STEREOSELECTIVE CONJUGATION OF OXAZEPAM BY HUMAN UDP-GLUCURONOSYLTRANSFERASES (UGTS): S-OXAZEPAM IS GLUCURONIDATED BY UGT2B15, WHILE R-OXAZEPAM IS GLUCURONIDATED BY UGT2B7 AND UGT1A9

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ABSTRACT:
(R,S)-Oxazepam is a 1,4-benzodiazepine anxiolytic drug that is metabolized primarily by hepatic glucuronidation. In previous studies, S-oxazepam (but not R-oxazepam) was shown to be polymorphically glucuronidated in humans. The aim of the present study was to identify UDP-glucuronosyltransferase (UGT) isozymes mediating R- and S-oxazepam glucuronidation in human liver, with the long term objective of elucidating the molecular genetic basis for this drug metabolism polymorphism. All available recombinant UGT isoforms were screened for R- and S-oxazepam glucuronidation activities. Enzyme kinetic parameters were then determined in representative human liver microsomes (HLMs) and in UGTs that showed significant activity. Of 12 different UGTs evaluated, only UGT2B15 showed significant S-oxazepam glucuronidation. Furthermore, the apparent $K_m$ for UGT2B15 (29-35 $\mu$M) was similar to values determined for HLMs (43-60 $\mu$M). In contrast, R-oxazepam was glucuronidated by UGT1A9 and UGT2B7. Although apparent $K_m$ values for HLMs (256-303 $\mu$M) were most similar to UGT2B7 (333 $\mu$M) rather than UGT1A9 (12 $\mu$M), intrinsic clearance values for UGT1A9 were 10 times higher than for UGT2B7. A common genetic variation results in aspartate (UGT2B15*1) or tyrosine (UGT2B15*2) at position 85 of the UGT2B15 protein. Microsomes from human embryonic kidney (HEK)-293 cells overexpressing UGT2B15*1 showed 5 times higher S-oxazepam glucuronidation activity than did UGT2B15*2 microsomes. Similar results were obtained for other substrates, including eugenol, naringenin, 4-methylumbelliferone, and androstane-3α-diol. In conclusion, S-oxazepam is stereoselectively glucuronidated by UGT2B15, whereas R-oxazepam is glucuronidated by multiple UGT isoforms. Allelic variation associated with the UGT2B15 gene may explain polymorphic S-oxazepam glucuronidation in humans.

Oxazepam is a 1,4-benzodiazepine derivative that is used in clinical practice for its anxiolytic, sedative, and anticonvulsant effects (Greenblatt et al., 1983). Oxazepam is formulated as a racemic preparation, with the R- and S-enantiomers having 40% and 60% bioavailability, respectively. The (S,R)-enantiomer (Mohler et al., 1978). Conjugation occurs via the hydroxyl group attached to the asymmetric 3-carbon position yielding diastereomeric glucuronides that are readily separated by routine high pressure liquid chromatography (HPLC) (Mascher et al., 1984; Patel et al., 1995a).

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1 Abbreviations used are: HPLC, high pressure liquid chromatography; HLM, human liver microsomes; UGT, UDP-glucuronosyltransferase; HEK, human embryonic kidney; TBS-T, Tris-buffered saline with 0.2% Tween 20; UDPGA, UDP-glucuronic acid.

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Interindividual variability in the pharmacokinetics and metabolism of (R,S)-oxazepam have been investigated in human volunteers (Patel et al., 1995a). S-Oxazepam glucuronide was found to be formed preferentially over R-oxazepam glucuronide with $S/R$ glucuronide diastereomeric ratios in the plasma and urine of volunteers averaging 3.5 ± 0.6 and 3.9 ± 0.8, respectively. Interestingly, in 2 of 11 subjects (18%), the $S/R$ ratio in the urine was relatively low (<1.9). Since the plasma clearance of oxazepam in these individuals was also very low (<0.6 ml/min/kg) compared with other individuals (0.9–1.4 ml/min/kg), it was concluded that these differences probably were the result of slower S-oxazepam clearance by glucuronidation in a significant minority of the study population (i.e., a “slow metabolizer” phenotype). Although pharmacodynamic measurements were not made, the relatively slow elimination of oxazepam would be expected to result in prolonged sedation in these individuals.

In vitro studies using human liver microsomes (HLMs) showed a similar picture in that S-oxazepam glucuronide was the predominant metabolite ($S/R$ ratios averaging 4.0), and 4 of 37 livers displayed relatively slow oxazepam glucuronidation activities coinciding with low $S/R$ metabolite ratios (<2.0) (Patel et al., 1995a). Enzyme kinetic analysis showed that the low glucuronidation activity was associated with higher apparent $K_m$ values and lower $V_{max}$ values for S-oxazepam glucuronidation in the four atypical livers compared with the other livers. A genetic polymorphism in the gene encoding for the
enzyme mediating this biotransformation was proposed to explain this phenomenon.

