Complementary Application of HSCCC and Semi-preparative HPLC for Rapid Separation of Phenylethanoid Glycosides from *Penstemon digitalis*

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Abstract: **Objective** Echinacoside and verbascoside are known for their excellent neuroprotective, anti-inflammatory, and anti-oxidative activities, therefore large amount of pure compounds are urgently needed as authentic standards for various *in vivo* and *in vitro* studies. Nowadays, they are abundenty extracted from endangered *Cistanche* spp. Phytochemical studies revealed that *Penstemon* spp., comprising about 280 species, were rich natural sources for the exploitation and large scale preparation of phenylethanoid glycosides. Thus, rapid isolation and purification of various phenylethanoid glycosides from *Penstemon* spp. were necessary. **Methods** The crude extract of *P. digitalis* was first enriched by AB-8, and then the high-speed counter-current chromatography (HSCCC) combined with semi-preparative high performance liquid chromatography (HPLC) method was adopted to isolate and purify echinacoside and verbascoside. **Results** Eventually, verbascoside (67.2 mg) with the purity of 92.6% and echinacoside (3.96 mg) with the purity of 98.9% were obtained from 5 g powdered leaves of *P. digitalis*. **Conclusion** The present mode of HSCCC coupled with semi-preparative HPLC could be a powerful method for the rapid isolation and purification of other phenylethanoid glycosides.

Keywords: echinacoside; high-speed counter-current chromatography; *Penstemon digitalis*; semi-preparative high performance liquid chromatography; verbascoside

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Introduction

Verbascoside and echinacoside (Fig. 1) are typical representatives of the phenylethanoid glycosides family. Owing to their excellent neuroprotective, anti-inflammatory, and anti-oxidative activities (Deepak *et al*, 2000; Funes *et al*, 2010; He *et al*, 2009; Liu *et al*, 2003), especially their potential activities for the intervention in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Kuang *et al*, 2009), large amount of pure compounds are urgently needed for various *in vivo* and *in vitro* studies related to the bioactivities of echinacoside and verbascoside. Phytochemical analysis demenstrated that various phenylethanoid glycosides (Ismail *et al*, 1995; Skrzypek *et al*, 1999; Zajdel and Glowniak, 2011; Zhou *et al*, 1998) were found in the plants of *Penstemon* Schmidel. Our latest research also revealed that *P. barbatus* (Cav.) Roth was an ideal source for the production of echinacoside and verbascoside (Xie *et al*, 2012). The genus *Penstemon* Schmidel belongs to the Scrophulariaceae family and comprises approximately 280 species. Assuming the phenylethanoid glycosides from *Penstemon* spp. shares the similar biosynthetic pathway, therefore we tentatively put forward there might be another alternative rich in echinacoside and verbascoside.

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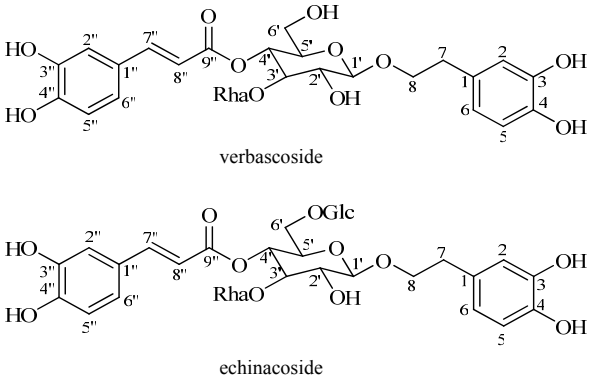

Fig. 1 Structures of verbascoside and echinacoside

High-speed counter-current chromatography (HSCCC) is a liquid-liquid partition chromatographic technique using no supportive matrix, and the liquid stationary phase is immobilized by centrifugal force, eliminating the irreversible adsorption of sample onto the solid matrix, and therefore resulting in marvelous sample recovery (Feng *et al*, 2011). However, the resolution of HSCCC is not as good as that of HPLC. HPLC is well known for its extraordinary efficiency, while it is relatively more expensive and requires a sample pretreatment before separation. Otherwise, plentiful raw materials would make columns polluted and overloaded. During the past decade, several applications of HSCCC combined with HPLC were carried out successfully to isolate and purify a number of natural products (Du *et al*, 2009; Hou *et al*, 2010; Yao *et al*, 2008; Zhu *et al*, 2009).

