A Direct Evidence of Stoichiometric Displacement between Insulin and Methanol in Reversed Phase Liquid Chromatography

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With four kinds of mobile phases, methanol-water, ethanol-water, 2-propanol and acetonitrile-water (all containing 0.1% trifluroacetic acid), the displacement between solute and solvent in RPLC was proved to be universal in frontal analysis (FA). Based on the measured Z value in usual RPLC to be a constant and the quantitative determination of methanol increment in mobile phase in FA, the stoichiometric displacement (SD) between insulin and methanol was directly proved by the experiment. The SD was also proved to occur only on about the one-fourth of the maximum amount of adsorbed methanol in the bonded phase layer (BPL) without any dynamic problem of mass transfer, while in FA, the SD firstly occurs on the surface of the BPL and then gradually sinks into the deeper sites companied with a dynamic problem. Although the displaced solvent by the same solute is less in the former case, the SD is independent of how deep of the solute enters the BPL. In addition, the adsorbed amount of solute on an adsorbent not only depends on the numbers of the adsorbed layer on the adsorbent surface, but also on the extent of the complete removal of the displaceable sölvent in the BPL. The physical fundamental of the SD and the methodology for investigation were also discussed.

Keywords liquid-solid system, reversed phase liquid chromatography, retention mechanism, stoichiometric displacement, adsorption mechanism, insulin, frontal analysis

Introduction

Fifteen years ago, a stoichiometric displacement model for retention (SDM-R) of solute in reversed phase liquid chromatography (RPLC) was presented.[1,2] It has been expanded to a unified retention model in all types of liquid chromatography (LC) except size exclusion chromatography.[3-5] The stoichiometric displacement (SD) concept not only was also employed and developed for an adsorption model of solute from solution in physical chemistry which is called the stoichiometric displacement model for adsorption (SDM-A),[5-9] but becomes a unified model for both adsorption mechanism in physical chemistry and retention mechanism in LC also.[5,9] In addition, the SDM has been also developed to be a powerful tool for protein refolding in life science.[5,10,11] The developments and applications of the SDM in biotechnolgy[10] and in more broad areas[8,11] were recently reviewed.[8,9,11,12]

If the SDM is really reasonable, it should be employed to answer each of four puzzles of retention mechanism of solute in RPLC.[13] In the previously first paper,[14] with a strictly designed experiment, one of the four puzzles, "does sample retention cause displacement of organic solvent from the stationary phase?" was definitely answered. In addition, based on the dynamics of trifluoroaceticacid (TFA) transfers from the interior of the bonded phase layer (BPL) into mobile phase,[15] another of them, "do sample molecules penetrate into the bonded phase and/or adsorb at the interface between the two phases?"[1] was also answered. However, the two answers still needs quantitative proving.

On one hand, a lot of experiments have measured the stoichiometric number of the SDM-R, Z, the total moles of the released solvent from the surfaces of the stationary phase and solute at the contact region between two phases when one molar solute is adsorbed by stationary phase in RPLC, but the measured Z value was obtained only by an indirect method. It still needs some direct experimental data to prove it. In this study, the SDM would be proved not only to have a strong theoretical basis, but a very reliable and simple methodology also. If the displaced organic solvent can be quantitatively measured and the ratio of the totally displaced organic solvent from the interface between the RPLC stationary phase and the solute to the totally adsorbed solute in mobile phase in FA is a constant, it would be direct experimental evidence to prove the SDM-R.

Theoretical

Thermodynamic characters of LC system

No matter whether a separation process of a solute actually occurs in an LC system or not, a valid chromatographic system in an equilibrium state involves a series of chemical equilibria.

Separation of solutes with LC is based on different activ-

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Received January 7, 2002; revised September 11, 2002; accepted September 27, 2002.

Project supported by the Key Laboratory of Modern Separation Science Foundation of Shaanxi Province (No. 2002—2003).

ity partition coefficients of the solutes, P_a, in two phases. A recognized characterization parameter of a solute retention, capacity factor k' of the solute, has been widely employed in LC. The relationship between the P_a and k' can be expressed as:

$$k' = P_a \phi \quad (1)$$

There, ϕ is the column phase ratio. The physical meaning of the ϕ in traditional LC is defined as the ratio of the solvent volume adsorbed on the stationary phase to that in the mobile phase. Its new definition for RPLC would be elucidated later.

The activity partition coefficient P_a in Eq. (1) is actually a special kind of chemical equilibrium constant describing the distribution of a solute in two phases and it is affected by any changes of components existing in the chromatographic system. It is equivalent to say that the magnitude of the P_a of the solute in LC relates to the chemical equilibrium of each step in a chromatographic system.

An assumption from the viewpoint of the partition mechanism in RPLC is that the injected amount of a solute can be referred to be infinitely low. Thus, it is as if the solute in the BPL did not alter its original equilibrium composition, resulting in no solvent leaving from the BPL. This assumption is actually questionable.

