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Fractionation and analysis of *Artemisia capillaris* Thunb. by affinity chromatography with human serum albumin as stationary phase

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Abstract

A method for the screening and analysis of biologically active compounds in traditional Chinese medicine is proposed. Affinity chromatography using a human serum albumin (HSA) stationary phase was applied to separate and analyze the bioactive compounds from *Artemisia capillaris* Thunb. Five major peaks and several minor peaks were resolved based on their affinity to HSA, two of them were identified as scoparone (SCO, 6,7-dimethoxycoumarin) and capillarisin (CAP). CAP shows a much higher affinity to HSA than SCO. The effects of acetonitrile concentration, eluent pH, phosphate concentration and temperature on the retention behaviors of several major active components were also investigated, and it was found that hydrophobicity and eluent pH play major roles in changing retention values. The results demonstrate that the affinity chromatography with a HSA stationary phase is an effective way for analyzing and screening biologically active compounds in traditional Chinese medicine.

Keywords: *Artemisia capillaris*; Scoparone; Capillarisin

1. Introduction

There is a long history for using traditional Chinese medicines (TCMs) to treat various diseases successfully. Based on thousands of years experience the theory behind TCMs has been gradually formed and improved, and the application of these drugs is becoming popular. Herbal drugs and natural products of plant origin are also widely used over all the world in other countries such as Japan, Korea and India. These drugs are usually complex mixtures containing up to hundreds or even thousands of different constituents, but only a few of the compounds are responsible for the pharmaceutical and/or toxic effects. Because the therapeutic principle of TCMs is completely different from that of synthetic medicines, TCMs could service human health in another way. TCMs contain large amount of proteins, sugars, mucilage and tannin in addition to their bioactive components, which makes the isolation and measurement of the active constituents as well as quality control of crude drugs and their medical preparations extremely difficult.

In a few cases, the biologically active compounds in TCMs are known, therefore, quantitative determination of the effective or principal constituents is crucial to quality control. Much attention has been drawn to the development of chromatographic methods in this field and a great number of such papers have been published [1–3]. Separation of TCMs by conventional chromatography such as gas chroma-

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tography (GC), reversed-phase high-performance liquid chromatography (RP-HPLC) and normal-phase (NP) HPLC is always based on the physico–chemical interactions between the known biologically active compounds and the mobile phase and stationary phase. There is no correlation between their retention and bioactivities.

In most cases, the biologically active compounds in TCMs which are responsible for the pharmaceutical effects are not known or only partially known, therefore, the key step for developing a rational method of quality control is to screen and determine the bioactive compounds. A conventional procedure for finding bioactive components is extraction of TCMs followed by pharmacological screening of the purified compounds. The way to increase the probability of success is still controversial, relegating most workers to trial-and-error experiments. Homma and co-workers introduced the concept of pharmacokinetics [4,5], despite the method is effective but laborious.

Affinity chromatography is based on the biological interactions between biologically active compounds and protein, enzymes and antibodies. It has been successfully applied to rapidly probe drug–protein binding and to study anticooperative, noncooperative and cooperative protein–ligand interactions [6–13]. Most synthetic drugs bind, more or less, to plasma protein [14–17]. Albumin is the most abundant plasma protein and often accounts for the entire drug binding in plasma. Binding to albumin can have physiological significance in the transport, modulation and inactivation of metabolites and drug activities, as well as serve as a protective device in the binding and inactivation of potential toxic compounds to which the body is exposed. The majority of drugs bind to serum albumin quantitatively. Since drug binding to albumin is readily reversible, the albumin–drug complex serves as a circulating drug reservoir that releases more drug as the free drug is biotransformed or excreted. Albumin binding thus decreases the maximum intensity but increases the duration of action of many drugs. But up to now, there has been no reported study of TCMs or natural products by the affinity chromatography. Therefore, it is valuable to study the possibility for screening and analysis of the biologically active components in TCMs by the affinity chromatography through their biological interaction with immobilized human serum albumin (HSA). For *Angelica sinensis* (Oliv.) Diels, two principal peaks identified as ferulic acid and ligustilide are the principal biological active components [18]. Based on these findings, the quantitative determination of ferulic acid and ligustilide by affinity chromatography has been developed for quality control of medicine from *Angelica sinensis* (Oliv.) Diels.

