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Involvement of UDP-glucuronosyltranferases and sulfotransferases in the liver and intestinal first-pass metabolism of seven flavones in C57 mice and humans *in vitro*

Lan Tang [1], Qian Feng [1], Jie Zhao, Lingna Dong, Wei Liu, Caihua Yang, Zhongqiu Liu *

Department of Pharmaceutics, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, Guangdong 510515, China

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ABSTRACT

The present study investigated the involvement of UDP-glucuronosyltransferase and sulfotransferase in the extensive liver and intestinal first-pass glucuronidation and sulfation of flavones in both mice and humans. Seven structurally similar mono- and di-hydroxyflavones were chosen as model compounds. Human liver, C57 mouse liver and intestinal S9 fraction, as well as C57 intestinal perfusion model were used. In human and C57 mouse, all selected flavones were found to be glucuronidated with the highest rates at the 7-OH group. In contrast, flavones with 3-OH group were not sulfated at all. Both glucuronidation and sulfation preferred 4′- and 7-OH in human and mouse *in vitro* and in situ. There were differences in glucuronidation and sulfation in human and mouse observed for all flavones and it is based on substitutional positions of the hydroxyl groups. The S9 fractions could accurately model glucuronidation (as the slope of correlation curve was 0.7988 for those flavones with 4′- or 7-OH) and sulfation (as the slope of correlation curve was 0.9834) in situ. Conclusively, the sulfation and glucuronidation of the flavones was regiospecific- and speciesdependent. Sulfation and glucuronidation in the mouse intestine *in vitro* were correlated well with those in situ.

1. Introduction

Flavones, widely found in food and herbal products, possess a variety of potentially significant biological properties, including anti-inflammatory, anti-allergenic, antiviral, antioxidant effects, and anti-carcinogenic effects, *in vitro* and *in vivo* (Benavente-Garcia and Castillo, 2008; Birt et al., 2001; Thomasset et al., 2007). Despite of these claimed bioactivities, flavonoids have poor bioavailability (less than 5%) and induce or inhibit some metabolic enzymes and transporters (Brand et al., 2006; Chi and Kim, 2005; Galijatovic et al., 2000). Consequently, the metabolism of flavonoids in humans is extremely complex and may affect their efficiency and safety.

The major extensive first-pass metabolism of flavonoids includes glucuronidation, sulfation, and methylation (Day et al., 2001; de Vries et al., 1998; Kroon et al., 2004; Manach and Donovan, 2004; Moon et al., 2000; Wang et al., 2006; Zhang et al., 2007). In rat plasma, a significant portion of absorbed aglycones, such as fisetin and 7-monohydroxyflavone (HF), are rapidly biotransformed into sulfates or glucuronides (Shia et al., 2009). In other flavonoids such as kaempferol (DuPont et al., 2004) and baicalein (Akao et al., 2000), glucuronides are also the predominant form in systemic circulation after oral administration. An intestinal absorption study of the flavone quercetin in rats has demonstrated that only conjugated forms (glucuronides and sulfates) are present in mesenteric vein blood (Crespy et al., 2001). Flavonoids could also be extensively metabolized via glucuronidation and sulfation (Jia et al., 2004; Setchell et al., 2001).

Much more attention is paid to the glucuronidation of flavonoids than to their sulfation (Joseph et al., 2007; Liu et al., 2007; Wang et al., 2006, 2009). Nevertheless, both pathways may be equally important, especially in the intestines. Although more glucuronides than sulfates are found in human plasma (Jeong et al., 2005), the flavonoid sulfates and glucuronides are often found in similar quantities in human intestinal Caco-2 cells and

Abbreviations: 3-HF, 3-hydroxyflavone; 4′-HF, 4′-hydroxyflavone; 5-HF, 5-hydroxyflavone; 7-HF, 7-hydroxyflavone; 3,5-diHF, 3,5-dihydroxyflavone; 3,7-diHF, 3,7-dihydroxyflavone; 3,4′-diHF, 3,4′-dihydroxyflavone; BCRP, breast cancer resistance protein; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EtOH, ethanol; HF, hydroxyflavone; IS, internal standard; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; P-gp, permeability glycoprotein; PMSF, phenylmethylsulfonyl fluoride; Sult, sulfotransferase; UDPGA, uridine-5′-diphosphoglucuronic acid; Ugt, UDP-glucuronosyltransferase; UPLC, ultra performance liquid chromatography.

