

Research Article

Analysis of Bovine Serum Albumin Ligands from *Puerariae flos* Using Ultrafiltration Combined with HPLC-MS

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Rapid screening techniques for identification of active compounds from natural products are important not only for clarification of the therapeutic material basis, but also for supplying suitable chemical markers for quality control. In the present study, ultrafiltration combined with high performance liquid chromatography-mass spectrometry (HPLC-MS) was developed and conducted to screen and identify bovine serum albumin (BSA) bound ligands from *Puerariae flos*. Fundamental parameters affecting the screening like incubation time, BSA concentration, pH, and temperature were studied and optimized. Under the optimum conditions, nine active compounds were identified by UV and MS data. The results indicated that this method was able to screen and identify BSA bound ligands from natural products without the need of preparative isolation techniques. Moreover, the method has more effective with easier operation procedures.

1. Introduction

Natural products have been used to cure human diseases and as dietary supplement for thousand years [1]. Therefore, the screening and identification of active compounds from natural products are rather important [2]. In the conventional bioassay guided fractionation approach of natural products, the complex mixture was extracted with different solvents. Then, active compounds in mixture were repeatedly isolated using different techniques like column chromatography and preparative HPLC until the separated compounds were pure enough. After structural characterization of the active compounds, pharmacological testing was finally carried out [3]. This approach was time consuming and labor intensive and often leads to loss of activity during the isolation and purification procedures [4]. Thus, many screening methods were developed for screening active compounds in natural products like equilibrium dialysis, cell membrane chromatography, ultrafiltration, and nanomaterials [5, 6].

Ultrafiltration is a facile method which utilizes both centrifugation force and a semipermeable membrane to

retain high molecular weight solutes [7]. This method could retain suspended solids and compounds of high molecular weight, while the liquid and low molecular weight compounds are allowed to pass through membrane depending on the nominal molecular weight cut-off of membrane. Based on these features, active compounds bound to protein could be retained by membrane together with protein after the incubation of natural product extract and protein [8]. Through high performance liquid chromatography-mass spectrometry (HPLC-MS) analysis, the structure of active compounds could be identified. Therefore, due to its time saving, easy operation, and high reliability, ultrafiltration was successfully applied in analysis of active compounds from complex mixtures binding with biomacromolecules like liposome, rat plasma protein, and enzymes [8, 9].

As a kind of extensively studied protein, bovine serum albumin (BSA) showed particular role in the research of active compounds. Because the interaction between BSA and active compounds could form a stable complex, it could be considered as a model for finding active compounds. Plenty of information of active compounds could be found through

the analysis of the compounds bound with BSA. *Puerariae flos*, the dry bud of *Pueraria lobata*, is a well-known Chinese medicine. In China and Japan, *Puerariae flos* has been used in treatment of diabetes mellitus, intoxication, hepatic lesion induced by alcohol, and problems associated with alcohol drinking [10]. While in North America and Southern China, it has been applied as a commercial food supplement for human health care [11]. However, related detail research is still lacking.

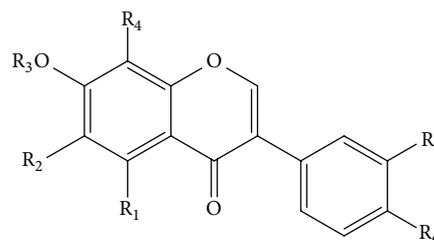
In this study, BSA ligands from *Puerariae flos* were analyzed by ultrafiltration combined with HPLC-MS. The experiment conditions including BSA concentration, incubation time, and centrifugal conditions were optimized. Nine compounds were identified as BSA ligands. The results indicated that this method permits quick analysis of BSA bound ligands without previous purification of natural product extracts.

