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An untargeted metabolomics-driven approach based on LC–TOF/MS and LC–MS/MS for the screening of xenobiotics and metabolites of *Zhi-Zi-Da-Huang* decoction in rat plasma

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ABSTRACT

Zhi-Zi-Da-Huang decoction (*ZZDHD*), a typical traditional Chinese medicine prescription, is widely used in clinical practice for the treatment of alcoholic liver disease. However, due to lack of holistic metabolic research, the active ingredients of *ZZDHD* have not been fully elucidated. It entails a huge obstacle for the quality evaluation, pharmacokinetic studies and clinical-safe medication administration of *ZZDHD*. In this work, an untargeted metabolomics-driven approach was proposed to rapidly screen and characterize xenobiotics and related metabolites *in vivo* conducted by LC–TOF/MS and LC–QqQ/MS. The t_R–m/z pairs which were present in the *ZZDHD*-dosed group and absent in the control group could be clearly displayed by XCMS Online platform combined with supervised orthogonal partial least squares discriminant analysis. Among them, a total of 61 *ZZDHD*-related xenobiotics and metabolites including 34 prototype components and 27 metabolites were rapidly identified or tentatively characterized in rat plasma. The results indicated that iridoid glycosides and monoterpenoids from *Gardenia jasminoides* Ellis, flavonoid glycosides from *Citrus aurantium* L., as well as anthraquinones from *Rheum palmatum* L. were the main absorbed chemical components of *ZZDHD*. Hydrolysis, glucuronidation and sulfation were the main metabolic pathways of *ZZDHD in vivo*. The present study provided a solid basis for further revealing the relationship between the xenobiotic metabolome and pharmacological activity of *ZZDHD*. In addition, the application of untargeted metabolomics-driven approach offers a fresh insight for rapid screening and identifying xenobiotics and metabolites of *ZZDHD* and other multiherb prescription.

1. Introduction

Zhi-Zi-Da-Huang decoction (*ZZDHD*) is a typical traditional Chinese medicine prescription (TCMP) which is made from four medicinal materials, including *Gardenia jasminoides* Ellis (*Zhi-Zi*), *Citrus aurantium* L. (*Zhi-Shi*), *Rheum palmatum* L. (*Da-Huang*) and Sojae Semen Praeparatum (*Dan-Dou-Chi*). *ZZDHD* has been widely used in clinical practice for the treatment of alcoholic liver disease (ALD) over a millennium and its therapeutic effect is generally considered credible. Modern pharmacological evaluations have demonstrated that *ZZDHD* exhibits promising hepatoprotective effect [1]. Although a total of 85 chemical components in *ZZDHD* were identified or tentatively characterized by LC–TOF/MS and LC–QqQ/MS [2,3], the active ingredients of *ZZDHD* which is crucial for further investigation of therapeutic effect of the formula are still not so clear. One feasible way is to clarify "what are absorbed" and "what are produced" of *ZZDHD in vivo* since only the xenobiotics and metabolites detected here could responsible for the therapeutic effects [4–6], in most cases.

It's an arduous task to comprehensive and systematic characterization of xenobiotics and metabolites of *ZZDHD* in complex biological matrices, due to their fairly trace concentrations *in vivo*, diverse structure types and physicochemical properties, unpredictable metabolites, interference from endogenous substances

Abbreviations: ALD, alcoholic liver disease; DAD, diode array detector; EICs, extracted ion chromatograms; m/z, mass-to-charge ratio; QqQ, triple quadrupole; RDA, retro diels-alder; SPE, solid phase extraction; TCMP, traditional Chinese medicine prescription; TIC, total ion chromatogram; TOF, time-of-flight; *ZZDHD*, *Zhi-Zi-Da-Huang* decoction.

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and lack of standards [7,8]. The conventional method of searching manually and intuitionally the differences between the control and treated group chromatograms may lose artificially some components, especially some metabolites *in vivo* [9]. Therefore, an unbiased and robust discrimination method should be developed to enhance the efficiency of analytical processes in xenobiotic metabolism research. By measuring and mathematically modelling changes in the levels of products of metabolism found in biological fluids and tissues [10–12], untargeted metabolomics provides fresh insight into subtle changes of endogenous components [13–15], and also could be extended to xenobiotics and related metabolites to which we are exposed. Applying untargeted metabolomics-driven approach, the interesting variables could be extracted easily from the xenobiotic metabolome without the need for priori knowledge of the compound structure. Thus it opens a new perspective in the field of TCMP metabolism. In this study, an untargeted metabolomics-driven approach was proposed to rapidly screen and characterize xenobiotics and metabolites of *ZZDHD in vivo* based on LC–TOF/MS and LC–QqQ/MS.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetic acid and methanol were purchased from Sigma Chemical (St. Louis, USA) and Merck (Darmstadt, Germany), respectively. Ultra high purity water was prepared 'in-house' using a Millipore-Q water purification system (Bedford, USA). The standards of chlorogenic acid, geniposide, naringin, hesperidin, neohesperidin, daidzein, naringenin, hesperetin, genistein, aloe-emodin, rhein and emodin (purity >98%) were purchased from Mansite Biotechnology Company (Chengdu, China).

