Journal of Chromatography A, 1233 (2012) 78–84

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Fast immobilized liposome chromatography based on penetrable silica microspheres for screening and analysis of permeable compounds

Cong Zhang [a], Jian Li [a], Li Xu [a,*], Zhi-Guo Shi [b,**]

[a] *Tongji School of Pharmacy, Huazhong University of Science and Technology, Wuhan 430030, China*
[b] *Key Laboratory of Analytical Chemistry for Biology and Medicine (Wuhan University), Ministry of Education, Wuhan 430072, China*

ARTICLE INFO

Article history:
Received 6 November 2011
Received in revised form 4 February 2012
Accepted 7 February 2012
Available online 13 February 2012

Keywords:
Immobilized liposome chromatography
Penetrable silica microspheres
Permeable compounds
Traditional Chinese medicine
Drug screening

ABSTRACT

In the present study, an immobilized liposome chromatography (ILC) stationary phase based on penetrable silica microspheres was prepared. The silica possessed mesopores and penetrable macropores, which afforded sufficient surface area and fast mass transfer, respectively. Compared with the ILC column based on common porous silica gels, the penetrable silica had larger capacity for liposomes' immobilization, lower backpressure and higher separation efficiency. Twenty-two kinds of drugs were used as tested analytes and their retention behaviors on newly prepared ILC column were investigated in detail. Column temperature and pH of mobile phase were both key factors affecting the retention of solutes. The retention of these drugs on ILC column reflected their permeability in vivo. Furthermore, the methanolic aqueous extracts of three traditional Chinese medicines (TCMs), *Radix Liquiritiae*, *Scutellaria Baicalensis* and *Flos Sophorae*, were screened on this novel ILC column. Effects of column temperature and eluent pH on chromatographic behaviors of components of the TCM methanolic extracts were also studied. Several permeable components can be found, indicating that they are potentially active components in the TCMs. It is also found that the as-prepared ILC column remained stable in at least 1 month and the relative standard deviations of adjusted retention times for solutes were lower than 9.0%. The proposed ILC based on the penetrable silica microspheres is promising for the fast and low-pressure separation, especially for fast screening of permeable compounds and modeling the drug–membrane interaction in vitro. It would be a useful approach to predict the permeability of potential active drugs.
© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Nowadays, new drugs are mainly screened from combinatorial chemistry data and natural products [1]. It is well known that limited substances in hundreds of synthetic compounds have potential biological activity and only a few constituents in natural products like traditional Chinese medicines (TCMs) are proved to be responsible for their pharmacological activity and/or toxicity. The drug–cell membrane interaction can greatly affect their pharmacokinetics, membrane permeability and access to receptor site. So the ability of a drug to interact with membrane has been identified as a very important factor influencing the biological behavior of the drug in the organism. Hence, to study drug–membrane interaction makes sense in the early stage of drug discovery [2–4].

There are several approaches to study drug–membrane interaction, such as hydrophobicity measurements [5,6], octanol–water partitioning [7,8], liposome partitioning [9,10], etc. Liposomes have similar lipid bilayer structure and fluid characteristic to real biological membranes. When used as stationary phases in chromatographic separation techniques, they can enhance selectivity, increase throughput of analysis and model the drug–membrane in vitro [11]. Additionally, they are simple and low-cost. Previous studies demonstrate that if a drug can be retained on liposomes, it may demonstrate permeability toward real biological membranes [12–15]. Generally, as the retention increases, the membrane permeability of the drug increases accordingly.

Hitherto, liposome sterically immobilized gels have been widely applied as the stationary phase in high performance liquid chromatography (HPLC) to study drug–membrane interaction of numerous drugs [16–18]. Besides, immobilized liposome chromatography (ILC) has been documented as a powerful tool in the screening and analysis of permeable components in TCMs [2,15]. Development of new ILC method, such as fast ILC, would be appreciated during the screening, since it may save time and enhance the efficiency. In our present work, the fast ILC based on a novel silica substrate was studied.

The penetrable silica microspheres were first synthesized by Shi et al. [19], which were featured with hierarchically porous structure, containing both mesopores and penetrable macropores. The

* Corresponding author. Tel.: +86 27 8369 2735; fax: +86 27 8369 2762.
** Corresponding author. Fax: +86 27 68754067.
E-mail address: xulpharm@mails.tjmu.edu.cn (Z.-G. Shi).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2012.02.013

mesopores guarantee large surface area of the material; the macropores, which traverse the microspheres, may realize fast mass transfer as well as provide low backpressure. The silica microspheres have been attempted for packed HPLC and proved to be promising in fast HPLC separation. In the present study, the penetrable silica was used as the substrate to immobilize liposome and further used as ILC stationary phase. ILC based on such novel material is supposed to have the advantages of both liposome membrane and the silica microspheres. It may provide a fast and low-pressure separation of solutes while modeling their drug–membrane interaction in vitro.

2. Experimental

2.1. Chemicals and reagents

Penetrable silica microspheres were prepared as reported [19]. The common porous silica (Kromasil) was bought from Akzo-Nobel (Amsterdam, Netherlands). Phosphatidylcholine (PC) was obtained from Lipoid Co. (Germany). Methanol was HPLC grade. Ultrapure water was produced by a Heal Fore NW system (Shanghai, China). Fiske–Subba Row reagent was freshly prepared weekly and stored in dark. All other chemicals were of analytical grade.

