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Short communication

Metabolites identification of bioactive licorice compounds in rats

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ABSTRACT

Licorice (*Glycyrrhiza uralensis* Fisch.) is one of the most popular herbal medicines worldwide. This study aims to identify the metabolites of seven representative bioactive licorice compounds in rats. These compounds include 22β-acetoxyl glycyrrhizin (**1**), licoflavonol (**2**), licoricidin (**3**), licoisoflavanone (**4**), isoglycycoumarin (**5**), semilicoisoflavone B (**6**), and 3-methoxy-9-hydroxy-pterocarpan (**7**). After oral administration of 250 mg/kg of **1** or 40 mg/kg of **2**–**7** to rats, a total of 16, 43 and 31 metabolites were detected in the plasma, urine and fecal samples, respectively. The metabolites were characterized by HPLC/DAD/ESI–MS^n and LC/IT-TOF-MS analyses. Particularly, two metabolites of **1** were unambiguously identified by comparing with reference standards, and 22β-acetoxyl glycyrrhizin-6′-methyl ester (**1-M2**) is a new compound. Compound **1** could be readily hydrolyzed to eliminate the glucuronic acid residue. The phenolic compounds (**4**–**7**) mainly undertook phase II metabolism (glucuronidation or sulfation). Most phenolic compounds with an isoprenyl group (chain or cyclized, **2–5**) could also undertake hydroxylation reaction. This is the first study on *in vivo* metabolism of these licorice compounds.

1. Introduction

Licorice, or Gan-Cao in Chinese, is derived from the roots and rhizomes of *Glycyrrhiza uralensis* Fisch. It is frequently used in traditional medicine to treat gastric ulcer, diabetes, cancer, hepatitis, influenza, and pulmonary diseases [1,2]. Its major bioactive constituents include triterpene saponins, flavonoids, and coumarins [3,4]. A systematic study on the *in vivo* metabolism of licorice could facilitate understanding its effective components and mechanism of action. However, metabolites identification of a multi-component herbal medicine is usually challenging, due to fairly low concentrations of the metabolites in biological samples, and poor understanding on the metabolic pathways of different types of compounds. Therefore, to establish the metabolic pathway of representative compounds could be helpful for fully elucidating the metabolism of a complex herbal medicine. In our previous reports, we had studied the rat metabolism of thirteen major flavonoids, saponins, and coumarins of licorice [2,5,6]. Here we report the metabolism of seven other licorice compounds with significant bioactivities.

These compounds include 22β-acetoxyl glycyrrhizin (**1**), licoflavonol (**2**), licoricidin (**3**), licoisoflavanone (**4**), isoglycycoumarin (**5**), semilicoisoflavone B (**6**), and 3-methoxy-9-hydroxy-pterocarpan (**7**), which belong to saponin, flavonol, isoflavan, isoflavanone, coumarin, isoflavone, and pterocarpan, respectively (Fig. 1). Little is known about oral absorption and *in vivo* metabolism of these compounds, so far. In this work, a total of 16, 43 and 31 metabolites were respectively detected in rats plasma, urine and fecal samples by liquid chromatography coupled with mass spectrometry (LC/MS). Their metabolic pathways were also proposed.

2. Materials and methods

2.1. Chemicals and reagents

The licorice compounds were isolated from *G. uralensis* Fisch. by the authors [3,4]. Their structures were characterized by NMR and mass spectrometry. The purities were above 98% according to HPLC/UV analysis. The metabolites **1-M2** and **1-M11** were also isolated from *G. uralensis*. The detailed separation procedures were described in Supplemental data. Heparin was purchased from Solarbio (Beijing, China). The other reagents were of analytical grade.

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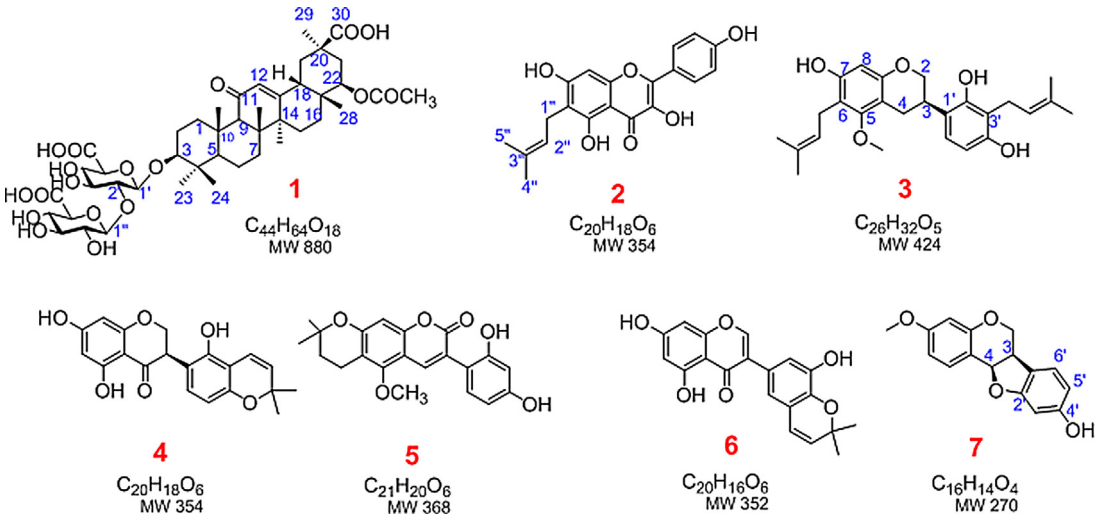

Fig. 1. Chemical structures of 22β-acetoxyl glycyrrhizin (**1**), licoflavonol (**2**), licoricidin (**3**), licoisoflavanone (**4**), isoglycycoumarin (**5**), semilicoisoflavone B (**6**), and 3-methoxy-9-hydroxy-pterocarpan (**7**).