In the present work, we use affinity chromatography with a HSA stationary phase to directly separate and analyze biologically active components in the *Artemisia capillaris* Thunb., a TCM that has been used to cure hepatitis A for a long period of time.

2. Experimental

2.1. Reagents and materials

Acetonitrile and methanol are of chromatographic grade, and distilled water was further purified with a Milli-Q ultrapure water system (Millipore, Milford, MA, USA). Scoparone (SCO) and capillarisin (CAP) standards were kind gift of Dr. Ban Yundong (Shenyang Pharmaceutical University, Shenyang, China). Other chemicals were of analytical grade.

2.2. Preparation of A. capillaris extract

The flowers of *A. capillaris* were collected in autumn at Donglin of Shengyang City, China, and identified by Dr. Ban Yundong at Shengyang Pharmaceutical University. The collected materials were crushed with a grinder, 0.1993 g was extracted in 30 ml methanol (HPLC grade) for 30 min, and the extract evaporated dry using N_2 gas. Methanol (0.3 ml) was added to the residue and the sample centrifuged at 3000 rpm for 5 min. The supernatant was then stored at 4°C in the dark for subsequent experiments.

2.3. Apparatus and instruments

The HPLC system comprises a LC-9A pump (Shimadzu, Kyoto, Japan), a Rheodyne-type injection valve with a 20-μl loop, a SPD-10AV UV

detector (Shimadzu), and a WDL-95 chromatographic workstation (National Chromatographic R&A Centre, Dalian, China). A column of 150×4.6 mm I.D., packed with HSA immobilized silica having a particle size of 7 μm (Hypersil, UK), was used to perform affinity chromatography. The column temperature was controlled by a temperature-regulating jacket (Elite, Dalian, China), and maintained at 35±0.1°C unless otherwise stated.

2.4. Chromatographic conditions

The mobile phases employed in this study were based on mixing potassium phosphate buffer with varying concentrations of acetonitrile. The flow-rate was 0.8 ml/min. All mobile phases were filtered through a 0.45-μm membrane and degassed by ultrasonication immediately prior to use. Detection wavelength was set to 238 nm.

2.5. Optimization of chromatographic conditions

The effects of gradient elution, concentrations of acetonitrile and phosphate buffer, the eluent pH and column temperature on the retention behaviors of principal active components in affinity chromatography were investigated. The chromatographic capacity factor (k') is calculated by following equation:

$$k' = (t_r - t_0)/t_0 \quad (1)$$

where t_r is the retention time of the solute of interest and t_0 is that of an unretained marker of water.

3. Results and discussion

3.1. Primarily separation of bioactive compounds in A. capillaris

The retention behaviors of the solutes on HSA immobilized silica packed column are influenced by the type and concentration of organic solvents, ionic strength, pH of phosphate buffer and temperature; so the selection of chromatographic conditions is very important in the process of separation and screening of TCMs. The column temperature and the pH of phosphate buffer selected for initial screening were near physiological conditions of 35°C and pH 7.4, respectively. The organic solvents usually used as the mobile phase for HSA immobilized silica stationary phase included *n*-propanol, isopropanol and acetonitrile, the maximum concentrations of *n*-propanol and isopropanol being 6% and that of acetonitrile being about 15%. As acetonitrile seems highly compatible with HSA, it was selected as the mobile phase. The concentration of phosphate buffer was set to 10 m*M* for the initial study.

