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A combination of representative compounds, metabolism platform and diagnostic extraction strategy for characterization of metabolites of Shuang-Huang-Lian oral liquid *in vivo* by ultra-performance liquid chromatography coupled with time-of-flight mass spectrometry

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ABSTRACT

Traditional Chinese medicines (TCMs) usually contained a large number of chemical components, which could be transformed into more complex metabolites *in vivo*. In this work, a "Representative compounds-Metabolism platform-Diagnostic extraction" strategy (RMD strategy) was proposed for comprehensively identification or characterization of xenobiotics in rat after oral administration of TCMs. Shuang-Huang-Lian oral liquid (SHL), a well-known traditional Chinese medicine preparation, was used as an example. The metabolic pathways of six representative compounds, bearing five different core structures in SHL, were elucidated and their metabolic reactions were employed for exploring metabolites of homologous components in metabolism platform. Meanwhile, diagnostic ions extraction were also used for screening more structural analogues in biofluids. All this work was completed by ultra-performance liquid chromatography coupled electrospray ionization quadruple time-of-flight mass spectrometry (UPLC/Qtof MS) and UNIFI metabolism platform. As a result, a total of 254 xenobiotics were identified or tentatively characterized in rat plasma and urine after oral administration of SHL and six representative compounds. The metabolism reaction included phase I reaction (hydroxylation, hydrolysis reaction, deglycosylation, hydrogenation, demethylation, dehydroxylation and ring opening reaction) and phase II reaction (glucuronidation, sulfation and methylation). This research provided useful information for further study of the pharmacology and mechanism of SHL *in vivo*. It also demonstrated that RMD strategy was an efficient approach for facilitate screening-out and rapid identification of xenobiotics in biological samples after oral administration of TCMs.

1. Introduction

Traditional Chinese medicines (TCMs) have attracted worldwide attention as their safety and efficiency have been confirmed for long-term clinical application in China [1]. The concept of multi-component and multi-target was put forward to explain the holistic therapeutic effects of TCMs [2]. However, chemical characterization of holistic components in the total extract of TCMs could not reveal *in vivo* therapeutic material basis. As an oral medicine, only the absorbed constituents and their metabolites exposed in plasma were possibly responsible for its clinical effects [3]. It was generally believed that the prototypes observed in the urine had existed in the plasma. Therefore, it had practical meaning to identify prototypes and metabolites in plasma and urine in order to reveal functional material basis of TCMs.

Xenobiotics detection and identification in biosamples was typically a labor-intensive and time-consuming process since complex and trace of absorbed constituents and metabolites of TCMs were usually hidden under endogenous components [1]. With

Abbreviations: TCMs, traditional Chinese medicines; UPLC/Qtof MS, ultra, performance liquid chromatography mass spectrometry; SHL, Shuang-Huang-Lian oral liquid; NMR spectroscopy, Nuclear magnetic resonance spectroscopy; FTA, Forsythoside A; CGA, Chlorogenic acid; RMD strategy, Representative compounds-metabolism platform-diagnostic extraction strategy; LC, liquid chromatography.

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the advancement of technology, this process had been speeded up with the help of modern spectroscopic techniques such as mass spectrometry [4,5] and NMR spectroscopy [6]. Among them, liquid chromatography coupled mass spectrometry was widely used in characterization of chemical components and related metabolites due to its high sensitivity and selectivity [7]. Recently, ultra-performance liquid chromatography (UPLC) was applied in chemical components profiling within less running time, which often combined with a quadrupole/time of flight-mass spectrometer (Q/tof-MS) to provide high resolution MS and MS/MS fragmentation information. It had been successfully applied in identification or characterization of TCMs related chemicals *in vitro* and *in vivo* [8–10]. However, analysts still had to face a serious challenges: low abundance of components of TCMs in biosamples, chemical diversity of ingredients with different core structures, and inevitable interference from endogenous substances [2]. To resolve these problems, many strategies were proposed, such as diagnostic ions extraction [11], dynamic background subtraction [12] and assistance of representative compounds [13]. These strategies certainly speeded up the characterization of metabolites in biofluids after ingestion of TCMs. However, each of them only focused on some part of the important MS information, more or less, which led to easily missing some part of metabolites when only one strategy was employed.