PBS was 10 mM sodium phosphate solution at pH of 7.4 containing 50 mM NaCl. Salicylic acid, para-aminosalicylic acid, acetylsalicylic acid, sulfadiazine, sulfamerazinum, sulfadimidine, procaine, nicotinamide were obtained from Alfa Aesar (Tianjin, China). Hydrocortisone, noradrenaline, phenylephrine, dopamine, atropine, benzocaine, chloromycetin, isoniazid and acetaminophen were purchased from Aladdin (Shanghai, China). Lidocaine, nifedipine and clonidine were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Terbutaline and captopril were bought from Sigma (St. Louis, MO, USA).

Three kinds of TCM, *Radix Liquiritiae* (RL), *Scutellaria Baicalensis* (SB) and *Flos Sophorae* (FS) were purchased from a local pharmaceutical store.

2.2. Instrument and measurement

The HPLC analysis was performed on a Hitachi (Tokyo, Japan) HPLC system. It consisted of a Model L-2130 pump, a Rheodyne 7725i injector (Cotati, CA, USA) and an L-2400 UV–vis spectrophotometric detector. Data were collected and processed by the T3000P (Hangzhou Hui Pu Technology Co. Ltd., Hangzhou, China) software. The screening of TCMs was performed on a Waters HPLC system equipped with a Waters 600 controller, a Waters 2996 photodiode array detector and a Waters 717 plus autosampler. Data were collected and processed by the Empower (Waters, Milford, MA, USA) data analysis software. The columns used for HPLC were 250 mm × 2.1 mm, packed with the homemade immobilized liposome silicas as specified. The mobile phase was 10 mM sodium phosphate solution containing 50 mM NaCl, adjusted to pH 7.4 (if not specified). The column temperature and the flow rate were described in the individual experiments. The pH values of mobile phases were measured with a Mettler Toledo Delta 320 pH meter (Shanghai, China). An ODS column from Shimadzu (Kyoto, Japan) was also used to evaluate the retention mechanism.

2.3. Sample preparation

The stock solutions of chemical drugs were prepared in pure methanol or the mixture of water and methanol at prescribed concentrations.

The methanolic aqueous extracts of RL, SB and FS were prepared as follows. The dried crude herbal drug was crushed with a grinder. One gram of powder was immersed in 100 mL 70% methanol–30% H_2O and ultrasonically extracted for 1 h. Then it was kept statically for 1 h. The mixture was centrifuged at 10,000 rpm and the supernatant was filtered through a membrane with pore size of 0.45-μm. The filtrate was collected for ILC analysis.

All the sample solutions were stored at 4 °C until use.

2.4. Preparation of ILC columns

The general procedure to prepare the liposome-immobilized silica was as follow [2,14]. A certain amount of PC dissolved in chloroform was placed in a round-bottomed flask, and different proportions of prescribed silica gel were added. The solid–liquid system was shaken to ensure the silica to interact with PC thoroughly; and the organic solvent was evaporated under reduced pressure at 55 °C using a rotary evaporator. In this way, a thin film was reported to form on the silica [2,14]. The PC film coated silica was dried overnight under high vacuum and hydrated with PBS. The suspension was kept statically for 3 h, and then was washed with PBS for three times to remove the unimmobilized liposome.

Each slurry of the above prepared silica microspheres was packed into a stainless steel column (250 mm × 2.1 mm i.d.). Specifically, the columns based on different silica microspheres were defined in Table 1.

2.5. Characterization

The amount of phosphorus in the stationary phases was determined by the method introduced by Bartlett [20]. Briefly, 2–3 mg accurately weighed, dried samples, 1 mL of H_2O and 0.5 mL of H_2SO_4 solution (5 mol L^{-1}) were placed in a glass bottle, and heated at 150–160 °C for 3 h. H_2O_2 (0.1 mL) was added and the solution was further heated for 1.5 h. Then 0.2 mL of ammonium molybdate (5%) and 0.2 mL of Fiske–Subba Row reagent were added. The total volume of the solution was fixed at 10 mL with H_2O, mixed thoroughly and heated at 100 °C for 7 min. The absorbance of final solution was detected at 830 nm. A standard curve was drawn using KH_2PO_4 as standard substance. The total phosphorus of ILC-1, ILC-2, ILC-3 and ILC-4 was calculated in this way, respectively.

A scanning electron microscope of JSM-35CF (JEOL, Tokyo, Japan) was used to study the morphology of the materials. A nitrogen porosimeter of SA 3100 Plus (Beckman Coulter, Miami, FL, USA) was used to determine the porous structure of the materials. The specific surface area value was calculated according to the BET (Brunauer–Emmett–Teller) method at P/P_0 between 0.05 and 0.2. Thermogravimetric analysis (TGA) was carried out using a Setaram (France) TG–DTA analyzer to investigate their thermal behavior. All the samples were heated progressively from 25 to 800 °C with a heating rate of 10 °C min^{-1}. Fourier transform infrared spectroscopy (FT-IR) spectra were obtained from AVATAR 360 instrument (Thermo, USA).

