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Short communication

Separation and purification of isoflavones from *Pueraria lobata* by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography (HSCCC) was applied to the semipreparative separation and purification of puerarin and related isoflavones from a crude extract of *Pueraria lobata*. Analytical HSCCC was used for the preliminary selection of a suitable solvent system composed of ethyl acetate–*n*-butanol–water (2:1:3, v/v/v). Using the above solvent system the preparative HSCCC was successfully performed yielding six relatively pure isoflavones including puerarin from 80 mg of the crude extract in one-step separation.

Keywords: *Pueraria lobata*; Pharmaceutical analysis; Plant materials; Isoflavones; Puerarins

1. Introduction

Pueraria lobata is a traditional Chinese medicinal herb. Its crude extract has been used for the treatment of hypertension and angina pectoris in China [1]. The studies on pharmacology and clinical practice have shown that the active constituents in the extract are isoflavones, mainly puerarin which significantly dilates coronary arteries, decreases myocardial oxygen consumption, and improves microcirculation in both animals and human patients suffering from cardiovascular disease [2]. Further studies on pharmacological and clinical effects of puerarin and other active constituents necessitate the development of an efficient preparative separation method of these drugs. Such a method will also facilitate quality control and improvement of the quality of existing pueraria radix products. Except for puerarin, pure standards of these isoflavones are not yet available.

The separation and purification of puerarin using the conventional methods such as column chromatography and high-performance liquid chromatography (HPLC) requires several steps resulting in low recoveries of products. High-speed counter-current chromatography (HSCCC) is a unique liquid–liquid partition chromatography which uses no solid matrix. Therefore, it eliminates irreversible adsorptive loss of samples onto the solid support used in the conventional chromatographic column. The method has been successfully applied to analysis and separation of various natural products [3–5].

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The present paper introduces a method for the separation and purification of puerarin and several related isoflavones from a crude extract of *Pueraria lobata* using HSCCC.

2. Experimental

2.1. Apparatus

HSCCC was performed with the following two models of multilayer coil planet centrifuge (CPC) manufactured by the Beijing Institute of New Technology Application, Beijing, China.

(1) Model GS-20 analytical CPC: The multilayer coil was prepared by winding 0.8 mm I.D. PTFE (polytetrafluoroethylene) tubing coaxially onto the column holder. The total capacity is 35 ml. The rotation speed is adjustable from 0 to 2000 rpm, and 1800 rpm was used in the present studies.

(2) Model GS10A2 preparative CPC: The multilayer coil was similarly prepared from 1.6 mm I.D. PTFE tubing. The total capacity is 260 ml. The rotation speed is adjustable from 0 to 1000 rpm but 800 rpm was used in the present studies.

These two HSCCC systems are equipped with an NS-1007 constant-flow pump, a Model 8823A UV monitor operating at 254 nm, a Yokogawa Model 3057 recorder, and a sample injection valve with a 2-ml or 10-ml sample loop.

2.2. Reagents

All solvents used for HSCCC and HPLC were of analytical grade and purchased from Beijing Factory, Beijing, China.

2.3. Preparation of crude sample and sample solution

Fresh roots of *Pueraria lobata* collected in Tibet were chopped and extracted with ethanol. The extract was concentrated to dryness and redissolved in water. The water soluble extract was concentrated and then chromatographed on polystyrene resin (NKA-0, 0.3–1.25 mm: NanKai Chemical Factory, Tianjing, China) with 20% ethanol to yield the crude sample for CCC separation.

The sample solution was prepared by dissolving the crude sample in the mobile phase of the solvent system used for separation.

2.4. Preparation of two-phase solvent system

For the present study, we selected a two-phase solvent system composed of ethyl acetate–*n*-butanol–water at various volume ratios. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature and the two phases separated shortly before use.

2.5. HSCCC separation procedure

Analytical HSCCC with its speedy separation and minimum solvent consumption offers a very promising way to carry out method development for preparative HSCCC separations [6]. In the present studies, the method is used for selecting a suitable solvent system for the separation of target compounds. The above solvent system composed of ethyl acetate–*n*-butanol–water was examined at different volume ratios such as 10:1:10, 1:4:5, 1:2:3, 1:1:2 and 2:1:3 using analytical HSCCC.

