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Short communication

Separation of tanshinones from *Salvia miltiorrhiza* Bunge by high-speed counter-current chromatography using stepwise elution

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Abstract

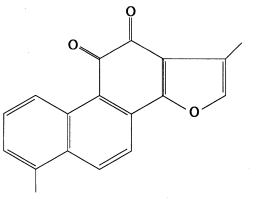
High-speed counter-current chromatography (HSCCC) was successfully used for isolation and purification of tanshinones from the roots of *Salvia miltiorrhiza* Bunge by stepwise elution. A set of three solvent systems and other experimental conditions were determined by analytical HSCCC. Using the optimized conditions, the preparative HSCCC separation was performed on 50 mg of crude light petroleum extract yielding pure tanshinones of tanshinone IIA (7 mg), tanshinone I (3 mg) and cryptotanshinone (4 mg) all at purities of over 95% in a single run.

Keywords: *Salvia miltiorrhiza*; Counter-current chromatography; Plant materials; Preparative chromatography; Tanshinones

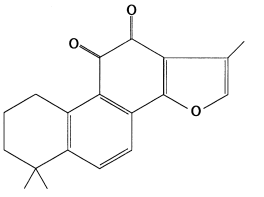
1. Introduction

Salvia miltiorrhiza Bunge, Dan-Shen in Chinese, is a well-known traditional Chinese medicinal herb used for treatment of various kinds of diseases especially for coronary disorders [1]. The major active constituents of this herb are tanshinones, including tanshinone I, tanshinone IIA and cryptotanshinone. Pharmacological tests revealed that all these components have an anti-cancer effect [2], and among these tanshinones, tanshinone IIA is most effective and has been used as a quality controller for some medicine. The chemical structures of these tanshinones are given in Fig. 1.

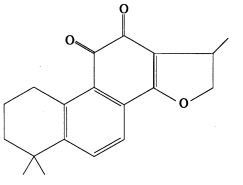
The separation and purification of tanshinones



tanshinone I



tanshinone II A



cryptotanshinone

Fig. 1. The chemical structures of tanshinones.

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using conventional methods such as column chromatography requires several steps resulting in low recoveries of the products. High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition chromatography, can eliminate irreversible adsorption of samples onto the solid support used in the conventional chromatographic column [3]. The method has been successfully applied to the analysis and separation of various natural products [4–6].

The present paper describes the separation and purification of tanshinones from a crude extract of *Salvia miltiorrhiza* Bunge by HSCCC.

2. Experimental

2.1. Apparatus

HSCCC separations were performed with the following two models of multilayer coil planet centrifuge (MLCPC) manufactured by the Beijing Institute of New Technology Application, Beijing, China.

(1) Model GS-20 analytical MLCPC: The multilayer coil was prepared by winding 0.8-mm I.D. PTFE (polytetrafluoroethylene) tubing coaxially onto the column holder. The total capacity is 35 ml. The β values varied from 0.4 at the internal terminal to 0.7 at the external terminal. ($\beta=r/R$ where r indicates the coil radius and R, the distance between the column holder shaft and the central axis of the centrifuge.) The rotation speed is adjustable from 0 to 2000 rpm, and 1700 rpm was used in the present studies.

(2) Model GS10A2 preparative MLCPC: The multilayer coil was similarly prepared from 1.6-mm I.D. PTFE tubing. The total capacity is 240 ml. The β values varied from 0.5 at the internal terminal to 0.75 at the external terminal. The revolution speed is adjustable from 0 to 1000 rpm, but 800 rpm was used in the present studies.

These two HSCCC systems are equipped with a Model MS-1007 constant-flow pump, a Model 8823A-UV monitor operating at 254 nm, a Yokogawa Model 3057 recorder, and a sample injection valve with a 2- or 20-ml sample loop.

2.2. Reagents

All organic solvents used for HSCCC and HPLC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. All standard samples were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China.

2.3. Preparation of sample solution

About 1.0-kg of roots of *Salvia miltiorrhiza* Bunge was extracted in a glass bottle (2.5-l capacity) with 1.5 l of methanol for three times (a total volume of 4.5 l) at room temperature. The extracts were combined, evaporated to dryness under reduced pressure, and then redissolved in 500 ml of water. The aqueous solution was again extracted with light petroleum (b.p. 30-60°C) (500 ml×six times), and these extracts were combined and evaporated to dryness to yield 201.0 g of the crude sample. Then, 5 mg of the extract was dissolved in 1 ml of each phase of the solvent system used for analytical HSCCC separation whereas 50 mg of the extract was dissolved in 5 ml of each phase for preparative HSCCC separation. The sample solutions were sonicated for several minutes before loading into the column.

