Characterization of interaction property of multicomponents in Chinese Herb with protein by microdialysis combined with HPLC

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Abstract
Interaction of traditional Chinese Herb Rhizoma Chuanxiong and protein was studied by microdialysis coupled with high performance liquid chromatography. Compounds in Rhizoma Chuanxiong, such as ferulic acid, senkyunolide A and 3-butylyphthalide, were identified by HPLC, HPLC–MS and UV–vis. Microdialysis recoveries and binding degrees of compounds in Rhizoma Chuanxiong with human serum albumin (HSA) and other human plasma protein were determined: recoveries of microdialysis sampling ranged from 36.7 to 98.4% with R.S.D. below 3.1%; while binding to HSA ranged from 0 to 91.5% (0.3 mM HSA) and from 0 to 93.5% (0.6 mM HSA), respectively. Compared with HSA, most of compounds bound to human blood serum more extensively and the results showed that binding of these compounds in Rhizoma Chuanxiong was influenced by pH. Two compounds were found to bind to HSA and human blood serum, their binding degrees were consistent with ferulic acid and 3-butylyphthalide, the active compounds in Rhizoma Chuanxiong.

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1. Introduction
Chinese herbs are treatments that are commonly advocated for a wide range of conditions in many Eastern countries and that have also become popular in the West [1,2]. Rhizoma Chuanxiong, the dried rhizome of Ligusticum chuanxiong HORT, is one of the most important crude drugs in TCMs and has been used to treat headaches, anemia, feelings of coldness and irregular menstrual cycles [3]. As a classic Chinese Herb, Rhizoma Chuanxiong has been studied for several decades. Some compounds, such as ferulic acid, phthalide analogues and aromatic compounds, have been isolated and identified [4]. Some of its essential components, like ferulic acid and phthalides analogues are regarded as biologically active components [5].

In principle, the pharmacological activity of many synthetic drugs is significantly affected by reversible interactions with plasma protein [6,7], such as α1-acid glycoprotein (AGP) and human serum albumin (HSA). These interactions play an important role in the drug distribution, efficacy and toxicity. A Chinese Herb usually contains up to hundreds or even thousands of different constituents, thus the pharmacological activity of Chinese Herbs on the human body can be resulted from the combined interaction of some active compounds, including the interaction between compounds and protein, especially blood plasma protein.

Microdialysis was introduced for sampling neurochemical substances from the extracellular fluid of the brain [8]. In recent years, microdialysis has become an important technique for the in vivo sampling of living systems. Because the typical molecular cut off is such that proteins are excluded,
microdialysis samples can be injected directly into the 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 [9–11]. Recently, we have successfully combined the microdialysis with HPLC to study the binding between small molecules and biopolymers. Bindings of various drugs including enantiomers to protein have also been studied [12–17].

In this study, the binding characteristics of compounds in an extract of Rhizoma Chuanxiong with HSA and human plasma proteins were studied by microdialysis coupled with HPLC.

2. Experimental

2.1. Reagent and materials

Rhizoma Chuanxiong was purchased from Merro Chinese Medicine Store (Dalian, China); human serum albumin (fatty acid and globulin free) was purchased from Sigma (St. Louis, MO, USA); healthy human blood serum (male) was purchased from Dalian Blood Center (Dalian, PR China); Ferulic acid was purchased from Sigma (St. Louis, MO, USA); 3-butylnithalide was ordered from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); other chemical reagents purchased as analytical or HPLC grade chemicals.

2.2. Apparatus and instruments

The HPLC system comprised two LC-10 ADvp pumps (Shimadzu, Kyoto, Japan), a Rheodyne type injection valve with a 20 μL loop, SPD-M10Avp diode array detector (Shimadzu, Kyoto, Japan) and the outlet of the diode array detector was directly connected to APCI/MS (Shimadzu, Kyoto, Japan). Mass spectrometers APCI/MS data are displaying using the control software LCMS Solution Version 2.02 (Shimadzu, Kyoto, Japan) supplied with the instruments.

Micro-injection pump with model 74900 was purchased from Cole-Parmer Corporation (USA). Regenerated cellulose, Spectro/Pro microdialysis hollow fibers (Spectrum Medical Industries, Los Angeles, CA) with a molecular weight cut-off 18,000 Da and length of 10 mm were used as dialysis membrane (0.200 mm i.d., 0.216 mm o.d.).

2.3. Preparation of the sample

2.3.1. Preparation of Rhizoma Chuanxiong extract

Six grams of Rhizoma Chuanxiong Hort was crushed with a grinder, and immersed in 60 mL of methanol (HPLC grade) for 1 h, then heated to boiling for 1 h. The methanol extract was filtered through a 0.45 μm membrane, diluted methanol to 60 mL and stored at 4 °C in the absence of light for further experiments.