2.6. Specific capacity factor (k_s)

The chromatographic retention of an analyte on immobilized liposome can be expressed as the specific capacity factor, k_s [3], defined as:

$$ks = \frac{k}{A} \tag{1}$$

$$k = \frac{V_R - V_0}{V_0} = \frac{t_R - t_0}{t_0} \tag{2}$$

where k is the retention factor and t_0 is the column dead time. A is the amount of phospholipids, which was determined as the average of phosphorus amount [20] in the washed suspension before packing into the column [3].

Table 1
Column denotation and characterization of the column packings.

Column denotation	Silica gel	Mass ratio of PC to silica	Weight loss (%)[a]	Amount of phosphorus (mg/g)[b]
ILC-1	Penetrable silica	1:4	18.5	8.67
ILC-2	Kromasil silica	1:4	18.8	8.23
ILC-3	Penetrable silica	1:2	28.9	12.09
ILC-4	Kromasil silica	1:2	24.2	8.38

[a] The weight loss was obtained from thermogravimetric analysis.
[b] The phosphorus content was determined by Bartlett's method.

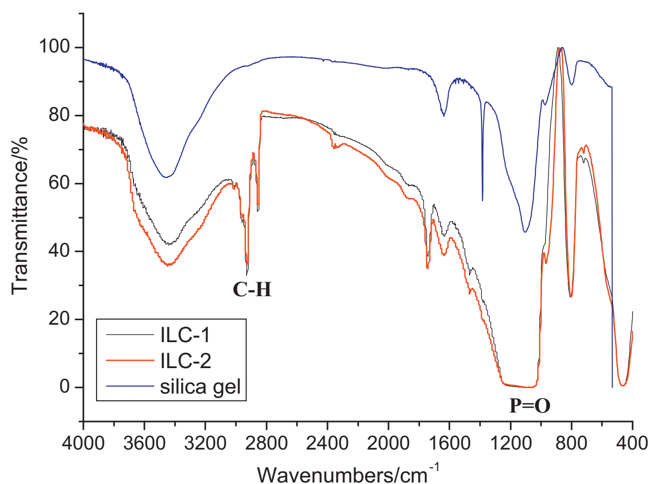

Fig. 1. FT-IR spectra of ILC-1, ILC-2 and silica gel.

Under the same experimental condition, k_s of each compound will remain constant. Hence, $(t_R - t_0)$ is directly proportional to A and the change of $(t_R - t_0)$ will reveal the change of A. Thus an online monitoring of the amount of phospholipids (A) could be realized.

3. Results and discussion

3.1. Characterization of the liposome-immobilized silicas

Previous researches demonstrated that liposomes were formed by self-assembly when hydrating PC in aqueous buffer solution [2,14]. In this study, the same preparation method was adopted, although the liposome structure on the silica surface was not confirmed. Fig. 1 shows the FT-IR spectra of ILC-1, ILC-2 and bare silica gel. The adsorption peaks at around 1100 cm^{-1} and 2900 cm^{-1} can be observed in the spectra of ILC-1 and ILC-2, while they are absent in the spectrum of bare silica gel. The adsorption should be ascribed to the vibration of P=O (~1100 cm^{-1}) and C—H (2900 cm^{-1}) of the liposome on the silicas, which suggests that liposome was successfully immobilized onto the silica gels.

The phosphorus content in the liposome-immobilized silicas was determined by the Bartlett's method (as described in Section 2.5), which reflected the amount of phospholipid loading on silica directly. As shown in Table 1, when the mass ratio of PC to silica was 1:4, the phosphorus content on penetrable silica was almost the same as that of Kromasil silica. In addition, the weight loss from TGA was also the same as far as these two silicas were concerned (~18%). When the mass ratio of PC to silica increased up to 1:2, the phosphorus content on the penetrable silica was about 40% higher than that on Kromasil silica. This result demonstrated that the penetrable silica may have higher loading capacity for liposome, which would be conducive to enhance the separation ability of ILC columns.

Fig. 2 shows the SEM images of the penetrable silica and Kromasil silica. It discloses that macropores exist in the penetrable silica, while these kinds of pores are absent in the Kromasil silica. From previous report [19], it is known that the macropores are evenly distributed within the penetrable silica, which would be helpful for ILC application. From the gauge bar in Fig. 2, it can be found that the particle sizes of the two silicas are quite similar (~5 μm).

Nitrogen sorption experiments were carried out on the penetrable silica, Kromasil silica and PC-coated ones to evaluate their porous characteristics. The surface area (SA), mean mesopore size (MMS) and mesopore volume (MV) of them were listed in Table 2. It can be found that, after being coated with PC, the SA, MMS and MV for both the penetrable silica and Kromasil silica decreased. Nevertheless, for Kromasil silica, these parameters decreased much more obviously than the penetrable one. Probably, during the coating process, some mesopores in Kromasil silica were blocked because of the absence of macropores.

3.2. Chromatographic performance of ILC

Since the same liposome content on penetrable and Kromasil silica were obtained when the ratio of PC to silica (1:4, w:w) were adopted, they were used for ILC analysis for further comparison.