In the present study, the HSCCC combined with semi-preparative HPLC method was adopted to isolate and purify echinacoside and verbascoside. Eventually, verbascoside (67.2 mg) with the purity of 92.6% and echinacoside (3.96 mg) with the purity of 98.9% were obtained from 5 g powdered leaves of *P. digitalis* for the first time. There could be other phenylethanoid glycosides including isoacteoside, cistanoside A, 2′-acetyl-acteoside, syringalide A, and so on. The present mode of HSCCC coupled with semi-preparative HPLC could be a powerful tool for the rapid isolation and purification of other phenylethanoid glycosides in our following studies.

Materials and methods

Materials and reagents

Authentic samples of echinacoside and verbascoside were purchased from Tauto Biotechnique Co., Ltd. The purities of echinacoside and verbascoside determined by HPLC were 99.4% and 99.6%.

Penstemon digitalis Nutt. ex Sims has been grown in School of Chemistry and Chemical Engineering, Anhui University of Technology, China, and was authenticated by Prof. BAI Lin-shan at the same school. The voucher specimens have been deposited in the Herbarium, Anhui University of Technology, China.

All solvents used for the preparation of enriched extract and for HSCCC separation were of analytical grade (Sinopharm Chemical Reagent Co., Ltd., China). Methanol and acetonitrile used for HPLC were made in Jackson Corporation, USA. The commercial macroporous resin AB-8 (0.3—1.25 mm) was purchased from NanKai Chemical Factory, China.

Apparatus

The HSCCC instrument was TBE—300A HSCCC (Tauto Biotechnique, China) with three multilayer coil separation columns connected in series (1.5 mm, total capacity of 260 mL), equipped with a 20 mL sample loop, an S—1007 pump (Beijing Shenyitong Technology Co., Ltd., China), an 8823B UV detector (Beijing Bindayingchuang Technology Co., Ltd., China), and a Model N2010 Workstation (Zhejiang University, China). Semi-preparative HPLC was carried out on a Shimadzu LC—6AD System (Shimadzu Co., Ltd., Japan). Analytical HPLC was achieved on a Shimadzu LC—20AD System (Shimadzu Co., Ltd., Japan). Rotational Vacuum Concentrator RVC 2—33 (Martin Christ Corporation, Germany) and Heidolph Rotary Evaporator Laborota 4001 (Heidolph Instruments GmbH & Co., Germany) were used for concentration and evaporation, respectively. The IR experiment was performed on Perkin-Elmer Spectrum One NTS (Perkin-Elmer Co., Ltd., USA) FT-IR Spectrometer. The FT-IR spectrum was detected with KBr tablet. ESI-MS experiments were carried out on a Bruker Esquire LC-MS Ion Trap Multiple Mass Spectrometer (Bremen, Germany). The ^{1}H-NMR and ^{13}C-NMR experiments were performed on a Varian Inova—600 (Varian Corporation, USA) NMR Spectrometer. NMR spectra were recorded in deuterated methanol as a solvent.

Extration and preenrichment of phenylethanoid glycosides

The leaves of *P. digitalis* were harvested on April

20th, 2011, dried at 40 ℃ in a forced-air oven, and then sifted through a 0.45 mm sieve. A weighed amount (5 g) of powdered leaves was extracted thrice with a total volume of 50 mL of 50% aqueous methanol. After centrifugation, the supernatant solution was combined, filtered, and evaporated to form a syrup.