In this study, a systematic strategy, called "Representative compounds-Metabolism platform-Diagnostic extraction", was proposed for screening-out and characterization of the absorbed constituents and related metabolites of TCMs in complicated systems based on UPLC/Qtof MS and UNIFI metabolism platform. It was a combination of some existed methods and techniques, with an additional homologous metabolites' predication module. This strategy included three steps. Firstly, metabolism pathway of representative components featured different chemical core structures in rats were quickly investigated based on metabolism prediction platform. Secondly, more xenobiotics, with the same core structures as the representative components, were screening out and then identified by setting metabolism reaction of absorbed components in metabolism platform (UNIFI, Waters) after oral administration of TCMs. Last, a diagnostic ions extraction was also used as a complementary to enrich the metabolites' characterization. In this case study, representative compounds were selected to discover the metabolism pathway of five chemical type's compounds in rats, and then all the MS fragmentation patterns and diagnostic ions were sorted to establish an in-house database for screening and characterizing more xenobiotics in biofluids. This strategy was successfully applied in characterized of prototypes and metabolites of a traditional Chinese preparations, Shuang-Huang-Lian oral liquid.

Shuang-Huang-Lian oral liquid (SHL), a well-known traditional Chinese medicine preparation, consisted of three herbs including *Scutellaria baicalensis*, *Forsythia suspensa* and *Lonicera japonica* as recorded in Chinese Pharmacopoeia [14]. Modern pharmacology study showed that SHL had activity of anti-virus, anti-bacterium and anti-inflammatory [15]. In clinic, SHL had been used for treatment of upper respiratory caused by virus or bacterium for many years based on its accuracy efficiency [16]. Moreover, it was selected as the recommended TCM preparations for the treatment of influenza in "Influenza Diagnosis and Treatment Guidelines (2011 edition)" based on its accuracy effect [17]. Chemical components of SHL was reported previously and it had five main chemical structure types including flavonoids, phenylethanoid glycosides, iridoid glycoside, lignans and organic acids [5,18]. As a preparation of TCMs, characterization of xenobiotics *in vivo* provided basis for future study of its pharmacology and revealed its functional basis. Thus, it had practical meaning to study metabolism of SHL. Up to till now, several published reports were available on analysis of metabolites and prototypes after oral administration of SHL. Yan *et al.* [5] characterized 68 interests in rat plasma after oral administration of SHL oral liquid, including 39 prototypes and 29 metabolites. Guo *et al.* [19] had characterized prototypes and metabolites in human plasma after intravenous infusion of SHL powder injection. These work improved our understanding of components which were absorbed into blood. However, this work ignore the biosample of urine to enrich the basis of SHL and oral liquid were easily accessible form of a drug to public. Moreover, the representative compounds' metabolism information was not used for elucidation of metabolic fate of SHL. Therefore, characterization of the metabolites in urine after ingestion were of great important to understand the basis which was the role in function of SHL oral liquid. The "Representative compounds-Metabolism platform-Diagnostic extraction" strategy (RMD strategy) was applied to characterize xenobiotics of SHL *in vivo*. As a result, a total of 254 SHL-related xenobiotics were identified or tentatively characterized in rat plasma and urine after oral administration of SHL and six representative compounds. Among them, 144 xenobiotics (29 prototypes and 115 metabolites) were tentatively characterized in rat plasma and urine after oral administration of SHL. Comparison with results in published works (39 absorbed prototypes and 29 metabolites) [5], more metabolites were characterized in bio-fluids. Notably, the metabolites of phenylethanoid glycosides, one type of main components in SHL which exerted anti-influenza activity [20], were characterized in biosamples after oral administration of SHL for the first time.

RMD strategy, proposed in this case, was time-saving and efficient method for characterization and rationalization of TCM related metabolites *in vivo*. Characterization of single compounds related metabolites was much easier than directly profiling TCM preparations related metabolites in biofluids due to complexity and diversity of chemical components in TCM. In addition, metabolites of these representative single compounds, featured core chemical structure types, provided useful information (retention time and mass fragmentation ions) for confirmation of reasonable metabolites after oral administration of TCM under the same UPLC–MS condition. The metabolic reactions and mass fragment information of these core compounds *in vivo* were also used for metabolism prediction and diagnostic ions extraction to explore more potential metabolites.

2. Material and methods

2.1. Reagents

Chlorogenic acid (**26**), baicalin (**89**) and luteoloside (**125**) were purchased from Chengdu Puruifa Medical Technological limited company (Chengdu, China). Forsythoside A (**1**), sweroside (**45**) and phillyrin (**55**) were purchased from Nanjing Dierge Medical Technological limited company (Nanjing, China). SHL oral liquid were purchased from SanJing Pharm Co.Ltd. (14022123, Harbin, China). Other reference standards were separated in author's laboratory and identified by analyzing their NMR and MS information [21]. Water, methanol and ethanol were all of HPLC grade. LC–MS grade acetonitrile and water were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). LC–MS grade formic acid was obtained from Sigma-Aldrich (St. Louis, USA).

2.2. Preparation of SHL samples and six representative compounds

For drug administration, SHL oral liquid (1.5 g crud drug/mL) was administrated to rats (n = 3) directly without any pretreatment twice a day at 16 mL/kg/day for two days. Phillyrin, forsythoside A,