Characterization of 1'-Hydroxymidazolam Glucuronidation in Human Liver Microsomes

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Running Title: Identification of 1'-Hydroxymidazolam N-glucuronide

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Abbreviations used are:
HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; NMR, nuclear magnetic resonance; UDPGA, uridine 5'-diphosphoglucuronic acid; UGT, uridine diphosphate glucuronosyltransferase; TMS, trimethylsilane.
Abstract

Midazolam is a potent benzodiazepine derivative with sedative, hypnotic, anticonvulsant, muscle-relaxant and anxiolytic activities. It undergoes oxidative metabolism catalyzed almost exclusively by the CYP3A subfamily to a major metabolite, 1'-hydroxymidazolam, which is equipotent to midazolam. 1'-Hydroxymidazolam is subject to glucuronidation followed by renal excretion. To date, the glucuronidation of 1'-hydroxymidazolam has not been evaluated in detail. In the current study, we identified an unreported quaternary N-glucuronide, as well as the known O-glucuronide, from incubations of 1'-hydroxymidazolam in human liver microsomes enriched with UDPGA. The structure of the N-glucuronide was confirmed by NMR analysis, which showed that glucuronidation had occurred at N-2 (the imidazole nitrogen that is not a part of the benzodiazepine ring). In a separate study, where midazolam was used as the substrate, an analogous N-glucuronide also was detected from incubations with human liver microsomes in the presence of UDPGA. Investigation of the kinetics of 1'-hydroxymidazolam glucuronidation in human liver microsomes indicated autoactivation kinetics (Hill coefficient, n = 1.2-1.5). The apparent S_50 values for the formation of O- and N-glucuronides were 43 and 18 µM, respectively, and the corresponding apparent V_max values were 363 and 21 pmol/mg microsomal protein/min. Incubations with recombinant human UGTs indicated that the O-glucuronidation was catalyzed by UGT2B4 and 2B7, while the N-glucuronidation was catalyzed by UGT1A4. Consistent with these observations, hecogenin, a selective inhibitor of UGT1A4, selectively inhibited the N-glucuronidation; while diclofenac, a potent inhibitor of UGT2B7, had a greater inhibitory effect on the O-glucuronidation than on the N-glucuronidation. In
summary, our study provides the first demonstration of $N$-glucuronidation of 1'-hydroxymidazolam in human liver microsomes.
Midazolam is a potent benzodiazepine derivative with sedative, hypnotic, anticonvulsant, muscle-relaxant and anxiolytic activities. It is widely used in the clinic for induction of anesthesia and for sedation of patients who are artificially ventilated in intensive care units (Dundee et al. 1984). Midazolam is rapidly eliminated from the body, almost exclusively by metabolism (Heizmann, et al. 1983). It undergoes oxidative metabolism mainly catalyzed by the CYP3A subfamily to a major metabolite, 1'-hydroxymidazolam, which is equipotent to midazolam, and two minor metabolites, 4-hydroxymidazolam and 1',4-dihydroxymidazolam, which are quantitatively unimportant (Dundee et al. 1984). 1'-Hydroxymidazolam is subject to further glucuronidation, followed by renal excretion. In humans, urinary recovery of 1'-hydroxymidazolam glucuronide accounted for 60-70% of an administered dose of [14C]midazolam (Heizmann and Ziegler, 1981). It has been reported that elevated serum levels of 1'-hydroxymidazolam glucuronide were found in patients with renal failure after administration of midazolam, and may account for the prolonged sedation observed in those patients (Bauer et al. 1995; Hirata et al. 2003).

Glucuronidation represents one of the major Phase II conjugation reactions in the conversion of both exogenous and endogenous compounds to polar and water-soluble metabolites that can be eliminated from the body in urine or bile (Sipes and Gandolfi, 1991). The reaction is catalyzed by a family of enzymes, UDP-glucuronosyltransferases (UGTs), which transfer glucuronic acid from UDP-glucuronic acid to the aglycone substrate. UGTs comprise of two subfamilies, UGT1 and UGT2. The substrate specificity of individual UGTs has been partially characterized. UGT subfamily 1 is responsible for
glucuronidation of bilirubin, amines, planar and bulky phenols, whereas subfamily 2 enzymes catalyze glucuronidation of a diverse chemical base including steroids, bile acids, and opioids (King et al. 2000).

