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Preparative isolation and purification of six diterpenoids from the Chinese medicinal plant *Salvia miltiorrhiza* by high-speed counter-current chromatography

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Abstract

A high-speed counter-current chromatography (HSCCC) method was developed for the preparative separation and purification of six diterpenoids, dihydrotanshinone I, cryptotanshinone, methylenetanshiquinone, tanshinone I, tanshinone IIA and danshenxinkun B from the Chinese medicinal plant *Salvia miltiorrhiza* Bunge. The crude diterpenoids were obtained by extraction with ethanol–*n*-hexane (1:1, v/v) from *S. miltiorrhiza* Bunge. Preparative HSCCC with the two-phase solvent systems A composed of *n*-hexane–ethanol–water (10:5.5:4.5, v/v) and B composed of *n*-hexane–ethanol–water (10:7:3, v/v) was successfully performed in a stepwise elution yielding six relatively pure diterpenoids from 300 mg of the crude extract in a single run. The purities of dihydrotanshinone I, cryptotanshinone, methylenetanshiquinone, tanshinone I, tanshinone IIA and danshenxinkun B were 88.1, 98.8, 97.6, 93.5, 96.8 and 94.3%, respectively.

Keywords: *Salvia miltiorrhiza*; Counter-current chromatography; Plant materials; Pharmaceutical analysis; Terpenoids; Tanshinones

1. Introduction

Dan-shen, the dried roots of *Salvia miltiorrhiza* Bunge, is one of the most popular traditional Chinese medicines and is officially listed in the Chinese Pharmacopoeia. It has been widely used for promoting blood circulation to remove blood stasis, clearing away heat, relieving vexation, nourishing blood and tranquilizing the mind and cooling blood to relieve carbuncles [1–4]. The major active constituents of this traditional Chinese medicine are tanshinones, a group of diterpenoids [5]. The chemical structures of six tanshinones are shown in Fig. 1.

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography that uses no solid support matrix. Therefore, it eliminates irreversible adsorptive loss of samples onto the solid support matrix used in the conventional chromatographic column. The method has been successfully applied to the analysis and separation of several natural products [6–10]. It was recently proposed for the separation and purification of tanshinones from the herbal medicine *S. miltiorrhiza* [11]. However, only three tanshinones were

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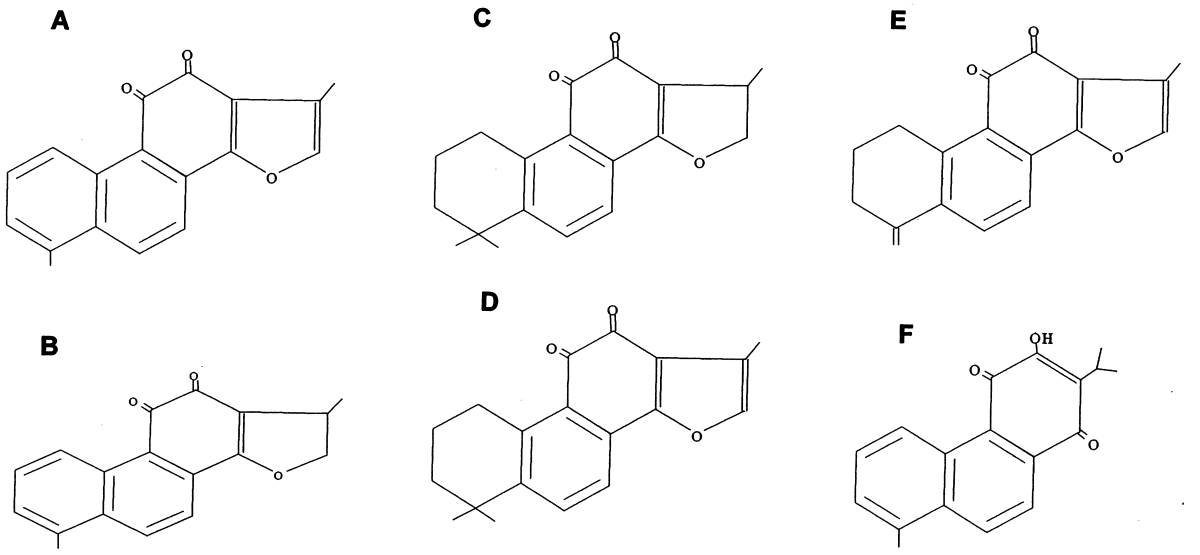

