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Analysis of interaction property of bioactive components in Danggui Buxue Decoction with protein by microdialysis coupled with HPLC–DAD–MS

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Abstract

Interaction of a commonly used combined prescription of Danggui Buxue Decoction (CPDBD) with protein was studied by microdialysis coupled with HPLC–DAD–MS. Nine compounds in CPDBD were unequivocally identified by comparing with their t_R, MS data and UV spectra with those of reference compounds, and simultaneously quantified. Microdialysis recoveries and binding degrees of 20 compounds in CPDBD with bovine serum albumin (BSA) were determined. Recoveries of microdialysis sampling ranged from 66.9 to 91.5% with RSD below 3.0%, and the binding degrees of those to BSA ranged from 6.3 to 59.8% (0.3 mM BSA) and from 6.9 to 86.6% (0.6 mM BSA). The results were determined at pH 7.4 and the influence of different pH value was investigated. Furthermore, the binding degrees of eight reference compounds were determined separately under the same conditions, indicating a significant effect of the interaction of compounds with each other on their binding degrees to BSA. By comparing their binding degrees with BSA with those of proven active compounds in CPDBD, i.e. chlorogenic acid (**3**), ferulic acid (**6**), ononin (**12**) and calycosin (**16**), other five compounds were found to possess potential activities, which were tentatively identified as calycosin-7-*O*-β-D-glucoside-6-*O*-malonate (**9**), senkyunolide I or H (**10**), formononetin-7-*O*-β-D-glucoside-6-*O*-malonate (**17**), and two unknown compounds.

Keywords: Protein; Microdialysis; HPLC–DAD–MS; Bioactive components; Combined prescription of Danggui Buxue Decoction

1. Introduction

Traditional Chinese medicines (TCMs), applied by TCMs practitioners for thousands of years, have been attracting ever-increasing attention for their therapeutic effects to western medicines with few side effects [1,2]. Although many TCMs have been proven effective by modern pharmacological studies and clinical trials, their bioactive components and the remedial mechanism are still not well understood. So far it is widely accepted that TCMs are mostly used in combination, and the composite formulae will produce a synergistic effect or antagonistic action [3]. So, the clarification of bioactive ingredients of TCMs needs an integrative method that can make it possible to perform bioactive assay, chemical isolation and identification of captured compounds almost simultaneously.

Microdialysis was introduced for sampling neurochemical substances from the extracellular fluid of the brain [4]. In recent years, microdialysis is becoming a standard technique for in vivo and in vitro analysis of drug and biochemical concentrations [5]. Because the typical molecular cutoff is such that proteins are excluded, microdialysis samples can be injected directly into HPLC system. This technique is being widely used in physiological, pharmacological, toxicological and behavioral studies for the recovery of exogenous substances, such as drugs and toxicants [6,7]. Some investigators have used the technique to determine degree of binding of drugs to plasma protein in vitro and in vivo [8–14].

Recently, the characterization of interaction property of TCMs with biological systems (DNA, protein, cell, etc.) is proposed for the screening and analyzing of multiple bioactive components [15–17]. Our laboratory has previously established some methods to screen potential bioactive components in combined prescription of TCMs, such as equilibrium dialysis with HPLC [18] and live cell extraction with LC–MS [19]. In present paper, we developed a new method to study the interaction

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property of bioactive components with protein by microdialysis coupled with HPLC–DAD–MS, and applied it into a combined prescription of Danggui Buxue Decoction (CPDBD)

2. Experimental

2.1. Herbal materials and chemicals

Radix Angelica sinensis and Radix Astragali were, respectively, collected from Min County of Gansu Province and Hunyuan County of Shanxi Province, China, and were authenticated by one of us, Prof. Ping Li.

