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Application of high-speed counter-current chromatography to the preparative separation and purification of baicalin from the Chinese medicinal plant *Scutellaria baicalensis*

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Abstract

Baicalin was separated and purified for the first time from the traditional Chinese medicinal plant *Scutellaria baicalensis* Georgi by high-speed counter-current chromatography. Crude baicalin was obtained by extraction with methanol–water (70:30, v/v) from *S. baicalensis* Georgi. The separation was performed in two steps with a two-phase solvent system composed of *n*-butanol–water (1:1, v/v), in which the lower phase was used as the mobile phase at a flow-rate of 1.0 ml min^{-1} in the head-to-tail elution mode. A total of 37.0 mg of baicalin at 96.5% purity was yielded from 200 mg of the crude baicalin (containing 21.6% baicalin) with 86.0% recovery as determined by HPLC analysis.

Keywords: Counter-current chromatography; Preparative chromatography; *Scutellaria baicalensis*; Pharmaceutical analysis; Plant materials; Baicalin; Flavones

1. Introduction

Scutellaria baicalensis (*S. baicalensis*) Georgi (Chinese name: Huang Qin) is a traditional Chinese herbal medicine. Its roots have been used for cleaning away heat, moistening aridity, purging fire, detoxifying toxicosis, reducing the total cholesterol level and decreasing blood pressures. This herb also possesses cholagogic, diuretic, and cathartic actions. Some concentrated composite herbal preparations that contain *S. baicalensis* Georgi as a major ingredient in their prescriptions are widely used in oriental countries. Baicalin is one of the main bioactive flavone constituents in *S. baicalensis* Georgi with a wide range of beneficial activities. Its chemical structure is shown in Fig. 1. In recent years, researches have shown that baicalin has not only detoxifying, inhibiting peripheral capillary permeability and antitumor effects but also a chemoprevention effect [1–3]. Thus, it is important to develop an efficient method to separate and purify baicalin.

Conventional chromatographic methods use solid stationary phases and need multiple chromatographic steps. In contrast, high-speed counter-current

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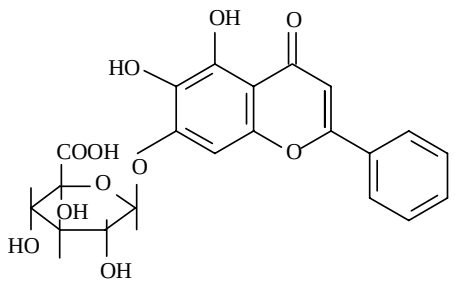

Fig. 1. Chemical structure of baicalin.

chromatography (HSCCC) is a support-free all-liquid chromatographic technique that is widely used due to the gentle operation conditions. As no solid stationary phase is needed, no irreversible adsorption on active surfaces take place. A diverse spectrum of solvent systems is readily available which renders the technique very versatile [4]. Therefore, this technique has been recently successfully used for the preparative separation of a number of Chinese medicinal products [5–16]. However, no report has been published on the use of HSCCC for the separation and purification of baicalin from Chinese herbs. The aim of the present work was to develop an efficient method for the preparation of high-purity baicalin from the Chinese medicinal plant *S. baicalensis* Georgi by HSCCC.

2. Experimental

2.1. Apparatus

The equipment employed for the preparative HSCCC was a Model CCC-1000 high-speed countercurrent chromatograph (Pharma-Tech Research, Baltimore, MD, USA). This apparatus had three preparative coils, connected in series (diameter of the tube, 2.6 mm; total volume, 342 ml). The revolution radius between the holder axis and central axis of the centrifuge (R) was 7.5 cm, and the β value varied from 0.47 at the internal terminal to 0.73 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The revolution speed of the apparatus could be regulated with a speed controller in a range between 0 and 2000 rpm. The HSCCC system was equipped with a Model Series II HPLC pump (Parma-Tech Research), a Model SPD-10 Avp UV-Vis detector (Shimadzu, Japan), a Model L 120 E flat-bed recorder (Linseis, Germany), and a sample injection valve with a 5 ml sample loop.

