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Identification of Human UGT2B7 as the Major Isoform Involved in the *O*-Glucuronidation of Chloramphenicol

Mei Chen, Barbara LeDuc, Stephen Kerr, David Howe, and David A. Williams

Massachusetts College of Pharmacy and Health Sciences, Boston, Massachusetts (M.C., B.L., S.K., D.A.W.); and Vertex Pharmaceuticals Incorporated, Cambridge, Massachusetts (D.H.)

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ABSTRACT:

Chloramphenicol (CP), a broad spectrum antibiotic, is eliminated in humans by glucuronidation. The primary UGT enzymes responsible for CP *O*-glucuronidation remain unidentified. We have previously identified the 3-*O*-CP (major) and 1-*O*-CP (minor) glucuronides by β-glucuronidase hydrolysis, liquid chromatography-tandem mass spectrometry, and 1D/2D H NMR. Reaction phenotyping for the glucuronidation of CP with 12 expressed human liver UGT isoforms has identified UGT2B7 as having the highest activity for 3-*O*- and 1-*O*-CP glucuronidation with minor contributions from UGT1A6 and UGT1A9. The kinetics of CP 3-*O*-glucuronidation by pooled human liver microsomes (HLMs) exhibited biphasic Michaelis-Menten kinetics with the apparent high-affinity K_{m1} and low-affinity K_{m2} values of 46.0 and 1027 μM, whereas expressed UGT2B7 exhibited Michaelis-Menten kinetics with the apparent K_m value of 109.1 μM. The formation of 1-*O*-CP glucuronide by pooled HLM and expressed UGT2B7 exhibited substrate inhibition kinetics with apparent K_m values of 408.2 and 115.0 μM, respectively. Azidothymidine (AZT) and hyodeoxycholic acid (substrates of UGT2B7) inhibited 3-*O*- and 1-*O*-CP glucuronidation in pooled HLMs. In 10 donor HLM preparations, both CP 3-*O*- and CP 1-*O*-glucuronidation showed a significant correlation with AZT glucuronidation (UGT2B7) (r_s = 0.85 and r_s = 0.83, respectively) at 30 μM CP, whereas no significant correlation was observed between CP 3-*O*-glucuronidation and serotonin glucuronidation (UGT1A6) or propofol glucuronidation (UGT1A9) at this CP concentration. These results suggest that UGT2B7 is the primary human hepatic UDP-glucuronosyltransferase isoform catalyzing 3-*O*- and 1-*O*-CP glucuronidation with minor contributions from UGT1A6 and UGT1A9.

Chloramphenicol (CP) (Fig. 1) is a broad-spectrum antibiotic that has been used to treat serious infections in humans, often when other antibiotics have failed. However, the use of this antibiotic is limited because of its potentially life-threatening side effects, such as aplastic anemia in adults or Gray Baby syndrome in premature and newborn infants (Suhrland and Weisberger, 1963). Despite these dangers, CP is still widely used in developing countries due to the increased cost and decreased availability of newer antibiotics (Brook, 2004).

Although CP has a complex metabolic pattern, glucuronidation has been recognized as the major pathway for adult human elimination with up to 85% of CP appearing in the urine as its glucuronide (Glazko et al., 1950; Glazko, 1966). Nonetheless, the UDP-glucuronosyltransferase (UGT) isoforms responsible for this glucuronidation remain unidentified. CP is rapidly absorbed with an oral bioavailability of 80 to 90%, indicating little presystemic glucuronidation, and with 5 to 15% of CP doses excreted unchanged in the urine. The drug is distributed into all tissues including the brain and spinal cord. Its elimination half-life of 4 to 6 h suggests that no enterohepatic cycling occurs.

The incidence of toxic effects of CP in neonates was found to be strongly related to age, because 9 of 10 babies who showed toxic effects were less than 9 days old (Kaufmann et al., 1981). Subsequent pharmacokinetic studies in these neonates confirmed an elevation of plasma CP levels, suggesting that the parent drug was responsible for the toxic effects of the Gray Baby syndrome (Weiss et al., 1960; Kaufmann et al., 1981). These clinical findings illustrate the importance of understanding the impact of age-dependent developmental differences on the activity of drug-metabolizing liver enzymes and drug clearance, which can markedly alter the pharmacokinetics of a drug in infants compared with adults (Kaufmann et al., 1981; Kearns et al., 2003). Because the mechanisms underlying these age-dependent developmental differences are largely unknown, the identification of the main human liver UGT isoforms involved in drug clearance is crucial to understanding the age-related variations in plasma drug concentration that lead to altered drug efficacy and/or toxicity in infants, which may necessitate an age-based dose adjustment.

Glucuronidation is inefficient at birth (Kaufmann et al., 1981; Kearns et al., 2003). As a result of low UGT isoform activity at birth, the clearance half-life for CP in newborns <3 months postnatal is ~10 h, at 6 months 4 to 6 h, and at 4 years of age 4 to 6 h (Miles, 1983). The reduced glucuronidation and thus slow elimination of CP has been cited as being responsible for the delayed systemic clearance and subsequent toxicity of this drug in newborns (Lambdin et al., 1960; Weiss et al., 1960; Suhrland et al., 1963).

Despite the widespread clinical use of CP for over five decades, the role of the liver in human CP *O*-glucuronidation has not been char-

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ABBREVIATIONS: CP, chloramphenicol; UGT, UDP-glucuronosyltransferase; HLM, human liver microsome; UDPGA, UDP-glucuronic acid; AZT, azidothymidine; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; CL_{int}, intrinsic clearance; CL_{max}, maximum clearance.

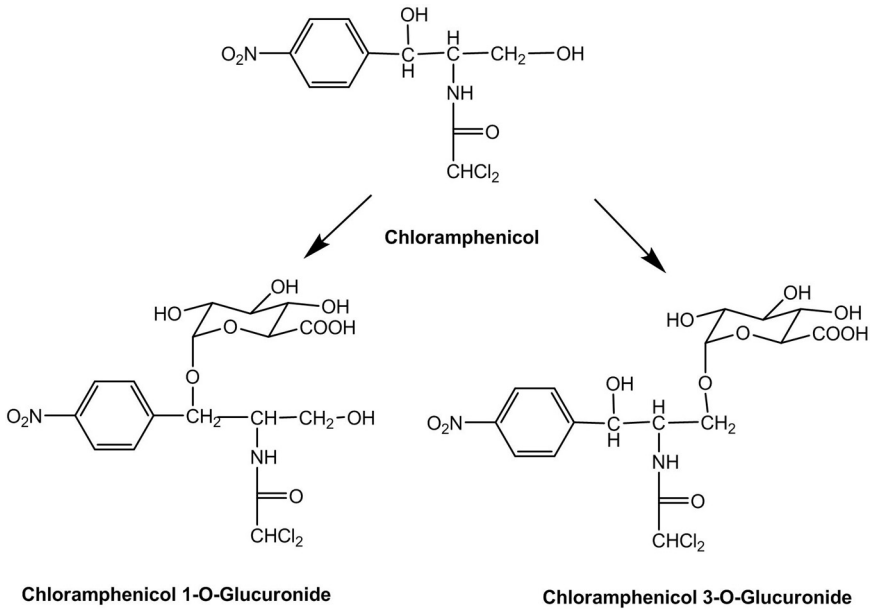

FIG. 1. Chloramphenicol and its major 3-*O*-glucuronide and minor 1-*O*-glucuronides.

