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The metabolism of 1'- and 4-hydroxymidazolam by glucuronide conjugation is largely mediated by UDP-glucuronosyltransferases 1A4, 2B4, and 2B7

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Running title: UGT isoforms involved in 1'- and 4-hydroxymidazolam glucuronides

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ABBREVIATIONS:

P450, cytochrome P450; HLM, human liver microsome; LC-MS/MS, liquid chromatography-tandem mass spectrometry; UGT, UDP-glucuronosyltransferase

Abstract

Midazolam undergoes oxidative hydroxylation by cytochrome P450 (CYP) 3A to its metabolites, which are excreted mainly as glucuronidated conjugates into the urine. In this study, we examined the glucuronidation of hydroxymidazolam in human liver microsomes (HLMs) and characterized the UDP-glucuronosyltransferases (UGTs) involved in 1'- and 4-hydroxymidazolam glucuronidation. Among the twelve UGT isoforms tested, the *O*- and *N*-glucuronidation of 1'-hydroxymidazolam was mediated by UGT2B4/2B7 and 1A4, respectively. In contrast, the glucuronidation of 4-hydroxymidazolam was mediated by UGT1A4. Consistent with these observations, the UGT1A4 inhibitor hecogenin and the UGT2B7 substrate diclofenac potently inhibited the *N*- and *O*-glucuronidation of 1'-hydroxymidazolam in HLMs, respectively. A correlation analysis of UGT enzymatic activity and the formation rate of glucuronide metabolites from 1'- and 4-hydroxymidazolam in 25 HLMs showed that hydroxymidazolam glucuronidation is correlated with UGT1A4-mediated lamotrigine glucuronidation and UGT2B7-mediated diclofenac glucuronidation activity. Taken together, these findings indicate that UGT1A4, 2B4, and 2B7 are major isoforms responsible for glucuronide conjugate formation from 1'- and 4-hydroxymidazolam, which are the two major oxidative metabolites of midazolam.

Introduction

Glucuronidation is an important detoxification and elimination pathway for endogenous and exogenous compounds, and it accounts for ~35% of all phase II drug metabolism (Evans and Relling, 1999). UDP-glucuronosyltransferases (UGTs) mediate glucuronidation. In humans, the UGT superfamily is comprised of two families (UGT1 and UGT2) and three subfamilies (UGT1A, 2A, and 2B) (Mackenzie et al., 2005). The lone UGT1A locus on Chromosome 2q37 encodes nine functional proteins: UGT1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, and 1A10. The nomenclature of UGT1A is based on the relative position of the first variable exon with respect to four common exons in the unspliced mRNA. The UGT2A subfamily has multiple loci located on Chromosome 4q13 and is comprised of the following functional proteins: UGT2A1, 2B4, 2B7, 2B10, 2B11, 2B15, 2B17, and 2B28 (Guillemette, 2003).

Individual UGTs exhibit distinct, but overlapping, substrate selectivities and differ in their regulation of expression, genetic polymorphisms, and other factors known to influence the activity of drug-metabolizing enzymes in humans (Miners et al., 2002, 2004). Despite recognition of the importance of glucuronidation as a clearance and detoxification mechanism for drugs, environmental chemicals, and endogenous compounds, relatively few selective substrates and inhibitors useful for the phenotyping of glucuronidated compounds have been identified (Court, 2005).

Midazolam is a potent benzodiazepine with sedative activities. It is metabolized to 1'- and 4-hydroxymidazolam by cytochrome P450 (CYP) 3A4 and CYP3A5 (Kronbach et al., 1989), and is further metabolized to glucuronide conjugates by UGT (Fig. 1). Glucuronide conjugates of 1'-hydroxymidazolam account for 60-70% of the

midazolam dose (Heizmann and Ziegler, 1981). Recently, Zhu et al. (2008) identified an *N*-linked glucuronide conjugate of 1'-hydroxymidazolam by incubation with human liver microsomes (HLMs). Although the *O*- and *N*-glucuronidation of 1'-hydroxymidazolam is catalyzed by UGT2B4/2B7 and 1A4, respectively (Zhu et al., 2008), the specific hepatic UGT isoforms involved in 4-hydroxymidazolam glucuronidation have not been identified.

The objective of this study was to identify and kinetically characterize and compare the UGT isoforms responsible for 1'- and 4-hydroxymidazolam glucuronidation *in vitro*. Our data will allow a better understanding of the factors affecting midazolam pharmacokinetics and drug interactions.

Materials and Methods

Chemicals and reagents. Midazolam, 1'-hydroxymidazolam, and 4-hydroxymidazolam were purchased from Ultrafine Chemical Co. (Manchester, UK). Alamethicin (from *Trichoderma viride*), uridine diphosphoglucuronic acid (UDPGA), saccharic acid-1, 4-lactone, diclofenac, hecogenin, and lamotrigine were obtained from Sigma-Aldrich (St. Louis, MO). The solvents were of HPLC grade (Fisher Scientific Co.); all other chemicals were of the highest quality available. Recombinant human UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) and pooled and ten single-donor HLMs were purchased from BD Gentest Co. (Woburn, MA). The vendor supplied information regarding the protein concentration. Fifteen single-donor HLMs were obtained from the Inje PGRC tissue bank (Busan,

Korea).

***In vitro* metabolism of hydroxymidazolam by HLMs.** For the microsomal phase II metabolism of hydroxymidazolam, 0.25 mg of pooled HLMs (H161, Gentest), 1'- or 4-hydroxymidazolam (100 μM), and 1.5 mg/mL alamethicin were reconstituted in 0.5 M Tris-HCl buffer (pH 7.5) and preincubated on ice for 15 min. The reaction was initiated by adding UDPGA (5 mM) and run for 60 min at 37°C in a shaking water bath. In this study, 60 min incubation time was selected because the reaction showed linearity till 60 min from the preliminary experiments. The reaction was terminated by the addition of 100 μL of acetonitrile on ice, and then centrifuged at 10,000 x *g* for 5 min at 4°C. Aliquots of the supernatant were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) for the identification of glucuronide conjugate metabolites of 1'- and 4-hydroxymidazolam.

