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Original article

Purification of Puerarin from *Pueraria lobata* by FCPC versus HSCCC Using Small-volume Columns

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ABSTRACT

Objective To develop an efficient method for separating and purifying puerarin from the roots of *Pueraria lobata*. **Methods** Separation by fast centrifugal partition chromatography (FCPC) was processed with a biphasic solvent system composed of ethyl acetate-*n*-butanol-water (2:1:3). The separation conditions were determined as follows: sample loading of 10 mg, flow rate of 2 mL/min, rotation speed of 2200 r/min, ascending mode, and detection wavelength of 254 nm. High speed countercurrent chromatography (HSCCC) was used as a comparative method with the rotation speed of 800 r/min, flow rate of 2.0 mL/min, and sample loading of 10 mg in tail-to-head mode. **Results** Puerarin was obtained by FCPC with a resolution of 0.90 and a purity above 99%, while a resolution below 0.50 and a purity below 90% by HSCCC. Compared with HSCCC, FCPC has the advantages with higher purity and better resolution. **Conclusion** FCPC is a powerful method to separate and purify puerarin.

Key words

fast centrifugal partition chromatography; high speed countercurrent chromatography; *Pueraria loata*; puerarin

1. Introduction

Puerarin (4,7-dihydroxy-8-β-*D*-glucosylisoflavone) is a major isoflavone glycoside isolated from the roots of *Pueraria lobata* (Willd) Ohwi (Figure 1) which has been used as traditional Chinese medicinal herb for centuries. Its comprehensive biological actions have been well-documented by numerous studies, regarding the protective effects on pancreatic islet cells (Xiong et al, 2006), myocardial fibrosis (Chen et al, 2012), liver fibrosis (Li et al, 2013; Zhang et al, 2006), liver injury (Liu et al, 2011; 2012), and retinal micro vascular dysfunction (Kim et al, 2012). Puerarin could attenuate the endothelial insulin resistance through inhibiting the inflammatory response (Huang et al, 2012). Therefore, puerarin has attracted a lot of attention and the preparation of puerarin is of significance for not only the further pharmacological and clinical effects research (Zhang et al, 2012), but also improvement of the quality. However, the separation and purification of puerarin using the conventional methods, such adsorption chromatography (He et al, 2004),

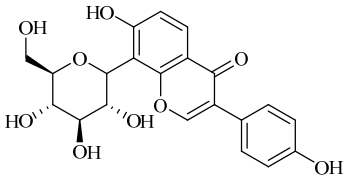

Figure 1 Chemical structure of puerarin

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require several steps resulting in a low recovery.

Countercurrent chromatography (CCC) is a liquid-liquid partition chromatography that uses no solid stationary phase support. The stationary phase is retained inside the column due to the centrifugal field, while the mobile phase is pumped through the stationary phase. The solutes are separated through the column according to their respective partition coefficient. Therefore, the CCC has some advantages: (1) irreversible adsorption, (2) massive amounts of sample injection, (3) less solvent consumption, and (4) time saving (Berthod, 2002). Two types of CCC apparatuses were used in CCC separation: (1) apparatuses containing coiled tubes, the hydrodynamic CCC machines, and (2) those with rotary seals and channels, the hydrostatic CCC machines. The hydrodynamic machines would be called high speed countercurrent chromatography (HSCCC) using a variable gravity field (Hu et al, 2013a; 2013b). The hydrostatic machines would be called centrifugal partition chromatography (CPC) using a constant gravity field (Foucault, 1995). Puerarin was isolated and purified from the roots of *P. lobata* by HSCCC (Cao et al, 1999), while CPC has not been used to separate puerarin yet. In this study, fast centrifugal partition chromatography (FCPC) with analytical column was used to separate and purify puerarin from the roots of *P. lobata* in one step. The analytical CPC column allowed for quick and easy optimization of the separation conditions, and comparison with HSCCC in separation effects.

2. Materials and methods

2.1 Reagents and materials

All organic solvents used for FCPC were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Methanol of HPLC grade from J & K Scientific Ltd. (Beijing, China) was used in the HPLC analyses.

