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Identification of Flavone Glucuronide Isomers by Metal Complexation and Tandem Mass Spectrometry: Regioselectivity of Uridine 5′-Diphosphate−Glucuronosyltransferase Isozymes in the Biotransformation of Flavones

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Supporting Information

ABSTRACT: Flavone glucuronide isomers of five flavones (chrysin, apigenin, luteolin, baicalein, and scutellarein) were differentiated by collision-induced dissociation of [Co(II) (flavone-H) (4,7-diphenyl-1,10-phenanthroline)$_2$]$^+$ complexes. The complexes were generated via postcolumn addition of a metal−ligand solution after separation of the glucuronide products generated upon incubation of each flavone with an array of uridine 5′-diphosphate (UDP)−glucuronosyltransferase (UGT) isozymes. Elucidation of the glucuronide isomers allowed a systematic investigation of the regioselectivity of 12 human UGT isozymes, including 8 UGT1A and 4 UGT2B isozymes. Glucuronidation of the 7-OH position was the preferred site for all the flavones except for luteolin, which possessed adjacent hydroxyl groups on the B ring. For all flavones and UGT isozymes, glucuronidation of the 5-OH position was never observed. As confirmed by the metal complexation/MS/MS strategy, glucuronidation of the 6-OH position only occurred for baicalein and scutellarein when incubated with three of the UGT isozymes.

KEYWORDS: *human UDP−glucuronosyltransferase, flavonoid, regioselectivity, mass spectrometry, metal complexation, glucuronidation*

INTRODUCTION

The biotransformation of flavonoids has been a topic of increasing research activity over the past decade due to the interest in mapping the correlation between the beneficial chemopreventive properties of flavonoids and the structures of the active compounds in the body.[1,2] Moreover, understanding the bioavailabilities of flavonoids demands consideration of the metabolism of native flavonoids upon consumption.[3−5] In this context, there have been a number of strategies aimed at elucidating the structures of the biotransformation products of flavonoids. This task is challenging due to the number of ways that flavonoids can be metabolized and the number of isomeric structures that may defy facile differentiation.[6] Although flavonoids are typically glycosylated in fruits and vegetables, they are readily enzymatically deglycosylated by β-glucosidases or lactose phloridzin hydrolases in the small intestine after ingestion.[7] Once the sugar side chain is removed, flavonoids are most frequently modified by addition of a glucuronic acid or sulfate group or in some cases by methyl or hydroxyl groups.[7,8] These processes are mainly carried out by phase II enzymes found in the small intestine, kidneys, and liver.[8] It is these conjugated flavonoid species that are absorbed by the body. In fact, it has been shown that flavonoid aglycones in general have poor bioavailability. The poor availability has motivated many investigations of metabolism in order to rationalize how inactive compounds or ones with poor bioavailability may exert positive health benefits.[6]

One of the most common conjugates formed during metabolism are *O*-glucuronides. Glucuronidation arises from the uridine 5′-diphosphate (UDP)−glucuronosyltransferase (UGT) family of enzymes.[8] This family is split into three main subgroups and contains a total of 19 different isoforms including 9 UGT1As, 3 UGT2As, and 7 UGT2Bs. Currently, it is known that the UGT1A group and the UGT2B group play a major role in phase II metabolism; however, little is known about the function of the UGT2A group.[9] With respect to their biotransformative role, UGT enzymes catalyze the addition of glucuronic acid to any hydroxyl group, resulting in formation of *O*-glucuronide products.[8] This rather ubiquitous glucuronidation process makes it particularly difficult to identify the products with confidence as flavonoids may have multiple hydroxyl groups. Reports have shown that the addition of glucuronic acid to a flavone can greatly alter the bioactivity of flavones. The apparent impact of glucuronidation on bioactivity has stimulated efforts to unravel the formation and distribution of the glucuronides as well as the effects of glucuronidation on the bioactivities of the flavonoids.[10,11]

Flavones, a subclass of flavonoids, are distinguished from other subgroups of flavonoids by a double bond between the 2 and 3 positions on the C ring and a lack of a hydroxyl group at the 3 position. The basic structure is shown in Figure 1. Flavones are found in various types of fruits and vegetables, as well many different herbs.[12] This subclass of flavonoids has

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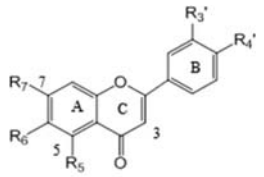