Analysis of the glucuronide conjugates of hydroxymidazolam by LC-MS/MS. For the identification of the glucuronide metabolites of hydroxymidazolam, an API 3000 LC-MS/MS system (Applied Biosystems, Foster City, CA), coupled with an Agilent 1100 series HPLC system (Wilmington, DE), was used. Separation was performed on a Polar-RP column (2 mm i.d. × 250 mm, 4-μm particle size; Phenomenex, Torrance, CA) with a mobile phase consisting of water (A) and acetonitrile (B) containing 0.1% formic acid. The analytes were eluted at a flow rate of 0.2 mL/min using a linear gradient. Solvent B started at 30%, then increased as follows: 30 (1 min), 50 (6-8 min), and 30% (9 min). The Turboion Spray interface was operated in the positive ion mode at 5,500 V and 500°C. The operating conditions were as follows: nebulizing gas flow, 7 psi; curtain

gas flow, 12 psi; and collision energy, 35 eV. Quadrupoles Q1 and Q3 were set on unit resolution. Multiple-reaction-monitoring (MRM) mode using specific precursor/product ion transition was employed for quantification. Detection of the ions was performed by monitoring the transitions of *m/z* 518 → 324 and 518 → 342 for the hydroxymidazolam glucuronides, and 339 → 163 for 7-hydroxycoumarin glucuronide (IS). Peak areas for all compounds were automatically integrated using Analyst software (version 1.2, Applied Biosystems).

Glucuronidation of hydroxymidazolam in HLMs or recombinant UGT isoforms. The optimal conditions for microsomal incubation were determined in the linear range for the formation of glucuronide metabolites from hydroxymidazolam. In all experiments, 1'- and 4-hydroxymidazolam were dissolved and serially diluted with methanol to the required concentrations. The final concentration of organic solvent did not exceed 1% in the final incubation mixtures. A typical incubation mixtures (total volume, 125 μL), containing either 0.25 mg of HLMs or recombinant UGT (0.1 mg), 1'- or 4-hydroxymidazolam (1-200 μM), and 1.5 mg/mL alamethicin were reconstituted in 0.5 M Tris-HCl buffer (pH 7.5) and preincubated on ice for 15 min. The reaction was initiated by adding UDPGA (5 mM) and run for 60 min at 37°C in a shaking water bath. The reaction was terminated by the addition of 100 μL of acetonitrile on ice, and the mixture was centrifuged at 10,000 x *g* for 5 min at 4°C. Aliquots of the supernatant were used for LC-MS/MS.

Chemical inhibition studies with HLMs. The inhibitory effects of the known UGT1A4 inhibitor hecogenin (final concentration, 10 μM) or the UGT2B7 substrate

diclofenac (50 μM) on the formation of 1'- and 4-hydroxymidazolam glucuronides were evaluated to determine the UGT isoforms involved in this metabolic pathway. Hecogenin was dissolved in DMSO and diclofenac was dissolved in methanol. The final concentration of the organic solvents in the reaction mixture was < 1% (v/v). The formation ratio of 1'- and 4-hydroxymidazolam glucuronides from 1'- and 4-hydroxymidazolam were determined from reaction mixtures incubated in the presence or absence of hecogenin or diclofenac. Except for the addition of hecogenin or diclofenac, all other incubation conditions were similar to those described previously.

Correlation experiments. For the correlation analysis, the comparative metabolic rates of hydroxymidazolam in 25 different HLMs were investigated by the incubation of 1'- and 4-hydroxymidazolam (50 μM) with 0.25 mg/mL microsomal protein for 60 min. The activity of each UGT isoform was determined by LC-MS/MS as described previously for lamotrigine (Rowland et al., 2006) and diclofenac glucuronidation (King et al., 2001). The correlation coefficients between the formation rates of glucuronide metabolites of 1'- and 4-hydroxymidazolam and the activity of each UGT isoform in the various HLMs were calculated by Spearman's correlation coefficient analysis (SAS version 8.01, SAS Institute Inc., Cary, NC). A p-value < 0.05 was considered to be statistically significant.

Data analysis. All results are expressed as the mean ± S.D. of estimates obtained from three different HLMs in triplicate experiments. Because of the absence of authentic standards for the hydroxymidazolam glucuronides, the conjugates were semiquantified using a hydroxymidazolam calibration curve. The apparent kinetic parameters for the

biotransformation of 1'- and 4-hydroxymidazolam (K_m and V_{max}) were determined by fitting an one-enzyme Michaelis-Menten equation or a Hill equation ($V = V_{max} \cdot [S]^n/[K_m+(S)^n]$). The calculated parameters include the maximum rate of formation (V_{max}), the Michaelis-Menten constant (apparent K_m), the intrinsic clearance (CL_{int} = V_{max}/apparent K_m), and Hill coefficient (n). All calculations were performed using WinNonlin software (Pharsight, Mountain View, LA). The percent inhibition was calculated from the ratio of the amount of metabolite formed with and without the specific inhibitor.

Results

Identification of glucuronide metabolites of hydroxymidazolam in HLMs. After the incubation of 1'-hydroxymidazolam with HLMs in the presence of UDPGA, two metabolites were profiled. These metabolites were confirmed as glucuronide conjugates by full-scan analysis, which showed the addition of 176 Da (glucuronic acid) to the parent (*m/z* 518). These two metabolites were tentatively identified as 1'-hydroxymidazolam *O*- and *N*-glucuronide based on the MS/MS fragmentation pattern. A fragment ion at *m/z* 324 (loss of glucuronic acid and a water molecule) was the base peak observed with 1'-hydroxymidazolam *O*-glucuronide, whereas that at *m/z* 342 (loss of glucuronic acid) was the major fragment ion for 1'-hydroxymidazolam *N*-glucuronide (Fig. 2). These results are consistent with those from previous reports (Zhu et al., 2008). When 4-hydroxymidazolam was incubated with the HLMs, two glucuronide conjugates (*m/z* 518) were detected. On the basis of the structures of the 1'-hydroxymidazolam *O*- and *N*-glucuronides, which were reported by Zhu et al. (2008), it

is reasonable to assume that glucuronic acid was conjugated to 4-hydroxymidazolam at the same N and OH positions. These two metabolites were designated as 4-hydroxymidazolam glucuronide I and II because we could not distinguish 4-hydroxymidazolam *O*-glucuronide from the *N*-glucuronide.