To date the glucuronidation of 1’-hydroxymidazolam has not been studied in detail. In the literature, 1’-hydroxymidazolam glucuronide is often assumed to be an $O$-glucuronide. In the current study, we have identified an unreported quaternary $N$-glucuronide, as well as the known $O$-glucuronide, from incubations of 1’-hydroxymidazolam in human liver microsomes enriched with UDPGA. Further studies were conducted to determine the isozymes responsible for the formation of $N$- and $O$-glucuronic acid conjugates of 1’-hydroxymidazolam in human liver microsomes.
Materials and Methods

Materials. 1′-Hydroxymidazolam was synthesized by the Labeled Compound Synthesis Group, Department of Drug Metabolism, Merck Research Laboratories, Rahway, NJ. Recombinant human UDP-glucuronosyltransferases (UGTs) were purchased from BD Gentest, Woburn, MA. UDPGA (uridine 5′-diphosphoglucuronic acid), alamethicin, hecogenin, and diclofenac were purchased from Sigma Chemicals, St. Louis, MO. All solvents were of HPLC grade and were obtained from Fisher Scientific, Pittsburgh, PA.

Glucuronidation of 1′-Hydroxymidazolam. Pooled human liver microsomes (batch: #452161) were purchased from BD Gentest, Woburn, MA. A pool of 50 male Sprague-Dawley rat (~225-250g, ~8 weeks old) liver microsomes was prepared in-house following procedures described in the literature (Raucy and Lasker, 1991). Microsomal protein concentrations of 0.25 to 1 mg/mL and incubation times of 10 to 60 min were used to optimize the conditions of the assay. The reaction mixture consisted of 1′-hydroxymidazolam (5 to 200 µM), 100 mM potassium phosphate buffer (pH 7.5) with 2 mM MgCl₂, and human/rat liver microsomes (0.5 mg protein/mL) or recombinant UGTs (1 mg protein/mL) treated with alamethicin at 50 µg/mg microsomal protein. The reactions were initiated by the addition of UDPGA (2 mM), incubated at 37°C, and terminated with ice-cold acetonitrile containing 0.2% formic acid and phenolphthalein-β-D-glucuronide as the internal standard. Samples were centrifuged at 3,220 g for 15 min and supernatants were subjected to LC-MS/MS analysis.
**Enzyme Kinetics Analysis.** The apparent kinetic parameters $K_m/S_{50}$, $V_{max}$ and $n$ (Hill coefficient; where appropriate) were calculated using nonlinear regression analysis (Sigma Plot, Systat Software Inc., San Jose, CA). Each set of data was fitted to both the Michaelis-Menten and the Hill equations. The quality of fit to a particular model was determined by evaluation of three criteria that are listed in decreasing order of importance: 1) the randomness of the residuals; 2) the size of the sum of the square of the residuals; and 3) the standard error of the parameter estimates (Soars et al. 2003). The maximal intrinsic clearance due to autoactivation kinetics was calculated based on the following equation (Houston and Kenworthy, 2000):

$$\frac{\varphi}{[S]} = \frac{V_{max}}{S_{50}} \times \frac{(n - 1)}{n(n - 1)^{1/n}}$$

