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Characterization of metabolic profile of honokiol in rat feces using liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry and ^{13}C stable isotope labeling

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ABSTRACT

As fecal excretion is one of important routes of elimination of drugs and their metabolites, it is indispensable to investigate the metabolites in feces for more comprehensive information on biotransformation *in vivo*. In this study, a sensitive and reliable approach based on ultra-performance liquid chromatography/quadrupole-time-of-flight-mass spectrometry (UHPLC-Q-TOF-MS) was applied to characterize the metabolic profile of honokiol in rat feces after the administration of an equimolar mixture of honokiol and [$^{13}C_6$]-labeled honokiol. Totally 42 metabolites were discovered and tentatively identified in rat feces samples, 26 metabolites were first reported, including two novel classes of metabolites, methylated and dimeric metabolites of honokiol. Moreover, this study provided basic comparative data on the metabolites in rat plasma, feces and urine, which gave better understanding of the metabolic fate of honokiol *in vivo*.

1. Introduction

Honokiol (3′,5-di-a-propenyl-1, 1′-biphenyl-2,4′-diol), a small-molecule polyphenol, is one of the main bioactive constituents of *Magnolia officinalis* Rehd et Wils, a traditional Chinese medicine. Honokiol has been proved to have anti-cancer effect in various cell types, including melanoma, chronic lymphocytic leukemia, myeloma, lung, prostate and colon cancer cell lines [1–6]. Because of its significant bioactivities, honokiol was widely investigated as a potential anticancer drug candidate. Metabolites are important indicators of predicting safety and explaining the toxicity of drug candidate, therefore a good understanding of metabolites is necessary.

After metabolism in liver, drug candidates and their metabolites excreted from the body mainly via urine and feces. Fecal excretion occurs in two ways: (1) the bile drugs and metabolites, if not reabsorbed by intestine, are excreted from the body through feces; (2) direct intestinal excretion, drugs and metabolites, particularly lipophilic compounds secreted from mesenteric blood into the intestinal lumen to be eliminated in feces. Feces excretion was a predominant route of elimination for a number of drugs [7–12]. For the HIV-1 integrase inhibitors dolutegravir and raltegravir, fecal excretion was the primary route of elimination with 64.0% and 51.2% of the dose recovered in feces, respectively [7,8]. With regard to apixaban, an anticoagulant, accounting for >54% of the dose were excretion in animals feces [9]. Furthermore, there might be significant difference between the metabolites in feces and urine. Möhle et al. [13] reported that the majority of urinary metabolites of testosterone were conjugates whereas fecal metabolites were mainly un-conjugates in long-tailed macaque and chimpanzee. In Prakash and Cui's study [14], one of metabolites of a candidate drug CP-93,393 used for anti-anxiety was presented as aglycone, whereas in urine was glucuronide conjugate. Pyribenzoxim, a new herbicide, the metabolites discovered in urine and feces were completely different [15]. In conclusion, investigation of metabolites in feces is an integral part of the safety evaluation and is critical to gain better insight into the fate of the drug candidate.

Until recently, there have been several reports available on metabolism and excretion of honokiol. Böhmdorfer et al. [16] investigated the metabolism of honokiol *in vitro* and discovered

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3 metabolites. In our laboratory, Liu et al. [17] and Lai et al. [18] have recognized 37 and 18 metabolites of honokiol from rat urine and plasma, respectively. However, the metabolites of honokiol in feces were still unknown.

In the present study, a reliable, sensitive UHPLC/Q-TOF-MS/MS approach [17–20] was applied for the separation and identification of the metabolites of honokiol in rat feces after administration of a mixture of honokiol and [$^{13}C_6$]-labeled honokiol via caudal vein. Finally, 42 metabolites were separated and tentatively identified and 26 metabolites were discovered for the first time.

2. Experimental

2.1. Chemicals and materials

Chemicals and materials for this study were prepared as described previously [17,18]. Honokiol (purity ≥ 98%) was separated and purified in our laboratory [21]. [$^{13}C_6$]-labeled honokiol (purity ≥ 98%) was obtained from the Wuxi Beita Company (Jiangsu, China).

2.2. Animals and administration

Male Sprague-Dawley rats (200–250 g) were obtained from HFK Bio-Technology, Co., Ltd. (Beijing, China). Animals were kept in an environmentally controlled breeding room for 3 days and were fed with food and water ad libitum. Before the experiment, all the rats were fasted overnight but free access to water.

Equimolar honokiol and honokiol-[$^{13}C_6$]-labeled packaged within a lipid nanoparticle [22] and dissolved in water (4 mg/mL each) before use. Then honokiol and [$^{13}C_6$]-labeled honokiol solution was administered to 3 rats at a dose of 40 mg/kg via caudal vein. A blank feces sample was collected before administration. Whole procedures involving animals had been approved by the Institute Guidelines on Animal Experimentation of Sichuan University in China.