Identification of the UGT isoforms involved in the metabolism of hydroxymidazolams. Two concentrations of 1'- and 4-hydroxymidazolam (20 and 100 μM) were incubated with a panel of recombinant UGT isoforms. The formation rates of hydroxymidazolam glucuronides after the incubation of 1'- or 4-hydroxymidazolam with the UGT isoforms are shown in Fig. 3. UGT1A4 and 2B4/2B7 metabolized 1'-hydroxymidazolam to 1'-hydroxymidazolam *N*- and *O*-glucuronide, respectively, more efficiently than any other UGT (Fig. 3A and B), whereas UGT1A4 metabolized 4-hydroxymidazolam to its glucuronide metabolites with little contribution from UGT1A3, 1A6, and 2B7 (Fig. 3C and D). Hydroxymidazolam glucuronide formation from 1'- and 4-hydroxymidazolam (50 μM) was also studied using a UGT isoform inhibitor or substrate (Fig. 4). 1'-Hydroxymidazolam *N*-glucuronidation was inhibited by the UGT1A4 inhibitor hecogenin (IC_{50} = 1.5 μM), whereas 1'-hydroxymidazolam *O*-glucuronidation was inhibited by the UGT2B7 substrate diclofenac (IC_{50} = 2.0 μM). The formation of 4-hydroxymidazolam glucuronide I and II was also inhibited by hecogenin (IC_{50} = 0.8 μM) and diclofenac (IC_{50} = 46.6 μM), respectively (Table I). The formation rates of 1'-hydroxymidazolam *N*-glucuronide and 4-hydroxymidazolam glucuronide I were significantly correlated with the activity of UGT1A4, whereas that of 1'-hydroxymidazolam *O*-glucuronide was correlated with the activity of UGT2B7

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and 1A4 (Fig. 5).

Enzyme kinetic analysis. Kinetic analyses of 1'- and 4-hydroxymidazolam metabolite formation were performed using three different HLM preparations. Under the experimental conditions used, the metabolism of 1'- and 4-hydroxymidazolam by the HLMs was best-fitted by a Hill equation (Fig. 6). The fitting of the data to the Michaelis-Menten two-enzyme model did not significantly improve the regression, as compared with fitting of the data to a one-enzyme model. The kinetic parameters estimated from the three HLMs are shown in Table 2. The sums of the formation (CL_{int}) of both metabolites were 346.2 and 90.3 μL/min/mg protein for 1'- and 4-hydroxymidazolam, respectively (Table 2).

Next, we examined the enzyme kinetic parameters for the formation of glucuronide conjugates from each hydroxymidazolam (0-200 μM) upon incubation with recombinant human UGT1A4, 2B4, and 2B7 (Fig. 7). Under the experimental conditions used, the metabolism of 1'- and 4-hydroxymidazolam by these UGT isoforms was also best described by a Hill equation. The UGT1A4-mediated *N*-glucuronidation of 1'-hydroxymidazolam showed a lower K_m and higher V_{max} than did UGT2B4- and 2B7-catalyzed *O*-glucuronidation, resulting in a higher CL_{int} (29.5 vs. 6.8 μL/min/mg protein, Table 3). The *in vitro* intrinsic clearance for glucuronide conjugate formation from 1'-hydroxymidazolam by UGT1A4 was similar to that for 4-hydroxymidazolam by UGT1A4 (29.5 vs. 26.1 μL/min/mg protein).

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Discussion

The glucuronidation of midazolam and its oxidized metabolites is an important part of midazolam metabolism in humans. It has been reported that the amount of hydroxymidazolam *O*-glucuronide excreted in the urine is quite variable (Heizmann and Ziegler, 1981). This variability could be one factor responsible for the large inter-individual differences seen in midazolam metabolism. In the present study, the glucuronidation of 1'- and 4-hydroxymidazolam in HLMs and the recombinant UGT isoforms mediating this metabolism were thoroughly evaluated.

In this study, we reconfirmed that 1'-hydroxymidazolam is metabolized to *N*- and *O*-glucuronide in the presence of UDPGA in HLMs, and that UGT1A4 and 2B4/2B7 are the major enzymes responsible for 1'-hydroxymidazolam *N*- and *O*-glucuronidation, respectively. Recombinant human UGT2B4/2B7 and 1A4 metabolized 1'-hydroxymidazolam to 1'-hydroxymidazolam *O*- and *N*-glucuronide, respectively, whereas the other UGT isoforms did not (Fig. 3). The formation rates of 1'-hydroxymidazolam *N*-glucuronide were strongly inhibited by hecogenin (10 μM, ~90%), a potent UGT1A4-selective inhibitor (Fig. 4) (Uchaipichat et al., 2006), while those of 1'-hydroxymidazolam *O*-glucuronide were inhibited by diclofenac (50 μM, ~95%), a selective UGT2B7 substrate (King et al., 2001). We found a significant correlation between 1'-hydroxymidazolam *N*-glucuronidation in HLMs (n = 25) and the glucuronidation activity of the UGT1A4 substrate lamotrigine (Rowland et al., 2006), whereas 1'-hydroxymidazolam *O*-glucuronidation was correlated with glucuronidation activity mediated by the UGT2B7 substrates diclofenac (King et al., 2001) and lamotrigine. The significant correlation observed between the activity of UGT1A4 and 1'-hydroxymidazolam *O*-glucuronidation in the panel of HLMs tested may not directly

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involve UGT1A4. Because our inhibition and recombinant experiments do not support a significant role for UGT1A4 in 1'-hydroxymidazolam *O*-glucuronidation, the observed correlation may be derived from the involvement of UGT2B7 in lamotrigine glucuronidation (Rowland et al., 2006). These results are compatible with those from a previous report by Zhu et al. (2008).

In addition, we demonstrated for the first time that 4-hydroxymidazolam is transformed to two glucuronide metabolites (I and II). Formation of the major metabolite, 4-hydroxymidazolam glucuronide I, was catalyzed by UGT1A4 but not the other UGT isoforms (Fig. 3C). The other isoforms, including UGT1A3, 1A6, 2B4, and 2B7, contributed to the formation of the minor metabolite 4-hydroxymidazolam glucuronide II (Fig. 3D). We also found that the formation rates of 4-hydroxymidazolam glucuronide I were strongly inhibited by hecogenin (10 μM, ~95%) (Fig. 4). Additionally, diclofenac, a well-known UGT2B7 substrate, inhibited 4-hydroxymidazolam glucuronide II formation (50 μM, ~50%). Our data significantly correlated 4-hydroxymidazolam glucuronide I formation in HLMs with UGT1A4-mediated lamotrigine *N*-glucuronidation (Fig. 5). Taken together, these results suggest that 4-hydroxymidazolam glucuronide I is the major glucuronide metabolite of 4-hydroxymidazolam and was formed by UGT1A4.

