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Profiling and identification of the metabolites of baicalin and study on their tissue distribution in rats by ultra-high-performance liquid chromatography with linear ion trap-Orbitrap mass spectrometer

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ABSTRACT

Baicalin (baicalein 7-*O*-glucuronide), which is one of the major bioactive constituents isolated from Scutellariae Radix, possesses many biological activities, such as antiallergic, antioxidation, and anti-inflammatory activities. In the present study, an efficient strategy was established using ultra-high-performance liquid chromatography coupled with linear ion trap-Orbitrap mass spectrometer (UPLC-LTQ-Orbitrap MS) to profile the *in vivo* metabolic fate of baicalin in rat plasma, urine, and various tissues. A combination of post-acquisition mining methods including extracted ion chromatogram (EIC) and multiple mass defect filters (MMDF) was adopted to identify the common and uncommon baicalin metabolites from the full mass scan data sets. Their structures were elucidated based on the accurate mass measurement, relevant drug biotransformation knowledge, the characteristic collision induced fragmentation pattern of baicalin metabolites, and bibliography data. Based on the proposed strategy, a total of 32 metabolites were observed and characterized. The corresponding reactions *in vivo* such as methylation, hydrolysis, hydroxylation, methoxylation, glucuronide conjugation, sulfate conjugation, and their composite reactions, were all discovered in the study. The results demonstrated that the rat liver and kidney are the most important organs for the baicalin metabolites presence. Six metabolites might play an important role in exerting pharmacological effects of baicalin *in vivo*. The newly discovered baicalin metabolites significantly expanded our understanding on its pharmacological effects, and could be targets for future studies on the important chemical constituents from herbal medicines.

1. Introduction

Scutellariae Radix (the root of *Scutellariae baicalensis* Georgi) has been widely used as an important medicinal herb in China and other East Asian countries for treatment of various ailments including fevers, ulcers, cancers, and inflammation [1–3]. Baicalin (baicalein 7-*O*-glucuronide) (shown in Fig. 1), which is one of the major bioactive constituents isolated from Scutellariae Radix, possesses many biological activities, such as antiallergic, antioxidation and anti-inflammatory activities [4]. With the increasing attention on baicalin, it is important to extensively study the information regarding absorption, distribution, metabolism, and excretion (ADME), among which the characterization of metabolites can help to explain and predict a variety of events related to the efficacy and toxicity of baicalin [5,6]. However, to our best knowledge, the biotransformation of baicalin has been poorly understood, although some works on the metabolism of baicalin have already been performed [7–10]. For example, only 9 metabolites were isolated and identified from urine and feces of Wistar rats, which is due in part to the difficulties in obtaining enough amounts for NMR detection to identify more trace metabolites [8].

Abbreviations: ADME, absorption, distribution, metabolism and excretion; HRMS, high-resolution mass spectrometry; UPLC-LTQ-Orbitrap, ultra-high-performance liquid chromatography coupled with a linear ion trap-Orbitrap mass spectrometer; SD, Sprague-Dawley; EIC, extracted ion chromatogram; MDF, mass defect filtering; MMDF, multiple mass defect filtering; NLF, neutral loss filtering; IPF, isotope pattern filtering; CMC-Na, carboxymethyl cellulose sodium; RDB, ring and double bond; CID, collision induced dissociation; CE, collision energy; TIC, total ion chromatogram.

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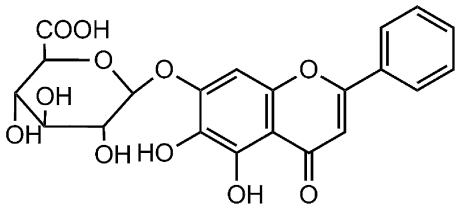

Fig. 1. Structure information of baicalin.

Recently, with the development of various data acquisition methods, liquid chromatography–mass spectrometry, especially for high-resolution mass spectrometry (HRMS), has exhibited excellent performances in metabolites detection owing to its high-speed and high detection sensitivity [11]. For example, linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap) used in the present study has combined high trapping capacity and MS^n scanning function of the linear ion trap along with accurate mass measurements within 5 ppm and a resolving power of up to 100,000. Particularly, the Orbitrap facilitates fast data-dependent acquisition of accurate MS^n spectra on an LC timescale, which could increase the throughput and identification efficiency of metabolites. Moreover, a number of off-line LC–MS data mining methods are developed to identify the trace metabolites overwhelmed by interferences from the background or the matrix. For example, multiple data processing technologies including extracted ion chromatogram (EIC), mass defect filter (MDF), multiple mass defect filters (MMDF), product ion filtering (PIF), neutral loss filtering (NLF), and isotope pattern filtering (IPF) have been successfully applied to the identification of complex compounds or metabolites [12–15].

