In Vitro–In Vivo Extrapolation Predicts Drug–Drug Interactions Arising from Inhibition of Codeine Glucuronidation by Dextropropoxyphene, Fluconazole, Ketoconazole, and Methadone in Humans

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ABSTRACT

Because codeine (COD) is eliminated primarily via glucuronidation, factors that alter COD glucuronide formation potentially affect the proportion of the dose converted to the pharmacologically active metabolite morphine. Thus, in vitro–in vivo extrapolation approaches were used to identify potential drug–drug interactions arising from inhibition of COD glucuronidation in humans. Initial studies characterized the kinetics of COD-6-glucuronide (C6G) formation by human liver microsomes (HLM) and demonstrated an 88% reduction in the Michaelis constant (Km) (0.29 versus 2.32 mM) for incubations performed in the presence of 2% bovine serum albumin (BSA). Of 13 recombinant UDP-glucuronosyltransferase (UGT) enzymes screened for COD glucuronidation activity, only UGT1B4 and UGT2B7 exhibited activity. The respective S50 values (0.32 and 0.27 mM) generated in the presence of BSA were comparable with the mean Km observed in HLM. Known inhibitors of UGT2B7 activity in vitro or in vivo and drugs marketed as compound formulations with COD were investigated for inhibition of C6G formation by HLM. Inhibition screening identified potential interactions with dextropropoxyphene, fluconazole, ketoconazole, and methadone. Inhibitor constant values generated for dextropropoxyphene (3.5 μM), fluconazole (202 μM), ketoconazole (0.66 μM), and methadone (0.32 μM) predicted 1.60- to 3.66-fold increases in the area under the drug plasma concentration–time curve ratio for COD in vivo. Whereas fluconazole and ketoconazole inhibited UGT2B4- and UGT2B7-catalyzed COD glucuronidation to a similar extent, inhibition by dextropropoxyphene and methadone resulted largely from an effect on UGT2B4. Interactions with dextropropoxyphene, fluconazole, ketoconazole, and methadone potentially affect the intensity and duration of COD analgesia.

The opioid codeine (COD) is one of the most widely used drugs worldwide. COD is used extensively in the treatment of mild to moderate pain, either alone or in combination with other analgesics. Furthermore, COD is used as an antitusive and for the treatment of diarrhea. It is generally accepted that COD analgesia arises from CYP2D6 catalyzed O-demethylation to form morphine (Somogyi et al., 2007). Approximately 4 to 10% of a COD dose is converted to morphine in CYP2D6-extensive metabolizers (Chen et al., 1991; Yue et al., 1991). Other elimination pathways include glucuronidation, N-demethylation, and renal clearance of unchanged drug. Of these, glucuronidation, to form COD-6-glucuronide (C6G), is the dominant metabolic pathway, accounting for 80 to 85% of the COD dose recovered in urine (Yue et al., 1991).

Accumulating evidence indicates that the relative formation of morphine plays a pivotal role in COD response. In
particular, variability in COD O-demethylation caused by genetic polymorphism of CYP2D6 is known to influence both analgesia and the occurrence of morphine-related adverse effects (Gasche et al., 2004; Somogyi et al., 2007; Madadi et al., 2009). Because glucuronidation is the dominant route of COD metabolism, changes in C6G formation will potentially affect the proportion of the dose metabolized via the O-demethylation pathway and hence the intensity and duration of pharmacological response. However, factors that influence COD glucuronidation in humans are poorly understood.

It has been reported that COD 6-glucuronidation is catalyzed by UGT2B7, with a possible contribution of UGT2B4 (Court et al., 2003). UGT2B7 is arguably the most important drug-metabolizing UGT enzyme in humans (Miners et al., 2010). Apart from COD, UGT2B7 also glucuronidates other opioids (e.g., morphine, naloxone), many nonsteroidal anti-inflammatory agents, valproic acid, and zidovudine. A relatively common coding region polymorphism, UGT2B7*2 (H268Y), seems not to affect the glucuronidation of opioids, including COD (Bhasker et al., 2000; Court et al., 2003). Compelling evidence linking other UGT2B7 variants and opioid disposition and response is similarly lacking (Thorn et al., 2009). In contrast, data from both in vitro and in vivo studies indicate that inhibition of UGT2B7 may potentially result in significant drug–drug interactions (DDIs), with reduced clearance via glucuronidation. For example, DDIs in vivo have been reported between fluconazole and zidovudine (Sahai et al., 1994) and methadone and zidovudine (McCance-Katz et al., 1998), whereas dextropropoxyphene, fluconazole, ketoconazole, methadone, and valproic acid have been shown to inhibit human liver microsomal morphine or zidovudine glucuronidation (Trappnell et al., 1998; Morrish et al., 2005; Takeda et al., 2006; Uchaipichat et al., 2006b).

Our recent studies have demonstrated that the magnitude of an in vivo inhibitory DDI with a UGT2B7 substrate as the object drug may be predicted accurately from an inhibitor constant ($K_I$) generated in vitro when incubations of human liver microsomes (HLM) are conducted in the presence of bovine serum albumin (BSA). Long-chain unsaturated fatty acids released from the microsomal membrane during the course of an incubation act as potent competitive inhibitors of UGT2B7 and UGT1A9, resulting in overestimation of the $K_m$ and $K_I$ values of substrates and inhibitors of these enzymes (Rowland et al., 2007, 2008b). BSA sequesters the inhibitory unsaturated long-chain fatty acids and, as a consequence, $K_I$ (and $K_m$) values are reduced by approximately one order of magnitude compared with data generated in the absence of albumin (Miners et al., 2006, 2010). It is noteworthy that in vitro $K_I$ values obtained in the presence of 2% BSA accurately predicted the magnitude of the fluconazole–zidovudine and valproic acid–lamotrigine interactions in vivo (Rowland et al., 2006; Uchaipichat et al., 2006b).