acterized. Furthermore, the UGT isoforms contributing to CP *O*-glucuronide formation have yet to be identified. We have previously identified the CP 3-*O*- (major) and CP 1-*O*-glucuronide (minor) conjugates (Chen et al., 2007). The determination of the specific human UGT isoforms responsible for the *O*-glucuronidation of CP could provide evidence into the reasons for the newborn toxicity of CP.

Therefore, in this study we sought to gain insight into the in vivo hepatic CP clearance by 1) phenotyping the specific UGT isoforms involved in the in vitro CP 3-*O*- and 1-*O*-glucuronidation by expressed human UGTs, 2) determining the kinetics of CP 3-*O*- and 1-*O*-glucuronidation in pooled human liver microsomes (HLMs) and by expressed human UGTs, and 3) confirming the contribution of the UGTs for CP *O*-glucuronidation from inhibition studies with UGT-selective substrates as inhibitors and correlation studies in donor human HLMs. We now report our results from phenotyping with expressed UGTs and from inhibition and correlation studies, which indicate that UGT2B7 is the primary UGT isoform catalyzing CP 3-*O*- and 1-*O*-glucuronidation.

Materials and Methods

Chemicals and Reagents. Unless otherwise indicated, reagents including CP, Tris-HCl, UDP-glucuronic acid (UDPGA), magnesium chloride, alamethicin, dimethyl sulfoxide, azidothymidine (AZT), serotonin, propofol, and hyodeoxycholic acid were purchased from Sigma-Aldrich (St. Louis, MO). AZT glucuronide sodium salt was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Pooled mixed gender HLM ($n = 20$) and expressed UGTs are microsomes from baculovirus-infected insect cells expressing human UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, UGT2B17 (Supersomes), and UGT control Supersomes were purchased from BD Gentest (Woburn, MA). The expressed UGT2A enzymes as well as UGT1A5, UGT2B10, UGT2B11, and UGT2B28 were not available from BD Gentest and thus were not tested. The pooled HLMs and expressed enzymes were kept frozen at −80°C and used shortly after receiving the enzymes. The protein content of all Supersomes was 5 mg/ml. Human liver microsomes from 10 individual donors were purchased from BD Gentest. All other chemicals were of reagent grade or of the highest purity available commercially.

Chloramphenicol *O*-Glucuronidation by Pooled HLMs. CP (300 μM) was incubated with alamethicin-activated pooled HLMs fortified with 20 mM UDPGA as described previously (Chen et al., 2007). The CP 3-*O*- and CP 1-*O*-glucuronide concentrations in the incubation mixture were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using biosynthesized CP 3-*O*-glucuronide as the standard (Chen et al., 2007). In brief, the quantitation of the CP glucuronides was achieved by using a LC-MS/MS system, which was equipped with an Agilent model 1100 series HPLC system and a Sciex API 4000 tandem mass spectrometer (Applied Biosystems, Foster City, CA). Liquid chromatography separation was performed on a Synergi Polar-RP column (Phenomenex, Torrance, CA) with a gradient consisting of mobile phase A water/formic acid (100:0.1, v/v) and mobile phase B acetonitrile/formic acid (100:0.1, v/v) at a flow rate 0.5 ml/min. Negative multiple reaction monitoring (MRM) mode was used to monitor CP, CP glucuronides, and warfarin (internal standard) at mass transitions *m/z* of 321.2/152.1 for CP, 497.2/193.1 for CP glucuronides, and 307.1/250.1 for warfarin (Chen et.al., 2007). Incubations without UDPGA or with boiled pooled HLMs were performed simultaneously and served as negative controls. Zero-time, zero-protein, and zero-substrate incubations served as blanks.

Chloramphenicol *O*-Glucuronidation by Expressed Human UGT Isoforms. CP 3-*O*- and 1-*O*-glucuronidation were measured in alamethicin-activated Supersomes expressing human UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15 (all 5 mg of protein/ml), or UGT2B17 (0.5 mg of protein/ml) Supersomes with 30 or 300 μM CP and 20 mM UDPGA. UGT control Supersomes (0.5 mg of protein/ml) served as the negative control. Incubations were done in duplicate, and samples were directly analyzed by the described LC-MS/MS method for pooled HLMs. All of the incubations were performed at 37°C for 50 min.

Enzyme Kinetic Data Analysis. All data points represent the mean of triplicate estimations. The kinetic parameters for CP 3-*O*- and 1-*O*-glucuronidation were calculated by fitting the untransformed experimental data to the following enzyme kinetic equations using Prism 5.2 (GraphPad Software Inc., San Diego, CA), designed for nonlinear regression analysis. The selection of the "best-fit" kinetic model was based on comparison of the sum-of-squared residuals, SD of fit, coefficient of determination (r^2), and *F* test (Prism 5.2).

The Michaelis-Menten equation (eq. 1),

$$v = (V_{max} \times [S])/(K_m + [S]) \quad (1)$$

where v is the rate of metabolite formation, V_{max} is the maximum velocity (as pmol product/min/mg microsomal or cell lysate protein), K_m is the Michaelis constant (substrate concentration at 0.5 V_{max}), and $[S]$ is the substrate concentration.

The two-enzyme Michaelis-Menten equation (eq. 2),

$$v = \{(V_{max1} \times [S])/(K_{m1} + [S])\} + \{(V_{max2} \times [S])/(K_{m2} + [S])\} \quad (2)$$

where K_{m1} and K_{m2} are the apparent high- and low-affinity components and V_{max1} and V_{max2} are the apparent maximum velocities for the high- and low-affinity components, was used when the Eadie-Hofstee plot exhibited a biphasic shape (Houston and Kenworthy, 2000; Court et al., 2001; Yamanaka et al., 2005).

