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Short communication

Preparative high-speed counter-current chromatography for purification of shikonin from the Chinese medicinal plant *Lithospermum erythrorhizon*

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Abstract

The bioactive compound shikonin was successfully isolated and purified from the crude extract of the traditional Chinese medicinal plant *Lithospermum erythrorhizon* Sieb. et Zucc. by preparative high-speed counter-current chromatography (HSCCC). The preparative HSCCC was performed using a two-phase solvent system composed of *n*-hexane–ethylacetate–ethanol–water (16:14:14:5 (v/v)). A total amount of 19.6 mg of shikonin at 98.9% purity was obtained from 52 mg of the crude extract (containing 38.9% shikonin) with 96.9% recovery. The preparative isolation and purification of shikonin by HSCCC was completed in 200 min in a one-step separation.

Keywords: Preparative chromatography; Counter-current chromatography; *Lithospermum erythrorhizon*; Plant materials; Pharmaceutical analysis; Shikonin

1. Introduction

Lithospermum erythrorhizon Sieb. et Zucc., a boraginaceous medicinal plant, is the main source of shikonin that has many beneficial effects in wound healing, antiinflammation, antibacteria, antitumour, antidiabetes and antivirus. It is an important traditional Chinese medicinal, perennial herb. Shikonin accumulates solely in the roots of the plant and is one of the major bioactive components that have been used clinically [1,2]. The preparative separation and purification of shikonin from plant materials by conventional methods such as column chromatography and high-performance liquid chromatography (HPLC) is tedious and usually requires multiple chromatography steps. High-speed counter-current chromatography (HSCCC) uses no solid support, so the adsorbing effects on stationary phase material and artifact formation can be eliminated. This technique has the maximum capacity with an excellent sample recovery as compared to HPLC. Furthermore, it permits introduction of crude samples directly into the hollow column. Therefore, HSCCC has recently been investigated to effectively separate and purify a number of natural products [3–15]. The aim of the present paper was to investigate the preparative separation and purification of shikonin from the crude extract of the traditional Chinese medicinal plant *L. erythrorhizon* Sieb. et Zucc. by HSCCC.

2. Experimental

2.1. Reagents and materials

HPLC grade *n*-hexane, acetonitrile, methanol, acetic acid, dichloromethane, ethyl acetate, benzene, light petroleum (bp 60–90 °C), ethanol, hydrochloric acid and sodium hydroxide were obtained from BDH (Poole, UK). Shikonin was obtained from Wako (Japan).

The roots of *L. erythrorhizon* Sieb. et Zucc. were purchased from Beijing Tong-Ren-Tang drug retail outlet in Hong Kong.

Stock solution of shikonin (0.5 mg ml^{-1}) was prepared in methanol solution and diluted to the desired concentration prior to use.

2.2. Apparatus

2.2.1. HSCCC

Preparative HSCCC was performed with a Model CCC-1000 high-speed counter-current chromatograph

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(Pharma-Tech Research, Baltimore, MD, USA) equipped with three preparative coils (diameter of tube, 1.6 mm) with a total capacity of 342 ml, which were connected in series. The β value of the preparative column varied from 0.47 at the internal layer to 0.73 at the external layer ($\beta = r/R$, where r is the distance from the coil to the holder shaft, and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The revolution speed of the apparatus could be regulated with a speed controller in a range between 0 and 2000 rpm. The two-phase solvent system was pumped into the column with a Model Series II HPLC pump (Parma-Tech Research). Continuous monitoring of the effluent was achieved with a Model SPD-10 Avp UV-Vis detector (Shimadzu, Japan). A manual injection valve with a 10 ml loop was used to introduce the sample into the column. A Model L 120 E flat-bed recorder (Linseis, Germany) was used to draw the chromatogram.

