

Regular Article

Glucuronide-sulfate Diconjugate as a Novel Metabolite of Glycyrrhetic Acid in Rat Bile

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Summary: To study the metabolites of glycyrrhetic acid (GA) in rat bile, an analytical method was developed to identify GA and its metabolites by liquid chromatography mass spectrometry (LC-MS). Rat bile was collected after i.v. injection of GA. Three major GA-related peaks were detectable in rat bile by high-performance liquid chromatography (HPLC) analysis at 254 nm. LC-MS spectra showed their protonated molecular ions at m/z 727, 647, and 551. Furthermore, the three metabolites were also confirmed to exist in rat bile on LC-MS total ion chromatogram (TIC). Taken together with the susceptible nature to β -glucuronidase digestion and alkaline conditions, they were identified to be a novel sulfate-glucuronide diconjugate and the known monoglucuronide and sulfate conjugate, respectively.

Keywords: metabolites; glycyrrhetic acid; bile; diconjugate; LC-MS

Introduction

Glycyrrhizin (GZ) is a triterpene saponin and major component of licorice root, which has a long history of use in both Eastern and Western cultures as a remedy in the treatment for a number of ailments including gastric ulcers,¹⁾ allergic disorders,^{2,3)} chronic hepatitis^{4,5)} and inflammations.⁶⁾ GZ is metabolized to the active metabolite, glycyrrhetic acid (GA, **Fig. 1**), by human intestinal bacteria prior to absorption.⁷⁾

In the liver, GA is almost completely metabolized to glucuronide and sulfate conjugates. Only a minimal fraction of an i.v. dose is excreted via the bile as unchanged GA. Previous studies have revealed the presence of three products of GA in rat bile: 18 β -glycyrrhetyl-30-glucuronide, 18 β -glycyrrhetyl-3-O-glucuronide, and 18 β -glycyrrhetyl-3-O-sulfate (**Fig. 2**).^{8–11)} Generally, O-glucuronides are formed from phenols, alcohols, and carboxylic acids. Sulfation is also a major conjugation path for alcohols and amines.¹²⁾ GA possesses both alcohol (C₃-OH) and carboxylic acid (C₃₀-OOH) groups which are likely sites for conjugation (chemical structure shown in **Fig. 1**). Hence, it is possible that GA can form a glucuronide-sulfate diconjugate and/or diglucuronide(s), but to date there is no experimental evi-

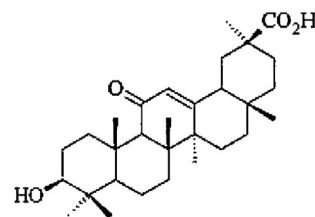


Fig. 1. Chemical structure of glycyrrhetic acid

dence to support this hypothesis. In order to characterize the metabolites of GA, rat bile was collected and the metabolites were investigated.

Materials and Methods

Chemicals and Reagents: Glycyrrhetic acid (>98% purity) was supplied by Henan Shuai Ke Pharmaceutical Co. Ltd. (Henan, China). *Escherichia coli* β -glucuronidase was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol (HPLC grade) was supplied by Tedia Co. Inc. (Fairfield, Ohio, USA). All other reagents were analytical grade.

Instrumentation: Analyses were performed using a Shimadzu 2010 LC-MS system equipped with a SIL-20AC

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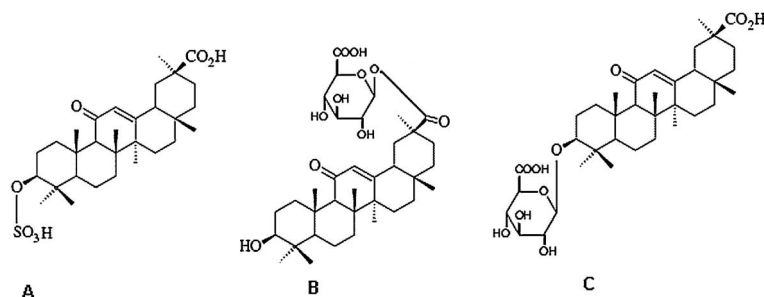


Fig. 2. Metabolites of glycyrrhetic acid

A: 18β-glycyrrhetyl-3-O-sulfate; B: 18β-glycyrrhetyl-30-glucuronide; C: 18β-glycyrrhetyl-3-O-glucuronide.