A few of the active components, mainly coumarins and chromones, have been found in *A. capillaris*, and the difference in their physico–chemical properties suggested that gradient elution procedure was most appropriate for initial screening. Fig. 1 shows the separation of biologically active components in *A. capillaris* under gradient elution, with six principal peaks including SCO, S2, S3, S4, CAP, C1 and C2, and more than 10 minor peaks. Peaks of SCO and CAP were identified with standard compounds as scoparone and capillarisine, respectively. The structures of them were illustrated in Fig. 2. The mechanism of protein–drug binding is complicated, with at least four types of noncovalent forces involved: ionic interactions, hydrogen bonding, hydrophobic interactions and Van der Waals force. Steric match also plays important role in this process.

3.2. Effect of acetonitrile concentration

The effect of acetonitrile concentrations from 0.0 to 15% (v/v) on the retention of the principal components was examined by keeping the phosphate buffer concentration at 10 m*M* (pH 7.4) and the column temperature at 35±0.1°C. It can evidently be observed that retention times of all these components are gradually reduced with increasing concentration of acetonitrile as shown in Fig. 3, which is very similar to the pattern observed in the reversed-phase liquid chromatography (LC). It is well known that the retention of solutes in reversed-phase LC can be simply expressed as follows [19]:

$$\ln k' = a + cC_B \quad (2)$$

where C_B is the volume fraction of organic modifier in the mobile phase, k' is the capacity factor, a and c

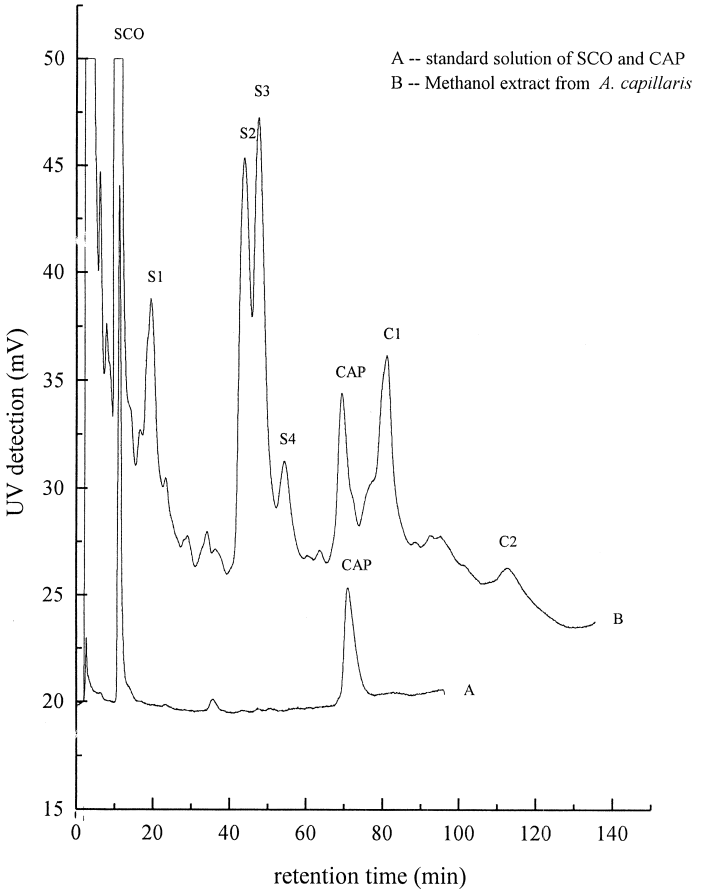
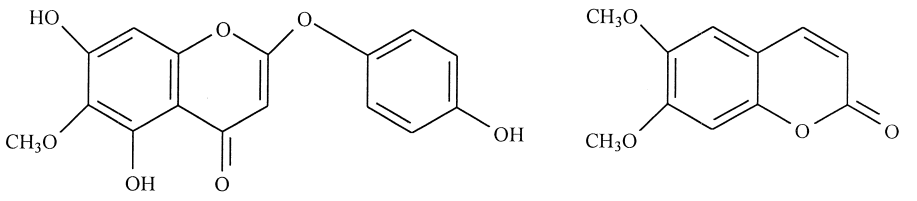


