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Short Communication

Structure–metabolism relationships for the glucuronidation of flavonoids by UGT1A3 and UGT1A9

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Abstract

Objectives This study tries to find structure–metabolism relationships between flavonoids and human UGT1A3 and UGT1A9.

Methods The glucuronidation of flavonoids was studied with recombinant UGT1A3 and UGT1A9, and the glucuronidation activity was determined by HPLC.

Key findings Of the flavonoids studied, it was shown for the first time that baicalein, quercetin-3-OCH_2OCH_3, quercetin-4′-CH_3, quercetin-3′-OCH_3 and quercetin-3′-Br are substrates of UGT1A3. Wogonin, baicalein, quercetin-4′-Cl, quercetin-3-OCH_2OCH_3, quercetin-3-*O*-arabinoside, quercetin-4′-CH_3, quercetin-3′-OCH_3 and quercetin-3′-Br are the newly reported substrates of UGT1A9. The preferred substrates for UGT1A3 and UGT1A9 contain the hydroxyl group at the C7-position. The glycon and the position of the B ring have conspicuous influences on the glucuronidation activity, and other chemical structures of flavonoids have minor effects.

Conclusions From the quantitative study, UGT1A9 in general has higher glucuronidation efficiency than UGT1A3.

Keywords flavonoids; glucuronidation; structure–metabolism relationships; UGT1A3; UGT1A9

Introduction

Glucuronidation is a major conjugation reaction that is catalysed by numerous UDP-glucuronosyltransferase (UGT) isoforms, a group of phase II drug-metabolizing enzymes that are resident in the endoplasmic reticulum. To date, at least twenty different UGT isoforms have been characterized in human beings. Recent studies have suggested that only UGT1A1, 1A3, 1A4, 1A6 and 1A9 of the UGT1 gene complex are expressed in human liver.[1] From previous studies, we consider that UGT1A3 and 1A9 are the main enzymes catalysing the glucuronidation of flavonoids in human liver, and their study would increase the understanding of high-throughput screening technologies for drug glucuronidation.[2,3] Recombinant human drug-metabolizing enzymes have become a widely used alternative for the study of human metabolism, particularly when testing substrate specificity of UGTs. Their identification is essential for understanding why individuals vary in their rates of glucuronidation, which may alter the pharmacological and toxicological responses to different agents.[4] Comparison of the kinetic parameters of substrates can be used to assess the potential risk of interaction between substrates at the single enzyme level, while sufficient information on the substrate specificities of the responsible drug-metabolizing isoforms is available.[5]

Flavonoids are a group of low-molecular-weight polyphenolic compounds with diverse chemical structures and characteristics. They occur naturally in fruits, vegetables, nuts, seeds, wine and tea, are an integral part of the human diet and have long been believed to have beneficial health effects against many diseases, in particular cardiovascular disease and cancer.[6,7] However, it has become clear that the bioactive forms of most flavonoids *in vivo* are not necessarily the natural phytochemical forms, but the conjugates and metabolites arising from these on absorption, and some conjugates retain biological activity.[8] Conjugation reactions seem to be the most common type of metabolic pathway for the flavonoids.[9] In particular, there are now many reports of extensive phase II metabolism of the aglycones, such as quercetin, kaempferol, baicalein, wogonin and apigenin, to glucuronides.[10,11]

The structure–metabolism relationship between flavonoids and human UGT1A10 has been reported; the preferred substrates of UGT1A10 contain the hydroxyl group to be

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glucuronidated at C6 or C7, but not C5, of the A-ring or on C4′ of the B-ring—up to two additional hydroxyl groups on the A-ring enhance activity, whereas the presence of other groups, notably sugar groups, decreases activity.[12] An aromatic ring attached to the hydroxyl group was found to increase the likelihood of glucuronidation by UGT1A6, UGT1A7 and UGT1A9.[13] These results suggest that a molecular length less than 14.5 Å may be required for a substrate to interact with the active site of UGTs.[14] Currently, pharmacophore models developed using Catalyst (Accelrys, San Diego, USA) indicate that substrates of the UGT1A family share two key hydrophobic regions 3 and 6–7 Å from the site of glucuronidation in a well-defined spatial geometry.[15]

