

Short communication

Two-step purification of scutellarin from *Erigeron breviscapus* (vant.) Hand. Mazz. by high-speed counter-current chromatography

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Abstract

Scutellarin, a flavone glycoside, popularly applied for the treatment of cardiopathy, has been purified in two-step purification by high-speed counter-current chromatography (HSCCC) from *Erigeron breviscapus* (vant.) Hand. Mazz. (Deng-zhan-hua in Chinese), a well-known traditional Chinese medicinal plant for heart disease. Two solvent systems, *n*-hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1:4, v/v/v/v/v) and ethyl acetate–*n*-butanol–acetonitrile–0.1% HCl (5:2:5:10, v/v/v/v) were used for the two-step purification. The purity of the collected fraction of scutellarin was 95.6%. This study supplies a new alternative method for purification of scutellarin.

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1. Introduction

Erigeron breviscapus (vant.) Hand. Mazz. is one of the most important traditional Chinese medicinal plants for cardiopathy [1], and is also used for expelling the cold, relieving exterior syndrome, removing stagnancy of indigested food and relieving pain [2–4]. Scutellarin, the major active component of *E. breviscapus*, is a flavone glucuronide whose structure is shown in Fig. 1. Scutellarin is drawing particular interests because it significantly dilates blood vessel, improves microcirculation, increases cerebral blood flow and inhibits platelet aggregation activity [5,6].

Normally, the separation and purification of scutellarin from the extract of *E. breviscapus* are performed by polyamide chromatography and macroporous resin adsorption chromatography [7,8]. It is pretty easy to enlarge the scale of separation by these two methods. However, it is impossible to eliminate the irreversible adsorptive loss of samples onto the solid support matrix, and column deterioration is a popular problem for gel chromatography, even for preparative reversed phase chromatogra-

phy [9]. High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography without any solid matrix, which eliminates the irreversible adsorption of samples on solid support leading to no column deterioration for HSCCC. HSCCC has been applied to the separation of different kinds of natural products, including flavonoids and flavone glycosides [10–12]. By now, purification of scutellarin from *E. breviscapus* by HSCCC has not been reported. The present study describes successful semi-preparative separation and purification of scutellarin from the crude extract of *E. breviscapus* by HSCCC.

2. Experimental

2.1. Apparatus

HSCCC (TBE-300) is from Tauto Biotech, Shanghai, China, with three preparative coils connected in series (diameter of 2.6 mm, total volume 300 ml) and a 20 ml sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β value varied from 0.5 at internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The HSCCC systems are equipped with a Model S constant-flow

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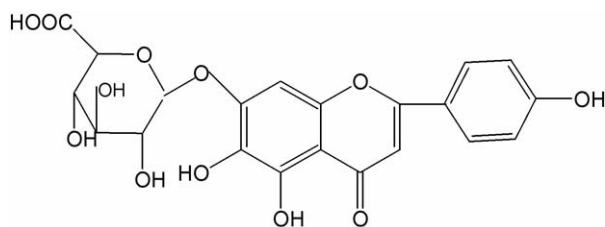


Fig. 1. The chemical structure of scutellarin.

pump, a Model 8823A UV monitor operating at 280 nm and a Model 3057 recorder.

2.2. Reagents

Analytical-grade methanol, *n*-butanol, acetic acid, ethyl acetate, acetonitrile and *n*-hexane were from Atoz Fine Chemicals Co. Ltd., Tianjin, China. Methanol of HPLC grade was obtained from Concord Tech. Co. Ltd., Tianjin, China. All aqueous solutions were prepared with pure water produced by Milli-Q system (18 M Ω , Milipore, Bedford, MA, USA).

The whole plant of *E. breviscapus* was collected from Qu-bei, Yun-nan province, China in October 2004. The air-dried material was stored under room temperature. The standard sample of scutellarin was supplied by the State Food and Drug Administration of China (SFDA). Another crude sample of scutellarin with the purity of 80% was supplied by Wanfang Co., Yun-nan province.

2.3. Preparation of two-phase solvent system for HSCCC separation

Two-step separation strategy was used in the present study on HSCCC with two solvent systems composed of *n*-hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1:4, v/v/v/v/v) and ethyl acetate–*n*-butanol–acetonitrile–0.1% HCl (5:2:5:10, v/v/v/v). Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use.

2.4. Preparation of crude sample and sample solution

The whole herb of *E. breviscapus* was dried at room temperature and then ground into powder (100 g) that was extracted with 400 ml 60% methanol for 3 h at 60 °C. The sample extraction procedure was repeated twice and the supernatants were combined together. The extract was filtered and evaporated to dryness by rotary vaporization. Crude sample (24.8 g) was obtained and stored in a refrigerator for the subsequent HSCCC separation.

