Characters of the Plateau of Methanol Increment in Frontal Analysis in Reversed Phase Liquid Chromatography

GENG, Xin-Du*,[a](耿信笃) REGNIER, Fred E[b](弗莱德 依 瑞格涅尔)
[a]*Institute of Modern Separation Science, Northwest University, Xi'an, Shaanxi 710069, China*
[b]*Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA*

With insulin methanol-water, and the ion-pairing agent, hydrochloric acid and trifluroacetic acid (TFA), the character of the first plateau (FP) on the elution curve of frontal analysis in reversed phase liquid chromatography (RPLC) was investigated by on-line UV-spectrometry and identified with nuclear magnetic resonance (NMR) spectrometry and mass spectrometry. The profile of the FP is the same as that of a usual elution curve of methanol in frontal analysis (FA). When the insulin concentration was limited to a certain range, the height of the FP was found to be proportional to the insulin concentration in mobile phase and its length companying to shorten. The FP profile on the intersection of two tangents reflects the components of the microstructure in the depth direction of the bonded stationary phase layer and the desorption dynamics of the displaced components. The displaced methanol was quantitatively determined by NMR and on-line UV spectrometries.

TFA with high UV absorbance can not be used as an ion-pairing agent for the investigation of the FP in RPLC, but it can be used as a good marker to investigate the complicated transfer process of components in the stationary phase in RPLC. A stoichiometric displacement process between solute and solvent was proved to be valid in both usual and FA in RPLC. From the point of view of dynamics of mass transfer, the solutes can only contact to the surface of stationary phase in usual RPLC, while solute can penetrate into it in FA of RPLC. The solvation of insulin in methanol and water solution as an example indicating the usage of the FP in the FA was also investigated in this paper.

Keywords displacement mechanism, frontal analysis, ion-pairing agent, methanol increment, partition mechanism, reversed phase liquid chromatography

Introduction

The argument about the retention mechanism of solute in reversed phase liquid chromatography (RPLC) mainly involves the interfacial process. Four theoretical puzzles in RPLC have not been solved yet.[1] Two of them are: (1) whether solute is of partition, adsorption, or the mixture of both. (2) Do sample molecules penetrate into the bonded phase and/or adsorb at the interface between the two phases? With the extensive investigation of the interfacial process, the difficult problems may not only be solved, but some new high technologies can also be explored. The facts that the total changes in thermodynamic functions of component during interfacial process were recently reported to divide into independently net adsorbed and net desorbed one's are good examples to indicate the significance of the interface process.[2-5] This point strongly supports the evaluation of free energy of protein folding on the interface of stationary phase in hydrophobic interaction chromatography and thus contributes to understanding the mechanism of protein folding.[6-10] In the previous study, the first plateau (FP) before the main plateau of insulin elution curve by frontal analysis (FA) in RPLC was reported. It was confirmed that when a solute was adsorbed, organic solvent must leave from the stationary phase in RPLC.[11] This point proves the retention mechanism of solute in RPLC to be of displacement mechanism. One of the authors reported that no matter whether the retention mechanism of solute in RPLC is of partition mechanism, or displacement mechanism, even the mixture of

* E-mail: xdgeng@nwu.edu.cn
Received June 21, 2001; revised November 28, 2001; accepted January 7, 2001.
Project supported by the National Natural Science Foundation of China (Nos. 39880003 and 20175016).

both, but they totally obey the stoichiometric displacement model for retention of solute (SDM-R).[12-14] But it still needs to be proved by a direct evidence.

Because the FP actually causes from solute adsorption, the character of the FP may reflect the retention behavior of the solute from the other side. The solute retention in RPLC occurs in a very complicated bonded stationary phase layer (bonded layer) consisting of ligands bonding to silica gel with one end but flexibly swinging with other end and liquid mixture of organic solvent, ion-pair agent and water. From the profile of the FP, it is possible to provide some information to understand not only the composition and microstructure of the bonded layer, but also the dynamics of the solute sorption. In addition, the exploring the applications of the FP would also be significant. In this study, with insulin, methanol-water, and the ion-pair agent, trifluroacetic acid (TFA) and HCl, the character of the FP in FA was investigated by on-line UV spectrometry, nuclear magnetic resonance (NMR) spectrometry, and mass spectrometry.

Experimental

Equipment and chemicals

A Hewlett Packerd 1090 liquid chromatograph from Hewlett Packerd Co. (CA, USA) consisting of three pumps with a diode-array detector was used for the frontal chromatographic FC analysis and an on-line UV spectrometry. A color Pro plotter from the same manufacturer was used for plotting the merged elution curves. The solvent deliver system employed was the same as that in the previous paper.[11] A SynChrompak RP-P (100 mm × 4.6 mm, particle size 5.6 μm, pore diameter 30 nm) was purchased from SynChrom Inc (West Lafayette, IN, USA), RPLC column temperature was controlled at 25 ± 0.50 ℃ with a water bath. A Gemini 200, nuclear magnetic resonance spectrometer (NMR) from Varian Co. (Palo Alto, CA, USA) and a NMR tube (Kontes, diameter 5 mm, grade 6528 pp) from Scientific Glassware/Instruments were used for NMR analysis.