2. Experimental

2.1. Materials. The dried *Puerariae flos* was purchased from Wan Hua Cao Healthcare Products Co., Ltd. (Anhui, China), and identified by Professor Mijun Peng, Key Laboratory of Hunan Forest Products and Chemical Industry Engineering, Jishou University. Bovine serum albumin (BSA) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The HPLC grade acetonitrile was bought from Tedia Company Inc. (Fairfield, Ohio, USA). Ultrapure water (18.2 MΩ resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Nine authentic isoflavones, puerarin, daidzin, 3'-methoxy daidzin, ononin, tectorigenin, biochanin A, genistein, 3'-methoxy daidzein, and irisolidone were isolated and characterized from *Puerariae* genus in our laboratory (Figure 1). Their structures were identified by UV, MS/MS, 1D, and 2D NMR experiments. The purity of each compound was determined to be $\geq 97\%$ by HPLC analysis.

2.2. Preparation of *Puerariae flos* Extract. Air-dried *Puerariae flos* (50.0 g) were powdered and extracted three times (each for 3 h) with 95% ethanol under reflux. The filtrate was concentrated under reduced pressure to yield a dried residue (3.38 g), which was further dissolved in 50 mL water and filtered through a 0.45 μm membrane (Acrodisc Syringe Filter, Pall, Ann Arbor, MI, USA). The filtrate was finally stored at 4°C for further experiments.

2.3. BSA Ligands Screening with Ultrafiltration. BSA solution (1 mg mL⁻¹ in phosphate buffer, pH 7.0, 0.01 M) was added in 1 mL of *Puerariae flos* extract (67.6 mg mL⁻¹) with equal volume. The mixture was shaken for 2 h at 35°C for fully interaction. Then, the mixture was transferred into Nanosep MF Centrifugal Devices (≤ 10 kDa, Pall, Ann Arbor, MI, USA) and centrifuged at 8000 rpm for 10 min with an Eppendorf centrifuge 5417R (Eppendorf, Hamburg, Germany), and the filtrate was stored at 4°C for analysis.



Number	Compounds	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
1	Puerarin	-H	-H	-H	-glc	-H	-OH
2	Daidzin	-H	-H	-glc	-H	-H	-OH
3	3'-Methoxy daidzin	-H	-H	-glc	-H	-OCH ₃	-OH
4	Ononin	-H	-H	-glc	-H	-H	-OCH ₃
5	Tectorigenin	-OH	-OCH ₃	-H	-H	-H	-OH
6	Biochanin A	-OH	-H	-H	-H	-H	-OCH ₃
7	Genistein	-OH	-H	-H	-H	-H	-OH
8	3'-Methoxy daidzein	-H	-H	-H	-H	-OCH ₃	-OH
9	Irisolidone	-OH	-OCH ₃	-H	-H	-H	-OCH ₃

FIGURE 1: Chemical structures of nine investigated compounds.

2.4. Optimization of Experimental Conditions. Different concentration of BSA solution (0.5, 1, 2, 3, and 4 mg mL⁻¹ in phosphate buffer, pH 7.0, 0.01 M) was added in 1 mL of *Puerariae flos* extract (67.6 mg mL⁻¹) with equal volume. The mixture was shaken for different binding time (1, 2, 3, 4, and 5 h) at 25°C. Then, the mixture was transferred into Centrifugal Devices and centrifuged at 8000 rpm for 10 min. Then, the filtrate was detected by UV-2450 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) and the absorbance was measured at 254 nm. To obtain the optimum pH and temperature in screening, the experiments were conducted at different pH (5.0, 6.0, 7.0, 8.0, and 9.0) and temperatures (5, 15, 25, 35, and 45°C). The lower absorbance means the higher screening degree because more compounds were retained by the membrane. Hence the screening efficiency of sample was defined as SE, which could be calculated using

$$\text{SE}\% = 1 - \left(\frac{A'_{254}}{A_{254}} \right), \quad (1)$$

where A_{254} is the absorbance of sample at 254 nm and A'_{254} is the absorbance of filtrate at 254 nm. And the binding strength of a compound to BSA was defined as the binding degree, BD, which could be calculated using

$$\text{BD}\% = 1 - \left(\frac{A_b}{A_a} \right), \quad (2)$$

where A_a and A_b are the peak areas of a compound in the HPLC chromatograms before and after screening, respectively. All experiments were conducted in triplicate and the results were obtained as the average of experimental values.