The two-site model (eq. 3) (Houston and Kenworthy, 2000; Uchaipichat et al., 2008),

$$v/V_{max} = (S/K_s + \beta S^2/\alpha K_s^2)/(1 + 2S/K_s + S^2/\alpha K_s^2) \quad (3)$$

where K_s represents the binding affinity and α and β are modifying factors that reflect changes in K_s and K_p (the catalytic rate constant), respectively.

The intrinsic clearance (CL_{int}) was calculated as $CL_{int} = V_{max}/K_m$, and expressed as l/min/mg protein. Enzyme activities were calculated by dividing metabolite concentration by protein concentration and incubation time and expressed as pmol/min/mg protein.

When the Eadie-Hofstee plot exhibited a sigmoidal "U" shaped curve, the Hill equation (eq. 4) was selected for estimating S_{50} and V_{max} (Weiss, 1997):

$$v = V_{max} \times S^n/(S_{50}{}^n + S^n) \quad (4)$$

where S_{50} is the substrate concentration resulting in 50% V_{max} (analogous to K_m in the Michaelis-Menten equation) and n is the Hill coefficient. The maximum clearance (CL_{max}) [$CL_{max} = V_{max} \times (n-1)/\{S_{50} \times n(n-1)^{1/n}\}$] provides an estimate of the highest clearance attained, i.e., when the enzyme is fully activated before saturation occurs (Houston and Kenworthy, 2000).

When the Michaelis-Menten plot showed a hyperbolic shape with no clearly defined plateau at high substrate concentrations and the Eadie-Hofstee plot showed a "hook" curve at high substrate concentrations, the uncompetitive substrate inhibition model (eq. 5) was used (Houston and Kenworthy, 2000; Venkatakrishnan et al., 2001).

$$v = V_{max} \times S/(K_m + S \times (1 + S/K_{si}) \quad (5)$$

where K_{si} is the constant describing the substrate inhibition interaction.

Inhibition of Chloramphenicol *O*-Glucuronidation by AZT, Serotonin, and Propofol. The 3-*O*- and 1-*O*-glucuronidation of CP in pooled HLMs was studied using known selective UGT isoform probe substrates as inhibitors (Miners et al., 2004; Miners et al., 2006). Of the expressed UGT isoforms glucuronidating CP at the 3-hydroxyl position, expressed UGT1A6, UGT1A9, and UGT2B7 exhibited significant activity and were therefore used for the inhibition studies. Because only expressed UGT2B7 exhibited activity for glucuronidating CP at the 1-hydroxyl group, it was used for the inhibition studies. The selective inhibitors were as follows: for UGT1A6, serotonin (Krishnaswamy et al., 2003; Court, 2005); for UGT1A9, propofol (Court et al., 2001; Yamanaka et al., 2005); and for UGT2B7, hyodeoxycholic acid (Pillot et al. 1993; Barre et al., 2007) and AZT (Court et al., 2003). Serotonin and AZT were each dissolved in water, hyodeoxycholic acid was dissolved in 1:1 acetonitrile and water, and propofol was dissolved in dimethyl sulfoxide. The concentration ranges were as follows: for hyodeoxycholic acid or propofol, 0.1 to 1000 μM; for serotonin, 100 to 40,000 μM; and for AZT, 0.1 to 5000 μM. All the incubations of inhibitors were carried out as described above for CP *O*-glucuronidation in pooled HLM. The 3-*O*-CP- and 1-*O*-CP-UGT activities at 100 μM CP in pooled HLMs in the absence of any probe substrate as inhibitor were used as controls. The uninhibited glucuronidation activities were calculated as a percentage of control activity.

Correlation Analysis. The 3-*O*- and 1-*O*-glucuronides produced from 30 or 3000 μM CP concentrations were measured in a bank of HLMs isolated from 10 individual donors, using the same incubation conditions as previously described for CP *O*-glucuronidation by pooled HLM. Other UGT activities measured included propofol glucuronidation (50 μM propofol; UGT1A9 substrate), AZT glucuronidation (2000 μM AZT; UGT2B7 substrate), and serotonin glucuronidation (4000 μM serotonin; UGT1A6 substrate). The concentrations of these probe substrates approximated their K_m values in pooled HLMs, which were 1331 μM for AZT, 90 μM for propofol, and 4854 μM for serotonin (data not shown). AZT, propofol, serotonin, and their glucuronides were analyzed by the previously described LC-MS/MS method for the glucuronidation of CP. The AZT glucuronide was analyzed with a standard curve of AZT glucuronide (Toronto Research Chemicals Inc.) spiked in the incubation matrix by LC-MS/MS in positive ionization MRM mode at mass transitions of m/z 444/268. The concentrations of serotonin, propofol, and their glucuronides in the incubation mixture were determined using the method as described by Yamanaka (2005) and analyzed by the described LC-MS/MS method with serotonin and propofol standard curves. Serotonin and propofol glucuronides were monitored in negative MRM mode at mass transitions of m/z 351/175 and m/z 353/177, respectively.

Correlation analyses between these CP-UGT activities and the other UGT activities in 10 individual donor HLMs were determined by Spearman's rank method. When the r_s value was greater than or equal to 0.5 and the P value was less than 0.05, the correlations were considered statistically significant.

Results

Chloramphenicol *O*-Glucuronidation by Expressed Human UGTs. UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17 were screened for CP 3-*O*- and 1-*O*-glucuronidation activity at 30 μM CP (Ogilvie et al., 2008), the approximate therapeutic plasma concentration (National Toxicology Program, 2002), and at 300 μM CP, the approximate toxic plasma concentration (National Toxicology Program, 2002) (Fig. 2). CP 3-*O*-glucuronidation was observed for UGT1A6, UGT1A9, UGT2B4, and UGT2B7 with activities >1 pmol/min/mg at 30 μM CP concentration. At 300 μM CP concentration, the additional isoforms UGT1A3 and UGT2B17 were observed to catalyze the 3-*O*-glucuronidation of CP (Fig. 2A). The isoforms UGT1A1, UGT1A4, UGT1A7, UGT1A8, UGT1A10, and UGT2B15 did not form the CP 3-*O*-glucuronide. Kinetic studies were not performed with UGT2B4 and UGT2B17 because these enzymes have exhibited low or negligible activity toward drugs and other xenobiotics (Miners et al., 2006). Thus, CP 3-*O*-glucuronidation kinetics were characterized for UGT1A6, UGT1A9, and UGT2B7. As shown in Fig. 2, A and B, UGT2B7 showed the highest CP 3-*O*- and 1-*O*-glucuronosyltransferase activities at 30 and 300 μM concentrations of CP. Negligible activities for the formation of CP 1-*O*-glucuronide were observed for all of the other expressed UGT isoforms.