Chromatographic separation of a solute is a process of mass transfer between two phases. No matter how many molecules of the solute involve in this transfer process, its equilibrium state, energy changes, *etc.*, are usually expressed as chemical potential (free energy/mole). In other words, any chromatographic parameter describes the statistical behaviors of an Avogadro's number of molecules of the solute. The previous experimental result does not support this assumption.[15] A question is how to explain the displacement, even stoichiometric displacement process from the viewpoint of theory, or what is the theoretical foundation of the SDM?

Two fundamental laws in physics and the stoichiometric displacement concept

As it is well known, there are two basic laws in physics. First, one space can never be occupied simultaneously by two objects. The second is the conservation of energy. Based on the former, a cavity in the BPL can not be simultaneously occupied by both of the injected solute and the organic solvent, originally residing in the cavity. If the original solvent still resides in the cave, solute would never be adsorbed by the stationary phase, *i.e.*, there will be no RPLC separation.

According to the law of conservation of energy, so long as the composition of the mobile phase employed is fixed, such as in isocratic elution, this law results in the free energy of the surface of the RPLC stationary phase to be a certain value. So, the adsorbed amount of solvent in the BPL also should be fixed. According to the law of energy conservation, the displaced solvent, therefore, has to leave from the stationary phase and returns into the mobile phase thus increasing the solvent concentration in the mobile phase. That was called methanol increment (if the solvent used is methanol) in the previous papers.[14,15] This is just a displacement process of methanol by solute. This process may involve the changes in thermal energy. Therefore, the absolute energy of solute adsorption may not exactly equal that of methanol desorption. However, so long as the chromatographic condition is fixed, the thermal energy would be also constant, resulting in that the moles of the desorbed methanol must be energetically equivalent to that of the adsorbed solute. In other words, the displacement between solute and methanol must be stoichiometric. Based on the foregoing theoretical analysis, an important conclusion is now obtained that the retention mechanism of solute in RPLC has to be a stoichiometric displacement process.

Chemical equilibrium constant and methodology of SDM-R

As it is well known, each special interaction among molecules could be expressed with an individually chemical equilibrium and its magnitude can be characterized by its equilibrium constant. In an equilibrium system of RPLC, no matter how many molecular interactions involve, a general equilibrium constant could include all of the individual equilibrium constants together to express all of those interactions. Except the theoretically derived the expression of the general equilibrium constant, it is unnecessary to do any kind of complicated calculation of interaction forces. Because the expression of the general equilibrium constant derived in this manner definitely contains the partition coefficient of solute, the retention model of solute and its expression in RPLC, such as Eq. (2), can be directly derived from the general equilibrium constant.[1,2] The SDM-R was just derived by the latter manner. The SDM-R was originally derived by five thermodynamic equilibria[1,2] and then a unified model derived with six equilibria.[3,4,8] The former is only a special form of the latter.

Simplified SDM-R

The original SDM-R was derived with five thermodynamic equilibria and can be expressed as Eq. (2),[1,2,5]

$$\log k' = \log I - Z \log a_D \quad (2)$$

where,

$$\log I = \log K_a + nr \log a_{Ld} + \log \phi \quad (3)$$

and

$$Z = nr + q \quad (4)$$

where, a_D is the displacer (organic solvent) activity in mobile phase, Z is the total moles of the displacer released at the contact region from the solute (q) and nr from the BPL, on the RPLC bonded phase represented. The term n denotes

to the moles of the displacer if the adsorbed layer is a monolayer and r represents the number of the adsorbed layers in which the solute actually arrives on the stationary phase. The term, K_a is the general equilibrium constant containing five individual step equilibrium constants for solute to displace solvent. a_{Ld} is the activity of the solvated ligands on the stationary phase. The term ϕ denotes the column phase ratio defined as the capacity factor when the activity partition coefficient of the solute is unity.[5] When a chromatographic system is given, each of K_a, a_{Ld}, n, r and ϕ is a constant. Thus, log I is a set of constants containing the final equilibrium constant K_a and relating to the affinity of the solute to the RPLC stationary phase. When the change in a_D is not very broad, both Z and log I are constants. So Eq. (2) is a linear equation. Another advantage of Eq. (2), as described above, is directly derived by five kinds of thermodynamic equilibrium to obtain the same expression as that usual one expressing the quantitative relationship between the logarithm of the capacity factor of solute, log k' and the concentration of the displacer (organic modifier) in mobile phase.

Although Eq. (2) is only valid for a system in which the mobile phase consists of a binary-component and the concentration range of the organic solvent is not very broad. For simplification, Eq. (2) was still employed in this study.

Z in usual LC and Z_{FA} in frontal analysis (FA)

With isocratic elution under the condition of various solvent concentrations, the slope of linear plot of log k' vs. log a_D shown in Eq. (2) is the Z value. In addition, as shown in Eq. (4), the Z value contains two fractions of the released solvents, nr and q. Thus, besides the determination of the Z value in usual RPLC is done by an indirect manner, the really displaced methanol from the surface of either BPL, or the solvated solute by solute displacing has not been known yet. From the dimension of Z to be the total moles of solvent per mole solute, the measured Z value in usual RPLC should be independent of both sample size and sample concentration.