Flavones (MW	R5	R6	R7	R3'	R4'
Chrysin (254)	OH	H	OH	H	H
Apigenin (270)	OH	H	OH	H	OH
Luteolin (286)	OH	H	OH	OH	OH
Baicalein (270)	OH	OH	OH	H	H
Scutellarein (286)	OH	OH	OH	H	OH
5-Hydroxyflavone (238)	OH	H	H	H	H
6-Hydroxyflavone (238)	H	OH	H	H	H
7-Hydroxyflavone (238)	H	H	OH	H	H
6,7-Dihydroxyflavone (238)	H	OH	OH	H	H

Figure 1. Structures of flavones.

been reported to be very biologically active and may play a role in countering diabetes mellitus, arteriosclerotic vascular disease and even breast cancer.[13,14] Numerous studies have demonstrated these positive chemopreventive properties in a variety of in vitro, in vivo, and case control studies.[15−17] As alluded to above, structural characterization of flavone-*O*-glucuronides is difficult. With UGT enzymes able to modify any hydroxyl group, the formation of different isomers is feasible. For many years, identification of these glucuronides isomers has been performed by the comparison of retention times with synthesized standards of the different isomers. However, this method is limited by the lack of availability of standards and the complexity of synthesizing and purifying such compounds.[17−24] NMR spectroscopy is arguably the most effective method for characterization of flavone glucuronide isomers, as demonstrated by Boutin et al.[25] for the structural differentiation of flavone glucuronides produced from UGT enzymes, but NMR requires scaled up sample quantities that make it less practical for broad scale in vivo studies.[25] Recently, methods have been developed to facilitate differentiation of these types of isomers based on advanced chromatographic methods with tandem mass spectrometry (MS/MS) and/or use of the UV-shift method to assign conjugation positions.[26−28] Of these methods, tandem mass spectrometry of flavonoid−metal complexes has proven to be extremely effective.[29−31] For example, we have shown that using metal complexation is an effective method for differentiation of isomeric flavonoids and their glucuronides, including ones in the subclass of flavones.[32−39] This metal complexation strategy has also been adapted for the identification of flavonoid glucuronide isomers in urine.[40,41] Most recently, this metal complexation method was applied to a large-scale systematic study that allowed detailed insight into regioselectivity of UGT isozymes for five common flavonoids including hesperetin, naringenin, isorhamnetin, kaempferol, and quercetin.[42] There has also been considerable progress in modeling human UGT quantitative structure−activity relationships (QSAR) and prediction of regioselectivity, as summarized in a recent review.[43] This type of QSAR modeling has already begun to shed light on understanding the complex substrate selectivity of human UGTs.[43]

In this present study, we have expanded our investigation of the selectivity of glucuronidation of the 12 most common UGT enzymes (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) for 5 flavones (apigenin, baicalein, chrysin, luteolin, and scutellarein) for which the glycoside forms are among the most common found in foods. These flavones are pervasive in common fruits, vegetables, and herbs, such as celery (apigenin), *Scutellariae radix* (baicalein), honey (chrysin), peppermint (luteolin), and *Scutellaria lateriflora* (scutellarein). While other studies have examined UGT isozyme regioselectivities, there has been little focus on how the base structure of flavones affects this selectivity.[25,42,44,45] The current systematic study provides insight into the regioselectivity of UGT isozymes for flavones and also shows the unique affect that a hydroxyl group at the 6 position of a flavone exerts on the regioselectivity of UGT isozymes. The metal complexation/MS/MS strategy is complementary to the UV-shift method[26] and sometimes gives confident differentiation of glucuronides not possible by other methods.