ILC-1 and ILC-2 were compared under the same chromatographic condition. The mobile phase was PBS, the flow rate was 0.2 mL min^{-1} and column temperature was 25 °C. The backpressure of ILC-1 and ILC-2 were 2.8 MPa and 9.3 MPa, respectively, which means ILC-1 had much lower backpressure than ILC-2. Since ILC-1 has higher permeability than ILC-2, it may be used at high flow rates to increase separation efficiency. Ten drugs were tested on these

Table 2
The surface areas, mean mesopore sizes and mesopore volumes of the penetrable silica, Kromasil silica and the PC-coated ones.

Porous characteristics	Silica gel			
	Penetrable silica		Kromasil silica	
	Before coated	After coated[d]	Before coated	After coated[d]
SA (m^2 g^{-1})[a]	310	130	296	43
MMS (nm)[b]	9.1	3.6	9.2	3.2
MV (cm^3 g^{-1})[c]	0.88	0.56	0.93	0.32

[a] Surface area.
[b] Mean mesopore size.
[c] Mesopore volume.
[d] The coated mass ratio of PC to silica is 1:4.

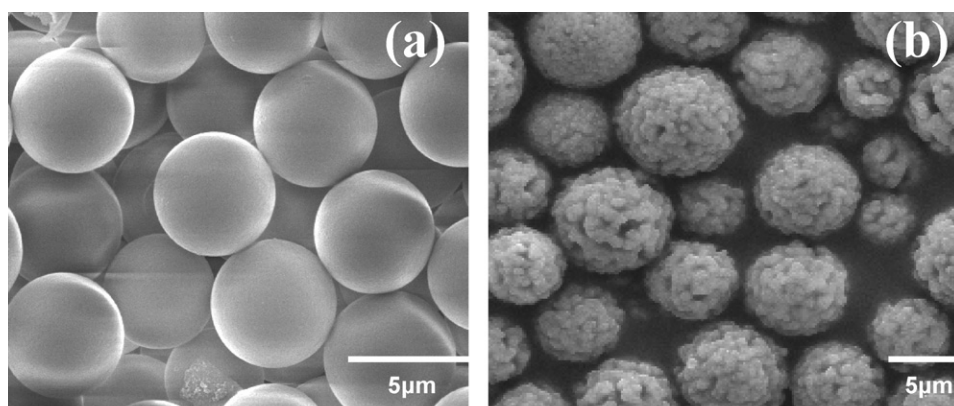

Fig. 2. The SEM images of Kromasil silica and the penetrable silica.

Table 3
The retention time of analytes and the column efficiency of ILC-1 and ILC-2.

Analytes	t_R (min)		N (m^{-1}) (×10^3)	
	ILC-1	ILC-2	ILC-1	ILC-2
Sulfadiazine	4.45	3.92	2.52	1.22
Sulfamerazinum	6.06	5.68	2.39	1.39
Sulfadimidine	9.93	9.82	2.08	1.66
Salicylic acid	5.08	5.86	2.81	2.69
Para-aminosalicylic acid	3.82	3.19	2.85	1.81
Noradrenaline	10.18	9.45	2.18	1.57
Chloromycetin	25.49	17.26	2.30	1.95
Terbutaline	54.01	31.74	1.75	1.03
Isoniazid	4.31	3.23	2.76	1.86
Nicotinamide	4.73	3.63	2.91	2.52

two columns. Retention time and theoretical plate number (N) of the individual analytes are listed in Table 3. The results demonstrate that ILC-1 had higher column efficiency than ILC-2.

The separation of four drugs was conducted on ILC-1 at flow rates of 1.0 and 1.5 mL min^{-1}, respectively. As shown in Fig. 3, the four compounds can be well separated with an acceptable backpressure. With the increasing flow rates from 1.0 to 1.5 mL min^{-1}, the analysis time were shortened from ∼29 min to ∼19 min. When the separation was conducted on ILC-2, flow rate of the mobile phase could not exceed 1 mL min^{-1} due to the high backpressure. As shown in Fig. 3, at the tolerated highest flow rate of 1 mL min^{-1}, 30 min were needed to separate the four drugs on ILC-2.

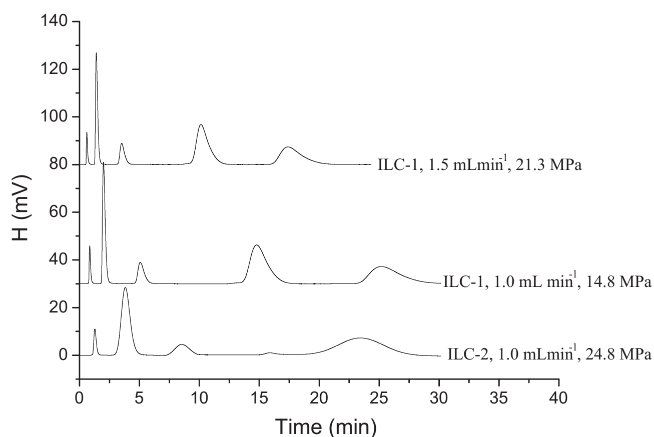

Fig. 3. Separation of four drugs on ILC-1 at different flow rates using PBS as mobile phase. Column temperature: 25 °C. The four compounds are sulfadimidine, phenylephrine, chloromycetin and hydrocortisone (in order of increasing retention time).