The glucuronidation of 1'- and 4-hydroxymidazolam in HLMs was characterized by Hill kinetics, suggesting the involvement of a single enzyme or more than one enzyme with similar affinities (Fig. 6). Eadie-Hofstee plots of 1'-hydroxymidazolam glucuronide formation showed convex relationships (Fig. 6B), indicating positive cooperativity ($n > 1$), whereas 4-hydroxymidazolam glucuronide I and II formation showed convex ($n = 1.2$) and concave ($n = 0.5$) relationships (Fig. 6D),

respectively. The intrinsic clearance for 1'-hydroxymidazolam *O*-glucuronidation was roughly three-fold higher than that for *N*-glucuronidation (Table 2). A similar kinetic profile was reported by Zhu et al. (2008), although the difference in intrinsic clearance for metabolite formation was higher than observed in this study. This may be accounted for by differences between the assay conditions in their study (100 mM phosphate buffer, 2 mM $MgCl_2$, 0.5 mg/mL HLMs, and 2 mM UDPGA) and in the present study. A different kinetic profile for nicotine *N*-glucuronidation has also been shown to be dependent on the incubation conditions (Ghosheh et al., 2001; Nakajima et al., 2002). In the current study, 4-hydroxymidazolam glucuronide I formation was a major metabolic pathway for 4-hydroxymidazolam glucuronidation (the CL_{int} values for 4-hydroxymidazolm glucuronide I and II formation were 87.0 and 3.3 μL/min/mg protein, respectively). In contrast to 4-hydroxymidazolam, 1'-hydroxymidazolam showed high enzyme affinity (low K_m values), resulting in relatively high metabolic clearance (Table 2).

We observed large inter-individual variability in 1'-hydroxymidazolam *O*-glucuronidation (roughly nine-fold), *N*-glucuronidation (~20 fold), and 4-hydroxymidazolam glucuronide I formation (about thirteen-fold) in the HLMs (n = 25). Large inter-individual differences in the percentage of glucuronide conjugates excreted in the urine may be due to inter-individual differences in the formation of these metabolites (i.e., differences in the catalytic properties of the UGT isoforms, rather than in the process of excretion) (Nakajima et al., 2002). There are genetic polymorphisms in certain UGT isoforms, including UGT1A1, 1A4, 1A6, 2B4, 2B7, and 2B15 (Tukey and Strassburg, 2000). Polymorphisms in the UGT1A4, 2B4, and 2B7 isoforms might cause

the inter-individual differences seen in 1'- and 4-hydroxymidazolam glucuronidation in humans.

In conclusion, we thoroughly characterized the glucuronidation of 1'- and 4-hydroxymidazolam in HLMs. Using UGT isoform-specific inhibitors, correlation analyses, and recombinant UGT isoforms, we found that UGT1A4 and 2B4/2B7 were responsible for the formation of 1'-hydroxymazolam *N*- and *O*-glucuronide, respectively, from 1'-hydroxymidazolam. In addition, the glucuronidation of 4-hydroxymidazolam was mainly metabolized by UGT1A4. The available data suggest that UGT enzymes exhibit overlapping substrate selectivity (Burchell et al., 1995; King et al., 2000). Therefore, identifying the substrate of each UGT isoform could be useful in UGT phenotyping. For this purpose, 4-hydroxymidazolam could be used *in vitro* as a probe substrate for UGT1A4 as has been suggested for 1'-hydroxymidazolam (Zhu et al., 2008).

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Footnotes

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Figure Legends

Figure 1. Proposed pathway of midazolam metabolism in HLMs.

Figure 2. MRM chromatogram of glucuronide metabolites obtained by LC-MS/MS using HLMs incubates of 1'- (A) and 4-hydroxymidazolam (B) in the presence of UDPGA.

Figure 3. Representative plots of the formation of 1'-hydroxymidazolam *N*- (A) and *O*-glucuronide (B) from 1'-hydroxymidazolam, 4-hydroxymidazolam glucuronides I (C) and II, and (D) from 4-hydroxymidazolam by recombinant human UGT isoforms.

Figure 4. Effects of the UGT isoform-selective inhibitor hecogenin (10 μM, UGT1A4) or the substrate diclofenac (50 μM, UGT2B7) on the formation of glucuronide metabolites from 1'- (A) and 4-hydroxymidazolam (B) by HLMs. Each bar represents the mean of duplicate determinations.

Figure 5. Simple regression analysis of lamotrigine or diclofenac glucuronidation and the rates of 1'-hydroxymidazolam *O*- (A) and *N*-glucuronidation (B) and 4-hydroxymidazolam glucuronide I formation (C) in 25 different HLMs.

Figure 6. Representative Michaelis-Menten (left panel) and Eadie-Hofstee (right panel) plots of the glucuronide conjugation of 1'- (A and B) and 4-hydroxymidazolam (C and D). HLMs were incubated with 0-200 μM 1'- or 4-hydroxymidazolam at 37°C for 60 min in the presence of UDPGA (5 mM). The data points represent the averages obtained from three different HLMs.

Figure 7. Representative Michaelis-Menten plots for glucuronide conjugate formation from 1'- (A and B) and 4-hydroxymidazolam (C) by recombinant UGT isoforms 1A4, 2B4, and 2B7. The recombinant UGT isoforms (0.25 mg/mL) were incubated with 0-100 μM 1'- or 4-hydroxymidazolam at 37°C for 60 min in the presence of UDPGA (5 mM). Each data point represents the average of triplicate incubations.

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Table 1.

IC_{50} values for the inhibition of UGT-mediated hydroxymidazolam glucuronidation by the UGT isoform-selective inhibitor or substrate in pooled HLMs.

UGT isoform	Inhibitor	IC_{50} values (μM)[a]	
		1'-Hydroxymidazolam *N*-glucuronide	1'-Hydroxymidazolam *O*-glucuronide
1A4	Hecogenin	1.5	> 5
2B7	Diclofenac	802.8	2.0
		4-Hydroxymidazolam Glucuronide I	4-Hydroxymidazolam Glucuronide II
1A4	Hecogenin	0.8	> 5
2B7	Diclofenac	941.1	46.6

[a]Averages of duplicate determinations

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Table 2.

