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Intestinal first-pass glucuronidation activities of selected dihydroxyflavones

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ABSTRACT

Flavonoids have low bioavailabilities due to extensive intestinal first-pass metabolisms, especially glucuronidation. The present study aimed to evaluate the intestinal glucuronidation of dihydroxyflavones and provide more information on their structure–activity relationships. Seven dihydroxyflavones, namely 3,7-, 5,7-, 6,7-, 7,8-, 2′,7-, 3′,7-, and 4′,7-dihydroxyflaovne and a monohydroxyflavone, 7-hydroxyflavone, were investigated by incubating each hydroxyflavone at various concentrations with either human jejunum microsome or rat intestinal microsome. Two mono-glucuronides were identified for each dihydroxyflavone. For human jejunum microsome, most of the studied dihydroxyflavones demonstrated greater glucuronidation activities than that of 7-hydroxyflavone except for 3,7-dihydroxyflavone and 4′,7-dihydroxyflavone. 3′,7-dihydroxyflavone had the greatest intrinsic clearance which was at least seven times greater than that of all other dihydroxyflavones. In addition, species difference in glucuronidation activity was observed with human jejunum microsome higher than rat intestinal microsome for all hydroxyflavones except for 3,7-dihydroxyflavone. The results further demonstrated that the hydroxyl group positions do affect the intestinal glucuronidation activity of hydroxyflavones. Increasing the number of hydroxyl groups on A- or B-ring (except for 4′-OH) would enhance the glucuronidation activity of flavones, whereas adding a 3-OH on C-ring might not. Furthermore, existence of hydroxyl group at 3′ position may enhance the glucuronidation activity of flavonoids.

1. Introduction

Flavonoids represent a large class of polyphenolic components that occur in most plant foods of plant origin. In general, flavonoids occur in plants either as aglycones or glycosides. However, the bioavailabilities of both forms have not been found as high as expected from their favorable lipophilic polyphenolic structures. The oral bioavailabilities of flavonoids were low and extensive first-pass metabolism, which mainly included glucuronidation, sulfation, and methylation, was proposed to be the main reason (Manach and Donovan, 2004). After oral intake of flavonoids, the small intestine is considered to be the first organ responsible for the first-pass metabolism. The considerable length of the intestine and characteristic villi and microvilli structure of intestinal epithelium provide an expansive surface for absorption and subsequent first-pass metabolism of the xenobiotics (Kaminsky and Zhang, 2003) including flavonoids. Extensive intestinal first-pass phase II metabolism and subsequent excretion of its conjugated metabolites were proposed to be the main factors contributing to the low oral bioavailabilities of flavonoids. Intestinal disposition may be more important than hepatic disposition in the first-pass metabolism of certain flavonoids such as apigenin, a trihydroxyflavone (triHF) (Chen et al., 2003). In addition, glucuronidation was reported to be highly favorable metabolic pathway for various flavonoids including galangin, kaempferol, baicalein, monohydroxyl flavones, etc. (Otake et al., 2002; Zhang et al., 2007a), which arouse our interest to further investigate the glucuronidation of flavonoids.

Flavone is one of the main sub-groups of flavonoids, with a C2–C3 double bond and a carbonyl group at C4. Previous *in vivo* studies on multihydroxyflavones (multiHFs) such as quercetin (Janisch et al., 2004) and kaempferol (DuPont et al., 2004) had demonstrated that their glucuronides were the dominant forms in human systemic circulation after their oral administration. Moreover, glucuronidation on hydroxyl group (–OH) at different substituted positions can affect the biological activity of their corresponding hydroxyflavone glucuronides (HFGs) as observed in quercetin (Janisch et al., 2004). Study on (−)-epigallocatechin gallate (EGCG) and (−)-epigallocatechin (EGC) also suggested that their glucuronides could retain radical scavenging ability and inhibitory effect on the release of arachidonic acid from HT-29 human colon cancer cells of the aglycone (Lu et al., 2003). Therefore

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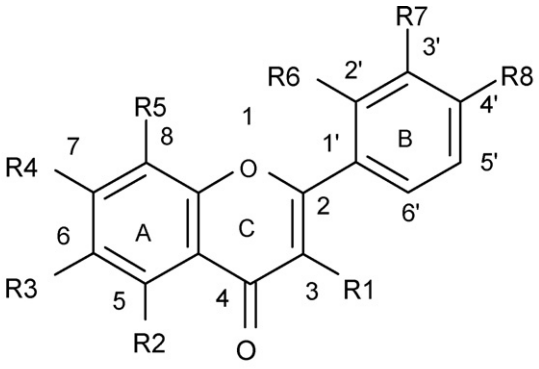
it is worth to investigate the intestinal glucuronidation of hydroxyflavones (HFs).

