Journal of Pharmaceutical and Biomedical Analysis 98 (2014) 287–295

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Metabolites identification of glycycoumarin, a major bioactive coumarin from licorice in rats

Qi Wang, Xue Qiao, Chun-fang Liu, Shuai Ji, Lin-min Feng, Yi Qian, De-an Guo, Min Ye*

State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China

ARTICLE INFO

Article history:
Received 21 February 2014
Received in revised form 6 May 2014
Accepted 2 June 2014
Available online 6 June 2014

Keywords:
Glycycoumarin
Glycyrrhiza uralensis
LC/MS
Metabolites identification
NMR

ABSTRACT

Glycycoumarin is a major bioactive coumarin of licorice (*Glycyrrhiza uralensis*), one of the most popular herbal medicines worldwide. In this work, the metabolism of glycycoumarin in rats was investigated. After oral administration of 40 mg/kg glycycoumarin, 4 and 10 metabolites were respectively detected in rats plasma and urine samples by liquid chromatography coupled with mass spectrometry (LC/MS). These metabolites were tentatively characterized by analyzing their tandem mass spectra and high-resolution mass spectra, and the structures of glucuronides were confirmed by β-glucuronidase hydrolysis. Glycycoumarin mainly undertakes hydroxylation and glucuronidation metabolism, accompanied by hydrogenation and dehydrogenation as minor reactions. Two hydroxylated metabolites, 4″-hydroxyl glycycoumarin and 5″-hydroxyl glycycoumarin, were obtained by microbial transformation of *Syncephalastrum racemosum* AS 3.264, and their structures were fully identified by 1D and 2D NMR. Both metabolites are new compounds. Furthermore, they were proved to be catalyzed by P450 enzymes by rat liver microsomes incubation experiments. Finally, a metabolic pathway of glycycoumarin in rats was proposed. This is the first systematic study on metabolites identification of glycycoumarin.

1. Introduction

Licorice (Gan-cao in Chinese), derived from the roots and rhizomes of *Glycyrrhiza uralensis* Fisch., is one of the most popular herbal medicines worldwide. It is mainly used to treat cough, gastric ulcer, inflammation, abdominal pain, cardiovascular diseases, and cancer [1]. The main bioactive chemical constituents in licorice include flavonoids, triterpene saponins, and coumarins [2]. Although the chemistry of licorice has been extensively studied, the "effective" compounds responsible for the treatment of different indications are far from clear, so far. *In vivo* metabolism and pharmacokinetic studies could play an important role in elucidating the effective compounds of multi-component herbal medicines [3]. In our previous reports, we had studied the metabolism of licorice flavonoids and saponins in rats [4,5]. However, little is known on the metabolism of licorice coumarins.

Most licorice coumarins are 3-arylcoumarins. Up to date, a total of 24 3-arylcoumarins have been isolated from *Glycyrrhiza* species [6]. Glycycoumarin (GCM) is a major coumarin in licorice, and accounts for 0.8 mg/g of the crude drug of *G. uralensis* [7]. GCM has been reported to show significant biological activities. For instance, GCM could significantly inhibit smooth muscle contraction induced by various types of stimulants including KCl and $BaCl_2$ (IC_{50} 2.93–7.39 μM), and has been proved to be the antispasmodic compound in licorice [8]. Recently, GCM has also been reported to have anti-hepatitis C virus, antioxidant, and anti-inflammatory activities [9,10].

The present study aims to elucidate the metabolic pathways of glycycoumarin after oral administration in rats. A total of 10 metabolites in the urine and 4 metabolites in the plasma were characterized by liquid chromatography coupled with mass spectrometry. Furthermore, two hydroxylated metabolites were prepared by microbial transformation as an *in vitro* model, and their structures were unambiguously identified by nuclear magnetic resonance. They were also confirmed to be catalyzed by P450 enzymes by incubating with rat liver microsomes. Finally, a metabolic pathway of glycycoumarin in rats was proposed.

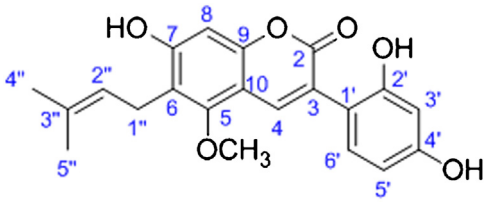
2. Materials and methods

2.1. Chemicals and reagents

Glycycoumarin was isolated from licorice (dried roots and rhizomes of *G. uralensis* Fisch.) by the authors [7]. Its structure was characterized by NMR and mass spectrometry (Fig. 1). The purity

* Corresponding author at: State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, 38 Xueyuan Road, Beijing 100191, China. Tel.: +86 10 82801516; fax: +86 10 82802024.
E-mail addresses: yemin@bjmu.edu.cn, yeminpku@yahoo.com (M. Ye).

http://dx.doi.org/10.1016/j.jpba.2014.06.001
0731-7085/



Glycycoumarin (GCM)

$C_{21}H_{20}O_6$

MW 368

Fig. 1. Chemical structure of glycycoumarin (GCM).

was above 98%, according to HPLC/UV analysis. HPLC-grade acetonitrile, methanol and formic acid were from Mallinkrodt Baker (Phillipsburg, NJ, USA). Ultra-pure water was prepared with a Millipore Milli-Q water purification system (Billerica, MA, USA). Other reagents were of analytical grade. β-glucuronidase (HP-1 type) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Heparin was from Solarbio (Beijing, China). β-Nicotinamide adenine dinucleotide phosphate hydrate (β-NADP), D-glucose 6-phosphate sodium salt (G-6-P), and glucose-6-phosphatedehydrogenase (G-6-P-DE) were purchased from Sigma–Aldrich.