Mean enzyme kinetic parameters for glucuronide conjugate formation from 1'- and 4-hydroxymidazolam in HLMs. The values are the means of estimates from three different HLM preparations.

		1'-Hydroxymidazolam		
Kinetic parameter	K_m[a]	V_{max}	n	CL_{int}
N-Glucuronide	9.1	790.0	1.6	86.8
O-Glucuronide	12.7	3,285.8	1.5	259.4
		4-Hydroxymidazolam		
Glucuronide I	41.5	3,609.9	1.2	87.0
Glucuronide II	976.9	3,210.5	0.5	3.3

[a]V_{max} is expressed as pmol/min/mg protein; K_m as μM; CL_{int} as V_{max}/K_m (μL/min/mg protein); and n as the Hill coefficient.

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Table 3.

Mean enzyme kinetic parameters for glucuronide conjugate formation from 1'- and 4-hydroxymidazolam by recombinant human UGT isoforms. Each data set is the average of triplicate incubations.

UGT isoform	Metabolite	1'-Hydroxymidazolam			
		K_m [a]	V_{max}	n	V_{max}/K_m
1A4	*N*-Glucuronide	15.9	468.3	1.31	29.5
2B4	*O*-Glucuronide	29.8	127.0	1.00	4.3
2B7		35.5	90.1	0.83	2.5
		4-Hydroxymidazolam			
1A4	Glucuronide I	40.8	1,064.2	1.23	26.1

[a] V_{max} is expressed as pmol/min/mg protein; K_m as μM; CL_{int} as V_{max}/K_m (μL/min/mg protein); and n as the Hill coefficient.

Figure 1.

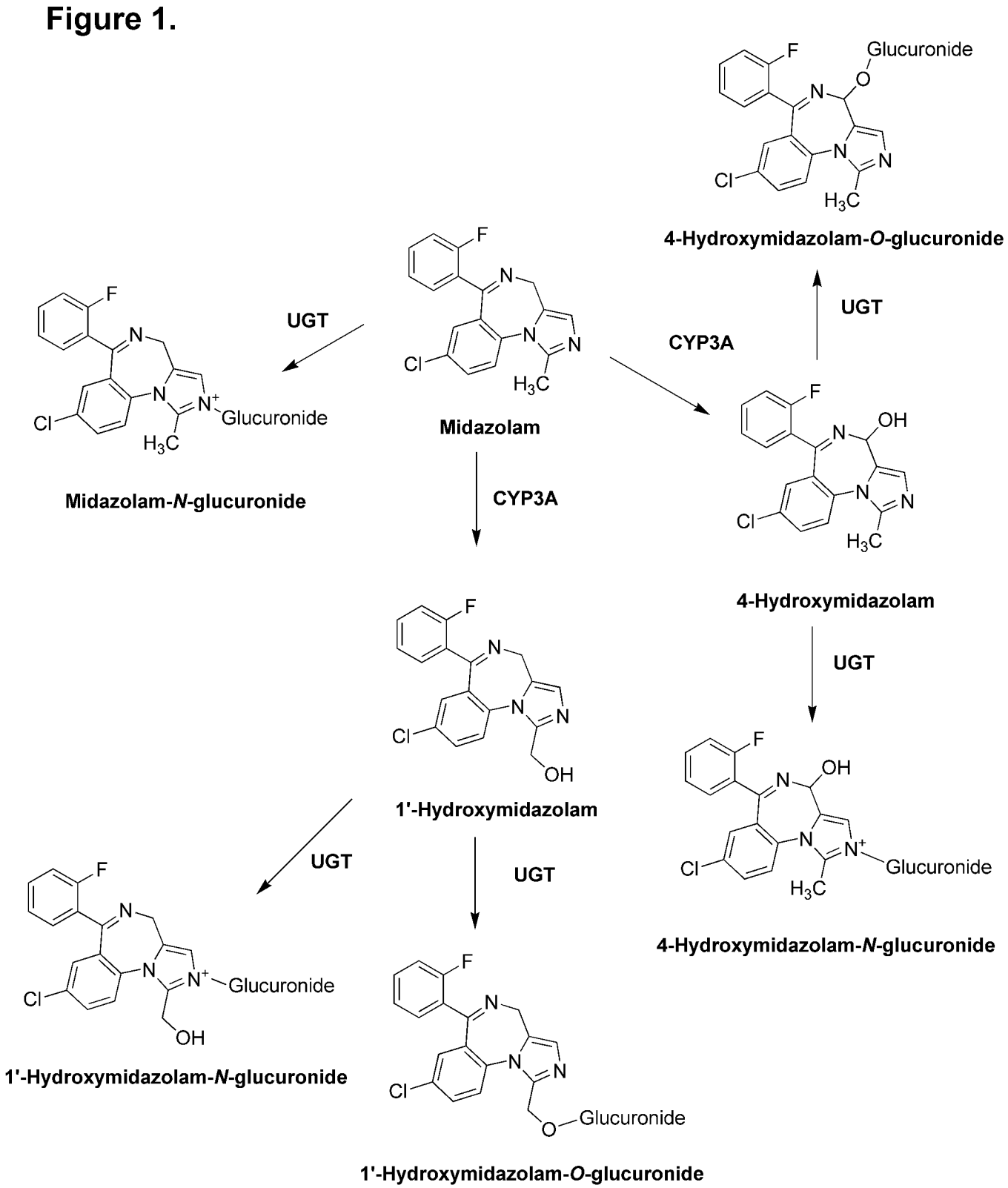
Midazolam-N-glucuronide
UGT
Midazolam
CYP3A
4-Hydroxymidazolam-O-glucuronide
UGT
CYP3A
4-Hydroxymidazolam
UGT
4-Hydroxymidazolam-N-glucuronide
1'-Hydroxymidazolam
UGT
UGT
1'-Hydroxymidazolam-N-glucuronide
1'-Hydroxymidazolam-O-glucuronide

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Figure 2.