Chrysin (5,7-dihydroxyflavone), one of the most extensively investigated dihydroxyflavones (diHFs), was found to be extensively metabolized to two conjugated metabolites including a glucuronide and a sulfate conjugate metabolite in Caco-2 cells model (Walle et al., 1999). Apigenin and genistein, also demonstrated extensive intestinal first-pass metabolism in the in situ multi-site perfusion model (Chen et al., 2003). Findings on flavones including chrysin, apigenin, luteolin and baicalein from our own group also indicated that flavones could be metabolized by the intestinal uridine 5′-diphospho-glucuronosyltransferase (UGT) enzyme during their oral absorptions (Ng et al., 2005). Our further study on the in situ intestinal perfusion of baicalein found that over 90% of baicalein was rapidly absorbed in the form of its glucuronide into the mesenteric blood (Zhang et al., 2005) and the extensive glucuronidation of baicalein was mediated by various human recombinant UGT isozymes (Zhang et al., 2007b). In addition, induction of UGTs by flavonoids such as chrysin, apigenin, luteolin, and diosmetin may further enhance first-pass metabolism of flavonoids (Walle et al., 2000; Galijatovic et al., 2001). In summary, findings from others and us consistently indicated the important role of intestinal first-pass metabolism, including glucuronidation, on flavonoid-containing products after their oral intake.

To explore the relationships between intestinal glucuronidation activity and the structures of flavonoids, we have established some preliminary investigations on the position preference on glucuronidation of monohydroxyflavones (monoHFs) using human jejunum S9 fraction (Zhang et al., 2006). The results demonstrated that the glucuronidation activities of 6- and 3′-HF were much greater than that of 3-, 4′-, 7-, and 2′-HF with 5-HF to be the lowest. Such finding implies that the nucleophilicity and stereoconformation of –OH groups, where glucuronidation takes place, are crucial for the intestinal glucuronidation of flavonoids. In order to further investigate the relationships between flavonoid structure and their glucuronidation activities and also to minimize the complexity of –OH groups in multiHFs, investigation on the flavones with two –OH groups were proposed for the current study. The purpose of the present study is to investigate further the structure–activity relationship (SAR) between the position of the –OH group and glucuronidation activity in some selected diHFs. In addition, with data from both monoHFs and diHFs we can get a clearer picture on the glucuronidation characteristics of HFs. Therefore, one monoHF (7-hyroxyflavone, 7-HF) and seven diHFs namely 3,7-diHF, 5,7-diHF (chrysin), 6,7-diHF, 7,8-diHF, 2′,7-diHF, 3′,7-diHF and 4′,7-diHF, which covered all possible diHFs, were selected for the current study (Fig. 1). These commercially available HFs were employed as model compounds because their chemical structures all contained 7-OH plus an additional –OH on either A-ring, B-ring or C-ring. It is expected that data obtained from HFs with similar structures and the monoHF (7-HF) as the control could demonstrate the SAR on the intestinal first-pass metabolism of flavonoids.



7-hydroxyflavone (7-HF): R4=OH; R1~R8=H,
3,7-dihydroxyflavone (3,7-diHF):R1, R4=OH; R2, R3, R5~R8=H
5,7-dihydroxyflavone (5,7-diHF, Chrysin): R2, R4=OH; R1,R3, R5~R8=H
6,7-dihydroxyflavone (6,7-diHF): R3, R4=OH; R1, R2, R5~R8=H
7,8-dihydroxyflavone (7,8-diHF): R4, R5=OH; R1~R3, R6~R8=H
2',7-dihydroxyflavone (2',7-diHF): R4, R6=OH; R1~R3, R5,R7, R8=H
3',7-dihydroxyflavone (3',7-diHF): R4, R7=OH; R1~R3, R5, R6, R8=H
4',7-dihydroxyflavone (3',7-diHF): R4, R8=OH; R1~R3, R5~R7=H

Fig. 1. Chemical structures of 7-hydroxyflavone and seven dihydroxyflavones.

2. Materials and methods

2.1. Materials

Chrysin was purchased from Aldrich Chemical Co. (WI, USA). 7-HF, 3,7-diHF, 6,7-diHF, 7,8-diHF, 2′,7-diHF, 3′,7-diHF and 4′,7-diHF were purchased from Indofine Chemical Co. (NJ, USA). Luteolin (5,7,3′,4′-tetrahydroxyflavone) used as an internal standard (IS) for the HPLC analysis was obtained from Lancaster Synthesis (Lancashire, UK). Uridine 5′-diphosphoglucuronic acid (UDPGA) was purchased from Sigma–Aldrich Co. (St. Louis, USA). The rat intestinal microsome was prepared as described previously (Zhang et al., 2007b) and the human jejunum microsome was purchased from Tissue Transformation Technologies (NJ, USA). Acetonitrile (ACN) (HPLC grade) was obtained from VWR (Leuven, Belgium) and methanol (HPLC grade) from Fisher Scientific (Leicestershire, UK). Sodium dihydrogen phosphate used for phosphate buffer was purchased from Riedel-de Haen AG (Seelze, Germany).

