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Metabolites identification of glycyrin and glycyrol, bioactive coumarins from licorice

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ABSTRACT

Coumarins are an important group of bioactive constituents in licorice (*Glycyrrhiza uralensis*), a worldwide popular herbal medicine. This study aims to elucidate the metabolism of two major licorice coumarins, glycyrin and glycyrol in rats. After oral administration of 40 mg/kg glycyrin, neither the parent compound nor its metabolites could be detected in rats plasma or urine samples, indicating that glycyrin had poor oral bioavailability. Two hydroxylated metabolites, 4″-hydroxyl glycyrin and 5″-hydroxyl glycyrin, were detected in rat liver microsome incubation system. Among them, the major metabolite 4″-hydroxyl glycyrin, which is a new compound, was obtained by microbial transformation of *Syncephalastrum racemosum* AS 3.264. Its structure was fully identified by 1D and 2D NMR. Meanwhile, glycyrol, together with three metabolites, were detected in rats urine and fecal samples after oral administration (40 mg/kg). Their structures were tentatively characterized by LC/MS. Glycyrol mainly undertakes hydroxylation metabolism, accompanied by hydration and dehydrogenation as minor reactions. This is the first systematic study on metabolism of glycyrin and glycyrol. The results could be valuable to evaluate druggability of these bioactive natural products.

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1. Introduction

Licorice, or Gan-Cao in Chinese, is a popular herbal medicine in China and many other countries. It is derived from the roots and rhizomes of *Glycyrrhiza uralensis*. Licorice is mainly used to treat cough, gastric ulcer, hepatitis, as well as pulmonary and skin diseases [1–5]. The major bioactive constituents of licorice include saponins, flavonoids, and coumarins. Glycycoumarin, glycyrin, and glycyrol are the major coumarins of licorice [6]. Significant bioactivities of these coumarins have been revealed in the recent years. For instance, glycyrin and glycyrol could inhibit HCV virus with IC_{50} values of 18.85 and 12.57 μM, respectively [7]. Glycyrin could lower the blood pressure of spontaneously hypertensive rats through PPAR-γ binding [8], and glycyrol could suppress collagen-induced arthritis in mice through decreasing NF-κB and NFAT transcriptional activities and inhibiting IL-2 expression [9]. Moreover, glycyrol could strongly inhibit neuraminidase (IC_{50} 3.1 μM), and down-regulate the inducible nitric oxide synthase at 5–50 μM [10,11]. However, little is known on oral bioavailability and *in vivo* metabolism of these coumarins, so far. Recently, we have reported the metabolic pathway of glycycoumarin in rats [12]. In this study, we report metabolites identification of glycyrin and glycyrol in rats and rat liver microsomes.

2. Materials and methods

2.1. Chemicals and reagents

Glycyrin and glycyrol were isolated from *G. uralensis* Fisch. by the authors [12]. Their structures were fully characterized by NMR spectroscopy and mass spectrometry (Fig. 1). The purities were above 98% as determined by HPLC/UV analysis. HPLC-grade acetonitrile, methanol and formic acid were from Mallinkrodt Baker (Phillipsburg, NJ, USA). Ultra-pure water was prepared with a Milli-Q water purification system (Millipore, Billerica, MA, USA). Other reagents were of analytical grade. Heparin was purchased from Solarbio (Beijing, China). β-Nicotinamide adenine dinucleotide phosphate hydrate (β-NADP), D-glucose 6-phosphate sodium salt (G-6-P), and glucose-6-phosphate dehydrogenase (G-6-P-DE) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Column chromatography was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Corporation, Qingdao, China), ODS (Fuji

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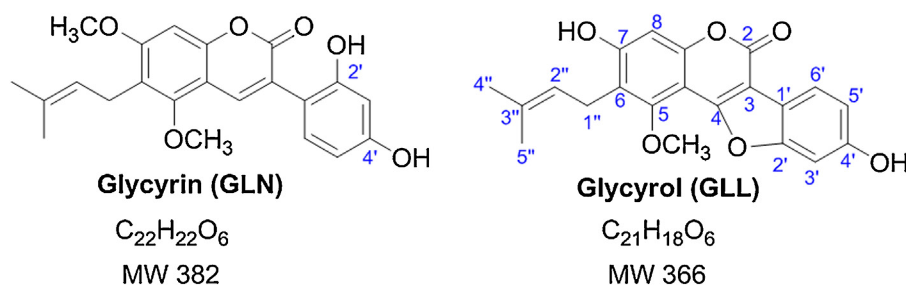

Fig. 1. Chemical structures of glycyrin (**GLN**) and glycyrol (**GLL**).

Silysia Chemical Ltd., Kasugai, Japan), and Sephadex LH-20 (Pharmacia Biotech AB, Sweden).

