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Original article

Involvement of UDP-glucuronosyltransferases in higenamine glucuronidation and the gender and species differences in liver

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ABSTRACT

Objectives: Higenamine (HG), an active ingredient of Aconite root in Chinese herbal medicine, is mainly metabolized by UDP-glucuronosyltransferases (UGT). However, the systematic glucuronidation of HG in humans remains unclear. The purpose of this study was to investigate the glucuronidation of HG.
Methods: 12 recombinant human UGT (rUGT) isozymes were used to characterize the HG glucuronidation. Liver microsomes from male and female mice, rats, guinea pigs, dogs, and humans were used to determine the species and gender differences using liquid chromatography-mass spectrometry.
Key findings: One monoglucuronide was detected in reactions catalyzed by rUGT1A6, rUGT1A8, rUGT1A9, also human and dog liver microsomes. UGT1A9 is the most important glucuronosyltransferase that metabolizes HG. Because carvacrol, a specific inhibitor of UGT1A9, can significantly decrease the glucuronidation of HG in Human liver microsomes and UGT1A9. HG metabolism by UGT1A9 described in Michaelis-Menten kinetics (K_m = 15.4 mM, V_{max} = 2.2 nmol/mg/min) and glucuronidation in liver microsomes were species dependent. Gender did not affect the kinetic parameters among species except in rats.
Conclusions: UGT1A9 is a major isoenzyme responsible for the glucuronidation of HG in Human liver microsomes (HLMs). Dog may be an appropriate animal model to evaluate HG metabolism.

1. Introduction

Higenamine (1-[(4-hydroxyphenyl)methyl]-1,2,3,4-tetrahydroisoquinoline-6,7-diol) (HG) is naturally found in various plants, including *Aconitum carmichaelii* (root), *Nelumbo nucifera* (lotus seeds), *Annona squamosa, Nandina domestica* (fruit), *Asarum heterotropoides*, and *Galium divaricatum* (stem and vine) [1]. Some of these natural plants are known as "medicine food homology." For example, *N. nucifera* is widely used in functional and nutritious foods and beverages in China. However, to date, the safety and the underlying mechanisms of HG metabolism have not been established. Thus far, data related to the potential efficacy of this herbal ingredient should be obtained.

HG is an active ingredient traditionally used as a cardiac inotropic and anti-inflammatory agent in Oriental countries. HG also protects brain cells against hypoxic damages by upregulating HO-1, indicating that this substance can be potentially used to treat ischemic injuries, such as stroke [2]. HG can also inhibit platelet aggregation and elicit anti-thrombotic effects [3]; these biological processes further demonstrate that HG may possibly exhibit beneficial effects on hypoxic injuries. This substance also reduces ischemic/reperfusion-induced myocardial damage in rats [4], stimulates cardiac β-adrenoceptor via vasodilation by interacting with α-adrenoceptor, and represents a new agent that can be used to evaluate pharmaceutical stress [3,5]. Considering these findings, researchers have been prompted to investigate the application of HG as a therapeutic ingredient. Now researchers have just accomplished the phase III clinical studies successfully in China. And well tolerability was shown among healthy Chinese subjects [6].

Despite these pre-clinical and clinical activities, significant challenges associated with the drug safety evaluation of HG

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remain. Limited information regarding the metabolic characteristics of HG is available. In another study, the pharmacokinetic and metabolic characteristics of HG in rabbits have been evaluated; results indicated that the hydrolysis of urine samples with beta-glucuronidase significantly increases HG concentrations in the urine of experimental groups regardless of the administration route [intravenous (i.v.) or oral] [7]. The parent drug and its conjugate excreted in urine are approximately 20% to 40% of the applied doses, respectively. After bolus containing 20 mg/kg HG is administered i.v. to rabbits, approximately 6% of HG as conjugated metabolites may be excreted by bile. Eight distinct urinary metabolites are isolated from pooled urine samples obtained at 24 h after oral administration; some of these metabolites include one diglucuronide and six HG monoglucuronide [8], and the major metabolite was a monoglucuronide. Thus, the transformation from HG to HG conjugates is possibly mediated by UDP-glucuronosyltransferases (UGT).

To our knowledge, no information regarding the specific UGT isoforms responsible for the glucuronidation of HG is available. Moreover, data showing the major isoforms metabolizing HG in humans have yet to be presented. The glucuronidation of HG should be further characterized because this process involves the detoxification of several compounds from therapeutic classes and xenobiotics. Glucuronidation reactions are catalyzed by the UGT superfamily; among the members of this superfamily, UGT1A and UGT2 B enzymes are the largest subfamilies. Individual UGT1A and UGT2 B enzymes exhibit distinct but overlapping aglycone substrate selectivities; these enzymes also differ in terms of their regulatory mechanisms involved in expression and tissue distribution. Among the 12 human UGT isoforms, UGT1A1 and UGT2B7 are well expressed in the intestines and the liver. UGT1A9 is well expressed in the liver and the kidneys [9] but unexpressed or poorly expressed in the intestines [10]. UGT1A8 and 1A10 are well expressed in the intestines but not in the liver [10,11]. Therefore, systematic metabolic profiling studies using well-expressed human UGT isoforms are necessary to determine the major isoform(s) responsible for HG metabolism; such studies can also help identify the metabolite(s) formed by particular isoforms and predict the major organ (site) of metabolism. Information may also be used to address safety and efficacy concerns, which may arise from potential drug–drug interactions and genetic polymorphisms.