In our previous study, nineteen flavonoids were investigated as substrates of UGTs,[16] and we found thirteen flavonoids were extensively metabolized *in vitro* by UGT1A3 and UGT1A9, while others were not. As we know, a large number of studies of flavonoid glucuronidation have been published. UGT1A3 and UGT1A9 are important in glucuronidation of flavanoids, but there have been few reports on the relationship between their structures and metabolism. Together with the previous study, we tried to elucidate the potential structure–metabolism relationships between flavonoids and UGTs. All the chemical structures of the flavonoids studied are shown in Figure 1.

Materials and Methods

Chemicals and reagents

Apigenin, astilbin, avicularin, baicalein, baicalin, daidzein, naringin, hesperidin, puerarin, rutin, rhoifolin, wogonin and compound (**2**) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Other flavonoids were obtained from the Department of Pharmaceutical Chemistry (Zhejiang University, Hangzhou, China). The purity of all the flavonoids was >99.5%. Uridine diphosphate glucuronic acid (UDPGA), alamethicin, D-saccharic acid 1,4-lactone and β-glucuronidase were purchased from Sigma (St Louis, USA). The PVDF membrane was purchased from Amresco (Solon, USA). Mouse anti-His antibody and peroxidase-conjugated goat anti-mouse secondary antibody were supplied by Amersham Biosciences (Little Chalfont, UK) and Zhongshan Biotechnology (Guangdong, China), respectively. Bac-to-Bac baculovirus expression system consisting of pFastBac vector, cellfectin reagent and DH10Bac-competent cells was supplied by Invitrogen (Carlsbad, USA). *Spodoptera frugiperda* Sf9 insect cells were obtained from CCTCC (Wuhan, China). All other chemicals and solvents were analytical reagent or chromatographic grade and obtained from common commercial sources.

Preparation of the homogenate of UGTs, which were expressed with the recombinant baculovirus in Sf9 cells

Spodoptera frugiperda (Sf9) insect cell were cultured at 27°C in Grace's medium containing 10% fetal bovine serum, 100 000 U/l penicillin and 100 000 U/l streptomycin. The recombinant baculovirus (Bacmid-UGT1A3 or Bacmid-UGT1A9) was amplified from the monolayer cultures of Sf9 insect cells, transfected to Sf9 insect cells at 27°C in Grace's medium. After 72 h, the cells were harvested, and then were disrupted by a standard optimized sonication method.[17] The concentration of protein was determined by the method of Lowry, and the protein was stored at −80°C until use.

Western blot analysis

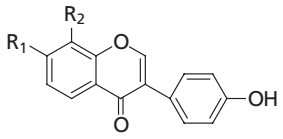
Expressed proteins were separated on a 10% SDS-polyacrylamide gel and transferred to 0.45 μm pore size PVDF membrane in Mini Protean II (Bio-Rad) by electroblotting in 15.6 mM Tris-HCl (pH 8.3) containing 120 mM glycine, with constant 100 V for 1 h. Membranes were incubated for 15 h in blocking buffer. Mouse anti-His antibody was added at a dilution of 1:3000 with blocking buffer, incubated for 1 h, and then followed by additional washes with phosphate-buffered saline (PBS) containing 0.05% Tween 20. The membrane was then soaked in blocking buffer with a 1:5000 peroxidase-conjugated goat anti-mouse IgG, incubated for 1 h, and washed free of the membrane by three washes in PBS and 0.05% Tween 20. Peroxidase activity was detected by incubating the membrane in 10 ml PBS buffer at pH 7.5 containing 0.15 mg/ml diaminobenzidine (DAB) and 60 μl 10% H_2O_2.