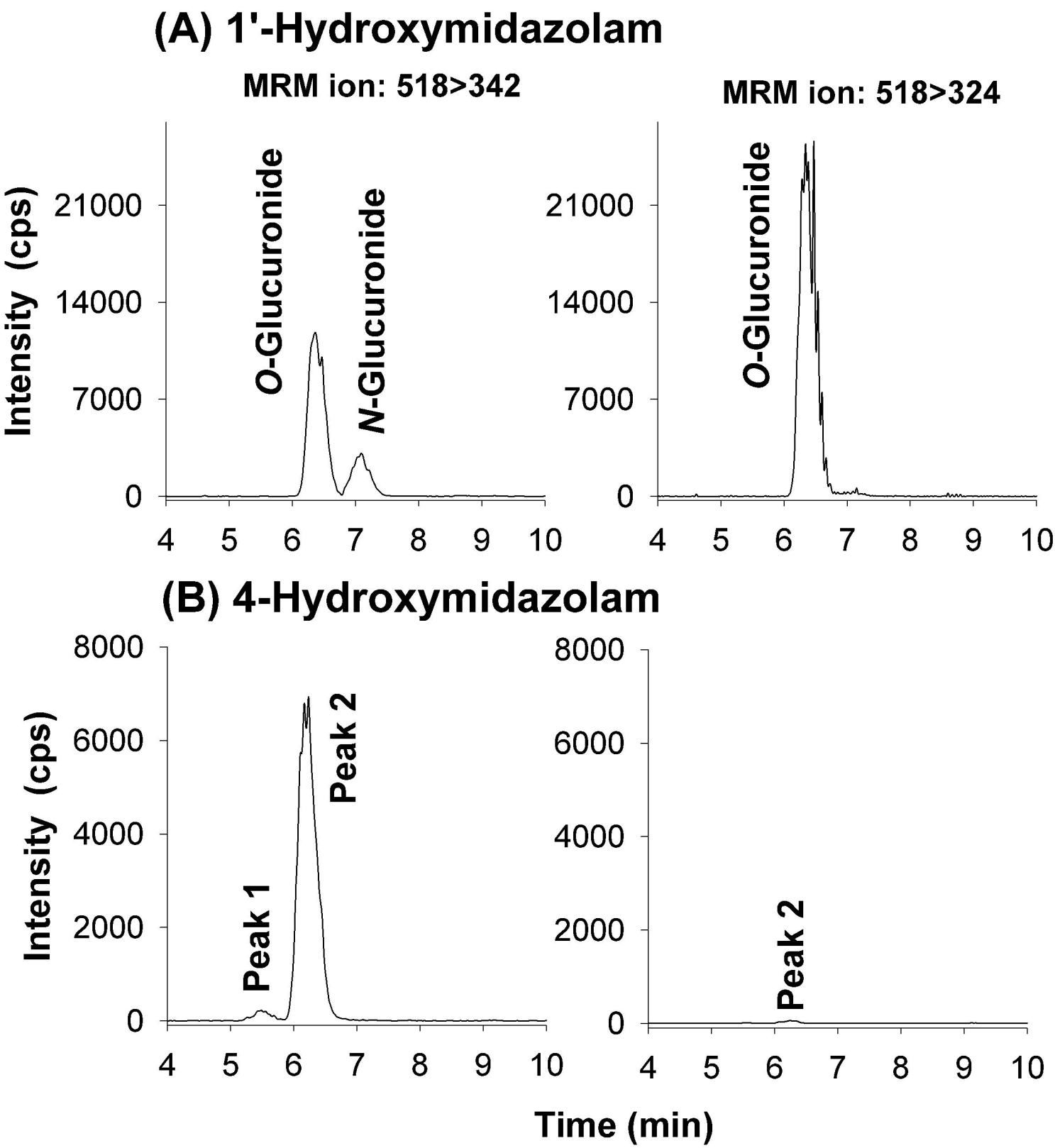

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Figure 3.

