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Short communication

Separation and purification of baicalin and wogonoside from the Chinese medicinal plant Scutellaria baicalensis Georgi by high-speed counter-current chromatography

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Abstract

A preparative high-speed counter-current chromatography (HSCCC) method for isolation and purification of baicalin and wogonoside from the Chinese medicinal plant Scutellaria baicalensis Georgi (Huang-qin in Chinese) was successfully established by using ethyl acetate–methanol–1% acetic acid water (5:0.5:5, v/v) as the two-phase solvent system. The upper phase of ethyl acetate–methanol–1% acetic acid water (5:0.5:5, v/v) was used as the stationary phase of HSCCC. Baicalin (58.1 mg) and wogonoside (17.0 mg) with the purity of 99.2 and 99.0%, respectively, were separated successfully in one-step separation from 120 mg of crude sample from S. baicalensis Georgi. The structures of baicalin and wogonoside were identified by ^{1}H NMR and ^{13}C NMR.

Keywords: Counter-current chromatography; Scutellaria baicalensis Georgi; Baicalin; Wogonoside

1. Introduction

Huang-qin, the roots of Scutellaria baicalensis (S. baicalensis) Georgi, is one of the most popular traditional medicinal plants in China and officially listed in the Chinese Pharmacopoeia [1]. Pharmacological test revealed that S. baicalensis Georgi could reduce the total cholesterol level, decrease blood pressures, clear away the fire of human body, and detoxify toxicosis [2]. Its roots have been used for cleaning away heat, relieving a cough and stanching blood in China. Baicalin and wogonoside are the main bioactive constituents in S. baicalensis Georgi. In recent years, researches have also shown that baicalin not only has the effects of reducing the total cholesterol level, detoxifying, and antitumor but also the effect of chemoprevention [2–4]. The chemical structures of baicalin and wogonoside are shown in Fig. 1.

High-speed counter-current chromatography (HSCCC) is a kind of support-free all-liquid partition chromatography that was first invented by Y. Ito [5]. Solute separation is based on partitioning between the two immiscible liquid phases: the mobile phase and the stationary phase. As no solid stationary phase is used, no irreversible adsorption of samples on the active surfaces takes place. Successful application of HSCCC for the separation and purification of baicalin from the root of S. baicalensis Georgi has been reported previously [6]. The separation was performed by a two-step HSCCC separation. The purity of baicalin in the first separation was 82%, and 96.5% at last. In present study, baicalin and wogonoside were purified successfully in one-step separation with the purity of 99.2 and 99.0%, respectively, as determined by HPLC. The present work developed a successful HSCCC method for the separation of wogonoside for the first time

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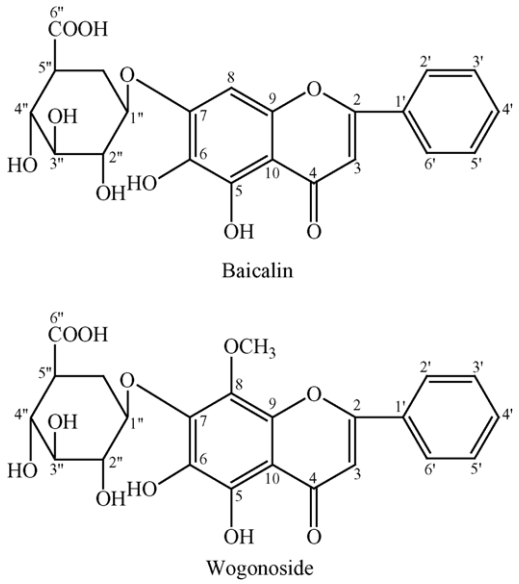

Fig. 1. Chemical structures of baicalin and wogonoside.

and ameliorated an efficient method for the separation of baicalin.

2. Experimental

2.1. Reagents and materials

All solvents used for preparation of crude sample and HSCCC separation were of analytical grade (Jinan Reagent Factory, Jinan, China). Methanol used for HPLC was of chromatographic grade (Yucheng Chemical Factory, Yucheng, China), and water used was distilled water. [$^2H_6$]dimethyl sulfoxide (DMSO-d_6) was used as the solvent for NMR determination.