2.4. Preparation of two-phase solvent system

The following three two-phase solvent systems were prepared:

solvent system A: *n*-hexane–EtOH–water (4:1.8:2, v/v),
solvent system B: *n*-hexane–EtOH–water (4:2.3:2, v/v),
solvent system C: *n*-hexane–EtOH–water (4:3:2, v/v).

Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

2.5. HSCCC separation procedures

Analytical HSCCC, because of its speedy sepa-

ration and minimum solvent consumption, is an ideal tool for optimizing the key parameters for preparative HSCCC separation [7]. In our studies, the method was used for selecting a suitable solvent system and optimizing the experimental conditions for the separation of target compounds. The solvent system composed of *n*-hexane–ethanol–water was examined at different volume ratios (see solvent systems A–C) using analytical HSCCC.

In each analytical separation, the coiled column was first entirely filled with the organic stationary phase, and the aqueous mobile phase was pumped into the column at a flow-rate of 1.0 ml/min, while the apparatus was rotated at 1700 rpm. After the mobile phase emerged from the outlet of the column and hydrodynamic equilibrium was established in the column, about 1 ml of the sample solution containing 5 mg of the light petroleum extract was injected through the injection valve. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were manually collected according to the chromatogram.

Preparative separation was performed using a stepwise elution with solvent systems A, B and C in sequence as follows: The coiled column was first entirely filled with the upper organic phase of solvent system A, and the lower aqueous phase was pumped into the column at a flow-rate of 2.0 ml/min, while

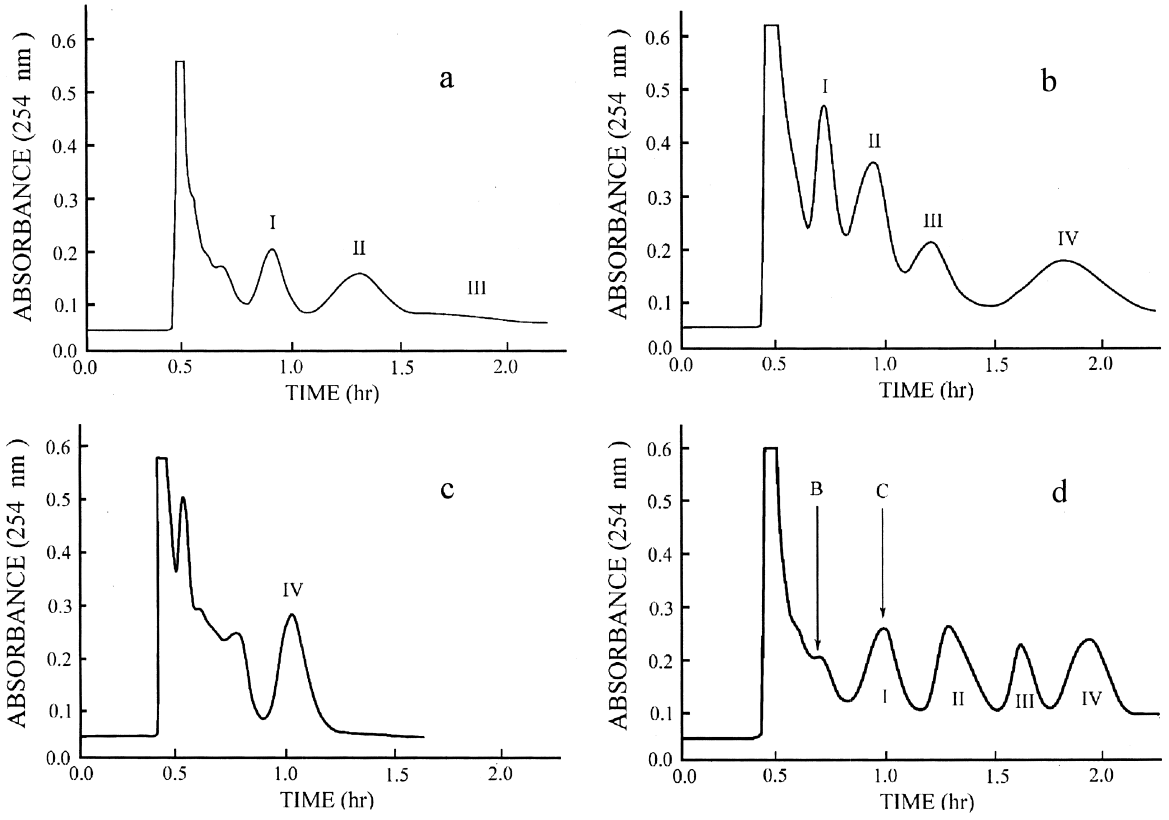

Fig. 2. Analytical HSCCC separation of the light petroleum extract of *Salvia miltiorrhiza* Bunge. (a) Solvent system A (*n*-hexane–ethanol–water, 4:1.8:2, v/v); (b) solvent system B (*n*-hexane–ethanol–water, 4:2.3:2, v/v); (c) solvent system C (*n*-hexane–ethanol–water, 4:3:2, v/v); (d) stepwise elution with the solvent systems A, B and C in sequence, i.e. the stationary phase was the upper organic phase of solvent system A, and the mobile phases including 60 ml of the lower phase of solvent system A, 20 ml of the lower phase of solvent system B, and 90 ml of the lower phase of solvent system C were sequentially eluted. Experimental conditions: revolution speed: 1700 rpm; stationary phase: upper organic phase of system A; mobile phase: lower aqueous phase of solvent system(s) A, B and/or C; flow-rate: 1 ml/min; sample size: 5 mg; retention of the stationary phase: 51%; detection: 254 nm.

