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Separation of compounds interacting with liposome membrane in combined prescription of traditional Chinese medicines with immobilized liposome chromatography

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Abstract

Immobilized liposome chromatography (ILC), the stationary phase of which has been regarded as a mimic biomembranes system was used to separate and analyze compounds interacting with liposome membrane in Danggui Buxue decoction, a combined prescription of traditional Chinese medicines (CPTCMs), and its compositions Radix Astragli and Radix Angelica Sinensis. More than 10 main peaks in the extract of Danggui Buxue decoction were resolved on the ILC column, suggesting that more than 10 components in the prescription have significant retention on ILC column. Ligustilide, astragaloside IV and formononetin, three main bioactive ingredients in Danggui Buxue decoction, were found to have relatively significant, while ferulic acid, another bioactive ingredient in the prescription, relatively weak retention on ILC column. Effects of the eluent pH and amount of immobilized phosphatidylcholine (PC) on separation of interactional compounds in the extract of Danggui Buxue decoction were also investigated. It was found that these two factors strongly affected the retention of some interactional compounds. In addition, the fractions partitioned with different solvents from water extract of this combined prescription were evaluated with this ILC column system.

Keywords: Immobilized liposome chromatography; Compounds interacting with liposome membranes; Danggui Buxue decoction; Radix Astragli; Radix Angelica Sinensis

1. Introduction

In traditional Chinese medicine (TCM) practice, the majority of herbal medicines are often prescribed in the form of mixtures called combined prescription of traditional Chinese medicines (CPTCMs) based on the TCM theoretical philosophy. Many strategies have been employed to investigate the bioactive components of the CPTCMs. For example, serum pharmacological [1] and high performance chromatographic methods [2] may be useful techniques for pharmacodynamics and chemical studies of CPTCMs. However, it is always a difficult task to study the absorption behaviors of CPTCMs, as CPTCMs often contain hundreds of known and unknown compounds.

Danggui Buxue decocotion, discovered historically by Dongyuan Li of the Jingyuan Dynasty of China, is a combined prescription of Radix Astragli:Radix Angelica Sinensis (5:1), and has been used to treat all kinds of ischemia.

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It was reported that the main bioactive ingredients of the combined prescription are polysaccharides, flavonoids (e.g. formononetin), phthtalides (e.g. ligustilide), saponins (e.g. astragaloside IV) and phenolics (e.g. ferulic acid) [3–7]. Although there have been many research papers on the clinical application, pharmacology and chemistry of the combined prescription, research has barely focus on its absorption.

Immobilized liposome chromatography (ILC) is regarded as a powerful tool to study drug–membrane interactions in vitro [8,9]. Liposomes [10–12] formed by phosphatidylcholine (PC), the main components found in cell membrane, or unilaminar phospholipids [13–16], were non-covalently or covalently immobilized on soft gel or porous silica stationary phases to probe the penetration ability of compounds through biological membranes, which has been considered as important parameters to evaluate their bioactivity [17,18]. Initial studies indicated that a good correlation between ILC and intestinal mucosa model was obtained for a series of drugs [19–22]. Previously, we investigated the application of ILC in separation and analysis of permeable components in single Chinese herb [23]. In this paper, we attempt to extend ILC to the separation and analysis of interactional components in more complex CPTCMs.

2. Experimental

2.1. Apparatus and instruments

The HPLC system is consisted of a 515 HPLC pump (Waters, Milford, USA) equipped with a Rheodyne injection valve with a 10 μl sample loop, a Waters 2487 dual λ absorbance detector (Waters, Milford, USA) and WDL-95 Work-station (Dalian Institute of Chemical Physics, Chinese Academy of Sciences, China). Distilled water was further purified with a water purification system (Millipore, Molsheim, France) for all experiments.

2.2. Reagents and materials

Silica gel (5 μm, 300 Å) from Chrom Expert (Sacramento, CA, USA), Phosphatidylcholine (PC) from Shanghai Chemical Reagents (Shanghai, China) were ordered. Ferucid acid and astragaloside IV were obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Radix Angelica Sinensis from Longgang Ltd. Corp. (Lanzhou, China), Radix Astragli from a local drug store were purchased. Formononetin was isolated and identified from Radix Astragli in our laboratory. Buffer A (pH 7.4) is 10 mM sodium phosphate buffer containing 50 mM NaCl. All other chemicals were analytical grade. All solvents and samples were filtered through 0.45 μm nylon membrane filter before use.

2.3. Preparation of ILC column

Preparations of the immobilized liposome columns have been reported elsewhere [23,24]. Briefly, PC (0.1–1 g) was dissolved in chloroform and 2–16 g silica gel was added to the solution, shaken for 30 min, and the solvent was removed in a round-bottomed flask by rotary evaporation, and then the dried silica was kept under high vacuum overnight to remove the remaining solvent. The PC film coated porous silica gel was swollen in buffer A for 2–4 h to form liposome, and then washed three times with buffer A to remove free and loosely coated liposome. Liposome immobilized stationary phase prepared was packed into a stainless steel column with the dimension of 50 mm × 4.6 mm i.d. using the slurry packing method in our laboratory.