The syrup was dissolved with water, subjected to column chromatographic separation with resin AB-8. Then, it was eluted with distilled water, 30% and 50% methanol in turn. Eluent of 50% methanolic solution was concentrated under reduced pressure and the residue (R1) was obtained and stored at 4 ℃ for further use.

HSCCC separation procedure

In HSCCC separation, the coil column was first entirely filled with the upper phase of the solvent system. Then the apparatus was rotated at 800 r/min, while the lower phase was pumped into the column at a flow rate of 2 mL/min. The temperature was set at 25 ℃. After the mobile phase front emerged and hydrodynamic equilibrium was reached in the column, R1 dissolved in 20 mL mobile phase was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV-vis detector at 280 nm.

Semi-preparative HPLC separation procedure

Fr. F1 in Fig. 2 was further purified by semi-preparative HPLC on a Shim-pack PRC-ODS column (25 cm × 30 mm, 15 μm), the mobile phase was 45% aqueous methanol, the injection was made through a 200 μL loop, the effluent was monitored at 330 nm, and the flow rate was 4 mL/min constantly.

Analytical HPLC conditions

The quantitative analyses were performed on a Phenomenex ODS column (150 mm × 4.6 mm, 5 μm) with a C_{18} guard column (Phenomenex Co., USA) using the following gradient elution: initially 10 min of 80% methanol-acetonitrile (3:2) (A) and 20% aqueous phosphoric acid solution (0.1%) (B); linear gradient to

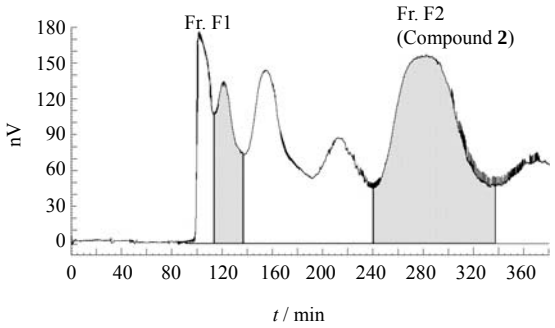

Fig. 2 HSCCC chromatogram of R1

72% A in 10 min and holding for 5 min; recycle to initial conditions in 5 min and holding for 5 min. The total running time was 30 min and the post-running time was 5 min. The flow rate was 1.0 mL/min. The eluent was monitored as 330 nm. The column temperature was set at 30 ℃. Each sample (20 μL) was injected into HPLC in triplicate.

Results and discussion

HSCCC and semi-preparative HPLC separation

Suitable two-phase solvent systems were screened following the procedure described in a previously published literature (Ito, 2005). The partition coefficients (*K* values) of echinacoside and verbascoside determined by analytic HPLC were summarized in Table 1 (Lei *et al*, 2001; Li *et al*, 2008; Han *et al*, 2012; Xie *et al*, 2012). A series of solvent systems were tested and finally, the most polar solvent system N4 (1:1) was chosen in the present research.

As natural plant secondary metabolites, echinacoside and verbascoside are known for their soaring medication values. An efficient method for large scale preparation is necessary. Moreover, *Penstemon* spp., comprising about 280 species (Albach, 2005), are rich natural sources for the exploitation and large scale preparation of phenylethanoid glycosides. Therefore, the rapid extraction, purification, and characterization of various phenylethanoid glycosides isolated from

Table 1 *K* values of echinacoside and verbascoside in different solvent systems

No.	Solvent systems	Ratios	*K* values	
			Echinacoside	Verbascoside
1	ethyl acetate-*n*-butanol-ethanol-water	5:5:1:10	0.84	4.21
2	ethyl acetate-*n*-butanol-ethanol-water	40:6:6:50	0.43	2.88
3	ethyl acetate-water	1:1	0.09	0.16
4	*n*-butanol-water	1:1	0.75	3.37