To better understand the retention mechanism on ILC, comparison was made between ILC-1 and a commercial ODS column. The mixture of PBS and methanol (70:30, v:v) was used as mobile phase for the ODS column. The flow rate was 1 mL min^{-1}. The elution order of different compounds on the ODS column was dissimilar to that on ILC-1. Taking seven representative compounds for example, tripolycyanamide, acetaminophen, sulfadimidine, phenylephrine, terbutaline, chloromycetin and hydrocortisone were eluted out in sequence on ILC-1. But on the ODS column, the elution order was tripolycyanamide, phenylephrine, terbutaline, acetaminophen, chloromycetin, sulfadimidine and hydrocortisone. This phenomenon indicated that the separation mechanism of ILC was not merely based on hydrophobic interaction. Extra complicated biological membrane interaction may be involved in the separation.

3.3. Retention behavior of drugs on ILC-1

3.3.1. Effects of the pH of mobile phase on the retention behavior of drugs

The pHs of the mobile phase varied from 4.0, 5.0, 7.4, 8.3 to 9.0. The retention factors of 22 drugs are listed in Table 4. The compounds had irregularly changing trends with the varying pH of the mobile phases. For example, the retention of salicylic acid increased when mobile phase pH value decreased, mainly due to decreased ionization of acid hydrophilic compounds, which may strengthen the interaction between the compound and the ILC stationary phase and then result in stronger retention. The basic compounds, like nifedipine and phenylephrine, had the opposite tendency to salicylic acid, probably because of the same reason. The basic compounds tend to be protonated at low pH, thus leading to a weak interaction with the stationary phase and consequently the weak retention. But some other compounds such as chloromycetin and clonidine, the retention of them changed irregularly. This may be due to the change of viscosity of the mobile phase and other factors caused by the pH variation [21]. Another possible explanation for this phenomenon may be ascribed to the properties of the liposome. As mentioned above, there are complicated interactions between liposome and the analytes, including hydrophobic interaction, electrostatic interaction, etc. It should be highly related to the nature of the tested drugs and the status of the liposome.

3.3.2. Effects of column temperature on the retention behavior of drugs

The influence of column temperature (20, 30, 37 and 45 °C) on the retention behaviors of 22 drugs was investigated. The results demonstrated that the retention of all the drugs on the column decreased when the temperature increased from 20 to 45 °C.

Table 4
Effect of the pH of mobile phase on retention of the drugs on ILC-1.[a]

Analytes	*k*					log *P*[b]
	pH 4.0	pH 5.0	PH 7.4	pH 8.3	pH 9.0	
Salicylic acid	3.31	1.48	0.48	0.48	0.39	2.011 ± 0.247
Para-aminosalicylic acid	1.82	0.62	0.13	0.12	0.03	1.242 ± 0.266
Acetylsalicylic acid	3.58	1.45	0.48	0.49	0.41	1.399 ± 0.226
Sulfadiazine	1.89	1.99	0.31	0.13	0.05	−0.074 ± 0.255
Sulfadimidine	2.98	2.97	2.18	0.45	0.48	0.296 ± 0.278
Sulfamerazinum	2.48	2.45	1.00	0.26	0.11	0.107 ± 0.267
Hydrocortisone	42.77	39.66	36.81	29.78	29.69	1.762 ± 0.417
Phenylephrine	1.08	1.96	6.74	7.85	9.47	0.117 ± 0.269
Noradrenaline	0.43	0.79	2.79	8.44	–	−1.260 ± 0.341
Dopamine	0.86	1.49	5.12	7.83	21.90	0.046 ± 0.219
Atropine	10.41	15.19	–	–	–	1.380 ± 0.365
Lidocaine	7.34	11.36	–	–	–	2.196 ± 0.386
Procaine	6.47	8.93	–	–	–	2.256 ± 0.532
Benzocaine	7.14	11.02	–	–	–	1.835 ± 0.251
Chloromycetin	23.06	25.23	21.39	20.18	19.89	1.103 ± 0.354
Nicotinamide	0.36	0.34	0.38	0.32	0.22	−0.386 ± 0.223
Isoniazid	0.14	0.15	0.22	0.07	0.13	−0.766 ± 0.254
Acetaminophen	1.35	1.41	1.21	1.05	0.87	0.475 ± 0.210
Terbutaline	2.30	5.46	15.72	17.58	26.80	0.696 ± 0.360
Clonidine	3.17	8.69	30.48	28.41	17.37	2.362 ± 0.603
Captopril	0.24	0.42	0.21	0.24	0.39	0.272 ± 0.469
Nifedipine	38.95	40.04	42.84	35.67	35.14	3.582 ± 0.588

–: the compound was retained strongly on ILC-1 and not detected within 90 min.

[a] Chromatographic conditions: mobile phase, 10 mM sodium phosphate solution containing 50 mM NaCl at different pH values; column, ILC-1; column temperature, 20 °C; flow rate, 1.0 mL min^{-1}; detection wavelength, 280 nm for salicylic acid, para-aminosalicylic acid, acetylsalicylic acid, chloromycetin and terbutaline; 254 nm for sulfadiazine, sulfadimidine, sulfamerazinum, nicotinamide and isoniazid; 242 nm for hydrocortisone; 238 nm for nifedipine; 220 nm for phenylephrine, noradrenaline, dopamine, atropine, lidocaine, procaine, benzocaine, acetaminophen, clonidine and captopril.

