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Short communication

Preparative isolation and purification of isoflavan and pterocarpan glycosides from *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by high-speed counter-current chromatography

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Abstract

(3*R*)-(−)-7,2′-Dihydroxy-3′,4′-dimethyl isoflavan-7-*O*-β-D-glucopyranoside and (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-3-*O*-β-D-glucopyranoside were separated from the ethyl acetate extract of the root of *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by high-speed counter-current chromatography (HSCCC). A two-phase system composed of ethyl acetate–ethanol–acetic acid–water (4:1:0.25:5, v/v) was selected by analytical HSCCC. Preparative HSCCC yielded, from 100 mg of the partially purified extract, 50 mg of isoflavan glycoside and 10 mg of pterocarpan glycoside each at over 95% purity by high-performance liquid chromatography (HPLC) analysis. Their structures were identified by MS, ^{1}H NMR and ^{13}C NMR.

Keywords: Counter-current chromatography; Preparative chromatography; *Astragalus membranaceus*; Plant materials; Pharmaceutical analysis; Isoflavan; Pterocarpan; Glycosides

1. Introduction

The roots of *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao, known as Huangqi in China, is the most important tonic in the traditional Chinese medicine to reinforce “q i” (vital energy), to strengthen the superficial resistance, and to promote the discharge of pus and the growth of new tissue. It has long been used as an anti-perspirant, anti-diuretic or atonic [1–4].

The flavonoids were well known as one group of the beneficial components [2–7], and have suitable chromophores for UV detection so that it has been chosen as marker compounds for the chemical evaluation or standardization of Huangqi and its products [4,8,9].

High-speed counter-current chromatography (HSCCC) is considered as a suitable alternative for the separation of phenolic compounds such as flavonoids and hydroxyanthraquinones [10–12].

The present paper introduced a method for the separation of (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-3-*O*-β-D-glucopyranoside and (3*R*)-(−)-7,2′-hydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucopyranoside, whose chemical structures are given in Fig. 1, from the extract of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao by HSCCC.

2. Experimental

2.1. Apparatus

The analytical HSCCC instrument employed in the present study is a Model GS 20 analytical high-speed counter-current chromatograph designed and constructed in Beijing Institute of New Technology Application (Beijing, China). The apparatus holds a pair of column holders symmetrically on the rotary frame at a distance of 5 cm

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R = β-D-glucose

Fig. 1. Chemical structures of (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-7-*O*-β-D-glucopyranoside and (3*R*)-(−)-7,2′-hydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucopyranoside from *A. membranaceus* var. *mongholicus*.

from the central axis of the centrifuge. The multilayer coil separation column was prepared by winding a 50 m long, 0.85 mm i.d. PTFE (polytetrafluoroethylene) tube directly on to the holder hub forming multiple coiled layers with a total capacity of 40 ml. The β-value varied from 0.4 at the internal terminal to 0.7 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder axis and central axis of the centrifuge). Although the revolution speed of the apparatus could be regulated with a speed controller in the range between 0 and 2000 rpm, an optimum speed of 1500 rpm was used in the present studies.

Preparative HSCCC was performed using a Model GS10A2 multilayer coil of 110 m long, 1.6 mm i.d. PTFE tubing with a total capacity of 190 ml. The β values of this preparative column range from 0.5 to 0.8. The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application, Beijing, China). Continuous monitoring of the effluent was achieved with a Model 8823A-UV Monitor (Beijing Institute of New Technology Application, Beijing, China) at 280 nm. A manual sample injection valve with a 1.0 ml loop (for the analytical HSCCC) or a 20 ml loop (for the preparative HSCCC) (Tianjin High New Science Technology Company, Tianjin, China) was used to introduce the sample into the column, respectively. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

The high-performance liquid chromatography (HPLC) equipment used was a Millennium^{32} system including a Waters 2487 dual-wavelength absorbance detecstor, a Waters 600E Multisolvent Delivery System, a Waters 600 system controller, a Waters Delta 600 pump, and a Millennium^{32} workstation (Waters, USA).

2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. Acetonitrile used for HPLC analysis was of chromatographic grade. Raw roots of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao were purchased from Shanxi Hunyuan Traditional Chinese Medicine Company, Shanxi, China.

2.3. Extraction of crude samples

Raw roots (15 kg) of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao were extracted three times with 50% aqueous ethanol. Then, the extract was combined and evaporated to dryness under reduced pressure, which yielded 4.1 kg of dry powder. Then, 1.2 kg of the residue obtained from the combined extract was dissolved with 6 l water. After filtration, the aqueous solution was extracted three times with 5 l of water-saturated chloroform, ethyl acetate and *n*-butanol successively which yielded 10 g of chloroform extract, 14.5 g of ethyl acetate extract and 180 g of *n*-butanol extract after being combined and evaporated to dryness under reduced pressure. The ethyl acetate extract was further subjected to silica gel chromatography (800 g of silica gel G 60, Qingdao Haiyang Chemica, Qingdao, China) by eluting stepwise with chloroform–methanol (100:1, 50:1, 30:1, 20:1, 15:1, 8:1, 4:1 and 2:1, v/v) to obtain eight fractions. Among those, fraction five yielded 200 mg of dried material. Portions of this partially purified sample of *A. membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao was subjected to HSCCC.

