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One-step Preparative Separation of Two Polyhydroxystilbenes from *Rheum likiangense* Sam. by High-speed Counter-current Chromatography

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ABSTRACT:

Introduction – The official rhubarb species have frequently been investigated for their hydroxyanthraquinone components and associated pharmacological properties. However, other unofficial rhubarb species were rarely studied until polyhydroxystilbenes (PHS), which commonly occur in the unofficial rhubarb, revealed a range of potential bioactivities. Hence, there has been increasing interest in the efficient preparation of high-purity PHS for pharmacological and clinical trials.

Objective – To develop a suitable method for large-scale preparative separation of PHS from the rhizome of *Rheum likiangense Sam*. by high-speed counter-current chromatography (HSCCC).

Results – Two PHS compounds were isolated successfully within 440 min using a solvent system consisting of methanol: *n*-butanol:chloroform:water (2:0.5:3:3, v/v/v/v). Eighty-four milligrams of desoxyrhaponticin with 98.2% purity and 148 mg of rhaponticin with 95.3% purity were respectively yielded from 1.5 g of crude extract in the single, one-step operation.

Conclusion – An optimised HSCCC method has been established for large-scale preparative separation of PHS compounds from *Rheum likiangense* Sam.

Supporting information may be found in the online version of this article.

Keywords: High-speed counter-current chromatography; one-step; preparative separation; polyhydroxystilbenes; *Rheum likiangense* Sam

Introduction

Rhei Rhizoma (rhubarb), commonly known as Da-huang in Chinese, is a purgative agent, which has been used in treatment of blood stagnation syndrome in traditional Asian medicines for 2000 yr (Xiao *et al*., 1984). In Chinese Pharmacopoeia (Shao *et al*., 2010), the official Da-huang is prescribed as the dried rhizome of *Rheum palmatum* L., *R. tanguticum* Maxim. ex Balf., and *R. officinale* Baill. of the family Polygonaceae. Hydroxyanthraquinones (HAQs) are generally considered as the major bioactive constituents of medical rhubarb due to their significant contributions to the cathartic and hemostasis effects. Five HAQs (e.g., rhein, emodin, aloe-emodin, physcion and chrysophanol) are officially used as the criteria for quality control of Da-huang products (Kashiwada *et al*., 1988). Among these, three official rhubarbs and the pharmacological properties of HAQs isolated from them have been studied intensively in recent years, whereas more than forty other rhubarb species of the genus *Rheum* distributed in China have rarely been studied.

Polyhydroxystilbenes (PHS) are believed to occur commonly in many unofficial Da-huang species and have raised increasing attention due to their anti-tumour (Jang *et al*., 1979; Lee *et al*., 2002), anti-diabetic (Li *et al*., 2007), coronary heart protecting (Renaud and de Lorgeril, 1992), anti-hyperlipidemic (Matsuda *et al*., 2001), liver damage reducing (Raal *et al*., 2009) and anti-adipositas (Tran *et al*., 2008) effects, as well as other potential bioactivities (Tadashi *et al*., 2001). Hence, the requirement of high-purity PHS for pharmacological and clinical trials has increased the interest in their preparation. Conventional methods for PHS preparative separation have low efficiency. Compared with traditional, liquid–solid separation methods, high-speed counter-current chromatography (HSCCC) without solid support matrix is advantageous and can eliminate the irreversible adsorptive sample loss, column deactivation, tailing of solute peaks and contamination (Ito, 2005). Successful applications of HSCCC have been reported previously for purifying different natural products (Deng *et al*., 2009; Yang *et al*., 2009; Guo *et al*., 2010).

In this study, we employed HSCCC in a forward–reverse alternate rotation mode for large-scale preparative separation of two PHS components (Fig. 1) from the rhizome of *Rheum likiangense* Sam., for which neither chemical nor pharmacological studies have been reported previously.