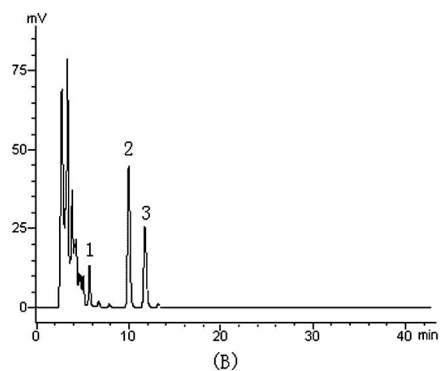
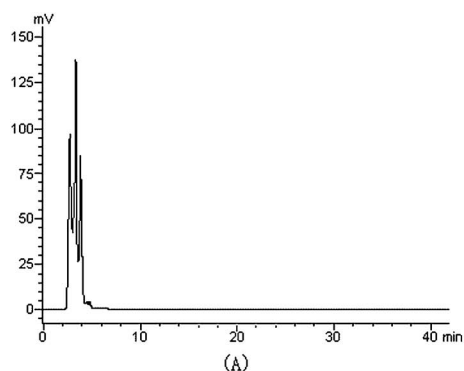


Fig. 3. HPLC chromatograms of bile from male Sprague-Dawley rats dosed i.v. with 2 mg/kg GA

A: control bile; B: rat bile collected during 2 h after i.v. injection of GA, peaks 1–3 were drug-related peaks.

auto-injector, a DGU-20A; online vacuum degasser, binary CBM-20A pumps, a Model SPD-10AVP ultraviolet detector, a CTO-20A temperature-controlled column oven, and a quadruple mass spectrometer equipped with an electrospray ionization interface (ESI) source, and a LC-MS workstation. (Shimadzu, Kyoto, Japan). The chromatographic separation was performed using a Hypersil ODS2 analytical column (5 μm, 4.6 mm × 200 mm, Elite CO., Dalian, China).

Animal experiments: The studies were approved by the Animal Ethics Committee of China Pharmaceutical University. Five male Sprague-Dawley rats (200 ± 20 g) were obtained from the Laboratory Animal Centre, Southeast University (Nanjing, China). The animals were fasted over-

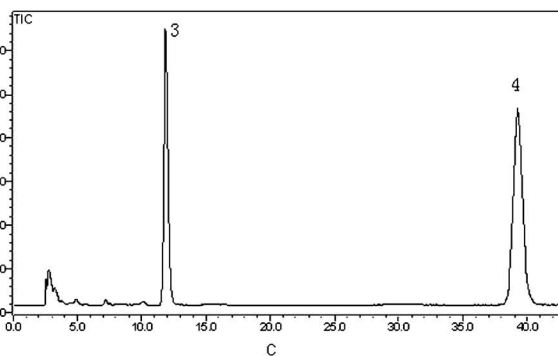
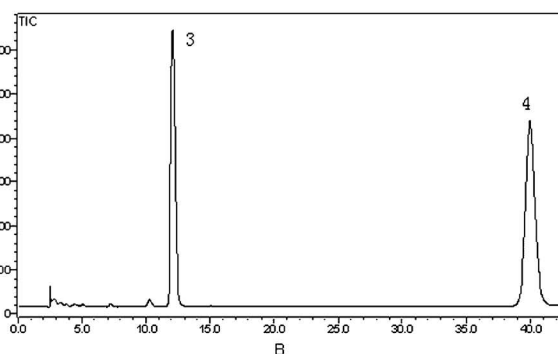
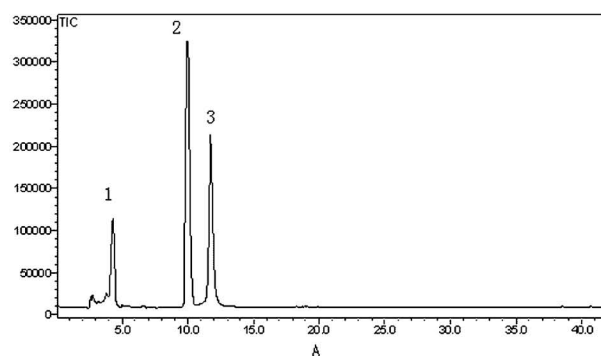