The primary aim of the present study was to use in vitro–in vivo extrapolation (IV-IVE) to identify potential DDIs resulting in inhibition of COD glucuronidation. In vitro inhibition data were generated by using HLM, with and without BSA, as the enzyme source. Drugs investigated included those previously identified from in vitro and in vivo inhibition studies with UGT2B7 substrates (namely, dextropropoxyphene, fluconazole, ketoconazole, methadone, and valproic acid), and acetaminophen, ibuprofen, and salicylic acid (the primary active metabolite of aspirin), which are marketed as compound formulations with COD for enhanced analgesia. The work additionally sought to confirm the involvement of both UGT2B7 and UGT2B4 in C6G formation and characterize the effect of BSA (2%) on the kinetics of COD glucuronidation in vitro and to assess the relative inhibition of these enzymes by inhibitors of human liver microsomal COD glucuronidation.

### Materials and Methods

**Materials.** Acetaminophen, alamethicin (from *Trichoderma viride*), codeine, BSA, dextropropoxyphene hydrochloride, *R*,*S*-ibuprofen, salicylic acid, UDP-glucuronic acid (UDPGLA; trisodium salt), and valproic acid were purchased from Sigma-Aldrich (Sydney, Australia); C6G was from Toronto Research Chemicals, Inc. (North York, ON, Canada); and Supersomes expressing UGT2B4, 2B7, and 2B15 were from BD Biosciences (San Jose, CA). Fluconazole was obtained from Pfizer Australia (Sydney, Australia); ketoconazole was from Janssen-Cigli Pty Ltd (Sydney, Australia); and *R*,*S*-methadone was from the National Institute on Drug Abuse (Rockville, MD). Solvents and other reagents were of analytical reagent grade.

**Human Liver Microsomes and Expression of UGT Proteins.** Human livers (HL 7, 10, 12, 13, and 40) were obtained from the human liver “bank” of the Department of Clinical Pharmacology, Flinders Medical Centre. Approval was obtained from the Flinders Medical Centre Research Ethics Committee for the use of human liver tissue in xenobiotic metabolism studies. HLM were prepared by differential centrifugation, as described by Bowalgaha et al. (2005). Before use in incubations, HLM were activated by the addition of the pore-forming peptide alamethicin (50 µg/mg protein) with preincubation on ice for 30 min (Boase and Miners, 2002).

UGT1A1, A3, A4, A6, A7, A8, A9, A10, B10, B217, and B28 CDNAs were stably expressed in a human embryonic kidney cell line (HEK293), as described previously (Uchaipichat et al., 2004). After growth to at least 80% confluence, cells were harvested, washed with 0.1 M phosphate-buffered saline, pH 7.4, and lysed by sonication (Heat Systems Ultrasasons, Plainview, NY) using a microtip limit of four, with four 1-s “bursts,” separated by 3 min with cooling on ice. Lysed samples were centrifuged at 12,000 g for 1 min at 4°C, and the supernatant fraction was subsequently separated and stored at −80°C until use. Given the relatively low activity of UGT2B4, B7, and 2B15 expressed in HER293 cells, Supersomes (BD Bioscience) expressing these enzymes were used in activity studies. The use of UGT2B enzymes from this source also allowed direct comparison of data from a previous study of codeine glucuronidation (Court et al., 2003).

Expression of each UGT was demonstrated by immunoblotting with an anti-UGT1A antibody (BD Bioscience), a nonselective UGT antibody (raised against purified mouse Ugt; see Uchaipichat et al., 2004), and an antibody that recognizes UGT2B7 and UGT2B10 (Kerdpin et al., 2009). In addition, activity measurements were performed with the recombinant proteins. Activities of recombinant UGT1A1, A3, A6, A7, A8, A9, A10, B4, B7, B15, 2B17, and 2B28 were confirmed by using the nonselective substrate 4-methylumbelliferone according to a previously published procedure (Rowland et al., 2007). UGT1A4 activity was demonstrated by using trifluoperazine as the substrate (Uchaipichat et al., 2006a), and UGT2B10 activity was confirmed by measurement of cotinine glucuronidation (Kerdpin et al., 2009).

**C6G Glucuronidation Assay.** Microsomal incubations, in a total volume of 100 µl (recombinant enzymes) or 200 µl (HLM), contained phosphate buffer (0.1 M, pH 7.4), MgCl$_2$ (4 mM), UDP-glucuronic acid (5 mM), COD (0.025–10 mM), and activated HLM (1 mg/ml) or recombinant UGT enzyme (1 mg/ml). After preincubation for 5 min, reactions were initiated by the addition of UDPGA (5 mM) and performed at 37°C in a shaking water bath for 60 min (HLM) or 120 min (UGT2B4 and UGT2B7). Reactions were terminated by the addition of HClO$_4$ (70% v/v; 2.5 µl) and cooling on ice for 20 min.
Samples were subsequently centrifuged at 5000g for 10 min at 10°C. A 120-μl aliquot of the supernatant fraction was transferred to a 1.5-ml Eppendorf tube containing 2 μl of KOH (4 M), and a 5-μl aliquot was injected directly into the HPLC column. For reactions performed in the presence of 2% (w/v) BSA, a lower range of COD concentrations (0.01–3 mM) was used as a consequence of the lower KD, in the presence of albumin (see Results). Conditions were as described for incubations performed in the absence of BSA except, because of the higher total protein content, reactions were terminated with 8 μl of HClO4 and the supernatant fraction was treated with 6 μl of KOH. UGT enzyme (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, 2B17, and 2B28) activity screening conditions were conducted at three COD concentrations (0.5, 2, and 10 mM), in the absence and presence of 2% BSA, using the incubation and assay conditions described above. C6G formation was not detected when UDPGA was incubated with lysate from untransfected HEK293 cells or control Supersomes. Likewise, there was no evidence for the formation of a glucoside conjugate when lysate from untransfected HEK293 cells or control Supersomes was incubated with UDPGA (which may contain UDP-glucose as an impurity).