UGT isoforms that mediate stereoselective glucuronidation of either 3'- or 3'-oxazepam in human liver have not yet been identified. 3'- and 3'-oxazepam glucuronidation formation by HLMs was shown to be differentially inhibited by a range of compounds suggesting that different enzymes catalyze the formation of the 2 metabolites (Patel et al., 1995b). 3'-Oxazepam glucuronidation was selectively inhibited by ketoprofen and morphine, which are known to be glucuronidated by UGT2B7, suggesting involvement of this isoform. However, the selectivity of these compounds as inhibitors of UGT2B7 has not been verified. Two other studies have investigated the possible role of UGT2B7 in oxazepam glucuronidation using recombinant enzyme (Jin et al., 1993; Coffman et al., 1998). In both instances very low oxazepam glucuronidation activities were reported, and both 3'-oxazepam and 3'-oxazepam were glucuronidated to an equal extent. Together these findings indicate that UGT2B7 may be involved in oxazepam glucuronidation but is not responsible for stereoselective conjugation of either 3'- or 3'-oxazepam.

The aim of the present study was to identify UGT isoforms mediating stereoselective glucuronidation of 3'- and 3'-oxazepam in liver using recombinant enzymes and HLMs. Recombinant allelic variants (UGT2B15*1 and UGT2B15*2) of UGT2B15, the principal isoform found to mediate 3'-oxazepam glucuronidation, were also used to evaluate whether this highly prevalent polymorphism could account for interindividual variability in 3'-oxazepam glucuronidation.

Materials and Methods

Reagents. Unless otherwise indicated, most chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile was from Fisher Scientific Co. (Fairlawn, NJ). Oxazepam and oxazepam glucuronide were gifts from Wyeth Pharmaceuticals (Philadelphia, PA). The oxazepam was a racemic mixture of 3'- and 3'-enantiomers as is found in clinical preparations. The oxazepam glucuronide was also a mixture of the two enantiomers that contained 86% 3'-oxazepam glucuronide and 14% 3'-oxazepam glucuronide by HPLC analysis.

Recombinant UGTs. Homogenates from baculovirus-insect cell expressed UGTs A1A, A1A, A4A, A6A, 1A9, B27, B215*1-BV and were obtained from BD Gentest (Woburn, MA), whereas UGTs 1A7 and 1A10 were from PanVera Corp. (Madison, WI). UGTs 2B4, 2B10, 2B17, and two UGT2B15 allelic variants (UGT2B15*1-HK and UGT2B15*2-HK) were obtained by stable expression in HEK-293 cells and membrane fractions were prepared as previously described (Guillemette et al., 2000). Briefly, cells were harvested using trypsin-EDTA treatment to release the cell monolayer, collected in centrifuge tubes, centrifuged at 500 g, and resuspended in ice-cold phosphate-buffered saline. After repeating the wash step, the cell pellets were resuspended in 0.25 M sucrose, homogenized using a Potter-Elvehjem glass homogenizer, and centrifuged at 20,000 g for 30 min to remove nuclei and other particulates. The membrane fractions were then collected by sedimentation at 100,000 g for 1 h, followed by resuspension of the membrane pellet in buffer containing 0.1 M potassium phosphate (pH 7.5), 1 mM EDTA, and 20% glycerol. Protein concentrations were determined by the bicinchoninic acid method (Smith et al., 1985). Protein concentrations were thawed once immediately prior to use. The quality of the liver samples used was ascertained by reference to at least 10 UGT and cytochrome P450 enzyme activities measured in this laboratory using the same set of liver samples. Livers with activities that were consistently less than 50% that of the median activity value for the entire liver set were excluded from the study.

Oxazepam Glucuronidation Assay. An in vitro UGT activity assay using oxidized oxazepam as a substrate was developed based on methods previously used in this laboratory (von Moltke et al., 1993; Court and Greenblatt, 1997a,b). Incubations (250 ml for HLMs, 100 ml for expressed UGTs) were performed at 37°C in 50 mM phosphate buffer (pH 7.5) with 5 mM MgCl2, 5 mM UDPGA, and (S,R)-oxazepam (10–1000 mM). Alamethicin was also included in incubations in an amount (50 lM of amelaminic/mg microsomal protein) determined in preliminary experiments using HLMs and UGT2B15 to result in maximal activation (100–200% increase in activity for HLMs; 50–100% increase for UGT2B15). Incubation time (up to 360 min) and protein concentration (up to 0.8 mg/ml) were also established in preliminary studies with both HLMs and expressed UGTs to be within initial linear rate conditions. Typically, protein concentration was 0.5 mg/ml, and incubation time was 300 min. Incubations were terminated by addition of acetonitrile (50% incubation volume), vortexed, and placed on ice. After addition of internal standard (1–2 lM of oxazepam), samples were centrifuged, and the supernatants were analyzed by HPLC.