2.2. Animals and drug administration

Male Sprague-Dawley rats (200 ± 20 g, body weight) were obtained from the Laboratory Animal Center of Peking University Health Science Center. The rats were bred in a cage (465 mm × 300 mm × 200 mm) in a breeding room at 25 °C, 60 ± 5% humidity, and a 12-h dark-light cycle. The rats were given access to tap water and normal chow *ad libitum*. All the animals were bred under the above conditions for 3-day acclimation, and were then fasted overnight before the experiments. The animal facilities and protocols were approved by the Animal Care and Use Committee of Peking University Health Science Center. All procedures were based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Animals were divided into six groups (GLN-A, GLN-B, GLL-A, GLL-B, Blank-A, Blank-B), and each group had two rats ($n = 2$). The pure compound glycyrin or glycyrol was suspended in 0.5% carboxymethylcellulose sodium, and was orally given to rats at 40 mg/kg, respectively. For the control group, the rats were administrated 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, 2, 6 h for groups GLN-A, GLL-A, Blank-A, and at 1, 4, 8 h for groups GLN-B, GLL-B, and Blank-B, respectively. The blood was centrifuged at 6000 rpm for 20 min to obtain the plasma. Plasma samples of the two rats 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 150 μL of methanol, and then filtered through a 0.22-μm membrane.

The rats were held in metabolism cages (DXL-D, Keke Medical Model Co. Ltd., Shanghai, China), and 0–24 h urine and fecal samples were collected. An aliquot of 4 mL of urine was loaded onto 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 150 μL of methanol and filtered through a 0.22-μm membrane for LC/MS analysis. Feces were dried in the 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 resulting solution was dried, and the residue was dissolved in 150 μL of methanol and filtered through a 0.22-μm membrane for analysis.

2.4. Rat liver microsome incubation

The rat liver microsome incubation experiments were carried out according to our recently reported procedure [13]. Glycyrin and glycyrol were dissolved in methanol respectively, and then diluted with PBS. The incubation mixture (300 μL) contained rat hepatic microsomes (0.6 mg/mL), potassium phosphate buffer (pH 7.4, 0.1 mM), $MgCl_2$ (5 mM), and nicotinamide adenine dinucleotide phosphate (NADPH)-generating system. The final concentration of glycyrin or glycyrol was 50 μM. The total amount of organic solvent was lower than 1% (v/v). PBS containing methanol was used as the blank control. The reaction was initiated by adding the NADPH-generating system, and was terminated by 1200 μL of cold acetonitrile at 4 °C after 2 h. For negative control samples, PBS was added instead of NADPH-generating system. The mixture was stored at 4 °C for 30 min, and the precipitated protein was removed by centrifugation (10,000 × *g* for 10 min at 4 °C).

2.5. Microbial transformation

Microbial transformation experiments were carried out according to our recent report [12]. The fungal strains were purchased from China General Microbiological Culture Collection Center. *Syncephalastrum racemosum* AS 3.264 was incubated at 25 °C on a rotary shaker (150 rpm) in the dark, and the fermentation was carried out in 1000 mL Erlenmeyer flasks containing 400 mL potato culture medium for scaled-up biotransformation. To each flask of 2-day-old cultures, 2 mL of glycyrin (10 mg/mL in methanol, a total of 120 mg) was added. After 6 days of incubation, the cultures were pooled and filtered, and an equal volume of ethyl acetate was used to extract the supernatant. The organic layer was concentrated to dryness, and was separated by silica gel column chromatography. The column was eluted with mixtures of petroleum ether-ethyl acetate (4:1, 3:1, 2:1, 1:1, 1:2, 1:4, 1:8, v/v) to obtain seven fractions. Fr. 5 was purified by semi-preparative HPLC and eluted with acetonitrile-water (30:70, v/v) to yield **GLN-M2** (18 mg). Semi-preparative HPLC was performed on an Agilent 1200 instrument equipped with a YMC Pack ODS-A column (10 mm × 250 mm, 5 μm, YMC Co. Ltd., Japan).

2.6. HPLC/DAD/ESI-MS^n analysis

The analysis was performed on an Agilent series 1100 HPLC instrument connected to a Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) through an ESI ion source. Samples were separated on an Agilent ZORBAX SB-C_{18} column (4.6 mm × 250 mm, 5 μm) protected with a ZORBAX Extend-C_{18} guard column (4.6 mm × 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; 25 min, 100% A; 30 min, 100% A. The flow rate was 1.0 mL/min, and the effluent was introduced into the 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 MS instrument was operated in positive ion mode for glycyrin and negative ion mode for glycyrol, respectively. The optimized parameters of

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