* Corresponding author. Address: 1838 North Guangzhou Avenue, Department of Pharmaceutics, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China. Tel./fax: +86 20 61648596.
E-mail address: liuzq@smu.edu.cn (Z. Liu).

[1] These authors contribute equally to this work.

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mouse intestines. Among all reported flavonoids, the first-pass metabolism of quercetin is the best studied. In an intestinal absorption study on quercetin in rats, only the conjugated forms (glucuronide or sulfate conjugate) are found in mesenteric vein blood (Crespy et al., 2001). Almost all the metabolites of quercetin in the plasma are its sulfate/glucuronide conjugates. After oral quercetin administration in humans, quercetin-3-glucuronide, quercetin-3′-sulfate, and isorhamnetin-3-glucuronide are mainly found in systemic blood with no significant amounts of free quercetin (Janisch et al., 2004). Nonetheless, sulfation is an important phase II reaction for the metabolism of numerous xenobiotics, drugs, and endogenous compounds. Therefore, the characteristics of both glucuronidation and sulfation *in vivo* and *in vitro* need to be investigated. Ronis reported the sulfation of the isoflavones, genistein, and daidzein in human and rat livers and their gastrointestinal tract (GI) and demonstrated that metabolism in the human GI tract has an important role in the generation of potentially bioactive isoflavone sulfates and a major role for the human phenolic sulfotransferase SULT1A family in genistein metabolism in the gut (Ronis et al., 2006). Van der found the identification of a wide range of phase II quercetin metabolites and opens the way for a better assessment of the biological activity of quercetin in humans by different *in vitro* models of rat and human liver and intestines, including cell lines, S9 samples, and hepatocytes (van der Woude et al., 2004). Our previous studies have revealed the characteristics of the glucuronidation of seven monoHFs (3-, 5-, 6-, 7-, 2′-, 3′-, and 4′-monohydroxyflavones) and five diHFs in expressed human UGTs and found that UGT1A1, 1A8, 1A9, 1A10, and 2B7 are the main UGT isoforms responsible for metabolizing flavones. Zhang et al. (2006) reported the positional preference of the glucuronidation of seven monoHFs in the S9 fraction of human intestines. However, information on the sulfation characteristics and comparison of the glucuronidation and sulfation of monohydroxyl and dihydroxyl flavones in human liver is very limited. The current study aims to investigate both the glucuronidation and sulfation of seven selected flavones in an *in vitro* human liver S9 fraction model.

The liver S9 fraction was used because it is a routine model system used in phase II metabolic studies involving sulfation, and the liver is rich in both Ugts and sulfotransferases or Sults.

Given that glucuronidation and sulfation of 7-hydroxycoumarin in liver matrices from humans, dogs, monkeys, rats, and mice are different (Wang et al., 2005), the S9 liver fraction of C57 mice were used to compare specific differences. In addition, the two conjugating pathways were further investigated using C57 mouse intestinal S9 fraction. The regional phase II metabolism of flavones in an in situ model in C57 mice were also compared with the glucuronidation and sulfation rates of flavones *in vitro* reaction systems to determine if S9 fractions accurately model glucuronidation and sulfation *in vivo*. Mice were chosen because earlier studies have indicated a higher formation of sulfate in their intestinal perfusate than in rats. Additionally, C57 is the most widely used inbred strain, and the first to have its genome sequenced. It has a permissive background for the maximal expression of most mutations. The seven flavones (3-, 4′-, 5-, and 7-monoHFs, and 3, 4′-, 3,5-, and 3,7-diHFs) were chosen because they share similar structural characteristics (Fig. 1). This similarity allowed us to determine how minor changes in chemical structures affected their metabolism and excretion of phase II conjugates. Hence, the present study investigates the potential characteristics of sulfation and glucuronidation in flavones in situ and *in vitro*.

