URIDINE DIPOSPHATE GLUCURONOSYLTRANSFERASES ISOFORM-DEPENDENT REGIOSPECIFICITY OF GLUCURONIDATION OF FLAVONOIDS

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Abstract

The objective of this study was to determine the regiospecificity of the important uridine diphosphate glucuronosyltransferase (UGT) isoforms responsible for the glucuronidation of flavones and flavonols. We systematically studied the glucuronidation of 13 flavonoids (7 flavones and 6 flavonols, with hydroxyl group at C-3, C-4', C-5, and/or C-7 positions in flavonoid structure) at a substrate concentration of 10μM by 8 recombinant human UGT isoforms mainly responsible for metabolism of flavonoids, UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10 and 2B7. At 10μM substrate concentration, different UGT isoforms gave different regiospecific glucuronidation patterns. UGT1A1 equally glucuronidated 3-*O* (glucuronic acid substituted at C-3 hydroxyl group), 7-*O* and 4'-*O*, whereas UGT1A8 and 1A9 preferably glucuronidated only 3-*O* and 7-*O* positions. UGT1A1 usually showed no regiospecificity for glucuronidating any position, whereas, UGT1A8 and UGT1A9 showed dominant, moderate or weak regiospecificity for 3-*O* or 7-*O* position, depending on the structure of the compound. UGT1A3 showed dominant regiospecificity for 7-*O* position, whereas 1A7 showed dominant regiospecificity for 3-*O* position. We also showed that the glucuronidation rates of 3-*O* and 7-*O* positions in flavones and flavonols were affected by the addition of multiple hydroxyl groups at different positions as well as by the substrate concentrations (2.5, 10 and 35μM). In conclusion, regiospecific glucuronidation of flavonols was isoform- and concentration- dependent, whereas flavones were dominantly glucuronidated at 7-*O* position by most UGT isoforms. We also concluded that UGT1A3 and UGT1A7 showed dominant regiospecificity for only 7-*O* and 3-*O* position, respectively. UGT1A8 and UGT1A9 showed moderate or weak preference on glucuronidating position 3-*O* over 7-*O* position, whereas other UGT isoforms did not prefer glucuronidating any particular positions.

Keywords

Regiospecificity; UGT isoforms; glucuronidation; flavonoids

INTRODUCTION

Uridine diphosphate glucuronosyltransferases (UDP-glucuronosyltransferases or UGTs) utilize uridine diphospho-glucuronic acid (UDPGA) as cofactor and transfer glucuronic acid to the usually lipophilic substrates, forming hydrophilic conjugates called glucuronides. Substrates of UGTs include endogenous compounds such as steroids, bile acids, bilirubin, hormones, dietary constituents like flavonoids, xenobiotics such as morphine and valproic acid, the products of phase I metabolism, and environmental toxins and carcinogens such as 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (1-3). UGT-catalyzed glucuronidation reactions are responsible for 35% of all drugs metabolized by phase II enzymes (4). Despite the important role of UGTs in the metabolism of drugs, environmental chemicals and endogenous compounds, the structural features of these enzymes responsible for substrate binding and formation of regioselective glucuronides remain poorly understood (5, 6).

Flavonoids are polyphenolic compounds with several hydroxyl groups on heterocyclic skeleton of flavan(2-phenylbenzopyrone). Therefore, conjugations of glucuronic acid mainly occur at different hydroxyl groups and *O*-glucuronides are formed (3). However, there is very limited information available in literature on the regiospecificity of glucuronidation of compounds in general and flavonoid in specific. The regiospecific glucuronides of certain flavonoids have shown to exhibit different pharmacological actions *in vivo* (7, 8). For example, quercetin,7-*O*-G had stronger antioxidative property than quercetin,3-*O*-G (8).

Also, it has been proposed that glucuronides in systemic circulation or after uptake into an organ such as liver, intestine and neutrophils may hydrolyze back into aglycone by β-glucuronidase enzyme for pharmacological action. However, the rate of flavonoid glucuronides hydrolysis depends on the position of conjugation, which could affect the subsequent re-conjugation rate (9, 10). Therefore, it becomes important to understand the regiospecificity of various UGT isoforms, as based on different expression levels of UGTs in various metabolic organs and mode of administration of drug, the exposure of regiospecific glucuronides to an organ may differ extensively.

Previously, we have shown that certain UGT isoforms were important for the glucuronidation of most flavonoids from different sub-classes (11-13). However, different UGT isoforms showed differences in their rate and position of glucuronidation of flavones and flavonols (12). Among the various UGT isoforms expressed in liver, UGT1A1, 1A3, 1A9 and 2B7 are the most important isoforms of our interest, based on the published expression levels of UGT isoforms in liver (14) as well as the rate of glucuronidation of various flavonoids by UGT isoforms (11, 12). UGT1A6 is another hepatic isoform which is highly expressed in Caco-2 cell lines (15), extensively used for disposition studies. Among extra-hepatic isoforms, UGT1A7, 1A8 and 1A10 are the most important isoforms responsible for glucuronidation of flavonoids (11, 12).

Therefore, we decided to study all major UGT isoforms for the preference of position of glucuronidation using 13 flavonoids (7 flavones and 6 flavonols) (Figure 1) with hydroxyl groups at C-3, C-4', C-5 and C-7 positions. Compounds from flavone and flavonol sub-classes were chosen for the study due to the commercial availability of congeneric compounds with hydroxyl group(s) at different positions on the same structural backbone in these sub-classes of flavonoids. The purpose of this study was to give insight into regiospecificity of various UGT isoforms, and predict the relative occurrence of various regiospecific glucuronides of flavonoids based on expression levels of various metabolic organs such as liver and intestine.

EXPERIMENTAL SECTION

Materials

3-hydroxyflavone or flavonol (Fnol), 3,4'-dihydroxyflavone or 4'-hydroxyflavonol (4'-HFnol), 3,7-dihydroxyflavone or 7-hydroxyflavonol (7-HFnol), 5,4'-dihydroxyflavone (5,4'DHF), 5,7-dihydroxyflavone (5,7DHF), 7,4'-dihydroxyflavone (7,4'DHF), 3,7,4'-trihydroxyflavone or resokaempferol or 7,4'-dihydroxyflavonol (7,4'DHFnol) and 3,5,7-trihydroxyflavone or galangin or 5,7-dihydroxyflavonol (5,7DHFnol), 5,7,4'-trihydroxyflavone or apigenin (Api or 5,7,4'THF), 3,5,7,4'-tetrahydroxyflavone or kaempferol or 5,7,4'-trihydroxyflavonol (5,7,4'THFnol or Kamp), were purchased from Indofine Chemicals (Somerville, NJ). 8 commercially available recombinant human UGT isoforms (Supersomes), UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7, were purchased from BD Biosciences (Woburn, MA). Uridine diphosphoglucuronic acid (UDPGA), alamethicin, D-saccharic-1,4-lactone monohydrate, magnesium chloride, and Hanks' balanced salt solution (powder form) were purchased from Sigma-Aldrich (St Louis, MO). All other materials (typically analytical grade or better) were used as received.

Identification of position of glucuronidation in the structure of flavones and flavonols by UV shift method

The sites of glucuronic acid substitutions in flavones and flavonols were established based on the online UV spectral shift method, optimized and validated in our lab based on the published literature (16, 17). Briefly, substitution of a single hydroxyl group by glucuronic acid in the structure of flavone at a specific position would result in diagnostic shifts or no shift in λ_{max} of Band I (300-380 nm) and/or Band II (240-280 nm) in the UV spectrum of the resulting glucuronide as compared to the UV spectrum of parent compound. Based on these differentiating diagnostic shifts for each position of glucuronidation, structure of a glucuronide can be estimated. The details of the method and its validation can be found in Singh *et al.* (2010) (18).

Methods

Solubility and stability of the tested flavonoids—Solubility and stability of the tested flavonoids were established at the experimental conditions. The results showed that these compounds were stable and soluble at the tested concentrations (not shown).

Glucuronidation activities of recombinant human UGTs—The incubation procedures for measuring UGTs' activities were essentially the same as published before (19, 20). Briefly, incubation procedures using supersomes were as follows: (1) supersomes (final concentration ≈ in range of 0.013~0.053 mg of protein per mL as optimum for the reaction), magnesium chloride (0.88 mM), saccharolactone (4.4 mM), alamethicin (0.022 mg/mL), different concentrations of substrates in a 50 mM potassium phosphate buffer (pH 7.4), and UDPGA (3.5 mM, add last) were mixed; (2) the mixture (final volume) 200 μL) was incubated at 37°C for a predetermined period of time (30 or 60 min); and (3) the reaction was stopped by the addition of 50μL of 94% acetonitrile/6% glacial acetic acid containing 100 μM of testosterone or 50 μM of 5-hydroxyflavone or formononetin as internal standard. Testosterone was used as internal standard for 4'HFnol, 7,4'THF, 7,4'DHFnol, 5,7,4'THF and 5,7,4'THFnol; formononetin for Fnol, 5,4'DHF, 5,7DHF and 5,7DHFnol; and 5-hydroxyflavone for 7HFnol. Three substrate concentrations, 2.5, 10 and 35 μM were used for the studying the UGT isoforms.

UPLC analysis of flavonoids and their glucuronides—We analyzed flavonoids and their respective glucuronides by using the following common method: system, Waters Acquity UPLC with photodiode array detector and Empower software; column, BEH C_{18},

1.7 μm, 2.1 × 50 mm; and injection volume, 10μL. Representative chromatograms were shown in Fig.S1 of Supporting Information

Quantification of glucuronides of flavonoids—The quantification of glucuronides of flavonoids was done using the standard curve of the parent compound with a correction factor for difference in extinction coefficient of the compound and its metabolites as shown in our previous publication (18). The correction factors were in the range of 0.5 (for 3-*O*-G of Fnol) to 2.5 (for 3-*O*-G for 7HFnol), as reported previously (18).