The Michaelis-Menten model for the formation of the CP 3-*O*-glucuronide by expressed UGT2B7 (Fig. 3A) was best fitted to the experimental data (Fig. 3A, Eadie-Hofstee plot insert), and the derived kinetic parameters were K_m 109.1 $\pm$ 5.2 μM and V_{max} 46.9 $\pm$ 0.4 pmol/min/mg protein and are summarized in Table 1. The apparent CL_{int} for the formation of 3-*O*-CP glucuronide by UGT2B7 is 0.43 l/min/mg protein. The sigmoidal model (Hill equation; Weiss, 1997) for the UGT1A9 catalyzed CP 3-*O*-glucuronidation was best fitted to the experimental data (Fig. 4A, Eadie-Hofstee plot inset), and the derived kinetic parameters for S_{50} were 714.4 $\pm$ 30.2 μM and 41.9 $\pm$ 0.6 pmol/min/mg expressed protein, with a positive Hill coefficient of 1.4, suggestive of positive cooperativity kinetics (Houston and Kenworthy, 2000), and are summarized in Table 1. The apparent CL_{max} for the formation of 3-*O*-CP glucuronide by UGT1A9 is 0.059 l/min/mg protein. Likewise, for UGT1A6-catalyzed CP 3-*O*-glucuronidation, the Hill equation was best fitted to the experimental data (Fig. 4B, Eadie-Hofstee plot inset), and the derived kinetic parameters for S_{50} were 1557.0 $\pm$ 122.0 μM, V_{max} 12.9 $\pm$ 0.4 pmol/min/mg protein, and Hill coefficient of 1.2, with an apparent CL_{max} of 0.0053 l/min/mg protein, and summarized in Table 1.

On the other hand, 1-*O*-CP glucuronidation by UGT2B7 exhibited atypical kinetics characteristic of substrate inhibition when initially fitted to the Michaelis-Menten equation (Fig. 3B, Eadie-Hofstee plot inset) (Houston and Kenworthy, 2000). Thus, the substrate inhibition equation was best fitted to the experimental data, and the derived

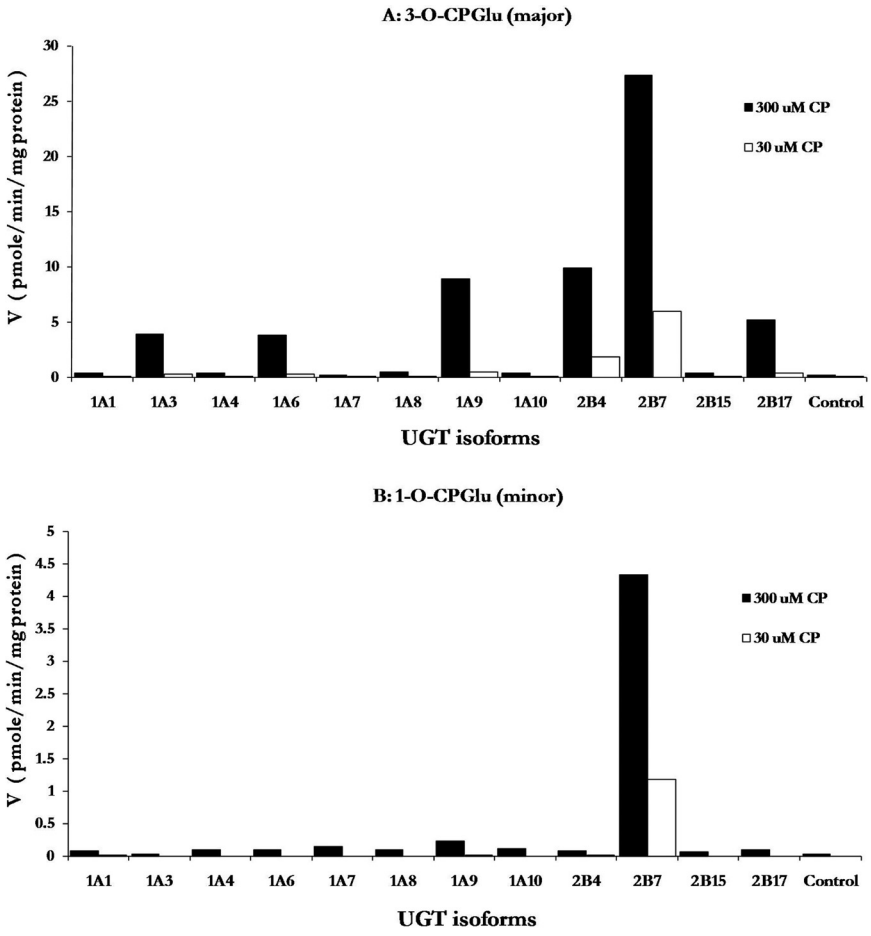

FIG. 2. CP-3-*O*-glucuronidation (A) and CP 1-*O*-glucuronidation (B) at 30 and 300 μM CP by a panel of 12 expressed human UGT isoforms in microsomes from baculovirus-infected insect cells. Each bar represents the mean of duplicate determinations.

kinetic parameters by UGT2B7 were for K_m 115.0 ± 14.2 μM and V_{max} 5.4 ± 0.2 pmol/min/mg expressed protein and are summarized in Table 1. The apparent CL_{int} value was 0.047 l/min/mg protein.

Kinetics of Chloramphenicol *O*-Glucuronidation by Pooled Human Liver Microsomes. CP 3-*O*-glucuronidation by pooled HLM exhibited atypical kinetics was characteristic of biphasic kinetics (Fig. 5A, Eadie-Hofstee plot inset) (Houston and Kenworthy, 2000). Therefore, the glucuronidation data were analyzed using the two-enzyme Michaelis-Menten equation (eq. 2), the two-site model equation (eq. 3), or the Hill equation (eq. 4). The two-enzyme Michaelis-Menten equation was best fitted to the experimental data, and the derived kinetic parameters for the high-affinity components (K_{m1} and V_{max1}) were 46.0 ± 27.0 μM and 37.9 ± 13.0 pmol/min/mg protein, respectively, whereas those for the low-affinity components (K_{m2} and V_{max2}) were 1027.0 ± 129.8 μM and 232.0 ± 11.2 pmol/min/mg protein, respectively, and are summarized in Table 1. The apparent intrinsic clearance CL_{int1} for the high-affinity component of CP 3-*O*-glucuronidation in pooled HLM was 0.82 l/min/mg protein.