2.2.2. HPLC

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

2.3. Preparation of crude shikonin

The roots of *L. erythrorhizon* Sieb. et Zucc. were dried to constant mass at 70 °C in a vacuum oven and then pulverized. Twenty-five grams of the pulverized sample was weighed and put into a 500 ml flask, to which 350 ml of benzene was added. After shaking extraction on a magnetic mixer at room temperature for 3 h, the mixture was filtrated. The extraction procedure was repeated twice (200 ml of benzene each time). All the filtrates were combined and evaporated to dryness under reduced pressure by rotatory evaporation. The residue was then dissolved in 300 ml of light petroleum. After filtration, the filtrate was added with 200 ml of 2% NaOH solution and stirred for 4 h. Then, the lower phase was separated and 3 mol l^{-1} hydrochloric acid was added until the solution turned red from blue. The solution was evaporated to dryness under reduced pressure in a rotatory evaporator. The residue was subsequently dissolved in 100 ml of light petroleum. After filtration, the filtrate was concentrated to dryness under a stream of nitrogen, which yielded 711 mg of crude shikonin. The crude shikonin was stored in a refrigerator for further purification by HSCCC.

2.4. Selection of two-phase solvent system

A number of two-phase solvent systems were tested by changing the volume ratio of the solvent to obtain the optimum composition that gave suitable partition coefficient (K) values. The partition coefficient values were determined according to the literature [16]. In brief, approximately 0.1 mg of shikonin 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. After the test tube was shaken vigorously for 10 min, the solution was separated by centrifugation at 4000 × g for 5 min. Then, the upper and lower phases were analyzed by HPLC to obtain the partition coefficient of shikonin. K was expressed as the peak area of shikonin in the upper phase divided by that in the lower phase.

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

The selected 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 upper phase was used as the stationary phase, while the lower phase was used as the mobile phase. The sample solution was prepared by dissolving the crude shikonin 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

The multilayer-coiled column was first entirely filled with the upper phase. The lower phase was then pumped into the head end of the inlet column at a flow-rate of 2.0 ml min^{-1} by an HPLC pump, while the apparatus was rotated at 1000 rpm. After hydrodynamic equilibrium was reached, as indicated by a clear mobile phase eluting at the tail outlet, the sample solution (52 mg of the crude shikonin in 10 ml of both phases) was injected through the sample port. The effluent from the tail end of the column was continuously monitored with a UV-Vis detector at 500 nm and the chromatogram was recorded. Each peak fraction was collected according to the elution profile and 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.

2.7. HPLC analysis and identification of shikonin

The crude extract, shikonin (standard) and each peak fraction from HSCCC were analyzed by HPLC. The analyses were performed with a C_{18} column at a column temperature of 30 °C. The mobile phase composed of methanol–water–acetic acid (70:28:2 (v/v/v)) was isocratically eluted at a flow-rate of 1.0 ml min^{-1}. The effluent was monitored at 245 nm to facilitate the simultaneous detection of shikonin and the other compounds. Chromatographic peaks were identified by comparing the retention time and the UV-Vis spectra from 220 to 620 nm by DAD against the standard. Routine sample calculations were made by comparison of the peak area with that of the standard.

