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Identification of human UDP-glucuronosyltransferase isoforms involved in the isofraxidin glucuronidation and assessment of the species differences of the reaction

Fanxing Meng [a], Yan Li [b], Guiyuan He [b], Guangbo Ge [b], Shumin Liu [a,*]

[a] Chinese Medicine Toxicological Laboratory, Institute of Traditional Chinese Medicine, Drug Safety Evaluation Center, Heilongjiang University of Chinese Medicine, Harbin 150040, China
[b] Laboratory of Pharmaceutical Resource Discovery, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

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ABSTRACT

Isofraxidin, 7-Hydroxy-6.8-dimethoxy-2*H*-1-benzopyran-2-one, is a major active component of *Acanthopanax senticosus*, which has been used as *Acanthopanax* (Ciwujia) injection to treat cardiovascular and cerebrovascular diseases in China for more than thirty years. The purpose of this study was to identify the roles of human UDP-glucuronosyltransferases (UGTs) in isofraxidin glucuronidation in the liver and intestinal microsomes and to reveal the potential species differences by comparing the liver microsomal glucuronidation from different experimental animals. One metabolite was biosynthesized and characterized as isofraxidin-7-O-glucuronide by liquid chromatography tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR). The intrinsic clearances in human liver and intestinal microsomes were 63.8 and 16.4 μL/min/mg, respectively. Human liver microsomes displays higher potential for isofraxidin elimination than human intestinal microsomes. The reaction phenotyping analysis was conducted using cDNA-expressed human UGTs and chemical inhibitors. The results indicated that UGT1A1 and UGT1A9 were the main isoforms involved in the formation of isofraxidin-7-O-glucuronide. The isofraxidin glucuronidation in liver microsomes from human (HLM), rat (RLM), mouse (MLM), dog (DLM), monkey (CyLM), minipig (PLM), and guinea pig (GpLM) followed the Michealis-Menten model. The isofraxidin glucuronidation displays species differences in terms of catalytic activities. GpLM had the highest clearance with the CL_{int} value of 152 μL/min/mg. CyLM, RLM and MLM exhibit similar catalytic activities in isofraxidin glucuronidation with the intrinsic clearance values of 54.6, 58.0 and 50.2 μL/min/mg, respectively, which are higher than those of PLM and DLM (23.9 and 37.7 μL/min/mg, respectively). Rat exhibits the most similar intrinsic metabolic clearance (CL_{int}) to human.

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1. Introduction

Acanthopanax senticosus is widely distributed in northeastern China, Korea, Japan, and far-eastern Russia [1]. The extracts of the active ingredients of *Acanthopanax senticosus* were prepared into *Acanthopanax* injection that was approved in 1983 in China [2]. The formulation can be applied to treat transient ischemic attack, intracranial arteriosclerosis, intracranial thrombosis, intracranial embolism, coronary disease, angina pectoris, neurasthenia, and menopausal syndrome in the clinic. Despite widespread use, the injection form could possibly lead to anaphylactic shock due to the complex chemical composition of *Acanthopanax*, which is suggested to be used in the oral dosage form as much as possible [3].

Isofraxidin, 7-hydroxy-6, 8-dimethoxy-2*H*-1-benzopyran-2-one, is a coumarin compound identified as one of the major active constituents in *Acanthopanax senticosus* [4,5]. The pharmacokinetic characteristics of isofraxidin as a pure compound after intravenous infusion were compared with those of the *Acanthopanax* injection in rats. The results showed that the area under the concentration-time curve (AUC) and clearance (CL) of the monomer are not statistically different with those of the injection at the equal isofraxidin concentration of 14 mg/L. [6]. However, the pharmacokinetic study has been demonstrated that the AUC of isofraxidin after oral administration of *Acanthopanax senticosus* extract is about two times more than that of the same dosage of pure isofraxidin at 7.5 and 15 mg/kg in rats. This is possibly due to the behavior of the precursors of isofraxidin in *Acanthopanax senticosus* extract. The precursors, such as eleutheroside *B*1, can be metabolized into isofraxidin in vivo [7]. It is indicated that this biotransformation of the precursors could increase the exposure and prolong the elimination of isofraxidin in the oral dosage form of

* Corresponding author.
E-mail address: keji-liu@163.com (S. Liu).

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Acanthopanax senticosus extract. Hence, more attention should be focused on the disposition of isofraxidin in vivo in further research.

Isofraxidin was reported to be metabolized mainly through *O*-demethylation, hydroxylation, and ring-opening metabolites by CYP3A, CYP2C and CYP2E1 in rat liver microsomes [8]. Two phase I metabolites were identified as 7.8-dihydroxyl-6-methoxycoumarin and 6.7-dihydroxyl-8- methoxycoumarin in rat plasma [7]. Isofraxidin glucuronidation and sulfonation metabolites were detected in rat plasma. The glucuronide, as one circulating metabolite of the orally administrated constituent, was also observed in rat bile, but not in rat urine and feces. This may be another reason for the AUC increasing of isofraxidin after orally administration which via the hydrolysis of intestinal bacteria on isofraxidin glucuronide [9]. Due to the structural characteristics of the coumarins, isofraxidin could be a good substrate for human UDP-glucuronosyltransferases (UGTs) [10–12]. However, no metabolism data are available on the glucuronidation of isofraxidin in human.

