Department of Pharmaceutical Analysis and Drug Metabolism, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang, People's Republic of China

Studies on the flavonoid substrates of human UDP-glucuronosyl transferase (UGT) 2B7

SHENGGU XIE, LINYA YOU, SU ZENG

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Su Zeng, Department of Pharmaceutical Analysis and Drug Metabolism, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, People's Republic of China
zengsu@zju.edu.cn

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Flavonoids are found in fruits, vegetables, nuts, seeds, herbs, spices, stems and flowers, as well as in tea and red wine. They are prominent components of citrus fruits and other food sources, are consumed regularly with the human diet, and have been shown to have many biological functions, including antioxidant and chelating properties. This study suggests features of the flavonoid structure necessary for it to act as a substrate of human UGT2B7. Generally speaking, flavonol has higher glucuronidation activity than flavones and isoflavones. Differences in C3′ position have an important effect on UGT2B7 glucuronidation activity, and the various substituents have different influences on glucuronidation activity. For flavonol, the bulky group at C4′ can enhance glucuronidation activity. Increasing the number of hydroxyl groups of flavonoids will increase their glucuronidation activity towards UGT2B7, while conjugation of glycon will weaken the activity, and hydroxyl position can also have an important role in activity. The high glucuronidation efficiency observed with many flavonoids suggests that the contribution of UGT2B7 to the metabolism of flavonoids may be significant. The results suggest that we should not only pay attention to glucuronidation activity, but should also attach importance to the regioselectivity of glucuronidation.

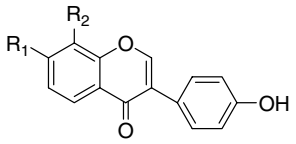
1. Introduction

Flavonoids are found in fruits, vegetables, nuts, seeds, herbs, spices, stems and flowers, as well as in tea and red wine. They are prominent components of citrus fruits and other food sources and are consumed regularly with the human diet (Aherne and O'Brien 2002). These low molecular weight substances, found in all vascular plants, are phenylbenzopyrones (phenylchromones) with an assortment of structures based on a common three-ring nucleus. More than 8000 compounds with a flavonoid structure have been identified (Hodek et al. 2002). This large number of compounds arises from the various combinations of multiple hydroxyl and methoxyl group substituents possible on the basic flavonoid skeleton.

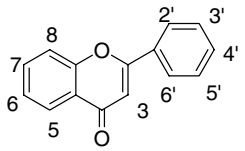
Flavonoid natural products exert a wide range of biochemical and pharmacological properties. They have been described as health-promoting, disease-preventing dietary supplements, and have activity as cancer preventive agents (Duarte et al. 1993; Huynh and Teel 2002). Additionally, they are extremely safe and are associated with low toxicity, making them excellent candidates for chemopreventive agents. The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including modulating enzyme activities resulting in decreased carcinogenicity of xenobiotics. It has become clear that the bioactive forms of flavonoids are not necessarily the natural phytochemical forms, but the conjugates and metabolites arising from these on absorption (Spencer et al. 2004). Conjugation reactions with glucuronic acid seem to be the most common type of metabolic pathways for flavonoids (Kuhnau 1996). In particular, there is now strong evidence for extensive phase II metabolism of the aglycones such as quercetin, kaempferol, baicalein, wogonin and apigenin to glucuronides (Oliveira and Watson 2000; Chen et al. 2006), although metabolism studies on individual isoforms are lacking.

UGTs are phase II biotransformation enzymes, localized in the endoplasmic reticulum, that catalyze the conjugation of glucuronic acid to a broad spectrum of endobiotic and xenobiotic substrates with diverse chemical structures, creating metabolites that are more water soluble and readily excreted. The role of glucuronidation has been generally considered to be a dominant feature of detoxification. Two main UGT gene families, UGT1 and UGT2, have been identified in humans, where they encode for proteins that catalyze the glucuronidation of xenobiotics and endobiotics (King et al. 2000). These enzymes are very important in drug metabolism, and UGT2B7 has a wide distribution in the human organism and reacts with a wide variety of chemical agents and drugs such as morphine, zidovudine, and nonsteroidal anti-inflammatory drugs (King et al. 2000).

