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Separation and purification of verticine and verticinone from *Bulbus Fritillariae Thunbergii* by high-speed counter-current chromatography coupled with evaporative light scattering detection

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Abstract

High-speed counter-current chromatography (HSCCC) coupled with evaporative light scattering detection (ELSD) was successfully applied to preparative separation and purification of verticine and verticinone from crude extracts of *Bulbus Fritillariae Thunbergii* by a one-step separation, using chloroform–ethanol–0.2 mol L^{-1} hydrochloric acid (3:2:2, v/v/v) as a solvent system. HPLC analysis of the fractions collected on the preparative HSCCC of 200 mg of crude extracts showed that the purity of verticine (25.6 mg) was 96.8% and that of verticinone (10.3 mg) was 95.4%. The chemical identities of these components were confirmed by ^{1}H NMR and EI–MS.

Keywords: Counter-current chromatography; *Bulbus Fritillariae Thunbergii*; Verticine; Verticinone; Evaporative light scattering detection

1. Introduction

Bulbus Fritillariae Thunbergii (Chinese name zhebeimu) is a famous Chinese medicine, which is derived from the bulbs of the genus *Fritillaria thunbergii* Miq. (Liliaceae) [1]. It has been used as one of the most important antitussive and expectorant drugs for more than 2000 years [2]. Extensive chemical studies have been conducted by many research groups [3–6]. A number of ingredients were found in *Bulbus Fritillariae Thunbergii* including isosteroidal alkaloids, steroidal alkaloids and non-alkaloids. Furthermore, pharmacological studies demonstrate that isosteroidal alkaloids are the primary active ingredients responsible for the antitussive activity. Among these alkaloids, verticine and verticinone (Fig. 1) are the most representative [7].

The conventional method of purifying verticine and verticinone was to utilize column chromatography, which required several steps, and resulted in low recovery yields [8]. High-speed counter-current chromatography (HSCCC), being as a support free liquid–liquid partition chromatography, eliminates irreversible adsorption of sample onto the solid support. This method has been successfully applied to the separation and purification of various natural products [9–12], and several reports have tried the HSCCC coupled with evaporative light scattering detection (ELSD) [13]. However, few studies have been focus on *Bulbus Fritillariae Thunbergii*. In this paper, high-speed counter-current chromatography is coupled with evaporative light scattering detection for separation and purification of verticine and verticinone from the extracts of *Bulbus Fritillariae Thunbergii* by a one-step HSCCC separation.

2. Experimental

2.1. Materials

Bulbus Fritillariae Thunbergii (Zhejiang, China) was purchased from Shanghai Kangqiao Medical Factory, authenticated by Shanghai Chinese Traditional Medicine Research Institute and fitted for Chinese Pharmacopoeia. All solvents used for the preparation of crude extracts and HSCCC separation were of

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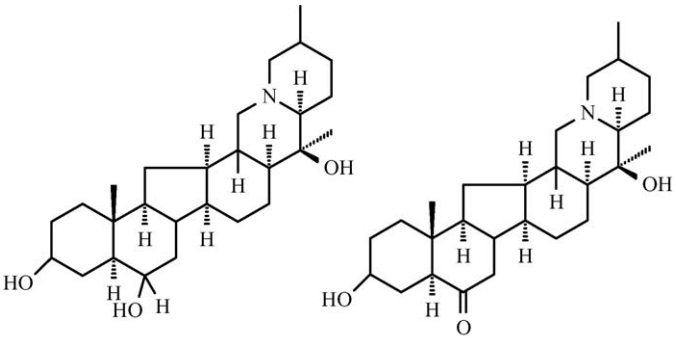

Fig. 1. Chemical structures of verticine and verticinone.

analytical grade (Chinese Medication Group Shanghai Chemical Reagent Company, Shanghai, China). Methanol (Merck), acetonitrile (Merck) and pure water used for HPLC was treated with SAGA-30D Super Pure Water System (Saga Electronic Technology, Co., Ltd., Shanghai, China). Verticine and verticinone standard samples were purchased from National Institute for the Control of Pharamaceutical and Biological Products, Ministry of Health, Beijing, China.