the apparatus was rotated at 800 rpm. After the mobile phase emerged from the tail outlet and hydrodynamic equilibrium was established in the column, 10 ml of the sample solution containing 50 mg of the light petroleum extract was injected through the injection valve. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were manually collected according to the chromatogram. After 2 h of elution with solvent system A, the mobile phase was switched to the lower phase of system B to elute the column for 50 min followed by the elution with the lower phase of system C to complete the separation.

2.6. HPLC analysis

HPLC analyses were performed with a Shimadzu LC-10AVP system including a LC-10AT liquid chromatograph, an SPD-M10AVP photodiode array detector, an SCL-OAVP system controller and a CTP-10SVP column oven using an Intersil ODS-3 column (150 mm H, 4.6 mm I.D.) at column temperature of 30°C. The mobile phase composed of methanol–water (80:20, v/v) was eluted at a flow-rate of 1.0 ml/min and the effluent monitored by a photodiode array detector. Identification of HSCCC peak fractions were carried out by comparing retention time and UV spectra with the standard samples.

3. Results and discussion

In the present study, the HPLC analysis revealed that tanshinones were present mainly in the light petroleum extract from the dried roots of *Salvia miltiorrhiza* Bunge. Consequently, the light petroleum extract was separated by analytical HSCCC to select suitable solvent systems and other experimental conditions. The solvent systems A–C were evaluated in terms of peak resolution of various tanshinones. The results indicated that cryptotanshinone and tanshinone I were well resolved by solvent system A (Fig. 2a), but the profile of unknown component was irregular and tanshinone IIA was retained in the column. When the relative volume of ethanol in the solvent system was increased (solvent systems B and C), the retention time of the unknown component decreased and tanshinone IIA was eluted from the column, however the resolution between cryptotanshinone and tanshinone I became worse (Fig. 2c).

In this situation, we selected a stepwise elution to improve the resolution of target compounds as follows: The crude sample was first separated by solvent system A for 40 min, then the mobile phase was changed to the lower phase of system B and 20 min later the mobile phase was again changed to the lower phase of system C to complete the separation. This stepwise elution produced resolution of all four target peaks (I, II, III and IV) as shown in Fig. 2d.

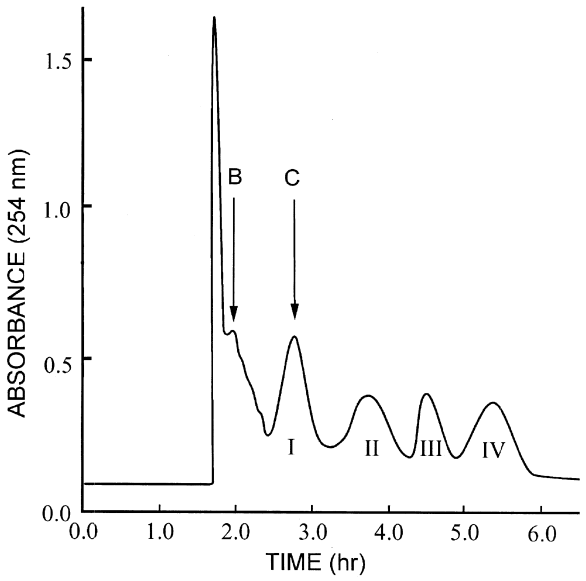

Fig. 3. Preparative HSCCC separation of the light petroleum extract of *Salvia miltiorrhiza* Bunge using stepwise elution with solvent system A, B and C. Experimental conditions: revolution speed: 800 rpm; stationary phase: the upper organic phase of solvent systems A; mobile phase: 250 ml of the lower phase of system A, 100 ml of the lower phase of system B and 450 ml of the lower phase of system C eluted in this sequence; flow-rate: 2 ml/min; sample size: 50 mg; retention of the stationary phase: 55%; detection: 254 nm. Peaks: I=cryptotanshinone; II=tanshinone I; III=unknown component; IV=tanshinone IIA.