Ferulic acid (**6**) was obtained from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, China); and L-tryptophan (**1**) was purchased from Amresco (St. Louis, MO, USA); other reference compounds, i.e. chlorogenic acid (**3**), calycosin-7-*O*-β-D-glucoside (**5**), ononin (**12**), (6α*R*,11α*R*)-9,10-dimethoxypterocarpan-3-*O*-β-D-glucoside (**14**), (3*R*)-2′-hydroxy-3′,4′-dimethoxy-isoflavan-7-*O*-β-D-glucoside (**15**), calycosin (**16**), and ligustilide (**21**) were isolated from Radix Angelica Sinensis and Radix Astragali in our laboratory. And their structures were elucidated by comparison of their spectral data (IR, MS, ^{1}H NMR and ^{13}C NMR) with those published references [20–25]. The purity of each compound was determined to be higher than 98% by normalization of the peak area detected by HPLC. The chemical structures of the reference compounds and other identified compounds in CPDBD are shown in Fig. 1.

BSA was purchased from Sigma (St. Louis, MO, USA) and acetonitrile was of HPLC grade from Merck (Darmstadt, Germany). Distilled water was further purified by a Milli-Q system (Millipore, Bedford, MA, USA). Other chemicals were of analytical grade. All solvents and samples were filtered through 0.45 μm filters before injecting into HPLC.

2.2. Apparatus and instruments

The LC–MS system consisted of an Agilent Series 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), equipped with a dual pump, an auto-sampler, a DAD detector, and an Agilent G1969 LC/MSD TOF system (Agilent Technologies) with an ESI source. Data were acquired and analyzed by LC–MS TOF Software Ver. A. 01.00 and PE Sciex Analyst QS (Agilent Technologies, USA).

Microdialysis was performed on a 3-syringe bracket microdialysis pump with Bee Syringe Pump Controller. MD-2310 10 mm 18,000 Da molecular weight cutoff polyacrylonitrile microdialysis probes (Bioanalytical System Inc., West Lafayette, IN, USA) were used for all experiments.

2.3. Sample preparation

2.3.1. Preparation of the extract of CPDBD

Thirty grams of mixed powder of Radix Astragali and Radix Angelica Sinensis (5:1, w/w) was immersed in 500 mL 75% ethanol for 1 h and then refluxed for 2 h at 85 °C, the solvent was removed with rotary evaporation under vacuum at 45 °C, and the residue was dissolved in 30 mL phosphate buffer and filtered through a 0.22 μm membrane, and the filtrate was stored at 4 °C in the absence of light for further experiments.

2.3.2. Preparation of BSA

BSA was prepared in 0.1 M phosphate buffer of pH 7.4 containing 0.1 M NaCl.