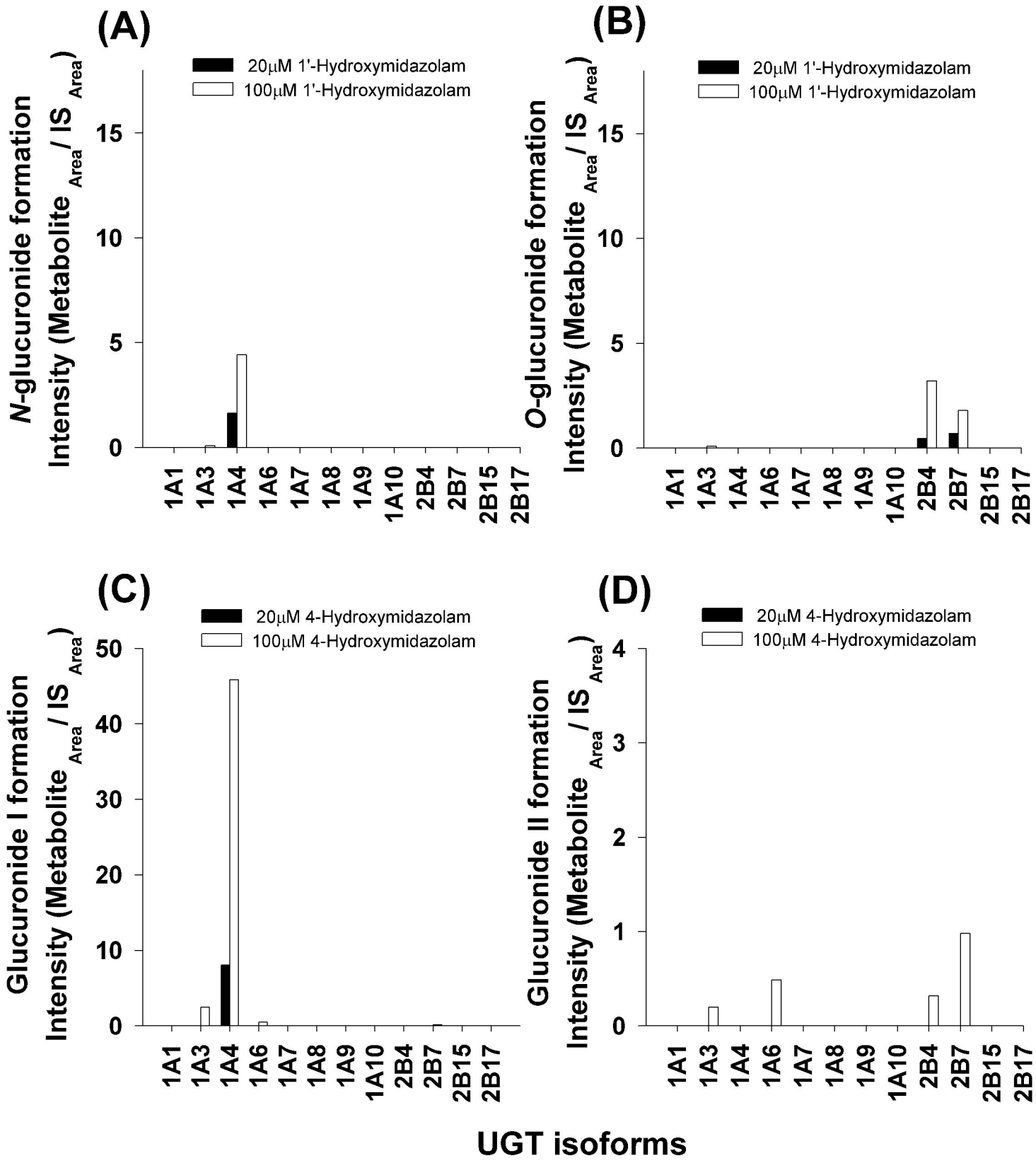

Figure 4.

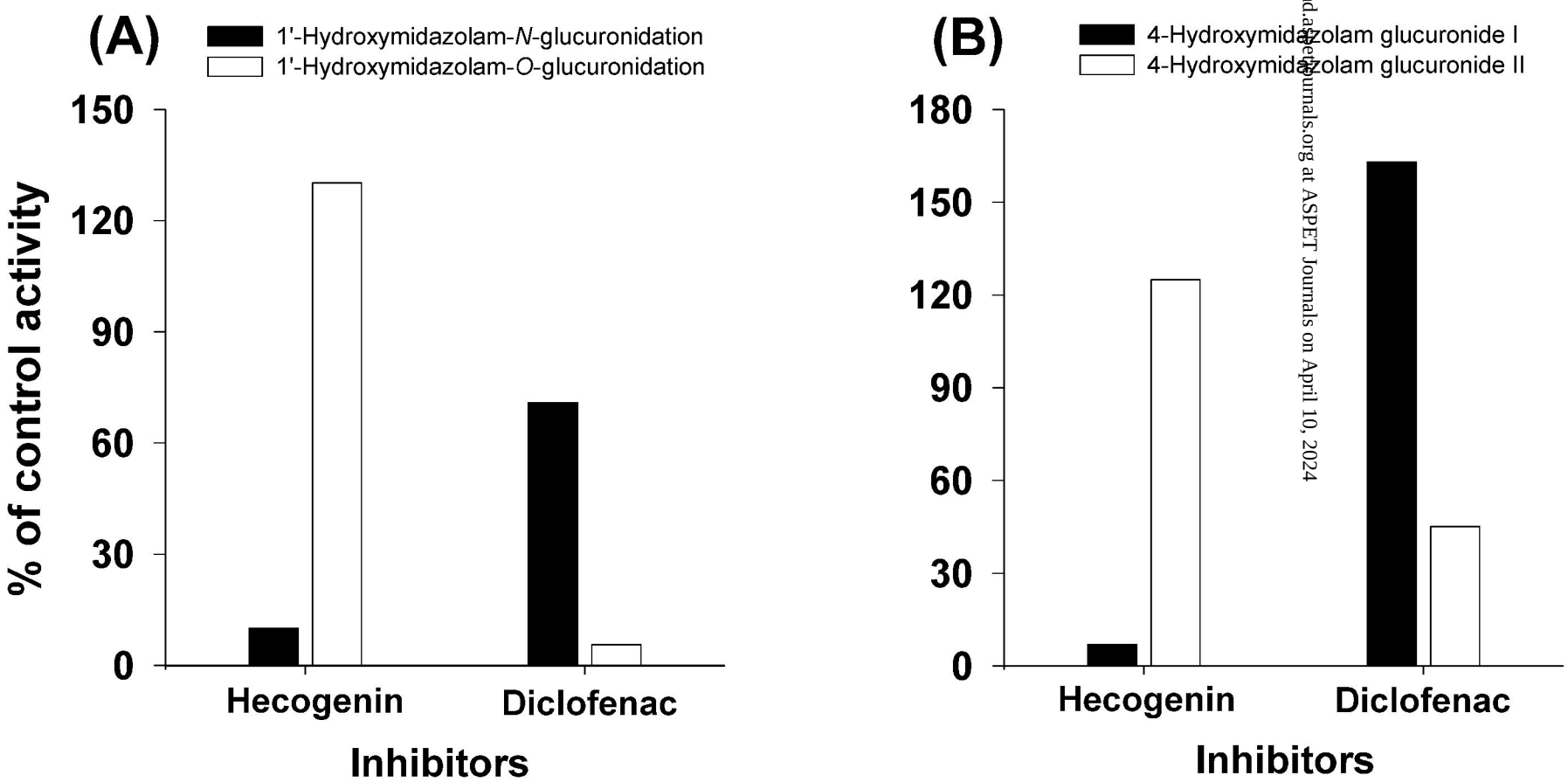

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Figure 5.