Materials and methods

Apparatus

The apparatus used for preparative separation was TBE-5000A HSCCC (Tauto Biotechnique, Shanghai, China) equipped with

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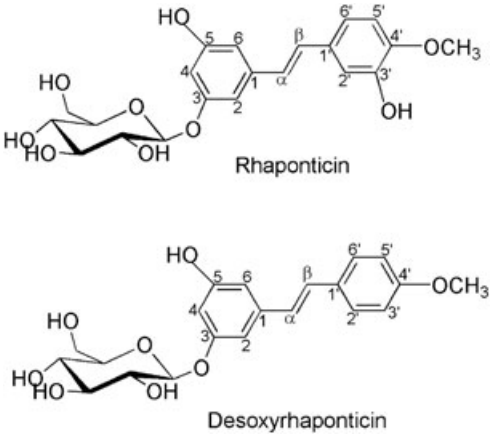

Figure 1. Chemical structures of two polyhydroxystilbenes from *Rheum likiangense* Sam.

three multilayer coil separation columns connected in series (tubing i.d. = 1.6 mm, total capacity = 4800 mL) and a sample injection valve with a 200 mL sample loop. The revolution speed of the apparatus was adjustable between 0 and 550 rpm using a speed controller. An A¨KTA prime system (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement at 313 nm. A HX 2050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China) was used to control the separation temperature. The data were collected using a Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Co. Ltd., Hangzhou, China).

For HPLC analysis, we used an Agilent 1200 HPLC system equipped with a G1311A solvent delivery unit, a G1332A degasser, a diode-array detector (DAD) and Agilent HPLC workstation. In addition, a Spherigel ODS C_{18}-column (250 mm × 4.6 mm, i.d. = 5 μm) and an Inova 400 MHz NMR spectrometer (Varian Inc., USA) were used.

Reagents and materials

Analytical-grade methanol, *n*-butanol, ethanol and chloroform were purchased from Atoz Fine Chemicals Co. Ltd. (Tianjin, China) and solvents of HPLC grade obtained from Concord Technology Co. Ltd. (Tianjin, China).

The whole plants of *R. likiangense* Sam. were collected from mountainside locations on the Qinghai–Tibetan Plateau (3500–4400 m above sea level) in September 2010. The plants were identified by Zhenlan Wu (Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining, China) and authenticated with the voucher specimen in the Qinghai–Tibetan Plateau Museum of Biology (Xining, China) (reference No. 0210139), as well as the Chinese Virtual Herbarium (http://qtpmb.cvh.org.cn).

Preparation of the two-phase solvent system

The HSCCC was used to test and select two-phase solvent systems composed of different reagents. All the solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature till the two phases were separated. Each phase was separately degassed by sonication for 20 min prior to use. The upper phase was used as the stationary phase.

Preparation of crude extract and sample solution

The air-dried rhizomes of *R. likiangense* Sam. were ground into powder (ca. 60 mesh) in a FZ102 plant disintegrator. Then, 50 g of powder were extracted with 250 mL of 50% ethanol for 3 h at 40 °C combined with ultrasonication. The extraction procedure was repeated twice. All supernatants of the ethanol extracts were combined together and evaporated under reduced pressure at 45 °C. The dried crude extract (7.6 g) was obtained and stored at 4 °C prior to use. The sample solution for HSCCC separation was prepared by dissolving 1.5 g of crude extract in 150 mL of the mobile phase.

Separation by HSCCC

The coiled column was filled with the upper phase and the apparatus rotated at 480 rpm. After the column was entirely filled, the lower phase was pumped through the column at a flow-rate of 25 mL/min as the mobile phase. When the separation column reached hydrodynamic equilibrium, 150 mL of sample solution containing 1.5 g of crude extract was injected. The forward–reverse alternate rotation mode was simultaneously employed for HSCCC separation. The temperature of the separation apparatus was controlled at 25 °C and the effluent continuously monitored with a UV detector at 313 nm. Each target peak fraction was collected manually according to the elution profile and evaporated under reduced pressure.

HPLC-DAD analysis and identification of HSCCC peak fractions

The crude extract of *R. likiangense* Sam. and peak fractions obtained via HSCCC separation were analysed using an Agilent 1200 HPLC system with a Spherigel ODS C_{18}-column (250 mm 4.6 mm, i.d. = 5 μm) at 25 °C. An optimised mobile phase of HPLC was used in the gradient mode. The effluent was monitored at 320 nm using a DAD and the flow rate constantly remained at 1.0 mL/min. Further identification of HSCCC target fractions was carried out using UV spectrum, ^{1}H-NMR and ^{13}C-NMR spectrum recorded by a Inova 400 MHz NMR.

