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

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Abstract

High-speed counter-current chromatography was applied to the isolation and purification of salvianolic acid B from the Chinese medicinal plant *Salvia miltiorrhiza* Bunge. The crude salvianolic acid B was obtained by extraction with ethanol–water from *S. miltiorrhiza* Bunge. Preparative high-speed counter-current chromatography with a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (3:7:1:9, v/v) was successfully performed yielding 342 mg salvianolic acid B at 98% purity from 500 mg of the crude extract in a one-step separation.

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

1. Introduction

Dan-shen, the dried roots of *Salvia miltiorrhiza* Bunge, has been used widely in traditional Chinese medicine for the treatment of various kinds of disorders such as coronary artery disease and angina pectoris [1,2]. The effective components of the roots could be classified as lipid-soluble and water-soluble. The lipid-soluble components are mainly tanshinones (diterpenoid quinones) while the water-soluble ones are mainly phenolic compounds. Recently, much attention has been paid to the pharmacological activities of the water-soluble phenolic compounds [3–5]. Among these phenolic compounds, salvianolic acid B (lithospermic acid B) exhibits endothelium-dependent vasodilation in the aorta and may be useful in the treatment of hypertension while magnesium salvianolic acid B has a potent hepatoprotective activity and shows an improved effect on uremic symptoms [6–9]. The chemical structure of salvianolic acid B is shown in Fig. 1.

High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography that uses no solid support matrix and the stationary phase is immobilized by centrifugal force. Therefore, it eliminates irreversible adsorptive loss of samples on to the solid support matrix used in the conventional chromatographic column, and is very suitable for separation and purification of active components from traditional Chinese medicinal herbs

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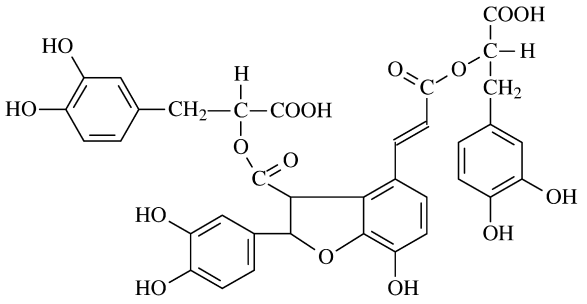

Fig. 1. The chemical structure of salvianolic acid B.

and other natural products [10–17]. The method has also been applied to the separation and purification of the lipid-soluble components, tanshinones from the herbal medicine *S. miltiorrhiza* [18,19]. However, no report has been published on the use of HSCCC for the isolation and purification of the water-soluble components from the plant. The purpose of this study, therefore, was to develop a method for the isolation and purification of salvianolic acid B, the important water-soluble compound from the Chinese medicinal plant *S. miltiorrhiza* Bunge by HSCCC.

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 Corp., USA), 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 20-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. Ethyl acetate, *n*-hexane, ethanol, methanol and formic acid were obtained from BDH (Poole, UK). Salvianolic acid B was obtained as a gift from Dr Y.M. Xu (Shanghai Institute of Materia Medica, Academia Sinica, Shanghai, China).

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

2.3. Preparation of crude salvianolic acid B from *S. miltiorrhiza Bunge*

Preparation of crude salvianolic acid B was carried out according to the literature [20]. In brief, the dried roots of *S. miltiorrhiza* Bunge were ground to powder. The powder (100 g) was extracted with 300 ml of 60% aqueous ethanol for 2 h. The mixture was filtered, then the residue was extracted with 300 ml of 30% aqueous ethanol for 2 h. Finally, the residue was extracted with 300 ml of water for 2 h. The filtrate was combined, and was extracted with *n*-hexane (150 ml×2). After two phases were separated with a separatory funnel, the organic phase was evaporated to dryness by rotary vaporization at 40°C to recover tanshinones, and the water phase was evaporated to about 100 ml at 60°C to remove ethanol. The concentrated liquid was diluted to about 500 ml with water, and was extracted with chloroform (100 ml×2) to remove fat-soluble compounds. After two phases were separated, the pH value of the water phase was adjusted to 2 with 10% hydrochloric acid. Then, the water phase was extracted with ethyl acetate (100 ml×3). After the two phases were separated, the organic phase was evaporated to dryness at 50°C, and the residue (5.425 g) was stored in a refrigerator for the subsequent HSCCC separation.