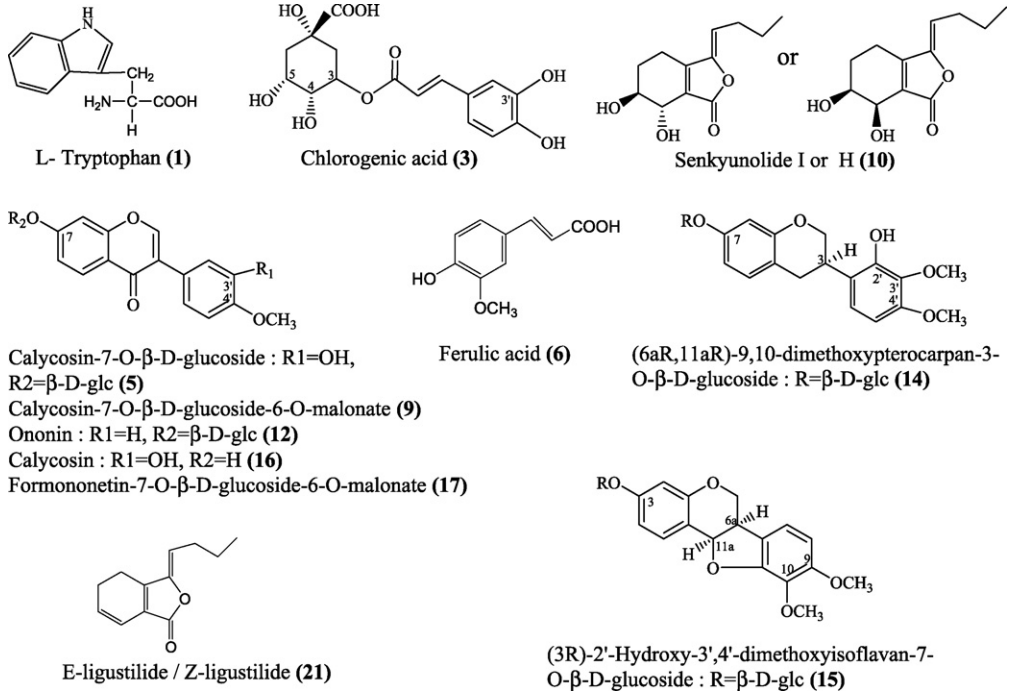

Fig. 1. Chemical structures of the reference compounds in combined prescription of Danggui Buxue Decoction.

2.3.3. Preparation of mixture

CPDBD solution was added with BSA (1:1, v/v), and then reacted for 10 h at 25 °C before sampling for fully balanced combination between the components in CPDBD and BSA.

Each standard solution with the same concentration in CPDBD was added with BSA, other conditions are the same.

2.3.4. Influence of ferulic acid on the interaction of calycosin with BSA

Firstly, various concentrations of calycosin standard solution from 1.0×10^{-5} to 1.0×10^{-4} M/L were added with BSA (0.6 mM/L), and then were dealt the same as the preparation of mixture. Secondly, various concentrations of ferulic acid were added to the balanced combination mixture between calycosin and BSA, and the system was kept for 10 h at 25 °C before sampling for the determination of free concentration of calycosin again.

2.4. Microdialysis sampling

The CPDBD–BSA mixture was incubated at 37.0 °C in a water bath over 10 min. Then the probe was placed into the solution (phosphate buffer solution at 1 μL/min) for microdialysis sampling. After proceeding for 20 min to reach an equilibration, the "CPDBD–BSA microdialysate" was collected for 50 min for HPLC analysis. Meanwhile, the "blank microdialysate", i.e. without BSA in CPDBD, was also collected by the same procedures.

The same microdialysis sampling procedures were performed on each reference compound to BSA.

2.5. HPLC–DAD–MS conditions

The HPLC analysis was performed on a ZORBAX C-18 column (250 mm × 4.6 mm I.D., 5 μm) and a ZORBAX C-18 guard column (12.5 mm × 4.6 mm I.D., 5 μm). The mobile phases consisted of 0.1% formic acid water (**A**) and acetonitrile (**B**) using a gradient elution of 8–10% (v/v) B at 0–10 min; 10–25% B at 10–35 min; 25–48% B at 35–60 min; 48–80% B at 60–85 min.

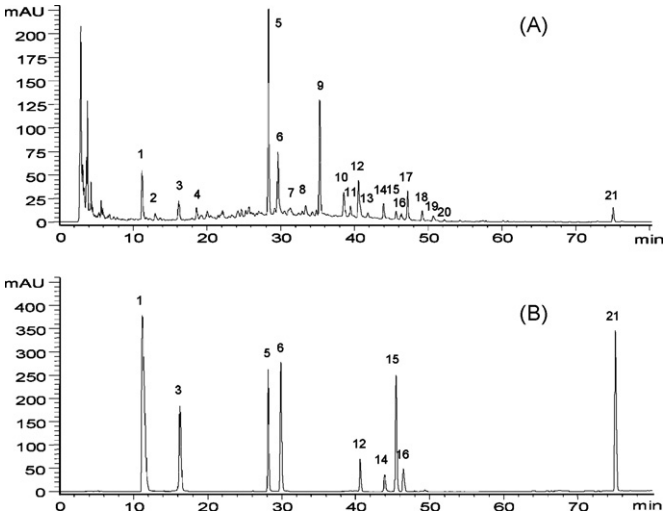

Fig. 2. (A) Representative chromatograms of combined prescription of Danggui Buxue Decoction. Chromatographic conditions are described in Section 2. (B) Representative chromatograms of the reference compounds. Chromatographic conditions are described in Section 2.

