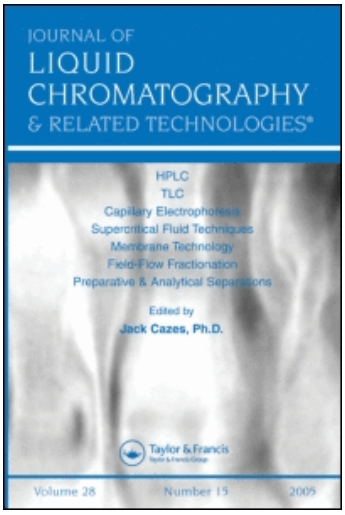

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Preparative Isolation and Purification of Prim-O-Glucosyl-Cinmifugin and 4′-O-β-D-Glucosyl-5-O-Methylvisamminol from *Radix saposhnikoviae* by High Speed Countercurrent Chromatography

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Abstract: A high-speed counter-current chromatography (HSCCC) method for separation and purification of prim-O-glucosyl-cinmifugin and 4′-O-β-D-glucosyl-5-O-methylvisamminol from the extract of *Radix saposhnikoviae* was developed by using chloroform-methanol-water (10 : 8 : 4, v/v) as the two-phase solvent system. Prim-O-glucosyl-cinmifugin (21.7 mg) and 44.7 mg of 4′-O-β-D-glucosyl-5-O-methylvisamminol were produced from 100 mg of the crude sample, both at over 99.0% purity, determined by HPLC. The structures of the isolated compounds were identified by ^{1}H-NMR and ^{13}C-NMR.

Keywords: Countercurrent chromatography, *Radix saposhnikoviae*, Prim-O-glucosyl-cinmifugin, 4′-O-β-D-Glucosyl-5-O-methylvisamminol

INTRODUCTION

Radix saposhnikoviae, the root of *Saposhnikovia divaricata (Turcz.) Schischk.*, Fangfeng in Chinese, is one of the well-known traditional Chinese medicinal herbs, which is listed in the Chinese pharmacopoeia and has been used as an anti-inflammatory, anti-febrile, and anodyne.[1,2] Present pharmacological studies and clinical practices demonstrated that Fangfeng

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also has various pharmaceutical effects on preventing agglomeration of blood platelets and as an immunoregulation.[3]Prim-O-glucosyl-cinmifugin and 4′-O-β-D-glucosyl-5-O-methylvisamminol are the most active ingredients of Fangfeng,[2] and both are used as standards in the quality control of Fangfeng products.[4] Their chemical structures are shown in Figure 1.

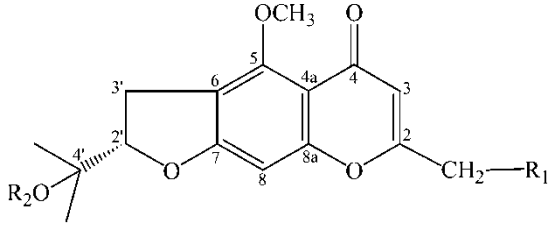
The separation and purification of prim-O-glucosyl-cinmifugin and 4′-O-β-D-glucosyl-5-O-methylvisamminol, using conventional methods such as column chromatography, requires several steps and results in low recoveries. High speed countercurrent chromatography (HSCCC), invented by Y. Ito,[5] is a support free all liquid partition chromatography. It avoids adsorption losses and the formation of artifacts due to the lack of active surfaces. So, it is suitable for separation and purification of active components from traditional Chinese medicinal herbs and other natural products.[6–8]

In the present paper, a successful HSCCC method for separation and purification of prim-O-glucosyl-cinmifugin and 4′-O-β-D-glucosyl-5-O-methylvisamminol from Fangfeng, with purities both over 99.0%, by using chloroform-methanol-water (10 : 8 : 4, v/v) as the two-phase system was described. The structures of the isolated compounds were identified by ^{1}H-NMR and ^{13}C-NMR.

