ELSEVIER

Journal of Chromatography B, 779 (2002) 331–339

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

# Screening and analysis of permeable compounds in *Radix Angelica Sinensis* with immobilized liposome chromatography

Xiqin Mao, Liang Kong, Quanzhou Luo, Xin Li, Hanfa Zou*

*National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 161 Zhongshan Road, Dalian 116011, China*

Received 5 March 2002; received in revised form 28 May 2002; accepted 4 June 2002

**Abstract**

Immobilized liposome chromatography (ILC) was used to screen and analyze permeable compounds in traditional Chinese medicines (TCMs), testing extracts from *Radix Angelica Sinensis*. More than 10 peaks were resolved based on their interactions with the ILC stationary phase, a system which mimics biomembranes; this means that more than 10 components in *Radix Angelica Sinensis* extract have significant retention on an ILC column. Two of them, ligustilide and ferulic acid, were identified from their MS spectrum and with standard samples. A possible molecular structure of another component retained on ILC was also preliminarily identified as 3-butylidene-4,5-dihydro-2(1,3*H*)-1-isobenzofuranol according to its MS spectrum, hydrophobicity and $^{1}$H NMR spectrum. Of all detected components, ligustilide had the best penetration ability through the biomembrane. The effects of pH, column temperature, and ionic strength on the chromatography of methanolic extracts of *Radix Angelica Sinensis* were also investigated. It was found that the separation selectivity on ILC is strongly affected by the eluent pH, but only slightly by the column temperature and ionic strength.


*Keywords:* *Radix Angelica Sinensis*; Immobilized liposome chromatography; Permeable compounds

## 1. Introduction

Although only a limited number of compounds in traditional Chinese medicines (TCMs) are responsible for their pharmaceutical and/or toxic effects, they are in fact complex mixtures containing up to hundreds or even thousands of different compounds, which makes the screening and analysis of bioactive components in them extremely difficult [1]. However, the screening and analysis of bioactive components in TCMs is very important not only for the quality control of crude drugs but also for elucidating the therapeutic principle of TCMs [2]. In traditional ways, screening of bioactive compounds is carried out on animal models [3], which are time-consuming, arduous, and inappropriate for directly screening bioactive components from TCMs. High-throughput screening methods using receptors and specific enzymes as targets have been extensively used in the field of screening candidates from synthetic compounds [4,5]. However, complex interactions between components, such as synergistic or antagonistic effects are responsible for the bioactivity or toxicity of TCMs and curative effect is also a

*Corresponding author. Tel.: +86-411-369-3409; fax: +86-411-369-3407.

*E-mail address:* zouhfa@mail.dlptt.ln.cn (H. Zou).

1570-0232/02/$ – see front matter 
PII: S1570-0232(02)00403-8

compositive result of all these interactions, therefore it is impossible for a mono-receptor model to perceive all these complex interactions clearly [6]. Biochromatography with immobilized protein stationary phases has been applied to probe the interaction between the group of compounds in TCMs and the protein [1,2,6–9].

Reversed-phase high-performance liquid chromatography (RP-HPLC) is the most frequently-used tool to model solute–membrane partitioning, now that drug–biological membrane interactions have been identified as very important factors influencing the bioactivity expression of drugs. However, it primarily models the hydrophobicity between solutes and membrane, whereas the interactions between solutes and the polar lipid head groups are not well included, while these are also believed to be important for the partition of drugs to biological membranes [10,11].

Immobilized liposome chromatography (ILC) developed in recent years is regarded as a powerful tool to study drug–membrane interactions in vitro [12,13]. Liposome [10,14,15] formed by phosphatidylcholine, the main components found in cell membrane, or unilaminar phospholipids [16–19], were noncovalently or covalently immobilized on soft gel particles or silica particles as chromatographic stationary phase to probe the penetration ability of compounds through biological membranes, which has been considered as one of most important parameters to evaluate their bioactivity [20–22]. In comparison to unilaminar phospholipids stationary phase, immobilized liposome is structurally more similar to biological membranes because of its lipid bilayer structure and better fluidity of lipid molecules. However, soft gel particles, such as sepharoses and sephadexes, are the main supports of liposome immobilization in liquid chromatography research at present. But they all suffer from some drawbacks, such as large particle size, wide size distribution and poor mechanical strength. We had developed a liposome immobilization method with porous silica as the support, which can effectively avoid these drawbacks. In addition, the ILC stationary phase had been used to predict the intestinal absorption of chemical drugs in our previous work [23,24].

