An Integrated Theory of Adsorption and Partition Mechanism and Each Contribution to Solute Retention in Reversed Phase Liquid Chromatography

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With the combination of the the stoichiometric displacement model for retention (SDM-R) in reversed phase liquid chromatography (RPLC) and the stoichiometric displacement model for adsorption (SDM-A) in physical chemistry, the total number of moles of the re-solvated methanol of stationary phase side, *nr*, and that of solute side in the mobile phase, *q*, corresponding to one mole of the desorbing solute, were separately determined and referred as the characterization parameters of the contributions of the adsorption mechanism and partition mechanism to the solute retention, respectively. A chromatographic system of insulin, using mobile phase consisting of the pseudo-homologue of alcohols (methanol, ethanol and 2-propanol)-water and trifluoroacetic acid was employed. The maximum number of the methanol layers on the stationary phase surface was found to be 10.6, only 3 of which being valid in usual RPLC, traditionally referred as a volume process in partition mechanism. However, it still follows the SDM-R. Both of *q* and *nr* of insulin were found not to be zero, indicating that the retention mechanism of insulin is a mixed mode of partition mechanism and adsorption mechanism. When methanol is used as the organic modifier, the ratio of *q*/*nr* was 1.13, indicating the contribution to insulin retention due to partition mechanism being a bit greater than that due to adsorption mechanism. A linear relationship between *q*, or *nr* and the carbon number of the pseudo-homologue in the mobile phase was also found. As a methodology for investigating the retention mechanism retention and behaviors of biopolymers, a homologue of organic solvents as the organic modifier in mobile phase has also been explored.

Keywords reversed phase liquid chromatography, retention mechanism, mixed mode, stoichiometric displacement, insulin

Introduction

In the reported papers,[1-3] two of four puzzles for retention mechanism of solute in reversed phase liquid chromatography (RPLC), *i.e.*, "does the sample retention cause displacement of organic solvent from the stationary phase?" and "do the sample molecules penetrate into the bonded phase and/or adsorb at the interface between the two phases?", were answered in a quantitative manner. The third puzzle "to what extent can the sample retention be described as a partition or an adsorption process (or some combination of the two?)" was also answered,[4] because the retention mechanism of solutes in all circumstances follows the stoichiometric displacement model for retention (SDM-R) of solute in usual RPLC,[5-7] and it is really unnecessary to distinguish the partition mechanism from the displacement mechanism.[3] However, this answer is not sufficient. If answer is requested from the traditional point of view, *i.e.*, to answer to what extent is the contribution of partition and adsorption, it would be helpful highly to understand the mechanism of solute in RPLC to make chromatographers more convinced of this answer.

Two extreme molecular processes, *i.e.*, displacement mechanism and partition mechanism, are used in liquid chromatography (LC) to represent the solute distribution between the mobile phase and stationary phase. Displacement processes are referred as a surface process occurring at the solid-liquid interface, while partition process is referred as a volume process. The former dominates in normal phase liquid chromatography in which the stationary phase is a monolayer or bilayer on a polar solid surface, while the latter is favored when the stationary phase is "thick" enough to accommodate solute molecules in its interier.[8] This criterion is fully satisfied in the traditional liquid-liquid chromatography system, in which the stationary phase and mobile phase are immiscible liquid. The stationary phase in RPLC system with chemically bonded ligands is relatively "thick" and suitable for accommodating the solute molecules for the partition mechanism.[9,10] However, the solute partition occurring in RPLC differs significantly from that taking place between two immiscible liquid phases because the chemically bonded phase with incorporated solvent molecules is by no means a bulk liquid.[11] A mixed mode of displacement mechanism, or adsorption mechanism and partition mechanism was reviewed by Jaroniec.[10]

The adsorption mechanism is also called competitive adsorption and controlled by the difference between the solute

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and solvent adsorption on the stationary phase, while the partition process is controlled by the differences in the molecular interactions of solute molecules in the stationary phase and mobile phase. Three differences between adsorption mechanism and partition mechanism were mentioned by Dorsey *et al*.[12] Thus, two common criteria to distinguish them are: (1) from the stand point of phenomenology, the distribution of solute is a surface process (monolayer or bilayer) for adsorption process, or volume process (adsorbed layer is deep enough for almost embedding solutes) for partition process. (2) from the stand point of molecular mechanism, the adsorption process is controlled by the difference between the interactions of the solute and solvent on the stationary phase, while the partition process is controlled by the differences in the molecular interactions of solute molecules in the mobile phase and stationary phase. It seems that the former involves the thickness of the bonded phase layer (BPL), the latter involves the kinds and magnitudes of molecular interactions among solute, solvent and stationary phases.

From previous studies,[1-3] no matter how deep the BPL is, the approached depth of solute depends not only on the depth of the BPL itself, but also on the dynamics of mass transfer in the BPL. In other words, even though the BPL is really deep enough for accommodating a solute, as long as the solute has not enough time either to enter the interior of the BPL, or to leave from the interior of it, it would be worthless to discuss the depth of the BPL. Additionally, it is also not enough only to consider some kind of molecular interactions either for adsorption process, or for partition process mentioned above. The SDM-R of a solute in RPLC can include five kinds of molecular interactions among solute, mobile phase and stationary phase, and express their contributions to solute retention by an equation. Thus the SDM-R should satisfy the foregoing requests from both the molecular interactions and depth of the BPL intensively and quantitatively investigating each contribution to solute retention from either adsorption process, or partition process in RPLC.