EXPERIMENTAL

Apparatus

The HSCCC instrument employed in the present study is a TBE-300A high speed countercurrent chromatograph (Shanghai Tauto Biotech Co., Ltd., Shanghai, China) with three multilayer coil separation columns connected in series (I.D. of the tubing = 1.6 mm, total volume = 260 mL), and a 20 mL sample loop. The revolution radius, or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β values of the multilayer coil varied from 0.5 at the internal terminal to 0.8



	R_1	R_2
prim-O-Glucosylcimifugin	O-Glc	H
4'-O-β-D-glucosyl-5-O-methylvisamminol	H	Glc

Figure 1. The chemical structures of target compounds.

at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1,000 rpm. An HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Instrument Company, Beijing, China) was used to control the separation temperature. An ÄKTA prime system (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. It contains a switching valve and a mixer, which were used for gradient formation. The data were collected with an Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Company, Hangzhou, China).

The HPLC equipment used was an Agilent 1100 HPLC system, including a G1311A QuatPump, G1315B UV-vis photodiode array detector, Rheodyne 7725i injection valve with a 20 mL loop, G1332 degasser, and an Agilent HPLC workstation (Agilent Technologies, Germany).

The nuclear magnetic resonance (NMR) spectrometer used here was a Mercury Plus 400 NMR system (Varian Inc., USA).

A FZ102 plant disintegrator (Taisite Instrument Company, Tianjin, China) was used for disintegration of the sample.

Reagents and Materials

All organic solvents used for preparation of crude sample and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). Methanol used for HPLC was chromatographic grade (Yucheng Chemical plant, Yucheng, China), and water was distilled water. [$^2H_6$]dimethyl sulfoxide (DMSO-d_6) was used as the solvent for NMR determinations.

Fangfeng was purchased from the Luan drug store (Jinan, China), and was identified as the roots of *Saposhnikovia divaricata (Turcz.)* Schischk by Professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

Preparation of Sample

About 1.10 kg of Fangfeng was crushed to powder (about 30 mesh). Then, the powder was percolated with 95% ethanol. When the outflow changed from yellow to colorless, the effluents were combined and evaporated to a cream by rotary vaporization at 45°C, under reduced pressure. The cream was then dissolved in 300 mL of water, and extracted with light petroleum, ethyl acetate, and n-butanol (saturated by water), successively. The n-butanol fraction was evaporated to 50 mL by rotary vaporization, under reduced pressure. The residual solution was then frozen at −4°C for 24 h. The

deposit was separated and 2.65 g of crude sample was obtained. It was stored in a refrigerator for subsequent HSCCC separation.

Selection of the Two-Phase Solvent Systems

A chloroform-methanol-water (10 : 8 : 4, v/v) two-phase system was used as the solvent system. The composition of the two-phase system was selected according to the partition coefficients (K) of the target compounds of crude sample from Fangfeng. The partition coefficients were determined by HPLC as follows: about 1 mg of sample was added to a test tube, to which 2 mL of each phase of the two-phase solvent system was added. The test tube was shaken vigorously for several minutes, and then the upper and the lower phase were analyzed by HPLC. The partition coefficients of all components in the sample were calculated according to the equation: $K = A_U/A_L$, where A_U is the peak area of the upper phase and A_L is the peak area of the lower phase.

Preparation of Two-Phase Solvent System and Sample Solution

Chloroform-methanol-water solvent systems with volume ratios of 10 : 8 : 4 were prepared by adding the solvents to a separatory funnel according to the volume ratios, and thoroughly equilibrated after shaking repeatedly. Then, upper phase and lower phase were separated and degassed by sonication for thirty minutes prior to use.

The crude sample (100 mg) was dissolved in 5 mL of the upper phase of chloroform-methanol-water (10 : 8 : 4, v/v) system.