However, the application of ILC in separation and analysis of permeable components in TCMs had not been reported. In this paper, we attempt to define an application of ILC in this field.

## 2. Experimental

### 2.1. Apparatus and instruments

The HPLC system consisted of a LC-9A pump (Shimadzu, Kyoto, Japan) equipped with a Rheodyne injection valve with a 10-μl sample loop, a photodiode array detector (Waters, Milford, USA) and Millennium Chromatographic Workstation (Waters, Milford, USA). An ODS column (150×4.6 mm I.D.) packed with $C_{18}$ stationary phase, particle size of 5 μm and pore size of 120 Å (Hypersil, UK) was used. Distilled water was further purified with a water purification system (Millipore, Molsheim, France) for all experiments.

GC–MS QP-5000 (Shimadzu, Kyoto, Japan) with electron impact (EI) source was used for MS analysis.

### 2.2. Reagents and materials

Silica (10 μm, 300 Å) was ordered from Chrom Expert (Sacramento, CA, USA). Ferulic acid was purchased from Sigma (St Louis, MO, USA). Phosphatidylcholine (PC), was obtained from Shanghai Chemical Reagents (Shanghai, China). Crude herbal drugs were all purchased from a local drug store. Methanol was HPLC grade. Buffer A is 10 m*M* sodium phosphate buffer (pH 7.0) containing 50 m*M* NaCl. All other chemicals were analytical grade. All solvents and samples were filtered through 0.45-μm pore-size nylon membrane filters before use.

### 2.3. Preparation of ILC stationary phase

PC (0.1–1 g) was dissolved in chloroform and 0.4–4 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, then the dried silica was kept under high vacuum overnight to remove any remaining solvent. The PC film coated porous silica gel was swollen in buffer A for 2–4 h to form liposome, then washed three times with

buffer A to remove free and loosely coated liposomes.

### 2.4. Preparation of methanolic extracts of Radix Angelica Sinensis

Crude herbal drug (15 g) was crushed with a grinder, and the powder with less than 30 mesh size was immersed in methanol (3 ml/g powder) for 2 h, then the mixture was boiled and circulation refluxed for 1 h. The methanolic extract was filtered through a 0.45-μm filter when it cooled, and stored in a refrigerator until use.

### 2.5. ILC of Radix Angelica Sinensis extract

Liposome immobilized stationary phase prepared as previously described was packed into a stainless steel column with the dimension of 50×4.6 mm I.D. using the slurry method in our laboratory. Methanolic extract (10 μl) of *Radix Angelica Sinensis* was injected for HPLC experiments on the ILC column. Buffer A was used as mobile phase, the flow-rate was kept at 1 ml/min, and column temperature was ambient unless otherwise stated.

### 2.6. Preparation of permeable components in Radix Angelica Sinensis extract

A methanolic extract of *Radix Angelica Sinensis* prepared was concentrated 10 times by rotary evaporation before being separated. A 100-μl aliquot of the sample was injected under a stepwise gradient elution with 30, 50, 60, 70, 80% (v/v) methanol–water passing through the ODS column for 15 min in turn, respectively. Flow-rate was 1 ml/min. Compounds which had strong retention on the ILC column were prepared on an ODS column and stored in a refrigerator before identification with MS.

## 3. Results and discussion

### 3.1. Separation of permeable compounds in Radix Angelica Sinensis

Drug absorption in the human body is influenced by many physiological factors but the solubility, particle size, chemical form, and other physicochemical characteristics of drugs are also important factors. The dissociation constant and hydrophobicity of a drug, as well as the pH at the absorption site, determine the absorption characteristics of a drug from physiological fluid [25]. Increased hydrophobicity is often correlated with improved absorption and thus may result in greater pharmaceutical activity. But other kinds of interactions, such as electrostatic interaction and hydrogen bonding interaction between drugs and biological membranes are also important influencing factors for drug absorption because the polar head group of lipids in biological membrane is the first contact site between drugs and the surface [18]. ILC can simulate the interactions of drugs with biological membranes resulting from a combination of hydrophobic, ion pairing, and hydrogen bonding occurring simultaneously, while ODS surfaces primarily model hydrophobic interactions. So ILC is a better chromatographic model than ODS surface to evaluate the permeability of compounds through biological membrane in the drug discovery process.