HPLC apparatus (model 1100; Agilent, Palo Alto, CA) consisted of an autoinjector, binary pump, column, and UV absorbance detector set at a wavelength of 214 nm. Chromatographic separation of the oxazepam glucuronide enantiomers was possible using a C8 column (4.6 mm × 25 cm, 10 mm, Luna; Phenomenex, Torrance, CA). The mobile phase was 20 mM, pH 4.5, potassium phosphate buffer in water (solution A) and acetonitrile (solution B). The solvent program consisted of an initial isocratic mobile phase mix (25% solution B) for 15 min, followed by a linear gradient from 25 to 60% solution B over 10 min. Approximate analyte retention times were 8 min for similar for all of the expressed UGT1A isoforms and for UGT2B7 and UGT2B15*1-BV.

Relative UGT2B15 protein content of UGT2B15*1 and UGT2B15*2 in membrane fractions from HEK-293 cells was determined by semiquantitative immunoblotting using the anti-human UGT2B antibody (EL-93), as previously described (Guillemette et al., 2000). Briefly, membrane proteins were resolved by electrophoresis through SDS-10% polyacrylamide gels and electroblotted onto nitrocellulose membranes. After the blocking step, blots were incubated at room temperature in UGT2B antisera diluted 1:2,000 in Tris-buffered saline with 0.2% Tween 20 (TBS-T) containing 5% dry milk. Blots were washed and then incubated overnight in a solution containing the secondary antibody (horseradish peroxidase-conjugated rabbit antimouse IgG; Amersham Biosciences Inc., Piscataway, NJ) diluted 1:20,000 in TBS-T containing 5% blocking reagent. Blots were washed extensively with several changes of TBS-T prior to detection of horseradish peroxidase. Immunocomplexes were visualized using enhanced chemiluminescence (Amersham Biosciences Inc.), exposed on hyperfilm for 30 s (Eastman Kodak Co., Rochester, NY), and quantified by BioImage Visage 110s (Genomic Solution Inc., Ann Arbor, MI). Blots were also probed for a second endoplasmic reticulum resident protein (Calnexin) to ascertain equal loading efficiency as described previously (Guillemette et al., 2000).

Human Liver Microsomes. Liver tissue from three donors were randomly selected from frozen blocks maintained at the Division of Clinical Pharmacology, Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston. Donors included a 36-year-old white male (LV12), a 74-year-old white male (LV21) and a 66-year-old white male (LV24). Tissues were from livers that had been donated for transplantation but had failed to match (LV21) or were from apparently normal tissue adjacent to tumors that were being surgically removed (LV21 and LV24). The donor of LV21 had a history of cigarette smoking, whereas the donor of LV12 had received morpheine. Pooled liver microsomes were also obtained by combining equal amounts of protein from HLMs prepared from 55 different donors.

Microsomes were prepared by differential centrifugation as previously described (Court and Greenblatt, 1997b). The resultant pellet was reconstituted in 20% glycerol/phosphate buffer, aliquoted, and stored at −80°C. Protein concentrations were measured by the bicinchoninic acid assay method (Smith et al., 1985). Frozen microsomes were thawed once immediately prior to use. The quality of the liver samples used was ascertained by reference to at least 10 other UGT and cytochrome P450 enzyme activities measured in this laboratory using the same set of liver samples. Livers with activities that were consistently less than 50% that of the median activity value for the entire liver set were excluded from the study.
Panel A shows a typical chromatogram of the purified glucuronide standards used for quantitation. The remaining panels show chromatograms of injectates from incubations performed in the presence of UDPGA, MgCl₂, oxazepam, and either HLMs from LV21 (panel B), UGT2B15*1-BV (panel C), UGT2B7 (panel D), and UGT1A9 (panel E). (R,S)-Oxazepam concentration was 100 μM except for UGT2B7 (panel D) in which it was 1000 μM (R,S)-oxazepam. Note the higher y-axis scale on panel B. Column effluent was monitored with a UV absorbance detector set at 214 nm. Phenacetin was used as the internal standard. Other details are given under Materials and Methods. mAUFs, arbitrary UV absorbance units.
R-oxazepam glucuronide, 9 min for S-oxazepam glucuronide, 17 min for phenacetin, and 25 min for oxazepam. Glucuronide enantiomer peaks were identified by relative retention time (R-eluting before S-oxazepam glucuronide) and relative peak size (S-approximately 5 times larger than the R-oxazepam glucuronide peak) in HLM incubates, as previously described (Fig. 1) (Patel et al., 1995a,b). Metabolite concentrations in the incubate were determined using a standard curve of peak area ratios (normalized to the internal standard) and generated by HPLC analysis of a series of known concentrations (0.1–50 ng/ml) of purified R- and S-oxazepam glucuronide dissolved in incubation buffer. Enzyme activities were then calculated by dividing metabolite concentration by protein concentration and incubation time and expressed as picomoles per minutes per milligram of protein. Intra- and interassay variability was less than 10 and 15%, respectively. The lower limit of quantitation for this assay was 0.1 pmol/min/mg of protein (signal to noise ratio of 4:1). All experiments were conducted in duplicate and results given as a mean value, except for comparisons of expressed UGT2B15*1 and UGT2B15*2, which were conducted in triplicate and results given as a mean ± standard deviation.

