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Short communication

Investigation of the biotransformation of osthole by liquid chromatography/tandem mass spectrometry

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ABSTRACT

Osthole is an active ingredient and one of the major coumarin compounds that were identified in the genus *Cnidium moonnieri* (L.) Cussion, the fruit of which was used as traditional Chinese medicine to treat male impotence, ringworm infection and blood stasis conventionally. Recent studies revealed that osthole has diverse pharmacological effects, such as improving male sexual dysfunction, anti-diabetes, and anti-hypertentions. The inhibition of thrombosis and platelet aggregation and protection of central nerve were also observed. On the other hand, the metabolism of osthole has not yet been investigated thoroughly. Herein the biotransformation of osthole in rat was investigated after oral administration of osthole by using efficient and sensitive ultra-performance liquid chromatography–tandem quadrupole-time of flight mass spectrometry (UPLC–QTOF/MS). Eighteen osthole metabolites and the parent drug were detected and identified in rat urine. Fourteen metabolites of osthole were identified and characterized for the first time. Structures of metabolites of osthole were elucidated by comparing fragment pattern under MS/MS scan and change of molecular weight with those of osthole. The main phase I metabolic pathways were summed as 7-demethylation, 8-dehydrogenation, hydroxylation on coumarin and 3,4-epoxide. Sulfate conjugates were detected as phase II metabolites of osthole.

1. Introduction

The dried fruit of *Cnidium moonnieri* (L.) Cussion, known as "Shechuangzi" in China and "Jashoshi" in Japan, is recognized as a traditional Chinese medicine (TCM) [1]. It has been used to treat male impotence, ringworm, asthma, fungal infection and blood stasis for thousands of years. Osthole, 7-methoxy-8-isopentenyl-coumarin, is one of the major coumarin derivatives found in *C. moonnieri* (L.) Cussion. Its concentration in dried fruit is estimated to range between 4 and 22 mg/g (0.05–2.14%) depending on the origin [2]. Osthole exhibits various pharmacological activities and has been proposed the possibility of its development as a promising lead compound for drug discovery [3,4].

Osthole has been shown to possess a relaxant effect on rabbit corpus cavernosum tissues in a dose-dependent manner, which is attributed by the release of NO from sinusoidal endothelium, suggesting improvement effect of osthole on male sexual dysfunction [4,5]. Moreover, recent studies demonstrated that osthole was effective on anti-diabetes by activation of nuclear receptor PPARα and PPARγ in a dose-dependent manner [6–8], and anti-hypertentions by block of $Ca_v1.2$ channel [2]. The inhibition of thrombosis and platelet aggregation, inhibitory effect on alcohol-induced fatty liver by reduction of hepatic oxidative stress, and protection of central nervous system diseases by improving neurobehavioral functions were observed [9–14]. Osthole is also used in Chinese composite formulation to enhance the strength of bones because of its effect on proliferation and differentiate of osteoblasts [15–17].

On the other hand, it is essential to conduct scientific evaluation of pharmacokinetics and biotransformation of herbal medicines to use them in the treat of various diseases [18]. Although osthole is used widely in different therapeutic applications, the *in vivo* metabolic profiling of osthole is obscure. Different suppositions with regard to phase I metabolism of osthole exist. Previous study by using *in vitro* microsome isolated from rats (RLM) has demonstrated that 8-dehydrogenation and 7-demethylation were the major metabolic pathways of phase I metabolism of osthole [19]. Recently, Lv et al. described the identification of phase I and II metabolites of osthole in male Sprague-Dawley rats using nuclear magnetic resonance (NMR) and mass spectrometry techniques [1]. They suggested that 7-demethylation and 8-hydroxylation were the main metabolic pathways of osthole in phase I metabolism *in vivo* and glycosylation happened during phase II metabolism process. It was anticipated that incubation by RLM was not a

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suitable approach to mimic phase I metabolic reactions *in vivo* since differences in phase I metabolism may exist between *in vivo* and *in vitro*.

In a recent microbial transformation study by researchers of the same research group [20], hydroxylation, dehydrogenation, demethylation and glycosylation reactions were observed as the major metabolic pathways of osthole in the fungus *Mucor spinosus*. The newly identified osthole derivatives with enhanced water solubility and bioactivities may direct further pharmacological studies as well as laying the foundation for both *in vitro* and *in vivo* metabolism studies.

To better understand the hepatic metabolism of osthole, we have investigated the urinary metabolites of osthole in rats by ultra-performance liquid chromatography–tandem quadrupole-time of flight mass spectrometry (UPLC–QTOF/MS). UPLC coupled to high accuracy mass spectrometry (QTOF/MS) plays an important role in the identifying unknown chemicals and has been proved to be a powerful and reliable approach to structure elucidation and identification of metabolites for its unmatched resolution, accuracy, and sensitivity [21–23]. Herein, *in vivo* study was performed to determine the metabolites by comparing the profiling of the control and dosed urine. The structures of metabolites were identified by comparing MS/MS fragment patterns and change of molecular mass with those of osthole. The metabolites of osthole *in vivo* were investigated using presented method. Eighteen metabolites and parent drug were found in rat urine. Except 7-demethylation, 8-hydroxylation and 8-dehydrogenation, hydroxylation on coumarin feature, and 3,4-epoxide were also identified to be the major metabolic pathways of osthole. Fourteen metabolites, including sulfate conjugates of osthole, were identified and characterized in rat urine for the first time.