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

For the present study, we selected a two-phase solvent system composed of ethyl acetate–ethanol–acetic acid–water at volume ratios of 4:1:0.25:5 (v/v). The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use.

The sample solutions were prepared by dissolving the partially purified extract in the lower phase at suitable concentrations according to the analytical or the preparative purpose.

2.5. Separation procedure

Analytical HSCCC was performed with a Model GS 20 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper phase. The lower

phase was then pumped into the head end of the column inlet at a flow-rate of 1.0 ml/min, while the apparatus was run at a revolution speed of 1800 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (10 mg in 1 ml of lower organic phase) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV detector at 280 nm. Each peak fraction was collected according to the chromatogram. Preparative HSCCC was similarly performed with a Model GS 10A2 HSCCC instrument as follows: the multilayer coiled column was first entirely filled with the upper phase as a stationary phase. Then the sample solution (100 mg in 20 ml of the upper organic phase) was injected through the sample port and the aqueous mobile phase was pumped through the column at a flow-rate of 2.0 ml/min while the column was rotated at 800 rpm. The effluent from the outlet of the column was monitored with a UV detector at 280 nm. Peak fractions were manually collected according to the chromatogram.

2.6. HPLC analysis and identification of CCC peak fractions

The partially purified extract of *A. membranaceus* var. *mongholicus* and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a SymmetryShield RP_{18} column (250 mm × 6 mm i.d.). The mobile phase composed of acetonitrile–water (25:75, v/v) was eluted at a flow-rate of 1.0 ml/min, and the effluent monitored by a dual-wavelength absorbance detector.

Identification of HSCCC peak fractions was carried out by MS, ^{1}H NMR and ^{13}C NMR spectra. NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer with TMS as internal standard. FABMS was obtained on a KYKY-ZHP-5# mass spectrometer.

3. Results and discussion

The partially purified sample of *A. membranaceus var. mongholicus* was analysed by HPLC. The result indicates that it contains several compounds including (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-3-*O*-β-D-glucopyranoside (about 50%) and (3*R*)-(−)-7,2′-dihydroxy-3′,4′-dimethylisoflavan-7-*O*-β-D-glucopyranoside (about 15%) and some unknown compounds (see Fig. 3A).

In order to achieve an efficient resolution of target compounds, the two-phase solvent system of ethyl acetate–ethanol–acetic acid–water was examined at different volume ratios such as 10:1:0:10, 5:1:0.1:5, 4:1:0.1:5, 2:1:0.25:3, 4:1:0.25:5 using analytical HSCCC. The result indicated that the volume ratio of 4:1:0.25:5 could separate (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-3-*O*-β-D-glucopyranoside and (3*R*)-(−)-7, 2′-dihydroxy-3′, 4′-dimethylisoflavan-7-*O*-β-D-glucopyranoside well.

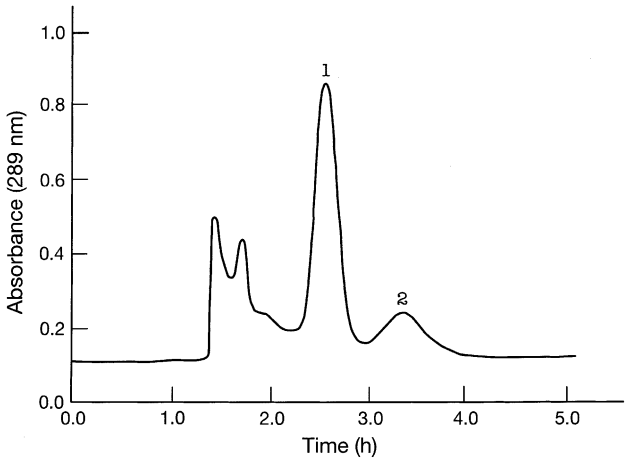

Fig. 2. Chromatogram of the partially purified extract from *Astragalus membranaceus* var. *mongholicus* by preparative HSCCC. Peak 1: (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-7-*O*-β-D-glucopyranoside; Peak 2: (3*R*)-(−)-7,2′-hydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucopyranoside. Solvent system: ethyl acetate–ethanol–acetic acid–water (4:1:0.25:5, v/v); stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2.0 ml/min; revolution speed: 800 rpm; sample: 100 mg dissolved in 20 ml lower phase; retention of the stationary phase: 60%.

