EVALUATION OF 5-HYDROXYTRYPTOPHOL AND OTHER ENDOGENOUS SEROTONIN (5-HYDROXYTRYPTAMINE) ANALOGS AS SUBSTRATES FOR UDP-GLUCURONOSYLTRANSFERASE 1A6

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ABSTRACT:

Serotonin is a specific in vitro substrate for human UDP-glucuronosyltransferase (UGT) 1A6. In this study, the contribution of UGT1A6 to the glucuronidation of endogenous structural analogs of serotonin, including 5-hydroxytryptophol, N-acetylsertotonin, and 6-hydroxymelatonin, was evaluated using available recombinant human UGT isoforms, human liver microsomes, and liver microsomes from animals that do not express functional UGT1A6 (Gunn rats and cats). Only UGT1A6 and UGT1A9 were found to glucuronidate 5-hydroxytryptophol at a concentration of 2 mM, although the glucuronidation rate with UGT1A6 was over 10 times that of UGT1A9. \( K_m \) values for human liver microsomes (156, 141, and 134 \( \mu M \)) were most similar to that of expressed UGT1A6 (135 \( \mu M \)) but vastly different from that of UGT1A9 (3674 \( \mu M \)). 5-Hydroxytryptophol glucuronidation by human liver microsomes (\( n = 54 \)) correlated well with serotonin glucuronidation (\( R^2 = 0.83 \)) and UGT1A6 protein content (\( R^2 = 0.85 \)). 5-Hydroxytryptophol also competitively inhibited serotonin glucuronidation by human liver microsomes (\( K_i = 291 \mu M \)) and UGT1A6 (\( K_i = 200 \mu M \)). N-acetylserotonin was glucuronidated most extensively by UGT1A6, although UGT1A9 and UGT1A10 showed moderate catalysis. 6-Hydroxymelatonin was glucuronidated largely by UGT1A9 and UGT1A10 but not at all by UGT1A6. Gunn rat liver glucuronidation rates for serotonin, 5-hydroxytryptophol, N-acetylsertotonin, and 6-hydroxymelatonin were 11, 5, 32, and 3%, respectively, of that of normal rat liver. Cat liver microsomes did not glucuronidate serotonin, whereas relatively low activities were observed for the other indole substrates. In conclusion, these results indicate that human UGT1A6 plays a predominant role in the glucuronidation of 5-hydroxytryptophol and N-acetylsertotonin, whereas 6-hydroxymelatonin is not a substrate for this enzyme.

Glucuronidation represents an important metabolic process in mammals involving the conjugation of drugs, xenobiotics, and endogenous compounds to glucuronic acid. This reaction is catalyzed by the UDP-glucuronosyltransferases (UGTs), a superfamily of enzymes whose members have distinct but overlapping substrate specificities. UGT1A6 is an important UGT isoform implicated in the glucuronidation of selectively low molecular weight molecules, including drugs such as acetaminophen and valproate (Ethell et al., 2003) and potential carcinogens chemically related to hydroxylated polycyclic aryl hydrocarbons (Bock et al., 1993). The only endogenous compound that has been identified to date as a substrate for human UGT1A6 is serotonin (5-hydroxytryptamine) (King et al., 1999). Furthermore, we have shown recently that serotonin is a highly selective in vitro probe substrate for UGT1A6 in human liver and extrahepatic tissues (Krishnaswamy et al., 2003a).

One limitation in using serotonin as a probe substrate is the relatively low affinity of UGT1A6 for this molecule, as evidenced by the relatively high \( K_m \) values for serotonin glucuronidation by recombinant UGT1A6 and human tissues (5 to 9 mM) (Krishnaswamy et al., 2003a). Consequently, serotonin is unlikely to be useful as a potent inhibitor of glucuronidation by UGT1A6. However, an array of structural analogs of serotonin of both endogenous and exogenous origin exists, constituting potential substrates for UGT1A6.