Enzyme Kinetic Analysis. For enzyme kinetic studies of oxazepam glucuronidation, substrate concentration (S) and velocity (V) data were fitted to the appropriate model (Venkatakrishnan et al., 2001) by nonlinear least-squares regression. The models were chosen initially based on the appearance of Michaelis-Menten and Eadie-Hofstee plots. These included the simple Michaelis-Menten model (eq. 1), the substrate activation model (eq. 2), and the competitive substrate inhibition model (eq. 3):

\[ V = \frac{V_{\text{max}} \times S}{K_m + S} \]  
\[ V = \frac{V_{\text{max}} \times S}{K_m + S + S^2} \]  
\[ V = \frac{V_{\text{max}} \times S}{K_m + S + (1 + S/K_m)} \]

where \( V_{\text{max}} \) is the maximal velocity, \( K_m \) is the substrate concentration at half-maximal velocity, \( N \) is an exponent indicative of the degree of curve sigmoidicity, and \( K_c \) is an inhibition constant. The model chosen to best represent the data were based on a number of criteria, including visual inspection of data plots (Michaelis-Menten and Eadie-Hofstee), distribution of residuals, size of the sum of squared residuals, and the standard error of the estimates. Kinetic parameters are given as an estimate ± the standard error of the estimate.

Other Glucuronidation Activities. Activities were also measured with microsomal fractions prepared from UGT2B15 allelic variants expressed in HEK-293 cells and pooled human liver microsomes using eugenol, naringenin, 4-methylumbelliferone, dihydrotestosterone, and androstane-3α-diol as substrates. Reactions typically contained 50 mM Tris, pH 7.5; 6 mM MgCl₂; 6 mM d-saccharo-1,4-lactone; 500 μM UDPGA containing 0.2 mCi ³H-UDPRA (PerkinElmer Life Sciences, Boston, MA); 0.4 to 0.6 mg/ml microsomal protein; and 200 μM aglycone concentration. Reaction mixtures were incubated with microsomes for 2 h at 37°C. Assays were terminated by addition of 100 μl of methanol. After centrifugation of samples, 100 μl was applied onto thin layer chromatography plates (0.25-mm-thick silica gel, Whatman, Maidstone, UK) and chromatographed in a mixture of toluene/methanol/acetic acid (7:3:1). Thin layer chromatography plates were exposed for 24 h, and the extent of glucuronidation was assessed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The lower limit of quantitation for this assay was approximately 1 pmol/min/mg of protein. Assays were conducted in triplicate and results given as a mean ± standard deviation.

Results

Oxazepam Glucuronidation by Expressed UGTs. All available expressed UGTs were screened for oxazepam-UGT activity using 100 μM and 1 mM substrate concentration (Fig. 1). At 100 μM oxazepam concentration UGT2B15 (+1–BV) was the predominant isofrom mediating S-oxazepam glucuronidation with more than 10 times greater activity than any other isofrom (Fig. 2A). Relatively low activities were observed for UGT1A1 and 1A6 but not for any other isofrom. Although UGT2B15 mediated S-oxazepam glucuronidation activity appeared to be lower at 1000 μM compared with 100 μM oxazepam concentration, it was still over 6 times more active than any other isofrom assayed. In addition to UGT1A1 and UGT1A6, UGT2B7 also showed a small amount of activity at this high concentration. In contrast, both UGT1A9 and UGT2B7 were found to mediate R-oxazepam glucuronidation (Fig. 2B). UGT1A9 showed relatively high activity at both of the substrate concentrations examined, whereas UGT2B7 was primarily active at the higher substrate concentration. All of the remaining UGTs showed no activity except for UGT1A7, which had low although measurable activities at both substrate concentrations (Fig. 2B).