Results and discussion

Optimisation of the HPLC-DAD method

The mobile phase with different components was selected and different elution modes were employed for analysing the crude extract of *R. likiangense* Sam. by HPLC-DAD. Results showed that each peak achieved baseline separation when acetonitrile (A), methanol (B) and pure water (C) were used as the mobile phase in the gradient elution mode (0–20 min, 5% A and 28% B; 20–28 min, 5% A and 45% B; 28–35 min, 5% A and 70% B). The HPLC chromatogram of the crude extract is shown in Fig. 2A.

Optimisation of HSCCC separation

The two-phase solvent system is essential for a successful HSCCC separation based on the liquid–liquid partition method. According to the polarity and general chemical properties of the two PHS components, several solvent systems composed of methanol, *n*-butanol, chloroform and water were tested by changing the volume ratio of *n*-butanol to obtain an optimal partition

coefficient (*K* value), appropriate retention of stationary phase, short settling time of the solvent system, and efficient separation and suitable running time. When the first solvent system of methanol:*n*-butanol:chloroform:water (2:2:3:3) was tested, the analytes were eluted close to the solvent front with poor separation, whereas gradual decrease in the volume ratio of *n*-butanol yielded a better resolution. Finally, good separation results were obtained using the solvent system of methanol:*n*-butanol:chloroform:water (2:0.5:3:3).

During the separation process, the forward–reverse alternate rotation mode was employed to shorten the elution time and prevent the broadening of the latter chromatogram peaks. However, the time point when to switch to the reverse rotation mode after the elution of analytes by the forward rotation mode is