Quantification of C6G Formation. HPLC was performed with an Agilent 1100 series instrument (Agilent Technologies, Sydney, Australia) fitted with a SecurityGuard C18 cartridge (4 × 2 mm; Phenomenex, Sydney, Australia) and a Synergi Hydro-RP C18 column (4 μm, 150 × 3 mm; Phenomenex). The mobile phase was a mixture of 2 mM triethylamine (pH adjusted to 2.7 with HClO4) and water (4 Phamex). The mobile phase was a mixture of 2 M triethylamine (pH adjusted to 2.7 with HClO4) and 14% acetonitrile, delivered at a flow rate of 1 ml/min. Column eluant was monitored by UV absorbance at 205 nm. Retention times of C6G and COD were 2.08 and 2.76 min, respectively. C6G formation in incubation samples was quantified by comparison of peak areas to those of a standard curve prepared over the concentration range 1 to 40 μM. The formation of C6G was linear with incubation times to at least 100 min and micromolar protein concentrations to at least 1.5 mg/ml. Overall within-day assay reproducibility was assessed by measuring C6G formation in nine separate incubations of the same batch of pooled HLM. Coefficients of variation were 2.1 and 2.6% for COD concentrations of 0.5 and 10 mM, respectively.

COD and Inhibitor Binding to HLM and BSA. The binding of drugs (COD and inhibitors) to HLM and to BSA plus HLM was characterized by equilibrium dialysis according to the method of McLure et al. (2000). One side of the dialysis cell contained the drug in phosphate buffer (0.1 M, pH 7.4); whereas the other compartment contained a suspension of either pooled HLM (1 mg/ml) or a combination of 2% BSA and HLM (1 mg/ml) in phosphate buffer (0.1 M, pH 7.4). Drug binding was characterized at five or six concentrations over the ranges shown in Table 1: COD, 25 to 10,000 μM; dextropropoxyphene, 2.5 to 80 μM; ketocozazole, 2.5 to 250 μM; methadone, 0.25 to 12 μM; and valproic acid, 500 to 6000 μM. The dialysis cell assembly was immersed in a water bath maintained at 37°C and rotated at 2 rpm for 4 to 5 h. Control experiments were performed with phosphate buffer or HLM, or a combination of 2% BSA with HLM on both sides of the cell, at low and high drug concentrations, to ensure that equilibrium was attained. A 200-μl aliquot was collected from each cell and treated with 500 μl of ice-cold methanol containing 4% glacial acid or 800 μl of ice-cold acetonitrile (samples containing ketocozazole). Samples were chilled on ice for 20 min and subsequently centrifuged at 13,000g for 5 min at 4°C. An aliquot of the supernatant fraction was analyzed by HPLC.

The HPLC system used was as described previously for the measurement of C6G formation. Chromatography conditions for each analyte are detailed in Supplemental Table 1. Drug concentrations of dialysis samples recovered from each side of the cell were calculated by reference to peak areas of standard curves that spanned both the bound and unbound concentrations of each compound. Binding to incubation components, calculated as the drug concentration in the buffer compartment divided by the drug concentration in the protein compartment, is expressed as the fraction unbound in incubations (fuinc). We have reported microsomal and BSA binding data for fluconazole previously (Uchaipichat et al., 2006b).

Inhibition of COD Glucuronidation. Inhibition experiments with pooled HLM, prepared by mixing equal protein amounts of microsomes from the five livers used in the C6G kinetic studies, were carried out in the absence and presence of 2% BSA. Initial inhibition screening studies were performed at COD concentrations corresponding to the KD for C6G formation (with and without BSA) at four inhibitor concentrations. Subsequent experiments conducted to determine the KD and inhibition mechanism included four inhibitor concentrations (see Results) at each of three COD concentrations: 1, 2, and 4 mM in the absence of BSA, and 0.15, 0.3, and 0.6 mM in the presence of 2% BSA. Inhibitors were added as aqueous solutions, except for ketocozazole and valproic acid, which were dissolved in methanol such that the final concentration of solvent in incubations was 1% v/v. This concentration of methanol has a negligible effect on UGT enzyme activity (Uchaipichat et al., 2004). Experiments that characterized the relative inhibition of recombinant UGT2B4 and UGT2B7 by dextropropoxyphene, fluconazole, ketocozazole, and methadone included 2% BSA. The COD concentration corresponded to the approximate S0.5 for C6G formation by these enzymes (0.3 mM), whereas the inhibitor concentrations corresponded to the KD value corrected for binding to incubation components.

Data Analysis. Data points represent the mean of duplicate estimates (<10% variance). The Michaelis-Menten, substrate inhibition, and Hill equations (see Uchaipichat et al., 2004 for expressions) were fit to kinetic data for C6G formation by using Enzfitter (Biosoft, Cambridge, UK). CTin was calculated as Vmax/Km, KI values for inhibition of COD glucuronidation were determined by fitting the expressions for competitive, uncompetitive, noncompetitive, and mixed inhibition to experimental data by using Enzfitter. Goodness of fit was assessed from comparison of the F statistic, r2 values, parameter standard error estimates, and 95% confidence intervals.

**IV-IVE.** The extent of inhibition of COD hepatic clearance (determined as the ratio of the areas under the plasma COD concentration–time curves with and without inhibitor coadministration) was predicted by using the equation for oral administration of a heptatically cleared drug (Miners et al., 2010);

\[
\frac{AUC}{AUC} = \frac{1}{1 + [I/F_m] + (1 - f_m)}
\]

where \([I]\) is the inhibitor concentration; \(f_m\) is the fraction of COD hepatic clearance via glucuronidation (taken here as 80%; Yue et al., 1991), and \(K_I\) is the inhibition constant generated in vitro. The inhibitor concentration \([I]\) was taken as the maximum hepatic maximum inlet concentration of the drug in vivo (Miners et al., 2010);

\[
[I/IVe] = \frac{[I_{max}] \times F_m \times Dose \times Q_{HF}}{Q_{HF}}
\]

where \([I_{max}], \ F_m, \ \text{and} \ Q_{HF}\) are the maximum total drug concentration in the systemic circulation associated with a given dose.