2.5. HPLC-MS Analysis. Analytical HPLC-DAD consisted of two LC-8A pumps, a Prominence SPD-M20A Diode Array Detector performing the wavelength scanning from 190 to 400 nm, a manual injection valve with a 20 μL loop, and an LC Solution workstation (Shimadzu, Kyoto, Japan). The target compounds were separated by using a reversed phase SunFire C_{18} (250 mm \times 4.6 mm i.d., 5 μm , Waters, Milford, MA, USA) column. The mobile phase consisted of A (0.4% acetic acid in water) and B (acetonitrile), which was programmed as follows: 0–12 min, 27% B; 15–30 min, 27–38% B; 32–55 min, 38–78% B. The flow rate was 0.8 mL min^{-1} while the ambient temperature was controlled at 20°C by air conditioner. Spectra were recorded from 200 to 400 nm (peak width 0.2 min and scanning data rate 1.25 s^{-1}) while the chromatogram was acquired at 254 nm.

For HPLC-MS experiments, HPLC was performed on Acquity UPLC system (Waters Corp., Milford, MA, USA) with cooling autosampler and column oven enabling temperature control of the analytical column. Separation was performed on a SunFire C_{18} (250 mm \times 4.6 mm i.d., 5 μm , Waters, Milford, MA, USA) column at 20°C. The stationary phase and the elution gradient were the same as those in the above HPLC analysis. Triple quadrupole tandem mass spectrometric detection was carried out on a Micromass Quattro Micro-API Mass Spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface. The ESI source was set in negative ionization mode. The following settings were applied to the instrument: capillary voltage, 3.00 kV; cone voltage, 40.0 V; extractor voltage, 3.00 V; source temperature, 120°C; desolvation temperature, 400°C; desolvation gas flow, 750 L h^{-1} ; cone gas flow, 50 L h^{-1} ; dwell time, 0.05 s. Nitrogen was used as the desolvation and cone gas. Mass detection was performed in full scan mode for m/z in the range 160–800. All data collected were acquired and processed using MassLynx NT 4.1 software with QuanLynx program (Waters Corp., Milford, MA, USA).

3. Results and Discussion

3.1. Optimization of HPLC Analysis. Optimum HPLC analytical conditions were required for all analyses in the present research. Thus, different mobile phases (methanol-water, acetonitrile-water, and methanol-acetonitrile-water) with different concentrations of acetic acid, different flow rates, and detection wavelength were tested. The results indicated that an excellent separation was achieved using a mixture of solvent acetonitrile containing 0.4% acetic acid (A) and water containing 0.4% acetic acid (B). The program was operated as follows: 0–12 min, 27% A; 15–30 min, 27–38% A; and 32–55 min, 38–78% A. The chromatogram of *Puerariae flos* extract was shown in Figure 2(a).

3.2. Optimization of Centrifugal Conditions. After incubation procedure, the mixture would be screened by centrifugal device. Experiments with different centrifugal conditions were investigated based on the condition described in the literature [12]. As a result, bioactive compounds from

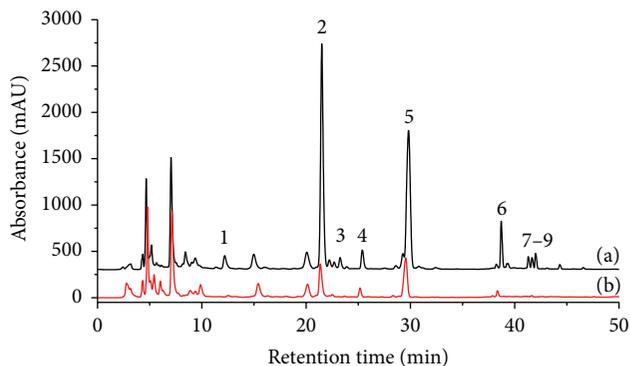


FIGURE 2: The chromatograms of (a) *Puerariae flos* extract and (b) the filtrate after ultrafiltration (1: puerarin, 2: daidzin, 3: 3'-methoxy daidzin, 4: ononin, 5: tectorigenin, 6: biochanin A, 7: genistein, 8: 3'-methoxy daidzein, and 9: irisolidone).