Because some organic solvents can destroy the bilayer structure of liposome coated on the surface of silica, not all organic solvents can be used in the mobile phase. In order to eliminate the effect of silanol groups on the surface of silica, an appropriate salt concentration was needed, although high salt concentrations will strongly affect the stability of liposome coated on silica. In our study, 10 m*M* phosphate buffer with pH 7.0 and lower than 150 m*M* NaCl was used as mobile phase.

A chromatogram of a methanolic extract of *Radix Angelica Sinensis* on ILC under the experimental conditions described above is shown in Fig. 1. It can be seen that the first peak is large and off-scale, which indicates that interactions of most compounds with the liposome are weak and they were eluted out of the column in a short time. There are eight principal peaks, $a_1$, $a_2$, $a_3$, $a_4$, $a_5$, $a_6$, $a_7$ and $a_8$ with significant retention on the ILC column when detected at 210 nm, and another peak named $a_9$ between $a_1$ and $a_2$ could be detected at 280 nm as shown in Fig. 1. The strength of their interaction with the ILC stationary phase is in the order $a_1 < a_9 < a_2 < a_3 < a_4 < a_5 < a_6 < a_7 < a_8$. In addition to those nine principal peaks, there are also a number of minor

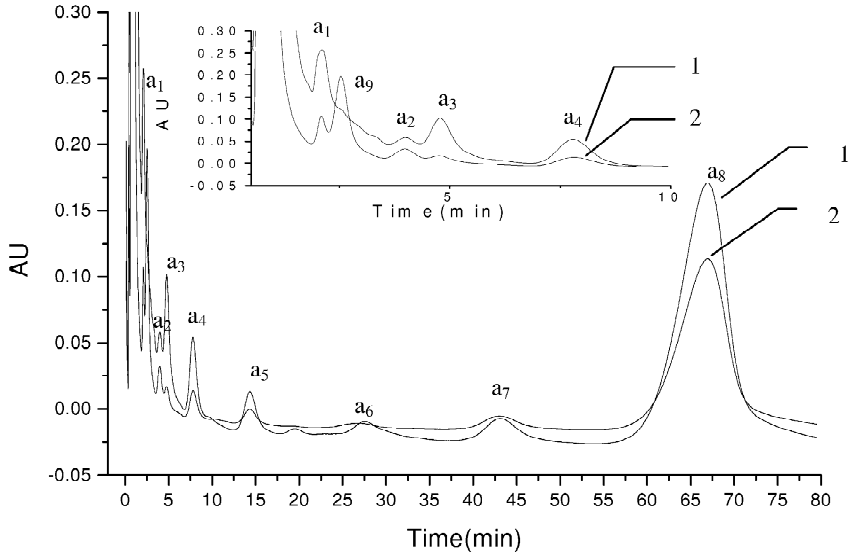


Fig. 1. Chromatograms of *Radix Angelica Sinensis* extract on ILC. Experimental conditions: mobile phase, buffer A; flow-rate, 1 ml/min; detection wavelength, 210 nm for chromatogram 1 and 280 nm for chromatogram 2. The inset shows an expansion of the first 10 min of the separation.

peaks. This means that some other components in the methanolic extract of *Radix Angelica Sinensis* have significant interactions with immobilized liposome. All these detected components can be regarded as permeable components; of course, it needs further study to prove whether or not they have practical pharmaceutical activity.

### *3.2. Identification of detected components*

MS is a powerful technique for identification of molecular structure. However, the fractions of peaks separated by ILC could not been directly applied for MS analysis because of the presence of a high concentration of inorganic salts. Thus we used RP-HPLC as complementary tool to prepare the components of interest as described previously.