2.2. Preparation of crude samples

The 100 g dried *Bulbus Fritillariae Thunbergii* was ground to powder (60 mesh) by a disintegrator (Shanghai Experimental Instrument Company, Shanghai, China) and loaded into microwave extractor (Model VIP 272, National Engineering Research Center for Chinese Traditional Medicine, Shanghai, China) and extracted at 680 W of microwave power in 90% ethanol (with a ratio of the powder to solvent 1:10) for 20 min. The extraction solution was filtered and concentrated to dryness by rotatory evaporator under reduced pressure and dried under vacuum (ZK 82J electrothermal vacuum desiccator, Shanghai Experimental Instrument Company). The residue was stored in a desiccator.

2.3. Selection of two-phase solvent system

The selection of the two-phase solvent system for the target compounds is the most important step in HSCCC, which may account for 90% of the entire work in HSCCC [14]. Two principles are generally followed. First, a suitable partition coefficient (K), which is the ratio of solute distributed between the mutually equilibrated two solvent phases. The suitable K-value for HSCCC are in the range of 0.5–1.0; second, higher retention of the stationary phase normally results in better peak resolution. If the setting time of the two-phase solvent system is less than 20 s, the solvent system would provide satisfactory retention of the stationary phase. A two-phase solvent system composed of chloroform–ethanol–0.2 mol L^{-1} hydrochloric acid (3:2:2, v/v/v) was chosen for the separation and purification of the crude extracts.

2.4. High-speed counter-current chromatography (HSCCC)

2.4.1. Instrumentation

The preparative HSCCC instrument (Model TBE-300A, Shanghai Tauto Biological Company, China) was equipped with three preparative coils connected in series (diameter of polytetrafluoroethylene (PTFE) tube, 2.6 mm; total volume, 119 mL) and a 10 mL sample loop. The revolution speed of the instrument is regulated with a speed controller in the range between 0 and 999 rpm. Constant temperature circulator (HX-1050, Beijing Boyikang Experimental Apparatus Company, China) was used to control the temperature. The solvent was pumped into the column with the AKTA purifier pump P-900 (Amersham, USA) at a flow rate of up to 10 mL/min and pressure up to 25 MPa. An optimum speed of 1.2 mL/min was used in the experiment. The continuous monitoring of the effluent was achieved with evaporative light scattering detection (Alltech ELSD 2000ES, Alltech, USA).

2.4.2. HSCCC separation procedure

The multiplayer coiled column was first fully filled with the upper phase (stationary phase) and lower phase (mobile phase) simultaneously at a flow rate of 10 mL/min. Then the lower phase alone was pumped at a flow rate of 1.2 mL/min. In the meantime, the HSCCC apparatus was rotated at 800 rpm, constant temperature was 25 °C. After 30 min, the lower phase emerged in the effluent and hydrodynamic equilibrium was established in the column. A sample solution containing 200 mg *Bulbus Fritillariae Thunbergii* extracts in 10 mL of the lower phase of the two-phase solvent system was injected through the injection valve. The effluent was continuously monitored by ELSD and each peak fraction was manually collected according to the chromatogram. After using thin layer chromatography (TLC) as a primary comparison with the standard sample of verticine and verticinone, HPLC analysis was carried out.

2.5. HPLC analysis, mass spectrometry and ^{1}H NMR identification of the fractions

HPLC analysis was performed with an Agilent/HP 1100 series (Agilent, USA) equipped with an Alltech ELSD 2000ES detector (Alltech USA). The Agilent/HP 1100 series HPLC was consisted of a vacuum degasser, quandary pump, thermostated column compartment, diode array detection and injection valve with a 20 μL loop. The crude extracts of *Bulbus Fritillariae Thunbergii* and each purified peak fraction from the preparative HSCCC separation were analyzed by HPLC (Agilent Exlipse XBD C_{18} column, 5 μm, 4.6 mm × 150 mm), eluted with acetonitrile–water–diethylamine (70:30:0.3, v/v/v) at a flow rate of 1.0 mL/min, and column temperature was 30 °C. ELSD parameters were as follows: drift tube temperature, 90 °C; gas flow, 2.4 L min^{-1}; impactor, off. The purified fractions of verticine and verticinone obtained from the preparative HSCCC separation were analyzed by electron impact mass spectroscopy (EI–MS) (GC-TOFMS, Micromass, UK) and ^{1}H NMR (Bruker Advance 500 MHz spectrometer referenced to tetramethylsilane), respectively.