MATERIALS AND METHODS

Reagents. All UGT isozymes were purchased from BD Biosciences (Woburn, MA, USA). Apigenin, baicalein, chrysin, luteolin, and scutellarein were all purchased from Indofine Chemical Co. (Hillsbrough, NJ, USA). UDP−glucuronic acid (UDPGA) trisodium salt, 4,7-diphenyl-1,10-phenanthroline (4,7-dpphen), and cobalt(II) bromide were purchased from Sigma−Aldrich (St. Louis, MO, USA). High-performance liquid chromatography (HPLC)-grade acetonitrile, HPLC-grade water, potassium phosphate, and methanol were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Synthesis of Flavonoid Glucuronides by UGT Enzymes. The procedure for the glucuronidation reactions was modified from the protocol reported in Davis et al.[40] Each enzyme was divided into 25 μL aliquots and stored at −80 °C until use. The following reaction procedure was used for each combination of UGT enzyme (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) and flavone (apigenin, baicalein, chrysin, luteolin, and scutellarein). All volumes were delivered using appropriate micropipets. The synthesis was carried out by adding 2 mM aqueous UDPGA (65 μL), 20 mM potassium phosphate buffer pH 7.0 (378.75 μL), and 10 mM methanolic solution of flavones (6.25 μL) to a microcentrifuge tube. The reaction was initiated by addition of 25 μL of a UGT enzyme (5 mg/mL). This concentration of enzyme was used based on a protocol reported by Plumb et al.[21] The mixture was incubated at 37 °C overnight. To quench the reaction, 1.5 mL of acetone was added. The final mixture was centrifuged for 10 min at 16 000*g*. The supernatant was removed, and the acetone was evaporated using a Savant DNA120 SpeedVac concentrator (Thermo Electron, Waltham, MA, USA) on low heat for 1 h 40 min. The remaining mixture was refrigerated until analysis. The activities of UGT enzymes were previously assessed in the presence of organic solvents at various concentrations, and it was reported that there were no significant changes in enzyme activities for solutions containing up to 2% methanol content.[43] Thus, the use of a minor portion of methanol (∼1.25% of the total volume of solution) in the present study was not expected to be a major detriment to enzymatic activity.[46] This low concentration of methanol enhanced the solubility of the flavones and led to more accurate concentrations in solution.

The reaction conditions used in this study were similar to those used in refs 25 and 42. Other glucuronidation procedures have been reported.[44,45] For example, previous studies have added magnesium chloride (0.88 mM), saccharolacton (4.4 mM), and alamethicin (0.022 mg/mL) to the incubates. The two major differences between the present study and previous studies[44,45] include the concentration of UDPGA added as well as the incubation time. The UDPGA concentration used in the present study was 260 μM, much less than the 3.5 mM used in refs 44 and 45. As for the incubation time, refs 44 and 45 used times between 5 and 60 min, whereas the reactions proceeded overnight in the present study. These changes in times and concentrations might cause some differences in the distributions of products in the present relative to former studies, but it is clear that the results agree in almost every case where they can be compared, leading to the conclusion that this change in incubation time does not affect the types of products formed.

HPLC-UV Analysis. HPLC of the flavone glucuronides was undertaken using a Prominence HPLC with a manual injector, a 50 μL loop (Shimadzu, Columbia, MA, USA), and a LCQ Duo quadrupole ion trap mass spectrometer (Thermo Electron, Waltham, MA, USA) with electrospray ionization (ESI). The column was a 50 mm × 2.1 mm i.d., 3.5 μm, Symmetry C_{18} column with a 10 mm × 2.1 mm i.d. guard column of the same material (Waters, Milford, MA). The injection volume was 50 μL. The mobile phase was 0.33% formic acid in water (A) and 0.33% formic acid in acetonitrile (B). The gradient used began at 15% B and increased to 40% B over 30 min. (This same setup and conditions are used for the companion LC-MS/MS analysis.) To evaluate the relative product distributions of different flavonoid glucuronides for each enzymatic reaction, the peak area for each resulting product was integrated based on its LC-UV chromatographic profile at 360 nm. The area of each product peak was divided by the total area of all product peaks in order to calculate the relative product distributions as percentages. Although absorption coefficients may vary for different products, in fact, the same products are being measured relative to each other for any flavone reacting with a series of UGT enzymes.

Mass Spectrometric Analysis. Samples were first analyzed by HPLC in the negative ESI mode in order to search for flavonoid glucuronides. The spray voltage was set at 4.5 kV, the heated capillary temperature was 200 °C, and the automatic gain control was set to 5 × 10^7 ions with a maximum injection time of 500 ms and 5 microscans averaging. All other parameters were set to obtain optimal signal. The positive ESI mode was used for LC-MS/MS analysis of the flavone−metal complexes. The metal complexes were formed by postcolumn addition of a methanolic solution of 10 mM $CoBr_2$ and 4,7-dpphen, which was infused at a rate of 20 μL/min controlled by a syringe pump. The spray voltage for the positive ion mode was set to 5 kV, and the heated capillary temperature was 200 °C. The automatic gain control for MS/MS was set to 2 × 10^7 ions with a maximum injection time of 500 ms and 5 microscan averaging; the isolation width was set to 4 Da, and a collision energy of 35% normalized collision energy was used for collision-induced dissociation (CID). For direct infusion of metal complex solutions, each flavone or flavone glucuronide was mixed in a methanolic solution with $CoBr_2$ and 4,7-dpphen in a 1:1:1 ratio. The solutions were made to have a final concentration of 10 μM. Samples were then infused at a rate of 5 μL/min via a syringe pump. The rest of the MS parameters were kept the same as those used for the samples analyzed by LC-MS.