The flow-rate was 0.8 mL/min, and the injection volume was 10 μL. The detection wavelength was set at 280 nm.

The ESI/MS conditions were set as follows: positive ion mode; drying gas (N_2), 9 L/min; nebulizer (N_2), 35 psi; drying gas temperature, 320 °C; capillary voltage, 4000 V; fragmentation voltage, 100 V; full scan range, 100–1500 *m/z*.

3. Results and discussion

3.1. Identification of constituents in CPDBD

The HPLC chromatogram of CPDBD extract monitored at 280 nm is shown in Fig. 2(A) and revealed that there were more than 21 components marked with numeral 1, 2, 3–21 in the chromatogram. Nine reference compounds were analyzed by HPLC–DAD–MS shown in Fig. 2(B) to provide their t_R, MS data, and UV λ_{max} which are summarized in Table 1 for the comparison with those of compounds present in CPDBD extract. With the comparison of the t_R, MS data, and UV λ_{max} with these

Table 1
The identification of components in combined prescription of Danggui Buxue Decoction

Peaks no.	t_R (min)	$[M+H]^+$ (*m/z*)	$[M+Na]^+$ (*m/z*)	Other ion (*m/z*)	W_{max} (nm)	Compound identity
1	11.2	431	453	–	281	L-Tryptophan
3	16.2	355	377	–	350	Chlorogenio acid
5	28.3	447	469	285	260, 290	Calycosin-7-*O*-β-D-glucoside
6	29.6	195	–	–	295	Ferulic acid
9	35.3	533	555	285	260, 290	Calycosin-7-*O*-β-D-glucoside-6-*O*-malonate
10	38.5	225	247	207	277	Senkyunolide I or H
12	40.6	431	453	269	255, 301	Ononin
13	41.6	549	571	–	265	Unknown compound
14	43.9	463	485	301	282	(6α*R*,11α*R*)-9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside
15	45.6	465	487	303	282	(3*R*)-2′-Hydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside
16	46.2	285	307	–	250, 290	Calyocosin
17	47.1	517	539	269	255, 301	Formononetin-7-*O*-β-D-glucoside-6-*O*-malonate
20	51.6	473	495	–	252	Unknown compound
21	75.2	191	213	–	280, 328	*E*-Ligustilide or *Z*-ligustilide

Table 2
The curves for the quantification of identified constituents

Peaks no.	Calibration curves	r^2	Test ranges (mM/L)	Dialysate concentration (mM/L)
1	$y = 2054.5x + 11.087$	0.9997	0.1030–5.15	1.161
3	$y = 2192x + 4.702$	0.9998	0.0537–2.68	0.252
5	$y = 3 \times 10^6 x + 4.512$	0.9995	0.0448–1.79	0.139
6	$y = 3434.4x + 20.674$	0.9997	0.1080–5.41	0.712
12	$y = 3 \times 10^6 x + 4.837$	0.9995	0.0116–0.58	0.025
14	$y = 92951x + 1.726$	0.9995	0.0108–0.54	0.032
15	$y = 75519x + 1.118$	0.9996	0.0106–0.53	0.038
16	$y = 5 \times 10^6 x + 5.595$	0.9996	0.0141–0.75	0.029
21	$y = 646.98x + 20.969$	0.9995	0.0980–8.20	Trace

reference compounds, peaks **1**, **3**, **5**, **6**, **12**, **14**, **15**, **16** and **21** in the chromatograms of CPDBD extract were unequivocally identified as L-tryptophan, chlorogenic acid, calycosin-7-*O*-β-D-glucoside, ferulic acid, ononin, (6α*R*,11α*R*)-9,10-dimethoxyp-terocarpan-3-*O*-β-glucoside, (3*R*)-2′-hydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside, calycosin, and ligustilide, respectively.