Penstemon spp. are necessary. Although the productivity of the recycling HSCCC, as reported in our previous study, is much higher than that of semi-preparative HPLC, it is relatively time-consuming. The rapidity of semi-preparative HPLC highlighted its superiority, which is particularly suitable for quick isolation, structural identification, and confirmation. By means of the complementary advantages of HSCCC and semi-preparative HPLC, an efficient separation method with the combination of HSCCC and semi-preparative HPLC was established to isolate and purify phenylethanoid glycosides from *P. digitalis.*

Analytical HPLC analysis

The contents of echinacoside and verbascoside in the leaves of *P. digitalis* were quantified by analytical HPLC. The amounts of echinacoside and verbascoside in the crude extract from *P. digitalis* were (1.47 ± 0.09) and (18.90 ± 0.21) mg/g. Initially, the recoveries of echinacoside and verbascoside of the 50% aqueous methanol extraction were 91.9% and 93.3%, respectively (Fig. 3A). Then, the recoveries of echinacoside and verbascoside in the crude extract pre-purified by AB-8 resin were 62.2% and 72.5%, respectively, and the purities of echinacoside and verbascoside in the crude extract were 3.4% and 52.1% (Fig. 3B). After HSCCC, compound **2** (67.2 mg) in Fig. 2 was basically isolated from R1. The purity was increased dramatically from 52.1% to 92.6% (Fig. 3C). With the help of HSCCC enrichment, semi-preparative HPLC was successfully introduced to the purification of compound **1** in Fig. 4. Eventually, 3.96 mg compound **1** with the purity of 98.9% (Fig. 3D) was obtained.

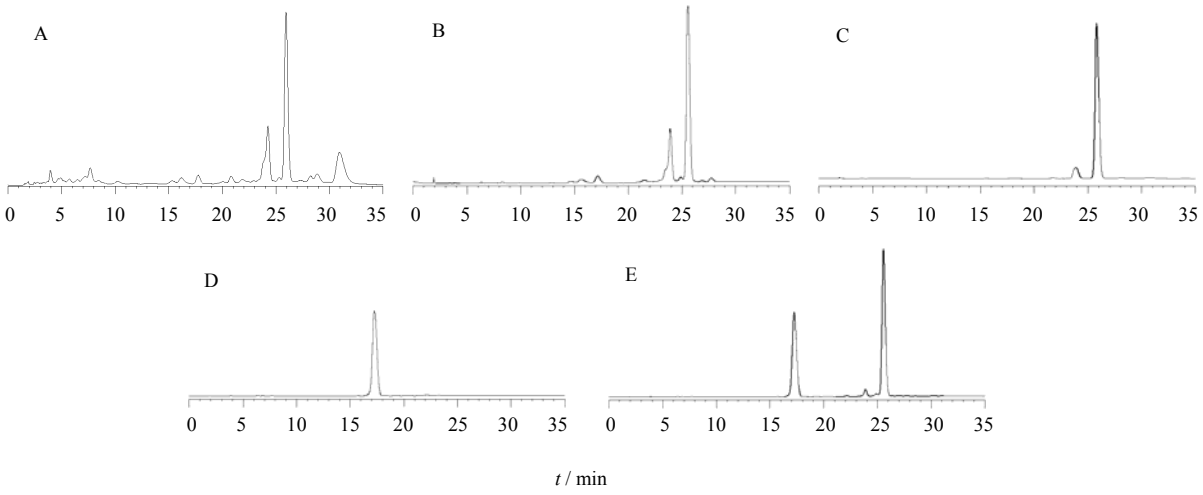

Fig. 3 HPLC chromatograms of methanolic extract from *P. digitalis* (A), sample enriched by AB-8 (B), compound 2 purified by HSCCC (C), compound 1 purified by semi-preparative HPLC (D), and reference solution of echinacoside and verbascoside (E)