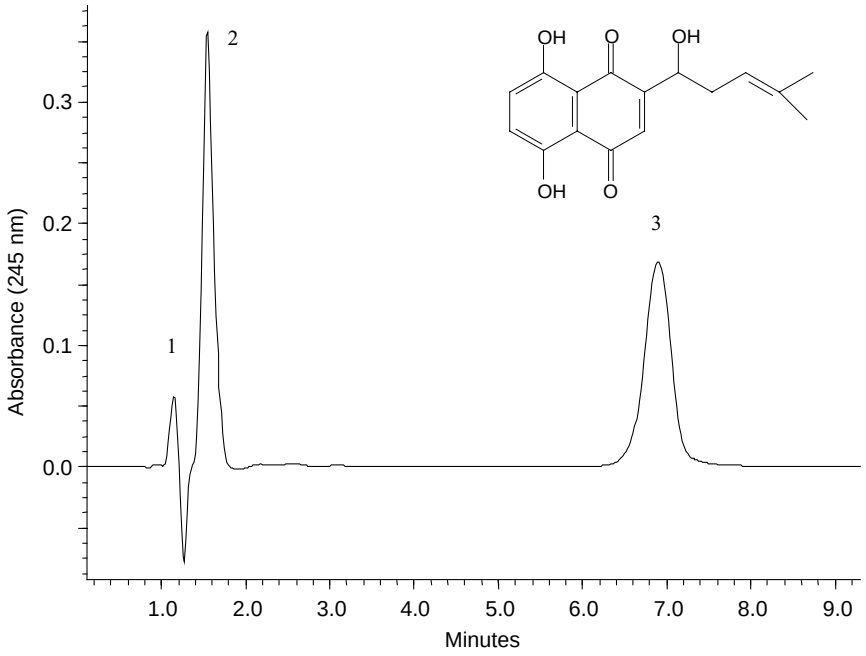

Fig. 1. Chromatogram of the crude shikonin extracted from *Lithospermum erythrorhizon* Sieb. et Zucc. by HPLC analysis, as well as the chemical structure of shikonin, 1: system peak, 2: unknown peak, 3: shikonin. Experimental conditions: column: reversed-phase symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm); column temperature: 30 °C; mobile phase: methanol–water–acetic acid (70:28:2 (v/v)); flow-rate: 1.0 ml min^{-1}; detection: 245 nm; injection volume: 20 μl.

3. Results and discussion

The crude extract from *L. erythrorhizon* Sieb. et Zucc. was analyzed by HPLC (Fig. 1). The result indicated that the crude sample contained several compounds among which shikonin represented 38.9% of the total.

3.1. Selection of suitable two-phase solvent system

Successful separation by HSCCC largely depends upon the selection of a suitable two-phase solvent system, which provides an ideal range of the partition coefficient (*K*) for the targeted sample. Several two-phase solvent systems were tested and their *K* values were measured and summarized in Table 1. When *n*-hexane–methanol or *n*-hexane–dicholormethane–acetonitrile was used as the two-phase solvent system, their *K* values were small. Shikonin and some other compounds were eluted together closely near the solvent front and resulted in a poor resolution. When *n*-hexane–ethanol–water at the ratios of 2:1:1 and 5:3:2 (v/v) was used as the two-phase solvent system, the *K* values were suitable and shikonin could be well separated from the other compounds. However, such solvent systems were not appropriate for separating a large amount of sample due to the poor solubility of shikonin at high concentrations. When *n*-hexane–ethylacetate–ethanol–water was used as the two-phase solvent system as shown in Table 1, all *K* values were suitable and shikonin could be well separated from the other compounds. Furthermore, shikonin is freely soluble in these solvent systems. From the above, the two-phase solvent system composed of *n*-hexane–ethylacetate–ethanol–water at a ratio of 16:14:14:5 was found to be the best.

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

Solvent system	*K* value
n-Hexane–methanol (1:1)	0.13
n-Hexane–ethanol–water (2:1:1)	1.20
n-Hexane–ethanol–water (10:7:3)	0.26
n-Hexane–ethanol–water (5:3:2)	0.49
n-Hexane–dicholormethane–acetonitrile (10:3:7)	0.08
n-Hexane–ethylacetate–ethanol–water (8:7:7:2)	0.92
n-Hexane–ethylacetate–ethanol–water (8:7:7:3)	1.34
n-Hexane–ethylacetate–ethanol–water (8:7:7:4)	1.83
n-Hexane–ethylacetate–ethanol–water (16:14:14:5)	1.05
n-Hexane–ethylacetate–ethanol–water (20:14:14:5)	1.05
n-Hexane–ethylacetate–ethanol–water (16:14:10:5)	2.04
n-Hexane–ethylacetate–ethanol–water (16:10:14:5)	0.93

3.2. Separation of shikonin by HSCCC

The crude sample (52 mg) was dissolved in 10 ml of both phases. The sample solution was separated and purified by HSCCC according to the procedure described above. The retention of the stationary phase was 34.5%, and the total separation time was 200 min. Fig. 2 shows preparative HSCCC separation of the crude shikonin sample, along with the HPLC chromatogram of purified shikonin from HSCCC. Based on the HPLC analysis and the elution curve of the preparative HSCCC, peak 3 corresponded to shikonin. Three