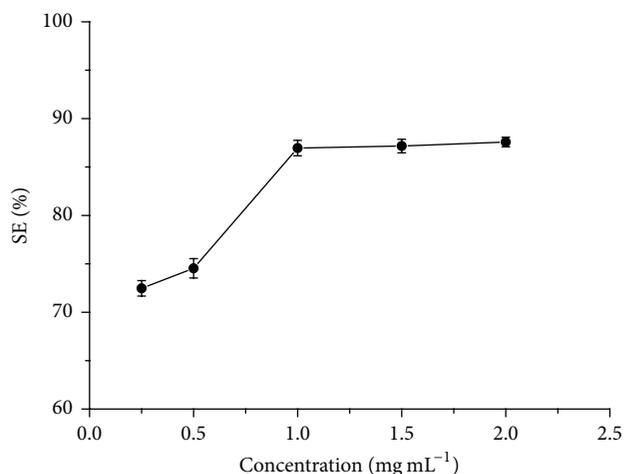


FIGURE 3: Effect of BSA concentration on SE.

the mixture were screened thoroughly when the mixture was centrifuged at 8000 rpm for 10 min.

3.3. Effect of BSA Concentration. The interaction between BSA and small molecules results in formation of a stable complex, which needs adequate concentration of BSA. For this reason, different concentrations of BSA were investigated to find the optimum condition. As a result, SE reached the highest when the concentration of BSA was 1 mg mL^{-1} (Figure 3). Therefore, the incubation was achieved when the concentration of BSA was 1 mg mL^{-1} .

3.4. Effect of Incubation Time. The interaction between BSA and small molecules also needs enough time. Sufficient incubation time could ensure that the screening was thorough. Different incubation time was investigated and the result was shown in Figure 4. When the incubation time was 2 h, the highest SE was observed. And no apparent increase appeared when the incubation time was prolonged. Therefore, the optimum incubation time was 2 h.

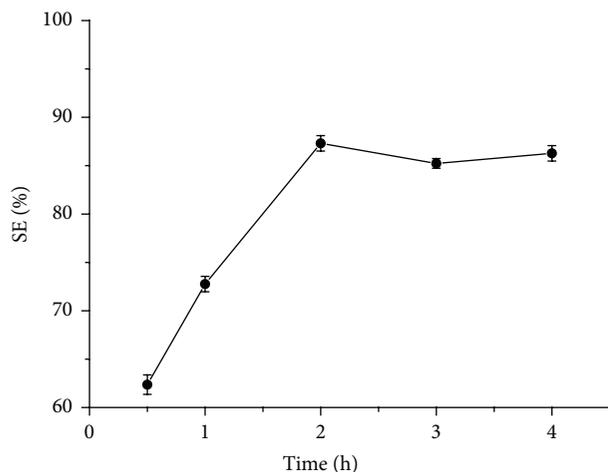


FIGURE 4: Effect of binding time on SE.