### Table 1

<table>
<thead>
<tr>
<th>Binding of codeine and inhibitors to human liver microsomes (1 mg/ml) in the absence and presence of bovine serum albumin (2% w/v)</th>
<th>HLM</th>
<th>HLM Plus 2% BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codeine</td>
<td>0.98 ± 0.013</td>
<td>0.96 ± 0.007</td>
</tr>
<tr>
<td>Dextropropoxyphene</td>
<td>0.69 ± 0.029</td>
<td>0.29 ± 0.017</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>1.04 ± 0.050</td>
<td>1.02 ± 0.027</td>
</tr>
<tr>
<td>Ketocozazole</td>
<td>0.27 ± 0.005</td>
<td>0.09 ± 0.003</td>
</tr>
<tr>
<td>Methadone</td>
<td>0.72 ± 0.039</td>
<td>0.56 ± 0.015</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>0.99 ± 0.007</td>
<td>0.29 ± 0.077</td>
</tr>
</tbody>
</table>

* fuinc concentration dependent in the range 2.5 to 20 μM.

* Data taken from Uchaipichat et al., 2006b.

* fuinc concentration dependent in the range 0.5 to 6 mM.
(see Table 6), absorption rate constant, fraction absorbed from the gastrointestinal tract, and liver blood flow (taken as 90 liter/h), respectively. The hepatic maximum unbound inlet concentration was calculated as the product of $I_{\text{inlet, max}}$ and fraction unbound in blood. Maximum hepatic inlet concentrations (total and unbound) were calculated from published pharmacokinetic data for dextropropoxyphene (Welling et al., 1976; Giacomini et al., 1978; Gram et al., 1979; Inturrisi et al., 1982), fluconazole (Sahai et al., 1994; Uchaipichat et al., 2006b), and methadone (Inturrisi et al., 1982; Foster et al., 2004). It was not possible to calculate $I_{\text{inlet, max}}$ for ketoconazole because reliable estimates of $k_{\text{u}}$ and $F_{\text{u}}$ are not available. Thus, IV-IVE was based on the reported maximum concentration of ketoconazole in plasma (total and unbound; Badcock et al., 1987; Daneshmend and Warnock, 1988).

Results

Binding of COD and Inhibitors to HLM and BSA. Nonspecific binding to HLM and binding to HLM plus BSA was characterized here for COD and the putative inhibitors dextropropoxyphene, ketoconazole, methadone, and valproic acid (Table 1). Our previous studies demonstrated that fluconazole does not bind nonspecifically to HLM, and binding of fluconazole to HLM plus 2% BSA is minor (Uchaipichat et al., 2006b). The binding of COD and valproic acid to HLM alone was negligible across the concentration ranges investigated. The binding of COD to the mixture of HLM and BSA (2%) was also minor. Dextropropoxyphene and methadone bound modestly to both HLM and HLM plus BSA. The binding of dextropropoxyphene to the HLM/BSA mixture was concentration-dependent over the range investigated (2.5–20 μM). Ketoconazole bound extensively to both HLM and HLM plus BSA, with mean $f_{\text{u,inc}}$ values of 0.27 ± 0.01 and 0.09 ± 0.01, respectively. Consistent with a previous report (Rowland et al., 2006), the binding of valproic acid to the HLM/BSA mixture was concentration-dependent; $f_{\text{u,inc}}$ values ranged from 0.29 at the lowest valproic acid concentration (0.5 mM) to 0.77 at the highest concentration (6 mM). Where observed, binding of inhibitors to HLM and to HLM plus BSA was accounted for in the calculation of $IC_{50}$ and $K_{i}$ values (i.e., parameters are based on the unbound concentration in the incubation mixture).

C6G Glucuronidation by HLM. Representative kinetic plots for C6G formation by HLM in the absence and presence of 2% BSA are shown in Fig. 1, and derived kinetic constants are given in Table 2. C6G formation by HLM in the absence of BSA was well described by the Michaelis-Menten equation, whereas weak substrate inhibition ($K_{i}$, approximately 40 times higher than $K_m$) was apparent for kinetic studies performed in the presence of BSA (2%). A transition from Michaelis-Menten to weak substrate inhibition kinetics in the presence of BSA has been observed previously for the glucuronidation of zidovudine (Uchaipichat et al., 2006b), another UGT2B7 substrate. The addition of BSA to incubations resulted in an 88% reduction in $V_{\text{m}}$, from 2.32 to 0.29 mM, without an effect on $V_{\text{max}}$ (Table 2). Microsomal $CL_{\text{int}}$ increased in proportion to the change in $K_m$. Kinetic constants for COD glucuronidation by pooled HLM, prepared by mixing equal protein amounts of microsomes from the five separate livers, were similar to

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Without BSA$^{ab}$</th>
<th>With 2% BSA$^{ab}$</th>
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<tbody>
<tr>
<td></td>
<td>$K_m$ mM</td>
<td>$V_{\text{max}}$ pmol/min/mg</td>
</tr>
<tr>
<td>H7</td>
<td>2.35 ± 0.01</td>
<td>440 ± 12</td>
</tr>
<tr>
<td>H12</td>
<td>2.68 ± 0.02</td>
<td>312 ± 0.9</td>
</tr>
<tr>
<td>H120</td>
<td>3.13 ± 0.18</td>
<td>920 ± 20.8</td>
</tr>
<tr>
<td>H13</td>
<td>2.29 ± 0.12</td>
<td>754 ± 13.2</td>
</tr>
<tr>
<td>H400</td>
<td>1.16 ± 0.01</td>
<td>438 ± 0.5</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>2.32 ± 0.73</td>
<td>573 ± 253</td>
</tr>
<tr>
<td>Pooled HLM</td>
<td>2.15 ± 0.13</td>
<td>584 ± 16</td>
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</table>

$^a$ Data presented as mean ± standard error of parameter fit.