The puerarin reference substance (Batch No. 752-200108) was purchased from The National Institutes for Food and Drug Control (Beijing, China). The roots of *Pueraria lobata* (Willd) Ohwi (Batch No. HP120511) were purchased from Ankang Health Element Pharmtech Co., Ltd. (Shaanxi, China).

2.2 Apparatus

Fast centrifugal partition chromatography (FCPC) was from Kromaton Technologies (Rousselet Robatel, France). The experimental FCPC was equipped with Shimadzu LC–10AD Pump, UV monitor operating at 254 nm, N2000 Work Station, and sample injection valve with 1 mL sample loop. The FCPC total column was 38 mL which is measured by two-phase liquid system of heptane-methanol-water (10:9:1) for three times. The rotation speed was adjustable from 3000 to 0 r/min. The flow rate was from 1.0 to 10 mL/min. And the sample injection was from 10 to 500 mg.

HSCCC was manufactured by Zhejiang University, and its total capacity is 47 mL. The rotation speed was adjustable from 1000 to 0 r/min.

HPLC analysis was performed on an Aglient Series 1260 HPLC Instrument (USA) equipped with a quaternary pump, a diode array detector, an autosampler, and a column compartment. Samples were separated on a Capcell Pak C_{18} column (250 mm × 4.6 mm, 5 μm, Shiseido, Japan). The mobile phase consisted of methanol-water (25:75). The flow rate was 1.0 mL/min. The effluent was monitored using a UV detector at 250 nm. The data were processed with Aglient 2.0 ChemStation software.

Ultrasonic and circulated extraction device CTGXZ–2B with the total capacity of 2 L was manufactured by Hongxianglong Biotechnology Co., Ltd. (Beijing, China).

2.3 Preparation of samples and crude sample solution

The standard puerarin was accurately weighed and dissolved in 30% ethanol to make the standard solution with concentration of 79 μg/mL.

The dried roots of *P. lobata* were crushed over 20 mesh and extracted twice by ultrasound and circulated extraction (800 W) with 40% ethanol for 30 min. The combined extracts were evaporated in vacuum to recover the solvent and then dried in vacuum at 60 ^{o}C to obtain the crude sample, in which the purity of puerarin was determined to be 15.821% by HPLC.

The sample solution was prepared by dissolving the crude sample in the solvent (mobile phase-stationary phase 4:1) for FCPC separation.

2.4 Solvent system selection

The partition coefficient (K_D) value of puerarin and the selectivity ($\alpha = K_1/K_2$, where $K_1 > K_2$) were used as evaluation parameters for selecting the solvent system. According to the golden rules in selecting solvent system (Ito, 2005), the suitable K values for CCC should be in the range of 0.5–2.0 and the α value should be greater than 1.5. According to the literature (Cao, 1999), the ethyl acetate-*n*-butanol-water system was used to separate and purify puerarin by FCPC.

2.5 Solvent system preparation and FCPC separation procedure

The FCPC separation was performed with a two-phase solvent system composed of ethyl acetate-*n*-butanol-water (2:1:3). When used for FCPC separation, the solvent system was prepared in a separatory funnel, allowing for full mixing and equilibration. The two phases were separated and sonicated for 15 min.

The FCPC column (38 mL) was fully filled in ascending mode with lower phase (stationary phase, 64 mL) at a flow rate of 8 mL/min, while the apparatus was rotated at 600 r/min. Then, the FCPC was filled in descending mode with lower phase (64 mL) at a flow rate of 8 mL/min in order to eliminate air bubbles. The upper phase (mobile phase) was

pumped into the column at a flow rate of 2.0 mL/min with a rotation speed of 2200 r/min. After the hydrostatic equilibrium was established, the sample solution was injected into the column. Puerarin fraction was collected from 11.5 to 12.5 min at a wavelength of 254 nm.