Separation Procedure of HSCCC

The procedure was carried out as follows: The upper phase and the lower phase of chloroform-methanol-water (10 : 8 : 4, v/v) were pumped into the multilayer-coiled column simultaneously by using an ÄKTA prime system, according to the volume ratio of 55 : 45. When the column was totally filled with the two phases, only the lower phase was pumped at a flow rate of 2.0 mL min^{-1}, and at the same time, the HSCCC apparatus was run at a revolution speed of 900 rpm. After hydrodynamic equilibrium was reached (about half an hour later), the sample solution was injected into the separation column. The separation temperature was controlled at 20°C. The effluent from the tail end of the column was continuously monitored with the ÄKTA prime system at 254 nm, and the chromatogram was recorded 50 min after the sample injection. Each peak fraction was manually collected according to the chromatogram and evaporated under reduced

pressure. The residuals were dissolved in methanol for subsequent HPLC analysis.

HPLC Analysis and Identification of HSCCC Peak Fractions

The crude sample, and each HSCCC peak fraction, were analyzed by HPLC. The analysis was accomplished with a Spherigel ODS C_{18} column (250 × 4.6 mm I. D, 5 μm) at room temperature. A methanol-water system was used as mobile phase, in gradient mode, as follows: 0–20 min, the ratio of methanol-water changed from 10 : 90 to 30 : 70; 20–40 min, 30 : 70 to 50 : 50. The flow rate was 0.7 mL min^{-1}, and the effluents were monitored at 254 nm by a photodiode array detector.

Identification of HSCCC peak fractions was performed by ^{1}H-NMR and ^{13}C-NMR. The UV spectra were taken from the HPLC three dimensional spectrum of absorbance versus time and wavelength.

RESULTS AND DISCUSSION

Optimization of HPLC Conditions

Several elution systems were tested in the HPLC separation of the crude sample, such as gradient elution of methanol-water, acetonitrile-water, etc. The results indicated that good separation results could be obtained when using a methanol-water system as follows: 0–20 min, the ratio of methanol-water changed from 10 : 90 to 30 : 70; 20–40 min, 30 : 70 to 50 : 50. The flow rate was 0.7 mL min^{-1}.

The crude sample and peak fractions separated by HSCCC were analyzed by HPLC. The chromatograms are shown in Figure 2.

Selection of Two-Phase Solvent System and Other Conditions of HSCCC

A series of experiments was performed to optimize the two-phase solvent system for HSCCC separation. An ethyl acetate-water system and ethyl acetate-methanol-water system were studied first. The results indicated that the target compounds mainly partitioned in the aqueous phase. So, the ethyl acetate-water system and ethyl acetate-methanol-water system were unsuitable for HSCCC separation. Then, a chloroform-methanol-water system was investigated and the partition coefficients of the target compounds are shown in Table 1. According to the partition coefficients shown in Table 1, some solvent systems were tested. When the chloroform-methanol-water