Fig. 1. Chromatogram for primary screening of methanol extract from *A. capillaris* on affinity chromatography column. Experimental conditions: the column used was of 150×4.6 mm I.D. packed with HSA immobilized on silica (7 μm), column temperature was 35°C, flow-rate was 0.8 ml/min, and UV detection wavelength was set to 238 nm. Elution was carried out with linear gradient from 10 m*M* phosphate buffer (pH 7.4) to 15% acetonitrile in 10 m*M* phosphate buffer (pH 7.4) in 90 min, and the latter eluent was ran for another 60 min.



Capillarisin

2-(p-hydroxyphenoxy)-6-methoxy-5,7-dihydroxy-chromone

Scoparone

6, 7-dimethoxy-coumarin

Fig. 2. Molecular structures of capillarisin and scoparone.

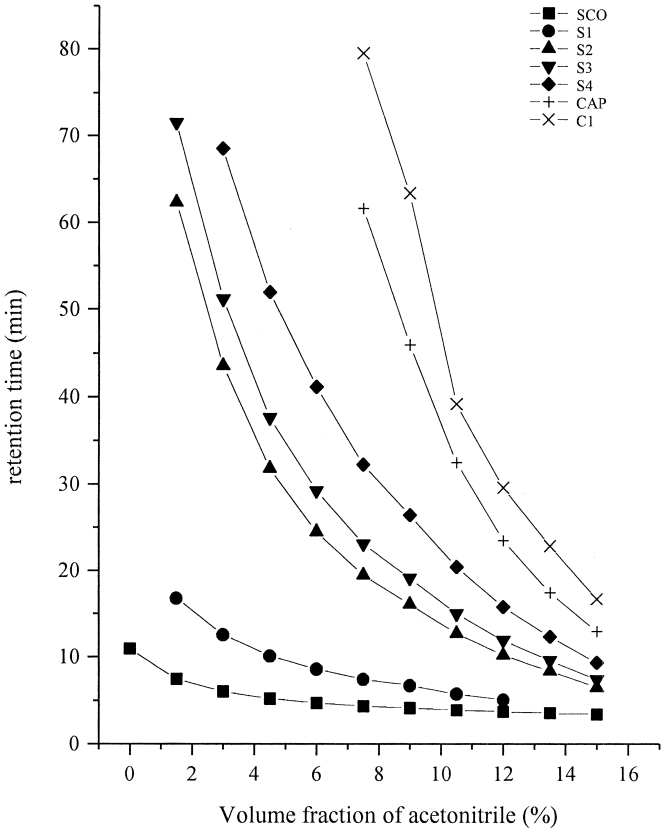


Fig. 3. Effect of acetonitrile on the retention behavior of principal components in *A. capillaris*.

are coefficients related with physico–chemical properties of the stationary and mobile phases. Eq. (2) was used to determine the values of *a*, *c* and the correlation coefficients for the six principal compounds (Table 1). The correlation coefficients of all compounds are above 0.99 with the exception of SCO at about 0.96. In this study, we have also found that all minor peaks, which could be traced from their position to other adjacent peaks, show excellent linear correlation (data not shown). It is apparent that Eq. (2) can well describe the effects of acetonitrile on the retention behaviors of the solutes in affinity

Table 1
The values of *a*, *c* and correlation coefficient for the elution of principal components in *A. capillaris* as described by Eq. (2) (for experimental conditions see text)