The HPLC system consisted of two Waters 510 pumps (Waters, Milford, MA, USA), a sample injector (Rheodyne, Cotati, CA, USA) with a 20 μl loop, a Waters temperature control module, an RCM-100/column heater, and a Waters 996 photodiode array detection (DAD) system. The column used was a reversed-phase Symmetry C_{18} column (150 × 3.9 mm i.d., 5 μm, Waters). Evaluation and quantification were made on a Millennium chromatography data system (Waters).

2.2. Reagents

Baicalin was purchased from Wako (Wako, Japan). HPLC-grade acetonitrile, chloroform, acetic acid, methanol, *n*-butanol, ethyl acetate, trifluoroacetic acid (TFA) and ethanol were purchased from BDH (Poole, UK).

S. baicalensis Georgi was purchased from Beijing Tong-Ren-Tang drug retail outlet in Hong Kong.

Stock solution of baicalin (1.0 mg ml^{-1}) was prepared in 80% methanol solution and diluted to the desired concentration prior to use.

2.3. Preparation of crude baicalin

Baicalin was extracted from the roots of *S. baicalensis* Georgi as described previously [17]. In brief, the roots of *S. baicalensis* Georgi were dried to constant mass at 60 °C and then pulverized. Thirty grams of the pulverized sample was weighed and put into a 1000 ml flask, to which 400 ml of methanol–water (70:30, v/v) was added. After the mixture had been extracted at ambient temperature under sonication for 30 min, the mixture was filtrated. The extraction procedure was repeated twice (200 ml each time). All the filtrates were combined and evaporated to dryness by rotatory evaporation. Crude baicalin (16.9 g) was yielded and stored in a refrigerator for the subsequent HSCCC separation.

2.4. Measurement of partition coefficient (K)

Approximately 0.1 mg of baicalin was weighed in a 10 ml test tube to which 2 ml of each phase of the pre-equilibrated two-phase solvent system was added.

The test tube was stoppered, and shaken vigorously for 10 min to thoroughly equilibrate baicalin with the two phases. The solution was then separated by centrifugation at 4000 × *g* for 10 min. Then, equal volumes of the upper and lower phases were evaporated to dryness, respectively. The residues were dissolved with 80% methanol to equal volumes and analyzed by HPLC to obtain the partition coefficient (*K*) of baicalin. *K* was expressed as the peak area of baicalin in the upper phase divided by that in the lower phase.

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

In the present study, different two-phase solvent systems at various ratios were selected. Each solvent system was thoroughly equilibrated in a separation funnel by repeatedly vigorously shaking at room temperature. The two phases were separated shortly and degassed by sonication prior to use. The sample solution was prepared by dissolving the crude baicalin in the mixture solution of lower phase and upper phase (1:1, v/v) of the solvent system used for HSCCC separation.

2.6. HSCCC separation

In each separation, the multilayer-coiled column was first filled entirely with the upper non-aqueous stationary phase, and then 200 mg of the sample dissolved in 5 ml of both phases was loaded. The centrifuge was rotated at 900 rpm, while the lower aqueous mobile phase was pumped into the head end of the inlet column at a flow-rate of 1.0 ml min^{-1} by an HPLC pump. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution was injected through the injection valve. The effluent from the outlet of the column was continuously monitored with a UV-Vis detector at 277 nm and the chromatogram was recorded by an L 120 E flat-bed recorder. Peak fractions were collected according to the elution profile as determined by HPLC. After the separation was completed, retention of the stationary phase was measured by collecting the column contents by forcing them out of the column with pressurized nitrogen gas in conjunction with slow rotation of the coil in the tail-to-head elution mode.

2.7. HPLC analysis and identification of baicalin

Analysis of baicalin in the crude sample and each peak fraction from HSCCC was performed on a C_{18} column at a column temperature of 30 °C. The mobile phase was a linear gradient of 0.1% phosphoric acid (A) and acetonitrile (B) that follows: A–B (75:25, v/v) to A–B (45:55, v/v) in 15 min, then to the initial condition A–B (75:25, v/v) immediately. The flow-rate was 1.0 ml min^{-1} and the effluent was monitored at 277 nm by DAD. The retention time was compared with that of standard baicalin. Routine sample calculations were made by comparison of the peak area with that of the standard.

