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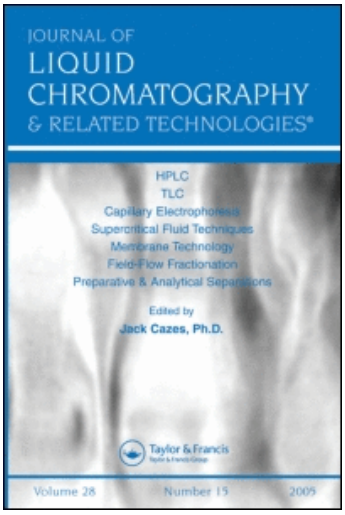

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Preparative Purification of Puerarin from Pueraria Flavones by Oligo-β-Cyclodextrin-Sepharose HP Matrix

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Preparative Purification of Puerarin from Pueraria Flavones by Oligo-β-Cyclodextrin-Sepharose HP Matrix

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Abstract: The matrix oligo-β-cyclodextrin-Sepharose HP was synthesized and successfully applied in the separation of puerarin from pueraria flavones. The optimal conditions of preparation were obtained. The separation was carried out with 7% acetic acid solution (V %) at a flow rate of 10 mL/min, after 300 mg flavones in 20 mL 5% ethanol (V %) were injected in a preparative column (350 mm × 44 mm i.d.). The puerarin fraction was collected and further purification was performed. The purity of puerarin obtained was higher than 97%. The recovery of the whole procedure reached 32.4%.

Keywords: Pueraria, Pueraria flavones, Preparative chromatography, β-Cyclodextrin

INTRODUCTION

Pueraria lobata is a traditional Chinese medicinal herb. Its extract has been widely used in the treatment of hypertension and angina pectoris in China. The studies on pharmacology and clinical practice have shown that the active compound in the extract is mainly puerarin, which significantly dilates coronary arteries, decreases myocardial oxygen consumption, and

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improves microcirculation in human patients suffering from cardiovascular disease.[1] Chromatography, using poly acrylamide as media, is the classical method for puerarin preparation with only 1% recovery and 97% purity.[2] In addition, a hydrolyzing method[3] and extraction procedure[4] also have been used in puerarin preparation recently.

Cyclodextrin (CD) has the shape of a hollow cavity of which the interior surface is hydrophobic compared to the hydrophilic hydroxyl-containing rims, and appropriate size and shape guest molecules can form inclusion complexes with it via hydrophobic interaction and hydrogen bonds.[5] So, it has been utilized in gas, liquid, and thin-layer chromatography as the stationary phases for the separations of enantiomers and isomers.[5–9]

Several methods for the immobilization of β-CD on agarose gel have been developed by Tanaka[9] and Per Vretblad.[10] Recently, we have presented the method of immobilization of oligo-β-CD on agarose and it has been pointed out that oligo-β-CD-allyl-Sepharose exhibited the best separation of puerarin from pueraria flavones.[11,12] In this paper we described a procedure for the preparation of puerarin with preparative chromatography column packed with oligo-β-CD-allyl-Sepharose.

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EXPERIMENTAL

Materials and Apparatus

Pueraria flavones (content of flavones $>49\%$) and puerarin (purity $>97\%$) were purchased from Luye Biology Limited Co., Huainan, Anhui province. Other reagents and solvents used in experiments were of analytical grade and purchased from Beijing Factory, Beijing, China.

Empty column (Huamei Biotechnology Limited Co., Shanghai, China), Pump LC-20C (Satellite Factory, Xinda Ltd., Beijing, China), 8823A–UV detector (Beijing Institute of New Technology Application, Beijing, China) and N2000 chromatography data system (Zhejiang Univ.) were used in the experiments. HPLC system (Shimadzu, 10TVAP) was used in analysis.

Preparation of Flavones Solution

6.0 g of Pueraria flavones powder was dissolved in 100 mL 20% ethanol (V %) followed by centrifugation to remove the possible dusts. Then the supernatant was pretreated by Al_3O_2 and Al_3O_2 was removed by centrifugation again. The resulting supernatant solution was diluted to 4-folder volume by distilled water and the diluted solution was injected into the preparative column at a flow rate of 10 mL/min after filtration through a 0.45 μm membrane.