[b] Logarithm of the partition coefficient between n-octanol and water. Available at: https://scifinder.cas.org.

Especially, at 20 °C, atropine, lidocaine, procaine and benzocaine were strongly retained, and the signals of them were not detected within 90 min. The explanation may be that the interaction between the solute and the immobilized liposome decreased or diminished with increasing temperature.

3.4. Screening three TCMs by ILC-1

The ILC based on penetrable silica may model drug–membrane interaction fast in vitro, affording a high throughput screening approach.

3.4.1. Chromatographic behavior of methanolic aqueous extracts of three TCMs

In this study, methanolic aqueous extracts of RL, SB and FS were individually investigated on ILC-1 with PBS as mobile phase at a flow rate of 1 mL min^{-1} at 25 °C. The 3D chromatogram of methanolic extract of RL is shown in Fig. 4(A). It is obvious that there were seven main peaks, which were denoted as A_1–A_7, in order of increasing retention time. Several minor peaks of RL extract were also detected besides the seven principal ones. Owing to the fact that the purity of each peak was difficult to tell on one-dimensional chromatography with UV detection, one peak may stand for a pure component or multi components. However, these nearly 10 components in the RL extract may have strong penetration ability through biological membrane based on their retention on ILC. Due to the correlation between the permeability of drugs and their bioactivity, the components which have marked retention on ILC should be considered as active ingredients in RL [3,14]. Using a more modern detector, such as mass spectrometry, the purity of peaks may be identified and the corresponding structure may be defined. Otherwise, multi-dimensional chromatography using ILC-1 as the first dimension, and another stationary phase such as ODS as the second one, more peaks may be obtained and the purity of the peaks may be enhanced. In addition, the possible active ingredients from ILC-1 may be prepared for further investigation. Combining these technologies, the penetrable components can be determined and confirmed, which would be conducive for the further studies about their activities.

Fig. 4(B and C) shows the characteristic 3D chromatograms of methanolic aqueous extracts of SB and FS, respectively. Many specific peaks are obtained from ILC of SB while fewer peaks are found in the other one. The different retention behaviors of different TCMs meant that various permeable active components may be obtained from them.

3.4.2. Effects of mobile phase pH

The pH value of human body is always stable. The normal pH value of blood and tissue fluid is 7.4. The pH of intracellular fluid is 7.0 and that of intestinal epithelial cell is 5.4. In this study, the three TCMs extracts were examined at these three different pH values to further investigate their possible active position in human body. The chromatograms obtained are shown in Fig. 5. Apparently, the chromatograms of every TCM under different eluent pH values are different from each other.

For example, in Fig. 5(A), the retention times of the principal peaks in RL extract all increased when the pH value decreased from 7.4 to 7.0. Besides, peak 1 was hard to identify at the pH of 7.4. Probably the analyte was eluted at the dead time. However, at the pH of 7.0, peak 1 was detected and had a good separation with peak 2. The reason for this trend may be that the degree of ionization of acid hydrophilic compounds reduced when the pH value was reduced, which may strengthen the interaction between the components and the stationary phase and then results in longer retention time. With the pH value continued to decrease to 5.4, the increasing tendency of retention was also obvious, but just six peaks could be observed while seven peaks were observed at the other two higher pHs. The explanation may be that the unionized compounds were easy to permeate lipid membrane. The component, which disappeared at the pH of 5.4, was supposed to have nearly no adsorption