Glucuronidation serves as an essential clearance and detoxification mechanism for exogenous compounds such as drugs, dietary constituents, and environmental pollutants, as well as endogenous compounds such as bile acids, bilirubin, steroids, and neurotransmitters [13]. In humans, approximately 35% of all drugs metabolized by phase II enzymes are subjected to glucuronidation reactions catalyzed by UGTs [14]. To date, 22 human UGTs are known [15]. The most clinically important hepatic and extra-hapatic UGTs are UGT 1A and 2B subfamilies [16]. Glucuronidation occurs to some extent in all mammalian species. Studies have indicated that glucuronidation exhibits remarkable species differences in the UGT expression and activity between humans, mice, rats, dogs and monkeys [17–19]. The in vitro metabolism study on glucuronidation is still the most effective and useful method to understand the factors affecting the pharmacokinetic behaviors of the chemical and to select the suitable experiment animal model to conduct pharmacokinetic or pharmacodynamic studies [20–22].

The aim of this study was to reveal the in vitro glucuronidation metabolism behaviors of isofraxidin. The glucuronide of isofraxidin was identified in vitro by liquid chromatography tandem mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR). The contribution of the involved UGT isoforms to glucuronidation was investigated using recombinant UGTs as well as human liver and intestinal microsomes. The species differences were compared with the intrinsic clearance of isofraxidin via the glucuronidation pathway by using liver microsomes from mouse, rat, dog, monkey, minipig and guinea pig.

2. Materials and methods

2.1. Chemicals and reagents

Isofraxidin (purity > 98%) was purchased from Weikeqi Biological Technology.

Co., Ltd. (Sichuan, China). Brij 58, β-glucuronidase (EC No. 3.2.1.31), uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), nilotinib, niflumic acid and fluconazole were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hecogenin was purchased from Tokyo Chemical Industry (TCI) Co. Ltd. (Tokyo, Japan). Human liver microsomes ($n = 50$), human intestinal microsomes ($n = 9$), Sprague-Dawley rat liver microsomes ($n = 100$), ICR/CD1 mouse liver microsomes ($n = 500$), Cynomolgus monkey liver microsomes ($n = 3$), Beagle dog liver microsomes ($n = 3$), Minipig liver microsomes ($n = 2$), Guinea pig liver microsomes ($n = 5$) and New Zealand rabbit liver microsomes ($n = 2$) were purchased from Research Institute for Liver Diseases (Shanghai, China). A panel of recombinant human UGT isoforms (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17) expressed in baculovirus-infected insect cells was purchased from Corning Life Sciences (Tewksbury, MA, USA). All the organic solvents with HPLC purity were of Fisher Scientific (Fair Lawn, NJ, USA). All other reagents were of analytical grade. The deionized water was purified using the Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Identification of isofraxidin glucuronidation

The incubation system contained 200 μM isofraxidin, 5 mM $MgCl_2$, 0.5 mg/mL microsomal protein and 4 mM UDPGA in 50 mM Tris-HCl buffer (pH 7.4). The microsomes were fully activated by the addition of Brij 58 (0.5 mg/mg protein) for 20 min. All samples were pre-incubated at 37 °C for 3 min before reactions were initiated by addition of UDPGA. After incubation for 120 min at 37 °C, the reactions were terminated with equal volume of ice-acetonitrile. Then the mixtures were centrifuged at 20,000 × *g* for 20 min at 4 °C. The supernatant was analyzed by UFLC-UV. The control groups without the cofactor or without substrate or without microsomes were also performed.

The formation of isofraxidin glucuronide was confirmed by co-incubating human liver microsomes with β-glucuronidase. After reaction for glucuronidation in 200 μL incubation mixture, the equal volume of 0.15 M acetate buffer (pH 4.5) with or without β-glucuronidase (4000 Fishman units) was added and then the reaction was carried out at 37 °C for 60 min. The reaction was terminated by the addition of 200 μL ice-acetonitrile and centrifuged at 20,000 × *g* for 20 min. The supernatant was analyzed by UFLC-UV. All incubations were performed in duplicate.

2.3. Biosynthesis of isofraxidin glucuronide

Isofraxidin glucuronide was biosynthesized by using rabbit liver microsomes and purified for structure elucidation and quantitative analysis. 100 mM isofraxidin was incubated with rabbit liver microsomes (0.5 mg/mL microsomal protein), 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, Brij 58 (0.5 mg/mg protein), and 4 mM UDPGA in 50 mL

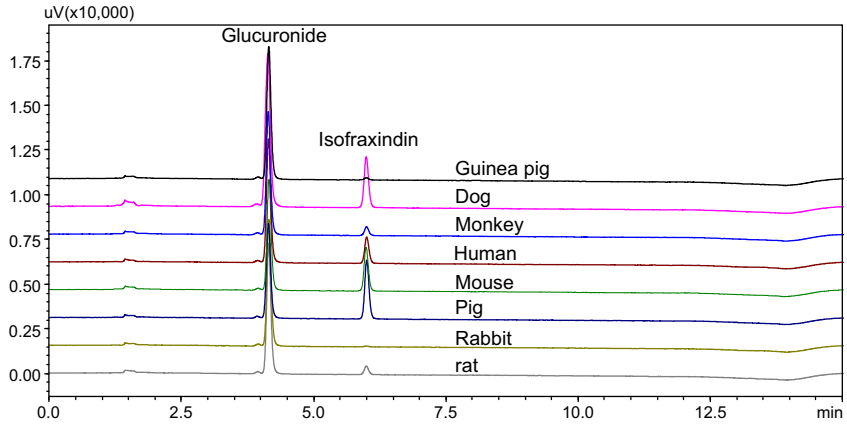

Fig. 1. Representative LC choromatogram of isofraxidin and its glucuronide in liver microsomes from different species.

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