Identification of Human UDP-Glucuronosyltransferase Isoforms Responsible for the Glucuronidation of Glycyrrhetinic Acid

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Summary: Glycyrrhetinic acid, the active metabolite of glycyrrhizin, is primarily eliminated by glucuronidation reaction in vivo. In spite of the widespread clinical use of glycyrrhizin, UDP-glucuronosyltransferase (UGT) isoforms involved in the glucuronidation of this drug are still unknown. This report identifies and characterizes the UGT isoforms responsible for glycyrrhetinic acid glucuronidation. In the enzymatic kinetic experiment performed with pooled human liver microsomes (HLMs), $K_m$ was 39.4 $\mu$M and $V_{max}$ was 609.2 pmol/min/mg protein. Of the baculosomes expressing 12 recombinant UGTs investigated, UGT1A1, 1A3, 2B4 and 2B7 showed catalytic activity and UGT1A3 exhibited the highest activity. $K_m$ values of recombinant UGT1A3 and 2B7 were 3.4 and 4.4 $\mu$M, respectively. Both imipramine (typical substrate of UGT1A3 and 1A4) and flurbiprofen (typical substrate of UGT2B7) inhibit the glucuronidation of glycyrrhetinic acid. Estimated IC$_{50}$ values were 138 $\mu$M for flurbiprofen and 207 $\mu$M for imipramine in the inhibition of the glucuronidation of glycyrrhetinic acid in HLMs. These results suggest that glycyrrhetinic acid glucuronidation is primarily mediated by UGT1A1, 1A3, 2B4 and 2B7.

Keywords: glucuronidation; UDP-glucuronosyltransferase; recombinant human UGT; glycyrrhetinic acid; enzyme kinetics; liver microsomes

Introduction

Glycyrrhizin is an active component of licorice (glycyrrhiza), a herbal medicine used worldwide for a long time. Previous studies suggest that glycyrrhizin has a number of pharmacological effects including antiinflammatory activity hepatoprotective effects, immunomodulatory effects, anticancer effects and it has been widely used in clinical practice for the treatment of chronic hepatitis.1,2 After administration, glycyrrhizin is hydrolyzed to glycyrrhetinic acid by intestinal bacteria and then absorbed as glycyrrhetinic acid.3 As the active metabolite of glycyrrhizin, glycyrrhetinic acid is almost completely metabolized through glucuronidation and sulfation in the liver.4

Glucuronidation is a major phase II metabolic pathway that enhances the elimination of many lipophilic xenobiotics (such as morphine) and endobiotics (such as bilirubin and steroids) to more water-soluble compounds by the formation of covalent linkages in the presence of UDP-glucuronic acid (UDPGA). Normally, glucuronidation serves as a detoxifying process, since glucuronides have greater polarity than the parent drugs and are thus more easily excreted. However, a number of glucuronides are reported to have pharmacological effects or toxicological effects.5 Glucuronidation reactions are mediated by the UDP-glucuronosyltransferase (UGT) superfamily.6 To date, at least nineteen human UGTs have been identified and classified into two superfamilies based on divergent evolution.7 As the enzymes catalyzing one of the major metabolic pathways, genetic polymorphism of UGTs has important implications for toxicological and physiological effects.8 For example, UGT1A1 polymorphism has been reported responsible for Crigler-Najjar syndromes type I and II, and Gilbert syndrome.9

Identification of the isoforms involved in drug metabolism is of great importance for predicting potential drug-drug interactions, and the information provided can be utilized to guide clinical practice and reduce side effects.
effects in clinical treatment. Recent years have witnessed an increasing interest in the study of interactions between herbal drugs and chemical drugs. Numerous interindividual differences in drug glucuronidation have been frequently observed. In spite of the widespread clinical use of glycyrrhizin, the UGT isoforms responsible for the glucuronidation of this drug are still unknown. Based on strategies typically used for definitive cytochrome P450 reaction phenotyping, this study characterized the UGT isoforms mediating the glucuronidation of glycyrrhetic acid by comparing the kinetic parameters in pooled human liver microsomes (HLMs), screening the active isoforms from 12 commercially available recombinant UGTs, and by inhibition experiment using typical UGT inhibitors.

