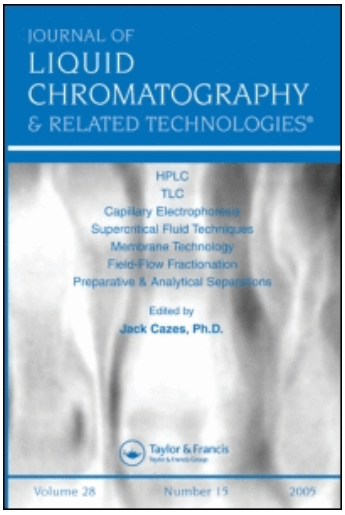

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HSCCC SEPARATION OF A STILBENE GLUCOSIDE FROM *POLYGONUM MULTIFLORUM*

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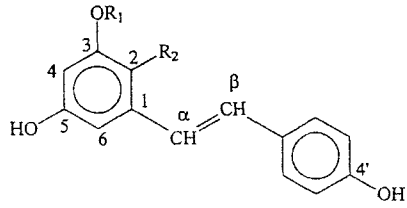
ABSTRACT

High-speed countercurrent chromatography (HSCCC) was applied to the separation and purification of 2,3,5,4'-tetrahydroxy stilbene-2-O-D-glucoside from dried roots (2.7g) of *Polygonum multiflorum*. The ethanol extract (240 mg) was first separated with the solvent system composed of ethyl acetate-ethanol-water at a volume ratio of 10:1:10 and the obtained fraction was repurified with the solvent system at the modified volume ratio of 50:1:50. The method produced stilbene glucoside (30mg) at a high purity of 96%. The chemical structure was identified by FAB-MS and ^{1}HNMR.



Resveratrol: R_1=R_2=H

Piceid: R_1=D-Glucose, R_2=H

2,3,5,4'-Tetrahydroxystilbene-2-O-D-glucoside: R_1=H, R_2=O-D-Glucose

Figure 1. The structure of stilbene components.

INTRODUCTION

The dried roots of *Polygonum multiflorum* have been used for treating a variety of inflammatory disorders such as suppurative dermatitis, gonorrhea, favus athlete's foot, and hyperlipidemia in Chinese and Japanese traditional medicine. The roots contain 2,3,5,4'-tetrahydroxy-stilbene-2-O-D-glucoside,[1] two new stilbene glucoside gallates and proanthocyanidins,[2] in addition to various anthraquinones including chrysophanol, emodin, rhein, and physcion, et al. These stilbene components (Fig. 1) are known to have useful pharmaceutical activities: Resveratrol possesses antibacterial and antifungal activities;[3] resveratrol and piceid have lipid-lowering activity in the oil mixture-fed rats;[4] piceid and 2,3,5,4'-tetrahydroxy stilbene reduce liver injury in peroxidized oil-fed rats.[5]

Although the roots of *P. multiflorum* contain a large amount (1.2%) of 2,3,5,4'-tetrahydroxy-stilbene-2-O-D-glucoside,[1] its standard required for the quality control of the product is not available. This may be due to various problems in the preparative-scale separation using the conventional chromatographic methods.[1,2,6,7]

The present paper describes a new simple method to separate 2,3,5,4'-tetrahydroxy-stilbene-2-O-D-glucoside from the root of *P. multiflorum* by high-speed countercurrent chromatography (HSCCC). This special type of liquid-liquid partition chromatography uses no solid support matrix and therefore eliminates irreversible adsorption of samples.[8] Recently, HSCCC has been increasingly used for purification of natural drugs.[9]

EXPERIMENTAL

Apparatus

HSCCC was performed with a model GS10A2 multilayer coil planet centrifuge fabricated at Beijing Institute of New Technology Application, Beijing, China. The multilayer coil separation column was prepared by winding a 1.6mm ID PTFE (polytetrafluoroethylene) tube coaxially onto the column holder hub at β = 0.5-0.75 (β=r/R where r is the distance from the holder axis to the coil and R, the distance from the holder axis to the centrifuge axis). The total column capacity measured 260 mL. The revolution speed is adjustable with a speed controller in a range of 0 - 1000 rpm with an 8cm radius. The system was also equipped with an NS-1007 constant-flow pump, a model 8823A-UV monitor operating at 254 nm, a Yokogawa model 3057 recorder, and a sample injection valve with 10 mL loop.

Reagents

All solvents used for HSCCC were of analytical grade and purchased from Beijing Chemical Factory, Beijing, China.

