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A targeted strategy to identify untargeted metabolites from *in vitro* to *in vivo*: Rapid and sensitive metabolites profiling of licorice in rats using ultra-high performance liquid chromatography coupled with triple quadrupole-linear ion trap mass spectrometry

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ABSTRACT

It is challenging to conduct *in vivo* metabolic study for traditional Chinese medicines (TCMs) because of complex components, unpredictable metabolic pathways and low metabolite concentrations. Herein, we proposed a sensitive strategy to characterize TCM metabolites *in vivo* at an orally clinical dose using ultra-high performance liquid chromatography-triple quadrupole-linear ion trap mass spectrometry (UHPLC-QTRAP-MS). Firstly, the metabolism of individual compounds in rat liver microsomes was studied to obtain the metabolic pathways and fragmentation patterns. The untargeted metabolites *in vitro* were detected by multiple ion monitoring-enhanced product ion (EPI) and neutral loss-EPI scans. Subsequently, a sensitive multiple reaction monitoring-EPI method was developed according to the *in vitro* results and predicted metabolites to profile the *in vivo* metabolites. Licorice as a model herb was used to evaluate and validate our strategy. A clinical dose of licorice water extract was orally administered to rats, then a total of 45 metabolites in urine, 21 metabolites in feces and 35 metabolites in plasma were detected. Among them, 18 minor metabolites have not been reported previously and 6 minor metabolites were first detected *in vivo*. Several isomeric metabolites were well separated and differentiated in our strategy. These results suggested that this new strategy could be widely used for the detection and characterization of *in vivo* metabolites of TCMs.

1. Introduction

Traditional Chinese medicines (TCMs) have been used for the treatment of various diseases and have aroused worldwide interests due to their significant efficacy in treating chronic and systemic diseases [1]. Comprehensive study on *in vivo* metabolism becomes an essential part of pharmaceutical research of TCMs, which facilitates understanding the potential therapeutic material basis *in vivo* [2]. However, metabolite identification of TCMs is quite difficult because of their complicated chemical composition, endogenous interferences, trace concentrations and unpredictable metabolic pathways [2–5].

Recently, various liquid chromatography-tandem mass spectrometric (LC-MS/MS) approaches have been developed for characterization of *in vivo* metabolites of TCMs. Among them, most reports focused on the optimization of various post-acquisition data mining techniques based on hybrid high resolution mass spectrometer (HRMS), such as quadrupole time-of-flight mass spectrometry (Q-TOF), linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap), *etc.* [2,6–8]. Q-TOF is widely used and suitable for non-targeted metabolites analysis, but is limited by its restricted scanning functions. The data mining techniques can help to discover and characterize metabolites of TCMs from complex MS data effectively, but cannot solve the shortcoming in data acquisition. Thus, signals of some trace metabolites may be missed during the data acquisition of QTOF. The more advanced hybrid-HRMS instruments such as LTQ-Orbitrap and LTQ-FTICRMS may have better sensitivity and selectivity, but they are extremely expensive which

Abbreviations: LC–MS/MS, liquid chromatography coupled with tandem mass spectrometry; QTOF, quadrupole time of flight; QTRAP, hybrid triple-quadrupole linear ion trap; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectrometry; UHPLC, ultra-high performance liquid chromatography; XIC, extracted ion chromatogram; MRM, multiple reaction monitoring; EPI, enhanced product ion scan

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makes them unavailable for most laboratories and routine analyses [8–10]. Therefore, most of the current reports about TCM metabolism often use single compound [11,12] or very high dose of TCM extracts (40–200 times higher than clinical dose) to ensure that the metabolites are abundant enough for detection in complex biological matrix [13–15]. However, the analysis of TCMs is a "complex system research" [16], thus the metabolism of single compound will be significantly different from multi-component synergistic effect. On the other hand, high dose administration will also lead to distinct metabolic pathways compared to that of clinical dose [13]. Therefore, it is important to develop a sensitive and rapid approach to systematically characterize the metabolites of TCMs at an orally clinical dose.

It is well known that the multiple reaction monitoring (MRM) mode of triple quadrupole mass spectrometer has excellent sensitivity and selectivity. The MRM can be used as a survey scan to trigger the acquisition of enhanced product ions (EPI) on a QTRAP when conducting a metabolite characterization study. This method is capable of targeting up to 500 transitions with sensitivity and selectivity better than QTOF [17] full scan mode. Whereas, MRM-EPI is not suitable for the identification of non-targeted metabolites of which the fragmentation patterns and metabolic pathways are unknown or unpredictable.

The main objective of this study was to develop a new strategy based on MRM-EPI to simultaneously perform targeted and untargeted detection of metabolites *in vivo* after oral administration of TCMs (Fig. 1). QTRAP-based multiple ion monitoring (MIM)-EPI and neutral loss (NL)-EPI scan are generally used to detect untargeted metabolites produced by phase I (oxidation, reduction and hydrolysis) and phase II (conjugation) reactions, respectively [6,18]. They are complementary with targeted MRM-EPI scan, therefore MIM-EPI and NL-EPI were initially used to detect *in vitro* metabolites of representative single compound of TCMs. Then, their fragmentation patterns and metabolic pathways were summarized. Based on the information of *in vitro* metabolites and reasonable predictions, an MRM-EPI approach was established to identify the metabolites *in vivo* after oral administration of TCMs. A pseudo untargeted analytical method based on MRM-EPI was implemented through this strategy.