Potential endogenous substrates of UGT1A6 that have been identified within the metabolic pathways of serotonin include \( L \)-5-hydroxytryptophan, 5-hydroxyindole-3-acetaldehyde, 5-hydroxyindole acetic acid, 5-hydroxytryptophol, \( N \)-acetylsertotonin, and 6-hydroxymelatonin (see Fig. 1). 5-Hydroxyindole acetic acid is excreted in free form, whereas the more hydrophobic 5-hydroxytryptophol undergoes conjugation prior to being excreted in urine. 5-Hydroxytryptophol is a relatively minor metabolite of serotonin that is excreted primarily as the glucuronide conjugate in human urine (Heiland et al., 1992a, 1995). 5-Hydroxytryptophol becomes more important quantitatively during alcohol intoxication, when a shift in the

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; UDPGA, UDP-glucuronic acid; HPLC, high-performance liquid chromatography; AZT, 3'-azido-2',3'-dideoxythymidine.
metabolism of serotonin occurs from 5-hydroxyindole acetic acid toward increased (15-fold higher) formation of 5-hydroxytryptophol due to the inhibition of aldehyde dehydrogenase by ethanol-derived acetaldehyde (Helander et al., 1993; Svensson et al., 1999). Urinary excretion of 5-hydroxytryptophol has also been shown to be markedly increased for several hours following intake of foods rich in serotonin, such as bananas (Helander et al., 1992b).

Wide interspecies variation has been reported in the metabolism serotonin to either 5-hydroxyindole acetic acid or 5-hydroxytryptophol (Some and Helander, 2002). 5-Hydroxytryptophol makes up 35% of the excreted serotonin metabolites in the rat on average and 10 to 20% in several other species (Some and Helander, 2002). The major metabolic products of the pineal hormone melatonin (also formed from serotonin) are N-acetylserotonin and 6-hydroxymelatonin, both of which are glucuronidated before being excreted into the urine (Young et al., 1985; Leone et al., 1987).

The aim of the present study was to determine whether the endogenously generated serotonin metabolites 5-hydroxytryptophol, N-acetylserotonin, and 6-hydroxymelatonin are selective and high-affinity substrates for UGT1A6 by using expressed human UGT isoforms, human liver microsomes, and liver microsomes from animals that do not express functional UGT1A6 (Gunn rats and cats). The serotonin precursor 5-hydroxytryptophan and the most abundant serotonin metabolite 5-hydroxyindole acetic acid were also evaluated as potential substrates for glucuronidation by human liver microsomes. Finally, 5-hydroxytryptophol was evaluated as a potential inhibitor of glucuronidation by UGT1A6.

Materials and Methods

Chemicals. Unless otherwise indicated, most reagents, including alamethicin, UDP-glucuronic acid (UDPGA; sodium salt), 5-hydroxytryptamine hydrochloride (serotonin hydrochloride), 5-hydroxytryptophol, 5-hydroxyindole acetic acid, 5-hydroxytryptophan, N-acetylserotonin, and 6-hydroxymelatonin,
were purchased from Sigma-Aldrich (St. Louis, MO). All reagents were of analytical or better grade.

**Liver Microsomes.** Liver samples from human donors with no known liver disease were provided by the International Institute for the Advancement of Medicine (Exton, PA), the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MN), or the National Disease Research Interchange (Philadelphia, PA). All livers were either intended for transplantation but had failed to tissue match or were normal tissue adjacent to surgical biopsies. Donors were primarily white (n = 48) but also included 4 African Americans and 2 Hispanics. Other available demographic information included gender (16 females, 38 males) and age (median, 41 years; range, 2–75 years). The procedure for preparation of microsomal fractions and their viability status have been described in detail elsewhere (Court et al., 2003). For animal species comparison, livers were obtained from 2 cats and 3 rats (one each of homozygous Gunn mutant J/J, heterozygous Gunn mutant J/+ and normal Wistar strain +/+). And their viability was ascertained by measuring morphine-3-glucuronidation activities as described below. The Human Investigation Review Committee and Animal Research Committee of Tufts University (Boston, MA) approved the collection and use of these tissues.

**Recombinant UGT Isoforms.** Microsomes from insect cells transfected with baculovirus containing cDNA for human UGT isoforms 1A1, 1A3, 1A4, 1A6, 1A8, 1A9, 2B4, 2B7, and 2B15 were obtained from BD Gentest (Woburn, MA), whereas microsomes containing UGTs 1A7 and 1A10 were obtained from PanVera Corp. (Madison, WI). Lymphoblast-derived microsomes containing cDNA-expressed human UGT1A6 were obtained from BD Gentest. Glucuronidation activities using positive control substrates and similarity in specific UGT protein content of these isoforms were verified by Western blotting as described previously (Court et al., 2003).

