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Short communication

Preparative isolation and purification of *trans*-3,5,4′-trihydroxystilbene-4′-*O*-β-D-glucopyranoside and (+)catechin from *Rheum tanguticum* Maxim. ex Balf. using high-speed counter-current chromatography by stepwise elution and stepwise increasing the flow-rate of the mobile phase

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Abstract

Preparative high-speed counter-current chromatography (HSCCC) was successfully used for isolation and purification of *trans*-3,5,4′-trihydroxystilbene-4′-*O*-β-D-glucopyranoside (compound 1) and (+)catechin (compound 2) from *Rheum tanguticum* Maxim. ex Balf. by stepwise elution with a pair of two-phase solvent system composed of ethyl acetate–ethanol–water (25:1:25, v/v) and (5:1:5, v/v), and stepwise increasing the flow-rate of the mobile phase from 0.8 to 2.0 ml min^{-1} after 5 h. The preparative HSCCC separation was performed on 250 mg of crude extract yielding pure compound 1 (10.2 mg) and compound 2 (26.7 mg) all at purities of over 96% in a single run. The structures of the two compounds have been elucidated by means of spectroscopic methods including MS and ^{1}H, ^{13}C nuclear magnetic resonance spectroscopy.

Keywords: *Trans*-3,5,4′-trihydroxystilbene-4′-*O*-β-D-glucopyranoside; (+)Catechin; High-speed counter-current chromatography; *Rheum tanguticum*

1. Introduction

Rheum tanguticum Maxim. ex Balf (Dahuang), as a traditional Chinese herb, is one of the three genuine Rhubarbs in Chinese Pharmacopoeia [1], which belongs to the family Rheum L. The roots and rhizome of this plant was traditionally used in the world as a folk medicine with strong antibacterial, antipyretic, antineoplastic and antipasmolytic action and has been also used as an agent to reduce blood-lipid, blood pressure, obesity and blood urea nitrogen [2]. Previous phytochemical studies on this plant revealed the presence of anthracene derivatives, stilbenes, phenylbutanone glucopyranosides, tannins, naphthalene derivatives, chromone derivatives, etc. [3–5]. Among these constituents, it has been made clear by pharmacological studies that 3,5,4′-trihydroxystilbene-4′-*O*-β-D-glucopyranoside can be used as an agent to reduce blood-lipid and blood pressure and (+)catechin has the function of decreasing urea nitrogen concentration in rat serum [5].

The chemical structures of 3,5,4′-trihydroxystilbene-4′-*O*-β-D-glucopyranoside and (+)catechin are shown in Fig. 1.

HPLC and column chromatography were used to separate and purify *trans*-3,5,4′-trihydroxystilbene-4′-*O*-β-D-glucopyranoside and (+)catechin from natural plant sources [4], but these methods were time-consuming and used large amounts of reagent. Furthermore, these methods caused a great loss of them. High-speed counter-current chromatography (HSCCC), being a support-free liquid–liquid partition chromatographic technique, eliminates the risk of irreversible adsorption of the

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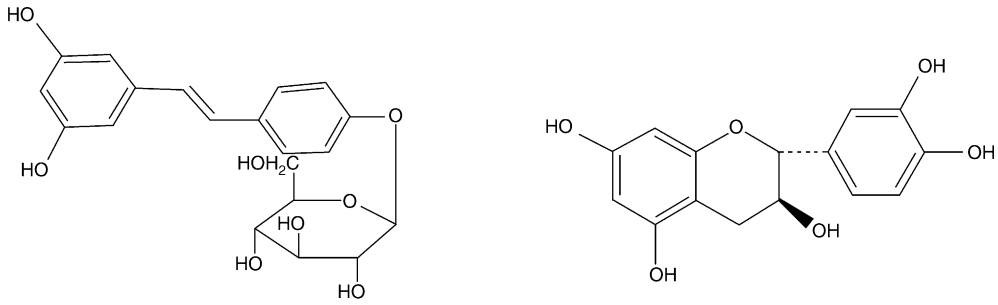

Fig. 1. Chemical structures of compounds 1 and 2.

sample onto the solid support [6], and has been widely used in preparative separation of natural products [7].