The half-life of HG in humans is 0.133 h [6], which differs from its half-life in rats and dogs [12], indicating the species differences of this substance. This result may initiate the possible variation of HG metabolism in different animals. To characterize the glucuronidation of HG in different species, we selected 10 types of liver microsomes, including those from male and female humans, rats, dogs, mice, and guinea pigs.

This study provided a detailed and systematic investigation of the UGT-catalyzed metabolism of HG by using expressed UGT isoforms and liver microsomes and further elucidated the effects of species and gender difference on HG metabolism. Our results may provide insights into the possible pathway associated with HG in clinical trials and the magnitude of drug–drug interactions.

2. Materials and methods

2.1. Reagents

Uridine diphosphoglucuronic acid (UDPGA), alamethicin, magnesium chloride (MgCl2), and β-glucuronidase were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents and other reagents used were of analytical reagent grade or higher. Pooled liver microsomes from female and male CD-1 mice, Sprague-Dawley rats, beagle dogs, guinea pigs, and humans were purchased from BD Bioscience (Woburn, USA). Twelve rUGT supersomes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, GT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) comparatively high-expressed in baculovirus-infected insect cells were purchased from BD Gentest (Woburn). All microsomes were stored at −80 °C until use. HG standards were prepared in methanol and stored in vials at −20 °C.

2.2. Microsomal incubation procedure

The incubation procedures for measuring UGT activities were essentially identical to those published (Joseph et al., 2007; Liu et al., 2007). Microsome suspensions (final concentration, 0.01–0.05 mg of protein) were dispensed into phosphate buffer solution (50 mM, pH 7.4) containing 0.88 mM magnesium chloride, 0.022 mg/mL alamethicin, and different concentrations of HG. The coenzyme UDPGA (3.5 mM) was added to the resultant mixture last. The mixture (final volume, approximately 400 μL) was incubated at 37 °C for predetermined periods of time (30–90 min) based on pilot experiments. The reaction was stopped by the addition of 60 μL of acetonitrile containing 60 μg/mL propiophenone as an internal standard (stop solution). All assays were terminated with an equal volume of phosphate buffer solution. The supernatants were centrifugated and then subjected to ultra-performance liquid chromatography (UPLC).

2.3. UPLC analysis for HG and its glucuronides

Analysis of HG and its metabolites was performed on a Waters Acquity UPLC system equipped with a photodiode array detector (λ_{max} = 284 nm) and Empower™ software. Samples were separated on a Waters BEH C18 column (2.1 × 100 mm, 1.7 μm particle diameter) that was maintained at 40 °C. Gradient elution at 0.2 mL/min with ACN and 0.1% formic acid was performed as follows: 0–2.0 min, 5%–10% ACN; 2.0–2.5 min, 10%–40% ACN; 2.5–4.5 min, 40%–65% ACN; 4.5–5.0 min, 65%–90% ACN; and 5.0–6.0 min, 90%–5% ACN. The HG standards were prepared in 400 μL of 70% phosphate buffer (50 mM):30% ACN and 4 μL of standard stock solution with 200 μL of 60 μg/mL propiophenone. Finally, it was diluted by equal volumes of phosphate buffer as previously mentioned.

2.4. HG glucuronide identification using LC–MS/MS and LC—HRMS

A Premier XE mass spectrometer triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) was operated in positive ion mode to identify HG and its metabolites. The main working parameters for the mass spectrometer were set as follows: capillary voltage of 3.0 kV, cone voltage of 35 V, ion source temperature of 120 °C, desolvation temperature of 330 °C, and cone gas rate of 50 L/h.

A quadrupole-time of flight (Q-TOF) tandem mass spectrometer (Bruker, USA) with an Agilent HPLC system was used to determine the molecular weight (MW) of HG and its metabolites. The applicable conditions were as follows: column, Agilent ZORBAX SB-C18, 5 μm, 4.6 × 150 mm; mobile phase B, 100% acetonitrile; mobile phase A, 100% aqueous buffer (0.1% formic acid); flow rate, 1 mL/min; gradient, 0 min to 9 min, 95%–65% A, 9–13 min, 65%–50% A, 13 to 15 min, 50%–95% A; wavelength, 340 nm; and injection volume, 200 μL. Ionization was achieved using electrospray ionization (ESI) in positive mode with a capillary voltage of 4500 V. The temperature of the dry heater was maintained at 200 °C, and the nebulizer voltage was set to 1.5 bar. The dry gas was set to a flow rate of 8.0 L/min. MS/MS spectra were produced by collision-induced dissociation of the selected precursor ions. Data