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Purification of paeoniflorin from *Paeonia lactiflora* Pall. by high-speed counter-current chromatography

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Abstract

High-speed counter-current chromatography was successfully used for the first time for the preparative separation and purification of paeoniflorin from the Chinese medicinal plant *Paeonia lactiflora* Pall. using a two-phase solvent system composed of *n*-butanol–ethyl acetate–water (1:4:5, v/v) in a single run. From 160 mg of the crude sample containing 22.0% paeoniflorin, 33.2 mg of paeoniflorin was yielded at 98.2% purity as determined by HPLC analysis. The recovery of paeoniflorin was 94.3%.

Keywords: Counter-current chromatography; *Paeonia lactiflora*; Pharmaceutical analysis; Plant materials; Preparative chromatography; Paeoniflorin

1. Introduction

The root of *Paeonia lactiflora* Pall. is a commonly used Chinese herbal drug which has been shown to possess antispasmodic, anti-inflammatory and analgesic effects. Paeoniflorin, a water-soluble compound from *P. lactiflora* Pall., is the principal bioactive component and has been reported to exhibit anti-coagulant, neuromuscular blocking, cognition-enhancing, immunoregulating and anti-hyperglycemic effects [1–3].

High-speed counter-current chromatography (HSCCC) is an all-liquid chromatographic technique, working without solid support and the separation is based on fast partitioning effects of the analytes between two immiscible liquid phases. Irreversible adsorbing effects and artifact formation are minimized, and consequently excellent sample recovery is guaranteed. Furthermore, this technique has the maximum capacity and permits introduction of the crude sample directly into the hollow column. Therefore, the application of HSCCC in preparative separation and purification of natural products is steadily growing, because of its superior separation abilities and excellent recovery rates [4–16]. The aim of the present study was to investigate the separation and purification of paeoniflorin from the root of *P. lactiflora* Pall. by HSCCC.

2. Experimental

2.1. Reagents and materials

n-Butanol, ethyl acetate, chloroform, methanol, *n*-hexane, ethanol and tetrahydrofuran (THF) were all of chromatographic grade and purchased from BDH (Poole, UK). Paeoniflorin was purchased from Wako (Japan). The roots of *P. lactiflora* Pall. were purchased from Beijing Tong-Ren-Tang drug retail outlet in Hong Kong.

2.2. Apparatus

Preparative HSCCC was performed on a Model CCC-1000 high-speed counter-current chromatograph (Pharma-Tech Research, Baltimore, MD, USA) equipped with three preparative coils, connected in series (1.6 mm i.d.; total capacity, 342 ml). 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

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coil to the holder shaft, and R (=7.5 cm) is the revolution radius or the distance between the holder axis and central axis of the centrifuge). The solvents were pumped into the column with a Model Series II HPLC pump (Parma-Tech Research). The effluent was continuously monitored with a Model SPD-10 Avp UV-Vis detector (Shimadzu, Japan), while the chromatogram was recorded with a Model L 120 E flat-bed recorder (Linseis, Germany). A manual sample injection valve with a 10 ml loop was used to introduce the sample into the column.

2.3. Selection of two-phase solvent system

The following four kinds of solvent systems at different volume ratios were tested in order to determine a suitable two-phase solvent system based on the partition coefficient (K) of paeoniflorin: (1) *n*-butanol–water, (2) *n*-butanol–ethyl acetate–water, (3) *n*-hexane–ethyl acetate–ethanol–water, (4) chloroform–methanol–water. The partition coefficient values were determined according to the literature [17]. In brief, 0.5 mg of pure paeoniflorin was mixed with 2 ml of each phase of the pre-equilibrated solvent system. After shaken vigorously, the mixture was separated by centrifugation at 4000 × g. An aliquot of each phase was pipetted and evaporated to dryness under nitrogen. Finally, the residues were diluted with methanol and analyzed using HPLC. K was expressed as the peak area of paeoniflorin in the upper phase divided by that in the lower phase.

The selected solvent system was thoroughly equilibrated in a separation funnel by vigorously shaking at room temperature. The two phases were separated shortly and degassed by sonication before use.