Although HSCCC separations of a stilbene derivative and catechins have been reported [8,9], preparative isolation and purification of them using HSCCC by a new method of combination of stepwise elution and stepwise increasing the flow-rate of the mobile phase is firstly achieved simultaneously in the present studies. The structures of compounds 1 and 2 have been elucidated by means of spectroscopic methods including MS, ^{1}H and ^{13}C NMR.

2. Experimental

2.1. Apparatus

Preparative HSCCC was performed using a Model GS10A2 multilayer coil of 110 m long, 1.6 mm I.D. polytetrafluoroethylene (PTFE) tube with a total capacity of 230 ml that was designed and fabricated at the Beijing Institute of New Technology Application, China. The $\beta\psi$ 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 20 ml loop (Tianjin High New Science technology Company, Tianjin, China) was used to introduce the sample into the column. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

The HPLC equipment used was Agilent 1100 HPLC system including G1311A solvent delivery unit, G1315B UV–vis photodiode array detector, Rheodyne 7725i injection valve with a 20 μl loop, G1332A degasser and Agilient HPLC workstation (Agilent Technologies Co. Ltd., Beijing, China).

The EI mass spectra were obtained with a Quattro II triple stage tandem mass spectrometer (Micromass UK, Altrincham, UK) equipped with a Z-spray atmospheric pressure ionization source. Sample solution was introduced into the mass spectrometer using a Model 100 syringe pump (KD Scientific, Boston MA, USA).

NMR spectra were measured using JEOL (Tokyo, Japan) JNM A300 NMR spectrometer operating at 300 MHz for ^{1}H and ^{13}C in CD_3OD.

2.2. Reagents

All organic solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China. Methanol used for HPLC analysis was of chromatographic grade. *R. tanguticum* Maxim. ex Balf. were collected in Qinghai Province, China. The plant was identified by Professor Pengfei Tu (School of Pharmaceutical Sciences, Peking University Health Science Center, Beijing, China).

2.3. Preparation of sample solution

Dry roots and rhizoma (4.3 kg) of *R. tanguticum* were ground into powder and extracted three times with 8 l of 95% EtOH altogether, the extraction time were 2 h, 2 h and 1 h, respectively. The extract was combined and evaporated to dryness by rotary vaporization at 50 °C in vacuum, which yielded 0.4 kg of dry powder. Then, the extract were suspended on distilled water (5 l) and extracted successively with light petroleum ether (b.p. 60–90 °C) (10 l), ethyl acetate (10 l) and *n*-butanol (10 l). The extract of *n*-butanol (250 g) was eluted on resin (2.5 kg of resin HPD 100, Changzhou Baoen Factory, Hebei, China) with H_2O (8 l), 20% EtOH (8 l), 40% EtOH (8 l), 60% EtOH (8 l) and 95% EtOH (8 l), respectively, and the elution of 20% EtOH was evaporated to dryness at 50 °C under reduced pressure.

Two hundred and fifty milligrams of the above 20% EtOH elution was dissolved in 10 ml of lower phases of the solvent system ethyl acetate–ethanol–water (25:1:25, v/v) according to the preparative purpose.

2.4. Preparation of the two-phase solvent system

For the present study, the two-phase solvent system used was composed of ethyl acetate–ethanol–water (25:1:25, v/v) and (5:1:5, v/v), respectively. The solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and two phases were separated shortly before use.

