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# Systematic Studies of Sulfation and Glucuronidation of 12 Flavonoids in the Mouse Liver S9 Fraction Reveal both Unique and Shared Positional Preferences

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**ABSTRACT:** Sulfation and glucuronidation are the principal metabolic pathways of flavonoids, and extensive phase II metabolism is the main reason for their poor bioavailabilities. The purpose of this study was to compare the similarities and differences in the positional preference of glucuronidation versus sulfation in the mouse liver S9 fraction. The conjugating rates of seven monohydroxyflavones (HFs) (i.e., 2′-, 3′-, 4′-, 3-, 5-, 6-, and 7-HF), and five dihydroxyflavones (diHFs) (i.e., 6,7-, 4′,7-, 3,7-, 5,7-, and 3,4′-diHF) were determined in three separate enzymatic reaction systems: (A) sulfation only, (B) glucuronidation only, or (C) simultaneous sulfation and glucuronidation (i.e., Sult−Ugt coreaction). In general, glucuronidation rates were much faster than sulfation rates. Among the HFs, 7-HF was the best substrate for both conjugation reactions, whereas 3-HF was rapidly glucuronidated but was not sulfated. As a result, the rank order of sulfation was very different from that of glucuronidation. Among the diHFs, regiospecific glucuronidation was limited to 7-OH and 3-OH positions, whereas regiospecific sulfation was limited to 7-OH and 4′-OH positions. Other positions (i.e., 6-OH and 5-OH) in diHFs were not conjugated. The positional preferences were essentially maintained in a Sult−Ugt coreaction system, although sulfation was surprisingly enhanced. Lastly, sulfation and glucuronidation displayed different regiospecific- and substrate-dependent characteristics. In conclusion, glucuronidation and sulfation shared the same preference for 7-OH position (of flavonoids) but displayed unique preference in other positions in that glucuronidation preferred the 3-OH position whereas sulfation preferred the 4′-OH position.

**KEYWORDS:** *sulfation, glucuronidation, flavonoids, mouse liver S9 fraction, structural preference*

## INTRODUCTION

Flavonoids have a variety of "claimed" biological activities, including anti-inflammatory, antiallergic, antiviral, anticancer, and antioxidant.[1−3] However, their bioavailabilities are poor due to rapid and extensive first-pass metabolism via the phase II metabolic pathways in the gut and liver. As a result, there are large amounts of sulfates and glucuronides in the plasma following oral administration of flavonoids or flavonoid-rich foods or diets.[4−7] For example, a significant portion of the absorbed flavonoid aglycones (e.g., fisetin and 7-hydroxyflavone or 7-HF) was rapidly biotransformed into sulfates or glucuronides in rats.[8] Separately, quercetin absorbed from the rat intestine was present in the conjugated forms (glucuronides or sulfates) in the mesenteric blood.[9] In humans, following the ingestion of quercetin-rich diets/foods, only quercetin metabolites (e.g., sulfate conjugates, glucuronide conjugates, or isorhamnetin conjugates) were found in the plasma,[10] and the major conjugates were identified as quercetin-3-glucuronide, 3′-methylquercetin-3-glucuronide, and quercetin-3′-sulfate.[7] In contrast, 5-hydroxyflavone (5-HF) was exclusively metabolized to glucuronide,[8] whereas chrysin (5,7-dihydroxyflavone, 5,7-diHF) and quercetin were both glucuronidated and sulfated.[11−13] Similarly, extensive intestinal sulfation and glucuronidation of apigenin revealed that most apigenins were not transported intact across the intestinal epithelium.[14]

Most of the published studies on flavonoid metabolism were focused on glucuronidation.[15−19] These studies have demonstrated that glucuronidation is regiospecific and isoform-dependent.[20,21] Furthermore, the concentrations of flavonoids used moderately affected the dominant isoforms for their metabolism, because UDP-glucuronosyltransferases 1As (or UGT1As, especially UGT1A1) may display substrate inhibition kinetics.[21] In contrast, much less is known about isoform-dependent regiospecific sulfation of flavones. To our knowledge, no information is available regarding the question as to whether rapidly glucuronidated flavonoids will be similarly sulfated. More importantly, there are no published data showing whether flavone metabolism via sulfation or glucuronidation pathway shares or displays unique structural requirements toward their substrates. The latter is important to elucidate if these two conjugation pathways are compensatory (the slower the glucuronidation is, the faster the sulfation is, or vice versa), competitive, or independent of each other.

Therefore, the purpose of this study is to determine if sulfation and glucuronidation pathways share or display unique structural requirements for their flavone substrates. The liver S9

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fraction was used here because the S9 fraction is routinely used in metabolism studies, especially for the phase II metabolic pathways involving sulfation. In addition, liver is enriched with both Ugts and sulfotransferases (Sults). Intact cells or organs were not used here because the focus is on the formation of the phase II conjugates, which cannot passively diffuse across the cell membrane.

## MATERIALS AND METHODS

**Materials.** Seven monohydroxyflavones (or HFs) and five dihydroxyflavones (or diHFs) were purchased from LC Laboratories (Woburn, MA, USA). Uridine diphosphoglucuronic acid (UDPGA), β-glucuronidase, alamethicin, magnesium chloride, and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other materials (typically of analytical grade or better) were used as received.

**Mouse Liver S9 Fraction Preparation.** Male FVB mouse (18−22 g) were obtained from Cyagen Biosciences Inc. (Guangzhou, China) and kept in an environmentally controlled room (temperature, 25 ± 2 °C; humidity, 50 ± 5%; 12 h dark−light cycle) for at least 3 days before the experiments. The animal experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* by the U.S. National Institutes of Health, and the procedures were approved by the Ethical Committee of the Southern Medical University (Guangzhou, China). Male mouse liver S9 fraction was prepared using a procedure published previously with minor modification.[22,23] Briefly, 10 freshly harvested mouse livers were washed and then perfused with ice-cold saline, weighed, and minced. Minced livers were homogenized using a motorized homogenizer (4 strokes) in ice-cold homogenization buffer (50 mM potassium phosphate, 250 mM sucrose, 1 mM EDTA, pH 7.4) and centrifuged at 7700*g* for 15 min at 4 °C. The fat layer was carefully aspirated, and the supernatant was collected with pasture pipets into microfuge tubes (1 mL each), which were stored at −80 °C until use. The concentration of the S9 fraction protein (normally 5−20 mg/mL) was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard.

**Ugt Activity Measurements.** The incubation procedures for measuring mouse Ugt activities in vitro were essentially the same as those published previously,[16,18] and all of the studies were conducted in triplicates. Briefly, different concentrations of flavone substrates, mouse liver S9 fraction protein (final concentration, 0.053−0.37 mg/mL), magnesium chloride (0.88 mM), alamethicin (0.022 mg/mL), and diphosphate glucuronic acid (UDPGA) at 3.5 mM (added last) were mixed in a 50 mM potassium phosphate buffer (pH 7.4, containing 0.1% (w/v) vitamin C as the stabilizing agent for flavones). Vitamin C (0.1%) was added to stabilize the substrate, and the reaction rates did not change because of its presence (not shown). The mixture (final volume ≈ 400 μL) was incubated at 37 °C for predetermined periods of time (5−90 min to limit the percent metabolism of substrate to <30%, allowing the accurate determination of initial rates) on the basis of the results of pilot experiments. Reaction was stopped by the addition of 60 μL of 94% acetonitrile plus 6% glacial acetic acid containing 50 μM testosterone as the internal standard (stop solution). For glucuronidation, three starting substrate concentrations were used: 5, 10, and 40 μM. We found that for phase II metabolism of flavonoids, 10 μM was considered to be a moderate concentration,[24−26] whereas 40 μM was considered to be the high test concentration because reaction rates at this concentration are generally above reported $V_{max}$ values of most flavonoid glucuronidation. A concentration of 5 μM (designated as low concentration in this paper) is achievable after a pharmacological dose of flavonoids (e.g., genistein).[27] We did not conduct studies at lower concentrations because of the quantification limit of our method was around 1.5 μM (30% of 5 μM) for model flavonoids with the lowest molar extinction coefficients (UV).

