Regular article

Identification and characterization of human UDP-glucuronosyltransferases responsible for the in vitro glucuronidation of ursolic acid

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A R T I C L E   I N F O

A B S T R A C T

This study aims to characterize the glucuronidation kinetics of ursolic acid (UA) in human liver microsomes (HLMs) and intestinal microsomes (HIMs) and identify the main UDP-glucuronosyltransferases (UGTs) involved. In our present study, only one type of UA glucuronide was observed after incubation with HLMs and HIMs respectively and was identified as a UA hydroxyl O-glucuronide. The glucuronidation of UA can be shown in HLMs and HIMs with K_m values of 3.29 ± 0.16 and 3.74 ± 0.22 μM and V_{max} values of 0.33 ± 0.03 and 0.42 ± 0.03 nmol/min/(mg protein). Among the 12 recombinant UGT enzymes investigated, UGT1A3 and UGT1A4 were identified as the major enzymes catalyzing the glucuronidation of UA [K_m values of 2.58 ± 0.12 and 4.66 ± 0.60 μM, V_{max} values of 0.72 ± 0.01 and 1.00 ± 0.06 nmol/min/(mg protein)]. The chemical inhibition study showed that the IC_{50} for hecogenin inhibition of UA glucuronidation was 51.79 ± 4.32 μM in HLMs. And chenodeoxycholic acid inhibited UA glucuronidation in HLMs with an IC_{50} of 28.26 ± 2.91 μM. In addition, UA glucuronidation in a panel of eight HLM was significantly correlated with telmisartan glucuronidation ($r^2 = 0.7660$, $p < 0.01$) and trifluoperazine glucuronidation ($r^2 = 0.5866$, $p < 0.01$) respectively. These findings collectively indicate that UGT1A3 and UGT1A4 were the main enzymes responsible for the glucuronidation of UA in human.

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1. Introduction

Ursolic acid (UA; 3β-hydroxy-12-urs-12-en-28-oic acid) is a natural pentacyclic triterpenoid carboxylic acid. It is the major pharmacologically active component of some traditional medicinal herbs (fructus crataegi, glossy privet fruit, gardenia jasminoides, etc.) and is well known to possess a wide range of biological functions such as antioxidative, anti-inflammatory, and antibacterial activities [1–5]. Recently, it is discovered that UA has a broad-spectrum antitumor activity, which has attracted especially more scholars' attention [6]. Hence, UA may be a promising clinical medication. Herb-drug interactions mediated by metabolizing enzymes have received increasing attention over the past few decades. However, it was little known about the interactions between UA and prescription drugs in clinical practice. Therefore, understanding the metabolic mechanism of UA is essential to ensure the safe administration of UA.

There are few studies available about the metabolism of UA in human. Xenobiotic metabolism has traditionally been considered in terms of "Phase I" and "Phase II" reactions and the most important enzymes are the cytochromes P450 (CYP) and UGTs, respectively [7]. Cheng et al. [8] and Wang et al. [9] studied the 'Phase I' reactions of UA and found that CYP3A4 and CYP2C9 were the main metabolic enzymes in the 'Phase I' reactions. In our preliminary studies, it was observed that UA was metabolized less than 30% in nicotinamide adenine dinucleotide phosphate (NADPH)-HLM incubation system and was metabolized about 80% in UGT reaction system (data not shown). The results demonstrated that the "Phase II" reactions mediated by UGT enzymes played a principal role in the metabolism of UA in vitro. It is well known approximately 35% of all clinical drugs

subjected to glucuronidation reactions were metabolized by UGTs [10]. However, the metabolic characteristics of UA and which UGT enzymes responsible for UA remain unclear. Identifying the UGT enzymes responsible for UA would help us to characterize the role of these particular enzymes in potential clinical herb and drug interactions and estimate the impact of genetic polymorphisms of interesting enzymes on drug disposition.

The purpose of this study is to characterize the glucuronidation kinetics of UA in HLMs and HIMs and identify the main UGT enzymes involved using a battery of recombinant human UGTs. Inhibition of UGT-glucuronidation activity and correlation in HLMs were also determined using known high-affinity UGT substrates or inhibitors to facilitate identification of the UGT enzymes involved in UA glucuronidation.

2. Materials and methods

2.1. Chemicals and reagents

Ursolic acid (99.8%) was supplied from Engineering Development Center of Yichun College, Jiangxi, and China. UDPGA, hecogenin, β-glucuronidase (Helix pomatia), and alamethicin [Sigma–Aldrich Corporation, Saint Louis, Missouri, and United States of America (USA)]. Pooled HLMs, HIMs and individual HLMs were provided from Research Institute for Liver Diseases (Shanghai Corporation Limited, Shanghai, and China). Recombinant human UGT Supersomes™ (UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, 2B4, 2B15, and 2B17) expressed in baculovirus-infected insect cells [BD Biosciences, Bedford, Massachusetts (MA), and USA]. Magnesium chloride (MgCl$_2$) (Tianjin Damao Chemical Reagent Factory, Tianjin, and China). Gliquidone, telmisartan, chenodeoxycholic acid, and trifluoperazine (TFP) hydrochloride (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, and China). Milli-Q water (Millipore Corporation, Billerica, MA, and USA) was used in all steps, and all other chemicals were of high-performance liquid chromatography (HPLC) grade or the Analytical grade which was commercially available.