Confirmation of flavonoids glucuronide structure by LC MS/MS—Flavonoids and their respective glucuronides were separated by the same UPLC system but using slightly different chromatographic conditions because of mass spectrometer requirements. Here, mobile phase A was ammonium acetate buffer (pH 7.5) and mobile phase B was 100% acetonitrile with the gradient as follows: 0-2.0 min, 10–35% B, 2.0-3.0 min, 35–70% B, 3.2-3.5 min, 70–10% B, 3.5-3.7 min, 10% B. The flow rate was 0.5 mL/min. The effluent was introduced into an API 3200 Qtrap triple-quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA) equipped with a TurboIonSpray™ source.

The mass spectrometer was operated in negative ion mode to perform the analysis of flavonoids and their respective glucuronides. The main working parameters for the mass spectrometers were set as follows: ion source temperature, 600°C; nebulizer gas (gas1), nitrogen, 40 psi; turbo gas (gas2), nitrogen, 40 psi; curtain gas, nitrogen, 20 psi; DP, -50 V; EP, -10V; CE, -30V; CXP, -3V and IS -4.5KV. Minor adjustments were then made for each flavonoid. Flavonoids mono-*O*-glucuronides were identified by MS and MS2 full scan modes.

The glucuronides were extracted by solid phase extraction from the glucuronidation experiment samples and re-constituted in small amount of 30% acetonitrile in water. The concentrated samples were then used to identify the glucuronides in UPLC/MS/MS.

Data analysis—Rates of metabolism in recombinant human UGT isoforms were expressed as amounts of metabolites formed per hour per mg protein or nmol/hr/mg.

Statistical analysis—One-way ANOVA or an unpaired Student's t-test (GraphPad Prism®, GraphPad software Inc., CA) with or without Tukey-Kramer multiple comparison (posthoc) tests was used to analyze the statistical significance among various data. The prior level of significance was set at 5%, or $p < 0.05$.

RESULTS

Confirmation of flavones and flavonols glucuronides structure by LC-MS/MS

LC-MS/MS studies of the glucuronides of flavonoids revealed that only mono-glucuronide was formed and position-specific mono-glucuronide was identified below.

Position of glucuronides in the structure of flavones and flavonols by UV shift method

We determined the position of glucuronidation based on the diagnostic shift in λ_{max} of Band I and Band II of UV spectra of glucuronides as shown in Figure S1 of Supporting Information.

For 4'HFnol, the first glucuronide was 4'-*O*-G while the second glucuronide was 3-*O*-G, whereas for 7HFnol, the first glucuronide was 3-*O*-G, while the second glucuronide was glucuronidated at position C-7 (Fig. S1 of Supporting Information). In case of 5,7DHFnol,

the position of glucuronidation of first, second and third glucuronides were 3-*O*-G, 7-*O*-G, and 5-*O*-G, respectively (Fig. S1 of Supporting Information). In case of both 7,4'DHFnol and 5,7,4'THFnol (kaempferol), the position of glucuronidation of first, second and third glucuronides were 7-*O*-G, 4'-*O*-G, and 3-*O*-G, respectively (Fig. S1 of Supporting Information).

First and second glucuronide of 5,4'DHF was 5-*O*-G and 4'-*O*-G respectively. Similarly for 5,7DHF, the first glucuronide was 5-*O*-G, while the second glucuronide was 7-*O*-G (Fig. S1 of Supporting Information). In case of 7,4'DHF, the position of conjugation of first and second glucuronides were 7-*O*-G, 4'-*O*-G, respectively, whereas, only one quantifiable glucuronide was observed in case of 5,7,4'THF (apigenin), glucuronidated at C-7 (Fig. S1 of Supporting Information).

Regiospecificity of flavonoid glucuronidation by UGT isoforms

We studied the regiospecificity of flavonols and flavones glucuronidation, i.e. preference of glucuronidating a particular hydroxyl group position by UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10 and 2B7. For the ease of understanding the data, the regiospecificity here is randomly defined into four categories: dominant, moderate, weak and none. *Dominant regiospecificity* means that one hydroxyl group position in the structure is dominantly glucuronidated, such that the ratio of the most prevalent glucuronide to other glucuronide(s) is equal to or greater than 9:1. *Moderate regiospecificity* means the same ratio is equal to or more greater than 3:1 but less than 9:1. *Weak regiospecificity* means that the same ratio is equal to or more than 2:1 but less than 3:1, whereas *no regiospecificity* means that the same ratio is less than 2:1. All UGT isoforms were studied for their regiospecificity for each compound based into these random categories.

Flavonols—In general, most isoforms preferably glucuronidated 3-*O* position in the structure of flavonols, followed by glucuronidation of 7-*O* position, except 1A3, which exclusively preferred glucuronidation of 7-*O* position (Figure 2, Table 1). However, different isoforms might display different regiospecificity depending upon structure of compounds. Fnol was a universal substrate and could be metabolized by any of the 8 UGT isoforms tested (Fig. 2a). For 7HFnol, different isoforms showed great differences in their regiospecificity. UGT1A3 and 2B7 were dominantly regiospecific by glucuronidating hydroxyl group only at C-7 position, whereas UGT1A7 dominantly glucuronidated hydroxyl group at C-3 position. UGT1A8 and 1A9 showed moderate regiospecificity for 3-*O* position, whereas UGT1A1 and 1A10 showed no preference (Fig. 2b, Table 1).

For 4'HFnol, most isoforms showed dominant regiospecificity by glucuronidating almost only the 3-*O* position, except UGT1A1 and 1A3 which showed no regiospecificity and glucuronidated both hydroxyl groups at C-4'position and C-3 positions comparably (Fig. 2c, Table 1).

In case of 7,4'DHFnol, UGT1A1, 1A10 and 2B7 glucuronidated all the three hydroxyl groups, whereas 1A3 and 1A9 glucuronidated hydroxyl groups at C-3 and C-7 positions only (Fig. 2d). UGT1A1 and 1A10 showed no regiospecific preference for any position, whereas UGT1A9 and 2B7 showed moderate and weak regiospecific preference of glucuronidating 3-*O* than 7-*O* position. On the other hand, UGT1A3 showed weak regiospecific preference for 7-*O* over 3-*O* position (Fig. 2d, Table 1).

In case of 5,7DHFnol, position(s) and regiospecificity of glucuronidation varied a lot for different isoforms. (Fig. 2e). UGT1A3 was dominantly regiospecific for 7-*O*, whereas UGT1A7 was dominantly regiospecific for 3-*O* (Fig. 2e, Table 1). UGT 1A1 weakly preferred the glucuronidation of 7-*O* over 3-*O* position, whereas 1A8 and 2B7 moderately

preferred the glucuronidation of 3-*O* and 5-*O*, respectively (Fig. 2e, Table 1). UGT1A9 and 1A6 did not show any regiospecific preference for any position, whereas 1A10 weakly preferred 3-*O* over 5-*O* (Fig. 2e, Table 1).

In case of 5,7,4'THFnol, 5-*O* position was not significantly glucuronidated by any isoform. UGT 1A1, 1A3, 1A7, 1A8 and 1A10 glucuronidated 3-*O*, 7-*O* and 4'-*O*, whereas 1A6 and 2B7 glucuronidated only 7-*O* and 4'-*O* and 1A9 glucuronidated only 3-*O* and 7-*O* positions (Fig. 2f). No isoforms showed any dominant or moderate regiospecificity for any position, except UGT1A6 which moderately preferred regiospecific glucuronidation of 4'-*O* over 7-*O* position (Fig. 2f, Table 1). UGT 1A1 and 1A3 showed no regiospecific preference between 3-*O* and 7-*O*, whereas 1A7, 1A8, 1A9 and 1A10 only weakly preferred 3-*O* position (Fig. 2f, Table 1).

In general, UGT1A1, 1A6, 1A10 and 2B7 did not show any dominant regiospecificity for any position, however in some cases, moderate or weak regiospecificity can be seen for glucuronidation of different positions of flavonols. UGT1A3 showed dominant regiospecificity for 7-*O* position, whereas 1A7 showed dominant regiospecificity for 3-*O* position. UGT1A8 and 1A9 preferably glucuronidated 3-*O* and 7-*O* positions only, with moderate or weak regiospecificity for 3-*O* position in most cases.

Flavones—In general, most isoforms preferably glucuronidated 7-*O* position in the structure of flavones, followed by glucuronidation of 4'-*O* position to some extent (Figure 3). In case of 5,4'DHF, except UGT2B7, all metabolizing isoforms glucuronidated both hydroxyl groups at both C-5 and C-4'- though 4'-*O* was the preferred position of glucuronidation in all cases. UGT1A1, 1A9, 1A10 and 2B7 showed dominant regiospecificity, whereas, 1A3 and 1A8 showed moderate regiospecificity for 4'-*O* position (Fig. 3a, Table 1).

In 5,7DHF, UGT1A1, 1A3, 1A7, 1A8 and 1A9 glucuronidated 7-*O* position only and hence showed dominant regiospecificity towards 7-*O* position (Fig. 3b, Table 1). On the other hand, UGT1A6, 1A10 and 2B7 glucuronidated both 7-*O* and 5-*O* positions, and showed only moderate regiospecificity towards 7-*O* position (Fig. 3b, Table 1).

In case of 7,4'DHF, except UGT1A6, all metabolizing isoforms glucuronidated both hydroxyl groups at C-7 and C-4' positions (Fig. 3c). UGT 1A3 and 1A6 showed dominant regiospecificity for glucuronidation of 7-*O* position, whereas UGT1A1, 1A8 and 1A9 showed only moderate regiospecificity for glucuronidation of 7-*O* position (Fig. 3c, Table 1). UGT1A10 did not show any regiospecificity and glucuronidated both positions comparably (Fig. 3c, Table 1). In case of 5,7,4'THF, all isoforms showed dominant regiospecific glucuronidation of hydroxyl group at C-7 position only (Fig. 3d, Table 1).