**Inhibition with Chemical Inhibitors of UGTs.** The incubations of 1'-hydroxymidazolam at a substrate concentration close to the apparent $K_m/S_{50}$ values (25 or 50 µM) were conducted in human liver microsomes, as described above, in the presence of diclofenac (9.3 to 500 µM) or hecogenin (2.5 to 400 µM). The reaction was allowed to proceed at 37°C for 45 min, and was terminated with ice-cold acetonitrile containing 0.2% formic acid and phenolphthalein-β-D-glucuronide as the internal standard. The assay was performed in duplicate. Samples were centrifuged prior to LC-MS/MS analysis. Similar inhibition studies were conducted using recombinant UGT1A4 or 2B4/2B7 in the presence of hecogenin or diclofenac (0.4 to 100 µM), respectively. The IC$_{50}$ values were calculated using nonlinear regression analysis (KaleidaGraph by Synergy Software).
Isolation of 1'-Hydroxymidazolam Glucuronides. The glucuronides of 1'-hydroxymidazolam (Glu-A and Glu-B) were isolated from human liver microsomal incubations. Briefly, the reaction mixture was precipitated with an equal volume of acetonitrile, followed by centrifugation. The supernatant was concentrated under N₂, and subjected to several centrifugations in preparation for injection onto HPLC for further purification. Two columns were used sequentially for isolating the glucuronides, Synergi Polar-RP column (4.6 x 250 mm, 4 µm; Phenomenex, Torrance, CA), and Zorbax RX C8 column (4.6 x 250 mm, 5 µm; Agilent, Wilmington, DE).

NMR Analysis. NMR Spectra were acquired using a Varian Inova 600 MHz spectrometer using CD₃CN as solvent. Signal assignments were obtained using ¹H-¹H COSY, NOESY, and ¹H-¹³C HSQC 2D NMR experiments. The ¹³C chemical shifts were obtained from the HSQC spectra. Chemical shifts are expressed in parts per million (ppm) downfield from TMS.

LC-MS/MS Analysis. LC-MS/MS was carried out on a Perkin Elmer Sciex API-3000 (Concord, Ontario) triple quadrupole mass spectrometer, interfaced to a Perkin-Elmer HPLC system (Norwalk, CT) equipped with two Series 200 pumps and a Perkin-Elmer Series 200 autosampler. The instrument was operated in the positive ion mode using a Turbo-ionspray interface. The chromatography was conducted using a Polar-RP column (4.6 x 250 mm, 4 µm particle size) purchased from Phenomenex (Torrance, CA). The mobile phase consisted of 5 mM ammonium acetate in water (A), and acetonitrile:methanol (70:30; v/v) (B). The analytes were eluted at 1 ml/min using a linear gradient. Solvent B started at 20%, then increased as follows: 40% (25 min), 50% (26 min), 60% (40 min), 95% (45 min). The column was washed at 95% B for 3 min, before
equilibrating with starting conditions. The retention times for the O- and N-glucuronides (Glu-A and Glu-B, respectively) were 10 and 21 min, respectively. For quantitation purposes, another gradient was used as follows. Solvent B started at 20% and increased as follows: 50% (8 min), 95% (17 min). The relative amounts of the glucuronides of 1'-hydroxymidazolam were determined using multiple reaction monitoring (MRM) of the transitions \(m/z\) 518 \(\rightarrow\) \(m/z\) 324 for the O-glucuronide, \(m/z\) 518 \(\rightarrow\) \(m/z\) 342 for the N-glucuronide of 1'-hydroxymidazolam, and \(m/z\) 495 \(\rightarrow\) \(m/z\) 319 for the internal standard. The amounts of the isolated O- and N-glucuronides were determined by quantitative NMR analysis (Pauli et al. 2005). The quantitation was accomplished by comparing the absolute integral values of a well-resolved proton signal in each glucuronide sample to the integral of the same signal in an external standard (1'-hydroxymidazolam). The precision and accuracy of the method was evaluated using known amounts of 1'-hydroxymidazolam, with coefficient of variations <5%, and accuracy of 97.4%. The analysis of the standards and the glucuronides was done back-to-back under identical conditions (i.e., using the same NMR spectrometer, receiver gain, probe tuning, acquisition and processing parameters). The isolated metabolites were used to construct standard curves for later LC-MS/MS quantitation.
Results

Glucuronidation of 1'-hydroxymidazolam and midazolam in human and rat liver microsomes. When 1'-hydroxymidazolam was incubated with human liver microsomes in the presence of UDPGA, two putative glucuronides (Glu-A and Glu-B) were detected, as confirmed by full scan analysis that showed addition of 176 Da to the parent (m/z 518). Only Glu-A was detected in incubations with rat liver microsomes (Fig. 1a). Subsequent product ion scan at m/z 518 revealed different fragmentation patterns for these two glucuronides. A fragment ion at m/z 324 (loss of 194 Da) was the most prominent fragment observed with Glu-A; while a fragment ion at m/z 342 (loss of 176 Da) was the major ion for Glu-B (Fig. 1a).