Insulin (bovine pancreas, HPLC) was obtained from Sigma Co.. Methanol was bought from EM Science (Gibbstone, NJ, USA). Hydrochloric acid (Ultrex, Ultrapure Reagent) was obtained from J. T. Baker Co.. Trifluroacetic acid (HPLC/spectro grade) was obtained from Pierce (Rockfold, IL, USA). Acetic acid (glacial, Fisher Chemical) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). The pure water employed was double-deionized water.

Mobile phase 1: 47.0% methanol-water ($V_{methanol}/V_{water}$) with 0.030% hydrochloric acid (HCl) ($V_{HCl}/V_{mobile\ phase}$). Mobile phase 2: 45.0% methanol-water ($V_{methanol}/V_{water}$) with 0.10% trifluroacetic acid (TFA) ($V_{TFA}/V_{mobile\ phase}$).

Experiment procedure

The method for obtaining the elution curves of the methanol increment in water with 0.10% TFA and 0.03% HCl was followed by Huang *et al*.[15] and the same procedure as that reported in the previous paper.[11] Except the methanol concentration in mobile phase was selected according to the capacity factor of insulin to be in the range of 2—10, the other experimental procedures including column cleaning and a special baseline were also as the same as that in the previous paper.[11]

For the FC analysis of insulin, the insulin concentration was adjusted by the ratio of the flow rate of insulin solution to that of mobile phase. The flow rate was 0.40 mL/min and the signal was recorded with an on-line UV spectrometry at a wavelength of 198 nm with a reference wavelength of 280 nm. The fraction of the eluate from 0.0 min to 11.77 min was collected as the blank for NMR determination, and the fraction between 11.77 min and the point just before insulin exited was collected for the determination of the methanol increment with NMR, while the plateau height in this range on the elution curve was used for measuring the amount of the methanol increment displaced by insulin. To obtain a complete elution curve of insulin, the adsorption had to be allowed to proceed until 5 to 10 min after breakthrough of insulin on the elution curve.

The NMR measurement was followed Ref. [11] and carried out by using deuterium oxide to be solvent.

Results and discussion

Stoichiometric displacement between methanol and TFA

It is well known that the presence of ion-pairing agents, such as TFA, formic acid and phosphate buffer solution with pH 2.5—3.0 in mobile phase is required for biopolymer separations by RPLC. Hydrochloric acid

(HCl) as an ion-pairing agent was reported for peptide separation[16,17] and the FA of insulin in previous paper.[11] TFA was reported to have three functions for biopolymer separations:[18] (1) to interact with proteins to make TFA concentration decreases in the sample solution of biopolymers; (2) TFA can be adsorbed by RPLC stationary phase and accumulated on it to modify its character; (3) to join in the stoichiometric displacement process between biopolymers and organic solvent as if it were a solute. As TFA has strong UV absorbance, the TFA displaced by methanol can be observed as a peak. In usual RPLC, the TFA displaced would come out together with solvent peak. Thus, it can not be easily identified.

Fig. 1 shows two sets of the elution curves of methanol increment with 0.10% TFA in 45.025% to 45.50% methanol solution shown in Fig. 1A, and 0.03% HCl in 47.05% to 47.50% methanol solution shown in Fig. 1B, respectively. To increase the UV absorbance of the methanol increment and diminish the interference in case of the presence of the residuary insulin and other biopolymers, the detection wavelength for the both was at 198 nm with reference wavelength of 280 nm.[11] The corresponding concentrations of the methanol increment in Fig. 1A are 0.025%, 0.05%, 0.10%, 0.20%, 0.30%, 0.40%, and 0.50%. Fig. 1B shows the corresponding concentrations of methanol increment are 0.05%, 0.10%, 0.20%, 0.30%, 0.40% and 0.50%.

Compared to Figs. 1A and 1B, as it was reported in the previous paper,[11] Fig. 1B is closer to the typical profile of elution curve of solute in FA and to be suitable for the quantitative determination of the methanol increment with an on-line UV spectrometry. Because each baseline in Fig. 1A even did not coincide with each other (in the region between the two dash lines) and the section of the elution curve on the intersection region of the two tangents performs an non smooth profile, it is difficult to determine of methanol increment with an on-line UV spectrometry.

A common point for Figs. 1A and 1B is that when the methanol increment is over a certain value, a peak or plateau exists on the intersection region, although this phenomenon is not very clear in Fig. 1B. From the comparison between Figs. 1A and 1B, a reduction can be obtained that TFA here looks as if it were a usual solute and it forms a peak as it is displaced by methanol.