RESULTS AND DISCUSSION

After reaction of the flavones in the presence of the glucuronysyltransferases, identification of the products as glucuronides is straightforward by LC-MS due to the characteristic mass shift (+176 Da) due to attachment of the glucuronyl moiety. However, the flavones with multiple hydroxyl groups produced one or more glucuronides upon incubation with each glucuronosyltransferase, yielding isobaric products. The MS/MS spectra of the resulting isobaric flavone glucuronides are too similar to allow their differentiation. An alternative approach utilizing the MS/MS spectra of the metal complexes $[Co(II)\ (FG-H)\ (4,7\text{-dpphen})_2]^+$ (where FG represents a flavone glucuronide) in conjunction with the absolute or relative chromatographic retention times allowed differentiation and assignment of the various flavone glucuronides, including isomers. A postcolumn complexation method was used to generate the $[Co(II)\ (FG-H)\ (4,7\text{-dpphen})_2]^+$ upon elution of each glucuronide, and these complexes gave distinctive, diagnostic fragmentation patterns upon CID, thus giving more confident identification than obtained for deprotonated or protonated flavone glucuronides.[27,40,42] For example, metal complexes incorporating 7-*O*-glucuronides exhibit losses of the auxiliary ligand (−Aux), the glucuronic acid moiety (−GlcA), or both (−(GlcA + Aux)), upon CID. The 7-*O*-glucuronides also demonstrate the loss of the flavone aglycone (−Agl) to a lesser extent. In contrast, the metal complexes of 5-*O*-glucuronides generally undergo a prominent loss of the glucuronic acid moiety along with the auxiliary ligand (−(GlcA + Aux)); additionally, the 5-*O*-glucuronide characteristically elutes before the 7-*O*-glucuronide.[40] The 6-*O*-glucuronides do not dissociate by elimination of the aglycone moiety, thus allowing them to be differentiated from the 7-*O*-glucuronides. B ring glucuronides dissociate via the loss of the auxiliary ligand (−Aux) as well as the combined losses of both the auxiliary ligand and glucuronide moiety (−(GlcA + Aux)).[27] 4′-*O*-Glucuronides elute prior to 3′-*O*-glucuronides and after the corresponding 7-*O*-glucuronides. As summarized briefly here, these characteristic elution orders and fragmentation patterns allow facile differentiation of isomeric flavonoid glucuronides.

With respect to the implementation of this approach, the flavone glucuronides were derived from the supernatants obtained after centrifugation of the enzymatic reaction incubates. The glucuronides were separated and then ionized by either negative ESI or via postcolumn metal complexation prior to introduction into the ion trap mass spectrometer. To pinpoint the elution of the flavone products of interest, specific m/z values corresponding to each unmodified flavone and its monoglucuronidated (aglycone +176) and diglucuronidated (aglycone +176 + 176) products were searched in the total ion chromatograms. In the present study, no diglucuronidated products were found. CID of the positively charged flavone glucuronide−metal complexes, not the deprotonated flavone glucuronides, yielded the most distinctive fragmentation patterns that confirmed the identity of each species. Examples of the MS/MS spectra for some of the metal complexes are shown in Figure 2 for the luteolin glucuronides produced from

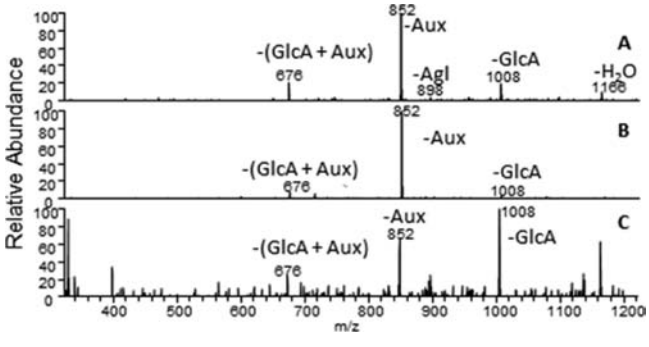

Figure 2. CID mass spectra of $[Co(II)\ (FG-H)\ (4,7\text{-dpphen})_2]^+$ for all UGT1A1−luteolin products: (A) 7-*O*-glucuronide, m/z 1184; (B) 3′-*O*-glucuronide, m/z 1184; (C) 4′-*O*-glucuronide, m/z 1184. −Aux (loss of auxiliary ligand); −GlcA (loss of glucuronic acid moiety); −Agl (loss of flavonoid aglycon); −(GlcA + Aux) (loss of glucuronic acid moiety and auxiliary ligand).