The stoichiometric number of this model, Z, was suggested to be a new characterization parameter not only for RPLC,[7,13-17] but also for all LC.[18-20] However, the fractions of Z, from both stationary phase and the surface of solute have not been exactly measured yet.

Ten years ago, based on the concept of mean active sites and also by means of chemical thermodynamic equilibrium, the stoichiometric displacement model for adsorption (SDM-A) of solute in liquid-solid adsorption system was developed to describe a quantitative adsorption model from the standpoint of pure physical chemistry,[19] and it was tested with calorimetry[20] and employed to calculate the fractions of thermodynamic functions.[21] Recently an extended Langmuir equation in liquid-solid system was theoretically derived and improved.[22] With the combination of the SDM-R and the SDM-A, the magnitudes of the fractions of Z may be obtained. As long as the fractions of Z values can be exactly measured, the contributions from each of adsorption mechanism and partition mechanism to solute retention may be also obtained. A book recently summarized the theoretical developments and applications of the SDM-R and SDM-A in a broad region.[7]

In this study, the idea that the largest amount of adsorbed methanol on the stationary phase is valid in FA and can accommodate the solute in usual RPLC will be evaluated by the experimental data. Furthermore the contributions of mobile phase and stationary phase to the solute retention in usual RPLC would be calculated.

Theoretical

Unification of adsorption and partition mechanisms

In the reported paper,[3] a conclusion was obtained that it is unnecessary to distinguish the partition mechanism from adsorption mechanism. Its theoretical foundation for explaining and unifying both should be further established.

The surface of a stationary phase in RPLC may be heterogeneous. For convenience, it is assumed that the interaction of solute and solvent molecules takes place only on specially active sites distributed over the surface of the stationary phase. No matter how different the nature of the interaction forces between the stationary phase of RPLC and the solute or solvent molecules is, or how heterogeneous the distribution of these active sites is, the numbers of mean active sites per unit surface area (or density) are assumed to be uniform. The "mean active site" is defined as a site able to adsorb one solvent molecule under a given chromatographic condition and denoted by $\bar{L}$. It is only an envisaged concept equivalent to the active site in connection with an adsorbed or desorbed solvent molecule.

The molecular interactions considered in partition mechanism and adsorption mechanism should be firstly discussed.

(1) *Partition mechanism only concerns two molecular interactions*

(A) A bare solute (P) and solvent (D)

$$P + mD = PD_m \qquad (1)$$

where PD_m is the solute-solvent complex and m is the moles of the solvent associated with one mole of the bare solute.

(B) Solute and stationary phase

When a bare solute molecule interacts with the naked ligand $\bar{L}$ of the stationary phase,

$$nr\,\bar{L} + P = P\bar{L}_{nr} \qquad (2)$$

where $P\bar{L}_{nr}$ is the solute-ligand complex, n is the moles of the "mean active sites" covered by one mole of solute and r is the numbers of the adsorbed layer of solvent where the solute actually arrives [see Eq. (8) below] on the stationary phase. Thus, the nr represents the potentially total moles of the solvent displaced by one mole of the solute on the stationary phase.

(2) *Molecular interactions considered in adsorption mechanism*

The interaction of solute and ligands of the stationary

phase is already shown in Eq. (2). The interaction of solvent and the ligands of the stationary phase is as:

$$\bar{L} + D = \bar{L}D \qquad (3)$$

where $\bar{L}D$ is the ligands-solvent complex.

Comparing with each molecular interaction in the SDM-R or SDM-A, Eqs. (1)—(3) are actually the three of step interactions, solute solvation, the formation of solute-ligand complex and the ligands solvation of the five molecular interactions considered in SDM-R.[5-7,19]

However, both adsorption process and partition process have some theoretical and experimental problems. Firstly, as reported in the previous paper,[3] the assumption in partition mechanism that the sample amount is so low that it can not change the composition of the BPL is unreasonable, because the partition process claims that the distribution of solute in the stationary phase and mobile phase is a volume process, the solute in the BPL is completely, or almost completely imbedded by the BPL. It does not consider that the solvent originally staying in the BPL must necessarily leave from the BPL, or solute displaces the solvent, as the solute distributes in the two phases. Thus, the traditional partition process conflicts to the two laws in physics, energy of conservation and that one space can never be simultaneously occupied by two objects.[3] Secondly, although the adsorption process considers the displacement of solute to solvent in the BPL, it neither considers the squeezing out of the solvent on the solute surface due to the solvated solute being adsorbed by the solvated ligands, nor considers the re-solvations of the solute and the stationary phase after the solute desorbs. To remedy these weaknesses of the adsorption mechanism and partition mechanism, the following two more interactions should be added.