In summary, for flavones with hydroxyl group at C-7 position, most UGT isoforms showed either dominant or moderate regiospecificity for glucuronidating 7-*O* position over 4'-*O* and 5-*O* positions. In absence of hydroxyl group at C-7 position, most UGT isoforms showed either dominant or moderate regiospecificity for glucuronidating 4'-*O* position over 5-*O* position.

Regiospecificity of UGT isoforms for flavonoids glucuronidation

We studied in detail the regiospecific glucuronidation of flavonoids by hepatic (1A1, 1A3, 1A9, 2B7 and 1A6) and extra-hepatic (1A7, 1A8 and 1A10) UGT isoforms using flavonoids (at 2.5 and 10 μM) with hydroxyl group(s) at different position(s) i.e. 3-*O*, 4'-*O*, 5-*O* and 7-*O*.

UGT1A1—UGT1A1 was able to effectively glucuronidate 3-*O*, 4'-*O* and 7-*O* position in selected flavones and flavonols, but 5-*O* position was not favored both at 2.5 and 10 μM substrate concentrations (Fig. 4A-4B). In case of mono-hydroxyflavonols (4'HFnol and 7HFnol), UGT1A1 did not show any regiospecific preference of glucuronidation between 3-*O* and 4'-*O* (at 2.5 and 10μM) or 3-*O* and 7-*O* positions (at 10μM), but weak regiospecificity for 7-*O* position at 2.5μM (Fig. 4A-4B, Table 1). In dihydroxyflavones, UGT1A1 showed moderate regiospecificity at 10 μM while weak at 2.5 μM for 7-*O* position in 7,4'DHF. It showed dominant regiospecificity for 7-*O* position in 5,7DHF and for 4'-*O* position in 5,4'DHF at both 2.5 and 10μM substrate concentrations (Fig. 4A-4B, Table 1).

UGT1A3—At 10 μM, UGT1A3 dominantly preferred glucuronidation of 7-*O* position in flavonols and flavones, wherever free hydroxyl group was present at C-7, except for 5,7,4'THFnol where UGT1A3 did not show dominant regiospecificity for 7-*O* position (Fig. 5A, Table 1). When 7-*O*H was absent, dominant regiospecificity was absent (Fig. 5A, Table 1).

UGT2B7—At 10 μM, UGT2B7 did not show strong preference at any particular position in flavonols (Fig. 5B, Table 1). However, number and position of hydroxyl group in the structure of flavonols affected which one (3-*O* or 7-*O*) or both (3-*O* and 7-*O*) are preferred position (Fig. 5B, Table 1). For flavones, moderate or dominant regiospecificity occurred at 7- hydroxyl group in 5,7DHF and 5,7,4'THF respectively (Fig. 5B, Table 1).

UGT1A6—At 10 μM, for flavonols, UGT1A6 did not show any pattern for glucuronidating any particular position. In 4'HFnol, UGT1A6 showed dominant regiospecificity for 3-*O* position, whereas in 7HFnol and 7,4'DHFnol, no position was glucuronidated (Fig. 6A, Table 1). However, UGT1A6 showed dominant or moderate regiospecificity for 7-*O* position in flavones (Fig. 6A, Table 1). Position of glucuronidation appeared to be highly affected by the number and position of hydroxyl group in the structure of flavonoids.

UGT1A7—At 10 μM, UGT1A7 dominantly preferred glucuronidation of 3-*O* position over 7-*O* position in all flavonols (Fig. 6B, Table 1), except for 5,7,4'THFnol where 3-*O* position was only weakly preferred. However in flavones, 7-*O* position was dominantly favored (Fig. 6B, Table 1). UGT1A7 did not glucuronidate 4'-*O* and 5-*O* positions at all (Fig. 6B).

UGT1A8—The glucuronidation of 4'-*O* and 5-*O* was not much favored by UGT1A8 at 10 μM and not at all favored at 2.5 μM (Fig. 7A-7B). At both 2.5 μM and 10 μM, UGT1A8 showed dominant or moderate regiospecificity for glucuronidating 3-*O* position in flavonols except in 5,7,4'THFnol where it showed only weak regiospecificity and 7,4'DHFnol which was not glucuronidated (Fig. 7A-7B, Table 1). UGT1A8 showed dominant or moderate regiospecificity for glucuronidating 7-*O* position in flavones at both concentrations (Fig. 7A-7B, Table 1).

UGT1A9—UGT1A9 mainly glucuronidated 3-*O* and 7-*O* positions in flavonols at both 2.5 μM and 10 μM, where 3-*O* was more favored position than 7-*O* for glucuronidation, except for 5,7THFnol at 10 μM, where 3-*O* and 7-*O* were glucuronidated at comparable rates (Fig. 8A-8B, Table 1). 7-*O* was dominantly preferred position of glucuronidation in flavones at both concentrations (Fig. 8A-8B, Table 1). Glucuronidation at 4'-*O* or 5-*O* position was not favored at all by UGT1A9 at both concentrations (Fig. 8A-8B).

UGT1A10—UGT1A10 was the only isoform which rapidly glucuronidated all the positions (3-*O*, 4'-*O*, 5-*O* and 7-*O*) in selected flavonoids. However, UGT1A10 did not show any

preference for glucuronidating any particular position (Fig. 9A-9B). Also, the degree of regiospecificity and position of preference of glucuronidation varied a lot for both flavonols and flavones at both 2.5 μM and 10 μM (Fig. 9A-9B, Table 1).

Effect of higher substrate concentration on the regiospecificity of UGT 1A1, 1A8 and 1A9

We also studied the effect of higher substrate concentration (35μM) on the regiospecificity of the three isoforms, UGT1A1, 1A8 and 1A9. Figure 10 represented the data of two flavanols (7HFnol and 5,7,4'THFnol) as examples. We found that the ratio of rates of glucuronidation of 3-O and 7-O positions in 7HFnol and 5,7,4'QHFnol by UGT1A1, 1A8 and 1A9 significantly changed with the concentration (p<0.05) (Figures 10A-10B).

For 7HFnol, UGT1A1 weakly preferred the glucuronidation of 7-O position at 2.5 μM, but showed no regiospecificity at 10 and 35μM substrate concentration (Fig. 10A). UGT1A8 showed moderate regiospecificity for 3-O position at all the three concentrations (Fig. 10A). UGT1A9 showed moderate regiospecificity for 3-O position at 2.5 and 10 μM, whereas no regiospecificity at 35μM substrate concentrations (Fig. 10A).

For 5,7,4'THFnol, UGT1A1 weakly preferred the glucuronidation of 7-O position at 2.5 μM, but showed no regiospecificity at 10 and 35μM substrate concentrations (Fig. 10B). UGT1A8 only showed weak regiospecificity at all the three concentrations; however 3-O was the preferred position of glucuronidation at 2.5 and 10 μM, while 7-O was the preferred position at 35μM substrate concentrations (Fig. 10B). UGT1A9 showed moderate regiospecificity for glucuronidation of 3-O position at 2.5μM, while weak regiospecificity for glucuronidation of 3-O position at 10μM substrate concentrations, but no regiospecificity at 35μM substrate concentration (Fig. 10B).

In conclusion, UGT1A1 showed similar regiospecificity at 10 and 35 μM for both compounds while behaved differently at 2.5 μM substrate concentration. UGT1A9 showed similar regiospecificity at 2.5 and 10 μM for both compounds while behaved differently at 35 μM substrate concentration. UGT1A8 also showed similar behavior for 5,7,4'THFnol as UGT1A9 but regiospecificity was independent of concentration for 7HFnol.

DISCUSSION

We concluded that regiospecific glucuronidation of flavonols was isoform- and concentration-dependent, whereas flavones were dominantly glucuronidated at 7-O position by most UGT isoforms (Figures 2-10). This is the first time that a study has demonstrated a UGT isoform-specific regiospecificity in glucuronidation of flavonoids, although the pattern of regiospecificity may be concentration-dependent. We showed that different UGT isoforms had differences in their regiospecificity of glucuronidation of flavonoids, however most isoforms preferred glucuronidation of 3-O position in flavonols followed by 7-O position and 7-O position in flavones, and 5-O position was hardly glucuronidated (Figures 2-10). Our findings on regiospecificity are consistent with conclusions of several published reports that investigated the glucuronidation of a particular or a few flavonoids. (21-23)

We also concluded that UGT1A3 and 1A7 showed dominant regiospecificity for only 7-O and 3-O position, respectively (Fig. 5A and 6B) (Table 1). UGT1A8 and 1A9 showed moderate or weak preference on glucuronidating position 3-O over 7-O position (Fig. 7 and 8) (Table 1), whereas other UGT isoforms, UGT1A1, 1A6, 1A10 and 2B7 showed either no positional preference or regiospecific pattern in most case (Fig. 4, 5B, 6A and 9) (Table 1). The regiospecificity patterns for UGT 1A3 and 1A6 were also confirmed at 2.5μM substrate concentration (data not shown).

The dominant and moderate regiospecificity of glucuronidation shown by different UGT isoforms can be useful in predicting the major glucuronide(s) formed in human plasma. For example, chrysin (5,7DHF) is shown to predominantly form 7-*O*-glucuronide in human volunteers (24). Chen *et al.* (2005) showed that the catalytic efficiency order for regiospecific glucuronidation of quercetin (3,5,7,3'-4'-pentahydroxyflavone or 5,7,3'-4'-tetrahydroxyflavonol) by UGT1A9 was 3- > 7- > 3′- > 4′-*O*-glucuronide.(21) The fact that 3-*O*-glucuronide of quercetin was also the main glucuronide found in bioavailability studies done in humans (8, 25, 26) showed that *in vitro* regiospecificity studies done using recombinant human UGT isoforms could give a reasonable prediction of the main glucuronide(s) to be found *in vivo*.

