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Metabolic profile of 5-hydroxy-4-methoxycanthin-6-one, a typical canthinone alkaloid, in rats determined by liquid chromatography-quadrupole time-of-flight tandem mass spectrometry together with multiple data processing techniques

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ABSTRACT

Picrasma quassioides (D. Don) Benn. is a traditional Chinese medicine used clinically to treat gastrointestinal disorders and as a vermifuge. 5-Hydroxy-4-methoxycanthin-6-one (CAN), a major canthinone alkaloid found in *P. quassioides*, has significant pharmacological activities. In the present study, a method using liquid chromatography-quadrupole time-of-flight tandem mass spectrometry together with multiple data processing techniques, including extracted ion chromatogram, multiple mass defect filter, precursor/product ion scanning and neutral loss scanning was developed to screen and characterize the phase I and II metabolites of CAN in plasma, bile, urine and feces of rats after a single oral dose of 20 mg/kg. A total of 17 metabolites were tentatively or conclusively identified. Pathways for the metabolism of CAN have been proposed, and include hydroxylation, *N*-decarbonylation, methylation, oxidation and sequential conjugation. A previously unknown metabolically active site at the C4-C6 position and a novel *N*-decarbonylation-oxidation metabolic pathway for the prototypical canthinone alkaloid, CAN, were discovered. Our results provide valuable information about the *in vivo* metabolism of CAN that can also be used as a comprehensive guide for the biotransformation of other canthinone alkaloids.

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1. Introduction

Picrasma quassioides (D. Don) Bennet (Simaroubaceae), known as ku-mu in Chinese, is a commonly used traditional Chinese medicine that is recorded in the Pharmacopeia of China (2015 Edition, Vol. 1) [1]. For thousands of years, ku-mu has been widely incorporated into heat-clearing and detoxification formulas for the treatment of diarrhea, eczema, sore throat and snake bite [1]. Phytochemically, a variety of chemical constituents, including alkaloids, quassinoids and triterpenoids, have been isolated from *P. quassioides*. Alkaloids, the main components of *P. quassioides,* show potent antipyretic, anti-inflammatory, antibacterial, and antitumor activities [2]. Because they are present in large amounts and have known therapeutic effects, some alkaloids have been used as marker compounds for quality control of *P. quassioides*-containing pharmaceutical preparations [2].

5-Hydroxy-4-methoxycanthin-6-one (CAN), a major canthinone alkaloid isolated from *P. quassioides*, has significant pharmacological properties, including antibacterial activity [3], cyclic adenosine monophosphate phosphodiesterase inhibitory activity [4] and the ability to increase intestinal blood flow rate [5]. CAN also has anti-inflammatory activity and has been shown to alleviate dextran sulfate sodium-induced ulcerative colitis in rats [6] and to significantly inhibit the development of carrageenan-induced paw edema and complete Freund's adjuvant-induced chronic arthritis in rats [7]. CAN and its analogs have also recently been shown to have potent cytotoxic activity against U937 and Hep G2 cell lines [8,9], suggesting that CAN, the prototypical canthinone alkaloid, is potentially a powerful anticancer agent. A potential anticancer compound should be assessed for druggability, which

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requires elucidation of its *in vivo* pharmacokinetic profile, including absorption, distribution, metabolism and excretion. A pharmacokinetic study of CAN in rats has recently been carried out by our team [10]. The T_{max}, C_{max}, AUC_{0-t} and $T_{1/2,z}$ (terminal elimination half-life) for CAN in rats were 1.10 ± 0.55 h, 341.04 ± 196.56 μg L^{-1}, 2235.06 ± 627.66 μg h L^{-1} and 6.21 ± 2.38 h, respectively, suggesting that it easily absorbed and rapidly metabolized. A detailed evaluation of the metabolites of CAN and other canthinone alkaloids has not, however, been undertaken. An understanding of the major metabolic and excretory pathways for CAN would likely be relevant to the metabolism of other canthinone alkaloids and the metabolites themselves could act as staring points for new anticancer agents.

Recently, liquid chromatography (LC) coupled with mass spectrometry (MS), especially high-resolution MS, has proved to be very useful for detecting and identifying drug metabolites. Data processing facilitates the identification of both target and non-target metabolites. The use of extracted ion chromatograms, which improves screening selectivity, is the most common way to search for target metabolites. This procedure can, however, produce many false positives when the metabolites are contained in complex sample matrices, such as feces [11]. Precursor/product ion scanning and neutral loss scanning have also been used to detect and identify certain types of target metabolites but prior knowledge of characteristic fragmentations is needed and minor metabolites may be overlooked [11]. A multiple mass defect filter (MMDF) technique, which identifies drug metabolites according to different biotransformations that have distinct mass defect values, has been used to detect minor metabolites [12], although this filtering process can only be performed on data acquired using high-resolution instruments [11]. These different technologies have all been successfully applied to the identification of metabolites [13,14].

