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Characterization of global metabolic profile of *Zhi-Zi-Hou-Po* decoction in rat bile, urine and feces after oral administration based on a strategy combining LC–MS and chemometrics

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ABSTRACT

Identification of metabolic profile of traditional Chinese medicine *in vivo* is always a challenge task. Usually, screening out and identifying the exogenous compounds manually from total ion chromatograms (TICs) of biologic samples is time-consuming and strenuous. In this study, a systematic identification strategy based on LC–MS was adopted to clarify the metabolic profiling of *Zhi-Zi-Hou-Po* decoction (*ZZHPD*) in rat. Bile, urine and feces samples of rat were obtained after oral administration and then analyzed by LC–MS after proper preparation. The xenobiotics were screened out from TICs globally and rapidly by untargeted metabolomics-driven strategy (UMDS) based on the combined of XCMS online (a web-based platform to process LC–MS data), MetAlign (a software to process LC–MS data) and SIMCA-P (a software for data analysis). Most of the xenobiotics were identified by means of series product ions filtering (sPIF), which was based on the database-hit of *ZZHPD* (including prototype and metabolites). Then the unmatched constituents were identified tentatively and their source and metabolic pathway were clarified by using diagnostic fragment ions strategy (DFIS). As a result, a total of 83 compounds including 44 prototype compounds and 39 metabolites were rapidly identified or tentatively characterized from the biologic samples, and among them, four of which were found for the first time. Further research on the correlations of these prototype compounds and metabolites revealed that glucuronidation is the main metabolic pathways of *ZZHPD* in rat bile and urine, while prototype compounds were the abundant ingredients detected in rat feces.

1. Introduction

Zhi-Zi-Hou-Po decoction (*ZZHPD*) is a famous traditional Chinese medicine formula described in *Shang-Han-Lun* (Treatise on Cold Damage Diseases), which is used to treat psychiatric illnesses in the clinic, especially depression [1]. Recently, reports about *ZZHPD* appeared more and more. Liu et al. identified 108 chemical components in *ZZHPD* [2], which had supplied global chemical components information of *ZZHPD in vitro*. In the aspect of *in vivo*, the pharmacokinetics of five major constituents in *ZZHPD* and different combinations of its constituent herbs were investigated [3–5], and the absorbed components and metabolites of *ZZHPD* in rat plasma after oral administration were identified using LC–MS in our previous study [6], which had revealed the metabolic profile of *ZZHPD in vivo* partly.

The therapeutic effect of decoction is the superimposed effect of the prototype components [7], metabolites [8,9] or both of them [10], which play treatment effect *in vivo* through different ways. So clarifying the metabolic profile of *ZZHPD in vivo* ulteriorly and globally is obviously in favor of revealing the crucial active ingredients and elucidating the potential antidepressant mechanism of *ZZHPD*. Rat bile, urine and feces are recognized as ideal biological

Abbreviations: *ZZHPD*, *Zhi-Zi-Hou-Po* decoction; HPLC-TOF/MS, High performance liquid chromatography time-of-flight mass spectrometry; HPLC-QqQ/MS, High performance liquid chromatography tandem-triple quadrupole mass spectrometry; TICs, total ion chromatograms; EICs, extracted ion chromatograms; *m/z*, mass-to-charge ratio; UMDS, untargeted metabolomics-driven strategy; sPIF, series product ions filtering; DFIS, diagnostic fragment ions strategy; TCM, traditional Chinese medicine; SPE, solid-phase extraction; OPLS-DA, orthogonal partial least squared discriminant analysis.

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samples to research *in vivo* metabolic patterns [11–14], as they are closely related to the excretion of drugs and their metabolites. However, to the best of our knowledge, almost no research on metabolic profiling of *ZZHPD* in these biologic samples has been reported till now.

The characterization of TCM formula's metabolic profile *in vivo* is always a challenge task [6,15], while some relevant approaches were reported along with the development of instruments and softwares in recent years. In our previous study [6], untargeted metabolomics-driven strategy (UMDS) was used to screen out the xenobiotics in plasma rapidly and globally, while datasets of chemical compounds in *ZZHPD* and exogenous components in rat plasma were acquired by analyzed with software at the same time, then the former were performed as constituents database of *ZZHPD* when differentiated the prototype constituents from metabolites in plasma before confirmed the structure. But in this study, due to our previous studies of chemical profiling *in vitro* [2] and metabolic profile in rat plasma [6] of *ZZHPD*, a database of prototype constituents of *ZZHPD* and related metabolites was set up, which could speed up the process of characterization. On the other hand, in the step of confirming the structures of the xenobiotics, however, there was still not a systematic and rapid strategy in our previous study [6]. Here, series product ions filtering (sPIF) and diagnostic fragment ions strategy (DFIS) were used in this study. Multiple product ions (series product ions of compound which were acquired in MS/MS following fragment rules) filtering, which had been successfully applied in the identification of main characteristic components *in vitro* [16] and metabolite identification *in vivo* [17,18] of TCM, was used for the rapid identification of the chemical compounds which has been reported and listed in the database. And for the uncharted metabolites, it is the primary task to ascertain the source of them. DFIS was used to identify the source of metabolites in the researches of metabolic profile of monomeric compound *in vivo* [19,20], and in recent years it had been applied in the studies of metabolic profiling of a series of bioactive phytochemicals of herbs *in vitro* [21] and *in vivo* [18].

