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Simultaneous separation and determination of four phenylethanoid glycosides in rat plasma sample after oral administration of *Cistanche salsa* extract by microemulsion liquid chromatography

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ABSTRACT

A simple, rapid and specific method was developed to separate as well as to determine the four phenylethanoid glycosides (PhGs) (echinacoside, tubuloside B, acteoside and isoacteoside) in rat plasma after oral administration of *Cistanche salsa* extract by reversed phase high performance liquid chromatography using a microemulsion as the mobile phase. The separations were performed on a Zorbax Extend-C_{18} column at 25 °C. Photodiode-array detector was conducted at 322 nm and with a flow rate of 0.8 mL min^{-1}. The optimized microemulsion mobile phase consisted of 0.3% triethylamine in 20 mM phosphoric acid at pH 6.0, 0.8% (v/v) ethyl acetate as oil phase, 1.5% (v/v) Genapol X-080 as surfactant, 2.5% (v/v) *n*-propanol as co-surfactant. Under the optimal conditions, the calibration curve for four PhGs was linear in the range of 10–1000 ng mL^{-1} with the correlation coefficients greater than 0.9994. The intra-day and inter-day precision (RSD) were below 8.64% and the limits of detection (LOD) for the four PhGs were 0.4–1.3 ng mL^{-1} (S/N = 3). The microemulsion liquid chromatography (MELC) method was successfully applied to separate and determine the four PhGs in rat plasma after oral administration of *C. salsa* extract.

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1. Introduction

The stem of *Cistanche salsa*, a parasitic plant native to northwest China, is a kind of traditional Chinese herbal medicine and used for the treatment of kidney deficiency, female infertility, morbid leucorrhea, neurataxia as well as senile constipation [1]. The phytochemical investigations showed that Echinacoside (ECH), Tubuloside B (TUBB), Acteoside (ACT) and Isoacteoside (ISO) are the four main Phenylethanoid glycosides of *C. salsa* extract, which were regard as the main active and marker components [2].

Quantification and pharmacokinetics studies on constituents of Traditional Chinese Medicine in plasma are required to offer suitable references in clinical application. Several methods, such as high-performance liquid chromatography (HPLC) [3–10] have been reported for the analysis of the four PhGs in a variety of sample matrices. However, to the best of our knowledge, there has been no publication on the simultaneous separation and determination of the four PhGs in plasma sample.

In HPLC analysis it has always been important to minimize the sample preparation in order to eliminate all the possible errors and losses. A direct injection of biological sample is preferred whenever possible. However, the presence of organic solvents in the conventional mobile phase in the concentration above 5% tend to precipitate proteins and makes direct HPLC analysis impossible. At the same time, the conventional HPLC methods may cause an environmental problem because of the large amounts of organic solvents needed. For overcoming these analytical problems in liquid chromatography, the microemulsion as mobile phase was employed in past few years.

Microemulsion is liquid disperse systems containing organic solvent (oil), water, surfactant and co-surfactant [11]. It can be formed spontaneously, constituting thermodynamically stable dynamic structures with the appearance of clear transparent liquids of relatively low viscosity. Many substances of hydrophilic and hydrophobic character can solve in microemulsion. In comparison to conventional hydro-organic mobile phase, the phases used in microemulsion liquid chromatography are less flammable,

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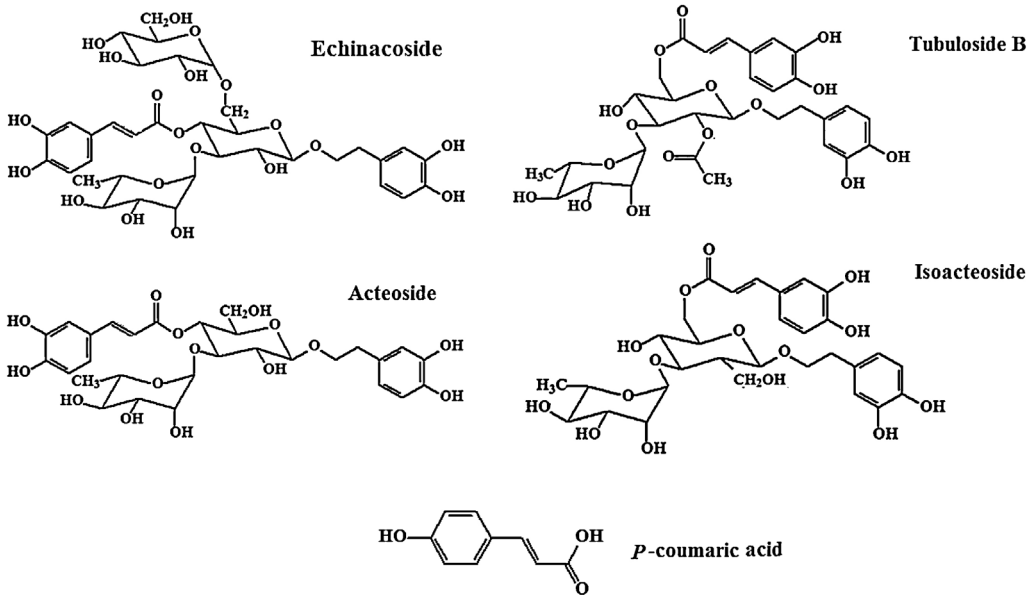