Enzyme Kinetics of Expressed UGTs Compared with HLMs. Enzyme kinetic studies were then performed using UGTs 2B15 (+1–BV), 1A9, and 2B7 as well as HLMs from three individuals. Kinetic data for S-oxazepam glucuronidation were best described by a single enzyme kinetic model with substrate inhibition for both HLMs and UGT2B15 (eq. 3; Fig. 3, A and B, respectively), whereas the simple Michaelis-Menten model best described UGT2B7 data (eq. 1; Fig. 3C). Similarly, apparent \( K_m \) values for S-oxazepam glucuronidation by HLMS (43, 58, and 60 μM) were most similar to UGT2B15 (35

![Image of S-oxazepam-UGT activity](https://example.com/image1.png)

![Image of R-oxazepam-UGT activity](https://example.com/image2.png)
Comparative Activities of UGT2B15 Allelic Variants and HLMs. Allelic variants of UGT2B15 (UGT2B15*1 and UGT2B15*2) were stably expressed in HEK-293 cells and microsomes prepared for comparison of activities using oxazepam, eugenol, naringenin, 4-methylumbelliferone, dihydrotestosterone, and androstane-3α,17β-diol as substrates. Immunoblotting of these preparations showed an average 3.3 times higher immunoreactive UGT2B protein in the UGT2B15*1 microsomes compared with the UGT2B15*2 microsomes (Fig. 4, A and B). No R-oxazepam glucuronidation activity was detected in any of the UGT2B15 preparations assayed. Enzyme kinetic analysis of S-oxazepam glucuronidation showed a similar apparent \(K_m\) value for HEK-293-expressed UGT2B15*1 (29 \(\mu M\); Fig. 4C and Table 1) compared with baculovirus-insect cell expressed UGT2B15*1 (35 \(\mu M\); Fig. 3B and Table 1). However, \(V_{\text{max}}\) values

\[ V_{\text{max}} = \frac{[S]}{K_m} \]

\[ \text{Intrinsic clearance (IC)} = \frac{V_{\text{max}}}{C_{\text{plasma}}} \]

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were about 3 times higher in baculovirus-insect cell expressed UGT2B15*1 compared with HEK-293-expressed UGT2B15*2 (17 versus 5 pmol/min/mg of protein, respectively; Table 1). S-Oxazepam glucuronidation activities measured with UGT2B15*2 over the same substrate concentrations (5–1000 µM) was only 3 to 8% (6 ± 2%) that of UGT2B15*1 (Fig. 4C). When corrected for immunoreactive UGT2B15 protein content, activities with UGT2B15*2 were still only 10 to 28% (20 ± 5%) that of UGT2B15*1. Unfortunately these low activities with UGT2B15*2 precluded determination of apparent $K_m$ values.

Substantial differences between these allelic variant preparations in activities were also observed for other substrates assayed. After correction for relative immunoreactive UGT2B15 protein content, activities for UGT2B15*2 were from 8 ± 3% (for naringenin) to 29 ± 6% (for 4-methylumbelliferone) that of UGT2B15*1 (Table 2). An exception was dihydrotestosterone activity in UGT2B15*2, which was 67 ± 13% that of UGT2B15*1. It should be acknowledged that naringenin, androstane-3a-diol, and dihydrotestosterone glucuronidation activities for UGT2B15*2 were so low that measured values were near to the lower limit of quantitation for the assay used. On the other hand, eugenol and 4-methylumbelliferone glucuronidation activities for UGT2B15*2 were at least 10 times higher, whereas oxazepam glucuronidation activities were 2 to 3 times higher, than the lower limit of quantitation for the respective assays.

Activities for all substrates were also measured using pooled HLMs and compared with activities for UGT2B15*1 (Table 2). Differences between expressed UGT2B15*1 and pooled HLMs were smallest for S-oxazepam-UGT activities in that UGT2B15*1 averaged 10% of the S-oxazepam-UGT activity of pooled HLMs. In contrast, UGT2B15*1 activities ranged from 3 to 5% that of pooled HLMs activities for the other five UGT substrates evaluated.

**Discussion**

To our knowledge, this is the first study to show that UGT2B15 stereoselectively glucuronidates S-oxazepam and is most likely the principal human UGT isofrom mediating this activity. Evidence in support of these conclusions include the markedly higher S-oxazepam glucuronidation activity displayed by expressed UGT2B15 compared with all other isoforms studied, lack of detectable R-oxazepam glucuronidation activity by UGT2B15, and the remarkable similarities between expressed UGT2B15 and HLMs in enzyme kinetic properties. In particular, decreasing activity at oxazepam concentrations over 100 µM, consistent with substrate inhibition, was consistently observed for both HLMs and UGT2B15 but not for any other UGT isoform studied.

