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Regioselective glucuronidation of the isoflavone calycosin by human liver microsomes and recombinant human UDP-glucuronosyltransferases

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ABSTRACT

Hepatic conjugation plays important roles in systemic exposure and drug interactions of flavonoids. In the present study, the hepatic metabolism of calycosin, a major isoflavone from Astragali Radix, was characterized and the regioselectivity in the predominant glucuronidation pathway was first delineated in human liver microsomes (HLMs) and a panel of recombinant human UDP-glucuronosyltransferases (UGTs). Calycosin underwent major glucuronidation and minor oxidation and sulfation in human liver subcellular fractions. The major glucuronide (G2) of calycosin was isolated and identified as calycosin 3′-glucuronide by NMR analysis, and thus, the minor glucuronide (G1) was tentatively assigned as calycosin 7-glucuronide. The formations of both glucuronides in HLMs fit typical Michaelis–Menten kinetics. HLMs exhibited higher affinity (K_m, G2 12.37 ± 1.20 μM *vs* G1 40.90 ± 5.51 μM) and velocity (V_{max}, G2 5.39 ± 0.13 nmol/min/mg protein *vs* G1 2.80 ± 0.13 nmol/min/mg protein) on G2 formation, leading to the intrinsic clearance of calycosin via 3′-glucuronidation 6 times that through 7-glucuronidation. UGT1A1, 1A3 and 1A10 showed activities on both 3′-OH and 7-OH, whereas UGT1A7, 1A8, 1A9, and 2B7 were only capable of catalyzing 3′-OH glucuronidation of calycosin. Among them, UGT1A9 exhibited the highest activity (Cl_{int}, 2169.50 μL/min/mg protein) for 3′-glucuronide formation followed by UGT1A7 (Cl_{int}, 396.38 μL/min/mg protein). UGT1A1 showed the highest activity towards 7-OH glucuronidation (Cl_{int}, 224.34 μL/min/mg protein), which was comparable to its activity on 3′-OH glucuronidation (Cl_{int}, 203.82 μL/min/mg protein). Propofol (UGT1A9 inhibitor) produced a complete inhibition of 3′-glucuronide formation accompanied by an increase of 7-glucuronide in HLMs, while bilirubin (UGT1A1 inhibitor) only partially (~60%) inhibited the 7-OH glucuronidation. These findings demonstrated the regioselective glucuronidation at the 3′-OH of the isoflavone calycosin in HLMs and shed light on potential drug interactions of calycosin with other UGT1A9 substrates.

1. Introduction

Flavonoids are ubiquitously present in fruits, vegetables, plants derived beverages, dietary supplements and herbal medicines. They have attracted considerable research interests due to high consumption in daily life and a growing body of evidence on their beneficial activities on human health [1–3]. Generally, flavonoid compounds exhibit very low bioavailability due to extensive first-pass metabolisms, in particular, intestinal and hepatic glucuronidation [4,5]. Recently, the reports demonstrating that glucuronidated metabolites of some flavonoid compounds, such as quercetin, genistein, and daidzein, were equally or even more pharmacologically active than the parent compounds [5–7] have boosted research interests on pharmacological evaluation of the phase II conjugates of the flavonoids. In the past two decades, the structure–glucuronidation relationship (SGR) of flavonoids has been extensively reported [8], which provides important information for biosynthesis of flavonoid glucuronides with desired biological activities for pharmaceutical purposes as well as prediction of drug interactions.

Isoflavones are a distinctive subclass of flavonoids of which the ring B is attached to the ring C at the C-3 position. Isoflavones are

Abbreviations: HLMs, human liver microsomes; UGTs, UDP-glucuronosyltransferases; SGR, Structure–glucuronidation relationship; HPLC, high performance liquid chromatography; NADPH, nicotinamide adenine dinucleotide phosphate reduced; CYP450s, cytochrome P450s; PAPS, 3′-phosphate 5′-phosphosulfate; UDPGA, uridine 5′-diphosphoglucuronic acid; ROESY, rotating frame nuclear Overhauser effect spectroscopy.

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well-known antioxidants with a wide spectrum of biological activities, such as anticancer, cardiovascular protection, and anti-inflammation [9–11]. Structurally similar to the estrogen estradiol, isoflavones are called phyto-oestrogens and have demonstrated health benefits to woman health [12,13]. However, comparing to other types of flavonoids, such as flavones and flavonols, there is relatively insufficient information on the SGR of isoflavones.

As one of the main isoflavones existing in nature, calycosin and its glycosides have been reported in several plants, including Astragali Radix, a well-known medicinal herb commonly prescribed in Asian countries for centuries due to its *Qi*-tonifying effects. A recent study in our laboratory revealed 421 flavonoids from Astragali using a multiple reaction monitoring (MRM)-based strategy [14], among which, more than 100 are isoflavones, including calycosin and its 81 glycosides. Calycosin-7-*O*-*β*-D-glucoside, the most abundant flavonoid in Astragali Radix, has been recently documented in China Pharmacopoeia as the chemical marker for quality control of the herb [15]. It has been well documented that [16,17] flavonoid glycosides usually subject to hydrolysis by microflora in gut lumen prior to absorption. Thus, the aglycone calycosin is likely one of the main forms of Astragali flavonoids that are absorbed across the intestinal epithelium and then extensively conjugated in the liver before eliciting the pharmacological activities *in vivo*. Characterization of the metabolism, in particular, glucuronidation of calycosin will definitely not only add valuable information to the existing literature on the SGR of flavonoids, but also help understand the beneficial activities and potential drug interactions of calycosin and its glycosides and the herbs containing these compounds as the main components, such as Astragali Radix.