2.2. Identification of metabolites by HPLC and LC/MS/MS

Due to lack of availability of standards for all hydroxyflavone glucuronides, the formed glucuronides in human jejunum microsomal incubates were first identified based on the HPLC retention times followed by further confirmation of their mass spectra by LC/MS/MS.

In order to obtain well-resolved peaks of the parent substrate, metabolites and IS, a Waters 2695 Separations Module equipped with a Waters 996 Photodiode Array Detector was employed. The HPLC separations were performed with a BDS C18 reversed phase column (4.6 mm ID × 250 mm, 5 μm, Thermo Hypersil) with a protective guard column (C18, 5 μm 300 Å, Delta-Pak™, Waters). The mobile phase was composed of methanol (A), acetonitrile (B), and 20 mM sodium phosphate (pH 4.6) (C). The gradient for the analysis of 4′,7-diHF began with 1% A, 2% B, and 97% C, and changed linearly to 8% A, 35% B, and 57% C from 0 to 27.5 min; then 0% A, 70% B, and 30% C from 27.5 to 33.5 min; and then back to the initial composition in 4 min followed by 5 min equilibrium. For the analysis of other HFs, the gradient began with 5% A, 15% B and 80% C, and changed linearly to 8% A, 35% B and 57% C from 0 to 10 min; then 0% A, 70% B, and 30% C from 10 to 16 min; and then back to the initial composition in 4 min followed by 5 min equilibrium. The flow rate was set as 1 ml/min. For the HPLC analyses of 7-HF, 3,7-diHF, 6,7-diHF, 2′,7-diHF, 4′,7-diHF and 5,7-diHF (chrysin), luteolin was used as IS. For the analysis of 3′,7-diHF and 7,8-diHF, 7-HF was used as IS instead. The HPLC chromatograms were obtained at the λ_{max} of the corresponding parent HF. UV spectra (wavelength ranged from 200 to 400 nm) of each HF and their glucuronides were also recorded.

After incubating the selected HF substrates with human jejunum microsome, the final incubation solutions were injected into LC/MS/MS for the identification of the metabolites. A Quadrupole QTrap LC/MS/MS spectrometer (Applied Biosystems, Toronto, Canada) coupled with PerkinElmer PE-200 series micro-pumps

and auto-sampler (PerkinElmer, Norwalk, CT, USA) was employed for the analysis. The separations were performed with a BDS C18 reversed phase column (4.6 mm ID × 250 mm, 5 μm, Thermo Hypersil) with a protective guard column (C18, 5 μm 300 Å, Delta-Pak™, Waters). The gradient consisting of acetonitrile (A) and 0.05% formic acid (B) began with 20% A and 80% B; changed linearly to 45% A and 55% B from 0 to 5 min; remained the composition from 5 to 10 min; and changed linearly to 70% A and 30% B from 10 to 12 min; then back to the initial composition in 2 min followed by 8 min equilibrium. A flow rate of 1 ml/min was used for the separation. Only 20% of eluent was introduced into mass spectrometer and the other 80% was splitted off. For MS setting, mass spectrometer was operated in electrospray ionization in negative mode with full scan and product ion scan at 429.0 *m*/*z* being utilized. The other mass spectrometer working parameters were as follows: curtain gas 40 psi, ion transfer voltage −4500 V, nebulizing gas and auxiliary gas 50 psi, interface heater turned on, collision gas 2.0 psi, declustering potential −51 V, entrance potential −6 V, collision energy −16 V, collision cell exit potential 0 V, channel electron multiplier 2200, turbospray ion source temperature 400 °C. For the identification of the metabolites of 7-HF, positive ion mode full scan and product ion scan at 415.0 *m*/*z* was conducted. The other mass spectrometer working parameters were as follows: curtain gas 40 psi, ion transfer voltage +4500 V, nebulizing gas and auxiliary gas 50 psi, interface heater turned on, collision gas medium, declustering potential 151 V, entrance potential 4.5 V, collision energy 25 V, collision cell exit potential 6 V, channel electron multiplier 2100, turbospray ion source temperature 400 °C.

2.3. Glucuronidation of selected hydroxyflavones by human jejunum microsome and rat intestinal microsome

Each diHF at final concentrations of 2.0, 3.9, 5.9, 11.8, 23.6 and 59.0 μM or 7-HF at 2.1, 4.2, 6.3, 12.6, 25.2 and 63.0 μM was pre-incubated with human jejunum microsome in 50 mM Tris–HCl (pH 7.5) buffer (containing 8 mM of $MgCl_2$ and 25 μg/ml of alamethicin) at 37 °C for 15 min.