2. Experimental

2.1. Chemicals and reagents

Osthole was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC/Spectro-grade methanol was purchased from Tedia (USA). Analytical reagent grade formic acid and ethyl acetate were purchased from Fisher Company (UK). Water was produced by a Milli-Q Ultrapure water system with the water outlet operating at 18.2 MΩ (Millipore, Billerica, USA).

2.2. Animal experiment

Sprague-Dawley rats were obtained from the Animal and Plant Care Facility, HKUST. The protocol for animal experiment was approved by the Committee on Research Practice, HKUST. In Brief, six female Sprague-Dawley rats weighting about 200–220 g were housed in metabolic cages with a 12-h light cycle and fasted for 12 h with free access to water before oral administration. Four rats were orally administrated of osthole suspension (40 mg/kg) by gavage and vehicle was administrated to the others. Urine was collected for a period of 0–24 h and centrifuged at 3000 rpm for 10 min. The supernatant was collected and stored at −40 °C until analysis.

2.3. Sample preparation

1 mL ethyl acetate (EA) was added to Eppendorf tube containing 1 mL urine sample, the mixture was vortex vigorously for about 30 s and then centrifuged at 13,800 rpm for 5 min. The supernatant was transferred to a clean tube and evaporated with stream of nitrogen gas. The residue was reconstituted with 50 μL methanol–water (50:50, v/v), and was vortex-mixed and centrifuged at same conditions mentioned above. Then the supernatant was transferred to insert and injected into LC–MS/MS for analysis.

2.4. Apparatus and conditions

The rapid gradient elution was performed using a Waters ACQUITY UPLC™ system (Waters Corp., Milford, MA, USA) with a Waters ACQUITY BEH C_{18} column (100 mm × 2.1 mm i.d., 1.8 μm). The temperature of column oven was maintained at 40 °C. The mobile phase consisted of water containing 0.4% formic acid (A) and methanol (B). The gradient program was: initial 10% (B); 0–5 min, the linear gradient from 10 to 100% (B); 5–7 min, 100% (B) isocratic; 7.0–6.1 min, the linear gradient from 100 to 10% (B); 7.1–10 min, 10% (B) isocratic and reconditioning of the column. The temperature of auto sampler was fixed at 5 °C and the injection volume was set to 2 μL with partial loop mode.

Mass spectrometry was performed in positive ion electrospray (ESI) mode. The capillary and cone voltages were set at 3 kV and 25 V. The desolvation and source temperature was set to 400 °C and 120 °C, respectively. Nitrogen was used as cone and desolvation gas and the flow rate was maintained at 50 L/h and 800 L/h, respectively. Accurate mass determination was corrected by a 2 ng/μL leucine enkephalin (556.2771 amu $[M+H]^+$) solution with flow rate at 5 μL/min used as lockmass. MS data were collected in centroid mode over a range of 100–800 *m*/*z*.

3. Results and discussion

3.1. UPLC–MS and UPLC–MS/MS analysis of osthole

The UPLC and MS conditions were optimized with osthole standard and the MS chromatogram and MS/MS spectrum of osthole were shown in Fig. 1. Osthole ($C_{15}H_{16}O_3$) was eluted at about 5.11 min under above experimental conditions (Fig. 1a). The pseudo-molecular ion $[M+H]^+$ (*m*/*z* 245) and the sodiated adduct $[M+Na]^+$ (*m*/*z* 267) were identified (Fig. 1b). The product ions at *m*/*z* 189 $[M+H-C_4H_8]^+$ and 131 $[M+H-C_4H_8-CH_2O-CO]^+$ were abundant in the MS/MS spectrum of the protonated ion at *m*/*z* 245.1170. Peak at *m*/*z* 159 $[M+H-C_4H_8-CH_2O]^+$ was also observed (Fig. 1c). The fragmentation pattern of osthole was consistent with previous works [24,25].

3.2. UPLC–MS and UPLC–MS/MS analysis of metabolites

The identification of metabolites was carried out according to following rules: in the first step, possible metabolites were predicted based on knowledge of metabolism and searched in full scan chromatogram of rat urine. Then, the formulas of potential metabolites were compared with that of parent drug to refer metabolic pathways. The high mass accuracy of QTOF analyzer enables an extraction of individual chromatograms in the narrow interval of *m*/*z* scale (±5 mDa), which is useful for the elimination of other compounds with similar masses [26]. Finally the collision-related fragments of proposed metabolites were studied and compared with that of parent drug. In this procedure, major fragments of protonated ions of metabolites were observed under different collision energy and compared with that of osthole.

According to the research of Born and Lewis on metabolism of coumarin [27,28], there are two pathways of coumarin metabolism, 7-demethylation (or 7-hydroxylation) and 3,4-epoxide. 3,4-Epoxide is the major pathway in rats and mice, which is also responsible for the toxicity induced by coumarin in rodents. We anticipated that osthole undergoes 7-demethylation (or *O*-demethylation), 8-dehydrogenation, hydroxylation on coumarin, and 3,4-epoxide in phase I metabolism procedure since osthole also has coumarin-structure feature.