2.4. Sample preparation

The sample pretreatment of CPTCMs is an important process affecting the results in chromatographic analysis. A sample prepared by the routine method of water decoction, which is in accordance with the method used in clinical application, will result in detectable issues for some of the compounds, as solubility of those components in water is very low. Thus, a certain concentration of ethanol is often added to the preparations to ensure the solubility of those components [25]. In this study, two extraction methods, refluxing with 75% ethanol and partitioning from water extracts with organic solvents of different polarity, were introduced.

- *Extract A*: Twenty-four grams mixed powder of Radix Astragli:Radix Angelica Sinensis (5:1) was immersed in 192 ml of 75% ethanol for 1 h and was refluxed for 1 h, and then the solvent was removed with rotary evaporation at 40 °C under vacuum; the residue was dissolved in 10 ml methanol, and then the solution was filtered through a 0.45 μm membrane, and the filtrate was used as a sample for ILC analysis.
- *Extract B*: Twenty grams powder of Radix Astragli was treated as the procedures for the Extract A.
- *Extract C*: Four grams powder of Radix Angelica Sinensis was treated as the procedures for the Extract A.
- *Fractions A-E*: One hundred and twenty grams mixed powder of Radix Astragli and Radix Angelica Sinensis (5:1) was immersed in 1200 ml of water for 1 h and was boiled for 1 h. The extraction was repeated two times. The combined solution was concentrated to about 200 ml with a rotary evaporator at 40 °C under vacuum. Then the residue was successively extracted with 200 ml of petroleum ether, ethyl ether, ethyl acetate and *n*-butanol saturated with water four times, respectively. Solvents from each fraction were removed with a rotary evaporator at 30–40 °C under vacuum, and the residues were respectively dissolved in 10, 15, 20 and 30 ml of methanol to obtain Fractions A-D corresponding to solvents of petroleum ether, ethyl ether,

ethyl acetate and *n*-butanol, respectively. The remaining water solution was referred to Fraction E. All samples were filtered through a 0.45 μm filter membrane for ILC analysis.

2.5. Chromatographic conditions

The mobile phases used in the ILC experiment were 10 mM sodium phosphate buffer (pH 7.4) containing 50 mM NaCl. The flow-rate of mobile phase was at 1.0 ml/min and the UV detection wavelength was set at 210 nm.

2.6. Specific capacity factor

The specific capacity factor (K_s) of a compound on ILC column was calculated by the following equation [8,20]

$$K_s = \frac{V_R - V_0}{A},$$

where V_R is the retention volume of the compound on ILC column; V_0 the void volume of a small and hydrophilic reference molecule ($NaNO_3$); and A is the amount of immobilized PC on ILC column, which was determined using the same method as in the reference [26].

3. Results and discussion

3.1. Separation of compounds interacting with liposome membrane in Danggui Buxue decoction and its compositions

ILC cannot only simulate the interactions of drugs with biological membranes resulting from a combination of hydrophobic, ion pairing and hydrogen bonding occurring simultaneously, but also separate different kinds of compounds in a complex mixture, especially for complex traditional Chinese medicines in which many ingredients have not yet been elucidated [23]. Therefore, ILC is a good chromatographic model to predict and evaluate the interaction of the components in TCMs with biological membrane.

Typical chromatograms for the extracts of Danggui Buxue decoction and its compositions Radix Astragli and Radix Angelica Sinensis on ILC under the present conditions are shown in Fig. 1. It was found that different TCMs had different chromatographic patterns on ILC. In all of the three chromatograms, the first peak is large and off-scale, they were eluted from the column near the void volume, indicating that most of components in Extracts A–C had weak interactions with the liposome. There are 14 main peaks for the extract