**Materials and Methods**

**Chemicals and reagents:** Glycyrrhetic acid (>98%, purity) was obtained from Henan Shuai Ke Pharmaceutical Co. Ltd. (Henan, China). Alamethicin (from Trichoderma viride), β-glucuronidase (from bovine liver), and D-Saccharic acid 1,4-lactone monohydrate were purchased from Sigma-Aldrich (St. Louis, MO). UDP-Glucuronic acid (UDPGA; trisodium salt) was purchased from Nacalai Tesque (Kyoto, Japan). Imipramine was purchased from Wako Pure Chemical Industries (Osaka, Japan). Pooled human liver microsomes (mixed gender, n = 10) were supplied by Shaanxi Lifegen Company (Shaanxi, China) and baculosomes of 12 recombinant human UGTs (recombinant 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, USA). All other reagents were of analytical grade.

**HPLC analysis of glycyrrhetic acid and its glucuronide:** Glycyrrhetic acid and its glucuronide were separated on a Hypersil ODS2 Column (5 μm, 4.6 × 200 mm) from Elite (Dalian, China) at a flow rate of 1.0 ml/min. The mobile phase consisted of 77% methanol and 23% water with the addition of 0.09 ml glacial acetic acid per 100 ml. The high performance liquid chromatography system consisted of a Shimadzu LC-10AT pump, a Shimadzu LC-10A UV detector and a column oven. The detector wavelength was 254 nm and column temperature was maintained at 30°C. Retention times of glycyrrhetic acid and its glucuronide were 18.5 min and 6.3 min, respectively. The concentration of the glucuronide of glycyrrhetic acid was quantified by a standard curve of glycyrrhetic acid ranging from 0.5–10 μM. The correlation coefficient for the standard curve was above 0.99.

**Glucuronidation of glycyrrhetic acid by pooled HLMs:** In vitro glucuronidation of glycyrrhetic acid by HLMs was carried out in a final volume of 0.2 ml, containing Tris buffer (50 mM, pH 7.5) 10 mM MgCl₂, 5 mM saccharolactone (D-Saccharic acid 1,4-lactone monohydrate), alamethicin (50 μg/mg protein), pooled HLMs (0.25 mg protein per ml), and glycyrrhetic acid at 2–100 μM. After pre-incubation for 5 min in a water bath maintained at 37°C, reactions were initiated by the addition of UDP-glucuronic acid to a final concentration of 5 mM. At 30 min incubation, the reactions were terminated by the addition of 200 μl cold acetonitrile containing 5% glacial acetic acid to enhance the stability of the glucuronide of glycyrrhetic acid. Quenched samples were then vortexed and centrifuged at 1200 g for 20 minutes. An aliquot of 20 μl of the supernatant was analyzed by HPLC. Rates of formation of the glycyrrhetic acid glucuronide were verified to be linear with respect to protein concentration and incubation time. Two blank determinations were performed: 1) incubation without substrate; 2) incubation at the absence of UDPGA.

**Screening experiment with recombinant human UGTs:** The screening test with recombinant UGTs was carried out with 12 human recombinant UGTs (recombinant UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17). Incubation conditions were similar to those of HLMs incubation except that the final protein concentration was 0.2 mg/ml and incubation time was 60 min. Substrate concentrations were 10 μM and 100 μM. Two blank determinations were conducted.
with the absence of substrate or cofactor UDPGA.

Identification of glucuronidation of glycyrrhetinic acid: Enzymatic hydrolysis was carried out to identify the glucuronide of glycyrrhetinic acid. An incubation mixture was terminated by adding 8 μl 50% phosphoric acid, followed by vortexed and centrifuged for 20 min at 12000 g. pH of the supernatant was adjusted to 4.4 with 1 M NaOH. An aliquot of the supernatant was transferred into a 1.5 ml plastic tube containing 5000 U/ml β-glucuronidase. Incubation was carried out overnight in a water bath maintained at 37°C. A control of the supernatant without β-glucuronidase was performed simultaneously.

Data analysis: Kinetic parameters were calculated by fitting data into Michaelis-Menten equation (eq. 1) and substrate inhibition (eq. 2) using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

The equations applied are:

\[
V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)
\]

\[
V = \frac{V_{max}[S]}{K_m + [S] + [S]^2/K_s} \quad (2)
\]

in which \( V \) is the velocity of the reaction, \( V_{max} \) is the maximum velocity of reaction, \( [S] \) is the concentration of the substrate, \( K_m \) is the Michaelis constant (the concentration of the substrate at half maximum velocity), and \( K_s \) is the constant describing the substrate inhibition interaction.