The roots of S. baicalensis Georgi was purchased from Limin-drugstore and identified by professor Yongqing Zhang (Shandong University of Traditional Chinese Medicine, Jinan, China).

2.2. Apparatus

The HSCCC instrument employed in the present study is TBE-300A high-speed counter-current chromatography (Tauto Biotechnique Company, Shanghai, China) with three multilayer coil separation column connected in series (i.d. of the tubing = 1.6 mm, total volume = 260 ml) and a 20 ml sample loop. The revolution radius was 5 cm, and the β-values of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 and 1000 rpm. An HX 1050 constant-temperature circulating implement (Beijing Boyikang Lab Instrument Co. Ltd., Beijing, China) was used to control the separation temperature. A ÄKTA prime (Amersham Pharmacia Biotechnique Group, Sweden) was used to pump the two-phase solvent system and perform the UV absorbance measurement. It contains a switch valve and a mixer, which were used for gradient formation. The data were collected with Sepu 3000 chromatography workstation (Hangzhou Puhui Science Apparatus Co. Ltd., Hangzhou, China).

A FZ102 plant disintegrator (Taisite Instrument Company, Tianjin, China) was used for disintegration of S. baicalensis Georgi sample.

The HPLC equipment used was Agilent 1100 HPLC system including a G1311A Quat pump, a G1315B DAD, a Rheodyne 7725i injection valve with a 20 μl loop, a G1332A degasser and Agilent HPLC workstation.

Nuclear magnetic resonance (NMR) spectrometer used here was Mercury Plus 400 NMR (Varian Inc., USA).

2.3. Preparation of crude sample

The roots of S. baicalensis Georgi was dried constant at 60 °C and then pulverized to about 30-mesh with a disintegrator. One hundred grams of the powder was extracted with 800 ml of boiling water for 30 min. The extraction procedure was then repeated twice again (700 ml each time) and the extracts were combined together. After filtrated with cotton, the pH of the filtrate was adjusted to about 2.0 with hydrochloric acid. The solution was heated nearly to boiling and then kept in a water bath with the temperature of 80 °C for 30 min in order to deposit completely. The upper liquid was spilled out. The deposit was filtrated and then dried at 60 °C under vacuum. Crude sample (24.2 g) was obtained and stored in a refrigerator for the subsequent HSCCC separation.

2.4. Selection of the two-phase solvent systems

Ethyl acetate–methanol–1% acetic acid water was used as the two-phase solvent system of HSCCC. The composition of it was selected according to the partition coefficient (K) of target compounds of crude sample extracted from the S. baicalensis Georgi. The K-values were determined by HPLC as follows: suitable amount of crude sample was dissolved in 5 ml of aqueous phase of the pre-equilibrated two-phase solvent system. The solution was determined by HPLC and the peak area was recorded as A_1. Then, equal volume of the organic phase was added to the solution and mixed thoroughly. After the equilibration was established, the aqueous phase was determined by HPLC again and the peak area was recorded as A_2. The partition coefficient (K) was obtained by the following equation: $K = (A_1 - A_2)/A_2$.

2.5. Preparation of two-phase solvent system and sample solution

Ethyl acetate–methanol–1% acetic acid water solvent system with the volume ratio of 5:0.5:5 (v/v) was prepared by adding the solvents to a separation funnel according to the volume ratios and thoroughly equilibrated by shaking

repeatedly. Then, the upper phase and the lower phase were separated and degassed by sonication for 30 min prior to use.

One hundred and twenty milligrams of crude extract sample was dissolved in 5 ml of the lower phase of ethyl acetate–methanol–1% acetic acid water system (5:0.5:5, v/v).

