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Journal of Chromatography A, 945 (2002) 281–285

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Short communication

Separation of tanshinones from *Salvia miltiorrhiza* Bunge by multidimensional counter-current chromatography

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Received 7 August 2001; received in revised form 6 November 2001; accepted 6 November 2001

Abstract

Analytical and preparative high-speed counter-current chromatography (HSCCC) was successfully used for the isolation and purification of tanshinones from the roots of *Salvia miltiorrhiza* Bunge. Using multidimensional HSCCC, four major components including tanshinone IIA (16 mg), tanshinone I (10 mg), dihydrotanshinone I (7 mg) and cryptotanshinone (11 mg) were isolated each at high purity of over 95%.

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

1. Introduction

Salvia miltiorrhiza Bunge, Dan-Shen in Chinese, is one of the herbs that were classified as ''Blood-invigorating'' in traditional Chinese herbal medicine and were thought by ancient Chinese physicians to make ''sluggish'' or ''stuck'' blood flow more freely. Recently, human and animal studies demonstrate that the herb has the effects of vasodilatation, protection of cardiac muscles from anoxia, reduced platelet aggregation and thrombus formation [1]. The major active constitutes of this herb are tanshinones, including tanshinone IIA, cryptotanshinone, dihydrotanshinone I and tanshinone I. Pharmacological tests revealed that tanshinone IIA sodium sulfate showed therapeutic effect on heart stroke, myocardial ischemia and infarction while cryptotanshinone showed strong inhibitory action against allergic and drug-resistant strains of *Staphylococcus aureus* [2]. The chemical structures of these tanshinones are given in Fig. 1.

The separation and purification of tanshinones 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, eliminates irreversible adsorption of samples onto the solid support used in the conventional chromatographic column [3]. The method has been successfully applied to analysis and separation of various natural products [4,5]. Recently, we have purified tanshinones from crude extract of *Salvia miltiorrhiza* using a stepwise elution [6].

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0021-9673/02/$ – see front matter
PII: S0021-9673(01)01495-9

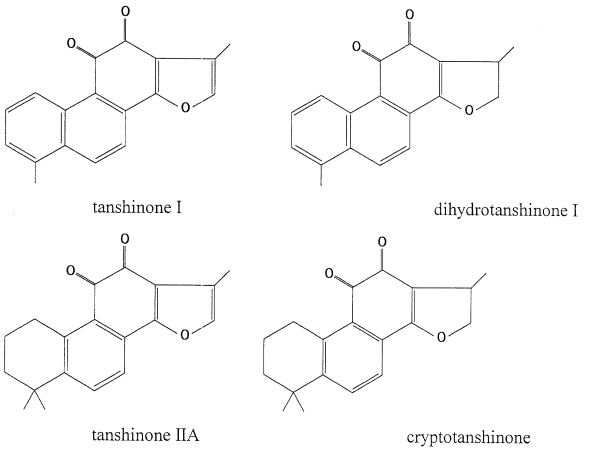

Fig. 1. The chemical structures of tanshinones.

In this paper a set of tanshinone analogs is separated using multidimensional high-speed counter-current chromatography [7] which is performed by introducing a peak fraction from the first column directly into the second column to elute with a different two-phase solvent system. The method substantially improved the previous results to yield five target compounds and one unknown impurity.

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 with 5 cm revolution radius: The multilayer coil was prepared by winding 0.8 mm I.D. PTFE (polytetrafluoroethylene) tubing coaxial onto the column holder. The total capacity is 35 ml. The β value varied from 0.4 at the internal terminal to 0.7 at the external terminal. The revolution speed is adjustable from 0 to 2000 rev./min, and 1700 rev./min was used throughout the present studies.

(2) Model GS10A2 preparative MLCPC with 10 cm revolution radius: The multilayer coil was similarly prepared from 1.6 mm I.D. PTFE tubing. The β value varied from 0.5 at the internal terminal to 0.75 at the external terminal. The total capacity was 240 ml. The revolution speed is adjustable from 0 to 1000 rev./min, but 800 rev./min was used in the present studies.

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

2.2. Reagents

All organic solvents used for HSCCC and high-performance liquid chromatography (HPLC) were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. All standard samples were purchased from National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Health, Beijing, China. Dried roots of *Salvia miltiorrhiza* were obtained from a local pharmacy store.