Although a previous study (Patel et al., 1995b) indicated that UGT2B7 could be the major enzyme responsible for S-oxazepam glucuronidation in HLMs, this is unlikely since activities and intrinsic clearance values for expressed UGT2B7 were very low, and apparent $K_m$ values were relatively high compared with UGT2B15. Furthermore, we found that UGT2B7 preferentially glucuronidated R-oxazepam over S-oxazepam, with more than 5 times higher activities for the R-stereoisomer, which is not consistent with the pattern of glucuronidation observed in HLMs (S-glucuronidation $\gg$ R-glucuronidation). This conclusion is also supported by results from a previous study, which showed that UGT2B7 poorly glucuronidated both of the oxazepam stereoisomers (Coffman et al., 1998).

R-Oxazepam glucuronidation was catalyzed by both UGT1A9 and UGT2B7. Apparent $K_m$ values for HLMs (256–303 µM) were most similar to UGT2B7 (333 µM) rather than UGT1A9 (12 µM). Although both isoforms may contribute to R-oxazepam glucuronidation in HLMs, the substantial differences in apparent $K_m$ values indicate that UGT1A9 would be most active at relatively low substrate concentrations (<100 µM), whereas UGT2B7 would contribute primarily at relatively high concentrations (<500 µM). This was also reflected by intrinsic clearance values for R-oxazepam glucuronidation, which were over 10 times higher for UGT1A9 compared with UGT2B7. Interestingly, oxazepam has been reported to inhibit 3'-azido-3'-deoxythymidine glucuronidation by HLMs (Rajaonarison et al., 1991), a conjugation reaction that is thought to be primarily mediated by UGT2B7 (Barbier et al., 2000). However, this only occurs at relatively high oxazepam concentrations (>500 µM) consistent with a low affinity interaction.

Plasma concentrations following the recommended dose of oxazepam (15–30 mg) to human subjects range from 1 to 5 µM (300–1500 ng/ml) (Greenblatt, 1981). Although plasma concen-

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**Table 1**

<p>| Enzyme kinetic parameters for oxazepam glucuronidation by HLMs and expressed UGTs |
|---------------------------------|-----------------|-----------------|-----------------|-------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (pmol/min/mg)</th>
<th>$V_{max}/K_m$ (mM/min/kg)</th>
<th>$N$</th>
<th>$K_i$ (µM)</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-Oxazepam Glucuronidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV12</td>
<td>58 ± 22</td>
<td>267 ± 58</td>
<td>4.6 ± 1.4</td>
<td>237 ± 113</td>
<td>3</td>
</tr>
<tr>
<td>LV21</td>
<td>43 ± 14</td>
<td>317 ± 42</td>
<td>7.3 ± 1.7</td>
<td>1469 ± 739</td>
<td>3</td>
</tr>
<tr>
<td>LV24</td>
<td>54 ± 20</td>
<td>325 ± 65</td>
<td>5.4 ± 1.4</td>
<td>256 ± 70</td>
<td>3</td>
</tr>
<tr>
<td>UGT2B15*1-BV</td>
<td>35 ± 5</td>
<td>17 ± 2</td>
<td>0.49 ± 0.06</td>
<td>1247 ± 308</td>
<td>3</td>
</tr>
<tr>
<td>UGT2B15*1-HK</td>
<td>29 ± 7</td>
<td>5 ± 1</td>
<td>0.16 ± 0.03</td>
<td>839 ± 516</td>
<td>3</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>203 ± 123</td>
<td>1.8 ± 0.3</td>
<td>0.0091 ± 0.00036</td>
<td>839 ± 516</td>
<td>3</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>R-Oxazepam Glucuronidation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV12</td>
<td>303 ± 25</td>
<td>95 ± 5</td>
<td>0.31 ± 0.02</td>
<td>3698 ± 1375</td>
<td>3</td>
</tr>
<tr>
<td>LV21</td>
<td>273 ± 122</td>
<td>46 ± 10</td>
<td>0.17 ± 0.06</td>
<td>3698 ± 1375</td>
<td>3</td>
</tr>
<tr>
<td>LV24</td>
<td>256 ± 15</td>
<td>163 ± 6</td>
<td>0.64 ± 0.03</td>
<td>3698 ± 1375</td>
<td>3</td>
</tr>
<tr>
<td>UGT2B15*1-BV</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>UGT2B15*1-HK</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>332 ± 82</td>
<td>7.0 ± 1.0</td>
<td>0.021 ± 0.0004</td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>12 ± 3</td>
<td>2.7 ± 0.2</td>
<td>0.22 ± 0.04</td>
<td>3698 ± 1375</td>
<td>3</td>
</tr>
</tbody>
</table>

N.D.: not determined since no activity was observed (quantitation limit 0.1 pmol/min/mg protein).