2. Materials and methods

2.1. Materials

All the four mono-hydroxyflavones and three di-hydroxyflavones were purchased from LC Laboratories (Woburn, MA). Uridine diphosphoglucuronic acid (UDPGA), β-glucuronidase, alamethicin, magnesium chloride, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), and Hanks' balanced salt solution (HBSS, powder form) were purchased from Sigma–Aldrich (St. Louis, MO). Human male pooled liver S9 fractions (Cat. No. 452961, 16 donors) were purchased from BD Gentest. All other materials were analytical grade or better.

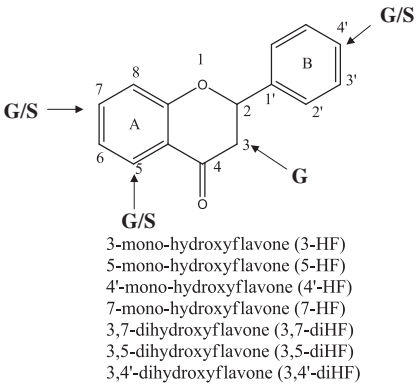

Fig. 1. Structure of model compounds. Shown in the scheme are the structures of the aglycone forms of the hydroxyflavone analogs. Conjugated phase II metabolites are formed on the 5,7-hydroxy group on the A ring, 4′-OH group on the B ring, and 3-OH group on the C ring. "G" and "S" represents the glucuronidation and sulfation sites for each hydroxyl group.

2.2. Mice and diets

Male C57 mice (7–8 wk old), 22–26 g body wt, were purchased from Southern Medical University (Guangzhou, China). The animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health, and the procedures were approved by the Ethical Committee of Southern Medical University. Mice were individually and kept in a controlled environment at 22 °C under a 12-h-light/12-h-dark cycle. All mice had free access to water and standard diet (from the Experimental Animal Center of Southern Medical University) for 1 week to get acclimated and were fasted for 8 h before each experiment.

2.3. Preparation of the solutions for experiments

The flavonoids compounds were weighed to pre-determined weights and dissolved in 20% dimethyl sulfoxide (DMSO) in 80% ethanol (EtOH) to the concentration of 5 mM then used as stock solutions. Before the mouse perfusion experiments, the stock solutions were diluted with HBSS. The formulation of the HBSS was as follows: HBSS powders 9.801 g, $NaHCO_3$ 0.372 g, glucose 3.502 g, HEPES 5.963 g, and NaCl 1.164 g, all the above were dissolved by distilled water and adjusted the pH to 6.5, and filtered with 0.2 μm filter before use.

For experiments determining the effects of concentrations on sulfation by liver Human S9 fraction, additional stock solutions were prepared using 20% DMSO (80% EtOH) so that these stock concentrations were 1000× more concentrated than the targeted concentrations used in the experiments. This is done to ensure that each reaction mixture has the same organic solvent composition.

2.4. Mouse liver S9 fraction preparation

Male mouse liver S9 fraction was prepared using a procedure published previously with minor modification (Chen et al., 2004; Okita et al., 1993). Briefly, 10 freshly harvested mouse livers were washed and then perfused with ice-cold saline, weighed and minced. Minced livers were homogenized using a motorized homogenizer (4 strokes) in ice-cold homogenization buffer (50 mM potassium phosphate, 250 mM sucrose, 1 mM EDTA, pH 7.4) and centrifuged at 7700g for 15 min at 4 °C. Fat layer was carefully aspirated, and the supernatant was collected with pasture pipettes into microfuge tubes (1 ml each), which were stored at −80 °C until use. The concentration of S9 fraction protein (normally 5–20 mg/ml) was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using the bovine serum albumin as the standard.

2.5. Mouse intestinal S9 fractions preparation

This method was adapted from a previously published method to prepare S9 fraction (Chen et al., 2003). Briefly, the small intestines of 10 mice were collected and washed in ice-cold solution containing 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 1.5 mM ethylenediaminetetraacetate (EDTA), and 0.5 mM DTT and 0.04 g/L PMSF. In a low temperature room, the mucosal surface of small intestines were scratched off gently and suspended into 10 mL homogenization buffer, which consists of