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Table 1
The *K*-values of verticine and verticinone in different two-phase solvent systems

Solvent system	*K*-value	
	Verticinone	Verticine
n-Butanol–ethyl acetate–water (2:3:5, v/v/v)	18	15
n-Butanol–ethyl acetate–water (1:2:3, v/v/v)	16	14
n-Butanol–ethyl acetate–water (2:1:3, v/v/v)	16	15
n-Hexane–diethyl ether–ethanol–water (1:5:2:5, v/v/v/v)	1.68	3
Chloroform–ethanol–water (4:2:2, v/v/v)	1.67	2.06
Chloroform–ethanol–0.2 mol L^{-1} hydrochloric acid (2:3:2, v/v/v)	0.98	1.02
Chloroform–ethanol–0.2 mol L^{-1} hydrochloric acid (3:2:2, v/v/v)	0.87	1.16

Experimental procedure: 5 mL of each phase of the pre-equilibrated two-phase solvent system was poured in a 20 mL test tube and 10 mg of the sample was added. The tube was ultrasonic for 2 min and waited the solvent to separate completely. A 100 μL of each layer was taken out and evaporated. The residue was dissolved in 1 mL methanol and analyzed by HPLC for the *K*-value. The *K*-value was expressed as the peak area of target compound in the upper phase vs. in the lower phase.

3. Results and discussion

3.1. HSCCC separation of verticine and verticinone from the crude extract

In the HSCCC experiment, a good solvent system can provide an ideal partition coefficient (*K*) for the target compounds. The most suitable *K*-value of the target compounds is close to 1. In our experiment, two compounds want to be separated by one solvent system, so they must have different *K*-values. According to solubility of verticine and verticinone, preliminary HSCCC studies were carried out with seven different two-phase solvent systems, such as *n*-butanol–ethyl acetate–water (2:3:5, 1:2:3, 2:1:3, v/v/v), *n*-hexane–diethyl ether–ethanol–water (1:5:2:5, v/v/v/v), chloroform–ethanol–water (4:2:2, v/v/v) and chloroform–ethanol–0.2 mol L^{-1} hydrochloric acid (2:3:2, 3:2:2, v/v/v). The measured *K*-values are shown in Table 1. The two-phase solvent systems of *n*-butanol–ethyl acetate–water and *n*-hexane–diethyl ether–ethanol–water did not give effective separation. The chloroform–ethanol–water system gave good result, however, it also led to emulsification. In the subsequent studies, this system was replaced with chloroform–ethanol–0.2 mol L^{-1} hydrochloric acid. After comparison of different ratios, chloroform–ethanol–0.2 mol L^{-1} hydrochloric acid (3:2:2, v/v/v) gave the best separation.

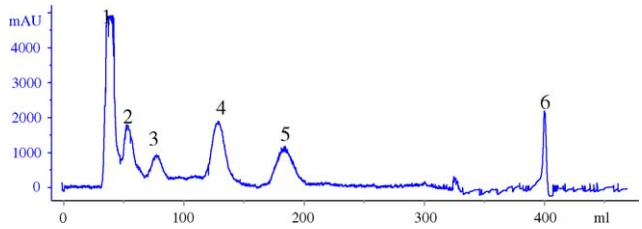

Fig. 2. Chromatogram of crude extracts of *Bulbus Fritillariae Thunbergii* by HSCCC. Peak 3, verticinone; peak 5, verticine. Experimental condition: two-phase solvent system; chloroform–ethanol–0.2 mol L^{-1} hydrochloric acid (3:2:2, v/v/v); stationary phase, upper phase; mobile phase, lower phase; flow-rate, 1.2 mL/min; rotary speed, 800 rpm; ELSD condition: drift tube temperature, 64.8 °C; gas flow, 2.1 L min^{-1}; impactor: off, sample size, 200 mg of crude extracts dissolved in 10 mL of the lower phase; retention of the stationary phase, 0.58; separation temperature, 25 °C.