In this study, a systematic identification strategy which integrated UMDS, sPIF and DFIS systematically based on LC–MS was adopted for the characterization of global metabolic profile of *ZZHPD* in rat. By this approach, the process of identification of exogenous components in rat was speeded up and the metabolic profile was characterized globally.

2. Experiment

2.1. Chemicals and reagents

Methanol (HPLC-grade) was purchased from Merk (Darmstadt, Germany), acetic acid (HPLC-grade) was purchased from Sigma Chemical (St. Louis, USA). Ultra-pure water was obtained from a Milli-Q system (Bedford, USA) freshly. The standards of chlorogenic acid, geniposide, naringin, hesperidin, neohesperidin, naringenin, hesperetin, honokiol and magnolol (purity > 98%) were purchased from Mansite Biotechnology Company (Chengdu, China). All other chemicals and reagents were of analytical grade and commercially available.

2.2. Plant material

All plant material were purchased from Jiangsu Simcere Pharmacy Ltd. (Jiangsu, China), while *Gardenia jasminoides* Ellis (*ZZ*) (nos. 150731) and *Citrus aurantium* L. (*ZS*) (nos. 150727) were collected in Jiangxi, China and *Magnolia officinalis* cortex (*HP*) (nos. 150803) was collected in Sichuan, China. They were authenticated by Professor Ming-Jian Qin (Department of Chinese Materia Medica, China Pharmaceutical University, Nanjing, China). For future reference, all voucher specimens were deposited in our laboratory. The quality of these crude drugs was controlled and processed according to the Chinese Pharmacopoeia (2015).

2.3. Preparation of ZZHPD sample

The *ZZHPD* sample was prepared according to the method developed in our laboratory [22]. The details of the preparation was shown in Text S1 (Supplementary material). An oral aqueous solution of *ZZHPD* with a concentration of 1.628 g crude drug per milliliter was obtained. For LC–MS analysis, an aliquot of 2.5 mL of *ZZHPD* was diluted into 10 mL with water and vortexed for 5 min. Then 3 mL of the diluent was diluted into 10 mL with ethanol and vortexed for 5 min. After 24 h on standing in 4 °C, the mixed liquor was centrifuged at 12000 rpm for 10 min and then the supernatant was filtered through a 0.45 μm membrane.

2.4. Animal experiments

All protocols and care of the rats were in accordance with the guide relevant national legislation and local guidelines. Ten male Sprague-Dawley rats (12–14 weeks, 180–220 g) were provided by Animal Multiplication Center of Qinglong Mountain (Nanjing, China). Before oral treatment, the rats were acclimated in an animal breeding room (temperature, 20 ± 2 °C; relative humidity, 50%; light/dark cycle, 12 h) for two weeks. Purified water and standard chow were provided *ad libitum*. All rats were fasted with free access to water for 12 h prior to the experiment.

Rats were randomly divided into two groups of five rats each. The number of rats in individual group was designed by the basic requirements of chemometrics analysis in UMDS [23]. *ZZHPD* was intragastric administrated to the treatment group (n = 5) at three continuous dose of 32 g/kg/d, and equal volume distilled water was intragastric administrated to the control group (n = 5). Each rat was held in a separate metabolic cage, urine and feces samples were collected from 0 to 24 h after oral administration of dosing or vehicle. For bile collection, rats were cannulated in the bile duct under general anesthesia, then they were allowed to recover from anesthesia before oral administration and bile samples were collected from 0 to 24 h after dosing or vehicle. The sample of each rat was collected in each glass tube, then individually labeled and stored at −20 °C until analysis.

2.5. Sample preparation

Bile samples were prepared by organic solvent precipitation. Three milliliter acetonitrile was added to an aliquot of 1.5 mL bile sample, and then vortexed for 5 min. The mixture was centrifuged at 4500 rpm for 10 min at 4 °C. The supernatant was transferred to another tube and evaporated to dryness at 30 °C under a gentle stream of nitrogen.

Urine samples were prepared by Alltech C_{18} solid-phase extraction (SPE) cartridge (500 mg/6.0 mL; Alltech Co., Kentucky, USA). Two milliliter urine sample was loaded onto SPE cartridges which had been preconditioned with 2 mL methanol and followed by 2 mL ultrapure water. Each cartridge was washed with 2 mL ultrapure water to remove the aqueous effluent and then eluted with 2 mL methanol. The eluent was collected and evaporated to dryness at 30 °C under a gentle stream of nitrogen.

Feces samples were dried in the dark on filter paper at ambient temperature and then slowly ground into powder. Then 0.5 g feces samples were extracted with 5 mL methanol by ultrasonic treatment for 30 min and subsequently centrifuged at 4500 rpm for 10 min, the supernatant was transferred to another tube and evaporated to dryness at 30 °C under a gentle stream of nitrogen.