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Application of preparative high-speed counter-current chromatography for isolation and separation of schizandrin and gomisin A from *Schisandra chinensis*

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Abstract

Following an initial cleaning-up step on the D101 macroporous resin, a preparative high-speed counter-current chromatography (HSCCC) with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:0.9:0.9:1, v/v) was used to isolate and separate schizandrin and gomisin A from *Schisandra chinensis*. A total of 107 mg schizandrin and 36 mg gomisin A with purities of 99.5% and 99.1% were obtained from 400 mg crude extract in one-step elution and less than 3 h, and the structure identification was performed by UV, IR, MS, ^{1}H NMR and ^{13}C NMR.

Keywords: Preparative chromatography; Counter-current chromatography; Schizandrin; Gomisin A; *Schisandra chinensis*

1. Introduction

Schisandra chinensis (*Beiwuweizi* in Chinese), a famous traditional Chinese medicine, grows wild in the most Eastern parts of Russia, the Kuril islands, southern Sachalin and also north-eastern China (Jilin, Liaoning, Heilongjiang and Hebei provinces), Korea and Japan [1], and is officially listed in the Chinese Pharmacopoeia [2]. It is irregularly round or oblate in shape and 5–8 mm in diameter. The surface of the fruit is red or dark red in color, wrinkly and glossy, and the pulp is very soft, faint in smell and sour in taste. This plant has been revealed a widely variety of active effects including antihepatotoxic effect, antioxidant and detoxificant effect, anticarcinogenic effect, activity on central nervous system and counteract the fatigue, increase the durance, and improve the physical performance of sportsmen [3–5]. The major active constituents of *Beiwuweizi* are considered to be lignans including schizandrin, gomisin A, schisantherin A, deoxyschizandrin, d-epigalbacine and so on. Now pharmacological tests revealed that schizandrin (Fig. 1) can protect the apoptosis of cultured PC12 cells induced by 6-OHDA [6] and liver against carbon tetrachlorode induced damage [7], and show scavenging effect on active oxygen radicals [8]; gomisin A (Fig. 1) can inhibit development of preneoplastic lesions in rat liver induced by 3′-methyl-4-dimethylamino-azobenzene (3′-MeDAB) [9], protect chronic liver injury induced by CCl_4 [10] and show preventive effect on acetaminophen-induced hexpatoxicity in rats [11].

In view of these beneficial effects, a large quantity of pure materials are urgently needed for further studies. The preparative separation of schizandrin and gomisin A from medicinal plants by classical methods are tedious, time consuming, requiring multiple chromatographic steps on silica gel, polyamide column, etc. So, an efficient method for the preparative separation and isolation of schizandrin and gomisin A from natural plants is warranted. High-speed counter-current chromatography (HSCCC), a support free liquid–liquid partition chromatographic technique, eliminates irreversible adsorption of the sample onto solid support

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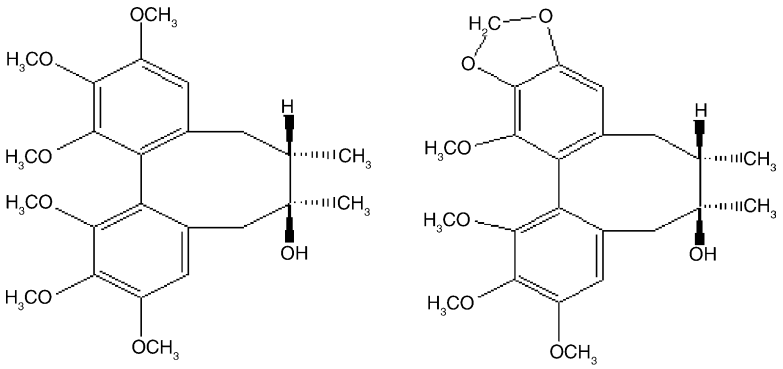

Fig. 1. The chemical structures of schizandrin and gomisin A.

[12], has an excellent sample recovery. The method permits directly introduction of crude samples into the column without more preparation, so it has been successfully applied to isolate and purify a number of natural products [13–17], but there have no reports of using HSCCC to isolate and separate schizandrin and gomisin A from *S. chinensis*.

The aim of the present paper, therefore, was to develop an efficient method to isolate and purify schizandrin and gomisin A from *S. chinensis* by HSCCC.