When the solute-ligand complex, $P\bar{L}_{nr}$ forms due to the interaction between a naked solute and the naked ligands on the stationary phase shown in Eq. (1), its surface exposed to the mobile phase would continue to solvate (remedying the weakness of adsorption mechanism) as:

$$P\bar{L}_{nr} + (m - q)D = P\bar{L}_{nr}D_{(m-q)} \qquad (4)$$

where $P\bar{L}_{nr}D_{(m-q)}$ is the formed ligand-solute-solvent complex and q is the solvent moles adsorbed on the exposed surface of one mole of the ligand-solute complex.

Finally, to remedy the weakness of partition mechanism, the Eqs. (1)—(4) are combined by way of Eqs. (2) + (4) − (1) − (3), and Eq. (5) is obtained as:

$$PD_m + nr\,\bar{L}D = P\bar{L}_{nr}D_{(m-q)} + (nr + q)D \qquad (5)$$

The Eq. (5) indicates that when a soluated solute (PD_m) is adsorbed by the solvated ligands ($nr\,\bar{L}D$) and forms the solute-ligands-solvent complex ($P\bar{L}_{nr}D_{(m-q)}$), the total moles of solvent, Z (the sum of nr and q) is squeezed out at the contact region between the stationary phase and solute in RPLC. The foregoing five molecular interactions [Eqs. (1)—(5)] are exactly the same as that in SDM-A or SDM-R.[5-7,19]

Several manners can be employed to express the magnitude of molecular interactions in the RPLC system. One way is exactly to calculate the magnitude of all kinds of molecular interactions. As pointed out in the previous paper,[3] it is very difficult and hardly practicable. The second way is to measure the activity coefficients of solute and solvent in both of mobile phase and stationary phase, and to express the magnitudes of solute-solvent interactions in the two phases. The third way is to use stepwise chemical equilibrium constant to express each individual molecular interaction in RPLC and then to unify all of these stepwise equilibrium constants together as a general equilibrium constant. As pointed out in the reported paper,[3] this is simple and practicable. As is well known, the SDM-R is theoretically derived by the last way and has been widely used in a broad region.[7] The SDM-R is employed to unify both adsorption mechanism and partition mechanism and to make an integrated theory of adsorption and partition of solute in RPLC in this study.

Many misunderstandings of the SDM-R have appeared in references. Firstly the r value representing the numbers of the adsorbed layer of solvent in Eq. (2) is taken as if it were 1 or 2, concluding that the SDM-R belongs to a surface process in adsorption mechanism. It is actually not true. The r value can be any positive integer, 1, even more. Thus, the SDM-R can still be referred as a volume process in partition mechanism. Secondly, the SDM-R is referred as the same mode as the displacement model presented by Snyder *et al*.[23] and Soczewinski.[24] The former is not only a displacement model, but also a quantitatively stoichiometric one including the fractions, nr, and q of Z, while the later is only a semi-quantitative model which may have a value like Z, but never have any fractions of Z. Thirdly, the interactions between solute and solvent in stationary phase and mobile phase are ignored because there are no activity coefficient in the expression of the SDM-R. As pointed out above, five molecular interactions include the activity coefficient of solute as concerned by Cheong *et al*.[25] and the simplified expression of the SDM-R is actually in activity form [see Eq. (9) below], but not in concentration. Because the SDM-R contains all of the molecular interactions including all considered in both of adsorption mechanism and partition mechanism, it would be possible to use SDM-R not only to remedy the weakness of the adsorption mechanism and partition mechanism, but to unify the adsorption mechanism and partition mechanism.

From the law of mass action and by using the methodology of chemical equilibrium, if the equilibrium constants of the above five equilibria are K_1, K_2, K_3, K_4 and K_5, respectively, then

$$K_5 = \frac{K_3K_4}{K_1K_2} = \frac{a_{P\bar{L}_{nr}D_{(m-q)}}\,a_D^{(nr+q)}}{a_{PD_m}\,a_{\bar{L}_{nr}D}} \qquad (6)$$

The term "a" denotes activity. The equilibrium constant K_5

is actually the general equilibrium constant containing four others and represents the final equilibrium constant denoted by K_a of the solute displacing solvent by means of a stoichiometric displacement model.

According to the SDM-R in RPLC,[5-7] the relationship between the capacity factor of solute, k' and each factors in Eq. (6) can be written as:

$$\log k' = \log K_a + nr\log a_{\overline{LD}} + \log \phi - (nr + q)\log a_D \quad (7)$$

Suppose, $$Z = nr + q \quad (8)$$

here, r represents the layer numbers of adsorbed solvent where the solute actually arrives, but may not be the really total layer numbers of the adsorbed solvent on the stationary phase. The term n denotes the moles of the displaced solvent, if r equals unity. The term nr is the moles of solvent released from stationary phase and q shows the moles from the solute.

The condensed form of Eq. (7) can be expressed as:

$$\log k' = \log I - Z\log a_D \quad (9)$$

The term $\log I$ denotes a constant relating to the affinity of one mole of the solute to a stationary phase, a_D stands for the activity of the strong solvent in a mobile phase, $a_{\overline{LD}}$ shows the activity of solvent adsorbed by the stationary phase, ϕ represents the column phase ratio and Z represents the total moles of solvent squeezed out at the interface between the solute and the stationary phase. It consists of three components, n, r and q.