2.6 Optimization of flow rate and rotation speed

The flow rate and rotation speed were optimized on the FCPC A. The optimization was to select the operating conditions for high stationary phase retention (S_f), the stationary phase retention equation must be adapted as follows

$$S_f = \frac{Vs}{Vc} \quad (1)$$

where the *Vs* represents the volume of stationary phase inside the FCPC column, and *Vc* is the FCPC column.

The flow rates were set as 2.0, 3.0, 5.0, and 7.0 mL/min, and the rotation speed was increased from 1600 to 2300 r/min to optimize the flow rate and rotation speed for high stationary phase retention.

2.7 Optimization of sample loading

Sample loading was optimized from 10 to 30 mg using FCPC A. At a constant sample volume of 1 mL, the sample loading was optimized from 10 to 30 mg in ascending mode with the rotation speed of 2200 r/min and the flow rate of 2.0 mL/min. Sample size was selected for good separation and high purity of puerarin.

2.8 HSCCC separation procedure and comparison with FCPC

The column of HSCCC was entirely filled with the lower phase as stationary phase and the elution mode was in tail to head mode. Then the apparatus was rotated at 800 r/min, while the upper organic phase was pumped at a flow rate of 2.0 mL/min. After the hydrodynamic equilibrium was established, the sample solution (10 mg/mL) was injected into the 1 mL injection valve.

The purities of puerarin and the separation resolution by FCPC were compared with that by HSCCC. In that case, the resolution equation must be adapted as follows:

$$R_S = (V_{R2} - V_{R1}) / [(W_1 + W_2) / 2] \quad (2)$$

$$V_R = V_C + V_S\ (1 - K_D) \quad (3)$$

where V_{R1} and V_{R1} are the retention volumes of solutes 1 and 2, and W_1 and W_2 are the peak widths of solutes 1 and 2 at the base.

3. Results

3.1 Selection of solvent system

Different volume ratios of the ethyl acetate-*n*-butanol-water system were tested for selecting a suitable solvent system. The solvent system of ethyl acetate-*n*-butanol-water (2:1:3) had better *K*, α, and S_f values. The results are shown in Tables 1 and 2. The solvent system of ethyl acetate-*n*-butanol-ethanol-water (4:1:0.5:5) had a suitable *K* value, while the selectivity ($\alpha = K_{puerarin} / K_3$) was less than 1.5.

Figure 2 shows HPLC separation of crude extract from the roots of *P. lobata*. The compounds **1**–**5** were the main compounds in the crude extract and the peak 2 was puerarin.

3.2 Optimization of flow rate and rotation speed

Figure 3 and Table 3 show the effect of mobile phase flow rate and rotation speed on S_f. It is observed a linear relationship with the highest rotation speed giving the highest stationary phase retention: 1600 r/min: $Y = 58.397 - 3.305X$,

Table 1 Solute distribution ratios (K_D) of five main compounds from roots of *P. lobata* in different solvent systems

ethyl acetate-*n*-butanol-ethanol-water	K_D				
	1	**2**	**3**	**4**	**5**
4:1:0:5	0.42	0.76	0.59	0.18	1.92
3:2:0:5	1.13	1.46	1.13	0.40	2.40
2:1:0:3	0.70	1.84	1.19	0.54	4.19
4:1:0.5:5	0.44	0.99	0.71	0.29	2.29
4:0.6:0.6:5	0.34	0.71	0.48	0.18	1.68

Table 2 Separation factors for puerarin between compounds 3 and 5

ethyl acetate-*n*-butanol-ethanol-water	K_i / K_j	
	$K_{puerarin} / K_3$	$K_5 / K_{puerarin}$
4:1:0:5	1.29	2.53
3:2:0:5	1.29	1.64
2:1:0:3	1.55	2.28
4:1:0.5:5	1.39	2.31
4:0.6:0.6:5	1.48	2.37