In order to quantify the total glucuronides formed, each of the above mixtures was divided into two equal portions. One portion (served as the reaction portion) was incubated after the addition of 2 mM of cofactor UDPGA to initiate the glucuronidation reaction. The other portion (served as the control portion) was added with an equivalent volume of water instead of UDPGA. Both portions were then incubated at 37 °C for 15 min. The reaction system (100 μl) was terminated by adding 40 μl of ACN/acetic acid (9:1, v/v, at −20 °C) containing 20 μg/ml of internal standard and 10 μl of 20% (v/v) vitamin C as antioxidant to both portions. The reaction time and protein concentration were all optimized to ensure linearity of metabolite formation. The resultant mixtures of these two portions were then centrifuged at 16,000 × *g* for 5 min and the supernatants were directly subjected to HPLC for analysis.

In order to compare the species difference in the glucuronidation of the studied diHFs, we also conducted the glucuronidation metabolisms of the selected HFs by the rat intestinal microsome using the same experimental conditions as that for the human studies with the HF concentration set at 6 μg/ml (equivalent to 23.6 μM diHFs, or 25.2 μM 7-HF).

2.4. Data analysis

2.4.1. Quantification of glucuronides formation for each hydroxyflavone

Data acquisition and integration of the HPLC/UV chromatograms were performed using the Millennium^{32} chromatography workstation. Peak areas were measured at the wavelength of maximal absorption (λ_{max}) of each parent HF substrates.

Calibration curve of each HF were prepared by plotting the peak area ratios (parent substrate versus the IS) against the substrate concentrations of each HF. Due to the lack of authentic standards for individual hydroxyflavone glucuronides, the total amount of metabolites formed at each substrate concentration was calculated from the corresponding calibration using the difference in peak area ratios (HF substrate/IS) between the control portion (the one without UDPGA) and the reaction portion (the one with UDPGA), with the consideration of the decrease in the parent substrate peak area ratio would correspond to the total amount of substrate converted into the metabolites.

2.4.2. Metabolism kinetic analyses for the glucuronidation of selected hydroxyflavones

The metabolic kinetic analyses of the glucuronidation activities of the selected HFs were determined based on total amount of glucuronides formed in individual HFs. To obtain the kinetic parameters such as V_{max} (maximal velocity, in nmol/(min mg)$^{-1}$) and K_m (substrate concentration at which half maximal velocity is achieved, in μM), the formation rates of the hydroxyflavone glucuronides at various concentrations were fitted to the Michaelis–Menten equation: $v = V_{max} \times [S]/(K_m + [S])$, where v is total glucuronides formation rate (nmol/(min mg)$^{-1}$) and [*S*] is the HF substrate concentration used for incubation (μM). The parameter of intrinsic clearance (Cl_{int}), V_{max}/K_m, (in μl/(min mg)$^{-1}$) was calculated to characterize the efficiency of glucuronidation of each HF. Reported values were presented as mean ± S.E. ($n = 3$). The kinetic parameters of glucuronidation were obtained by fitting the data to the Michaelis–Menten equation using the software of Prism (GraphPad Software, Inc.).

2.5. Statistical analysis

Statistically significant difference between two groups was evaluated by Student's *t*-test. A $P < 0.05$ was considered significant for all tests.

3. Results

3.1. Identification of glucuronides of selected hydroxyflavones

The characteristics of HPLC chromatograms, UV spectra and mass spectra for glucuronides formed from selected diHFs and 7-HF were summarized in Table 1. For all diHFs studied, no diglucuronide was detected from the full scan of LC/MS. LC/MS/MS analysis of seven diHFs and their metabolites using negative mode indicated that all metabolites were mono-glucuronic acid conjugates. The identification of these conjugates was based on a pseudo-molecular ion ($[M-H]^-$) at *m*/*z* 429, which corresponded to a glucuronic acid moiety (176 Da) added to diHF molecule (253 Da). Moreover, further MS/MS analyses of the pseudo-molecular ion produced both characteristic fragment ions of diHF ($[M-H-G]^-$) at *m*/*z* 253 and glucuronic acid moiety ($[G-H]^-$) at *m*/*z* 175 of each glucuronide conjugate.

For 7-HF, which is a monoHF, LC/MS/MS analysis of the metabolite using positive mode indicated that its metabolite was also a mono-glucuronic acid conjugate. Similarly, it was based on an protonated molecular ion ($[M+H]^+$ at *m*/*z* 415, which corresponded to the addition of a glucuronic acid moiety (176 Da) to the monoHF (239 Da). MS/MS analysis of the protonated molecular ion demonstrated an intense peak at *m*/*z* 239, which represented the fragment ion of monoHF ($[M+H-G]^+$). Consistent with our previous finding

Table 1
HPLC, UV and LC/MS/MS characteristics of glucuronides of selected dihydroxyflavones and 7-HF