There are very few reports addressing *in vitro* or *in vivo* metabolism of calycosin. Calycosin glucuronides and sulfates were detected in urine and plasma samples from rats receiving an oral dose of calycosin-7-*O*-*β*-D-glucoside [18,19]. When an Astragali Radix extract was orally administered to rats or a healthy volunteer, glucuronidated and sulfated calycosin were detected in the urine samples [20]. Our previous study [21] also revealed that calycosin dominantly underwent phase II reactions (glucuronidation, sulfation, glycosylation) in zebrafish larvae. A recent *in vitro* study [22] reported two hydroxylated metabolites of calycosin in HLMs. In another study using rat hepatic S9 fraction [18], a number of metabolites formed via hydroxylation, dimerization, dehydroxylation and glucuronidation were tentatively identified by IT-TOF-MS^n. To our best knowledge, a systematic investigation of the most important hepatic phase II biotransformation, in particular, the glucuronidation, of calycosin in humans is still lack.

Therefore, in the present study, the *in vitro* hepatic metabolism including cytochrome P450s (CYP450s)-mediated oxidation, sulfation and glucuronidation of calycosin in human liver subcellular fractions were preliminarily examined. The regioselectivity in the dominant glucuronidation pathway was further delineated through structural elucidation of the major glucuronide using NMR analysis. Finally, enzyme kinetics of calycosin in HLMs as well as several main human recombinant UDP-glucuronosyltransferase (UGT) enzymes was performed in order to understand the enzyme-catalyzing mechanism and predict drug interactions with calycosin.

2. Materials and methods

2.1. Materials

Calycosin (purity > 95%) was purchased from Shanghai Forever Biotech Co. Ltd. (Shanghai, China). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise mentioned. Formic acid and methanol of high performance liquid chromatography (HPLC) grade were purchased from Merck (Darmstadt, Germany). Deionized water was purified by a Milli-Q purification system (Millipore, Bedford, MA). Recombinant human UGT Supersomes (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17) expressed in baculovirus-infected insect cells were purchased from BD Gentest (Woburn, MA). HLMs and human liver S9 (cytomegalovirus-seronegative mixed gender pools) were obtained from Sigma (St. Louis, MO) and stored at −80 °C. Pooled rat liver microsomes used for the preparation of the major glucuronide of calycosin were prepared from 30 Sprague–Dawley rats (male, 250–300*g*) by differential centrifugation according to a standard procedure [23]. The protein content was determined by Lowry's method [24].

2.2. Metabolism of calycosin by human liver subcellular fractions

2.2.1. CYP450s-mediated biotransformation

The reaction mixture contained a nicotinamide adenine dinucleotide phosphate (NADPH)-regenerating system (4 mM $MgCl_2$, 1 mM $NADP^+$, 1 mM glucose-6-phosphate and 1 U/mL glucose-6-phosphate dehydrogenase) and pooled HLMs (1 mg/mL) in 100 mM potassium phosphate buffer (pH 7.4) in a total volume of 0.2 mL. Reactions were initiated by adding glucose-6-phosphate dehydrogenase and kept at 37 °C for 60 min.

2.2.2. Sulfation

Calycosin was incubated in a 0.2 mL of reaction mixture containing 5 mM $MgCl_2$, 8 mM dithiothreitol, 0.0625% bovine serum albumin and 0.8 mg/mL of pooled human liver S9 in 50 mM Tris–HCl buffer (pH 7.4) at 37 °C. The reaction was initiated by addition of 200 μM of 3′-phosphate 5′-phosphosulfate (PAPS) and kept for 60 min.

2.2.3. Glucuronidation

Calycosin was incubated with 8 mM $MgCl_2$, 25 μg/mL alamethicin, and 0.1 mg/mL HLMs in 50 mM Tris–HCl buffer (pH 7.4) in a total volume of 0.2 mL. The reaction was initiated by adding 2 mM uridine 5′-diphosphoglucuronic acid (UDPGA) and kept for 10 min at 37 °C.

Calycosin was dissolved in DMSO and the final concentration is 12.5 μM. DMSO is 1% (v/v) in all reactions. Zero-minute incubations and reactions without the cofactor (NADPH-regenerating system, PAPS, and UDPGA, respectively) or with denatured (heating at 95 °C for 10 min) human liver S9 or microsomal proteins served as controls. Three independent experiments were conducted for each reaction.

2.3. Glucuronidation activity of human UGTs towards calycosin

Glucuronidation activity of calycosin in 12 recombinant human UGTs (UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) was measured at two substrate concentrations (15 and 50 μM, the approximate K_m values for G1 and G2 formations in HLMs, respectively) in the aforementioned glucuronidation reaction system which contained 0.1 mg protein/mL individual recombinant human UGTs instead. All reactions were conducted at 37 °C for 10 min and repeated 3 times.

2.4. Kinetics of calycosin glucuronidation in pooled HLMs and recombinant human UGTs

Kinetics of calycosin glucuronidation was determined under the initial rate conditions. Briefly, calycosin (1–200 μM) was incubated with 0.1 mg/mL of pooled HLMs or selected individual UGT Supersomes (UGT 1A1, 1A3, 1A7, 1A8, 1A9 and 1A10) in the presence of