Results

Enzymatic kinetics of glycyrrhetic acid glucuronidation in pooled HLMs: After enzymatic hydrolysis, the peak of the glucuronide at 6.3 min was removed. The formation of glycyrrhetic acid glucuronide was verified to be linear with incubation time to at least 60 min and the final protein concentration to at least 1 mg/ml. Enzymatic kinetics of glycyrrhetic acid glucuronidation by pooled HLMs were determined by fitting data into Michaelis-Menten equation (Fig. 1). Based on a duplicate determination, \( K_m \) was 39.4 μM, and \( V_{max} \) 609.2 pmol/min/mg protein in pooled HLMs (Table 1).

Glucuronidation of glycyrrhetic acid by recombinant UGTs: Of the baculosomes expressing 12 recombinant human UGTs (recombinant UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17), UGT1A3 and UGT2B7, at 10 μM, showed catalytic ability of glycyrrhetic acid with glucuronidation velocity of 212.3 and 46.9 pmol/min/mg protein, respectively (Fig. 2). At 100 μM, recombinant UGT1A1, 1A3, 2B4 and 2B7 showed catalytic ability of glucuronidation of glycyrrhetic acid and recombinant UGT1A3 showed the highest catalytic capability (Fig. 2). Further studies were carried out to characterize the kinetics of the enzymes. At a protein concentration of 0.1 mg/ml, incubation with recombinant UGT1A3 was performed for 30 min and the concentration of glycyrrhetic acid ranged from 2–100 μM. Incubation with recombinant UGT2B7 was carried out for 60 min at the protein concentration of 0.1 mg/ml and the concentrations of glycyrrhetic acid ranged from 5–100 μM. Both UGT1A3 and 2B7 exhibited substrate-inhibition at high concentration (Fig. 3). Km values of recombinant UGT1A3 and 2B7 were 3.4 and 4.4 μM, respectively. Vmax values of recombinant UGT1A3 and 2B7 were 458.2 and 99.9 pmol/min/mg protein, respectively. Ka values of recombinant UGT1A3 and 2B7 were 54.6 and 397.4 μM, respectively (Table 1). Intrinsic clearance values of recombinant UGT1A3 and 2B7, calculated as Vmax/Km, were 136.5 and 22.9 μl/min/mg protein, respectively.

Inhibition of HLMs incubation with imipramine and flurbiprofen: Due to the relatively pronounced activity of recombinant UGT1A3 and 2B7 in glycyrrhetic acid glucuronidation, an inhibition experiment was conducted using the typical inhibitors of UGT1A3 and 2B7. Imipramine (for UGT1A3 and 1A4)12,13 and flurbiprofen (for UGT2B7)14 were used as potential inhibitors. The formation rate of glycyrrhetic acid glucuronide was tested at a substrate concentration of 40 μM and the final concentration ranges for imipramine and flurbiprofen were 10–750 μM and 1–200 μM, respectively. Incubation was conducted using the same conditions described previously. Two control vials were incubated simultaneously without the inhibitors. Estimated IC50 values were 138 μM for flurbiprofen and 207 μM for imipramine in the inhibition of glycyrrhetic acid glucuronidation in HLMs (Fig. 4).

Discussion

This research investigates the glucuronidation of glycyrrhetic acid by HLMs, baculosome of human recombinant UGTs and the inhibitory effects of related UGT-inhibitors so as to identify the UGT isoforms that mediate the glucuronidation of glycyrrhetic acid. Initially, kinetic parameters in pooled HLMs were obtained
Fig. 3. Kinetic study conducted in recombinant UGT 1A3 and 2B7.
(A) glycyrrhetinic acid (2–100 μM) was incubated with recombinant UGT1A3 (0.1 mg protein/ml) for 30 min. (B) the Eadie-Hofstee plot for recombinant UGT1A3. (C) glycyrrhetinic acid (5–100 μM) was incubated with recombinant UGT2B7 (0.1 mg protein/ml) for 60 min. (D) the Eadie-Hofstee plot for recombinant UGT2B7. Data were fitted to substrate inhibition equation.