2.5. Separation procedure

HSCCC was performed as follows. The multiple layer coiled column was first entirely filled with the upper phase. Then, the lower phase was pumped into the head end of the inlet column at a flow-rate of 0.8 ml min^{-1}, while the apparatus was run at 800 rpm. After hydrodynamic equilibrium was reached, indicated by a clear mobile phase eluting at the tail outlet. Ten millilitres sample solution (25 mg/ml) was injected using an injection valve. The effluent from the tail end of the column was continuously monitored with UV detection at 280 nm. Peak fractions were manually collected according to the chromatogram.

2.6. HPLC analysis

The crude 95% EtOH extract of *R. tanguticum* Maxim. ex Balf. and peak fractions from HSCCC were analyzed by HPLC. The analyses were performed with a kromasil 100A C_{18} column (250 mm × 4.6 mm I.D. 5 μm) at column temperature of 40 °C. Separation was performed under the following conditions: the mobile phase is as following (Table 1), at a flow-rate of 1.0 ml min^{-1}, and the effluent was continuously monitored at 280 nm, The concentrations of compounds 1 and 2 were estimated by the peak area percent at 280 nm (Fig. 2).

Table 1
Gradient conditions of HPLC mobile phase

Time	Acetonitrile (%)	0.05% H_3PO_4–Water (%)
0.00	4	96
10.00	11	89
25.00	13	87
50.00	15	85