We have also tested the chemical stability of the tested compounds and found that 2′-, 3′-, 4′-, 3-, 6-, and 7-hydroxyflavones (2′-HF, 3′-HF, 4′-HF, 3-HF, 6-HF, and 7-HF) and 6,7-, 4′,7-, 3,7-, 5,7-, and 3,4′-dihydroxyflavones (6,7-diHF, 4′,7-diHF, 3,7-diHF, 5,7-diHF, and 3,4′-diHF) were stable in 50 mM potassium phosphate buffer (pH 7.4) at 37 °C with 0.1% vitamin C for 30−90 min. The 5-HF flavone was stable for only 15 min in 50 mM potassium phosphate buffer (pH 7.4) at 37 °C with 0.1% vitamin C. We then conducted a pilot study to measure the formation of 6-*O*-glucuronide and 7-*O*-glucuronide (from 6HF and 7HF) as a function of time (at 0, 15, 30, 45, 60, and 90 min) using three starting concentrations (5, 10, and 40 μM), and the results indicated that formation was approximately linear for up to 90 min (not shown). The formation of 5-*O*-glucuronide (from 5-HF) was measured at 0, 5, 10, 15, and 20 min using three concentrations, and the results indicated the formation was approximately linear for up to 15 min (not shown). Therefore, the incubation time for 5-HF was 5−15 min and for other flavones was from 30 to 90 min.

**Sult Activity Measurements.** The S9 fraction (0.053−0.37 mg/mL protein), 0.1 mM adenosine 3′-phosphate 5′-phosphosulfate (PAPS), and 0.1% vitamin C in a 50 mM potassium phosphate buffer (pH 7.4) were mixed with different concentrations of a substrate. The mixture (final volume ≈ 400 μL) was incubated at 37 °C for a predetermined period of time (5−90 min), and the reaction was stopped by the addition of 60 μL of stop solution. For sulfation reactions, three substrate concentrations were used as well: 5, 10, and 40 μM. From pilot studies, the best incubation time for 5-HF was from 5 to 15 min and for other flavones was from 30 to 90 min.

**Coreaction System of Glucuronidation and Sulfation.** In the coreaction system, cofactors for Sult and Ugt were both present at the same time, and their concentration in the coreaction system was the same as in the single reaction system. Due to chemical stability consideration, incubation of 5-HF was limited to 5−15 min, whereas the incubation of other flavones was limited to 30−90 min. To minimize variability, experiments for two single-reaction systems (Sult and Ugt) and one coreaction system were conducted simultaneously for each of the flavones.

**Elimination Half-Life of Aglycone in Single and Coreaction Systems of Glucuronidation and Sulfation.** Amounts of 6,7-diHF (10 μM) remaining as a function time were determined at 0, 10, 15, 30, 45, 60, and 75 min for a single Ugt reaction system and a Sult−Ugt coreaction system or at 0, 10, 20, 40, 60, 80, and 120 min for a single Sult reaction system. The elimination half-lives of aglycone in single and coreaction systems of glucuronidation and sulfation were determined on the basis of the percent of flavones remaining when compared to the starting concentration.

**UPLC Analysis of Flavones and Their Glucuronides and Sulfates.** We analyzed seven HFs, five diHFs, and their respective glucuronides and/or sulfates by using the same method as published.[20] Briefly, the system comprised a Waters Acquity UPLC with a photodiode array detector and Empower software: column, BEH C18, 1.7 μm, 2.1 × 50 mm; mobile phase B, 100% acetonitrile; mobile phase A, 100% aqueous buffer (2.5 mM $NH_4Ac$, pH 7.4); flow rate, 0.45 mL/min; gradient, 0−2.0 min, 10−35% B, 2.0−3.0 min, 35−70% B, 3.2−3.5 min, 70−10% B, 3.5−4.0 min, 10% B; injection volume, 10 μL; detection wavelength, 254 nm for testosterone; other values were different depending on compounds (actual values listed in Table 1). Linearity was established over the range 1.5625−50 μM for all compounds. The lower limit of quantification (LLOQ) for all compounds was at least 1.5625 μM. The analytical method for each compound was validated for interday and intraday variations using six samples at each of three concentrations (50, 12.5, and 1.56 μM). Error for all flavones was <15%.

**LC-MS/MS Analysis of Flavonoid Glucuronides and Sulfates.** Seven HFs, five diHFs, and their respective glucuronides and sulfates were separated by the same UPLC system. The effluent was introduced into a Premier XE mass spectrometer triple-quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The mass spectrometer was operated in the negative ion mode to perform the analysis of flavones and their metabolites. The main working parameters for the mass spectrum were capillary voltage, 3.0 kV; cone voltage, 35 V; ion source temperature, 110 °C; and desolvation temperature, 500 °C. Flavone monoglucuronides and monosulfates were identified by MS and MS2 full-scan modes (Table 1).

**Table 1. UV Characteristics, LC-MS/MS Characteristics, and Conversion Factors ($K$) of Glucuronides and Sulfates of Selected Monohydroxyflavones and Dihydroxyflavones[a]**

| | flavones | | | | | glucuronides | | | | | | | sulfates | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | UV $\lambda_{max}$, nm | | | | | UV $\lambda_{max}$, nm | | GCI in LC-MS/MS | | | | | UV $\lambda_{max}$, nm | | SCI in LC-MS/MS | |
| | band I | band II | position | TW | $K$ | band I | band II | $[M + H]^+$ | $[M - G + H]^+$ | position | TW | $K$ | band I | band II | $[M + H]^+$ | $[M - S + H]^+$ |
| 2′-HF | 323.9 | 254.9 | 2′-O-G | 254 | 1.26 ± 0.08[b] | 321.5 | 254.9 | 415 | 239 | 2′-O-S | 254 | 0.72 ± 0.06 | 319.2 | 253.7 | 319 | 239 |
| 3′-HF | 298.9 | 240.7 | 3′-O-G | 254 | 0.93 ± 0.02[b] | 301.3 | 256.1 | 415 | 239 | 3′-O-S | 254 | 1.40 ± 0.09 | 306.0 | 243.1 | 319 | 239 |
| 4′-HF | 324.2 | 252.5 | 4′-O-G | 254 | 1.16 ± 0.02[b] | 318.0 | 253.7 | 415 | 239 | 4′-O-S | 254 | 1.21 ± 0.05 | 315.7 | 253.7 | 319 | 239 |
| 3-HF | 343.8 | 239.5 | 3-O-G | 254 | 0.96 ± 0.06[b] | 309.2 | 247.8 | 415 | 239 | 3-O-S | | | | | NA | NA |
| 5-HF | 335.8 | 269.1 | 5-O-G | 254 | 0.84 ± 0.13[b] | 324.1 | 262.0 | 415 | 239 | 5-O-S | 254 | 1.52 ± 0.03 | 294.1 | 257.3 | 319 | 239 |
| 6-HF | 304.8 | 268.0 | 6-O-G | 254 | 0.99 ± 0.03[b] | 306.0 | 260.8 | 415 | 239 | 6-O-S | 254 | 1.59 ± 0.04 | 306.0 | 257.5 | 319 | 239 |
| 7-HF | 309.0 | 252.3 | 7-O-G | 254 | 0.96 ± 0.03[b] | 309.0 | 252.3 | 415 | 239 | 7-O-S | 254 | 0.98 ± 0.04 | 308.4 | 252.5 | 319 | 239 |
| 3,4′-diHF | 356.9 | 232.4 | 3-O-G | 254 | 1.02 ± 0.03[b] | 334.7 | 232.4 | 415 | 239 | 3-O-S | | | | | NA | NA |
| | | | 4′-O-G | 254 | 0.87 ± 0.03[b] | 352.1 | 246.6 | 431 | 255 | 4′-O-S | 355 | 1.06 ± 0.076 | 348.5 | 246.6 | 335 | 255 |
| 3,7-diHF | 343.8 | 252.5 | 3-O-G | 263 | 1.29 ± 0.03[b] | 314.4 | 251.4 | 431 | 255 | 3-O-S | | | | | NA | NA |
| | | | 7-O-G | 254 | 0.86 ± 0.09[b] | 341.5 | 250.2 | 431 | 255 | 7-O-S | 340 | 0.97 ± 0.08 | 341.5 | 246.6 | 335 | 255 |
| 4′,7-diHF | 331.1 | 253.7 | 4′-O-G | | | | | NA | NA | 4′-O-S | 254 | 1.01 ± 0.014 | 314.4 | 253.7 | 335 | 255 |
| | | | 7-O-G | 254 | 1.09 ± 0.04 | 331.1 | 252.5 | 431 | 255 | 7-O-S | 254 | 1.18 ± 0.04 | 331.1 | 252.5 | 335 | 255 |
| 5,7-diHF | 312.2 | 266.8 | 7-O-G | 254 | 1.16 ± 0.02 | 306.2 | 266.8 | 431 | 255 | 7-O-S | 254 | 1.45 ± 0.10 | 308.1 | 268.0 | 335 | 255 |
| 6,7-diHF | 316.8 | 266.8 | 6-O-G | 309 | 1.61 ± 0.14 | 314.4 | 264.4 | 431 | 255 | 6-O-S | | | | | NA | NA |
| | | | 7-O-G | 309 | 1.22 ± 0.11 | 312.2 | 264.4 | 431 | 255 | 7-O-S | 309 | 1.21 ± 0.76 | 312.0 | 266.8 | 335 | 255 |