UGT1A1 and in Figure 3A,B for the baicalein glucuronides produced from UGT1A1. All products identified based on the unique MS/MS patterns of the metal complexes are summarized in Table 1 along with the quantitative distribution of products obtained by integration of the chromatographic peak areas of each product and unmodified flavone.

The glucuronide products of four additional flavones, including three with just a single hydroxyl group at the 5, 6, or 7 position, as well as one flavone with hydroxyl groups at the 6 and 7 positions, were also evaluated in order to provide

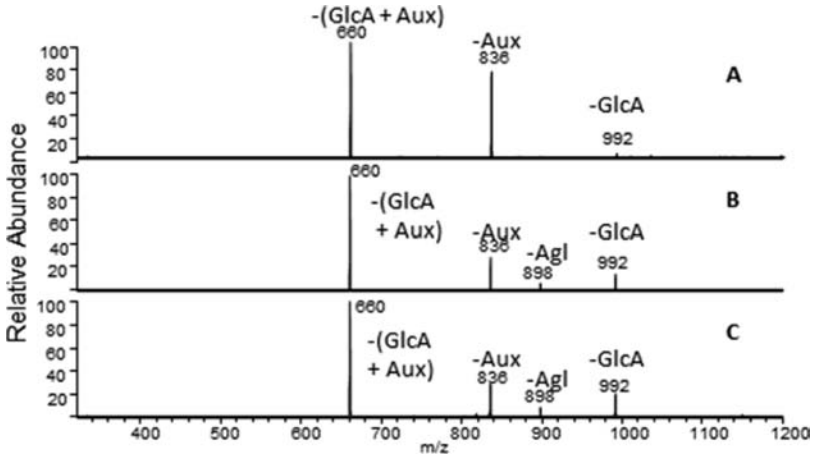

Figure 3. CID mass spectra of $[Co(II)\ (FG-H)\ (4,7\text{-dpphen})_2]^+$ for all UGT1A8−baicalein products compared to the CID of the baicalein 7-*O*-glucuronide standard: (A) 6-*O*-glucuronide, *m*/*z* 1168; (B) 7-*O*-glucuronide, *m*/*z* 1168; (C) 7-*O*-glucuronide standard, *m*/*z* 1168. −Aux (loss of auxiliary ligand); −GlcA (loss of glucuronic acid moiety); −Agl (loss of flavonoid aglycon); −(GlcA + Aux) (loss of glucuronic acid moiety and auxiliary ligand).

Table 1. Glucuronide Product Distributions[a]

chrysin	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
chrysin	35	trace	100	30	100	40	20	80	100	65	100	100
5-*O*-glucuronide	–	–	–	–	–	–	–	–	–	–	–	–
7-*O*-glucuronide	65	100	trace	70	trace	60	80	20	–	35	trace	trace
apigenin	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
apigenin	35	70	100	80	100	65	10	75	100	95	100	100
5-*O*-glucuronide	–	–	–	–	–	–	–	–	–	–	–	–
7-*O*-glucuronide	65	30	–	20	–	35	90	25	–	5	–	–
4′-*O*-glucuronide	–	–	–	–	–	–	–	–	–	–	–	–
luteolin	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
luteolin	40	80	100	100	70	55	5	85	100	70	100	100
5-*O*-glucuronide	–	–	–	–	–	–	–	–	–	–	–	–
7-*O*-glucuronide	10	10	–	–	10	10	40	5	–	5	–	–
3′-*O*-glucuronide	20	5	–	–	15	20	40	10	–	25	–	–
4′-*O*-glucuronide	30	5	–	–	5	15	15	trace	–	trace	–	–
baicalein	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
baicalein	5	5	100	trace	100	trace	trace	trace	100	100	100	100
5-*O*-glucuronide	–	–	–	–	–	–	–	–	–	–	–	–
6-*O*-glucuronide	–	–	–	–	–	70	5	20	–	–	–	–
7-*O*-glucuronide	95	95	–	100	–	30	95	80	–	–	–	–
scutellarein	1A1	1A3	1A4	1A6	1A7	1A8	1A9	1A10	2B4	2B7	2B15	2B17
scutellarein	30	trace	100	5	100	trace	trace	5	100	100	100	100
5-*O*-glucuronide	–	–	–	–	–	–	–	–	–	–	–	–
6-*O*-glucuronide	–	–	–	–	–	50	25	20	–	–	–	–
7-*O*-glucuronide	70	100	–	95	–	50	75	75	–	–	–	–
4′-*O*-glucuronide	–	–	–	–	–	–	–	–	–	–	–	–