Preparative Chromatography

According to our previous work,[11,12] the media were synthesized and the amounts of immobilized CD on allyl-Sepharose HP were 18 mg per gram of sucked-dry gel (calculated from the concentrations of CD before and after immobilization). The resulting gel was packed by slurry packing technique into the empty glass column (35 mm i.d.) to form a 440 mm long gel bed in which void volume was determined to be 45 mL by 1% acetone. Thereafter, preparative chromatography was performed with the gel column monitored with the UV detector at the wavelength of 254 nm. The flow rate of the mobile phase consisting of acetic acid and water at ratios ranging from 5:95 to 10:90 was 10 mL/min unless otherwise specified.

Crystallization of Puerarin

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The puerarin fraction was evaporated at 65°C in vacuum condition to afford a brown solid and the resulting solid was washed by 95% acetic acid (V/V) to remove the brown color.

Crystallization with 30% acetic acid solution (V %): To 100 mg puerarin powder obtained from the previous step, 1 mL 30% acetic acid (V/V) was added and the suspension was heated at 90°C until the powder was dissolved. The supernatant was separated by centrifugation and charged into a 5 mL crystallizer. The crystallization was done at room temperature for 24 h and the puerarin crystal was obtained by filtration.

Crystallization with distilled water: Distilled water was employed instead of 30% acetic acid (V/V) during the crystallization. The puerarin solution prepared in the similar method as that in 30% acetic acid (V/V) was poured into a 5 mL crystallizer again. Thereafter, the crystallization temperatures were set to decrease at 50, 40, and 30°C, and maintained for 5, 12, and 12 h at each point, respectively. After the crystallization, the puerarin crystal could be collected by filration.

Final Optimized Procedure

The final preparation procedure was shown in Figure 1.

HPLC Analysis of Puerarin

Puerarin content was assayed by the Shimadzu HPLC with a 250 mm × 4.6 mm (i.d.) RP-C18 column (Beijing Analytical Instrument Apparatus Factory, Beijing, China). The wavelength of the UV detector was 250 nm. The mixture of methanol-0.5% acetic acid solution (27:73, V/V) was used as the mobile phase at a flow rate of 0.8 mL/min.

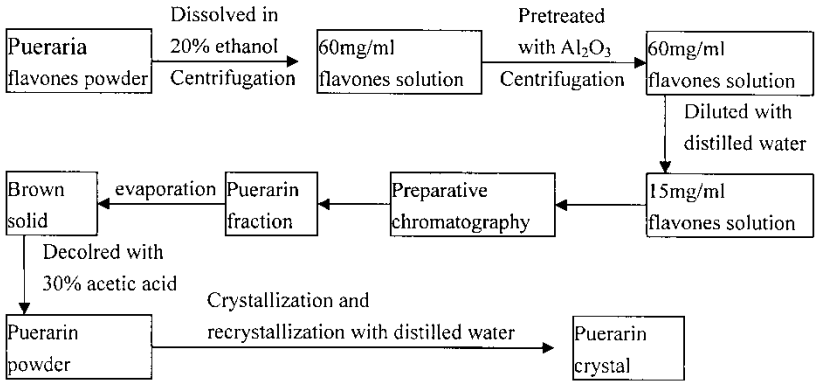

Figure 1. The plot of final preparation procedure.

RESULTS AND DISCUSSION

Optimizations of Mobile Phase and Flow Rate

Acetic acid solution was found to be the best mobile phase for the separation of puerarin on the small column.[11,12] So the mobile phases containing 10%, 7%, and 5% acetic acid (V/V) were investigated on the preparative runs for optimization. The chromatograms were shown in Figure 2 in which P presents the peak of puerarin.

As shown in Figure 2, separation between the frontal impurities and the puerarin was enhanced when 7% acetic acid solution (V/V) was applied in comparison to 10% acetic acid (V/V). The interaction between the stationary phase and the puerarin was increased by decreasing the proportion of acetic acid, which could be demonstrated by the retention times of the puerarin. However, continuous decreasing of the acetic acid could not contributed to better separation of preparative chromatography due to the extensive band broadening caused by stronger retention and even strongly adsorption, which was testified in column regeneration. With these considerations in mind, the mobile phase which consisted of acetic acid–water (7:93, V/V) was employed in the preparative chromatography runs.