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

About 1.0 kg of root material of *Salvia miltiorrhiza* Bunge was extracted in a glass bottle (2.5 l capacity) with 1.5 l of light petroleum (boiling point (b.p.) 30–60 °C) three times (a total volume of 4.5 l) at room temperature. The extracts were combined, and evaporated to dryness under reduced pressure to yield 97.0 g of the crude sample. In the analytical separation 5 mg of the extract was dissolved in 1 ml of each phase of the solvent system used for separation whereas 100 mg of the extract was dissolved in 10 ml of each phase for preparative HSCCC separation. The sample solutions were sonicated for several minutes before loading into the column.

For the present study, we selected a two-phase solvent system composed of light petroleum (b.p. 60–90 °C), ethyl acetate, methanol and water at various volume ratios. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases were separated shortly before use.

2.4. HSCCC Separation procedures

Analytical HSCCC, because of its speedy separation and minimum solvent consumption, is an ideal tool for optimizing the key parameters for preparative HSCCC separation [8]. In our studies, the method was used for selecting a suitable solvent system and optimizing the experimental conditions for the separation of target components. The above solvent system composed of light petroleum, ethyl acetate, methanol and water was examined at various volume ratios such as 1:1:1:1, 1:4:2:2, 2:3:3:2, 2:3:2.5:1.7 and 2:3:2.5:1.8 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 rev./min. 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.

Multidimensional preparative separation was performed with the similar procedure and instruments as described by Yang et al. [7]. In this CCC technique, the peak fraction eluted from the first column with insufficient resolution is directly introduced into the second column to elute with the second solvent system which provides a suitable partition coefficient values for the target compounds.

2.5. HPLC analysis

HPLC analyses were performed with a Shimadzu LC-10AVP system including an 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 (150×4.6 mm I.D.) at a column temperature of 30 °C. The mobile phase, composed of acetonitrile–water (61:39, v/v), was eluted at a flow-rate of 1.0 ml/min and the effluent monitored by photodiode array detection. HSCCC peak fractions were identified by comparing retention times 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. Therefore, the extract was separated by analytical HSCCC to select suitable solvent systems and other experimental conditions. The performance of the solvent systems composed of light petroleum, ethyl acetate, methanol and water at various volume ratios was evaluated in terms of peak resolution of tanshinones. The results indicated that all of these solvent systems could be used to separated tanshinone IIA and an unknown component from light petroleum extract. However, for the resolution of cryptotanshinone and tanshinone I, the solvent system at a volume ration of 2:3:2:1.7 was found to be most suitable. Fig. 2 shows the analytical HSCCC separation of the light petroleum extract obtained by the above solvent system.

Fig. 3 shows the preparative separation of 100 mg of the light petroleum extract by multidimensional

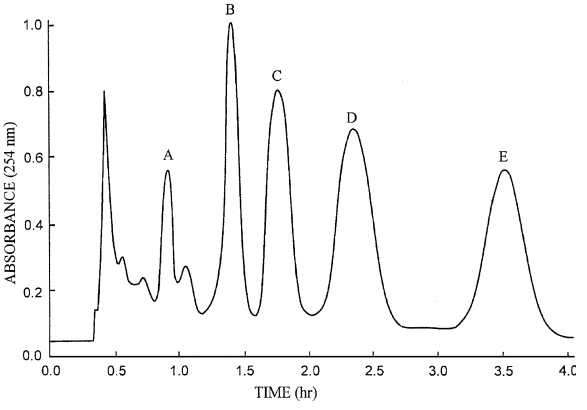

Fig. 2. Analytical HSCCC separation of the light petroleum extract of *Salvia miltiorrhiza* Bunge. Experiment conditions: rotation speed, 1700 rev./min; solvent system, light petroleum–ethyl acetate–methanol–water (2:3:2.5:1.7, v/v); mobile phase, lower aqueous phase; flow-rate, 1 ml/min; sample size, 5 mg; and retention of the stationary phase, 51%. Peaks: A, dihydrotanshinone I; B, cryptotanshinone; C, tanshinone I; D, unknown component; and E, tanshinone IIA.