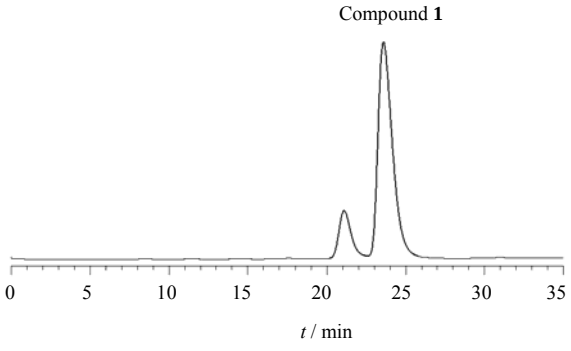

Fig. 4 Semi-preparative HPLC chromatogram of compound 1

Characterization

The structural identification was performed with FT-IR, ESI-MS, and NMR. The structure of compound **1** was confirmed as follows. IR ν_{max}^{KBr} (cm^{-1}): 3420 (OH), 2932 (C-H), 1692 (conj. ester), 1630 (CH=CH), 1604, 1524 (aromatic ring). Negative ESI-MS: *m/z* 785 (M-H), molecular formula: $C_{35}H_{46}O_{20}$. ^{1}H-NMR (CD_3OD, 600 MHz) δ: 7.59 (1H, d, J = 19.1 Hz, H-7″), 7.05 (1H, d, J = 2.1 Hz, H-2″), 6.94 (1H, dd, J = 8.3, 2.1 Hz, H-6″), 6.77 (1H, d, J = 8.3 Hz, H-5″), 6.70 (1H, d, J = 2.1 Hz, H-2), 6.69 (1H, d, J = 8.5 Hz, H-5), 6.58 (1H, dd, J = 8.5, 2.1 Hz, H-6), 6.27 (1H, d, J = 19.1 Hz,

H-8″), 5.17(1H, d, *J* = 1.2 Hz, Rha-H-1), 5.00 (1H, t, *J* = 9.5 Hz, H-4′), 4.38 (1H, d, *J* = 9.5 Hz, H-1′), 4.29 (1H, d, *J* = 9.3 Hz, Glc-H-1), 3.91 (1H, m, Hb-8), 3.79 (1H, dd, *J* = 10.5, 3.6 Hz, H-3′), 3.56 (1H, m, Ha-8), 2.79 (2H, t, *J* = 5.7 Hz, H-7), 1.07 (1H, d, *J* = 7.4 Hz, Rha-H-6). ^{13}C-NMR (CD_3OD) δ: 168.7 (C-9″), 149.9 (C-3″), 148.3 (C-7″), 146.9 (C-4″), 146.1 (C-4), 144.7 (C-3), 131.4 (C-1), 127.5 (C-1″), 123.3 (C-6″), 121.3 (C-6), 117.2 (C-5), 116.5 (C-2), 116.4 (C-5″), 115.3 (C-8″), 114.7 (C-2″), 104.7 (Glc-C-1), 104.2 (C-1′), 103.1 (Rha-C-1), 81.7 (C-3′), 78.0 (Glc-C-3), 77.9 (Glc-C-5), 76.2 (C-2′), 74.8 (C-5′, Glc-C-2), 73.8 (Rha-C-4), 72.4 (C-8, Rha-C-2), 72.1 (Rha-C-3), 71.5 (Glc-C-4), 70.6 (C-4′, Rha-C-5), 69.4 (C-6′), 62.7 (Glc-C-6), 36.6 (C-7), 18.5 (Rha-C-6).