$^b$ Kinetic constants derived from fitting with the Michaelis Menten equation.

$^c$ Kinetic constants derived from fitting with the substrate inhibition equation.

$^d$ $CL_{\text{int}}$ calculated as $V_{\text{max}}/K_m$ for both Michaelis-Menten and substrate inhibition kinetics.
the mean data obtained for the separate livers (Table 2). Although COD kinetic parameters were calculated based on the unbound concentration present in incubations, binding of COD to HLM plus BSA was minor (Table 1) and corrected and uncorrected $K_m$ values differed minimally.

**COD Glucuronidation by Recombinant UGT Enzymes.** UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, 2B17, and 2B28 were screened for C6G formation at three COD concentrations (0.5, 2, and 10 mM), both in the absence and presence of BSA (2%). As noted in Materials and Methods, all UGT enzymes except UGT 2B4, 2B7, and 2B15 were expressed in HEK293 cells. Given the relatively low expression of these enzymes in HEK293 cells, Supersomes were used as the recombinant enzyme source. The use of UGT 2B4 and 2B7 from this source further allowed direct comparison of data to a previously published study (Court et al., 2003). Although expression of all UGT enzymes was demonstrated by immunoblotting and activity measurements (data not shown), only UGT2B4 and UGT2B7 catalyzed the 6-glucuronidation of COD. Rates of C6G formation by UGT2B4 for COD concentrations of 0.5, 2, and 10 mM were 6, 34, and 59 pmol/min/mg in the absence of BSA, and 23, 37, and 39 pmol/min/mg in the presence of BSA. For UGT2B7, rates of C6G formation at COD concentrations of 0.5, 2, and 10 mM were 13, 33, and 39 pmol/min/mg in the absence of BSA, and 36, 46, and 48 pmol/min/mg in the presence of BSA. Although it is acknowledged that UGT expression may differ from enzyme to enzyme and between expression systems, the use of positive controls precluded absent activity as a reason for the inability of UGTs other than 2B4 and 2B7 to form C6G.

In contrast to HLM, COD glucuronidation by recombinant UGT2B4 and UGT2B7 exhibited sigmoidal kinetics (Fig. 2), which was modeled by using the Hill equation. Addition of BSA to incubations resulted in approximate 8- and 4-fold reductions in the respective $S_{50}$ values for UGT2B4 and UGT2B7 (Table 3). The respective $S_{50}$ values (0.32 and 0.27 mM) generated in the presence of BSA were comparable with the mean $K_m$ observed in HLM. Although the addition of BSA had no effect on the $V_{max}$ and Hill coefficients for UGT2B7-

![Fig. 2. Kinetic plots for codeine 6-glucuronidation by recombinant UGT2B4 and UGT2B7 generated in the presence and absence of 2% (w/v) bovine serum albumin. A and C, plots of the rate of product (C6G) formation versus substrate concentration for UGT2B7 (A) and UGT2B4 (C). B and D, Eadie-Hofstee plots for UGT2B7 (B) and UGT2B4 (D). Points are experimentally derived values, and curves are from model fitting.](image)

**TABLE 3.** Derived kinetic parameters for codeine glucuronidation by recombinant UGT2B4 and UGT2B7 determined in the absence and presence of 2% (w/v) bovine serum albumin.

<table>
<thead>
<tr>
<th></th>
<th>Without BSA</th>
<th>With 2% BSA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$S_{50}$</td>
<td>$V_{max}$</td>
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<tr>
<td></td>
<td>mM</td>
<td>pmol/min/mg</td>
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<tr>
<td>UGT2B4</td>
<td>2.61 ± 0.001</td>
<td>79 ± 0.02</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>1.07 ± 0.03</td>
<td>49 ± 0.63</td>
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$^a$ $CL_{max}$ calculated as $V_{max}S_{50} \times (n-1)/(n(n-1))^{1/n}$. 

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[44x677]COD Glucuronidation by Recombinant UGT Enzymes. UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B15, 2B17, and 2B28 were screened for C6G formation at three COD concentrations (0.5, 2, and 10 mM), both in the absence and presence of BSA (2%). As noted in Materials and Methods, all UGT enzymes except UGT 2B4, 2B7, and 2B15 were expressed in HEK293 cells. Given the relatively low expression of these enzymes in HEK293 cells, Supersomes were used as the recombinant enzyme source. The use of UGT 2B4 and 2B7 from this source further allowed direct comparison of data to a previously published study (Court et al., 2003). Although expression of all UGT enzymes was demonstrated by immunoblotting and activity measurements (data not shown), only UGT2B4 and UGT2B7 catalyzed the 6-glucuronidation of COD. Rates of C6G formation by UGT2B4 for COD concentrations of 0.5, 2, and 10 mM were 6, 34, and 59 pmol/min/mg in the absence of BSA, and 23, 37, and 39 pmol/min/mg in the presence of BSA. For UGT2B7, rates of C6G formation at COD concentrations of 0.5, 2, and 10 mM were 13, 33, and 39 pmol/min/mg in the absence of BSA, and 36, 46, and 48 pmol/min/mg in the presence of BSA. Although it is acknowledged that UGT expression may differ from enzyme to enzyme and between expression systems, the use of positive controls precluded absent activity as a reason for the inability of UGTs other than 2B4 and 2B7 to form C6G.