3. Results and discussion

The crude baicalin extracted from the roots of *S. baicalensis* Georgi was first analyzed by HPLC. The result indicated that it contained several compounds, including baicalin (21.6%), baicalein, wogonin and some unknown compounds as shown in Fig. 2.

In HSCCC, successful separation necessitates the careful search for a suitable two-phase solvent system, which provides an ideal range of the partition coefficient (*K*) for the applied sample [18]. Baicalin has both hydrophobic group (phenyl group) and hydrophilic group (hydroxyl group and glucuronyl group). Based on these physical properties of baicalin, a series of experiments were performed to determine suitable two-phase solvent systems for HSCCC. Their *K* values of baicalin were measured and are given in Table 1.

Table 1
The *K* (partition coefficient) values of baicalin in different solvent systems

Solvent system	*K* value
n-Hexane–ethyl acetate–ethanol–water (3:7:1:9)	0.04
Chloroform–methanol–water (4:3:2)	44.2
Chloroform–methanol–water (7:13:8)	21.0
Ethyl acetate–chloroform–methanol–water (1:3:3:2)	15.5
n-Butanol–acetic acid–water (4:1:5)	3.08
n-Butanol–methanol–water (4:1:4)	0.44
n-Butanol–ethanol–water (4:1:4)	0.48
n-Butanol–water(1:1)	0.42
n-Butanol–water (1:1, 0.1% TFA)	6.29

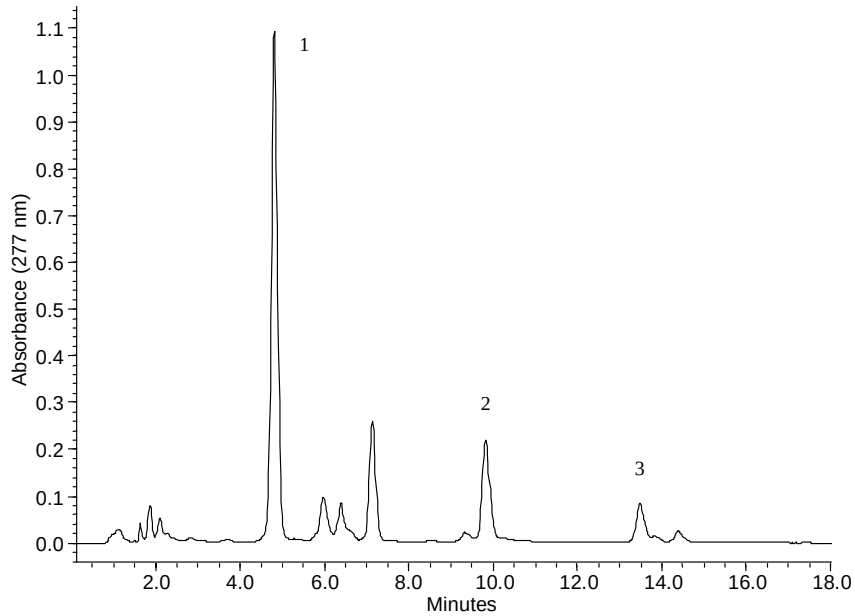

Fig. 2. Chromatogram of the crude baicalin extracted from *S. baicalensis* Georgi by HPLC analysis: 1 = baicalin, 2 = baicalein, 3 = wogonin. Experimental conditions: column: reversed-phase symmetry C_{18} column (150 × 3.9 mm i.d., 5 μm); column temperature: 30 °C; mobile phase: 0.1% phosphoric acid (A) and acetonitrile (B), linear gradient elution: A–B (75:25, v/v) to A–B (45:55, v/v) in 15 min, then to initial condition A–B (75:25, v/v) immediately; flow-rate: 1.0 ml min^{-1}; detection: 277 nm; injection volume, 20 μl.