Table 1. Kinetic parameters of glycyrrhetinic acid glucuronidation in pooled human liver microsomes (HLMs) and recombinant UGT isoforms.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg protein)</th>
<th>$K_i^a$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLMs$^b$</td>
<td>39.4</td>
<td>609.2</td>
<td>—</td>
</tr>
<tr>
<td>UGT1A3$^c$</td>
<td>3.4</td>
<td>458.2</td>
<td>54.6</td>
</tr>
<tr>
<td>UGT2B7$^c$</td>
<td>4.4</td>
<td>99.9</td>
<td>397.4</td>
</tr>
</tbody>
</table>

$^aK_i$ = substrate inhibition constant, $^b$Michaelis-Menten, $^c$Substrate inhibition

by fitting data into Michaelis-Menten equation. Based on the screening experiment conducted with baculosome of 12 recombinant UGTs commercially available, UGT1A1, 1A3, 2B4 and 2B7 were candidate isoforms responsible for the glucuronidation of glycyrrhetinic acid. Due to the relatively pronounced catalytic activity, kinetic experiments were carried out for recombinant UGT1A3 and 2B7. Kinetic profiles of both recombinant UGT1A3 and 2B7 followed substrate inhibition equation. $K_m$ value of recombinant UGT1A3 was 3.4 μM and $K_m$ value of recombinant UGT2B7 was 4.4 μM.

A separate inhibition experiment (Fig. 4) conducted on the effects of UGT inhibitors in pooled HLMs suggested that glycyrrhetic acid was not dependent on a single isoform, albeit recombinant UGT1A3 showed a relatively high catalytic activity among the UGTs screened. Intrinsinc clearance of recombinant UGT1A3 (136.5 μl/min/mg protein) was apparently larger than that of recombinant UGT2B7 (22.9 μl/min/mg protein). This indicates that recombinant UGT1A3 is more efficient on the clearance of glycyrrhetic acid in vitro. Apparently, $K_m$ (39.4 μM) for glycyrrhetic acid glucuronidation in pooled HLMs was approximately ten times that obtained from the incubation with recombinant UGT1A3 (3.4 μM) and UGT2B7 (4.4 μM). The study on many different UGT isoforms in the liver and method of immunoblot analysis with antibodies were useful to understand this differ-
ence.\textsuperscript{15–17} $K_m$ presented in pooled HLMs was apparent and attributed to the contribution of many isoforms expressed in the liver which mediate the glucuronidation of glycyrrhetic acid.

Substrate-inhibitory effect of glycyrrhetic acid glucuronidation at high substrate concentrations was observed when conducting the kinetic experiment for recombinant UGT1A3 and 2B7. Similar substrate-inhibitory effect was observed previously for the glucuronidation of nonsteroidal anti-inflammatory drugs\textsuperscript{18} and the glucuronidation of frusemide by UGT1A1, 1A3 and 1A6.\textsuperscript{19} These phenomena may be attributed to modification to UGTs in vitro.\textsuperscript{20} However, this phenomenon was not observed in HLMs using a substrate concentration up to 100 $\mu$M. This is because HLMs are a combination of different UGT isoforms and glycyrrhetic acid is not dependent on a single isoform as analyzed in the inhibition experiment.

The concomitant administration of drugs is becoming more and more popular. As a result, drug-drug interactions become a common clinical problem. Determination of the inhibitory potentials of glycyrrhetic acid against UGTs is crucial to the evaluation of possible drug-drug interactions. Previous studies report that glycyrrhetic acid inhibits the glucuronidation of SN-38 (the active metabolite of irinotecan) with IC$_{50}$ of 440 $\mu$M.\textsuperscript{21} The data we obtained in this study were consistent with these findings. The glucuronidation of SN-38 was mediated by UGT1A1 (highest catalytic activity) and 1A3 and 1A6.\textsuperscript{22} This exhibited catalytic activity of glycyrrhetic acid in the screening experiment with recombinant UGTs. Thus, glycyrrhetic acid may play a competitive role in the glucuronidation of SN-38. Due to the in vitro inhibition data previously observed in SN-38 glucuronidation (IC$_{50}$: 440 $\mu$M) and the low clinical plasma concentration (<0.4 $\mu$M),\textsuperscript{23} the inhibitory potential of glycyrrhetic acid on UGT 1A1 in clinical practice is low.

In summary, the methods in this study may be effectively applied in the identification of UGT isoforms involved in the glucuronidation of glycyrrhetic acid. Based on our results, the glucuronidation of glycyrrhetic acid is primarily mediated by UGT1A1, 1A3, 2B4 and 2B7.

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