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Profiling and identification of metabolites of isorhynchophylline in rats by ultra high performance liquid chromatography and linear ion trap Orbitrap mass spectrometry

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ABSTRACT

The searching of potentially bioactive metabolites in the biological body is an interesting and meaningful work for the drug study. However, the structural clarification of possible metabolites is one of the most challenging tasks in drug metabolism studies because of the variety of metabolic reactions and complexity of metabolites *in vivo*. Here, an ultra high performance liquid chromatography/linear ion trap-Orbitrap mass spectrometry (U-HPLC/LTQ-Orbitrap-MS) with combination of data post-processing techniques, including extracted ion chromatogram (EIC) and multiple mass defect filters (MMDF), was established for profiling and identification of metabolites of isorhynchophylline (IR) *in vivo and in vitro*, and the possible metabolic pathways were subsequently proposed after the oral dose of 20 mg/kg; A total of 47 metabolites of IR were tentatively identified, including 47, 21, 18, and 25 metabolites in rat urine, plasma, liver and rat liver microsomes (RLM) samples, respectively. To our knowledge, most of them were reported for the first time. Seven metabolic pathways, including dehydrogenation, oxidation, hydrolysis, reduction, demethylation, hydroxylation and glucuronide conjugation were involved in the metabolism. Among them, dehydrogenation, hydrolysis, hydroxylation and oxidation were considered as the main metabolic pathway of metabolism according to metabolic profile of *in vivo* and *in vitro*. The relative percentage of each metabolite and main metabolite types were also determined to better understand the metabolic behavior of IR in rats. The newly discovered IR metabolites significantly expanded our understanding and were going to be greatly helpful for the further pharmacokinetic study of IR *in vivo*.

1. Introduction

The tetracyclic oxindole alkaloids isorhynchophylline (IR), as shown in Fig. 1, has been reported to be the major alkaloid of *Uncaria rhynchophylla* (Gou-Teng in Chinese) [1]. Several reviews on the pharmacological actions and biological activities of IR have been reported [2,3]. It has been demonstrated that IR has the antimicrobial [4], anti-inflammatory [5,6], anti-hypertension activities [7] and confers protective effects on neuronal damage *in vivo* and *in vitro* [8,9]. Accumulating evidence showed that IR holds a beneficial effect on providing neuroprotective action against Aβ-induced neuronal injury toxicity, and plays an important role in the treatment of neurodegenerative diseases [10,11].

These beneficial functions of IR have been confirmed to be associated closely with its absorption, distribution, metabolism and excretion (ADME) *in vivo*. Recently, some studies have reported on the metabolism of IR, four aromatic hydroxyl and related metabolites of IR were found in rat plasma, bile, urine and feces [12,13]. Chen et al. isolated and identified four metabolites in rat urine after oral administration of IR [9]. It was also reported that IR mainly

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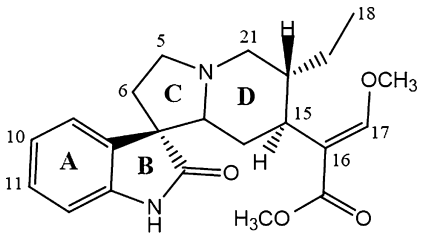

Fig. 1. The chemical structure of isorhynchophylline.

metabolize in liver after oral administration. However, there is no systematic metabolic pathway study of IR *in vivo*. Understanding the systematic metabolic behavior of IR contributes to clarifying the mechanism of its pharmacological action, therefore, the metabolism characteristic profile of IR needs to be more clarified comprehensively.

It is well known that the structural elucidation of metabolites is one of the most challenging tasks for their complex structure, lower content and various isomers. Tetracyclic oxindole alkaloid IR is a good example. Although some previous literatures reported various methods of analysis of IR and its metabolites with liquid chromatography–electrospray ionization mass spectrometry (LC–ESI–MS), less metabolite was detected due to the higher detection limitation and absence of high-resolution mass (HR-MS) instrument [13–15].

Nowadays, highly accurate, stability and sensitive HR-MS instrumentation have played the key role for the identification of drug metabolites which often have low detection in complex biological matrices [16]. Our work adopted ultra high performance liquid chromatography/linear ion trap-Orbitrap mass spectrometry (UHPLC/LTQ-Orbitrap-MS) with automated data processing techniques to elucidate and differentiate structure of the metabolites in rat urine, plasma, liver and rat liver microsomes (RLM). The results of this work provided effective information for the clarification of the metabolism, broaden the range of metabolites and deepen the cognition of metabolic behavior of IR *in vivo*. According to previous report [12], IR metabolized mainly in liver tissues after oral administration, and Cytochrome P450 in rat liver microsomes played important role in IR hydroxylation. In order to find out the significant value of rat liver microsomes on the metabolism of IR, the metabolic profile *in vitro* was also further studied corresponding to the profile *in vivo*. Additionally, relative percentage of each metabolite was analyzed and the possible biological metabolic pathway and main metabolite types of IR were proposed in our work.