Solute	*a*	*c*	Linear regression correlation[a]
SCO	0.92±0.10	−12.56±1.09	$r=-0.9679$, $P=1.1\cdot10^{-6}$, $n=11$
S1	1.94±0.05	−15.17±0.64	$r=-0.9948$, $P=3.5\cdot10^{-7}$, $n=8$
S2	3.43±0.04	−18.84±0.39	$r=-0.9983$, $P=3.9\cdot10^{-11}$, $n=10$
S3	3.59±0.03	−18.56±0.34	$r=-0.9987$, $P=1.4\cdot10^{-11}$, $n=10$
S4	3.90±0.04	−18.28±0.40	$r=-0.9983$, $P=6.8\cdot10^{-10}$, $n=9$
CAP	4.97±0.03	−23.17±0.24	$r=-0.9998$, $P=7.3\cdot10^{-8}$, $n=6$
C1	5.22±0.11	−22.84±0.99	$r=-0.9962$, $P=2.0\cdot10^{-5}$, $n=6$

[a] r represents sample correlation coefficient.

chromatography suggesting that the hydrophobic force is one of the important driving forces for protein–drug binding.

3.3. Effects of the eluent ionic strength and pH value

Biochromatography at different phosphate concentration at pH 7.4, 35±0.1°C and 15% acetonitrile was performed to investigate the effects of inorganic salt on the retention of principal active components. When examining the effect of the eluent pH, the concentration of phosphate buffer was kept at 10 m*M* and the eluent pH values were adjusted from 5.5 to 7.4. More extreme eluent pH values may eliminate the activity of the immobilized ligand.

The effect of ionic strength on the retention of bioactive compounds in affinity chromatography is shown in Fig. 4. It can be seen that the retention times of most solutes, such as SCO, S2, S3, S4, CAP and C1, increased very slightly with the concentration of phosphate buffer. This can probably be explained by an increase of the surface tension of the mobile phase with increasing concentration of inorganic salt, which promote the hydrophobic interaction between the solute and the stationary phase, thereby increasing the retention of the solute. However, a contrary phenomenon was observed for a high affinity component as the retention time of C2 decreased substantially with concentration of phosphate buffer. This phenomenon may be demonstrating a role for electrostatic interaction in drug–protein binding involving C2. When there was no inorganic salt in the mobile phase of acetonitrile–water (15:85), most peaks eluted from the column at the void volume(data not shown). We may hypoth-

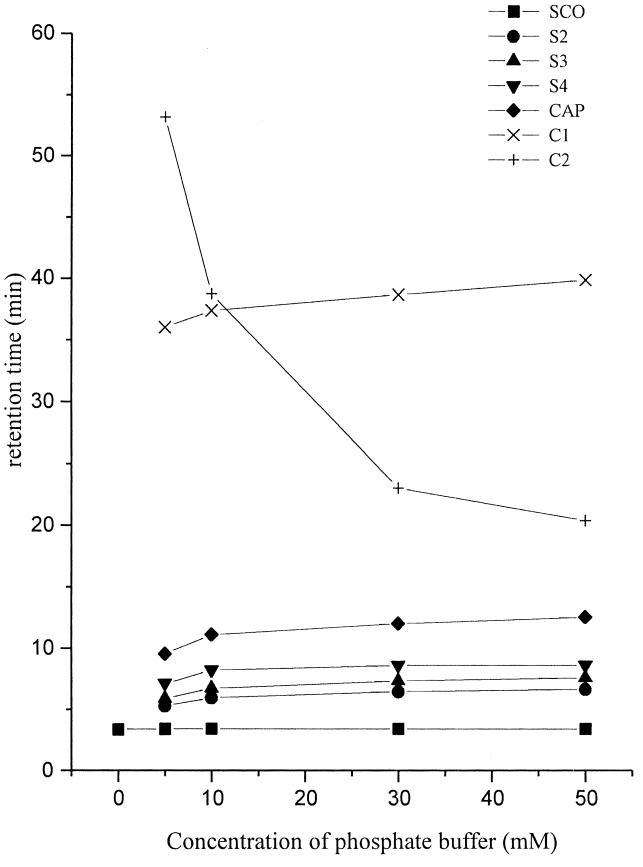


Fig. 4. Effect of the eluent ionic strength on the retention behaviors of some components in *A. capillaris*.

esize that the presence of inorganic salt in mobile phase might have a role in maintaining the native conformation of HSA during chromatography.