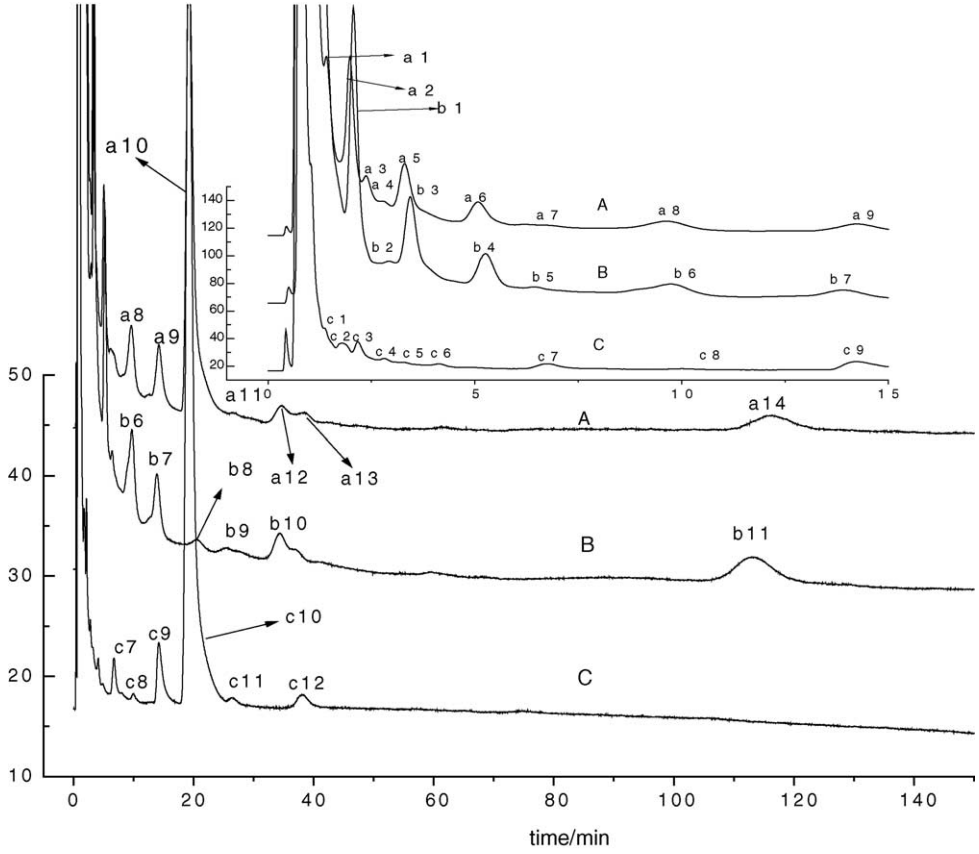

Fig. 1. Chromatograms of the extracts of Danggui Buxue decoction and its compositions. Experimental conditions: mobile phase, buffer A; flow-rate, 1 ml/min; detection wavelength, 210 nm. The inset shows an expansion of the first 15 min of the chromatograms. (A) Extracts of Danggui Buxue decoction, (B) Radix Astragli and (C) Radix Angelica Sinensis.

Table 1
Retention time and peak area of the main peaks in Danggui Buxue decoction and its compositions

Danggui Buxue decoction			Radix Astragli			Radix Angelica Sinensis		
Peaks[a]	t_R (min)	Peak area	Peaks[a]	t_R (min)	Peak area	Peaks[a]	t_R (min)	Peak area
a1	1.41	0.93				c1	1.36	0.07
a2	1.98	32.68	b1	2.06	88.15	c2	1.81	3.87
a3	2.37	1.97				c3	2.18	5.86
a4	2.76	0.29	b2	2.92	0.94	c4	2.81	0.71
a5	3.30	11.76	b3	3.44	53.79	c5	3.19	3.28
a6	5.07	20.65	b4	5.26	31.63	c6	4.13	6.18
a7	6.17	0.38	b5	6.43	0.86	c7	6.75	12.54
a8	9.62	9.25	b6	9.74	13.63	c8	10.00	3.44
a9	14.23	13.77	b7	13.86	12.45	c9	14.20	19.38
a10	19.26	144.1	b8	20.50	2.60	c10	19.23	364.6
a11	26.53	0.28	b9	26.53	1.64	c11	26.45	0.97
a12	34.56	5.94	b10	34.28	14.60			
a13	38.23	1.09				c12	38.10	7.72
a14	115.90	20.06	b11	112.57	49.64			

[a] Peaks indicated stand for the peaks in chromatograms shown in Fig. 1.

of Danggui Buxue decoction, 11 main peaks for the extracts of Radix Astragli and 12 main peaks for the extract of Radix Angelica Sinensis, respectively, with significant retention on the ILC column when detected at 210 nm. In addition to those principal peaks, there are also a number of minor peaks on three chromatograms. The longer the retention time is, the stronger its interaction with the ILC stationary phase is. All these reserved components are constituents capably interacting with liposome membrane, thus may be considered to be absorbed by the human body.

By comparing the three chromatograms, it can also be seen that all the main peaks on the chromatogram of Dang-