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

In the present study, we selected a two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–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.

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

2.5. HSCCC separation procedure

In each separation, the coiled column was first entirely filled with the upper phase (stationary phase), and then the apparatus was rotated at 1000 rpm, while the lower phase (mobile phase) 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 20 ml of the sample solution containing 500 mg of the crude salvianolic acid B was injected through the injection valve. 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.

2.6. HPLC analysis

The crude sample and each peak fraction obtained by HSCCC were analyzed by HPLC according to the literature [21]. 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, Milford, MA, USA). 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 (methanol–water–formic acid, 14:85.2:0.8, v/v) and solvent B (methanol–water, 65:35, v/v) in gradient mode as follows: 0% of B for 2 min; a linear gradient from 30 to 45% of B for 8 min. The flow-rate was 1.0 ml min^{-1}, and the effluent was monitored at 280 nm.

3. Results and discussion

Fig. 2A shows the HPLC analysis of the crude

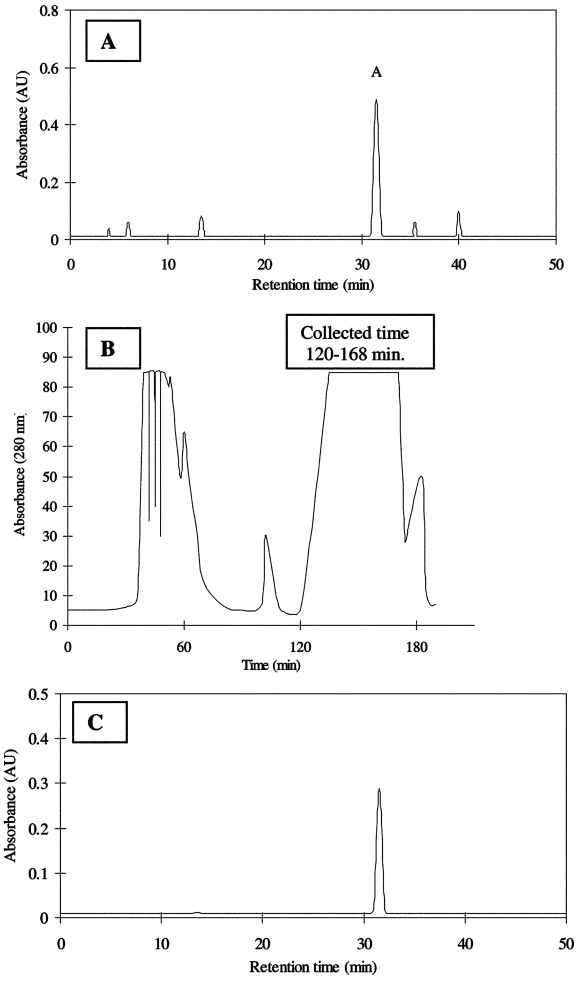

Fig. 2. (A) Chromatogram of crude salvianolic acid B from *S. miltiorrhiza* Bunge by HPLC analysis; A, salvianolic acid B. Conditions: column: reversed-phase Ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm); mobile phase: solvent A (methanol–water–formic acid, 14:85.2:0.8, v/v) and solvent B (methanol–water, 65:35, v/v) in gradient mode; flow-rate: 1.0 ml min^{-1}; detection: 280 nm; loaded amount, 100 ng. (B) Chromatogram of crude salvianolic acid B from *S. miltiorrhiza* Bunge by HSCCC separation; A, salvianolic acid B. Conditions: column: multilayer coil of 2.6 mm I.D. PTFE tube with a total capacity of 325 ml; rotary speed: 1000 rpm; solvent system: *n*-hexane–ethyl acetate–ethanol–water (3:7:1:9, v/v); mobile phase: lower phase (water phase); flow-rate: 2 ml min^{-1}; detection: 280 nm; sample size: 500 mg; retention of the stationary phase: 57%. (C) HPLC chromatogram of salvianolic acid B purified by HSCCC from *S. miltiorrhiza* Bunge. Conditions: column: reversed-phase Ultrasphere C_{18} column (250×4.6 mm I.D., 5 μm); mobile phase: solvent A (methanol–water–formic acid, 14:85.2:0.8, v/v) and solvent B (methanol–water, 65:35, v/v) in gradient mode; flow-rate: 1.0 ml min^{-1}; detection: 280 nm; loaded amount, 70 ng.

salvianolic acid B from *S. miltiorrhiza* Bunge. Peak A corresponds to salvianolic acid B.