As shown in Fig. 5, the eluent pH has great influence on the retention and selectivity of the solutes in affinity chromatography. The retention of S2, S3, S4 and CAP gradually increased with a decrease in eluent pH. For example, S2 and S3 were well separated, but CAP and S3 overlapped when eluent pH was at 5.5. The change in elution order of certain solutes was verified using the CAP standard. The overall results show that as eluent pH of 6.0 is suitable for good separation of principal components in *A. capillaris*.

3.4. Effect of column temperature

Separations of *A. capillaris* at different column temperatures, from 25°C to 45°C in 15% acetonitrile and 10 m*M* phosphate buffer (pH 6.0) were performed. Fig. 6 plots ln k' of principal components against the reciprocal of absolute temperature, while the ΔH of these components under these experimental conditions could be calculated according to the van 't Hoff equation:

$$\ln k' = A + \frac{\Delta H}{RT} \tag{3}$$

where R is the ideal gas constant, T is absolute temperature, ΔH is the enthalpy change of the solutes and A is a coefficient relating with the entropy change of the solutes and the phase ratio. The calculated ΔH values are listed in Table 2. The enthalpy changes (ΔH) of protein binding with S2, S3, S4, CAP, C1 and C2 were above 23 kJ/mol, and thus demonstrates strong noncovalent interaction of those components with HSA. It is evident that retention and selectivity of these components in-

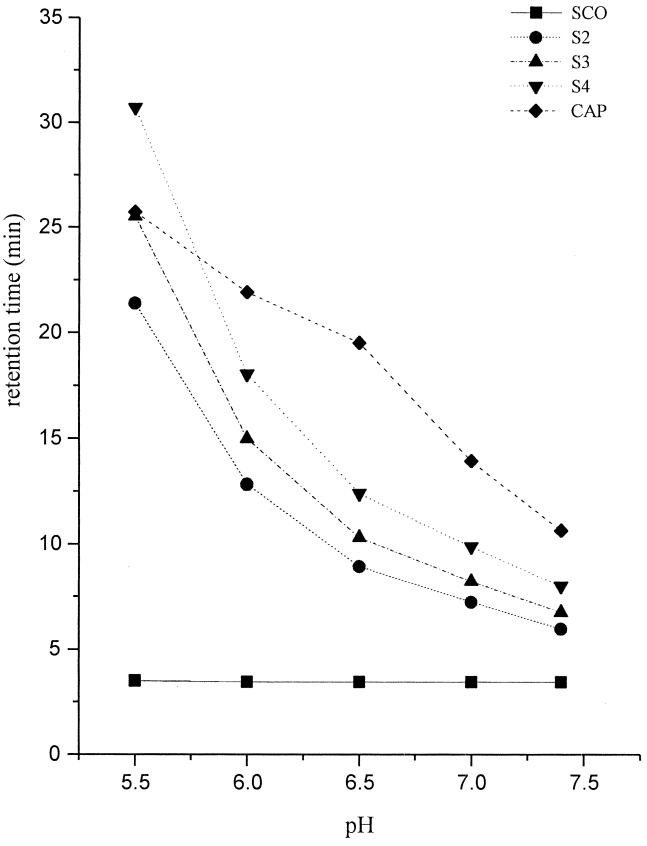


Fig. 5. Effect of the eluent pH values on the retention behaviors of principal components in *A. capillaris*.