2. Experimental

2.1. Apparatus

Preparative HSCCC was carried out with a model TBE-300A high-speed counter-current chromatography (Shenzhen, Tauto Biotech, China). The apparatus equipped with a polytetrafluoroethylene three preparative coils (diameter of tube, 2.6 mm, total volume, 300 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 value varied from 0.5 at the internal terminal to 0.8 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 S constant-flow pump, a model UV-II detector operating at 254 nm, and a model N2010 workstation (Zhejiang University, Hangzhou, China). The experimental temperature was adjusted by HX 1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China).

The analytical HPLC system used throughout this study consisted of 515 pump (Waters, USA), 2487 detector (Waters), and a model N2000 workstation (Zhejiang University, Hangzhou, China) and a 20 μl sample loop. D101 macroporous resin was purchased from the Chemical Plant of Nankai University (Tianjin, China).

2.2. Reagents

Ethyl acetate, *n*-hexane, methanol, ethanol, acetic acid were analytical grade and purchased from WuLian Chemical Factory (Shanghai, China). Acetonitrile was HPLC grade (Merck, Germany). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. The *S. chinensis* was purchased from a local drug store.

2.3. Preparation of the crude extract

The *S. chinensis* was ground into powder, 4000 ml volume of 80% aqueous ethanol was added to a bottle (5000 ml), which contained 500 g of the powder for the extraction. The extraction was reflux in a haven for two times and 1.5 h for each. United the filter and evaporated to no ethanol under reduced pressure at 60 °C and the residue was obtained, then the residue was redissolved in water, which was added into a glass column (5 cm × 80 cm, contained 600 g D101 macroporous resin). Water was first used to elute the resin until the elution was nearly no color, then 2500 ml 60% aqueous ethanol was used to elute the target compounds, and 10 elution fractions (250 ml for each) were collected and 6 (from 3 to 8 fraction) were united and evaporated to dryness according to HPLC analysis, which was used for further HSCCC isolation and separation.

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

Two-phase solvent systems were used in the present study, *n*-hexane–ethyl acetate–methanol–water (10:9:9:10, v/v) was prepared. The solvent mixture was thoroughly equilibrated in a separated funnel at room temperature and the two phases were separated shortly before use. The sample solution was prepared by dissolving the sample in the 15 ml lower phase of solvent system for isolation and purification.

2.5. HSCCC separation procedure

The coil column was first entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 800 rpm, while the lower phase was pumped into the column at a flow rate of 2.2 ml/min. After the mobile phase front

emerged and hydrodynamic equilibrium was established in the column, about 15 ml sample solution containing 400 mg of the crude extract was injected through the injection value. The effluent of the column was continuously monitored with a UV–vis detector at 254 nm. Peak fractions were collected according to the elution profile. The temperature of the apparatus was set at 25 °C.

2.6. Analysis and identification of HSCCC peak fractions

The crude extract after cleaning-up by macroporous resin and the fractions obtained by HSCCC were analyzed by HPLC. The analyses were performed with a reversed-phase LiChrospher C_{18} (150 mm × 6.0 mm I.D., 5 μm) (Hanbang Science, Jiang Su province, China) at a column temperature of 25 °C. The mobile phase composed of CH_3CN–H_2O–HAC (60:40:2, v/v/v) was eluted isocratically at a flow rate of 1.0 ml/min and the effluent monitored at 254 nm.

Identification of HSCCC fractions was carried out by UV (Cary-50), IR (Hitachi 275-50), MS (Finnigan MAT 711), ^{1}H NMR and ^{13}C NMR spectra (Varian Unity Inova-500).

3. Results and discussion

3.1. HPLC analysis of the crude extract

The crude extract was first analyzed by HPLC. An excellent separation of schizandrin and gomisin A could be achieved under the following conditions: the mobile phase composed of acetonitrile–water–acetic acid (60:40:2, v/v/v) was eluted isocratically at a flow rate of 1.0 ml/min, the column temperature and detection wavelength were set at 25 °C and 254 nm. No complex gradient of mobile phase and no buffer were necessary. The HPLC chromatogram of the crude extract is shown in Fig. 2A. Peaks 1 and 2 correspond to schizandrin and gomisin A, respectively.

