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Rapid identification of herbal compounds derived metabolites using zebrafish larvae as the biotransformation system

Chen Wang[a], Ying-Hao Yin[a], Ying-Jie Wei[b], Zi-Qi Shi[b], Jian-Qun Liu[c], Li-Fang Liu[a,*], Gui-Zhong Xin[a,**]

[a] State Key Laboratory of Natural Medicines, Department of Chinese Medicines Analysis, China Pharmaceutical University, Nanjing 210009, China
[b] Key Laboratory of New Drug Delivery Systems of Chinese Materia Medica, Jiangsu Provincial Academy of Chinese Medicine, Jiangsu, Nanjing 210028, China
[c] Key Laboratory of Modern Preparation of TCM, Ministry of Education, Jiangxi University of Traditional Chinese Medicine, No. 818 Xingwan Road, Nanchang 330004, Jiangxi Province, China

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ABSTRACT

Metabolites derived from herbal compounds are becoming promising sources for discovering new drugs. However, the rapid identification of metabolites from biological matrixes is limited by massive endogenous interference and low abundance of metabolites. Thus, by using zebrafish larvae as the biotransformation system, we herein proposed and validated an integrated strategy for rapid identification of metabolites derived from herbal compounds. Two pivotal steps involved in this strategy are to differentiate metabolites from herbal compounds and match metabolites with their parent compounds. The differentiation step was achieved by cross orthogonal partial least-squares discriminant analysis. Automatic matching analysis was performed on R Project based on a self-developed program, of which the number of matched ionic clusters and its corresponding percentage between metabolite and parent compound were taken into account to assess their similarity. Using this strategy, 46 metabolites screened from incubation water samples of zebrafish treated with total *Epimedium* flavonoids (EFs) could be matched with their corresponding parent compounds, 37 of them were identified and validated by the known metabolic pathways and fragmentation patterns. Finally, 75% of the identified EFs metabolites were successfully detected in urine samples of rats treated with EFs. These experimental results indicate that the proposed strategy using zebrafish larvae as the biotransformation system will facilitate the rapid identification of metabolites derived from herbal compounds, which shows promising perspectives in providing additional resources for pharmaceutical developments from natural products.

1. Introduction

In recent years, natural products (NPs) steadily contribute to new pharmaceutical developments [1,2]. Herbal medicines, as promising sources of NPs, are receiving increasing worldwide attention [3,4]. The importance of herbal compounds (NPs originating from herbs) was underlined in 2015 with the awarding of the Nobel Prize in Physiology or Medicine [5]. More importantly, it has been found that metabolites, rather than the parent compounds, are the active species for many herbal compounds [6,7]. Various herbal components such as flavonoids, saponins, and alkaloids have been shown to be characterized with extensive metabolism in biological systems [8–10]. Therefore, metabolites derived from herbs with reliable therapeutic efficacy are worth further exploration. Global detection and identification of metabolites derived from herbal compounds are pivotal for mapping their metabolic changes in biological systems. It is also a crucial step to provide additional resources for discovering new drugs from NPs.

However, it is still challenging to detect and identify exogenously sourced metabolites, especially for non-targeted compounds from complex matrixes [11–13]. The most critical difficulty lies in distinguishing and matching herbal compounds and metabolites in complex biological matrixes. The advance in high resolution mass spectrometers (MS)-based strategies has greatly facilitated the process of metabolite detection and identification [9,14–17]. The typical example is that Hao et al. have established a chemicalome-metabolome matching approach to characterize the global metabolites of herbal compounds from complex biological

* Corresponding author at: State Key Laboratory of Natural Medicines, Department of Chinese Medicines Analysis, China Pharmaceutical University, No. 24 Tongjia Lane, Nanjing, China.
** Corresponding author.
E-mail addresses: liulifang69@126.com (L.-F. Liu), xingz@cpu.edu.cn (G.-Z. Xin).

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matrixes [13,18]. This approach was demonstrated to be potent in matching herbal compounds and metabolites in complex matrixes. Conversely, we hold a hope that simple biological matrixes could facilitate the detection and identification of exogenously sourced metabolites.

More recently, zebrafish larvae are increasingly used as the biotransformation system in preliminary drug metabolism studies [19–21]. Unlike mammals, the development time of the zebrafish larvae is short (after about 2 days most common vertebrate specific body features can be seen including brain, eyes, ears and all internal organs) and the number of offspring is large (100–200 eggs per mating) [22]. Thus, zebrafish larvae are ideal whole organism model systems in high-throughput preliminary drug metabolism studies with representative advantages of low cost and short cycle [23–25]. More importantly, the simple biological matrix (incubated in water) can greatly reduce the endogenous interference in MS-based metabolite screening and identifying, which accelerate the process of detection and identification of exogenously sourced metabolites.

Thus, we put forward in this study an integrated strategy for rapid identification of metabolites derived from herbal compounds by using zebrafish larvae as the biotransformation system. Firstly, the UPLC-QTOF/MS method is utilized to the global detection of herbal components and metabolites from water matrix of zebrafish incubation system. Secondly, the aligned MS profiles are fed to SIMCA-P$^+$ for orthogonal partial least-squares discriminant analysis (OPLS-DA), which is utilized to screen variables belong to metabolites. Thirdly, an original computer program was developed for automatic matching analysis of herbal compounds and metabolites in the R Project. Finally, the matched metabolites were validated and identified by the known metabolic pathways and fragmentation patterns.