2.4. Sample preparation

The roots of *P. lactiflora* Pall. were dried to constant weight and pulverized. Thirty grams of the pulverized sample was then extracted for 20 min in a 500 ml flask with 400 ml of ethanol–water (70:30, v/v) at room temperature under sonication. The extraction procedure was repeated twice. All the filtrates were combined and evaporated to dryness under reduced pressure by rotary evaporation, which yielded 1.46 g of crude paeoniflorin sample.

The sample solution was prepared by dissolving the crude paeoniflorin in a 1:1 (v/v) mixture of each phase used for HSCCC separation at a suitable concentration.

2.5. HSCCC separation procedure

The multilayer-coiled column was first entirely filled with the upper organic stationary phase. The lower phase was then pumped into the column at 2.0 ml min^{-1} in the head-to-tail elution mode, 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, 10 ml of the sample solution containing 160 mg of the crude paeoniflorin was injected through the sample port. The effluent was continuously monitored with a UV–Vis detector at 273 nm at which maximum absorbance was detected, while the chromatogram was recorded. The peak fraction (48 ml) was collected from 123 to 147 min according to the HSCCC chromatogram and analyzed by HPLC. After all the desired peaks were eluted, the rotation and elution were stopped. Then, the column contents were collected with a graduated cylinder by forcing them out with pressurized nitrogen gas. The retention of the stationary phase relative to the total column capacity was computed from the volume of the stationary phase collected from the column.

2.6. HPLC analysis and identification of paeoniflorin

The HPLC system consisted of an HPLC 510 pump (Waters, Milford, MA, USA), a reversed-phase Symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm, Waters), a sample injector (Rheodyne, Cotati, CA, USA), a Waters 996 photodiode array detection (DAD) system, and a chromatogram processor (Waters). The analyses of the crude extract, paeoniflorin (standard) and an aliquot of the overall collection of the peak fraction from HSCCC were performed with an isocratic elution using methanol–THF–water (16:4:80, v/v) as the mobile phase at 30 °C. The flow-rate was 1.0 ml min^{-1}. The effluent was monitored at 231 nm which corresponded to the maximum absorbance when methanol–THF–water (16:4:80, v/v) was used as the mobile phase. The components were confirmed from their retention time and compared with that of the standard paeoniflorin. Routine sample calculations were made by comparison of the peak area with that of the standard.

3. Results and discussion

The chromatographic process in HSCCC is based on the partition of a solute between the two liquids that are used as the mobile phase and stationary phase, respectively. Successful separation requires the suitable choice of a two-phase solvent system, which provides an ideal range of the partition coefficient (K) [15,16]. Therefore, the K value

Table 1
The K (partition coefficient) values of paeoniflorin in different two-phase solvent systems

Solvent system	K value
Chloroform–methanol–water (5:6:4)	2.82
Chloroform–methanol–water (4:3:2)	6.43
n-Hexane–ethyl acetate–ethanol–water (1:1:1:1)	0.013
n-Hexane–ethyl acetate–ethanol–water (5:5:4:6)	0.014
n-Hexane–ethyl acetate–ethanol–water (5:5:6:4)	0.010
n-Butanol–water (1:1)	2.07
n-Butanol–ethyl acetate–water (3:2:5)	2.02
n-Butanol–ethyl acetate–water (1:1:2)	1.91
n-Butanol–ethyl acetate–water (2:3:5)	1.32
n-Butanol–ethyl acetate–water (1:4:5)	0.52

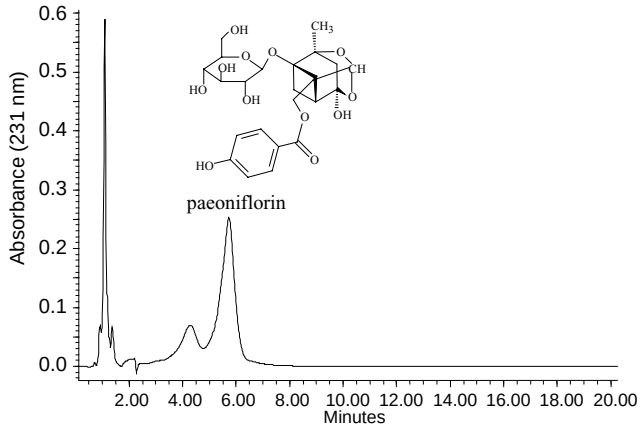

Fig. 1. Chromatogram of HPLC analysis of the crude sample from *Paeonia lactiflora* Pall., as well as the chemical structure of paeoniflorin, conditions: reversed-phase Symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm); column temperature: 30 °C; mobile phase: methanol–THF–water (16:4:80, v/v); flow-rate: 1.0 ml min^{-1}; detection: 231 nm; injection volume, 20 μl.