CP 1-*O*-glucuronidation by pooled HLM also exhibited substrate inhibition kinetics (Fig. 5B, Eadie-Hofstee plot inset) (Hutzler and Tracy, 2002). The substrate inhibition equation was fitted to the experimental data, and the derived kinetic constants for K_m were 408.2 ± 41.4 μM and V_{max} 16.7 ± 0.6 pmol/min/mg expressed protein (Table 1). The apparent CL_{int} value was 0.041 l/min/mg protein.

Inhibition of Chloramphenicol *O*-Glucuronidation by AZT, Hyodeoxycholic Acid, Serotonin, and Propofol. At 5000 μM, AZT inhibited the formation of 3-*O*-CP-glucuronide by approximately 30% and 76% of 1-*O*-CP glucuronide production by pooled HLM at 100 μM CP (Fig. 6A). When this inhibition study was repeated with expressed UGT2B7, >75% inhibition was observed for CP 3-*O*- and 1-*O*-glucuronidation (Fig. 6A). Hyodeoxycholic acid at 1000 μM exhibited >90% inhibition for both CP 3-*O*-glucuroniude and CP 1-*O*-glucuronide formation in pooled HLMs (Fig. 6B). For the other inhibitors, propofol inhibited CP 3-*O*-glucuronide formation ~60%, but <20% inhibition for CP 1-*O*-glucuronide formation, whereas serotonin exhibited insignificant inhibition (<5%) for both glucuronides by pooled HLMs (data not shown). The control activities of the UGTs for the formation of 3-*O*-CP and 1-*O*-CP glucuronides at 100 μM CP in pooled HLMs were determined to be 25.2 pmol/min/mg and 4.0 pmol/min/mg protein.

Correlation Study in Human Liver Microsomes. Correlation experiments were conducted with pooled alamethicin-activated HLM from 10 human donor livers at 30 and 3000 μM CP concentrations. As shown in Fig. 7, CP 3-*O*- and 1-*O*-glucuronidation in the individual donor HLMs were significantly correlated with AZT glucuronidation activity at 30 and 3000 μM (Fig. 7, A–D) (r_s = 0.85, 0.83 and r_s = 0.65, 0.79, respectively) and summarized in Table 2, but they were not correlated with propofol or serotonin glucuronidation activity at substrate concentration of 30 μM ($r_s < 0.5$), except for propofol glucuronidation activity at 3000 μM CP ($r_s = 0.71$) (Table 2). These results indicate that UGT2B7 is the primary source of 3-*O*- and 1-*O*-glucuronides with a lesser contribution by UGT1A9 at CP concentrations >30 μM.

Discussion

We have previously identified CP 3-*O*- and 1-*O*-glucuronides generated by pooled HLMs as the only glucuronide metabolites in humans (Chen et al., 2007). In this study, we have identified the main

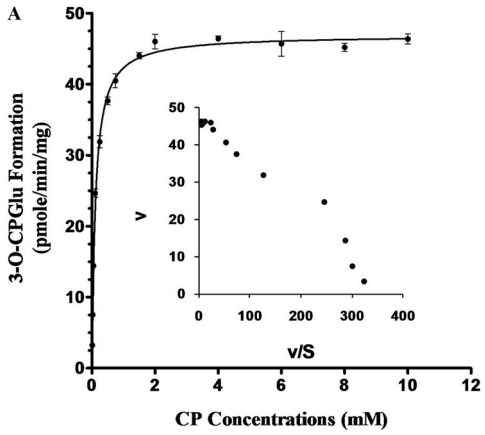

FIG. 3. Kinetics of CP-3-*O*-glucuronidation (A) and CP-1-*O*-glucuronidation (B) by expressed human UGT2B7 in microsomes from baculovirus-infected insect cells. For CP-3-*O*-glucuronidation (A), the solid line represents fitting the data to the single-enzyme Michaelis-Menten equation and the Eadie-Hofstee plot is shown as an inset. For 1-*O*-CP glucuronidation (B), the solid line represents fitting the data to the substrate inhibition model and the Eadie-Hofstee plot is shown as an inset. The CP-3-*O*- and 1-*O*-glucuronidation activity was determined as described under *Materials and Methods*. The kinetic parameters are summarized in Table 1. Each data point represents the mean of triplicate determinations ± S.D.

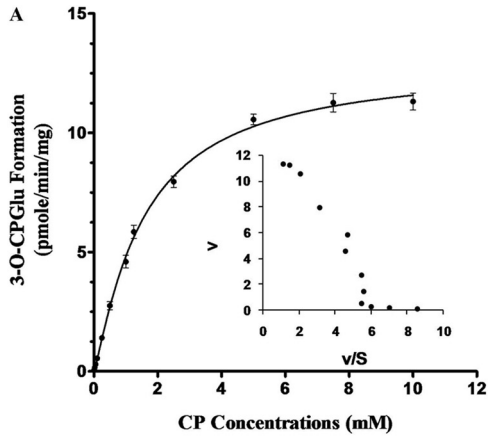

FIG. 4. Kinetics of CP-3-*O*-glucuronidation activity by expressed human UGT1A9 (A) and UGT1A6 (B) in microsomes from baculovirus-infected insect cells represent fitting the data to the Hill equation, and the Eadie-Hofstee plot is shown as an inset. The CP-3-*O*-glucuronidation activity was determined as described under *Materials and Methods*. The kinetic parameters are summarized in Table 1. Each data point represents the mean of triplicate determinations ± S.D.

UGT isoforms responsible for the CP 3-*O*- and 1-*O*-glucuronidations by HLM. Phenotyping with expressed UGT isozymes showed the highest activity for UGT2B7 as the primary UGT isoform catalyzing CP 3-*O*-glucuronidation with lesser contributions from UGT1A6, UGT1A9, and other UGT isoforms at 30 and 300 μM CP. Of the isoforms capable of metabolizing CP at physiological plasma concentrations, UGT1A6, UGT1A9, and UGT2B7 are expressed in the liver and may potentially contribute to its hepatic clearance. However, the low activities for UGT1A6, UGT1A9, and UGT2B4 suggest that involvement of these isoforms is likely to be minor at best. UGT2B7 was the only isoform catalyzing CP 1-*O*-glucuronidation at either CP concentration.

The kinetic profiles for CP 3-*O*-glucuronidation by expressed UGT2B7 are typical of the single-enzyme Michaelis-Menten

TABLE 1

Best-fit-derived kinetic parameters for CP O-glucuronidation by HLM and baculovirus-expressed human UGT isoforms

Each value represents best-fit values ± S.D.