[a]The metabolic positions of metabolites were determined by UV characteristics of relevant HFs. TW, test wavelength (nm); GCI, glucuronide characteristic ions; SCI, sulfate characteristic ions; NA, not available due to low intensity of the peak. [b]Using published data.[20]



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**Determination of Position of Conjugation by UV Spectra Shift Method.** The molecular positions of glucuronidation and sulfation on HFs and diHFs were identified by changes in UV spectra using the same $\lambda_{max}$ shift method published previously.[20] Details are shown in Table 1 and described under Results.

**Molar Extinction Coefficient Ratio of Flavone Conjugates.** To provide more accurate estimations of substrate and metabolite concentrations and of glucuronidation and sulfation rates, a conversion factor ($K$) representing the ratio between the molar extinction coefficient of the glucuronide, or sulfate and its aglycone, was determined for each flavone and its respective phase II metabolite. The conversion factors ($K$) of the glucuronides from the seven monohydroxyflavones and two dihydroxyflavones (3,4′- and 3,7-diHF) were taken from previously published results.[20] To calculate $K$ of sulfates, sulfates of flavones were prepared using the S9 fraction at flavone concentrations of 10 and 40 $\mu$M. The supernatant was then removed and extracted twice with methylene chloride (sample/dichloromethane = 1:3, v/v) to remove >95% of flavone aglycones and protein present in the S9 fraction. The resulting sample was then divided into two parts, one of which was analyzed following incubation with water and the other after hydrolysis with sulfatase (30 U/mL) at 37 °C for 2 h. The difference in the amount of aglycones found in these two samples was the amount of metabolite formed. The relationship between the spectral peak areas of the metabolites before hydrolysis and the peak areas of aglycones after the hydrolysis was used to establish the conversion factor required to quantify the amounts of flavone conjugates as described previously.[28,29]

**Statistical Analysis.** One-way ANOVA and factorial design ANOVA (univariate analysis of variance) with Tukey's multiple-comparison (posthoc) test or Student's $t$ test were used to evaluate statistical differences. Differences were considered to be significant when $p$ values were <0.05.

## RESULTS

**Confirmation of Flavone Conjugate Formation by LC-MS/MS.** We conducted LC-MS/MS studies of the metabolites of all flavones (Figure 1) used in the present study to show that all glucuronides and sulfates formed using FVB mouse S9 fraction were monoglucuronides and monosulfates (Table 1). The UPLC chromatograms and MS data of these flavones and their glucuronides (except 4′,7-diHF, 5,7-diHF, and 6,7-diHF) have been published in our earlier publication.[20] All of the HFs formed had one glucuronide and one sulfate moiety except for 3-HF, which formed only one glucuronide but no sulfate. There were no diglucuronides or disulfates of any diHFs. Conjugating reactions of 3,4′-, 3,7-, and 6,7-diHF all produced two monoglucuronide isomers but only one monosulfate. In contrast, conjugation of 4′,7-diHF resulted in two sulfate isomers but only one glucuronide, whereas conjugation of 5,7-diHF resulted in only one glucuronide and one sulfate (Figure 2).

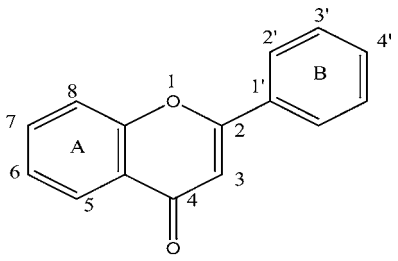


**Figure 1.** Structures of model flavones and aglycone forms of tested flavones used in the present study. Seven monohydroxyflavones and five dihydroxyflavones were chosen for these experiments. Available conjugating −OH positions include 3, 5, 6, 7, 2′, 3′, and 4′.

**Positions of Conjugation of Dihydroxyflavones.** There are no commercial standards for flavone glucuronides and sulfates, and therefore it was necessary to determine the likely molecular positions of monoglucuronides and monosulfates using the UV spectra and relative reaction rates. The site of glucuronidation of tested flavones was tentatively assigned using the UV peak ($\lambda_{max}$) shift method developed previously in our laboratory,[30] and actual position could be further confirmed by NMR. In general, the UV spectra of flavones have two $\lambda_{max}$ values at around 300 nm (band I) and at around 240 nm (band II). Glucuronidation at the 3-hydroxyls and 4′-hydroxyls resulted in band I hypsochromic shifts (or blue shift) of 13−30 and 5−10 nm, respectively. Glucuronidation of the 5-hydroxyl group caused a band II hypsochromic shift of 5−10 nm. In contrast, glucuronidation of the 7-hydroxyl group did not cause any change in band I or II.[31] Because we only have five diHFs with limited substitution pattern (five possible positions at 3, 5, 6, 7, and 4′), this method of positional identification is very accurate and reliable for every compound except for 6,7-diHF, the positional identification of which required additional factors (see below).[20] Therefore, the 3-O-G and 7-O-G of 3,7-diHF and the 3-O-G and 4′-O-G of 3,4′-diHF were tentatively assigned according to the published UV method (Table 1).[20] For 4′,7-diHF, the unique glucuronide showed no shift in band I, which indicated that the metabolite was likely the 7-*O*-glucuronide of 4′,7-diHF (Table 1). For 5,7-diHF, the unique glucuronide showed no shift in band I or II, which indicated that this metabolite was likely the 7-*O*-glucuronide of 5,7-diHF (Table 1). For 6,7-diHF, the unique glucuronide was presumed to be 7-*O*-glucuronide because the glucuronide production from 7-HF was much greater than that from 6-HF, indicating that 7-OH was much more active than 6-OH. This is consistent with our previous observation that the more slowly metabolized phenolic group in a diHF was always more similar to that of the more slowly metabolized HF than the more rapidly metabolized HF.[20]

Similarly, we also tentatively assigned the position of monosulfates from diHFs using the same UV $\lambda_{max}$ shift method, which could be further confirmed by NMR. 3-HF could not be sulfated by FVB mouse liver S9 fraction. For 4′-HF, sulfation at the 4′-OH position severely diminished the peak at 321.5 nm and caused a band I hypsochromic shift of 5.8 nm. For 7-HF, there was nearly no band I hypsochromic shift. Sulfation of the 5-hydroxyl group of 5-HF resulted in a major 41.7 nm hypsochromic shift in band I, suggesting sulfation at this site significantly altered electron conjugation of the flavone ring structure. Identifications of the sulfate positions using the shift in band I and II values are shown in Table 1, and the extinction coefficient ratios of all HFs and diHFs are also listed in Table 1.