The structure of compound **2** was confirmed as follows. IR ν_{max}^{KBr} (cm^{-1}): 3403 (OH), 2935 (C-H), 1693 (conj. ester), 1630 (CH=CH), 1607, 1522 (aromatic ring). Negative ESI-MS: *m/z* 623 [M – H], molecular formula: $C_{29}H_{36}O_{15}$. ^{1}H-NMR (CD_3OD, 600 MHz) δ: 1.09 (3H, d, *J* = 6 Hz, CH_3 of Rha), 2.78 (2H, t, *J* = 7 Hz, H-7), 4.37 (1H, d, *J* = 9.5 Hz, H-1′), 5.01 (1H, t, *J* = 9.5 Hz, H-4′), 5.16 (1H, d, *J* = 1.2 Hz, Rha-H-1), 6.25 (1H, d, *J* = 19.1 Hz, H-8″), 6.58, 6.69, 6.70, 6.77, 6.94, 7.05 (6H, aromatic H), 7.58 (1H, d, *J* = 19.1 Hz, H-7″). ^{13}C-NMR (MeOD) δ: 168.7 (C-9″), 149.9 (C-3″), 148.3 (C-7″), 146.9 (C-4″), 146.1 (C-4), 144.7 (C-3), 131.4 (C-1), 127.5 (C-1″), 123.3 (C-6″), 121.3 (C-6), 117.2 (C-5), 116.5 (C-2), 116.4 (C-5″), 115.3 (C-8″), 114.7 (C-2″), 104.2 (C-1′), 103.1 (Rha-C-1), 81.7 (C-3′), 76.2 (C-2′), 73.8 (Rha-C-4), 72.4 (C-8, Rha-C-2), 72.1 (Rha-C-3), 70.6 (C-4′, Rha-C-5), 69.4 (C-6′), 36.6 (C-7), 18.5 (Rha-C-6).

The spectral data of compounds **1** and **2** matched those of echinacoside and verbascoside in literatures (Han *et al*, 2012; Kobayashi *et al*, 1984).

HSCCC combined with semi-preparative HPLC is an efficient and superior separation mode for the isolation and purification of echinacoside and verbascoside. It is a feasible and economical technique for the rapid separation of phenylethanoid glycosides. Besides, to the best of our knowledge, this is the first report on discovering and isolating echinacoside from the plant of *P. digitalis.*

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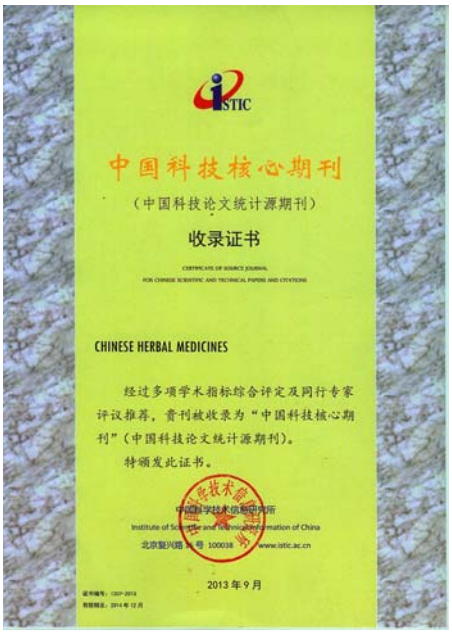

Latest Progress on *Chinese Herbal Medicines* (CHM)

Since the foundation of Chinese Herbal Medicines (CHM) in 2009, it has been included in China Academic Journals Integrated Online Database, Chemical Abstracts Service (CAS) in USA, Index of Copurnicus (IC) in Poland, Ulrich's Periodicals Directory (UPD) in USA, Centre for Agriculture and Bioscience International Abstracts (CABI), Global Health (GH), and EMBASE in Holland.

On Sep. 27, 2013, Institute of Scientific and Technical Information of China (ISTIC) revealed CHINESE S&T JOURNAL CITATION REPORTS (Expanded version). As reported, the total frequency of Chinese Herbal Medicines (CHM) being cited was 64, the expanded influence factor was 0.495, and 77.6% of all the papers were supported by fund.

We sincerely thank the readers, authors, reviewers, editorial board, and leadership at all levels of the majority for the care and support to CHM. All staff editorial department will continue to advance with the times, make a greater contribution to the development and internationalization of the cause of Chinese medicine.