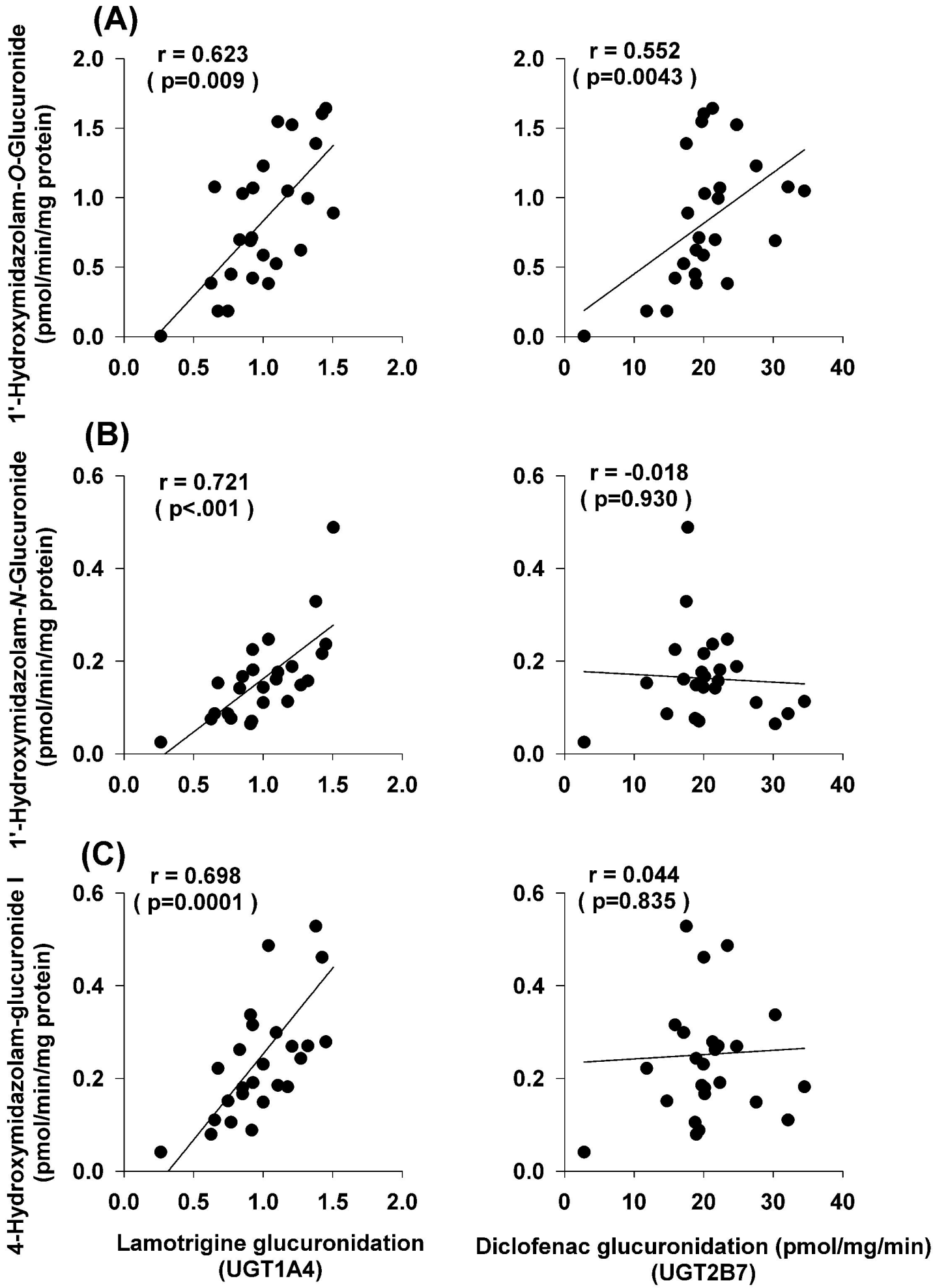

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Figure 6.

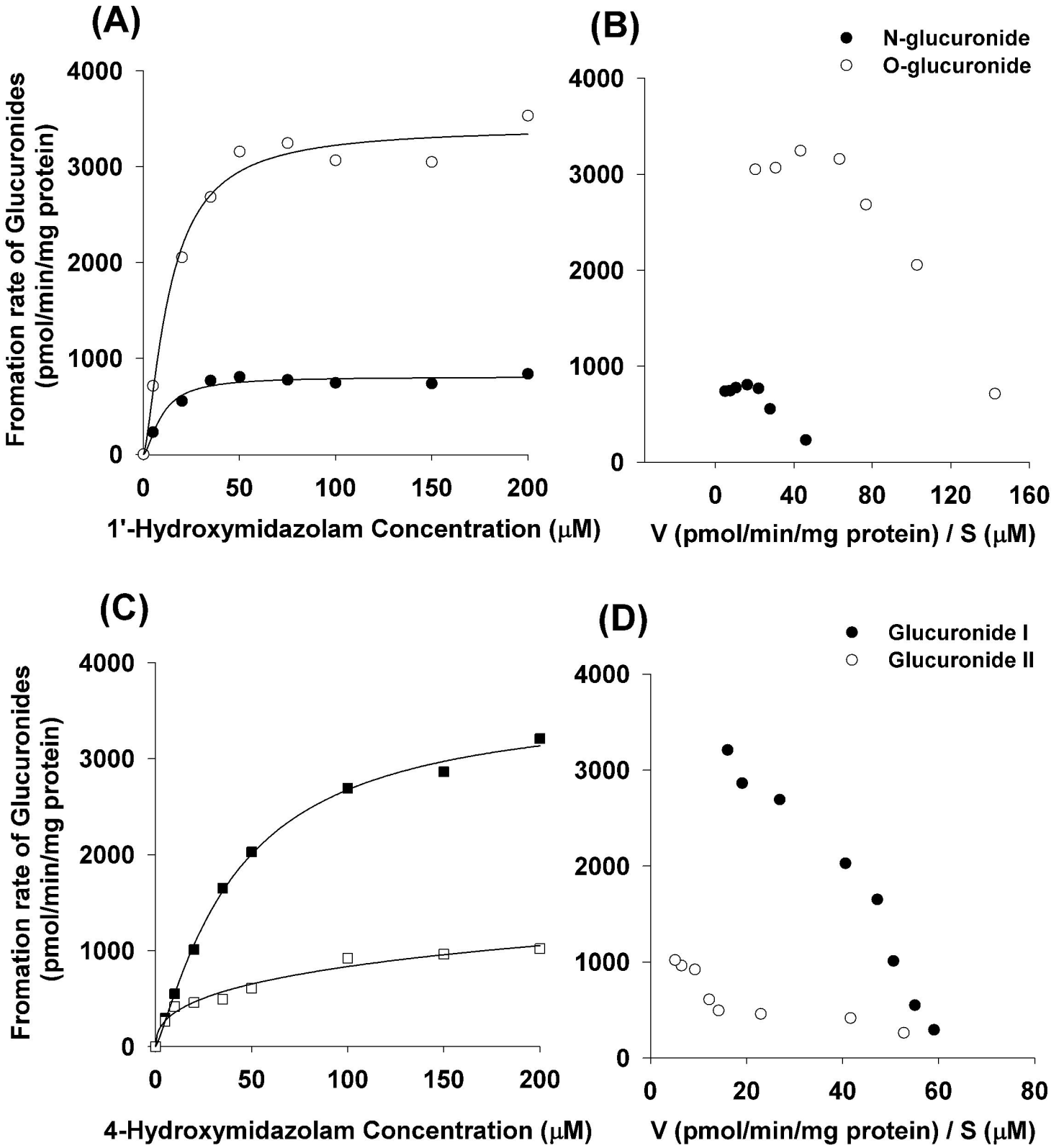

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Figure 7.