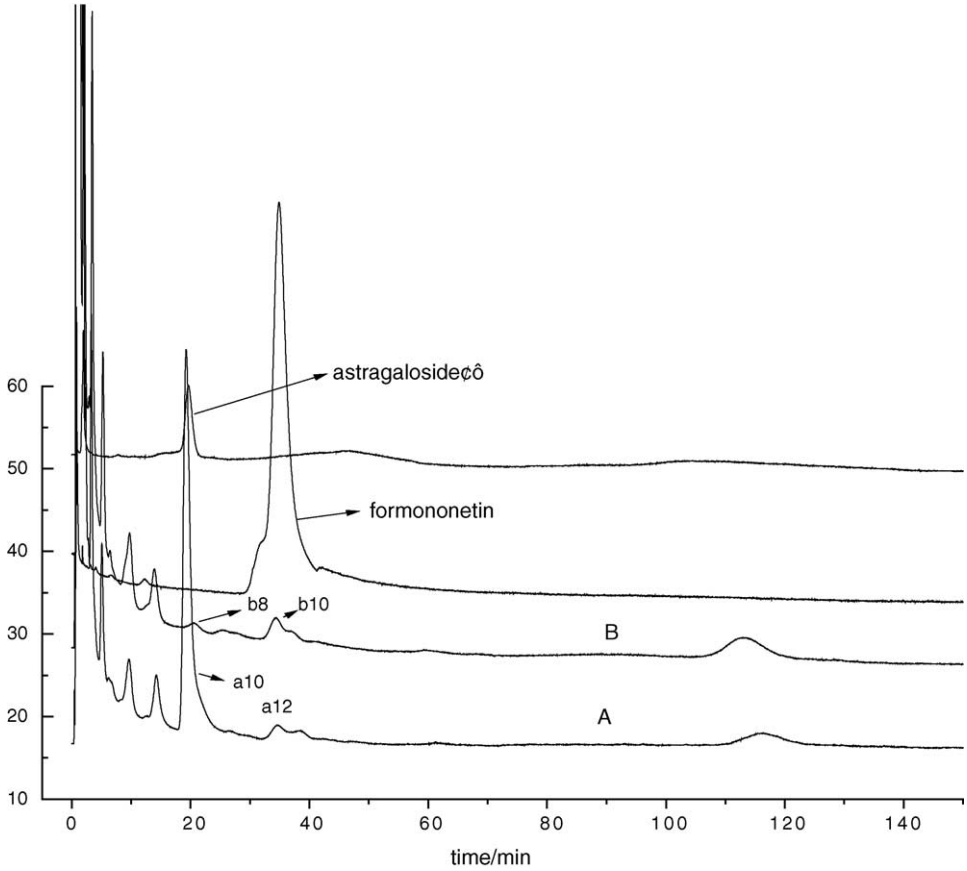

Fig. 2. Chromatograms of extracts of Danggui Buxue decoction, Radix Astragli and the reference compounds astragaloside IV and formononetin. (A) Extracts of Danggui Buxue decoction and (B) Radix Astragli. Experimental conditions are the same as those in Fig. 1.

gui Buxue decoction can be attributed to the peaks of Radix Astragli and Radix Angelica Sinensis according to their retention position and peak shape. The relative amounts of each compounds interacting with liposome membrane in the three extracts can be compared based on the peak area of each resolved peak on the three chromatograms. The retention times and peak areas for the main peaks detected in the three extracts are shown in Table 1. It was found that the peaks a1, a3 and a13 of the prescription extract were mainly contributed by c1, c3 and c12 of the extract of Radix Angelica Sinensis, and the peaks a12 and a14 were mainly contributed by b10 and b12 of the extract of Radix Astragli. However, the other nine peaks of the prescription extract might be contributed by the components from both of Radix Angelica Sinensis and Radix Astragli. There are no new peaks (components) detected in the extract of Danggui Buxue decoction, which are consistent with the results done with RP-HPLC-DAD analysis reported by Xiao et al. [2].

3.2. *Identification of astragaloside IV, formononetin, ferulic acid and ligustilide on ILC*

Astragaloside IV [27], formononetin [28], ferulic acid [29] and ligustilide [30] are the main bioactive ingredients in the combined prescription of Danggui Buxue, and the former two compounds only exist in Radix Astragli and the latter two compounds in Radix Angelica Sinensis, respectively. The quantity of them is often taken as the norm for quality control of Danggui Buxue decoction and its compositions [31–35]. Separation of standard samples of ferulic acid, astragaloside IV and formononetin on ILC was performed the chromatograms are shown in Fig. 2. It can be seen that there are strong retentions on ILC column for astragaloside IV and formononetin ($\log K_s$ = 2.5 and 2.9). Peaks b8 and b10 on the chromatogram of extract of Radix Astragli have the same retention as these two standards, thus can be identified as astragaloside IV and formononetin. But on the chromatogram of Danggui Buxue decoction, only peak a12 has the same retention as that of formononetin, and the peak of astragaloside IV is overlapped with another peak a10, which is considered as ligustilide according to the characteristic shape and peak area reported previously by our research group [23]. The chromatograms for standard ferulic acid and ligustilide are shown in Fig. 3. It can be seen that the interaction of ferulic acid with ILC stationary phase is weak under the present experimental conditions, but its retention increases with the decreasing of eluent pH value as shown in Fig. 3. The peak of ligustilide can be easily found in the chromatograms for the

