Food and Chemical Toxicology 64 (2014) 135–143

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Steviol glucuronidation and its potential interaction with UDP-glucuronosyltransferase 2B7 substrates

Meiyu Wang, Jia Lu, Jiajun Li, Huixin Qi, Yedong Wang, Hongjian Zhang *

College of Pharmaceutical Sciences, Soochow University, Suzhou, China

ARTICLE INFO

Article history:
Received 2 August 2013
Accepted 22 November 2013
Available online 1 December 2013

Keywords:
Steviol
Glucuronidation
UGT2B7
Inhibition
Botanical–drug interaction

ABSTRACT

Hydrolysis of stevioside and rebaudioside A in the gastrointestinal tract after oral intake leads to the formation of steviol, the aglycone, which is absorbed into the circulation. Although in vivo studies have shown that steviol is cleared from the body via glucuronidation, the role of liver vs. intestine in steviol glucuronidation has not been well defined and related UDP-glucuronosyltransferases (UGTs) have not been identified. The present study investigated steviol glucuronidation and obtained kinetic parameters in liver and intestinal microsomes of human and rat, as well as in recombinant human UGT systems. Results suggest that organ specificity exists in the intrinsic clearance of the glucuronidation reaction. Steviol glucuronidation was primarily mediated by UGT2B7 at low concentration and UGT2B7 and UGT1A3 at high concentration. Inhibition studies with selected UGT2B7 substrates indicate that diclofenac displayed a relatively strong inhibition (K_i, 4.2 μM) against steviol glucuronidation in human liver microsomes. Taken together, the identification of the involvement of UGT2B7 in steviol glucuronidation would provide a mechanistic basis for the evaluation of the interaction between steviol and diclofenac. As metabolic clearance of botanical-derived products can be the objects (victims) of botanical–drug interactions, further studies are needed to investigate the in vivo relevance of such interactions.

1. Introduction

UDP glucuronosyltransferase (UGT) is a family of enzymes that catalyze the transfer of glucuronic acid to molecules containing certain structure features such as carboxyl, hydroxyl and amino groups. The reaction, termed glucuronidation, is an integral part of body's mechanisms to remove endogenous and exogenous substances (Burchell et al., 1995). Increasing evidence indicates that UGT enzymes play a critical role in the metabolism of many drugs and active components of various natural products (Court, 2005; Li et al., 2012; Radominska-Pandya et al., 2005; Ritter, 2007). UGT enzymes are generally categorized into three major subfamilies based on sequence homology and each family contains many members of UGT isoforms (Mackenzie et al., 2005; Rowland et al., 2013; Tukey and Strassburg, 2000). Among those, UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B7, and 2B15 have been indicated to be important isoforms in drug metabolism and elimination (Miners et al., 2010).

Stevioside and rebaudioside A are major components of stevia extracts which have been used as natural sweeteners due to their intense sweetness (250–300 times sweeter than sucrose) (Crammer and Ikan, 1986). In addition, those natural occurring compounds have been implicated to have beneficial effects such as anti-hyperglycemic, anti-hypertensive, anti-diabetic, anti-inflammatory, and anti-tumor activities (Chen et al., 2005; Kinghorn and Soejarto, 2002; Nakamura et al., 1995; Yasukawa et al., 2002). Results from a number of in vitro and in vivo studies indicate that stevioside and rebaudioside A are hydrolyzed to steviol, the aglycone, in the gastrointestinal tract after oral intake (Gardana et al., 2003; Renwick and Tarka, 2008; Roberts and Renwick, 2008; Wheeler et al., 2008). In human pharmacokinetic studies of stevioside and rebaudioside A, steviol glucuronide was found to be the major circulating metabolite (Geuns et al., 2006). Because of the low permeability of stevioside and rebaudioside A (Geuns et al., 2003; Koyama et al., 2003), it is generally believed that steviol is the key component that is absorbed into the circulation as the related substance and it is eliminated from the body by glucuronidation and subsequent urinary secretion of steviol glucuronide (Koyama et al., 2003; Wheeler et al., 2008).

Abbreviations: HLM, human liver microsomes; IS, internal standard; SVG, steviol glucuronide; UGT, UDP glucuronosyltransferase.

* Corresponding author. Address: College of Pharmaceutical Sciences, Soochow University, 199 RenAi Road, Suzhou Industrial Park, Suzhou 215123, China. Tel.: +86 (512) 6588 2659; fax: +86 (512) 6588 2089.

E-mail address: zhanghongjian@suda.edu.cn (H. Zhang).