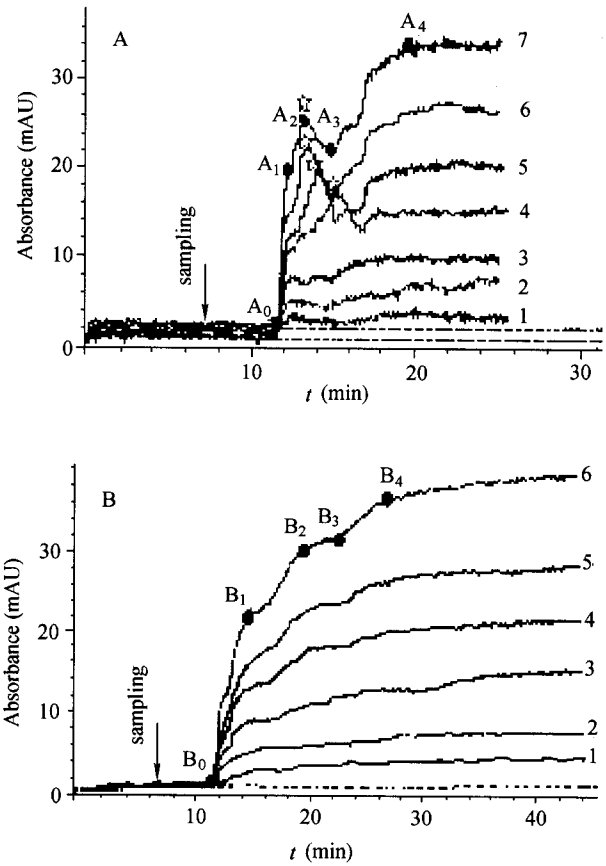

Fig. 1 Elution curves of various concentration of methanol with two kinds of ion-paring agents. (A) Methanol and water with 0.10% TFA. The methanol concentrations (%, *V/V*) are: (1) 45.025, (2) 45.050, (3) 45.10, (4) 45.20, (5) 45.30, (6) 45.40 and (7) 45.50. (B) Methanol and water with 0.03% HCl. The methanol concentrations (%, *V/V*) are: (1) 47.05, (2) 47.10, (3) 47.20, (4) 47.30, (5) 47.40 and (6) 47.50. SynChropak, RP-C18; flow rate, 0.40 mL/min. Detection and reference wavelengths to be 198 nm vs. 280 nm; sampling time, 7.50 min.

To improve this point further, the difference of UV absorbance between TFA and HCl needs to be compared. Fig. 2 shows a set of chromatograms of TFA with various concentrations by usual RPLC with mobile phase of 45.0% methanol and water solution. The curves 1, 2, 3, 4 and 5 show the chromatograms obtained from 25 μL of TFA with concentrations 0.05%, 0.10%, 0.20%, 0.30% and 0.40%, respectively. As shown in Fig. 2, the UV absorbance of TFA at wavelength 198 nm with reference wavelength 280 nm is very strong. For example,

the change in the average UV absorbance intensity of 25 μL of 0.1% TFA in usual RPLC is equivalent to ten times of that from methanol changing in 0.30% (*V*/*V*) in FA. However, the UV absorbances of 0.03% HCl and phosphate (0.03 mol/L) buffer solution with pH 2.0 of 25 μL are only 4.5 mAU for the former and 4.3 mAU for the latter. That is only 1/50 UV absorbance from 0.1% TFA detected at the same wavelength.

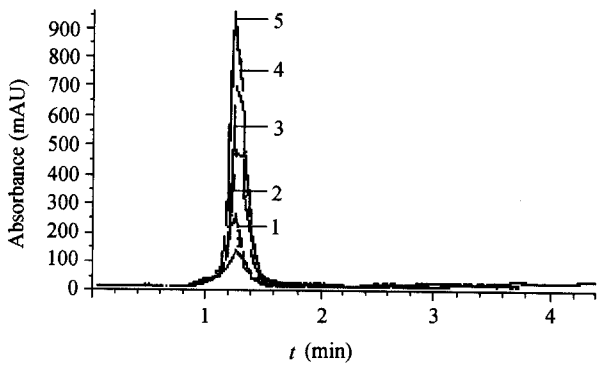

Fig. 2 Peak height of different concentration of TFA. Methanol/water (45%, *V*/*V*) solution; detection wavelength, 198.4 nm with reference wavelength 280.20 nm; flow rate, 1.0 ml/min. Sample volume, 25 μL. Peaks 1, 2, 3, 4 and 5 represent the TFA concentrations are 0.05%, 0.10%, 0.20%, 0.30% and 0.40% (*V*/*V*), respectively.

A very interesting thing observed was that the appear time of the TFA peak shown in Fig. 1A increases with the decreases in the methanol concentration in the mobile phase. If one of the function of TFA is as a solute, the appear time of TFA peak here may be simply called "retention" time. Because the retention times of TFA in the range of methanol concentration covering 0.20% to 0.50% changed not much, the peak height (from the bottom of each valleys to the top of the corresponding peaks) was measured to be almost invariable, 2.58 ± 0.24 mAU.