2.3.2. Interaction of Rhizoma Chuanxiong extracts with protein

Hundred microliter Rhizoma Chuanxiong extract was placed in a glass vial and heated by water bath at 60 °C to remove the methanol. After it cooling human blood serum or HSA solution, which was dissolved in 0.1 M phosphate buffer, containing 0.1 M NaCl, was added and mixed together. The mixture was stored at 4 °C for 12 h for further perfusion and HPLC analysis. The volume of solution of perfusion sample was 3 mL.

2.3.3. HPLC analysis

The HPLC analysis conditions were as follows: column was an ODS column (200 mm × 4.6 mm i.d., 5 μm, Kromasil, Sweden). The mobile phases were: (A) acetonitrile and (B) water. Using a gradient of 15% A for 10 min, a linear gradient eluted from 15 to 30% A over 20 min, then maintain the final mixture for 30 min. The flow rate was set at 0.8 mL min⁻¹. The detection wavelength was set at 215 nm.

The APCI/MS conditions were as follows: nitrogen gas flow rate was 25 L min⁻¹, APCI temperature was 400 °C, CDL temperature was 250 °C, block temperature was 200 °C and detector voltage was 1.8 kV.

3. Results and discussion

3.1. Identification of constituents in Rhizoma Chuanxiong

In this experiment, gradient elution was adopted after optimization of chromatographic condition. The HPLC chromatogram using UV detect at 215 nm was shown in Fig. 1 and this revealed that there were more than 20 components marked with letters A, B, C to M in the chromatogram.

![Fig. 1. Chromatograms of components in Rhizoma Chuanxiong extract and in dialysate separated on C18 column. Experimental condition were referenced to the text. CX represent the Rhizoma Chuanxiong and CX perfurate represents the dialysate after microdialysis probe. Peaks with letters represent the contents in Rhizoma Chuanxiong. Peaks A, F and K had been identified and the molecule formulas were listed under the name of these peaks.](image-url)
Some of these components have been identified using HPLC and HPLC–MS (APCI) and PDA. The molecular ion of peak A is 194.18 and its maximum UV absorption wavelength is 313 nm. Comparison with ferulic acid standard and reference [18], peak A was identified as ferulic acid. The molecular ion of peak F is 192.2 and its maximum UV absorption wavelength is 280 nm, again comparison with reference [18,19], peak F can be identified as senkyunolide A. The molecular ion of peak K is 190.23 and its maximum UV absorption wavelength is 280 nm, again comparison with reference [18], peak K was identified as 3-butylphthalide. The structures of these three compounds were shown in Fig. 1.

3.2. Quantification of ferulic acid and 3-butylphthalide

The curves for the quantitative of ferulic acid and 3-butylphthalides have been measured and could be presented as follows:

Ferulic acid : \[ C = 9.448 + 5.643 \times 10^{-5} A \]
\[ r = 0.9995; 0.0231-0.528 \text{ mM} \]

3-Butylphthalide : \[ C = 2.278 + 5.375 \times 10^{-5} A \]
\[ r = 0.9998; 0.0362-0.257 \text{ mM} \]

where \( C \) represents the concentration of ferulic acid and 3-butylphthalide in solution and \( A \) represents peak areas ferulic acid and 3-butylphthalide. The concentrations of ferulic acid and 3-butylphthalide in solution can be calculated based on the actual concentration of the solution. The probe recovery is defined as the ratio between the concentration of compound in dialysate and the actual concentration in solution and is calculated by Eq. (1).

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

where \( R \) is the concentration recovery, \( C_d \) the concentration in the dialysate and \( C_s \) is the concentration in the sample. Thus, the unbounded concentration \( (C_s) \) of in the mixture of Rhizoma Chuanxiong and protein can be calculated by Eq. (2).

\[ C_s = \frac{C_d}{R} \quad (2) \]

Recoveries of compounds in Rhizoma Chuanxiong were listed in Table 1. During microdialysis sampling, some factors influence the recovery, such as the type and length of membrane, the geometry of the probe, system temperature and the perfusion flow rate [20]. In this experiment, homemade microdialysis probes with 10 mm length membrane, whose molecular weight cutoff was 18,000 Da were used. Perfusion flow rate and system temperature were critical factors to influence the precision of recovery. In this experiment, the perfusion flow rate and temperature were 1 \( \mu \text{L min}^{-1} \) and 37 \( ^\circ \text{C} \).

It could be seen that the recovery of ferulic acid was 58.7% (Table 1). In general, neutral molecules typically exhibit higher recovery than ionized. The smaller and more hydrophilic the higher recoveries are found [20,21]. The molecular weight of ferulic acid is 194.18, as an ionic molecule, its recovery was lower than other small molecules in the Rhizoma Chuanxiong.