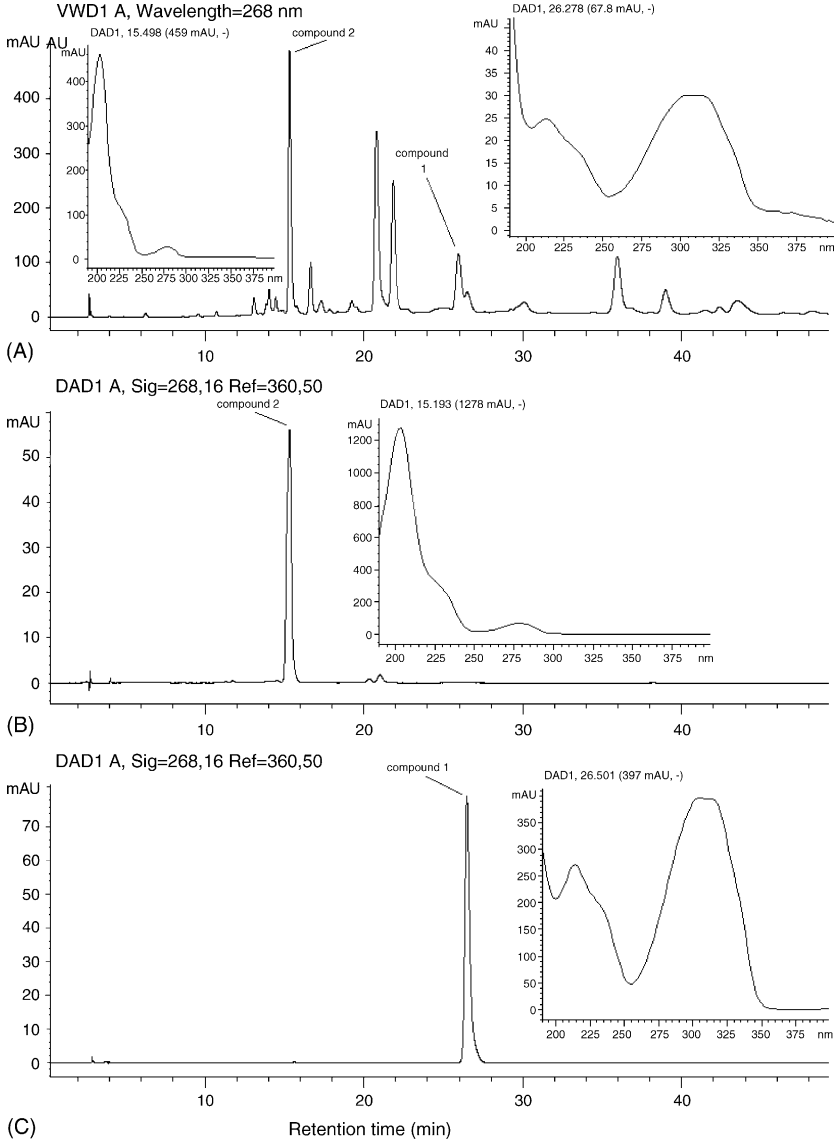

Fig. 2. Chromatograms of HPLC analyses of the crude sample of *R. tanguticum* and its HSCCC fractions. Column: kromasil 100A C_{18} column (250 mm × 4.6 mm I.D. 5 μm); the mobile phase is as following (Table 1); flow-rate: 1 ml min^{-1}. (A) The crude sample; (B) HSCCC fraction of compound 2; (C) HSCCC fraction of compound 1.

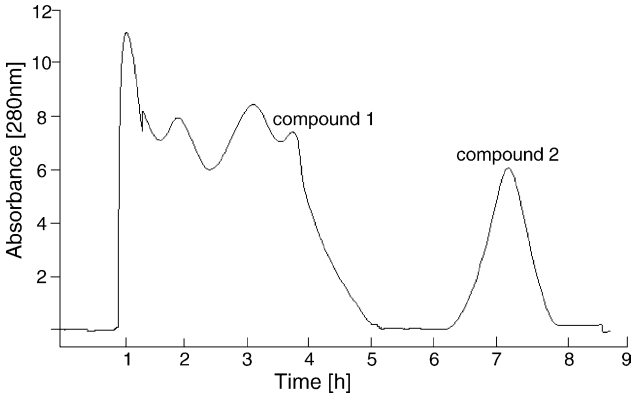

Fig. 3. HSCCC of fraction of the 95% EtOH extract of *R. tanguticum*. Solvent system: ethyl acetate–ethanol–water (5:1:5, v/v), stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2 ml min^{-1}, revolution speed: 800 rpm; sample: 250 mg dissolved in 10 ml lower phase; retention of the stationary phase: about 60%.

3. Results and discussion

3.1. Selection of the two-phase solvent system

HSCCC is a very effective tool for the preparative separation and purification of natural products. The extracts from plant materials usually contain a high number of different compounds with a broad range of hydrophobicity. Most often, only one component needs to be separated from the others, and the standard HSCCC method, which uses a single of solvent system and a constant flow-rate of the mobile phase, is used. In order to separate more different hydrophobic compounds, stepwise elution or stepwise increasing the flow-rate of the mobile phase might be adopted [10,11]. Preliminary HSCCC experiments were carried out with the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water at a volume ratio of 5:5:5:5, 4:6:4:6 or 2:8:4:6, It took a long period of time (>20 h) to separate compound 2 from other impurities and it was also difficult to separate compound 1. In the subsequent studies, another two-phase solvent system was tested.

Performance of the new two-phase solvent system composed of ethyl acetate–ethanol–water was evaluated in terms of peak resolution. With the two-phase solvent system at volume ratio of 5:1:5, although the separation of compound 2 could be achieved and the time of separation became shorter, it was hardly to separate compound 1 from other impurities (Fig. 3). The separation of compound 1 was still poor and the separation time of compound 2 was also long, when the two-phase solvent system at volume ratios of 10:1:10 was adopted. At a ratio of 25:1:25, with the same two-phase solvent system, compound 1 could be separated, but the peak resolution is not satisfactory. Furthermore, compound 2 was still retained in the column for a long period of time (>15 h) (Fig. 4).