Table 1
The partition coefficient and separation factor of schizandrin and gomisin A in different solvent systems

Solvent system (*n*-hexane–ethyl acetate–methanol–water)	Schizandrin (K_1)	Separation factor (α)	Gomisin A (K_2)
10:5:5:10	2.07	1.77	3.66
10:7:7:10	1.35	2.10	2.83
10:9:9:10	0.55	2.20	1.21
10:11:11:10	0.12	2.83	0.34
10:13:13:10	0.08	3.12	0.25

3.2. Selection of two-phase solvent system and other conditions of HSCCC

In our research, the solvent system composed of *n*-hexane–ethyl acetate–methanol–water at different volume ratios was investigated. This two-phase solvent system could be used to isolate and separate a broad range of hydrophobicity by modifying the volume ratio of the four solvents. Table 1 shows that *n*-hexane–ethyl acetate–methanol–water ratios ranging from 10:5:5:10, 10:7:7:10, 10:9:9:10, 10:11:11:10, 10:13:13:10 can be used to isolate the crude extract. Lots of experiments were performed to optimized the best suitable

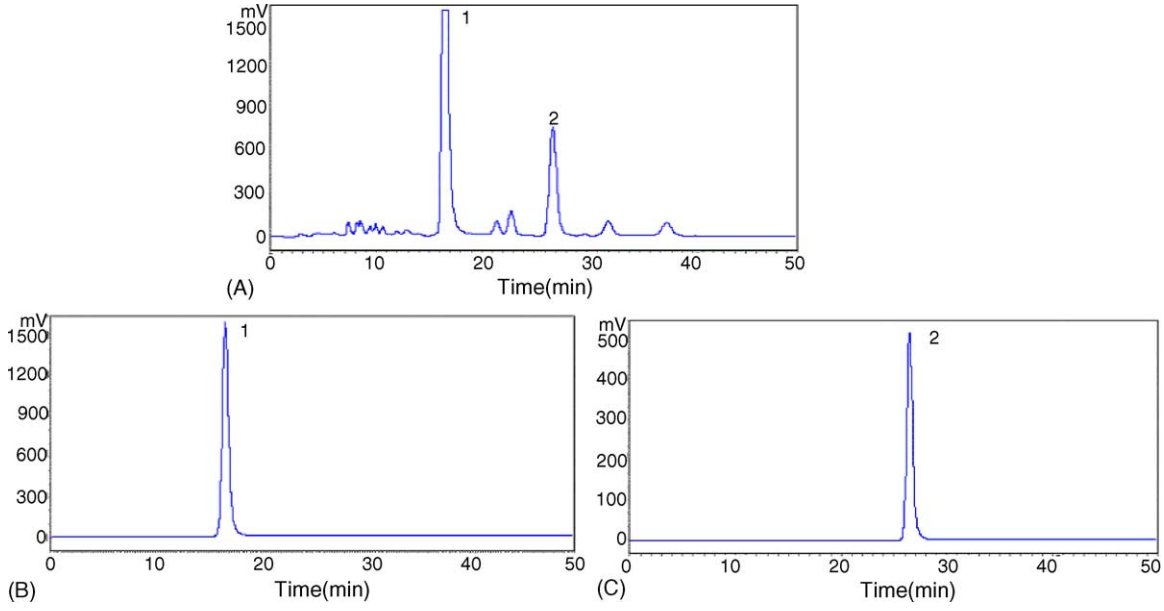

Fig. 2. HPLC chromatograms of crude extract from *Schisandra chinensis* after cleaning-up by D101 macroporous resin and the fractions obtained by HSCCC. Column: reversed-phase LiChrospher C_{18} (150 mm × 6.0 mm I.D., 5 μm); mobile phase: CH_3CN–H_2O–HAC (60:40:2, v/v/v); flow rate: 1.0 ml/min; UV wavelength: 254 nm; column temperature: 25 °C; A: crude extract; B: fraction "I"; C: fraction "II"; 1: schizandrin; 2: gomisin A.

using the above solvent systems. The K and α values of the target compounds in different solvent systems are shown in Table 1. The results indicated that the solvent system composed of *n*-hexane–ethyl acetate–methanol–water at the volume ratios of 10:5:5:10 (v/v) and 10:7:7:10 (v/v) had large K values, while small K values would be produced at the volume ratios of 10:11:11:10 (v/v) and 10:13:13:10 (v/v), but appropriate k and α values can be obtained at a the volume ration of 10:9:9:10 (v/v). So, the two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (10:9:9:10, v/v) was selected to isolate and purity the target compounds in the present paper.