The sample solution was prepared by dissolving 20 mg of the dried crude sample in 3 ml of the lower phase of the first solvent system.

Stock solution of scutellarin (1.0 mg/ml) was prepared in methanol. A series of standard working solutions with concentrations in the range of 10–200 μ g/ml for scutellarin were

obtained by further dilution of the stock solution with mobile phase.

2.5. Measurement of partition coefficient (*K*)

Scutellarin (2.0 mg) was weighed and put in a 10 ml test tube to which 3 ml of pre-equilibrium upper phase and lower phase solvent was added with the proportion of the two phases is 1:1 (v/v). The test tube was stoppered and shaken vigorously for 10 min to thoroughly equilibrate scutellarin with the two phases. The solution was then separated by centrifugation. The upper and lower phases were then determined by UV at 335 nm to obtain the partition coefficient (*K*) of scutellarin. *K* was expressed as the absorbency of scutellarin in the upper phase divided by that in the lower phases.

2.6. HSCCC separation procedure

In each run, the coiled column was first filled with the upper phase of solvent system. Then, the apparatus was rotated at 850 rpm, and at the same time, the lower phase was pumped through the column at a flow-rate of 1.5 ml/min. After the mobile phase emerged in the effluent, hydrodynamic equilibrium was established in the column, 3 ml of the sample solution containing 20 mg of crude scutellarin was injected through the valve. The effluent was monitored with a UV–vis detector at 280 nm and the peak fractions were collected according to the elution profile.

2.7. HPLC analysis and identification of HSCCC peak fractions

The crude sample and each peak fraction obtained by HSCCC were analyzed by Agilent 1100 HPLC system. An Agilent 1100 system is composed of a quaternary pump with a degasser, a thermostatted column compartment, a variable wavelength detector, an auto-sampler and 1100 ChemStation software. Sample analyses were performed on an Alltech C₁₈ column (250 mm \times 4.6 mm I.D., 5 μ m.) with a gradient elution of 0.1% phosphoric acid (A) and methanol (B) as follows: A–B (90:10) to A–B (10:90) in 70 min. The flow-rate was 0.8 ml/min and the effluent was monitored at 335 nm by UV detector.

An Agilent 1100 system was applied for LC–MS with the same conditions as in sample analysis except that the phosphoric acid was changed to TFA. A UV6000LP photodiode array detector (Finnigan MAT, San Jose, MA, USA) was used to monitor continuously at 335 nm. The outlet of the flow cell was connected to a splitting valve and a flow of 100 μ l/min was achieved and induced to the electrospray ion source via a short length of fused silica tubing. Electrospray ionization–mass spectrometry (ESI–MS) was performed on a Finnigan LCQ DecaXP ion trap mass spectrometry (Thermo Finnigan, San Jose, CA, USA). A spray voltage of 4.5 kV was employed and the temperature of heated transfer capillary was set to 275 °C. Nitrogen was used as collision and drying gas with the flow-rate of 7 l/min and the collision energy was 35%. The mass spectrometer was scanned from *m/z* 100 to 1000 with full scan mode.

Scutellarin and HSCCC purified scutellarin was scanned by a UV6000 LP photodiode array detector from 600 to 200 nm.

3. Results and discussion

3.1. Optimization of HPLC method

HPLC and high-performance capillary electrophoresis (CE) have been reported in the determination of scutellarin [13], but HPLC is a popular method to analyze crude extract and HSCCC fractions. Optimization was performed on the crude extract, and composition and pH value of mobile phase were discussed. Methanol led to a better resolution of separation than acetonitrile, which was selected as mobile phase with water. Phosphoric acid was used to adjust the pH value. The mobile phase at higher than pH 4 caused peak tailing. However, the peaks were sharp and symmetric with the application of pH 3.0. Also this pH value is helpful to suppress ionization of the weak acidic phenolic group and interactions of these groups with residual traces of metals in the stationary phase [14]. The crude extract was separated efficiently under the optimized conditions as shown in Fig. 2. To identify the scutellarin in the crude sample, 0.50 mg/ml reference material was added to the crude sample and the mixture was performed on HPLC. The peak area of peak 1 increased evidently and peak 1 was shown to be scutellarin.