Selected HFs	Glucuronides found	Chromatographic R_t (min)	UV λ_{max} (nm)		Characteristic ions in LC/MS/MS		
					$[M-H]^-$	$[M-G-H]^-$	$[G-H]^-$
3,7-diHF	3,7-diHF-M1	6.94	251.0	313.8	429	253	175
	3,7-diHF-M2	8.19	251.0	316.1	429	253	175
5,7-diHF	Chrysin-M1	9.29	267.5	305.4	429	253	175
(chrysin)	Chrysin-M2	10.02	260.4	317.3	NA	NA	NA
6,7-diHF	6,7-diHF-M1	6.44	265.2	312.6	429	253	175
	6,7-diHF-M2	9.11	259.2	313.8	429	253	175
7,8-diHF	7,8-diHF-M1	6.19	256.9	311.2	429	253	175
	7,8-diHF-M2	7.54	264.0	–	429	253	175
2′,7-diHF	2′,7-diHF-M1	5.50	252.2	304.2	429	253	175
	2′,7-diHF-M2	6.68	–	312.6	429	253	175
3′,7-diHF	3′,7-diHF-M1	5.55	254.5	311.4	429	253	175
	3′,7-diHF-M2	8.14	225.1	309.0	NA	NA	NA
4′,7-diHF	4′,7-diHF-M1	13.49	253.3	329.2	429	253	175
	4′,7-diHF-M2	14.10	253.3	324.5	429	253	175
Selected HFs	Glucuronides found	Chromatographic R_t (min)	UV λ_{max} (nm)		Characteristic ions in LC/MS/MS		
					$[M+H]^+$	$[M+H-G]^+$	$[G-H]^-$
7-HF	7-HF-M	6.98	252.2	307.8	415	239	

NA: not available due to low intensity of the peak; ND: not detectable; M1: metabolite 1; M2: metabolite 2.

on 7-HF (Zhang et al., 2006), no di-glucuronide of 7-HF was detected from the full scan of mass spectra.

3.2. Metabolic kinetics analyses for the glucuronidation of selected hydroxyflavones

As shown in Table 2, eight studied HFs showed different kinetic profiles. The rank order of the Cl_{int} (V_{max}/K_m) of the eight HFs was as follows: 3′,7-diHF ≫ 5,7-diHF (chrysin), 2′,7-diHF, 6,7-diHF, 7,8-diHF > 7-HF, 4′,7-diHF, and 3,7-diHF. The results suggested that HFs with higher Cl_{int}, i.e., 3′,7-diHF and chrysin are expected to be subject to more significant intestinal first-pass metabolism than those with lower Cl_{int}, i.e., 7-HF, 4′,7-diHF, and 3,7-diHF.

Fig. 2 shows the formation rate of hydroxyflavone glucuronides (HFGs) at various substrate concentrations for all selected diHF and 7-HF. The saturable glucuronidation characteristics were observed for 3′,7-diHF, chrysin, 2′,7-diHF, 7-HF, 4′,7-diHF, and 3,7-diHF. At low substrate concentrations, the total glucuronides formation rates increased proportionally with the substrate concentrations. As the substrate concentrations increased, the increment in formation rates became smaller and a plateau was approached, possibly due to the saturation of active site(s) of UGT. The metabolisms of these compounds with human jejunum microsome could be described by a simple Michaelis–Menten equation. However, no such saturation of HFGs formation was observed for 6,7-diHF and 7,8-diHF in the studied substrate concentration range (2–59 μM), i.e., the total HFGs formation rate kept increasing linearly over the whole selected concentration range, with no plateau observed. In fact, we conducted an additional testing on their glucuronide formations at the substrate concentration of 118 μM (data not shown), i.e., a double of the maximal concentration (59 μM) tested in the kinetic analyses, but still no significant sign of saturation was observed for both diHFs. Hence, their K_m and V_{max} could not be obtained by directly fitting the kinetic data into the Michaelis–Menten equation. Assuming the selected substrate concentrations were still far lower than the concentration that would cause saturation of UGT enzymes ($[S] \ll K_m$), the term "$[S]$" in the denominator of the Michaelis–Menten equation $v = V_{max} \times [S]/(K_m + [S])$ could be simplified to $v = V_{max} \times [S]/K_m$. V_{max}/K_m will represent the slope of the plot of glucuronide formation rates against substrate concentration (Tracy, 2003). In this way the Cl_{int} (V_{max}/K_m) of 6,7-diHF and 7,8-diHF could be estimated from the slopes of their corresponding plots, and the values are shown in Table 2.