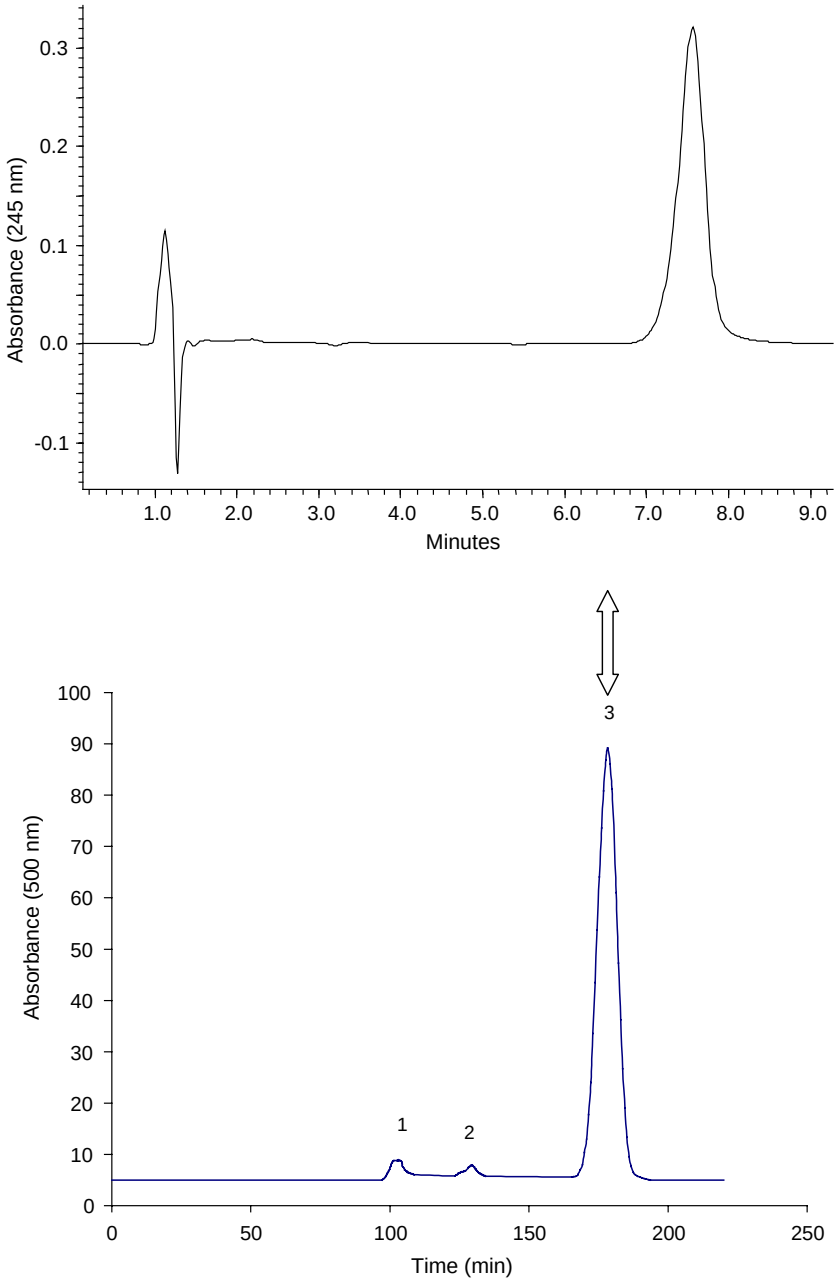

Fig. 2. Chromatogram of the crude shikonin extracted from *Lithospermum erythrorhizon* Sieb. et Zucc. by HSCCC separation, along with the HPLC chromatogram of purified shikonin from HSCCC, 1: unknown peak, 2: unknown peak, 3: shikonin. Conditions: column: multilayer coil of 1.6 mm i.d. PTFE tube with a total capacity of 342 ml; rotary speed: 1000 rpm; solvent system: *n*-hexane–ethylacetate–ethanol–water (16:14:14:5 (v/v)); mobile phase: the lower phase; flow-rate: 2.0 ml min^{-1}; detection: 500 nm; sample size: 52 mg; injection volume: 10 ml; retention of the stationary phase: 34.5%.

spectra from 220 to 620 nm were taken from the apex and the upslope and downslope points from the shikonin peak. They completely overlapped each other indicating that the peak of shikonin was pure. A total amount of 19.6 mg of shikonin (98.9% purity) was yielded with 96.9% recovery.

In order to save solvents and time, the slow eluting compounds after shikonin 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 applied to separate and purify shikonin, the main bioactive component of the Chinese medicinal herb *L. erythrorhizon* Sieb. et Zucc. with *n*-hexane–ethylacetate–ethanol–water (16:14:14:5) as solvent system. A total amount of 19.6 mg of shikonin was obtained from 52 mg crude sample. The purity of shikonin was increased from 38.9 to 98.9% after only one-step separation. The overall results of the present

study indicate that HSCCC is a powerful technique in separating and purifying bioactive compounds from natural sources.

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