When midazolam was incubated with liver microsomes enriched with UDPGA, one glucuronide (Glu; m/z 502) was detected in human liver microsomes, but not in rat liver microsomes (Fig. 1b).

Structure identification of 1’-hydroxymidazolam glucuronides (Glu-A and Glu-B) by NMR. Glu-A and Glu-B were isolated and their structures were determined by comparing their NMR spectra to that of 1’-hydroxymidazolam (Fig. 2a). The major glucuronide (Glu-A) was confirmed to be the O-glucuronide (Fig. 2c). The minor (Glu-B), a hitherto unknown glucuronide, was shown to be a quaternary N-glucuronide (Fig. 2b). The distinction between O- vs. N-glucuronide structures was evident from both 1H and 13C chemical shifts of the anomeric position (4.46 and 101.0 ppm in Glu-A vs 5.53 and 87.8 ppm in Glu-B, respectively; see supplemental materials). The exact location of the N-glucuronide moiety was determined on the basis of NOE observed between the anomeric proton and the imidazole proton (as indicated by the arrow in Fig. 3).
Enzyme kinetics of 1’-hydroxymidazolam glucuronidation in human liver microsomes. The rates of formation of O- and N-glucuronides were proportional to microsomal protein concentration from 0.25 to 0.5 mg protein/mL, and were approximately linear with time of incubation from 5 to 60 min (data not shown). For most of the microsomal incubations, 0.5 mg/mL protein and 30 min incubation time were used. The effect of substrate concentration (5 to 200 µM) on the glucuronidation of 1’-hydroxymidazolam was shown in Fig. 4. Both the O- and N-glucuronidation seemed to best fit the Hill model yielding an n value of ~1.2 and 1.5, respectively. The apparent S50 values for the formation of O- and N-glucuronides were ~43 and 18 µM, respectively, in human liver microsomes. The corresponding apparent Vmax values were 363 and 21 pmol/mg/min (Fig. 4). Thus, the maximal intrinsic clearance for the O-glucuronidation was ~9-fold higher than that for the N-glucuronidation.

Glucuronidation of 1’-hydroxymidazolam by recombinant UGTs. Incubations of 1’-hydroxymidazolam with 12 recombinant human UGTs (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, 2B17) revealed that O-glucuronidation was catalyzed by UGT2B4 and 2B7, whereas N-glucuronidation was catalyzed by UGT1A4. No metabolism was observed with other UGT enzymes (the limit of assay sensitivity was ~0.01 µM). The kinetic parameters of the glucuronidation catalyzed by UGT2B4, 2B7 and 1A4 are presented in Table 1. Incubations of various concentrations of 1’-hydroxymidazolam with UGT2B4 and 2B7 demonstrated a similar apparent Km value of ~35 µM for the formation of the O-glucuronide. The corresponding Vmax values were 37 and 27 pmol/mg protein/min. The N-glucuronidation by UGT1A4 exhibited non
Michaelis-Menten kinetics, with an apparent $S_{50}$ value of 40 $\mu$M, apparent $V_{\text{max}}$ value of 19 pmol/mg protein/min, and an $n$ value of 1.5 (Table 1).