Table 2
Apparent enzyme kinetic parameters for the glucuronidation of eight hydroxyflavones (HF) by human jejunum microsome ($n = 3$)

Selected HFs	V_{max} (nmol/(min mg)$^{-1}$)	K_m (μM)	Cl_{int} (μl/(min mg)$^{-1}$)
7-HF	4.79 ± 0.50	49.04 ± 9.18	97.6
3,7-diHF	5.65 ± 0.73	70.86 ± 14.50	79.7
5,7-diHF (chrysin)	10.24 ± 1.36	43.62 ± 10.53	234.8
6,7-diHF	NA	NA	163.4
7,8-diHF	NA	NA	150.6
2′,7-diHF	5.10 ± 0.39	29.91 ± 4.60	170.3
3′,7-diHF	18.38 ± 0.54	10.58 ± 0.85	1737.2
4′,7-diHF	12.34 ± 2.88	132.9 ± 41.75	92.9

Note: data are expressed as mean ± S.E. ($n = 3$). NA: not applicable.

3.3. Comparison of glucuronidation activities between rat intestinal microsome and human jejunum microsome

The comparison was conducted with the same HF substrate concentration (6 μg/ml). It was found that the total glucuronides formation rate of all studied diHFs with rat intestinal microsome was generally lower than that from human jejunum microsome except for 3,7-diHF, in which the measured activity of rat intestinal microsome was similar to that of human jejunum microsome (Fig. 3).

4. Discussions

To explain the structure–activity relationship of flavonoids and glucuronidation, we do need a series of systematically designed studies. Our previous study (Zhang et al., 2006) on monoHFs using human jejunum S9 fraction demonstrated that stereoconformation and nucleophilicity appeared to be crucial for the intestinal glucuronidation of flavones, which supports the S_N2 mechanism of glucuronidation by UGTs (Yin et al., 1994). Hence, the present study represents our continuous effort to improve our understanding of the SAR through investigating some selected diHFs.

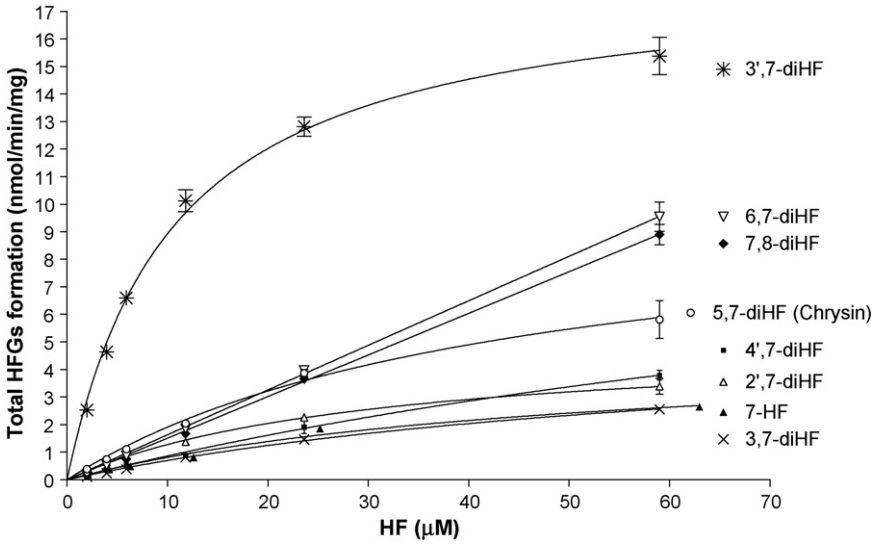


Fig. 2. Total glucuronidation rate in human jejunum microsome at various substrate concentrations of eight HFs. Data are expressed as mean ± S.E. ($n = 3$).

In the current study, saturable glucuronidations were observed for 3′,7-diHF, chrysin, 7,2′-diHF, 7HF, 7,4′-diHF, and 3,7-diHF in the selected substrate concentration range. It is suggested that intestinal first-pass metabolism may be saturated during oral absorption of xenobiotics when drug concentrations in the enterocytes exceed the K_m value and saturable first-pass metabolism is more likely to occur in the small intestine than in the liver because of the substantial drug concentration gradient which exists during absorption (Lin et al., 1999). However, the extent of intestinal first-pass metabolism is highly dependent on the oral dose and could be substantial when a very low oral dose is given. It has been demonstrated that the rate of biotransformation and clearance are concentration-dependent prior to the saturation with higher clearance obtained for lower drug concentration (Lin et al., 1999). Since the daily intake of flavones are only 1–2 mg (Erdman et al., 2007), the intestinal first-pass metabolisms of flavones are expected to be greater than that of other flavonoids like flavonols, of which the daily intake can be up to 30 mg (Erdman et al., 2007). In other words, flavones may actually be less prone to experience saturable intestinal first-pass glucuronidation when compared with other dietary flavonoids.