In addition, separation flow rates of 5 mL/min and 10 mL/min had been investigated, which indicated no obvious changes of resolutions between the puerarin and neighboring peaks. So the flow rate of 10 mL/min was selected to shorten the time of separation.

It is well known in literatures,[13,14] that cyclodextrins trend to acidic hydrolysis in the present of a strong acid such as sulfuric acid. However, the media containing β-cyclodextrin in our experiments showed perfect stability after a year of thousands of runs with mobile phase containing acetic acid. It was tentatively interpreted based on the weak acidity of acetic acid and lower operation temperature (room temperature). Further works of separation, mechanism, and stability of media are in progress.

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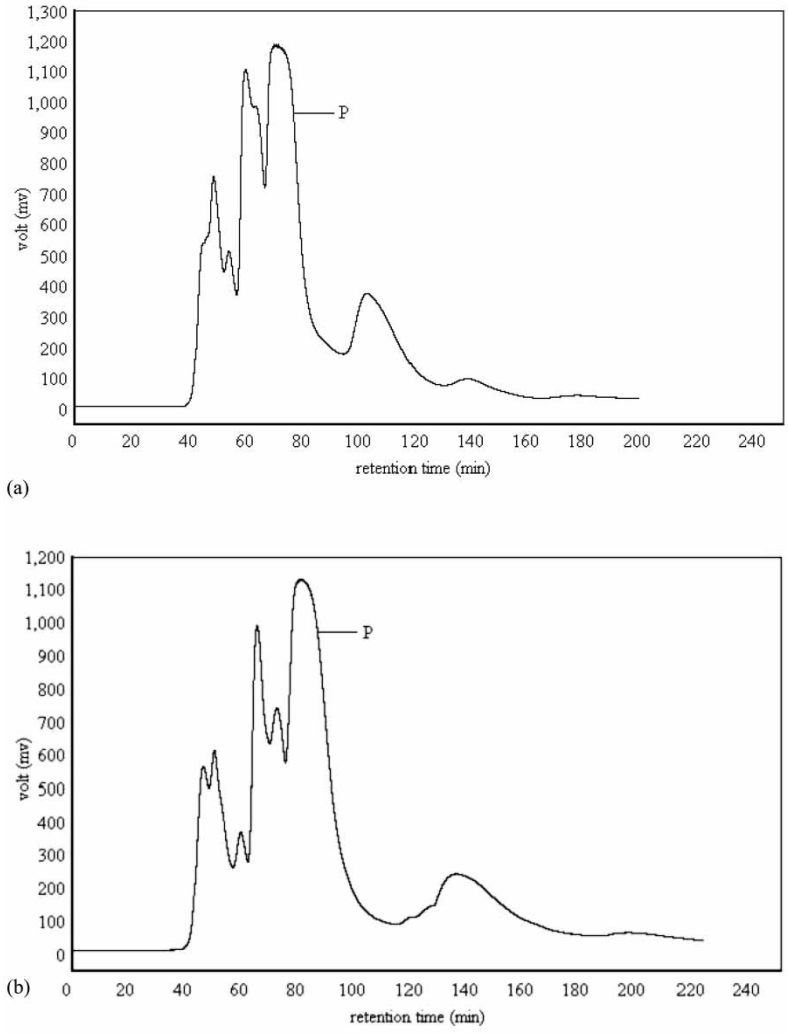

Figure 2. Influence of acetic acid concentration in mobile phase. Separations were performed with different acetic acid solutions after injection of 300 mg flavones in 20 mL 5% ethanol (V %). (a) 10% acetic acid (V %), (b) 7% acetic acid (V %), (c) 5% acetic acid (V %).

(continued)

Optimization of the Preparation of the Sample

As could be seen from Figure 3, the increase of ethanol concentration improved the recovery of puerarin in the pretreatment procedure with Al_2O_3. However, the higher proportion of ethanol in sample solution resulted in the "finger effect" during the preparative runs (see later section).