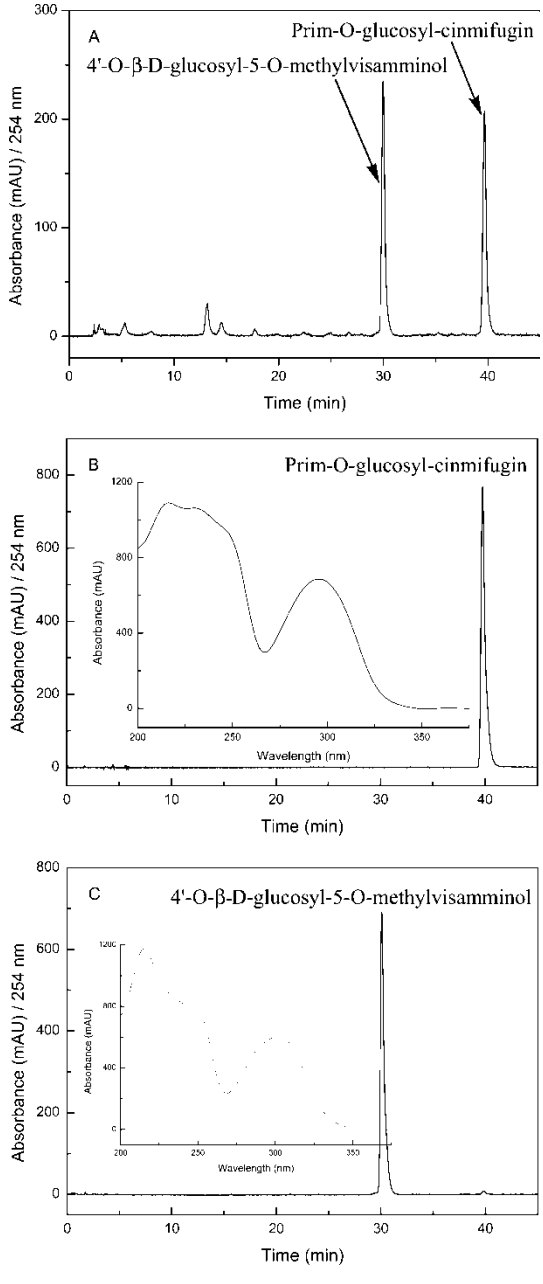


Figure 2. HPLC chromatograms. A: crude extract from Fangfeng, B: fraction of peak I of HSCCC, C: fraction of peak II of HSCCC. Column: SPHERIGEL ODS C_{18} column (250 mm × 4.6 mm I.D, 5 μm); mobile phase: methanol-water system as follows: 0–20 min, 10 : 90 → 30 : 70; 20–20 min, 30 : 70 → 50 : 50; flow rate: 0.7 mL min^{-1}; detection wavelength: 254 nm.

Table 1. The partition coefficients of target compounds in chloroform-methanol-water system

	K	
Chloroform-methanol-water (v/v)	Prim-O-glucosyl-cinmifugin	4′-O-β-D-glucosyl-5-O- methylvisamminol
10 : 5 : 4	1.79	11.43
10 : 6 : 4	1.66	9.32
10 : 7 : 4	1.24	6.70
10 : 8 : 4	1.05	3.48

(10 : 8 : 4, v/v) system was applied to the HSCCC, good results were realized and the separation time was acceptable.

The influence of the flow rate of mobile phase, the separation temperature, and revolution speed were also investigated. The results indicated that, when the flow rate was 2 mL min^{-1}, the separation temperature was 20°C, and the revolution speed was 900 rpm, good separation results were obtained.

The crude samples from Fangfeng were separated and purified under the optimum HSCCC conditions. The HSCCC chromatogram is shown in Figure 3. Prim-O-glucosyl-cinmifugin (21.7 mg) (Peak I, collected during 78–96 min), and 44.7 mg of 4′-O-β-D-glucosyl-5-O-methylvisamminol (Peak II, collected during 246-297 min), were obtained from 100 mg of the

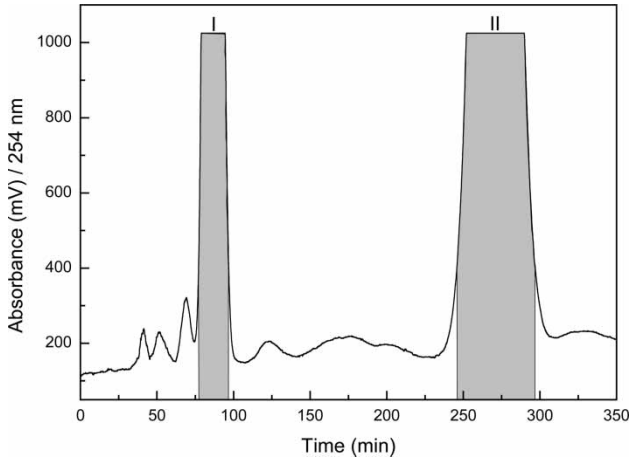


Figure 3. HSCCC chromatogram of crude extract from Fangfeng. Solvent system: Chloroform-methanol-water (10 : 8 : 4, v/v); mobile phase: lower phase; flow rate: 2.0 mL min^{-1}; revolution speed: 900 rpm; temperature: 20°C; retention of the stationary phase: about 55%; detection wavelength: 254 nm.