In contrast to HLM, COD glucuronidation by recombinant UGT2B4 and UGT2B7 exhibited sigmoidal kinetics (Fig. 2), which was modeled by using the Hill equation. Addition of BSA to incubations resulted in approximate 8- and 4-fold reductions in the respective $S_{50}$ values for UGT2B4 and UGT2B7 (Table 3). The respective $S_{50}$ values (0.32 and 0.27 mM) generated in the presence of BSA were comparable with the mean $K_m$ observed in HLM. Although the addition of BSA had no effect on the $V_{max}$ and Hill coefficients for UGT2B7.
catalyzed COD glucuronidation, the $V_{\text{max}}$ and Hill coefficient obtained for UGT2B4 in the presence of BSA were decreased and increased, respectively, compared with experiments performed in the absence of albumin (Table 3). It should be noted that, in contrast to the known effects of alamethicin on human liver microsomal UGT activities (Boase and Miners, 2002), preliminary experiments showed that preincubation of Supersomes expressing UGT2B4 and UGT2B7 with alamethicin (50 μg/ml protein) had no effect on the rate of COD glucuronidation (data not shown). Thus, alamethicin preincubation of Supersomes expressing UGT enzymes was not routinely performed.

**Inhibition of Human Liver Microsomal COD Glucuronidation.** Experiments conducted to calculate IC$_{50}$ and $K_i$ values used pooled HLM as the enzyme source, with and without 2% BSA. The effects of four concentrations of each putative inhibitor were assessed initially at the COD concentrations corresponding to the approximate mean $K_m$ values for C6G formation in the absence (2 mM) and presence (0.3 mM) of 2% BSA (Table 4). Inhibitor binding to HLM and BSA was accounted for in the calculation of inhibition parameters (IC$_{50}$ and $K_i$). Acetaminophen, fluconazole, ibuprofen, salicylic acid, and valproic acid were weak to moderate inhibitors of COD glucuronidation, with estimated IC$_{50}$ values >2 mM in the absence of BSA (Table 4). Potent inhibition was observed for dextropropoxyphene, ketoconazole, and methadone, with IC$_{50}$ values ranging from 4.5 to 25 μM. Addition of BSA (2%) to incubations typically resulted in an 6- to 12-fold reduction in the IC$_{50}$ (Table 4). It is noteworthy that IC$_{50}$ values for dextropropoxyphene, ketoconazole, and methadone ranged from 0.7 to 2.9 μM. It should be noted that the high binding of ibuprofen and salicylic acid to BSA precluded inhibition studies in the presence of albumin.

Based on data from the inhibition screening studies, kinetic experiments were performed to determine $K_i$ values for dextropropoxyphene, fluconazole, ketoconazole, and methadone. Results are shown in Fig. 3 as Dixon plots. Inhibition data for fluconazole, ketoconazole, and methadone were well modeled by using the expression for competitive inhibition, whereas the equation for noncompetitive inhibition provided the best fit for dextropropoxyphene inhibition of human liver microsomal COD glucuronidation. Consistent with the IC$_{50}$ data, $K_i$ values generated in the presence of BSA were lower (approximately 7- to 15-fold) compared with $K_i$s obtained in the absence of albumin. Potent inhibition of C6G formation was observed for dextropropoxyphene, ketoconazole, and methadone (Table 5).

**Inhibition of UGT2B4- and UGT2B7-Catalyzed COD Glucuronidation.** Effects of dextropropoxyphene, fluconazole, ketoconazole, and methadone on UGT2B4- and UGT2B7-catalyzed C6G formation were determined to assess whether these compounds selectively inhibited the two enzymes involved in COD glucuronidation. Effects of inhibitors were measured in the presence of BSA (2% w/v) at the COD concentration corresponding to the approximate $S_{40}$ value observed for C6G formation by recombinant UGT2B4 and UGT2B7 (namely, 0.3 mM; see Table 3). The concentration of each inhibitor added to incubations corresponded to the $K_i$ value (Table 5) corrected for the binding of the compound to HLM plus BSA (i.e., $K_i$/ff$_{\text{HLM/BSA}}$; see Fig. 4 legend). Although it is acknowledged that binding to HLM and Supersomes may not be identical because of differences in membrane composition, similarities in the predicted and observed inhibition pattern (see below) suggest comparable drug binding between HLM plus BSA and Supersomes plus BSA. Whereas fluconazole and ketoconazole inhibited COD glucuronidation by each enzyme to a similar extent, the inhibition by dextropropoxyphene and methadone arose predominantly via an effect on UGT2B4. By reference to the equations for competitive and noncompetitive inhibition (Segel, 1993) when the substrate and inhibitor concentrations correspond to $K_m$ and $K_i$, respectively, it can be shown that 33% inhibition is expected for a competitive inhibitor whereas 50% inhibition is expected for a noncompetitive inhibitor. The data shown in Fig. 4 are broadly consistent with the degree of inhibition predicted for the competitive (fluconazole, ketoconazole, and methadone) and noncompetitive (dextropropoxyphene) inhibitors of UGT2B4- or UGT2B7-catalyzed C6G formation under the experimental conditions used.

**IV-IVE for DDI Potential.** Predicted changes in the AUC for COD when coadministered with dextropropoxyphene, fluconazole, ketoconazole, or methadone are given in Table 6. Based on total inhibitor concentration in blood for the doses indicated in Table 6, a 60% or more increase in AUC was predicted for the four interactions; increases in the AUC ratio ranged from 1.60- to 3.66-fold. When considered in terms of unbound inhibitor concentration in blood, the magnitude of the predicted interactions with methadone and dextropropoxyphene decreased by approximately 70%, whereas no interaction was predicted with ketoconazole. The predicted change in the AUC ratio based on unbound fluconazole concentration did not change appreciably given the minor plasma protein binding of this compound.