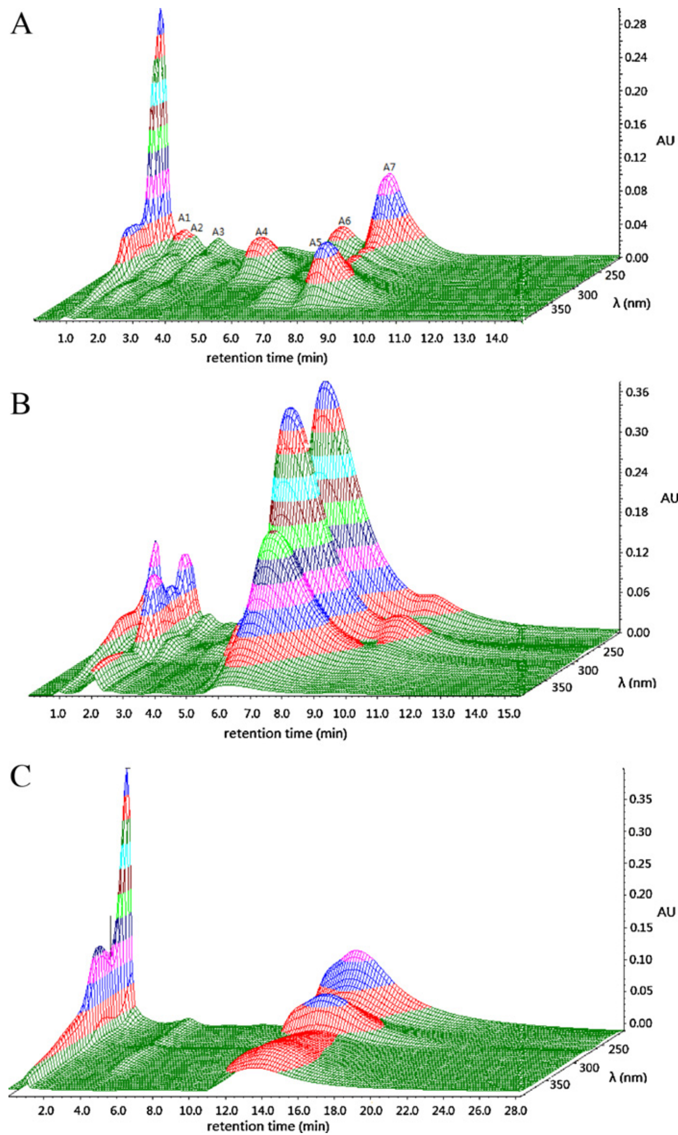

Fig. 4. 3D chromatogram of methanolic aqueous extracts of three TCMs on ILC-1 using PBS as the mobile phase. Column temperature: 25 °C; flow rate: 1 mL min^{-1}; TCMs: (A) RL; (B) SB; (C) FS.

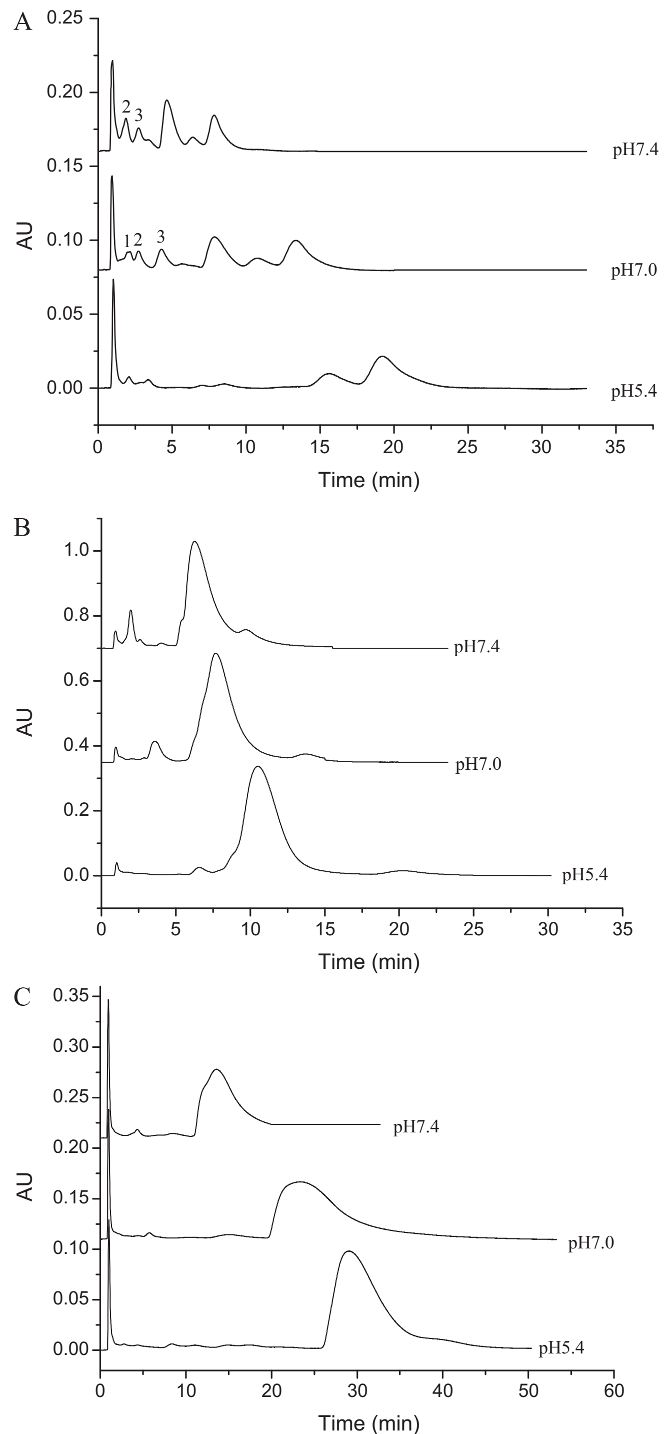

Fig. 5. Effect of mobile phase pH on the analysis of methanolic extracts of three ordinary TCMs using 10 mM sodium phosphate solution at different pH values. Column temperature: 25 °C; flow rate: 1 mL min^{-1}; sample: (A) RL; (B) SB and (C) FS.

or weak absorption in vivo due to its ionization. The above observations may indicate that the effective components of RL may be well absorbed in intestines.

As the pHs of the mobile phase were changed, SB and FS had the same trends with RL, as shown in Fig. 5(B and C).