Day *et al.* (2000) showed that hydroxyl group on C-5 position did not glucuronidate (22) which confirmed our findings. This along with the fact that there has been no published reports of detecting *in vivo* 5-*O*-glucuronides of flavonoids supported our prediction that 5-*O* was not a favored site for glucuronidation of flavonoids. Even the weak regiospecificity can give useful prediction, if most of the UGT isoforms favors the glucuronidation of only a particular position. For example, after oral intake of kaempferol (5,7,4'THFnol) in human, 3-*O*-glucuronide of kaempferol was found to be the predominant form in plasma (27), which matched with our prediction based on the regiospecific glucuronidation of 5,7,4'THFnol in our study.

We also showed that the pattern and degree of regiospecificity of glucuronidation of flavonols was significantly affected by the substrate concentrations (Figures 4 and 7-9). For flavones, the preferred site of glucuronidation was independent of concentration used; however the degree of regiospecificity could change in certain cases (Figures 4 and 7-9). The differences in the *in vivo* cellular substrate concentration and the UGT isoforms expression in various organs (14) could significantly affect the various regiospecific glucuronide(s) formed in an organ. Yang *et al* (2010) showed that the C_{max} of 7-*O*-glucuronide was four times higher than that of 4'-*O*-glucuronide during intravenous administration of genistein where liver was the first-pass metabolic organ. On the other hand, the C_{max} of 7-*O*-G was only 1.85 times higher than that of 4'-*O*-glucuronide during per-oral administration of genistein where intestine was the first-pass metabolic organ.(28) This could have therapeutic implications in case of localized treatment, as the total concentration of a particular flavonoid available in an organ will be dependent on the rates of glucuronidation of the flavonoid and the rate of hydrolysis of various regiospecific glucuronide(s) back into the flavonoid in that organ (9, 10). For example, O'Leary *et al.* (2001) showed that the activity of β-glucuronidase found in human liver extract in hydrolyzing quercetin glucuronides was twice than the activity of β-glucuronidase found in human intestinal extract.(29) Also, the ratio of kcat/km of the human recombinant β-glucuronidase against hydrolysis of 3-*O*-glucuronide of quercetin was the highest, followed by 7-*O*-G and 4'-*O*-G.(29)

In conclusion, regiospecificity of glucuronidation of flavonols was an isoform- and concentration- dependent phenomenon, while most UGT isoforms showed dominant to moderate regiospecificity for 7-*O* position in glucuronidating flavones. For flavonols, certain hepatic and extra-hepatic UGT isoforms showed dominant regiospecificity for one position such as UGT1A3 for 7-*O* and UGT1A7 for 3-*O* position, whereas isoforms such as UGT1A8 and UGT1A9 mostly preferred glucuronidation at 3-*O* position followed by 7-*O* position. Rest of the UGT isoforms did not prefer glucuronidating any particular phenolic group. We believe that these insights about isoform-specific regiospecificity will be a great help in isoform-specific future *in silico* modeling of structure-metabolism relationship of glucuronidation of flavonoids/substrates by UGT enzymes. In a recent publication, Wu *et al.* (2011) successfully developed pharmacophore-based CoMFA model for prediction of

UGT1A9-mediated formation of 3-*O*-Glucuronide of flavonols using recombinant human UGT1A9 isoform.(30) Further studies are required to delineate the protein structural differences among the UGT isoforms which are responsible for the dominant or preferred regiospecificity for a particular OH group in the flavonoids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ABBREVIATIONS USED

UGT	Uridine diphosphate glucuronosyltransferases
UDP	Uridine diphosphate
UDPGA	uridine diphosphoglucuronic acid
UPLC	ultraperformance liquid chromatography
MS	mass spectroscopy
UV	ultraviolet
HF	Hydroxyflavone
DHF	dihydroxyflavone
THF	trihydroxyflavone
Fnol	flavonol
HFnol	hydroxyflavonol
DHFnol	dihydroxyflavonol
THFnol	trihydroxyflavonol
O	glucuronide

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Flavonol	R₁	R₂	R₃
Fnol	H	H	H
4'HFnol	H	H	OH
7HFnol	H	OH	H
7,4'DHFnol	H	OH	OH
5,7DHFnol	OH	OH	H
5,7,4'THFnol	OH	OH	OH

Flavone	R₁	R₂	R₃
4'HF	H	H	OH
5HF	OH	H	H
7HF	H	OH	H
5,4'DHF	OH	H	OH
5,7DHF	OH	OH	H
7,4'DHF	H	OH	OH
5,7,4'THF	OH	OH	OH

Figure 1.
Structure of flavonols and flavones used in the study
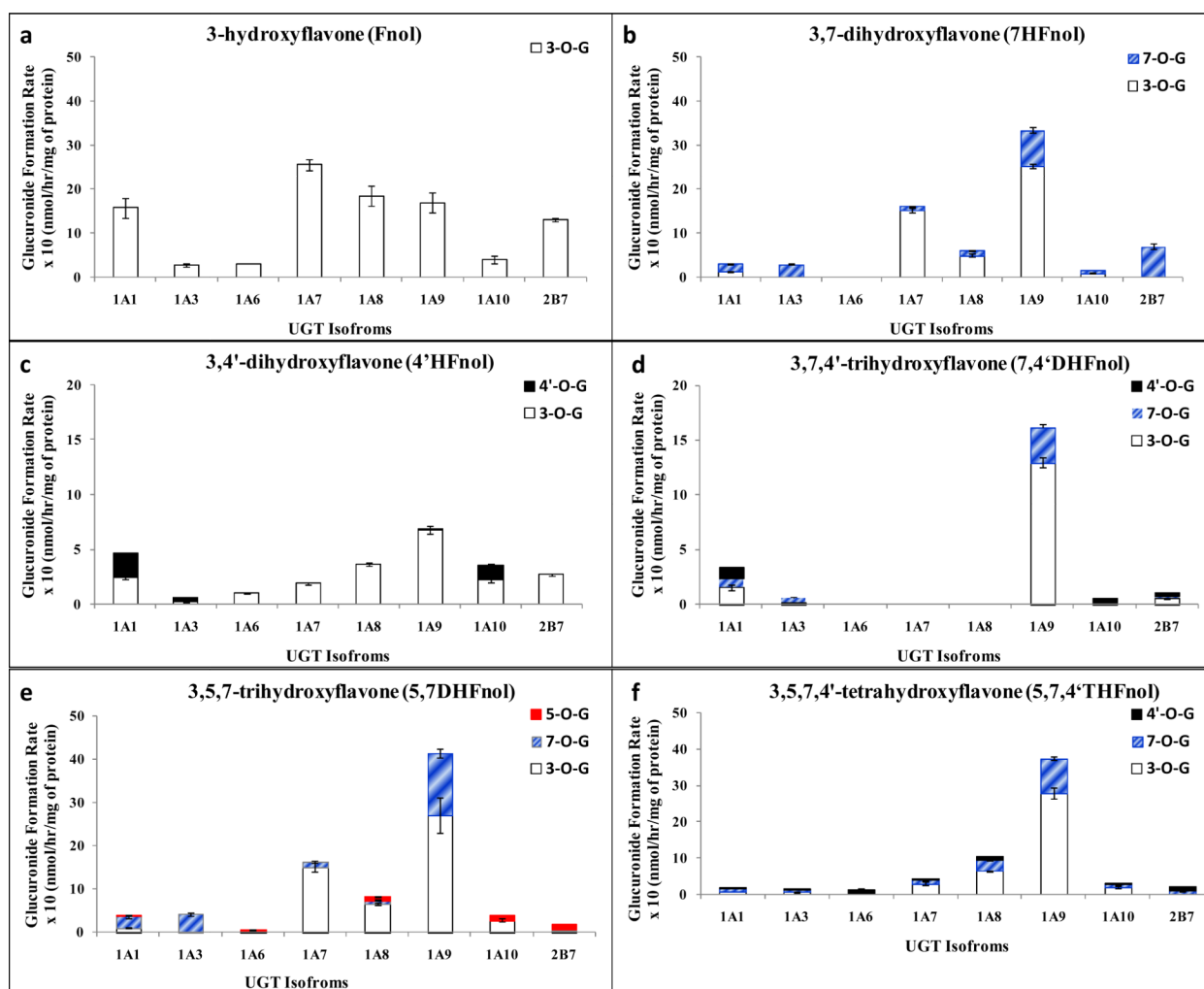
Figure 2. Regiospecific glucuronidation of flavonols by UGTs