2. Experimental

2.1. Chemicals and reagents

Isorhynchophylline (IR), rhynchophylline (R), isocorynoxeine (IC), corynoxeine (C) (purity ≥ 98%) were purchased from Shanghai Standard Biotech Co., Ltd. (Shanghai, China). Male Sprague-Dawley (SD) rat liver microsomes (17 mg protein/mL) and a nicotinamide adenine dinucleotide phosphate (NADPH) – regenerating system were purchased from RILD Research Institute for Liver Diseases Co., Ltd (Shanghai, China). HPLC-grade acetonitrile used in the mobile phase was from Merck KGaA (Darmstadt, Germany). Analytical grade ammonium hydroxide (Sigma-Aldrich, St. Louis, MO, USA) was used as modifiers in the mobile phase for the UHPLC. Ultrapure water(18.2 MΩ cm at 25 °C) was prepared by a Millipore Alpha-Q water purification system (Millipore, Bedford, MA, USA). In addition, other reagents and chemicals were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Animal and drug administration

Male Wistar rats (at weight of 200 ± 20 g) were purchased from SLAC Lab Animal Center (Shanghai, China). Rat studies were conducted according to protocols approved by the Review Committee of Animal Care and Use at the Shanghai Institute of Materia Medica (Shanghai, China). All rats used in our experiment were housed in rat cages in a unidirectional airflow room under controlled temperature (20–24 °C), relative humidity (40–70%), and a 12-h light/dark cycle. Food and water were provided ad libitum and rats were acclimated to the facilities and environment for 7 days before the experiments.

Next day all rats were randomly assigned to four groups: three administration groups (A, B and C) and blank control group (three rats each for group A and group B, six rats for group C). The rats in group A were used for the collection of urine, while group B for blood and C for liver. IR was dissolved into 0.1 M HCl and then diluted to a concentration of 2 mg/ml with distilled water (pH 5–6). Rats of each group were fasted 12 h with free access to drinking water before oral administration IR at a dose of 20 mg/kg to groups A, B and C.

2.3. Biological samples collection and pretreatment

Plasma samples: The serial blood samples were collected from orbital sinus under isoflurane anesthesia in heparinized tube at 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h, and the obtained samples were centrifuged at 5000 rpm for10 min, respectively. The plasma samples were mixed, and an aliquot of 2 mL was treated with 8 mL acetonitrile to precipitate protein. After centrifuging at 14,000 rpm for 10 min, the supernatant was dried under nitrogen gas at room temperature. The residue was dissolved in 200 μL methanol.

Urine samples: rats were housed in Nalgene metabolic cages (one rat per cage) and received oral administration IR (via gavage), and urine samples were collected for 0–24 h. The urine sample (2 mL) was collected and dried under nitrogen gas at room temperature. The residue was reconstituted in 200 μL methanol.

Liver samples: The rats were anesthetized with an intra peritoneal (i.p.) injection of 15 mg (50 mg/ml) of pentobarbital sodium. Under anesthesia rats were sacrificed by bleeding at 1, 2, 4 h (two rats per time point). Liver were excised and then rinsed in ice-cold saline before gently blotting on absorbent paper and weighing. 0.2 g mixed samples were homogenized in 5 vols of ice-cold saline and centrifuged at 14,000 rpm for 10 min to get the supernatant as liver samples.

Rat liver microsomes samples: microsomal incubations of IR were conducted by the method described previously [17]. The reaction mixture (final volume: 0.2 mL), consisting of substrate 100.0 mM IR, rat liver microsomes (protein 1.0 mg), and 5.0 mM $MgCl_2$ in 0.1 M phosphate buffered saline (PBS) buffer (pH 7.4), was mixed well and pre-incubated for 3 min in a shaking water bath at 37 °C. The reaction was initiated by adding 10 μL of NADPH solution (final concentration: 1 mM) in the mixture. After undergoing incubation for 60 min, the reactions were terminated by adding an equal volume of ice-cold ethyl acetate, and then the incubation mixture was extracted three times with ethyl acetate (1 mL each time, including the terminator). The extracts were dried by evaporation under gradual nitrogen flow at 40 °C. The dried residue was reconstituted in 200 μL of methanol for analysis.

After the remaining rats were administrated orally with physiological saline; their blood, urine and liver samples were collected and used as blank control according to the above description without collection points. While for rat liver microsomes, control samples contained no substrates. Finally, all the samples were stored at −80 °C until analysis.