In the present study, a method combining liquid chromatography-quadrupole time-of-flight tandem mass spectrometry (LC-Q-TOF-MS) with multiple data processing techniques, including extracted ion chromatograms, MMDF, precursor/product ion scanning and neutral loss scanning, was developed to screen and characterize the phase I and II metabolites of CAN in plasma, bile, urine and feces of rats after a single oral dose. The steric hindrance of conjugated moieties was taken into account to determine the positions of substituents in the phase II metabolites of CAN. A total of 17 metabolites were observed and three of these were conclusively identified by comparison with authentic standards and literature MS^2 fragmentation data. Metabolic pathways for CAN and fragmentation patterns of metabolites are summarized.

2. Experimental

2.1. Chemicals and materials

CAN ($C_{15}H_{10}N_2O_3$) was isolated from *P. quassioides* in our laboratory and identified by MS and NMR spectra. The purity of the sample was >98.0%. HPLC-grade acetonitrile was purchased from Shanghai Xingke Biochemistry Co., Ltd. (Shanghai, China). Deionized water was prepared using a Milli-Q water purification system (Merck KGaA, Darmstadt,German). All other reagents were of analytical grade and were obtained from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China). C18 EC Bag cartridges (3 mL, 200 mg) were bought from Agilent Technologies, Inc. (Santa Clara, CA, USA).

2.2. Animals and drug administration

Animal experiments were conducted with the approval of the Animal Ethics Committee of the China Pharmaceutical University (Nanjing, China) and conformed to *the Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health [15]. Eighteen male Sprague-Dawley rats (age: 7–8 weeks, weight: 200 ± 20 g) were obtained from Shanghai Super-B&K Laboratory Animal Co., Ltd. (Shanghai, China) and housed with free access to standard food and water. The animals were kept under controlled conditions (temperature: 22 ± 2 °C; relative humidity: 50 ± 10%) with a 12 h light/12 h dark cycle and acclimated for at least 1 week prior to the experiment. Before drug administration, all rats were placed in metabolism cages and fasted for 12 h with free access to water. Blank biosamples were collected from each rat. CAN dissolved in 0.5% sodium carboxymethylcellulose was administered by intragastric gavage at a dose of 20 mg/kg, based on earlier rat pharmacology studies [7].

2.3. Biological sample collection

2.3.1. Plasma collection

Blood samples (~0.2 mL) were collected from six rats by orbital sinus bleeding into heparinized 2 mL Eppendorf tubes at 0, 0.08, 0.25, 0.5, 0.75, 1, 2 h after administration of CAN and were centrifuged at 3000 rpm for 10 min to yield plasma. Before analysis, the plasma samples taken from all animals at six equivalent time points (from 0.08 to 2 h) were pooled.

2.3.2. Bile collection

Six rats had a polyethylene-10 tube (internal diameter 0.08 cm, Becton Dickinson, Franklin Lakes, New Jersey, USA) implanted into the bile duct under 20% ethyl carbamate (1 g/kg) anesthesia for bile collection. Air conditioning was used to maintain body temperatures during the experimental procedures and prevent hypothermic alterations in bile flow. Bile samples from each animal were collected on ice from the cannula at 15 min intervals for 12 h after oral administration of CAN.

2.3.3. Urine and feces collection

The 12 h pre-dose and 0–12, 12–24, 24–36 and 36–48 h post-dose urine and feces samples from six different rats were collected separately using the metabolism cages. The rats were allowed free access to water and food during the period of sample collection.

All of the collected plasma, bile, urine and feces samples were stored at −80 °C until analysis.

2.4. Sample pretreatment

All biosamples were thawed at room temperature before analysis.

2.4.1. Plasma samples

Ethyl acetate (1 mL) was added to the plasma sample (100 μL) in a 2 mL Eppendorf tube. The mixture was vortexed for 10 min and centrifuged at 16,000 rpm for 5 min. An aliquot of 900 uL of supernatant was carefully transferred to a clean tube and evaporated to complete dryness at 37.5 °C under a stream of nitrogen. The residue was reconstituted in methanol (100 μL) and, following centrifugation (16,000 rpm for 2 min), an aliquot (10 μL) of the supernatant liquor was injected into the LC-Q-TOF-MS instrument.

2.4.2. Bile and urine samples

Bile (1 mL) and urine (2 mL) samples were centrifuged at 3000 rpm for 10 min. The supernatants were applied to activated C18 EC Bag cartridges (washed before use with methanol (6 mL) followed by water (6 mL)). The columns were washed with formic acid-water (6 mL, 1:1000, v/v) and eluted with methanol (6 mL). The eluates were evaporated to dryness at 37.5 °C under a stream of nitrogen and redissolved in methanol (200 μL). Following centrifu-

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