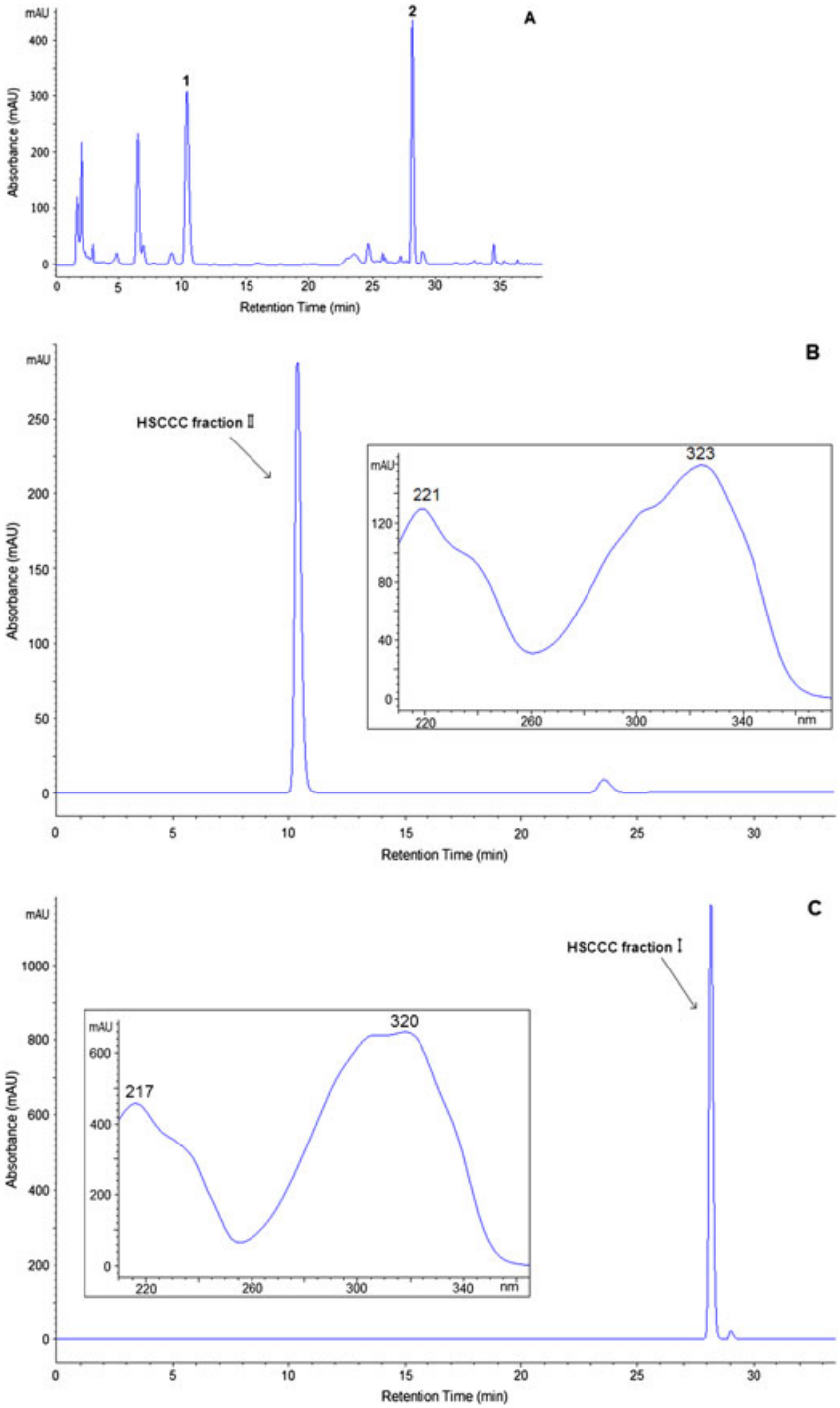

Figure 2. HPLC chromatograms of crude extract from *Rheum likiangense* Sam. and HSCCC peak fractions. (A) Crude extract from *R. likiangense* Sam. (1, rhaponticin, 10.4 min; 2, desoxyrhaponticin, 28.1 min.); (B and C) HSCCC peak fractions II and I of Fig. 3. Conditions: a Spherigel ODS C_{18}-column (250 mm × 4.6 mm i.d., 5 μm); column temperature: 25 °C; mobile phase: acetonitrile (A), methanol (B) and pure water (C) in gradient mode (0–20 min, 5% A and 28% B; 20–28 min, 5% A and 45% B; 28–35 min, 5% A and 70% B); flow rate: 1.0 mL/min; detection wavelength: 320 nm.

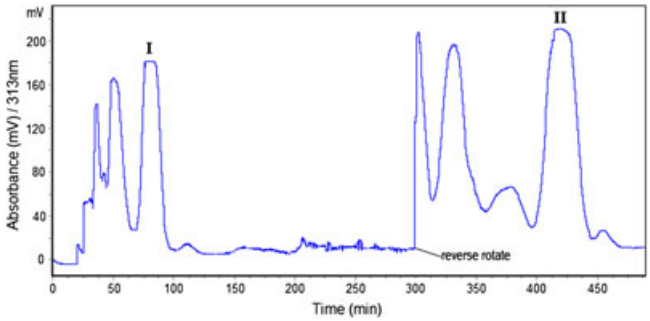

Figure 3. HSCCC chromatogram of crude extract from *Rheum likiangense* Sam.Solvent systems: methanol:*n*-butanol:chloroform:water (2:0.5:3:3); revolution speed: 480 rpm; flow rate: 25 mL/min; temperature: 25 °C; mode: forward rotation (0–300 min), reverse rotation (300–440 min); retention of the stationary phase: 59%; 1.5 g crude extract; detection wavelength: 313 nm; fraction I: desoxyrhaponticin; fraction II: rhaponticin.

critical for complete separation and sharp target peaks. Therefore, we tested three different starting times (at 160, 245 and 300 min) of reverse rotation and found that for an efficient separation of crude sample by HSCCC, the reverse rotation mode should be started at 300 min after forward rotation. In addition, the flow rate of the mobile phase was set to 25 mL/min and the revolution speed was 480 rpm to improve the retention of the stationary phase and the separation.

Despite the influence of temperature on the partition coefficient (*K* value) and the mutual solvency of the two phases, the temperature of this preparative separation was constantly controlled at 25 °C to meet the energy-saving need for further industrial application.

Under the optimum HSCCC conditions indicated above, the stationary phase retention was 59%, the *K* value was 1.75 and the total running time of preparative separation was approximately 440 min. Two target HSCCC fractions were obtained, including 84 mg of fraction I and 148 mg of fraction II from 1.5 g of crude extract. The HSCCC separation chromatogram of crude extract from *R. likiangense* Sam. is shown in Fig. 3.