UGT Isoform	*1-O*-CP Glucuronide Kinetics ± S.D. (n = 3)			*3-O*-CP Glucuronide Kinetics ± S.D. (n = 3)					
	K_m [c]	V_{max} [c]	CL_{int} [c]	K_m or S_{50} [e]	V_{max}	CL_{int1} or CL_{max} [b]	K_m	V_{max}	CL_{int2}
	μM	*pmol/min/mg protein*	*l/min/mg protein*	μM	*pmol/min/mg protein*	*l/min/mg protein*	μM	*pmol/min/mg protein*	*l/min/mg protein*
Pooled HLM[d]	408.2 ± 41.4	16.7 ± 0.6	0.041	46.0 ± 27.0[d]	37.9 ± 13.0[d]	0.82[d]	1027 ± 129.8[d]	232 ± 11.2[d]	0.226[d]
2B7	115.0 ± 14.2	5.4 ± 0.2	0.047	109.1 ± 5.2	46.9 ± 0.4	0.43[a]			
1A6		<LOQ		1557.0 ± 122.1[e]	12.9 ± 0.3[e]	0.008[b]			
1A9		<LOQ		714.4 ± 30.2[e]	41.9 ± 0.6[e]	0.059[b]			

<LOQ, below limit of quantitation.
[a] CL_{int} calculated from Michaelis-Menten kinetics.
[b] CL_{max} calculated from Hill equation fitted kinetics.
[c] Best-fit kinetic parameters for 1-*O*-CP glucuronide calculated with substrate inhibition equation K_{si} (HLM) was 28500 ± 8200 μM and K_{si} (UGT2B7) was 8090 ± 1240 μM.
[d] Best-fit K_{m1} and K_{m2} and V_{max1} and V_{max2} kinetic parameters calculated for 3-*O*-CP glucuronide with two-enzyme Michaelis-Menten equation.
[e] Best-fit kinetic parameters for 3-*O*-CP glucuronide calculated using Hill equation.

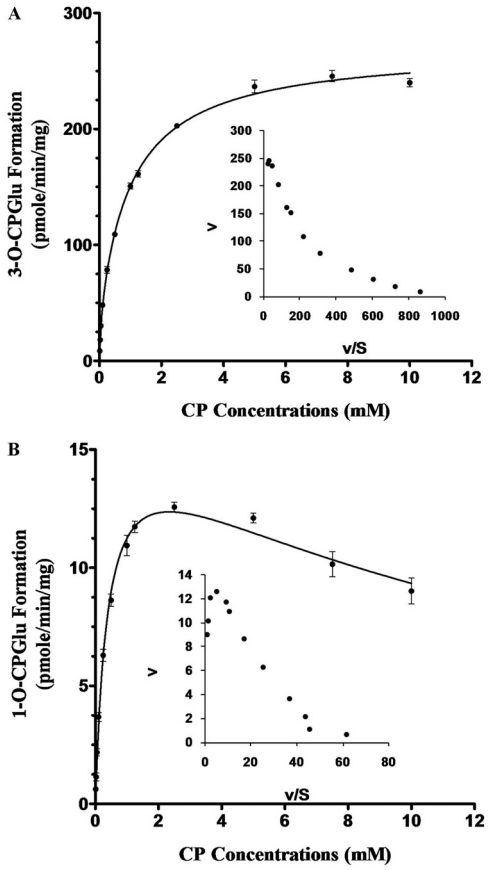

FIG. 5. Kinetics of CP-3-*O*-glucuronidation (A) and CP-1-*O*-glucuronidation (B) in pooled HLMs. For CP-3-*O*-glucuronidation (A), the solid line represents fitting the data to the two-enzyme Michaelis-Menten equation, and the Eadie-Hofstee plot is shown as an inset. For CP-1-*O*-glucuronidation (B), the solid line represents fitting the data to the substrate inhibition model and the Eadie-Hofstee plot is shown as an inset. The CP-3-*O*- and 1-*O*-glucuronidation activity was determined as described under *Materials and Methods*. The kinetic parameters are summarized in Table 1. Each data point represents the mean of triplicate determinations ± S.D.

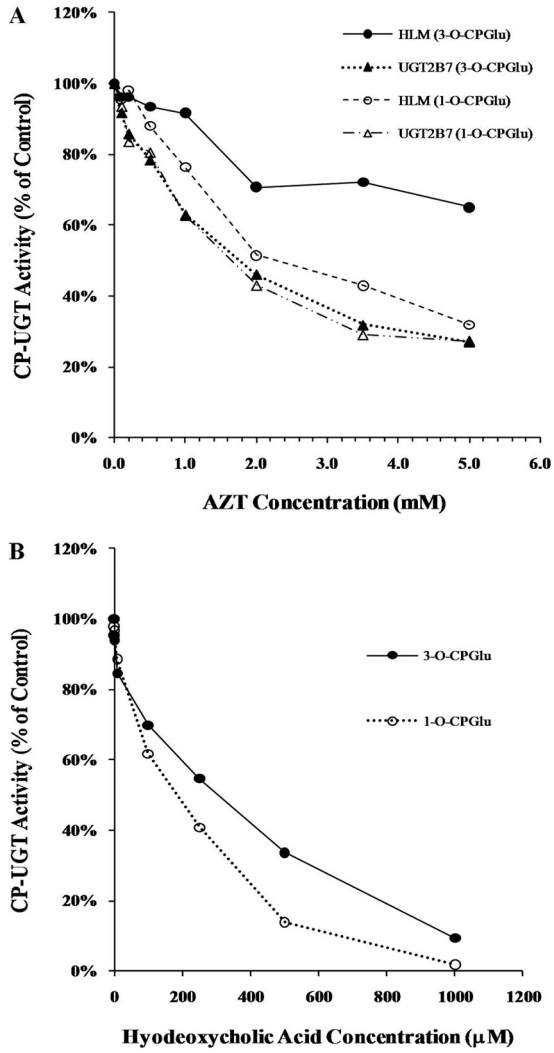

FIG. 6. The inhibitory effects of AZT (A) and hyodeoxycholic acid (B) on the CP 3-*O*- and 1-*O*-glucuronidation activity were determined in pooled HLMs at 100 μM CP. Each data point represents the mean of duplicate determinations.

model, whereas expressed UGT1A6 and UGT1A9 are characteristic of the sigmoidal (Hill) model. CP 1-*O*-glucuronidation by expressed UGT2B7 displayed substrate inhibition, and the kinetics were derived using the substrate inhibition equation. The CL_{int} for 1-*O*-CP glucuronide in pooled HLMs was similar to that for UGT2B7, suggesting that this isoform was the only contributor. The 10- to 20-fold greater CL_{int} value for the CP 3-*O*-glucuronide than that for the 1-*O*-CP glucuronide by pooled HLM and expressed UGT2B7 implies that the 3-OH site of CP is glucuronidated more readily than the more sterically hindered benzylic OH (Fig. 1). The K_m values for CP glucuronidation by expressed UGT1A6 and UGT1A9 were helpful for confirming the minor involvement of these isoforms in CP *O*-glucuronidation.