Fig. 4. LC-MS TIC profiles of GA and its metabolites in rat bile after i.v. administration of GA (2 mg/kg)

A: GA and metabolites in untreated bile; B: bile after β-glucuronidase digestion at 37°C for 2 h; C: bile after base hydrolysis with NaOH (2 M) at 90°C for 1 h.

night (12 h) and had free access to water throughout the experimental period. The abdomen was opened by median incision under anesthesia with i.p. administered urethane (1 g/kg). The common bile duct was cannulated with PE10 polyethylene tubing inner diameter, 0.28 mm; outer diameter, 0.61 mm; Becton Dickinson & Co. (Parsippany, NJ, USA). Two hours after the surgery, the rats were dosed i.v. via the caudal vein with a single dose of GA (2 mg/kg). Bile samples were collected in pre-weighed vials over the periods 0 to 2 h after the GA administration. All samples were frozen over dry ice immediately and stored at -20°C for further analysis.

Bile Sample Preparation: 20 μL bile was used for

hydrolysis in each condition. Alkaline hydrolysis was carried out in sodium hydroxide solution (2 M) at 90°C for 1 hour. Enzymatic hydrolysis was achieved by incubation with β -glucuronidase (250 U/mL) at pH 7.0 at 37°C for 2 hours.¹³⁾ After hydrolysis (either with alkaline, glucuronidase, or control buffer), the reaction solution (20 μL) was diluted with 0.5 mL mobile phase. After centrifugation for 3 min at 12,000 g, an aliquot of 10 μL of each sample was injected onto the HPLC-UV and LC-MS system for analysis.

HPLC Condition and MS parameters: The mobile phase was consisted of methanol-5 mM ammonium acetate buffer (pH 4.0, 75:25, v/v) and the flow rate was set at 1 mL/min. The flow rate after the UV detector was split off to