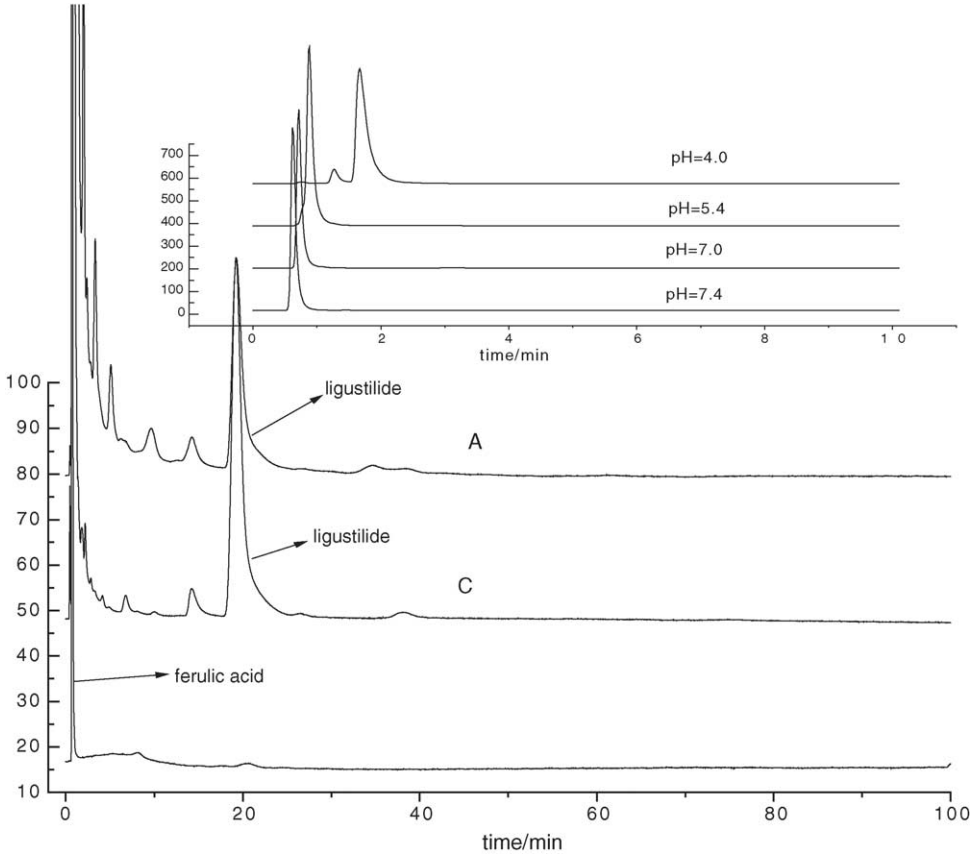

Fig. 3. Chromatograms for the extracts of Danggui Buxue decoction, Radix Angelica Sinensis and the reference compound ferulic acid. (A) Extracts of Danggui Buxue decoction and (B) Radix Angelica Sinensis. The inset shows the influence of the eluent pH on separation of ferulic acid on ILC. Experimental conditions are the same as those in Fig. 1.

extracts of Radix Angelica Sinensis and Danggui Buxue decoction, and its retention on ILC column is very strong with $\log K_s = 2.5$.

3.3. Effects of eluent pH value

Previous study in our laboratories [8,23] and Beigi et al. [20] indicated that the retention and selectivity of components interacting with liposome membrane on ILC column were affected by the eluent pH value. In this study, effect of the eluent pH value on the separation of the extract of Danggui Buxue decoction was investigated with the pH range from 4.0 to 7.4, and the representative chromatograms are shown in Fig. 4. It was found that the chromatogram patterns under different eluent pH values are obviously different from each other. For example, in addition to peaks 2–5, whose retention time are unchanged with the change of pH values, the retention of some peaks, such as peak 6 decreases with the decreasing of the eluent pH values, whereas the retention of other peaks such as ferulic acid increases in the forms of both pure compound (Fig. 3) and in the extract of Danggui Buxue decoction (Fig. 4). Besides, some peaks are separated with the change of eluent pH values. For example, peak 1 was resolved into two peaks (peak 1′ and peak 1″) when the eluent pH value changed from 5.4 to 4.0 (Fig. 4). It is evident that the eluent pH value plays an important role in the retention and separation selectivity for interactional solutes on the ILC column.

3.4. Effect of immobilized amount of PC

The chromatograms for the extract of Danggui Buxue decoction under the different amount of immobilized PC are shown in Fig. 5. It can be seen that only one large off-scale peak observed for the prescription extract on a column without PC (Fig. 5A), indicating that there are very weak interactions, if any, between the prescription extracts and the silica gel. By increasing the amount of immobilized PC, the retention time of interactional compounds in the prescription extracts increased. For example, peak 7 with a retention time of 115 min on ILC column with 43 μmol/g immobilized PC eluted at about 450 min on the ILC column with 171 μmol/g immobilized PC. In addition, some overlapped peaks can be resolved as the amount of immobilized PC increases. For example, peak 3 is separated into peaks 3′ and 3″ when the amount of immobilized PC changes from 126 to 171 μmol/g,