The flavonoid substrates for UGT1A1, 1A3, 1A8, 1A10 and 2B15 have been widely reported (Oliveira and Watson 2000; Chen et al. 2006; King et al. 2000; Green et al. 1994;

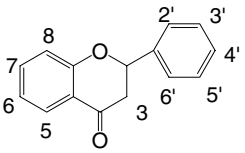


	R1	R2
Daidzein	OH	H
2	OCH_3	H



	3	5	6	7	8	2′	3′	4′	5′	6′
Apigenin	H	OH	H	OH	H	H	H	OH	H	H
Quercetin	OH	OH	H	OH	H	H	OH	OH	H	H
Quercetin-3-OCH_2OCH_3	R1	OH	H	OH	H	H	OH	OH	H	H
Quercetin-4′-CH_3	OH	OH	H	OH	H	H	OH	CH_3	H	H
Isorhamnetin	OH	OH	H	OH	H	H	OCH_3	OH	H	H
Quercetin-4′-Cl	OH	OH	H	OH	H	H	OH	Cl	H	H
Wogonin	H	OH	H	OH	OCH_3	H	H	H	H	H
Kaempferol	OH	OH	H	OH	H	H	H	OH	H	H
3	H	OH	H	R2	H	H	H	OH	H	H
Luteolin	H	OH	H	OH	H	H	H	OH	OH	H
Morin	OH	OH	H	OH	H	OH	H	OH	H	H
Luteolin-7-glucoside	H	OH	H	R3	H	H	H	OH	OH	H
1	H	H	H	OCH_3	H	H	OH	H	H	H
Quercetin-3-*O*-arabinoside	R4	OH	H	OH	H	H	OH	OH	H	H

R1 = -OCH_2OCH_3
R2 = -*O*-neohesperidose
R3 = -*O*-glucoside
R4 = -*O*-arabinoside



	3	5	6	7	8	2′	3′	4′	5′	6′
Astilbin	R5	OH	H	OH	H	H	OH	OH	H	H

R5 = -*O*-Rha

Cheng et al. 1999; Lewinsky et al. 2005; Otake et al. 2002; Boersma et al. 2002), but the substrates for human UGT2B7 have been less extensively studied. It has been reported that human UGT2B7 has an important role in quercetin and luteolin glucuronidation (Boersma et al. 2002), while other published data clearly demonstrate the minor importance of UGT2B7 for glucuronidation of galangin in humans (Otake et al. 2002). Because of the presence of large amounts of structurally diverse flavonoids in fruits, vegetables, and plant-derived beverages, it is important to characterize the features in the flavonoid structure necessary for them to be human UGT2B7 substrates. And through the study of the structure-metabolism relationships, we can predict the importance of human UGT2B7 for flavonoid glucuronidation *in vivo* from the chemical structure of its substrate. The current study was undertaken to investigate the flavonoid substrates of UGT2B7. We found that many flavonoids are extensively catalyzed by UGT2B7, and tried to elucidate the potential structure-metabolism relationships between flavonoids and UGT2B7. The high glucuronidation efficiency observed towards many flavonoids suggests that the contribution of UGT2B7 to the metabolism of these flavonoids may be significant.

2. Investigations and results

2.1. Activity of UGT2B7 to catalyze flavonoid glucuronidation

In order to demonstrate the UGT2B7 glucuronidation activity of the flavonoids, incubation mixtures with the respective substrate were incubated in the presence of the cofactor UDPGA at 37 °C in a water bath for 60 min and then subjected to HPLC, when metabolite peaks were observed, which were not present in the absence of the cofactor UDPGA. These peaks were identified by β-glucuronidase and HPLC/MS as conjugates of flavonoids (e.g. apigenin, Fig. 2). They could all be hydrolyzed completely by sufficient β-glucuronidase at 37 °C in a water bath for 12 h, when the metabolite peaks in the HPLC chromatogram disappeared. Furthermore, these metabolite peaks all had molecular ions $[M-H]^-$ GlucA of m/z (flavonoid +

Table 1: Glucuronidation assay of flavonoids by human 2B7, HPLC conditions and LC/ESI-MS analysis