When the a_D range in Eq. (9) is not very broad, $a_{\overline{LD}}$ is referred as a constant. The other parameters K_a, n, q, r and ϕ in Eq. (7) are also constants. Thus, Eq. (7) or Eq. (9) is a linear equation. Two constants, $\log I$ and Z can be easily obtained from the intercept and slope, respectively, of the linear plot of $\log k'$ vs. $\log a_D$ with experimental data.

Based on Eq. (9), solute retention is dominated by two terms, $\log I$ and $Z\log a_D$. From the physical meaning, the former represents the magnitude of the solute adsorption from the mobile phase, and the latter stands for the solute desorption from the stationary phase. The competition between them leads its reversed process resulting the solute to have retention. With the increase of a_D in the mobile phase, k' decreases and *vice versa*. Because many terms in each of $\log I$ and Z, as shown in Eqs. (7) and (8), contain the contributions of the stationary phase and mobile phase, obviously it is very difficult to differentiate these contributions from each other. However, another basic equation of the SDM-R relating to $\log I$ and Z in RPLC is as:[7,26]

$$\log I = Zj + \log \phi \quad (10)$$

where, j is a set of constants relating to the affinity of one mole solvent to the stationary phase and independent of types of solutes. The theoretical j value equals the logarithm of the pure organic solvent (100%).[26] Eq. (10) is derived and proved to be only linear in the circumstance of the interaction between solute and stationary phase to be non-selective, such as that in RPLC and hydrophobic interaction chromatography (HIC).[7,26,27]

Combining Eq. (10) with Eq. (8), another expression of the SDM-R in RPLC and HIC is obtained as:

$$\log k' = Z(j - \log a_D) + \log \phi \quad (11)$$

From Eq. (11), the capacity of solute k' only depends on Z and a_D. If the chromatographic run is done by an isocratic elution, k' is only dominated by Z value.

The SDM-R indicates that without Z moles of the solvent released at the interface between the stationary phase and the solute, or its reversed process, the solute never has chromatographic separation. In other words, without the accomplishing the re-adsorbed nr moles of the solvent by the stationary phase and the re-solvated q moles solvent from the mobile phase by the solute, during the reverse process of solute adsorption, the solute is unable to leave from the stationary phase and to go to the mobile phase. Therefore, a conclusion is that Z can become a parameter to characterize the total contributions of a solute from the stationary phase and mobile phase. Additionally, though the magnitudes of both nr and q also depend on the molecular structure of the solute, the effects from the same solute can offset with each other. An more important conclusion can also be obtained that nr becomes a parameter to characterize the contribution of the stationary phase to the solute retention, while q does that of the mobile phase, respectively.

As described above, two extreme circumstances usually to consider the retention mechanism of solute in RPLC are that adsorption mechanism is dominated by stationary phase, while partition mechanism is controlled by mobile phase.[10] If this point is reasonable, three important conclusions would be obtained. First, based on the fact that the value of either nr, or q in SDM-R is never zero, thus, a pure mechanism of either adsorption mechanism, or partition mechanism never occurs in RPLC. Therefore, a mixed mode of adsorption mechanism and partition mechanism of solute retention exists forever in RPLC. Second, the magnitude of the parameter nr can be used to characterize the contribution to solute retention from adsorption mechanism, while that of q can express the contribution to solute retention from partition mechanism. Additionally, from the reported paper,[3] the magnitudes of both nr and q are independent of the largest depth of the BPL, but depends on the place where the solute is actually able to arrive. Third, as long as the both nr and q can be exactly determined, the net contributions from the adsorption mechanism and partition mechanism, respectively, would be obtained.

Determination of nr and q

The SDM-A of solute was theoretically derived and experimentally tested only from the point of view of pure physical chemistry.[7,19] The SDM-A not only proves the SDM-R

shown in Eq. (9) to be reasonable, but also is a supplement to the SDM-R. The expression of the SDM-A can be shown in two forms as:

$$\log a_{\mathrm{P\bar{L}}_{nr}\mathrm{D}_{(m-q)}} = \beta_a + (nr/Z)\log a_{\mathrm{PD}_m} \quad (12)$$

and

$$\log P_a = \beta_a - (q/Z)\log a_{\mathrm{PD}_m} \quad (13)$$

where,

$$\beta_a = \log K_a + nr\log P_{a'} \quad (14)$$

$a_{\mathrm{PL}_{nr}\mathrm{D}_{(m-q)}}$ denotes the activity of the solute adsorbed on the stationary phase, or in the BPL. The term a_{PD_m} represents the solute activity in the mobile phase employed. P_a and P_a' are the activity partition coefficients of solute and solvent in the two phases, respectively. The term β is a constant containing a set of constants, K_a, representing the stoichiometric displacement equilibrium constant of solute displacing solvent. The physical meaning of β_a is a constant relating to the affinity of one mole of the solute to the BPL. The other parameters, n, r, q and Z have the same physical meaning as that shown in the SDM-R described above.

