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Short communication

Purification of astilbin and isoastilbin in the extract of *smilax glabra rhizome* by high-speed counter-current chromatography

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Abstract

Purification of astilbin from the rhizome extract of *Smilax glabra* was conducted using a high-speed counter-current chromatograph equipped with a 700 mL column. In a single operation, 1.5 g of crude sample was separated to yield 105 mg of component astilbin and 48 mg of isoastilbin while the upper phase of the two-phase solvent system composed of *n*-hexane–*n*-butanol–water (1:1:2, v/v/v) was used for stationary phase. The chemical structures of the two flavonoid glycosides were confirmed by electrospray ionization ion trap multiple mass spectrometry and nuclear magnetic resonance experiments.

Keywords: Counter-current chromatography; *Smilax glabra*; Liliaceae; Astilbin; Purification

1. Introduction

The rhizome of *Smilax glabra* (Liliaceae) has been used extensively in traditional Chinese medicine to treat syphilis, acute bacterial dysentery and chronic nephritis [1–3]. Reports show flavonoid glycosides such as astilbin are the bioactive components in the rhizome of *Smilax glabra* [4–6]. During preparative processing of pure astilbin, isomerization easily lead to the 3-epimer isoastilbin caused by hot solvent extraction and vacuum-evaporation previous the final freeze-drying step.

High-speed counter-current chromatography (HSCCC) is a feasible separation technique of flavonoids [7–9] based on fast partitioning of the analytes between two immiscible liquid solvent phases, which can be performed at a lower temperature to prevent isomerization of compounds sensitive to temperature. The present paper describes a very convenient method to prepare astilbin by high-speed counter-current chromatographic separation using a suitable solvent system that the mobile phase can be directly freeze-dried preventing thermally induced isomerization.

2. Experimental

2.1. Reagents and preparation of two-phase solvent system

Analytical grade solvents, *n*-hexane and *n*-butanol were purchased from Shanghai Chemical Company (China). The HSCCC experiments were performed with a two-phase solvent system composed of *n*-hexane–*n*-butanol–water (1:1:2, v/v/v). After thorough equilibration of the mixture in a separatory funnel (ambient temperature, about 20 °C), the two phases were separated shortly before use. The upper organic phase was used as stationary phase, and the lower aqueous phase as mobile phase.

2.2. Preparation of sample

Freeze-dried rhizome of *Smilax glabra* (5 kg) was twice macerated with 30 L 95% methanol. The crude extract was

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evaporated at 35 °C in vacuum to 3 L. The concentrated solution was treated with active charcoal to remove pigments and partitioned with 3 L ethyl acetate to take out less polar components, then lyophilized to yield 91 g of the crude extract which was subjected to a silica gel column chromatography. After elution with chloroform–methanol–water (15:4:1), all fractions containing astilbin were combined, evaporated and lyophilized to yield 7.6 g crude astilbin for subsequent HSCCC separation.

2.3. HPLC analysis

An Agilent 1100 HPLC system composed of a quaternary pump with a degasser, a thermostatted column compartment, a variable wavelength detector, a manual injector, and 1100 ChemStation software. The HPLC separation was performed on a 5 μm, 250 mm × 4.6 mm Zorbax-ODS column in isocratic elution mode at ambient temperature. The mobile phase consisted of methanol–water–glacial acid (37:63:0.5, v/v/v) at a flow rate of 1.0 mL/min. The detector was operated at λ 291 nm.

2.4. Selection of solvent system

The solvent system for HSCCC separation was selected by comparing the difference of partition coefficients of astilbin and isoastilbin in various solvent systems. Partition coefficient was calculated by HPLC peak area of astilbin or isoastilbin in the upper phase and lower phase when a crude sample was added into an isovolumetric upper phase and lower phase of a two-phase solvent system.