Fig. 1. Chemical structures of ECH, TUBB, ACT, ISO and IS.

inexpensive, non-toxic and biodegradable. Both hydrophilic and hydrophobic samples are often easily dissolved in these phases. Solutes with different polarities can be separated in a single run owing to the interactions with the stationary and mobile phase components, then the separations being highly reproducible [12]. Microemulsion is classified as either oil-in-water (O/W) or water-in-oil (W/O), where the O/W microemulsion is the preferred for HPLC [13]. Recently, it has been proved that the use of microemulsions as mobile phases in HPLC is feasible and provides selectivity and separation efficiency comparable to or greater than those of conventional HPLC systems [13–18]. The advantage of the microemulsion systems which has also been proved for MELC is short retention time and low cost as it avoids procedures such as protein precipitation used in advance of a conventional HPLC analysis [19].

In the present work, we investigated the possibility of separation and determination of the four PhGs in rat plasma after oral administration of *C. salsa* extract by reversed phase high performance liquid chromatography using a microemulsion as the mobile phase. Moreover, the effect of operating parameters on the separation and determination performance was studied.

2. Experimental

2.1. Chemicals and reagents

ECH ($C_{35}H_{46}O_{20}$, MW = 786.73), TUBB ($C_{32}H_{40}O_{16}$, MW = 680.65), ACT ($C_{29}H_{36}O_{15}$, MW = 624.59), ISO ($C_{29}H_{36}O_{15}$, MW = 624.59) and *p*-coumaric acid (internal standard) (IS) ($C_9H_8O_3$, MW = 164.16) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Their structures are shown in Fig. 1. Non-ionic surfactant $C_{13}E_8$ (Genapol X-080) was obtained from Sigma (St. Louis, MO, USA) and used without further purification. Various concentrations (v/v) of aqueous surfactant solutions were prepared by weighing certain amounts of the surfactant and by directly dissolving the surfactant in distilled water. Heptanes, ethyl acetate, *n*-octanol, toluene, *n*-hexane and *n*-propanol were obtained from Merck (Darmstadt, Hessen, Germany). Phosphoric acid and triethylamine (Beijing Chemical Factory, AR Beijing, China) were prepared before the experiment. All other reagents used in this work were of analytical grade. Distilled water (Millipore, Bedford, MA, USA) was used throughout the study.

2.2. Preparation of standard solutions

Stock solutions (50 μg mL^{-1}) of the four PhGs and IS solutions (10.0 μg mL^{-1}) were got by dissolving suitable amounts of each pure substance in microemulsion mobile phase and kept stable for 2 months when stored at 4 °C in the refrigerator (assessed by HPLC).

2.3. Preparation of C. salsa extract

The crude drug of *C. salsa* was purchased from local drug stores. Fifty grams of crude drug of *C. salsa* were extracted twice by refluxing with 75% ethanol for 1.5 h, and the extract solution was filtered. The filtered extract was then concentrated under reduced pressure and lyophilized to give an extract (4.26 g), which was stored at 4 °C before use.

In order to calculate the administered dose of four PhGs, their contents in *C. salsa* extract were quantitatively determined. The extract of *C. salsa* was dissolved in microemulsion mobile phase and diluted to the concentration of 225 ng mL^{-1}. Then 10 μL of this solution was injected into HPLC system for analysis. The contents of four PhGs (ECH, TUBB, ACT and ISO) in the extract of *C. salsa* were determined to be 12.94, 1.23, 6.75 and 3.47%, respectively.

2.4. Application of the method

Sprague-Dawley male rats (200 ± 20 g) were purchased from the Experimental Animal Center of Fourth Military Medical University (Xi'an, China). The animals were pathogen-free and housed in an environmentally controlled breeding room (temperature maintained at 24 ± 1 °C and a 12:12 h light–dark cycle) for at least 1 week before experimentation. Standard laboratory food and water were available at all times except that food was withdrawn 18 h prior to initiation of the experiments. Before drug administration, the animals were fasted overnight. All animal procedures were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China.

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