Fig. 1. Chemical structures of tanshinones. Structures of (A) tanshinone I; (B) dihydrotanshinone I; (C) cryptotanshinone; (D) tanshinone IIA; (E) methylenetanshiquinone; and (F) danshenxinkun B.

purified, and the sample size was small (only 50 mg) [11]. The purpose of this study was to develop a more efficient HSCCC method for the preparative separation and purification of diterpenoids from the medicinal plant *S. miltiorrhiza* Bunge on a relatively larger scale (300 mg).

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA). The apparatus consisted of three preparative coils, connected in series (diameter of tube=2.6 mm, total volume=325 ml). The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 7.5 cm, and the β value varied from 0.47 at the internal terminal to 0.73 at the external terminal ($\beta=r/R$ where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with a Model Series II HPLC pump (Pharma-Tech Research), a Model SPD-10Avp UV–Vis detector (Shimadzu, Japan), a Model L 120 E flat-bed recorder (Linseis, Germany), and a sample injection valve with a 10-ml sample loop.

2.2. Reagents

All solutions were prepared with analytical-grade compounds. Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. Ethanol, *n*-hexane and acetonitrile were obtained from BDH (Poole, UK). The tanshinone stock solutions were 0.100 mg ml^{-1} which were prepared by dissolving 1.00 mg of tashinones (obtained from various sources [12]) in 10.00 ml of the mobile phase and then stored in a refrigerator. The working solutions were prepared by suitable dilution of the stock solutions with the mobile phase. All other solutions were prepared by dissolving appropriate amounts of commercially available chemicals in water.

The dried roots of *S. miltiorrhiza* Bunge were purchased from a local drug store.

2.3. Preparation of crude diterpenoids from S. miltiorrhiza Bunge

The dried roots of *S. miltiorrhiza* Bunge were ground into powder. A 250 ml volume of *n*-hexane–

ethanol (1:1, v/v) was added to a bottle (800 ml) containing 100 g of the powder for the extraction of tanshinones, and the mixture was shaken. After 30 min, the mixture was separated by centrifugation at 10 000 *g* for 10 min, and the extract was collected. The extraction procedure was repeated until the extract was almost colorless, and all extracts were combined. Then, the extract was diluted 1:2 with water, and the two phases were separated with a separatory funnel. The organic phase (*n*-hexane phase) was washed with 30% aqueous ethanol until the water phase was almost colorless. After separation, the organic phase was evaporated to dryness by rotary vaporization at 40°C, and the dry extract (2.157 g) was stored in a refrigerator for the subsequent HSCCC separation.

2.4. Preparation of two-phase solvent system and sample solution

The following two two-phase solvent systems were prepared: (1) solvent system A: *n*-hexane–ethanol–water (10:5.5:4.5, v/v), and (2) solvent system B: *n*-hexane–ethanol–water (10:7:3, v/v). Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

The sample solution was prepared by dissolving the crude sample in the lower phase of solvent system B used for separation.

2.5. HSCCC separation procedure

Preparative separation was performed using a stepwise elution with solvent systems A and B in sequence as follows: the coiled column was first entirely filled with the upper phase of solvent system A. Then, the apparatus was rotated at 1000 rpm, while the lower phase of solvent system A was pumped into the column at a flow-rate of 2.0 ml min^{-1}. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 10 ml of the sample solution containing 300 mg of the crude tanshinones was injected through the injection value. The effluent of the column was continuously monitored with a UV–Vis detector at 280 nm. Peak fractions were collected according to the elution profile. After 260 min of elution with the lower phase of solvent system A, the mobile phase was switched to the lower phase of system B to complete the separation.