0278-6915/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2013.11.028

Because of the increasing use of nutritional supplements and functional food components, botanical–drug interactions have received considerable attention in recent years. While consumers use a variety of products to promote health and/or manage certain disease conditions (Dog, 2009; Roy et al., 2010), the likelihood of concurrent use with many drugs (particularly over-the-counter medications) would inevitably increases, thus leading to potential pharmacokinetic and/or pharmacodynamic interactions between botanicals and therapeutic drugs. Indeed, numerous literature reports have indicated that naturally occurring compounds can affect activities of drug metabolizing enzymes, resulting in clinically significant botanical–drug interactions (Vieira and Huang, 2012; Zhou et al., 2007). A well known example is St. John's wart, a popular remedy that is used to manage mild or moderate depression, which has been shown to affect the pharmacokinetics of several drugs via the induction of cytochrome P450 enzyme and membrane transporter activities (Mathijssen et al., 2002; Rahimi and Abdollahi, 2012; Ruschitzka et al., 2000). Grapefruit juice is another example whose ingredients (furanocoumarins) are capable of inhibiting CYP3A4 activity (Paine et al., 2005; Schmiedlin-Ren et al., 1997).

Although the pharmacokinetics of stevioside and rebaudioside A has been well studied, the glucuronidation of steviol, their hydrolytic metabolite, has not been fully understood and related UGT enzymes have not been identified. In the present study, the authentic steviol glucuronide standard was first synthesized and a corresponding LC–MS/MS method was developed for the quantitation of steviol glucuronide in biological matrices. The in vitro glucuronidation of steviol was examined in various preparations of microsomes and recombinant human UGT enzyme systems, followed by the characterization of enzyme kinetics and identification of UGT isoforms responsible for steviol glucuronidation. Further studies were carried out to investigate UGT2B7-mediated steviol glucuronidation and its potential to interact with representative UGT2B7 substrates such as diclofenac, haloperidol, lamotrigine and zidovudine. Our results suggest that while haloperidol, lamotrigine and zidovudine are weak UGT2B7 inhibitors, diclofenac displays noticeable inhibition at clinically relevant concentrations. The present findings provide a mechanistic basis for future in vivo investigation on the potential of UGT2B7-mediated interactions between diclofenac and steviol (the metabolite of stevioside or rebaudioside A), during which steviol glucuronidation can be used as a probe reaction.

2. Materials and methods

2.1. Materials

Steviol glucuronide was synthesized according to the procedures previously described (Chaturvedula et al., 2011). The purity and structural integrity of steviol glucuronide were confirmed by HPLC, LC–MS and proton NMR. Steviol was obtained from Shanghai Zhanshu Chemical Technology Co., Ltd. (Shanghai, China), and scutellarein-7-glucuronide (IS, internal standard for steviol glucuronide) was purchased from Shanghai Standard Biotech Co., Ltd. (Shanghai, China). Haloperidol, lamotrigine, zidovudine, disodium hydrogen phosphate, and sodium dihydrogen phosphate were purchased from Sigma–Aldrich (St. Louis, MO). Diclofenac (>99% purity by HPLC) was obtained from Shanghai Oriental Pharmaceutical Science and Technology Co., Ltd. (Shanghai, China). UDPGA was purchased from Roche (Basel, Switzerland). Human liver microsomes (Catalog number 452161, pooled from 20 different organ donors), human intestine microsomes (Catalog number 452210) and recombinant human UGT isoforms (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) were purchased from BD Gentest (Woburn, MA). Pooled Sprague–Dawley rat liver microsomes were prepared as described previously (Pearce et al., 1996). All other reagents and chemicals were of analytical grade and of the highest quality available commercially.

2.2. Assays and methods

2.2.1. Glucuronidation of steviol in microsomes

Steviol was incubated with microsomes isolated from rat and human liver and intestinal tissues, and the linearity of steviol glucuronide formation as a function of time and protein concentration was first examined. The reaction mixtures consisted of microsomes, 100 mM phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 5 mM D-saccharic acid 1,4-lactone, steviol (2 μM) and alamethicin (50 μg/mg microsomal protein). The final volume of the incubation mixture was 100 μL. The various components were mixed and placed on ice for 15 min to allow alamethicin-induced pore formation. Prior to the start of the incubation, the mixture was warmed up at 37 °C for 2 min, and then the reaction was initiated by the addition of UDPGA to a final concentration of 2 mM. The incubations were terminated at specified time points with the addition of 100 μL cold acetonitrile containing the IS. Samples were centrifuged using a refrigerated bench-top centrifuge and aliquots of 10 μL of the supernatants were transferred into HPLC sample vials. Levels of steviol glucuronide were determined by LC–MS/MS. Based on preliminary data, the microsomal protein concentration and incubation time were set to 0.3 mg/mL (or lower) and 5 min, respectively, to ensure linearity of the formation of steviol glucuronide.