Compd.	UV (nm)	Mobile phase[a]	Substrate peak (retention time/min)	$[M-H]^-$ (m/z)	Metabolite peaks M1, M2, M3, ... (retention time/min)	$[M-H]^-$ (m/z)	Major fragment ion in LC/MS (m/z)
Apigenin	334	50 : 50	34.8	269	9.9, 10.7	445	269 $[M-H]^-$-GlucA [b]
Daidzein	300	50 : 50	13.9	253	5.9	429	253 $[M-H]^-$-GlucA,
Quercetin	368	55 : 45	14.8	301	3.4, 6.6,11.8	477	$[M-H]^-$-GlucA
Quercetin-3-OCH_2OCH_3	357	60 : 40	13.5	345	6.7	521	345 $[M-H]^-$-GlucA
Quercetin-4′-CH_3	268	70 : 30	20.6	299	6.6, 7.7	475	299 $[M-H]^-$-GlucA
Isorhamnetin	267	70 : 30	16.6	315	5.8, 6.2, 6.6	491	315 $[M-H]^-$-GlucA
Quercetin-4′-Cl	270	70 : 30	22.4	319	7.1, 8.3	495	319 $[M-H]^-$-GlucA
Wogonin	275	70 : 30	12.1	293	5.4, 6.0	469	293 $[M-H]^-$-GlucA
Kaempferol	365	60 : 40	16.6	285	6.2, 6.5	461	285 $[M-H]^-$-GlucA
3	332	45 : 55	15.8	577	5.2, 5.5	753	577 $[M-H]^-$-GlucA 445 $[M-H]^-$-neohesperidose
Luteolin	350	55 : 45	17.7	285	5.1, 12.6	461	285 $[M-H]^-$-GlucA
Morin	355	55 : 45	11.5	301	3.4, 5.4, 6.4, 8.6	477	301 $[M-H]^-$-GlucA
Astilbin	289	45 : 55	10.5	–	nd	–	–
Luteolin-7-glucoside	283	45 : 55	12.1	–	nd	–	–
1	308	70 : 30	19.5	–	nd	–	–
Quercetin-3-*O*-arabinoside	359	45 : 55	15.1	–	nd	–	–
2	290	50 : 50	40.9	–	nd	–	

[a] mobile phase consisted of methanol and 0.2% phosphoric acid (pH = 2), and flow rate was 1.0 mL·min^{-1},
[b] GlucA, glucuronic acid,
nd, indicates no glucuronides were detected,
– did not analyse using HPLC/MS in negative ion mode

176) (Table 1). All the conjugates were identified by β-glucuronidase and HPLC/MS.

A series of flavonoids were investigated for their glucuronidation activity with human UGT2B7 and the results are shown in the Table 1. After overnight incubation, we detected no metabolite peaks from the chromatograms of five flavonoid compounds in contrast to the control incubation without UDPGA. In the incubation chromatograms of astilbin, luteolin-7-glucoside, compound **1**, quercetin-3-*O*-arabinoside and compound **2**, respectively, no metabolite peaks were detected, while the incubation chromatograms of the other twelve flavonoids had metabolite peaks. These results suggest that human UGT2B7 has no glucuronidation activity toward these five compounds, namely astilbin, luteolin-7-glucoside, compound **1**, quercetin-3-*O*-arabinoside and compound **2**. The other twelve compounds have glucuronidation activity. All of these flavonoid glucuronidation assay results are shown in Tables 1 and 2, with detailed HPLC conditions, HPLC/MS data and substrate conversion rates, respectively. Representative HPLC/MS spectra for glucuronidation activity by human UGT2B7 towards flavonoids (apigenin) are shown in the Fig 2.

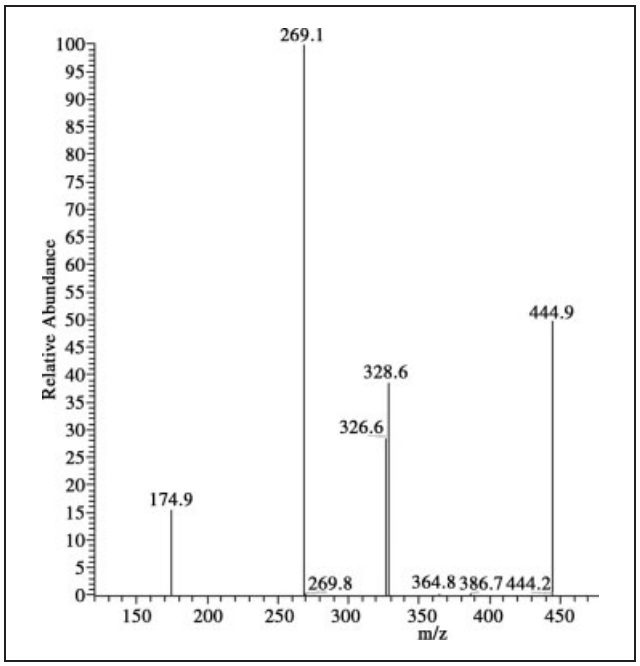


Fig. 1: Representative mass spectrum of apigenin glucuronide formed from apigenin by human UGT2B7