If the TFA is really a solute, the displacement between methanol and TFA should be stoichiometric and follows the expression of the SDM-R [Eq. (1)],

$$\log k' = \log I - Z\log a_D \qquad (1)$$

Where k' is the capacity factor of TFA. $\log I$ is a batch of constant relating to the affinity of TFA to the RPLC stationary phase. Z is the total moles of the methanol displaced at the contact region between TFA and the stationary phase, as one mole of TFA is adsorbed by the stationary phase. The a_D is the methanol activity in the mobile phase.

With the linear plot by Eq. (1), the linear correlation coefficient being 0.98 indicates that the displacement relationship between methanol and TFA is really stoichiometric. $\log I$ and Z were measured to be 130.5 and 122.5, respectively. It means that if 1 mol of TFA desorbs from the stationary phase, 122.5 mol of methanol has to be adsorbed by the stationary phase. To satisfy this condition, it requires either high enough methanol increment, or a long enough time to accomplish TFA desorption. It should be that the higher the methanol increment, the shorter the time takes. When methanol increment is $\geqslant 0.20\%$, although the "retention" of the TFA increases with the decreases in methanol increment in mobile phase, each TFA peak (the elution curves 4—7) can be clearly observed and, as pointed above, has almost the same height, but the depth of each valley (from the valley bottom to the plateau top of the methanol) is getting lower and lower until to zero (see elution curves 1—3). When the concentration of the methanol increment is $\leqslant 0.10\%$, the retention of the displaced TFA is so long that it likes the eluted out of biopolymers by isocratic elution by usual RPLC, resulting the peak to become very broad with very low peak height until it can not be observed.

It is surprising that the magnitudes of $\log I$ and Z of TFA can be comparable to that of biopolymers.[13,14] We used the same experimental condition to measure both $\log I$ and Z of TFA with usual RPLC. As we expected, TFA had no retention as the methanol concentration changed from the same concentration range from 47.0% to 47.50%. This fact shows that the displacement process between TFA and methanol may be different between usual RPLC and FA in RPLC. We now need to answer two questions. (1) Why does TFA peak in the intersection region of each elution curve appear and each peak has almost the same height? (2) Why is the peaks clearer for TFA than for HCl?

When the mobile phase employed in a chromatographic system only has three components, one of ion-pair agents, methanol and water, and establishes equilibrium between two phases, the stationary phase contains the same components also. If the concentration of anyone of the three components changes, the supposed methanol concentration increases in the mobile phase and the ad-

sorbed methanol in the stationary phase should increase also. According to the two principles in physics that conservation of energy and one space can not simultaneously occupied by two objects, both of the ion-pair agent and water have to leave from the bonded layer and then reestablish a new equilibrium between the two phases. The fact that the peak height of the displaced TFA was measured to be 2.58 ± 0.24 mAU indicates that the displaced amount of TFA or water is believed to be independent of the concentration of methanol in the mobile phase. In other words, a stoichiometric displacement process among the three components definitely occurs.

From the stand point of polarity of those components, the polarity increases in the order as: TFA < methanol < water < HCl. If the four components were washed out in a usual RPLC, it would be eluted as peaks and the elution order would be the same as the decreases in the order of their polarity. However, the order of the washed out of the displaced components from the bonded stationary phase layer (bonded layer) depends on the resided regions of them in the depth direction of the bonded layer. Because the methanol increment (*i. e.* water decrement in same solution) in this study entered the FA system as a continuous matter, the methanol increment or water decrement should not form a peak, but form a plateau, while the desorbed ion-pair agent should form a peak on the intersection region of the two tangents, or the break-though region. Based on the intensity of their UV absorbance, the profiles of peak or plateau may either increase the heights of peak and plateau, or decrease them. The UV absorbance of TFA is much higher than that of HCl, resulting to appear a TFA peak in Fig. 1A, but not in Fig. 1B.

From the foregoing discussion, a conclusion can be obtained here. The fact that when the methanol concentration is greater than 0.20%, the peak height of TFA is independent of the concentration of methanol increment shown in Fig. 1A and the good linearity of Eq. (1) further proves the displaced process between methanol and the ion-pair agents to be a stoichiometric. Two more questions still need to answer: (1) why does a valley appear in the intersection of the elution curve of methanol increment in Fig. 1A and do two plateaus occur in Fig. 1B? (2) why is the Z value obtained from FA significant difference from that with usual RPLC method?

To answer the two questions, we should firstly know the complicated microstructure of the bonded layer and then do the comparisons of the UV-absorbance and the polarity among TFA, HCl, water and methanol. Secondly, we should discuss the comparisons of distribution rate of components in two phases, or the dynamics of mass transfer at the interface between the two phases. Lastly, we need to know whether solute enters into the bonded layer, or just contacts to it in usual RPLC. The last problem, as pointed above, is also the one of the four theoretical puzzles which have not been solved yet.[1]

Discontinuous change of the displaced components and the curve profile on the intersection region between two tangents

The formation of the FP in FA was reported to come from the solvated bonded phase and solute.[11] Based on the results from electron spin resonance spectrometry (ESR) and NMR that the polarity of the bonded phase solvation layer varies with the composition of the bulk mobile phase as well as the spatial position (depth) along the alkyl chain.[19,20] It means that the RPLC bonded phase layer or adsorption layer is inhomogeneous in its depth direction.