Sample Preparation

The dried milled roots (2.7g) of *P. multiflorum* collected from Yunnan, China were extracted with 30 mL chloroform for three times under ultrasonication to remove anthraquinones. Then, the residual was similarly extracted with 30 mL ethanol for three times. The ethanol extracts were combined and concentrated to dryness. The dried extract was dissolved in the mobile phase for HSCCC separation.

HCCC Separation

In each separation the coiled column was first entirely filled with the upper organic stationary phase. Then the apparatus was rotated at 800 rpm, while the lower aqueous mobile phase was pumped into the column at a flow-rate of 2.0 mL/min. After the mobile phase front emerged and the system established a steady state hydrodynamic equilibrium, the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the chromatogram.

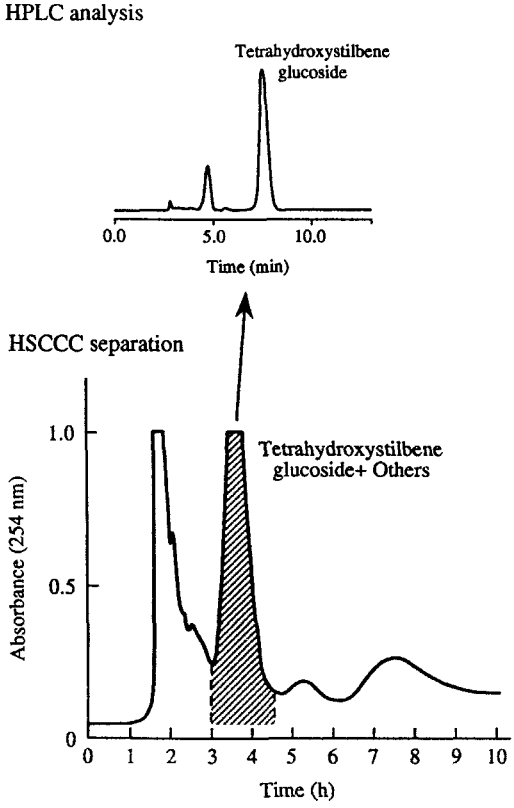


Figure 2. HSCCC separation of crude root extract of *P. multiflorum*. Experimental conditions: apparatus: model GS10A2 multilayer coil planet centrifuge with an 8cm revolution radius; column: multilayer coil prepared from 1.6 mm ID PTFE tubing with a total capacity of 260 mL (β=0.5-0.75); sample: 240mg of crude root extract of *P. multiflorum* in 10mL solvent; solvent system: ethyl acetate-ethanol-water (10:1:10, v/v/v); mobile phase: lower phase; flow rate: 2 mL/min; revolution: 800 rpm; detection: 254 nm. HPLC analysis: Rainin SD-200 HPLC system; column: Microsob-MV C_{18} column (250 x 4.6mm ID, 5μ); mobile phase: methanol-water (40:60, v/v), isocratic elution; flow-rate: 1.0 mL/min; detection: 254 nm.

In the present study, a solvent system composed of ethyl acetate-ethanol-water (10:1:10, v/v/v) was used in the first separation to harvest an enriched fraction of stilbene glucoside from the crude extract. Then, this partially purified fraction was concentrated and subjected to HSCCC separation using the same solvent composition at a different volume ratio of 50:1:50.

HPLC Analysis

The HSCCC peak fractions were analyzed by HPLC. The HPLC instrument used for analysis was a Rainin SD-200 HPLC system (Rainin Instrument Company, Mack road, Woburn, MA, USA). The analysis was performed with a Microsob-MV C_{18} column (250 x 4.6 mm ID, 5μ diameter, Rainin Instrument Company). The mobile phase composed of methanol and water (40:60, v/v) was isocratically eluted at a flow rate of 1.0 mL/min, and the effluent monitored by a UV detector at 254 nm.

FAB-MS and ^{1}HNMR Identification

The stilbene glucoside purified by HSCCC was identified by FAB-MS and ^{1}HNMR. FAB-MS was taken on a Finnigan MAT711, Tabspec instrument, and ^{1}HNMR spectra on a Bruker AM-500 spectrometer (in D_2O).

RESULTS AND DISCUSSION

In the present study, 240mg of the ethanol crude extract (10 mL) from the dried roots of *P. multiflorum* was separated by HSCCC using a solvent system composed of ethyl acetate-ethanol-water at a volume ratio of 10:1:10 (Fig. 2). In this separation, the fraction corresponding to the shadowed peak in the HSCCC chromatogram contained stilbene glucoside at about 80% purity by HPLC analysis. This partially purified fraction was further subjected to HSCCC separation using the same set of solvents at a modified volume ratio of 50:1:50 (Fig. 3). In this second separation the stilbene glucoside fraction showed a high purity of over 96% by HPLC analysis. The recovery of the final product was 30mg or 1.1% (w/w) from the dried roots (2.7g) of Yunnan *P. multiflorum*.

