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Identification of metabolites of oridonin in rats with a single run on UPLC-Triple-TOF-MS/MS system based on multiple mass defect filter data acquisition and multiple data processing techniques

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ABSTRACT

Oridonin (ORI) is an active natural *ent*-kaurane diterpenoid ingredient originating from well-known traditional Chinese herb medicine and is expected to be pursued as a new anticancer agent. In the present study, a novel and efficient approach was developed for *in vivo* screening and identification of ORI metabolites using ultra high performance liquid chromatography coupled with hybrid triple quadrupole time-of-flight mass spectrometry (UPLC-Triple-TOF-MS/MS). This analytical strategy was as follows: an effective on-line data acquisition method multiple mass defect filter (MMDF) combined with dynamic background subtraction (DBS), was developed to trace all of potential metabolites of ORI. The MMDF and DBS method could trigger an information dependent acquisition scan, which could give the information of low-level metabolites masked by background noise and endogenous components in complex matrix. Moreover, the sensitive and specific multiple data-mining techniques including extracted ion chromatography, mass defect filtering, product ion filtering and neutral loss filtering were employed to identify the metabolites of ORI. Then, structures for the metabolites were successfully assigned based on accurate masses, the mass fragmentation of ORI and metabolic knowledge. Finally, an important parameter Clog P was used to estimate the retention time of isomers. Based on the proposed strategy, 16 phase I and 2 phase II metabolites were detected in rats after oral administration of ORI. The main biotransformation route of ORI was identified as reduction, oxidation, dehydroxylation and glucuronic acid conjugation. This is the first study of ORI metabolism *in vivo*. This study not only proposed a practical strategy for rapidly screening and identifying metabolites, but also provided useful information for further study of the pharmacology and mechanism of ORI *in vivo*. At the same time this methodology can be widely applied for the structural characterization of the metabolites of other *ent*-kaurane diterpenoid.

1. Introduction

Isodon rubescens (Donglingcao in Chinese), the dried aerial of *I. rubescens* (Hemsl.) Hara, is widely used as well-known traditional Chinese medicine and have been officially listed in the Chinese Pharmacopeia [1]. The herb has long been used as a folk remedy for respiratory and gastrointestinal bacterial infections, inflammations and cancer. Diterpenoids are reported to be the bioactive components of Isodon rubescens, and most of the diterpenoids belong to the *ent*-kaurane diterpenoids. Oridonin (ORI) (Fig. 1), one of the most abundant diterpenoids of Isodon rubescens, has lots of pharmacological and biological activities, including anti-inflammatory, anti-viral, anti-tumor, anti-microbial, anti-catastrophe and anti-oxidation actions [2–4]. It has aroused high interest especially in cancer researchers due to its potential to be developed into tumor chemotherapeutic drug [5,6]. Inhibition of tumor cell growth by ORI was ascribed to the ability of the drug to affect cell cycle progression and/or to promote cell death by apoptosis and autophagy [7]. Depending on cell type, ORI has been shown to induce cell cycle arrest in G1/S, S/G2 or G2/M and to modulate the expression/activity of different cell cycle regula-

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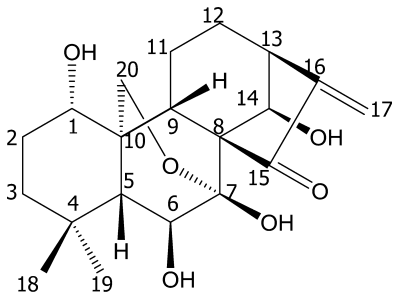

Fig. 1. Chemical structure of ORI.

tory proteins [8,9]. Notably, many studies reported ORI showed strong cytotoxicity against murine B16-F10 melanoma [10] and induced cell killing in A375 melanoma cell line [11]. These accumulating evidences demonstrate that ORI has significant anticancer and remarkable anti-tumor activity. With the development, ORI is expected to become a novel anti-cancer and anti-tumor drug [12]. Pharmacokinetic profiles of ORI in rats indicate a fast absorption and an extensive metabolic elimination after oral administration [13–21]. Pre-experimental results in our laboratory show that ORI rapidly undergo significant metabolism in the body. All of these results indicated that ORI was rapidly and thoroughly metabolized *in vivo* and excreted mainly as metabolites. There is no report on ORI metabolism and probable drug–drug interaction in rats is still unclear so far. Metabolite study is an integral part of drug discovery and development, so metabolites should to be identified, and their toxicity must be evaluated, which is an important part of drug discovery and development.