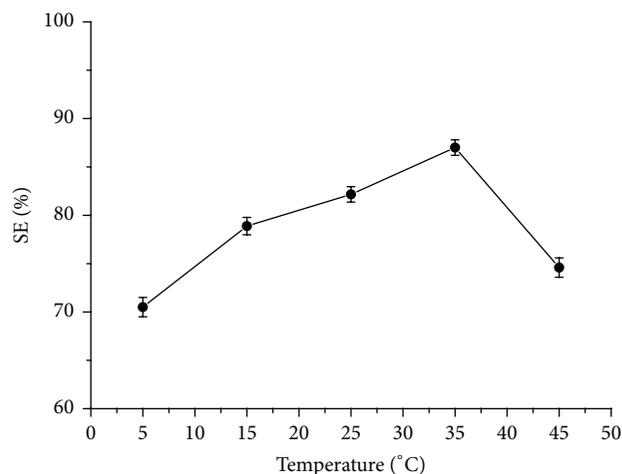


FIGURE 6: Effect of temperature on SE.

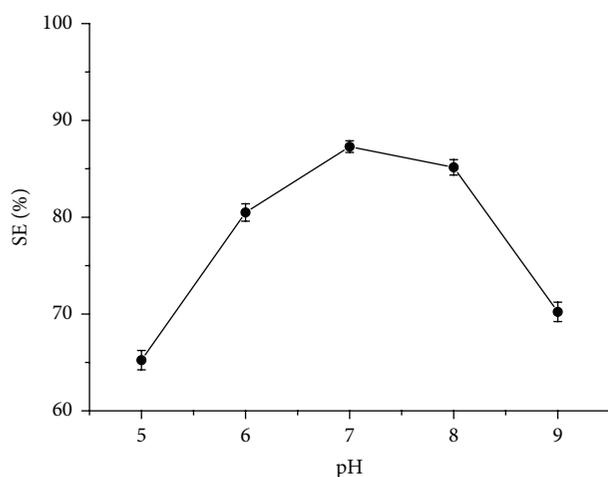


FIGURE 5: Effect of pH on SE.

3.5. Effect of pH and Temperature. Although BSA was relatively stable, it was still sensitive about pH and temperature. The pH value and temperature could affect the status of a protein [13]. The effect of pH on SE was studied at different pH values. As shown in Figure 5, the maximum SE of sample was obtained at pH 7.0. The effect of temperature on SE was also investigated and the results were shown in Figure 6. It was found that the highest SE was achieved at 35°C. These results were consistent with the reports in literature [14]. Therefore, the pH value and temperature in screening were set at 7.0 and 35°C.

3.6. Precision and Stability Test. Intraday and interday variability were measured to determine the repeatability of method. The same sample was analysed with ultrafiltration six times in one day and the relative standard deviation (RSD) of BD results was less than 1.8% ($n = 6$), while the same sample was analysed with ultrafiltration three times in three days in a row and the RSD of BD results was less than 3.7% ($n = 3$). These results indicated that the screening procedure

was reproducible and sufficiently precise for the qualitative evaluation of the active compounds [15]. In stability test, the experiment was carried out when the sample was stored for 48 h. The RSD was less than 2.9%. Those results demonstrated that the compounds in *Puerariae flos* sample were stable for 48 h.

3.7. Identification of Bound Ligands. Figure 2 showed the chromatograms of *Puerariae flos* extract and filtrate after ultrafiltration. By comparing the area of each peak in chromatograms, nine compounds were found binding to BSA. The structures of these compounds were identified by HPLC-MS experiment and the analysis of their retention times, UV data, and MS data with those of authentic samples (Table 1). By analysis of the UV spectra, all of the compounds typically had a maximum absorbance near 260 nm with a second maximum between 300 and 330 nm, which were the typical spectra of isoflavone derivatives.