crude sample. The purities of prim-O-glucosyl-cinmifugin and 4′-O-β-D-glucosyl-5-O-methylvisamminol were 99.6% and 99.2%, respectively, as determined by HPLC. The HPLC chromatograms and UV spectra of these compounds are shown in Figure 2.

Structural Identification

The chemical structure of each peak fraction of HSCCC was identified according to ^{1}H-NMR and ^{13}C-NMR data.

Peak I: ^{1}H-NMR (400 MHz, DMSO-d_6): δ 6.30 (1H, s, H-3), 6.65 (1H, s, H-8), 4.71 (1H, t, $J = 9.0$ Hz, H-2′), 3.32 (2H, m, H-3′, overlapped), 1.15 (3H, s, C-4′-CH_3), 1.14 (3H, s, C-4′-CH_3), 3.82 (3H, s, CH_3O-), 4.52 (2H, br s, C_2-CH_2-O-), 4.28 (1H, d, $J = 8.0$ Hz, Glc H-1), 3.02–3.66 (4H, m, sugar protons). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 164.6 (C-2), 110.0 (C-3), 175.5 (C-4), 111.4 (C-4a), 162.4 (C-5), 117.5 (C-6), 158.9 (C-7), 93.3 (C-8), 155.1 (C-8a), 91.1 (C-2′), 27.0 (C-3′), 70.0 (C-4′), 65.1 (C_2-CH_2-), 60.3 ($-OCH_3$), 25.8 (C-4′-CH_3), 24.9 (C-4′-CH_3), 102.4 (Glc C-1), 73.4 (Glc C-2), 76.6 (Glc C-3), 70.0 (Glc C-4), 77.1 (Glc C-5), 61.1 (Glc C-6). Comparing the above data with Ref. [9,10] the obtained product was identified as prim-O-glucosyl-cinmifugin.

Peak II: ^{1}H-NMR (400 MHz, DMSO-d_6): δ 5.96 (1H, br s, H-3), 6.65 (1H, s, H-8), 4.84 (1H, dd, $J = 8.5$, 9.0 Hz, H-2′), 3.32 (2H, m, H-3′, overlapped), 2.26 (3H, s, C-2-CH_3), 1.26 (3H, s, C-4′-CH_3), 1.24 (3H, s, C-4′-CH_3), 3.81 (3H, s, CH_3O-), 4.40 (1H, d, $J = 7.6$ Hz, Glc H-1), 3.02–3.40 (4H, m, sugar protons). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 163.9 (C-2), 111.2 (C-3), 175.9 (C-4), 111.4 (C-4a), 164.5 (C-5), 117.8 (C-6), 159.4 (C-7), 93.6 (C-8), 155.5 (C-8a), 90.5 (C-2′), 27.6 (C-3′), 77.3 (C-4′), 19.6 (C_2-CH_3), 60.7 ($-OCH_3$), 22.3 (C-4′-CH_3), 23.5 (C-4′-CH_3), 97.7 (Glc C-1), 73.9 (Glc C-2), 76.9 (Glc C-3), 70.3 (Glc C-4), 77.1 (Glc C-5), 61.2 (Glc C-6). Comparing the above data with Ref. [10,11] the obtained product was identified as 4′-O-β-D-Gluxosyl-5-O-methylvisamminol.

In conclusion, the results of this study clearly demonstrated that HSCCC is very successful for separation and purification of prim-O-glucosyl-cinmifugin and 4′-O-β-D-glucosyl-5-O-methylvisamminol from the root of *Saposhnikovia divaricata (Turcz.) Schischk.*

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