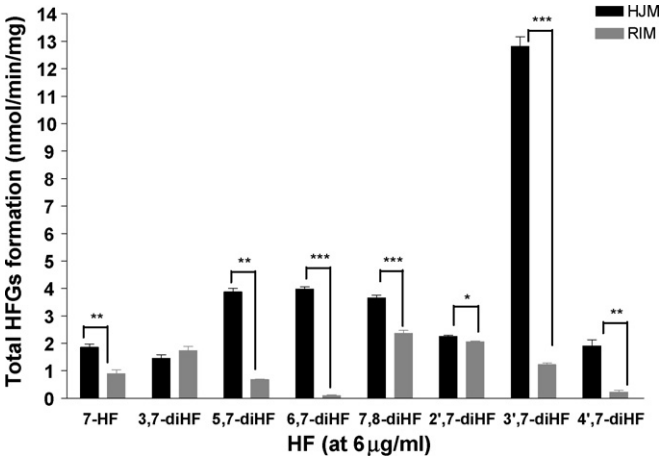


Fig. 3. Comparison of glucuronidation rate of eight HFs between human jejunum microsome and rat intestinal microsome. HJM, human jejunum microsome; RIM, rat intestinal microsome. Data are expressed as mean ± S.E.M. ($n = 3$). $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$.

Differences in the kinetic parameters obtained from the current study with human jejunum microsome and from our previous study on monoHFs with human jejunum S9 fraction were observed (Zhang et al., 2006). S9 is one of the models adapted to obtain the UGTs from intestinal cells. Comparing the kinetic parameters on 7-HF obtained in the current study from human microsome with that from our previous study using human jejunum S9 fraction (Zhang et al., 2006), the K_m values obtained for 7-HF were comparable (about 55 μM). However, a sixfold lower V_{max} (0.81 ± 0.09 vs. 4.97 ± 0.61 nmol/(min mg)$^{-1}$) and thus a sixfold lower Cl_{int} (14.5 vs. 89.6 μl/(min mg)$^{-1}$) were observed in the previous study. Similarly, in our previous study on baicalein glucuronidation (Zhang et al., 2007b), the V_{max} and Cl_{int} values with the human jejunum microsome were higher than that with human jejunum S9. The higher V_{max} value of glucuronidation in microsome than in S9 fraction is expected because the membrane-bound UGT isozymes are enriched in the microsomal preparation with a relatively high content.

As the expression of UGT isoforms is tissue specific (Fisher et al., 2001), using a particular UGT isoform in glucuronidation assays has its value in establishing their specificities to different substrates at different sites. It was found that chrysin (5,7-diHF) was an efficient substrate of UGT1A1 which is significantly expressed in small intestine and thus chrysin might experience significant intestinal first-pass glucuronidation, as supported previously by an *in vivo* study (Walle et al., 2001). In addition, as different UGTs display different regioselectivities and conjugation activities (Boersma et al., 2002), using UGT isoforms also help the generation of pharmacophore models and the establishment of enzyme conformations. However, as demonstrated from our previous study on baicalein, flavonoids experience extensive glucuronidation by various human recombinant UGT isozymes in different extent (Zhang et al., 2007b). Therefore, intestinal microsome was used for the current study aiming to provide the overall glucuronidation activity of intestine.

Being a S_N2 nucleophilic substitution reaction, glucuronidation is initiated by the attack of the –OH group from the phenolic substrate to the C1 of pyranose acid ring of UDPGA (Yin et al., 1994). Therefore, the position of the –OH groups on HFs together with its nucleophilicity and steric characteristics may affect the overall glucuronidation efficacy of HF as demonstrated from our previous study on monoHFs (Zhang et al., 2006). Based on the results obtained in the current study, the effects of the addition of

–OH groups in A-ring/B-ring/C-ring (Fig. 1) on the glucuronidation activity of diHFs could be interpreted. According to the estimated Cl_{int} values, the glucuronidation activity of the selected HFs was as follows: 3′,7-diHF ≫ 5,7-diHF (chrysin), 2′,7-diHF, 6,7-diHF, 7,8-diHF > 7-HF, 4′,7-diHF, and 3,7-diHF. Compared with the mono-hydroxyl substituted 7-HF, an addition –OH group on A-ring at C5, C6 or C8, and B-ring at C2′, C3′ increased the overall glucuronidation activity. This might be due to the presence of an extra –OH group acting as an additional site that also undergoes S_N2 attack on the glucuronic acid for glucuronidation. Moreover, the additional electron-donating –OH group at A-ring enhanced the nucleophilicity of 7-OH group by electron resonance and thus increased the glucuronidation activity. This hypothesis was supported by ours and others findings in chrysin. Chrysin has two –OH groups on A-ring, and previous study identified that it was 7-OH glucuronidated as 5-OH formed intra-molecular hydrogen bond with oxygen at C4 carbonyl group, reducing its nucleophilicity (Boersma et al., 2002; Galijatovic et al., 1999). In the current study, the HPLC chromatogram of incubated chrysin sample indicated that the major metabolite 1 (M1) and a minor metabolite 2 (M2) formed were likely to be 7-glucuronide and 5-glucuronide of chrysin, respectively. The overall glucuronidation activity of chrysin was much higher than that of 7-HF (Cl_{int} 234.8 vs. 97.6 μl/(min mg)$^{-1}$), although the contribution of M2 to the total amount of chrysin glucuronides was negligible. These results suggested that the additional –OH on A ring might enhance the overall glucuronidation activity of HF through the electron-donating mechanism to both –OH substituents on A-ring which is consistent with the findings by Lewinsky that UGT1A10 activity increased with increasing numbers of –OH groups in the A-ring (Lewinsky et al., 2005).