The expression of the SDM-A can be described as that the logarithm of the activity partition coefficient of solute in two phases shown in Eq. (13), $\log P_a$, or the logarithm of the solute activity on a stationary phase shown in Eq. (12), $\log a_{\mathrm{PL}_{nr}\mathrm{D}_{(m-q)}}$, is proportional to the logarithm of the solute activity in the mobile phase, $\log a_{\mathrm{PD}_m}$.

The physical meanings of the nr/Z and q/Z can be described with the ratios of the moles of solvent desorbed at the contact region from the solvated stationary phase side and the solvated solute side to the total moles of the desorbed solvent, respectively. The similarities and differences between Eqs. (12) and (13) were reported in details before.[28]

It is easy to understand that the sum of nr/Z in Eq. (12) and q/Z in Eq. (13) equals to unity and the two equations have the same intercept, β_a. The term β_a contains four parameters K_a, $P_{a'}n$, and r at a given stationary phase and mobile phase. Thus, both Eqs. (12) and (13) are linear equations. The two terms, nr/Z and q/Z, can be actually measured by experiment. If we use the same chromatographic conditions, *i.e.*, stationary phase, composition of mobile phase, and solute in usual RPLC or that in frontal analysis (FA) of RPLC to determine Z with Eq. (9) and nr/Z by Eq. (12), or q/Z by Eq. (13), and then by combining the two of the three kinds of values, the components of Z, nr and q can be obtained.

During the foregoing process of deriving equations, each concentration term must be theoretically expressed with activity. However, it is very hard to obtain each accurate activity coefficient needed on the stationary phase and the mobile phase. Specially, the definition of the activity coefficient on the stationary phase has not been recognized. Fortunately, some mobile phases, for example, methanol-water solution, were measured to be identical to an ideal solution.[29] With this original data of activity coefficient of n-alcohols, such as methanol, ethanol, and n-propanol, the logarithm of the activity coefficient of n-alcohols was found to be linear to the logarithm of the molar concentration of the homologue in solution.[29] In other words, without considering the activity coefficient of solvent, at least, Z value in Eq. (9), nr/Z in Eq. (12), and q/Z in Eq. (13) would not significantly change. Thus, for convenience, we referred all of mobile phases as an ideal solution and all adsorbed layers to be an ideally adsorbed layer, resulting in all of the activity coefficients in this study to be taken as unity.

If Eq. (12) is expressed as its concentration form, then

$$\log [c_{\mathrm{P\bar{L}}_n\mathrm{D}_{(m-q)}}] = \beta + nr/Z\log [c_{\mathrm{PD}_m}] \quad (15)$$

For simplicity of symbol, both $[c_{\mathrm{P\bar{L}}_n\mathrm{D}_{(m-q)}}]$ and $[c_{\mathrm{PD}_m}]$ can be replaced by c_s and c_m, and β_a by β, respectively. Thus, Eq. (15) is now written as

$$\log c_s = \beta + nr/Z\log c_m \quad (16)$$

And in the same way, Eq. (13) becomes:

$$\log P_a = \beta - (q/Z)\log c_m \quad (17)$$

Eqs. (16) and (17) are the two concentration expressions of the SDM-A.

Eq. (16) can now be described as that the logarithm of the concentration of a component on absorbent is proportional to the logarithm of the equilibrium concentration of the component in bulk solution. Eq. (16) is mathematically the same as the empirical Freundlich Equation. Though this equation is an empirical equation only valid for gas-solid adsorption, it has been widely used in many adsorption circumstances of liquid-solid adsorbed system.

Scientists have not known the physical meanings of each constant in the empirical Freundlich equation and the mystery why the slope of the linear plot with Freundlich equation is always less than unity. Now we can fully understand all of these mysteries that nr and q are only the fractions of Z. Thus, it could be concluded that the originally empirical Freundlich Equation can be theoretically derived with the SDM-A, and it is valid in some complicated liquid-solid systems and each parameter has exact physical meaning.[28]

The SDM-A has two concentration expressions, Eqs. (16) and (17). Though both equations can be mathematically converted into each other and have the same intercept, as it was explained before, both equations have different physical meanings.[28]

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 HPLC column RP-P, C-18 (100 mm × 4.6 mm; particle size, 5.6 μm; pore diameter, 30

nm; specific surface area, 53 m^2/g; ligand density, 4 $\mu mol/m^2$; . packings weight of the column, 1.0 g) was purchased from SynChrom Inc. (West Lafayette, IN, USA). The column temperature was controlled at (25 ± 0.50) ℃ with a water bath.

Insulin (bovine pancreas, HPLC) was bought from Sigma Co. Methanol and 2-propanol were obtained from EM Science (Gibbstone, NJ, USA). Absolute alcohol was bought from McCormick Distilling Co., Inc. (Perkin, IC, USA). Trifluoroacetic acid (HPLC/spectro grade) was obtained from Pierce (Rockfold, IL, USA). Hydrochloric acid (Ultrex, Ultrapure Reagent) was obtained from T. Baker. Acetic acid (glacial, Fisher Chemical) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pure water is double-deionized water.

