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Simultaneous determination of fourteen compounds of *Hedyotis diffusa Willd* extract in rats by UHPLC–MS/MS method: Application to pharmacokinetics and tissue distribution study

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ABSTRACT

A rapid, sensitive and selective ultra high-performance liquid chromatography-tandem mass spectrometry UHPLC–MS/MS method has been developed and validated for the simultaneous determination of fourteen bioactive ingredients (gallic acid, geniposidic acid, protocatechuic acid, caffeic acid, ferulic acid, scopoletin, apigenin-7-*o*-glucuronide, daidzein, apigenin, ursolic acid, oleanolic acid, β-sitosterol, coniferin, and stigmasterol) in the plasma and tissues of rats. Danshensu and icariin were used as internal standards (IS1 and IS2). The chromatographic separation was achieved by using an Agilent ZORBAX RRHD Eclipse Plus C18 column (2.1 mm × 50 mm, 1.8 μm) with gradient elution using mobile phase, which consisted of 0.1% acetic acid water (solvent A) and methanol (solvent B) and pumped at a flow rate of 0.3 mL/min. Mass spectrometric detection was performed in multiple reaction monitoring (MRM) mode utilizing electrospray ionization (ESI) in positive and negative mode. The plasma samples were pretreated via protein precipitation with 300 μL of methanol containing 0.1% (v/v) formic acid and organ homogenates were processed by solid-phase extraction (SPE) with Waters Oasis HLB 3 cc (60 mg), respectively. The intra- and inter- day precisions (RSD%) were less than 10.3%, while the accuracy was ranged from –7.34% to 9.10%. Extraction recovery ranged from 85.02 to 112.0% and the matrix effects ranged from 85.12% to 109.6%. The present method exhibited excellent linearity and the lower limits of quantification (LLOQ) were 30.0 ng/mL, 15.0 ng/mL, 80.0 ng/mL, 30.0 ng/mL, 10.0 ng/mL, 3.0 ng/mL, 2.5 ng/mL, 2.5 ng/mL, 1.5 ng/mL, 15.0 ng/mL, 75.0 ng/mL, 15.0 ng/mL, 30.0 ng/mL, and 20.0 ng/mL for gallic acid, protocatechuic acid, geniposidic acid, caffeic acid, ferulic acid, scopoletin, apigenin-7-*o*-glucuronide, daidzein, apigenin, ursolic acid, oleanolic acid, β-sitosterol, coniferin, and stigmasterol, respectively. This analytical method was verified by the FDA guidelines for bioanalytical method validation and applied to investigate the pharmacokinetics and biodistribution of fourteen constituents of *Hedyotis diffusa Willd* extract in rats. These results provide useful information for improving the pharmacokinetics and biodistribution of fourteen bioactive ingredients of *Hedyotis diffusa Willd* extract in SD rats, supporting additional clinical application and Chinese herbal medicine safety evaluations.

1. Introduction

Hedyotis diffusa Willd (*H. diffusa*), a traditional Chinese medicine, has been commonly used in folk medicines and widely distributed in east and Southeast Asia [1], such as China, Japan and Indonesiahas [2,3]. In addition, modern research have shown that the chemical constituents of *Oldenlandia diffusa* not only contain anthraquinones, terpenes, flavonoids, organic acids, alkaloids, sterols, alkanes, polysaccharides, hedyotis diffusa, cardiac glycosides, and other ingredients, but also involve some trace elements and amino acid [4–6]. In vitro and in vivo studies shown these phytochemicals and plant extracts exhibit a range of pharmacological activities of anti-cancer, anti-fibroblast, immunomodulatory, and neuroprotective effects [7–10], for example, treatment of antioxidant, anti-inflammatory, hepatitis, tonsillitis, sore throat,

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appendicitis, urethral infection, and malignant tumors of the liver, lung, and stomach [3,5,7,10,11]. Up to now, three major classes of compounds, including iridoid glucosides, flavonoids, and anthraquinones, have been reported as bioactive compounds of this herb [12–17]. Phytochemical investigations of this plant revealed that it contained gallic acid, geniposidic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, scopoletin, hesperidin, apigenin-7-*o*-glucuronide, quercitrin, daidzein, luteolin, apigenin, ursolic acid, oleanolic acid, β-sitosterol, coniferin, stigmasterol, and others, which are strikingly represented various of pharmacological characters [4,17–19]. Such as, gallic acid is helpful for the treatment of lung cancer [14], ferulic acid has anti-inflammatory effects [19], the iridoids geniposidic acid, ursolic acid, and oleanolic acid have anti-oxidant, anti-inflammatory, anti-liver cancer, and blood pressure-lowering effects [4,6,7,12]. According to reports in the literature, flavonoids and sterols in the extract of *Hedyotis diffusa Willd* have anti-breast cancer and anti-inflammatory effect [4,20,31,32]. In addition, phenolic acids are very common and important secondary metabolites in nature.

In recent years, the research of pharmacokinetics has been highly valued which not only can explain the absorption, distribution, metabolism, and excretion of drugs, but also provide the understanding of the mechanisms of action and the causes of toxicity. The commonly used research methods for the pharmacokinetics include blood concentration and biological effect. For instance, the pharmacokinetics of a novel anticancer AMPK activator hernandezine, icaritin, and Yinchenzhufu decoction in rats were validated via UHPLC–MS/MS [21–24]. The same as to other Chinese herbal medicine, many reports have been published to focus on pharmacological activities of *Hedyotis diffusa Willd*, owing to its mild effectiveness and low toxicity [10].