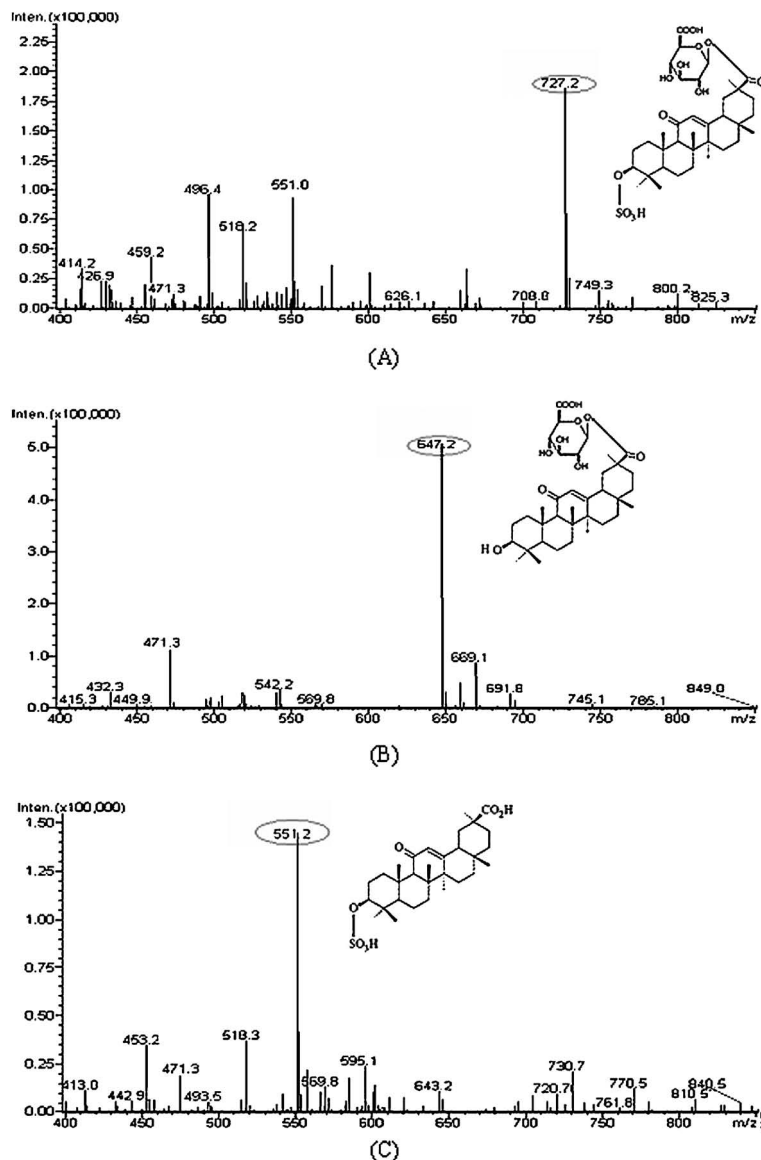


Fig. 5. A: ESI⁺ mass spectra of peak 1 in Fig. 4A, the most prominent stick was consistent with the molecular weight ($m/z = 727$) of GA-gluc-sulfate; B: ESI⁺ mass spectra of peak 2 in Fig. 4A, the most prominent stick was consistent with the molecular weight ($m/z = 647$) of GA-gluc; C: ESI⁺ mass spectra of peak 3 in Fig. 4A, the most prominent stick was consistent with the molecular weight ($m/z = 551$) of GA-sulfate.

the mass spectrometer at the ratio of 1:4. The column oven and autosampler tray temperature were kept constant at 40 and 4°C, respectively. Peaks were detected at 254 nm. Analysis by LC-MS was performed in positive electrospray ionization (ESI⁺) with the detector voltage set at 1.6 kV. The scan range in LC-MS analysis was from *m/z* 400 to 900.

Results

HPLC Analysis of Biliary Metabolites: Representative HPLC chromatograms of rat bile collected during 0–2 h post dose are presented in **Figure 3**. Compared with controls (**Fig. 3A**), there were three drug-related peaks at retention times of 5.6, 9.8, and 11.5 min in the bile of rats dosed with GA (**Fig. 3B**). The parent compound GA, which has a retention time of approximately 40 min (corresponding to peak 4 in **Fig. 4B, 4C**) was not detectable in bile.

Hydrolysis and LC-MS Analysis of Biliary Metabolites: The metabolites corresponding to peaks 1, 2 and 3 in **Figure 3B** were also confirmed by LC-MS total ion chromatogram (TIC) (**Fig. 4A**). Peak 3 in **Figure 4A** showed resistance to both glucuronidase (**Fig. 4B**) and alkaline hydrolysis (**Fig. 4C**), suggesting it is a sulfate conjugate. Its protonated molecular ion was recorded at *m/z* 551 (**Fig.**

5C), which was consistent with the molecular weight of GA-sulfate. These combined observations allowed the identification of peak 3 as 18β-glycyrrhetyl-3-*O*-sulfate (GA-sulfate).

The protonated molecular ion of the metabolite corresponding to peak 2 in **Figure 4A** was observed at *m/z* 647 (**Fig. 5B**) which is consistent with the molecular weight of the monoglucuronide conjugate of GA. **Figure 4C** shows that the metabolite in peak 2 is hydrolysable under alkaline conditions, with concomitant increase in the area of peak 4 (GA) suggesting that this is an acyl glucuronide. GA has only one carboxy group (C₃₀-OOH) available for conjugation to form an acyl glucuronide. This peak was therefore tentatively assigned as 18β-glycyrrhetyl-3-*O*-glucuronide (GA-gluc).