**5-Hydroxytryptophol Glucuronidation Assay.** 5-Hydroxytryptophol glucuronidation activities were measured by using an HPLC method similar to the serotonin glucuronidation assay we have described previously (Krishnaswamy et al., 2003b), with slight modifications. Incubation mixtures contained 50 mM phosphate buffer (pH 7.5), 5 mM MgCl2, and UDPGA (20 mM for kinetic studies, otherwise 5 mM) in a final volume of 100 μl. Amalthein (0.0025% w/v) was also included in incubations at a concentration determined in preliminary experiments to result in maximal activation (approximately 2-fold increase). Microsomes at concentrations of 0.05 to 0.25 mg/ml of protein were used. Incubations were performed in a water bath for 20 min at 37°C and terminated by the addition of 100 μl of acetonitrile containing acetalaminophen as an internal standard. After centrifugation at 16,000g for 10 min, the supernatants were dried down in a vacuum oven at 45°C and reconstituted with 100 μl of water and used for HPLC. The HPLC instrument was an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) with a 250 × 4.6-mm C18 column (Phenomenex, Torrance, CA). 5-Hydroxytryptophol glucuronide was detected by a fluorescence detector set at an excitation wavelength of 225 nm and emission wavelength of 330 nm; however, for quantitation purposes, UV absorption at 225 nm was used. The identity of the metabolite peak as a glucuronide for each substrate was substantiated by spectral analysis or better grade. Glucuronide formation rates were expressed as nanomole equivalents per minute per milligram of microsomal protein assuming similar UV absorbance of parent and glucuronide molecules. The identity of the metabolite peak as a glucuronide was substantiated by a requipment of UDPGA in the incubation mixture and peak disappearance following treatment with β-glucuronidase. Further structural characterization of the glucuronide products was not carried out. Initial rate conditions with respect to time (up to 60 min) and protein concentration (up to 0.8 mg/ml) for the formation of 5-hydroxytryptophol glucuronide for both human liver microsomes and expressed UGT1A6-containing microsomes were established in preliminary studies at a 5-hydroxytryptophol concentration of 1 mM. Less than 10% substrate consumption was observed. 5-Hydroxytryptophol glucuronidation activities of recombinant UGT isoforms and vector controls were measured at a 5-hydroxytryptophol concentration of 2 mM using 5 mM UDPGA and a microsomal protein concentration of 1 mg/ml. For correlation analyses, 0.1 mM 5-hydroxytryptophol and 0.1 mg/ml of human liver microsomal protein were used. Kinetic studies using human liver microsomes and expressed UGT isoforms were performed using a fixed high UDPGA (20 mM) and varying 5-hydroxytryptophol concentrations (0.01–4 mM) in the incubation mixture.

**Other Glucuronidation Activities and UGT1A6 Protein Content.** N-Acetylseryl serotonin glucuronidation by expressed UGT isoforms was assayed using a method similar to that described above for 5-hydroxytryptophol glucuronidation, with minor changes. The reaction mixture contained 1 mM N-acetylseryl serotonin and 0.5 mg/ml of the respective expressed UGT isoform. Incubation at 37°C was carried out for 1 h. 6-Hydroxymelatonin glucuronidation by expressed UGT isoforms was assayed using a method similar to that described for 5-hydroxytryptophol glucuronidation assay, except that the reaction mixture contained 1 mM substrate and 0.5 mg/ml of microsomal protein, and the incubation time was 1 h. N-Acetylseryl serotonin glucuronide and 6-hydroxymelatonin glucuronide were detected by a fluorescence detector (excitation, 225 nm; emission, 330 nm), but for quantitation purposes, absorption by UV detector at 225 nm was used. Glucuronide concentrations were determined using standard curves generated using respective substrates assuming similar UV absorbance. Results are therefore given as nanomole equivalents per minute per milligram of microsomal protein. The identity of the metabolite peak as a glucuronide for each substrate was substantiated by spectral analysis or better grade. Further structural characterization of the glucuronide products was not attempted.

Morphine-3-glucuronide formation by animal liver microsomes was assayed as described previously (Court et al., 2003). Glucuronidation activities measured by using the substrates serotonin, 4-nitrophenol, estradiol, trifluoperazine, propofol, acetalaminophen, AZT, morphine, and S-oxazepam, and the relative UGT1A6 protein content measured by Western blotting for the same set of human liver microsomes, have been published (Krishnaswamy et al., 2003a). The same results were used here for correlational analysis.