To obtain kinetic parameters of steviol glucuronidation, steviol was incubated with respective biological matrices with steviol concentrations ranging from 0 to 80 μM. After 5 min of incubation, the reaction was terminated and samples were processed as described above and levels of steviol glucuronide were determined by LC–MS/MS.

2.2.2. Glucuronidation of steviol by recombinant human UGT isoforms

To identify which human UGT enzymes that might be involved in the glucuronidation of steviol, steviol was incubated separately with twelve recombinant human UGT isoforms (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17). The reaction mixtures consisted of hUGT (0.5 mg/mL), 100 mM phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 5 mM D-saccharic acid 1,4-lactone, steviol (2 and 20 μM) and alamethicin (50 μg/mg protein). The final volume of the incubation mixture was 100 μL. The mixtures were placed on ice for 15 min to allow alamethicin-induced pore formation. Prior to the start of the incubation, the mixtures were warmed up at 37 °C for 2 min and UDPGA (a final concentration of 2 mM) was then added to start the reaction. The incubations were terminated at specified time points with the addition of 100 μL cold acetonitrile containing the IS. Samples were prepared and analyzed as described above for the formation of steviol glucuronide.

2.2.3. Inhibition of steviol glucuronidation by selected UGT2B7 substrates

The interaction potential of steviol glucuronidation with UGT2B7 substrates was investigated in human liver microsomes under the conditions described above. Briefly, the reaction mixtures consisted of human liver microsomes (0.2 mg/mL), 100 mM phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 5 mM D-saccharic acid 1,4-lactone, steviol (2 μM) and alamethicin (50 μg/mg protein). The final volume of the incubation mixture was 100 μL. The inhibition potency (IC_{50} values) was first estimated in the presence of diclofenac, lamotrigine, haloperidol and zidovudine. Based on the observed potency, K_i value and inhibition mechanism of diclofenac were further investigated with the substrate concentrations of 2, 5, and 10 μM and the inhibitor concentrations ranging from 0 to 300 μM. After 5 min of incubation, the reaction was terminated and samples were prepared and analyzed by LC–MS/MS.

Inhibition of the glucuronidation of diclofenac by steviol was also investigated in human liver microsomes. Because of the lack of authentic standard for diclofenac glucuronide, the method of substrate depletion was employed to monitor enzyme activity changes in the presence or absence of steviol (2 and 20 μM). Briefly, the reaction mixtures consisted of human liver microsomes (0.2 mg/mL), 100 mM phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 5 mM D-saccharic acid 1,4-lactone, diclofenac (1 μM), steviol (2 and 20 μM) and alamethicin (50 μg/mg protein). The final volume of the incubation mixture was 100 μL. After a brief warming up at 37 °C, UDPGA (a final concentration of 2 mM) was added to start the reaction. Aliquots of samples were taken at specified time points and were then processed and analyzed for the disappearance of diclofenac by LC–MS/MS. Inhibition of the glucuronidation of haloperidol, lamotrigine, and zidovudine by steviol was also examined in human liver microsomes. Unfortunately, there was no appreciable substrate depletion observed during the testing period, making it difficult to quantify inhibition potency of steviol. The findings suggested that the current conditions could not be universally applied to different UGT2B7 substrates and further optimization would be needed for individual substrate using respective glucuronide standards.

2.2.4. Quantitation of steviol glucuronide by LC–MS/MS

All samples were analyzed by a LC–MS/MS system consisting of an API4000 Qtrap mass spectrometer equipped with a turbo-V ionization source (Applied Biosystems, Foster City, CA, USA), two LC-20AD pumps with a CBM-20A controller, DGU-20A solvent degasser and a SIL-20A autosampler (Shimadzu, Columbia, MD, USA). An Agela Venusil XBP C18 column (50 × 2.1 mm; 5 μm particle size) was used to achieve HPLC separation. Column temperature was held at 40 °C. Gradient elution at a flow rate of 0.3 mL/min was performed using the following mobile phase: A, 0.1% formic acid solution and B, 0.1% formic acid in methanol. The total run time was 6 min.

For MS/MS quantitation, the API 4000 Qtrap mass spectrometer was operated in the ESI negative mode with multiple reaction-monitoring (MRM). Both steviol glucuronide and the internal standard were monitored with a dwell time set to 100 ms. The MS/MS parameters were set as follows: curtain gas, 30 psi; nebulizer gas (GS1), 55 psi; turbo gas (GS2), 55 psi; ion spray voltage, 4500 V; and ion source temperature, 500 °C. For the selected ion transitions, the declustering potential (DP) and the