In FA, however, it is a different circumstance. Sample solution in FA is continuously entering the RPLC column. No matter how low the equilibrium concentration of a solute is employed, once a section of the chromatographic column is covered, or saturated by the solute, this section would be "permanently covered" by the solute in terms of dynamic equilibrium. This process goes forward until the whole surface of the stationary phase of the column is saturated by the solute.

Suppose the solvent is methanol and the solute is insulin. After FA is accomplished, the ratio of the total moles of the displaced methanol, $M_{\text{methanol(T,D)}}$ (T represents the totally desorbed methanol or totally adsorbed insulin, and D denotes desorption) to the total moles of the adsorbed solute $M_{\text{insulin(T,A)}}$ (A denotes adsorption) is the moles of the displaced methanol corresponding to one mole of the adsorbed insulin and is expressed by Z_{FA}.

The squeezed methanol from the surface of the solvated solute is actually instantaneous, because the stationary phase can provide high enough energy at molecular level to the solvated insulin molecules.[16] It should be emphasized here, the decreases in methanol concentration in insulin solution due to the insulin solvation should be almost completely compensated by the increases in the desolvation of the solvated insulin adsorption during FA process. So long as the insulin concentration in the mobile phase is not very high, the decreases in the methanol increment due to the interactions among the solvated insulin molecules with each other can be ignored. Thus, the directly measured $M_{\text{methanol(T,D)}}$ only represents the methanol from the BPL, which would be the same physical meaning as the term nr shown in Eq. (4) in usaual RPLC. Because both of $M_{\text{methanol(T,D)}}$ and $M_{\text{insulin(T,A)}}$ can be directly measured by experiment,[14,15] compared to the indirect method for the determination of the Z value in usual RPLC, the determined $M_{\text{methanol(T,D)}}$ with a direct method in the FA is the displaced amount of the methanol just from the RPLC stationary phase by insulin and denoted as Z_{FA}.

From the foregoing discussion, it seems that when experimental conditions are the same, the measured Z_{FA} in FA of RPLC should also be a fraction of the Z in usual RPLC, or $Z_{FA} < Z$. It is actually not true, because, as the report in the previous paper, some dynamical problems of methanol transfer exist in FA making displace more solvent from the BPL.

From the experimental data obtained from the previous paper, a small solute, such as trifluroacetic acid (TFA), takes at least 40 min to accomplish a cycle of adsorption and desorption, so that any small solutes could dynamically have not enough time to penetrate into the interior of the BPL in usual RPLC.[15] Compared to usual small solutes, insulin is a relatively large molecule. It is impossible for insulin to insert into octodecylsilane (ODS) ligands, indicating insulin molecules are unable to enter the interior of the BPL. In other words, the $M_{\text{methanol(T,D)}}$ depends on the insulin concentration in the mobile phase. However, a dynamic process still exists here. The velocity of the sinking down on the surface of BPL depends on that of the mass diffusion of methanol and water, and so on from the interior to surface of the BPL.

As long as the displacement between insulin and methanol is stoichiometric, a linear relationship between the $M_{\text{methanol(T,D)}}$ to the $M_{\text{insulin(T,A)}}$ should exist and is expressed as Eq. (5):

$$M_{\text{methanol(T,D)}} = Z_{FA} \times M_{\text{insulin(T,A)}} + c_{FA} \quad (5)$$

The physical meaning of the term, $Z_{FA} \times M_{\text{insulin(T,A)}}$ is of the contribution the displaced methanol only from insulin adsorption in FA, while the term, c_{FA} is independent of insulin concentration in mobile phase in FA. In other words, no matter whether an FA is running or not, the c_{FA} represents an intrinsically existed methanol on the BPL on the stationary phase in RPLC. An FA running means that it takes long enough time to make a mobile phase containing a solute and go though the column and gradually saturate the stationary

phase. Thus, both solute concentration (thermodynamic factor) and enough time (dynamic factor) are the necessary conditions in FA and the term, $Z_{FA} \times M_{insulin(T,A)}$ in Eq. (5) denotes this contribution. The term, c_{FA} in Eq. (5) represents a really existed methanol on the BPL which can be potentially displaced by solutes by means of either FA, or usual RPLC. The unit of the c_{FA} may be mass, or concentration which can be converted into a thermodynamic quantity, and it is independent of any effect of dynamic factor. In other words, the methanol amount, c_{FA}, on the stationary phase in RPLC can be instantaneously displaced even in usual RPLC. We know that as long as FA is running, with the gradually increasing amount of the adsorbed insulin, the displaced methanol firstly comes from the surface of the BPL and then gradually expands into the deep place in the interior of the BPL.[15] It is easy to understand that the methanol amount of c_{FA} should exist on the surface of the BPL, or the boundary between the two phases.

When c_{FA} equals to zero, the straight line of the linear plot by Eq. (5) goes through the origin. It means that there is not any potentially existed methanol on the BPL.