**Enzyme Kinetic Analyses.** For enzyme kinetic studies, substrate concentration (S) and velocity (V) data were fitted to the appropriate model by nonlinear least-squares regression (SigmaPlot 8.0; SPSS Inc., Chicago, IL). Since the appearance of Eadie-Hofstee (V/S versus V) plots for all sets of data in this study was consistent with single enzyme kinetics, the standard Michaelis-Menten model was used to derive the kinetic parameter values Vmax (maximal velocity) and apparent Ks (substrate concentration at half-maximal velocity). Parameters are given as the estimate ± the standard error of the estimate from the nonlinear regression analysis.

**Inhibition Kinetics.** For inhibition studies, substrate (serotonin) and inhibitor (5-hydroxytryptophol) were dried down together from respective stock solutions in methanol. The reaction mixture was preincubated at 37°C for 20 min before the addition of microsomes, and serotonin glucuronidation was determined as described previously (Krishnaswamy et al., 2003b). To determine IC50 (inhibitor concentration resulting in 50% inhibition of the enzymatic activity observed in the absence of the inhibitor) values, activity data were analyzed by nonlinear regression analysis as described previously (Court et al., 1997; von Moltke et al., 1998) using the following model (eq. 1): V/No = IC50/(1 + IC50) × 100, where V/No is the serotonin glucuronidation formation rate as a percentage of control activity (with no inhibitor) measured at the inhibitor concentration of I. Studies using multiple inhibitor and substrate concentrations were then performed to identify the type of inhibition through the appearance of the Dixon plots. Since the appearance of plots were consistent with competitive inhibition, inhibition constant (Ki) values were derived by nonlinear regression (SigmaPlot 8.0; SPSS Inc.) using the following competitive inhibition model (eq. 2): V/No = Vmax × S/(Km + S).

**Statistical Analyses.** Statistical analyses were performed using the SigmaStat 2.03 software package (SPSS Inc.). Spearman rank order correlation analysis was used to determine the relationship between UGT1A6 protein content and the glucuronidation activities measured. For this determination, a Spearman correlation rank order correlation coefficient (R) of greater than 0.5 and a P value of less than 0.001 were considered statistically significant.

**Results**

Glucuronidation by Human and Animal Liver Microsomes. As shown in Table 1, cat liver microsomes had no measurable serotonin.

<ref>Figure 1</ref>, <ref>Figure 2</ref>, <ref>Table 1</ref>
glucuronidation activity and relatively little 5-hydroxytryptophol (0.7% of human activity), N-acetylsertotonin (1.2% of human activity), and 6-hydroxymelatonin (1.7% of human activity) glucuronidation activities compared with pooled human liver microsomes. In contrast, morphine (primarily a UGT2B substrate in the species evaluated) was glucuronidated much more extensively by cat liver microsomes (23% of that of human activity). Homozygous mutant (J/J) Gunn rat showed activities that were 11, 5, 32, and 3% of that of the homozygous normal (+/+; Wistar background) rat for serotonin, 5-hydroxytryptophol, N-acetylsertotonin, and 6-hydroxymelatonin, respectively. Glucuronidation activities for the heterozygous (+/+) Gunn rat tended to be intermediate between homozygous mutant and normal animals. Morphine-3-glucuronidation activity in the homozygous mutant Gunn rat was 71% of that of the normal rat. 5-hydroxytryptophan and 5-hydroxyinole acetic acid were not found to be glucuronidated by human liver microsomes despite performing incubations under highly favorable conditions (up to overnight incubations and millimolar substrate concentrations).

**Glucuronidation by Recombinant UGTs.** Glucuronidation of the endogenous structural analogs of serotonin, including 5-hydroxytryptophol, N-acetylsertotonin, and 6-hydroxymelatonin, were measured by using commercially available recombinant human UGT isoforms (UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17). 5-Hydroxytryptophol, at a concentration of 2 mM, was glucuronidated by recombinant UGT1A6- and UGT1A9-containing insect cell microsomes with activities of 5.5 and 0.33 nmol Eq/min/mg protein, respectively (Fig. 2A). None of the other isoforms or vector controls had any measurable activity. Lymphoblast-expressed UGT1A6 (L) had similar 5-hydroxytryptophol glucuronidation activity (4.9 nmol/min/mg protein) as insect cell-expressed UGT1A6. Glucuronidation of N-acetylsertotonin was measured at a substrate concentration of 1 mM using the same set of recombinant UGT isoforms (Fig. 2B). N-acetylsertotonin glucuronidation was over 5-fold higher for UGT1A6 (0.7 nmol Eq/min/mg protein) compared with UGT1A9 and UGT1A10 (0.13 and 0.11 nmol Eq/min/mg protein, respectively). UGT1A8, UGT2B7, and UGT2B15 had less than 5% of the activity of UGT1A6, whereas other isoforms tested showed no measurable activity. 6-Hydroxymelatonin glucuronidation activity was measured at a substrate concentration of 1 mM for the same set of UGT isoforms (Fig. 2C). In contrast to the other substrates evaluated, UGT1A10 and UGT1A9 showed the highest glucuronidation activities (0.36 and 0.27 nmol Eq/min/mg protein, respectively), with lower activities (less than 25% of that of UGT1A10) for UGT1A8, UGT2B15, UGT2B7, and UGT2B4. Furthermore, UGT1A6 showed no detectable 6-hydroxymelatonin glucuronidation activity.