2.6. HPLC analysis

The crude sample and each peak fraction obtained by HSCCC were analyzed by high-performance liquid chromatography (HPLC) according to Chen and Chen [12]. The HPLC system used throughout this study consisted of a Waters 510 pump (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20-μl loop, and a Waters 996 photodiode array detector. Evaluation and quantification were made on a Millenium chromatography data system (Waters). The column used was a reversed-phase Ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm, Beckman, Fullerton, CA, USA). The mobile phase was solvent A (0.075% aqueous trifluoroacetic acid) and solvent B (acetonitrile) in the gradient mode as follows: 0–5 min, 0% B; 5–25 min, 0–70% B; 25–40 min, 70% B; 40–41 min, 70–0% B. The flow-rate was 1.0 ml min^{-1}, and the effluent was monitored at 280 nm.

3. Results and discussion

Fig. 2 shows the HPLC chromatogram of the crude tashinones from *S. miltiorrhiza* Bunge.

Preliminary HSCCC studies were carried out with the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water. It required a long elution time to elute tashinones with the two-phase solvent system at ratios 5:5:5:5 and 7:3:7:3, respectively. In subsequent studies, another two-phase solvent system was tested.

Performance of the two-phase solvent system composed of *n*-hexane–ethanol–water at various volume ratios (10:5:5, 10:5.5:4.5 and 10:6:4) was evaluated in terms of peak resolution. It required a long elution time to elute all tashinones with the two-phase solvent system at ratios 10:5:5 and 10:5.5:4.5. When the two-phase solvent system at ratio 10:6:4 was used, the resolution of the former several peaks was not satisfactory, and it also required a long elution time to elute all tashinones. In subsequent studies, a stepwise elution was tested

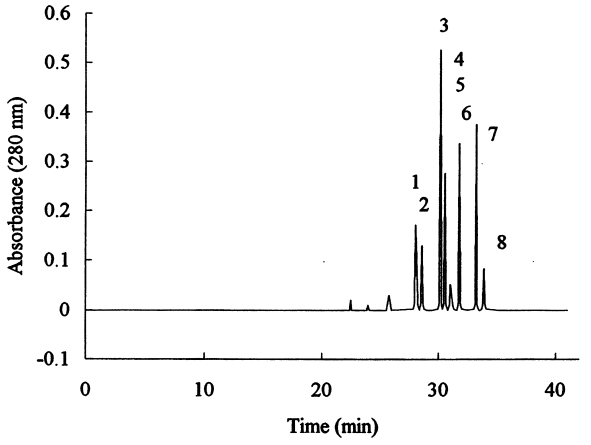

Fig. 2. Chromatogram of crude tanshinones from *Salvia miltiorrhiza* Bunge by HPLC analysis. Peaks: 1=dihydrotanshinone I, 2=unknown component, 3=cryptotanshinone, 4=tanshinone I, 5=unknown component, 6=methylenetanshiquinone, 7=danshenxinkun B, and 8=tanshinone IIA. Conditions: column: reversed-phase Ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm); mobile phase: solvent A (0.075% aqueous trifluoroacetic acid) and solvent B (acetonitrile) in gradient mode; flow-rate: 1.0 ml min^{-1}; detection at 280 nm.