2.6. HSCCC separation

HSCCC separation procedure was carried out as follows: the upper phase (stationary phase) and the lower phase (mobile phase) of ethyl acetate–methanol–1% acetic acid water (5:0.5:5, v/v) were pumped into the separation column simultaneously using ÄKTA prime system, according to the volume ratio of 50:50. After the column was entirely filled with the two phases, only the lower phase was pumped at a flow rate of 1.5 ml min^{-1}, and at the same time, the HSCCC apparatus was run at the revolution speed of 900 rmp. After hydrodynamic equilibrium was reached (about half an hour later), the sample solution (120 mg of crude sample dissolved in 5 ml of the lower phase) was injected into the separation column. The separation temperature was controlled at 25 °C. The effluent from the tail end of the column was monitored at 254 nm. The chromatogram was recorded 30 min after sample injection. Different fractions were collected according to the obtained chromatogram and evaporated to dryness. The residuals were dissolved in methanol for subsequent HPLC analysis.

2.7. HPLC analysis and identification of HSCCC peak fractions

The crude sample and each HSCCC peak fraction were analyzed by HPLC. Analysis was accomplished with YWG C_{18} column (200 mm × 4.6 mm i.d., 10 μm) at room temperature. The mobile phase was methanol–0.05% acetic acid water (41:59) and the flow rate was 1.0 ml min^{-1}. The effluents were monitored at 254 nm by a photodiode array detector.

Identification of HSCCC peak fraction was performed by ^{1}H NMR and ^{13}C NMR, which were recorded on Mercury Plus 400 NMR with TMS as internal standard.