2.5. HSCCC procedure

A J-type HSCCC instrument made in Institute of Food and Biological Engineering, Zhejiang Gongshang University, Hangzhou, China, was used for the separation of polar flavonoid glycosides from *Smilax glabra* rhizome. The machine holds a separation column at a distance of 10 cm from the center of the centrifuge. The column revolves around the central axis of the centrifuge and simultaneously rotates about its own axis at the same angular velocity in the same direction. The column holder hub was 25 cm in length and 6 cm in O.D. The multilayer coils was prepared by winding 100 m of a PTFE (polytetrafluoroethylene)-tubing (3.0 mm I.D., Zeus Industrial Products, Raritan, NJ, USA) onto the holder hub. The β value of the coils from the inner layer to the outer layer is 0.48–0.78. The capacity of the column was determined to be 700 mL. The mobile phase was delivered using a Waters 510 HPLC pump (Millipore Corporation, Milford, MA, USA). An injection loop (100 mL) was used for sample loading. In each separation, the HSCCC column was first filled with stationary phase, then the apparatus was started at 700 rpm, and the sample, 1.5 g crude extract in 70 mL lower phase, was injected into the column through an injection loop. Afterward, the mobile phase was delivered into the column at flow rate of 2.0 mL/min to elute the components monitored with a UV–vis detector (Model UV-752, Shanghai Instrument Factory, Shanghai, China) set at 254 nm. The effluent was collected with a BS-100 mode fraction collector (Shanghai Instrument Factory, Shanghai, China), each fraction 15 mL.

2.6. Spectroscopic analysis

^{1}H and ^{13}C NMR spectra were recorded in [$^{2}H_6$]dimethyl sulfoxide (DMSO-d_6) on a Bruker AMX 400 nuclear magnetic resonance (NMR) spectrometer (Bruker BioSpin, Germany). Electrospray ionization ion trap multiple mass spectrometry (ESI-MS) experiments were performed on a Bruker Esquire LC–MS in positive mode using a syringe pump.

3. Results and discussion

3.1. HSCCC separation

Relatively, *n*-butanol is a good solvent for dissolving astilbin and isoastilbin. Thus, various solvent systems based on *n*-butanol and water were conducted partition coefficient tests. Among the solvent systems, *n*-hexane–*n*-butanol–water (1:1:2) gave suitable partition coefficients for astilbin (0.65) and isoastilbin (1.23) (Table 1). Eventually, *n*-hexane–*n*-butanol–water (1:1:2) was used for the HSCCC separation of the crude astilbin. Fig. 1 showed the result of the HSCCC separation of 1.5 g crude sample, which resulted in four peaks I–IV. HPLC analysis demonstrated components III and IV corresponded the peaks a (astilbin) and b (isoastilbin) in the

Table 1
Partition coefficients for astilbin and isoastilbin in various solvent systems

Solvent system	Ratio	Partition coefficient[a]	
		Astilbin	Isoastilbin
n-Hexane–*n*-butanol–water	1:4:5	4.51	5.94
n-Hexane–*n*-butanol–water	2:4:6	2.53	3.36
n-Hexane–*n*-butanol–water	2.7:4:7	1.72	2.39
n-Hexane–*n*-butanol–water	4:4:8	0.65	1.23
n-Hexane–*n*-butanol–water	5.3:4:10	0.21	0.39

[a] Expressed as: peak area$_{stationary}$/peak area$_{mobile}$.

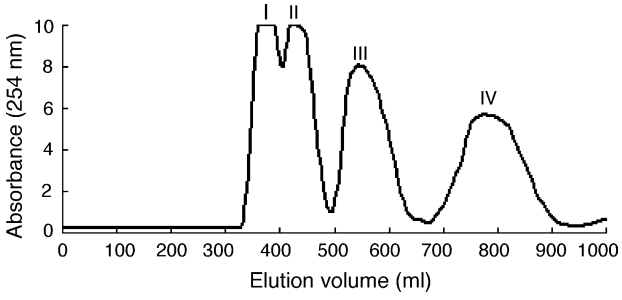

Fig. 1. HSCCC separation chromatogram of 1.5 g crude astilbin sample. Two-phase solvent system: *n*-hexane–*n*-butanol–water (1:1:2, v/v/v); stationary phase: upper organic phase; flow-rate of mobile phase: 2.0 mL/min; revolution speed: 700 rpm; retention of stationary phase: 55%.