In the negative mode, all the isoflavones revealed deprotonated molecular ion $[M-H]^-$ in the MS spectrum. According to the studies on the isoflavone glycosides in *Puerariae lobata* [16, 17], the neutral loss of 120 Da in negative mode is believed to be indicative of C-glycoside [18, 19]. The $[M-120-H]^-$ ion was observed in the MS spectra of compound 1, and this compound was identified as puerarin (1). The $[M-162-H]^-$ ion was observed in the MS spectrum of compound 2–4, which corresponded to the presence of hexose sugar, and $[M-162-15-H]^-$ ion was observed in the fragments, which is attributed to the neutral loss of CH_3 caused by the cleavage of methoxyl. These compounds were identified as daidzin (2), 3'-methoxy daidzin (3), and ononin (4) [20, 21]. According to MS spectra of the product ions in compounds 5, 6, 8, and 9, the $[M-15-H]^-$ ion observed corresponded to the neutral loss of CH_3 caused by the cleavage of methoxyl from the $[M-H]^-$. Based on the differences existed in the molecular weight, these compounds were characterized as tectorigenin (5), biochanin A (6), 3'-methoxy daidzein (8), and irisolidone (9) [22, 23]. Compound 7 exhibited $[M-H]^-$ ion at m/z 269 and it was characterized as genistein (7) by comparing

TABLE 1: The identification, retention time, UV, and MS characteristics of active compounds in *Puerariae flos*.

Number	Identification	t_R (min)	Proposed ions	(m/z)	UV λ_{max} (nm)
1	Puerarin	12.19	[M-H] ⁻	415	253, 319
			[M-C ₄ H ₈ O ₄ -H] ⁻	295	
2	Daidzin	21.50	[M-H] ⁻	415	248, 300
			[M-glc-H] ⁻	253	
3	3'-Methoxy daidzin	23.26	[M-H] ⁻	445	256, 317
			[M-glc-H] ⁻	283	
			[M-glc-CH ₃ -H] ⁻	268	
4	Ononin	25.40	[M-H] ⁻	429	256, 320
			[M-glc-H] ⁻	267	
			[M-glc-CH ₃ -H] ⁻	252	
5	Tectorigenin	29.82	[M-H] ⁻	299	263
			[M-CH ₃ -H] ⁻	284	
6	Biochanin A	38.71	[M-H] ⁻	283	261
			[M-CH ₃ -H] ⁻	268	
7	Genistein	41.30	[M-H] ⁻	269	268, 337
			[M-H] ⁻	283	
8	3'-Methoxy daidzein	41.65	[M-CH ₃ -H] ⁻	268	258, 317
			[M-H] ⁻	313	
			[M-H] ⁻	313	
9	Irisolidone	41.99	[M-CH ₃ -H] ⁻	298	260
			[M-2CH ₃ -H] ⁻	283	

TABLE 2: Comparison of ultrafiltration and conventional bioassay method in analysis of active compounds.

Item	Ultrafiltration	Conventional bioassay [3]
Theory	Protein-drug binding Membrane separation	Solvent extraction Chromatographic separation
Procedure	Incubation of mixture Ultrafiltration HPLC-MS analysis	Solvent extraction Repeated fractionation and isolation Structural characterization Pharmacological test
Time	About three hours	Several days
Reusable	The devices could be reused less than 5 times	The recycle of reagents needs much time and energy
Temperature	Room temperature	Room temperature

the elution order and UV spectra with that reported in literature [24].

3.8. Interaction of Bound Ligands with BSA. The BD of compounds in *Puerariae flos* extract were calculated and shown as follows: puerarin (94.54%), daidzin (93.82%), 3'-methoxy daidzin (99.72%), ononin (86.97%), tectorigenin (91.81%), biochanin A (94.96%), genistein (97.89%), 3'-methoxy daidzein (98.16%), and irisolidone (97.54%). In this method, the higher BD of the compound means the more bioactivity it has. According to this principle, the qualitative activity study of each compound could be obtained. The results indicated that 3'-methoxy daidzin, 3'-methoxy daidzein, genistein, and irisolidone expressed relatively higher BD values and activities.

4. Conclusions

In this paper, nine BSA bound ligands from *Puerariae flos* were successfully screened and identified by ultrafiltration combined with HPLC-MS. The interaction of bound ligands with BSA was investigated as well. This method showed good repeatability and stability. Compared with the mentioned conventional bioassay approach, the proposed method reduced the difficulty of experiments with only three operation steps and enabled analysis of active compounds from complex mixture without prior purification (Table 2).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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