3.4.3. *Effects of column temperature*

The effects of column temperature on the retention of the three TCMs' extracts on ILC were investigated in the temperature range of 25–45 °C. The retention time of every component decreased when the temperature increased, mainly because the interaction between the solute and the immobilized liposome decreased or diminished with the increasing temperature. In addition, peak 6 in RL methonic extract nearly could not be detected at the temperature of 45 °C. This phenomenon indicated that, if someone has got a fever, some components may be less effective because of their decreased absorption into human body. Thus, temperature is also an important factor to be considered during the process of fast screening of TCMs on ILC.

3.5. *Stability of ILC-1 column*

As described in Section 2.6, the change of the adjusted retention times ($t'_R = t_R - t_0$) of analytes can reveal the change of the amount of phospholipids (A) of the stationary phase. In this study, t'_R of six different drugs, acetaminophen, sulfadimidine, noradrenaline, terbutaline, chloromycetin, hydrocortisone, were monitored during series of runs over 1 month. t'_R of each drug was calculated and the relative standard deviations (RSDs) of t'_R are shown in Table 5. The adjusted retention times varied little, which means the loss of

Table 5
Evaluation of the stability of ILC-1.

Drugs	Acetaminophen	Sulfadimidine	Phenylephrine	Terbutaline	Chloromycetin	Hydrocortisone
RSD of adjusted retention time (%)	5.7	7.9	8.8	4.4	7.2	6.1

lipids from the penetrable silica spheres was neglectable and the ILC-1 column remained stable in at least 1 month.

4. Conclusions

Combined the superiorities of both liposome and penetrable silica microspheres, the novel ILC column was developed for the fast separation of solutes while modeling their drug–membrane interaction in vitro. The chromatographic conditions, column temperature and the pH of mobile phase, would influence the retention and the separation of solutes. Permeable compounds in methanolic aqueous extracts of *Radix Liquiritiae*, *Scutellaria Baicalensis* and *Flos Sophorae* were screened on the novel ILC. These marked retentive components were regarded as bioactive compounds in TCMs. This silica based ILC column is a promising alternative in the fast drug screening process.

Acknowledgments

The authors gratefully acknowledge the financial support of this research by the Nature Science Foundation of China (Nos. 81173018, 21105033 and 21075091), the Fundamental Research Funds for the Central Universities (Nos. 114027 and 01-09-514944), and New Teachers' Fund for Doctor Stations, Ministry of Education (No. 20110142120029) and fund of Huazhong University of Science and Technology (No. 0124514009).

References

[1] W.N. Zhang, Z.X. Hu, Y. Liu, Y.Q. Feng, S.L. Da, Talanta 67 (2005) 1023.
[2] Y. Wang, L. Kong, X. Lei, L. Hu, H. Zou, E. Welbeck, S.W. Bligh, Z. Wang, J. Chromatogr. A 1216 (2009) 2185.
[3] F. Beigi, I. Gottschalk, C. Lagerquist Hägglund, L. Haneskog, E. Brekkan, Y. Zhang, T. Österberg, P. Lundahl, Int. J. Pharm. 164 (1998) 129.
[4] W.N. Zhang, J. Sun, Y.J. Wang, X.H. Liu, Y.H. Sun, R. Lu, Z.G. He, Chromatographia 70 (2009) 1321.
[5] D.E. Schmidt, J.R. Votaw, R.M. Kessler, T. de Paulis, J. Pharm. Sci. 83 (1994) 305.
[6] T. Braumann, J. Chromatogr. 373 (1986) 191.
[7] C. Giaginis, A. Tsantili-Kakoulidou, J. Pharm. Sci. 97 (2008) 2984.
[8] C. Hansch, J.M. Clayton, J. Pharm. Sci. 62 (1973) 1.
[9] K. Balon, B.U. Riebesehl, B.W. Muller, Pharm. Res. 16 (1999) 882.
[10] Y. Katz, J.M. Diamond, J. Membr. Biol. 17 (1974) 101.
[11] Z.X. Hu, W.N. Zhang, H.B. He, Y.Q. Feng, S.L. Da, J. Chromatogr. B 827 (2005) 173.
[12] Y. Zhang, C. Zeng, Y. Li, S. Hjertén, P. Lundahl, J. Chromatogr. A 749 (1996) 13.
[13] E. Boija, G. Johansson, Biochim. Biophys. Acta 1758 (2006) 620.
[14] X. Mao, L. Kong, Q. Luo, X. Li, H. Zou, J. Chromatogr. B 779 (2002) 331.
[15] L. Sheng, S. Li, L. Kong, X. Chen, X. Mao, X. Su, H. Zou, P. Li, J. Pharm. Pharmacol. 38 (2005) 216.
[16] P. Lundahl, F. Beigi, Adv. Drug Deliv. Rev. 23 (1997) 221.
[17] X. Liu, C. Nakamura, Q. Yang, N. Kamo, J. Miyake, J. Chromatogr. A 961 (2002) 113.
[18] A. Gómez-Hens, J. Manuel Fernández-Romero, TrAC, Trends Anal. Chem. 24 (2005) 9.
[19] J. Wei, Z. Shi, F. Chen, Y. Feng, Q. Guo, J. Chromatogr. A 1216 (2009) 7388.
[20] G.R. Bartlett, J. Biol. Chem. 234 (1959) 466.
[21] Z.X. Hu, W.N. Zhang, H.B. He, Y.Q. Feng, S.L. Da, Chromatographia (69) (2009) 637.