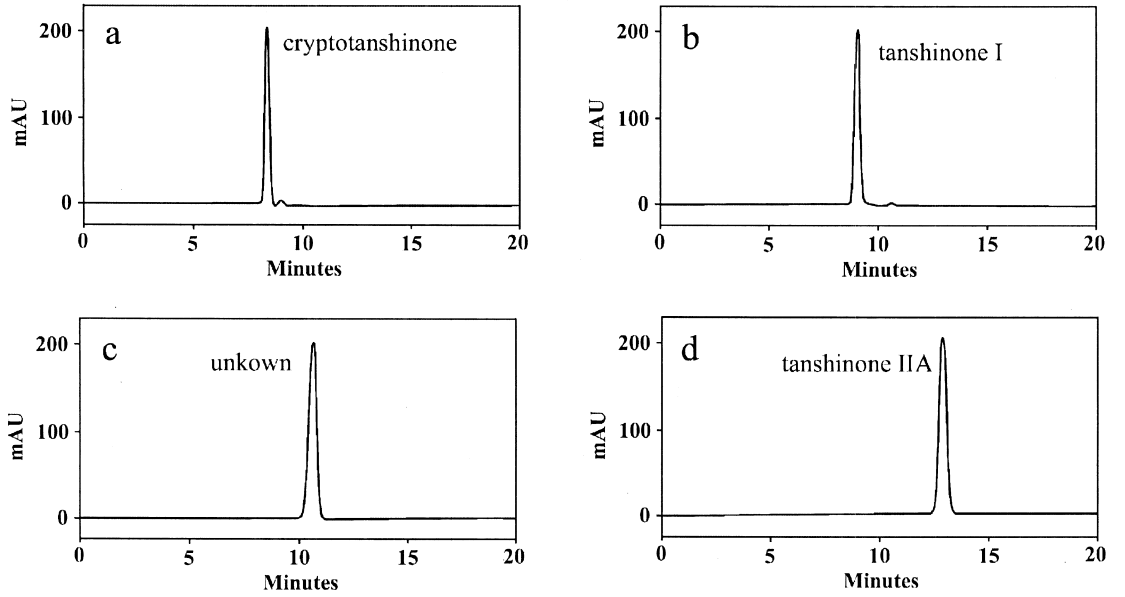

Fig. 4. HPLC analyses of the HSCCC fractions of tanshinones from *Salvia miltiorrhiza* Bunge. (a) Peak I; (b) peak II; (c) peak III; (d) peak IV of the preparative HSCCC separation shown in Fig. 3. Column: Intersil ODS-3 (150 mm×4.6 mm I.D.); column temperature: 30°C; mobile phase: methanol–water (80:20, v/v); flow-rate: 1.0 ml/min; detection: 254 nm.

Fig. 3 shows the preparative HSCCC separation of 50 mg of the light petroleum extract using the optimized stepwise elution determined by analytical HSCCC. The crude sample was first separated by solvent system A for 2 h, and the mobile phase was changed to the lower phase of system B to elute for 50 min, followed by elution with the lower phase of system C to complete the separation. Although the sample size was increased ten times that used in the analytical HSCCC run, an equivalent separation was achieved. Each peak fraction of this preparative HSCCC was analyzed by HPLC (Fig. 4) which indicates that the purity of tanshinone IIA corresponding to peak IV was over 97% and other tanshinones also gave high purities of over 95%. The yields of tanshinone IIA, tanshinone I and cryptotanshinone were 7, 4 and 3 mg, respectively.

The results of our studies indicate that HSCCC is a powerful tool for both analytical and preparative separations of tanshinones from a crude extract of *Salvia miltiorrhiza* Bunge. Using a stepwise elution with three solvent systems, preparative HSCCC can yield several milligrams of four compounds at high purity in a single run.

Acknowledgements

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References

[1] H.-W. Lou, B.-J. Wu, M.-Y. Wu, Z.-G. Yong, M. Niwa, Y. Hirata, Phytochemistry 24 (1985) 815.
[2] X. Wang, S. Yuan, R. Huang, Y. Song, Hua. Hsi. I. Ko. Ta. Huseh. Huseh. Pao. 27, No.4 (1996) 388 (Chinese).
[3] Y. Ito, Crit. Rev. Anal. Chem. 17 (1986) 65.
[4] T.-Y. Zhang, X. Hua, R. Xiao, S. Kong, J. Liq. Chromatogr. 11 (1988) 233.
[5] F.-Q. Yang, T.-Y. Zhang, G.-L. Tian, H.-F. Cao, Q.-H. Liu, Y. Ito, J. Chromatogr. A 858 (1999) 103.
[6] X.-L Cao, Yu Tian, T.-Y. Zhang, Y. Ito, Prep. Biochem. Biotechnol. 28 (1998) 79.
[7] D.E. Schaufelberger, in: Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography, Chemical Analysis, Vol. 132, Wiley-Interscience, New York, 1996, pp. 45–70, Chapter 2.