Millere *et al.* concluded[20,21] the whole bonded phase layer in RPLC can, at least, be divided into four regions dependenting on the concentration of methanol in mobile phase. The region IV has a solvation environment characteristic of organic modifier regardless of bulk mobile phase composition. The region III is surface-bond water and silanols, hydrogen bonding solutes interact with this region and it has aqueous character and a high microviscosity. The compositions of other two regions, region I at the interface between the two phases as methanol content of the bulk mobile is $\geqslant 50\%$ and region II near by the silica surface which depends on the composition of the mobile phase as the aqueous content was $\geqslant 50\%$ in it.

Miller *et al.* used a continuous manner to enter solutes through a RPLC column with methanol-water solution and tested it by ESR.[21,22] This methodology is very similar to that of FA employed in this study. In addition, the composition of mobile phase used for the two circumstances of Miller *et al.* method and this study is almost the same, methanol concentration to be approximate of 50%. The difference between the two methods is that Miller *et al.* used the test solutes with various polarities adsorbed on the RPLC packing materials, while we tested the eluted components by methanol and detected with online UV spectrometry. Miller *et al.*'s conclusion should

be valid for our study here.

For convenience, the elution curves on the breakthough region in Figs. 1A and 1B can be divided into four regions. According to the conclusion by Miller *et al.*, the order of the region from silica surface to mobile phase should be the regions III, II, IV and I. This order is not the same as that of the polarity of the components existing in the bonded layer. Except the water bonding to silanols in region III may never be displaced by means of elution, the other three regions would correspond to the curves as: the region I to the curves A_0A_1 and B_0B_1, the region IV to curves A_1A_2 and B_1B_2, and the region II to the curves A_2A_3 and B_2B_3. The curves A_3A_4 and B_3B_4 correspond to the differences of the methanol concentration between the final equilibrium in the mobile phase and that in the region II.

Some common points between Figs. 1A and 1B should be firstly compared with each other. The two lines in the two figures before the points A_0 and B_0 and that after A_4 and B_4 separately represent the original equilibrium concentrations of methanol (45.0% methanol in Fig. 1A and 47.0% methanol in Fig.1 B) and the final equilibrium methanol concentrations (45.50% methanol in Fig. 1A and 47.50% methanol in Fig. 1B) in the mobile phases. Because the stationary phase in RPLC is known to have very strong non-polarity, the non-polarity in the region I should be much higher than that in the mobile phase. In addition, the difference of the original methanol concentrations between the two mobile phases is only 2%, resulting the difference of the methanol concentration between the mobile phase and the region I for the two circumstances is almost the same, so does the two jump height.

Second, some differences existed in Figs. 1A and 1B must be explained. A sharp peak, ($A_1A_2A_3$) followed by a deep valley ($A_2A_3A_4$) with shorter "retention" time (~ 13 min) appears in Fig. 1A, while a very broad "peak" ($B_1B_2B_3$) with a shallow valley ($B_2B_3B_4$) exists in Fig. 1B. The curves from A_1 to A_4 and B_1 to B_4 correspond to the regions IV and II. As it pointed above, TFA in Fig. 1A has the strongest non-polarity and UV absorbance among TFA, methanol, HCl and water and should stay in the region IV, while HCl in Fig. 1B has the strongest polarity and very weak UV absorbance among them and, thus should not stay in region IV. Because the very weak UV absorbance and very low concentration in the mobile phase, for convenience, its contribution to the profile of the curves, from B_1 to B_4 may be ignored. The compositions in the region IV are mainly TFA in methanol in Fig. 1A, while that is only methanol in Fig. 1B. The stronger UV absorbance of TFA than methanol causes a big UV jump (A_1A_2) and a slowly little increase in UV absorbance (B_1B_2). From the point of view of the absolute peak heights from A_1 to A_2 and from B_1 to B_2, the both is almost comparable. However, the absolute height includes the contribution of the base line caused from the equilibrium on the half way. If the connection straight lines between A_1A_3 and B_1B_3 can be referred to be the base line on the half way, the absolute contribution of the region IV in Fig. 1B is actually much lower than that in Fig. 1A. The force driving methanol in the region IV to come out for the two circumstances is only due to the diffusion of methanol from high concentration to low concentration. However, for Fig. 1A, except methanol diffusion, the difference of chemical potential between TFA and methanol drives TFA come out, resulting to have a shorter "retention" time.