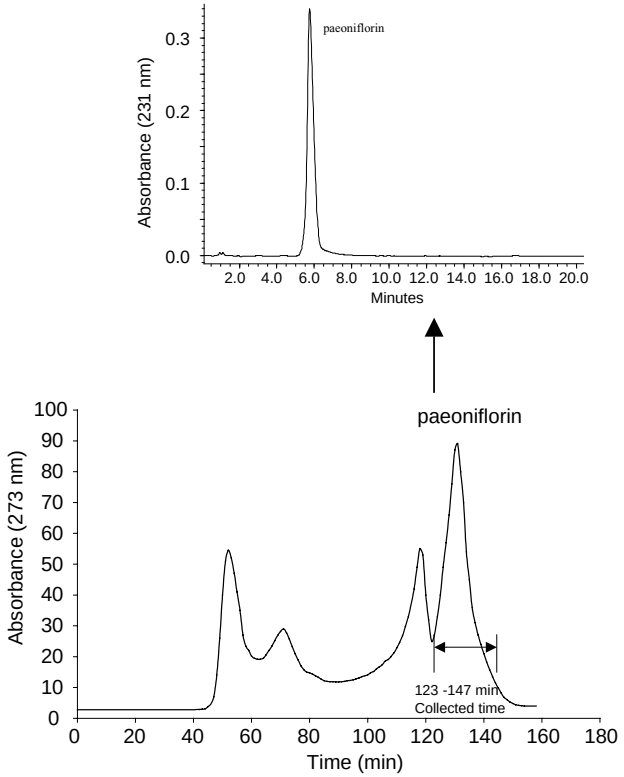

Fig. 2. Chromatogram of the crude sample from *Paeonia lactiflora* Pall. by HSCCC separation, along with the HPLC chromatogram of the purified paeoniflorin from HSCCC. HSCCC conditions: column: multilayer coil of 1.6 mm i.d. PTFE tube with a total capacity of 342 ml; rotary speed: 1000 rpm; solvent system: *n*-butanol–ethyl acetate–water (1:4:5, v/v); mobile phase: the lower phase; flow-rate: 2.0 ml min^{-1}; detection: 273 nm; sample size: 160 mg; injection volume: 10 ml; retention of the stationary phase: 52.9%. HPLC conditions: reversed-phase Symmetry C_{18} column (150 mm × 3.9 mm i.d., 5 μm); column temperature: 30 °C; mobile phase: methanol–THF–water (16:4:80, v/v); flow-rate: 1.0 ml min^{-1}; detection: 231 nm; injection volume, 20 μl.

is the most important parameter in HSCCC. In order to achieve efficient separation of paeoniflorin from the crude sample, the *K* value was determined in a series of solvent systems with a broad range of hydrophobicities by modifying the relative volume ratios, such as polar *n*-butanol, chloroform and ethyl acetate systems. The *K* values of paeoniflorin in the various two-phase solvent systems are summarized in Table 1. Among them, the two-phase solvent system consisting of *n*-butanol–ethyl acetate–water at a ratio of 1:4:5 was found to be the best.

The crude sample was analyzed by HPLC, which indicated that it contained several compounds among which the paeoniflorin represents the major component accounting for 22.0% of the total (Fig. 1). HSCCC was then used for the preparative separation and purification of paeoniflorin from the sample solution in which 160 mg of crude sample was dissolved in 10 ml of the both phases (Fig. 2). The retention of the stationary phase was 52.9%, and the total separation time was only 160 min. Based on the HPLC analysis and the elution curve of the preparative HSCCC, the peak of paeoniflorin was confirmed. A total of 33.2 mg of paeoniflorin at 98.2% purity was yielded with 94.3% recovery (Fig. 2).

The results of our studies clearly demonstrate that HSCCC is a very powerful tool in the preparative separation and purification of bioactive compounds from traditional Chinese medicinal plants including *P. lactiflora* Pall.

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