The chromatogram of a methanolic extract of *Radix Angelica Sinensis* on ODS column with methanol/water (50:50, v/v) as mobile phase is shown in Fig. 2, in which more than 10 obvious peaks can be observed. We found the corresponding position of the peaks on HPLC and ILC through comparing their UV spectra obtained by photodiode array detection as shown in Fig. 3. Although there are some minor differences between some members of the two groups of spectra, for example, $a_6$ and H in Fig. 3, the different mobile phase used in RP-HPLC and ILC may be the main reason for these nuances. Preliminarily, the peaks $a_1$, $a_3$, $a_4$, $a_5$, $a_6$, $a_7$, $a_8$ and $a_9$ in Fig. 1 correspond to peaks A, F, E, G, H, I, J, and D in Fig. 2, respectively. The result was further proven by the retention behavior of the fractions of eight peaks purified with RP-HPLC on ILC as shown in Fig. 4, in which only peak D and $a_9$ was not shown because they could not been detected at 210 nm, but their retention also coincided with each other when detected at 280 nm. But there is no corresponding peak of peak $a_2$ in Fig. 2; a possible reason for this was because this component had no retention on the ODS column, so it was not shown in Fig. 3. The component of peak B retained on the ODS column did not strongly retain on ILC at pH 7.0, so it was also not shown in Fig. 3, but its retention increased at pH 5.4 and could be detected from *Radix Angelica Sinensis* extract as shown in Fig. 8. A similar phenomenon happened for ferulic acid, which is also marked in Fig. 8. As for peak C with obvious retention on RP-HPLC as shown in Fig. 2, it was not present in Fig. 1 and experiments

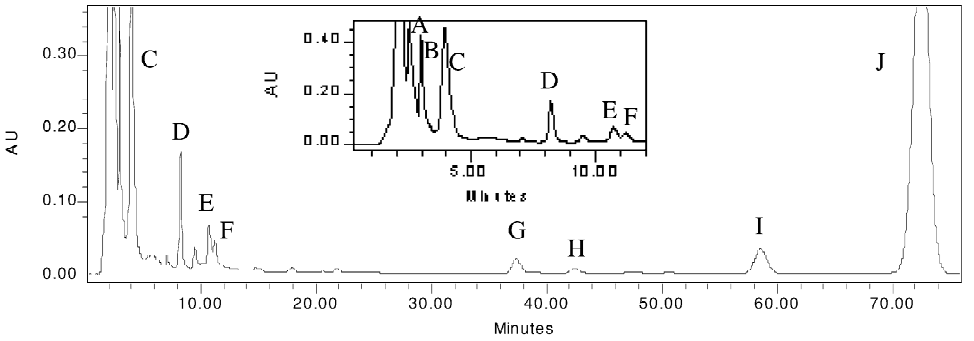


Fig. 2. Chromatogram of *Radix Angelica Sinensis* extract on RP-HPLC with ODS stationary phase. Experimental conditions: mobile phase, methanol/water (50:50, v/v); flow-rate, 1 ml/min; detection wavelength, 280 nm. The inset shows an expansion of the first 12 min of the separation.

showed that the retention of component for peak C prepared by RP-HPLC on ILC was very weak, and it was also not present in Fig. 3.

The molecular masses ($M_W$) of components for peaks D ($a_9$), F ($a_3$), G ($a_5$), H ($a_6$), I ($a_7$) and J ($a_8$) were determined by MS as 190, 224, 192, 194, 238 and 190, respectively. The MS spectrum of component for peak J as shown in Fig. 5 coincided with that of ligustilide reported in literature [26], and its UV spectrum shows three characteristic absorption peaks at 210, 280, and 328 nm, which also well matches with ligustilide [27]. So the component for peak J can be identified as ligustilide and its structure is shown in Fig. 6. The MS spectrum of component for peak G is also shown in Fig. 5. It can be seen that the component for peak G has a very similar fission behavior to ligustilide, so it is very possible that the former is a structural analog of the latter but with a molecular mass greater with the addition of two protons, i.e. one of the four double bonds of ligustilide may be saturated with two protons. Among all the four double bonds on ligustilide molecule marked as 1*, 2*, 3* and 4*, 3* and 4* have more possibility of being saturated than 1* and 2*. However, the hydrophobicity of the compound saturated with hydrogen on bond 3* will be changed very little compared with the original compound, while it will be greatly changed when bond 4* is saturated because of the formation of the hydroxyl group. The change in retention behavior of the component for peak G on RP-HPLC as shown in Fig. 2 showed that bond 4* is very possibly saturated. This result was further supported by its $^1$H NMR 400 MHz spectrum as shown in Fig. 7. As can be seen from the spectrum, there are three groups of peaks of hydrogen of ethylenic link, which always have a higher chemical shift (δ). The only explanation for this result is that the double bond 3* is intact. So the structure of the component for peak G may be identified as shown in Fig. 6, but its name could not be found in the literature, so a chemical name was given for component G as 3-butylidene-4,5-dihydro-2(1,3*H*)-1-isobenzofuranol according to the chemical name of ligustilide, 3-butylidene-4,5-dihydro-1(3H)-isobenzofuranone.