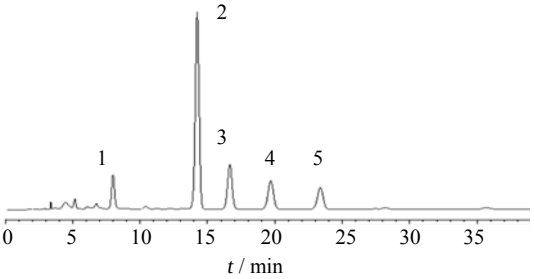

Figure 2 HPLC separation of crude extract from *P. lobata* roots

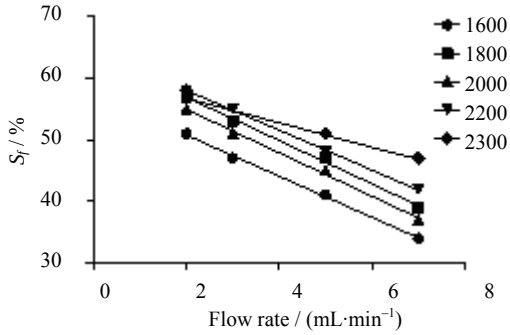

Figure 3 Relationships between S_f and flow rate with different rotation speeds

Table 3 Variety of S_f with different flow rates and rotation speeds in ascending mode (n = 3)

Rotation speeds/ (r·min^{-1})	Flow rates / (mL·min^{-1})	Elution volumes / mL	Back pressures / bar	S_f / %
1600	2.0	18.5	25	51
	3.0	20	24	47
	5.0	22.5	23	41
	7.0	25	23	34
1800	2.0	16.2	32	57
	3.0	17.8	30	53
	5.0	20.3	29	47
	7.0	23	28	39
2000	2.0	17	36	55
	3.0	18.5	35	51
	5.0	21	34	45
	7.0	23.9	33	37
2200	2.0	16	47	58
	3.0	17	45	55
	5.0	19.8	44	48
	7.0	22	42	42
2300	2.0	16	52	58
	3.0	18	49	53
	5.0	18.5	25	51
	7.0	20	24	47

r^2 = 0.996; 1800 r/min: Y = 64.286 – 3.305X, r^2 = 0.997; 2000 r/min: Y = 62.442 – 3.586X, r^2 = 0.999; 2200 r/min: Y = 64.422 – 3.217X, r^2 = 0.998; 2300 r/min: Y = 62.956 – 3.466X, r^2 = 0.994. The operating parameters were as follows: flow rate of 2.0 mL/min, rotation speed of 2200 r/min, and 58% of stationary phase retention.

3.3 Optimization of sample loading

The effects of sample concentration are shown in Table 4. With the sample concentration increasing, the retention time of puerarin would be delayed due to some loss of stationary phase. This would result in lower resolution of puerarin. As the concentration was 10 mg/mL, the purity and the recovery of puerarin were better than those at other concentration. While the concentration increased to 30 mg/mL, the resolution of puerarin fell down, with the purity of 93.353% and recovery of 34.6%, respectively. Therefore, the optimal concentration of 10 mg/mL was chosen.

3.4 FCPC separation of puerarin

Figure 4 shows a chromatogram of puerarin separated by FCPC with flow rate of 2.0 mL/min, rotation speed of 2200 r/min, and the sample size of 10 mg/mL. The puerarin (1 mg) was obtained from 10 mg extract with the recovery rate of 65%.

The HPLC of purified target and puerarin reference substances separated by FCPC are shown in Figure 5, which indicated that the purified target was puerarin. For further identification of the target, the TOF-MS mass spectrum gave the ions of m/z of 439.2 $[M + Na]^+$ and 417.2 $[M + H]^+$ with a molecular mass of 416.2.