**5-Hydroxytryptophol Glucuronidation Kinetics.** Enzyme kinetic studies were performed using microsomes from three different human livers (LV22, LV35, and LV41) and expressed human UGT1A6 and UGT1A9. A simple one-enzyme Michaelis-Menten kinetic model described all of the data adequately. As shown in Table 2, liver microsomes had $V_{max}$ values ranging from 11.5 to 26.4 nmol Eq/min/mg protein, whereas apparent $K_m$ values varied between 134 and 156 $\mu$M. The $K_m$ value for expressed UGT1A6 (135 ± 36 $\mu$M) was essentially identical to that of human liver microsomes, whereas the $K_m$ for UGT1A9, 3674 ± 470 $\mu$M, was over 20-fold higher. Furthermore, the calculated intrinsic clearance ($V_{max}/K_m$) for UGT1A6 was over 300-fold higher compared with UGT1A9.

**Correlation of 5-Hydroxytryptophol Glucuronidation Activities.** 5-Hydroxytryptophol glucuronidation activities measured by using liver microsomes from 54 individuals at a substrate concentration of 0.1 mM ranged from 1.8 to 12.0 nmol Eq/min/mg protein, representing more than a 6-fold variation. Mean and median activity values were 5.0 and 4.8 nmol/min/mg, respectively. The distribution of these data were normal (Kolmogorov-Smirnov normality test; K-S Dist. = 0.103; $P = 0.155$). Correlational analyses indicated highly significant relationships of 5-hydroxytryptophol glucuronidation activities determined at 0.1 mM with relative UGT1A6 protein content ($R_s = 0.848$; $P < 0.001$; $n = 52$) and serotonin glucuronidation activities ($R_s = 0.832$; $P < 0.001$; $n = 54$) (Fig. 3). Of the various UGT isoforms expressed in insect cell microsomes, we observed the highest correlation with the activity of UGT1A6 and the two UGT1A6 marker activities (serotonin and 4-nitrophenol glucuronidations). Lower but significant correlations were also seen with UGT1A1 (estradiol-3-glucuronidation), UGT1A4 (trifluoperazine glucuronidation), and UGT1A9 (propofol glucuronidation) isoform marker activities but not for other activities (Table 3).

**Inhibition of Serotonin Glucuronidation by 5-Hydroxytryptophol.** Inhibition of serotonin glucuronidation by 5-hydroxytryptophol was measured by co-incubating 4 mM serotonin with increasing concentrations of 5-hydroxytryptophol using both expressed UGT1A6 and pooled human liver microsomes. Expressed UGT1A6 and pooled human liver microsomes had IC$_{50}$ values of 402 and 481 $\mu$M, respectively (Fig. 4). Inhibition of serotonin glucuronidation by 5-hydroxytryptophol was competitive based on the appearance of Dixon plots (Fig. 5). $K_i$ values for 5-hydroxytryptophol inhibition were 290 ± 0.02 and 200 ± 5.12 $\mu$M for pooled human liver microsomes and expressed UGT1A6, respectively.