As to the other five components in CPDBD (**9**, **10**, **13**, **17**, and **20**) with suitable binding degrees to BSA, their identities were tentatively achieved by comparing their MS and UV data with the published ones. The peak **9** mainly giving the $[M+H]^+$ ion (*m*/*z* 533), the same fragment ion corresponding to the protonated aglycone at *m*/*z* 285, and similar UV spectrum with the peak **5**, was assigned to calycosin-7-*O*-β-D-glucoside-6-*O*-malonate [26]. Similarly, the peak **17** was assigned to formononetin-7-*O*-β-D-glucoside-6-*O*-malonate, the malonate **12**. For compound **10**, the observed diagnostic ion $[M+H-H_2O]^+$, the adduct ions $[M+H]^+$ and $[M+Na]^+$ corresponded to the structure of senkyunolide I or H [27]. The identities of the other two components need further investigation. The t_R, $[M+H]^+$ ions, $[M+Na]^+$ ions, UV λ_{max} values and the identification of components in CPDBD were listed in Table 1.

3.2. Quantification of nine identified constituents

The stock solution of nine standards was diluted to make different concentration ranges. The calibration curve of each compound was performed with at least six appropriate concentrations in triplicate, their regression equations were calculated in the form of $y = a \times x + b$, where y represents peak areas and x represents the concentration of identified constituents Table 2 lists linear calibration curve with r^2 and linear range of each compound, and also shows the dialysate content of each compound in CPDBD determined by microdialysis.

3.3. Recovery of microdialysis

Recovery is the most important factor for determining the free drug concentration in the mixtures solution. The unbounded

Table 3
Relative microdialysis recovery of compounds in combined prescription of Danggui Buxue Decoction

Peaks	Retention time (min)	Relative recovery (%)			RSD (%) ($n = 5$)		
		6.0	7.4	8.9 (pH)	6.0	7.4	8.9 (pH)
1	11.21	81.8	86.5	82.7	2.5	1.4	1.4
2	12.98	76.6	83.8	71.2	2.2	0.3	2.4
3	16.17	82.7	75.0	73.9	2.7	1.9	1.2
4	18.59	76.8	75.8	79.0	2.9	0.4	2.7
5	28.31	66.6	69.6	61.7	2.0	1.8	1.1
6	29.59	82.6	79.1	76.2	0.3	1.3	1.2
7	31.28	63.6	81.1	65.2	2.2	3.0	2.6
8	33.40	63.5	58.9	69.4	2.5	1.3	1.7
9	35.29	64.4	70.2	66.5	1.8	1.6	2.0
10	38.54	87.2	89.0	86.2	0.4	2.1	0.3
11	39.44	68.8	76.6	75.1	2.5	1.3	1.1
12	40.55	69.4	72.9	69.9	2.3	2.3	2.8
13	41.81	71.0	72.0	71.8	2.5	2.9	2.1
14	43.94	72.3	78.0	72.5	1.1	2.0	1.6
15	45.56	84.6	91.5	79.9	0.4	2.1	2.9
16	46.16	70.9	68.9	66.0	0.5	2.4	1.4
17	47.12	70.3	70.7	68.8	1.8	2.5	1.2
18	49.10	71.0	73.1	66.2	2.6	1.2	1.7
19	50.62	70.3	73.1	65.5	1.1	2.1	1.2
20	52.12	62.0	66.9	63.9	2.6	1.8	2.1

concentration (C_s) in the mixture of CPDBD extract and BSA solution can be calculated by Eq. (1) [5].

$$C_s = \frac{C_d}{R} \quad (1)$$

where R is the concentration recovery, C_d is the concentration in the dialysate and C_s is the concentration in the sample. Recoveries of compounds in CPDBD were calculated and are listed in Table 3.

As shown in Table 3, the recoveries of compounds at different pH (6.0, 7.4 and 8.9) are not the same. The influence of pH on recovery mainly results from the change of the molecular state of compounds. In general, neutral molecules typically exhibit higher recovery than ionized. The smaller and more hydrophilic, higher the recoveries are found [28,29]. For instance, the recoveries of chlorogenic acid (**3**) and ferulic acid (**6**) at pH 6.0 are higher than those at pH 7.4 and 8.9, for the two acid compounds are mostly in molecular state at pH 6.0 while in ionized state at pH 7.4. In addition, RSD of recoveries for all compounds at pH 6.0, 7.4 and 8.9 was below 3.0%, which indicated that the method of microdialysis coupled with HPLC could be reliable and stable to simultaneously determine compounds in CPDBD.