**Discussion**

Initial studies aimed to characterize the effect of BSA on COD glucuronidation by HLM and confirm the contributions of UGT2B4 and UGT2B7 to C6G formation. Kinetic param-
eters for C6G formation in HLM in the absence of BSA were similar to those reported previously by Court et al. (2003). Addition of BSA (2%) to incubations resulted in an 88% reduction in $K_m$ without an effect on $V_{max}$. A similar effect has been reported for the glucuronidation of several other UGT2B7 substrates by HLM (Rowland et al., 2007; Kilford et al., 2009), confirming that $K_m$ or microsomal intrinsic clearance values for UGT2B7 substrates are overestimated and underestimated, respectively, by approximately an order of magnitude when HLM are used as the enzyme source in the absence of albumin supplementation.

Also consistent with previous published data (Court et al., 2003), the screening of 13 recombinant enzymes demonstrated that only UGT2B4 and UGT2B7 glucuronidated...
COD. In contrast to the Michaelis-Menten (or weak substrate inhibition) kinetics observed for C6G formation by HLM, COD glucuronidation by UGT2B4 and UGT2B7 exhibited sigmoidal kinetics. Differences in kinetic behavior between HLM and recombinant UGTs have been observed in other studies (e.g., Bowalgaha et al., 2005). Reasons for the inter-system differences remain unknown, but may reflect membrane effects on protein function (Miners et al., 2006). The difference in the kinetic model (sigmoidal versus hyperbolic) between present and previously (Court et al., 2003) reported data for UGT2B7 may arise from our use of more points at lower substrate concentrations, which favors detection of sigmoidal kinetics. $S_{50}$ values for COD glucuronidation by both UGT2B4 and UGT2B7 were reduced by BSA, and $S_{50}$ values generated in the presence of BSA were close in value. However, in contrast to HLM and UGT2B7, the $V_{\text{max}}$ for UGT2B4 was also reduced by BSA. Although it is not possible to determine the relative contribution of UGT enzymes to a metabolic pathway in the absence of relative protein expression data, it is noteworthy that UGT2B4 mRNA expression in human liver exceeds that of UGT2B7 9-fold (Ohno and Nakajin, 2009). Collectively, these data suggest a significant, perhaps major, contribution of UGT2B4 to human liver microsomal COD glucuronidation. UGT2B4 and UGT2B7 seem to exhibit overlapping substrate selectivities (Jin et al., 1997; Court et al., 2003), although activities toward most aglycones are usually higher with UGT2B7.

Drugs previously identified as potential UGT2B7 inhibitors in either in vitro or in vivo studies (namely, dextropropoxyphene, fluconazole, ketoconazole, methadone, and valproic acid) along with acenaptomen, ibuprofen, and salicylic acid (the primary active metabolite of aspirin), which are marketed as compound formulations with COD for enhanced analgesia, were screened for inhibition of human liver microsomal COD glucuronidation. Acetaminophen, ibuprofen, and salicylic acid inhibited C6G formation to a minor extent only, indicating that inhibition of COD glucuronidation by drugs present in compound formulations is unlikely. In contrast, $IC_{50}$ values generated for dextropropoxyphene, fluconazole, ketoconazole, and methadone in the presence of BSA were in the ranges of plasma concentrations observed for therapeutic doses in vivo. Thus, $K_i$ values were determined for these compounds. As with the $IC_{50}$, $K_i$ values generated from incubations supplemented with BSA (and corrected for binding to HLM and albumin) were 6.5- to 17-fold lower than the corresponding parameters determined in the absence of BSA. A similar effect of BSA was observed in studies of the fluconazole–zidovudine and valproic acid–lamotrigine interactions in vitro (Rowland et al., 2006; Uchaipichat et al., 2006b) and confirm that, like the $K_m$, $K_i$ values for UGT2B7 inhibitors are underestimated when BSA is not present in incubations of HLM. It has been reported recently that fluconazole and ketoconazole are glucuronidated by hepatic UGTs (Bourcier et al., 2010), although a contribution of glucuronidation to dextropropoxyphene and methadone elimination seems not to have been shown to date.

It is noteworthy that dextropropoxyphene, fluconazole, ketoconazole, and methadone differentially inhibited UGT2B4 and UGT2B7-catalyzed C6G formation. Whereas fluconazole and ketoconazole inhibited each enzyme to a similar extent, dextropropoxyphene and methadone inhibition may cause lesser inhibition of drugs that are selectively glucuronidated by UGT2B7. Indeed, $K_i$ values obtained here for fluconazole (from incubations with and without BSA) of human liver microsomal COD glucuronidation were approximately 30% higher than the corresponding $K_s$ we previously reported for inhibition of zidovudine glucuronidation (Uchaipichat et al., 2006b). $K_i$ values determined here for metha-

### Table 5

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ Without BSA</th>
<th>$K_i$ With 2% BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextropropoxyphene</td>
<td>29.1 ± 1.2*</td>
<td>3.55 ± 0.3*</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>1341 ± 0.04</td>
<td>202 ± 0.001*</td>
</tr>
<tr>
<td>Ketoclonazole</td>
<td>11.3 ± 1.5*</td>
<td>0.32 ± 0.03*</td>
</tr>
<tr>
<td>Methadone</td>
<td>2.95 ± 0.05*</td>
<td>0.32 ± 0.03*</td>
</tr>
</tbody>
</table>

* $K_i$ value based on the unbound concentration in the incubation medium (i.e., corrected for binding to HLM and BSA).