Analysis of the activity of UGT1A3 and UGT1A9

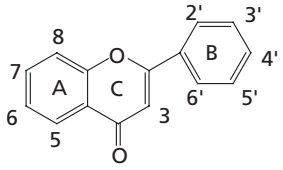
A typical incubation mixture (100 μl total volume) contained 100 mM Tris-HCl (pH 7.81), 10 mM $MgCl_2$, 10 mM alamethicin, 5 mM D-saccharic acid 1,4-lactone, 1 g/l cell homogenate containing individual UGTs, including UGT1A3 or UGT1A9, and flavonoids. Rate of product formation was optimized for linearity with respect to protein concentration and incubation time. The mixture without UDPGA was pre-incubated at 37°C in a water bath for 5 min, and the reaction was initiated by the addition of 5.0 mM UDPGA and incubated under the same conditions for 30 min. Controls were incubated in the absence of UDPGA. To terminate the reaction, an equal volume of cold methanol was added, and the samples were centrifuged at 10 000 rev/min for 6 min. Then 20 μl of supernatant was subjected to HPLC analysis. The glucuronides of the flavonoids were identified by hydrolysis with β-glucuronidase at 37°C in a shaking water bath overnight. A volume of 100 μl incubation mixture without D-saccharic acid 1,4-lactone was divided into two equal samples. One sample was hydrolysed at 37°C for 12 h by adding 200 units β-glucuronidase in KH_2PO_4 buffer, pH 5.0. The other sample was treated similarly but without β-glucuronidase in buffer. After incubation, both samples were centrifuged at 10 000 rev/min for 10 min. A fraction of each supernatant was analysed by HPLC as described above. An LC-MS system was also used to identify glucuronidation. The same batch of stable expressed human UGT1A3 or UGT1A9 was used for the flavonoid glucuronidation study.

HPLC analysis

Analysis was performed on a Shimadzu LC-10A (Kyoto, Japan) system, equipped with LC-10AD pumps, a SPD-10AVP UV detector and a Diamonsil C_{18} column (25 cm × 4.6 mm, 5 μm). The mobile phase consisted of an appropriate ratio of methanol and 0.2% phosphoric acid (pH 2), and the flow rate was 1.0 ml/min. Each flavonoid and its metabolites were detected at the same wavelength. HPLC conditions of flavonoids are shown in detail in Table 1.

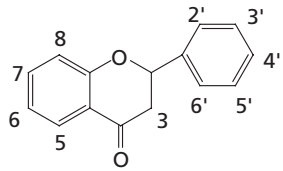


	R1	R2
daidzein	OH	H
puerarin	OH	glucose
compound(2)	OCH_3	H



	3	5	6	7	8	2'	3'	4'	5'	6'
apigenin	H	OH	H	OH	H	H	H	OH	H	H
wogonin	H	OH	H	OH	OCH_3	H	H	H	H	H
hesperidin	H	OH	H	R3	H	H	OH	OCH_3	H	H
baicalein	H	OH	OH	OH	H	H	H	H	H	H
baicalin	H	OH	OH	R4	H	H	H	H	H	H
quercetin-4'-Cl	OH	OH	H	OH	H	H	OH	Cl	H	H
quercetin-4'-CH_3	OH	OH	H	OH	H	H	OH	CH_3	H	H
quercetin-3',4'-OCHO	OH	OH	H	OH	H	H	-OCH_2-	-O-	H	H
quercetin-3'-Br	OH	OH	H	OH	H	H	Br	OH	H	H
quercetin-3'-OCH_3	OH	OH	H	OH	H	H	OCH_3	OH	H	H
quercetin-3-OCH_2OCH_3	R5	OH	H	OH	H	H	OH	OH	H	H
quercetin-3-*β*-*D*-xylopyranoside	R6	OH	H	OH	H	H	OH	OH	H	H
quercetin-3-*O*-arabinoside	R7	OH	H	OH	H	H	OH	OH	H	H
avicularin	R8	OH	H	OH	H	H	OH	OH	H	H
rhoifolin	H	OH	H	R9	H	H	H	OH	H	H
rutin	R10	OH	H	OH	H	H	OH	OH	H	H
compound 1	H	H	H	OCH_3	H	H	OH	H	H	H

R3=-*O*-rutinose
R4=-*O*-glucuronide
R5=-OCH_2OCH_3
R6=*β*-D-xylopyranoside
R7=-*O*-arabinoside
R8=-*O*-arabinose
R9=-*O*-neohesperidose
R10=-*O*-rutinose



	3	5	6	7	8	2'	3'	4'	5'	6'
astilbin	R11	OH	H	OH	H	H	OH	OH	H	H
naringin	H	OH	OH	R12	H	H	H	OH	H	H

R11=-*O*-Rha
R12=-*O*-neohesperidose

Figure 1 Chemical structures of flavonoids.