When the term $c_{FA} > 0$, there are two circumstances. First, in presence of insulin in mobile phase, the displacement between insulin and methanol in FA of RPLC never occurs. It would be the circumstance in which the methanol concentration in the mobile phase is too high for either insulin adsorption in FA, or insulin retention in usual RPLC. Second, the methanol concentration is suitable for chromatographic investigation in the two circumstances, but insulin is absent. The former is in the case of column cleaning, while the latter is just for an established dynamical equilibrium before any kind of LC including the usual and FA in RPLC. From the viewpoint of dynamics of methanol adsorption and desorption and the physical meaning of the c_{FA} described above, a conclusion would be obtained that the methanol amount, c_{FA} on the surface of the BPL can freely and instantaneously adsorb and desorb without any dynamic problem.

Supposing that a solute has molecular size to be comparable to that of methanol, such as TFA, and can be separated in usual RPLC. TFA can accomplish the process of displacing methanol just in the c_{FA} region instantaneously. On the contrary, if a displacement process between a solute and solvent carries out instantaneously, this SD process has to take place only from the c_{FA} region on the surface of the BPL.

When $c_{FA} < 0$, the BPL needs more methanol to compensate the insufficiency of methanol as FA is running. That would be the circumstance that equilibrium between two phases of a chromatographic system has not been yet established.

From the physical meaning of the Z_{FA}, the product of Z_{FA} and $M_{insulin(T,A)}$, $Z_{FA} \times M_{insulin(T,A)}$ in Eq. (5) represents a fraction of the totally displaced methanol existing underneath the c_{FA} region of the BPL, or the methanol existing only in the interior of the BPL.

As long as Eq. (5) is reasonable, a very important conclusion could be obtained. In terms of dynamics of mass transfer, the adsorbed organic solvent on the stationary phase, or that in the BPL in RPLC can be divided into two parts, zero term only occurring in the region c_{FA} or on the surface of the BPL and non-zero term only occurring underneath the c_{FA} region, the deep place of the BPL, or the interior of the BPL. The former can be instantaneously displaced by solute only in usual RPLC alone, while the latter can take place in the c_{FA} region firstly and then gradually expand into the interior of the BPL.

Experimental

Equipment and chemicals

A Hewlett Packard 1090 liquid chromatograph with a diode-array detector and a Hewlett Packard Color Pro plotter were used. SynChrompak column RP-C_{18} (100 mm × 4.6 mm, particle size 5.6 μm, pore diameter, 30 nm) was purchased from SynChrom Inc. (West Lafayette, IN, USA). The column temperature was controlled at (25 ± 0.50) ℃ with a water bath. A Nuclear Magnetic Resonance Spectroscomets (NMR, Gemini 200, Varian Co., Palo, CA, USA) and NMR tubes (Kontes, diameter 5 mm) were used for NMR determination.

Insulin (bovine pancreas, HPLC) and lysozyme (chicken egg white) were bought from Sigma Co. Acetonitrile and 2-propanol were obtained from EM Science (Gibbstone, NJ, USA). Absolute alcohol was bought from McCormick Distilling Co., Inc. (Perkin, IL, USA) and trifluroacetic acid (HPLC/spectro grade, TFA) was obtained from Pierce (Rockfold, IL, USA). The other chemicals used in this study were the same as that in the previous paper.[15]

Mobile phases were: (1) 47.0% methanol-water ($V_{methanol}/V_{water}$) with 0.030% hydrochloric acid ($V_{HCl}/V_{mobile\ phase}$), and four other organic solvents-water ($V_{solvent}/V_{water}$) with 0.1% TFA ($V_{TFA}/V_{mobile\ phase}$) as: (2) 45.0% methanol; (3) 32.0% ethanol; (4) 18.0% 2-propanol and (5) 27.50% acetonitrile.

Experimental procedure

The procedure of frontal analysis in RPLC in this study was followed that of Huang *et al.*[17] and by the equipment scheme employed in the previous paper.[15] Insulin was dissolved in each of the five mobile phases, respectively. It is absolutely necessary to gain a smooth and a horizontal base line of the blank of run for accurate measurement of plate height of the methanol increment, or NMR determination of the methanol increment with pure deuterium oxide as solvent. The length of the base line was specially designed as that reported in the previous papers.[14,15]

The elution curves of the increments of four organic solvents, methanol, ethanol, 2-propanol and acetonitrile in water with 0.10% TFA were obtained according to the same procedure as that from 47.0% methanol-water with 0.03% hydrochloric acid,[14] except their concentrations in the mobile phase employed were selected according to the capacity factor

of insulin to be in range of 2 to 10. The other experimental procedure were selected as the same as that in the previous papers.[14,15]

Result and discussion

Elution curves of insulin and the increment of organic solvents in different mobile phases with TFA