2.2. Qualitative analysis of UA glucuronide by LC-MS/MS

UA (8 μM) was incubated in a final volume of 200 μl incubation mixture containing 0.5 mg/ml of microsomal protein (from HLMs or HIMs), alamethicin (50 μg/mg protein), UDPGA (3 mM), MgCl$_2$ (8 mM), Tris-hydrochloric acid (50 mM, pH 7.4). Microsomes were preincubated with alamethicin for 20 min on ice before incubation. After preincubation at 37 °C for 5 min, the reaction was initiated by the addition of UDPGA at 37 °C for 30 min, the reaction was terminated with 1 ml of cold ethyl acetate (containing 8 μM gliquidone used as internal standard for the subsequent quantitative determination of UA). Then the incubation mixtures were centrifuged at 20,000× g and 4 °C for 10 min, the supernatant was collected and dried using concentrated drying apparatus at 55 °C for 25 min. The dried residue was redissolved in 200 μl of mobile phase and 5 μl aliquots were analyzed by high performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS) for metabolite profiling. Incubations without UDPGA and substrate served as negative controls.

LC-MS/MS was performed using Agilent 6430A LC-MS/MS system coupled with an Agilent 1200 series HPLC system. Chromatographic separation was achieved on a Luna C$_{18}$ (50 mm × 2.0 mm, 5 μm) column with the mobile phase of ammonium formate (5 mM, pH 3.41) and acetonitrile (20:80, V/V) at a flow rate of 0.2 ml/min. The pH of ammonium formate (5 mM) was adjusted by formic acid. Electrospray ionization (ESI) was performed to identify UA glucuronide operating in both negative and positive ion mode from m/z

100–700, with full and product ion scans, and selected ion monitoring. The operating parameters of the MS were as follows: gas flow, 8 L/min; gas temperature, 350 °C; capillary voltage, −4000 V; nebulizer pressure, 40 psi; and fragmentor voltage, 135 V.

2.3. Quantitative analysis of glucuronidation assay of UA

The glucuronidation of UA by HLMs or HIMs was performed as described above. The quantitative analysis of UA glucuronide was achieved by injecting 5 μl of the dried residue redissolved in mobile phase into an LCMS-2010 EV system consisting of a Shimadzu LC-20AB (Shimadzu Corporation, Kyoto, and Japan) system equipped with a Luna C$_{18}$ column (50 mm × 2.0 mm, 5 μm). Mobile phase of ammonium formate (5 mM, pH 3.41: acetonitrile, 20:80, V/V) was run at a flow rate of 0.2 ml/min. SIM was performed in the negative ion mode. The detector voltage was put at −1.85 kV. The block heater and the curved desolation line temperature were put at 200 °C and 250 °C, respectively. Other MS detection conditions were set as follows: interface voltage, 40 V; voltage, 4 kV, drying gas (N$_2$) pressure,60 Pa; and nebulizing gas (N$_2$) flow, tuned to be 1.5 L/min. SIM was performed by monitoring the [M−H]$^-$ m/z of 455.35 for UA and 526.25 for gliquidone (IS).

As standards of the UA glucuronides were not commercially available, the amount of each UA glucuronide formed in vitro was calculated from the removal of parent compound UA. Incubations with inactivated HLMs or HIMs served as control group. The calibration curve of UA in HLMs or HIMs incubation system were constructed over 0.5–64 μM and 0.125–16 μM, respectively. There was a good linearity for the determination of UA in HLMs incubation system from 0.5 to 64 μM (r^2 = 0.998) and in HIMs incubation system from 0.125 to 16 μM (r^2 = 0.994).

For the confirmation of the site of glucuronidation of UA, the dried residue from above incubation system in HLMs was hydrolyzed in 200 μl of 0.01 mol/L sodium carbonate solution at 45 °C for 2 h. The peak area of UA glucuronidation production after hydrolysis was detected and compared that without alkaline pretreatment.

2.4. Hydrolysis with β-glucuronidase

UA was treated with HLMs as described above, and the supernatant was collected, dried and redissolved in 200 μl of 50 mM sodium acetate buffer (pH 5.27). Each sample was incubated in the absence or presence of 4000 units of β-glucuronidase at 37 °C for 4 h. Each incubation reaction was terminated as described above, and the supernatant was injected for LC-MS analysis.

2.5. Glucuronidation by recombinant UGTs

Twelve commercially available recombinant human UGTs (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) were used to screen the glucuronidation of UA at three concentrations (4, 8, and 16 μM). Incubation conditions and analyses were similar to those HLMs except that the protein concentration was 0.2 mg/ml.

2.6. Kinetic analysis

UA concentration series from 1 to 64 μM were performed in HLMs, recombinant UGT1A3 and UGT1A4, and UA at different concentrations from 0.5 to 40 μM were treated with pooled HIMs. Kinetic parameters were evaluated from the suitable curves using Graphpad Prism (Graphpad Software Inc., California (CA), and USA) followed by nonlinear regression analysis. The following equation was used for assuming a Michaelis–Menten equation: $V = V_{max} \times [S]/([K_m + [S])$. Where V is the rate of reaction, V_{max} is