Other factors such as the revolution speed of the separation column, the flow rate of the mobile phase and the separation temperature, were also investigated. Emulsification seemed to occur when the revolution speed is higher than 800 rpm. Ultimately, a flow rate of 1.2 mL/min, a revolution speed of 800 rpm and separation temperature of 25 °C gave the best separation. Fig. 2 showed the preparative HSCCC separation chromatogram. A 25.6 mg of verticine and 10.3 mg of verticinone were obtained from the 200 mg crude extracts.

3.2. HPLC analysis

The method of HPLC analysis was referenced in Chinese Pharmacopoeia (2005). The crude samples and peak fractions separated by HSCCC were analyzed by HPLC under the analytical conditions described in Section 2.5. The chromatograms

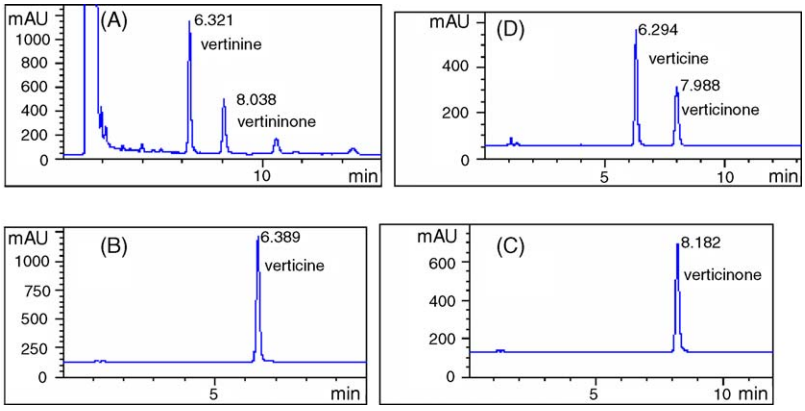

Fig. 3. HPLC chromatogram of crude extracts of *Bulbus Fritillariae Thunbergi* (A), peak 5 from preparative HSCCC (B), peak 3 from preparative HSCCC (C) and the standard sample of verticine and verticinone (D). Experimental condition: Aglient Exlipse XBD C_{18} column (5 μm, 4.6 mm × 150 mm); mobile phase, acetonitrile–water–diethylamine (70:30:0.3); flow rate, 1.0 mL/min; temperature, 30 °C; ELSD condition: drift tube temperature, 90 °C; gas flow, 2.4 L min^{-1}; impactor, off.

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were shown in Fig. 3. The purified samples of verticine and verticinone calculations were made by comparison of the peak area with the standard. The results showed that peak 3 corresponded to verticinone and peak 5 corresponded to verticine, and their purity were 95.4 and 96.8%, respectively.

3.3. Structural identification

The identities of peaks 3 and 5 in HSCCC were determined by electron impact mass spectroscopy (EI–MS) and ^{1}H NMR. Peak 3 ^{1}H NMR ($CDCl_3$) δ (ppm): 0.78 (s, 3H, 19-CH_3), 1.02 (s, 3H, 21-CH_3), 1.08 (d, $J = 7.0$ Hz, 3H, 27-CH_3), 3.58 (m, 1H, 3-CH); EI–MS: *m*/*z*, 429 (M^+), 414, 384, 372, 154, 124, 112 (100%). Peak 5 ^{1}H NMR ($CDCl_3$) δ (ppm): 0.82 (s, 3H, 19-CH_3), 1.05 (s, 3H, 21-CH_3), 1.10 (d, $J = 7.1$ Hz, 3H, 27-CH_3), 1.25 (m, 1H, 5-CH), 3.60 (m, 1H, 3-CH); EI–MS: *m*/*z*, 431 (M^+), 412, 386, 154, 112 (100%). Compared with the data given in reference [15], the compounds in peaks 3 and 5 were verticinone and verticine, respectively.

4. Conclusion

The overall results of our studies indicated that HSCCC coupled with evaporative light scattering detection was successfully used for separation and purification of verticine and verticinone from *Bulbus Fritillariae Thunbergii*. The present study also demonstrated that HSCCC coupled with ELSD is a powerful tool to monitor separation and purification of non-chromophoric active substances from natural medicines.

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