For diHFs, a similar approach was used to tentatively assigned the position of sulfation, which was based on the UV peak ($\lambda_{max}$) shift in band I and II regions. Using a similar methodology for 4′,7-diHF, the sulfate 1 had a shorter retention time and a major hypsochromic shift of 16.7 nm of the band I

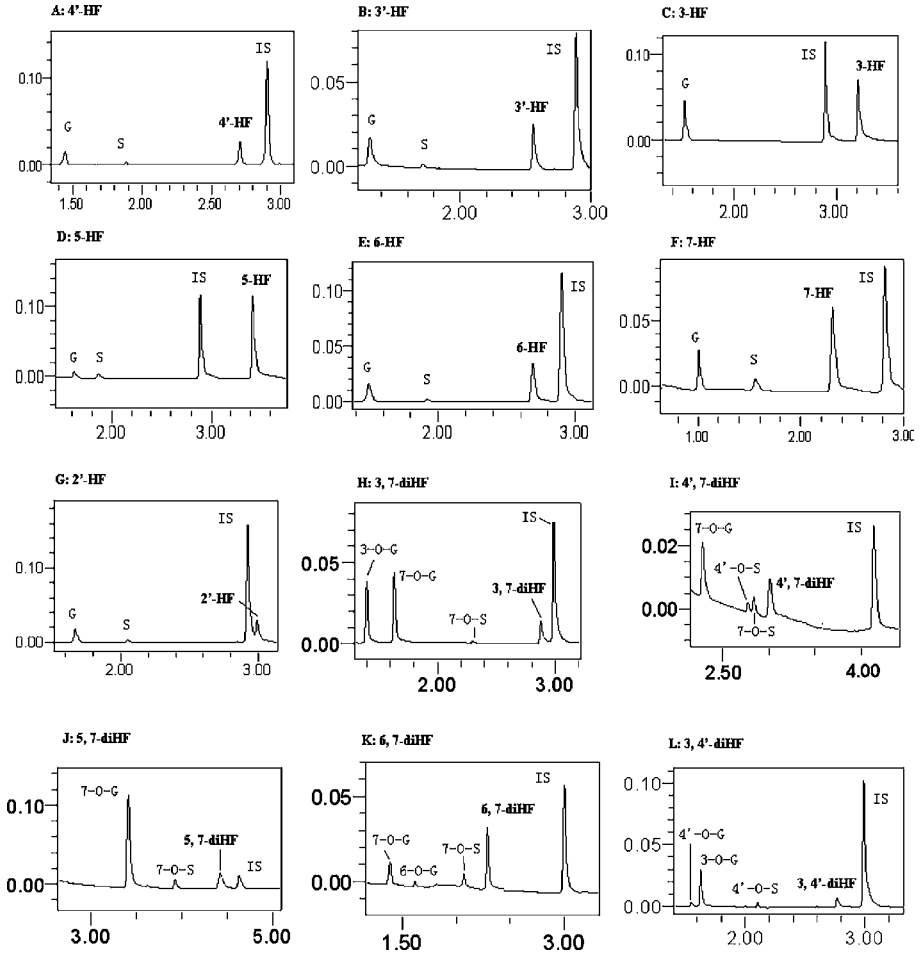


**Figure 2.** UPLC chromatograms of HFs and di-HFs, and their phase II metabolites in Sult- and Ugt-associated reactions. The UPLC chromatograms were used to quantify the flavones and their respective metabolites, which were obtained after incubation of flavones with the FVB mouse liver S9 fraction.

region. It was assigned as 4′-*O*-sulfate, whereas sulfate 2 showed nearly no change at either band I or band II and was assigned as 7-*O*-glucuronide (Table 1). For 3,4′-diHF, the unique sulfate produced at band I induced a $\lambda_{max}$ hypsochromic shift of 8.4 nm, so it was assigned as 4′-*O*-sulfate. For 3,7-diHF and 5,7-diHF, both had only one sulfate, and each sulfate produced nearly no change in the UV spectrua at either band I or II regions compared to aglycones, and so it was identified as a 7-*O*-sulfate. This identification of the sulfate position is constructive and necessary because it provided the basis for determining the molar extinction coefficient ratios as shown in Table 1. On the other hand, both 3,7-diHF and 3,4′-diHF had only one sulfate, and we assigned the sulfates of 3,7-diHF and 3,4′-diHF as 7-*O*-sulfate and 4′-*O*-sulfate, respectively, because 3-HF was not sulfated. The sulfation reaction results for monohydroxyflavones demonstrated that 7-OH was more active than 6-OH and that the amount of sulfate 1 of 6,7-diHF was greater than that of sulfate 2. As shown in our previous studies, the more slowly metabolized phenolic group in a diHF was always similar to that of the more slowly metabolized HF than to the more rapidly metabolized HF.[20] Therefore, we presumed that sulfate 1 of 6,7-diHF with a shorter retention time was 7-*O*-sulfate and the other rare sulfate was 6-*O*-sulfate.

**Sulfation Rates of Monohydroxyflavones.** The rates of sulfation of seven monohydroxyflavones by FVB mouse liver S9 fraction in Sult single reaction were determined at 5, 10, and 40 $\mu$M (Figure 3A). The results showed that the sulfation rate of 7-HF was always the fastest of all monohydroxyflavones at all substrate concentrations, whereas 3-HF was not sulfated by FVB mouse liver S9 fraction at all. At 10 $\mu$M substrate concentration, the rank order of the sulfation rates (in nmol per min per mg of protein) was 7-HF ($0.0998 \pm 0.0061$) > 4′-HF ($0.0793 \pm 0.0031$) > 2′-HF ($0.0719 \pm 0.0030$) > 5-HF ($0.0524 \pm 0.0019$) > 3′-HF ($0.0312 \pm 0.0095$) > 6-HF ($0.0211 \pm 0.0002$) > 3-HF ($0.00 \pm 0.00$). At 5 $\mu$M substrate, the rank order was the same, whereas the rank order in the middle changed slightly at 40 $\mu$M. The results clearly indicated that there were significant differences in the sulfation rates with respect to different positions of a hydroxyl group ($p < 0.05$, one-way ANOVA) (Figure 3A).

