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Characterization of metabolites of worenine in rat biological samples using liquid chromatography–tandem mass spectrometry

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ABSTRACT

The *in vivo* and *in vitro* metabolites of worenine in rat were identified or characterized using a specific and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method. *In vivo* samples including rat urine, feces, and plasma samples were collected after ingestion of 25 mg/kg worenine to healthy rats. The *in vivo* and *in vitro* samples were cleaned up by a solid-phase extraction procedure (C18 cartridges) and a liquid–liquid extraction procedure, respectively. Then these pretreated samples were injected into a reversed-phase C18 column with mobile phase of methanol–ammonium acetate (2 mM, adjusted to pH 3.5 with formic acid) (60:40, v/v) and detected by an on-line MS/MS system. As a result, at least twenty-seven metabolites and the parent medicine were found in rat urine after ingestion of worenine. Seven metabolites and the parent medicine were identified or characterized in rat feces. Three metabolites and the parent medicine were detected in rat plasma. One metabolite was found in the rat intestinal flora incubation mixture, and three metabolites were characterized in the homogenized liver incubation mixture. The main phase I metabolism of worenine in rat was dehydrogenization, hydrogenation, hydroxylation, and demethylene reactions, and that of phase II was sulfation and glucuronidation.

1. Introduction

Worenine (structure shown in Fig. 1, existed isomerism) is one of the bioactive components isolated from *Coptidis rhizoma* (Huanglian), a widely used Traditional Chinese Medicine, which has been used for centuries for the treatment of dysentery, hypertension, inflammation, and liver diseases [1,2]. Despite its important therapeutic value, its *in vivo* or *in vitro* metabolism is not clear yet. Up till now, the works only focused on the qualitative determination of worenine in plant [3,4].

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been proven to be a powerful analytical tool for the identification of medicine and its metabolites in biological matrices due to its high sensitivity and specificity [5–8]. It is especially suitable for the analysis of thermolabile, highly polar and non-volatile metabolites. MS/MS technique has made possible the acquisition of structurally informative data from protonated molecules of analytes of interest, even when they are not resolved chromatographically [9,10]. Structural elucidation of medicine metabolites using LC–MS/MS is based on the premise that metabolites retain the substructures of the parent medicine molecule. MS–MS product ion spectrum of each metabolite provides detailed substructural information of its structure. So, using the product ion spectrum of parent medicine as a substructural template, metabolites presented in crude mixtures may be rapidly identified and detected based on their changes in molecular masses (ΔM) and spectral patterns of product ions, even without standards for each metabolite [11,12].

For studying the metabolism of worenine comprehensively, this work presents the metabolism of worenine in rat urine, feces and plasma. The present study also involves incubation of worenine with intestinal flora and homogenized liver in order to clarify its *in vivo* and *in vitro* metabolic pathway. The parent medicine and its twenty-seven metabolites were detected in rat urine after administration of worenine orally. Seven metabolites and the parent medicine were identified or characterized in rat feces. Three metabolites and the parent medicine were detected in rat plasma. Only one metabolite was found in the rat intestinal flora incubation mixture, and three metabolites were characterized in the homogenized liver incubation mixture. All the results were reported for the first time. These results will be useful for future studies involving worenine, such as clinical therapy.

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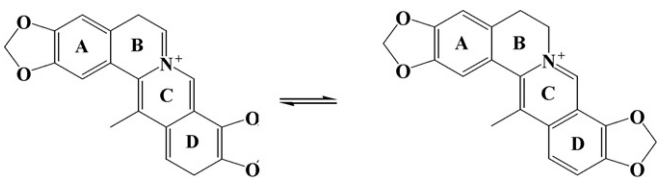

Fig. 1. The structure of worenine.

2. Experimental

2.1. Reagents and chemicals

Worenine was purchased from Beijing Hengye Zhongyuan Chemical Co., Ltd. (China). β-Glucuronidase (from *Escherichia coli*) was purchased from Sigma (St. Louis, MO, USA). Methanol was of HPLC grade (Fisher Chemical Co. Inc., CA, USA). Distilled water, prepared from demineralised water, was used throughout the study. Other reagents used were of analytical grade.

2.2. Instrumentation

LC–MS and LC–MS/MS experiments were performed on an LCQ Duo quadrupole ion trap mass spectrometer with a TSP4000 HPLC pump and a TSP AS3000 autosampler using positive electrospray as the ionization process (all components from Finnigan, Austin, TX, USA). A high-speed desk centrifuge (TGL-16C, Shanghai Anting Scientific Instrument Factory, Shanghai, China) was used to centrifuge samples. The urine samples were extracted on a C18 solid-phase extraction (SPE) cartridge (3 ml/200 mg, AccuBond, Agilent, Washington, DC, USA). The intestinal incubation experiments were carried out in anaerobic incubation bags (AnaeroPouchTM-Anaero 08G05A-23, Mitsubishi Gas Chemical Company Inc., Chiyoda-ku, Japan) using anaerobic-generating bags (Mitsubishi Gas Chemical Company Inc.).