Finally, we combined the two solvent systems at volume ratios of 25:1:25 and 5:1:5 to perform a successful separation of the two compounds in a single run by stepwise elution and stepwise increasing the flow-rate of the mobile phase (Fig. 5). Using a solvent system composed of ethyl acetate–ethanol–water

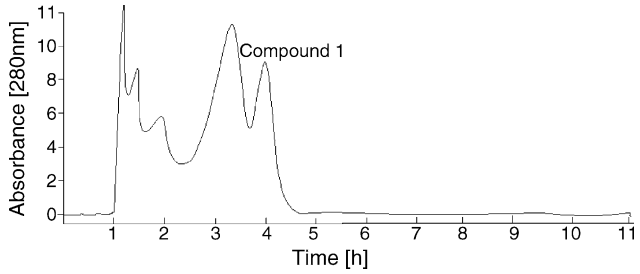

Fig. 4. HSCCC of fraction of the 95% EtOH extract of *R. tanguticum.* Solvent system: ethyl acetate–ethanol–water (25:1:25, v/v), stationary phase: upper organic phase; mobile phase: lower aqueous phase; flow-rate: 2 ml min^{-1}, revolution speed: 800 rpm; sample: 250 mg dissolved in 10 ml lower phase; retention of the stationary phase: about 65%.

(25:1:25, v/v), the separation was started at a slow flow-rate of 0.8 ml min^{-1} to separate the compound 1. Then, the mobile phase was switched to the lower phase of the solvent system composed of ethyl acetate–ethanol–water (5:1:5, v/v) at 5 h. At the same time, the flow-rate was increased stepwise to 2.0 ml min^{-1} to elute the compound 2. The separation of them was achieved with satisfactory peak resolution, and the retention of the stationary phase was good (about 53%).

The crude 95% EtOH extract of *R. tanguticum* was analyzed by HPLC, and the chromatogram is shown in Fig. 2A. The contents of compounds 1 and 2 were 10.8% and 22.3% based on HPLC peak area percentage, respectively.

Fig. 5 shows the preparative HSCCC separation of 250 mg of 95% EtOH rude extract using the solvent system composed of ethyl acetate–ethanol–water. This separation yielded 10.2 mg of compounds 1 and 26.7 mg of compound 2 all at purities of over 96% purity according to HPLC analysis and the chromatograms of HPLC analysis were shown in Fig. 2B and C.

Although it has been reported that stepwise elution or stepwise increasing the flow-rate of the mobile phase was adopted in preparative HSCCC [12–14], it is the first time to develop a new method of combination of stepwise elution and stepwise increasing the flow-rate of the mobile phase applied in the present studies. It is worthwhile to mention the advantage of combination of them. When one target compound could not be

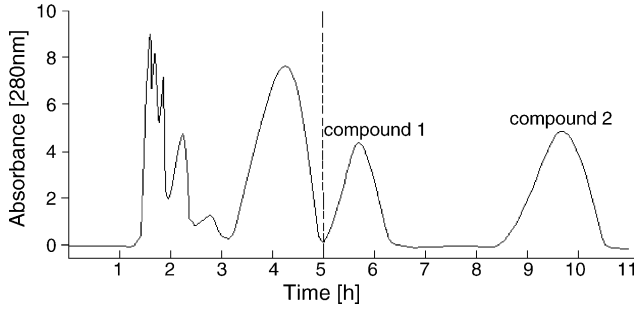

Fig. 5. HSCCC of fraction of the 95% EtOH extract of *R. tanguticum.* solvent system: ethyl acetate–ethanol–water (25:1:25, v/v) and (5:1:5, v/v), stationary phase: upper organic phase of ethyl acetate–ethanol–water (25:1:25, v/v); mobile phase: lower aqueous phase of ethyl acetate–ethanol–water (25:1:25, v/v) before 5 h and ethyl acetate–ethanol–water (5:1:5, v/v) after 5 h; flow-rate: 0.8 ml min^{-1} before 5 h and 2 ml min^{-1} after 5 h, revolution speed: 800 rpm; sample: 250 mg dissolved in 10 ml lower phase; retention of the stationary phase: about 53%.