In addition, it could be seen from Figs. 1A and 1B that it must take, at least, 20 min for methanol in Fig. 1A and 30 min for TFA in Fig. 1B to reestablish the new equilibrium among the regions III, II, IV and I, and the mobile phase, even though the methanol concentration in the mobile phase only changes a very narrow range (0.50%). This is very important fact to explain the retention mechanism of solute in RPLC. It indicates that we must pay attention to the dynamics of mass transfer at the interface between stationary and mobile phases in RPLC. Suppose solute could penetrate into the interior of the bonded layer. Although we do not know how long the solute takes to arrive at the resided the interior of the bonded layer, we now know that it, at least, takes 20 min to move out of the bonded layer. In usual RPLC, the retention time of solute is usually only a few min. A very important conclusion can be obtained that solute has no enough time to penetrate into the interior of the bonded layer and then is washed out. It is believed that solute only contacts to the surface, or, at least, only can reside in the surface region of the bonded layer to displace the organic modifier molecules originally residing in the surface region of the bonded layer in RPLC. However, TFA in FA of RPLC, can penetrate into the interior of the bonded layer, because it has enough time to make equilibrium between two phases before doing chromatographic separations.

TFA in the interior of the bonded layer means that it is embraced by alkyl chains, methanol and water. It needs more methanol molecules to squeeze TFA out of the bonded layer. This is the reason why Z determined by Eq. (1) is very large. On the other hand, whole TFA molecules interact with the embraced components, and thus, the affinity to the stationary phase is also very large. It is easy to understand why $\log I$ evaluated by Eq. (1) is so large. Traditionally, the mechanism of solute retention here belongs to partition mechanism. We know that in this circumstance, the solute retention still follows the stoichiometric displacement model for retention of solute.

From the foregoing discussion, several very important conclusions can be obtained as the following:

(1) In RPLC, it takes 20—30 min to set up a new equilibrium. This is true even though the difference between two concentrations is only 0.20%.

(2) Based on the dynamics of the desorption of TFA and water, solute in usual RPLC has no enough time to leave from the interior of the bonded layer.

(3) So long as an adsorption of component and its reversed process, its desorption on a solid surface is dominated by chemical equilibrium, no matter whether the component can penetrate into the interior of the bonded phase layer, or just contact to the surface of the stationary phase, any components in anywhere in the stationary phase have to leave from the stationary phase and return into the mobile phase, as a solute is adsorbed. This is just the displacement between solute and solvent. Thus, it is not important to argue whether the retention mechanism is of partition, adsorption, even the mixture of both.

(4) The displacements between solute and organic solvent in usual RPLC and FA are proved to be true. However, the both process may not be same. For usual RPLC, the displacement only occurs in the contact surface, or called direct displacement, while for FA it occurs everywhere in the whole bonded layer. It includes direct and indirect, or induced displacement.

(5) TFA, as an ion-pairing agent for protein separation, is a mark to investigate the retention mechanism of small solute in RPLC. TFA in this study really penetrates into the bonded layer of RPLC and joins in the displacement among water and methanol.

A set of elution curve of insulin

Corresponding to a group of elution curves of methanol shown in Fig. 1B, a set of elution curve of the FP with insulin adsorption was also accomplished. The concentrations of insulin in the mobile phase separately are 0.025, 0.050, 0.075, 0.100, 0.200, 0.300 and 0.400 mg/mL. With zoom techniques, a corresponding FP were obtained. To see those clearly, only two of them, 0.025 (curve 4) and 0.100 mg/mL (curve 5), were shown in Fig. 3. In convenience for comparing to the elution curves of the FP to that of methanol, three of the elution curves of methanol with the concentrations

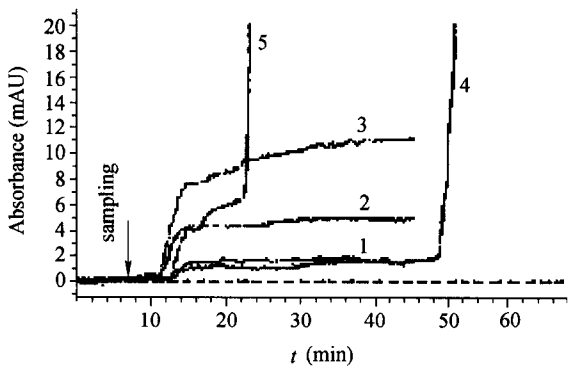

Fig. 3 Inserting the small plateaus of the methanol increament displaced by insulin into the elution curves of pure methanol. All of experimental conditions are the same as shown in Fig. 1. Methanol increments are: (1) 0.05%, (2) 0.10% and (3) 0.20%. Insulin concentrations are: (4) 0.025 mg/mL and (5) 0.10 mg/mL.