**Glucuronidation Rates of Monohydroxyflavones.** The rates of glucuronidation of seven monohydroxyflavones by the FVB mouse liver S9 fraction were again determined at 5, 10, and 40 $\mu$M substrate concentrations (Figure 3B). The glucuronidation rates of 7-HF were still the fastest of all monohydroxyflavones at all three substrate concentrations, and 3-HF was glucuronidated the second fastest at 5 and 10 $\mu$M. However, when the concentration was 40 $\mu$M, 4′-HF and 3′-HF jumped ahead of 3-HF, whereas 5-HF was always glucuronidated most slowly. This was consistent with our previous study showing that 5-HF was the least reactive among the seven monohydroxyflavones when the reaction was catalyzed by

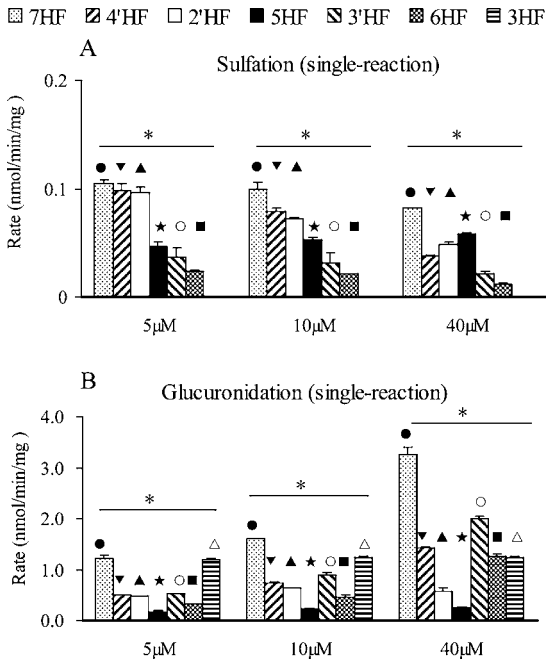


**Figure 3.** Glucuronidation and sulfation of seven monohydroxyflavones in a single Sult or Ugt reaction system. Three different concentrations (5, 10, and 40 $\mu$M) were used in the experiments done in triplicates ($n$ = 3). The rates of glucuronidation and sulfation were calculated in nmol/min/mg protein. The error bar is the SD of three determinations. The data were analyzed by one-way ANOVA with Tukey's posthoc test. The asterisk (*) indicates a statistically significant difference for the glucuronidation (A) and sulfation (B) rates among seven monohydroxyflavones ($p$ < 0.05, one-way ANOVA). Significant differences between three concentrations for each flavone are marked as follows: ●, $p$ < 0.05 for 7-HF; ▼, $p$ < 0.05 for 4′-HF; ▲, $p$ < 0.05 for 2′-HF; ★, $P$ < 0.05 for 5-HF; ○, $p$ < 0.05 for 3′-HF; ■, $p$ < 0.05 for 6-HF; △, $p$ < 0.05 for 3-HF.

human recombinant UGT1A1 or UGT1A10 and in human liver and intestinal microsomes at concentrations of 2.5, 10, and 35 $\mu$M.[20] Also, the rank orders of glucuronidation were the same at concentrations of 5 and 10 $\mu$M: 7-HF > 3-HF > 3′-HF > 4′-HF > 2′-HF > 6-HF > 5-HF. At the 40 $\mu$M substrate concentration, the rank order in the middle changed except for 7-HF and 5-HF. The results clearly indicated that the glucuronidation of the seven hydroxyflavones was structure-dependent ($p$ < 0.05, one-way ANOVA) (Figure 3B). However, the rank order for glucuronidation was very different from that of the sulfation (Figure 3), especially for 3-HF, which was rapidly glucuronidated but not sulfated at all.

**Sulfation Rates of Dihydroxyflavones.** The rates of sulfation of the five dihydroxyflavones were determined at 5, 10, and 40 $\mu$M (Figure 4A). All of the diHFs formed only one monosulfate, each of which was determined to be 7-*O*-sulfate, except for 4′,7-diHF, which formed two monosulfates. The formation rate of 7-*O*-sulfate from 4′,7-diHF was always higher than the formation rate at the 4′-OH position at all three concentrations, consistent with reaction rates of monohydroxyflavones from 4′-HF and 7-HF.

At a 5 $\mu$M substrate concentration, the fastest formation rate (in nmol per min per mg of protein) was for the formation of 7-*O*-sulfate of 6,7-diHF (0.2217 ± 0.0102), followed closely behind by 7-*O*-sulfate of 4′,7-diHF (0.1826 ± 0.0033), then 7-*O*-sulfate of 4′,7-diHF (0.1036 ± 0.0051), 7-*O*-sulfate of 3,7-diHF (0.0486 ± 0.0083), 7-*O*-sulfate of 5,7-diHF (0.0387 ± 0.0025), and 4′-*O*-sulfate of 3,4′-diHF (0.0210 ± 0.0022). At 10 $\mu$M substrate concentration, the rank order was the same as the order observed at 5 $\mu$M. The fastest sulfation rate occurred at 7-OH for 4′,7-diHF with 40 $\mu$M substrate. These results clearly indicated that the sulfation tendency at 7-OH position was much greater than those at the other positions ($p$ < 0.05, one-way ANOVA), and rank order of positional preferences in diHF followed that of HFs.

**Glucuronidation Rates of Dihydroxyflavones.** The glucuronidation rates of five diHFs were determined to rank order the activity of different hydroxyl groups. Among the diHFs, there are four flavones containing 7-OH, two containing 3-OH, two containing 4′-OH, one containing 5-OH, and one containing a 6-OH group. The glucuronidation rate at 7-OH was always faster than that at other −OH groups for all dihydroxyflavones at all three substrate concentrations. The glucuronidation rates at 3-OH were again the second fastest among the other −OH groups. The glucuronidation activity of −OH groups in diHFs were 7-OH > 3-OH > 4′ −OH ($p$ > 0.05) ≈ 6-OH > 5-OH (univariate analysis of variance, $p$ < 0.05). For example, at 10 $\mu$M, the glucuronidation rate rank order (nmol per min per mg of protein) was 1.1059 ± 0.1311 (7-O-G of 6,7-diHF) ≈ 1.0763 ± 0.0399 (7-O-G of 5,7-diHF) ≈ 1.0621 ± 0.0413 (7-O-G of 4′,7-diHF) ≈ 1.0201 ± 0.0288 (7-O-G of 3,7-diHF) > 0.7564 ± 0.0018 > (3-O-G of 3, 7-diHF) > 0.0642 ± 0.0010 (4′-O-G of 4′,7-diHF) ≈ 0.0644 ± 0.0078 (6-O-G of 6,7-diHF) > 0.00 ± 0.00 (5-O-G of 5, 7-diHF). This was also consistent with the previous results that the glucuronidation rate of 7-HF was always the highest, followed by that of 3-HF, 4′-HF, and 6-HF. Also, the activity rank orders of glucuronidation were the same at 5 $\mu$M and at 10 $\mu$M (Figure 4B).

Taken together, for diHFs, 7-OH was a preferred position for both sulfation and glucuronidation, whereas 3-OH was a preferred position for glucuronidation but a nonreactive position for sulfation. In contrast, 4′-OH was a preferred position for sulfation, but not for glucuronidation.

**Conjugation of HFs in the Sult−Ugt Coreaction System.** We investigated the sulfation and glucuronidation in a Sult−Ugt coreaction system to determine if effects of structural changes observed in a single-reaction system seen above will be affected in a Sult−Ugt coreaction system, which somewhat better mimics cellular conditions where both sulfation (in cytosol) and glucuronidation (at endothelium reticulum) occur albeit at different cellular compartments. The experiments were conducted in the Sult−Ugt coreaction system using 5, 10, and 40 $\mu$M substrate concentrations. We did not see significant changes with respect to the rank order of sulfation or glucuronidation in a coreaction system (Figure 5) when compared to the single-reaction systems (Figure 3), although there was some minor shift in one or two of the compounds at one concentration.