Rate of glucuronidation of regiospecific glucuronides of Fnol (a), 4'HFnol (b), 7HFnol (c), 7,4'DHFnol (d), 5,7DHFnol (e) and 5,7,4'THFnol (f) with UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10 and 2B7. Flavonols (at 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) hr with UGTs (using optimum final protein concentration ~ 0.25, 0.5 or 1 mg/ml). The amounts of each regiospecific mono-glucuronide formed were measured using UPLC. Rates of mono-glucuronide formation were calculated as nmol/hr/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n=3). UGT stands for Uridine diphosphate glucuronosyltransferases).
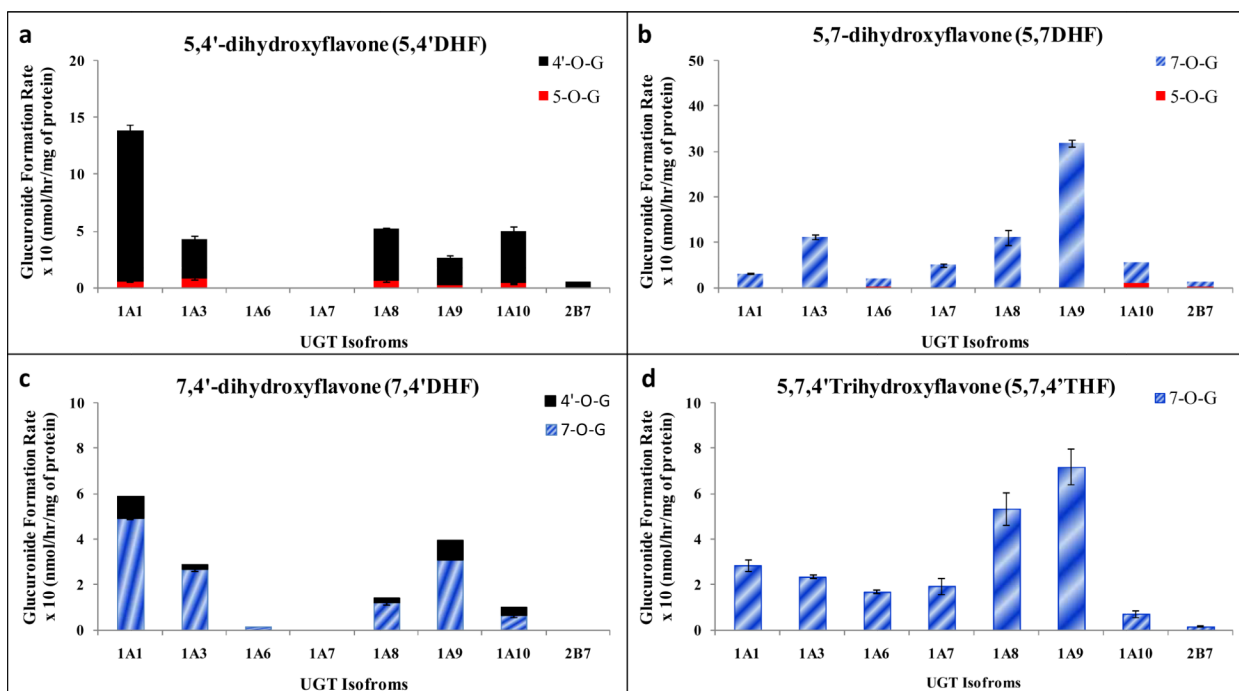
Figure 3. Regiospecific glucuronidation of flavones by UGTs
Rate of glucuronidation of regiospecific glucuronides of 5,4'DHF (a), 5,7DHF (b), 7,4'DHF (c), and 5,7,4'THF (d) with UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10 and 2B7. Flavones (at 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) hr with UGTs (using optimum final protein concentration ~ 0.25, 0.5 or 1 mg/ml). The amounts of each regiospecific mono-glucuronide formed were measured using UPLC. Rates of mono-glucuronide formation were calculated as nmol/hr/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n=3). UGT stands for Uridine diphosphate glucuronosyltransferases).
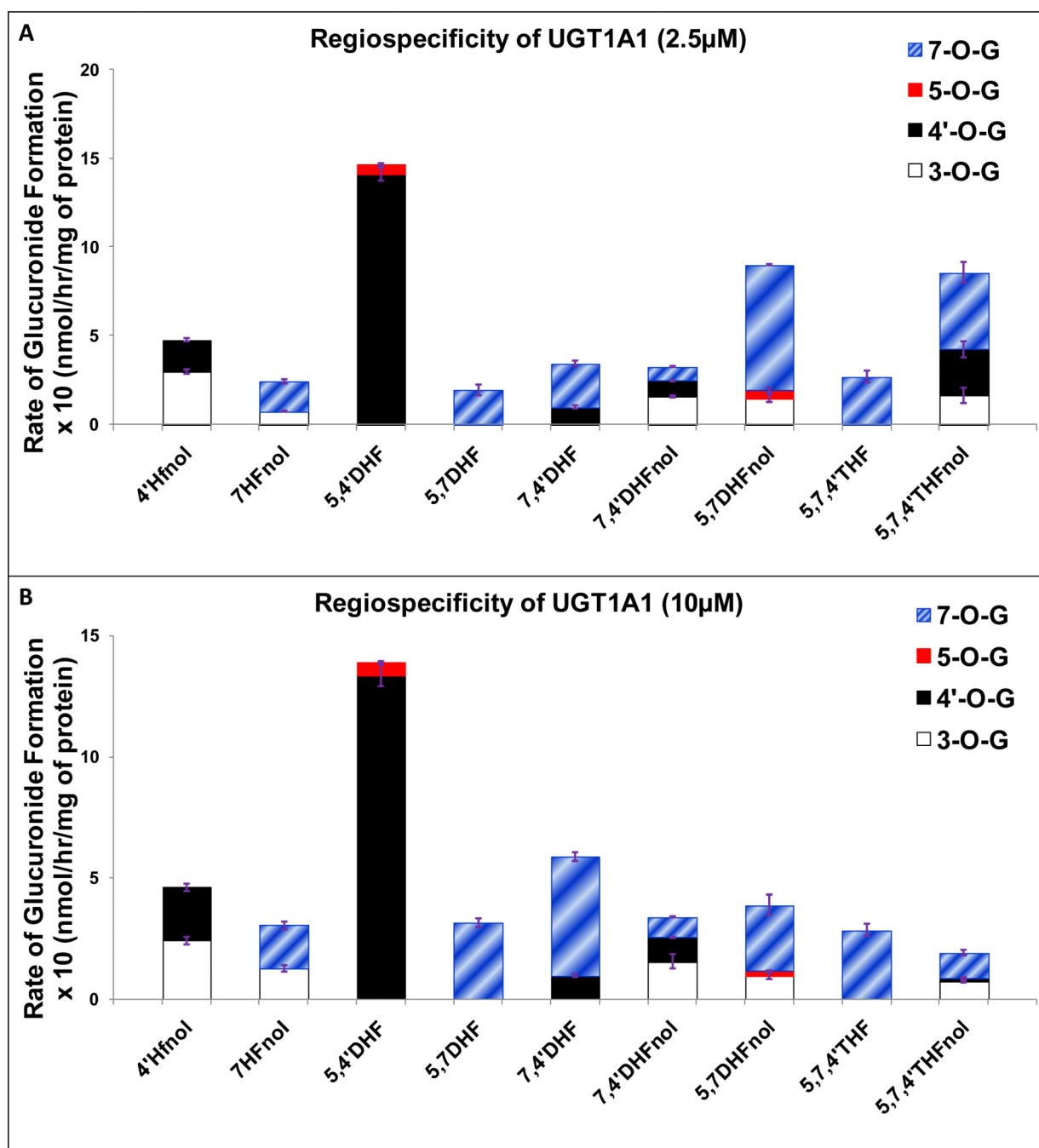
Figure 4. Regiospecific glucuronidation of flavonoids by UGT1A1 at 2.5 μM (A) and 10 μM (B)
Flavonoids (flavones and flavonols at 2.5 or 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) hr with UGT1A1 (using optimum final protein concentration ~ 0.25, 0.5 or 1 mg/ml). The amounts of mono-glucuronide(s) formed were measured using UPLC. The amounts of each regiospecific mono-glucuronide formed were measured using UPLC. Rates of mono-glucuronide formation were calculated as nmol/hr/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n=3). The regiospecific data of the flavonoids at 10 μM has been re-plotted from Figures 2 and 3. UGT stands for Uridine diphosphate glucuronosyltransferases).
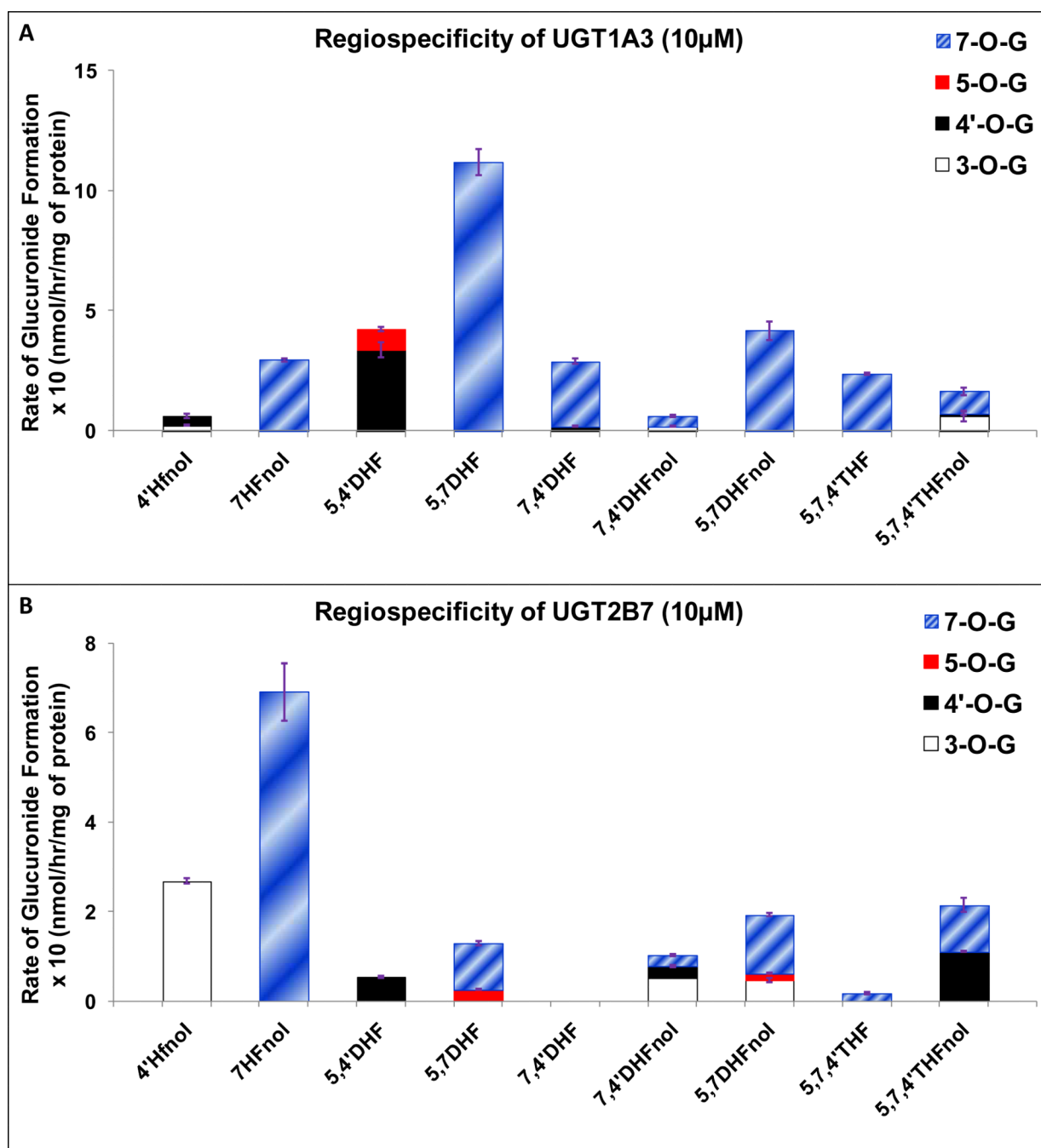
Figure 5. Regiospecific glucuronidation of flavonoids by UGT1A3 (A) and UGT2B7 (B)
Flavonoids (flavones and flavonols at 10 µM concentration) were incubated at 37 °C for 1
(or 0.5) hr with UGT1A3/UGT2B7 (using optimum final protein concentration ~ 0.25, 0.5
or 1 mg/ml). The amounts of mono-glucuronide(s) formed were measured using UPLC. The
amounts of each regiospecific mono-glucuronide formed were measured using UPLC. Rates
of mono-glucuronide formation were calculated as nmol/hr/mg of protein. Each bar is the
average of three determinations, and the error bars are the standard deviations of the mean
(n=3). The regiospecific data of the flavonoids has been re-plotted from Figures 2 and 3.
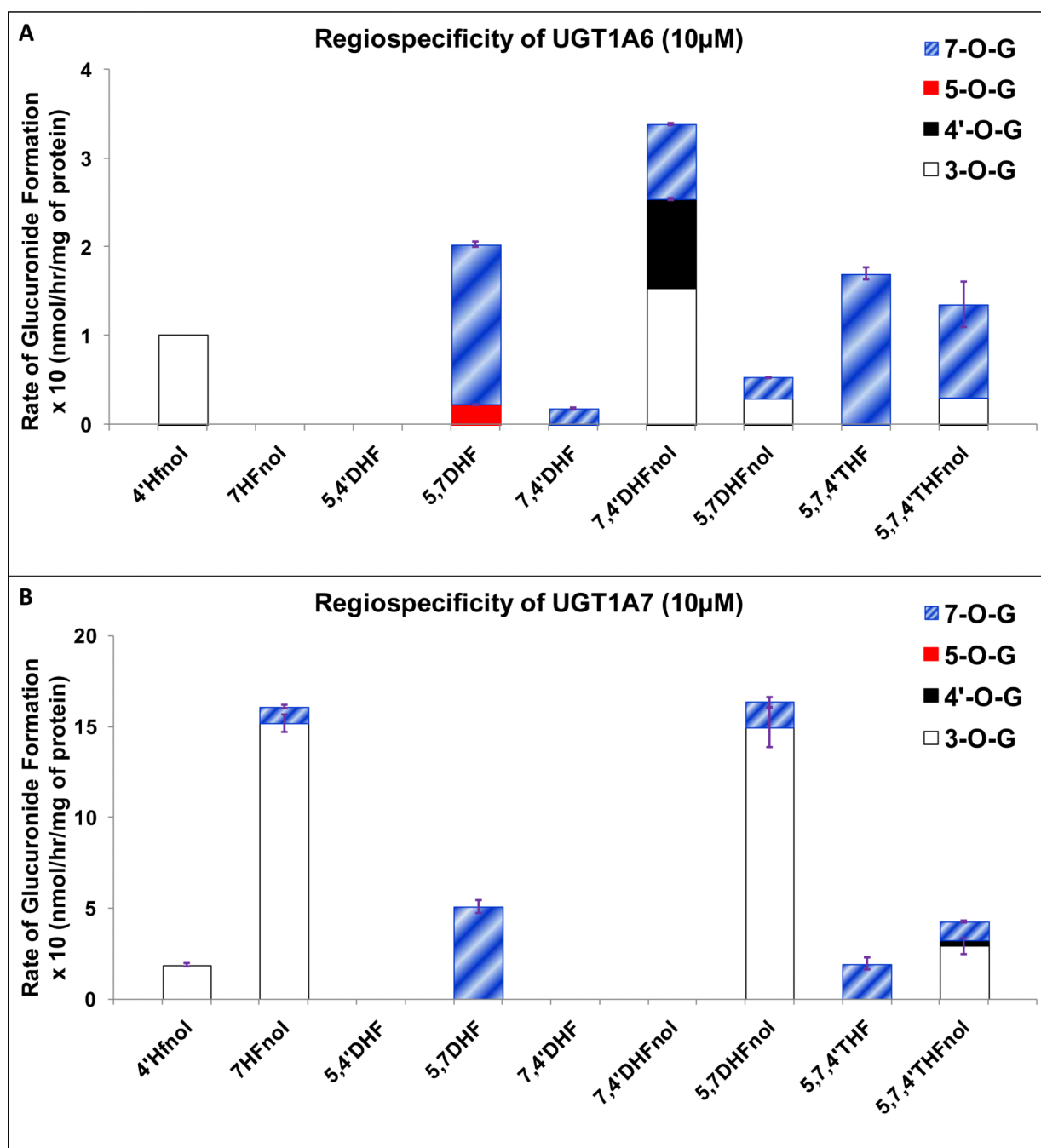UGT stands for Uridine diphosphate glucuronosyltransferases).

