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In vivo study of erysolin metabolic profile by ultra high performance liquid chromatography coupleded to Fourier transform ion cyclotron resonance mass spectrometry

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ABSTRACT

An ultra high performance liquid chromatography coupled to Fourier transform ion cyclotron resonance mass spectrometry (UHPLC-FT-ICR-MS) method was developed for the first time to study the *in vivo* metabolism of erysolin, a compound derived from cruciferous plants which has a definite effect of anti-tumor and anti-nerve injury. In this research, the chromatographic separation was performed on an ACQUITY UPLC® BEH C18 column (2.1 mm × 100 mm, 1.7 μm, Waters, USA) and eluted by a gradient program, the identification work was achieved on a Bruker ultra-high resolution spectrometer in positive ion mode. Plasma, urine, feces and bile samples were collected from rats to screen metabolites after an intragastric administration of erysolin at the dose of 100 mg/kg. As a result, the parent drug and a total of six phase II metabolites were detected and preliminarily identified by analyzing their MS and MS/MS spectrometry profiles. Our results indicated that erysolin mainly metabolized via the mercapturic acid metabolic pathway, erysolin first react with glutathione to form glutathione conjugate, followed by taking off the glutamic acid and glycine to form cysteine conjugate, then the N-acetylation reaction occurs, the product would be excreted out of the body at last. In conclusion, results obtained in our study may contribute to a better understanding of the metabolism process and characteristics of erysolin *in vivo*, and provide an important reference for future research.

1. Introduction

Erysolin (4-methylsulfonlybutyl isothiocyanate) is a hydrolysate of glucoerysolin which was first identified from the extracts of *Erysimum perofskianum* Fisch. et Mey, one kind of cruciferous plants [1]. It also can be found in some other cruciferous plants such as cabbage *Brassica oleracea* L. [2], *Cardaria draba* (L.) Desv. [3], and *erysimum allionii* hort. [4]. Numerous studies have confirmed that erysolin has a strong ability to inhibit tumorigenesis through various pathways, including holistic cytotoxicy to tumor cells [5–7], inhibition of Phase I enzymes and inducing Phase II enzymes to accelerate the inactivation of carcinogens [8–10], regulation of multidrug resistance proteins [11,12], protective effects of genotoxic based on ATR-Chk1 pathway [13,14], inhibition of tumor metastasis by Notch signaling pathway [15], increasing reactive oxygen species to induce apoptosis [16–19] and inhibiting the expression of NF-κB by regulating TLR3 signaling pathway [20]. In addition, it also has antibacterial activities [21] and potential anti-nerve injury effect [22].

In consideration of the outstanding pharmacological values and edible characteristics from cruciferous vegetables, erysolin can be considered as a promising drug candidate. Broader and more in-depth study of the efficacy, especially the specific treatment mechanism research of erysolin, need the metabolism data *in vivo*. Metabolism research plays an important role in drug discovery, the metabolites of the drug may have a new pharmacological activity or toxicity and its original pharmacological activity may be increased or decreased [23]. However, the metabolic profile of erysolin is still unclear up to now, and there has no paper showed whether it can be found in the blood or what it can be transformed *in vivo*.

In order to obtain abundant information about metabolites from complex biological samples, sensitive and reliable analytical methods should be established with highly modernized equipment. Nowadays, high-performance liquid chromatography coupled to mass spectrometry (LC–MS) is an universal means in the study of metabolic profile for the

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efficient detection and identification capability [24,25]. Compared with conventional triple quadrupole, time of flight and ion trap mass analyzer, fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) has ultra-high mass accuracy (below 2 ppm in most cases) and unsurpassed resolving power (1,000,000, m/z = 400) [26]. For the mass accuracy of precursor ions or parent ion (MS mode) and fragment ions (MS/MS) have sub-ppm levels, combined with unambiguous isotope pattern matching technology of Smart Formula 3D™, FT-ICR-MS can provide the most reliable elemental composition and formula, Such high confidence levels can be easily obtained without internal calibration or repeated correction. Ultra high resolution data can reduce the complexity of data itself caused by other chemical interferences [27,28]. FT-ICR-MS is becoming the most powerful commercially available instrument and it has been used to identify metabolites successfully by many researchers in recent years [29–31].

In this study, an ultra high performance liquid chromatography combined with Fourier transform ion cyclotron resonance mass spectrometry (UHPLC-FT-ICR-MS) method was firstly established for the research of metabolic profile in rats after a single intragastric gavage. The major metabolites of erysolin in plasma, urine, bile and feces from rats were identified and the probable metabolic pathways *in vivo* were illuminated. Results of this reserch could provide reference for subsequent studies related to efficacy, pharmacokinetics and other *in vivo* projects of erysolin in the future.

2. Experimental

2.1. Materials and reagents

Erysolin (purity > 99%,) was synthesized in School of Pharmacy, Shenyang Pharmaceutical University (Shenyang, China) and School of Pharmacy, Taishan Medical University (Tai'an, China). HPLC-grade acetonitrile and formic acid were obtained from Fisher Scientic (Fair Lawn, NJ, USA). The distilled water was purchased from Wahaha Co., LTD. (Hangzhou, China) and all other chemicals were of analytical grade and obtained commercially.