Table 1 Glucuronidation assay of flavonoids, HPLC conditions and LC/ESI-MS analysis

Compound	UV (nm)	Mobile phase[a]	Substrate peak		Metabolite peaks		Major fragment ion in LC/MS (*m/z*)
			retention time (min)	$[M-H]^-$(*m/z*)	retention time (min)	$[M-H]^-$(*m/z*)	
Apigenin	334	50:50	25.9	269	7.2	445	269$[M-H]^-$-GlucA
Avicularin	355	50:50	10.2	433	2.9,3.5,6.4,7.8	609	433$[M-H]^-$-GlucA, 477 $[M-H]^-$-arabinose
Daidzein	300	50:50	17.5	253	4.9	429	253$[M-H]^-$-GlucA
Quercetin-4′-Cl	270	70:30	31.4	319	8.4, 11.7	495	319$[M-H]^-$-GlucA
Wogonin	275	70:30	14.2	293	7.7	469	293$[M-H]^-$-GlucA
Quercetin-3′,4′-OCHO	339	70:30	16.2	313	3.8, 6.6	489	357$[M-H]^-$-GlucA
Quercetin-3-OCH2OCH3	357	60:40	10.7	345	4.4,7.3	521	345$[M-H]^-$-GlucA
Quercetin-3-β-D-xylopyranoside	358	45:55	16.9	433	9.7, 11.6, 12.5,15.0	609	433$[M-H]^-$-GlucA 477$[M-H]^-$-xylopyranoside
Quercetin-3-*O*-arabinoside	359	45:55	19.1	433	15.0[b]	609	433 $[M-H]^-$-GlucA 477$[M-H]^-$-arabinoside
Quercetin-4′-CH3[c]	268	70:30	24.7	299	8.1	475	299$[M-H]^-$-GlucA
Quercetin-3′-OCH3[c]	267	70:30	19.0	315	7.5	491	315$[M-H]^-$-GlucA
Quercetin-3′-Br[c]	268	72:28	26.7	363	9.0	539	363$[M-H]^-$-GlucA
Baicalein[c]	273	63:37	15.0	269	7.7	445	269$[M-H]^-$-GlucA
Compound **1**	308	70:30	26.5	–	nd	–	–
Compound **2**	290	70:30	9.3	–	nd	–	–
Astilbin	289	45:55	12.6	–	nd	–	–
Rhoifolin	332	45:55	18.6	–	nd	–	–
Rutin	380	43:57	17.5	–	nd	–	–
Naringin	282	38:62	25.0	–	nd	–	–
Puerarin	250	25:75	30.0	–	nd	–	–
Hesperidin	283	45:55	11.9	–	nd	–	–
Baicalin	276	48:52	22.0	–	nd	–	–

[a]Mobile phase consisted of methanol and 0.2% phosphoric acid (pH 2), and the flow rate was 1.0 ml/min. [b]No glucuronides were detected for UGT1A3. [c]The concentrations of these flavonoids was <10 μM, the other compounds concentrations were in the range 20–60 μM. –, did not analyse using HPLC/MS in negative ion mode; GlucA, glucuronic acid; nd, no glucuronides were detected.

The LC-MS apparatus (Thermo Finnigan, San Jose, USA) was equipped with an electrospray mass spectrometric detector. The same column was used, and the mobile phase consisted of appropriate ratio of methanol and formic acid solution (pH 2) at a flow rate of 0.5 ml/min. MS conditions used were as follows: capillary voltage, 5 kV; cone voltage, –14.9 kV with nitrogen as sheath and auxiliary gas. MS spectra were obtained in the range of m/z 150–800.