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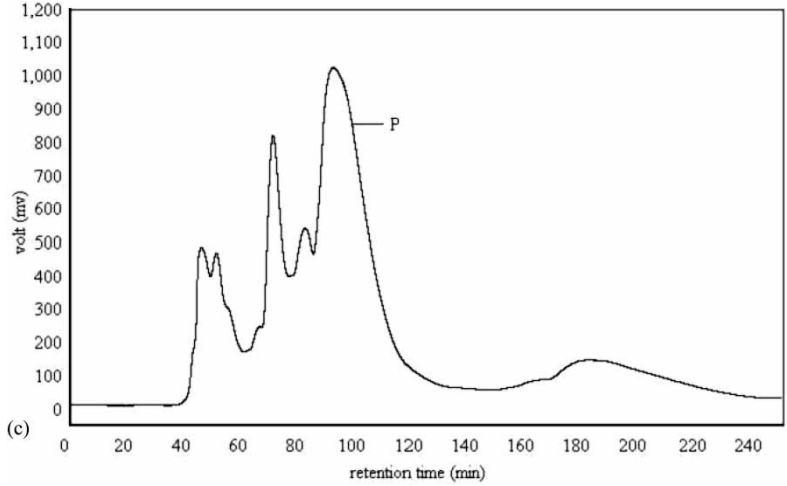

Figure 2. Continued.

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Hence, the compromising proportion of ethanol (20%, V/V) was employed to keep an acceptable recovery of puerarin (89.3%).

If 300 mg flavones sample in 20 mL 20% ethanol (V %) was chromatographed on the preparative column directly, the "finger effect", which seriously deteriorated the resolution between the puerarin and the frontal impurities, occurred because of the great viscosity difference between the sample zone and the mobile phase. Decreasing the sample concentration to half of the origin, however, could not eliminate the phenomenon. So it was reasonable to assume that the "finger effect" was correlated to the higher concentration of ethanol in sample solution. Hence, the sample solution,

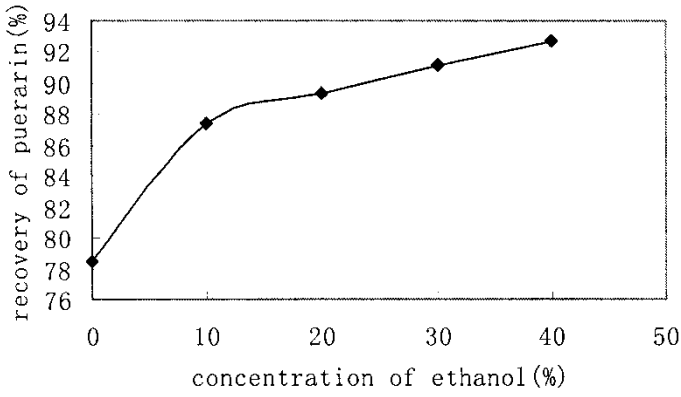

Figure 3. Relation between the ethanol concentration and the puerarin recovery in the pretreatment.

after pretreatment with Al_2O_3, was diluted gradually by distilled water. When the resulting diluted solution containing 300 mg flavones in 20 mL 5% ethanol (V/V) was applied to preparative runs, the "finger effect" was avoided, which could be affirmed from the piston-like shape of a brown zone of the sample in the elution.

Regeneration of Preparative Column

The preparative column needed to be regenerated after 15 cycles due to the sharp decrease of the separation efficiency. The regeneration was performed with 2-folder column volume 50% acetic acid (V/V) and the separation capability could be restored to the origin.

Relationship between Puerarin Recovery and Its Purity

A ratio of puerarin peak area obtained from the HPLC analysis of preparative eluate to that of impurities was used to indicate the purity of the fraction. In preparative chromatography, the relation between the ratio and the puerarin recovery was shown as Figure 4 in which the numbers of X axis represented the fractions collected between the different time intervals. Following the strategy of reducing impurities, compromising the peak cut from 79.6 min