### Table 6

<table>
<thead>
<tr>
<th>Drug (In Vivo Dose)</th>
<th>Predicted Fold Increase in AUC Ratio Based On:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Inhibitor Concentration</td>
</tr>
<tr>
<td>Dextropropoxyphene</td>
<td>1.83</td>
</tr>
<tr>
<td>8-hourly(+)</td>
<td>1.60</td>
</tr>
<tr>
<td>Fluconazole (400 mg once daily(+)</td>
<td>2.97</td>
</tr>
<tr>
<td>Ketoclonazole (200 mg once daily)</td>
<td>3.66</td>
</tr>
</tbody>
</table>

(+) Dose calculated as free base where drug was administered as a salt.
(+) Hepatic maximum input concentration, except for ketoclonazole (maximum plasma concentration; see Materials and Methods).
(+) Inturrisi et al., 1982.
(+) Sahal et al., 1994.
(+) Badcock et al., 1987.
(+) Foster et al., 2004.
done and ketoconazole inhibition of COD glucuronidation also differ from inhibition studies with the predominant UGT2B7 substrate morphine, which reported less potent inhibition (Morrish et al., 2005; Takeda et al., 2006). While this may be caused in part by the differing contributions of UGT2B4 and UGT2B7 to COD and morphine (3- and 6-) glucuronidation and the differential inhibition of each enzyme by methadone, binding to HLM was not accounted for in the morphine inhibition studies and effects of BSA were not investigated.

Based on total inhibitor concentration in blood, extrapolation of the $K_i$ values for dextropropoxyphene, fluconazole, ketoconazole, and methadone predicted 1.60- to 3.66-fold increases in the AUC ratio for COD when coadministered with each inhibitor at the doses shown in Table 6. When considered in terms of unbound inhibitor concentration in blood, the inhibition potential of fluconazole was unaltered and a lesser interaction (1.72-fold increase in AUC) was predicted with methadone, whereas predicted inhibitory effects of dextropropoxyphene were minor or negligible. Although there is no consensus whether DDI potential should be predicted based on total or unbound concentration of the perpetrator drug in plasma, optimum prediction of the magnitude of inhibitory interactions involving both cytochrome P450 and UGT substrates is achieved when total maximum hepatic input concentration is used in eq. 2 (Ito et al., 2004; Brown et al., 2005; Rowland et al., 2006; Uchaipichat et al., 2006b; Miners et al., 2010). On this basis, significant DDIs involving inhibition of COD glucuronidation by dextropropoxyphene, fluconazole, ketoconazole, and methadone would be predicted in vivo. Consistent with these predictions, it has been reported in abstract form that plasma COD concentrations were 3-fold higher in subjects receiving methadone compared with those on buprenorphine (Somogyi et al., 2009). Furthermore, the urinary metabolic ratio (COD/C6G) for COD glucuronidation was substantially higher in the methadone-treated subjects. It is noteworthy that the 3-fold increase in COD plasma concentration observed in vivo is close to the 3.6-fold increase in the AUC ratio predicted here (Table 6). In addition to effects on UGT2B7-catalyzed drug glucuronidation, recent in vitro data suggest ketoconazole may inhibit the elimination of drugs cleared by UGT1A1 and UGT1A9 (Yong et al., 2005).

Like the prediction of DDI potential, IV-IVE may be used to determine in vivo hepatic clearance (CLH) and extraction ratio from the experimentally measured microsomal intrinsic clearance, calculated as $\text{CL}_{\text{int}} = V_{\text{max}}/K_m$ (see Miners et al., 2006, 2010 for approach). Estimates of CLH for COD clearance via hepatic glucuronidation were derived with the equation for the well stirred model of hepatic clearance using microsomal data from Rowland et al. (2008a), 0.93 as the fraction of COD unbound in blood, and the mean $K_m$ and $V_{\text{max}}$ values generated here for human liver microsomal COD glucuronidation (Table 2). Predicted hepatic clearances were 0.8 and 6 liter/h for kinetic constants obtained in the absence and presence of BSA, respectively. The approximate 10-fold increase in predicted CLH from in vitro $\text{CL}_{\text{int}}$ values determined from incubations supplemented with BSA is consistent with previous studies kinetic studies of UGT1A9, UGT2B7, and cytochrome P450 2C9 substrates conducted in this and other laboratories (Rowland et al., 2007, 2008a,b; Kilford et al., 2009). Despite this, the extrapolated CLH from experiments performed in the presence of BSA still underpredicts the known in vivo CLH for COD via glucuronidation (approximately 36 liter/h; assuming a systemic clearance of 45 liter/h (Soars et al., 2002) and $f_m = 0.8$; see Materials and Methods). The data are consistent with the 2- to 5-fold underprediction observed for the predicted in vivo clearances of UGT2B7 substrates from in vitro data obtained in the presence of BSA (Rowland et al., 2007; Kilford et al., 2009), but contrasts to the near-exact prediction of in vivo CLH for the UGT1A9 substrate propofol and the cytochrome P450 2C9 substrate phenytoin when in vitro kinetic data generated in the presence of BSA are used for IV-IVE (Rowland et al., 2008a). Because it is believed that the Michaelis constant obtained from experiments with HLM supplemented with BSA reflects “true” hepatocellular $K_m$ (Rowland et al., 2007), other factors such as underprediction of $V_{\text{max}}$, extrahepatic glucuronidation or uptake barriers presumably contribute to the accuracy of in vivo clearance prediction for moderately polar UGT2B7 substrates.

In summary, IV-IVE predicts significant DDIs arising from inhibition of COD metabolic clearance via glucuronidation by coadministered dextropropoxyphene, fluconazole, ketoconazole, and methadone. Available evidence is generally consistent with superior pain relief from COD in CYP2D6-extensive metabolizers, and absent or minor COD analgesia in poor metabolizers (Somogyi et al., 2007). Conversely, the relative conversion of COD to morphine is approximately 2- to 3-fold higher in CYP2D6 ultra-rapid metabolizers compared with extensive metabolizer and this may result in an exaggerated response, including sedation and respiratory depression. Thus, it may be speculated that inhibition of COD glucuronidation by coadministered dextropropoxyphene, fluconazole, ketoconazole, and methadone will potentially result in enhanced and prolonged analgesia caused by increased formation of morphine. Furthermore, marked inhibition of the glucuronidation of high-dose COD could conceivably result in morphine toxicity.

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