3.4. Interaction of compounds in CPDBD with BSA

The binding degrees of compounds in CPDBD with BSA were related to their activities in some sense. Several factors influenced the binding degrees, including pH values and the interaction of compounds with each other.

3.4.1. Binding at pH 7.4

Binding results of the CPDBD to BSA with different concentration compared with blank microdialysate at pH 7.4 are shown in Fig. 3 The binding degrees of the compounds in CPDBD with BSA was defined as Eq. (2):

$$\text{binding degree} = \frac{(C_0 - C_S)}{C_0} \quad (2)$$

where C_0 is the concentration of compound in the solution.

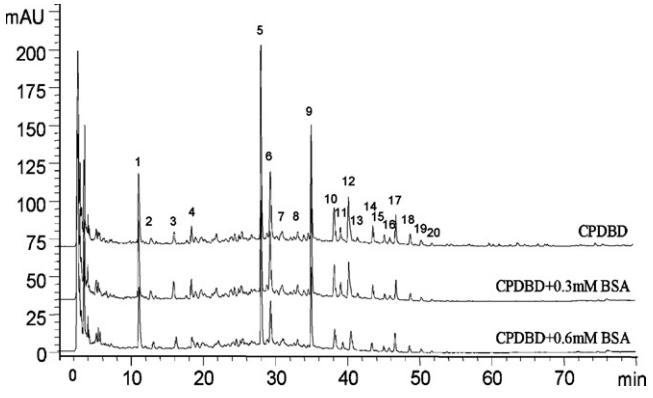

Fig. 3. Chromatograms of combined prescription of Danggui Buxue Decoction with BSA. CPDBD + 0.3 mM BSA means that the interaction of combined prescription of Danggui Buxue Decoction with BSA the concentration is 0.3 mM.

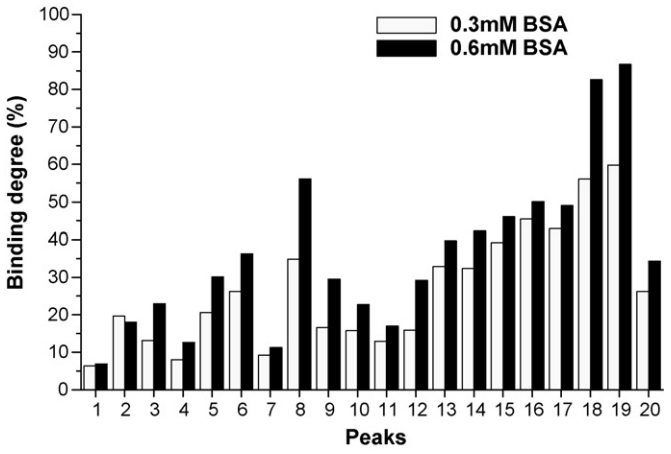

Fig. 4. Binding degrees of CPDBD react with BSA. The left bar represents the binding degree of CPDBD with 0.3 mM BSA. The right bar represents binding with 0.6 mM BSA.

As clearly shown in Fig. 4, the binding degrees were influenced by the polarity of compounds, i.e. weaker the polarity, higher the binding degree. So the binding degree of peak **1** was the lowest, while peak **19** was the highest. The result might be explained by the configuration of BSA including two hydrophobic pockets which easily bind with compounds of weak polarity. On the other hand, the binding degree may be associated with the molecular configuration. Peaks **8** and **20** were just the instances in case. Though strong was the polarity of peak **8**, its binding degrees were up to 34.8% (0.3 mM BSA) and 56.1% (0.6 mM BSA); while the binding degrees of peak **20** were only 26.3% (0.3 mM BSA) and 34.3% (0.6 mM BSA) in spite of the weakest polarity among all compounds. The content of **21** in microdialysate could not be quantified because of its volatility.