Fig. 2 shows the result obtained from 100 mg of the partially purified extract of *A. membranaceus* var. *mongholicus* by preparative HSCCC. After this separation, the fractions containing (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-3-*O*-β-D-glucopyranoside and (3*R*)-(−)-7,2′-dihydroxy-3′,4′-dimethylisoflavan-7-*O*-β-D-glucopyranoside were collected, respectively. The analysis of these fractions indicated that the Peak 1 fraction contained (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-3-*O*-β-D-glucopyranoside which weighs 50 mg at over 95% purity and the Peak 2 fraction contained (3*R*)-(−)-7, 2′-dihydroxy-3′, 4′-dimethylisoflavan-7-*O*-β-D-glucopyranoside which weighs 10 mg at over 95% purity as determined by HPLC (Fig. 3B and C).

The structural identification of the first component (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-3-*O*-β-D-glucopyranoside, was carried out by MS, ^{1}H NMR and ^{13}C NMR spectra as follows:

MS: 463 (M^{+} + 1), 301 (M^{+} + 1-glucose unit). ^{1}H NMR{500 MHz, [$^{2}H_6$] dimethyl sulfoxide (DMSO-d_6)} δ ppm: 3.40–3.90 (6H), 3.42 (1H, m, H-6), 3.70 (1H, m, H-6a), 3.77 (1H, s, C_{10}-OMe), 3.78 (3H, s, C_9-OMe), 4.29 (1H, d, J = 6.5 Hz, H-6), 4.95 (1H, d, J = 6.5 Hz, anomeric), 5.58 (1H, d, J = 6.5 Hz, H-11a), 6.53 (1H, d, J = 8.5 Hz, J = 1.5 Hz, H-8), 6.58 (1H, d, J = 1.5 Hz, H-2), 6.98 (1H, d, J = 8 Hz, H-7), 7.44 (1H, d, J = 8.5 Hz, H-1). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm: 132.0 (C-1), 110.5 (C-2), 158.5 (C-3), 104.0 (C-4), 156.2 (C-4a), 65.7 (C-6), 39.8 (C-6a), 121.6 (C-6b), 118.8 (C-7), 105.1 (C-8), 152.7 (C-9), 133.3 (C-10), 151.0 (C-11), 78.2 (C-11a), 114.0 (C-11b), 100.3 (C-1′), 73.2 (C-2′), 76.5 (C-3′), 69.6 (C-4′), 77.0 (C-5′), 65.7 (C-6′), 59.9 (C_9-OMe), 56.1 (C_{10}-OMe). Comparing with the reported data, the ^{1}H NMR

