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An integrated strategy for rapid discovery and identification of the sequential piperine metabolites in rats using ultra high-performance liquid chromatography/high resolution mass spectrometery

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ABSTRACT

Piperine, one of the major bioactive constituents isolated from natural flavorings and medicinal-culinary herbs, possesses various biological activities. In the present study, an integrated strategy based on ultra high-performance liquid chromatography/high resolution mass spectrometry was established to reveal piperine metabolism in rats. First of all, post-acquisition data-mining methods, including high resolution extracted ion chromatograms (HREICs) and multiple mass defect filtering (MMDF), were used to screen piperine metabolite candidates in a full-scan HRMS^1 level. Then, parent ion list-dynamic exclusion coupled with data-dependent data-acquisition method was utilized to acquire MS^n datasets. In addition, the established reverse molecular assembly (RMA) approach based on paired diagnostic product ions (*p*DPIs) coupled with neutral loss fragments (NLFs) was used to ascertain and identify the major-to-trace piperine metabolites efficiently. And then, the calculated Clog*P* values were utilized to distinguish the positional isomers. As a result, a total of 148 piperine metabolites were detected and characterized tentatively. The results demonstrated that piperine mainly underwent hydrogenation, dehydrogenation, hydroxylation, glucuronide conjugation, sulfate conjugation, ring-cleavage, and their composite reactions. Our results not only provided novel and useful data to better understand the safety, toxicity and efficacy of this potential therapeutic agent, but also indicated that the proposed strategy was reliable for a rapid discovery and identification drug-related constituents *in vivo*.

1. Introduction

Piperine is an active natural cinnamamide-type alkaloid found in many medicinal-culinary herbs, such as *Piper nigrum* L. (Black pepper and White pepper), *Zingiber officinale* (ginger) (Jiang in Chinese), *Piper longum* L. (Biba, mongolian medicine), *Piper sarmentosum* Roxb, and Dangzuo (Tibetan medicine) [1–5]. It has been reported to have anti-carcinogenic, anti-hyperlipidemia, immunomodulatory, anti-depressant, anti-inflammatory, anti-diarrheal, anti-ulcer, anti-ischemic and anti-oxidative activities [6–14]. Meanwhile, piperine could also enhance the *in vivo* absorption of other drugs such as aulin, amoxicillin, and phenytoin due to its potential effects on regulating microsomal metabolic enzymes [15]. However, although 12 metabolites had been tentatively identified in SD rats recently, the *in vivo* metabolic pathways of piperine might not be holistically proposed due to the absence of scientific analytical strategy [16]. And thus, it still plays a towing role to comprehensively profile piperine metabolism which will benefit the researches for its further therapeutic applications [17,18].

Xenobiotics are metabolized into various forms *via* different metabolic pathways. The metabolites are often found in small amount and hence their signals are usually masked by background noise produced by endogenous substances. In past decades, with the rapid developing of liquid chromatography/mass spectrometry (LC/MS) sensitivity, to establish a comprehensive and efficient analytical method to obtain useful information from complex backgrounds is made feasible and become a major project [19–21]. Thus, various post-acquisition data-mining methods emerged to acquire

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information from high resolution mass spectrometry (HRMS) and multiple stage-mass spectrometery (MS^n) datasets of the major-to-trace metabolites [22–24]. Among them, high resolution extracted ion chromatogram (HREIC) [21], mass defect filtering (MDF) to multiple mass defect filtering (MMDF) [25], isotope pattern filtering, neutral loss filtering and background subtraction could be utilized to facilitate metabolites detection.

Herein, an ultra high-performance liquid chromatography (UHPLC)/HRMS based strategy was established to perform a comprehensive profiling and identification of metabolites in plasma, urine and feces of Sprague-Dawley (SD) rats followed oral administration of piperine. The full scan-parent ion list-dynamic exclusion (FS-PIL-DE) data-acquisition method coupled with multiple data-mining techniques, including HREICs, MMDF, NLFs [26] and diagnostic product ions (DPIs) [27,28] were applied to improve the efficiency of metabolites identification. Especially, as it is known that the product ions and neutral fragments in CID mode are usually emerged complementation, the proposed strategy-reverse molecular assembly (RMA) that combined the paired-DPIs (*p*DPIs) with NLFs was developed to accomplish a much more efficient structural elucidation. In the end, the *in vivo* metabolic pathways of piperine were proposed based on these detected metabolites.

2. Experimental

2.1. Chemicals and materials

Piperine reference standard was purchased from Chengdu Must Biotechnology Co. Ltd (Sichuan, China). Its structure was fully elucidated by comparing its spectra data (ESI–MS and ^{1}H, ^{13}C NMR) with published literatures. Its purity was acceptable (≥98%) according to HPLC-UV analysis.

HPLC grade acetonitrile, methanol and formic acid (FA) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). All the other chemicals of analytical grade are available at the work station, Beijing Chemical Works (Beijing, China). Deionized water used throughout the experiment was purified by a Milli-Q Gradient Å 10 System (Millipore, Billerica, MA, USA). Grace Pure ^{TM} SPE C_{18}-Low solid-phase extraction cartridges (200 mg/3 mL, 59 μm, 70 Å) were purchased from Grace Davison Discovery Science (Deerfield, IL, USA).