High recoveries were discovered for compounds from peaks B to J, with recoveries higher than 87.4%, even up to 98.4%. Compounds eluting from the C18 column in this time range probably are neutral compounds like the identified senkyunolide A and 3-butylphthalide. However, one compound in peak L, has a low recovery of 36.7%. Suggesting it is other a relative larger molecule. Recoveries of peaks K and M, 68.9 and 69.5%, respectively, were lower than those corresponding peaks B to J. Based on the retention times of these two peaks, it would be seemed that their hydrophilic property were lower.

Given that R.S.D. of recoveries were below 3.1%. It can be concluded that this method of microdialysis coupled with HPLC provided accurate determination of the concentrations of compounds of Rhizoma Chuanxiong extract.

3.4. Interaction of compounds in extract of Rhizoma Chuanxiong with HSA

Recoveries of compounds in Rhizoma Chuanxiong were listed in Table 1. During microdialysis sampling, some factors influence the recovery, such as the type and length of membrane, the geometry of the probe, system temperature and the perfusion flow rate [20]. In this experiment, homemade microdialysis probes with 10 mm length membrane, whose molecular weight cutoff was 18,000 Da were used. Perfusion flow rate and system temperature were critical factors to influence the precision of recovery. In this experiment, the perfusion flow rate and temperature were 1 \( \mu \text{L min}^{-1} \) and 37 \( ^\circ \text{C} \).

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Fig. 2. Chromatograms of Rhizoma Chuanxiong extract react with HSA. CX represents the Rhizoma Chuanxiong, and CX + 0.3 mM HSA means that the interaction of Rhizoma Chuanxiong with HSA the concentration is 0.3 mM. The ular protein consists of a single polypeptide chain of 585 amino acids, and it has many important physiological functions [22–24]. HSA contributes to osmotic blood pressure and realize transport and distribution of many molecules and metabolites, such as fatty acids, amino acids, hormones, cations and anions and many diverse drugs [22,23]. It also enables the transport of many drugs that are poorly soluble in water, through the bloodstream [24]. It has been shown that the distribution, free concentration and the metabolism of various drugs can be significantly altered as a result of their binding to HSA [25]. Therefore, the interaction of drugs with human serum albumin has major biochemical importance and can be used as a model for elucidation of the drug protein complexation.

3.4.1. Binding at pH 7.4

Binding results of the extract of Rhizoma Chuanxiong with HSA at pH 7.4 were analyzed by HPLC and the results were shown in Figs. 2 and 3. The binding strength of the compounds in Rhizoma Chuanxiong extract with HSA was defined as Eq. (3):

\[ \text{binding degree} = \frac{C_0 - C_s}{C_0} \]

where \( C_0 \) is the concentration of compound in the solution.

The binding degree of ferulic acid (peak A) was 23.0% (using 0.3 mM HSA) and 36.7% (using 0.6 mM HSA).

Abundant studies have been done on the binding of ligand and albumin, indicating that most ligands are bound reversibly [26,27]. It is generally recognized that there is a small number of distinct binding locations. Although controversy remains about the exact number of discrete binding locations on albumin the general consensus is as follows [28]: these sites are consistent with the two long-chain fatty acid sites of sites III and IV; the two metal-binding sites of sites V and VI. The two principal binding sites I and II for small heterocyclic or aromatic carboxylic acids like aspirin, warfarin, ibuprofen, etc. [28]. The study has not reported binding sites of Chinese Herb compounds in Rhizoma Chuanxiong. As an aromatic carboxylic acids analog, ferulic acid may bind relative strong to HSA, which showed a preference for small aromatic carboxylic acid.

The binding degrees of senkyunolide A (peak F) was just 6.5% at 0.3 mM HSA and 5.4% at 0.6 mM HSA. Rather lower than most other compounds, senkyunolide A contains a reduced aryl ring, so it is possible that it does not bind to special binding sites on HSA. Binding degrees of 3-butyphthalide (peak K) was 27.7 and 30.2%, using 0.3 and 0.6 mM HSA, respectively, and this is significantly higher than senkyunolide A. It can be explained that aromatic nature and the presence of special binding sites for such compounds on HSA. At the same time, HSA binds easily with hydrophobic molecule.

The binding degrees of peaks I and M were 22.2 and 32.5% using 0.6 mM HSA. 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 [29]. So molecules with higher or lower binding degree would not be the bioactive compounds in Chinese medicine. As we know, ferulic acid and 3-butyphthalide are active compounds in Rhizoma Chuanxiong [4]. Molecules with similar binding degrees as ferulic acid and 3-butyphthalide, such as peaks I and M, should be worthy of further study as potential drugs.

3.4.2. Influence of pH on binding degrees

pH is a critical factor affecting the molecular state of both biopolymers and drugs especially those ionic compounds. When the solution pH is higher than the isoelectric point of HSA (pH 4.0), HSA bears negative charge. Fig. 4 showed how binding degrees of Rhizoma chuanxiong were influenced with pH.