Figure 6. Regiospecific glucuronidation of flavonoids by UGT1A6 (A) and UGT1A7 (B)
Flavonoids (flavones and flavonols at 10 µM concentration) were incubated at 37 °C for 1 (or 0.5) hr with UGT1A6/UGT1A7 (using optimum final protein concentration ~ 0.25, 0.5 or 1 mg/ml). The amounts of mono-glucuronide(s) formed were measured using UPLC. The amounts of each regiospecific mono-glucuronide formed were measured using UPLC. Rates of mono-glucuronide formation were calculated as nmol/hr/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n=3). The regiospecific data of the flavonoids has been re-plotted from Figures 2 and 3. UGT stands for Uridine diphosphate glucuronosyltransferases).
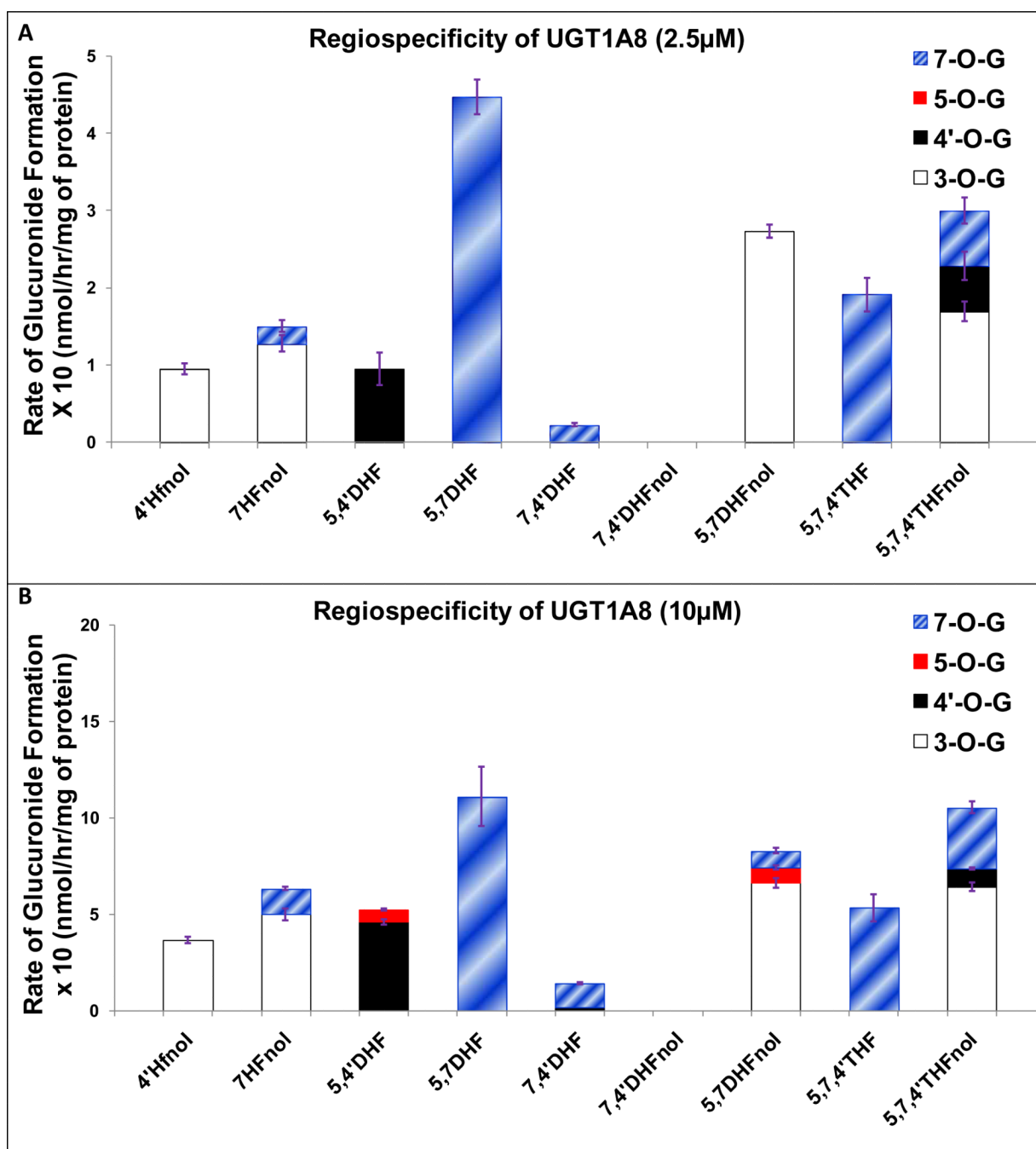
Figure 7. Regiospecific glucuronidation of flavonoids by UGT1A8 at 2.5 µM (A) and 10 µM (B)
Flavonoids (flavones and flavonols at 2.5 or 10 µM concentration) were incubated at 37 °C for 1 (or 0.5) hr with UGT1A8 (using optimum final protein concentration ~ 0.25, 0.5 or 1 mg/ml). The amounts of mono-glucuronide(s) formed were measured using UPLC. The amounts of each regiospecific mono-glucuronide formed were measured using UPLC. Rates of mono-glucuronide formation were calculated as nmol/hr/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n=3). The regiospecific data of the flavonoids at 10 µM has been re-plotted from Figures 2 and 3. UGT stands for Uridine diphosphate glucuronosyltransferases).
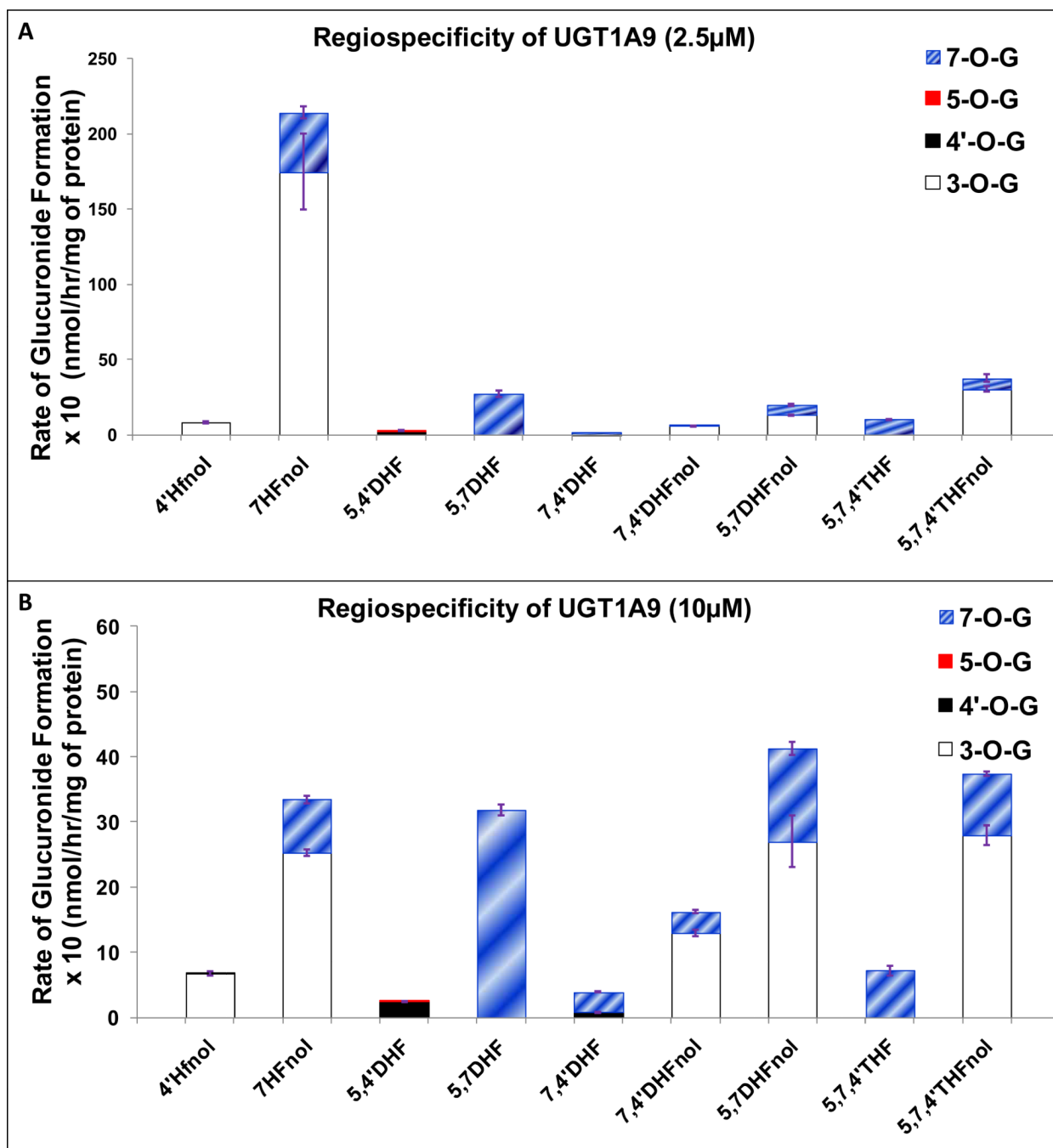