**Discussion**

After recently discovering the high specificity of serotonin as a UGT1A6 substrate (Krishnaswamy et al., 2003a), it was logical to predict that a number of metabolic derivatives and structural analogs of serotonin might also be glucuronidated by the same UGT isoform. Hence, we evaluated a series of endogenously generated indole derivatives found in the serotonin metabolic pathway, including 5-hydroxytryptophan, 5-hydroxyindole acetic acid, 5-hydroxytryptophol, N-acetylsertotonin, and 6-hydroxymelatonin, as potential substrates for glucuronidation by human liver microsomes. 5-Hydroxytryptophan...
and 5-hydroxyindole acetic acid, despite each having an available hydroxyl group for conjugation, were found not to be glucuronidated by human liver microsomes. Although it is possible that these compounds are glucuronidated by other tissues, this finding is consistent with a lack of reports for identification of the glucuronide conjugates of these compounds in human biological fluids. The structural basis for the apparent lack of reactivity of these compounds may relate to increased hydrophilicity as the result of a carboxyl group on the indolealkyl side chain of each compound, which is not present on any of the other compounds evaluated (see Fig. 1). Recent work using a

**Fig. 2.** Glucuronidation of serotonin analogs by expressed UGTs. Glucuronidation of 5-hydroxytryptophol (A), N-acetylserotonin (B), and 6-hydroxymelatonin (C) were measured at a substrate concentration of 1 mM (5-hydroxytryptophol at 2 mM) using microsomes containing UGT isoforms expressed in baculovirus-infected insect cells, except for UGT1A6 (L), which was from a lymphoblast cell expression system. The height of each bar represents the mean of duplicate determinations.
pharmacophore structure-activity modeling approach suggests that the presence of a hydrophilic group on the substrate molecule 6 Å distance from the nucleophilic conjugation site negatively impacts the likelihood of glucuronidation by UGT1A6 (Sorich et al., 2004). Whether the indolealkyl side chain carboxyl group that we have identified represents such an inhibitory moiety would need to be confirmed by the use of similar sophisticated molecular modeling techniques. On the other hand, 5-hydroxytryptophol, N-acetylsertotonin, and 6-hydroxymelatonin were extensively glucuronidated by human liver microsomes, which agrees with previous reports describing the identification of glucuronides of each of these compounds in human urine (Young et al., 1985; Leone et al., 1987; Helander et al., 1995).

Although we did not perform structural characterization of the formed glucuronides in this particular study, as we had done previously for serotonin glucuronide (Krishnaswamy et al., 2003b), it is likely that conjugation occurred via the hydroxyl moiety of each compound. This is based on the structural similarity of each of the evaluated compounds with serotonin and our previous finding by nuclear magnetic resonance analysis that serotonin is specifically glucuronidated at the hydroxyl position (Krishnaswamy et al., 2003b). The other possible conjugation sites are the side chain or aromatic nitrogens; however, to our knowledge, N-glucuronidation has never been reported for UGT1A6. Furthermore, we only found single glucuronide peaks for each compound on HPLC chromatograms indicating that only one conjugation site was utilized.

Available expressed UGTs were used to identify the major isoforms responsible for glucuronidation of the evaluated substrates. Consistent with the structural similarities to serotonin, 5-hydroxytryptophol and N-acetylsertotonin were glucuronidated largely, although not exclusively, by UGT1A6. UGT1A9 was also found to glucuronidate both compounds to a small extent, which is not surprising since UGT1A9 frequently shows significant overlap in substrate specificity with UGT1A6 (Ebner and Burchell, 1993; Ethell et al., 2002). On the other hand, 6-hydroxymelatonin was glucuronidated largely by UGT1A9 and UGT1A10 and not at all by UGT1A6. This difference probably relates to the 5-methoxy group on the indole ring of 6-hydroxymelatonin, which may block access to the more limited catalytic site of UGT1A6 compared with other isoforms. Whereas UGT1A9 is expressed in liver, UGT1A10 is an extrahepatic isoform and so may contribute to the glucuronidation of 6-hydroxymelatonin in other tissues, such as the gastrointestinal tract (Strassburg et al., 2000).

Liver microsomes from animals that are known to have deficient expression of UGT1A6 were also evaluated for glucuronidation activity. As we have shown previously, serotonin glucuronidation was undetectable in cat liver microsomes, consistent with UGT1A6 being encoded by a nonfunctional pseudogene in this species (Court and Greenblatt, 2000). Relatively low levels of activity were observed for compounds to a small extent, which is not surprising since UGT1A9 frequently shows significant overlap in substrate specificity with UGT1A6 (Ebner and Burchell, 1993; Ethell et al., 2002). On the other hand, 6-hydroxymelatonin was glucuronidated largely by UGT1A9 and UGT1A10 and not at all by UGT1A6. This difference probably relates to the 5-methoxy group on the indole ring of 6-hydroxymelatonin, which may block access to the more limited catalytic site of UGT1A6 compared with other isoforms. Whereas UGT1A9 is expressed in liver, UGT1A10 is an extrahepatic isoform and so may contribute to the glucuronidation of 6-hydroxymelatonin in other tissues, such as the gastrointestinal tract (Strassburg et al., 2000). Liver microsomes from animals that are known to have deficient expression of UGT1A6 were also evaluated for glucuronidation activity. As we have shown previously, serotonin glucuronidation was undetectable in cat liver microsomes, consistent with UGT1A6 being encoded by a nonfunctional pseudogene in this species (Court and Greenblatt, 2000). Relatively low levels of activity were observed for