Data analysis

Kinetic constants for flavonoids glucuronidation by each enzyme were obtained by fitting untransformed experimental data to the following kinetic model (the Michaelis–Menten equation):

$$V = \frac{V\max \times [S]}{Km + [S]} \quad (1)$$

where V is the rate of reaction, Vmax is the maximum velocity, Km is the Michaelis constant, and [S] is the substrate concentration. The velocity of glucuronidation was determined by flavonoid substrates depletion. The results were shown as mean ± SD from three independent experiments, and all data were calculated using Microsoft Excel. The kinetic parameters were determined from each experiment and then the mean and SD were calculated.

Statistical analysis

Statistical analysis of the UGT1A9 and UGT1A3 on the rate of glucuronidation, *Vmax/Km* in Table 2, was performed using the Kruskal–Wallis test (SPSS, CA, USA). In all cases, post-hoc comparisons of the means of individual groups were performed using Dunn's test. A significance level of $P < 0.05$ denoted significance in all cases.

Results

Analysis of the recombinant UGT1A3 and UGT1A9 by Western blot

The whole cell homogenate was probed by Western blot using anti-His antibody and the appropriate second antibody according to the method described above, and Sf9 cells transfected with the blank bacmid were used as the blank controls. UGT1A3 had a higher expression level than UGT1A9.

Flavonoid glucuronidation assay

As in the previous study,[16] a series of flavonoids were investigated for glucuronidation by UGTs. To confirm the glucuronidation activity, incubation mixtures with respective compounds in the presence of the cofactor UDPGA were incubated at 37°C in a water bath for 12 h and then subjected to HPLC; metabolite peaks were observed, which were not

Table 2 Kinetic parameters of flavonoid glucuronidation with human recombinant UGT1A3 and UGT1A9[a]

Substrate	UGT1A3			UGT1A9		
	Km[b] (μM)	*Vmax*[c] (ρmol/min/mg)	*Vmax/Km* (μl/min/mg)	*Km* (μM)	*Vmax* (ρmol/min/mg)	*Vmax/Km* (μl/min/mg)
Apigenin	28.88 ± 2.47	224.19 ± 21.11	7.75 ± 0.29	45.38 ± 10.05	1721.48 ± 222.93	38.60 ± 4.84
Quercetin-4′-Cl	57.00 ± 5.14	966.55 ± 59.51	16.99 ± 0.57	65.79 ± 4.52	2877.97 ± 121.61	43.80 ± 1.25
Wogonin	50.50 ± 1.93	1732.48 ± 33.68	34.32 ± 0.64	32.27 ± 1.73	2306.99 ± 42.63	71.60 ± 2.52
Quercetin-3-β-D-xylopyranoside	419.42 ± 5.00	514.01 ± 39.94	1.23 ± 0.09	132.51 ± 11.46	1250.90 ± 45.72	9.47 ± 0.49
Daidzein	599.87 ± 53.72	721.81 ± 149.88	1.19 ± 0.14	62.27 ± 6.71	539.76 ± 22.31	8.06 ± 0.51

[a]Assay was performed at 37°C for 30 min using 1.0 mg/ml protein. The concentration of apigenin was in the range 9–45 μM, quercetin-4′-Cl was 20–100 μM, wogonin and daidzein were 35–175 μM, quercetin-3-β-D-xylopyranoside was 45–225 μM. The rates of glucuronidation were determined by the disappearance of the substrates. [b]*Km,* Michaelis constant. [c]*Vmax,* maximum velocity. Data are presented as mean ± SD, $n = 9$.