**Conjugation of diHFs in the Sult−Ugt Coreaction System.** The results indicated that effects of structural changes on sulfation and glucuronidation were essentially maintained in the coreaction system (Figure 6) when compared to the single-reaction systems (Figure 4). Specifically, 7-OH sulfation was once again preferred over 4′-OH sulfation (Figure 6), whereas other groups were not sulfated. The results again showed that the sulfation rate was unexpectedly enhanced ($p$ < 0.05) in the presence of an Ugt reaction system (Figure 6A) ($p$ < 0.05), whereas the formation rates of glucuronides were usually

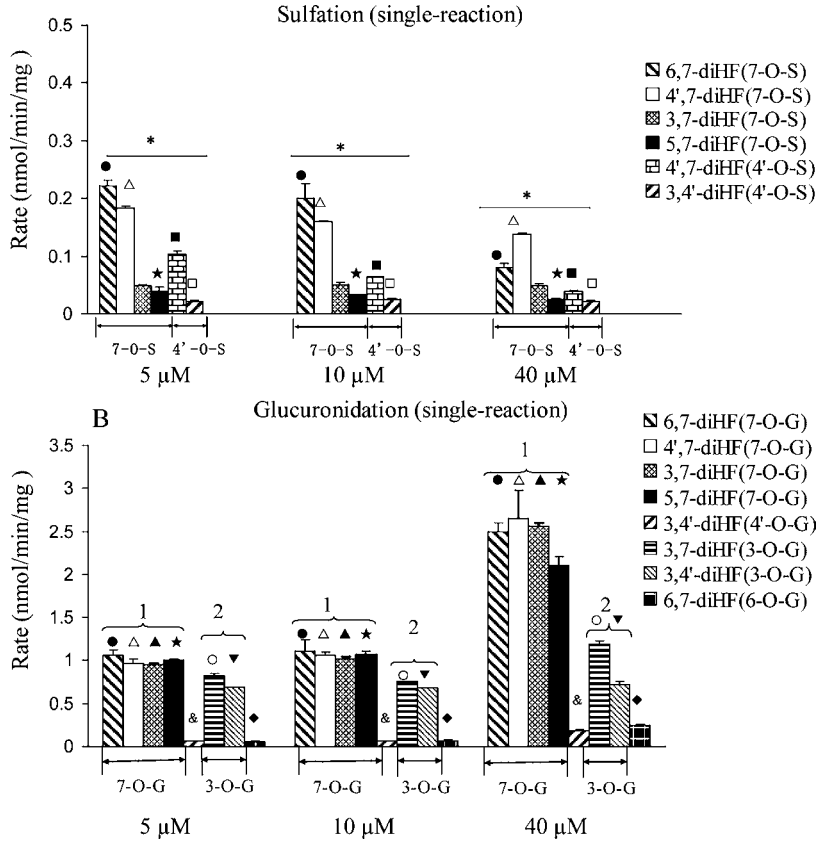


**Figure 4.** Glucuronidation and sulfation of five dihydroxyflavones by FVB mouse liver S9 in a single Sult (A) or Ugt (B) reaction system. Concentrations used, number of experiments, and calculation of the rates of glucuronidation and sulfation are the same as in the caption to Figure 3. The data were analyzed by one-way ANOVA with Tukey's posthoc test (A) or Univariate ANOVA with Tukey's test (B). The asterisk (∗) in panel A indicates a statistically significant difference in the sulfation rates among five diHFs ($p < 0.05$, one-way ANOVA). The number "1" in panel B shows that the 7-OH position is the group with the fastest glucuronidation rates, and the number "2" shows the −OH group with the second fastest glucuronidation rates at 3-OH. Significant differences between three concentrations for each flavone are marked as follows: ●, $p < 0.05$ for 6,7-diHF (7-O-S or 7-O-G); △, $p < 0.05$ for 4′,7-diHF (7-O-S or 7-O-G); ▲, $p < 0.05$ for 3,7-diHF (7-O-G); ★, $p < 0.05$ for 5,7-diHF (7-O-S or 7-O-G); ■, $p < 0.05$ for 4′,7-diHF (4′-O-S); □, $p < 0.05$ for 3,4′-diHF (4′-O-S) $p < 0.05$ for 3,4′-diHF (4′-O-G); ○, $p < 0.05$ for 3,7-diHF (3-O-G); ▼, $p < 0.05$ for 3,4′-diHF (3-O-G) 6-HF; ◆, $p < 0.05$ for 6,7-diHF (6-O-G).

unchanged or slightly decreased in the presence of a Sult reaction system.

**Interactions of Conjugation of HFs in the Sult−Ugt Coreaction System.** To determine whether there was an expected competitive interaction between glucuronidation and sulfation for these flavones, we made a direct comparison plot for sulfation or glucuronidation in Sult/Ugt single and Sult−Ugt coreaction systems at 10 $\mu$M (Figure 7). Unexpectedly, the sulfations of HFs and diHFs were enhanced significantly in the Ugt−Sult coreaction system ($p < 0.05$, Student's $t$ test). We found that the higher the substrate concentration, the higher the percentage increases in sulfation rates (with the exemption of 2′-HF) in a coreaction system. In contrast to the sulfation reaction, the formation rates of glucuronides from HFs and diHFs were usually unchanged or only mildly affected in the coreaction system. The phenomenon was almost the same at the other two concentrations (i.e., 5 and 40 $\mu$M).

**Disappearance Half-Life of 6,7-diHF in Single and Coreaction Systems of Glucuronidation and Sulfation.** To determine if an increase of the sulfation in the coreaction system would enhance the elimination, we compared the elimination half-life ($t_{1/2}$) values of 6,7-diHF in the single system versus the coreaction system. We chose 6,7-diHF because the formation rate of its 7-*O*-sulfate was enhanced significantly in the Sult−Ugt coreaction system. The $t_{1/2}$ values of 6,7-diHF were 244.31 ± 36.81, 26.78 ± 1.32, and 21.18 ± 2.03 min in Sult reaction, Ugt reaction, and Sult−Ugt coreaction systems, respectively. The results indicated that the increase of sulfation in the coreaction system lead to only a moderate albeit significant change on rate of elimination of the flavones by the mouse liver S9 fraction.

**Concentration-Dependent Conjugation in the Single and Coreaction Systems.** The data from the above plots were reorganized to display concentration dependence of various conjugating reactions. In both the Sult single-reaction and coreaction systems, sulfation rates for all selected monohydroxflavones (besides 5-HF) decreased as substrate concentration increased (substrate inhibition) (Figures 3A and 5A). This was also observed for diHFs in single and coreaction systems (Figures 4A and 6A), although in the coreaction system, the rank order of sulfation at the 7-hydroxyl position of 3,7- and 4′,7-diHF was also slightly altered as the result of concentration change. Therefore, the data appear to suggest that most of the selected flavones cause substrate inhibition at high concentrations. In contrast, the glucuronidation rate for 11 hydroxyflavones (besides 2′-HF) increased with increasing substrate concentration, regardless if the Sult reaction system was present or not (Figures 3B, 4B, 5B, and 6B).


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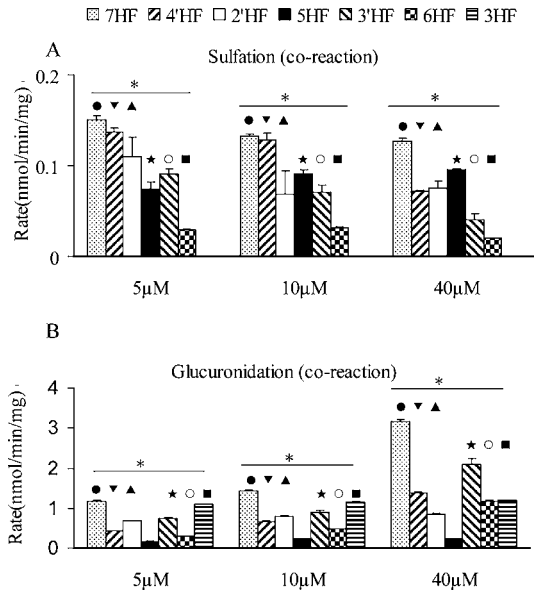


**Figure 5.** Glucuronidation and sulfation of seven monohydroxyflavones in Sult−Ugt coreaction system. Concentrations used, number of experiments, calculation of the rates of glucuronidation and sulfation, and data analysis are the same as in the caption to Figure 3. The asterisk (∗) indicates a statistically significant difference for the glucuronidation and sulfation rates among seven monohydroxyflavones ($p < 0.05$, one-way ANOVA). Significant differences between three concentrations for each flavone are the same as in the caption to Figure 3.