The kinetics for the formation of CP 3-*O*-glucuronide by pooled HLM were characteristic for the two-enzyme Michaelis-Menten model with high- and low-affinity components. The mean K_m value for the high-affinity component was 20-fold lower than the K_m for the low-affinity reaction, and the apparent Cl_{int} difference was 4-fold. Comparisons of the enzyme kinetic parameters between pooled HLMs and expressed UGT2B7 were made to further determine the relative contribution of UGT2B7 to CP 3-*O*-glucuronidation. A substrate that is glucuronidated solely by a single expressed UGT isoform should have an apparent K_m similar to the apparent K_m for pooled HLM (Court et al., 2003). Based on the results of the present study, the apparent high-affinity K_{m1} value (~46 μM) was similar to the apparent K_m for UGT2B7 (~109 μM), which suggests that in human liver, UGT2B7 may be the predominant UGT isoform catalyzing CP 3-*O*-glucuronidation. When the mean K_m and V_{max} values for the high- and low-affinity components of CP 3-*O*-glucuronidation by HLM were substituted into the two-enzyme Michaelis-Menten equation, the high-affinity component (UGT2B7) is responsible for ~90% of CP 3-*O*-glucuronidation activity at 30 μM and ~18% of activity at 3000 μM CP, and as a result it contributes significantly to the higher CL_{int} value by pooled HLM at low CP concentrations. Hence, CP was 3-*O*-glucuronidated in pooled HLM much more rapidly by UGT2B7 than by either UGT1A6 or UGT1A9, the CL_{int} of which were 10- to 100-fold less, consistent with their minor role in the clearance of CP at therapeutic plasma concentrations. It is likely that the low-affinity component of hepatic CP 3-*O*-glucuronidation, which comprises the minor UGT isoform activities, are also collectively contributing to CP 3-*O*-glucuronidation at CP concentrations >30 μM and would therefore be expected to make minor contributions (<10%) to CP clearance in patients receiving this drug. Furthermore, UGT1A6 and UGT1A9 have S_{50} values of similar order to the low-affinity component of HLM CP 3-*O*-glucuronidation. It should be noted that CP exhibits minor nonspecific binding to pooled HLMs (M. Chen, un-

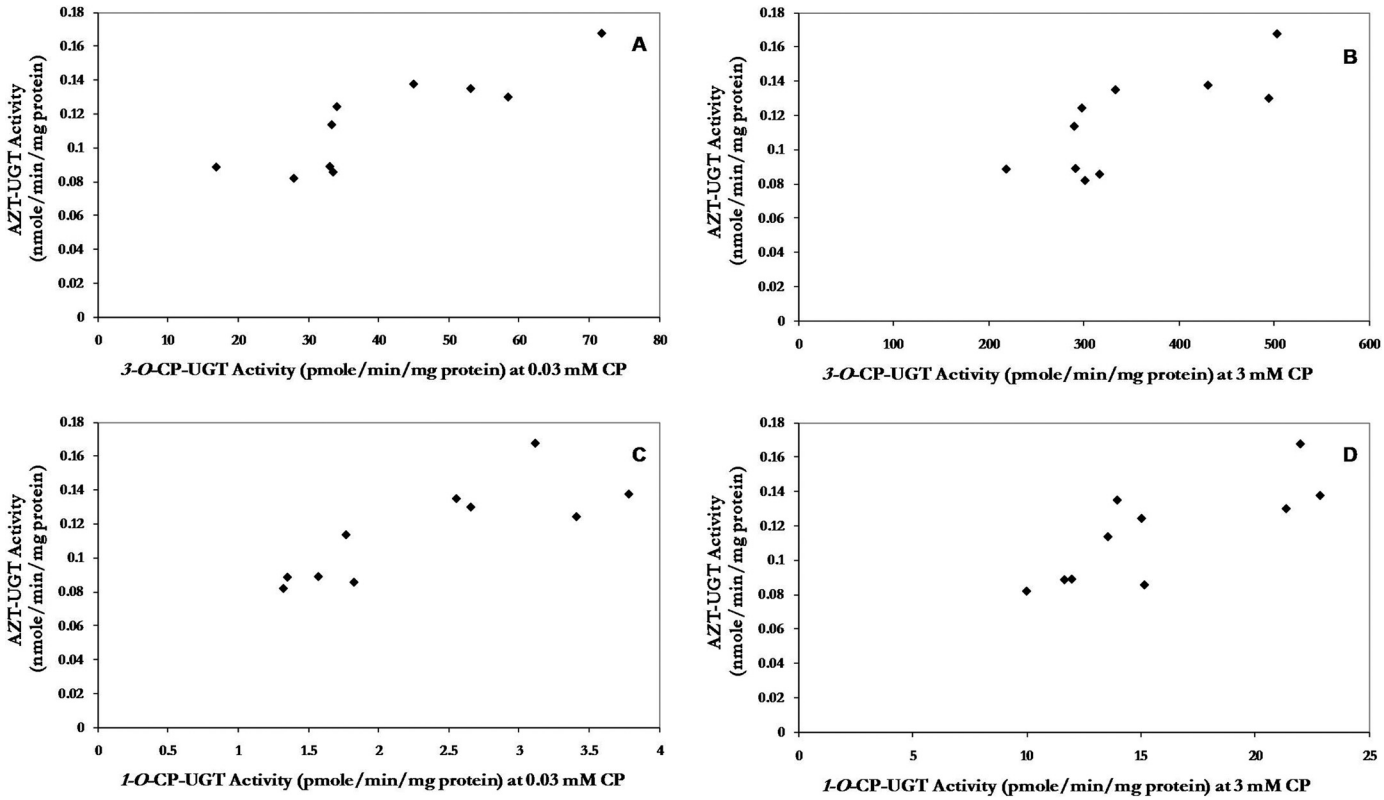

FIG. 7. The correlation between 3-*O*-CP (A and B) and 1-*O*-CP glucuronosyltransferase (C and D) activities in a bank of HLMs from 10 individual donors at 30 and 3000 μM CP and AZT glucuronosyltransferase activities at 2000 μM AZT. Each data point represents the mean of duplicate determinations.