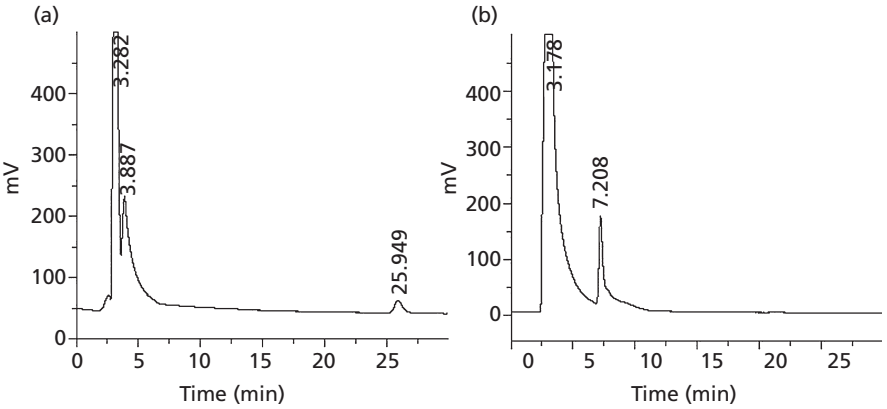


Figure 2 Representative chromatograms of apigenin glucuronidation catalysed by UGT1A3 and UGT1A9 with high activity. Retention times: 25.9 min, substrate; 7.2 min, glucuronide. (a) Before glucuronidation metabolism; (b) substrate incubated with UGT1A3 or UGT1A9 overnight.

present in the blank control without UDPGA (Figure 2). These peaks were identified by β-glucuronidase and LC-MS as conjugates of flavonoids (Table 1). All glucuronide peaks disappeared after β-glucuronidase hydrolysis. Total ion chromatograms were collected by fully scanning ions over a mass range of m/z 150–800, encompassing the m/*z*-values of both diglucuronides and monoglucuronides for each flavonoid. Major daughter ion transitions of peaks identified as flavonoid glucuronides are described in Table 1. All of them were monoglucuronides.

On daidzein, avicularin, quercetin-3-*O*-arabinoside and quercetin-3-β-D-xylopyranoside incubation chromatograms, the metabolite peaks were small compared with the substrate peaks, respectively, while other flavonoid incubation chromatograms had only metabolite peaks, and no substrate peaks were detected. The result showed that daidzein, avicularin, quercetin-3-*O*-arabinoside and quercetin-3-β-D-xylopyranoside had minor activity; other flavonoids, except nine compounds discussed below, showed high glucuronidation activity with UGT1A3 and UGT1A9.

There was only one conjugate detected for apigenin, daidzein, wogonin and quercetin-3-*O*-arabinoside glucuronidation via UGT1A3 and UGT1A9, while quercetin-4′-Cl, quercetin-3′, 4′-OCHO, quercetin-3-OCH_2OCH_3, avicularin and quercetin-3-β-D-xylopyranoside each had two or more metabolites detected (retention times are shown in Table 1). In this study, it was impossible to determine the position at which glucuronidation occurs, but since conjugates had different retention times we concluded that apigenin, daidzein, wogonin and quercetin-3-*O*-arabinoside had to form at least one conjugate, quercetin-4′-Cl, quercetin-3′, 4′-OCHO, quercetin-3-OCH_2OCH_3 at least two metabolites and avicularin and quercetin-3-β-D-xylopyranoside at least four conjugates.

With overnight incubation, no metabolite peaks were detected on HPLC chromatograms of nine flavonoid compounds compared with blank control incubation. This result showed that both UGT1A3 and UGT1A9 had no glucuronidation activity toward these nine compounds (compound **1**, compound **2**, puerarin, naringin, hesperidin, astilbin, rutin, rhoifolin and baicalin). All results of these flavonoid glucuronidation assays are shown in Table 1, with detailed HPLC conditions and LC-MS data, respectively. The representative chromatograms of glucuronidation of flavanoids catalysed by UGT1A3 and UGT1A9 are shown in Figures 2 and 3.

Enzymatic kinetic study

To determine the enzymatic kinetic parameters for UGT1A3 and UGT1A9, a glucuronidation assay was performed at 37°C for 30 min using 1.0 mg/ml homogenate.[18] The rate of glucuronide formation was determined according to depletion of substrate. Preliminary experiments indicated that the glucuronidation reaction was linear for 30 min incubation, and the