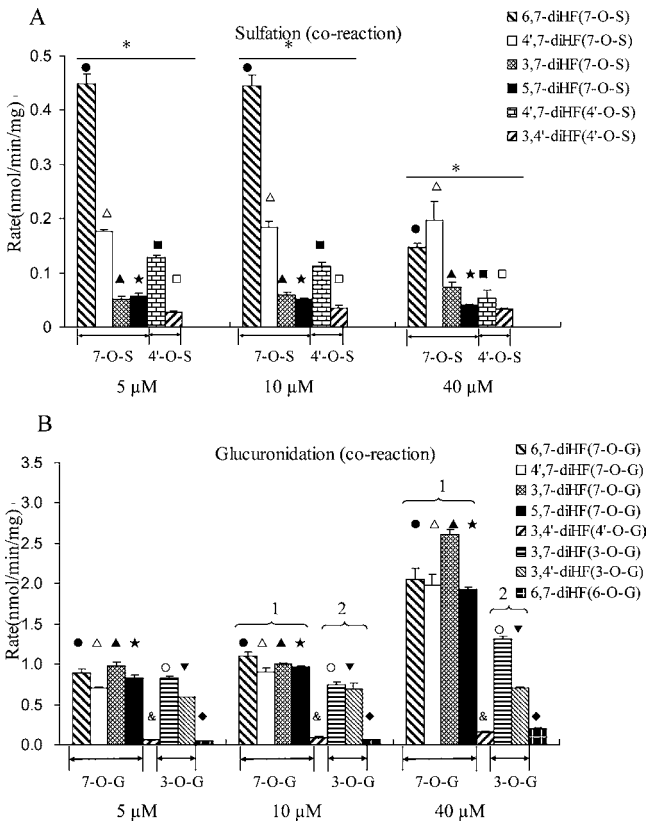


**Figure 6.** Glucuronidation and sulfation of five dihydroxyflavones by FVB mouse liver S9 in a Sult−Ugt coreaction system. Concentrations used, number of experiments, and calculation of the rates of glucuronidation and sulfation are the same as in the caption to Figure 3. The data were analyzed by one-way ANOVA with Tukey posthoc test (A) or Univariate ANOVA with Tukey's test (B). The asterisk (∗) in panel A indicates a statistically significant difference for the sulfation rates among five dihydroxyflavones ($p < 0.05$). The number "1" and the number "2" are the same as in the caption to Figure 4. Significant differences between three concentrations for each flavone are the same as in the caption to Figure 4.

## DISCUSSION

This is the first systematic study determining the positional preference of Sult-catalyzed sulfation of flavonoids. We also compared this preference with that of Ugt-catalyzed glucuronidation. The results reveal that the structure−activity relationship (or SAR) with respect to sulfation is quite different from that for the glucuronidation when using the mouse liver S9 fraction. The rank order of reaction was shown to be very different between sulfation and glucuronidation, although sulfation and glucuronidation at the 7-OH position was favored among the 12 flavonoids studied here. Moreover, the Sult−Ugt coreaction system unexpectedly enhanced the sulfation of flavonoids, suggesting that the two metabolic pathways are unlikely to compete with each other for substrates as long as the substrates are not exhausted.

The distinctive SAR displayed by Sult suggests that the sulfation pathway will not compensate for the glucuronidation pathway or vice versa in limiting the bioavailability of flavonoids. In other words, there is no relationship between a flavonoid's susceptibility to sulfation versus glucuronidation, and independent SARs are needed if the goal is to predict sulfation versus glucuronidation. This new finding suggests that we will need to consider a flavonoid's potential for sulfation and glucuronidation separately if we are to improve its bioavailability.

The rank order of the sulfation between seven individual HFs and five individual diHFs suggests that a more slowly sulfated phenolic group in a dihydroxylflavone was always predictable using results derived from the monohydroxyflavones (positional preferences for HF and diHF were similar), which was also shown previously for glucuronidation.[20] For example, the sulfation reaction results for all HFs had shown that 7-OH was more active than 4′-OH and, therefore, the formation of 7-*O*-sulfate from 4′,7-diHF was greater than that of the 4′-*O*-sulfate (Figures 3A and 4A). As expected, sulfation at the 5-OH position of diHFs was below the detection limit (Figures 3 and 4). On the other hand, the glucuronidation relationship shown previously for a different group of compound holds true for the current group of 12 flavonoids. For example, the hydroxyl group activity of diHFs for glucuronidation had the same order as HFs (7-OH > 3-OH > 4′-OH ≥ 6-OH). As expected, more glucuronide was produced from 7-HF than from 5-HF, indicating that 7-OH was more reactive. For 5,7-diHF, the unique glucuronide was determined to be 7-*O*-glucuronide (Figures 3B and 4B), and 5-*O*-glucuronide was not found. Taken together, 5-OH is not usually glucuronidated as long as another −OH position was available.

The fact that Sult could not metabolize 3-HF is consistent with the small size of reported active binding cavity of the sulfotransferases revealed in crystallography studies. For example, it has been reported that human SULT1A3 selectively sulfates only the D-Dopa (not L-Dopa) and prefers the 3-OH position over the 4-OH position of D-Dopa.[32] Our own study showed that human SULT1A3 only metabolizes flavonoids with a 7-OH group (not shown). The small size of the active pocket means that a sterically hindered 3-OH group is not