TABLE 2

Correlation of chloramphenicol glucuronosyltransferase activity with the glucuronosyltransferase activities of marker substrates

Marker Substrate Activity	*3-O*-CP UGT Activity				*1-O*-CP UGT Activity			
	CP = 30 μM		CP = 3000 μM		CP = 30 μM		CP = 3000 μM	
	r_s	P	r_s	P	r_s	P	r_s	P
AZT glucuronidation	0.85	0.000[a]	0.65	0.038[a]	0.83	0.000[a]	0.79	0.0038[a]
Propofol glucuronidation	0.44	0.180	0.71	0.018[a]	0.16	0.63	0.31	0.36
Serotonin glucuronidation	−0.01	0.97	0.31	0.36	−0.10	0.76	−0.079	0.81

r_s, Spearman rank order correlation coefficient.
[a] The correlations were considered statistically significant when the r_s value was greater than or equal to 0.50 and the P value was less than 0.05.

published data), precluding nonspecific microsomal binding as a cause of atypical kinetics. CP 1-*O*-glucuronidation in both pooled HLM and UGT2B7 exhibited substrate inhibition kinetics (Hutzler and Tracy, 2002), which implies binding of more than one substrate molecule in the active site.

The coincubation of CP with 5000 μM AZT in pooled HLM was observed as 30 and 76% concentration-dependent inhibition for the 3-*O*- and 1-*O*-glucuronidation of CP, respectively. We attribute the partial inhibition observed for CP 3-*O*-glucuronidation to its inhibition by the long-chain unsaturated fatty acids (oleic acid, linoleic acid, and arachidonic acid) present (as phospholipids) in pooled HLMs, which when released from the microsomal membrane during the course of an incubation, act as potent inhibitors of hepatic UGT2B7 (K_i 0.2–2 μM), competing with AZT (K_m 1331 μM) for the inhibition of hepatic UGT2B7-catalyzed CP 3-*O*-glucuronidation (Tsoutsikos et al., 2004; Rowland et al., 2007). However, when CP was coincubated with AZT and expressed UGT2B7 under the same incubation conditions as for HLMs, >75% inhibition was observed for both CP 3-*O*- and 1-*O*-glucuronidation, consistent with the lower content of inhibitory unsaturated long-chain fatty acids in the expressed UGT2B7 (Tsoutsikos et al., 2004; Rowland et al., 2007), resulting in a decreased inhibition of CP 3-*O*-glucuronidation by the unsaturated fatty acids. Because of the complexity of the 1-*O*-glucuronidation substrate inhibition kinetics of CP by UGT2B7, its inhibition kinetics were not studied further (Tsoutsikos et al., 2004). The 10- to 20-fold greater CL_{int} for UGT2B7-catalyzed *O*-glucuronidation in pooled HLMs and expressed UGT2B7 of the primary 3-hydroxyl group than the more sterically hindered 1-hydroxyl group may also be contributing factors to the observed differences in the inhibition kinetics between CP 3-*O*- and 1-*O*-glucuronidation. When CP is coincubated with hyodeoxycholic acid in pooled HLM, >90% inhibition was shown at 1000 μM hyodeoxycholic acid for both the 3-*O*- and 1-*O*-glucuronidation of CP. Coincubation of CP with propofol demonstrated moderate inhibition (~60%) of CP 3-*O*-glucuronidation and <20% for CP 1-*O*-glucuronidation in pooled HLM and no inhibition (<5%) for serotonin. These results help to support UGT2B7 as the primary UGT isoform catalyzing both CP 3-*O*- and 1-*O*-glucuronide formations in pooled HLMs and UGT1A9 as having a minor role in the *O*-glucuronidation of CP.

To assess the contribution of UGT2B7 to CP 3-*O*- and 1-*O*-

glucuronidation, a correlation analysis was carried out between CP 3-*O*-glucuronidation versus AZT (UGT2B7), propofol (UGT1A9), or serotonin (UGT1A6) glucuronidation by 10 donor HLMs. A significant correlation occurred between CP 3-*O*- and 1-*O*-glucuronidation and AZT glucuronidation at 30 and 3000 μM CP, but only a significant correlation for propofol (UGT1A9) occurred at 3000 μM CP concentration. No correlation was observed for serotonin (UGT1A6) at these CP concentrations. These correlation results help to confirm that UGT2B7 is the likely isoform catalyzing the hepatic 3-*O*- and 1-*O*-glucuronidation of CP at therapeutic concentrations.

When CP was incubated with pooled human kidney microsomes, the apparent CL_{int} for CP 3-*O*-glucuronide was an order of magnitude less than pooled HLMs (data not reported), indicating the contribution of renal 3-*O*-glucuronidation to systemic CP clearance in the neonate is much less than hepatic 3-*O*-glucuronidation.

In summary, our data from incubation of CP with human expressed UGT isoforms, inhibition studies with UGT-selective substrates, and correlation studies clearly indicate that CP was efficiently *O*-glucuronidated to its major CP 3-*O*-glucuronide by pooled HLMs and expressed UGT2B7, and to a lesser extent by UGT1A9, confirming that hepatic 3-*O*-glucuronidation by UGT2B7 plays a major role in the systemic clearance of CP at therapeutic plasma concentrations. As a result of low UGT isoform activity in infants <6 months postnatal, clinical studies have shown that hepatic glucuronidation undergoes significant changes during neonatal development requiring age-related changes in drug therapy and dosages as a result of delayed systemic clearance of the drug. For example, the clearance of AZT by UGT2B7 is approximately 50% lower in neonates than found in children 14 to 99 days of age (Boucher et al., 1993), whereas the clearance of morphine by UGT2B7 in postoperative infants was significantly reduced during the neonatal period and in infants and children under the age of two years, which required an adjustment in the intravenous morphine dosage (Bouwmeester et al., 2003). The results from our study indicate that the clearance of CP primarily by UGT2B7 suggests that the systemic clearance of CP is likely to be minimal in neonates and infants <6 months postnatal, which is consistent with the argument that reduced *O*-glucuronidation of CP is responsible for its delayed clearance and subsequent toxicity in newborns. Available evidence suggests that UGT2B7 polymorphism has little effect on drug glucuronidation (Coffman et al., 1998; Bhasker et al., 2000), but an influence on CP elimination cannot be discounted in this age group. This study highlights the fact that the identification of the liver UGT isoform(s) involved in drug clearance is crucial to understanding the age-dependent variations in plasma drug concentration that lead to altered drug efficacy and/or toxicity in infants, which might require an age-based dosage adjustment.

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Address correspondence to: Dr. David A. Williams, Massachusetts College Pharmacy and Health Sciences, Department of Pharmaceutical Sciences, 179 Longwood Ave., Boston, MA 02115. E-mail: david.williams@mcphs.edu