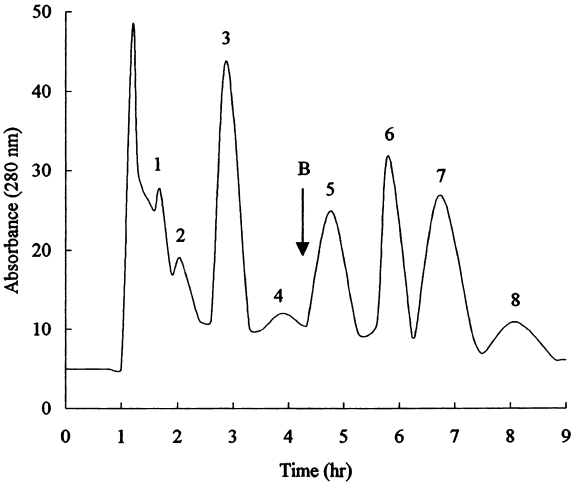

Fig. 3. Chromatogram of crude tanshinones from *Salvia miltiorrhiza* Bunge by HSCCC separation using stepwise elution with solvent systems A and B. Peaks: 1=dihydrotanshinone I, 2=unknown component, 3=cryptotanshinone, 4=tanshinone I, 5=unknown component, 6=methylenetanshiquinone, 7=danshenxinkun B, and 8=tanshinone IIA. Conditions: column: multilayer coil of 2.6 mm I.D. PTFE tube with a total capacity of 325 ml: rotary speed: 1000 rpm; stationary phase: the upper phase of solvent system A; mobile phase: 0–260 min, the lower phase of solvent system A, and 260–560 min, the lower phase of solvent system B; flow-rate: 2 ml min^{-1}; detection at 280 nm; sample size: 300 mg; retention of the stationary phase: 59%.

with solvent systems A and B as follows: the stationary phase was the upper phase of solvent system A, and the mobile phase was first the lower phase of solvent system A. After some time, the mobile phase was switched to the lower phase of system B to complete the separation.

When the two-phase solvent systems A composed of *n*-hexane–ethanol–water at a ratio of 10:4:6 and B composed of *n*-hexane–ethanol–water at a ratio of 10:6:4 were used in stepwise elution after 490 min, it took a long time (about 12 h) to elute all tashinones. When the two-phase solvent systems A at a ratio of 10:4:6 and B at a ratio of 10:7:3 were used in stepwise elution after 170 min, the resolution of the last several peaks was not good. When the two-phase solvent systems A at a ratio of 10:5.5:4.5 and B at a ratio of 10:7:3 were used in stepwise elution after 260 min, all tashinones could be eluted out in 9 h, and the peak resolution was satisfactory. Fig. 3 shows the preparative HSCCC separation of 300 mg of the crude sample using the solvent systems A at a ratio of 10:5.5:4.5 and B at a ratio of 10:7:3 after 260 min in a stepwise elution. HPLC analysis of each peak fraction of this preparative HSCCC revealed that six relatively pure tashinones could be obtained from the crude extract in a one-step separation. The purities of dihydrotanshinone I, peak 2 (unknown component), cryptotanshinone, tanshinone I, peak 5 (unknown component), methylenetanshiquinone, danshenxinkun B and tanshinone IIA were 88.1, 89.2, 98.8, 93.5, 95.1, 97.6, 94.3 and 96.8%, respectively. The HPLC chromatograms of tashinones as purified from the preparative HSCCC are shown in Fig. 4.

In conclusion, a method for the preparative separation and purification of six diterpenoids from the Chinese medicinal plant *S. miltiorrhiza* Bunge was developed by preparative HSCCC. The six relatively pure tanshinones were obtained from 300 mg of the crude extract in a one-step separation. Compared with the previous method [11], the method developed in the present study could be used to obtain more tanshinones (six known and two unknown compounds) in larger amounts in a single run.