Preliminary HSCCC was carried out with the two-phase solvent system composed of *n*-hexane–ethyl acetate–ethanol–water. The polarity of this system could be adjusted by varying the amount of *n*-hexane, ethyl acetate and ethanol. If the amount of *n*-hexane was increased and the amounts of ethyl acetate and ethanol were reduced, the polarity of the organic phase would decrease. Conversely, if the amount of *n*-hexane was reduced and the amounts of ethyl acetate and ethanol were increased, the polarity of the organic phase would increase. Nevertheless, the *K* values under these conditions were very small. Baicalin and some other compounds were eluted together closely near the solvent front and resulted in a poor resolution. It was difficult to separate baicalin from the other compounds. Because the hydrophilicity of baicalin is stronger than its hydrophobicity, baicalin should have a higher solubility in a strongly polar solvent. Consequently, baicalin is mostly distributed into the lower aqueous phase. Thus, the two-phase solvent systems composed of *n*-hexane–ethyl acetate–ethanol–water were not suitable for the separation of baicalin from *S. baicalensis* Georgi.

Then, we tested a medium polarity solvent system, such as chloroform–methanol–water and ethyl acetate–chloroform–methanol–water, which was often used for the separation of polar glycosides. However, their *K* values in the two-phase solvent systems were very big with which baicalin was eluted in excessively broad peak with long elution time. Thus, the two-phase solvent systems composed of chloroform–methanol–water and ethyl acetate–chloroform–methanol–water were neither suitable for the separation of baicalin from *S. baicalensis* Georgi.

Finally, we tested the polarity solvent system *n*-butanol–water, which was added with acetic acid, TFA, methanol or ethanol. The solvent system of *n*-butanol–acetic acid–water showed the distribution of the lower phase was on the head side and the upper phase was on the tail side. Although this solvent system had an appropriate partition coefficient (*K*), it was difficult to separate baicalin from the other compounds. *n*-Butanol–methanol–water, *n*-butanol–ethanol–water and *n*-butanol–water also had appropriate partition coefficients, but the settling