47.05% (curve 1), 47.10% (curve 2) and 47.20% (curve 3) methanol originally shown in Fig. 1B are now merged with that two FP of insulin and compared in the Fig. 3. From Fig. 3, the plateau height of the FP from 0.025 mg/mL insulin is a bit lower than that from 47.05% methanol. Additionally, the two top lines are almost parallel to the common base line denoted with dash line. It means that the concentration of the methanol increment from 0.025 mg/mL insulin is almost comparable to 0.050% extra. methanol. In fact, its concentration is of $(0.033 \pm 0.002)\%$ (see Table 1). However, the FP profile from 0.10 mg/mL insulin is much alike to that from 47.50% shown in Fig. 1B. The average methanol increment from two determinations is $(0.164 \pm 0.006)\%$ (Table 1). With the increases in the insulin concentra-

tion, the FP would decrease gradually until both of the FP and the main plateau of insulin itself unifying into one plateau, *i.e.*, the main plateau. That is the maximum concentration of the produced extra methanol which can be determined by UV spectrometry. The main problem is that the FP is not parallel to the common base line. If both h_1 and h_2 reported in the previous paper can be used for the measurement of the FP when insulin concentration is 0.025 mg/mL, only h_2 can be used for that when insulin concentration is 0.10 mg/mL.[11] All of the average results of the displaced methanol increment due to insulin adsorption by on-line UV-spectrometry were listed in Table 1.

Table 1 Comparisons between found methanol increment by UV-spectrometry and NMR[a,b]

$c_{insukin}$(mg/mL)	$c_{methanol}$ found (%, *V/V*)		
	UV-spectrometry[c]	NMR	
	0.03% HCl	0.03% HCl	0.1% TFA
0.025	0.033 ± 0.002	------	------
0.050	0.049 ± 0.003	------	------
0.075	0.064 ± 0.006	------	------
0.10	0.122 ± 0.009	0.17 ± 0.04	0.29 ± 0.03
0.20	0.164 ± 0.007	0.20 ± 0.04	0.32 ± 0.02
0.30	0.177 ± 0.018	0.24 ± 0.02	0.33 ± 0.02
0.40	0.198 ± 0.022	0.19 ± 0.02	0.25 ± 0.02

[a] SynChropak-RP C18, methanol/water (1, 47.0% + 0.03% HCl; 2, 45.0% + 0.10% TFA), (25 ± 0.5) ℃. [b] Average values of three continuously individual determinations for both UV-spectrometry and NMR.

The UV-absorbance of methanol measured with on-line, without doing the correction of evaporation, adsorption or desorption from containers, compared to NMR, its correction is much simpler and easier. Based on this point, the result obtained from UV-absoption should be better than that from NMR, so long as the system cleaned is very well and all of chemicals used must be very pure. However, without NMR identification, we can not conclude that UV spectrometry can be used to determine the extra methanol with very low concentration in the presence of an aqueous methanolic solution with a very high concentration.

Identifications with mass spectrometry and NMR

Mass spectrometry (MS) is very reliable and sensitive method to identify whether any kind of biopolymers exists in the fraction covering 11.77—34 min. To collect this fraction in the range and to dry in vacuum. Its residue in it was tested with MS. The result from MS showed that any substances in the range of molecular weight covering 1000—6000 Dalton was not detected.

In the previous paper,[11] the displaced methanol by insulin was determined by NMR and conformed with UV-spectrometry, when methanol with 0.03% hydrochloric acid as the mobile phase was used. NMR should be used for the quantitative determination as TFA being its ion-pair agent in the mobile phase used here.

With the presented method,[11] the calibration curve for UV-spectrometry was obtained from Fig. 1A. The displaced methanol by insulin in the presences of either HCl or TFA determined by NMR and in the presence of only HCl by UV-spectrometry were compared to each other. Table 1 actually shows two kinds comparisons. (1) NMR results between 45.0% methanol with 0.10% TFA and 47.0% methanol with 0.03% HCl; (2) between UV-spectrometry and NMR in the same mobile phase consisting of 47.0% methanol with 0.03% HCl. For the former, the NMR result in the presence of 0.10% TFA (shown in the last column) is always higher about 0.10% than that with the presence of 0.03% HCl (shown in the left column). This may be explained with a TFA peak with almost the same peak height and probably the same peak area as shown in Fig. 1A on the intersection region of the elution curve of methanol, which means that the accumulation of TFA on the RPLC stationary phase before

insulin is adsorbed and it desorbs and returns to the bulk mobile phase when insulin is adsorbed. Consequently, it produces a TFA increment. It should be pointed out that TFA is a very useful agent in NMR. It causes a higher result, as discussion above, due to the adsorption and desorption by the RPLC stationary phase. For the latter, except insulin concentration is 0.10 mg/mL, the other results from both on UV-spectrometry and NMR coincide very well, because that the HCl concentration in the mobile phase is very low and its adsorption by the stationary phase is also weaker than TFA. Thus, the effect on the NMR result with HCl to be ion-pairing agent may be ignored.

For the comparison between UV-spectrometry and NMR with ion-pairing agent HCl, as shown in Table 1, NMR result is still systematic higher than that with UV-spectrometry.