HPLC-DAD analysis and HSCCC fraction identification

The HSCCC target fractions of the two PHS components were analysed by HPLC-DAD. As shown in Fig. 2B and C, fraction II was located at the retention time of 10.4 min with 95.3% purity (Fig. 2B) and fraction I at 28.1 min with 98.2% purity (Fig. 2C).

Final structural evidence for identification of the two HSCCC fractions were obtained by ^{1}H-NMR, ^{13}C-NMR and UV spectrum and is detailed below.

HSCCC fraction I. Colourless needles, m.p. 226–228 °C, showing strong bluish-purple fluorescence by UV irradiation, UV [MeOH, nm (log ε)]: 217(4.31), 307(4.43), 320(4.35) (Fig. 2C), ^{1}H-NMR (DMSO-d_6): δ: 3.1–3.8 (sugar-H), 7.50 (2 H, d, *J* = 8.8 Hz, H-2′, 6′), 7.09 (1 H, d, *J* = 16.4 Hz, H-α), 7.05 (1 H, d, *J* = 16.4 Hz, H-β), 6.92 (2 H, d, *J* = 8.8 Hz, H-3′,5′), 6.73 (1 H, br.s, H-2), 6.57(1 H, br.s, H-6), 6.33(1 H, br.s, H-4), 4.79 (1 H, d, *J* = 7.6 Hz, anomeric-H), 3.75 (3 H, s, $-OCH_3$); ^{13}C-NMR(DMSO-d_6): δ: 159.1 (C-4″), 158.9 (C-3), 158.3 (C-5), 139.3 (C-1), 129.6 (C-1′), 128.3 (C-α), 127.9 (C-2′), 127.9 (C-6′), 126.3 (C-β), 114.2 (C-3′), 114.2 (C-5′), 107.3 (C-2), 105.0 (C-6), 102.9 (C-4), 100.7 (C-1″), 77.1 (C-5″), 76.6 (C-3″), 73.2 (C-2″), 69.7 (C-4″), 60.7 (C-6″), 55.2 ($-OCH_3$).

Based on the chemical evidence, HSCCC fraction I was characterised as 3,5-dihydroxy-4′-methy-loxystilbene-3-*O*-β-D-glucoside (desoxyrhaponticin). This was confirmed by comparing its physical and spectral data with those in the literature (Sung 2000).

HSCCC fraction II. Yellow powder, m.p. 245–247 °C, showing strong bluish-purple fluorescence by UV irradiation, UV [MeOH, nm (log ε)]: 221(4.30), 305(4.40), 323(4.38) (Fig. 2B), ^{1}H-NMR (DMSO-d_6): δ: 3.76–3.11 (sugar-H), 7.01 (1 H, d, *J* = 1.6 Hz, H-2′), 6.98 (1 H, d, *J* = 16.4 Hz, H-α), 6.83 (1 H, d, *J* = 16.4 Hz, H-β), 6.93 (1 H, dd, *J* = 8.4, 1.6 Hz, H-6′), 6.89 (1 H, d, *J* = 8.4 Hz, H-5′), 6.72 (1 H, br.s, H-2), 6.56 (1 H, br.s, H-6), 6.33 (1 H, br.s, H-4), 4.79 (1 H, d, *J* = 7.6 Hz; anomeric-H), 3.76 (3 H, s, $-OCH_3$); ^{13}C-NMR (DMSO-d_6): δ: 158.9 (C-5), 158.3 (C-3), 147.7 (C-4′), 146.5 (C-3′), 139.3 (C-1), 130.0 (C-1′), 128.6 (C-β), 126.2 (C-α), 118.7 (C-6′), 112.9 (C-2′), 112.2 (C-5′), 107.2 (C-2), 105.0 (C-6), 102.9 (C-4), 100.7 (C-1″), 77.1 (C-5″), 76.5 (C-3″), 73.2 (C-2″), 69.7 (C-4″), 60.7 (C-6″), 55.7 ($-OCH_3$).

Compared with the literature data (Yoshiki *et al*., 1984), HSCCC fraction II corresponded to 3,3′,5-trihydroxy-4′-methy-loxystilbene-3-*O*-β-D-glucoside (rhaponticin).

Summary

Our study demonstrated that HSCCC was an efficient method for large-scale preparative separation of two PHS compounds from *R. likiangense Sam*.

Supporting information

Supporting information may be found in the online version of this article.

Acknowledgement

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