In each analytical separation, the coiled column was first entirely filled with the organic stationary phase, and then the apparatus was rotated at 1800 rpm, while the aqueous mobile phase was pumped into the column at a flow-rate of 1.0 ml/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, about 1 ml of the sample solution containing 2 mg of the crude extract was injected through the injection valve. The effluent of the column was continuously monitored with a UV detector at 254 nm. Peak fractions were collected according to the elution profile.

The preparative separation was similarly performed using a 10 ml sample volume (80 mg crude extract) at a flow-rate of 2.0 ml/min at 800 rpm.

2.6. HPLC analysis

The crude sample and each peak fraction obtained by HSCCC were analyzed using a Rainin SD-200 HPLC system (Rainin Instruments, Woburn, MA, USA) equipped with a Supelcosil LC-18 column

(250×4.6 mm I.D., 5 μm, Supelco, Bellefonte, PA, USA). The mobile phase composed of methanol–water (25:75, v/v) was eluted at a flow-rate of 1.0 ml/min, and the effluent was monitored using a UV detector at 254 nm.

3. Results and discussion

Fig. 1 shows the HPLC separation of the crude extract from *Pueraria lobata*. Peak 2 corresponds to puerarin, the major component amounting approximately 30% of the total extract. Peaks 1, 4, 5 were identified as 3′-hydroxypuerarin, and two puerarin xylosides, respectively, by fast atom bombardment mass spectrometry (FAB-MS), ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR. Peaks 3 and 6 were identified as 3′-methoxypuerarin and daidzin, respectively, by comparing the retention time with the reference standards provided by the Institute of Materia Medica, Chinese Academy of Medical Sciences, Shanghai, China. The structures of these isoflavones are shown in Fig. 2.

Performance of the two-phase solvent system composed of ethyl acetate–*n*-butanol–water at various volume ratios (10:1:10, 1:4:5, 1:2:3, 1:1:2 and 2:1:3) was evaluated by analytical HSCCC in terms of peak resolution of various isoflavones. The results indicated that all of these solvent systems except for 10:1:10 (v/v/v) could be used to separate puerarin, 3′-methoxypuerarin, and daidzin from the crude sample. However, for the resolution of other three components and the separation time, the solvent system at a volume ratio of 2:1:3 was found to be most suitable.

Fig. 3 shows the preparative HSCCC separation of 80 mg of the crude sample using the solvent system composed of ethyl acetate–*n*-butanol–water (2:1:3,

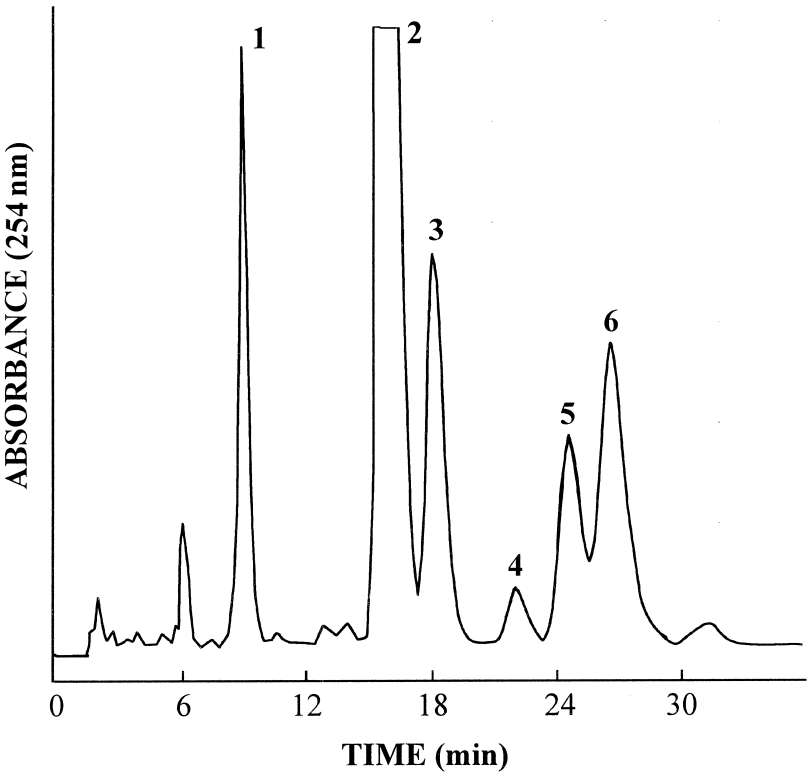
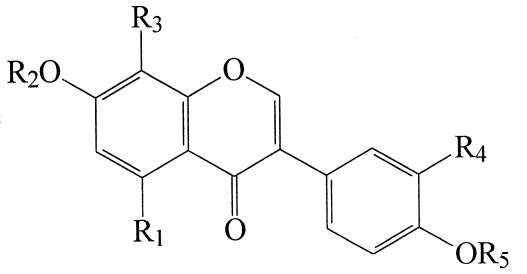