2.3. Sample preparation

2.3.1. In vivo samples

Six male Wistar rats (200 ± 5 g, 2 months old, Hubei Experimental Animal Research Center, China) were housed in metabolic cages for the collection of urine, feces and plasma. The rats were fasted for 24 h but with access to water, and then administered a single dose of worenine (25 mg/kg) by oral gavage. Urine samples were collected during the time period 0–48 h and centrifuged at 3000 × *g* for 10 min. Feces were collected individually during the time period of 0–24 h. The urine and feces samples were stored at −20 °C until analysis. Heparinized blood samples of 200 μl were collected at 1, 2, 4, 8, and 12 h from the ophthalmic veins of the rats by sterile capillary tube, then, shaken up and centrifuged at 2000 × *g* for 10 min. The supernatants were decanted, and immediately frozen at −20 °C until analysis.

2.3.1.1. Urine extraction. Free fraction. An aliquot of 1 ml of mixed 0–48 h urine samples was loaded onto a C18 SPE cartridge that was preconditioned with 2 ml of methanol and 1 ml of water. Then, the SPE cartridge was washed with 2 ml of water and the analytes were eluted with 1 ml of methanol. The effluent was filtered through 0.45 μm membrane and an aliquot of 10 μl was used for LC–MS/MS analyses.

Acidic hydrolysis. After optimizing the acidity and the heated time, 0.8 ml of 6 M HCl and 50 mg of cysteine were added to 1 ml of mixed 0–48 h urine samples. The mixture was heated at 100 °C for 60 min. After cooling to room temperature, it was neutralized to pH 8 with 6 M NaOH and extracted with SPE cartridge immediately, just like the procedure mentioned above.

Enzymatic hydrolysis. After optimizing the acidity, temperature, enzymatic content and the time of hydrolysis, 1 ml of mixed 0–48 h urine samples was adjusted to pH 5.0 with a few drops of glacial acetic acid. Then, 0.5 ml of acetate buffer (0.5 mol/L, pH 5.0) and 0.2 ml of β-glucuronidase from *E. coli* (10,000 units/ml) were added to the solution prior to enzymatic hydrolyses. It took 5 h at 37 °C. After cooling, the solution was adjusted to pH 8 with 6 M NaOH and extracted with SPE cartridge immediately, just like the procedure mentioned above.

Free fraction was used for the comprehensive LC–MS/MS analyses of worenine and its metabolites. The target solutions after acidic and enzymatic hydrolyses were only used to assist in the investigation of phase II metabolites.

2.3.1.2. Feces and plasma extraction. The feces sample was homogenized with water. An aliquot of 500 μl feces homogenate was extracted twice with 1 ml of ethyl acetate after adding 50 μl of 0.001% Na_2CO_3 solution. The supernatant ethyl acetate layers were decanted, pooled and evaporated at 37 °C under nitrogen stream. The residue was redissolved in 500 μl of mobile phase and filtered through 0.45 μm membrane and an aliquot of 10 μl was used for LC–MS/MS analyses.

The plasma samples were added 300 μl of methanol to precipitate plasma proteins, and then centrifuged at 2000 × *g* for 10 min. The supernatant was filtered through 0.45 μm membrane and an aliquot of 10 μl was used for LC–MS/MS analyses.

2.3.2. In vitro samples

Preparation of anaerobic cultural solutions [13]. 37.5 ml A solution (0.78% K_2HPO_4), 37.5 ml B solution (0.47% KH_2PO_4, 1.18% NaCl, 1.2% $(NH_4)_2SO_4$, 0.12% $CaCl_2$, 0.25% $MgSO_4 \cdot H_2O$), 50 ml C solution (8% Na_2CO_3), 0.5 g L-cysteine, 2 ml 25% L-ascorbic acid, 1 g eurythrol, 1 g tryptone and 1 g nutrient agar were mixed together, and diluted to 1 l with distilled water. HCl (2 M) was used to adjust the solution to pH 7.5–8.0.

Metabolism in intestinal bacteria. The fresh intestinal contents were obtained from male Wistar rats (200 g, 2 months old). Samples were homogenized with a glass rod in anaerobic cultural solution at the rate of 0.5 g:1.5 ml immediately. Then, the homogenate was filtrated using gauze. Worenine was added into the above intestinal flora cultural solution to a final concentration of 50 μg/ml. The culture dishes were put in anaerobic incubation bags. The out bags of anaerobic-generating bags were opened, and put into anaerobic incubation bags immediately, then sealed. Incubations were carried out in a shaking water-bath at 37 °C anaerobically. The incubation was continued for 4 and 24 h, terminated and extracted (twice) with ethyl acetate. The organic extracts were merged and evaporated at 37 °C under nitrogen stream. The residues were reconstituted in 0.6 ml of mobile phase, centrifuged at 13,000 × *g* for 10 min. The supernatant was used for LC–MS/MS analyses.

Preparation and incubation of liver homogenate. Male Wistar rats (200 g, 2 months old) were fasted for 24 h and killed by decapitation between 10 a.m. and noon. A weighed amount of liver was rapidly placed on ice. It was rinsed twice with saline and immediately minced with scissors and homogenized in ice-cold Krebs–Henseleit buffer (pH 7.4) [14] after sterilization to yield liver homogenate (0.4 g/ml). All the above steps were carried out at 0–4 °C. The concentration of P450 was detected by spectrophotometry [15]. Worenine was added to liver homogenate at a concentration of 50 μg/ml. The mixture was incubated at 37 °C with shaking. The incubation time was varied from 0, 30, 60, 90, 120 to 240 min. The gas phase was oxygen. After the termination of incubation the mixture was extracted twice with equal volumes of ethyl acetate. The organic extracts were merged and evaporated at 37 °C under a slow