Fig. 1 shows four sets of elution curves of insulin with various concentrations in FA-RPLC with four kinds of mobile phases. They consisted of 45.0% methanol (Fig. 1A), 32.0% ethanol (Fig. 1B), 18.0% 2-propanol (Fig. 1C), and 27.5% acetonitrile (Fig. 1D), which include water and 0.10% TFA respectively. The detection wavelength was selected at 254 nm with reference wavelength 550.10 nm. Except the solvents, all experimental conditions shown in Fig. 1 were the same. As mentioned in the previous papers,[14,15] a long base line was taken as 7.50 min in this study. Comparing with the four sets of elution curves, some common points and differences exist. First, a small plateau for the first plateau appears before the main plateau of insulin in each elution curve. Second, each of the first plateau height increases with the increase of the equilibrium insulin concentration in their corresponding mobile phases. Third, when insulin concentration is given, their ratios of each first plateau heights to their own corresponding main plateaus depend on the kinds of solvent themselves. That is the largest for acetonitrile and the smallest for methanol. The first plateau height from acetonitrile is even about 30% of that of its main plateau. Although the others do not show so clearly as acetonitrile, the first plateau unexceptionally occurs in each cases. It can be concluded now that no matter what kinds of organic solvents and ion-pairing agents are used, the existence of the displacement between solute and organic solvent in FA-RPLC is a universal phenomenon, at least, it is true for insulin as a solute.

Z and sample size in usual RPLC

In usual RPLC, to make chromatographic behavior of solute follow that of linear chromatography, the injected sample size should be as low as possible. A new question is whether sample size has an effect on Z value, or not. Table 1 shows the measured Z values of both insulin and lysozyme in various sample sizes and concentrations. The stationary phase and mobile phase employed here were the same as that in the FA mentioned above. The result shown in Table 1 indicates Z values of insulin and lysozyme to be basically independent of sample size and concentration. It elucidates the displacement between insulin and methanol or that between lysozyme and methanol in usual RPLC is really stoichiometric. Additionally, the injected insulin amount in usual RPLC shown in Table 1 was actually raised up to milligram scale which was almost comparable to the adsorbed amount of insulin in its saturation state when the insulin concentration is 0.10 mg/mL in the mobile phase employed in FA (Table 2).

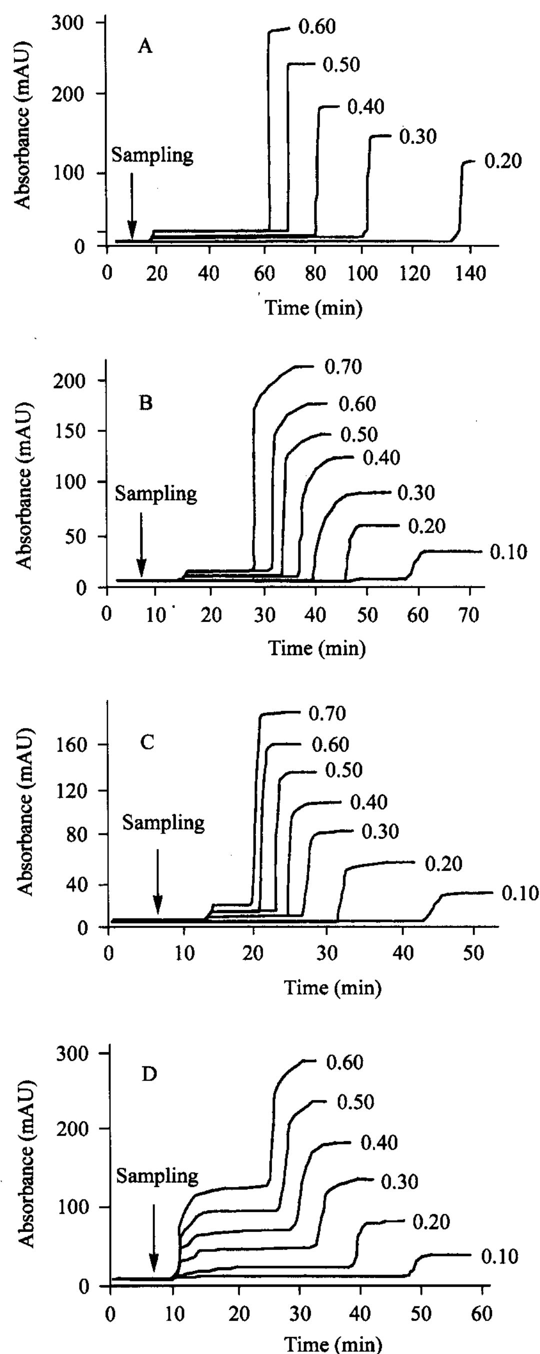

Fig. 1 Elution curves of insulin in different mobile phases of FA-RPLC [SynChrompak, RP-C18; flow rate, 0.40 mL/min; detection wavelength at 254 nm with reference wavelength 550.10 nm with a diode-array detector; sampling time, 7.50 min. (A) Insulin concentrations (mg/mL): (1) 0.20, (2) 0.30, (3) 0.40, (4) 0.50 and (5) 0.60 in 45.0% (V/V) methanol-water solution with 0.10% TFA; (B) Insulin concentrations (mg/mL): (1) 0.10, (2) 0.20, (3) 0.30, (4) 0.40, (5) 0.50, (6) 0.60 and (7) 0.70 in 32.0% (V/V) ethanol-water solution with 0.10% TFA; (C) Insulin concentrations (mg/mL): (1) 0.10, (2) 0.20, (3) 0.30, (4) 0.40, (5) 0.50, (6) 0.60 and (7) 0.70 in 18.0% 2-propanol-water solution with 0.10% TFA; (D) Insulin concentrations (mg/mL): (1) 0.10, (2) 0.20, (3) 0.30, (4) 0.40, (5) 0.50 and (6) 0.60 in 27.5% (V/V) acetonitrile-water solution with 0.10% TFA].