Herein, the comprehensive profiling and identification of *in vivo* metabolism of baicalin in plasma and urine of Sprague-Dawley rats was performed. A metabolism identification strategy based on the integration of ultra-high-performance liquid chromatography (UPLC)-LTQ-Orbitrap mass spectrometer with multiple data processing techniques was developed for characterizing the major-to-trace metabolites of baicalin in the rat plasma and urine. At the same time, the established method was further applied to elucidate the distribution of baicalin metabolites in various rat tissues.

2. Experimental

2.1. Chemicals and materials

Authentic standards, *viz.*, baicalin, baicalein, scutellarin, wogonoside, and oroxin A, were purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Their structures were fully elucidated by the comparison of their spectra data (ESI-MS and ^1H, ^{13}C NMR) with those published literature values. The purities were higher than 98% according to HPLC/UV analysis.

HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (99%) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Grace Pure™ SPE C18-Low solid-phase extraction cartridges (200 mg/3 mL, 59 μm, 70 Å) were purchased from Grace Davison Discovery Science™ (Deerfield, IL, USA). All the other chemicals of analytical grade from Beijing Chemical Works (Beijing, China) are commercially available. Deionized water used throughout the experiment was purified by a Milli-Q Gradient A 10 System (Millipore, Billerica, MA, USA). The 0.22 μm membranes were purchased from Xinjinghua Co. (Shanghai, China).

2.2. Animals

Eight male Sprague-Dawley (SD) rats (220–240 g) were purchased from Beijing Weitong Lihua Biotechnology Co., Ltd. (Beijing, China). The animals were housed individually under a constant temperature of 22 ± 1 °C and humidity of 50 ± 10%. The rats were randomly divided into two groups: Group A (n = 4), drug group for plasma, urine, and tissues; Group B (n = 4), control group for blank plasma, urine, and tissues. They were fasted for 12 h with free access to water prior to experiments. 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 strictly consistent with the Guide for the Care and Use of Laboratory Animals (U.S. National Research Council, 1996).

2.3. Drug administration and biological samples preparation

Baicalin was suspended in 0.5% carboxymethylcellulose sodium (CMC-Na) aqueous solution and orally administered to rats of Group A at a dose of 400 mg/kg body weight. A 2 mL aliquot of 0.5% CMC-Na aqueous solution was administrated to each rat in Group B. 0.8 mL blood samples were withdrawn from the suborbital venous plexus of rats at 0.5, 1, 2, and 4 h post-dosing. Each sample was transferred to a heparinized micro-centrifuge tube and centrifuged at 14,000 rpm for 10 min to obtain plasma. The organs including heart, liver, spleen, lung, kidney, and brain, were respectively removed from two rats in Group A and Group B at 4 h post-dosing and washed with cold biological saline. Homogenates of heart, liver, spleen, lungs, kidneys, and brain were prepared by a homogenizer (DY89-11, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) at 1000 rpm for 3 min at 4 °C, which were suspended in biological saline at a ratio of 1.0 g of tissue to 5 mL of biological saline. Then the homogenates were centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was separated out and processed using the below described method. Urine samples were collected over 0–24 h after the oral administrations (two rats in each group were sacrificed at 4 h). Finally, all biological samples from the same group were merged into one sample.

All the biological samples were prepared by a solid-phase extraction (SPE) method. An SPE cartridge was pretreated with 5 mL of water, 5 mL of methanol and 5 mL of water, successively. 1 mL sample of plasma or urine or the tissue solution was vortexed, loaded, and allowed to flow through the SPE cartridge with gravity, respectively. The SPE cartridge was washed with 5 mL of water and 3 mL of methanol, successively. The methanol eluate was collected and evaporated to dryness under N_2 at room temperature. The residue was re-dissolved in 100 μL of acetonitrile/water (10:90, v/v) and centrifuged at 14,000 rpm for 10 min. A volume of 5 μL supernatant was injected into UPLC-ESI-LTQ-Orbitrap MS for analysis.

2.4. UPLC analysis

An Accela 600 pump LC system (Thermo Scientific, Bremen, Germany) was used equipped with a binary pump and an autosampler. A Waters ACQUITY BEH C_{18} column (2.1 × 100 mm i.d., 1.7 μm) was used for separation of the metabolites at room temperature. Acetonitrile/methanol 3:1 (solvent B) and 0.5% formic acid aqueous solution (solvent A) were used as mobile phase. The flow rate was 0.3 mL/min applied with a linear gradient as follows: 0–15 min, 10–45% B; 15–18 min, 45–68% B; 18–20 min, 68–70% B; 20–21 min, 70–10% B; 21–24 min, 10% B.