**Inhibition of 1'-hydroxymidazolam glucuronidation in human liver microsomes.**

The inhibition study was conducted at a substrate (1'-hydroxymidazolam) concentration of 25 or 50 $\mu$M in the presence of UGT inhibitors, hecogenin or diclofenac. Hecogenin showed potent and selective inhibitory effect on the $N$-glucuronidation ($IC_{50}$ value = 1.1 $\mu$M), but had little or no inhibitory effect on the $O$-glucuronidation in human liver microsomes ($IC_{50}$ value > 500 $\mu$M) (Fig. 5a). This inhibitory effect of hecogenin was further confirmed using recombinant UGT1A4 ($IC_{50}$ value = 0.77 $\mu$M) (Fig. 5b). Diclofenac, on the other hand, had a greater inhibitory effect on the formation of $O$-glucuronide than on the formation of $N$-glucuronide by human liver microsomes ($IC_{50}$ values: 16 vs 151 $\mu$M) (Fig. 6a). Further inhibition studies with recombinant UGT isozymes indicated that diclofenac exhibited similar inhibitory effect toward UGT2B4- and 2B7-mediated $O$-glucuronidation of 1’-hydroxymidazolam, with $IC_{50}$ values of 7.4 and 17.2 $\mu$M, respectively (Fig. 6b).
Discussion

Following the incubations of 1'-hydroxymidazolam with human liver microsomes in the presence of UDPGA, two glucuronides (Glu-A and Glu-B) were detected. Based on the different fragmentation patterns, we assumed that Glu-A, which had a protonated aglycone with further loss of water (m/z 324) as the prominent fragment ion, was an O-glucuronide, and that Glu-B, which had a protonated aglycone corresponding to 1'-hydroxymidazolam (m/z 342) as the major fragment ion, was an N-glucuronide, given that more energy would be required to cleave a C-N bond than a C-O bond. Glu-A and Glu-B were isolated from microsomal incubations, and their structures were confirmed by NMR analysis. The NMR data indicated that the exact location of the glucuronic acid moiety in 1'-hydroxymidazolam N-glucuronide was at N-2. To the best of our knowledge, this is the first demonstration of a quaternary N-glucuronide of 1'-hydroxymidazolam in human liver microsomes, and the first rigorous structural proof of the O-glucuronide, which is often referred to as the 1'-hydroxymidazolam glucuronide. The maximal intrinsic clearance for O-glucuronidation was ~9-fold higher than that for N-glucuronidation, suggesting that O-glucuronidation could be the major conjugation pathway in human liver microsomes.

In a separate study, where midazolam was used as the substrate, an analogous N-glucuronide also was detected in human liver microsomal incubations in the presence of UDPGA (Fig 1b). It was reported previously that a quaternary N-glucuronide of midazolam was detected from human liver preparations in vitro, but no structure elucidation was presented (Siddle et al. 2003). Based on the structure of 1'-
hydroxymidazolam N-glucuronide presented here, it is reasonable to assume that glucuronic acid was conjugated to midazolam at the same N-2 position.

The O- and N-glucuronidation of 1'-hydroxymidazolam in human liver microsomes exhibited non-Michaelis-Menten kinetics consistent with autoactivation, which has been reported for in vitro glucuronidation of several substrates (Soars et al. 2003; Fisher et al. 2000) as well as in vivo (Wong et al. 2007). The mechanism of autoactivation kinetics observed for UGTs is currently unknown.

In vitro incubations with twelve different recombinant human UGT isoforms showed that UGT1A4 was the only isoform catalyzing N-glucuronidation of 1'-hydroxymidazolam. UGT1A4 has been identified in human liver and stomach. It is a major UGT isoform involved in the glucuronidation of many tertiary amines (Green and Tephly 1996; King et al. 2000). But there is no UGT1A4 ortholog in rat tissues (King et al. 2000). Accordingly, when 1'-hydroxymidazolam was incubated with rat liver microsomes enriched with UDPGA, only the O-glucuronide (Glu-A) but not the N-glucuronide (Glu-B) was detected, consistent with the observation that UGT1A4 is the only UGT isoform catalyzing the N-glucuronidation. Similar studies with midazolam also showed that an analogous N-glucuronide formed in human liver microsomes was not observed in rat liver microsomes enriched with UDPGA, and only UGT1A4 was able to catalyze the formation of midazolam N-glucuronide.