2.2. Animals and drug administration

Male Sprague-Dawley rats (180–220 g) were obtained from the Laboratory Animal Center of Peking University Health Science Center. The rats were bred in a cage (465 × 300 × 200 mm) in a breeding room at 25 °C, 60 ± 5% humidity, and a 12-h dark-light cycle for 3 days. The rats had free access to water and normal chow. All animals were fasted overnight before experiments. The animal facilities and protocols were approved by the Animal Care and Use Committee of Peking University Health Science Center. All procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). The pure compounds were separately suspended in 0.5% carboxy-methyl cellulose sodium (4 mg/mL), and were given to rats (n = 2, 2 mL for each rat) orally at 40 mg/kg. As an exception, **1** was dissolved in water and was orally administered to rats (n = 2) at 250 mg/kg. The control was administered with 2 mL normal saline.

2.3. Preparation of plasma, urine and fecal samples

Blood (1 mL) was collected into heparinized tubes from the angular vein at 0.5, 1, 2, 4, 6 and 8 h after oral administration (2 rats for each time point; for each rat, blood was collected at 0.5, 2 and 6 h, or at 1, 4 and 8 h), respectively, and was then centrifuged at 6000 rpm for 10 min to obtain the plasma. The plasma samples for different time points were mixed, and an aliquot of 3 mL was treated with 4 volumes of methanol to precipitate protein. The mixture was vortexed (2200 rpm) for 5 min, and centrifuged at 9000 rpm for 10 min. The supernatant was separated, dried in vacuum at 37 °C, dissolved in 200 μL of methanol, and then filtered through a 0.22-μm membrane for LC/MS analysis.

For urine and fecal samples, the rats were held in metabolism cages (DXL-D, Keke Medical Model Co. Ltd., Shanghai, China), and 0–24 h samples were collected. An aliquot of 4 mL of urine was loaded on a pretreated SPE column (Oasis HLB, 6 mL, Waters, Milford, MA, USA), washed with 5 mL of water, and then successively eluted with 5 mL of 5% methanol and 5 mL of methanol. The methanol eluate was collected and dried in vacuum at 37 °C. The residue was dissolved in 200 μL of methanol and filtered through a 0.22-μm membrane for LC/MS analysis. Feces were dried in air and then ground into a crude powder. The powder (1.0 g) was extracted with methanol (20 mL) in an ultrasonic bath for 30 min. The solution was dried, and the residue was dissolved in 200 μL of methanol and filtered through a 0.22-μm membrane for LC/MS analysis.

2.4. HPLC/DAD/ESI–MS^n analysis

HPLC/DAD/ESI–MS^n analysis was performed on an Agilent series 1100 HPLC instrument connected to a Finnigan LCQ Advantage ion trap mass spectrometer (ThermoFisher Scientific, Waltham, MA). Samples were separated on an Agilent Zorbax SB-C_{18} column (4.6 × 250 mm, 5 μm) protected with a Zorbax Extend-C_{18} guard column (4.6 × 12.5 mm, 5 μm). The column temperature was 30 °C. The mobile phase consisted of acetonitrile (A) and water containing 0.1% (v/v) formic acid (B). A linear gradient elution program was used as follows: 0 min, 12% A; 30 min, 39% A; 40 min, 44% A; 50 min, 95% A; 55 min, 95% A. The flow rate was 1.0 mL/min, and the effluent was introduced into ESI source of the mass spectrometer at 0.25 mL/min via a T-union splitter. UV spectra were obtained by scanning from 200 to 400 nm. The ESI source was operated in the negative ion mode. Ultra-high purity helium and high-purity nitrogen was used as the collision gas and nebulizing gas, respectively. The optimized parameters were as follows: ion spray voltage, 3.5 kV; sheath gas, 50 arbitrary units; auxiliary gas, 5 arbitrary units; capillary temperature, 350 °C; capillary voltage, −30 V; tube lens offset voltage, −30 V. Mass spectra were recorded in the range of m/z 150–1000. MS^n (n = 2–4) was triggered by a data-dependent threshold. The collision-induced dissociation energy was adjusted to 35% for **2–7** and 38% for **1**. The isolation width of precursor ions was 2.0 mass units. Data were processed by Xcalibur 2.0.7 software (ThermoFisher).

2.5. HPLC/ESI-IT-TOF-MS analysis

High-resolution mass spectra were obtained on an IT-TOF mass spectrometer connected to an LC-20 system (Shimadzu, Tokyo, Japan). The HPLC conditions were the same as described in Section 2.4. The ESI source was operated in the negative ion mode. The collision and cooling gas was high-purity argon (Ar), and the nebulizing gas was high-purity nitrogen (N_2, 1.5 L/min). Curved desolvation line (CDL) temperature, 200 °C; interface voltage, 3.5 kV; detector voltage, 1.70 kV; drying gas pressure, 100 kPa; heat block temperature, 200 °C; MS full scan range, m/z 200–1000. MS data were processed by LCMS Solution software (Shimadzu).