Applications

When solute dissolves in solvent, the interactions between the solute and the solvent would form solvate-solvent. We know that insulin can interact with either water or methanol but do not know it in very detail. Methanol can destroy its three-dimensional structure of insulin molecules and can also polymerize it to form dimer, trimer, and so on. As a result, the methanol concentration would decrease during these process. Though the insulin solvation in terms of water or methanol may be different, as long as the experimental condition to be same, the changeable extent in the concentration of methanol and water would depend on the time delay after insulin dissolving, due to kinetic mechanism, the polymerizing and the denaturation. Fig. 4 shows the merged two elution curves of 0.050 mg/mL insulin with mobile phase consisting of 47.0% methanol and 0.03% HCl with standing 3 (curve 2) and 5 (curve 1) hours after insulin dissolving in 47.0% methanol and 0.03% HCl solution, respectively. The two elution curves are so closed the latter that without the zoom enlarging, it may be simply referred to one elution curve. This fact indicates that the reproducibility of our experimental data is really reliable. The fact that the measured concentrations of methanol separately depends on their delay times after insulin dissolving into the mobile phase indicates that insulin molecules react with water and methanol with different rates. The higher concentration of methanol measured with a shorter time delay indicates that insulin interacts with water, or hydration is faster than that of methanol, while the lower concentration of methanol measured with a longer time delay shows that the source of methanol due to the interaction between methanol and insulin, and the methanol solvation becomes more significantly.

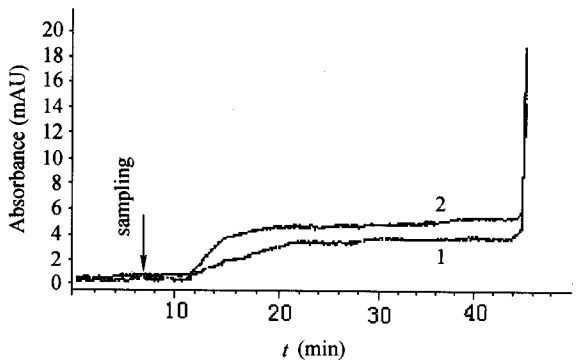

Fig. 4 Dependance of the time of insulin solvation. Two elution curve of insulin (0.050 mg/mL) with 3 h and 5 h standing after dissolving. (1) 5 h, (2) 3 h.

References

1 Carr, P. W.; Martire, D. E.; Snyder, L. R. *J. Chromatogr.* **1993**, *656*, 1.

2 Geng, X. D. *Acta Chim. Sinica* **1995**, *53*, 369 (in Chinese).

3 Geng, X. P. *Thermochim. Acta* **1998**, 131.

4 Geng, X. P.; Han, T. S.; Cao, C. J. *Thermal. Anal.* **1995**, *45*, 157.

5 Zhang, J.; Mao, Z. K.; Geng, X. D. *Acta Chim. Sinica* **1999**, *57*(9), 963 (in Chinese).

6 Geng, X. D.; Zhang, J.; Wei, Y. M. *Chin. Sci. Bull.* **2000**, *45*(3), 237.

7 Geng, X. D.; Chang, X. *J. Chromatogr.* **1992**, *599*, 185.

8 Geng, X. D.; Feng, W. K.; Chang, J. H.; Chang, X. Q.; Ma, F.; Yin, J. N.; Li, H. R.; Bian, L. J. *High Technol. Lett.* **1991**, *7*, 1.

9 Guo, L, A.; Geng, X. D. *Chin. J. Biotechnol.* **2000**, *16* (6), 661 (in Chinese).

10 Liu, T.; Geng, X. D. *J. Northwest Univ.* (*Nat. Sci. Ed.*) **1999**, *29*(2), 123 (in Chinese).

11 Geng, X. D.; Regnier, F. E. *Chin. J. Chem.* in press.

12 Geng, X. D. *J. Northwest Univ.* (*Nat. Sci. Ed.*) **1994**, *24*(4), 337 (in Chinese).

13 Geng, X. D.; Regnier, F. E. *J. Chromatogr.* **1984**, *296*, 15.

14 Geng, X. D.; Regnier, F. E. *J. Chromatogr.* **1985**,

332, 147.

15 Huang, J. X.; Horvath, C. *J. Chromatogr.* **1987**, *406*, 275.

16 Corradini, D.; Cannarsa, G. *J. Liq. Chromatogr.* **1995**, *18 & 19*, 3919.

17 Yamada, H.; Imoto, T. In *CRC Handb. HPLC Sep. Amino Acids, Pept., Proteins*, Vol. 1, Ed.: Hancock, W. S. **1984**, 167.

18 Shi, Y. L.; Geng, X. D. *Chem. J. Chin. Univ.* **1992**, *15*, 207 (in Chinese).

19 Ellison, E. H.; Marshall, D. B. *J. Phys. Chem.* **1991**, *C95*, 808.

20 Miller, C.; Joo, C.; Roh, S.; Gorse, J.; Kooser, R. In *Chemically Modified Oxide Surfaces* (*Chemically Modified Surfaces*), Vol. 3, Eds.: Leyden, D. E.; Collins, W. T., Gorden & Breach, New York, **1990**, p. 251.

21 Miller, C.; Dadoo, R.; Kooser, R. G.; Gorse, J. *J. Chromatogr.* **1988**, *458*, 255.

22 Sentell, K. B. *J. Chromatogr.* **1993**, *656*, 231.

(E0106201 LI, L. T.; DONG, L. J.)