Recently, high-resolution mass spectrometry (HRMS) such as time of flight (TOF) and orbital trapping analyzers have been used for metabolite identification due to their high mass accuracy and resolution. Unfortunately, orbital trapping involves a compromise between resolution and speed. Hybrid triple quadrupole time-of-flight mass spectrometry instruments have been the workhorse instrument for the quantitative portion of this application due to its excellent sensitivity, high throughput sensitivity, accuracy and comprehensive nature of metabolite detection [22]. In TOF systems, it can provide the acquisition of full-scan MS spectra and product-ion spectral data sets for the metabolites by means of information dependent acquisition (IDA). Then, the MS and MS/MS spectra data were used for the identification of both target and non-target compounds. In previous studies, non-target and minor metabolites from the full-scan mass chromatograms using LC-TOF/MS have been likely to be overwhelmed by interferences from the background or the matrix because excess quantities of endogenous components are co-eluted, so their product-ion mass spectra acquisitions have not been triggered. This deficiency leads to many trace metabolites can not be detected. The mass defect filter (MDF) technique was developed for on-line data acquisition to trace all probable metabolites. Different bio-transformations have distinct mass defect values. Almost all of the mass defect values of metabolites fall within a defined narrow and national window around that of the parent drug. The MDF technique was first reported in 2003 for the identification of drug metabolite ions [23]. The method could automatically remove background interference and monitor the minor metabolites through setting the initial mass defect filtering algorithm [24–26]. At the same time, there have been many studies on the development and application of data mining technologies, such as MetabolitePilot™ software (AB Sciex) provided multiple data processing technologies, including extracted ion chromatogram (XIC), product ion filter (PIF), neutral loss filter (NLF), MDF and isotope pattern filter. To the best of our knowledge, these technologies have been successfully applied to identify complex compounds or metabolites [27,28]. Although HRMS in the identification of compounds has incomparable superiority, there is a great challenge when isomers are intended to identify. With regard to this, the Clog P, an important parameter, which was used to estimate the isomers of the metabolites.

In the present study, the novel approach of an on-line data acquisition method dependent on MMDF and DBS combined with multiple data processing techniques, including XIC, MDF, PIF and NLF, was developed on a Triple TOF 5600+ system (AB Sciex). Only a single run was needed for the analysis and the data acquisition process was proposed in detail. The method was utilized for the detection and structure characterization of ORI metabolites in a rat urine and bile sample. Based on an accurate mass measurement, the fragmentation patterns of the parent drug, relevant drug biotransformation knowledge and the value of Clog P, a total of 18 metabolites with different structures were identified or tentatively assigned. The metabolic pathway of ORI and the fragmentation patterns of ORI metabolites were also proposed. This was the first metabolic investigation of ORI using UPLC-Triple TOF-MS and multiple data processing techniques for the rapid identification of ORI metabolites in rat bile and urine.

2. Experimental

2.1. Chemicals and materials

ORI (purity >99%) was purchased from Nanjing Zelang Co., Ltd. (Jiangsu, People's Republic of China). HPLC-grade methanol (Fisher Chemicals, USA) and formic acid (Diamond Technology Corporation, USA) were of HPLC grade. De-ionized water was prepared in our lab using a Milli-Q water purification system (Millipore, ELIX100, USA) for preparing the mobile phase. Analytical grade sodium carboxymethyl cellulose (CMC–Na) and other chemicals were of analytical grade (Tianjin Chemical Corporation, People's Republic of China).

2.2. UPLC-Triple TOF-MS conditions

HPLC experiments were performed on an Agilent (Agilent, USA) UPLC system equipped with a quaternary solvent delivery system, an auto-sampler and a column compartment. The chromatographic separation was carried on a C18 reversed phase LC column (Phenomenex Kinetex C18 100 × 2.1 mm, 2.6 μm) with a Security Guard UPLC C18 column (4.0 × 3.0 mm i.d., 5 mm; Phenomenex, Torrance, CA, USA). The column temperature was maintained at 25 °C. The mobile phase consisted of methanol (A) and water containing 0.1% formic acid (B). The gradient elution program was optimized for the separation, and the program was as follows: 5–30% A from 0 to 10 min, 30–100% A from 10 to11 min. After holding the composition of 100% solvent A for the next 2 min, the column was returned to it starting conditions until 15 min for column balance. The mobile phase flow rate was set at 0.4 mL/min and the injection volume was 5 μL.

A Triple TOF™ 5600+ system with Duo-Spray™ ion sources operating in the negative electrospray ionization (ESI) mode was used for the detection. The following MS/MS conditions were used: ion spray voltage, −4.5 kV; the turbo spray temperature, 550 °C and declustering potential (DP), 60 V. Nitrogen was used as the nebulizer and the auxiliary gas, and the nebulizer gas (gas 1), the heater gas (gas 2) and the curtain gas were set to 55, 55 and 35 L/min, respectively. For the full MS-IDA (information dependent acquisition)-8MS/MS analysis, the scan range was operated with the mass *m/z* 100 to *m/z* 1000 and with a 200 ms accumulation time. The collision energy (CE) was set at −40 eV and the collision energy spread (CES) was 15 eV, enabling us to obtain an average enhanced