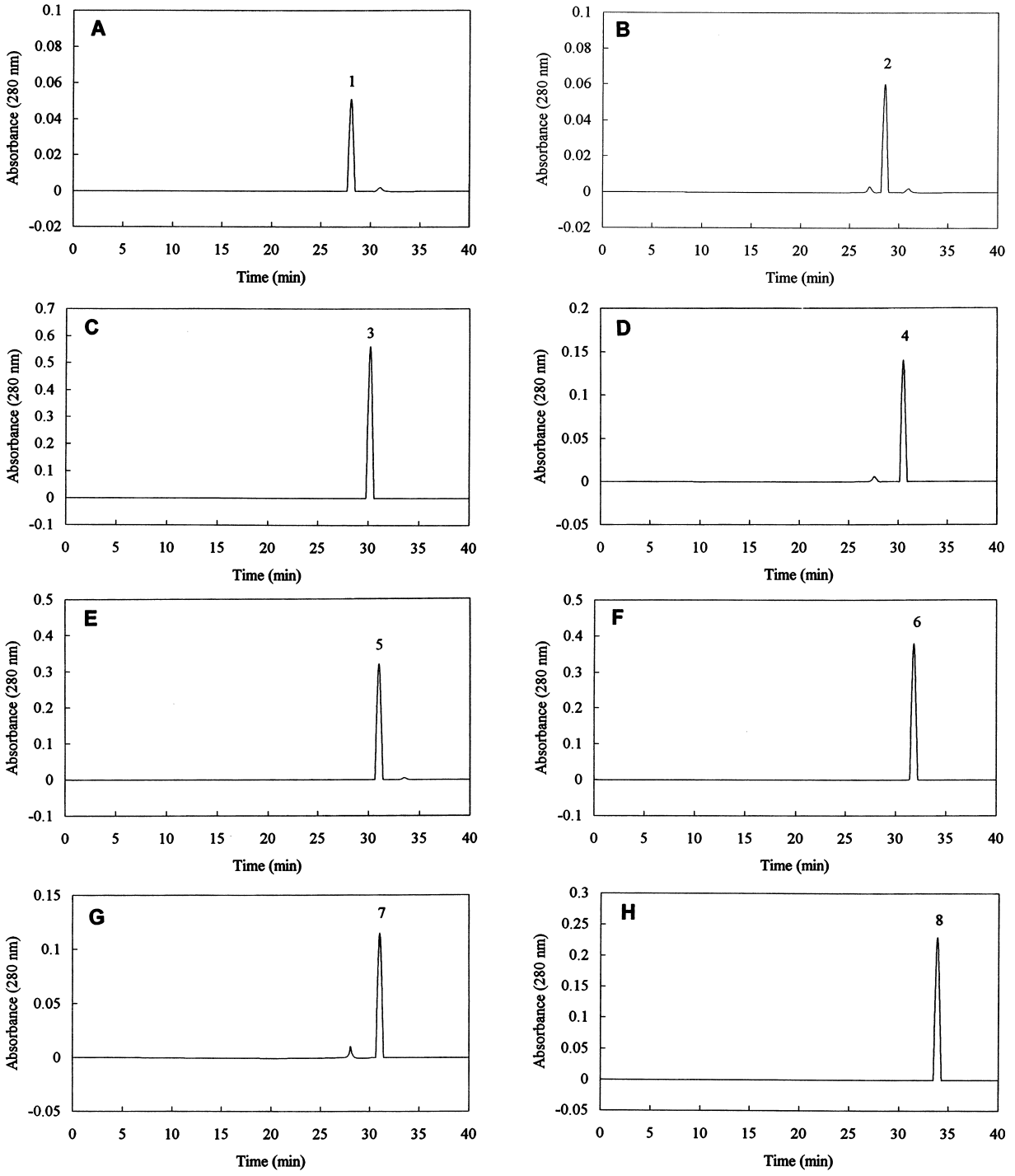

Fig. 4. HPLC chromatogram of tashinones purified from *Salvia miltiorrhiza* Bunge. Peaks: (A) 1=dihydrotanshinone I, (B) 2=unknown component, (C) 3=cryptotanshinone, (D) 4=tanshinone I, (E) 5=unknown component, (F) 6=methylenetanshiquinone, (G) 7=danshenxinkun B, and (H) 8=tanshinone IIA. Conditions: column: reversed-phase Ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm): mobile phase: solvent A (0.075% aqueous trifluoroacetic acid) and solvent B (acetonitrile) in gradient mode; flow-rate: 1.0 ml min^{-1}; detection at 280 nm.

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