Preliminary HSCCC studies were carried out with the two-phase solvent system composed of *n*-butanol–ethyl acetate–water. It required a long elution time to elute salvianolic acid B with the two-phase solvent system at a volume ratio of 1:1:2 (data not shown). As shown in Table 1, the *K* (partition coefficient) value of salvianolic acid B in the two-phase solvent system composed of *n*-butanol–ethyl acetate–water was too large. Thus, the two-phase solvent system composed of *n*-butanol–ethyl acetate–water was not suitable for the separation of salvianolic acid B from plant. Consequently, another two-phase solvent system was then tested.

It also required a long elution time to elute salvianolic acid B with the two-phase solvent system composed of *n*-hexane–*n*-butanol–ethanol–water at various volume ratios (3:7:1:9 and 3:7:2:8). As shown in Table 1, the *K* values of salvianolic acid B in the two-phase solvent system composed of *n*-hexane–*n*-butanol–ethanol–water were also too large. Thus, the two-phase solvent system composed of *n*-hexane–*n*-butanol–ethanol–water was also not suitable for the separation of salvianolic acid B from plant. In subsequent studies, another two-phase solvent system was thus tested.

Performance of the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at various volume ratios (4:6:2:8, 3:7:1:9 and 3:7:2:8) was evaluated in terms of peak resolution. It was very difficult to separate salvianolic acid B from other substances with the two-phase solvent system at a ratio of 4:6:2:8 (data not shown). When the two-phase solvent system at a ratio of 3:7:1:9 was used, the peak resolution were satisfactory, and the retention of the stationary phase was also very good (about 57%). When the two-phase solvent system at a ratio of 3:7:2:8 was also tested, the peak resolution was poor. Fig. 2B shows the preparative HSCCC separation of 500 mg of crude sample using the solvent system composed of *n*-hexane–ethyl acetate–ethanol–water (3:7:1:9, v/v). After salvianolic acid B was eluted, in order to save solvents and time, the remaining compounds in the column were removed by pumping out the stationary phase instead of eluting them with the mobile phase because the stationary phase was not to be reused. HPLC analysis of each peak fraction of this preparative HSCCC revealed that salvianolic acid B corresponding to peak A was over 98% pure. The yield of salvianolic acid B was 342 mg. The HPLC chromatogram of salvianolic acid B as purified from the preparative HSCCC is shown in Fig. 2C.

The *K*-values of salvianolic acid B in several solvent systems were measured according to the literature [22], and are given in Table 1.

In conclusion, HPCCC was successfully used for the isolation and purification of salvianolic acid B from *S. miltiorrhiza* Bunge, and yielded 342 mg salvianolic acid B at 98% purity from 500 mg of the crude extract in a one-step separation.