Hecogenin is a known substrate and a selective inhibitor of UGT1A4 (Green and Tephly 1996; Al-Zoughool and Talaska 2006; Uchaipichat et al. 2006). In the current inhibition study, hecogenin essentially abolished the N-glucuronidation of 1'-hydroxymidazolam in human liver microsomes, while it had little or no inhibitory effect
on the \(O\)-glucuronidation. In a follow-up inhibition study using recombinant UGT1A4, hecogenin inhibited UGT1A4-mediated \(N\)-glucuronidation at an IC\(_{50}\) value less than 1 \(\mu\)M. Taken together, these data confirmed the involvement of UGT1A4 in the formation of \(1'\)-hydroxymidazolam \(N\)-glucuronide.

Our study demonstrated that UGT2B4 and 2B7 are the two isoforms responsible for the in vitro formation of \(1'\)-hydroxymidazolam \(O\)-glucuronide in human liver microsomes. UGT2B7 is a very important human UGT isoform in that it appears to be expressed in many tissues besides liver, and it catalyzes the glucuronidation of a wide range of xenobiotics, including polycyclic aromatic hydrocarbons, phenols, opioids, aliphatic alcohols, carboxylic acids, tetrazoles (King et al. 2000). Recently, UGT2B7 was reported to catalyze the \(N\)-glucuronidation of an amide and a primary amine (Zhang et al. 2004; Staines et al. 2004). In contrast, UGT2B4 catalyzes only a limited number of substrates, and the data so far are not consistent among different labs. For example, the glucuronidation of hyodeoxycholic acid was reported to be catalyzed by UGT2B4; yet in another study, UGT2B4 showed no activity for hyodeoxycholic acid (King et al. 2000). The best substrate for this isoform has yet to be identified. Diclofenac is a substrate of UGT2B7, and also inhibits the glucuronidation of dihydrocodeine and morphine catalyzed by UGT2B7 (King et al 2001). In the present study, diclofenac selectively inhibited \(O\)-glucuronidation of \(1'\)-hydroxymidazolam in human liver microsomes, and also showed similar inhibitory effect toward UGT2B4- and 2B7-mediated \(O\)-glucuronidation of \(1'\)-hydroxymidazolam.

Available data suggest that UGT enzymes exhibit distinct but overlapping substrate selectivities (King et al. 2000; Burchell et al. 1995). Thus, identifying selective substrates
of certain UGT isoforms could be very useful when evaluating drug-drug interaction potential of a given compound at the UGT level. For this purpose, 1'-hydroxymidazolam could be used as an in vitro probe substrate for UGT1A4 and UGT2B4/2B7 by monitoring the formation of N- and O-glucuronide, respectively. In clinical drug-drug interaction studies, midazolam is often used as a probe substrate for CYP3A (Thummel et al. 1994a; Thummel et al. 1994b; Huang et al. 2007). The exposure to midazolam, if altered, will inform on the potential of an investigational drug as a perpetrator of CYP3A. On the other hand, given that the levels of 1'-hydroxymidazolam in plasma are the result of formation by CYP3A and subsequent metabolism to glucuronides by UGTs, by monitoring plasma concentration of 1'-hydroxymidazolam from the same study, we may be able to get additional insights into the potential of the investigational drug to affect selected UGT isoforms (UGT1A4, 2B4, 2B7), assuming the exposure to midazolam is not altered.

In summary, we have demonstrated, for the first time, the formation of a quaternary N-glucuronide of 1'-hydroxymidazolam, in addition to the well-known O-glucuronide in human liver microsomes enriched with UDPGA. Also, we identified the corresponding enzymes responsible for the glucuronidations as UGT1A4 and UGT2B4/2B7, respectively.
Acknowledgements

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References


Footnotes

Part of this work was presented at the 3\textsuperscript{rd} Pharmaceutical Sciences World Congress, April 2007 (Amsterdam, the Netherlands).

\textsuperscript{a} these authors contributed to this work equally.