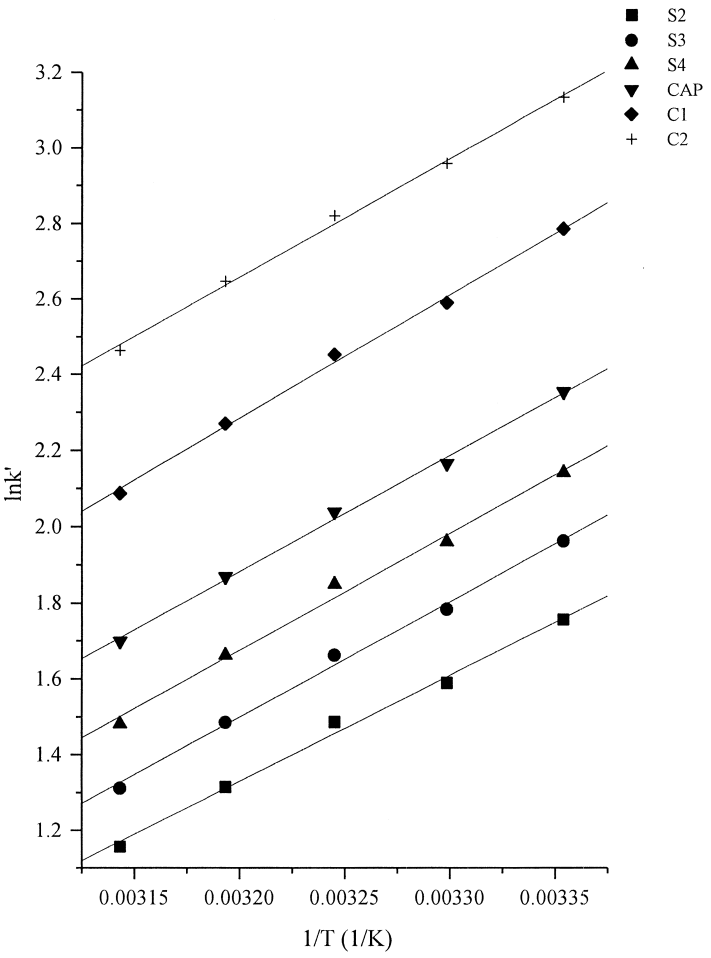


Fig. 6. The Van 't Hoff plot of some principal components in *A. capillaris*.

creased with decreasing column temperature, Column temperature above ambient temperature can be used to reduce the time required for each analysis, although this may comprise selectivity and lifetime of the column.

3.5. Analysis of SCO and CAP in A. capillaris

Conditions for gradient elution were designed according to the above results for the separation and analysis of SCO and CAP. The chromatogram for the

Table 2
Enthalpy change for binding of principal components in *A. capillaris* to HSA[a]

	Sample No.					
	S2	S3	S4	CAP	C1	C2
ΔH (kJ/mol)	23.3±1.1	25.3±1.0	25.5±1.3	25.3±0.8	27.2±0.9	26.1±0.9

[a] Experimental conditions: the mobile phase is 15% acetonitrile in 10 m*M* phosphate buffer (pH 6.0); column temperature is varied between 25 and 45°C.

analysis of the methanol extract from *A. capillaris* under these conditions is shown in Fig. 7. The peaks of S4 and CAP show asymmetry, and similar phenomenon have been observed on some strongly retained compounds as tanshinones from *Salvia miltiorrhiza* Bge., but the reason for peak tailing is not yet clear. One reason may be from the damage of column performance after a long use. The principal components are resolved within 60 min. Comparing the C1 peak shown in Fig. 7 with that in Fig. 1, it became smaller. Apparently it was interfered with by other peaks in Fig. 1, which made the peak look bigger. If this interference is discounted from C1 in Fig. 1, the peak area ratio of CAP, C1 and C2 is 1:1.01:0.403, and that in Fig. 7 is about 1:0.98:0.410, the two values agree well. Therefore, it could be said that a better separation for C1 was achieved under present conditions. The amounts of SCO and CAP determined in *A. capillaris* by this method were as much as 1.2% and 0.05% (w/w), respectively.