3.2. Optimization of HSCCC separation

Since HSCCC is a liquid–liquid partition separation method, two-phase solvent system is essential for the successful separation. Partition coefficient (K) is the most important parameter in solvent system selection, which should be in the range of 0.5–2.5 to get an efficient separation and a suitable run time. Based on the chemical properties of scutellarin that has hydrophobic group and hydrophilic group at the same time, a series of experiments were tried and K values were calculated as shown in Table 1. In solvent system with very high polarity of *n*-butanol–water, most part of scutellarin was in the

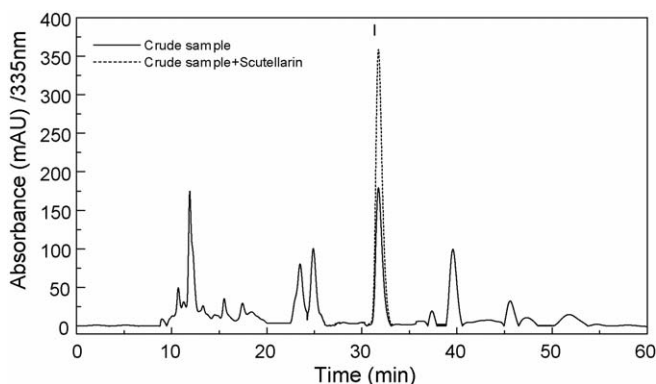


Fig. 2. HPLC chromatography of the crude extract from *E. breviscapus*: reversed-phase Alltech C_{18} (4.6 mm \times 250 mm I.D., 5 μ m); mobile phase, gradient elution of 0.1% phosphoric acid (A) and methanol (B) as follows: A–B (90:10) to A–B (10:90) in 70 min; flow-rate, 0.8 ml/min; detection wavelength, 335 nm.

Table 1

The K (partition coefficient) values of the scutellarin in different solvent system

Solvent system (v/v)	K
<i>n</i> -Butanol–water (1:1)	8.63
<i>n</i> -Butanol–acetic acid–water (4:1:5)	2.30
<i>n</i> -Butanol–methanol–water (4:1:5)	1.09
<i>n</i> -Butanol–ethanol–water (4:1:5)	0.96
Chloroform–methanol–water (5:6:4)	13.7
Chloroform–methanol–water (2:2:1)	7.51
Chloroform–methanol–acetic acid–water (5:6:1:4)	6.52
Chloroform–methanol–acetic acid–water (2:1:1:2)	4.19
Ethyl acetate– <i>n</i> -butanol–acetonitrile–water (5:1:7:10)	0.82
Ethyl acetate– <i>n</i> -butanol–acetonitrile–water (5:2:5:10)	0.91
Ethyl acetate– <i>n</i> -butanol–acetonitrile–0.1% HCl (5:2:5:10)	1.48
<i>n</i> -Hexane–ethyl acetate–methanol–water (1.5:5:1.5:5)	0.29
<i>n</i> -Hexane–ethyl acetate–methanol–water (1:6:1.5:7.5)	0.29
<i>n</i> -Hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1:4)	0.573
<i>n</i> -Hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:2:3)	0.418