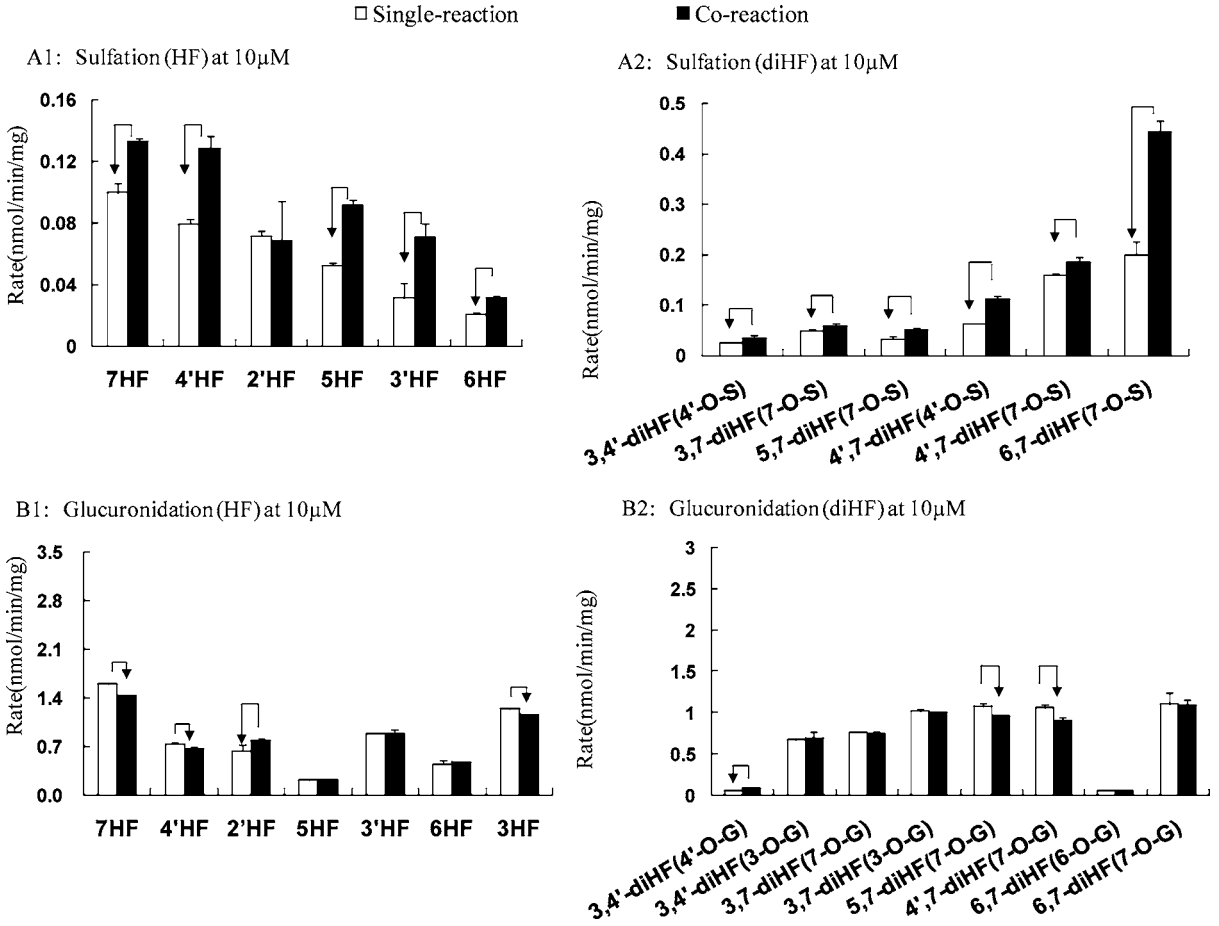


**Figure 7.** Interaction between sulfation and glucuronidation for hydroxyflavones in a Sult−Ugt coreaction system. Concentration of 10 $\mu$M is shown. Sulfations in the Sult single reaction system and the Sult−Ugt coreaction system were compared (A1, A2). Glucuronidations in the single Ugt reaction system and the Sult−Ugt coreaction system were also compared (B1, B2). The data were analyzed by Student's $t$ test. The arrow indicates a statistically significant difference ($p < 0.05$).

available for sulfation. In contrast, human UGT1A9 has broad substrate specificity against flavonoids, and almost all flavonoids chosen are its substrates; no hydroxyl position is off-limits for UGTs, although some positions (e.g., 7-OH and 3-OH) are clearly preferred over other positions (e.g., 5-OH).[33] An analysis of the literature suggested that the 3-OH group was the most reactive for glucuronidation, even in the more complex flavones with two phenolic groups. In addition, the 7-OH group was also highly reactive.[7,20,34] The latter is consistent with published data showing that 7-HF was sulfated, whereas 3-HF was not by SULTs in human liver and duodenum S9 fractions.[35] This analysis provided the structural basis for why Sults and Ugts will have different SARs.

We found strong evidence that mouse Sults and Ugts would not compete for the same substrate as would have been expected, suggesting that the binding of the substrate to the enzyme is not the rate-limiting step in the conjugation process. We were surprised that sulfation rates were enhanced in the Sult−Ugt coreaction system because we thought the more rapid metabolism via the glucuronidation pathway would have taken the substrate away from sulfation, thereby decreasing its sulfation. The fact that sulfation profiles of most flavonoids displayed substrate inhibition (Figure 7) provided a plausible explanation for this surprising finding, but more studies are needed to identify the actual causes.

This surprising discovery suggests that Ugt and Sult conjugation pathways could display kinetic interplay capable of changing the kinetics of conjugation reaction and profiles. For example, the elimination half-life ($t_{1/2}$) values of 6,7-diHF in the coreaction were moderately shorter than in the single-reaction system. It is not entirely clear what the basis of this interplay is and whether this will happen in intact cells where flavone aglycones are to encounter Sult (cytosol) first and then Ugt (endoplasmic reticulum). If there are enough Sults to bind and metabolize aglycones, Ugt will never encounter the substrate. On the other hand, if there is an excess amount of flavones, they will interfere with Sult functions by substrate inhibition. It appears that Ugts are capable of facilitating the sulfation reaction by reacting with excess flavone aglycone. At any rate, the interplay is consistent with a previous observation that excretion rates of genistein, daidzein, and glycitein sulfates in intact cells where both metabolic pathways are present were all significantly higher than predicted on the basis of their formation rates in the Caco-2 cell lysates using a single-reaction system.[15] Additionally, higher percentages of apigenin conjugates are present as sulfates at lower loading concentration than at higher loading concentration (of apigenin) in the Caco-2 cells.[14]

The S9 fraction was employed for the present study because the focus was on the metabolite formation processes. The whole cells were not used for this purpose because phase II

metabolites of flavonoids (i.e., glucuronides and sulfates) are too hydrophilic to passively diffuse across a cell membrane. In other words, excretion rates from whole cell or organ model systems would not have represented the formation rates of hydrophilic phase II metabolites. Therefore, the imperfect S9 fraction model is the best model available for the determination of the formation rates. This model is not perfect because cellular production of sulfates is likely to be more favored in a whole cell system than the S9 fraction used in the present study, perhaps because in mammalian cells flavonoids encounter Sults (in cytosolic domain) first and then UDP-glucuronosyltransferase or Ugt (at the endoplasmic reticulum). In this liver S9 fraction model, a substrate will have equal access to Sults and Ugts, and hence glucuronidation could have been overestimated. Therefore, it was highly unexpected that the presence of a glucuronidation reaction system in the mixed reaction system actually increased the sulfonation rates for some flavonoids.

In conclusion, Ugts and Sults have different and independent SARs, and there appears to be limited kinetic interplay between the sulfation and glucuronidation pathways in the mouse liver S9 fraction. Both sulfation and glucuronidation pathways showed stronger preference for the 7-OH group. Besides the 7-OH group, glucuronidation also showed a similar preference for 3-OH, whereas sulfation also showed a preference for the 4′-OH position. Unexpected enhancement of the sulfation reaction in the coreaction system is likely the result of lowering of the substrate concentrations, because most sulfation displayed substrate inhibition kinetic profiles. The latter deserves more serious investigation to confirm our preliminary finding here because the S9 fraction used in the present study does not have cellular structures, which separate Sults and Ugts into different compartments inside cells.

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### Notes

The authors declare no competing financial interest.


## ABBREVIATIONS USED

3-HF, 3-hydroxyflavone; 5-HF, 5-hydroxyflavone; 6-HF, 6-hydroxyflavone; 7-HF, 7-hydroxyflavone; 2′-HF, 2′-hydroxyflavone; 3′-HF, 3′-hydroxyflavone; 4′-HF, 4′-hydroxyflavone; 3,7-diHF, 3,7-dihydroxyflavone; 3,4′-diHF, 3,4′-dihydroxyflavone; 5,7-diHF, 5,7-dihydroxyflavone; 6,7-diHF, 6,7-dihydroxyflavone; 4′,7-diHF, 4′,7-dihydroxyflavone; KPI, potassium phosphate buffer (pH7.4); UDPGA, uridine diphosphoglucuronic acid; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; UPLC, ultraperformance liquid chromatography; Ugt, UDP-glucuronosyltransferase; Sult, sulfotransferase.

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