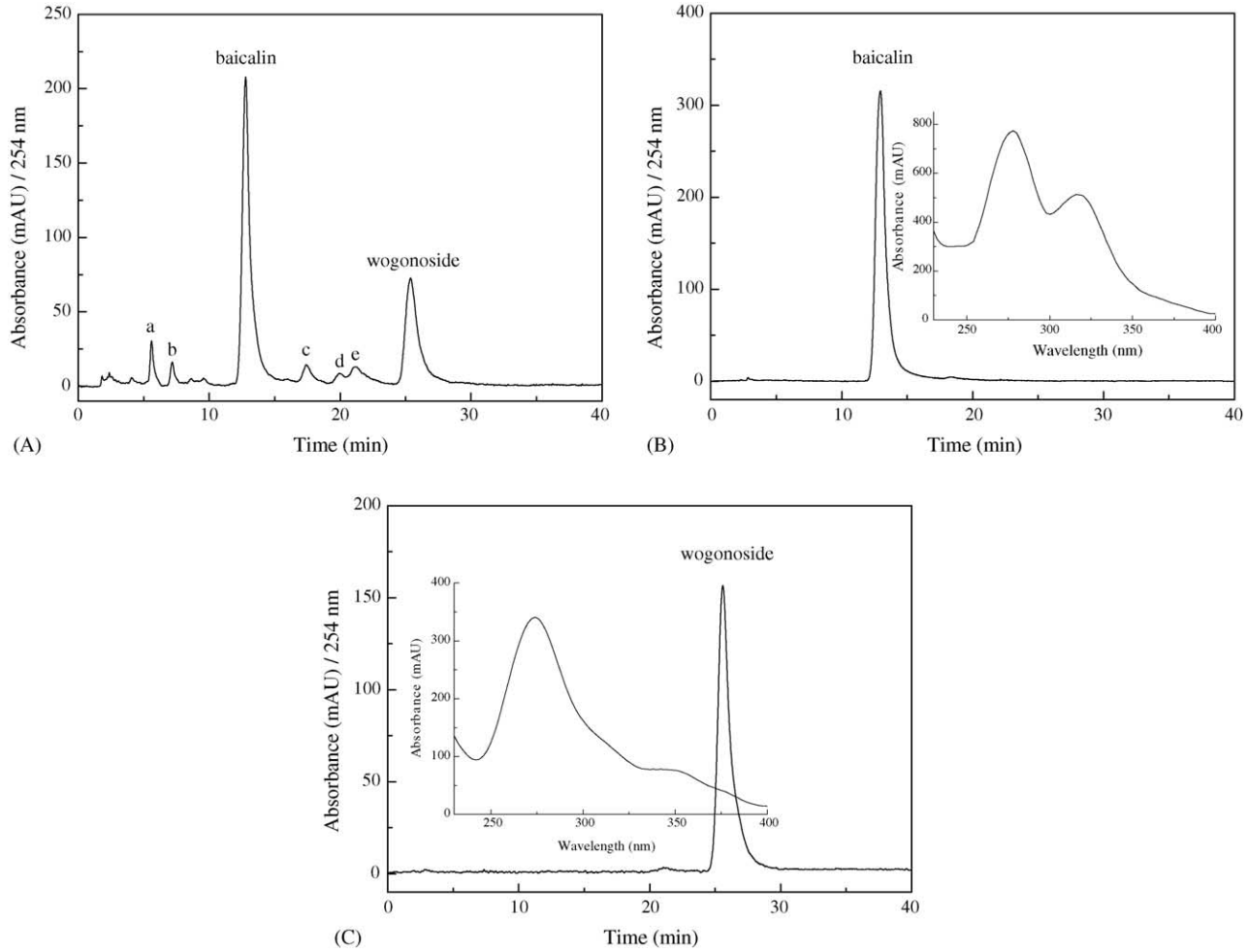

Fig. 2. HPLC chromatogram of crude extract from S. baicalensis Georgi and HSCCC peak fractions. *Conditions:* column, reversed phase YWG C_{18} column (200 mm × 4.6 mm i.d., 10 μm); mobile phase, methanol–0.05% acetic acid water (41:59, v/v); flow rate, 1.0 ml min^{-1}; detection wavelength, 254 nm. (A) Crude extract from S. baicalensis Georgi; (B) HSCCC peak fraction I in Fig. 3; (C) HSCCC peak fraction II in Fig. 3.

3. Results and discussions

3.1. Optimization of HPLC method

In the present work, HPLC method for analysis of crude sample and peak fractions of HSCCC was established at first. In order to select an appropriate elution system for the HPLC separation of baicalin and wogonoside, different kinds of solvents were employed to analyze crude sample. The results indicated that when methanol–0.05% acetic acid water was used as the mobile phase, major peaks can be obtained and each peak got baseline separation. The crude sample and peak fractions separated by HSCCC were also analyzed by HPLC. The chromatograms are shown in Fig. 2.

3.2. Optimization of two-phase solvent system and other HSCCC conditions of HSCCC

In the present study, different solvent systems such as ethyl acetate–water, ethyl acetate–methanol–water, ethyl acetate–acetic acid water, and ethyl acetate–methanol–acetic acid water were used as the two-phase solvent system to optimize the HSCCC separation condition. The results indicated that when ethyl acetate–water and ethyl acetate–methanol–water were used as the solvent systems, the target compounds mainly partitioned in the aqueous phase. So these systems were unsuitable for HSCCC separation. But when 1% acetic acid water was used instead of the water of ethyl acetate–methanol–water solvent system, the partition of the compounds between the upper phase and the lower phase could be improved greatly. The partition coefficient (K) of baicalin and wogonoside and other compounds present in the crude sample in ethyl acetate–methanol–1% acetic acid water solvent systems were shown in Table 1. The solvent systems listed in Table 1 were also tested in HSCCC separation. The results indicated that when ethyl acetate–methanol–1% acetic acid water (5:0.5:5, v/v) was used as the two-phase solvent system, baicalin and wogonoside with high purity could be separated successfully.

The influence of the flow rate of mobile phase was also investigated. The results indicated that reducing flow rate could improve the reservation of the stationary phase in some degree, but the chromatogram peaks were extended at the same time. At last, a flow rate of 1.5 ml min^{-1} was employed in the experiment and the retention of the stationary was about 46%.

The crude samples from S. baicalensis Georgi were separated and purified under the optimum HSCCC conditions.