Figure 8. Regiospecific glucuronidation of flavonoids by UGT1A9 at 2.5 μM (A) and 10 μM (B)
Flavonoids (flavones and flavonols at 2.5 or 10 μM concentration) were incubated at 37 °C for 1 (or 0.5) hr with UGT1A9 (using optimum final protein concentration ~ 0.25, 0.5 or 1 mg/ml). The amounts of mono-glucuronide(s) formed were measured using UPLC. The amounts of each regiospecific mono-glucuronide formed were measured using UPLC. Rates of mono-glucuronide formation were calculated as nmol/hr/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n=3). The regiospecific data of the flavonoids at 10 μM has been re-plotted from Figures 2 and 3. UGT stands for Uridine diphosphate glucuronosyltransferases).
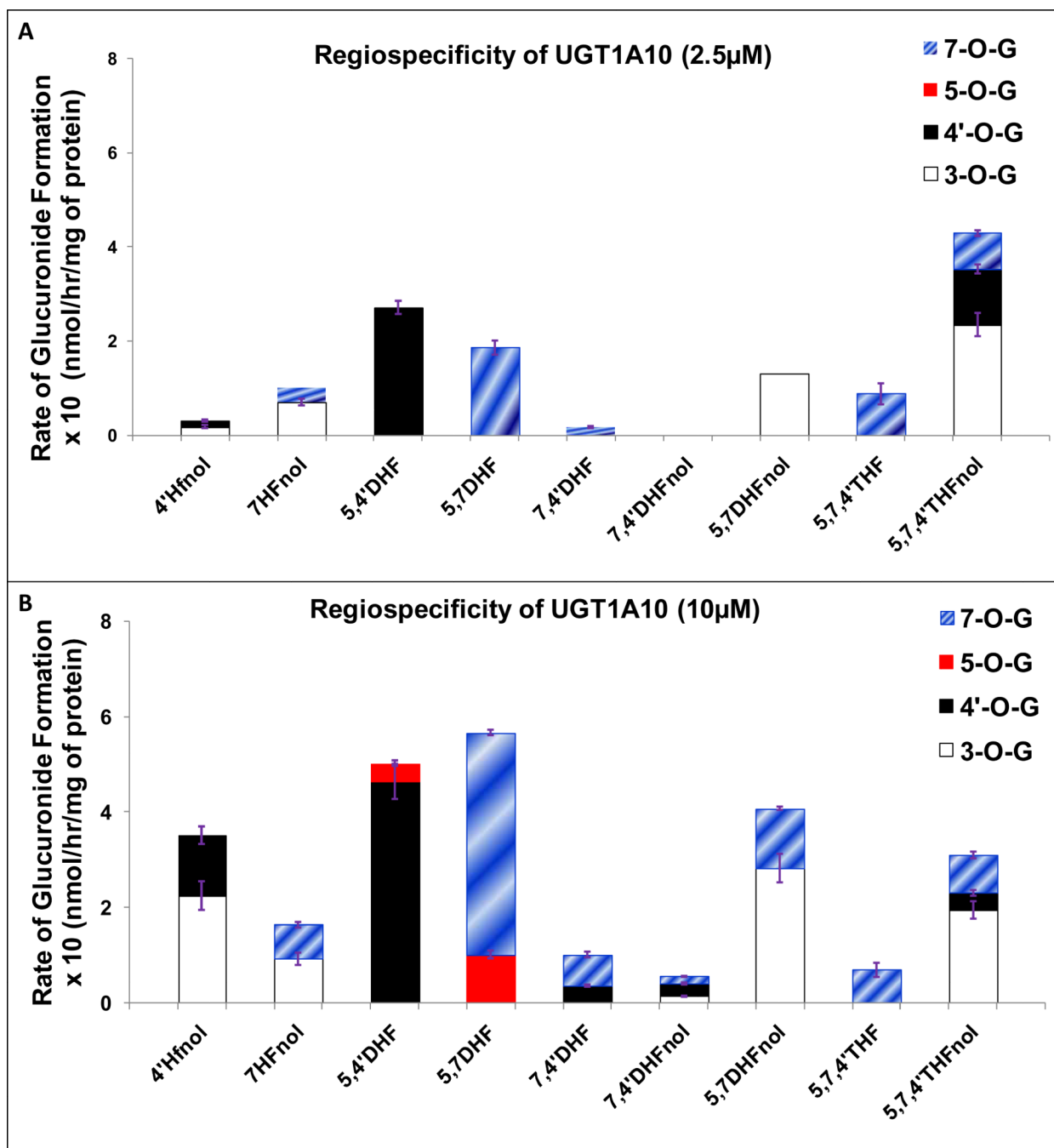
Figure 9. Regiospecific glucuronidation of flavonoids by UGT1A10 at 2.5 μM (A) and 10 μM (B)
Flavonoids (flavones and flavonols at 2.5 or 10 μM concentration) were incubated at 37 °C
for 1 (or 0.5) hr with UGT1A10 (using optimum final protein concentration ~ 0.25, 0.5 or 1
mg/ml). The amounts of mono-glucuronide(s) formed were measured using UPLC. The
amounts of each regiospecific mono-glucuronide formed were measured using UPLC. Rates
of mono-glucuronide formation were calculated as nmol/hr/mg of protein. Each bar is the
average of three determinations, and the error bars are the standard deviations of the mean
(n=3). The regiospecific data of the flavonoids at 10 μM has been re-plotted from Figures 2
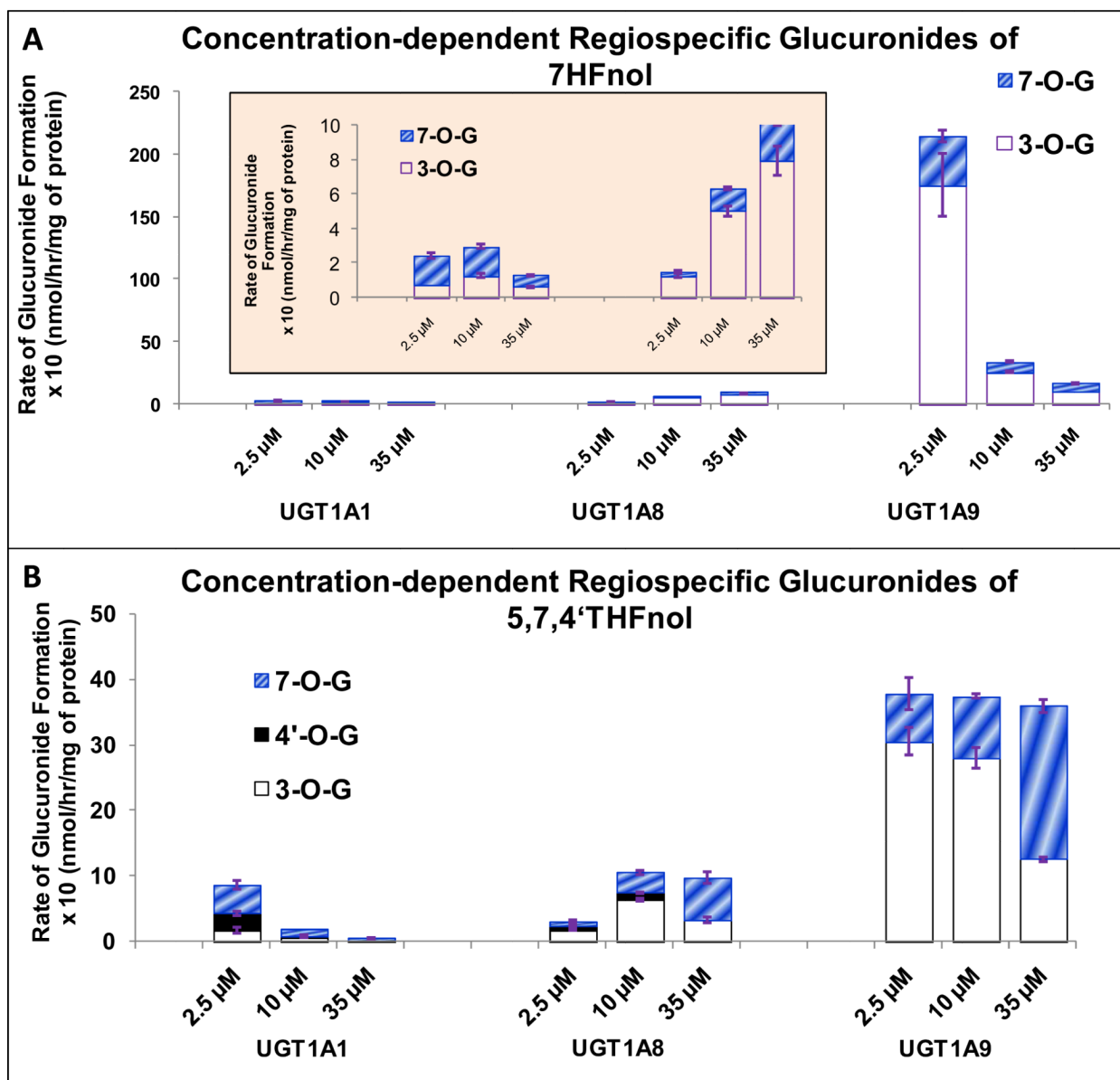and 3. UGT stands for Uridine diphosphate glucuronosyltransferases).