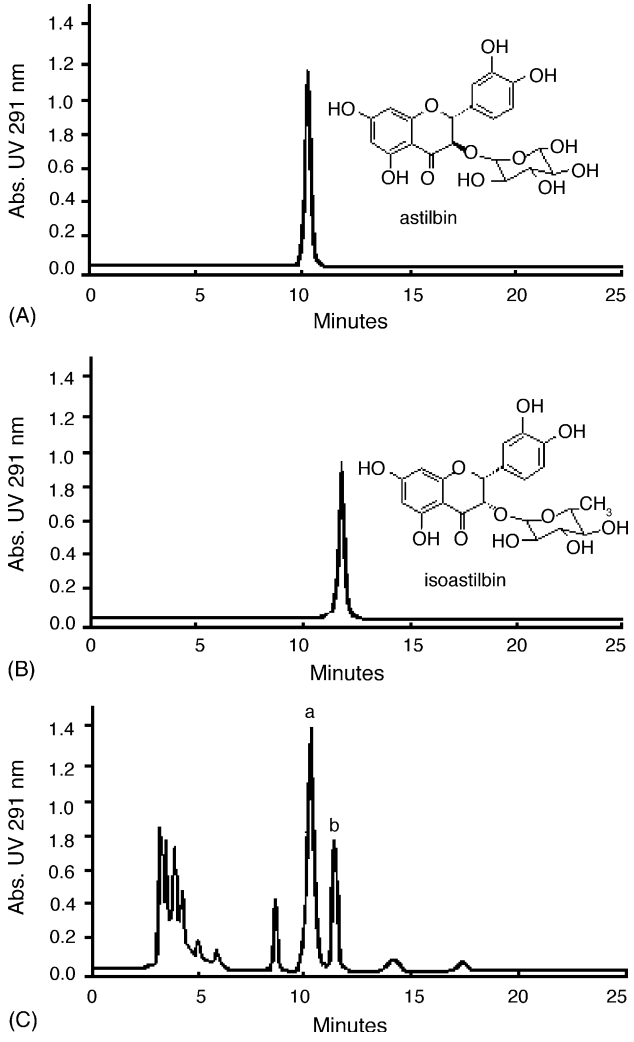

Fig. 2. HPLC analysis of the crude astilbin extract from *Smilax glabra* rhizome and the purified components obtained from HSCCC separation. Experimental conditions: HPLC-column: 5 μm, 250 mm × 4.6 mm Zorbax-ODS; mobile phase: methanol–water–acetic acid (37:63:0.5, v/v/v); flow-rate: 1.0 mL/min. peak a: astilbin; peak b: isoastilbin. (A) Component III from HSCCC separation; (B) component IV from HSCCC separation; (C) the crude extract.

HPLC analysis of the crude sample, respectively (Fig. 2). The two sections of the fractions corresponding peaks III and IV were directly freeze-dried to yield 105 mg component III and 48 mg component IV. The purified components were directly used for structure elucidation by ESI-MS and NMR analysis.

3.2. Elucidation of chemical structures

3.2.1. Component III

White powder, specific optical rotation $[\alpha]_D$—13.5° (c, 0.76, MeOH) is in accordance to literature [12]. ESI-MS (pos): *m/z* 473.5 $[M + Na]^+$. ^1H and ^{13}C NMR (DMSO-d_6) data are listed in Table 2. The ^{13}C-chemical shifts of the aglycone moiety are in very good accordance to the ppm-values of the 3-hydroxy flavanol structure [10,11]. The ^1H and ^{13}C NMR data indicate a α-L-rhamnopyranosid sugar unit. A *trans*-diaxial coupling constant observed for H-2 and H-3 demonstrates the relative stereochemistry as *trans*-orientation. Together with the results of specific optical rotation, component III of the HSCCC run is astilbin.