[a]All values are percentages of total product distribution. A dash is used to indicate the absence of a product. The average standard deviation is ±6%. All values are rounded to the nearest 5%. Trace indicates a value that falls below 2.5%.

additional confirmatory evidence about the relative retention times of flavones modified at the 5, 6, or 7 position. The additional flavones are listed in Figure 1, and the MS/MS patterns of the $[Co(II)\ (FG-H)\ (4,7\text{-dpphen})_2]^+$ are in the Supporting Information. Interestingly, the fragmentation patterns of the glucuronide products generated from the simple 6-hydroxy and 7-hydroxy flavones do not display the identical multiple pathways noted for the flavones that possess multiple hydroxyl groups. For example, the only pathway for the glucuronidated 6-hydroxyflavone is the loss of the auxiliary ligand (−Aux), and the most dominant fragmentation pathway for the glucuronidated 7-hydroxyflavone is the loss of the glucuronic acid group (−GlcA). This notable simplification of the fragmentation patterns is not surprising. The streamlined monohydroxyl flavones do not have multiple metal coordination sites like the other multihydroxyl flavones, and thus, the array of possible metal-chelation structures that lead to diagnostic fragment ions is correspondingly reduced, thus yielding simpler MS/MS patterns. This point is clearly demonstrated by comparison of the MS/MS patterns of the glucuronide of 6-hydroxyflavone, 7-hydroxyflavone, and the two glucuronides of 6,7-dihydroxyflavone (Figure 4). Whereas the

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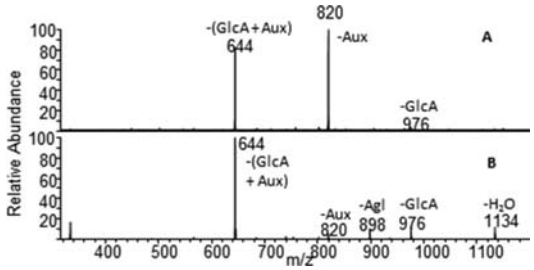

Figure 4. CID mass spectra of $[Co(II)(FG-H)(4,7\text{-dpphen})_2]^+$ for all UGT1A9–6,7-dihydroxyflavone products: (A) 6-*O*-glucuronide, *m/z* 1152; (B) 7-*O*- glucuronide, *m/z* 1152. –Aux (loss of auxiliary ligand); –GlcA (loss of glucuronic acid moiety); –Agl (loss of flavonoid aglycon); –(GlcA + Aux) (loss of glucuronic acid moiety and auxiliary ligand).

fragmentation patterns for 7-*O*-glucuronide from 7-hydroxyflavone show only a single product, the 7-*O*-glucuronide formed from 6,7-dihydroxyflavone shown in Figure 4A exhibits a richer series of diagnostic fragment ions (i.e., loss of glucuronic acid moiety, loss of aglyone, loss of auxiliary ligand, and loss of both glucuronic acid and auxiliary ligand) that allow ready differentiation of the 6-*O*- and 7-*O*-glucuronides. In short, the relative retention times but not the MS/MS patterns of the simplest monohydroxyl flavones are useful for supporting the assignment of glucuronide products of the multihydroxylated flavones. With respect to the retention times, the 7-*O*-glucuronides consistently elute sooner than the 6-*O*-glucuronides, thus providing important confirmatory evidence.

The 5-hydroxyflavone formed one detectable glucuronide product upon incubation with the UGT isozymes, as indicated based on observation of a presumed deprotonated glucuronide upon LC-MS. However, this 5-*O*-glucuronide did not form stable metal complexes. Glucuronidation at the 5-O position inhibits metal coordination between that position and the nearby keto group, thus explaining the lack of metal complexes. Glucuronidation at the 5-O position was not found for any of the multihydroxylated flavones described in this study, suggesting that the 5-O position is the least favorable when other sites are available.