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List of Figures

Fig. 1. LC-MS/MS analysis of microsomal incubations of 1’-hydroxymidazolam in the presence of UDPGA. Left two panels: product ion scan of $m/z$ 518 from human and rat liver microsomal incubations. Right two panels: product ion spectra of Glu-A and Glu-B (a). LC-MS/MS analysis of microsomal incubations of midazolam in the presence of UDPGA from human and rat liver microsomal incubations. Left two panels: product ion scan at $m/z$ 502. Right panel: product ion spectra of Glu (b).

Fig. 2. $^1$H NMR spectra of 1’-hydroxymidazolam (a); 1’-hydroxymidazolam N-glucuronide (b); 1’-hydroxymidazolam O-glucuronide (c).

Fig. 3. $^1$H-$^1$H 2D NOE (ROESY) NMR spectrum of 1’-hydroxymidazolam N-glucuronide.

Fig. 4. Concentration-dependent rate of formation of 1’-hydroxymidazolam glucuronides in human liver microsomes in the presence of UDPGA. Right two panels: Eadie-Hofstee plots of O- and N-glucuronidation in human liver microsomes.

Fig. 5. Inhibition of 1’-hydroxymidazolam glucuronidation in human liver microsomes (a) or recombinant UGT1A4 (b) by hecogenin.

Fig. 6. Inhibition of 1’-hydroxymidazolam glucuronidation in human liver microsomes (a) or recombinant UGT2B4/2B7 (b) by diclofenac.
Table 1  Kinetics of 1’-hydroxymidazolam glucuronidation by UGT2B4, 2B7 and 1A4.

<table>
<thead>
<tr>
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<th>O-Glucuronidation</th>
<th>N-Glucuronidation</th>
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<tbody>
<tr>
<td></td>
<td>UGT 2B4</td>
<td>UGT 2B7</td>
</tr>
<tr>
<td>$K_m^{\text{app}}$ (µM)</td>
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<td>32</td>
</tr>
<tr>
<td>$V_{\text{max}}^{\text{app}}$ (pmol/mg/min)</td>
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<td>27</td>
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<tr>
<td>$n^a$</td>
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</tr>
<tr>
<td>Model</td>
<td>Michaelis-Menten</td>
<td>Michaelis-Menten</td>
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Values represent average obtained from duplicate incubations.

$^a$ n: Hill coefficient; when n=1, Hill equation = Michaelis-Menten equation.
Fig. 1

a)

- **HLM** with Glu-A and Glu-B markers.
- **RLM** with Glu-A marker.

<table>
<thead>
<tr>
<th>Relative Intensity (%)</th>
<th>Time (min)</th>
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<tbody>
<tr>
<td>0%</td>
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<tr>
<td>20%</td>
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<td>80%</td>
<td>25-30</td>
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<tr>
<td>100%</td>
<td>30-45</td>
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</tbody>
</table>

- **Glu-A (O-Glu)**
  - m/z (amu): 324, 342 (518-176)

- **Glu-B (N-Glu)**
  - m/z (amu): 342 (518-176), 324
Fig. 1

b)
Fig. 3

[Chemical structure and 2D NMR spectrum with annotations]

* = atropisomers
Fig. 4

**O-Glu**

- $S_{50} = 43 \, \mu M$
- $V_{max} = 363 \, \text{pmol/mg/min}$
- $n = 1.2$

**N-Glu**

- $S_{50} = 18 \, \mu M$
- $V_{max} = 21 \, \text{pmol/mg/min}$
- $n = 1.5$
Fig. 5

a).

Hecogenin Concentration (µM)

IC$_{50}$ = 1.1 µM

b).

Hecogenin Concentration (µM)

IC$_{50}$ = 0.77 µM
Fig. 6

a).

- O-Glu
- N-Glu

IC₅₀ = 151 μM

IC₅₀ = 16 μM

Diclofenac Concentration (uM)

b).

- UGT2B4
- UGT2B7

IC₅₀ = 17.2 μM

IC₅₀ = 7.4 μM

Diclofenac Concentration (uM)