### *3.3. Effects of mobile phase composition*

It was found that the retention and selectivity of permeable components on ILC columns are strongly affected by the eluent pH. Separations of *Radix Angelica Sinensis* extract were carried out at the eluent pH from 4.5 to 7.4, and the observed results are shown in Fig. 8. It can be seen that the chromatogram patterns of *Radix Angelica Sinensis* extract on ILC at different pH values are somewhat different from each other. The number of peaks is highest at pH 5.4 and least at pH 7.4. For example, peak B in Fig. 2 could not be detected at pH 7.0 because it does not retain on ILC in this condition, but it was detected at pH 5.4 as shown in Fig. 8. In addition, ferulic acid identified with standard sample could not be detected until pH 4.5. It is evident that the eluent pH value obviously plays an important

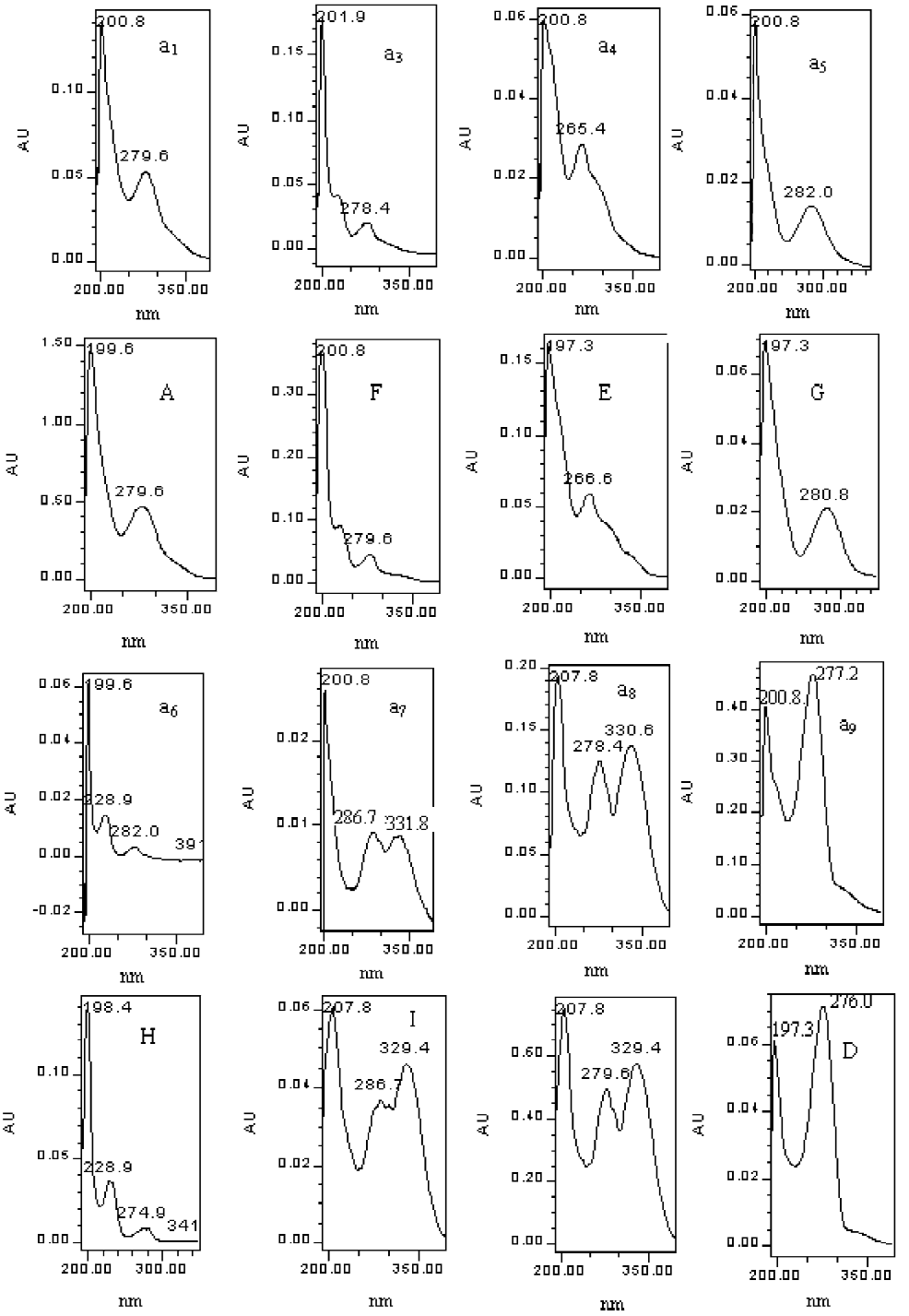


Fig. 3. Comparison of UV spectra of corresponding peaks on ILC and RP-HPLC; the alphabetical order corresponds to Figs. 1 and 2, respectively.

