexpected phenomenon (Ogilvie et al., 2006). One can also speculate that glucuronidation of warfarin derivatives produces biologically active glucuronides, like the 6-O-glucuronide of morphine (Ritter, 2000). Interactions between glucuronides and specific transporters are another potentially mitigating but unexplored factor in the clearance of these compounds.

This report characterizes human UGTs associated with warfarin metabolism and provides vital information that will be necessary to fully explore the importance of this metabolic pathway. UGT deficiency due to epigenetic factors and/or polymorphisms could significantly alter the glucuronidation of warfarin metabolites and result in a compromised capacity for warfarin metabolism. Overall, these in vitro studies provide insight for understanding the phase II metabolic pathways that glucuronidate cytochrome P450 warfarin metabolites and facilitate their excretion. This study shows that UGT activity is controlled through specific P450 oxidations while adjusting warfarin dosing algorithms. We thank Richard Bonner, Benjamin Jefferson, Suzanne Owen, and Amanda Fincher (Arkansas Department of Health) as well as Bob Kobelski (Centers for Disease Control) for valuable contributions while preparing this manuscript and Grazyna Nowak (University of Arkansas for Medical Sciences) for insightful comments during the preparation of this manuscript.
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