Table 1 Z values of insulin and lysozyme with various sample sizes[a]

Sample sizes (μg)	Concentration (mg/mL)	Z	
		Insulin	Lysozyme
2.0	0.10	21.3	44.8
5.0	1.0	19.8	42.7
20	5.0	16.9	40.2
1000	10.0	18.4	48.4
Average		19.1 ± 1.5	44.05 ± 4.9

[a] methanol-water (V/V, 47%) with HCl (V/V, 0.03%).

Table 2 Totally adsorbed insulin, $M_{\text{insulin(T,A)}}$ and totally desorbed methanol, $M_{\text{methanol(T,D)}}$[a,b]

c_{insulin} (mg/mL)	$M_{\text{Insulin(T,A)}}$ (mmols × 10^5)	$M_{\text{methanol(T,D)}}$ (mmols × 10^2)	Fractions of the maximum of $M_{\text{methanol(T,D)}}$ (%)
0.025	8.75	12.7	48.7
0.050	12.0	15.4	59.0
0.075	14.5	17.7	67.8
0.100	16.3	18.9	72.4
0.200	23.5	23.4	88.5
0.300	27.6	26.1	100
0.400	29.6	24.1	92.1

[a] SynChrompak, RPLC-C18, methanol/water (V/V, 47%) + 0.03% HCl (V/V, 0.03%), (25 ± 0.5) ℃. [b] Averaged result from three continuous individual determinations.

It means that no matter how large the sample size, or how high the concentration of insulin is, insulin molecules have not enough time to penetrate, to sink down into the deeper place, underneath the surface of the BPL, therefore the stoichiometric displacement process between insulin and methanol only occurs on the surface of the BPL.

Stoichiometric displacement between insulin and methanol in FA

Table 3 shows the comparison of the determined average amount of methanol displaced by insulin between on-line UV spectrometry and NMR by three continuous individual determinations, as the mobile phase consisted of 47.0% methanol-water solution with 0.03% hydrochloric acid. As reported before,[14,15] both methods shown in Table 3 have also a good consistent result with each other.

The totally average displaced methanol, $M_{\text{methanol(T,D)}}$ shown in Table 2 and the measured methanol increment, $c_{\text{methanol,found}}$ shown in Table 3, while the totally average adsorbed insulin, $M_{\text{insulin(T,A)}}$ shown in Table 2 would be the products of the same break-though volume and the concentration of insulin, c_{insulin} shown in Table 3.

From Table 2, it can be found that when the insulin concentration in the mobile phase increases, the adsorbed $M_{\text{insulin(T,A)}}$ by the RPLC column raises up. However, when insulin concentration increases to 0.40 mg/mL, although the $M_{\text{insulin(T,A)}}$ still increases, the measured methanol increment shown in Table 3 and the $M_{\text{methanol(T,D)}}$ shown in Table 2 de-

Table 3 Comparisons of the methanol diplaced by insulin by UV-spectrometry and NMR[a,b]

c_{insulin} (mg/mL)	$c_{\text{methanol,found}}$ (V/V)	
	UV-spectrometry	NMR
0.025	0.033 ± 0.010	—
0.050	0.046 ± 0.004	—
0.075	0.060 ± 0.006	—
0.100	0.089 ± 0.006	0.17 ± 0.04
0.200	0.139 ± 0.025	0.20 ± 0.04
0.300	0.186 ± 0.020	0.24 ± 0.02
0.400	0.192 ± 0.013	0.19 ± 0.02

[a] SynChropak-RP C_{18}, methanol-water (V/V, 47%) + HCl (V/V, 0.03%), (25 ± 0.5) ℃. [b] Averaged concentrations of three continuous individual determinations.

crease. The reason was explained in the previous paper to be the interactions among insulin molecules due to the formation of a double layer, even multi layer of the adsorbed insulin on the RPLC stationary phase.[15] The maximum $M_{\text{methanol(T,D)}}$ is actually approached when the insulin concentration in the mobile phase is 0.30 mg/mL.