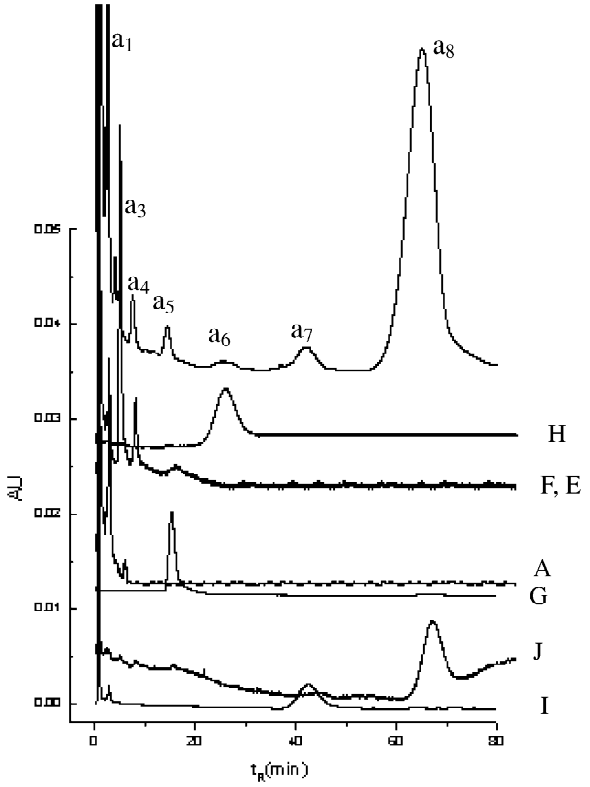


Fig. 4. Retention of compounds for the fractions of peaks prepared with RP-HPLC from *Radix Angelica Sinensis* extract on ILC. Experimental conditions: mobile phase, buffer A; flow-rate, 1 ml/min; detection wavelength, 210 nm.

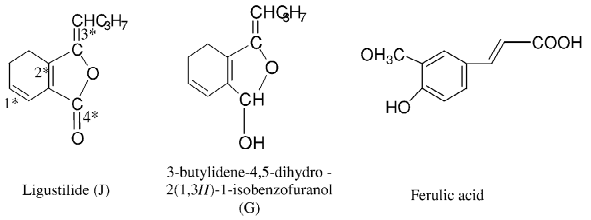


Fig. 6. Molecular structures of compounds identified in *Radix Angelica Sinensis.*

role in the retention and selectivity of solutes on the ILC column.

The effect of eluent ionic strength on the chromatogram of the extract of *Radix Angelica Sinensis* was investigated by adjusting the NaCl concentration to 50, 100, and 150 m*M*. The retention times of all retained solutes did not change significantly in the range of the salt concentration studied although all were slightly less retained with increase in salt concentration, indicating that ionic interactions affected the retentions of drugs to only a slight degree.

The effect of column temperature on the chromatogram of the *Radix Angelica Sinensis* extract was investigated by changing the column temperatures from 25 to 45 °C. Retention increased when reducing column temperature, but the selectivity was nearly unchanged.

## 4. Conclusion

Our study has demonstrated that ILC is a potentially powerful technique for screening and analysis