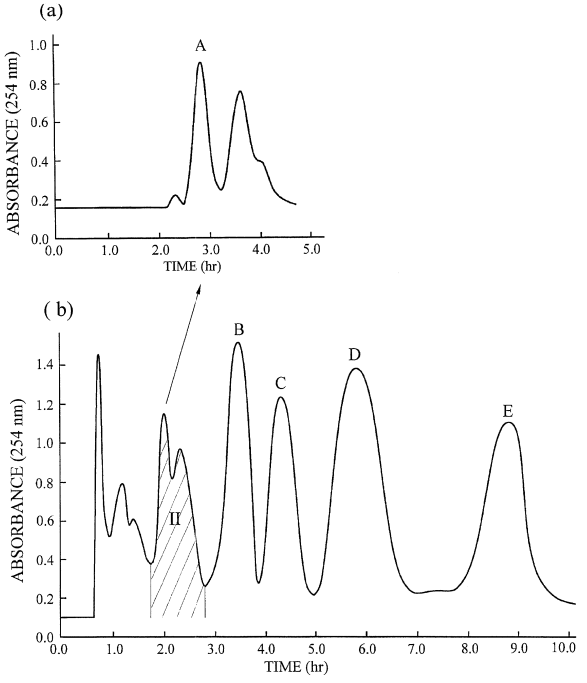

Fig. 3. Multidimensional preparative HSCCC separation of the light petroleum extract of *Salvia miltiorrhiza* Bunge. Experimental conditions: rotation speed, 800 rev./min; solvent system, (a) light petroleum–ethyl acetate–methanol–water (2:3:2.5:1.7, v/v); (b) light petroleum–ethyl acetate–methanol–water (2:3:2.5:1.8, v/v); mobile phase, lower aqueous phase; flow-rate, 2 ml/min; detection, 254 nm; and sample size, 100 mg. (a) Chromatogram obtained by the second HSCCC; (b) chromatogram obtained by the first HSCCC. Peaks: A, dihydrotanshinone I; B, cryptotanshinone; C, tanshinone I; D, unknown component; and E, tanshinone IIA.

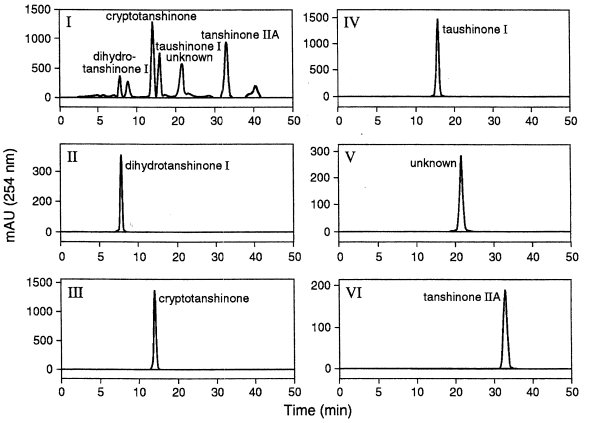

Fig. 4. HPLC analyses of the crude sample and HSCCC fractions of tanshinones from *Salvia miltiorrhiza* Bunge. I, crude sample; II, peak A; III, peak B; IV, peak C; V, peak D; and VI, peak E of the preparative HSCCC separation shown in Fig. 3. Column, Intersil ODS-3 (150×4.6 mm I.D.); column temperature, 30 °C; mobile phase, acetonitrile–water (61:39, v/v); flow-rate, 1.0 ml/min; and UV wavelength, 254 nm.

HSCCC using the optimized solvent system selected by analytical HSCCC. Cryptotanshinone, tanshinone I, unknown component and tanshinone IIA can be well resolved in the first preparative separation, but the resolution of dihydrotanshinone I is unsatisfactory. In this situation we employed a two-step elution strategy called multidimensional HSCCC [7] in which the peak fraction of the first separation containing dihydrotanshinone I was introduced into the second HSCCC system to elute with a two-phase solvent system which provides a suitable partition coefficient values for the target compounds. Consequently, dihydrotanshinone I—which was not resolved in the stepwise elution [6]—was successfully isolated from other components. Four major components including tanshinone IIA (16 mg), tanshinone I (10 mg), dihydrotanshinone I (7 mg) and cryptotanshinone (11 mg) were isolated each at high purity of over 95% (Fig. 4).

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. Multidimensional preparative HSCCC can yield several milligrams of the five components at high purity.

Acknowledgements

Financial support from Beijing Commission of Science and Technology and Beijing is gratefully acknowledged. We also thank senior engineer Xining Li, Baoxin Han, and Dr Xueli Chao for their excellent technical assistance.

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