In the present study, *Epimedium koreanum* was taken as a model herb to validate our proposed strategy. *Epimedium koreanum* is one of the most valuable herbs for osteoporosis [26]. *Epimedium* flavonoids (EFs) have been well documented as the major active ingredients of *Epimedium koreanum* [27–29]. Some metabolites of EFs are of even better activity and bioavailability than their parent compounds [30–33]. Using the proposed strategy, 37 metabolites were identified and linked to their parent compounds after biotransformation of EFs in the zebrafish larvae system. These results were also supported by the final validation via detecting metabolites of EFs in urine samples of rats. Thus, the integrated strategy is expected to be widely applicable in rapid identification of herbal metabolites using zebrafish larvae as the biotransformation system, which also shows promising perspectives in providing additional resources for pharmaceutical developments from NPs.

2. Experimental and methods

2.1. Chemicals and reagents

The total EFs was prepared from *Epimedium koreanum* Nakai according to previous publication [34]. The composition and structural information of EFs were determined by UPLC-QTOF/MS. Authentic standards of *Epimedium* flavonoids (Epimedin A, Epimedin B, Epimedin C, Icariin, Sagittatoside B, 2″-*O*-rhamnosylicariside II, Baohuoside I) and Internal Standard (Naringenin) were purchased from National Institutes for Food and Drug Control (Beijing, China) (Figs. S1 and S2).

LC–MS grade acetonitrile and formic acid were purchased from TEDIA (Fairfield, OH, USA). Deionized water was produced by Milli-Q system (Millipore, Merck, Germany) with the water outlet operating at 18.20 MΩ. Other reagents involved were all analytical grade.

2.2. Zebrafish larvae husbandry and sample preparation

Zebrafish embryos were collected from mating of adult zebrafish and reared in embryo medium (0.33 mM $CaCl_2$, 5 mM NaCl, 0.33 mM Mg_2SO_4, 0.17 mM KCl, 10^{-5}% Methylene Blue) under standard criteria [35]. At 3 days postfertilization (dpf), zebrafish larvae were transferred into 24-well plates for continued incubation at 28.5 °C (10 fishes per well). At 7 dpf, embryo medium was replaced with 1 mL 0.5% DMSO aqueous solution and three experimental groups were included in current study: zebrafish larvae exposed to 25 μg/mL EFs dissolved in 0.5% DMSO aqueous solution (Dosed group, n = 10); zebrafish larvae exposed to 0.5% DMSO aqueous solution (vehicle group, n = 10); 25 μg/mL EFs dissolved in 0.5% DMSO aqueous solution without zebrafish larvae (EFs group, n = 10). At 8 dpf, the culture solution of each well was collected individually and dried under nitrogen, stored at −80 °C before analysis.

Dried zebrafish larvae culture solution sample was re-dissolved in 150 μL acetonitrile containing 4.8 μg/mL IS, which were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was then transferred to vials for further UPLC-QTOF/MS analysis (Detailed workflow of the present study was shown in Fig. 1).

2.3. Instrument, parameters and condition

Chromatographic separation was performed on a Shimadzu LC-30A Series UPLC system (Shimadzu, Duisburg, Germany), which consists of a LC-30A binary pump, a SIL-30AC autosampler, and a CTO-30AC column oven. C_{18} reversed phase LC column (1.9 μm particles, 100 mm × 2.1 mm, Hypersil Gold, Thermo Scientific, USA) was applied for all analyses at 35 °C. The mobile phase was composed of water containing 0.1% formic acid (solvent A, v/v) and acetonitrile (solvent B) at a flow rate of 0.3 mL/min. A 16-min binary gradient elution as follows was performed for the separation: 0–2 min, 15% → 35% solvent B; 2–10 min, 35% → 70% solvent B; 10–11 min, 70% → 100% solvent B; 11–13 min, 100% solvent B for isocratic elution; 13–13.1 min, 100% → 15% solvent B, followed by 3 min of column re-equilibration.

Mass spectrometry experiments were carried out on an AB SCIEX TripleTOF^{TM} 5600+ system (AB SCIEX Technologies, USA) equipped with an electrospray ionization (ESI) source in negative ion mode. The MS parameters were set as follows: ESI temperature, 500 °C; curtain gas pressure, 35 psi; nebulizer gas (Gas 1) pressure, 60 psi; heater gas (Gas 2) pressure, 60 psi; ion spray voltage, −4.5 KV; collision energy, −35 V. Nitrogen was kept as nebulizer and auxiliary gas. The MS scan range was 100–1000 (m/z).

2.4. Quality control (QC) sample preparation

A quality control (QC) sample was prepared by pooling small aliquots of each sample to ensure broad metabolite coverage. QC specimen was analyzed every five samples throughout the whole UPLC-QTOF/MS analysis procedure, which was used to monitor the reproducibility and stability of the acquisition system.

2.5. The proposed strategy for herbal components and metabolites matching analysis

After UPLC-QTOF/MS-based data acquisition of samples from the above three groups, the proposed strategy was carried out as following steps: (i) background subtracting, peak finding and alignment, normalizations and constructing data matrix from these three groups; (ii) differentiating herbal components and metabolites by cross OPLS-DA analysis; (iii) structural characterization of herbal components and constructing product ions datasets of herbal components and metabolites, respectively; (iv) matching analysis of these two product ions datasets of herbal components