It is widely accepted that in the pharmaceutical industry the overall distribution, metabolism and efficiency of many drugs can be altered based on their affinity to serum albumin. In addition, many promising new drugs are rendered ineffective because of their unusually high affinity for this abundant protein [30]. So molecules with higher or lower binding degree would not be the bioactive compounds in Chinese medicine. Compounds **3**, **5**, **6** and **16** with suitable binding degrees to BSA were proven active components in CPDBD by chemical and pharmacological investigations [31–34]. Therefore, other compounds in CPDBD with similar binding degrees such as peaks **9**, **10**, **13**, **17** and **20**, would be worthy of further study as bioactive candidates.

3.4.2. Influence of pH on binding degrees

PH is a critical factor affecting the molecular state of both biopolymers like proteins and drugs especially those ionic compounds. For one thing, when the solution pH is higher than the isoionic point of BSA (pH 4.7), BSA bears negative charge. For another, the molecular state of the compounds in CPDBD is altered as the variation of pH, and neutral molecules typically exhibit higher binding degrees than ionized.

Binding degrees of chlorogenic acid (**3**) and ferulic acid (**6**) were 29.1 and 39.4% at pH 6.0, 22.9 and 36.2% at pH 7.4, 16.8 and 32.3% at pH 8.9, when the concentration of BSA was

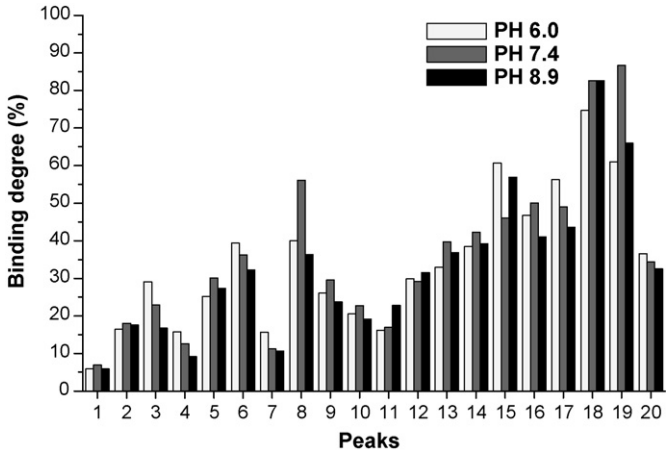

Fig. 5. Binding degrees of components in CPDBD react with BSA under different pH. The left bar represents the binding degree of CPDBD with 0.6 mM BSA at pH 6.0. The middle bar represents the binding degree of CPDBD with 0.6 mM BSA at pH 7.4. The right bar represents the CPDBD binding with 0.6 mM BSA at pH 8.9.

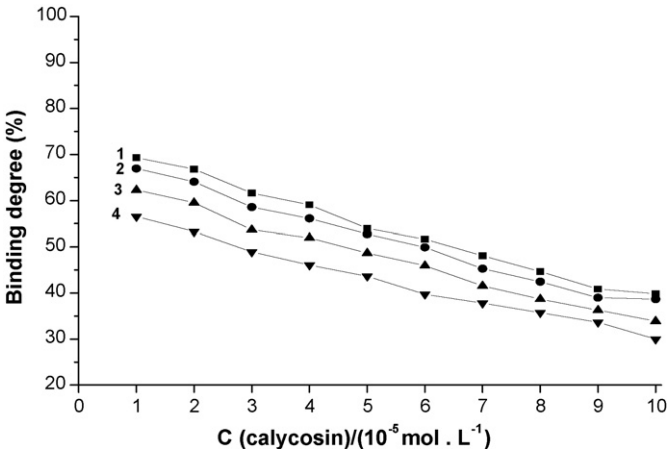

Fig. 6. Variation curves of binding degree of Calycosin with protein after ferulic acid were added. 1: blank (without ferulic acid), 2: 3×10^{-5} M/L ferulic acid was added, 3: 6×10^{-5} M/L ferulic acid were added, 4: 12×10^{-5} M/L ferulic acid was added.