Three kinds of mobile phases consisted of organic solvents-water ($V_{solvent}/V_{water}$) with 0.1% TFA ($V_{mobile\ phase}/V_{TFA}$) as: (1) 45.0% methanol, (2) 32.0% ethanol and (3) 18.0% 2-propanol. Two solutions were used as the strong elution solution to do gradient elution for the column cleaning: (1) 50% acetic acid and (2) 90% methanol/water + 0.03% ($V_{mobile\ phase}/V_{HCl}$) HCl. The insulin solution of 1.0 mg/mL was separately dissolved into the three above mentioned mobile phases.

Experimental procedure

The procedure of frontal analysis (FA) in RPLC in this study was followed to the equipment scheme employed in the previous paper[2] and insulin was dissolved in each of the three mobile phases. It is absolutely necessary to gain a smooth and horizontal base line for the blank of the measurement of either the first plateau height, or NMR determination of methanol increment with pure deuterium oxide as solvent. Thus, the length of the base line was specially designed as that reported in the previous papers.[1,2]

The elution curves of the increments of three organic solvents, methanol, ethanol and 2-propanol in water with 0.10% TFA were obtained according to the same procedure as the previous paper,[2] 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 procedures were the same as in previous papers.[1,2]

The same stationary phase and mobile phase used in frontal analysis (FA) were employed for the determinations of k' of insulin in usual RPLC with isocratic elution at a flow rate of 1.0 mL/min. With Eq. (9), both of Z and log I could be obtained by the linear plot of log k' versus log [D].

All data were recorded at wavelength 254 nm with reference wavelength 550 nm.

Results and discussion

All calculations were done with a computer. The distance between atoms was taken from the software ACD/Chemsketch (1998).

Evaluation of layer numbers of methanol molecule for accommodating solute in BPL

In the reported paper,[3] the largest amount of the adsorbed methanol, $M_{methanol(T,A)}$ is 26.1 mmol/column (4.14 $\mu mol/m^2$) and only 27.2% (1.34 $\mu mol/m^2$) of it has not any dynamic problem for mass transfer. The former is important for FA in RPLC, while the latter is significant for usual RPLC. Because the argument of solute retention to be adsorption mechanism, partition mechanism, or their mixed one is only limited in usual RPLC, not for FA, how many layer numbers of the adsorbed methanol form to be 1.34 $\mu mol/m^2$ methanol would be an important criterion to judge whether a solute distribution in two phases belongs to an adsorption mechanism, or a partition mechanism.

Many authors reported the thickness of the BPL to cover in the range of 1.7—3.0 nm.[12,30,31,33,34] Although Miller *et al.* and Sentell[32,35,36] reported the composition of the BPL in the deep direction to be inhomogeneous, they did not measure the exact compositions and thickness of each region. Therefore, it is difficult to calculate the regions in which a solute can arrive. Even though we know the specific area of the stationary phase employed to be 80 m^2/g and the total surface area of the column to be 53 m^2/column and the bonding density to be 4 $\mu mol/m^2$, the exact layer number of methanol is still hardly calculated. Although we know the ligands of octadecyl bonded as densely as possible, and the rest of the 4 $\mu mol/m^2$ is silanol, we still do not know how much space can be occupied by methanol together with water. Fortunately, our measured ratio 27.2% was only methanol in the previous paper, resulting in our ignoring the presence of water in the BPC in this study.[3]

For convenience, we assume that only methanol molecules exist and their distribution in the depth direction to be homogeneous in the BPL. Although the thickness of the stationary phase of octadecyl covering 1.7—3.0 nm was reported,[12,30,31,33,34] it was recognized as 3.0 nm.[12,30] It seems reasonable to take 3.0 nm to be the thickness of the BPL in this study. The longest distance between two atoms of methanol molecule is 0.258 nm, and the numbers of methanol layer on the stationary phase, or in BPL can be calculated to be 10.6.

As shown in Fig. 1, the whole thickness of methanol layer is 10.6 including two parts of dynamic and non-dynamic layers of mass transfer during solute retention. From the experimental result, the latter 2.9 layers are only valid in usual RPLC. In other words, the former 7.7 layers are not available in usual RPLC, but valid for FA in RPLC. The longest distance of two hydrogen atoms of benzene molecule is 0.497 nm. It can be completely embedded in approximate 3.0 molecular layers of methanol. According to the criteria by Jaroniec[10] and Dorsey *et al.*[12] a surface process only occurs in monolayer or bilayer, while volume process means that BPL is "thick" enough to accommodate solute molecules. If these criteria can be acceptable, benzene retention in RPLC should belong to a volume process, or partition process.