Table 1
The *K* (partition coefficient) values of salvianolic acid B in several solvent systems

Solvent system	*K*
n-Butanol–ethyl acetate–water (1:1:2)	3.28
n-Hexane–*n*-butanol–ethanol–water (3:7:1:9)	4.16
n-Hexane–*n*-butanol–ethanol–water (3:7:2:8)	3.57
n-Hexane–ethyl acetate–ethanol–water (4:6:2:8)	0.92
n-Hexane–ethyl acetate–ethanol–water (3:7:1:9)	1.25
n-Hexane–ethyl acetate–ethanol–water (3:7:2:8)	1.05

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References

[1] L.N. Li, Pure Appl. Chem. 70 (1998) 547.
[2] W. Tang, G. Eisenbrand, Chinese Drugs of Plant Origin: Chemistry, Pharmacology, and Use in Traditional and Modern Medicine, Springer, Berlin, 1992, pp. 891–902.
[3] G.T. Liu, T.M. Zhang, B.E. Wang, Y.W. Wang, Biochem. Pharmacol. 43 (1992) 147.
[4] Y.J. Wu, C.Y. Hong, S.L. Lin, P.L. Wu, M.S. Shiao, Arterioscler. Thromb. Vasc. Biol. 18 (1998) 481.
[5] T. Yokozawa, J.J. Zhou, H. Oura, T. Tanaka, G. Nonaka, I. Nishioka, Phytother. Res. 9 (1995) 105.
[6] K. Hase, R. Kasimu, P. Basnet, S. Kadota, T. Namba, Planta Med. 63 (1997) 22.
[7] K. Kamata, M. Noguchi, M. Nagai, Gen. Pharmacol. 25 (1994) 69.

[8] K. Kamata, T. Iizuka, M. Nagai, Y. Kasuya, Gen. Pharmacol. 24 (1993) 977.
[9] T. Tanaka, S. Morimoto, G. Nonaka, I. Nishioka, T. Yokozawa, H.Y. Chung, H. Oura, Chem. Pharm. Bull. 37 (1989) 340.
[10] H.B. Li, F. Chen, T.Y. Zhang, F.Q. Yang, G.Q. Xu, J. Chromatogr. A 905 (2001) 151.
[11] L. Lei, F.Q. Yang, T.Y. Zhang, P.F. Tu, L.J. Wu, Y. Ito, J. Chromatogr. A 912 (2001) 181.
[12] L. Chen, Y.S. Han, F.Q. Yang, T.Y. Zhang, J. Chromatogr. A 907 (2001) 343.
[13] F.Q. Yang, T.Y. Zhang, Y. Ito, J. Chromatogr. A 919 (2001) 443.
[14] H.B. Li, F. Chen, J. Chromatogr. A 925 (2001) 133.
[15] G.L. Tian, T.Y. Zhang, F.Q. Yang, Y. Ito, J. Chromatogr. A 886 (2000) 309.
[16] F.Q. Yang, T.Y. Zhang, Q.H. Liu, G.Q. Xu, Y.B. Zhang, S. Zhang, Y. Ito, J. Chromatogr. A 883 (2000) 67.
[17] X.L. Cao, Y. Tian, T.Y. Zhang, X. Li, Y. Ito, J. Chromatogr. A 855 (1999) 709.
[18] G.L. Tian, Y.B. Zhang, T.Y. Zhang, F.Q. Yang, Y. Ito, J. Chromatogr. A 904 (2000) 107.
[19] H.B. Li, F. Chen, J. Chromatogr. A 925 (2001) 109.
[20] J. Li, L.Y. He, W.Z. Song, Acta Pharmacol. Sin. 28 (1993) 543.
[21] J.P. Yuan, H. Chen, F. Chen, J. Agric. Food Chem. 46 (1998) 2651.
[22] Y. Ito, in: Y. Ito, W.D. Conway (Eds.), High-Speed Countercurrent Chromatography (Chemical Analysis, Vol. 132), Wiley–Interscience, New York, 1996, p. 3, Ch. 1.