separated from other impurities and the resolution of target peak is not satisfactory because of its too short retention time, the separation of low flow-rate of the mobile phase is well achieved. On the other hand, if one target compound is retained in the column for a long period of time, stepwise elution or stepwise increasing the flow-rate of the mobile phase is an efficient way to achieve the separation. Thus, the two different kinds of compounds can be well separated simultaneously in a short time using HSCCC by stepwise elution and stepwise increasing the flow-rate of the mobile phase. However, general speaking, the low flow-rate of the mobile phase, which can improve the peak resolution and be beneficial to increase the volume of station phase, will lead to a long time of separation; the increasing of the flow-rate of the mobile phase, which can speed up the elution of peaks, will lead to the decline of the peak resolution and the reduction of the volume of the station phase. Considering this, the choice of a suitable flow-rate of the mobile phase which will not alter the volume of the station phase and prolong the time of separation obviously needs several tries. And besides, it is also not easy to choose an appropriate second mobile phase so that the retained compound can be eluted easily and the volume of the stationary phase in the column will not be significantly altered. This can be examined by mixing the upper phase of the first solvent system with the lower phase of the second solvent system in a small test tube, observing the settling time and the volume of upper and lower phase, and measuring the partition coefficient K of the targeted compound in the new solvent system according to the ratio of HPLC peak area.

3.2. Identification of compounds 1 and 2

Compound 1 was obtained as a pale brown needle, The ^{1}H NMR spectrum of it in CD_3OD exhibits nine-proton signals in the olefinic proton region, among which a pair of doublets signals at δ 6.85 and δ 6.97 are assigned to a pair of *trans* olefinic protons as shown by the coupling constant (J = 16.2 Hz). An A_2B_2-type quartet signal at δ 7.04 and δ 7.4 (J = 8.7 Hz) represents the presence of *p*-substituted benzene ring. The AX_2-type signals appeared at δ 6.15(1 H, t, J = 2.1 Hz) and δ 6.43(2 H, d, J = 2.1 Hz) are assignable to the protons of the 1,3,5-trisubstituted benzene ring. ^{13}C NMR (300 MHz, CD_3OD) δ: 62.47, 71.30, 74.87, 77.93, 78.09, 102.14, 102.93, 105.94, 117.87, 128.48, 128.57, 128.87, 133.14, 140.98, 158.61, 159.63. From these spectral data, the structure of compound 1 is determined to be *trans*-3,5,4′-trihydroxystilbene-4′-*O*-β-D-glucopyranoside.

Compound 2 was obtained with a pale white needle. In CD_3OD, the ^{1}H, ^{13}C NMR (300 MHz) data were as following. ^{1}H NMR δ: 2.52 (1 H, dd, J = 8.1 Hz), 2.86 (1 H, dd, J = 5.4 Hz), 3.98 (1 H, m), 4.57 (1 H, d, J = 7.5 Hz), 5.86 (1 H, d, J = 2.4 Hz), 5.93 (1 H, d, J = 2.4 Hz), 6.72 (1 H, dd, J = 8.4 Hz, J = 1.8 Hz), 6.76 (1 H, d, J = 8.4 Hz), 6.83 (1 H, d, J = 1.8 Hz). ^{13}C NMR δ: 28.39, 68.73, 82.74, 95.48, 96.27, 100.79, 115.19, 116.07, 120.03, 132.13, 146.14, 156.83, 157.18, 157.72. From these data, it was easy to confirm that compound 2 is (+)catechin. Moreover, the EI-MS shows molecular ion at m/z 290, which is in agreement with the molecular formula $C_{15}H_{14}O_6$ of (+)catechin [15].

4. Conclusions

Since the properties of the two compounds were different, it was difficult to separate the two compounds simultaneously using the standard HSCCC technique with a single of solvent system and a constant flow-rate of the mobile phase, but the separation of them could be achieved by stepwise elution and stepwise increasing the flow-rate of the mobile phase. The present results of our studies indicate that the new HSCCC technique by varying solvent system and flow-rate of the mobile phase is a powerful technique for the separation of natural products.

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