2.3. Sample preparation

Feces samples were collected using metabolic cages and collected at 0–4, 4–10, 10–24, 24–36, and 36–48 h after drug administration. The feces samples were left in a cool and dry place to dryness, and then stored at −20 °C before analysis.

Feces samples were crushed with liquid nitrogen, and then a 100 mg aliquot of the feces sample were mixed with 1 mL ethyl acetate and centrifuged at 13,000 rpm for 10 min. In order to extract more metabolites, the entire upper layer was removed to a clean test tube, and then 1 mL methanol was added to the lower layer. After centrifugation, the two upper layers were combined and evaporated to dryness under nitrogen at 45 °C. The dried residues containing metabolites were dissolved in 100 μL of mobile phase (methanol/water = 25/75, v/v).

2.4. Ultra-performance-LC-UV-MS/MS

The samples were separated at a linear gradient of 25% B (methanol) to 80% B within 30 min at a flow rate of 0.25 mL/min. A 5 μL volume of sample was injected.

The Waters Q-TOF Mass Spectrometer (Waters Corporation, USA) was connected to the UHPLC system via an ESI interface. As honokiol contains two hydroxyls, the ESI source was operated in negative ionization mode. The source parameters included capillary voltage at 2.8 kV, source temperature of 90 °C, desolvation temperature of 200 °C. The desolvation gas flow was 300 L/h. The cone voltage was set at 20 V. All data collected in continuum mode were acquired using Masslynx^{TM} 4.1 software. For Q-TOF-MS/MS experiment, the flow rate of the collision gas was 0.45 L/h, and collision energy was 15–40 eV. The MS and MS/MS acquisition rate was set at 1.0 s with a 0.02 s inter-scan delay. Honokiol was used as the lock mass, generating an $[M–H]^-$ ion (*m/z* 265.1229) at a concentration of 2 μg/mL and flow rate of 5 μL/min to ensure accuracy during the MS analysis.

3. Results and discussion

3.1. Screening of metabolites

Owing to an equal aliquot of honokiol and [$^{13}C_6$]-labeled honokiol was administered to the rats, the isotope mixture resulted in double peaks with mass difference of 6 Da and an intensities ratio 1:1, as shown in Fig. 1(a) and (b). This characteristic isotopic pattern was used as a marker for screening the metabolites [17,18].

Additionally, another characteristic isotopic pattern was also observed in the mass spectra, which had three peaks with mass difference of 6 Da and an intensities ratio 1:2:1, as shown in Fig. 1(c) and (d). The typical pattern of compound with two bromine atoms isotope correspond to the intensity ratio 1:2:1 and the mass difference of 2 Da [23]. As for honokiol and [$^{13}C_6$]-labeled honokiol (Δ = 6 Da), we proposed that compounds which contained two honokiol isotope would meet the above isotopic pattern. So compounds with this isotopic pattern were identified as the dimeric metabolites of honokiol.

Comparing the feces samples collected in different time period with blank feces sample, no metabolite was found in the feces sample at 0–4 h. After 4 h, honokiol metabolites started to be found. All 42 metabolites can be found in the feces sample at 10–24 h. 27of 42 metabolites were found in the feces sample at 4–10 h. However, only 15 and 5 metabolites were found in the feces sample at 24–36 and 36–48 h, respectively.

Because the intensity of ion current was positively correlated with the concentration of metabolite, it was easy to obtain the fraction at which the concentration of each metabolite was highest through comparing the ion intensity of each metabolite in different time period. The metabolites detected in rat feces at different time period and the sample with the highest concentration of each metabolite are listed in Table 1.

As the sample at 10–24 h presented the most metabolites, so 10–24 h sample was selected for further analysis. The base peak intensity chromatography of feces sample collected in 10–24 h and blank feces are presented in Fig. 2. Table 2 summarizes the *m/z* values, retention times and MS/MS fragment data of the metabolites, the theoretical masses and molecular formulas were also included.

3.2. Identification of metabolites

Identification and elucidation of these metabolites were performed by comparing fragment ions, changes of the deprotonated molecular masses, and retention times with those of honokiol. The predicted structures of these 42 metabolites are offered in Fig. 3.

3.2.1. Phase I metabolites

In this study, 6 phase I metabolites (M1, M2a, M2b, M3, M4 and M5) were observed in feces sample, as shown in Table 2. And M1, M2a, M2b were first reported *in vivo*.

The mass spectra of M1, which was detected at a retention time of 10.52 min gave a deprotonated molecular ion $[M–H]^-$ at *m/z* 269.0818. M1 was 4 Da higher than honokiol (M0), based on the measured accurate *m/z* data. Hence, structure formula of M1 was assigned to be $C_{16}H_{14}O_4$ (calcd. 269.0814, oxidation) rather than $C_{18}H_{22}O_2$ (calcd. 269.1542, reduction of double bond). The fragment ion at *m/z* 225 corresponded to loss of CO_2 (44 Da) from M1