Fig. 1. HPLC separation of the crude extract from *Pueraria lobata*. Experimental conditions: apparatus: Rainin SD-200 HPLC system with a Supelcosil LC-18 column (250 mm×4.6 mm I.D., 5 μm); mobile phase: methanol–water (25:75, v/v); flow-rate: 1.0 ml/min; detection: 254 nm.



	R_1	R_2	R_3	R_4	R_5
1. 3'-hydroxyl-puerarin	H	H	glc	OH	H
2. puerarin	H	H	glc	H	H
3. 3'-methoxy-puerarin	H	H	glc	OCH_3	H
4. puerarin-xyloside	H	H	glc→6xyl	H	H
5. puerarin-xyloside	H	H	glc→2xyl	H	H
6. daidzin	H	glc	H	H	H

Fig. 2. The structures of six isoflavones.

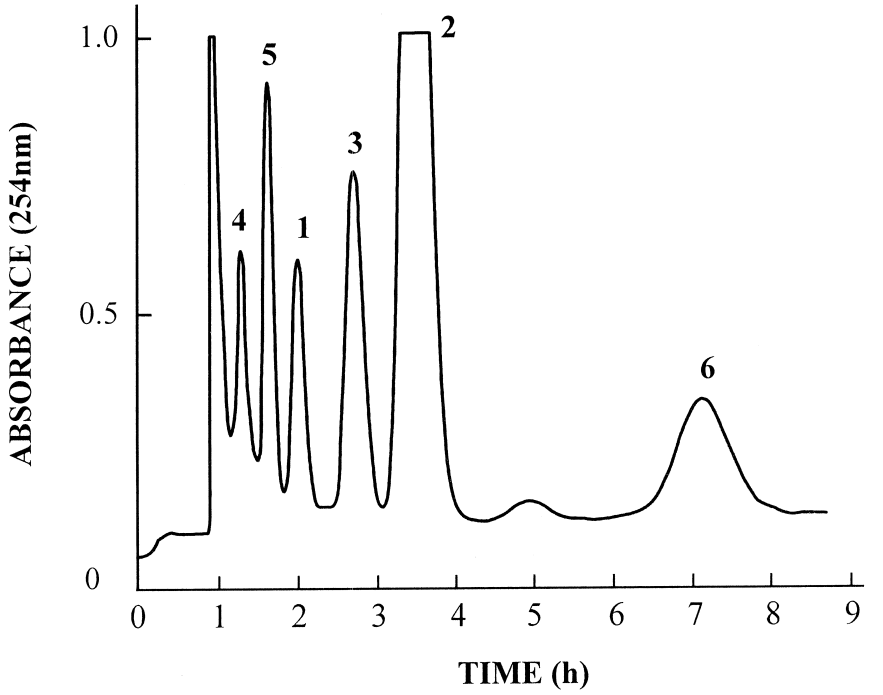


Fig. 3. Preparative HSCCC separation of the crude extract of *Pueraria lobata*. Experimental conditions: apparatus: Model GS10-A2 preparative CPC; column: multilayer coil of 1.6 mm I.D. PTFE tubing with a total capacity of 260 ml; rotation speed: 800 rpm; solvent system: ethyl acetate–*n*-butanol–water (2:1:3, v/v/v); mobile phase: lower aqueous phase; flow-rate: 2 ml/min; detection: 254 nm; sample size: 80 mg; retention of the stationary phase: 56%.

v/v/v). Although the sample size is as large as 40-times that used in the analytical HSCCC, an equivalent or even better separation is achieved. HPLC analysis of each peak fraction of this preparative HSCCC revealed that puerarin corresponding to peak 2 was over 98% pure while other isoflavones also showed high purities of over 95% except for those corresponding to peaks 1 and 4 which were about 90% pure.

The overall results of the present studies indicate that HSCCC is a powerful tool for both analytical and semipreparative separations of isoflavones from the crude extract of *Pueraria lobata*.

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