Table 2
^{13}C, and ^1H NMR spectral data of components III and IV: (δ (ppm); *J* (Hz)) measured in DMSO-d_6)

	Component III (astilbin)		Component IV (isoastilbin)	
	Position ^{13}C	Position ^1H	Position ^{13}C	Position ^1H
2	81.5	5.24 (d, 9.8)	79.9	5.53 (d, 2.6)
3	75.6	4.63 (d, 9.8)	73.3	4.22 (d, 2.6)
4	194.3		192.6	
5	163.3		163.7	
6	96.0	5.90 (d, 2.1)	96.2	5.93 (d, 2.0)
7	166.9		167.4	
8	95.0	5.88 (d, 2.1)	95.2	5.90 (d, 2.0)
9	162.1		162.4	
10	101.0		100.1	
1′	126.8		126.4	
2′	114.7	6.88 (s)	114.1	6.86 (d, 1.6)
3′	145.8		144.8	
4′	145.1		145.1	
5′	115.3	6.74 (s)	115.0	6.71 (d, 8.3)
6′	118.7	6.74 (s)	117.5	6.73 (dd, 8.3,1.6)
Rha				
1″	100.0	4.07 (s)	98.8	4.78 (d, 1.4)
2″	70.1	3.36 (br, s)	70.2	3.48 (dd, 3.4,1.4)
3″	70.4	3.42 (dd, 9.4, 2.8)	70.2	3.21 (dd, 9.4,3.4)
4″	71.6	3.15 (dd, 9.4, 9.4)	71.4	3.05 (dd, 9.4,9.4)
5″	68.9	3.88 (dq, 9.4, 6.2)	68.8	2.50 (overlapped)
6″	17.6	1.05 (d, 6.2)	17.5	0.85 (d, 6.2)

3.2.2. Component IV

White powder, $[\alpha]_D$—85.6° (c, 0.85, MeOH) is in accordance to literature [12]. ESI-MS (pos): *m/z* 473.9 $[M + Na]^+$. ^1H and ^{13}C NMR (DMSO-d_6) data are listed in Table 2. ^{13}C NMR chemical data of compound IV are almost identical to III, but there is a shift difference of $\sim\Delta$ 2 ppm for in position C-2 and C-3, indicating a different relative stereochemistry in the aglycone moiety. This is conformed by small coupling constants of H-2 and H-3 showing the relative *cis*-configuration. The α-L-rhamnose unit is in accordance to the data of 1. Together with the value for specific optical rotation, component IV of the HSCCC separation is the 3-epimer isoastilbin.

3.3. HSCCC versus preparative HPLC

Preparative HPLC experiment was performed with a Zorbax-ODS column (15 μm, 20 mm × 300 mm) using the same mobile phase as for the analytical HPLC analysis at a flow rate of 16 mL/min. A complete separation could be obtained while less than 120 mg of crude sample was injected. However, about 15% astilbin isomerized into isoastilbin while the HPLC effluent containing astilbin was evaporated at 35 °C which is necessary to remove methanol for

freeze-dryness. The HPLC preparation of 120 mg crude sample yielded 7.9 mg of 84.1% astilbin plus 14.7% isoastilbin and 3.3 mg of 98.3% isoastilbin. Obviously, the HPLC purification of astilbin possessed the disadvantages-a less sample load and isomerization from heat processing such as evaporation, while HSCCC could load larger amount sample (1.5 g) in single separation and the effluent of HSCCC could be directly freeze-dried to prevent isomerization of astilbin to isoastilbin, since the very little amount of *n*-hexane and *n*-butanol in the effluent had almost no influence on the freezing of the effluent at a routine freezing temperature (−30 °C) and no evaporating was needed for the collected fractions previous freeze-drying.

4. Conclusion

Astilbin could be separated from isoastilbin by high-speed counter-current chromatography in which direct freeze-drying of the HSCCC effluent to yield pure astilbin. Also, isoastilbin can be obtained from the HSCCC separation of the rhizome extract of *Smilax glabra*, in case it was generated in consequence of hot solvent extraction.

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