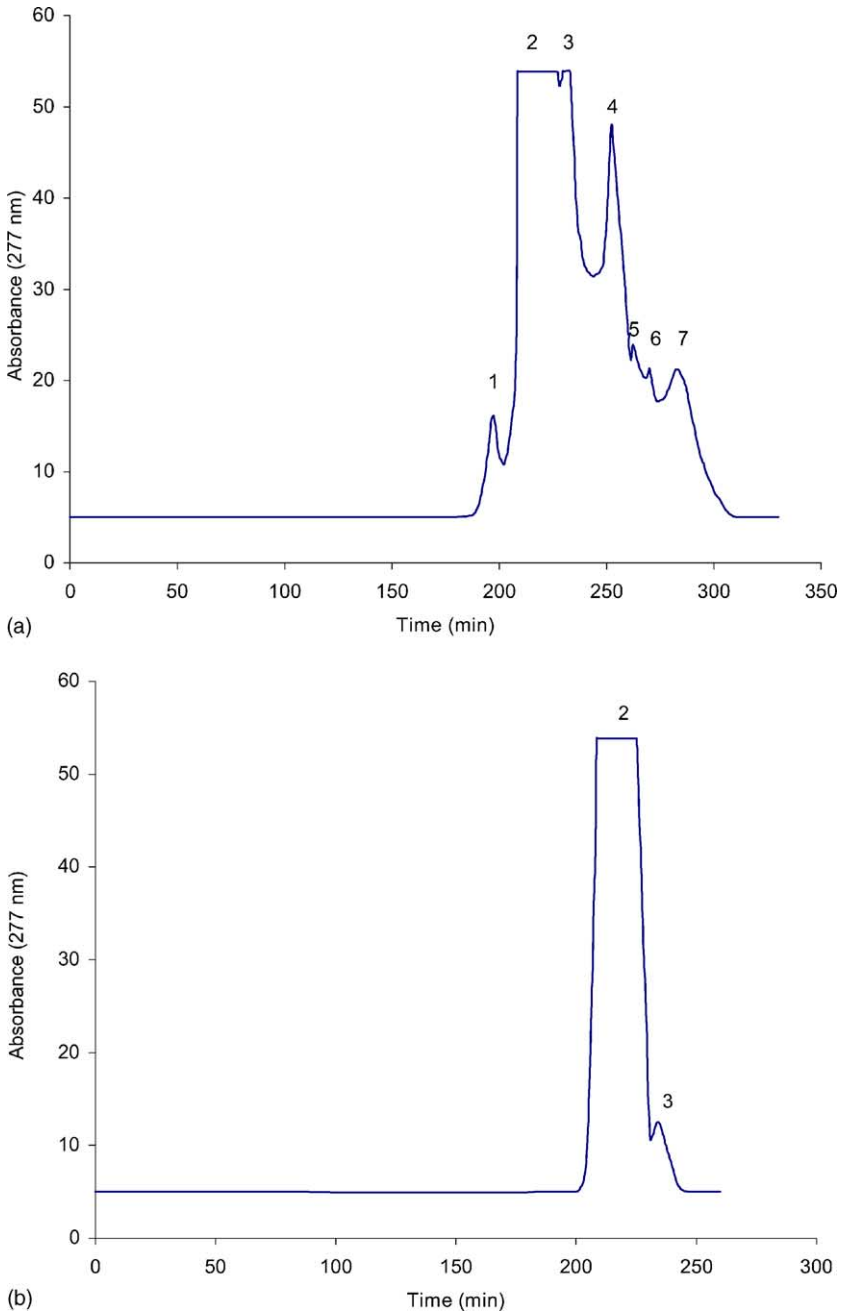

Fig. 3. Chromatogram of the crude baicalin extracted from *S. baicalensis* Georgi by HSCCC separation: 2 = baicalin. Conditions: column—multilayer coil of 1.6 mm i.d. PTFE tube with a total capacity of 342 ml; rotary speed—900 rpm; solvent system—*n*-butanol–water (1:1, v/v); mobile phase—the lower phase; flow-rate—1.0 ml min^{-1}; detection—277 nm; sample size—200 mg; injection volume—5 ml; retention of the stationary phase—41.8%; (a) the first separation; (b) the second separation.

times of these solvent systems were rather long. In order to get a satisfactory retention of stationary phase, the flow-rate of the mobile phase must be less than 1.0 ml min^{-1}. When a trace amount of TFA was added into these solvent systems, although their settling times became shorter, their partition coefficients were high which required a long time to elute baicalin.