Peak 1 in **Figure 4A** was abolished after being treated with glucuronidase (**Fig. 4B**) and under alkaline conditions (**Fig. 4C**), resulting in the increase in the areas of both peak 3 (identified above as GA-sulfate) and peak 4 (GA). Its protonated molecular ion was detected at *m/z* 727 (**Fig. 5A**), which is consistent with the molecular weight of GA-gluc-sulfate. Based on its base-lability and MS spectral information, peak 1 was tentatively identified as a novel metabolite of GA: 18β-glycyrrhetyl-3-*O*-sulfate-3-*O*-glucuronide (GA-gluc-sulfate).

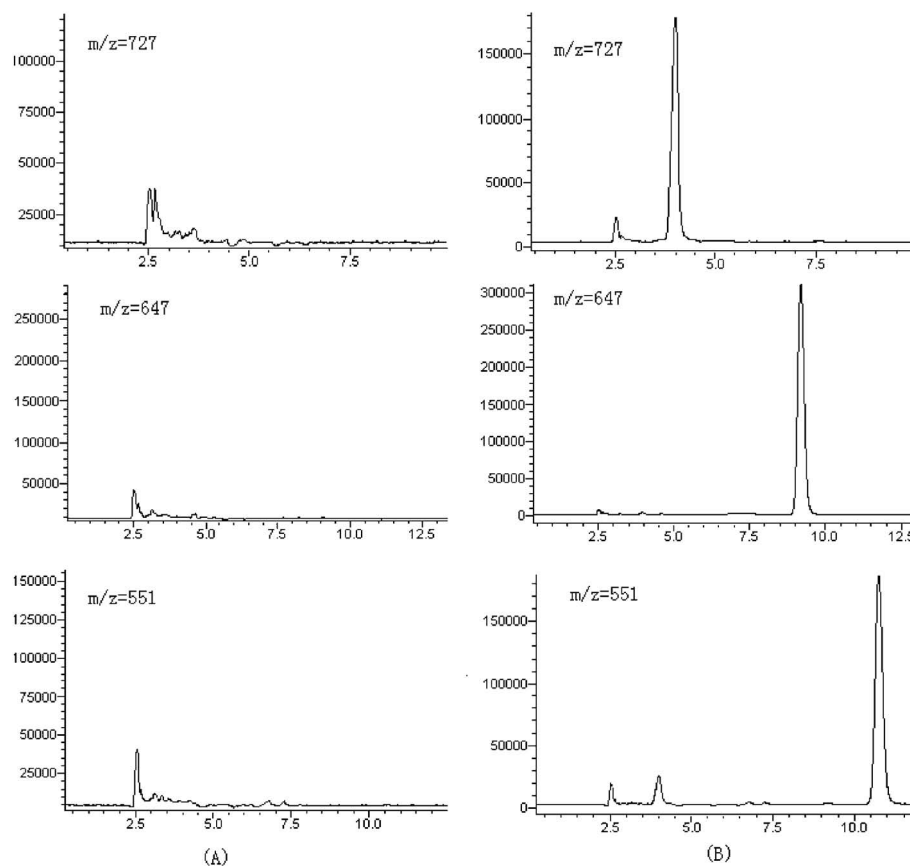


Fig. 6. Typical SIM chromatograms of metabolites in control rat bile (A) and rat bile during 2 h after i.v. injection of GA at a dose of 2 mg/kg (B)