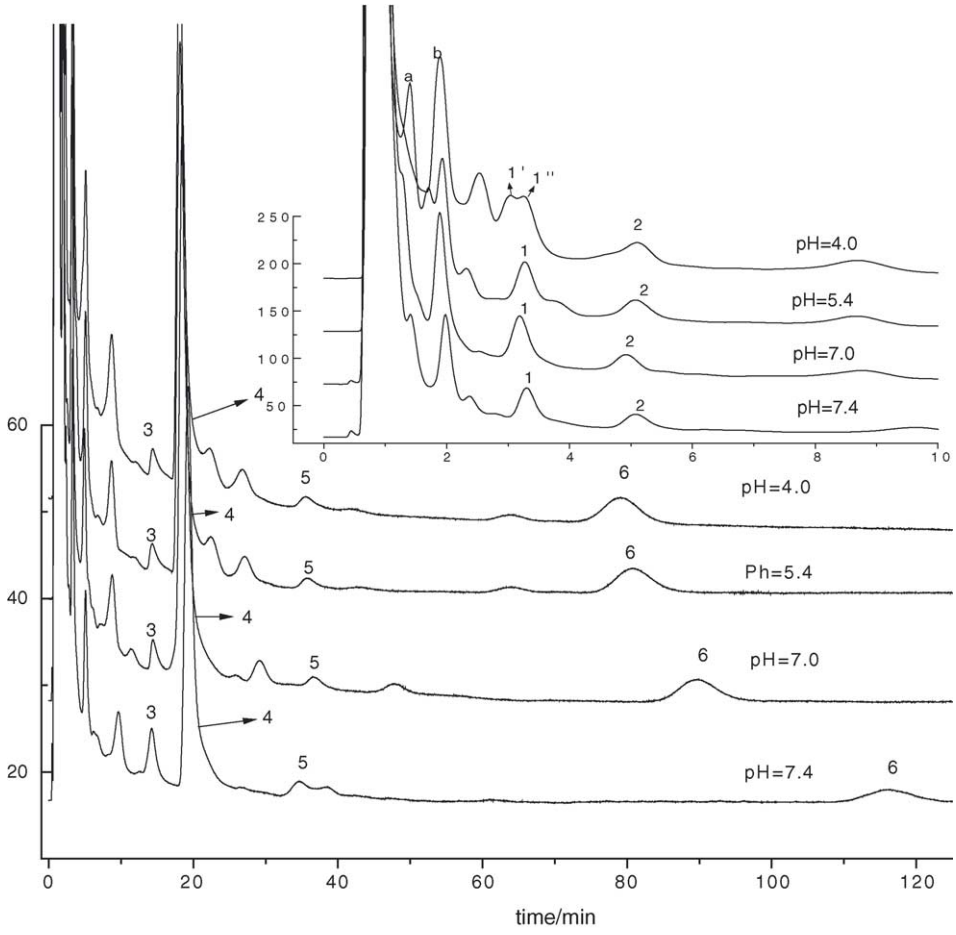

Fig. 4. Separation of the extract of Danggui Buxue decoction on ILC at different eluent pH values. Experimental conditions: mobile phase, buffer A with different pH values; other experimental conditions are the same as those in Fig. 1. The inset shows an expansion of the first 10 min of the chromatogram.