2.2. Glucuronide formation study for flavonoid glucuronidation by UGT2B7

To determine the rates of glucuronidation, a UGT2B7 glucuronidation assay was performed in a water bath at 37 °C for 60 min using 1.0 mg·mL^{-1} UGT2B7. Substrate concentrations were all 1.0 mg·mL^{-1}. The rates of glucurone formation were determined from the removal of the substrate by conversion. Preliminary experiments also indicated that the glucuronidation reactions were linear over 60 min incubation, and that the methods used had satisfactory accuracy and precision. The glucuronidation conver-

Table 2: Glucuronidation assay of flavonoids by human 2B7 and regioselectivity of flavonoids for human UGT2B7 based on peak area measured by HPLC equipped with UV detector

Compd.	Substrate conversion (%)	Regioselectivity (M1/M2/M3 . . . %)
Apigenin	14.9 ± 4.1%	89.4/10.6
Daidzein	11.1 ± 3.7%	100
Quercetin	35.1 ± 2.8%	4.5/71.8/23.7
Quercetin-3-OCH_2OCH_3	27.4 ± 5.7%	100
Quercetin-4′-CH_3	68.1 ± 10.9%	26.1/73.9
Isorhamnetin	36.0 ± 4.1%	32.5/10.3/57.2
Quercetin-4′-Cl	41.4 ± 3.6%	32.4/67.6
Wogonin	34.6 ± 6.7%	18.0/82.0
Kaempferol	22.2 ± 2.7%	22.2/77.8
Compound (3)	13.0 ± 4.9%	46.6/53.4
Luteolin	24.8 ± 2.2%	9.7/91.3
Morin	37.0 ± 8.7%	39.4/44.5/9.4/6.7

sions of substrates with UGT2B7 (n = 3) are shown in Table 2. Quercetin-4′-CH_3 and quercetin-4′-Cl substrate conversions were conspicuous, with rates of substrate conversion of more than 40%, while apigenin, daidzein and compound **3** showed minor glucuronidation activity, less than 20%. Other substrates were moderately catalyzed by human UGT2B7, with substrate conversions between 20% and 40%.

2.3. Regioselectivity of human UGT2B7 in flavonoid glucuronidation

Only one conjugate was detected for daidzein and quercetin-3-OCH_2OCH_3, while the other flavonoid substrates studied here all had two or more conjugates (Tables 1, 2). In this study, it was not possible to determine at which position glucuronidation occurs, but since all the conjugates had different retention times we concluded that daidzein and quercetin-3-OCH_2OCH_3 had to form at least one conjugate. Apigenin, quercetin-4′-CH_3, quercetin-4′-Cl, wogonin, kaempferol, luteolin and compound **3** had at least two conjugates, the percentages of the metabolites being 89.4, 10.6%; 26.1, 73.9%; 32.4, 67.6%; 18.0, 82.0%; 22.2, 77.8%; 9.7, 91.3% and 46.6, 53.4%, respectively based on peak area measured by HPLC-UV. Quercetin and isorhamnetin had at least three metabolites, the percentages of each conjugate being 4.5, 71.8, 23.7% and 32.5, 10.3, 57.2%, respectively. Morin had at least four metabolites detected, percentages being 39.4, 44.5, 9.4, and 6.7% (Table 2).

3. Discussion

In the past, most studies of flavonoid metabolism have been based on human liver microsome or animal experiments (Kim et al. 2004; Hu and Yao 2004), and there are also many reports using recombinant UGT isoforms (Otake et al. 2002; Boersma et al. 2002), but there are few reports about flavonoid substrates for human UGT2B7. The structural differences between the different groups of flavonoids, together with substrate conversion rates, were used to provide insights into the structural elements associated with glucuronidation by UGT2B7. In this study, we showed the possible structural features required for substrate specificity of UGT2B7, and this structure-metabolism relationship was established by visual inspection of the chemical structures.