Previous glucuronidation study on human extrahepatic UGT1A10 activity (average of two experiments, in nmol/(min mg)$^{-1}$) demonstrated an order of Chrysin (6.5) > 7-HF (4.4) » 3,7-diHF (1) (Lewinsky et al., 2005). Our findings from the human intestinal microsome in the present study were consistent with this trend. Moreover, the investigators from the same study also suggested that –OH groups in particular positions and orientations are factors determining whether a flavonoid is a good substrate of UGT1A10. They indicated that mono-hydroxyl flavones/flavonols with –OH groups at C3, C6 or C7, but not C5, were glucuronidated. The same study also found that UGT1A10 activity increased with increasing numbers of –OH groups in the A-ring: 7HF < chrysin (5,7-diHF) < baicalein (5,6,7-triHF), which was also true with respect to hydroxylation of the B-ring of flavones: chrysin (5,7-diHF) < apigenin (5,7,4′-triHF) < luteolin (5,7,3′,4′-tetraHF). Another study comparing the intestinal metabolism of isoflavones also demonstrated that the glucuronidation rate were lower when an additional A-ring electron-donating group (–OH or $–OCH_3$) was absent (such as daidzein and formononetin) (Chen et al., 2005). Our finding on the greater glucuronidation activities of diHFs in comparison with 7-HF was consistent with the others (Lewinsky et al., 2005). However, in the current study no such enhanced effect was observed when –OH group was added on C-ring at C3 position. Although no similar studies on the 3,7-diHF was reported, previous investigation on UGT1A10 activity reported that the C2–C3 double bond could confer a planar conformation of the flavonoids (Lewinsky et al., 2005). In particular, the flavonols, which are flavones having 3-OH group including 3,7-diHF, exhibit a planar structure because of the intramolecular hydrogen bond between the C3-OH and the C6′-H. In general, flavonols has the lowest glucuronidation activities among different groups of flavonoids and the activity was observed in a decreased order from flavanones (without C2–C3 double bond) then flavones (with C2–C3 double bond but no 3-OH) to flavonol (with both C2–C3 double bond and 3-OH). These phenomena could be attributed to the increasing tendency of planar structure (Lewinsky et al., 2005). The same reason could be the explanation of the lowered glucuronidation activity observed in 3,7-diHF when compared with 7-HF in both the previous study and the current study. Future work is necessary to evaluate this phenomenon. For hydroxyl substitution in B-ring, additional 3′-OH group seemed enhancing the glucuronidation activity of diHF the most with an order of 3′,7-diHF ≫ 2′,7-diHF > 4′,7-diHF. Interestingly, 3′-HF was reported to be much more active than 2′-HF or 4′-HF in the glucuronidation (Zhang et al., 2006). Both our current and previous studies consistently indicated the importance of 3′-OH for the glucuronidation of flavonoids. On the other hand, the additional 4′-OH did not seem to significantly enhance the reactivity of 4′7-diHF when comparing with 7-HF.

In addition to the investigation of SAR, species differences in the metabolism of flavonoids were also investigated. In the present study, both rat intestinal microsome and human jejunum microsome produced two glucuronide conjugates of diHFs but only one glucuronide of 7-HF. Moreover, the retention times and UV spectra of all glucuronide conjugates found in rat and human preparations were the same, indicating that these two preparations generated the same glucuronides of all HFs tested. However, the formation rate of total glucuronides was significantly different in two species. At the same concentration of the same HF substrate, the human jejunum microsome demonstrated higher total metabolic rates for all HFs studied except for 3,7-diHF. Moreover, different rank order of the metabolic rate among selected HFs was observed between human and rat microsomes. The difference in metabolic rates and their rank order might be due to the differences in the expression of specific UGTs mediating the glucuronidation of individual HF in two different preparations. In addition, different rates of intestinal metabolism may also be ascribed to the possible uneven distribution of UGT isoforms along small intestine (Shelby et al., 2003), thus further studies are warranted to identify the detailed enzyme selectivity.

5. Conclusions

The current study demonstrated the effect of additional –OH substitution on the glucuronidation activity of HFs by human jejunum microsome. Except for 3,7-diHF and 4′7-diHF, all the studied diHFs with 7-OH and an additional –OH group on either A-ring or B-ring demonstrated higher glucuronidation activities than 7-HF. The additional 3′-OH may be critical for the extensive glucuronidation activity of flavonoids. Together with our previous investigations on various monoHFs, our findings provide further understandings of the SAR between flavonoids and their intestinal glucuronidation.

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