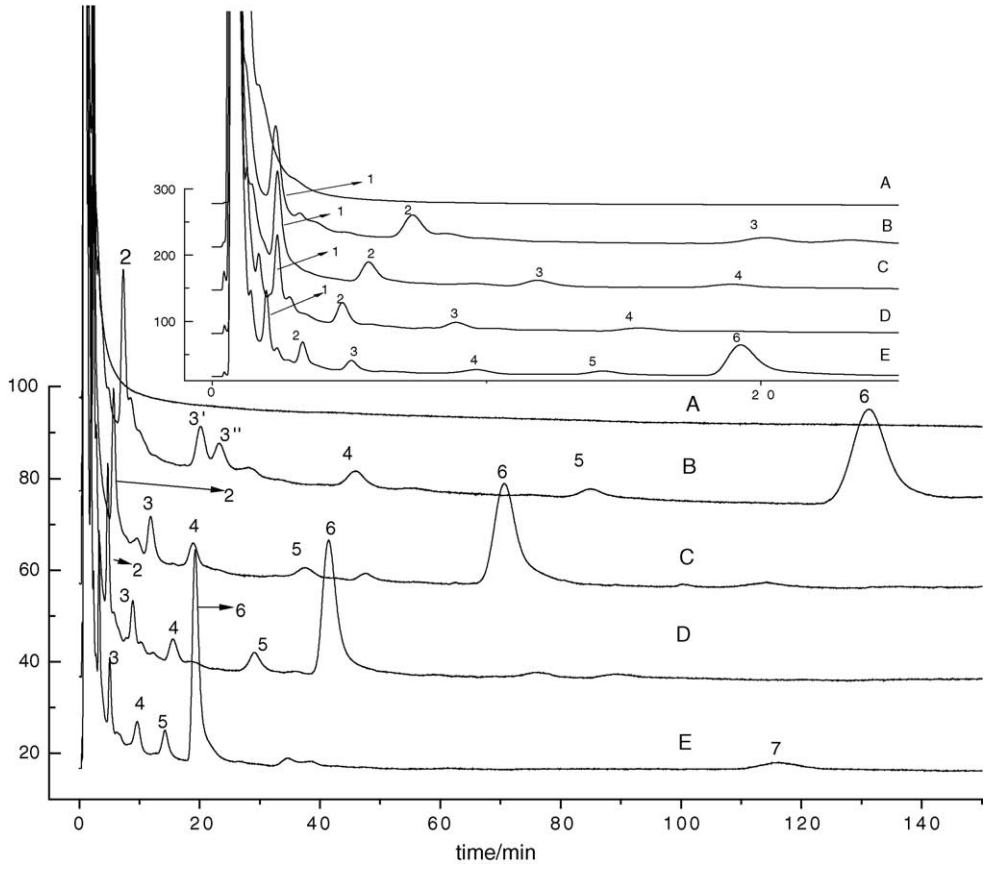

Fig. 5. Chromatograms of the extract of Danggui Buxue decoction on ILC with different amount of immobilized PC. Experimental conditions: mobile phase, buffer A; flow-rate, 1 ml/min; detection wavelength, 210 nm. The inset shows an expansion of the first 25 min of the chromatogram. (A) Column without PC; (B) column coated with 171 μmol/g PC; (C) column coated with 126 μmol/g PC; (D) column coated with 81 μmol/g PC and (E) column coated with 43 μmol/g PC.

whereas they were nearly not separated when the amount of immobilized PC changes from 43 to 126 μmol/g. Thus, an appropriate amount of immobilized PC on silica gel should be one of important issues for the study of different TCMs.

It was reported [19,20] that the $\log K_s$ value for a series of drugs was independent of the amount of coated PC in the range of 15–55 mmol on Superdex 200. We have investigated the effect of immobilized PC amount on $\log K_s$ value

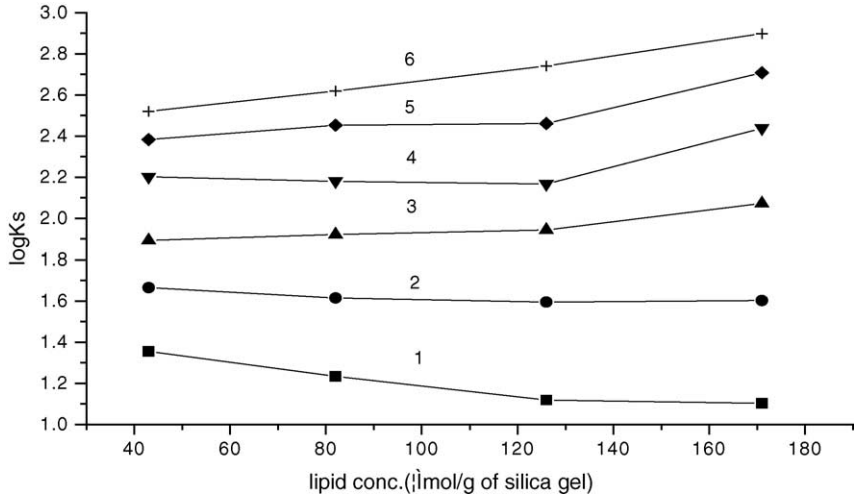

Fig. 6. Correlation of the specific capacity factor K_s with the amount of immobilized PC on ILC columns for six main peaks of the extract of Danggui Buxue decoction. Solutes: numbers indicated on solid lines stand for the peaks in chromatograms shown in Fig. 5.

of main peaks in the extract of Danggui Buxue decoction; the results are shown in Fig. 6. It can be seen that the log K_s values of six main peaks were slightly changed by increasing the amounts of immobilized PC from 43 to 126 μmol/g, but much more significantly changed when the amounts changed from 126 to 171 μmol/g. A possible reason is that the PC are not completely covered on silica gel when the amount of immobilized PC is lower than 126 μmol/g, and thus the exposed silanol groups of silica gel will influence the retention of these peaks on ILC columns. In addition, the true void volume of ILC columns (V_0) cannot be accurately measured, which is different from each other on column without PC [20], and thus resulting in a difference of log K_s values of a compound, especially for those early-eluted peaks.