$^a$: parameter not present in kinetic model used to fit data.

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Substantial involvement of UGT2B15 in the glucuronidation of oxazepam may provide a satisfactory mechanistic explanation for a pharmacokinetic phenomenon first described almost 20 years ago (Abernethy et al., 1983). Then it was observed that oxazepam clearance values were over 3 times higher in obese individuals compared with nonobese controls. The authors of that study (Abernethy et al., 1983) speculated that there might be enhanced extrahepatic glucuronidation of oxazepam perhaps resulting from the increase in adipose tissue mass in obese individuals. The recent finding (Tchernof et al., 1999) that UGT2B15 mRNA (but not UGT2B17mRNA) is highly expressed in adipose tissue supports this hypothesis, although further work is needed to verify that the appropriate enzyme activities are also present in this tissue.

$S$-Oxazepam glucuronidation activities for expressed UGT2B15 were from 5 to 20 times lower than activities for HLMs. Although all UGTs known to be expressed in human liver were screened for activity (except for UGT2B11 and UGT2B28, which were not available at the time of study), it is possible that there is another UGT isofrom present in liver with a higher turnover rate for oxazepam than UGT2B15. However, it is more likely that this difference simply reflects a difference in relative abundance of enzyme between the two preparations. In support of this, we found greater differences (20 to 33 times lower activity) when comparing expressed UGT2B15*1 and pooled HLMs for each of the five other activities measured.

UGT2B15 shares amino acid sequence homology (>$92\%$ identity), tissue distribution, and substrates with UGT2B17 (Hum et al., 1999). Consequently it was surprising to find no detectable oxazepam glucuronidation activity with recombinant UGT2B17, despite evidence of activity with this preparation for other substrates. This contrasts with the other substrates identified to date for UGT2B15 (such as dihydrotestosterone and androstane-3α-diol), which are also glucuronidated by UGT2B17 (Turgeon et al., 2001). Although this finding needs to be confirmed, it indicates that $S$-oxazepam may have substantial utility as a selective substrate probe for UGT2B15 in both in vitro and human studies.

As discussed previously (Patel et al., 1995a,b), although racemic oxazepam is the only preparation currently available, there are limitations to the use of a racemic drug for these investigations. In particular, interpretation of our results could be confounded by either stereoselective hydrolysis of the glucuronides or inhibitory interactions. For instance, $S$-oxazepam glucuronidation activity relative to $R$-oxazepam glucuronidation activity in HLMs could be explained by an inhibitory effect of $S$-oxazepam on $R$-oxazepam glucuronidation by the same UGT isofrom, rather than there being different UGT isofroms that preferentially conjugate one stereoisomer over the other. Although this could be resolved (theoretically) by the use of purified stereoisomers, both $R$- and $S$-oxazepam (and other 3-hydroxylated benzodiazepines) have been shown to spontaneously racemize with a half-life of less than 4 min in aqueous solution at 37°C (Yang and Lu, 1991).

The UGT2B15 gene and associated regulatory region appears to be the relevant candidate gene for investigating the molecular genetic basis of polymorphic $S$-oxazepam glucuronidation previously identified in human studies (Patel et al., 1995a). A single nucleotide polymorphism has been identified in the amino acid coding portion of the UGT2B15 gene, which results in either an aspartate (UGT2B15*1) or tyrosine (UGT2B15*2) at position 85 of the protein (Levesque et al., 1997). This polymorphism appears to be highly prevalent with frequencies of the UGT2B15*1 allele ranging from 0.45 in white Americans to 0.63 in Hispanic Americans (Lampe et al., 2000; Riedy et al., 2000). Furthermore, this coding difference was shown to have functional consequence, in that 2-fold higher
Glucuronidation activities for five different substrates were determined as described in Materials and Methods using pooled HLMs and UGT2B15*1 and UGT2B15*2 stably expressed in HEK-293 cells. Shown are the mean values ± S.D. of triplicate determinations. In addition to absolute values, activities are given as a percentage of HLM activity for UGT2B15*1 and as a percentage of UGT2B15*1 (for UGT2B15*2).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pooled HLM Activity</th>
<th>UGT2B15*1 Activity</th>
<th>% HLM</th>
<th>UGT2B15*2 Activity</th>
<th>% HLM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min/mg</td>
<td>pmol/min/mg</td>
<td></td>
<td>pmol/min/mg</td>
<td></td>
</tr>
<tr>
<td>5-Oxazepam</td>
<td>32 ± 2</td>
<td>3.4 ± 0.3</td>
<td>99.9 ± 0.8</td>
<td>0.21 ± 0.08</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>Eugenol</td>
<td>7271 ± 1266</td>
<td>229 ± 45</td>
<td>3.2 ± 0.6</td>
<td>2.2 ± 1.0*</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>Naringenin</td>
<td>2856 ± 258</td>
<td>87 ± 6</td>
<td>3.0 ± 0.2</td>
<td>13 ± 5</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>4-Methylumbelliferone</td>
<td>3182 ± 277</td>
<td>152 ± 8</td>
<td>4.8 ± 0.3</td>
<td>5.9 ± 1.5*</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>Androstane-3,14-diol</td>
<td>1763 ± 144</td>
<td>83 ± 10</td>
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</tr>
<tr>
<td>Dihydrotestosterone</td>
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<td>3.1 ± 0.5</td>
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<td>26 ± 13</td>
</tr>
</tbody>
</table>