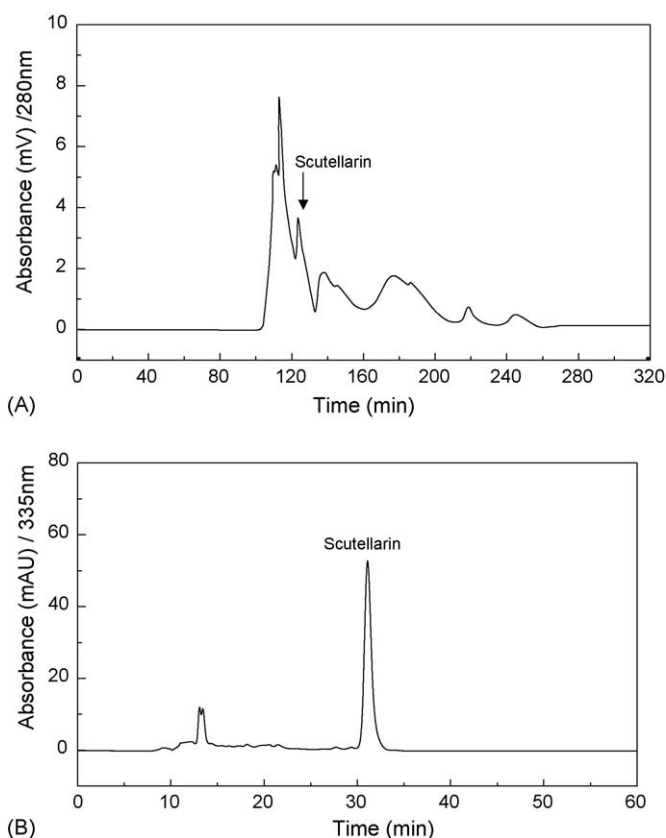


Fig. 3. (A) HSCCC chromatography of the crude sample. *n*-Hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1:4, v/v/v/v/v); station phase, upper phase; mobile phase, lower phase; flow-rate, 1.5 ml/min; revolution speed, 850 rpm; separation temperature, 20 °C; sample size, 20 mg; retention of station phase, 61%; sample loop, 20 ml; detection wavelength, 280 nm. (B) HPLC chromatography of the fractions obtained by HSCCC reversed-phase Alltech C_{18} (4.6 mm \times 250 mm I.D., 5 μ m); mobile phase, gradient elution of 0.1% phosphoric acid (A) and methanol (B) as follows: A–B (90:10) to A–B (10:90) in 70 min; flow-rate, 0.8 ml/min; detection wavelength, 335 nm.

upper phase and K value was very high. When acetic acid or methanol or ethanol was added, better K value was got, but it was difficult to separate scutellarin from the other compound on HSCCC with the above three solvent systems. The solvent systems with medium polarity, such as chloroform–methanol–water were tried, however the K values were much bigger than 2.5. Although K values of the solvent systems composed of ethyl acetate–*n*-butanol–acetonitrile–water were appropriate, it was difficult to separate scutellarin from other compounds because of low retention of stationary phase leading to low resolution. Subsequently the solvent systems composed of *n*-hexane–ethyl acetate–methanol–water were tried, which showed a little lower K value. To improve the polarity of the solvent systems, acetic acid was added and finally K values were in the suitable range of 0.5–2.5. The solvent system composed of *n*-hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1:4, v/v/v/v/v) was performed on HSCCC to separate the crude sample of *E. breviscapus*. The influence of the flow-rate of mobile phase and the revolution speed were also investigated to improve the retention of the stationary phase. The results indicated that reducing flow-rate and increasing the revolution speed could improve the retention of the stationary phase leading to better resolution. The flow-rate of 1.5 ml/min and the revolution speed

of 850 rpm were used. The chromatogram of HSCCC separation is shown in Fig. 3A. Each fraction was analyzed by reversed phase chromatography with C_{18} column and the target fraction was primarily located at the retention time of 120 min with a purity of 81.3% according to HPLC analysis (Fig. 3B). For further confirmation, HSCCC fraction of supposed scutellarin was mixed with the sample solution of standard sample of scutellarin and the peak area of scutellarin increased evidently (data not shown).

To get better purity of scutellarin, the solvent system with better K value (close to 1) composed of ethyl acetate–*n*-butanol–acetonitrile–0.1% HCl (5:2:5:10, v/v/v/v) was used for the second step HSCCC separation (Fig. 4A). Compared with reference material of scutellarin, the target fraction was located at the retention time of 150 min with a purity of 95.6% analyzed by HPLC C_{18} column (Fig. 4B). The HSCCC separation was stopped after the fraction of scutellarin was eluted. The yield of scutellarin after two-step HSCCC separation is 89.12%. For further identification, HSCCC fraction of supposed scutellarin and reference material were analyzed by HPLC–MS. HSCCC target fraction was identified as scutellarin based on three major