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 housed 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, and were given access to water and normal chow *ad libitum*. 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). Glycycoumarin was suspended in 0.5% sodium carboxy-methyl cellulose and was orally administrated to rats at 40 mg/kg, respectively. Saline (2 mL) was given to rats (n = 2) for the control group.

2.3. Preparation of plasma and urine samples

Blood (1 mL) was collected from the angular vein of rats at 0.15, 0.25, 0.5, 1, 2, and 4 h (n = 2 for each time point), respectively, and was centrifuged at 6000 rpm for 20 min to obtain the plasma. The plasma samples were mixed, and an aliquot of 3 mL was treated with 12 mL of methanol to precipitate protein. The mixture was vortexed for 5 min, and centrifuged at 9000 rpm for 10 min. The supernatant was dried in vacuum at 40 °C, dissolved in 300 μL of methanol, and then filtered through a 0.22-μm membrane.

To collect urine and feces samples, rats were held in metabolism cages (DXL-D; Keke Medical Model Co. Ltd., Shanghai, China) for 24 h. An aliquot of 4 mL of urine was loaded on an Oasis HLB SPE column (Waters, Milford, MA, USA), and eluted with 5 mL of water, 5 mL of 5% methanol, and 5 mL of methanol, successively. The methanol eluate was collected and dried in vacuum at 40 °C. The residue was dissolved in 300 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 by 20-fold of methanol in an ultrasonic bath for 30 min. The resulting solution was dried, and the residue was dissolved in 300 μL of methanol and filtered through a 0.22-μm membrane for analysis.

2.4. Enzyme hydrolysis

A 50-μL aliquot of plasma or urine sample was dried under a gentle flow of nitrogen and was mixed with 200 μL of β-glucuronidase solution (containing 19.86 U/μL, in sodium acetate buffer, pH 5.5). The mixture was vortexed for 5 min, and incubated in a 37 °C water bath for 2 h. Then it was added with 1 mL of methanol to precipitate protein, and then centrifuged at 9000 rpm for 10 min. The supernatant was dried under a gentle nitrogen flow and then dissolved in 300 μL of methanol. The solution was filtered through a 0.22-μm membrane for chemical analysis.

2.5. Microbial transformation

Microbial transformation was used as an *in vitro* model to prepare rat metabolites of glycycoumarin. The fungal strains were obtained from China General Microbiological Culture Collection Center. The microbial transformation experiments were carried out according to our previous report [11]. For preparative scale biotransformation, *Syncephalastrum racemosum* AS 3.264 was incubated at 25 °C with rotary shaking at 150 rpm in 1000 mL Erlenmeyer flasks containing 400 mL of potato culture medium. Two days after inoculation, glycycoumarin (10 mg/mL in methanol, 2 mL for each flask) was added to the cultures, and the incubation continued for six days. A total of 180 mg of glycycoumarin was used. Then the cultures were pooled and filtered, and the supernatant was extracted with an equal volume of ethyl acetate. The organic layer was concentrated to dryness, and was separated on a silica gel column. The column was eluted with mixtures of chloroform–methanol (30:1, 20:1, 10:1, v/v) to obtain seven fractions. Fr. 4 was purified by semi-preparative HPLC and eluted with acetonitrile–water (30:70) to obtain M7 (1 mg) and M8 (10 mg). Semi-preparative HPLC was performed on an Agilent 1200 instrument with a YMC Pack ODS-A column (250 mm × 10 mm, 5 μm, YMC Co. Ltd., Japan).

2.6. Rat liver microsomes incubation

Rat liver microsomes incubation of glycycoumarin was conducted following the procedure described in our recent paper [12]. Glycycoumarin was dissolved in methanol, and then diluted with PBS. The final concentration of glycycoumarin in the 300-μL incubation mixture was 50 μM, and the amount of organic solvent in the mixture was lower than 1% (v/v). Reactions were initiated by adding the NADPH-generating system, and PBS was added as the negative control. The incubation was conducted at 37 ± 1 °C. The reaction was terminated after 2 h of incubation by adding 1200 μL of cold acetonitrile. The mixture was then kept at 4 °C for 30 min, and the precipitated protein was removed by centrifugation at 10,000 × g for 10 min at 4 °C.

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

HPLC/DAD/ESI-MS^n analysis was performed on an Agilent series 1100 HPLC instrument coupled with a Finnigan LCQ Advantage ion trap mass spectrometer (Thermo Fisher 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 the ESI source of mass spectrometer at 0.25 mL/min *via* a T-union splitter. UV spectra were obtained by scanning from 200 to 400 nm. For MS analysis, the