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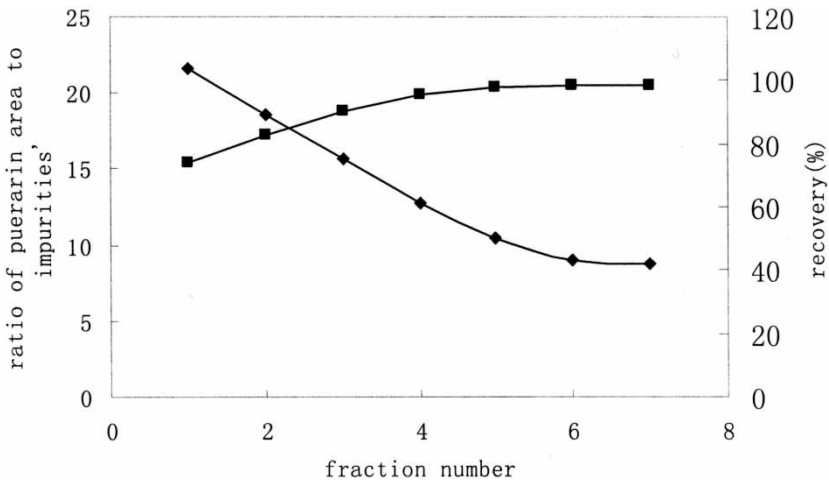

Figure 4. The relation between purity and recovery of puerarin fraction. —◆— and —■— represented the recovery of puerarin and the ratio of puerarin area in HPLC analysis to that of impurities. The time intervals (min) of the fractions indicated by the X axis were given as follows: 1, 80.6–102.2; 2, 79.6–102.2; 3, 78.6–102.2; 4, 77.6–102.2; 5, 76.6–102.2; 6, 75.6–102.2; 7, 75.1–102.2. Separation conditions are shown in Figure 2b.

to 102.2 min was employed for the further procedures, which, as indicated in Figure 4, offered the recovery of 82.7%.

Decoloring and Crystallization

The decorloring step was performed with 3.5 mL 95% acetic acid (V/V) added to 1 g of the brown solid obtained from the vacuum evaporation, and the resulting puerarin powder exhibited a higher saturation point in 30% acetic acid compared to distilled water. It was interesting that crystallization was performed successfully at room temperature; in distilled water, however, sharp a decrease of crystallization temperature could result in the solution forming gels. So the crystallization in distilled water was performed with decreased gradient temperature, as described in the experimental section. Table 1 showed the purities and the recoveries of puerarin in the crystallizations using 30% acetic acid and distilled water. Finally, distilled water was employed due to the higher purity of 91.7% obtained. Recrystallization was performed with the same condition, which offered the purity of 97% to the puerarin crystal.

In crystallization and recrystallization, the filtrate was recycled twice and three times, respectively, for the sake of improvement of recoveries, and the amounts of recovered puerarin were 80%, as well as 64.9% in each step, with slight changes of purities.

Total Recovery and Purity of the Process

Table 2 showed the total recovery and purity of our preparation of puerarin. From Table 3, in which the items of other preparations were listed, it could be seen that using preparative chromatography based on oligo-β-cyclodextrin-Sepharose exhibited the advantage of higher recovery, while the purity of the puerarin was maintained in the same level.

CONCLUSIONS

The matrix oligo-β-cyclodextrin-Sepharose HP was synthesized and successfully applied in the separation of puerarin from pueraria flavones. The

Table 1. Comparisons of different crystallization solvents

Crystallization solvent	Purity (%)	Recovery in crystallization (%)
30% acetic acid solution (V %)	81.7	57.2
Distilled water	91.7	54

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Table 2. Total recovery and purity of the process

Process	Recovery (%)	Purity (%)
Pretreatment of sample	89.3	—
Puerarin fraction	73.9	—
Decoloring	62.6	71.4
Crystallization	50.0	91.7
Recrystallization	32.4	>97
Total	32.4	>97

Table 3. Comparison of different preparations of puerarin

Process	Recovery (%)	Purity (%)
Poly acrylamide chromatography[a][2,15]	1	97
Hydrolysis[3]	1.36	98.32

[a]Puerarin was purified from *Pueraria lobata* in the process, so the recovery of the procedure in which pueraria flavone was extracted from *Pueraria lobata* is taken into consideration.

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preparative chromatography was performed with the mobile phase consisting of acetic acid–water (7:93, V/V) at the flow rate of 10 mL/min. Under the optimal preparative conditions, the eluate of puerarin was collected for further procedures including evaporation, decoloring, crystallization, and recrystallization. The purity of the puerarin obtained was higher than 97% and the total recovery reached 32.4%.

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