To our knowledge, the quantitative detection of pharmacokinetics and biodistribution of fourteen compounds (gallic acid, geniposidic acid, protocatechuic acid, caffeic acid, ferulic acid, scopoletin, apigenin-7-*o*-glucuronide, daidzein, apigenin, ursolic acid, oleanolic acid, β-sitosterol, coniferin, and stigmasterol) of *Hedyotis diffusa Willd* extract in the plasma and tissues of rats have not been reported. Hence, it is necessary to establish the rapid and accurate approaches to investigate its pharmacokinetics and biodistribution. UHPLC–MS/MS, is one of dominant tools to apply in pharmaceutical analysis, clinical diagnostics, and environmental monitoring, which has high separation efficiency, high sensitivity, high accuracy, and short analysis time [21–24].The aim of the present investigation is to develop a reliable and sensitive method based on UHPLC–MS/MS to quantify the fourteen constituents of *Hedyotis diffusa Willd* extract distribution in the plasma and organs of rats. It is the first time to study the simultaneous determination of fourteen constituents of *Hedyotis diffusa Willd* extract in rats by UHPLC–MS/MS. Validation of the method was based on the US FDA guideline [25].

2. Material and methods

2.1. Chemicals and reagents

Reference standards of gallic acid and caffeic acid were provided from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). The standards of geniposidic acid, daidzein, scopoletin, apigenin-7-*o*-glucuronide, apigenin, and protocatechuic acid, were purchased from Chengdu Pufeide Biological Technology Co., Ltd (Sichuan, China). The standards of oleanolic acid, ursolic acid, stigmasterol, β-Sitosterol, and the internal standard danshensu (IS1) were purchased from National Institute for Food and Drug Control (Beijing, China). The standards of coniferin, ferulic acid, and the internal standard icariin (IS2) were supplied from Sichuan Weikeqi Biological Technology Co., Ltd. (Si chuan, China). All standards, which the purity was more than 98.0%, were suitable for UHPLC–MS/MS analysis. The structure of analytes and IS are presented in Fig. 1. Acetonitrile and formic acid of HPLC grade were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Methanol and acetic acid of HPLC grade were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade. Ultra-pure water used in the experiment was prepared by a Milli-Q water purification system (Millipore, Billerica, USA). Solid-phase extraction columns (SPE) Waters Oasis HLB 3 cc (60 mg) and Oasis MAX 3 cc (60 mg) were purchased from Waters (Wexford, Ireland). Agilent Bond Elut 1cc (50 mg) C18 were obtained from Agilent (Agilent Co., USA).

2.2. Preparation of H. Diffusa extract

The dried herb of *Hedyotis diffusa Willd* (Batch No: 160801), purchased from Guangxi Jizhao Pharmaceutical Co., Ltd (Nanning, China), which was crushed to a uniform size, then passed through a 50-mesh sieve. 360 g of powder was accurately weighed and soaked in methanol (1:50, w/v) for 12 h, then extracted by ultrasound (200 W, 40 kHz, 45 °C) for 4 h. The mixed extract was placed in centrifugate at 4000 rpm for 20 min at 4 °C. The supernatant was evaporated to dryness using the evaporator at a constant temperature of 40 °C. To calculate the administered dose, the contents of forteen ingredients in *Hedyotis diffusa Willd* extract were measured quantitatively under the optimized conditions of UHPLC–MS/MS. The contents of gallic acid, geniposidic acid, protocatechuic acid, caffeic acid, ferulic acid, scopoletin, apigenin-7-*o*-glucuronide, daidzein, apigenin, ursolic acid, oleanolic acid, β-sitosterol, coniferin, and stigmasterol were 0.0776, 2.466, 0.561, 0.615, 1.362, 0.528, 0.0778, 0.0258, 0.0543, 0.3414, 4.929, 0.201, 0.164, and 0.131 mg/g, which were extracted from the herbal medicine, respectively.

2.3. Animal treatments and pharmacokinetic analysis

2.3.1. Animal

All procedures involving animals were approved by the Laboratory Animal Care Committee of Guangxi Department of Science and Technology. Twenty–four healthy Sprague–Dawley (SD) rats weighing 260 ± 20 g were supplied by the Experimental Animal Center of Guangxi Medical University (Nanning, China). The rats were housed individually in cages in an air-conditioned environment (temperature 25 °C, relative humidity 50–60%) with a natural light–dark cycle and allowed free access to food and water. Twenty–four SD rats were randomly divided into two groups and fasted overnight with free access to water prior to oral administration.

2.3.2. Oral administration of H. Diffusa extract

The twenty–four healthy SD rats received a single 4.837 g/kg dose of *H. diffusa* extract (equivalent to 4.477 mg/kg of gallic acid, 142.3 mg/kg of geniposidic acid, 32.38 mg/kg of protocatechuic acid, 35.49 mg/kg of caffeic acid, 78.58 mg/kg of ferulic acid, 30.45 mg/kg of scopoletin, 4.491 mg/kg of apigenin-7-*o*-glucuronide, 1.645 mg/kg of daidzein, 3.135 mg/kg of apigenin 19.69 mg/kg of ursolic acid, 284.3 mg/kg of oleanolic acid, 11.56 mg/kg of β-sitosterol, 9.466 mg/kg of coniferin, and 7.545 mg/kg of stigmasterol). Two parallel oral administration groups which one used for blood collection and the other one for organ tissues collection. Extract of *H. diffusa* was dissolved in normal saline which used to intragastric dose for rats. After administration, blood samples (about 250 μL) were taken at 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h by ocular fundus veins of rats. The plasma samples were immediately cen-