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**TABLE 2**

<table>
<thead>
<tr>
<th>Microsomal Preparation</th>
<th>Apparent $K_m$ (μM)</th>
<th>$V_{max}$ (nmol Eq/min/mg)</th>
<th>Intrinsic Clearance ($V_{max}/K_m$)</th>
</tr>
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<tbody>
<tr>
<td>LV22</td>
<td>156 ± 16</td>
<td>12 ± 0.3</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>LV35</td>
<td>141 ± 6</td>
<td>25 ± 0.3</td>
<td>0.18 ± 0.05</td>
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<tr>
<td>LV41</td>
<td>134 ± 12</td>
<td>26 ± 0.7</td>
<td>0.20 ± 0.06</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>135 ± 36</td>
<td>8 ± 0.2</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>3674 ± 470</td>
<td>0.6 ± 0.0</td>
<td>0.00002 ± 0.0</td>
</tr>
</tbody>
</table>

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**FIG. 3.** Correlation of 5-hydroxytryptophol glucuronidation activities to serotonin glucuronidation activities and immunoreactive UGT1A6 protein in human liver microsomes. 5-Hydroxytryptophol glucuronidation activities were determined in human liver microsomes ($n = 54$) and correlated with serotonin glucuronidation activities (A) and relative UGT1A6 protein contents (B) ($n = 52$) in the same set of human liver microsomes. Also shown are the Spearman rank order correlation coefficient ($R_s$) and $P$ value for each comparison. 5-hydroxytryptophol and N-acetylsertotonin, and slightly higher levels for 6-hydroxymelatonin, probably as a result of glucuronidation by UGT isoforms other than UGT1A6. Similarly, glucuronidation activities for all of the evaluated indoles were significantly lower in the mutant Gunn rat compared with the homozygous normal animal. Since the Gunn rat mutation results in abrogation of all the UGT1A isoforms, this residual activity most likely results from UGT2B isoforms.
Considering the high 5-hydroxytryptophol glucuronidation activities shown by human liver microsomes, we proceeded to further evaluate the selectivity of this substrate for human UGT1A6, as well as its potential use as a UGT1A6 inhibitor. The essentially identical \( K_m \) values for expressed UGT1A6 and human liver microsomes and greatly higher \( K_m \) for UGT1A9 suggest that UGT1A6 but not UGT1A9 is the principle enzyme responsible for 5-hydroxytryptophol glucuronidation in human liver. This conclusion is further supported by the over 300-fold higher intrinsic clearance value calculated for UGT1A6 compared with UGT1A9. However, it should be pointed out that this difference was determined using recombinant enzyme preparations, and UGT1A9 and UGT1A6 levels in liver (or other) tissues could differ substantially. Finally, the significant correlations of 5-hydroxytryptophol with serotonin glucuronidation activities and relative UGT1A6 protein content substantiate the predominant role of UGT1A6 in determining interindividual variability in 5-hydroxytryptophol glucuronidation. It should be pointed out that, for these correlation analyses, 5-hydroxytryptophol glucuronidation in human liver microsomes was measured by using a substrate concentration of 100 \( \mu \)M, and so it is possible that, at higher concentrations (in the millimolar range), there may be a greater contribution from low-affinity isoforms such as UGT1A9; however, physiologically relevant concentrations of 5-hydroxytryptophol are likely to be much lower. Although serum concentrations have not been reported, urine concentrations of total (free plus conjugated) 5-hydroxytryptophol average 0.18 \( \mu \)M but may increase to as much as 1.2 \( \mu \)M following consumption of bananas (a serotonin source) or 6.2 \( \mu \)M with alcohol ingestion (Helander et al., 1995). Levels of free and total 5-hydroxytryptophol were 10 to 390 and 15 to 820 nM, respectively, in regions of human brain (Beck et al., 1984). Consequently, such low concentrations greatly favor a selective role for UGT1A6 in 5-hydroxytryptophol glucuronidation in vivo.