* Corrected for 3.3 times higher immunoreactive UGT2B15 protein in UGT2B15*1 compared with UGT2B15*2 microsomal preparations.

In conclusion, we have shown that oxazepam is glucuronidated by several UGTs normally expressed in human liver. Whereas R-oxazepam is glucuronidated by multiple UGTs (UGT1A9 and 2B7), S-oxazepam is preferentially and selectively glucuronidated by UGT2B15. Consequently, S-oxazepam may be a specific substrate for UGT2B15 and have utility for pharmacogenetic studies of UGT2B15 in human subjects and tissues. Furthermore, allelic variation associated with the UGT2B15 gene is likely to explain polymorphic S-oxazepam glucuronidation in humans. Preliminary studies indicate that the UGT2B15*2 polymorphism in the coding region of the UGT2B15 gene could be responsible, although further study is needed to confirm this finding.

TABLE 2
Comparative glucuronidation activities for UGT2B15*1 and UGT2B15*2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pooled HLM Activity</th>
<th>UGT2B15*1 Activity</th>
<th>% HLM</th>
<th>UGT2B15*2 Activity</th>
<th>% HLM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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* Corrected for 3.3 times higher immunoreactive UGT2B15 protein in UGT2B15*1 compared with UGT2B15*2 microsomal preparations.

The discrepancy in results between the current study and the previous report (Levesque et al., 1997) appears to relate to whether intact cells (with substrate added to the cell media) or disrupted cell homogenates (E. Levesque, unpublished data) were used for the glucuronidation assay. One possible explanation is that UGT2B15*2 is less stable than UGT2B15*1 under the conditions used to prepare the microsomes from the HEK-293 cells. In the present study, we found no evidence for stability differences between the variants (after microsome preparation) as indicated by a linear increase in glucuronide formation with increasing incubation time.

Although we did see substantially higher absolute dihydrotestosterone glucuronidation activities, UGT2B15*2 were also found for most other substrates assayed, including androstane-3α-diol, naringenin, eugenol, and 4-methylumbelliferone. Although we did see substantially higher absolute dihydrotestosterone glucuronidation activities in UGT2B15*1 compared with UGT2B15*2, correction for UGT2B15 protein content essentially eliminated the difference, indicating that the disparity could be substrate dependent.

Similar results (UGT2B15*1 showing greater activity than UGT2B15*2) have been obtained independently with HEK-293 cell homogenates (E. Levesque, unpublished data). Consequently, the discrepancy in results between the current study and the previous report (Levesque et al., 1997) appears to relate to whether intact cells (with substrate added to the cell media) or disrupted cell preparations (homogenates or microsomes) are used for the glucuronidation assay. One possible explanation is that UGT2B15*2 is less stable than UGT2B15*1 under the conditions used to prepare the microsomes from the HEK-293 cells. In the present study, we found no evidence for stability differences between the variants (after microsome preparation) as indicated by a linear increase in glucuronide formation with increasing incubation time.

Protein stability studies using intact cells indicate that there are substantial differences between some UGT2B isoforms, although the exact mechanism for this has not been elucidated (Turgeon et al., 2001). Interestingly, in both this study and in the previous report (Levesque et al., 1997), 3- to 4-fold more UGT2B15 protein was present in cells expressing the UGT2B15*1 variant compared with the UGT2B15*2 variant, suggesting that the single amino acid difference could affect protein stability, although it could simply reflect differences in expression plasmid copy number. Further studies using genotyped human liver microsomes and human sub-