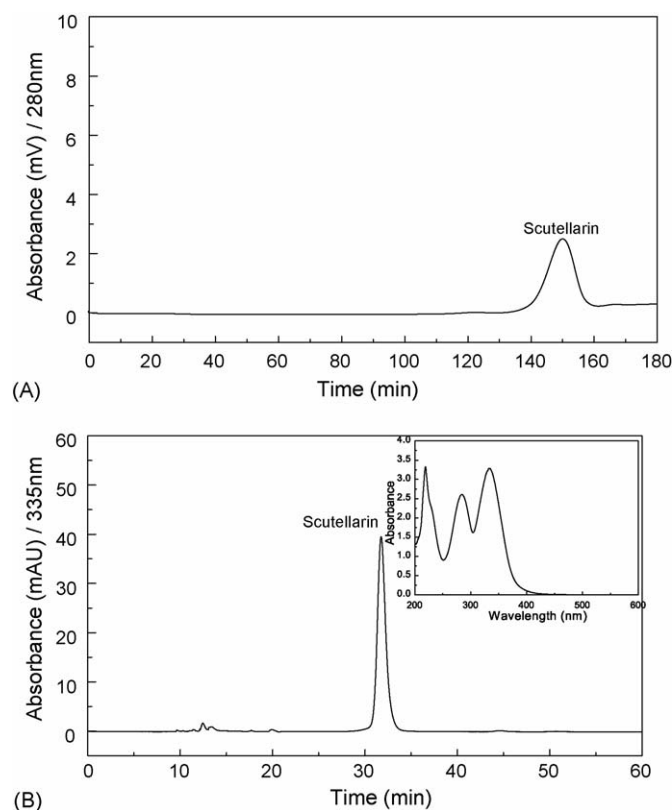


Fig. 4. (A) HSCCC chromatography of peak fraction of one-step separation. Ethyl acetate–*n*-butanol–acetonitrile–0.1% HCl (5:2:5:10, v/v/v/v); station phase, upper phase; mobile phase, lower phase; flow-rate, 1.5 ml/min; revolution speed, 850 rpm; separation temperature, 20 °C; sample size, 5 mg; retention of station phase, 31%; sample loop, 20 ml; detection wavelength, 280 nm. (B) HPLC chromatography of the fractions obtained by two-step HSCCC (with the same condition of Fig. 3B).

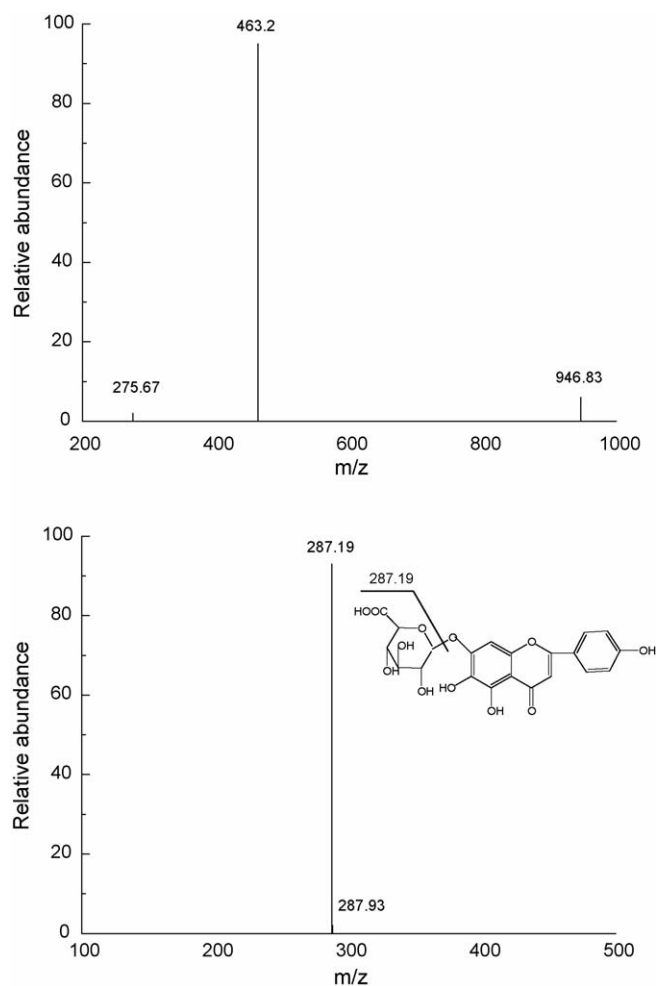


Fig. 5. (A) Positive-ion mass spectrum of scutellarin; (B) the MS/MS spectra of scutellarin. MS conditions: nitrogen gas, 50 unit/min; spray voltage, 4.5 kV; capillary temperature, 275 °C; positive mode; collision energy, 35%.

parameters were included as follows: retention time of HPLC analysis, UV spectrum and MS spectrum. HPLC–MS showed an $[M + 1]^+$ peak at m/z 463.2 corresponding to the molecular formula $C_{21}H_{18}O_{12}$ (Fig. 5A). The MS/MS spectrum of scutellarin showed a fragmentation pattern at m/z 287.2 corresponding to the aglycone of scutellarin (Fig. 5B).

4. Conclusions

A two-step separation method was developed for the semi-preparative purification of scutellarin from *E. breviscapus* on HSCCC with two solvent systems, *n*-hexane–ethyl acetate–methanol–acetic acid–water (1:6:1.5:1:4, v/v/v/v/v) and ethyl acetate–*n*-butanol–acetonitrile–0.1% HCl (5:2:5:10, v/v/v/v). The purity of the scutellarin fraction was 95.6%, which served an alternative method to yield high-purity of scutellarin.

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