Licorice, the roots and rhizomes of *Glycyrrhiza uralensis*, *Glycyrrhiza inflata* and *Glycyrrhiza glabra*, was selected as the model herb because it is widely used in most traditional Chinese medicine prescriptions [19]. It has been used to treat various diseases, such as cough, hepatitis and peptic ulcer [20]. Triterpenoids and flavonoids are the major active components of licorice [21]. In particular, glycyrrhetinic acid (GA) and glycyrrhizic acid (GL), which belong to triterpenoid and glycoside derivative, are regarded as the most important active constituent of licorice [21,22]. In addition, liquiritigenin (LG), isoliquiritigenin (ILG) and their glycoside derivatives, liquiritin (LQ) and isoliquiritin (ILQ), are the most abundant flavonoids [21] and exhibit excellent pharmacological activities [20,23]. In this work, LG, ILG, LQ, ILQ, 18β-GL and 18β-GA were therefore chosen as representative compounds of licorice to establish the MRM-EPI method for *in vivo* metabolite identification.

2. Material and methods

2.1. Chemicals, reagents and materials

The reference standards of 18β-glycyrrhizic acid, 18β-glycyrrhetinic acid, liquiritigenin, isoliquiritigenin, liquiritin and isoliquiritin, with a purity of ≥98%, were purchased from National Pharmaceutical Engineering Center for Solid Preparation in Chinese Herbal Medicine (Nanchang, China). The licorice raw material was acquired from Hubei Pharmaceutical Group Co. Ltd. (Wuhan, China). Male rat liver microsomes (RLM) were obtained from BD Biosciences (Woburn, MA, USA). β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) and dimethyl sulfoxide (DMSO) were purchased from

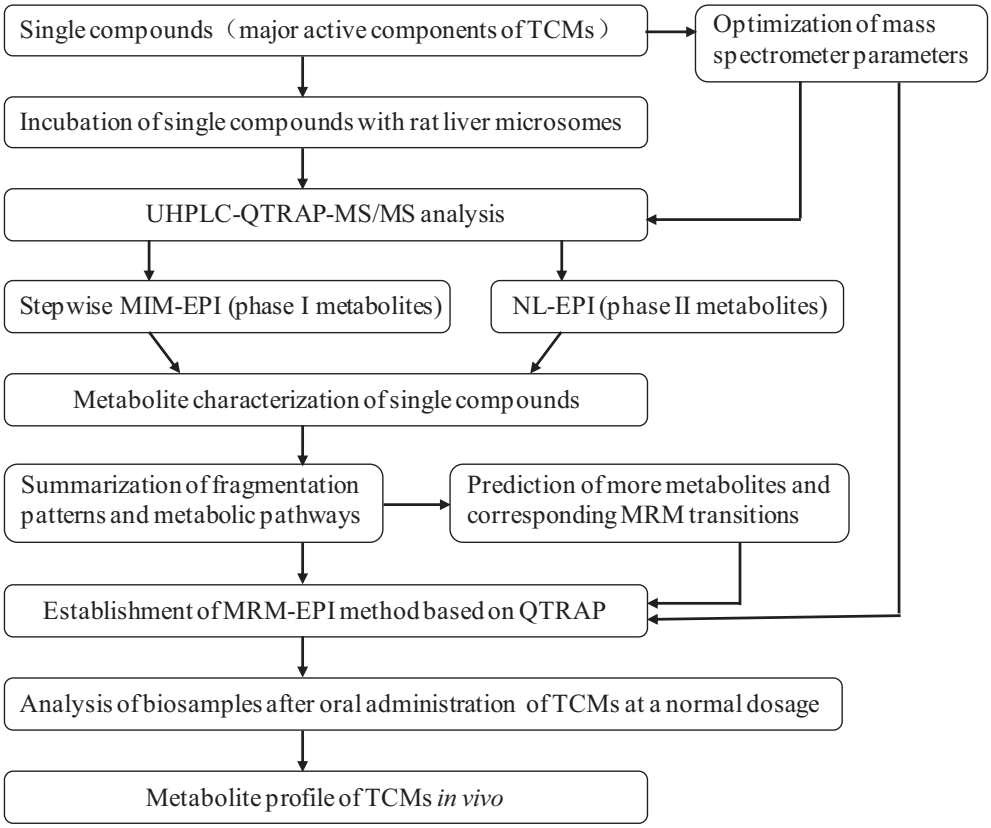

Fig. 1. A novel strategy for the systematic detection and identification of the *in vivo* metabolites of TCMs at a normal dosage using MRM-EPI scan method based on QTRAP.