Identification of Flavone Glucuronides. Two flavones, chrysin and apigenin, produced at most one characteristic monoglucuronide when reacted in the presence of each UGT isozyme. The reactions with apigenin resulted in the same monoglucuronide product for UGT1A1, 1A3, 1A6, 1A8, 1A9, 1A10, and UG2B7 but no products for UGT1A4, 1A7, 2B4, 2B15, and 2B17. The sole product exhibited losses of GlcA, Agl, and Aux, a pattern that is characteristic of 7-*O*-glucuronide products. On the other hand, chrysin formed a single product for all glucuronosyltransferases except for UGT2B4 that resulted in no products. The single monoglucuronidated product from chrysin dissociated by pathways characteristic of a 7-*O*-glucuronide (losses of GlcA, Agl, and Aux).

Luteolin, with three hydroxyl groups, generated three different products upon reaction in the presence of UGT1A1, 1A3, 1A7, 1A8, 1A9, 1A10, and 2B7. The first eluting product dissociated by losses of GlcA, Agl, and Aux, all which are consistent with a 7-*O*-glucuronide product. The next two products each displayed losses of Aux, (Aux + GlcA), and GlcA upon CID. Since 4′-*O*-glucuronide products typically elute before 3′-*O*-glucuronide products, the two species are identified as the 4′-O and 3′-O products, respectively. (Figure 2).

A single product was observed for baicalein when modified in the presence of UGT1A1, 1A3, and 1A6, and two glucuronides were produced upon reactions with UGT1A8, 1A9, and 1A10. The product formed from the 1A1, 1A3, and 1A6 reactions showed characteristic losses of Aux, GlcA, and (GlcA + Aux). These are also the same fragments observed for the first eluting product of the 1A8, 1A9, and 1A10 reactions. The (GlcA + Aux) ion is the dominant fragment ion in the spectra, in addition to less abundant ions representing losses of Aux and GlcA. This MS/MS pattern is the same fragmentation pattern seen for the 7-*O*-glucuronide of the 6,7-dihydroxyflavone discussed earlier. The second product from the reactions of baicalein with UGT1A8, 1A9, and 1A10 showed prominent fragments attributed to the loss of Aux or the loss of (Aux + GlcA) that matches the dissociation pattern of the 6-*O*-glucuronide product from the 6,7-dihydroxyflavone reaction. The structural assignment of these two bacalein glucuronides was confirmed by comparing these fragmentation patterns to that obtained for a commercially available reference compound, baicalein 7-*O*-glucuronide. Upon CID, the latter exhibited the loss of Aux or GlcA as well as a dominant fragment corresponding to the loss of (Aux + GlcA) (Figure 3C). On the basis of this evidence as well as the retention time of baicalein 7-*O*-glucuronide (Figure 5A) relative to the retention

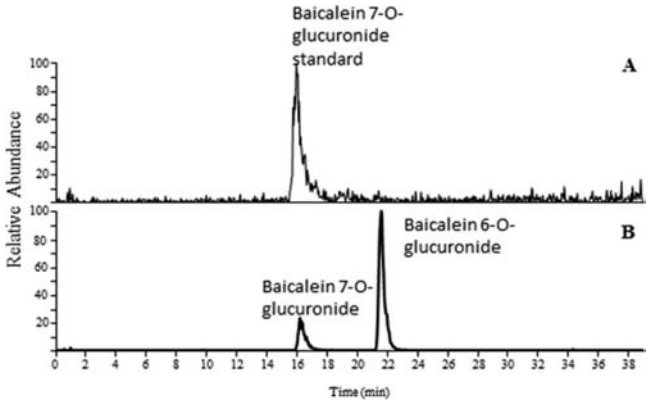

Figure 5. Selective ion chomatogram (*m/z* 445) for (A) baicalein 7-*O*-glucuronide and (B) UGT1A8–baicalein products.

times of the two baicalein–glucuronide products (see Figure 5B), it is clear that the single product formed by bacalein upon reaction with UGT1A1, 1A3, and 1A6 and the first eluting product upon reaction of bacalein with UGT 1A8, 1A9, and 1A10 corresponds to glucuronidation of the 7-O position. This also allows the confident identification of the second product as a 6-*O*-glucuronide based on its greater retention time relative to the 7-*O*-glucuronide, as described earlier for the model flavones.