The binding degree of ferulic acid was 23.0% at pH 7.4, 12.1% at pH 6.0 and 11.5% at pH 8.9. When the concentration of HSA was 0.3 mM. We attribute this to the changes in the structure of the binding site of HSA with pH and also the ionic state of ferulic acid.
Fig. 4. Binding degrees of components in Rhizoma Chuanxiong extract react with HSA under different pH. The left bar represents the binding degree of Rhizoma Chuanxiong with 0.3 mM HSA at pH 7.4. The middle bar represents the binding degree of Rhizoma Chuanxiong with HSA at pH 6.0. The right bar represents the Rhizoma Chuanxiong binding with 0.3 mM HSA at pH 8.9.

Binding of 3-butylphthalide to HSA were observed to decrease at pH 6.0 and 8.9. The reason for this is the same as that for ferulic acid, because 3-butylphthalide may be binding at the same binding sites with ferulic acid.

In Fig. 4, binding degrees of other peaks were changed at different pH. We can conclude that pH influences the interaction between compounds in Rhizoma Chuanxiong and HSA as a result of changes in the binding site structures of HSA and molecular state of active compounds.

3.5. Interaction of Rhizoma Chuanxiong with human blood serum

Blood contains nearly 300 different proteins. In China and Japan, a major method of studying the principle of Chinese medicine lies with the pharmacology of blood serum. As there are multiple binding sites of drugs in blood serum, the binding of Chinese medicine with blood serum reveals the whole drug-protein interaction not the single interaction of one drug to one protein.

3.5.1. Binding at pH 7.4

The results of binding of Rhizoma Chuanxiong to blood serum at pH 7.4 were shown in Figs. 5 and 6. The binding degree of ferulic acid was 33.3% in 1:1 blood serum (i.e. 1:1 blood serum that has been diluted 1:1 with phosphate buffer pH 7.4, containing 0.5 M NaCl). However, in whole blood serum, it became 47.9%. Comparing these data with binding to HSA, it could be seen that binding to blood serum was more stronger. Presumably, this is because there are also other proteins like α1-acid polyprotein, IgG, etc., in blood serum.

Binding degrees of senkyunolide A was 9.1% with 1:1 blood serum, and 13.7% with whole blood serum. Again, these values were all higher than binding to HSA. Similar results were observed for 3-butylphthalalide, for which binding degrees were 56.8 and 55.7%, respectively.

All other compounds in Rhizoma Chuanxiong bound to blood serum were more stronger than to HSA except for the peak E. Binding degrees of E to HSA were 51.5 and 51.6% for HSA concentration was 0.3 and 0.6 mM, while binding to 1:1 blood serum and whole blood serum were 49.2 and 50.5%. The same binding degree means that the compounds of peak M would bind only to HSA and not to other proteins in blood serum.

3.5.2. Influence of pH on binding degrees

Results of the binding of Rhizoma Chuanxiong to blood serum at different pH were shown in Fig. 7. This shows that binding degree of ferulic acid decreased at pH 6.0 and 8.9, as seen with HSA. Possibly this implies that HSA is the major binding site of ferulic acid in blood serum.

A similar trend of decreased binding was observed for 3-butylphthalalide. However, for 3-butyphthalide binding degree to 1:1 blood serum was 58.6% at pH 7.4, 25.5% at pH 6.0 and 23.4% at pH 8.9. For binding to HSA, the
Fig. 7. Binding degrees of components in Rhizoma Chuanxiong extract react with human blood serum under different pH. The left bar represents the binding degree of Rhizoma Chuanxiong with 1:1 human blood serum at pH 7.4. The middle bar represents the binding degree of Rhizoma Chuanxiong with 1:1 human blood serum, at pH 6.0. The light bar represents the Rhizoma Chuanxiong binding with 1:1 human blood serum at pH 8.9.

results were 27.7, 2.9 and 6.5% to 0.3 mM HSA, respectively. Thus, binding degrees on blood serum were not decreased the same extent to HSA. Presumably, there are other binding sites on blood serum for 3-butylphthalide and binding was obviously influenced by the different pH.

4. Conclusion

In this experiment microdialysis-HPLC was successfully used for studying the interaction of Chinese Herb Rhizoma Chuanxiong and protein. It shows that there are some suitable binding degrees on HSA for bioactive components ferulic acid and 3-butylphthalide, and the peaks I and M have similar binding degrees to HSA and human blood serum as these two compounds. It means this method can be used for the screening of bioactive components from Chinese Herb extracts. Further applications of microdialysis combined with HPLC/HPLC–MS to screen Chinese medicines and to the study of biological interactions of bioactive compounds from Chinese medicines with appropriate targets will be carried out in the near future.

References