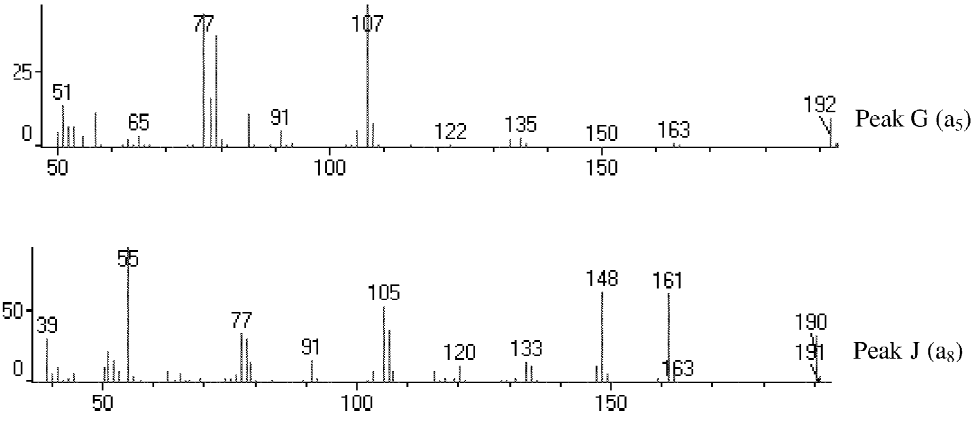


Fig. 5. MS spectra of components for peaks G ($a_5$) and J ($a_8$) prepared from methanolic extract of *Radix Angelica Sinensis* by RP-HPLC.

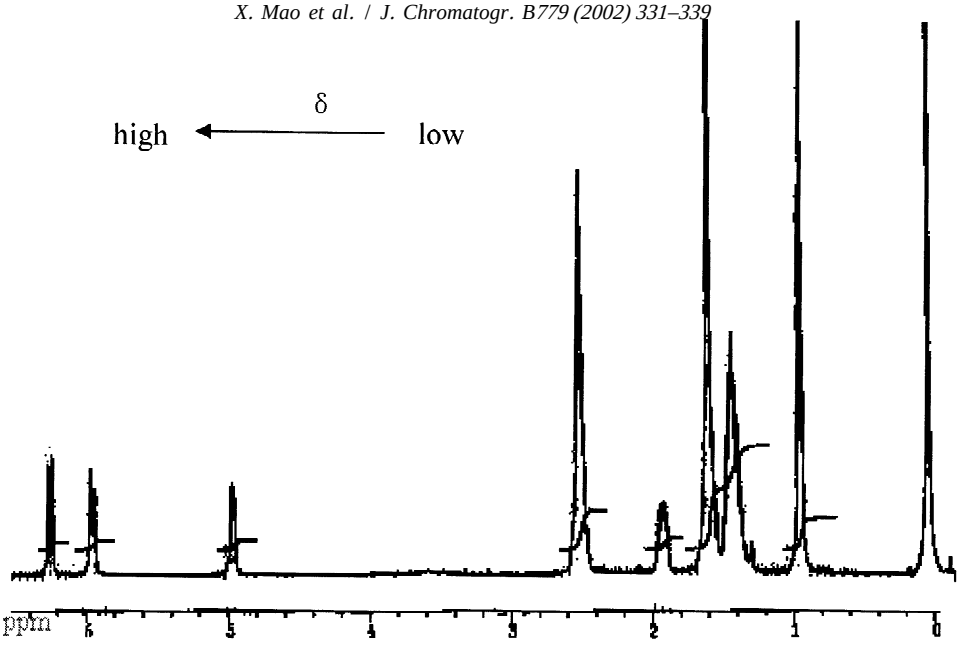


Fig. 7. $^{1}$H NMR (400 MHz) spectrum of component for peak G.

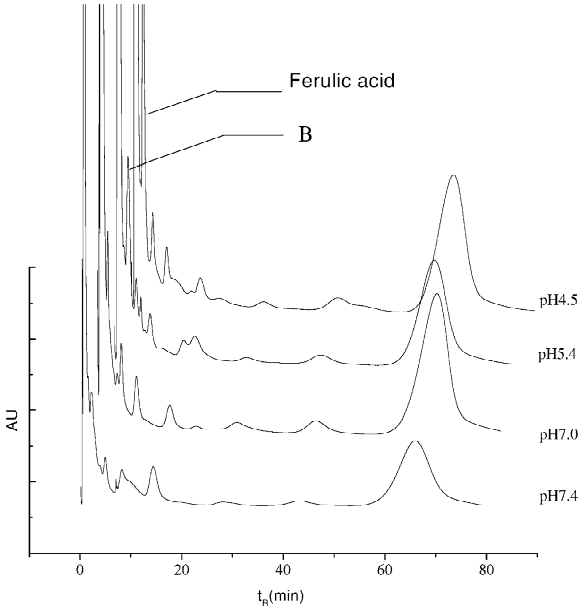


Fig. 8. Influence of eluent pH on the separation of *Radix Angelica Sinensis* extract on ILC. Experimental conditions: mobile phase, 10 m*M* phosphate buffer, containing 50 m*M* NaCl; flow-rate, 1 ml/min; detection wavelength, 210 nm.

of permeable components in TCMs. Because the pharmaceutical activity of TCMs may result from the combination of a number of bioactive compounds, ILC may be applied to predict the penetration ability of multiple compounds in TCMs simultaneously. Combination with other chromatographic models, such as biochromatography with immobilized protein stationary phases and RP-HPLC, as well as other complementary techniques, such as photodiode array detection, MS, and NMR will further improve the resolution and structural identification of permeable compounds in TCMs.