0.6 mM. We attribute this to the changes in the structure of the binding site of HSA with pH and also the ionic state of peak **3** and **6**.

In Fig. 5, binding degrees of other peaks were changed at different pH. Some compounds had high binding degrees at pH 7.4, such as peak **1**, **2**, **5**, **8**, **9**, **10**, **13**, **14**, **16** and **19**; while some other compounds had high binding degrees at pH 6.0, such as peak **5**, **7**, **15**, **17** and **20**; and still some had high binding degrees at pH 8.9, such as peak **11**, **12**, and **18**. We can conclude that pH influences the interaction between compounds in CPDBD and BSA as a result of changes in the binding site structures of BSA and molecular state of active compounds.

3.4.3. Comparison of the binding degrees of single reference solution with that in the extract of CPDBD

Though most drugs were nonspecific when binding with the plasma protein, unlike the definite acceptor to plasma protein, their interactions were usually generated at the relatively stable binding sites, and with some of conformation altering in certain sense. Therefore, the suppressing, inductive and synergistic effects were probably produced between drugs when binding at multiple sites. Many different kinds of compounds consisted in the TCM at various molecular weights and polarity degrees were bound to influence each other in the course of binding with plasma protein and resulted in an altered binding degree.

The binding degrees of reference materials were determined in each solution, respectively, and then compared with those in the decoction under the same conditions. The results of comparison showed differences of binding degrees in certain ways shown in Table 4, The binding degrees of **1**, **5** and **12** were 10.6, 39.8 and 36.8% in each solution while 6.9, 30.1, 29.2% in the decoction. The decrease might be caused by competitive effect. On the other hand, the binding degrees of **3**, **6**, **15** and **16** were increased by synergistic effect.

3.4.4. Influence of ferulic acid on the interaction of calycosin with BSA

As shown in Fig. 6, the binding degree of calycosin decreased with the concentration of ferulic acid increasing, which suggested that there was competitive binding with BSA between calycosin and ferulic acid. However, the binding degree of calycosin in CPDBD with BSA is higher than that binding separately, which indicated other components or the matrix might have a synergistic effect on calycosin.

The results above indicated that, compared with those of single reference compounds, the binding degrees of compounds in CPDBD reflected the interaction and influence with each other,

Table 4
Comparison of binding degrees of reference solution with those in combined prescription of Danggui Buxue Decoction

Peaks no.	Compound	Binding degrees (%)	
		Reference solution	CPDBD[a]
1	L-Tryptophan	10.6	6.9
3	Chlorogenio acid	19.6	22.9
5	Calycosin-7-*O*-β-D-glucoside	39.8	30.1
6	Ferulic acid	25.6	36.2
12	Ononin	36.8	29.2
14	(6α*R*,11α*R*)-9,10-Dimethoxypterocarpan-3-*O*-β-D-glucoside	40.6	42.3
15	(3*R*)-2′-Hydroxy-3′,4′-dimethoxyisoflavan-7-*O*-β-D-glucoside	42.9	46.0
16	Calyocosin	39.8	50.1

[a] Refers to combined prescription of Danggui Buxue Decoction.

which may thought to be more suitable to evaluate the activity of different components.

4. Conclusion

In this experiment, microdialysis–HPLC–DAD–MS was successfully used for studying the interaction of combined prescription of TCM and protein. It shows that there are some suitable binding degrees on BSA for bioactive components chlorogenic acid (**3**), ferulic acid (**6**), ononin (**12**) and calycosin (**16**), and the peaks calycosin-7-*O*-β-D-glucoside-6-*O*-malonate (**9**), senkyunolide I or H (**10**), **13**, formononetin-7-*O*-β-D-glucoside-6-*O*-malonate (**17**), and **20** have similar binding degrees to BSA. The results indicated that the newly proposed strategy might be a useful approach as a first step in the screening of potential bioactive candidates in TCMs with high probability.

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