The influence of flow rate of mobile phase, the separation temperature and the revolution speed were also investigated. The result indicated that slow flow speed can produce a good separation, but more time and more mobile phase will be needed, and the chromatogram peak was extended. Considering these aspects, the flow rate was selected 2.2 ml/min in the present study. The temperature has significant effect on K values, the retention of stationary phase and the mutual solvency of the two phase. After tested at 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 40 °C, it can be seen that good result can be obtained when the separation temperature was controlled at 25 °C. The revolution speed has a great influence to the retention of stationary phase, high rotary speed can increase the retention of the stationary phase. In our experiment, the revolution speed was set at 800 rpm.

Under the optimized conditions, three fractions ("I", "II", "III") were obtained in one-step elution and less than 3 h (HSCCC chromatogram shown in Fig. 3), and the retention of the stationary phase was 65%. The isolation yielded 107 mg schizandrin (fraction "I") and 36 mg gomisin A (fraction "II") with the purities of 99.5% and 99.1%, respectively determined by HPLC (HPLC chromatogram is shown in Fig. 2B and C). As expected, the HPLC analysis of each fraction revealed that the components eluted in the order of peaks 1 (schizandrin) and 2 (gomisin A). But, the fraction "III" was un-pure fraction and contained several peaks (HPLC chromatogram was not shown).

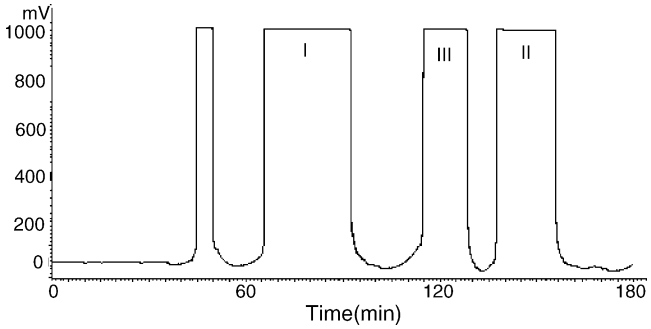

Fig. 3. HSCCC chromatogram of the crude extract from *Schisandra chinensis* after cleaning-up by D101 macroporous resin. Solvent system: *n*-hexane–ethyl acetate–methanol–water (10:9:9:10, v/v); stationary phase: upper phase; mobile phase: lower phase; flow rate: 2.2 ml/min; revolution speed: 800 rpm; separation temperature: 25 °C; sample size: 400 mg; retention of stationary phase: 65%; detection wavelength: 254 nm.

3.3. The structural identification

The structural identification of the isolated components was performed UV, IR, MS, ^{1}H NMR and ^{13}C NMR as follows. Peak "I" (schizandrin): a white amorphous powder; mp 128–130 °C; UV λ_{max}^{MeOH} (log ε): 217.5 (4.64), 251.2 (4.20), 276.5 (3.53). IRυ_{max} (KBr) cm^{-1}: 3490 (OH), 1593 (aromatic); MS *m/z* (%): 432 (M$^+$, 100), 414 (19), 361 (28), 330 (83), 44 (100). Peak "II" (gomisin A): a white amorphous powder; mp 86–88 °C; UV λ_{max}^{MeOH} (logε): 219.0 (4.61), 251.5 (4.06), 273.0 (3.59); IRυ_{max} (KBr) cm^{-1}: 4360 (OH), 1615, 1595 (aromatic); MS *m/z* (%): 416 (M$^+$, 72), 398 (2), 314 (47), 299 (10), 172 (8), 101(32), 59 (59). Comparing with the reported data, the UV, IR, ^{1}H NMR and ^{13}C NMR data are in agreement with those of schizandrin and gomisin A in literatures [18–22].

4. Conclusion

Our study demonstrates that HSCCC is a powerful method in separating and isolating bioactive components from natural sources. Using HSCCC two lignans including schizandrin and gomisin A are separated from *S. chinensis* with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (10:9:9:10, v/v). One hundred and seven milligrams schizandrin and 36 mg gomisin A with high purities were obtained from 400 mg crude extract in one-step elution and less than 3 h. The method is simple, fast and without complex solvent system or gradient elution.

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