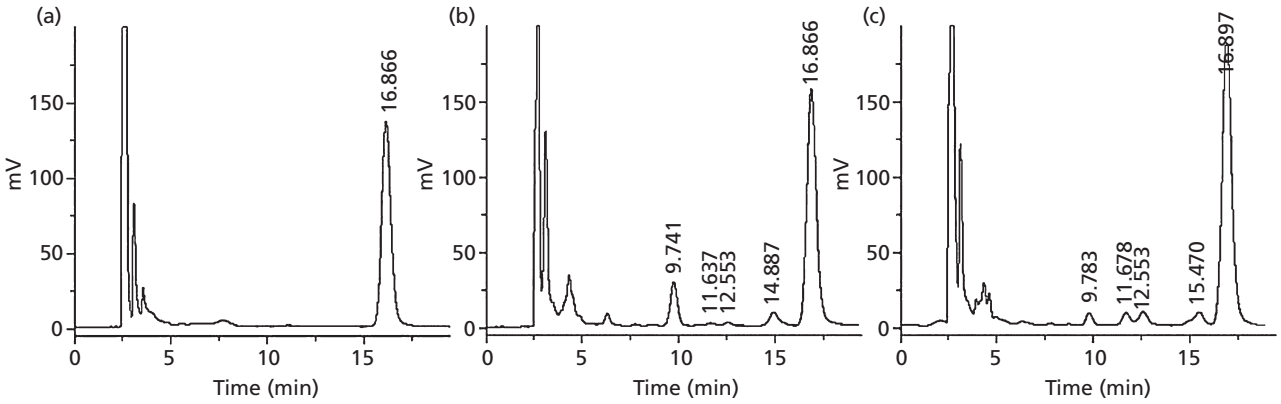


Figure 3 Representative chromatograms of glucuronidation of quercetin-3-β-D-xylopyranoside catalysed by UGT1A3 and UGT1A9 with low activity. Retention times: 16.9 min, substrate; 9.7, 11.6, 12.5, 15.0 min, glucuronides. (a) Before glucuronidation metabolism; (b) substrate incubated with UGT1A3 overnight; (c) substrate with UGT1A9 overnight.

methods used here had satisfactory accuracy (recovery 85.0–110.0%, $n = 6$) and precision (<5.0%, $n = 3$). The linear ranges, calibration curves and limit of quantitation (LOQ) of the HPLC methods were within an acceptable level. The glucuronidation of four other flavonoids was also fitted to the Michaelis–Menten model. Kinetic parameters of flavonoid glucuronidation with recombinant UGT1A3 and UGT1A9 ($n = 9$) are shown in Table 2.

From the UGTs expression level analysis and enzymatic kinetic parameter studies (Table 2), the *Vmax/Km* of UGT1A9 was found to be higher than for UGT1A3, and its ability to catalyse these five flavonoids was obviously greater than that of UGT1A3.

Discussion

There are some reports on structure–metabolism relationships between UGTs and flavonoid glucuronidation. The structural differences between the different groups of flavonoids, together with catalytic kinetic parameters, were used to provide insights into the structural elements associated with glucuronidation by UGT1A3 and UGT1A9. In this study, we showed the possible structural features required for substrate specificity of UGT1A3 and UGT1A9 activity toward flavonoids, and this structure–metabolism relationship was mostly established by visual inspection of the chemical structures.

Flavonoid glycosides studied here are poor or non-substrates for UGT1A3 and UGT1A9. Daidzein can be converted by UGT1A3 and UGT1A9, while puerarin cannot. The only difference is that puerarin has a C8 glucose. A similar situation arises with baicalein and baicalin. While quercetin-3-β-*D*-xylopyranoside and avicularin are substrates for UGT1A3 and UGT1A9, quercetin-3-*O*-arabinoside is a substrate for UGT1A9. These three compounds, which have glycon groups, have obviously less glucuronidation activity than other quercetin derivatives. Focusing attention on their sugar groups, it can be seen that flavonoid glycosides catalysed by UGT1A3 and UGT1A9 have five carbon sugar groups, i.e. less than six. Once the number of glycon carbons exceeds five, flavonoids with the glycon will lose glucuronidation activity, such as hesperidin, in which the C7 position has a rutinose. Five carbons with sugar groups may be the critical point of glucuronidation activity catalysed by UGT1A3 and UGT1A9. Quercetin-3-β-D-xylopyranoside and avicularin sugar group volumes are smaller than quercetin-3-*O*-arabinoside, and are the substrates for UGT1A3 and UGT1A9, while quercetin-3-*O*-arabinoside is only catalysed by UGT1A9. Even so, the conjugation of five carbon groups, such as in pentoses and arabinoside, will weaken the glucuronidation activity compared with other flavonoids. Flavonoid glycosides with six or more carbon sugar groups are non-substrates for UGT1A3 and UGT1A9.