2.2. Animals

Eight male SD rats weighted 220 ± 20 g were obtained from Beijing Weitong Lihua Experimental Animals Company (Beijing, China). The rats were housed in a controlled room at standard temperature (24 ± 2 °C) and humidity (70 ± 5%), and kept on a 12 h light/12 h dark regime. After a week acclimation, rats were randomly divided into two groups: Drug Group ($n = 4$) for test plasma, urine and feces; Control Group ($n = 4$) for blank plasma, urine and feces. They were fasted for 12 h with free access to water prior to the experiment. The animal protocols were approved by the institutional Animal Care and Use Committee at Beijing University of Chinese Medicine. The animal facilities and protocols were complied with the Guide for the Care and Use of Laboratory Animals (USA National Research Council, 1996).

2.3. Drug administration and biological samples preparation

Piperine was suspended in 0.5% carboxymethylcellulose sodium (CMC-Na) solution. Rats in Drug Group were given a dose of 250 mg/kg body weight orally. 0.5% CMC-Na aqueous solution (2 mL) was administrated to rats in Control Group. Blood samples (0.5 mL) were taken from the suborbital venous plexus of rats at 0.5, 1, 2 and 4 h post-administration. Each sample was centrifuged at 3500 rpm for 10 min to separate plasma samples. Urine and feces samples were collected 0–24 h after oral administration. All homogeneous biological samples from the same group were merged into a collective sample.

An approach involved protein and solid residue precipitation and concentration was performed to prepare biological samples. Plasma and urine samples (1 mL) were respectively added into SPE cartridge pretreated with methanol (5 mL) and deionized water (5 mL). And then, the SPE cartridges were successively washed with deionized water (5 mL) and methanol (3 mL). The methanol eluate was collected and evaporated in nitrogen at room temperature. The residue was redissolved in 100 μL of acetonitrile/water (10:90, *v*/*v*) and then centrifuged at 14,000 rpm for 15 min. Meanwhile, feces sample (1.0 g) was ultrasonic extracted with deionized water (5.0 mL) for 15 min and then filtered. Supernatant (1 mL) was added into the pretreated SPE cartridge, and then the same process described above was conducted. All the supernatants were used for further instrumental analysis.

2.4. Instrument and conditions

LC analysis was performed on an Accela 600 pump UHPLC system (Thermo Electron, Bremen, Germany), equipped with a binary pump and an auto-sampler. The chromatographic separation was carried out using Waters ACQUITY BEH C18 column (2.1 × 100 mm i.d., 1.7 μm; Waters Corporation, Milford, MA, USA) at room temperature. Acetonitrile/methanol (3:1, *v*/*v*, solvent B) and 0.1% FA aqueous solution (solvent A) were used as mobile phase. The flow rate was set to 0.25 mL/min with a linear gradient as follows: 0–2 min, 5% B; 2–3 min, 5–10% B; 3–17 min, 10–30% B; 17–28 min, 30–60% B; 28–30 min, 60–80% B; 30–35 min, 80% B. The injection volume was 2 μL.

HR-ESI–MS and MS/MS spectra were obtained using LTQ-Orbitrap MS (Thermo Electron, Bremen, Germany) connected to the UHPLC instrument *via* ESI interface. Samples were analyzed in positive ion mode with the tune method set as follows: sheath gas (nitrogen) flow rate of 30 arb, aux gas (nitrogen) flow rate of 5 arb, capillary temperature of 350 °C, spray voltage of 4.0 kV, capillary voltage of 25 V, tube lens voltage of 110 V. Accurate mass analysis were calibrated according to the manufacturer's guidelines. Centroided mass spectra were acquired within the mass range of m/z 50–800.

In the FS experiment, HRMS data were recorded at mass resolving power of 30,000 full width at half maximum (FWHM, calculated for m/z 200). To minimize the total analysis time, data-dependent MS/MS scanning to trigger fragmentation spectra of target ions was performed. The collision energy for collision induced dissociation (CID) was adjusted to 40% of maximum. The dynamic exclusion to prevent repetition was employed, and the repeat count was set at 5 with the dynamic repeat time at 30 s and dynamic exclusion duration at 60 s. In addition, the PIL-dependent acquisition mode was employed as a complementary tool to obtain the ESI–MS/MS datasets.

2.5. Peak selections and data processing

Thermo Xcalibur 2.1 workstation was used for data acquisition and processing. In order to obtain as many ESI–MS/MS fragment ions of piperine metabolites as possible, the peaks detected with intensity over 40000 were selected for identification. The accurate mass of chemical formulas attributed to all parent ions of the selected peaks were calculated using a formula predictor by setting the parameters as follows: C [10–25], H [20–40], O [0–15], S [0–4], N [0–4], Cl [0–4] and ring double bond (RDB) equivalent value [0–15].