3.5. *Fractions partitioned with different solvents from water extract of Danggui Buxue decoction*

The water extract of Danggui Buxue decoction cannot be well analyzed due to the limited solubility of many compounds in water; however, this problem can be overcome by means of organic solvent extraction and concentration. The chromatograms of fractions partitioned with different solvents, i.e. petroleum ether, ethyl ether, ethyl acetate and *n*-butanol, as well as the remnant water solution from water extract of Danggui Buxue decoction are shown in Fig. 7. It can be seen that the number of peaks for the petroleum ether fraction, *n*-butanol fraction, are very small, indicating that majority of components in water extract of the prescription are hardly dissolved in petroleum ether or *n*-butanol. On the other hand, the number of peaks is large for ethyl ether and ethyl acetate fractions. In fact, some peaks are identical components among these fractions. The four main bioactive ingredients, astragaloside IV, formononetin, ferulic acid and ligustilide, can be seen in the chromatograms of different fractions. For example, the peaks 1–3 were confirmed as astragaloside IV, ligustilide and formononetin, respectively (Fig. 7).

Another class of the bioactive ingredients in Danggui Buxue decoction is polysacchrides, and it largely exists in Radix Astragli and Radix Angelica Sinensis. They are different from the other bioactive ingredients, as their solubility in water is very high, and thus can be readily extracted with water. They may be adsorbed by the transcytosis routes and the active carrier-mediated transcellular [22,36] in human body, and thus have no significant retention on ILC column, which can only mimic the passive transcellular [22]. A large and off-scale peak with weak retention, which might be mainly contributed by polysaccharides, can be observed for the *n*-butanol extract and remnant water extract as shown in Fig. 7.

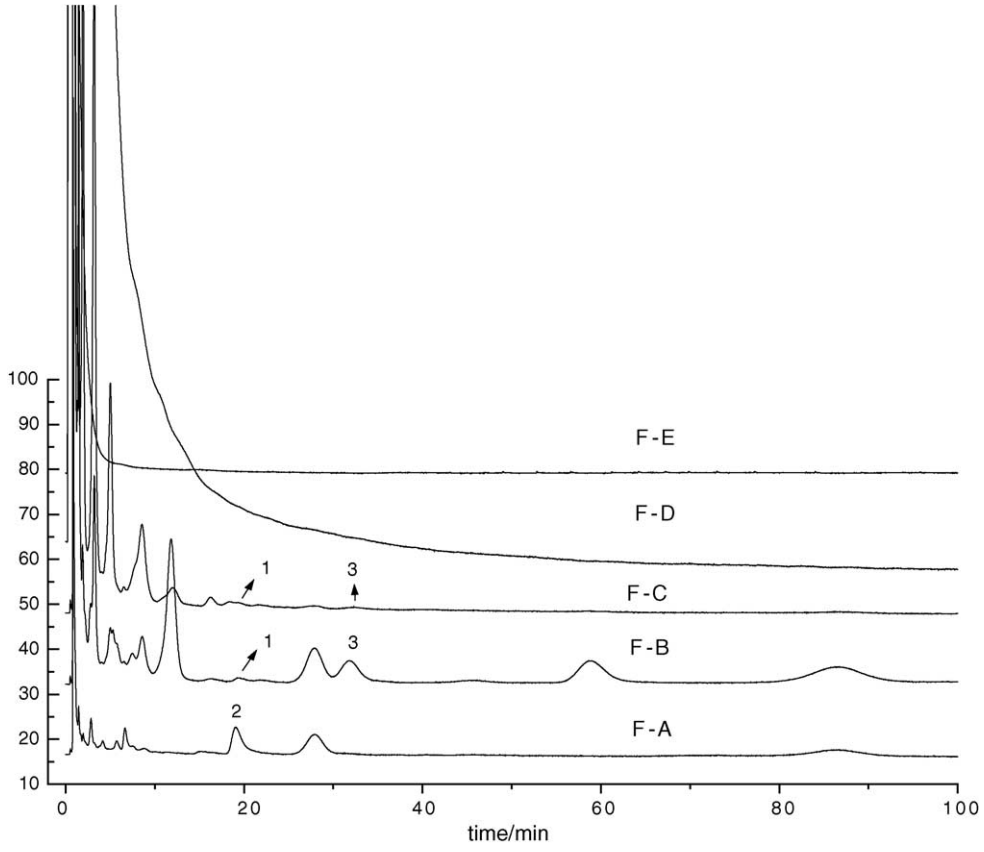

Fig. 7. Chromatograms of fractions partitioned with different solvents from water extract of Danggui Buxue decoction. (F-A) Fraction partitioned with petroleum ether, (F-B) fraction partitioned with ethyl ether, (F-C) fraction partitioned with ethyl acetate, (F-D) fraction partitioned with *n*-butanol, (F-E) the remaining water solution; (1) astragaloside IV, (2) ligustilide, (3) formononetin. Experimental conditions are the same as those in Fig. 1.

This assumption may be confirmed by purifying and analyzing the weakly retained fractions with other analytical tools in the future.

4. Conclusions

The present study shows that ILC is a potentially powerful technique for the analysis of components interacting with liposome membrane in CPTCMs. It can offer useful information about the absorption of compounds in CPTCMs. Furthermore, the combination of ILC with other chromatography models, such as immobilized protein and cell membrane stationary phases, will further clarify the pharmacological activity of compounds interacting with liposome membrane in CPTCMs.

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