Stilbene glucoside isolated by HSCCC, which emits bright fluorescence under UV light, was identified by both FAB-MS and ^{1}HNMR. FAB-MS showed two peak fragments with [MH^+] of 407 and 244 which correspond to tetrahydroxystilbene glucoside and tetrahydroxy-stilbene parent, respectively. The ^{1}HNMR spectrum exhibited in the aromatic and olefinic field a pair of meta-coupled doublets δ6.09 (1H, d, J=2Hz, 4-H), δ6.29 (1H, d, J=2Hz, 6-H), transolefic proton signals δ6.36 (1H, d, J=16Hz, β-H), δ7.01 (1H, d, J=16Hz, α-H), and an A_2B_2-type signal δ6.52 (2H, d, J=8Hz, 2',6'-H), δ6.91 (2H, d, J=8Hz, 3',5'-H). In the high field, the spectrum exhibited C-H signals on glucose, δ3.99 (1H, d, J=8Hz, C_1-H), δ2.88-3.52 (6H, m, other C-H). Considering these spectra together with its biological origin, it is concluded that the above compound should be 2,3,5,4'-tetrahydroxy-stilbene-2-O-D-glucoside.

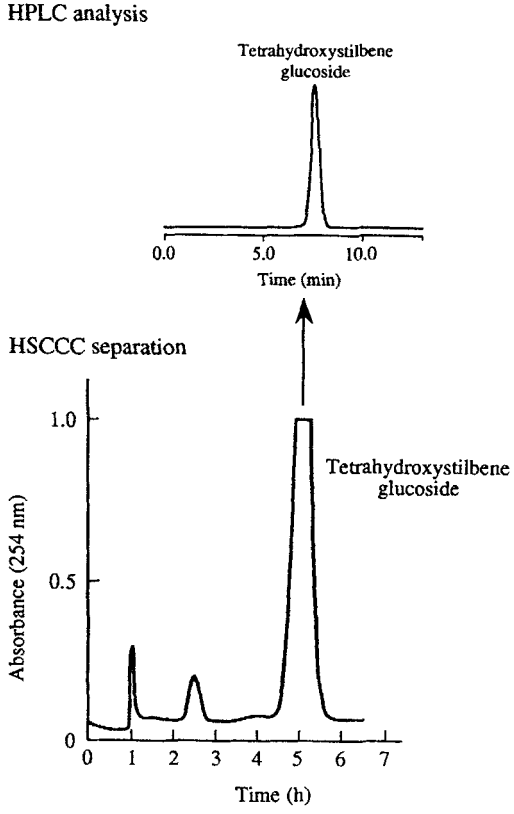


Figure 3. HSCCC separation of partially purified extract of *P. multiflorum*. Experimental conditions: apparatus: model GS10A2 multilayer coil planet centrifuge with an 8cm revolution radius; column: multilayer coil prepared from 1.6 mm ID PTFE tubing with a total capacity of 260 mL (β=0.5-0.75); sample: partially purified fraction of root extract of *P. multiflorum* (all shaded portion of chromatogram in Fig. 2); solvent system: ethyl acetate-ethanol-water (50:1:50, v/v/v); mobile phase: lower phase; flow rate: 2 mL/min; revolution: 800 rpm; detection: 254 nm. HPLC analysis: Rainin SD-200 HPLC system; column: Microsob-MV C_{18} column (250 x 4.6mm ID, 5μ); mobile phase: methanol-water (40:60, v/v), isocratic elution; flow-rate: 1.0 mL/min; detection: 254 nm.

This final product, 2,3,5,4'-tetrahydroxy-stilbene-2-O-D-glucoside, was found to be unstable under exposure to light at room temperature. Therefore, the preparation should be made under low temperature and analyzed immediately. The stability of this compound needs further study.

The results of our experiments indicate that HSCCC can be used as a suitable method for the preparative separation and purification of 2,3,5,4'-tetrahydroxy-stilbene-2-O-D-glucoside from the crude root extract of *P. multiflorum.*

The sample size processed by HSCCC can be greatly increased using a large multilayer column, hence the method is ideal for preparing the standard of stilbene glucoside for quality control.

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