2.2. Plant material

G. jasminoides Ellis and *C. aurantium* L. (collection in Jiangxi, China), *R. palmatum* L. (collection in Gansu, China) and Sojae Semen Praeparatum (collection in Henan, China) were purchased from Xiansheng drug store (Nanjing, China). The medicinal plants used in the experiment were authenticated by Professor Minjian Qin (Department of Chinese Materia Medica, China Pharmaceutical University, Nanjing, China). All voucher specimens were deposited at the Herbarium of China Pharmaceutical University, Nanjing, China (nos. 140528, 140406, 140301, 140312).

2.3. Preparation of ZZDHD

ZZDHD was prepared according to a prior report from our laboratory [1], which was shown in Supporting information Text S1. An oral aqueous solution of *ZZDHD* with a concentration of 1.0 g/mL was obtained. For LC–MS analysis, an aliquot of 4.0 mL of *ZZDHD* was diluted into 10 mL with water, and then 2.5 mL aliquot of the mixture was added with 7 mL of 95% ethanol, centrifuged at 8000 × *g* for 5 min and filtered through a 0.45 μm membrane.

2.4. Animals and treatment

All protocols and care of the rats were in accordance with the guide relevant national legislation and local guidelines. Male Sprague-Dawley rats (12–14 weeks) weighing 180–220 g were acclimated at a relative humidity of 50% and a temperature of 20 ± 2 °C with a 12 h light/dark cycle in an animal breeding room for two weeks before oral treatment. Purified water and standard chow were provided *ad libitum*. All rats were fasted for 12 h with free access to water prior to the experiment. The rats were randomly divided into the control group and *ZZDHD*-dosed group ($n = 8$). *ZZDHD* was dosed to rats at 12 g/kg/day for two consecutive days, while 0.9% physiological saline (12 mL/kg/day) was dosed to the rats of the control group in the same way.

2.5. Plasma sample collection and preparation

On the third day, the rats were anesthetized by intraperitoneal injection of 20% urethane after the last oral administration. Blood samples were collected from hepatic portal vein into heparinized tubes at 1 h after administration. Then, the plasma was isolated by centrifugation at 5000 × *g* for 10 min at 4 °C. An aliquot of 2 mL plasma was deproteinized with 6 mL acetonitrile. After vortex mixed for 3 min, centrifugation at 12,500 × *g* was performed for 10 min at 4 °C. The supernatant was transferred to a fresh centrifuge tube and dried under nitrogen gas at room temperature, then reconstituted in 2 mL deionized water and loaded on the pre-activated Poly-Sery HLB SPE columns (3 cm^3/60 mg, CNW Technologies GmbH, Germany). Before that, the columns were preconditioned with 3 mL of methanol and 3 mL of deionized water. The analytes retained in the cartridge were eluted with 6 mL methanol. Then, the methanol elutes were collected and dried under nitrogen gas at room temperature. The residue reconstituted in 100 μL methanol. After centrifuged at 12,500 × *g* for 10 min at 4 °C, a 20 μL aliquot was injected for LC–MS analysis.

2.6. Instrumentation and conditions

LC–MS systems consisted of Agilent-1260 LC coupled with DAD, Agilent-6224 TOF mass spectrometer (Agilent Corp., Santa Clara, CA, USA) and TSQ Quantum Ultra AM QqQ/MS (Thermo Finnigan, San Jose, CA, USA) were used for the accurate mass and structure information of xenobiotic metabolome of *ZZDHD in vivo*. The mobile phase consisted of 0.1% acetic acid (A) and methanol (B) was carried with linear elution gradient as follows: 0 min, 5% B; 12.5 min, 20% B; 35 min, 32% B; 54 min, 46% B; 62 min, 45% B; 75 min, 50% B; 85–92 min, 60% B; 95–105 min, 75% B; 115–125 min, 95% B, which was delivered at 1.0 mL min^{-1}. A Lichrosper C18 column (250 mm × 4.6 mm, 5 μm, Hanbon, China) with the column temperature at 30 °C was used. Re-equilibration duration was 15 min between individual runs. The LC effluent was split and introduced into the inlet of two MS in parallel. Each mass detector equipped with an ESI and operated in positive and negative ionization mode, respectively.

The optimized conditions of TOF/MS were: capillary voltage, 4.0 kV/−3.5 kV; drying gas (N_2) temperature, 350 °C; drying gas (N_2) flow rate, 10.0 L min^{-1}; skimmer, 65 V; nebulizer pressure, 30 psi; scan range, 100–1200 *m/z*. The acquisition and analysis of data were controlled by Mass Hunter B.04.00 software.

QqQ/MS controlled by Xcalibur software was utilized to determine the product ion mass spectra (MS/MS). The spray voltage was 4.0 kV and −3.5 kV for positive and negative MS scan mode respectively, assisted by nitrogen sheath and auxiliary gas flow rate were 35 Arb and 5 Arb, respectively. The collision energy of 10–35 eV was used at a pressure of 1.3 mTorr for collision-induced dissociation to get distinct fragmentation for certain analyte.

2.7. Strategy for comprehensive and systematic analysis of ZZDHD metabolome

The first step of this strategy was to acquire global MS data and extract three-dimensional data matrix, comprising of sample code, retention time (t_R)–*m/z* pairs and ion intensity. The LC–TOF/MS data from the plasma samples of the control group and *ZZDHD*-dosed group were analyzed to identify potential discriminant signals. Data from positive and negative ionization modes were included in two separate data sets in order to analyze them individually