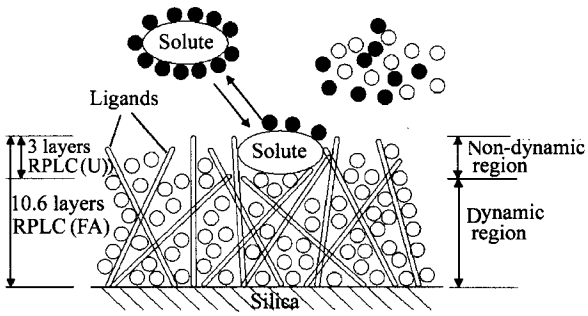

Fig. 1 Scheme of unification of adsorption and partition mechanism of solute retention by SDM-R and each contributions from both in RPLC. RPLC (U)—usual RPLC; RPLC (FA)—frontal analysis RPLC; ●—q, contribution from adsorption mechanism (47%); ○—nr, contribution from partition mechanism (53%).

The stationary phase was emphasized to play an important role in governing the solute retention mechanism.[12,14,37] In some reports the mixed mode of adsorption mechanism and partition mechanism was discussed, but the exact extent of the adsorption mechanism and partition mechanism has not been discussed. From the traditional point of view, for adsorption mechanism, a stationary phase dominates solute retention, while for partition mechanism, the mobile phase controls solute retention. However, it was reported that even though many scientists claim that solute retention likely belongs to partition mechanism, stationary phase is still referred as a significant contribution to the solute retention.[38]

Many researchers investigated the contributions of the stationary phase[30,33,38,39] and mobile phase[14,30,40,41] to solute retention in RPLC, but they only reported the investigation of either stationary phase, or mobile phase alone.

Two linear plots by SDM-A *and* SDM-R

The adsorption isotherms of insulin (not shown here) were obtained from the aqueous pseudo-homologue of n-alcohol with 0.10% TFA solutions. With Eqs. (16) and (17), the linear parameters of insulin for the plots by the two equations were listed in Table 1. The concentration ranges of the three kinds of the organic modifiers and insulin were listed in the notes of Table 1.

From Table 1, each linear regression coefficient R values are greater than 0.995. It indicates that both Eqs. (16) and (17) fit the obtained experimental data well. In addition, each standard deviation for nr/Z and corresponding q/Z value in Table 1 is less than ± 0.02. As expected in the theoretical, the sum of the nr/Z and q/Z is really equal to unity and the intercept β for the two linear plots is exactly the same. This fact further proves that the SDM-A is very close to the real circumstance of adsorption from solution.

The adsorbed amount of insulin on the stationary phase and the concentration partition coefficient of insulin depend on, as shown in Eqs. (16) or (17), both the constant β and product term of $nr/Z\log c_m$, or $q/Z\log c_m$. The term β is a constant contributing to insulin adsorption, while the term, $nr/Z\log c_m$, or $q/Z\log c_m$ dominates insulin desorption. The competition between β and either $nr/Z\log c_m$, or $q/Z\log c_m$ decides the finally adsorbed amount of insulin.

The shape of the adsorption isotherm of insulin is dominated by the nr/Z or q/Z value. From Table 1, both nr/Z and q/Z indicate a regular rule. With the increases in the chain length of the pseudo-homologue, each nr/Z decreases but q/Z increases.

The β value theoretically depends on, the molecular structure of solute, the character of the stationary phase and the kind of solvents. The experimental result shown in Table 1 proves this point. However, the β values of insulin obtained from the aqueous solutions of methanol and 2-propanol are almost the same, but that from the aqueous ethanol solution is the greatest one. It seems that the β value does not follow the homologous rule. It may be attributed to the various competitions between the self-association, the changes in molecular conformation, and the polymerization of insulin molecules in various kinds of organic solvents.[42]

Contributions of adsorption mechanism and partition mechanism to solute retention

With the combination of the Z value obtained from Eq. (9) and nr/Z in Eq. (16) or q from Eq. (17), nr and q were obtained and listed in Table 2. Table 2 also indicates the comparison between nr and q values which separately represent the contributions of the partition mechanism and adsorption mechanism to insulin retention. The fact that each nr shown in Table 2 is less than its corresponding q elucidates that the contribution of adsorption mechanism to insulin retention is less than partition mechanism. For example, when methanol is as the organic modifier, the contributions are 47% for adsorption mechanism and 53% for partition mechanism, respectively. This is also shown in Fig. 1.

Table 1 Linear parameters of insulin for the plots of log c_s and log P_a vs. log c_m[a]

Solvents in mobile phase	Parameter					
	log c_s vs. log c_m			log P vs. log c_m		
	R	nr/Z	β	R	q/Z	β
Methanol	0.9958	0.472 ± 0.02	-3.32 ± 0.02	0.9966	0.528 ± 0.02	-3.32 ± 0.02
Ethanol	0.9981	0.448 ± 0.01	-3.10 ± 0.01	0.9987	0.552 ± 0.01	-3.10 ± 0.01
2-Propanol	0.9985	0.419 ± 0.01	-3.37 ± 0.01	0.9991	0.582 ± 0.01	-3.37 ± 0.01

[a] The concentration range of insulin for the aqueous methanol (45.0%), ethanol (32%) and 2-propanol (18%) are 0.050—0.60 mg/mL, 0.10—0.60 mg/mL and 0.10—0.70 mg/mL, respectively.