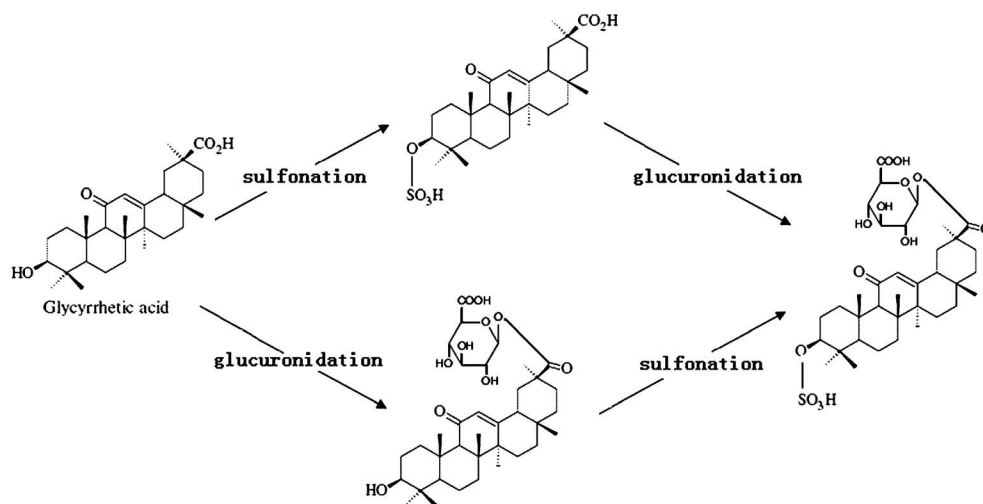


Fig. 7. Hepatic biotransformation of GA through a two-step intermediary process

Selected ion monitoring (SIM) of Biliary Metabolites: LC-MS was also performed in positive ion selected ion monitoring (SIM) mode using target ions at m/z 727, 647, 551 and 471 (Fig. 6). It was confirmed that the 3 metabolites existed in rat bile collected during the 2h after intravenous injection of GA but not in the bile of controls.

Discussion

This study has identified three major metabolites of GA in bile samples from GA-dosed rats, two of which were identified as the known 18 β -glycyrrhetyl-30-glucuronide and 18 β -glycyrrhetyl-3-O-sulfate. The third metabolite, a novel 18 β -glycyrrhetyl-3-O-sulfate-30-glucuronide (GA-glu-sulfate) diconjugate of GA was identified in rat bile in the current study. When GA (2 mg/kg) is administered i.v. to duct-cannulated rats, 18-glycyrrhetyl-3-O-glucuronide is formed as a minor metabolite of which excretion into bile is less than 1% of dose.¹⁴⁾ However, we failed to detect this metabolite. In this study, bile samples used for qualitative analysis were diluted with mobile phase. This might relate to the failure of 18 β -glycyrrhetyl-3-O-glucuronide.

The identification of GA-glu-sulfate raises questions about the mechanisms of diconjugate formation. Sulfate-glucuronide diconjugates have been identified for other compounds. For example, an acyl glucuronide-sulfate conjugate of naproxen has been identified as a major biliary metabolite in male SD rats.¹³⁾ The species differences in the hepatic formation of diconjugates have also been previously reported for bisphenol A (BPA) using hepatocytes from humans, mice, and rats. BPA-glucuronide is a major metabolite in hepatocytes from all species except for male Fischer-344 rats. In this case, BPA-glucuronide-sulfate diconjugate is a major metabolite formed.¹⁵⁾ The same has been reported for the *in vitro* metabolism of the suspected pro-oestrogenic compound, methoxychlor (MXC). In liver slices from male rats, the bis-demethylated MXC glucuronide and glu-

ronide-sulfate diconjugate are major metabolites, whereas the mono and bis-demethylated MXC glucuronides predominate in the female.¹⁶⁾

According to evidence that sulfate and glucuronidated conjugates can serve as substrates for further metabolism,¹⁷⁻²⁰⁾ a two-step pathway of phase II metabolism of GA in the rat is proposed as that GA undergo both initial sulfonation and subsequent glucuronidation, or alternatively initial glucuronidation followed by further sulfonation to form GA-glu-sulfate (Fig. 7).

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