Table 4 Results of separation in different sample loadings

Sample loadings / mg	Purities / %	Recovery rates / %	Retention time / min
10	99.396	65.9	12.721
15	99.567	50.0	13.072
20	93.385	45.6	14.239
25	93.381	41.1	14.472
30	93.353	34.6	13.538

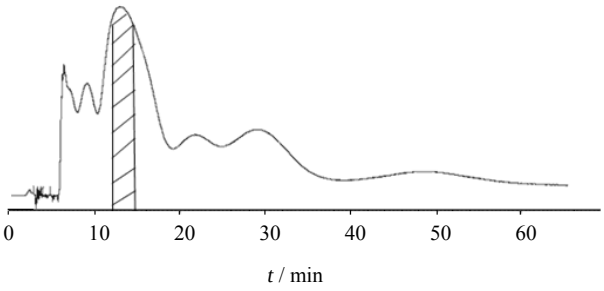

Figure 4 FCPC of puerarin separation

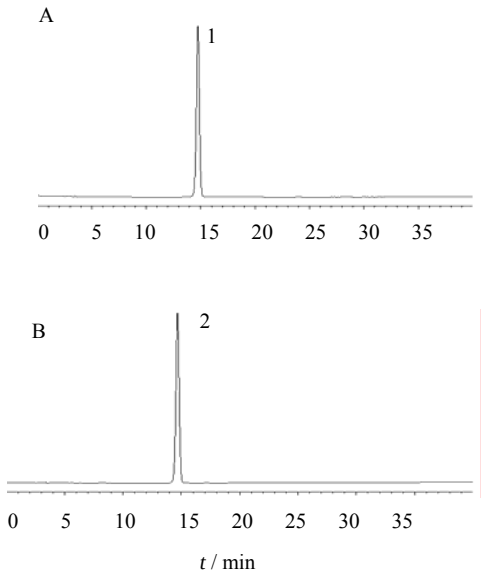

Figure 5 HPLC of puerarin (A) and reference substance (B)
1: puerarin 2: puerarin reference substance

3.5 Puerarin separation by HSCCC and comparison with FCPC

Figure 6 shows a chromatogram of puerarin separation by HSCCC. The separation parameters of HSCCC were similar to those of FCPC: flow rate: 2.0 mL/min, rotation speed: 800 r/min, and sample loading: 10 mg/mL (1 mL injection valve), which gave 47% of stationary phase retention.

Table 5 shows the results of HSCCC compared with FCPC on resolution (R_s), the purity of puerarin, S_f and retention time. Puerarin fraction was collected from 25 to 29 min using HSCCC, and the purity of puerarin was below 90% due to lower R_s. Therefore, FCPC is a time-saving and effective method for separating puerarin compared with HSCCC.

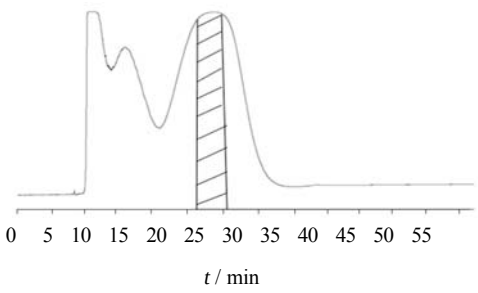

Figure 6 HSCCC of puerarin separation

Table 5 Results of puerarin separation using FCPC and HSCCC

Methods	Purity of puerarin / %	Retention time / min	S_f / %	R_s
FCPC	> 99	13.306	58	0.90
HSCCC	< 90	27.502	47	< 0.5

4. Discussion

A time-saving and effective separating method has been developed for the preparation of puerarin from the roots of *P. lobata*. Fast centrifugal partition chromatography A (FCPC A) with total capacity of 38 mL can be helpful for the quickly optimizing experimental conditions as follows: flow rate of 2.0 mL/min, rotation speed of 2200 r/min, ascending mode, sample concentration of 10 mg/mL, S_f of 58%, purity above 99%, and recovery rate above 60% within 20 min.

The large polar solvent ethyl acetate-*n*-butanol-water system can be better retained on hydrostatic CPC column with S_f of 58% and resolution of 0.90 while obtained S_f of 47% and resolution below 0.50 by HSCCC. Therefore, FCPC is more suitable for separating puerarin with large polar solvent system (ethyl acetate-*n*-butanol-water) compared with HSCCC.

Overall, FCPC is an efficient method for puerarin preparation from the crude extract at one step. The results of the research can provide the reference for scaling up on a larger CPC column, and can be helpful for improving the quality of puerarin.

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