Table 2 Contributions of adsorption mechanism (nr) and partition mechanism (q) to insulin retention[a]

Alcohols	Range (V/V, %)	R	Z	log I	nr	q
Methanol	50—58	0.9965	22.4 ± 1.3	26.3 ± 0.1	10.6	11.8
Ethanol	32—37	0.9944	17.1 ± 1.0	13.8 ± 0.1	7.66	9.44
2-Propanol	18—22.5	0.9975	14.0 ± 0.5	26.2 ± 1.0	5.87	8.13

[a] Alcohols/water + 0.10% TFA, (25 ± 0.5) ℃.

Linear plots of Z, nr, and q vs. N_c

The homologous rule is usually used to test a model and to find some regularities only for small molecules. Unfortunately, it has not found the existence of protein homologue in the nature.

The kernel of the SDM-R, as stated in the theoretical section, is that when one mole solute is adsorbed by the stationary phase, a stoichiometric moles, Z, of solvent would release. It should be equivalent to say that when Z moles of solvent are adsorbed by the same stationary phase, one mole solute would leave from the stationary phase. So long as this concept is reasonable, we may investigate the behavior of a protein retention by means of the chromatographic character of a pseudo-homologue. If homologous rule can be used for studying proteins, the selected organic solvent must be a homologue. When 1-propanol has high viscosity, it is replaced by 2-propanol as organic solvent, so they are referred as a pseudo-homologue.

The plots either nr or q vs. the carbon number of the pseudo-homologue, N_c, are expressed as Eqs. (18) and (19), respectively:

$$nr = -2.37N_c + 12.8 \quad (R = 0.9903) \qquad (18)$$

$$q = -1.84N_c + 13.46 \quad (R = 0.9866) \qquad (19)$$

Two slopes from the two linear plots are negative with greater absolute value for nr than q. The negative sign means that both nr and q would decrease with carbon number increasing. This should be reasonable, because the number of the displaced solvent molecules by insulin would linearly decrease with the increases in the size of the solvent molecule. From Eqs. (18) and (19), the two intercepts show that when the carbon number is zero, or the organic solvent is absent in the mobile phase, both have a comparable average value, (13.15 ± 0.35) with a positive sign. They represent the maximum values of nr and q, when alcohol in mobile phase is absent, *i.e.*, pure water is as the mobile phase. It has no physical meaning, because there is not any chromatographic separation for insulin in this circumstance.

Because both nr and q are linear to N_c, the relationship between nr and q should be linear also and express as Eqs. (20) and (21), respectively.

$$q = 0.779nr + 3.53 \quad (R = 0.9997) \qquad (20)$$

$$rn = 1.28q - 4.52 \quad (R = 0.9997) \qquad (21)$$

The Eqs. (20) to (21) indicate an excellent linear relationship between the two components, nr and q. The two slopes of these equations show that nr is greater than q, indicating the extent of nr value decreasing is more rapid than q. This fact also shows that compared to the contribution of the adsorption mechanism, that of the partition mechanism of RPLC to insulin retention increases with solvent molecular size increasing. The latter again indicates the contribution to insulin retention is greater from partition mechanism than that from adsorption mechanism by using this pseudo-homologue.

Conclusions

1. With the combination the stoichiometric displacement model for retention (SDM-R) and the stoichiometric displacement model for adsorption (SDM-A) and the test by the reversed phase liquid chromatographic system consisting of insulin with a mobile phase of a pseudo-homologue alcohol, water and trifluoroacetic acid (TFA), the moles of the displaced methanol from the stationary phase side, nr and that from solute side, q, or the fractions of Z (the sum of nr and q) were separately and successfully determined.

2. The retention mechanism of solute in RPLC never exists either pure adsorption mechanism or partition mechanism. The two terms, nr and q of insulin express the contributions of the adsorption mechanism and partition mechanism to the solute retention, respectively. The number of layers of methanol molecules in the stationary phase surface were also determined and can be divided into two parts, 2.9 layers without any dynamic problem of mass transfer being available of solute distribution in usual RPLC and 7.7 layers with dynamic problem only available for solute distribution in frontal analysis in RPLC. Although solute distributes only in the former about three layers being a volume process in partition process, a stoichiometric displacement process was definitely proved to take place.

3. The SDM-R of solute covers five kinds of molecular interactions among solute, solvent and stationary phase. Thus, the adsorption mechanism, partition mechanism and the mixed mode of both could be theoretically unified by the SDM-R.

4. For the RPLC system consisting of insulin and methanol/water with 0.1% TFA, the adsorption mechanism and partition mechanism were found to contribute to insulin retention 47% and 53%, respectively. The unification of adsorption mechanism and partition mechanism by the SDM-R was also proved by quantitative data.

5. The contributions of the adsorption mechanism and

partition mechanism to insulin retention were found to change with the chain length of the pseudo-homologue of alcoholas organic modifier in mobile phase. Compared to the adsorption mechanism, with the increases in the carbon number of the pseudo-homologue, the contribution from the partition mechanism is getting more and more.

6. As a methodology for investigating the retention mechanism and retention behaviors of biopolymers in RPLC, a homologue, even a pseudo-homologue can be employed as the organic modifier in mobile phase. Based on the homologue rule, much information about biopolymer retention can be obtained.

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