The retention, thus the affinity, of the solutes on HSA stationary phase was in the increasing order of

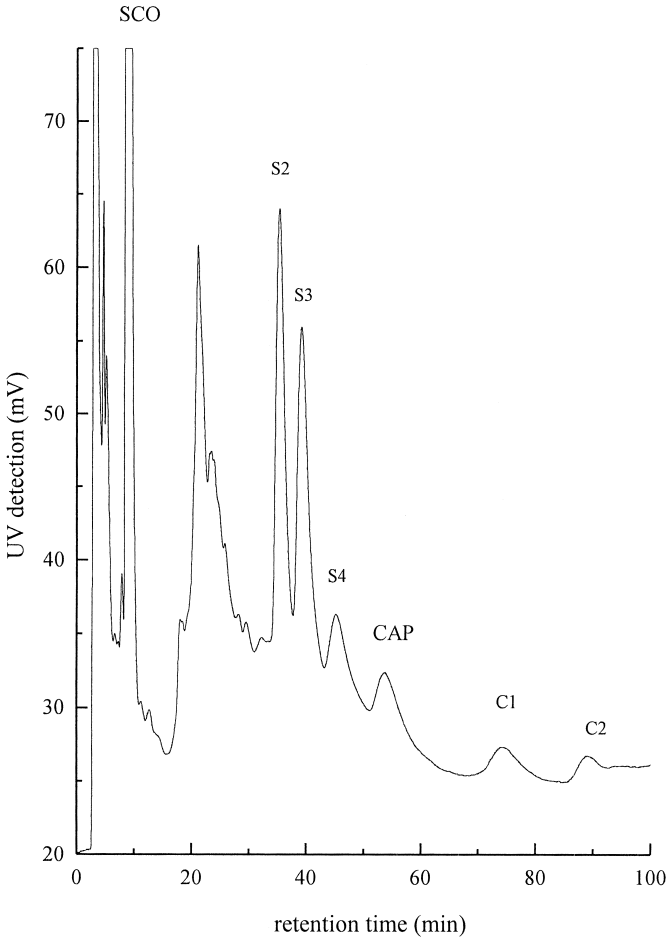


Fig. 7. Chromatogram for the analysis of the methanol extract from *A. capillaris* under optimized conditions. Experimental conditions: 10 min isocratic elution with mobile phase of 1.5% acetonitrile in 10 m*M* phosphate buffer (pH 6.0); then 5 min linear gradient elution from 1.5% to 12% acetonitrile in 10 m*M* phosphate buffer (pH 6.0) with the elution of the latter mobile phase kept for an additional 45 min; and finally another 45 min linear gradient elution from 12% acetonitrile in 10 m*M* phosphate buffer (pH 6.0) to 15% acetonitrile in 10 m*M* phosphate buffer (pH 7.4). Other conditions as in Fig. 1.

SCO, S1, S2, S3, S4 and CAP. This order was not influenced by organic modifier and ionic strength, but was changed by the pH. The identity of the various unknown compounds in *A. capillaris* could be determined using affinity chromatography in combination with mass spectrometry, diode array detection or nuclear magnetic resonance (NMR).

Application of affinity chromatography to studies of TCMs and natural products have significant advantages. First, the interactions of biologically active compounds with proteins, enzymes and DNA can be probed, even if these compounds have not yet been identified. Second, the biologically active components and their biochemical change during the process of Chinese medicines can be rapidly evaluated and monitored. Third, the interactions occurring between biologically active compounds can be studied by adding some effective components screened from Chinese remedies or endogenous compounds to the mobile phase. According to the theory of TCMs, at least a few biologically active components may act together in curing the disease. Therefore, it might be expected that affinity chromatography should play important role in disclosing the mystery of TCMs.

4. Conclusion

HSA stationary phase based affinity chromatography was successfully and rapidly used for analyzing two principal bioactive components, SCO and CAP, from *A. capillaris*. CAP shows much higher affinity to HSA than SCO. This study also demonstrates that the mechanism of drug–protein binding is complicated, involving hydrophobic effect, hydrogen bonding, Van der Waals forces, electrostatic interaction and steric match. Further applications of affinity chromatography for screening and analyzing TCMs and to study interaction of biologically active compounds with proteins will be carried out in the near future.

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