This LC-MS/MS strategy also allows confident assignment of the products of the scutellarein glucuronidation reactions. Similar to bacalein, scutellarein also formed a single glucuronide when incubated with UGT1A1, 1A3, and 1A6 and two products when incubated with UGT1A8, 1A9, and 1A10. On the basis of the MS/MS patterns and relative retention times, the single product from the UGT1A1, 1A3, and 1A6 reactions and the first eluting species from reaction of UGT1A8, 1A9, and 1A10 is attributed to a 7-*O*-glucuronide product. The second eluting product of the UGT1A8, 1A9, and 1A10 reactions shows losses of both Aux and (Aux + GlcA), analogous to the pattern seen for the 6-O modification of

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baicalein, so this product can be identified as a modification of the 6-O position of scutellarein

Selectivity Trends. 7-Hydroxyflavone, chrysin, apigenin, and luteolin afford an interesting series for comparison of how additional hydroxyl groups affect glucuronidation site selectivity because each of these flavones has an increasing number of hydroxyl groups, starting with the standard 7-OH, then adding the 5-OH, then adding the 4′-OH, and then adding the 3′-OH for each flavone in the series. Singh et al.[45] investigated the selectivity of UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, and 2B7 with chrysin and apigenin by using a UV-shift method.[45] They reported that apigenin and chrysin were glucuronidated solely at the 7-O position, along with minor modification of the 5-O position of apigenin for UGT1A6 and 1A10. Our results agree with these findings with the exception that we observed no glucuronidation reactions with UGT1A7 and no glucuronidation at the 5-O position for apigenin. Comparing the results obtained for chrysin and apigenin, it appears that a hydroxyl group at the 4′ position of a flavone has little to no effect on the selectivity of the UGT isozymes as there is virtually no difference in the product distributions for chrysin and apigenin for all the UGT isozymes. Luteolin provides a unique opportunity to observe how UGT selectivity changes upon addition of another hydroxyl group at the 3′ position. Interestingly, the addition of the second hydroxyl to the B ring of flavone results in modification of both B-ring sites for all active UGT isoforms.

Baicalein and scutellarein each have three potential glucuronidation sites on the A ring (5-OH, 6-OH, and 7-OH) and are also the only two flavones that have the 6-OH group. Chen et al.[18] reported that no glucuronidation of baicalein occurred for reactions with UGT1A3 and UGT1A9.[18] In the current study, we found baicalein does in fact form abundant monoglucuronide products in the presence of all UGT1A isozymes except for UGT1A4 and 1A7 and exhibits no reaction with any of the UGT2B isozymes. Scutellarein showed similar activity to baicalein with all UGT isozymes. The site selectivity for bacalein and scutellarein are similar, suggesting that the extra hydroxyl group at the 4′ position for scutellarein is a nonreactive site. For baicalein and scutellarein, incubation with UGT 1A8, 1A9, and 1A10 results in the formation of 6-*O*-glucuronides.

All flavones show limited reactivity with the UGT2B isozymes. This same trend was noticed previously for glucuronidation of flavonols in our earlier study in which it was hypothesized that the planar nature of these compounds restricted their modification by the UGT2B isozymes. This outcome contrasts with the ample glucuronidation observed for flavanones (a class of flavonoids that lack the 2−3 double bond, thus allowing greater conformational flexibility of the C ring).[42]

This study provides insight into the regioselectivity of 12 UGT isozymes for 5 naturally occurring flavones and demonstrates the differentiation of glucuronide isomers that is essential for bioavailability and biotransformation studies. The formation and CID analysis of metal complexes of the type ($[Co(II)\ (FG-H)\ (4,7\text{-dpphen})_2]^+$) via postcolumn addition of a metal−ligand solution was a key analytical strategy that allowed differentiation of isobaric products, most of which give identical MS/MS fragmentation patterns for the conventional deprotonated species. For example, this approach allows differentiation of 6-*O*- and 7-*O*-glucuronides and complements the UV-shift strategy used by others.[44,45] UGT isozyme selectivity is affected by the presence of a hydroxyl group at the 3′ position, as luteolin is the only flavone that exhibited glucuronidation of the B ring. For baicalein and scutellarein, three of the UGT1A isozymes (1A8, 1A9, and 1A10) resulted in the formation of 6-*O*-glucuronides, enabling the fragmentation rules for the metal complexation/MS/MS strategy to be expanded. Consistent with our previous results for flavonols, the planar structure of the flavones decreases their glucuronidation by the UGT2B isozymes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

MS/MS spectra for chrysin, apigenin, scutellarein, 6-hydroxyflavone, and 7- hydroxyflavone glucuronides complexed with cobalt(II) and 4,7-dpphen. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

CID, collision-induced dissociation; UGT, UDP−glucuronosyltransferase; UDPGA, UDP−glucuronic acid; 4,7-dpphen, 4,7-diphenyl-1,10-phenanthroline

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