2.2. Animal experiments

Specific pathogen free (SPF) degree Sprague Dawley rats (male, 200–220 g) were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All rats were housed in a standardized animal room with temperature (22 ± 2 °C), humidity (50 ± 10%), and a natural light/dark cycle with free access to standard food and water, there were at least 7 days for the rats to adapt to the current environment. Before administration, the rats were fasted for 12 h but with water freely available. The whole experimental procedures were complied with the principles of animal care which National Institute of Health's Guidelines promulgated and approved by the Medical Ethic Committee of Shenyang Pharmaceutical University (No. SYPU-IACUC-0685-201). All surgery was operated under standard conditions and all efforts were made to reduce animal suffering, the experiment was also complied with the practice guide to the animal administration issued by EFPIA (the European Federation of Pharmaceutical Industries Associations) and ECVAM (the European Centre for the Validation of Alternative Methods) [32].

The rats were randomly divided into three groups with six rats in each group. Erysolin (suspended in 0.5% sodium carboxymethyl cellulose) was orally administered to the three groups at a single dose of 100 mg/kg. Since different metabolites may produced in the different time, our collection plan of biological samples covered 24 h. In group I, blood samples were collected from tail tip into heparinized tubes at 0.5, 1, 2, 4, 8, 12 and 24 h after intragastric gavage administration, about 0.2 mL blood was collected at each time point, then the samples were immediately centrifuged at 13,000 rpm for 10 min under 4 °C using refrigerated centrifuge to obtain plasma. Rats in group II were held in metabolic cages to collect the urine and feces samples within 0 ~ 24 h after administration. Rats in group III were cannulated in the bile duct under common anesthesia, bile samples were also collected from 0 to 24 h after dosing. Blank samples of plasma, urine, feces and bile were also collected from rats using the method above without dosing. The tubes used to collect urine and bile were placed in ice bath, urine, feces and bile samples were collected as timely as posssible, all samples were frozen at −80 °C until analysis.

2.3. Sample preparation

The collected plasma samples were mixed together by equal volumes from each time point while the urine, feces and bile samples were mixed directly. For the plasma, urine and bile samples, 1200 μL of acetonitrile was added to a volume of 400 μL mixed biological samples and vortexed for 2 min, then the samples were centrifuged at 13,000 rpm for 10 min under 4 °C using refrigerated centrifuge, the organic layer was transferred to a new tube and evaporated to dryness under the stream of nitrogen gas in ice bath in the next step. The mixed feces samples were lyophilized and added to tenfold (volume) acetonitrile, then made feces homogenate in ice bath, ultrasonic treatment to be carried out for 20 min and vortexed for 2 min subsequently, the next centrifugation and solvent drying operations were the same as the preparation of plasma, urine and bile residues. In order to conserve the polar metabolites as much as possible in the sample, no further purification measures were taken. The residue was dissolved in 200 μL mobile phase (acetonitrile/water = 50:50, v/v) and filtered with organic membrane of 0.22 μm, at last, the filtrate was transferred to a sample vial to run the UHPLC-FT-ICR-MS analysis.

2.4. Instrument and analytical conditions

The UHPLC-DAD-FT-ICR-MS system contains an Agilent 1260 system (including an online degasser, a quadruple pump, a thermostatically controlled column compartment, an automatic sampler and a diode array detector, Agilent, USA), a Bruker Solarix 7.0T FT-ICR-MS system (Bruker, Germany) and a Bruker Compass-Hystar workstation (Bruker, Germany). Data Analysis Software (Bruker, Germany) was used to control the instrument, data acquisition and analysis. LC separation was achieved on an ACQUITY UPLC® BEH C18 column (2.1 mm × 100 mm, 1.7 μm) (Waters, USA), which was protected by an ACQUITY UPLC® Column online filtration system (Waters, USA) and the column temperature was maintained at 30 °C. The mobile phase contained water-0.1% formic acid (A) and acetonitrile-0.1% formic acid (B), the gradient condition of mobile phase was: 90–85% (A) in 0–8 min, 85–50% (A) in 8–12 min, 50–90% (A) in 12–15 min, the column was equilibrated for 10 min. The flow rate was 0.25 mL/min and the injection volume was 2 μL.

The mass spectra were performed with electrospray ionization (ESI) source, positive ion mode was used, the main parameters are shown as follows: capillary voltage, −4.5 kV; end plate offset, −500 V; dry gas flow, 8 L/min; dry gas temperature, 200 °C; nebulizer gas pressure, 4 bar; collision gas, high-purity argon; nebulizing gas, high-purity nitrogen. Full-scan mode data were recorded from m/z 50–1500 amu, and the collision energy was ranged from 5 eV to 30 eV for MS/MS experiments.

2.5. Method validation

In line with the purposes and procedures of this study, the method was validated elastically according to the currently accepted FDA Bioanalytical Method Validation Guidance [33] and European Medicines Agency Guideline on Bioanalytical Method Validation [34] with respect to specificity, instrument precision, repeatability and stability of the sample after treatment. Because the bile contains the parent drug and all its metabolites, so we chose bile as the representative sample of