Figure 10. Concentration-dependent regiospecific glucuronidation of 7HFnol (A) and 5,7,4'THFnol (B) by UGT 1A1, 1A8 and 1A9

7HFnol and 5,7,4'THFnol (at 2.5, 10 and 35 μM concentration) were incubated at 37 °C for 1 (or 0.5) hr with UGT1A1, 1A8 or 1A9 (using optimum final protein concentration ~ 0.25, 0.5 or 1 mg/ml). The amounts of mono-glucuronide(s) formed were measured using UPLC. The amounts of each regiospecific mono-glucuronide formed were measured using UPLC. Rates of mono-glucuronide formation were calculated as nmol/hr/mg of protein. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean (n=3). UGT stands for Uridine diphosphate glucuronosyltransferases).

Table 1

Degree of regiospecificity (dominant, moderate, weak or no) of various UGT (uridine diphosphate glucuronosyltransferases) isoforms for glucuronidating flavones and flavonols. The position shown in bracket stands for major glucuronide. In case of di-hydroxyflavones and hydroxyflavonols, degree of regiospecificity was determined based on ratio of rates of formation of two glucuronides. In case of tri-hydroxyflavones, di-hydroxyflavonols and tri-hydroxyflavonols, degree of regiospecificity was determined based on ratio of rates of formation of two faster glucuronides.

UGT Isoforms	Substrate Concentration (μM)	4'HFnol	7HFnol	5,4'DHF	5,7DHF	7,4'DHF	5,7DHFnol[1]	7,4'DHFnol[1]	5,7,4"THF[2]	5,7,4"THFnol[1]
1A1	2.5	No (3-O, 4'-O)	Weak (7-O)	Dominant (4'-O)	Dominant (7-O)	Weak (7-O)	Weak (7-O)	No (3-O, 7-O)	Dominant (7-O)	Weak (7-O)
	10	No (3-O, 4'-O)	No (3-O,7-O)	Dominant (4'-O)	Dominant (7-O)	Moderate (7-O)	Weak (7-O)	No (3-O, 7-O)	Dominant (7-O)	No (3-O, 7-O)
1A3	2.5	Dominant (3-O)	Moderate (7-O)	Dominant (4'-O)	Dominant (7-O)	Dominant (7-O)	Dominant (7-O)	Dominant (7-O)	Dominant (7-O)	No (3-O, 7-O)
	10	No (3-O, 4'-O)	Dominant (7-O)	Moderate (4'-O)	Dominant (7-O)	Dominant (7-O)	Dominant (7-O)	Weak (7-O)	Dominant (7-O)	No (3-O, 7-O)
1A6	2.5	Dominant (3-O)	*	*	Moderate (7-O)	*	-	*	Dominant (7-O)	Moderate (3-O)
	10	Dominant (3-O)	*	*	Moderate (7-O)	Dominant (7-O)	#	*	Dominant (7-O)	##
1A7	10	Dominant (3-O)	Dominant (3-O)	*	Dominant (7-O)	*	Dominant (3-O)	*	Dominant (7-O)	Weak (3-O)
1A8	2.5	Dominant (3-O)	Moderate (3-O)	Dominant (4'-O)	Dominant (7-O)	Dominant (7-O)	Dominant (3-O)	*	Dominant (7-O)	Weak (3-O)
	10	Dominant (3-O)	Moderate (3-O)	Moderate (4'-O)	Dominant (7-O)	Moderate (7-O)	Moderate (3-O)	*	Dominant (7-O)	Weak (3-O)
1A9	2.5	Dominant (3-O)	Moderate (3-O)	Dominant (4'-O)	Dominant (7-O)	Moderate (7-O)	Weak (3-O)	Moderate (3-O)	Dominant (7-O)	Moderate (3-O)
	10	Dominant (3-O)	Moderate (3-O)	Dominant (4'-O)	Dominant (7-O)	Moderate (7-O)	No (3-O, 7-O)	Moderate (3-O)	Dominant (7-O)	Weak (3-O)
1A10	2.5	No (3-O, 4'-O)	Weak (3-O)	Dominant (4'-O)	Dominant (7-O)	Dominant (7-O)	Dominant (3-O)	*	Dominant (7-O)	Moderate (3-O)
	10	Dominant (3-O)	No (3-O, 7-O)	Dominant (4'-O)	Moderate (7-O)	No (7-O, 4'-O)	#	No (3-O, 7-O)	Dominant (7-O)	Weak (3-O)
2B7	10	Dominant (3-O)	Dominant (7-O)	Dominant (4'-O)	Moderate (7-O)	*	#	Weak (3-O)	Dominant (7-O)	##

The highlighted cells in the table represent any change in regiospecificity with change from lower (2.5μM) to higher (10μM) substrate concentration.

[1] stands for ratio of rates of formation of 3-O and 7-O glucuronides.

[2] stands for ratio of rates of formation of 5-O and 7-O glucuronides.

* No glucuronidation was detected at any hydroxyl group.

Two faster glucuronides were 3-O and 5-O, instead of 3-O and 7-O.

Two faster glucuronides were 4'-O and 7-O, instead of 3-O and 7-O.