Fig. 2 shows a linear plot of $M_{\text{methanol(T,D)}}$ versus $M_{\text{insulin(T,A)}}$ in the concentration range of insulin from 0.025 mg/mL to 0.30 mg/mL. The linear equation and deviations can be expressed with Eq. (6) as:

$$M_{\text{methanol(T,D, mmol)}} = 0.071 \pm 0.0043 + (694.4 \pm 27.2) M_{\text{insulin(T,A, mmol)}} \quad (6)$$

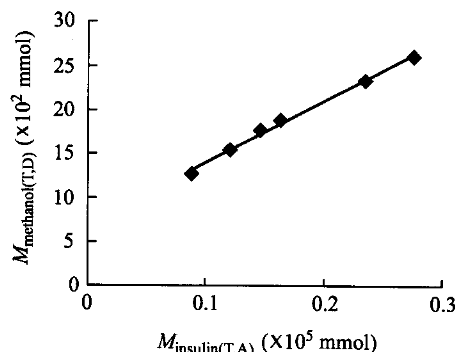

Fig. 2 Scheme of a stoichiometric displacement relationship between $M_{\text{insulin(T,A)}}$ and $M_{\text{methanol(T,D)}}$ in FA-RPLC. Experimental conditions are the same as shown in Fig. 1.

The linear correlation coefficient of this plot being 0.9970 indicates that the stoichiometric relationship between methanol and insulin really exists. The slope, Z_{FA} of the linear plot, 694.4 represents the moles of the displaced methanol by one mole of the adsorbed insulin. Compared to the Z value to be 19.1 shown in Table 1, the Z_{FA} being 699.4 is 36.4 folds of Z. This confirms to that expected in theoretical part and explained in detail as follows.

A reasonable explanation should be that compared to the existed amount of the methanol, c_{FA} on the surface of the BPL, the whole BPL has not enough thickness in the depth

direction to immerse more parts of insulin molecules. As pointed out in theoretical part, it is impossible for insulin molecules with large size to enter the interior of the BPL. Insulin molecules only can "lie down" on the surface of the BPL and gradually sink down into the interior of the BPL by the pushing force due to the difference of the chemical potential of insulin between two phases. It could be thought that as methanol of the c_{FA}, the saturated adsorption of insulin with the low concentration of insulin in the mobile phase can only displace that part of the methanol underneath the surface of the BPL.

Based on the report by Miller *et al*.[18,19] and Sentell *et al*.,[20] the structure involving the components and viscosities of four regions of the BPL in the depth direction is inhomogeneous. No matter how different the concentration of methanol in a mobile phase is employed, the region IV underneath the region I is almost pure organic methanol. From the linear relationship between $M_{\text{methanol}(T,D)}$ and $M_{\text{insulin}(T,A)}$ shown in Fig. 2, the fact that even though insulin molecules descend the same distance in the both of regions I and IV, the displaced methanol would be much more for the region IV than that for the region I. This is the reason why the measured Z_{FA} of insulin from FA is much greater than that from usual RPLC, even though the chromatographic conditions in both are completely the same. It also indicates that the SD is dominated by energy conservation, and not due to a displacement process by an equal volume.

From Table 3, the calculated c_{FA} 0.071 mmol on the surface of the BPL from Eq. (6) accounts for 27.2% of 0.261 mmol of the displaced maximum methanol from the BPL in FA. In other words, the existed methanol amount c_{FA} on the surface of the BPL without any dynamic problems in both usual RPLC and FA in RPLC is only about one-forth of the maximum adsorbed methanol in the whole BPL.

It is as if the result obtained in this study were conflict to that reported also from FA in RPLC[8,9] in which the measured Z values of the aromatic alcohol homologue coincide well between usual RPLC and FA in RPLC. It is actually not true. Although both studies are carried out with FA in RPLC, from the standpoint of methodology, the measured methanol in this study is of a direct method, while another one, the Z value measured is still indirect method. The former includes the contributions of both thermodynamics and dynamics to the methanol displaced by solute, and the displaced methanol by solute, as shown in Eq. (5), is directly measured when methanol concentration in mobile phase is invariable. The directly measured amount of methanol depends on the composition of BPL and how deep solute enters in it. However, the later only involves five thermodynamic equilibria, and the Z value is obtained by calculating the changes in the partition coefficient of solute in both FA in RPLC and usual RPLC [k' in Eq. (2)]. The partition coefficient of solute in the two circumstances depends on the compositions of both BPL and mobile phase, but Z value is independent of the both. Thus, the effect of Z value on the partition coefficient of solute is by means of the product of $Z \times \log a_D$, shown in Eq. (2). In other words, no matter whether usual RPLC, or FA in RPLC is, as long as the Z value is measured by indirect method, it merely and indirectly reflects the stoichiometric displacement relationship between solute and solvent in RPLC.

It should be pointed out here that a complete FA process means the saturated adsorption of insulin on the RPLC stationary phase with the presence of insulin in the mobile phase employed and the corresponding methanol is simultaneously displaced by insulin from the BPL. From the traditional viewpoint of physical chemistry that the adsorbed amount of solute depends on the solute concentration in bulk solution, the increase in the adsorbed amount of insulin is attributed to the formation of the multi layer of the solute on absorbent. However, it is hard to explain the linear relationship between $M_{\text{methanol}(T,D)}$ and $M_{\text{insulin}(T,A)}$ shown in Eq. (6) and Fig. 2. Thus, we now should consider a new point that the adsorbed amount of a solute also depends on what fraction of the totally adsorbed solvent is displaced. A conclusion is obtained that the extent complete removal of the displaceable organic solvent originally resided in the BPL by solute is also to dominate the adsorbed amount of solute.