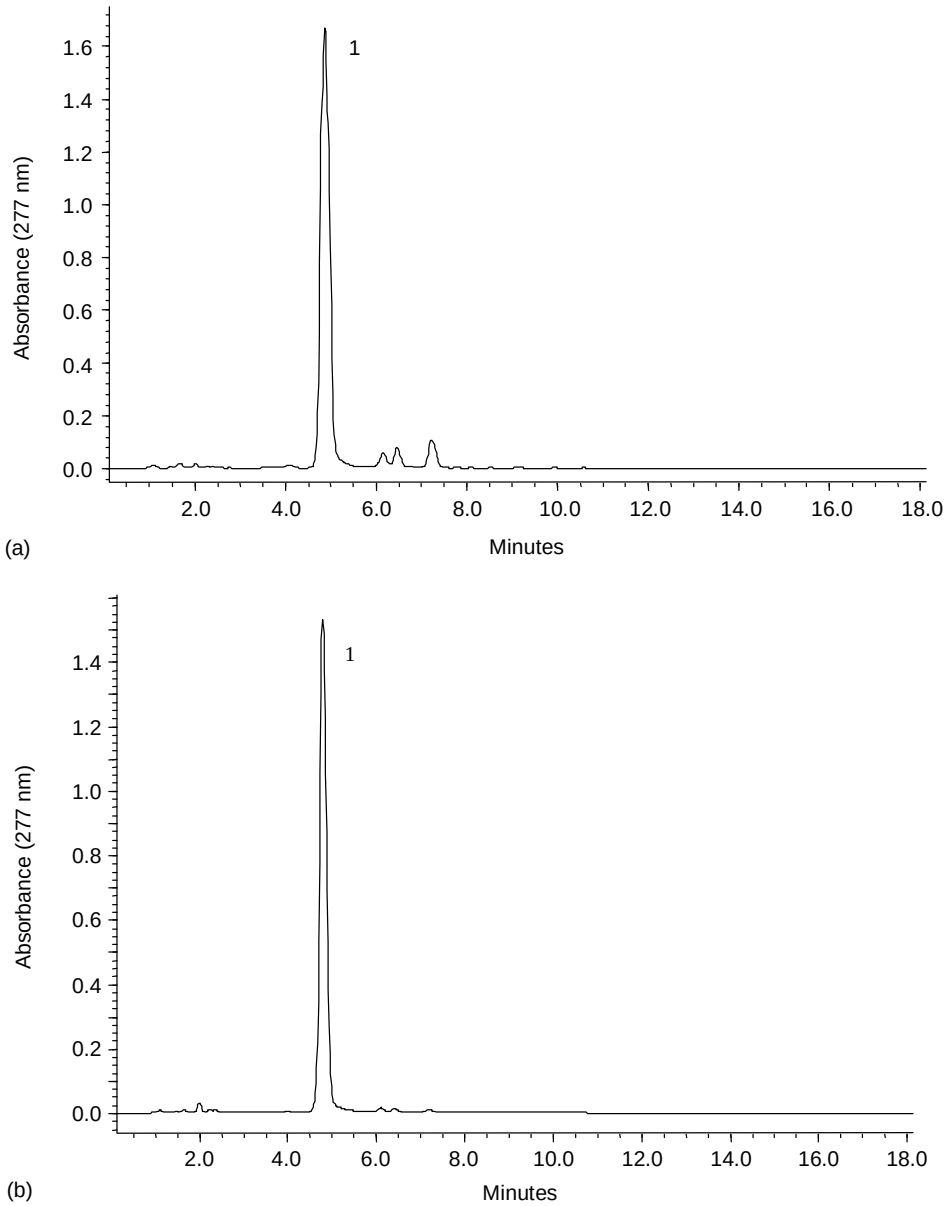

Fig. 4. HPLC chromatogram of baicalin purified by the preparative HSCCC: 1 = baicalin. Experimental conditions: column—reversed-phase symmetry C_{18} column (150 × 3.9 mm i.d., 5 μm); column temperature—30 °C; mobile phase—0.1% phosphoric acid (A) and acetonitrile (B), linear gradient elution—A–B (75:25, v/v) to A–B (45:55, v/v) in 15 min, then to initial condition A–B (75:25, v/v) immediately; flow-rate—1.0 ml min^{-1}; detection—277 nm; injection volume—20 μl; (a) the first separation; (b) the second separation.

After testing the above solvent systems, the two-phase solvent system composed of *n*-butanol–water was found to be the best. The retention of the stationary phase was 41.8%.Fig. 3a shows preparative HSCCC separation of the crude baicalin sample (200 mg) using this solvent system. HPLC analysis of each fractionated effluent of the preparative HSCCC revealed that peak 2 corresponded to baicalin. However, the peak of baicalin could not be well separated from another compound. As shown in Fig. 4a, there were still impurities in the baicalin fraction. This partially purified fraction was dried, and 48.5 mg of baicalin (82.0% purity) was obtained. This product was further redissolved in the both phases and purified again by HSCCC with the same solvent system (Fig. 3b). Finally, 37.0 mg baicalin (96.5% purity) was yielded with 86.0% recovery. The HPLC chromatogram of baicalin as purified from the preparative HSCCC is shown in Fig. 4b.

In the crude baicalin, the content of baicalin was approximately 21.6%. The purity of baicalin was increased to 96.5% after two steps of separation by HSCCC. This result demonstrated the high resolving power of HSCCC that could be achieved by the careful selection of proper solvent systems.

In order to save solvents and time, the slow eluting compounds after baicalin were removed by forcing out the stationary phase with pressurized nitrogen gas instead of eluting them with the mobile phase because the stationary phase was used only once.

4. Conclusion

HSCCC was successfully used to separate and purify baicalin (96.5%) from the roots of *S. baicalensis* Georgi. The present study clearly indicates that HSCCC is a valuable method in separating, purifying and identifying bioactive components from Chinese herbal medicinal products.

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