Apigenin, wogonin, daidzein, baicalein, luteolin, naringenin, 7-hydroxyflavone, genestein, FLAP and EGCE all do not have a C3 hydroxyl group, but the results show these flavonoids can be catalysed by both UGT1A3 and UGT1A9, so we presume that the flavonoids' C3 hydroxyl is not a necessary group for the substrates of UGT1A3 and UGT1A9.[19–22] Most flavonoids discussed here have a 5-position hydroxyl. Daidzein lacks the 5-position hydroxyl, but qualitative results show that daidzein is slowly catalysed by UGTs. It was reported that the C5 position did not appear to be a site for glucuronidation catalysed by UGT1A3 and UGT1A9, such as in quercetin and luteolin.[19,23–25] However, from Table 1, both avicularin and quercetin-3-β-D-xylopyranoside have C5 hydroxyl conjugates; both of them have four conjugates while having only four hydroxyl groups. This may indicate that the C5 hydroxyl appears to be a site for conjugation under the influence of sugar groups. Avicularin and quercetin-3-β-D-xylopyranoside are flavonoid glycosides; if there are no sugar groups, the substrates will not have C5 conjugates for steric reasons. The same situation does not occur for quercetin-3-*O*-arabinoside.

The flavonoids in our study that have a 7-position hydroxyl group and no large glycon are all substrates of human UGT1A3 and UGT1A9. It was reported that quercetin, naringenin, galangin, fisetin, 7-hydroxyflavone, genistein, kaempferol, luteolin, FLAP, EGCE and EGC are substrates for human UGT1A3 and UGT1A9.[18,22–24] All of these flavonoids have a hydroxyl group in the 7-position, particularly the 7-hydroxyflavone has no hydroxyl except in the C7 position. Compounds **1** and **2**, the C7 positions of which have a

-OCH_3 group instead of -OH, have no glucuronidation activity. In brief, although the C7 position may be not be the most efficient conjugation site, most flavonoids with a C7-position hydroxyl group are UGT1A3 and UGT1A9 substrates.[24]

Wogonin has a higher glucuronidation efficiency than other flavonoids (the kinetics parameters are shown in Table 2). The conspicuous difference in chemical structures is that wogonin has a -OCH_3 group in the C8 position while the other flavonoids have a hydrogen instead. The difference in glucuronidation efficiency indicates that bulky groups in the 8-position, which are near a 7-hydroxyl, can enhance glucuronidation activity. Interestingly, the presence of bulky groups adjacent to the phenol is also a feature of the structure of propofol and makes it a specific substrate of UGT1A9. The effects of bulky groups adjacent to phenol are validated by Ethell's research.[5]

Conclusions

All quercetin derivatives evidently can be catalysed, but daidzein has very low glucuronidation efficiency when compared with other flavonoids. This result indicates substituent groups in the B ring have a minor effect on activity, but the position of the B ring may have an important influence. Smith *et al*.[26] reported that common-feature pharmacophores for UGT1A9 had been generated using the program Catalyst, and quercetin compatibly mapped to the respective UGT1A9 pharmacophores. When the B ring is on the C3 position, the substrate chemical structures will lose the ability, or be less able, to map to the respective pharmacophores, so this illustrates the importance of the B ring position. Data on glucuronide formation of six flavonoids is also evidence.[2] The rate of glucuronide formation for apigenin, galangin, fisetin, 7-hydroxylflavone, genistein and quercetin was 612, 508, 610, 276, 604, 131 and 550 pmol/min/mg, respectively. Genistein glucuronidation rate was evidently lower than for the other five flavonoids, and the major difference is the B ring position; genistein is an isoflavone and others are flavones or flavonols.

In summary, the flavonoids that have a C7 hydroxyl group are good substrates for human UGT1A3 and UGT1A9, and the position of the B ring and the glycon have conspicuous influences on the glucuronidation activity, while other structural aspects of flavonoids have minor effects. From Western blot analysis and the quantitative study, human UGT1A9 has a higher glucuronidation efficiency than UGT1A3.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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