5-Hydroxytryptophol was found to competitively inhibit serotonin glucuronidation by both pooled human liver microsomes and expressed UGT1A6. The similarities in inhibition constants (\( IC_{50} \) and \( K_i \)) between liver and expressed UGT1A6 provide further evidence

<table>
<thead>
<tr>
<th>5-Hydroxytryptophol Glucuronidation vs.</th>
<th>( R_s )</th>
<th>( P ) value</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A6 protein</td>
<td>0.85</td>
<td>&lt;0.001</td>
<td>52</td>
</tr>
<tr>
<td>Serotonin glucuronidation (UGT1A6)</td>
<td>0.83</td>
<td>&lt;0.001</td>
<td>54</td>
</tr>
<tr>
<td>4-Nitrophenol glucuronidation (UGT1A6)</td>
<td>0.74</td>
<td>&lt;0.001</td>
<td>54</td>
</tr>
<tr>
<td>Estradiol-3-glucuronidation (UGT1A1)</td>
<td>0.67</td>
<td>&lt;0.001</td>
<td>54</td>
</tr>
<tr>
<td>Trifluoperazine glucuronidation (UGT1A4)</td>
<td>0.62</td>
<td>&lt;0.001</td>
<td>54</td>
</tr>
<tr>
<td>Propofol glucuronidation (UGT1A9)</td>
<td>0.50</td>
<td>&lt;0.001</td>
<td>54</td>
</tr>
<tr>
<td>Acetaminophen glucuronidation (UGT1A)</td>
<td>0.39</td>
<td>0.042</td>
<td>27</td>
</tr>
<tr>
<td>AZT glucuronidation (UGT2B7)</td>
<td>0.22</td>
<td>0.12</td>
<td>54</td>
</tr>
<tr>
<td>S-Oxazepam glucuronidation (UGT2B15)</td>
<td>0.33</td>
<td>0.015</td>
<td>54</td>
</tr>
</tbody>
</table>

**TABLE 3**

**Correlation of 5-hydroxytryptophol glucuronidation with UGT1A6 protein content and marker activities for the major hepatic UGT isoforms measured by using the same set of human liver microsomes**

Values represent the Spearman rank order correlation coefficient (\( R_s \)), \( P \) value, and number of livers (\( n \)) for the indicated comparisons.

![Inhibition of serotonin glucuronidation by 5-hydroxytryptophol.](image)

**FIG. 4.** Inhibition of serotonin glucuronidation by 5-hydroxytryptophol. Inhibition of serotonin glucuronidation activity (at a fixed serotonin concentration of 4 mM) was measured by increasing concentrations of 5-hydroxytryptophol with either pooled human liver microsomes or insect cell-expressed UGT1A6. Each data point represents the mean of duplicate determinations. \( IC_{50} \) values were determined by nonlinear regression curve fitting using Eq. 1.

![Dixon plots of serotonin glucuronide formation in the presence of 5-hydroxytryptophol by pooled human liver microsomes (A) and cDNA-expressed UGT1A6 (B).](image)

**FIG. 5.** Dixon plots of serotonin glucuronide formation in the presence of 5-hydroxytryptophol by pooled human liver microsomes (A) and cDNA-expressed UGT1A6 (B). Serotonin glucuronidation was measured at serotonin concentrations of 1, 2, and 6 mM, with each coincubated with 5-hydroxytryptophol concentrations of 0, 0.25, 0.5, and 2 mM. \( K_i \) values were determined by nonlinear regression fitting using the competitive inhibition model (Eq. 2).
for the selectivity of serotonin as a substrate for UGT1A6 in human liver. However, substantiation of 5-hydroxytryptophol as a selective UGT1A6 inhibitor will require verification of a lack of effect of this compound on glucuronidation activities mediated by other UGT forms.

In conclusion, results from these studies indicate that the endogenous serotonin structural analogs 5-hydroxytryptophol and N-acetylsertotonin are glucuronidated primarily by UGT1A6, whereas 6-hydroxymelatonin is glucuronidated by UGT1A9 in human liver. 5-hydroxytryptophol is also identified as a competitive inhibitor of glucuronidation by UGT1A6 in vitro.

References


Krishnaswamy S, Duan SX, von Mollike LL, Greenblatt DJ, and Court MH (2003a) Validation of serotonin (5-hydroxytryptamine) as an in vitro substrate probe for human UDP-glucuronosyltransferase (UGT) 1A6. Drug Metab Dispos 31:133–139.


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