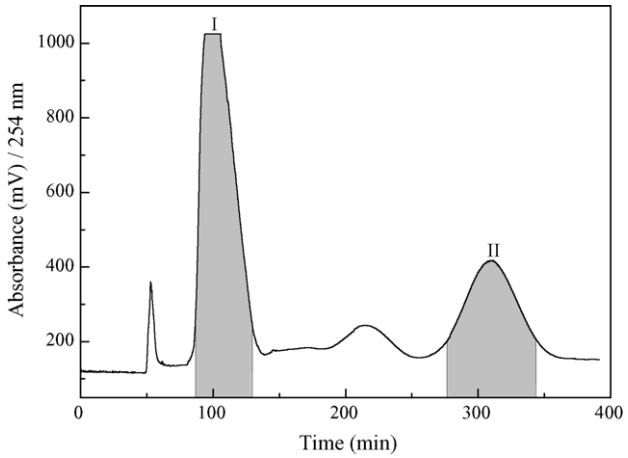

Fig. 3. HSCCC chromatogram of crude extract from S. baicalensis Georgi. Two-phase solvent system: ethyl acetate–methanol–1% acetic acid water (5:0.5:5, v/v); mobile phase: the lower phase; flow rate: 1.5 ml min^{-1}; revolution speed: 900 rpm; detection wavelength: 254 nm; sample size: 120 mg of crude sample dissolved in 5 ml of the lower phase; separation temperature: 25 °C; retention percentage of the stationary phase: 46%.

The HSCCC chromatogram was shown in Fig. 3. Baicalin (58.1 mg) (peak I, collected during 86–129 min) and wogonoside (17.0 mg) (peak II, collected during 276–345 min) could be obtained from 120 mg crude sample by one-step HSCCC separation. The purity of baicalin and wogonoside was 99.2 and 99.0%, respectively, as determined by HPLC. The chromatograms of HPLC and UV spectra of these compounds are shown in Fig. 2.

3.3. The structural identification

The chemical structure of each peak fraction separated by HSCCC was identified according to its ^{1}H NMR and ^{13}C NMR data.

Peak I: ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.60 (1H, s, 5-OH), 8.70 (1H, s, 6-OH), 8.07 (2H, m, H-2′, 6′), 7.61 (3H, m, H-3′, 4′, 5′), 7.05 (1H, s, H-3), 7.01 (1H, s, H-8). ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.5 (C-2), 106.1 (C-3), 182.5 (C-4), 146.8 (C-5), 130.6 (C-6), 151.2 (C-7), 93.7 (C-8), 149.2 (C-9), 104.7 (C-10), 130.8 (C-1′), 126.3 (C-2′, 6′), 129.1 (C-3′, 5′), 132.0 (C-4′), 100.1 (C-1″), 72.8 (C-2″), 75.2 (C-3″), 71.3 (C-4″), 75.4 (C-5″), 170.0 (C-6″). Compared with the data given in reference [7,8], peak I was identified as baicalin.

Peak II: ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.54 (1H, s, 5-OH) 8.09 (2H, m, H-2′, 6′), 7.64 (3H, m, H-3′, 4′, 5′), 7.08 (1H, s, H-3), 6.68 (1H, s, 6-OH), 3.90 (1H, s, 7-OCH_3).

Table 1
The K-values of baicalin, wogonoside and other compounds in ethyl acetate–methanol–1% acetic acid water

Solvent system (v/v)	K (a*)	K (b*)	K (baicalin)	K (c*)	K (d*)	K (e*)	K (wogonoside)
5:0.5:5	0.03	0.12	0.54	0.99	1.62	1.86	2.78
5:1:5	0.11	0.19	0.68	1.12	1.43	1.64	1.96
5:2:5	0.24	0.27	0.84	1.26	1.27	1.40	1.43

* Peak labelled in Fig. 2A.

^{13}C NMR (100 MHz, DMSO-d_6): δ 163.6 (C-2), 105.2 (C-3), 182.4 (C-4), 156.1 (C-5), 98.7 (C-6), 156.2 (C-7), 12.93 (C-8), 149.3 (C-9), 105.2 (C-10), 61.3 (8-OMe), 130.7 (C-1′) 126.3 (C-2′), 129.2 (C-3′), 132.2 (C-4′), 129.2 (C-5′), 126.3 (C-6′), 100.1 (C-1″), 73.0 (C-2″), 75.3 (C-3″), 71.4 (C-4″), 76.1 (C-5)″, 169.5 (C-6″). Compared with the data given in reference [7,8], peak II corresponded to wogonoside.

In conclusion, HSCCC was successfully used for the separation and purification of baicalin and wogonoside from the root of S. baicalensis Georgi. Baicalin (58.1 mg) and wogonoside (17.0 mg) were yielded from 120 mg of the crude extract in one-step separation.

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