Of all the flavonoids tested, flavonols generally had higher glucuronidation activity than flavones and isoflavones for human UGT2B7. For example, the substrate conversion rates of quercetin and its derivates were generally higher than 30%, while apigenin and daidzein were lower, and compound **1** and compound **2** had no detectable glucuronidation activity. For flavonols, a bulky group in the C4′ position can enhance glucuronidation activity: quercetin-4′-CH_3 and quercetin-4′-Cl > quercetin, isorhamnetin, quercetin-3-OCH_2OCH_3, kaempferol and morin. Quercetin and isorhamnetin had similar substrate conversion rates, the only difference between the chemical structures being at C3′, which has $-OCH_3$ instead of $-OH$ in the latter. The number of metabolites with UGT2B7 were the same for these two compounds, both having three metabolite peaks detected. The only difference between quercetin and kaempferol is that the latter has $-H$ instead of $-OH$ in the C3′ position, and the glucuronidation conversion rates are conspicuously different, kaempferol being 22.2% while quercetin is 35.1%. This indicates that differences at the C3′ position have an important role in UGT2B7 glucuronidation activity, the various substituents having different influences on glucuronidation activity.

Overall, hydroxyl groups in particular positions and orientations are the factors determining whether a flavonoid is a good substrate for UGT2B7. Generally speaking, increasing the number of hydroxyl groups of flavonoids will increase their glucuronidation activity towards UGT2B7, both for flavones and flavonol: luteolin > apigenin, morin and quercetin > kaempferol, the latter having less hydroxyl groups. Quercetin, luteolin and morin have similar chemical structures including the number of hydroxyls in the B ring, except for their position. Their glucuronidation activity is different: morin (2′ and 4′ $-OH$) > quercetin (3′ and 4′ $-OH$) > luteolin (4′ and 5′ $-OH$). The results show the importance of the position of the flavonoid hydroxyl for UGT2B7 glucuronidation activity. With quercetin, quercetin-3-OCH_2OCH_3 and quercetin-3-*O*-arabinoside, the former had three metabolite peaks detected and the next had only one, while the latter had no activity detected. The only difference between the compounds is at the C3 position: $-OH$, $-OCH_2OCH_3$ and -*O*-arabinoside, respectively. It indicates that a bulky group in this position will weaken glucuronidation activity, and the volume of the bulky group has an important effect on the activity.

In general, binding with a sugar group, such as glucuronic acid, glucose, xylose, rhamnose, or oligosaccharides, gives the aglycone greater water-solubility. Also the glucuronidation catalyzed by UGTs is the process of conjugating with glucuronic acid. The flavonoid glycosides studied here are poor or non-substrates for human UGT2B7. Luteolin-7-glucoside, quercetin-3-*O*-arabinoside and astilbin are non-substrates for human UGT2B7 except for compound **3**, while all have a sugar group. Luteolin can be catalyzed by UGT2B7, but luteolin-7-glucoside cannot. The only difference between the two compounds is the C7-position glucose, the compound with C7 glucose having no glucuronidation activity towards UGT2B7. And the same situation occurs in quercetin and quercetin-3-*O*-arabinoside. It has been reported that the 5-position did not appear to be a site for conjugation (Boersma et al. 2002; Chen et al. 2005), but from Table 1, compound **3** had two conjugates detected while it has only one conjugation site compared with apigenin. This result indicates that the C5 hydroxyl shows conjugate activity as there are only two hydroxyl groups in compound **3**. Thus it may indicate that C5 hydroxyl appears to be a site for glucuronidation under the influence of sugar groups. If there is no sugar group, the substrate will not activate the C5 conjugate site for steric reasons. But the reason why a small volume sugar group can activate C5 hydroxyl conjugation activity requires further research. Even so, binding with glycon, such as pentoses and arabinoside, will weaken glucuronidation activity compared with other flavonoids as indicated in this study.

Quercetin and luteolin have been reported to be effective substrates for UGT2B7 (Boersma et al. 2002). In this experiment, only three glucuronides of quercetin and two of luteolin were detected after incubation with recombinant UGT2B7, although it has been reported that four quercetin monoglucuronides corresponding to the four hydroxyl groups (3-, 7-, 4′-, 3′-, respectively), and three for luteolin (7-, 4′-, 3′-, respectively), were produced by incubation with UGT2B7. The percentages of glucuronides of quercetin and luteolin are also slightly different. This could be explained by differences in recombinant enzyme activity or limits of detection sensitivity, as the 4′-monoglucuronide was very small, less than 5%, and different recombi-

nant enzyme expression systems. For this purpose, more effective expression systems and more sensitive activity detection technologies need to be developed.