Partition and/or adsorption retention mechanism of solute in RPLC

As pointed above whether the retention mechanism of solute in RPLC is a partition or/and adsorption mechanism has not been solved yet.[1] Jaroniec gave a review paper on the development of the investigation of partition and adsorption mechanism of solute in RPLC.[21] A surface process in adsorption mechanism and a volume process in partition mechanism were referred to be the significant difference between the two mechanisms. Three differences between adsorption and partition mechanisms were suggested to distinguish them.[22] Two questions are raised: (1) How is the strict boundary in terms of the thickness of the BPL to distinguish surface process of adsorption mechanism from the volume process in partition mechanism in RPLC? and (2) Can the one-fourth of the maximum amount of displaced methanol by insulin from BPL, *i.e.*, c_{FA} be referred to be the criteria to distinguish a surface process from a volume process?

Geng reported in another paper that there is no need to distinguish the retention mechanism of solute to be partition or/and adsorption, it was only based on a theoretical analysis.[23] In the previous paper,[15] based on the transfer time of the components, such as TFA, methanol, or water from the interior of the BPL to the mobile phase being, at least, 20 min, a postulate was obtained that the displacement process between solute and solvent in usual RPLC can only occur on the surface of the BPL. Although some experimental data support this postulate, but that is only a qualitative explanation.

The fact that the SD between insulin and methanol only takes place in the region of 27.2% of the maximum amount of the displaced methanol in the BPL indicates that the SD in usual RPLC is really carried out on the surface region of the

BPL. According to the recognized criteria proposed by Sentell and Dorsey,[22] the retention mechanism of insulin in usual RPLC belongs to adsorption. According to the same criteria, as insulin molecules can sink down (not penetrate) into the deep place of the BPL to displace much more solvent, even all of solvent in the BPL, the retention mechanism of insulin in FA in RPLC belongs to partition mechanism.

Conclusions

1. A direct experimental evidence in frontal analysis (FA) proved the process between insulin and methanol in RPLC is a stoichiometric displacement.

2. The two basic laws, the conservation of energy and that one space can not be simultaneously occupied by two objects in physics, require that a solute adsorption from solution has to be accompanied with a stoichiometric number of solvent molecules to leave from the bonded phase layer (BPL) and return to the mobile phase. Thus, the two laws are the theoretical foundation of the stoichiometric displacement model for retention (SDM-R) of solute.

3. Although various kinds of interactions among molecules in the SDM-R are considered, the methodology of chemical equilibrium that is employed to derive the SDM-R is simple and broadly applied in many areas. The SDM-R is only derived by means of thermodynamics. It is impossible to include any effect of dynamic factors in the SD process. If it takes a certain time to approach to the final equilibrium, the solute has not enough time to displace the same amount of solvent in the deep place of the BPL, resulting in less solvent would be displaced than that occurring in FA process.

4. With the observation of the first plateau formed before the main plateau of insulin on its elution curve, the SD between the solvent and insulin was proved to be universal in RPLC.

5. Although the structure of the BPL is inhomogeneous, the ratio, Z_{FA} of the total amount of the displaced methanol $M_{methanol(T,D)}$, in frontal analysis (FA) to the totally adsorbed insulin, $M_{insulin(T,A)}$, is a constant, coinciding with that expected in the theoretical analysis. The Z in usual RPLC is also a constant independent of either the solute mass or solute concentration. In both circumstances, the retention mechanism of solute in RPLC follows the SDM-R. With a quantitative determination in FA, only one-fourth of the maximum amount of the absorbed methanol in the BPL can be provided the displacement by solute in usual RPLC. This circumstance is usually referred to be a surface process in adsorption mechanism. However, in FA, the SD occurs on the c_{FA} region firstly and then expands underneath the c_{FA} region, or into the deeper place in the BPL which is usually referred to be a volume process in partition mechanism. Although in this study, the SD between methanol and insulin was investigated only by FA, this conclusion can be valid in both usual RPLC and FA of RPLC. Thus, it is really unnecessary to argue whether the retention mechanism of solute belongs to adsorption, partition, or the mixed mode of both in FA and in usual RPLC.

6. The saturated adsorption of insulin in FA does not mean that all of methanol in the BPL completely desorbs, or is displaced by insulin. The adsorbed amount of solute in usual liquid-solid system depends not only on the layer numbers of the adsorbed solute on the surface of absorbent, from the traditional viewpoint of physical chemistry, but also depends on the extent of complete removal of the originally resided solvent on the absorbent surface from the result obtained in this study.

Acknowledgement

The authors are much obliged to Dr. Don Zebolsky of the Chemistry Department, Creighton University, Omaha for proof-reading.

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(E0201072 SONG, J. P.; FAN, Y. Y.)