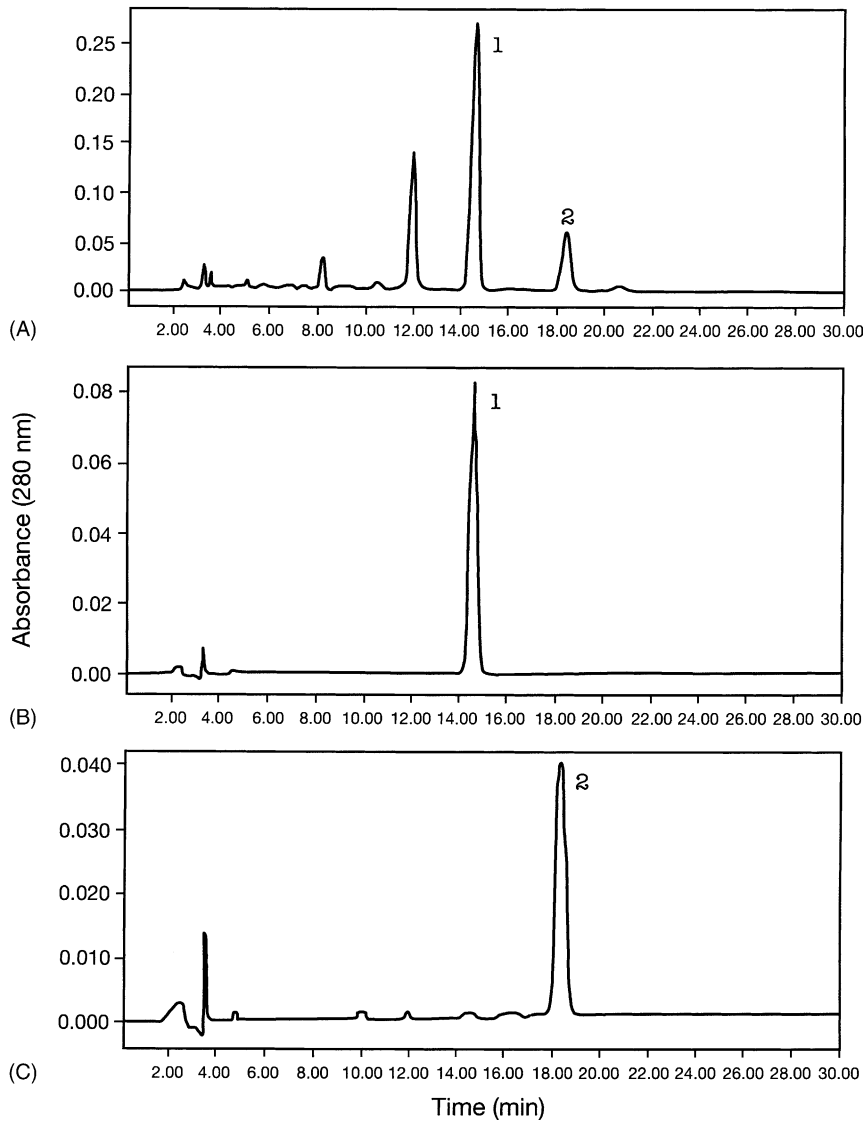

Fig. 3. The result of HPLC analyses of the partially purified sample from *Astragalus membranaceus* var. *mongholicus*. Column: SymmetryShield RP_{18} column (250 mm × 6 mm i.d.); mobile phase: acetonitrile–water (25:75, v/v); flow-rate: 1.0 ml/min (A) The original sample; (B) HSCCC fraction from Peak 1 (Fig. 2); (C) HSCCC fraction from Peak 2 (Fig. 2).

and ^{13}C NMR data are in agreement with those of (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-3-*O*-β-D-glucopyranoside [13].

The structural identification of the second component (3*R*)-(−)-7,2′-dihydroxy-3′,4′-dimethylisoflavan-7-*O*-β-D-glucopyranoside, was carried out by ^{1}H NMR and ^{13}C NMR spectra as follows: ^{1}H NMR (500 MHz, DMSO-d_6) δ ppm: 3.96 (1H, t, $J = 10.5$ Hz, H-2), 4.20 (1H, ddd, $J = 10.5$ Hz, $J = 3.5$ Hz, $J = 1.5$, H-2), 3.36 (1H, dddd, $J = 10.5$ Hz, $J = 11$ Hz, $J = 5$ Hz, $J = 3.5$ Hz, H-3), 2.81 (1H, ddd, $J = 16.5$ Hz, $J = 5$ Hz, $J = 1.5$ Hz, H-4), 2.93 (1H, dd, $J = 16.5$ Hz, $J = 11$ Hz, H-4), 7.00 (1H, d, $J = 8.5$ Hz, H-5), 5.55 (1H, dd, $J = 8.5$ Hz, 2.5 Hz, H-6), 6.47 (1H, d, $J = 2.5$ Hz, H-8), 6.46 (1H, d, $J = 9$ Hz, H-5, 6.78 (1H, d, $J = 9$ Hz, H-6, 4.76 (1H,d, H-1), 3.20 (1H, dd, $J = 9$ Hz, $J = 8$ Hz, H-2), 3.26 (1H, d, $J = 9$ Hz, H-3), 3.15 (1H, t, $J = 9$ Hz, H-4), 3.29 (1H, ddd, $J = 9$ Hz, $J = 6$ Hz, $J = 2$ Hz, H-5), 3.46 (1H, dd, $J = 12$ Hz, $J = 6$ Hz, H-6), 3.69 (1H, dd, $J = 12$ Hz, $J = 2$ Hz, H-6″), 3.69 (3H, s, $C_{3'}$-OMe), 3.75 (3H, s, $C_{4'}$-OMe). ^{13}C NMR (500 MHz, DMSO-d_6) δ ppm: 69.2 (C-2), 31.3 (C-3), 29.7 (C-4), 115.7 (C-4a), 130.0 (C-5), 108.7 (C-6), 156.7 (C-7), 103.8 (C-8), 154.5 (C-8a), 120.8 (C-1′), 148.1 (C-2′), 136.1 (C-3′), 151.6 (C-4, 103.1 (C-5, 121.4 (C-6, 100.7 (C-1), 73.2 (C-2), 76.6 (-3), 69.7 (C-4), 77.0 (C-5), 60.7 (C-6), 60.2 (C_3 OMe), 55.6 (C_4 OMe). Comparing with the reported data, the ^{1}H NMR and ^{13}C NMR data are in agreement with those of (3*R*)-(−)-7,2′-dihydroxy-3′,4′-dimethylisoflavan-7-*O*-β-D-glucopyranoside [14].

The result of our studies described above clearly demonstrated that HSCCC is very successful in the preparative separation of (6a*R*, 11a*R*) 9,10-di-methoxypterocarpan-3-

O-β-D-glucopyranoside and (3*R*)-(−)-7,2′-dihydroxy-3′,4′-dimethylisoflavan-7-*O*-β-D-glucopyranoside from *A. membranaceus* var. *mongholicus*.

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