In this study it was not possible to determine the position at which glucuronidation occurs, but we can conclude that apigenin, quercetin-4′-CH_3, quercetin-4′-Cl, wogonin, kaempferol, luteolin and compound **3** formed at least two conjugates, quercetin and isorhamnetin each have three conjugates, and morin had at least four metabolites. Flavonoids showed regioselective glucuronidation activity toward UGT2B7, and Day et al. reported that quercetin glucuronides which have different conjugation positions have evidently distinct biological activity (Day et al. 2000). These results suggest that we should not only pay attention to glucuronide formation rates, but also attach importance to the regioselective glucuronidation of flavonoids. Unfortunately for lack of glucuronide standard samples, this study does not propose quantitative regioselectivity of flavonoids. Despite its preliminary character, this study clearly indicates some structure-metabolism relationships in the glucuronide conjugation of flavonoids by human UGT2B7.

4. Experimental

4.1. Chemicals and reagents

Apigenin, daidzein, quercetin, wogonin, kaempferol, luteolin, morin, isorhamnetin, astilbin, luteolin-7-glucoside, quercetin-3-*O*-arabinoside and compound **2** were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and other compounds were obtained from the Department of Pharmaceutical Chemistry (Zhejiang University, Hangzhou, China). Uridine diphosphateglucuronic acid (UDPGA), alamethicin, D-saccharic acid 1,4-lactone, β-glucuronidase were purchased from Sigma (St. Louis, Mo, USA). The Bac-to-Bac baculovirus expression system and Sf9 insect cells were supplied by Invitrogen™. All other chemicals and solvents were of analytical reagent or chromatographic grade and were obtained from common commercial sources.

4.2. Preparation of human UGT2B7 expressed with recombinant baculovirus in Sf9 cells

Spodoptera frugiperda (Sf9) insect cells, were cultured at 27 °C in Grace's medium containing 10% fetal bovine serum, 100000 $U \cdot L^{-1}$ penicillin and 100000 $U \cdot L^{-1}$ streptomycin. The recombinant baculovirus (Bacmid-UGT2B7) was amplified from monolayer cultures of Sf9 insect cells, transfected to Sf9 insect cells at 27 °C in Grace's medium and incubated for 72 h, and the cells were then harvested. Cells were disrupted by a standard optimized sonication method (Qian et al. 2004). The concentration of protein was determined by the method of Lowry et al. (1951), and the protein was stored at −80 °C until use.

4.3. HPLC analysis

Analysis was performed on a Shimadzu (Kyoto, Japan) LC-10A 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 appropriate proportions of methanol and 0.2% phosphoric acid (pH = 2), and the flow rate was 1.0 $mL \cdot min^{-1}$. Each flavonoid and its metabolites were detected at the same wavelength. HPLC conditions of the flavonoids are shown in detail in Table 1. The HPLC/MS system (Esquire, Bruker, Germany) was equipped with an electrospray mass spectrometric detector, the same column was used, and the mobile phase consisted of appropriate proportions of methanol and acetic acid solution (pH = 2) at a flow rate of 0.5 $mL \cdot min^{-1}$.

4.4. Analysis of the glucuronidation activity of human UGT2B7

A typical incubation mixture (100 μL of total volume) contained 100 $mmol \cdot L^{-1}$ Tris-HCl (pH 7.81), 10 $mmol \cdot L^{-1}$ $MgCl_2$, 10 $mmol \cdot L^{-1}$ alamethicin, 5 $mmol \cdot L^{-1}$ D-saccharic acid 1,4-lactone, 1 $g \cdot L^{-1}$ recombinant UGT2B7. Rate of substrate conversion was optimized for linearity with respect to incubation time. The mixture without UDPGA was perincubated at 37 °C in a water bath for 5 min, and then the reaction was initiated by the addition of 5.0 $mmol \cdot L^{-1}$ UDPGA and incubated for 60 min. Controls were incubated in the absence of UDPGA in parallel at the same time. To terminate the reaction, 100 μL of cold methanol was added, and the samples were centrifuged at 10,000 rpm for 6 min. Then 20 μL of the supernatant was subjected to HPLC analysis. The glucuronides of the flavonoids were confirmed by hydrolysis with β-glucuronidase overnight and HPLC/MS.

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