## Acknowledgements

Financial support from the Chinese Academy of Sciences (STZ-00-09) and Knowledge Innovation Program of DICP to Dr Hanfa Zou is gratefully acknowledged.

## References

[1] H. Wang, H. Zou, L. Kong, J. Ni, J. Basic Clin. Physiol. Pharmacol. 11 (2000) 155.

[2] L. Kong, H. Zou, H. Wang, J. Ni, Y. Zhang, Chem. J. Chin. Univ. 21 (2000) 36.
[3] G.H. Du, J.J. Hu, L.J. Xia, J.X. Huang, J.T. Zhang, Acta Pharmacol. Sin. 33 (1998) 876.
[4] A. Koltermann, U. Kettling, J. Bieschke, T. Winkler, M. Eigen, Proc. Natl. Acad. Sci. USA 95 (1998) 1421.
[5] M.H. Pausch, Trends Biotechnol. 15 (1997) 487.
[6] H. Wang, H. Zou, J. Ni, B. Guo, Chromatographia 52 (2000) 459.
[7] H. Wang, L. Kong, H. Zou, J. Ni, Y. Zhang, Chromatographia 50 (1999) 439.
[8] H. Wang, L. Kong, H. Zou, J. Ni, Y. Zhang, Chin. J. Chromatogr. 17 (1999) 119.
[9] H.L. Wang, H.F. Zou, J.Y. Ni, L. Kong, S. Gao, B.C. Guo, J. Chromatogr. A 870 (2000) 501.
[10] S. Ong, H. Liu, C. Pidgeon, J. Chromatogr. A 728 (1996) 113.
[11] H. Liu, S. Ong, L. Glunz, C. Pidgeon, Anal. Chem. 67 (1995) 3550.
[12] T.H. Lee, M.I. Aguilar, Adv. Chromatogr. 41 (2001) 175.
[13] X. Mao, L. Kong, H. Wang, H. Zou, Chin. J. Anal. Chem. 30 (2002) 231.
[14] P. Lundahl, F. Beigi, Adv. Drug Deliv. Rev. 23 (1997) 221.
[15] X. Liu, Q. Yang, N. Kamo, J.J. Miyake, J. Chromatogr. A 913 (2001) 123.
[16] C. Pidgeon, S. Ong, H. Chol, H. Liu, Anal. Chem. 66 (1994) 2701.
[17] C. Pidgeon, S. Ong, CHEMTECH 25 (1995) 38.
[18] S. Ong, H. Liu, X. Qiu, C. Pidgeon, Anal. Chem. 67 (1995) 755.
[19] C.Y. Yang, S.J. Cai, H. Liu, C. Pidgeon, Adv. Drug Deliv. Rev. 23 (1996) 229.
[20] P. Artursson, J. Karlsson, Biochem. Biophys. Res. Commun. 175 (1991) 880.
[21] C. Altomare, R. Tsai, N.E. Tayar, B. Testa, A. Carotti, S. Cellamare, P.G.D. Benedetti, J. Pharm. Pharmacol. 43 (1991) 191.
[22] P. Artursson, J. Pharm. Sci. 79 (1990) 476.
[23] X. Mao, H. Zou, Q. Luo, L. Kong, X. Li, N. Sun, Chin. J. Chromatogr. 19 (2001) 433.
[24] X. Mao, H. Zou, Q. Luo, L. Kong, X. Li, N. Sun, Chin. J. Anal. Chem. 29 (2001) 1135.
[25] Q.-M. Wang (Ed.), Pharmacology, Shanghai Science and Technology Press, Shanghai, China, 1994, p. 7.
[26] Y. Chen, N. Chen, X. Ma, H. Li, Chem. J. Chin. Univ. 5 (1984) 125.
[27] J. Tao, Y. Ruan, Q. Mei, Acta Pharmacol. Sin. 19 (1984) 561.